**On the cover:**

Flavonoid fluorescence in stellate trichomes of *Degenia velebitica*, a Croatian endemic species, is reported by Stamenković and Tkalec (pp. 247–255).

# ACTA BOTANICA CROATICA

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# ACTA BOTANICA CROATICA

The journal originally entitled *Acta Botanica Instituti Botanici Regalis Universitatis Zagrebensis* was founded in 1925. In 1957 its name was changed to Acta Botanica Croatica. In 1998, it became an entirely English-language journal.

The journal covers field (terrestrial and aquatic) and experimental research on plants and algae; including plant viruses and bacteria; from the subcellular level to ecosystems. Manuscripts focusing upon the lowland and karstic areas of southern Europe, karstic waters, other types of fresh water, and the Adriatic (Mediterranean) Sea are particularly welcome. More detailed information is available on the link

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# Foreword

It is a great honour and privilege to celebrate the centennial of *Acta Botanica Croatica*, a journal that has played a pivotal role in the advancement of botanical science in the past 100 years. Since its founding in 1925 by botanists from the Department of Botany at the Faculty of Philosophy of the University of Zagreb led by Professor Vale Vouk, the journal has served as a vital platform for the dissemination of high-quality research in the different botanical disciplines, encompassing studies ranging from sub-cellular levels to entire ecosystems.

The establishment of *Acta Botanica Croatica* in the years following the First World War was a visionary endeavour, driven by the need to enhance communication within the international scientific community and to facilitate the exchange of knowledge. Over the decades, the journal has undergone significant transformations under the guidance of esteemed editors, each contributing to its continued growth and scientific relevance. From its early years as *Acta Botanica Instituti Botanici Regalis Universitatis Zagrebensis*, through the post-war consolidations, to its modern-day presence in globally recognized indexing databases, the journal has remained committed to scientific excellence.

A major milestone in the journal's history was its transition into an entirely English-language publication in 1998, a step that greatly expanded its international reach and impact. The subsequent inclusion in prestigious indexing services such as the Web of Science and SCOPUS, along with partnerships with renowned publishers, has reinforced the journal's reputation as a leading European botanical publication. Today, *Acta Botanica Croatica* attracts contributions from authors across the globe, fostering a diverse and interdisciplinary approach to botanical research.

Throughout its century-long journey, *Acta Botanica Croatica* has not only chronicled the evolution of botanical science but has also contributed significantly to its development. The journal has been instrumental in shedding light on the flora of karstic and arid regions, particularly in southern Europe, and has facilitated critical discussions on emerging topics in plant ecology, taxonomy, physiology, and molecular biology. By consistently upholding rigorous peer-review standards and embracing technological advancements in scholarly publishing, the journal has remained at the forefront of scientific dissemination.

The success of *Acta Botanica Croatica* is largely attributed to the dedication and expertise of its editors over the past century. Each editor has played a crucial role in shaping the journal's identity, ensuring its scientific rigour, and expanding its reach. We acknowledge their invaluable contributions: Vale Vouk (1925–1956), Stjepan Horvatić (1957–1968), Ljudevit Ilijanić (1969–1992), Ljerka Marković (1993–1997), Damir Viličić (1998–2013), Branka Salopek-Sondi (2014–2018), Nenad Jasprica (2019–2025), and Mirta Tkalec (2022–present day).

As we look to the future, *Acta Botanica Croatica* remains steadfast in its mission to advance botanical knowledge, support interdisciplinary collaboration, and provide a distinguished platform for researchers worldwide. The 100th anniversary of the journal is not just a commemoration of its rich history but also an inspiration for continued scientific progress. We extend our deepest gratitude to all editors, reviewers, authors, and readers who have contributed to the journal's enduring success.

With great anticipation, we embark on the next century of Acta Botanica Croatica, aiming to further strengthen its role as a hub for botanical research, expand collaboration across diverse scientific disciplines, and embrace emerging technologies in scholarly publishing. Our goal is to enhance both accessibility and impact while remaining dedicated to excellence in botanical research and global scientific exchange.

*Acta Botanica Croatica*
*Nenad Jasprica*
*Mirta Tkalec*
Editors-in-Chief

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Review article

# Marine benthic diatoms from the Adriatic Sea (NE Mediterranean): review of investigations and checklist with updated nomenclature

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**Abstract** – Despite more than 30 years of investigations, the knowledge of the composition and spatial distribution of marine benthic diatoms along both coasts of the Adriatic Sea is still limited. The distribution and a checklist of marine benthic diatom taxa in the Mediterranean sub-basin of the Adriatic Sea are presented based on literature data. The nomenclature is brought up to date and synonyms are included. Eight hundred and twenty-two species belonging to 70 families and 163 genera have been reported. The number of pennate species represented was 719. Families with the highest number of genera were: Naviculaceae (8), Bacillariaceae (7), Surirellaceae (6), Fragilariaceae (6). The highest number of taxa belonged to the Naviculaceae (95) and Bacillariaceae (88). The most numerous genera were *Mastogloia* (65 taxa), *Navicula* (62), *Nitzschia* (53), *Amphora* (52), *Diploneis* (43), *Cocconeis* (37), *Halamphora* (24), *Achnanthes* (22), *Licmophora* (22), *Tryblionella* (19), *Fallacia* (15), *Surirella* (14), *Grammatophora* (13), and *Lyrella* (12). This study focused on the areas of investigation according to the literature reviewed and showed that many areas have to be investigated. Nevertheless, this first comprehensive checklist of marine benthic diatoms in the Adriatic Sea, updated with recent literature, is a valuable tool. However, it is important that this update has been progressive over time. Incorporating data on diatom taxa will improve the overall diversity of microalgae and our knowledge of the ecology of coastal ecosystems.

**Keywords**: Adriatic Sea, Bacillariophyta, biogeography, epilithic, epipelic, epiphytic, Mediterranean Sea, taxonomy

## Microphytobenthos

The microphytobenthos (MPB) colonises the surface sediment layer (2 to 3 mm thick) where strong chemical-physical gradients exist (MacIntyre et al. 1996). In temperate regions, the microphytobenthic community is mainly composed of benthic diatoms, which are either colonial or unicellular, either free-living or attached to a substrate through gelatinous protrusions and play an important role in primary production in marine ecosystems (Falkowski et al. 2004). Classification based on sediment typology reveals distinct groups: (i) epipelon, diatoms living on sediment, (ii) epipsammon, diatoms adhering to sand grains, and (iii) epiphyton, diatoms living on other photosynthetic organisms (Round et al. 1990). The microphytobenthic community plays a key ecological role within the aquatic ecosystem, regulating nutrient and oxygen fluxes at the water-sediment interface and contributing significantly to primary production (MacIntyre et al. 1996). Due to their ecophysiological characteristics, these microorganisms serve as valuable indicators for water quality assessment and in paleoecological reconstructions (Stevenson and Pan 1999, Cibic and Blasutto 2011, Barinova et al. 2019, B-Béres et al. 2023).

Microphytobenthic populations, particularly in shallow aquatic environments, are frequently enriched by phytoplanktonic species that settle on sediments in conditions devoid of turbulent movement, tidal currents, or water column stratification. Conversely, under conditions of pronounced turbulence in the water column, microphytobenthic organisms may be resuspended, infiltrating the phytoplankton community (Delgado et al. 1991, MacIntyre et al. 1996). Furthermore, identical species may be present both in the water column and in the surface sediments, making it difficult to characterize the typical species in each habitat ecologically (Cibic et al. 2022). In a two-year study

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(2003-2004) on MPB biodiversity conducted in Northern Adriatic Sea, on average, 9% of the species in the sediment were planktonic (Cibic et al. 2007a); however, they were predominantly in poor condition. Only species with robust frustules, such as *Pseudo-nitzschia seriata*, were identifiable and likely still photosynthetically active (Cibic et al. 2007b).

In recent years, the application of light microscopy (LM) and scanning electron microscopy (SEM) has enabled the discovery of numerous diatom species in marine coastal waters, greatly improving our understanding of diatom communities in different coastal regions, such as the Black Sea, the Mediterranean Sea or Yellow Sea (Park et al. 2018, Kaleli and Akçaalan 2021, Zidarova et al. 2022). While various authors have previously compiled checklists (Hendey 1974, Viličić et al. 2002, Cibic and Facca 2010, Caraus 2017), the recent and progressing systematics of diatoms has resulted in many synonyms and the transfer of taxa to a new species of a genus (Li et al. 2018, Morales et al. 2019). A considerable proportion of the studies conducted has predominantly focused on planktonic forms of diatoms, thus the publications considered herein generally include both benthic and planktonic diatoms.

## Mediterranean Sea

The Mediterranean Sea is a semi-enclosed continental sea that is almost entirely landlocked between Europe, Africa and Asia and consists of several sub-zones, including the Alboran Sea, the Balearic Sea, the Ligurian Sea, the Tyrrhenian Sea, the Ionian Sea, the Adriatic Sea, the Aegean Sea and the Levantine Basin. It is a concentration basin that receives relatively low saline Atlantic Water (AW), which flows eastwards in the surface layer and gains salt due to the positive Evaporation-Precipitation balance over the Mediterranean (Menna et al. 2022). The northeastern Mediterranean Sea is influenced by the brackish waters of the Black Sea through the Sea of Marmara. The shallowest part of the Mediterranean Sea is the northernmost third of the Adriatic Sea (i.e., the Northern Adriatic Sea), a shelf with depths up to 100 m (Vilibić et al. 2023). The Mediterranean Sea is considered an oligotrophic basin with a few biological production and biodiversity hotspots (Myers et al. 2000). The meadows of *Posidonia oceanica* L. Delile, a species of seagrass endemic to the Mediterranean Sea, are the most important ecosystem in the Mediterranean in terms of biodiversity. However, many alien and invasive species have recently been reported along the eastern coasts of the Mediterranean and Aegean Sea, originating from the Red Sea through the Suez Canal (Zenetos et al. 2005). The Mediterranean Sea is an important basin, and its coasts have been heavily influenced by humans through tourism, urbanization and maritime transport. Additionally, the Mediterranean Sea is considered a hot spot of global warming, as it has been changing faster than the global ocean, with recent studies agreeing on increases in sea level, sea surface temperature and salinity over the past two decades, which have had a strong impact on the marine environment (Menna et al. 2022).

As far as modern diatom studies in the Mediterranean Sea are concerned, one of the pioneering studies was published by Witkowski et al. (2000). While Witkowski et al. (2000) published a monograph of diatom taxa from various marine coastal sites worldwide, Blanco and Blanco's monograph (2014) showed widespread taxa alongside site-specific taxa in the Mediterranean region. In addition, several checklists with a combination of planktonic species in the Mediterranean Sea have been published. Viličić et al. (2002) published a phytoplankton checklist for the eastern Adriatic Sea, while Kaleli and Akçaalan (2021) showed the diatom flora of the Turkish coasts, Tas (2013) investigated the phytoplankton composition in the Aegean Sea (Tas 2013), and Velâsquez and Cruzado (1995) investigated the diatom flora of the northwestern Mediterranean Sea. Most of the studies used a similar sampling technique, specifically the use of a plankton net designed for phytoplankton sampling, with incidental observations of benthic diatoms. Consequently, the use of the plankton net resulted in the observation of relatively large cell sizes for the most commonly cited taxa.

In recent decades, the literature on the distribution of MPB species in the Mediterranean Sea has been limited to the coastal regions of Spain and France (Delgado 1989, Barranguet et al., 1996, Barranguet 1997, Riaux-Gobin et al. 1998). While these publications highlight the importance of MPB as a primary producer within coastal or lagoon ecosystems, and report the total MPB expressed as chlorophyll and pigment concentrations, they do not provide information on the taxonomic composition or present a comprehensive taxonomic list. In recent study, Pérez-Burillo et al. (2022) evaluated the advantages and limitations of LM and DNA metabarcoding for the identification of benthic diatom communities in shallow coastal environments of the Mediterranean Sea using biofilm samples from different substrates in the Ebro delta bays (Spain). The compilation of the historic records of marine and brackish diatom species and genera reported for the French coastal areas since 1888 to 2019, with information on the geographical locations and ecology (e.g. sampled substrate, habitat, growth forms) for most taxa by Ribeiro et al. (2022), provides a great dataset for the benthic marine diatoms of French Mediterranean coast.

Before the compilation of the MPB checklist for the Italian seas (northern and western Middle Adriatic, and Ligurian Sea) by Cibic and Facca (2010), there were no time series data (no continuous observations at specific sites over an extended period with constant abundance) on the MPB in the Italian seas. The knowledge of MPB in Italian seas is low compared to longer-term studies on phytoplankton (Cibic and Facca 2010). The list of Cibic and Facca (2010) encompasses taxa derived from natural sediment samples collected from various locations, including the Gulf of Trieste (Sdrigotti et al. 1999, Welker et al. 2002, Cibic et al. 2007a), the Venice Lagoon (Tolomio et al. 1999, 2002, Facca et al.

2002a, b, 2003, 2004, Tolomio 2004, Facca and Sfriso 2007), the Adriatic coast from Ancona to the Po Delta (Totti 2003), the Lesina Lagoon (Gambi et al. 2003) as well as epibiontic microalgae on marine hydroids *Eudendrium racemosum* in the Ligurian Sea (Romagnoli et al. 2007, 2014), epilithic diatoms on different artificial hard substrates in the Conero area (Totti et al. 2007), and the first colonisation stages of diatoms on artificial hard substrates at two stations in the Gulf of Trieste (Bartole et al. 1991-94) and the Venice Lagoon (Tolomio and Andreoli 1989, Tolomio et al. 1991). Additionally, several recent papers provided benthic diatom checklists either in the main text or as Supplementary Material. These studies cover the Gulf of Trieste (Franzo et al. 2014, Rogelja et al. 2018) and the Grado Lagoon (Natali et al. 2023), the Adriatic coast from Ancona to the Po Delta (Accoroni et al. 2016, Cibic et al. 2019), and the Venice Lagoon (Baldassarre et al. 2023). Additionally, microbenthic community structure and trophic status of sediments of the Ionian Sea were studied in the period 2013–2014 (Rubino et al. 2016). The only available information regarding the microphytobenthic community of the Ligurian Sea refers exclusively to epibiontic species associated with marine hydroids (Romagnoli et al. 2007, 2014), showing that diatoms living on this marine invertebrate derive an advantage from both the host and the environmental conditions due to the availability of nutrients and organic compounds (Cibic and Facca 2010). Diatom assemblages from the Tyrrhenian Sea have been used to study the effects of heavy metal contamination, where this group showed the presence of teratogenic forms in response to this kind of pollution (Rogelja et al. 2016).

The epiphytic diatom communities of the endemic Mediterranean seagrass *P. oceanica* are among the most thoroughly studied (Mazzella et al. 1994, De Stefano et al. 2000). Mazzella et al. (1994) identified *Cocconeis* Ehrenberg species as the most abundant and frequent diatoms on the leaves in all seasons and throughout the depth range of the seagrass distribution. De Stefano et al. (2000) analysed eight species of *Cocconeis* on the leaves of *P. oceanica* using SEM on samples collected around the island of Ischia (Gulf of Naples, Italy). Their results showed that certain *Cocconeis* species such as *C. scutellum* var. *posidoniae* M.De Stefano, D.Marino & L.Mazzella and *C. neothumensis* var. *marina* M.De Stefano, D.Marino & L.Mazzella exhibit high abundance, forming a continuous, almost monospecific layer on the colonised segments of the leaves (Mazzella et al. 1994). In a comparative study, De Stefano et al. (2008) provided additional insights into *C. scutellum* Ehrenberg and its varieties and presented information on the geographical distribution of all the analysed *C. scutellum* taxa, including from the leaves of *Posidonia* in the Mediterranean region collected in spring 2005 and 2006 (the season when the density of epiphytic macroalgae is low) and covering almost the entire Mediterranean Sea (including sampling sites along the coasts of Spain, Italy, Slovenia, Croatia, Greece and Türkiye).

A comprehensive review of the existing literature reveals that there is only a limited number of studies focused on the coastal waters of Türkiye (Kaleli and Akçaalan 2021). The Sea of Marmara and the Aegean Sea were the most intensively studied areas, with 20 and 19 studies, respectively. Studies on benthic and planktonic species composition and biogeographic distribution in the Mediterranean Sea and the Black Sea were rather sparse with only 11 and 14 studies, respectively (Kaleli and Akçaalan 2021). On the Turkish coast of the Aegean Sea, Altuğ et al. (2011) and Aslan et al. (2018) investigated the composition of phytoplankton in the northeastern Aegean Sea. Koray (2001) published a checklist of phytoplankton in the Turkish seas. These studies focused mainly on the planktonic forms of diatoms. Several studies have also been carried out on the benthic diatoms of the shores of Greece (Economou-Amilli 1980, Belegratis 2002, Belegratis and Economou-Amilli 2002, Louvrou 2007). Foged (1985a, b) published monographs on the diatom communities of the islands of Samos, Kos and Kalymnos. In the southern Aegean Sea, periphyton colonisation in hydrothermal marine areas of the island of Milos has been studied (Louvrou 2007). The author later described *Detonia dobrinae* Louvrou, Danielidis & Economou-Amilli (Louvrou et al. 2006) and several taxa of *Meloneis* I.Louvrou, D.B.Danielidis & A.Economou-Amilli (Louvrou et al. 2012). Loir (2010-2014) published micrographs of diatoms from the Mediterranean Sea, including on the island of Crete. Tas (2013) observed that diatoms and dinoflagellates were the dominant groups of phytoplankton groups on the Datça peninsula. *Cocconeis* and *Amphicocconeis* M.De Stefano & D.Marino have been studied extensively in the Aegean Sea (De Stefano and Marino 2003, De Stefano et al. 2006, 2008, Majewska et al. 2014). These studies introduced several new species and expanded knowledge of these genera using both LM and SEM. Recently, Konucu and Develi (2021) reported two new monoraphid diatom species, *Cocconeis sigillata* Riaux-Gobin et Al-Handal and *Amphicocconeis rodriguensis* Riaux-Gobin et Al-Handal, from samples collected in the northeastern Mediterranean Sea.

Morphological studies by Pennesi et al. (2011, 2012) investigated the ultrastructure of some marine Mastogloia Thwaites ex W.Smith species collected during opportunistic sampling of benthic diatoms from diverse geographical regions, including tropical, subtropical including Indonesia and the Red Sea, and temperate zones, such as Patmos Island in the Aegean Sea, Greece, where epiphytic diatom samples were collected from seagrasses and macroalgae in September 2000. The genus *Mastogloia* has been reported in Turkish temperate inland waters, exhibiting a presence in both pelagic and benthic diatom flora (Elmacı and Obalı 1998, Akbulut and Yıldız 2002, Çelekli and Külköylüoğlu 2006, Sıvacı et al. 2007, Çolak Sabancı 2013). Recently, several papers have been published on diatom communities in the Aegean Sea; Li et al. (2018) described *Gedaniella flavovirens* (H.Takano) Chunlian Li, A.Witkowski & M.P. Ashworth in the Fragilariaceae, and several taxa were introduced from the Aegean Turkish coasts in the Iztuzu coast of Muğla (Kaleli et al. 2020). Additionally, diatoms from Iztuzu beach and Iztuzu coastal lake (Kaleli 2019, Kaleli 2022), as well as the benthic diatom composition in Homa Lagoon (Izmir) (Çolak Sabancı 2010, 2011, 2012a, b, 2013, Çolak Sabancı and Koray 2010), have been studied. Some of these studies introduced new records for the Turkish flora, while others documented diverse compositions.

In the African Mediterranean region, on the other hand, there have been very few studies. Aleem (1950) contributed new taxa from the Levantine Basin of Egypt. Voigt (1963) worked on *Mastogloia* species in the Mediterranean. Zalat (2001, 2002) and Zalat et al. (2019) investigated the community structure of the Suez Canal and Egyptian coasts, describing new species and showing their spatial distribution, focussing on habitat preferences and life forms. Their studies revealed that most species are cosmopolitan, with a significant presence of freshwater forms. More recently, a study on the distribution of diatoms on the Algerian coasts has been published (Kaddeche et al. 2022).

## Hydrographic and oceanographic characteristics of the Adriatic Sea

The Adriatic Sea, the northernmost embayment of the Mediterranean Sea, 800 km long and 150 km wide, divided into three sections (Northern, Middle and Southern), is under considerable continental influence and characterised by diverse water masses (Viličić et al. 2002, Vilibić et al. 2023, Verri et al. 2024). Water exchange with the Mediterranean Sea takes place through the 800 m deep Strait of Otranto, with significant inflows into the Southern Adriatic Sea in winter, driven by the "Bimodal Oscillating System" (BiOS). This internal mechanism influences the biogeochemistry and biology of both the Adriatic and Ionian Seas (Civitarese et al. 2010, 2023, Ljubimir et al. 2017). The BiOS connects the deep thermohaline cell of the eastern Mediterranean, which originates in the Southern Adriatic, with the Northern Ionian Gyre (NIG) and causes decadal shifts in circulation and redistribution of salinity (Civitarese et al. 2023). The eastern Adriatic coast is influenced by high-salinity and nutrient-poor inflows from the Ionian Sea and freshwater discharges from oligotrophic karstic rivers (Batistić et al. 2014, Ljubimir et al. 2017). Saline waters enter the Adriatic Sea in intermediate layers on the eastern side of the strait, while the Adriatic water mainly exits via the western shelf and the deep section of the strait (Gačić et al. 1996, Vilibić et al. 2023). The nutrient levels in the Ionian Sea and the Adriatic Sea show similar variability, although the Adriatic Sea generally has a lower nutrient content. By contrast, anticyclonic NIG enhances the inflow of fresher, nutrient-rich Modified Atlantic Water (MAW) originating in the Western Mediterranean, causing nutricline upwelling at the NIG border, decreasing Southern Adriatic temperature, salinity, and density, and reducing winter convection, thereby increasing ecosystem productivity (Civitarese et al. 2010). An overview of productivity across the geographical regions of the Adriatic Sea shows that the Northern Adriatic, particularly the Gulf of Trieste, Po River delta, and Rovinj area, has been the focus of most studies on primary production (N=46). The Middle Adriatic has been notable for *in situ* primary production measurements since the 1960s. Annual primary production ranges from 87.4–260.0 g C $m^{-2}$ $y^{-1}$ in the Northern Adriatic and 70.0–177.4 g C $m^{-2}$ $y^{-1}$ in the Middle Adriatic. The Southern Adriatic is the least studied, with only daily estimates available (236–374 mg C $m^{-2}$ $d^{-1}$) (Matek and Ljubešić 2024). The occurrence of organisms from the Atlantic/Western Mediterranean and the Eastern Mediterranean/temperate zones in the Adriatic correlates with the decadal circulation changes (the anticyclonic and cyclonic circulations) of the NIG (Civitarese et al. 2010, 2023).

## Review of diatom investigations in the Adriatic Sea

The study of diatoms in the Adriatic Sea began in the late 1800s and several species were included in the diatom flora like *Bacillaria adriatica* Lobarzewski (Lobarzewski 1840). One of the pioneering studies in the Adriatic region was conducted by Kützing, who described *Berkeleya adriatica* Kützing (Kützing 1844) and *Amphitetras adriatica* Kützing (Kützing 1845). This was followed by the work of Grunow (1860), Cleve and Grunow (1880), and Van Heurck (1881), who discovered additional new species. In the 1900s, *Mastogloia adriatica* Voigt was described by Voigt (1963), and these records were followed by local studies in the 2000s.

In recent decades, several authors have published studies on phytoplankton composition, such as Viličić et al. (2002) with a checklist of phytoplankton in the eastern Adriatic Sea, Viličić (2014) with a book on the ecology and composition of phytoplankton in the Adriatic, Neri et al. (2024) with a comparative analysis of phytoplankton diversity in the Northern Adriatic Sea using microscopy and metabarcoding etc. According to Viličić et al. (2002), marine diatoms, comprising 518 species (330 pennates and 174 centric), were the predominant phytoplankton group in the eastern Adriatic. The study highlighted a regional distribution, with pennate diatoms more abundant in the Northern Adriatic and centric diatoms dominating the offshore Southern Adriatic.

In the Adriatic Sea, benthic diatoms have been reported from several areas, including: the Gulf of Trieste (Bartole et al. 1991-94, Sdrigotti et al. 1999, Munda 2005, Cibic et al. 2007a, b, Cibic and Blasutto 2011, Franzo et al. 2014, Rogelja et al. 2018), Venice Lagoon (Tolomio and Andreoli 1989, Tolomio et al. 1999, Facca et al. 2002a, b, 2003, 2004, Tolomio et al. 2002, Facca and Sfriso 2007, Baldassarre et al. 2023), the northwestern Adriatic coast (Totti 2003, Totti et al. 2007, Franzo et al. 2015, Accoroni et al. 2016, Pennesi and Danovaro 2017, Cibic et al. 2019, Natali et al. 2023) and the eastern Adriatic coast (Burić et al. 2004, Miho and Witkowski 2005, Caput Mihalić et al. 2008, Levkov et al. 2010, Car et al. 2012, 2019a, b, 2020, 2021, Mejdandžić et al. 2015, Nenadović et al. 2015, Hafner et al. 2018a, b, Kanjer et al. 2019, Seveno et al. 2023, 2024).

The Northern Adriatic Sea, the most intensively studied area (e.g. Sdrigotti et al. 1999, Totti 2003, Munda 2005, Totti et al. 2007, Facca and Sfriso 2007, Mejdandžić et al. 2015), is a shallow sub-basin of the Adriatic Sea characterized by a morphologically complex coastline leading to the formation of variable hydrodynamic and sedimentary environments. The hydrology in this region is influenced by various factors such as winds and river discharge, and the general circulation is cyclonic. Freshwater (mean flow of 1496 $m^3 s^{-1}$ in the period 1917-2008, Cozzi and Giani 2011) originates from major rivers along the northern and northwestern coasts, with the Po River contributing nutrient-rich water, constituting approximately one-third of the total freshwater input into the Adriatic Sea. The Po's freshwater flow, characterized by relatively fresh and mesotrophic water, might form a thin surface layer over the northern sub-basin in summer, while it is reduced in winter and flows directly south along the Italian coast (Poulain 2001 and references therein). An analysis of the most recent decade (2013–2022) shows a pronounced negative trend across the entire Adriatic Sea, indicating a consistent annual reduction of –4.2% in freshwater input throughout the river basin (Aragão et al. 2024). The sedimentation pattern is consistent with the hydrodynamic circulation (Ravaioli et al. 2003) and shows a narrow strip of recent sand along the coast, followed by a broad belt of muddy sediments. Offshore, there is a muddy transition zone characterized by a gradual increase in sand content, dominating a wide-open sandy shelf area with minimal recent sedimentation (known as relict sands) (Franzo et al. 2015 and references therein). Although the Northern Adriatic shelf is a relatively low-energy environment with low tidal ranges and wave heights, ephemeral deposition of sediments occurs after high-tide events and is subsequently remobilized by waves in dense currents (Traykovski et al. 2007). Sediments migrate southward in a series of wind-induced resuspension events promoted by the Bora and Scirocco winds (Fain et al. 2007). The main sources of biogenic elements are both autochthonous (plankton) and allochthonous (atmospheric inputs and soil organic matter transported by rivers) factors (Franzo et al. 2015). In line with the findings of Totti (2003), Franzo et al. (2015) also reported the presence of the centric diatom *Paralia sulcata* (Ehrenberg) Cleve in sediments off the coast of Emilia-Romagna (Northern Adriatic) in a study aimed at improving the understanding of offshore benthic communities in the Adriatic Sea. Cibic et al. (2012) studied benthic diatom community dynamics in relation to temperature, salinity, nutrient concentrations, freshwater inflow, and mucilage in the Gulf of Trieste (Northern Adriatic Sea, Italy) over seven years (1999–2005) at two sublittoral stations. They found that *Nitzschia* and *Navicula* showed a positive trend with increasing temperature, while *Pleurosigma* exhibited a negative trend. *Navicula* and *Nitzschia* appeared to be negatively affected by mucilage events in the summers of 2000 and 2004, while *Diploneis* occupied the ecological niche temporarily vacated by *Navicula* and *Nitzschia*. *Diploneis* had a negative relationship with temperature at the shallower site. Although no significant correlations between bin-averaged salinity and diatom abundance were found, *Cylindrotheca* was observed at high salinity levels. Despite the general seasonal variability observed in benthic diatom communities, characterized by distinct summer and winter assemblages (Cibic et al. 2012), Franzo et al. (2015) did not observe significant temporal variability in microalgal composition.

In the Venice Lagoon, benthic diatoms from the surface sediment layer were studied to investigate possible relationships between epiphytic diatoms and water quality in shallow coastal areas characterised by marked physical and chemical gradients and significant anthropogenic impacts (Facca and Sfriso 2007). In addition, Cibic and Blasutto (2011) investigated the response of diatoms to different nutrient concentrations under nutrient-rich conditions in a benthic diatom study at three sublittoral sites in the Gulf of Trieste (Italy). It can be observed that the dominance of a single species leads to a decline in diversity. Under oligotrophic conditions, without providing a competitive advantage for a single species when diatoms faced limitations by more than one nutrient, diversity remained high (Cibic and Blasutto, 2011). This study suggests that benthic diatoms, like macrobenthos, can serve as useful indicators of nutrient enrichment and represent a potential and innovative tool for biomonitoring.

The most interesting environments along the Middle Adriatic are highly stratified estuaries formed by several small karstic rivers (Zrmanja, Krka), which are maintained by sufficient river discharge and low tides (Viličić et al. 2002). In the oligotrophic and highly stratified Zrmanja estuary, the composition and abundance of diatoms in biofilm formed on artificial substrates exposed at different depths were studied in July 2000 (Burić et al. 2004, Caput Mihalić et al. 2008). The study conducted by Caput Mihalić et al. (2008) focused on the composition and abundance of diatoms within biofilms on artificial substrates exposed at different depths in the Zrmanja estuary in July 2000. The predominant periphyton species identified were *Amphora coffeaeformis* (C.Agardh) Kützing and *Navicula veneta* Kützing. Shannon-Wiener index ranged from 0.87 to 2.08 at the upper estuary station and from 1.1 to 2.7 at the other station. Additionally, Burić et al. (2004) investigated the abundance of periphytic pennate diatoms, in particular *Cocconeis scutellum*, attached to artificial substrates in the karstic Zrmanja estuary in the summer of 2000. The authors focused on comparing the abundance of attached *Cocconeis* cells with those suspended in the planktonic phase.

Previous research focused mainly on the Northern Adriatic and estuaries (e.g. Burić et al. 2004, Caput Mihalić et al. 2008, Levkov et al. 2010), resulting in a significant knowledge gap regarding the diatom flora of the Southern Adriatic, especially before 2008. Although part of the material for the taxonomic study by Levkov et al. (2010) came from the Ombla River estuary near Dubrovnik (the Southern Adriatic), it focused mainly on 15 species of the genus *Rhoicosphenia* based on LM and SEM. A literature review revealed not only a lack of studies in the coastal waters of the Southern Adriatic before 2008, but also the absence of continuous long-term observations of benthic diatoms at a consistent location. There was also a shortage of information on the seasonal distribution and succession of epiphytic diatoms on macroalgae. The most comprehensive study of diatom community structure in the Southern Adriatic was conducted between autumn 2008 and autumn 2010 in areas inhabited by invasive *Caulerpa* species (Car et al. 2012, 2019a, b).

Prior to the studies by Car et al. (2012, 2019a, b), there were no studies on diatom communities associated with the marine green macroalga *Caulerpa taxifolia* (M.Vahl) C.Agardh ("killer seaweed", Bryopsidales, Chlorophyta), which outcompetes native seaweeds and seagrasses in the Mediterranean Sea by forming dense carpets, leading to biodiversity loss. The study by Kanjer et al. (2019) provided valuable insights into the biodiversity of threatened *P. oceanica* meadows, affected by human activities and invasive species, while also providing crucial information for the identification of taxa-specific epiphytic diatom communities in the eastern Adriatic seagrass meadows. This study describes the epiphytic diatom community living on the blades of the *P. oceanica* in the coastal region of the eastern Adriatic Sea (Dugi Otok island). LM analysis of 21 samples revealed a diverse epiphytic diatom community with 68 taxa belonging to 30 genera across all depths (43 from 10 m depth, 41 from 15 m depth and 39 from 20 m depth), dominated by the genus *Cocconeis*, which is typical of the epiphytic growth on the leaves of *P. oceanica*. All identified species are common in other marine benthic periphytic and epiphytic habitats. Although there was no positional influence on community structure, ANOSIM tests ($P < 0.05$) indicated significant depth-related differences.

In contrast to the study of Kanjer et al. (2019), the study by Car et al. (2019a) focused on the taxonomy of epiphytic diatoms associated with invasive macroalgae of the genus *Caulerpa*. These macroalgae are characterised by secondary metabolites such as caulerpenyne (CYN), which act as chemical defence mechanisms against herbivores and epiphytes (Box et al. 2008, 2010). It is interesting to note that the CYN content varies between the different *Caulerpa* species, e.g. *Caulerpa prolifera* has higher CYN concentrations than invasive *Caulerpa* species under similar climatic conditions (Box et al. 2010). Epiphytic diatoms were studied in the summer and autumn of 2010 on the eastern Adriatic coast (Hvar Island) on fronds of *C. taxifolia* and, for comparison, on the autochthonous macroalgae *Padina* sp. and *Halimeda tuna* (J.Ellis & Solander) J.V.Lamouroux. Qualitative analysis was performed using LM and SEM. The Shannon-Wiener diversity index determined for *C. taxifolia* showed a wide range of values (3.11-4.88), with a maximum in August and a minimum in October. While the number of taxa on the fronds of *C. taxifolia* increased from June (41) to August (88), it decreased in autumn due to the high relative abundance of *Cocconeis caulerpacola* Witkowski, Car and Dobosz, a diatom typical of *Caulerpa*. On the other hand, the highest number of taxa on *Padina* sp. was observed in September (82). The detailed composition of the epiphytic diatoms and the seasonal dynamics in the area affected by the invasive macroalga *Caulerpa taxifolia* were determined for the first time.

In a study by Car et al. (2012), a new diatom species, *C. caulerpacola*, was observed on the green alga *C. taxifolia* over a wide geographical range; from the Adriatic (Stari Grad Bay, Hvar Island, Middle Adriatic, Croatia), on the Mediterranean coasts (Saint Raphaël, west of Cannes, France), and on the east coast of Australia (Moreton Bay, southeast Queensland). It has also been observed on *Caulerpa cylindracea* Sonder (syn. *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman & Boudouresque), another Mediterranean invasive alga. LM, SEM and TEM (transmission electron microscopy) were used for morphological and fine structure analyses. The new species was compared with closely related species (*C. borbonica*, *C. diruptoides* and *C. pseudodiruptoides*). Surprisingly, *C. caulerpacola* was commonly found on *C. taxifolia* despite its reputation as a 'killer seaweed' in very high abundance, but its occurrence seems to be very patchy. Indeed, *C. taxifolia* seems to be a suitable host for epiphytic diatoms, especially for the tiny *Cocconeis caulerpacola*.

Furthermore, a study by Car et al. (2019b) compared the epilithic diatom community structures at three sites on the eastern Adriatic coast (Croatia) in the presence of two different invasive *Caulerpa* species, *C. taxifolia* and *C. cylindracea*, which have spread rapidly in the Mediterranean in recent decades. Between November 2008 and October 2010, 40 samples were collected seasonally at three sites (Hvar Island, Mljet Island and Dubrovnik). Qualitative analyses using LM and EM identified 310 epilithic taxa from 65 genera. The predominant genera were *Mastogloia* (48), *Amphora* (32), *Diploneis* (24), *Nitzschia* (23), *Navicula* (22) and *Cocconeis* (19). A similar seasonal shift in growth forms was observed at all sampling sites, with a doubling (Hvar, Mljet) or tripling (Dubrovnik) of the number of erect diatoms in spring. Apart from erect forms, Dubrovnik and Mljet were characterised by adnate forms, while Hvar was characterised by tube-dwelling forms. The highest values of the Shannon-Wiener diversity index occurred in autumn and ranged from 5.26 to 5.34. Significant differences in diatom communities among sampling sites were correlated with the presence of invasive macroalgae (Hvar – *C. taxifolia*; Mljet and Dubrovnik - *C. cylindracea*). The main taxa contributing to the community variance were *Cocconeis scutellum* var. *scutellum*, *Rhopalodia pacifica* Krammer, *Navicula ramosissima* (C.Agardh) Cleve, and *Berkeleya rutilans* (Trentepohl ex Roth) Grunow. While the spatial variation may reflect unmeasured environmental factors, the results suggest the possible influence of invasive *Caulerpa* algae affecting the habitat through competition with native algae. This study improves the understanding of diatom diversity in challenging environments at both regional and broader scales.

A diatom community similar to the epilithic diatom in a study of Car et al. (2019b) has also been reported for various hard substrates in the Adriatic Sea (Munda 2005, Totti et al. 2007). In a study examining seasonal fouling of artificial substrates by diatoms in the heavily polluted and eutrophic area near Piran in the Gulf of Trieste, with concrete plates (50 × 50 cm) used to observe the changes in diatom assemblages, Munda (2005) reported that the genera *Berkeleya*, *Navicula* and *Licmophora* dominated and covered most of the experimental surfaces. *Achnanthes* taxa were among the initial colonizers, while those belonging to *Nitzschia* and some epipelic taxa settled in autumn. Regarding the depth distribution, the highest taxa richness was observed in spring at 3 m depth and in autumn at 7 m depth (Munda 2005).

Additionally, the community structure of epilithic diatoms reported in Car et al. (2019b) study is in agreement with the results from the Northern Adriatic Sea (Ancona region) in the study by Totti et al. (2007), which was conducted on artificial hard substrates (marble, quartzite and slate) to evaluate substrate-dependent differences in colonisation and to characterise the seasonal variation of micro-epilithic communities in terms of abundance, biomass and community structure from April 2003 to April 2004 on a seasonal basis, and showed a dominance of motile taxa (*Navicula* spp., *Nitzchia* spp.), followed by erect (*Grammatophora marina*, *G. oceanica*,), adnate (*Halamphora coffeiformis* and *C. scutellum*) and tube-dwelling diatoms (*B. rutilans*). However, no significant differences in abundance and biomass were observed in the three substrates. Although Totti et al. (2007) reported high seasonal variability in epilithic microalgal communities, with lower cell densities in winter, the Car et al. (2019b) data suggested that the variation in community structure in terms of relative abundances of diatom taxa was related to sampling sites and possibly to the presence of the two invasive macroalgae considered, rather than to seasonal effects.

In addition, studies on benthic diatoms in Neum Bay (Bosnia and Herzegovina) in 2010 and 2011 (Hafner et al. 2018a, b) contributed significantly to the knowledge of diatoms in the southeastern Adriatic. The study by Hafner et al. (2018a) aimed to expand the knowledge of the MPB in the Adriatic Sea by investigating, for the first time the benthic diatom communities in the small semi-enclosed oligotrophic Neum Bay (Bosnia and Herzegovina), where benthic diatoms growing naturally on rocks and macroalgae were identified at a single station in 2010 and 2011. A total of 24 samples were taken at two different depths, 0.5 m and 8 m, and analysed by LM and SEM. A total of 425 pennate and 58 centric taxa (species and infraspecific taxa) belonging to 60 families and 115 genera were identified. The genera with the largest number of taxa were: *Mastogloia* (46 taxa), *Navicula* (36), *Diploneis* (35), *Nitzschia* (34), *Amphora* (31), *Cocconeis* (27), *Achnanthes* (14), *Halamphora* (12), *Lyrella* (11), and *Surirella* and *Licmophora* (10 each). *Amphora bigibba* var. *interrupta* and *Cocconeis scutellum* were the most common taxa, occurring in 87.5% of the samples. A total of 142 taxa were found only once (sporadic taxa). Although the benthic diatom richness in the bay was high, the taxa were not evenly distributed in time. Consistent quantitative and qualitative data are still needed to better determine the seasonal and spatial changes of the epilithic assemblages in the region.

Two recent studies were conducted to understand the development of diatom communities in relation to physico-chemical parameters (Hafner et al. 2018b, Car et al. 2020). In the study by Hafner et al. (2018b) in Neum Bay (Bosnia and Herzegovina), the taxonomic composition and structure of a marine epilithic diatom community was sampled from the bottom of the two sites at monthly intervals from January to December 2011. While the complete list of species and infraspecific taxa of benthic diatoms in Neum Bay from 2010 and 2011 (Hafner et al. 2018a) included 483 diatom taxa within 115 genera, the list of marine benthic diatoms found in Neum Bay in 2011 included 264 taxa (species and infraspecific taxa) belonging to 69 genera (Hafner et al. 2018b). Of these, 149 and 203 taxa occurred in the samples from shallow (0.5 m depth) and deep (8 m depth) sites, respectively. The monthly distribution of most diatoms was irregular, and many sporadic taxa were found. The difference between the shallow and the deep sites was mainly due to the diatom taxa that were frequently found and whose percentage abundance was high. These were *Halamphora coffeiformis*, *Caloneis excentrica*, *Cocconeis scutellum* var. *scutellum*, *Licmophora flabellata*, *Licmophora gracilis*, *Licmophora* sp., *Navicula abunda*, *Rhabdonema adriaticum*, and *Striatella unipunctata*. Canonical correspondence analysis (CCA) showed that temperature, oxygen saturation (O2/O2'), silicate concentration ($SiO_4$) and salinity were the most important factors influencing diatom community structure in the bay.

While recent studies have focused on benthic diatoms from natural substrates in the Middle and Southern Adriatic (Car et al, 2012, 2019a, b, 2021; Hafner et al. 2018a, b, Kanjer et al. 2019, Seveno et al. 2024), some earlier studies on benthic diatoms in the Adriatic Sea were conducted on artificial substrates (e.g. Tolomio and Andreoli 1989, Tolomio et al. 1991, Bartole et al. 1991-1994, Burić et al. 2004, Munda 2005, Totti et al. 2007, Caput Mihalić et al. 2008, Mejdandžić et al. 2015). Mejdandžić et al. (2015) investigated biofilm formation on an artificial substrate made of Plexiglas (polymer of methyl methacrylate) in the Northern Adriatic Sea (Rovinj). The study, conducted in autumn 2013, at a depth of 5 m above the sea floor, analyzed diatom and bacterial succession over a one-month exposure. Samples for algological analysis were taken after 1 hour, half a day, a day or a few days, a week to one month. Selective agar plates, epifluorescence, LM, electron microscopy (EM), and high-performance liquid chromatography (HPLC) were used for pigment analysis. During the exposure, all biofilm components increased in abundance; the bacterial community was dominated by heterotrophic marine bacteria with $1.96 \pm 0.79 \times 10^4$ colony forming units (CFU) $cm^{-2}$, the phototrophic component was dominated by diatoms ($6.10 \times 10^5$ cells $cm^{-2}$), and fucoxanthin was the dominant pigment (up to 110 ng $cm^{-2}$). The diatom community, led by *Cylindrotheca closterium* (Ehrenberg) Reimann & J.C.Lewin and other pennate benthic diatoms, comprised 30 different taxa, as revealed by detailed EM analysis. The study confirmed that a Plexiglas surface in a marine environment was susceptible to biofouling within 30 days of contact. Bacteria and cyanobacteria were initially involved in the colonisation processes, followed by diatoms, which formed a primary biofilm in the sea.

The use of artificial substrates in diatom studies offers advantages such as reduced effort and cost for sampling and processing, less habitat disturbance and higher sampling accuracy (Lamberti and Resh 1985, Lane et al. 2003). The main advantage of using artificial substrates over sampling natural habitats is the consistent standardisation between replicates. In addition, the use of artificial substrates for monitoring purposes has no impact on algal colonisation and allows global applicability without restrictions due to the natural life cycle and distribution range of macroalgae (Carreira-Flores et al. 2020, Natali et al. 2023). Although artificial substrates have been used for diatom studies for almost 100 years (Naumann 1915; cited in Tuchman and Stevenson 1980, Hoagland et al. 1986, Barbiero 2000), there is still concern about whether diatom communities that develop on artificial substrates accurately represent communities that develop on natural substrates (Lane et al. 2003). Ideally, artificial substrates should have community composition and abundance representative of natural substrates at the same site (Tuchman and Stevenson 1980, Lamberti and Resh 1985, Lane et al. 2003). But usually, the colonization patterns of artificial substrates differ from those observed on natural substrates (Mejdandžić et al. 2015). For example, diatom communities that develop on artificial substrates may better represent the diatom community of a particular natural substrate (Lane et al. 2003). Therefore, further comparative studies of diatom community structure on different artificial and natural substrates are needed.

Since 2015, only two studies, conducted by Nenadović et al. (2015) in the Middle Adriatic, and Car et al. (2021) in the Southern Adriatic, have explored the differences in composition between artificial and natural substrates. The study by Nenadović et al. (2015) focused on the initial colonization of diatoms in the periphytic community on various submerged artificial substrates, characterized by diverse physico-chemical properties, was situated in the coastal region of the Middle Adriatic Sea (Zadar region). The study analysed the abundance and genus composition of diatoms in newly formed biofilms. Additionally, comparisons were made with the diatom community on a natural substrate, specifically the seagrass *P. oceanica* sampled from a nearby meadow, to evaluate differences in abundance and composition on artificial substrates compared to an already established community on a natural substrate. This study aimed to determine the differences in abundance and composition of the diatom community on diverse artificial substrates and to discuss these differences by analysing the affinity of diatoms for specific artificial materials. Comparative investigations indicate that, while living organisms (macrophytes) and organic materials (wood, leaves) serve as supplementary nutrient sources for attached communities, the introduction of newly deployed inorganic artificial substrates (such as glass and plastic) into marine environments provides an opportunity to study the initial development and the succession of diatoms in periphyton communities (Nenadović et al. 2015). Furthermore, although Mejdandžić et al. (2015) and Nenadović et al. (2015) studied the development of periphytic diatoms on various artificial substrates (plexiglass, asbestos, painted iron, wood, concrete, glass, plastic, etc.), their results referred mostly to the generic level. The relevance of studying diatom affinity as an important fouling community on different artificial materials is to gain a comprehensive understanding of the impact of marine debris. This understanding is essential for systematic work on alleviating the negative impacts of litter on the marine environment.

In addition, to understand the differences in composition on artificial and natural substrates, three habitats (epilithon, epiphyton and artificial substrate) were compared in a recent diatom study by Car et al. (2021). Weekly samples were taken in August and September 2016 from the marine lake Mrtvo More in the Southern Adriatic. The study involved detailed LM and, for the first time, SEM analyses of benthic diatoms from a marine lake. A total of 97 taxa within 42 genera were identified, with *Cocconeis scutellum* and *Halamphora coffeiformis* being the most frequent. Shannon-Wiener diversity index (H') values varied from 1.78 to 4.52 throughout the year. Two groups were recognized: (1) epilithon and artificial glass substrate, (2) macroalgae. The analysis indicated that diatom communities on artificial substrates corresponded exactly to those on rock substrates, suggesting their utility as representative alternatives for studying epilithic diatoms in future experiments.

The influence of physico-chemical parameters on the initial colonisation of bacteria and diatoms on a submerged artificial substrate and the development of diatom communities was studied weekly from April to October 2016 at a station in the marine lake Mrtvo More in southern Croatia (Car et al. 2020). The physico-chemical parameters (temperature, salinity, oxygen saturation, Chl-*a* concentration, nutrients: silicate, phosphate, nitrate, nitrite, ammonium) varied significantly according to month and season. According to the TRIX trophic index, the lake showed different trophic characteristics: (i) oligotrophic (at the beginning and end of the study), (ii) mesotrophic (end of June to mid-July), (iii) eutrophic (end of July to mid-September). Heterotrophic bacteria peaked (69,268 cells $\text{cm}^{-2}$) in early June when the diatom abundance began to increase. Among the diatoms, adnate forms were the primary colonizers, particularly *Cocconeis dirupta* W.Gregory var. *flexella* (Janisch and Rabenhorst) Grunow and *Cocconeis scutellum* Ehrenberg var. *scutellum*, while motile taxa joined the fouling communities from July to September. The lake has a high diatom species richness, with the species diversity index being highest in August. The total number of diatom taxa (285 within 72 genera detected on an artificial glass substrate is comparable to some studies of epilithic diatoms in the

Southern Adriatic (Hafner et al. 2018b, Car et al. 2019b), but higher than in previous studies of periphytic diatoms growing on artificial substrates in the Northern Adriatic (Mejdandžić et al. 2015, Nenadović et al. 2015) or in a study of the surface sediment layer in the Venice Lagoon (Facca and Sfriso 2007). However, we believe that this could be due, at least in part, to differences in the methodology used. This study has shown that, among the physico-chemical parameters, temperature, salinity and nitrate concentration have the greatest influence on the abundance of diatom species and that species diversity increases with nutrient enrichment. Strong correlations between environmental variables and diatoms were found and shifts in dominance at the species level were observed.

Two of the most recent studies on benthic diatoms in the Adriatic Sea (Seveno et al. 2023, 2024) focused on blue *Haslea* species, known for the synthesis of blue-green water-soluble pigments such as marennine, which have allelopathic, antioxidant, antiviral and antibacterial properties. Recent descriptions of new species (*H. karadagensis*, *H. nusantara*, *H. provincialis* and *H. silbo*) have deepened our understanding of these pigments. While their beneficial properties have been demonstrated under laboratory conditions, the dynamics of blooms of blue *Haslea* spp. in its natural habitat have been limited to oyster ponds. The study by Seveno et al. (2023) is the first documentation of benthic blooms of *Haslea* spp. in open environments, particularly on periphyton covering turf and macroalgae-like *Padina*. Two blue *Haslea* species – *H. ostrearia* and *H. provincialis* – were recorded at monitoring sites in the Mediterranean (Corsica, France and Croatia). The blooms followed the spring phytoplankton bloom and occurred in shallow, calm waters, suggesting that light plays an important role in their formation. The end of the blooms, marked by a warming of the upper water masses, coincided with shifts in the biofilm community, highlighting the importance of environmental conditions in regulating these unique benthic phenomena (Seveno et al. 2023).

Most work on diatoms in the Adriatic Sea is based on LM and EM, while molecular biology techniques are only sporadically used to analyse benthic diatom samples (Lobban et al. 2015, Witkowski et al. 2016, Li et al. 2019, Baldassarre et al., 2023). New molecular techniques can expand our knowledge of a species and enhance the understanding of its morphological range, biogeography and reproductive isolation (Medlin 2018). DNA sequencing has had a significant impact on the phylogeny, evolution and systematics of diatoms at the species and population level (Medlin and Kaczmarska 2004, Sorhannus 2007, Souffreau et al. 2011, Theriot et al. 2010, Witkowski et al. 2016). DNA metabarcoding has established itself as an alternative to LM-based identifications due to its speed, reproducibility and cost (Zimmermann et al. 2015). However, it is well known that a species can produce different diatom morphologies depending on the season or habitat (see references in Cox 2014), and it has been proposed that the taxonomic level of forma be used to reflect morphologies that change with specific environmental conditions (Cox 2014, Medlin 2018). Morphological changes are particularly striking in pennate diatoms, but they may be more subtle in centric species (Medlin 2018). Conflicts may arise between phylogenetic/molecular speciation and morphological speciation. It is recommended that a combination of species-level methods (both molecular and traditional) be used to resolve the species boundary issues. It cannot be assumed that the barcode of a single individual of a species is representative of the species, as different individuals in a population and individuals in different geographic populations may have slightly different barcode sequences (Medlin 2018). To get a semi-quantitative overview of the diversity of an environmental sample, we would theoretically first need to know the barcodes of all possible organisms in the biosphere. Unfortunately, we are still very far away from such knowledge and it is questionable whether we will ever know them. GenBank provides sequences for perhaps five hundred identified species of marine and freshwater diatoms (out of an estimated 100,000 species). The problem is that these sequences are underpinned with few voucher images and environmental data. A larger dataset of voucher sequences would improve DNA barcoding and allow easier and faster identification of diatoms. This is otherwise a time-consuming process, requiring diatom taxonomists to spend considerable time identifying and characterizing assemblages under the microscope. Currently, the traditional LM method for identifying diatoms in a mixed sample is still faster and more reliable than DNA barcoding of environmental samples for an experienced diatom researcher (Jahn et al. 2007). However, the combination of LM and DNA approaches provides stronger support for ecological studies of benthic microalgal communities in shallow coastal environments than either approach alone (Pérez-Burillo et al. 2022). In the study on the impact of MOSE (Experimental Electromechanical Module) flood barriers on the microphytobenthic community of the Venice Lagoon in 2019 and 2020, the integration of classical taxonomy with 18S rRNA gene metabarcoding enabled a more comprehensive understanding of the potential of the whole community and underlines the complementarity of these methods in ecological research (Baldassarre et al. 2023).

Generally, SEM has revolutionised the systematics of diatoms by revealing ultrastructural features visible that are invisible to LM, becoming essential for the delimitation of taxa; it is an essential aid to diatom classification (Round et al. 1990). The introduction of multivariate statistical methods has further advanced diatom studies by revealing complex morphometric features. More recently, molecular biology techniques have provided even more insight into variation and greatly facilitated species discovery and delimitation (Alverson 2008). Overall, these tools have improved our ability to recognise subtle differences. In addition, collections support the ongoing discovery, classification and revision of diatom species and genera, contributing to the complex and dynamic taxonomy of diatoms. The first institutional collection of permanent microscopic specimens of diatoms in Croatia, the Croatian National Diatom

Collection, was established in March 2018 at the University of Zagreb, Faculty of Science. The collection contains, among other things, type specimens of newly discovered species in Croatia, which are no longer sent to other European collections, but are kept in Croatia as national biodiversity treasures. Collections such as this represent an invaluable wealth of information for biological research, providing insights into the diversity of diatom taxonomy, the biodiversity of specific areas, species distribution and temporal and spatial changes. Taken together, this information facilitates the effective protection of water ecosystems in Croatia (Gligora Udovič and Ljubešić 2021).

Since studies on the distribution of benthic diatom species along the Adriatic coast are scattered in space and time and provide rather heterogeneous information in terms of methodology, sample size and organisms studied, this review aims to provide an updated and integrated picture of Mediterranean benthic diatoms in the coastal waters of the Adriatic Sea, based on the studies conducted over the last 35 years. This paper aimed to compile a list of benthic diatom species detected in the Adriatic coasts based on the results of the most important studies carried out so far on marine diatoms and includes an updated nomenclature. The list was created to consolidate all previously scattered data for future research on benthic diatoms in Croatia, other coastal areas and beyond. This list aims to expand the knowledge of marine diatoms in the Adriatic Sea by reviewing previous studies and including an updated nomenclature.

## Methodological approach

This study is based on literature records of marine diatoms recorded in the Adriatic Sea. The literature search resulted in 52 references from 1989 to 2024 (On-line Suppl. Tab. 1), including planktonic diatoms and marine benthic diatoms in the coasts, coastal lakes and lagoons of the Adriatic Sea. References included articles, reviews, checklists, and PhD theses.

The diatom checklist was compiled using information from publications dating from 1999 to 2021. The old published data of diatoms from the Adriatic Sea by Viličić et al. (2002) were supplemented with more recent information. The main sources for the diatom checklist, besides Viličić et al. (2002), are Sdrigotti et al. 1999, Cibic et al. 2007a, Hafner et al. (2018a, b), Rogelja et al. 2018, and Car et al. (2019b, 2020, 2021).

The results of this study depend on the correct identification by the authors of each reference. In most cases, there are no photographs or illustrations of the taxa, so verification of the records is difficult and some identifications may be incorrect. Despite efforts to avoid synonyms or the changed names of longer established taxa, some taxa may be listed here twice under different names.

For each species, information on its study areas was provided (Tab. 1, Fig. 1). The freshwater species reported in some studies were not excluded due to river pressure and altered salinity at different sites.

**Tab. 1.** Checklist of diatoms found in the Northern (N), Middle (M) and Southern (S) Adriatic Sea. Taxa found in all regions of the Adriatic Sea are indicated with asterisk (*).

***Achnanthes** apiculat*a (Greville) Riaux-Gobin, Compère, Hinz & Ector [M]
*Achnanthes brevipes* var. *brevipes* Agardh*
*Achnanthes brevipes* var. *intermedia* (Kützing) Cleve [M, S]
*Achnanthes brockmannii* Hustedt [M, S]
*Achnanthes* cf. *ceramii* Hendey [S]
*Achnanthes* cf. *javanica* Grunow [M]
*Achnanthes cuneata* Grunow [S]
*Achnanthes curvirostrum* J.Brun [S]
*Achnanthes danica* (Flögel) Grunow [N, S]
*Achnanthes fimbriata* (Grunow) R.Ross [M]
*Achnanthes gracilis* Lee [M]
*Achnanthes groenlandica* (Cleve) Grunow [M, S]
*Achnanthes hyperboreoides* A.Witkowski, Metzeltin & Lange-Bertalot [S]
*Achnanthes javanica* Grunow [M, S]
*Achnanthes kuwaitensis* Hendey [M, S]
*Achnanthes lemmermannii* Hustedt [M]
*Achnanthes longipes* C. Agardh*
*Achnanthes marginalis* Hendey [M]
*Achnanthes parvula* Kützing [M]
*Achnanthes pseudogroenlandica* Hendey [M, S]
*Achnanthes separata* Hustedt ex Simonsen [M, S]
*Achnanthes septata* var. *incurvata* (Østrup) A. Cleve [M]
***Actinocyclus** alienus* Grunow [N]
*Actinocyclus gallicus* F.Meister [M]
*Actinocyclus ingens* var. *irregularis* (Van Heurck) D.M.Harwood & T.Maruyama [M]
*Actinocyclus normanii* (W.Gregory ex Greville) Hustedt [M]
*Actinocyclus ochotensis* A.P.Jousé [M]
*Actinocyclus octonarius* Ehrenberg [N, S]
*Actinocyclus roperi* (Brébisson) Grunow ex Van Heurck [S]
*Actinocyclus splendens* J.Rattray [M]
*Actinocyclus subtilis* (W. Gregory) Ralfs [M, S]
***Actinoneis** lorenziana* (Grunow) Mereschkowsky [N]
***Actinoptychus** adriaticus* Grunow [N, M]
*Actinoptychus heliopelta* Grunow [M]
*Actinoptychus senarius* (Ehrenberg) Ehrenberg [N, M]
*Actinoptychus splendens* (Shadbolt) Ralfs [N, M]
*Actinoptychus vulgaris* Schumann [M]
***Amphicocconeis** disculoides* (Hustedt) Stefano & Marino [S]
***Amphiprora** pseudoduplex* (Osada & Kobayasi) Hällfors [M]
***Amphitetras** antediluviana* Ehrenberg [N, S]
*Amphitetras subrotundata* Janisch [S]
***Amphora** abludens* Simonsen [M, S]
*Amphora acuta* Gregory [N]
*Amphora amoena* Hustedt [M]
*Amphora angusta* Gregory [N]
*Amphora arcus* Gregory [N]
*Amphora arenaria* Donkin*

**Tab. 1.** Continued

*Amphora arenicola* Grunow ex Cleve [M]
*Amphora beaufortiana* Hustedt [M]
*Amphora bigibba* var. *interrupta* (Grunow) Cleve [M, S]
*Amphora binodis* Gregory [N]
*Amphora* cf. *amoena* Hustedt [M, S]
*Amphora* cf. *gacialis* W.Smith [M]
*Amphora* cf. *ramsbottomi* M.M.Salah [M]
*Amphora cingulata* Cleve*
*Amphora constricta* (Ehrenberg) W. Carruthers [M]
*Amphora copulata* (Kützing) Schoeman & R.E.M.Archibald [M]
*Amphora corpulenta* var. *capitata* Tempère & Peragallo [M]
*Amphora crassa* W.Gregory*
*Amphora delicatissima* Krasske [M, S]
*Amphora dubia* Gregory [N]
*Amphora exilitata* M.H.Giffen [S]
*Amphora gracialis* W. Smith [M, S]
*Amphora gracilis* Ehrenberg [M, S]
*Amphora graeffeana* Hendey [M]
*Amphora graeffii* Grunow [N]
*Amphora granulifera* Cleve [N]
*Amphora grevilleana* Gregory [M]
*Amphora helenensis* Giffen [M, S]
*Amphora holsaticoides* T.Nagumo & H.Kobayasi [M, S]
*Amphora immarginata* Nagumo [M, S]
*Amphora insecta* Grunow ex Schmidt*
*Amphora janischii* A. Schmidt [N]
*Amphora jostesorum* Witkowski. Lange-Bertalot & Metzeltin [S]
*Amphora laevis* W.Gregory [N, M]
*Amphora laevissima* W.Gregory*
*Amphora lineolata* (Ehrenberg) Ehrenberg*
*Amphora lunata* Østrup [M, S]
*Amphora marina* W.Smith [N]
*Amphora mexicana* A.W.F.Schmidt [N]
*Amphora obtusa* W.Gregory [N, M]
*Amphora ocellata* Donkin [S]
*Amphora ovalis* (Kützing) Kützing [N, M]
*Amphora pannucea* Giffen [M, S]
*Amphora pediculus* (Kützing) Grunow [M]
*Amphora piper* Cholnoky [S]
*Amphora praelata* Hendey [S]
*Amphora proteus* W.Gregory*
*Amphora pusio* Cleve [N]
*Amphora robusta* W.Gregory [N]
*Amphora staurophora* (Castracane) Cleve [N]
*Amphora turgida* var. *parallela* Heiden [S]
*Amphora weissflogii* A. Schmidt [N]
***Aneumastus*** *maculosus* (Donkin) Lange-Bertalot. [N]
***Anorthoneis*** *hyalina* Hustedt [M]
***Ardissonea*** *formosa* (Hantzsch) Grunow*
*Ardissonea robusta* (Ralfs ex Pritchard) De Notaris*
***Astartiella*** *stroemii* (Hustedt) Bukhtiyarova [N]
***Asterolampra*** *marylandica* Ehrenberg [M, S]
***Asteromphalus*** *flabellatus* (Brébisson ) Greville [N]
*Asteromphalus heptactis* (Brébisson) Ralfs [N, M]
*Asteromphalus hookeri* Ehrenberg [M]
*Asteromphalus robustus* Castracane [N, M]
***Aulacoseira*** *granulata* (Ehrenberg) Simonsen [M, S]
***Auliscus*** *sculptus* (W.Smith) Brightwell [N, M]
***Auricula*** *adriatica* H. Peragallo & M. Peragallo [N, S]
*Auricula amphitritis* Castracane [N, M]
*Auricula complexa* (Gregory) Cleve [N]
*Auricula decipiens* (Grunow) & Cleve [N]
*Auricula intermedia* (F.W.Lewis) Cleve [S]
***Azpeitia*** *nodulifera* (A.W.F.Schmidt) G.A.Fryxell & P.A.Sims [N, M]
*Azpeitia obscura* (Greville) P.A. Sims [N]
*Azpeitia tabularis* (Grunow) Fryxell et P.A. Sims [N]
***Bacillaria*** *paxillifera* (O.F.Müller) T.Marsson*
*Bacillaria socialis* (Gregory) Ralfs [M, S]
***Berkeleya*** *micans* (Lyngbye) Grunow [N]
*Berkeleya rutilans* (Trentepohl ex Roth) Grunow [M, S]
*Berkeleya scopulorum* (Brébisson ex Kützing) E.J.Cox [N, M]
***Biddulphia*** *biddulphiana* (J.E.Smith) Boyer*
*Biddulphia catenata* Schussnig [N, M]
*Biddulphia spinosa* (Bailey) Boyer [N]
*Biddulphia titiana* Grunow [N, S]
***Biddulphiella*** *tridens* (Ehrenberg) P.A.Sims & M.P.Ashworth [N, M]
***Biremis*** *ambigua* (Cleve) D.G.Mann [M, S]
***Bleakeleya*** *notata* (Grunow) Round [S]
***Brachysira*** *aponina* Kützing [M]
*Brachysira estonarium* Witkowski, Lange-Bertalot & Metzeltin [M]
***Brebissonia*** *lanceolata* (C.Agardh) R.K.Mahoney & Reimer [M, S]
***Caloneis*** *bicuneata* (Grunow) C.S.Boyer [M, S]
*Caloneis bioculata* (Grunow) Hustedt [N]
*Caloneis clavicula* Z.Levkov & Metzeltin [M]
*Caloneis egena* (A.F.W.Schmidt) Cleve [N, M]
*Caloneis elongata* (Grunow) Boyer [M, S]
*Caloneis excentrica* (Grunow) Boyer [M, S]
*Caloneis fusioides* (Grunow) Heiden & Kolbe [N, S]
*Caloneis latefasciata* Grunow [N]
*Caloneis liber* (W.Smith) Cleve*
*Caloneis linearis* (Cleve) Boyer [N]
***Campylodiscus*** *adriaticus* Grunow*
*Campylodiscus biangulatus* Greville*
*Campylodiscus clevei* Leuduger-Fortmorel*
*Campylodiscus clypeus* (Ehrenberg) Ehrenberg ex Kützing [M]
*Campylodiscus echeneis* Ehrenberg ex Kützing [N, M]
*Campylodiscus fastuosus* Ehrenberg [N, M]
*Campylodiscus giffenii* Lobban & J.S.Park [M, S]
*Campylodiscus hodgsonii* W. Smith [N]
*Campylodiscus imperialis* Greville [N]
*Campylodiscus innominatus* R.Ross & Abdin [M, S]
*Campylodiscus neofastuosus* Ruck & Nakov*
*Campylodiscus punctulatoides* M.Ricard [M]
*Campylodiscus thuretii* Brébisson*
***Carinasigma*** *latum* (E.J.Cox) G.Reid [M]
*Carinasigma rectum* (Donkin) G.Reid [N, S]
***Catacombas*** *obtusa* (Pantocsek) Snoeijs [S]
***Chamaepinnularia*** *truncata* (D.König) A.Witkowski, Lange-Bertalot & Metzeltin [M]
***Cistula*** *lorenziana* (Grunow) Cleve [M]
***Climacosphenia*** *moniligera* Ehrenberg*
***Cocconeiopsis*** *orthoneoides* (Hustedt) Witkowski, Lange-Bertalot & Metzeltin [M]
***Cocconeis*** *britannica* Naegeli in Kützing [N, M]
*Cocconeis californica* Grunow [N, M]
*Cocconeis californica* var. *kerguelensis* H.Heiden [M]
*Cocconeis capensis* (Cholnoky) Witkowski [M]

**Tab. 1.** Continued

*Cocconeis* cf. *distantula* Giffen [M, S]
*Cocconeis* cf. *irregularis* (Schulz) Witkowski [S]
*Cocconeis convexa* M.H.Giffen [S]
*Cocconeis costata* var. *hexagona* Grunow [M, S]
*Cocconeis costata* W.Gregory [M, S]
*Cocconeis dirupta* var. *flexella* (Janisch & Rabenhorst) Grunow [M, S]
*Cocconeis dirupta* W.Gregory [M, S]
*Cocconeis diruptoides* Hustedt [S]
*Cocconeis discrepans* A.W.F.Schmidt [M]
*Cocconeis distans* W.Gregory [N, M]
*Cocconeis fasciolata* (Ehrenberg) N.E.Brown [M]
*Cocconeis fimbriata* Ehrenberg [N]
*Cocconeis fluminensis* (Grunow) H. Peragallo & M. Pergallo [N]
*Cocconeis guttata* Hustedt in Aleem & Hustedt [M, S]
*Cocconeis irregularis* (P.Schulz) A.Witkowski in Witkowski [S]
*Cocconeis krammeri* Lange-Bertalot & Metzeltin [M]
*Cocconeis latecostata* Hustedt [M]
*Cocconeis maxima* (Grunow) H. Peragallo & M. Peragallo [N, M]
*Cocconeis molesta* Kützing [N, M]
*Cocconeis molesta* var. *crucifera* Grunow [M]
*Cocconeis notabilis* Pantocsek [M]
*Cocconeis nugalas* M.H.Hohn & J.Hellerman [M]
*Cocconeis pelta* A. Schmidt [M]
*Cocconeis peltoides* Hustedt [M, S]
*Cocconeis pinnata* Gregory ex Greville [N]
*Cocconeis placentula* var. *euglypta* (Ehrenberg) Cleve [M]
*Cocconeis pseudodiruptoides* Foged [S]
*Cocconeis pseudomarginata* W.Gregory [N, S]
*Cocconeis schmidtii* Heiden [M, S]
*Cocconeis scutellum* Ehrenberg*
*Cocconeis speciosa* W.Gregory [M, S]
*Cocconeis stauroneiformis* H.Okuno [S]
*Cocconeis woodii* Reyes-Vasquez [M, S]
***Coronia*** *decora* (Brébisson) Ruck & Guiry*
*Coronia impressa* (Grunow) Ruck & Guiry [N]
*Coronia limbata* (Brébisson) Ruck & Guiry [N, M]
*Coronia samoensis* (Grunow) Ruck & Guiry [N]
***Coscinodiscus*** *asteromphalus* Ehrenberg [N]
*Coscinodiscus centralis* Ehrenberg*
*Coscinodiscus cinctus* Kützing [N]
*Coscinodiscus curvatulus* Grunow [N, S]
*Coscinodiscus fimbriatus* Ehrenberg [N]
*Coscinodiscus gigas* Ehrenberg [S]
*Coscinodiscus granii* Gough [S]
*Coscinodiscus hauckii* var. *mesoleius* Cleve [M]
*Coscinodiscus janischii* Schmidt [S]
*Coscinodiscus marginatus* Ehrenberg [N]
*Coscinodiscus obscurus* A. Schmidt [N]
*Coscinodiscus oculus iridis* (Ehrenberg) Ehrenberg [N]
*Coscinodiscus perforatus* Ehrenberg [N]
*Coscinodiscus radiatus* Ehrenberg [N]
*Coscinodiscus thorii* Pavillard [S]
***Craspedostauros*** *decipiens* (Hustedt) E.J.Cox [S]
*Craspedostauros indubitabilis* (Lange-Bertalot & S.I.Genkal) E.J.Cox [S]
***Craticula*** *cuspidata* (Kutzing) D.G.Mann [N]
*Craticula halophila* (Grunow) D.G.Mann [N]
***Ctenophora*** *pulchella* var. *lanceolata* (O'Meara) L.Bukhtiyarova [M]
***Cyclophora*** *tenuis* Castracane [M, S]
***Cyclotella*** *kützingiana* var. *pelagica* Grunow [N]
*Cyclotella striata* (Kützing) Grunow [N]
***Cylindrotheca*** *closterium* (Ehrenberg) Reimann & J.C.Lewin [N, M]
***Cymatoneis*** *sulcata* (Greville) Cleve [M]
***Cymatosira*** *belgica* Grunow [N]
*Cymatosira lorenziana* Grunow [M]
***Delphineis*** *minutissima* (Hustedt) Simonsen [M]
*Delphineis surirella* (Ehrenberg) G.W.Andrews [N]
***Diatoma*** *elongata* (Lyngbye) C.Agardh [M]
*Diatoma vulgaris* Bory [M]
***Dickieia*** *subinflata* (Grunow) D.G.Mann [M]
***Dimeregramma*** *marinum* (W.Gregory) Ralfs [N, M]
***Diploneis*** *aestuarii* Hustedt [N]
*Diploneis adonis* (Brun) Cleve [N]
*Diploneis advena* (A. Schmidt) Cleve [M]
*Diploneis bombus* (Ehrenberg) Ehrenberg*
*Diploneis caffra* (M.H.Giffen) Witkowski, Lange-Bertalot & Metzeltin [M]
*Diploneis campylodiscus* (Grunow) Cleve [N]
*Diploneis* cf. *parca* (A.W.F.Schmidt) Boyer [S]
*Diploneis* cf. *smithii* (Brébisson) Cleve [N, M]
*Diploneis chersonensis* (Grunow) Cleve [M, S]
*Diploneis coffaeiformis* (A.W.F.Schmidt) Cleve [M]
*Diploneis crabro* (Ehrenberg) Ehrenberg*
*Diploneis decipiens* var. *parallela* A.Cleve [N]
*Diploneis dalmatica* (Grunow) Cleve [M, S]
*Diploneis didyma* (Ehrenberg) Ehrenberg*
*Diploneis dilatata* (M.Peragallo) Lange-Bertalot & Fuhrmann [M]
*Diploneis distortum* W. Smith [N]
*Diploneis divergens* (A.W.F.Schmidt) Cleve [N, M]
*Diploneis fusca* (W.Gregory) Cleve [N, M]
*Diploneis incurvata* var. *dubia* Hustedt [M, S]
*Diploneis incurvata* (W.Gregory) Cleve [M]
*Diploneis littoralis* (Donkin) Cleve [M]
*Diploneis littoralis* var. *clathrata* (Østrup) Cleve [M]
*Diploneis mediterranea* (Grunow) Cleve [N]
*Diploneis mirabilis* D.König [M]
*Diploneis nitescens* (W.Gregory) Cleve [M, S]
*Diploneis notabilis* (Greville) Cleve*
*Diploneis novaeseelandiae* (A.Schmidt) Hustedt [M]
*Diploneis papula* (A.W.F.Schmidt) Cleve [N, M]
*Diploneis parca* (A. Schmidt) Boyer [M]
*Diploneis rex* S.J.M.Droop [M]
*Diploneis sejuncta* f. *constricta* Hustedt [M]
*Diploneis smithii* (Brébisson) Cleve*
*Diploneis smithii* var. *dilatata* (Peragallo) Lange-Bertalot & Fuhrmann [M]
*Diploneis smithii* var. *recta* Peragallo [M, S]
*Diploneis splendida* Cleve*
*Diploneis stroemii* Hustedt [M, S]
*Diploneis subovalis* Cleve [N]
*Diploneis subcincta* (A. Schmidt) Cleve [N]
*Diploneis suborbicularis* (Gregory) Cleve [M]
*Diploneis vacillans* (A.W.F.Schmidt) Cleve*
*Diploneis vacillans* var. *renitens* (A.Schmidt) Cleve [M, S]
*Diploneis vetula* (A.W.F.Schmidt) Cleve [S]
*Diploneis weissflogii* (A.W.F.Schmidt) Cleve [M]
***Divergita*** *toxoneides* (Castracane) Theriot [S]
***Ehrenbergiulva*** *granulosa* (Grunow) Witkowski, Lange-Bertalot & Metzeltin [N, M]

**Tab. 1.** Continued

*Ehrenbergiulva hauckii* (Grunow) Witkowski, Lange-Bertalot & Metzeltin [N, M]
***Encyonema*** *ventricosum* (C.Agardh) Grunow [M]
***Entomoneis*** *alata* (Ehrenberg) Ehrenberg [N, S]
*Entomoneis corrugata* (Giffen) Witkowski, Lange-Bertalot & Metzeltin [M]
*Entomoneis decussata* (Grunow) K.Osada & H.Kobayasi [S]
*Entomoneis gigantea* (Grunow) Nizamuddin [N]
*Entomoneis gigantea* var. *sulcata* (O'Meara) Moreno, Licea & Santoyo [N, S]
*Entomoneis paludosa* (W. Smith) Reimer*
*Entomoneis pulchra* (Bailey) Reimer [S]
***Epithemia*** *gibba* (Ehrenberg) Kützing [N]
*Epithemia pacifica* (Krammer) Lobban & J.S.Park [M, S]
***Fallacia*** *aequorea* (Hustedt) D.G.Mann [M]
*Fallacia bioculata* (Grunow ex A.W.F.Schmidt) D.G.Mann [N]
*Fallacia* cf. *vittata* (Cleve) D.G. Mann [M, S]
*Fallacia floriniae* (M.Møller) Witkowski [M, S]
*Fallacia forcipata* (Greville) Stickle & D.G.Mann*
*Fallacia hyalinula* (De Toni) Stickle & D.G.Mann [N]
*Fallacia litoricola* (Hustedt) D.G.Man [M]
*Fallacia nummularia* (Greville) D.G.Mann [N, M]
*Fallacia ny* (Cleve) D.G.Mann [M, S]
*Fallacia nyella* (Hustedt) D.G.Mann [M, S]
*Fallacia pygmaea* (Kützing) Stickle & D.G.Mann*
*Fallacia reichardtii* (Grunow) Witkowski, Lange-Bertalot & Metzeltin [N]
*Fallacia rogalli* Voigt [M]
*Fallacia spatiata* (Østrup) Witkowski & Lange-Bertalot & Metzeltin [M]
*Fallacia versicolor* (Grunow) D.G.Mann [N]
***Fogedia*** *acuta* (Salah) Witkowski, Lange-Bertalot & Metzeltin [S]
*Fogedia christensenii* A.Witkowski, Metzeltin & Lange-Bertalot [S]
*Fogedia finmarchica* (Cleve & Grunow) A.Witkowski, Metzeltin & Lange-Bertalot [S]
***Fragilaria*** *barbatula* (Kützing) Lange-Bertalot [N]
*Fragilaria capensis* Grunow [M, S]
*Fragilaria improbula* Witkowski & Lange-Bertalot [M]
*Fragilaria quebecensis* (Poulin. Hudon & Cardinal) A.Witkowski. Lange-Bertalot & Metzeltin [M]
***Glyphodesmis*** *distans* (Gregory) Grunow in Van Heurck [N]
*Glyphodesmis williamsonii* (W. Smith) Grunow [N]
***Gomphonella*** *olivacea* (Hornemann) Rabenhorst [M]
***Gomphonema*** *apicatum* Ehrenberg [M]
*Gomphonema coronatum* Ehrenberg [M]
***Grammatophora*** *angulosa* Ehrenberg [N, S]
*Grammatophora angulosa* var. *islandica* (Ehrenberg) Grunow [S]
*Grammatophora arcuata* Ehrenberg [M, S]
*Grammatophora gibberula* Kützing [N, M]
*Grammatophora hamulifera* Kützing [N, M]
*Grammatophora longissima* Petit [N]
*Grammatophora macilenta* W. Smith*
*Grammatophora marina* (Lyngbye) Kützing*
*Grammatophora maxima* Grunow [S]
*Grammatophora oceanica* Ehrenberg*
*Grammatophora oceanica* var. *subtilissima* (Bailey) De Toni [M, S]
*Grammatophora serpentina* Ehrenberg [M, S]
*Grammatophora undulata* Ehrenberg*
***Grunowago*** *bacillaris* (Grunow) Lobban & Ashworth [N]
***Gyrosigma*** *acuminatum* (Kützing) Rabenhorst [N]
*Gyrosigma attenuatum* (Kützing) Rabenhorst [N, S]
*Gyrosigma balticum* (Ehrenberg) Rabenhorst [N, S]
*Gyrosigma balticum* var. *diminutum* (Grunow) A.Cardinal, Poulin & Bérard-Therriault [N]
*Gyrosigma fasciola* (Ehrenberg) J. W. Griffith & Henfrey [N]
*Gyrosigma lineare* (Grunow) Cleve [N]
*Gyrosigma macrum* (W.Smith) J.W.Griffith & Henfrey [N, S]
*Gyrosigma prolongatum* (W.Smith) J.W.Griffith & Henfrey [N]
*Gyrosigma scalproides* (Rabenhorst) Cleve [M]
*Gyrosigma strigilis* (W.Smith) J.W.Griffin & Henfrey [N]
*Gyrosigma wansbeckii* (Donkin) Cleve [N]
***Halamphora*** *acutiuscula* (Kützing) Levkov*
*Halamphora borealis* (Kützing) Levkov [M]
*Halamphora coffeiformis* (C. Agardh) Levkov*
*Halamphora costata* (W.Smith) Levkov*
*Halamphora cuneata* (Cleve) Levkov [M, S]
*Halamphora cymbifera* (Gregory) Levkov [M]
*Halamphora exigua* (W.Gregory) Levkov [M, S]
*Halamphora granulata* (Gregory) Levkov [M, S]
*Halamphora holsatica* (Hustedt) Levkov [M]
*Halamphora hyalina* (Kützing) Rimet & R.Jahn*
*Halamphora hybrida* (Grunow) Levkov [M]
*Halamphora interrupta* (Heiden) Levkov [M]
*Halamphora kolbei* (Aleem) Álvarez-Blanco & S.Blanco [M, S]
*Halamphora lineata* (Gregory) Levkov [M]
*Halamphora luciae* (Cholnoky) Levkov [M, S]
*Halamphora pseudoholsatica* (T.Nagumo & H.Kobayashi) J.G.Stepanek & Kociolek [M, S]
*Halamphora pseudohyalina* (Simonsen) J.G.Stepanek & Kociolek [M, S]
*Halamphora staurophora* (Juhlin-Dannfelt) Álvarez-Blanco & S.Blanco [M, S]
*Halamphora subacutiuscula* (Schoeman) J.G.Stepanek & Kociolek [M, S]
*Halamphora subangularis* (Hustedt) Levkov [M, S]
*Halamphora subholsatica* (Krammer) Levkov [M, S]
*Halamphora terroris* (Ehrenberg) P.Wang [N]
*Halamphora turgida* (Gregory) Levkov [S]
*Halamphora veneta* (Kützing) Levkov [N]
***Hantzschia*** cf. *distinctepunctata* Hustedt [S]
*Hantzschia* cf. *marina* (Donkin) Grunow [S]
*Hantzschia elongata* (Hantzsch) Grunow [M]
*Hantzschia leptocephala* (Østrup) Lange-Bertalot & Metzeltin [S]
*Hantzschia marina* (Donkin) Grunow [N]
*Hantzschia virgata* (Roper) Grunow [S]
***Haslea*** *britannica* (Hustedt & Aleem) Witkowski, Lange-Bertalot & Metzeltin [S]
*Haslea* cf. *balearica* Witkowski [M]
*Haslea crucigera* (W.Smith) Simonsen [S]
*Haslea duerrenbergiana* (Hustedt) F.A.S.Sterrenburg [M, S]
*Haslea spicula* (Hickie) Bukhtiyarova [M, S]
*Haslea stundlii* (Hustedt) Blanco, Borrego-Ramos & Olenici [M]
*Haslea vitrea* (Cleve) Simonsen [N]
***Hendeyella*** *dubia* (Grunow) Li, Witkowski & Ashworth*
***Hemiaulus*** *hauckii* Grunow ex Van Heurck [N]
***Hippodonta*** *caotica* Witkowski, Lange-Bertalot & Witkowski [S]
***Homoeocladia*** *angularis* (W.Smith) Kuntze [N, M]
*Homoeocladia distans* (W.Gregory) Kuntze [N, M]

**Tab. 1.** Continued

***Hyalodiscus*** *radiatus* (O'Meara) Grunow [M, S]
*Hyalodiscus scoticus* (Kützing) Grunow [M, S]
*Hyalodiscus subtilis* Bailey [N]
***Hyalosira*** *delicatula* Kützing [N]
***Hyalosynedra*** *laevigata* (Grunow) D. M. Williams & Round*
***Iconella*** *linearis* (W.Smith) Ruck & Nakov [M]
*Iconella spiralis* (Kützing) E.C.Ruck & T.Nakov [M]
***Karayevia*** *submarina* (Hustedt) Bukhtiyarova [M]
***Lampriscus*** *shadboltianus* (Greville) Peragallo & Peragallo [M]
***Licmophora*** *abbreviata* C.Agardh [M, S]
*Licmophora communis* (Heiberg) Grunow [N]
*Licmophora dalmatica* (Kützing) Grunow [N]
*Licmophora debilis* (Kützing) Grunow [N, M]
*Licmophora ehrenbergii* (Kützing) Grunow*
*Licmophora flabellata* (Greville) C. Agardh*
*Licmophora gigantea* Mereschkowsky [N]
*Licmophora gracilis* (Ehrenberg) Grunow*
*Licmophora grandis* (Kützing) Grunow [N]
*Licmophora hastata* Mereschkowsky [N, M]
*Licmophora hyalina* (Kützing) Grunow [N]
*Licmophora lyngbyei* (Kützing) Grunow [N]
*Licmophora juergensii* Agardh [N, S]
*Licmophora mediterranea* Mereschkowsky [N]
*Licmophora paradoxa* (Lyngbye) C. Agardh*
*Licmophora parasitica* Mereschowsky [N]
*Licmophora pfannkuckae* Giffen [M, S]
*Licmophora reichardtii* Grunow [N, S]
*Licmophora remulus* (Grunow) Grunow*
*Licmophora robusta* H.Peragallo & M.Peragallo [N, S]
*Licmophora tenuis* (Kützing) Grunow [N]
*Licmophora tincta* (C.Agardh) Grunow [S]
***Lindavia*** *glomerata* (H.Bachmann) Adesalu & Julius [N]
***Luticola*** *ventricosa* (Kützing) D.G.Mann [M]
***Lyrella*** *abrupta* (W.Gregory) D.G.Mann [N, M]
*Lyrella amphoroides* D.G.Mann [M]
*Lyrella* cf. *lyroides* (Hendey) D.G.Mann [M]
*Lyrella clavata* (W.Gregory) D.G.Mann [N, M]
*Lyrella david-mannii* Witkowski, Lange-Bertalot & Metzeltin [M]
*Lyrella fogedii* Witkowski, Lange-Bertalot & Metzeltin [M]
*Lyrella hennedyi* (W.Smith) Stickle & D.G.Mann*
*Lyrella lyra* (Ehrenberg) Karayeva [M, S]
*Lyrella lyroides* (Hendey) D.G.Mann [N, M]
*Lyrella spectabilis* (Gregory) D. G. Mann [N, M]
*Lyrella subforcipata* (Hustedt) Gusliakov & Karayeva [M]
*Lyrella sulcifera* (Hustedt) Witkowski [M]
***Martyana*** *schulzii* (C.Brockmann) Snoeijs [M, S]
***Mastogloia*** *adriatica* Voigt [M, S]
*Mastogloia affinis* Cleve [M]
*Mastogloia angulata* F.W.Lewis [N, S]
*Mastogloia apiculata* W. Smith [N]
*Mastogloia baldjikiana* Grunow [M]
*Mastogloia beaufortiana* Hustedt [M]
*Mastogloia belaensis* Voigt [S]
*Mastogloia binotata* (Grunow) Cleve*
*Mastogloia biocellata* (Grunow) G.Novarino & A.R.Muftah [S]
*Mastogloia borneensis* Hustedt [M, S]
*Mastogloia braunii* Grunow*
*Mastogloia* cf. *affirmata* (Leudiger-Fortmorel) Cleve [S]
*Mastogloia corsicana* (Grunow) H.Peragallo & M.Peragallo*
*Mastogloia crucicula* var. *alternans* Zanon [M, S]
*Mastogloia crucicula* (Grunow) Cleve*
*Mastogloia cuneata* (F.Meister) Simonsen [M, S]
*Mastogloia cyclops* Voigt [M, S]
*Mastogloia decipiens* Hustedt [M, S]
*Mastogloia decussata* Grunow [M, S]
*Mastogloia emarginata* Hustedt [M, S]
*Mastogloia erythraea* Grunow*
*Mastogloia erythraea* var. *grunowii* Foged [M]
*Mastogloia exigua* F.W.Lewis [M, S]
*Mastogloia exilis* Hustedt [M, S]
*Mastogloia fallax* Cleve [M, S]
*Mastogloia fimbriata* (T.Brightwell) Grunow*
*Mastogloia foliolum* Brun [M]
*Mastogloia grunowii* A.W.F.Schmidt [M, S]
*Mastogloia horvathiana* Grunow [M, S]
*Mastogloia jelineckii* (Grunow) Grunow [M, S]
*Mastogloia ignorata* Hustedt [M]
*Mastogloia inaequalis* Cleve [N]
*Mastogloia laminaris* (Ehrenberg) Grunow [S]
*Mastogloia lanceolata* Thwaites ex W. Smith [N, S]
*Mastogloia latecostata* Hustedt [M]
*Mastogloia linearis* Simonsen [M]
*Mastogloia macdonaldii* Greville*
*Mastogloia manokwariensis* Cholnoky [M, S]
*Mastogloia marginulata* Grunow [N]
*Mastogloia mauritiana* Brun*
*Mastogloia mediterranea* Hustedt [M]
*Mastogloia obliqua* Hagelstein [S]
*Mastogloia ovalis* A.W.F.Schmidt [M, S]
*Mastogloia ovata* Grunow [N, M]
*Mastogloia ovulum* Hustedt*
*Mastogloia ovum-paschale* (A. Schmidt) Mann [N]
*Mastogloia paradoxa* Grunow [N, M]
*Mastogloia parva* Hustedt [M, S]
*Mastogloia peragalloi* Cleve*
*Mastogloia pseudoexigua* Cholnoky [M]
*Mastogloia pseudolatecostata* T.A.Yohn & R.A.Gibson [M, S]
*Mastogloia pumila* (Grunow) Cleve [N, M]
*Mastogloia punctifera* Brun [N]
*Mastogloia pusilla* Grunow*
*Mastogloia quinquecostata* Grunow [N, M]
*Mastogloia recta* Hustedt [M]
*Mastogloia regula* Hustedt [M, S]
*Mastogloia robusta* Hustedt [S]
*Mastogloia similis* Hustedt [M, S]
*Mastogloia smithii* Thwaites ex W.Smith [N, M]
*Mastogloia splendida* (Gregory) H.Pergallo*
*Mastogloia subaspera* Hustedt [N]
*Mastogloia undulata* Grunow [N]
*Mastogloia varians* Hustedt [M, S]
*Mastogloia vasta* Hustedt [M, S]
***Melosira*** *lineata* (Dillwyn) C.Agardh [N]
*Melosira moniliformis* var. *octogona* (Grunow) Hustedt [M]
*Melosira moniliformis* C.Agardh*
*Melosira nummuloides* C.Agardh*
*Melosira varians* C.Agardh [M]
***Meridion*** *circulare* (Greville) C.Agardh [M]
***Metascolioneis*** *tumida* (Brébisson ex Kützing) Blanco & Wetzel [N]

**Tab. 1.** Continued

***Microtabella*** *interrupta* (Ehrenberg) Round*
***Nanofrustulum*** cf. *sopotense* (Witkowski & Lange-Bertalot) E.Morales, C.E.Wetzel & L.Ector [S]
*Nanofrustulum krumbeinii* (A.Witkowski, Witak & K.Stachura) E. Morales [M]
*Nanofrustulum shiloi* (J.J.Lee, Reimer & McEnery) Round, Hallsteinsen & Paasche [S]
*Nanofrustulum sopotense* (Witkowski & Lange-Bertalot) E.Morales, C.E.Wetzel & Ector [M, S]
***Navicula*** *abunda* Hustedt [M]
*Navicula agnita* Hustedt [M, S]
*Navicula ammophila* Grunow [N]
*Navicula apiculata* Brébisson [M]
*Navicula aproximata* Greville [N]
*Navicula arenaria* Donkin [N, M]
*Navicula arenaria* var. *rostellata* Lange-Bertalot [M]
*Navicula avenacea* (Rabenhorst) Brébisson ex Grunow [N]
*Navicula bipustulata* A.Mann [M, S]
*Navicula borneoensis* Hustedt [M, S]
*Navicula cancellata* Donkin*
*Navicula* cf. *fauta* Hustedt [M]
*Navicula cincta* (Ehrenberg) Ralfs [M, S]
*Navicula cluthensis* f. *rostrata* (Simonsen) Hustedt [M]
*Navicula cluthensis* var. *cluthensis* Gregory [M]
*Navicula crucifera* Grunow [M]
*Navicula dehissa* Giffen [S]
*Navicula digitoradiata* (W.Gregory) Ralfs [N, M]
*Navicula directa* (W.Smith) Brébisson*
*Navicula distans* (W.Smith) Brébisson [N, S]
*Navicula eidrigiana* J.R.Carter [M, S]
*Navicula erifuga* Lange-Bertalot [S]
*Navicula flagellifera* Hustedt [M, S]
*Navicula frigida* Grunow [N]
*Navicula formentere* Cleve [S]
*Navicula gregaria* Donkin [S]
*Navicula grevilleana* Hendey [N]
*Navicula grippii* Simonsen [S]
*Navicula hauckii* Cleve [N]
*Navicula johanrossii* Giffen [S]
*Navicula kariana* Grunow [S]
*Navicula libellus* Gregory [N]
*Navicula longa* (W.Gregory) Ralfs [M]
*Navicula longa* var. *irregularis* Hustedt [M]
*Navicula luciae* A.Witkowski & Lange-Bertalot [M]
*Navicula lusoria* M.F.Giffen [M, S]
*Navicula mediterranea* Cleve et Brun [N]
*Navicula meniscus* Schumann [N]
*Navicula nortumbrica* Donkin [N]
*Navicula palpebralis* Brébisson ex W.Smith*
*Navicula palpebralis* var. *angulosa* (W.Gregory) Van Heurck [M]
*Navicula palpebralis* var. *minor* Grunow [S]
*Navicula pavillardii* Hustedt [M]
*Navicula pennata* f. *maxima* (Cleve) Frenguelli [M]
*Navicula pennata* A.W.F.Schmidt [N, M]
*Navicula peregrina* (Ehrenberg) Kützing [N]
*Navicula phyllepta* Kützing [N]
*Navicula pinnata* Pantocsek [M]
*Navicula quarnerensis* Grunow [S]
*Navicula ramosissima* (C.Agardh) Cleve [N, M]
*Navicula recurvata* Gran [S]
*Navicula rolandii* S.Wunsam, A.Witkowski, & Lange-Bertalot [M]
*Navicula rostellata* Kützing [S]
*Navicula salinarum* Grunow [M]
*Navicula salinarum* var. *rostrata* (Hustedt) Lange-Bertalot [S]
*Navicula salinicola* Hustedt [S]
*Navicula subagnita* Proshkina-Lavrenko [M, S]
*Navicula subrostellata* Hustedt [M]
*Navicula superimposita* A. Schmidt [N]
*Navicula vara* Hustedt [M]
*Navicula viridula* (Kützing) Ehrenberg [N]
*Navicula zostereti* Grunow [N, M]
***Neidium*** *trigonocephalum* (Ehrenberg) A.Tuji & Y.Tanimura [M]
***Neobrightwellia*** *alternans* (Bailey) M.P.Ashworth & Sims [N, M]
***Neofragilaria*** *burchardtiae* (Witkowski, Metzeltin & Lange-Bertalot) E.Morales [M, S]
*Neofragilaria nicobarica* Desikachary, Prasad & Prema [M]
***Neohuttonia*** *reichardtii* (Grunow) Hustedt [S]
***Nitzschia*** *acuminata* var. *subconstricta* Grunow [M]
*Nitzschia agnewii* Cholnoky [S]
*Nitzschia agnita* Hustedt [M, S]
*Nitzschia bartholomei* (Grunow) Lange-Bertalot & Krammer [M]
*Nitzschia behrei* Hustedt [M]
*Nitzschia bilobata* W.Smith [N, M]
*Nitzschia bulnheimiana* (Rabenhorst) H.L.Smith [S]
*Nitzschia capitellata* Hustedt [M, S]
*Nitzschia carnicobarica* Desikachary & Prema [S]
*Nitzschia* cf. *bulnheimiana* (Rabenhorst) H.L.Smith [M]
*Nitzschia* cf. *commutata* Grunow [N]
*Nitzschia* cf. *sigma* (Kützing) W. Smith [M, S]
*Nitzschia commutatoides* Lange-Bertalot [M]
*Nitzschia distans* var. *tumescens* Grunow [M]
*Nitzschia fasciculata* (Grunow) Grunow [N]
*Nitzschia fluminensis* Grunow [N, M]
*Nitzschia frustulum* (Kützing) Grunow*
*Nitzschia fusiformis* Grunow [M, S]
*Nitzschia gazellae* Karsten [N]
*Nitzschia grossestriata* Hustedt [M, S]
*Nitzschia hybrida* Grunow [M, S]
*Nitzschia improvisa* Simonsen [S]
*Nitzschia incerta* (Grunow) M.Peragallo*
*Nitzschia incurva* Grunow*
*Nitzschia incurvata* var. *lorenziana* R.Ross [M, S]
*Nitzschia insignis* W.Gregory*
*Nitzschia laevis* Frenguelli [S]
*Nitzschia lanceola* Grunow [M]
*Nitzschia lanceolata* var. *minor* (Grunow) H.Peragallo & M.Peragallo [M, S]
*Nitzschia lanceolata* W. Smith [M]
*Nitzschia liebethruthii* Rabenhorst [M, S]
*Nitzschia longissima* (Brébisson) Ralfs*
*Nitzschia macilenta* W.Gregory*
*Nitzschia marginulata* var. *didyma* Grunow [M, S]
*Nitzschia microcephala* Grunow [N]
*Nitzschia normannii* Grunow [M]
*Nitzschia obtusa* W. Smith [N]
*Nitzschia ovalis* H.J.Arnott [M, S]
*Nitzschia peisonis* Pantocsek [M]
*Nitzschia pellucida* Grunow [M, S]

**Tab. 1.** Continued

*Nitzschia plioveterana* Lange-Bertalot [M]
*Nitzschia reversa* W.Smith [S]
*Nitzschia scalpelliformis* Grunow [M]
*Nitzschia sicula* (Castracane) Hustedt [N, S]
*Nitzschia sigma* (Kützing) W.Smith [N, M]
*Nitzschia sigma* var. *rigida* Grunow ex Van Heurck*
*Nitzschia sigma* var. *rigidula* (H.Peragallo & M.Peragallo) Grunow [N]
*Nitzschia sigma* var. *sigmatella* Grunow [N]
*Nitzschia sigmaformis* Hustedt [M]
*Nitzschia subconstricta* Desikachary & Prema [M, S]
*Nitzschia valdestriata* Aleem & Hustedt [S]
*Nitzschia ventricosa* J.L.Palmer [M, S]
*Nitzschia vermicularis* (Kützing) Hantzsch [N]
***Odontella** aurita* (Lyngbye) C.Agardh [N, M]
***Oestrupia** ergadensis* (W.Gregory) Witkowski, Lange-Bertalot & Metzeltin [M]
*Oestrupia kinkeriana* (Truan) Heiden [N]
*Oestrupia powellii* var. *vidovichii* (Grunow) Heiden [M]
*Oestrupia powellii* (F.W.Lewis) Heiden [N, M]
***Okedenia** inflexa* (Brébisson ex Kützing) Eulenstein ex De Toni [N]
***Opephora** marina* (W.Gregory) Petit [N, M]
*Opephora mutabilis* (Grunow) Sabbe & Wyverman [M, S]
*Opephora pacifica* (Grunow) Petit*
*Opephora schwartzii* (Grunow) Petit ex Pelletan [M]
***Pantocsekiella** ocellata* (Pantocsek) K.T.Kiss & E.Ács [M]
***Paralia** sulcata* (Ehrenberg) Cleve*
***Parlibellus** bennikei* Witkowski, Metzeltin & Lange-Bertalot [M, S]
*Parlibellus berkeleyi* (Kützing) E.J.Cox [S]
*Parlibellus calvus* A.Witkowski, Metzeltin & Lange-Bertalot [S]
*Parlibellus* cf. *cruciculoides* (C.Brockmann) Witkowski, Lange-Bertalot & Metzeltin [S]
*Parlibellus delognei* (Van Heurck) E.J.Cox [M, S]
*Parlibellus hamulifer* (Grunow) E.J.Cox [N]
*Parlibellus plicatus* (Donkin) E.J.Cox [N]
*Parlibellus rhombicula* (Hustedt) Witkowski, Lange-Bertalot & Metzeltin [M, S]
*Parlibellus rhombicus* (W.Gregory) E.J.Cox [N]
***Petrodictyon** gemma* (Ehrenberg) D.G.Mann [M, S]
***Petroneis** besarensis* (Giffin) Witkowski, Lange-Bertalot & Witkowski [M, S]
*Petroneis humerosa* (Brébisson ex W.Smith) Stickle & D.G.Mann [M]
***Pinnularia** claviculus* Schulz [M, S]
*Pinnularia quadratarea* var. *cuneata* Østrup [S]
*Pinnularia rectangulata* (Gregory) Rabenhorst [N]
*Pinnularia trevelyana* (Donkin) Rabenhorst [M]
***Placoneis** flabellata* (F.Meister) Kimura, H.Fukushima & Ts.Kobayashi [S]
***Plagiodiscus** martensianus* Grunow & Eulenstein [M]
*Plagiodiscus nervatus* Grunow [M]
***Plagiogramma** atomus* Greville [M]
*Plagiogramma interruptum* (W.Gregory) Ralfs [N, M]
*Plagiogramma laeve* (Gregory) Ralfs [N]
*Plagiogramma minus* (W.Gregory) Chunlian Li, Ashworth & Witkowski [N, M]
*Plagiogramma minus* var. *nanum* (W.Gregory) Chunlian Li, Ashworth & Witkowski [N, M]
*Plagiogramma pulchellum* Greville [N]
*Plagiogramma staurophorum* (W.Gregory) Heiberg*
***Plagiogrammopsis** vanheurckii* (Grunow) Hasle, Stosch & Syvertsen [M]
***Plagiotropis** elegans* (W.Smith) Grunow [N]
*Plagiotropis gibberula* Grunow [N]
*Plagiotropis lata* (Cleve) Kuntze [N]
*Plagiotropis lepidoptera* (W. Gregory) Kuntze*
*Plagiotropis tayrecta* T.B.B.Paddock [M, S]
*Plagiotropis vitrea* (W. Smith) Kuntze [N]
***Planothidium*** aff. *engelbrechtii* (Cholnoky) Round & L.Bukhtiyarova [M]
*Planothidium* cf. *campechianum* (Hustedt) Witkowski, Lange-Bertalot & Metzeltin [M]
*Planothidium delicatulum* (Kützing) Round & Bukhtiyarova [M]
*Planothidium dispar* (Cleve) Witkowski, Lange-Bertalot & Metzeltin [N]
*Planothidium quarnerense* (Grunow) Witkowski. Lange-Bertalot & Metzelin [N, M]
***Platessa** conspicua* (Ant.Mayer) Lange-Bertalot [M]
***Pleurosigma** aestuari* (Brébisson) W. Smith [N]
*Pleurosigma angulatum* (J.T.Quekett) W.Smith*
*Pleurosigma decorum* W.Smith [M, S]
*Pleurosigma elongatum* W. Smith [N, S]
*Pleurosigma formosum* W.Smith*
*Pleurosigma itium* Ricard [M]
*Pleurosigma longum* Cleve [N]
*Pleurosigma nicobaricum* Grunow [N, M]
*Pleurosigma normanii* Ralfs [N]
*Pleurosigma rigidum* W. Smith [N]
*Pleurosigma speciosum* W. Smith [N]
*Pleurosigma strigosum* W. Smith [N]
***Pleurosira** laevis* (Ehrenberg) Compère [M]
***Podocystis** adriatica* (Kützing) Ralfs*
***Podosira** dubia* (Kützing) Grunow [N]
***Prestauroneis** protracta* (Grunow) Kulikovskiy & Glushchenko [M]
***Proschkinia** complanata* (Grunow) D.G.Mann [N]
***Protokeelia** cholnokyi* (Giffen) Round & Basson [M, S]
***Protoraphis** atlantica* R.A.Gibson [M]
***Psammodictyon** constrictum* (W.Gregory) D.G.Mann [M]
*Psammodictyon panduriforme* (W. Gregory) D.G.Mann*
*Psammodictyon panduriforme* var. *continuum* (Grunow) Snoeijis [M]
*Psammodictyon roridum* (Giffen) D.G.Mann [M, S]
*Psammodictyon rudum* (Cholnoky) D.G.Mann [M, S]
***Psammodiscus** nitidus* (W.Gregory) Round & D.G.Mann [N, M]
***Pseudostaurosira** perminuta* (Grunow) Sabbe & Wyverman [N]
***Pseudopodosira** westii* (W.Smith) Sheshukova-Poretskaya & Glezer [N]
***Pseudostaurosiropsis** geocollegarum* (Witkowski) E.A.Morales [M]
*Pseudostaurosiropsis punctiformis* (Witkowski, Metzeltin & Lange-Bertalot) Witkowski, Seddon & Pliński [M]
***Rhabdonema** adriaticum* Kützing*
*Rhabdonema arcuatum* (Lyngbye) Kützing [S]
*Rhabdonema minutum* Kützing [N, M]
***Rhaphoneis** amphiceros* (Ehrenberg) Ehrenberg [N, M]
*Rhaphoneis nitida* (Gregory) Grunow [N]
***Rhizosolenia** styliformis* T.Brightwell [N, M]
***Rhoicosigma** compactum* (Greville) Grunow [N]
***Rhoicosphenia** abbreviata* (C.Agardh) Lange-Bertalot [M, S]
*Rhoicosphenia linearis* Østrup [M]

**Tab. 1.** Continued

*Rhoicosphenia marina* (Kützing) M. Schmidt [M, S]
***Rhopalodia*** *acuminata* Krammer [M]
*Rhopalodia gibberula* (Ehrenberg) Müller [N]
*Rhopalodia musculus* (Kützing) Otto Müller*
*Rhopalodia sterrenburgii* Krammer [M]
***Sarcophagodes*** *mutabilis* E.A.Morales [S]
***Seminavis*** *barbarae* Witkowski, Lange-Bertalot & Metzeltin [M]
*Seminavis obtusiuscula* (Grunow) Danieledis & D.G.Mann [S]
***Shionodiscus*** *oestrupii* (Ostenfeld) A.J.Alverson, S.-H.Kang & E.C.Theriot [N]
***Skabitschewskia*** *borealis* (A.Cleve) Lange-Bertalot & Kulikovskiy [M]
***Stauroneis*** *plicata* C.Brockmann [M, S]
*Stauroneis smithii* Grunow [M]
*Stauroneis undata* Hustedt [S]
***Staurophora*** *amphioxys* (W.Gregory) D.G.Mann [N]
***Staurosirella*** *guenter-grassii* (Witkowski & Lange-Bertalot) E.A.Morales, C.E.Wetzel & Ector [M, S]
*Staurosirella martyi* (Héribaud) Morales & Manoylov [M, S]
***Stephanocyclus*** *meneghiniana* (Kützing) Kulikovskiy, Genkal & Kociolek [M]
***Stephanodiscus*** *hantzschii* Grunow [S]
***Stoermeria*** *americana* (Bailey) M.P.Ashworth & P.A.Sims [M]
***Striatella*** *unipunctata* (Lyngbye) C.Agardh*
***Surirella*** *baldjicki* Norman [N]
*Surirella brebissonii* Krammer & Lange-Bertalot [M]
*Surirella ceylanensis* var. *oblongistriata* F.Hustedt [M]
*Surirella comis* A. Schmidt [N]
*Surirella crumena* Brébisson ex Kützing [M]
*Surirella gemma* var. *reniformis* Proshkina-Lavrenko [N]
*Surirella fluminensis* Grunow [N]
*Surirella hybrida* Grunow [N, M]
*Surirella intercedens* Grunow [N]
*Surirella lata* W.Smith [M]
*Surirella minuta* Brébisson ex Kützing [N]
*Surirella ovalis* Brébisson [N]
*Surirella striatula* Turpin [M]
*Surirella venusta* Østrup [S]
***Synedra*** *brockmannii* Hustedt [N]
*Synedra commutata* Grunow [M]
*Synedra distinguenda* Hustedt [N]
*Synedra gaillonii* (Bory) Ehrenberg [M, S]
*Synedra laevis* Kützing [S]
*Synedra pulcherrima* Hantzsch [N]
***Synedrosphenia*** *baculus* (W.Gregory) Lobban & Ashworth [N]
*Synedrosphenia crystallina* (C.Agardh) Lobban & Ashworth*
*Synedrosphenia fulgens* (Greville) Lobban & Ashworth*
***Tabularia*** *fasciculata* (C.Agardh) D.M.Williams & Round [M, S]
*Tabularia gaillonii* (Bory) Bukhtiyarova [N]
*Tabularia investiens* (W.Smith) D.M.Williams & Round*
*Tabularia ktenoeides* M.Kuylenstierna [M, S]
*Tabularia tabulata* (C.Agardh) Snoeijs [N]
***Talaroneis*** *furcigera* (Grunow) Sterrenburg [N, M]
***Tetramphora*** *decussata* (Grunow) Stepanek & Kociolek*
*Tetramphora ostrearia* (Brébisson) Mereschkowsky [N, S]
*Tetramphora rhombica* (Kitton) Stepanek & Kociolek [N, M]
*Tetramphora securicula* (Peragallo & Peragallo) Stepanek & Kociolek [N, M]
*Tetramphora sulcata* (Brébisson) Stepanek & Kociolek*
***Thalassionema*** *frauenfeldii* (Grunow) Tempère & Peragallo*
*Thalassionema nitzschioides* (Grunow) Mereschkowsky*
***Thalassiophysa*** *hyalina* (Greville) Paddock & P.A.Sims [M]
***Thalassiosira*** *angulata* (Gregory) Hasle*
*Thalassiosira eccentrica* (Ehrenberg) Cleve*
*Thalassiosira gravida* Cleve [N, S]
*Thalassiosira hyalina* (Grunow) Gran [M]
*Thalassiosira leptopus* (Grunow) Hasle et Fryxell*
*Thalassiosira mediterranea* (Schröder) Hasle [N]
*Thalassiosira nordenskioeldii* Cleve [S]
*Thalassiosira subtilis* (Ostenfeld) Gran [N]
***Toxarium*** *hennedyanum* (Gregory) Pelletan*
*Toxarium undulatum* J.W.Bailey*
***Toxonidea*** *balearica* Grunow [N, S]
*Toxonidea insignis* Donkin [N]
***Trachyneis*** *aspera* (Ehrenberg) Cleve*
***Trachysphenia*** *australis* Petit [N]
***Triceratium*** *broeckii* Lauduger – Fortmorel [S]
*Triceratium favus* Ehrenberg [N, M]
*Triceratium finnmarchicum* Grunow [S]
*Triceratium pellucidum* (Castracane) Y.C.Guo, J.Ye & H.Zhou [N]
*Triceratium pelagicum* (J.L.B.Schröder) Sournia [N, M]
*Triceratium pentacrinus* Wallich [M, S]
Triceratium reticulum Ehrenberg [M, S]
***Trieres*** *mobiliensis* (J.W.Bailey) Ashworth & Theriot [M, S]
***Trigonium*** *arcticum* (Brightwell) Cleve [S]
*Trigonium formosum* (Brightwell) Cleve [M, S]
***Tropidoneis*** *maxima* (W.Gregory) Cleve [N]
*Tropidoneis longa* (Cleve) Cleve [N]
***Tryblionella*** *acuminata* W.Smith [N, M]
*Tryblionella acuta* (Cleve) D.G.Mann [N]
*Tryblionella apiculata* W.Gregory [N, M]
*Tryblionella* cf. *littoralis* (Grunow) D.G.Mann [N]
*Tryblionella coarctata* (Grunow) D.G.Mann [N, S]
*Tryblionella compressa* (Bailey) Poulin [M, S]
*Tryblionella debilis* Arnott ex O'Meara [N]
*Tryblionella didyma* (Hustedt) D.G.Mann [M, S]
*Tryblionella hantzschiana* Grunow*
*Tryblionella hungarica* (Grunow) Frenguelli [M, S]
*Tryblionella littoralis* (Grunow) D.G.Mann [M]
*Tryblionella marginulata* (Grunow) D.G.Mann [N, M]
*Tryblionella navicularis* (Brébisson) Ralfs*
*Tryblionella pararostrata* (Lange-Bertalot) Clavero & Hernández-Mariné [M]
*Tryblionella persuadens* (Cholnoki) K.P.Cavalcante. P.I.Tremarin & T.A.V.Ludwig [M, S]
*Tryblionella plana* (W.Smith) Pelletan [N]
*Tryblionella punctata* W.Smith [M, S]
*Tryblionella punctata* var. *elongata* Grunow [N]
*Tryblionella scalaris* (Ehrenberg) Siver & P.B.Hamilton [M]
***Ulnaria*** *longissima* (W.Smith) Van de Vijver & D.M.Williams [S]
*Ulnaria ulna* (Nitzsch) Compère [N, S]
***Undatella*** *lineata* (Greville) Paddock & P.A.Sims [M]
*Undatella quadrata* (Brébisson ex Kützing) Paddock & P.A.Sims [M]
***Vikingea*** *promunturi* (Giffen) Witkowski, Lange-Bertalot & Metzeltin [S]
***Zygoceros*** *rhombus* Ehrenberg [N]

Taxonomic classification was done according to Round et al. (1990). Previously, some species were given names that differed from those of the latest taxonomic order. An attempt was made to classify the species with updated current names according to Guiry and Guiry (2024), Kociolek et al. (2024) and the website Algaebase (http://www. algaebase.org/), which is constantly updated and therefore has a different taxonomic hierarchy than the one previously used.

## The current status of marine benthic diatoms in Adriatic

The present work represents the first detailed list of marine benthic diatoms in the Adriatic Sea. An overview of all 52 publications included in this review shows that diatoms from the Northern Adriatic were analysed in most studies (N=32), followed by Middle Adriatic (N=15) and Southern Adriatic (N=12). Only two studies analysed diatoms on a larger spatial scale covering all regions of the Adriatic (On-line Suppl. Tab. 1). The literature was unevenly distributed, with 30 papers for the Italian coast and 18 for the Croatian coast. The coast of Bosnia and Herzegovina, on the other hand, has been the subject of 2 studies, while Slovenia and Albania are represented by 1 study each (On-line Suppl. Tab. 1). The number of publications on diatom research fluctuated, with the most productive period being that of 2000-2024, during which 47 papers were published (On-line Suppl. Tab. 1). In certain periods, research activity has increased considerably in particular areas, such as the recent intensive studies of diatoms in the Southern Adriatic. Since the beginning of diatom studies mostly in Northern Adriatic, several authors have made significant contributions to the composition of diatoms over the years.

In this paper, we merge the data with the most recent nomenclature, focusing on the current names of the cited taxa (On-line Suppl Tab. 2). A list of updated species nomen-

**Fig. 1.** A) Major seas of the Mediterranean, B) Locations of the studies of diatom taxa from the Adriatic Sea presented in Suppl. Tab. 1.

clature (On-line Suppl Tab. 2) cited in the previous studies (Cited Name) was given (Tab. 1) and the species/genus names were checked for validity using AlgaeBase (Guiry and Guiry, 2024).

Marine diatoms were represented by 822 species (163 genera and 70 families) (Tab. 1). The number of pennate species represented was 719. Families with the highest number of genera were: Naviculaceae (8), Bacillariaceae (7), Surirellaceae (6), Fragilariaceae (6). Additionally, the highest number of species and infraspecific taxa belonged to the Naviculaceae (95) and followed by Bacillariaceae (88). The taxonomic composition of benthic diatoms in the Adriatic may be defined as the community characterized by genera such as; *Mastogloia* (65 taxa), *Navicula* (62), *Nitzschia* (53), *Amphora* (52), *Diploneis* (43), *Cocconeis* (37), *Halamphora* (24), *Achnanthes* (22), *Licmophora* (22), *Tryblionella* (19), *Fallacia* (15), *Surirella* (14), *Grammatophora* (13), *Lyrella* (12).

Altogether, 381 taxa were found in the Northern, 524 taxa in the Middle and 373 taxa in the Southern Adriatic Sea. Among the total 822 diatom taxa, 177 taxa were found exclusively in the Northern Adriatic, 195 were found exclusively in the Middle and 94 occurred only in Southern Adriatic (Tab. 1). The studies used in this checklist have shown that many species were documented as first records in the Adriatic.

The variation in findings of benthic diatoms across different geographical areas may also be attributed to differing research methodologies. In the Adriatic Sea, benthic diatom analyses are often conducted within the framework of other research studies, such as phytoplankton investigations. Additionally, studies of benthic diatoms make use of various substrates, both natural and artificial.

However, the number of diatom species on the Adriatic coasts is similar to the checklist of marine diatoms from Turkish coastal waters (Kaleli and Akçaalan 2021), and the reason for the low number of marine diatoms (767 taxa belonging to 183 genera, of which 567 taxa belonged to Bacillariophyceae in Türkiye) may be attributed to the restricted scope of benthic studies along these coastlines, as highlighted by Kaleli and Akçaalan (2021). However, recent findings (Kaleli et al. 2020) suggest a potential increase in the number of taxa in forthcoming studies for both the Turkish coastal waters and the Adriatic Sea, particularly with those specifically geared towards the detection of benthic species.

This paper revealed the benthic diatom composition in the Adriatic coastal waters with the latest nomenclature and could be used as a fundamental list for later studies.

