Biochemical Systematics and Ecology 61 (2015) 312–318

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Chloroplast and nuclear DNA studies in Iberian Peninsula endemic *Silene scabriflora* subspecies using cpSSR and ISSR markers: Genetic diversity and phylogenetic relationships

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ARTICLE INFO

Article history:
Received 16 December 2014
Received in revised form 18 June 2015
Accepted 27 June 2015
Available online xxx

Keywords:
Silene scabriflora
Molecular markers
Genetic differentiation
Geographic variation
Refugia

ABSTRACT

The Iberian Peninsula is considered to be a center of natural distribution and diversity for several species of flora. In this study, the genetic diversity and phylogenetic relationships of *Silene scabriflora* from the Iberian Peninsula were analyzed using ISSR and cpSSR markers. A total of 161 ISSR markers were produced, with a percentage of polymorphic loci of 99.4%. A high level of genetic differentiation among *S. scabriflora* subspecies (*S. scabriflora* spp. *scabriflora*, *S. scabriflora* spp. *megacalycina*, *S. scabriflora* spp. *gallaecica* and *S. scabriflora* spp. *tuberculata*) was observed (Gst = 0.3685), which was illustrated by UPGMA dendrogram. Molecular results are in agreement with subspecies morphological characterization, particularly supporting the morphological similarities between *S. scabriflora* spp. *scabriflora* and *S. scabriflora* spp. *megacalycina*. Three of the five cpSSR loci analyzed were polymorphic and the two different alleles found in each polymorphic locus were combined in two different haplotypes. The results obtained in this study provide evidences for a long distance dispersion theory, Southern of Spain as the biodiversity center of *S. scabriflora*; for speciation, Southern of Spain as a refugia-within-refugia considering the speciation that occurs in *S. scabriflora* spp. *scabriflora* found in this region and the possibility of *S. scabriflora* spp. *scabriflora* as being ancestral of the species.

1. Introduction

The genus *Silene* belongs to the Caryophyllaceae family and has been recognized since the early days of evolutionary biology as an important model system in ecology and evolution with remarkably interesting features for studying sexual and

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http://dx.doi.org/10.1016/j.bse.2015.06.029
0305-1978/

mating systems (Bernasconi et al., 2009). This genus comprises about 23 sections and 700 species worldwide, 194 of which are reported to be present in Europe (Bratteler et al., 2006).

The variety of ecological habitats of *Silene* distribution in the Mediterranean region of Eastern Europe and their strong differentiation makes this region a natural global centre of this genus and a site for diversity.

The Iberian Peninsula is described as one of the most important Pleistocene glacial refugia in the European subcontinent (Hewitt, 2000, 1999) and the high level of endemism in Iberian plants and animals (Doadrio, 1988; Garcia-Barros et al., 2002; Gómez-Campo et al., 1984; Moreno Saiz et al., 1998; Ribera, 2000) indirectly suggest *in situ* long-term survival, differentiation and speciation in this area.

Silene scabriflora Brot. is an endemic species of the Iberian Peninsula and includes four subspecies, *Silene scabriflora* spp. *scabriflora*, *Silene scabriflora* spp. *tuberculata* (Ball) Talavera, *Silene scabriflora* spp. *megacalycina* Talavera and *Silene scabriflora* spp. *gallaecica* Talavera.

S. scabriflora spp. *scabriflora* is the most widespread subspecies throughout the Iberian Peninsula, although absent in the northwestern (NW) region. The remaining three subspecies are narrowly endemic, with more restricted distribution. *S. scabriflora* spp. *megacalycina* is confined to the Spanish provinces of León, Lugo and Ourense in the NW Iberian Peninsula. *S. scabriflora* spp. *gallaecica* is also endemic to the NW of Iberian Peninsula and is restricted to the Atlantic coast (La Coruña and Pontevedra provinces). *S. scabriflora* spp. *tuberculata* mainly appears in the southern part of the Iberian Peninsula, in the Portuguese Algarve and in the Spanish provinces of Cádiz, Granada, Malaga and Seville and with sporadic occurrences in NW Morocco.

