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Genetic variability of the narrow endemic *Rhamnus persicifolia* Moris (Rhamnaceae) and its implications for conservation

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ABSTRACT

Rhamnus persicifolia Moris is an endemic small tree belonging to the *Rhamnus cathartica* group, growing along mountainous streams of Central-Eastern Sardinia (Italy). ISSR markers were used to detect the genetic diversity within and among six populations representative of the species distribution range. In spite of the limited distribution of this endemic taxon, fairly high levels of genetic diversity were detected. Percentage of polymorphic bands (PPB), gene diversity (H_S and H_T) and Shannon information measure (Sh) were calculated both at population (PPB = 30.70%, $H_S = 0.1105$, $Sh = 0.1646$) and at species level (PPB = 68.42%, $H_T = 0.2066$, $Sh = 0.3139$).

The existence of a spatial distribution of genetic diversity in *R. persicifolia* was revealed by a low gene flow, a fairly high level of genetic differentiation ($G_{ST} = 0.4583$) among populations and a positive correlation between genetic and geographic distances (Mantel test, $r = 0.71$, $p = 0.016$). The spatial genetic structure was also confirmed with BAPS analysis. Our results show that a certain level of isolation by distance and sex-ratio bias may explain the distribution of genetic diversity among populations.

Conservation measures are suggested on the basis of the genetic diversity detected, by implementing an integrated *in situ* and *ex situ* conservation program for each population, in order to ensure effective protection for this endemic species.

1. Introduction

Extinction is the consequence of a mutually accelerating demographic and genetic decline (Washitani et al., 2005). Therefore, an understanding of the genetic diversity within and among populations of rare plant species is a fundamental prerequisite for developing conservation programs (e.g. Mahalovich and McArthur, 2004; Doede, 2005). In particular, data on the genetic structure of the extant populations should be gathered over the entire range of the species distribution (Honjo et al., 2004). On the other hand, endemic plant species may exhibit equivalent or higher levels of diversity when compared to their more widely distributed congeners (Gitzendanner and Soltis, 2000; Ellis et al., 2006).

Rhamnus persicifolia Moris is a small tree or shrub closely related to *Rhamnus cathartica* L., from which it differs by its elliptic-lanceolate leaves and reddish ripe drupes. It is endemic to Central-Eastern Sardinia (Italy), and occurs at about 500–1500 m a.s.l. on both limestone and siliceous substrata. This species grows in scattered groups or as single trees, in riparian

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woods or hygrophilous scrub along mountainous streams (Tutin, 1968; Arrigoni, 1977; Snogerup, 1985) and deep gorges (Fenu et al., 2010).

Like many other species in this genus, *R. persicifolia* is a dioecious plant probably pollinated by insects although wind pollination can also take place, as detected for *Rhamnus ludovici-salvatoris* by Traveset et al. (2003) and Gulías et al. (2004). The flowering period occurs from May to June, while fruits develop from August to October. Drupes contain 3(4) pyrenes each, the fourth being generally abortive. Mattana et al. (2009) detected high seed viability, suggesting that this species has no reproductive problems, unlike the Balearic endemic *R. ludovici-salvatoris* (Traveset et al., 2003). However, no exhaustive data are available yet on its reproductive biology, chorology and ecology.

R. persicifolia is included in the Italian Red Book as vulnerable (Conti et al., 1992, 1997), considering its estimated narrow distribution area (ca. 20 × 10 km; Snogerup, 1985) and population decline induced by human activities, which generated a continued degradation of the riparian forest vegetation of Sardinia, and by climate change (Arrigoni, 1977). To date, the number of populations is six while in other locations single plants have been found. Moreover, half of the populations are threatened owing to low plant numbers or unbalanced sex bias.

In recent years, a number of molecular techniques have been used widely to detect genetic diversity or phylogenetic relationships in endemic species. In particular, Inter-Simple Sequence Repeat (ISSR) markers can be highly variable within a species and have the advantage over Random Amplified Polymorphic DNA (RAPD) of employing longer primers that allow more stringent annealing temperatures, increasing the reproducibility of tests (Jones et al., 1997).