## Conclusion

The study of marine diatoms on the coasts of the Adriatic Sea began more than 100 years ago, and in the last 35 years, extensive studies have been carried out. However, many geographical areas have not yet been studied and their diatom composition remains unknown. Therefore, this checklist aims to give an overview of the benthic diatoms that have been studied in the last 35 years and to create a database with updated nomenclature to fill the gap regarding marine benthic diatoms along the Adriatic coasts.

So far, however, there has been very little information on benthic diatoms in the Adriatic Sea. The knowledge of benthic diatom communities in the Adriatic Sea is poor compared to longer-term studies on phytoplankton. In this work, studies on marine plankton and benthos were combined to create a dataset that could be comparable for future studies in the Adriatic and other regions. Although checklists existed for marine plankton, this study provided a list of diatoms that included both forms from the Adriatic coasts. The inclusion of data on benthic taxa improves the knowledge of overall microalgal diversity and of coastal and transitional ecosystem ecology. The list included many benthic diatoms as well as the planktonic forms mentioned above but showed that not all the taxa on the Adriatic coasts are fully covered. Future studies should focus on the composition of benthic diatoms using LM and EM to provide additional data for further implications. Morphological details of benthic diatoms would not only increase the knowledge of taxonomy but also of species diversity and geographical distribution. However, as many of the species were not illustrated, future studies may increase the knowledge of accurate identification based on illustrated studies in the region. It should be noted that there have been no changes in the taxonomy of genera and species. The nomenclature has been updated for several taxa that have been transferred or are currently no longer in use. Therefore, further studies should be carried out to clarify the problems of diatom systematics in the Adriatic coastal waters.

This is the first exhaustive checklist of benthic diatoms of the Adriatic Sea, including the latest papers, updated with very recent books and websites, and can be used as a valuable tool for further studies. However, it should be noted that the checklist should be updated over time. Further studies on the structure of the diatom flora would provide additional information that would lead to a better ecological understanding and an approach to coastal ecosystem monitoring to prevent damage to marine biodiversity, especially for the disturbed Adriatic coasts. Nevertheless, knowledge of the composition and spatial distribution of marine benthic diatoms along the coast of the Adriatic coast remains limited.

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and kind person who was extremely active internationally and whose network spanned numerous countries on most continents.

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## References

Accoroni, S., Romagnoli, T., Pichierri, S., Totti, C., 2016: Effects of the bloom of harmful benthic dinoflagellate *Ostreopsis* cf. *ovata* on the microphytobenthos community in the northern Adriatic Sea. Harmful Algae 55, 179–190. https://doi.org/10.1016/j.hal.2016.03.003

Akbulut, A., Yıldız, K., 2002: The planktonic diatoms of Lake Çıldır (Ardahan-Turkey). Turkish Journal of Botany 26(2), 55–75. https://journals.tubitak.gov.tr/botany/vol26/iss2/1

Aleem, A. A., 1950: Some new records of marine algae from the Mediterranean Sea. With reference to their geographical distribution. Acta Horti Gothoburgensis 18, 276–288.

Altuğ, G., Aktan, Y., Oral, M., Topaloğlu, B., Dede, A., Keskin, Ç., İşinibilir, M., Çardak, M., Çiftçi, P. S., 2011: Biodiversity of the northern Aegean Sea and southern part of the Sea of Marmara, Turkey. Marine Biodiversity Records 4, E65. https://doi.org/10.1017/S1755267211000662

Alverson, A. J., 2008: Molecular systematics and the diatom apecies. Protist 159, 339–353. https://doi.org/10.1016/j.protis.2008.04.001

Aragão, L., Mentaschi, L., Pinardi, N., Verri, G., Senatore, A., Di Sabatino, S., 2024: The freshwater discharge into the Adriatic Sea revisited. Frontiers in Climate 6: 1368456. https://doi.org/10.3389/fclim.2024.1368456

Aslan, H., Gönülal, O., Can-Yılmaz, E., Çamur Elipek, B., Baytut, Ö., Tosunoğlu, M., Karabacak, E. Kurt, Y., 2018: Species diversity in lentic, lotic, marine and terrestrial biotopes of Gokceada Salt Lake wetland (Canakkale, Turkiye). Fresenius Environmental Bulletin 27(5), 2853–2866.

Baldassarre, L., Natali, V., De Pascale, F., Vezzi, A., Banchi, E., Bazzaro, M., Relitti, F., Tagliapietra, D., Cibic, T., 2023: The Impact of MOSE (Experimental Electromechanical Module) flood barriers on microphytobenthic community of the Venice Lagoon. Microorganisms 11(4), 936. https://doi.org/10.3390/microorganisms11040936

Barbiero, R. P., 2000: A multi–lake comparison of epilithic diatom communities on natural and artificial substrates. Hydrobiologia 438, 157–170. https://doi.org/10.1023/A:1004182231973

Barinova, S., Bondarenko, A., Ryabushko, L., Kapranov, S., 2019: Microphytobenthos as an indicator of water quality and organic pollution in the western coastal zone of the Sea of Azov. Oceanological and Hydrobiological Studies 48(2), 125–139. https://doi.org/10.1515/ohs-2019-0013

Barranguet, C., 1997: The role of microphytobenthic primary production in a Mediterranean mussel culture area. Estuarine, Coastal and Shelf Science 44(6), 753–765.

Barranguet, C., Plante-Cuny, M. R., Alivon, E., 1996: Microphytobenthos production in the Gulf of Fos, French Mediterranean coast. Hydrobiologia 333, 181–193. https://doi.org/10.1007/BF00013432

Bartole, L., Welker, C., Dellavalle, G., Bressan, G., 1991–1994: Primi stadi di colonizzazione "a diatomee" di un substrato duro artificiale in due stazioni fisse del Golfo di Trieste (nord Adriatico). Nova Thalassia 12, 163–189.

Batistić, M., Garić, R., Molinero, J. C., 2014: Interannual variations in Adriatic Sea zooplankton mirror shifts in circulation regimes in the Ionian Sea. Climate Research 61(3), 231–240. https://doi.org/10.3354/cr01248

B-Béres, V., Stenger-Kovács, C., Buczkó, K., Padisák, J., Selmeczy, G. B., Lengyel, E., Tapolczai, K. 2023: Ecosystem services provided by freshwater and marine diatoms. Hydrobiologia 850, 2707–2733. https://doi.org/10.1007/s10750-022-04984-9

Belegratis, M. R., 2002: Studies on periphytic algae in marine sites of Evoikos Gulf. PhD Thesis. University of Athens, Greece.

Belegratis, M. R., Economou-Amilli, A., 2002: Seasonal and vertical distribution of marine epiphytic algae in the Evoikos Gulf (Aegean Sea, Greece). Algological Studies 107, 131–151. https://doi.org/10.1127/algol_stud/107/2002/131

Blanco, I. A., Blanco, S., 2014: Benthic diatoms from Mediterranean coasts. Bibliotheca Diatomologica 60, 1–409.

Box, A., Sureda, A., Tauler, P., Terrados, J., Marbà, N., Pons, A., Deudero, S., 2010: Seasonality of caulerpenyne content in native *Caulerpa prolifera* and invasive *C. taxifolia* and *C. racemosa* var. *cylindracea* in the western Mediterranean Sea. Botanica Marina 53(4), 367–375. https://doi.org/10.1515/BOT.2010.034

Box, A., Sureda, A., Terrados, J., Pons, A., Deudero, S., 2008: Antioxidant response and caulerpenyne production of the alien *Caulerpa taxifolia* (Vahl) epiphytized by the invasive algae *Lophocladia lallemandii* (Montagne). Journal of Experimental Marine Biology and Ecology 364, 24–28. https://doi.org/10.1016/j.jembe.2008.06.029

Burić, Z., Caput, K., Viličić, D., 2004: Distribution of the diatom *Cocconeis scutellum* in the karstic estuary (Zrmanja, eastern Adriatic Sea). Biologia, Bratislava 59(1), 1–7.

Caput Mihalić, K., Viličić, D., Ahel, M., Burić, Z., Carić, M., 2008: Periphytic algae development in the upper reach of the Zrmanja estuary (eastern Adriatic coast). Vie et Milieu 58(3-4), 203–213.

Car, A., Hafner, D., Dupčić Radić, I., Kaleli, A., Ljubimir, S., Solak, C. N., 2021: Comparison of benthic diatom community structures on natural and artificial substrates in marine lake (Adriatic Sea). Acta Adriatica: international journal of Marine Sciences 62(1), 21–44. https://doi.org/10.32582/aa.62.1.1

Car, A., Hafner, D., Ljubimir, S., Dupčić Radić, I., Bobanović-Ćolić, S., Jasprica, N., 2020: Colonization of bacteria and diatoms on an artificial substrate in a marine lake (eastern Adriatic Sea, NE Mediterranean). Acta Botanica Croatica 79(2), 212–227. https://doi.org/10.37427/botcro-2020-028

Car, A., Witkowski, A., Dobosz, S., Burfeind, D.D., Meinesz, A., Jasprica, N., Ruppel, M., Kurzydłowski, K. J., Płociński, T., 2012: Description of a new marine diatom, *Cocconeis caulerpacola* sp. nov. (Bacillariophyceae), epiphytic on invasive *Caulerpa* species. European Journal of Phycology 47(4), 433–448. https://doi.org/10.1080/09670262.2012.735255

Car, A., Witkowski, A., Dobosz, S., Jasprica, N., Ljubimir, S., 2019a: Epiphytic diatom assemblages on invasive algae *Caulerpa taxifolia* and autochthonous *Halimeda tuna* and *Padina* sp. from the Adriatic Sea – summer/autumn aspect. Oceanological and Hydrobiological Studies 48(3), 209–226. https://doi.org/10.2478/ohs-2019-0019

Car, A., Witkowski, A., Jasprica, N., Ljubimir, S., Čalić, M., Dobosz, S., Dupčić Radić, I., Hrustić, E., 2019b: Epilithic diatom communities from areas of invasive *Caulerpa* species (*Caul-*

*erpa taxifolia* and *Caulerpa cylindracea*) in the Adriatic Sea, NE Mediterranean. Mediterranean Marine Science 20(1), 151–173. https://doi.org/10.12681/mms.14330

Caraus, I., 2017: Algae of Romania. A distributional checklist of actual algae. Version 2.4. Studii si Cercetari Biologie 7, 1–1002.

Carreira-Flores, D., Neto, R., Ferreira, H., Cabecinha, E., Díaz-Agras, G., Gomes, P. T., 2020: Artificial substrates as sampling devices for marine epibenthic fauna: A quest for standardization. Regional Studies in Marine Science 37, 1–11. https://doi.org/10.1016/j.rsma.2020.101331

Cibic, T., Baldassarre, L., Cerino, F., Comici, C., Fornasaro, D., Kralj, M., Giani, M., 2022: Benthic and pelagic contributions to primary production: experimental insights from the Gulf of Trieste (Northern Adriatic Sea). Frontiers in Marine Science 9, 877935. https://doi.org/10.3389/fmars.2022.877935

Cibic, T., Blasutto, O., 2011: Living marine benthic diatoms as indicators of nutrient enrichment: a case study in the Gulf of Trieste. In: Compton, J. C. (ed.), Diatoms: ecology and life cycle, 169–184. Nova Science Publishers, Hauppauge, New York.

Cibic, T., Blasutto, O., Falconi, C., Fonda Umani, S., 2007a: Microphytobenthic biomass, species composition and nutrient availability in sublittoral sediments of the Gulf of Trieste (northern Adriatic Sea). Estuarine, Coastal and Shelf Science 75(1-2), 50–62. https://doi.org/10.1016/j.ecss.2007.01.020

Cibic, T., Blasutto, O., Fonda Umani, S., 2007b: Biodiversity of settled material in a sediment trap in the Gulf of Trieste (northern Adriatic Sea). Hydrobiologia 580, 57–75. https://doi.org/10.1007/s10750-006-0465-9

Cibic, T., Comici, C., Bussani, A., Del Negro, P., 2012: Benthic diatom response to changing environmental conditions. Estuarine, Coastal and Shelf Science 115, 158–169. https://doi.org/10.1016/j.ecss.2012.03.033

Cibic, T., Facca, C., 2010: Microphytobenthos. In: Relini, G. (ed.), Checklist della flora e della fauna dei mari italiani. Parte II. Biologia Marina Mediterranea 17 (suppl. 1), 754–800.

Cibic, T., Fazi, S., Nasi, F., Pin, L., Alvisi, F., Berto, D., Viganò, L., Zoppini, A., Del Negro, P., 2019: Natural and anthropogenic disturbances shape benthic phototrophic and heterotrophic microbial communities in the Po River Delta system. Estuarine, Coastal and Shelf Science 222, 168–182. https://doi.org/10.1016/j.ecss.2019.04.009

Civitarese, G., Gačić, M., Batistić, M., Bensi, M., Cardin, V., Dulčić, J., Garić, R., Menna, M., 2023: The BiOS mechanism: History, theory, implications. Progress in Oceanography 216, 103056. https://doi.org/10.1016/j.pocean.2023.103056

Civitarese, G., Gačić, M., Lipizer, M., Eusebi Borzelli, G. L., 2010: On the impact of the Bimodal Oscillating System (BiOS) on the biogeochemistry and biology of the Adriatic and Ionian Seas (Eastern Mediterranean). Biogeosciences 7, 3987–3997. https://doi.org/10.5194/bg-7-3987-2010

Cleve, P.T., Grunow, A. 1880: Beiträge zur kenntniss der arctischen diatomeen. Kongliga Svenska vetenskaps-Akademiens Handlingar 17(2), 1–121.

Çelekli, A., Külköylüoğlu, O., 2006: Net planktonic piatom (Bacillariophyceae) composition of Lake Abant (Bolu). Turkish Journal of Botany 30(5), 331–347. https://journals.tubitak.gov.tr/botany/vol30/iss5/2

Çolak Sabancı, F., 2010: Contributions to the knowledge of algal flora of Homa Lagoon (Aegean Sea, Turkey). Journal of Black Sea / Mediterranean Environment 16(3), 311–327. Retrieved October 16, 2023 from https://dergipark.org.tr/en/pub/jbme/issue/9843/121871

Çolak Sabancı, F., 2011: Relationship of epilithic diatom communities to environmental variables in Homa lagoon (Izmir, Turkey). Aquatic Biology 13(3), 233–241. https://doi.org/10.3354/ab00367

Çolak Sabancı, F., 2012a: An illustrated survey on the morphological characters in three species of the diatom genus *Mastogloia* (Bacillariophyceae). Turkish Journal of Botany 36(6), 727–737. https://doi.org/10.3906/bot-1111-18

Çolak Sabancı, F., 2012b: Taxonomic survey of benthic diatoms on natural substrata from coastal lagoon (Aegean Sea, Turkey). Turkish Journal of Fisheries and Aquatic Sciences 12(4), 841–849. https://doi.org/10.4194/1303-2712-v12_4_12

Çolak Sabancı, F., 2013: Species of *Mastogloia* (Bacillariophyceae) - new for the Aegean coast of Turkey. Mediterranean Marine Science 14(1), 129–140. https://doi.org/10.12681/mms.331

Çolak Sabancı, F., Koray, T., 2010: Four new records for the benthic diatoms (genera *Cocconeis*, *Seminavis*, *Synedra*, and *Trachysphenia*) from the Aegean Sea. Turkish Journal of Botany 34(6), 531–540. https://doi.org/10.3906/bot-0912-285

Cox, E. J., 2014: Diatom identification in the face of changing species concepts and evidence of phenotypic plasticity. Journal of Micropalaeontology 33, 111–120, https://doi.org/10.1144/jmpaleo2014-014

Cozzi, S., Giani, M., 2011: River water and nutrient discharges in the Northern Adriatic Sea: Current importance and long term changes. Continental Shelf Research 31(18), 1881–1893. https://doi.org/10.1016/j.csr.2011.08.010

Delgado, M., 1989: Abundance and distribution of microphytobenthos in the bays of Ebro Delta (Spain). Estuarine, Coastal and Shelf Science 29(2), 183–194. https://doi.org/10.1016/0272-7714(89)90007-3

Delgado, M., De Jonge, V. N., Peletier, H., 1991: Effect of sand movement on the growth of benthic diatoms, Journal of Experimental Marine Biology and Ecology 145(2), 221–231. https://doi.org/10.1016/0022-0981(91)90177-X

De Stefano, M., Marino. D., 2003: Morphology and taxonomy of *Amphicocconeis* gen. nov. (Achnanthales, Bacillariophyceae, Bacillariophyta) with considerations on its relationship to other monoraphid diatom genera. European Journal of Phycology 38(4), 361–370. https://doi.org/10.1080/09670260310001612646

De Stefano, M., Marino, D., Mazzella, L., 2000: Marine taxa of *Cocconeis* on leaves of *Posidonia oceanica*, including a new species and two new varieties. European Journal of Phycology 35(3), 225–242. https://doi.org/10.1080/09670260010001735831

De Stefano, M., Romero, O. E., Totti, C., 2008: Ultrastructure and taxonomy of *Cocconeis scutellum* and its varieties. Botanica Marina 51(6), 506–536. https://doi.org/10.1515/BOT.2008.058

De Stefano, M., Sacchi, U., Totti, C., Romero, O. E., 2006: *Cocconeis distans* Gregory and *Amphicocconeis debesi* (Hustedt) De Stefano comb. nov. (Bacillariophyta), an intricate taxonomical history. Botanica Marina 49(5-6), 438–449. https://doi.org/10.1515/BOT.2006.055

Economou-Amilli, A., 1980: Marine diatoms from Greece. I. Diatoms from the Saronikos Gulf. Nova Hedwigia 32(1), 63–104.

Elmacı, A., Obalı, O., 1998: Aksehir gölü kıyı bölgesi alg florası (The algal flora of the littoral region in Lake Akşehir). Turkish Journal of Biology 22(1), 81–98.

Facca, C., Sfriso, A., 2007: Epipelic diatom spatial and temporal distribution and relationship with the main environmental parameters in coastal waters. Estuarine, Coastal and Shelf Science 75(1-2), 35–49. https://doi.org/10.1016/j.ecss.2007.03.033

Facca, C., Sfriso, A., Ghetti, P. F., 2004: Abbondanza e diversità del fitoplancton e delle diatomee bentoniche in laguna di Venezia. Biologia Ambientale 18(2), 19–24. Retrieved October

16, 2023 from http://www.cisba.eu/images/rivista/biologia_ambientale/Ba2004-2/Ba_2004-2_Facca_Diatomee_VE.pdf

Facca, C., Sfriso, A., Socal, G., 2002a: Temporal and spatial distribution of diatoms in the surface sediments of the Venice Lagoon. Botanica Marina 45(2), 170–183. https://doi.org/10.1515/BOT.2002.016

Facca, C., Sfriso, A., Socal, G., 2002b: Changes in abundance and composition of phytoplankton and microphytobenthos due to increased sediment fluxes in the Venice Lagoon, Italy. Estuarine, Coastal and Shelf Science 54(5), 773–792. https://doi.org/10.1006/ecss.2001.0848

Facca, C., Sfriso, A., Socal, G., 2003: Diatomee planctoniche e bentoniche: abbondanza e diversità nella laguna di Venezia. Biologia Marina Mediterranea 10(2), 1006–1009.

Fain, A. M. V., Ogston, A. S., Sternberg, R. W., 2007: Sediment transport event analysis on the western Adriatic continental shelf. Continental Shelf Research 27(3-4), 431–451.

Falkowski, P. G., Schofield, O., Katz, M. E., Van De Schootbrugge, B., Knoll, A. H., 2004: Why is the land green and the ocean red? In: Thierstein, H. R. and Young, J. R. (eds.), Coccolithophores: from molecular processes to global impact, 429–453. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-662-06278-4_16

Foged, N., 1985a: Diatoms in Samos, a Greek island in the Aegean. Bibliotheca Diatomologica 10, 1-119.

Foged, N., 1985b: Diatoms in Kos and Kalymnos, two Greek islands in the Aegean. Bibliotheca Diatomologica 10, 1–105.

Franzo, A., Cibic, T., Del Negro, P., De Vittor, C., 2015: Spatial distribution of microphytobenthos, meiofauna and macrofauna in the north-western Adriatic Sea: a synoptic study. Advances in Oceanography and Limnology 6(1-2), 58–75. https://doi.org/10.4081/aiol.2015.5470

Franzo, A., Cibic, T., Del Negro, P., Solidoro, C., 2014: Microphytobenthic response to mussel farm biodeposition in coastal sediments of the northern Adriatic Sea. Marine Pollution Bulletin 79(1-2), 379–388. https://doi.org/10.1016/j.marpolbul.2013.11.002

Gačić, M., Kovačević, V., Manca, B., Papageorgiou, E., Poulain, P.-M., Scarazzato, P., Vetrano, A., 1996: Thermohaline properties and circulation in the Otranto Strait. Bulletin de l'Institut oceanographique 17, 117–145.

Gambi, C., Totti, C., Manini, E., 2003: Impact of organic loads and environmental gradients on microphytobenthos and meiofaunal distribution in a coastal lagoon. Chemistry and Ecology 19(2-3), 207–223. https://doi.org/10.1080/0275754031000119951

Gligora Udovič, M., Ljubešić, Z., 2021: The Croatian National Diatom Collection. Acta Botanica Croatica, 80(1). Retrieved October 7, 2024 from https://www.abc.botanic.hr/index.php/abc/article/view/3184

Grunow, A., 1860: Ueber neue oder ungenügend gekannte Algen. Erste folge, diatomeen, familie Naviculaceen. Verhandlungen der Kaiserlich-Königlichen Zoologisch-Botanischen Gesellschaft in Wien 10, 503–582.

Guiry, M. D., Guiry, G. M., 2024: AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. Retrieved October 18, 2024 from http://www.algaebase.org

Hafner, D., Jasprica, N., Car, A., 2018a: Taxonomic survey of benthic diatoms in Neum Bay, southeastern Adriatic. Natura Croatica 27(1), 1–26. https://doi.org/10.20302/NC.2018.27.1

Hafner, D., Car, A., Jasprica, N., Kapetanović, T., Dupčić Radić, I., 2018b: Relationship between marine epilithic diatoms and environmental variables in oligotrophic bay, NE Mediterranean. Mediterranean Marine Science 19(2), 223–239. https://doi.org/10.12681/mms.14151

Hendey, N. I., 1974: A revised check-list of British marine diatoms. Journal of the Marine Biological Association of the United Kingdom 54, 277–300.

Hoagland, K. D., Zlotsky, A., Peterson, C. G., 1986: Chapter 2 The source of algal colonizers on rock substrates in a freshwater impoundment. In: Evans, L. V., Hoagland, K. D. (eds.), Algal biofouling, 21–39. Elsevier, Amsterdam, https://doi.org/10.1016/S0166-1116(08)72168-X

Jahn, R., Zetzsche, H., Reinhardt, R., Gemeinholzer, B., 2007: Diatoms and DNA barcoding: a pilot study on an environmental sample. In: Kusber W. H., Jahn R. (eds.), Proceedings of the 1st Central European Diatom Meeting 2007. Berlin, 63–68. Botanic Garden and Botanical Museum Berlin-Dahlem, Freie Universität Berlin, Berlin.

Kaddeche, H., Noune, F., Dzizi, S., Chaib, N., Boudjellab, Z. E., Blanco, S., 2022: Determinant factors of diatom assemblage's distribution along the coastal Central Constantine (Northeastern Algeria). Aquatic Ecology 56, 1245–1269. https://doi.org/10.1007/s10452-022-09980-8

Kaleli, A., 2019: Benthic diatom composition of Iztuzu coastal lake, Dalyan (Aegean Sea, Turkey). Aquatic Sciences and Engineering 34(4), 122–130. https://doi.org/10.26650/ASE2019575987

Kaleli, A., 2022: Contributions to the diatom flora of the Aegean Sea and the Mediterranean Sea from the turkish coasts with remarks on rare taxa. Mediterranean Marine Science 23(4), 952–967. https://doi.org/10.12681/mms.28075

Kaleli, A., Akçaalan, R., 2021: Checklist of marine diatoms from Turkish coastal waters with updated nomenclature. Aquatic Research 4(1), 88–115. https://doi.org/10.3153/AR21008

Kaleli, A., Car, A., Witkowski, A., Krzywda, M., Riaux-Gobin, C., Solak, C. N., Kaska, Y., Zgłobicka, I., Płociński, T., Wróbel, R., Kurzydłowski, K., 2020: Biodiversity of carapace epibiont diatoms in lggerhead sea turtles (*Caretta caretta* Linnaeus 1758) in the Aegean Sea Turkish coast. PeerJ 8: e9406. https://doi.org/10.7717/peerj.9406

Kanjer, L., Mucko, M., Car, A., Bosak, S., 2019: Epiphytic diatoms on *Posidonia oceanica* (l.) Delile leaves from eastern Adriatic Sea. Natura Croatica 28(1), 1–20. https://doi.org/10.20302/NC.2019.28.1

Kociolek, J. P., Balasubramanian, K., Blanco, S., Coste, M., Ector, L., Liu, Y., Kulikovskiy, M., Lundholm, N., Ludwig, T., Potapova, M., Rimet, F., Sabbe, K., Sala, S., Sar, E., Taylor, J., Van De Vijver, B., Wetzel, C. E., Williams, D. M., Witkowski, A., Witkowski, J., 2024, DiatomBase. Retrieved February 23, 2024 from http://www.diatombase.org

Konucu, M., Develi, E. E., 2021: New records of pennate diatom species from the Mediterranean Sea. Mediterranean Fisheries and Aquaculture Research 4(2), 43–51. Retrieved October 16, 2023 from https://dergipark.org.tr/en/pub/medfar/issue/62622/868097

Koray, T., 2001: Türkiye denizleri fitoplankton türleri kontrol listesi (A check-list for phytoplankton of Turkish seas). Ege Journal of Fisheries and Aquatic Sciences 18(1-2), 1–23. Retrieved October 16, 2023 from https://dergipark.org.tr/en/pub/medfar/issue/62622/868097

Kützing, F. T., 1844: Die Kieselschaligen. Bacillarien oder Diatomeen. Nordhausen, Germany.

Kützing, F. T. 1845: Phycologia germanica, d.i. Deutschlands algen in bündigen beschreibungen. Nebst einer anleitung zum untersuchen und bestimmen dieser gewächse für anfäger. Nordhausen, Germany.

Lamberti, G. A., Resh, V. H., 1985: Distribution of benthic algae and macroinvertebrates along a thermal stream gradient. Hydrobiologia. 128, 13–21. https://doi.org/10.1007/BF00008935

Lane, C. M., Taffs, K. H., Corfield, J. L., 2003: A comparison of diatom community structure on natural and artificial substrates. Hydrobiologia 493, 65–79. https://doi.org/10.1023/A:1025498732371

Levkov, Z., Caput Mihalić, K., Ector, L., 2010: A taxonomical study of *Rhoicosphenia* Grunow (Bacillariophyceae) with a key for identification of selected taxa. Fottea 10(2), 145–200. https://doi.org/10.5507/fot.2010.010

Li, C., Gastineau, R., Turmel, M., Witkowski, A., Otis, C., Car, A., Lemieux, C., 2019: Complete chloroplast genome of the tiny marine diatom *Nanofrustulum shiloi* (Bacillariophyta) from the Adriatic Sea. Mitochondrial DNA Part B: Resources 4(2), 3374–3376. https://doi.org/10.1080/23802359.2019.1673245

Li, C. L., Witkowski, A., Ashworth, M. P., Dąbek, P., Sato, S., Zgłobicka, I., Witak, M., Khim, J. S., Kwon, C. J., 2018: The morphology and molecular phylogenetics of some marine diatom taxa within the Fragilariaceae, including twenty undescribed species and their relationship to *Nanofrustulum*, *Opephora* and *Pseudostaurosira*. Phytotaxa 355(1), 1–104. https://doi.org/10.11646/phytotaxa.355.1.1

Lobarzewski [Lobarzewsky], H. R. von, 1840: Einige neue diatomaceen Agardh et auct. der ostküste des Adriatischen meeres. Linnaea 14: 265–277.

Lobban, C. S., Ashworth, M. P., Car, A., Herwig, W., Ulanova, A., 2015: *Licmosphenia* revisited: transfer to *Licmophora*, redescription of *L. clevei* Mereschkowsky and descriptions of three new species. Diatom research 30(3), 227–236. https://doi.org/10.1080/0269249X.2015.1045037

Loir, M., 2010–2014. Marine benthic diatoms. Retrieved August 16, 2023 from http://www.diatomloir.eu/Site Diatom/Communities.html.

Louvrou, I., 2007: Periphyton and its colonization in marine hydrothermal regions of island Milos (Greece). PhD, University of Athens, Greece.

Louvrou, I, Danielidis, D. B., Economou-Amilli, A., 2006. Taxonomic status of *Detonia* Frenguelli and the establishment of *Detonia dobrinae* sp. nov. (Bacillariophyceae). In: Ognjanova Rumenova, N., Manoylov, K. (eds.), Advances in Phycological Studies: Festschrift in honour of Prof. Dobrina Temniskova-Topalova. 123–131. Pensoft Publisher, St. Kliment Ohridski University Press, Sofia, Bulgaria.

Louvrou, I., Danielidis, D. B., Economou-Amilli, A., 2012: *Meloneis* gen. nov., a new epipsammic genus of Rhaphoneidaceae (Bacillariophyceae). PLoS ONE 7(3), e32198. https://doi.org/10.1371/journal.pone.0032198

Ljubimir, S., Jasprica, N., Čalić, M., Hrustić, E., Dupčić Radić, I., Car, A., Batistić, M., 2017: Interannual (2009–2013) variability of winter-spring phytoplankton in the open South Adriatic Sea: effects of deep convection and lateral advection. Continental Shelf Research 143, 311–321. https://doi.org/10.1016/j.csr.2017.05.007

MacIntyre, H. L., Geider, R. J., Miller, D. C., 1996: Microphytobenthos: The ecological role of the "secret garden" of unvegetated, shallow-water marine habitats. I. Distribution, abundance and primary production. Estuaries 19, 186–201. https://doi.org/10.2307/1352224

Majewska, R., D'alelio, D., De Stefano, M., 2014: *Cocconeis* Ehrenberg (Bacillariophyta), a genus dominating diatom communities associated with *Posidonia oceanica* Delile (monocotyledons) in the Mediterranean Sea. Aquatic Botany 112, 48–56. https://doi.org/10.1016/j.aquabot.2013.07.008

Matek, A., Ljubešić, Z., 2024: Primary production research in the Adriatic Sea - a review. Acta Adriatica 65(2). https://doi.org/10.32582/aa.65.2.1

Mazzella, L., Buia, M. C., Spinoccia, L., 1994: Biodiversity of epiphytic diatom community on leaves of *Posidonia oceanica*. In: Marino, D., Montresor, M. (eds.), Proceedings of the 13th International Diatom Symposium, 241–251. Biopress, Bristol.

Medlin, L. K., 2018: Mini review: diatom species as seen through a molecular window. Brazilian Journal of Botany 41, 457–469. https://doi.org/10.1007/s40415-018-0444-1

Medlin, L. K., Kaczmarska, I., 2004: Evolution of the diatoms: V. Morphological and cytological support for the major clades and a taxonomic revision. Phycologia 43(3), 245–270. https://doi.org/10.2216/i0031-8884-43-3-245.1

Mejdandžić, M., Ivanković, T., Pfannkuchen, M., Godrijan, J., Marić Pfannkuchen, D., Hrenović, J., Ljubešić, Z., 2015: Colonization of diatoms and bacteria on artificial substrates in the northeastern coastal Adriatic Sea. Acta Botanica Croatica 74(2), 407–422. https://doi.org/10.1515/botcro-2015-0030

Menna, M., Gačić, M., Martellucci, R., Notarstefano, G., Fedele, G., Mauri, E., Gerin, R., Poulain, P.-M., 2022: Climatic, decadal, and interannual variability in the upper layer of the Mediterranean Sea using remotely sensed and in-situ data. Remote Sensing. 14, 1322. https://doi.org/10.3390/rs14061322

Miho, A., Witkowski, A., 2005: Diatom (Bacillariophyta) flora of Albanian coastal wetlands taxonomy and ecology: A review. Proceedings of the California Academy of Sciences 56(12), 129–145.

Morales, E. A., Wetzel, C. E., Novais, M. H., Buczkó, K., Morais, M. M., Ector, L., 2019: Morphological reconsideration of the araphid genus *Pseudostaurosira* (Bacillariophyceae), a revision of *Gedaniella*, *Popovskayella* and *Serratifera*, and a description of a new*Nanofrustulum* species. Plant Ecology and Evolution 152(2), 262–284. https://doi.org/10.5091/plecevo.2019.1604

Munda, I. M., 2005: Seasonal fouling by diatoms on artificial substrata at different depths near Piran (Gulf of Trieste, Northern Adriatic). Acta Adriatica 46(2), 137–157. https://hrcak.srce.hr/86

Myers, N., Mittermeier, R. A., Mittermeier, C. G., Da Fonseca, G. A., Kent, J., 2000: Biodiversity hotspots for conservation priorities. Nature 403(6772), 853–858. https://doi.org/10.1038/35002501

Natali, V., Malfatti, F., Cibic, T., 2023: Ecological effect of differently treated wooden materials on microalgal biofilm formation in the Grado Lagoon (Northern Adriatic Sea). Microorganisms 11, 2196. https://doi.org/10.3390/microorganisms11092196

Nenadović, T., Šarčević, T., Čižmek, H., Godrijan, J., Marić Pfannkuchen, D., Pfannkuchen, M., Ljubešić, Z., 2015: Development of periphytic diatoms on different artificial substrates in the Eastern Adriatic Sea. Acta Botanica Croatica 74(2), 377–392. https://doi.org/10.1515/botcro-2015-0026

Neri, F., Ubaldi, M., Accoroni, S., Ricci, S., Banchi, E., Romagnoli, T., Totti, C., 2024: Comparative analysis of phytoplankton diversity using microscopy and metabarcoding: insights from an eLTER station in the Northern Adriatic Sea. Hydrobiologia. https://doi.org/10.1007/s10750-024-05692-2

Park, J. S., Lobban, C. S., Lee, K. W., 2018: Diatoms associated with seaweeds from Moen Island in Chuuk Lagoon, Micronesia. Phytotaxa 351(2), 101–140. https://doi.org/10.11646/phytotaxa.351.2.1

Pennesi, C., Danovaro, R., 2017: Assessing marine environmental status through microphytobenthos assemblages colonizing the Autonomous Reef Monitoring Monitoring Structures (ARMS) and their potential in coastal marine restoration. Marine Pollution Bulletin 125(1-2), 56–65. https://doi.org/10.1016/j.marpolbul.2017.08.001

Pennesi, C., Poulin, M., De Stefano, M., Romagnoli, T., Totti, C., 2011: New insights to the ultrastructure of some marine *Mastogloia* species section Sulcatae (Bacillariophyceae), including

*Mastogloia neoborneensis* sp. nov. Phycologia 50(5), 548–562. https://doi.org/10.2216/10-39.1

Pennesi, C., Poulin, M., De Stefano, M., Romagnoli, T., Totti, C., 2012: Morphological studies of some marine *Mastogloia* (Bacillariophyceae) belonging to the section sulcatae, including the description of new species. Journal of Phycology 48(5), 1248–1264. https://doi.org/10.1111/j.1529-8817.2012.01215.x

Pérez-Burillo, J., Valoti, G., Witkowski, A., Prado, P., Mann, D. G., Trobajo, R., 2022: Assessment of marine benthic diatom communities: insights from a combined morphological–metabarcoding approach in Mediterranean shallow coastal waters. Marine Pollution Bulletin 174, 113183. https://doi.org/10.1016/j.marpolbul.2021.113183

Poulain, P. M., 2001: Adriatic Sea surface circulation as derived from drifter data between 1990 and 1999. Journal of Marine Systems 29, 3–32. https://doi.org/10.1016/S0924-7963(01)00007-0

Ravaioli, M., Alvisi, F., Menegazzo-Vitturi, L., 2003: Dolomite as a tracer for sediment transport and deposition on the northwestern Adriatic continental shelf (Adriatic Sea, Italy). Continental Shelf Research 23, 1359–1377. https://doi.org/10.1016/S0278-4343(03)00121-3

Riaux-Gobin, C., Vétion, G., Neveux, J., Duchêne, J.-C., 1998: Microphytobenthos et phytoplancton en baie de Banyuls (Golfe du Lion): Biomasses pigmentaires et facteurs hydroclimatiques. Vie et Milieu 48(1), 1–13.

Ribeiro, L., Figueira, R., Oiry, S., Buchet, R., Sabbe, K., Barillé, L., 2022: Benthic marine diatoms from French coastal and transitional waters. Version 1.5. Université de Nantes. Retrieved October 10, 2024 from https://doi.org/10.15468/byyhxu accessed via GBIF.org

Rogelja, M., Cibic, T., Belmonte, M., Del Negro, P., 2018: Active and resting microbenthos in differently contaminated marine coastal areas: insights from the Gulf of Trieste (northern Adriatic, Mediterranean Sea). Hydrobiologia 806, 283–301. https://doi.org/10.1007/s10750-017-3366-1

Rogelja, M., Cibic, T., Pennesi, C., De Vittor, De, 2016: Microphytobenthic community composition and primary production at gas and thermal vents in the Aeolian Islands (Tyrrhenian Sea, Italy), Marine Environmental Research 118, 31–44. https://doi.org/10.1016/j.marenvres.2016.04.009

Romagnoli, T., Bavestrello, G., Cucchiari, E. M., De Stefano, M., Di Camillo, C. G., Pennesi, C., Puce, S., Totti, C., 2007: Microalgal communities epibiontic on the marine hydroid *Eudendrium racemosum* in the Ligurian Sea during an annual cycle. Marine Biology 151, 537–552. https://doi.org/10.1007/s00227-006-0487-x

Romagnoli, T., Totti, C., Accoroni, S., De Stefano, M., Pennesi, C., 2014: SEM analysis of the epibenthic diatoms on *Eudendrium racemosum* (Hydrozoa) from the Mediterranean Sea. Turkish Journal of Botany 38(3), 566–594. https://doi.org/10.3906/bot-1305-52

Round, F. E., Crawford, R. M., Mann, D. G., 1990: The Diatoms. Biology and morphology of the genera. Cambridge University Press, Cambridge.

Rubino, F., Cibic, T., Belmonte, M., Rogelja, M., 2016: Microbenthic community structure and trophic status of sediments in the Mar Piccolo of Taranto (Mediterranean, Ionian Sea). Environmental Science and Pollution Research 23, 12624–12644. https://doi.org/10.1007/s11356-015-5526-z

Sdrigotti, E., Barbariol, V., Welker, C., 1999: Diatom assemblages in coastal shallow waters at the water-sediment interface (Gulf of Trieste, North Adriatic Sea). Annales. Series historia naturalis 9(17), 191–202. Retrieved October 16, 2023 from https://www.dlib.si/details/URN:NBN:SI:DOC-BW6NZCZP

Seveno, J., Car, A., Sirjacobs, D., Fullgrabe, L., Dupčić Radić, I., Lejeune, P., Leignel, V., Mouget, J-L., 2023: Benthic diatom blooms of blue *Haslea* spp. in the Mediterranean Sea. Marine Drugs 21(11), 583. https://doi.org/10.3390/md21110583

Seveno, J., Witkowski, A., Car, A., Gastineau, R., Sirjacobs, D., Leignel, V., Mouget, J-L., 2024: Impact of blue *Haslea* spp. blooms on benthic diatom and bacterial communities. Phycology 4(3), 465–507. https://doi.org/10.3390/phycology4030027

Sıvacı, E. R., Kilinc, S., Dere, Ş., 2007: Relationships between epipelic diatom taxa (Bacillariophyceae) and nutrients in two karstic lakes in central Anatolia, Turkey. Journal of Freshwater Ecology 22(3), 515–520. https://doi.org/10.1080/02705060.2007.9664181

Sorhannus, U., 2007: A nuclear-encoded small-subunit ribosomal RNA timescale for diatom evolution. Marine Micropaleontology 65(1-2), 1–12. https://doi.org/10.1016/j.marmicro.2007.05.002

Souffreau, C., Verbruggen, H., Wolfe, A. P., Vanormelingen, P., Siver, P. A., Cox, E. J., Mann, D. G., Van de Vijver, B., Sabbe, K., Vyverman, W., 2011: A time-calibrated multi-gene phylogeny of the diatom genus *Pinnularia*. Molecular Phylogenetics and Evolution 61(3), 866–879. https://doi.org/10.1016/j.ympev.2011.08.031

Stevenson, R. J., Pan, Y., 1999: Assessing environmental conditions in rivers and streams with diatoms. In: Stoermer, E. F., Smol, J. P. (eds.), The Diatoms: applications for the environmental and earth sciences. 11–40. Cambridge University Press, New York. https://doi.org/10.1017/CBO9780511613005.003

Tas, S., 2013: Phytoplankton composition and abundance in the coastal waters of the Datça and Bozburun Peninsulas, southeastern Aegean Sea (Turkey). Mediterranean Marine Science 15(1), 84–94. https://doi.org/10.12681/mms.418

Theriot, E. C., Ashworth, M., Ruck, E., Nakov, T., Jansen, R. K., 2010: A preliminary multigene phylogeny of the diatoms (Bacillariophyta): challenges for future research. Plant Ecology and Evolution 143(3), 278–296. https://doi.org/10.5091/plecevo.2010.418

Tolomio, C., 2004: Approche expérimentale pour l'étude des diatomées des sédiments de la lagune de Venise, Italie. Diatom Research 19(1), 81–101. https://doi.org/10.1080/0269249X.2004.9705609

Tolomio, C., Andreoli, C., 1989: Recherches sur le periphyton à diatomées dans un vivier de la Lagune de Venise (mai 1984-mai 1985). Diatom Research 4(1), 151–162. https://doi.org/10.1080/0269249X.1989.9705060

Tolomio, C., Andreoli, C., Avignone, M. T., 1991: Contribution a la connaissance du periphyton diatomique des eaux saumâtres de la Valle Sparesera (Suite). Diatom Research 6(2), 391–399. https://doi.org/10.1080/0269249X.1991.9705183

Tolomio, C., Moro, I., Moschin, E., Valandro, A., 1999: Résultats préliminaires sur les diatomées benthiques de substrats meubles dans la lagune de Venise, Italie (Mars 1994-Janvier 1995). Diatom Research 14(2), 367–379. https://doi.org/10.1080/0269249X.1999.9705478

Tolomio, C., Moschin, E., Duzzin, B., 2002: Distribution des diatomées benthiques de substrats meubles dans le bassin sud de la lagune de Venise, Italie. Diatom Research 17(2), 401–414. https://doi.org/10.1080/0269249X.2002.9705557

Totti, C., 2003: Influence of the plume of the river Po on the distribution of subtidal microphytobenthos in the northern Adriatic Sea. Botanica Marina 46(2), 161–178. https://doi.org/10.1515/BOT.2003.017

Totti, C., Cucchiari, E., De Stefano, M., Pennesi, C., Romagnoli, T., Bavestrello, G., 2007: Seasonal variations of epilithic diatoms on different hard substrates, in the northern Adriatic

Sea. Journal of the Marine Biological Association of the United Kingdom 87(3), 649–658. https://doi.org/10.1017/S0025315407054665

Traykovski, P., Wiberg, P. L., Geyer, W. R., 2007: Observations and modelling wave-supported sediment gravity flows on the Po prodelta and comparison to prior observations from the Eel shelf. Continental Shelf Research 27(3-4), 357–399. https://doi.org/10.1016/j.csr.2005.07.008

Tuchman, M. L., Stevenson, R. J., 1980: Comparison of clay tile, sterilized rock, and natural substrate diatom communities in a small stream in Southeastern Michigan, USA. Hydrobiologia 75, 73–79. https://doi.org/10.1007/BF00006564

Van Heurck, H., 1881: Synopsis des Diatomées de Belgique Atlas. Ducaju et Cie, Anvers, Belgium.

Velâsquez, Z. R., Cruzado, A., 1995: Inventory of the diatom flora of the Nw Mediterranean Sea. Vie et Milieu / Life & Environment 249–263.

Verri, G., Furnari, L., Gunduz, M., Senatore, A., Santos da Costa, V., De Lorenzis, A., Fedele, G., Manco, I., Mentaschi, L., Clementi, E., Coppini, G., Mercogliano, P., Mendicino, G., Pinardi, N., 2024: Climate projections of the Adriatic Sea: role of river release. Frontiers in Climate 6, 1368413. https://doi.org/10.3389/fclim.2024.1368413

Vilibić, I., Pranić, P., Denamiel, C., 2023: North Adriatic Dense Water: lessons learned since the pioneering work of Mira Zore-Armanda 60 years ago, Acta Adriatica 64, 53–78, https://doi.org/10.32582/aa.64.1.11, 2023

Viličić, D., 2014: Ecology and composition of phytoplankton in the Adriatic Sea. Koeltz Scientific Books, Konigstein.

Viličić, D., Marasović, I., Mioković, D., 2002: Checklist of phytoplankton in the eastern Adriatic Sea. Acta Botanica Croatica 61(1), 57–91. Retrieved October 16, 2023 from https://hrcak.srce.hr/file/5822

Voigt, M., 1963: Some new and interesting *Mastogloia* from the Mediterranean Area and the Far East. Journal of the Royal Microscopical Society 82(2), 111–121. https://doi.org/10.1111/j.1365-2818.1963.tb05308.x

Welker, C., Sdrigotti, E., Covelli, S., Faganeli, J., 2002: Microphytobenthos in the Gulf of Trieste (Northern Adriatic Sea): Relationship with Labile Sedimentary Organic Matter and Nutrients. Estuarine, Coastal and Shelf Science 55(2), 259–273. https://doi.org/10.1006/ecss.2001.0901

Witkowski, A., Lange-Bertalot, H., Metzelin, D., 2000: Diatom flora of marine coasts I. In: Lange-Bertalot, H. (ed.), Iconographia diatomologica, vol. 7, Diversity-taxonomy-identification, 1–925. A.R.G. Gantner, Ruggell, Germany.

Witkowski, A., Li, C., Zgłobicka, I., Yu, S., Ashworth, M., Dabek, P., Qin, S., Tang, C., Krzywda, M., Ruppel, M., Theriot, E. C., Jansen, R. K., Car, A., Płociński, T., Wang, Y., Sabir, J.S.M., Daniszewska-Kowalczyk, G., Kierzek, A., Hajrah, N. H., 2016: Multigene assessment of biodiversity of diatom (Bacillariophyceae) assemblages from the littoral zone of the Bohai and Yellow Seas in Yantai Region of northeast China with some remarks on ubiquitous taxa. Journal of Coastal Research. 74(sp1), 166–195. https://doi.org/10.2112/SI74-016.1

Zalat, A. A., 2001: New araphid diatom species of the genus *Trachysphenia* Petit from recent coastal sediments of the eastern side of the Gulf of Suez, Egypt. Diatom Research 16(2), 417–432. https://doi.org/10.1080/0269249X.2001.9705528

Zalat, A. A., 2002: Distribution and origin of diatoms in the bottom sediments of the Suez Canal lakes and adjacent areas, Egypt. Diatom Research 17(1), 243–266. https://doi.org/10.1080/0269249X.2002.9705542

Zalat, A. A., El-Sheekh, M. M., Gaballa, M., 2019: Distribution pattern of diatom flora in the surface sediments of Bardawil Lagoon (North Sinai), Egypt. Thalassas: An International Journal of Marine Sciences volume 35, 531–539. https://doi.org/10.1007/s41208-019-00160-4

Zenetos, A., Cinar, M. E., Pancucci-Papadopoulou, M. A., Harmelin, J. G., Furnari, G., Andaloro, F., Bellou, N., Streftaris, N., Zibrowius, H, 2005: Annotated list of alien marine species in the Mediterranean with emphasis on worst invasive species. Mediterranean Marine Science 6(2), 63–118. https://doi.org/10.12681/mms.186

Zidarova, R., Ivanov, P., Hristova, O., Dzhurova, B., Hineva, E., 2022: The unexpected diversity in *Amphora* sensu lato (Bacillariophyta) at Sozopol Bay, the western Black Sea. Phytotaxa 544(2), 103–127. https://doi.org/10.11646/phytotaxa.544.2

Zimmermann J., Glöckner G., Jahn R., Enke N., Gemeinholzer B., 2015: Metabarcoding vs. morphological identification to assess diatom diversity in environmental studies. Molecular Ecology Resources 15(3), 526–542. https://onlinelibrary.wiley.com/doi/full/10.1111/1755-0998.12336

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Review Article

# The Croatian National Diatom Collection – an overview and future challenges

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**Abstract** – Knowledge of the importance of algae in the Earth's hydrosphere emerged after the discovery of the microscope and microorganisms at the beginning of the 18th century. Due to the beauty and morphological distinctiveness of diatoms, the majority of protist research has been focused on those intriguing silicate cells. Today, the vast species diversity of diatoms can only be estimated, but the taxa that have been explored over the years are stored in collections that represent an important resource for modern taxonomy, phylogeny and evolution, ecology, paleoecology, biogeography, phylogeography, applied biology, monitoring and conservation. The Croatian National Diatom Collection (HRNDC) was established in 2018 on the basis of long-term work by Croatian algologists. Today it represents a collection of 2883 permanent specimens that store valuable information on the diatom biodiversity of all waterbodies and store important holotypes, isotypes and paratypes of described new species for science. The future brings the challenge of keeping the collection sustainable, digitalized, and more accessible to the scientific community as well as capable of meeting the challenge of the omics era. The HRNDC is a priceless contribution to the legacy of the Croatian natural heritage.

**Keywords:** diatoms, collection, freshwater and marine environment, natural heritage

## Introduction

Over the years, scientists have tried to gather as much information about nature as they possibly could. The biodiversity of the different ecosystems on Earth has always been one of the main topics in biological studies. In order to preserve their findings about different species, biologists organise the collected specimens in biological collections that help us to understand the taxonomic and phylogenetic background of each published species. Collections such as herbaria or zoological collections in their core keep records of extant and extinct species through a certain period of time and serve as historical evidence that will teach generations about the characteristics of examined samples. Collections will allow easier identification of any specimen found in the future. They can also provide more accurate categorisation of newly discovered species through the morphological or molecular studies of already recorded specimens. Collections enable biodiversity in the ecosystems studied to be tracked by providing evidence of living species and comparing them with deposited specimens. All of these benefits contribute to the fact that the study of the life-form diversity is considerably improved by the systematisation that biological collections provide.

## Diatom collections

Diatom collections are an important resource for modern taxonomy, phylogeny and evolution, ecology, biogeography, phylogeography, applied biology, monitoring and conservation. Diatom specimens provide valuable information for taxonomists to be able to answer scientific questions about basic morphological characteristics and variation of species and genera as well as providing a basis for the establishment of the phylogenetic relationships of diatom species and their geographic distribution and ecology. They contain representatives of diatoms from freshwater, brackish water and marine environments, both fossil and recent, therefore representing an irreplaceable treasure and biorepositories of diatom diversity.

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The documentation of diatoms requires the preparation of permanent specimens, and in the older diatom collections consisted mainly of permanent glass slides and micas. Today, the term diatom collection is also used for collections of living cultures, and thus the collection of diatoms has gained a new dimension in the research processes. Having a live culture allows not only the study of morphological variations to find breaks in the pattern of variations leading to definition and naming of diagnosable entities (Mann and Droop 1996), but also allows us to perform molecular research and resolve phylogenetic relationships, metabolic pathways in order to screen specific compounds and to evaluate their biotechnological potential and to use diatoms as model organisms (Rotter et al. 2021).

Diatoms are not usually collected in the same way as other organisms, due to their microscopic size and spatial and temporal occurrence, patchiness in the environment. Instead, they are often analysed using water samples or sediment/periphyton composites from aquatic environments containing a span of different organisms, which are then deposited as samples and permanent slides. Renowned universities, botanical gardens and institutes around the world maintain collections of diatoms. In addition to Husted's, the most extensive diatom collection, notable collections include: the Natural History Museum Diatom collection, Macedonian National Diatom Collection, Van Heurck diatom collection housed at the National Botanic Garden of Belgium, Academy of Natural Sciences of Drexel University, the diatom collection of Albert Grunow at the herbarium of the Natural History Museum Vienna, the Szczecin Diatomological Collection of the University of Szczecin, Poland. Some of them are prominent in terms of the number of samples, some of them for the quantity of different habitats and others have collections of more significant authors. Motivated by awareness of diatom biodiversity in the diverse waterbodies in Croatia, the importance of research conducted to date and the importance of keeping the national heritage in the country the Croatian National Diatom Collection (HRNDC) was established on 1st March 2018 at the University of Zagreb, Faculty of Science (Gligora Udovič and Ljubešić 2021).

## Historical highlights – two important diatomologists for the Croatian diatom flora

### Dr. Friedrich Hustedt (Bremen, 15 December 1886 – Bremen, 1 April 1968)

Friedrich Hustedt is one of the best-known diatom researchers and has described around 2000 new diatom taxa worldwide. During the excursion organised after the 7th Congress of Limnologists in 1934, he visited Croatia and collected samples from the River Krka and the Plitvice Lakes. From these samples, Hustedt (1945) described many new species for science such as *Achnanthes fonticola* Hustedt, *Cocconeis semiaperta* Hustedt, *Cyclotella stelligeroides* Hustedt, *Cyclotella plitvicensis* Hustedt, *Navicula exiguiformis* Hustedt, *Navicula obsita* Hustedt, etc. All these diatom taxa were illustrated and documented in Simonsen (1987) and type slides have been deposited in the Hustedt collection, which was previously located at the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven but was recently transferred to the Botanical Garden and Museum in Berlin. Hustedt (1945) is one of the most important papers in the diatom taxonomy of the whole of SE Europe and emphasizes the significance of karstic areas in Croatia as centres for the diversity and endemism of diatoms.

### Dr. Anto Jurilj (Komarica, Bosnia and Herzegovina, 19 February 1910 – Zagreb, 1 April 1981)

Anto Jurilj was a Croatian botanist and phycologist. He graduated from grammar school in 1933, obtained a diploma in biology in 1937 and finally completed his PhD in Zagreb in 1949 on the subject of New diatoms of Lake Ohrid and their phylogenetic importance. In the 1950s, he worked first as an assistant and then as a professor at the Faculty of Science in Zagreb. In 1961, Jurilj began working in the Laboratory of Biology with Technical Microscopy at the Technical Faculty of Zagreb, where he became head of the laboratory. He retired in 1980. One of Jurilj's most important contributions to science was his research on diatoms in Lake Ohrid. He described 50 new endemic taxa for the lake and recorded 30 relict taxa that were only considered fossils from the Miocene and Pliocene. Particularly important for phylogeny and evolution was an array of six branching events between the genera *Surirella* and *Campylodiscus*. He described five new genera and one of these, *Iconella*, is now widely accepted for many members of *Surirella sensu lato* (Ruck et al. 2016). His influence on diatom research can also be recognised in the naming of 13 different species described by various authors (e.g. *Actinoptychus juriljii* Molinari & Guiry (Molinari-Novoa and Guiry 2021), *Campylodiscus jurilji* Hajós (Hajós 1973), *Cyclotella juriljii* Skvortsov (Skvortsov 1971), *Cymbella jurilji* Husted (Hustedt 1955), *Cymbopleura juriljii* Z. Levkov & D. Metzeltin (Levkov et al. 2007) *Placoneis juriljii* A. Miho & Lange-Bertalot (Miho and Lange-Bertalot 2006). A part of his diatom collection concerning Lake Ohrid diatoms has been found and is now deposited in the Croatian National Diatom Collection. Although he published his work in various scientific journals such as Acta Biologica, Acta Botanica Croatica, Archiv für Hydrobiologie und Mikroskopie, his most comprehensive work on fossil diatoms called Diatomophyta fossilia, which was commissioned for the first part of the palaeobotanical encyclopaedia Traite de paleobotanique, remains unpublished. In this work, Jurilj studied 320 genera of diatoms with 12,000 species and discovered nine new genera with 30 new species during his research. His original handwritten paper is kept in the National Library of the University of Zagreb.

## Diatom diversity

Diatoms are considered one of the most diverse groups of algae, with estimates of the number of diatom species ranging from 20,000 to 2.000,000 (Mann and Droop 1996, Spaulding et al. 2021). Some of the most recent works estimate the number of diatoms at 100,000–200,000 species, divided into the three classes Bacillariophyceae, Mediophyceae and Coscinodiscophyceae (Medlin and Kaczmarska 2004). There are currently 14,867 diatom species listed in the global algae database AlgaeBase (Guiry and Guiry 2024, https://www.algaebase.org/), while the Diatoms of North America database (Spaulding et.al. 2021, https://diatoms.org/) contains 1,099 described species. The wide range of diatom diversity is due to the continuous discovery and classification of new species and/or revisions of already described species and genera and the complex taxonomy of diatoms, which frequently leads to revisions and reclassifications. In addition, the use of different methods contributes to this complexity. In the 1970s, the scanning electron microscope revolutionized the systematics of diatoms by revealing taxonomically important ultrastructural features that were not visible with the light microscope (Hasle and Fryxell 1970, Alverson 2008). For some species of diatoms there was the necessity of introducing transmission electron microscopy (TEM), especially for those lightly silicified cells often found in marine environments (e.g., *Pseudo-nitzschia*, *Entomoneis*) (Burić et al. 2008, Mejdandžić et al. 2018). Finally, molecular methodology such as environmental DNA (eDNA) sequencing and genotyping of specific marker genes are now providing new insights into the systematics of diatoms and helping in the daily discovery of new species (Alverson 2008).

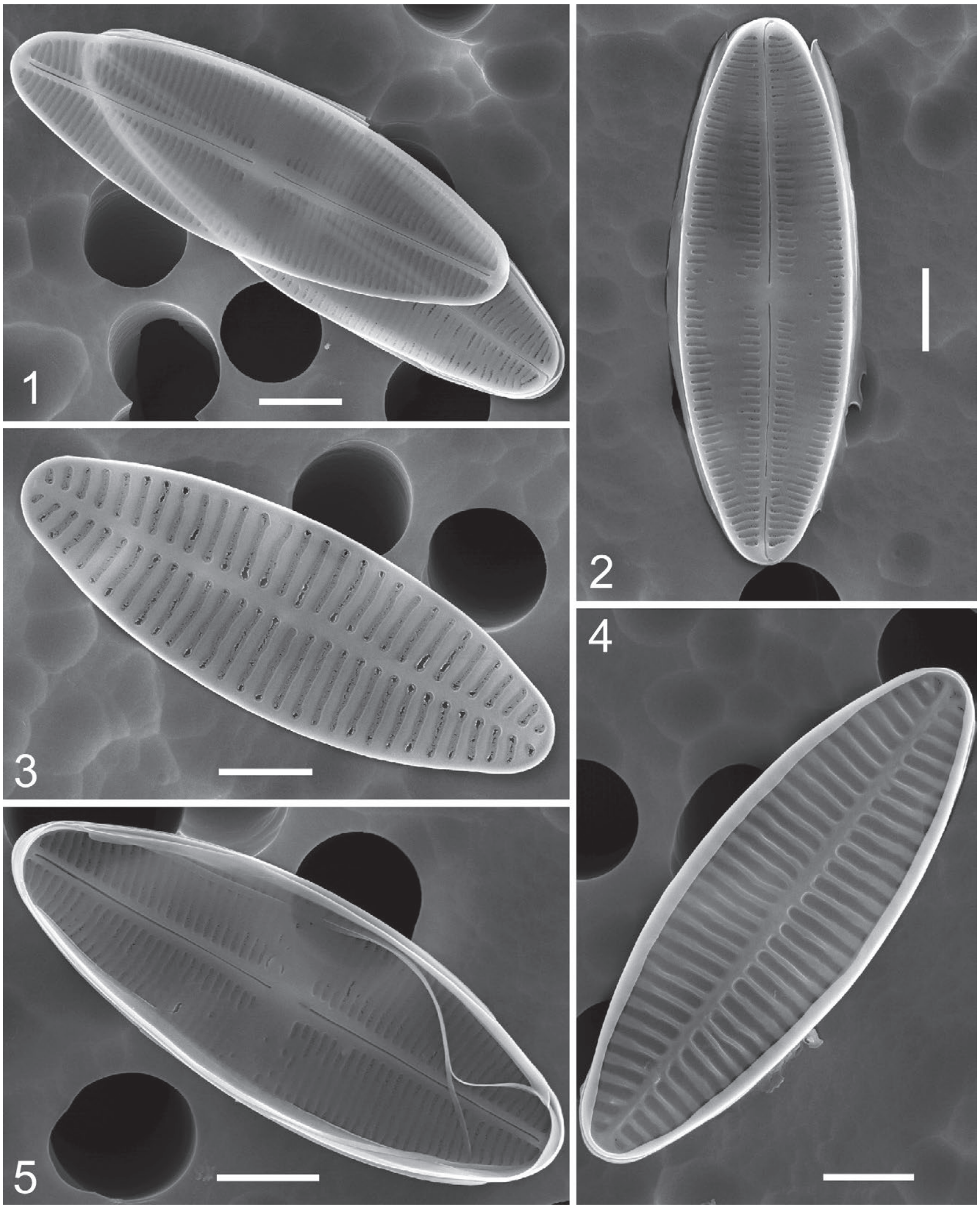
## Overview of diatom research in the Eastern Adriatic Sea

Diatom research in the Adriatic Sea started very early, and some of the first described diatoms were *Bacillaria adriatic* Lobarzewski (von Lobarzewski 1840), *Berkeleya adriatica* Kützing (Kützing 1844) and *Amphitetras adriatica* Kützing (Kützing 1845). The beginnings of Adriatic research were focused on the northern Adriatic, where due to the influence of Italy and the Austro-Hungarian Monarchy, researchers came from different European countries (Casellato 2008). The first record of phytoplankton in the Adriatic Sea was at the end of the 19th century and it reports on the diatom *Nitzschia closterium* (Ehrenberg) W. Smith bloom in the northern Adriatic (Hauck 1872). Some of the pioneering important work of phytoplankton investigation, but focused on diatoms, is the results of the research expeditions "Rudolf Virchow" (Schröder 1911), "Naiads" (Schiller 1913a, b, 1925a, b, 1933, Schussnig 1914), and "Thor" (Jörgensen 1920, 1923) which resulted in numerous publications that are to a large extent still valuable keys for determining diatom species. But the main step in diatom research was the work of German botanist Friedrich Hustedt. He published the determination key for planktonic diatoms that was partly based on the analysis of Adriatic samples and many editions of that work are still relevant today (Hustedt 1927-1966). Croatian diatomologist Anto Jurilj, and Friedrich Hustedt collaborated and exchanged publications.

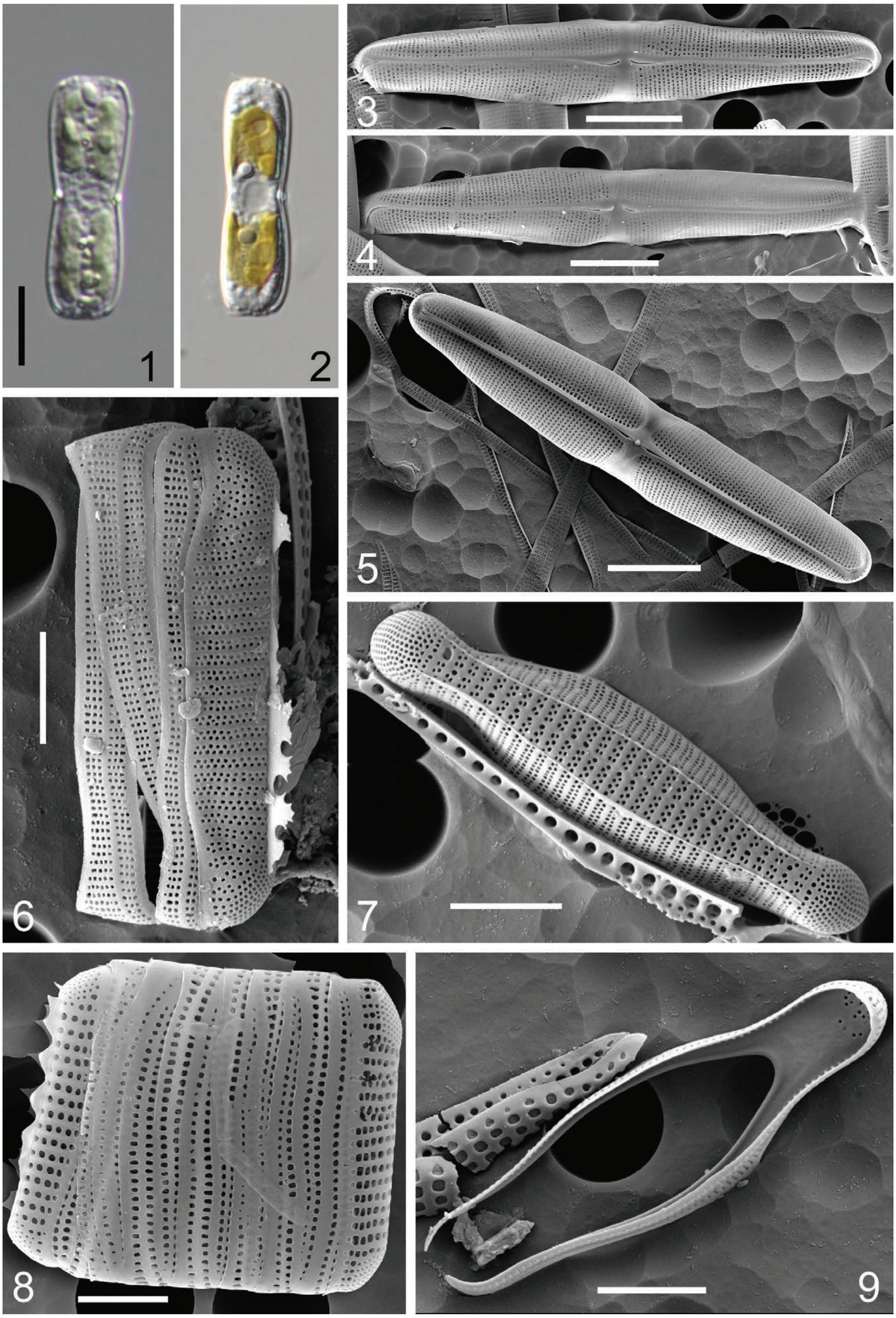
The research of phytoplankton taxonomy and ecology in the Adriatic Sea was continuous during the 20th century following the up-to-date literature and methods (Ercegović 1936, Viličić and Ljubešić 2017 and references therein). A comprehensive overview of eastern Adriatic phytoplankton taxonomy research and an updated checklist was published by Viličić et al. (2002) showing the great diatom diversity, of 518 diatom species. It must be added that 266 diatom species from this list, mostly benthic were recorded only by Revelante (1985). More recently an updated checklist of phytoplankton from 12 ports in the Adriatic was published recording 330 diatom taxa (Mozetič et al. 2019). Diatom diversity in the Adriatic Sea is high, probably due to its shallow parts and habitat heterogeneity and the long tradition of skilled diatomists. New species are constantly described not only those from the water column, but also those attached to a substrate (Car et al. 2012, Van De Vijver et al. 2020, Lobban et al. 2021). The diversity of periphytic diatoms has been investigated on natural and artificial substrates and submerged plants (Burić et al. 2004, Caput Mihalić et al. 2008, Car et al. 2012, 2019a, b, 2020, 2021, Mejdandžić et al. 2015, Nenadović et al. 2015, Hafner et al. 2018a, b, Kanjer et al. 2019, Seveno et al. 2023, 2024).

By the beginning of the 2010s, molecular methods came into traditional diatom taxonomy research. The first published sequences of diatoms from the Adriatic Sea were of the well-established species *Cylindrotheca closterium* (Ehrenberg) W. Smith (Pletikapić et al. 2012), and of the newly described species *Bacteriastrum jadranum* Godrijan, Maric & Pfannkuchen (Godrijan et al. 2012). In 2019 a complete chloroplast genome sequence was published of the small pennate diatom *Nanofrustulum shiloi* (J.J.Lee, Reimer & McEnery) Round, Hallsteinsen & Paasche collected from the Adriatic Sea (Li et al. 2019). It is also important to mention studies that used quick molecular identification for species that were difficult to resolve with traditional microscopy or that were treated as a threat for human health during harmful algal blooms (HABs): toxic specimens of the genus *Pseudo-nitzschia* (Penna et al. 2013, Grbin et al. 2017, Arapov et al. 2020, Smodlaka Tanković et al. 2022). Electron microscopy has proved a great tool for obtaining an insight into this genus heterogeneity (Burić et al. 2008, Ljubešić et al. 2011). Processes of diatom cultivation significantly endorsed the use of the molecular approach in taxonomical research into planktonic diatoms in the Adriatic Sea. With cultures established through several projects in the past decade, the number of cultivated diatoms from the Adriatic Sea increased exponentially, and consequently, multiple sequences were deposited in primary genetic databases such as GenBank, European Nucleotide Archive (ENA) or within in-house databases (Ruđer Bošković Institute, Marine Research Centre Rovinj; Institute of Oceanography and Fisheries, Split). One newly described diatom genus *Majewska* (Van de Vijver et al., 2020), with type species *Majewska istriaca* (Pl. 1) and several other genera were revised and supplemented with new taxa decribed from the Adriatic Sea, including *Entomoneis* (Mejdandžić et al. 2017, 2018, Al-Handal et al. 2020, 2020a, 2023), *Proschkinia* (Majewska et al. 2019), *Nitzschia* (Mucko et al. 2020), *Hyalosira* (Lobban et al. 2021) and *Craspedostauros* (Majewska et al. 2021) (Pl. 1-8). Description of images in plates are shortened and with commonly known diatom terminology. For detailed terminology explanation and description of each species please see original publications. eDNA sequencing and genotyping of cultivated diatoms in parallel enabled the first metabarcoded diatom checklist of North Adriatic Sea to be published (Grižančić et al. 2023).

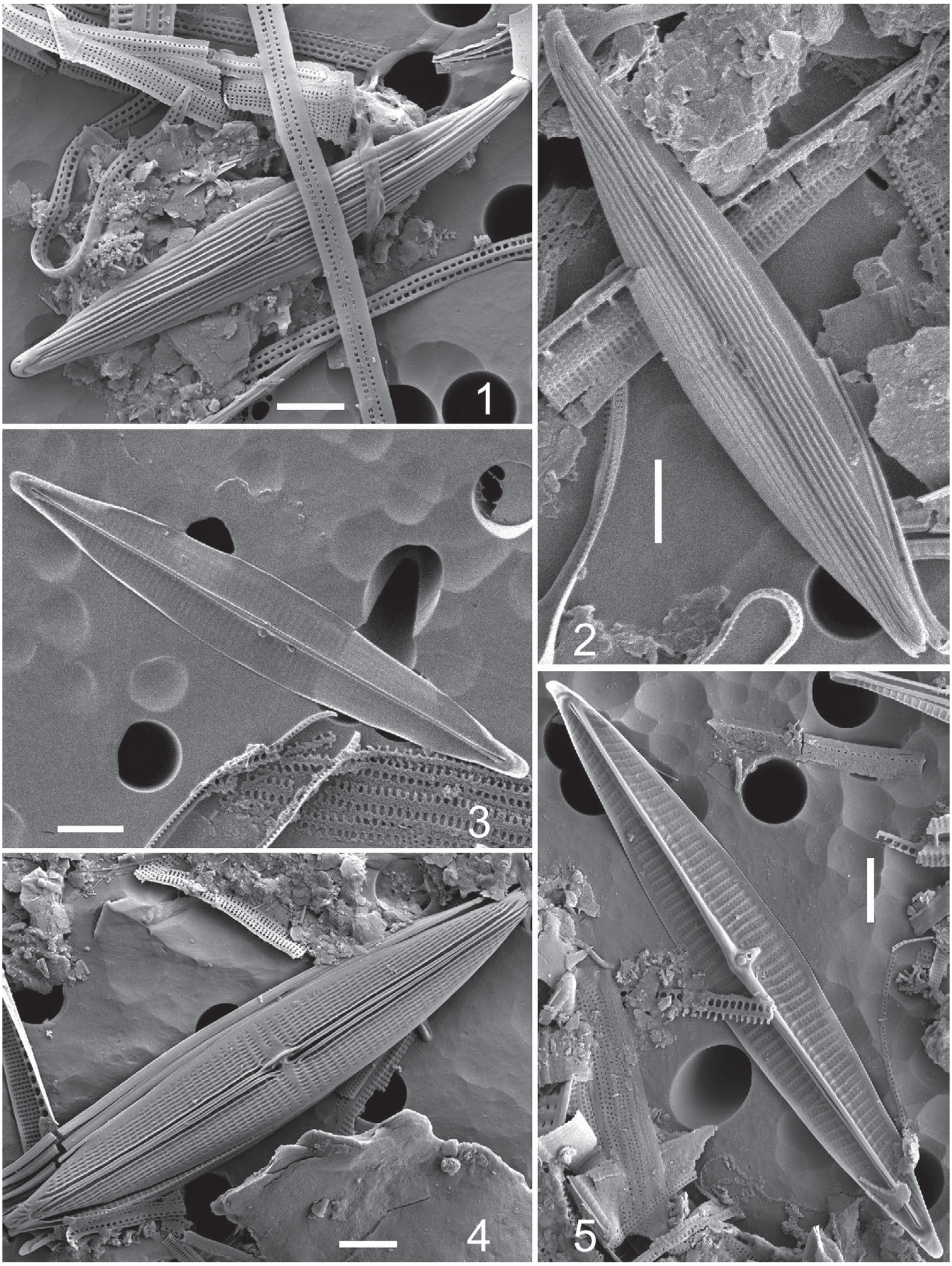
Today, phytoplankton in the Adriatic Sea is continuously metabarcoded and investigated with traditional microscopy for the purposes of whole-phytoplankton community and diatom community research (Mucko et al. 2018, Matek et al. 2023, Turk Dermastia et al. 2023, Baricevic et al. 2024), which is generating knowledge for future studies.



**Pl. 1.** SEM micrographs of *Majewskaea istriaca* from the PMFTB0125 culture material (Figs. **1–5**): **1** – Two overlaying raphe valves with the upper valve in the internal valve view and the lower in the external valve view; **2** – Internal view of the raphe valve; **3** – External view of the sternum valve; **4** – Internal view of the sternum valve with well-developed silica ridge surrounding the entire valve; **5** – Internal view of the raphe valve with partially attached open, narrow, unperforated girdle bands. SEM scale bar = 2 µm. Abbreviations: SEM–Scanning electron microscopy.

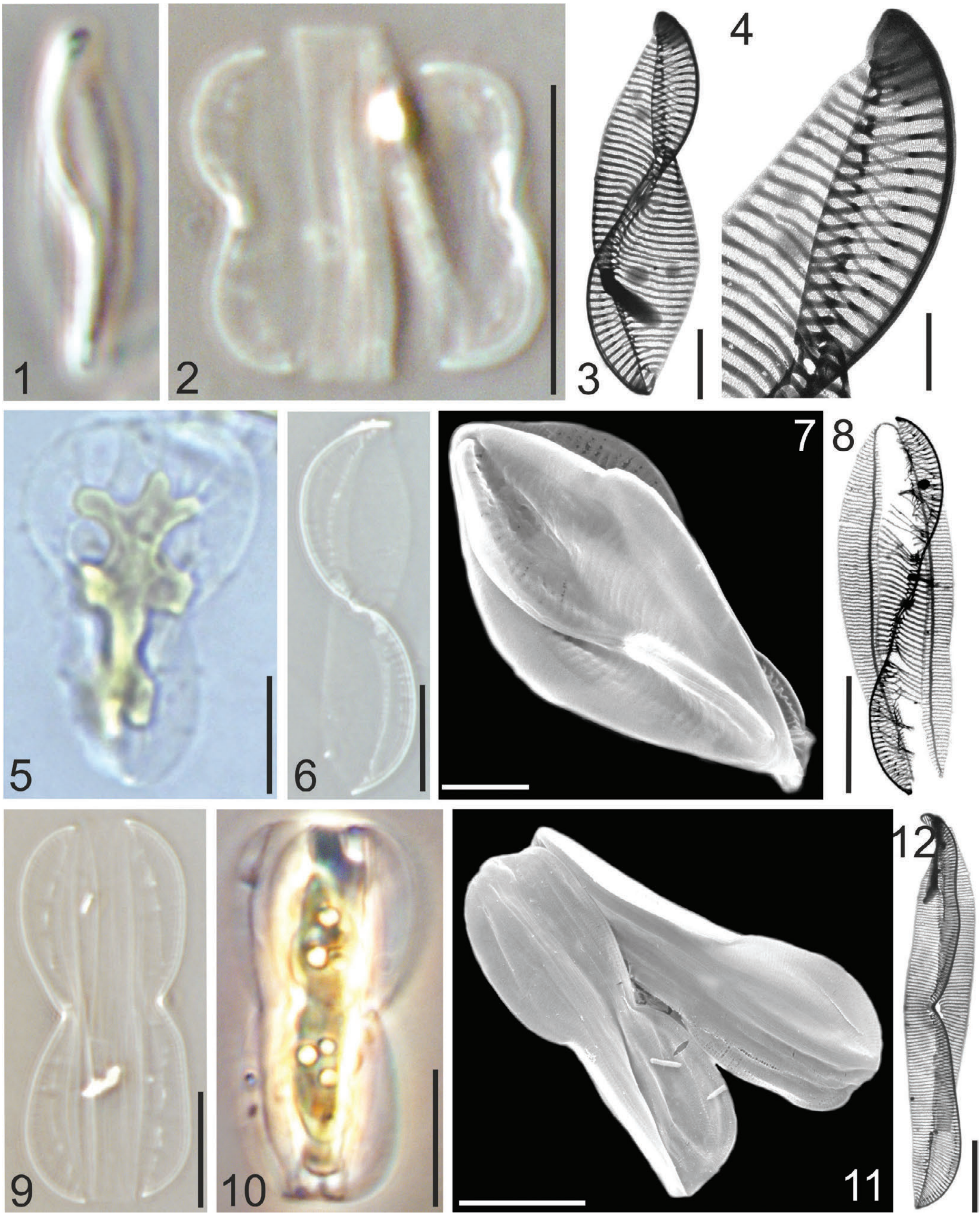


**Pl. 2.** *Craspedostauros legouvelloanus* PMFTB0003 culture material (Figs. **1–5**): **1,2** – LM micrographs of live cells in girdle view; **3,4** – SEM micrographs of external valve view showing the raphe and stria morphology; **5** – SEM micrograph of complete valve showing the internal valve structure (modified from Majewska et al. 2020.). SEM micrographs of *Hyalosira septata* from the type material (Figs. **6–9**): **6** – Girdle view of valve with prominent cristae and partially detached girdle bands (modified from Lobban et al. 2021.); **7** – External valve view showing stria morphology; **8** – Girdle view of a complete frustule; **9** – Open girdle band with prominent septa. LM scale bar = 10 µm (Figs. **1, 2**). SEM scale bar = 5 µm (Figs. **3–5**) and 2 µm (Figs. **6–9**). Abbreviations: LM–Light microscopy, SEM–Scanning electron microscopy.

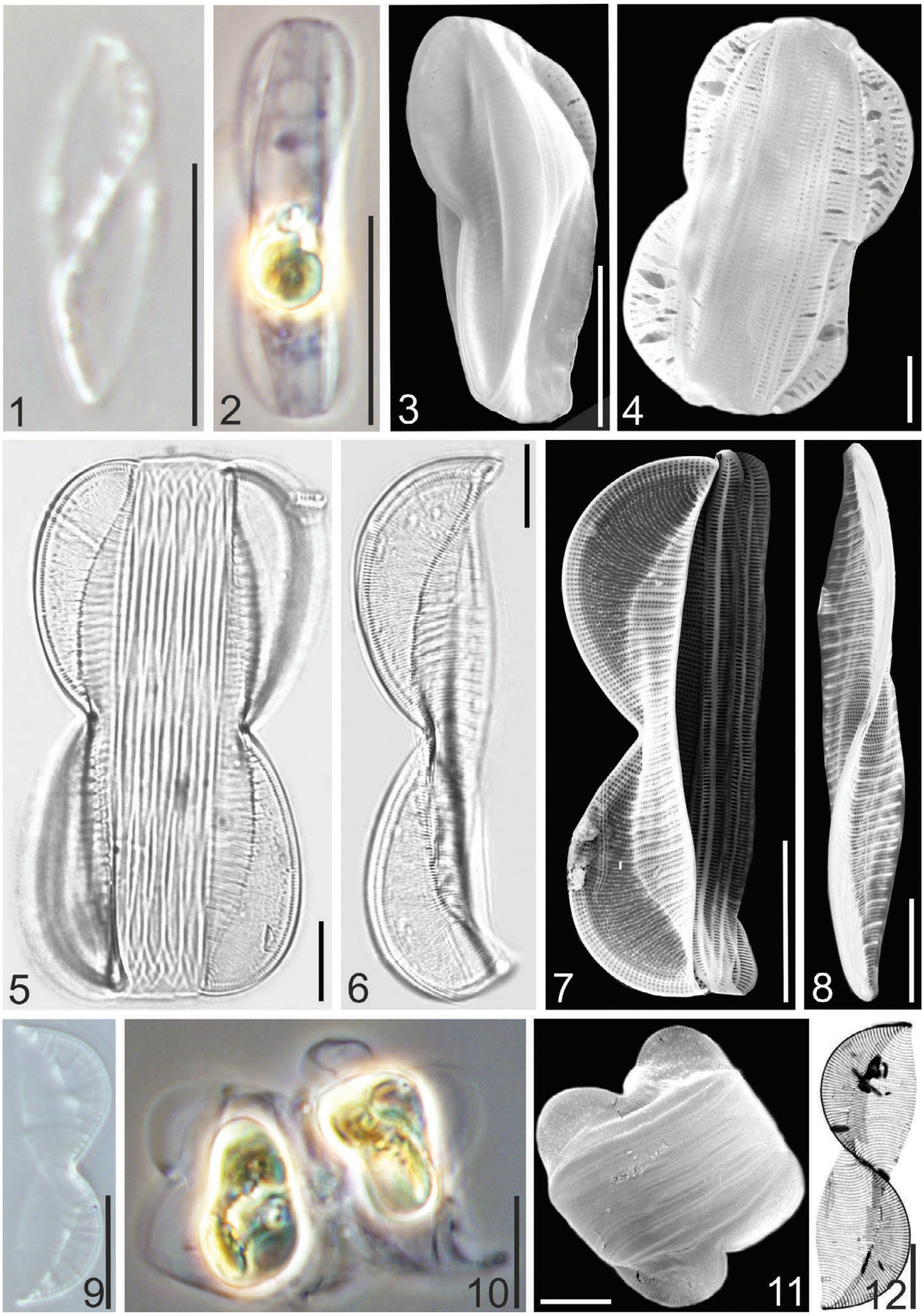


**Pl. 3.** SEM micrographs of *Proschkinia sulcata* from the type material (Figs. **1–3**): **1,2** – External valve view, note the valve central area with fistula opening hidden beneath the pocket-like silica thickening; **3** – Internal valve view showing stria pattern and a central fistula occluded by one domed hymen forming a ball-like structure. SEM micrographs of *Proschkinia torquata* from the type material (Figs. **4–5**): **4** – External valve view showing the longitudinal silica strips over the valve central area and a pocket-like rimmed silica flap obscuring the external fistula opening; **5** – Internal valve view with a distinctive fistula opening composed of several circular domed hymenes raised on the lateral expansion of the central nodule. SEM scale bar = 2 µm. Abbreviations: SEM–Scanning electron microscopy.

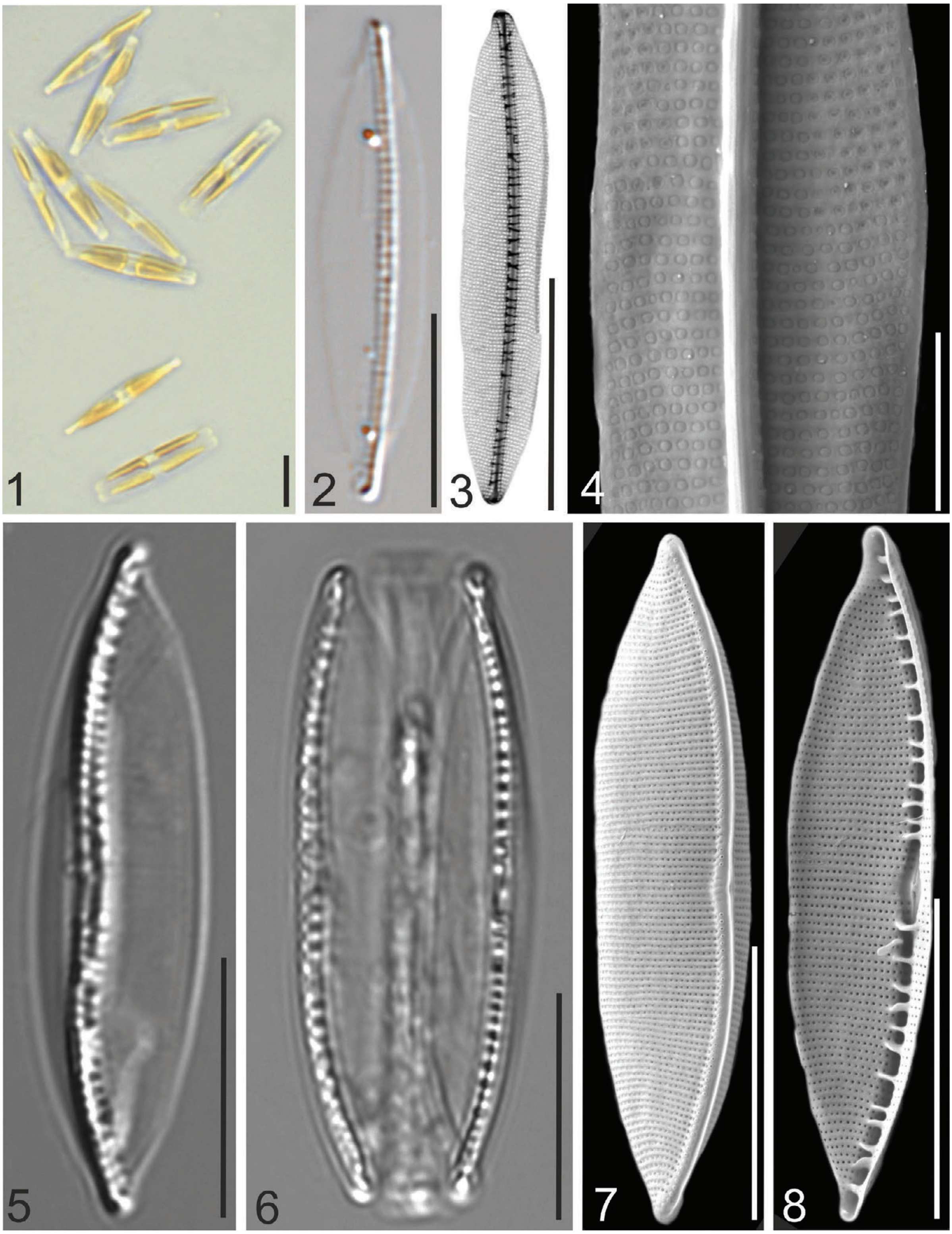
**Pl. 4.** LM and SEM micrographs of *Entomoneis annagodheae* from the field and type material (Figs. **1–4**): **1** – LM micrograph of live cell from field material; **2** – LM micrograph of external valve with prominent oblique transapical fascia; **3,4** – SEM micrographs of external and internal valve view. LM and SEM micrographs of *Entomoneis tenera* from culture and type material (Figs. **5–8**): **5** – LM micrograph of live apically torsioned cell from culture material, strain PMFEN1; **6** – LM micrograph of torsioned frustule in girdle view; **7** – SEM micrograph of torsioned frustule in girdle view; **8** – SEM micrograph of external valve with elevated bilobate keel. LM, SEM and TEM micrographs of *Entomoneis umbratica* from the type material (Figs. **9–12**): **9** – TEM micrograph of valve in girdle view; **10** – LM micrograph of valve with sigmoid raphe-bearing keel; **11** – LM micrograph of valve in girdle view with valvocopulae and copulae; **12** – SEM micrograph of showing girdle details. LM scale bar = 10 µm (Figs. **1,2,10,11**) and 5 µm (Figs. **5,6**), SEM scale bar = 10 µm (Figs. **3,4**), 5 µm (Fig. **12**) and 2 µm (Figs. **7,8**), TEM scale bar = 4 µm (Fig. **9**). Micrographs **1–4** are modified from Al-Handal et al. 2020; Micrographs **5–8** are modified from Mejdandžić et al. 2017; Micrographs **9–12** are modified from Mejdandžić et al. 2018. Abbreviations: LM–Light microscopy, SEM–Scanning electron microscopy.



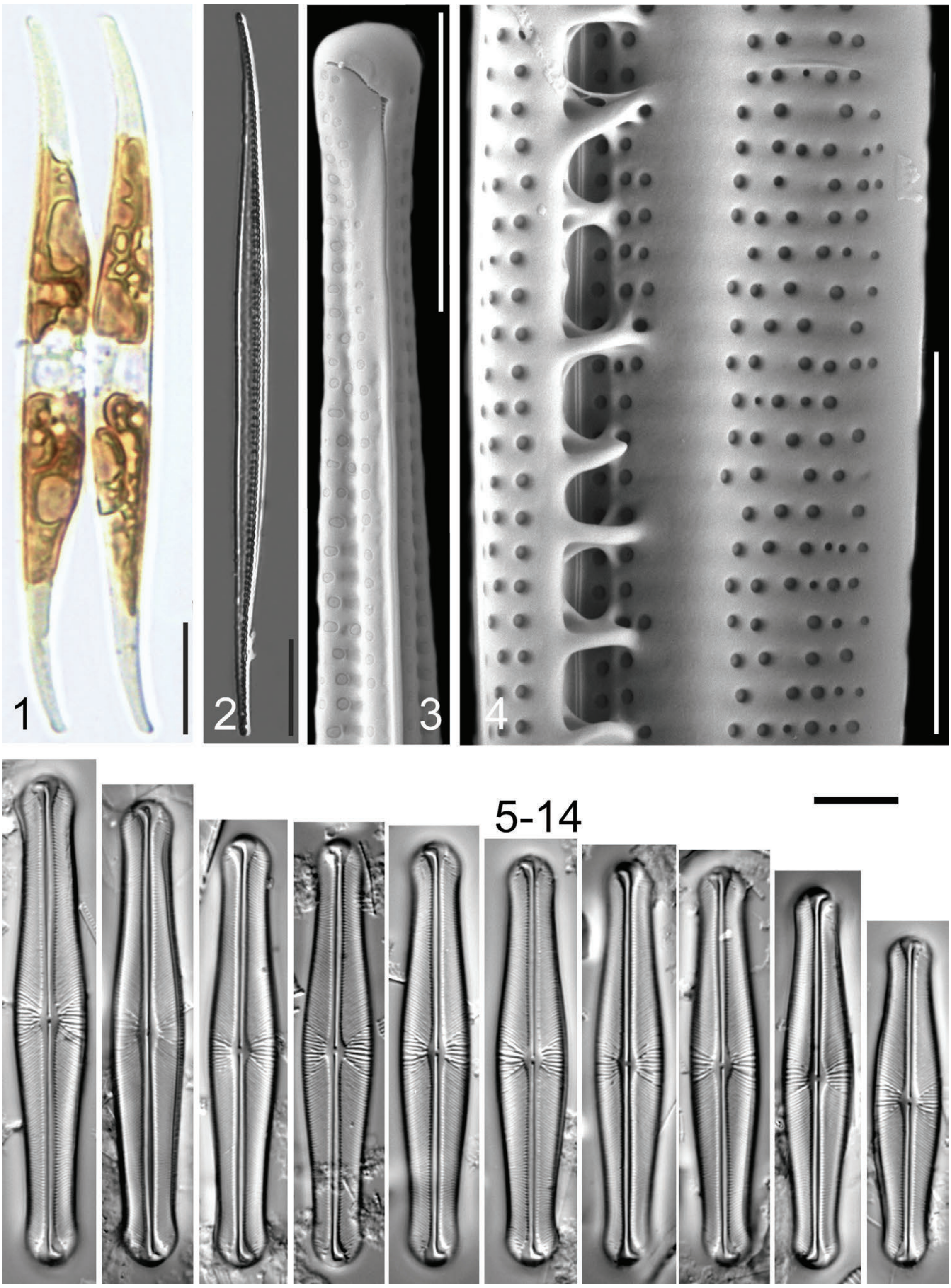
**Pl. 5.** LM and TEM micrographs of *Entomoneis pusilla* from the type material (Figs. **1–4**): **1** – LM micrograph of valve with sigmoid raphe-bearing keel; **2** – LM micrograph of frustule in girdle view; **3, 4** – TEM micrographs of valves with striae and keel details. LM, SEM and TEM micrographs of *Entomoneis adriatica* from the type material (Figs. **5–8**): **5** – LM micrograph of live apically torsioned cell from culture material, strain BIOTAII-49; **6** – LM micrograph of valve with sigmoid raphe-bearing keel; **7** – SEM micrograph of external valve; **8** – TEM micrograph of valve with valvocopulae. LM, SEM and TEM micrographs of *Entomoneis vilicicii* from culture and type material (Figs. **9–12**): **9** – LM micrograph of frustule in girdle view; **10** – LM micrograph of live cell from culture material, strain PMFBION4A; **11** – SEM micrograph of two partially torsioned frustules; **12** – TEM micrograph of valve with acute valve apices. LM scale bar = 10 µm (Figs. **1,2,5,6,9**), SEM scale bar = 2 µm (Fig. **7**) and 10 µm (Fig. **11**), TEM scale bar = 5 µm (Figs. **8,12**), 2 µm (Fig. **3**) and 1 µm (Fig. **4**). Micrographs **1,3,4,5,7** and **9–12** are modified from Mejdandžić et al. 2018. Abbreviations: LM–Light microscopy, SEM–Scanning electron microscopy.



**Pl. 6.** LM and SEM micrographs of *Entomoneis gracilis* from culture and type material (Figs. **1–4**): **1** – LM micrograph of valve with sigmoid raphe-bearing keel; **2** – LM micrograph of live cell from culture material, strain BIOTAII-60a; **3** – SEM micrograph of torsioned frustule with elevated raphe-bearing keel*;* **4** – SEM micrograph of frustule in girdle view. LM and SEM micrographs of *Entomoneis grisslehamnensis* from the type material (Figs. **5–8**): **5** – LM micrograph of frustule in girdle view; **6** – LM micrograph of valve with scalpelliform apices in girdle view; **7** – SEM micrograph of valve and accompanying valvocopulae in girdle view; **8** – SEM micrograph of external valve with sigmoid keel. LM, SEM and TEM micrographs of *Entomoneis infula* from culture and type material (Figs. **9–12**): **9** – LM micrograph of valve in girdle view; **10** – LM micrograph of two live cells from culture material, strain BIOTAII-68; **11** – SEM micrograph of frustule in girdle view; **12** – TEM micrograph of valve with scalpelliform apices. LM scale bar = 10 µm (Figs. **1,2,5,6,9,10**), SEM scale bar = 10 µm (Figs. **7,8**), 5 µm (Figs. **3,11**) and 2 µm (Fig. **4**), TEM scale bar = 10 µm (Fig. **12**). Micrographs **1–4** and **9–12** are modified from Mejdandžić et al. 2018; Micrographs **5–8** are modified from Al-Handal et al. 2023. Abbreviations: LM–Light microscopy, SEM–Scanning electron microscopy.



**Pl. 7.** LM, SEM and TEM micrographs of *Nitzschia adhaerens* from culture and type material (Figs. **1–4**): **1** – LM micrograph of live cells in culture material, strain BIOTAII-60; **2** – LM micrograph of spindle-shaped valve with cuneate apices; **3** – TEM micrograph of with pronounced raphe sternum and fibulae; **4** – SEM micrograph of with details of continuous raphe and striae areolae. LM and SEM micrographs of *Nitzschia dalmatica* from the type material (Figs. **5–8**): **5** – LM micrograph of linear to lanceolate valve; **6** – LM micrograph of frustule in girdle view; **7,8** – SEM micrographs of external and internal valves with eccentric elevated keel. LM scale bar = 10 µm (Figs. **1,2,5,6**), SEM scale bar = 10 µm (Figs. **4,7,8**), TEM scale bar = 10 µm (Fig. **3**). Micrographs **1–8** are modified from Mucko et al. 2020. Abbreviations: LM–Light microscopy, SEM–Scanning electron microscopy.



**Pl. 8.** LM and SEM micrographs of *Nitzschia inordinata* from culture and type material (Figs. **1–4**): **1** – LM micrograph of live cells from culture material, strain BIOTAII-44; **2** – LM micrograph of sigmoid valve with protracted apices; **3** – SEM micrograph of external valve with details of protracted apex with bent raphe fissure; **4** – SEM micrograph of internal valve with fibulae and areolae details. LM micrographs of *Envekadea hedinii* (Figs. **5–14**): LM micrographs of external view of the valves. LM scale bar = 10 µm (Figs. **1**,**2**), SEM scale bar = 5 µm (Figs. **3**,**4**). Micrographs **1–4** modified from Mucko et al. 2020. Abbreviations: LM–Light microscopy, SEM–Scanning electron microscopy.

## Overview of the diatom research in the freshwater bodies in Croatia

Research on freshwater algae in Croatia began in the early 20th century (Brunnthaler 1900, Car 1906). Initially, the focus was on the floristic recording, taxonomy and description of the species (Krmpotić 1914, Pevalek 1919, 1925, 1935, 1938, Ercegović 1925, Vouk 1936, 1947, 1948, Jurilj 1957, Emili 1958), but with the acquisition of new knowledge, research expanded to the ecology of species, seasonal dynamics, spatial distribution, role in water treatment processes and primary organic production. Most diatom research was conducted in the context of comprehensive community diversity studies. This led to more comprehensive research on the diversity of algae, including diatoms, in the second half of the 20th century, and the first list of diatoms was published in 1995 (Plenković-Moraj 1995).

Firstly, algal and diatom research in Croatia was conducted mainly in karstic lakes and the tufa barriers of karst rivers, but today, due to the mandatory monitoring programme for surface waters under the Water Framework Directive (WFD) (European Community, 2000), research has expanded to the whole country and contributed to the discovery of new species. More advanced approaches to the study and biomonitoring of freshwaters brought new findings on diatoms and on periphyton community colonisation on natural and artificial substrates in the Zrmanja, Krka and Ombla rivers (Kralj et al. 2006, Caput Mihalić et al. 2008, Levkov et al. 2010) as well as on the study of the epiphytic community on sawgrass in the Plitvice Lakes National Park (Caput and Plenković-Moraj 2000). Studies have used a range of methods to assess species composition, abundance and distribution, often emphasising high species diversity and unique community structures. Therefore, general research on periphytic diatoms has focussed on various aspects such as diversity and community structure (Plenković et al. 1989, Plenković-Moraj 1996, Plenković-Moraj et al. 2008) in different freshwater habitats, including rivers, lakes and streams.

Diatoms were further analysed and recorded as part of extensive ecological studies on the taxonomy and ecology of phytoplankton in the Danube floodplains and along the Danube River, regarding the influence of the flooding of the Danube on species composition (Mihaljević et al. 2009, 2010, 2013, 2015, Stević et al. 2013). Phytoplankton was also studied in other large rivers such as the Mura, Drava and Sava (Stanković et al. 2012) and karst lakes (Žutinić et al. 2014, Gligora Udovič et al. 2015, 2017) using a novel morpho-functional approach and functional groups. The phytoplankton community in the context of Reynolds functional groups is well researched in Croatia and used as a bioindicator for assessing the water quality and ecological status of freshwater systems (Žutinić et al. 2020, Hanžek et al. 2021, 2023, Šimunović et al. 2022). All these phytoplankton studies included lists of diatom species and expanded the knowledge of diatom diversity and ecology, from phytobenthos to phytoplankton. The exploration of new habitats, such as mires with a narrow pH gradient, has also led to an increase in the list of recorded diatom species in Croatia (Stanković et al. 2022)

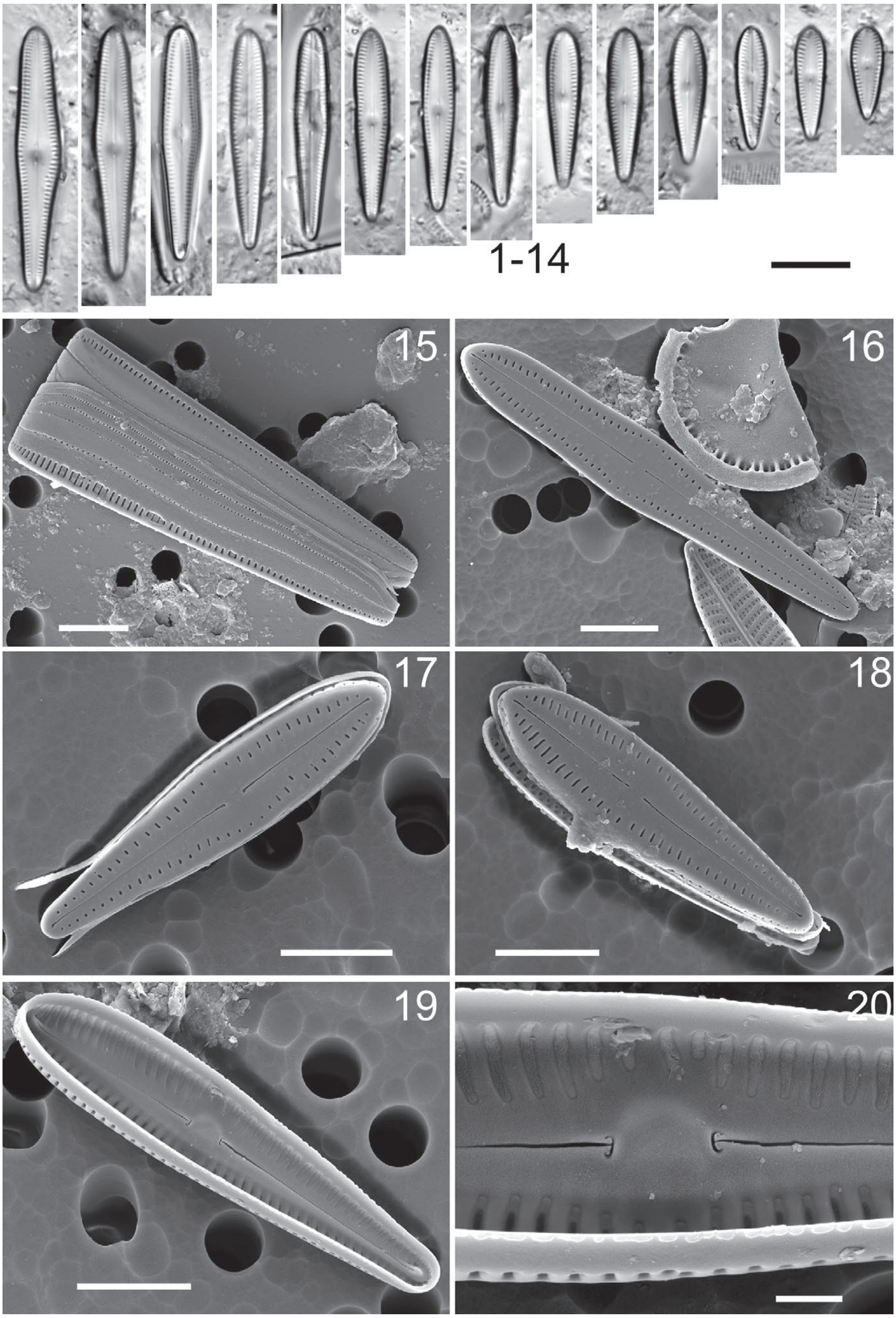
Today, the list of diatom species comprises 897 species and is used as the official operational list of diatoms in the national freshwater monitoring system and in assessing the ecological status of Croatian freshwater ecosystems. Studies in Croatia have developed and applied methods based on phytobenthos and phytoplankton to assess the ecological status of rivers and lakes and thus contribute to effective water management and protection strategies resulting in scientific articles in the field of ecology and benthic diatom assemblages along freshwater typology (Kralj Borojević et al. 2017).

The use of molecular methods and new genomic tools as well as the combination of morphological and molecular approaches in the analysis of phytoplankton and phytobenthos has provided new insights into algal diversity, including diatoms and also greatly improved phytobenthos and phytoplankton research in freshwater, embedded in the standardised bioassessment of the water quality and ecological status of regularly monitored water bodies (Kolda et al. 2019, Hanžek et al. 2021, Kulaš et al. 2021, 2022).

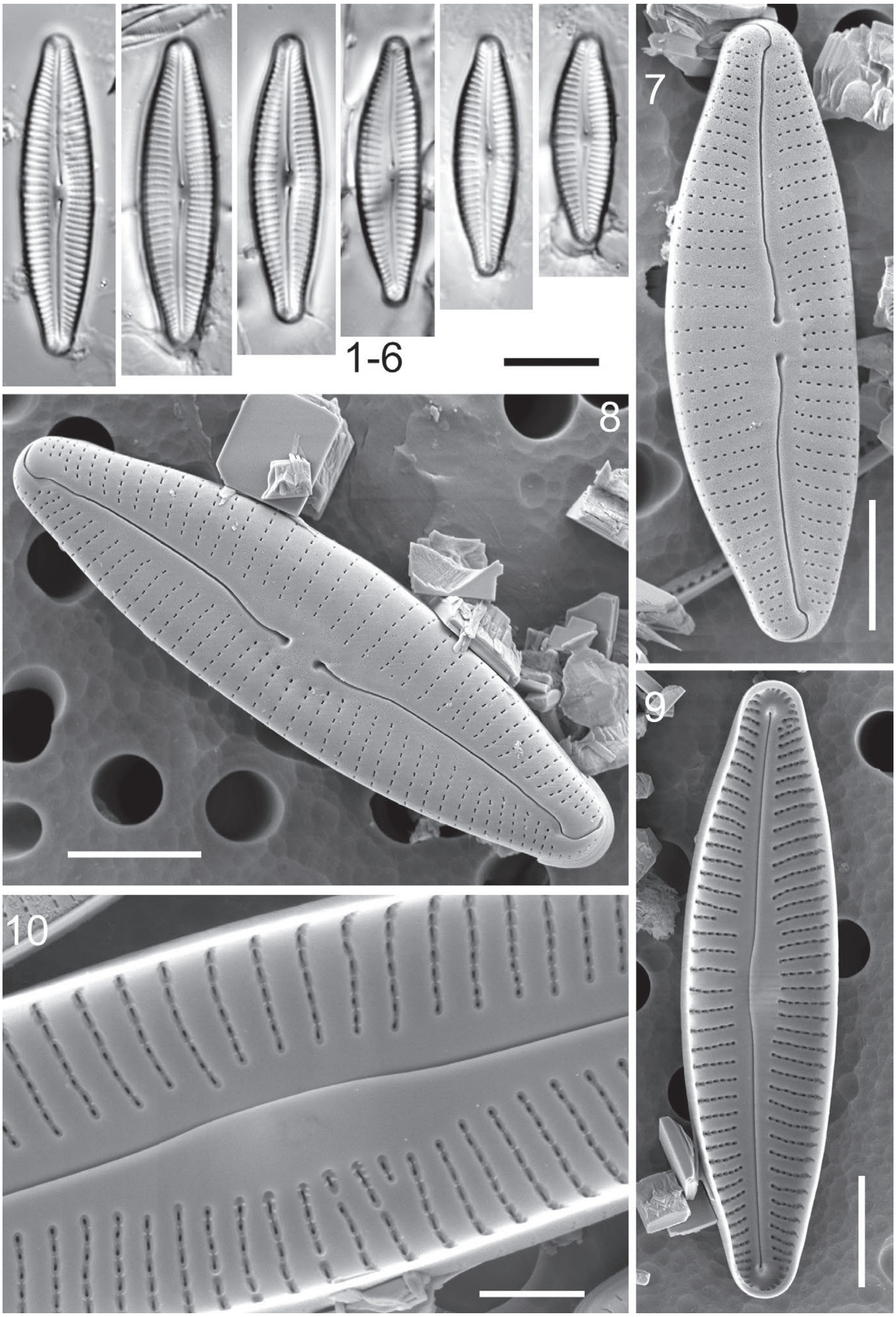
Analyses of the diatom material in all of these comprehensive studies led to comparison of some species to the type material (Kulaš et al. 2020, Trábert et al. 2019), to more detailed morphological studies (Gligora Udovič et al. 2017) and also the description of several new freshwater species. The ecological study of the phytoplankton community in the shallow Vrana Lake resulted in the study by Gligora et al. (2009) of the population of an unknown naviculoid diatom and the characterisation of the new genus *Envekadea* in 2009 in Vrana Lake near the town of Biograd, Dalmatia (Pl. 8).

The description was based on morphological characteristics of *Navicula hedinii* Hustedt. Furthermore, extensive paleo research was conducted on Vrana Lake in Croatia, which resulted in the identification of a new species, *Envekadea vranaensis* Caput Mihalić, Galović & Levkov (Caput Mihalić et al. 2020), which was described from Holocene sediments. Another new species *Tetramphora croatica* Gligora Udovič, Caput Mihalić, Stanković & Levkov, was observed and described from contemporary and core samples of Lake Vransko (Caput Mihalić et al. 2019). The first successful dive to the bottom of the karstic lake Crveno jezero, near the town of Imotski in the southern part of Croatia, resulted in the discovery of a diatom species new to science *Gomphosphenia plenkoviciae* Gligora Udovič & Žutinić (Gligora Udovič et al. 2018) (Pl. 9).

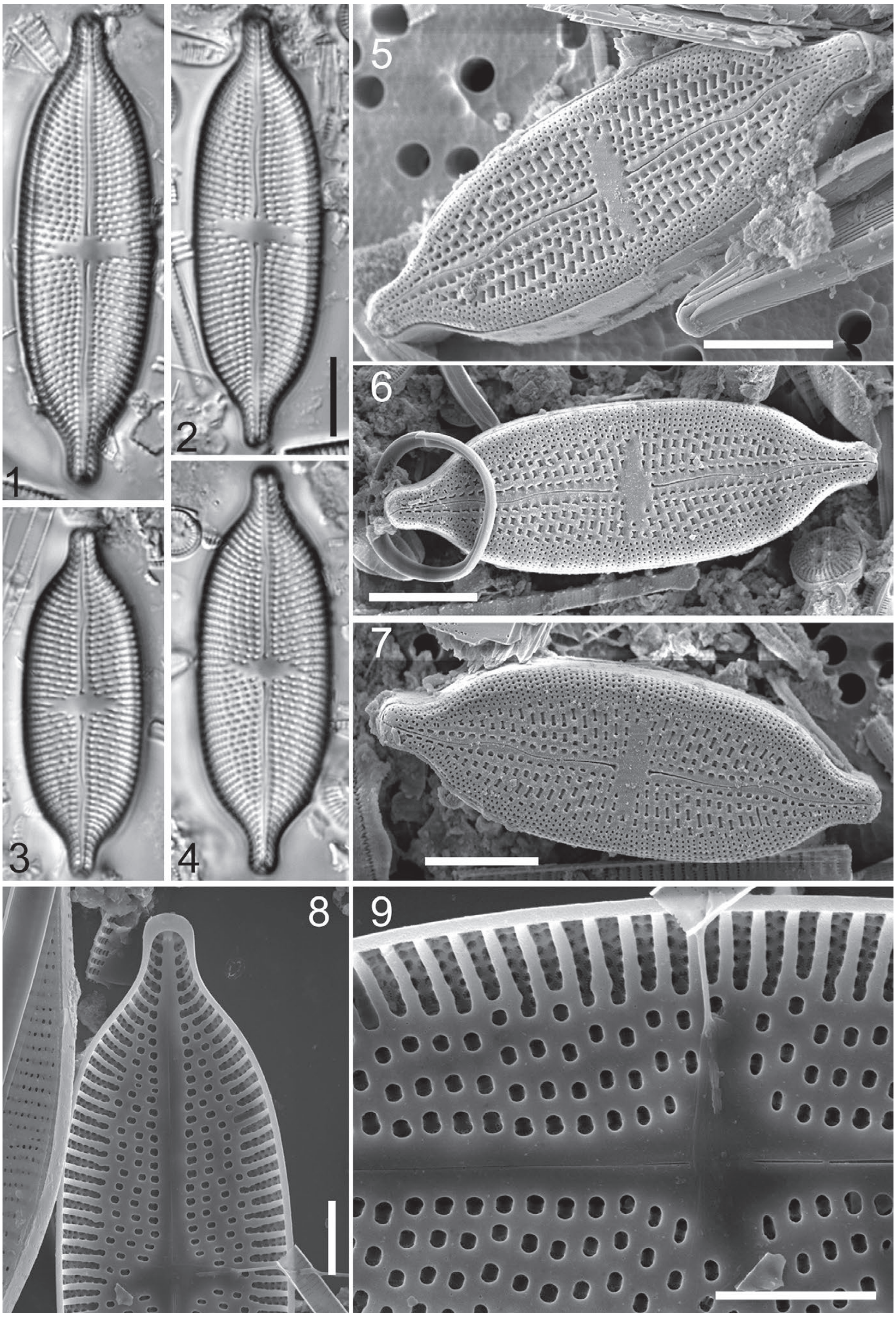
During the research in Krka National Park and establishment of the Krka diatom collection two new species were described *Cymbopleura amicula* Gligora Udovič, Kulaš, Šušnjara, Arapov, Blanco & Levkov (Gligora Udovič et al. 2022) and *Aneumastus visovacensis* Gligora Udovič et Levkov (Gligora Udovič et al. 2023) (Plate 10, 11).



**Pl. 9.** Light micrographs of *Gomphosphenia plenkoviciae* from the type material (Figs. **1–14**). External girdle view of the whole frustule (Fig. **15**). External view of the whole valve. Note the simple proximal and distal raphe ends and striae composed of a single areola located near valve margin (Figs. **16–18**). Internal view of the whole valve. Internally, areolae are occluded by hymens. Distally, raphe terminates in helictoglossae (Fig. **19**). Internal detailed view of mid-valve showing the internal structure of proximal raphe ends (T-shaped) and areolae (Fig. **20**). LM scale bar = 10 µm (Figs. **1–14**). SEM scale bar = 5 µm (Figs. **15–19**); 2 µm (Fig. **20**). Abbreviations: LM–Light microscopy, SEM–Scanning electron microscopy.



**Pl. 10.** Light micrographs of *Cymbopleura amicula* from river Krka (Figs. **1–6**). External view of the whole valve showing the areola and raphe structure. Areolae have slit-like external opening. Distal raphe ends are dorsally deflected. Proximally raphe terminates with expanded central pores (Figs. **7, 8**). Internal view of the whole valve. Note the small struts present across the vimines (Fig. **9**). Internal detailed view of mid-valve showing the internal structure of raphe and areolae. Raphe “lacking an intermissio” or proximal raphe ends are covered by overgrowth of silica that hides the deflected endings (Fig. **10**). LM scale bar = 10 µm (Figs. **1–6**). SEM scale bar = 5 µm (Figs. **7–9**) and 2 µm (Fig. **10**). Abbreviations: LM–Light microscopy, SEM–Scanning electron microscopy.



**Pl. 11.** Light micrographs of *Aneumastus visovacensis* from the type material (Figs. **1–4**). External view of the whole valve. Striae near raphe uniseriate are composed of large elongated, bone–shaped or X–shaped areolae, becoming biseriate near the valve margin and composed of small, round areolae. Areolae are complex, occluded, opened into deep pits via sieves of small pores (Figs. **5–7**). Internal valve view. Raphe is very narrow linear with indistinct and straight proximal raphe ends (Fig. **8**). Internal detailed view of midvalve showing the internal structure of raphe and areolae. Areola openings round to ellipsoidal near axial area becoming elongate towards valve margin (Fig. **9**). LM scale bar =10 µm (Figs. **1–4**). SEM scale bar = 10 µm (Figs. **5–7**) and 5 µm (Figs. **8, 9**). Abbreviations: LM–Light microscopy, SEM–Scanning electron microscopy.

