Genetic Fingerprinting of Germplasm Accessions as an Aid for Species Conservation: A Case Study with Borderea chouardii (Dioscoreaceae), One of the Most Critically Endangered Iberian Plants

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• Background and Aims Molecular markers have changed previous expectations about germplasm collections of endangered plants, as new perspectives aim at holding a significant representation of all the genetic diversity in the studied species to accomplish further conservation initiatives successfully. Borderea chouardii is a critically endangered allotetraploid dioecious member of Dioscoreaceae, known from a single population in the Iberian pre-Pyrenees. This population was reported to be highly structured into two genetically distinct groups of individuals corresponding to their spatial separation along the vertical cliff where it grows. In 1999, the Spanish Government of Aragón launched the first conservation programme for the ex situ preservation of this species, and since then a seed collection has been conserved at the Germplasm Bank of the Universidad Politécnica de Madrid. However, as some seed samples had not been labelled clearly at the time of collection, their origin was uncertain.
• Methods Genetic variation in germplasm accessions of B. chouardii was investigated using microsatellite (simple sequence repeat; SSR) markers.
• Key Results The 17 primer pairs used detected 62 SSR alleles in the 46 samples analysed from five different germplasm stocks. Eight alleles scored from the wild population were not detected in the germplasm samples analysed. The relatedness of the germplasm samples to the wild subpopulations through neighbour-joining clustering, principal coordinates analysis (PCO) and assignment tests revealed a biased higher representation of the genetic diversity of the lower cliff (43 samples) subpopulation than that of the upper cliff (three samples).
• Conclusions The collection of additional samples from the upper cliff is recommended to achieve a better representation of the genetic diversity of this subpopulation. It is also recommended that these stocks should be managed separately according to their distinct microspatial origin in order to preserve the genetic substructuring of the wild population.

Key words: Borderea chouardii, endangered plants, ex situ conservation strategies, genetic diversity, germplasm stocks, microsatellites, polyploids, population structure, SSRs.

INTRODUCTION
Seed storage in germplasm banks is one of the best established ex situ techniques available for plant conservation, and, so far, at least three Mediterranean endemic taxa (Diplotaxis siettiana Maire, Lysimachia minoricensis J.J. Rodr. and Tulipa sprengeri Baker) have been preserved from extinction by this means (Greuter, 1994). However, these are extreme cases, and preventive germplasm stocks for many other endemic taxa are being maintained in botanical gardens (Wyse-Jackson and Sutherland, 2000) and scientific institutions (e.g. universities). These collections can aid the conservation of endangered taxa, e.g. by reintroduction to previous localities and reinforcement of declining populations. Most existing plant germplasm banks were established before molecular genetic information became available for many endangered taxa, thus compromising the sampling schemes, which may not have been adapted to fulfil an adequate genetic representation of the genetic make up of species (Fay, 2003). A prerequisite to the use of these stocks is therefore the evaluation of their genetic make up relative to the allelic representation of the target population. Seed accessions derived from inadequate sampling schemes may be genetically impoverished because of artificial bottlenecks in the material held in reserve (von Bothmer and Seberg, 1995; Brown and Marshall, 1995), thus becoming unsuitable for a successful recovery plan. This has been especially true for rupicolous endemics that have fragmented ranges with low genetic interchange because of discontinuous habitat availability (Mateu-Andrés and Segarra-Moragues, 2000; Freville et al., 2001; Jiménez et al., 2002; Prentice et al., 2003; Torres et al., 2003). Some recent studies have added a point of caution on the issue, suggesting that the ex situ strategy may not be as straightforward as expected in the absence of evaluation of the genetic representation (Calero et al., 1999; Ibáñez et al., 1999). Therefore, in order to accomplish a successful ex situ conservation management plan for a given endangered species, we need both knowledge of the genetic diversity present in the wild populations to design appropriate sampling schemes and an assessment of the genetic representation of the material collected from the source population.

