Spatial genetic structure of Aquilegia taxa endemic to the island of Sardinia

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† Background and Aims The Mediterranean Basin is one of the most important regions for the Earth’s plant biodiversity; however, the scarcity of studies on fine scale patterns of genetic variation in this region is striking. Here, an assessment is made of the spatial genetic structure of all known locations of the three Sardinian endemic species of Aquilegia in order to determine the relative roles of gene flow and genetic drift as underlying evolutionary forces canalizing the divergence of Sardinian Aquilegia taxa, and to see if the spatial genetic structure found fits the current taxonomic differentiation of these taxa.

† Methods DNA from 89 individuals from all known locations of Aquilegia across Sardinia was analysed by means of amplified fragment length polymorphism (AFLP) markers. Both principal co-ordinates analysis (PCoA) and Bayesian clustering analyses were used to determine the spatial genetic structure irrespective of any taxonomic affiliation. Historical effects of gene flow and genetic drift were assessed by checking for the existence of isolation-by-distance patterns.

† Key Results STRUCTURE and PCoA analyses revealed a pattern of genetic variation geographically structured into four spatial genetic groups. No migration–drift equilibrium was detected for Aquilegia in Sardinia, when analysed either as a whole or in individual groups. The scenario approached a Case III pattern sensu Hutchinson and Templeton, which is associated with extreme isolation conditions where genetic drift has historically played a dominant role over gene flow.

† Conclusions The pattern of genetic variation of Sardinian taxa of Aquilegia indicates that genetic drift has been historically more influential than gene flow on population structure of Sardinian species of Aquilegia. Limited seed dispersal and divergent selection imposed by habitat conditions have been probably the main causes reinforcing post-Pleistocene geographical isolation of Aquilegia populations. The spatial genetic structure found here is not fully compatible with current taxonomic affiliations of Sardinian Aquilegia taxa. This is probably a consequence of the uncoupling between morphological and genetic patterns of differentiation frequently found in recently radiated taxa.

Key words: Mediterranean Basin, Sardinia Island, endemic species, Aquilegia barbaricina, Aquilegia nugorensis, Aquilegia nuragica, biodiversity hotspot, AFLP markers, spatial genetic structure, genetic drift, divergent selection, habitat specialization.

INTRODUCTION

The Mediterranean Basin is one of the most important regions for the Earth’s plant biodiversity (Médail and Myers, 2004; Thompson, 2005). The Tyrrhenian Islands (Sardinia and Corsica), together with the rest of the western Mediterranean Islands (Sicily and Balearic Islands), constitute one of the most important biodiversity hotspots within this region (Blondel and Médail, 2009). Large Mediterranean islands, due to high long-term climate stability, combine a high richness of plant lineages and species with the persistence of many endemic taxa (Médail and Diadema, 2009). The outstanding levels of biodiversity and endemism turn this area into an optimal scenario for the study of the effects of spatial isolation on taxa diversification through the interplay between evolutionary forces and geographic isolation. As continental islands (sensu Whittaker, 1998) they are also considered as the best natural laboratories in which to study the effects of geographical isolation on allopatric speciation via selection and/or genetic drift (Bittkau and Comes, 2005).

Sardinia is, after Sicily, the largest island in the Mediterranean Sea. The Central, Eastern and Northern mountain ranges of the island have been identified as one of the 52 putative glacial refugia within the Mediterranean region (Médail and Diadema, 2009). Its flora consists of 2295 species (Conti et al., 2005) and 347 of these are endemics (e.g. Sardinian, Corso-Sardinian, Corso-Sardinian-Balearic endemics), with 45.8% being exclusive Sardinian endemics (Bacchetta et al., 2005). Among the later, three species of the genus Aquilegia are distributed across these mountain ranges: A. barbaricina, A. nugorensis and A. nuragica (Arrigoni and Nardi, 1977, 1978). The genus Aquilegia has become a model for the study of adaptive radiations from the knowledge acquired from American lineages (Hodges and Arnold, 1994; Hodges et al., 2004; Whittall and Hodges, 2007); however, the Euroasiatic counterpart of this model genus has been studied only recently. In a recent study by Bastida et al. (2010), phylogenetic analyses of Euroasiatic taxa revealed that the most recently radiated species are endemic to South European mountain ranges (dated approx. 1 million years ago). Among these species are the Sardinian
Despite the widely recognized ecological and evolutionary relevance, the scarcity of studies on fine scale patterns of genetic variation of species inhabiting Mediterranean islands is striking (but see, Affre et al., 1997; Quilichini et al., 2004; Molins et al., 2009). Following theoretical expectations, endemic species including island endemics (Frankham, 1997) should exhibit lower levels of genetic diversity than widespread species (Hamrick and Godt, 1989; Gitzendanner and Soltis, 2000). Island endemic species worldwide usually exhibit spatially structured patterns of genetic variation [e.g. Nigella arvensis in the Aegean region (Bittkau and Comes, 2005) or Aster asa-grayi in Japan (Maki, 1999)]. High spatial structuring is assigned to geographical isolation coupled to the limited dispersal abilities of the species, circumstances that restrict gene flow among populations and result in subsequent population differentiation by drift and selection (see Edh et al., 2007). Evaluating the relative influences of gene flow and genetic drift on the distribution of genetic variation at different spatial scales provides invaluable insights into the evolutionary interpretation of how patterns of genetic variation have been historically established (Hutchison and Templeton, 1999).

The study of recently radiated taxa, such as the genus Aquilegia (Ro and McPheron, 1997; Bastida et al., 2010), often entails taxonomic and phylogenetic discrepancies, since it usually leads to the uncoupling of morphological and genetic patterns of differentiation (Wang et al., 2005; Whitfield and Lockhart, 2007; Parks et al., 2009; Rymer et al., 2010). Specifically, the rapid speciation which occurred in Aquilegia has led, in both the North American (Hodges and Arnold, 1994; Ro and McPheron, 1997; Whittall et al., 2006; Whittall and Hodges, 2007) and the Euroasiatic taxa (Bastida et al., 2010), to a high number of species with marked morphological differences but low genetic divergence. This circumstance makes the usual approaches of studying intra- and interspecific genetic variation among populations based on a priori defined species and populations difficult. Our approach to evaluate the spatial genetic structure does not consider any taxonomic affiliation.