In spite of the large number of species belonging to the *Rhamnus* L. genus, very few molecular studies have been undertaken in the recent past on species belonging to this genus. At genus level the clear separation from *Frangula* Mill. has been defined (Bolmgren and Oxelman, 2004), but investigations on diversity levels within individual species have been performed only on *Rhamnus alaternus* L. and *R. ludovici-salvatoris* Chodat (Ferriol et al., 2009) and on *Rhamnus glaucophylla* Sommier (Bedini et al., 2011).

In the present study the ISSR technique was used to examine natural populations of *R. persicifolia* for the following purposes: (1) to estimate how much genetic diversity is maintained in the species, (2) to describe how genetic diversity is distributed within and among populations, and (3) to provide suggestions and possible guidelines for effective conservation programs.

2. Material and methods

2.1. Sampling strategy and DNA extraction

All six known populations of *R. persicifolia* were identified on the basis of literature data (Arrigoni, 1977; Fenu et al., 2010) together with our field observations (Fig. 1; Table 1). In this study, by population we mean a plant group made up by more than 10 individuals, while single or few scattered plants were not included in the sampling. Three populations are located in the Supramontes region [Palumbrosa (PB), Rio Olai (OL), Pischina Urtaddala (UR)] and the other three in the Gennargentu area [Ponte Crobine (CR), Rio Correboi (CO) and S'Olziada (SO)].

For each population, the number of male and female individuals was counted during the fruiting periods (late September 2008) and the area they occupy was evaluated using GPS (Suunto X9i) (Table 1).

In summer 2009, a variable number of plants ($7 < N < 9$) according to the availability and accessibility of individuals were randomly sampled ($n = 48$), without taking into account sex. 2–4 leaves per plant were dried *in situ* in silica gel, washed in the laboratory and total genomic DNA was extracted by using DNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions.

2.2. ISSR amplification

The ISSR-PCR mixture (25 μl) contained 50 ng of total plant DNA, 0.5 mM primer, 0.2 mM of each dNTP, 0.5U Dynazyme II (Finnzymes, Finland) and Dynazyme buffer. Amplification was carried out in a Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany). The following thermal profile was used: initial denaturation at 94 °C for 3 min, followed by 35 cycles: denaturation at 94 °C for 45 s, primer annealing for 30 s, and elongation at 72 °C for 130 s. Amplification products were resolved electrophoretically on 2.0% agarose gels run at 100 V in TAE buffer, visualized by staining with ethidium bromide, and photographed under ultraviolet light. A set of fifteen primers complementary to simple sequence repeats were tested. Table 2 summarizes the primer sequences.

2.3. Genetic diversity analysis

Since ISSR markers are dominant, and regarding the species as a diploid, we assumed that each band represented the phenotype at a single biallelic locus. ISSR electrophoretic profiles were evaluated by visual inspection and amplified fragments were scored for presence (1) or absence (0) of comigrating bands.

The percentage of polymorphic bands (PPB) was measured at population and species levels. Likewise, Gene Diversity (*GD*) was determined according to Nei (1987) as $GD = [n/(n\text{-}1)](1 - \Sigma p_i^2)$, where p_i is the frequency of a given ISSR fragment, and was calculated at two levels: average intra-population diversity (H_S) and total genetic diversity for the species (H_T). Both the