Borderea chouardii (Gaussen) Heslot is one of two extant species of this relict Pyrenean genus of the largely...
pantropical family Dioscoreaceae. This species was described in the middle of the last century (Gaus sen, 1952) based on individuals from a single population located close to Sopeira (Huesca province, Spain), in one of the southernmost pre-Pyrenean mountain ranges (Moreno-Saiz, 1990; Villar and Lazare, 1991; Franco-Mugica, 1993). To date, it is the only known site for the species despite several years of extensive search (García et al., 2002). *Borderea chouardii* is a dioecious, strictly sexually reproducing geophyte. It has one of the longest life spans reported for herbaceous plants, with some individuals reported to be >300 years old (García, 1996, 1997, 2003; García et al., 2002). It grows in rather inaccessible crevices on a single limestone cliff. Recent estimations indicate a population size close to 2220 individuals, with a 2 : 1 male-to-female sex-biased ratio (García et al., 2002; García, 2003; Göni and Guzmán, 2003). In this species, males flower yearly whereas females alternate flowering years with non-flowering years (García, 2003). Individuals in this single population are spatially separated into two subpopulations on the cliff, accounting for 30 and 70% of the total population in the upper and lower cliff groups, respectively.

Because of its extreme rarity, *B. chouardii* was the first Iberian plant for which a Species Recovery Plan was devised (García 1996, 1997). Subsequently, several conservation initiatives were funded by a LIFE Project of the European Union (García et al., 2002) and by the Aragon Regional Government (DGA). This species was also included in several lists on endangered species (Barreno et al., 1984; VVAA, 2000) as ‘Critically Endangered’ (García et al., 2002; Göni and Guzmán, 2003).

Studies of population dynamics have revealed that the population of *B. chouardii* is demographically stable, with a finite rate of increase close to one, and that this trend is expected to continue throughout the next century unless a recurrent increase of 10% mortality occurs (García, 2003). In the absence of any other extrinsic factors, this mortality rate could be reached in the event of catastrophic events (e.g. fires), and habitat protection is a top priority for survival of the species. However, demographic changes are often difficult to avoid due to demographic and environmental stochasticity, and, therefore, additional conservation strategies are required.

The genetic diversity of the wild population of *B. chouardii* has so far been surveyed with three different molecular tools. Of these, allozymes were the least polymorphic and did not allow any fine-scale assessment of the population structuring (Segarra-Moragues and Catalán, 2002). Conversely, the more polymorphic RAPD (random amplified polymorphic DNA) and, to a greater extent, microsatellites were able to distinguish two genetically divergent subpopulations within the small range of this taxon, corresponding to their spatial separation (Segarra-Moragues et al., 2005). The origin of these spatial subpopulations is probably related to past genetic bottlenecks coupled with the inefficient seed dispersal mechanism shown by this species (post-carpotropism) and by the short dispersal of pollen, which is mediated by ants (García, 2003), that promotes mating among close individuals. The strong genetic substructuring detected in *B. chouardii* prompted several novel recommendations for the conservation management plan of the species, including the screening of genetic diversity of the seed bank (Segarra-Moragues et al., 2005).

A seed collection of *B. chouardii* was established in 1999 and has been maintained at the Germplasm Bank of the Polytechnic University of Madrid (UPM). As these stocks were established before the development of genetic studies on this species, genetic information was not considered in the sampling schemes. Furthermore, since different collectors contributed to this seed collection, the spatial provenance of the seeds is, in some cases, unclear.

The maintenance of genetic diversity is considered crucial in preventing the loss of evolutionary potential and ensuring the survival of a given species, because of its association with fitness traits and increased biological success (Paschke et al., 2002; Pluess and Stöcklin, 2004). This is a special matter of concern for narrowly distributed taxa that, like *B. chouardii*, have often been associated with low levels of genetic diversity due to the concurrence of several factors including small population size, inbreeding depression and specialized habitat adaptation (Loveless and Hamrick, 1984; Hamrick et al., 1991; Ellstrand and Elam, 1993; Hamrick and Godt, 1996; Segarra-Moragues et al., 2005). Despite the fact that genetic diversity levels at neutral loci may not necessarily be linked to those of adaptive loci, several studies have demonstrated the association between higher fitness rates and higher levels of variability at neutral loci (Paschke et al., 2002; Pluess and Stöcklin, 2004), suggesting that preserving adequate amounts of genetic diversity scored at neutral loci could also result in the preservation of similar levels of genetic diversity at adaptive loci.