In this study, we assess the spatial genetic structure of all known Sardinian populations of the genus Aquilegia to determine the relative roles of genetic drift and gene flow as underlying evolutionary forces that canalize the divergence of Sardinian Aquilegia taxa. Specifically, we asked the following questions. (1) How is the spatial pattern of genetic variation of Sardinian Aquilegia populations spatially structured irrespective of their taxonomic affiliation? (2) What is the main evolutionary force underlying this spatial genetic structure? (iii) Does the current taxonomic differentiation of Sardinian Aquilegia taxa fit to the spatial genetic structure found?

MATERIALS AND METHODS

Study species, sites and plant material

Aquilegia species (Ranunculaceae) are perennial rhizomatous herbs widely distributed across Asia, Europe and North America (23, 21 and 22 taxa, respectively). Mature plants consist of an erect, usually pubescent stem growing from a basal rosette with bi- or tritermate compound leaves. Hermaphrodite, pendant flowers are radially symmetrical and exhibit five sepals and five petals, each petal consisting of a flat limb and a backwardly directed, nectar-secreting spur. Basal rosettes grow during April and May and develop one or several inflorescences, eventually flowering from May to August (Cullen and Heywood, 1964; Whittemore, 1997; Nold, 2003).

In Sardinia, three narrow endemic species have been described. Aquilegia barbaricina and A. nugorensis are similar in height (30–80 cm), while A. nuragica is usually smaller (20–45 cm). As in almost all Euroasiatic taxa of the genus, flowers are pale blue to purple, with the exception of the whitish ones of A. barbaricina. Flower size is larger in A. nugorensis (40–56 mm diameter) than in the other two species (25–30 mm). Spurs exhibit different degrees of curvature among species, being straighter in A. nugorensis (Arrigoni and Nardi, 1977, 1978).

Sardinian Aquilegia taxa are exclusively distributed across the central and eastern mountain ranges of the island, in the regions of Gennargentu massif, Tacchi and Supramontes (Fig. 1). They inhabit different environments; A. barbaricina appears in wet woodlands, meadows and stream margins, mainly occurring on siliceous substrates and secondarily on limestone. Aquilegia nugorensis inhabits wet rocky outcrops and appears mainly on limestone and secondarily on siliceous substrates. Finally, A. nuragica is known from a unique population on a wet limestone cliff extremely difficult to access (Arrigoni and Nardi, 1978). They are all highly threatened species, with scattered distributions and small population sizes. Aquilegia barbaricina and A. nuragica are listed both as ‘critically endangered’ in the IUCN red list (Camarda, 2006a, b; IUCN, 2010), and also as one of the 50 most endangered plants of the Mediterranean islands (de Montmollin and Strahm, 2007). Aquilegia nugorensis is included as ‘endangered’ in the Italian regional red list (Conti et al., 1997).

During June and July of 2009 and 2010, plant material from a total of 89 wild individuals was collected in all 19 locations where any species of Aquilegia is known to occur in the whole Sardinia Island (see Fig. 1 and Table 1 for further geographic and ecological details of sampling sites). Plant material samples were homogeneously distributed across population area and, in the case of proximity, a minimum 2 m distance between individuals was always taken into account to ensure sample independence. Fresh young leaves were immediately desiccated in the field with abundant silica gel at room temperature until DNA extraction. During the sampling, extreme care was taken to minimize the damage to these highly threatened species.

DNA extraction and amplified fragment length polymorphism (AFLP) protocol

Total genomic DNA was extracted from approx. 30 mg of dried leaf material by means of the DNeasy Plant Mini Kit (Qiagen) and using the Quiacube extraction robot (Qiagen). In general, amplified fragments were obtained following the protocol of Vos et al. (1995) with some modifications related to the use of fluorescent dye-labelled selective
Fig. 1. Study area and results of the genetic structure analyses. The 19 known locations of Aquilegia in Sardinia are distributed over the Central and Eastern mountain systems. Four locations at Monte Corrasi (North): Arco Corrasi (8), Punta Corrasi (11), Ahottadoglios (14) and Palumbrosa (15). Four Eastern locations: Gorropu Canyon (19), Pischina Urtaddala (18), Flumineddu (7) and Codula Orbisi (1). Five Central locations: Badde Enis (2), Talana (3), Rio Correboi (4), Rio Oli (5) and Monte Spada (6). Three locations at SE-Gennargentu Massif: Baccu Seardu (12), Su Furciddu (17) and Calavrighe Grossu (9). Finally, three locations in the Tacchi region: Monte Arquerì (10), Funtana Dorada (13) and Funtana Sa Ceraxia (16). Filled symbols represent populations already described as A. barbaricina (circles), A. nugorensis (squares) and A. nuragica (diamond). Open symbols (triangles) represent Aquilegia locations not previously described.

Spatial genetic structure is inferred by a model-based clustering method implemented in STRUCTURE. At each location, pie charts indicate the mean proportion of membership of individuals at each location for the K=4 genetic groups. The right-hand bar diagram indicates membership of each individual for the 4 genetic groups. Spatial genetic groups are indicated to the right of the figure.

primers and the use of a different restriction enzyme as rare cutter. Although EcoRI and MseI (Eco-AFLPs) are the most widely used restriction enzymes for the generation of AFLP markers, here we employ PstI and MseI (Pst-AFLPs) as rare and frequent cutter enzymes, respectively. Recent works suggest that PstI–MseI produces a higher number of bands, with a higher proportion of these bands being informative, a higher efficiency in detecting polymorphisms, a more random distribution of fragments along chromosomes instead of clustered in centromeric regions as in Eco-AFLPs, and clearer and more easily scorable fragments (Castiglioni et al., 1999; Vuylsteke et al., 1999; Young et al., 1999; Yuan et al., 2004; Ojha, 2005).

Digested DNA was ligated to the PstI and MseI adaptors. Fragments were selectively amplified with MseI and PstI primers bearing one selective nucleotide. The final selective amplification was carried out using PstI and MseI primers with two and three selective nucleotides primers, respectively. In total 48 selective primers combinations were assayed in a pilot study carried out on a random sub-sample of 15 individuals. Eight primer combinations were finally selected based on fragment abundance and polymorphism (Table 2). Fragment separation and detection were carried out in an ABI PRISM 3100 DNA automatic sequencer (Applied Biosystems). The presence or absence of fragments in each individual profile was scored with the software GeneMapper v.3.7 (Applied Biosystems).