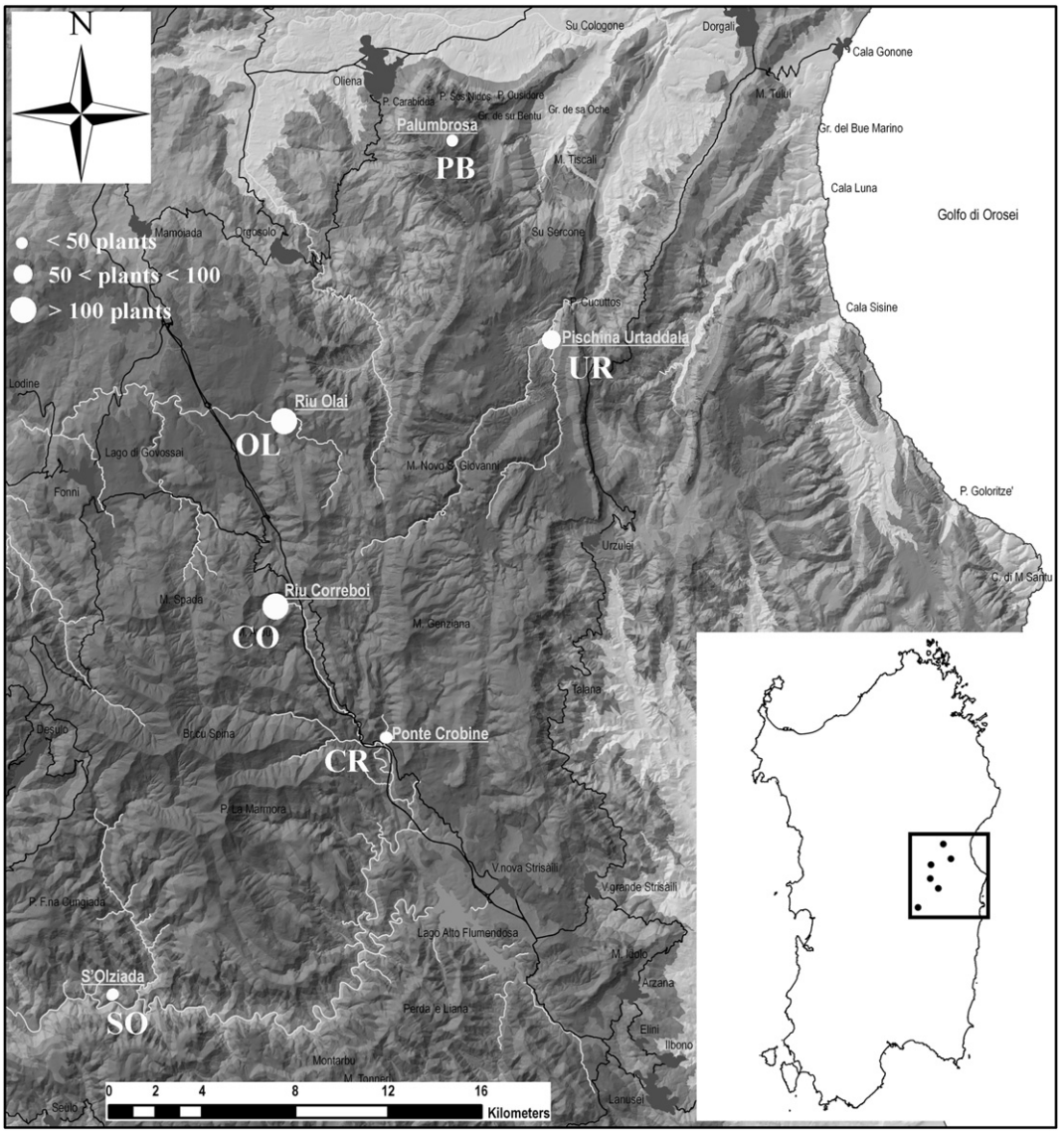

Fig. 1. Geographic locations of the six sampled *R. persicifolia* populations. The following population codes were used: Palumbrosa (PB), Rio Correboi (CO), Rio Olai (OL), S'Olziada (SO), Pischina Urtaddala (UR), Ponte Crobine (CR).

coefficient of genetic differentiation $G_{ST} = (H_T - H_S)/H_T$ (Nei, 1973) and the level of gene flow $N_m = (1 - G_{ST})/4G_{ST}$ (Nei, 1977; Slatkin and Barton, 1989) were estimated. Shannon information measure (Lewontin, 1972) $Sh = 1 - \Sigma p_i \log_2 p_i$, was computed in order to quantify the genetic diversity within populations (H_S), and within the species as a whole (H_T). The resulting binary data matrix of ISSR phenotypes was analyzed using POPGENE version 1.32 (Yeh et al., 1997) to estimate genetic diversity parameters assuming Hardy–Weinberg equilibrium. The matrix is available from the corresponding author.