These four subspecies are currently recognized as independent subspecies by the Spanish Flora book (Talavera, 1991). However, this classification is based only on morphological characters which are very similar between some of them: *S. scabriflora* spp. *scabriflora*, *S. scabriflora* spp. *megacalycina* and *S. scabriflora* spp. *tuberculata*, differ only in a few morphological characters. To date, no systematic studies are available and more detailed studies are important for providing new insights into the phylogenetic relationship between these *S. scabriflora* subspecies.

Different types of molecular markers have been successfully used in phylogenetics, systematic, evolutionary and conservation biology and molecular ecology. Nuclear inter-simple sequence repeats (ISSR) are a powerful tool for investigating genetic variation within species (Coutinho et al., 2014; Mao and Fang, 2014; Zietkiewicz et al., 1994). However, these markers have limitations due to their biparental inheritance. In contrast, uniparental inherited organelle DNA markers, such as chloroplast simple sequence repeats (cpSSRs), allow overcoming this limitation and complement information given by nuclear markers. An important advantage of ISSR and cpSSR marker systems is that no knowledge of the target species' genome sequence is required for analyses (Wang et al., 2008).

The Iberian Peninsula has been described as a biodiversity hotspot (Arroyo, 1997; Medail and Quezel, 1997), and as a melting pot for plant biodiversity (Rodríguez-Sánchez et al., 2008). Although some reports on endemic *Silene* species are available, none of them include the Iberian Peninsula *S. scabriflora* subspecies. In the present study the *S. scabriflora* subspecies were analyzed by ISSRs and cpSSRs in order to for the first time, assess the genetic diversity of *S. scabriflora* (1), estimate the genetic differentiation and phylogenetic relationships among *S. scabriflora* subspecies (2) and understand possible glacial refugia of *S. scabriflora* subspecies on the Iberian Peninsula (3).

2. Material and methods

2.1. Plant material and sampling

Young intact leaves of 4–8 individual plants, sometimes the total number of plants found, of each *S. scabriflora* subspecies – *Silene scabriflora* spp. *scabriflora*, *Silene scabriflora* spp. *megacalycina*, *Silene scabriflora* spp. *tuberculata* and *Silene scabriflora* spp. *gallaecica* were collected in their natural habitat in the Iberian Peninsula, then immediately dried in silica gel and stored at −80 °C until DNA extraction. The distance between the plants sampled was at least 1 m in order to avoid collecting leaves from the same individual. *S. scabriflora* spp. *scabriflora* was collected in Southern Spain and Central Portugal (HVR 19519, HVR 20901); the remaining three subspecies have a more restricted distribution and so were collected only in the provinces where their specimens exist: *S. scabriflora* spp. *tubeculata* in Southern Spain (HVR 19578), *S. scabriflora* spp. *megacalycina* in NW Spain (HVR 20902; HVR20903) and *S. scabriflora* spp. *gallaecica* in NW Spain (HVR 19534, HVR 19565; HVR20904; HVR20905) Atlantic coast (Fig. 1). All these specimens are included in the HVR herbarium (http://sweetgum.nybg.org/ih/herbarium.php?irn=126327).

2.2. DNA isolation

Total DNA was extracted from 100 mg of frozen leaf tissue following the protocol supplied in the DNeasy® Plant Mini Kit (QIAGEN, Düren, Germany). Extracted DNA was quantified by UV spectrometer (Nanodrop® ND-1000, Thermo Fisher Scientific, Waltham, MA, USA) followed by a quality check in 1.0% agarose gel electrophoresis and necessary dilutions were done (about 10 ng/μL).

