Conservation genetics of the rare Pyreneo-Cantabrian endemic
Aster pyrenaeus (Asteraceae)

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Abstract

Background and aims
Aster pyrenaeus (Asteraceae) is an endangered species, endemic to the Pyrenees and Cantabrian Mountain ranges (Spain). For its long-term persistence, this taxon needs an appropriate conservation strategy to be implemented. In this context, we studied the genetic structure over the entire geographical range of the species and then inferred the genetic relationships between populations.

Methodology
Molecular diversity was analysed for 290 individuals from 12 populations in the Pyrenees and the Cantabrian Mountains using inter simple sequence repeats (ISSRs). Bayesian-based analysis was applied to examine population structure.

Principal results
Analysis of genetic similarity and diversity, based on 87 polymorphic ISSR markers, suggests that despite being small and isolated, populations have an intermediate genetic diversity level ($P\% = 52.8\%$, $H_E = 0.21 \pm 0.01$, genetic similarity between individuals $= 49.6\%$). Genetic variation was mainly found within populations (80–84%), independently of mountain ranges, whereas 16–18% was found between populations and <5% between mountain ranges. Analyses of molecular variance indicated that population differentiation was highly significant. However, no significant correlation was found between the genetic and geographical distances among populations ($R_s = 0.359$, $P = 0.140$). Geographical structure based on assignment tests identified five different gene pools that were independent of any particular structure in the landscape.

Conclusions
The results suggest that population isolation is probably relatively recent, and that the outbreeding behaviour of the species maintains a high within-population genetic diversity. We assume that some long-distance dispersal, even among topographically remote populations, may be determinant for the pattern of genetic variation found in populations. Based on these findings, strategies are proposed for genetic conservation and management of the species.

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Introduction

In alpine environments, the distribution of species is often fragmented due to pronounced mountainous topography and associated abiotic heterogeneity on small spatial scales (Kudo 1991; Molau 1993; Körner 2003). Alpine plant species usually form local populations of various sizes, exhibiting a marked ability for extended local persistence due to perenniality and/or clonality (Bliss 1971; Körner 2003). The characteristics of fragmented populations have profound consequences on the species genetic patterns, which are crucial to elucidate for adequate management of endangered populations and species. Genetic variation within plant species is determined by a number of different factors such as reproductive mode (sexual vs. clonal), breeding system (outcrossing vs. selfing), life-history traits, population history, geographical range or selective constraints (Loveless and Hamrick 1984). These factors are also mainly responsible for the way the total genetic variation of a species is partitioned between and within populations (Hamrick et al. 1992).

The spatial isolation that is often accompanied by a reduction in the levels of gene flow leads to isolation by distance and to a high genetic differentiation among populations. However, small-scale heterogeneity and spatially differentiated selective constraints can lead to high levels of diversity within populations (Gugerli et al. 1999; Till-Bottraud and Gaudeul 2002). For entomophilous plant species, small, isolated populations may provide too few mates and little attraction or reward for pollinators (Kunin 1997; Dauber et al. 2010), leading to a reduction in the quality and quantity of pollination services (Wilcock and Neiland 2002), particularly exacerbated when rare plants are surrounded by other flowering species (Duncan et al. 2004; Lazaro et al. 2009). This will reduce seed set and gene flow within and between populations. Such factors combine to erode genetic diversity within populations and enhance between-population differentiation (Rathcke and Jules 1993; Steffan-Dewenter and Tscharntke 1999). Moreover, species in small, isolated populations may lose genetic diversity through stochastic processes such as genetic drift and become less fit due to increased inbreeding (Ellstrand and Elam 1993; Byers and Waller 1999) and Allee effects, which can eventually lead to extinction (Groom 1998). Increasing population size and maximizing genetic diversity are among the primary goals of conservation management (Frankham et al. 2002; Van Dyke 2008).

The pattern of geographical variation in population genetic diversity and differentiation will be influenced by both historical and contemporary changes in population size and gene flow (Vucetich and Waite 2003). The effect of population history is especially significant for species that have survived the long glacial episodes of the Pleistocene because their current distribution and genetic pattern is the result of successive range shifts during glacial and interglacial cycles (Hewitt 2004).

Unlike plants from the Alps, very few studies have focused on the genetic diversity of plant populations in the Pyrenees (Segarra-Moragues and Catalán 2003, 2010; Segarra-Moragues et al. 2007; Lauga et al. 2009) and even less on those in the Cantabrian Mountains (Peredo et al. 2009); thus the present study provides new insights into genetic diversity patterns across the Pyrenees and the relationship between Pyrenean and Cantabrian Mountain ranges.

