INTRODUCTION

The Mediterranean Basin displays such an abundance of endemic species (about 13,000 species, corresponding to 4.3% of the plant species described worldwide) that it may be considered one of the biodiversity hotspots at a global level (Myers et al., 2000). At the local level, ten more biodiversity hotspots have been recognized (Medail and Quézel, 1999) in the Mediterranean region. In particular, the larger islands may have played a key role in the conservation of mid-tertiary floras (Greuter, 1995). Many species are characterized by disjoint distributions, thus leading to high levels of differentiation between geographically isolated populations (Quilichini et al., 2004). The extent of genetic differentiation among con-specific populations relative to the extent of differentiation present between closely related endemic taxa in the Mediterranean flora is an issue which has recently attracted attention (Debussche and Thompson, 2002). This issue is of particular importance for species delimitation in biodiversity inventories and in order to identify and delimit taxa for specific conservation measures (Olfelt et al., 2001).

Studies on the amount and distribution of genetic diversity of endemic and protected Mediterranean plant species are still scarce and very limited information is available on their genetic structure. Many species live in harsh environments, such as cliffs and steep slopes characterized by the presence of drought and wind, thus forming patches that are isolated by other environments which they cannot populate because of their limited dispersal ability. This is the case for Centaurea horrida and other plants of the Asteraceae family, which have recently been studied: the congener Centaurea corymbosa has a very low colonizing ability and survives in six small populations (Fréville et al., 2001), while Femeniasia balearica (formerly C. balearica) now lives in a very restricted habitat (Vilatersana et al., 2007). Both have been analysed for their genetic composition by means of allozymes, microsatellites and amplified fragment length polymorphism (AFLP) and display quite high levels of genetic variation and genetic differentiation between populations. In this paper we undertake the first study of the genetic structure of the remaining populations of the endangered species Centaurea horrida, by means of microsatellite markers. Centaurea horrida (Fig. 1) is a long-living spinous dwarf scrub that grows to a height of 70 cm (Valsecchi, 1977). Its distribution is limited to sea-cliffs on islands and peninsulas where it forms patches of isolated populations, both in primary and secondary dwarf communities (Desole, 1956; Valsecchi, 1977). Centaurea horrida is a diploid taxon with 2n = 18 (Desole, 1954), that reproduces sexually, by way of cross-pollination carried out by insects. It flowers in late spring (April–May) and bears fruit in summer (July–August; Pisanu, 2007). It is a protected species according to the Berne Convention (Appendix I), a priority species according to the EU Directive 43/92 ‘Habitat’ (Annex II) and a vulnerable species according to the EU Directive 92/43/EEC.
studied, and were stored at –80°C. A total of 385 individuals (Table 1) throughout the seven populations of the plants living on that island.

The study was conducted on two populations from the island of Asinara (FOR and STR), two from Stintino (FAL and DON), two from Alghero (LIO and BAR) and one from Tavolara (TAV), the latter consisting of the total number of the plants living on that island.

Samples of fresh leaves were collected from a total of 385 individuals (Table 1) throughout the seven populations studied, and were stored at –80°C until DNA extraction. Total DNA was extracted by grinding the frozen leaves in a mortar in liquid N2 and by using the DNeasy Plant Mini Kit (Qiagen, Italy), according to the manufacturer’s instructions. The average concentration of the extracted DNA was 20 ng μL⁻¹.

Amplification reactions were modified with respect to Fréville et al. (2000). They were performed in a total volume of 15 μL, containing HotMasterTaq (Eppendorf®) buffer 1X, 2.5 mM MgCl2, 2 μM of each dNTP, 0.5 μM of each forward and reverse primer, 25 ng genomic DNA and one unit of Taq polymerase HotMasterTaq (Eppendorf®). Amplification was carried out in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA), under the following conditions: an initial cycle at 94°C for 2 min, followed by 30 amplification cycles, at 94°C for 1 min, annealing temperature (Tₐ) for 2 min, and a final step of extension at 65°C for 5 min.

The amplification products were run on a capillary MegaBACE® DNA sequencer (Amersham). The raw data were analysed using allied MegaBACE Fragment Profiler software, to score the single-plant genotypes.

