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On the verge of extinction: genetics of the critically endangered Iberian plant species, *Borderea chouardii* (Dioscoreaceae) and implications for conservation management

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Abstract

Borderea chouardii* is a relictual and dioecious, strictly sexually reproducing, long-living geophyte of the Dioscoreaceae family. Previous biological and demographic studies have indicated the existence of a uniformly distributed panmictic population of this taxon at the southernmost Spanish pre-Pyrenean mountain ranges where it occurs in rather inaccessible crevices of a single limestone cliff. However, individuals of *B. chouardii* are spatially subdivided into two subpopulations located, respectively, on the upper and lower parts of the cliff, and vertically separated 150 m. Because of its extreme rarity, *B. chouardii* was the first Iberian taxon to have a specific conservation plan and has been included in several red lists under the category of critically endangered (CR). However, no previous attempts have been conducted to analyse the fine scale evolutionary mechanisms involved in its present microspatial distribution. Genetic diversity and population structure have been investigated through the analysis of neutral hypervariable markers such as simple sequence repeats (SSRs) and randomly amplified polymorphic DNAs (RAPDs) to unravel the impact of life history traits in the differentiation of the two subpopulations. Both types of molecular markers were unequivocal in distinguishing two genetically distinct groups of individuals corresponding to their spatial separation. However, SSRs detected a higher level of subpopulation differentiation (F_{ST} = 0.35, R_{ST} = 0.32) than RAPDs (F_{ST} = 0.21). SSR data indicated significant deviation from random dispersal of genes and genotypes between the two groups, suggesting that mating occurs mainly among individuals within subpopulations, thus, favouring the divergence between the two groups. This microevolutionary differentiation scenario might have been caused by a coupled effect of past genetic drift and reproductive isolation, as a result of strong glacial age bottlenecks and inefficient dispersal system of pollen and seeds, respectively. The identification of such genetic structure in this narrow endemic prompts a modification of the management strategies of its single extant population.

Keywords: allotetraploids, conservation of endangered plants, genetic diversity, microsatellites, population structure, RAPD

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Introduction

An increasing number of studies have demonstrated the value of genetic data in addressing issues for the investigation of plant microevolutionary processes at fine scale levels (i.e. Mateu-Andrés & Segarra-Moragues 2000; Fréville *et al*. 2001; Hardy *et al*. 2003). Genetic data can be important additions to demographic data and information about reproductive biology for an adequate management of endangered populations and species. Genetic depletion, characteristic of species with a history of fragmented

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populations and small population sizes, is believed to have dramatic consequences on the ability of a species to survive environmental changes (Ellstrand & Elam 1993; Wise *et al*. 2002), as could be associated with certain fitness traits (Gautschi *et al*. 2002; Paschke *et al*. 2002). In this respect, neutral markers are useful to estimate the relative evolutionary importance of genetic factors such as mutation rates, gene flow, and genetic drift. Because of their narrow ranges, endemics are commonly characterized by low levels of genetic diversity (Hamrick *et al*. 1979, 1991; Ellstrand & Elam 1993; Gitzendanner & Soltis 2000). However, several exceptions have been reported (Ranker 1994; Batista *et al*. 2001; Hoebe & Young 2001) indicating the need to study each particular case.

Borderea chouardii (Gaussen) Heslot is a relictual and dioecious, long-living (up to 300 years old), apparently strictly sexually reproducing geophyte (García 1996, 1997; García *et al*. 2002) of the Dioscoreaceae family. It is known from a single population in the vicinity of the Sopeira Dam, Huesca Province (Spain) located in one of the southernmost pre-Pyrenean mountain ranges (Gaussen 1952; Moreno-Saiz 1990; Villar & Lazare 1991; Franco-Mugica 1993; García 1996, 1997). It grows on rather inaccessible crevices in a single limestone cliff. Rough estimates of its population size range from *c*. 500 (Gómez-Campo & Malato-Beliz 1985; Sainz-Ollero *et al*. 1996) to 2200 individuals (García *et al*. 2002; García 2003). Under the more optimistic estimates however, it has been observed that only one-half of these individuals are reproductive and that, as consequence of the earlier sexual maturity of males and the alternation of flowering and nonflowering years of females, there exists a 2:1 male-biased sex ratio (García *et al*. 2002; García 2003). The population is subdivided into two groups that are vertically separated *c*. 150 m from each other (García *et al*. 2002). One subpopulation is located on the upper part of the cliff, accounting for approximately 30% (*c*. 660 individuals, of which 300 are reproductive) of the total population, and the second is on the lower part of the cliff, near the Noguera Ribagorzana river brook, which accounts for the remaining 70% (*c*. 1540 individuals, of which 700 are reproductive). No other traces of *B. chouardii* have been found beyond its *'locus classicus'* (Gaussen 1952) after several decades of extensive searching for potential sites (García *et al*. 2002; García 2003). The extremely reduced distribution range of this long-lived perennial has been interpreted as the consequence of the limited dispersal capability and relictual distribution of a formerly more widespread range severely affected by climatic changes (only five species out of more than 600 Dioscoreaceae are present in Europe). The flowers of *B. chouardii* are predominantly pollinated by ants (García 2003) as in its congener *B. pyrenaica* Miégeville (García *et al*. 1995) and, in consequence, pollen is not expected to be dispersed over long distances. Moreover, *B. chouardii* has developed a particular seed dispersal mechanism, denoted as postcarpotropism, where the pedicel turns backwards while increasing its length to set the ripe capsules into the rock crevice. This mechanism is also present in other genera of rupicolous plants such as *Cymbalaria* Hill. (Scrophulariaceae), *Petrocoptis* A. Br. (Caryophyllaceae, cf. Mayol-Martínez 1998), and *Sarcocapnos* DC. (Papaveraceae). Because of this, seed dispersal must be ensured to an appropriate habitat, but this would strongly reduce the probability of long-distance dispersal and the ability to colonize other habitats, thus, increasing the risk of extinction in case of dramatic alterations of the population size. This mechanism has been reported as largely inefficient for *B. chouardii*, where 90% of the seeds are lost, and provides almost no opportunities for successful long-distance dispersal (García 2003). However, some seeds falling from the upper cliff may reach suitable crevices in its lower part and contribute to the linkage of the two subpopulations in a source-sink manner.