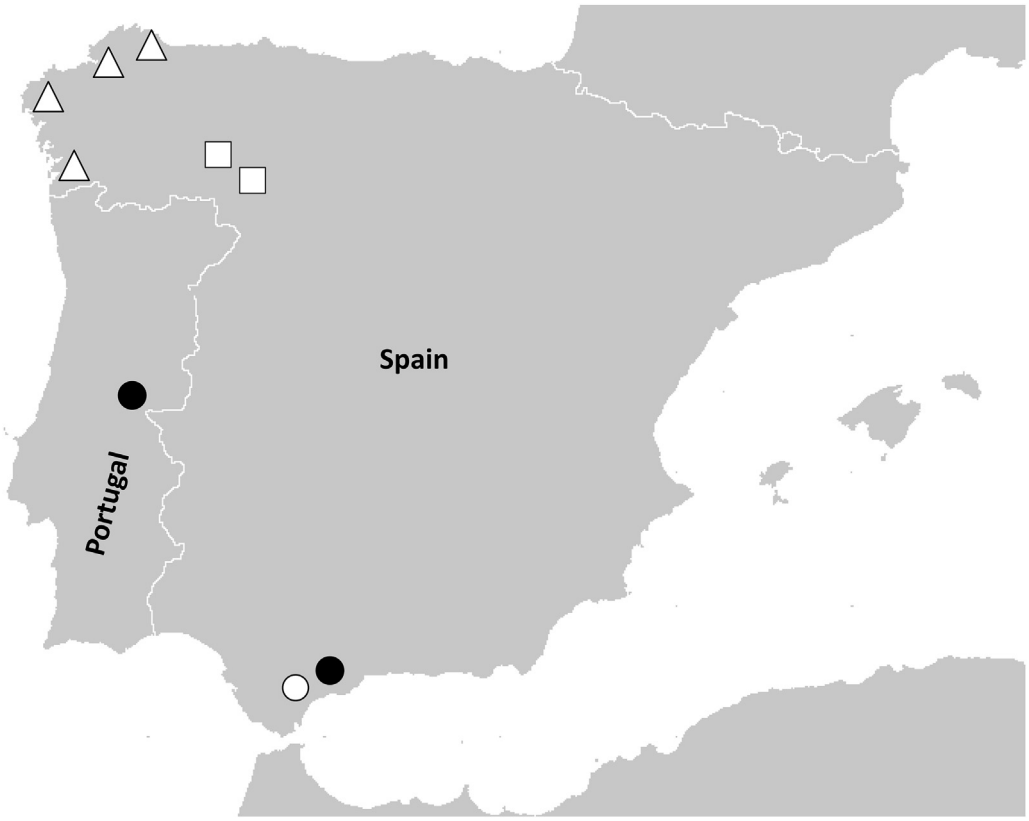

Fig. 1. Geographic location of *S. scabriflora* subspecies studied (white triangles – *S. scabriflora* spp. *gallaecica*; white squares – *S. scabriflora* spp. *megacalycina*; black circles – *S. scabriflora* spp. *scabriflora*; white circles – *S. scabriflora* spp. *tuberculata*).

2.3. ISSR amplification

After pre-screening 14 ISSR primers from the UBC#100/9 set (University of British Columbia, Vancouver, BC, Canada) tested in DNA bulks from each subspecies, 10 primers were selected *(UBC807*, *UBC811*, *UBC827*, *UBC835*, *UBC848*, *UBC853*, *UBC857*, *UBC886*, *UBC888* and *UBC889*) for further analysis, which combined high polymorphism with clear and reproducible fragments ($\geq$2 replications).

Each amplification for ISSR primers was performed in a reaction volume of 20.0 μL containing 10 ng of genomic DNA, 0.5 μM of primer, 10 mM of dNTPs, 25 mM of $MgCl_2$ and 1.0 U of Taq DNA polimerase in the manufacture's buffer (Thermo Fisher Scientific, Waltham, MA, USA).

The ISSR-PCR reactions were carried out using the T-Professional Basic thermocycler (Biometra, Germany). After an initial denaturation of 5 min at 94 °C, 45 cycles for 30 s at 94 °C, 45 s at 52 °C, 2 min at 72 °C were performed, followed by a final 10 min extension at 72 °C. PCR products were separated in 1.8% (p/v) agarose gels using TBE buffer, stained with ethidium bromide, and the molecular weights were estimated using the GeneRulerTM 100 bp DNA Ladder Plus (Thermo Fisher Scientific, Waltham, MA, USA). The electrophoretic patterns of the PCR products were digitally recorded using a Molecular Image Gel-Doc™ XR+ with Image Lab™ Software (BIO RAD, Hercules, CA, USA). The images were analysed using the same software, which assigns a fragment size to each band using an algorithm based on the 100 bp ladder. These fragment sizes were used to assign loci for each primer.

