

Life history variation between species of the relictual genus *Borderea* (Dioscoreaceae): phylogeography, genetic diversity, and population genetic structure assessed by RAPD markers

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The genus *Borderea* consists of two species, *B. pyrenaica* and *B. chouardii*, taxa which have been previously considered as conspecific due to their overall close morphology. These two sole species of the rare genus of Dioscoreaceae are endemic to the Pyrenees (Spain, France). This mountain range likely operated as a refugium for these plants during the last glaciations. *B. chouardii* is only known from a single population in the Spanish Prepyrenees and has been classified as at risk of extinction in the Red List of Endangered Species (IUCN); *B. pyrenaica* shows a narrow distribution range in the central Pyrenees and Prepyrenees. We analysed genetic variation, population structure and differentiation in these two taxa using RAPD markers. Our study was conducted on the same seven populations for which very low levels of genetic differentiation were detected previously through allozyme analysis. By contrast, high levels of genetic variability were detected through the RAPD hypervariable markers. Twelve RAPD primers produced 112 distinct bands in the 397 surveyed individuals, totalling 395 different RAPD phenotypes. Only four bands were monomorphic across all samples of *Borderea*, whereas 21 of the polymorphic bands were species-specific (20 for *B. chouardii*, and one for *B. pyrenaica*). The largest genetic distances were those between the *B. chouardii* and the *B. pyrenaica* phenotypes. An analysis of molecular variance showed greater variance between groups (*B. chouardii* vs. *B. pyrenaica*, 76.08%) than within groups (3.60%). RAPD band specificity, phenotypic distances, and the partitioning of variance all support the taxonomic separation of the two species. Statistical evaluation of within- and among-population RAPD genetic variability in *B. pyrenaica* showed that genetic variability was higher within populations (>80%) than among them. No clear pattern of RAPD differentiation could be observed among the six studied populations of this taxon though slight differences in genetic diversity could be observed in the more isolated Prepyrenean populations compared with the more widespread Pyrenean ones. These results suggest a recent postglacial origin of the present *B. pyrenaica* populations. © 2003 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2003, 80, 483–498.

ADDITIONAL KEYWORDS: *Borderea chouardii* – *Borderea pyrenaica* – conservation of endangered plants – *Dioscorea* – narrow endemics – postglacial diversification – Pyrenean flora – tertiary flora.

INTRODUCTION

Southern European refugia have been considered centres of speciation and reservoirs of genetic variation for many plant taxa during the oscillatory climatic changes of the late Tertiary and the Quaternary (Webb & Bartlein, 1992; Hewitt, 1993, 1996; Comes & Kad-

ereit, 1998). The Pyrenees, the mountain divide between the Iberian peninsula and the Western European plains, played a key role during the postglacial colonizations of the Pleistocene, operating both as a migratory northbound route for some species and as a natural barrier that prevented the expansion of others (Taberlet *et al.*, 1998). This mountain range became a hybrid zone for close parapatric taxa that rejoined there after the last ice melt (Hewitt, 1993; Vasquez *et al.*, 1994; Taberlet *et al.*, 1998). The southern terri-

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tories of Europe have also been the site of different speciation events during the contraction-expansion cycles that dominated the latter ice ages (Hewitt, 1996; Gutiérrez-Larena, Fuertes-Aguilar & Nieto-Feliner, 2002). In these regions periodic advances and retreats of glaciers likely forced species to descend and ascend the mountain ranges with the consequent genetic isolation and bottlenecking of their populations followed by potential secondary contacts between some of them. The genus *Borderea* fits well into this scenario; the Pyrenees were the likely place of speciation of its two sole extant species, *B. chouardii* and *B. pyrenaica*, that now occupy different habitats. The mountain range may also have served as a refuge for populations of the more widespread *B. pyrenaica* during upslope and downslope migrations.

The *Borderea* species are orophytes, endemic to the central Pyrenean and Prepyrenean mountains. They possess some of the longest lifespans for herbaceous plants that have been reported (García & Antor, 1995a; García, 1997; García, Guzman & Goñi, 2002), with some individuals having been aged at over 300 years old. Both species are dioecious, mainly ant-pollinated (García, Antor & Espadaler, 1995), and strictly sexually reproducing geophytes, with a male-biased sex ratio of 2 : 1 (García & Antor, 1995b; García *et al.*, 2002). The two species are different in several

morphological quantitative characters, they are geographically separated (Fig. 1), and they show distinct ecological preferences (Gaussen, 1952, 1965). However, their morphological similarity has moved some authors to speculate about the taxonomic distinctness of the single-population taxon *B. chouardii* from its congener *B. pyrenaica*, suggesting that the former could be a subspecies of the latter (Burkill, 1960). The scarcity of material available from *B. chouardii* for comparative studies (collection is prevented by Spanish law) contributes to their uncertain taxonomic status.

Borderea chouardii is a threatened chasmophytic species that has been classified as being 'in danger of extinction' in Annex II of the Habitats Directive of the European Union (García, 1996) and as 'critically endangered' in the Spanish Red List of Endangered National Plants (V.V.A.A., 2000). It is known from a single population at one of the southernmost Spanish Prepyrenean mountain ranges (Sopeira, Huesca province) (Fig. 1) growing on limestone cliffs at low elevation (c. 800 m above sea-level). Studies of this taxon have shown that only half of the approximately 2000 individuals of this population are reproductive and that only one third of these are females (García *et al.*, 2002). The extremely low effective population size and narrow geographical range (about 1000 m²) of

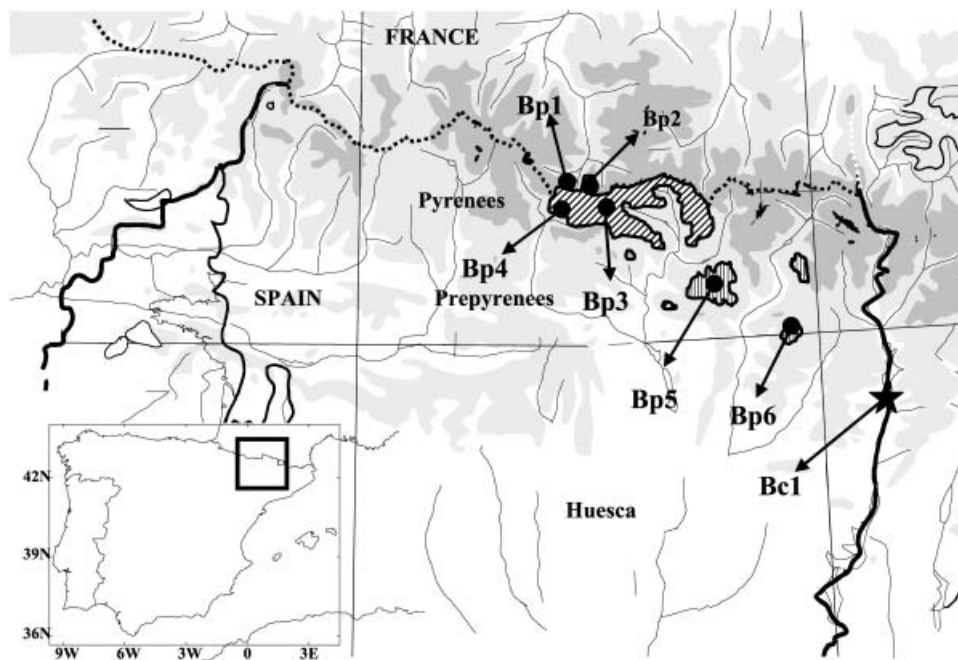


Figure 1. Map of the area of distribution of *Borderea* and the studied populations: Bc = *B. chouardii*; Bp = *B. pyrenaica*. Diagonal shading corresponds to Pyrenean range (Monte Perdido massif) and vertical shading to Prepyrenean range (Cotiella and Turbón massifs). Bp1, France: Gavarnie, La Planette; Bp2, France: Gavarnie, Les Rochers Blancs; Bp3, Spain: Huesca, Pineta; Bp4, Spain: Huesca, Ordesa; Bp5, Spain: Huesca, Saravillo, La Vasa Mora; Bp6, Spain: Huesca, Turbón. Bc1, Spain: Huesca, Sopeira.