Based on the presented long-term research on diatoms in Croatia, the Department of Biology founded the Croatian National Diatom Collection (HRNDC), the first institutional collection of permanent microscopic specimens of diatoms in Croatia, currently comprising 2,883 microscopic slides and 23 type specimens (Tab. 1).

**Tab. 1.** List of newly (2009-2023) described species deposited in the Croatian National Diatom Collection (HRNDC).

| Species | Habitat | Described by | Region | Reference | Accession number |
|---|---|---|---|---|---|
| *Entomoneis pusilla* | marine plankton | Sunčica Bosak, Maja Mejdandžić | Adriatic Sea, south–eastern coast, Croatia | Mejdandžić et al. 2018 | HRNDC000001 |
| *Entomoneis gracilis* | marine plankton | Sunčica Bosak, Maja Mejdandžić | Adriatic Sea, south–eastern coast, Croatia | Mejdandžić et al. 2018 | HRNDC000002 |
| *Entomoneis vilicicii* | marine plankton | Sunčica Bosak, Maja Mejdandžić | Adriatic Sea, south–eastern coast, Croatia | Mejdandžić et al. 2018 | HRNDC000003 |
| *Entomoneis infula* | marine plankton | Sunčica Bosak, Maja Mejdandžić | Adriatic Sea, south–eastern coast, Croatia | Mejdandžić et al. 2018 | HRNDC000004 |
| *Entomoneis adriatica* | marine plankton | Sunčica Bosak, Maja Mejdandžić | Adriatic Sea, south–eastern coast, Croatia | Mejdandžić et al. 2018 | HRNDC000005 |
| *Entomoneis umbratica* | marine plankton | Sunčica Bosak, Maja Mejdandžić | Adriatic Sea, south–eastern coast, Croatia | Mejdandžić et al. 2018 | HRNDC000006 |
| *Proschkinia torquata* | sea water | Sunčica Bosak, Bart Van de Vijver, Roksana Majewska | Marine Turtle Rescue Centre, Pula, Croatia | Majewska et al. 2019 | HRNDC000007 |
| *Proschkinia sulcata* | sea water | Roksana Majewska, Bart Van de Vijver, Sunčica Bosak | Marine Turtle Rescue Centre, Pula, Croatia | Majewska et al. 2019 | HRNDC000007 |
| *Gomphosphenia plenkoviciae* | freshwater | Marija Gligora Udovič, Petar Žutinić | Crveno jezero, Croatia | Gligora Udovič et al. 2018 | HRNDC000008 |
| *Tetramphora croatica* | brackish water | Marija Gligora Udovič, Katarina Caput Mihalić, Igor Stanković, Zlatko Levkov | Lake Vransko, Croatia | Mihalić et al. 2019 | HRNDC000009 |
| *Nitzschia dalmatica* | marine plankton | Maja Mucko, Sunčica Bosak | Southeast Adriatic Sea, Croatia | Mucko et al. 2020 | HRNDC000010 |
| *Nitzschia adhaerens* | marine plankton | Maja Mucko, Sunčica Bosak | Southeast Adriatic Sea, Croatia | Mucko et al. 2020 | HRNDC000011 |
| *Nitzschia inordinata* | marine plankton | Maja Mucko, Sunčica Bosak | Southeast Adriatic Sea, Croatia | Mucko et al. 2020 | HRNDC000012 |
| *Cymbopleura amicula* | freshwater | Marija Gligora Udovič, Antonija Kulaš, Mirela Šušnjara, Jasna Arapov, Saul Blanco, Zlatko Levkov | Krka River, Croatia | Gligora Udovič et al. 2022 | HRNDC000026 |
| *Aneumastus visovacensis* | freshwater | Marija Gligora Udovič, Zlatko Levkov | Lake Visovac, Croatia | Gligora Udovič et al. 2023 | HRNDC000046 |
| *Entomoneis tenera* | marine plankton | Maja Mejdandžić, Sunčica Bosak | Adriatic Sea, Croatia | Mejdandžić et al. 2017 | HRNDC000435 |
| *Entomoneis annagodheae* | marine plankton | Adil Y. Al-Handal, Maja Mucko | Kungälv fjord, West coast of Sweden | Al-Handal et al. 2020 | HRNDC000436 |
| *Majewskaea istriaca* | marine benthos | Bart Van de Vijver, Käthe Robert, Andrzej Witkowski, Sunčica Bosak | Adriatic Sea, Croatia | Van De Vijver et al. 2020 | HRNDC000437 |
| *Craspedostauros legouvelloanus* | marine benthos | Roksana Majewska, Sunčica Bosak | Kosi Bay, South Africa Adriatic Sea | Majewska et al. 2021 | HRNDC000502 |
| *Hyalosira septata* | marine benthos | Sunčica Bosak, Bart Van de Vijver, Nihavet Bizsel | Pula Aquarium, Croatia | Lobban et al. 2021 | HRNDC000850 |
| *Entomoneis grisslehamnensis* | sea water | Adil Y. Al-Handal, Maja Mucko | Sweedish coast of Baltic Sea: Grisslehamn and Juniskär | Al-Handal et al. 2023 | HRNDC001665 |
| *Pinnularia furatensis* | brackish water | Adil Y. Al-Handal | Euphrates River, Southern Iraq | Al-Handal 2022 | HRNDC001666 |
| *Envekadea hedinii sp.nov* | brackish water | Van de Vijver, Gligora, F. Hinz, Kralj & Cocquyt | Lake Vransko, Croatia | Gligora et al. 2009 | HRNDC002157 |

## Future challenges

Having in mind the variety and quantity of data being published every year and considering the intensity of diatom research (especially new species descriptions), the existence and professional sustainability and curation of diatom collections is of the utmost importance. However, neither a database nor a diatom collection is free from daily and long-term challenges that need to be overcome with professional staff (scientists and collaborators). One of the main challenges we would like to address is the stability of funding and staff employment to ensure that the collection is continuously available for research, teaching and public use. We often face uncertain funding and rely on scientific (or professional monitoring) projects that have an expiry date, but the collection is here to stay. The frequent turnover of personnel working for a collection introduces uncertainty and potential errors within the database and provides fertile ground for errors in publications that collect data from the same collections.

Considering the omics era and the description of new species with the deposition of material, collections should also be careful to register peer-reviewed data only, ensuring the quality of taxonomy. However, we can still register one specimen as species A and revise it further to a strain of species B, subspecies of species A or entirely other species C, as long as personnel keeps track of the annotation. Eukaryotic tree of life (Adl et al. 2019) is expanding daily and currently establishes nine taxonomic levels (according to pr2 database, Vaulot et al. 2022) and many taxa are changing in their higher systematic ranks, as well as shifting from one genus to other. Phylogenetic papers often confront key aspects of species definition to be faulty when dealing with monophyly vs. paraphyly and support in clade differentiation spanning one species or species complex. There is also sensitivity in cryptic species descriptions that needs to be addressed, and diatoms have many examples of such behaviour (Pinseel et al. 2019) and plasticity in terms of morphological *vs.* phylogenetical concept of species (Alverson 2008 and references therein). Thus, revisions of existing historical material deposited in collections often can shed light on complex species relationships.

The availability of a collection directly depends on its housing the collection if it is not digitalized. Well into the technological era, most research, in view of the exploitation and comparison of data, can be done online, through available, well curated collections such as the Croatian National Diatom Collection (HRNDC). To meet this challenge, the collection is set to be digitalized in 2025 as part of the newly established BIOFIT data platform. BIOFIT is an internal web database application designed for the collection and management of ecological research data. The information about a collection(s) housed within BIOFIT will be made publicly accessible in a curated format on the Flora Croatia Database (Nikolić 2024) and the HRNDC web page https://www.diatoms.biol.pmf.hr/collection. Once this process is done, valuable data will be ready for use by members of academia and scholars. However, digital availability is not sufficient if the collections not well curated. Experts in taxonomy, phylogeny and evolution, whether they act as authors of a publication and depositors, or as second-hand curators, have a tremendously important job – to correct the taxonomical annotation of specimens. Biocurators need to ensure that relevant specimens are reliable, reusable and accessible by other researchers. With this in mind, every revised taxonomy annotation must be tracked and acknowledged on every collection milestone, ensuring up-to-date lists and systematics. Many collections have therefore established workflows which ensure fulfilment of the tasks biocurators do, so nothing is left unattended (Odell et al. 2017). Within this process of curation, newly established collections such as HRNDC can benefit from networking, sharing of best practices, developing collaboration and promoting interactions with other similar (and more experienced) diatom collections.

Finally, it is important to emphasise that collections often help generate solutions to societal challenges by stimulating interaction among academia, the public and bioindustry. The popularization of science through collections as valuable resource in which parties can observe, experience, touch and acknowledge scientific work ensures that the efforts required in their establishment have been worthwhile within the framework of the Croatian natural heritage.

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## Author contribution statement

Z. LJ. – conceptualization, writing - original draft; D. J. – writing - original draft, writing - review and editing; T.P.

– data curation, investigation, writing - original draft; M.Ž. – data curation, investigation, writing - original draft; Z. Le. – visualization, writing - original draft; M.M. – writing - review and editing; S.B. – writing - review and editing; M.G.U. – conceptualization, writing - original draft.

## References

Adl, S. M., Bass, D., Lane, C. E., Lukeš, J., Schoch, C. L., Smirnov, A., Agatha, S., Berney, C., Brown, M. W., Burki, F., Cárdenas, P., Čepička, I., Chistyakova, L., del Campo, J., Dunthorn, M., Edvardsen, B., Eglit, Y., Guillou, L., Hampl, V., Heiss, A. A., Hoppenrath, M., James, T. Y., Karnkowska, A., Karpov, S., Kim, E., Kolisko, M., Kudryavtsev, A., Lahr, D. J. G., Lara, E., Le Gall, L., Lynn, D. H., Mann, D. G., Massana, R., Mitchell, E. A. D., Morrow, C., Park, J. S., Pawlowski, J. W., Powell, M. J., Richter, D. J., Rueckert, S., Shadwick, L., Shimano, S., Spiegel, F. W., Torruella, G., Youssef, N., Zlatogursky, V., Zhang, Q., 2019: Revisions to the classification, nomenclature, and diversity of eukaryotes. Journal of Eukaryotic Microbiology 66(1), 4–119. https://doi.org/10.1111/jeu.12691

Al-Handal, A. Y., 2022: *Pinnularia furatensis* sp. nov. (Bacillariophyceae): a new brackish water diatom from the Euphrates River, Southern Iraq. Fundamental and Applied Limnology 196(1), 75–81. https://doi.org/10.1127/fal/2022/1462

Al-Handal, A. Y., Mucko, M., Peharec Štefanić, P., Wulff, A., 2023: *Entomoneis grisslehamnensis*, a new diatom (Bacillariophyceae) from the Baltic Coast of Sweden with taxonomic and ecological notes on *Entomoneis paludosa* (W. Smith) Reimer. Diatom Research 38(1), 55–68. https://doi.org/10.1080/0269249X.2023.2215110

Al-Handal, A.Y., Mucko, M., Wulff, A., 2020a: *Entomoneis annagodhei* sp. nov., a new marine diatom (Entomoneidaceae, Bacillariophyta) from the west coast of Sweden. Diatom Research 35(3), 269–279. https://doi.org/10.1080/0269249X.2020.1787229

Al-Handal, A. Y. 2020. *Entomoneis annagodheae* Al-Handal & Mucko, the correct name for *E. annagodhei* Al-Handal & Mucko. Diatom Research 35(4), 395. https://doi.org/10.1080/0269249X.2020.1842253

Alverson, A. J., 2008: Molecular systematics and the diatom species. Protist 159(3), 339–353. https://doi.org/10.1016/j.protis.2008.04.001

Arapov, J., Bužančić, M., Penna, A., Casabianca, S., Capellacci, S., Andreoni, F., Skejić, S., Bakrač, A., Straka, M., Mandić, J., Ninčević Gladan, Ž., 2020: High proliferation of *Pseudo-nitzschia* cf. *arenysensis* in the Adriatic Sea: ecological and morphological characterisation. Mediterranean Marine Science 21(3), 759–774. https://doi.org/10.12681/mms.22932

Baricevic, A., Kogovsek, T., Smodlaka Tankovic, M., Grizancic, L., Knjaz, M., Vlasicek, I., Podolsak, I., Kuzat, N., Pfannkuchen, M., Maric Pfannkuchen, D., 2024: Coastal eukaryotic plankton diversity of the Southern Adriatic as revealed by metabarcoding. Diversity 16(5), 293. https://doi.org/10.3390/d16050293

Bosak, S., Šupraha, L., Nanjappa, D., Kooistra, W. H. C. F., Sarno, D., 2015: Morphology and phylogeny of four species from the genus *Bacteriastrum* (Bacillariophyta). Phycologia 54(2), 130–148. https://doi.org/10.2216/14-62

Brunnthaler, J., 1900: Plankton - Studien. Prošćansko jezero (Croatien). Verhandlungen der Zoologisch-Botanischen Gesellschaft in Wien 50, 382–383.

Burić, Z., Caput, K., Viličić, D., 2004: Distribution of the diatom *Cocconeis scutellum* in the karstic estuary (Zrmanja, eastern Adriatic Sea). Biologia, Bratislava 59(1), 1–7.

Burić, Z., Viličić, D., Caput Mihalić, K., Kralj, K., Ljubešić, N., 2008: *Pseudo-nitzschia* blooms in the Zrmanja River estuary (eastern Adriatic Sea). Diatom Research 23(1), 51–63. https://doi.org/10.1080/0269249X.2008.9705736

Caput, K., Plenković-Moraj, A., 2000: Epiphytic diatoms on sawgrass (*Cladium mariscus*) in the karstic waters Plitvice Lakes, Croatia. Biologia 55, 343–350.

Caput Mihalić, K., Galović, I., Wetzel, C. E., Ector, L., Ilijanić, N., Miko, S., Wishkerman, A., Hamilton, P. B., Levkov, Z., 2020: *Envekadea vranaensis* sp. nov. a new diatom species (Bacillariophyta) from the lacustrine Holocene sediments of Lake Vrana, Croatia. Nova Hedwigia 110(1-2), 1–19. https://doi.org/10.1127/nova_hedwigia/2019/0561

Caput Mihalić, K., Gligora Udovič, M., Galović, I., Stanković, I., Šušnjara, M., Žutinić, P., Kulaš, A., Špoljarić, I., Levkov, Z., 2019: *Tetramphora croatica* sp. nov. –A new brackish-water species from lake vransko, Croatia. Phytotaxa 401(4), 276–286. https://doi.org/10.11646/phytotaxa.401.4.5

Caput Mihalić, K., Viličić, D., Ahel, M., Burić, Z., Carić, M., 2008: Periphytic algae development in the upper reach of the Zrmanja Estuary (eastern Adriatic coast). Vie et Milieu 58(3-4), 203–213.

Car, A., Hafner, D., Dupčić Radić, I., Kaleli, A., Ljubimir, S., Solak, C. N., 2021: Comparison of benthic diatom community structures on natural and artificial substrates in marine lake (Adriatic Sea). Acta Adriatica 62(1), 21–44. https://doi.org/10.32582/aa.62.1.1

Car, A., Hafner, D., Ljubimir, S., Dupčić Radić, I., Bobanović-Ćolić, S., Jasprica, N., 2020: Colonization of bacteria and diatoms on an artificial substrate in a marine lake (eastern Adriatic Sea, NE Mediterranean). Acta Botanica Croatica 79(2), 212–227. https://doi.org/10.37427/botcro-2020-028

Car, A., Witkowski, A., Dobosz, S., Burfeind, D. D., Meinesz, A., Jasprica, N., Ruppel, M., Kurzydłowski, K. J., Płociński, T., 2012: Description of a new marine diatom, *Cocconeis caulerpacola sp. nov.* (Bacillariophyceae), epiphytic on invasive *Caulerpa* species. European Journal of Phycology 47(4), 433–448. https://doi.org/10.1080/09670262.2012.735255

Car, A., Witkowski, A., Dobosz, S., Jasprica, N., Ljubimir, S., 2019a: Epiphytic diatom assemblages on invasive algae *Caulerpa taxifolia* and autochthonous *Halimeda tuna* and *Padina* sp. from the Adriatic Sea – summer/autumn aspect. Oceanological and Hydrobiological Studies 48(3), 209–226. https://doi.org/10.2478/ohs-2019-0019

Car, A., Witkowski, A., Jasprica, N., Ljubimir, S., Čalić, M., Dobosz, S., Dupčić Radić, I., Hrustić, E., 2019b: Epilithic diatom communities from areas of invasive *Caulerpa* species (*Caulerpa taxifolia* and *Caulerpa cylindracea*) in the Adriatic Sea, NE Mediterranean. Mediterranean Marine Science 20(1), 151–173. https://doi.org/10.12681/mms.14330

Car, L., 1906: Das Microplancton der Seen des Karstes. Annales de biologie Lacustre 1, 50–56.

Casellato, S., 2008: Marine biology in the Adriatic Sea: An historical review. Proceedings of the California Academy of Sciences 59, 191–203.

Emili, H., 1958: Hidrološka istraživanja na Plitvičkim jezerima (Hydrological research on the Plitvice Lakes). In: Šafar, J. (ed.), Plitvička jezera, 173–226. Nacionalni park Plitvička jezera, Grafički zavod Hrvatske, Zagreb.

Ercegović, A., 1925: Litofitska vegetacija vapnenaca i dolomita u Hrvatskoj (Lithophytic vegetation of limestone and dolomite in Croatia). Acta Botanica Croatica 1(1), 64–114.

Ercegović, A., 1936: Études qualitative et quantitative du phytoplancton dans les eaux côtiéres de l'Adriatique oriental moyen au cours de l'année 1934. Acta Adriatica 1, 1–124.

European Community, 2000: Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. Official Journal of the European Communities L327, 1–72.

Gligora, M., Kralj, K., Plenković-Moraj, A., Hinz, F., Acs, E., Grigorszky, I., Cocquyt, C., Van de Vijver, B., 2009: Observations on the diatom *Navicula hedinii* Hustedt (Bacillariophyceae) and its transfer to a new genus *Envekadea* Van de Vijver *et al.* gen. nov. European Journal of Phycology 44(1), 123–138. https://doi.org/10.1080/09670260802389783

Gligora Udovič, M., Cvetkoska, A., Žutinić, P., Bosak, S., Stanković, I., Špoljarić, I., Mršić, G., Kralj Borojević, K., Ćukurin, A., Plenković-Moraj, A., 2017: Defining centric diatoms of most relevant phytoplankton functional groups in deep karst lakes. Hydrobiologia 788, 169–191. https://doi.org/10.1007/s10750-016-2996-z

Gligora Udovič, M., Kulaš, A., Šušnjara, M., Arapov, J., Blanco, S., Levkov, Z., 2022: *Cymbopleura amicula* stat nov. et. nom. nov. (Bacillariophyceae) - a rare diatom species from a karst river in Croatia. Phytotaxa 532(2), 139–151. https://doi.org/10.11646/phytotaxa.532.2.2

Gligora Udovič, M., Ljubešić, Z., 2021: The Croatian National Diatom Collection. Acta Botanica Croatica 80(1). Retrieved October 24, 2024 from https://www.abc.botanic.hr/index.php/abc/article/view/3184

Gligora Udovič, M., Šušnjara, M., Kulaš, A., Goreta, G., Arapov, J., Levkov, Z., 2023: One new species of *Aneumastus* D.G. Mann et Stickle (Bacillariophyceae) from Krka River, Croatia. Fottea 23(1), 21–29. https://doi.org/10.5507/fot.2022.010

Gligora Udovič, M., Žutinić, P., Kralj Borojević, K., Plenković-Moraj, A., 2015: Co-occurrence of functional groups in phytoplankton assemblages dominated by diatoms, chrysophytes and dinoflagellates. Fundamental and Applied Limnology 187(2), 101–111. https://doi.org/10.1127/fal/2015/0759

Gligora Udovič, M., Žutinić, P., Piltaver, I. K., Kulaš, A., Ozimec, R., Tofilovska, S., 2018: *Gomphosphenia plenkoviciae* sp. nov., a new species from Crveno jezero, Croatia. Phytotaxa 351(3), 229–238. https://doi.org/10.11646/phytotaxa.351.3.4

Godrijan, J., Marić, D., Imešek, M., Janeković, I., Schweikert, M., Pfannkuchen, M., 2012: Diversity, occurrence, and habitats of the diatom genus *Bacteriastrum* (Bacillariophyta) in the northern Adriatic Sea, with the description of *B. jadranum* sp. nov. Botanica Marina 55(4), 415–426. https://doi.org/10.1515/bot-2011-0021

Grbin, D., Pfannkuchen, M., Babić, I., Mejdandžić, M., Mihanović, H., Marić Pfannkuchen, D., Godrijan, J., Peharec Štefanić, P., Olujić, G., Ljubešić, Z., 2017: Multigene phylogeny and morphology of newly isolated strain of *Pseudo-nitzschia mannii* Amato & Montresor (Adriatic Sea). Diatom Research 32(1), 127-131. https://doi.org/10.1080/0269249X.2017.1284158

Grižančić, L., Baričević, A., Smodlaka Tanković, M., Vlašiček, I., Knjaz, M., Podolšak, I., Kogovšek, T., Pfannkuchen, M. A., Marić Pfannkuchen, D., 2023: A metabarcode based (species) inventory of the northern Adriatic phytoplankton. Biodiversity Data Journal 11, e106947. https://doi.org/10.3897/BDJ.11.e106947

Guiry, M. D., Guiry, G. M., 2024: AlgaeBase. World-wide electronic publication, University of Galway. Retrieved September 25, 2024 from https://www.algaebase.org

Hafner, D., Car, A., Jasprica, N., Kapetanović, T., Dupčić Radić, I., 2018a: Relationship between marine epilithic diatoms and environmental variables in oligotrophic bay, NE Mediterranean. Mediterranean Marine Science 19(2), 223–239. https://doi.org/10.12681/mms.14151

Hafner, D., Jasprica, N., Car, A., 2018b: Taxonomic survey of benthic diatoms in Neum Bay, southeastern Adriatic. Natura Croatica 27(1), 1–26. https://doi.org/10.20302/NC.2018.27.1

Hajós, M., 1973: Faciological and stratigraphic importance of the Miocene diatoms in Hungary. Beihefte zur Nova Hedwigia 45, 365–390.

Hanžek, N., Gligora Udovič, M., Kajan, K., Borics, G., Várbíró, G., Stoeck, T., Orlić, S., Stanković, I., 2023: Comparative identification of phytoplankton taxonomic and functional group approach in karst lakes using classical microscopy and eDNA metabarcoding for ecological status assessment. Hydrobiologia 851, 1015–1034. https://doi.org/10.1007/s10750-023-05344-x

Hanžek, N., Gligora Udovič, M., Kajan, K., Borics, G., Várbíró, G., Stoeck, T., Žutinić, P., Orlić, S., Stanković, I., 2021: Assessing ecological status in karstic lakes through the integration of phytoplankton functional groups, morphological approach and environmental DNA metabarcoding. Ecological Indicators 131, 108166. https://doi.org/10.1016/j.ecolind.2021.108166

Hasle, G. R., Fryxell, G. A., 1970: Diatoms: Cleaning and mounting for light and electron microscopy. Transactions of the American Microscopical Society 89(4), 469–474. https://doi.org/10.2307/3224555

Hauck, T., 1872: Ueber das Massenauftreten der Nitzschia closterium (Ehr.) Sm. in der Adria. Oesterreichische Botanische Zeitschrift 37, 253–254.

Hustedt, F., 1927-1966: Die Kieselalgen Deutschlands, Österreichs und der Schweiz mit Berücksichtigung der übrigen Länder Europas sowie der angrenzenden Meeresgebeite. In: Dr. L. Rabenhorst›s Kryptogramen-Flora von Deutschland, Österreich und der Schweiz. Band 7, 1–920, Akademische Verlagsgeselschaft, Leipzig.

Hustedt, F., 1945: Diatomeen aus Seen und Quellgebieten der Balkan-Halbinsel. Archiv für Hydrobiologie 40(4), 867–973.

Hustedt, F., 1955: Neue und wenig bekannte Diatomeen. VIII. Abhandlungen des Naturwissenschaftlichen Verein zu Bremen 34, 47–68.

Jörgensen, E., 1920: Mediterranean Ceratia. Report on the Danish oceanographical expeditions 1908-10 to the Mediterranean and adjacent seas. II Biology J1, 1–110. Copenhagen.

Jörgensen, E., 1923: Mediterranean Dinophysiaceae - Report on the Danish oceanographical expeditions 1908–1910 to the Mediterranean and adjacent seas. II Biology. 2., 1–48. Copenhagen.

Jurilj, A., 1957: Flora of Diatoms of Sarmatic Sea in Environs of Zagreb. Acta Biologica I, 28, 5–153.

Kanjer, L., Mucko, M., Car, A., Bosak, S., 2019: Epiphytic diatoms on *Posidonia oceanica* (L.) Delile leaves from eastern Adriatic Sea. Natura Croatica 28(1), 1–20. https://doi.org/10.20302/NC.2019.28.1

Kolda, A., Petrić, I., Mucko, M., Gottstein, S., Žutinić, P., Goreta, G., Ternjej, I., Rubinić, J., Radišić, M., Gligora Udovič, M., 2019: How environment selects: Resilience and survival of microbial mat community within intermittent karst spring Krčić (Croatia). Ecohydrology 12(2), 16. https://doi.org/10.1002/eco.2063

Kralj, K., Plenković-Moraj, A., Gligora, M., Primc-Habdija, B., Šipoš, L., 2006: Structure of periphytic community on artificial substrata: influence of depth, slide orientation and colonization time in karstic Lake Visovačko jezero, Croatia. Hydrobiologia 560, 249–258. https://doi.org/10.1007/s10750-005-1330-y

Kralj Borojević, K., Gligora Udovič, M., Žutinić, P., Várbíró, G., Plenković Moraj, A., 2017: Do benthic diatom assemblages reflect abiotic typology: a case study of Croatian streams and rivers. Acta Botanica Croatica 76(1), 1–11. https://doi.org/10.1515/botcro-2016-0051

Krmpotić, J., 1914: Prilog zimskoj flori i fauni Plitvičkih jezera (A contribution to the winter flora and fauna of the Plitvice Lakes). Prirodoslovna istraživanja Hrvatske i Slavonije, Jugoslavenska Akademija Znanosti i Umjetnosti 3, 26–32.

Kulaš, A., Gligora Udovič, M., Ector, L., Van de Vijver, B., 2020: Analysis of the type material of *Achnanthes hauckiana* Grunow (Achnanthaceae, Bacillariophyceae). Botany Letters 167(4), 439–452. https://doi.org/10.1080/23818107.2020.1808527

Kulaš, A., Gligora Udovič, M., Tapolczai, K., Žutinić, P., Orlić, S., Levkov, Z., 2022: Diatom eDNA metabarcoding and morphological methods for bioassessment of karstic river. Science of The Total Environment 829, 154536. https://doi.org/10.1016/j.scitotenv.2022.154536

Kulaš, A., Marković, T., Žutinić, P., Kajan, K., Karlović, I., Orlić, S., Keskin, E., Filipović, V., Gligora Udovič, M., 2021: Succession of microbial community in a small water body within the alluvial aquifer of a large river. Water 13(2), 115. https://doi.org/10.3390/w13020115

Kützing, F. T., 1844: Die Kieselschaligen. Bacillarien oder Diatomeen. Nordhausen.

Kützing, F. T., 1845: Phycologia Germanica, d.i. Deutschlands Algen in bündigen Beschreibungen. Nebst einer Anleitung zum Untersuchen und Bestimmen dieser Gewächse für Anfänger. Nordhausen: zu finden bei Wilh. Köhne.

Levkov, Z., Krstić, S., Metzeltin, D., Nakov, T., 2007: Diatoms of Lakes Prespa and Ohrid, about 500 taxa from ancient lake system. Iconographia Diatomologica 16, 1–613.

Levkov, Z., Mihalić, K. C., Ector, L., 2010: A taxonomical study of *Rhoicosphenia* Grunow (Bacillariophyceae) with a key for identification of selected taxa. Fottea 10(2), 145–200. https://doi.org/10.5507/fot.2010.010

Li, C., Gastineau, R., Turmel, M., Witkowski, A., Otis, C., Car, A., Lemieux, C., 2019: Complete chloroplast genome of the tiny marine diatom *Nanofrustulum shiloi* (Bacillariophyta) from the Adriatic Sea. Mitochondrial DNA. Part B, Resources 4(2), 3374–3376. https://doi.org/10.1080/23802359.2019.1673245

Ljubešić, Z., Bosak, S., Viličić, D., Borojević, K. K., Marić, D., Godrijan, J., Ujević, I., Peharec, P., Đakovac, T., 2011: Ecology and taxonomy of potentially toxic *Pseudo-nitzschia* species in Lim Bay (north-eastern Adriatic Sea). Harmful Algae 10(6), 713–722. https://doi.org/10.1016/j.hal.2011.06.002

Lobarzewski, H. R., 1840: Einige neue Diatomaceen Agardh et auct. der Ostküste des Adriatischen Meeres. Linnaea 14, 265–277

Lobban, C. S., Majewska, R., Ashworth, M., Bizsel, N., Bosak, S., Kooistra, W. H. C. F., Lam, D. W., Navarro, J. N., Pennesi, C., Sato, S., Van de Vijver, B., Witkowski, A., 2021: Diatom genus *Hyalosira* (Rhabdonematales emend.) and resolution of its polyphyly in Grammatophoraceae and Rhabdonemataceae with a new genus, *Placosira*, and five new *Hyalosira* species. Protist 172(3), 125816. https://doi.org/10.1016/j.protis.2021.125816

Majewska, R., Ashworth, M. P., Bosak, S., Goosen, W. E., Nolte, C., Filek, K., Van de Vijver, B., Taylor, J. C., Manning, S. R., Nel, R., 2021: On sea turtle-associated *Craspedostauros* (Bacillariophyta), with description of three novel species. Journal of Phycology 57(1), 199–218. https://doi.org/10.1111/jpy.13086

Majewska, R., Bosak, S., Frankovich, T. A., Ashworth, M. P., Sullivan, M. J., Robinson, N. J., Lazo-Wasem, E. A., Pinou, T., Nel, R., Manning, S. R., Van de Vijver, B., 2019: Six new epibiotic *Proschkinia* (Bacillariophyta) species and new insights into the genus phylogeny. European Journal of Phycology 54(4), 609–631. https://doi.org/10.1080/09670262.2019.1628307

Mann, D. G., Droop, S. J. M., 1996: 3. Biodiversity, biogeography and conservation of diatoms. Hydrobiologia 336(1), 19–32. https://doi.org/10.1007/BF00010816

Matek, A., Mucko, M., Casotti, R., Trano, A. C., Achterberg, E. P., Mihanović, H., Čižmek, H., Čolić, B., Cuculić, V., Ljubešić, Z., 2023: Phytoplankton diversity and co-dependency in a stratified oligotrophic ecosystem in the south Adriatic Sea. Water 15(12), 2299. https://doi.org/10.3390/w15122299

Medlin, L. K., Kaczmarska, I., 2004: Evolution of the diatoms: V. Morphological and cytological support for the major clades and a taxonomic revision. Phycologia 43(3), 245–270. https://doi.org/10.2216/i0031-8884-43-3-245.1

Mejdandžić, M., Bosak, S., Nakov, T., Ruck, E., Orlić, S., Gligora Udovič, M., Peharec Štefanić, P., Špoljarić, I., Mršić, G., Ljubešić, Z., 2018: Morphological diversity and phylogeny of the diatom genus *Entomoneis* (Bacillariophyta) in marine plankton: six new species from the Adriatic Sea. Journal of Phycology 54(2), 275–298. https://doi.org/10.1111/jpy.12622

Mejdandžić, M., Bosak, S., Orlić, S., Gligora Udovič, M., Peharec Štefanić, P., Špoljarić, I., Mršić, G., Ljubešić, Z., 2017: *Entomoneis tenera* sp. nov., a new marine planktonic diatom (Entomoneidaceae, bacillariophyta) from the Adriatic Sea. Phytotaxa 292(1), 001–018. https://doi.org/10.11646/phytotaxa.292.1.1

Mejdandžić, M., Ivanković, T., Pfannkuchen, M., Godrijan, J., Pfannkuchen, D. M., Hrenović, J., Ljubešić, Z., 2015: Colonization of diatoms and bacteria on artificial substrates in the northeastern coastal Adriatic Sea. Acta Botanica Croatica 74(2), 407–422. https://doi.org/10.1515/botcro-2015-0030

Mihaljević, M., Stević, F., Horvatić, J., Hackenberger Kutuzović, B., 2009: Dual impact of the flood pulses on the phytoplankton assemblages in a Danubian floodplain lake (Kopački Rit Nature Park, Croatia). Hydrobiologia 618, 77–88. https://doi.org/10.1007/s10750-008-9550-6

Mihaljević, M., Stević, F., Špoljarić, D., Pfeiffer, T. Ž., 2015: Spatial pattern of phytoplankton based on the morphology-based functional approach along a river–floodplain gradient. River Research and Applications 31(2), 228–238. https://doi.org/10.1002/rra.2739

Mihaljević, M., Špoljarić, D., Stević, F., Cvijanović, V., Hackenberger Kutuzović, B., 2010: The influence of extreme floods from the River Danube in 2006 on phytoplankton communities in a floodplain lake: Shift to a clear state. Limnologica - Ecology and Management of Inland Waters 40(3), 260–268. https://doi.org/10.1016/j.limno.2009.09.001

Mihaljević, M., Špoljarić, D., Stević, F., Žuna Pfeiffer, T., 2013: Assessment of flood-induced changes of phytoplankton along a river–floodplain system using the morpho-functional approach. Environmental Monitoring and Assessment 185, 8601–8619. https://doi.org/10.1007/s10661-013-3198-z

Miho, A., Lange-Bertalot, H., 2006: Diversity of the genus *Placoneis* in Lake Ohrid and other freshwater habitats of Albania. In: Witkowski, A. (ed.), Proceedings of the Eighteenth International Diatom Symposium, 301–313. Biopress Ltd., Bristol.

Molinari-Novoa, E. A., Guiry, M. D., 2021: Nomenclatural notes on algae. V. New names for various algal taxa, mostly at the species rank. Notulae Algarum 184, 1–17.

Mozetič, P., Cangini, M., Francé, J., Bastianini, M., Bernardi Aubry, F., Bužančić, M., Cabrini, M., Cerino, F., Čalić, M., D'Adamo, R., Drakulović, D., Finotto, S., Fornasaro, D., Grilli, F., Kraus, R., Kužat, N., Marić Pfannkuchen, D., Ninčević Gladan, Ž., Pompei, M., Rotter, A., Servadei, I., Skejić, S., 2019: Phytoplankton diversity in Adriatic ports: Lessons from the port baseline survey for the management of harmful algal species. Marine Pollution Bulletin 147, 117–132. https://doi.org/10.1016/j.marpolbul.2017.12.029

Mucko, M., Bosak, S., Casotti, R., Balestra, C., Ljubešić, Z., 2018: Winter picoplankton diversity in an oligotrophic marginal sea. Marine Genomics 42, 14–24. https://doi.org/10.1016/j.margen.2018.09.002

Mucko, M., Bosak, S., Mann, D. G., Trobajo, R., Wetzel, C. E., Peharec Štefanić, P., Ljubešić, Z., 2020: A polyphasic approach to the study of the genus *Nitzschia* (Bacillariophyta): Three new planktonic species from the Adriatic Sea. Journal of Phycology 57(1), 143–159. https://doi.org/10.1111/jpy.13085

Nenadović, T., Šarčević, T., Čižmek, H., Godrijan, J., Marić Pfannkuchen, D., Pfannkuchen, M., Ljubešić, Z., 2015: Development of periphytic diatoms on different artificial substrates in the Eastern Adriatic Sea. Acta Botanica Croatica 74(2), 377–392. https://doi.org/10.1515/botcro-2015-0026

Nikolić, T. (ed.), 2024: *Flora Croatica Database*. Faculty of Science, University of Zagreb. Retrieved September 15, 2024 from https://hirc.botanic.hr/fcd/

Odell, S. G., Lazo, G. R., Woodhouse, M. R., Hane, D.L., Sen, T. Z., 2017: The art of curation at a biological database: Principles and application. Current Plant Biology 11–12, 2–11 https://doi.org/10.1016/j.cpb.2017.11.001

Penna, A., Casabianca, S., Perini, F., Bastianini, M., Riccardi, E., Pigozzi, S., Scardi, M., 2013: Toxic *Pseudo-nitzschia* spp. in the northwestern Adriatic Sea: characterization of species composition by genetic and molecular quantitative analyses. Journal of Plankton Research 35(2), 352–366. https://doi.org/10.1093/plankt/fbs093

Pevalek, I., 1919: Prilog poznavanju alga Hrvatske i Slavonije (A contribution to the knowledge of the algae of Croatia and Slavonia). Prirodoslovna istraživanja Hrvatske i Slavonije, Jugoslavenska Akademija Znanosti i Umjetnosti 14, 153–162.

Pevalek, I., 1925: Oblici fitogenih inkrustacija i sedre na Plitvičkim jezerima i njihovo geološko znamenovanje (Forms of phytogenic encrustations and tufa on Plitvica lakes and their geological designation). Glasnik Hrvatskog Prirodoslovnog Društva 37/38, 101–110.

Pevalek, I., 1935: Der Travertin und die Plitvice Seen. Verhandle Internationalen Verein Limnologie 7, 165–181.

Pevalek, I., 1938: Biodinamika Plitvičkih jezera i njihova zaštita (Biodynamics of the Plitvice Lakes and their protection). Zaštita Prirode 1, 40–61.

Pinseel, E., Kulichová, J., Scharfen, V., Urbánková, P., de Vijver, B. V., Vyverman, W., 2019: Extensive cryptic diversity in the terrestrial diatom *Pinnularia borealis* (Bacillariophyceae). Protist 170(2), 121–140. https://doi.org/10.1016/j.protis.2018.10.001

Plenković, A., Marčenko, E., Srdoč, D., 1989: Periphyton growth on glass slides in aquatic ecosystems of the National Park Plitvice Lakes. Periodicum Biologorum 91/1, 88–90.

Plenković-Moraj, A., 1995: Diatoms (Bacillariophyceae) of the Croatian freshwaters. Acta Botanica Croatica 54(1), 22–33.

Plenković-Moraj, A., 1996: Croatian freshwater Cyanophyceae. Natura Croatica 5(4), 299–315.

Plenković-Moraj, A., Kralj, K., Gligora, M., 2008: The effect of current velocity on diatom colonization on the glass slides in unpolluted headwater creek. Periodicum Biologorum 110(3), 291–295.

Pletikapić, G., Berquand, A., Radić, T. M., Svetličić, V., 2012: Quantitative nanomechanical mapping of marine diatom in seawater using peak force tapping atomic force microscopy. Journal of Phycology 48(1), 174–185. https://doi.org/10.1111/j.1529-8817.2011.01093.x

Revelante, N., 1985: A catalogue of phytoplankton reported for the Rovinj area of the northern Adriatic. Thalassia Jugoslavica 21, 139–169.

Rotter, A., Barbier, M., Bertoni, F., Bones, A. M., Cancela, M. L., Carlsson, J., Carvalho, M. F., Cegłowska, M., Chirivella-Martorell, J., Conk Dalay, M., Cueto, M., Dailianis, T., Deniz, I., Díaz-Marrero, A. R., Drakulovic, D., Dubnika, A., Edwards, C., Einarsson, H., Erdoğan, A., Eroldoğan, O. T., Ezra, D., Fazi, S., FitzGerald, R. J., Gargan, L. M., Gaudêncio, S. P., Gligora Udovič, M., Ivošević DeNardis, N., Jónsdóttir, R., Kataržytė, M., Klun, K., Kotta, J., Ktari, L., Ljubešić, Z., Lukić Bilela, L., Mandalakis, M., Massa-Gallucci, A., Matijošytė, I., Mazur-Marzec, H., Mehiri, M., Nielsen, S. L., Novoveská, L., Overlingė, D., Perale, G., Ramasamy, P., Rebours, C., Reinsch, T., Reyes, F., Rinkevich, B., Robbens, J., Röttinger, E., Rudovica, V., Sabotič, J., Safarik, I., Talve, S., Tasdemir, D., Theodotou Schneider, X., Thomas, O. P., Toruńska-Sitarz, A., Varese, G. C., Vasquez, M. I., 2021: The essentials of marine biotechnology. Frontiers in Marine Science 8, 1–53. https://doi.org/10.3389/fmars.2021.629629

Ruck, E. C., Nakov, T., Alverson, A. J., Theriot, E. C., 2016: Phylogeny, ecology, morphological evolution, and reclassification of the diatom orders Surirellales and Rhopalodiales. Molecular Phylogenetics and Evolution 103, 155–171. https://doi.org/10.1016/j.ympev.2016.07.023

Schiller, J., 1913a: Bericht über die bot. Unters, und deren vorl. Ergebnisse der III. KreuzungS. M. S. „Najade« im Sommer 1911. Glasnik Hrvatskog Prirodoslovnog Društva za godinu 1913. I. Rasprave, 216–217.

Schiller, J., 1913b: Vorläufige Ergebnisse der Phytoplankton Untersuchungen auf den Fahrten S.M.S. «Najade« in der Adria 1911–1912. I Die Coccolithophoriden. Sitzungsberichte der kaiserlichen akademie der wissenschaften mathematisch-naturwissenschaftliche klasse 122, 597–617.

Schiller, J., 1925a: Die planktischen Vegetationen des Adriatischen Meeres. A. Die Coccolithophoriden Vegetation in den Jahren 1911–14. Archiv für protistenkunde 51, 1–130.

Schiller, J., 1925b: Die planktischen Vegetationen des Adriatischen Meeres. B. Chrysomonadina, Heterokontae, Cryptomonadina, Eugleninae, Volvocales. 1. Systematischer Teil. Archiv für protistenkunde 53, 59–124.

Schiller, J., 1933: Dinoflagellatae. Rabenhorst's Kryptogamen-Flora von Deutschland, Österreich und der Schweiz. Akademische Verlagsgeselschaft, Leipzig.

Schröder, B., 1911: Adriatisches Phytoplankton. Sitzungsberichte der Kaiserlichen akademie der wissenschaften mathematisch-naturwissenschaftliche klasse 120, 601–657.

Schussnig, B., 1914: Aus der Biologie des adriatischen Phytoplanktons. Verhandlungen der zoologischbotanischen gesellschaft in Wien 64, 299–309.

Simonsen, R., 1987: Atlas and catalogue of the diatom types of Friedrich Hustedt. J. Cramer, Berlin, Stuttgart.

Šimunović, M., Kulaš, A., Žutinić, P., Goreta, G., Gligora Udovič, M., 2022: Phytoplankton metrics for trophic and ecological status assessment of a natural karstic lake. Acta Botanica Croatica 81(2), 185–196. https://doi.org/10.37427/botcro-2022-016

Seveno, J., Car, A., Sirjacobs, D., Fullgrabe, L., Dupčić Radić, I., Lejeune, P., Leignel, V., Mouget, J-L., 2023: Benthic diatom blooms of blue *Haslea* spp. in the Mediterranean Sea. Marine Drugs 21(11), 583. https://doi.org/10.3390/md21110583

Seveno, J., Witkowski, A., Car, A., Gastineau, R., Sirjacobs, D., Leignel, V., Mouget, J-L., 2024: Impact of blue *Haslea* spp. blooms on benthic diatom and bacterial communities. Phycology 4(3), 465–507. https://doi.org/10.3390/phycology4030027

Skvortsov, B. V., 1971: Diatoms from Yenisei River and its tributaires, middle part of Siberia, western Asia. Philippine Journal of Science 98, 57–113.

Smodlaka Tanković, M., Baričević, A., Gerić, M., Domijan, A. M., Pfannkuchen, D. M., Kužat, N., Ujević, I., Kuralić, M., Rožman, M., Matković, K., Novak, M., Žegura, B., Pfannkuchen, M., Gajski, G., 2022: Characterisation and toxicological activity of three different *Pseudo-nitzschia* species from the northern Adriatic Sea (Croatia). Environmental Research 214(4), 114108. https://doi.org/10.1016/j.envres.2022.114108

Spaulding, S. A., Potapova, M. G., Bishop, I. W., Lee, S. S., Gasperak, T. S., Jovanoska, E., Furey, P. C., Edlund, M. B., 2021: Diatoms.org : supporting taxonomists, connecting communities. Diatom Research 36(4), 291–304. https://doi.org/10.1080/0269249X.2021.2006790

Stanković, I., Udovič, M.G., Šušnjara, M., Alegro, A., Levkov, Z., 2022: Diversity and ecology of benthic diatoms in a mire with narrow pH gradient. Fottea 22(1), 39–55. https://doi.org/10.5507/fot.2021.015

Stanković, I., Vlahović, T., Gligora Udovič, M., Várbíró, G., Borics, G., 2012: Phytoplankton functional and morpho-functional approach in large floodplain rivers. Hydrobiologia 698, 217–231. https://doi.org/10.1007/s10750-012-1148-3

Stević, F., Mihaljević, M., Špoljarić, D., 2013: Changes of phytoplankton functional groups in a floodplain lake associated with hydrological perturbations. Hydrobiologia 709, 143–158. https://doi.org/10.1007/s10750-013-1444-6

Trábert, Z., Buczkó, K., Borics, G., Gligora Udovič, M., Karthick, B., Ector, L., Földi, A., Kiss, K.T.; Ács, É., 2019: Biogeography and morphology of a poorly known diatom *Dorofeyukea rostellata* (Hustedt) Kulikovskiy & Kociolek. Limnetica 38(2), 743–758. https://doi.org/10.23818/limn.38.43

Turk Dermastia, T., Vascotto, I., Francé, J., Stanković, D., Mozetič, P., 2023: Evaluation of the rbcL marker for metabarcoding of marine diatoms and inference of population structure of selected genera. Frontiers in Microbiology 14, 1071379. https://doi.org/10.3389/fmicb.2023.1071379

Van De Vijver, B., Robert, K., Witkowski, A., Bosak, S., 2020: *Majewskaea* gen. nov. (Bacillariophyta), a new marine benthic diatom genus from the Adriatic Sea. Fottea 20(2), 112–120. https://doi.org/10.5507/fot.2020.001

Vaulot, D., Sim, C. W. H., Ong, D., Teo, B., Biwer, C., Jamy, M., Lopes dos Santos, A., 2022: metaPR2: A database of eukaryotic 18S rRNA metabarcodes with an emphasis on protists. Molecular Ecology Resourses 22(8), 3188–3201. https://doi.org/10.1111/1755-0998.13674

Viličić, D., Ljubešić, Z., 2017: Razvoj metoda istraživanja fitoplanktona u Jadranskom moru (Development of phytoplankton research methods in the Adriatic Sea). Hrvatske vode 2, 49–58.

Viličić, D., Marasović, I., Mioković, D., 2002: Checklist of phytoplankton in the eastern Adriatic Sea. Acta Botanica Croatica 61(1), 57–91.

Vouk, V., 1936: Komparativno-biološke studije o termama (Comparative-biological studies on thermae). Jugoslavenska Akademija Znanosti i Umjetnosti 256/80, 195–228.

Vouk, V., 1947: Biologijska svojstva termalnih voda i njihovo značenje za balneologiju (Biological properties of thermal waters and their significance for balneology). Jugoslavenska Akademija Znanosti i Umjetnosti 273, 5–25.

Vouk. V., 1948: Thermal-vegetation and ecological valences theory. Hydrobiologia 1, 90–95.

Žutinić, P., Gligora Udovič, M., Kralj Borojević, K., Plenković-Moraj, A., Pádisak, J., 2014: Morpho-functional classifications of phytoplankton assemblages of two deep karstic lakes. Hydrobiologia 740, 147–166. https://doi.org/10.1007/s10750-014-1950-1

Žutinić, P., Kulaš, A., Levkov, Z., Šušnjara, M., Orlić, S., Kukić, S., Goreta, G., Valić, D., Gligora Udovič, M., 2020: Ecological status assessment using periphytic diatom communites - case study Krka River. Macedonian Journal of Ecology and Environment 22(1), 29–44. https://doi.org/10.59194/MJEE20221029zh

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Review Article

# Post-fire succession of black pine (*Pinus nigra*) forest vegetation under different fire regimes

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**Abstract** – The black pine is a characteristic Mediterranean pine species and extends from Spain eastward to Southern France, Italy, Austria, the Balkans and Türkiye. Black pine is a fire tolerator and has a distinctive natural fire regime differently from the serotinous red pine and Aleppo pine forests in Mediterranean. Black pine forests are one of the ecosystems that are most affected by changing fire regimes. Fires in these forests generally occur as low intensity surface fires. The black pine is a light-demanding species and in pure stands it forms single layer structure with self-pruning. It also has a thick bark. Therefore, heat during surface fires does not have a lethal effect on the trees and crown fires will not be generated due to self-pruning. However, with changing climatic conditions and forest structure based on human use and management, the fires in black pine forests have begun to become crown fires in large areas. Since the black pine does not have any adaptation to crown fires, the trees are killed and no regeneration occurs. This process results in the degradation of black pine forests into other vegetation types such as oak woodlands, rock rose (*Cistus* spp.) scrublands or bracken fern (*Pteridium aquilinum* L. (Kuhn)) herbaceous vegetation. This holds true for the whole of the distribution of the black pine in the Mediterranean. Therefore, a silvicultural approach ensuring fires occur as surface fires only should be employed in the existing black pine forests and the areas degraded after fires should be restored in an ecological way to re-establish the black pine.

**Keywords:** black pine, crown fire, degradation, fire regime, Mediterranean, succession, surface fire, vegetation

## Introduction

The genus *Pinus* is represented by 122 species worldwide (POWO 2024). They are mainly distributed in the northern hemisphere and form forests in a wide geographical belt between the 2nd and 70th latitudes (Nobis et al. 2012). A widely distributed pine species is the black pine (*Pinus nigra* J.F.Arnold). It is a characteristic Mediterranean pine species with a natural geographical distribution extending from Spain eastward to Southern France, Italy, Austria, the Balkans and Türkiye (Christopoulou et al. 2014). It is also found in parts of north-west Africa, some Mediterranean islands (Corsica, Sicily, Cyprus and some Aegean islands) and the Crimean Peninsula in the Black Sea (Moreira et al. 2011). Within the Mediterranean basin, it has the widest distribution in Türkiye (Sevgi and Akkemik 2007).

The taxonomy of the species has a very complicated history (Akkemik 2020), but currently it includes five subspecies: *Pinus nigra* J.F. Arnold subsp. *nigra*, *P. nigra* subsp. *laricio* Palib. ex Maire, *P. nigra* subsp. *dalmatica* (Vis.) Franco, *P. nigra* subsp. *salzmannii* (Dunal) Franco and *P. nigra* subsp. *pallasiana* (Lamb.) Holmboe (Farjon 2010, POWO 2024). The distribution of these subspecies, which are scattered throughout the Mediterranean basin, varies geographically (Alptekin 1986, Enescu et al. 2016). *Pinus nigra* subsp. *nigra*, known as the Austrian black pine, extends from Austria to central Italy and has a natural distribution in Greece and the Balkans. The Corsican black pine (*P. nigra* subsp. *laricio*) is distributed in Southern Italy, Sicily and Corsica. The Dalmatian black pine (*P. nigra* subsp. *dalmatica*) is found in the north-east of the Adriatic coast and on the Croatian islands. The Pyrenean black pine (*P. nigra* subsp. *salzmannii*) occurs naturally in the Pyrenees Mountains, central and southern Spain and North Africa. The Anatolian black pine (*P. nigra* subsp. *pallasiana*) is distributed mainly in Anatolia, the Southern Carpathians, Crimea, Cyprus and Syria.

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The species is generally located in the supra-Mediterranean and sub-Mediterranean zones of the Mediterranean region and is a characteristic pine species of this region (Enescu et al. 2016). Forest fires have a decisive role in shaping the vegetation in the Mediterranean region (Pausas and Keeley 2009). Forests of the red pine (*P. brutia* Ten.), Aleppo pine (*P. halepensis* Mill.) and maritime pine (*P. pinaster* Aiton), which are widely distributed in the Mediterranean region (Bonari et al. 2021), are frequently exposed to fires and rapidly revegetate by means of their serotinous cones (Ne'eman and Arianoutsou 2021). Indeed, what is important here is not the ability of the species to adapt to fire, but their adaptation to the natural fire regime of the ecosystem (Sugihara et al. 2006, Keeley et al. 2011).

Fire regime is a concept that describes the mean and range of variation in characteristics like frequency, periodicity, intensity, severity and patchiness of fire (Krebs et al. 2010). There is a mutual interaction between the fire regime and vegetation; while the fire regime depends on the type of vegetation and its flammable load capacity, the vegetation can also tolerate the effects of fire with its adaptation capability (Sugihara et al. 2006). Therefore, if the fire regime experiences a major change, it may have a negative impact on the ecosystem's post-fire regeneration ability and changes in vegetation structure may occur (Keeley et al. 2011).

The majority of fires (more than 90%) in the Mediterranean basin are caused by human activities (San-Miguel-Ayanz et al. 2023). Ecosystems dominated by species that rejuvenate from seeds (obligate seeders) are particularly sensitive to changes in fire frequency (Pausas et al. 2008). The increased frequency of fire recurrence in red pine and Aleppo pine forests prevents these forests from reaching the maturity needed to create a sufficient crown seed bank. This causes species to fail to rejuvenate after fire, and forest ecosystems can be replaced by maquis or other vegetation types (Kavgacı et al. 2016).

Black pine forests too have a natural fire regime. However, this regime is characterized by a relatively high fire frequency and low intensity fires, unlike the thermo- and meso-Mediterranean pine ecosystems of red pine and Aleppo pine, which support highly flammable foliage and require fires with higher temperature to regenerate, but which cannot survive at high fire frequencies due to lack of sufficient time for canopy seed banking (Ne'eman and Arianoutsou 2021). As a result of the fire adaptation characteristics of black pine, it also differs notably from red pine and Aleppo pine. The triggering effects of both human activities and climate change on fires (Meehl and Tebaldi 2004, Dequé 2007, Beniston et al. 2007) are causing changes in the fire regime of black pine forests that affect vegetation recovery. This effect can be expected to be harsher than thermo- and meso-Mediterranean pine ecosystems, whose recovery after fire depends on a crown seed bank.

This study was prepared in this context and an assessment was done on the post-fire recovery of black pine forests in Mediterranean under the current fire regime and potential effects of a changing fire regime on post-fire vegetation dynamics. In order to accurately understand the interaction between the changing fire regime and black pine forests, it is important to know their biological, ecological and vegetative characteristics. Therefore, first the biology, ecology, general vegetation characteristics and structural features are explained. Subsequently, the interactions between black pine forests and fire are discussed, the changes that may occur in the vegetation structure under changing conditions are evaluated, and based on those, suggestions for post-fire restoration practices are submitted.

## Biology and ecology of black pine forests

The black pine is a tree species that can reach 30-35 m height; mature stems have deep cracks and very thick bark (Yaltırık and Efe 1994, Fig. 1). Its resinous buds are cylindrical

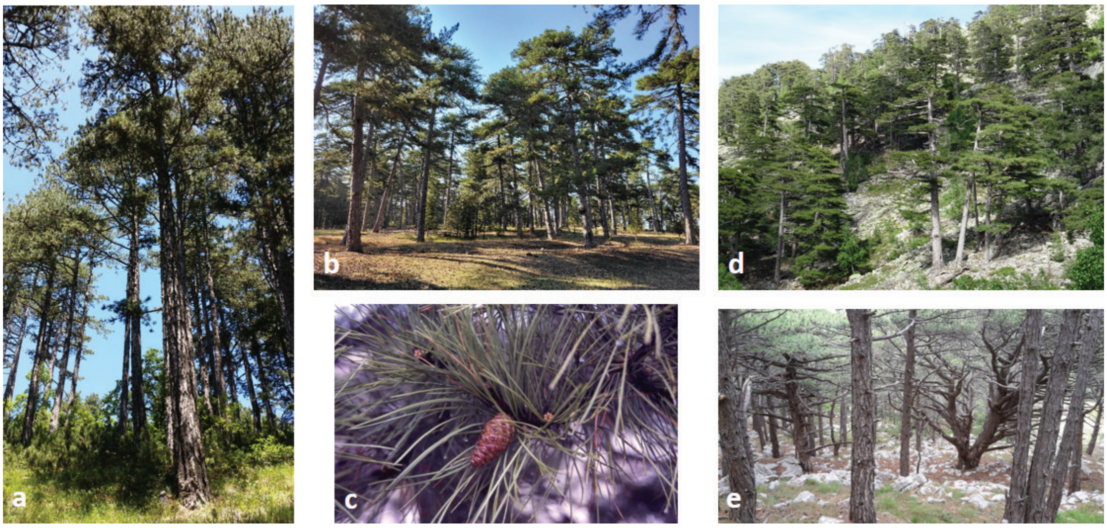

**Fig. 1.** Physiognomy of black pine forests in the Mediterranean: a – *Pinus nigra* subsp. *nigra* forests from Northern Macedonia, b – *P. nigra* subsp. *pallasiana* from Mugla in Türkiye, c – cone and shoot of *P. nigra* subsp. *salzmannii* from Spain, d – *P. nigra* subsp. *laricio* from Italy, e – *P. nigra* subsp. *dalmatica* from Croatia. Photo: D. Mandžukovski (a), E.S. Keleş (b), J. Loidi (c), G. Bonari (d), D. Krstonošić (e).

in shape and have a suddenly tapering structure at the ends. Leaves are 8-15 cm long, dark green and quite hard. The leaves at the ends of the shoots turn towards the bud, forming a bowl. Black pine is a monoecious species. The cones, 4-8 cm long and 2-4 cm wide, are stemless. Cones mature in two years and scatter their seeds from late autumn to early spring. When seeds are exposed to high temperatures for a long time, they lose their ability to germinate (Turna and Bilgili 2006). It is a relatively light-demanding tree, but it can tolerate shading to some extent at high productive sites.

Although the black pine is a species of the Mediterranean basin, it can appear under different ecological conditions within the basin. In addition to the Mediterranean distribution, it is also widely distributed in temperate-humid or cold-humid climates (Enescu et al. 2016). The coastal areas of the Black Sea, the mountainous regions of Northern Anatolia, the Balkans, central Europe and the Pyrenees are characterized by humid climate conditions, in which the black pine is abundant. The black pine also grows extensively in semi-humid and semi-arid climates such as the inner parts of the Black Sea and Sea of Marmara (Atalay and Efe 2012). The species is also distributed in the regions close to the steppe and has become evident as one of the pine species most frequently introduced into the steppe (Mayer and Aksoy 1986). Its optimum distribution appears to be between 700-1400 m above sea level (a.s.l.), but, it is also found in lower and higher elevation zones. It is resistant to both summer drought and heat and winter cold, grows on very different bedrocks such as limestone, ultramafic, dolomite and silicate. It is a moderate species in terms of soil requirements. The black pine is also widely planted outside of its natural distribution range (Mikulová et al. 2019, Čahojová et al. 2024).

## Vegetation characteristics of black pine forests

As noted above the black pine has a broad geographic distribution and it is found in different climate zones, on different bedrocks and in an elevational gradient from meso- and supra-Mediterranean to sub-Mediterranean. These environmental differences cause a variation in species composition and thus the formation of different vegetation types. Since the post-fire regeneration capability of species differ, variation in species composition is important for post-fire vegetation dynamics.

According to Mucina et al. (2016), black pine dominated forests are classified in two classes. These are *Junipero-Pinetea sylvestris*, forming relict oro-Mediterranean and sub-Mediterranean orotemperate dry pine forest and *Erico-Pinetea* representing relict pine forests on calcareous and ultramafic substrates of the Balkans, the Alps, the Carpathians and Crimea.

Within *Junipero-Pinetea sylvestris*, the alliance *Berberido aetnensis-Pinion laricionis* represents the acidophilous dry pine forests in the supra-Mediterranean belt of Corsica, Sardinia, Sicily and Calabria. As an order the alliance is grouped under *Berberido creticae-Juniperetalia excelsae*.

Within *Erico-Pinetea*, there are six alliances, in which black pine dominated forests are classified. These are *Fraxino orni-Pinion nigrae* representing relic black pine forests on calcareous substrates of the South Adriatic, Central and Southern Balkans; *Junipero hemisphaericae-Pinion nigrae* for the natural black pine forests on calcareous substrates of the central-southern Apennines in the supratemperate thermotype; *Erico-Fraxinion orni*, which forms relict black pine forests on dolomite and ultramafic substrates of the Dinarides; *Chamaecytiso hirsuti-Pinion pallasianae* indicating relict black pine forests on calcareous, dolomitic and ophiolithic rocky slopes of the Southern Balkans and finally the Anatolian alliances *Cisto laurifolii-Pinion pallasianae* and *Adenocarpo complicati-Pinion pallasianae* distributed in temperate and Mediterranean Anatolia respectively (Kavgacı et al. 2021, 2023).

Apart from the Central and Southern Balkan and Apennine alliances, the others are classified under the order *Erico-Pinetalia*. *Fraxino orni-Pinion nigrae*, *Chamaecytiso hirsuti-Pinion pallasianae* and *Junipero hemisphaericae-Pinion nigrae* are classified under a newly defined order *Junipero communis-Pinetalia nigrae* (Biondi and Allegrezza 2019). Differently from all these vegetation units, the black pine forests in Spain were grouped within the *Querco-Fagatea* order (Regato-Pajeres and Elana-Rosselló 1995).

## Effects of fire on black pine forest vegetation

Black pine forests are found in relatively fire-prone areas. With changing climatic conditions, these areas are likely to experience more frequent and potentially more intensive fires. And indeed, data show that in recent years, these forests have been exposed to fires more frequently and across larger areas than in the past (Christopoulou et al. 2013).

Pines are generally classified under two groups, as fire-avoiders and fire-adapted pines (Keeley 2012). Fire-adapted pines are also subdivided in accordance with their syndromes: fire-tolerator pine syndrome, fire-embracer pine syndrome

**Tab. 1.** Fire adaptation traits of pines (*Pinus*) frequently exposed to forest fires in the Mediterranean. + and – indicate whether the trait is present for the tree species or not. Based on Keeley (2012) and Ne'eman and Arianoutsou (2021).

| Fire adaptation trait | *P. nigra* | *P. brutia* | *P. halepensis* | *P. pinaster* |
|---|---|---|---|---|
| Bark thickness | + | +/- | +/- | + |
| Self-pruning | + | – | – | + |
| Serotinous cones | – | + | + | – |
| Heat stimulated germination | – | + | + | – |

and fire-refugia pine syndrome. Under these groups, the black pine is a fire tolerator species exposed to surface fires where trees survive following repeated fires by means of their height, self-pruning of lower branches, thick bark, and where their long, highly flammable needles foment frequent fire that kills competitors and reduces surface fuels (Tab. 1). Bark thickness in pines is a fire adaptation trait that originated during the Cretaceous Period at least 126 Ma ago in association with surface fires (He et al. 2012).

Within the natural fire regime, fires in black pine forests generally occur as surface fires. At this point a number of tree-ring based fire reconstructions have been carried out in black pine forests. All of these studies show active fire histories in black pine forests over many centuries, with fire return intervals linked to climate variation and human activities, and ranging from ca. 5-40 years (Touchan et al. 2012, Christopoulou et al. 2013, Şahan et al. 2021, 2022). Since the black pine is light-demanding and intolerant of shade, it generally forms a single-layer stand structure. However, it can also form layered stand structures depending on the site history, productivity, canopy and light conditions, and tree species mixture (Sevgi et al. 2022). Due to the presence of surface fuels, laddering from tall understory vegetation, and crown continuity, such sites are susceptible to crown fires, which are deleterious to black pines.

The black pine is an important tree species in Europe and the Mediterranean for commercial, restoration and conservation purposes (e.g. Isajev et al. 2004, Beltrán et al. 2018, Barčić et al. 2022, Vacek et al. 2023). Numerous studies have highlighted the threat that crown fires pose to black pine populations across its range (Christopoulou et al. 2013, Beltrán et al. 2018, Şahan et al. 2022). Suppression of low intensity fires, land abandonment, and inappropriate management practices have led to densification of black pine forests, invasion of fire intolerant species, and accumulation of live and dead fuels that in combination are changing the fire regime in these ecosystems (Moreira et al. 2020). In addition, the increasing risk of high severity fire in black pine forests is greatly exacerbated by current and projected climate trends (Moriondo et al. 2006). Below, we summarize the impacts of surface and crown fires on black pine forests and consider management implications of these important changes to black pine habitats. To conclude we suggest a set of management principles that will help ameliorate the threats posed to the black pine by the growing threat of catastrophic wildfire.

## Surface fires in black pine forests

The main fire adaptation characteristics of black pine are thick stem bark, self-pruning and a dense shoot structure that protects the bud (Retana et al. 2002). The thick bark structure prevents the energy released in a surface fire from having a lethal effect on the cambium. Due to the self-pruning and the absence of an understory layer in a stand, surface fires cannot turn into crown fires. Additionally, owing to the dense shoot structure, fire intensity cannot have a negative effect on the buds. Thus, in a surface fire, the fire intensity remains low and the trees can continue their existence while preserving their vitality (Keleş and Kavgacı 2022, Tab. 2, Fig. 2a).

**Tab. 2.** Percentage and fidelity synoptic table of 2nd year vegetation sampling after the 2021 fire in Yılanlı, Muğla – Türkiye. First and second columns of each study area represent the percentage frequencies and fidelity measure of each species, respectively. Phi coefficient for fidelity is 0.30. Fidelity was calculated by using Juice program (Tichý 2002). Grey shaded species represent the diagnostic ones for each study area. The black pine either fails to rejuvenate in crown fire areas or shows very weak regeneration, insufficient to re-establish a forest. The nomenclature of species follows Euro+Med 2006+.

| Study area number | **1** | | **2** | | **3** | | **4** | | **5** | | **6** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fire severity | Surface | | Surface | | Crown | | Crown | | Unburned | | Unburned | |
| Aspect | Northern | | Southern | | Northern | | Southern | | Northern | | Southern | |
| Nr. of relevés | 10 | | 10 | | 10 | | 10 | | 10 | | 10 | |
| Nr. of species | 45 | | 41 | | 97 | | 86 | | 30 | | 33 | |
| *Pinus nigra* | 100 | 25.8 | 100 | 25.8 | 40 | | 10 | | 100 | 25.8 | 100 | 25.8 |
| **Diagnostic species** | | | | | | | | | | | | |
| *Pteridium aquilinum* | 90 | 61.2 | | | 10 | | | | 50 | | 20 | |
| *Crepis reuteriana* | 40 | 59.8 | | | | | | | | | | |
| *Lathyrus cicera* | 30 | 41.8 | | | | | 10 | | | | | |
| *Securigera varia* | 30 | 41.8 | | | | | 10 | | | | | |
| *Digitalis cariensis* | 70 | 36.9 | | | 50 | | 60 | 27.2 | 10 | | | |
| *Trifolium tomentosum* | | | 20 | 41.5 | | | | | | | | |
| *Poaceae* | | | 20 | 41.5 | | | | | | | | |
| *Acanthus spinosus* | 10 | | 10 | | 100 | 85 | 10 | | | | | |
| *Buglossoides arvensis* | | | 10 | | 80 | 81.4 | | | | | | |
| *Rubus canescens* | | | | | 90 | 78.3 | | | 30 | | | |
| *Trifolium tomentosum* | | | | | 60 | 74.5 | | | | | | |

**Tab. 2.** Continued.

| | | | | | | |
|---|---|---|---|---|---|---|
| *Hypericum perforatum* | | | 60$^{74.5}$ | | | |
| *Arabis verna* | 20 | | 90$^{70.5}$ | | 30 | |
| *Veronica cymbalaria* | | | 60$^{61.4}$ | 10 | 10 | |
| *Verbascum cheiranthifolium* | 40 | 40 | 100$^{60.9}$ | 30 | | |
| *Trifolium arvense* | | | 40$^{59.8}$ | | | |
| *Senecio vulgaris* | | 10 | 50$^{59.6}$ | | | |
| *Nepeta nuda* | 10 | | 70$^{55.9}$ | 40 | | |
| *Silene italica* | 30 | | 60$^{52}$ | 10 | | |
| *Galium setaceum* | | | 30$^{51.3}$ | | | |
| *Arrhenatherum palaestinum* | | | 30$^{51.3}$ | | | |
| *Cerastium brachypetalum* | 10 | | 60$^{48.2}$ | 30 | | 10 |
| *Bromus sterilis* | | | 60$^{44.7}$ | 40 | | 20 |
| *Campanula lyrata* | 30 | | 70$^{43.8}$ | | 50 | 10 |
| *Allium scorodoprasum* | | | 30$^{41.8}$ | 10 | | |
| *Filago arvensis* | | 10 | 30$^{41.8}$ | | | |
| *Lactuca intricata* | | | 30$^{41.8}$ | 10 | | |
| *Bromus tectorum* | | | 30$^{41.8}$ | 10 | | |
| *Teucrium chamaedrys* | | | 20$^{41.5}$ | | | |
| *Cistus salviifolius* | | | 20$^{41.5}$ | | | |
| *Trifolium scabrum* | | | 20$^{41.5}$ | | | |
| *Centaurea virgata* | | | 20$^{41.5}$ | | | |
| *Vulpia myuros* | | | 20$^{41.5}$ | | | |
| *Asyneuma virgatum* | | | 20$^{41.5}$ | | | |
| *Bromus squarrosus* | | | 20$^{41.5}$ | | | |
| *Erysimum smyrnaeum* | | | 20$^{41.5}$ | | | |
| *Rhus coriaria* | | 10 | 40$^{39.5}$ | 20 | | |
| *Chondrilla juncea* | | 10 | 40$^{35.1}$ | 30 | | |
| *Briza humilis* | | | | 90$^{93.9}$ | | |
| *Valerianella species* | | 10 | | 80$^{81.4}$ | | |
| *Alyssum simplex* | | | | 70$^{81.3}$ | | |
| *Trigonella spruneriana* | | | | 70$^{81.3}$ | | |
| *Velezia pseudorigida* | | 10 | | 70$^{74.5}$ | | |
| *Ononis reclinata* | | 20 | 40 | 100$^{74.2}$ | | |
| *Astragalus oxytropifolius* | | 10 | 10 | 70$^{68.9}$ | | |
| *Glaucium corniculatum* | | | | 50$^{67.4}$ | | |
| *Linum corymbulosum* | | 10 | 40 | 80$^{63.3}$ | | |
| *Caryophyllaceae* | | | | 40$^{59.8}$ | | |
| *Alkanna tubulosa* | | | | 40$^{59.8}$ | | |
| *Origanum sipyleum* | 20 | 30 | 30 | 80$^{53.9}$ | | |
| *Euphorbia falcata* | | | | 30$^{51.3}$ | | |
| *Polygonum species* | | | | 30$^{51.3}$ | | |
| *Clypeola jonthlaspi* | | | | 30$^{51.3}$ | | |
| *Echinops ritro* | | 10 | | 40$^{51.2}$ | | |
| *Verbascum cariense* | | | 10 | 40$^{51.2}$ | | |
| *Picnomon acarna* | 10 | 50 | 10 | 70$^{46.5}$ | 10 | |
| *Sanguisorba minor* | 40 | | 30 | 70$^{46.5}$ | | 10 |
| *Tragopogon porrifolius subsp. longirostris* | | | 10 | 30$^{41.8}$ | | |
| *Centaurea urvillei* | | | 10 | 30$^{41.8}$ | | |
| *Ziziphora tenuior* | | | 10 | 30$^{41.8}$ | | |

**Tab. 2.** Continued.

| | | | | | | |
|---|---|---|---|---|---|---|
| *Malva cretica* | | | | 20 $^{41.5}$ | | |
| *Minuartia multinervis* | | | | 20 $^{41.5}$ | | |
| *Astragalus condensatus* | | | | 20 $^{41.5}$ | | |
| *Origanum minutiflorum* | | | | 20 $^{41.5}$ | | |
| *Euphorbia rigida* | | | 10 | 30 $^{35.1}$ | | 10 |
| *Populus tremula* | | | | | 60 $^{74.5}$ | |
| *Quercus cerris* | | | | | 30 $^{51.3}$ | |
| *Turritis laxa* | 10 | | | | 30 $^{41.8}$ | |
| *Populus x canescens* | 50 | 30 | 60 | | 80 $^{40.2}$ | |
| *Cistus laurifolius* | 40 | 10 | 40 | 30 | 70 $^{36.9}$ | |
| *Doronicum orientale* | 30 | 10 | 10 | | 40 $^{31.3}$ | |
| *Quercus coccifera* | | | | | | 80 $^{87.7}$ |
| *Genista anatolica* | | | | | | 70 $^{81.3}$ |
| *Dactylis glomerata* | | | 40 | | | 80 $^{67.1}$ |
| *Trifolium grandiflorum* | | | | | | 40 $^{59.8}$ |
| *Trifolium lucanicum* | | | | | | 30 $^{51.3}$ |
| *Stipa bromoides* | | | | | | 20 $^{41.5}$ |
| *Pilosella hoppeana* | | | | | | 20 $^{41.5}$ |
| *Lolium rigidum* | | | | | | 20 $^{41.5}$ |
| *Rosa canina* | 20 | | | | 20 | 40 $^{35.1}$ |
| *Cephalanthera longifolia* | 100 $^{54.8}$ | 80 $^{36.5}$ | | 20 | 30 | 10 |
| *Vicia cracca* | 90 $^{51.6}$ | 10 | 70 $^{32.8}$ | 30 | | 10 |
| *Minuartia hybrida* | | | 80 $^{59.9}$ | 60 $^{38.8}$ | | |
| *Trifolium campestre* | 10 | | 80 $^{53.9}$ | 60 $^{33.7}$ | 10 | |
| *Sonchus asper* | 50 | 40 | 100 $^{47.8}$ | 90 $^{38.8}$ | | |
| *Cichorium intybus* | | | 50 $^{43.8}$ | 40 $^{31.3}$ | | |
| *Ajuga chamaepitys* | | | 70 $^{43.8}$ | 90 $^{64}$ | | |
| *Lactuca serriola* | 60 | 70 | 100 $^{41.8}$ | 90 $^{32.9}$ | | |
| *Verbascum glomeratum* | 10 | | 60 $^{38.8}$ | 70 $^{49.3}$ | | |
| *Anthyllis vulneraria* | | 20 | 60 $^{38.8}$ | 60 $^{38.8}$ | | |
| *Crepis foetida* | 20 | 40 | 80 $^{38.3}$ | 90 $^{47.5}$ | | |
| *Capsella bursa-pastoris* | | | 40 $^{35.1}$ | 40 $^{35.1}$ | | |
| *Conyza canadensis* | 10 | 60 | 70 $^{32.8}$ | 70 $^{32.8}$ | | |
| **Other species appearing at more than 4 relevés** | | | | | | |
| *Lens culinaris* | 30 | 50 | 50 | 40 | 10 | |
| *Poa bulbosa* | 10 | 10 | 30 | 50 | 10 | 50 |
| *Crataegus monogyna* | 30 | | | | | 30 |
| *Crepis sancta* | | 20 | 30 | 10 | | |
| *Polycarpon tetraphyllum* | | | 30 | 30 | | |
| *Clinopodium vulgare* | 20 | | 20 | | 10 | |
| *Pilosella piloselloides* | | | 20 | 20 | | 10 |

On the other hand, after surface fires, the floristic composition shows differences from unburned forests (Fig. 3).

However, cases where trees are damaged after a surface fire may occur. In this case, the reestablishment of the forest can be supplied by natural regeneration depending on the occurrence of the appropriate germination conditions in soil and the seed reserve in which seeds are still alive and germinate after dispersal (Fig. 2b). The important issue in this case is the amount of trees remaining alive and the size of the fire area. If there are not enough living trees in the burned area, the only seeding source are the trees at unburned areas. Since their seed dispersal distances would

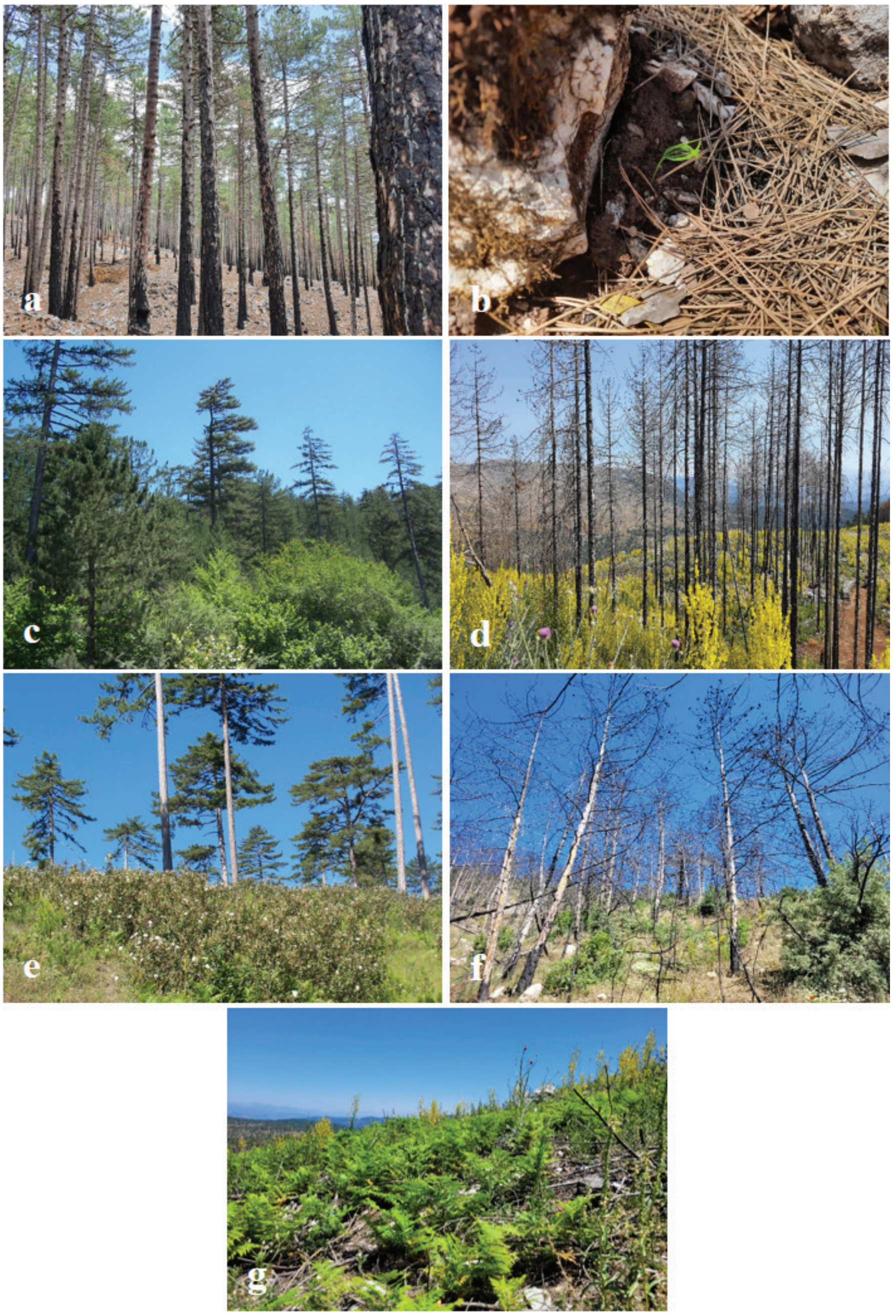

**Fig. 2.** Physiognomy of black pine forests before and after fire: a – surface fire, b – post-fire regeneration, c – black pine forest with dense underground layer, d – crown fire, e – black pine forest with *Cistus laurifolius*, f – crown fire with oak coppice after fire, g – *Pteridium aquilinum* vegetation after crown fire.

remain at a certain rate, there may be a possibility that the field will not be completely seeded. And this may cause a change in vegetation structure.

Stand age preventing tree bark from forming a sufficient thickness also has an effect on the lethality of fires on trees when the fire occurs as a surface fire. This reveals the importance of the age of a stand exposed to fire and indicates that mature black pine stands are more resistant to surface fires than young stands.

The lethal effect of surface fires may also closely be related to whether there is a dense shrub and herb layer in the forest. In relation to the increasing amount of fuel load, a dense shrub and herb layer may cause a higher intense surface fire resulting in a negative impact on trees. Although black pine forests generally do not include a dense scrub and herb layer, as is seen in the Tab. 2, it is possible to encounter such conditions especially at highly productive sites (Fig. 2c).

A sufficient post-fire regeneration of the black pine depends not only on the stand age but also the optimum number of trees and therefore crown coverage fostering the constitution of large amount of seed. The higher or lower number of trees necessary may have negative consequences in terms of post-fire regeneration. If the number is low, there may not be a sufficient cone reserve to seed the area, which

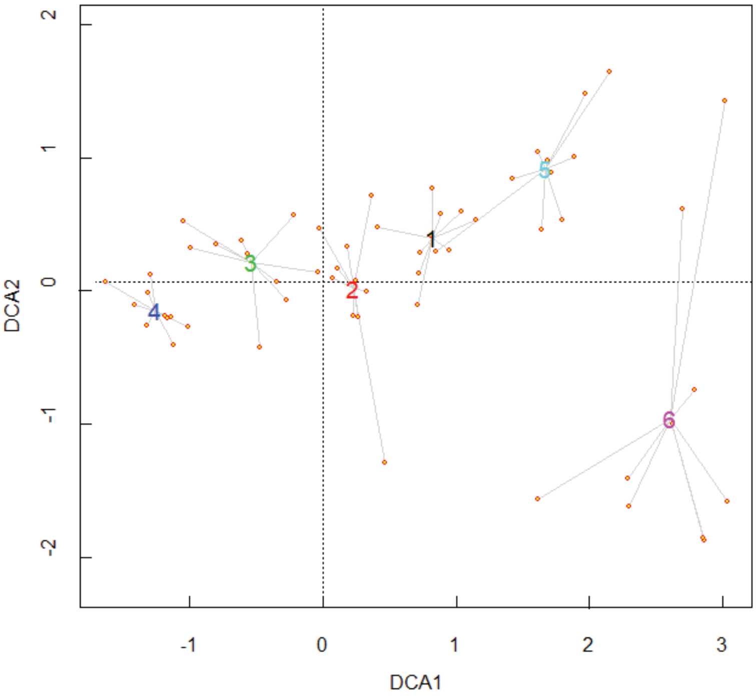

**Fig. 3.** Detrended correspondence analysis (DCA) ordination of 2nd year vegetation sampling after the 2021 fire in Yılanlı, Muğla - Türkiye. There is a clear floristic differentiation between crown fire (3, 4), surface fire (1, 2) and unburned areas (5, 6). The crown fire area is characterized by the low frequency of black pine juveniles which would not be sufficient to establish a forest canopy and a higher number of ruderal plants. DCA was done with R project (R Core Team 2021).

may lead to insufficient regeneration. On the other hand, in stands where the number of trees is higher, crown coverage may be weak due to competition among trees, and therefore inadequacies may occur in terms of cone and seed reserves (Christopoulou et al. 2014). This shows the importance of having an optimum number of trees with crowns that support high cone and seed production in stands. In a study by Arista and Talavera (1996), denser stands were found to have lower cone and seed production. This was associated with the reduced photosynthetic surface and increased competition between trees. Ordóñez et al. (2005) also reached similar results and detected a decrease in the amount of cones per tree and the amount of seeds per cone in too dense stands. This indicates the importance of silvicultural tending in stands to support the post-fire regeneration.

In terms of seed retention of black pine, there are differences between years (Odabaşı et al. 2004). Rich seed years of the species occur every 2-3 years. If the fire coincides with a seedless or poor seed year, it is not possible to re-establish a black pine forest in the area naturally, since there will be no seed reserve, even if there are viable trees in the burned area. In the case of natural regeneration after a fire, there may be variations in the amount of regeneration depending on site condition differences. In addition to the topographic conditions such as aspect, elevation and slope (Tavşanoğlu 2008), herbaceous plant competition, soil depth (Christopoulou et al. 2014), the presence of seed pests (Ordóñez and Retana 2004), branches, logs, burnt trees and other woody residues left on the field (Harmon et al. 1986, Harmon and Hua 1991) have an impact on these variations.

Briefly, if black pine forests are mature enough and the fire occurs as a surface fire, they can generally continue their existence. Natural regeneration mechanisms may also work in cases where the trees are even damaged. However, in addition to the changing climate conditions, forestry practices causing vertical and horizontal layered stand structures can cause the surface fires to turn into the crown fires covering large areas.

## Crown fires in black pine forests

As a result of the heat generated by the fire, serotinous cones, which are a fire adaptation trait of coniferous species like red pine, Aleppo pine, maritime pine and cypress, open, and mass germination occurs after the fire (Thanos and Daskalakou 2000, Kavgacı et al. 2016). Unlike these species, the black pine, which is distributed in the supra- and sub-Mediterranean zones, does not have serotinous cones (Lanner 1998). Therefore, as a crown fire appears, the heat during the fire has a lethal effect on the seed reserves in the canopy and causes problems for the black pine to join and dominate the post-fire vegetation composition (Retana et al. 2002, Savage and Mast 2005, Pausas et al. 2008). An example of this kind of floristic change (Tab. 2, Fig. 3) and degradation (Fig. 2d) has been recently observed by Keleş and Kavgacı (2022) after forest fires occurred in black pine forests in Türkiye.

The opening of mature cones of black pine and seed dispersal occur from the late winter to early spring (Bolós and Vigo 1984, Laguna 1993, Skordilis and Thanos 1997). In the

Mediterranean region, fires occur frequently in the summer months. During this period, the seeds in cones are not mature enough or capable of germination (Peix 1999). Therefore, the only seed source that can contribute to regeneration after summer fire is the soil seed bank. Although black pine seeds are resistant to temperature shocks at certain levels (Ayan et al. 2020, Turna and Bilgili 2006), seeds in the soil cannot withstand the high temperatures reached during intense summer fires (Habrouk et al. 1999). Young black pine individuals in the understory layers (scrub and herb layers) are also burned during the fire. Therefore, after a crown fire, neither the crown seed bank, nor the soil seed bank, or young individuals can make a contribution to the survival of black pines after a fire, which prevents the natural regeneration of black pine.

In addition to the fact that, fires are beginning to occur as crown fires in mature black pine forests, the increase in fire frequency causes the stands to be exposed to fires during young stand ages and therefore in much drier site conditions (Gracia et al. 2002). Since sufficient stand height cannot be achieved in such forests, fires consume the entire fuel load including all the canopy and negatively affect seed stocks. The change of fire regime along with fire frequency, intensity and therefore severity causes the fire adaptation abilities of black pine to fail and different post-fire vegetation dynamics to emerge (Fyllas and Troumbis 2009, Keleş and Kavgacı 2022, Tab. 2, Fig. 3). Under these conditions, trees die completely and the seeds are damaged by fire due to the lack of the serotinous cone feature (Tapias et al. 2004). In such cases, the reemergence of a black pine forest depends only on the unburned stands at the edge of the burned area. This is also related to the size of the fire area, since most of the area would be not seeded in an optimum way after large fires.

The number of black pine seedlings decreases in the burnt area as the distance from the edge of unburned areas increases towards the core of the burnt area. Black pine seeds, like many other pine species, are dispersed by wind (Nathan et al. 2001). Seeds with wings can be dispersed over large area (Klaus 1989). Therefore, the seeds dispersed from unburned neighboring stands may seed the burned areas (Christopoulou et al. 2014). However, the optimal seed dispersal distance of trees is limited and for black pine, this is about 20-40 m (Odabaşı et al. 2004). In areas outside this distance, it does not seem possible for a forest structure to re-occur naturally. Therefore, if a crown fire area exceeds the optimum seed dispersal distance and there is not a sufficient amount of unburned patches (Christopoulou et al. 2014), the black pine may not realize a homogenous germination to survive in all the area of the fire and it will be replaced by a vegetation structure dominated by different species.

With the changes in the fire regime, especially with the increase in fire frequency, the obligate seeding tree species cannot naturally regenerate due to the damage in the crown seed bank and the vegetation is formed by other species (Pausas et al. 2003, Broncano and Retana 2004). One of the most important fire adaptation abilities of Mediterranean type ecosystems is obligate resprouting (Pausas and Keeley 2014). Many shrub and tree species dominating maquis and sclerophyllous forests are obligate resprouters. Since these species are also found in the underground layers of forests dominated by obligate seeding tree species, they can regenerate rapidly after fire and cause the formation of maquis and sclerophyllous forests instead of coniferous forests (Kavgacı et al. 2016). It is possible to accept that many maquis and sclerophyllous forests in the Mediterranean today, especially in high productive sites, were formed in this way.

A similar vegetation conversion is valid for black pine forests. In order to make an assessment of how this conversion happens or could happen, it is necessary to know the plants forming and dominating the underground layer of black pine forest and their fire adaptation characteristics. As the black pine vegetation composition in different geographical and environmental areas is examined, it is seen that there are other trees, shrubs and herbaceous plants that are obligate resprouterss, as well as obligate seeders based on a soil seed bank (Tavşanoğlu and Pausas 2018).

Post-fire survival through the soil seed bank is one of the most characteristic fire adaptations of plants in Mediterranean-type ecosystems. *Cistus laurifolius* L., which is a common element of black pine forest vegetation in the Eastern Mediterranean, is one of the species with this type of feature (Fig. 2e). It is known that *Cistus* species regenerate quickly after fire thanks to the soil seed bank (Tavşanoğlu 2011). If the black pine cannot regenerate due to fire frequency or crown fires, *C. laurifolius* germinates quickly and if there is no obligate resprouter tree or taller shrub species, it can dominate the vegetation (Ocak et al. 2007). As a matter of fact, currently, much of the *C. laurifolius* shrubland distributed in large areas in sub-Mediterranan and subeuxine regions are ecosystems that have replaced black pine forests that have suffered fires (Atalay and Efe 2010, Kavgacı et al. 2021).

Although black pine forest vegetation varies regionally, it can be seen that both deciduous and evergreen oak species are included in the floristic composition. Oak species are generally obligate resprouters and easily survive after fire (Pausas and Darwin 2001, Rodríguez-Trejo and Myers 2010, Kim et al. 2020). Deciduous oak species such as *Quercus cerris* L., *Q. pubescens* Willd. and *Q. infectoria* G. Olivier and evergreen ones like *Q. coccifera* L. often form mixed stands with black pine. In cases where black pine cannot regenerate after a fire, these species can resprout and dominate the vegetation (Keleş and Kavgacı 2012, Fig. 2f). Such a mixture of stands is not typical only for the natural distribution of black pine but also for plantations as, established in Croatia and Slovenia where deciduous were converted to black pine forests (Čahojová et al. 2024).

In the absence of woody species that accompany black pine or show vigorous renewal after fire, a herbaceous vegetation dominated by obligate resprouter grasses may occur. In fact, in humid environmental sites, *Pteridium aquilinum* L. (Kuhn) can be found densely in the herb layer of black pine forests (Kavgacı et al. 2013). The species regenerates quickly after a fire thanks to its underground rhizomes and covers the area (Keleş and Kavgacı, 2022, Fig. 2g). Vegetation conversion is managed not only by obligate seeders or resprouters but also colonizers like *Verbascum* ssp. as seen in Fig. 2d.

## Conclusion

Studies indicate that there will be changes in fire regimes in the Mediterranean basin and fires will probably become more severe (Arca et al. 2010). Changing climatic conditions play a triggering role in fires occurring more frequently and at higher intensities. Under these conditions, it is expected that nonserotinous species will be more affected by the changes than serotinous ones. Therefore, it is predicted that black pine forests will be one of the most affected ecosystems due to the changing fire regime (Espelta et al. 2002).

Black pine forest ecosystems can continue to exist after low-intensity surface fires due to their adaptation. On the other hand, the decrease in fire frequency and the occurrence of high intensity crown fires create a problematic situation in terms of the continuity of black pine and cause the formation of different vegetation types because the fire adaptation capacity of the black pine is inadequate to overcome these changes.

In summary, changes in the fire regime have negative consequences for the continuity of black pine forests. Therefore, post-fire restoration of these forests becomes an important issue. It is crucial to adopt an ecologically based restoration approach after fire. A restoration plan that is appropriate to the ecology and biology of black pine, which prevents soil loss and erosion, promotes biodiversity and includes fire prevention measures, is necessary for the continuity of black pine forests. Since these forests are getting more prone to high-intensity fires, the black pine plantations, which are out of the species' natural distribution range and are exposed to crown fires should be converted to natural deciduous forests. This can easily be done by fostering the natural regeneration of post-fire resprouters, which are natural element of plantation flora and immediately emerge after fires, like oaks (Bergmeier et al. 2021, Čahojová et al. 2024).

Additionally, silvicultural treatments in the existing black pine forests should be carried out to promote a stand structure that is inclined to surface fires. Therefore, extending the harvesting period, fostering self-pruning, creating single layer stand structures but also a canopy supporting mass seed production should be the targets of silviculture. Additionally, to decrease the fuel load at ground layer, moderate grazing (Christopoulou et al. 2014) and prescribed burning (Ferrat et al. 2021) can be applied. This can reduce the fire intensity and ensure the black pine continuity not only by resistance but also by resilience.

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## Author contribution statement

E.S.K. and A.K. jointly conceived and wrote this paper.

## References

Akkemik, Ü., 2020: Pinaceae. In: Akkemik, Ü. (ed.), Türkiye'nin Bütün Ağaçları ve Çalıları (All trees and shrubs of Türkiye), 161–212. Türkiye İş Bankası Kültür Yayınları, İstanbul.

Alptekin, C. Ü., 1986: Anadolu karaçamı (*Pinus nigra* Arn. subsp. *pallasiana* Lamb. Holmboe)' nın coğrafik varyasyonları (Geographical variations of Anatolian black pine (*Pinus nigra* Arn. subsp. *pallasiana* Lamb. Holmboe). Journal of the Faculty of Forestry, Istanbul University, 132–165. https://doi.org/10.17099/jffiu.57982

Arca, B., Pellizzaro, G., Duce, P., Salis, M., Bacciu, V., Spano, D., Ager, A., Finney, M., 2010: Climate change impact on fire probability and severity in Mediterranean areas. In: Viegas D. X. (ed.), VI International Conference on Forest Fire Research, ADAI, Coimbra, Portugal.

Arista, M., Talavera, S., 1996: Density effect on the fruit-set, seed crop viability and seedling vigour of *Abies pinsapo*. Annals of Botany 77(2), 187–192. https://doi.org/10.1006/anbo.1996.0021

Atalay, İ., Efe, R., 2010: Anadolu karaçamı (*Pinus nigra* Arnold subsp. *pallasiana* (Lamb.) Holmboe)'nın ekolojisi ve tohum nakli açısından bölgelere ayrılması. (Regional division of Anatolian black pine (*Pinus nigra* Arnold subsp. *pallasiana* (Lamb.) Holmboe) in terms of ecology and seed transplantation). T.C. Çevre ve Orman Bakanlığı Orman Ağaçları ve Tohumları Islah Araştırma Müdürlüğü, Meta Basımevi, Ankara.

Atalay, I., Efe, R., 2012: Ecological attributes and distribution of Anatolian black pine (*Pinus nigra* Arnold. subsp. *pallasiana* Lamb. Holmboe) in Turkey. Journal of Environmental Biology 33(29), 509–519.

Ayan, S., Erkan, C., Çelik, E. N. Y., Gülseven, O., Akın, Ş. S., Yılmaz, E., 2020: Anadolu karaçamının (*Pinus nigra* JF Arnold subsp. *pallasiana* (Lamb.) Holmboe) bazı popülasyonlarında yüksek sıcaklık şoku uygulamalarının tohum canlılığı ve çimlenmesi üzerine etkileri (Effects of high temperature shocks applications on seed viability and germination in some Anatolian Black Pine (*Pinus nigra* J.F. Arnold ssp. *pallasiana* (Lamb.) Holmboe) populations). Turkish Journal of Forestry 21(2), 106–112. https://doi.org/10.18182/tjf.677339

Barčić, D., Hršak, V., Rosavec, R., Ančić, M., 2022: Ecological potential of Mediterranean habitats for black pine (*Pinus nigra*

J.F.Arnold) in Croatia. Forests 13(11), 1900. https://doi.org/10.3390/f13111900

Beltrán, M., Piqué, M., Cervera, T., Palero, N., Camprodon, J. 2018: Best management practices for the conservation of black pine (*Pinus nigra*) forests. Making compatible forest production and habitat conservation. Life+ PINASSA Project. Forest Ownership Center, Barcelona.

Beniston, M., Stephenson, D. B., Christensen, O. B., Ferro, C. A. T., Frei, C., Goyette, F., Halsnaes, K., Holt, T., Jylha, K., Koffi, B., 2007: Future extreme events in European climate: an exploration of regional climate models projections. Climatic Change 81, 71–95. https://doi.org/10.1007/s10584-006-9226-z

Bergmeier, E., Capelo, J., Di Pietro, R., Guarino, R., Kavgacı, A., Loidi, J., Tsiripidis, I., Xystrakis, F., 2021: 'Back to the Future'-Oak wood-pasture for wildfire prevention in the Mediterranean. Plant Sociology 58(2), 41–48. https://doi.org/10.3897/pls2021582/04

Biondi, E., Allegrezza, M., 2019: Syntaxonomy of *Pinus nigra* s.l. communities in the *Erico-Pinetea* class and their distribution in the central Apennines and Balkan province. Plant Biosystems 154(2), 1–17. https://doi.org/10.1080/11263504.2019.1701128

Bolós, O., Vigo, J., 1984: Flora dels Països Catalans Vol I. Editorial Barcino, Barcelona.

Bonari, G., Fernández-González, F., Çoban, S., Monteiro-Henriques, T., Bergmeier, E., Didukh, Xystrakis, F., Angiolini, C., Chytrý, K., Acosta, A. T. R., Agrillo, E., Costa, J. C., Danihelka, J., Hennekens, S. M., Kavgacı, A., Knollová, I., Neto, C. S., Sağlam, C., Škvorc, Ž., Tichý, K., Chytrý, M., 2021: Classification of the Mediterranean lowland to submontane pine forest vegetation. Applied Vegetation Science 24(1), e12544. https://doi.org/10.1111/avsc.12544

Broncano, M. J., Retana, J., 2004: Topography and forest composition affecting the variability in fire severity and post-fire regeneration occurring after a large fire in the Mediterranean basin. International Journal of Wildland Fire 13(2), 209–216. https://doi.org/ 10.1071/WF03036

Čahojová, L., Jakob, A., Valjavec, M. B., Čarni, A., 2024: Response of vulnerable karst forest ecosystems under different fire severities in the Northern Dinaric Karst mountains (Slovenia). Fire Ecology 20, 38. https://doi.org/10.1186/s42408-024-00267-x

Christopoulou, A., Ful´e, P. Z., Andriopoulos, P., Sarris, D., Arianoutsou, M., 2013: Dendrochronology-based fire history of *Pinus nigra* forests in Mount Taygetos, Southern Greece. Forest Ecology and Management 293, 132–139. https://doi.org/10.1016/j.foreco.2012.12.048

Christopoulou, A., Fyllas, N. M., Andriopoulos, P., Koutsias, N., Dimitrakopoulos, P. G., Arianoutsou, M., 2014: Post-fire regeneration patterns of *Pinus nigra* in a recently burned area in Mount Taygetos, Southern Greece: The role of unburned forest patches. Forest Ecology and Management 327, 148–156. https://doi.org/10.1016/j.foreco.2014.05.006

Dequé M., 2007: Frequency of precipitation and temperature extremes over France in an anthropogenic scenario: Model results and statistical correction according to observed values. Global Planet Change 57, 16–26. https://doi.org/10.1016/j.gloplacha.2006.11.030

Enescu, C. M., de Rigo, D., Caudullo, G., Mauri, A., Houston Durrant, T., 2016: *Pinus nigra* in Europe: Distribution, habitat, usage and threats. In: San-Miguel-Ayanz, J., de Rigo, D., Caudullo, G., Houston Durrant, T., Mauri, A. (eds.), European atlas of forest tree Species. Publication of the European Union, e015138+, Luxembourg.

Espelta, J. M., Rodrigo, A., Habrouk, A., Meghelli, N., Ordóñez, J. L., Retana, J., 2002: Land use changes, natural regeneration patterns and restoration practices after a large wildfire in NE Spain: Challenges for fire ecology and landscape restoration. In: Fire and Biological Processes, 315–324. 1th edition. Backhuys Publishers, Leiden.

Euro+Med 2006+:Euro+Med PlantBase – the information resource for Euro-Mediterranean plant diversity. Retrieved June 1, 2024 from http://www.europlusmed.org

Farjon, A., 2010: A Handbook of the World's Conifers. 2 vols. (Vol. 1), Brill, Leiden, Boston.

Ferrat, L., Morandini, F., Lapa, G., 2021: Influence of prescribed burning on a *Pinus nigra* subsp. *laricio* forest: Heat transfer and tree vitality. Forests 12, 915. https://doi.org/10.3390/f12070915

Fyllas, N. M., Troumbis, A. Y., 2009: Simulating vegetation shifts in north-eastern Mediterranean mountain forests under climatic change scenarios. Global Ecology and Biogeography 18(1), 64–77. https://doi.org/10.1111/j.1466-8238.2008.00419.x

Gracia, M., Roig, P., Retana, J., 2002: Mid-term successional patterns after fire of mixed pine-oak forests in NE Spain. Acta Oecologica 23(6), 405–411. https://doi.org/10.1016/S1146-609X(02)01169-4

Habrouk, A., Retana, J., Espelta, J. M., 1999: Role of heat tolerance and cone protection of seeds in the response of three pine species to wildfires. Plant Ecology 145, 91–99. https://doi.org/10.1023/A:1009851614885

Harmon, M. E., Franklin, J. F., Swanson, F. J., Sollins, P., Gregory, S. V., Lattin, J. D., Anderson, N. H., Cline, S. P., Aumen, N. G., Sedell, J. R., Lienkaemper, G. W., Cromack Jr., K., Cummins, K. W., 1986: Ecology of coarse woody debris in temperate ecosystems. Advances in Ecological Research 15, 133–302. https://doi.org/10.1016/S0065-2504(08)60121-X

Harmon, M. E., Hua, C., 1991: Coarse woody debris dynamics in two old-growth ecosystems. BioScience 41(9), 604–610. https://doi.org/10.2307/1311697

He, T., Pausas, J. G., Belcher, C. M., Schwilk, D. W., Lamont, B. B., 2012: Fire-adapted traits of *Pinus* arose in the fiery Cretaceous. New Phytologist 194(3), 751–759. https://doi.org/10.1111/j.1469-8137.2012.04079.x

Isajev, V., Fady, B., Semerci, H., Andonovski, V., 2004: EUFORGEN Technical Guidelines for genetic conservation and use for European black pine (*Pinus nigra*). International Plant Genetic Resources Institute, Rome, Italy.

Kavgacı, A., Sevgi, O., Tecimen, H., Yılmaz, O., Carus, S., Dündar, T., 2013: Classification and ordination of *Pinus nigra* dominated forests at Alacam mountains (NW Anatolia-Turkey). Eurasian Journal of Forest Science 1(1), 38–50.

Kavgacı, A., Örtel, E., Torres, E., Safford, H., 2016: Early postfire vegetation recovery of *Pinus brutia* forests: effects of fire severity, prefire stand age, and aspect. Turkish Journal of Agriculture and Forestry 40(5), 723–736. https://doi.org/10.3906/tar-1601-21

Kavgacı, A., Balpınar, N., Öner, H. H., Arslan, M., Bonari, G., Chytrý, M., Čarni, A., 2021: Classification of forest and shrubland vegetation in Mediterranean Turkey. Applied Vegetation Science 24(2), e12589. https://doi.org/10.1111/avsc.12589

Kavgacı, A., Karaköse, M., Keleş, E. S., Balpınar, N., Arslan, N., Novak, P., Čarni, A., 2023: Classification of forest and shrubland vegetation in central and eastern Euxine Turkey and SW Georgia. Applied Vegetation Science 26(4), e12753. https://doi.org/10.1111/avsc.12753

Keeley J. E., Pausas J. G., Rundel P. W., Bond W. J., Bradstock R. A., 2011: Fire as an evolutionary pressure shaping plant traits. Trends in Plant Science 16(8), 406–411. https://doi.org/10.1016/j.tplants.2011.04.002

Keeley, J. E., 2012: Ecology and evolution of pine life histories. Annals of Forest Science 69, 445–453. https://doi.org/10.1007/s13595-012-0201-8

Keleş, E. S., Kavgacı, A., 2022: Anadolu karaçamı (*Pinus nigra* J.F.Arnold subsp. *pallasiana* Lamb. Holmboe) ormanlarında yangın sonrası vejetasyon dinamiği (Post-fire vegetation dynamics in Anatolian black pine (*Pinus nigra* J.F.Arnold subsp. *pallasiana* Lamb. Holmboe) forests). Ongoing PhD Thesis. Karabük University, Karabük.

Kim, J., Lim, J. H., Shin, M., Han, S. H., Kang, W., 2020: Oak resprouting survival and competition for 19 years after wildfire in the Republic of Korea. Forests 11(5), 515. https://doi.org/10.3390/f11050515

Klaus, W., 1989: Mediterranean pines and their history. Plant Systematics and Evolution 162, 133–163. https://doi.org/10.1007/978-3-7091-3972-1_8

Krebs, P., Pezzatti, G. B., Mazzoleni, S., Talbot L. M., Conedera, M., 2010: Fire regime: history and definition of a key concept in disturbance ecology. Theory in Biosciences. 129, 53–69. https://doi.org/10.1007/s12064-010-0082-z

Laguna, M., 1993: Flora forestal española. Galicia Editorial S. A., La Coruña.

Lanner, R. M., 1998: Seed dispersal in *Pinus*. In: Richardson, D. M. (ed.), Ecology and biogeography of *Pinus*, 281–295. Cambridge University Press, Cambridge.

Mayer, H., Aksoy, H., 1986: Forests of Turkey. Gustav Fischer Verlag, Stuttgart.

Meehl, G. A., Tebaldi, C., 2004: More intense, more frequent, and longer lasting heat waves in the 21st century. Science 305(5686), 994–997. https://doi.org/10.1126/science.1098704

Mikulová, K., Jarolímek, I., Bacigál, T., Hegedüšová, K., Májeková, J., Medvecká, J., Slabejová, D., Šibík, J., Škodová, I., Zaliberová, M., Šibíková, M. 2019: The effect of non-native black pine (*Pinus nigra* J.F.Arnold) plantations on environmental conditions and undergrowth diversity. Forests 10, 548. https://doi.org/10.3390/f10070548

Moreira, F., Arianoutsou, M., Corona, P., De las Heras, J. (Eds.)., 2011: Post-fire management and restoration of southern European forests. vol. 24, Springer Science & Business Media, https://doi.org/10.1007/978-94-007-2208-8

Moreira, F., Ascoli, D., Safford, H., Adams, M. A., Moreno, J. M., Pereira, J. M. C., Catry, F. X., Armesto, J., Bond, W., González, M. E., McCaw, L., Price, O., Pausas, J. G., Curt, T., Koutsias, N., Rigolot, E., Stephens, S., Tavsanoglu, Ç., Vallejo, R., Van-Wilgen, BW., Xanthopoulos, G., Fernandes, P. M., 2020: Wildfire management in Mediterranean-type regions: paradigm change needed. Environmental Research Letters 15(1), 011001. https://doi.org/10.1088/1748-9326/ab541e

Moriondo, M., Good, P., Durao, R., Bindi, M., Giannakopoulos, C., Corte-Real, J., 2006: Potential impact of climate change on fire risk in the Mediterranean area. Climate Research 31(1), 85–95. https://doi.org/10.3354/cr031085

Mucina, L., Bültman, H., Dierssen, K., Theurillat, J.-P., Dengler, J., Čarni, A., Šumberová, K., Raus, T., Di Pietro, R., Gavilán Garcia, R., Chytrý, M., Iakushenko, D., Schaminée, J. H. J., Bergmeier, E., Santos Guerra, A., Daniëls, F. J. A., Ermakov, N., Valachoviĉ, M., Pigantti, S., Rodwell, J. S., Pallas, J., Capelo, J., Weber, H. E., Lysenko, T., Solomeshch, A., Dimopoulos, P., Aguiar, C., Freitag, H., Hennekens, S. M., Tichý, L., 2016: Vegetation of Europe: Hierarchical floristic classification system of plant, lichen, and algal communities. Applied Vegetation Science 19(S1), 3–264. https://doi.org/10.1111/avsc.12257

Nathan, R., Safriel, U. N., Noy-Meir, I., 2001: Field validation and sensitivity analysis of a mechanistic model for tree seed dispersal by wind. Ecology 82(2), 374–388. https://doi.org/10.1890/0012-9658(2001)082[0374:FVASAO]2.0.CO;2

Ne’eman, G., Arianoutsou, M., 2021: Mediterranean pines–adaptations to fire. In: Ne’eman, G., Osem, Y. (eds.), Pines and their mixed forest ecosystems in the Mediterranean Basin. Managing Forest Ecosystems, 457–480. Springer Nature, Switzerland.

Nobis, M. P., Traiserb, C., Roth-Nebelsicks, A. R., 2012: Latitudinal variation in morphological traits of the genus *Pinus* and its relation to environmental and phylogenetic signals. Plant Ecology & Diversity 5(1), 1–11. https://doi.org/10.1080/17550874.2012.687501

Ocak, A., Kurt, L., Oz, M., Tug, G. N., 2007: Floristical and ecological studies on burned blackpine (*Pinus nigra* Arn. subsp. *pallasiana* (lamb) Holmboe) forest area at central Anatolia. Asian Journal of Plant Sciences 6(6), 892–905. https://doi.org/10.3923/ajps.2007.892.905

Odabaşı, T., Çalışkan, A., Bozkuş, H. F., 2004: Silvikültür tekniği (Silviculture technique). İstanbul Üniversitesi Orman Fakültesi Yayını, İstanbul.

Ordóñez, J. L., Retana, J., 2004: Early reduction of post-fire recruitment of *Pinus nigra* by post-dispersal seed predation in different time-since-fire habitats. Ecography 27(4), 449–458. https://doi.org/10.1111/j.0906-7590.2004.03886.x

Ordóñez, J. L., Retana, J., Espelta, J. M., 2005: Effects of tree size, crown damage, and tree location on post-fire survival and cone production of *Pinus nigra* trees. Forest Ecology and Management 206(1-3), 109–117. https://doi.org/10.1016/j.foreco.2004.10.067

Pausas, J. G., Darwin, C., 2001: Resprouting vs seeding–a Mediterranean perspective. Oikos 94(1), 193–194. https://doi.org/10.1034/j.1600-0706.2001.t01-1-10979.x

Pausas, J. G., Keeley, J. E., 2009: A burning story: the role of fire in the history of life. Bioscience 59(7), 593–601. https://doi.org/10.1525/bio.2009.59.7.10

Pausas, J. G., Keeley, J. E., 2014: Evolutionary ecology of resprouting and seeding in fire-prone ecosystems. New Phytologist 204(1), 55–65. https://doi.org/10.1111/nph.12921

Pausas, J. G., Llovet, J., Rodrigo, A., Vallejo, R., 2008: Are wildfires a disaster in the Mediterranean basin? A review. International Journal of Wildland Fire 17(6), 713–723. https://doi.org/10.1071/WF07151

Pausas, J. G., Ouadah N., Ferran A., Gimeno T., Vallejo R., 2003: Fire severity and seedling establishment in *Pinus halepensis* woodlands, eastern Iberian Peninsula. Plant Ecology 169, 205–213.

Peix, J., 1999: Foc verd II: Programa de gestió del risc d’incendi forestal. Primersegona Edicions, Barcelona.

Powo, R. B. G., 2024: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Retrieved May 4, 2024 from www. plantsoftheworldonline.org

R. Core Team, 2021: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org.

Regato-Pajeres, P., Elana-Rosselló R., 1995: Natural black pine (*Pinus nigra* subsp. *salzmannii*) forests of the Iberian eastern mountains: Development of the phytoecological basis for their site evaluation. Annals of Forest Science 52, 589–606.

Retana, J., Maria Espelta, J., Habrouk, A., Luis Ordonez, J., de Solà-Morales, F., 2002: Regeneration patterns of three Mediterranean pines and forest changes after a large wildfire in northeastern Spain. Ecoscience 9(1), 89–97. https://doi.org/10.1080/11956860.2002.11682694.

Rodríguez-Trejo, D. A., Myers, R. L., 2010: Using oak characteristics to guide fire regime restoration in Mexican pine-oak and oak forests. Ecological Restoration 28(3), 304–323. https://doi.org/10.3368/er.28.3.304

San-Miguel-Ayanz, J., Durrant, T., Boca, R., Maianti, P., Liberta`, G., Jacome Felix Oom, D., Branco, A., De Rigo, D., Suarez-Moreno, M., Ferrari, D., Roglia, E., Scionti, N., Broglia, M., Onida, M., Tistan, A., Loffler, P., 2023: Forest Fires in Europe, Middle East and North Africa 2022, 1–187. Publications Office of the European Union, Luxembourg. https://doi:10.2760/871593, JRC135226.

Savage, M., Mast, J. N., 2005: How resilient are southwestern ponderosa pine forests after crown fires? Canadian Journal of Forest Research 35(4), 967–977. https://doi.org/10.1139/x05-028

Sevgi, O., Akkemik, U., 2007: A dendroecological study on *Pinus nigra* Arn. at different altitudes of northern slopes of Kazdaglari, Turkey. Journal of Environmental Biology 28(1), 73–75.

Sevgi O., Yılmaz O. Y., Kavgacı A., Tecimen H. B., Carus S., 2022: Alaçam dağları (Balıkesir)'nın iki katlı saf karaçam meşcereleri ve yetişme ortamı özellikleri (Two layered stands of pure black pine stands in Alaçam mountains (Balıkesir) and their site charecteristics). In: Sevgi, O., Tecimen, H. B., Okan, T., (eds.). Karaçam, 116–130, Türkiye Ormancılar Derneği Yayını, Ankara.

Skordilis, A., Thanos, C. A., 1997: Comparative ecophysiology of seed germination strategies in the seven pine species naturally growing in Greece. In: Ellis, R. H., Black, M., Murdoch, A. J., Hong, T. D. (eds.), Basic and applied aspects of seed biology, Current Plant Science and Biotechnology in Agriculture 30, 623–632. Springer, Dordrecht. https://doi.org/10.1007/978-94-011-5716-2_69

Sugihara, N. G., van Wagtendonk J. W., Fites-Kaufman J., 2006: Fire as ecological process. In: Sugihara, N. G., van Wagtendonk, J. V., Shaffer, K. E., Fites-Kaufman, J., Andrea, E. T., (eds.). Fire in California's ecosystems, 58–74, University of California Press, Berkeley, CA, USA.

Şahan, E. A., Köse N., Akkemik, Ü., Güner H. T., Tavşanoğlu, Ç., Bahar, A., Trouet, V., Dalfes, H. N., 2021: Fire history of *Pinus nigra* in Western Anatolia: A first dendrochronological study. Dendrochronologia 69, 125874. https://doi.org/10.1016/j.dendro.2021.125874

Şahan, E. A., Köse N., Güner H. T., Trouet, V., Tavşanoğlu, Ç., Akkemik, Ü., Dalfes, H. N., 2022: Multi-century spatiotemporal patterns of fire history in black pine forests, Turkey. Forest Ecology and Management 518, 120296. https://doi.org/10.1016/j.foreco.2022.120296

Tapias, R., Climent, J., Pardos, J. A., Gil, L., 2004: Life histories of Mediterranean pines. Plant Ecology 171(1), 53–68. https://doi.org/10.1023/B:VEGE.0000029383.72609.f0

Tavşanoğlu, C., 2008: The effect of aspect on post-fire recovery of a mixed Lebanon cedar-Anatolian black pine. Asian Journal of Plant Sciences 7(7), 696–699. https://doi.org/ 10.3923/ajps.2008.696.699

Tavşanoğlu, C., 2011: Fire-related cues (heat shock and smoke) and seed germination in a *Cistus creticus* population in southwestern Turkey. Ekoloji 20(79), 99–104.