**Aster pyrenaeus** DC (Asteraceae) is a critically endangered perennial species, endemic to the French Pyrenees and Cantabrian Mountains (Cambecèdes and Largier 2003). The species was first identified and collected from an unknown Pyrenean population and planted in the Royal Gardens of the Kingdom of France around 1685. Native populations were extensively harvested by botanical collectors until the early 20th century (Cambécèdes and Largier 2003) and the species was thought to be nearly extinct in the early 1990s with only three known populations. However, because it prefers very steep mountain slopes, often with difficult access, its current distribution remained unknown. Today, 14 isolated populations (sometimes very small) are known in France and Spain (Cambécèdes and Largier 2009). The species has been protected since 1982 in France and 1990 in Spain. Recently, the main threat to the species has changed from collectors to the decrease in grazing animals, favouring the expansion of competitive species and habitat closure (Cambécèdes and Largier 2009). Thus, during the last decade, *A. pyrenaeus* has been a high priority for conservation efforts from both the French government (Directive Habitats 92/43/CEE) and the autonomous region of Asturias (Decreto 65/1995), increasing the urgency to document and understand the genetic structure of this endangered plant species. Previous field studies indicated that *A. pyrenaeus* is mainly an outcrossing species and produces wind-dispersed achenes with a pappus (Guzman et al. 2003; García 2004).

Given its biology, history and current distribution, we expect the populations of *A. pyrenaeus* to exhibit low levels of genetic diversity and high population differentiation and, consequently, smaller populations at greater risk of extinction. Indeed, many rare endemic species show low genetic diversity compared with widespread taxa [i.e. *Cycas guizhouensis* K.M. Lan & R.F. Zou (Xiao et al. 2004), *Chamaecrista semaphore* Moench (Da Silva et al. 2007)]. However, other rare species have been...
shown to present rather high levels of genetic diversity [i.e. *Nouelia insignis* Franch. (Luan et al. 2006), *Physeria bellii* Mulligan (Kothera et al. 2007)]. Nevertheless, the current genetic pattern of *A. pyrenaeus* could mainly result from the response of the species to glacial/post-glacial climatic changes. Basic knowledge on the past history and population dynamics of this species is indispensable to implement a preservation programme. The geographical structure of the genetic diversity still needs to be characterized in order to define appropriate sampling strategies for conservation purposes.

To characterize the genetic pattern of *A. pyrenaeus*, we used the inter simple sequence repeat (ISSR) technique, which has been widely applied in conservation genetics (Xiao et al. 2004; García-Gonzales et al. 2008; Crema et al. 2009; Su et al. 2009) and to resolve phyleogeographical issues (Graves and Schrader 2008; Li et al. 2008). We first studied the within- and among-population genetic diversity in the Pyrenees and Cantabrian Mountains. We then inferred the genetic relationships between these populations with respect to their geographical locations. We used the results to establish recommendations for conservation, management and restoration of this endangered species. Finally, we propose a scenario describing the history of *A. pyrenaeus* populations during the last postglacial period.

Materials and methods

**Studied species**

*Aster pyrenaeus* (*2n = 18*) is a perennial herb 40–100 cm in height. It grows on calcareous rocky north- and east-facing slopes between 500 and 2400 m a.s.l. The flowering period extends from mid-July to mid-October (Guzman et al. 2003). It is a gynomonoecious species (the inflorescence has both hermaphroditic protandrous yellow disc florets and pistillate blue–lilac ray florets) mainly visited by *Cephus* sp. (Cephidae, Hymenoptera), *Neoascia podagrica* (Syrphidae, Diptera) and *Odontomya ornata* (Stratiomydidae, Diptera) (Guzman et al. 2003). Autonomous self-pollination has occasionally been shown to result from the response of the species to glacial/post-glacial climatic changes. Basic knowledge on the past history and population dynamics of this species is indispensable to implement a preservation programme. The geographical structure of the genetic diversity still needs to be characterized in order to define appropriate sampling strategies for conservation purposes.

To characterize the genetic pattern of *A. pyrenaeus*, we used the inter simple sequence repeat (ISSR) technique, which has been widely applied in conservation genetics (Xiao et al. 2004; García-Gonzales et al. 2008; Crema et al. 2009; Su et al. 2009) and to resolve phyleogeographical issues (Graves and Schrader 2008; Li et al. 2008). We first studied the within- and among-population genetic diversity in the Pyrenees and Cantabrian Mountains. We then inferred the genetic relationships between these populations with respect to their geographical locations. We used the results to establish recommendations for conservation, management and restoration of this endangered species. Finally, we propose a scenario describing the history of *A. pyrenaeus* populations during the last postglacial period.