Table 1
Ecological information about the six sampled populations of *Rhamnus persicifolia*. Sample size (N), latitude (Lat N), longitude (Long E), elevation range in m asl (Elev), substrate (Sub. - Lm = limestone and Si = siliceous), exposure (Exp.), estimated population extention in m^2 (Ext), and sex bias (prevailing sex is showed ♂ = male or ♀ = female near the value) are reported.

Population (code)	N	Lat N	Long E	Elev	Sub.	Exp.	Ext	Sex bias
Palumbrosa (PB)	8	40°14′ 52.29	9°26′ 21.83	1300–1350	Lm	NE	5000	0.82 ♂
Pischina Urtaddala (UR)	8	40°10′ 15.29	9°29′ 26.86	700–750	Lm	NNE	12000	0.80 ♀
Rio Olai (OL)	8	40°08′ 29.56	9°21′ 22.46	830–1230	Si	NW	50000	0.78 ♂
Rio Correboi (CO)	9	40°04′ 06.71	9°21′ 02.50	1045–1369	Si	N	50000	0.36 ♂
Ponte Crobine (CR)	8	40°00′ 24.91	9°24′ 42.09	875–930	Si	W	10000	0.26 ♂
S'Olziada (SO)	7	39°55′ 08.05	9°16′ 43.17	530–565	Si	ESE	5000	1 ♂

Table 2
Sequence of ISSR primer, optimal annealing temperature, size range of the amplified products, total number of bands and number of polymorphic bands.

Sequence	Annealing temperature (°C)	Size range (bp)	N° of bands	N° of polymorphic bands
$(GA)_8C$	47.5	600–1,600	6	6
$(GA)_8YC$	47.5	500–700	2	2
$(CAG)_5$	47.5	700–1,700	7	6
$(CAA)_5$	47.5	500–1,000	3	0
$(GA)_9T$	47.5	800–1,700	6	3
$(CTA)_6$	47.5	400–1,000	3	2
$(TC)_8C$	47.5	500–1,600	6	6
$(AC)_8G$	47.5	500–1,500	5	3
$(GA)_8RGY$	47.5	100–400	2	0
$(CT)_8RG$	47.5	400–1,500	9	9
$(ATG)_6$	47.5	400–1,700	8	6
$(GACA)_4$	46.5	700–1,500	2	1
$(GA)_8YG$	46.5	300–1,600	6	3
$(CT)_8A$	46.5	400–1,700	7	4
$(CA)_8YC$	46.5	700–1,600	4	0
15	–	–	76	51

For each population, the relationship between genetic diversity and both sex bias (considering all individuals) and population size was calculated with Statistica 8.0 (StatSoft, 2007) using Kendall's Tau correlation coefficient. The sex bias of each population (i.e. the deviation from sex ratio 1:1) was calculated as the absolute value of the difference in the number of individuals of both sexes divided by the total number of plants in the population. The sex bias values obtained vary from 0 (equal frequency of both sexes and high genetic diversity expected) to 1 (only one sex present in the population and low genetic diversity expected). We applied the formula (1 - sex bias) in order to score high sex bias values where high genetic diversity was expected and the contrary for low expected genetic diversity (Table 1).

A UPGMA dendrogram was performed on Nei's genetic distance using POPGENE version 1.32 to visualize the genetic relationships among all populations. A Mantel test with 100,000 random permutations was conducted with Arlequin 3.11 (Excoffier et al., 2005) to determine whether the matrix of genetic distances between populations was correlated with the matrix of their geographic distances (Mantel, 1967).