To assess the reproducibility and reliability of our AFLP fragments, we replicated 25% (18 individuals) of the samples. Instead of replicating just the AFLP production, we also replicated DNA isolations. Doing so provides more reliable replicates, since repeating only the process of AFLP production does not generate true replicates (Bonin et al., 2007; Holland et al., 2008).

AFLP data analyses

Since there seem to be taxonomic inconsistencies in the delimitation of species in the Sardinian Aquilegia, we did not perform analyses separately for each taxon. To avoid
<table>
<thead>
<tr>
<th>Locality (Municipality, Province)</th>
<th>ID</th>
<th>Co-ordinates (N/E)</th>
<th>Altitude</th>
<th>Habitat</th>
<th>Substrates</th>
<th>Attributed taxa</th>
<th>Collector</th>
<th>N_p</th>
<th>N_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codula Orbisi (Orgosolo, NU)</td>
<td>4</td>
<td>08°08′/09°29′</td>
<td>800–885</td>
<td>Rocky outcrops</td>
<td>Limestones</td>
<td>NP</td>
<td>AC, GF, EM</td>
<td>53</td>
<td>5</td>
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<tr>
<td>Badde Enis (Orgosolo, NU)</td>
<td>2</td>
<td>07°07′/09°26′</td>
<td>840–1000</td>
<td>Rocky outcrops</td>
<td>Limestones</td>
<td>NP</td>
<td>GB, RB, AC, GF</td>
<td>76</td>
<td>5</td>
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<tr>
<td>Ponte Crobine (Talana, OG)</td>
<td>3</td>
<td>02°02′/09°24′</td>
<td>870–930</td>
<td>Riparian wood of Alnus glutinosa</td>
<td>Granites</td>
<td>NP</td>
<td>GB, RB, AC, GF</td>
<td>400</td>
<td>10</td>
</tr>
<tr>
<td>Rio Correboi (Vilagrande Strisaili, OG)</td>
<td>4</td>
<td>03°03′/09°20′</td>
<td>1100–1370</td>
<td>Riparian wood of A. glutinosa and Rhamnus persicifolia</td>
<td>Metamorphites</td>
<td>A. barbaricina</td>
<td>GB, RB, AC, GF</td>
<td>321</td>
<td>5</td>
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<tr>
<td>Rio Olai (Orgosolo, NU)</td>
<td>5</td>
<td>08°08′/09°21′</td>
<td>950–985</td>
<td>Riparian wood of A. glutinosa and R. persicifolia</td>
<td>Granites</td>
<td>A. barbaricina</td>
<td>GB, RB, EM</td>
<td>134</td>
<td>5</td>
</tr>
<tr>
<td>Monte Spada (Fonni, NU)</td>
<td>6</td>
<td>04°04′/09°16′</td>
<td>1340–1350</td>
<td>Riparian wood of A. glutinosa and Flex aqufiloum</td>
<td>Metamorphites</td>
<td>A. barbaricina (LC)</td>
<td>GB, EM</td>
<td>110</td>
<td>5</td>
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<tr>
<td>Rio Flumineddu (Urzulei, OG)</td>
<td>7</td>
<td>09°09′/09°28′</td>
<td>738–780</td>
<td>Mesophyloous wood and shady rocky outcrops</td>
<td>Limestones</td>
<td>NP</td>
<td>GB, AC</td>
<td>.</td>
<td>10</td>
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<tr>
<td>Arco Corras (Oliena, NU)</td>
<td>8</td>
<td>14°09′/09°25′</td>
<td>1370–1420</td>
<td>Shady rocky outcrops</td>
<td>Limestones</td>
<td>A. nugorensis</td>
<td>AC, GF</td>
<td>156</td>
<td>5</td>
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<td>Calavrighe Grussu (Vilagrande Strisaili, OG)</td>
<td>9</td>
<td>00°00′/09°21′</td>
<td>1095–1105</td>
<td>Riparian wood of A. glutinosa</td>
<td>Metamorphites</td>
<td>A. nugorensis</td>
<td>RB, GF, EM</td>
<td>1209</td>
<td>5</td>
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<tr>
<td>Monte Arcuer (Ussassai, OG)</td>
<td>10</td>
<td>49′/09°22′</td>
<td>920–925</td>
<td>Mesophyloous wood of Taxus baccata and L. aquiloum</td>
<td>Travertines</td>
<td>GB, GF</td>
<td>19</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Punta Corras (Oliena, NU)</td>
<td>11</td>
<td>14°04′/09°25′</td>
<td>1380–1410</td>
<td>Shady rocky outcrops</td>
<td>Limestones</td>
<td>A. nugorensis</td>
<td>GB, EM</td>
<td>97</td>
<td>5</td>
</tr>
<tr>
<td>Baccu Sardu (Vilagrande Strisaili, OG)</td>
<td>12</td>
<td>00′00′/09°19′</td>
<td>1500–1520</td>
<td>Riparian wood of A. glutinosa</td>
<td>Granites</td>
<td>A. nugorensis</td>
<td>GB, RB, EM</td>
<td>250</td>
<td>5</td>
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<td>Funtana Dorada (Seui, OG)</td>
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<td>52′/09°22′</td>
<td>890–915</td>
<td>Riparian wood of Ostrya carpinifolia and T. baccata</td>
<td>Travertines</td>
<td>A. nugorensis</td>
<td>GB, RB, AC, GF</td>
<td>152</td>
<td>5</td>
</tr>
<tr>
<td>Ahottadoglio (Oliena, NU)</td>
<td>14</td>
<td>14°09′/09°25′</td>
<td>1345–1355</td>
<td>Shady rocky outcrops</td>
<td>Limestones</td>
<td>A. nugorensis</td>
<td>AC, GF</td>
<td>87</td>
<td>5</td>
</tr>
<tr>
<td>Palumbrosa (Oliena, NU)</td>
<td>15</td>
<td>14°09′/09°25′</td>
<td>1330–1332</td>
<td>Shady rocky outcrops</td>
<td>Limestones</td>
<td>A. nugorensis</td>
<td>GB, RB, AC, GF</td>
<td>139</td>
<td>5</td>
</tr>
<tr>
<td>Funtana sa Cerasia (Seui, OG)</td>
<td>16</td>
<td>53′/09°23′</td>
<td>900–1060</td>
<td>Riparian wood of O. carpinifolia and L. aquiloum</td>
<td>Travertines</td>
<td>A. nugorensis (LC)</td>
<td>GB, EM</td>
<td>553</td>
<td>5</td>
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<tr>
<td>Su Furciddu (Vilagrande Strisaili, OG)</td>
<td>17</td>
<td>00′00′/09°20′</td>
<td>1230–1375</td>
<td>Riparian wood of A. glutinosa</td>
<td>Metamorphites</td>
<td>A. nugorensis</td>
<td>RB, EM</td>
<td>1016</td>
<td>5</td>
</tr>
<tr>
<td>Piscina Urtaddala (Urzulei, OG)</td>
<td>18</td>
<td>10′/09°29′</td>
<td>700–705</td>
<td>Shady rocky outcrops</td>
<td>Limestones</td>
<td>A. barbaricina</td>
<td>AC, MD, EM</td>
<td>102</td>
<td>5</td>
</tr>
<tr>
<td>Gorropu Canyon (Urzulei-Orgosolo, OG-NU)</td>
<td>19</td>
<td>11′/09°30′</td>
<td>620–680</td>
<td>Shady rocky outcrops</td>
<td>Limestones</td>
<td>A. nuragica (LC)</td>
<td>GB, FF, GF, EM</td>
<td>61</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 1. Localities, identification numbers and geographic co-ordinates of the sampled locations.

