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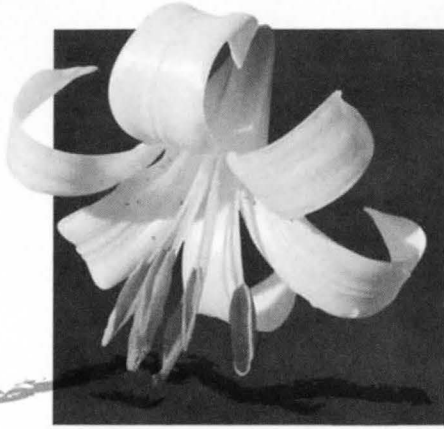
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SPECIES BOUNDARIES AND POPULATION DIVERGENCE IN THE PYRENEAN ENDEMIC RELICT GENUS *BORDEREA* (DIOSCOREACEAE) AS REVEALED BY MICROSATELLITE (SSR) AND OTHER HYPERVARIABLE MARKERS

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ABSTRACT

Microsatellite alleles were used to delimit the genetic boundaries and divergence of the two relictual endemic Pyrenean taxa *Borderea chouardii* and *B. pyrenaica* (Dioscoreaceae), and to infer the different life histories followed by each species. Our study was conducted on the same populations previously analyzed with allozymes and RAPD markers. The three studied data sets were congruent in the inference of a single evolutionary scenario for the split of the two *Borderea* taxa from a common Tertiary ancestor in the Prepyrenees, thus supporting their taxonomic treatment as separate species. However, the more variable SSR and RAPD data provided better resolution for a stepping-stone model of local colonization of *B. pyrenaica* populations from southern Prepyrenean refugia to the northern Pyrenees. SSR markers proved to be more robust than RAPD markers in assessing the genetic structure of recently diverged populations of *B. pyrenaica* and thus qualified as the best molecular markers for fine-scale evolutionary investigations of Dioscoreaceae. Furthermore, microsatellites rendered unique clues to decipher the mechanisms involved in the origin of these relictual species and their genetic background. *Borderea* was shown to be a tetraploid genus of hybrid origin with a chromosome base number of $x = 6$. Phylogenetic data, karyological evidence, and our present knowledge based on microsatellite analyses allowed us to speculate that the Pyrenean endemic genus *Borderea* and its sister taxon, the Mediterranean genus *Tamus*, represent some of the oldest paleopolyploid lineages of the mostly pantropical yam family.

Key words: *Borderea chouardii*, *B. pyrenaica*, Dioscoreaceae, microsatellites, molecular divergence, polyploidy, RAPD, SSR.

INTRODUCTION

The genus *Borderea* Miègev., endemic to the central Pyrenean and Prepyrenean mountain ranges, has been considered to be a Tertiary relictual lineage of Dioscoreaceae (Gaussen 1965) based on the fact that the vast majority of its family members (more than 600 spp.) show a present pantropical distribution with only a few taxa growing outside of that range (Knuth 1924; Burkill 1960; Dahlgren et al. 1985).

The species of this genus have been subjected to several taxonomic rearrangements throughout history. The two currently accepted taxa (*Borderea pyrenaica* and *B. chouardii*; cf. Heywood 1980; Villar et al. 2001) were first described within the large pantropical genus *Dioscorea* L. as *D. pyrenaica* Bubani & Bordère ex Gren. and *D. chouardii* Gaussen, respectively (Gaussen 1952, 1965). The name *Borderea* arose in the mid-nineteenth century (Miègeville 1866) to describe specimens from the Pyrenees (*B. pyrenaica* Bubani ex Miègev.), which differed from *Dioscorea* mainly in their dwarf habit and wingless seeds. Karyological analyses of the two species demonstrated that both taxa shared not only these remarkable morphological attributes, but also a chromosome number of $2n = 24$, which was assumed to represent a chromosome base number of $x = 12$, distinct from that shown for most *Dioscorea* taxa ($x = 10$). In turn, this chromosome value was used as a further argument to classify both species under the same genus *Borderea* (Heslot 1953). On the basis of morphological similarities with *Bor-*

derea, other taxa native to geographical regions apart from the Pyrenean range were also transferred to this genus. Thus, the Chilean endemic *Dioscorea humilis* Bert. ex Colla was renamed as *B. humilis* (Bert. ex Colla) Pax. However, this and two other Andean dwarf endemics show certain distinctive morphological features within Dioscoreaceae (i.e., the possession of prominent pistillodes in the male flowers, round capsules, and strongly emarginate leaves) that favored their separate treatment as members of the independent genus *Epipetrum* Phil. (Knuth 1924). Milne-Redhead (1963) described from Kenya *Dioscorea gilletii* Milne-Redh., a taxon potentially close to *Borderea* based on its wingless seeds and other less consistent traits. The extreme taxonomic importance given to the wingless seed morphological character moved Huber (1998) to classify the east African *D. gilletii* and the two Pyrenean endemics as the disjunct members of the small section *Borderea* within the large genus *Dioscorea*. A careful examination of the type material for *D. gilletii* (K! H3644/83, H3645/83) revealed several morphologically distinctive features with respect to those exhibited by *Borderea* (Segarra-Moragues and Catalán 2005, unpubl. data), suggesting that *D. gilletii* was more closely related to other *Dioscorea* species than to *Borderea*. Recent phylogenetic studies of Dioscoreaceae (Caddick et al. 2002a) based on *rbc*L, *atp*B, 18S ribosomal DNA (rDNA) sequences, and morphological characters demonstrated that *Dioscorea* s.l. (Huber 1998) is paraphyletic and that the dioecious Dioscoreaceae (i.e., *Borderea*, *Tamus* L.) are embedded within a
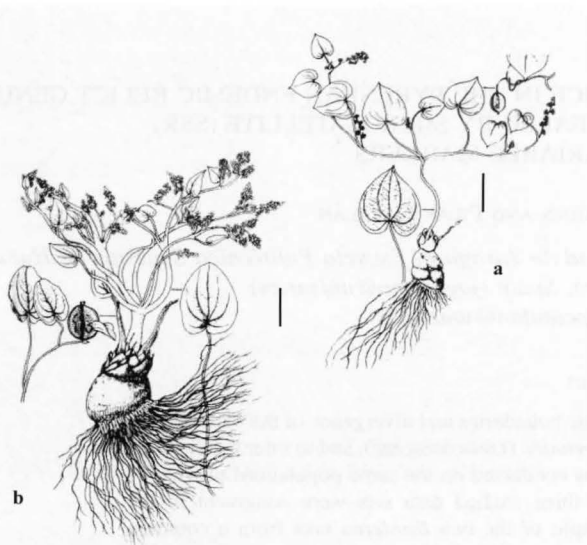
Fig. 1.—Habit of *Borderea* taxa: (a) *B. chouardii,* male plant and detail of fruiting branch of female plant; (b) *B. pyrenaica,* male plant and detail of fruiting branch of female plant. Scale bar = 1 cm. Drawings reproduced by courtesy of M. Saule (1991).
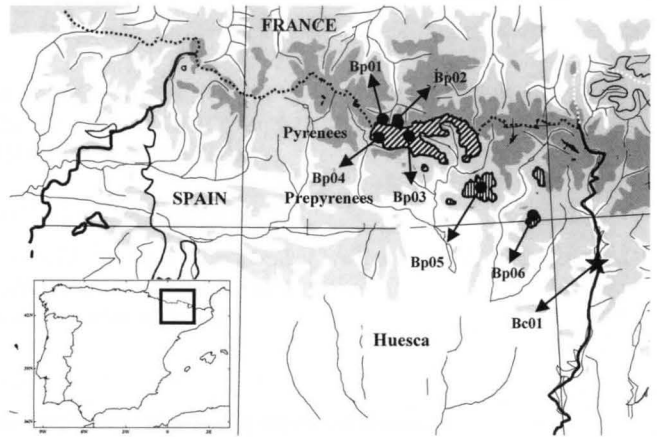

Fig. 2.—Map of the studied populations of *Borderea.* Bc = *B. chouardii* (Bc01, Sopeira, Huesca, Spain). Bp = *B. pyrenaica* (Bp01, La Planette, Gavarnie, France; Bp02, Les Rochers Blancs, Gavarnie, France; Bp03, Pineta, Huesca, Spain; Bp04, Ordesa, Huesca, Spain; Bp05, Cotiella [La Vasa Mora], Huesca, Spain; Bp06, Turbón, Huesca, Spain).

clade of monoecious species. These findings, supported by evidence that the putatively distinctive traits of these genera are not as unique as previously thought, prompted the inclusion of *Borderea* and its Mediterranean sister genus *Tamus,* within *Dioscorea* (Schols et al. 2001; Caddick et al. 2002*b*).