In the present study, we used microsatellites to determine the levels of genetic variation preserved in the ex situ collection of *B. chouardii* and to determine the geographical origin of these accessions. This knowledge benefits the conservation strategy led by the Aragón Government for this species by identifying the genetic provenance of the germplasm stocks in case they are required for future recovery actions, such as reinforcements or reintroductions.

**MATERIALS AND METHODS**

*Germplasm samples*

Three hundred *B. chouardii* seeds, collected from 1999 to 2002 and kept in the germplasm bank of the UPM, were used in the present study. Because most of seedlings germinated under conventional conditions died well before reaching the minimum growth required to obtain enough DNA, all the germinated seedlings were micropropagated in order to obtain suitable DNA concentrations for the amplification of all simple sequence repeat (SSR) loci. Despite some occasional somaclonal mutations caused by this technique (Palombi and Damiano, 2002), *in vitro* culture has proven to be very stable in other Dioscoreaceae grown under similar conditions (Ahuja et al., 2002; Dixit et al., 2003).
From the 264 seeds that did germinate, only 46 (17%) reached the stage of 5–6 young leaves necessary for in vitro propagation and subsequent DNA isolation. These 46 samples belong to five different stocks of the *B. chouardii* seed bank: Barranco (B), 11 samples; Cantera (CA), one sample; Cueva (CU), 14 samples; Mina (M), two samples; and Rappel (R), 18 samples, and represent approx. 6% of the total samples included in each stock. Fresh material obtained from the micropropagated plantlets was dried in silica gel (Chase and Hills, 1991) and used for DNA isolation. DNA was extracted following the 2·silica gel (Chase and Hills, 1991) and used for DNA isolation. The DNA concentration was estimated by comparing it with the brightness of ethidium bromide-stained marker VII (Roche) on agarose gels. Samples were diluted to a final concentration of approx. 5 ng L⁻¹ in 0·1x TE buffer.

**SSR amplification**

Seventeen SSR primer pairs previously characterized in Segarra-Moragues *et al.* (2003, 2004) were used in this study. Ten of them were specifically developed for this species (Segarra-Moragues *et al.*, 2003), and the remaining seven were transferred from the sister taxon *B. pyrenaica* Miégeville (Segarra-Moragues *et al.*, 2004). All primer pairs were developed to amplify trinucleotide (CTT) SSR regions. Polymerase chain reactions (PCRs) were performed as described in Segarra-Moragues *et al.* (2005). Products were run 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 SSR data**

Because of the hybrid allopolyploid origin of *B. chouardii* (P. Catalán *et al.*, unpubl. res.), 14 of the 17 analysed SSR regions resolved a total of 18 digenic disomic loci and five single disomic SSR loci for which genotypes could be confidently ascertained and encoded as for conventional diploids (Table 1). The remaining three SSR regions (Bc1159, Bc166 and Bp2292) showed overlapping size ranges of allele sets from both parental genomes, precluding the correct assignment of genotypes due to allele size homoplasy (P. Catalán, J. G. Segarra-Moragues, M. Palop-Esteban *et al.*, unpubl. res.).

Genotypic data were then collected for SSR loci derived from the 14 SSR regions totalling 23 non-linked disomic loci (P. Catalán, J. G. Segarra-Moragues, M. Palop-Esteban *et al.*, unpubl. res.). SSR alleles of these 23 loci were assigned to the corresponding genomic complement (A or B) from which they were amplified (Table 1). Some of the SSR primer pairs amplified a single SSR region, from only one parental subgenome (Bc1551, Bc1644, Bp126, Bp1286 and Bp2214), whereas others amplified homologous SSR loci from both parental subgenomes (Bc1145b, Bc1169, Bc1258, Bc1274, Bc1357, Bc1422, Bp2256, Bp2290 and Bp2391). This information was used to calculate genetic distances considering genotypic information for individual samples. For this purpose, the 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). In order to include the information produced by the remaining three microsatellite regions studied, an alternative binary coding of SSR bands was performed for all 17 SSR regions where the linear combination of multilocus phenotypes was analysed as for dominant genetic markers (Mengoni *et al.*, 2000; Staub *et al.*, 2000; Hormaza, 2002; Palombi and Damiano, 2002). The genetic data matrices encoded with either genotypes or phenotypes were then used to calculate the genetic distances between samples.