Altitude, habitat and substrate characteristics are also shown.

The attributed taxa for already known populations are reported, as well as new populations discovered in this study (NP). Locations 4 and 5 attributed to A. barbaricina by Arrigoni (2006). Location 6 described as A. barbaricina by Arrigoni and Nardi (1977) and location 18 attributed to the same taxa by Fenu et al. (2010). Locations 8, 11, 14 and 15 attributed to A. nugorensis by Fenu et al. (2010). Locations 9, 12, 13, 16 and 17 described as A. nugorensis by Arrigoni and Nardi (1978). Location 19 described as A. nuragica by Arrigoni and Nardi (1978).

LC, the locus classicus for each species; N_p, population size; N_i, the number of sampled individuals (direct field count).

Collectors abbreviations: G. Bacchetta (GB), G. Broi (GBR), R. Buttau (RB), A. Congiu (AC), M. Daws (MD), F. Fele (FF), G. Fenu (GF) and E. Mattana (EM).

possible fragment size homoplaspy (Vekemans et al., 2002) we evaluated the correlation between locus frequency and fragment size by means of the program AFLP-surv 1.0 (Vekemans, 2002). Only fragments within the 150–500 bp size range were finally considered since the non-significant correlation indicated that they were not prone to size homoplasy (Herrera and Bazaga, 2008).

Genetic diversity within locations was estimated by the total number of markers, percentage of polymorphic loci (p_PIL) and Nei’s gene diversity (H; Nei, 1987). Three parameters of marker rarity provided indirect estimates of population divergence and isolation: unique markers (markers present only in one population; M_u), diagnostic markers (unique markers present in all the individuals of a population; D) and frequency-down-weighted marker values (D_w; Schönswetter and Tribsch, 2005). Since populations which have been isolated for a long time accumulate rare markers by mutations, estimates of marker rarity may indicate divergence and isolation of populations. Specifically, D_w marker values may be a standardized measure of divergence and long-term isolation (Schönswetter and Tribsch, 2005). Several software packages were used to calculate these parameters; H and D_w were determined by means of R Statistical Software v.2.11.1 (R Development Core Team, 2010) and AFLPdat (Ehrich, 2006), while p_PIL, M_u and D were obtained using FAMD v.1.25 (Schlitter and Harris, 2006). To avoid the possible
In the study of Aquilegia diversity, we performed a simple regression of the number of individuals per population on the estimate of Nei’s genetic diversity and pPL values for four randomly selected samples per location (Schönswetter and Tribsch, 2005; Ronikier et al., 2008), except at locations 2, 4 and 10, with two, two and three samples, respectively.

To explore the possible effect of population size on genetic diversity, we performed a simple regression of the number of Aquilegia individuals per population on the estimate of Nei’s genetic diversity and pPL values. To reduce potentially confounding effects of geographical range sub-structure on IBD patterns, we performed both across the whole distribution range and for each spatial genetic group (cf. Bittkau and Comes, 2005). IBD patterns were checked by regressing pairwise FST values and geographic distances, and significance was assessed by Mantel tests using 9999 random permutations in Genalex 6.4 (Peakall and Smouse, 2006). Using this approach, acceptance of the null hypothesis of regional drift–migration does not rely just on the mere significant positive and monotonic correlation between genetic and geographic distances. Additionally, variance in differentiation should increase with distance (Hutchison and Templeton 1999; Griffin and Barrett, 2004). Thus, we also regressed the residuals from the former regressions on the pairwise geographic distances.

**RESULTS**

Primer combinations averaged a very low scoring error rate (1.29 %, Table 2) which stressed the suitability and repeatability of our AFLP data set. Overall, 590 loci were consistent and unambiguously scored for 89 individuals from all known 19 locations occurring in Sardinia. A total of 519 loci (88 %) were found to be polymorphic, and primer combinations resolved on average 73 loci.

Genetic diversity and marker rarity across populations

In general, genetic diversity measures within locations were low as estimated by pPL and H, which averaged 13.23 and 0.0281, respectively (Table 3). These measures ranged from extremely low values at location 19 (1.02, 0.0016), to the highest values found at location 16 (19.38, 0.0401). Average
Table 3. Estimates of genetic diversity and marker rarity for Sardinian populations of Aquilegia

