

# Genetic structure of peripheral, island-like populations: a case study of *Saponaria bellidifolia* Sm. (Caryophyllaceae) from the Southeastern Carpathians

Anna-Mária Csergő · Peter Schönswetter ·  
Gyöngyvér Mara · Tamás Deák · Nicolae Boşcaiu ·  
Mária Höhn

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**Abstract** Geographically peripheral populations often experience a reduction of genetic diversity and divergence from the core populations. Habitat geometry and quality can induce a local genetic diversity pattern, which overlies the regional variability issued from the range-wide phylogeography. We evaluated the genetic variation and genetic

divergence of *Saponaria bellidifolia* Sm. on limestone outcrops within peripheral island-like populations from the Southeastern Carpathians, using RAPD markers. We also determined the degree of isolation related to other European populations, using AFLP. The Romanian populations had a decreased overall genetic diversity shared among populations, with lower level in small populations. Potential habitat size had a positive effect on genetic diversity estimates. Fisher's exact tests of genetic differentiation revealed significant divergences only between the geographically most distant populations. Romanian populations were genetically pauperised as compared to Bulgarian and Italian populations and our results suggest that they might have originated from a recent range expansion from southern glacial refugia.

N. Boşcaiu is deceased.

A.-M. Csergő (✉)  
Department of Horticulture, Faculty of Technical and Human Sciences, Sapientia Hungarian University of Transylvania, Sighişoarei 1C, Op. 9. Cp. 4, 540485 Târgu-Mureş, Romania  
e-mail: csergo.anna.maria@gmail.com

P. Schönswetter  
Department of Biogeography and Botanical Garden, Faculty Centre Botany, University of Vienna, Rennweg 14, 1030 Vienna, Austria  
e-mail: peter.schoenswetter@univie.ac.at

Gy. Mara  
Faculty of Technical and Social Sciences, Sapientia Hungarian University of Transylvania, Libertăţii 1, 530104 Miercurea-Ciuc, Romania  
e-mail: maragyongyver@sapientia.siculorum.ro

T. Deák  
Department of Plant Genetics, Faculty of Horticulture, Corvinus University, Ménesi 44, 1118 Budapest, Hungary  
e-mail: tamas.deak@uni-corvinus.hu

N. Boşcaiu  
Department of Cluj-Napoca, Institute of Biology of the Romanian Academy of Science, Republicii 9, 400015 Cluj-Napoca, Romania

M. Höhn  
Department of Plant Sciences, Faculty of Horticulture, Corvinus University, Ménesi 44, 1118 Budapest, Hungary  
e-mail: maria.hohn@uni-corvinus.hu

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## Introduction

Peripheral populations are important components of biodiversity and have recently come into the focus of conservation efforts (Lesica and Allendorf 1995; Hatcher et al. 2004). The peculiarity of peripheral populations is mainly due to three processes. Firstly, the populations' size and density tend to decrease from the centre towards the margins of the distribution area simultaneously with intensified environmental constraints (e.g. decline of habitat quality; Brown 1984; but see Sagarin et al. 2006). Consequently, reduction of genetic diversity in the same direction may be anticipated, due to continuously reduced gene flow (Antonovics 1976; Eckstein et al. 2006), and higher

frequency of extinction and recolonisation events (Holt 2003). The latter effect might lead to a decrease of genetic diversity within populations and an increase of differentiation among populations (Hatcher et al. 2004). Secondly, as a result of the reduction or absence of gene flow over long periods of time, small peripheral populations may have gradually diverged from the core of the distribution area due to the accumulation of mutations and random genetic drift (Ellstrand and Elam 1993; Young et al. 1996). Thirdly, ecological conditions at the edge of the distribution range may differ from those in the central part, thereby fostering local adaptation and eventual speciation (Herlihy and Eckert 2005). However, not all evidence supports the predictions of this centre-periphery model (Eckert et al. 2008). It has been shown that even small sized, isolated populations can hold important reserves of genetic variation and high evolutionary potential, hence there is a need for considering them in conservation strategies (Lammi et al. 1999; Jones et al. 2001; Van Rossum et al. 2003; Leimu and Mutikainen 2004).

