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Variability of pollen aperture heteromorphism in annual pansies (*Viola* Section *Melanium*)

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Abstract – Pollen heteromorphism is frequent in the section *Melanium* of the genus *Viola,* in which over 80% of the species produces pollen morphs with 3 to 6 apertures. Some authors have pointed out that many factors can affect the proportion of the different pollen types in perennial species, and that this proportion can change among populations. This work focuses on the study of the polymorphic pollen assemblage of three annual pansies: *Viola arvensis*, *V. kitaibeliana,* and *V. hymettia*, and on the assessment of its variability both within a population and within the same plant. In all the species, with both large and small flowers, 3-, 4- and 5-aperturate pollen grains were observed, with a large prevalence of 4-aperturate types. No pollen grains with 6 apertures were found. No significant variability of the pollen assemblage among flowers of the same plant was observed. In addition, in these three *Viola* species the frequencies of the various pollen morphs are also fairly constant among plants of the same population.

Keywords: Annual pansies, pollen morph, pollen heteromorphism, section *Melanium*, variability, *Viola*

Introduction

Viola L. section *Melanium* Ging. is a derived, monophyletic and morphologically well-defined group of about 80–100 perennial and annual species showing highly-reduced genetic divergence (Yockteng et al. 2003). Its geographical distribution extends over Europe and westernmost Asia, with a few species in Northern Africa and one disjointed and probably native species in North America (Clausen et al. 1964, Yockteng et al. 2003).

Pollen heteromorphism, defined as the production of several pollen grain morphs with different aperture numbers by the same plant (Till-Bottraud et al. 1995), occurs in over 30% of angiosperm species (Mignot et al. 1994). It is particularly common in this section of the genus *Viola*, in which over 80% of the species produce pollen morphs with 3 to 6

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apertures (Dajoz et al. 1993, Till-Bottraud et al. 1999, Nadot et al. 2000). Generally, 4- or 5-aperturate pollen grains represent more than 85% and very often more than 95% (Till-Bottraud et al. 1999). The most frequent pollen type is always 4-aperturate for perennial pansies and for typically large-flowered annuals like *Viola tricolor* L., *V. hymettia* Boiss. & Heldr., and *V. roccabrunensis* Espeut (Clapham et al. 1987, Till-Bottraud et al. 1999, Espeut 2004), as it is related to entomophilous pollination. On the other hand, in *V. arvensis* Murray and *V. kitaibeliana* Schult. 5-aperturate pollen grains prevail (Pettet 1964, Clapham et al. 1987, Randall 2004), which is related to autogamous pollination strategy. Still, 4-aperturate pollen grains were also observed by other authors (Erdtman 1952, Dajoz et al. 1995, Espeut 1999).

Dajoz et al. (1991), Mignot et al. (1994) and Till-Bottraud et al. (1999) pointed out that many genetic and environmental factors can affect the proportion of the different pollen types in heteromorphic perennial species, and that this can change among populations. On the other hand, to our knowledge, studies of the polymorphic pollen assemblage of annual pansies and the assessment of its variability within a population, or within the same plant, have not been previously reported in literature.

Furthermore, Scoppola and Lattanzi (2012), in accordance with Kristofferson (1923) and Erben (1985), observed an increasing reduction in the flower size from the lower to the upper parts of the specimen in all the analyzed species, both autogamous and heterogamous, and in *V. arvensis* and *V. tricolor* also from the main axis to the branches. As pollen heteromorphism is related to pollination strategy, can the main pollen type change within the same plant in such variable flowers?

The aims of this paper were: 1) to analyze the pollen assemblage of three annual pansies, *Viola arvensis*, *V. kitaibeliana* and *V. hymettia*; 2) to assess its variability among flowers within the same plant and among plants within the same population; and particularly, 3) to assess whether the main pollen type is directly correlated with the increasing reduction of the flower size in those species, like *V. arvensis,* that show both autogamous and heterogamous pollination, and in entomophilous species like *V. hymettia*.

Materials and methods

Study species

Viola arvensis (2n = 34), the field pansy, is the most common of the annual species closely related to *V. tricolor,* the wild pansy. It is a Mediterranean-Eurasiatic element, regarded as an archaeophyte and widespread throughout almost the whole of Europe and SW Asia, from 0 to 1,500–1,800 m a.s.l., and as a weed linked to open shrubland and synanthropic habitats (Gams 1926, Valentine et al. 1968, Marcussen and Karlsson 2010, Vollrath 2011). The corolla is shorter than or equal to the calyx (or, rarely, a little longer), usually pale creamy-yellow (Fig. 1A). It is a mostly autogamous species with a very small stylar flap (*labellum*) and the entrance of the stigmatic cavity, in a front view, is obliquely forward directed (Scoppola and Lattanzi 2012).