<table>
<thead>
<tr>
<th>Spatial genetic groups</th>
<th>Locality ID</th>
<th>pPL</th>
<th>H</th>
<th>Mpr, (D)</th>
<th>DW</th>
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<tbody>
<tr>
<td>Corrasi</td>
<td>Arco Corrasi</td>
<td>8</td>
<td>0.0213 (+0.1048)</td>
<td>2</td>
<td>19.63</td>
</tr>
<tr>
<td></td>
<td>Punta Corrasi</td>
<td>11</td>
<td>0.0182 (+0.0987)</td>
<td>1</td>
<td>19.72</td>
</tr>
<tr>
<td></td>
<td>Ahottadoglios</td>
<td>14</td>
<td>0.0247 (+0.1144)</td>
<td>0</td>
<td>21.78</td>
</tr>
<tr>
<td></td>
<td>Palumbrosa</td>
<td>15</td>
<td>0.0281 (+0.1231)</td>
<td>1</td>
<td>21.18</td>
</tr>
<tr>
<td></td>
<td>Average (+s.d.)</td>
<td>19</td>
<td>0.0231 (+0.1102)</td>
<td>1 (+0.81)</td>
<td>20.57 (+1.07)</td>
</tr>
<tr>
<td>Gorropu</td>
<td>Gorropu Canyon</td>
<td>19</td>
<td>0.0016 (+0.0290)</td>
<td>2 (2)</td>
<td>34.44</td>
</tr>
<tr>
<td>CCD</td>
<td>Codula Orbisi</td>
<td>1</td>
<td>0.0320 (+0.1292)</td>
<td>3</td>
<td>19.64</td>
</tr>
<tr>
<td></td>
<td>Badde Enis*</td>
<td>2</td>
<td>0.0286 (+0.1670)</td>
<td>2</td>
<td>10.43</td>
</tr>
<tr>
<td></td>
<td>Ponte Crobini</td>
<td>3</td>
<td>0.0334 (+0.1330)</td>
<td>3</td>
<td>22.37</td>
</tr>
<tr>
<td></td>
<td>Rio Correblu*</td>
<td>4</td>
<td>0.0421 (+0.2011)</td>
<td>3</td>
<td>10.60</td>
</tr>
<tr>
<td></td>
<td>Rio Olai</td>
<td>5</td>
<td>0.0326 (+0.1272)</td>
<td>5 (1)</td>
<td>25.44</td>
</tr>
<tr>
<td></td>
<td>Monte Spada</td>
<td>6</td>
<td>0.0351 (+0.1336)</td>
<td>1 (1)</td>
<td>24.08</td>
</tr>
<tr>
<td></td>
<td>Pischina Urtaddala</td>
<td>18</td>
<td>0.0224 (+0.1055)</td>
<td>3 (2)</td>
<td>32.21</td>
</tr>
<tr>
<td></td>
<td>Average (+s.d.)</td>
<td>19</td>
<td>0.0323 (+0.1423)</td>
<td>3 (+1.63)</td>
<td>20.68 (+7.93)</td>
</tr>
<tr>
<td>GTF</td>
<td>Flumineddu</td>
<td>7</td>
<td>0.0362 (+0.1422)</td>
<td>1</td>
<td>24.41</td>
</tr>
<tr>
<td></td>
<td>Calavrighe Grussu</td>
<td>9</td>
<td>0.0387 (+0.1439)</td>
<td>2</td>
<td>33.18</td>
</tr>
<tr>
<td></td>
<td>Monte Arquer*</td>
<td>10</td>
<td>0.0056 (+0.0610)</td>
<td>1</td>
<td>17.15</td>
</tr>
<tr>
<td></td>
<td>Baccu Sardu</td>
<td>12</td>
<td>0.0345 (+0.1313)</td>
<td>3 (1)</td>
<td>33.37</td>
</tr>
<tr>
<td></td>
<td>Fontana Dorada</td>
<td>13</td>
<td>0.0255 (+0.1136)</td>
<td>3</td>
<td>19.82</td>
</tr>
<tr>
<td></td>
<td>Fontana Ceraxia</td>
<td>16</td>
<td>0.0401 (+0.1434)</td>
<td>3 (1)</td>
<td>26.29</td>
</tr>
<tr>
<td></td>
<td>Su Furciddu</td>
<td>17</td>
<td>0.0340 (+0.1353)</td>
<td>2</td>
<td>20.76</td>
</tr>
<tr>
<td></td>
<td>Average (+s.d.)</td>
<td>17</td>
<td>0.0349 (+0.1349)</td>
<td>2.33 (+0.79)</td>
<td>26.30 (+10.48)</td>
</tr>
</tbody>
</table>

Spatial genetic groups in which the whole distribution can be clustered (see Results) are also indicated. pPL, percentage of polymorphic loci; H, Nei’s gene diversity; Mpr, markers unique for each population; D, diagnostic markers, occurring in all the individuals in a population; DW, frequency-down-weighted marker values. Average values of each parameter for the locations at each spatial genetic group are also indicated. Parameters estimated for populations marked with asterisks, excluded from average values, should be considered cautiously due to sub-sampling (see the Materials and Methods).

Spatial structuring of genetic variation

Four genetic clusters could be clearly recognized in the PCoA analysis (Fig. 2). The main axis strongly segregated locations 8, 11, 14 and 15, all situated at Monte Corrasi, which explained 34.7% of the total genetic variation. The secondary axis, which explained 23.2% of the total genetic variation, segregated location 19 (Gorropu Canyon) and location 18 (Pischina Urtaddala). The last cluster grouped the rest of individuals without a clear genetic structure inside.

STRUCTURE analyses provided further structuring insights into the PCoA pattern. The occurrence of four distinct genetic lineages in the Aquilegia of Sardinia was the most likely partition found in the analysis (Fig. 1): Monte Corrasi (locations 8, 11, 14, 15; Corrasi group, hereafter); Gorropu Canyon (location 19; Gorropu group, hereafter), both groups identified in PCoA; central (locations 1–6, 18; ‘CCD’ group, hereafter); and SE-Gennargentu/Tacchi + Flumineddu (locations 9, 12, 17/10, 13, 16 + 7; ‘GTF’ group, hereafter). It is also noteworthy that within CCD, individuals from location 18...
Table 4. Analyses of molecular variance of Sardinian populations of Aquilegia

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>% Total variance</th>
<th>FSC</th>
<th>FST</th>
<th>FCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>No groups</td>
<td></td>
<td></td>
<td></td>
<td>0.70</td>
<td>0.70</td>
<td>0.70</td>
</tr>
<tr>
<td>Among populations</td>
<td>17</td>
<td>1733.39</td>
<td>70.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within populations</td>
<td>70</td>
<td>567.91</td>
<td>29.97</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among groups</td>
<td>3</td>
<td>795.80</td>
<td>31.66</td>
<td>0.59</td>
<td>0.72</td>
<td>0.31</td>
</tr>
<tr>
<td>Among populations</td>
<td>15</td>
<td>937.60</td>
<td>40.90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within populations</td>
<td>70</td>
<td>567.91</td>
<td>27.45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taxonomic structuring (A. barbaricina, A. nugorensis, A. nuragica)</td>
<td></td>
<td>0.63</td>
<td>0.73</td>
<td>0.28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Genetic variation was partitioned into the groups of populations resulting from PCoA and STRUCTURE analyses. Partition without considering any grouping is also shown. All P-values were at least <0.0003.