Because of its extreme rarity (Morillo & Gómez-Campo 2000), *B. chouardii* was the first Iberian taxon for which a specific conservation plan was developed by the Aragón Regional Government in Spain (García 1996, 1997; Moreno-Saiz *et al*. 2003). It continues to be subjected to the Conservation Recovery Plan managed by the Aragón Government (DGA) and the LIFE project of the European Union (García *et al*. 2002). In addition, it was included in the National Catalogue of Endangered Species and in the Annex II of the Habitats Directive of the European Union under the category of 'in danger of extinction' (García 1996). More recently, it has been reclassified by García *et al*. (2002) as CR ('critically endangered') under subcategories B + B1 + B2 (IUCN 1994) and F (Keith 1998) and in the Red List of Endangered National Plants (VVAA 2000) and has been chosen as the logo of the IUCN-Spanish vascular commission on flora (cf. Moreno-Saiz *et al*. 2003).

The main threats to this species are its reduced range, small population size, and habitat specialization. These factors coupled with almost no possibilities of new colonizations and dioecy make *B. chouardii* very sensitive to disturbance. Models of ecological change under a variety of conditions suggest that the persistence of populations of *B. chouardii* may be compromised in the future century if they experience an increase in mortality of 10% (García 2003). Additionally, a population growth rate close to one and the slow dynamics of the species accentuate the risk of extinction in case of catastrophic events (García 2003). The monitoring of population dynamics, population reinforcements, and the maintenance of germplasm in seed banks conform the main current conservation strategies aimed at preventing potential population decline and reducing the risk of extinction of this species. The availability of genetic data for *B. chouardii* might prompt changes in its ongoing management plans (Segarra-Moragues & Catalán 2002, 2003).

Fréville *et al*. (2001) and Torres *et al*. (2003) suggested the use of combined information obtained from different neutral molecular markers for a reliable assessment of fine scale genetic processes in relictual, narrowly distributed plants. Therefore, in order to study the effects of long-term reduced population size on the present genetic variation and population structuring of *B. chouardii*, we have conducted a study using both microsatellites simple sequence repeats (SSRs) (Tautz 1989; Morgante & Olivieri 1993) and randomly amplified polymorphic DNAs (RAPDs) (Williams *et al*. 1990) with the following goals: (i) to assess the existing levels of genetic variability in the species; (ii) to analyse the distribution of this genetic diversity in the population; and (iii) to infer potential microevolutionary processes that could have led to present genetic microdifferentiation between the two subpopulations. Additionally, these results could be of paramount importance to assess the current conservation management plan for this highly endangered species.

Materials and methods

Collection of samples and DNA extraction

Sampling covered both subpopulations of the extant population of *Borderea chouardii*. Twenty-six individuals were sampled from the upper part of the cliff and 21 individuals from the lower part to make up a total of 47 individuals previously screened for variability in 21 allozyme loci (Segarra-Moragues & Catalán 2002). Our reduced sample size obeyed both to the limited accessibility of individuals (most of them out of reach) and, more important, to the Spanish laws that limit the collection of this protected species. Individuals were sampled at intervals > 1 m and collected from different crevices to avoid the sampling of closely related individuals. The same set of individuals was used for both RAPD and microsatellite surveys.

Fresh leaves from all sampled individuals were dried in silica gel (Chase & Hills 1991) and used for DNA isolation. DNA was extracted following the cetyltrimethyl ammonium bromide (CTAB) protocol of Doyle & Doyle (1987) adapted for miniprep extractions. DNA concentration was estimated by comparing it to the brightness of an ethidium bromide stained marker VII (Roche) on agarose gels; samples were diluted to a final concentration of about 5 ng/μL in Tris-EDTA 0.1× buffer.

PCR amplifications

Forty RAPD primers from Operon Technologies (kits A and B) were assayed in a pilot sample of three individuals from each of the two subpopulations. Amplifications were carried out in 20-μL total volume containing 1× buffer (Ecogen), 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 4 pmol of primer, 1.0 unit of *Taq* DNA polymerase (Ecogen), and 2–5 ng template DNA. Polymerase chain reactions (PCRs) were performed using both positive and negative controls in order to detect the efficiency of the enzyme and the absence of contamination. The amplification program consisted of a step of DNA melting of 4 min at 94 °C, followed by 40 cycles of 94 °C for 1 min, 39 °C for 1 min, and 72 °C for 1.5 min, and a final elongation step of 72 °C for 7 min. The amplified products were resolved in 2% agarose gels stained with ethidium bromide; electrophoreses were set at 100 V during 4 h in 0.5× TBE buffer. RAPD bands were visualized with UV transmitted light and captured with Gel Doc 1000 (Bio-Rad). RAPD amplifications were repeated at least twice in order to check the reproducibility of the banding profiles. Twelve of the 40 assayed primers that rendered repeatable, strongly stained and easily scorable amplicons were selected for the analysis of the whole set of samples.

Ten primer-pairs for the amplification of trinucleotide SSRs developed for this species (Segarra-Moragues *et al*. 2003) and seven additional ones developed for the congener *Borderea pyrenaica* (Segarra-Moragues *et al*. 2004) were used to amplify microsatellite loci in the same 47 samples after transferability tests were established. Some of these were amplified simultaneously (multiplex, Mitchell *et al*. 1997) in the same PCR (group I: *Bc1274*, *Bc1357*, *Bc1551*, *Bp2214*, and *Bp2292*; group II: *Bp126*, *Bp2256*, *Bp2290*, and *Bp2391*; group III: *Bc1159*, *Bc1169* and *Bc1644*; and group IV: *Bc1258*, *Bc1422* and *Bc166*). *Bc1145B* and *Bp1286* were amplified separately and their products were combined with those of other primer-pairs of groups I and II, respectively, for simultaneous electrophoreses. PCRs were performed in 20-μL mix containing 3–5 pmol each of the fluorescein-labelled forward and unlabelled reverse primers, 1× *Taq* buffer (Promega), 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 1 unit of *Taq* DNA polymerase (Promega), and approximately 5–8 ng DNA. The PCR program consisted of an initial melting step (94 °C, 4 min) followed by 30 cycles (94 °C, 45 s, annealing temperature, 45 s and 72 °C, 1 min–1 min 20 s) and a final extension step (72 °C, 7 min). PCRs, as in RAPDs, were carried out in a PE GeneAmp 9700 (Applied Biosystems). The products of up to six SSR primer-pairs were run simultaneously on an ABI 310 automated sequencer (Applied Biosystems). Fragment lengths were assigned with GENESCAN and GENOTYPER software (Applied Biosystems) using ROX-500 as the internal lane standard.

Genetic analysis of RAPD data

RAPD bands were scored by their presence or absence into a data matrix analysed using different methods. Genetic diversity was estimated from the frequencies of the RAPD bands using Nei's (1973) method: $h = 1 - 1/m \ \Sigma_l \Sigma_u \ p_{lu}^2$, where p_{lu} is the frequency of the u-th band at the l-th locus and m is the number of loci (Peever & Milgroom 1994).