Regardless of its taxonomic attribution, *Borderea* represents an evolutionary split from an old Dioscoreaceae lineage that successfully adapted to and colonized the central region of the Pyrenees and, as currently circumscribed, only includes two taxa, *B. pyrenaica* Miègev. and *B. chouardii* (Gaussen) Heslot (Fig. 1). These two species are endemic of the central Pyrenean and Prepyrenean mountain ranges that present some of the longest life spans reported for herbaceous plants (García and Antor 1995*a*; García 1997; García et al. 2002), including some individuals over 300 years old. Both taxa are dioecious geophytes and apparently only reproduce sexually (García and Antor 1995*b*; García et al. 1995, 2002). *Borderea chouardii* is a chasmophytic species that has been classified as "in danger of extinction" in the Annex II of the Habitats Directive of the European Union and as "critically endangered" in the Spanish Red List of Endangered National Plants (García 1996; Varios Autores 2000; Moreno-Saiz et al. 2003). It is only known from a single population of approximately 2000 individuals (García et al. 2002), located in one of the southernmost Spanish Prepyrenean mountain ranges (Sopeira, Huesca province: Fig. 2) growing on limestone cliffs at lower elevations (ca. 800 meters above sea level [m.a.s.l.]). The extremely limited effective population size and reduced area of occupancy (less than 1000 m²) of *B. chouardii,* coupled with its reduced capability to colonize new habitats caused by its limited seed dispersal system (postcarpotropism), could drive this plant into extinction because of either biological stochastic events or anthropogenic action. *Borderea pyrenaica,* though more widespread than its congener *B. chouardii,* is confined to a narrow geographic area of 160 km² in the central Pyrenean

and Prepyrenean region (Fig. 2), where it inhabits mobile calcareous screes above 1800 m.a.s.l. Its populations are distributed in three main mountain "islands," the largest one expanding around the Monte Perdido massif in the Pyrenean axial divide and two more reduced population cores located in the Prepyrenean Cotiella and Turbón massifs, respectively (Fig. 2). The geographical map distances among the populations of *B. pyrenaica* growing along the Pyrenean range are short (less than 15 km), however, they are separated by some of the highest peaks of this mountain chain. These peaks constitute natural barriers between the more abundant Spanish populations on the southern side of the Pyrenees (around the Ordesa and Pineta valleys) and the more sporadic populations that grow to the north in France around the Gavarnie Valley. By contrast, the Prepyrenean populations of *B. pyrenaica* are located further away, in two isolated Spanish mountain massifs that are separated by deep valleys and by more than 30 km (Cotiella) and 50 km (Turbón) from the Pyrenean core, respectively. These geographic features currently prevent any gene flow between the Pyrenean and the Prepyrenean population cores. On the other hand, the southernmost Prepyrenean *B. pyrenaica* population of Turbón is located at almost the same geographic distance from its conspecific Prepyrenean core at Cotiella (20 km), as from its congener *B. chouardii* at Sopeira (25 km) (Fig. 2). Populations of *B. pyrenaica* are somewhat larger—some comprising more than 10,000 reproductive individuals that inhabit wide, almost pristine high-mountain areas. Yet, despite its restricted geographic distribution, populations of *B. pyrenaica* are less threatened from intrinsic or extrinsic factors than *B. chouardii.*

The two species are divergent for several morphological characters related to the size and shape of the fruit and the seeds, the thickness and color of the leaf, and the shape of the leaf apex (Gaussen 1952) (Fig. 1). They are also geographically separated (Fig. 2) and show distinct ecological preferences (Gaussen 1952, 1965). However, they share a close morphology (Fig. 1) that moved some authors to speculate about the taxonomic distinctness of *B. chouardii* from

its congener *B. pyrenaica,* suggesting that *B. chouardii* could be a subspecies of *B. pyrenaica* (Burkill 1960). However, no formal proposals for taxonomic change were suggested. The scarcity of available material for *B. chouardii* for comparative studies (i.e., collection is prevented by Spanish laws) contributes to their uncertain taxonomic status, although a monographic study of the genus is currently under way (Segarra-Moragues and Catalán 2005, unpubl. data).

Previous molecular studies based on allozymes 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 and Catalán 2002). Nonetheless, the greatest genetic distances were those between *B. chouardii* and all the *B. pyrenaica* populations, but the relationships among *B. pyrenaica* populations could not be ascertained with confidence due to the low levels of polymorphism detected by these markers. To address this issue, a further population genetic analysis of *B. chouardii* and *B. pyrenaica* was conducted using highly variable random amplified polymorphic DNA (RAPD) markers (Segarra-Moragues and Catalán 2003). This study revealed a strong molecular distinctness for the two taxa and allowed a molecular characterization of most studied individuals. However, in spite of the larger amounts of genetic diversity detected within *B. pyrenaica,* very few population-unique bands were detected, resulting in an intermingled hierarchy of RAPD phenotypes. These results were interpreted as being the consequence of historical events, supporting a recent post-glacial expansion evolutionary scenario for the *B. pyrenaica* populations, rather than the homogenizing effect resulting from present-day gene flow among populations, which we presumed should be low in view of the large geographical distances, natural barriers that separate the three main population cores (Fig. 2), and related biological factors such as the type of pollination vectors in *B. pyrenaica* (mainly ants; García et al. 1995) and the limited seed dispersal capability.

Simple sequence repeats (SSRs; Tautz 1989) are codominant markers of the nuclear genome that are useful for population genetic studies (Degen et al. 1999; Naito et al. 1999; Perera et al. 2000; Sun et al. 2001; Al-Rabab'ah and Williams 2002), molecular identification of closely related taxa or populations (Bruschi et al. 2000; Macaranas et al. 2001), and estimation of dates of origin of hybrid species (Welch and Rieseberg 2002). Different types of microsatellite alleles have been broadly used in plant genomic analyses (Morgante and Olivieri 1993; Wang et al. 1994), even for the assessment of genetic relationships between wild relatives and derived cultivars of species of agronomic interest (Anthony et al. 2002; Hormaza 2002; Palombi and Damiano 2002). The use of microsatellites, however, is still limited among Dioscoreaceae where they have only been used in the characterization of individuals of the wild yam species *Dioscorea tokoro* Makino (Terauchi and Konuma 1994) and for the characterization of germplasm stocks of the white yam (*D. rotundata* Poir.) (Mignouna et al. 2003). Comparative studies between RAPD and SSRs demonstrated better performance of the latter markers in detecting the genetic structure of populations and in providing a higher number of polymorphisms able to characterize close species, infraspecific taxa, populations, individuals, and even clonal plant sports (Bech-

er et al. 2000; Mengoni et al. 2000; Staub et al. 2000; Palombi and Damiano 2002; Mignouna et al. 2003).

In order to gather further information on the genome divergence and past evolutionary histories of these two species, we assessed the genetic differentiation and population structure of the palaeoendemic *Borderea* taxa through SSR analysis and compared these results to the data previously obtained from allozymes and RAPD markers. Since conservation resources are often limited, the identification of *B. chouardii* as an independent taxonomic entity and evolutionary lineage from *B. pyrenaica* was imperative. The highly variable codominant single-locus SSR alleles could also help to unravel other biological features of *Borderea* that have passed undetected in our previous molecular surveys. Because of the potential risk that the use of a single molecular marker could result in misleading data and the benefits derived from the performance of combined studies with congruent molecular markers, we conducted a further combined analysis of RAPD and microsatellite markers in the *Borderea* populations with the intention of obtaining a better picture on the genomic characteristics of the studied taxa. A further goal of our investigation was to evaluate the reliability of the SSR markers in resolving the population structure of *B. pyrenaica* compared to that obtained from RAPDs, in order to perform a large-scale population genetic study of this taxon.

MATERIALS AND METHODS

Population Sampling, DNA Extraction

The present study was conducted on the same populations and individuals previously analyzed for allozyme and RAPD markers (Segarra-Moragues and Catalán 2002, 2003). A total of 407 individuals collected from seven populations of *Borderea* were included in the survey (Fig. 2). The ratio of male to female of 1: 1 was kept in the original sampling scheme. Sampling included the only known population of *B. chouardii* (Bc01: Sopeira, Huesca, Spain, $n = 47$), and six populations of *B. pyrenaica* (60 individuals each) 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 (Bp01: La Planette, Gavarnie, France; Bp02: Les Rochers Blancs, Gavarnie, France), and the other two grow on the southern face of this mountain range (Bp03: Pineta, Huesca, Spain; Bp04: Ordesa, Huesca, Spain). The remaining *B. pyrenaica* populations inhabit the more distant Prepyrenean massifs (Bp05: Cotiella [La Vasa Mora], Huesca, Spain; Bp06: Turbón, Huesca, Spain).

Fresh leaves from all sampled individuals were dried in silica gel and used for DNA isolation. DNA was extracted following the hexadecyltrimethylammonium bromide (CTAB) protocol of Doyle and Doyle (1987) adapted for miniprep extractions. DNA concentration was calculated by comparison to marker VII (Roche, Barcelona, Spain) concentration on agarose gel. Samples were diluted to a final concentration of ca. 5 ng/μl in 0.1× TE buffer and used for further DNA amplifications.

RAPD and SSR Amplifications

The RAPD analyses corresponded to that described in Se-garra-Moragues and Catalán (2003). In brief, 12 RAPD primers out of 40 assayed (Operon Technologies, Alameda, California, USA, kits A and B) in a previous pilot study were selected for the screening of all studied individuals. Amplifications were carried out in 20 μl total volume containing 1× buffer (Ecogen, Machynlleth, Powyes, UK), 2.5 mM MgCl$_2$, 0.4 mM of each dNTP, 4 pmoles of primer, 1.0 unit of Taq DNA polymerase (Ecogen), and 2 ng template DNA. The amplification program consisted of an initial step of DNA melting of 4 min at 94°C, followed by 40 cycles at 94°C for 1 min, 39°C for 1 min, and 72°C for 1.5 min, followed by an 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 100V during 4 hr in 0.5× TBE buffer. RAPD bands were visualized with UV transmitted light and captured with Gel Doc 1000 (BioRad, Hercules, California, USA). RAPD amplifications were repeated at least twice in order to check the reproducibility of the banding profiles. Ten individuals of *B. pyrenaica* failed to produce RAPD amplicons and the number of samples was reduced accordingly for these markers as follows (Bp01, $n = 58$; Bp02, $n = 56$; Bp03, $n = 58$ and Bp05, $n = 58$).