An earlier study conducted with samples collected from the wild population of *B. chouardii* allowed us to conclude that genetic differentiation between the two spatially separated subpopulations of this species was better explained by genetic distances based on allele identity, such as DA (Nei *et al.*, 1983), which better described the genetic relationships among individuals than those based on mutation rates (e.g. ȳu², Goldstein *et al.*, 1995; Average Distance, Goldstein and Pollock, 1997) (cf. Segarra-Moragues *et al.*, 2005). Consequently, we used this same distance index (DA) to depict the genetic relationships among the *B. chouardii* germplasm genotypes as implemented in POPULATIONS (Langella, 2000). On the other hand, the matrix of linear combination of multilocus microsatellite phenotypes, encoded as binary data, were used to compute D (Dice, 1945) and J (Jaccard, 1908) similarity coefficients with NTSYSpc v. 2·11a (Rohlf, 2002) and the pairwise distance difference (PD) with ARLEQUIN v. 2000 (Schneider *et al.*, 2000). Correlation between these different metrics was obtained through 1000 Mantel test replicates (Mantel, 1967) using NTSYSpc. As genetic distances between SSR phenotypes showed significantly high correlation among them (D/J r = 0·998 P < 0·001; D/PD r = −0·987, P < 0·001; J/PD r = −0·992, P < 0·001), the PD that is also the standard distance used for AMOVA (see below) was chosen for subsequent analyses. The selected genetic distances were used for clustering analyses to visualize the relationships between all the germplasm samples. Samples from the wild subpopulations were also included in these analyses in order to investigate the origin of the germplasm accessions. Neighbour-joining trees (Saitou and Nei, 1987) were constructed using MEGA2 (Kumar *et al.*, 2001). Principal coordinates analysis (PCO) analyses, where eigenvectors and eigenvalues were computed with the same distance indices, were produced using NTSYSpc.

The partitioning of the variance among germplasm stocks was studied using analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) in ARLEQUIN. Two different groups of germplasm accessions were established based on the preliminary results obtained from clustering analyses. One group consisted of Barranco (B), Cueva (CU) and Rappel (R) accessions, whereas the other group was formed by Mina (M) accessions. The Cantera (CA) accession was considered to pertain either to the first group or to the second group, and hierarchical AMOVA analyses were performed.
Table 1. Allele sets detected across 23 SSR loci in the wild population and in the five analysed germplasm stocks of B. chouardii

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<th>No.</th>
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<th>No.</th>
<th>Cueva (CU) n = 14</th>
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<th>Cantera (CA) n = 1</th>
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Alleles are indicated by their amplification product size (bp). Numbers in bold and in italics indicate exclusive alleles from the upper and the lower cliff subpopulations, respectively, and those in parentheses correspond to new alleles not previously detected in the wild population. A and B designate SSR loci for each of the two subgenomes of B. chouardii. An asterisk indicates those SSR regions that were not genotyped because of size homoplasy. N = sample size. No. = number of alleles.
accordingly. AMOVA was also performed between individual genotypes and phenotypes from the natural population and the germplasm bank as a method to test for percentages of differentiation and levels of significance among the two groups (Phippen et al., 1997).

STRUCTURE (Pritchard et al., 2000) was used to infer the proportion of membership of each individual from the germplasm bank to each of the two wild subpopulations. We used two ancestry models in our analyses: (1) the admixture model, that considers that individuals may have acquired a fraction of their genome from ancestors from each subpopulation; and (2) the non-admixture model, that considers that each individual comes purely from one of the subpopulations. The admixture model was applied to the genotypic data and the non-admixture model to the phenotypic data, as recommended by Pritchard et al. (2000). Because germplasm samples were all collected from the only extant subpopulations of B. chouardii and as only two genetic groups have been identified across the spatial range of the species so far (Segarra-Moragues et al., 2005), we assume that all the individuals analysed from the germplasm stocks belong to one of these two groups. Therefore, STRUCTURE runs were performed for two \((K = 2)\) inferred clusters using the allele frequencies correlated model. A burn-in period and a run length of \(10^5\) and \(10^6\) Monte Carlo Markov chain iterations were used, respectively, in order to reach an approximate stabilization of the summary statistics (Pritchard et al., 2000). We examined the probabilities of each germplasm sample belonging to each of the two predefined clusters. To assist the clustering, the individuals sampled from the wild subpopulations were also incorporated into the analyses and taken as reference using the USEPOPINFO option implemented in STRUCTURE (Pritchard et al., 2000).