**Sampling procedure**

We studied 10 populations in the French Pyrenees and two populations in the Cantabrian Mountain range in northern Spain (Fig. 1, Table 1). Population sizes are highly variable, ranging from 11 to ~2500 individuals (Table 1). Distances between populations varied from 1 km (PANL and PANH) to 400 km (CAU and DUJ; CAU and BUL). The number of individuals sampled in each population varied from 5 to 37, depending on population size and the difficulty of accessing the study site (Table 1). Sampling effort for populations TAC and CAU (i.e. five individuals sampled) was low due to the difficult access. Leaf material (one leaf per individual) was collected from a total of 290 individuals and stored in silica gel. DNA extraction was performed with the DNeasy Plant Mini Kit (Qiagen, Paris, France) according to the manufacturer’s protocol, using 40 mg of dried leaf material. DNA concentration was determined by spectrophotometry with the NanoDrop™ ND-1000 (Thermo Scientific, Courtaboeuf, France).

**ISSR procedure**

Six of 49 ISSR primers produced clear and reproducible bands, and were hence selected for further study. Polymerase chain reaction (PCR) amplification was performed in a total volume of 25 μL, consisting of 20 ng of DNA template, 1 × PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 2 mM primer, 0.625 U Go Taq DNA (Promega, France) and purified water. Each PCR cycle consisted of the following steps: initial denaturation at 95 °C for 3 min, 38 cycles of 40 s denaturation at 95 °C, 40 s annealing at the primer’s Tₘ (Table 2), 1 min extension at 72 °C and a final 5 min extension at 72 °C. For each primer, we determined the best annealing temperature by performing a gradient PCR. The PCR products were separated on 2% agarose gels buffered with 1 × TAE for 2 h 30 min at 100 V, detected by staining with ethidium bromide and photographed under ultraviolet light. Molecular weights were estimated using 50- and 100-bp DNA ladders (Promega). For all samples, PCR reactions were carried out using the same thermocycler. To assess the reproducibility of the ISSR patterns for each primer, PCR reactions were repeated twice for 50 samples. No band variation was detected when the two runs of a given DNA sample were compared. Positive controls were systematically included in each PCR run and in each electrophoresis gel to facilitate intergel comparisons, to check the efficiency of PCR, and to test for the reproducibility of ISSR patterns. Negative controls (without template DNA) were also included in every run to test for contamination in the reagents.

**Data analysis**

Inter simple sequence repeat bands were scored as present (1) or absent (0) and a binary matrix was manually constructed. Assuming that populations are in Hardy–Weinberg equilibrium (Fₛ = 0), the software program AFLP-SURV v.1.0 (Vekemans et al. 2002) was used to estimate within-population genetic diversity.

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through the following parameters: percentage of polymorphic loci ($P\%$), Nei’s (1978) unbiased expected heterozygosity ($H_e$). Similarity of ISSR profiles between individuals was calculated using Nei and Li’s (1979) similarity index ($S = \frac{2n_{xy}}{n_x + n_y}$), where $n_x$ and $n_y$ refer to the number of ISSR bands in individuals $x$ and $y$, respectively, and $n_{xy}$ is the number of bands shared by both individuals $x$ and $y$. Only polymorphic bands were considered in the index calculation. We checked the whole dataset for private fragments in populations.

To analyse the genetic differentiation and geographic structure of $A.\ pyrenaeus$ populations, variation in ISSR patterning was examined with analysis of molecular variances (AMOVA) using ARLEQUIN v. 3.01 (Excoffier et al. 2005). Analysis of molecular variances was performed at different hierarchical levels: (i) between all the 12 populations included in a global analysis, (ii) between mountain ranges (Pyrenees vs. Cantabrians), and (iii) between populations in a given mountain range (either Pyrenees or Cantabrians). $F$-statistics were computed under the random mating hypothesis with ARLEQUIN v. 3.01 (Excoffier et al. 2005). This provided the unbiased $F_{ST}$ estimator $\theta$, following Weir and Cockerham (1984) for which 95\% confidence intervals were obtained by bootstrapping 1000 replicates over loci. Fisher’s exact tests were performed, using Genepop v. 4 (Raymond and Rousset 1995; Rousset 2008), on marker frequencies at each locus between all pairs of populations to determine whether significant differences in marker frequencies existed between groups of individuals. To determine the genetic relationships among populations, the AFLP-SURV v. 1.0 (Vekemans et al. 2002) and the PHYLIP packages