### Materials and Methods

#### Plant material

The distribution range of *Centaurea horrida* Badarò is highly fragmented and consists of only four coastal locations, from north-west to north-east Sardinia (western Mediterranean), the characteristics of which are reported in Table 1; its geographical position is shown in Fig. 2. The study was conducted on two populations from the island of Asinara (FOR and STR), two from Stintino (FAL and DON), two from Alghero (LIO and BAR) and one from Tavolara (TAV), the latter consisting of the total number of the plants living on that island.

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### Amplification conditions

Simple sequence repeat (SSR) primers from *Centaurea corymbosa* (Fréville et al., 2000) were tested for their ability to amplify single genomic regions in *Centaurea horrida*. Four out of seven were selected because they yielded an unambiguous amplification pattern. The SSRs chosen, their primer sequences and the fluorophore used are listed in Table 2.

Amplification reactions were modified with respect to Fréville et al. (2000). They were performed in a total volume of 15 μL, containing HotMasterTaq (Eppendorf®) buffer 1X, 2.5 mM MgCl₂, 2 μM of each dNTP, 0.5 μM of each forward and reverse primer, 25 ng genomic DNA and one unit of Taq polymerase HotMasterTaq (Eppendorf®). Amplification was carried out in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA), under the following conditions: an initial cycle at 94°C for 2 min, followed by 30 amplification cycles, at 94°C for 1 min, annealing temperature (Tₐ) for 2 min, and a final step of extension at 65°C for 5 min.

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### Data analysis

Allele frequencies and observed and expected heterozygosities (Hₒ and Hₑ, respectively) were estimated at each locus for all populations. Fisher’s exact test using the Markov Chain algorithm (Guo and Thompson, 1992) was used to assess deviations from the Hardy–Weinberg equilibrium for each population and each locus. Genotypic disequilibrium between pairs of loci was tested at the single population level by Fisher’s exact test. Weir and Cockerham’s (1984) estimators of F-statistics were used to analyse

<table>
<thead>
<tr>
<th>Location area</th>
<th>Co-ordinates</th>
<th>Status</th>
<th>Sample number</th>
<th>Population size (no. of individuals)</th>
<th>Population name (code)</th>
<th>Surface area</th>
<th>Lithology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asinara Isle</td>
<td>40°59’07&quot;N–41°07’N</td>
<td>National park</td>
<td>59</td>
<td>&gt;300</td>
<td>Fornelli (FOR)</td>
<td>30.83 ha</td>
<td>Schist and granite</td>
</tr>
<tr>
<td>Stintino Peninsula</td>
<td>40°50’58”N–8°10’15”E</td>
<td>Natura 2000 site</td>
<td>60</td>
<td>&gt;300</td>
<td>Stretti (STR)</td>
<td>12.42 ha</td>
<td>Schist</td>
</tr>
<tr>
<td>Capo Caccia Peninsula</td>
<td>40°33’37”N–8°08’10”E</td>
<td>Regional park</td>
<td>59</td>
<td>&gt;300</td>
<td>Coscia di Donna (DON)</td>
<td>2.1 ha</td>
<td>Limestone</td>
</tr>
<tr>
<td>Tavolara Isle</td>
<td>40°53’55”N–9°40’44”E</td>
<td>Marine reserve</td>
<td>33</td>
<td>&lt;300</td>
<td>Cala della Barca (BAR)</td>
<td>&lt;1 ha</td>
<td>Limestone and granite</td>
</tr>
</tbody>
</table>
genetic diversity both within and between populations. In particular, \(F_{IS}\) was calculated in order to estimate which proportion of the total genetic variation was due to a departure from the Hardy–Weinberg equilibrium at the population level. \(F_{ST}\) was calculated in order to estimate the proportion of the total genetic variation due to differentiation between populations. \(F_{ST}\) was also used to estimate gene flow by calculating the number of migrants per generation (\(Nm\)). The \(F_{ST}\) analogue for microsatellites \(R_{ST}\) (Slatkin, 1995) was also used, so as to include molecular information relating to the size of differences between the alleles in the differentiation estimates. The statistical methods implemented by BOTTLENECK (Piry et al., 1999) were used for detecting genetic bottlenecks in the study populations either under the infinite allele model (IAM) or the stepwise mutation model (SMM). The two-phased model of mutation (TPM) was also tested, because most microsatellite data better fit the TPM than the SMM or IAM. The TPM is intermediate to the SMM and IAM.

