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PRIMER NOTE

Characterization of 10 trinucleotide microsatellite loci in the Critically Endangered Pyrenean yam *Borderea chouardii* (Dioscoreaceae)

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Abstract

The low levels of allozymic variability found in the Critically Endangered *Borderea chouardii* prompted us to develop microsatellite markers to assess the genetic variability and population structure for the adequate conservation management of this species. A $(CTT)_n$-enriched partial genomic library was constructed. Ten polymorphic microsatellite loci were isolated from it, rendering 51 alleles in 47 individuals analysed. The allelic pattern observed for all of the loci with more than two alleles suggests that *B. chouardii* is tetraploid.

Keywords: *Borderea choauardii*, conservation biology, enriched library, trinucleotide repeats

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Borderea Miégeville is a small genus of the Dioscoreaceae, endemic to the central Pyrenees. It comprises only two species, *B. pyrenaica* Miégeville and *B. chouardii* (Gaussen) Heslot. *Borderea chouardii* is only known from a single population at Sopeira (Huesca, Spain), located in the southern Prepyrenean mountain ranges (Moreno-Saiz 1990), with only a few hundred individuals counted (García 1996; García *et al*. 2001). Because it grows on limestone cliffs, most of the population is out of reach for collection. Its poor colonizing ability, due to biological and anthropogenic problems, further increases its risk of extinction and consequently it is considered as one of the most endangered species of the Iberian Peninsula, having been recently reclassified as 'Critically Endangered' (VVAA 2000).

This study is the first step towards future studies aimed at: (i) assessing levels of genetic variability and population structure in *B. chouardii*; (ii) checking whether the genetic depletion detected with allozyme markers (Segarra-Moragues & Catalán 2002) is a general feature of the *B. chouardii* genome or only a consequence of the technique employed; and (iii) addressing these results to the conservation plans of the species.

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DNA from 2 g of *in vitro*-cultivated individuals was extracted following the cetyltrimethyl ammonium bromide protocol (Doyle & Doyle 1990) to screen for microsatellites. After digestion with *Sau*3A, fragments in the 300–900 bp range were ligated into dephosphorylated *Bam*HI-digested pBluescript II (+/–) vector. The construction of the enriched library followed a modification of Kijas *et al*. (1994).

Single-stranded copies of inserts were obtained by asymmetric polymerase chain reaction (PCR; 10× pmol of forward primer/reverse primer) and bound to streptavidin-coated M-280 magnetic beads (Dynal) following the manufacturer's instructions. After five washes in solutions of decreasing concentrations of SSC (2×, 1× and 0.5×), 10 µL of this enriched DNA were used as template for a symmetric PCR, containing 50 pmol each of T_3 and T_7 pBluescript primers. The PCR products were purified (QIAquick; Qiagen) and digested with *Sau*3A. Fragments ranging from 400 to 900 bp were isolated, purified and ligated to the vector. The whole ligation volume (20 µL) was transformed into 100 µL of XL10-Gold ultracompetent cells (Stratagene). A total of 700 recombinant clones (35%) were transferred to Hybond-N+ nylon filters (Amersham) and screened for $(CTT)_n$ motifs with a 5′-digoxigenin-labelled $(CTT)_8$GC oligonucleotide.

Forty-seven positive clones, all containing $(CTT)_n$ motifs ($n \geq 7$), were sequenced yielding 26 different clones.

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Table 1 Ten microsatellite loci in *Borderea chouardii*

