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Genetic characterization of *Genista sericea* Wulfen (Cytiseae – Fabaceae) as revealed by nuclear DNA content and ITS nrDNA region analysis

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Summary – *Genista sericea* Wulfen, a northern Illyrian amphi-Adriatic species, presents a certain morphological variability. To clarify whether the genetic variations support the morphological differences among accessions of different geographic origin, analysis of nuclear DNA content and polymorphism of the internal transcribed spacer (ITS) dataset was studied. The variation in nuclear DNA content of *G. sericea* var. *sericea* and var. *rigida* is minimal (2.09 and 2.08 pg/nucleus respectively) and is correlated with equal chromosome numbers in both varieties. Intraspecific variability of the ITS region was studied on 13 accessions of *G. sericea*, 6 belonging to var. *sericea* and 7 to var. *rigida*. These accessions were analyzed in comparison to closely related species already studied. ITS sequences of *G. sericea* revealed large polymorphism and formed two main clusters. One cluster (6 accessions) comprehends var. *sericea* of northern Italy, Slovenia and northern Croatia; the other cluster (7 accessions) includes five accessions of var. *rigida* from southern Croatia and Montenegro and two from the Pollino massif (southern Italy). The later two accessions considerably differed from other accessions of var. *rigida*. This genetic analysis supports the previous assumptions, which subdivided *G. sericea* into at least two taxa. On the basis of the results presented, it is here suggested that the subdivision of *G. sericea* into var. *sericea* and var. *rigida* should be maintained.

Key words: Fabaceae, *Genista sericea*, genome size, ITS, phylogenic analysis.

Abbreviations: DAPI – 4, 6-diamidino-2-phenylindole, ITS – internal transcribed spacer, PI – propidium iodide.

Introduction

Genista sericea Wulfen, a northern Illyrian amphi-Adriatic species, has a range from northeastern Italy to Albania, with a disjunct distribution on the Pollino massif, Calabria, Italy (PAMPANINI 1912, GIBBS 1966, CONTI et al. 2005).

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Within the species, PAMPANINI (1912) distinguished var. *sericea* and var. *rigida*. In the first taxon the non-flowering branches are elongated and flexuous; the leaves are lanceolate and acute with a glabrous upper surface. In the second taxon the branches are shorter, more strongly ribbed and rigid; the leaves are ovate, broader and thicker, especially the young, with a sparsely pubescent upper surface. Whereas var. *sericea* is distributed in the northern part of the range (northeastern Italy and northern part of the Illyrian region), var. *rigida* is spread in the southern part of the Illyrian region (Dalmatia, Bosnia-Hercegovina, Montenegro, Albania) and on the Pollino massif (Italy). In recent literature, var. *rigida* is not considered (GIBBS 1966, PIGNATTI 1982, CONTI et al. 2005).

In northern Italy, var. *sericea* occurs along the southern slopes of the Alps; it is frequent in the east (Friuli Venezia Giulia, particularly in the karst area near Trieste), while it becomes rarer towards the west, in southern Veneto and southern Trentino. In Slovenia, var. *sericea* is present only in the south west. In Croatia it occurs in the northwestern area, especially in Kvarner (PAMPANINI 1912, PIGNATTI 1982, FEOLI CHIAPELLA and RIZZI LONGO 1987, CONTI et al. 2005).

Genista sericea belongs to sect. Spartioides Spach. According to its morphological and phytochemical characters, it is known as a heterogeneous section that does not form a monophyletic group. Because of the molecular data, the species of this section are included in separate groups intermingled with species of other sections and several intersectional hybrids are also present (PARDO et al. 2004). This heterogeneity was confirmed also by karyological and palynological data (CANTÓ et al. 1997; CUSMA VELARI et al. 2003, 2009; RIZZI LONGO and FEOLI CHIAPELLA 2009). The species of this section occur mostly in the Mediterranean region, with two main distribution centers: a Western (southern Spain and northwestern Africa) and an Eastern one (Balkan Peninsula and Anatolia) (GIBBS 1966, CANTÓ et al. 1997).

RIZZI LONGO and FEOLI CHIAPELLA (1993) carried out a pollen morphology analysis of *G. sericea* by light microscope and SEM, where var. *sericea* and var. *rigida* differ in some quantitative (grain dimensions) and qualitative (shape of amb and furrows) characters. CUSMA VELARI et al. (1996) have determined the chromosome number of *G. sericea* var. *sericea* and var. *rigida*: 2n=4x=48.