A Mantel test (Mantel, 1967) was applied to the matrices of pairwise \(F_{ST}/(1–F_{ST})\) and log-transformed geographical distances between populations to assess isolation-by-distance, i.e. the presence of migration-drift equilibrium between populations.

Analysis of molecular variance (AMOVA) was performed to partition the total genetic variation among regions and between populations within regions (Excoffier et al. 1992). The test of significance for the AMOVA was carried out on 1000 permutations of the data.

The problem of inferring the number \(K\) of clusters present in a data set has been addressed by Pritchard et al. (2000) by using the Bayesian paradigm and ad hoc software called STRUCTURE. They placed a prior distribution on \(K\) and based inference for \(K\) on the posterior distribution \(Pr(\mathbf{X}|K) = Pr(K|\mathbf{X}) \cdot Pr(K)\), where \(\mathbf{X}\) is the multilocus genotype of individuals. More recently, it has been suggested that a better estimator of \(K\) is the modal value of \(\Delta K\) (Evanno et al., 2005), the second-order rate of change of the likelihood function with respect to \(K\). The latter approach was used in the present work to estimate \(K\). The analysis was based on the admixture model, correlated allele frequencies between populations, and was run with a length of burn-in period of \(10^5\) and the same number of MCMC replications. Twenty runs were carried out for each \(K\) value from 1 to 10 (the number of real populations plus three) tested.

The software packages used to analyse the genetic data were GENEPOP (Raymond and Rousset, 1995), GENETIX (Belkhir et al., 1996), BOTTLENECK (Piry et al., 1999), GenAlEx v.6 (Peakall and Smouse, 2001), RST CALC (Goodman, 1997) and STRUCTURE 2.1 (Pritchard et al., 2000).

RESULTS

Genetic variability

A total of 385 plants of Centaurea horrida were analysed using four microsatellite markers, identifying a total of 80

![Fig. 2. Schematic map of Sardinia (western Mediterranean Sea) showing the geographic localization of the populations of C. horrida studied (see also Table 1).](image)
alleles. All the loci studied are highly polymorphic: the number of detected alleles per locus across all the populations ranged from 15 (locus 21D9) to 25 (locus 13D10). There were no indications for null alleles at any of the loci. No alleles were found fixed at any of the loci; neither was evidence found that a given population harboured specific alleles.

Genetic diversity (Table 3) was measured using Nei’s heterozygosity ($H_e$) and ranged from 0.449 (locus 21D9, TAV population) to 0.925 (locus 13D10, DON population). The high estimates of genetic variability are confirmed by the average $H_e$ values, ranging from 0.603 (LIO) to 0.854 (FAL and DON). These values are higher for the populations of the Stintino–Asinara region than for the two populations of the Alghero region and the isolated population of Tavolara.

The Hardy–Weinberg equilibrium was tested for all the loci and populations by testing the departure of the expected heterozygosities was compared to the observed specific alleles. The presence of correlation between genetic differentiation and geographic distance (log km) between populations was demonstrated by a Mantel test ($P = 0.004, G = 2.41, Z = 10.6$), indicating that the present distribution of genetic variation among the remnant populations of *Centaurea horrida* is, at least in part, the result of an equilibrium between drift and gene flow. Gene flow was estimated on the basis of either $F_{ST}$ or $R_{ST}$. The maximum value of $Nm$ was 8.37 (populations FAL and DON), whereas the minimum value was 1.33 (populations LIO and TAV).