Fig. 1 Geographical structure of 12 populations of $A.\ pyrenaeus$ in the Pyrenees (1) and Cantabrian Mountains (2) calculated using the STRUCTURE software v. 2.3.3. The pie charts give the proportions of the gene pools present in the population.
(NEIGHBOR and CONSENSE; Felsenstein 1989) were used to calculate pairwise Nei’s genetic distance (Nei 1978) between each population, and to construct a neighbour-joining (NJ) tree based on 10,000 permuted trees, bootstrapped across loci. Isolation by distance was tested by Mantel tests (10,000 permutations) performed between pairwise estimates of $F_{ST}$ ratio and the logarithm of geographical distance (natural logarithm scale) for all samples (Rousset 1997) using ARLEQUIN v. 3.01 (Excoffier et al. 2005). We next applied a model-based clustering method to infer genetic structure and define the most adequate number of clusters in the whole dataset using the software STRUCTURE v. 2.3.3 (Pritchard et al. 2000). We set the number of clusters ($K$) from 1 to 14 and ran 20 independent runs for each $K$ value. Each run consisted of a burn-in period of $10^5$ steps followed by $10^6$ Markov chain Monte Carlo repetitions, assuming an admixture model, a uniform prior for alpha and correlated allele frequencies with prior population information. We used the ad hoc statistic $\Delta K$ to identify the most likely number of clusters in the dataset (Evanno et al. 2005). Because independent runs can produce different permutations of the group labels, we used CLUMPP v. 1.1.1 (Jakobsson and Rosenberg 2007) to align the membership coefficient matrices from the 20 highest likelihood runs for each $K_{\text{max}}$ (Full Search algorithm with random input order and $10^5$ permutations to align the runs). The CLUMPP output consists of the same permuted matrices so that all replicates are as closely matched as possible. In order to detect substructure, we again applied the same Bayesian-based analysis within each predefined cluster. We then assigned each individual to a gene pool if the membership probability was $>0.6$ (Coulon et al. 2008).

Results

Within-population diversity

The six primer pairs used in the study generated a total of 102 reliable ISSR bands, of which 87 were polymorphic.

### Table 1 Name, location, altitude and size of the 12 studied *A. pyrenaeus* populations. The PAN site consists of two populations separated by $\sim1$ km (PANL and PANH).

| Population location | Population code | Elevation (m) | Longitude/latitude | Sample size | Population size |
|---------------------|-----------------|--------------|--------------------|-------------|-----------------|
| Cauterets           | CAU             | 1150         | 2°27'W/42°53'N     | 5           | 11              |
| Cirque du Litor—Béost | LIT             | 1400         | 2°38'W/42°57'N     | 29          | 33              |
| Vallon de Tachet—Arrens valley | TAC | 1500         | 2°35'W/42°55'N     | 5           | 20              |
| Gerbe—Ossau valley  | GER             | 1350         | 2°47'W/43°00'N     | 30          | 1000            |
| Montagne de Pan (low part)—Ossau valley | PANL | 600          | 2°46'W/42°58'N     | 26          | 50              |
| Montagne de Pan (high part)—Ossau valley | PANH | 900          | 2°46'W/42°58'N     | 28          | 1000            |
| Pic de Bergon—Aspe valley | BER | 1340         | 2°52'W/42°58'N     | 29          | 500              |
| Laberouat—Aspe valley | LAB            | 1615         | 3°00'W/42°57'N     | 27          | 100             |
| Refuge de Laberouat—Aspe valley | RELAB | 1450         | 2°59'W/42°57'N     | 31          | 100             |
| Piquet de Lhurs—Aspe valley | LHU | 1480         | 3°01'W/42°55'N     | 28          | 50              |
| French Pyrenees      |                 |              |                    |             |                 |
| Cantabrian Mounts (Spain) |     |              |                    |             |                 |
| Bulne               | BUL             | 800          | 4°50'W/43°14'N     | 22          | 2500            |
| Val del Duje        | DUJ             | 450          | 4°48'W/43°15'N     | 30          | 450             |

### Table 2 Primers used in ISSR analyses of *A. pyrenaeus*, annealing temperature ($T_m$ in °C) and number of reliable and polymorphic bands for each primer. $B = (C, G or T)$, $D = (A, G or T)$, $R = (A or G)$, $W = (A or T)$ and $Y = (C or T)$.

| ISSR sequence | $T_m$ | No. of bands | % of polymorphic |
|---------------|-------|--------------|------------------|
| ISSR sequence | $5' \to 3'$ | analysed | bands |
| BDB(ACA)$_4$  | 50.6  | 20           | 90               |
| WB(GACA)$_4$  | 46.0  | 10           | 70               |
| (GT)$_8$C     | 46.0  | 9            | 78               |
| (AC)$_8$YG    | 54.8  | 18           | 100              |
| (AG)$_8$C     | 56.5  | 17           | 82               |
| (TG)$_8$RC    | 46.0  | 28           | 96               |
(85.29%; Table 2). Within populations the mean percentage of polymorphic loci (P%) reached 63.2%, ranging from 33.3% (CAU and TAC) to 75.8% (LHU; Table 3). The expected heterozygosity (He) reached on average 0.21 ± 0.02, and was between 0.13 ± 0.01 (CAU and TAC) and 0.23 ± 0.02 (GER and LHU). At the species and the Pyrenees mountain region levels, P% and He were very similar (Table 3) but were lower in the Cantabrians. The mean genetic similarity reached 49.7% within populations. The LAB population had the lowest genetic similarity (44.0%) while the GER population had the highest (55.7%). No private fragments were found in any population. In each population, P% and He were significantly and positively correlated (R² = 0.939, P < 0.001). Furthermore, we detected no influence of population size on molecular diversity (P%; R² = 0.072, P = 0.400; He; R² = 0.084, P = 0.360).