B. chouardii coupled with the reduced capability to colonize new habitats due to its limited seed dispersal system could cause the extinction of this plant due to either stochastic events or anthropogenic action.

B. pyrenaica is a more widespread taxon than *B. chouardii*, though it is confined to a narrow geographical area in the Central Pyrenean and Prepyrenean region (Fig. 1). This endemic plant lives on mobile calcareous screes above 1800 m above sea-level and occupies an area of approximately 160 km². Most of its populations are located in Spain, on the southern side of the Pyrenees and in the Prepyrenees. The only known populations of *B. pyrenaica* on the northern side of the Pyrenees are at Gavarnie, in France. The geographical map distances among the *B. pyrenaica* populations are short for those located around the axial divide of the Pyrenean mountain chain (less than 15 km from each other). They are, however, separated by some of the highest peaks of this mountain range – the Monte Perdido massif – which borders the Gavarnie, Ordesa and Pineta valleys (Fig. 1). Conversely, the Prepyrenean populations of *B. pyrenaica* are located farther away, in the Cotiella and Turbón mountains, which are separated by deep valleys and by more than 30 km and 50 km from the Pyrenean core populations, respectively (Fig. 1). Populations of *B. pyrenaica* are large compared with that of its congener; some of them include more than 10 000 reproductive individuals that inhabit wide, almost unaltered high-mountain areas. Thus, in spite of their restricted geographical range, populations of *B. pyrenaica* are less threatened by either intrinsic or extrinsic factors.

The genus *Borderea* is considered to be a relict of a Tertiary lineage of the Dioscoreaceae (Burkill, 1960; García *et al.*, 1995), as the vast majority of the family (more than 600 spp.) show a pantropical distribution with only few taxa presently occurring outside that range. A recent phylogenetic study of Dioscoreaceae (Caddick *et al.*, 2002a) based on *rbcl*, *atpB*, and 18S rDNA sequences and morphological characters demonstrated that the genus *Dioscorea s.l.* (Brummitt, 1992; Huber, 1998) is paraphyletic and that the dioecious Dioscoreaceae (i.e. *Borderea*, *Tamus*) are embedded within a clade of monoecious species. According to these findings, supported by the evidence that the putative synapomorphic traits of these genera are not unique to them, Caddick and coworkers decided to include *Borderea* and its sister Mediterranean genus *Tamus* within *Dioscorea* (Caddick *et al.*, 2002b).

Regardless of its taxonomic classification, *Borderea* likely represents an evolutionary split from an old Dioscoreaceae lineage that successfully adapted and colonized the central mountains of the Pyrenees (Burkill, 1960). In this study we assess the possible impact of climatic changes experienced during the late

Tertiary and Quaternary ages in these mountain ranges on the present genetic variability of the *B. pyrenaica* populations and consider how such changes may have led to genetic isolation of populations and speciation to produce *B. chouardii*. As the area currently occupied by *Borderea* (especially *B. pyrenaica*) was primarily covered by ice during the last glaciations (Chueca & Lampre, 1994), its populations are expected to have suffered several changes in their habitat conditions. As glaciated regions became larger, populations of *B. pyrenaica* would be expected to have developed at lower elevations, and probably these retreats caused the extinction or the decimation of some populations. The genetic bottlenecks experienced by the remnant populations would have contributed to the genetic impoverishment of the species before the current habitats were recolonized. Declines in population sizes, coupled with local population extinctions, are likely arguments that have been postulated to explain the loss of genetic diversity found in several present-day endangered plants (Pleasants & Wendel, 1989; Godt & Hamrick, 1998). However, a postglacial colonizing success would have caused the expansion of geographical range of the new populations, ultimately allowing increases in genetic variability and the possibility of secondary contacts between them. This last scenario could fit well with the life history of the more widespread *B. pyrenaica*, differentiating it from that of the narrow endemic *B. chouardii*.

Under this hypothesis we might expect strong genetic divergence between the two *Borderea* taxa and higher levels of genetic variability in *B. pyrenaica* than in *B. chouardii*. Regarding the populations of *B. pyrenaica*, we would expect to find a clear genetic differentiation between those populations distributed in the Pyrenean axial divide and those found in the more isolated Prepyrenean ranges in the absence of recent gene flow. Alternative hypotheses on distinct origin times of these two taxa and different colonizing pathways to their presently occupied areas have been evaluated through analysis of genetic parameters and statistical correlation between genetic and geographical distances.

An allozyme analysis conducted on six populations of *B. pyrenaica* and on the only known population of *B. chouardii* detected very low levels of genetic variability in these taxa (Segarra-Moragues & Catalán, 2002). These results strongly contrasted with the levels of genetic variability expected for *Borderea* based on its reproductive traits. Nonetheless, the largest genetic distances were those between *B. chouardii* and all the *B. pyrenaica* populations.

As the allozyme markers detected so few polymorphisms within *Borderea*, we conducted a population genetic analysis of *B. chouardii* and *B. pyrenaica*

using highly variable RAPD markers (Williams *et al.*, 1990). RAPD markers have been applied satisfactorily both in the assessment of genetic divergence and genetic variability of closely related Dioscoreaceae taxa (Ramser *et al.*, 1996, 1997) and to ascertain the historical population dynamics of arctic and montane flora subjected to glaciations (Gabrielsen *et al.*, 1997; Bauert *et al.*, 1998; Tollefsrud *et al.*, 1998).

Although there are some technical difficulties, such as dominance, that hinder the determination of allelic frequencies at RAPD loci in natural populations (Lynch & Milligan, 1994), the advantages offered by these markers which can give robust population estimates of genetic diversity (Borowsky, 2001), and the large number of studies published for comparison, make them suitable for population genetic surveys when the potential pitfalls of the technique are avoided by an accurate and reproducible PCR protocol and by increasing the number of primers screened and the number of loci scored.

MATERIAL AND METHODS

POPULATION SAMPLING, DNA EXTRACTION AND AMPLIFICATION

Sampling of populations and individuals was conducted on the same localities and individuals as those used for the allozyme analysis (Fig. 1). A total of 397 individuals were used for the RAPD analysis. A male : female ratio of 1 : 1 was maintained in this RAPD sampling scheme. Sampling included the only known population of *B. chouardii* (Bc1: Sopeira, Huesca, Spain, $N = 47$), and six populations of *B. pyrenaica* distributed along its geographical range. Four of the *B. pyrenaica* populations are located in the Pyrenean axial divide, two of them occur on the northern face of the Monte Perdido massif (Bp1: La Planette, Gavarnie, France, $N = 58$; Bp2: Rochers Blancs, Gavarnie, France, $N = 56$), and the other two grow on the southern face of this mountain range (Bp3: Pineta, Huesca, Spain, $N = 58$; Bp4: Ordesa, Huesca, Spain, $N = 60$). The remaining *B. pyrenaica* populations inhabit the more distant Prepyrenean massifs (Bp5: Cotiella, Huesca, Spain, $N = 58$; Bp6: Turbón, Huesca, Spain, $N = 60$).

Fresh leaves from all sampled individuals were dried in silica gel and used for DNA isolation. DNA was extracted following the CTAB protocol of Doyle & Doyle (1987) adapted for miniprep extractions. DNA concentration was estimated by comparing with the brightness of ethidium bromide stained marker VII (Roche) on agarose gels; samples were diluted to a final concentration of c. 5 ng/ μ L in 0.1 \times Tris-EDTA buffer. Forty RAPD primers from Operon Technologies (kits A and B) were assayed in a pilot sample of 28

individuals selected from the seven studied populations. Amplifications were carried out in 20 μ L total volume containing 1 \times buffer (Ecogen), 2.5 mM MgCl₂, 0.4 mM each dNTP, 4 pmol primer, 1.0 unit Taq DNA polymerase (Ecogen), and 2 ng template DNA. The PCR was conducted in a GenAmp 9700 Thermalcycler (Applied Biosystem) using both positive and negative controls in order to detect the efficiency of the enzyme and the absence of contamination. The amplification programme consisted of a single step of DNA denaturation 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; electrophoresis was set at 100 V for 4 h in 0.5 \times 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 primers out of the 40 assayed that rendered repeatable, strongly stained and easily scorable amplicons were selected for the analysis of the whole set of samples.