Tavşanoğlu, Ç., Pausas, J. G., 2018: A functional trait database for Mediterranean Basin plants. Scientific Data 5, 180135. https://doi.org/10.1038/sdata.2018.135

Thanos C. A., Daskalakou E. N., 2000: Reproduction in *Pinus halepensis* and *P. brutia*. In: Ne'eman G, Trabaud L (eds.), Ecology, biogeography and management of *Pinus halepensis* and *P. brutia* forest ecosystems in the Mediterranean Basin, 79–90. Backhuys Publishers, Leiden.

Tichý, L., 2002: JUICE, Software for Vegetation Classification. Journal of Vegetation Science 13(3), 451–453. https://doi.org/10.1111/j.1654-1103.2002.tb02069.x

Touchan R., Baisani, C., Mitsopoulos, I. D., Dimitrakopoulos, A. D., 2012: Fire history in European black pine (*Pinus nigra* arn.) forests of the Valia Kalda, Pindus Mountains, Greece. Tree Ring Research 68(1), 45–50. https://doi.org/10.3959/2011-12.1

Turna, I., Bilgili, E., 2006: Effect of heat on seed germination of *Pinus sylvestris* and *Pinus nigra* ssp. *pallasiana*. International Journal of Wildland Fire 15(2), 283–286. https://doi.org/10.1071/WF05069

Vacek, Z., Cukor, J., Vacek, S., Gallo, J., Bažant, V., Zeidler, A., 2023: Role of black pine (*Pinus nigra* J. F. Arnold) in European forests modified by climate change. European Journal of Forest Research 142, 1239–1258. https://doi.org/10.1007/s10342-023-01605-5

Yaltırık, F., Efe, A., 1994: Dendroloji Ders Kitabı, Gymnospermae-Angiospermae. [Dendrology Textbook, Gymnospermae–Angiospermae]. İstanbul Üniversitesi Orman Fakültesi Yayını, İstanbul.

## Appendix: List of syntaxa mentioned in the text

- *Junipero-Pinetea sylvestris* Rivas-Mart. 1965
  - *Berberido creticae-Juniperetalia excelsae* Mucina 2016
    - *Berberido aetnensis-Pinion laricionis* (S. Brullo et al. 2001) Mucina et Theurillat 2016
- *Erico-Pinetea* Horvat 1959
  - *Erico-Pinetalia* Horvat 1959
    - *Erico-Fraxinion orni* Horvat 1959
    - *Cisto laurifolii-Pinion pallasianae*
    - *Adenocarpo complicati-Pinion pallasianae*
  - *Junipero communis-Pinetalia nigrae* Biondi et Allegrezza 2020
    - *Fraxino orni-Pinion nigrae* Em 1978
    - *Chamaecytiso hirsuti-Pinion pallasianae* Barbero et Quezel 1976
    - *Junipero hemisphaericae-Pinion nigrae* Biondi et Allegrezza 2020

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Review article

# Connecting the dots: Epigenetics, ABA, and plant stress tolerance

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**Abstract** – Extreme changes in environmental conditions are the main cause of abiotic stress and pose a major challenge for plant survival. In addition to controlling stress-related gene expression through a signal transduction cascade, epigenetic regulation plays an important role in orchestrating rapid and precise responses of plants to dynamic changes in environmental conditions. Complex mechanisms such as DNA methylation, especially via the *de novo* pathway, and histone tail modifications such as methylation, acetylation, phosphorylation, ubiquitination, and SUMOylation are involved in plant stress responses. In addition, histone variants and chromatin structure contribute to the dynamic regulation of chromatin state and determine whether it remains open or closed. Here, we summarise the complex combination of epigenetic modifications and factors that together regulate plant response to climate stress, focusing on the interplay between epigenetic mechanisms and the phytohormone abscisic acid.

**Keywords:** ABA, DNA methylation, histone modifications, histone variants, plant stress response, RdDM, small non-coding RNA

## Introduction

Changing and often extreme environmental conditions are the main cause of abiotic stress and pose a major challenge to plant survival. The inability of plants to relocate exacerbates this challenge. Abiotic stress has negative effects on essential plant processes such as metabolism, growth, and development, as has been extensively documented (Lichtenthaler 1998, Shulaev et al. 2008, Zandalinas et al. 2022). Primary sources of climate-related abiotic stress such as drought, salinity, heat stress, cold, and flooding lead to an overproduction of reactive oxygen species (ROS) in plant cells. ROS include molecules such as hydrogen peroxide ($H_2O_2$), superoxide ions ($O_2^{\bullet -}$) and hydroxyl radicals ($OH^{\bullet}$), which act as signalling molecules in triggering the cellular response to stress (Mandal et al. 2022). At high concentrations, however, ROS outweigh the enzymatic and non-enzymatic antioxidant system and put the cell in a state of oxidative stress (Cramer et al. 2011, Mandal et al. 2022), which leads to the inactivation of cellular mechanisms and, ultimately, to the death of the plant cell.

Epigenetic mechanisms enable plants to respond quickly and precisely to changes in abiotic conditions in the environment. When plants are exposed to different sources of abiotic stress, changes in methylation patterns and chromatin modifications occur both at individual loci and globally at the whole genome level (Zhang et al. 2018, Liu and He 2020); in some cases, meiotic inheritance of stress-induced changes is transmitted to subsequent generations (Liu and He 2020, Ramakrishnan et al. 2022).

This review elucidates the basic epigenetic mechanisms utilised by plants exposed to abiotic stress, thus enhancing our understanding of these intricate processes. By altering DNA methylation patterns and histone modifications, plants can dynamically regulate gene expression in response to different environmental conditions. Small non-coding RNAs, such as small interfering RNAs (siRNAs), are also involved in the fine-tuning of gene expression and mediate RNA-directed DNA methylation (RdDM) to silence transposable elements (TEs) under stress conditions. A comprehensive understanding of these mechanisms and their associated functions (Fig. 1) holds considerable potential for further development of epigenetic-based plant protection strategies and the production of crops that are resistant to abiotic stress.

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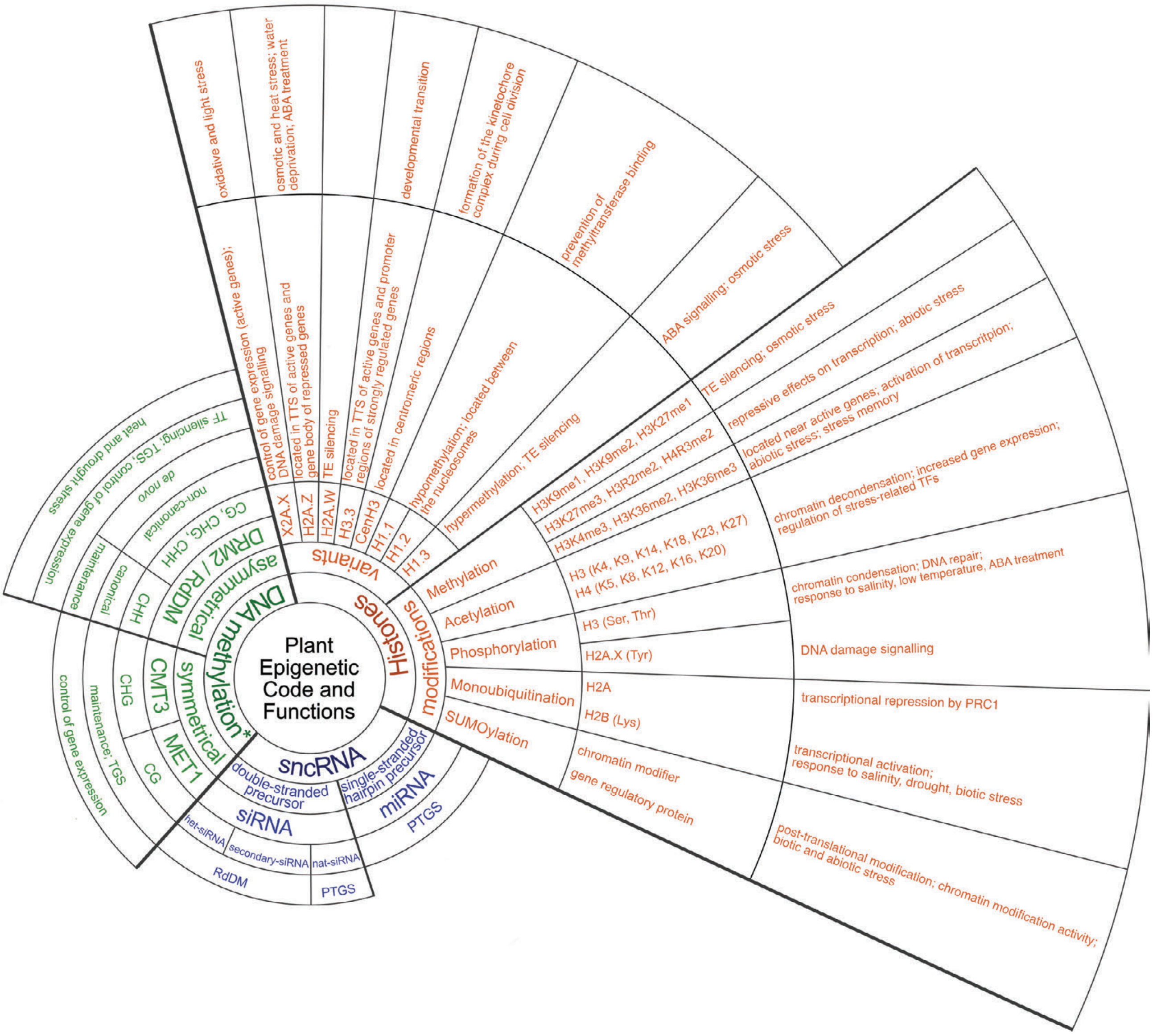

**Fig. 1.** Schematic diagram illustrating epigenetic mechanisms involved in plant stress response. The diagram includes histone variants and modifications, DNA methylation, and small non-coding RNAs, each annotated with their putative functions in stress response. Abbreviations: ABA – abscisic acid; CMT3 – Chromomethylase 3; DRM2 – Domains rearranged methyltransferase 2; Lys – Lysine; MET1 – Methyltransferase 1; miRNA – micro RNA; PRC1 – Polycomb repressive complex 1; PTGS – Post-transcriptional gene silencing; RdDM – RNA-directed DNA methylation; ROS1 – Repressor of Silencing 1; Ser – Serine; siRNA – small interfering RNA; sncRNA – small non-coding RNA; TE – Transposable element; TF – Transcription factor; TGS – Transcriptional gene silencing; Thr – Threonine; TTS – Transcription termination site; Tyr – Tyrosine. *in balance with DNA demethylation mediated by ROS1.

## Epigenetic code in plants

Epigenetic changes include all changes in the chromatin structure that lead to increased or decreased gene expression without altering the underlying DNA sequence. The complex combination of different epigenetic modifications and factors that collectively regulate gene expression and cellular identity, known as the epigenetic code, consists of histone variants and histone modifications (methylation, acetylation, phosphorylation, ubiquitination and SUMOylation), DNA methylation and non-coding RNAs (Duan et al. 2018, Chung et al. 2022). The effects of epigenetic control on gene activity depend on the specific types of epigenetic modifications and their proximity to neighbouring genes (Chang et al. 2020). The presence of heterochromatin marks such as DNA methylation and the methylated histone variant H3K9me2 can have an inhibitory effect on the expression of downstream genes if the marks are positioned within the promoter (Lei et al. 2015, Shi et al. 2023). Furthermore, in some cases, these features within the gene body can impair the efficiency of full-length transcript generation (Wang et al. 2013a, Duan et al. 2017). In addition, epigenetic marks of DNA and histone on TEs located within the promoters of stress-responsive genes play an important role in the dynamics of stress response regulation. By activating TEs under stressful conditions, these marks lead to upregulation of stress-related genes and facilitate adaptive responses to environmental challenges (Chang et al. 2020).

## Histone variants

The canonical histones H2A, H2B, H3, and H4, which are part of the nucleosome histone octamer, and the histone H1, which additionally stabilises it, can show variations in their primary structure. Different "subtypes" of the individual histones are referred to as histone variants. While histones H2A, H2B, H3, and H4 are expressed in the S-phase of the cell cycle and bind to the DNA molecule after replication, histone variants are expressed throughout the cell cycle and can replace the basic histones in the nucleosome at any point in the cell cycle (Jiang and Berger 2017). Different histone variants contribute to the dynamic chromatin landscape and exert a significant influence on essential nuclear processes. In addition, each of these variants has a different effect on the regulation of gene expression during development or stress response. Consequently, the abundance of histone variants has an immense potential to answer numerous unresolved questions in the field of eukaryotic epigenetics (Foroozani et al. 2022). The most important histone variants in plants (H2A.X, H2A.Z, H2A.W, H3.3, CenH3, H1.1, H1.2, and H1.3; Jiang and Berger 2017) are, together with their general functions, presented in Fig. 1 (orange). Sequences of expressed plant genes contain the canonical histone H2A and the histone variant H2A.X in their gene body (Lei and Berger 2020). The histone variant H2A.X plays a role in signalling damage to the DNA molecule that can occur due to light or oxidative stress (Nisa et al. 2019). Phosphorylation of H2A.X at serine residues is a signal that damage to the DNA molecule has occurred at a specific site (Lei and Berger 2020). The histone variant H2A.Z is predominantly found in euchromatic regions of the DNA molecule, either at transcription start sites of transcriptionally active genes or along the bodies of transcriptionally repressed genes (Sura et al. 2017, Lei and Berger 2020). The presence of H2A.Z in the first nucleosome downstream of the transcription start site (position +1) promotes transcription by preventing the stalling of RNA polymerase (Pol) II (Sura et al. 2017).

Histone variant H2A.W promotes chromatin condensation and is involved in the silencing of TEs in the pericentromeric heterochromatic region (Yelagandula et al. 2014). Although H2A.W colocalises with regions of the DNA molecule containing the histone modification H3K9me2 and cytosine methylation, the mechanism of deposition of H2A histone variant W in nucleosomes is independent of histone or DNA methylation processes (Yelagandula et al. 2014). The canonical histone H3, also known as histone variant H3.1, is predominantly located in the pericentromeric chromatin region where it is involved in silencing expression of TEs (Mito et al. 2005), and in the euchromatic regions of chromosomes (Jiang and Berger 2017). The histone chaperone Histone cell cycle regulator (HIRA) is involved in the deposition of the histone variant H3.3 instead of H3.1 in nucleosomes (Nie et al. 2014). The H3.3 variant predominates in nucleosomes of actively transcribed genes (Fig. 1, orange). Transcription termination sites contain the highest concentration of histone H3.3, which correlates with increased expression of these genes (Jiang and Berger 2017, Wollmann et al. 2017). Genes that contain H3.3 histone variant only in promoter regions are less strongly expressed and are subject to strong regulation (Jiang and Berger 2017). Interestingly, the presence of the H2A.Z histone variant in genes shows an inverse correlation with the presence of histone H3.3 (Nie et al. 2014, Jiang and Berger 2017). The CenH3 histone variant is located in the nucleosomes of the centromeric region of the chromosome and plays a key role in the formation of the kinetochore complex during cell division (Fig. 1, orange; Keçeli et al. 2020). Histone variants H1.1 and H1.2 are constitutively expressed in all cells and tightly bind part of the DNA molecule between the nucleosomes, preventing methyltransferases from accessing the DNA molecule (Jiang and Berger 2017). Under normal conditions the histone variant H1.3 is only expressed in the guard cells, where it is responsible for the correct opening of the stomata (Rutowicz et al. 2015). The expression of histone variant H1.3 together with H2A.Z is induced under conditions of water deprivation or after treatment with abscisic acid (ABA), when it is no longer restricted to the guard cells (Fig. 2A; Jiang and Berger 2017). Histone H1.3 is thought to cause hypermethylation of the DNA molecule because, unlike histone variants H1.1 and H1.2, it allows methyltransferases access to sequences that need to be methylated (Rutowicz et al. 2015, Jiang and Berger 2017).

## Histone modification

The dominant histone modifications are methylation (me), acetylation (ac), phosphorylation, ubiquitination and SUMOylation (Fig. 1, orange). Since histone methylation marks play a crucial role in regulating chromatin structure and gene expression, changes in histone methylation are an important epigenetic regulatory factor in the response of plants to abiotic and biotic stress (Bobadilla and Berr 2016). Histones can be methylated at specific amino acid residues within their N-terminal tail domains. The most common methylated amino acid residues are lysine (K) and arginine (R). Histones can be mono-, di- and trimethylated on lysine residues, while on arginine residues they can be mono- or dimethylated (Liu et al. 2010). Histone H3 methylation marks such as H3K9me1, H3K9me2, and H3K27me1 are usually characteristic of silenced TEs in the heterochromatic regions of chromosomes (Chung et al. 2022). The histone modification H3K27me3 has a repressive effect on transcription in the euchromatic regions of chromosomes (Bobadilla and Berr 2016, Chung et al. 2022). The histone modifications H3R2me2 and H4R3me2, which are anticorrelated in their position with the modification H3K4me3, have a repressive effect on transcription (Bobadilla and Berr 2016). The histone modifications H3K4me3, H3K36me2, and H3K36me3 are located in the vicinity of actively expressed genes in the euchromatin region of the chromosome (Chung et al. 2022, Shi et al. 2023).

The targets of histone acetylation are specific lysine side branches at the N-terminus of histones H3 (K4, K9, K14, K18, K23, K27) and H4 (K5, K8, K12, K16, K20) (Liu et al.

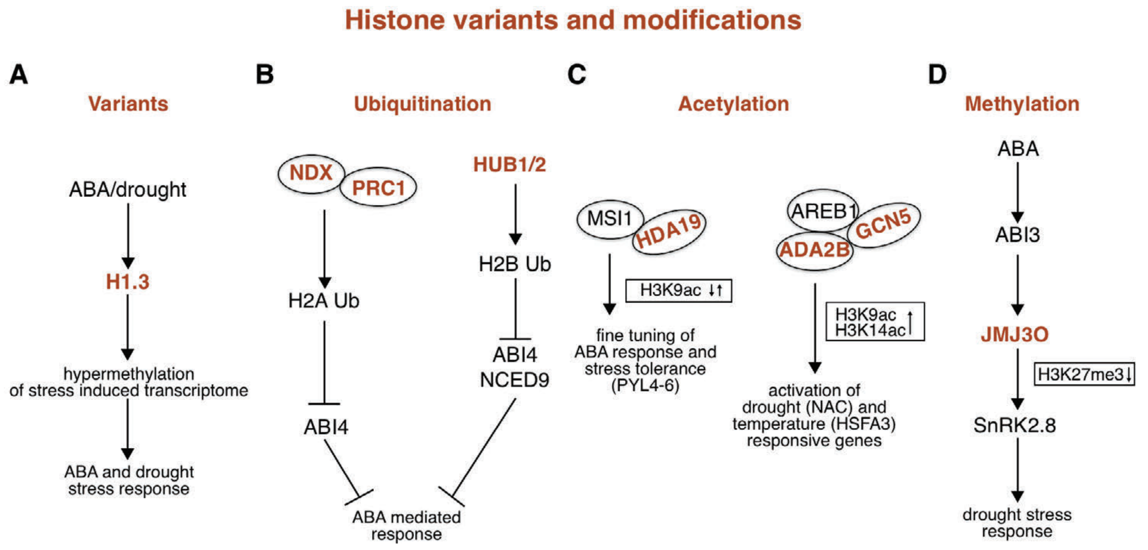

**Fig. 2.** ABA-related epigenetic role mediated by histone variants and modifications.
(**A**) Water deprivation and ABA treatment induce the expression of histone variant H1.3. In contrast to the tightly bound histone variants H1.1 and H1.2, H1.3 allows access to methyltransferases, resulting in hypermethylation of DNA loci involved in the response to ABA and drought stress. (**B**) Under normal conditions (without ABA), NDX interacts with PRC1 and binds to the downstream regions of certain ABA-responsive genes, including *ABI4*. This interaction promotes monoubiquitination of H2A and thus represses the expression of ABA-responsive genes (left). HUB1/2 repress the ABA-signalling gene *ABI4* and the ABA-biosynthesis gene *NCED9* by H2B ubiquitination (right). (**C**) The MSI1-HDA19 protein complex binds to the chromatin of ABA receptor genes, including *PYL4-6*, and controls the fine-tuning of ABA signalling by maintaining a controlled level of histone H3K9ac mark (left). The AREB1-GCN5-ADA2b interaction complex binds to the ABRE motif of drought-responsive genes of the *NAC* family, where it increases H3K9ac levels and Pol II accumulation. GCN5 is also involved in controlling the expression of HSPs through H3K9 and H3K14 acetylation of temperature-responsive *HSFA3* (right). (**D**) ABA induces the expression of the transcription factor ABI3, which leads to the activation of the histone demethylase JMJ30. JMJ30 removes repressive histone modifications H3K27me3 from the promoter of *SnRK2.8* kinase and promotes its expression, leading to plant response to abiotic stress and water deficit tolerance. Abbreviations: ABA – abscisic acid; ABI3/4 – ABA insensitive 3 and 4; ABRE – ABA-responsive elements; ADA2b – Alteration/deficiency in activation 2b; AREB1 – Abscisic acid-responsive element binding protein 1; GCN5 – General control non-depressible 5; HDA19 – Histone deacetylase 19; HSFA3 – Heat shock factor 3; HSPs – Heat shock proteins; HUB1/2 – Histone monoubiquitination 1 and 2; MSI1 – Multicopy supressor of IRA1; NCED9 – 9-cis-epoxycarotenoid dioxygenase 9; NDX – Nodulin Homeobox Factor; Pol II – Polymerase II; PRC1 – Polycomb repressive complex 1; PYL4-6 – Pyr1-like 4-6; SnRK2.8 – SNF1-related protein kinase 2-8; ↑ – increase; ↓ – decrease.

2016). Like methylation, histone acetylation is involved in regulating the expression of transcription factors (TFs) involved in plant response to biotic and abiotic stress (Hu et al. 2019). In general, histone acetylation increases gene expression by promoting chromatin decondensation (Luo et al. 2017).

Protein kinases most frequently phosphorylate serine, threonine, and tyrosine residues of histones H3 and H2A.X. Phosphorylation of histone H3 plays a role in chromosome condensation, stimulation of gene expression, DNA damage repair (Chung et al. 2022), and response to stress caused by increased salinity, low temperature, or ABA treatment (Wang et al. 2015). Phosphorylation of the histone variant H2A.X, as previously mentioned, plays a role in DNA damage signalling (Lei and Berger 2020).

The monoubiquitination of histones in plants is a specific type of histone modification in which a single ubiquitin molecule binds with its C-terminus to a lysine residue in the histone. This post-translational modification occurs mainly in histones H2A and H2B (Feng and Shen 2014, Nunez-Vazquez et al. 2022). Monoubiquitination of histone H2A is associated with transcriptional repression controlled by Polycomb repressive complex 1 (PRC1), while monoubiquitination of histone H2B is associated with transcriptionally active loci (Chung et al. 2022) and is known to activate genes involved in plant responses to abiotic and biotic stress such as drought, salt, and fungal diseases (Fig. 2B; Dhawan et al. 2009, Chen et al. 2019, Sun et al. 2020).

The binding of small ubiquitin-like modifier (SUMO) proteins to lysine residues of histones is called SUMOylation and represents a post-translational regulation of protein function. The *Arabidopsis* proteome contains numerous proteins affected by SUMOylation, suggesting that this post-translational modification is as important as phosphorylation and ubiquitination (Augustine and Vierstra 2018). Many of the known SUMO substrates are nuclear proteins, including gene regulatory proteins, transcriptional coactivators/repressors, and chromatin modifiers, emphasising the crucial role of SUMOylation in the regulation of nuclear processes (Singh et al. 2022). An increased SUMOylation of nuclear proteins is a notable and rapid action in response to various stressors. However, the exact mechanism by which this modification contributes to stress resilience is still unclear. Several studies have reported the role of SUMOylation in altering gene expression in plant response to biotic and abiotic stress (Chen et al. 2011, Park et al. 2011, Ghimire et al. 2020). For example, Niu et al. (2019) observed that SUMOylation of Topless-related protein 1 (TPR1), a transcriptional co-repressor of immune responses in *Arabidopsis*, reduces its association with histone deacetylase 19 (HDA19) and decreases its activity in transcriptional co-repression, resulting in suppressed immunity.

### Small non-coding RNAs

One of the greatest discoveries in RNA biology was the discovery of small non-coding RNAs (sncRNAs), which play a crucial role in various cellular processes. These sncRNAs, which are typically 20-30 nucleotides (nt) long, act at multiple levels, including chromatin remodelling and segregation, RNA processing and stability, transcription and translation. In addition, sncRNA molecules are actively involved in the process of DNA methylation known as RdDM (Borges and Martienssen 2015, Huang and Jin 2022). In plants, two main categories of sncRNAs are distinguished according to their origin and structure: microRNAs (miRNAs), which are usually 21-22 nt long (Axtell and Meyers 2018), and siRNAs, which are usually between 21 and 24 nt long (Borges and Martienssen 2015). MiRNAs, a subgroup of hairpin RNAs (Axtell 2013), are non-coding single-stranded RNA molecules that are involved in the process of post-transcriptional gene silencing (PTGS) in the cytoplasm (Meister 2013, Matzke and Mosher 2014). The second group of sncRNAs, known as siRNAs, is derived from double-stranded RNA precursors. Based on their function, siRNA molecules are divided into heterochromatic siRNAs (het-siRNAs), natural antisense transcript siRNAs (nat-siRNAs) and secondary siRNAs. While nat-siRNAs are involved in PTGS (Moldovan et al. 2010, Borges and Martienssen 2015), het-siRNAs and secondary siRNAs are involved in canonical RdDM (Ji and Chen 2012) and non-canonical RdDM (Borges and Martienssen 2015), respectively (Fig. 1, purple).

### DNA methylation

DNA methylation is the addition of a methyl group from S-adenosylmethionine to the fifth carbon atom of cytosine, resulting in 5-methylcytosine (5-meC), catalysed by a family of methyltransferases (Fig. 1, green). Methylation in gene promoters generally leads to repression of nearby gene expression (Zhang et al. 2018), with some exceptions such as the promoter of the gene encoding the DNA demethylase Repressor of Silencing 1 (ROS1; Lei et al. 2015, Williams et al. 2015). In most cases, methylation prevents the binding of transcriptional activators and enhances the binding of transcriptional repressors (Kumar and Mohapatra 2021). In addition, methylation of promoter regions can influence the formation of post-translational modifications of histone tails that inhibit transcription (Zhang et al. 2018). Methylation in the gene body is a characteristic of constitutively expressed genes and occurs mainly within exons in the CG context that are far from the start and end site of transcription (Bewick and Schmitz 2017). It is hypothesised that the main role of methylation of the gene body is to prevent the binding of the histone variant H2A.Z, which influences gene expression depending on the developmental stage of the plant and environmental signals (Zilberman et al. 2008, Zhang et al. 2018). Intron methylation can influence the alternative processing of mRNA transcripts (Zhang et al. 2018).

During the life cycle of a plant, two types of DNA methylation alternate. One is responsible for the maintenance and inheritance of the established methylation state, and the other for *de novo* DNA methylation. The context of the sequence that will be methylated by a specific DNA methyltransferase depends on whether the role of that DNA methyltransferase is to preserve the existing DNA methylation marks or to methylate DNA *de novo* (Matzke and Mosher 2014). Methyltransferase 1 (MET1), a homologue of the mammalian protein DNA (cytosine-5)-methyltransferase 1 (DNMT1), is responsible for the maintenance of CG methylation. Methyltransferase Chromomethylase 3 (CMT3), a homologue of mammalian DNMT3, is responsible for the maintenance of methylation in the CHG context. The third plant-specific methyltransferase, Domains rearranged methyltransferase 2 (DRM2), is the primary methyltransferase responsible for the CHH context, but also for the establishment of *de novo* methylation in all three contexts (He et al. 2011).

While methylation in symmetric contexts is inherited after DNA replication and can be passively maintained, the maintenance of methylation in an asymmetric CHH context is more complex and requires an active signal. In plants, this signal is mediated by siRNA molecules that direct the methyltransferase DRM2 to the target site and perform their function as part of the RdDM. RdDM is primarily involved in TEs silencing (Matzke and Mosher 2014, Zhang et al. 2018). TEs methylation reduces the expression of downstream genes (Hirsch and Springer 2017). The first step to control the activity of the newly inserted TEs is PTGS by activation of the non-canonical RdDM. Immediately after insertion, TEs are active and are transcribed by the enzyme Pol II. However, to counteract the potentially deleterious effects of TEs activity, some of the TEs RNA transcripts are recognised and bound by RNA-dependent RNA polymerase 6 (RDR6) that converts these single-stranded RNA molecules into double-stranded RNAs, which are then processed into 21-22 nt long siRNA molecules by the proteins Dicer-like 2 (DCL2) and Dicer-like 4 (DCL4). These siRNAs form a complex with the Argonaute 1 (AGO1) protein, which targets the complementary transposon transcripts for cleavage and degradation (Matzke and Mosher 2014). In addition to AGO1, siRNAs can also interact with Argonaute 2 (AGO2), the methyltransferase DRM2, Pol V and the protein Needed for RDR2-independent DNA methylation (NERD; Pontier et al. 2012). This interaction leads to DNA methylation being initiated *de novo*, which serves as a signal for the canonical pathway (Nuthikattu et al. 2013).

Canonical RdDM begins with the recruitment of Pol IV to specific target sites in the genome, primarily regions containing TEs. Once Pol IV is recruited, it first produces single-stranded P4-RNA, also known as Pol IV-dependent RNA, with the help of the chromatin-remodelling factor Classy 1 (CLSY1; Law et al., 2011). These single-stranded RNA molecules are then converted into long double-stranded RNAs by the activity of RNA-dependent RNA polymerase 2 (RDR2). In the next phase, the dsRNAs are processed into siRNAs, which are about 24 nt long. This processing is mediated by the protein Dicer-like 3 (DCL3). The small RNAs are then methylated at their 3' end (Ji and Chen 2012) and transferred to the cytoplasm before being incorporated into the Argonaute 4 (AGO4) protein, forming the RNA-induced silencing complex (RISC). As soon as they are loaded into AGO4, they are directed back into the cell nucleus. The second part of the mechanism begins with the activity of Pol V, which produces long non-coding RNAs. Once Pol V is recruited, it interacts with AGO4 via the C-terminal domain of its largest subunit – Nuclear RNA polymerase E1 (NRPE1). Due to their complementarity, the long non-coding RNA transcripts produced by Pol V pair with siRNA molecules from the RISC. Furthermore, the protein RNA-directed DNA methylation 1 (RDM1) binds AGO4 to methyltransferase DRM2 (Matzke and Mosher 2014).

In addition to methylation, demethylation of the DNA molecule is an important mechanism in the regulation of gene expression, TEs activity and the plant's response to stress. Passive demethylation occurs during replication of the DNA molecule by inactivating or reducing the concentration of enzymes involved in the maintenance of methylation (Li et al. 2018). Active demethylation occurs through the activity of DNA-glycosylases – ROS1, Demeter (DME) and DEMETER-like protein 2 and 3 (DML2/3), which actively cleave the methyl group from 5-meC (Li et al. 2018). This demethylation of TEs prevents the spread of methylation marks to nearby protein-coding genes and thus preserves the integrity of gene expression patterns (Tang et al. 2016). Moreover, an increased level of DNA methylation promotes the expression of *ROS1* and counteracts the effect of RdDM on a considerable number of loci (Lei et al. 2015, Williams et al. 2015, Zhang et al. 2018). For this reason, ROS1 plays an important role in the activation of TEs, and the induction of genes involved in the stress response. Interestingly, the methylation status of the *ROS1* gene promoter regulates the expression of the ROS1 protein, creating a feedback loop (Yang et al. 2022).

## Epigenetic response to temperature stress

When plants are exposed to temperatures that are 10 to 15 °C above the optimal temperature for their growth, this is referred to as heat stress (Wahid et al. 2007). At the cellular and molecular level, heat stress leads to damage of membrane and cytoskeletal proteins, as well as the accumulation of misfolded proteins and reduced enzyme activity. Such changes lead to alterations in the transcriptome, proteome, and metabolome, which in turn lead to reduced rates of photosynthesis and cellular respiration and an overall reduction in plant growth and development (Zhao et al. 2020, Chung et al. 2022). The initial response of plants to heat stress is usually a rapid and transient increase in the production of heat shock proteins (HSPs) and the induction of an antioxidant network to overcome the deleterious effects of ROS accumulation (Mittler et al. 2012, Hasanuzzaman et al. 2020). HSPs are considered heat-induced molecular chaperones, i.e., they help in the proper folding, stabilisation, and refolding of other proteins in the cell. Their main function is to protect cellular components and maintain cellular homeostasis under elevated temperature conditions.

At the epigenetic level, the response of plants to elevated temperatures is mainly controlled by histone dynamics and RdDM (Popova et al. 2013, Lämke et al. 2016, Yang et al. 2018). Some of the most important histone modifications involved in heat stress response are H3K4me3 and H3K9ac. These modifications are known as active or permissive marks and are associated with open chromatin, making the underlying genes more accessible to the transcription machinery. Indeed, in heat-treated *Arabidopsis*, H3K4me3 and H3K9ac were strongly induced in the 5'-region of genes encoding the heat shock proteins HSP18.2, HSP22.0 and Ascorbate peroxidase 2 (APX2) and were associated with hyperinduction of these genes upon heat stress (Lämke et al. 2016). In addition, histone deacetylases 6 and 2C (HDA6/HD2C) are involved in the plant response to heat stress. Heat stress induces the expression of HD2C (Buszewicz et al. 2016), which interacts with other histone deacetylases, HDA6 and HDA19, as well as with methyltransferases and chromatin remodelling complexes (CRCs) from the SWItch/Sucrose Non-Fermentable (SWI/SNF) subfamily. This interaction suggests that histone deacetylation may be functionally linked to chromatin remodelling and DNA methylation, indicating that a complex regulatory network is involved in the response to heat stress (Chung et al. 2022). On the other hand, a histone acetyltransferase (HAT) called General control non-depressible 5 (GCN5) plays an important role in the generation of permissive histone modifications, such as H3K9ac and H3K14ac (Hu et al. 2015). These modifications occur in the promoter regions of certain genes, including *HSFA3*, which regulates the expression of HSPs (Fig. 2C; Schramm et al. 2007), and the gene encoding Ultraviolet hypersensitive 6 (UVH6), which is involved in DNA damage repair (Liu et al. 2003). Acetylation of histones in these promoter regions enhances gene expression and contributes to the plant's ability to cope with heat stress. In *gcn5* mutants in which GCN5 activity is impaired, the expression of key genes such as *UVH6*, *MBF1C* and *HSFA2/3* is reduced, resulting in lower thermotolerance (Hu et al. 2015). Studies on rice (*Oryza sativa* L.) have shown that heat stress activates the expression of the protein Fertilization-Independent Endosperm1 (FIE1), which is part of the Polycomb repressive complex 2 (PRC2; Folsom et al. 2014, Miryeganeh 2021). When FIE1 is overexpressed, this leads to reduced seed size and premature cellularisation. Interestingly, the study also showed that the repressive histone modification H3K9me2 and methylation status are temperature sensitive. This suggests that the thermal sensitivity of seed enlargement may be influenced by changes in the epigenetic regulation of endosperm development during early embryogenesis.

In *Arabidopsis*, heat stress leads to globally increased methylation of the genome, while in other plants heat stress causes different trends and patterns of DNA methylation (Chung et al. 2022). However, the presence and proper function of the RdDM pathway is crucial for heat stress tolerance in plants, as mutants with non-functional canonical RdDM show a reduced response to heat stress (Popova et al. 2013). Under normal growth conditions, the Suppressor of drm1 drm2 cmt3 (SDC) protein is silenced by RdDM (Miryeganeh 2021). Heat stress activates the expression of SDC, leading to the activation of genes involved in the response to long-term heat stress and recovery. In other words, this activation suggests that the transcriptional response to heat stress must counteract the silencing effect of RdDM at specific genomic sites (Sanchez and Paszkowski 2014, Chang et al. 2020).

The RdDM pathway has also been shown to act as a protection against the activation and transposition of TEs during heat stress by maintaining their silencing and preventing possible genomic instability. Heat stress leads to the activation of certain TEs, such as the retrotransposon *ONSEN* in *Arabidopsis*, and mutants impaired in siRNA biogenesis show increased accumulation of *ONSEN* transcripts (Ito et al. 2011). In addition, *ONSEN* transposition has been detected in the progeny of heat-treated *Arabidopsis* with a non-functional RdDM pathway (Cavrak et al. 2014).

Two types of low-temperature stress can be distinguished: cold stress, which occurs at temperatures between 0 °C and 20 °C, and freezing stress, which occurs below 0 °C (Ritonga and Chen 2020). Cold stress triggers a series of complex changes in plant cells that affect photosynthesis, cellular respiration, water relations, mineral supply, and various metabolic activities (Guo et al. 2018, Ritonga and Chen 2020). The first response to cold stress occurs at the plasma membrane, where low temperatures can lead to reduced fluidity, conformational changes in membrane proteins and altered metabolite accumulation, which in turn results in changes in the redox state of the cell (Plieth et al. 1999, Orvar et al. 2000). As a result, receptors for $Ca^{2+}$ ions, ROS and phytohormones are activated, and protein kinase cascades that regulate gene expression to increase cold tolerance are initiated (Theocharis et al. 2012, Guo et al. 2018, Guo et al. 2019). At temperatures below 0 °C, ice crystals form in the apoplastic solution, leading to a reduction in water potential and causing water to flow out of the cells, resulting in cell dehydration. The formation of ice crystals can lead to electrolyte leakage and changes in membrane lipids, resulting in further stress (Ritonga and Chen 2020). To survive, plants utilise cold acclimation, in which they accumulate cold-protective polypeptides (e.g., the Cold-regulated 15A protein) and osmolytes (e.g., soluble sugars and proline) to prevent intracellular ice crystal formation and cell damage (Ritonga and Chen 2020, Hassan et al. 2021).

C-repeat binding factors (CBF) are TFs that belong to the family of Dehydration response element binding factors (DREB; Medina et al. 1999) and are the most important regulators of plant responses to cold stress. They activate *Cold regulated* (*COR*) genes, which are responsible for plant tolerance to low temperatures (Chang et al. 2020). ABA is also involved in this mechanism by inducing the expression of both *CBF* and *COR* genes, further enhancing the plant's ability to cope with low temperatures (Kidokoro et al. 2021).

The chromatin-remodelling factor Pickle (PKL) is involved in the CBF-dependent stress response in *Arabidopsis*. The *pkl* mutants have reduced expression of *C-repeat binding factor 3* (*CBF3*) and *COR* genes and show hypersensitivity to low temperatures (Yang et al. 2019). In their previous study, Yang et al. (2017) observed that *pkl* mutants have reduced methylation at a large number of RdDM target loci, but only a small number of these loci are responsible for controlling TEs activity. It has been proposed that PKL plays a role in shaping the chromatin landscape in RdDM target regions and thus acts as a chromatin remodelling factor that supports RdDM function (Yang et al. 2017). Interestingly, PKL in cooperation with Photoperiod independent early flowering 1 (PIE1), which belongs to the SWI2/SNF2-Related1 (SWR1) family of CRCs responsible for the exchange of H2A-H2B to H2A.Z-H2B dimer (Jiang and Berger 2017), and with the H3K27me3 methyltransferase Curly leaf (CLF) in the PRC2 complex also facilitates deposition of the repressive histone modification H3K27me3 near genes marked with H2A.Z histone variants (Carter et al. 2018).

It is noteworthy that cold stress reduces the deposition of H3K27me3 in the promoters of two *COR* genes, *COR15A* and *Galactinol synthase 3* (*GOLS3*), even after a return to normal growth temperature, indicating the role of H3K27me3 as a memory marker for recent transcriptional activity in *Arabidopsis* (Kwon et al. 2009). Consequently, PKL could influence the plant response to cold stress by supporting the functionality of the RdDM pathway and modulating the chromatin state of *COR* genes through H3K27me3-dependent mechanisms.

In addition to histone methylation, several other histone modifications play an important role in the response of plants to cold stress. The exposure of maize (*Zea mays* L.) to low temperatures leads to an increased expression of histone deacetylases (HDACs) and thus to a reduced acetylation of repetitive sequences in the heterochromatic regions of the genome. The increased acetylation is associated with reduced DNA methylation and the reduced number of repressive H3K9me2 histone modifications in this part of the genome. However, when the plants are exposed to prolonged stress, methylation patterns are re-established (Hu et al. 2012). In *Arabidopsis*, overexpression of Histone deacetylase 2D (HD2D) increases the plant's tolerance to low temperatures. Another histone deacetylase, HD2C, is degraded after exposure to stress by the protein High expression of osmotically responsive genes 15 (HOS15), which is part of the E3-ubiquitin ligase complex. The proteasomal degradation of the ubiquitinated deacetylase HD2C leads to higher acetylation levels in the promoters of the *COR* genes, resulting in their activation. In addition, HOS15 facilitates the recruitment of the CBF TFs to the promoters of the *COR* genes, which further increases their expression (Chang et al. 2020). It appears that the balance between histone acetylation and deacetylation is crucial for the fine-tuning of gene expression during plant response to cold stress. Some genes involved in cold tolerance may need to be activated, while others need to be repressed to maintain cellular function. The interplay of acetylation and deacetylation allows plants to regulate the expression of specific genes in response to changing environmental conditions, facilitating their adaptation and survival under cold stress.

Several studies have demonstrated a positive correlation between upregulated anthocyanin biosynthesis and tolerance to low temperatures (Ahmed et al. 2015, Li et al. 2015, Xiang et al. 2021). A recent study by Sicilia et al. (2020) reported for the first time that genes involved in anthocyanin biosynthesis exhibit different levels of DNA methylation in response to cold stress. One of these genes is *Ruby*, which is regulated by a retrotransposon element. In the study by Sicilia et al. (2020), oranges with higher anthocyanin content were found to have lower DNA methylation levels in the promoter region of the *Ruby* gene after exposure to low temperatures. In other words, under conditions of low methylation, the activity of the retrotransposon increases, leading to higher expression of the *Ruby* gene and consequently higher anthocyanin content. These results provide valuable insights into how anthocyanin biosynthesis is epigenetically regulated in response to cold stress, which could contribute to the improvement of cold tolerance in plants.

## Epigenetic control of drought and water deficiency

Water is an essential element for the growth and survival of plants. Its deficiency has negative effects on various plant processes, such as seed germination, photosynthesis, transpiration, and metabolite transport (Hao et al. 2019, Fan et al. 2020). Insufficient water availability or an increase in the osmotic potential in the soil poses a challenge for plant water uptake. Therefore, when plants perceive water deficiency, they undergo complicated reprogramming at multiple levels, including epigenetic, transcriptional, post-transcriptional and post-translational processes (Shinozaki and Yamaguchi-Shinozaki 2007, Manna et al. 2021). Recent studies have highlighted two key factors that contribute to this reprogramming. First, induction of gene expression in the ABA biosynthetic pathway, leading to an increase in ABA levels and expression of ABA-dependent genes (Osakabe et al. 2014). Second, widespread transcriptional changes in the expression of numerous genes that are not under the regulatory control of ABA but are associated with changes in chromatin organisation and structure (Khan and Zinta 2016).

In the early 2000s, a study showed that drought stress leads to increased expression of the enzyme 9-cis-epoxycarotenoid dioxygenase 3 (NCED3), which plays a crucial role in the biosynthesis of the hormone ABA (Iuchi et al. 2001). This increased expression is driven by the accumulation of H3K4me3 in the gene body. In another study by Ding et al. (2011), a protein called Arabidopsis trithorax 1 (ATX1) was found to be responsible for the accumulation of H3K4me3 modification and to play a role in both ABA-dependent and ABA-independent signalling pathways related to dehydration stress. In addition, genes encoding Responsive to desiccation 29A (RD29A), Low-temperature-induced 65 (LTI65), Responsive to desiccation 22 (RD22) and Related to AP2 4 (RAP2.4) undergo activating histone modifications such as H3K4me3 and H3K9ac in their promoter regions when induced under water-deficit conditions (Chang et al. 2020). The number of these histone modifications increases the longer and more intense the stress is. However, in the recovery period after the stress, the above-mentioned histone modifications are removed again (Chang et al. 2020). Under normal growth conditions, the repressive histone modification H3K27me3 is regulated by the polycomb group protein complexes, in particular PRC1 and PRC2. A study by Ramirez-Prado et al. (2019) investigated a PRC1-like protein in *Arabidopsis*, termed Like heterochromatin protein-1 (LHP1), and its involvement in the repression of MYC2 TF, a master regulator of the interplay between the ABA, jasmonic acid and ethylene response pathways. Loss of LHP1 resulted in reduced H3K27me3 levels in the gene bodies of *NAC domain containing proteins 19 and 55* (*ANAC019/055*), which are part of the transcriptomic network downstream of MYC2. These two TFs have been characterised as positive regulators of drought tolerance and their upregulation increases tolerance to this environmental stress (Tran et al. 2004). Indeed, the *lhp1* mutant showed phenotypes similar to the *MYC2*- and *ANAC019/055*-overexpressing plants, including increased resistance to aphids, sensitivity to ABA and improved drought tolerance (Ramirez-Prado et al. 2019).

In addition to methylation, drought stress often leads to changes in histone acetylation of drought-responsive genes, resulting in widespread changes in histone acetylation patterns throughout the plant genome. Histone acetylation is dynamically regulated by HATs and HDACs. Under drought stress, the expression of certain *HAT* genes, such as *TaHAG2*, *TaHAG3* and *TaHAC2*, was upregulated in a drought-resistant wheat variety compared to other drought-sensitive varieties, suggesting that *HAT* genes play a role in improving drought tolerance (Li et al. 2022). The gene *GCN5*, which belongs to the *HAT* family, is a widely recognised enzymatic factor responsible for the acetylation of lysine residues at histones H3 and H4 (Gan et al. 2021). Li et al. (2019) showed that drought stress in *Populus trichocarpa* induces changes in histone acetylation at the whole genome level. H3K9ac mark was associated with upregulated genes, while downregulated genes showed reduced H3K9ac levels. Analysis of differentially expressed genes revealed a significant enrichment of the ABRE motif, which corresponds to the ABA-responsive element binding protein (AREB1), within H3K9ac-associated promoters (Fujita et al. 2005). AREB1 has been shown to interact with the HAT complex, which consists of GCN5 and the transcriptional adaptor Alteration/deficiency in activation 2b (ADA2b). This interaction leads to the recruitment of the complex to drought-responsive genes, including the *NAC* gene family, by binding to ABRE motifs. As a result, there is an increase in H3K9ac and the accumulation of Pol II, leading to the activation of drought-responsive genes (Fig. 2C). Interestingly, this process enables *P. trichocarpa* to effectively cope with drought stress and thrive. As for *HAT* genes, several studies reported that drought stress affects the expression of *HDAC* genes, resulting in altered histone acetylation in drought-related genes. However, the specific expression pattern was not reported. For example, in soybean (*Glycine max* (L.) Merr.), drought treatment decreased the expression of several *HDACs* (Yang et al. 2018), while in rice (*O. sativa* L.), certain *HDAC* genes were upregulated (Hou et al. 2021). In *Arabidopsis*, *hda19* mutants exhibited increased drought resistance (Ueda et al. 2018), while overexpression of the *HDAC* gene *HD2D* increased tolerance to abiotic stresses, including drought (Han et al. 2016). Studies on the response of *HDACs* to drought stress have shed light on specific mechanisms involving *HDA6* in *Arabidopsis*. *HDA6* has been proposed to act as an ON/OFF switch for a complex drought-responsive metabolic pathway that leads to a metabolic switch from glycolysis to acetate synthesis, thereby stimulating the jasmonic acid signalling pathway for drought tolerance (Kim et al. 2017). Under normal conditions, *HDA6* suppresses the expression of key enzymes of the acetic acid biosynthetic pathway by reducing the acetylation of histone H4. However, under drought conditions, *HDA6* dissociates from these genes, allowing increased H4 acetylation and upregulation of transcription. This initiates the pathway of acetic acid biosynthesis, which ultimately increases drought tolerance through the accumulation of acetate (Kim et al. 2017).

DNA methylation is another important factor in the response of plants to water deficiency. Correlation analyses have shown that DNA methylation has differential effects on gene expression under drought stress, suggesting that it is involved in multiple regulatory pathways that directly or indirectly affect gene expression (Sun et al. 2022). Stress increases genome methylation of *P. trichocarpa* compared to wild-type plants, which in turn regulates the expression of numerous TFs. Methylation of genes encoding TFs generally increases their expression, while reduced methylation decreases the transcription rate (Liang et al. 2014). In rice (*O. sativa* L.), it has been observed that cultivars sensitive to water deficit are hypomethylated, while those resistant to drought are hypermethylated (Gayacharan and Joel 2013). TEs are also subject to changes in DNA methylation caused by abiotic stress, including drought. In *P. trichocarpa*, about 65% of transposons located in the promoters of TFs were hypomethylated in response to water deficit, and the remaining 35% were hypermethylated (Liang et al. 2014). In maize (*Z. mays* L.), transposons were discovered to regulate 33% of genes involved in the response to abiotic stress, and this regulation is not always stimulatory (Makarevitch et al. 2015). One example is the maize gene *NAC111*, where the insertion of miniature inverted-repeat transposable elements (MITEs) in the promoter region correlates with lower gene expression, leading to increased drought sensitivity (Mao et al. 2015). Interestingly, the same study showed that MITEs, when heterologously expressed in *Arabidopsis*, suppress the expression of *ZmNAC111* via the RdDM pathway and the deposition of the repressive histone modification H3K9me2.

It is assumed that the SNF2/Brahma-type protein Chromatin remodelling 12 (CHR12) is responsible for the temporary interruption of plant growth after drought and heat stress. A study by Mlynárová et al. (2007) showed that overexpression of *CHR12* in *Arabidopsis* leads to stunted growth of typically active primary buds and diminished growth of the primary stem, particularly under stress conditions. In contrast, the *CHR12* knockout mutant shows less growth inhibition when exposed to moderate stress than the wild type. This result is particularly interesting when considering another study by Han et al. (2012), which showed that loss of function of the SWI2/SNF2 chromatin remodelling ATPase Brahma (BRM) leads to increased drought tolerance, suggesting that BRM plays a crucial role in balancing growth and stress responses in plants.

## ABA is a central mediator of the epigenetic code of climate-related abiotic stress in plants

Since the stressors associated with climate change (extreme temperatures, water deficiency, drought, and soil salinisation) often occur simultaneously in the context of climate change, the most effective responses of plant organisms should overlap or even be shared. The best-known common mediator of plant responses to water deficit, drought, extreme temperatures, and soil salinity is ABA (Vishwakarma et al. 2017). The ABA signalling pathway is activated by the recognition of phytohormone molecules by cell receptors, which initiate downstream signalling cascades that trigger various physiological effects (Wang and Zhang 2008). Briefly, ABA binds to Pyrabactin resistance 1, Pyr1-like and Regulatory components of ABA receptors (PYR1/PYL/RCAR) due to accumulation induced by stress signalling, resulting in inhibition of Protein phosphatases type 2C (PP2Cs). This inhibition in turn releases Sucrose nonfermenting 1-related protein kinases 2 (SnRK2s) and activates them by autophosphorylation. Once activated, SnRK2s play an important role in a number of vital biological processes, such as transcriptional regulation, RNA processing and epigenetic modifications (Wang et al. 2013b, Chang et al. 2020, Fidler et al. 2022).

Over the last twenty years, considerable efforts have been made to unravel the epigenetic mechanisms underlying ABA responses (Bulgakov et al. 2019, Chang et al. 2020). In the plant kingdom, there is a unique subset of minor H1 variants that respond to both drought and ABA. This subset, known as H1.3, is thought to play an important role in facilitating adaptive responses to stressful environmental conditions (Jiang and Berger 2017). Extensive studies of *h1.3* null mutants have shown that the presence of H1.3 is essential for two important aspects: maintaining optimal stomatal functionality under normal growth conditions and enabling adaptive developmental responses when plants encounter the dual challenge of reduced light availability and water deficiency (Rutowicz et al. 2015). H1.3 may play a role in orchestrating changes in the stress-induced transcriptome by participating in DNA hypermethylation processes (Fig. 2A). This hypothesis is supported by the discovery that stress-induced DNA hypermethylation is significantly reduced in *h1.3* mutants (Rutowicz et al. 2015). It has already been established that both H1.1 and H1.2 inhibit the interaction of DNA methyltransferases with DNA molecules (Zemach et al. 2013). Under stress conditions, H1.3 could compete with histone variants H1.1 and H1.2 that modulate DNA accessibility to DNA methyltransferases and associated epigenetic changes (Rutowicz et al. 2015).

New findings indicate that histone modifications are also involved in the ABA-mediated response to stress conditions. Histone ubiquitination regulates many genes associated with seed dormancy. The absence of the E3 ligase enzymes Histone monoubiquitination 1 and 2 (HUB1/2) reduces seed dormancy and represses genes such as *9-cis-epoxycarotenoid dioxygenase 9* (*NCED9*) and *ABA insensitive 4* (*ABI4*; Chang et al. 2020). In *Arabidopsis*, Nodulin Homeobox Factor (NDX) interacts with components of the PRC1 complex, and together they lead to the silencing of ABA-dependent genes, such as *ABI4*, through histone H2A monoubiquitination (Zhu et al. 2020). In turn, ABA reduces the expression of the NDX protein and enables the activation of *ABI4* gene expression, but it is also involved in the regulation of histone demethylation (Fig. 2B; Zhu et al. 2020).

A study by Mehdi et al. (2016) found that the WD40 repeat-containing protein Multicopy suppressor of IRA1 (MSI1) in *Arabidopsis* forms a complex with HDA19, a histone deacetylase. This MSI1-HDA19 complex controls the fine-tuning of ABA signalling transduction. It exerts its influence by binding to the chromatin of ABA receptor genes and maintaining a controlled level of histone H3 acetylation at lysine 9. This delicate balance ultimately influences ABA receptor gene expression (Fig. 2C). Interestingly, decreased levels of MSI1 or HDA19 were associated with increased salt stress tolerance, suggesting increased sensitivity of the genes to ABA. In addition, the complex was observed to target the promoters of the key ABA receptor genes, namely *PYL4*, *PYL5* and *PYL6*, leading to repression of their expression (Mehdi et al. 2016). Histone deacetylases HDA6 and HD2C are involved in regulating the expression of two ABA-responsive genes, *ABA insensitive 1* and *2* (*ABI1/2*; Luo et al. 2012). These genes encode two phosphatases from the PP2C protein family, which negatively regulates the ABA response (Merlot et al. 2001). Indeed, *hda6* and *hd2c* mutants were found to have increased *ABI1/2* gene expression and lower resistance to aqueous NaCl and ABA treatment (Luo et al. 2012). Furthermore, histone methylation affects the expression of the *SNF1-related protein kinase 2-8* (*SnRK2.8*) gene, as treatment with ABA induces the expression of the *ABA insensitive 3* (*ABI3*) gene, leading to the activation of histone demethylase JMJ30. Subsequently, JMJ30 removes the repressive histone modifications H3K27me3 from the promoter of the *SnRK2.8* gene and promotes its expression (Wu et al. 2019, Chang et al. 2020, Shi et al. 2023). This regulatory cascade is particularly important for the response of young seedlings to water deficiency (Fig. 2D; Wu et al. 2019).

It has been shown that after treatment with ABA, the chromatin structure undergoes CRC-mediated changes. The Switch/sucrose nonfermenting 3B (SWI3B) subunit of SWI/SNF CRCs interacts with Hypersensitive to ABA1 (HAB1), a member of the PP2C phosphatase family (Fig. 3A).

Under normal growth conditions, HAB1 inhibits SWI3B and thus prevents the expression of ABA-responsive genes. However, treatment with ABA counteracts the effect of HAB1 and allows SWI3B to activate the expression of ABA-responsive genes (Saez et al. 2008). Another CRC subunit, BRM from the SWI2/SNF2 family, suppresses ABA-responsive genes in the absence of stress (Fig. 3B; Han et al. 2012). The activity of BRM is regulated by the interaction of SnRK2 kinases and PP2C phosphatases. In the signalling pathway, BRM is located downstream of SnRK2s. Phosphorylation of BRM by SnRK2s leads to the release of BRM-mediated repression of ABA-responsive genes. Conversely, PP2C-mediated dephosphorylation of BRM maintains the repressive role of BRM in the response to ABA (Peirats-Llobet et al. 2016). SWR1 CRCs are involved in the exchange of H2A-H2B for the H2A.Z-H2B dimer in nucleosomes (Jiang and Berger 2017), while CRCs from the INO80 subfamily are involved in the reverse process (Fig. 3C; Han et al. 2015). The exchange of H2A.Z-H2B dimers is faster than that of H2A-H2B dimers (Brahma et al. 2017). As a result, genes covered by H2A.Z-H2B nucleosomes can respond faster to stimuli. Indeed, histone H2A.Z plays a role in the response of plants to osmotic stress and is removed from induced genes under conditions of water deficit (Sura et al. 2017). Under heat stress conditions Heat shock factor 1 (HSF1) TF is involved in the removal of histone H2A.Z from genes induced at a temperature of 27 °C (Cortijo et al. 2017).

ABA-mediated responses synergise with other regulatory mechanisms, including the plant-specific epigenetic pathway of RdDM. A study by Kim et al. (2019) has shown that the expression of several ABA-dependent genes is regulated by the RdDM pathway and ROS1-dependent DNA demethylation (Fig. 4A). In *ros1* mutants treated with ABA, certain ABA-dependent genes are hypermethylated in a promoter region, leading to reduced gene expression. Examples of such genes are *Nicotinamidase 3* (*NIC3*), whose gene product is involved in the recycling of the cofactor NAD$^+$ (Kim et al. 2019), and RD29A (Gong et al. 2002),

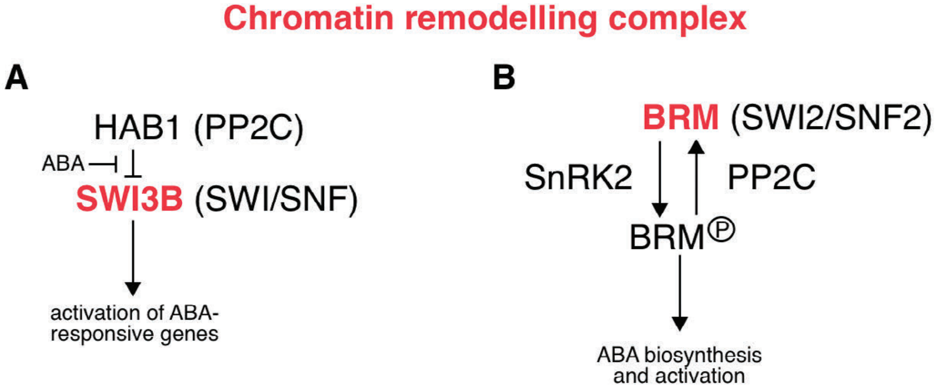

**Fig. 3.** ABA-related epigenetic role mediated by the activity of the chromatin remodelling complex. (**A**) The PP2C phosphatase HAB1 mediates the inhibition of SWI3B, the subunit of SWI/SNF CRC, and thus prevents the expression of ABA-responsive genes. Under stress conditions, ABA accumulation neutralises the effect of HAB1 and allows SWI3B to activate the expression of ABA-responsive genes. (**B**) The activity of the BRM is regulated by the process of phosphorylation and dephosphorylation. SnRK2 kinase-mediated phosphorylation of BRM leads to the release of BRM-mediated repression of ABA-responsive genes. In contrast, PP2C-mediated dephosphorylation of BRM serves to maintain its repressive role in the ABA response. Abbreviations: BRM – SWI2/SNF2 chromatin remodelling ATPase Brahma; CRC – chromatin remodelling complex; HAB1 – Hypersensitive to ABA1; PP2C – Protein phosphatases type 2C; SnRK2 – Sucrose nonfermenting 1-related protein kinases 2; SWI/SNF – SWItch/Sucrose Non-Fermentable subfamily; SWI3B – Switch/sucrose nonfermenting 3B.

which is involved in responses to abiotic stress. In addition, DNA methylation in response to ABA can influence the localisation of proteins in cells. *RdDM* and *hd2c* mutants show defective cellular localisation of two DEAD-box RNA helicases, Stress response suppressor 1 and 2 (STRS1/2), in response to abiotic stress, including treatment with ABA (Khan et al. 2014). Moreover, treatment with ABA reduces the expression of STRS1/2 helicases, while *strs1/2* mutants exhibit increased tolerance to heat, water and salt stress, and upregulation of stress-responsive genes (Fig. 4B; Khan et al. 2014). A recent study on maize has shown that loss of Mediator of paramutation 1 (MOP1) protein activity leads to

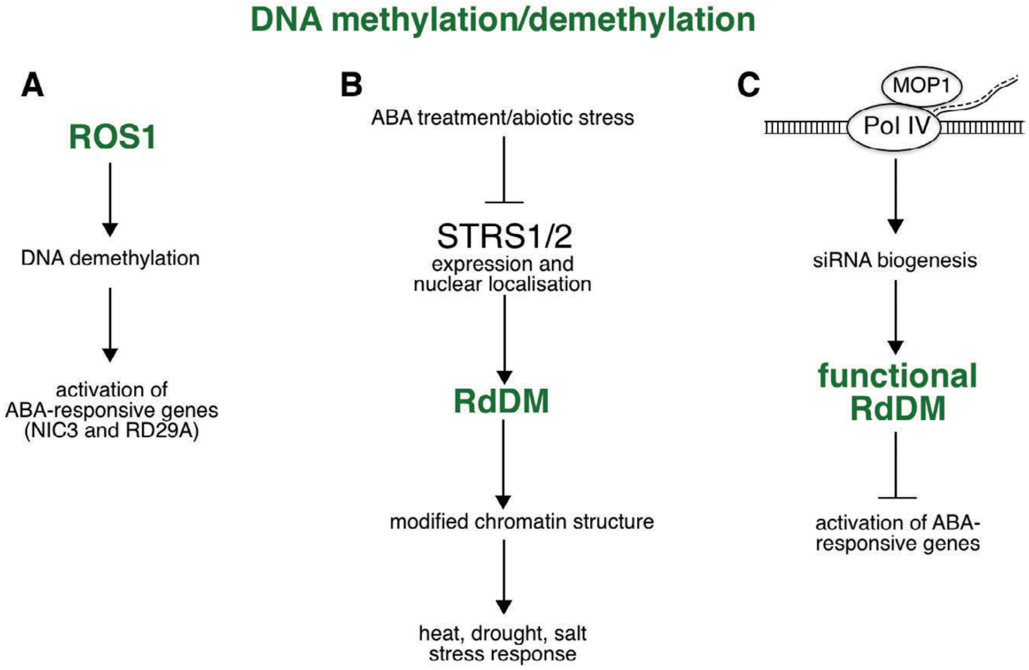

**Fig. 4.** ABA-related epigenetic role mediated by RdDM. (**A**) Increased level of ROS1-mediated DNA methylation is required for expression of ABA- and stress-responsive genes such as *RD29A* and *NIC3*, suggesting a role for ROS1-dependent DNA demethylation in ABA-mediated stress responses. (**B**) RdDM in response to ABA reduces the expression and affects the localisation of two DEAD-box RNA helicases, STRS1/2, thereby enhancing the expression of stress-responsive genes and increasing tolerance to heat, water, and salt stress. (**C**) MOP1, the maize orthologue of *Arabidopsis* RDR2, represses expression of ABA-responsive genes *via* Pol IV-dependent siRNA biogenesis of RdDM. Abbreviations: MOP1 – Mediator of paramutation 1; NIC3 – Nicotinamidase 3; Pol IV – Polymerase IV; RD29A – Responsive to desiccation 29A; RdDM – RNA-directed DNA methylation; RDR2 – RNA-dependent RNA polymerase 2; ROS1 – Repressor of Silencing 1; siRNA – small interfering RNA; STRS1/2 – Stress response suppressor 1 and 2.

increased expression of ABA-responsive genes (Vendramin et al. 2020). Interestingly, MOP1 is an orthologue of *Arabidopsis* RDR2, a protein known for its interaction with Pol IV (Haag et al. 2014). This interaction is essential for Pol IV-dependent siRNA biogenesis and the functionality of the RdDM pathway (Fig. 4C). Madzima et al. (2021) discovered a list of genes that were uniquely up- and downregulated upon both ABA treatment and loss of MOP1. By comparing these unique genes to genes whose promoters share homology with MOP1-dependent siRNAs, the group found that a quarter of the genes are directly regulated by MOP1. In addition, a comprehensive Gene Ontology (GO) analysis revealed that there were more significantly enriched GO terms associated with upregulated genes than with downregulated genes. Since GO terms are associated with various biological processes, including signal transduction, cell communication, regulation of gene expression, and response to abiotic stimuli, the authors propose that MOP1-dependent activity and siRNAs are directly related to the regulation of certain biological processes in response to ABA. On the other hand, siRNAs associated with downregulated genes are probably independent of MOP1 and may have a less specific biological role associated with ABA responses (Madzima et al. 2021).

## Conclusion and future perspectives

Numerous studies have shown that epigenetic processes, including DNA methylation, histone modifications and variants, and chromatin remodelling contribute to the response to stressors and enable plants to defend themselves and thrive under difficult conditions. These mechanisms also play a crucial role in hormonal signalling, regulation of antioxidant enzyme levels, and activation of stress resistance genes. Of particular interest is the fact that some epigenetic patterns can be inherited, increasing the adaptability of future generations to a stressful environment. Understanding the intricate regulatory processes involving epigenetic regulation as a target of stress and a force of adaptation can lead to the development of tools to protect crops from the growing challenges posed by pervasive stressors in global agriculture. ABA is involved in numerous aspects of stress responses, including almost all epigenetic mechanisms, and there are examples of overlap between the regulatory mechanisms of ABA and epigenetic adaptations to the environment. Therefore, elucidating the interplay between stressors, ABA and the epigenetic code could serve as a basis for the development of tools to optimise the synergistic responses of plants to global environmental change. Using the link between ABA-responsive element binding factors (ABFs) and ABA-responsive elements (ABREs) to make specific epigenetic marks at genetic loci and modulate gene expression is a promising way to improve breeding programmes. This approach enables precise and controlled adjustment of epigenetic modifications at key genetic loci and enables crops to develop a customised and efficient response to various stress factors. By strategically utilising this molecular partnership, researchers can contribute to the development of crops that are not only highly adaptable to adverse conditions, but also meet the growing demands of global agriculture in the face of changing environmental conditions. This advance has the potential to transform plant breeding and usher in an era of stress-resistant and high-yielding crop varieties.

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## References

Ahmed, N.U., Park, J.-I., Jung, H.-J., Hur, Y., Nou, I.-S., 2015: Anthocyanin biosynthesis for cold and freezing stress tolerance and desirable color in *Brassica rapa*. Functional and Integrative Genomics 15, 383–394. https://doi.org/10.1007/s10142-014-0427-7

Augustine, R.C., Vierstra, R.D., 2018: SUMOylation: re-wiring the plant nucleus during stress and development. Current Opinion in Plant Biology 45(A), 143–154. https://doi.org/10.1016/j.pbi.2018.06.006

Axtell, M.J., 2013: Classification and comparison of small RNAs from plants. Annual Review of Plant Biology 64, 137–159. https://doi.org/10.1146/annurev-arplant-050312-120043

Axtell, M.J., Meyers, B.C., 2018: Revisiting criteria for plant microRNA annotation in the era of big data. The Plant Cell 30(2), 272–284. https://doi.org/10.1105/tpc.17.00851

Bewick, A.J., Schmitz, R.J., 2017: Gene body DNA methylation in plants. Current Opinion in Plant Biology 36, 103–110. https://doi.org/10.1016/j.pbi.2016.12.007

Bobadilla, R., Berr, A., 2016: Histone methylation – a cornerstone for plant responses to environmental stresses? In: Shanker, A. K., Shanker, C. (eds.), Abiotic and biotic stress in plants – recent advances and future perspectives, 31–62. InTech, Rijeka.

Borges, F., Martienssen, R.A., 2015: The expanding world of small RNAs in plants. Nature Reviews Molecular Cell Biology 16, 727–741. https://doi.org/10.1038/nrm4085

Brahma, S., Udugama, M.I., Kim, J., Hada, A., Bhardwaj, S.K., Hailu, S.G., Lee, T.-H., Bartholomew, B., 2017: INO80 exchanges H2A.Z for H2A by translocating on DNA proximal to histone dimers. Nature Communications 8, 15616. https://doi.org/10.1038/ncomms15616

Bulgakov, V.P., Wu, H.-C., Jinn, T.-L., 2019: Coordination of ABA and chaperone signaling in plant stress responses. Trends in Plant Science 24(7), 636–651. https://doi.org/10.1016/j.tplants.2019.04.004

Buszewicz, D., Archacki, R., Palusiński, A., Kotliński, M., Fogtman, A., Iwanicka-Nowicka, R., Sosnowska, K., Kuciński, J., Pupel, P., Olędzki, J., Dadlez, M., Misicka, A., Jerzmanowski, A., Koblowska, M.K., 2016: HD2C histone deacetylase and a SWI/SNF chromatin remodelling complex interact and both are involved in mediating the heat stress response in *Arabidopsis*.

Plant, Cell and Environment 39(10), 2108–2122. https://doi.org/10.1111/pce.12756

Carter, B., Bishop, B., Ho, K.K., Huang, R., Jia, W., Zhang, H., Pascuzzi, P.E., Deal, R.B., Ogas, J., 2018: The chromatin remodelers PKL and PIE1 act in an epigenetic pathway that determines H3K27me3 homeostasis in Arabidopsis. The Plant Cell 30(6), 1337–1352. https://doi.org/10.1105/tpc.17.00867

Cavrak, V.V., Lettner, N., Jamge, S., Kosarewicz, A., Bayer, L.M., Mittelsten Scheid, O., 2014: How a retrotransposon exploits the plant's heat stress response for its activation. PLoS Genetics 10, e1004115. https://doi.org/10.1371/journal.pgen.1004115

Chang, Y., Zhu, C., Jiang, J., Zhang, H., Zhu, J., Duan, C., 2020: Epigenetic regulation in plant abiotic stress responses. Journal of Integrative Plant Biology 62(5), 563–580. https://doi.org/10.1111/jipb.12901

Chen, C.-C., Chen, Y.-Y., Tang, I.-C., Liang, H.-M., Lai, C.-C., Chiou, J.-M., Yeh, K.-C., 2011: Arabidopsis SUMO E3 ligase SIZ1 is involved in excess copper tolerance. Plant Physiology 156(4), 2225–2234. https://doi.org/10.1104/pp.111.178996

Chen, H., Feng, H., Zhang, X., Zhang, C., Wang, T., Dong, J., 2019: An *Arabidopsis* E3 ligase *HUB 2* increases histone H2B monoubiquitination and enhances drought tolerance in transgenic cotton. Plant Biotechnology Journal 17(3), 556–568. https://doi.org/10.1111/pbi.12998

Chung, S., Kwon, C., Lee, J.-H., 2022: Epigenetic control of abiotic stress signaling in plants. Genes and Genomics 44, 267–278. https://doi.org/10.1007/s13258-021-01163-3

Cortijo, S., Charoensawan, V., Brestovitsky, A., Buning, R., Ravarani, C., Rhodes, D., van Noort, J., Jaeger, K.E., Wigge, P.A., 2017: Transcriptional regulation of the ambient temperature response by H2A.Z nucleosomes and HSF1 transcription factors in Arabidopsis. Molecular Plant 10(10), 1258–1273. https://doi.org/10.1016/j.molp.2017.08.014

Cramer, G.R., Urano, K., Delrot, S., Pezzotti, M., Shinozaki, K., 2011: Effects of abiotic stress on plants: a systems biology perspective. BMC Plant Biology 11, 163. https://doi.org/10.1186/1471-2229-11-163

Dhawan, R., Luo, H., Foerster, A.M., AbuQamar, S., Du, H.-N., Briggs, S.D., Scheid, O.M., Mengiste, T., 2009: HISTONE MONOUBIQUITINATION1 interacts with a subunit of the mediator complex and regulates defense against necrotrophic fungal pathogens in *Arabidopsis*. The Plant Cell 21(3), 1000–1019. https://doi.org/10.1105/tpc.108.062364

Ding, Y., Avramova, Z., Fromm, M., 2011: The Arabidopsis trithorax-like factor ATX1 functions in dehydration stress responses via ABA-dependent and ABA-independent pathways. The Plant Journal 66(5), 735–744. https://doi.org/10.1111/j.1365-313X.2011.04534.x

Duan, C.-G., Wang, X., Zhang, L., Xiong, X., Zhang, Z., Tang, K., Pan, L., Hsu, C.-C., Xu, H., Tao, W.A., Zhang, H., Zhu, J.-K., 2017: A protein complex regulates RNA processing of intronic heterochromatin-containing genes in *Arabidopsis*. Proceedings of the National Academy of Sciences 114(35), E7377-E7384. https://doi.org/10.1073/pnas.1710683114

Duan, C.-G., Zhu, J.-K., Cao, X., 2018: Retrospective and perspective of plant epigenetics in China. Journal of Genetics and Genomics 45(11), 621–638. https://doi.org/10.1016/j.jgg.2018.09.004

Fan, X., Cao, X., Zhou, H., Hao, L., Dong, W., He, C., Xu, M., Wu, H., Wang, L., Chang, Z., Zheng, Y., 2020: Carbon dioxide fertilization effect on plant growth under soil water stress associates with changes in stomatal traits, leaf photosynthesis, and foliar nitrogen of bell pepper (*Capsicum annuum* L.). Environmental and Experimental Botany 179, 104203. https://doi.org/10.1016/j.envexpbot.2020.104203

Feng, J., Shen, W.-H., 2014: Dynamic regulation and function of histone monoubiquitination in plants. Frontiers in Plant Science 5, 83. https://doi.org/10.3389/fpls.2014.00083

Fidler, J., Graska, J., Gietler, M., Nykiel, M., Prabucka, B., Rybarczyk-Płońska, A., Muszyńska, E., Morkunas, I., Labudda, M., 2022: PYR/PYL/RCAR receptors play a vital role in the abscisic-acid-dependent responses of plants to external or internal stimuli. Cells 11(8), 1352. https://doi.org/10.3390/cells11081352

Folsom, J.J., Begcy, K., Hao, X., Wang, D., Walia, H., 2014: Rice *Fertilization-Independent Endosperm1* regulates seed size under heat stress by controlling early endosperm development. Plant Physiology 165(1), 238–248. https://doi.org/10.1104/pp.113.232413

Foroozani, M., Holder, D.H., Deal, R.B., 2022: Histone variants in the specialization of plant chromatin. Annual Review of Plant Biology 73, 149–172. https://doi.org/10.1146/annurev-arplant-070221-050044

Fujita, Y., Fujita, M., Satoh, R., Maruyama, K., Parvez, M.M., Seki, M., Hiratsu, K., Ohme-Takagi, M., Shinozaki, K., Yamaguchi-Shinozaki, K., 2005: AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. The Plant Cell 17(12), 3470–3488. https://doi.org/10.1105/tpc.105.035659

Gan, L., Wei, Z., Yang, Z., Li, F., Wang, Z., 2021: Updated mechanisms of GCN5 – The monkey king of the plant kingdom in plant development and resistance to abiotic stresses. Cells 10(5), 979. https://doi.org/10.3390/cells10050979

Gayacharan, Joel, A.J., 2013: Epigenetic responses to drought stress in rice (*Oryza sativa* L.). Physiology and Molecular Biology of Plants 19, 379–387. https://doi.org/10.1007/s12298-013-0176-4

Ghimire, S., Tang, X., Zhang, N., Liu, W., Si, H., 2020: SUMO and SUMOylation in plant abiotic stress. Plant Growth Regulation 91, 317–325. https://doi.org/10.1007/s10725-020-00624-1

Gong, Z., Morales-Ruiz, T., Ariza, R.R., Roldán-Arjona, T., David, L., Zhu, J.-K., 2002: ROS1, a repressor of transcriptional gene silencing in Arabidopsis, encodes a DNA glycosylase/lyase. Cell 111(6), 803–814. https://doi.org/10.1016/S0092-8674(02)01133-9

Guo, J., Ren, Y., Tang, Z., Shi, W., Zhou, M., 2019: Characterization and expression profiling of the *ICE-CBF-COR* genes in wheat. PeerJ 7, e8190. https://doi.org/10.7717/peerj.8190

Guo, X., Liu, D., Chong, K., 2018: Cold signaling in plants: Insights into mechanisms and regulation. Journal of Integrative Plant Biology 60(9), 745–756. https://doi.org/10.1111/jipb.12706

Haag, J.R., Brower-Toland, B., Krieger, E.K., Sidorenko, L., Nicora, C.D., Norbeck, A.D., Irsigler, A., LaRue, H., Brzeski, J., McGinnis, K., Ivashuta, S., Pasa-Tolic, L., Chandler, V.L., Pikaard, C.S., 2014: Functional diversification of maize RNA polymerase IV and V subtypes via alternative catalytic subunits. Cell Reports 9(1), 378–390. https://doi.org/10.1016/j.celrep.2014.08.067

Han, S.-K., Sang, Y., Rodrigues, A., BIOL425 F2010, Wu, M.-F., Rodriguez, P.L., Wagner, D., 2012: The SWI2/SNF2 chromatin remodeling ATPase BRAHMA represses abscisic acid responses in the absence of the stress stimulus in *Arabidopsis*. The Plant Cell 24(12), 4892–4906. https://doi.org/10.1105/tpc.112.105114

Han, S.-K., Wu, M.-F., Cui, S., Wagner, D., 2015: Roles and activities of chromatin remodeling ATPases in plants. The Plant Journal 83(1), 62–77. https://doi.org/10.1111/tpj.12877

Han, Z., Yu, H., Zhao, Z., Hunter, D., Luo, X., Duan, J., Tian, L., 2016: *AtHD2D* gene plays a role in plant growth, development, and response to abiotic stresses in *Arabidopsis thaliana*. Frontiers in Plant Science 7, 310. https://doi.org/10.3389/fpls.2016.00310

Hao, S., Cao, H., Wang, H., Pan, X., 2019: The physiological responses of tomato to water stress and re-water in different growth periods. Scientia Horticulturae 249, 143–154. https://doi.org/10.1016/j.scienta.2019.01.045

Hasanuzzaman, M., Bhuyan, M.H.M., Zulfiqar, F., Raza, A., Mohsin, S., Mahmud, J., Fujita, M., Fotopoulos, V., 2020: Reactive oxygen species and antioxidant defense in plants under abiotic stress: Revisiting the crucial role of a universal defense regulator. Antioxidants 9(8), 681. https://doi.org/10.3390/antiox9080681

Hassan, M.A., Xiang, C., Farooq, M., Muhammad, N., Yan, Z., Hui, X., Yuanyuan, K., Bruno, A.K., Lele, Z., Jincai, L., 2021: Cold stress in wheat: plant acclimation responses and management strategies. Frontiers in Plant Science 12, 676884. https://doi.org/10.3389/fpls.2021.676884

He, X.-J., Chen, T., Zhu, J.-K., 2011: Regulation and function of DNA methylation in plants and animals. Cell Research 21, 442–465. https://doi.org/10.1038/cr.2011.23

Hirsch, C.D., Springer, N.M., 2017: Transposable element influences on gene expression in plants. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1860(1), 157–165. https://doi.org/10.1016/j.bbagrm.2016.05.010

Hou, J., Ren, R., Xiao, H., Chen, Z., Yu, J., Zhang, H., Shi, Q., Hou, H., He, S., Li, L., 2021: Characteristic and evolution of HAT and HDAC genes in Gramineae genomes and their expression analysis under diverse stress in *Oryza sativa*. Planta 253, 72. https://doi.org/10.1007/s00425-021-03589-1

Hu, Y., Lu, Y., Zhao, Y., Zhou, D.-X., 2019: Histone acetylation dynamics integrates metabolic activity to regulate plant response to stress. Frontiers in Plant Science 10, 1236. https://doi.org/10.3389/fpls.2019.01236

Hu, Y., Zhang, L., He, S., Huang, M., Tan, J., Zhao, L., Yan, S., Li, H., Zhou, K., Liang, Y., Li, L., 2012: Cold stress selectively unsilences tandem repeats in heterochromatin associated with accumulation of H3K9ac. Plant, Cell and Environment 35(12), 2130–2142. https://doi.org/10.1111/j.1365-3040.2012.02541.x

Hu, Z., Song, N., Zheng, M., Liu, X., Liu, Z., Xing, J., Ma, J., Guo, W., Yao, Y., Peng, H., Xin, M., Zhou, D., Ni, Z., Sun, Q., 2015: Histone acetyltransferase GCN 5 is essential for heat stress-responsive gene activation and thermotolerance in Arabidopsis. The Plant Journal 84(6), 1178–1191. https://doi.org/10.1111/tpj.13076

Huang, C.-Y., Jin, H., 2022: Coordinated epigenetic regulation in plants: a potent managerial tool to conquer biotic stress. Frontiers in Plant Science 12, 795274. https://doi.org/10.3389/fpls.2021.795274

Ito, H., Gaubert, H., Bucher, E., Mirouze, M., Vaillant, I., Paszkowski, J., 2011: An siRNA pathway prevents transgenerational retrotransposition in plants subjected to stress. Nature 472, 115–119. https://doi.org/10.1038/nature09861

Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., Tabata, S., Kakubari, Y., Yamaguchi-Shinozaki, K., Shinozaki, K., 2001: Regulation of drought tolerance by gene manipulation of 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. The Plant Journal 27(4), 325–333. https://doi.org/10.1046/j.1365-313x.2001.01096.x

Ji, L., Chen, X., 2012: Regulation of small RNA stability: methylation and beyond. Cell Research 22, 624–636. https://doi.org/10.1038/cr.2012.36

Jiang, D., Berger, F., 2017: Histone variants in plant transcriptional regulation. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1860(1), 123–130. https://doi.org/10.1016/j.bbagrm.2016.07.002

Keçeli, B.N., Jin, C., Van Damme, D., Geelen, D., 2020: Conservation of centromeric histone 3 interaction partners in plants. Journal of Experimental Botany 71(17), 5237–5246. https://doi.org/10.1093/jxb/eraa214

Khan, A., Garbelli, A., Grossi, S., Florentin, A., Batelli, G., Acuna, T., Zolla, G., Kaye, Y., Paul, L.K., Zhu, J.-K., Maga, G., Grafi, G., Barak, S., 2014: The Arabidopsis STRESS RESPONSE SUPPRESSOR DEAD-box RNA helicases are nucleolar- and chromocenter-localized proteins that undergo stress-mediated relocalization and are involved in epigenetic gene silencing. The Plant Journal 79(1), 28–43. https://doi.org/10.1111/tpj.12533

Khan, A., Zinta, G., 2016: Drought stress and chromatin: an epigenetic perspective. In: Hossain, M. A., Wani, S. H., Bhattacharjee, S., Burritt, D. J., Tran, L.-S. P. (eds.), Drought stress tolerance in plants, 571–586. Springer Cham.

Kidokoro, S., Hayashi, K., Haraguchi, H., Ishikawa, T., Soma, F., Konoura, I., Toda, S., Mizoi, J., Suzuki, T., Shinozaki, K., Yamaguchi-Shinozaki, K., 2021: Posttranslational regulation of multiple clock-related transcription factors triggers cold-inducible gene expression in *Arabidopsis*. Proceedings of the National Academy of Sciences 118(10), e2021048118. https://doi.org/10.1073/pnas.2021048118

Kim, J.-M., To, T.K., Matsui, A., Tanoi, K., Kobayashi, N.I., Matsuda, F., Habu, Y., Ogawa, D., Sakamoto, T., Matsunaga, S., Bashir, K., Rasheed, S., Ando, M., Takeda, H., Kawaura, K., Kusano, M., Fukushima, A., Endo, T.A., Kuromori, T., Ishida, J., Morosawa, T., Tanaka, M., Torii, C., Takebayashi, Y., Sakakibara, H., Ogihara, Y., Saito, K., Shinozaki, K., Devoto, A., Seki, M., 2017: Acetate-mediated novel survival strategy against drought in plants. Nature Plants 3, 17097. https://doi.org/10.1038/nplants.2017.97

Kim, J.-S., Lim, J.Y., Shin, H., Kim, B.-G., Yoo, S.-D., Kim, W.T., Huh, J.H., 2019: ROS1-dependent DNA demethylation is required for ABA-inducible *NIC3* expression. Plant Physiology 179(4), 1810–1821. https://doi.org/10.1104/pp.18.01471

Kumar, S., Mohapatra, T., 2021: Dynamics of DNA methylation and its functions in plant growth and development. Frontiers in Plant Science 12, 596236. https://doi.org/10.3389/fpls.2021.596236

Kwon, C.S., Lee, D., Choi, G., Chung, W.-I., 2009: Histone occupancy-dependent and -independent removal of H3K27 trimethylation at cold-responsive genes in Arabidopsis. The Plant Journal 60(1), 112–121. https://doi.org/10.1111/j.1365-313X.2009.03938.x

Lämke, J., Brzezinka, K., Altmann, S., Bäurle, I., 2016: A hit-and-run heat shock factor governs sustained histone methylation and transcriptional stress memory. The EMBO Journal 35, 162–175. https://doi.org/10.15252/embj.201592593

Law, J.A., Vashisht, A.A., Wohlschlegel, J.A., Jacobsen, S.E., 2011: SHH1, a homeodomain protein required for DNA methylation, as well as RDR2, RDM4, and chromatin remodeling factors, associate with RNA polymerase IV. PLOS Genetics 7, e1002195. https://doi.org/10.1371/journal.pgen.1002195

Lei, B., Berger, F., 2020: H2A variants in Arabidopsis: versatile regulators of genome activity. Plant Communications 1(1), 100015. https://doi.org/10.1016/j.xplc.2019.100015

Lei, M., Zhang, H., Julian, R., Tang, K., Xie, S., Zhu, J.-K., 2015: Regulatory link between DNA methylation and active demethylation in *Arabidopsis*. Proceedings of the National Academy of Sciences 112(11), 3553–3557. https://doi.org/10.1073/pnas.1502279112

Li, H., Liu, H., Pei, X., Chen, H., Li, X., Wang, J., Wang, C., 2022: Comparative genome-wide analysis and expression profiling of histone acetyltransferases and histone deacetylases involved in the response to drought in wheat. Journal of Plant Growth Regulation 41, 1065–1078. https://doi.org/10.1007/s00344-021-10364-9

Li, S., Lin, Y.-C.J., Wang, P., Zhang, B., Li, M., Chen, S., Shi, R., Tunlaya-Anukit, S., Liu, X., Wang, Z., Dai, X., Yu, J., Zhou, C., Liu, B., Wang, J.P., Chiang, V.L., Li, W., 2019: The AREB1 transcription factor influences histone acetylation to regulate drought responses and tolerance in *Populus trichocarpa*. The Plant Cell 31(3), 663–686. https://doi.org/10.1105/tpc.18.00437

Li, S.-J., Bai, Y.-C., Li, C.-L., Yao, H.-P., Chen, H., Zhao, H.-X., Wu, Q., 2015: Anthocyanins accumulate in tartary buckwheat (*Fagopyrum tataricum*) sprout in response to cold stress. Acta Physiologiae Plantarum 37, 159. https://doi.org/10.1007/s11738-015-1913-9

Li, Y., Kumar, S., Qian, W., 2018: Active DNA demethylation: mechanism and role in plant development. Plant Cell Reports 37, 77–85. https://doi.org/10.1007/s00299-017-2215-z

Liang, D., Zhang, Z., Wu, H., Huang, C., Shuai, P., Ye, C.-Y., Tang, S., Wang, Y., Yang, L., Wang, J., Yin, W., Xia, X., 2014: Single-base-resolution methylomes of *Populus trichocarpa* reveal the association between DNA methylation and drought stress. BMC Genetics 15(1), S9. https://doi.org/10.1186/1471-2156-15-S1-S9

Lichtenthaler, H.K., 1998: The stress concept in plants: an introduction. Annals of the New York Academy of Sciences 851(1), 187–198. https://doi.org/10.1111/j.1749-6632.1998.tb08993.x

Liu, C., Lu, F., Cui, X., Cao, X., 2010: Histone methylation in higher plants. Annual Review of Plant Biology 61, 395–420. https://doi.org/10.1146/annurev.arplant.043008.091939

Liu, J., He, Z., 2020: Small DNA methylation, big player in plant abiotic stress responses and memory. Frontiers in Plant Science 11, 595603. https://doi.org/10.3389/fpls.2020.595603

Liu, X., Yang, S., Yu, C.-W., Chen, C.-Y., Wu, K., 2016: Histone acetylation and plant development. In: Lin, C., Luan, S. (eds.), The enzymes volume 40, 173–199. Elsevier.

Liu, Z., Hong, S.-W., Escobar, M., Vierling, E., Mitchell, D.L., Mount, D.W., Hall, J.D., 2003: Arabidopsis *UVH6*, a homolog of human *XPD* and yeast *RAD3* DNA repair genes, functions in DNA repair and is essential for plant growth. Plant Physiology 132(3), 1405–1414. https://doi.org/10.1104/pp.103.021808

Luo, M., Cheng, K., Xu, Y., Yang, S., Wu, K., 2017: Plant responses to abiotic stress regulated by histone deacetylases. Frontiers in Plant Science 8, 2147. https://doi.org/10.3389/fpls.2017.02147

Luo, M., Wang, Y.-Y., Liu, X., Yang, S., Lu, Q., Cui, Y., Wu, K., 2012: HD2C interacts with HDA6 and is involved in ABA and salt stress response in *Arabidopsis*. Journal of Experimental Botany 63(8), 3297–3306. https://doi.org/10.1093/jxb/ers059

Madzima, T.F., Vendramin, S., Lynn, J.S., Lemert, P., Lu, K.C., McGinnis, K.M., 2021: Direct and indirect transcriptional effects of abiotic stress in *Zea mays* plants defective in RNA-directed DNA methylation. Frontiers in Plant Science 12, 694289. https://doi.org/10.3389/fpls.2021.694289

Makarevitch, I., Waters, A.J., West, P.T., Stitzer, M., Hirsch, C.N., Ross-Ibarra, J., Springer, N.M., 2015: Transposable elements contribute to activation of maize genes in response to abiotic stress. PLoS Genetics 11, e1004915. https://doi.org/10.1371/journal.pgen.1004915

Mandal, M., Sarkar, M., Khan, A., Biswas, M., Masi, A., Rakwal, R., Agrawal, G.K., Srivastava, A., Sarkar, A., 2022: Reactive oxygen species (ROS) and reactive nitrogen species (RNS) in plants – maintenance of structural individuality and functional blend. Advances in Redox Research 5, 100039. https://doi.org/10.1016/j.arres.2022.100039

Manna, M., Thakur, T., Chirom, O., Mandlik, R., Deshmukh, R., Salvi, P., 2021: Transcription factors as key molecular target to strengthen the drought stress tolerance in plants. Physiologia Plantarum 172(2), 847–868. https://doi.org/10.1111/ppl.13268

Mao, H., Wang, H., Liu, S., Li, Z., Yang, X., Yan, J., Li, J., Tran, L.-S.P., Qin, F., 2015: A transposable element in a *NAC* gene is associated with drought tolerance in maize seedlings. Nature Communications 6, 8326. https://doi.org/10.1038/ncomms9326

Matzke, M.A., Mosher, R.A., 2014: RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. Nature Reviews Genetics 15, 394–408. https://doi.org/10.1038/nrg3683

Medina, J., Bargues, M., Terol, J., Pérez-Alonso, M., Salinas, J., 1999: The Arabidopsis *CBF* gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. Plant Physiology 119(2), 463–470. https://doi.org/10.1104/pp.119.2.463

Mehdi, S., Derkacheva, M., Ramström, M., Kralemann, L., Bergquist, J., Hennig, L., 2016: The WD40 domain protein MSI1 functions in a histone deacetylase complex to fine-tune abscisic acid signaling. The Plant Cell 28(1), 42–54. https://doi.org/10.1105/tpc.15.00763

Meister, G., 2013: Argonaute proteins: functional insights and emerging roles. Nature Reviews Genetics 14, 447–459. https://doi.org/10.1038/nrg3462

Merlot, S., Gosti, F., Guerrier, D., Vavasseur, A., Giraudat, J., 2001: The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. The Plant Journal 25(3), 295–303. https://doi.org/10.1046/j.1365-313x.2001.00965.x

Miryeganeh, M., 2021: Plants' epigenetic mechanisms and abiotic stress. Genes 12(8), 1106. https://doi.org/10.3390/genes12081106

Mito, Y., Henikoff, J.G., Henikoff, S., 2005: Genome-scale profiling of histone H3.3 replacement patterns. Nature Genetics 37, 1090–1097. https://doi.org/10.1038/ng1637

Mittler, R., Finka, A., Goloubinoff, P., 2012: How do plants feel the heat? Trends in Biochemical Sciences 37(3), 118–125. https://doi.org/10.1016/j.tibs.2011.11.007

Mlynárová, L., Nap, J.-P., Bisseling, T., 2007: The SWI/SNF chromatin-remodeling gene *AtCHR12* mediates temporary growth arrest in *Arabidopsis thaliana* upon perceiving environmental stress. The Plant Journal 51(5), 874–885. https://doi.org/10.1111/j.1365-313X.2007.03185.x

Moldovan, D., Spriggs, A., Dennis, E.S., Wilson, I.W., 2010: The hunt for hypoxia responsive natural antisense short interfering RNAs. Plant Signaling and Behavior 5(3), 247–251. https://doi.org/10.4161/psb.5.3.10548

Nie, X., Wang, H., Li, J., Holec, S., Berger, F., 2014: The HIRA complex that deposits the histone H3.3 is conserved in *Arabidopsis* and facilitates transcriptional dynamics. Biology Open 3(9), 794–802. https://doi.org/10.1242/bio.20148680

Nisa, M.-U., Huang, Y., Benhamed, M., Raynaud, C., 2019: Thep-Plant DNA damage response: signaling pathways leading to growth inhibition and putative role in response to stress conditions. Frontiers in Plant Science 10, 653. https://doi.org/10.3389/fpls.2019.00653

Niu, D., Lin, X.-L., Kong, X., Qu, G.-P., Cai, B., Lee, J., Jin, J.B., 2019: SIZ1-mediated SUMOylation of TPR1 suppresses plant immunity in Arabidopsis. Molecular Plant 12(2), 215–228. https://doi.org/10.1016/j.molp.2018.12.002

Nunez-Vazquez, R., Desvoyes, B., Gutierrez, C., 2022: Histone variants and modifications during abiotic stress response. Frontiers in Plant Science 13, 984702. https://doi.org/10.3389/fpls.2022.984702

Nuthikattu, S., McCue, A.D., Panda, K., Fultz, D., DeFraia, C., Thomas, E.N., Slotkin, R.K., 2013: The initiation of epigenetic silencing of active transposable elements is triggered by RDR6 and 21-22 nucleotide small interfering RNAs. Plant Physiology 162(1), 116–131. https://doi.org/10.1104/pp.113.216481

Orvar, B.L., Sangwan, V., Omann, F., Dhindsa, R.S., 2000: Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. The Plant Journal 23(6), 785–794. https://doi.org/10.1046/j.1365-313x.2000.00845.x

Osakabe, Y., Yamaguchi-Shinozaki, K., Shinozaki, K., Tran, L.P., 2014: ABA control of plant macroelement membrane transport systems in response to water deficit and high salinity. New Phytologist 202(1), 35–49. https://doi.org/10.1111/nph.12613

Park, H.J., Kim, W.-Y., Park, H.C., Lee, S.Y., Bohnert, H.J., Yun, D.-J., 2011: SUMO and SUMOylation in plants. Molecules and Cells 32(4), 305–316. https://doi.org/10.1007/s10059-011-0122-7

Peirats-Llobet, M., Han, S.-K., Gonzalez-Guzman, M., Jeong, C.W., Rodriguez, L., Belda-Palazon, B., Wagner, D., Rodriguez, P.L., 2016: A direct link between abscisic acid sensing and the chromatin-remodeling ATPase BRAHMA via core ABA signaling pathway components. Molecular Plant 9(1), 136–147. https://doi.org/10.1016/j.molp.2015.10.003

Plieth, C., Hansen, U.-P., Knight, H., Knight, M.R., 1999: Temperature sensing by plants: the primary characteristics of signal perception and calcium response. The Plant Journal 18(5), 491–497. https://doi.org/10.1046/j.1365-313X.1999.00471.x

Pontier, D., Picart, C., Roudier, F., Garcia, D., Lahmy, S., Azevedo, J., Alart, E., Laudié, M., Karlowski, W.M., Cooke, R., Colot, V., Voinnet, O., Lagrange, T., 2012: NERD, a plant-specific GW protein, defines an additional RNAi-dependent chromatin-based pathway in Arabidopsis. Molecular Cell 48(1), 121–132. https://doi.org/10.1016/j.molcel.2012.07.027

Popova, O.V., Dinh, H.Q., Aufsatz, W., Jonak, C., 2013: The RdDM pathway is required for basal heat tolerance in Arabidopsis. Molecular Plant 6(2), 396–410. https://doi.org/10.1093/mp/sst023

Ramakrishnan, M., Zhang, Z., Mullasseri, S., Kalendar, R., Ahmad, Z., Sharma, A., Liu, G., Zhou, M., Wei, Q., 2022: Epigenetic stress memory: A new approach to study cold and heat stress responses in plants. Frontiers in plant science 13, 1075279. https://doi.org/10.3389/fpls.2022.1075279

Ramirez-Prado, J.S., Latrasse, D., Rodriguez-Granados, N.Y., Huang, Y., Manza-Mianza, D., Brik-Chaouche, R., Jaouannet, M., Citerne, S., Bendahmane, A., Hirt, H., Raynaud, C., Benhamed, M., 2019: The Polycomb protein LHP 1 regulates *Arabidopsis thaliana* stress responses through the repression of the MYC 2-dependent branch of immunity. The Plant Journal 100(6), 1118–1131. https://doi.org/10.1111/tpj.14502

Ritonga, F.N., Chen, S., 2020: Physiological and molecular mechanism involved in cold stress tolerance in plants. Plants 9(5), 560. https://doi.org/10.3390/plants9050560

Rutowicz, K., Puzio, M., Halibart-Puzio, J., Lirski, M., Kroteń, M.A., Kotliński, M., Kniżewski, Ł., Lange, B., Muszewska, A., Śniegowska-Świerk, K., Kościelniak, J., Iwanicka-Nowicka, R., Żmuda, K., Buza, K., Janowiak, F., Jõesaar, I., Laskowska-Kaszub, K., Fogtman, A., Zielenkiewicz, P., Tiuryn, J., Kollist, H., Siedlecki, P., Ginalski, K., Świeżewski, S., Koblowska, M., Archacki, R., Wilczyński, B., Rapacz, M., Jerzmanowski, A., 2015: A specialized histone H1 variant is required for adaptive responses to complex abiotic stress and related DNA methylation in Arabidopsis. Plant Physiology, 169(3), pp.00493.2015. https://doi.org/10.1104/pp.15.00493

Saez, A., Rodrigues, A., Santiago, J., Rubio, S., Rodriguez, P.L., 2008: HAB1–SWI3B interaction reveals a link between abscisic acid signaling and putative SWI/SNF chromatin-remodeling complexes in *Arabidopsis*. The Plant Cell 20(11), 2972–2988. https://doi.org/10.1105/tpc.107.056705

Sanchez, D.H., Paszkowski, J., 2014: Heat-induced release of epigenetic silencing reveals the concealed role of an imprinted plant gene. PLoS Genetics 10, e1004806. https://doi.org/10.1371/journal.pgen.1004806

Schramm, F., Larkindale, J., Kiehlmann, E., Ganguli, A., Englich, G., Vierling, E., Von Koskull-Döring, P., 2007: A cascade of transcription factor DREB2A and heat stress transcription factor HsfA3 regulates the heat stress response of Arabidopsis. The Plant Journal 53(2), 264–274. https://doi.org/10.1111/j.1365-313X.2007.03334.x

Shi, L., Cui, X., Shen, Y., 2023: The roles of histone methylation in the regulation of abiotic stress responses in plants. Plant Stress, 100303.

https://doi.org/10.1016/j.stress.2023.100303

Shinozaki, K., Yamaguchi-Shinozaki, K., 2007: Gene networks involved in drought stress response and tolerance. Journal of Experimental Botany 58(2), 221–227. https://doi.org/10.1093/jxb/erl164

Shulaev, V., Cortes, D., Miller, G., Mittler, R., 2008: Metabolomics for plant stress response. Physiologia Plantarum 132(2), 199–208. https://doi.org/10.1111/j.1399-3054.2007.01025.x

Sicilia, A., Scialò, E., Puglisi, I., Lo Piero, A.R., 2020: Anthocyanin biosynthesis and DNA methylation dynamics in sweet orange fruit [*Citrus sinensis* L. (Osbeck)] under cold stress. Journal of Agricultural and Food Chemistry 68(26), 7024–7031. https://doi.org/10.1021/acs.jafc.0c02360

Singh, M., Singh, A., Yadav, N., Yadav, D.K., 2022: Current perspectives of ubiquitination and SUMOylation in abiotic stress tolerance in plants. Frontiers in Plant Science 13, 993194. https://doi.org/10.3389/fpls.2022.993194

Sun, M., Yang, Z., Liu, L., Duan, L., 2022: DNA methylation in plant responses and adaption to abiotic stresses. International Journal of Molecular Sciences 23(13), 6910. https://doi.org/10.3390/ijms23136910

Sun, Y., Zhao, J., Li, X., Li, Y., 2020: E2 conjugases UBC1 and UBC2 regulate MYB42-mediated SOS pathway in response to salt stress in *Arabidopsis*. New Phytologist 227(2), 455–472. https://doi.org/10.1111/nph.16538

Sura, W., Kabza, M., Karlowski, W.M., Bieluszewski, T., Kus-Slowinska, M., Pawełoszek, Ł., Sadowski, J., Ziolkowski, P.A., 2017: Dual role of the histone variant H2A.Z in transcriptional regulation of stress-response genes. The Plant Cell 29(4), 791–807. https://doi.org/10.1105/tpc.16.00573

Tang, K., Lang, Z., Zhang, H., Zhu, J.-K., 2016: The DNA demethylase ROS1 targets genomic regions with distinct chromatin modifications. Nature Plants 2, 16169. https://doi.org/10.1038/nplants.2016.169

Theocharis, A., Clément, C., Barka, E.A., 2012: Physiological and molecular changes in plants grown at low temperatures. Planta 235, 1091–1105. https://doi.org/10.1007/s00425-012-1641-y

Tran, L.-S.P., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K., Fujita, M., Seki, M., Shinozaki, K., Yamaguchi-Shinozaki, K., 2004: Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the *early responsive to dehydration stress 1* promoter. The Plant Cell 16(9), 2481–2498. https://doi.org/10.1105/tpc.104.022699

Ueda, M., Matsui, A., Nakamura, T., Abe, T., Sunaoshi, Y., Shimada, H., Seki, M., 2018: Versatility of HDA19-deficiency in increasing the tolerance of Arabidopsis to different environmental stresses. Plant Signaling and Behavior 13(8): e1475808. https://doi.org/10.1080/15592324.2018.1475808

Vendramin, S., Huang, J., Crisp, P.A., Madzima, T.F., McGinnis, K.M., 2020: Epigenetic regulation of ABA-induced transcriptional responses in maize. G3 Genes|Genomes|Genetics 10(5), 1727–1743. https://doi.org/10.1534/g3.119.400993

Vishwakarma, K., Upadhyay, N., Kumar, N., Yadav, G., Singh, J., Mishra, R.K., Kumar, V., Verma, R., Upadhyay, R.G., Pandey, M., Sharma, S., 2017: Abscisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. Frontiers in Plant Science 8, 164. https://doi.org/10.3389/fpls.2017.00161

Wahid, A., Gelani, S., Ashraf, M., Foolad, M., 2007: Heat tolerance in plants: An overview. Environmental and Experimental Botany 61(3), 199–223. https://doi.org/10.1016/j.envexpbot.2007.05.011

Wang, P., Xue, L., Batelli, G., Lee, S., Hou, Y.-J., Van Oosten, M.J., Zhang, H., Tao, W.A., Zhu, J.-K., 2013b: Quantitative phosphoproteomics identifies SnRK2 protein kinase substrates and reveals the effectors of abscisic acid action. Proceedings of the National Academy of Sciences 110(27), 11205–11210. https://doi.org/10.1073/pnas.1308974110

Wang, X., Duan, C.-G., Tang, K., Wang, B., Zhang, H., Lei, M., Lu, K., Mangrauthia, S.K., Wang, P., Zhu, G., Zhao, Y., Zhu, J.-K., 2013a: RNA-binding protein regulates plant DNA methylation by controlling mRNA processing at the intronic heterochromatin-containing gene *IBM1*. Proceedings of the National Academy of Sciences 110, 15467–15472. https://doi.org/10.1073/pnas.131539911

Wang, X.-F., Zhang, D.-P., 2008: Abscisic acid receptors: multiple signal-perception sites. Annals of Botany 101(3), 311–317. https://doi.org/10.1093/aob/mcm284

Wang, Z., Casas-Mollano, J.A., Xu, J., Riethoven, J.-J.M., Zhang, C., Cerutti, H., 2015: Osmotic stress induces phosphorylation of histone H3 at threonine 3 in pericentromeric regions of *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences 112(27), 8487–8492. https://doi.org/10.1073/pnas.1423325112

Williams, B.P., Pignatta, D., Henikoff, S., Gehring, M., 2015: Methylation-sensitive expression of a DNA demethylase gene serves as an epigenetic rheostat. PLOS Genetics 11, e1005142. https://doi.org/10.1371/journal.pgen.1005142

Wollmann, H., Stroud, H., Yelagandula, R., Tarutani, Y., Jiang, D., Jing, L., Jamge, B., Takeuchi, H., Holec, S., Nie, X., Kakutani, T., Jacobsen, S.E., Berger, F., 2017: The histone H3 variant H3.3 regulates gene body DNA methylation in *Arabidopsis thaliana*. Genome Biology 18, 94. https://doi.org/10.1186/s13059-017-1221-3

Wu, J., Ichihashi, Y., Suzuki, T., Shibata, A., Shirasu, K., Yamaguchi, N., Ito, T., 2019: Abscisic acid-dependent histone demethylation during postgermination growth arrest in *Arabidopsis*. Plant, Cell and Environment 42(7), 2198–2214. https://doi.org/10.1111/pce.13547

Xiang, M., Ding, W., Wu, C., Wang, W., Ye, S., Cai, C., Hu, X., Wang, N., Bai, W., Tang, X., Zhu, C., Yu, X., Xu, Q., Zheng, Y., Ding, Z., Lin, C., Zhu, Q., 2021: Production of purple Ma bamboo (*Dendrocalamus latiflorus* Munro) with enhanced drought and cold stress tolerance by engineering anthocyanin biosynthesis. Planta 254, 50. https://doi.org/10.1007/s00425-021-03696-z

Yang, C., Shen, W., Chen, H., Chu, L., Xu, Y., Zhou, X., Liu, C., Chen, C., Zeng, J., Liu, J., Li, Q., Gao, C., Charron, J.-B., Luo, M., 2018: Characterization and subcellular localization of histone deacetylases and their roles in response to abiotic stresses in soybean. BMC Plant Biology 18, 226. https://doi.org/10.1186/s12870-018-1454-7

Yang, L., Lang, C., Wu, Y., Meng, D., Yang, T., Li, D., Jin, T., Zhou, X., 2022: ROS1-mediated decrease in DNA methylation and increase in expression of defense genes and stress response genes in *Arabidopsis thaliana* due to abiotic stresses. BMC Plant Biology 22, 104. https://doi.org/10.1186/s12870-022-03473-4

Yang, R., Hong, Y., Ren, Z., Tang, K., Zhang, H., Zhu, J.-K., Zhao, C., 2019: A role for PICKLE in the regulation of cold and salt stress tolerance in Arabidopsis. Frontiers in Plant Science 10, 900. https://doi.org/10.3389/fpls.2019.00900

Yang, R., Zheng, Z., Chen, Q., Yang, L., Huang, H., Miki, D., Wu, W., Zeng, L., Liu, J., Zhou, J.-X., Ogas, J., Zhu, J.-K., He, X.-J., Zhang, H., 2017: The developmental regulator PKL is required to maintain correct DNA methylation patterns at RNA-directed DNA methylation loci. Genome Biology 18, 103. https://doi.org/10.1186/s13059-017-1226-y

Yelagandula, R., Stroud, H., Holec, S., Zhou, K., Feng, S., Zhong, X., Muthurajan, U.M., Nie, X., Kawashima, T., Groth, M., Luger, K., Jacobsen, S.E., Berger, F., 2014: The histone variant H2A.W defines heterochromatin and promotes chromatin condensation in Arabidopsis. Cell 158(1), 98–109. https://doi.org/10.1016/j.cell.2014.06.006

Zandalinas, S.I., Balfagón, D., Gómez-Cadenas, A., Mittler, R., 2022: Plant responses to climate change: metabolic changes under combined abiotic stresses. Journal of Experimental Botany 73(11), 3339–3354. https://doi.org/10.1093/jxb/erac073

Zemach, A., Kim, M.Y., Hsieh, P.-H., Coleman-Derr, D., Eshed-Williams, L., Thao, K., Harmer, S.L., Zilberman, D., 2013: The Arabidopsis nucleosome remodeler DDM1 allows DNA methyltransferases to access H1-containing heterochromatin. Cell 153(1), 193–205. https://doi.org/10.1016/j.cell.2013.02.033

Zhang, H., Lang, Z., Zhu, J.-K., 2018: Dynamics and function of DNA methylation in plants. Nature Reviews Molecular Cell Biology 19, 489–506. https://doi.org/10.1038/s41580-018-0016-z

Zhao, J., Lu, Z., Wang, L., Jin, B., 2020: Plant responses to heat stress: physiology, transcription, noncoding RNAs, and epigenetics. International Journal of Molecular Sciences 22(1), 117. https://doi.org/10.3390/ijms22010117

Zhu, Y., Hu, X., Duan, Y., Li, S., Wang, Y., Rehman, A.U., He, J., Zhang, J., Hua, D., Yang, L., Wang, L., Chen, Z., Li, C., Wang, B., Song, C.-P., Sun, Q., Yang, S., Gong, Z., 2020: The Arabidopsis nodulin homeobox factor AtNDX interacts with AtRING1A/B and negatively regulates abscisic acid signaling. The Plant Cell 32(3), 703–721. https://doi.org/10.1105/tpc.19.00604

Zilberman, D., Coleman-Derr, D., Ballinger, T., Henikoff, S., 2008: Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. Nature 456, 125–129. https://doi.org/10.1038/nature07324

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# Past, present and future of an alien fungus *Clathrus archeri* in Croatia

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**Abstract** – *Clathrus archeri* (Berk.) Dring, an alien saprotrophic fungus originating from Australia and New Zealand has been present in Europe for over 100 years. From its first observation in 1920 in France, it has spread all over Europe, and is among the better surveyed alien saprotrophic fungi with respect to its spread and distribution. Despite this, data about *C. archeri* in Croatia in the scientific literature are very scarce. To fill the current gap on the presence and distribution of *C. archeri* in Croatia, we collected reliable available data on its presence, analysed several environmental factors (climate, soil acidity, topography) on those localities, and developed habitat suitability models using Maxent software. We found out that the fungus has been present in Croatia for over 20 years, so we synthesized information on 25 observations, which were all but one placed in the Continental NATURA 2000 biogeographical region of the country. Localities were situated between 104 and 532 m a.s.l., with mean annual temperatures ranging from 8.1 to 10.9 °C, and annual precipitation ranging between 815 and 1620 mm. On all localities acid soil reactions were present with pH values below 6.8. Although the current number of observations is moderate, in terms of its spatial distribution we can consider *C. archeri* a widespread alien fungus in Croatia. Based on the habitat suitability models developed here, and data about its ecology from the literature, we can expect more and spatially broader observations of *C. archeri* in the future.

**Keywords:** GBIF, iNaturalist, octopus stinkhorn, Phallaceae, stinkhorns, velika polipovka

## Introduction

For a long time, fungi have been overlooked in the invasion science literature, apart from the fungal pathogens that have strong negative impacts on biodiversity and the economy (e.g. in the agriculture). Symbiotic fungi have been to some extent surveyed, but saprotrophic fungi rarely (Desprez-Loustau et al. 2007, 2010). According to Desprez-Loustau et al. (2007), lack of the quantification of the impacts of non-pathogenic alien fungi may be a major reason for the scarcity of surveys and the data on them. In last 15 years, a number of European countries have compiled lists of alien fungi (see Voglmayr et al. 2023, for countries and references), but as shown from the example of Austria in which they expanded their first checklist (Voglmayr and Krisai-Greilhuber 2002) with an almost five-fold increase in the second edition (Voglmayr et al. 2023), it is an ongoing process. As highlighted by Voglmayr et al. (2023) plant pathogens are the dominant ecofunctional group in the list of Austrian alien fungi, which is the case in other countries as well. Saprobionts are still generally underrepresented in alien fungi lists. It should be noted that biogeography of fungi is much less known and clear than that of plants and animals (Desprez-Loustau et al. 2007).

Among the alien saprotrophic fungi in Europe, *Clathrus archeri* (Berk.) Dring is one of the better known, with lots of papers reporting on its distribution across Europe (see the review by Parent and Thoen 1986, and Parent et al. 2000). One reason for this may be the peculiar appearance of its sporocarps (Fig. 1), making it easier for identification, and attracting more attention, even from non-fungi experts and the general public.

According to Parent and Thoen (1986) the first record in Europe of this fungus, originating from Australia and New Zealand, is from 1920 in Vosges, France (Lemasson 1923 in Parent and Thoen 1986), where it is believed to have most probably arrived with wool products. From there it gradually spread all over Europe. It has been recorded in great variety of habitats including different types of forests (deciduous, coniferous – natural and plantations, mixed coniferous-deciduous), grasslands, orchards, disturbed habitats, gardens (Parent and Thoen 1986, Bîrsan et al. 2014, Pietras et al. 2016, Brännhage and Gross 2020). However, although it can be found in a diversity of habitats it does prefer humid and acidic soil (Parent and Thoen 1986, Pietras et al. 2016).

Published data on *C. archeri* in Croatia are extremely scarce and to our knowledge, with respect to the scientific lit-

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**Fig. 1.** *Clathrus archeri* observed by authors in November 2023 in Donji Stupnik (left image – eggs; middle image – sporocarp) and Donje Ladanje (right image – sporocarp). Pen in the left image is 14 cm long. Photo: Sven D. Jelaska.

erature, these are: Tkalčec et al. (2005) in which the species is mentioned for Croatia for the first time in the scientific literature; Kranjčev (2008) who reports on its presence in two locations in northwest Croatia; Pietras et al. (2021) who included in their analysis one locality (out of four) on the fungus's presence in Croatia deposited on GBIF.org on October 26th, 2018. Apart from the scientific literature, *C. archeri* was included in all three editions of the "Ordinance on Protection of Fungi" (Official Gazette 1998–2002), but was omitted from the subsequent "Ordinance on Designation of Wild Taxa as Protected or Strictly Protected" (Official Gazette 2006) and "Red Book of Croatian Fungi" (Tkalčec et al. 2008). Preliminary inclusion of *C. archeri* on the list of protected fungi was probably a result of a lack of data and information on the fungus' origin, which happened in other countries as well, in the Eastern part of Europe where *C. archeri* arrived and was observed later (e.g. Romania – Tănase and Pop 2005, Bulgaria – Gyosheva et al. 2006, Ukraine – Didukh 2009). Increased knowledge and data collected resulted in its removal from the Red Book of Ukraine (Heluta et al. 2022).