One of the most important factors influencing fitness and survival of marginal and small populations is the habitat geometry and quality (Booy et al. 2000; Wolf and Harrison 2001; Lönn and Prentice 2002; Torres et al. 2003). Many rare plant species are confined to naturally fragmented habitats or ecological islands such as rock outcrops, peat bogs or primary grasslands, which might be separated by large inhospitable areas. In the light of population genetic theory, plant populations are able to respond to the fragmentation events by certain life-history traits like breeding system, life form, seed dispersal mechanisms or persistent seed bank (Nybom and Bartish 2000).

Several studies evaluated the genetic structure of peripheral vs. central populations, including species from naturally patchy habitats (e.g. Lammi et al. 1999; Lönn and Prentice 2002; Eckstein et al. 2006; Wróblewska and Brzosko 2006; Yakimowski and Eckert 2007). However, few referred to species native to the Southeastern Carpathians and discussed them only in the context of a range-wide phylogeography (e.g. Trewick et al. 2002).

The geographical distribution and habitat specificity of *Saponaria bellidifolia* Sm. (Caryophyllaceae) offered a good opportunity for the analysis of naturally fragmented populations located in island-like habitats at the periphery of a species' range. *S. bellidifolia* is a herbaceous perennial that is presumably outcrossing (given its protandrous flowers) and is being pollinated by hawk moths, burnet moths, beetles and bees (A.-M. Csergő, personal observation). The seeds lack conspicuous adaptations to dispersal. The species is a submediterranean mountain plant with a pronounced disjunct distribution area in Southern Europe, being mostly widespread in the Balkan Peninsula (Jalas and Suominen 1986). The northernmost island-like populations are found in the Apuseni Mountains (Southeastern Carpathians, Romania). Here, *S. bellidifolia* is confined to dry, warm, south-exposed slopes of crystalline limestone and dolomite outcrops that protrude from a landscape matrix of beech, Scots pine or larch forests, valleys and pastures. Such a fragmented landscape can enhance the isolation of the populations, despite relatively small distances separating the outcrops (1–13 km).

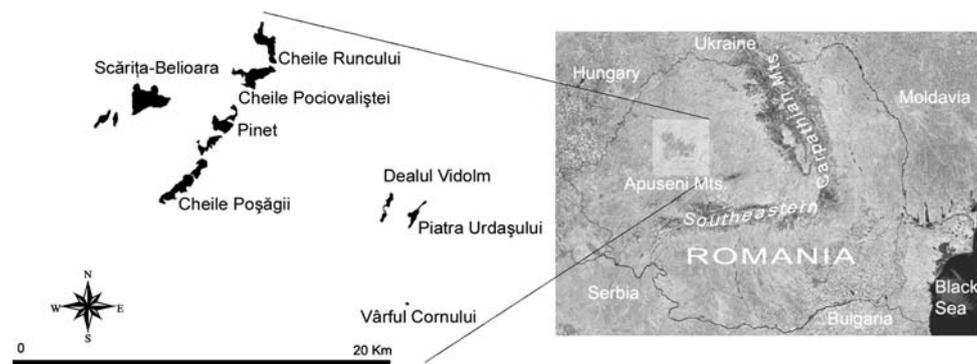
In the present study, two datasets were used to tackle different spatial and temporal horizons. We asked the following main questions: (1) To what extent has the genetic pattern of *S. bellidifolia* populations been shaped by the naturally fragmented habitats along the periphery of the species' distribution range? (2) What is the position of the studied island-like Romanian populations in the range-wide phylogeography of *S. bellidifolia* and how strongly do they diverge from other European populations?

## Materials and methods

### Study site and plant material

Ten individuals per population were sampled from seven of eight known localities in the Apuseni Mts (Southeastern Carpathians, Romania) (Fig. 1). Five populations (Scărița-Belioara, Cheile Poșăgii, Pinet, Cheile Pociovaliștei and

**Fig. 1** Geographic distribution of *S. bellidifolia* populations on eight limestone outcrops in the Apuseni Mts (Southeastern Carpathians, Romania)



Cheile Runcului) belong to Gilău-Muntele Mare Mountains and the other two (Dealul Vidolm and Piatra Urdaşului) are situated in Trascău Mountains. A large river separates the two mountain ranges. Vârful Cornului (Fig. 1) was omitted because of the low sample size (it contained only six individuals).