(Pischina Urtaddala; Pischina, hereafter), segregated by PCoA, showed a particularly high genetic admixturing. All detected groups were spatially cohesive except for GTF. The Flumineddu population was located fairly far apart from the rest of the populations, which in turn were spatially segregated in three locations at SE-Gennargentu (9, 12 and 17) and three in the Tacchi region (10, 13 and 16) (see Fig. 1).

Genetic differentiation among groups

Estimates of genetic diversity and marker rarity across genetic groups supported the results obtained by the PCoA and Bayesian grouping analyses and reflected strong genetic differentiation among groups (Table 3).

The AMOVAs showed significant differences in the proportion of variance explained at all levels and for all grouping schemes tested (all P-values <0.0003, Table 4). Dealing with the four spatial genetic groups (Corrasi, Gorropu, CCD and GTF), genetic variation among groups was high (31.66 %, Table 4). Grouping populations according to their taxonomic affiliation resulted in a lower genetic variation among groups (28.42%). Irrespective of the grouping scheme, genetic variation among populations within groups was always higher than variation among groups, suggesting that historical rates of gene flow among populations have been limited, probably as a consequence of stronger selection processes among populations than among groups.

Spatial genetic structure

To test the null hypothesis of regional drift—migration equilibrium, correlations of genetic distance (FST) and its variance vs. geographic distance were performed, both for the whole Sardinian geographical range and for each spatial genetic group.

Over the whole of Sardinia, pairwise FST values were not significantly correlated with geographic distances (rM ¼ 0.038, P ¼ 0.312; Fig. 3A). For the CCD group, correlation was significantly positive (rM ¼ 0.686, P ¼ 0.003; Fig. 3C). The Corrasi group exhibited a positive, but just marginally significant correlation (rM ¼ 0.893, P ¼ 0.042; Fig. 3B). For the GTF group (excluding the Flumineddu population, located apart from the group), correlation was not significant (rM ¼ 0.038, P ¼ 0.312; Fig. 3D). Finally, correlation for the Gorropu group could not be performed since it was defined by a single population. Correlation between residuals and geographical distance was not significant in any case (data not shown), suggesting the same scattering degree over all the range of geographical separations.

Despite the positive and significant correlation for CCD and Corrasi, in these groups variance in genetic differentiation did not increase with distance. Therefore, the results reject the null hypothesis of regional drift—migration equilibrium, both for the whole island and for all spatial genetic groups.

Discussion

Genetic diversity

Our analyses across the whole distribution range of the three Sardinian exclusive and endangered taxa of Aquilegia revealed extremely low levels of genetic diversity. This result is in accordance with the general expectation that endemic species, and particularly island endemics (Frankham, 1997), exhibit lower levels of genetic diversity than widespread species (Hamrick and Godt, 1989, 1996).

First suggested by Stebbins (1942), it has been almost universally assumed that endemic species show reduced genetic variation compared with widespread congeners, but this relationship is not apparent nowadays. Gitzendanner and Soltis (2000) provided only slight evidence (or contradictory evidence) for differences in genetic diversity between rare and widespread plant species, and concluded that, although there is an overall trend associating rare species with low levels of genetic variation, endemic species do not define a homogeneous group of reduced genetic diversity (but see Nybom et al., 2004). In the Mediterranean Basin, empirical results also show this controversy, with both lower (e.g. Affre and Thompson, 1997a; Affre et al., 1997; Toumi and Lumaret, 2001) and higher (e.g. Affre and Thompson, 1997b; Mateu-Andrés, 1999) levels of genetic diversity observed in endemics than in their related widespread congeners.

Several causes have been proposed to explain the reduced genetic variation of endemic species (Thompson, 2005). Endemic species are usually specialized to local ecological conditions, and natural selection may reduce variation by eliminating non-adapted alleles (Babbel and Selander, 1974; Brown and Schoen, 1992). Additionally, small population sizes, founder effects and genetic bottlenecks may also induce high levels of inbreeding and subsequent reductions in genetic variability (Barrett and Kohn, 1991; Ellstrand and Elam, 1993).

Here, we found a weak effect of population size on diversity estimates (marginal on H and not significant on pPL), which does not seem strong enough to be the main cause of the observed reduced genetic diversity. Across Sardinia, Aquilegia populations are found in different and restricted habitat types, ranging from riverside meadows to rocky outcrops (Mattana et al., 2012). Reduced diversity values of Sardinian Aquilegia might be the consequence of local adaptation (divergent selection) to particular environmental conditions, with selection eroding genetic variability. In this sense, the available
phylogeny of the Euroasiatic taxa of the genus establishes that habitat specialization and geographic isolation were the main drivers of species divergence in the process of adaptive radiation of the Euroasiatic taxa of the genus (Bastida et al., 2010). Furthermore, Alcántara et al. (2010) have found divergent selection imposed by environmental habitat parameters on phenotypic divergence between Aquilegia species and on ecotypic differentiation within species.

Geographical structure of the pattern of genetic variation

STRUCTURE results from individual multilocus profiles, also endorsed by PCoA analyses, revealed that the pattern of genetic variation of Sardinian endemic taxa of Aquilegia is structured geographically into four spatial genetic groups: Gorropu, Corrasi, CCD and GTF. However, AMOVAs revealed that, although differences within populations still explained a high proportion, the highest amount of genetic variation was explained by differences among populations. In general, our results reveal a pattern of genetic variation spatially structured under a classical perspective of a priori-defined populations and also under the ‘blind’ individual-based approach by STRUCTURE.

