Journal of Heredity 2004:95(2):177–183
DOI: 10.1093/jhered/esh028

Characterization of Seven $(CTT)_n$ Microsatellite Loci in the Pyrenean Endemic *Borderea pyrenaica* (Dioscoreaceae): Remarks on Ploidy Level and Hybrid Origin Assessed through Allozymes and Microsatellite Analyses

J. G. Segarra-Moragues, M. Palop-Esteban, F. González-Candelas, and P. Catalán

From the Departamento de Agricultura y Economía Agraria, Escuela Superior Politécnica de Huesca, Universidad de Zaragoza, Ctra. de Cuarte Km. 1, E-22071, Huesca, Spain (Segarra-Moragues and Catalán), and the Instituto Cavanilles de Biodiversidad y Biología Evolutiva, Genética Evolutiva. Universitat de València, Apdo, Correos, 22085, E-46071, Valencia, Spain (Palop-Esteban and González-Candelas). We thank A. Valadon, D. Fallour, and the forest guards at the National Park of the Pyrenees for their help during the sampling of populations of *B. pyrenaica*, and thanks to three anonymous referees, whose suggestions greatly improved an earlier version of the manuscript. Financial support was provided by both a project grant [P105/99-AV (229–142)] to P.C. and a Ph.D. grant to J.G.S. from Diputación General de Aragón. M.P. and F.G.-C. were supported by a Ministerio de Educación y Ciencia/Comisión Interministerial de Ciencia y Technologia (MEC/CICYT) (P98-1436) grant.

Address correspondence to José Gabriel Segarra-Moragues at the address above, or e-mail: jogasemo@posta.unizar.es.

We present the identification and characterization of microsatellite loci in the Pyrenean endemic *Borderea pyrenaica* Miégeville (Dioscoreaceae). Seven microsatellite loci were isolated from a $(CTT)_n$-enriched partial genomic library. Electropherograms patterns suggest that *B. pyrenaica* is a tetraploid species, as is its congener *B. chouardii*. One microsatellite locus was monomorphic, whereas the remaining ones presented from 2 to 10 alleles when analyzed in a sample of 60 individuals. Microsatellites have revealed higher levels of genetic variability than those in previous studies based on allozymes. Levels of genetic diversity are discussed in terms of tetrasomic (autotetraploidy) or duplicated disomic (allotetraploidy) modes of allele segregation. According to the first hypothesis, mean levels of genetic variability (H_{min}–H_{max}) range between 0.36 and 0.41, whereas, according to the second hypothesis, the 7 primer pairs amplified 11 chromosomal loci, and mean levels of observed and expected heterozygosities were 0.217 and 0.229, respectively, and did not differ significantly from HW expectations. These results suggest a hybrid allopolyploid origin for the *Borderea* taxa.

Borderea Miégeville is a small genus of the Dioscoreaceae, endemic to the Pyrenees and Prepyrenees. *Borderea* is considered a Tertiary relict of the pantropically distributed family of yams (Knuth 1924), which was successfully adapted and colonized the central region of the Pyrenean mountain ranges. It includes only two species: *Borderea chouardii* (Gaussen) Heslot, for which only a single population is presently known (García et al. 2002), and *Borderea pyrenaica* Miégeville. Both taxa share an overall close morphology (Burkill 1960; Huber 1998), though they differ in several vegetative, fruit, and seed traits (Gaussen 1952; Segarra-Moragues and Catalán, unpublished data).

Borderea pyrenaica Miégeville is a small dioecious, long-lived [up to 305 years (García and Antor, 1995)], and strictly sexually reproducing geophyte. It grows on alpine north-facing mobile limestone screes, mostly over 1800 meters above sea level (m.a.s.l), confined to a narrow area of approximately 160 km^2. It is mainly located on the Spanish side of the Central Pyrenees (Huesca province), being present in both the Pyrenean and Pre-Pyrenean mountain ranges reaching also the French side of the Pyrenees at

Gavarnie. Despite the small number of known populations of *B. pyrenaica* and its restricted area of distribution, some populations are very large and widespread in isolated areas, therefore lowering its endangered situation and risk of extinction.