Viola kitaibeliana (2n = 16), the dwarf pansy, is a Mediterranean-Caucasian species which extends to central Europe where it is a component of early stages of grassland, stony slopes and screes, found also on sandy soils, fallow land, fields and other open places, from 0 to approximately 1,850 m a.s.l. (Werner 1988, Randall 2004, Vollrath 2011, Magrini

and Scoppola 2013). It is a small-flowered and mostly autogamous species, with a corolla cup-shaped, not (or lightly) exceeding the calyx (Fig. 1B); the stylar flap is absent and the entrance of the stigmatic cavity, in a front view, is obliquely forward directed (Erben 1985, Scoppola and Lattanzi 2012).

Viola hymettia (2n = 16) is a SE-European species which is widespread mainly in Greece and the Aegean Islands, in Central and Southern Italy, where it has a disjointed distribution, and in Sicily; it is an early element of stony pastures, dry open habitat and scrub fringes, occurring from 200 to 800–1,000 m a.s.l. (Merxmüller 1982, Erben 1985, Raus 1986, Davis et al. 1988). This relatively large-flowered species has a corolla distinctly exceeding its calyx, creamy and yellow colored, often suffused with violet (Fig. 1C). The stigmatic opening, in a front view, is obliquely upwards directed (Scoppola and Lattanzi 2012).

Fig. 1. Flowers of A) *Viola arvensis*, B) *V. kitaibeliana*, and C) *V. hymettia* from wild populations of Central Italy.

Collection and study sites

Seedlings of *V. arvensis* and *V. kitaibeliana* were collected in wild populations in Central Italy, in February and March 2013, respectively (Tab. 1). They were transplanted in pots, together with the soil collected *in situ*, and cultured *ex situ* until May 2013. *Viola hymettia* plants were studied *in situ* from January to April 2013. About 100 specimens belonging to these taxa were used for palynological investigations. Samples of each species are deposited in Herbarium UTV, Tuscia University (Viterbo, Italy).

Tab. 1. Locations of the studied *Viola* populations.

Species	Localities	Elevation	Coordinates	Habitat
Viola arvensis	Acque Albule, Tivoli (Roma, Italy)	66 m a.s.l.	41°57'47"N 12°42'53"E	shrubby grassland in uncultivated land (calcareous soil)
Viola kitaibeliana	Le Vigne, Ofena (L'Aquila, Italy)	481 m a.s.l.	42°18'03"N 13°44'05"E	arid and stony shrubby grassland (calcareous soil)
Viola hymettia	Riello, Viterbo (Viterbo, Italy)	323 m a.s.l.	42°25'38"N 12°05'38"E	uncultivated escarpment with pine trees and shrubs (volcanic soil)

Palynological study

Pollen grains were sampled from 10–30 living plants per population that had been previously labeled and numbered. Flowers were harvested possibly before full blooming to collect pollen from closed anthers, from 1 to 9 different flowers per plant (only from the main axis) (Fig. 2). All the flowers were dissected immediately after harvesting to avoid pollen loss. The plant code (species and number) and the flower position were registered for each one.

Anthers were removed from the flower and put directly on a microscope slide, immersing them in a drop of lactic acid to let out all the pollen grains. Observations of the entire pollen assemblage were carried out under a light microscope Leitz HM-LUX3 with a magnification of 100× (resolution: 0.25 μm) and the different pollen morphs were counted according to this scheme:

- triangular shape = 3-aperturate grain,
- squared shape = 4-aperturate grain,
- pentagonal shape = 5-aperturate grain,
- hexagonal shape = 6-aperturate grain,
- round, elliptical, irregular shape = immature, aborted or unidentifiable grains.

Fig. 2. Sample of *Viola hymettia* plant with the progressive numbering of the flowers according to their arrangement from the lower to the upper parts of the stem (I–IV). The fourth flower is a bud suitable for pollen collection.

This usually entailed the counting of 100–1,600 grains, or even more.

For each species, microphotographs of pollen grains were taken using a digital camera, Fujifilm FinePix S2980.

Statistical analysis

For statistical analysis only the sets of data with more than 150 pollen grains and with percentages of immature or aborted grains less than 20% were used. Data were analyzed by one-way ANOVA using GraphPad Prism 5.1, followed by Tukey's multiple comparison test to test the significant differences among flowers in each plant and among plants in the same population. Pearson's correlation test was performed to test for correlation between the percentages of the main pollen morph and the position of the flowers at 95% confidence interval.