The SSR analyses were based on the previous loci-characterization surveys conducted by Segarra-Moragues et al. (2003, 2004). Enriched genomic libraries in trinucleotide (CTT) motifs were separately constructed for *B. chouardii* (Segarra-Moragues et al. 2003) and *B. pyrenaica* (Segarra-Moragues et al. 2004). A total of 10 and 7 primer-pairs were designed to amplify the corresponding microsatellite regions in *B. chouardii* and *B. pyrenaica,* respectively. Transferability tests under multiplexed conditions were then assayed for the 17 microsatellite loci in both species resulting in successful cross amplifications for all 407 studied individuals (Catalán et al., unpubl. data). PCRs (polymerase chain reactions) were performed in 20 μl reactions containing 3–5 pmoles each of the fluorescein labeled forward and unlabelled reverse primers, 1× Taq buffer (Promega, Barcelona, Spain), 2 mM MgCl$_2$, 0.4 mM of each dNTP, and 1 unit of Taq DNA polymerase (Promega), and approximately 5–8 ng DNA. The PCR program for the transferred loci consisted of an initial melting step (94°C, 4 min) followed by 30 cycles (94°C, 45 sec; annealing temperature [55–60°C], 45 sec; and 72°C, 1 min–1min 20 sec) and a final extension step (72°C, 7 min). PCR conditions for the loci developed and amplified in each separate source species are described in Segarra-Moragues et al. (2003) for *B. chouardii* and in Segarra-Moragues et al. (2004) for *B. pyrenaica.* Products were run on an ABI 310 automated DNA sequencer (Applied Biosystems, Madrid, Spain). Fragment lengths were assigned with GENESCAN and GENOTYPER software (Applied Biosystems) using ROX-500 as the internal lane standard.

Data Analysis

Borderea was recently discovered to be a tetraploid genus on the basis of molecular microsatellite data (Segarra-Moragues et al. 2003, 2004) and the present study (Fig. 3; see also Discussion); this is in contrast to previous chro-

mosome counts by Heslot (1953) that suggested *Borderea* could be a diploid taxon with $2n = 24$. Allelic inheritance analyses are currently underway (Catalán et al. unpubl. data) in order to determine whether disomic (amphidiploid) or tetrasomic (autotetraploid) inheritance occur in *Borderea*; thus, information on genotypes of individuals is not yet available. Microsatellite alleles have been coded as binary data in polyploid plant species (Mengoni et al. 2000) based on the impossibility of distinguishing duplex and simplex diallelic combinations and different triallelic combinations in some cases. The linear combination of presence/absence of codominant SSR bands generates phenotypic binary microsatellite patterns for each individual that can be compared with similar binary patterns obtained from the dominant RAPD markers (Mengoni et al. 2000; Staub et al. 2000; Hormaza 2002; Palombi and Damiano 2002). We applied the binary code system (1/0) to both the distinct RAPD phenotypes obtained from the 12 selected RAPD primers and the different SSR phenotypes resulting from the scoring of alleles at the 17 microsatellite loci across all investigated individuals. SSR bands that presented the same electrophoretic mobility were assumed to be homologous based on the success of all attempted cross-amplifications carried out between the two closely related *Borderea* species and the conserved trinucleotide changes observed between alleles of the same loci. Both separate (SSR, RAPD) and combined (SSR + RAPD) data matrices were constructed and used for further genetic analyses using different computer programs.

Genetic distances between phenotypes were calculated through several metric distances. The simple matching (SM) metric based on shared presences and absences of bands was used to compute distances between SSR phenotypes. Dice's (D) and Jaccard's (J) similarity coefficients both excluding shared absences of bands and the pairwise difference (PD) distance (Excoffier et al. 1992) were used to compute distances between SSR and RAPD phenotypes in the separate data matrices and in the combined one. SM, D, and J coefficients were calculated with NTSYSpc vers. 2.11a (Rohlf 2002) and the PD distance was computed with ARLEQUIN vers. 2.000 (Schneider et al. 2000). Correlation between these metrics was calculated through a Mantel test with 1000 replicates (Mantel 1967) using NTSYSpc. Genetic distances between RAPD phenotypes based on D, J, and PD showed significant high correlations among them (Segarra-Moragues and Catalán 2003). These metrics and the SM metric were also highly correlated when analyzing the SSR phenotypes (PD/J $r = -0.992$ $P < 0.001$; PD/D $r = -0.981$ $P < 0.001$; PD/SM $r = -0.993$ $P < 0.001$; J/D $r = 0.992$ $P < 0.001$; J/SM $r = 0.987$ $P < 0.001$; D/SM $r = 0.991$ $P < 0.001$) and the combined data matrix of RAPD + SSR phenotypes in *B. pyrenaica* (PD/J $r = -0.980$ $P < 0.001$; PD/D $r = -0.973$ $P < 0.001$; J/D $r = 0.998$ $P < 0.001$). As all coefficients were highly correlated with each other the pairwise difference distance was chosen for subsequent analyses.

Population Structure

The genetic structure of the taxa and populations of *Borderea* was first studied through the analysis of the molecular variance (AMOVA; Excoffier et al. 1992) using ARLEQUIN. Although AMOVA was originally designed for re-
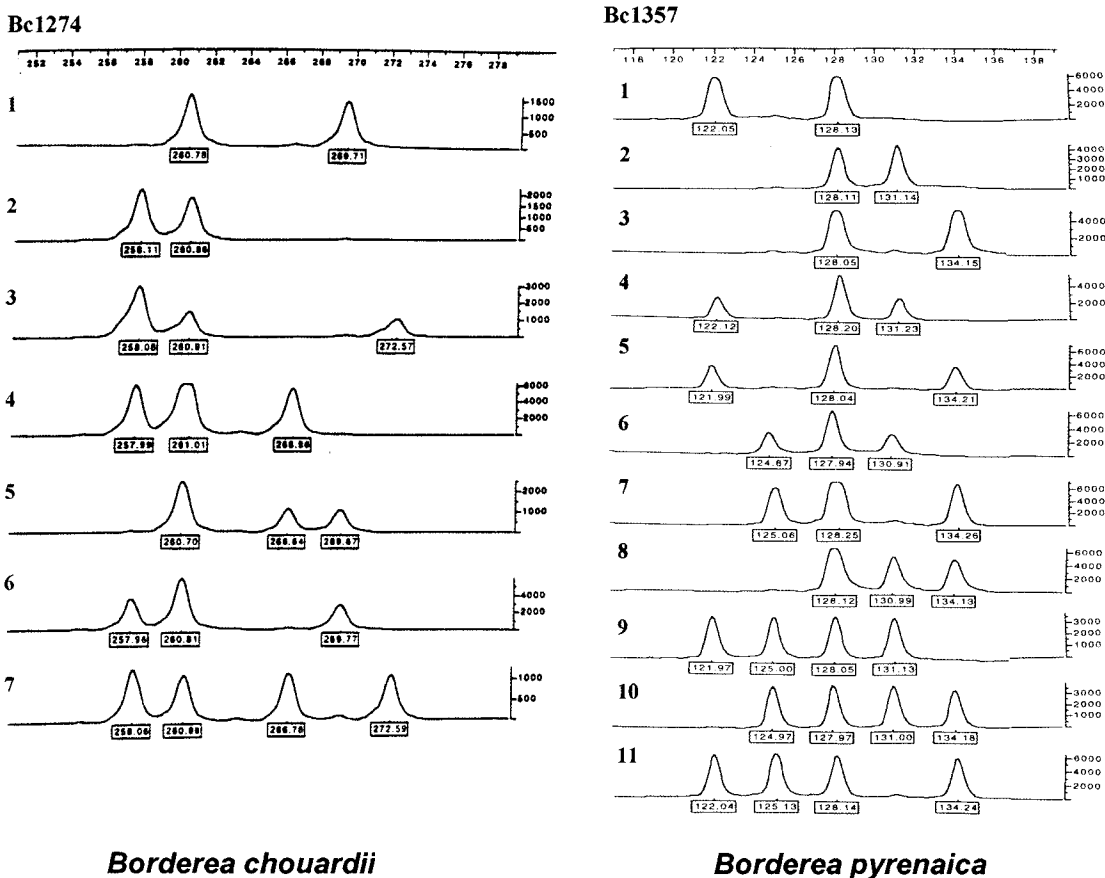
Bc1274

Borderea chouardii

Bc1357

Borderea pyrenaica

Fig. 3.—Electropherograms obtained with GENOTYPER for locus Bc1274 and Bc1357 in populations of *B. chouardii* and *B. pyrenaica*, respectively, showing individual patterns with up to four alleles.

striction fragment length polymorphism (RFLP) haplotypes it has been widely used to analyze binary coded phenotypes (i.e., RAPDs, cf. Steward and Excoffier 1996; Gabrielsen et al. 1997; Martin et al. 1997; Palacios and Gonzalez-Candelas 1997; Wolff et al. 1997; AFLP, cf. Palacios et al. 1999; and SSRs, cf. Bruschi et al. 2000). AMOVA analysis was performed at different hierarchical levels within *Borderea*: (i) all samples considered as belonging to the same species (*Borderea* s.l.; cf. Burkill 1960); (ii) between species (*B. chouardii* vs. *B. pyrenaica*); (iii) within and among populations of *B. pyrenaica* with no geographical ranges; and (iv–vii) within and among populations and among five different geographical divisions of *B. pyrenaica* ([1]: Pyrenees vs. Prepyrenees; [2]: northern Pyrenees vs. southern Pyrenees and Prepyrenees; [3]: northern Pyrenees vs. southern Pyrenees vs. Prepyrenees; [4]: northern Pyrenees vs. southern Pyrenees vs. Cotiella massif vs. Turbón massif; [5]: Pyrenees and Cotiella massif vs. Turbón massif). Significance levels of the variance components estimated for each case were obtained by non-parametric permutations procedures using 1000 replicates.