DATA ANALYSIS

RAPD bands were scored by their presence/absence into a data matrix that was analysed using different methods. Three different metric distances were computed: Dice's and Jaccard's similarity coefficients, both excluding shared absences of bands, were computed with an implementation of RAPDISTANCE version 1.04 (Armstrong *et al.*, 1996), adapted for large sample sizes and the pairwise difference distance (Excoffier, Smouse & Quattro, 1992) was computed with ARLEQUIN vs. 2.000 (Schneider, Roessli & Excoffier, 2000). Correlations between Dice's, Jaccard's, and pairwise difference values were calculated through a Mantel test with 1000 replicates (Mantel, 1967) using NTSYSpc vs. 2.11a (Rohlf, 2002). As genetic distances between phenotypes based on Dice's (D) and Jaccard's (J) coefficients showed significant high correlation among them and with the pairwise difference (PD) distance (D/J $r = 0.996$, $P < 0.002$; D/PD $r = -0.993$, $P < 0.001$; J/PD $r = -0.985$, $P < 0.001$), the pairwise difference distance was chosen for subsequent analyses.

Genetic diversity was estimated from the frequencies of the RAPD bands using Nei's (1973) algorithm $h = 1 - 1/m \sum_i \sum_u plu^2$, where plu is the frequency of the u^{th} band at the l^{th} locus and m is the number of loci (Peever & Milgroom, 1994). Genetic diversity values were calculated at both the species and population levels, both including or excluding monomorphic bands.

The genetic structure of the populations of *Borderea* was studied by means of the analysis of the molecular

variance (AMOVA; Excoffier *et al.*, 1992) using ARLEQUIN. Although AMOVA was originally designed for RFLP haplotypes, it has been widely used to analyse RAPD phenotypes (Steward & Excoffier, 1996; Gabrielsen *et al.*, 1997; Martin, Gonzalez-Benito & Iriondo, 1997; Palacios & González-Candelas, 1997; Wolf, El-Akkad & Abbott, 1997). AMOVA analysis was performed at different hierarchical levels: (i) all samples considered as one species (*Borderea s.l.*) (ii) among species (*B. chouardii* vs. *B. pyrenaica*) (iii) within and among populations of *B. pyrenaica* with no geographical ranges, (iv-vi) within and among populations and among groups of populations of *B. pyrenaica* separated into four geographical ranges (1: Pyrenees vs. Prepyrenees; 2: northern Pyrenees vs. southern Pyrenees vs. Prepyrenees; 3: northern Pyrenees and Cotiella massif vs. Turbón massif; 4: French Pyrenees vs. Spanish Pyrenees and Cotiella massif vs. Turbón massif; 6: French Pyrenees vs. Spanish Pyrennes vs. Cotiella massif vs. Turbón massif). Significance levels of the variance components estimated for each case were obtained by non-parametric permutation using 1000 replicates.

The relationship among all RAPD phenotypes was visualized by a Neighbour-Joining tree constructed with MEGA vs. 2.0 (Kumar *et al.*, 2001) in which statistical robustness of the groupings was assessed by bootstrap analysis with 1000 replicates (Felsenstein, 1985) using PAUP* version 4.0beta10 (Swofford, 2002), and by a principal coordinate analysis (PCO) using NTSYSpc (Rohlf, 2002). Two different approaches were followed in the PCO analysis: (i) with the whole data matrix, to visualize the multidimensional relationships of the RAPD phenotypes of both taxa, and (ii) with a subset of the matrix, containing only the phenotypes of *B. pyrenaica*, to test for differences in the spatial distribution of RAPD phenotypes among populations of this taxon.

Genetic distances between populations were based on F_{ST} statistics among RAPD phenotypes obtained from the AMOVA. The resulting distance data matrix was used to construct a phenogram applying the UPGMA algorithm in NTSYSpc and bootstrapped with POPULATIONS version 1.2.28 (Langella, 2000). The correlation between RAPD genetic distances and geographical distances between populations was assessed by means of a 1000 replicate Mantel test using NTSYSpc.

RESULTS

RAPD DIVERSITY IN *BORDEREA*

The 12 primers used in this survey rendered 112 bands for the 397 surveyed individuals (Table 1). Only four (3.57%) out of the 112 markers were monomorphic.

Eighty (71.42%) of these bands were present in *B. chouardii* and 81 (72.32%) in *B. pyrenaica*. Fifty (44.64%) bands were shared between both taxa whereas 58 (51.78%) bands were exclusive to one species or the other. Twenty-one of these private loci were monomorphic within species (20 for *B. chouardii* and one for *B. pyrenaica*) and therefore useful for the discrimination between these taxa. Among the loci present in both taxa 47.5% were polymorphic in *B. chouardii* and 92.59% in *B. pyrenaica*. Within *B. chouardii* polymorphic loci ($N = 38$) almost equalled the monomorphic ones ($N = 42$) whereas in *B. pyrenaica* the number of polymorphic bands ($N = 75$) largely outnumbered the invariable ones ($N = 6$).

Measurements of genetic diversity are shown in Table 2. *Borderea s.l.* showed relatively high levels of genetic diversity (mean diversity = 0.574), ranging from 0.190 to 0.843 depending on the primer. Levels of genetic diversity at species rank ranged from 0.112 to 0.762 in *B. pyrenaica* and from 0 to 0.323 in *B. chouardii*. Most of the genetic variation contained in *Borderea s.l.* could be attributed to the more widespread species *B. pyrenaica* (mean diversity = 0.379) whereas the restricted endemic *B. chouardii* was less variable (mean diversity = 0.139). All *B. pyrenaica* populations showed similar levels of genetic diversity, the large Pyrenean population of Pineta (Bp3) being the most variable one (0.414).

The distribution of frequencies of band presences across the 112 positions scored also showed remarkable differences between taxa (Fig. 2). In all populations of *B. pyrenaica* the bands appeared homogeneously distributed in different classes of frequency, exhibiting a similar sigmoid graph pattern that overlapped with that calculated for the species. Conversely, in the single population of *B. chouardii* there was a high proportion of fixed or high-frequency bands and a low number of low-frequency bands as depicted in its semisigmoid graph pattern (Fig. 2).

RELATIONSHIPS AMONG RAPD PHENOTYPES

The RAPD markers generated by the 12 primers provided 395 distinct phenotypes across the 397 studied individuals. Only two phenotypes were shared, one between two individuals of *B. pyrenaica* (Bp1) and the other between two individuals of *B. chouardii*. Most of the band patterns clearly differentiated individuals from each species (Fig. 3). The genetic distances between phenotypes were considerably larger between species than within them, hence supporting the high levels of genetic diversity found between them.