We used BAPS 5.3 (Corander et al., 2009) to detect clusters of genetically similar populations and to test the gene flow among them. The method determines clusters of population samples minimizing Hardy–Weinberg and linkage disequilibria within the clusters and treating the number of clusters as an unknown parameter.

In this work we adopted the spatial option, which uses population locations when estimating the number of clusters (K) and considers genetically distinct clusters of populations as spatially separated. This spatial organization was showed using a Voronoi tessellation. We ran 10 replicates for values of $K_{max} = 10$ (K_{max} is the maximum number of clusters). The admixture analysis was performed ignoring clusters with fewer than five individuals, simulating 200 individuals in 20 iterations, to determine the number of population clusters. The strength of gene flow was estimated via a stochastic characterization of the rates of admixture between the K identified populations. The rate of exchange events was represented by the proportion of individuals in a population showing significant admixture from a particular source (Tang et al., 2009). The p value in the gene flow graph was set to 0.05.

The hierarchical ISSR frequency distribution was described using molecular variance analysis (AMOVA). Components of variance partitioned among BAPS clusters, among populations within clusters and within populations were estimated from a Euclidean distance matrix using Arlequin 3.11 (Excoffier et al., 2005) with 1000 random permutations.

3. Results

For the 48 R *persicifolia* samples the 15 selected primers generated a total of 76 bands, corresponding to an average of 5.07 bands per primer (Table 2). The size range of PCR fragments was 100–1700 bps. The number of bands and the percentage of polymorphic fragments produced by each primer varied. Primer $(CT)_8RG$ produced the highest number of bands (9), all of which were polymorphic. Among the 76 bands, 51 (67.11%) were polymorphic at the species level (Tables 2, 3). The PPB for a single population ranged from 13.16 to 50.00%, with an average of 30.70% within populations (Table 3).

GD of populations varied between 0.0546 and 0.1792, with mean values of 0.1105 and 0.2066 at population (H_S) and species level (H_T), respectively (Table 3). The extreme values of *Sh* in populations were 0.0790 and 0.2687, while the mean estimates were 0.1646 at population level and 0.3139 at species level (Table 3). Among the six populations, OL exhibited the greatest level of variability, whereas SO exhibited the lowest one. G_{ST} was 0.4583 as estimated by partitioning total gene diversity. N_m was estimated to be 0.5910 individuals per generation among populations (Table 3).

Kendall's Tau correlation coefficient revealed a relationship between genetic diversity and sex bias ($r = 0.73$; $P < 0.05$), but no relationship was found between genetic diversity and population size ($r = 0.60$; P not significant).

In the UPGMA dendrogram the SO population turned out to be the most differentiated (Fig. 2). The other five populations were split in two main branches: the first including UR and CR and the second encompassing CO, PB and OL divided into further sub-branches.

Table 3
Genetic diversity in *R. persicifolia* determined by ISSR markers at population and at species level (* = H_S, ** = H_T). PPB: percentage of polymorphic bands; *GD*: Nei gene diversity; *Sh*: Shannon information index; H_T: genetic diversity within the whole species; H_S: genetic diversity within populations; G_{ST}: coefficient of genetic differentiation among populations; N_m: gene flow.