Three different metric distances were computed between individuals: Dice (Dice 1945; Nei & Li 1979) and Jaccard's (Jaccard 1908) similarity coefficients, both excluding shared absence of bands, were computed with NTSYSPC version 2.11a (Rohlf 2002), and the pairwise-difference distance (Excoffier *et al*. 1992) was computed with ARLEQUIN version 2.000 (Schneider *et al*. 2000). Correlations between Dice and Jaccard's and pairwise difference values were calculated through a Mantel test with 1000 replicates (Mantel 1967) using NTSYSPC. As the three genetic distances between phenotypes showed highly significant correlations (*D*/*J* $r = 0.9997$, $P < 0.001$; *D*/*PD* $r = -0.9848$, $P < 0.001$; *J*/*PD* $r = -0.9882$, $P < 0.001$), the pairwise difference distance was chosen for subsequent analyses.

The genetic structure of the population was studied by means of analyses of the molecular variance (AMOVA; Excoffier *et al*. 1992) using ARLEQUIN. As only one population is available for the AMOVA, sources of variation were investigated among subpopulations (upper cliff vs. lower cliff) and within subpopulations. Significance levels of the variance components were obtained by permutation tests using 1000 replicates.

The relationships among all RAPD phenotypes were visualized by a principal coordinate analysis (PCO) with the superimposition of a minimum spanning tree (MST) using NTSYSPC (Rohlf 2002) to test for differences in the spatial distribution of RAPD phenotypes along the species' range and by a neighbour-joining (Saitou & Nei 1987) tree constructed with MEGA version 2.0 (Kumar *et al*. 2001), where statistical robustness of the groupings was assessed by 1000 bootstrap replicates (Felsenstein 1985) using PAUP* version 4.0b10 (Swofford 2002).

Genetic analysis of microsatellite data

The *Borderea* species have been recently discovered to be allotetraploid on the basis of molecular data (Segarra-Moragues *et al*. 2003, 2004, and unpublished), contradicting previous statements based on chromosome counts that suggested that *Borderea* was diploid (Heslot 1953). Because of the addition of divergent genomes, inheritance in allopolyploids is duplicate disomic and, consequently, if alleles can be assigned confidently to their corresponding locus, then it is possible to encode genotypes as for standard diploids. However, difficulties arise when alleles of different genetic complements present overlapping allele sizes (size homoplasy; Estoup *et al*. 2002), thus hindering a correct assignment of genotypes. This occurred with alleles obtained from three out of the 17 primer-pairs used, two developed for this species (*Bc1159* and *Bc166*; Segarra-Moragues *et al*. 2003) and a third one was transferred from *B. pyrenaica* (*Bp2292*; Segarra-Moragues *et al*. 2004), which will be referred hereafter as nongenotyped loci. These three primer-pairs rendered polymorphic products and altogether accounted for 20 alleles, but because of the impossibility to encode the corresponding diploid genotypes, they were considered only in the clustering analysis of SSR phenotypes (see succeeding discussions).

For the genotyped loci, the mean number of alleles (*A*), the proportion of polymorphic loci at the 95% (*P*95) and 99% (*P*99) criteria, the observed (H_O), and unbiased expected (H_E) heterozygosities (Nei 1978) were computed using GENETIX version 4.04 (Belkhir *et al*. 2003) both for the population as a whole and for the two subpopulations. Genotypic linkage disequilibrium was tested using Fisher exact test both for each pair of loci and within each population using GENEPOP version 3.3 (Raymond & Rousset 1995a) following the Markov chain method with 500 batches and 10 000 iterations per batch. This software was also used to calculate *F*-statistics (Wright 1951), according to Weir & Cockerham (1984), and *R*-statistics (Slatkin 1995), according to Rousset (1996), to estimate genic and genotypic differentiation between subpopulations and deviations from Hardy–Weinberg equilibrium(HWE) both within each subpopulation and for each locus using the probability test (Guo & Thompson 1992). The estimation of the number of migrants (Nm) between subpopulations was based on the corrected estimate of the private alleles method (Barton & Slatkin 1986). For this purpose, allele sizes were converted into repeat units by dividing the observed allele sizes by the reported repeat motif length of the microsatellites (three in all cases).

Several metrics were used to assess alternative evolutionary assumptions about the relationships between individuals (Slatkin 1995). The corresponding indices are based on the infinite alleles model (IAM; Kimura & Crow 1964), D_A (Nei *et al*. 1983) and D_{SW} (Shriver *et al*. 1995; following Destro-Bisol *et al*. 2000), and on the stepwise mutation model (SMM, Kimura & Ohta 1978), $\delta\mu^2$ (Goldstein *et al*. 1995) and average square distance (Goldstein & Pollock 1997), as implemented in POPULATIONS (Langella 2000). The resulting distance matrices among individuals were used to construct neighbour-joining (NJ) phenograms using MEGA2 (Kumar *et al*. 2001). In order to include information from nongenotyped loci, we performed a binary coding of SSR data to assess the relationships of individual SSR phenotypes in a manner similar to dominant data but with the added benefit of reproducibility. All PCO were conducted accordingly with the selected distance matrices as for RAPDs.

Hierarchical analyses of molecular variance (AMOVAS) were based on allele frequency information and conducted according with the mutation model assumed, with F_{ST} and R_{ST} following IAM and SMM, respectively. These results were compared with those of genetic structure obtained from RAPDs. PCOs were conducted accordingly with the selected distance matrices as for RAPDs.

STRUCTURE (Pritchard *et al*. 2000) was used to infer the number of subpopulations (*K*), in which the samples could

be grouped according to their genetic background and to estimate the membership of each individual to the predefined clusters. We examined the probabilities for a range of values of *K* starting from 1 to 5. These analyses were based on an admixture ancestry model with correlated allele frequencies, using a burn-in period and a run length of the Markov chain Monte Carlo of 10^5 and 10^6 iterations, respectively, in order to reach an approximate stabilization of the summary statistics (Pritchard *et al*. 2000). GENECLASS (Cornuet *et al*. 1999) was used to check how indicative an individual's multilocus genotype was of its subpopulation of origin. The methods used were (i) likelihood approaches based on a frequency analysis (Paetkau *et al*. 1995), which assign an individual to the population in which the individual's genotype is most likely to occur, and on a Bayesian analysis, where the probability of subpopulation allele frequencies was derived from sample subpopulation frequencies; and (ii) based on D_A and $\delta\mu^2$ distances, where individuals were assigned to the 'closest' matching subpopulation according to their genotype frequencies (Cornuet *et al*. 1999). Significance of analyses was tested over 100 000 permutations.