### Table 3. Observed and expected heterozygosity measured at each locus for each population, and averages over loci and populations

<table>
<thead>
<tr>
<th>Locus</th>
<th>STR</th>
<th>FOR</th>
<th>FAL</th>
<th>DON</th>
<th>LIO</th>
<th>BAR</th>
<th>TAV</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>21D9</td>
<td>$H_e$</td>
<td>0.741</td>
<td>0.746</td>
<td>0.431</td>
<td>0.475</td>
<td>0.328</td>
<td>0.393</td>
<td>0.250</td>
</tr>
<tr>
<td>13D10</td>
<td>$H_e$</td>
<td>0.856</td>
<td>0.866</td>
<td>0.842</td>
<td>0.798</td>
<td>0.287</td>
<td>0.610</td>
<td>0.449</td>
</tr>
<tr>
<td>28A7</td>
<td>$H_e$</td>
<td>0.696</td>
<td>0.847</td>
<td>0.683</td>
<td>0.763</td>
<td>0.517</td>
<td>0.900</td>
<td>0.939</td>
</tr>
<tr>
<td>12B1</td>
<td>$H_e$</td>
<td>0.431</td>
<td>0.475</td>
<td>0.883</td>
<td>0.810</td>
<td>0.287</td>
<td>0.614</td>
<td>0.576</td>
</tr>
<tr>
<td>Average $H_e$</td>
<td>0.831</td>
<td>0.852</td>
<td>0.854</td>
<td>0.854</td>
<td>0.603</td>
<td>0.774</td>
<td>0.688</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4. $F_{ST}$ (below diagonal) and $R_{ST}$ (above diagonal) values for each population pair

<table>
<thead>
<tr>
<th>Locus</th>
<th>STR</th>
<th>FOR</th>
<th>FAL</th>
<th>DON</th>
<th>LIO</th>
<th>BAR</th>
<th>TAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>21D9</td>
<td>0.153</td>
<td>0.127</td>
<td>0.131</td>
<td>0.162</td>
<td>0.285</td>
<td>0.244</td>
<td></td>
</tr>
<tr>
<td>13D10</td>
<td>0.062</td>
<td>0.136</td>
<td>0.052</td>
<td>0.125</td>
<td>0.327</td>
<td>0.141</td>
<td></td>
</tr>
<tr>
<td>28A7</td>
<td>0.071</td>
<td>0.110</td>
<td>0.076</td>
<td>0.246</td>
<td>0.202</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12B1</td>
<td>0.072</td>
<td>0.046</td>
<td>0.025</td>
<td>0.190</td>
<td>0.023</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIO</td>
<td>0.183</td>
<td>0.178</td>
<td>0.196</td>
<td>0.250</td>
<td>0.111</td>
<td>0.082</td>
<td></td>
</tr>
<tr>
<td>BAR</td>
<td>0.137</td>
<td>0.160</td>
<td>0.140</td>
<td>0.240</td>
<td>0.176</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>TAV</td>
<td>0.155</td>
<td>0.137</td>
<td>0.160</td>
<td>0.140</td>
<td>0.240</td>
<td>0.176</td>
<td>0.039</td>
</tr>
</tbody>
</table>
BOTTLENECK software, sign test, Wilcoxon test and standardized differences test, the latter was not employed, because it requires at least 20 polymorphic loci to be reliable. Even so, the four polymorphic SSRs do not guarantee high statistical power. The presence of genetic bottlenecks was tested under the IAM, the SMM and the TPM models of evolution. In neither case was evidence of a recent (within approx. the past $2N_e-4N_e$ generations) bottleneck found.

Analysis of the population structure

Since this study concerns a rare and endangered species, it was of paramount importance to estimate $K$, the most probable number of ‘genetic units’ or ‘gene pools’ present in the data, in order to be able to suggest possible mechanisms that have shaped their genetic variability, and to reach conservation recommendations. This was done by applying the Bayesian clustering method as implemented by STRUCTURE (Pritchard et al., 2000). The estimate of $K$ was based on $\Delta K$, the second-order rate of change of the likelihood function with respect to $K$, as suggested by Evanno et al. (2005). A sharp signal was found at $K=2$ (see Supplementary Information Table, available online), therefore suggesting that two homogeneous gene pools shaped the genetic structure of the populations analysed. To check the composition of each individual population and each plant with respect to the inferred populations, further analysis was conducted based on $K=2$. The results are shown for the populations in Fig. 3. Analysis of the genetic components of the populations shows that the STR, FOR, FAL, DON and TAV populations derive the major component of their genetic composition from the first inferred population and the LIO and BAR populations from the second. Quantitative analysis of this process is also shown in in a supplementary figure (Supplementary Information, available online), where the contribution of the two inferred gene pools is reported in graphical form for each of the plants analysed.