To fill the current gap in the knowledge of the presence and distribution of *C. archeri* in Croatia, we collected reliable available data on its presence, based on which we analysed the ecological preferences of the species. Furthermore, we developed habitat suitability models to get some indication on its potential overall distribution in Croatia, which can be also used for planning future research on the species.

## Material and methods

To gather information on the presence of *C. archeri* in Croatia we have searched for fungus's records in GBIF.org (https://www.gbif.org/); iNaturalist.org (https://www.inaturalist.org/), Mushroomobserver.org (https://mushroomobserver.org/) and Wood (2008). Furthermore, we have used several query terms in different combinations ("*Clathrus*", "*archeri*", "*Anthurus*", "Devil's fingers", "Octopus stinkhorn", "velika polipovka", "Croatia", "Hrvatska") in the following sources:

– Portal of Croatian scientific and professional journals (https://hrcak.srce.hr/en)

– Croatian Research Information System (https://www.croris.hr/)

– Scopus (https://www.scopus.com)

– Internet web pages

Queryings scientific literature sources revealed just two publications in which *C. archeri* was mentioned: Tkalčec et al. (2005) and Kranjčev (2008). Only the latter gave locations (two) for the fungus in Croatia. GBIF.org did not contain all the localities present in iNaturalist, because of licensing restrictions assigned to some observation records. We have contacted owners of these observations and obtained permission to use their observations in this analysis. They are presented in Tab. 1 with corresponding links, while data present in GBIF on April, 24th 2024 can be found in the GBIF.org (2024) downloaded data set.

From the Internet web pages search, we have included three results (No. 1, 5, and 19 in Tab. 1) that mentioned month and year of observation, in addition to a locality that could be georeferenced. For such location in the Karlovac City area (No. 1 in Tab. 1) there was an observation in GBIF.org (2024) with exact coordinates, hence we used that location but kept the web source presented in Tab. 1 for being among the first data of the presence of the fungus in Croatia. Descriptions of two locations of *C. archeri* in Kranjčev (2008) were sufficiently precise in just one case, the presence of which we confirmed in November 2023 (No. 3 in Tab. 1) and assigned coordinates using the Global Navigation Satellite System (GNSS). Overall, we have synthesized data on 25 locations of *C. archeri* in Croatia (Fig. 2) with the oldest data originating in 2004.

To develop habitat suitability models, we used Maxent software (Phillips et al. 2024), 22 data with exact coordinates on the occurrences of *C. archeri* in Croatia (Tab. 1) and two sets of environmental variables. In the first iteration, we used eight climatic variables (mean temperature and amount of precipitation per meteorological season) based on a 30-year period (1960–1990, Croatian Meteorological and Hydrological Service). In the second iteration, in addition to climatic variables, pH value (in $H_2O$) of the topsoil (0–5 cm depth) was used. For latter we used a dataset available for Europe (Poggio et al. 2021). All environmental variables were prepared as 300 m resolution grids.

**Tab. 1.** Localities of *Clathrus archeri* in Croatia with WGS84 coordinates, month and year of observation, and source of data (all web sources accessed on April 24, 2024.). * localities observed by the Authors, ** – coordinates approximated, localities not used for the Maxent models

| No. | X-coordinate | Y-coordinate | Month | Year | Source |
|---|---|---|---|---|---|
| 1 | 15.59496 | 45.52655 | December | 2019 | GBIF.org (2024) |
| | | | October | 2004 | https://www.svijet-gljiva.com/katalog-gljiva/item/91-clathrus-archeri |
| 2** | 16.91227 | 46.14785 | October | 2007 | Kranjčev (2008) |
| 3* | 16.14177 | 46.28702 | November | 2023 | GBIF.org (2024) |
| | | | October | 2007 | Kranjčev (2008) |
| 4 | 16.13286 | 46.26402 | September | 2010 | https://mushroomobserver.org/52525 |
| 5** | 16.12500 | 45.63459 | June | 2012 | https://www.24sata.hr/reporteri/u-sumi-kod-busevca-pronasao-je-rijetku-vrstu-smrdljive-gljive-271077 |
| 6 | 15.59853 | 45.52442 | October | 2012 | GBIF.org (2024) |
| 7 | 16.03694 | 45.66291 | October | 2017 | GBIF.org (2024) |
| 8 | 17.60421 | 45.35970 | September | 2018 | https://www.inaturalist.org/observations/193238448 |
| 9 | 17.60344 | 45.35771 | September | 2018 | https://www.inaturalist.org/observations/193238557 |
| 10 | 15.20384 | 45.27234 | October | 2018 | GBIF.org (2024) |
| 11 | 15.74351 | 45.63000 | September | 2019 | GBIF.org (2024) |
| 12 | 17.49942 | 45.55693 | November | 2019 | https://www.inaturalist.org/observations/36022378 |
| 13 | 17.63949 | 45.57991 | August | 2020 | GBIF.org (2024) |
| 14 | 17.31514 | 45.41640 | September | 2022 | https://www.inaturalist.org/observations/134201619 |
| 15 | 16.31555 | 46.43399 | October | 2022 | https://www.inaturalist.org/observations/197127579 |
| 16 | 15.78877 | 45.76543 | October | 2022 | https://www.inaturalist.org/observations/139409586 |
| 17 | 15.91247 | 45.31635 | October | 2022 | GBIF.org (2024) |
| 18 | 15.91247 | 45.31624 | October | 2022 | GBIF.org (2024) |
| 19** | 16.01816 | 45.56576 | October | 2022 | https://institutzeleni.wordpress.com/2022/10/09/prica-iz-vukomerickih-gorica/ |
| 20 | 17.69796 | 45.55104 | November | 2022 | https://www.inaturalist.org/observations/141909715 |
| 21 | 17.67791 | 45.62665 | November | 2022 | https://www.inaturalist.org/observations/142387360 |
| 22 | 14.31846 | 45.08774 | April | 2023 | GBIF.org (2024) |
| 23 | 16.15927 | 46.29094 | August | 2023 | GBIF.org (2024) |
| 24* | 15.82043 | 45.72737 | November | 2023 | GBIF.org (2024) |
| 25 | 15.99926 | 45.66489 | November | 2023 | https://www.inaturalist.org/observations/206601927 |

Given the number of occurrence data, we have used all as a training set. Consequently, only Area Under Curve (AUC) for the training set was available. Three localities to which exact coordinates could not be assigned (No. 2, 5, and 19 in Tab. 1), were used as an independent test data set to evaluate the habitat suitability models developed.

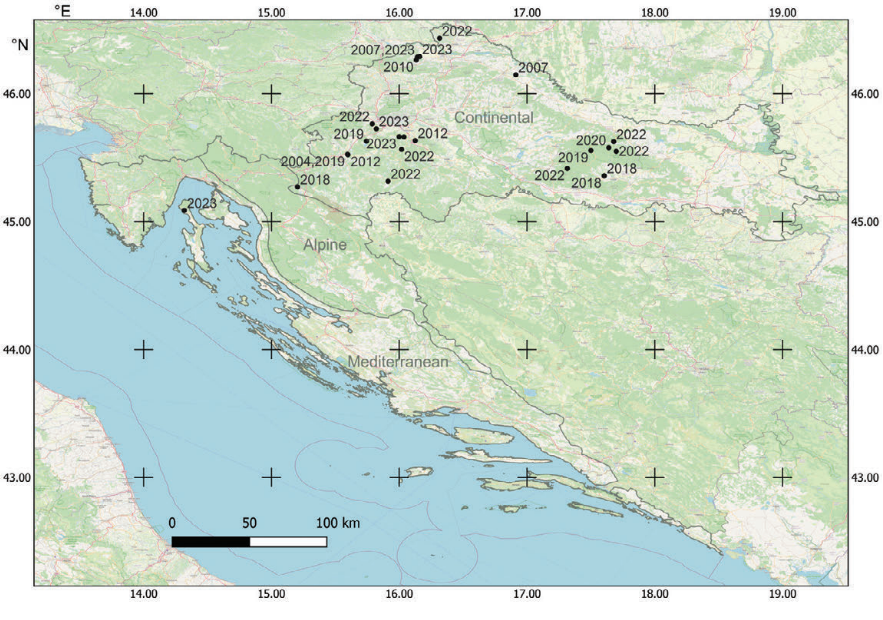

**Fig. 2.** Distribution of *Clathrus archeri* in Croatia (details of localities in Tab. 1), with NATURA2000 biogeographical regions indicated. Background map is OpenStreetMap (Base map and data from OpenStreetMap and OpenStreetMap Foundation (CC-BY-SA). https://www.openstreetmap.org and contributors)

In addition to climatic and pH variables mentioned above, we assigned values of elevation and slope (based on a 300 meter resolution digital elevation model of Croatia) to all 25 localities, and calculated several descriptive statistical measures (minimum, maximum, mean, 10th and 90th percentile) to get some insight into the ecological preferences of *C. archeri* in Croatia. Elevation and slope were not included in habitat suitability modelling since elevation is correlated to climate (positively with precipitation, and negatively with temperature) and value of slope can vary significantly on short distances, and hence could increase uncertainty of the habitat suitability model.

At two locations (No. 3 and 24 in Tab. 1, Donje Ladanje and Donji Stupnik respectively) where we observed *C. archeri* in November 2023 we collected soil samples. After removing the leaf litter, upper 5 cm of the soil was sampled with a cylindrical corer (8 cm in diameter) in triplicates around the observed sporocarp (with a distance of 1 m between the subsamples) and stored as a composite sample representative for the location. Samples were transported to the laboratory where they were air dried at room temperature. To measure pH values, a combined electrode pH meter (HANNA HI 99121, Direct soil pH meter) was used on suspensions composed of 10 g of soil and 25 mL of distilled water. Prior to measuring, the suspensions were covered for 30 min with occasional stirring. Organic matter content was determined by annealing in a muffle furnace (INKO LP-08). According to the NRM Laboratories methodology (Davies 1974) ground soil samples were first dried in an oven at 110 °C for 24 h, then weighed for 5 g samples, transferred to porcelain bowls, and heated in the muffle furnace at 430 °C for 2 h. After annealing, the samples were transferred into a desiccator for cooling and weighed again the next day. The organic matter content was determined using mass differences. Both pH and organic matter content measurements were made of three random subsamples from the composite soil sample of each locality, and expressed as mean values.

## Results

Except for one in the Mediterranean region, all localities of *C. archeri* were situated in the Continental NATURA 2000 biogeographical region, with no localities recorded in the Alpine region (Fig. 2). Localities were situated between 104 and 532 m a.s.l., with 10th and 90th inter-percentile ranging from 109 to 381 m a.s.l. (Tab. 2).

The 10–90 inter-percentile range for mean annual temperature was 9.6 to 10.8 °C, and for annual precipitation 831

**Tab. 2.** Environmental variables values for *Clathrus archeri* localities (Tab. 1) in Croatia. pH – pH value of topsoil in 0 – 5 cm depth, elevation (m a.s.l.), slope (°), –prec – precipitation (mm) and –temp – temperature (°C) for four seasons (sp – spring, su – summer, a – autumn, w – winter) and annual (y–). min – minimum, max – maximum, mean – average, perc10 and perc90 – 10% and 90% percentiles for each environmental variable.

| No. | Approximate locality | pH | elevation | slope | spprec | suprec | aprec | wprec | yprec | sptemp | sutemp | atemp | wtemp | ytemp |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Karlovac | 5.4 | 108 | 0.0 | 242 | 282 | 312 | 230 | 1066 | 10.7 | 19.8 | 11.2 | 1.7 | 10.8 |
| 2 | Podravski bregi | 5.5 | 125 | 0.0 | 199 | 273 | 221 | 157 | 850 | 10.7 | 19.4 | 10.4 | 0.4 | 10.2 |
| 3 | Donje Ladanje | 5.4 | 217 | 3.8 | 216 | 298 | 247 | 174 | 935 | 10.1 | 18.9 | 10.1 | 0.3 | 9.9 |
| 4 | Veliki Novaki | 6.2 | 215 | 1.0 | 218 | 300 | 249 | 176 | 943 | 10.1 | 18.8 | 10.1 | 0.3 | 9.8 |
| 5 | Buševec, Velika Gorica | 5.5 | 104 | 0.0 | 207 | 262 | 259 | 182 | 909 | 10.9 | 20.0 | 11.1 | 1.3 | 10.8 |
| 6 | Karlovac | 5.3 | 108 | 0.0 | 241 | 282 | 311 | 230 | 1063 | 10.7 | 19.8 | 11.2 | 1.7 | 10.8 |
| 7 | Gudci | 5.4 | 121 | 0.0 | 208 | 264 | 262 | 184 | 917 | 10.7 | 19.8 | 11.0 | 1.2 | 10.7 |
| 8 | Jaguplije | 6.0 | 214 | 5.1 | 192 | 257 | 216 | 150 | 815 | 10.4 | 19.5 | 10.4 | 0.3 | 10.2 |
| 9 | Jaguplije | 5.9 | 213 | 5.1 | 192 | 257 | 216 | 150 | 815 | 10.5 | 19.5 | 10.4 | 0.3 | 10.2 |
| 10 | Sveti Petar (Ogulin) | 6.2 | 365 | 7.4 | 368 | 380 | 484 | 387 | 1620 | 9.0 | 18.2 | 10.1 | 0.8 | 9.5 |
| 11 | Donja Zdenčina | 5.7 | 111 | 0.4 | 218 | 267 | 280 | 200 | 966 | 10.8 | 19.9 | 11.2 | 1.6 | 10.9 |
| 12 | Brestovac | 5.8 | 509 | 5.5 | 248 | 338 | 253 | 181 | 1021 | 8.5 | 17.6 | 8.7 | –1.1 | 8.4 |
| 13 | Sekulinci | 6.0 | 229 | 4.4 | 199 | 270 | 217 | 153 | 840 | 10.3 | 19.3 | 10.2 | 0.1 | 10.0 |
| 14 | Gornja Šumetlica | 5.5 | 532 | 10.1 | 253 | 338 | 265 | 190 | 1046 | 8.1 | 17.2 | 8.4 | –1.4 | 8.1 |
| 15 | Gornji Mihaljevec | 6.3 | 231 | 0.8 | 207 | 292 | 230 | 162 | 891 | 10.2 | 18.8 | 10.0 | 0.1 | 9.8 |
| 16 | Mala Gorica | 6.3 | 151 | 0.7 | 224 | 284 | 282 | 201 | 991 | 10.4 | 19.4 | 10.7 | 1.0 | 10.4 |
| 17 | Blatuša | 5.8 | 149 | 2.8 | 248 | 286 | 317 | 237 | 1088 | 10.3 | 19.5 | 10.8 | 1.2 | 10.5 |
| 18 | Blatuša | 5.8 | 149 | 2.8 | 248 | 286 | 317 | 237 | 1088 | 10.3 | 19.5 | 10.8 | 1.2 | 10.5 |
| 19 | Gornji Hruševec | 5.4 | 153 | 2.4 | 214 | 269 | 272 | 192 | 947 | 10.4 | 19.5 | 10.7 | 1.0 | 10.4 |
| 20 | Čačinci | 6.1 | 235 | 5.9 | 198 | 270 | 216 | 152 | 835 | 10.3 | 19.3 | 10.1 | 0.1 | 9.9 |
| 21 | Bokane | 6.1 | 165 | 4.1 | 196 | 264 | 215 | 153 | 828 | 10.7 | 19.7 | 10.5 | 0.4 | 10.4 |
| 22 | Dragozetići, Cres | 6.8 | 392 | 5.3 | 339 | 281 | 493 | 410 | 1524 | 9.8 | 19.1 | 11.6 | 2.8 | 10.8 |
| 23 | Maruševec | 6.0 | 202 | 3.7 | 213 | 294 | 244 | 172 | 923 | 10.3 | 19.0 | 10.2 | 0.4 | 10.0 |
| 24 | Donji Stupnik | 5.1 | 128 | 0.0 | 218 | 275 | 276 | 196 | 964 | 10.6 | 19.7 | 10.9 | 1.2 | 10.6 |
| 25 | Donja Lomnica | 5.3 | 120 | 1.0 | 208 | 264 | 262 | 184 | 919 | 10.6 | 19.7 | 10.9 | 1.1 | 10.6 |
|  | min | 5.1 | 104 | 0.0 | 192 | 257 | 215 | 150 | 815 | 8.1 | 17.2 | 8.4 | –1.4 | 8.1 |
|  | max | 6.8 | 532 | 10.1 | 368 | 380 | 493 | 410 | 1620 | 10.9 | 20.0 | 11.6 | 2.8 | 10.9 |
|  | mean | 5.8 | 209.9 | 2.9 | 228.6 | 285.3 | 276.6 | 201.6 | 992.1 | 10.2 | 19.2 | 10.5 | 0.7 | 10.2 |
|  | perc10 | 5.3 | 109 | 0.0 | 197 | 262 | 216 | 152 | 831 | 9.3 | 18.4 | 10.1 | 0.1 | 9.6 |
|  | perc90 | 6.3 | 381 | 5.7 | 251 | 323 | 317 | 237 | 1088 | 10.7 | 19.8 | 11.2 | 1.6 | 10.8 |

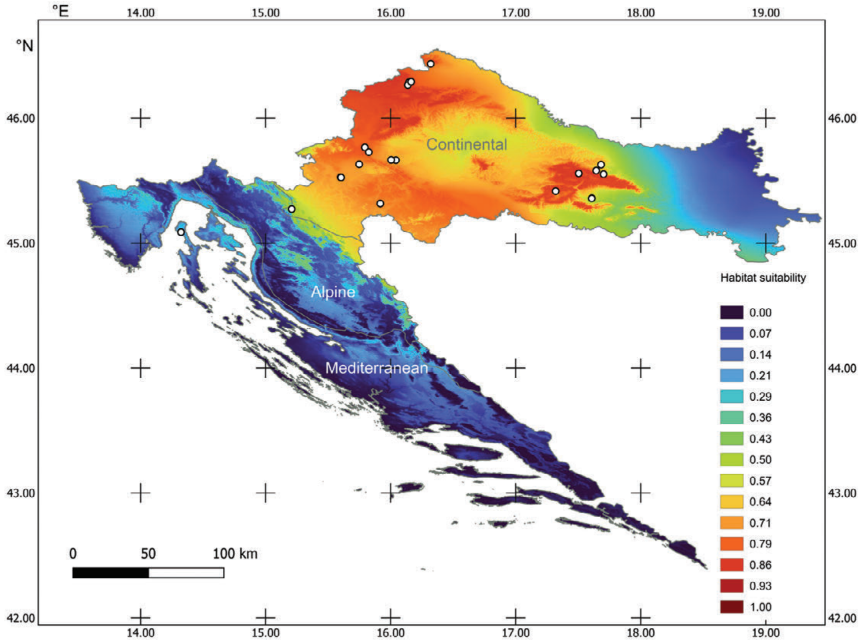

**Fig. 3.** Maxent habitat suitability model for *Clathrus archeri* in Croatia based on eight climatic variables (mean temperature and amount of precipitation per meteorological season). Colours on the map, with corresponding number indicates habitat suitability for *C. archeri* in range from 1 (red – presumed 100% suitable habitat) to 0 (blue – 0% suitable habitat). White circles indicate *C. archeri* occurrences in Croatia used to build the model, NATURA 2000 biogeographical regions indicated.

to 1088 mm. With respect to the seasonal precipitation amounts, there were no seasons with less than 150 mm at any locality (Tab. 2). Soil reaction ranged from 5.1 to 6.8, with 10–90 inter-percentile range being 5.3 to 6.3 (Tab. 2), both corresponding to the acidic part of the pH scale. Our measurements of soil reaction sampled at two localities in a stand of planted Eastern White Pine *Pinus strobus* L. (No. 3 in Tab. 1) and in the *Epimedio-Carpinetum betuli* (Horvat 1938) Borhidi 1963 forest (No. 24 in Tab. 1) have shown an even lower pH, of 4.3 and 4.1, respectively. Regarding organic matter content, the mean value for Donje Ladanje (No. 3 in Tab. 1) was 19.14% and for Donji Stupnik (No. 24 in Tab. 1) 16.75%, indicating that soils at both localities were rich in organic matter.

Climate-only habitat suitability model (Fig. 3) and Climate-pH habitat suitability model (Fig. 4) yielded a very

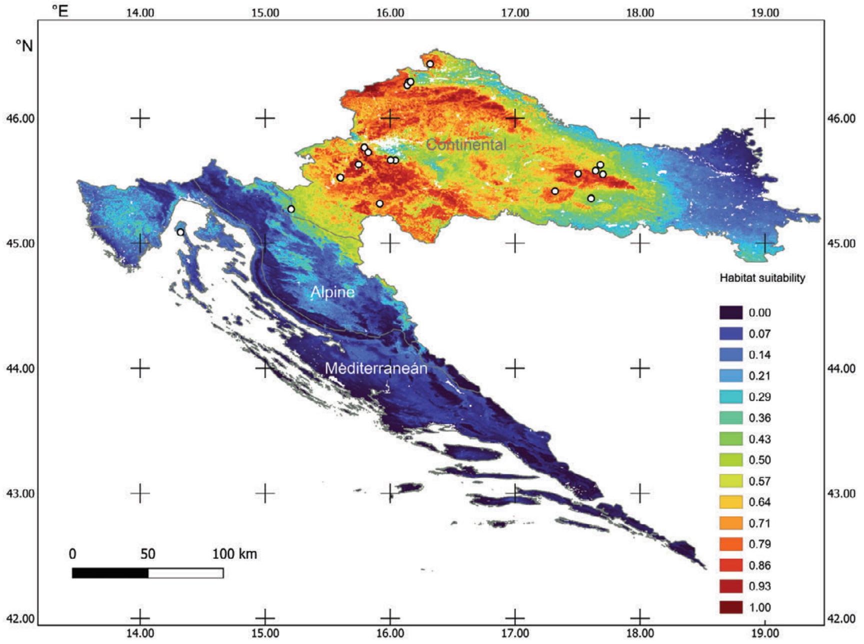

**Fig. 4.** Maxent habitat suitability model for *Clathrus archeri* in Croatia based on eight climatic variables (mean temperature and amount of precipitation per meteorological season) and pH value of soil in 0–5 cm depth. Colours on the map, with corresponding number indicates habitat suitability for *C. archeri* in range from 1 (red – presumed 100% suitable habitat) to 0 (blue – 0% suitable habitat). White circles indicate *C. archeri* occurrences in Croatia used to build the model, NATURA 2000 biogeographical regions indicated.

**Tab. 3.** Variable contributions to Maxent habitat suitability models for *Clathrus archeri* in Croatia. Abbreviations: w – winter, a – autumn, su – summer, sp – spring, temp – mean temperature, prec – total amount of precipitation, pH – pH value of topsoil in 0 – 5 cm depth.

| **Model with climate data** | | | **Model with climate data and pH value of topsoil** | | |
|---|---|---|---|---|---|
| Variable | Percent contribution | Permutation importance | Variable | Percent contribution | Permutation importance |
| | | | pH | 53.1 | 12.5 |
| suprec | 48.9 | 9.2 | wprec | 19.4 | 58.8 |
| wprec | 22.5 | 47.5 | spprec | 8.5 | 11.5 |
| aprec | 19.3 | 12.6 | aprec | 8.4 | 0 |
| sptemp | 5.4 | 0 | suprec | 7.2 | 1.4 |
| spprec | 2.2 | 14.5 | wtemp | 3.2 | 13.5 |
| wtemp | 1.7 | 16.2 | sptemp | 0.3 | 2.4 |
| atemp | 0 | 0 | atemp | 0 | 0 |
| sutemp | 0 | 0 | sutemp | 0 | 0 |

similar general spatial pattern at country level, with Climate-pH based model seemingly being spatially more specific in terms of areas with high suitability.

The latter model also contains some no-data areas, as a consequence of the lack of pH data for water body areas and human settlements. The majority of predicted suitable habitats are in the Continental biogeographical region where most of the recorded localities are situated. There are some moderately suitable areas in the Alpine region, in which, however, there are currently no records of *C. archeri.* Those areas are mostly in parts adjacent to the Continental region around the Kupa, Dobra and Una rivers, and in lower elevation parts corresponding to the presence of flat depressions within the karst known as "polje" (e.g. Cerničko polje, Gacko polje, Krbavsko polje). Within the Mediterranean region, very low habitat suitability was predicted by both Maxent models, with slightly higher values in the Northern Adriatic where one locality exists (No. 22 in Tab. 1). For both habitat suitability models, area under curve (AUC) of the receiver operating curve (ROC) had similar values, AUC = 0.817 for Climate-only and AUC = 0.827 for Climate-pH model (Tab. 3).

Precipitation variables contributed more to the models than the temperature variables. Summer precipitation had the highest percentage of contribution to the Climate-only model, while the pH of topsoil had the highest percentage to the Climate-pH model. Winter precipitation was second best in terms of the percentage of the contribution to the models, but had by far the highest permutation importance indicating that models were heavily dependent on it (Tab. 3). On three localities that were not used for habitat suitability modelling (No. 2, 5 and 20 in Tab. 1), both models predicted high values of suitability: 0.68, 0.67 and 0.75, respectively, for Climate-only model; 0.75, 0.79 and 0.89 for Climate-pH model.

## Discussion

The history and introduction of *C. archeri* in Croatia remain unclear. Its first mention in Božac (1984) in his popular guide to fungi cannot be considered the first evidence of its presence in Croatia because the exact geographical extent covered by the guide is not defined. At that time Croatia was a part of former Socialist Federative Republic of Yugoslavia. Furthermore, in the book's preface the author explains that the most frequently used criterion for inclusion of fungi species in the book was their high frequency in "our regions" (which could be referred to other constituent republics of the former country beside Croatia) and in Central Europe. The latter could mean that *C. archeri* found its place in the book without actually being present in Croatia. However, at that time *C. archeri* had already been recorded (1966) in Slovenia (also part of the former country) for quite a long time (Vrščaj et al. 2004, Stanič 2024). In 1998, *C. archeri* was included in the first list of protected fungi in Croatia (Official Gazette 1998), and remained there in the second (published also in 1998) and third edition (published in 2002). We could not trace back the background for this inclusion, since the first mention of *C. archeri* in the literature is that of Tkalčec et al. (2005) in their paper on gasteral Basidiomycota of Croatia in which they note that their reference for *C. archeri* was the first for Croatia. Tkalčec (personal communication) found it in 1999 in two locations in the western part of Croatia. Without new evidence, this could be regarded as the year of the fungus's first observation in Croatia. Nevertheless, given the pace of spread eastward from its initial introduction to France in 1920 (Lemasson 1923 in Parent and Thoen 1986), and subsequent first observations in Switzerland in 1942 (Vischer 1943), Austria in 1948 (Lohwag 1948) and Slovenia in 1966 (Vrščaj et al. 2004, Stanič 2024), it is possible that the fungus was present in Croatia from the 1980s or early 1990s, but remained unnoticed. Although we cannot be sure of the exact time of its arrival, with a fair amount of certainty we can consider that it arrived from the Slovenia.

The data given here show that *C. archeri* is much more widespread in Croatia than was considered previously, according to the scientific literature. Both habitat suitability models imply that it could occupy even larger areas than presently, at least across the majority of the NATURA 2000 Continental biogeographical region in Croatia. High values of predicted habitat suitability models at locations that were not used in building the models, as well as reasonably high AUC values work in favour of the predicted models' reliabilities. In both models, precipitation turned out to be more important than temperature in defining the suitability for *C. archeri*. In habitat suitability models for Romania (Bîrsan et al. 2021) and on a global level (Pietras et al. 2021) there were the same findings, with the coldest quarter of the year's precipitation contributing the most to their models. Here, winter precipitation (which corresponds to the precipitation of the coldest quarter) was the most important among the climate variables in the Climate-pH model, while it was second most important, after summer precipitation, in the Climate-only model (Tab. 3). The Mediterranean part of Croatia has a distinct dry season during the summer, hence this could play an important role in defining potential niche of the species in Croatia. Pietras et al. (2016) found that in Poland *C. archeri* is mostly present in areas with annual precipitation greater than 600 mm. They confirmed its presence in part of Poland with low mean annual precipitation (550 mm), but during the year with an exceptionally wet season. The only location so far known in the Mediterranean part of Croatia is in the Northern Adriatic, which does not have such dry summer periods as those in Central and Southern parts of the Adriatic, where summer precipitation is mostly below 150 mm, which in combination with highly permeable limestone bedrock leads to dry soil conditions.

Another important aspect that could limit *C. archeri* in the Mediterranean part of Croatia is the soil reaction. In their review of the fungus's presence in Europe, Parent and Thoen (1986) found it to favour acidic soils, reporting a pH range in France from 4.5 to 6, which matches our own measurements from two localities having pH value of 4.1 and 4.3, and pH values (Tab. 2) obtained from the dataset available for Europe (Poggio et al. 2021). The Croatian coast is characterised by limestone bedrock and the consequent alkaline soil reaction. In Giovanetti et al. (2014), soil reactions measured across the Croatian part of Adriatic coast, except for one sample with a pH value of 6.2, ranged from 7.7 to 8.1. The importance of the soil reaction for *C. archeri* is supported by the fact that it was the most important variable in the Climate-pH model. Nevertheless, given the fact that the fungus is present in the Mediterranean region, and that there is one observation (iNaturalist. org 2018) from May 2018 in the Slovenian part of the Istria peninsula very close (1.2 km) to the Croatian border, it is reasonable to expect the fungus' presence in Istria, although neither of the models (Fig. 3 and Fig. 4) predicted high suitability there. Consequently, both models seem to underestimate the suitability in the Mediterranean region, although we believe that only the Northern part of the Adriatic, which is not characterised by as low summer precipitation and alkaline soil reaction as the more Southern areas, is concerned.

The presence of highly suitable areas in the Alpine region in which there are no known localities, does not necessarily mean that the models overestimate the suitability for *C. archeri* in these parts of Croatia. Indeed, there are no ecological constraints for the fungus there, since that area has a lot of precipitation as well as parts with acid soils. Although the maximum elevation of localities of *C. archeri* presented here is just 532 m a.s.l., with 90% of locations appearing below 400 m a.s.l. (Tab. 2), the species can be found at much higher elevations e.g. 1200 m a.s.l. in Romania (Bîrsan et al. 2014), or as reported by Parent and Thoen (1986) even up to 2000 m a.s.l. The fact that currently known localities in Croatia are mostly below 400 m a.s.l., could be merely a consequence of prevalence of incident observations during field visits that were not targeted specifically to survey the presence of *C. archeri*. Some of those observations could be from edible fungi collectors who prefer this elevation belt that corresponds to distribution of pedunculate (*Quercus robur* L.) and sessile (*Q. petraea* (Matt.) Liebl.) oak forests, known for their fungi richness. An additional explanation could be the very short duration of the fungus' visible presence which lasts approximately just one week per individual sporocarp. This includes the egg phase, which can be observed up to 5 days. However, without dissection it is impossible to record an observation, since other stinkhorn fungi also develop eggs. We expect that sampling effort (in this case actually simply the number of field days, given the non-targeted nature of it) is much higher in lower elevations, hence the higher probability of observing *C. archeri* there.

In the future, we could expect an increase in the presence of *C. archeri* in Croatia in terms of its abundance and occupancy area. Even at present, on various social networks photographs of the fungus from Croatia can be found, but without details on its geographical origin, hence they could not be used here. It is not easy to predict how much this increase might be, and how long it would last. In their analysis of the impact of climate change on *C. archeri*, Pietras et al. (2021) used three scenarios for the year 2080 expecting the fungus' range contraction in Central Europe, including the Continental region of Croatia from which currently the majority of observations derive. Depending on the climate change scenario employed, there could be some range expansion in the areas that overlap with the Mediterranean region in Croatia. However, Pietras et al. (2021) developed their models exclusively with bioclimatic variables, without data on soil acidity, which could play a significant role in shaping the future of *C. archeri* distribution.

Almost 20 years after its first mention for Croatia in the scientific literature (Tkalčec et al. 2005) and just one short note in the meantime reporting on two localities (Kranjčev 2008) we reveal that *C. archeri* is nowadays, in terms of its spatial distribution, a widespread alien fungus in Croatia. Open access data provided by international networks like GBIF and iNaturalist were indispensable for this analysis. Consequently, these data should be an integral part in further efforts in increasing our knowledge on alien fungi, and other taxa, presence and distribution in Croatia, that are otherwise poorly covered in the scientific literature. In addition, efforts to raise the awareness of such species among colleagues and the general public, as well as of the importance of observations being deposited on platforms like GBIF and/or iNaturalist could significantly contribute to the amount of available observational records. Habitat suitability models can help in planning field surveys, which is especially useful for taxa with low cost-efficiency field work, as *C. archeri* is, due to its short sporocarp longevity.

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## References

Bîrsan, C., Cojocariu, A., Cenuşă, E., 2014: Distribution and ecology of *Clathrus archeri* in Romania. Notulae Scientia Biologicae 6(3), 288–291. https://doi.org/10.15835/nsb639389

Bîrsan, C., Mardari, C., Copoţ, O., Tănase, C., 2021: Modelling the potential distribution and habitat suitability of the alien fungus *Clathrus archeri* in Romania. Botanica Serbica 45(2), 241–250. https://doi.org/10.2298/BOTSERB2102241B

Božac, R., 1984: Šest stotina gljiva naših krajeva (Six hundred mushrooms of our regions). Mladost, Zagreb.

Brännhage, J., Gross, A., 2020: Factsheet Neomyceten. Tintenfischpilz und Roter Gitterling. Eidg. Forschungsanstalt WSL, Birmensdorf, 3 S.

Davies, B. E., 1974: Loss-on-ignition as an estimate of soil matter. Soil Science of America. Proceedings, 38, 150–151.

Desprez-Loustau, M. L., Robin, C., Buée, M., Courtecuisse, R., Garbaye, J., Suffert, F., Sache, I., Rizzo, D. M., 2007: The fungal dimension of biological invasions. Trends in Ecology and Evolution 22(9), 472–480. https://doi.org/10.1016/j.tree.2007.04.005

Desprez-Loustau, M. L., Courtecuisse, R., Robin, C., Husson, C., Moreau, P., Blancard, D., Selosse, M., Lung-Escarmant, B., Piou, D., Sache, I., 2010: Species diversity and drivers of spread of alien fungi (*sensu lato*) in Europe with a particular focus on France. Biological Invasions 12, 157–172. https://doi.org/10.1007/s10530-009-9439-y

Didukh, Y. P., 2009: The Red Data Book of Ukraine Flora. Global consulting, Kyiv.

GBIF.org, 2024: GBIF Occurrence Download. Retrieved April 24, 2024 from https://doi.org/10.15468/dl.6mreqj

Giovanetti, M., Vuković, N., Jelaska, S. D., 2014: Australian acacias across eastern Adriatic – abundant but not aggressive. Periodicum Biologorum 116(3), 275–283.

Gyosheva, M. M., Denchev, C. M., Dimitrova, E. G., Assyov, B., Petrova, D. R., Stoichev, G. T., 2006: Red List of fungi in Bulgaria. Mycologia Balcanica 3, 81–87.

Heluta, V. P., Zykova, M. O., Hayova, V. P., Prydiuk, M. P., Shevchenko, M. V., 2022: An update to the species list of fungi in the Red Data Book of Ukraine. Ukrainian Botanical Journal 79(3), 154–168. https://doi.org/10.15407/ukrbotj79.03.154

iNaturalist.org, 2018: Devil's-Fingers (*Clathrus archeri*). Retrieved April 24, 2024 from https://www.inaturalist.org/observations/12907117

Kranjčev, R., 2008: Velika polipovka, Čekinjava pločašica, Vijugavo-mesnata mirisnica (Devil's fingers, Eyelash cup, White false truffle). Šumarski list 9–10, 492.

Lohwag, K., 1948: *Anthurus muellerianus* Kalchbr., var. *aseroëformis* Ed. Fischer, ein neuer Pilz für Österreich. Österreichische Mykologische Gesellschaft. 20.

Official Gazette, 1998–2002: Pravilnik o zaštiti gljiva (Ordinance on protection of fungi). NN 79/1998, NN 115/1998, NN 34/2002.

Official Gazette, 2006: Pravilnik o proglašavanju divljih svojti zaštićenim i strogo zaštićenim (Ordinance on designation of wild taxa as protected or strictly protected). NN 7/2006.

Parent, G. H., Thoen, D., 1986: État actuel de l'extension de *Clathrus archeri* (Berkeley) Dring (syn.: *Anthurus archeri* (Berk.) Ed. Fischer) en Europe et particulièrement en France et au BÉnÉlux. Bulletin trimestriel de la Société mycologique de France 102(3), 237–272.

Parent, G. H., Thoen, D., Calonge, F. D., 2000: Nouvelles données sur la répartition de *Clathrus archeri* en particulier dans l'Ouest et le Sud-Ouest de l'Europe. Bulletin trimestriel de la Société mycologique de France 116(3), 241–266.

Phillips, S. J., Dudík, M., Schapire, R. E., 2024: Maxent software for modelling species niches and distributions (ver. 3.4.1). Retrieved April 24, 2024 from http://biodiversityinformatics.amnh.org/open_source/maxent/

Pietras, M., Rudawska, M., Iszkuło, G., Kujawa, A., Leski, T., 2016: Distribution and molecular characterization of an alien fungus *Clathrus archeri* in Poland. Polish Journal of Environmental Studies 25(3), 1197–1204. https://doi.org/10.15244/pjoes/61230

Pietras, M., Kolanowska, M., Selosse, M., 2021: Quo vadis? Historical distribution and impact of climate change on the worldwide distribution of the Australasian fungus *Clathrus archeri* (Phallales, Basidiomycota). Mycological Progress 20, 299–311. https://doi.org/10.1007/s11557-021-01669-w

Poggio, L., de Sousa, L. M., Batjes, N. H., Heuvelink, G. B. M., Kempen, B., Ribeiro, E., Rossiter, D., 2021: SoilGrids 2.0: producing soil information for the globe with quantified spatial uncertainty. SOIL 7(1), 217–240. https://doi.org/10.5194/soil-7-217-2021

Stanič, I., 2024: Karta razširjenosti za *Clathrus archeri*. Podatkovna zbirka gliv Slovenije Boletus informaticus (Distribution map of *Clathrus archeri*. Data collection of Slovenian fungi Boletus informaticus). In: Ogris, N. (ed.), Web portal Boletus informaticus. Retrieved April 24, 2024 from https://boletusinformaticus.si/sre_karta.aspx?idorg=b59ac192-6ef6-4504-b0e6-234c1643c481

Tănase, C., Pop, A., 2005: Red List of Romanian Macrofungi Species. In: Mihăilescu, S. (ed.), Bioplatform – Romanian National Platform for Biodiversity, 101–107. Academiei Române, Bucureşti.

Tkalčec, Z., Mešić, A., Antonić, O., 2005: Survey of the Gasteral Basidiomycota (Fungi) of Croatia. Natura Croatica 14(2), 99–120.

Tkalčec, Z., Mešić, A., Matočec, N., Kušan, I., 2008: Crvena knjiga gljiva Hrvatske (Red Book of Croatian fungi). Ministry of Culture, State Institute for Nature Protection, Zagreb.

Vischer, W., 1943: Ein für die Schweiz neuer Pilz *Anthurus aseroeformis* Ed. Fischer. Schweizerische Zeitschrift für Pilzkunde 21, 17–20.

Voglmayr, H., Krisai-Greilhuber, I., 2002: Pilze. In: Essl, F., Rabitsch, W. (eds.), Neobiota in Österreich, 181–195. Umweltbundesamt, Vienna.

Voglmayr, H., Schertler, A., Essl, F., Krisai-Greilhuber, I., 2023: Alien and cryptogenic fungi and oomycetes in Austria: an annotated checklist (2nd edition). Biological Invasions 25, 27–38. https://doi.org/10.1007/s10530-022-02896-2

Vrščaj, D., Erjavec, Š., Boh, A., Stanič, I., Piltaver, A., Seljak, G., Tratnik, B., Malovrh, B., Tortić, M., Čebulec, M., Šerod, S., Vrhovnik, F., Arko, J., Dolenc, F., Oblak, V., Vrhovec, B., Kavčič, Z., Seršen, J., Kavrečič, B., Bertosi, M., Hočevar, S., Haas, H., Ivanovič, A., Kaiser, S., Ujčič, M., Moser, M., Kosec, J., Koprek, F., Munda, A., Šmauc, A., Dolenc, A., Hudoklin, V., Mohorčič, V., Arzenšek, B., Kovač, A., Velikonja, P., Markovič, D., Kelhar, M., Podgornik, G., Lekše, J., Jurc, M., Goršak, B., Hudohmet, M., Jurc, D., Poličnik, M., Grebenc, T., Bertoncelj, F., Bračko, B., Bohte, T., Gerhold, A., Voss, W., Oblak, F., Kovše, J., Miller, O., Tkalčeč, Z., Došler, B., Fink, A., Pirnat, F., Kalchbrenner, K., Krajnc, J., Škulj, M., Hrovatič, M., Klemenčič, J., Jenko, V., Knoll, L., Tratnik, V., Dolšak, F., Kraigher, H., Kranjc, M., Breitenbach, J., Kotlaba,F., Petkovšek, M., Wettstein, R., Agerer, R., Loose A., Pec, M., Potokar, A., Beenken, L., Cortese, D., Černigoj, F., Klun, J., Oblak, N., Rozman, J., Schild, E., Šajn, V., Šarič, Zaletel, A., Arko, V., Babšek, J., Batič, F., Bech, W., Berden, J., Bračun, F., Celič, I., Cesar, M., Cetto, B., Doganoc, N., Godec, B., Goleš, L., von Höhnel, F. X. R., Hvastja, C., Jurc, B., Kocjančič, K., Korošec, V., Kovačič, F., Lindtner, V., Ljubič, F., Novak, J., Palfner, G., Pavlič, J., Perušek, R., Petkovšek, V., Pilat, A., Pouzar, Z., Rižnar, I., Rupel, M., Schinner, F., Sonc, M., Svrček, M., Toplak, I., 2004: Priloga Glive v Sloveniji (Contribution to Fungi in Slovenia). In: Jurc, D. (ed.), Seznam vrst in razširjenost makromicet v Sloveniji z analizo stopnje ogroženosti, 52–52. Gozdarski inštitut Slovenije, Ljubljana.

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# Leaf morphological variability of *Pyrus spinosa* and *Crataegus monogyna* and their potential hybridization

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**Abstract** – This paper is aimed at investigating leaf morphological variability and possible hybridization between two species within the Rosaceae family: the one-seed hawthorn (*Crataegus monogyna* Jacq.) and the almond-leaved pear (*Pyrus spinosa* Forssk.). These two genera are taxonomically related and meet many prerequisites for successful hybridization between them, which prompted us to investigate the possibility of the presence of a long-described yet uninvestigated hybrid called ×*Pyrocrataegus*. The research was conducted along the Eastern Adriatic coast, where both species are widespread and often grow together in open woodlands, forest edges and abandoned agricultural areas. The examination of morphological variability was based on a morphometric analysis of seven populations using ten phenotypic traits of leaves. In general, our results showed great variability of leaf morphological traits within and between the studied populations, as well as a clear differentiation between the two species. The results of principal component analysis (PCA) showed a few intermediate individuals between the two species, indicating possible hybridization. However, as heteroblasty is present in *P. spinosa*, with its seedlings reported to have lobed, hawthorn-like leaves, dimorphism could also result from the reappearance of juvenile leaves on adult trees by means of rejuvenation. In order to draw a definitive conclusion about the existence of hybrid individuals in the next study, DNA markers and a much larger sample, especially morphologically intermediate individuals per population should be included.

**Keywords:** Croatia, hybrids, heteroblasty, morphometric analysis, morphological variability, Rosaceae

## Introduction

With over 3000 species in more than 90 genera, the rose family (Rosaceae) is one of the most diverse angiosperm families (Zhang et al. 2017). The family includes many ecologically and economically important species that contain the whole spectrum of beneficial properties for biodiversity, as well as for human nutrition and healthcare. Phylogenetic relationships within Rosaceae are complicated and have not been fully clarified, as homoplasy of morphological characters, frequent hybridization and apomixis complicate their classification and phylogenetic reconstruction (Zhang et al. 2017). Within the family, some genera engage in interspecific hybridization more easily than others, like *Malus* Mill. (Larsen et al. 2008), *Sorbus* L. (Németh et al. 2020) and *Pyrus* L. (Bell and Hough 1986). However, hybridization in Rosaceae is not limited only to that between species within the same genus, but crosses between species from different genera are also possible (Postman 2011). Intergeneric hybridization in Rosaceae often results in highly fertile individuals that appear repeatedly in nature (Campbell et al. 2007).

Among the genera with the largest number of intergeneric hybrids are *Pyrus* and *Crataegus* L. Successful hybridization has been reported between *Pyrus* species and *Sorbus* (Postman 2011), *Cydonia* L. (Shimura et al. 1983) and *Malus* (Pasqualetto et al. 2022), resulting in new hybrid genera like ×*Sorbopyrus* C.K.Schneid. and ×*Pyronia* Veitch ex. Trab. In

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addition, the best-known intergeneric hybrid of *Crataegus* is *Crataemespilus* Camus, a sexual hybrid between *Crataegus* and *Mespilus* L. (Phipps 2016). These hybrid individuals usually display new, intermediate forms of vegetative and generative traits (Pasqualetto et al. 2022). However, most of these hybrids have been obtained artificially in attempts to obtain individuals with superior morphological, sensory or physiological characteristics, as hybridization is recognized as the most important source of genetic variation in fruit breeding (Van Tuyl and de Jeu 1997). When successful, intergeneric hybridization allows the introduction of chromosomal genomic regions of one taxon into that of another taxon through subsequent backcrossing, enabling the introduction of favourable traits to improve flavour, texture or disease resistance (Fischer et al. 2014).

In addition to the aforementioned intergeneric hybrids, sporadic mentions of a hybrid between *Pyrus* and *Crataegus*, named ×*Pyrocrataegus* Rehder (Rehder 1949, McNeill et al. 2016) can be found in the literature. This proposed hybrid was described as the result of hybridization between *Crataegus oxyacantha* and *Pyrus communis* L., as well as between *C. monogyna* Jacq. and *P. pollveria* Lej (Rehder 1949). It is important to note that the author of *C. oxyacantha* was not noted, and therefore the exact species is not clear, as by present taxonomy it could be synonymous with any of the following accepted taxa, depending on the author: *C.* × *polyacantha* Jan, *C. laevigata* (Poir.) DC. or *C. marshallii* Eggl. Furthermore, according to the World Flora Database (WFO 2024), the taxonomic classification of *P. pollveria* is still unclear. Unfortunately, no further investigations were conducted on this hybrid, nor was it described in more detail in the available literature. Therefore, an intergeneric hybrid between *Pyrus* and *Crataegus* remains a botanical curiosity and is yet to be confirmed by modern taxonomical methods.

During field research in 2021, we observed almond-leaved pear (*Pyrus spinosa* Forssk.) individuals with peculiar, hawthorn-like leaves on a few branches, which aroused our interest in the long-described hybrid between these two genera. In this particular area, along the eastern Adriatic coast, both the almond-leaved pear and the one-seed hawthorn (*Crataegus monogyna* Jacq.) can be found. These are deciduous shrubs or small trees that reach up to 10 m in height (Zamani et al. 2012, Nabavi et al. 2015). However, they differ significantly in leaf morphology. Almond-leaved pear leaves are narrowly lanceolate or elliptic in shape, up to 7 cm long and 3 cm wide. The leaf edge is entire, sometimes moderately crenate. Leaves are shiny and vary in colour, from green to dark green, greyish- to bluish-green from below, initially hairy on both sides, later glabrous or only hairy below (Zamani et al. 2012). On the other hand, one-seed hawthorn leaves are 3-5 cm wide and long, broadly ovate to rhombic, deeply lobed, with pointed tips of the lobes. Lobes sometimes reach up almost to the midrib. Colour-wise, the leaves of common hawthorn are dark green, glabrous and shiny, lighter from below, hirsute only in the vein corners (Idžojtić 2009). Both species have up to 2 cm-long petioles. The almond-leaved pear is native to xerophytic habitats of Southern and South-eastern Europe and of Asia Minor, where it grows in discontinuous bush associations and open spaces, on a wide range of soil and habitat conditions (Vidaković et al. 2021). On the other hand, the one-seed hawthorn is widely spread across most of Europe and western Asia (Nabavi et al. 2015).

Considering their overlapping natural distribution and occasional reproductive compatibility described in the literature (Rehder 1949, McNeill et al. 2016), in this study we aimed to investigate the possibility of the presence of a long-described yet uninvestigated hybrid between *Crataegus* and *Pyrus* called ×*Pyrocrataegus* along the eastern Adriatic coast. In addition, the variability of their respective leaf morphologies is set to be studied, along with the population variability of both species. These data would provide valuable insight into the diversity of leaf sizes and shapes of these species, which can provide additional knowledge about their plasticity and adaptation processes.

## Material and methods

### Plant material and morphometric analysis

The plant material for morphometric analysis was collected in three *C. monogyna* (P1-P3) and four *P. spinosa* (P4-P7) populations (Tab. 1, Fig. 1).

It is important to note that *P. spinosa* samples were subsampled from a larger study oriented towards research into genetic diversity and population genetics (Vidaković et al. 2024). The collection area encompasses the regions of Istria and Northern Dalmatia, where these two species have overlapping natural distribution areas, and where dimorphic *P. spinosa* individuals were observed. At each location, 10 shrubs/trees were selected for the analysis. From each shrub/tree, 20 fully developed leaves with no signs of disease or damage were collected from the short shoots in the sunlit part of the canopy. The leaves were collected during the vegetation period of 2022. Upon collection, leaves were herbarized, scanned using Microtek ScanMaker 9800XL scanner, measured using WinFolia software (WinFolia$^{TM}$ 2001) and stored at the Faculty of Forestry and Wood Technology of the University of Zagreb.

In total, 10 leaf morphological traits were measured: leaf area (LA), perimeter (P), form coefficient (FC), leaf length (LL), maximum leaf width (MLW), leaf length, measured from the leaf base to the point of maximum leaf width (PMLW), leaf blade width at 90% of leaf blade length (LW90); angle closed by the main leaf vein and the line defined by the leaf blade base and a point on the leaf margin, at 10% (LA10) and 25% (LA25) of leaf blade length and petiole length (PL). In total, 1400 leaves were measured, 800 of *P. spinosa* and 600 of *C. monogyna*.

### Statistical analysis

Following the procedure described in Sokal and Rohlf (2012), descriptive statistical parameters for all of the stud-

**Tab. 1.** Populations, sampling sites, taxa, geographic coordinates, and multivariate diversity index (MDI) for seven studied populations. The significance level of differences in the average values of MDI between groups according to the Kruskal-Wallis test is marked by asterisk (*).

| Population ID | Sampling site | Taxa | Latitude (N) | Longitude (E) | Multivariate diversity index (MDI)* |
|---|---|---|---|---|---|
| P1 | Buje | *Crataegus monogyna* | 45.4328 | 13.7775 | 2.474 |
| P2 | Pula | *Crataegus monogyna* | 44.8756 | 13.9005 | 1.639 |
| P3 | Nin | *Crataegus monogyna* | 44.2120 | 15.3341 | 1.816 |
| P4 | Škropeti | *Pyrus spinosa* | 45.2732 | 13.8272 | 2.529 |
| P5 | Pula | *Pyrus spinosa* | 44.8756 | 13.9005 | 1.727 |
| P6 | Nin | *Pyrus spinosa* | 44.2120 | 15.3341 | 2.712 |
| P7 | Obrovac | *Pyrus spinosa* | 44.2143 | 15.6628 | 2.440 |
| | | *Crataegus monogyna* | | | 2.326 |
| | | *Pyrus spinosa* | | | 2.632 |
| | | P (KW)* | | | 0.002 |

ied traits were calculated, including arithmetic mean (M), standard deviation (SD) and coefficient of variability (CV). These parameters were calculated at individual population level and in total and gave insights into morphological characteristics and range of variation for each population and trait.

In addition, principal component analysis (PCA) was conducted in order to assess population structure and to reveal interactions between individuals and studied morphometric traits. To enhance the analysis, a biplot was constructed by first two principal components. The principal component analysis was conducted using the "MorphoTools" R scripts in R v.3.2.2 (R Core Team 2016).

The Euclidean distance matrix was calculated between all pairs of individuals based on the scores of the first two principal components (PC) considering 10 leaf traits. The average Euclidean distances were calculated for each population and species and used as the multivariate diversity index (MDI) of a population (or species) (Poljak et al. 2024). The Kruskal-Wallis test between species was performed using the STATISTICA version 13 software package (STATISTICA version 13, 2018).

In addition, the Euclidean distance matrix was also used in the analysis of molecular variance (AMOVA; Excoffier et al. 1992) to partition the total morphological variance between species, among populations within species and with-

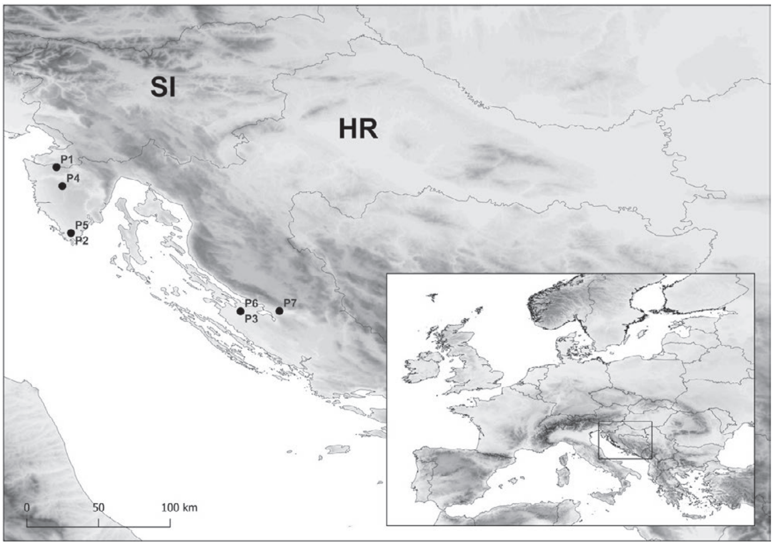

**Fig. 1.** Geographic locations of the studied *Crataegus monogyna* (P1-P3) and *Pyrus spinosa* (P4-P7) populations. Populations: P1 – Buje; P2 – Pula; P3 – Nin; P4 – Škropeti; P5 – Pula; P6 – Nin; P7 – Obrovac. The rectangle on the map in the lower right corner indicates the research area in the SE European context. Abbreviations: SI – Slovenia, HR – Croatia.

in populations (two-way AMOVA) and to partition the total morphological variance among and within populations of each species (one-way AMOVA). The significance levels of the variance components were determined after 10.000 permutations. The calculations were performed in Arlequin ver. 3.5.2.2 (Excoffier and Lischer 2010).

## Results

The results of descriptive statistics for both species are shown in Tab. 2, individually per population and in total.

On average, one-seed hawthorn leaves were 1.88 cm long, 1.77 wide, with 1.10 cm long petioles. The morphometric trait that refers to leaf shape, i.e., form coefficient (FC) had an average value of 0.50. The most variable trait was leaf area (LA), with CV value of 35.87%, followed by petiole length (PL) with CV value of 25.36%. On the other hand, the least variable traits were the angles closed by the main leaf vein and the line defined by the leaf blade base and a point on the leaf margin, at 10% (LA10) and 25% (LA25) of leaf blade length, with CV values of 11.33 and 9.58%, respectively. On an individual population level, population P1 (Buje) was characterized by on average the largest leaves, with seven out of 10 maximal values (LA, P, LL, MLW, PMLW, LW90, PL). In contrast, the largest number of minimal values was found in P2 (Pula) (LA, P, LL, MLW, LW90, LA25, PL), which characterizes this population as the one with the smallest leaves. By far the most variable population was P1 (Buje), with the highest CV values for all of the measured leaf traits except petiole length (PL), ranging from 10.09 (LA25) to 34.12% (LA). On the other hand, leaf traits were the least variable in P3 (Nin), with six minimal CV values (P, FC, MLW, LW90, LA25, PL).

The average leaf of the almond-leaved pear was 3.44 cm in length, 1.39 cm in width and had a 1.12 cm-long petiole. Form coefficient has an average value of 0.63. Coefficients

**Tab. 2.** Results of the descriptive statistical analysis for the studied populations and morphometric traits. Morphometric traits analysed: LA – leaf area (cm$^2$); P – leaf perimeter (cm); FC – form coefficient; LL – leaf blade length (cm); MLW – maximum leaf width (cm); PMLW – leaf blade length measured from the leaf base to the point of maximum leaf width (cm); LW90 – leaf blade width at 90% of the leaf blade length (cm); LA10 – angle closed by the main leaf vein and the line defined by the leaf blade base and the point on the leaf margin, at 10% (°); LA25 – angle closed by the main leaf vein and the line defined by the leaf blade base and the point on the leaf margin, at 25% (°); PL – petiole length (cm). Descriptive parameters: M – arithmetic mean, SD – standard deviation and CV – coefficient of variation (%). Populations: P1-P7 as in Tab. 1.

| Taxa | Population ID | Descriptive parameters | Morphometric traits analysed | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | LA | P | FC | LL | MLW | PMLW | LW90 | LA10 | LA25 | PL |
| *Crataegus monogyna* | P1 | M | 2.63 | 8.59 | 0.46 | 2.14 | 2.05 | 1.04 | 0.66 | 54.81 | 52.53 | 1.21 |
| | | SD | 0.90 | 1.69 | 0.10 | 0.41 | 0.37 | 0.32 | 0.17 | 7.45 | 5.30 | 0.29 |
| | | CV (%) | 34.12 | 19.64 | 22.40 | 19.10 | 17.87 | 30.58 | 25.47 | 13.60 | 10.09 | 23.94 |
| *Crataegus monogyna* | P2 | M | 1.59 | 6.07 | 0.57 | 1.70 | 1.49 | 0.95 | 0.48 | 61.07 | 49.78 | 0.92 |
| | | SD | 0.31 | 1.09 | 0.10 | 0.21 | 0.22 | 0.16 | 0.10 | 4.85 | 4.61 | 0.22 |
| | | CV (%) | 19.52 | 17.96 | 17.68 | 12.31 | 14.67 | 16.68 | 19.89 | 7.95 | 9.25 | 24.30 |
| *Crataegus monogyna* | P3 | M | 1.88 | 7.21 | 0.47 | 1.79 | 1.76 | 0.85 | 0.55 | 60.79 | 53.34 | 1.18 |
| | | SD | 0.42 | 1.22 | 0.08 | 0.24 | 0.20 | 0.19 | 0.10 | 6.04 | 4.74 | 0.24 |
| | | CV (%) | 22.28 | 16.92 | 17.54 | 13.40 | 11.32 | 21.95 | 18.93 | 9.94 | 8.89 | 20.73 |
| *Crataegus monogyna* | Total | M | 2.03 | 7.29 | 0.50 | 1.88 | 1.77 | 0.95 | 0.57 | 58.89 | 51.88 | 1.10 |
| | | SD | 0.73 | 1.68 | 0.11 | 0.35 | 0.35 | 0.24 | 0.14 | 6.67 | 4.97 | 0.28 |
| | | CV (%) | 35.87 | 23.02 | 21.23 | 18.54 | 19.75 | 24.94 | 25.55 | 11.33 | 9.58 | 25.36 |
| *Pyrus spinosa* | P4 | M | 4.58 | 9.15 | 0.68 | 3.77 | 1.66 | 1.93 | 0.86 | 39.79 | 33.02 | 1.35 |
| | | SD | 1.46 | 1.58 | 0.07 | 0.72 | 0.26 | 0.41 | 0.13 | 7.29 | 5.50 | 0.23 |
| | | CV (%) | 31.79 | 17.28 | 10.11 | 19.04 | 15.87 | 21.34 | 15.21 | 18.33 | 16.67 | 17.17 |
| *Pyrus spinosa* | P5 | M | 3.57 | 8.72 | 0.59 | 3.62 | 1.37 | 1.90 | 0.72 | 35.09 | 28.03 | 1.18 |
| | | SD | 0.84 | 1.14 | 0.06 | 0.50 | 0.19 | 0.22 | 0.16 | 3.61 | 2.78 | 0.31 |
| | | CV (%) | 23.64 | 13.06 | 9.85 | 13.73 | 13.59 | 11.74 | 22.07 | 10.30 | 9.93 | 26.38 |
| *Pyrus spinosa* | P6 | M | 2.70 | 7.50 | 0.59 | 3.14 | 1.16 | 1.57 | 0.58 | 36.11 | 29.03 | 0.92 |
| | | SD | 1.23 | 1.92 | 0.06 | 0.85 | 0.23 | 0.53 | 0.11 | 7.33 | 4.91 | 0.30 |
| | | CV (%) | 45.72 | 25.60 | 9.52 | 26.99 | 19.97 | 33.51 | 19.08 | 20.30 | 16.91 | 32.95 |
| *Pyrus spinosa* | P7 | M | 3.29 | 7.83 | 0.65 | 3.23 | 1.38 | 1.61 | 0.72 | 39.86 | 32.25 | 1.05 |
| | | SD | 1.24 | 1.64 | 0.08 | 0.70 | 0.31 | 0.38 | 0.16 | 4.86 | 4.06 | 0.21 |
| | | CV (%) | 37.50 | 20.92 | 11.78 | 21.74 | 22.52 | 23.70 | 23.01 | 12.20 | 12.60 | 20.50 |
| *Pyrus spinosa* | Total | M | 3.53 | 8.30 | 0.63 | 3.44 | 1.39 | 1.75 | 0.72 | 37.71 | 30.58 | 1.12 |
| | | SD | 1.35 | 1.67 | 0.07 | 0.73 | 0.30 | 0.42 | 0.17 | 6.15 | 4.76 | 0.31 |
| | | CV (%) | 38.32 | 20.14 | 11.91 | 21.10 | 21.64 | 23.90 | 23.57 | 16.32 | 15.57 | 27.17 |

**Tab. 3.** The results of two-way (between species) and one-way (within species) analysis of molecular variance (AMOVA). df – degrees of freedom; $f_{ST}$ – measure of differentiation among populations; *** significant at P < 0.001; ** significant at 0.001 < P < 0.01.

| Analysis | Source of variation | df | % Variation | $f_{ST}$ | P |
|---|---|---|---|---|---|
| Between *Crataegus monogyna* and *Pyrus spinosa* | Between species | 1 | 44.89 | 0.449 | *** |
| | Among population within species | 5 | 8.90 | 0.162 | *** |
| | Within populations | 63 | 46.21 | 0.538 | *** |
| Within *Crataegus monogyna* | Among populations | 2 | 20.39 | 0.204 | *** |
| | Within populations | 27 | 79.61 | | |
| Within *Pyrus spinosa* | Among populations | 3 | 13.41 | 0.134 | ** |
| | Within populations | 36 | 86.59 | | |

of variability between the studied leaf traits ranged from 15.57 (LA25) to 38.32% (LA). The second most variable trait, as for *C. monogyna*, was petiole length, with CV value of 27.17%. When observing individual populations, P4 (Škropeti) had the highest values in all of the measured leaf traits except LA10. In contrast, population P6 (Nin) had eight out of 10 the lowest average values of leaf morphometric traits (LA, P, FC, LL, MLW, PMLW, LW90, PL). In addition, P6 (Nin) had also the most variable leaf morphology, with seven maximal CV values (LA, P, LL, PMLW, LA10, L25, PL). On the other hand, population P5 (Pula) was the least variable, with seven minimal CV values (LA, P, LL, MLW, PMLW, LA10, LA25).

The multivariate diversity index (MDI) values, based on leaf morphological traits, ranged from 1.639 to 2.474 in *C. monogyna*, and from 1.727 to 2.712 in *P. spinosa* (Tab. 1). On overall individual species level, *P. spinosa* had a significantly larger MDI (2.632) than *C. monogyna* (2.326), as demonstrated by the Kruskal-Wallis test (P = 0.0023). The results of two-way AMOVA conducted for both species showed statistically significant differences between the two species, among populations within species and within populations (Tab. 3). The analysis also revealed that within-population and between-species variabilities contributed almost equally to the total variability, with 46.21% and 44.89%, respectively. One-way AMOVA conducted on individual species showed significant differences among populations within both species. In addition, within-population variability accounted for most of the total variability in both species.

Principal component analysis (PCA) was conducted, based on 10 morphological leaf traits. The two first principal components explained 83.52% of the total variability, with additional 9.95 and 3.36% explained by the third and fourth principal components, respectively. Five traits were in a high negative correlation with the first principal component (LA, P, LL, PMLW, LW90) and two traits were in a high positive correlation with the same component (LA10, LA25). The second principal component was in a high negative correlation with MLW, while the third principal component was highly positively correlated with FC (Tab. 4).

The biplot constructed by the first two principal components is shown in Fig. 2. Clear separation of the two species can be observed along the first axis, where barycentres of *P. spinosa* are separated on the left, and those of *C. monogyna* on the right side of the first axis. *Crataegus monogyna* was characterized by generally wider leaf base angles (LA10,

**Tab. 4.** Pearson's correlation coefficients between morphometric traits and scores of the first four principal components. Morphometric traits' acronyms as in Tab. 2.

| Trait | PC – Principal Component | | | |
|---|---|---|---|---|
| | PC1 | PC2 | PC3 | PC4 |
| LA | –0.9433 | –0.2295 | 0.1209 | –0.0556 |
| P | –0.8023 | –0.5137 | –0.2630 | –0.0863 |
| FC | –0.3207 | 0.5363 | 0.7648 | 0.0647 |
| LL | –0.9728 | 0.1209 | –0.1116 | 0.0283 |
| MLW | –0.1090 | –0.9468 | 0.1315 | –0.2323 |
| PMLW | –0.9673 | 0.1584 | –0.0773 | –0.0419 |
| LW90 | –0.8083 | –0.2400 | 0.4336 | –0.1344 |
| LA10 | 0.8123 | –0.4630 | 0.2536 | 0.0392 |
| LA25 | 0.7515 | –0.6198 | 0.1916 | –0.0309 |
| PL | –0.5192 | –0.6914 | 0.0390 | 0.4944 |
| Eigenvalue | 5.68 | 2.67 | 0.99 | 0.34 |
| Variance (%) | 56.78 | 26.74 | 9.95 | 3.36 |
| Cumulative Variance (%) | 56.78 | 83.52 | 93.47 | 96.83 |

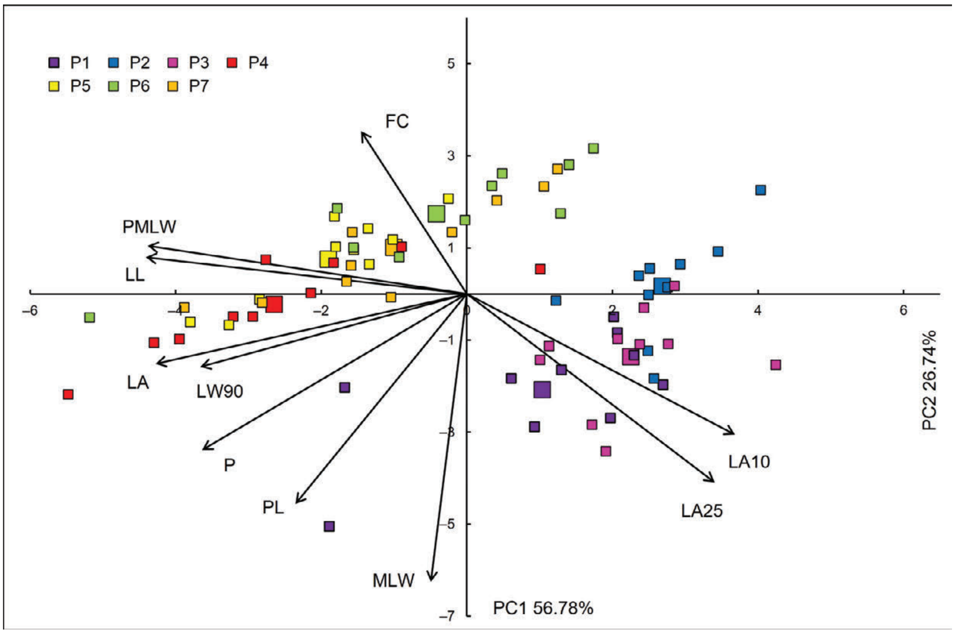

**Fig. 2.** Biplot of the principal component analysis (PCA) based on ten leaf morphometric traits in the studied *Crataegus monogyna* (P1-P3) *and Pyrus spinosa* (P4-P7) populations. Each individual shrub/tree is indicated by a small sign, while the population barycenters are represented by larger ones. Morphometric traits' acronyms: LA – leaf area; P – leaf perimeter; FC – form coefficient; LL – leaf blade length; MLW – maximum leaf width; PMLW – leaf blade length measured from the leaf base to the point of maximum leaf width; LW90 – leaf blade width at 90% of the leaf blade length; LA10 – angle closed by the main leaf vein and the line defined by the leaf blade base and the point on the leaf margin, at 10%; LA25 – angle closed by the main leaf vein and the line defined by the leaf blade base and the point on the leaf margin, at 25%; PL – petiole length. Populations acronyms: P1 – Buje; P2 – Pula; P3 – Nin; P4 – Škropeti; P5 – Pula; P6 – Nin; P7 – Obrovac.

LA25), while *P. spinosa* was characterized by longer and wider leaves.

However, a few individuals of both species ended up on the opposite side of the axis. For instance, two individuals in P1 were separated on the left side of the first axis, and they were characterized by long petioles and high perimeter value, while a few individuals in P4, P6 and P7 were separated on the right side of the first axis. These few individuals from *P. spinosa* populations, with unusual, hawthorn-like leaves indicate possible hybridization between these two genera (Fig. 3).

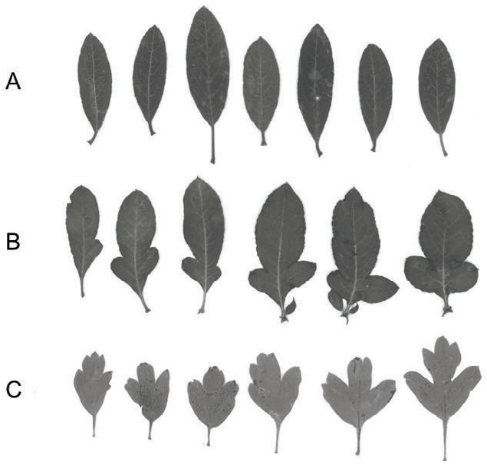

**Fig. 3.** Leaf variability of *Pyrus spinosa* (A), possible hybrid between *P. spinosa* and *Crataegus monogyna* (B) and *C. monogyna* (C) from the Nin in the eastern Adriatic.

## Discussion

Leaf dimensions of *C. monogyna* obtained in this research were slightly lower compared to the length and width of 3-5 cm listed by Schuck (2008), and within the range of 1-6 cm stated by Fichtner and Wissemann (2021) and Khadivi et al. (2019). The petiole length of 1.10 cm fits within the ranges listed by all the above mentioned authors (1-3 cm). On the other hand, leaf dimensions of *P. spinosa* were in accordance with previous descriptions of 2.5-7 cm long and 1-3 cm wide leaves with a petiole of 1-2 cm (Idžojtić 2009, Zamani et al. 2012, Vidaković et al. 2021). In both species, the leaf area (LA) and petiole length (PL) were the most variable traits, with CV values above 30% in LA and 25% in PL. Such a pattern of variability is very common among woody species (Khadivi-Khub et al. 2015, Kumar et al. 2018). In *C. monoygna*, the coefficients of variability in the majority of the measured leaf traits were very similar to that obtained by Khadivi et al. (2019), but significantly higher than in Khadivi-Khub et al. (2015). However, their respective studies did not include leaf area. Coefficients of variability in *P. spinosa* traits ranged from 11.91 to 38.32%, which is lower than the range of 18.02-45.62% obtained by Vidaković et al. (2021).

According to AMOVA, the majority of total variability in both species could be attributed to within-population variability, while a much smaller percentage was associated with among-population variability. Such a distribution of variability is expected, as it was previously confirmed in many other insect-pollinated and animal-dispersed species (Vidaković et al. 2021, 2022). However, *C. monogyna* populations were somewhat better differentiated than those of *P. spinosa*, which is also supported by weak genetic differentiation of *P. spinosa* populations in the area (Vidaković et al. 2024). Furthermore, significant differences in morphological variability between the two species were confirmed by MDI values, which demonstrated greater morphological variability of *P. spinosa*. This result is supported by the greater overall variability of leaf morphological traits in *P. spinosa* (Vidaković et al. 2021) than in *C. monogyna* (Khadivi-Khub et al. 2015, Khadivi et al. 2019). This could be the result of adaptation to microhabitat conditions, but also of phylogenetic and evolutionary processes. Namely, for *P. spinosa* exhibits greater morphological variability than the phylogenetically older *P. pyraster* (L.) Burgsd. (Korotkova et al. 2018, Vidaković et al. 2021, 2022). This may indicate still ongoing evolutionary speciation and morphological differentiation, resulting in more diverse leaf morphology.

As mentioned in the Introduction section, during field research in 2021, conducted along the eastern Adriatic coast, we observed a few *P. spinosa* individuals with unusual, hawthorn-like leaves on numerous branches. Hybridization between *Pyrus* and *Crataegus* could indeed be possible, as they both belong to the tribe Maleae (Sun et al. 2024), which indicates their close taxonomic relationship. Furthermore, both genera possess the basal chromosome number of 17 (Evans and Campbell 2002), which is thought to have originated from aneuploidization events approximately 50 million years ago (Considine et al. 2012), with *Gillenia* Moench as a probable common ancestor (Sun et al. 2024). A common chromosome number, along with coordinated flowering phenology, reproductive compatibility and common pollinators, is one of the main prerequisites for successful hybridization (Rieseberg and Carney 1998).

Our results based on the morphological analysis of the leaves did indeed show a few intermediate individuals, indicating possible hybridization between the two species. Alternatively, the dimorphic leaves of *P. spinosa* individuals could be explained by the sporadic appearance of juvenile leaves in the adult stage. Namely, almond-leaved pear seedlings were reported to have lobed, hawthorn-like leaves (Dostálek 1980), which was also observed by personal observation of young plants in an ongoing outdoor seed germination experiment carried out on the Faculty of Forestry and Wood Technology in Zagreb. Such substantial differences between juvenile and subsequent or adult forms in plant species are known as heteroblasty (Zotz et al. 2011). This botanical phenomenon of distinct morphological phenotypes in juvenile, transitional and adult stages is present in many agricultural species, as well as in some woody species like *Acacia confusa* Merr., *A. colei* Maslin et L.A.J.Thomson, *Eucalyptus globulus* Labill., *Hedera helix* L., *Quercus acutissima* Carruth. and *Populus* spp. (Manuela and Xu 2020).

The reappearance of juvenile leaves in the adult stage could be explained by the process of rejuvenation. This process enables plants to reverse the adult phase characteristics and recover some juvenile traits (Zhang et al. 2020). Small RNA profiling revealed an increase in microRNA156 (miR156) during plant rejuvenation (Chen et al. 2013), which maintains juvenile traits by repressing a group of SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors (Ye et al. 2019). Additionally, miR156 is subject to epigenetic regulation (Manuela and Xu 2020), which makes epigenetics one of the main factors controlling plant development and rejuvenation (Zhang et al. 2020). For instance, new sprouts from the adult tree collar or water sprouts, which are very common in pears, are considered to be ontogenetically juvenile, compared to their parent tree (del Tredici 2001). Among other juvenile traits that occur on such sprouts are dimorphic leaves, usually larger and more variable in shape (del Tredici 2017). In our case, this could be an alternative explanation for the occurrence of dimorphic leaves in this *P. spinosa*. However, further genetic and morphometric studies should be conducted in order to draw a definite conclusion about hybridization between *P. spinosa* and *C. monogyna*.

## Conclusions

Both the almond-leaved pear and the one-seed hawthorn are widespread in the coastal areas of Southern Europe and play a vital role in local ecosystems and the maintenance of biodiversity. This study was aimed at supplementing knowledge on the morphological variability of these two sympatric species. The results showed great variability of leaf morphological traits within and between studied populations, as well as a clear differentiation between the two species. However, hawthorn populations were better differentiated than those of the almond-leaved pear, but the latter had generally more diverse leaf morphology. High variability of almond-leaved pear leaves was also manifested through the presence of dimorphic, hawthorn-like leaves on some individuals, which raised the suspicion of the presence of a long-described but under-investigated hybrid between the two genera. Although the results showed several intermediate individuals, a possible explanation for dimorphic leaves on almond-leaved pear individuals, apart from hybridization, could be the reappearance of juvenile leaves on adult trees by means of rejuvenation. In order to draw a definitive conclusion about the existence of hybrid individuals, in the next study, DNA markers and a much larger sample, especially of morphologically intermediate individuals per population, should be included.

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## Author contribution statement

A.V., Z.Š., Z.L., I.P. – conceptualization and study design; A.V., I.P. – sampling; M.J. – morphometric analysis; Z.Š., I.P. – data analysis; A.V., I.P. – writing original draft preparation; Z.Š., Z.L., M.J. – review and editing. All authors have read and agreed to the published version of the manuscript.

## References

Bell, R. L., Hough, L. F., 1986: Interspecific and intergeneric hybridization of *Pyrus*. HortScience 21(1), 62–64. https://doi.org/10.21273/HORTSCI.21.1.62

Campbell, C. S., Evans, R. C., Morgan, D. R., Dickinson, T. A., Arsenault, M. P., 2007: Phylogeny of subtribe Pyrinae (formerly the Maloideae, Rosaceae): Limited resolution of a complex evolutionary history. Plant Systematics and Evolution 266(1/2), 119–145. https://doi.org/10.1007/s00606-007-0545-y

Chen, Y.-T., Shen, C.-H., Lin, W.-D., Chu, H.-A., Huang, B.-L., Kuo, C.-I., Yeh, K.-W., Huang, L.-C., Chang, I.-F., 2013: Small RNAs of *Sequoia sempervirens* during rejuvenation and phase change. Plant Biology 15(1), 27–36. https://doi.org/10.1111/j.1438-8677.2012.00622.x

Considine, M. J., Wan, Y., D'Antuono, M. F., Zhou, Q., Han, M., Gao, H., Wang, M., 2012: Molecular genetic features of polyploidization and aneuploidization reveal unique patterns for genome duplication in diploid *Malus*. PloS One 7(1), e29449. https://doi.org/10.1371/journal.pone.0029449

del Tredici, P., 2001: Sprouting in temperate trees: A morphological and ecological review. The Botanical Review 67(2), 121–140. https://doi.org/10.1007/BF02858075

del Tredici, P., 2017: Aging and rejuvenation in trees. Arboricultural Consultant 50(1), 7–11.

Dostálek, J., 1980: *Pyrus spinosa* und ihre Hybriden in Südwestbulgarien. Folia Geobotanica et Phytotaxonomica 15, 59–73. https://doi.org/10.1007/BF02853139

Evans, R. C., Campbell, C. S., 2002: The origin of the apple subfamily (Maloideae; Rosaceae) is clarified by DNA sequence data from duplicated GBSSI genes. American Journal of Botany 89(9), 1478–1484. https://doi.org/10.3732/ajb.89.9.1478

Excoffier, L., Lischer, H. E. L., 2010: Arlequin suite ver. 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10(3), 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Excoffier, L., Smouse, P. E., Quattro, J. M., 1992: Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitocondrial DNA restriction sites. Genetics 131(2), 479–491. https://doi.org/10.1093/genetics/131.2.479

Fichtner, A., Wissemann, V., 2021: Biological flora of the British Isles: *Crataegus monogyna*. Journal of Ecology, 109(1), 541–571. https://doi.org/10.1111/1365-2745.13554

Fischer, T. C., Malnoy, M., Hofmann, T., Schwab, W., Palmieri, L., Wehrens, R., Schuch, L. A., Müller, M., Schimmelpfeng, H., Velasco, R., Martens, S., 2014: F1 hybrid of cultivated apple (*Malus × domestica*) and European pear (*Pyrus communis*) with fertile F2 offspring. Molecular Breeding 34, 817–828. https://doi.org/10.1007/s11032-014-0077-4

Idžojtić, M., 2009: Dendrologija – list (Dendrology - leaf). University of Zagreb, Faculty of Forestry and Wood Technology, Zagreb.

Khadivi, A., Heidari, P., Rezaei, M., Safari-Khuzani, A., Sahebi, M., 2019: Morphological variabilities of *Crataegus monogyna* and *C. pentagyna* in northeastern areas of Iran. Industrial Crops and Products 139, 111521. https://doi.org/10.1016/j.indcrop.2019.111531

Khadivi-Khub, A., Karimi, S., Kameli, M., 2015: Morphological diversity of naturally grown *Crataegus monogyna* (Rosaceae, Maloideae) in Central Iran. Brazilian Journal of Botany 38, 921–936. https://doi.org/10.1007/s40415-015-0187-1

Korotkova, N., Parolly, G., Khachatryan, A., Ghulikyan, L., Sargsyan, H., Akopian, J., Borsch, T., Gruenstaeudl, M., 2018: Towards resolving the evolutionary history of Caucasian pears (*Pyrus*, Rosaceae) - Phylogenetic relationships, divergence times and leaf trait evolution. Journal of Sytematics and Evolution 56(1), 35–47. https://doi.org/10.1111/jse.12276

Kumar, C., Singh, S. K., Pramanick, K. K., Verma, M. K., Srivastav, M., Singh, R., Bharadwaj, C., Naga, K. C., 2018: Morphological and biochemical diversity among the *Malus* species including indigenous Himalayan wild apples. Scientia Horticulturae 233, 204–219. https://doi.org/10.1016/j.scienta.2018.01.037

Larsen, A. S., Jensen, M., Kler, E. D., 2008: Crossability between wild (*Malus sylvestris*) and cultivated (*M.* ? *domestica*) apples. Silvae Genetica 57(3), 127–130. https://doi.org/10.1515/sg-2008-0019

Manuela, D., Xu, M., 2020: Juvenile leaves or adult leaves: Determinants for vegetative phase change in flowering plants. International Journal of Molecular Science 21(24), 9753. https://doi.org/10.3390/ijms21249753

McNeill, J., Shaw, J. M. H., Wiersema, J. H., 2016: Proposal to preclude homonymy of generic names with names of intergeneric graft-hybrids (chimaeras). Taxon 65(5), 1198–1199. https://doi.org/10.12705/655.39

Nabavi, S. F., Habtemariam, S., Ahmed, T., Sureda, A., Daglia, M., Sobarzo-Sánchez, E., Nabavi, S. M., 2015: Polyphenolic composition of *Crataegus monogyna* Jacq.: From chemistry to medical applications. Nutrients 7(9), 7708–7728. https://doi.org/10.3390/nu7095361

Németh, C., Papp, N., Nosková, J., Höhn, M., 2020: Speciation by triparental hybridization in genus *Sorbus* (Rosaceae). Biologia Futura 71, 209–222. https://doi.org/10.1007/s42977-020-00003-x

Pasqualetto, G., Palmieri, L., Martens, S., Bus, V. G. M., Chagné, D., Wiedow, C., Malnoy, M. A., Gardiner, S. E., 2022: Molecular characterization of intergeneric hybrids between *Malus* and *Pyrus*. Horticulture Research 10(1), uhac239. https://doi.org/10.1093/hr/uhac239

Phipps, J. B., 2016: Studies in *Mespilus*, *Crataegus*, and ×*Crataemespilus* (Rosaceae), i. differentiation of *Mespilus* and *Crataegus*, Expansion of ×*Crataemespilus*, with supplementary observations on differences between the *Crataegus* and *Amelanchier* clades. Phytotaxa 257(3), 201–229. https://doi.org/10.11646/phytotaxa.257.3.1

Poljak, I., Šatović, Z., Vidaković, A., Tumpa, K., Idžojtić, M., 2024: Population variability of rosemary willow (*Salix eleagnos* Scop.) based on leaf morphometry: Evidence of small and large-leaf morphotypes. Šumarski List 148(5-6), 219–236. https://doi.org/10.31298/sl.148.5-6.1

Postman, J. D., 2011: Intergeneric hybrids in Pyrinae (=Maloideae) subtribe of Pyreae in family Rosaceae at USDA genebank. Acta Horticulturae 918, 937–943. https://doi.org/10.17660/ActaHortic.2011.918.123

R Core Team, 2016: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/. Retrieved June 15, 2024.

Rehder, A., 1949: Biobliography of cultivated trees and shrubs, hardy in the cooler temperate regions of the northern hemisphere. The Arnold arboretum of Harvard University, Jamaica Plain, Massachusetts.

Rieseberg, R. H., Carney, S. E., 1998: Tansely review No. 102 Plant hybridization. New Phytologist 140, 599–624.

Schuck, D. J., 2008: *Crataegus monogyna* Jacq. In: Roloff, A., Weisgerber, H., Lang, U., Stimm, B. (eds.), Enzyklopädie der Holzgewdchse, 1–12. Wiley-VCH, Weinheim.

Shimura, I., Ito, Y., Seike, K., 1983: Intergeneric hybrid between Japanese pear and quince. Journal of the Japanese Society for Horticultural Science 52(3), 243–249. https://doi.org/10.2503/jjshs.52.243

Sokal, R. R., Rohlf, F. J., 2012: Biometry: The principles and practice of statistics in biological research. W.H. Freeman and Co., New York.

Statistica (Data Analysis Software System), 2018: Statistica, ver. 13, TIBCO Software Inc., Palo Alto. http://www.statsoft.com.

Sun, J., Zhao, D., Qiao, P., Wang, Y., Wu, P., Wang, K., Guo, L., Huang, L., Zhou, S., 2024: Phylogeny of genera in Maleae (Rosaceae) based on chloroplast genome analysis. Frontiers in Plants Science, 15. https://doi.org/10.3389/fpls.2024.1367645

Van Tuyl, J. M., de Jeu, M. J., 1997: Methods for overcoming interspecific crossing barriers. In: Sawhney, V. K., Shivanna, K. R. (eds.), Pollen biotechnology for crop production and improvement, 273–292. Cambridge University Press, New York.

Vidaković, A., Liber, Z., Šatović, Z., Idžojtić, M., Volenec, I., Zegnal, I., Pintar, V., Radunić, M., Poljak, I., 2021: Phenotypic diversity of almond-leaved pear (*Pyrus spinosa* Forssk.) along Eastern Adriatic coast. Forests 12, 1630. https:// doi.org/10.3390/f12121630

Vidaković, A., Šatović, Z., Liber, Z., Radunić, M., Skendrović Babojelić, M., Poljak, I., 2024: Genetic diversity of *Pyrus pyraster* (L.) Burgsd. and *P. spinosa* Forssk.: evidence of introgression from cultivated into wild pear populations. Trees. https://doi.org/10.1007/s00468-024-02553-2

Vidaković, A., Šatović, Z., Tumpa, K., Idžojtić, M., Liber, Z., Pintar, V., Radunić, M., Runjić, T. N., Runjić, M., Rošin, J., Gaunt, D., Poljak, I., 2022: Phenotypic variation in European wild pear (*Pyrus pyraster* (L.) Burgsd.) populations in the North-Western part of the Balkan Peninsula. Plants 11(3), 335. https://doi.org/10.3390/plants11030335

WFO, 2024: World Flora Online. Retrieved June 15, 2024 from https://www.worldfloraonline.org/

WinFolia™, 2001: WinFolia ver. PRO 2005b. Regent Instruments Inc., Quebec City.

Ye, B.-B., Zhang, K., Wang, J.-W., 2019: The role of miR156 in rejuvenation in *Arabidopsis thaliana*. Journal of Integrative Plant Biology 62(5), 550–555. https://doi.org/10.1111/jipb.12855

Zamani, A., Attar, F., Maroofi, H., 2012: A synopsis of the genus *Pyrus* (Rosaceae) in Iran. Nordic Journal of Botany 30(3), 310–332. https://doi.org/10.1111/j.1756-1051.2011.00989.x

Zhang, S. D., Jin, J. J., Chen, S. Y., Chase, M. W., Soltis, D. E., Li, H. T., Yang, J. B., Li, D. Z., Yi, T. S., 2017: Diversification of Rosaceae since the Late Cretaceous based on plastid phylogenomics. New Phytologist 214(3), 1355–1367. https://doi.org/10.1111/nph.14461

Zhang, Z., Sun, Y., Li, Y., 2020: Plant rejuvenation: from phenotypes to mechanisms. Plant Cell Reports 39(10), 1249–1262. https://doi.org/10.1007/s00299-020-02577-1

Zotz, G., Wilhelm, K., Becker, A., 2011: Heteroblasty – A review. The Botanical Review 77, 109–151. https://doi.org/10.1007/s12229-010-9062-8

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# Distribution, ecology and variability of *Galanthus reginae-olgae* Orph. along the northern limit of its Balkan distribution (Croatia)

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**Abstract** – *Galanthus reginae-olgae* Orph. occurs in mainland Greece, the Ionian Islands, Corfu, Albania, Italy, Sicily, Montenegro, Bosnia and Herzegovina, and Croatia. This paper aims to extend the knowledge of the distribution and ecology of *G. reginae-olgae* in Croatia and the northwestern Balkans. A field study in 2023 revealed as many as 35 populations in southern Croatia, some of them counting up to 100,000 individuals. Most of the populations exhibit semi-sciophilic behaviour within transitional habitats (ecotones) from insolated grassland to shaded eu- and sub-Mediterranean thickets and forests, and are found in the altitudinal range from sea level to 664 m a.s.l., at an average distance of 5.44 km from the coastline. Analysis of the bioclimatic variables reveals that the populations studied find an optimum in Mediterranean conditions with a mean annual air temperature of 14.61 °C and mild winters. The annual precipitation is high, exceeding 1600 mm, although it is limited to the winter period. The analyses of the temperature data provided insight into the environmental conditions that might serve as triggers for the initiation and duration of anthesis. In view of the the observed leaf length and flowering time, the populations are designated *G. reginae-olgae* subsp. *vernalis* Kamari. Since the Croatian populations are sparsely scattered along the northern limit of the species' distribution on the Balkan Peninsula, future research should investigate the adaptability of this species to habitat and climate changes in the eastern Mediterranean region.

**Keywords**: bioclimatic variables, flowering period, *Galanthus reginae-olgae* subsp. *vernalis*, morphology, taxonomy, transitional habitats

## Introduction

The genus *Galanthus* L. (Amaryllidaceae) comprises 23 species of bulbous, petaloid monocots native to Europe, Asia Minor, and the Near East (Davis 1999, 2001, Zubov and Davis 2012, Tan et al. 2014, Zubov et al. 2019, Timukhin and Tuniyev 2022). Centres of species diversity are found in Greece and regions adjacent to the Balkans, Turkey, and the Caucasus. Species of this genus commonly occur in woodland and forests, on fertile, base-rich soils, on limestone or other calcareous substrates, at altitudes up to 2700 m (Davis 1999, 2001). They rank among the finest garden plants and have long been used for pharmaceutical purposes due to their content of bioactive compounds (e.g., alkaloid galanthamine) (Jovanović et al. 2016, 2018). As a result of illegal collection, habitat destruction and climate change, *Galanthus* is threatened in the wild; all species have been listed in Appendix II of CITES, and several of them are included in one of the threatened categories of the IUCN Red List (IUCN 2023).

Queen Olga's snowdrop (*G. reginae-olgae* Orph.) was first discovered in the Taigetos Mountains in southern Greece by the botanist T. G. Orphanides in 1876 (Boissier 1882). Closely related to the common snowdrop (*G. nivalis* L.), it was treated as its subspecies by many authors (e.g., Gottlieb-Tannenhain 1904, Stern 1956, Webb 1978). Both species have applanate vernation, narrow linear leaves and a single green mark at the apex of each inner perianth segment, but the leaves of *G. reginae-olgae* are typically absent at the onset of flowering, or only 1–3 cm long, with a conspicuous glaucous stripe running down the middle of the upper leaf surface. In addition, it is predominantly an autumn- to early winter-flowering species, whereas *G. nivalis* is characterized by its late winter- to early spring-flowering

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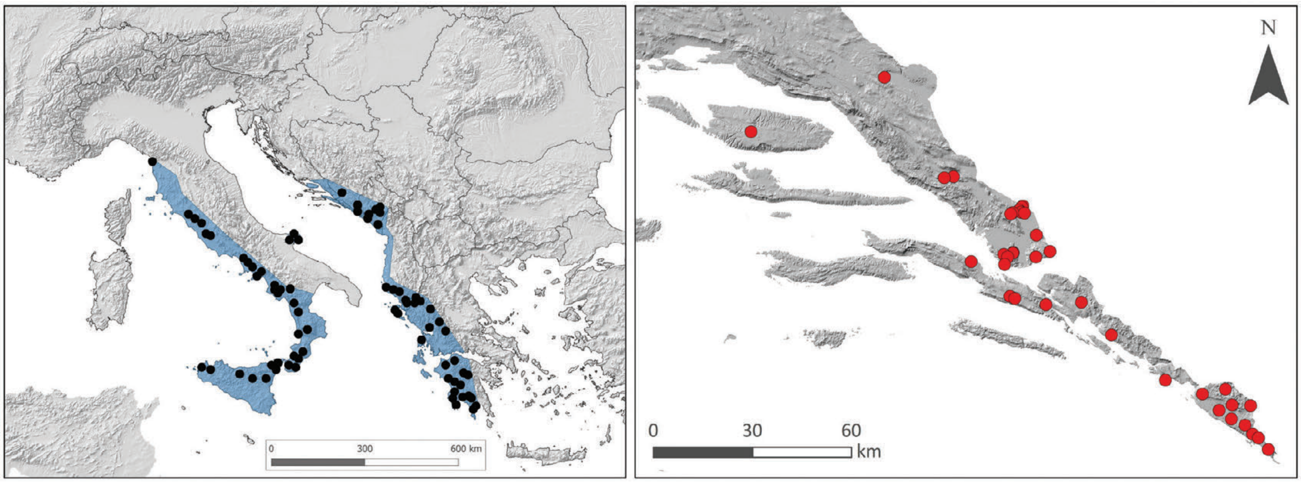

**Fig. 1.** The area of distribution of *Galanthus reginae-olgae* based on literature, herbaria, online databases and personal communications (left). Distribution of *G. reginae-olgae* in Croatia based on our reserch (right).

time (Davis 1999, 2001). To date, *G. reginae-olgae* has been found in mainland Greece (Delipawlow and Angeliew 1970, Davis 1999, 2001, Vladimirov et al. 2011), Corfu (Stern 1956, Delipawlow and Angeliew 1970, Davis 1999, 2001), the Ionian Islands (Trigas et al. 2021), Albania (Delipawlow and Angeliew 1970, Malo and Shuka 2008, Shuka et al. 2011, Mahmutaj et al. 2015, Barina, 2017), Montenegro (Davis 1999, 2001, Jovanović 2019), Bosnia and Herzegovina (Šilić 1996, Davis 2001, Tomović et al. 2023), Croatia (Davis 1999, 2001, Čičovački and Alegro 2013, Jovanović et al. 2013), mainland Italy (Davis 1999, 2001, Conti et al. 2005, Bartolucci et al. 2018, Rosati et al. 2020) and Sicily (Stern 1956, Gramuglio et al. 1978, Davis 1999, 2001, Conti et al. 2005, Di Marco et al. 2012, Bartolucci et al. 2018) (Fig. 1).

It is most common in or near woodlands, in damp, north-facing shady locations, among rocks or in narrow gorges, on moist, humus-rich and deep soils overlaying limestone, at a wide range of altitudes, but usually above 500 m (Davis 1999, 2001). Along with chorological and ecological research, this species has been the subject of numerous studies, including morphological and taxonomical (Kamari 1982, Davis 1999), karyological (Kamari 1981, Papanicolaou and Zacharof 1983), anatomical (Davis and Barnett 1997), phytochemical (Conforti et al. 2010), palynomorphological (Jovanović et al. 2022) and molecular investigations (Zonneveld et al. 2003, Lledó et al. 2004, Meerow et al. 2006, Larsen et al. 2010, Rønsted et al. 2013).

Based on flowering time and leaf length at the onset of flowering, *G. reginae-olgae* is divided into two subspecies. *Galanthus reginae-olgae* subsp. *reginae-olgae* flowers in autumn with leaves either absent or only 1–3 cm long, whereas *G. reginae-olgae* subsp. *vernalis* Kamari flowers in winter or early spring with leaves that are always present and usually several centimetres long (Davis 1999, 2001). However, as the degree of leaf development is not a fully reliable character (Shuka et al. 2011), and the flowering period varies greatly depending on the environmental conditions, the infraspecific differentiation of this species is considered difficult to ascertain (Trigas et al. 2021).

In Croatia, *G. reginae-olgae* was recorded by Davis (1999, 2001), then confirmed on Mt. Snježnica by Čičovački and Alegro (2013), and thus presented to the Croatian botanical community. In addition, several localities in southern Croatia (i.e., Konavle, Lokrum, Metković, Brač, and Donji Proložac) were recorded and published in the Flora Croatica on-line database (Nikolić et al. 2025). Due to the morphological similarities with *G. nivalis* and the challenging delimitation of its subspecies, the actual patterns of the diversity and distribution of *G. reginae-olgae* have remained uncertain. Hence, the present paper aims to extend the knowledge of the distribution and ecology of *G. reginae-olgae* in Croatia and the northwestern Balkans.

## Material and methods

Field inspection was conducted regularly once a month from November 2022 to March 2023, in order to detect snowdrop populations and to monitor their flowering period. An extended field survey, performed in January and February 2023, included population size assessment, habitat type record and estimation of canopy coverage. Population size was estimated by counting the number of individuals in smaller populations (up to 1,000 individuals). The same was implemented in larger populations, however using five smaller 10 × 10 m subplots (managed by five botanists) and performing extrapolation on entire population area. The habitat types were described using dominant tree or shrub species, while canopy coverage (%) was assessed visually.

To determine parameters that define the ecological preferences of *G. reginae-olgae* along its northern limit of Balkan distribution, 14 bioclimatic variables from CHELSA climatological datasets (vers. 2.1) at spatial resolution 1 × 1 km were used (Karger et al. 2017, 2021). The variables were selected according to relevant literature data for *Galanthus* (Artjushenko 1963, Davis 1999, 2001, Cox 2013). Additionally, for each population, the distance from the coastline, altitude and inclination were obtained from the datasets of the European Environmental Agency – EEA Coastline

(EEA 2020) and EU-DEM (vers. 1.0) (CLMS 2020). Basic descriptive statistics of the climatic and geographic parameters were calculated and shown in the standard box-plot diagrams using Past software (vers. 4.15) (Hammer et al. 2001).

The populations discovered were identified according to the observed flowering time and leaf development, using the keys proposed by Davis (1999, 2001). The average leaf length was assessed during peak flowering using the following categories: 0–3 cm, 3–6 cm, 6–9 cm, and 9–12 cm. In addition, the variation in flower morphology and markings, and other possibly informative taxonomic features, were inspected in each studied population.

## Results

A total of 35 populations of *G. reginae-olgae* were found or confirmed in southern Croatia (On-line Suppl. Tab. 1) over a range of more than 160 km (Fig. 1). The largest populations with an estimated number of up to 100,000 individuals were recorded in only six locations (Vidonje, Rosni Do North, Rosni Do South, Putnikovići, Mutni Do (Pelješac) and Kuna Konavoska), whereas 72% of the populations (25) had fewer than 10,000 individuals. Among the latter, almost half counted no more than 1,000 individuals (On-line Suppl. Tab. 1).

The populations of *G. reginae-olgae* in Croatia are mostly recorded in semi-shaded habitats (the average canopy coverage 60%; min 10%, max 100%) (Fig. 2). At higher altitudinal positions, this species was found growing in semi-shade of deciduous forests and thickets of downy oak (*Quercus pubescens* Willd.), oriental hornbeam (*Carpinus orientalis* Mill.) and European hop-hornbeam (*Ostrya carpinifolia* Scop.). At lower altitudes (and closer to the sea), it occurs in the vegetation of transitional thickets and low forests of evergreen oak (*Q. ilex* L.) and other eu-Mediterranean shrubs. Some parts of particular populations (e.g. Pločice, Osobjava (Pelješac), Komarna) also spread to cultivated areas (e.g., olive groves). Most of the recorded populations grow on deep soils with sporadic surface rocks.

The investigated populations were found in an altitudinal range from sea level up to 664 m a.s.l., with an average altitude of 203.27 ± 29.44 m a.s.l. (Fig. 3, On-line Suppl. Tab. 2). Most of the populations recorded in Croatia occurred on flat or slightly sloping terrain (5.86° ± 0.85°). The average distance from the coastline was 5.44 km ± 0.68 km (min 0.06 km, max 16.42 km) (Fig. 3, On-line Suppl. Tab. 2).

Based on bioclimatic data, Croatian habitats of *G. reginae-olgae* are climatologically characterised by typical Mediterranean conditions with a mean annual air temperature of 14.61 ± 0.18 °C, and mild winters with the mean daily minimum air temperature of the coldest month (bio 6) (2.31 ± 0.34 °C), of the coldest quarter (bio 11) (6.66 ± 0.21 °C) and of the wettest quarter (bio 8) (11.16 ± 0.20 °C) all above zero (Fig. 3, On-line Suppl. Tab. 2). According to the bioclimatic data, frost events in even the coldest month are very rare, occurring at only three population sites. The annual precipitation in the study area exceeds 1600 mm, most of the pre-

**Fig. 2.** Habitats of *Galanthus reginae-olgae* in Croatia: A – Mediterranean deciduous *Quercus pubescens* low forests and thickets (Kuna Konavoska, 663 m a.s.l.), B – Mediterranean evergreen *Q. ilex* low forests and thickets (Vratar North, 8 m a.s.l.), C – semi-shaded habitats on the edge of *Paliurus spina-christi* thickets (Majkovi, 391 m a.s.l.), D – semi-shaded habitats within cultivated land – olive groves (Pločice, 164 m a.s.l.).

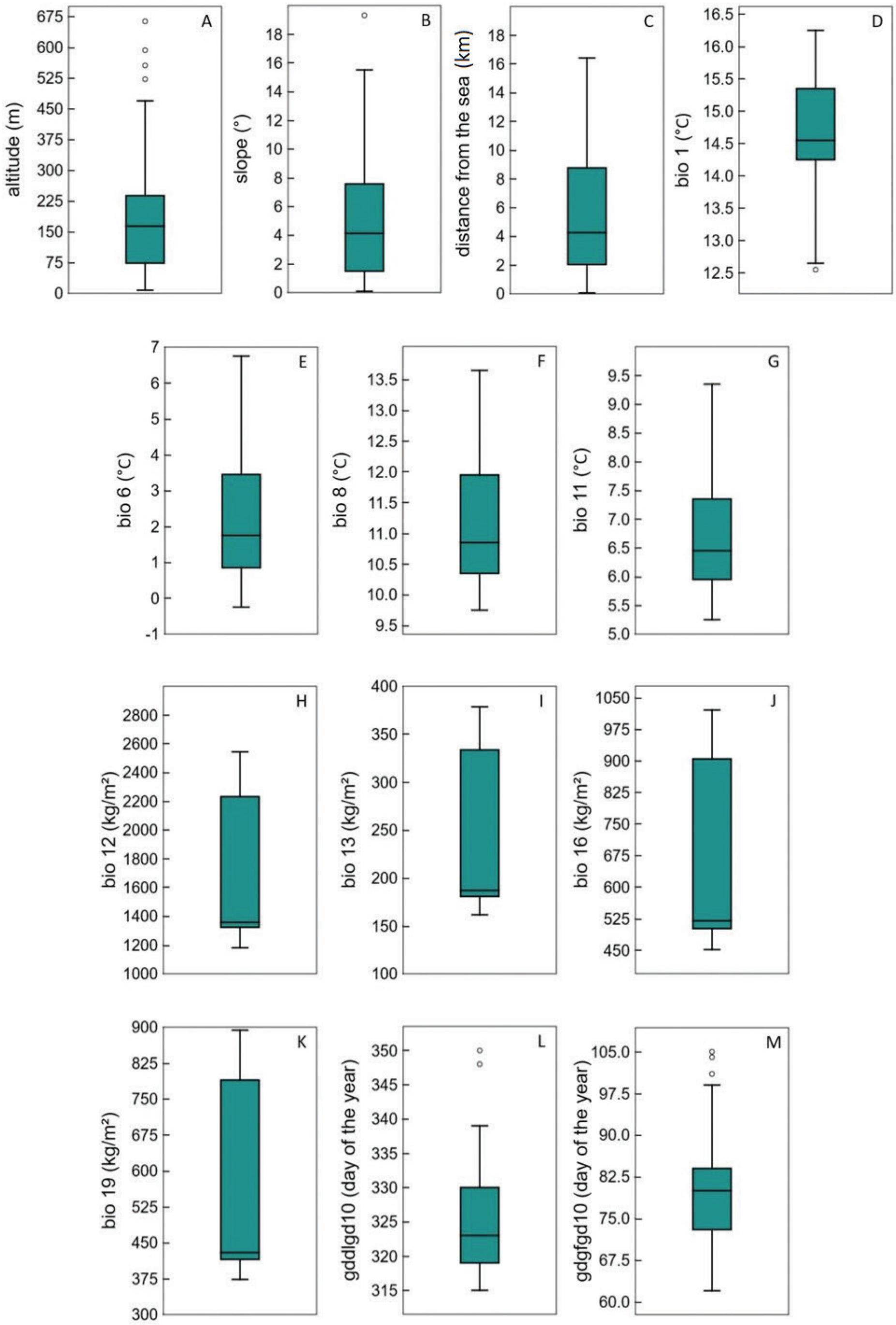

**Fig. 3.** The box-plot diagrams of geographical, terrain, temperature and precipitation parameters for populations of *Galanthus reginae-olgae* (N = 35) in Croatia. A – altitude (m), B – slope (°), C – distance from the sea (km), D – mean annual air temperature (Bio 1), E – mean daily minimum air temperature of the coldest month (bio 6), F – mean daily mean air temperatures of the wettest quarter (bio 8), G – mean daily mean air temperatures of the coldest quarter (bio 11), H – annual precipitation amount (bio 12), I – precipitation amount of the wettest month (bio 13), J – mean monthly precipitation amount of the wettest quarter (bio 16), K – mean monthly precipitation amount of the coldest quarter (bio 19), L – last day of the year above 10 °C (gddlgd10), M – first day of the year above 10 °C (gdgfgd10). The upper and lower box limits show upper and lower quartile values, while whiskers show minimum and maximum data values.

cipitation occurring only in the wettest and coldest quarter of the year (i.e., the winter period) (Fig. 3, On-line Suppl. Tab. 2).

The observation of the onset of anthesis revealed that some of the populations started to flower as early as the beginning of December, but the majority of them opened the flowers later on in December. On average, the flowering terminated by the end of February, whereas some populations at higher altitudes flowered until the first half of March. Occasional observations of several Croatian populations in the growing season of 2021/2022 showed that the onset of anthesis was ten days later than in the season of 2022/2023. Based on bioclimatic data, the mean first day of the year with a temperature below 10 °C (e.g. the last day of the year

**Fig. 4.** *Galanthus reginae-olgae* subsp. *vernalis* in Croatia: A – flower, B – pale median stripe on the adaxial leaf surface, C – leaves, D – individuals growing in a dense population.

above 10 °C – gddlgd10) is November 20, whereas the mean last day of the year with the temperature below 10 °C (e.g. first day of the year above 10 °C – gdgfgd10) is March 20 (Fig. 3, On-line Suppl. Tab. 2). When this period was restricted to temperatures beneath 5 °C (gddlgd5, gdgfgd5), a narrower temporal frame was obtained – from January 3 to February 9, based, however only on seven (20%) population sites in which the winter temperature drops beneath 5 °C. According to these data and field observations, the flowering period in the investigated populations of *G. reginae-olgae* begins and continues when winter temperatures are beneath 10 °C.

During the peak flowering in our study, the average leaf length within all populations exceeded 3 cm. Nearly two-thirds of populations (60%) had an average leaf length from 3 cm to 6 cm, while more than one-third of the populations had longer leaves (6-9 cm). Based on the observed leaf length and flowering time, the populations are designated *G. reginae-olgae* subsp. *vernalis* (Fig. 4), with the possible presence of intermediate forms between the two subspecies.

Besides typical flower morphology (Fig. 5A), variation in individual specimens was observed, including the following atypical variants (on different sites):

- variant with a single apical abaxial green mark on each inner perianth segment, covering c. 2/3 of the segment (Lisac) (Fig. 5B);
- variant with slender perianth segments of equal length and width, i.e., a poculiform perigone (all segments in one circle, cup-shaped) (Vidonje) (Fig. 5C);
- variant with broadly ovate perianth segments of equal length and width, a poculiform perigone with a diffuse abaxial green mark near the apex (up to 1/3 of the segments) (Vidonje) (Fig. 5D);
- variant with an abaxial green mark consisting of parallel lines at the apex of outer perianth segments (Rosni Do, and Vidonje) (Fig. 5E, F);
- variant with an abaxial green mark consisting of discontinuous surfaces at the apex of outer perianth segments (Vidonje) (Fig. 5G);
- variant with an abaxial green mark at the apex of each outer perianth segment, i.e., viridiapice markings (Vidonje) (Fig. 5H).

**Fig. 5.** Variation in flower morphology and markings of *Galanthus. reginae-olgae* subsp. *vernalis* in Croatia: A – typical flower, B – a single apical abaxial green mark on inner perianth segments, covering c. 2/3 of the segment, C – slender perianth segments of equal length and width (poculiform perigone), D – broadly ovate perianth segments of equal length and width, a poculiform perigone with a diffuse abaxial green mark near the apex (up to 1/3 of the segments), E, F – an abaxial green mark consisting of parallel lines at the apex of outer perianth segments, G – an abaxial green mark consisting of discontinuous surfaces at the apex of outer perianth segments, H – an abaxial green mark at the apex of outer perianth segments (viridiapice).

## Discussion

*Galanthus reginae-olgae* in Croatia is found in 35 populations, in a range of more than 160 km in the southern part of the country. Most of the recorded populations numbered fewer than 10.000 individuals. Considering that *G. reginae-olgae* was confirmed for Croatia in 2012 (Čičovački and Alegro 2013), the results represent a significant contribution to the insufficient knowledge of the distribution of this species in Croatia and along its northern distributional limit on the Balkan Peninsula (Davis 1999, 2001). Given that the investigated populations are mostly in semi-shaded habitats, it can be stated that this species exhibits semi-sciophilic behaviour in Croatia. At higher altitudes, it was found growing in the semi-shade of deciduous forests and thickets of *Quercus pubescens*, *Carpinus orientalis* and *Ostrya carpinifolia*, confirming Čičovački and Alegro (2013). At lower altitudes (closer to the sea), it occurs in the vegetation of transitional thickets and low forests of *Q. ilex* and other eu-Mediterranean shrubs. Most of the recorded populations grow on deep soils with sporadic surface rocks. These habitats are similar to those in Bosnia and Herzegovina (Tomović et al. 2023), and Albania (Malo and Shuka 2008, Shuka et al. 2011). According to Davis (1999, 2001), *G. reginae-olgae* is mostly found in or near woodlands of oriental plane (*Platanus orientalis* L.), pine (*Pinus* spp.) and Greek fir (*Abies cephalonica* Loudon), growing in damp, north-facing shady places, among rocks or in narrow gorges, on moist, humus-rich, and deep soils overlaying limestone. *G. reginae-olgae* subsp. *vernalis* occurs in similar habitats, including deciduous woodlands of European beech (*Fagus sylvatica* L.), oak (*Quercus* spp.), hornbeam (*Carpinus* spp.) and other species, and occasionally in coniferous woodland (*Abies* spp.). The populations in Croatia occur in the altitudinal range from sea level to 664 m a.s.l., with an average altitude of 203.27 m a.s.l. On Mt. Snježnica, the species occurs at altitudes from 650 m to 750 m a.s.l. (Čičovački and Alegro 2013). In Bosnia and Herzegovina, it was found at altitudes of 260–330 m a.s.l. (Tomović et al. 2023), and in Albania from 300 m to 1200 m a.s.l. (Malo and Shuka 2008, Shuka et al. 2011). In the overall area of distribution, *G. reginae-olgae* was recorded at an even wider range of altitudes, from near sea level to 1300 m a.s.l., but more commonly above 500 m a.s.l. (Davis 2001).

Considering the climatological characterization of Croatian habitats of *G. reginae-olgae* based on bioclimatic data, it is evident that this snowdrop prefers typical Mediterranean conditions, with a mean annual air temperature of 14.61 °C, and mild winters. Although the annual precipitation in the study area exceeds 1600 mm, seemingly unexpected for the Mediterranean region, the most of the rainfall comes in the winter period, providing favourable conditions for the growth and development of *G. reginae-olgae*. Depending on the altitudinal position, the populations in Croatia flower between early December and early March, but on average the flowering period begins in late December and terminates by the end of February, similar to the reports for other areas of the species' range (Davis 2001, Malo and Shuka 2008, Shuka et al. 2011, Vladimirov et al. 2011). According to literature, the flowering time of *Galanthus* species is genetically determined and influenced by air and soil temperature, and precipitation (Davis 1999). In addition, flower opening is not induced by light but by air temperature (Church 1908). The analysis of the temperature data could provide insights into the environmental circumstances that might serve as triggers for the onset and duration of anthesis. Based on temperature data and field observations in Croatia, and assuming that temperature is a key trigger for the onset of flowering in *Galanthus* (Davis 1999), it may be argued that the flowering period in the researched populations of *G. reginae-olgae* begins and continues when winter temperatures drop beneath 10 °C.

According to Davis (1999, 2001), *G. reginae-olgae* is divided into two subspecies based on flowering time and leaf length at the onset of flowering. Namely, *G. reginae-olgae* subsp. *reginae-olgae* is autumn-flowering (September to December) with leaves absent or 1–3 cm long, whereas *G. reginae-olgae* subsp. *vernalis* is characterized by winter- to spring-flowering time (December to March), and leaf length of 3–7 cm at the onset of flowering (Davis 1999, 2001). Based on the observed leaf length and flowering time, the populations in Croatia are designated *G. reginae-olgae* subsp. *vernalis*, but intermediate forms with subsp. *reginae-olgae* might also be present. The variability of these taxonomic characters, as stated by several authors (e.g., Shuka et al. 2011, Trigas et al. 2021, Tomović et al. 2023), suggests that the infraspecific division of *G. reginae-olgae* needs to be thoroughly reconsidered.

As previously shown, *G. reginae-olgae* in Croatia prefers semi-shaded habitats, inhabiting transitional habitats (ecotones) from insolated grassland to shaded thickets and forests. Being a eurivalent species in terms of light exposure, it is not particularly endangered, as are, for example, typical heliophilous species of grassland habitats. However, to maintain these transitional habitats, it is necessary to maintain both habitat types in which it occurs. Namely, it is important to conserve grasslands, preferably by traditional grazing, to prevent them from becoming overgrown. On the other hand, it is also important to preserve thickets and young forests, preventing them from being cleared and burned. Finally, although the species is well adapted to changes in both habitat types, sudden disturbances caused by logging, fire or grassland encroachment could have detrimental effects on populations. Although *G. reginae-olgae* subsp. *vernalis* is not protected under national legislation or by the EU Habitat Directive, it is strongly advised that a monitoring scheme for this taxon be developed and implemented. Considering that the Croatian populations are rare and scattered along the northern limit of the distribution on the Balkan Peninsula, the monitoring would provide insights into the adaptability of the species to habitat changes (fires and vegetation succession), as well as into the way the species adapts its phenology (proliferation, flowering, pollination, and fruiting) to the climate changes in the eastern Mediterranean region.

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## Author contribution statement

V.Š., F.J. – conceptualization and methodology; V.Š., F.J., B.I., M.D., M.B. – data collection and sampling; V.Š., A.R. – data analysis; V.Š., F.J. – manuscript preparation. All authors have read and agreed to the published version of the manuscript.

## References

Artjushenko, Z. T., 1963: Lukovichnie i klubnelukovichnie rastenia dla otkritogo grunta [Bulbous and corm plants for outdoor space]. Akademia Nauk SSSR, Botanicheski institut V. L. Komarova, Leningrad (in Russian).

Barina, Z. (ed.), 2017: Distribution atlas of vascular plants in Albania. Hungarian Natural History Museum, Budapest.