### Genetic differentiation and geographical structure

Whatever the hierarchical level considered, genetic variation was always much higher within populations (80.68–84.02%, AMOVA; Table 4) than among populations (15.98–18.01%). ΦST values were substantially similar (0.16–0.18%), indicating a moderate among-population differentiation. Pairwise ΦST ranged from 0.075 to 0.351; all values differed from zero (Table 5), which was confirmed by global Fisher’s exact tests (χ² = 1227.8, d.f. = 174, P < 0.001). In contrast, genetic variation among mountain ranges was very low (~5%) but still highly significant (P < 0.001). The result of the Mantel test indicated a limited isolation by distance, because the pairwise genetic distances measured as FST/(1 − FST) and the logarithm of the distance between pairs of populations were not significant (Rs = 0.359, P = 0.140). Neighbour-joining analysis based on Nei’s genetic distances failed to support geographical clustering. Only three groups were significantly identified, with moderate bootstrap support (>50%; Fig. 2), i.e. Cantabrian populations, PANH and GER, and RELAB and LHU from Ossau and Aspe Valley, respectively. Geographical structure based on STRUCTURE revealed that two genetic clusters (K = 2) had the best ad hoc statistical fit (Fig. 3). A substructure within each previous cluster was found with ΔK = 2 and 3 for the initial cluster A and B, respectively (Fig. 1). As a result, five different gene pools (A1, A2, B1, B2 and B3) were identified. Eight individuals out of 290 were not assigned to a genetic group with a membership probability >0.6. For the smallest populations (CAU and TAC), the average membership coefficients (q_mean) were very high (0.99 and 0.98, respectively), indicating nearly perfect assignment of individuals. Only gene pool A2 was detected in these small populations (Fig. 1). For larger populations, the pattern was not well structured since we detected two, three and four gene pools whatever the geographical distribution of the populations. In LHU and RELAB populations, A2 was the dominant gene pool (q_mean = 0.99), and A1 was the prevalent one in LIT, GER and PANH (q_mean = 0.98, 0.89 and 0.96, respectively). For LAB and BER populations, A1 (q_mean = 0.99 and 0.95, respectively) and B1 (q_mean = 0.97 and 0.90, respectively) were the dominant gene pools, while for PANL, population B3 was the main gene pool (q_mean = 0.95). Interestingly, Cantabrian populations presented four gene pools in nearly equal proportions (Fig. 1).

### Discussion

#### Within-population diversity

Endemic and narrowly distributed plants usually show lower levels of genetic diversity and higher levels of genetic structure compared with their relatives with wider distribution areas (Hamrick and Godt 1989; Nybom 2004). This is probably caused by the more accentuated effects of genetic drift and restricted gene flow in the rarer plants (Hamrick and Godt...
For the endemic *A. pyrenaeus*, we revealed that whatever the mountain range or the population within a mountain range, most of the genetic diversity was found within populations. The same trend was commonly reported in outcrossing and/or perennial species (Hamrick et al. 1992).  

Table 4 Results of AMOVA based on 87 ISSR loci in two mountain ranges, Pyrenees and Cantabrians, at different hierarchical levels.

| Source of variation                        | d.f. | SS    | Variance components | % of the total variance | $\Phi$ values | $P$    |
|-------------------------------------------|------|-------|----------------------|-------------------------|---------------|--------|
| Global analysis                           |      |       |                      |                         |               |        |
| Among populations                         | 11   | 443.529 | 1.620               | 18.01                   | $\Phi_{ST} = 0.180$ | <0.001 |
| Within populations                        | 278  | 2067.018 | 9.220               | 81.99                   |               |        |
| Total                                     | 289  | 2510.547 | 10.850              | 100                     |               |        |
| Pyrenees                                  |      |       |                      |                         |               |        |
| Among populations                         | 9    | 360.209 | 1.504               | 15.98                   | $\Phi_{ST} = 0.159$ | <0.001 |
| Within populations                        | 226  | 1859.866 | 9.253               | 84.02                   |               |        |
| Total                                     | 235  | 2220.076 | 10.757              | 100                     |               |        |
| Cantabrian Mountains                      |      |       |                      |                         |               |        |
| Among populations                         | 1    | 18.128  | 1.727                | 17.09                   | $\Phi_{ST} = 0.171$ | <0.001 |
| Within populations                        | 53   | 207.152 | 9.006                | 82.91                   |               |        |
| Total                                     | 54   | 225.280 | 10.734              | 100                     |               |        |
| Pyrenees vs. Cantabrian Mountains         |      |       |                      |                         |               |        |
| Among mountain range                      | 1    | 65.191  | 0.559                | 4.96                    | $\Phi_{CT} = 0.049$ | <0.013 |
| Within population among mountain range    | 10   | 378.338 | 1.510                | 14.37                   | $\Phi_{SC} = 0.140$ | <0.001 |
| Within populations                        | 278  | 2067.018 | 9.227               | 80.67                   | $\Phi_{ST} = 0.183$ | <0.001 |
| Total                                     | 289  | 2510.547 | 11.298              | 100                     |               |        |

d.f., degree of freedom; SS, sum of squares.