The nuclear ribosomal DNA (nrDNA) internal transcribed spacer region (ITS) has proven a useful source of characters for phylogenetic studies in many Angiosperm families including Leguminosae (ÁLVAREZ and WENDEL 2003). Many genera of *Cytiseae* (KAESS and WINK 1997b), *Lupinus* (BADR et al. 1994, KAESS and WINK 1997a), *Genista* (DE CASTRO et al. 2002), *Genista* and related genera as *Teline*, *Echinospartum*, *Retama*, *Stauracanthus* (PARDO et al. 2004), *Cytisus* and allied genera such as *Argyrocytisus*, *Calicotome*, *Cytisophyllum* (CUBAS et al. 2002), *Adenocarpus*, *Genista* and *Teline* (PERCY and CRONK 2002) and *Ulex* (AINOUCHE et al. 2003), have already been analyzed.

Although molecular data are considered the most convincing data set, which can be used to identify phylogenetic relationships, a combination of different data sets, including genome size data, provides useful information on genetic similarity and taxonomic position (VIŽINTIN et al. 2006). Most reports (LEITCH and BENNETT 2002) confirmed that genome size is usually a value with limited variations within a single species and could be considered characteristic of the species. Flow cytometry could be used for estimations of genome size in absolute units using intercalating fluorochrome PI stained nuclei (NOIROT et

al. 2002) or for an intraspecific comparison in relative units using DAPI, an AT base specific dye (BUITENDIJK et al. 1997). Genome size data concerning the taxa *Genista*, *Cytisus*, *Lembotropis* and *Argyrocytisus* are rather scarce.

The aim of our study is to assess the intraspecific variability of *G. sericea*, highlighting the possible differences between var. *sericea* and var. *rigida* at a molecular level through the analyses of nrDNA regions ITS1 and ITS2. Furthermore, as genome size has not yet been determined for *G. sericea*, our aim is to provide data on genome size in both varieties.

Materials and methods

Plant material

Genome size determination was performed with fresh plant material. Accessions used for the flow cytometry analyses were: *G. sericea* var. *sericea*, collected at Opicina and Monrupino (Trieste – Italy) and G. *sericea* var. *rigida*, collected on the Pollino massif (Calabria, Italy) (Tab. 1).

Tab. 1. Propidium iodide and DAPI flow cytometric genome size determination in the examined populations of *Genista sericea*.

Staining method	Taxon	Accession	No. plant	Average 2C (pg) ± SD
PI staining				
	G. sericea var. *sericea*	Opicina (Trieste, Italy) 90579 (TSB)	5	2.09 ± 0.03
	G. sericea var. *rigida*	M. Pollino (Cosenza, Italy) 90582 (TSB)	5	2.08 ± 0.03
DAPI staining				
	G. sericea var. *sericea*	Opicina (Trieste, Italy) 90579 (TSB)	4	1.96 ± 0.02
		Monrupino (Trieste, Italy) 90580 (TSB)	4	1.95 ± 0.02
	G. sericea var. *rigida*	Pollino (Cosenza, Italy) 90582 (TSB)	4	1.95 ± 0.03

The extraction of DNA for molecular analyses was performed from herbarium plant material. Different accessions of *G. sericea* were examined: six belonging to var. *sericea* and seven to var. *rigida*. The studied accessions, with their geographical origin and NCBI Genbank number are listed in table 2 and figure 1. Voucher specimens are deposited in the Herbarium of the Department of Life Sciences, University of Trieste, Italy (TSB). The classification of PAMPANINI (1912) was followed.

Determination of nuclear DNA content by flow cytometry

The relative and absolute value of DNA content was assessed by flow cytometry using *Trifolium pratense* L. as standard. The genome size (0.85 pg) was previously estimated for this species using *Trifolium repens* L. cv. Milo as standard (VIŽINTIN et al. 2006).

For the determination of absolute DNA content, tissues from green and young stems of the analyzed samples, together with leaf tissues of the standard species, were chopped with a razor blade in plastic Petri dishes in cold LB01 buffer, according to a technique adapted

Tab. 2. Accession data of the populations of *Genista sericea* sampled for phylogenetic analyses in the ITS region of nrDNA: origin, collector and NCBI GeneBank accession numbers.