The relationships among all SSR phenotypes were visualized by a neighbor-joining (NJ) tree constructed with MEGA vers. 2.0 (Kumar et al. 2001) in which statistical robustness of the groupings was assessed by a 1000-replicates bootstrap analysis (Felsenstein 1985) using PAUP* vers. 4.0 beta 10 (Swofford 2002), and by multivariate prin-

cipal coordinate analyses (PCO) conducted with NTSYSpc (Rohlf 2002). Three different approaches were performed in the PCO analyses: (i) with the whole set of samples, to visualize the multidimensional relationships of the SSR phenotypes of both taxa; (ii) with a subset of the matrix, containing only the SSR phenotypes of *B. pyrenaica*, to search for differences in the molecular spatial distribution of phenotypes among populations of this taxon; and, (iii) with the combined SSR + RAPD data matrix to assess the consistency of the spatial distribution of populations depicted by the two sorts of molecular markers in *B. pyrenaica*. The results rendered by these analyses were compared with those reported in the previous RAPD survey (Segarra-Moragues and Catalán 2003). Genetic distances based on pairwise F_{ST} statistics between populations were used to construct unweighted pair-group method with arithmethic averaging (UPGMA) phenograms using NTSYSpc and bootstrapped with POPULATIONS vers. 1.2.28 (Langella 2000). Correlations between genetic and geographic distances between populations were assessed by means of a 1000 replicates Mantel test using NTSYSpc.

RESULTS

Relationships Between Borderea *Phenotypes*

As stated in Segarra-Moragues and Catalán (2003), the 12 RAPD primers generated 112 bands, of which only four

Table 1.   Alleles found in *B. chouardii* and *B. pyrenaica* for the 17 SSR loci studied. For each species and locus, the number of alleles (N_A) and the allele sizes in Bp. In bold, the alleles common to both species.

| Locus | *B. chouardii* | | *B. pyrenaica* | |
	N_A	Allele sizes	N_A	Alleles sizes
Bc166	12	**182**, 185, 188, 191, 194, 197, 203, 206, 215, 218, 221, 224	3	175, 178, **182**
Bc1145b	2	91, **103**	7	85, 88, 94, 97, **103**, 106, 109
Bc1159	4	**120, 123, 126**, 132	3	**120, 123, 126**
Bc1169	4	**123**, 142, 145, 152	2	**123**, 126
Bc1258	7	159, **162, 171, 180**, 183, 186, 189	7	145, 156, **162**, 165, 168, **171, 180**
Bc1274	5	**258, 261, 267, 270, 273**	22	249, 255, **258, 261**, 264, **267, 270, 273**, 276, 279, 282, 285, 288, 291, 294, 297, 303, 309, 312, 315, 318 321
Bc1357	6	**125, 134, 137**, 146, 157, 160	7	122, **125**, 128, 131, **134, 137**, 140
Bc1422	5	**162, 195, 216, 219, 222**	18	159, **162**, 177, 180, 186, 189, 192, **195**, 198, 201, 204, 207, 210, 213, **216, 219, 222**, 225
Bc1551	2	**264, 267**	24	**264, 267**, 270, 273, 276, 279, 282, 285, 288, 297, 303, 306, 309, 312, 315, 318, 321, 324, 327, 330, 333, 336, 339, 342
Bc1644	4	166, 169, **175, 178**	9	160, 163, 172, **175, 178**, 181, 184, 187, 190
Bp126	2	220, **226**	5	**226**, 235, 238, 241, 244
Bp1286	1	**123**	1	**123**
Bp2214	1	213	2	204, 216
Bp2256	2	**226**, 232	3	220, 223, **226**
Bp2290	3	**130**, 133, 140	11	127, **130**, 143, 149, 152, 155, 158, 161, 164, 167, 170
Bp2292	4	**202, 205, 211, 214**	7	199, **202, 205**, 208, **211, 214**, 217
Bp2391	1	**126**	11	123, **126**, 129, 133, 136, 140, 143, 146, 149, 153, 156
Total	65		142	

were monomorphic, whereas 108 (96.43%) were polymorphic across all *Borderea* samples. Thirty-one bands (27.67%) were exclusive to *B. chouardii* and, of them, 20 (64.52%) were fixed in this taxon, whereas 31 bands (27.68%) were exclusive to *B. pyrenaica* and, of them, only one (3.23%) was fixed. All these fixed private bands constitute diagnostic molecular markers useful to differentiate the two taxa. On the other hand, 50 out of 112 bands were shared between the two species. Thirty-nine polymorphic markers in *B. chouardii* (48.15%) and 75 in *B. pyrenaica* (92.59%) provided 395 distinct phenotypes across the 397 studied samples (Segarra-Moragues and Catalán 2003). Only two RAPD phenotypes were shared, one between two individuals of *B. pyrenaica* (Bp01) and the other between two individuals of *B. chouardii*.

The microsatellite genetic study of *Borderea* s.l. conducted here for the first time, detected similar levels of genetic variability to that provided by the RAPD markers; however, the resolving power of these SSR markers was notably higher than that shown by the less reliable RAPD markers. The 17 reproducible SSR loci detected a total of 172 bands (alleles) across the 407 studied individuals. Thirty out of 65 bands present in *B. chouardii* were exclusive to this taxon (46.15%) and, of these, three (10%) were fixed and diagnostic, separating it from its congener, whereas 107 out of 142 bands were exclusive to *B. pyrenaica* (75.35%), and three of these (2.83%) were diagnostic for this species. Thirty-five (20.35%) out of 172 total bands were shared between the two taxa. A summary of the SSR alleles detected in *Borderea* s.l. and in each of the independent species *B. chouardii* and *B. pyrenaica* is shown in Table 1. SSR markers were even more precise than RAPD markers in identifying each of the 407 individuals studied by their own SSR phenotypes.

Principal coordinate analysis of the whole SSR data matrix (*Borderea* s.l.) showed a complete differentiation between the *B. chouardii* and the *B. pyrenaica* phenotypes that clustered separately on the space delimited by the first two axes that accumulated 32.92% of the variance (Fig. 4), a result similar to that obtained from RAPD analysis (Segarra-Moragues and Catalán 2003). However, in contrast to the poor genetic population structuring shown by RAPDs (cf. Segarra-Moragues and Catalán 2003), subsequent PCO analysis of SSR phenotypes restricted to the *B. pyrenaica* data set distinguished a clear-cut clustering among phenotypes belonging to the five geographical regions (Fig. 5). The 3D
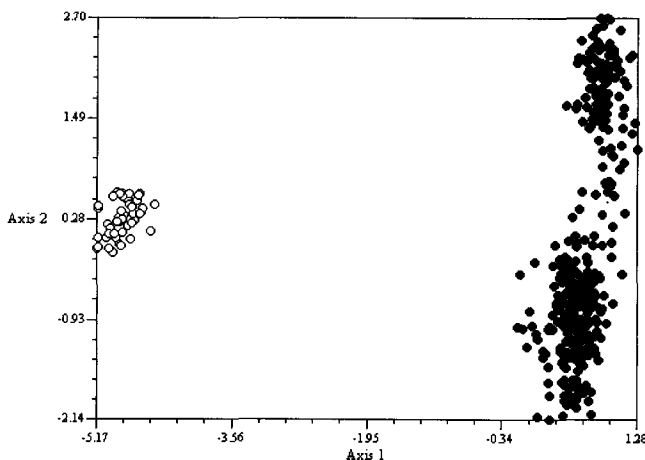

Fig. 4.—PCO plots of *Borderea*. The 407 plants of *Borderea* (360 of *B. pyrenaica*) rendered 407 SSR phenotypes (360 of *B. pyrenaica*). The first two axes explained 21.45 and 11.47%, respectively, of the total variance. ○ *B. chouardii*; ● *B. pyrenaica*.
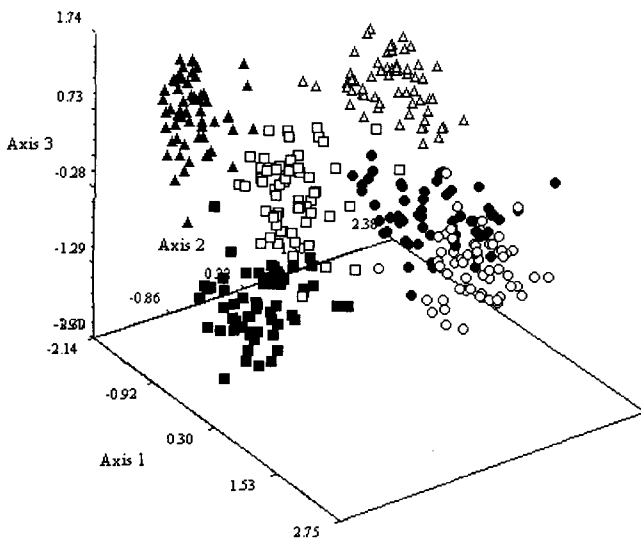
Fig. 5.—PCO plots of 360 *B. pyrenaica* plants (360 SSR phenotypes). The first three axes accounted for 15.60, 9.47, and 5.87%, respectively, of the total variance. ● Bp01, ○ Bp02, ■ Bp03, □ Bp04, ▲ Bp05, and △ Bp06.

projection of the SSR phenotypes in the space defined by the first three axes that accumulated 30.94% of the variance separated the more isolated southernmost Prepyrenean population of Turbón (Bp06) from the rest along the negative extreme of axis 1 and the positive extreme of axis 3. Other clusters corresponding to the Prepyrenean populations of Cotiella (Bp05) and the Pyrenean Spanish populations of Ordesa (Bp04) and Pineta (Bp03) are located in intermediate positions on axis 1, but separate along positive to negative positions on axis 2. Finally, a mixed cluster of phenotypes from the French Pyrenean populations of Gavarnie (Bp01 and Bp02) differentiated along the positive extreme of axis 1 (Fig. 5). Phenotypes of the two French northern populations of the Pyrenees partially intermixed in their common cluster due to the close proximity of these populations, which is less than 3 km apart. The spatial pattern shown by the *B. pyrenaica* population clusters in this molecular PCO space is in agreement with their geographical distribution (Fig. 2).