![Fig. 3. Analysis of population structure according to a Bayesian clustering method. The populations studied derive their genetic structure from two inferred populations (‘gene pools’ 1 and 2) of origin. A pie diagram indicates the proportion of membership of each inferred population (black or white) in the real populations studied.](image)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Percentage of variation</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Two regions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among regions</td>
<td>1</td>
<td>840</td>
<td>0.050</td>
</tr>
<tr>
<td>Among populations</td>
<td>5</td>
<td>9.33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Within regions</td>
<td>763</td>
<td>82.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(B) Three regions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among regions</td>
<td>2</td>
<td>10.01</td>
<td>0.009</td>
</tr>
<tr>
<td>Among populations</td>
<td>4</td>
<td>7.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Within regions</td>
<td>763</td>
<td>82.63</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

$P$ values are estimated based on a permutation test (1000 randomizations).

AMOVA

The total amount of genetic variation was also partitioned by AMOVA into components according to the geographic subdivision of the populations. First, based upon the analysis of the population structure, the hypothesis that the populations fall into two geographic regions was tested, separating the Alghero area from the rest of the range. The AMOVA results (Table 5A) show that the within-population component accounts for 82 % of the total variance and that both the differences between regions and the differences between populations within a region account for smaller, but significant, amounts of the total genetic variation. Second, the hypothesis that all three geographic areas (Fig. 1) harbour significant amounts of variation was tested. This partitioning of the data revealed that 10 % of the genetic variance resided between regions and 7 % between populations within regions (Table 5B).

DISCUSSION

Genetic variability

*Centaurea horrida* is the only species belonging to the *Horridae* section of subgenus *Acrolophus* (Dostál, 1976), which previously included also *C. balearica* now re-classified as *Femeniasia balearica* Susanna. Today, this species is rare and survives only in a few scattered populations in northern Sardinia, occupying $<50$ ha ($0.5$ km$^2$) of Real Area of Occupancy (RAOO) in four different areas. In this paper, seven natural populations of *C. horrida* covering the entire distribution range of the species were analysed using four microsatellite genetic markers. This represents the first attempt at assessing the amount and distribution of genetic variability of this species and therefore constitutes a first step towards the planning of sound conservation strategies.

The amount of genetic variability found was medium-high, as indicated by the values of $H_e$, ranging from $0.603$ (LIO) to $0.854$ (FAL and DON). The north-western populations were those showing the highest levels of heterozygosity, while the lowest value was observed in the Alghero area. In the congener species *C. corymbosa*, estimates of $H_e$ by means of SSR markers in six natural
populations yielded values in the range of 0.36–0.62 (Fréville et al., 2001). It is to be noted that the four SSRs used in the present work are the same used by Fréville and colleagues, making these results directly comparable.

In another rare species belonging to Asteraceae, Femeniasia balearica, with a lifestyle very similar to that of C. horrida and a comparably small habitat, quite high levels of genetic variation were found by means of AFLP (Vilatersana et al., 2007). Allozyme analysis of seven species of the Centaurea genus endemic to Sicily (Bancheva et al., 2006) revealed heterozygosity values ranging from $H_e = 0.126$ for Centaurea cineraria subsp. Cineraria to $H_e = 0.276$ in Centaurea todari. All these species grow on limestone cliffs. In another endemic species, Centaurea tenorei, in the Sorrentina peninsula, in southern Italy, which has populations irregularly located in an area including coastal zones and internal ridges, the amount of genetic variability was assessed again by means of allozymes (Palermo et al., 2002). The lowest $H_e$ value was observed in C. tenorei subsp. tenorei (0.08), while the highest was observed in C. parlatoris (0.34). It is noted that estimates of genetic diversity obtained with AFLP, microsatellite, and allozyme markers are not directly comparable due to differences in mutation rates. Nevertheless, the data at hand suggest that high genetic diversity values may have played a role in allowing the survival of these species in a harsh and (presumably) stressful highly stressed environment. This is particularly true for C. horrida, which lives on shallow soil on rocky sea cliffs and is exposed to strong winds and high levels of salinity.