The evaluation of population size was difficult because of some hardly accessible sites, thus we considered the potential habitat size (area of south-exposed slopes) as a surrogate for the analysis (Table 1). Measurements were made using 1:25.000 maps and ArcView GIS 3.1 software (ESRI Inc., New York).

To estimate the position of the Romanian populations in the range-wide phylogeography, seven European populations were compared in the analysis (Pirin, Rodopi, Biokovo, Gran Sasso, Hautes-Pyrénées, Gilău-Muntele Mare and Trascău) (Fig. 2, Table 3). Five individuals per

population were sampled (except Hautes-Pyrénées, Pirin and Gilău-Muntele Mare with four samples).

Voucher specimens have been deposited in the Herbarium of Babeş-Bolyai University from Cluj-Napoca, Romania (CL).

#### DNA isolation, RAPD amplification and electrophoresis

Total DNA was extracted from young leaves using the DNeasy Plant Mini Kit (Qiagen Inc.), according to the manufacturer's instructions. GeneQuant II RNA/DNA calculator was used for spectrophotometric determination of the DNA concentration. The yield of DNA was generally good, ranging from 8 to 46.5 µg/ml.

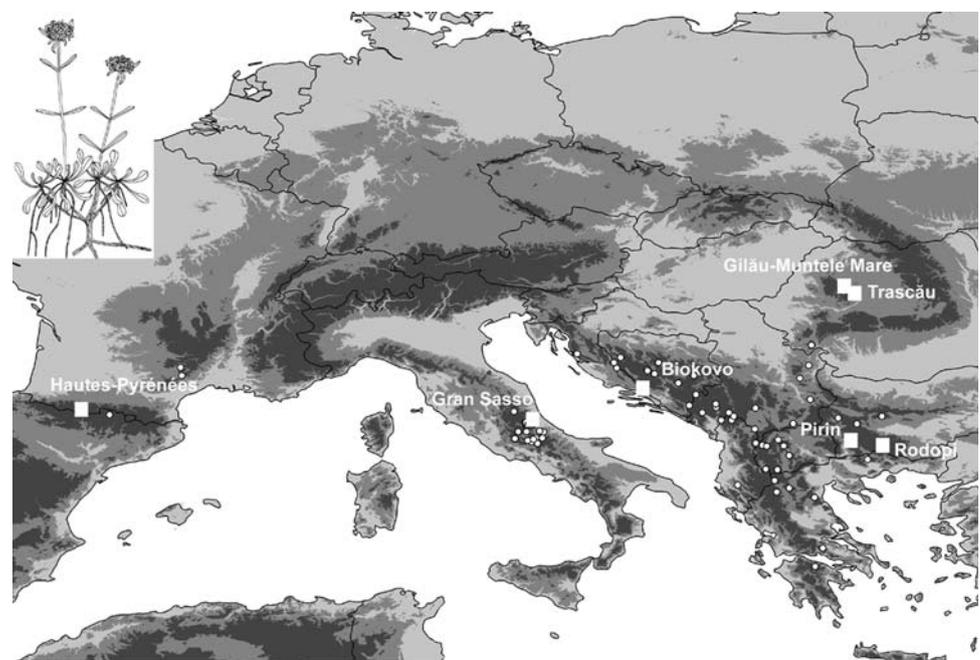
The genetic analysis of populations within Apuseni Mts was performed using the RAPD technique. Reactions were

**Table 1** Affiliation of Romanian *S. bellidifolia* populations from the Apuseni Mts (Southeastern Carpathians) to the two main mountain ranges, outcrops area and population diversity estimates

Main mountain range	Population	Outcrop area (ha)	$H_{exp}$	% <sub>PL</sub>	No. of unique markers
Gilău-Muntele Mare	Scăriţa-Belioara	1,613	0.101	23.863	–
	Cheile Poşăgii	663	0.062	15.909	–
	Pinet	555	0.089	19.318	–
	Cheile Pociovaliştei	796	0.068	17.045	–
	Cheile Runcului	649	0.087	23.863	1
	Overall			0.131	34.090
Trascău	Dealul Vidolm	20	0.042	10.227	–
	Piatra Urdaşului	27	0.022	4.545	–
	Overall		0.042	10.227	–

$H_{exp}$  denotes Nei's unbiased heterozygosity, %<sub>PL</sub> denotes percentage polymorphic loci

**Fig. 2** Geographic distribution of *S. bellidifolia* in Europe. Circles represent herbarium and literature data, squares represent the studied populations



carried out in 25 µl volumes, containing 1× enzyme buffer (10 mM tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 1.6 µM primer, 1 U *Taq* polymerase and ~50 ng DNA. Reactions were performed with the following profile: initial denaturation at 94°C for 3 min, followed by five cycles at 94°C for 20 s, 33°C for 1 min, 72°C for 1 min, 36 cycles at 94° for 20 s, 36°C for 1 min and 72°C for 1 min, followed by a final extension step at 72°C for 7 min.