Results

The 12 RAPD primers used in this survey rendered 81 bands for the 47 surveyed individuals (see Supplementary material). All these bands but two were shared between the two subpopulations. Thirty-nine (48%) of the 81 markers were polymorphic at the species level (Table 1). The subpopulation from the upper cliff showed a higher proportion of polymorphic loci (43%) than that of the lower part (30%). Levels of genetic diversity detected by RAPDs ranged from 0 to 0.36 depending on the primer and the subset of individuals considered. Mean values of genetic diversity were 0.14 at the species level, which is higher for the upper (0.15) than for the lower cliff subpopulation (0.11). There was also an increase in value notably when only polymorphic bands were considered (0.29, species level; 0.34, upper cliff and 0.35, lower cliff; Table 1).

The 14 primer-pairs used in SSR analysis, for which genotypes could be scored unambiguously, detected 47 alleles in this same set of samples. These primer-pairs showed differential banding profiles. For five of them (*Bc1551*, *Bc1644*, *Bp126*, *Bp1286*, and *Bp2214*), we assumed that only one genomic complement was being amplified (from one of the putative parental genomes) as only up to two alleles per individual were observed in those microsatellite regions. Two of these loci (*Bp1286* and *Bp2214*) were monomorphic across the 47 samples studied and the remaining ones were polymorphic, showing two to four alleles per locus. Conversely, genetic dosages for the two putative parental genomes were amplified with the remaining nine primer-pairs (*Bc1145B*, *Bc1169*, *Bc1258*, *Bc1274*, *Bc1357*, *Bc1422*, *Bp2256*, *Bp2290*, and *Bp2391*) and alleles could be assigned to their corresponding genomic complement and encoded as for conventional diploid taxa. These loci were renamed as *Bc1145Ba*,*b*, *Bc1169a*,*b*, etc., respectively, to designate each of the two subgenomes. A total of 23 SSR loci were scored from the 14 primer-pairs, 11 of which were monomorphic across all 47 samples. The remaining ones showed 2 (i.e. *Bp2290a*) to 5 (i.e. *Bc1258b*) alleles each (see Supplementary material). The mean number of alleles per locus (*A*) was 2.05 ± 0.28 with similar values for the upper and lower cliff subpopulations (1.70 ± 0.22 vs. 1.70 ± 0.21, respectively, Table 1). The percentage of polymorphic loci was 52%, both at *P*95 and *P*99 criteria (Table 1). Both subpopulations showed slight differences for this statistic at the 95% criterion (35% vs. 39%), but showed the same percentage at the 99% one (43%) (Table 1). Percentages of polymorphic loci detected by RAPDs and SSRs between both subpopulations were nonsignificantly different from each other (Mean values across markers: 43.34 ± 0.19, upper cliff vs. 36.93 ± 9.26, lower cliff) (Mann–Whitney's *U*-test, $P = 0.683$).

Eight exclusive alleles from the genotyped loci were present in each of the two subpopulations but with lower mean frequencies in the upper cliff (0.10 vs. 0.28). Additionally, 10 and 3 exclusive alleles were detected from the nongenotyped loci for the upper and lower cliff subpopulations, respectively (results not shown). The upper cliff

Table 1 Mean genetic diversity indices obtained for 12 RAPD primers and 23 microsatellite loci in *Borderea chouardii* at the species and subpopulation levels

	RAPDs			Microsatellites				
	P	*h*	h_p	$A \pm \sigma$	P_{95}	P_{99}	$H_O \pm \sigma$	$H_E \pm \sigma$
Species level	48	0.14	0.29	2.05 ± 0.28	52.2	52.2	0.14 ± 0.03	0.17 ± 0.05
Upper cliff	43	0.15	0.34	1.70 ± 0.22	34.8	43.5	0.14 ± 0.05	0.13 ± 0.04
Lower cliff	30	0.11	0.35	1.70 ± 0.21	39.1	43.5	0.14 ± 0.04	0.14 ± 0.04

For RAPDs, the proportion of polymorphic bands (*P*) and the mean genetic diversity over loci (*h*) and over polymorphic loci (h_p) are given. For microsatellites, the average number of alleles (*A*), the proportion of polymorphic loci at 95% (*P*95) or 99% (*P*99) criterion and the observed (H_O) and unbiased expected (H_E) heterozygosities are given.

subpopulation has a larger and more evenly represented number of alleles than the lower cliff subpopulation except for a few shared allelic variants (see Supplementary material). Estimates of the number of migrants per generation between both subpopulations based on the frequencies of private alleles (Barton & Slatkin 1986) were 0.23, suggesting a strong isolation between the two groups.

Mean unbiased estimates for sample size (Nei 1978) of observed (H_O) and expected (H_E) heterozygosities over the total population were 0.14 ± 0.03 and 0.17 ± 0.05, respectively, with similar values of observed and expected heterozygosites for both subpopulations ($H_O = 0.14 \pm 0.05$, $H_E = 0.13 \pm 0.04$, upper cliff; and $H_O = 0.14 \pm 0.04$, $H_E = 0.14 \pm 0.04$, lower cliff). Total genetic diversity of the species was $H_T = 0.20 \pm 0.06$. None of the 506 tests for linkage disequilibrium performed between pairs of genotyped SSR loci within subpopulations was significant at $P < 0.05$, whereas when tests were conducted across all samples, only two were significant, which involve the pairs *Bp2290a* and *Bc1258b* ($P < 0.05$), and *Bc1258b* and *Bc1422b* ($P < 0.01$), but none was significant after applying Bonferroni-type correction for multiple tests. Significant heterozygote deficiency was detected for three of the most polymorphic loci (*Bc1274b*, *Bc1422b*, $P < 0.01$ and *Bc1258b*, $P < 0.001$) at the species level. These differences were less marked ($P < 0.01$) and maintained only for locus *Bc1258b* in the lower cliff subpopulation (Table 2). These results could be explained by Wahlund effect, indicating that both subpopulations constitute independent panmictic units.