The unrooted NJ tree constructed from pairwise distances between the 407 SSR phenotypes also revealed the differentiation of two main clusters (Fig. 6) that corresponded to phenotypes of *B. chouardii* and *B. pyrenaica*, respectively, with branch divergence showing 100% bootstrap support. In contrast to previous results based on RAPD analysis (Segarra-Moragues and Catalán 2003), hierarchy of SSR phenotypes across the six studied populations of *B. pyrenaica* was resolved in this NJ tree (Fig. 6). Most of the phenotypes from each of the studied populations of *B. pyrenaica* joined in separate clusters indicating a certain degree of genetic isolation although their respective branches were not supported. Several phenotypes of the French populations from the north side of the Pyrenees (Bp01 and Bp02) appeared intermingled in a less differentiated cluster, a direct consequence of their close geographical proximity and the likely existence of present day gene flow between them. On the other hand, some phenotypes from the Ordesa population

(Bp04), from the south side of the Pyrenees, clustered together with those from the French north side (Bp01 and Bp02), whereas the vast majority of the remaining phenotypes clustered with the other Spanish population of Pineta (Bp03) on the south side of the mountains. Phenotype clusters corresponding to the Prepyrenean populations of *B. pyrenaica* at Cotiella (Bp05) and Turbón (Bp06) shared less genetic affinities to the Pyrenean population cores (Bp01, Bp02, Bp03, and Bp04), paralleling their geographically isolated distribution (Fig. 2, 5). The southernmost Prepyrenean population of Turbón (Bp06) showed a basal diverging clustering within the *B. pyrenaica* group and represented the population of this taxon most similar to that of the congener *B. chouardii*.

Multivariate PCO analysis was also conducted on the combined SSR + RAPD data matrix (results not shown); the tridimensional plotting of phenotypes in the space defined by the first main axes was less hierarchically structured than that obtained from SSRs. The same spatial differentiation pattern was observed for the clusters of phenotypes corresponding to the Pyrenean and Prepyrenean populations of *B. pyrenaica* in that projection as in the one obtained from the SSR markers (Fig. 5). However, in the latter, the first three axes accumulated a lower percentage of variance (23.81%) and the separation among clusters was not as neat. This lack of resolution was caused by the inclusion of a poorly resolved set of RAPD phenotypes (Segarra-Moragues and Catalán 2003) into the combined data matrix. A similar loss of hierarchical resolution was observed in the NJ tree based on the combined data set (results not shown) indicating that the RAPD markers are less valuable in differentiating the genetic structure of recently diverged populations in contrast to the powerful discriminating value demonstrated by microsatellites.

Population Genetic Structure

Partitioning of genetic variance within *Borderea* was obtained through AMOVA analysis (Table 2). The genetic differentiation between *B. chouardii* and *B. pyrenaica* previously detected by RAPD markers was corroborated by the statistical analysis of the SSR phenotypes. The first distribution analysis attributed 48.52% 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 the samplings were treated as separate species in the second analysis (*B. chouardii* vs. *B. pyrenaica*), the differences among taxa accumulating 48.99% of the total variation, whereas differences among populations and within populations were only of 18.73 and 32.28%, respectively. The F_{ST} values for the RAPD and SSR differentiation between the two species were highly significant (0.79 and 0.68, respectively; $P < 0.001$) in both cases.

AMOVA analyses conducted at different hierarchical levels within *B. pyrenaica* always revealed higher genetic diversity within populations than either between populations or between geographical regions regardless of the data matrix used for comparison (Table 2; SSRs, RAPDs). However, the SSR markers always detected lower levels of genetic variability within populations, but higher levels of genetic
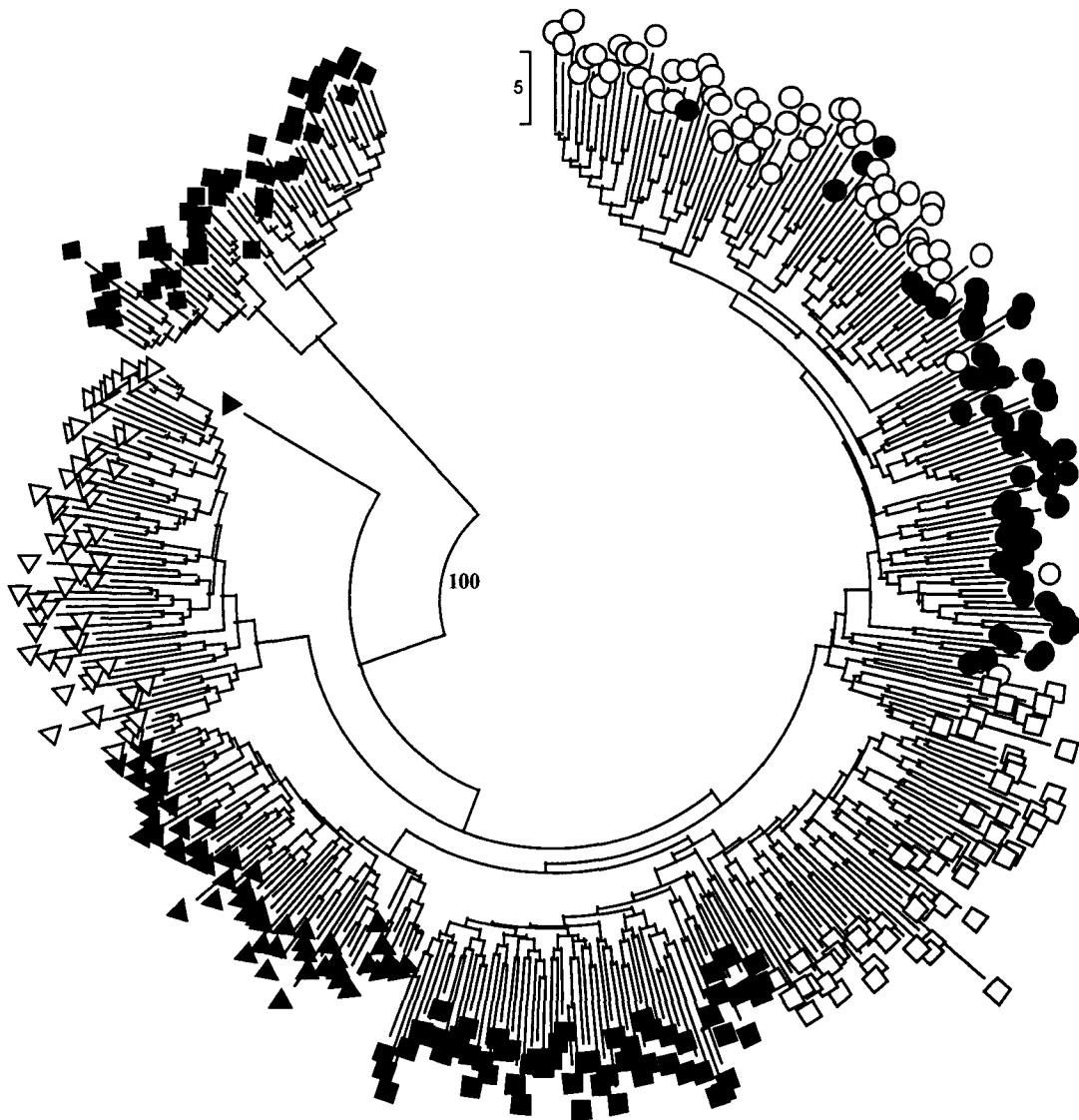
Fig. 6.—Neighbor-joining (NJ) tree of the 407 SSR phenotypes observed in *Borderea*. Bp01 ● and Bp02 ○, correspond to French populations of *B. pyrenaica*; Bp03 ■ and Bp04 □ to populations in the axial ranges on the Spanish side of the Pyrenees; Bp05 ▲ and Bp06 △ to populations in the Prepyrenean ranges; Bc01 ◆ corresponds to *B. chouardii*. Support for the grouping is indicated on the branch by the bootstrap value.

differentiation among populations and among regions than the RAPD markers in all assayed cases, indicating a more conserved nature and pointing toward their suitability for a better assessment of the genetic relationships among recently diverged populations.

The lowest value for the percentage of genetic variation accumulated among populations from the same area (15.14, 8.80, and 12.63%, for the SSRs, RAPDs, and combined analyses, respectively) was that obtained when the populations were divided into four geographical ranges (northern Pyrenees [Bp01, Bp02] vs. southern Pyrenees [Bp03, Bp04] vs. Prepyrenean Cotiella [Bp05] vs. Prepyrenean Turbón [Bp06]), suggesting a close genetic relationship and homogeneity of populations from the same region. At the same time, the highest percentage of partitioning of variance among regions also was obtained for this subdivision of populations, both for the SSRs (23%) and the combined analyses

(17.28%). Whereas for RAPDs, the highest value of divergence among regions (8.59%) was obtained when the southernmost Prepyrenean population of Turbón (Bp06) was considered separate from the rest. These results indicated that SSRs are more precise in depicting genetic relationships among closely related populations. Nonetheless, the two molecular markers are coincident in showing the populations of the southern Prepyrenean ranges as the most genetically distant and those from the northern Pyrenean range as the most recently derived.