Population	PPB (%)	*GD* ± SD	*Sh* ± SD	G_{ST}	N_m
PB	25	0.0791 ± 0.047	0.1214 ± 0.071		
UR	26.32	0.1057 ± 0.059	0.1539 ± 0.084		
OL	50	0.1792 ± 0.061	0.2687 ± 0.089		
CO	35.53	0.1167 ± 0.057	0.1761 ± 0.082		
CR	34.21	0.1279 ± 0.061	0.1885 ± 0.088		
SO	13.16	0.0546 ± 0.035	0.079 ± 0.065		
Average	30.70	0.1105*	0.1646		
Species level	68.42	0.2066 ± 0.026**	0.3139 ± 0.037	0.4583	0.5910

BAPS analysis identified four genetic clusters (Fig. 3a). The Voronoi tessellation highlighted the spatial organization of genetic clusters, with clusters containing multiple populations usually comprising geographically proximate populations. In particular, the first (1) consisted of UR and CR; the second (2) contained PB and CO; the other two populations (OL, SO) were completely separated in autonomous groups. The potential gene flow graph among genetic clusters (Fig. 3b) evidenced the presence of a weak gene flow only between clusters 1 – 3 (6.00%) and clusters 2 – 3 (2.40%). AMOVA further confirmed that the variation component increased from BAPS clusters to population levels (Table 4). The Mantel test revealed no highly significant correlation between matrices of genetic and geographic distances ($r = 0.64$; $P < 0.028$).

4. Discussion

4.1. Genetic diversity at species level

In contrast to the expectation that endemic plant species are genetically depauperate (Karron, 1991; Ellstrand and Elam, 1993), it has been demonstrated that many endemic species maintain high levels of genetic variability compared to their widespread congeners (Gitzendanner and Soltis, 2000). In spite of the extremely small geographic range of *R. persicifolia*, its genetic diversity level (*GD* 0.0546–0.1792) was as high as its congeneric *R. alaternus* (*GD* 0.082–0.165; Ferriol et al., 2009), widespread in the Mediterranean region. The genetic diversity of *R. persicifolia* resulted also considerably higher than other narrow endemics species such as *R. ludovici-salvatoris* (*GD* 0.033–0.054; Ferriol et al., 2009) and *R. glaucophylla* Sommier (*GD* 0.0664–0.1003; Bedini et al., 2011). However, *R. persicifolia* possesses a level of genetic diversity comparable to that recorded in other cross-pollinating plant species endemic to the Mediterranean basin [*Armeria maderensis* (Mill.) Willd. (*GD* 0.060–0.070; Piñeiro et al., 2009); *Lamyropsis microcephala* (Moris) Dittrich & Greuter (*GD* 0.056–0.098; Bacchetta et al., submitted); *Medicago citrina* (Font Quer) Greuter (*GD* 0.035–0.143; Juan et al., 2004); *Moehringia lebrunii* Merxm. (*GD* 0.112–0.159; Minuto

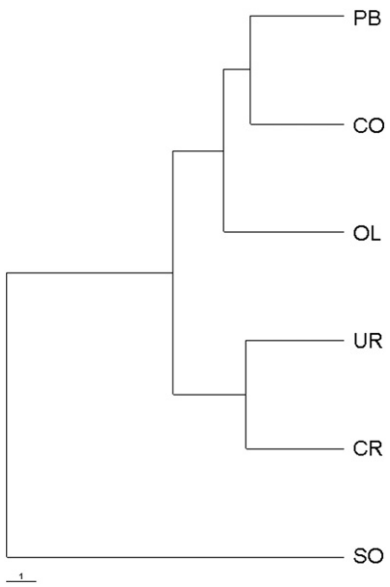

Fig. 2. Dendrogram based on the Nei's genetic distance of the six *R. persicifolia* populations sampled. Population codes are shown in Table 1.