Table 5 Pairwise estimated $\Phi_{ST}$ values among 12 populations of *A. pyrenaeus*. All values differed significantly from zero.

|       | CAU | LIT | TAC | GER | PANL | PANH | BER | LAB | RELAB | LHU | BUL | DUJ |
|-------|-----|-----|-----|-----|------|------|-----|-----|-------|-----|-----|-----|
| CAU   |     |     |     |     |      |      |     |     |       |     |     |     |
| LIT   | 0.207|     |     |     |      |      |     |     |       |     |     |     |
| TAC   | 0.351| 0.213|     |     |      |      |     |     |       |     |     |     |
| GER   | 0.170| 0.095| 0.210|     |      |      |     |     |       |     |     |     |
| PANL  | 0.207| 0.160| 0.240| 0.125|     |      |     |     |       |     |     |     |
| PANH  | 0.175| 0.080| 0.135| 0.147| 0.136|     |     |     |       |     |     |     |
| BER   | 0.200| 0.129| 0.208| 0.140| 0.124| 0.103|     |     |       |     |     |     |
| LAB   | 0.130| 0.147| 0.176| 0.108| 0.134| 0.114| 0.075|     |       |     |     |     |
| RELAB | 0.228| 0.154| 0.190| 0.191| 0.181| 0.150| 0.153| 0.164|       |     |     |     |
| LHU   | 0.203| 0.126| 0.160| 0.149| 0.139| 0.130| 0.145| 0.118| 0.089|     |     |     |
| BUL   | 0.286| 0.194| 0.286| 0.230| 0.180| 0.150| 0.185| 0.156| 0.219| 0.191|     |     |
| DUJ   | 0.268| 0.129| 0.256| 0.276| 0.129| 0.148| 0.192| 0.134| 0.197| 0.149| 0.161|     |

For the endemic *A. pyrenaeus*, we revealed that whatever the mountain range or the population within a mountain range, most of the genetic diversity was found within populations. The same trend was commonly reported in outcrossing and/or perennial species (Hamrick et al. 1992).
*A. pyrenaeus* exhibits an intermediate level of mean intrapopulation genetic diversity (0.21 for Nei’s expected heterozygosity *H*<sub>E</sub>), which conforms to the value (*H*<sub>E</sub> = 0.20) found by Nybom (2004) in a literature survey for endemic species using dominant markers. Comparisons with other studies are difficult since genetic diversity depends on numerous factors, such as life history, breeding system, growth life forms, geographical range and even the type of molecular method used (Powell et al. 1996; Nybom 2004). In spite of these complications, if we compare the results of studies using dominant markers it appears that the genetic diversity of *A. pyrenaeus* is similar to that of other alpine species: *Eryngium alpinum* L. (*H*<sub>E</sub> = 0.20; Gaudeul et al. 2000), *Trollius europaeus* L. (*H*<sub>E</sub> = 0.22 in the Alps and 0.197 in the Pyrenees; Despres et al. 2002), *Epilobium fleischeri* Hochst., *Geum reptans* L. and *Campanula thyrsoides* L. (*H*<sub>E</sub> = 0.19, 0.21 and 0.20, respectively; Kuss et al. 2008) and *Senecio boissieri* DC (*H*<sub>E</sub> = 0.19 in the Cantabrian Mountains; Peredo et al. 2009). The values of *H*<sub>E</sub> and *P* % suggest that even small populations can maintain a high level of genetic diversity. Thus, we suggest that no recent severe bottlenecks occurred or that genetic diversity may not respond immediately to reduction in population size (Young et al. 1996). However, these small populations could also result from recently dispersed individuals from different population sources. We did not find a significant correlation between population size and genetic diversity despite the theoretical prediction that small populations might lose genetic variation due to genetic drift, founder effects or population bottlenecks (Ellstrand and Elam 1993; Young et al. 1996). Therefore, we have no indication that natural fragmentation resulted in a pronounced loss of genetic diversity within *A. pyrenaeus* populations. Other studies on alpine plants showed an inconsistent pattern for genetic diversity and population size relationships. Some have detected a significant correlation (*E. alpinum*, Gaudeul et al. 2000; *T. europaeus*, Despres et al. 2002; *E. fleischeri*, Kuss et al. 2008) while others found no correlation (*Hypericum nummularium* L., Gaudeul 2006; *G. reptans*, Kuss et al. 2008; *C. thyrsoides*, Kuss et al. 2008; Ægisdóttir et al. 2009), depending on the sampling parameters used (i.e. sample size, breeding system, marker system; Nybom 2004). The within-population diversity is also likely to be influenced by some life-history traits of the species such as the type of breeding system. Outcrossing plant species tend to have higher genetic variation within populations, whereas populations of selfing species or species with a mixed mating system are often genetically less variable (Hamrick and Godt 1996; Till-Bottraud and Gaudeul 2002; Nybom 2004). The within-population diversity is also likely to be influenced by some life-history traits of the species such as the type of breeding system. Outcrossing plant species tend to have higher genetic variation within populations, whereas populations of selfing species or species with a mixed mating system are often genetically less variable (Hamrick and Godt 1996; Till-Bottraud and Gaudeul 2002; Nybom 2004). In our study, genetic variability was mostly observed within population (80–84 %). On this basis, *A. pyrenaeus* can be considered as an outcrosser as previously found by Garcia (2004), which contributes to maintaining within-population genetic variability.
Genetic and geographical structure