Results

Pollen heteromorphism

In the examined flowers, 3-, 4- and 5-aperturate grains were observed (Fig. 3), while no 6-aperturate grains were found. In all the species, a statistically significant prevalence of the

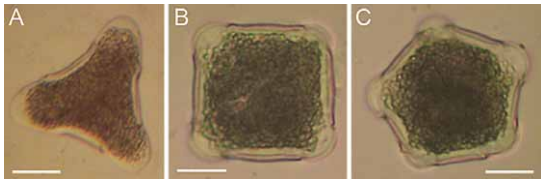

Fig. 3. Microphotographs of the observed pollen morphs: A) 3-aperturate, B) 4-aperturate, and C) 5-aperturate grains of *V. hymettia* (scale bar = 10 μm).

4-aperturate grains (on average more than 93%; $p < 0.0001$) over the other morphs was observed. Particularly, in *V. arvensis* it ranges from 81% to 99% (93.58 ± 4.99%, mean ± standard deviation), with 1–18% of 5-aperturate grains (6.18 ± 5.11%) and with less than 3% of 3-aperturate ones (0.25 ± 0.57%) (Fig. 4). In *V. kitaibeliana* almost all the grains observed were 4-aperturate (98.41 ± 1.13%) while 3- and 5-aperturate grains accounted for fewer than 2% (0.47 ± 0.76% and 1.12 ± 1.13%, respectively) (Fig. 4). *V. hymettia* showed the greater variability with about 77–98% of 4-aperturate grains (90.74 ± 6.55%), a little greater percentage of 3-aperturate grains than the other species, up to 15.5% (1.34 ± 3.53%), and with 5-aperturate grains that range from 0 to 21% (7.92 ± 6.16%) (Fig. 4).

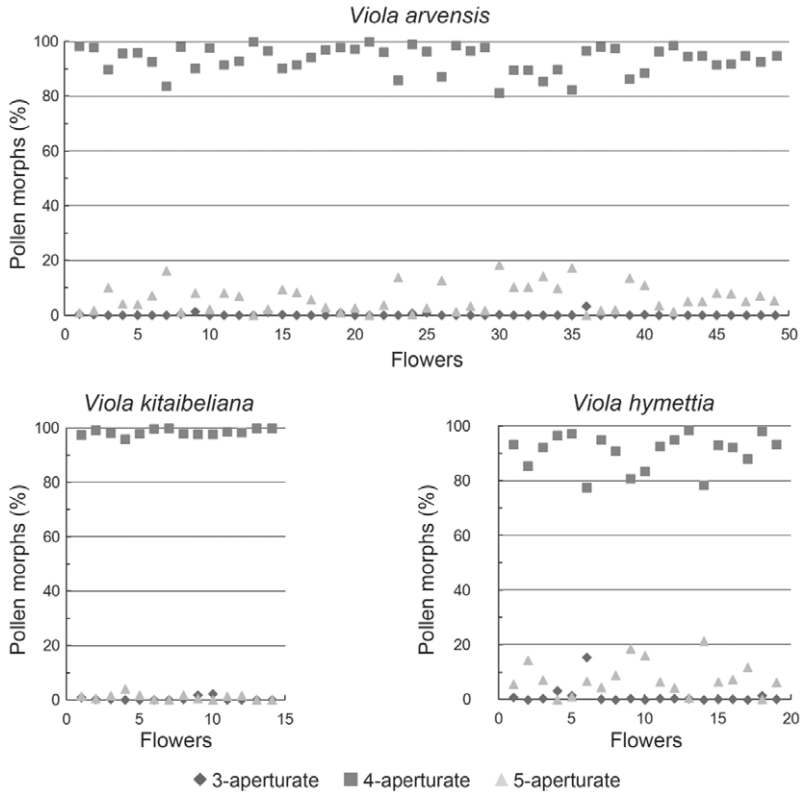

Fig. 4. Frequencies of the different pollen morphs in all the analyzed flowers of *Viola arvensis*, *V. kitaibeliana*, and *V. hymettia*. Data are ordered from the first to the last flowers.

Pollen heteromorphism variability

No significant differences in the proportions of the various morphs were observed among flowers of the same plant in these three species (Tabs. 2, 3, 4). *Viola hymettia* showed the larger variability in the polymorphic pollen assemblage, independently of flower position (Fig. 5, Tab. 4), as no trend in the variation of the percentages of the different pollen morphs was observed. On the other hand, a certain variability in the pollen assemblage of the first 2 flowers was observed in *V. arvensis*, with 4-aperturate morphs ranging from about 81% to 100% and with 5-aperturate morphs from 0% to 18%. This variability was progressively and strongly reduced up to the last flowers (Fig. 5, Tab. 2). *Viola kitaibeliana* developed a maximum of 3 flowers per plant showing less variability in the pollen assemblage, with 4-aperturate morphs ranging from about 96% to 100% and 5-aperturate from 0 to 4% (Fig. 5, Tab. 3).