The two *Borderea* species were initially surveyed with allozymes (Segarra-Moragues and Catalán, 2002). This study rendered low levels of genetic diversity within taxa and between populations of *B. pyrenaica*, precluding a fine-scale assessment of their population genetic structure. However, intriguing patterns of allozyme profiles with fixed heterozygosity were found for some loci (*IDH* and *PGI2*), which were alternatively interpreted as the consequence of differential natural selection pressures for heterozygous (*versus* homozygous) individuals, of gene duplications, or of a potential hybrid origin of the genus, believed to be composed by diploid taxa (Gaussen 1965; cf. Heslot 1953), from ancestors with complementary alleles for these loci.

A better assessment of the genetic relationships among the two *Borderea* taxa and of populations of *B. pyrenaica* was obtained with random amplified polymorphic DNA (RAPD) (Segarra-Moragues and Catalán, 2003), which allowed us to correlate the severe demographic changes and preferential postglacial colonization pathways experienced by the *Borderea* populations with the oscillatory climatic changes of the Quaternary, but did not clarify the genomic dosages of these taxa, because of the dominant nature of these markers.

The present work is a precursor of future population genetics studies designed to assess the genetic diversity, population structure, and genetic divergence of *Borderea* populations and to compare these results to those obtained for *Borderea*'s close relative, the narrow endemic *Borderea chouardii*. Our aim is to analyze the genetic structure and patterns of inheritance in *Borderea*, as well as to investigate the microevolutionary processes of territory contraction-expansion and recolonization experienced by the *B. pyrenaica* populations during the late glaciations.

Materials and Methods

DNA was extracted from 2 g of fresh leaves of *B. pyrenaica* following the CTAB protocol (Doyle and Doyle, 1990). One μg of DNA digested with 17.5 U of *Sau*3A (Boehringer Mannheim) in a 40 μl volume was used for the construction of a partial CTT-enriched genomic library. This enzyme was selected because it shares the same four-bp recognition sequence of *Mbo*I, which rendered good results in other Dioscoreaceae (Terauchi and Konuma 1994). Fragments ranging from 300 to 900 bp were selected and ligated (3:1 insert/vector molar ratio) with 3 U of T_4 DNA ligase (Promega) into dephosphorylated *Bam*HI-digested pBluescript II (+/−) vector (Stratagene). Single-stranded copies of the inserts were obtained by asymmetric polymerase chain reaction (PCR) in 100 μl mix containing 50 pmol of "17m" (forward) and 5 pmol of "Reverse" primers, 0.2 mM of each dNTP, 1X *Taq* buffer (Promega), 0.2 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Promega), and 20 μl of the previous ligation. All PCRs were carried out in a PE GeneAmp 9700 (Applied Biosystems). The PCR program consisted of an initial denaturation step (94°C, 4 min) followed by 35 cycles (94°C, 1 min; 50°C, 1 min; and 72°C, 1 min).

One mg of streptavidin-coated M-280 magnetic beads (Dynal) were washed five times with 100 μl of a solution containing 0.14 M NaCl, 2 mM KCl, 4 mM Na_2HPO_4, 1 mM KH_2PO_4, and 0.1% BSA, pH 7.4, and then twice with 100 μl and 200 μl, respectively, of 2X B&W buffer (10 mM Tris-HCl, pH 7.5; 1mM EDTA; 2M NaCl). Two hundred pmol of 5′-biotinylated $(CTT)_8GC$ oligonucleotide were attached to the Dynabeads (Dynal; 15 min, room temperature), washed three times to remove unbound oligonucleotides with 400 μl of B&W buffer, and resuspended in 100 ml of a solution of 3X SSC (20 × SSC: 6 M NaCl; 0.6 M Na_3-citrate, pH 7), 0.1% SDS, and 2% PEG 6000 and kept at hybridization temperature (50°C). Fifteen μl of the asymmetric PCR product was denatured (94°C, 4 min), cooled for 5 min on ice, and then added to the preheated bead suspension and incubated at 50°C for hybridization (30 min). The hybridized beads were washed five times each with 100 μl of three decreasing-concentration solutions of SSC [each containing 2X SSC, 1X SSC, or 0.5X SSC, respectively, plus 10% SDS, and 5 pmol each of "17m" (forward) and "Reverse" primers]. Bound sequences were eluted in 100 μl of 0.2X SSC (94°C, 10 min) and desalted with QIAex II (Qiagen). Ten μl of this enriched DNA was used as template for a symmetric PCR (same conditions as for asymmetric PCR, but with an additional extension step of 7 min at 72°C) containing 50 pmol each of T_3 and T_7 pBluescript primers. PCR products were purified (QIAquick, Qiagen) and digested with *Sau*3A. Fragments ranging from 400 to 900 bp were isolated and purified (QIAquick, Qiagen) and ligated to the vector as described above in 20 μl total volume. The whole ligation volume was transformed into 100 μl of XL10-Gold ultracompetent cells (Stratagene). A total of 1,200 recombinant clones (75%) were transferred to Hybond-N+ nylon filters (Amersham) and screened for $(CTT)_n$ motifs with a 5′-DIG labeled $(CTT)_8GC$ oligonucleotide.