Eighty random oligonucleotide primers from Operon Technologies that previously worked on Caryophyllaceae were tested. Amplification products were run on 1.2% TBE agarose gels, and stained with ethidium bromide. The banding pattern was visualised under UV light and photographed. DNA fragments were compared to a 100-bp molecular marker with standard size fragments ranging from 100 to 1,500 bp. Primers that gave distinct, variable banding patterns were selected and tested on all samples. Some individuals were replicated at least five times and only reproducible bands were taken into consideration.

#### Analysis of the RAPD data

To gain insights into the partitioning of population's genetic diversity within Apuseni Mts, we subdivided the populations into those from Gilău-Muntele Mare and from Trascău Mts. Given the life-history traits of the study species (outcrossing perennial, several pollinators, limited or no self-compatibility), we assumed populations to be in Hardy-Weinberg equilibrium. Nei's (1978) overall and partitioned expected heterozygosity and the percentage of polymorphic loci were calculated using TFPGA 1.3 (Miller 1997, available from <http://www.marksgeneticssoftware.net/tfpga.htm>). We performed an analysis of molecular variance (AMOVA) with Arlequin 3.1 (Excoffier et al. 2005) to evaluate the variance components and their significance levels within and among populations and groups. Fisher's exact test was performed (Sokal and Rohlf 1995) to obtain the degree of differentiation between populations. A UPGMA tree was constructed based on a matrix of Nei's (1978) similarities with 1,000 bootstrap replicates. A linear regression was performed in order to estimate the effect of potential habitat size (area of south-exposed slopes) on the obtained genetic diversity estimates of *S. bellidifolia* (R software version 2.61, R Development Core Team 2007).

#### AFLP-production

The AFLP procedure followed Gaudel et al. (2000), but with PCR reaction volumes halved. The following two selective primer combinations were chosen (fluorescent dye in brackets): (6-FAM)—*EcoRI* ACA and *MseI* CAC;

(VIC)—*EcoRI* AGG and *MseI* CTG. For each individual, 1.2 µl 6-FAM-, and 2 µl VIC-labelled selective PCR products were precipitated with 30 µl 96% ethanol and 1 µl sodium acetate (3 N, pH 4.6), washed with 70% ethanol, dried, and re-suspended in 0.2 µl GeneScan ROX 500 (size standard; Applied Biosystems, Foster City, CA, USA) and 9.8 µl formamide. Blind samples and ten replicates were included to test for contamination and to identify non-reproducible fragments, which were excluded from further analysis. Electrophoresis was carried out with an ABI 3100 capillary sequencer (Applied Biosystems); raw data were collected and aligned with the internal size standard using ABI Prism GeneScan 3.7.1 (Applied Biosystems). AFLP bands were scored using the program Genographer (version 1.6.0, Montana State University 1999; available from <http://hordeum.msu.montana.edu/genographer/>), and the results were exported as a presence/absence matrix.

#### Analysis of the AFLP data

Nei's (1978) expected heterozygosity, the percentage of polymorphic loci and Fisher's exact test were calculated from the AFLP dataset with TFPGA 1.3 (Miller 1997). We performed a neighbour-joining analysis of a Nei and Li (1979) genetic distance matrix with TREECON 1.3b (Van de Peer and De Wachter 1997). The tree was rooted with *S. lutea* L. Branch support was estimated with 2,000 bootstrap replicates.

## Results

#### RAPD analysis of Romanian populations

In the 70 analysed individuals, the 17 used primers produced 88 markers, of which 31 (35.23%) were polymorphic. The overall genetic diversity (Nei's unbiased heterozygosity 1978) was  $H_{exp} = 0.133$ . One unique marker was found in the population from Cheile Runcului and 21 region-specific markers have been detected in Gilău-Muntele Mare Mts, but none in Trascău Mts. Nei's unbiased heterozygosity and percentage of polymorphic loci varied substantially among populations. It was higher in Gilău-Muntele Mare Mts (0.131 and 34.09%), compared with Trascău Mts (0.042 and 10.23%). The highest level of polymorphism was found in Scărița-Belioara (0.101 and 23.86%) and the lowest in Piatra Urdaşului (0.022 and 4.55%) (Table 1).