The partitioning of genetic variance within *Borderea chouardii* calculated through AMOVA attributed 20.7% of the variance to differences between subpopulations, whereas a higher percentage of the genetic variance was distributed within subpopulations (79.3%) based on RAPD data (Table 3). SSRs revealed even higher percentage of variation attributed between subpopulations than RAPDs. Depending on the mutation model assumed (IAM or SMM), percentages of variance among subpopulations were 35.2% and 30.8%, respectively, suggesting that genetic drift may be an important factor, but not the sole cause of genetic differentiation between the two subpopulations of *B. chouardii*. Percentages of within subpopulation genetic diversity were always higher and could be related to the obligate outcrossing of the species. Average $F_{ST} = 0.35$ and $R_{ST} = 0.32$ values were significantly different from zero ($P < 0.001$); however, both statistics were not significantly different from each other in a rank correlation test ($P > 0.05$) (Sokal & Rohlf 1994). The same F_{ST} and R_{ST} value was obtained for six of the 12 analysed loci, whereas for the other six, three presented a higher value of F_{ST} than R_{ST} and the remaining ones gave opposite result. The higher mean value of F_{ST} stemmed out from two loci, *Bc1258b* ($F_{ST} = 0.31$, $R_{ST} = 0.06$) and *Bc1422b* ($F_{ST} = 0.65$, $R_{ST} = 4 \times 10^{-4}$). Assuming homogeneity in mutation rates at microsatellite loci in the two subpopulations of individuals, these analyses agreed with genetic drift as

Table 2 F_{IS} statistics calculated at the species level and for each subpopulation

Locus	Species level	Upper cliff	Lower cliff
Bc1145Ba	−0.211	−0.316	−0.081
Bc1169b	+0.134	—	−0.084
Bc1258a	−0.122	−0.111	−0.111
Bc1258b	+0.436***	+0.206	+0.409**
Bc1274a	−0.058	−0.111	—
Bc1274b	+0.246**	−0.250	−0.254
Bc1357b	+0.031	−0.264	—
Bc1422b	+0.451**	−0.047	−0.084
Bc1551	−0.045	—	−0.111
Bc1644	−0.051	0.000	−0.116
Bp126	+0.084	+0.079	−0.026
Bp2290a	−0.082	−0.020	−0.143
Total	+0.167***	−0.105	−0.013

Significant values are indicated as $^{**}P < 0.01$, $^{***}P < 0.001$. Dash indicates that a locus was monomorphic in the corresponding subpopulation.

Table 3 Comparative AMOVA based on (a) 46 RAPD phenotypes and (b, c) 47 SSR genotypes of *Borderea chouardii*. (b) IAM model, F_{ST} (c) SMM model, R_{ST}

Source of variation (groups)	Sum of squared deviations (SSD)	d.f.	Variance components	Total variance %
(a) Upper cliff vs. lower cliff				
Between subpopulations	25.361	1	Va = 0.93682	20.7
Within subpopulations	161.788	45	Vb = 3.59528	79.3
Total	187.149	46	Vc = 4.53210	
(b) Upper cliff vs. lower cliff				
Between subpopulations	39.323	1	Va = 0.81385	35.2
Within subpopulations	138.422	92	Vb = 1.50459	64.8
Total	177.745	93	Vc = 2.31844	
(c) Upper cliff vs. lower cliff				
Between subpopulations	363.783	1	Va = 7.46745	30.8
Within subpopulations	1544.196	92	Vb = 16.78474	69.2
Total	1907.979	93	Vc = 24.25219	

being the likely agent responsible for the differentiation of the two groups. However, the relative influence of mutation on the genetic differentiation between both subpopulations could not be evaluated statistically as the number of alleles detected in the polymorphic loci (< 5) was not large enough to conduct tests with sufficient statistical power (cf. Hardy *et al*. 2003). The small distribution range of the species seems a likely argument against differences in mutation rates between both groups of individuals. In here, we presume that genetic differentiation could be caused by the coupled effects of small population size and reproductive isolation, although the accumulation of different alleles by mutation in each geographical unit cannot be ruled out completely.

Although only 48% of the RAPD bands were polymorphic, most individuals were characterized by a unique RAPD phenotype. Only one multilocus RAPD phenotype was shared between two individuals from the lower part of the cliff (Figs 1a and 2a). SSRs identified 43 distinct genotypes, three of them shared by two individuals from the upper cliff and another one shared by two from the lower cliff (Figs 1b and 2b). PCO of RAPD data showed a nearly complete differentiation between *B. chouardii* subpopulations. Individual samples clustered separately along the first axis, which accumulated 18.32% of the variance (Fig. 1a). Most RAPD phenotypes corresponding to individuals from the upper cliff clustered on the right side of the plot, whereas phenotypes corresponding to individuals from the lower cliff clustered on the left side. A cleaner differentiation between these two groups was obtained from SSR data (Fig. 2b). PCO of these data accumulated a higher percentage of variance in the first axis (67.06%).

Neighbour-joining (NJ) clustering of RAPD phenotypes (Fig. 2a) showed similar results as those from PCO. While the two major clades of this tree separated most phenotypes from the upper and the lower cliff subpopulations, some phenotypes clustered within the other clade. A variety of genotypic relationships were observed in the NJ trees based on microsatellite data. In those constructed from distances assuming SMM ($\delta\mu^2$ and ASD), some genotypes appeared intermingled; in contrast, those constructed assuming IAM (D_A, Fig. 2b, and D_{SW}) were well resolved. The better performance of IAM based distances, in which, as opposed to the SMM, genetic drift contributes more than new mutations to the evaluation of the relationships among recently derived populations, agree with the previous results by Pérez-Lezaun *et al*. (1997) and Destro-Bisol *et al*. (2000), which were reviewed in Balloux & Lugon-Moulin (2002). Poor bootstrap values (< 50%) were obtained for the major clades of the RAPD and SSR trees. However, additional analyses conducted with the whole SSR data (67 SSR bands) encoded as binary characters (1/0), and thus considering individual SSR phenotypes as in Mengoni *et al*. (2000), Staub *et al*. (2000) and Palombi & Damiano (2002), revealed a relatively good bootstrap support for the clades grouping the upper and lower cliff phenotypes (bootstrap 81%) (Fig. 2c). The higher precision of the F_{ST} value (0.49) obtained from this larger data set also points to the phenotypic distinctiveness of the two subpopulations of *B. chouardii*. This result was also supported by Fisher exact tests (Raymond & Rousset 1995b) that detected highly significant