Twenty-five positive clones were sequenced. Twenty-two of them contained $(CTT)_n$ motifs ($n \geq 5$), whereas the other 3 appeared to be false positives. Among the positive clones, 6 corresponded to the same or different allele variant of a previously sequenced clone. Primers were designed for 8 of the 16 different clones with PRIMER3 (Rozen and Skaletsky 1996; Table 1); the remaining 8 clones had flanking regions too short or unsuitable for primer design. One locus failed to amplify throughout all assayed samples. PCR reactions were performed in 20 μl mix containing 3–4 pmol each of the unlabeled forward and reverse primers, 1X *Taq* buffer (Promega), 2 mM $MgCl_2$, 0.4 mM of each dNTP, 1 U of *Taq* DNA polymerase (Promega), and approximately 20 ng DNA. The PCR program consisted of an initial denaturation step (94°C, 4 min) followed by 30 cycles (94°C, 1 min; annealing temperature, 1 min; and 72°C, 45 s) and a final extension step (72°C, 7 min). PCRs were carried out in a PE GeneAmp 9700 (Applied Biosystems).

Several groupings of primer pairs were performed in the

Table 1. Seven microsatellite loci in *Borderea pyrenaica*

Locus	Repeat motif	Primer sequence (5′–3′)	T (°C)	Size (bp)	N_A	Allele sizes (bp)	$H_{min–max}$	H_o	H_e	GenBank number
Bp126	$(CTT)_8$	F: NED-TTTGGCCGTACTTCCTCCACC R: CCATCTTCTTCTCATCACTACACACC	60	235	3	235, 238, 241	0.193–0.256	0.450	0.490	AY214383
Bp1286	$(CTT)_8$	F: HEX-GGACTTCGATGAGCTTTCTTTGGA R: TCCCAAACTACGTAGGAAACGATG	60	124	1	124	0	0	0	AY214384
Bp2214	$(CTT)_7$	F: HEX-CAGACATGAAGGACGATGACGA R: TCCAGAATCCCAGCATGAACAA	57	216	2	204, 216	0.007–0.009	0.017	0.017	AY214385
Bp2256	$(CTT)_8$	F: 6-FAM-GCTCAACTCAACACCCTTCCCTT R: CTAGGCCACTTATCCCTCCTCCT	57	235	2	220, 223	0.430–0.570	0–0	0–0	AY214386
Bp2290	$(CTT)_7$	F: NED-TCTGGAAGAGATGTTCATGTTGTTG R: TCTGAGCTCTTAGATGCCATTGTT	60	142	6	127, 130, 152, 155, 158, 161	0.632–0.693	0.250–0.467	0.289–0.487	AY214387
Bp2292	$(CTT)_7$	F: 6-FAM-AGGAGATGGTCATGGCTAATGGG R: CATCCAACACCTCATGAATCTCAAA	60	201	4	205, 211, 214, 217	0.628–0.663	—	—	AY214388
Bp2391	$(CTT)_8$	F: 6-FAM-GATTTCGGTAGCCCATCGCTC R: CCAATTCAATCAGAATCCGCAA	60	135	10	126, 129, 133, 136, 140, 146, 149, 153, 156, 159	0.645–0.677	0.017–0.750	0.017–0.764	AY214389
Mean (± SD)							0.36 ± 0.29–0.41 ± 0.31	0.217 ± 0.279	0.229 ± 0.290	
Mean of polymorphic loci (± SD)							0.42 ± 0.27–0.48 ± 0.28	0.325 ± 0.287	0.344 ± 0.295	