Analysis of molecular variance estimates revealed that the amount of the variation retained within populations (49.20%,  $P < 0.0001$ ) was low relative to other plants with similar extent of fragmentation (Cotrim et al. 2003). We have found notable, significant levels of variation between

**Table 2** Analysis of molecular variance of RAPD data of 70 individuals of *S. bellidifolia* in the Apuseni Mts (Southeastern Carpathians)

Source of variation	df	Sum of squares	Variance components	Percentage variation	P-value
Among groups	1	49.979	1.268	26.73	<0.05
Among populations within groups	5	68.750	1.142	24.07	<0.0001
Within populations	63	147.000	2.333	49.20	<0.0001
Total	69	265.729	4.743	100	

the two mountain ranges and between populations (Table 2).

Fisher's exact test resulted in significant difference between the two mountain ranges ( $\chi^2 = 259.03$ ,  $df = 176$ ,  $P < 0.0001$ ), and between Cheile Runcului and Dealul Vidolm ( $\chi^2 = 215.54$ ,  $df = 176$ ,  $P < 0.0226$ ).

In the UPGMA dendrogram, the two populations of Trascău Mts were placed in a distinct cluster (bootstrap value 98%). Cheile Runcului was separated within the cluster of the Gilău-Muntele Mare Mts, but this was supported only by a weak bootstrap value (56%) (Fig. 3). Nei's (1978) genetic distances were very low, ranging between 0.017 and 0.119.

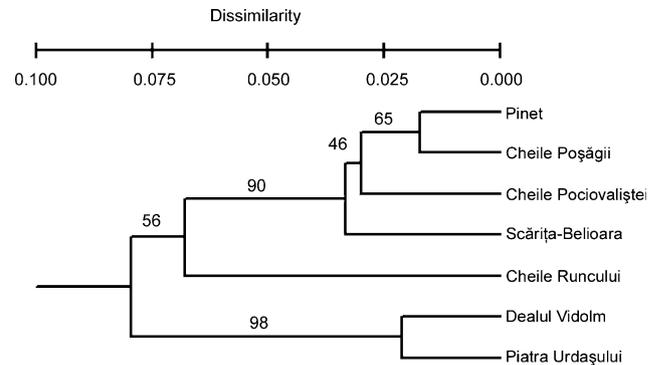
Expected heterozygosity and percentage polymorphic loci responded positively to potential habitat size:  $R^2 = 0.771$ ,  $P = 0.009$  (Fig. 4) and respectively  $R^2 = 0.777$ ,  $P = 0.009$  (results not shown).

#### Range-wide AFLP-analysis

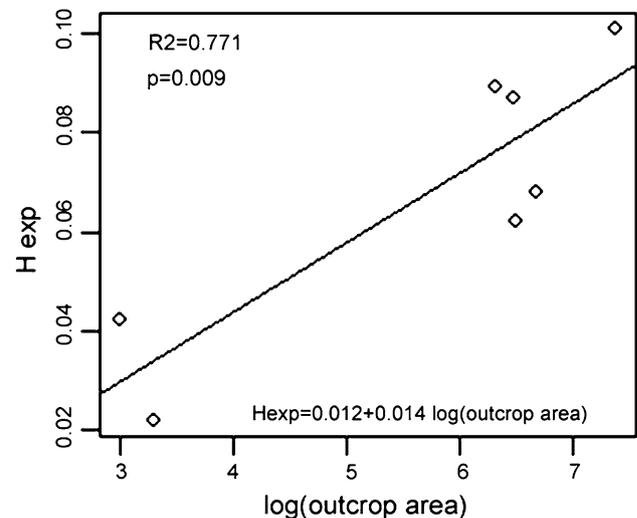
We scored 91 reproducible AFLP fragments of which 15 were monomorphic (including the outgroup *S. lutea*). Nei's unbiased heterozygosity varied between 0.059 in Rodopi and 0.010 in the Hautes-Pyrénées. The Romanian populations were among the genetically most impoverished ones ( $H_{exp} = 0.016$  and 0.014 for Gilău-Muntele Mare and Trascău Mountains, respectively; Table 3). The percentage of polymorphic loci varied strongly among populations, being highest in Rodopi and lowest in the Carpathians and Hautes-Pyrénées. The number of unique bands was highest in Gran Sasso and Rodopi (Table 3).