2.4. cpSSR amplification

Five chloroplast SSR loci (ccmp3, ccmp4, ccmp6, ccmp7 and ccmp10), were amplified in all samples using the consensus primer pairs designed by Weising and Gardner (1999). The forward primer of each pair was fluorescently labeled with 6-FAM (ccmp3 and ccmp4), HEX (ccmp6) or NED (ccmp7 and ccmp10). PCR reactions were performed in a 20 μL final volume containing 10 ng of template DNA, 0.15 mM of each dNTP, 1 U FIREpol® DNA polymerase in the manufacturer's buffer (Solis BioDyne, Estonia), 2 mM $MgCl_2$ and 0.4 μM of forward and reverse primers. Amplifications were carried out in a T-Professional Basic thermocycler (Biometra, Germany) initially set for 3 min at 94 °C, followed by 30 cycles of 94 °C for 45 s, 50 °C (ccmp4,

ccmp6, ccmp7 and ccmp10) or 55 °C (ccmp3) for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. PCR products were visualized by electrophoresis on 2.5% agarose gels (p/v), followed by ethidium bromide staining.

Amplified products were run on the ABI Prism® 3730 Genetic Analyzer using the GeneScan™ 500 LIZ® size standard (PE Applied Biosystems, Foster City, CA, USA).

2.5. Data analysis

For the genetic similarity analysis, ISSR DNA bands were scored as present (1) or absent (0) on the basis of size comparison with an external standard (GeneRulerTM 100 bp DNA Ladder Plus) (Thermo Fisher Scientific, Waltham, MA, USA) to produce a set of binary data. Due to dominancy of these markers, it was assumed that each DNA fragment position corresponds to a locus with two alleles revealed by band absence or presence (Powell et al., 1996). Only data from intensely stained, unambiguous, clear and reproducible bands ($\geq$2 replications) were scored and used for statistical analysis.

The resulting binary data matrix of ISSR was analyzed using POPGENE version 32 (Yeh et al., 2000) to estimate genetic diversity parameters assuming Hardy–Weinberg equilibrium: the number (NPL) and percentage of polymorphic loci (%PL) were measured at species level. Likewise, genetic diversity was determined according to Nei (1973) and calculated at two levels: total genetic diversity within *S. scabriflora* subspecies as a whole (h) and genetic diversity among subspecies (Hs). The coefficient of genetic differentiation (Gst) (Nei, 1973) and Shannon information index (I) (Lewontin, 1972) were also estimated.

Based on the same data, a dendrogram was constructed by cluster analysis using the Unweighted Pair Group Method of the Arithmetic Averages (UPGMA) using the Simple Matching Coefficient (SM) as implemented in the software package NTSYS-pc, version 2.02g (Rohlf, 2008).

Labeled products of cpSSRs were analysed and sized by means of Peak Scanner™ v1.0 free software (PE Applied Biosystems, Foster City, CA, USA).

3. Results and discussion

This is the first study concerning *S. scabriflora* genetic diversity and differentiation. *S. scabriflora* spp. *scabriflora* is widespread on the Iberian Peninsula. However, the remaining three subspecies are scarce with a very narrow distribution, and are currently restricted to a few specific provinces of the Iberian Peninsula.

The greater the genetic diversity of a species is, the more complex is its genetic background, the stronger its evolutionary potential and ability to withstand adversity and the more easily it will expand its distribution range and adapt to new environments. Therefore, research on the genetic diversity within a species is fundamental to understanding its origin and evolution (Zhao et al., 2014).

In this study, unambiguous, reproducible and polymorphic ISSR patterns were obtained using ten selected primers. A total of 161 markers were scored, which 160 were polymorphic among the four *S. scabriflora* subspecies (Table 1) and 17 subspecies-specific. UBC835 and UBC853 primers produced the largest number of markers – 22 and 21, respectively. Analysis of the data showed that the percentage of polymorphic loci (%PL) ranged from 85.7 % to 100 %, with a mean value of 99.4% (Table 1). These data reveal that ISSR markers are highly polymorphic, permitting a deduction about the genetic diversity of *S. scabriflora* species and estimate of the genetic relationship between *S. scabriflora* subspecies.