The PCO of the whole data matrix (*Borderea s.l.*) showed a complete differentiation between *B. chouardii* and *B. pyrenaica* phenotypes which clustered separately on the space defined by the first two

Table 1. Details of primers used

Primer	Sequence (5'–3')	bp range	Number of bands observed		<i>Borderea s.l.</i>		<i>B. chopardii</i>		<i>B. pyrenaica</i>	
			Monomorphic	Polymorphic	Monomorphic	Polymorphic	Monomorphic	Polymorphic	Monomorphic	Polymorphic
			OPA12	TCGGCGATAG	250–800	0	6	3 (2)	3 (3)	0
OPB3	CATCCCCCTG	250–1800	0	10	3 (2)	5 (1)	0	7 (2)	0	7 (2)
OPB5	TGCGCCCTTC	400–1700	0	12	7 (2)	3	0	10 (2)	0	10 (2)
OPB6	TGCTTGCC	425–1900	0	12	5 (2)	4 (3)	0	7 (3)	0	7 (3)
OPB7	GGTGACGCAG	500–1400	1	11 (1 singleton)	3	2 (1)	1	10 (7, 1 singleton)	1	10 (7, 1 singleton)
OPB8	GTCCACACGG	500–1500	0	9	1	8	0	9	0	9
OPB9	TGGGGGACTC	250–1030	0	10	7 (7)	0	0	3 (3)	0	3 (3)
OPB10	CTGCTGGGAC	800–1400	0	11	1	6 (1, singleton)	0	10 (4)	0	10 (4)
OPB11	GTAGACCCGT	250–1600	2	5	4 (1)	0	2	4 (3)	2	4 (3)
OPB12	CCTTGACGCA	500–1100	0	4	0	4	0	4	0	4
OPB15	GGAGGGTGT	475–1300	1	9	6 (3)	1 (1)	1	5 (3)	1	5 (3)
OPB18	CCACAGCAGT	700–1300	0	9 (1 singleton)	2 (1)	2 (1)	2 (1)	5 (3, 1 singleton)	2 (1)	5 (3, 1 singleton)
Total			4	108 (2 singleton)	42 (20)	38 (11, 1 singleton)	6 (1)	75 (30, 2 singleton)	6 (1)	75 (30, 2 singleton)

Numbers in brackets are the number of private bands among the scored ones.

axes that accounted for 47.81% of the variance (Fig. 4A). Subsequent PCO analysis restricted to the *B. pyrenaica* dataset did not show any clear-cut clustering of phenotypes (Fig. 4B). Only 19.57% of the variance was accounted for by the first two axes. This analysis revealed a general intermingled distribution of phenotypes from all of the six populations of *B. pyrenaica* studied; however, a clinal grouping from south to north could be observed as phenotypes corresponding to populations of the Prepyrenean ranges (Bp5 and Bp6) appeared predominantly on the right side of the plot and those of the axial divide (Bp1, Bp2, Bp3 and Bp4) on the left side. Phenotypes from the southernmost Prepyrenean population of Turbón (Bp6) differentiated most from the rest.

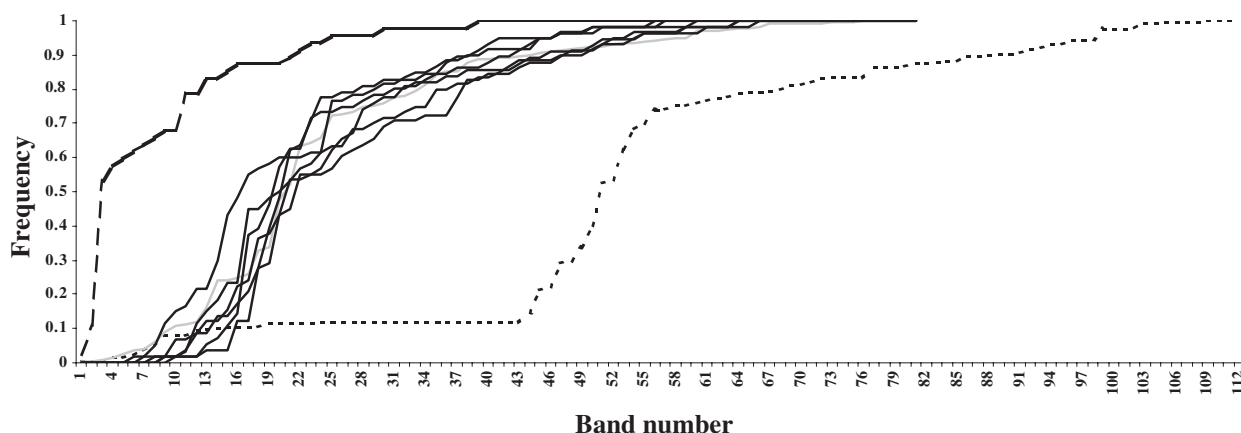
The unrooted Neighbour-Joining tree constructed from pairwise distances between the 395 phenotypes (Fig. 5) also revealed the differentiation of two main clusters, corresponding to phenotypes of *B. chopardii* and *B. pyrenaica*, which were supported by a 100% bootstrap value. The hierarchy of RAPD phenotypes across the six studied populations of *B. pyrenaica* was low and unsupported; however, some differentiation could be observed for some populations and geographical regions of this taxon that showed exclusive clusters of phenotypes. Most phenotypes from the northern Pyrenees populations (Bp1, Bp2) linked together in one of the main clusters of the subtree whereas phenotypes from the southern Pyrenees populations fell mostly in the other cluster (Fig. 5). A high number of phenotypes of the isolated Prepyrenean population of Turbón (Bp6) formed an exclusive subcluster which was close to a few phenotypes from the other Prepyrenean population of Cotiella (Bp5); the remaining Turbón phenotypes were nested within a subcluster of predominantly southern Pyrenean Ordesa phenotypes (Bp4). The southern Pyrenean populations of Pineta and Ordesa (Bp3, Bp4) shared phenotypic affinities with the Prepyrenean populations of Cotiella and Turbón (Bp5, Bp6) and, to a lesser extent, with the northern Pyrenean populations at Gavarnie (Bp1, Bp2). Notably, some northern Pyrenean phenotypes of population Bp1 were closer to phenotypes of southern Pyrenean population Bp4 whereas some phenotypes of northern Pyrenean population Bp2 were closer to those of southern Pyrenean population Bp3. The distinct phenotypic affinities shown by northern to southern Pyrenees populations could imply the existence of different migration pathways of *B. pyrenaica* during the postglacial expansions.

POPULATION GENETIC STRUCTURE

Partitioning of genetic variance within *Borderea* obtained by means of AMOVA analysis (Table 3), also

Table 2. Values for the genetic diversity index obtained from the 12 RAPD primers within *Borderea s.l.*, *B. chouardii*, and at species and population levels of *B. pyrenaica*

RAPD primer\taxon	<i>B. pyrenaica</i>								
	<i>Borderea s.l.</i>	<i>B. chouardii</i>	Species level	Population level					
				Bp1	Bp2	Bp3	Bp4	Bp5	Bp6
OPA12	0.843	0.218	0.127	0	0.171	0.133	0.190	0.101	0.160
OPB3	0.519	0.234	0.258	0.230	0.250	0.230	0.190	0.250	0.370
OPB5	0.457	0.091	0.333	0.210	0.290	0.460	0.280	0.310	0.390
OPB6	0.621	0.046	0.330	0.340	0.310	0.450	0.270	0.250	0.250
OPB7	0.617	0.156	0.551	0.530	0.550	0.570	0.460	0.580	0.510
OPB8	0.460	0.281	0.475	0.490	0.480	0.530	0.460	0.400	0.440
OPB9	0.778	0	0.090	0.020	0.070	0.030	0.010	0.030	0.320
OPB10	0.772	0.323	0.762	0.616	0.720	0.784	0.776	0.754	0.750
OPB11	0.367	0	0.197	0.218	0.142	0.218	0.164	0.167	0.183
OPB12	0.190	0.112	0.200	0.168	0.200	0.210	0.218	0.244	0.133
OPB15	0.515	0.006	0.112	0.220	0.187	0.102	0.046	0.028	0.064
OPB18	0.537	0.145	0.316	0.295	0.320	0.332	0.326	0.319	0.287
Proportion of polymorphic loci	96.43	47.50	92.59	62.96	69.13	70.37	58.02	60.49	71.60
Genetic diversity over all loci	0.574	0.139	0.379	0.345	0.367	0.414	0.340	0.355	0.377
Genetic diversity over polymorphic loci	0.595	0.293	0.409	0.373	0.397	0.447	0.368	0.384	0.407

**Figure 2.** Observed positive allele frequencies for 112 RAPD loci in *Borderea s.l.* (dotted line), 80 loci in *B. chouardii* (dashed line) and 81 loci in *B. pyrenaica* (species level = grey line, population level = solid black lines).

corroborated the genetic differentiation between *B. chouardii* and *B. pyrenaica*. The first distribution analysis (not shown) attributed 52.16% of the variance to differences among populations of *Borderea s.l.* when all samples were considered to be one species, indicating a strong heterogeneity in that group. This was further confirmed when they were separated into two groups (*B. chouardii* vs. *B. pyrenaica*); the differences between the species accounted for 76.08% of the total

variation whereas differences among populations (within taxa) and within populations were only 3.6% and 20.31%, respectively. The F_{ST} value for the RAPD differences between both species was high (0.79) and significant ($P < 0.001$).