The results of ANOVAs conducted on flowers of different plants showed that, in *V. arvensis, V. kitaibeliana*, and *V. hymettia,* no significant differences in the proportions of the different pollen morphs could be detected among individuals from the same population (p values vary from 0.0759 to 0.7834). In the three species, no statistically significant correla-

Tab. 2. Polymorphic pollen assemblages of flowers of *Viola arvensis.* Mean percentage ± standard deviation and results of one-way ANOVA for three pollen morph, with statistically significance at $p < 0.05$ (post hoc Tukey's test).

Flower position	3-aperturate	4-aperturate	5-aperturate
I	0.25 ± 0.42%	94.05 ± 4.20%	5.70 ± 4.26%
II	0.16 ± 0.32%	92.77 ± 6.21%	7.07 ± 6.30%
III	0.69 ± 1.32%	93.98 ± 5.11%	5.34 ± 5.58%
IV	0.03 ± 0.06%	96.11 ± 2.15%	3.86 ± 2.12%
V	0.11 ± 0.02%	91.73 ± 0.04%	8.16 ± 0.05%
VI	–	91.94 ± 0.02%	8.06 ± 0.01%
p	0.4292	0.8652	0.8491
$F_{5,48}$	1.001	0.3717	0.3955
R^2	0.1064	0.04237	0.04496

Tab. 3. Polymorphic pollen assemblages of flowers of *Viola kitaibeliana.* Mean percentage ± standard deviation and results of one-way ANOVA for three pollen morph, with statistically significance at $p < 0.05$ (post hoc Tukey's test).

Flower position	3-aperturate	4-aperturate	5-aperturate
I	0.29 ± 0.38%	98.13 ± 1.31%	1.59 ± 1.35%
II	0.88 ± 1.09%	98.41 ± 0.98%	0.70 ± 0.80%
III	–	99.23 ± 1.09%	0.77 ± 1.10%
p	0.3000	0.5309	0.4226
$F_{2,13}$	1.361	0.6750	0.9400
R^2	0.2140	0.1189	0.1582

Tab. 4. Polymorphic pollen assemblages of flowers of *Viola hymettia.* Mean percentage ± standard deviation and results of one-way ANOVA for three pollen morph, with statistically significance at $p < 0.05$ (post hoc Tukey's test).

Flower position	3-aperturate	4-aperturate	5-aperturate
I	0.43 ± 0.40%	90.42 ± 4.30%	9.15 ± 4.69%
II	6.77 ± 7.64%	90.54 ± 11.27%	2.69 ± 3.71%
III	0.18 ± 0.18%	89.09 ± 7.33%	10.73 ± 7.20%
IV	0.28 ± 0.24%	90.54 ± 6.08%	9.18 ± 6.31%
V	0.22 ± 0.28%	90.12 ± 10.44%	9.67 ± 10.68%
VI	0.27 ± 0.01%	92.25 ± 0.10%	7.48 ± 0.09%
VII	–	88.08 ± 0.01%	11.92 ± 0.02%
VIII	1.46 ± 0.07%	98.22 ± 0.02%	0.32 ± 0.02%
IX	0.15 ± 0.02%	93.41 ± 0.10%	6.44 ± 0.08%
p	0.0817	0.6869	0.1877
$F_{8,19}$	2.174	0.7010	1.621
R^2	0.4914	0.2376	0.4187