The genetic diversity of *S. scabriflora* species was assessed by Nei's gene diversity coefficient (Nei, 1973) and was performed at two levels; total genetic diversity of *S. scabriflora* subspecies as a whole (h) and genetic diversity among *S. scabriflora* subspecies (Hs), with values of 0.2831 and 0.1788, respectively. Shannon's diversity index (I) (Lewontin, 1972) was determined, being 0.4451. This level of genetic diversity has also been observed in other species using ISSR markers, namely *Tuberaria major* (h = 0.197, I = 0.324, %PL = 97.7) (Trindade et al., 2012) and *Plantago algarbiensis* (h = 0.2309; I = 0.3520; % PL = 83.1) (Ferreira et al., 2013), both of which are restricted to the southern Iberian Peninsula.

Table 1
DNA profile and polymorphism in *S. scabriflora* using 10 ISSR primers (TL – Total Loci; PL – Polymorphic Loci; EL – Exclusive Loci; %PL – Percentage of polymorphic Loci).

	Primer sequence (5′–3′)	TL	PL	ML	EL	%PL
UBC807	$(AG)_8T$	16	16	0	0	100.00
UBC811	$(GA)_8C$	14	14	0	0	100.00
UBC827	$(AC)_8G$	13	13	0	0	100.00
UBC835	$(AG)_8YC$	22	22	0	1	100.00
UBC848	$(CA)_8RG$	17	17	0	0	100.00
UBC853	$(TC)_8RT$	21	21	0	0	100.00
UBC857	$(AC)_8YG$	19	19	0	0	100.00
UBC886	$VDV(CT)_7$	14	14	0	0	100.00
UBC888	BDB $(CA)_7$	18	18	0	0	100.00
UBC889	DBD $(AC)_7$	7	6	1	0	85.7
Total		161	160	1	1	99.4

The great number of endemic species in this region may be due to the Iberian Peninsula having been important Pleistocene glacial refugia. Due to the dramatic climatic changes, this period caused considerable migration, fragmentation and extinction of populations (Bennett, 1996; Comes and Kadereit, 1998; Dynesius and Jansson, 2000), and Southern Europe maintained a great genetic diversity. In this refugia region there was a tendency for higher accumulation of genetic diversity with persistence and relative stability. The varied topography of the mountains in the southern refugia may have split a species range into isolated disjuncts (with restricted gene flow) that over time diverged and differentiated into unique genetic variants with distinct features (Hewitt, 1999). Populations in the South may also have survived glacial cycles by ascending the mountains during warm interglacial periods and descending during the cold glacial periods. Moreover, the Iberian Peninsula physiographic complexity and geographical position favored survival throughout the region in the Pleistocene, leading to the emergence of several endemic species.

The Gst is a widely used statistical measure of genetic differentiation. It is not affected by the reproductive system of a species, by the number of alleles per locus or by the effect of evolutionary pressures. The genetic differentiation (Gst) among *S. scabriflora* subspecies was 0.3685, a value which is considered high and supports the idea of a fragmented refugia structure in the past. This value was reflected in the dendrogram based on the genetic similarity obtained using the SM coefficient since the four subspecies are differentiated into two main clusters (Fig. 2). The SM coefficient is considered the more appropriate coefficient of similarity for dominant markers when considering closely related taxa (Halldén et al., 1994). Cluster analyses places the plants into groups on a hierarchical structure: the maximal similarity for plants belonging to the same group and minimal for plants sorted in different groups (Laurentin, 2009). Cluster I in the dendrogram comprises three of the four subspecies, *S. scabriflora* spp. *scabriflora*, *S. scabriflora* spp. *megacalycina* and *S. scabriflora* spp. *tuberculata*, and cluster II only *S. scabriflora* spp. *gallaecica*. The coefficient of similarity (0.46) between them indicates a considerable differentiation level regarding an analysis at subspecies level (Fig. 2). Cluster I is further divided into two subgroups, with a coefficient of similarity of 0.58 (Fig. 2). Subgroup Ia) includes *S. scabriflora* spp. *scabriflora* and *S. scabriflora* spp. *megacalycina* with a coefficient of similarity of 0.70 and subgroup Ib) *S. scabriflora* spp. *tuberculata* (Fig. 2). This clustering analysis of *S. scabriflora* subspecies by ISSR markers was in agreement with their morphological traits described in Flora Ibérica, particularly supporting the morphologic similarities between *S. scabriflora* spp. *scabriflora* and *S. scabriflora* spp. *megacalycina*.