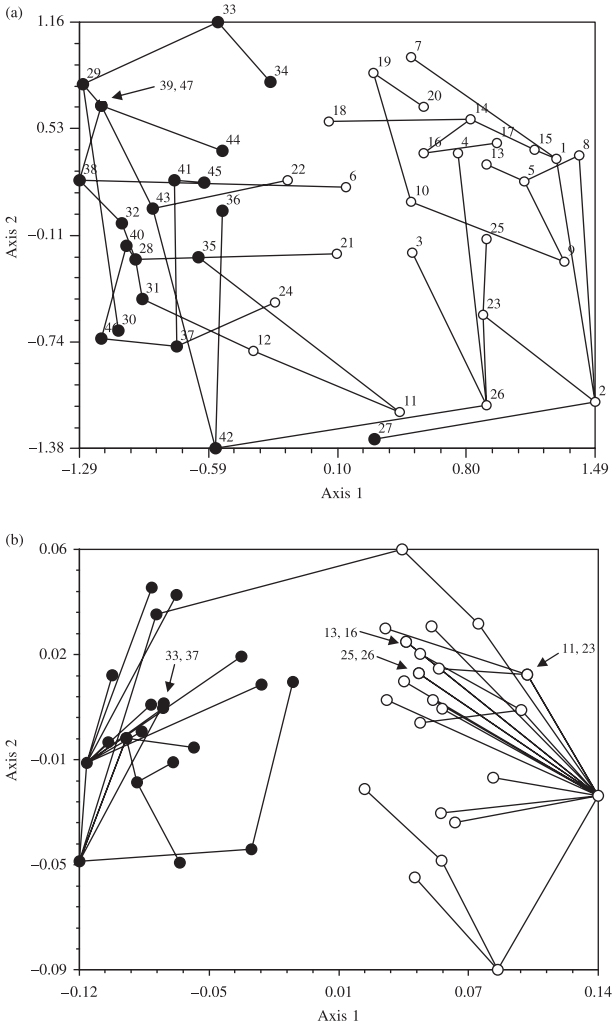

Fig. 1 Principal coordinates analyses (PCOs) plot with the minimum-spanning tree superimposed based on the pairwise-difference distance matrix (RAPDs) and D_A matrix (SSR) showing the relationship among RAPDs phenotypes and SSR genotypes found in *Borderea chouardi*. (a) 46 RAPD phenotypes. The first two axes accounted for 18.32% and 9.93%, respectively, of the total variance. (b) 43 different SSR genotypes. The first two axes accounted for 67.06% and 13.10%, respectively, of the total variance. Numbers of the individuals have been removed for a clearer representation. Arrows indicate individuals with the same RAPD phenotype or SSR genotype in the population. ○, individuals from the upper cliff and ●, individuals from the lower cliff.

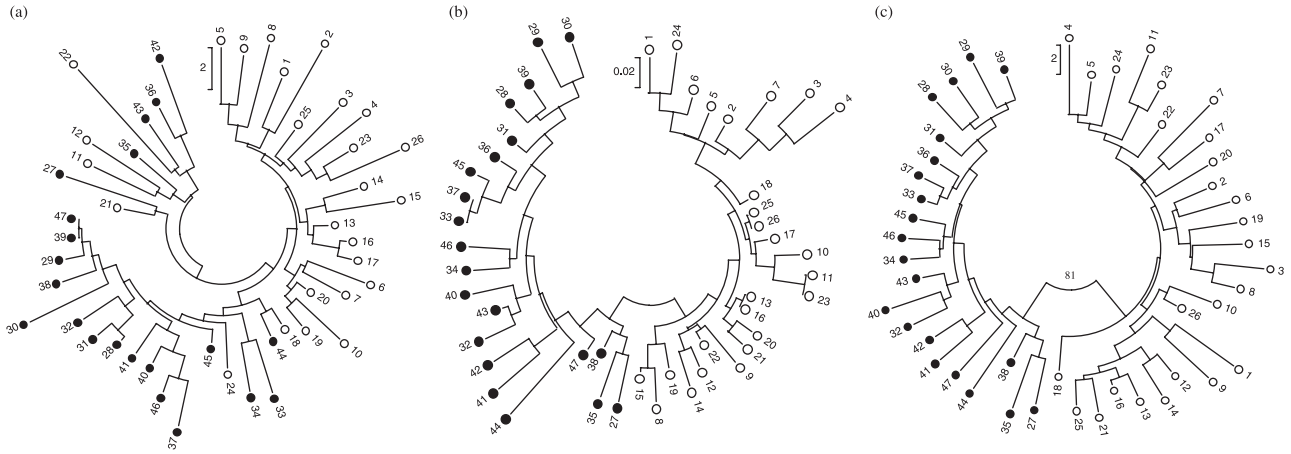

Fig. 2 Neighbour-joining trees showing the relationships among individuals of *Borderea chouardii*. (a) RAPD phenotypes with Pairwise-difference (PD) distance method. (b) SSR genotypes with D_A distance method. (c) SSR phenotypes with PD distance method (all bands scored by their presence or absence), where the support of branch is indicated by bootstrap value. ○, individuals from the upper cliff and ●, individuals from the lower cliff.

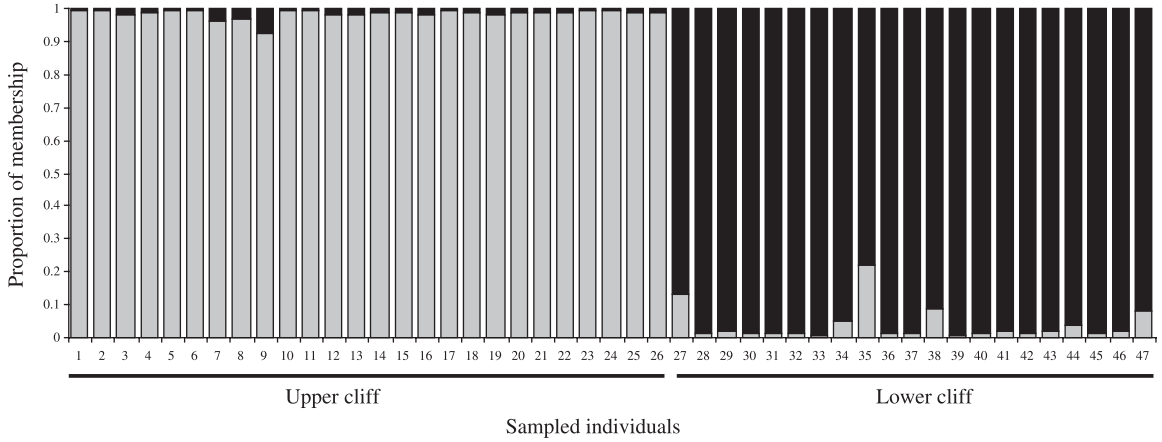

Fig. 3 Estimated membership of the 47 sampled individuals to the predefined subpopulations inferred with STRUCTURE. Grey shading, upper cliff; black shading, lower cliff.

($P < 0.001$) departures from random distribution of alleles and genotypes across both subpopulations and by the estimation of the number of subpopulations (K) that produced a higher probability for the hypothesis of two subpopulations (probability of ($K = 2$) = 0.997) than for a single one (prob($K = 1$) = 1.74×10^{-60}). All 26 and 21 individuals sampled from the upper and lower cliffs, respectively, showed a higher estimated membership to their corresponding predefined subpopulation group according to their genetic background (Fig. 3).