High levels of spatial genetic structure have been described for other island endemics, such as Brassica insularis (Hurtrez-Bousses, 1996), Cyclamen balearicum (Affre et al., 1997), Anchusa crispa (Quilichini et al., 2004) and Senecio rodriquezii (Molins et al., 2009), in the Western Mediterranean sea; Nigella arvensis complex (Bittkau and Comes, 2005) and Brassica cretica (Edh et al., 2007) in the Aegean sea; Silene struthiolidoides and S. hawaiiensis in the Hawaiian archipelago (Westerbergh and Saura, 1994); and Aster asa-grayi in Japan (Maki, 1999). In our case, the level of spatial genetic structuring is likely to be the consequence of geographical isolation, probably as the result of the interplay between the complex Sardinian topography and geology and the variation of climate characteristics within the island, and limited dispersal abilities of the species. These circumstances promote restricted gene flow among populations and subsequent differentiation by drift and selection (Edh et al., 2007). Major paleogeographic and paleoclimatic processes may be discarded here due to the recent dating of this group of Aquilegia taxa. European Aquilegia taxa began to diversify...
around 2.54 million years ago. Of these, the clade including Sardinian taxa is the most recent (Bastida et al., 2010).

**Gene flow and drift within spatial genetic groups**

In fact, we have detected no migration–drift equilibrium for the Aquilegia in Sardinia (neither when analysed as a whole nor when analysed in individual groups), approaching a Case III pattern sensu Hutchinson and Templeton (1999) (Fig. 3A). This scenario is associated with extreme isolation conditions where drift historically has played a dominant role in the absence of significant gene flow.

Some degree of association between genetic and geographic distances can be found even in the absence of regional equilibrium especially at shorter geographic distances and, in general, over geographical ranges where gene flow may be acting to some extent (Hutchinson and Templeton, 1999). This seems to be the case for the Corrasi and CCD groups. The Corrasi group is made up of four locations situated over a reduced geographic range (~1 km²). This proximity makes it problematic to draw an inference of the roles of gene flow and genetic drift as per Hutchinson and Templeton’s Cases. Despite this, Corrasi showed a significant positive relationship between pairwise FST values and geographic distance which indicates a higher relevance of gene flow over genetic drift. Pollen flow might be the main agent connecting these locations. Bumble-bees usually forage at distances of 400–600 m, reaching altitudes of 800–1400 m (Walther-Hellwig and Frankl, 2000; Osborne et al., 2008), which is in the range of distances separating these populations (581 m).

The genetic group CCD, which encompasses central populations of the Island, approximates a positively sloped Case II, where patterns of isolation by distance are beginning to form, with gene flow reducing differentiation among neighbouring populations. This scenario suggests two hypothetical evolutionary trajectories: (1) an emerging Case IV pattern where gene flow will exert a predominant influence, eventually defining a range of higher separation distances where genetic drift will predominate; or (2) an emerging Case III pattern where environmental and/or geographic conditions enhance isolation levels, and genetic drift will start to predominate over gene flow.

The group GTF approximated a typical Case III pattern, with a wide divergence in the scatter and a non-significant correlation between genetic and geographic distances. This suggests a high degree of isolation between the three locations of SE-Gennargentu (9, 12 and 17), highly differentiated with nearly no admixture, and the three locations at Tacchi region (10, 13 and 16), where genotypes from other genetic groups (particularly from Corrasi) are present.

Gorropu and Pischina are both constituted by a single population and cannot be evaluated in the present context. Nevertheless, Gorropu is highly isolated spatially. It presents no admixture and only shares some genetic information with Pischina. Thus it is probably subject to high levels of genetic drift.

The heavily admixedtured location of Pischina harbours genetic profiles from Gorropu, CCD and GTF (probably from Flumineddu location 7). Gene flow is presumably playing a relevant role in its genetic composition.

Thus, as in other geographic areas and island-related species (e.g. Bittkau and Comes, 2005), our evidence indicates that genetic drift has historically been more influential than gene flow on the population structure of the Sardinian species of Aquilegia. However, in some areas with less isolated populations, such as the group CCD, the influence of gene flow has also played a relevant role.

**Differentiation of spatial genetic groups**

The Aquilegia population at Gorropu Canyon is probably the most geographically isolated in Sardinia. It hangs 25–50 m away from the soil level, over one of the walls of a deep rocky canyon. The nearest Aquilegia location, Pischina Urtaddala, is 1–67 km upstream, in a temporal water course flowing to Gorropu Canyon. It exhibits the highest Dw marker value and a complete fixation of unique markers (i.e. exclusive markers fixed for each one of the individuals; Table 3), which indicates a long-term isolation of individuals. Further, it shows no admixture from any other genetic group and only shares some genetic information with Pischina. The latter suggest a genetic link from Gorropu to Pischina where seed dispersal could be responsible for this connection. The distance separating these locations (1–67 km) is at the upper limit of the distance that pollinators can cover in their foraging activity, and further topographic barriers may magnify this straight line separation distance (Herrera and Bazaga, 2008). Seed dispersal by ungulates is the most probable cause. Wild European mouflon (Ovis musimon) and domestic sheep, goats and cows are abundant in these mountain systems (Ciuti et al., 2009). Although long-distance dispersal of dry-fruited shrubs by ungulates has been traditionally assumed to entail the complete destruction of seeds, recent evidence indicates that herbivore endozoochory may be of potential relevance for colonization of distant sites, because some seeds may survive ungulate digestion (Manzano et al., 2005; Manzano and Malo, 2006).

The genetic admixture shown by individuals at the Pischina Urtaddala location from CCD, Gorropu and GTF groups is particularly interesting. During the wettest time of the year, waters from Orbisi canyon reach Pischina Urtaddala (a small pond) and then flow into the Flumineddu river, just before Gorropu Canyon. This scheme reflects to some extent the pivotal geographic location interconnecting the Gorropu group (discussed above) with GTF and CCD. Genotypes from GTF probably reached Pischina from Flumineddu location (7) and genotypes from CCD probably from Codula Orbisi (location 1) situated upstream, to which it is connected by river flow during the wettest season. The particular admixture scheme and the pivotal position in the spatial confluence of different spatial genetic groups make this location an important one for hybridization; a circumstance that will be discussed in the next section when we incorporate taxonomic information.