The genetic structure of plant populations reflects the interactions of various evolutionary processes including the long-term evolutionary history, such as shifts in distribution, habitat fragmentation, and population isolation, mutation, genetic drift, breeding system, gene flow and selection (Schaal et al. 1998). Factors such as isolation, small populations and gene flow may have a major influence on the levels of genetic diversity within and among populations (Hamrick et al. 1992). Continuous distribution of plants usually weakens the differentiation among populations (Wright 1951).

Our analyses of genetic structure revealed a moderate differentiation among populations ($\Phi_{ST} = 0.18$). The pattern of population differentiation is confirmed by Fisher’s exact test, but the Mantel test revealed no correlation between geographical and genetic distances. In the two smallest populations, sampling effort was low; however, it represented 45.5 and 25% of the entire population for CAU and TAC, respectively. Thus, it is possible that not all the genetic variability was sampled, but as the level of genetic diversity within these populations is similar to that of larger populations it could be assumed that the sampled individuals may reflect the variability of the population.

In a meta-analysis of RADP-based estimates of $\Phi_{ST}$ values, Nybom and Bartish (2000) and Nybom (2004) demonstrated that $\Phi_{ST}$ values for long-lived perennial, endemic and outcrossed species with wind-dispersed seeds have the lowest $\Phi_{ST} (~0.25)$. The values for $\Phi_{ST}$ found in A. pyrenaicus populations, although slightly lower, were similar to these. Moreover, compared with other alpine perennials, pairwise $\Phi_{ST}$ values among populations ranging from 0.07 to 0.35 (Table 5) are widely reported (Gugerli et al. 1999; Young et al. 2002; Pluess and Stöcklin 2004). This intermediate level of population differentiation, coupled with the fact that most of the genetic variance occurs within populations (>80%), suggests that population isolation occurred recently (Gugerli et al. 1999; Segarra-Moragues and Catalán 2003; Pluess and Stöcklin 2004). This pattern of population differentiation is commonly described in other alpine plants and endemic species (Gugerli et al. 1999; Segarra-Moragues and Catalán 2003; Wesche et al. 2006). Genetic variation among all the populations or among the populations within a given mountain range was nevertheless highly significant ($P < 0.001$), which led us to conclude the occurrence of an impact of isolation. The Mantel test failed to reveal isolation by distance; therefore, geographical distance is not responsible for the reduction in gene flow between the different geographical locations. Although insect visitors to A. pyrenaicus flowers have been identified (Guzman et al. 2003), their effectiveness in pollination is unknown and there are no available data concerning pollen dispersal. However, given the remoteness of the populations, their isolation in valleys and the mountain barriers to pollinator displacements, pollen flow between populations appears unlikely. But given the inaccessibility of the A. pyrenaicus populations, currently sampled populations could be linked through isolated populations or individuals throughout the unexplored areas. The identification of new populations would tend to support this hypothesis. Between-population gene flow through seed dispersal is difficult to evaluate since no data on seed dispersal are available. Dispersal of achenes in the Asteraceae may be species dependent, and even in a single genus there may be variation in pappus length, weight and their associated dispersal capability (Andersen 1993). Thus, comparison with other species is problematic and more studies on seed dispersal in this species are needed. Yet, regarding the results obtained here, it may be considered that predominant wind flow in mountain areas could provide further opportunities for long-distance dispersal.

The Bayesian-based analysis performed by STRUCTURE allowed the detection of five different gene pools which did not reflect a particular structure in the landscape. Individuals were clustered in locations with variable estimated membership coefficients (0.60–0.99). The five genetic clusters included individuals belonging to different locations. Only small populations (CAU and TAC) were assigned to a single genetic group (A1). This pattern is supported by the pattern of the NJ tree (Fig. 2), which reveals little congruence to the geographical distance between populations.