Bartolucci, F., Peruzzi, L., Galasso, G., Albano, A., Alessandrini, A., Ardenghi, N. M. G., Astuti, G., Bacchetta, G., Ballelli, S., Banfi, E., Barberis, G., Bernardo, L., Bouvet, D., Bovio, M., Cecchi, L., Di Pietro, R., Domina, G., Fascetti, S., Fenu, G., Festi, F., Foggi, B., Gallo, L., Gottschlich, G., Gubellini, L., Iamonico, D., Iberite, M., Jiménez-Mejías, P., Lattanzi, E., Marchetti, D., Martinetto, E., Masin, R. R., Medagli, P., Passalacqua, N. G., Peccenini, S., Pennesi, R., Pierini, B., Poldini, L., Prosser, F., Raimondo, F. M., Roma-Marzio, F., Rosati, L., Santangelo, A., Scoppola, A., Scortegagna, S., Selvaggi, A., Selvi, F., Soldano, A., Stinca, A., Wagensommer, R. P., Wilhalm, T., Conti, F., 2018: An updated checklist of the vascular flora native to Italy. Plant Biosystems 152(2), 179–303. https://doi.org/10.1080/11263504.2017.1419996

Boissier, E., 1882: Flora Orientalis. V. H. Georgh, Genevae et Basiliae.

Church, A. H., 1908: Types of Floral Mechanism: A Selection of Diagrams and Descriptions of Common Flowers I. Clarendon Press, Oxford.

Čičovački, J. Alegro, A., 2013: Visibaba – vijesnik zime(?) [Snowdrop – a harbinger of winter(?)]. Priroda 3(13), 42–44.

CLMS 2020: Copernicus Land Monitoring Service. EU-DEM v1.0. Retrieved September 30, 2020 from https://land.copernicus.eu/imagery-in-situ/eu-dem/eu-dem-v1–0-and-derived-products/eu-dem-v1.0

Conforti, F., Loizzo, M. R., Marrelli, M., Menichini, F., Statti, G. A., Uzunov, D., Menichini, F., 2010: Quantitative determination of Amaryllidaceae alkaloids from *Galanthus reginae-olgae* subsp. *vernalis* and *in vitro* activities relevant for neurodegenerative diseases. Pharmaceutical Biology 48(1), 2–9. https://doi.org/10.3109/13880200903029308

Conti, F., Abbate, G., Alessandrini, A., Blasi, C., 2005: An annotated checklist of the Italian vascular flora. Palombi, Rome.

Cox, F., 2013: A gardener's guide to snowdrops. The Crowood Press, Ramsbury.

Davis, A. P., 1999: The Genus *Galanthus*. Timber Press Inc., Portland.

Davis, A. P., 2001: The genus *Galanthus* – snowdrops in the wild. In: Bishop, M., Davis, A. P., Grimshaw, J. (eds.), Snowdrops: A monograph of cultivated *Galanthus*, 9–64. The Griffin Press, Maidenhead.

Davis, A. P., Barnett J. R., 1997: The leaf anatomy of the genus *Galanthus* L. (Amaryllidaceae J. St. Hil.). Botanical Journal of the Linnean Society123(3), 333–352. https://doi.org/10.1006/bojl.1996.0087

Delipawlow, D., Angeliew, W., 1970: Die Arten der Gattung *Galanthus* L. auf der Balkanhalbinsel. Archiv fur Gartenbau 18(8), 427–433.

Di Marco, F., Bernardo, L., Peruzzi, L., 2012: Contribution to the vascular flora of Papasidero (north-western Calabria, Italy). Atti della Società Toscana di Scienze Naturali, Memorie, Serie B 119, 33–50. https://doi.org/10.2424/ASTSN.M.2012.06

EEA 2020: European Environmental Agency. EEA coastline for analysis. Retrieved September 30, 2020 from https://www.eea.europa.eu/

Gottlieb-Tannenhain, P. von, 1904: Studien uber die Formen der Gattung *Galanthus*. Abhandlungen der Zoologisch-Botanischen Gesellschaft in Wien 2(4), 1–93.

Gramuglio, G., Rossitto, M., Arena, M., Villari, R., 1978: Nuova stazione di *Woodwardia radicans* (L.) SM. in Sicily. Il Naturalista Siciliano 2(3-4), 127–134.

Hammer, Ø., Harper, D. A. T., Ryan, P. D., 2001: PAST: Paleontological statistics software package for education and data analysis. Paleontologica Electronica 4(1), 1–9.

IUCN 2023: The IUCN Red list of threatened species. October 22, 2023 Retrieved on September 30, 2020 from https://www.iucnredlist.org/

Jovanović, F., 2019: Taksonomija i ekologija vrsta roda *Galanthus* L. (Amaryllidaceae) u Srbiji [Taxonomy and ecology of the genus *Galanthus* L. (Amaryllidaceae) in Serbia]. PhD thesis. Univesity of Belgrade – Faculty of Forestry, Belgrade (in Serbian).

Jovanović, F., Obratov-Petković, D., Bjedov, I., Mačukanović-Jocić, M., Braunović, S., Rakonjac, Lj., Nikolić, B., 2022: Pollen micromorphology of *Galanthus reginae-olgae* subsp. *vernalis* Kamari from the eastern Adriatic coast. In: Vuković, N., Šegota, V. (eds.), Book of Abstracts–7th Croatian Botanical Symposium with international participation, 46–47. Croatian Botanical Society, Zagreb.

Jovanović, F., Obratov-Petković, D., Bjedov, I., Živanović, I., Braunović, S., Ćirković-Mitrović, T., Tomović, G., 2018: Morphological variability of snowdrops in the central part of the Balkan Peninsula. HortScience 53(8), 1119–1124. https://doi.org/10.21273/HORTSCI13135-18

Jovanović, F., Obratov-Petković, D., Mačukanović-Jocić, M., 2013: Nectar production in *Galanthus reginae-olgae* subsp. *vernalis* Kamari from the Adriatic Sea coast. In: Alegro, A., Boršić, I. (eds.), Book of Abstracts–4th Croatian Botanical

Symposium with international participation, 213–213. Croatian Botanical Society, Zagreb.

Jovanović, F., Obratov-Petković, D., Niketić, M., Vukojičić, S., 2016: Distribution of the genus *Galanthus* L. (Amaryllidaceae) in Serbia. Botanica Serbica 40(1), 69–81. https://doi.org/10.5281/zenodo.48864

Kamari, G., 1981: A biosystematic study of the genus *Galanthus* L. in Greece II (Cytology). Botanika Chronika. Patras 1(2), 60–98.

Kamari, G., 1982: A biosystematic study of the genus *Galanthus* L. in Greece. Part I. Taxonomy. Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie 103(1), 107–135.

Karger, D. N., Conrad, O., Böhner, J., Kawohl, T., Kreft, H., Soria-Auza, R. W., Zimmermann, N. E., Linder, H. P., Kessler, M., 2017: Climatologies at high resolution for the earth's land surface areas. Scientific Data 4, 170122. https://doi.org/10.1038/sdata.2017.122

Karger, D. N., Conrad, O., Böhner, J., Kawohl, T., Kreft, H., Soria-Auza, R. W., Zimmermann, N. E., Linder, H. P., Kessler, M., 2021: Climatologies at high resolution for the earth's land surface areas. EnviDat. https://doi.org/ 10.16904/envidat.228.v2.1

Larsen, M. M., Adsersen, A., Davis, A. P., Lledó, M. D., Jäger, A. K., Rønsted, N., 2010: Using a phylogenetic approach to selection of target plants in drug discovery of acetylcholinesterase inhibiting alkaloids in Amaryllidaceae tribe Galantheae. Biochemical Systematics and Ecology 38(5), 1026–1034. https://doi.org/10.1016/j.bse.2010.10.005

Lledó, D. M., Davis, A. P., Crespo, M. B., Chase, M. W., Fay, M. F., 2004: Phylogenetic analysis of *Leucojum* and *Galanthus* (Amaryllidaceae) based on plastid matK and nuclear ribosomal spacer (ITS) DNA sequences and morphology. Plant Systematics and Evolution 246, 222–243. https://doi.org/10.1007/s00606-004-0152-0

Mahmutaj, E., Shuka, L., Xhulaj, M., Hoda, P., Mersinllari, M., 2015: Rare and endemic plants in the southern mountain ecosystems of Albania, their threats and diversity. Albanian Journal of Agricultural Sciences 14(1), 1–10.

Malo, S., Shuka, L. 2008: New records on the flora of the Gjirokastra region (South Albania). Natura Montenegrina, Podgorica 7(3), 369–373.

Meerow, A. W., Francisco-Ortega, J., Kuhn, D. N., Schnell, R. J., 2006: Phylogenetic relationships and biogeography within the Eurasian clade of Amaryllidaceae based on plastid ndhF and nrDNA ITS sequences: Lineage sorting in a reticulate area? Systematic Botany 31(1), 42–60. https://doi.org/10.1600/036364406775971787

Nikolić T., Bogdanović, S., Vuković, N., Šegota, V. (eds) 2025: Flora Croatica Database. Department of Botany, Faculty of Science, University of Zagreb. Retrieved January 30, 2025 from http://hirc.botanic.hr/fcd/

Papanicolaou, K., Zacharof, E., 1983: Cytological notes and taxonomic comments on four *Galanthus* L. taxa from Greece. Israel Journal of Botany 32(1), 23–32.

Rønsted, N., Zubov, D., Bruun-Lund, S., Davis, A. P., 2013: Snowdrops falling slowly into place: An improved phylogeny for *Galanthus* (Amaryllidaceae). Molecular Phylogenetics and Evolution 69(1), 205–217. https://doi.org/10.1016/j.ympev.2013.05.019

Rosati, L., Fascetti, S., Romano, V. A., Potenza, G., Lapenna, M. R., Capano, A., Nicoletti, P., Farris, E., Lange, P. J. de, Del Vico, E., Facioni, L., Fanfarillo, E., Lattanzi, E., Cano-Ortiz, A., Marignani, M., Fogu, M. C., Bazzato, E., Lallai, E., Astrid Laface, V. L., Musarella, C. M., Spampinato, G., Mei, G., Misano, G., Salerno, G., Esposito, A., Stinca, A. (2020). New chorological data for the Italian vascular flora. Diversity 12(1), 22. https://doi.org/10.3390/d12010022

Shuka, L., Malo, S., Tan, K., 2011: New chorological data and floristic notes for Albania. Botanica Serbica 35(2), 157–162.

Šilić, Č., 1996: Spisak biljnih vrsta (Pteridophyta i Spermatophyta) za Crvenu knjigu flore Bosne i Hercegovine [List of botanical species (Pteridophyta and Spermatophyta) for the Red book of Bosnia and Herzegovina]. Glasnik Zemaljskog muzeja Bosne i Hercegovine u Sarajevu 31, 323–367 (in Bosnian).

Stern, F. C., 1956: Snowdrops and snowflakes. The Royal Horticultural Society, London.

Tan, K., Biel, B., Siljak-Yakovlev, S., 2014: *Galanthus samothracicus* (Amaryllidaceae) from the island of Samothraki, northeastern Greece. Phytologia Balcanica 20(1), 65–70.

Timukhin, I. N., Tuniyev, B. S., 2022: *Galanthus lebedevae* (Amaryllidaceae) – A new species from Sochi. Turczaninowia 25(4), 58–68. https://doi.org/10.14258/turczaninowia.25.4.9

Tomović, G., Sabovljević, M. S., Assyov, B., Kutnar, L., Boycheva, P., Ivanov, D., Papp, B., Pantović, J., Sabovljević, A. D., Šabanović, E., Jovanović, F., Šovran, S., Knežević, A., Aleksić, G. R., Niketić, M., Shivarov, V. V. , Yaneva, G., Ştefănuţ, S., Bîrsan, C. C., Szeląg, Z., Djordjević, V., Kabaš, E., Dudáš, M., Kolarčik, V., 2023: New records and noteworthy data of plants, algae and fungi in SE Europe and adjacent regions, 12. Botanica Sebica 47(1), 173–182. https://doi.org/10.2298/BOTSERB2301173T

Trigas, P., Kalpoutzakis, E., Kalogiannis, E., Valli, A.-T., Kougioumoutzis, K., Katopodis, K., Constantinidis, T., 2021: Noteworthy new floristic records from Greece. Botanica Serbica 45(2), 321–331. https://doi.org/10.2298/BOTSERB2102321T

Vladimirov, V., Dane, F., Matevski, V., Stevanović, V., Tan, K., 2011: New floristic records in the Balkans: 15. Phytologia Balcanica 17(1), 129–156.

Webb, D. A., 1978: The European species of *Galanthus*. In: Heywood V. H. (ed.), Flora Europaea: Notulae Systematicae ad Floram Europaeam spectantes: No. 20. Botanical Journal of the Linnean Society 76(4), 307–313. https://doi.org/10.1111/j.1095-8339.1978.tb01817.x

Zonneveld, B. J. M., Grimshaw, J. M., Davis, A. P., 2003: The systematic value of nuclear DNA content in *Galanthus*. Plant Systematics and Evolution 241, 89–102. https://doi.org/10.1007/s00606-003-0016-z

Zubov, D. A., Davis, A. P., 2012: *Galanthus panjutinii* sp. nov.: A new name for an invalidly published species of *Galanthus* (Amaryllidaceae) from the Northern Colchis area of Western Transcaucasia. Phytotaxa 50(1), 55–63.
https://doi.org/10.11646/phytotaxa.50.1.5

Zubov, D. A., Konca, Y., Davis, A. P., 2019: *Galanthus bursanus* (Amaryllidaceae): A new species of snowdrop from the Marmara Sea region, NW Turkey. Kew bulletin 74(2), 18. https://doi.org/ 10.1007/s12225-019-9806-5

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# Retrodunal dry grassland vegetation in the hinterland of Velika Plaža (Montenegro)

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**Abstract** – The article presents the results of a study on retrodunal dry grassland communities in the hinterland of Velika Plaža (town of Ulcinj, Montenegro). They are documented with 47 relevés, which were compared with 177 dry grassland and 107 sand dune vegetation plots. Using hierarchical cluster analysis (flexible Beta method), and ordination (non-metric multidimensional scaling), the dry grasslands from the hinterland of Velika Plaža were grouped into three clusters, representing three new associations: *Junco bufonii-Vulpietum ligusticae*, *Tuberario guttatae-Avellinietum festucoidis*, *Helinathemo jonii-Artemisietum campestris.* The first association develops at the transition from wet grassland on gleyic soil to xerophilous grassland over sands. It is characterized by relatively high biomass production and is thus regularly mowed. The second association develops on somewhat higher and better drained sands, and due to very low biomass production, it is used exclusively as pasture. The *Helinathemo jonii-Artemisietum campestris* is also a pasture, evolved as a result of progressive natural succession of dune sands on the highest part of the dune system. It is represented by two sub-associations, one of which is characterized by the extremely high coverage of *Asphodelus ramosus*. Stands with *A. ramosus* primarily grow in the form of narrow strips along roads and around fences, sheltered from the wind, and on more developed and humus-rich soils. This suggests that *Asphodeletosum ramosi* present the terminal stage of the dune community *Helianthemo jonii-Artemisietum campestris*. The latter association is classified within the *Crucianellion maritimae* (Ammophiletea arundinaceae), whereas the other two belong to the *Vulpio-Lotion* (*Helianthemetea guttati*).

**Keywords:** dry grasslands, *Crucianellion maritimae*, *Helinathemo jonii-Artemisietum campestris*, *Junco bufonii-Vulpietum ligusticae*, retrodunal grasslands, syntaxonomy, *Tuberario guttatae-Avellinietum festucoidis*, *Vulpio-Lotion*

## Introduction

In the last decade, when the mapping of NATURA 2000 habitats in Montenegro began, the research on vegetation was intensified, especially regarding rare and endangered communities. The wider area of Ulcinj, specifically the Velika Plaža dunes, stands out in terms of the number of vegetation relevés in the coastal region (Stanišić-Vujačić et al. 2023). This is the largest sandy beach on the eastern Adriatic and though it is a very popular tourist destination it still has well-developed dune vegetation (Šilc et al. 2016, Stešević et al. 2020, Milanović et al. 2021). The main focus of vegetation research has been on the narrow coastal zone, embryonic dunes, and shifting sand dunes (Mijović 1994, 2006, 2012, Stešević et al. 2020). Part of the area was declared a natural monument in 1968 (Official Gazette of the Republic of Montenegro 30/1968). However, the accompanying documentation did not specify the boundaries of the protected area, so a review was later proposed (CGIS 2017). According to the IUCN categorization of protected areas, Velika Plaža belongs to category III (CGIS 2017).

Recent new findings of rare, endangered and important plant species in the hinterland of Velika Plaža (e.g. *Isoetex histrix*, *I. gymnocarpa*, *Solenopsis aurentia*, *Avellinia festucoides*,

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*Corynephorus divaricatus*, Stešević et al. 2025) and its habitats (Mediterranean temporary ponds, *Brachypodietalia* dune grasslands with annual plants) indicate its biological value and provide an argument for extending the boundaries of the protected area deeper inland. In the hinterland, dry and wet grasslands alternate with wet forests, shrubs, and bodies of water, forming a unique mosaic (Šilc et al. 2020, Milanović et al. 2021). In terms of species diversity, Mediterranean dry grasslands are particularly notable (Apostolova et al. 2014) and are listed in the European Union Council Directive 92/43/EEC, as well as on the European Red List of Habitats (Jansen et al. 2016). These are important from the perspectives of synecology, distribution, and classification, as the delimitation of some dry grassland syntaxa is not always clear. Certain plant communities show intermediate characteristics between two syntaxa, such as *Vulpio-Lotion* and *Psammo-Vulpion* (Mucina et al. 2016). The literature on dry grassland communities in the coastal part of Montenegro lists the following associations: *Holoschoeno-Scabiosetum albae* Hodak 1974, *Laguro-Corynephoretum divaricatae* Horvatić 1974, *Lupino-Laguretum ovati* Lakušić 1965 (Blečić and Lakušić 1976), *Onobrychido caput-galli-Vulpietum fasciculatae* Stešević et al. 2020 (Stešević et al. 2020) and *Artemisietum campestris* prov. (Milanović et al. 2021). The first three associations, which are not documented with relevés, were originally classified in the *Vulpio-Lotion* alliance; the fourth in *Laguro ovati-Vulpion fasciculatae*, while the fifth association is considered a vegetation equivalent of the NATURA 2000 habitat type 2240 *Brachypoditealia* dune grasslands.

It is a major challenge for phytosociologists to understand the regularity of occurrence and spatial variation of plant communities that have evolved over more or less stabilized dune sands, their syntaxonomic position and ecological characterization. Addressing this challenge requires sufficient information on numerous natural phenomena: origin and age of the sand, pH reaction, depth and moisture of the sand, humus and clay content in the upper soil layers where most plants develop their roots, degree of sand stabilization (especially in the upper soil layers), exceptional seasonal dynamics with pronounced change of phenophases, pathways of natural succession and regression, impact of different human activities on the floristic composition of communities, etc. (Marcenò et al. 2018). This is particularly true for sandy deposits, which are well drained and rich in organic matter and therefore unsuitable for many plant species. They are often only colonized by a small number of highly specialized taxa that have adapted to be able to thrive in these unfavorable natural conditions (Iserman 2011). In these environments, the appearance of only one new plant species on the surface may reflect important evolutionary processes in the soil and within plant communities. Such changes may require a revision of the syntaxonomic position of the community within higher classification units.

Although this phenomenon is clearly recognizable ecologically and explainable in the field, it often presents challenges for objective classification based on statistical parameters. Moreover, it is often the case that many species considered diagnostic of some more widespread alliances and higher vegetation units that have developed over sands do not occur in certain geographical localities. This ultimately drives the need to classify the studied plant associations into appropriate syntaxonomic schemes based more on ecology and physiognomy than on floristic composition (Pignatti et al. 1995, Mucina et al. 2016, Marcenò et al. 2018). This problem is further exacerbated by the frequent and diverse influences of humans, who have used sand for various purposes since the earliest epochs (EEA 1999).

In this paper, we present 3 new dry grassland associations from the hinterland of Velika Plaža and describe them in detail. We discuss different approaches to the classification of Mediterranean dry grassland communities and propose a syntaxonomic classification.

## Material and methods

The wider area of the hinterland of Velika Ulcinjska Plaža is an alluvial plain in a flysch zone that extends in a WNW-SSE direction from the mouth of the Port Milena channel to the Bojana River and its mouth into the Adriatic Sea (Fig. 1).

Immediately behind the sea coast, up to 3.5 m high drifting sand deposits, known as Breg mora, have formed due to the constant impact of sea waves and wind. Various dry dune communities on the mild southern slopes and wetlands in dune slacks, sporadically overgrown with scattered willow trees or well-developed floodplain riparian ash and oak forests, develop in this belt just after the open dunes. After this swamp area, on the highest part of the dunes, various retrodunal grasslands emerge. These areas are protected from the direct influence of the winds from the sea, and the dune sands are almost fixed with closed communities, though they still experience some shifting sand, mainly due to direct or indirect human impact. Often, they are fenced, used for grazing and mowing, or partly converted into agricultural land. Behind this zone there is a depression running parallel to the seashore with a bottom about 2.2 meters above sea level, containing fragmented remnants of floodplain riparian ash and oak forests. Alongside these forests, wet grasslands have developed in a narrower or wider zone, gradually transitioning into more xerophytic grasslands as the terrain rises, eventually forming a sand deposit up to 5.5 meters high called Štojska Greda. On its embankment, the main road to Ada Bojana was built. Between the settlements in Gornji Štoj and the road, the terrain is almost flat, yet interspersed with shallow depressions and gentle, barely perceptible rises where grassy vegetation develops, ranging from temporary ponds to low therophyte meadows. In the far east, along the course of the Bojana River, the sandy clayey soils rise again (sometimes with deep loam and peat), and extensive forest complexes develop on them, although these have been cleared in places and converted into hay meadows.

The phytosociological relevés were made according to the Braun-Blanquet (1964) approach, using plots of 25 m$^2$, a plot size commonly employed for sampling grassland communities (Chytrý and Otýpková 2003). The plots were

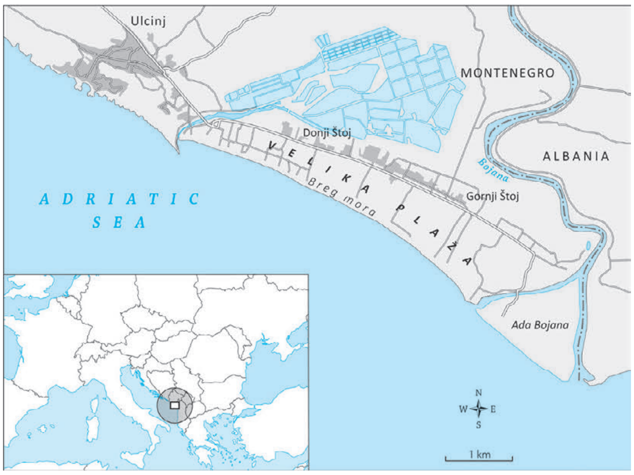

**Fig. 1.** Geographic position of Velika Plaža in Montenegro. The circle on the map in the lower left corner indicates the research area in the SE European context.

located in the retrodunal sand deposits, within stands displaying various physiognomies of dry grasslands in the hinterland of Velika Plaža. The plant material was deposited in the Herbarium collection of the University of Montenegro (TGU) and the Herbarium of the Faculty of Forestry of the University of Banja Luka (BALU). All relevés were entered into the Turboveg database (Hennekens and Schaminée 2001). The original relevés are stored in the Vegetation database of Montenegro (GIVD EU-ME-001, Stanišić-Vujačić et al. 2023).

The entire data collection consisted of 331 relevés: 47 original ones (sandy soils), 177 relevés of dry grassland associations in Montenegro and Croatia (on different substrata), and 107 to the sand dune vegetation on Velika Plaža (On-line Suppl. Tab. 1).

Species cover was estimated using the seven-degree cover-abundance scale of Braun-Blanquet (1964) and converted to the percentage midpoints of the estimated cover classes (r – 0.1%, + – 2%, 1 – 3%, 2 – 13%, 3 – 38%, 4 – 63%, 5 – 88%). The hierarchical cluster analysis was performed on a dataset comprising 224 relevés (47 original ones and 177 from the literature or unpublished of dry grassland communities (different substrata) from Montengro and Croatia), by PC-ORD 4 (McCune and Mefford 1999) incorporated in JUICE 7.0 software package (Tichý 2002). Relative Sørensen was used as distance measure (McCune and Grace 2002) and the flexible Beta (–0.25) as a linkage method. The species cover values were square-root transformed. Diagnostic species were identified according to their fidelity values (Chytrý et al. 2002). All groups were standardized to equal sizes (Tichý and Chytrý 2006), and Fisher's exact test ($P < 0.01$) was applied. To identify constant and dominant species, a frequency threshold of 50% and a coverage threshold of 25% were applied, respectively.

Diagnostic, constant, and dominant species of the retrodunal communities from Velika Plaža were identified on the smaller dataset (47 relevés), while diagnostic species of the major Clusters (I-V) of dry grassland communities (different substrata) from Montenegro and Croatia and dry retrodunal communities (on sandy soil) from Velika Plaža were identified on the large dataset (224 relevés).

A three-letter code (e.g., BUL = *Poetea bulbosae* Rivas Goday et Rivas-Mart. in Rivas-Mart. 1978) in brackets after a taxon name indicates the vegetation class of which the taxon is a diagnostic species, as reported in the FloraVeg.EU database (https://floraveg.eu/, Chytrý et al. 2024).

NMDS ordination analyses were conducted using the R 4.3.2 programming environment (R Core Team 2023), using the *vegan* package on different datasets: (i) 224 relevés (47 original ones and 182 from the literature or unpublished), (ii) 154 relevés of dune and retrodune dry grassland vegetation. The ecological indicator values of the relevés (Pignatti 2005) were calculated in JUICE (Tichý 2002) and passively projected onto a non-metric multidimensional scaling (NMDS) diagram (using Bray-Curtis distance measures) to illustrate the ecological relationships between the relevés in these three datasets and to explain the environmental gradients underlying the main ordination axes. Percentage cover data were transformed using the Hellinger transformation.

Life forms according to Raunkiaer (Pignatti 1982) were obtained for each species, and the proportion of particular life forms was calculated for each relevé. The nomenclature of taxa follows POWO (2024). Syntaxonomical concepts and nomenclature of higher syntaxa follow the Database of European Vegetation (Chytrý et al. 2024).

## Results

In the hierarchical cluster analyses, performed on the dataset of 224 relevés the dry grassland communities (on different substrata) from Montenegro and Croatia and retrodunal grasslands from Velika Plaža were differentiated into five main clusters (I-V) and 16 subclusters (1-16), which corresponds to 13 associations (Fig. 2A).

Cluster I is characterized by perennials *Chrysopogon gryllus, Centaurea deusta, Eryngium amethystinum* and includes 2 associations: *Chrysopogoni-Airetum capillaris* (MNE), and *Bromo-Chrysopogonetum grylli* (MNE); Cluster II by annuals *Bromus hordeaceus*, *Bunias erucago*, *Vulpia ligustica*, *Trifolium nigrescens*, and three plant communites: *Dasypyrum villosum* comm. (MNE), *Bunio erucagi-Vulpietum ligusticae* (MNE), and *Junco bufonii-Vulpietum ligusticae* ass. nova (Velika Plaža hinterland, MNE); Cluster III by psammophytes *Onobrychis caput-galli*, *Helianthemum jonium*, *Lagurus ovatus*, *Artemisia campestris*, *Hedypnois rhagadioloides*, and *Helianthemo jonii-Artemisietum campestris* ass. nova from the Velika Plaža hinterland, MNE; Cluster IV by perennials *Bromus erectus*, *Phleum bertolonii*, and *Helichrysum italicum*, and four associations: *Chrysopogoni-Airetum* (HRV), *Psiluro-Trifolietum* (HRV), *Agrostietum maritimae arenosum* (HRV), *Haynaldio-Phleetum* (HRV), Cluster V by *Trifolium stellatum*, *Ornithopus compressus*, *Anthyllis circinnata* and three associations: *Ornithopodo-Vulpietum* (HRV), *Trifolio-Brachypodietum rupestris* (HRV), and *Gastridio-Brachypodietum retusii* (HRV).

NMDS ordination of the relevés shows that along the first axis, clusters are divided into two big groups that cor-

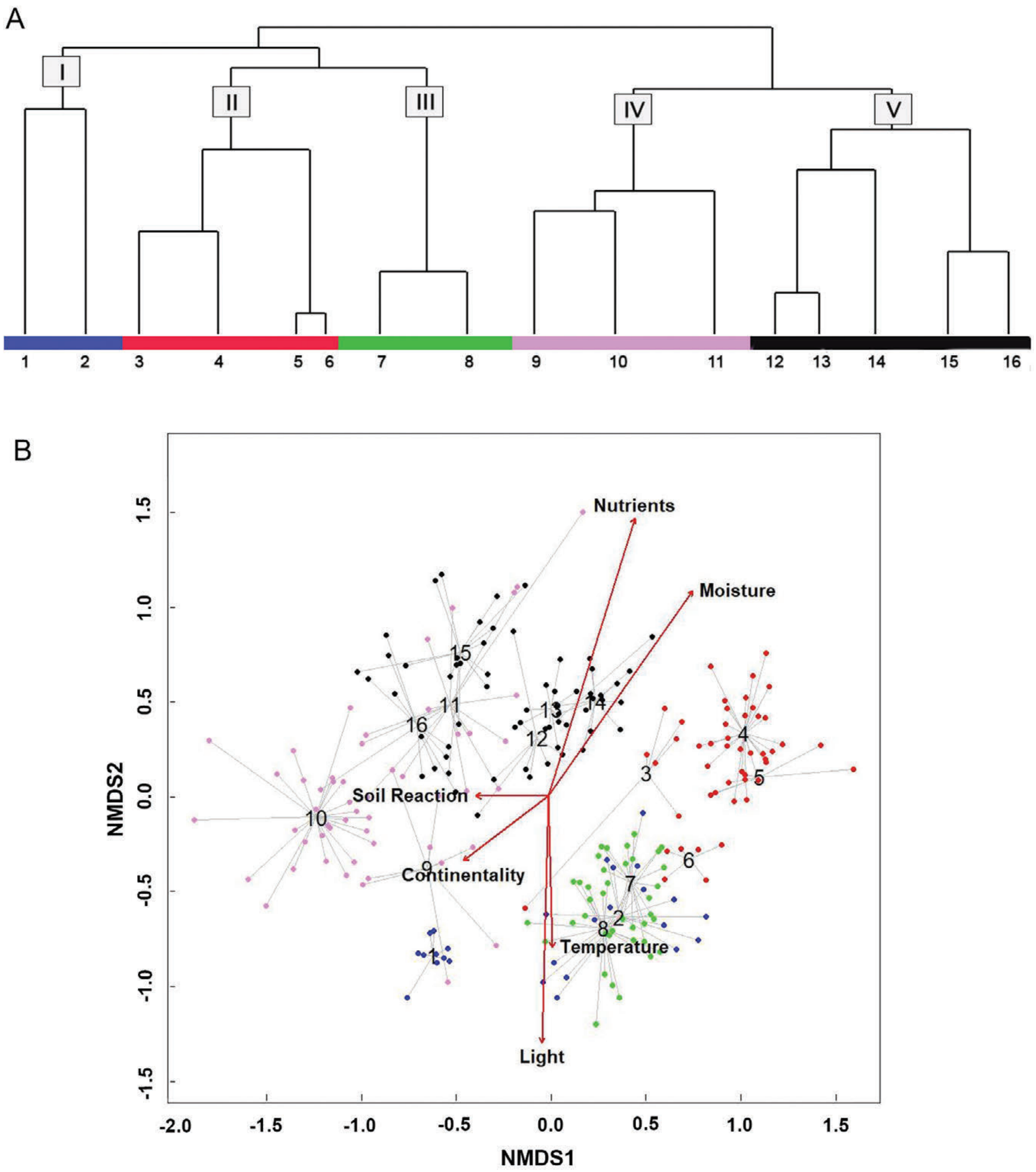

**Fig. 2.** Hierarchical cluster analysis (A), and ordination analysis: NMDS (B) of the dataset with 224 relevés of dry grassland communities (different substrata) from Montenegro and Croatia and retrodunal communities (on sandy soil) from Velika Plaža; Cluster I (blue): 1 – *Chrysopogoni-Airetum capillaris* (MNE), 2 – *Bromo-Chrysopogonetum grylli* (MNE), Cluster II (red): 3 – *Dasypyrum villosum* comm. (MNE), 4 – *Bunio erucagi-Vulpietum ligusticae* (MNE), 5 – *Junco bufonii-Vulpietum ligusticae* (Velika Plaža hinterland, MNE), 6 – *Tuberario guttatae-Avellinetum festucoidis* (Velika Plaža hinterland, MNE), Cluster III (green): 7 – *Helianthemo jonii-Artemisietum campestris asphodeletosum ramosi* (Velika Plaža hinterland, MNE), 8 – *Helianthemo jonii-Artemisietum campestris typicum* (Velika Plaža hinterland, MNE), Cluster IV (pink): 9 – *Chrysopogoni-Airetum* (HRV), 10 – *Psiluro-Trifolietum* (HRV), 11 – *Agrostietum maritimae arenosum* and *Haynaldio-Phleetum* (HRV), Cluster V (black): 12, 13, 14 – *Ornithopodo-Vulpietum* (HRV), 15 – *Trifolio-Brachypodietum rupestris* (HRV), 16 – *Gastridio-Brachypodietum retusii* (HRV). Stress value: 0.23.

respond to the classification in Fig. 2A and the first axis is correlated with soil reaction. The second axis strongly correlates with nutrients, moisture, light, and temperature (Fig. 2B). The retrodunal communities of Cluster II are placed in the right part of the diagram, as more nitrophilous and moist, while on the left are communities of Clusters IV and V, which grow on nutrient poorer and less humid substrates. Communities of Clusters I and III are discriminated on the gradients of light and temperature.

NMDS ordination analysis was performed on the 154 relevés of sand dune communities and retrodunal dry communities documented at Velika Plaža (On-line Suppl. Fig. 1). The first axis is also correlated with soil reaction, and the second with light, continentality, moisture and temperature. Retrodunal communities are discriminated along the gradient of continentality, and placed in the right of the diagram (On-line Suppl. Fig. 1, clusters 7-10). Retrodunal grasslands from the hinterland of Velika Plaža were grouped into Clusters II and III, subclusters 5-8 (Fig. 2A), Clusters 5-8 (Fig. 2B), and Clusters 7-10 (On-line Suppl. Fig. 1) which correspond to the newly reported associations: therophyte-dominated grasslands *Junco bufonii-Vulpietum* *ligusticae* ass. nova and *Tuberario guttatae-Avellinietum festucoidis* ass. nova, and perennial grasslands *Helianthemo jonii- -Artemisietum campestris* ass. nova (On-line Suppl. Fig. 2).

***Junco bufonii-Vulpietum ligusticae*** ass. nova
(relevés 1-7 in Tab. 1, holotypus: relevé 6, hinterland of Velika Plaža, subcluster 5 in Fig. 2A; On-line Suppl. Fig. 3A)

**Diagnostic species:** *Vulpia ligustica*, *Plantago lanceolata*, *Lotus corniculatus*, *Juncus bufonius*, *Anthoxanthum odoratum*, *Ranunculus marginatus*

**Constant species:** *Vulpia ligustica*

**Dominant species:** *Vulpia ligustica*, *Vulpia ciliata*

**Ecology, distribution and use:** This association represents the hygro-xerophilous type of grassland, whose stands occur under extremely hygrophilous conditions in winter and spring, and extremely xerophilous conditions in summer (Fig. 2B, cluster 5; On-line Suppl. Fig. 1, cluster 7). This type of vegetation thrives on flat terrain, covering nearly 100% of the soil and reaching a height of approximately 30 cm. Due to the relatively high biomass production, these are the only xerophilous retrodunal grasslands in the Velika Plaža hinterland that are regularly mowed. They usually develop at the transition from wet grassland on gley soil (*Alopecuro-Ranunculetum marginati* Zeidler 1954) to xerophilous grasslands on sandy soils, in the zone of primary distribution of *Quercus robur*. The soils in these areas have a higher clay and humus content due to the persistent moisture resulting from the influence of groundwater or a high water table in spring. As a result, the soils dry out more slowly, allowing a dense vegetation cover to develop, with a combination of mesohygrophilous and xerophilous plants. It is optimally developed in May and is characterized by a very high cover and the nearly absolute dominance of the annual grass *Vulpia ligustica* (TUB).

Anthropogenic floristic elements also have absolute or high frequency in this association, including *Trifolium nigrescens* (BUL, CHE), *Anthemis arvensis* (PAR), *Avena barbata* (CHE), *Bromus hordeaceus* (SIS). Species of *Stipo-Trachynietea distachyae* and *Helianthemetea guttati* are also present, such as *Hypochaeris glabra*, *Vulpia myuros*, *Trifolium campestre*, *Cerastium pumilum* var. *glutinosum*, and *Petrorhagia dubia*. Due to its transitional nature, many species of hygrophilous grasslands (mainly of *Alopecuro-Ranunculetum marginati*) are also present, including *Alopecurus rendlei*, *Ranunculus marginatus*, *Juncus bufonius*, *Linum bienne*, *Trifolium subterraneum*, and *Briza minor*. Therophytes dominate the biological spectrum (On-line Suppl. Fig. 2).

Recently, the spatial continuity of these grasslands has been interrupted by the conversion of land into construction sites or arable land.

In the study area, this spring and early summer ephemeral association forms narrow or wider strips along wet grasslands in the central depression of the hinterland, and in favorable spots on slight elevations on the terrace of the Bojana River. According to current knowledge, the distribution of this association is limited to the hinterland of Velika Plaža in Ulcinj (On-line Suppl. Fig. 4). However, given the ecological similarity with Velipoja in Albania, it is possible that *Junco bufonii-Vulpietum ligusticae* also occurs there.

***Tuberario guttatae-Avellinietum festucoidis* ass. nova**
(relevés 8-13 in Tab. 1, holotypus: relevé 12, hinterland of Velika Plaža, subcluster 6 in Fig. 2A, On-line Suppl. Fig. 3B)

**Diagnostic species:** *Tuberaria guttata*, *Lotus angustissimus*, *Filago gallica*, *Aira elegans*, *Trifolium nigrescens*, *Avellinia festucoides*, *Crepis neglecta*

**Constant species:** *Sherardia arvensis*, *Linum bienne*, *Lagurus ovatus*, *Cynodon dactylon*, *Arenaria leptoclados*, *Anthemis arvensis*

**Dominant species:** *Vulpia ciliata*, *Trifolium nigrescens*, *Anthemis arvensis*, *Poa bulbosa*, *Medicago minima*, *Bromus hordeaceus*

**Ecology, distribution and use:** This association develops on slightly higher and better-drained sands in the zone of built-up areas and in their immediate vicinity, on flat terrain, covering 100% of the soil and reaching a height of 15-20 cm. Compared to the *Junco bufonii-Vulpietum ligusticae,* it is xerophilous (Fig. 2B, cluster 6; On-line Suppl. Fig. 1, cluster 8). It is characterized by a marked change in phenology of plant community and very low biomass production, which is why it is used exclusively as pasture.

Due to the low biomass production and the grazing, the surface sands are weakly bound, brownish-grey, with little humus and almost no carbonates. They develop at the highest points of the seemingly flat terrain, where they are replaced in slightly deeper depressions by the vegetation of temporary ponds and the *Scirpoides holoschoenus* community. Depending on the intensity of trampling and grazing and the humus content of the surface layer, these communities can range from poorer to richer in the total species number and participation of ruderal elements. Consequently, they exhibit numerous transitions to ruderal Mediterranean ephemeral communities.

In early spring, *Poa bulbosa, Avelinia festucoides* and *Tuberaria guttata* typically dominate, and are later replaced by *Crepis neglecta*, *Aira elegans*, *Vulpia ciliata*, *Trifolium nigrescens* or *Anthemis arvensis*. They are optimally developed in May during and after the frequent spring rains, in the summer they are completely dry, and in autumn some characteristic geophytes and late-flowering annual introduced grasses appear.

Among the elements of anthropogenic vegetation, the following species are particularly common: *Erodium cicutarium* (BUL, SIS), *Silene gallica* (CHE, TRA), *Bromus hordeaceus* (SIS), as well as species of *Helianthemetea guttati* and *Stipo-Trachynietea distachyae*: *Tuberaria guttata*, *Filago gallica*, *Aira elegans*, *Corynephorus divaricatus*, *Lagurus ovatus*, *Vulpia ciliata*, *Crepis neglecta*, *Hypochaeris glabra*,

**Tab. 1.** Analytical table of dry grassland communities in the hinterland of Velika Plaža: *Junco bufonii-Vulpietum ligusticae* (rels. 1-7, holotypus* rel. 6), *Tuberario guttatae-Avellinietum festucoidis* (rels. 8-13, holotypus* rel. 12), *Helianthemo jonii-Artemisietum campestris asphodeletosum ramosi* (rels. 14-34, holotypus* rel. 20), *Helianthemo jonii-Artemisietum campestris typicum* (rels. 35-47, holotypus* rel. 42).

| Relevè No | 1 | 2 | 3 | 4 | 5 | 6* | 7 | 8 | 9 | 10 | 11 | 12* | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20* | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42* | 43 | 44 | 45 | 46 | 47 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plot size ($m^2$) | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| Altitude (m) | 2 | 5 | 2 | 2 | 2 | 1 | 1 | 4 | 3 | 3 | 3 | 3 | 10 | 2 | 1 | 2 | 2 | 1 | 1 | 0 | 2 | 1 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 2 | 1 | 1 | 0 | 1 | 2 |
| Total vegetation cover (%) | 100 | 95 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 90 | 85 | 80 | 95 | 90 | 90 | 90 | 75 | 85 | 95 | 75 | 85 | 90 | 80 | 80 | 80 | 70 | 90 | 75 | 95 | 90 | 100 | 100 | 100 | 95 | 100 | 90 | 100 | 100 | 90 | 100 | 100 | 100 |
| Cover of shrub layer (%) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 45 | 45 | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Cover of herb layer (%) | 100 | 90 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 85 | 100 | 100 | 90 | 80 | 80 | 95 | 90 | 90 | 85 | 75 | 80 | 95 | 70 | 80 | 80 | 80 | 80 | 80 | 70 | 90 | 50 | 60 | 90 | 100 | 100 | 100 | 90 | 80 | 90 | 95 | 100 | 90 | 100 | 100 | 100 |
| Cover of moss layer (%) | . | 10 | . | . | . | . | . | . | . | . | . | 70 | 60 | 10 | 2 | 15 | 1 | 2 | 5 | . | 20 | . | 5 | 3 | 5 | 20 | 20 | 2 | 2 | 1 | . | 1 | 10 | 1 | 3 | 1 | . | 1 | 5 | 30 | 0 | 10 | 2 | . | . | . | 1 |
| ***Junco bufonii-Vulpietum ligusticae*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Vulpia ligustica* | 5 | 4 | 5 | 2 | 5 | 5 | 5 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Anthoxanthum odoratum* | . | . | . | 1 | 1 | 1 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Juncus bufonius* | 1 | 1 | + | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Lotus corniculatus* | 1 | . | + | . | . | 1 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Plantago lanceolata* | + | 1 | 1 | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Ranunculus marginatus* | + | + | . | . | . | 2 | 2 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . |
| ***Tuberario guttatae-Avellinietum festucoides*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Tuberaria guttata* | . | . | . | . | . | . | . | r | r | + | + | 2 | + | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Avellinia festucoides* | . | . | . | . | . | . | . | . | . | + | + | 2 | 1 | . | . | . | + | . | + | . | + | . | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Crepis neglecta* | . | . | . | . | . | . | . | . | r | + | + | 2 | 2 | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | r | 1 | + | 1 | + | 2 | . | . | . | . | . | . | . | . |
| *Filago gallica* | . | . | . | . | . | . | . | + | + | + | 1 | + | + | . | . | . | + | + | + | . | . | . | + | + | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | + | . | . | . | . | . | . | . |
| *Lotus angustissimus* | + | . | . | . | . | . | . | + | 2 | + | + | + | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Trifolium nigrescens* | 2 | 2 | 2 | 2 | 1 | 2 | 1 | 2 | 3 | 2 | 1 | 2 | 3 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | + | . | . |
| *Aira elegantissima* | 1 | . | + | . | 1 | . | . | r | r | + | 2 | 2 | 2 | . | . | . | + | . | 1 | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| ***Helinathemo jonii-Artemisietum campestris typicum*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Artemisia campestris* | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | + | 3 | 4 | 4 | 3 | 3 | 4 | 3 | 3 | 3 | 4 | 3 | 3 | 3 | 4 | 3 | 3 | 4 | 2 | 2 | 3 | 2 | + | 1 | + | . | 1 | 2 | 1 | 1 | 2 | . | 1 |
| *Helianthemum jonium* | . | . | . | . | . | . | . | . | . | . | . | . | . | + | 1 | 2 | . | + | 3 | 3 | 1 | 1 | 2 | 2 | 2 | 2 | 3 | 3 | + | 1 | . | 2 | 3 | 3 | . | . | . | . | . | 2 | 2 | 2 | 2 | 1 | 2 | 1 | . |
| *Erigeron canadensis* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | + | + | + | + | . | + | + | + | + | . | + | . | . | + | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| ***Helinathemo jonii-Artemisietum campestris asphodeletosum ramosi*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Asphodelus ramosus* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | + | . | . | . | . | 1 | . | 2 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 4 |
| *Crepis sancta* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | + | + | + | . | . | + | 1 | 1 | + | 1 | . |
| ***Helianthemetea guttati*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Medicago littoralis* | . | . | . | . | . | . | . | . | . | . | . | . | . | 2 | . | . | . | 2 | 1 | 1 | . | + | . | . | 1 | 1 | + | 1 | 1 | 1 | 2 | 1 | . | 1 | . | . | . | . | . | + | 1 | 2 | . | + | . | + | . |
| *Vulpia fasciculata* | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | 2 | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Trifolium stellatum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . |
| *Asterolinon linum-stellatum* | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | + | . | . | . | . | . | . | + | + | . | . | . | . | . | + | + | . | + | . | . | . | . | . | 1 | + | . | . | + | . | . | . |
| *Plantago bellardii* | . | . | . | . | . | . | . | . | . | + | 1 | + | . | . | . | . | . | 1 | . | + | . | . | + | 2 | . | . | . | . | . | 1 | . | . | . | . | 1 | . | . | . | 2 | 1 | . | . | . | . | . | . | . |
| *Galium divaricatum* | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Corynephorus divaricatus* | . | . | . | . | . | . | . | + | . | + | + | + | r | . | . | + | + | 1 | + | 1 | . | . | . | . | + | 1 | + | 1 | 1 | 1 | 1 | 1 | . | 1 | . | . | . | . | . | . | . | . | 1 | + | . | . | . |
| *Linaria pelisseriana* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |

**Tab. 1.** Continued

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Helianthemum salicifolium* | . | . | . | . | . | . | . | . | r | . | . | . | . | . | . | + | . | + | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | + | + | . |
| *Valerianella dentata* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Onobrychis caput-galli* | . | . | . | . | . | . | . | . | . | . | . | . | . | 2 | 1 | 1 | . | . | 2 | 2 | 1 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 3 | 2 | . | 3 | 3 | 4 | + | 2 | 1 | 2 | 1 | 1 | + | 2 | 2 | 2 | 1 | + | + |
| *Minuartia mediterranea* | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Trifolium tenuifolium* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | + | . | . | . | . | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Valerianella eriocarpa* | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . |
| *Lagurus ovatus* | . | 1 | . | . | . | . | . | . | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 1 | 2 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 2 | . | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | + | 2 | 1 | 1 | 1 | . | 1 |
| *Hedypnois rhagadioloides* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | + | + | . | 1 | . | 1 | 1 | + | 1 | . | + | . | + | + | 1 | 1 | . | . | 1 | + | + | + | . | + | 1 | . | . |
| *Vulpia ciliata* | . | . | . | 3 | . | . | . | 2 | 1 | 3 | 3 | 1 | . | + | . | + | 1 | + | + | + | + | 1 | + | . | . | + | . | . | + | + | 1 | . | . | . | + | + | + | + | + | + | + | . | + | + | 1 | + | . |
| *Lathyrus sphaericus* | . | . | . | . | . | . | . | r | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Scorpiurus muricatus* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | + | 1 | . | . | . | . | . | . | . | . |
| *Trifolium cherleri* | . | . | . | . | . | . | . | + | . | . | + | 1 | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | + | 2 | 1 | + | 1 | + | . | . | . | . | . | . | . |
| *Medicago minima* | . | + | . | + | + | . | . | 4 | . | 2 | . | r | . | 1 | . | 1 | . | + | . | . | . | + | 1 | + | . | . | . | . | . | . | . | . | . | . | + | 1 | + | + | + | . | . | 1 | . | . | . | 1 | . |
| *Hypochaeris glabra* | 1 | + | . | 1 | + | 1 | . | + | + | + | . | + | 1 | + | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . | . | + | . | + | 1 | . | . | . | + | . | + | + | . | + | + | + |
| *Trifolium arvense* | . | . | . | . | + | . | . | . | . | . | . | 1 | . | 1 | . | . | . | . | + | 1 | + | . | 1 | . | + | . | . | + | + | 1 | + | 1 | . | . | . | + | + | . | . | 1 | . | . | 1 | + | 2 | + | . |
| ***Helichryso-Crucianielletea maritimae*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Teucrium capitatum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . | 1 | + | . | . | . | . | 1 | . | . | + | . | . | . | . | . |
| *Silene conica* | . | . | . | + | . | . | . | . | . | . | . | + | . | + | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . |
| *Verbascum sinuatum* | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | + | . | . | + | + | + | 1 | + | . | . | + | + | . | . | 1 | + | + | . | + | . | + | . | + | . | 1 | . | 1 | + | . | . | + | + |
| *Echium plantagineum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | + | + | + | . |
| *Phleum arenarium* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . |
| ***Stipo-Trachynietea distachyae*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Linum strictum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | + | . | + | 1 | . | + | 1 | 1 | + | . | . | . | + | . | . | . | r | . | + | + | + | + | . | 1 | 1 | . | . | 1 | + | . | 1 |
| *Petrorhagia dubia* | + | + | + | + | + | . | . | r | 1 | 1 | + | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Cynosurus echinatus* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | 1 | . | . | . | . | . | . | . | 1 | . |
| *Sideritis romana* ssp. *purpurea* | . | . | . | . | . | . | . | + | + | + | . | + | . | . | . | . | + | + | . | . | . | + | 1 | + | . | . | . | . | . | + | + | . | . | . | 1 | . | . | + | . | + | . | . | . | . | . | . | . |
| *Briza minor* | 1 | + | + | . | 1 | . | . | . | . | . | + | . | . | . | + | . | . | . | . | . | + | . | + | . | . | + | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . |
| *Festuca incurva* | . | . | . | . | . | . | . | + | . | + | + | 1 | 2 | . | + | 1 | 1 | 1 | 1 | 1 | 1 | 1 | + | + | 1 | 1 | + | + | . | 1 | . | . | 1 | . | + | . | . | + | . | + | . | . | . | . | 1 | . | . |
| *Briza maxima* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | 1 | + | . | + | + | 1 | . | . | + | + | + | . | . | . | + | + | + | . | . | + | + | + | . | . | + | + | + | + | + | . | + | . |
| *Saxifraga tridactylites* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . |
| *Clypeola jonthlaspi* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . |
| *Hornungia petraea* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . |
| *Trifolium campestre* | 1 | 2 | 1 | 2 | 2 | . | . | 1 | 1 | 1 | 1 | 1 | 2 | + | + | . | . | 1 | 2 | 1 | 2 | 2 | 2 | 1 | . | 1 | . | . | 1 | . | 1 | 1 | . | . | 1 | 1 | + | . | 1 | . | . | . | . | + | . | . | . |
| *Filago germanica* | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | + | 2 | + | . | . | . | + | . | . | . | . | . | + | + | + | . | . | . | + | . | . | . | . | . | + | . | + | . | . | . | . |
| ***Ammophiletea*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Leontodon saxatilis* | + | 1 | + | . | . | . | . | + | + | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . |
| *Sonchus bulbosus* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | r | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Cyperus capitatus* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | 1 | 1 | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| ***Poetea bulbosae*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Trifolium subterraneum* | . | 1 | 1 | 1 | . | 1 | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |

**Tab. 1.** Continued

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Trifolium scabrum* | 1 | + | . | . | + | . | . | . | . | + | + | 2 | 2 | 1 | . | 2 | + | 1 | 1 | 1 | 1 | + | 1 | 1 | 1 | 1 | 1 | . | + | + | . | . | . | + | 1 | + | + | + | 1 | + | . | + | . | . | + | 1 | . |
| *Erodium cicutarium* | + | + | . | . | . | + | . | + | + | + | + | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | + | + | 1 | . | . | . | . | . | . | . | . |
| *Poa bulbosa* | + | . | . | . | . | . | . | + | 2 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | + | 1 | 1 | 1 | + | 1 | . | . | + | . | . | + | . | . | . | . | 1 | 1 | . | . | 1 | + | . | 1 | . | + | + | + | . |
| *Rumex acetosella* | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Romulea bulbocodium* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | + | + | . | . | . | . | . | . | . | . |
| *Lolium perenne* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . |
| ***Molinio-Arrhenatheretea*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Linum bienne* | . | 1 | + | + | + | 1 | + | + | 1 | . | 2 | + | 1 | . | . | . | . | . | . | 1 | 1 | 1 | + | + | . | . | . | . | + | . | + | 1 | + | . | + | + | + | + | + | + | + | . | . | 1 | + | . | + |
| *Alopecurus rendlei* | + | . | 1 | . | . | 1 | 1 | . | r | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Trifolium resupinatum* | . | + | 1 | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Gaudinia fragilis* | . | . | . | . | 1 | + | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | + | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Serapias vomeracea* | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Trifolium lappaceum* | + | . | . | . | . | . | . | . | . | r | + | . | 1 | . | + | . | . | . | . | . | 1 | 1 | . | + | . | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | + | . | + | . | . | . |
| *Poa trivialis* | . | . | + | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Lolium multiflorum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . |
| *Orobanche minor* | . | . | . | + | . | . | . | . | 2 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . |
| *Carex flacca* ssp. *serrulata* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | r | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Dactylis glomerata* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | + | + | . | . | . | + | . | . | + | . | . | . | . | . | . | . | + | + | . | . | + | + | . | + | . | . | + | 1 |
| *Narcissus tazetta* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 2 | 2 | . | + | . | . | . | . | . | . | . | . | . |
| ***Chenopodietea*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Silene gallica* | . | + | + | 1 | + | . | + | + | 2 | 1 | 1 | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . | . | . | . | . |
| *Avena barbata* | + | . | + | 1 | 1 | . | . | . | 1 | . | . | . | 1 | 1 | . | 1 | + | + | 1 | 1 | 1 | + | 1 | . | + | + | 1 | + | 1 | . | 1 | 1 | + | . | + | 1 | 1 | 1 | + | 1 | + | . | 1 | 1 | 1 | 1 | 1 |
| *Nigella damascena* | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Medicago arabica* | + | . | 1 | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . |
| *Catapodium rigidum* | . | + | . | . | . | . | . | . | . | . | . | . | + | + | 1 | + | + | 1 | + | + | 1 | . | + | + | + | 1 | . | + | . | 1 | + | 1 | . | + | + | + | + | + | + | + | . | + | 1 | . | + | + | . |
| *Medicago rigidula* | . | . | + | . | . | . | . | . | . | + | + | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Aegilops neglecta* | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | + | + | . | + | + | . | . | . | + | . | + | + | . | . | . | . | . | . | . | 1 | 1 | 2 | 1 | 1 | + | + | . | . | + | + | 1 | 1 |
| *Cerastium glomeratum* | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | + | . |
| *Aegilops triuncialis* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . |
| *Capsella rubella* | . | . | . | . | . | . | . | r | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . |
| *Bellardia trixago* | + | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Lolium rigidum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | + | . | + | + | . | . | . | + | . | . | + | . | . | . | . | . | + | . | . | + | + | . | . | + | + | + | + | . | + |
| *Bunias erucago* | . | . | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | + | . |
| *Cardamine hirsuta* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | + | . |
| *Rostraria cristata* | . | . | . | . | . | . | . | 1 | + | . | . | . | + | . | . | . | 1 | 1 | + | + | . | + | + | . | . | . | + | . | . | + | 1 | + | . | . | + | . | + | + | + | . | . | . | + | + | + | . | . |
| *Knautia integrifolia* | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | + | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | 1 | . | 1 | . | . | . | . | . | . | . | . | 1 |
| *Medicago polymorpha* | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Geranium purpureum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | + | . |
| *Carduus pycnocephalus* | . | . | . | . | . | . | . | 1 | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | + | . | . | . | . | . |
| *Trisetaria panicea* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . |

**Tab. 1.** Continued

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Cuscuta australis* ssp. *cesatiana* | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Vicia sativa agg.* | + | + | . | . | . | + | . | . | . | . | . | . | . | 1 | + | + | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | + | . | + | . | + | . | . | . | + | . |
| *Urospermum picroides* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | + | . | . | . | . | . | . | 1 |
| *Trifolium patens* | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . |
| *Lysmachia foemina* | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Valerianella locusta* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . |
| *Parentucellia viscosa* | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Aegilops geniculata* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . |
| *Alyssum simplex* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Anisantha madritensis* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . | . | . | . | . | . |
| *Dasypyrum villosum* | . | . | . | . | . | . | . | 2 | + | . | . | . | . | 3 | 1 | 2 | + | + | 1 | + | 1 | + | 1 | + | + | + | + | + | + | + | 1 | + | + | 1 | 1 | 2 | 2 | 1 | 1 | 1 | + | 1 | 1 | + | + | 1 | 2 |
| *Tordylium apulum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | 1 | . | 1 | . | . | . | . | . | . | . | . | + |
| *Trifolium angustifolium* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | + | . | . | + | + | . | + | + | . | . | + | . | . | . | . | . | . | r | + | + | + | 1 | + | + | . | + | + | . | + | + | . |
| *Gastridium ventricosum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Medicago orbicularis* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | 1 | . | . | . | . | . | . | . | . | 1 |
| *Anisantha rigidus* | . | . | . | . | . | . | . | . | . | . | . | . | . | 2 | . | . | . | . | . | + | . | + | + | . | . | . | . | . | . | + | + | . | . | + | . | . | . | . | . | + | . | 1 | . | . | . | . | . |
| ***Artemisietea vulgaris*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Daucus carota* | . | . | . | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Scolymus hispanicus* | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | + | + | . | . | . | . | . | . | . | + | . | . | . | . | . | 2 | + | + | . | . | + | . | . | . | + | . |
| *Elymus repens* | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . |
| *Chondrilla juncea* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Daucus guttatus* | . | . | . | . | . | . | . | . | + | . | r | 1 | + | 1 | + | . | + | 2 | 1 | 1 | 1 | . | 1 | + | . | + | + | . | + | + | + | . | . | . | + | + | 1 | 1 | 1 | + | . | + | + | + | . | 1 | . |
| *Carthamus lanatus* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | 1 | . | . | . | . | . | . | . | + | + |
| *Salvia verbenaca* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . |
| *Crepis setosa* | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Rubus ulmifolius* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . |
| ***Papaveretea rhoeadis*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Anthemis arvensis* | 1 | 1 | + | 1 | + | + | + | 4 | 3 | + | 2 | + | . | . | . | + | + | 1 | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | 1 | 1 | + | + | + | . | + | . | . | . | . | + | . |
| *Lysimachia arvensis* | . | 2 | + | + | + | . | . | r | + | . | + | . | + | . | . | . | . | . | + | + | 1 | 1 | + | 1 | . | . | . | . | + | + | 1 | 1 | . | . | + | + | . | . | + | + | + | . | . | + | . | + | . |
| *Sherardia arvensis* | . | + | + | + | + | . | + | + | + | + | . | + | + | + | . | . | . | + | . | . | . | + | . | . | . | . | + | . | . | . | 1 | + | . | . | + | + | 1 | + | 1 | + | + | . | + | . | + | 1 | . |
| *Lamium purpureum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | + | . |
| *Myosotis arvensis* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Nigella arvensis* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Ajuga chamaepitys* | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | + | + | + | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . |
| *Euphorbia helioscopia* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | + | + | + | + | 1 | + |
| *Euphorbia peplus* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | + | + | + | + | . |
| *Veronica arvensis* | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Sonchus oleraceus* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + |
| *Stellaria media* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . |
| *Euphorbia falcata* | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . | . |

**Tab. 1.** Continued

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Euphorbia exigua* | . | . | . | . | . | . | . | . | . | . | . | . | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | + | + | . | . | . | . | . | . | . | . | . | . |
| ***Sisymbrietea*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Geranium dissectum* | . | . | + | + | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | + | . | . | . | . | . | . | . | . | . | 1 |
| *Geranium columbinum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Geranium pusillum* | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Crepis foetida* | . | . | . | . | . | . | . | + | . | + | + | 1 | . | . | . | 1 | + | 2 | + | + | + | + | 1 | 1 | . | 1 | + | + | + | + | + | + | . | + | . | + | + | 1 | . | . | . | + | . | 1 | + | . | . |
| *Geranium rotundifolium* | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | r | . | . | . | + | . | . | . | . | 1 | + | + | . | . |
| *Vicia villosa* ssp. *varia* | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 2 | + | 1 | . | . | . | . | 1 | . | + | 1 | 1 |
| *Geranium molle* | . | + | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | + | + | . | + | . | . | . | 1 | . |
| ***Festuco-Brometea*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Chrysopogon gryllus* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 5 | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Bothriochloa ischaemum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 2 | . | + | + | . | . | . | . | . | . | . | + |
| *Anacamptis coriophora* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | + | + | . | + | . | . | + | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . |
| *Cuscuta epithymum* ssp. *epithymum* | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Anacamptis pyramidalis* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . |
| *Carex caryophyllea* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Ophrys sphegodes* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . |
| *Sanguisorba minor* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | + | + | + | + | + | 1 | + | . | 1 | 1 | . | . | . | . | 1 | . | . | . | . | . | . | . | 2 | + | . | + | + | . | . |
| *Arenaria leptoclados* | . | + | + | . | . | . | . | 1 | + | 1 | . | + | + | 1 | + | 1 | + | 1 | + | + | 1 | + | + | + | + | + | + | + | + | + | + | + | . | . | + | + | + | . | + | 1 | + | 2 | + | + | + | + | . |
| *Muscari comosum* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . |
| *Orobanche nana* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Teucrium chamaedrys* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . |
| *Satureja montana* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | 2 | . | . | . | . | . | . | . |
| *Linum tenuifolium* | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Allium sphaerocephalon* ssp. *arvense* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | + | . | + | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | + | 1 | . | + | . | . | + | . |
| *Melica ciliata* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Hypericum perforatum* | . | . | . | . | 1 | + | . | . | . | . | . | . | + | . | . | + | 1 | . | + | + | . | + | + | + | + | + | + | . | + | . | + | + | . | . | + | 1 | + | + | . | + | . | . | . | + | . | . | + |
| *Blackstonia perfoliata* | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | + | . | 1 | + | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . |
| *Centaurium pulchellum* | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | 1 | + | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | + | . | . | . | . | . | . | . |
| *Sagina apetala* | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Sonchus maritimus* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Scirpoides holoschoenus* | . | . | . | . | + | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | + | . | . | . | . | . | + | . | . | . | . | . | + | + | . | . | . | 1 | . | + | . | . | . | . | . | 1 | + |
| *Imperata cylindrica* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . |
| *Anemone hortensis* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | + | . |
| *Allium chamaemoly* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | + | . | . | + | . | . | . |
| **Others** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Parentucellia latifolia* | 1 | 1 | + | + | 1 | . | . | . | 1 | + | . | 1 | 1 | . | . | . | . | . | . | + | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Vulpia myuros* | + | + | + | 1 | 1 | . | . | . | . | . | . | . | 2 | . | . | + | . | . | . | . | . | . | . | . | . | + | . | . | . | . | + | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *Cerastium pumilum* ssp. *glutinosum* | + | 1 | 1 | + | . | . | 1 | r | . | + | + | 1 | + | 1 | . | 1 | + | . | + | . | + | . | 1 | . | . | . | . | . | + | . | . | . | . | . | 1 | . | + | . | . | 1 | . | 1 | 1 | . | 1 | + | . |
| *Cynodon dactylon* | 1 | + | + | . | . | + | + | + | r | . | + | + | 1 | + | . | 1 | + | . | 1 | + | + | + | 1 | + | . | 2 | 1 | 1 | 1 | + | 1 | 1 | 1 | + | 1 | . | 1 | + | + | . | 1 | 1 | + | 2 | 1 | + | . |

**Tab. 1.** Continued

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Bromus hordeaceus* (incl. *molliformis)* |2|1|.|+|+|.|+|+|+|+|3|r|+|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|
| *Serapias bergonii* |.|.|+|.|+|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|1|+|+|+|+|+|1|+|1|1|1|+|.|
| *Poa jubata* |.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|
| *Ochlopoa annua* |.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|
| *Moenchia mantica* |.|.|.|2|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Carex divisa* |.|.|.|.|1|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Centaurium tenuiflorum* ssp. *acutiflorum* |.|.|.|.|.|+|.|.|.|.|.|+|.|.|.|.|.|.|+|.|.|.|+|1|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Anacamptis laxiflora* |.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Vicia grandiflora* |.|.|+|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|+|.|
| *Pinus halepensis* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Fumana ericoides* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|1|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Serapias lingua* |+|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Polypogon maritimus* |.|.|.|.|.|.|.|.|.|1|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|
| *Petrorhagia saxifraga* |.|.|.|.|.|.|.|+|.|.|.|1|+|.|+|+|1|1|1|1|+|+|+|+|+|1|+|1|1|1|1|1|1|+|+|.|+|+|+|+|+|+|+|.|.|+|+|
| *Clinopodium arvensis* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|
| *Satureja cuneifolia* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|1|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Polypogon monspeliensis* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Trigonella balansae* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|+|.|
| *Lomelosia argentea* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|+|+|.|.|.|.|.|.|.|+|.|.|.|.|.|
| *Alkanna tinctoria* |.|.|.|.|.|.|.|+|.|.|.|.|.|1|.|+|+|+|.|1|+|.|1|1|+|.|.|.|1|1|1|+|+|+|.|.|.|.|.|1|+|1|1|+|+|.|.|
| *Hypochaeris radicata* |.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Myosotis ramosissima* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|
| *Equisetum ramosissimum* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|r|
| *Plantago coronopus* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|
| *Euphorbia maculata* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Tragus racemosus* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Plantago indica* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|+|.|
| *Minuartia hybrida* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Polycarpon tetraphyllum* ssp. *alsinifolium* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|
| *Arum italicum* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|1|+|.|.|.|.|.|+|+|1|
| *Ornithogalum sibthorpii* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|+|.|.|.|.|.|.|.|.|.|
| *Coronilla securidaca* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|+|.|
| *Noccaea perfoliata* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|1|.|.|.|.|.|.|.|.|.|.|.|.|
| *Hypochaeris cretensis* |.|.|.|.|.|.|.|.|.|.|.|r|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|+|.|.|.|.|.|.|.|.|
| *Euphorbia terracina* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|+|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Medicago doliata* |.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|
| *Anchusella cretica* |.|.|.|.|.|.|.|.|.|.|.|.|.|+|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|.|

*Petrorhagia dubia*, *Trifolium campestris*, *Cerastium pumilum* var. *glutinosum*. In some stands, extremely high moss coverage (70%) was observed, while in others it was completely absent. Therophytes dominate the biological spectrum (On-line Suppl. Fig. 2).

According to current knowledge, the distribution area of this association is limited to the hinterland of Velika Plaža in Ulcinj (On-line Suppl. Fig. 4). However, fragments are expected in Velipoja, Albania.

***Helianthemo jonii-Artemisietum campestris* ass. nov.**
(relevés 14–47 in Tab. 1, holotype: relevé 42, cluster III (subclusters 7 and 8) in Fig. 2A; On-line Suppl. Fig. 3C-E).

**Diagnostic species:** *Artemisia campestris*, *Onobrychis caput-galli*, *Helianthemum jonium*

**Constant species:** *Artemisia campestris*

**Dominant species:** *Artemisia campestris*, *Asphodelus ramosus*, *Helianthemum jonium*, *Onobrychis caput-galli*

**Ecology, distribution and use:** It is a pasture-like retrodunal xerophilous association that has developed as a result of the progressive natural succession of the highest part of the dune system and the regression stage on artificially destabilized sands in the hinterland of Velika Plaža. The terrain is mostly flat, only occasionally sloping 15–20° and facing north. Due to the dynamics of the relief and the conversion of land and infrastructure works, it is often fragmented and interspersed in a mosaic with dune depressions, mown meadows or anthropogenic habitats. The stands are characterized by a total plant cover of (50-)70 to 90(-95)%, with the proportion of mosses ranging from 0 to 30%. The June aspect of the association is dominated by *Helianthemum jonium* and the July-August aspect by *Artemisia campestris*. In addition to species of *Helianthemetea guttati*, *Helichryso-Crucianielletea maritimae* and *Stipo-Trachynietea distachyae* (e.g. TRA, TUB – *Medicago littoralis*, *Lagurus ovatus*, *Vulpia ciliata*, TRA – *Linum strictum*, TUB – *Onobrychis caput-galli*, *Corynephorus divaricatus*), elements of the anthropogenic vegetation of *Chenopodietea* (*Avena barbata*, *Catapodium rigidum*, *Dasypyrum villosum*), *Poetea bulbosae* (*Poa bulbosa*, *Trifolium scabrum*) and *Sisymbrietea* (*Crepis foetida*), are common and a sign of strong zoo-anthropogenic pressure. In the spectrum of life forms, annual species have the largest share, but perennials dominate in the physiognomy of this community.

As with *Junco bufonii-Vulpietum ligusticae*, the fragmentation of these communities has recently become apparent due to the conversion of land into construction sites or arable land.

The range of the association is restricted to the hinterland of Velika Plaža, but its fragments may be expected in the Velipoja reserve (Albania), although Fanelli et al. (2015) did not mention psammophytic communities dominated by the eponymous species *Artemisia campestris* and *Helianthemum jonium*.

The association is represented by two subassociations.

***Helinathemo jonii-Artemisietum campestris typicum* subass. nova**
(relevés 35-47 in Tab. 1, holotypus: relevé 42, Hinterland of Velika Plaža, subcluster 8 in Fig. 2A, On-line Suppl. Fig. 3C)

**Diagnostic species:** *Helianthemum jonium*, *Artemisia campestris*, *Erigeron canadensis*

**Constant species:** *Artemisia campestris*

**Dominant species:** *Artemisia campestris*, *Helianthemum jonium*, *Onobrychis caput-galli*, *Dasypyrum villosum*, *Chrysopogon gryllus*

**Ecology, distribution and use:** This subassociation has developed primarily as a result of progressive natural succession of dune sands on the highest part of the dune system (Breg Mora) which is usually protected by a windbreak belt of floodplain riparian ash and oak forest fragments or pine plantations, and mostly situated in fenced areas. It is also found in degraded areas in the highest parts of the sand deposits in the hinterland (Štojska Greda) as a regression stage on artificially destabilized sands. reaching a height of 15-20 cm. Community is characterized by a total cover of (50-)70-90(-95)%, reaching a height of 35-40 cm, with mosses accounting for (1-)3-15(-20)%. The invasive species *Erigeron canadensis* was frequently found in these stands, which is expected given the zoo-anthropogenic pressure, grazing, increased traffic and visits during the tourist season, and infrastructure works. In the physiognomy of this community, the chamaephyte form dominates (On-line Suppl. Fig. 3C), while in the spectrum of life forms, annual species have the largest share (On-line Suppl. Fig. 2).

***Helinathemo jonii-Artemisietum campestris asphodeletosum ramosi* subass. nova**
(relevés 14-34 in Tab. 1, holotypus: relevé 20, Hinterland of Velika Plaža, subcluster 7 in Fig. 2A, On-line Suppl. Fig. 3D, E)

**Diagnostic species:** *Asphodelus ramosus*, *Crepis sancta*

**Constant species:** *Asphodelus ramosus*

**Dominant species:** *Asphodelus ramosus*, *Artemisia campestris*

**Ecology, distribution and use:** This subassociation represents the final stage of development of the association during the progressive natural overgrowth of dune sands, driven by sand stabilization and the accumulation of humus in the upper soil layers. It typically occurs as linear strips in wind-protected areas along forest edges, roads, or fences, mostly on flat terrain, occcasionally sloped 15–20°, with a northern exposure. It is characterized by a high total vegetation cover of (75-)80(-90%), reaching a height of 95-100 cm, and a moss cover ranging from 0 to 30%. *Asphodelus ramosus* dominates the spring aspect, and *Artemisia campestris* dominates the summer aspect.

Compared to the *typicum* subassociation, grazing intensity is low, and the increased humus in the surface layer leads to sand stabilization. This is reflected in the colonization by more geophytes and species that do not typically inhabit the sand, as exemplified by the spread of *Asphodelus ramosus*. The ecological ambience in these stands is more mesophilous (Fig. 2B, Clusters 7, 8; On-line Suppl. Fig. 2, Clusters 9, 10). Species of contact habitats, such as forest species, are found in these stands: *Anemone hortensis* (PUB), *Arum italicum* (PUB, POP, QUI). Anthropogenic and dry species include: *Avena barbata*, *Dasypyrum villosum*, *Crepis sancta* (CHE), *Sherardia arevnsis*, *Euphorbia helioscopia*, and *E. peplus* (PAR). Moist habitat species include: *Linum bienne* (MOL). Elements of *Stipo-Trachynietea distachyae* and *Helianthemetea guttati* are common: *Lagurus ovatus*, *Vulpia ciliata*, *Briza maxima*, *Linum strictum*, *Onobrychis caput-galli*.

The physiognomy of this community is dominated by the perennial forms of geophytes and chamaephytes (On-line Suppl. Fig. 3D, E), while in the overall spectrum of life forms, annual species have the largest share (On-line Suppl. Fig. 2).

According to current knowledge, the distribution of this subassociation is also limited to the hinterland of Velika Plaža (On-line Suppl. Fig. 4). Its fragments might also occur in the Velipoja Reserve (Albania), although Fanelli et al. (2015) did not mention psammophytic communities dominated by *Asphodelus ramosus*.

## Discussion

### Relation of the *Junco bufonii-Vulpietum ligusticae* to other therophyte dry grassland communities

A recent study of sub-Mediterranean dry grassland communities in the sub-Mediterranean parts of Montenegro documented a novel association dominated by *Vulpia ligustica,* provisionally named *Bunio erucagi-Vulpietum ligusticae* (Stanišić-Vujačić 2023, Fig. 2A). This community is ecologically and syntaxonomically similar to mown meadows that have developed on former nutrient-rich arable land. It exhibits two phenological aspects: early spring, characterized by species such as *Bunias erucago*, *Calepina irregularis*, *Erodium cicutarium*, *Sherardia arvensis*, *Tordylium apulum;* and late spring, dominated by *Vulpia ligustica*. The community is widespread in the area of the Zeta Plain at altitudes of 25-73 m a.s.l. and is associated with the *Vulpio-Lotion*.

*Calepina irregularis* and *Tordylium apulum* are completely absent in the newly described retrodunal community *Junco bufonii-Vulpietum ligusticae* from the hinterland of Velika Plaža, while *Bunias erucago* occurs only sporadically. The community has a rather mesophilic character, as indicated by the presence of species such as *Alopecurus rendlei*, *Ranunculus marginatus* and *Linum bienne* in addition to *Juncus bufonius* (Tab. 1). Typical psammophytic elements common in the surroundings of the Velika Plaža hinterland, such as *Lagurus ovatus*, *Onobrychus caput-galli*, and *Medicago littoralis* (Stešević et al. 2020) are almost completely absent. This absence may be related to the complete stabilization of the sand and the competitive dominance of dry grasslands and exclusively non-psammophytic species. This floristic feature differentiates *Junco bufonii-Vulpietum ligusticae* from the nearby dune grasslands *Onobrychido caput-galli-Vulpietum fasciculatae*, part of the *Laguro ovati-Vulpion fasciculatae* alliance (On-line Suppl. Fig. 1).

According to the *Prodromus of Plant Communities of Montenegro* (Blečić and Lakušić 1976), Mediterranean grasslands in the coastal region include three associations: *Holoschoeno-Scabiosetum albae, Laguro-Corynephoretum divaricatae* and *Lupino-Laguretum ovati*. However, these associations are known only by name, and data on their floristic composition and structure are unavailable. They were classified into *Vulpio-Lotion*, *Thero-Brachipodietalia* and *Thero-Brachyodietea*. In modern syntaxonomy, the order *Thero-Brachypodietalia* is considered synonyomous with *Cymbopogono-Brachypodietalia ramosi*, and the position of *Vulpio-Lotion* has been reclassified into *Vulpietalia* and *Helianthemetea guttati* (Chytrý et al. 2024). Because of the aforementioned lack of relevés or textual descriptions of the mentioned communities, it was not possible to perform a critical revision of the classification or a comparison of the floristic composition or its ecology with *Junco bufonii-Vulpietum ligusticae.*

In Croatia and Albania, no communities are known where *Vulpia ligustica* is a dominant, name-giving or differential species (Dring et al. 2002, Trinajstić 2008, Fanelli et al. 2015).

In Italy, *Vulpio ligusticae-Dasypyretum villosii* (Fanelli 1998) is characterized as a non-littoral pseudosteppe grassland dominated by therophytes growing on acid sands located more than 5 km inland from the coast (Fanelli 1998, 2011). *Dasypyrum villosum* and *Hordeum bulbosum* dominate, while *Vulpia ligustica* as a characteristic species has low coverage. As in the community on the retrodunal sands of Velika Plaža, *Molinio-Arrhenatheretea* elements are represented in a considerable number of stands (Fanelli 1998), but this community also contains ecologically drier variants and is variable. It is classified in the order *Brometalia rubenti-tectorum* and the class *Thero-Brachypodietea* and not in the class *Chenopodietea*, which is thought to be floristically more similar (Fanelli 1998). In the modern classification (Chytrý et al. 2024), *Thero-Brachypodietea* is synonymous with *Lygeo sparti-Stipetea tenacissimae*, while the order *Brometalia rubenti-tectorum* retains its position in the class *Chenopodietea*.

Also, in Italy, De Pace et al. (2011) documented a low-density *Dasypyrum villosum-Lagurus ovatus-Vulpia ligustica* association in a pine forest near sand dunes on the Ionian Sea coast in the Apulia region. However, the classification of this community was not discussed.

In northwestern Sardinia, Farris et al. (2013) described an agro-pastoral, semi-nitrophilous, therophytic community dominated by *Vulpia ligustica*: *Cynosuro polybracteati-Vulpietum ligusticae.* It is a ploughed pasture divided into 4 ecological variants distributed across the altitudinal range from 250-1000 m a.s.l. The association is classified into the anthropogenic vegetation *Stellarietea mediae*, *Thero-Brometalia*, and *Echio plantaginei-Galactition tomentosae.*

In the recent classification, *Stellarietea mediae* is synonymous with *Papaveretea rhoeadis* and *Thero-Brometalia* with *Brometalia rubenti-tectorum* (Chytrý et al. 2024).

Mainly due to the affiliation of the dominant and characteristic species *Vulpia ligustica* with the class *Helianthemetea guttati*, the newly described retrodunal association *Junco bufonii-Vulpietum ligisticae* from the hinterland of Velike Plaže is included in *Helianthemetea guttati*, *Vulpietalia* and *Vulpio-Lotion*, whereby the specificity of this community is emphasized by the proportion of anthropogenic vegetation elements (*Poetea bulbosae* and *Chenopodietea*) and anthropogenically managed pastures and meadows (*Molinio-Arrhenatheretea*).

### Relation of the *Tuberario guttatae-Avellinietum festucoidis* to other therophyte dry grassland communities

*Avellinia festucoides*, the differential and name-giving species of this community, was recently recorded as new in the flora of Montenegro (Stešević et al. 2025). Although it prefers dry, open, sandy habitats, usually near the sea (Tutin 1980, Pignatti 1982), in the hinterland it has also been observed in occasional wet habitats, such as Mediterranean temporary ponds, wet dune slacks and wooded dunes with *Pinus pinea* and/or *Pinus pinaster* (Stešević et al. 2025). In hierarchical cluster analysis and ordination analyses of selected grassland and dune communities, the association *Tuberario guttatae-Avellinetum festucoidis* is clearly distinguished (Fig. 2A, B, On-line Suppl. 1).

With *Avellinia festucoides* (syn. *Avellinia michelii* (Savi) Parl.), the endemic community *Sileno conicae-Avellinietum michelii* part of the alliance *Laguro ovati-Vulpion membranaceae* (Géhu and Biondi 1994), *Malcomietalia*, *Tuberiarietea guttatae*, were described in the inner north Adriatic fixed dunes (Sbrulino et al. 2013). Similar to our community, its physiognomy is partly determined by perennial plants from anthropogenic vegetation, *Poa bulbosa* and *Cynodon dactylon*, as well as by typically psammophytic elements such as *Lagurus ovatus* (Sbrulino et al. 2013).

Given these finding and considering the basic ecological and floristic principles that compare our association with others outside the southeastern Adriatic, the *Laguro ovati-Vulpion membranaceae* and *Vulpio-Lotion* should be vicarious alliances that share a considerable number of common species. The proposed solution that *Laguro ovati-Vulpion membranaceae* belongs to segetal and ruderal vegetation (*Brometalia rubenti-tectorum, Chenopodietea*) and that *Vulpio-Lotion* belongs to Mediterranean and sub-Mediterranean-Atlantic annual low-growing ephemeral herbaceous and grass-rich vegetation on acidic substrates, sometimes developed over sands in dunes (*Vulpietalia, Helianthemetea guttati*), seems to be unfounded. Rather than classifying them into completely different orders and classes, we are of the opinion that *Laguro ovati-Vulpion membranaceae* should find its place among the dune ephemeral grasses of the order *Vulpietalia*.

The retrodunal association *Tuberario guttatae-Avellinietum festucoidis* is quite distant from all associated communities of the *Vulpio-Lotion* from Croatia (*Agrostetum maritimae arenosum*, *Chrysopogoni-Airetum capillaris*, *Ornithopodi-Vulpietum myuri*, *Gastridio-Brachypodietum retusi*, *Psiluro-Trifolietum cherleri*, *Haynaldio-Phleetum* and *Trifolio cherleri-Brachypodietum rupestris*) and is much more closely related to *Junco bufonii-Vulpietum ligusticae*, despite significant ecological, floristic, and physiognomical differences (Fig. 2A, B). In addition to the markedly different floristic composition of the Croatian associations, they are separated by a significantly lower number of perennial plants characteristic of the *Cymbopogono-Brachypodietalia ramosi*, into which this alliance was originally classified (Horvatić 1963), and also by the absence of species that prefer slightly moist habitats traditionally understood as characteristic of the alliance (*Linaria pelisseriana*, *Trifolium subterraneum*). This is to be expected as the dune systems in Croatia are very limited, and typical psammophitic decalcified low Mediterranean grasslands are very rare.

The above characteristics define our association as a terrophytic-geophytic and a dry association with washed-out carbonate sands. This makes it similar to some dry alliances of the classes *Koelerio-Corynephoretea canescentis* and *Stipo-Trachynietea distachyae*. Despite the significant participation of elements of anthropogenic vegetation, we classify this community within *Helianthemetea guttati*, *Vulpietalia* and *Vulpio-Lotion*.

The community develops on retrodunal sands (Štojska Greda), at higher positions which are well drained and very dry in summer. Settlements have been founded in these areas, and stands are now greatly diminished in extent. Due to these unfavorable hydrological conditions, these areas have been used as pastureland, primarily for small livestock (sheep and goats). This usage has led to the appearance of species that can tolerate trampling and grazing, but at the same time do not allow further pedogenesis and natural succession.

### Relation of the *Helianthemo jonii-Artemisietum campestris* to other dry grassland communities

In view of the physiognomic similarity between the spring aspect of *Helianthemo jonii-Artemisietum campestris asphodeletosum ramosi* from the retrodunal sands of Velika Plaža and the stands of the *Bromo-Chrysopogonetum grylli* pasture community dominated by *Asphodelus ramosus* from Ćemovsko Polje near Podgorica, a comparison of these communities was conducted. Both are heliophilous and thermophilous, with a pronounced dominance of perennials (Stanišić-Vujaćić et al. 2022, Fig. 2A, B). They are pasture-type and spatially alternate with mown meadows dominated by *Vulpia ligustica*. These communities are also linked by their syntaxonomy, while *Artemisia campestris* is considered a diagnostic species of the *Festuco-Brometea* class (Mucina 1997), to which *Bromo-Chrysopogonetum grylli* also belongs. However, due to its extensive ecological niche, *Artemisia campestris* is also included in the *Sedo-Scleranthetea* and *Koelerio-Corynephoretea canescentis*

(Chytrý et al. 2024), while its communities developed on shifting and stabilized dunes are classified as *Ammophiletea* (Babalónas et al. 1995, Sỳkora 2003, Tzonev et al. 2005, Mahdavi 2017) and *Helichryso-Crucianelletea maritimae* (Mahdavi 2017, Marcenò et al. 2018) as well as the psammophilous garrigues community in *Ononido-Rosmarinetea* (Choisnet et al. 2014). The ordination analysis revealed no significant similarity between *Helianthemo jonii-Artemisietum campestris* and *Bromo-Chrysopogonetum grylli* (Fig. 2B) and assigned the first community to other grasslands developed on sandy substrates. The second name-giving taxon of this community, *Helianthemum jonium*, which Brullo et al. (2001) considered as one of the characteristic species of the order *Helianthemo ionici-Scabiosetalia albae* Brullo et al. 2001 (from the class *Helichryso-Crucianelletea maritimae*). This order includes chamaephytic-hemicryptophytic communities of mesophytic character colonizing the inner, weakly mobile parts of the dunes of the Adriatic and Ionian coasts of the Italian Peninsula.

Tomaselli et al. (2024) identified *Helianthemum jonium* as a characteristic species of garrigue vegetation in the Apulia region, within the following syntaxa: order *Cisto-Micromerietalia julianae* (class *Ononido-Rosmarinetea*) and associations *Helianthemo jonii-Thymetum capitati*, *Vicio giacominianae-Helianthemetum jonii*, and *Helianthemo jonii-Fumanetum thymifoliae*. Data on the synecology of this species are also available for Albania (Hamallaj, north of Durrës), where it grows on sandy dunes in open areas between *Juniperus macrocarpa, Cistus salviifolius, Medicago marina, Alkanna tinctoria*, *Juncus acutus* and others. In addition, there are data from Croatia (Dalmatia), where it grows on sandy soils of abandoned vineyards within garrigues of *Cisto-Micromerietalia julianae*, then along roads and abandoned vineyards, on calcareous rocks in open grasslands mixed with garrigues, on sandy slopes of abandoned excavations under *Alkanna tinctoria*, *Ononis natrix*, *Foeniculum vulgare*, *Brachypodium retusum*, *Helichrysum italicum* and others (Bogdanović et al. 2020). As this species was discovered relatively recently in the eastern Adriatic (Bogdanović et al. 2020), communities with this species have not yet been documented.

The *Helianthemo jonii-Artemisietum campestris* from the retrodunal sands of Velike Plaža could be classified into *Helianthemo jonici-Scabiosetalia albae* (Brullo et al. 2001), but in FloraVeg.EU (Chytrý et al. 2024) this order is not mentioned, even as a synonym.

As part of mapping habitat types of community interest according to the 92/43 EEC Directive in Montenegro, stands of communities with *Artemisia campestris* and *Helianthemum jonium* from the hinterland of Velika Plaža are listed as a vegetation equivalent of the habitat type 2240 *Brachypodietalia* dune grasslands with annuals (Milanović et al. 2021), although according to the syntaxonomic classification they cannot be classified in *Lygeo sparti-Stipetea tenacissimae*. The analysis of the syntaxonomic affiliation of species to vegetation classes (Pignatti et al. 1995, Mucina 1997, FloraVeg.EU 2024) revealed a relatively low proportion of dune species from the class *Ammophiletea arundinaceae* (Tab. 1), instead containing species of *Chenopodietea*, *Helianthemetea guttati* and *Stipo-Trachynietea distachyae.* Nevertheless, referring to the evaluation of physiognomic and ecological criteria for syntaxonomy (Pignatti et al. 1995), we consider that it belongs to the class *Ammophiletea arundinaceae*, the order *Crucianelletalia maritimae*, and the alliance *Crucianellion maritimae*. The same approach was followed by Šilc et al. (2016) when classifying the *Scabiosa argentea-Ephedra distachya* community from Albania. Although the community has several diagnostic species of *Ammophiletalia arundinaceae* and only one – the name-giving taxon *Ephedra distachya* of *Crucianellion maritimae* – is classified in *Crucianielletalia maritimae* based on its ecology and physiognomy.

Regarding grazing intensity, *Helianthemo jonii-Artemisietum campestris* is represented by two sub-associations. One is characterized by an extremely high coverage of *Asphodelus ramosus* (On-line Suppl. Fig. 3D, E), the other by its almost complete absence (On-line Suppl. Fig. 3C). It is known that this species is unpalatable to most domestic animals and that it can be a sign of overgrazing (Stanišić-Vujačić et al. 2022, Terzi 2023), and its presence can also be indicative of underuse or abandonment of agro-pastoral activities (Biondi et al. 2016). The fact that stands with *Asphodelus ramosus* grow mainly in the form of narrow strips along roads and around fences, sheltered from the wind, on better developed and more humus-rich soil, suggests that they represent the final stage of the *Helianthemo jonii-Artemisietum campestris* dune community. In this stage, the vitality and coverage of the name-giving species decline due to natural succession followed by soil development on stabilized dunes. Therefore, this syntaxon should be considered a developmental stage (sub-association) of the previous association, leading to further natural succession.

In recent years, decreasing engagement in agriculture and livestock farming has led to the overgrowth of pastures and arable land. Meanwhile, the transformation of land into construction sites poses a serious threat to the continuity of this association.

## Syntaxonomical classification

TUB *Helianthemetea guttati* Rivas Goday et Rivas-Mart. 1963

TUB-02 *Vulpietalia* Pignatti 1953

TUB-02D *Vulpio-Lotion* Horvatić 1963

***Junco bufonii-Vulpietum ligusticae* ass. nov.,** *Holotypus*: rel. 6, Tab. 1

***Tuberario guttatae-Avellinietum festucoidis* ass. nov.;** *Holotypus*: rel. 12, Tab. 1

AMM *Ammophiletea arundinaceae* Br.-Bl. et Tx. ex Westhoff et al. 1946

AMM-02 *Crucianelletalia maritimae* Sissingh 1974

*CRU-02A Crucianellion maritimae* Rivas Goday et Rivas-Mart. 1958

***Helianthemo jonii-Artemisietum campestris* ass. nov.;** Holotypus: rel. 42, Tab. 1

**subass. *typicum***

**subass. *asphodeletosum ramosi;*** *Holotypus*: rel. 20, Tab. 1

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## Author contribution statement

D.S. – conceptualization, conducted the relevés, funding acquisition, M.S.V., Đ.M. – conducted the relevés, writing, U.Š. – funding acquisition, methodology validation, writing. All authors reviewed the paper and agreed to the published version of the manuscript.

## References

Apostolova, I., Dengler, J., Di Pietro, R., Gavilán, R. G., Tsiripidis, I., 2014: Dry grasslands of Southern Europe: syntaxonomy, management and conservation. Hacquetia 13(1), 1–14. https://doi.org/10.2478/hacq-2014-0015

Babalonas, D., Sykora, K. V., Papastergiadou, E. S., 1995: Review of plant communities from Greek dunes and salt marshes, a preliminary summarizing list. Annali di Botanica 53, 107–117.

Blečić, V., Lakušić, R., 1976: Prodromus biljnih zajednica Crne Gore (List of plant communities of Montenegro). Glasnik Republičkog Zavoda za Zaštitu Prirode i Prirodnjačkog Muzeja (Titograd) 9, 57–98.

Bogdanović, S., Holcer, D., Holcer, N. J., Shuka, L., 2020: *Helianthemum jonium* (Cistaceae), a new species in the eastern Adriatic. Natura Croatica 29(2), 9–18. https://doi.org/10.20302/NC.2020.29.2

Biondi, E., Pesaresi, S., Galdenzi, D., Gasparri, R., Biscotti, N., del Viscio, G., Casavecchia, S., 2016: The post-abandonment dynamic in Mediterranean perennial grasslands: the heliophilous edge vegetation of the new class *Charybdido pancratii-Asphodeletea ramosi*. Plant Sociology 53(2), 3–18. https://doi.org/10.7338/pls2016532/01

Braun-Blanquet, J., 1964: Pflanzensoziologie. Grundzüge der Vegetationskunde. 3 ed. Springer Verlag, Wien.

Brullo, S., Giusso del Galdo, G., Siracusa, G., Spampinato, G., 2001: Considerazioni fitogeografiche sulla vegetazione psammofila dei litorali italiani. Biogeographia 22, 93–136. https://doi.org/10.21426/B6110009

Choisnet, G., Bioret, F., Boullet, V., 2014: Contribution à l'étude des associations et sigmassociations végétales des levées alluvionnaires de la vallée de l'Ardèche (France, département de l'Ardèche). Documents Phytosociologiques 9, 215–306.

Chytrý, M., Tichý, L., Holt, J., Botta-Dukat, Z., 2002: Determination of diagnostic species with statistical fidelity measures. Journal of Vegetation Science 13(1), 79–90. https://doi.org/10.1111/j.1654-1103.2002.tb02025.x

Chytrý, M., Otýpková, Z., 2003: Plot sizes used for phytosociological sampling of European vegetation. Journal of Vegetation Science 14(4), 563–570. https://doi.org/10.1111/j.1654-1103.2003.tb02183.x

Chytrý, M., Řezníčková, M., Novotný, P., Holubová, D., Preislerová, Z., Attorre, F., Biurrun, I., Blažek, P., Bonari, G., Borovyk, D., Čeplová, N., Danihelka, J., Davydov, D., Dřevojan, P., Fahs, N., Guarino, R., Güler, B., Hennekens, S. M., Hrivnák, R., Kalníková, V., Kalusová, V., Kebert, T., Knollová, I., Knotková, K., Koljanin, D., Kuzemko, A., Loidi, J., Lososová, Z., Marcenò, C., Midolo, G., Milanović, Đ., Mucina, L., Novák, P., von Raab-Straube, E., Reczyńska, K., Schaminée, J. H. J., Štěpánková, P., Świerkosz, K., Těšitel, J., Těšitelová, T., Tichý, L., Vynokurov, D., Willner, W., Axmanová, I., 2024: FloraVeg.EU – An online database of European vegetation, habitats and flora. Applied Vegetation Science 27(3), e12798. https://doi.org/10.1111/avsc.12798

CGIS (Bioportal Crne Gore), 2017: Web portal za nacionalno zaštićena područja (Web portal for protected areas in Montenegro). Retrieved April 15, 2024 from http://prirodainfo.me/

De Pace, C., Vaccino, P., Cionini, P., Pasquini, M., Bizzarri, M., Qualset, C., 2011: *Dasypyrum*. In: Kole, C. (ed.), Wild crop relatives: genomic and breeding resources, cereals, 185–292. Vol. 4. Springer, Heidelberg.

Dring, J., Hoda, P., Mersinllari, M., Mullaj, A., Pignatti, S., Rodwell, J., 2002: Plant communities of Albania – a preliminary overview. Annali di Botanica (Roma) 2, 7–30.

EEA (European Environment Agency), 1999: Environment in the European Union at the turn of the century: Environmental Assessment Report No 2. EEA, Copenhagen.

Fanelli, G., 1998: *Dasypyrum villosum* vegetation in the territory of Rome. Rendiconti Lincei. Scienze Fisiche e Naturali 9(9), 149–170. https://doi.org/10.1007/BF02904397

Fanelli, G., 2011: The ecology of *Dasypyrum villosum* in Italy: highlights from phytosociology. Accademia nazionale delle scienze detta dei XL – Scritti e Documenti 44, 105–120.

Fanelli G., De Scantis M., Gjeta E., Mullaj A., Attorre, F., 2015: The vegetation of the Buna River protected landscape (Albania). Hacquetia 14(2), 129–174. https://doi.org/10.1515/hacq-2015-0008

Farris, E., Secchi, Z., Rosati, L., Filigheddu, R., 2013: Are all pastures eligible for conservation? A phytosociological survey of the Sardinian–Corsican Province as a basic tool for the Habitats Directive. Plant Biosystems 147(4), 931–946. http://dx.doi.org/10.1080/11263504.2013.778911

FloraVeg.EU, 2024: Database of European vegetation, habitats and flora. Retrieved August 15, 2024 from https://www.floraveg.eu

Géhu, J. M., Biondi, E., 1994: Végétation du littoral de la Corse. Essai de synthèse phytosociologique. Braun-Blanquetia 13, 1–149.

Hennekens, S. M., Schaminée, J. H. J., 2001: TURBOVEG, a comprehensive data base management system for vegetation data. Journal of Vegetation Science 12(4), 589–591. https://doi.org/10.2307/3237010

Horvatić, S., 1963: Vegetacijska karta otoka Paga s općim pregledom vegetacijskih jedinica Hrvatskog primorja (Vegetation map of the island of Pag with a general overview of vegetation units of Hrvatsko Primorje). Prirodoslovna Istraživanja JAZU 33, 1–187.

Isermann, M., 2011: Patterns in species diversity during succession of coastal dunes. Journal of Coastal Research 27(4), 661–671. http://doi.org/10.2307/41315839

Janssen, J. A. M., Rodwell, J. S., Criado, M. G., Gubbay, S., Haynes, T., Nieto, A., Sanders, N., Landucci, F., Loidi, J., Ssymank, A., 2016: European red list of habitats. 2. Terrestrial and freshwater habitats. Publications Office of the European Union, Luxembourg.

Mahdavi, P., 2017: Vegetation and functional diversity of sand dune habitats between and within different bioclimatic regions. PhD Thesis. Georg-August University School of Science (GAUSS), Göttingen.

Marcenò, C., Guarino, R., Loidi, J., Herrera, M., Isermann, M., Knollová, I., Tichý, T., Tzonev, R. T., Acosta, A. T. R., Chytrý, M., 2018: Classification of European and Mediterranean coastal dune vegetation. Applied Vegetation Science 21(3), 533–559. https://doi.org/10.1111/avsc.12379

McCune, B., Grace, J. B., 2002: Analysis of ecological communities. MjM Software Design, Glenden Beach.

McCune, B., Mefford, M. J., 1999: PC-ORD for Windows, Multivariate analysis of ecological data, Version 4. MjM Software Design, Gleneden Beach.

Mijović, A., 1994: Kserohalofilne psamofitske zajednice Velike ulcinjske plaže (The xerohalophytic sand communities of the Long beach). Glasnik Instituta za Botaniku i Botaničke Bašte Univerziteta u Beogradu 28, 147–157.

Mijović, A., Popović, Z., Karadžić, B., Mijatović, M., Perišić, S., 2006: Distribution of xerohalophytic vegetation along the seaward and landward zone in south-Adriatic sandy beach (Montenegro). Biotechnology & Biotechnological Equipment 20(1), 30–35.

Mijović, A., Popović, Z., Vukov, T., Smiljanić, M., Matić, R., Bojović, S., 2012: Sand topography influences the distribution of xerohalophytic vegetation on a southern Adriatic beach in Montenegro. Plant Biosystems 146(3), 664–673. https://doi.org/10.1080/11263504.2012.687403

Milanović, Đ., Caković, D., Hadžiablahović, S., Vuksanović, S., Mačić, V., Stešević, D., Stanišić-Vujačić, M., Biberdžić, V., Lakušić, D., 2021: Priručnik za identifikaciju tipova staništa Crne Gore od značaja za Evropsku uniju sa obrađenim glavnim indikatorskim vrstama (Manual for identification of habitat types of Montenegro of importance for the European Union with processed main indicator species). Agencija za zaštitu životne sredine Crne Gore, Univerzitet u Banjoj Luci – Šumarski fakultet, Podgorica, Banja Luka, Beograd.

Mucina, L., 1997: Conspectus of classes of European vegetation, Folia Geobotanica & Phytotaxonomica 32, 117–172. https://doi.org/10.1007/BF02803738

Mucina, L., Bültman, H., Dierssen, K., Theurillat, J.-P., Dengler, J., Čarni, A., Šumberová, K., Raus, T., Di Pietro, R., Gavilán Garcia, R., Chytrý, M., Iakushenko, D., Schaminée, J. H. J., Bergmeier, E., Santos Guerra, A., Daniëls, F. J. A., Ermakov, N., Valachovič, M., Pigantti, S., Rodwell, J. S., Pallas, J., Capelo, J., Weber, H. E., Lysenko, T., Solomeshch, A., Dimopoulos, P., Aguiar, C., Freitag, H., Hennekens, S. M., Tichý, L., 2016: Vegetation of Europe: Hierarchical floristic classification system of vascular plant, bryophyte, lichen, and algal communities. Applied Vegetation Science 19(S1), 3–264. https://doi.org/10.1111/avsc.12257

Official Gazette of the Republic of Montenegro, 1968: Rješenje o zaštiti objekata prirode (Decision on the protection of natural objects). 30.

Pignatti, S., 1982: Flora d'Italia. Edagricole, Bologna.

Pignatti, S., Oberdorfer, E., Schaminée, J. H. J., Westhoff, V., 1995: On the concept of vegetation class in phytosociology. Journal of Vegetation Science 6(1), 143–152. https://doi.org/10.2307/3236265

Pignatti, S., 2005: Valori di bioindicazione della piante vascolari della flora d'Italia. Braun Blanquetia 39, 1–97.

POWO (Plants of the World Online), 2024: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Retrieved April 15, 2024 from http://www.plantsoftheworldonline.org/

R Core Team, 2023: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Sburlino, G., Buffa, G., Filesi, L., Gamper, U., Ghirelli, L., 2013: Phytocoenotic diversity of the N-Adriatic coastal sand dunes – The herbaceous communities of the fixed dunes and the vegetation of the interdunal wetlands. Plant Sociology 50(2), 57–77. https://doi.org/10.7338/pls2013502/04

Stešević, D., Küzmič, F., Milanović, Đ., Stanišić Vujačić, M., Šilc, U., 2020: Coastal sand dune vegetation of Velika Plaža (Montenegro). Acta Botanica Croatica 79(1), 43–54. https://doi.org/10.37427/botcro-2020-003

Stešević, D., Stanišić Vujačić, M., Milanović, Đ., Šilc, U., 2025: On the distribution and conservation status of some Mediterranean species new for the flora of Montenegro. Hacquetia 24(1), 83–94. https://doi.org/10.3986/hacq-2025-0004

Stanišić-Vujačić, M., Stešević, D., Hadžiablahović, S., Caković, D., Šilc, U., 2022: An *Asphodelus ramosus* dominated plant community in Montenegro: fringe or grassland? Acta Botanica Croatica 81(1), 12–22. https://doi.org/10.37427/botcro-2022-027

Stanišić-Vujačić, M., Stešević, D., Caković, D., Šilc, U., 2023: Vegetation database of Montenegro, Phytocoenologia 51(4), 303–311. https://doi.org/10.1127/phyto/2023/0406

Stanišić-Vujačić, M., 2023: Vegetacija suvih travnjaka u sumbediteranskom dijelu Crne Gore (Vegetation of dry grasslands in submediterranean part of Montenegro). PhD Thesis. University of Montenegro, Podgorica.

Sýkora, K. V., Babalonas, D., Papastergiadou, E. S., 2003: Strandline and sand-dune vegetation of coasts of Greece and of some other Aegean countries, Phytocoenologia 33(2-3), 409–446. https://doi.org/10.1127/0340-269X/2003/0033-0409

Šilc, U., Mullaj, A., Alegro, A., Ibraliu, A., Dajić Stevanović, Z., Luković, M., Stešević, D., 2016: Sand dune vegetation along the eastern Adriatic coast. Phytocoenologia 46(4), 339–355. https://doi.org/10.1127/phyto/2016/0079

Šilc, U., Stešević, D., Luković, M., Caković, D., 2020: Changes of a sand dune system and vegetation between 1950 and 2015 on Velika Plaža (Montenegro, E Mediterranean). Regional Studies in Marine Science 35, 101139. https://doi.org/10.1016/j.rsma.2020.101139

Tomaselli, V., Sciandrello, S., Minissale, P., Forte, L., Costanzo, E., Giusso del Galdo, G., Carruggio, F., Pazienza, G., Brullo, S., 2024: Syntaxonomical remarks on the garrigues from Apulia (S Italy) and neighboring territories. Plants 13(13), 1800. https://doi.org/10.3390/plants13131800

Terzi, M., 2023: A new *Asphodelus ramosus*-dominated association from the Murge Plateau (SE Italy). Hacquetia 22(2), 179–195. https://doi.org/10.2478/hacq-2022-0020

Trinajstić, I., 2008: Biljne zajednice Republike Hrvatske (Plant communities of Croatia). Akademija Šumarskih Znanosti, Zagreb.

Tzonev, R., Dimitrov, M., Roussakova, V., 2005: Dune vegetation on the Bulgarian Black Sea coast. Hacquetia 4(1), 7–32.

Tichý, L., 2002: JUICE, software for vegetation classification. Journal of Vegetation Science 13(3), 451–453. https://doi.org/10.1111/j.1654-1103.2002.tb02069.x

Tichý, L., Chytrý, M., 2006: Statistical determination of diagnostic species for site groups of unequal size. Journal of Vegetation Science 17(6), 809–818. https://doi.org/10.1111/j.1654-1103.2006.tb02504.x

Tutin, T. G., 1980: Genus *Avellinia* Parl. In: Tutin, T. G., Heywood, V. H., Burges, N. A., Moore, D. M., Valentine, D. H., Walters, S. M., Webb, D. A. (eds.), Flora Europaea, Vol. 5, 232. Cambridge University Press, Cambridge.

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# Micropropagation and optimisation of *in vitro* production of the rare and threatened moss *Entosthodon pulchellus* (Funariaceae)

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**Abstract** – The establishment of axenic cultures and the propagation of the rare moss *Entosthodon pulchellus* (H. Philip.) Brugués (Funariaceae), as well as the optimisation of its *ex situ* growth conditions and rapid biomass production were the goals of this study. The results obtained also provide insights into the developmental biology of this moss in *in vitro* conditions, particularly regarding basal media contents with or without supplements of selected plant growth regulators or sugars, i.e. its nutritional needs. The procedure for establishing axenic cultures of this rare and threatened moss species is described. The optimisation protocol of the growth and production conditions is elaborated. The spore germinability of dried herbarium samples remained rather high in the tested laboratory conditions. Suitability tests on different media types showed KNOP basal media to be the best fit for the rapid biomass production of both secondary protonemata and leafy gametophores, without the addition of sugars or plant growth regulators. However, sugar, namely sucrose, induced rapid and massive protonemal development and can be used when this developmental stage is needed.

**Keywords**: bryophyte, conservation, development, *ex situ*, growth

## Introduction

Bryophytes, like other plants, suffer from environmental changes and habitat destruction (Löbel et al. 2018). Rapid climate alternations have led to a decrease in bryophyte populations despite their dispersal potential (Zanatta et al. 2020), especially those living at the edge of their overall ranges. These facts along with their fast reaction to small changes (good indicators) make this group of plants very vulnerable and susceptible to high extinction risks both regionally and globally. Although bryophytes are included in general and national conservation initiatives (Hallingbäck and Tan 2010), active protection is rarely applied (Rowntree et al. 2011, Sabovljević et al. 2014a), especially long-term. Another problem is the lack of knowledge concerning the biology and ecology of rare and threatened bryophyte species, which makes it rather difficult to apply active measures prior to thorough studies. Recently, in order to overcome these problems, the emerging discipline of conservation physiology has begun to address species protection, offering the possibility to study species outside their natural populations and habitats and to apply new knowledge to their propagation and species protection. Pioneering work on bryophytes in this direction has been conducted recently (Sabovljević et al. 2022).

*Entosthodon pulchellus* (H. Philip.) Brugués is a funaroid moss species found in areas of the sub-Mediterranean-sub-Atlantic regions of Europe, and is also known from North

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Africa, South-west, central and eastern Asia. Although it is rarely reported, Hodgetts et al. (2019a,b) consider its overall European population stable, while the range-edge populations seem to be declining, like those in Germany. The same authors also reported the populations to be extremely rare in Austria and Switzerland, and stable in Hungary and Portugal.

The species is known to inhabit thin soil on calcareous terraces, with scarce vegetation and has an ephemeral and shuttle life strategy. Sporophytes are often present since the species is autoicous. According to known reports it is altitudinally rather indifferent, also appearing in sunny and warm places in high mountains, usually in spring to early summer when it completes its short life cycle, culminating in spore dispersal.

Priority in conservation management is given to more rare genotypes and subpopulations (e.g. Jin et al. 2020). Thus, in this study we have focused on the Russian accession of *E. pulchellus* with the aim of establishing an *ex situ* population to learn more about its biology and to optimise captive propagation and large-scale biomass production. This should enable reintroduction to potentially suitable habitats and/or population strengthening in declining subpopulations. Additionally, since the species from this genus often hybridise naturally (Ostendorf et al. 2021) and the ploidy status is unclear throughout its populations, this approach will provide clean material for further studies.

In Russia this species is known mainly from its Asian part, including southern Siberia (the Altai Republic, Zabaikalsky Territory, the Republic of Buryatia), the Amur Province in the Russian Far East, and Yakutia. It grows at altitudes ranging from 100 to 1650 m a.s.l. in various habitats, such as the niches and cracks of rock outcrops, screes, and on bare soil on steep slopes with steppe vegetation (the latter kind of habitat is sporadic in Yakutia, occurring up to 68° N). However, in European Russia *E. pulchellus* is much rarer, found only in xeric SE regions in the Astrakhan and Volgograd Provinces, where it grows on the slopes of ravines and karst sinkholes, in slightly wetter conditions than *E. hungaricus* (Boros) Loeske which appears not to be rare on soil among grasses in steppe communities.

The species is critically endangered (IUCN: CR) in Switzerland, endangered (IUCN: EN) in Slovenia, Czechia, Germany and Ireland, and at serious risk of extinction in Austria (Hodgetts et al. 2019b, Martinčič 2024). In Britain and Hungary, it is considered near threatened (IUCN: NT), although rare but stable (Hodgetts et al. 2019b), while in many other countries it is a data deficient species (IUCN: DD) due to its rarity, ephemeral life span and/or underrecording (e.g. Croatia, Serbia, and Albania).

## Material and methods

### Plant material

The plant material for the present study was collected in the Altai Republic, Ulagan District, on the left bank of the Chulyshman River, 1 km downstream, at the Chulcha Creek mouth, 51°5'32" N – 87°58'51" E, altitude 520 m a.s.l., in *Rhododendron* L. thickets on a steep mossy slope, on the soil ledge, 17 June 2021, coll. M.S. Ignatov & E.A. Ignatova #21-251 (MW9092068, MHA9130772). Herbaria acronyms follow Thiers (2024).

Sporophytes from the deposited herbarium material were used to establish the *in vitro* culture of *E. pulchellus*. Mature sporophyte capsules were carefully separated from the dry plant material, thoroughly cleaned of mechanical impurities and rinsed in distilled water. Then the cleaned capsules were sterilised with 7% sodium hypochlorite solution (NaOCl). In addition to the different NaOCl concentrations, the exposure time was also varied, with durations up to 120 sec to obtain uncontaminated spores prior to their dispersal into the KNOP basal medium under the flow chamber as previously elaborated (e.g. Jadranin et al. 2023). After the spores were spread on the medium, spore germinability and the presence of possible contamination by remnants of the cohabitants were tested and then subcultivated to fresh medium until a fully axenic culture was achieved. The Petri dishes containing the spores were cultured for two months under sterile conditions at a constant temperature (18 ± 2 °C) and humidity (60 - 70%) in a long-day light regime (16 h light/8 h dark). Light with a flux density of 50 µmol $m^{-2} s^{-1}$ was provided by fluorescent tubes (Tesla Pančevo, Serbia). The axenic cultures thus obtained were subsequently micropropagated and used as starting material for the experiments presented here.

### *In vitro* micropropagation of the plant material

After the achievement of axenic plantlets, the plants were propagated for six weeks on a minimal KNOP nutrient medium (Reski and Abel 1985) until the development of gametophores and their optimal size and biomass for the experimental treatments had been achieved. For further investigation, the explants were placed in Petri dishes with the appropriate media type depending on the experiment. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 45 min. A single gametophore of 5 mm in length was used as the starting explant in all the treatments. Each experimental group consisted of 20 individual gametophores. The experiment was performed axenically under controlled conditions as described in the plant material chapter.

### Experimental design

In this study, two different experiments were conducted. Experiment type I was used to investigate the effects of nutrient media and exogenously added sugars on the morphogenesis of *E. pulchellus*. The explants were grown on a minimal KNOP medium (Reski and Abel 1985), a Murashige and Skoog (MS) half-strength medium (designated MS/2) (Sabovljević et al. 2009), and a BCD medium (Sabovljević et al. 2009) to which 15 g $L^{-1}$ of sucrose or fructose were subsequently added. The concentrations of sucrose and

**Tab. 1.** Experimental design summary for investigating *Entosthodon pulchellus* morphogenesis.

| | | |
|---|---|---|
| **Experiment type I** | Sugar-free | KNOP (control): KNOP minimal medium, sugar-free |
| | | MS/2 (control): MS mineral salts, half strength, sugar-free |
| | | BCD (control): BCD mineral salts, sugar-free |
| | Enriched with sucrose | KNOP+S: KNOP minimal medium enriched with 15 g $L^{-1}$ sucrose |
| | | MS/2+S: MS mineral salts, half strength, enriched with 15 g $L^{-1}$ sucrose |
| | | BCD+S: BCD mineral salts, enriched with 15 g $L^{-1}$ sucrose |
| | Enriched with fructose | KNOP+F: KNOP minimal medium enriched with 15 g $L^{-1}$ fructose |
| | | MS/2+F: MS mineral salts, half strength, enriched with 15 g $L^{-1}$ fructose |
| | | BCD+F: BCD mineral salts, enriched with 15 g $L^{-1}$ fructose |
| **Experiment type II** | Enriched with plant growth regulators | KNOP (control): KNOP minimal medium, PGR-free |
| | | KNOP: supplemented with 0.1 μM IBA |
| | | KNOP: supplemented with 0.1 μM BAP |
| | | KNOP: supplemented with 0.03 μM IBA and 0.1 μM BAP |
| | | KNOP: supplemented with 0.1 μM IBA and 0.1 μM BAP |
| | | KNOP: supplemented with 0.3 μM IBA and 0.1 μM BAP |

fructose were used at half-strength; sugars are usually added at full strength (30 g $L^{-1}$) in media for vascular plant cultivation (see Tab. 1 for details).

Since bryophytes grow predominantly photoautotrophically compared to photomixotrophic vascular plants under axenic conditions, the addition of a lower sugar concentration seemed to be more suitable for mosses, as shown in previous studies (e.g. Sabovljević et al. 2005).

In experiment type II, the effects of exogenous plant growth regulators (PGRs) on the morphogenesis of *E. pulchellus* were investigated. The plants were grown on a KNOP minimal medium supplemented with different concentrations of indole-3-butyric acid (IBA) and 6-benzylaminopurine (BAP) both individually and combined. The concentrations of IBA and BAP used in this experiment are listed in Tab. 1.

Morphological parameters such as the index of multiplication and the diameter of the secondary protonema were measured after four weeks. The index of multiplication (IM) represents the newly formed shoots which emerge from the new buds of the initial explant. All morphological changes were carefully measured and documented using a Leica stereomicroscope (Leica MZ 7.5 Bi-Optic Inc. Santa Clara, CA, USA) and a conventional light microscope (Leica DMLS, Santa Clara, CA, USA).