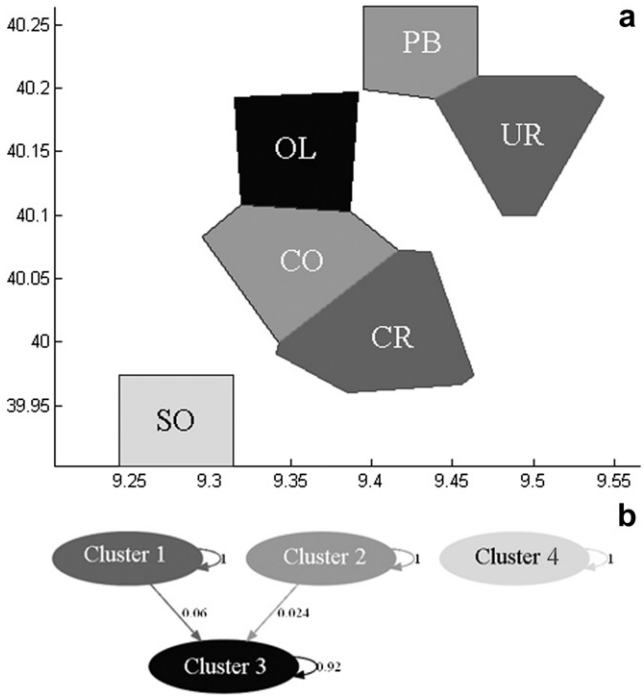

Fig. 3. Results of BAPS analyses for *R. persicifolia*. a) Voronoi tessellation showing spatial organization of populations in four clusters delineated by BAPS. Population codes are shown in Table 1. Latitude and longitude are reported respectively in *x* and *y* axes. The four clusters are marked with different colours. b) Tentative gene flow graph among the four clusters. The genetic exchange levels are reported. The numbers associated to the arrows show the rate of exchange events expressed as the proportion of individuals in a population showing significant admixture from a particular source. The p value was set to 0.05. Colours correspond to the clusters shown in (a).

et al., 2006); *Moehringia sedoides* (Pers.) Loisel. (*GD* 0.072–0.290; Minuto et al., 2006); *Primula apennina* Widmer (*GD* 0.176–0.231; Crema et al., 2009)] but higher than other autogamous widespread plants like *Pancratium maritimum* L. (*GD* 0.001–0.008; Grassi et al., 2005).

R. persicifolia is a dioecious species and its fairly high level of genetic diversity might be explained by its obligate outcrossing, assuring a minimum genetic flow even in populations with a low number of individuals (i.e. CR and PB) attenuating the genetic drift effect.

4.2. Genetic differentiation among and within populations

GD was higher in populations living around the core of the distribution range, located on the eastern slopes of the Gennargentu massif (particularly OL with gene flow from other clusters). In fact, the lowest gene diversity values were recorded in PB and SO (Table 1). These populations suffer both a genetic drift due to the small number of individuals and to a male-biased sex ratio (0.82 and 1 for PB and SO, respectively). This observation is consistent with the result of Kendall's Tau correlation coefficient that sex ratio is the main determinant of genetic diversity within populations. The result is in line with the findings of many authors who assert that the breeding system has a highly significant impact on genetic diversity and its distribution (Ranker, 1994; Hamrick and Godt, 1996; Maguire and Sedgley, 1997). Habitat conditions may play a role in influencing sex bias as observed in other dioecious plants (Bierzychudek and Eckhart, 1988). Similarly, in other dioecious plant species, males are associated with more xeric habitats and females with more mesic ones (Fox and Tyrone Harrison, 1981). The same pattern may apply to this species and in particular to populations growing in extreme xeric conditions (PB and SO in Table 1).

Spatial analysis among populations (Mantel test) showed a moderate correlation between genetic distance and geographic distance: the level of isolation by distance partially explains the distribution of genetic diversity among populations, even if

Table 4
Analysis of molecular variance among four BAPS clusters, among populations within clusters and within six populations of *R. persicifolia* based on ISSR data (*P* value <0.000001).

Source of variation	*df*	SS	Variance components	% variation
Among BAPS clusters	2	21.107	0.35799	19.26
Among pops within clusters	3	17.269	0.58993	31.73
Within pops	42	38.270	0.91119	49.01
Total	47	76.646	1.75757	100

two pairs of the nearest populations (PB and UR, CO and CR) were separated into different clusters according to the UPGMA dendrogram.