RESULTS

The 17 primer pairs detected 62 different microsatellite alleles in the 46 samples analysed from the germplasm stocks of B. chouardii (Table 1). The number of alleles detected within each germplasm stock was similarly high for those with a similar moderate number of samples analysed, ranging from 46 (CU) to 49 (R), but lower for those stocks with a more reduced sample set, ranging from 37 (M) to 33 (CA) (Table 1). Eight alleles (Bc1169B-145, Bc1357B-134, Bc1644-166, 175, Bc166-185, Bc166-203, Bc166-206 and Bc166-224) previously scored in the wild population of B. chouardii (Segarra-Moragues et al., 2005) were not detected in any of the analysed germplasm samples. Two of these alleles (Bc1169B-145 and Bc1644-166) were only found in the lower cliff wild subpopulation, whereas the remaining six were private alleles of the upper cliff wild subpopulation (Table 1). Conversely, three novel SSR alleles, not scored from the wild samples, were detected in the germplasm stocks (Table 1). One of these (Bc1258A-165) was found in a homozygous individual of the R stock, one (Bc1274-279) was found in two homozygous and two heterozygous individuals of the CU stocks, and the third one (Bc166-233) was found in two triallelic individuals of the R stock (Table 1). These novel alleles deviated one, three and five mutational steps, respectively, from the largest alleles scored for those microsatellite loci/regions within the individuals of the wild population (Table 1).

Neighbour-joining trees were constructed either with genotypic data derived from 23 SSR loci, which included information from 49 SSR alleles (Fig. 1), or with multilocus phenotypic data (70 microsatellite bands) (not shown). The samples clustered in two main groups, corresponding to

**Fig. 1.** Neighbour-joining tree showing the relationships among germplasm accessions and wild individuals of B. chouardii based on genotypic data of 23 SSR loci with the \(D_\alpha\) distance method.
the upper and lower cliff subpopulations, retaining the previously reported microspatial separation between the two *B. chouardii* geographical groups (Segarra-Moragues et al., 2005) after the inclusion of the germplasm samples. All samples apart from two (from the R stock) were characterized by unique molecular genotypic or phenotypic fingerprints. These two samples, that were labelled as having been collected from the same mother plant in the wild population and that also share their molecular fingerprints, could be full sibs. All germplasm samples derived from stocks B, CU and R were embedded within samples from the lower cliff subpopulation, whereas those from stock M clustered with those from the upper cliff subpopulation. The single CA sample showed differential genetic affinities depending on the data set used for clustering. Whereas in the tree constructed with the genotypic data set, this sample was embedded in a cluster formed by R and B stocks, closer to the lower cliff subpopulation (Fig. 1), in the tree constructed with the phenotypic data set, this sample clustered with those from the upper cliff subpopulation. The inclusion of the CA sample as a reference, succeeded in assigning most of the germplasm samples to their subpopulation source (Fig. 2). However, these analyses proved more informative when information from all phenotyped SSR alleles was considered (Fig. 2B). In both cases, the M samples were inferred to have a high proportion of membership (>90%) of the upper cliff subpopulation, whereas those from B, CU and R showed high proportions of membership of the lower cliff subpopulation (Fig. 2A). Nonetheless, when only genotypic information was considered, some samples from these stocks (B, CU and R) showed a moderate proportion of membership (approx. 30%) of the upper cliff cluster (Fig. 2A). This result is consistent with some individuals from the lower cliff wild subpopulation being descended from individuals from the upper cliff wild subpopulation (Fig. 2A) as a consequence of downward dispersal of seeds, possibly by gravity. Assignment of the CA samples varies from one analysis to the other; the genotypic data attributed a relatively high percentage of membership (approx. 70%) of this sample to the lower cliff subpopulation, whereas the phenotypic data attributed
a higher percentage of membership (>98%) to the upper cliff subpopulation.