### Statistical analysis

Statistical analysis was conducted using the R programming language (v. 4.3.1) (R Core Team 2022). The preliminary data exploration was done using the Shapiro-Wilk normality test and Levene's test of homogeneity of variance. Such analysis revealed that nonparametric statistics should be applied. Therefore, the Kruskal-Wallis rank sum test was used for a comparison of the experimental groups, after which Dunn's multiple comparison test with the Benjamini-Hochberg P-value adjustment method was applied, with one exception, in experiment type II for the comparison of the diameters of the secondary protonema patches, where the Wilcoxson rank-sum test was used in order to compare the two groups. The significance level (α) was set at 0.05 for all experiment.

## Results

### The influence of different growth medium types and exogenously added sugars on the morphogenesis of *Entosthodon pulchellus*

In experiment type I, the largest number of newly formed gametophores was documented in those plants grown on the sugar-free MS/2 medium (Fig. 1A). Numerous shoots were also developed in the plants grown on the KNOP medium with exogenously added sucrose (KNOP+S) and the sugar-free KNOP medium (Fig. 1A).

However, no statistically significant differences were observed between these three experimental groups (MS/2, KNOP, and KNOP+S), suggesting that these media may be equally suitable for the *in vitro* cultivation of the species. Compared to the KNOP and MS/2 sugar-free media, IM was significantly lower in the plants grown on the BCD medium ($P < 0.05$) (Fig. 1A).

In general, the addition of sugars negatively affected the formation of new buds and shoots in *E. pulchellus*, except when sucrose was combined with the minimal KNOP medium (KNOP+S). When fructose was added to the medium, the IM decreased significantly in all three types of media used in experiment type I ($P < 0.05$) (Fig. 1A). A similar effect was observed when the BCD medium was supplemented with sucrose (BCD+S) at the same concentration (15 g $L^{-1}$).

As for the development of the secondary protonema patch, a similar pattern was recorded, i.e. those plants grown on sugar-free media developed regular protonemal patches although different in size (Fig. 1B). The largest diameter of secondary protonema was observed in the

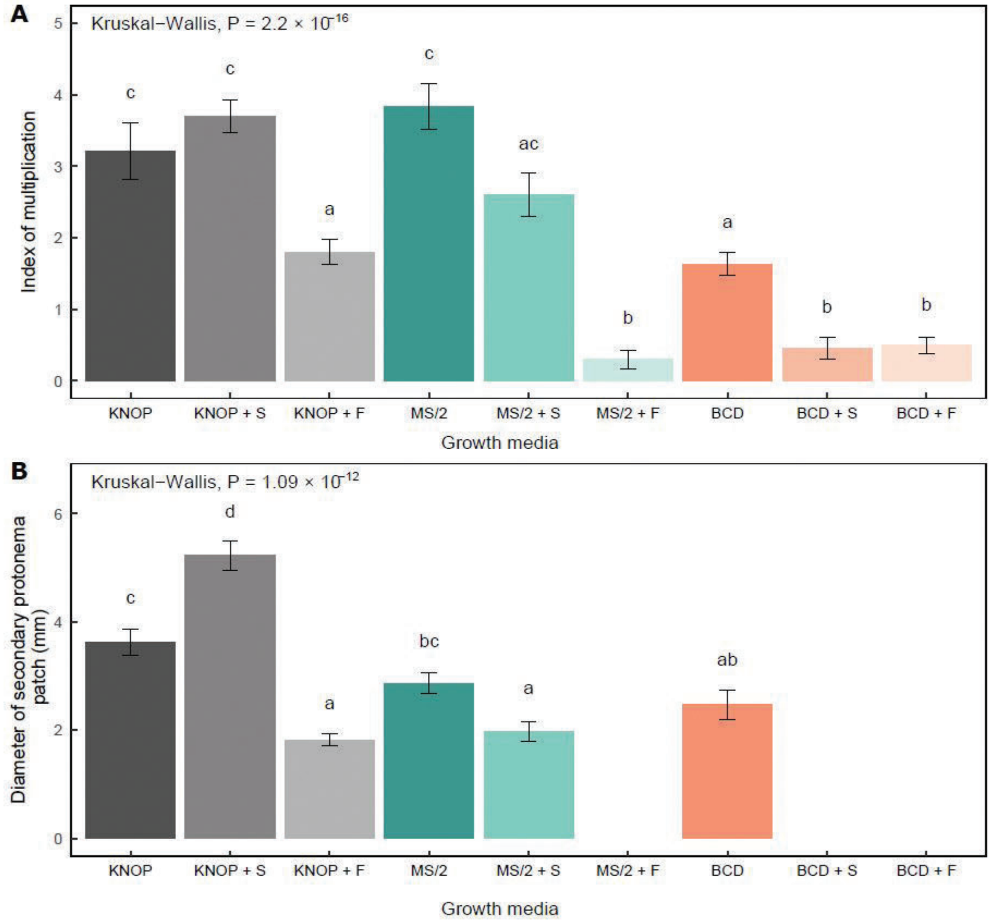

**Fig. 1**. The influence of the plant growth medium types and exogenously added sugars on the *Entosthodon pulchellus* index of multiplication (A) and diameter of secondary protonema patch (B) in experiment type I. The data are presented as the mean ± standard error. Different letters above the bars denote statistically significant differences ($P < 0.05$) between the experimental groups.

plants grown on the KNOP medium containing 15 g $L^{-1}$ sucrose (KNOP+S) (Fig. 1B). However, the addition of sucrose exerted different effects on the development of the secondary protonema depending on the growth media type. Although sucrose had a positive effect on protonemal development in the plants grown on the KNOP+S medium ($P < 0.05$), when added to the MS/2 medium, sucrose negatively affected the protonemal growth ($P < 0.05$). Furthermore, sucrose completely inhibited the development of secondary protonema when present in the BCD medium (BCD+S) (Fig. 1B), i.e. no plants developed measurable protonemal patches. On the other hand, fructose significantly decreased the protonemal diameter in *E. pulchellus* when added to the KNOP medium (KNOP+F) ($P < 0.05$), while it completely inhibited the formation of protonemata in the plants grown on the MS/2 and BCD media at a concentration of 15 g $L^{-1}$ (Fig. 1B).

In addition to the evaluation of the two morphogenesis parameters, the morphological appearance of the plants was also taken into account in order to determine the optimal medium for the *in vitro* micropropagation of *E. pulchellus* (Fig. 2).

Overall, according to the results of experiment type I, the sugar-free KNOP and MS/2 media appeared to be equally suitable for the *in vitro* micropropagation of the investigated species (Fig. 2A, B). However, since the addition of sucrose to the KNOP medium promoted the development of protonemata, this medium could be used for the purpose of growing more protonemata than shoots (Fig. 2D), and for easier and rapid multiplication. Compared to the KNOP and MS/2 media, the BCD medium was generally inadequate for the micropropagation of *E. pulchellus*, as the plants did not develop a sufficient number of new shoots and secondary protonema patches (Fig. 2C, F, I).

An interesting phenomenon was observed in those plants grown on the fructose-enriched MS/2 medium (MS/2+F) (Fig. 3). The phylloid cells underwent dedifferentiation and multiple secondary protonemal threads emerged from the individual cells (shown by arrows in Fig. 3), indicating possible sublethal conditions for *E. pulchellus* upon addition of fructose to the MS/2 medium.

## The influence of the plant growth regulators on the morphogenesis of *Entosthodon pulchellus*

To assess the effects of the plant growth regulators, the plants were grown on the KNOP medium supplemented with different concentrations of IBA and BAP as described in the Material and Methods section (Tab. 1). The minimal

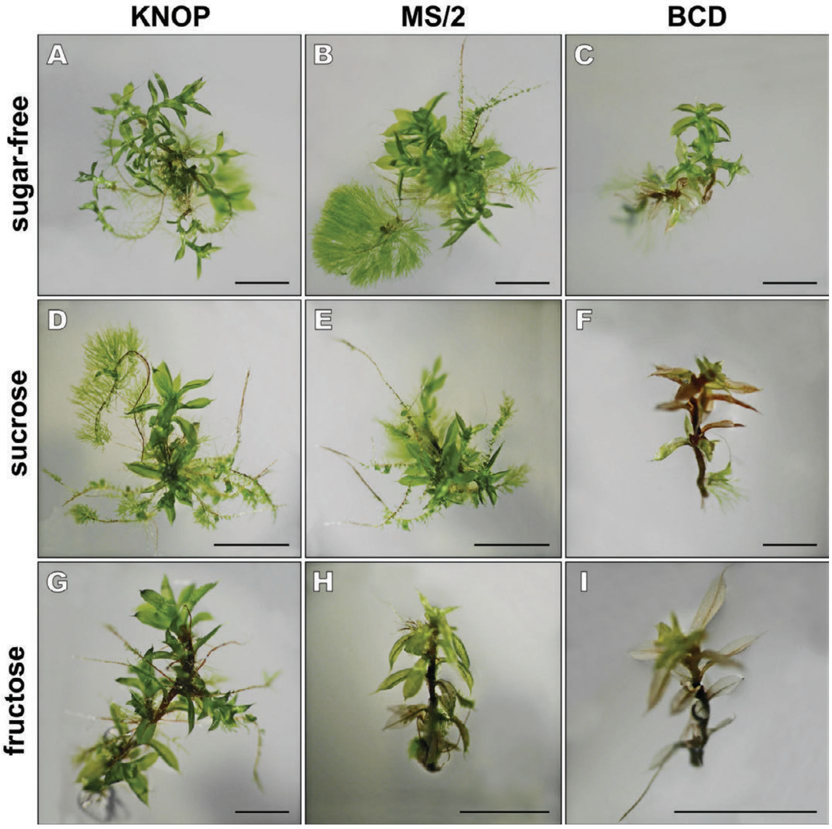

**Fig. 2**. The appearance of *Entosthodon pulchellus* explants grown on sugar-free media (A-C) and media supplemented with 15 mg L$^{-1}$ sucrose (D-F) and 15 mg L$^{-1}$ fructose (G-I) in experiment type I. The bars represent the size of 2 mm in accordance with the magnification (0.63× for A-C, F and G; 0.8× for D and E; 1× for H; 1.6× for I).

KNOP medium was selected according to the results obtained in experiment type I as the most suitable minimal medium for the propagation of the examined species.

The highest IM was recorded in the plants grown on the KNOP medium without exogenously added PGRs (control experimental group), although a large number of small shoots were also observed in the plants grown on the medium supplemented with 0.1 µM BAP or 0.1 µM IBA (Fig. 4A).

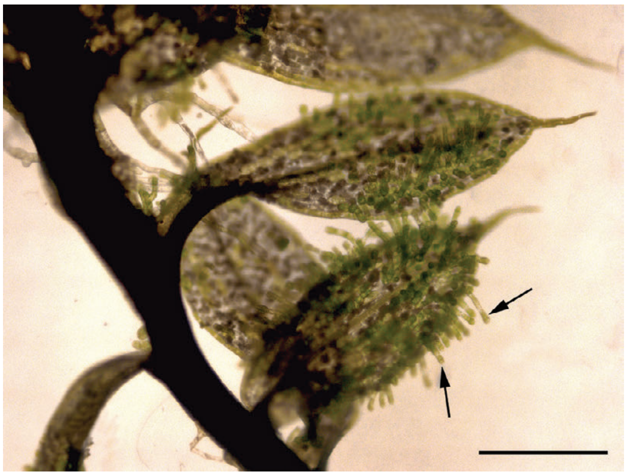

**Fig. 3**. The formation of secondary protonemal threads from phylloid cells (arrows) in *Entoshtodon pulchellus* explants grown on MS/2 medium supplemented with fructose. Scale bar = 200 µm (magnification 10×).

It is important to note that there were no statistically significant differences between those three experimental groups, i.e. between the control plants and the plants grown on the KNOP medium enriched with IBA and BAP separately. However, when combinations of IBA and BAP were applied, there was a significant decrease in IM compared to the control group ($P < 0.05$), which was especially observed in those plants grown on the KNOP medium supplemented with the highest concentration of IBA (0.3 µM) in combination with 0.1 µM BAP (Fig. 4A). These results may indicate that the potential synergistic effect of the two PGRs exerted a negative impact on the formation of new shoots in the examined species.

A peculiar situation was observed regarding the development of secondary protonemal patches in experiment type II. Protonemata were recorded in only two experimental groups, i.e. the plants developed measurable protonemal patches (Fig. 4B). Specifically, such protonemal patches were documented in the control group and in those plants grown on the medium supplemented with 0.3 µM IBA and 0.1 µM BAP, with the latter experimental group exhibiting a significantly smaller diameter of secondary protonema ($P < 0.05$) (Fig. 4B).

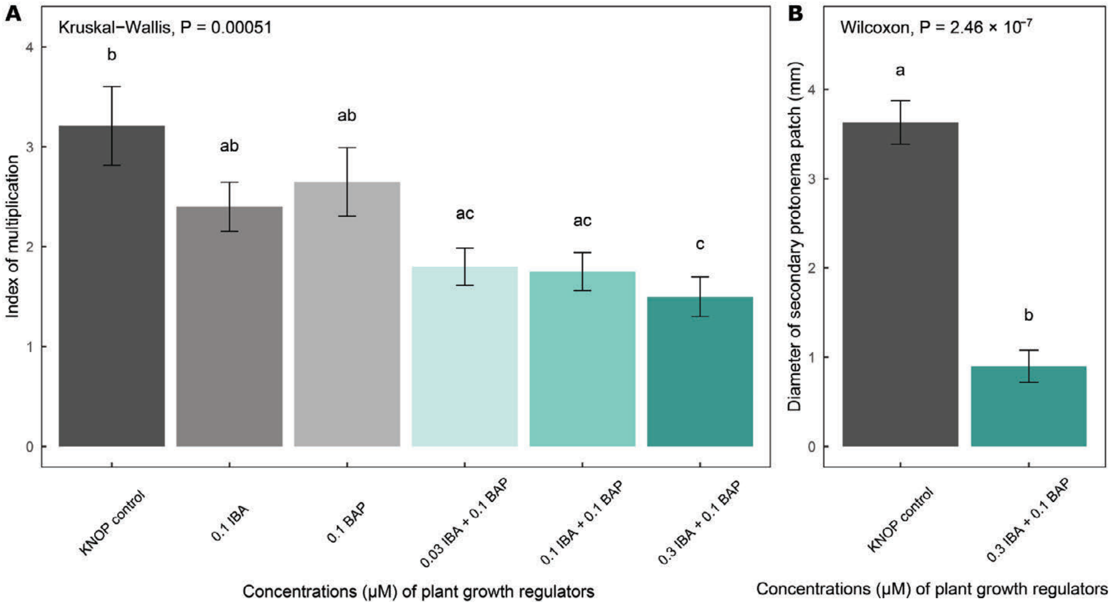

**Fig. 4**. The effect of KNOP growth media supplementation with the plant growth regulators on the index of multiplication (A) and diameter of secondary protonema patch (B) in experiment type II. The data are presented as the mean ± standard error. Different letters above the bars denote statistically significant differences ($P < 0.05$) between the experimental groups.

The plants grown on the KNOP media supplemented with PGRs developed normal phylloids and protonema, albeit smaller than the plants in the control group (Fig. 5).

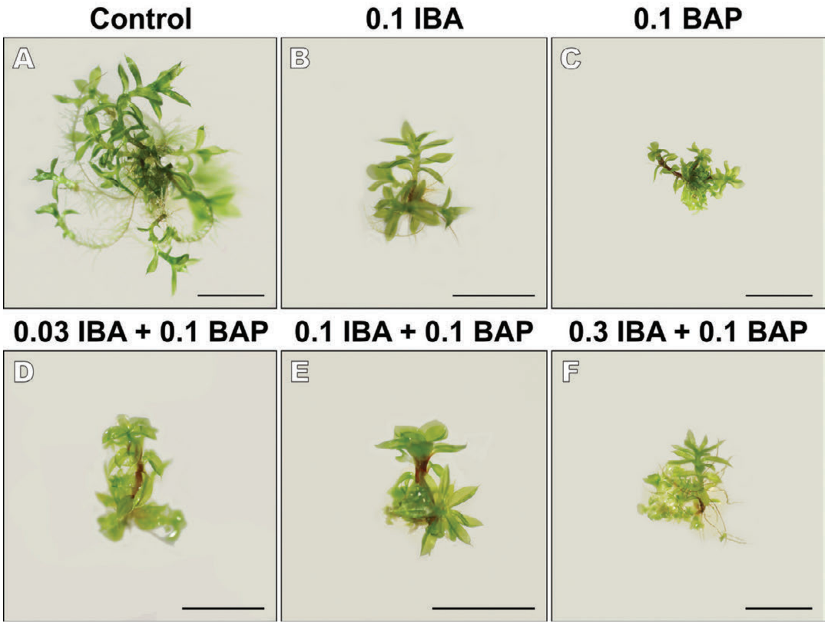

**Fig. 5**. The appearance of *Entosthodon pulchellus* explants grown on PGR-free KNOP media (A) and KNOP media supplemented with 0.1 µM IBA (B) and 0.1 µM BAP (C) individually or combined (D-F) in experiment type II. The bars represent the size of 2 mm in accordance with the magnification (0.63× for A, C and F; 0.8× for B and D; 1× for E).

The formation of small buds which did not develop into long shoots were documented in the plants grown on the medium with a combination of IBA and BAP (Fig. 5D-F).

Conversely, measurable secondary protonema only formed when IBA was used at a higher concentration than BAP (Fig. 4B and Fig. 5F). Overall, these results indicate that the applied concentrations of PGRs were not necessary for the micropropagation of *E. pulchellus* and that this species developed better in the absence of exogenously added IBA and BAP.

## Discussion

### The influence of different growth medium types and exogenously added sugars on the morphogenesis of *Entosthodon pulchellus*

In order to establish the conditions for the micropropagation of *E. pulchellus*, three different types of media were used in this study. According to the results obtained in experiment type I, both the MS/2 and KNOP media are equally suitable for long-term propagation (Fig. 1 and Fig. 2). In addition, the sucrose-enriched KNOP medium (KNOP+S) can also be used for the massive biomass production of secondary protonema, thus contributing to the vegetative growth and multiplication of this species *in vitro*. On the other hand, the BCD medium had negative effects on the development of shoots and protonemal filaments both when it was used sugar-free (Fig. 1A and Fig. 2C) and when enriched with sucrose (BCD+S) or fructose (BCD+F) (Fig. 1A and Fig. 2F, I). One of the possible reasons for such results may be the composition of the growth media used. Unlike the KNOP and half-strength MS media, the BCD medium contains potassium nitrate ($KNO_3$) in a rather high concentration, which may affect the development of *E. pulchellus*. However, the BCD medium has been shown to be suitable for the micropropagation of certain species *in vitro*, such as the bryo-halophyte *Hennediella heimii* (Hedw.) R.H.Zander (Ćosić et al. 2022), *Molendoa hornschuchiana* (Hook.) Lindb. ex Limpr. (Vujičić et al. 2012), and *Thamnobryum alopecurum* (Hedw.) Gangulee (Sabovljević et al. 2012). In addition, the bryo-halophyte *Entosthodon hungaricus* (Boros) Loeske formed more numerous new buds when cultured on a BCD medium than on a MS medium, although it did not form large patches of secondary protonema (Sabovljević et al. 2012a). Therefore, BCD is one of the common media types used in experiments for the *in vitro* propagation of mosses, but remains a rather peculiar one. Half-strength MS and KNOP minimal media, on the other hand, contain all essential nutrients in optimal amounts and are more suitable for bryophytic growth as bryophytes often live on mineral-poor substrates and do not have high nutrient requirements, nor do they absorb very much from the substrate in natural conditions.

In addition, numerous studies to date have shown that certain types of media are more suitable for the cultivation of mosses *in vitro* and that some species develop better on solidified media without sugars or PGRs (e.g. Sabovljević et al. 2022, Jadranin et al. 2023). In this study, the results clearly indicate that the addition of sugar was not necessary to promote the growth of *E. pulchellus* (Fig. 1 and Fig. 2), and that the addition of fructose in combination with BCD media can be detrimental (Fig. 5). In fact, the plants propagated better on sugar-free media, except in the case when sucrose was added in the minimal KNOP medium (KNOP+S) (Fig. 1 and Fig. 2). Stimulatory effects of moderate sucrose concentrations have already been documented for several moss species such as *Dicranum scoparium* Hedw. (Vujičić et al. 2009), *Atrichum undulatum* (Hedw.) P. Beauv. (Sabovljević et al. 2005), and *Pogonatum urnigerum* (Hedw.) P. Beauv. (Cvetić et al. 2007). In addition, some species did not develop buds in the absence of sucrose in the medium, such as *Leptobryum pyriforme* (Hedw.) Wilson and *Barbula gregaria* (Mitt.) A. Jaeger (Mitra & Allsopp 1959). Besides sucrose, other sugars have also been frequently used as stimulants for developmental processes. In *Bryum argenteum* Hedw., for example, fructose had a positive effect on the growth of protonemal patches (Bijelović et al. 2004) and on the formation of sex organs (Sabovljević et al. 2005) when applied in low to moderate concentrations. Sugars such as glucose, fructose and sucrose are essential components of plant metabolism and serve as energy suppliers (Hassid and Putman 1950). Although they are necessary for plant growth and development and play a role in responses to environmental stresses (Klavina 2014), high sugar concentrations may have negative effects on bryophyte growth and development *in vitro* since bryophytes are rather autotrophic in axenic conditions and do not require additional carbon sources for proper vegetative growth like tracheophytes (Sabovljević et al. 2022). In this study, fructose in combination with the MS/2 medium (MS/2+F) caused the occurrence of an interesting phenomenon (Fig. 3). The plantlets grown under these conditions showed a high degree of dedifferentiation of thread cells of secondary protonemata from which the new protonemal filaments emerged. This response to unfavourable conditions was previously documented for the phylogenetically related species *E. hungaricus* grown on a KNOP medium supplemented with 250 mM NaCl (unpublished data). It was hypothesised that this might be the strategy used by funaroid mosses to avoid conditions similar to osmotic stress which fructose could promote in combination with other compounds from MS medium. Nevertheless, there is a wide range of data in the literature on the influence of exogenous sugars on the formation of new shoots and buds, suggesting a *species*-specific response to additional carbon sources directly related to specific nutritional requirements and developmental strategies. According to the contradictory results in the literature, the results obtained for *E. pulchellus* are not unexpected, but further studies with more sugar species and a wider range of concentrations are needed to clarify their role in developmental processes in axenic conditions. Thus, the effects of sugars remain obscure both generally and specifically and they could have multiple roles in bryophytes when externally added, such as serving as a carbon source, or playing signalling, structural or functional roles.

### The influence of the plant growth regulators on the morphogenesis of *Entosthodon pulchellus*

Exogenously applied PGRs have different effects on developmental processes in bryophytes, as shown by studies conducted to date on various moss species (Sabovljević et al. 2014b, 2022). Some species develop spontaneously in axenic conditions, such as *Physcomitrella patens* (von Schwartzenberg 2009), while other species require exogenous PGRs for the induction of secondary protonema and bud formation. However, there is still a lack of data on how synthetic auxins and cytokinins in combination affect the morphogenesis of bryophytes and what the optimal concentration range is for the initiation and development of specific structures in bryophytes (Jadranin et al. 2023). This information is crucial for understanding the developmental process and requirements of bryophytes *in vitro* to ensure the optimal micropropagation and conservation of endangered and rare species.

In this study, the effects of IBA and BAP, applied both individually and in combination, on the vegetative growth and development of *E. pulchellus* were investigated (Fig. 4A, B). The plantlets responded to exogenous PGRs as expected, since all bryophytes synthesise essential phytohormones and possess receptors for them (Sabovljević et al. 2014b). When applied individually at low concentrations, both IBA and BAP led to normal gametophore development, albeit smaller in size than in the control plants (Fig. 4A and Fig. 5B, C). In certain species, BAP at low concentrations can promote the formation of new shoots, as in *Pterygoneurum sibiricum* Otnyukova (Jadranin et al. 2023) or *A. undulatum* (Bijelović et al. 2004). In contrast, when BAP is applied in high concentrations, it can induce the development of abnormal gametophores with small numbers of buds and shoots. This has been documented for *B. argenteum* (Bijelović et al. 2004), *H. heimii* (Ćosić et al. 2022), and *P. sibiricum* (Jadranin et al. 2023). In general, cytokinins influence bud formation at the caulonemal threads, dictating the formation of new gametophores in the protonemal patches (Ashton et al. 1979). Therefore, when applied at optimal concentrations, BAP may be effective in the vegetative propagation of some moss species.

However, when applied in conventional concentrations, BAP was not favourable for the propagation of *E. pulchellus*, as the plants spontaneously formed a large number of new, larger shoots in the absence of exogenous cytokinins (Fig. 5A). As in the BAP treatment, the plants developed normally when grown on the medium supplemented with 0.1 µM IBA, but failed to develop measurable secondary protonemata and a sufficient number of new shoots (Fig. 4B and Fig. 5B). It appears that prolonged treatment with auxins (Bopp 1963, Cvetić et al. 2007) or high concentrations of auxins (Ashton and Cove 1990) have negative effects on the development of protonemal patches *in vitro*. In addition, some forms of synthetic auxins may have positive effects on morphogenesis, while others have negative ones (Sarla and Chopra 1987). Therefore, more experiments should be conducted to identify the optimal concentrations of PGRs for the micropropagation of certain species.

On the other hand, when a combination of IBA and BAP was added to the medium, this had a negative effect on the development of new shoots and secondary protonemata of *E. pulchellus* (Fig. 4A and Fig. 5D-F). The plants mostly formed caulonemal cells or rhizoid-like structures, but no new buds. Generally, combinations of auxins and cytokinins are essential for proper plant development, but the optimal concentrations are difficult to achieve because responses to exogenous PGRs are often species-specific. The negative effects of the combination of IBA and BAP are well documented in the literature for the index of multiplication or secondary protonema diameter or both (e.g. Bijelović et al. 2004, Vujičić et al. 2011, Ćosić et al. 2022, Jadranin et al. 2023). In addition to the negative effects on protonemal development, IBA in combination with BAP may also lead to the formation of rhizoids *in vitro* (Sakakibara et al. 2003), which was also the case in this study. Interestingly, the plants grown on the medium supplemented with higher concentrations of IBA (0.3 µM) and a constant concentration of BAP (0.1 µM) developed protonemata and shoots, albeit to a much lesser extent than the plants in the control group. These results indicate that *E. pulchellus* regenerates better spontaneously and reproduces vegetatively without exogenously added PGRs.

Conversely, in some species cultivated under axenic conditions, the combination of auxin and cytokinins were found to stimulate the formation of new shoots, as in *M. hornschuchiana* (Vujičić et al. 2012), while only a low concentration of both hormones positively influenced the spreading of secondary protonemata. The moss *Drummondia stricta* (Mitt.) Müll. Hal. also developed better with the addition of auxins and cytokinins (Singh et al. 2017) than control plants grown on a KNOP medium. A positive effect on secondary protonema formation was also documented for *Bruchia vogesiaca* Nestl. ex Schwägr. (Sabovljević et al. 2012b) when IBA and BAP were applied in combination. Therefore, a possible solution for the negative effects of exogenous PGRs in *E. pulchellus* micropropagation could be to use other types of synthetic auxins or cytokinins or to vary the applied concentration range. It has already been suggested that IBA should be applied over a shorter period of time and at a lower concentration to achieve a positive effect in combination with BAP and avoid the negative results related to novel gametophore formations.

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## References

Ashton, N. W., Cove, D. J., 1990: Mutants as tools for the analytical dissection of cell differentiation in *Physcomitrella patens* gametophytes. In: Chopra, R. N., Bahtla, S. C. (eds.), Bryophyte development: physiology and biochemistry, 17–31. CRC Press, Boca Raton, FL.

Ashton, N. W., Grimsley, N. H., Cove, D. J., 1979: Analysis of gametophyte development in the moss *Physcomitrella patens*, using auxin and cytokinin resistant mutants. Planta 144, 427–435. https://doi.org/10.1007/BF00380118

Bijelović, A., Sabovljević, M., Grubišić, D., Konjević, R., 2004: Phytohormone influence on the morphogenesis of two mosses *Bryum argenteum* Hedw. and *Atrichum undulatum* (Hedw.) P. Beauv. Israel Journal of Plant Sciences 52, 31–36. https://doi.org/10.1560/RX30-U7QH-DEMQ-GL7P

Bopp, M., 1963: Development of the protonema and bud formation in mosses. Botanical Journal of Linnean Society 58(373), 305–309. https://doi.org/10.1111/j.1095-8339.1990.tb00899.x

Ćosić, M. V., Sabovljević, M. S., Papp, B., Giba, Z. S., Šinžar-Sekulić, J. B., Sabovljević, A. D., Vujičić, M. M., 2022: Micropropagation of rare bryo-halophyte *Hennediella heimii*. Botanica Serbica 46(2), 187–195. https://doi.org/10.2298/BOTSERB2202187C.

Cvetić, T., Sabovljević, A., Sabovljević, M., Grubišić, D., 2007: Development of the moss *Pogonatum urnigerum* (Hedw.) P. Beauv. under *in vitro* culture conditions. Archives of Biological Sciences 59(1), 57–61. https://doi.org/10.2298/ABS0701057C

Hallingbäck, T., Tan, B., 2010: Past and present activities and future strategy of bryophyte conservation. Phytotaxa 9, 266–274. https://doi.org/10.11646/phytotaxa.9.1.15.

Hassid, W. Z., Putman, E. W., 1950: Transformation of sugars in plants. Annual Review of Plant Biology 1, 109–124. https://doi.org/10.1146/annurev.pp.01.060150.000545

Hodgetts, N., Calix, M., Englefield, E., Fettes, N., Garcia Criado, M., Patin, L., Nieto, A., Bergamini, A., Bisang, I., Baisheva, E., Campisi, P., Cogoni, A., Hallingback, T., Konstantinova, N., Lockhart, N., Sabovljevic, M., Schnyder, N., Schrock, C., Sergio, C., Sim Sim, M., Vrba, J., Ferreira, C. C., Afonina, O., Blockeel, T., Blom, H., Caspari, S., Gabriel, R., Garcia, C., Garilleti, R., Gonzalez Mancebo, J., Goldberg, I., Hedenas, L., Holyoak, D., Hugonnot, V., Huttunen, S., Ignatov, M., Ignatova, E., Infante, M., Juutinen, R., Kiebacher, T., Kockinger, H., Kučera, J., Lonnell, N., Luth, M., Martins, A., Maslovsky, O., Papp, B., Porley, R., Rothero, G., Soderstrom, L., Ştefănuţ, S., Syrjanen, K., Untereiner, A., Vaňa, J., Vanderpoorten, A., Vellak, K., Aleffi M., Bates, J., Bell, N., Brugues, M., Cronberg, N., Denyer, J., Duckett, J., During, H. J., Enroth, J., Fedosov, V., Flatberg, K.-I., Ganeva, A., Gorski, P., Gunnarsson, U., Hassel, K., Hespanhol, H., Hill, M., Hodd, R., Hylander, K., Ingerpuu, N., Laaka-Lindberg, S., Lara, F., Mazimpaka, V., Mežaka, A., Muller, F., Orgaz, J. D., Patino, J., Pilkington, S., Puche, F., Ros, R. M., Rumsey, F., Segarra-Moragues, J. G., Seneca, A., Stebel, A., Virtanen, R., Weibull, H., Wilbraham, J., Żarnowiec, J., 2019a: A miniature world in decline: European Red List of Mosses, Liverworts and Hornworts. Brussels, Belgium: IUCN. ISBN 978-2-8317-1993-1 (PDF), 978-2-8317-1994-8 (print). https://doi.org/10.2305/IUCN.CH.2019.ERL.2.en

Hodgetts, N., Blockeel, T., Konstantinova, N., Papp, B., Schnyder, N., Schröck, C., Sergio, C., & Untereiner, A., 2019b: *Entosthodon pulchellus* (Europe assessment). The IUCN Red List of Threatened Species 2019b: e.T85841585A87794990. Retrieved May 18, 2024 from https://www.iucnredlist.org/species/85841585/87794990

Jadranin, B. Z., Ćosić, M. V., Božović, Đ. P., Vujičić, M. M., Ignatov, M. S., Ignatova, E. A., Sabovljević, A. D., Sabovljević, M. S., 2023: An insight into the biology of the rare and peculiar moss *Pterygoneurum sibiricum* (Pottiaceae): A conservation physiology approach. Plants 12(6), 1359. https://doi.org/10.3390/plants12061359

Jin, C., Huixia, K., Shubin, D., 2020: Population genetic structure and gene flow of rare and endangered *Tetraena mongolica* Maxim. reveald by reduced representation sequencing. BMC Plant Biology 20, 391. https://doi.org/10.1186/s12870-020-02594-y

Klavina, L., 2014: Polysaccharides from lower plants: Bryophytes. In: Ramawat, K. G., Mérillon, J. M. (eds.), Polysaccharides, 146–158. Springer, Berlin.

Löbel, S., Mair, L., Lönnell, N., Schröder, B., Snäll, T., 2018: Biological traits explain bryophyte species distributions and responses to forest fragmentation and climatic variation. Journal of Ecology 106(4), 1700-1713. https://doi.org/10.1111/1365-2745.12930

Martinčič, A. 2024: New checklist and the red list of the mosses (Bryophyta) of Slovenia. Hacquetia 23(1), 69–118. https://doi.org/10.2478-hacq-2023-0006

Mitra, G., Allsopp, A., 1959: Effects of kinetin, gibberellic acid and certain auxins on the development of shoot buds on the protonema of *Pohlia nutans*. Nature 183, 974–975. https://doi.org/10.1038/183974b0

Ostendorf, A. K., van Gessel, N., Malkowsky, Y., Sabovljevic, M. S., Rensing, S. A., Roth-Nebelsick, A., Reski, R., 2021: Polip-loidization within the Funariaceae – a key principle behind speciation, sporophyte reduction and the high variance of spore diameters? Bryophyte diversity and evolution 43(1), 164–179. https://doi.org/10.11646/bde.43.1.13

R Core Team, R. A., 2022. Language and environment for statistical computing; R Foundation for statistical computing, Vienna. Retrieved December 25, 2022 from https://www.R-project.org/.

Reski, R., Abel, W. O., 1985: Induction of budding on chloronemata and caulonemata of the moss *Physcomitrella patens*, using isopentenyladenine. Planta 165, 354–358. https://doi.org/10.1007/BF00392232

Rowntree, J. K., Pressel, S., Ramsay, M. M., Sabovljevic, A., Sabovljevic, M., 2011: In vitro conservation of European bryophytes. In Vitro Cellular and Developmental Biology – Plant 47(1), 55–64. https://doi.org/10.1007/s11627-010-9326-3

Sabovljević, A., Sabovljevic, M., Grubisic, D., Konjevic, R., 2005: The effect of sugars on development of two moss species (*Bryum argenteum* and *Atrichum undulatum*) during *in vitro* culture. Belgian Journal of Botany 138, 79–84. https://doi.org/10.2307/20794569

Sabovljević, A., Sabovljević, M., Jocković, N., 2009: In vitro culture and secondary metabolite isolation in bryophytes. In: Jain, S. M., Saxena, P.K. (eds.), Methods in molecular biology, protocols for *in vitro* cultures and secondary metabolite analysis of aromatic and medicinal plants, vol. 547, 117–128. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-60327-287-2_10

Sabovljević, A., Vujičić, M., Skorić, M., Bajić-Ljubičić, J., Sabovljević, M., 2012: Axenically culturing the bryophytes: Establishment and propagation of the pleurocarpous moss *Thamnobryum alopecurum* Nieuwland ex Gangulee (Bryophyta, Neckeraceae) in *in vitro* conditions. Pakistan Journal of Botany 44, 339–344.

Sabovljević, M., Papp, B., Sabovljević, A., Vujičić, M., Szurdoki, E., Segarra-Moragues, J. G., 2012a: *In vitro* micropropagation of rare and endangered moss *Entosthodon hungaricus* (Funariaceae). Bioscience Journal 28, 632–640.

Sabovljević, M., Vujičić, M., Šinžar-Sekulić, J., Segarra-Moragues, J. G., Bapp, B., Skorić, M., Dragačević, L., Sabovljević, A., 2012b: Reviving, in vitro differentiation, development and micropropagation of the rare and endangered moss *Bruchia vogesiaca* (Bruchiaceae). HortScience 47(9), 1347–1350. https://doi.org/10.21273/HORTSCI.47.9.1347

Sabovljević, M., Vujicic, M., Pantovic, J., Sabovljevic, A., 2014a: Bryophyte conservation biology: *in vitro* approach to the ex situ conservation of bryophytes from Europe. Plant Biosystems 148(4), 857–868. https://doi.org/10.1080/11263504.2014.949328

Sabovljević, M. S., Vujičić, M. M., Sabovljević, A. D., 2014b: Plant growth regulators in bryophytes. Botanica Serbica 38(1), 99–107.

Sabovljević, M. S., Ćosić, M. V., Jadranin, B. Z., Pantović, J. P., Giba, Z. S., Vujičić, M. M., Sabovljević, A. D., 2022: The conservation physiology of bryophytes. Plants 11(10), 1282. https://doi.org/10.3390/plants11101282

Sakakibara, K., Nishiyama, T., Sumikawa, N., Kofuji, R., Murata, T., Hasebe, M., 2003: Involvement of auxin and a homeodomain-leucine zipper I gene in rhizoid development of the moss *Physcomitrella patens*. Development 130(20), 4835–4846. https://doi.org/10.1242/dev.00644

Sarla, X., Chopra, R. N., 1987: Effect of some auxins and antiauxins on protonemal growth and bud formation in *Bryum pallescens* Schleich. ex Schwaegr. grown *in vitro*. Plant Science 51(2–3), 251–256. https://doi.org/10.1016/0168-9452(87)90200-7

Singh, V. J., Gupta, D., Sahu, V., Asthana A., K., 2017: *In vitro* growth pattern of moss *Drummondia stricta* (Mitt.) Müll. Hal. (Orthotrichaceae) in different hormonal concentrations. Recent Advances in Botanical Science 1, 1–7. https://doi.org/10.2174/9789811433788120010005

Thiers, B., 2024: Index Herbariorum: A global directory of public herbaria and associated staff, New York Botanical Garden's Virtual Herbarium. Retrieved May 8, 2024 from https://sweetgum.nybg.org/science/ih/

von Schwartzenberg, K., 2009: Hormonal regulation of development by auxin and cytokinin in moss. In: Knight, C., Perroud, P.-F., Cove, D. (eds.), The moss *Physcomitrella patens*, vol. 36, 246–281. Wiley Online Library, Hoboken, NJ.

Vujičić, M., Sabovljević, A., Sabovljević, M. 2009: Axenically culturing the bryophytes: a case study of the moss *Dicranum scoparium* Hedw. (Dicranaceae, Bryophyta). Botanica Serbica 33(2), 137–140.

Vujičić, M., Sabovljević, A., Sabovljević, M., 2011: Axenically culturing the bryophytes: Establishment and propagation of the moss *Hypnum cupressiforme* Hedw. (Bryophyta, Hypnaceae) in *in vitro* conditions. Botanica Serbica 35, 71–77.

Vujičić, M. M., Sabovljević, A. D., Šinžar-Sekulić, J., Skorić, M., Sabovljević, M., 2012: *In vitro* development of the rare and endangered moss *Molendoa hornschuchiana* (Hook.) Lindb. ex Limpr. (Pottiaceae, Bryophyta). Hort Science 47(1), 84–87. https://doi.org/10.21273/HORTSCI.47.1.84

Zanatta, F., Engler, R., Collart, F., Broennimann, O., Mateo, R. G., Papp, B., Munoz, J., Baurain, D., Guisan, A., Vanderpoorten, A., 2020: Bryophytes are predicted to lag behind future climate change despite their high dispersal capacities. Nature Communications 11, 5601. https://doi.org/10.1038/s41467-020-19410-8

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# Flavonoid composition and localization in trichomes and leaves of *Degenia velebitica* (Brassicaceae)

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**Abstract** – *Degenia velebitica* (Degen) Hayek, the most important representative of the Croatian endemic flora, is a heliophyte and xerophyte highly specialized for survival in extreme living conditions characterised by intense solar radiation. In this work, the composition and *in situ* localization of flavonoids in the epidermal trichomes and leaf tissues of *D. velebitica* were investigated. Flavonoids were localized in the lumens of stellate trichomes, vacuoles and cell walls of epidermal cells, but only in peripheral parts of spongy and palisade parenchyma cells, on both the abaxial and adaxial sides of the leaf. Quercetin, kaempferol and isorhamnetin were the main flavonoid aglycones in extracts of trichomes and leaves. The results show the important physiological role of epidermal trichomes and flavonoids in protecting *D. velebitica* against insolation.

**Keywords:** fluorescence microscopy, isorhamnetin, kaempferol, quercetin, stellate trichomes, UV protection, xeromorphysm

## Introduction

*Degenia velebitica* (Degen) Hayek is a monotypic plant genus from the Brassicaceae family and a stenoendemic in the Croatian flora, found in only a few locations along the Velebit and the Kapela mountains (Nikolić et al. 2015). It grows as a low, cushion-like plant whose vegetative parts are silver-gray (Fig. 1A). The branched, perennial rootstock bears numerous shoots, both fertile and sterile. The fertile shoots, up to 10 cm high, are unbranched, with densely packed leaves and a terminal inflorescence (Fig. 1B) of several relatively large flowers (up to 1 cm) intensely yellow in colour (Domac 1993). The sterile shoots are short, with a rosette of narrowly outlined leaves that are densely covered with trichomes, i.e. hairs (Fig. 1C). In July, the characteristic fruits are formed – ellipsoidal, spongy, swollen capsules, which are also silvery-gray due to the hair coverage (Fig. 1D). Upon ripening, the fruit opens and releases two flat seeds (Fig. 1E). With its characteristic and decorative leaves, flowers and fruits, *D. velebitica* is well known to amateur gardeners, professional growers, experts and botanists around the world, making it the main representative of the rich Croatian national flora and a symbol of its mountains.

*Degenia velebitica* was discovered by Hungarian botanist Arpad Degen during his botanical research on Velebit in July 1907; he clasified it in the genus *Alyssum* or *Vesicaria* (Degen 1909). The following year, Austrian botanist August Hayek concluded that it was a completely new genus and species in the European flora and named it *Degenia velebitica* (Hayek 1910) in honor of his colleague and its discoverer. Phylogenetic studies based on nuclear and plastid DNA sequence data nested *D. velebitica* in a clade consisting of five genera (*Alyssoides* Mill., *Clastopus* Bunge ex Boiss., *Degenia* Hayek, *Resetnikia* Španiel, Al-Shehbaz, D.A.German & Marhold, and *Physoptychis* Boiss.) within a monophyletic tribe Alysseae DC (Rešetnik et al. 2013). Because of its small population and distribution, estimated at 4.8 ha with 37,000 individuals (Liber et al. 2020), it has been a strictly protected species since 1964 and currently has the status of endangered species (Official Gazette 2013, Nikolić 2015-2025). Since it has a high percentage of seed germination (Naumovski 2005), classical cultivation has become the basis for *ex situ* conservation of this taxon as part of the Program for the Protection of Croatian Endemic Plant Taxa in the Botanical Garden of the Biology Department of the Faculty of Science in Zagreb (Stamenković et al. 2010).

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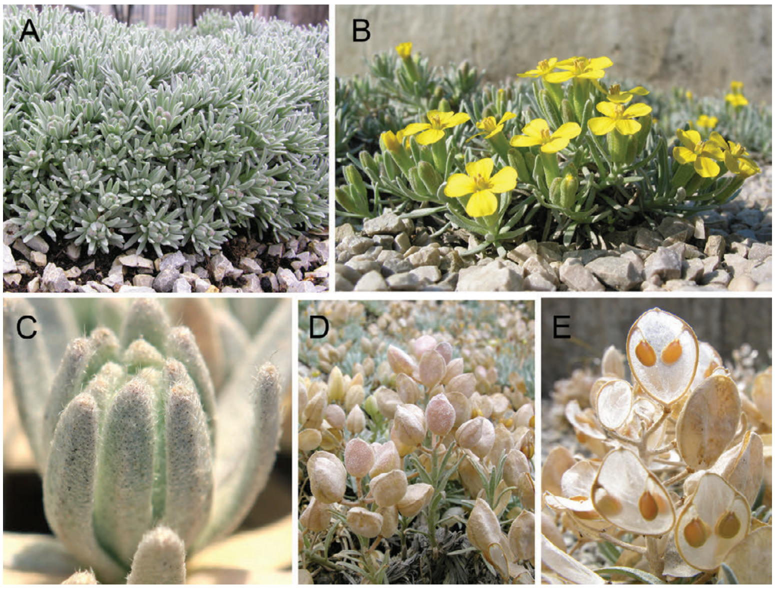

**Fig 1.** *Degenia velebitica*: A – plant habit, B – flowers, C – leaves densely covered with trichomes, D – ripe fruits, E – seeds.

*Degenia velebitica* grows in limestone screes and in rock crevices (Horvat 1930, 1931) exposed to intense insolation in summer and cold northeast winds in winter. Such extreme conditions define the ecological niche of this species, and account for its specific anatomical and morphological adaptations. Among the most important of these are the xeromorphic and heliomorphic structure of the leaves and stems including a dense coverage of stellate hairs, compact leaf mesophyll, numerous small stomata and short and thickened stem internodes, well-developed central conductive elements of the xylem and an extremely branched and long root (Stevanović and Vujnović 1990). Analysis of volatile compounds in *D. velebitica* leaves revealed eugenol, 2-methoxy-4-vinylphenol and benzyl alcohol as the main volatile O-aglycones, (9Z,12Z)-octa-9,12-dienic acid, hexadecanoic acid and phytol as the main free volatiles while the $H_2O$-soluble volatile fraction contained mostly glucosinolate degradation products and 3,4,5-trimethylpyrazole (Mastelić et al. 2010). However, more detailed research on the leaf structure and its flavonoid composition is lacking. Flavonoids are a diverse group of hydroxylated phenolic compounds with an aromatic ring structure performing a vast range of biological functions, including stress protection (Singh et al. 2021). The aim of this work was to investigate the anatomical characteristics of the *D. velebitica* leaf and the composition and localization of flavonoids in the leaf and epidermal trichomes as a posible protection against unfavorable environmental conditions.

## Materials and methods

### Plant material

Analyses were done on *D. velebitica* adult plants that have been grown from seeds in the Botanical Garden for several years. The daily solar radiation values, measured at the station for permanent monitoring Zagreb-1, which is close to the Botanical Garden, were from 5 W $m^{-2}$ in the winter to the 45 W $m^{-2}$ in the summer while temperatures were from -3 °C to 30 °C.

### Leaf and hair structure

The basic anatomical features of the leaf structure were determined on medium-thick freehand transverse sections of fresh *D. velebitica* leaves incubated in a drop of 100 mM potassium phosphate buffer (pH 7.0). The sections were observed with an Olympus CX21 light microscope and the images were visualized and digitized using a DinoEye Eyepiece Camera (Dino-Lite, Netherlands) controlled by the Dinocapture 2.0 computer program. The shape, size, arrangement and surface composition of epidermal trichomes were determined using a scanning electron microscope (SEM) TS5136 (TeScan, Czech Republic) with an integrated energy dispersive spectrometer (Oxford, USA) and the associated computer program for visualization, digitization and data processing.

### Localization of flavonoids by epifluorescence microscopy (EFM)

Flavonoid compounds in the leaves of *D. velebitica* were localized according to a modified method of Hutzler et al. (1998) and Tattini et al. (2000). Fresh leaves of the plants grown in the sun were transferred to the laboratory and immediately used for microscopy. Transverse and paradermal sections of the leaves were incubated in a drop of 100 mM potassium phosphate buffer (pH 7.0). The autofluorescence of individual structures was observed using a BX51 epifluorescence microscope (Olympus, Japan) with a WU-BP330-385 excitation filter (exc = 330 – 385 nm) and a BA-420 cutoff filter (em = 420 nm), and the micrographs were visualized and digitized using an Olympus DP70 digital camera controlled by the Olympus DP Controller 2002 program. After observation of the autofluorescence, a drop of 0.5% (w/v) aqueous ammonia solution or 0.5% (w/v) 2-amino-ethyl diphenyl boronic acid solution (“Naturstoff” reagent, NR) in 100 mM potassium phosphate buffer was added. After incubation for 1 to 5 min, excess solution was removed by rinsing in the buffer. The secondary fluorescence of phenolic compounds after treatment with NR and fluorescence enhanced after alkalization with ammonia were observed at the same cross-section positions as autofluorescence.

### Localization of flavonoids by confocal laser scanning microscopy (CLSM)

Flavonoids were also localized in medium-thick cross-sections of fresh *Degenia* leaves by a TCS-SP2-AOBS confocal microscope (Leica, Germany) at an excitation wavelength of an argon laser of 488 nm (exc = 488 nm) and 514 nm (exc = 514 nm) according to the method of Hutzler et al. (1998) and Agati et al. (2009). The leaf sections were treated in the same way as for fluorescence microscopy with the autofluorescence of the tissue observed before incubation and the secondary fluorescence after NR treatment. Detection of chlorophyll fluorescence was in the range 670 - 750 nm (exc = 514 nm), and flavonoid secondary fluorescence was in the range from 560 to 600 nm (exc = 488 nm) using the Leica Confocal Software 2.61 computer program.

### Determination of flavonoids by high-performance liquid chromatography (HPLC)

Major flavonoid aglycones were analyzed in methanolic extracts of lyophilised leaves, detrichomized leves and trichomes incubated for 30 min at +80 °C and acidified with HCl until the final concentration of 5 M. Analytes were separated on a reverse-phase C18 Bischoff Zorbax ODS column (5 µm, 250 × 4.6 mm) with pre-column (5 × 4.6 mm) using a Perkin Elmer Series 200 system with a UV/VIS diode-array detector. The elution with mobile phase consisting of 20% (v/v) methanol with 0.5% (v/v) $H_3PO_4$ (A) and 100% methanol (B) was performed with a 28 min linear gradient from 100% to 25% A, 4 min linear gradient from 75% to 100% B and a 4 min isocratic with 100% B (Stamenković et al. 2015). The flow rate was 1 mL min$^{-1}$, and elution was monitored at 280 and 374 nm. Identification and quantification of flavonoid aglycones was made by comparing retention times in combination with UV spectral data using standard solutions of quercetin, isorhamnetin and kaempferol. The results were expressed as mg per gram of dry weight and shown as the mean value of at least three replicates ± standard error. For statistical analysis Fisher's least significant difference (LSD) post hoc test was used to compare flavonoid content in different tissues.

## Results

### General characteristics of leaf

The linear lanceolate leaves of *D. velebitica* are relatively thick, between 400 and 600 µm. The adaxial and abaxial leaf blades are densely covered with unicellular epidermal stellate trichomes – hairs (Fig. 2A, B). A single hair consists of a short stalk and numerous branches at the apex. The cell wall of a hair is markedly thickened, while in the interior of the stalk and branches there is a cavity without noticeable cell structures, but with visible droplet inclusions (Fig. 2C, D).

Beneath the layer of hair covering is a single layer of epidermal cells with moderately thickened cell walls (Fig. 2C). The cuticle is poorly developed and thin. Numerous stomata are located on both sides of the leaf (Fig. 2C). In the epidermis, enlarged cells with thickened walls are also visible – basal epidermal cells from which unicellular hairs develop (Fig. 2D). The mesophyll of the leaf (Fig. 2E) is differentiated into a very well-developed palisade parenchyma and a

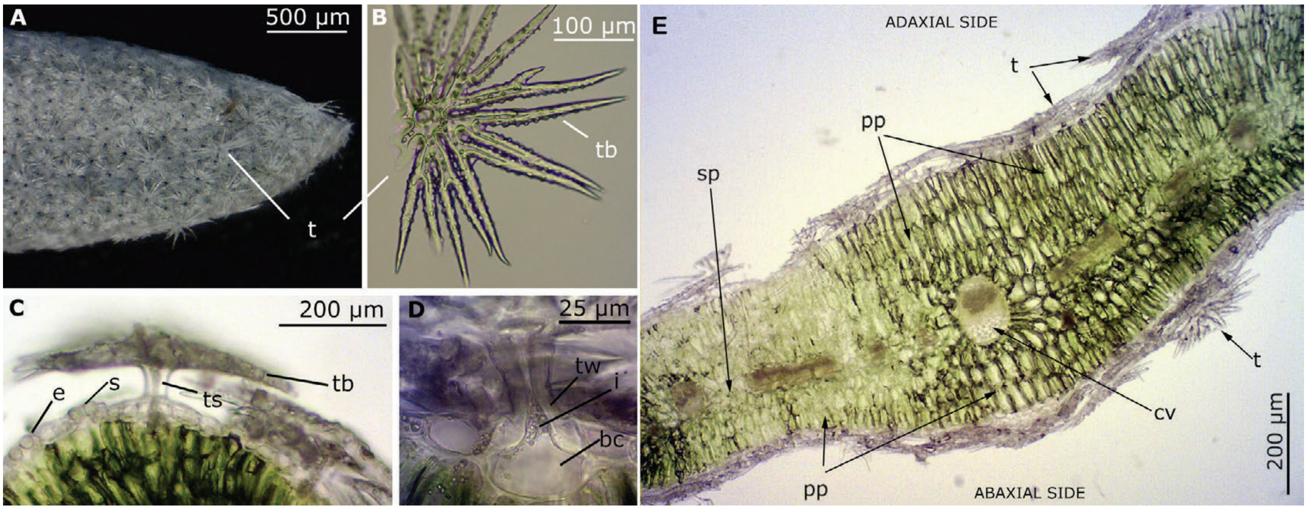

**Fig. 2.** Light micrographs of a *Degenia velebitica* leaf: leaf tip covered with stellate trichomes (A), isolated trichome (B), cross-section of the leaf margin (C) with enlarged detail of the hair shaft (D) and cross-section of a leaf (E). t – trichome, tb – trichome branch, ts – trichome stalk, tw – trichome cell wall, e – epidermis, bc – basal epidermal trichome cell, i – inclusions in the trichome lumen, s – stomata, pp – palisade parenchyma, sp – spongy parenchyma, cv – central vascular vessel.

less developed spongy parenchyma. The leaf is of the isobilateral type with three layers of elongated (cylindrical) palisade parenchyma cells on the adaxial side and two layers of palisade cells on the abaxial side. The spongy parenchyma is reduced and limited to the central part of the leaf, and is made up of several layers of small, round cells. The mesophyll cells, especially the palisade cells, are very compact, so the intercellular spaces are difficult to see. In the central part of the leaf there is a central conducting vein (Fig. 2E).

### Morphology of epidermal trichomes by a scanning electron microscope (SEM)

The trichomes are densely arranged on the adaxial and abaxial sides of the lamina and cover the green parts of the leaf as a shield in at least two layers (Fig. 3A, B). The trichomes are of the stellate type, composed of a short stalk with a thickened slightly protruding center from which a dozen branches radiate, dichotomously divided into most often two (sometimes three) long tips. The tips of the branches are straight and very slightly bent to follow the leaf shape (Fig. 3B, C, D). The diameter of the upper, branched part of the hair is usually very large, up to 400 µm, so the hairs on the leaf surface are visible even to the naked eye. The adaxial surface of the trichome branches is tuberculate – covered with numerous small, elongated tubercles (Fig. 3E) up to 10 µm long, while the abaxial surface facing the epidermis is smooth (Fig. 3D, F).

The trichome stalks are short, 30 to 40 µm high and about 20 µm in diameter at the base. Micrographs of the stalk show a thickened, lignified cell wall and a cavity, which is filled with cell debris to a lesser (Fig. 3F) or greater (Fig. 3G) extent. Precipitated calcium carbonate crystals of non-biogenic origin were visible on some trichomes (Fig. 3F). Spectrometric analysis with a measurement point on the surface of the trichome showed high contents of oxygen (49.87%), carbon (38.08%) and calcium (12.04%).

### Localization of flavonoids by epifluorescence microscopy (EFM)

In micrographs of the adaxial leaf section (Fig. 4A), the autofluorescence of cellular structures was clearly visible: light blue of the thick cell walls of the trichomes, bluish-green of the cuticle and outer walls of the epidermal cells with the rest of the cell being blue-violet. In the palisade cells, green fluorescence of cell walls, red fluorescence of chloroplasts and blue-violet fluorescence of vacuoles was observed.

After a 3-minute incubation with NR, secondary yellow fluorescence of flavonoids appeared having the most intensive signal within the cuticule (Fig. 4B) and guard cells of stomata (Fig. 4D). The epidermal cells showed intense yellow fluorescence of the walls and weaker fluorescence of vacuole content. In contrast, in the palisade parenchyma, yellow fluorescence was observed only in the peripheral parts of the cells corresponding to the cell wall, plasmalemma or cytosol while the blue-violet fluorescence of vacuoles and the red fluorescence of chlorophyll in the chloroplasts remained unchanged. In the trichomes the change in fluorescence occurred after 7-minute incubation with NR, with the central parts of the stalk and branches fluorescencing yellowish green, and the thick walls remaining blue (Fig. 4C).

On the transverse section of a leaf incubated for two minutes in ammonium hydroxide solution (Fig. 4E) an increase in green fluorescence of flavonoids was observed. Their localization partially corresponded to the sections

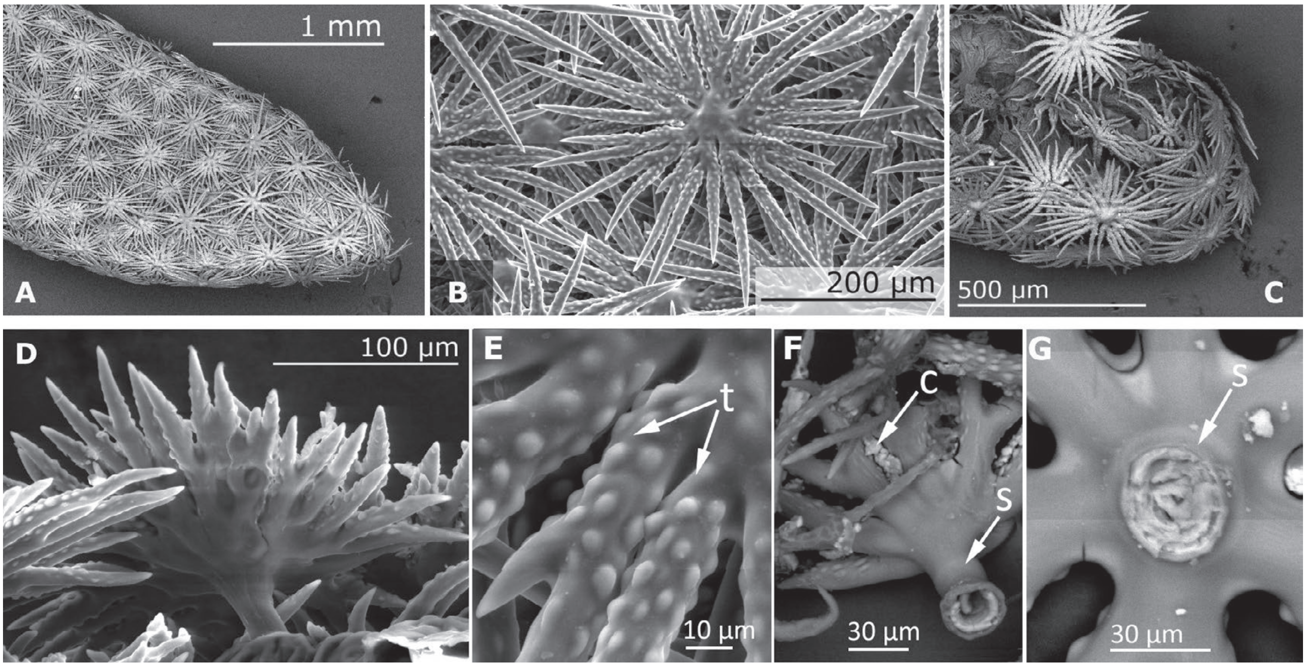

**Fig. 3.** Scanning electron micrograph of epidermal trichomes of *Degenia velebitica*: leaf tip covered with trichomes (A), trichomes from above (B) and from the side (C), trichome adhered to epidermis (D), tuberculate adaxial surface of trichome branch (E), trichome stalk from the abaxial side (F) and basal part of trichome stalk (G). t – tubercle or nodule, s – trichome stalk and c – calcium carbonate crystals.

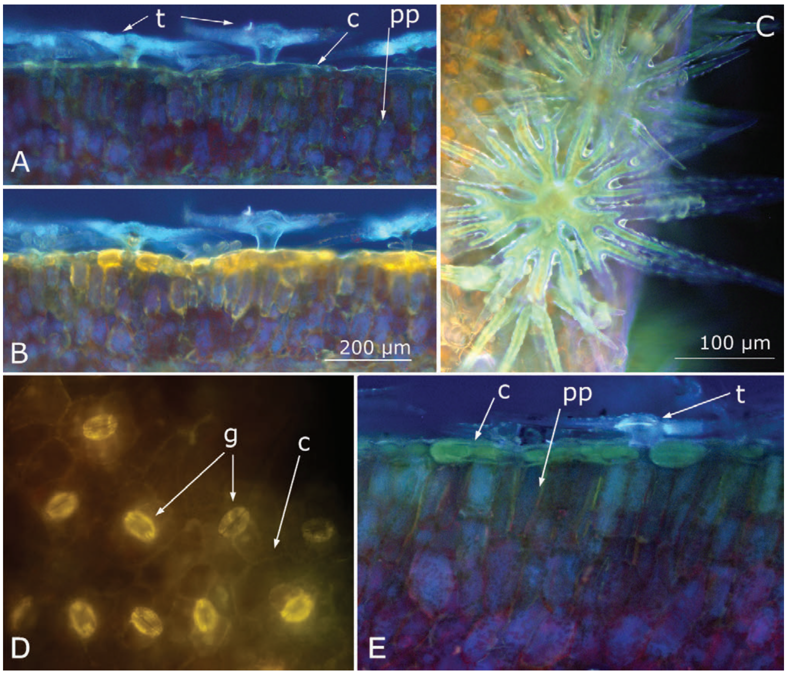

**Fig. 4.** Histochemical visualization of flavonoids in *Degenia velebitica* leaves by epifluorescence microscope: the autofluorescence of trichomes, epidermis and upper layers of palisade parenchyma in untreated leaf (A), secondary yellow fluorescence of the same tissue section (B), trichomes (C), and paradermal leaf section with guard cells (D) after incubation with "Naturstoff" reagent, and induced green fluorescence of epidermal and palisade cells after incubation with ammonium hydroxide solution (E). t – trichome, c – cuticle, e – epidermis, pp – palisade parenchyma, g – guard cell.

treated with NR – the epidermal cells fluoresced most intensely, but in addition to the fluorescence of the cell walls, intense fluorescence of the vacuolar contents of almost all cells was also visible. Cells of the upper layers of the palisade parenchyma in the area of the cell wall and cytosol also fluoresced, especially on the adaxial side of the leaf.

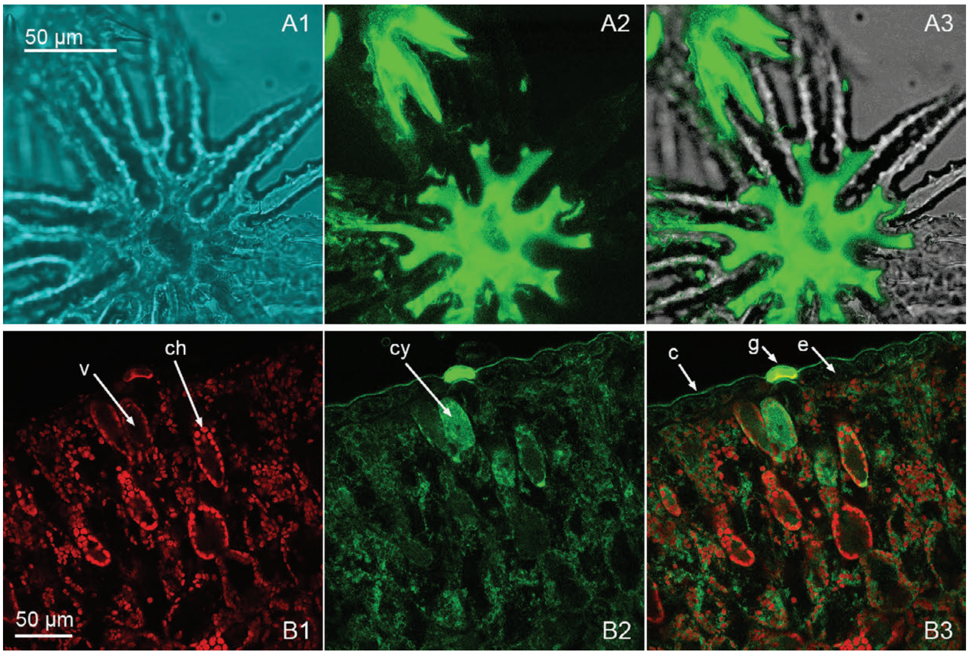

**Fig. 5.** Histochemical visualization of flavonoids in *Degenia velebitica* trichomes and leaf tissues by confocal laser scanning microscopy: the trichome in bright field (A1), the secondary fluorescence of flavonoids inside the trichome in pseudo-green color after the overlapping of seven separate micrographs that were obtained by scanning at different depths (A2), and an overlapping image of the previous two (A3); cross-section of a leaf first excited for autofluorescence of chlorophyll and visualized in pseudo-red color (B1), then excited at 488 nm after incubation with "Naturstoff" reagent, and monitoring the secondary fluorescence of flavonoids visualized in pseudo-green color (B2) and after overlapping the images of the two channels (B3). c – cuticle of the outer epidermal cell wall, ch – chloroplasts, cy – cytoplasm, e – epidermis, g – guard cells, v – vacuole.

### Localization of flavonoids by confocal laser scanning microscopy (CLSM)

Secondary fluorescence of flavonoids was visible in the interior of the stalk and branches of epidermal trichomes that were scraped from the surface of fresh leaves and incubated with NR for 5-6 minutes. The most intense signal was visible in the area of the stalk and in the initial, wider parts of the canal of the branches (Fig. 5A2, A3).

The micrographs of leaf sections incubated with NR show cuticle (Fig. 5B2, B3) with an intense yellow fluorescence, in contrast to the epidermal cells, where the fluorescence was weaker. The most intense signal was observed in guard cells, where all parts of the cells fluoresced uniformly. The palisade cells had clearly visible chloroplasts (red fluorescence, Fig. 5B1, B3) located around a large, central vacuole while the secondary flavonoid fluorescence was visible in the peripheral area of the cells between the vacuole and the cell wall (Fig. 5B2, B3). No significant fluorescence was observed in the vacuoles.

### Determination of flavonoids

Acidic methanolic extracts of *D. velebitica* leaves and trichomes were analysed by HPLC to detect flavonoid aglycons resulting from acid hydrolysis. In both extracts, one major peak at retention time 23.99 min and two minor peaks at retention times 26.81 and 27.4 min were detected (Fig. 6A).

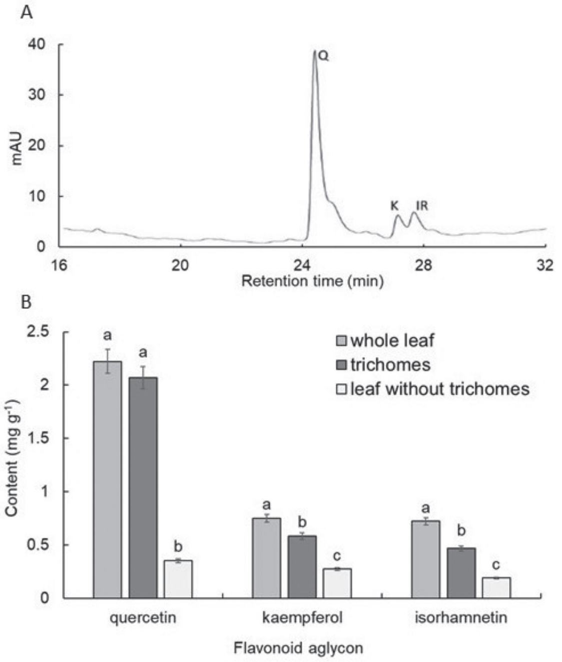

**Fig. 6.** *Degenia velebitica* flavonoid aglycones: chromatogram of acidic methanolic leaf extract showing peaks of quercetin - Q, kaempferol – K and isorhamnetin – IR (A) and content of flavonoids measured in whole leaf, trichomes or leaf with removed trichomes (B). The results are the mean value of three replicates ± standard error. Different letters indicate a significant difference between flavonoids in whole leaf, trichomes or leaf with removed trichomes according to LSD test ($P \leq 0.05$).

According to the retention times and UV spectra of pure standards the major peak was identified as quercetin while the two minor peaks were identified as kaempferol and isorhamnetin. Quantification showed that leaves and trichomes mostly contained quercetin, while kaempferol and isorhamnetin were less represented (Fig. 6B). Flavonoids were most abundant in trichomes. The concentration of quercetin in the trichome extract was even six times higher than that in the extract of leaves from which the hairs were removed. Also, the trichome extract contained two times more kaempferol and 2.5 times more isorhamnetin than the extract of detrichomized leaves.

## Discussion

The leaves of *D. velebitica* are equifacial and amphistomatic, densely covered with epidermal trichomes (hairs) on both sides, which is visible even macroscopically. The stellate hairs are branched forming as many as two protective layers through which the epidermis is almost not visible. They are dead, filled with air and silvery-white because of the total reflection of light. Non-glandular trichomes are always composed of dead cells and this »hairy layer« prevents the sun falling directly on the epidermis, reducing its temperature which consequently reduces the level of transpiration (Dunkić et al. 2001). These anatomical features are typical of many xerophytes and heliophytes, i.e. they reflect the adaptation of plants to life in dry habitats with extreme insolation (Fahn and Cutler 1992). Trichomes are one of the first lines of defence against abiotic stresses such as UV exposure, water loss, temperature extremes and herbivore damage (Watts and Kariyat 2021). Similar morphological and anatomical features of stellate trichomes have been identified in leaves of the closest relatives within the tribe Alysseae, *Alyssoides utriculata* (Ančev and Goranova 2006) and *Resetnikia triquetra* (as *Fibigia t.*, Damjanović and Stevanović 1993). Brassicaceae trichomes are diverse in morphology, including malpighiaceous (or T-shaped) trichomes and stellate trichomes which could be radiate-stellate or peltate-stellate trichomes with dendritic branches as in the tribe Alysseae and are associated with arid habitats (Karabourniotis et al. 2020). Trichomes of *D. velebitica* have thickened, lignified walls and a spectrometric analysis in our study determined a high percentage of oxygen, carbon and calcium, which indicates the presence of calcium carbonate. It is known that silica, calcium carbonate and calcium phosphate play a prominent role in the biomineralization of trichomes, and the Brassicaceae family has trichomes mineralized with both calcium carbonate and calcium phosphate (Hopewell et al. 2021). Across different species of Brassicaceae from the tribe Alysseae to which *D. velebitica* belongs, the most common trichome was radiate-stellate with forked to dendritic branches, usually with a very rough surface and mineralized with calcium carbonate (Hopewell et al. 2021).

In cross-sections of *D. velebitica* leaves, the light blue autofluorescence (primary fluorescence) observed by EFM in thickened trichome walls, cell walls of outer epidermal cells and cuticle originates from UV-excited polyphenolic compounds such as lignin (Sylvester et al. 1991). The intense yellow fluorescence observed by EFM in the central cavities of the hairs when the cross-section is excited with UV light after 7 min incubation in NR is evidence of the presence of flavonoids. Flavonoids play many roles in plant adaptation to changing living conditions, including the regulation of growth and development, active antioxidant activity and removal of reactive oxygen species (ROS) from the cell as well as attenuation of harmful UV radiation (Agati and Tattini 2010, Shen et al. 2022). Naturstoff reagent, i.e. 2-amino ethyl diphenylboronic acid, is a fluorescent dye that forms specific adducts with flavonoids, whose absorption-emission spectra are shifted towards longer wavelengths resulting in yellow to yellow-green secondary fluorescence (Hutzler et al. 1998). For the flavonoid localization in living plant tissues using NR, the most appropriate incubation sample time was from 1.5 min (Tattini et al. 2000) to a maximum of 5 min (Hutzler et al. 1998, Agati et al. 2009). The longer incubation time required for the appearance of secondary fluorescence in *D. velebitica* trichomes can be explained by the difficulty of NR penetration into the central cavities of the trichomes, because they are firmly attached to the epidermis, and the cavities are partially filled with cellular debris. The secondary fluorescence of flavonoids in isolated fresh trichomes was confirmed by CLSM under blue light excitation. Required incubation time in NR was shorter, probably because the isolated trichomes were more accessible to the reagent. Due to the complexity of the identification of flavonoids in their glycoside forms, HPLC analysis of methanolic extracts after acid hydrolysis was performed, revealing the flavonols quercetin, kaempferol and isorhamnetin (3-methylquercetin), the main aglycones in trichomes as well as in leaves. According to our knowledge, within the tribe Alysseae, which includes *Degenia*, flavonoids have been investigated in the genera *Alysum* and *Aurinia* revealing highly glycosylated flavonols, di- and triglycosides of kaempferol, quercetin, and their methyl ethers (Bucar et al. 2005). In *Alyssum alyssoides* kaempferol 3-O-β-D-glucopyranoside (astragalin), kaempferol 3-O-(6″-α-L-rhamnopyranosyl)-β-D-glucopyranoside (nicotiflorin), quercetin 3-O-β-D-glucopyranoside (isoquercetin), quercetin 3-O-β-D-galactopyranoside (hyperoside), isorhamnetin 3-O-β-D-glucopyranoside, isorhamnetin 3-O-β-D-galactopyranoside and isorhamnetin 3-O-(6″-α-L-rhamnopyranosyl)-β-D-glucopyranoside (narcissin) were identified (Tsiftsoglou et al. 2019). Interestingly the concentration of flavonoids, especially quercetin, was several times higher in trichome extracts than in extracts of detrichomized *Degenia* leaf. It is known that simple hairs, i.e. non-glandular trichomes, may contain different flavonoids (Karabourniotis et al. 2020). Methylated kaempferol glycosides were identified in non-glandular trichomes of holm oak leaves (Skaltsa et al. 1994) while kaempferol glycoside derivatives were identified in dendritic trichomes of *Cistus salvifolius* leaves and their ability to absorb UVB and UVA radiation was proven (Tattini et al. 2007). In a study in which *D. velebitica* response to UV stress was investigated, the concentrations of aglycones quercetin, kaempferol and isorhamnetin were significantly higher in UV-exposed plants than in plants grown without UV stress (Stamenković et al. 2015). UV radiation, as well as intense PAR radiation, causes a strong increase in the concentration of flavonoids, specifically of dihydroxy-B-substituted flavonols such as quercetin derivatives which besides their photoprotective role also have a strong antioxidant property (Laoué et al. 2022). Quercetin aglycone and quercetin 3-O-rhamnoside were found in olive leaf trichomes (Liakopoulos et al. 2006). Moreover, the highest density of trichomes and the highest concentration of flavonoids were found in olive leaves on southern exposure and in the outer part of the crown the most exposed to the sun (Liakoura et al. 1997), confirming the role of trichomes in defense against UV radiation in plants with heliomorphic and xeromorphic characteristics. The importance of "hairiness" in the defense against UVB radiation was also demonstrated by the significantly reduced photosynthesis in detrichomized leaves compared to whole leaves in holm oak (Skaltsa et al. 1994) and *Verbascum* species (Manetas 2003).

In *D. velebitica* leaf sections, intense fluorescence after a short incubation in NR, observed by EFM and CLSM, showed the presence of flavonoids in guard cells of stomata while the fluorescence was weaker in the walls and cytoplasm of outer epidermal cells. NR-induced fluorescence was observed in guard cells of *Arabidopsis thaliana* (Brassicaceae) along with the accumulation of ROS that may act as a second messenger during stomatal opening (Watkins et al. 2017). These authors suggested that flavonols, acting as antioxidants, suppress ROS levels in guard cells and thus modulate the dynamics of the stomatal aperture. On transversal sections of *D. velebitica* leaves treated with aqueous ammonia and observed by EFM under UV light excitation, intense green fluorescence of almost all epidermal cells, including vacuoles was visible. It is known that only dihydroxy-B-substituted flavonoids, such as quercetin and isorhamnetin derivatives, after treatment with NR, create adducts that fluoresce intensely when co-excited with blue light (Agati et al. 2009), while glycosides of kaempferol, which is a monohydroxy-B-substituted flavonoid, fluoresce intensely only in alkaline solutions (Hutzler et al. 1998). Therefore, it can be concluded that kaempferol derivatives prevail in the vacuoles of the epidermal cells of *D. velebitica* leaves while quercetin derivates are present in guard cells, walls and cytoplasm of other epidermal cells. In palisade cells, intense fluorescence was limited only to the peripheral parts of the cells in the area of the cell wall, plasma membrane and cytoplasm while the vacuoles did not fluoresce either after incubation in NR, or in alkalized sections. Biosynthesis of flavonoids occurs mainly in the cytoplasm and flavonols are transported and stored in glycosylated form mainly in the vacuoles of guard cells, epidermal and subepidermal cells, but also in the cell walls of the epidermal cells as methylated flavonol glucosides and within the leaf cuticle which optimizes their role as UV-screeners or ROS scavengers (Laoué et al. 2022). Flavonoids are additionally associated to chloroplasts and the cell nucleus where they can reduce the ROS levels and preserve oxidative damage in various stress conditions (Agati et al. 2020). The above findings indicate that flavonoid accumulation during UV and PAR stress increases the attenuation of excess radiation, but also enhances the antioxidant capacity of the cell to resist oxidative stress.

## Conclusion

The localization of flavonoids in the leaf tissue, especially in epidermal cells of *Degenia velebitica*, emphasizes their role as UV filters, but also as non-enzymatic antioxidants. The presence of flavonoids, especially quercetin inside trichomes highlights the important role of this dense covering layer in protecting the sensitive leaf structures of *D. velebitica* from intense radiation, overheating and water loss.

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## References

Agati, G., Brunetti, C., Fini, A., Gori, A., Guidi, L., Landi, M., Sebastiani, F., Tattini, M., 2020: Are flavonoids effective antioxidants in plants? Twenty years of our investigation. Antioxidants 9(11), 1098. https://doi.org/10.3390/antiox9111098

Agati, G., Stefano, G., Biricolti, S., Tattini, M., 2009: Mesophyll distribution of 'antioxidant'flavonoid glycosides in *Ligustrum vulgare* leaves under contrasting sunlight irradiance. Annals of Botany 104(5), 853–861. https://doi.org/10.1093/aob/mcp177

Agati, G., Tattini, M., 2010: Multiple functional roles of flavonoids in photoprotection. New Phytologist 186(4), 786–793. https://doi.org/10.1111/j.1469-8137.2010.03269.x

Ančev, M., Goranova, V., 2006: Trichome morphology of eleven genera of the tribe Alysseae (Brassicaceae) occurring in Bulgaria. Willdenowia 36(1), 193–204.

Bucar, F., Plazibat, M., Knauder, E., Maleš, Ž., 2005: LC/PDA/MS/MS analysis of flavonoids in some taxa of *Alyssum* and *Aurinia* (Brassicaceae) from Croatia. In: Zorc, B. (ed.), Book of Abstracts of the Third Croatian Congress on Pharmacy with International Participation, 188–188. Faculty of Pharmacy and Biochemistry, Zagreb.

Damjanović, O., Stevanović, B., 1993: Morpho-anatomical adaptations of endemic species *Fibigia triquetra* (DC.) Boiss. (Brassicaceae). Bulletin de l'Institut et du Jardin Botaniques de l'Université de Beograd 24-25, 9–19.

Degen, A., 1909: Bemerkungen über einige orientalische Pflanzarten. LIII. Über die Entdeckung eines Vertreters der Gattung Lesquerella im Velebitgebirge. Magyar Botanikai Lapok 8, 3–24

Domac, R., 1993: *Degenia* Hayek. In: Tutin, T. G., Burges, N. A., Chater A. O., Edmondson J. R., Heywood, V., Moore, D. M., Valentine, D. H., Walters, S. M., Webb, D. A. (eds.), Flora Europaea, 359, vol. 1. 2nd ed. Cambridge University Press, Cambridge.

Dunkić, V., Bezić, N., Mileta, T., 2001: Xeromorphism of trichomes in Lamiaceae species. Acta Botanica Croatica 60(2), 277–283.

Fahn, A., Cutler, F. D., 1992: Xerophytes. Encyclopedia of Plant Anatomy, vol. 3. Gebrüder Bornträger, Berlin, Stuttgart.

Hayek, A., 1910: Die systematische Stellung von *Lesquerella velebitica* Degen. Oesterreichische botanische Zeitschrift 60, 89–93

Hopewell, T., Selvi, F., Ensikat, H. J., Weigend, M., 2021: Trichome biomineralization and soil chemistry in Brassicaceae from Mediterranean ultramafic and calcareous soils. Plants 10(2), 377. https://doi.org/10.3390/plants10020377

Horvat, I., 1930: Vegetacijske studije o hrvatskim planinama. Zadruge na planinskim goletima [Vegetation studies on Croatian mountains. Communities on mountain bare areas]. Rad Jugoslavenske Akademije Znanosti 238, 1–96.

Horvat, I, 1931: Vegetacijske studije o hrvatskim planinama. II. Zadruge na planinskim stijenama i točilima [Vegetation studies on Croatian mountains. II. Communities on mountain rocks and screes]. Rad Jugoslavenske Akademije Znanosti 241, 147–206.

Hutzler, P., Fischbach, R., Heller, W., Jungblut, T., Reuber, S., Schmitz, R., Veit, M., Weissenböck, G., Schnitzler, J., 1998: Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. Journal of Experimental Botany 49(323), 953–965. https://doi.org/10.1093/jxb/49.323.953

Karabourniotis, G., Liakopoulos, G., Nikolopoulos, D., Bresta, P., 2020: Protective and defensive roles of non-glandular trichomes against multiple stresses: structure–function coordination. Journal of Forestry Research 31(1), 1-12. https://doi.org/10.1007/s11676-019-01034-4

Laoué, J., Fernandez, C., Ormeño, E., 2022: Plant flavonoids in mediterranean species: A focus on flavonols as protective metabolites under climate stress. Plants 11(2), 172. https://doi.org/10.3390/plants11020172

Liakopoulos, G., Stavrianakou, S., Karabourniotis, G., 2006: Trichome layers versus dehaired lamina of *Olea europaea* leaves: differences in flavonoid distribution, UV-absorbing capacity, and wax yield. Environmental and Experimental Botany 55(3), 294–304. https://doi.org/10.1016/j.envexpbot.2004.11.008

Liakoura, V., Stefanou, M., Manetas, Y., Cholevas, C., Karabourniotis, G., 1997: Trichome density and its UV-B protective potential are affected by shading and leaf position on the canopy. Environmental and Experimental Botany 38(3), 223–229. http://dx.doi.org/10.1016/S0098-8472(97)00005-1

Liber, Z., Surina, B., Nikolić, T., Škrtić, D., Šatović, Z., 2020: Spatial distribution, niche ecology and conservation genetics of *Degenia velebitica* (Brassicaceae), a narrow endemic species of the north-western Dinaric Alps. Plant Systematics and Evolution 306(4), 64. https://doi.org/10.1007/s00606-020-01695-3

Manetas, Y., 2003: The importance of being hairy: the adverse effects of hair removal on stem photosynthesis of *Verbascum speciosum* are due to solar UV-B radiation. New Phytologist 158, 503–508.

Mastelić, J., Blažević, I., Kosalec, I., 2010: Chemical composition and antimicrobial activity of volatiles from *Degenia velebitica*, a European stenoendemic plant of the Brassicaceae family. Chemistry & Biodiversity, 7(11), 2755–2765. https://doi.org/10.1002/cbdv.201000053

Naumovski, D., 2005: Germination ecology of seeds of endemic species *Degenia velebitica* (Degen) Hayek (Brassicaceae). Acta Botanica Croatica 64(2), 323–330.

Nikolić, T., Milović, M., Bogdanović, S., Jasprica, N., 2015: Endemi u hrvatskoj flori [Endemics in Croatian flora]. Alfa d.d., Zagreb.

Nikolić, T. (ed.), 2015-2025: Flora Croatica Database. Retrieved April 5, 2025 from http://hirc.botanic.hr/fcd. Prirodoslovno-matematički fakultet, Sveučilište u Zagrebu.

Official Gazette (Narodne Novine), 2013: Pravilnik o strogo zaštićenim vrstama [Ordinance of strictly protected species]. 144. Croatian Government, Zagreb.

Rešetnik, I., Satovic, Z., Schneeweiss, G. M., Liber, Z., 2013: Phylogenetic relationships in Brassicaceae tribe *Alysseae* inferred from nuclear ribosomal and chloroplast DNA sequence data. Molecular Phylogenetics and Evolution 69(3), 772–786. https://doi.org/10.1016/j.ympev.2013.06.026

Shen, N., Wang, T., Gan, Q., Liu, S., Wang, L., Jin, B., 2022: Plant flavonoids: Classification, distribution, biosynthesis, and antioxidant activity. Food Chemistry 383, 132531. http://dx.doi.org/10.1016/j.foodchem.2022.132531

Singh, P., Arif, Y., Bajguz, A., Hayat, S., 2021: The role of quercetin in plants. Plant Physiology and Biochemistry, 166, 10–19. http://dx.doi.org/10.1016/j.plaphy.2021.05.023

Skaltsa, H., Verykokidou, E., Harvala, C., Karabourniotis, G., Manetasi, Y., 1994: UV-B protective potential and flavonoid content of leaf hairs of *Quercus ilex*. Phytochemistry 37(4), 987–990. http://dx.doi.org/10.1016/S0031-9422(00)89514-X

Stamenković, V., Kovačić, S., Juretić, B., Mihelj, D., 2010: *Ex-situ* protection program for Croatian endemic plant taxa in the Botanical Garden of the Department of Biology, Faculty of Science. In: Jasprica, N., Pandža, M., Milović, M. (eds.), Abstract Book of the Third Croatian Botanical Congress, 180–181. Croatian Botanical Society, Murter, Zagreb.

Stamenković, V., Likić, S., Tkalec, M., 2015: Defence response of *Degenia velebitica* (Brassicaceae) to solar UV radiation. In: Bogdanović, S., Jogan, N. (eds.), Book of Abstracts of the Sixth Balkan Botanical Congress, 109–110. Natural History Museum Rijeka, Croatian Botanical Society, Botanical Society of Slovenia, University of Rijeka, Rijeka.

Stevanović, B., Vujnović, K., 1990: Morpho-anatomical adaptations of the endemic species *Degenia velebitica* (Deg.) Hay. Feddes Repertorium 101(7-8), 385–389. http://dx.doi.org/10.1002/fedr.19901010710

Sylvester, A. W., Cande, W. Z., Freeling, M., 1991: Developmental morphology of maize leaves using UV light induced autofluorescence of cell wall compounds. Maize Genetics Cooperation Newsletter 65, 34–35.

Tattini, M., Gravano, E., Pinelli, P., Mulinacci, N., Romani, A., 2000: Flavonoids accumulate in leaves and glandular trichomes of *Phillyrea latifolia* exposed to excess solar radiation. New Phytologist 148(1), 69–77. http://dx.doi.org/10.1046/j.1469-8137.2000.00743.x

Tattini, M., Matteini, P., Saracini, E., Traversi, M. L., Giordano, C., Agati, G., 2007: Morphology and biochemistry of non-glandular trichomes in *Cistus salvifolius* L. leaves growing in extreme habitats of the Mediterranean basin. Plant Biology 9(03), 411–419. http://dx.doi.org/10.1055/s-2006-924662

Tsiftsoglou, O. S., Lazari, D. M., Stefanakis, M. K., Kokkalou, E. L., 2019: Flavonoids and phenolic acids from the aerial parts of *Alyssum alyssoides* L. (Brassicaceae). Biochemical Systematics and Ecology 83, 51–53. http://dx.doi.org/10.1016/j.bse.2018.12.004

Watkins, J. M., Chapman, J. M., Muday, G. K., 2017: Abscisic acid-induced reactive oxygen species are modulated by flavonols to control stomata aperture. Plant Physiology 175(4), 1807–1825. http://dx.doi.org/10.1104/pp.17.01010

Watts, S., Kariyat, R., 2021: Morphological characterization of trichomes shows enormous variation in shape, density and dimensions across the leaves of 14 *Solanum* species. AoB Plants 13(6), plab071. https://doi.org/10.1093/aobpla/plab071

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# Construction of the *Arabidopsis* isogenic lines containing dually localized protein TROL only in the inner chloroplast envelope membrane

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**Abstract** – The thylakoid rhodanese-like protein (TROL) is located at the end of the photosynthetic electron transport chain, at the vicinity of photosystem I, where it dynamically interacts with the ferredoxin:NADP$^+$ oxidoreductase (FNR) and is postulated to facilitate the transfer of electrons from reduced ferredoxin (Fd) to NADP$^+$. TROL is one of the few so far known dually localized chloroplast proteins. Besides being localized in the thylakoid membranes as the 66 kDa mature form, it has also been found in the inner envelope membrane of chloroplasts as the 70 kDa precursor. In thylakoids, the interaction between TROL and FNR acts like a switch, prioritizing the photosynthetic electron destination sinks according to the organellar needs. The role of TROL in the chloroplast inner envelope membrane is, however, presently unknown. By engineering the presequence protease processing site, a single amino acid exchange of Ala67 to Ile67 has been introduced to TROL, leading to inhibited cleavage of the presequence and resulting in protein incorporation at the inner envelope membrane. In this work, we engineered the *Arabidopsis* mutant plants that contain TROL almost exclusively in the inner envelope membrane (TROL-IE). To facilitate studying the role of this protein in this chloroplast compartment, we also produced the antiserum specific for the IE form of the TROL.

**Keywords:** dual localization, inner envelope membrane, mutagenesis, photosynthesis, presequence processing, thylakoids

## Introduction

Photosynthesis is an essential energy-converting process for producing complex carbohydrates and sustaining oxygenic life on Earth by releasing molecular oxygen. It involves light reactions taking place in the thylakoid membranes, and carbon fixation reactions in chloroplast stroma. Photosynthetic charge separation, electron transfer, and redox reactions, in synchrony, generate the proton motive force necessary for the synthesis of adenosine triphosphate (ATP) and directing electrons towards the production of stromal reducing equivalent nicotinamide adenine dinucleotide phosphate (NADPH). The transport of photosynthetic electrons terminates at the stromal side of photosystem I (PSI), where the electrons are being primarily handed over from reduced ferredoxin (Fd) to ferredoxin:NADP$^+$ oxidoreductase (FNR). Although in chloroplasts the main sink for electrons from ferredoxin is NADPH, depending on the organelle needs, other stromal acceptors can receive photosynthetic electrons as well. Such acceptors are oxygen in pseudocyclic electron transport (Allen 2003), ferredoxin-thioredoxin reductase, which is important for the regulation of carbon assimilation (Buchanan and Balmer 2005), nitrogen and sulfur assimilation reactions, the biosynthesis of chlorophyll, phytochrome and fatty acids (Hanke and Mulo 2013). Cyclic electron transport (CET) around Photosystem I, where the electrons are returned from PS I to the cytochrome $b_6f$ complex (Joliot and Joliot 2006) is also possible.

The important regulator of this final linear electron transfer step is the thylakoid-membrane incorporated thylakoid rhodanese-like protein (TROL) that interacts with the FNR by dynamic tethering (Vojta et al. 2012, Vojta and Fulgosi 2012, Vojta and Fulgosi 2016, Kekić et al. 2020), and consequently influences the end point of photosynthetically derived electrons. TROL contains the binding region for the FNR (Jurić et al. 2009, Kekić et al. 2020) and seems to be the exclusive docking site for this protein (McKenzie et al. 2020). However, it has been shown that TROL is not essential for *Arabidopsis* growth and development (Jurić et al. 2009, Vojta et al. 2015). In TROL knock-out (KO) mutants, because there is no TROL to dock FNR, the photosynthetic electron transfer is not primarily directed to the production of

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NADPH, which is an electron donor for the Calvin-Benson cycle, but other electron acceptors are more likely to receive the produced electrons. Such acceptors are nitrite reductase, sulfite reductase, fatty acid desaturase, glutamine-2-oxoglutarate amino transferase, and ferredoxin-thioredoxin reductase (FTR). The FTR pathway constitutes an indispensable redox-signaling cascade for light-dependent reduction of chloroplast stromal proteins (Yoshida et al. 2022). FNR may also act as the direct ferredoxin:plastoquinone reductase (Fd:PQ), establishing redox regulation and antioxidant defense point (Jurić et al. 2013). Therefore, in the absence of TROL, the distribution of high-energy electrons is directed towards ROS scavenging pathways, rather than to the NADP$^+$ reduction (Vojta et al. 2015). TROL KO plants show increased stress resistance along with significantly enhanced ROS scavenging (Vojta et al. 2015). Also, the absence of TROL leads to the complete loss of the dynamic FNR-binding and release to/from the thylakoid membrane (Vojta and Fulgosi 2016). Therefore, the TROL-FNR interaction seems to be an important mechanism for the regulation and prioritization of energy-conserving and energy dissipating pathways in vascular plant photosynthesis (Fulgosi and Vojta 2020).

Recently, the topology of TROL has been investigated in detail to define the localization and orientations of its domains (Vojta and Fulgosi 2019). TROL spans the thylakoid membranes with the two segments, one at the N-terminus of the protein and the other close to its C-terminal part. The C-terminal polyproline (PEPE) domain and FNR-interacting ITEP domain protrude to the chloroplast stroma and provide a flexible binding site for the FNR (Jurić et al. 2009, Vojta and Fulgosi 2019, Kekić et al. 2020). In the thylakoid lumen, encompassed by the two transmembrane helices, resides inactive rhodanese (RHO) domain, which most probably has the regulatory role (Jurić et al. 2009, Vojta and Fulgosi 2019).

TROL is one of the few known dually localized chloroplast proteins. Seventeen other proteins have been identified in both chloroplast envelope and thylakoid membranes (Klasek and Inoue 2016). They all carry an N-terminal signal for import into chloroplasts. These proteins are involved in protein transport, tetrapyrrole biosynthesis, membrane dynamics, transport of nucleotides and inorganic phosphate (Klasek and Inoue 2016). One of these proteins is Tic62, a redox sensor in the inner envelope membrane and the thylakoids, proposed to anchor FNR, just like TROL (Benz et al. 2010) and is involved in chloroplast protein import (Küchler et al. 2002). Besides the thylakoids-envelope, there can also be dual localization in the stroma-envelope compartments, as in the case of protein CPSAR1, which is dually localized in the stroma and the inner envelope membrane and is involved in thylakoid biogenesis (Garcia et al. 2010). Also, chloroplast-nucleus localization has been confirmed, as for the protein NPR1 which switches its location as adaptive response to salt stress (Seo et al. 2020). Finally, it was reported that approximately 5% of mitochondrial and chloroplast proteins are dually targeted in plants (Sáiz-Bonilla et al. 2023).

In addition to being mainly located at the stroma thylakoids, in its fully processed form of 66 kDa, TROL can also be found embedded in the chloroplast inner envelope membrane (IE) in its non-processed form of 70 kDa (Jurić et al. 2009, Vojta et al. 2018), with an as yet unraveled role. This dual localization has been proven by the proteome analysis of chloroplast envelopes and thylakoid membranes (Peltier et al. 2004). Both forms remain protected after thermolysin digestion of intact chloroplasts, indicating their complete incorporation into these membranes (Vojta et al. 2018).

During protein import into chloroplasts, the information contained in targeting sequences are sequentially decoded resulting in localization of the polypeptide to the appropriate organelle subcompartment (Cline and Henry 1996). TROL is a nuclear-encoded protein, synthesized with the cleavable N-terminal 69 amino acids long presequence that directs the protein to the chloroplasts. TROL is, like most of the IE membrane proteins, directed through the general import pathway (Toc and Tic complex) to cross the envelope membranes. This transfer might end at the level of the IE membrane, where we suppose TROL is released from the translocon machinery, as instructed by a hydrophobic stop-transfer signal in its sequence (Vojta et al. 2007). The majority of the TROL protein pool continues its way towards the thylakoids, by entering the chloroplast stroma where the preprotein is recognized and processed by the stromal processing peptidase (SPP), the mature protein being finally incorporated into thylakoid membranes (Vojta et al. 2007, Vojta et al. 2018). Proteins targeted to the thylakoid membrane require dual targeting signals that direct their import across the chloroplast envelope membranes and subsequent incorporation into the thylakoids. The targeting signal for thylakoids can be in the form of a single bipartite transit sequence that carries the information for targeting to stroma at the N-terminal region and to the thylakoids at the C-terminal region of the presequence (Cline and Henry 1996, Schnell 1998). Since the single targeting domain is predicted for the transit sequence of TROL, the signals for the targeting of TROL to the thylakoids seem to reside within the primary sequence of the mature polypeptide, as reported for other integral thylakoid membrane proteins, such as light-harvesting chlorophyll a/b binding protein (preLHCP) and the precursor to the 20-kDa subunit of the CP24 complex (Schnell 1998, Lamppa 1988).

In our previous experiments, we determined that a single amino acid mutation around the stromal protease processing site of the presequence of TROL, Ala67Ile, directs the entire pool of *in vitro* synthesized and imported TROL to the chloroplast IE membrane, without incorporation in the thylakoids, and if so, then to a very low extent (Vojta et al. 2018). This result led to the idea of creating *Arabidopsis* plants that would accumulate TROL located at the single intrachloroplastic site – the IE. Such plants could unravel the still unknown function of TROL in this membrane. The importance of constructing TROL-IE plants lies in the fact that chloroplast envelopes make up only around 3% of total chloroplast membranes. The vast majority of membranes are thylakoids and by investigations performed on chloroplasts or total membrane extracts we are not able to properly investigate the role of TROL in the IE. By constructing TROL-IE plants we will be able, for the first time, to properly assess the role/s of TROL in the IE and the interactions with its IE-associated partners.

## Materials and methods

### Plant material and breeding conditions

Several *Arabidopsis* lines have been used in this research. *Arabidopsis thaliana* (L.) ecotype Columbia (Col-0) plants, or wild-type (WT); TROL knock-out plants (KO), that do not accumulate protein TROL due to the T-DNA insertion into the gene *At4g01050* (SAIL_27_B04, obtained from NASC) Col background (Jurić et al. 2009); TROL overexpression line (OX) that was constructed by transformation of TROL KO *A. thaliana* plants with the plasmid vector pH7WG2.0 (35S promoter) containing TROL-HA-FLAG construct, in which the HA and FLAG tags were added C-terminally (Vojta and Fulgosi 2019); and finally TROL-IE plants, that contain TROL only in the inner envelope membrane of chloroplasts and were constructed in this research. All plants were grown under 16/8h light/dark period with a photosynthetic light intensity of 25 μmol photons $m^{-2}$ $s^{-1}$ (Osram Flora lamps) at 22 °C, relative humidity 60% during the day and 70% during the night. Plants were grown on a potting substrate (A400, Stender GmbH, Schermbeck, Germany) in a growth chamber (Kambič, Slovenia).

### TROL presequence mutagenesis

Using the QuikChange Multi Site-Directed Mutagenesis method (Agilent Technologies, Santa Clara, California, United States), 67Ala → 67Ile substitution was introduced into the TROL presequence. Amino acids 67–78 of the sequence of TROL, AKSLTYEEALQQ, represent a partially conserved N-terminal part of the polypeptide, around the predicted transit peptide cleavage site. The substitution 67Ala→67Ile leads to the absence of processing of the transit peptide and the subsequent arrest of the protein TROL in the inner envelope membrane of chloroplasts (Vojta et al. 2018). Besides the Ala67 to Ile67 amino acid exchange FLAG (DYKDDDDK) and HA (Human influenza hemagglutinin, YPYDVPDYA) tags have been added to the C-terminus of the protein for easier downstream detection and purification. The whole construct was cloned into the pH7WG2.0 vector under the control of 35S promoter (Vojta et al. 2018).

### Cultivation of *Agrobacterium tumefaciens* bacteria for transformation

Competent *Agrobacterium tumefaciens* EHA 105 bacteria were transformed chemically with the TROL(A/I)-FLAG-HA construct in the binary vector pH7WG2.0. Transformed Agrobacteria were multiplied by growth in liquid YEB medium with addition of rifampicin and spectinomycin in a horizontal shaker at 28 °C and 250 rpm until $OD_{600}$ reached 1-1.5. The bacterial suspension was then transferred to tubes and centrifuged for 15 min at 1460 g. The centrifugation procedure was repeated to collect the remaining precipitate. The bacterial pellet was resuspended in infiltration medium with the addition of 0.03% (v/v) of the mild surfactant Silwet L-77 (Momentive Performance Materials GmbH & Co KG, Leverkusen, Germany) to lower surface tension and to enable bacteria to enter hard-to-reach parts of the flower during transformation by "Floral-dip" method.

### Cultivation of *A. thaliana* TROL KO plants for transformation

Eighteen *Arabidopsis* TROL KO plants were grown in jars in a climate chamber as described. When the plants began to develop the primary inflorescence, it was necessary to cut it at the base, in order to break the apical dominance and allow the development of the secondary inflorescence. After 5-7 days, the stems with the secondary inflorescences were 2-10 cm long, representing the optimal stage for transformation.

### *In planta* transformation of *A. thaliana* TROL KO plants by the "Floral-dip" method

*Arabidopsis* plants with developed secondary inflorescences were immersed in a suspension of Agrobacteria transformed with the TROL(A/I)-FLAG-HA construct in pH7WG2 vector, without using a vacuum (Clough and Bent 1998). This way, the transformation of the entire plants or seeds occurs, without the need for *in vitro* tissue culture. The pots with the plants were turned upside down and the inflorescences were immersed in a glass containing 500 mL of infiltration solution for 20-30 seconds, with gentle stirring of the solution with a glass rod. The plants were then placed horizontally on a plastic mat, covered with baking paper, and stored overnight in a climate chamber under low light conditions. On the next day, the jars with the plants were placed upright, filled with water, and exposed to light (~25 μmol photons $m^{-2}$ $s^{-1}$). The floral-dip procedure was repeated after 8 days. The plants were watered for the next two weeks, then the watering was stopped, and the plants were allowed to dry in order to collect the seeds. Seeds from each of the transformed plants were collected separately, and represented the T1 generation of the transformants.

### Selection of transformed *A. thaliana* seeds

Sterilization of the seeds was carried out using 70% EtOH and 0.05% Triton X-100. Seeds were mixed in the solution by shaking for 5 min. Supernatant was discarded, followed by subsequent incubation with 70% EtOH for 10 min. The sterilization was finished by raising the seeds in sterile water. Following surface sterilization, seeds were selected for hygromycin resistance on solid MS medium (Moorashige and Skoog basal salt mixture, Sigma) containing 15μg/mL hygromycin. Seeds were stratified for 2 days in the dark at 4 °C and then transferred to the growth chamber for 4-6 h at 22 °C in continuous light (PAR 25 μmol photons $m^{-2}$ $s^{-1}$) to stimulate germination. Plates were wrapped in aluminum foil and incubated for 2 days at 22 °C. The foil was then removed, and seeds were incubated for a further 48 h in continuous light (PAR 25 μmol photons $m^{-2}$ $s^{-1}$). Seedlings with short hypocotyls were dismissed, while those with long hypocotyls were transferred to the soil to produce T1 plants and T2 seeds. The T2 seeds were selected by transferring seeds from each individual plant to the MS medium + hygromycin and treated as described previously. Only seeds from T1 plants (T2 seeds) that produced 70% seedlings with long hypocotyls and 30% with short ones were used in further selection steps. They developed into T2 plants and their T3 seeds were further selected on selective hygromycin plates. Only those with all long hypocotyls were supposed to be homozygous for the Ala67Ile67 mutation and were used further. T3 seeds and plants were tested by PCR, DNA sequencing and Western blotting. DNA isolation was performed using Phire Plant Direct PCR kit (Thermo Scientific™), and PCR was performed using the forward primer for TROL (5' ggaatTCATGGAAGCTCTGAAAACCGCA 3') and the reverse primer for FLAG_linker_HA tag (5' CTTGTAGTCTAACTTGACAGCAGCAGCGTA). This way, only TROL from the transformed plants (containing FLAG and HA tags on the 3' end of the gene) should multiply. Western blotting analyses were performed on total leaf extracts, intact chloroplasts, and chloroplast membranes from T2 plants by using anti-TROL (1:2000, Agrisera AS194257) and anti-HA antibodies (1:1000, Sigma H9658) and anti-rabbit IgG peroxidase (1:50000, Sigma A0545) as a secondary antibody for anti-TROL, and anti-rat IgG peroxidase (1:30000, Sigma A9073) as a secondary antibody for anti-HA. Plants with a stronger signal that were also PCR-positive were used for further investigations. Those were plants enumerated 8 and 18. Their sequence (mutation Ala67 to Ile67 GCT to ATT and C-terminal tags) was also successfully checked and confirmed by DNA sequencing. Therefore, the two mutant lines used in this study were designated TROL-IE8 and TROL-IE18, and these two lines were used for future experiments.

### Production of the preTROL antiserum

The first 66 aminoacids of the presequence of TROL (MEALKTATFSPMSVLSEKRSEPRKPFSLPNLFPPKSQRPISQESFLKRFNGGLALLTSVLSSATAP) were custom synthetized and prepared for the immunization of two rabbits by the company Davids Biotechnologie Gmbh. (Regensburg, Germany). 15 mg of the peptide of purity > 85% was synthetized, quality checked by HPLC and MS and conjugated to KLH carrier for immunization. The antigen in the concentration of 10 mg $mL^{-1}$ was used for the immunization of two experimental animals. 35 days after immunization test, sera were taken from the rabbits and verified, together with the preimmune serum, for sensitivity and specificity to our plant extracts. Antiserum produced in this way (anti-preTROL) was expected to detect only the inner envelope membrane form of the TROL and not the thylakoid mature one. The immunization continued until day 63 to increase the titer of antibodies in the serum. From both sera samples, a part was purified on affinity matrix with the antigen. Final products were four different sera: serum 1 (anti-preTROL1), serum 1 affinity purified (anti-preTROL1AP), serum 2 (anti-preTROL2), and serum 2 affinity purified (anti-preTROL2AP). They were all tested (together with the preimmune sera) for specificity and sensitivity of TROL-IE detection in isolated chloroplasts of TROL OX and TROL IE plants. The optimal dilution of the anti-preTROL antisera was determined to be 1:100.

### Isolation of total leaf extracts from *Arabidopsis* plants

Leaves of four-week-old *Arabidopsis* plants (WT, OX, IE-8, and IE-18) were cut and ground in liquid nitrogen using mortar and pestle. Laemmli buffer (Laemmli 1970) was added to the extract, samples were vortexed and heated at 70 °C for 5-10 min. Centrifugation at 13000 g for 5 min at room temperature pelleted the insoluble leftovers and the extracts were stored at -20 °C (for faster use) or at -80 °C (for later use).

### Isolation of intact chloroplasts from *Arabidopsis* plants

Leaves of four-week-old *Arabidopsis* plants (WT, OX, IE-8, and IE-18) were cut and ground in ice-cold isolation medium (330 mM sorbitol, 20 mM TRIS/HCl, 5 mM EDTA, 10 mM $Na_2CO_3$, 0.1% BSA), filtered through 4-layered gauze and Miracloth filter (Sigma-Aldrich) and sedimented by centrifugation at 1500 g for 5 min, at 0 °C. The pellet was resuspended in a small amount of isolation medium and purified through 55%/35% Percoll density gradient. Centrifugation at 5000 g was performed for 10 min at 0 °C, in the swing-out rotor, with the slowest possible deceleration rate. Intact chloroplasts were collected from the interphase between 55% and 35% Percoll, washed twice in isolation medium, sedimented at 1500 g for 5 min at 0 °C, and chlorophyll concentration was measured spectrophotometrically.

### Isolation of total chloroplast membranes from *Arabidopsis* plants

After the determination of chlorophyll concentration, intact chloroplasts corresponding to 2 μg chlorophyll were pelleted at 1500 g for 1 min at 0 °C, resuspended, and incubated in lysis buffer (10 mM Tris-base, 5 mM $MgCl_2$, pH 7.9) for 30 min on ice. By subsequent centrifugation at 25000 g for 20 min at 4 °C the sample was separated to supernatant (stroma) and membrane pellet (consisting mostly of thylakoids). The pellet was resuspended in Laemmli buffer to the final concentration of 2 mg chlorophyll/mL buffer and stored at –20 °C until further use.

### Western blot analysis

10-30 μg of isolated chloroplasts or thylakoids of the corresponding *Arabidopsis* line were loaded on 10% or 12% SDS-polyacrylamide gels and separated by electrophoresis (SDS-PAGE). Afterwards, Western transfer to the nitrocellulose membranes was performed, and membranes were immunodecorated with the anti-TROL (1:3000, Agrisera AS194257), anti-preTROL (1:100), and anti-HA antibodies

(1:1000, Sigma H9658). As secondary antibody, anti-rabbit IgG peroxidase (Sigma A0545) was used for anti-TROL (1:50000) and anti-preTROL (1:10000) and anti-rat IgG peroxidase (1:30000, Sigma A9073) for anti-HA. Final detection was carried out by enhanced chemiluminescence (ECL) and exposure to X-ray films.

## Results

### Construction of *Arabidopsis* TROL-IE plants containing TROL only in the inner envelope membrane

In our previous work we exchanged several amino acids around the protease processing site of the TROL presequence. The mutation of Ala67 to Ile67 was the only one that influenced the processing of the protein, by arresting the TROL in the chloroplast inner envelope membrane (Vojta et al. 2018). In order to investigate *in vivo* the role of the TROL in this compartment, we had to construct the *Arabidopsis* plants containing TROL at this single location. Therefore, we have cloned the construct of TROL with the Ala67 to Ile67 amino acid exchange and the C-terminal FLAG (DYKDDDDK) and HA (Human influenza hemagglutinin, YPYDVPDYA) tags in the pH7WG2.0 vector. This construct was used to transform TROL KO *Arabidopsis* plants. Seeds from transformed plants were collected (T1 generation) and homozygous TROL-IE plants were selected up to the T3 generation. Selection for hygromycin resistance, PCR, and testing for expression of recombinant proteins by Western blot analysis, in combination, enabled us to select the homozygous plants carrying TROL only in the IE of chloroplasts. The two homozygous mutant lines that we selected for future investigations were named TROL-IE8 and TROL-IE18.

As controls, we used *At*TROL containing the entire wildtype sequence (WT), as well as plants overexpressing TROL, with C-terminal FLAG and HA tags (OX). Whole leaf extracts, isolated chloroplasts and chloroplast membranes were tested for the presence of TROL and HA-tags in these lines. Both antibodies (anti-TROL and anti-HA) could detect the newly transformed protein in the inner envelope membrane (IE8 and IE18, Fig. 1A lanes 6-9 and 17-20). Also, TROL was successfully recognized by the anti-TROL

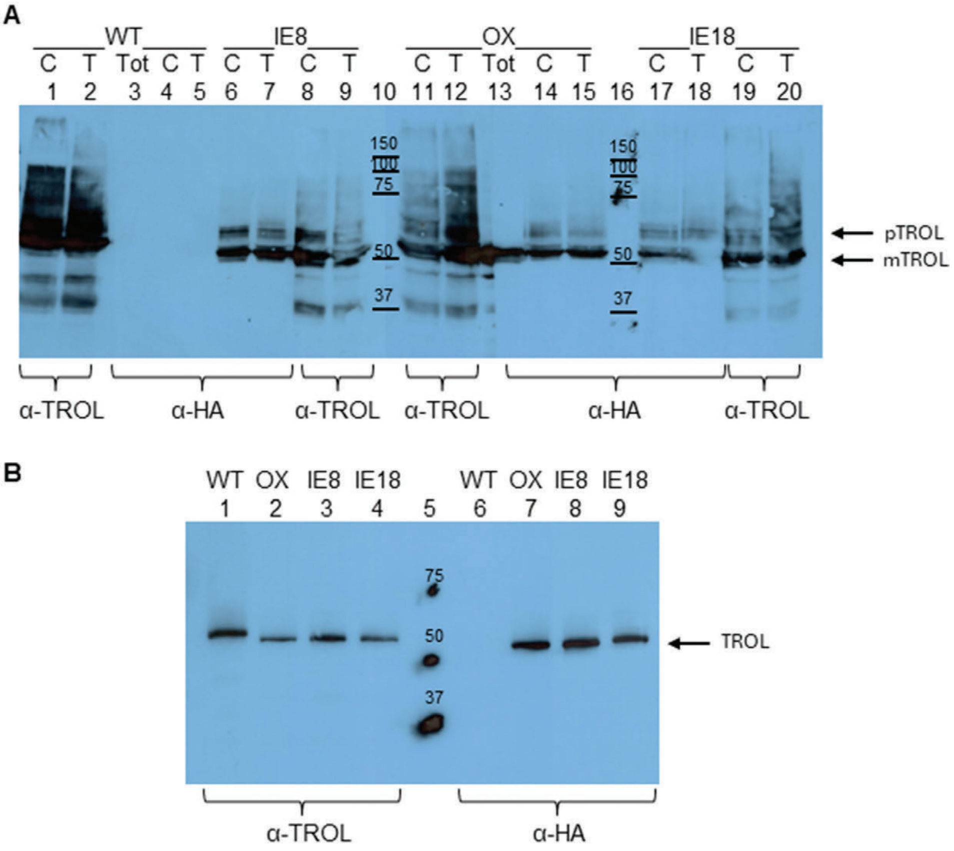

**Fig. 1.** Western blot analysis of *A. thaliana* TROL KO plants transformed with the TROL(A/I)-FLAG-HA construct. (**A**) Wild type (WT, Lanes 1-5), TROL overexpressor (OX, Lanes 11-15) and TROL inner envelope lines of *Arabidopsis* (IE8, Lanes 6-9, and IE18, Lanes 17-20) have been analyzed. Total leaf extract (Tot), intact chloroplasts (C) and total chloroplast membranes (T) in the amount corresponding to 30 µg chlorophyll were analyzed by 12% SDS-PAGE and Western blotting. Membranes were immunodetected with anti-TROL (α-TROL) and anti-HA antibodies (α-HA). Conjugated secondary antibodies anti-rabbit IgG peroxidase for anti-TROL, and anti-rat IgG peroxidase for anti-HA, have been used. Results were visualized by enhanced chemiluminescence (ECL) and exposure to X-ray films. Lanes 10 and 16 represent protein markers in kDa. Arrows denote the positions of TROL preprotein (pTROL) and mature TROL (mTROL). (**B**) Intact chloroplasts of WT, OX, IE8 and IE18 lines in the amounts corresponding to 5 µg chlorophyll were analyzed by 12% SDS-PAGE and Western blotting, as described for A. Lane 5 represents protein marker in kDa. Arrow denotes the position of TROL (since there is less sample loaded on gel, preprotein is not visible).

and anti-HA in the overexpressor line (Fig. 1A, lanes 11-15). Anti-HA antibody did not recognize TROL or any other protein in the WT plants, since in those plants there was no HA-epitope (Fig. 1A, lanes 3-5). Due to a stronger background in all tested extracts, we attempted to analyze the lower amount of intact chloroplast samples (Fig. 1B). In this way, we greatly reduced unnecessary background bands and once again confirmed that the IE plants express the modified TROL.

Although we have successfully confirmed the presence of TROL in the IE plants, we did not manage to confirm the exact molecular weight of these proteins on the SDS-PAGE, even when using strongly denaturating urea gels (data not shown, Sirpiö et al. 2011). The calculated size difference between the precursor and the mature form of the TROL is only 4 kDa. To prove that the protein TROL that we detected in the IE plants was indeed the precursor, and indeed only in the inner envelope membrane, we had to develop a method that would recognize only the precursor of the TROL and not its processed form.