N_m value (0.5910) indicated a low rate of gene flow among six populations of *R. persicifolia*. In accordance to this fact a consistent amount of inter-population differentiation ($G_{ST} = 0.4583$) was recorded. In particular, AMOVA analysis detected an increasing value from the cluster to the intra-population point of view. However, the moderate value of variation detected within populations confirms a low level of genetic exchange.

The UPGMA dendrogram based on Nei's genetic distances revealed three main branches (one of which split in two sub-branches) showing a substantial congruence with the Bayesian clustering which resolved four differentiated groups. The first cluster formed by CO and PB was closely linked to the second (OL), with whom a connection is geographically possible and gene flow was observed. However, OL also showed a low gene flow with the third cluster formed by CR and UR. The low genetic distance detected for these two populations, in contradiction with their long geographic distance, might be explained by their recent separation. Among riparian woods in intermediate basins such as Baccu Pedrigoni, Riu Is Eras, Riu Pauli, Riu Crobu, Codula Sa Mela, Rio Flumineddu, the species is present as few single trees that may have acted as a link between CR and UR. The fourth cluster was characterized by the SO population which is both genetically and geographically the most isolated one and has no contact with the others.

The overall genetic structure and the low gene flow characterizing the study populations are not easy to interpret. Simple seed dispersal mediated by birds may be assumed as observed in other Mediterranean species of this genus such as *R. ludovici-salvatoris* (Traveset et al., 2003) and *R. alaternus* (Gulías et al., 2004). However, in the two years of our study no bird presence around the plants during the fruiting period was recorded. Nevertheless, as in closely related *R. cathartica* (Knight et al., 2007), stochastic fruit dispersal by small frugivorous mammals might take place. Finally, as *R. persicifolia* grows in riparian woods or hygrophilous scrub along mountainous rivers/streams, we may also speculate fruit flotation from different populations. However, the majority of populations (i.e. CO and PB, or UR and CR) were located in different hydrographic basins and the other three (CO, CR, and SO) live in the same hydrographic basin (Flumendosa river) but along different tributaries of the river. Thus, fruit flotation from different populations is very unlikely to occur and, as a consequence, the geographical separation may lead to low gene flow and high genetic differentiation among populations. A further variable in gene flow evaluation might be pollen dispersal. However, no data are available on the pollination syndrome of *R. persicifolia.*

4.3. Implications for conservation

Our analysis based on molecular markers is the first attempt to uncover the levels of genetic variability and differentiation in the natural populations of *R. persicifolia*. The high genetic variability detected at population level suggests an integrated *in situ* and *ex situ* conservation program for each investigated population, in order to ensure effective protection for this vulnerable species. In particular, in the light of its seed biology (Mattana et al., 2009), *ex situ* seed conservation and the cultivation of plants in nurseries, already started at the Sardinian Germplasm Bank (BG-SAR), should be implemented for each population (except for the SO population where no female individuals were found) in order to conserve the highest genetic variability and obtain plant material from the original populations for future reinforcement activities. The high level of genetic variability indicates the lack of genetic bottlenecks in this species, as detected by Crema et al. (2009) in *P. apennina* Widmer, endemic to the mountain tops of the Apennines (Italy), suggesting that crosses within population do not lead to inbreeding depression.

In *R. persicifolia* a possible conservation strategy could be to avoid transfer of reproductive material among populations to balance sex ratio and to maintain local genetic adaptation, as already highlighted for *R. ludovici-salvatoris* (Ferriol et al., 2009). However, considering the small population sizes and male-biased sex ratios of the SO and PB populations, additional measures should be taken for these two very threatened populations. *In situ* measures such as habitat protection and management should be applied in order to preserve the few remaining individuals of the PB and SO populations. These male-biased populations should also be reinforced by introducing female individuals obtained from other populations, considering both their genetic similarity and their habitat affinities, particularly in terms of substrate (i.e. OL for PB and CO for SO). In addition, as a precautionary measure, the cloning by cuttings and their *ex situ* conservation should also be carried out for the few individuals present.

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