DISCUSSION

Genetic data can be used to design sampling strategies that maximize the genetic diversity of collections and to manage restoration of populations using ex situ stocks (Maunder et al., 2001; McGlaughlin et al., 2002; Kephart, 2004). However, the genetic characterization of populations is required prior to the establishment of any ex situ accession (Caujapé-Castells and Pedrola-Monfort, 2004; Mateu-Andrés and Segarra-Moragues, 2004). On the other hand, if collections such as those of B. chouardii have already been established, then molecular markers can be used to assess the genetic representation of the wild subpopulations in the germplasm bank and to identify suitable source–target combinations to avoid the introduction of alien material in the natural subpopulations (Maunder et al., 2001). Empirical sources of evidence have demonstrated that highly structured populations may show a degree of local adaptation and that the introduction of alien material may not only disrupt the current genetic architecture but also reduce the overall population fitness in unforeseen ways (Gustafson et al., 2004a, b; Hardy et al., 2004). In this sense, genetic typing of available ex situ stocks of B. chouardii of undocumented origin is an important prerequisite for their use in undertaking restoration tasks for this endangered species. Appropriate management schedules should be proposed for this taxon to ensure no further loss of genetic diversity and adaptive capability (Robert et al., 2004).

Genetic diversity of the germplasm accessions of B. chouardii

Genetic diversity measured in terms of allele richness indicates that only 88% of the alleles detected in the wild subpopulations of B. chouardii are represented in the germplasm accessions analysed so far. Nonetheless, three alleles, previously unrecorded from the natural population, were scored from the analysed germplasm stocks (CU and R, Table 1). In vitro culture can induce somaclonal variation in propagated material in terms of gain or loss of genetic variability with respect to the source material (Palombi and Damiano, 2002). However, the occurrence of somaclonal variation seems unlikely in our case because two of the three novel alleles were three (Bc1274-279) and five (Bc166-233) mutational steps larger than the largest allele scored either within the wild population or within the germplasm stocks. Therefore, it is improbable that somaclonal variation produced these new alleles during the first generation of
in vitro culture that usually involves a single mutation step (Xu et al., 2000). The other allele (Bc1258A-165) was scored from a homozygous individual, and it is also improbable that somaclonal mutation would account for duplicate acquisition of a novel allele. The fact that two of these new alleles appeared in the R stock suggests that they could occur in rather inaccessible individuals from the upper cliff that have not been sampled previously except for this germplasm stock collected during a rappel descent of the cliff.

Despite the low genotypic and phenotypic differences detected between the natural population and the germplasm bank, the percentage of genetic variation observed in the germplasm stocks analysed is below the recommended percentage (99–99.9%) of the desired genetic variation to be preserved in narrow endemics (Caujapé-Castells and Pedrola-Monfort, 2004; López-Pujol et al., 2004). Most of this allelic representation is related to that of the lower cliff subpopulation as 47 (96%) of the 49 alleles of this group are represented, in contrast to only 50 (89%) of the 56 alleles from the upper cliff. Furthermore, these figures dramatically decrease when percentages are calculated for the exclusive SSR alleles; only 67 and the 82% of the private SSR alleles from the upper and lower cliff subpopulations were represented, respectively, in the analysed samples of the germplasm bank. Even if the observed values of allelic representation may be biased by the number of accessions analysed, this number approached that of the samples studied from the wild population and represents 6% of the available germplasm stocks for this species. Increasing the number of accessions analysed would probably result in the detection of more alleles but would reduce the scarce ex situ material of B. chouardii, which would be more accentuated given the low survival rate (17%) of seedlings after germination. Moreover, the additional detection of new alleles in the germplasm stocks at otherwise very low frequencies also makes these collections unsuitable for restoring accidental loss of genetic variation in the wild population. In this sense, microsatellites could serve to target suitable wild plants as seed donors to improve the quantity of under-represented alleles in the germplasm stocks.