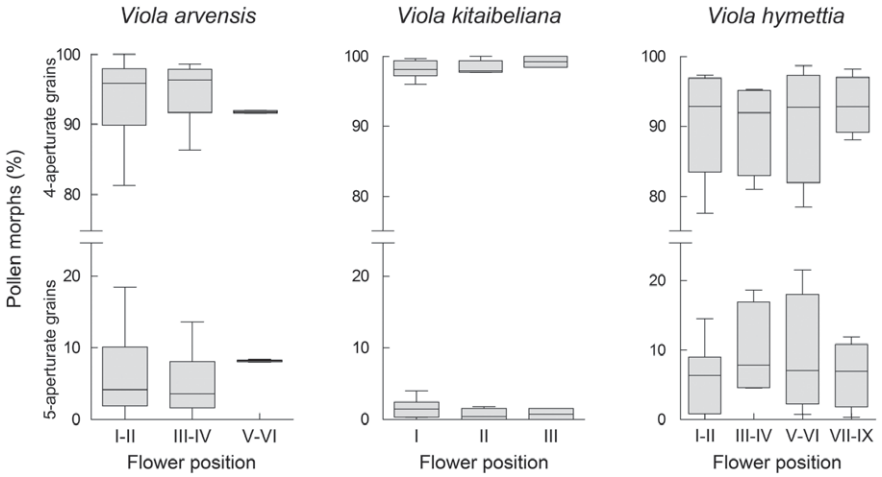

Fig. 5. Box-and-whisker graphs of the main pollen morphs in the flowers of *Viola arvensis*, *V. kitaibeliana*, and *V. hymettia*. The ordinal numbers of the flowers, from I to IX, are reported according to their arrangement on the main stem from the lower to the upper parts of the plant.

tion was observed between the percentages of the main pollen morph and the position of the flowers (Pearson's correlation test, two-tailed p values vary from 0.1174 to 0.9362).

Discussion

In all three species, our results show a highly significant prevalence of the 4-aperturate morph over the other two morphs. In *V. hymettia* the relative proportion of some pollen

morphs varies quite considerably: in some flowers, from sunny areas, there were no 3-aperturate pollen grains at all, while in more pigmented flowers and in others with upper petals suffused with violet, in the shade of the trees, more than 15% were 3-aperturate pollen grains and only 77% 4-aperturate ones. Variability in the color of the corolla related to light gradient is already known for *V. tricolor*, an annual pansy with creamy to bluish or violet large petals occurring in meadows and habitats linked to woodlands (Pettet 1964). Till-Bottraud et al. (1999) proved that pollen-type proportions of a perennial pansy, *V. calcarata* L., vary among populations along altitudinal gradients. On the other hand, to our knowledge there is no information regarding pollen heteromorphism variability within the same population of annual pansies linked to gradient.

The pollen assemblage of the two other species is more uniform than in *V. hymettia*. *Viola arvensis* shows a certain variability in the percentages of the main morph, especially in the first flowers, where 4-aperturate grains range from 81% to 100%. A greater variability, ranging from 69% to 96%, was observed among those populations with mostly 5-aperturate grains (Pettet 1964). In the pollen assemblage of *V. kitaibeliana* almost all the grains are 4-aperturate, in accordance with a recent study from Iran (Saeidi Mehrvarz et al. 2014). By contrast, our recent unpublished data highlight a rather wide variability among other populations from Central Italy where 5-aperturate grains prevail (from 83 to 92%). The pollen assemblages of the last two species show less variability when 4-aperturate pollen grains predominate.

Our study was focused on the assessment of the pollen heteromorphism variability in these three *Viola* species, taking into account differences within an individual and within a population. Pollen heteromorphism is related to pollination strategy: in heterogamous plants there is a prevalence of pollen grains with a few apertures (generally 4 or less), while in the autogamous ones 5-aperturate morphs prevail (Dajoz et al. 1993). So, we hypothesized a variability among flowers related to the increasing reduction of their size from the lower to the upper parts of the plant in those species, like *V. arvensis,* that show both autogamous and heterogamous pollination. We even expected to observe the main pollen type changing among such variable flowers within the same plant. On the other hand, we did not expect to find this change in species like *V. hymettia* showing flower features so adapted to entomophilous pollination (large and flattened corolla, scent, stigmatic opening up-wards directed, protruding *labellum*, etc.). Our results have only confirmed the last hypothesis. In fact, no significant correlation was detected between the number of apertures and the position of the flower, either in *V. hymettia* or in two other species.

No significant differences were recorded even among individuals. Our results are not in accordance with data reported by Dajoz et al. (1995) for some *V. arvensis* populations where the percentages of the different pollen morphs vary significantly among plants of the same population so much as to change the main morph. These conflicting findings are probably due to differences in sampling methods that may often lead to biased estimates, especially the analysis of a maximum of only 200 pollen grains per flower without any indication about the developmental stage of the collected flowers. There is a large difference in the number of pollen grains between flowers in full bloom and flower buds (from about 60 to over 2,500). In fact, only in the closed anthers collected from buds can all the pollen assemblage be found, although with a variable percentage of unidentifiable grains.

Finally, according to our results, even when there is variability in the relative frequencies of the different pollen morphs, the same type always prevails within both a single plant

and the population. Then, it is possible to identify the main pollen morph by analyzing just a few flowers per plant or per population. However, in order to avoid bias due to sampling methods, we suggest the collection of flower buds in well developed plants, with at least three or more flowers, and the analysis of the whole pollen assemblage rather than a few hundred grains.

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