Taxon	Sample	Origin	Collector	GeneBank accession No.	
				ITS1	ITS 2
G. sericea var. *sericea*	GS17	Vivaro, Pordenone, Italy, 15/10/2000, 84731 (TSB)	L. Feoli Chiapella	EU525894	EU525906
	GS19	Pinedo di Claut, Pordenone, Italy, 23/5/1999, 84728 (TSB)	C. Coran	EU525896	EU525908
	GS18	Mt. Čaven, Nova Gorica, Slovenia, 10/5/1999, 84730 (TSB)	L. Feoli Chiapella	EU525895	EU525907
	GS20	Mt. Nanos, Nova Gorica, Slovenia, 29/5/1999, 84726 (TSB)	L. Feoli Chiapella	EU525893	EU525909
	GS2	Baščanska Draga – Punat, Krk, Croatia, 24/4/1966, 84138 (TSB)	M. Tarabocchia	EU525897	EU525910
	GS15	Capo Pax Tecum – Plomin, Istria, Croatia, 70 m, 26/4/1964, 84139 (TSB)	T. Mozenich	EU525898	EU525911
G. sericea var. *rigida*	GS12	Biokovo, Kozica, Croatia, 600 m, 30/4/2001, 84457 (TSB)	L. Feoli Chiapella	EU525904	EU525917
	GS9	Kuči, Donji Medun, Podgorica, Montenegro, 3/6/1998, 84143 (TSB)	V. Karaman	EU525900	EU525913
	GS10	Kakarička gora, Masline, Podgorica, Montenegro, 173 m, 3/6/1998, 84456 (TSB)	V. Karaman	EU525901	EU525914
	GS11	Piperi, Podgorica, Montenegro, 300 m, 17/5/1998, 90581 (TSB)	V. Karaman	EU525902	EU525915
	GS13	Cijevna reka, Montenegro, 27/5/2000, 84451 (TSB)	S. Hadžiablahovič	EU525903	EU525916
	GS4	Timpone Dolcetti, Pollino, Cosenza, Italy, 9/6/1997, 84141 (TSB)	D. Puntillo	EU525899	EU525912
	GS16	Morano Calabro, Pollino, Cosenza, Italy, 3/6/1997, 84140 (TSB)	A. Vaccaro	EU525905	EU525918

from DOLEŽEL et al. (1989). The suspension was passed through a 30 µm nylon-mesh filter and nuclei were stained with 50 µg mL^{-1} of PI and 50 µg mL^{-1} ribonuclease.

For the determination of relative DNA content, the staining was performed with DAPI and the procedure was modified according to OTTO (1988). Nuclei of our sample and of the standard species were released in 0.1 M citric acid containing 0.5% Tween 20. The suspension was filtered through a 30 µm nylon-mesh filter. A 4x volume of staining buffer containing 4 µg mL^{-1} DAPI and 0.4 M disodium hydrogen phosphate was added.

Measurements were done on a Partec PAS flow cytometer using a linear scale. For PI staining, samples were analyzed using an argon laser tuned to 488 nm, with emissions measured through an RG 590 long-pass optical filter. For DAPI staining, the UV spectrum ex-

Fig. 1. Geographycal origin of the examined accessions of *Genista sericea*. ▲ *G. sericea* var. *sericea*, ● *G. sericea* var. *rigida*

cited with a HBO lamp was used and emissions were measured through a GG 435 long-pass filter. Seven thousand nuclei per sample were measured and at least four repetitions of different nuclear isolations were performed for each species. Flomax® software (Partec, Münster) was used to calculate the positions of G0/G1 peaks of standard and investigated accessions. The analysis was completed in a short period to minimize the seasonal variation effect.

Analysis of the ITS region of rDNA

Genomic DNA was extracted from herbarium plant material applying CTAB mini DNA extraction protocol according to COMPTON et al. (1998) with slight modifications. The ribosomal ITS 1 and 5.8 S -ITS 2 regions were separately amplified using, respectively, ITS1-ITS2 and ITS3-ITS4 primers designated by WHITE et al. (1990). Amplification was carried out by means of a polymerase chain reaction in 50 µl reaction mixture containing 1 x Taq polymerase buffer (Promega, Madison, WI, USA), 200 µM of each dNTP, 0.5 µM of each primer, 100 ng DNA and 2.5 units of Taq polymerase (Promega). Optimized amplification was performed in a PTC-150-16E-25 M.J. Research Inc. thermal minicycler programmed as follows: (A) an initial 3 min denaturation at 95 °C; (B) 35 cycles of denaturation at 93 °C for 35 sec, annealing at 49 °C for 35 sec and polymerisation at 72 °C for 2 min; (C) final extension at 72 °C for 7 min. The amplified DNA products were sequenced from both sides by Macrogen Inc. (Seoul, Korea) using the same primers on an ABI 3730 XL DNA analyser (Applied Biosystems, Renton, USA). Sequence results from each amplified fragment were visualized and edited to obtain an optimized consensus sequence using CodonCode Aligner ver. 1.4.6 (CodonCode Corporation, USA). Nucleotide sequences were deposited in the GenBank database with the accession numbers reported in table 2.