Certain patterns of genetic and phenotypic differentiation across a species' geographic distribution can be attributed to local adaptation, which is expected to increase quantitative genetic differentiation at phenotypic traits above the range of genetic differentiation values obtained at neutral marker loci (Pujol et al., 2008; Whitlock, 2008). The overlapping of *S. scabriflora* spp. *scabriflora* and *S. scabriflora* spp. *megacalycina* distributions can explain their genetic and morphological similarities.

Summing up, *S. scabriflora* spp. *scabriflora* and *S. scabriflora* spp. *megacalycina* are more closely related and *S. scabriflora* spp. *tuberculata* and, particularly, *S. scabriflora* spp. *gallaecica* are more distant (Fig. 2). These latter two subspecies are located closer to the sea; the Mediterranean and Atlantic coasts, respectively, and are influenced by very specific and characteristic environments which can partially explain their divergence compared to the other two subspecies. These results suggest some sort of isolation by distance pattern that probably reflects a very ancient expansion which likely occurred during the Pleistocene. Another alternative explanation is that the *Silene scabriflora* subspecies distribution may have been affected by the

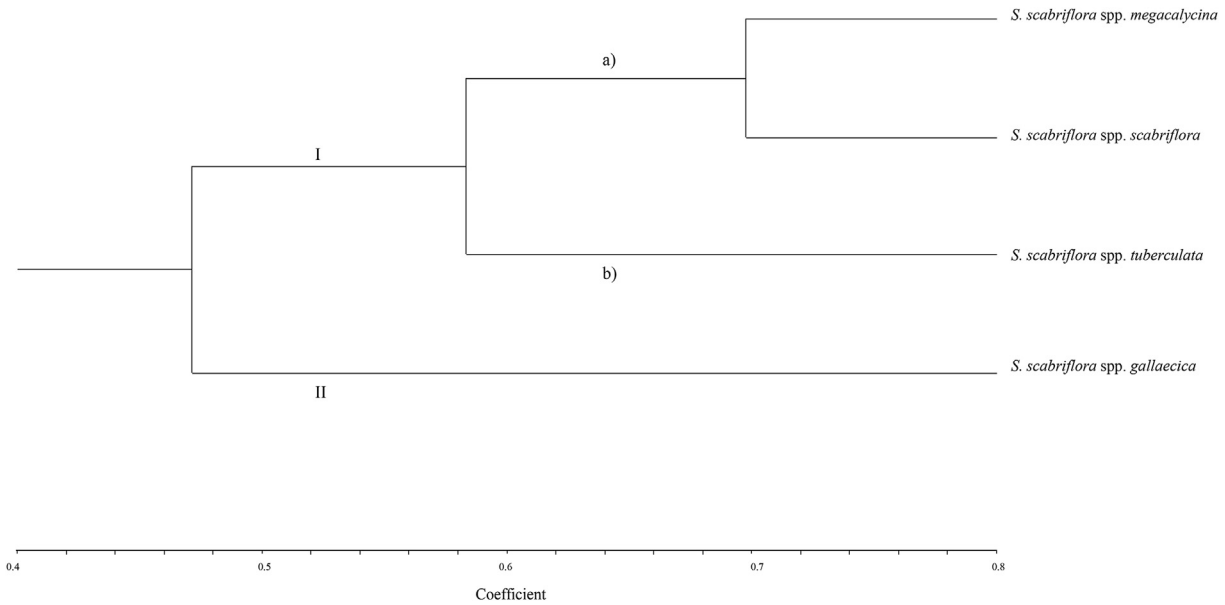

Fig. 2. Dendrogram of *S. scabriflora* subspecies showing genetic similarity, based on ISSR data, using the UPGMA method and SM coefficient.

Table 2
Size of the alleles for the five cpSSR loci analyzed in *S. scabriflora* subspecies and their haplotypes.

Subspecies	ccmp3 (bp)	ccmp4 (bp)	ccmp6 (bp)	ccmp7 (bp)	ccmp10 (bp)	cpSSR haplotype
S. scabriflora spp. *megacalycina*	101	112	109	136	88	I
S. scabriflora spp. *scabriflora*	101	112	109	136	88	I
	101	113	100	136	87	II
S. scabriflora spp. *tuberculata*	101	112	109	136	88	I
S. scabriflora spp. *gallaecica*	101	112	109	136	88	I

particular conditions of one much overlooked period when studying recolonization – the Mid-holocene period which occurred after the last glacial maximum and is described by summers up to five degrees warmer but with colder winters.