Repeat motif of the sequenced clone, 5′ fluorescent label of the forward primer, temperature of annealing used in the PCR, product size of the sequenced clone, number of alleles (N_A), and minimum and maximum ($H_{min–max}$) observed heterozygosities, calculated according to Palop et al. (2000) under the assumption of autotetraploidy. Observed and expected heterozygosities (H_o, H_e) calculated under the assumption of allotetraploidy for single chromosomal and double (separated by dashes) chromosomal loci. GenBank accession numbers are indicted for each locus. SD = Standard Deviation. HEX, NED, and 6-FAM are fluorescent dyes from Perkin Elmer–Applied Biosystems.

same PCR cocktail (multiplexing). The choice of the fluorescent-dye label for each microsatellite primer was based on its observed allele size range. Loci with overlapping alleles were multiplexed by labeling them with different fluorescent dyes, whereas amplified products with non-overlapping alleles were labeled with the same dye (Table 1). Loci were amplified in 60 individuals sampled from one population of *B. pyrenaica*. The products were run on an ABI 310 automated sequencer (Applied Biosystems), and the profiles were checked for reproducibility with those detected in acrylamide gels. Amplified fragment lengths were assigned to allelic classes with GENESCAN and GENOTYPER software (Applied Biosystems) using ROX-500 (Applied Biosystems) as the internal lane standard.

Results and Discussion

Six of the 7 microsatellite loci developed in *B. pyrenaica* were polymorphic, yielding a total of 28 alleles. The number of alleles in the polymorphic loci ranged from 2 to 10 (Table 1). Intriguing patterns of individual allele distribution suggested that *B. pyrenaica* is a tetraploid species (Figure 1), a finding previously documented for its congener, *B. chouardii* (Segarra-Moragues et al. 2003). Three (*Bp2290, Bp2292*, and *Bp2391*; Figure 1B,C,D) of the 6 polymorphic loci presented up to 4 alleles in some individuals, whereas the other 3 loci showed up to 2 alleles per individual. Fixed heterozygous diallelic profiles were observed for locus *Bp2256* (Figure 1E) in all studied individuals. Cross-amplifications of the 10 microsatellite loci characterized for *B. chouardii* (Segarra-Moragues et al. 2003) in this same *B. pyrenaica* sample set revealed similar results. Up to 4 alleles per individual were found for 8 loci (*Bc1145b, Bc1159, Bc1258, Bc1274, Bc1357, Bc1422, Bc1551*, and *Bc1644*); one locus (*Bc1169*) was monomorphic, and another one (*Bc166*) showed fixed diallelic heterozygosity (results not shown). In summary, individuals with up to 4 bands were found at 11 out of 17 screened microsatellite loci, whereas 2 loci showed fixed heterozygous profiles. This result constitutes a robust data set that does not comply with the hypothesis of isolate gene duplication reported in other typically diploid plant taxa (Zwettler et al. 2002).

In *B. pyrenaica*, microsatellite loci revealed higher levels of genetic diversity than allozymes (Segarra-Moragues and Catalán 2002) and levels similar to those of RAPDs (Segarra-Moragues and Catalán, 2003), despite the limited number of loci analyzed. A detailed study of the inheritance patterns of microsatellite alleles is currently underway. Here we have calculated heterozygosities under the assumption of two alternative hypotheses, autotetraploidy versus allopolyploidy (Table 1).

In autopolyploids, allele inheritance is tetrasomic because of the chromosome doubling of similar genomes (Stebbins 1950). In this case, since no information on the segregation of the progeny is available, the genotype of individuals presenting two or three bands for a given locus cannot be ascertained with full confidence. Hence, minimum and maximum observed heterozygosities were calculated according to Palop et al. (2000). The real value for this parameter (H_o) should fall between the interval of the minimum and maximum heterozygosities reported in Table 1. Among the polymorphic loci, minimum values ranged from 0.007 to 0.645, with a mean of 0.42, while the maximum ones varied from 0.009 to 0.693, with a mean of 0.48.