Assignment of genotypes conducted using GENECLASS also corroborated the results of these analyses. Likelihood methods of assignment based on Bayesian and frequency analyses gave similar results but better resolution than some of the distance-based methods (Table 4). For the Bayesian and frequency methods, only two and three individuals from the upper and lower cliff, respectively, were misclassified but they also presented a high probability of assignment to their subpopulation of origin. Distance-based methods gave highly contrasting results: while the application of D_A (IAM) resulted in the correct assignment of all genotypes, $\delta\mu^2$ (SMM) resulted in a very poor resolution and misclassification of many individuals from both subpopulations.

Discussion

Genetic diversity of Borderea chouardii

Reproductive systems and the history of a species have been often regarded as the main factors affecting levels of genetic diversity, genetic divergence, and genetic structure within and among plant populations (Loveless & Hamrick

Table 4 Assignment tests based on (1) likelihood estimates of multilocus frequencies calculated with Bayesian (a) and frequency (b) methods and on (2) genetic distances [D_A (c), $\delta\mu^2$ (d)]

Sampled populations/method	Assigned populations							
	a) Bayesian				b) Frequency			
	Upper cliff	Lower cliff	Ambiguous	Total	Upper cliff	Lower cliff	Ambiguous	Total
Upper cliff	24	0	2	26	23	0	3	26
Lower cliff	0	21	0	21	0	21	0	21
	(c) D_A				(d) $\delta\mu^2$			
	Upper cliff	Lower cliff	Ambiguous	Total	Upper cliff	Lower cliff	Ambiguous	Total
Upper cliff	26	0	0	26	6	0	20	26
Lower cliff	0	21	0	21	1	0	20	21

1984; Hamrick & Godt 1989, 1996; Hamrick *et al*. 1991; Karron 1991). Outcrossing perennials generally exhibit higher levels of genetic diversity and lower levels of population differentiation, indicating the influence of the species traits on these parameters (Hamrick & Godt 1989, 1996). However, long-time isolated populations could accumulate private alleles reflecting their genetic differences because of isolation by distance (Prentice *et al*. 2003). *Borderea chouardii* represents a singular case. In spite of the low to moderate levels of genetic variability detected within the single population of this narrow endemic and obligated outcrosser, a strong spatial genetic structuring has been detected for its two close subpopulations.

Dioscorea tokoro Makino, a dioecious diploid taxon from Japan, is the only *Dioscorea* species, in which a population genetic study has been conducted with allozymes (Terauchi 1990) and microsatellites (Terauchi & Konuma 1994) that showed higher genetic variability values for allozymes, and for microsatellites than *B. chouardii* (Table 5). However, the scarcity of molecular surveys in wild Dioscoreaceae precludes a wide generalization of the genetic depletion status of *B. chouardii* compared to the family of the yams. Nonetheless, a relatively good assessment can be obtained from the comparison with the only extant congener, *Borderea pyrenaica*. In this respect, the three types of molecular markers that have been investigated in *B. chouardii* have proved highly congruent and have detected lower values for all genetic descriptors in this species than in its more widespread congener *B. pyrenaica* (Table 5).

These low levels of genetic diversity and the high proportion of monomorphic loci exhibited by *B. chouardii* could be attributed to diverse factors, including the reduced population size and short-distance dispersal of its seeds, which likely enhance inbreeding and the effect of genetic drift. Severe historical local extinctions and genetic bottlenecks could also have contributed to the genetic impoverishment of the species. However, neither the upper nor the lower cliff subpopulations are genetically homogeneous for RAPD and SSR markers, possibly because of dioecy and the high longevity of the plants (> 300 years old; cf. García 2003), the attributes that together may buffer against gene loss.

Table 5 Comparison of genetic diversity values obtained from three wild Dioscoreaceae studied with three molecular techniques

	Allozymes			RAPD		Microsatellites		
	A	*P*	H_T	*P*	*h*	*A*	*P*	H_T
D. tokoro	2.5[1]	100[1]	0.28[1]	—	—	6.2[2]	100[2]	0.68[2]
B. pyrenaica	1.4[3]	15.9[3]	0.07[3]	93[4]	0.38[4]	4.3[6]	72.2[6]	0.30[6]
B. chouardii	1.1[3]	9.5[3]	0.05[3]	48[5]	0.14[5]	2.1[5]	52.0[5]	0.20[5]

A, mean number of alleles per locus; *P*, proportion of polymorphic loci; *h* and H_T, total genetic diversities.
[1]Data from Terauchi (1990); [2]data from Terauchi & Konuma (1994); [3]data from Segarra-Moragues & Catalán (2002); [4]data from Segarra-Moragues & Catalán (2003). [5]This study: [6]summarized data from 18 SSR loci amplified in *B. pyrenaica*, unpublished results.

Additionally, the success in hybridization between presently extinct progenitors of the allotetraploid *B. chouardii*, corroborated by allozyme data (Segarra-Moragues & Catalán 2002) and microsatellite data (Segarra-Moragues *et al*. 2004; this study and unpublished) could have played an important role in increasing the evolutionary potential and success rate of the newly arisen species (Levin 1983; Ramsey & Schemske 1998; Soltis & Soltis 2000) as documented for several other tetraploid plants (Ness *et al*. 1989; Soltis & Soltis 1989; Wolf *et al*. 1990; López-Pujol *et al*. 2004).

Population subdivision

An earlier allozyme study of *B. chouardii* (Segarra-Moragues & Catalán 2002), detected only four different genotypes in

this same set of individuals. Three of them were shared between both subpopulations, whereas the remaining one was exclusive of a single individual sampled from the upper cliff. This low allozyme variation precluded a fine scale assessment of the population genetic structure. However, the more variable RAPD and microsatellite markers proved useful for this purpose. All but one of the RAPD markers was shared between the two subpopulations, indicating that all individuals were derived from a common gene pool preserved over time. In contrast, SSRs, which accumulate variation faster than base-substitution dependent markers (such as RAPDs), indicate a clear-cut differentiation among the isolated patches of the *B. chouardii* population. Nevertheless, both markers agree in identifying a common gene pool as many SSR loci are fixed for the same allele in both subpopulations, although the remnant stocks have acquired or maintained some private alleles independently. F_{IS} values also indicate nonrandom mating among individuals in the population but almost random within each subpopulation (Table 2), probably reflecting Wahlund effect. Nonsignificant deviation of F_{IS} within subpopulations indicates that both groups are at HWE that agreed with the demographic stability depicted in population dynamics surveys (García 2003). The genotypic differentiation of the two groups is particularly evident from the PCO and the minimum-spanning tree analysis obtained from SSR data (Fig. 1b), where most of the individuals belonging to each of the two subpopulations appear to be directly related to a few ancestors, indicating a recent divergence among them possibly caused by a strong population bottleneck in the past.