An objective of this study was to identify possible glacial refugia of *Silene scabriflora* subspecies in the Iberian Peninsula. ISSR markers, despite being a suitable molecular marker to assess genetic diversity since it amplifies many different regions of a genome and is simple, reproducible and cost-effective, as a nuclear dominant marker, it doesn't allow for reconstruction of colonization patterns (Bellucci et al., 2011; Wang et al., 2008, 2011). For this reason, and in order to explore exchanges between refugia in *S. scabriflora* species, chloroplast SSR markers were also performed.

From the set of 10 ccmp pairs designed by Weising and Gardner (1999) to amplify cpSSR loci, ccmp3, ccmp4, ccmp6, ccmp7 and ccmp10 were chosen to amplify all of the *S. scabriflora* subspecies plants analyzed. Eight different alleles at the five loci amplified were detected (Table 2). Three loci (ccmp4, ccmp6 and ccmp10) out of the five chloroplast microsatellites screened were polymorphic, the remaining two loci (ccmp3 and ccmp7) being monomorphic in all of *S. scabriflora* subspecies plants analyzed (Table 2). Each locus revealed two different size variants: 112 and 113 bp in ccmp4; 100 and 109 bp in ccmp6 and 87 and 88 bp in ccmp10. Among the polymorphic loci, three alleles (113 in ccmp4, 100 in ccmp6 and 87 in ccmp10), with a frequency of 12.5%, were found only in *S. scabriflora* spp. *scabriflora* collected in the South of Spain. Therefore, considering the eight allelic variants obtained with cpSSR markers, two different haplotypes were detected and designated herein as haplotype I and II. Haplotype II was found to be unique to *S. scabriflora* spp. *scabriflora* of Southern Spain while haplotype I was common to all subspecies, including the *S. scabriflora* subsp. *scabriflora* from Portugal.

The present results, despite the current limitation of the *Silene scabriflora* subspecies numbers allows investigation of Southern Spain as the biodiversity hotspot for the species from where it would have spread to the North, since Southern Spain is the only Iberian Peninsula region where the two cpSSR haplotypes are found. This region could also be considered as an *S. scabriflora* spp. *scabriflora* refugia, since some kind of speciation seems to have occurred in this subspecies which resulted in a new haplotype (haplotype II).

Taking into account all the molecular data obtained in the present study, *S. scabriflora* spp. *scabriflora* seems to be the ancestral of *S. scabriflora*. *S. scabriflora* spp. *scabriflora* represents the most widespread of *S. scabriflora* and shares many morphological characters with two of the other subspecies, *S. scabriflora* spp *megacalycina* and *S. scabriflora* spp *tuberculata* which is reflected by their genetic similarity. Two different SSR chlorotypes were detected within *S. scabriflora* spp. *scabriflora* corroborating the larger genetic variability compared to the remaining subspecies, all possessing a single chlorotype.

The present data provides information on the genetic diversity of *S. scabriflora* and phylogenetic relationships among its subspecies, which could significantly affect the long-term survival and evolution of this species.

Subspecies assignment by morphology described in Flora Ibérica was backed by the molecular results obtained in regard to genetic similarity between subspecies, suggesting some sort of isolation that probably reflects a very ancient expansion.

In this way, this study presents an efficient tool for further molecular studies in this species. The results obtained in this study provide more evidence for some proposed theories, namely; long distance dispersion, with Southern Spain as the biodiversity center of *S. scabriflora* from which this species spread to the Northern Iberian Peninsula; about speciation, Southern Spain as a refugia-within-refugia considering the speciation that occurs in *S. scabriflora* spp. *scabriflora* found in this region and the strong possibility of *S. scabriflora* spp. *scabriflora* being the ancestral of the species.

Acknowledgments

This work was supported by the Fundação para a Ciência e Tecnologia (FCT) SFRH/BD/43167/2008 (to J.R.) and SFRH/BD/96400/2013 (to V.F.) grants.

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