Genetic and Geographical Distances between Taxa and Populations

Genetic distances among populations of *Borderea* were based on F_{ST} values, the analogue of Φ_{ST} values, calculated from the Euclidean Distance. The F_{ST} coefficients were used

Table 2. Analysis of Molecular Variance (AMOVA) based on 395 RAPD phenotypes of *Borderea* (349 of *B. pyrenaica* and 46 of *B. chouardii*), 407 SSR phenotypes (360 of *B. pyrenaica* and 47 of *B. chouardii*), and combined 360 RAPD + SSR phenotypes of *B. pyrenaica*. SSD = Sum of Squares Difference.

| Source of variation (groups)	SSRs				RAPDs (data from Segarra-Moragues and Catalán 2003)				Combined RAPDs + SSRs			
	SSD	d.f.	Variance components	% of the total variance	SSD	d.f.	Variance components	% of the total variance	SSD	d.f.	Variance components	% of the total variance
Borderea s.l.												
Among populations	2714.85	6	7.65	48.52	2268.37	6	6.56	52.16	—	—	—	—
Within populations	3246.97	400	8.12	51.48	2348.32	390	6.02	47.8	—	—	—	—
B. chouardii vs. *B. pyrenaica*												
Among taxa	1260.872	1	12.32	48.99	1926.66	1	22.55	76.08	—	—	—	—
Among populations within taxa	1413.981	5	4.71	18.73	341.71	5	1.07	3.60	—	—	—	—
Within populations	3246.966	400	8.12	32.28	2348.32	390	6.02	20.31	—	—	—	—
B. pyrenaica s.l.												
Among populations	1453.98	3	4.71	36.18	341.71	5	1.06	14.48	1752.64	5	5.76	28.27
Within populations	2939.81	354	8.30	63.82	2161.18	344	6.28	85.52	5025.65	344	14.61	71.73
B. pyrenaica (geographical ranges)												
1. Pyrenees (Bp01 to Bp04) vs. Prepyrenees (Bp05, Bp06)												
Among regions	389.14	1	0.77	5.75	97.07	1	0.23	3.04	473.83	1	0.97	4.66
Among populations within regions	1064.84	4	4.30	32.13	244.63	4	0.94	12.65	1278.82	4	5.24	25.16
Within populations	2939.82	354	8.30	62.11	2161.18	344	6.28	84.31	5025.65	344	14.61	70.18
2. N Pyrenees (Bp01, Bp02) vs. S Pyrenees and Prepyrenees (Bp03 to Bp06)												
Among regions	602.09	1	2.43	17.19	112.85	1	0.37	4.87	689.51	1	2.78	12.82
Among populations within regions	851.89	4	3.41	24.11	228.85	4	0.87	11.58	1063.13	4	4.29	19.80
Within populations	2939.80	354	8.30	58.70	2161.18	344	6.28	83.55	5025.65	344	14.61	67.38
3. N Pyrenees (Bp01, Bp02) vs. S Pyrenees (Bp03, Bp04) vs. Prepyrenees (Bp05, Bp06)												
Among regions	834.42	2	1.76	13.14	166.59	2	0.21	2.89	971.95	2	1.93	9.32
Among populations within regions	619.56	3	3.30	24.72	175.12	3	0.89	12.09	780.69	3	4.21	20.29
Within populations	2939.82	354	8.30	62.14	2161.18	344	6.28	85.02	5025.65	344	14.61	70.39
4. N Pyrenees (Bp01, Bp02) vs. S Pyrenees (Bp03, Bp04) vs. Cotiella (Bp05) vs. Turbón (Bp06)												
Among regions	1193.46	3	3.09	23.00	259.68	3	0.53	7.22	1418.13	3	3.60	17.28
Among populations within regions	260.52	2	2.03	15.14	82.02	2	0.60	8.08	334.51	2	2.63	12.63
Within populations	2939.82	354	8.30	61.86	2161.18	344	6.28	84.70	5025.65	344	14.61	70.09
5. Pyrenees and Cotiella (Bp01 to Bp05) vs. Turbón (Bp06)												
Among regions	431.30	1	1.76	12.38	122.65	1	0.67	8.59	548.81	1	2.41	10.98
Among populations within regions	1022.68	4	4.12	29.07	219.05	4	0.83	10.74	1203.83	4	4.94	22.49
Within populations	2939.82	354	8.30	58.55	2161.18	344	6.28	80.68	5025.65	344	14.61	66.54
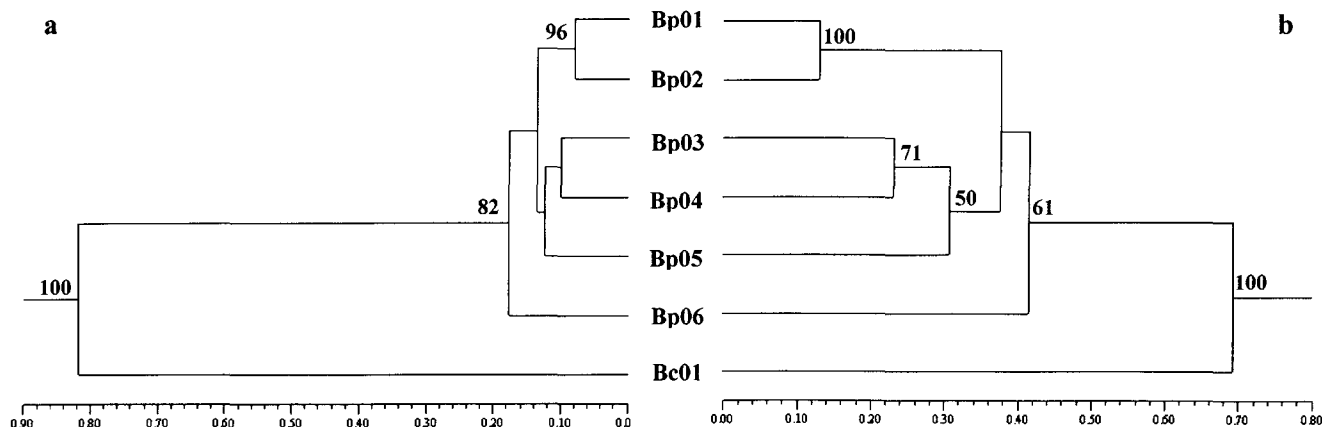
Fig. 7.—UPGMA clusterings based on F_{ST} statistics showing the relationships among the seven populations of *Borderea*. Support for branches (>50%) are indicated by bootstrap values: (a) RAPD, (b) SSR.

to construct UPGMA phenograms based on both SSR and RAPD distance matrices. The two types of molecular markers were concordant in depicting the same linkage among populations (Fig. 7); however, patristic distances and levels of bootstrap support were higher in the SSR phenogram than in the RAPD phenogram. In both cases, the greatest distances were those observed between the single population of *B. chouardii* and all six populations of *B. pyrenaica* (ca. 0.7 for SSRs and >0.8, RAPDs). These results were supported in both clusterings by full bootstrap percentages (100%). Distances between populations of *B. pyrenaica* ranged from moderate (>0.4, SSR) to low (<0.2, RAPD) values. The two closest populations were those from the northern Pyrenees at Gavarnie (Bp01, Bp02), which are also geographically close and show strong bootstrap support (100% SSR and 96% RAPD). Another tight cluster was composed of the southern Pyrenean populations (Bp03, Bp04), which are well supported in the SSR clustering (71%) and, in turn, linked to the northernmost Prepyrenean population of Cotiella (Bp05). The greatest distance (ca. 0.42, SSRs; ca. 0.20, RAPDs) was that between the southernmost Prepyrenean population of Turbón (Bp06) and the rest of the *B. pyrenaica* populations. The genetic affinities among populations of *B. pyrenaica* are mostly concordant with geographic distances. Nevertheless, populations from the French side of the Pyrenees (Bp01 and Bp02) appear to be less closely related to those of the Spanish side (Bp03 and Bp04) although the map distance between them is shorter than that of the latter populations to Prepyrenean population Bp05. 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 geographic distances computed for *B. pyrenaica* populations showed significant values of 0.71 ($P < 0.01$) and 0.68 ($P < 0.05$) for SSR and RAPD markers, respectively, indicating that some populations located in closer geographic proximity are genetically less closely related than other populations that are geographically further apart, but that show higher genetic affinities. These results point toward past climatic oscillatory changes and geography as the main factors intervening in the postglacial colonization pathways followed by the populations of *B. pyrenaica* and in the maintenance of their present genetic relationships.

DISCUSSION

Microsatellite Markers and the Attributes and Relationships of Genus Borderea with Respect to other Dioscoreaceae

Comparative studies from a large pool of different molecular markers are the most accurate way to test the validity of potential evolutionary scenarios for any group of living organisms (Avise 1994). Of the three molecular marker systems assayed in *Borderea* (allozymes, RAPDs, SSRs; cf. Segarra-Moragues and Catalán 2002, 2003; Segarra-Moragues et al. 2003, 2004, and the present study) microsatellites—due to their codominant nature and their capacity to detect high, but stable levels of polymorphism—have provided the most robust data set to investigate the past evolutionary history of the *Borderea* taxa and populations. These properties qualify SSRs as the best molecular tools for detailed fine-scale evolutionary and taxonomic investigations of little-differentiated populations of Dioscoreaceae. Nonetheless, microsatellite data are mostly congruent with allozyme and RAPD data in depicting a similar evolutionary scenario for the ancestral Pyrenean yams. SSR markers have also rendered unique clues to decipher the mechanisms involved in the origin of these relictual species and their genetic background.