Divergence among populations was strong, with several significantly differentiated populations (Fisher's exact test, Table 4). Gilău-Muntele Mare showed significant genetic distance to Rodopi and Gran Sasso populations and no significant genetic distance to Pirin, Biokovo and Hautes-Pyrénées. Trascău had a similar pattern, but it was significantly differentiated also from Pirin (Fisher's exact test, Table 4). The southernmost Bulgarian population (Rodopi) was significantly different from all but Pirin population, whereas Pirin showed no differentiation from Scărița-Belioara and Hautes-Pyrénées.

Based on the genetic distance matrix of AFLP markers, the neighbour-joining tree (Fig. 5) placed the population



**Fig. 3** UPGMA analysis of seven populations of *S. bellidifolia* from the Apuseni Mts (Southeastern Carpathians). Bootstrap values (1,000 replicates) are shown above the branches



**Fig. 4** Response of Nei's expected heterozygosity (1978) as a function of outcrops area in seven populations of *S. bellidifolia* in the Apuseni Mts (Southeastern Carpathians)

from Gran Sasso in a separate cluster with high bootstrap support (100%). A well-supported cluster (bootstrap value 98%) contained populations originating from Bulgaria (Pirin and Rodopi). A large cluster comprised geographically distant populations from Romania, the Biokovo and the Hautes-Pyrénées also with high bootstrap support (99%).

**Table 3** Geographic and genetic attributes for seven populations of *S. bellidifolia* in Europe

Population	Locality	Country of origin	Geographic coordinates	$H_{\text{exp}}$	%PL	No. of unique bands
Hautes-Pyrénées	Gavarnie	France	42° 45' N, 0° 02' W	0.010	2.564	2
Biokovo	Makarska	Croatia	43° 18' N, 17° 02' E	0.019	3.846	2
Gran Sasso	Villa Santa Lucia	Italy	42° 20' N, 13° 47' E	0.033	6.410	12
Gilău-Muntele Mare	Belioara	Romania	46° 29' N, 23° 22' E	0.016	3.846	0
Trascău	Vidolm	Romania	46° 27' N, 23° 30' E	0.014	2.564	1
Pirin	Banderica	Bulgaria	41° 47' N, 23° 27' E	0.018	5.128	5
Rodopi	Trigrad	Bulgaria	41° 35' N, 24° 22' E	0.059	12.821	10

$H_{\text{exp}}$  denotes Nei's unbiased heterozygosity, %PL denotes percentage polymorphic loci

**Table 4** Matrix of combined probabilities for pairwise comparisons of the Fisher's exact test of population differentiation for seven European populations of *S. bellidifolia*

Population	Hautes-Pyrénées	Biokovo	Gran Sasso	Gilău-Muntele Mare	Trascău	Pirin	Rodopi
Hautes-Pyrénées	0						
Biokovo	1.000	0					
Gran Sasso	0.000	0.001	0				
Gilău-Muntele Mare	1.000	1.000	0.002	0			
Trascău	0.998	1.000	0.002	1.000	0		
Pirin	0.281	0.001	0.000	0.463	0.000	0	
Rodopi	0.000	0.000	0.000	0.000	0.000	0.953	0

## Discussion

### Genetic structure of Romanian populations of *Saponaria bellidifolia*

The level of genetic diversity encountered in *S. bellidifolia* populations within the Romanian Carpathians was low compared with populations of other perennial (mean  $H_{\text{exp}} = 0.242$ ), regionally distributed (mean  $H_{\text{exp}} = 0.222$ ) or outcrossing (mean  $H_{\text{exp}} = 0.260$ ) plant species (Nybom and Bartish 2000). In fact, our values ( $H_{\text{exp}} = 0.101$ – $0.022$ ) were closer to those reported for annual (mean  $H_{\text{exp}} = 0.125$ ), endemic (mean  $H_{\text{exp}} = 0.191$ ) or autogamous (mean  $H_{\text{exp}} = 0.091$ ) plant species (Nybom and Bartish 2000). The percentage of polymorphic loci (4.55–23.86%) was similar to that observed in rare perennials with fragmented populations (15–25%, Cotrim et al. 2003; 22.3%, Tero et al. 2005).