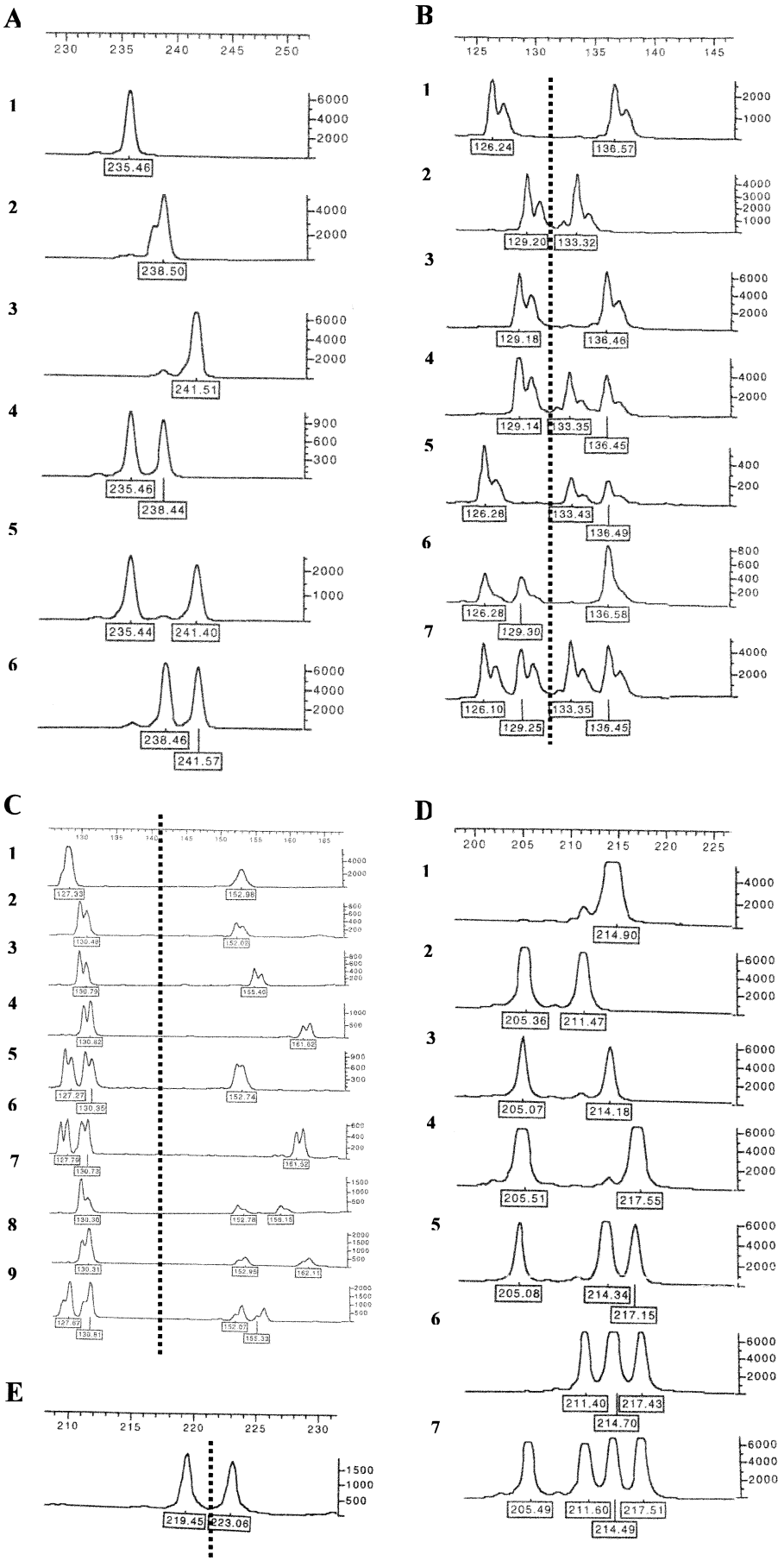
By contrast, in allopolyploids, inheritance is disomic, because of the addition of divergent genomes (Stebbins 1950). Under such a hypothesis, the alleles necessarily have to be assigned to their correspondent duplicated locus. This was successfully accomplished for six of the loci (three of them showed alleles from a single chromosomal locus, and another three showed alleles from two chromosomal loci each). There was only one case (locus *Bp2292*, Figure 1D) for which alleles could not be satisfactorily assigned to separate chromosomal loci. A total of nine loci were screened under this hypothesis, three of which were monomorphic. Among the polymorphic ones heterozygosity levels (H_o) ranged from 0.017 to 0.750, with a mean of 0.325. Observed and expected heterozygosities did not differ significantly (Table 1).