The AMOVA conducted at different hierarchical levels within *B. pyrenaica* always revealed a higher genetic diversity within populations than either between populations or between regions (Table 3). In

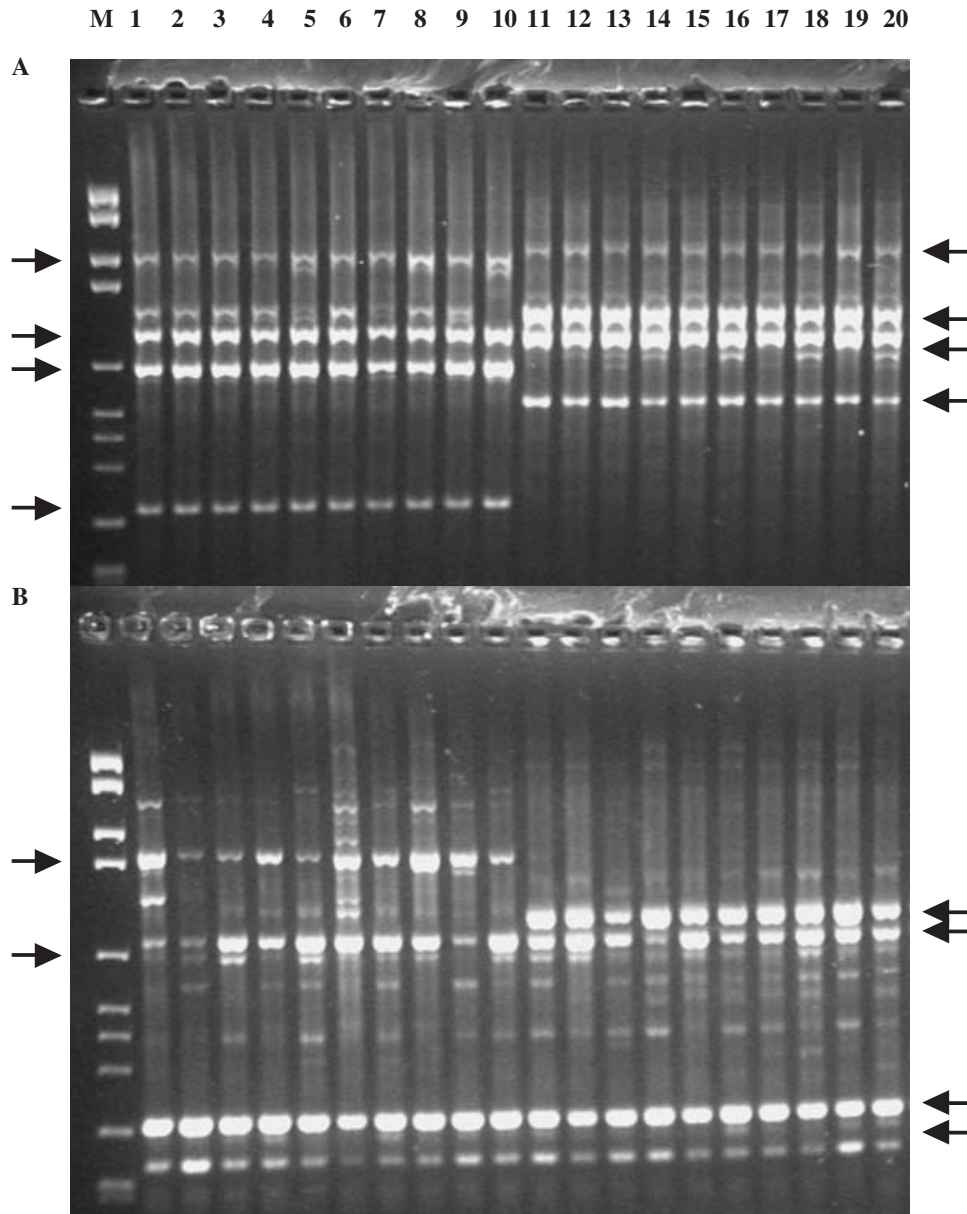


Figure 3. Example of RAPD differentiation profiles in *Borderea*, obtained with two different primers: OPB18 (A) and OPB11 (B). M, corresponds to molecular size marker VI (Roche Diagnostics). Lanes 1–10 correspond to *B. pyrenaica* and 11–20 to *B. chouardii*. Arrows indicate scored bands.

all studied cases partitioning of RAPD variance was higher than 80% within populations and lower than 15% among populations within groups or lower than 9% between distinct geographical divisions. F_{ST} values were markedly low in all cases (<0.16) although significantly different from zero at $P < 0.001$. The lowest value of the percentage of genetic variation accumulated among populations of the same area (8.08%) was obtained when the populations were divided into four geographical ranges (northern

Pyrenees (Bp1, Bp2), southern Pyrenees (Bp3, Bp4), Prepyrenean Cotiella (Bp5), and Prepyrenean Turbón (Bp6)), suggesting a close genetic relationship and homogeneity of populations from the same region. The highest value of partitioning of variance between regions (8.59%) was that of the group that separated the southernmost Prepyrenean population of Turbón (Bp6) from the rest (Bp1–Bp5), indicating a distinct genetic composition of individuals from this isolated area.

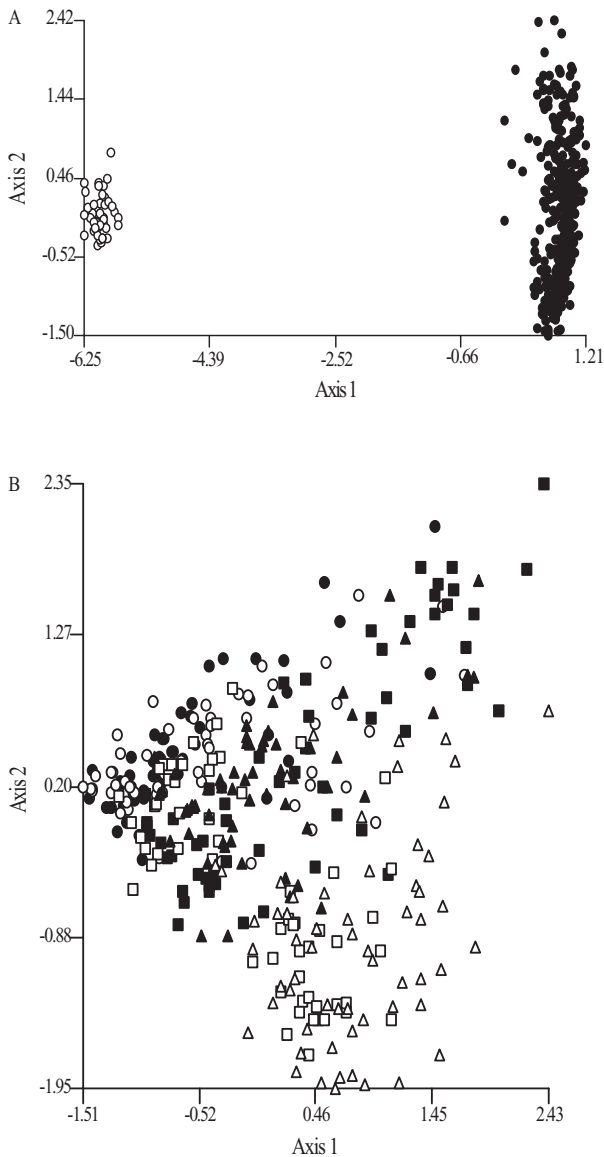


Figure 4. Two-dimensions principal coordinate analysis plottings of *Borderea*. (A) 397 plants of *Borderea* (350 of *B. pyrenaica*) that rendered 395 RAPD phenotypes (349 of *B. pyrenaica*). The first two axes explained 41.98% and 5.83%, respectively, of the total variance. ○ = *B. chouardii*; ● = *B. pyrenaica*. (B) The 350 plants of *B. pyrenaica* (349 RAPD phenotypes). The first two axes accounted for 10.66% and 8.91%, respectively, of the total variance. ● = Bp1, ○ = Bp2, ■ = Bp3, □ = Bp4, ▲ = Bp5 and △ = Bp6.