The geographical position of populations as well as outcrop size are important determinants of genetic diversity within the Apuseni Mts. As expected, the populations from Gilău-Muntele Mare Mts exhibit a higher level of genetic diversity compared to those from Trascău Mts that are reciprocally distant, have smaller habitat size and lower habitat quality (Table 1).

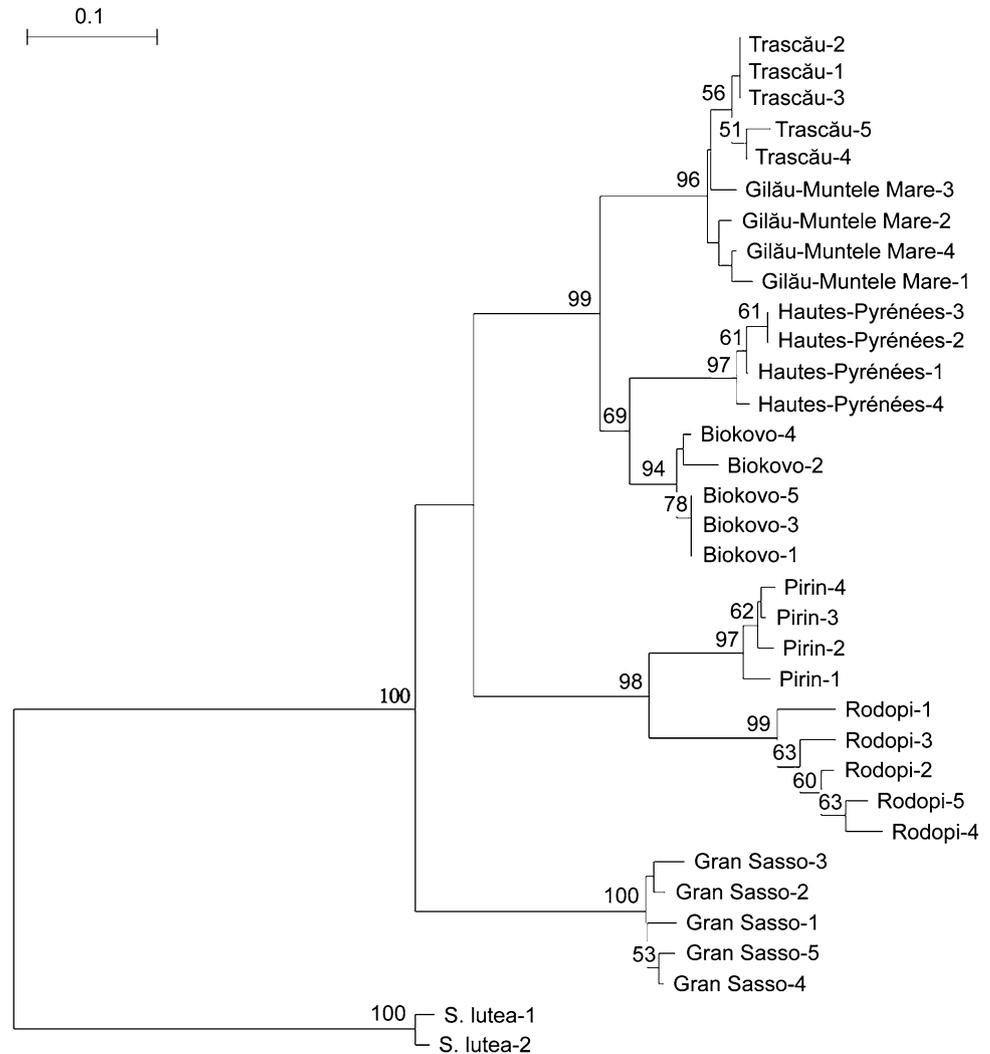
The population from Scărița-Belioara shows the highest genetic diversity ( $H_{\text{exp}} = 0.101$ , 23.86%) despite the low density of individuals. This outcrop is the largest and

geomorphologically the most diverse in the area, and probably induces a specific spatial structure of populations, which could influence genetic diversity (Travis and Ezard 2006). For instance, gaps between occupied patches might act as barriers to gene flow by restricting movement of pollinators and thus promote differentiation (Loveless and Hamrick 1984). On the contrary, high density of individuals registered in other populations (Cheile Poșăgii and Cheile Pociovaliștei) could increase the chance of interaction between neighbouring plants and thus foster inbreeding.