Linkage disequilibrium (LD) was pronounced in the populations studied, all loci being in LD, with a few exceptions. LD can arise as a consequence of a reduction in effective population size that enhances drift. However, evidence of a relatively recent and severe genetic bottleneck, which could have been the result of habitat fragmentation, was not detected. The results obtained need to be confirmed on a larger data set, because a low number of genetic markers greatly reduces the power of the statistical tests used, under both IAM and SMM (Cornuet and Luikart, 1996). It is recommended (Piry et al., 1999) that at least ten polymorphic loci are analysed to achieve a statistical power higher than 0.8. Even under the TPM, arguably the more appropriate model of evolution for SSRs (Di Rienzo et al., 1994), the present data failed to display any evidence of reduction in $N_e$.

The possibility cannot be ruled out that LD has arisen as a consequence of physical linkage between the loci, since no genetic map is available. A third explanation is that LD has arisen as a result of positive selection acting on loci linked to the SSRs used (Kim and Stephan, 2000). However, the presence of LD is an indication that further investigation into the mating system of C. horrida is needed, in order to assess the relationship between $N$ and $N_e$ in this species. A likely explanation for the observed LD is inbreeding within local populations of C. horrida, as also suggested by frequent heterozygote deficits (Table 3; see Results).

Despite the strong LD signal in the populations studied, the species does not display a reduction of genetic variability, as shown by the very high values of $H_e$ and by the absence of private alleles. This behaviour is peculiar, since other rare and endangered species of the Mediterranean basin, such as F. balearica (Vilatersana et al., 2007), are characterized by both a lower amount of genetic variability and by higher differentiation between populations. This issue will probably be clarified by the use of a larger set of genetic markers on the population studied.

**Genetic structure**

When dealing with conservation issues, it is often necessary to detect $K$, the number of panmictic units or ‘gene pools’ in the data, in order to be able to suggest possible mechanisms that have shaped the genetic variability observed. The use of a Bayesian approach to the detection of $K$ has become increasingly popular in the last decade (Bertorelle and Excoffier, 1998; Pritchard et al., 2000). In the present study it was possible to estimate $K = 2$ as the number of inferred populations from which the studied populations derive. The most precise interpretation of this value is that two homogeneous gene pools contributed to the seven populations sampled. The LIO and BAR populations may have originated from the same ancestral population (see below; analysis of genetic differentiation between populations).

**Genetic differentiation**

The genetic divergence between populations, as estimated by $F_{ST}$ and $R_{ST}$, was high ($F_{ST} = 0.123$ and $R_{ST} = 0.158$) even though lower than that observed in Femeniasia balearica, where the amount of genetic variation found between populations was 30% of the total genetic variation observed, based on an AMOVA analysis of AFLP genotypes and in C. corymbosa, where an overlapping set of microsatellite markers estimated $F_{ST} = 0.23$. The high levels of genetic differentiation observed are those expected for a species characterized by a scattered distribution pattern, which may well limit gene flow, thus determining the differentiation values observed in C. horrida populations. In a similar study conducted on the rare Eryngium alpinum (Umbelliferae), a species which bears evidence of comparable biological and ecological traits (seed set production and short distance dispersion), the differentiation observed was $F_{ST} = 0.23$ between 12 populations genotyped by seven SSRs (Gaudeul et al., 2005).