Locus	Repeat motif	Primer sequence (5′–3′)	Label	T (°C)	Size (bp)	N_A	H_{Min}	H_{Max}	GenBank Accession no.
Bc1145b	$(CTT)_8$	F: TCTAATCCACTATGTCTCCTCC R: TTACTGCGACTTCTGGTGCTCTT	HEX	60	109	2	0.155	0.206	AY116172
Bc1159	$(CTT)_8$	F: TTCTTCCGCCGTGAGACCTCC R: TCCAGAAGCTTACCTTCCTAGCTC	6-FAM	60	133	4	0.561	0.611	AY116173
Bc1169	$(CTT)_{14}$	F: GGTCTCCAGCCATTAGAAACAA R: TCGATATTGTGAAACGAGAGCA	NED	60	154	4	0.501	0.606	AY116174
Bc1258	$(CTT)_{12}$	F: ATTCTCCTGCTCCTCCTCCACC R: CCTTCCTTCATTCTTGCTGTCTT	6-FAM	60	183	7	0.610	0.675	AY116175
Bc1274	$(CTT)_{12}$	F: ATAATCCACCGCCCTGTAAACC R: CCCTTTCTATCTCTATTTCAACCC	6-FAM	55	273	5	0.591	0.657	AY116176
Bc1357	$(CTT)_{10}$	F: CTCAACGGCCACGCTCTCTT R: ACCACCACCTCCTCATCCTCTT	NED	63	138	6	0.561	0.635	AY116177
Bc1422	$(CTT)_7$	F: CTCAGGCTTCTTGTGCTGCTT R: AATTACACCACTCTCACCATTACC	NED	60	162	5	0.507	0.609	AY116178
Bc1551	$(CTT)_{13}$-$(CAA)_5$	F: CACTTTTATTTCACTCATCCCTATC R: ATATGCAAAGCCACAGTAACCA	NED	58	279	2	0.046	0.061	AY116179
Bc1644	$(CTT)_{14}$	F: GCCTTCCATTTTGTGTCTCTTTGG R: GTTGAGCAGGGAGCTTGGACTT	HEX	60	181	4	0.064	0.085	AY116180
Bc166	$(CTT)_{13}$	F: ACAGAGAGAGTTAACAAGACAACAAC R: GGTGAGGACTGACAGGATAGAGAAGA	HEX	60	203	12	0.597	0.663	AY116181
Mean ± σ							0.42 ± 0.22	0.48 ± 0.24	

Repeat motif of the sequenced clone, forward and reverse primers, 5′ fluorescent label (forward primer), temperature of annealing used in the polymerase chain reaction (T), product size of the sequenced clone, no. of alleles (N_A), minimum (H_{Min}) and maximum (H_{Max}) observed heterozygosity and GenBank Accession no. are indicated for each locus.
HEX, 6-FAM, NED,

Primers could be designed for 20 clones using PRIMER 3 (Rozen & Skaletsky 1996). The remainder had too short or unsuitable flanking regions for primer design. The 10 loci with the best primer pair parameters were selected (Table 1) and tested across 47 individuals sampled from the only extant population of *B. chouardii*.

The PCR reactions were performed in a 20 µL mix containing 3–5 pmol each of the unlabelled forward and reverse primers, 1× *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 programme consisted of an initial denaturation step of 94 °C for 4 min followed by 30 cycles of 94 °C for 1 min, 1 min at the annealing temperature and 72 °C for 45 s and a final extension step of 72 °C for 7 min. The PCRs were carried out in a PE GeneAmp 9700 (Applied Biosystems). Independent PCR products for each primer pair were electrophoresed in 8% acryl-bisacrylamide sequencing gels and visualized by silver staining. Alternatively, several groupings of primer pairs in the same PCR cocktail (multiplex) were performed, allowing their combination in the same PCR reaction according to the product sizes and the fluorescent dye used (Table 1). These 10 loci were amplified in all of the samples and their products were run on an ABI 310 automated sequencer (Applied Biosystems). The corresponding electropherogram profiles were checked for reproducibility with those obtained with the acrylamide gels. Fragment lengths were assigned with GENESCAN and GENOTYPER software (Applied Biosystems) using ROX-500 as the internal lane standard.

The microsatellite loci revealed higher levels of polymorphism (0.42–0.48) than allozymes (0.12) (Segarra-Moragues & Catalán 2002) providing a total of 51 alleles (Table 1). The 10 loci were polymorphic, with the number of alleles ranging from two to 12. The peak patterns observed in all of the loci with more than two alleles (except for Bc1644 which was nearly fixed for the most common one) suggest that *B. chouardii* is a tetraploid species. As 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 heterozygosities were calculated following Palop *et al*. (2000). Most loci show similar heterozygosity levels ranging from 0.501 to 0.610 and 0.606 to 0.675 minimum and maximum heterozygosity values, respectively. These values are not correlated with the number of alleles present in each locus. Only three loci, Bc1145b, Bc1551 and Bc1644, detected

lower levels of heterozygosity. These 10 loci are, therefore, suitable for a broader population genetic structure study, currently underway, of this Critically Endangered species.

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