## Phenotype of TROL-IE plants

*Arabidopsis* IE8 and IE18 lines grew well under the same conditions as the WT plants. Their hypocotyls developed later (Fig. 2A); they grew somewhat slower due to the delayed germination (Fig. 2). At 3.5 to 4 weeks of age, before flowering starts, the plants are most suitable for biochemical experiments. At this stage TROL-IE plants had smaller rosettes than the WT, and smaller biomass. As they grew, their stems seemed to be slightly thinner than the stems of WT. The flowering was slightly delayed, and they produced a significantly smaller number of siliques and seeds than the WT. Otherwise, they resembled the phenotype of WT *Arabidopsis*.

## Production of the antibody against the presequence of TROL

Since anti-TROL antibody recognizes both the inner envelope membrane (precursor) form and the thylakoid membrane-located (mature) TROL protein, we decided to produce additional antibody against the N-terminal presequence of the protein (not recognized by existing anti-TROL). For that purpose, synthetic polypeptide of 66 N-terminal amino acids of TROL presequence was produced by custom peptide synthesis (Davids Biotechnologie Gmbh., Regensburg, Germany). There were two sera produced: serum 1 (anti-preTROL1) from rabbit 1 and serum 2 (anti-preTROL2) from rabbit 2. Both sera were rather unspecific with strong background (Fig. 3, Lanes 3, 4, 10, 11). Therefore, both sera were purified on affinity matrix with the bound antigen (synthetic polypeptide of 66 N-terminal amino acids of TROL presequence). Final products after purification were: serum 1 affinity purified (anti-preTROL1AP) and serum 2 affinity purified (anti-

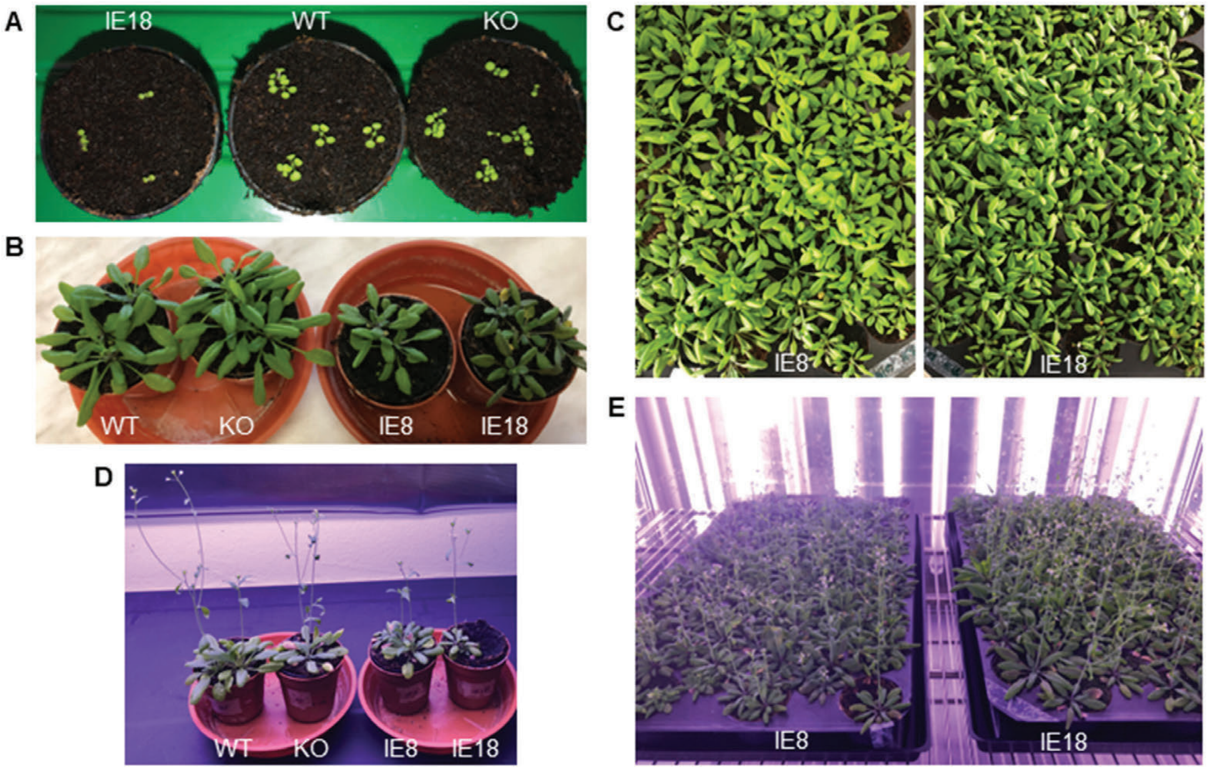

**Fig. 2.** The phenotype of *Arabidopsis* plants that contain protein TROL only in the inner envelope membrane of chloroplasts (TROL-IE plants). (**A**) 12 days old TROL-IE plants in comparison to the WT and TROL KO. (**B**) and (**C**) 4 weeks old plants. (**D**) 5 weeks old plants that started flowering. (**E**) 9 weeks old plants. WT denotes wild-type *Arabidopsis* plants ecotype Columbia (Col-0); KO denotes *Arabidopsis* TROL knock-out mutant; IE8 and IE18 are *Arabidopsis* mutant lines that contain TROL only in the inner envelope membrane of chloroplasts.

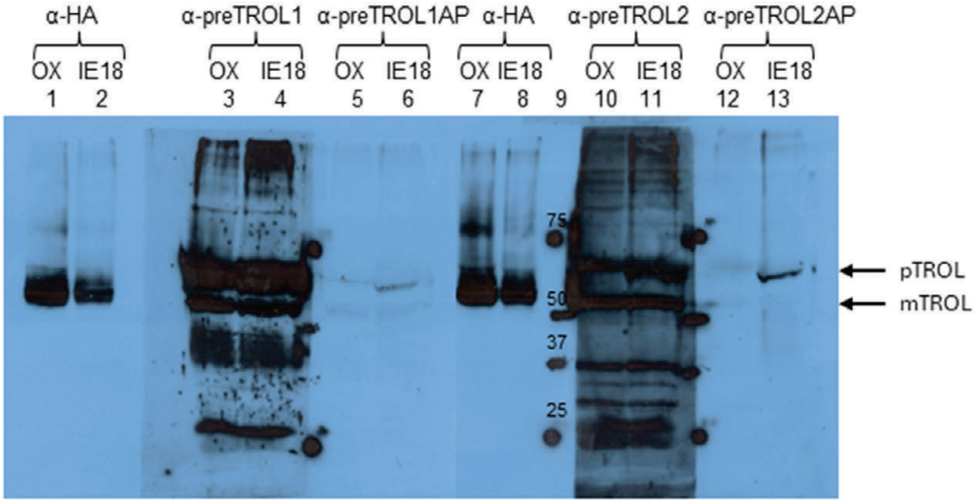

**Fig. 3.** Testing the anti-preTROL sera on OX and IE18 *Arabidopsis* lines by Western blot analysis. TROL overexpressor (OX) and TROL inner envelope membrane IE8 lines of *Arabidopsis* have been analyzed. Intact chloroplasts in an amount corresponding to 10 μg chlorophyll for anti-HA analyses and 40 μg chlorophyll for anti-preTROL analyses were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and immunodecorated with the anti-preTROL (α-preTROL) and anti-HA antibodies (α-HA), as a control. Conjugated secondary antibodies anti-rabbit IgG peroxidase for anti-preTROL, and anti-rat IgG peroxidase for anti-HA have been used. Results were visualized by enhanced chemiluminescence (ECL) and exposure to X-ray films. Lane 9 represents the protein marker in kDa. Arrows denote the positions of TROL preprotein (pTROL) and mature TROL (mTROL). Non-purified anti-preTROL antisera (α-preTROL1 and α-preTROL2, Lanes 3, 4, 10, 11) seem to be unspecific. Affinity purified anti-preTROL antisera (α-preTROL1AP and α-preTROL2AP, Lanes 5, 6, 12, 13) seem to be very specific, showing a single clear band in TROL-IE plants, indicating TROL precursor.

preTROL2AP). They seem highly purified and specific (Fig. 3, Lanes 5, 6, 12, 13), just of a very low titer. Therefore, we determined experimentally that a dilution of 1:100 was the highest required for the proper detection of the TROL precursor. Between those two purified sera, anti-preTROL2AP proved to be a bit stronger and therefore we decided to use it in further experiments.

After the preTROL antiserum was successfully produced, we tested the chloroplasts from OX and IE lines by SDS-PAGE and Western blot analysis, as described previously. We used anti-HA and anti-preTROL antisera. Affinity purified anti-preTROL clearly recognized only a single band in TROL-IE8 and TROL-IE18 lines (Fig. 3, Lanes 6 and 13; Fig. 4, Lanes 2 and 3) and not in the TROL OX line (Fig. 3, Lanes 5 and 12; Fig. 4, Lane 1). Anti-preTROL that was not affinity purified did not differentiate between TROL-IE and TROL OX lines (Fig. 3, Lanes 3, 4, 10, 11), except that the resulting signal/band was more intensive in TROL-IE line (Fig. 3, Lanes 4, 11). When compared to the anti-HA blots on TROL-IE lines, it was clear that the affinity purified anti-preTROL recognized the precursor and not the mature form of TROL. A very weak signal was observed on the TROL OX line (Fig. 3, Lanes 5 and 12). It corresponded to the IE form of TROL in this line, which was in the vast minority in comparison to the thylakoidal form of TROL. This weak signal was visible in TROL OX extracts only when a sample corresponding to 40 or more mg chlorophyll was loaded on gel. These experiments confirmed that TROL is located exclusively in the inner envelope membrane in TROL-IE8 and TROL-IE18 *Arabidopsis* lines, and that the affinity purified preTROL antiserum can be further used to exclusively detect the inner envelope membrane form of TROL.

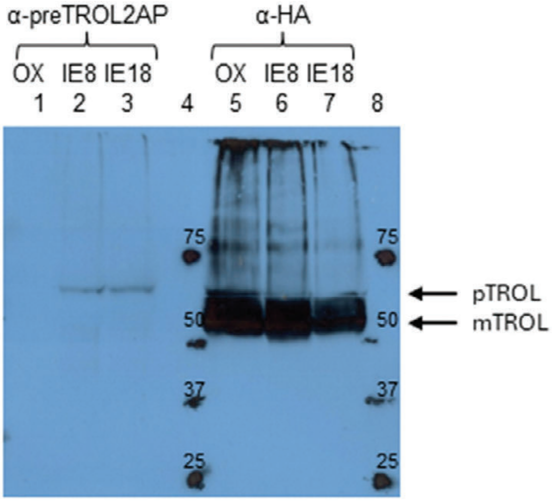

**Fig. 4.** Detection of TROL in OX, IE8, and IE18 *Arabidopsis* lines by using anti-preTROL serum. Intact chloroplasts in an amount corresponding to 40 μg chlorophyll for anti-preTROL analyses (Lanes 1-3) and 10 μg chlorophyll for anti-HA analyses (Lanes 5-7) were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane and immunodecorated with anti-preTROL (α-preTROL2AP) and anti-HA antibodies (α-HA), as a control. Conjugated secondary antibodies anti-rabbit IgG peroxidase for anti-preTROL, and anti-rat IgG peroxidase for anti-HA, have been used. Results were visualized by enhanced chemiluminescence (ECL) and exposure to X-ray films. Lanes 4 and 8 represent a protein marker in kDa. Arrows denote the positions of TROL preprotein (pTROL) and mature TROL (mTROL). Anti-preTROL2AP serum seems to be very specific, showing the single clear band in TROL-IE plants (lanes 2 and 3), indicating TROL precursor. Anti-preTROL serum does not show visible signal on OX line because there is too little amount (undetectable) of the TROL precursor in this sample.

**Analysis of TROL localization in TROL-IE plants**

By newly produced anti-preTROL affinity purified sera we confirmed that the TROL in TROL-IE plants was found only in precursor form in the inner envelope membrane (Fig. 4). TROL OX line was a good control for determination of the localization of TROL since, just as TROL-IE8 and TROL-IE18 lines, it was under the control of a strong constitutive promotor 35S and has C-terminally added FLAG and HA tags for easier detection and purification. Therefore, when using anti-HA antibody, we managed to detect TROL in all three lines (OX, IE8, and IE18, Fig. 4, Lanes 5-7). However, when using anti-preTROL serum, TROL was detected only in the IE lines (Fig. 4, Lanes 2 and 3). This observation confirmed that the accumulation of the precursor due to mutagenesis occurred in the inner envelope membrane. OX line lacks the signal because the majority of the TROL in this line is in the thylakoids, as a mature form of the protein, which cannot be recognized using antibodies against the presequence.

## Discussion

The role of the TROL in the thylakoid membranes has been investigated for more than 15 years, since the protein was characterized in 2009 (Jurić et al. 2009). TROL has been shown to be involved in photosynthetic electron distribution by docking the FNR to the thylakoid membranes and therefore bringing it to the vicinity of PSI, namely ferredoxin (Fd) (Jurić et al. 2009, Kekić et al. 2020). Fd distributes high-energy electrons to a multitude of enzymes involved in chloroplast metabolism. By the interaction of TROL and FNR, electrons are preferentially directed to carbon assimilation, which requires NADPH, and so the majority of Fd is immediately oxidized by the enzyme ferredoxin:NADP$^+$ oxidoreductase (FNR), which associates with the thylakoid membrane (Jurić et al. 2013).

Apart from thylakoids, TROL resides in the inner envelope membrane (IE) and its role in this compartment has remained unexplored to date. This is because the smaller portion of TROL precursor in the IE is being masked by the majority of the mature form of TROL located in the thylakoids. Dual localization has been shown for other chloroplast proteins of various functions (Klasek and Inoue 2016). In some cases, protein is dually localized because its activity is needed in more than one location. Cells usually either use multiple gene products (differently located isoforms) or target a single gene product to multiple locations by various mechanisms (Klasek and Inoue 2016). At this stage we can only speculate on the role of TROL in the IE membrane. It might represent a "storage" from which TROL is mobilized to thylakoids when needed. There is also a possibility that TROL in the IE membrane is someway involved in the electron transfer at this membrane. Mass spectroscopy analyses (Vojta et al. 2023) indicated its possible interaction with Tic62 and Tic20, proteins that build a Translocon at the chloroplastic inner envelope membrane. This might indicate involvement of TROL in a protein translocation process across the chloroplast envelope, probably as a possible additional redox regulator through interaction with FNR. There is also a possibility that IE membrane TROL is involved in metabolic regulation by docking FNR and regulating NADPH production in this compartment. To test these hypotheses and to investigate its role in the inner envelope membrane of chloroplasts *in vivo*, we had to "remove" TROL from the thylakoids.

In our previous research, by Western blotting of isolated outer and inner chloroplast envelope membranes, thylakoids, and stroma, we confirmed that TROL preprotein is associated almost exclusively with the inner envelope membrane and that the mature part is located in the thylakoids (Jurić et al. 2009). We managed to find the determinants for the dual localization of TROL around its presequence protease processing site (Vojta et al. 2018). We mutated Ala to Ile at the position 67 in TROL precursor and by performing *in vitro* protein import experiments we proved that the majority of such preprotein was not processed and transferred to its destination in the thylakoid membrane (Vojta et al. 2018). These results provided us with the tool to "remove" TROL from the thylakoids for the purpose of studying its function in the inner envelope membrane. By applying the "Floral dip" method of Agrobacterial transformation of *Arabidopsis* plants with the TROL knock-out background with the mutated construct TROL(A/I)-FLAG-HA in pH7WG2.0 vector we successfully managed to create plants that contain TROL only in the inner envelope membrane (TROL-IE plants). These plants were selected on selective growth medium with the addition of specific antibiotic and tested by PCR, DNA sequencing, and Western blotting at different stages of the selection process (Fig. 1). Plants that were selected as homozygous were named TROL-IE8 and TROL-IE18.

TROL-IE8 and TROL-IE18 plants grow well throughout the whole life cycle and produce viable seeds. In comparison to the WT *Arabidopsis*, their development is delayed. Their hypocotyls develop several days later, and they grow slower due to delayed germination (Fig. 2). TROL-IE plants have smaller rosettes and weaker stems, and their flowering is delayed. Otherwise, they resemble the phenotype of the wild type of *Arabidopsis*. The life cycle of the TROL-IE plants surprisingly does not resemble the TROL KO plant cycle. TROL KO plants, in which there is no production of TROL, follow the WT developmental cycle, and their hypocotyls, rosettes and seeds are all slightly bigger than the WT (Jurić et al. 2009, Vojta et al. 2015).

Although we confirmed which plants were successfully transformed, by using SDS-PAGE and subsequent Western blotting using anti-TROL or anti-HA antibodies, it was not possible to differentiate the precursor and the mature form of TROL according to the difference in size and therefore not possible to determine whether it was localized in the inner envelope membrane or/and in thylakoids. The reason for this is that when using anti-TROL or anti-HA in most cases a bulky signal was visible on gel. The addition of 8 M urea in the samples and/or in the gel itself (data not shown) also did not help to reliably differentiate the thylakoidal (66 kDa) vs inner envelope membrane form of TROL (70 kDa). Therefore, it was essential to produce the antiserum that would recognize only the precursor form of TROL. We selected N-terminal 66 amino acids of TROL to custom produce a polypeptide, an antigen that would, by immunizing experimental animals, give a specific antiserum. Anti-preTROL antiserum (antiserum against the N-terminal presequence of TROL) was successfully produced (by Davids Biotechnologie Gmbh., Regensburg, Germany). While testing it, it was clear that the antiserum must be further affinity purified, since it showed a strong background signal when we assessed the plant extracts. Anti-preTROL serum that was not affinity purified did not differentiate between TROL-IE and TROL OX lines, except that the resulting signal/band was more intensive in the TROL-IE line. Affinity purified antiserum proved to be highly specific, recognizing a single clear band in TROL-IE lines, but not in the TROL OX line. A weak visible signal in the TROL OX line was present only when there was a lot of sample loaded on gel. This is because TROL preprotein in TROL OX line was present in extremely small amounts, while the vast majority of TROL was in thylakoids, as a mature form.

We analyzed the chloroplasts from WT, TROL OX, TROL KO, and TROL-IE lines by SDS-PAGE and Western blotting, as described in Materials and Methods section. We used anti-TROL, anti-HA, and anti-preTROL sera. It was clearly visible that there was no signal using any of the antisera in TROL KO plants (data not shown). Affinity purified anti-preTROL clearly recognized only a single band in TROL-IE 8 and TROL-IE 18 lines and not in the TROL overexpression line (Fig. 3 and Fig. 4). When compared to the anti-HA blots on TROL-IE lines, it was clear that affinity purified anti-preTROL recognized the precursor form of TROL and not the mature one (Fig. 4). When using the anti-HA antibody, mature form of TROL could be detected also in the IE lines (Fig. 4 Lanes 6 and 7), however in lesser amount than in the OX (Fig. 4 Lane 5). In the IE lines processing of TROL still occurred to some extent (Vojta et al. 2018) and mature TROL was visible because of the much larger amount of thylakoids, although the prevailing amount of TROL was in the inner envelope membrane. Therefore, when taken together with the previous localization results (Jurić et al. 2009, Vojta et al. 2018), these experiments confirmed that in the TROL-IE8 and TROL-IE18 *Arabidopsis* lines TROL is located almost exclusively in the inner envelope membrane, and that the affinity purified anti-preTROL antiserum can be further used to exclusively detect the inner envelope form of TROL. By these experiments we provided the tool to investigate TROL in the inner envelope membrane of chloroplasts: its role in organelle metabolism and energetics, abiotic stress response, its interaction partners, and finally, its possible role in electron transfer at the level of the IE.

## Acknowledgements

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## Author contribution statement

L. V. devised the research concept, acquired funding, designed, and performed all experiments except the floral dip transformation, analyzed results, and wrote the manuscript. H. F. co-designed experiments and discussed results. A. T. P. performed floral dip transformation. E. D. grew experimental plants. All authors reviewed the paper and agreed to the published version of the manuscript.

## References

Allen, J. F., 2003: Cyclic, pseudocyclic and noncyclic photophosphorylation: new links in the chain. Trends in Plant Science 8(1), 15–19. https://doi.org/10.1016/S1360-1385(02)00006-7

Benz, J. P., Lintala, M., Soll, J., Mulo, P., Bölter, B., 2010: A new concept for ferredoxin-NADP(H) oxidoreductase binding to plant thylakoids. Trends in Plant Science 15(11), 608–613. https://doi.org/10.1016/j.tplants.2010.08.008

Buchanan, B. B., Balmer, Y., 2005: Redox regulation: a broadening horizon. Annual Review of Plant Biology 56, 187–220. https://doi.org/10.1146/annurev.arplant.56.032604.144246

Cline, K., Henry, R., 1996: Import and routing of nucleus-encoded chloroplast proteins. Annual Review of Cell and Developmental Biology 12, 1–26. https://doi.org/10.1146/annurev.cellbio.12.1.1

Clough, S. J., Bent, A. F., 1998: Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. The Plant Journal 16(6), 735–743. https://doi.org/10.1046/j.1365-313x.1998.00343.x

Fulgosi, H., Vojta, L., 2020: Tweaking photosynthesis: FNR-TROL interaction as potential target for crop fortification. Frontiers in Plant Science 11, 318. https://doi.org/10.3389/fpls.2020.00318

Garcia, C., Khan, N. Z., Nannmark, U., Aronsson, H., 2010: The chloroplast protein CPSAR1, dually localized in the stroma and the inner envelope membrane, is involved in thylakoid biogenesis. The Plant Journal 63(1), 73–85. https://doi.org/10.1111/j.1365-313X.2010.04225.x

Hanke, G., Mulo, P., 2013: Plant type ferredoxins and ferredoxin-dependent metabolism. Plant, Cell & Environment. 36(6), 1071–1084. https://doi.org/10.1111/pce.12046

Joliot, P., Joliot, A., 2006: Cyclic electron flow in C3 plants. Biochimica et Biophysica Acta 1757(5–6), 362–368. https://doi.org/10.1016/j.bbabio.2006.02.018

Jurić, S., Hazler-Pilepić, K., Tomašić, A., Lepeduš, H., Jeličić, B., Puthiyaveetil, S., Bionda, T., Vojta, L., Allen, J. F., Schleiff, E., Fulgosi, H., 2009: Tethering of ferredoxin:NADP$^+$ oxidoreductase to thylakoid membranes is mediated by novel chloroplast protein TROL. The Plant Journal 60(5), 783–794. https://doi.org/10.1111/j.1365-313X.2009.03999.x

Jurić, S., Vojta, L., Fulgosi, H., 2013: Electron transfer routes in oxygenic photosynthesis: regulatory mechanisms and new perspectives. Photosynthesis, 23-46. InTech, Rijeka.

Kekić, T., Fulgosi, H., Vojta, L., Bertoša, B., 2020: Molecular basis of ferredoxin:NADP(+) reductase interactions with FNR binding domains from TROL and Tic62 proteins. Journal of molecular structure 1215, 128281. https://doi.org/10.1016/j.molstruc.2020.128281

Klasek, L., Inoue, K., 2016: Dual protein localization to the envelope and thylakoid membranes within the chloroplast. International Review of Cell and Molecular Biology 323, 231–263. https://doi.org/10.1016/bs.ircmb.2015.12.008

Küchler, M., Decker, S., Hörmann, F., Soll, J., Heins, L., 2002: Protein import into chloroplasts involves redox-regulated proteins. The EMBO Journal 21(22), 6136–6145. https://doi.org/10.1093/emboj/cdf621

Laemmli, U. K., 1970: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685. https://doi.org/10.1038/227680a0

Lamppa, G. K., 1988: The chlorophyll a/b-binding protein inserts into the thylakoids independent of its cognate transit peptide. The Journal of Biological Chemistry 263(29), 14996–14999. https://doi.org/10.1016/S0021-9258(18)68137-2

McKenzie, S. D., Ibrahim, I. M., Aryal, U. K., Puthiyaveetil, S., 2020: Stoichiometry of protein complexes in plant photosynthetic membranes. Biochimica et Biophysica Acta Bioenergetics. 1861(2), 148141. https://doi.org/10.1016/j.bbabio.2019.148141

Peltier, J.-B., Ytterberg, A.-J., Sun, Q., van Wijk, K.-J., 2004: New functions of the thylakoid membrane proteome of *Arabidopsis thaliana* revealed by a simple, fast, and versatile fractionation strategy. Journal of Biological Chemistry 279(47), 49367–49383. https://doi.org/10.1074/jbc.M406763200

Sáiz-Bonilla, M., Martín-Merchán, A., Pallás, V., Navarro, J. A., 2023: A viral protein targets mitochondria and chloroplasts by subverting general import pathways and specific receptors. Journal of Virology 97(10), e0112423. https://doi.org/10.1128/jvi.01124-23

Schnell, D. J., 1998: Protein targeting to the thylakoid membrane. Annual Review of Plant Physiology and Plant Molecular Biology 49, 97–126. https://doi.org/10.1146/annurev.arplant.49.1.97

Seo, S. Y., Wi, S. J., Park, K. Y., 2020: Functional switching of NPR1 between chloroplast and nucleus for adaptive response to salt stress. Scientific Reports 10, 4339. https://doi.org/10.1038/s41598-020-61379-3

Sirpiö, S., Suorsa, M., Aro, E.-M., 2011: Analysis of thylakoid protein complexes by two-dimensional electrophoretic systems. Methods in Molecular Biology 775, 19–30. https://doi.org/10.1007/978-1-61779-237-3_2

Vojta, L., Carić, D., Cesar, V., Antunović Dunić, J., Lepeduš, H., Kveder, M., Fulgosi, H., 2015: TROL-FNR interaction reveals alternative pathways of electron partitioning in photosynthesis. Scientific Reports 5, 10085. https://doi.org/10.1038/srep10085

Vojta, L., Čuletić, A., Fulgosi, H., 2018: Effects of TROL presequence mutagenesis on its import and dual localization in chloroplasts. International Journal of Molecular Sciences 19(2), 569. https://doi.org/10.3390/ijms19020569

Vojta, L., Fulgosi, H., 2012: Energy conductance from thylakoid complexes to stromal reducing equivalents. Advances in Photosynthesis - Fundamental Aspects, 175–190. InTech, Rijeka.

Vojta, L., Fulgosi, H., 2016: Data supporting the absence of FNR dynamic photosynthetic membrane recruitment in *trol* mutants. Data in Brief 7, 393–396. https://doi.org/10.1016/j.dib.2016.02.044

Vojta, L., Fulgosi, H., 2019: Topology of TROL protein in thylakoid membranes of *Arabidopsis thaliana*. Physiologia Plantarum 166(1), 300–308. https://doi.org/10.1111/ppl.12927

Vojta, L., Horvat, L., Fulgosi, H., 2012: Balancing chloroplast redox status – regulation of FNR binding and release. Periodicum Biologorum 114, 25–31.

Vojta, L., Rac-Justament, A., Zechmann, B., Fulgosi, H., 2023: Thylakoid rhodanese-like protein–ferredoxin:NADP$^+$ oxidoreductase interaction is integrated into plant redox homeostasis system. Antioxidants 12(10), 1838. https://doi.org/10.3390/antiox12101838

Vojta, L., Soll, J., Bölter, B., 2007: Requirements for a conservative protein translocation pathway in chloroplasts. FEBS Letters 581(14), 2621–2624. https://doi.org/10.1016/j.febslet.2007.05.004

Yoshida, K., Yokochi, Y., Tanaka, K., Hisabori, T., 2022: The ferredoxin/thioredoxin pathway constitutes an indispensable redox-signaling cascade for light-dependent reduction of chloroplast stromal proteins. Journal of Biological Chemistry 298(12):102650. https://doi.org/10.1016/j.jbc.2022.102650

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# Physiological and molecular response of *Brassica rapa* to moderate and extreme heat

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**Abstract** – One of the key factors affecting plant survival and agricultural yield production is temperature. The magnitude of temperature extremes is increasing as a result of global climate change. The present study evaluated the impact of elevated temperature treatments on *Brassica rapa* seed germination, as well as of prolonged exposure of seedlings to temperatures of 37 °C and short-term exposure to the temperature of 45 °C. Elevated temperatures reduced seed germination rate and affected germination pattern. Both applied heat stresses negatively affected seedling development and root growth, and showed a differential physiological and molecular response. Under prolonged exposure to 37 °C seedling growth and development patterns were impaired but with no sign of oxidative stress, which could be related to increased indole-3-acetic acid (IAA), abscisic acid, enhanced heat shock protein 90 (HSP90) and reduced 1-aminocyclopropane-1-carboxylate levels. The short-term exposure to a temperature of 45 °C, a treatment mimicking a heat wave event, had more negative effects on seedling growth, which correlated with the appearance of oxidative stress. The extreme temperature significantly stimulated the gene expression of heat stress transcription factors *HSFs* and dehydration-responsive element-binding protein *DREB2A*, and induced the accumulation of auxin IAA and HSP90 proteins. Our study confirms the great importance of phytohormones and HSP90 in the heat stress response of *B. rapa* and emphasizes the potential for their manipulation in phytoprotection and breeding programs for adaptation to climate change.

**Key words:** cabbage, germination, heat, phytohormones, root growth, seedling growth, stress markers

## Introduction

Plants have the ability to adapt their phenotype in response to different environmental influences. To withstand adverse conditions, they have evolved numerous molecular strategies to adapt to environmental challenges and deal effectively with all types of stressful conditions. Environmental and endogenous signals precisely regulate plant growth and development (Peleg and Blumwald 2011) and ambient temperature is one of the most important parameters influencing all aspects of plant development, such as germination, seedling establishment, vegetative growth and reproductive development (Begcy et al. 2018, Angadi et al. 2000). Germination is a particularly strictly regulated stage in the life cycle of plants. It is the first step in the successful establishment of plant growth and influences species distribution (Yamauchi et al. 2004, Heschel et al. 2007). Although all plant growth stages can be affected by heat stress, high temperatures can negatively impact seed germination and limit subsequent plant growth, sometimes leading to irreversible damage and plant death (Yeh et al. 2012). To adapt to elevated temperatures, plants undergo a series of changes in their transcriptome, proteome, metabolome and lipidome (Mittler and Blumwald 2015, Raza et al. 2023), while vulnerability depends on temperature intensity and duration. Recently, great progress has been made in research into the response mechanisms of plants to elevated temperatures (Kan et al. 2023). However, further research is needed as the ways in which heat stress is perceived and transduced into physiological and morphological responses are still unclear.

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Important players in the adaptation to high temperatures are phytohormones, which influence growth in a dose-dependent manner and whose fine-tuning and interactions are particularly important in the regulation of a variety of developmental processes under heat stress (Peleg and Blumwald 2011, Vanstraelen and Benková 2012, Ahammed et al. 2016). Heat exposure particularly influences the metabolism and accumulation of so-called stress hormones such as auxin, ethylene, abscisic acid (ABA), salicylic acid and jasmonic acid, which is reflected in plant growth and development (Vanstraelen and Benková 2012, Qin et al. 2019). But our knowledge of how exposure to different temperatures affects the metabolism and accumulation of phytohormones and how this fine-tuning affects the development and growth of specific plant is still limited.

The frequency and magnitude of temperature extremes are increasing as a result of global climate change, which is expected to result in ever more intense and frequent extreme weather events (Seneviratne et al. 2021). The optimal growth temperature, as well as an upper threshold at which development stagnates or stops have significant effects on plant distribution, performance and yield (Ahmad et al. 2022). *Brassica rapa* has a long history of human cultivation and is a feral plant found around the world. Due to its high nutritional value, health-promoting effects and mild flavour, it is a very popular leafy vegetable. It prefers cool weather (15-20 °C), so climate change and the associated rise in temperature have a negative impact on growth and seed yield (Angadi et al. 2000). Still, little is known about how different types of heat stress, such as prolonged exposure to moderately elevated temperature or short exposure to extreme temperature affect morphological, the physiological and molecular responses of *B. rapa* seedlings. The temperatures applied in our experiments reflect the current rise in global temperature and are of ecological importance. The temperature of 37 °C is commonly measured during the summer season in areas with a continental climate, while short exposures to 45 °C reflect the extreme heat wave events encountered intermittently during summer. Therefore, we investigated the effects of elevated temperatures on the germination and emergence of *B. rapa* seedlings. Additionally, we analysed growth, proline (Pro) and malondialdehyde (MDA) content, as well as heat-inducible HSP90 protein and *HSFA7A*, *HSFB2A* and *DREB2A* genes in seedlings exposed to prolonged moderately elevated temperature or to an extreme and short heat wave. Special attention was devoted to the accumulation of the major stress related phytohormones since, as far as we know, there are currently no data available on their heat-induced changes in *B. rapa* seedlings.

## Materials and methods

### Plant material and sterilization

For all experiments, *B. rapa* L. subsp. *pekinensis* (purchased from ISP-International Seeds Processing GmbH, Germany) was used. Seeds were sterilized by soaking in 70% ethanol for 1 min, and then incubated in 3% Izosan G (100% sodium dichloroisocyanurate dihydrate, Pliva) for 10 min. After a 5-fold rinsing step with sterile distilled water, seeds were sown on Murashige and Skoog (1962) medium (MS) and stratified at 4 °C for 5 days. Plates with seeds were then moved to a growth chamber (PHC Corporation, Tokyo, Japan), positioned horizontally (for the germination assay) or vertically (for the seedling growth assay) and incubated for 16 h with light at 150 µmol $m^{-2}$ $s^{-1}$ and 8 h in darkness.

### Seed heat treatment and germination

To examine temperature effects on seed germination, plates with seeds were incubated at constant temperature of 24 °C (control) and at elevated temperatures (up to 41 °C). After 3 days, the germination rate was evaluated as percentage (%) of seeds with a visible radicle tip and hypocotyl length was measured.

### Seedling heat treatments

Seven-day-old *B. rapa* seedlings were exposed to a heat stress treatment at two different temperatures: 45 °C for 2 h and 15 min followed by a recovery at 24 °C, or 37 °C for 24 h. A preliminary experiment showed that 2 h and 15 min exposure to 45 °C did not result in immediate lethality to the seedlings (On-line Suppl. Fig. 1).

For the growth parameters, the plant material was analysed seven days (168 h) after the start of the heat treatment. Gene expression, HSP90 protein levels and phytohormone concentrations were assessed at a single time point, 24 h after the start of treatment. Pro and MDA content were measured at three time points: 2 h and 15 min, 24 h and 48 h after the start of treatment at 37 °C and 45 °C. Control seedlings were kept at 24 °C and analysed at the same time points as the treated seedlings.

### Seedling growth assay

Seedling growth was monitored 7 days from the beginning of treatments (until the root tip of control seedlings reached the bottom of the dish). Root length of heat-treated and control seedlings was determined by ImageJ v.1.49 (Schneider et al. 2012). Lateral root number was counted manually. Total root length was measured as the sum of primary and lateral root lengths. Primary root growth rate was expressed as the difference between the final (168 h after the start of heat treatment) and initial (immediately prior to heat treatment) primary root length. Average fresh weight per seedling was calculated by weighing 10-12 whole seedlings at the end of the experiment.

### Malondialdehyde and proline evaluation

MDA and Pro contents were determined as markers of oxidative damage during heat exposure and after recovery from heat treatments at 24 °C. Whole seedlings exposed to heat or control conditions were sampled, weighed and immediately frozen in liquid nitrogen before analyses.

MDA determination was done according to Hodges et al. (1999) and Sunkar et al. (2003) and Pro determination was done according to Bauer et al. (2022).

### Gene expression evaluation

Whole seedlings exposed to heat-treated or control conditions were sampled, weighed and frozen in liquid nitrogen. RNA was isolated from 40-50 mg of frozen homogenized seedlings using the MagMaxTM Plant RNA Isolation Kit according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 µg of isolated RNA using 200 U of RevertAid H Minus Reverse Transcriptase and Oligo(dT)18 primer (Thermo Fisher Scientific) in a total volume of 20 µL. The RT-qPCR was performed in technical duplicates on the MIC platform (Bio molecular Systems) by using GoTaq® qPCR Master Mix reagent (Promega), gene specific primers (On-line Suppl. Tab. 1) and 10 ng of cDNA. Thermocycling conditions were set to 5 min at 95 °C, followed by 40 cycles at 95 °C for 10 s and 60 °C for 10 s. Specific amplification was verified by no template controls and melting curves generated by increasing the temperature from 55 °C to 95 °C at 0.5 °C s$^{-1}$. Quantification cycle (Cq) values and primer efficiencies were calculated with MIC qPCR Cycler software (Bio Molecular Systems). Relative gene expression of heat stress-related genes *HSFA7A*, *HSFB2A* and *DREB2A* was calculated with the ΔΔCq method (Pfaffl 2001, Vandesompele et al. 2002). The *B. rapa* genes *OGIO* (2-oxoglutarate and Fe (II)-dependent oxygenase) and *PUX* (plant UBX domain-containing protein) were used as references. Genes, accession numbers and primer sequences are listed in On-line Suppl. Tab. 1.

### HSP90 determination

Total soluble proteins were extracted from 150-200 mg frozen heat-treated or control seedlings. The seedlings were homogenized in 400-800 µL Staples and Stahmann (1964) extraction buffer (92.5 mM TRIS-HCl, 500 mM sucrose, 6.48 mM dithiothreitol, pH 7.6). After sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide gels, proteins were transferred onto a polyvinylidene difluoride (PVDF; Immobilon-P, Sigma-Aldrich) membrane. HSP90 was detected by using anti-HSP90-1 (Agrisera) and anti-Rabbit IgG-horse raddish peroxidase (Sigma Aldrich) antibodies and Immobilon® Forte Western HRP substrate (Millipore). PVDF membranes were stained with Coomassie brilliant blue (0.1% (w/v) Coomassie R-250 in 40% methanol and 10% acetic acid). Images were analysed in ImageJ v.1.49 (Schneider et al. 2012) as described in Taylor and Posch (2014). To calculate the changes in protein levels (FD), the respective control values were set to one.

### Phytohormone determination

Free endogenous indole-3-acetic acid (IAA), abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylate (ACC) were measured by gas chromatography–mass spectrometry (GC–MS) according to previously adapted protocols (Villas-Bôas et al. 2003, Rawlinson et al. 2015, Ludwig-Müller et al. 2021). Heat-treated and control seedlings were sampled, weighed, frozen in liquid nitrogen and homogenised. After homogenization, at least 100 mg of frozen plant tissue was transferred to 2 mL tubes. A hundred nanograms of labelled standards $^{13}C_6$-IAA (Cambridge Isotope Laboratories, Andover, MA, USA; 10 ng µL$^{-1}$) and $^{2}H_4$-ACC (Eurisotop GmbH, Saarbrücken, Germany; 10 ng µL$^{-1}$), and 200 ng of $^{2}H_6$-ABA (Cambridge Isotope Laboratories, Andover, MA, USA; 10 ng µL$^{-1}$) were added to each tissue sample as internal standards. Samples were weighed, processed and measured in technical duplicates. ABA, IAA and ACC concentrations were determined using principles of isotope dilution (Cohen et al. 1986) from diagnostic ion ratios of endogenous and labelled hormones at a m/z of 190/194 for ABA, 130/136 for IAA and 141/145 for ACC, at respective retention times of 11.8-11.9 min, 10.5-10.6 min and 6.9-7.0 min, using the formula: $Y=\left(\frac{Ci}{Cf}-1\right)\times X$. Ci is the sum of peak areas of standard and endogenous hormone, Cf the peak area of the standard, X the amount of labelled standard added, and Y the hormone concentration. Values obtained by this formula were then divided by the fresh weight and expressed as such.

### Statistical methods

At least three biological replicates per treatment or control were analysed. Germination rates were compared between treatments and control using a Chi-square test of independence. For other parameters, normality of data was assessed using the Shapiro-Wilk test and homogeneity of variances using the Levene test. Normality and equal variances were assumed if $P > 0.05$. Although the data were normally distributed, the variances were not always homogeneous. Therefore, depending on the homogeneity of variances, a one-way ANOVA followed by Tukey's HSD post hoc test or Welch's ANOVA followed by a Games-Howell post hoc test was performed. Statistical significance was set at $P \leq 0.05$. Statistical analysis was performed in the TIBCO Statistica 13.5.0.17 software package (TIBCO Software, USA).

## Results

### Heat significantly affects seed germination

Seeds of *B. rapa* were exposed to a set of rising temperatures and germination rate (% of seeds with visible radicle tips) was monitored (Fig. 1). In control conditions, *B. rapa* germination started as early as after 8 h (On-line Suppl. Fig. 2) and after 3 days all seeds (100%) had developed into green seedlings. At slightly elevated temperature (28.5 °C), hypocotyls elongated significantly (Fig. 1A, C) while a further temperature rise (e.g. 32.7 °C) significantly inhibited hypocotyl elongation. At temperatures above 38 °C, the germination rate was significantly reduced compared to the control. In addition, these temperatures blocked hypocotyl elongation, apical hook opening and cotyledon opening, ex-

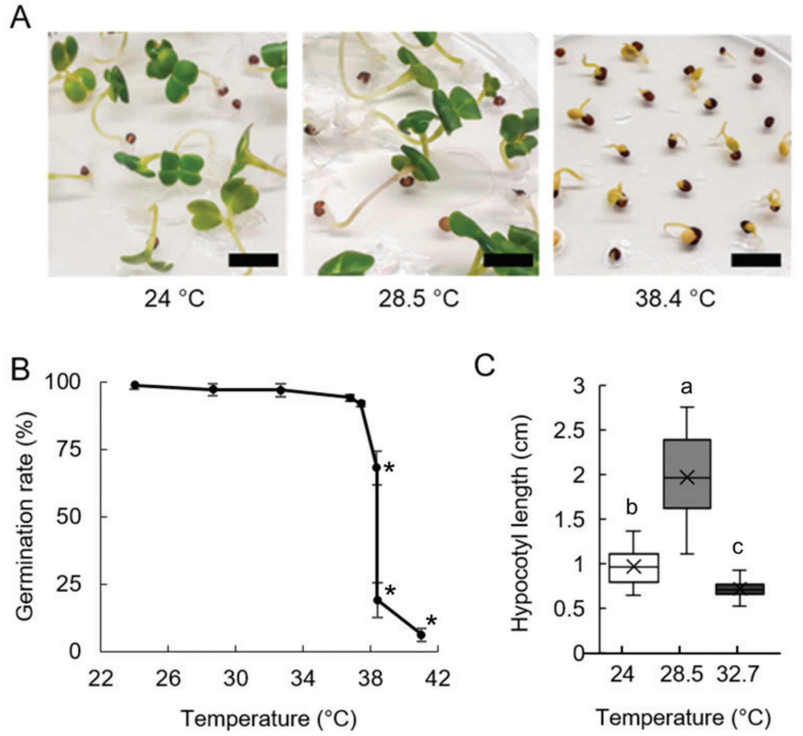

**Fig. 1.** Effect of elevated temperatures on *Brassica rapa* seed germination. Following stratification, seeds were exposed for three days to a set of temperatures when seedlings were documented (A), the germination rate (% of seeds with visible radicle tip) determined (B), and hypocotyl length measured (C). Data in B are the average of three biological replicates (mean ± SD) each consisting of ~100 seeds. Asterisks (*) indicate a significant difference between the control (24 °C) and the different temperatures at $P \leq 0.05$ (Chi-square test of independence). Data in C are represented as boxes that indicate the lower and upper quartiles while means and medians are denoted with a horizontal line and a cross in the box, respectively. Whiskers represent the highest and lowest scores in the data set. Different letters indicate a significant difference at $P \leq 0.05$ (Welch's ANOVA, Games-Howell test). Scale bar in (A) = 1 cm.

pansion and greening (Fig. 1A, B). The highest temperature at which a minimal number of *B. rapa* seeds still germinated was 40 °C.

### Heat affects seedling growth, morphology and biomass accumulation

Although both prolonged exposure (24 h) to 37 °C and short-term (2 h and 15 min) heat treatment at 45 °C, influenced the growth of *B. rapa* seedlings, the short exposure to 45 °C had more severe effects. A heat wave at 45 °C blocked lateral root development and affected primary root growth rate, total root length and biomass accumulation significantly more than the prolonged exposure to 37 °C (Fig. 2). Exposure to 37 °C induced hypocotyl elongation (Fig. 2A) while both heat treatments induced leaf yellowing, inhibited leaf growth (On-line Suppl. Fig. 3) and significantly reduced biomass accumulation (Fig. 2E).

### Heat wave affects proline and malondialdehyde accumulation

To determine the severity of the applied heat treatments, Pro and MDA levels as markers of oxidative damage were

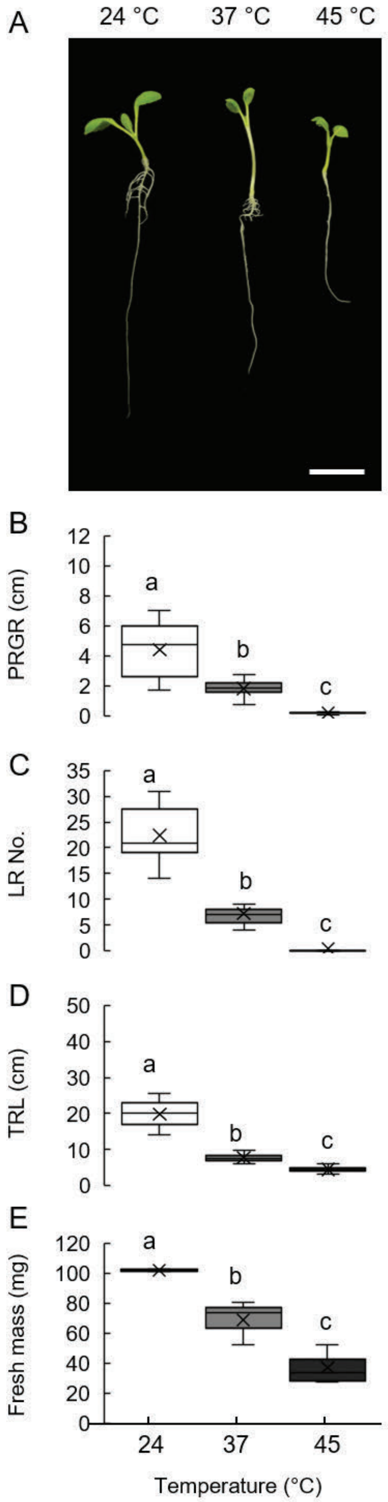

**Fig. 2.** The effect of heat stress on *Brassica rapa* seedling development. Seedlings were treated continuously at 37 °C for 24 h, or at 45 °C 2 h and 15 min followed by recovery at 24 °C. Control plants were kept at 24 °C. Seedlings were photographed (A) and analyzed 7 days after the start of heat stress treatments. Primary root growth rate, PRGR (B), lateral root number, LR No. (C), total root length, TRL (D) and biomass accumulation (E) were determined. Data in B-E are represented as boxes that indicate the lower and upper quartiles while means and medians are denoted with a horizontal line and a cross in the box, respectively. Whiskers represent the highest and lowest scores in the data set. Different letters indicate a significant difference at $P \leq 0.05$ (Welch's ANOVA, Games-Howell test). Scale bar in (A) = 2 cm.

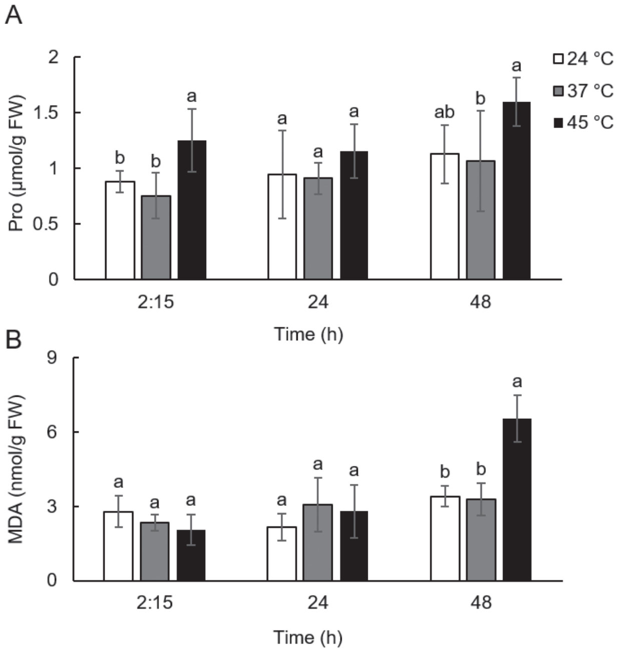

**Fig. 3.** Proline, Pro (A) and malondialdehyde, MDA (B) accumulation following heat stress in *Brassica rapa*. Pro and MDA levels were measured in seedlings either treated at 37 °C for 24 h or exposed to 45 °C for 2 h and 15 min (2:15) followed by recovery. Control plants were kept at 24 °C. Pro and MDA levels were measured at 2:15 h, 24 h and 48 h from the start of the treatments and expressed per fresh weight (FW). Data represent the average of six replicates with standard deviations denoted by vertical bars. Different letters indicate a significant difference at P ≤ 0.05 (ANOVA, Tukey's test).

measured in heat treated (37 °C for 24 h, or 45 °C for 2 h and 15 min followed by recovery) *B. rapa* seedlings and compared to the corresponding control. Pro and MDA levels were measured in three time points – 2 h and 15 min, 24 h and 48 h from the start of the treatments. Exposure to 45 °C immediately induced Pro accumulation by 42% and elevated Pro levels were also detected even 48 h after heat wave treatment (Fig. 3A). In accordance, MDA content was significantly higher in seedlings 48 h after heat wave treatment (Fig. 3B). Exposure to 37 °C did not affect Pro and MDA accumulation indicating that the stress response of *B. rapa* seedlings to the heat wave-like treatment was more pronounced than that to prolonged exposure to a moderately elevated temperature.

### Heat wave induces expression of heat stress-related genes and proteins

Plant stress response is mediated by transcription factors that perceive stress signals and direct downstream defence gene expression. To further investigate the effect of heat treatments, we quantified gene expression of major heat stress-responsive transcription factors *HSFA7A*, *HSFB2A* and *DREB2A* by RT-qPCR. Short-term exposure to 45 °C revealed a drastic induction of the expression of the examined genes, while a prolonged exposure to 37 °C only moderately enhanced *HSFA7A*, *HSFB2A* and *DREB2A* gene expression (Fig. 4A). HSP90 protein accumulation was evaluated after immunodetection and normalized to the RuBisCO protein (Fig. 4B, On line Suppl. Fig. 4). Both heat stress treatments induced accumulation of HSP90 proteins. Under control conditions, a low level of one HSP90 protein form (Fig. 4B, white arrowhead) was detected, while under heat stress enhanced accumulation as well as additional forms of HSP90 proteins were observed (black arrowheads).

### Heat treatments affect phytohormone levels

Evident morphological changes of heat-treated *B. rapa* seedlings encouraged us to explore the fluctuations of phytohormones IAA, abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylate (ACC, the precursor of ethylene)

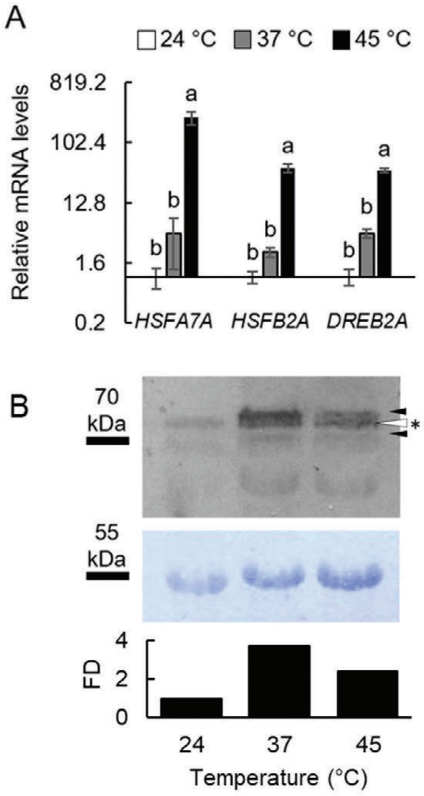

**Fig. 4.** Expression of heat stress-related transcription factor genes and proteins in *Brassica rapa*. Seedlings were treated at 37 °C for 24 h or exposed to 45 °C for 2 h and 15 min followed by recovery. Control plants were kept at 24 °C. Gene expression of heat stress-responsive transcription factors (A) and HSP90 proteins (B) were measured 24 h after the start of the treatments. For the relative quantification of *HSFA7A*, *HSFB2A* and *DREB2A* genes were normalized to the reference genes *OGIO* and *PUX*. Gene expressions are ΔΔCq values (with corresponding controls taken as one) on a log2 scale and presented as averages of three biological replicates with standard deviations denoted by vertical bars. Different letters indicate significant differences at P ≤ 0.05 (ANOVA, Tukey's test). Immunoassay signals of HSP90 were normalized to the RuBisCO protein intensities on PVDF membranes after staining with Coomassie brilliant blue and expressed as fold difference (FD), where corresponding control (24 °C) is set to one. The asterisk/white arrowhead indicates HSP90 present at control conditions and black arrowheads indicate heat-induced HSP90 forms.

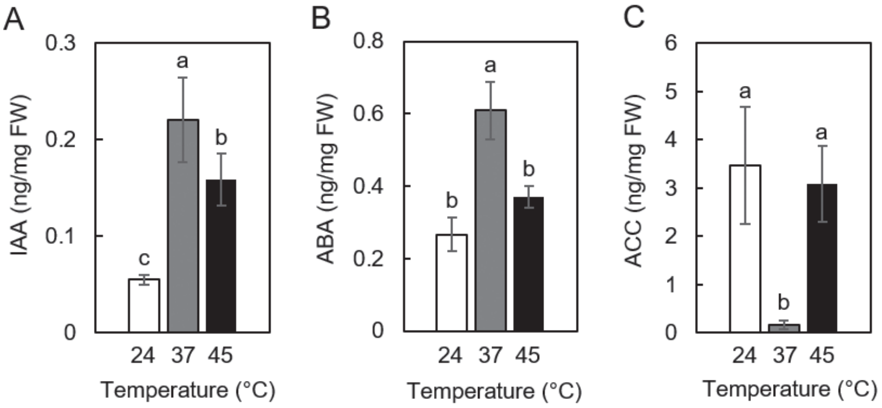

**Fig. 5.** Phytohormone levels following heat stress in *Brassica rapa*. Seedlings were either treated at 37 °C for 24 h or exposed to 45 °C for 2 h and 15 min followed by recovery. Control plants were kept at 24 °C. Indol-3-acetic acid, IAA (A), abscisic acid, ABA (B), and 1-aminocyclopropane-1-carboxylic acid, ACC (C) were measured by GC-MS from whole seedlings harvested 24 h from the start of the treatments and expressed per fresh weight (FW). Data represent the average of three replicates with standard deviations denoted by vertical bars. Different letters indicate a significant difference between control and heat treatments at P ≤ 0.05 (ANOVA, Tukey's test).

(Fig. 5). Plant growth regulators were affected more severely by prolonged exposure to 37 °C than by short-term exposure to 45 °C followed by recovery. IAA content was significantly upregulated by both heat treatments (Fig. 5A) while exposure to 37 °C, but not to 45 °C, significantly induced ABA (Fig. 5B) and reduced ACC levels (Fig. 5C).

## Discussion

The magnitude of temperature extremes is increasing as a result of global climate change and is accompanied by more frequent heat waves. Elevated temperatures may alter germination timing and rate, decrease biomass accumulation and reduce species' performance and yields by impairing reproduction (Anderson et al. 2020, Ahmad et al. 2022). Since temperature is one of the most important factors in plant growth and development, significant changes in plant distribution, abundance and yield are expected in the near future. Temperature increases of 1 °C cause a 17% yield reduction in *Brassica* species (Kaushal et al. 2016). Here, we studied the heat effects on *B. rapa* seed germination, and seedlings' performance under a moderately elevated temperature of 37 °C, reflecting the commonly measured temperature during the summer season in areas with a continental climate, and the effect of a short exposure to an extreme temperature of 45 °C, reflecting extreme heat wave events that occasionally occur during summer days. We analysed early stages of seedling establishment when plants are more vulnerable and temperature particularly affects growth rate and development. Successful seed germination is the major prerequisite of species' survival and distribution (Heschel et al. 2007) and may significantly impact crop yield (Boter et al. 2019). Several works have shown a negative correlation between rising temperatures and *Brassica* species seed germination rates. Wilson et al. (1992) showed that 40 °C is a critical temperature decreasing germination rates significantly in 11 different cultivars of *B. rapa*, *B. oleracea* and *B. napus*, while Motsa et al. (2015) showed that 36 °C is the maximum germination temperature for *B. rapa* ssp. *chinensis*. In accordance with these results, we showed that although *B. rapa* seed radicule protrusion occurred under heat, post-germination seedling development was strongly impaired indicating 36 °C as the temperature threshold for *B. rapa* distribution and seedling establishment in the field. Once germinated, *B. rapa* seedlings tolerated 37 °C and could survive for as long as 2 h and 15 min at 45 °C. However, the accumulation of biomass, as well as root growth and development were significantly disturbed by exposure to both temperature regimes (Fig. 2), and the effect was more pronounced when seedlings were exposed to a heat wave at 45 °C. Indeed, short exposure to 45 °C temporarily blocked root growth of *B. rapa* seedlings. In other *Brassica* species heat stress often reduces growth, especially of aboveground organs, which is an adaptation that can help plants reduce transpiration and conserve water (Gunasekera et al. 2006, Rodríguez et al. 2015, Munns and Millar 2023), but heat response and tolerance can vary greatly among species, varieties or even accessions (Pavlović et al. 2018, 2019, Bauer et al. 2022). For example, exposure to elevated temperature reveals accession-specific diversity in *B. oleracea* var. *acephala* and *B. napus* (Bauer et al. 2022, Boter et al. 2023), with some having enhanced growth and accumulation of biomass, while most remain sensitive to heat, which fits with our results. Moreover, a significant difference in the phenotypic root system architecture of *B. napus* varieties is associated with differences in the transcriptional dynamics of the heat shock and hormonal response genes (Boter et al. 2023). Furthermore, comprehensive analyses of *B. rapa* genes expressed only at one type of heat treatment, such as 45 °C (Dong et al. 2015, Quan et al. 2023), 42 °C (Yu et al. 2023) or 40 °C (Zhang et al. 2022) have been performed. We compared how different types of heat treatments affect the gene expression of heat-related *HSF* and *DREB2A* transcription factors and further determined HSP90 protein and phytohormone levels, which to our knowledge, has not previously been done in heat stress treated *B. rapa* seedlings.

At 37 °C, *HSFA7A*, *HSFB2A* and *DREB2A* were slightly enhanced, while the 45 °C-treatment significantly upregulated these genes (Fig. 4A). Both types of heat treatment enhanced HSP90 protein accumulation and induced the expression of new HSP90 forms (Fig. 4B). Additional HSP90 proteins recognised by immunodetection may be heat-induced splice variants, post-translationally modified HSP90 proteins or newly induced HSP90 proteins, as 17 genes encoding *HSP90* have been reported for *B. rapa* (Wang et al. 2022). Differential expression of HSP90s participates in the responses of *B. napus* to salt stress and *Sclerotinia sclerotiorum* infection (Wang et al. 2022), and in the response of *B. oleracea* to cold (Sajad et al. 2022), but as far as we know, no accurate expression analyses of individual HSP90 genes and proteins have been determined in *Brassica* species under heat stress.

In many plant species, Pro accumulates under heat stress and mediates tolerance by participating in signalling networks, helping to maintain redox balance as an ROS scavenger, and serving as an osmoprotectant (Raza et al. 2023). If they are not neutralised, ROS can damage proteins, lipids, carbohydrates and nucleic acids. Lipid peroxidation, characterised by the accumulation of MDA, destabilises cell membranes by increasing their fluidity. Increased Pro is confirmed for different *B. juncea* cultivars (Hayat et al. 2011) and rapeseed *B. napus* (Mohamed et al. 2020) subjected to abiotic stress. When *B. rapa* seedlings are exposed to 45 °C for 4 h, the MDA content increases significantly under heat stress (Rai et al. 2021). Here, *B. rapa* seedlings exposed to 37 °C showed no changes in Pro and MDA levels, while exposure to 45 °C immediately caused an accumulation of Pro that remained elevated for at least 2 days after the applied stress (Fig. 3). MDA accumulated with a delay of 2 days, indicating a heat-induced overproduction of ROS and consequent lipid peroxidation (Mittler et al. 2012), which led to an extreme reduction of plant growth after the short-term exposure to 45 °C (Fig. 2). Our results indicate that Pro and MDA are not suitable markers of heat stress when examining a single species or cultivar and their use as indicators of stress state depends on the type of temperature applied. However, comparing Pro and MDA accumulation among different cultivars for the purpose of screening and selecting those more resistant to a particular type of abiotic stress has been proven to be a good indicator for *Brassica* species (Pavlović et al. 2019, Bauer et al. 2022).

Although seedlings treated at 37 °C also showed significant growth deviations, they were accompanied by only small changes in Pro, MDA and heat-related gene expression levels so we further analysed heat treatment effects on phytohormone fluctuations that are known to be involved in *Brassica* species' stress tolerance mechanisms (Pavlović et al. 2018, 2019). Indeed, an exposure to either 37 °C or 45 °C had significant and differential effects on IAA, ABA and the immediate ethylene precursor ACC accumulation. These changes in phytohormone levels may be responsible for detected heat-related root growth disturbances (Figs. 2, 5). Phytohormones affect growth in a dose-dependent manner and their finetuning and interactions are especially important under stress exposure (Vanstraelen and Benková 2012). The perturbation in phytohormone levels under heat exposure mediates plant adaptive responses by impacting nutrient synthesis and allocation and guiding growth and developmental changes known as thermomorphogenesis (Casal and Balasubramanian 2019). Auxin, ABA and ethylene have a central role in determining root architecture, guiding the induction and growth of the main roots, lateral roots, adventitious roots and root hairs (Olatunji et al. 2017). They also promote hypocotyl elongation at elevated temperature (Emenecker and Strader 2020), which is in accordance with our results. Elevated IAA and ABA, and a decrease of the ethylene precursor ACC may be the main reasons for the changes induced at 37 °C in *B. rapa* seedling morphology, while significant changes of IAA seem to govern growth and developmental changes of seedlings exposed to short extreme heat stress (Figs. 1, 2). In accordance with previous studies (Gray et al. 1998, Bianchimano et al. 2023), IAA level was significantly enhanced after both heat treatments and IAA can be considered a good heat stress marker in *B. rapa* seedlings. ACC was barely detectable at 37 °C and showed no significant change upon short-term exposure to 45 °C, which could be due to the imbalanced activity of the ACC synthase and ACC oxidase enzymes as described by Antunes and Sfakiotakis (2000) for kiwifruit grown at 40 °C. ABA content increased at 37 °C but after exposure to 45 °C (followed by recovery) was close to the control value. We assumed that a strong heat-induced rise in ABA level during treatment at 45 °C would instantly suppress seedling root growth and time-dependent screening of ABA levels under heat will be considered in our future research.

Heat-induced fluctuations of different hormones are not unequivocal and uniform among different species and may vary under different heat treatments (Prerostova et al. 2020, Poór et al. 2022) which is in agreement with our results. *In planta* auxin and ethylene synergistically affect root elongation and root hair formation, but act antagonistically in lateral root initiation and hypocotyl elongation (Muday et al. 2012). ABA induces primary root growth and suppresses lateral roots at low concentrations (Tiwari et al. 2022) and is important in mediating plant adaptation to stress (Baron et al. 2012). ABA confers thermotolerance by supporting photosystem II (PSII), switching on antioxidants, producing osmolytes and inducing gene expression of HSFs and HSPs (Ahammed et al. 2016, Jha et al. 2022). We measured a significant induction of ABA levels at 37 °C, and control-like ABA levels after short-term heat treatment at 45 °C followed by a recovery period at 24 °C (Fig. 5B) indicating that ABA accumulation is possibly instantly and strongly affected by heat. Similar ABA perturbances are noticed in *S. lycopersicum* and *A. thaliana* plants treated at 40 °C. After initial heat-induced ABA accumulation, a post-treatment ABA decline was observed (Dobrá et al. 2015). ABA may also have a crucial role in heat-induced *B. rapa* seed germination arrest observed here (Fig. 1). Exposure to extreme heat blocked hypocotyl elongation, apical hook opening, cotyledon opening, expansion and greening of *B. rapa* seedlings (Fig. 1). ABA is known to mediate inhibition of post-germination seedling establishment in the dark, while under light conditions, ABA is degraded enabling seedling greening and growth (Yadukrishnan and Datta 2021). Whether heat disturbs ABA degradation and induces seed germination and seedlings growth arrest of *B. rapa* should be further investigated in the future.

The results of our work highlight *B. rapa* vulnerability at early developmental stages. Prolonged, moderately elevated temperature changed the growth and development pattern by significant perturbation of phytohormone level and HSP90 protein accumulation. Short exposure to extreme heat significantly stimulates the expression of *HSFs* and *DREB2A* transcription factors, induces the accumulation of auxin IAA and HSP90 proteins, and almost stops seedling growth. The above confirms the great importance of phytohormones, especially IAA and ABA, in regulating *B. rapa* growth under heat stress and indicates the potential of targeting auxin and ABA metabolic and signalling pathways for phytoprotection or for use in breeding programs with aim to enhance stress tolerance and improve climate change adaptation.

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## References

Ahammed, G. J., Yu, J. Q., Li, X., Zhou, J., Zhou, Y. H., Yu, J. Q., 2016: Role of hormones in plant adaptation to heat stress. In: Ahammed, G., Yu, J. (eds.), Plant hormones under challenging environmental factors, 1–21. Springer, Dordrecht. https://doi.org/10.1007/978-94-017-7758-2

Ahmad, M., Imtiaz, M., Shoib Nawaz, M., Mubeen, F., Imran, A., 2022: What did we learn from current progress in heat stress tolerance in plants? Can microbes be a solution? Frontiers in Plant Science 13, 1–19. https://doi.org/10.3389/fpls.2022.794782

Anderson, R., Bayer, P. E., Edwards, D., 2020: Climate change and the need for agricultural adaptation. Current Opinion in Plant Biology 56, 197–202. https://doi.org/10.1016/j.pbi.2019.12.006

Angadi, S. V., Cutforth, H. W., Miller, P. R., McConkey, B. G., Entz, M. H., Brandt, S. A., Volkmar, K. M., 2000: Response of three *Brassica* species to high temperature stress during reproductive growth. Canadian Journal of Plant Science 80(4), 693–701. https://doi.org/10.4141/P99-152

Antunes, M., Sfakiotakis E. M., 2000: Effect of high temperature stress on ethylene biosynthesis, respiration and ripening of `Hayward` kiwifruit. Postharvest Biology and Technology. 20(3), 251-259. https://doi.org/10.1016/S0925-5214(00)00136-8

Baron, K. N., Schroeder, D. F., Stasolla, C., 2012: Transcriptional response of abscisic acid (ABA) metabolism and transport to cold and heat stress applied at the reproductive stage of development in *Arabidopsis thaliana*. Plant Science 188–189, 48–59. https://doi.org/10.1016/j.plantsci.2012.03.001

Bauer, N., Tkalec, M., Major, N., Talanga Vasari, A., Tokić, M., Vitko, S., Ban, D., Ban, S. G., Salopek-Sondi, B., 2022: Mechanisms of kale (*Brassica oleracea* var. *acephala*) tolerance to individual and combined stresses of drought and elevated temperature. International Journal of Molecular Science 23(19), 11494. https://doi.org/10.3390/ijms231911494

Begcy, K., Sandhu, J., Walia, H., 2018: Transient heat stress during early seed development primes germination and seedling establishment in rice. Frontiers in Plant Science 9:1768, 1–13. https://doi.org/10.3389/fpls.2018.01768

Bianchimano, L., De Luca, M. B., Borniego, M. B., Iglesias, M. J., Casal, J. J., 2023: Temperature regulation of auxin-related gene expression and its implications for plant growth. Journal of Experimental Botany 74(22), 7015–7033. https://doi.org/10.1093/jxb/erad265

Boter, M., Calleja-Cabrera, J., Carrera-Castaño, G., Wagner, G., Hatzig, S. V., Snowdon, R. J., Legoahec, L., Bianchetti, G., Bouchereau, A., Nesi, N., Pernas, M., Oñate-Sánchezm L., 2019: An integrative approach to analyze seed germination in *Brassica napus*. Frontiers in Plant Science 10:1342.. https://doi.org/10.3389/fpls.2019.01342

Boter, M., Pozas, J., Jarillo, J. A., Piñeiro, M., Pernas, M., 2023: *Brassica napus* roots use different strategies to respond to warm temperatures. International Journal of Molecular Sciences 24(2), 1143. https://doi.org/10.3390/ijms24021143

Casal, J., Balasubramanian, S., 2019: Thermomorphogenesis. Annual Review of Plant Biology 70. 321–346. https://doi.org/10.1146/annurev-arplant-050718-095919

Cohen, J. D., Baldi, B. G., Slovin, J. P., 1986: 13C6-[benzene ring]-indole-3-acetic acid. Plant Physiology 80(1), 14–19. https://doi.org/10.1104/pp.80.1.14

Dobrá, J., Černý, M., Štorchová, H., Dobrev, P., Skalák, J., Jedelský, P. L., Lukšanová, H., Gaudinová, A., Pešek, B., Malbecka, J., Vanek, T., Brzobohatý, B., Vanková, R., 2015: The impact of heat stress targeting on the hormonal and transcriptomic response in *Arabidopsis*. Plant Science 231, 52–61. https://doi.org/10.1016/j.plantsci.2014.11.005

Dong, X., Yi, H., Lee, J., Nou, I. S., Han, C. T., Hur, Y., 2015: Global gene-expression analysis to identify differentially expressed genes critical for the heat stress response in *Brassica rapa*. PLoS One 10, e0130451. https://doi.org/10.1371/journal.pone.0130451

Emenecker, R. J., Strader, L. C., 2020: Auxin-abscisic acid interactions in plant growth and development. Biomolecules 10(2), 281. https://doi.org/10.3390/biom10020281

Gray, W. M., Östin, A., Sandberg, G., Romano, C. P., Estelle, M., 1998: High temperature promotes auxin-mediated hypocotyl elongation in *Arabidopsis*. Proceedings of the National Academy of Sciences 95(12), 7197–7202. https://doi.org/10.1073/pnas.95.12.7197

Gunasekera, C., Martin, L., Siddique, K. H. M., Walton, G. H., 2006: Genotype by environment interactions of Indian mustard (*Brassica juncea* L.) and canola (*B. napus* L.) in Mediterranean-type environments. European Journal of Agronomy 25(1), 1–12. https://doi.org/10.1016/j.eja.2005.08.002

Hayat, S., Mir, B. A., Wani, A. S., Hasan, S. A., Irfan, M., Ahmad, A., 2011: Screening of salt-tolerant genotypes of *Brassica jun-*

*cea* based on photosynthetic attributes. Journal of Plant Interactions 6(1), 53–60. https://doi.org/10.1080/17429145.2010.521592

Heschel, M. S., Selby, J., Butler, C., Whitelam, G. C., Sharrock, R. A., Donohue, K., 2007: A new role for phytochromes in temperature-dependent germination. New Phytologist 174(4), 735–741. https://doi.org/10.1111/j.1469-8137.2007.02044.x

Hodges, D. M., DeLong, J. M., Forney, C. F., Prange, R. K., 1999: Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207, 604–611. https://doi.org/10.1007/s004250050524

Jha, U. C., Nayyar, H., Siddique, K. H. M., 2022: Role of phytohormones in regulating heat stress acclimation in agricultural crops. Journal of Plant Growth Regulation 41, 1041–1064. https://doi.org/10.1007/s00344-021-10362-x

Kan, Y., Mu, X. R., Gao, J., Lin, H. X., Lin, Y., 2023: The molecular basis of heat stress responses in plants. Molecular Plant 16(10), 1612–1634. https://doi.org/10.1016/j.molp.2023.09.013

Kaushal, N., Bhandari, K., Kadambot, H. M., Siddique, K. H. M., Nayyar, H., 2016: Food crops face rising temperatures: An overview of responses, adaptive mechanisms, and approaches to improve heat tolerance. Cogent Food & Agriculture, 2(1). https://doi.org/10.1080/23311932.2015.1134380

Ludwig-Müller, J., Rattunde, R., Rößler, S., Liedel, K., Benade, F., Rost, A., Becker, J., 2021: Two auxinic herbicides affect *Brassica napus* plant hormone levels and induce molecular changes in transcription. Biomolecules 11(8), 1153–1175. https://doi.org/10.3390/biom11081153

Mittler, R., Blumwald, E., 2015: The roles of ROS and ABA in systemic acquired acclimation. Plant Cell 27(1), 64–70. https://doi.org/10.1105/tpc.114.133090

Mittler, R., Finka, A., Goloubinoff, P., 2012: How do plants feel the heat? Trends in Biochemical Sciences 37(3), 118–125. https://doi.org/10.1016/j.tibs.2011.11.007

Mohamed, I. A. A., Shalby, N., Bai, C., Qin, M., Agami, R. A., Jie, K., Wang, B., Zhou, G., 2020: Stomatal and photosynthetic traits are associated with investigating sodium chloride tolerance of *Brassica napus* L. cultivars. Plants 9(1), 62. https://doi.org/10.3390/plants9010062

Motsa, M. M., Slabbert, M. M., van Averbeke, W., Morey, L., 2015: Effect of light and temperature on seed germination of selected African leafy vegetables. South African Journal of Botany 99, 29–35. https://doi.org/10.1016/j.sajb.2015.03.185

Muday, G. K., Rahman, A., Binder, B. M., 2012: Auxin and ethylene: collaborators or competitors? Trends in Plant Science 17(4), 181-195. https://doi.org/10.1016/j.tplants.2012.02.001

Munns, R., Millar, A. H., 2023: Seven plant capacities to adapt to abiotic stress. Journal of Experimental Botany 74(15), 4308–4323. https://doi.org/10.1093/jxb/erad179

Murashige, T., Skoog, F., 1962: A revised medium for rapid growth and bio assays with tobacco tissue cultures. Plant Physiology 15, 474–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Olatunji, D., Geelen, D., Verstraeten, I., 2017: Control of endogenous auxin levels in plant root development. International Journal of Molecular Sciences 18(12), 2587. https://doi.org/10.3390/ijms18122587

Pavlović, I., Mlinarić, S., Tarkowská, D., Oklestková, J., Novák, O., Lepeduš, H., Vujčić Bok, V., Radić Brkanac, S., Strnad, M., Salopek-Sondi, B., 2019: Early Brassica crops responses to salinity stress: a comparative analysis between Chinese cabbage, white cabbage and kale. Frontiers in Plant Science, 10, 450. https://doi.org/10.3389/fpls.2019.00450

Pavlović, I., Petřík, I., Tarkowska, D., Lepeduš, H., Vujčić, V., Radić Brkanac, S., Novák, O., Salopek-Sondi, B. 2018: Correlations between phytohormones and drought tolerance in selected Brassica crops: Chinese cabbage, white cabbage and kale. International Journal of Molecular Sciences, 19(10), 2866; https://doi.org/10.3390/ijms19102866

Peleg, Z., Blumwald, E., 2011: Hormone balance and abiotic stress tolerance in crop plants. Current Opinion in Plant Biology 14(3), 290–295. https://doi.org/10.1016/j.pbi.2011.02.001

Pfaffl, M. W., 2001: A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research 29(9), 45. https://doi.org/10.1093/nar/29.9.e45

Poór, P., Nawaz, K., Gupta, R., Ashfaque, F., Khan, M. I. R., 2022: Ethylene involvement in the regulation of heat stress tolerance in plants. Plant Cell Reports 41, 675–698. https://doi.org/10.1007/s00299-021-02675-8

Prerostova, S., Dobrev, P. I., Kramna, B., Gaudinova, A., Knirsch, V., Spichal, L., Zatloukal, M., Vankova, R., 2020: Heat acclimation and inhibition of cytokinin degradation positively affect heat stress tolerance of *Arabidopsis*. Frontiers in Plant Science 11, 1–14. https://doi.org/10.3389/fpls.2020.00087

Qin, H., He, L., Huang, R., 2019: The Coordination of ethylene and other hormones in primary root development. Frontiers in Plant Science 10, 874. https://doi.org/10.3389/fpls.2019.00874

Quan, J., Li, X., Li, Z., Wu, M., Zhu, B., Hong, S. B., Shi, J., Zhu, Z., Xu, L., Zang, Y., 2023: Transcriptomic analysis of heat stress response in *Brassica rapa* L. ssp. *pekinensis* with improved thermotolerance through exogenous glycine betaine. International Journal of Molecular Sciences 24(7), 6429. https://doi.org/10.3390/ijms24076429

Rai, A. N., Mandliya, T., Kulkarni, P., Rao, M., Suprasanna, P., 2021: Evolution and transcriptional modulation of lipoxygenase genes under heat, drought, and combined stress in *Brassica rapa*. Plant Molecular Biology Reporter 39, 60–71. https://doi.org/10.1007/s11105-020-01234-x

Rawlinson, C., Kamphuis, L. G., Gummer, J. P. A., Singh, K. B., Trengove, R. D., 2015: A rapid method for profiling of volatile and semi-volatile phytohormones using methyl chloroformate derivatisation and GC–MS. Metabolomics 11, 1922–1933. https://doi.org/10.1007/s11306-015-0837-0

Raza, A., Charagh, S., Abbas, S., Hassan, M. U., Saeed, F., Haider, S., Sharif, R., Anand, A., Corpas, F.J., Jin, W., Varshney, R.K., 2023: Assessment of proline function in higher plants under extreme temperatures. Plant Biology 25(3), 379–395. https://doi.org/10.1111/plb.13510

Rodríguez, V. M., Soengas, P., Alonso-Villaverde, V., Sotelo, T., Cartea, M. E., Velasco, P., 2015: Effect of temperature stress on the early vegetative development of *Brassica oleracea* L. BMC Plant Biology 15(1), 145. https://doi.org/10.1186/s12870-015-0535-0

Sajad, S., Jiang, S., Anwar, M., Dai, Q., Luo, Y., Hassan, M. A., Tetteh, C., Song, J., 2022: Genome-wide study of *Hsp90* gene family in cabbage (*Brassica oleracea* var. *capitata* L.) and their imperative roles in response to cold stress. Frontiers in Plant Science 13, 908511. https://doi.org/10.3389/fpls.2022.908511

Schneider, C. A., Rasband, W. S., Eliceiri, K. W., 2012: NIH Image to ImageJ: 25 years of image analysis HHS public access. Nature Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089

Seneviratne, S. I., Zhang, X., Adnan, M., Badi, W., Dereczynski, C., Di Luca, A., Ghosh, S., Iskandar, I., Kossin, J., Lewis, S., Otto, F., Pinto, I., Satoh, M., Vicente-Serrano, S. M., Wehner, M., Zhou, B., 2021: Weather and climate extreme events in a changing climate. doi: 10.1017/9781009157896.013. In: Masson-Delmotte, V., Zhai, P., Pirani, A., Connors, S. L., Péan, C., Berger, S., Caud, N., Chen, Y., Goldfarb, L., Gomis, M. I., Huang, M., Leitzell, K., Lonnoy, E., Matthews, J. B. R., Maycock, T. K., Waterfield, T., Yelekçi, O., Yu, R., Zhou B. (eds.), Climate change 2021: The physical science basis. Contribution of working group i to the sixth assessment report of the intergovernmental panel on climate change. Cambridge University Press, 1513–1766. Cambridge, United Kingdom and New York.

Staples, R. C., Stahmann, M. A., 1964: Changes in proteins and several enzymes in susceptible bean leaves after infection by the bean rust fungus. Phytopathology 54, 760–764.

Sunkar, R., Bartels, D., Kirch, H. H., 2003: Overexpression of a stress-inducible aldehyde dehydrogenase gene from *Arabidopsis thaliana* in transgenic plants improves stress tolerance. Plant Journal 35(4), 452–464. https://doi.org/10.1046/j.1365-313X.2003.01819.x

Taylor, S. C., Posch, A., 2014: The design of a quantitative western blot experiment. BioMed Research International 2014, 361590–361598. https://doi.org/10.1155/2014/361590

Tiwari, M., Kumar, R., Min, D., Jagadish, S. V. K., 2022,: Genetic and molecular mechanisms underlying root architecture and function under heat stress—A hidden story. Plant, Cell & Environment 45(3), 771–788. https://doi.org/10.1111/pce.14266

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3, https://doi.org/10.1186/gb-2002-3-7-research0034

Vanstraelen, M., Benková, E., 2012: Hormonal interactions in the regulation of plant development. Annual Review of Cell and Developmental Biology 28, 463-487. https://doi.org/10.1146/annurev-cellbio-101011-155741

Villas-Bôas, S. G., Delicado, D. G., Åkesson, M., Nielsen, J., 2003: Simultaneous analysis of amino and nonamino organic acids as methyl chloroformate derivatives using gas chromatography-mass spectrometry. Analytical Biochemistry 322(1), 134–138. https://doi.org/10.1016/j.ab.2003.07.018

Wang, L., Liu, F., Ju, L., Xue, B, Wang, Y., Wang, D., Hou, D., 2022: Genome structures and evolution analysis of *HSP90* gene family in *Brassica napus* reveal the possible roles of members in response to salt stress and the infection of *Sclerotinia sclerotiorum*. Frontiers in Plant Science 13, 854034. https://doi.org/10.3389/fpls.2022.854034

Wilson, R. E., Jensen, E. H., Fernandez, G. C. J., 1992: Seed germination response for eleven forage cultivars of *Brassica* to temperature. Agronomy Journal 84(2), 200–202. https://doi.org/10.2134/agronj1992.00021962008400020015x

Yadukrishnan, P., Datta, S., 2021: Light and abscisic acid interplay in early seedling development. New Phytology 229, 763-769. https://doi.org/10.1111/nph.16963

Yamauchi, Y., Ogawa, M., Kuwahara, A., Hanada, A., Kamiya, Y., Yamaguchi, S., 2004: Activation of gibberellin biosynthesis and response pathways by low temperature during imbibition of *Arabidopsis thaliana* seeds. Plant Cell 16(2), 367–378. https://doi.org/10.1105/tpc.018143

Yeh, C. H., Kaplinsky, J. N., Hu, C., Charng, Y., 2012: Some like it hot, some like it warm: Phenotyping to explore thermotolerance diversity. Plant Science 195, 10–23. https://doi.org/10.1016/j.plantsci.2012.06.004

Yu, J., Li, P., Tu, S., Feng, N., Chang, L., Niu, Q., 2023: Integrated analysis of the transcriptome and metabolome of *Brassica rapa* revealed regulatory mechanism under heat stress. International Journal of Molecular Sciences, 24(18), 13993. https://doi.org/10.3390/ijms241813993

Zhang, L., Dai, Y., Yue, L., Chen, G., Yuan, L., Zhang, S., Li F., Zhang, H., Li, G., Zhu, S., Hou, J., Tang, X., Zhang, S., Wang, C., 2022: Heat stress response in Chinese cabbage (*Brassica rapa* L.) revealed by transcriptome and physiological analysis. PeerJ 10:e13427. https://doi.org/10.7717/peerj.13427

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Short communication

# *Stellaria ruderalis* (Caryophyllaceae) in the Caucasus, new records and species habitat preferences

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**Abstract** – *Stellaria ruderalis* (Caryophyllaceae) is an annual herb of the *S. media* group, recently described in Central Europe and later discovered in other regions of the continent and beyond. Here, we provide novel data on its occurrence in the Caucasus Biodiversity Hotspot, where the species has not been collected since its description as new to science. *Stellaria ruderalis* was discovered at 14 sites in eastern and central Georgia (2022–2023) and northern Armenia (2023). It inhabited various ruderal habitats as well as deciduous and mixed forests. It is presumably a common component of the regional flora, although overlooked to date. Thus, its tentative IUCN classification is proposed here as Data Deficient (DD).

**Keywords:** biogeography, Caucasus, flora, habitat, *Stellaria media* agg.

## Introduction

The Caucasus is a mountainous region forming a boundary between Europe and Asia. For its extraordinarily diverse biota, it is considered one of the 36 global biodiversity hotspots. Its rich vascular plant flora (~6400 species) has an endemism rate of ~25%, and the related vegetation and habitat diversity is also considerably high. Armenia and Georgia are countries in the South Caucasus, whose floras harbour a significant portion of the taxonomic richness of the hotspot (Mittermeier et al. 2004). Despite intensive research of the regional flora, which has a two-century-long tradition, many neophytes have been discovered only recently (e.g. Kikodze et al. 2010). Examples of regionally novel species are those recently described as new to science (e.g. Piwowarczyk et al. 2023), belonging to which is *Stellaria ruderalis* M. Lepší, P. Lepší, Z. Kaplan et P. Koutecký. This allotetraploid taxon of the *Stellaria media* group (*Caryophyllaceae*, Alsinoideae) is an annual or winter annual herb, mainly flowering from March to May. Individuals then finish their life cycle, persisting in the dry summer and autumn periods exclusively in a seed bank. Members of the group are native to Eurasia and North Africa. However, regional differences in their inner taxonomic division do not allow adoption of individual species' distribution without careful investigation of herbarium material or living plants. The current concepts of European members of the group recognize *S. media* (L.) Vill. (s. str.; $2n = 4x = 40$), *S. pallida* (Dumort.) Crép. ($2n = 2x = 22$), *S. neglecta* Weihe ($2n = 2x = 22$), *S. cupaniana* (Jord. & Fourr.) Béguinot ($2n = 2x = 22$; sometimes merged with the previous species), and the most recently described species *S. ruderalis* ($2n = 4x = 44$) (Lepší et al. 2019, El Mokni et al. 2023). Plants of the *Stellaria media* group are a frequent component of various habitats in the Caucasus, including various natural (forests) as well as artificial habitats (trampled sites, road margins, railways, fields), shaded or open (Novák et al. 2023). Our knowledge of the global distribution of *S. ruderalis* is still far from complete. The species was described from the Czech Republic, and was known to occur in Central Europe (Lepší et al. 2019, Novikov et al. 2020, Dudáš et al. 2022), but it has also

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been recorded in the European Mediterranean Basin, including Sardinia, the Balkans (Lepší et al. 2019) and the Iberian Peninsula (Sáez 2020), as well as Tunisia (El Mokni et al. 2023), Algeria and Morocco (GBIF 2024) in North Africa. There have been no published reports on *S. ruderalis* for the Caucasus to date, nor there are any entries for this species in databases Euro+Med (2006–2023) and POWO (2024). On the other hand, four vouchers collected in Transcaucasia (Armenia, Georgia) and stored under the name *S. media* in the herbarium of the Natural History Museum in Vienna (W) have recently been revised as *S. ruderalis* by J. Walter (GBIF 2024). Moreover, plants with similar seed morphology and chromosome number of 2n = 44 from Tbilisi were mentioned by Peterson (1936). This evidence suggests the long-standing occurrence of this species in the area. Still, field observations of the species in the Caucasus are missing since it was described as new to science, and there are no data on its distribution, abundance, and habitat preferences in that area.

The main goal of our contribution is to report new sites of *Stellaria ruderalis* discovered in Armenia and Georgia since 2022, provide their ecological circumstances and discuss the species' position in the context of regional biodiversity.

## Material and methods

We collected *Stellaria ruderalis* in Armenia and Georgia during botanical field expeditions in 2022 and 2023. We documented each site of the species with a herbarium specimen and acquired its precise geographical position (WGS 84) and elevation by a GPS receiver. Phytosociological relevés were recorded in several sites and stored in the Transcaucasian Vegetation Database (Novák et al. 2023). Soil pH is provided for these sites, which were analysed from dried samples of topsoil (upper 15 cm) in a suspension with deionized water (2:5) by a portable Greisinger instrument. The herbarium specimens were studied morphologically in detail and afterwards stored in the herbaria of Masaryk University, Brno (BRNU), Moravian Museum, Brno (BRNM), and the National Herbarium of Georgia, Botanical Institute of Ilia State University, Tbilisi (TBI). Revisions of all reported herbarium specimens were carried out by Z. Kaplan (Institute of Botany, Czech Academy of Science), one of the authors of the *S. ruderalis* description (Lepší et al. 2019). The nomenclature follows Euro+Med PlantBase (Euro+Med 2006–2023) for plants and EuroVegChecklist (Mucina et al. 2016) for vegetation syntaxa. To provide basic climatic characteristics, we extracted climatic data for the sites from Worldclim 2.1 Bioclim (spatial resolution of 30 seconds). They included BIO1 Annual Mean Temperature and BIO12 Annual Precipitation (Fick and Hijmans 2017).

## Results and discussion

*Stellaria ruderalis* has been discovered at 14 sites (Fig. 1) within the Caucasus Biodiversity Hotspot in Armenia and Georgia since 2022. Based on the aforementioned sources, it should be considered a new member of the regional flora. Below, we provide an overview of our findings of the species in both countries (Fig. 1, On-line Suppl. Tab. 1).

### Sites and their environmental conditions

In Armenia, *Stellaria ruderalis* was discovered in the forested northeastern part of the country (860–1060 m a.s.l.). The measured topsoil pH at its sites was 5.29–6.82, i.e. moderately to slightly acidic reaction. The site annual mean temperature was 9.1–9.6 °C (median 9.6 °C), while annual precipitation was amounted to 507–537 mm (median 518 mm). In Georgia, we report *S. ruderalis* from ten sites across the central and eastern parts of the country in steppes as well as within deciduous and coniferous forest zones (410–1600 m a.s.l.). Its populations thrive in contrasting climatic conditions, including sub-Mediterranean and mountain continental types. The sites' annual mean temperature was 5.6–12.3 °C (median 8.6 °C), while the amount of annual precipitation was 516–926 mm (median 727 mm). Our observations indicate that the species may grow along the whole elevation gradient of natural forests.

### Habitats

Armenian sites consisted of deciduous mesophilous (phytosociological class *Carpino-Fagetea*) and xeromesophilous forests (*Quercetea pubescentis*). The species was collected in forests dominated by *Carpinus betulus* and *C. orientalis* where it preferred spots with a topsoil disturbed by freely-grazing cows, like other annuals (e.g. *Galium aparine*, *Geranium lucidum*, *Viola arvensis*). Analogically, it was revealed in Georgian deciduous and mixed mesophilous forests (*Carpino-Fagetea*) with *Acer velutinum*, *Carpinus*

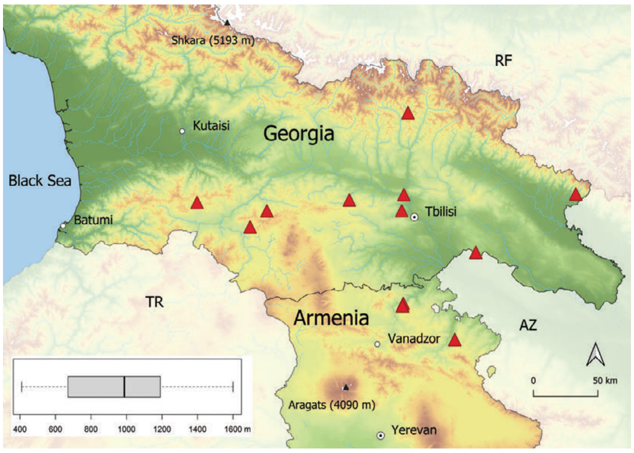

**Fig. 1.** Distribution of the reported *Stellaria ruderalis* sites (red triangles) in Georgia and Armenia. A boxplot provides median (bold line), interquartile range (box) and range of values (whiskers) of elevation of the sites.

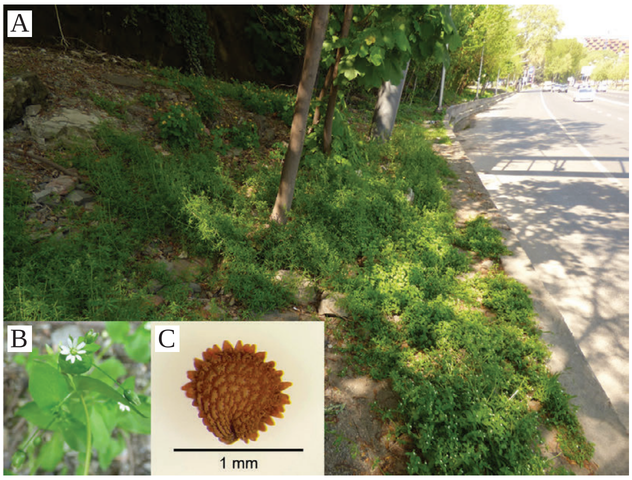

**Fig. 2.** Stands of *Stellaria ruderalis* in the understory of a disturbed nutrient-rich forest, dominated by *Acer* spp. (A) with inflorescence (B) and seed details (C), Georgia, Tbilisi, Zviad Gamsakhurdia Embankment, April 26, 2022. (Photo: P. Novák – A, B, P. Hubatka – C).

*betulus*, *C. orientalis* and *Picea orientalis*. They included both ruderalized types with understory dominated by shade-tolerant nitrophytes in Tbilisi (Fig. 2) and well-preserved forest types in the Lagodekhi (Greater Caucasus) and Rkoni (Lesser Caucasus) surroundings.

However, most of the Georgian sites consisted of various frequently disturbed and slightly shaded artificial habitats like road embankments, from the colline to the upper mountain belt, with the highest site of the whole dataset at the elevation of 1600 m (Lesser Caucasus). Sparse thermophilous trampled vegetation in a city pavement (*Digitario sanguinalis-Eragrostietea minoris*) harboured *S. ruderalis* in Mtskheta near Tbilisi. Our observations are aligned with the reported species' ecology (Lepší et al. 2019).

### Conservation status and prospective for further research

*Stellaria ruderalis* used to be mentioned as *S. media* (Lepší et al. 2019). The latter species was also found during our surveys in Georgia (e.g. Levitana village, Shida Kartli Region; BRNM 845570). The distribution and ecological differentiation of both species remain unclear in the Caucasus due to this confusion. *S. ruderalis* appears widespread in the investigated regions, having a broad habitat and climatic niche, although overlooked to date. Further investigations in the field and public herbaria are needed to support this assumption. Therefore, we propose a tentative classification of *S. ruderalis* as Data Deficient (DD) according to the IUCN Red List guidelines (IUCN 2024) for Armenia and Georgia.

### Acknowledgement

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### Author contribution statement

P.N. and V.T. share the first authorship, they wrote the initial draft of the manuscript, the former additionally compiled the figures and managed data from GIS layers while the latter solved the taxonomic and biogeographic issues. V.K., P.H., J.R. and G.F. assisted in the fieldwork and processing of the herbarium specimens. P.H. photographed the detail of the seed. All the authors revised the manuscript and approved its final version.

## References

Dudáš, M. (ed.), Ďurišová, Ľ., Eliáš, P. Jr., Eliášová, M., Kobiv, Y., Kšiňan, S., Malovcová-Staníková, M., Pliszko, A., Taraška, V., 2022: New floristic records from Central Europe 10 (reports 134–148). Thaiszia – Journal of Botany 32(2), 179–192. https://doi.org/10.33542/TJB2022-2-05

Euro+Med, 2006–2023: Euro+Med PlantBase – the information resource for Euro-Mediterranean plant diversity. Retrieved December 12, 2023 from http://www.europlusmed.org

El Mokni, R., del Guacchio, E., Iamonico, D., 2023: Further insights into the *Stellaria media* aggregate (Caryophyllaceae, *Alsinoideae, Alsineae*) in Africa: first reports of *S. ruderalis* in North Africa and *S. cupaniana* in Tunisia, with nomenclatural notes on the name *Alsine cupaniana*. Phytotaxa 584(4), 264–274. https://doi.org/10.11646/phytotaxa.584.4.3

Fick, S. E., Hijmans, R. J., 2017: WorldClim 2: new 1km spatial resolution climate surfaces for global land areas. International Journal of Climatology 37(12), 4302–4315. https://doi.org/10.1002/joc.5086

GBIF, 2024: *Stellaria ruderalis* M. Lepší, P. Lepší, Z. Kaplan & P. Koutecký. Retrieved July 20, 2024 from https://www.gbif.org/occurrence/search?offset=0&continent=AFRICA&taxon_key=10860042

IUCN, 2024: Guidelines for Using the IUCN Red List Categories and Criteria. Version 16. Prepared by the Standards and Petitions Committee. Retrieved March 24, 2024 from https://www.iucnredlist.org/documents/RedListGuidelines.pdf

Kikodze, D., Memiadze, N., Kharazishvili, D., Manvelidze, Z., Müller-Schärer, H., 2010: The alien flora of Georgia. 2nd ed. Joint SNSF SCOPES and FOEN publication, Ascona and Tbilisi.

Lepší, M., Lepší, P., Koutecký, P., Lučanová, M., Koutecká, E., Kaplan, Z., 2019: *Stellaria ruderalis*, a new species in the *Stellaria media* group from central Europe. Preslia 91(4), 391–420. https://doi.org/10.23855/preslia.2019.391

Mittermeier, R. A., Robles Gil, P., Hoffmann, M., Pilgrim, J., Brooks, T., Goettsch Mittermeier, C., Lamoreux, J., Da Fonseca, G. A. B., 2004: Hotspots revisited: Earth's biologically richest and most endangered terrestrial ecoregions. CEMEX, Mexico City.

Mucina, L., Bültman, H., Dierssen, K., Theurillat, J.-P., Dengler, J., Čarni, A., Šumberová, K., Raus, T., Di Pietro, R., Gavilán Garcia, R., Chytrý, M., Iakushenko, D., Schaminée, J. H. J., Bergmeier, E., Santos Guerra, A., Daniëls, F. J. A., Ermakov, N., Valachovič, M., Pignatti, S., Rodwell, J. S., Pallas, J., Capelo, J., Weber, H. E., Lysenko, T., Solomeshch, A., Dimopoulos, P., Aguiar, C., Freitag, H., Hennekens, S. M., Tichý, L., 2016: Vegetation of Europe: Hierarchical floristic classification system of plant, lichen, and algal communities. Applied Vegetation Science 19(S1), 3–264. https://doi.org/10.1111/avsc.12257

Novák, P., Kalníková, V., Szokala, D., Aleksanyan, A., Batsatsashvili, K., Fayvush, G., Kolbaia, S., Nakhutsrishvili, S., Sedláček, V., Štěrba, T., Zukal, D., 2023: Transcaucasian Vegetation Database – a phytosociological database of the Southern Caucasus. Vegetation Classification and Survey 4, 231–240. https://doi.org/10.3897/VCS.105521

Novikov, A., Sup-Novikova, M., Pachschwöll, C., 2020: *Stellaria ruderalis* M. Lepší, P. Lepší, Z. Kaplan et P. Koutecký, a new species record for the flora of Ukraine. Webbia 75(2), 355–358. https://doi.org/10.36253/jopt-9613

Peterson, D., 1936: *Stellaria* Studien. Zur Zytologie, Genetik, Ökologie und Systematik der Gattung *Stellaria*, insbesondere der *media*-Gruppe. Botaniska Notiser 88, 281–419.

Piwowarczyk, R., Pedraja, Ó. S., Moral, G. M., Khutsishvili, M., Kharazishvili, D., 2023: Holoparasitic Orobanchaceae in Georgia (Caucasus): taxonomic revision, diversity, distribution, habitats and host range. Phytotaxa Monograph, 604(1), 1–103. https://doi.org/10.11646/phytotaxa.604.1.1

POWO, 2024: *Stellaria ruderalis* M. Lepší, P. Lepší, Z. Kaplan & P. Koutecký. Retrieved July 20, 2024 from https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:77205182-1

Sáez, L., 2020: *Stellaria ruderalis* M. Lepší, P. Lepší, Z. Kaplan & P. Koutecký (Caryophyllaceae), new for the Iberian Peninsula and the Balearic Islands. Acta Botanica Malacitana 45, 203–205. https://doi.org/10.24310/abm.v45i.10133

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Short communication

# *Stachys ocymastrum* (Lamiaceae) – a new plant species in Croatia

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**Abstract** – *Stachys ocymastrum* (L.) Briq. (Lamiaceae) is an annual species native to the Western Mediterranean. During fieldwork, it was found in a ruderal site in the village of Veli Iž on the island of Iž (Zadar archipelago, Croatia) in May 2024. Since there are no earlier records of this species in Croatia, it should be considered a new taxon of this country's flora and included in the current Flora Croatica Database. Additionally, this study reports *S. ocymastrum* for the first time on the eastern part of the Adriatic Basin.

**Keywords**: Dalmatia, Eastern Adriatic, island, Italian hedge nettle, vascular flora

## Introduction

The genus *Stachys* is one of largest genera of Lamiaceae and includes 372 accepted species (POWO 2024). It is distributed mainly in the Mediterranean area and Southwest Asia, in North America, South America and southern Africa (Bhattacharjee 1980, Tundis et al. 2014, Salmaki et al. 2019). For the Euro-Mediterranean region 153 species of the *Stachys* genus have been listed (Euro+Med 2006-2024). They have different plant life forms, e.g. annual and perennial herbs and subshrubs (Bhattacharjee 1980).

The Italian hedge nettle, *Stachys ocymastrum* (L.) Briq. (syn.: *Galeopsis hirsuta* L., *Sideritis ocymastrum* L.) is an annual plant distributed in the western part of the Mediterranean region, i.e. is native to Portugal, Spain, the Balearic Islands, France including Corsica, Italy including Sardinia, Sicily, Malta, Greece including Crete, Morocco, Algeria, Tunisia, Libya, Madeira and the Canary Islands, as well as Lebanon and it is introduced to North America (New Jersey) (Hassler 1994-2024, POWO 2024).

*Stachys ocymastrum* is a scapose therophyte with an erect shoot (12-70 cm high) and simple or branched stems. The entire plant is covered with long silky, shiny hairs, with the indumentum consisting of both glandular and non-glandular trichomes. Leaves are from 1.6-6.5 cm long and 1.4-5 cm wide, oblong-ovate to broadly ovate, obtuse, and slightly cordate at base, with crenate-serrate margin. The leaves are opposite; the basal ones have a long (1.7-5.5 cm) petiole. The inflorescence consists of 4-18 verticillasters, with 2-6 flowers each, below rather remote but usually crowded above. Bracts are ovate (0.8-1.9 cm long and 0.4-1.1 cm wide) sessile, the upper ones entire, densely hairy on the margin; flowers have 1-1.5 mm long pedicel. Calyx is 8-12 mm long, hirsute, campanulate, with equal teeth (5-7 mm), as long as, or slightly longer than the tube, long pointed, almost spiny, with yellow apices. Corolla is up to 16 mm long, white. Upper lip 4-8 mm long, bifid, its lobes 2–3 mm long, erect; lower lip with a large central lobe of 5-8 × 2.5-5.5 mm, spatulate, pale yellow with violet pattern. Stamens exserted from the tube, with thecae aligned with the filament. Mericarps 1.5-2 mm, smooth to warty, grey, or dark brown (Ball 1972, Morales and Pardo de Santayana 2010, Martin Mosquero et al. 2000).

According to the Flora Croatica Database (FCD, Nikolić 2024), 18 *Stachys* species and 9 subspecies currently occur in Croatia. The presence of *S. ocymastrum* has never been reported for the country, including the eastern coast and islands of the Adriatic Sea. Here we present the first record of *S. ocymastrum* for Croatia, discovered during fieldwork on the island of Iž in the Middle Adriatic (Dalmatia).

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## Materials and methods

The plant occurrence was documented with geographic coordinates, photographs as well as with herbarium sheets. Herbarium specimens were collected on 9 May 2024, and a voucher specimen was deposited in the Herbarium of the Faculty of Agronomy in Zagreb (ZAGR). For the identification we used Flora Europaea (Ball 1972), Flora d'Italia (Pignatti et al. 2017-2019) and Flora Croatica (Nikolić 2019).

## Study area

The island of Iž is situated in the Zadar archipelago, surrounded by the islands of Ugljan, Rava and Dugi Otok (Fig. 1). It is 12.3 km long and up to 2.6 km wide, with an area of 16.51 km$^2$ and a coastline length of 35.22 km (Duplančić Leder et al. 2004). The main soil types are *terra rossa* developed above limestone and sandy soils developed on the dolomite substrate. The island has a typical Mediterranean climate: a moderately warm rainy winter climate with a dry warm summer. The annual average temperature is 15 °C and the annual average precipitation is 880 mm (Zaninović et al. 2008). According to Köppen's climate classification, the island lies within the Mediterranean climate zone of *Csa*-type, with hot-summers and mild winters (Köppen and Geiger 1954). According to the 2021 census, the island has 516 permanent residents, living in two settlements: Mali Iž and Veli Iž (Croatian Bureau of Statistics 2024). The island is a popular tourist destination in the summer.

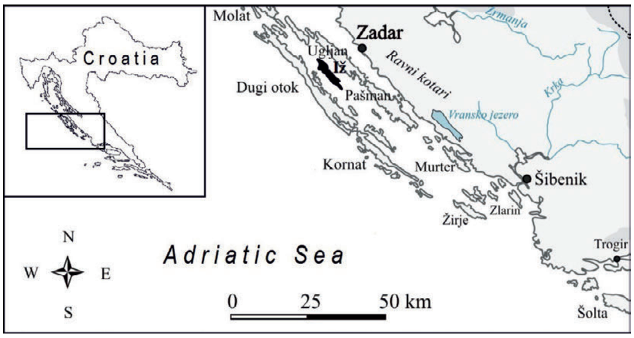

**Fig. 1.** The location of the island of Iž in the Zadar archipelago, Middle Adriatic (Dalmatia).

## Results and discussion

During our fieldwork on the island of Iž, plant specimens were collected and identified as *Stachys ocymastrum* (Fig. 2). It was found as follows: Croatia, northern Dalmatia, Zadar County, Island of Iž, Veli Iž village, main street (riva), 44.051767 N, 15.109506 E, elevation 1 m, 9 May 2024, leg. et det. D. Purger, herbarium ID number: ZAGR-79697. The population consisted of six individuals in the late stages of blooming (Fig. 2A). The morphological features of the plants corresponded to the description in the literature (Ball 1972, Martin Mosquero et al. 2000, Morales and Pardo-de-Santayana 2010).

The plants were growing over a total area of about 20 m$^2$ in grassland surrounded by shrubs near the path in the main street of the village of Veli Iž, maintained as the village's green area. In the immediate surroundings, *Oxalis articulata* Savigny, *Bromus sterilis* L. and *Geranium molle* L. were recorded. According to the first observations about circumstances on the field, we consider the occurrence of the species native. Nevertheless, the possibility that it is the result of recent introduction cannot be excluded, since the plants were discovered at a ruderal site in the village's main street, frequently visited by tourists. Indeed, the species occupies similar ruderal habitats and, in general, it is not fre-

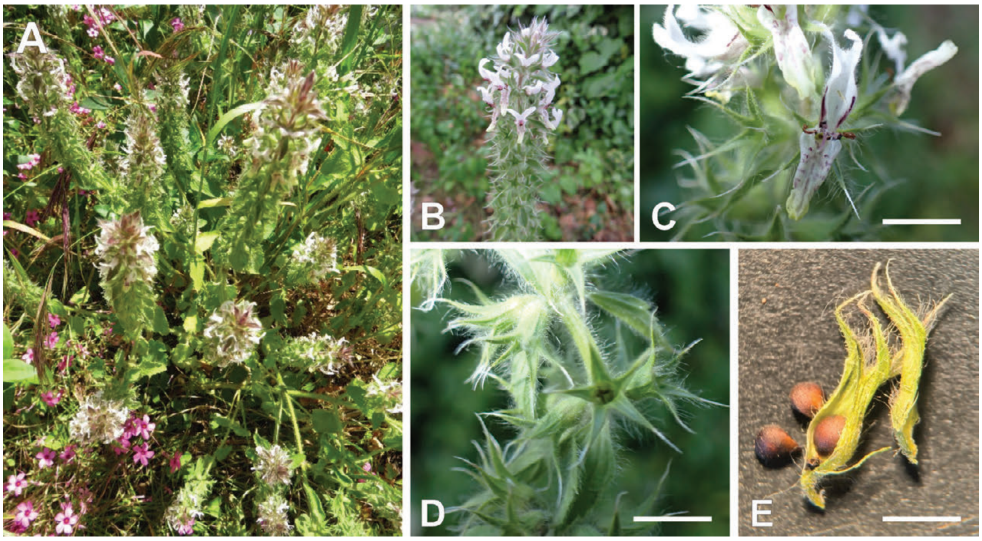

**Fig. 2.** *Stachys ocymastrum* in the Veli Iž village, the island of Iž, Zadar County, northern Dalmatia, Croatia. A – habitus, B – inflorescence, C – flower with a bifid erect upper lip and a violet pattern on the lower lip, D – calyx, E –mericarps and calyx. Scale bars: 5 mm (photos: A, B, E – D. Purger; C, D – M. Pandža).

quent and abundant in its natural habitats. In its entire distribution area, *S. ocymastrum* is found from sea level up to lower mountains (up to 1000 m a.s.l.), along paths and roadsides, bordering paths, in grasslands, and scrublands. It grows on nutrient-rich soils and base-rich rocks, it is edaphic indifferent (Morales and Pardo-de-Santayana 2010, Acta plantarum 2024). In Spain and Italy, *S. ocymastrum* grows in dry and hot rocky places, mostly within dry grasslands and ruderal vegetation (Martin Mosquero et al. 2000, Biondi et al. 2012, Pignatti et al. 2017-2019). In the suburban environments of southern Italy, it is found within the vegetation of the *Chenopodietea* class (Laface et al. 2022). It is also found in the evergreen Mediterranean cork-oak forests (Wojterski 1990), in both human-influenced and natural Mediterranean grasslands, e.g. grasslands dominated by *Asphodelus ramosus* L. (Zangari et al. 2023), as well as in agricultural land (e.g. in olive groves, Borkowsky 1994). *Stachys ocymastrum* flowers from February to June, rarely to July (Morales and Pardo-de-Santayana 2010). No estimation about status in its global range, but in Spain, this species is not rare; it belongs to the LC (Least concern) IUCN Category (Anonymous 2024).

Our find is the first record of *S. ocymastrum* on the eastern coast and islands of the Adriatic Sea. The northeastern limit of the distribution of this predominantly western Mediterranean species stretches to Italy (Pignatti et al. 2017-2019). The population recorded in this study is located between those reported from the province of Ancona in the central Adriatic side of the Italian peninsula (Biondi et al. 2012) and Ionian Islands (Greece) in the south (Borkowsky 1994). Since our survey was conducted on a botanically unexplored small island, this record reflects the lack of sufficient research on the Adriatic islands to date and highlights the need for further field surveys, which are essential for determining the actual distribution and ecology of *S. ocymastrum* in the eastern part of the Adriatic Basin.

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## Author contribution statement

D.P. and J.J.P. conceived the floristic study on the island of Iž, D.P. and M.P. collected and identified of plant samples, D.P. and N.J. wrote the manuscript. All authors critically revised and approved its final version.

## References

Acta Plantarum - Flora delle regioni italiane, 2024: *Stachys ocymastrum* (L.) Briq. Retrieved July 20, 2024 from: https://www.actaplantarum.org/flora/flora_info.php?id=504540

Anonymous, 2024: *Stachys ocymastrum*. Herbal Virtual del Mediterrani Occidental. Retrieved October 10, 2024 from https://herbarivirtual.uib.es/en/general/268/especie/stachys-ocymastrum-l-briq-

Ball, P. W., 1972: *Stachys*. In: Tutin, T. G., Heywood, V. H., Burges, N. A., Moore, D. M., Valentine, D. H., Walters, S. M., Webb, D. A. (eds.), Flora Europaea, vol. 3, 151–157. Cambridge University Press, Cambridge.

Bhattacharjee, R., 1980: Taxonomic studies in *Stachys* II. A new infrageneric classification of *Stachys* L. Notes from the Royal Botanic Garden, Edinburgh 38, 65–96.

Biondi, E., Gubellini, L., Pinzi, M., Casavecchia, S., 2012: The vascular flora of Conero Regional Nature Park (Marche, Central Italy). Flora Mediterranea 22, 67–167. https://doi.org/10.7320/FlMedit22.067

Borkowsky, O., 1994: Übersicht der Flora von Korfu. Floristic Investigations of Corfu Ionian Islands / Greece. Braunschweiger Geobotanische Arbeiten 3. Universitätsbibliothek der Technischen Universität Braunschweig, Braunschweig.

Croatian Bureau of Statistics, 2024: Census of population, households and dwellings in 2021 – population by settlements. Retrieved July 16, 2024, from: https://dzs.gov.hr/u-fokusu/popis-2021/popisni-upitnik/english/results/1501

Duplančić Leder, T., Ujević, T., Čala, M., 2004: Coastline lengths and areas of islands in the Croatian part of the Adriatic Sea determined from the topographic maps at the scale of 1:25.000. Geoadria 9(1), 5–32. https://doi.org/10.15291/geoadria.127

Euro+Med, 2006–2024: Euro+Med PlantBase – the information resource for Euro-Mediterranean plant diversity. Retrieved July 16, 2024 from http://www.europlusmed.org

Hassler, M., 1994–2024: World Plants. Synonymic Checklist and Distribution of the World. Retrieved July 16, 2024 from: https://www.worldplants.de/world-plants-complete-list/complete-plant-list/?name=Stachys-ocymastrum#plantUid-407511

Köppen, W., Geiger, R., 1954: Klima der Erde. Justus Perthes, Darmstadt.

Laface, V. L. A., Musarella, C. M., Sorgonà, A., Spampinato, G., 2022: Analysis of the population structure and dynamic of endemic *Salvia ceratophylloides* Ard. (Lamiaceae). Sustainability 14(16), 10295. https://doi.org/10.3390/su141610295

Martin Mosquero, M. A., Juan, R., Pastor, J., 2000: Morphological and anatomical studies on nutlet of *Stachys* (Lamiaceae) from Southwest Spain. Thaiszia – Journal of Botany 10, 27–38.

Morales, R., Pardo de Santayana, M., 2010: *Stachys* L. In: Castroviejo, S., Morales, R., Quintanar, A., Cabezas, F., Pujadas, A., Cirujano, S. (eds.), Flora Iberica. Plantas Vasculares de la Península Ibérica e Islas Baleares, Verbenaceae-Labiatae-Callitrichaceae, vol. 12, 216–232. Real Jardín Botánico, CSIC, Madrid.

Nikolić, T., 2019: Flora Croatica – vaskularna flora Republike Hrvatske, vol. 4. Ekskurzijska flora [Flora Croatica – vascular flora of the Republic of Croatia, vol. 4. Excursion flora]. Alfa d. d., Zagreb.

Nikolić, T. (ed.), 2024: Flora Croatica database. Retrieved July 16, 2024 from http://hirc.botanic.hr/fcd

Pignatti, S., Guarino, R., La Rosa, M., 2017–2019: Flora d'Italia, 2ª edizione. Edagricole di New Business Media, Bologna.

POWO, 2024: Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Retrieved July 20, 2024 from http://www.plantsoftheworldonline.org

Salmaki, Y., Heubl, G., Weigend, M., 2019: Towards a new classification of tribe Stachydeae (Lamiaceae): Naming clades using molecular evidence. Botanical Journal of the Linnean Society 190(4), 345–358. https://doi.org/10.1093/botlinnean/boz021

Tundis, R., Peruzzi, L., Menichini, F., 2014: Phytochemical and biological studies of *Stachys* species in relation to chemotaxonomy: A review. Phytochemistry 102, 7–39. https://doi.org/10.1016/j.phytochem.2014.01.023

Wojterski, T. W., 1990: Degradation stages of the oak forests in the area of Algiers. Vegetatio 87, 135–143. https://doi.org/10.1007/BF00042950

Zangari, G., Bartoli, F., Lucchese, F., Caneva, G., 2023: Plant diversity in archaeological sites and its bioindication values for nature conservation: Assessments in the UNESCO site Etruscan Necropolis of Tarquinia (Italy). Sustainability 15(23), 16469. https://doi.org/10.3390/su152316469

Zaninović, K., Gajić-Čapka, M., Perčec Tadić, M., Vučetić, M., Milković, J., Bajić, A., Cindrić, K., Cvitan, L., Katušin, Z., Kaučić, D., Likso, T., Lončar, E., Lončar, Ž., Mihajlović, D., Pandžić, K., Patarčić, M., Srnec, L., Vučetić, V., 2008: Klimatski atlas Hrvatske [Climate atlas of Croatia] 1961–1990: 1971–2000. Državni hidrometeorološki zavod, Zagreb.

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