Relationship of germplasm accessions to the wild subpopulations of B. chouardii

Assignment tests have a highly discriminative capability to determine the origin of an individual when the reference population samples have a clear differentiation at the molecular level. They have proved useful in relating a sample to its geographical source with high probability once as many reference populations as possible have been genetically typed (Waser and Strobeck, 1998). However, the accuracy of these methods is affected by the number of samples analysed and by the number of loci scored (Cornuet et al., 1999; Davies et al., 1999). Despite the narrow vertical 150 m spatial separation between the samples of the wild population of B. chouardii, the reduced population size and limited seed dispersal capability of this species have favoured the molecular divergence of the two upper and lower cliff subpopulations characterized by 18 and 11 exclusive SSR alleles, respectively (Segarra-Moragues et al., 2005). The clear genetic split between these two groups of individuals allows inferences about the origin of the germplasm accessions. As a consequence, the geographical source of the germplasm accessions could be traced back to one of these two natural groups when information from the 23 genotyped loci (Fig. 2A) and, to a greater extent, from all 70 scored microsatellite bands (Fig. 2B) was considered.

The germplasm accessions showed different affinities from the wild subpopulations of B. chouardii in terms of both qualitative allelic composition and multilocus SSR profiles. Comparison of exclusive alleles previously scored from the wild subpopulations and those detected from the germplasm accessions revealed that the stocks of B, CU and R presented a higher proportion of the alleles assigned exclusively to the lower cliff subpopulation (81–8%), whereas those exclusive to the upper cliff subpopulation showed a higher representation in the reduced M stock (38.9%). The single CA accession showed intermediate results, with 12.2 and 27.3% of its alleles representing the upper and lower cliff, respectively.

These observations were consistent with PCO and clustering analyses of samples based on both genotypic and phenotypic SSR data, for which the accessions of M stocks were related to the upper cliff subpopulation and those of the remaining B, CU and R stocks to the lower cliff subpopulation. Further insights into the relationships between germplasm accessions and the wild subpopulations could be extracted from the clustering analyses. Whereas samples of B and CU were deeply embedded among individuals collected from the lower cliff, those of the R stock form sister subclusters linking both microgeographical areas. This position in the neighbour-joining trees could be related to their presumed intermediate collection site. These R stock samples were probably collected from mother plants that could have been located in the upper part of the lower cliff and perhaps could have maintained low levels of gene flow with individuals from the upper cliff subpopulation. This is consistent with the high proportion of membership of some of these samples to the upper cliff cluster (Fig. 2A). Nonetheless, although they could have retained a certain degree of mixed ancestry, their closer relationship to individuals from the lower cliff was clearer (Figs 1 and 2). More samples of the CA stock would be needed to resolve the intermediate position of the unique studied sample with respect to the wild subpopulations. The alternative linkage of this accession to the two groups obtained through genotypic assignment tests could be explained by a mixed ancestry of this particular sample (Rannala and Mountain, 1997), but a preliminary conclusion extracted from our larger phenotypic data suggests a closer affinity for the upper cliff subpopulation (Fig. 2B).

Our study provides valuable information for the management of the ex situ stocks of B. chouardii. One direct conclusion that can be extracted from our study is that more samples are required for the complete allelic representation of the wild population in this genetic collection, especially from the upper cliff subpopulation. The genetic
characterization of both the germplasm accessions and the wild individuals allows for the identification of the microspatial origin of these stocks and provides supplementary information for their potential use in reinforcement of the subpopulations. Microsatellite data allowed us to identify a common origin for the B, CU and R stocks of the subpopulations. The M and CA stocks should be managed separately solely for the reinforcement of the lower cliff subpopulation if necessary, in order to keep intact the genetic structure of the wild subpopulations of B. chouardii. The use of native material in the restoration of targeted populations is a management scheme that has been highly recommended in recent conservation genetics literature as it combines intraspecific phylogenetic relationships of coalescence with the maintenance of the genetic structure observed in wild populations (Gustafson et al., 2004b). This procedure may result in a higher success rate of recovery plans in cases where there exists a degree of association between certain genotypic combinations and their relative adaptive capability to microecological conditions (Gustafson et al., 2004a).

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