Individuals from Corrasi, representing four locations, show no evidence of admixture. However, Corrasi genotypes do occur in other groups of the island, especially within the central location 4 (Río Correboi, CCD) and within the southern location 16 (Funtana Sa Ceraxia, GTF). This observation is consistent with more recent gene flow between these localities than is seen with CCD and GTF. The spread of Corrasi genotypes to other areas may be due to the aforementioned seed dispersal events by different ungulates.
GTF genotypes are spread across all central locations situated from 3 to 18 (Fig. 1). That is, these genotypes occur in all CCD locations (1–6), in Piscina (18) and in Flumineddu (7; whose high proportion of GTF genotypes led it to be clustered within the GTF genetic group). This high occurrence of GTF genotypes in all locations, except Gorroppu and Corrasi (a circumstance particularly evident at Flumineddu), may also be explained by seed dispersal events by ungulates. An alternative but tentative explanation could be an ancestral generalized occurrence of this group of genotypes across the majority of the current distribution range of Aquilegia in Sardinia, except Corrasi. Nowadays it is relegated to SE-Gennargentu, Tacchi, Flumineddu and Piscina (which in turn may be subject to dispersal events from Flumineddu, as discussed earlier).

Potential implications for taxonomy

The results obtained here from the analyses of AFLP multilocus individual profiles are not fully compatible with the current taxonomic affiliations of Sardinian Aquilegia taxa. Our analyses revealed the existence of four genetic lineages in the Aquilegia genus in Sardinia, while current taxonomic treatments (Arrigoni and Nardi, 1977, 1978) consider three species of Aquilegia in Sardinia: A. barbaricina, A. nugorensis and A. nuragica (Fig. 1). Aquilegia barbaricina is described from locations 4–6 and 18, with location 6 (Monte Spada) being the locus classicus (Arrigoni and Nardi, 1977). Aquilegia nugorensis is described from locations 8, 9 and 11–17, with location 16 being the locus classicus (Arrigoni and Nardi, 1978). Aquilegia nuragica is uniquely described from population 19 (Arrigoni and Nardi, 1978). Detailed taxonomic insights are beyond the scope of this article but, given the genetic evidence presented here, the spatial genetic structure does not match the taxonomically defined entities.

This genetic/taxonomic uncoupling is reflected by the AMOVAs which indicated that the proportion of variance explained when adopting the taxonomic grouping was lower than that obtained when considering the spatial genetic groups from the STRUCTURE results (28.4% vs. 34.7%).

The group GTF includes all populations currently ascribed to A. nugorensis (9, 12–13 and 16–17; Fig. 1) with the exception of populations at Monte Corrasi, that constitute a well differentiated spatial genetic group. This group shows a high correspondence to what is currently described as A. nugorensis, including the two populations not previously described.

The CCD group includes all the populations of what is currently ascribed to A. barbaricina (4–6 and 18), and three populations not formerly known (1–3). Our analyses agree with this view and also consider that the three populations not previously described should also be potentially ascribed to this taxon, given also the high morphological similarity of their individuals to those from locations 4–6. Location 18, Piscina Urtaddala, exhibits a high genotypic contribution of this CCD cluster, attributed to A. barbaricina, but also presents important contributions from Gorroppu, the group associated with A. nuragica, and from the group corresponding to A. nugorensis (see below), which may indicate that this location could be subject to recent interspecific introgressive hybridization involving three different lineages. Future research should be devoted to the study of genetic diversity at this specific location.

Locations of the Corrasi group, described as A. nugorensis (Arrigoni, 2006), constitute a well differentiated spatial genetic group, distinct from the rest of the A. nugorensis populations. The strong segregation signal exhibited in the STRUCTURE analyses suggests that the designation of the populations of Monte Corrasi as A. nugorensis deserves further study, as previously suggested by Fenu et al. (2010). Gorroppu constitutes the most well-differentiated group in the Island. Its unique population (19) was described as A. nuragica (Arrigoni and Nardi, 1978). The fact that it is described as a single-population species, together with evidence commented on in preceding sections, suggest extremely high values of isolation and low genetic diversity. These observations help to explain its problematic conservation status (‘critically endangered’; IUCN, 2010).

Concluding remarks

The only phylogenetic study available to date of the Euroasiatic taxa of Aquilegia (Bastida et al., 2010) establishes that habitat specialization and geographic isolation are the main drivers of species divergence in the process of adaptive radiation of these taxa. Furthermore, Alcántara et al. (2010) have found divergent selection imposed by environmental habitat parameters on phenotypic divergence between several South European species of Aquilegia and on ecotypic differentiation within them. Evidence presented here also points towards this. Across Sardinia, Aquilegia occurs in different and restricted habitat types, ranging from riverside meadows to rocky outcrops (Mattana et al., 2012), and also from limestone to granites and metamorphic substrates (Table 1). This intra- and interspecific diversity of habitats may indicate that habitat specialization by divergent selection may have been a very relevant force contributing to population differentiation and species divergence, although this needs to be specifically studied and demonstrated. In addition, overall reduced levels of genetic diversity, as commented on at the beginning of Discussion, are also in agreement with this possibility, since divergent selection would be eroding genetic variability.

Together with divergent selection imposed by habitat environments, the limited dispersal ability of these species would also contribute to reinforce post-Pleistocene geographical isolation of Sardinian Aquilegia populations. This ecological and evolutionary scenario determines the historical dominant influence of genetic drift over gene flow on population structure of Sardinian species of Aquilegia.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Figure S1: population graph analysis conducted on the four spatial genetic groups pre-defined by STRUCTURE.

ACKNOWLEDGEMENTS

We thank Rafael G. Albaladejo and Carlos M. Herrera for helpful comments on earlier drafts. Two anonymous reviewers provided valuable suggestions to improve the paper. Additionally, we also benefited from the valuable help
provided by C. Pérez and E. López with lab work and also from the helpful advice of P. Bazaga. A. Congiu and R. Buttai helped in the field work, and two rock-climbers, F. Fele and G. Broi, provided us with the plant material from the otherwise inaccessible population at Gorropu Canyons. J.L.G. also thanks A. Sariego for providing crucial keys during the work. We also thank the Remote Sensing and Geographic Information Systems Laboratory of Estación Biológica de Doñana (LAST-EBD). All bioinformatic analyses regarding STRUCTURE were carried out on the freely available Bioportal (www.bioportal.uio.no). The work was partially supported by grant PIE-200730I012 (Consejo Superior de Investigaciones Científicas) and by the RNM-154 research group (Junta de Andalucía).

LITERATURE CITED