A first insight into the hybrid polyploid nature of *Borderea* was provided by allozymes (Segarra-Moragues and Catalán 2002). Fixed diallelic heterozygous profiles were observed for the two species in one variable enzymatic system (PGI, Figure 2) and for *B. pyrenaica* in the also variable IDH system. Allozyme PGI analysis in pollen grains of *Borderea* demonstrated that the two alleles found at the *PGI2* locus were coinherited in the same gamete (Segarra-Moragues and Catalán, unpublished results). The typical three-banded pattern was repeatedly recovered, rather than the two-banded profile that would be expected as consequence of the independent expression of each gene, in the gamete pool of the anther (Weeden and Gottlieb 1979). This result suggests a complete departure from Mendelian segregation and points toward a disomic inheritance of duplicated homozygous loci for these alternative allozyme alleles.

These previous findings based on allozymes have been corroborated by microsatellites. We have observed that

→

Figure 1. Sample electropherograms obtained with GENOTYPER for five microsatellite loci in *B. pyrenaica*. **(A)** Locus *Bp126*. Lanes 1–3 correspond to homozygous individuals; 4–6, to heterozygous individuals. **(B)** Locus *Bp2391*. Lanes 1–3 correspond to individuals with two alleles; lanes 4–6, to individuals with three alleles; lane 7, to one individual with four alleles. **(C)** Locus *Bp2290*. Lanes 1–4 correspond to heterozygous individuals with two alleles; lanes 5–8, to individuals with three alleles; lane 8, to one individual with four alleles. **(D)** Locus *Bp2292*. Lane 1 corresponds to one homozygous individual; lanes 2–4, to heterozygous individuals with two alleles; lanes 5–6, to individuals with three alleles; lane 7, to one individual with four alleles. **(E)** Locus *Bp2256*. All individuals studied are fixed for this heterozygous profile. Dashed lines separate allelic zones of the corresponding locus.


A
230 235 240 245 250
1
6000
4000
2000
235.46
2
4000
2000
238.50
3
6000
4000
2000
241.51
4
900
600
300
235.46
238.44
5
2000
1000
235.44
241.40
6
6000
4000
2000
238.46
241.57
B
125 130 135 140 145
1
2000
1000
126.24
136.57
2
4000
3000
2000
1000
129.20
133.32
3
6000
4000
2000
129.18
136.46
4
4000
2000
129.14
133.35
136.45
5
400
200
126.28
133.43
136.49
6
800
600
400
200
126.28
136.58
129.30
7
4000
2000
126.10
133.35
129.25
136.45
C
1
4000
2000
127.33
152.98
2
600
400
200
130.48
152.03
3
800
600
400
200
130.79
155.40
4
1000
800
130.62
161.62
5
900
600
300
127.57
130.36
152.74
6
600
400
200
127.78
130.73
161.32
7
1500
1000
500
130.30
152.78
159.15
8
2000
1500
1000
500
130.31
152.95
162.11
9
2000
1500
1000
500
127.67
130.61
152.67
155.33
D
200 205 210 215 220 225
1
4000
2000
214.90
2
6000
4000
2000
205.36
211.47
3
6000
4000
2000
205.07
214.18
4
6000
4000
2000
205.51
217.55
5
6000
4000
2000
205.08
214.34
217.15
6
6000
4000
2000
211.40
217.43
214.70
7
6000
4000
2000
205.49
211.60
217.51
214.49
E
210 215 220 225 230
1500
1000
500
219.45
223.06