Genetic differentiation was evaluated also between pairs of populations and proved significant in all cases, based on a permutation test. The lowest differentiation was found for the population pair FAL–DON (0.046), which are located close to each other in the Stintino area. In general, the populations of the Asinara–Stintino groups display lower levels of differentiation. Their isolation is in fact recent: given the shallow nature of the sill between Stintino peninsula and Asinara island, which is only about 20 m deep, it dates back only to the end of the Würmian, about 13 ka cal BP (Antonioli et al., 2004).
The highest \( F_{ST} \) values were found for the populations LIO–TAV (0.24), which are at the extremes of the distribution on an east–west axis, but also for the populations FOR–LIO (0.23), which are separated by about 30 kilometres of coastline. While in the first case it can be assumed that geographic distance is responsible for the high differentiation, in the second case an alternative explanation must be found. Most of the area between FOR and LIO is an unsuitable habitat for \textit{C. horrida}, and has been so for the last 100 000 years (S. Andreucci, University of Sassari, Italy, pers. comm.), as it hosts dense juniper woods and more competitive shrub communities. Taking into account both the very low dispersal ability and the habitat specificity of \textit{C. horrida}, it could be argued that genetic differentiation is more affected by biological barriers than by geographical distance.

The genetic divergence between populations was also estimated by \( R_{ST} \), the \( F_{ST} \) analogue based on the stepwise mutation model. The highest \( R_{ST} \) value was again found between the Tavolara island population and that of the Alghero area; the lowest was observed between the pairs DON–LIO and DON–TAV. All the \( R_{ST} \) values were significantly different from zero, and consistently higher than those for \( F_{ST} \). An exception to this trend is presented by the LIO population; for five out of six pairwise population comparisons involving LIO, \( F_{ST} \) was greater than \( R_{ST} \). This can be interpreted as ongoing differentiation because of recent genetic drift, due to the peculiar ability of \( R_{ST} \) to detect differentiation events older than those revealed by \( F_{ST} \). This hypothesis is at least in part corroborated by the presence, in the LIO population, of two out of five loci pairs showing linkage disequilibrium, a characteristic typical of small isolated populations.

Mantel’s test, used to confirm the presence of isolation-by-distance (IBD) between the populations studied, was significant, thus IBD played a role in shaping the present distribution of genetic variability. This is in agreement with the separation of the populations studied in different geographical regions, as indicated also by the AMOVA results. The amount of gene flow, however, is quite low, estimated at about 1.7 migrants/generation. This is probably due to both restricted pollen dispersal and to the poor ability of \textit{C. horrida} to disperse achenes (Pisanu et al., 2007).

**Implications for conservation**

The current distribution area of \textit{Centaurea horrida} consists of tracts of land that have neither been below sea level nor subjected to volcanic or sedimentary events since the Miocene (Carmignani et al., 2001). The divergence observed between the populations studied is therefore to be ascribed to events linked to the life-cycle, the mating system and, in recent years, anthropogenic impacts on the species. The position of re-assessing what is meant by a ‘population’ is of the utmost importance, especially when dealing with conservation problems and in cases where the geographical proximity of individuals is not always indicative of their provenance from a single Mendelian unit. The combined results of Mantel’s test, Bayesian analysis and AMOVA that were obtained suggest that three distinct conservation units exist, from the point of view of management. To successfully preserve the genetic diversity of the species, special regard should be given to in situ strategies, since the amount of genetic variation harboured in each population is still high and the number of individuals, with the exception of the Tavolara population, is not low. However, fragmentation of the populations should be avoided, to prevent problems due to loss of diversity. All the areas where \textit{C. horrida} grows are included in the \textit{Natura 2000} network, each at different levels of protection.

A more thorough characterization of the ecological features of \textit{Centaurea horrida} is under way, which should provide further useful insights for conservation. For example, a significant effect of the site on seed production and germination has been found (Pisanu, 2007), which could affect patterns of genetic diversity. Given the changes in climate that the Mediterranean area is likely to undergo in the future, the genetic composition of the populations of \textit{C. horrida}, a plant adapted to harsh conditions, could also provide us with an interesting model to understand ecological and evolutionary responses to drought stress due to climate change.

**SUPPLEMENTARY INFORMATION**

Supplementary information is available online at http://aob. oxfordjournals.org/ and consists of a table giving estimates of \( K \), the number of inferred populations of origin, based upon the ‘\( \Delta K \)’ method for \textit{Centaurea horrida}, and a figure showing quantitative analysis of the genetic structure in the seven populations of \textit{C. horrida} studied in this work.

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