Phylogenetic analysis of ITS sequences

The initial data matrix was aligned using ClustalX (ver. 1.83). A phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura 3-parameter model (TAMURA 1992) on Mega 5.0 software (TAMURA et al. 2011). Bootstrapping was performed at one thousand replicates to assess the confidence values of the clusters formed. Bootstrap data are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 508 positions in the final dataset.

An accession of *Cytisus* was used as an outgroup. Several sequences of closely related *Genista* species from the section *Spartioides*, already available in the GenBank, have also been included in the ITS analyses to obtain a more comprehensive picture. Accession numbers and sources of these sequences are indicated in table 3.

Tab. 3. Accession data and sequences of ITS region obtained from NCBI GenBank.

Taxon	References	GeneBank accession No.
Genista cinerea subsp. *speciosa* Rivas Mart. et al.	PARDO et al. 2004	AY263637
Genista cinerea subsp. *ausetana* O. Bolož s et Vigo	PARDO et al. 2004	AY263638
Genista cinerea (Vill.) DC. subsp. *cinerea*	PARDO et al. 2004	AY263636
Genista majorica Canto et M.J. Sanchez	PARDO et al. 2004	AY263652
Genista ramosissima (Desf.) Poir.	PARDO et al. 2004	AY263662
Genista valentina (Sprengel) Steud.	PARDO et al. 2004	AY263676
Cytisus scoparius (L.) Link subsp. *scoparius*	CUBAS et al. 2002	AF351120

Results

Analysis of nuclear DNA content

Genome size values and base composition were determined for five plants of both varieties of *G. sericea* (var. *sericea* and var. *rigida*) using PI staining and for four plants of each accession of the two varieties using DAPI (Tab. 1). Determination using PI serves as an estimation of absolute nuclear DNA content, while determination using DAPI serves as a comparison for further intraspecific analysis. The absolute value of nuclear DNA content for var. *sericea* is 2.09 pg DNA per nucleus. The value obtained for var. *rigida* is 2.08 pg DNA per nucleus.

Because lower CV values cause higher resolution, DAPI staining was additionally used to test more accurately the intraspecific genome size variation: *G. sericea* var. *sericea* presents a relative nuclear DNA content of 1.95 – 1.96 pg per nucleus and var. *rigida* 1.95 pg per nucleus. Results obtained with DAPI staining confirmed that the variability of genome size is rather small between the two varieties of *G. sericea* and is not useful for the characterization of the varieties.

It should be noted that the values obtained by PI staining have given higher estimations of DNA content, suggesting unequal AT/GC content.

Polymorphism of the ITS region of rDNA

The internal transcribed spacer (ITS1 and ITS2) region of ribosomal DNA was sequenced from 13 accessions of *G. sericea* (Tab. 2). Each examined sequence produced a slightly different sequence profile; their alignment resulted in a matrix of 508 characters. A high variability of ITS nucleotide sequences was discovered exhibiting 22 variable sites.

The nucleotide sequences of amplified ITS regions were used to construct a phylogenetic tree (Fig. 2) to assess the relationships among the analyzed accessions. The ITS tree reveals two major clades, bootstrap support was 74. One clade is formed by all the accessions of var. *sericea*, from Friuli – Venezia Giulia (Italy), Slovenia and northern coast of Croatia. The other clade includes the accessions of var. *rigida* from southern Croatia coast and Montenegro. The two accessions of var. *rigida* from Pollino massif (southern Italy) stand at the base, but the bootstrap support is low.