GENETIC AND GEOGRAPHICAL DISTANCES BETWEEN TAXA AND AMONG POPULATIONS

Genetic distances between populations of *Borderea* were based on their F_{ST} values which were used to construct an UPGMA phenogram (Fig. 6). The largest

patristic distances (>0.8) were those observed between the single population of *B. chouardii* and all six populations of *B. pyrenaica*. Therefore, *B. chouardii* separated well (100% bootstrap) from the *B. pyrenaica* populations as expected from its genetic distances. Patristic distances between populations of *B. pyrenaica* were considerably lower (<0.2). The most similar populations were the two northern Pyrenean ones from Gavarnie (Bp1, Bp2), which are also geographically close and showed strong bootstrap support (96%). Another cluster of similar populations consisted of the southern Pyrenean ones (Bp3, Bp4), which were linked to the Prepyrenean population of Cotiella (Bp5). The largest patristic distance was that between the southernmost Prepyrenean population of Turbón (Bp6) and the rest of the populations, a separation that was relatively well supported (82% bootstrap). In *B. pyrenaica* the genetic linkages among populations were mostly concordant with geographical distances. Nevertheless populations from the French side of the Pyrenées (Bp1 and Bp2) appeared less related to those of the Spanish side (Bp3 and Bp4) although the map distance among them is shorter than that of the latter populations to Prepyrenean population Bp5. This suggests a certain degree of isolation and reduced gene flow between the two sides of the Pyrenean axial divide due to geography. The Mantel correlation test between genetic and geographical distances computed for *B. pyrenaica* populations showed a significant value of 0.68, indicating that some populations located at short geographical distances are not so genetically related and, conversely, some populations that are spatially more separated show closer genetic affinities. These results suggest that geography and climatic changes played an important role in the colonization routes followed by the populations of *B. pyrenaica* and in the maintenance of their present genetic relationships.

DISCUSSION

GENETIC DIVERSITY IN *BORDEREA*

The main factors that affect the levels of genetic diversity, genetic divergence and distribution of genetic variability within and among plant populations usually have been interpreted as the result of a balanced combination between reproductive systems and the past history of the species under study (Loveless & Hamrick, 1984; Hamrick & Godt, 1989, 1996; Hamrick *et al.*, 1991). Outcrossing perennials generally exhibit higher levels of genetic diversity and lower levels of population differentiation, indicating the influence of their biological traits on these parameters. However, long-term isolated populations could accumulate exclusive alleles reflecting their genetic differences.

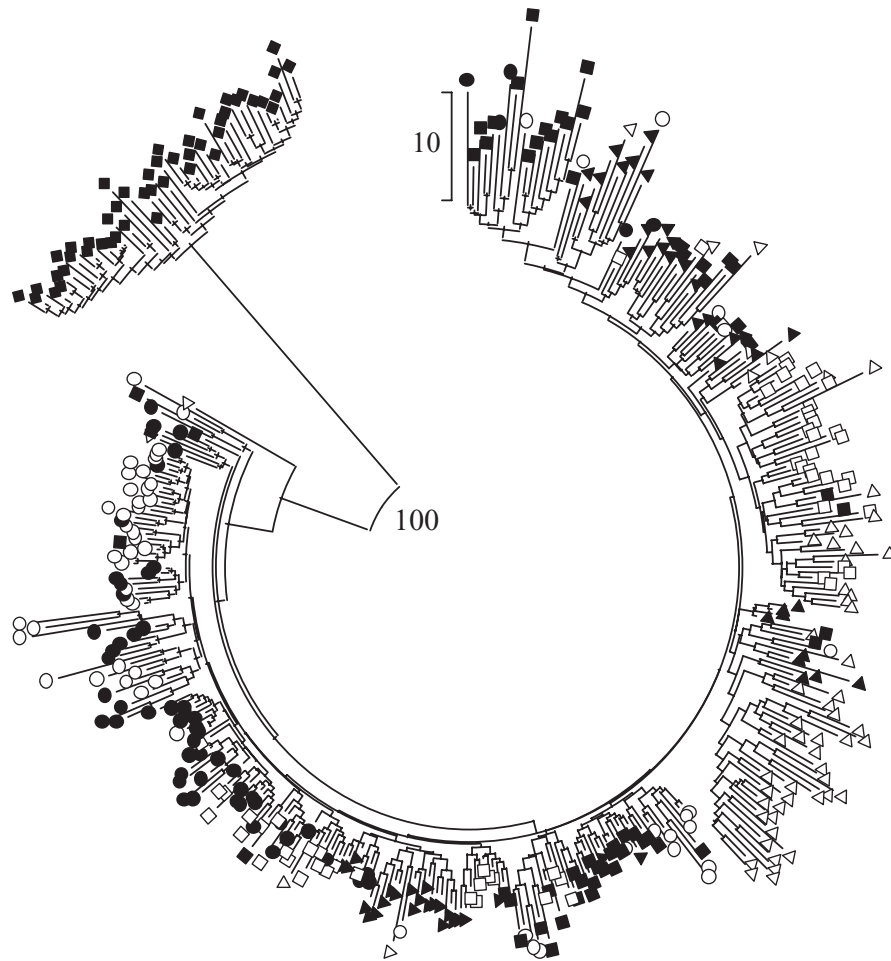


Figure 5. Neighbour-Joining tree of the 395 RAPD phenotypes observed in *Borderea*. ● = Bp1 and ○ = Bp2 correspond to French populations of *B. pyrenaica*, ■ = Bp3 and □ = Bp4 to populations on the axial ranges of the Spanish side of the Pyrenees, and ▲ = Bp5 and △ = Bp6 to populations on the Prepyrenean ranges. ◆ = Bc1 corresponds to *B. chouardii*. Support of the grouping is indicated in the branch by the bootstrap value.

Several authors (Karron, 1991; Edwards & Wyatt, 1994; Godt, Walker & Hamrick, 1997; Maki & Horie, 1999) have also pointed out the higher levels of genetic diversity and lower levels of population differentiation shown by widespread species compared with their more narrowly distributed relatives.

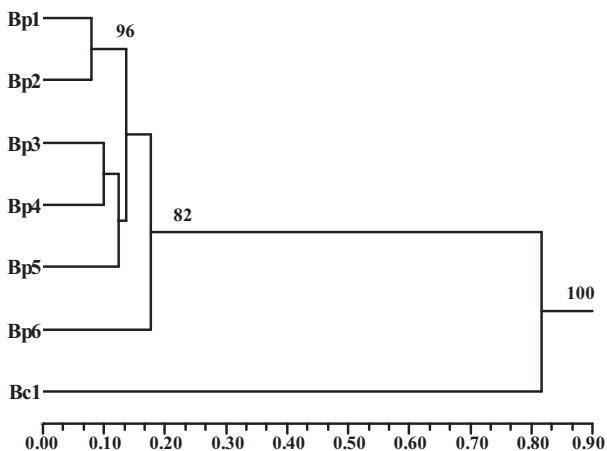
According to these generalizations it would be plausible to find high levels of genetic diversity in *Borderea* favoured by their biological traits. At the same time, the expected levels of genetic variability should be higher in *B. pyrenaica* than in *B. chouardii* given the differences in their distribution ranges and population sizes. Other contrasting results may reflect the separate life histories of these palaeoendemic species followed after their split by a potential series of population expansions and contractions during the oscillatory climatic changes of the late ice ages.