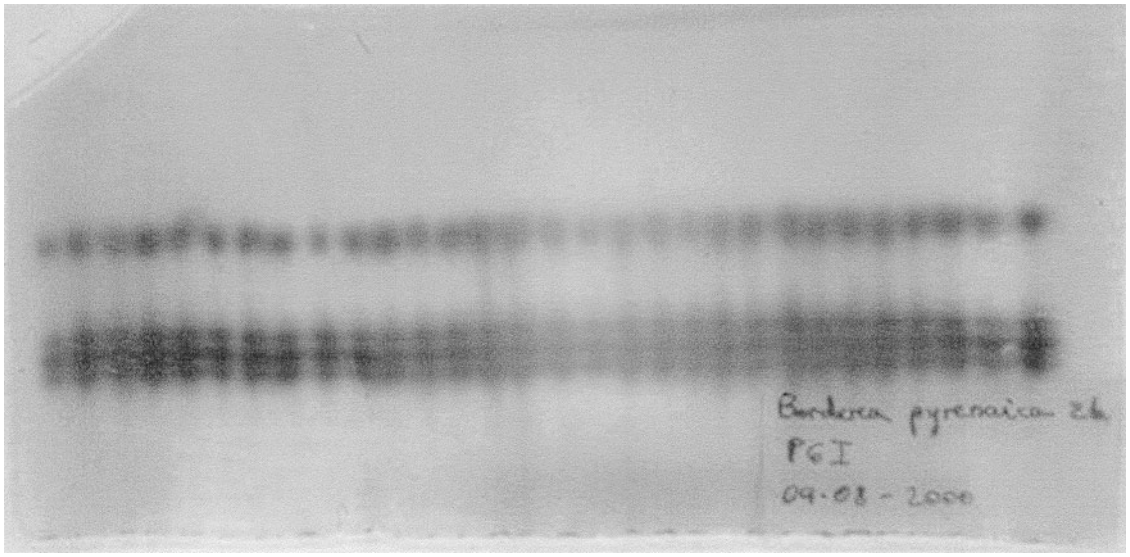


Figure 2. Sample of a PGI-stained gel of 30 individuals of *B. pyrenaica* showing the two enzymes of this system, PGI1, above, and PGI2, below. Note that all individuals at PGI2 are heterozygous, showing a typical three-banded profile.

alleles of variable loci with up to two bands (*Bp126*, Figure 1A, and *Bp2214*) are in Hardy–Weinberg equilibrium, suggesting that primers are amplifying single loci and that the duplicated ones would likely have been lost by the divergence of the primer target sequences. The fixed heterozygous profile of the *Bp2256* locus (and also that reported from the cross-amplification of *Bc166*) is interpreted as the amplification of two duplicate loci that are fixed for one allele each. The monomorphic *Bp1286* locus is better explained as the amplification of a single locus rather than the coincidence in size of two separate loci, given the high mutation rate of microsatellites. For two of the more variable loci (*Bp2290* and *Bp2391*), alleles could be easily attributed to their correspondent duplicated loci as their respective spanning-size ranges do not overlap; thus, two different allelic zones are distinguished for each of these loci (127–130 and 152–161 for *Bp2290*, and 126–129 and 133–159 for *Bp2391*; Figure 1B,C). All these loci were in Hardy–Weinberg equilibrium, and all of the 60 screened individuals had either one or two alleles for each one of these zones. However, individuals with only one allele were found at the variable locus *Bp2292* (Figure 1D, lane 1), which showed individuals with up to four alleles (Figure 1D, lanes 2–7), therefore bringing up the question on the polyploid nature and the inheritance patterns of *Borderea*. If the species is autopolyploid, individuals with one band are likely to occur as a consequence of random segregation of alleles from tetrasomic inheritance, whereas, if the species is allopolyploid and experiences disomic inheritance, individuals with a single band can be explained only by the coexistence of different alleles with equal sizes (size homoplasy; Estoup et al. 2002) or by the presence of null alleles in one or both loci. A more detailed study with a larger number of individuals and populations is currently underway, aiming to clarify the inheritance pattern of microsatellite alleles in *Borderea*. Nonetheless, our preliminary results, based on the finding of individuals with up to four alleles at 11 out of the 17 assayed microsatellite loci, and of two SSR and two allozyme loci with fixed heterozygous profiles, suit better the assumption of allotetraploidy.