In two initial SSR assays conducted on single populations of *B. chouardii* (Segarra-Moragues et al. 2003) and of *B. pyrenaica* (Segarra-Moragues et al. 2004) each separate set of loci showed individuals with up to four alleles. Cross amplifications of these 17 loci along all 407 studied individuals have confirmed the previous findings (Fig. 3), thus reaffirming the tetraploidy of the two *Borderea* taxa. This is the first record concerning the polyploid nature of this relict Pyrenean genus. The two species of *Borderea* were considered to be diploid by Heslot (1953) who counted $2n = 24$ and $n = 12$ chromosomes in *B. pyrenaica* and $2n = 24$ chromosomes in *B. chouardii*. We assumed *Borderea* was diploid and had a chromosome base number of $x = 12$, close to that presented by most *Dioscorea* taxa ($x = 10$), and used this karyological character as a further criterion to distinguish *Borderea* from *Dioscorea*. No other cytogenetic studies have been conducted in *Borderea* since those of Heslot (1953), and all later authors have accepted $x = 12$ as the

chromosome base number of the Pyrenean yams (Burkill 1960; cf. Gaussen 1965; Huber 1998). On the other hand, the Mediterranean genus *Tamus,* with a chromosome number of $2n = 48$, was believed to be a tetraploid taxon that shared with *Borderea* the chromosome base number $x = 12$ indicative of a close evolutionary relationship between them (Burkill 1960; Huber 1998). Other karyological surveys of Dioscoreaceae have shown that the most common chromosome base numbers in the family are $x = 10$, present in nine paleotropical sections of *Dioscorea* and in the holarctic section *Macropoda* Uline, and $x = 9$, present in four out of nine tropical sections of *Dioscorea* and in *Rajania* L., whereas the American *D. mexicana* Scheidw., shows $x = 8$ (Huber, 1998).

An immediate conclusion from our microsatellite survey is that if *Borderea* is a tetraploid genus with $2n = 24$ chromosomes, then its chromosome base number is not $x = 12$, but $x = 6$. Up to now $x = 6$ is the smallest chromosome base number recorded in the mostly pantropical Dioscoreaceae (Burkill 1960; Dahlgren et al. 1985; Huber 1998). However, *Borderea* may well represent a case of a secondary base number ($x = 12$) of polyploid derivation (Stebbins 1971) where the original base number $x = 6$ was doubled by tetraploidization giving rise to the gametic number $n = 12$ observed by Heslot (1953) and interpreted as a functionally $x = 12$.

Further insights into the genomic inheritance of nuclear chromosome markers in *Borderea* have also been obtained from the analysis of microsatellite alleles. A more detailed statistical study on SSR inheritance patterns in the *Borderea* species is presently underway. However, for most of the studied SSR loci both *B. pyrenaica* and *B. chouardii* show predominant duplicate disomic inheritance (Catalán et al. unpubl. data) reinforcing the hypothesis of a hybrid origin of these polyploid taxa (Segarra-Moragues and Catalán 2002). Fixed heterozygous microsatellite profiles as well as variable, but cosegregating allelic patterns in SSR loci are concordant with previous findings based on fixed heterozygous patterns for some allozyme loci (PGI-2, IDH) in the likely existence of a past hybridization event that resulted in the present known genus *Borderea*. Amphipolyploidy is recognized as the more common polyploidization mechanism in flowering plants (Stebbins 1950, 1956, 1971; Stace 1987; Soltis and Soltis 1993, 1999) and is of special relevance in relictual lineages of many families of angiosperms (Stebbins 1971; Soltis and Soltis 1993). *Borderea* fits well within an archaic amphidiploid scenario whereas other polyploid *Dioscorea* taxa (with several multiples of $2n = 10$) seem to have had a more recent origin—especially those concerned with the highly polyploid cultivated yams (Huber 1998).

The phylogenetic studies of Caddick et al. (2002a) based on analysis of *rbc*L sequences demonstrated that polyploid Mediterranean *Tamus* was the closest relative of the Pyrenean endemic *Borderea*. According to these results, and based on our present knowledge about ploidy levels and inheritance patterns in *Borderea,* we speculate that these two sister genera could also have a common amphidiploid origin derived from common ancestors with $x = 6$. Thus, tetraploid *Borderea* ($2n = 24$) and octoploid *Tamus* ($2n = 48$) could constitute some of the oldest extant paleopolyploid lineages of Dioscoreaceae.

Borderea and *Tamus* are sympatric in the Pyrenees; these two ancient genera belong to the same biogeographical Mediterranean region, although *Tamus* is widespread in the pan-Mediterranean area, whereas *Borderea* is restricted to the central Pyrenean zone. Chloroplast sequence data, karyological evidence, and our present microsatellite data reinforce the hypothesis of a common hybrid origin of these two genera from Tertiary Dioscoreaceae ancestors that likely became extinct with the advent of glaciation, but whose descendants survived as newly arisen polyploids in Mediterranean refugia during the coldest periods of the Quaternary and successfully adapted to the newly deserted areas during the postglacial warming era.

Whereas the close relationship of *Borderea* and *Tamus* is undisputedly supported by various data sources in spite of some apparent morphological differences—the twining habit and berry fruit shown by *Tamus* in contrast to the non-twining dwarf habit and capsule fruit present in *Borderea*—the purported close affinity of *Borderea* to other taxa of Dioscoreaceae seems to be less reliable. Thus, the attributed affinity of the *Borderea* taxa to the east African *Dioscorea gilletii,* classified under the same sect. *Borderea* of a large genus *Dioscorea* s.l. by Burkill (1960) and Huber (1998), and their putative past ancestry from a pan-Thetyan tropical Dioscoreaceae lineage (Burkill 1960) are likely spurious. New phylogenetic analyses based on chloroplast sequence data (*rbc*L, *mat*K) (P. Wilkins and P. Scholz pers. comm.) indicate that *Borderea* and *Tamus* are sister group to an isolated Mediterranean clade whereas *D. gilletii* is nested within an independent African *Dioscorea* clade. Similarly, the presumed closeness of *Borderea* to the Andean endemic orophyte genus *Epipetrum* and their hypothesized origin from a common pan-Atlantic Dioscoreaceae ancestor (Braun-Blanquet 1948) are not sustainable based on current biogeographical knowledge (even with the lack of molecular data for *Epipetrum*). Thus, the crucial shared morphological characters that most frequently have been used for classification purposes—such as, the possession of unwinged seeds, a feature shared by *Borderea, D. gilletti,* and *Epipetrum,* as well as by *Tamus* and *Nanarepenta* Matuda (cf. Caddick et al. 2002b), and the acquisition of a dwarf mountain habit, common in *Borderea* and *Epipetrum* and, to a lesser extent, in *D. gilletii* as well as in other North American *Dioscorea* species (cf. Burkill 1960)—appear to be the consequence of parallel convergent evolutionary processes leading to independent Dioscoreaceae lineages in different geographical regions, and probably, at different evolutionary times.

The Species of Borderea and Their Evolutionary History

Microsatellite data also support the genetic distinctness of the two *Borderea* taxa. In concordance with previous molecular studies based on allozyme and RAPD markers (Segarra-Moragues and Catalán 2002, 2003), the SSR analyses have provided three new private alleles for each of *B. chouardii* and *B. pyrenaica* that account for the characterization and the separation of the two species. These markers, together with a private allozyme allele (PGM1–2) detected for *B. chouardii* (Segarra-Moragues and Catalán 2002) and 20 and 1 private RAPD markers detected for *B. chouardii* and *B. pyrenaica,* respectively, (Segarra-Moragues and Cat-
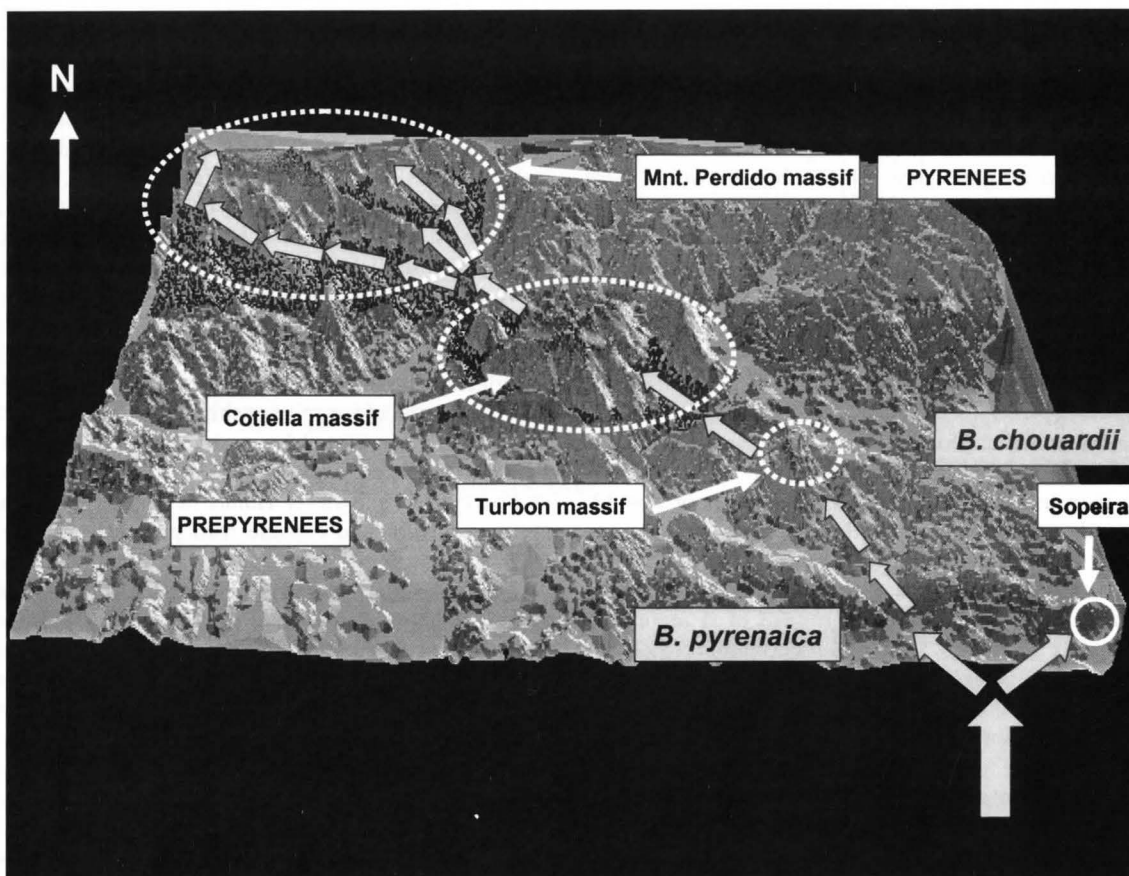
Fig. 8.—Hypothesized *Borderea* split from a Tertiary ancestor that gave rise to the presently known *Borderea* taxa and potential postglacial colonization route followed by *B. pyrenaica* from the southern Prepyrenean ranges to the northern Pyrenees. Circled areas indicate current areas occupied by *B. chouardii* (solid line) and *B. pyrenaica* (dashed line), respectively.