Both RAPDs ($F_{ST} = 0.21$) and SSRs ($F_{ST} = 0.35$, $R_{ST} = 0.32$) indicate significant differentiation between the two subpopulations. Accordingly, highly significant departures from random dispersal of genes and genotypes were found between the isolated subpopulations of individuals (Raymond & Rousset 1995b). This is a surprising result for a single-population taxon, with subpopulations of individuals separated only by 150 m; in such case, the dioecious nature of *B. chouardii* that forces pollen to be transferred to a female plant and is responsible for its long lifespan (García 1997, 2003) would tend to prevent population substructuring. However, the short distance dispersal of both pollen and seeds along with the male-skewed sex ratio, which promotes breeding between spatially close individuals, seems to have had a high impact in shaping the genetic structure of this species by a coupled effect of genetic drift and inbreeding (Nei *et al*. 1975; Rich *et al*. 1979; Brakefield 1989; Leberg 1992). Similar or higher levels of between population genetic diversity are often reported in the literature (see for example *Centaurea corymbosa* Poutrret, $F_{ST} = 0.35$ for allozymes and $F_{ST} = 0.23$ for microsatellites, Fréville *et al*. 2001) and appear to be the most frequent genetic make-up for narrow range rupicolous taxa (Mateu-Andrés & Segarra-Moragues 2000; Fréville *et al*. 2001; Jiménez *et al*. 2002) characterized by small population sizes, habitat fragmentation and reduced gene flow between geographically separate populations. The case of *B. chouardii* is especially remarkable because such level of genetic differentiation has occurred between two subpopulations only separated by 150 m of vertical distance.

Conservation implications

Our study on the genetic structuring of the narrow endemic *B. chouardii* can provide valuable information for the management and conservation of the only extant population of this taxon. It is difficult to assess to what extent the genetic impoverishment detected in *B. chouardii* may compromise the survival of the species at the short-term as population dynamics studies have demonstrated a high degree of population stability in the absence of dramatic alterations of population size (García 2003). Although, the relevance of genetic variability to buffer against environmental stochasticity (Ellstrand & Elam 1993) and to increase the fitness (Gautschi *et al*. 2002; Paschke *et al*. 2002; Wise *et al*. 2002; Kephart 2004) cannot be directly investigated with neutral markers, the latter have proven useful to decipher levels of gene flow between populations that may counteract the potential deleterious effects of genetic drift and inbreeding. In this sense, the low average number of migrants per generation inferred from microsatellites (0.23) and the large proportion of exclusive alleles in both subpopulations are indicative of genetic isolation between the two groups of individuals which could ultimately lead to local adaptation.

Although the species seems to be 'naturally rare', most of the documented recent population decline is attributable to human activity, increasing the risk of extinction as a result of its reduced population size. For instance, the construction of a dam and a public road seriously altered the population size (Sainz-Ollero *et al*. 1996). Other biological factors, such as plant longevity, absence of significant levels of herbivore predation, and a high fruit set (García *et al*. 2002; García 2003) do not appear to compromise the species survival in the short term. However, the low levels of genetic diversity found in *B. chouardii* indicate a degree of genetic peril that may produce an incapability to adapt to severe population declines because of gene loss, genetic drift, and inbreeding depression or may reduce its colonising ability of new habitats in competition with other rupicolous plants. Hence, under the smaller population census estimates (Sainz-Ollero *et al*. 1996), *B. chouardii* would be on the theoretically smaller limit range (500 individuals) to be buffered against genetic drift (Franklin 1980), which, together with the biased sex ratio, the alternation of reproductive and nonreproductive

years of females and of the subpopulation genetic structure reported here, would tend to reduce the effective population size.

These genetic analyses have distinct implications for the conservation management of *B. chouardii*. We suggest that a successful management program could incorporate genetic data on this species in the following manner: (i) As most individuals sampled are characterized by unique molecular RAPD phenotypes and SSR genotypes, all extant individuals of *B. chouardii* should be actively protected in the field. (ii) From 1999, a germplasm bank of seeds and tissue culture of *B. chouardii* is being maintained at the Universidad Politécnica de Madrid (UPM). However, this *ex situ* strategy has been developed without information on the genetic structuring revealed in this work and it is uncertain whether the genetic diversity sampled in those stocks is representative of the genetic diversity present in the population. Indeed inadequate sampling strategies may result in induced bottlenecks in the preserved material (Maunder *et al*. 2001). Given the genetic uniqueness of both subpopulations, we recommend future collections of seeds to be done from both groups of individuals as separate stocks. (iii) At the same time, we recommend the exploration of the genetic diversity contained in the germplasm bank of seeds and tissue culture of *B. chouardii*. This is especially important if this material is to be used in future restoration or reinforcement of subpopulations to guarantee the maintenance of the genetic structuring revealed here. Deliberate undocumented transfer of material from one subpopulation to another would alter present population structure and, in the absence of a demonstration of the fitness consequences of such introductions, we recommend that deposition of seeds at random within the population (García 2003; García & Guzmán, personal communication) should be avoided. (iv) Experimental crosses between individuals from the upper cliff and from the lower cliff could result in a highly heterozygous progeny array. This could lead to highly vigorous individuals if there is no local adaptation or outbreeding depression (Templeton 1986; Lynch 1991; Fenster & Galloway 2000). These artificial progeny arrays could be used to establish experimental populations, whose adaptive fitness could be monitored and eventually used for the reinforcement of local subpopulations once deleterious genetic effects are ruled out.

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Supplementary material

The supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2482/MEC2482sm.htm

Table S1 Genetic diversity index (h) obtained from the 12 RAPD primers within *Borderea chouardii* at the species and subpopulation levels

Table S2 Allele frequencies for the 12 polymorphic genotyped microsatellite loci in the two subpopulations of *B. chouardii*

Table S3 Genetic variability in 23 genotyped microsatellite loci in *B. chouardii*. Observed (H_O) and unbiased expected (H_E) heterozygosities are given for each locus

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Our research is focussed on evolutionary and population genetic studies of Iberian and Pyrenean plant endemics and their implications on conservation programs. J.G. Segarra-Moragues and M. Palop-Esteban work as postdoctorial scientists, and F. González-Candelas and P. Catalán as senior lecturers at the Universities of Zaragoza and València, Spain.