The tetraploidy of both species of this relictual genus has been revealed for the first time through two different microsatellite analyses (Segarra-Moragues et al. 2003, and the current study). Because the chromosome counts given for the *Borderea* taxa are $2n = 24$ (Heslot 1953), our results indicate that *Borderea* should have a lower chromosome base number of $x = 6$, which was not previously recorded within the Dioscoreaceae ($x = 8, 9, 10, 12$; cf. Burkill 1960; Dahlgren et al. 1985; Huber, 1998), and support the hypothesis of a hybrid origin for the genus. In addition, these new sets of markers are valuable for obtaining a broader perspective on the genetic variability, genetic structure, and taxonomy of *Borderea*, and for inferring phylogeographic processes and differences in life histories among this palaeoendemic genus.

References

Burkill IH, 1960. The organography and the evolution of Dioscoreaceae, the family of the yams. Bot J Linn Soc 56:319–412.

Dahlgren RMT, Clifford HT, and Yeo PF, 1985. The families of the monocotyledons. Berlin: Springer-Verlag.

Doyle JJ, and Doyle JL, 1990. Isolation of plant DNA from fresh tissue. Focus 12:13–15.

Estoup A, Jarne P, and Cornuet JM, 2002. Homoplasy and mutation model at microsatellite loci and their consequences for population genetic analysis. Mol Ecol 11:1591–1604.

García MB, and Antor RJ, 1995. Age and size structure in populations of a long-lived dioecious geophyte: *Borderea pyrenaica* (Dioscoreaceae). Int J Plant Sci 156(2):236–243.

García MB, Guzman D, and Goñi D, 2002. An evaluation of the status of five threatened plant species in the Pyrenees. Biol Cons 103(2):151–161.

Gaussen H, 1952. Une nouvelle espèce de *Dioscorea* aux Pyrénées: *D. chouardii*. Bull Soc Bot France 99:23–25.

Gaussen H, 1965. Révision de *Dioscorea* (*Borderea*) Pyrénéens. Bull Soc Hist Nat Toulouse 100:392–398.

Heslot H, 1953. Le nombre chromosomique des Dioscoréacées pyrénéennes et leur rattachement au genre *Borderea* Miégeville. Compt Rend Acad Sci Paris 237:433–434.

Huber H, 1998. Dioscoreaceae. In: The families and genera of vascular plants, vol. 3, Monocotyledons, Lilianae (except Orchidaceae) (Kubitzki K ed). Berlin: Springer; 216–235.

Knuth R, 1924. Dioscoreaceae. In: Das Pflanzenreich, vol. 87 (IV) (Engler HGA ed). Leipzig: Engelmann; 1–387.

Palop M, Palacios C, and González-Candelas F, 2000. Development and across-species transferability of microsatellite markers in the genus *Limonium* (Plumbaginaceae). Cons Genet 1(2):177–179.

Rozen S, and Skaletsky HJ, 1996. Primer 3. Whitehead Institute for Biomedical Research, Cambridge, MA.

Segarra-Moragues JG, and Catalán P, 2002. Low allozyme variability in the critically endangered *Borderea chouardii* and in its congener *Borderea pyrenaica* (Dioscoreaceae), two paleoendemic relicts from the central Pyrenees. Int JPl Sci 163:159–166.

Segarra-Moragues JG, and Catalán P, 2003. Life history variation between species of the relictual genus *Borderea* (Dioscoreaceae): phylogeography, genetic diversity, and population genetic structure assessed by RAPD markers. Biol J Linn Soc 80:483–498.

Segarra-Moragues JG, Palop-Esteban M, González-Candelas F, and Catalán, P, 2003. Characterization of ten trinucleotide microsatellite loci in the critically endangered Pyrenean yam *Borderea chouardii* (Dioscoreaceae). Mol Ecol Notes 3(2):265–267.

Stebbins GL, 1950. Variation and evolution in plants. New York: Columbia University Press.

Terauchi R, and Konuma A, 1994. Microsatellite polymorphism in *Dioscorea tokoro*, a wild yam species. Genome 37:794–801.

Weeden NF, and Gottlieb LD, 1979. Distinguishing allozymes and isozymes of phosphoglucoisomerases by electrophoretic comparisons of pollen and somatic tissues. Biochem Genet 17:287–296.

Zwettler D, Vieira CP, and Schlötterer C, 2002. Polymorphic microsatellites in *Antirrhinum* (Scrophulariaceae), a genus with low levels of nuclear sequence variability. J Hered 93(3):217–221.

Received March 28, 2003
Accepted December 11, 2003

Corresponding Editor: James Hamrick