In contrast to the low levels of genetic diversity found in *Borderea* by allozyme analysis (Segarra-Moragues & Catalán, 2002), RAPD analysis detected higher levels of polymorphism for the same set of individuals, demonstrating the capability of this technique to detect genetic variability. These results agree with other comparative studies in which RAPD markers are always more variable than allozymes (Peakall, Smouse & Huff, 1995; Staub *et al.*, 1997; Papa *et al.*, 1998). However, care should be taken when extending the inferences on levels of diversity found by one or the other marker due to their different genetic natures.

Most of the RAPD molecular variation corresponds to bands that were exclusive to individuals or that were shared by few of them resulting in individual phenotypic fingerprinting patterns for almost all of the studied samples. The uniqueness of individual

Table 3. Analysis of molecular variance (AMOVA) based on 395 RAPD phenotypes of *Borderea* (349 of *B. pyrenaica* and 46 of *B. chouardii*)

Source of variation (groups)	Sum of squared deviations (SSD)	d.f.	Variance components	% of total variance
<i>B. chouardii</i> vs. <i>B. pyrenaica</i>				
Among taxa	1926.66	1	22.55	76.08
Among populations within taxa	341.71	5	1.07	3.60
Within populations	2348.32	390	6.02	20.31
<i>B. pyrenaica</i> s.l.				
Among populations	341.71	5	1.06	14.48
Within populations	2161.18	344	6.28	85.52
<i>B. pyrenaica</i> (geographical ranges)				
1. Pyrenean (Bp1 to Bp4) vs. Prepyrenean (Bp5, Bp6)				
Among regions	97.07	1	0.23	3.04
Among populations within regions	244.63	4	0.94	12.65
Within populations	2161.18	344	6.28	84.31
2. North Pyrenees (Bp1, Bp2) vs. South Pyrenees (Bp3, Bp4) vs. Prepyrenees (Bp5, Bp6)				
Among regions	166.59	2	0.21	2.89
Among populations within regions	175.12	3	0.89	12.09
Within populations	2161.18	344	6.28	85.02
3. Pyrenees and Cotiella massif (Bp1 to Bp5) vs. Turbón massif (Bp6)				
Among regions	122.65	1	0.67	8.59
Among populations within regions	219.05	4	0.83	10.74
Within populations	2161.18	344	6.28	80.68
4. North Pyrenees (Bp1, Bp2) vs. South Pyrenees (Bp3, Bp4) vs. independent Prepyrenean populations (Bp5 vs. Bp6)				
Among regions	259.68	3	0.53	7.22
Among populations within regions	82.02	2	0.60	8.08
Within populations	2161.18	344	6.28	84.70

**Figure 6.** UPGMA clustering based on F_{ST} statistics showing the relationships among the seven populations of *Borderea*. Support of branches (>50%) are indicated by bootstrap values.

RAPD profiles has been recorded in other dioecious plants (Peakall *et al.*, 1995). In spite of the high level of genetic variability detected by RAPDs, genetic distances between phenotypes were low, except for the

divergence between phenotypes of the single population of *B. chouardii* and all the *B. pyrenaica* ones.

MOLECULAR DIVERGENCE BETWEEN *B. CHOUARDII* AND *B. PYRENAICA*

RAPD markers have been satisfactorily applied to evaluate the taxonomic status of closely related taxa (Van Buren *et al.*, 1994; González-Andrés & Ortíz, 1995; Gillies & Abbott, 1998; Le Duc, Adams & Zhong, 1999; Adams, 2000), including the Dioscoreaceae (Lebot *et al.*, 1998; Terauchi, 1990). In this respect, our present study confirms the suitability of the technique to differentiate *B. chouardii* from *B. pyrenaica*.

The occurrence of 31 (20 fixed) private alleles in *B. chouardii* and 31 (one fixed) alleles in *B. pyrenaica* (Table 1; Fig. 3) strongly suggests the taxonomic distinctness of both species. These raw data are also supported by the subsequent analyses performed in this study. Partition of molecular variance was high between the two taxa (76.08%) and levels of heterogeneity were low among their populations (3.60%) when they were considered as separate species (Table 3); in contrast, higher levels of heterogeneity were observed

among populations when all populations of *Borderea* were treated as a single taxon (52.16%). The molecular differences between *B. chouardii* and *B. pyrenaica* can be graphically observed in both the PCO plot represented in Figure 4A, in which RAPD phenotypes from each taxon clearly separate spatially, and in the Neighbour-Joining tree of Figure 5, in which the separate clustering of their respective phenotypes is supported by the highest bootstrap value. The UPGMA phenogram constructed from the genetic distances between populations (Fig. 6) also shows that the largest distances are those between *B. chouardii* and *B. pyrenaica* (>0.8).

Genetic variation detected by both the more conservative allozyme markers and by the highly variable RAPD markers support the distinctness of the two *Borderea* species. A single exclusive allozyme allele (Segarra-Moragues & Catalán, 2002) and 20 private RAPD bands characterize *B. chouardii* and separate it from its congener *B. pyrenaica*. This result is concordant with a preliminary RAPD study by Mayol & Roselló (2001) based on a much more limited sampling of *Borderea* accessions. All the phenetic analyses conducted here with our broadly sampled population-genetic study of RAPD markers in *Borderea*, together with the allozyme data (Segarra-Moragues & Catalán, 2002), have definitively demonstrated the genetic divergence between these two species.

LIFE HISTORIES OF THE *BORDEREA* TAXA IN THE PYRENEES

Plants of relictual distribution in the southern European mountains have been regarded as glacial survivors (Küpfer, 1974; Bauert *et al.*, 1998). A number of palaeoendemic taxa from the Pyrenees, such as the *Borderea* taxa, are the likely descendants of Tertiary ancestors (Braun-Blanquet, 1948; Gaussen & Lerede, 1948; Gaussen, 1965). The recent phylogenetic study (Caddick *et al.*, 2002a) demonstrated the close and isolated relationships of the broadly distributed late Mediterranean genus *Tamus* and its sister mountain taxon *Borderea* from other *Dioscorea* lineages. In spite of the lack of reliable fossil records or an accurate molecular clock, it is presumed that the ancestral *Borderea* stocks expanded in the Tertiary and colonized a restricted mountain area in the Pyrenees, their populations separating from their lowland closest relatives. Similar adaptive changes by warm tropical lineages that climbed the high mountains and adopted a dwarf form have occurred elsewhere within the Dioscoreaceae, for instance in the orophyte taxon *Epipetrum*, in the Chilean Andean cordillera (Burkill, 1960; Huber, 1998). The independent acquisition of the mountain habit by these unrelated lineages of Dioscoreaceae is likely connected with the severe cli-

matic changes that affected the tropical flora adjacent to the high mountains of both hemispheres since the late Tertiary.

Quaternary refugia were presumably frequent in the Pyrenees where the ice cover did not reach below altitudes of about 1000 m even during the colder glaciations (Riss, Würm) and where some mountain slopes apparently protruded beyond the ice-sheet (Llopis-Llado, 1955; Chueca & Lampre, 1994). Palynological data have confirmed the presence of areas with vegetation during the late Pleistocene in the central Pyrenees (Jalut *et al.*, 1992). The life history of the palaeoendemic *Borderea* species can be traced back from our molecular data. The hypothesis of a Tertiary origin of the genus seems plausible as the RAPD genetic distances observed between the two taxa indicate a long divergence time between them.