The UPGMA tree (Fig. 3) as well as the genetic diversity estimates (Table 1) confirms our hypothesis that distance between suitable habitat fragments enhances population isolation. The two mountain ranges separated by only 13 km form two distinct clusters that are significantly differentiated (Fisher's exact test,  $\chi^2 = 259.0296$ ,  $df = 176$ ,  $P < 0.0001$ ). One population (the geographically outlying Cheile Runcului) shows signs of isolation, being the only population with a unique marker and sharing other two markers with only one or two populations from the study area.

In Gilău-Muntele Mare Mts, four polymorphic markers are shared only by a limited number of populations, but the majority of markers are common to all populations. This fact suggests either enhanced gene flow between populations, or recent colonisation (implying that little time was available for significant genetic divergence within the

**Fig. 5** Neighbour-joining analysis of AFLP phenotypes observed in 32 plants of *S. bellidifolia* from seven populations in Europe. *Saponaria lutea* L. was used as outgroup. Bootstrap values (2,000 replicates) are shown above the branches



area). AMOVA analysis revealed that only half of the genetic variation is retained within populations while the remaining half was due to between-population variability and by the divergence between the two mountain ranges (24.07 and 26.73% respectively). This strikingly high value can be attributed to the absence of otherwise common markers from the Trascău Mts.

Altogether, despite the low genetic variability of the species in this island-like habitat complex, due to local fragmentation events and habitat specificity, the populations hold a specific genetic diversity pattern provided mainly by the high amounts of among-population divergence.

#### Relation to European populations

The AFLP results (Fig. 5) support the predictions of the centre-periphery model, in agreement with the findings of other empirical studies (Lammi et al. 1999; Lönn and Prentice 2002; Eckstein et al. 2006). The peripheral

populations of *S. bellidifolia* in the Southeastern Carpathians, as well as those in the Hautes-Pyrénées and Biokovo Mts have lower diversity than the southernmost ones from Bulgaria and Italy. According to our data, their genetic impoverishment might be attributed to a genetic bottleneck occurring during range expansion of the species. Higher levels of genetic diversity and the higher number of unique markers of the Bulgarian and Italian populations suggest that *S. bellidifolia* survived the cold stages of the Pleistocene in southern European refugia but that only the Balkan populations served as source for postglacial range expansion, a pattern often seen in phylogeographical studies of both plants and animals (Taberlet et al. 1998; Hewitt 1999). Low genetic diversity and lack of significant differentiation among Romanian, Croatian and one Bulgarian population (Pirin) suggest a recent range expansion of *S. bellidifolia*, probably through long-distance dispersal. This spreading strategy has often been documented in plants (e.g. Cain et al. 2000; Schönswetter et al. 2002) and could be also the case of *S. bellidifolia*. By contrast, for

*Anthyllis montana*, another submediterranean mountain plant, Kropf et al. (2002) suggested only small-scale migration after glaciations that promoted genetic divergence and differentiation of infraspecific lineages.

We hypothesise that the expansion of *S. bellidifolia* towards the Apuseni Mts might have been favoured by a chain of suitable rocky habitats that link the Northwestern Balkan Mountains and the Carpathians. Their role in the biogeography of submediterranean species was recognised earlier, based on floristic evidence (Boşcaiu 1971; Boşcaiu and Marossy 1980–1981). Similarly, a geographic and floristic analysis of the Dinaric Mountains placed these mountains in an intermediate position between the Balkans and the Carpathians (Lakušić 1970) and might provide support for the revealed genetic similarity of Romanian and Croatian *S. bellidifolia* populations.

Altogether, we consider that the genetic pattern revealed in the Carpathian populations of *S. bellidifolia* shows local imprints, which resulted from its phylogeographic history as well as from its ecological specificity. Outcrops size and the natural habitat fragmentation are important determinants of genetic diversity within these northern isolated populations and therefore habitat preservation seems the most appropriate conservation strategy of this species within the Southeastern Carpathians.

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