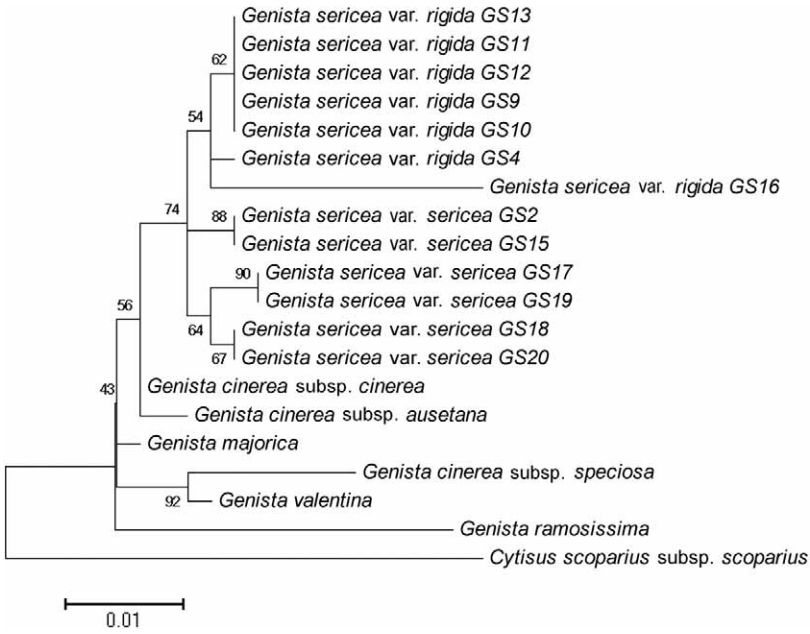

Fig. 2. Phylogram of 13 populations of *Genista sericea* and of 6 related species of the genus *Genista*. *Cytisus scoparius* subsp. *scoparius* was added as an outgroup.

Discussion

The accessions of *G. sericea* var. *sericea* and var. *rigida* present a very similar nuclear DNA content (2.09 and 2.08 pg per nucleus); also the relative nuclear DNA content is similar in the two varieties (1.95 – 1.96 and 1.95 pg per nucleus). The nuclear DNA content in a wild population of a species of *Genista sericea* is presented for the first time in this study, as the only data in literature regarding this genus are those of BELLENOT-KAPUSTA et al. (2006), who studied some ornamental brooms (as *G. tinctoria*, *G. lydia*, *G. pilosa*, *G. hispanica* and *G. aetnensis*) the 2C DNA content of which varies from 1.60 to 3.56, and of SUDA et al.

(2005) who determined the nuclear DNA content value for *G. benehoavensis* as 4.60 pg. In particular the 2C DNA content of *G. pilosa*, the only species previously examined of sect. *Spartioides*, is 1.99 pg, while in *G. tinctoria* and *G. lydia* of the affine sect. *Genista is* 1.73 and 1.74 pg. Data concerning other genera of *Cytiseae*, wild and cultivated plants of *Cytisus*, *Lembotropis*, *Argyrocytisus* and *Laburnum*, were published by BELLENOT-KAPUSTA et al. (2006) and OLSZEWSKA and OSIECKA (1983, 1984): the analyzed 2C DNA content varies from 1.10 to 4.40. MISSET and GOURRET (1996) determined the genome size of *Ulex* (7.7 pg/2C). OBERMAYER et al. (1999) and NAGANOWSKA et al. (2006) have studied many species of *Lupinus* (2C content from 1.15 to 2.68), a genus distant from the others of Cytiseae.

Because of the obtained genome size data, morphological differences between the two varieties of *G. sericea* are not correlated with significant differences in genome size and this led us to suppose that no major insertions or deletions of DNA segments occurred in any of varieties. This result is correlated with chromosome studies which showed the same number in both varieties (2n=4x=48; CUSMA VELARI et al. 1996).

Both varieties of *G. sericea* formed clearly separated clades (74 bootstrap supports) which did not intermingle with other related *Genista* species. Although analysis of ITS region is mainly used for interspecific phylogeny, this study indicates that genetic variation of ITS region in *G. sericea* was in this case sufficient to resolve both subspecies. In *G. sericea* the data presented reflect the separation of two distinct groups of accessions, the first comprehending all the accessions of var. *sericea* from northern Italy, Slovenia and northern Croatia, the second including all the accessions of var. *rigida* collected in southern Croatia, Montenegro and in southern Italy.

Molecular data are in accordance with morphological characteristics and pollen differences (RIZZI LONGO and FEOLI CHIAPELLA 1993), as well as seed dimensions (CUSMA VELARI and FEOLI CHIAPELLA, personal observations). Our analysis supports the subdivision of *G. sericea* into two taxa (var. *sericea* and var. *rigida*). On the basis of molecular data, the accessions of the Pollino massif (Calabria, Italy) attributed by PAMPANINI (1912) to var. *rigida* are related to those of southern Croatia and Montenegro, but do show diversification within var. *rigida* that could be further studied.

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