The *Borderea* species likely split from a common ancestor present in the area before the drastic advents of the late ice ages and then colonized different ecological habitats. *B. chouardii* became established at lower altitudes as a chasmophytic plant growing in steep calcareous cliffs. In spite of its more temperate habitat, this taxon did not expand after the glaciations, probably due to its inefficient seed dispersal system which almost took the species to extinction. Conversely, *B. pyrenaica*, adapted to mobile screes, survived the ice ages, and successfully recolonized the open territories at higher altitudes.

Genetic diversity, as detected by RAPDs, was approximately three times lower in the only known population of *B. chouardii* than it was across the six studied populations of *B. pyrenaica*, an expected result for a single-population taxon with a small effective population size. *B. chouardii* is suspected to have passed through severe genetic bottlenecks in the past that would have forced inbreeding (Segarra-Moragues & Catalán, 2002). Moreover, the distribution of RAPD band frequencies (Fig. 2) also differed from that observed in *B. pyrenaica*. More than 50% of the bands were fixed in *B. chouardii* in contrast to a much lower percentage of fixed bands observed in *B. pyrenaica*; among the polymorphic ones, the frequencies of positive alleles tended also to be higher in the single population of *B. chouardii*. These low levels of genetic diversity and the high percentage of fixed loci exhibited by *B. chouardii* may be due to an accumulation of diverse factors, including the reduced population size, the short dispersal distance of the seeds, which enhanced inbreeding and increased the effect of genetic drift, and the occurrence of severe historical extinctions and genetic bottlenecks that homogenized the only remnant population of this taxon. Despite the low values of genetic diversity found in *B. chouardii*, dominant RAPD markers distinguished almost all sampled individuals by their distinct phenotypes.

A more detailed population genetic study of this threatened plant using microsatellites is presently underway.

GENETIC STRUCTURE, HISTORICAL DEMOGRAPHY, AND POSTGLACIAL COLONIZATION EVENTS IN *B. PYRENAICA*

Since all the populations of *B. pyrenaica*, either considered separately or combined at species level, exhibited the same frequency distribution pattern of bands (Fig. 2), we suspect that they share a recent common gene pool. Low levels of genetic divergence between populations coupled with high levels of genetic diversity within them could also be explained by extensive gene flow (Hamrick & Godt, 1996). Some of the biological features of *B. pyrenaica*, such as its perennial habit and obligate outcrossing, would favour this hypothesis, whereas other spatial and biological characters related to the isolated location of its populations and short dispersal distance of its pollen and seeds (García *et al.*, 1995) would inhibit present-day genetic interchange. This is particularly true for the more isolated populations of *B. pyrenaica* from the Prepyrenean mountain massifs (Turbón and Cotiella, Fig. 1), which are located far away from the main core range of *B. pyrenaica* in the Pyrenees. It is therefore unlikely that gene flow might account for the observed genetic relatedness between populations from the two geographically separated areas. This is different for populations from either side of the Pyrenean axial divide around the Monte Perdido massif (La Planette, Rochers Blancs, Pineta, Ordesa; Fig. 1), which could be connected via seed dispersal by some unknown agent as they are less than 10 km apart from each other and showed some of the lowest genetic distances (Fig. 4).

In spite of the high number of phenotypic variants found within *B. pyrenaica*, and the clear geographical isolation of some populations, no private bands were detected in any population of *B. pyrenaica*. Analyses based on distances between RAPD phenotypes (Figs 2 and 3, Table 3) indicated that most of the rare polymorphic RAPD alleles probably have been recently acquired. Apart from the single species-specific band, most of the remaining markers that were detected among the 350 studied individuals of *B. pyrenaica* were shared by individuals from different populations. The genetic distances among phenotypes of *B. pyrenaica* were low and some of them appeared more closely related to phenotypes from other populations than to those of the same population resulting in an intermingled clustering of population variants (Figs 4B and 5). This was corroborated by the AMOVA that found most of the genetic diversity within populations rather than among populations or among geographical regions (Table 3). Similar results in the partitioning of the genetic variance have been reported in several popu-

lation genetic and phylogeographical studies of angiosperms conducted with RAPDs (Dawson *et al.*, 1993; Smith & Pham, 1996; Gabrielsen *et al.*, 1997; Gillies *et al.*, 1997; Martin *et al.*, 1997; Wolff *et al.*, 1997) although other studies have detected the opposite pattern (Bauert *et al.*, 1998; Buso, Rangel & Ferreira, 1998). Bauert *et al.* (1998) reported a complete lack of within-population and within-region genetic variability but contrasting levels of genetic differentiation among three studied regions of the Alps where the arctic relict *Saxifraga cernua* lives. According to their results, the authors hypothesized on the existence of three isolated glacial refugia in the Alps and on the extreme genetic drift caused by the glaciations in the *S. cernua* populations of each region.

The scenario proposed for *B. pyrenaica* is that of a severe contraction of its populations during the last glaciation at lower altitudes followed by a rapid postglacial expansion at higher altitudes. Both Pyrenees and Prepyrenees could have sheltered *B. pyrenaica* stocks, the Prepyrenees being a likely warmer refuge that also preserved stocks of its congener *B. chouardii*. Local extinctions and genetic bottlenecks likely impoverished the genetic pool of the surviving populations of *B. pyrenaica*. The time elapsed since the start of the postglacial colonization events to the present day appears to have been insufficient for the generation of population-unique variants. The genetic structure of *B. pyrenaica*, as shown by our results, seems to be characteristic for recently established populations, like those belonging to alpine and arctic plants that invaded new areas after the retreat of the Pleistocene glaciers (Gabrielsen *et al.*, 1997; Bauert *et al.*, 1998; Gabrielsen & Brochmann, 1998; Tollefsrud *et al.*, 1998).

RAPD genetic distances between populations of *B. pyrenaica* were significantly correlated with their geographical distances showing an intermediate value ($R = 0.689$). Both the Neighbour-Joining tree constructed from distances among phenotypes and the UPGMA phenogram based on F_{ST} statistics between populations showed some interesting clusterings (Figs 5, 6). The most isolated Prepyrenean population from Turbón (Bp6) was the most genetically distant one (Fig. 6); more than half of its phenotypes clustered separately from the rest (Fig. 5). This result agrees with the fact that due to its geographical isolation from the main core distribution range of the species (Fig. 1) this population may have accumulated the highest number of genetic differences. Intermingled clustering of the remnant populations suggests a different genetic relatedness from that which would be inferred from their geographical proximity. Populations from the southern Pyrenees (Bp3, Bp4) were found to be more closely related to the Prepyrenean population from Cotiella (Bp5) than they were to

northern Pyrenean populations (Bp1, Bp2) (Fig. 6) despite the shorter linear geographical distances between populations on both sides of the Pyrenean axial divide (Fig. 1); this unexpected result may be due to the barrier caused by the high peaks of the Monte Perdido massif that prevents present gene-flow via seed or pollen dispersal. Genetic exchange among southern populations of the axial divide and some close Prepyrenean populations is likely to occur through a continuous distribution of mobile calcareous scree habitats.

Recolonization of the habitats presently occupied by *B. pyrenaica* may have followed different pathways. Although more conserved allozyme markers were unable to detect enough genetic variability within *B. pyrenaica*, the presence of a rare allele at locus PGI-2 shared among the Prepyrenean populations (Bp5, Bp6) and one of the most distant northern Pyrenean populations of the axial divide (Bp1), along with the different patterns of shared RAPD phenotypes between populations from the Spanish and the French ranges, could indicate a postglacial colonizing route from south to north. This expansion wave was likely followed by the present isolation of *B. pyrenaica* in the Prepyrenean island massifs and the establishment of a large metapopulation on both sides of the Pyrenean axial divide.

The evolutionary scenario depicted by RAPD markers for *B. pyrenaica* is concordant with that found through allozyme analysis (Segarra-Moragues & Catalán, 2002). The two approaches indicate a recent origin of the present-day core of populations of *B. pyrenaica* and a rapid colonizing process of its present territories after the glaciations. The genetic divergences expected among geographically and reproductively isolated Tertiary relict populations, if they ever existed, may have been swamped by the repeated and overlapping migration dispersals experienced throughout time.

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