alán 2003) constitute a large molecular set of identifiers for these Pyrenean endemics. Reconstruction of phenotypic relationships among populations of both species and distance-based methods conducted with allozymes, RAPD, and SSR markers always discriminate *B. chouardii* from *B. pyrenaica* as two distinct genetic entities. Thus, molecular evidence based on three different data sets indicates an old divergence of the two *Borderea* taxa and favors their taxonomic recognition as separate species. In contrast to the taxonomic treatment proposed by Burkill (1960) and in agreement with that defended by Gaussen (1952, 1965), *Borderea chouardii* should be considered a different species from *B. pyrenaica* and not a mere subspecies of it.

The molecular data correlate well with the morphological traits in the distinction of the two taxa. Apart from traditional features used to differentiate *B. chouardii* from *B. pyrenaica* (Gaussen 1952; Fig. 1), a careful examination of new materials allowed us to find new distinctive traits for these species including the coat, shape, and color of the seeds. Hence, whereas *B. chouardii* bears brownish fusiform seeds with an apical caruncle, those of *B. pyrenaica* possess dark horizontally-compressed seeds covered by an extended thin-layered caruncle (Segarra-Moragues and Catalán 2005, unpubl. data). Thus, the new morphological characters contribute to the identification of *B. chouardii* as a singular species and posit further arguments for its separate classification from *B. pyrenaica*.

Reconstruction of the life history of *Borderea* was first attempted with the more variable RAPD markers (Segarra-Moragues and Catalán 2003) due to the low variability detected with allozymes (Segarra-Moragues and Catalán 2002); however, the two markers agree on the old divergence of the two *Borderea* species and on a recent postglacial diversification of the present *B. pyrenaica* populations (Segarra-Moragues and Catalán 2003). The microsatellite data also support this evolutionary scenario for *Borderea* and add more precise data for a colonizing postglacial migratory route of *B. pyrenaica* from southern Prepyrenean refugia towards the northern Pyrenean ranges (Fig. 8).

Patterns and models of plant evolution during the oscillatory climatic changes of the late Tertiary and Quaternary ages in Europe and in the Mediterranean basin are diverse (Hewitt 1996; 2000). There is a general acceptance that the northern territories of Europe were mostly covered by ice during the glaciations without any trace of potential refugia (nunataks) for plants and that postglacial colonization followed a general trend from south to north (Gabrielsen et al. 1997; Tollefsrud et al. 1998). Concomitant with the retreat and advances of the ice sheets (Hewitt 1996; Comes and Kadereit 1998; Gutiérrez-Larena et al. 2002), the sheltering mountains of southern Europe are believed to have hosted intermittent phases of expansion and contraction of plant populations derived from locally ascending and descending migratory movements on the mountains. Under such circum-

stances further scenarios have been postulated for different plant species ranging from long-term isolation processes in geographically separate refugia to recent colonization events from single or limited shelters (Bauert et al. 1998; Taberlet et al. 1998; Zhang et al. 2001). *Borderea* represents a typical example of the latter case and this scenario is also supported by the more accurate microsatellite data (Fig. 8).

Despite concordance of phylogeographic patterns exhibited by several angiosperm groups in the southern European mountains, molecular studies have shown that each plant group has followed its own evolutionary history. The present day distribution of species and populations could be due, in part, to factors as different as the ecological affinities of the original founders; their ability to successfully invade newly vacated areas or zones already occupied by other pioneers; and, their differential rates of extinction in sheltered areas (Comes and Kadereit 1998). Hybridization likely played a crucial role in the conquest of newly vacated areas at higher altitudes—land made available by glacial retreat—as it increased the capability to invade more inhospitable environments (Soltis and Soltis 1999; Ellstrand and Schierenbeck 2000; Zhang et al. 2001). The case of *Borderea* well illustrates the dramatic differences in adaptive fitness shown by two closely related species of common hybrid origin. In spite of the potential advantages acquired through hybridization, the less successful *B. chouardii*—due to its inefficient seed dispersal mechanisms (postcarpotropism) described by Segarra-Moragues et al. (2005)—was nearly rendered extinct during the glacial periods and failed to colonize new habitats at postglacial times. The SSR data also confirm the strong genetic bottlenecking experienced by the only surviving population of *B. chouardii* where levels of genetic variability were considerably lower than those of its congener *B. pyrenaica*. By contrast, the aggressive hybrid *B. pyrenaica* evolved into a postglacial subalpine species that successfully colonized the barren scree of the central Prepyrenees and Pyrenees where competition with other plants was less stringent and seed dispersal could be mediated by grazing animals.

The SSR data favor a stepping-stone colonization hypothesis (Hewitt 2000) for *B. pyrenaica*, previously envisaged through RAPD analyses (Segarra-Moragues and Catalán 2003), and add further evidence for a fine-scale reconstruction of a saltatory migration route from southern Prepyrenean to northern Pyrenean massifs (Fig. 8). The relatively low levels of genetic differentiation found among populations of this taxon, in contrast to the higher levels of genetic variation detected within populations indicate (in the absence of present gene flow between the three main Prepyrenean and Pyrenean mountain ranges) that the present *B. pyrenaica* populations are of recent origin and that they were likely derived from a pre-Quaternary lineage subjected to severe population declines during the coolest glacial phases. The short time that has elapsed since the beginning of the postglacial colonization and expansion of *B. pyrenaica* until today (ca. 10,000 yr) has prevented the genetic isolation of these recently arisen populations which do not show any private SSR alleles. Previous hypotheses, based on RAPD data, pinpoint the Prepyrenees as the likely place of speciation and divergence of the two *Borderea* taxa and as the starting point for the *B. pyrenaica* migratory way to the

north (Segarra-Moragues and Catalán 2003) have been confirmed and reassessed by more solid data provided by microsatellites.

From our analyses of SSR alleles (Fig. 2, 6–8) we derived a more robust scenario for the successive advances and divergences of the *B. pyrenaica* populations. Our research corroborates that populations from an initial stock, isolated at the southernmost Prepyrenean massif of Turbón, diverged to form the northernmost Prepyrenean populations at the Cotiella massif, and eventually, were distributed within the Pyrenean range. Moreover, microsatellites have confirmed the presence of three relictual SSR alleles, shared with its ancestral congener *B. chouardii*, in the populations of the two Prepyrenean massifs and their absence in those distributed in the Pyrenean core (Table 1). The ancillary data strongly support the single south-to-north migratory cline followed by *B. pyrenaica* from older and first established populations in the warmer and lower latitude refugia of the Prepyrenees toward the younger and more recently settled populations that colonized the cooler and higher mountain ranges of the Pyrenees concomitantly with the gradual retreat of the glaciers. The more discrete genetic structure found in the Prepyrenean populations (Fig. 6) indicates their relative genetic isolation from those of the Pyrenees. As a consequence of the postglacial warming trends, the Prepyrenean populations were left behind during the up-slope migration toward the higher northern massifs of the Pyrenees. These populations ended geographically isolated at the highest points of the southern Prepyrenean in mountain "isles" surrounded by foothills containing new montane vegetation (Fig. 8).

According to the microsatellite data, a small-scale colonization process can be proposed for the *B. pyrenaica* populations that reached the main Pyrenean core from the south. The expansion along the southern side of the central Pyrenees was favored by the availability of suitable habitats, whereas northward ascents were probably impeded by the higher altitudes of the Monte Perdido massif, which presently maintains some of the few relictual glaciers of the Pyrenean chain. However, this natural barrier was likely circumvented through lower-altitudinal mountain passes that facilitated the ultimate colonization of the northern side of the Pyrenees at the Gavarnie Valley (Fig. 2, 8). The close correspondence between some SSR phenotypes of the southern-Pyrenean populations of Ordesa and those of the northern-Pyrenean populations of Gavarnie confirms this connecting migratory pathway, whereas the limited differentiation observed among the northern-Pyrenean populations indicates a recent divergence and the likely existence of present gene flow among these populations.

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