Other studies conducted in the Pyrenees on threatened endemic species showed the same genetic structure (Borderea pyrenaica Miégev. and B. chouardii Gaussen Heslot, Segarra-Moragues and Catalán 2003; Delphinium montanum DC, Simon et al. 2001). Past demographic events, and current gene flow, are likely to be responsible for this present-day structure of genetic variation.

A number of palaeoendemic taxa from the Pyrenees, like A. pyrenaicus, are the likely descendants of Tertiary ancestors (Gaussen and Lerede 1948). Ice sheets rarely reached altitudes <1000 m in the Pyrenees and Cantabrians (García-Ruiz and Martí-Bono 1994); thus, as there is no apparent isolation by distance, it is likely that the species became established in large populations at lower altitudes during Pleistocene glaciations. The large and perhaps continuous distribution of ancestral populations over lowland areas could have been favoured by both the calcareous habit of A. pyrenaicus and the
almost continuous presence of limestone in the Pyrenean piedmonts and Cantabrian Mountains with no significant geological barriers preventing gene flow through the population. Then the species successfully recolonized the open territories at higher altitudes with the retreat of the glaciers.

**Implications for conservation**

Within-population genetic diversity is usually needed to ensure population establishment and long-term persistence as well as long-term evolutionary potential of restored populations (McKay *et al.* 2005). Even small populations of *A. pyrenaeus* appear to maintain high genetic diversity. Thus, from a genetic point of view, they do not seem endangered. However, primarily allogamous species like this often experience chronic pollen limitation due to the scarcity of both pollinators and mates and heterospecific pollen deposition (Eckert *et al.* 2010), which, in turn, reduces seedling recruitment within populations. Moreover, the populations are geographically isolated with limited gene flow between locations. Thus, for such small allogamous populations, management decisions need to be taken to prevent population extinction. Besides close monitoring of the size and the changes in the genetic structure of all populations, two main management proposals for long-term conservation of *A. pyrenaeus* populations can be suggested.

The first should concentrate on small populations, which may suffer from pollen limitation due to scarcity of both pollinators and mates (further investigations are needed to confirm this hypothesis). In order to increase reproductive success of individuals in small populations and promote natural recruitment, it is possible to saturate stigmas with cross pollen from the same population. We also suggest that seeds be collected from each individual, to maintain growing seedlings offsite and use the ex situ plants materials for in situ reintroduction. This requires a good understanding of the regeneration niche of the species by a detailed study of sites where individuals are currently present (soil, exposure, etc.).

Closely related self-incompatible species may suffer biparental inbreeding depression. Although, in the studied *A. pyrenaeus* populations, individuals seem to be distantly related (mean genetic similarity = 49.6%), we have no information on the genetic and spatial structures within populations (spatial autocorrelation). Therefore, pollen incompatibility between mates may occur between the more closely related individuals. The second management proposal could be the reinforcement of populations by seeds collected from surrounding populations with the same gene pool to avoid the possible negative effects of outbreeding depression. These include collecting plants or seeds locally, or from genetically close populations, matching climatic and environmental conditions between collection and restoration sites (McKay *et al.* 2005).

*Aster pyrenaeus* is located in areas where pastoral, forestry and hunting practices have long influenced the dynamics of the ecosystems. In the past, open areas were maintained within the range of *A. pyrenaeus* by livestock, agricultural burning and mowing practices. The abandonment of pastoralism, accentuated in recent years, leads to habitat closure due to forest propagation (and favours the expansion of the bracken, i.e. the rhizomatous fern *Pteridium aquilinum*, which could locally outcompete *A. pyrenaeus*) (Wencewiez 2002). A national action plan, drafted with scientists and managers, will lead to a better understanding of the threats, in particular, competition and the vegetation dynamics, and should lead to the proposal of management actions favourable to the species.

**Conclusions and forward look**

A high within-population genetic diversity is reported for the rare endemic *A. pyrenaeus* throughout its distribution range in the Pyrenees and Cantabrians, which can be explained by the outbreeding behaviour of the species. Despite the fact that it lives in isolated populations of different sizes, neither isolation of habitats nor population size affected genetic variability within the studied populations. Population differentiation was moderate, suggesting a restricted gene flow between populations and indicating that population isolation is probably relatively recent. Geographical distance was not found to be responsible for the reduction in gene flow between the different locations; in this context, we assume that some long-distance dispersal mechanisms, even among topographically remote populations, may be the crucial determinant for the pattern of genetic variation found. Further research should focus on the pollen and seed dispersal strategies in the *A. pyrenaeus* populations, and studies based on other markers from cp DNA and/or nr DNA might help to elucidate the comprehensive phylogeography of the species.

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Contributions by the authors
All the authors contributed to a similar extent overall.

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Conflicts of interest statement
None declared.

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