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# Characterization of microsatellite loci in the endangered species of fern *Marsilea strigosa* Willd. (Marsileaceae, Pteridophyta)

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## Abstract

**Absence of enzymatic polymorphism in the endangered species of fern *Marsilea strigosa* prompted us to develop microsatellite loci in this species. We used an enriched partial genomic library, from which six polymorphic microsatellite loci were obtained. From a previous attempt to develop microsatellite markers with standard techniques, a single long and polymorphic locus was obtained. This highlights the benefits from using enrichment techniques in those species where microsatellite loci may be rare in the genome.**

*Keywords:* conservation biology, dinucleotide repeats, enriched library, *Marsilea strigosa*, microsatellites

*Marsilea strigosa* Willd. (Marsileaceae, Pteridophytae) is a rare aquatic fern found in the Mediterranean basin and in the north of the Volga delta (Tutin *et al.* 1964). This species is listed in the French Red Book of endangered species (Olivier *et al.* 1995) and in the European 'Habitat' directive. This species completes its life-cycle in temporary ponds (Colas *et al.* 1996). Understanding the dispersal capacity and the actual reproductive system of this species may give important information for conservation and management purposes. The complete absence of enzymatic polymorphism (Vitalis *et al.* 1998) prompted us to identify microsatellite loci.

A partial genomic library was enriched with (CT)<sub>n</sub> and (GT)<sub>n</sub> microsatellite motifs following a modified protocol of Kijas *et al.* (1994). Fragments ranging from 400 to 900 bp of *Sau3A*-digested genomic DNA were ligated into dephosphorylated, *Bam*HI-digested pUC19 vector (Appligene). Single-stranded copies of inserts were obtained by asymmetric polymerase chain reaction (PCR), in 50 µL mix containing 12.5 pmoles of modified M13 Forward primer (5'-GTTTCCCAGTCACGACGTTG-3'), 0.5 pmoles of modified M13 Reverse primer (5'-TTGTGAGCGGA-

TAACAATTC-3'), 0.2 mM of each dNTP, 1.5 mM MgCl<sub>2</sub>, 2.5 units of *Taq* polymerase (Goldstar, Eurogentec), 75 mM Tris-HCl pH 9.0, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% (w/v) Tween 20. PCR reaction was performed using a PTC-100 thermocycler (MJ Research Inc.): initial denaturation was at 94 °C for 4 min, followed by 30 cycles (94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min) and 4 min at 72 °C. Three hundred pmoles of 5'-biotinylated oligonucleotides ATA-GAATAT(CT)<sub>15</sub> and ATAGAATAT(GT)<sub>15</sub> were attached separately to 60 µg of streptavidin-coated magnetic beads (MagneSphere, Promega), in 60 µL of 5× SSPE (20× SSPE: 3 M NaCl, 200 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM EDTA, pH 7.4) for 15 min at room temperature. The beads were washed five times in 100 µL 5× SSPE to remove unbound oligonucleotides, and were resuspended in 50 µL of 10× SSPE. Thirty-five µL of denatured (98 °C for 10 min) single-stranded inserts were hybridized to 50 µL of beads-bounded oligonucleotides at 55 °C overnight in 100 µL of a solution of 1 M NaCl. The beads were washed four times in 100 µL of 2× SSPE at room temperature, and four times in 100 µL 2× SSPE at 55 °C to remove unbound inserts. They were resuspended in 20 µL of H<sub>2</sub>O and bound sequences were eluted at 98 °C for 10 min. Ten µL of this enriched DNA was used as a template in PCR (see asymmetric PCR conditions above, with 12.5 pmoles of both pUC19 primers, same thermal profile). PCR products were purified (QIAquick, Qiagen) and digested with *Sau3A*. Fragments ranging from 400 to

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**Table 1** Seven microsatellite loci in *Marsilea strigosa*. For each locus, the core sequence, the size of the product (known from sequenced clones) obtained from amplification with the given forward (F) and reverse (R) primers and the GenBank accession nos are indicated. Annealing temperatures used for PCR are also given, as well as the number of alleles ( $N_A$ ), the observed heterozygosity ( $H_O$ ) and Nei's expected heterozygosity (Nei 1973) under random mating within subpopulations ( $\bar{H}_S$ )

Locus	Core sequence	Primer sequences (5'-3')	T (°C)	Size (bp)	$N_A$	$H_O$	$\bar{H}_S$	Accession no.
IA10	(CT) <sub>26</sub>	F: AGAGATGCTTAACAAAGTCC R: TATAGCATTTCGGTTAGAGAG	55	100	11	0.03	0.16	AF317636
IE3	(GT) <sub>9</sub> TT(GT) <sub>7</sub>	F: GATCGGTATTTTATATGTG R: TATCCAGCAACTACAATAC	47	102	2	0.00	0.00	AF317637
IF7	(AG) <sub>22</sub>	F: GCCCAAATTTGGAGCCTAAT R: AGCCTATGATTGAGTCACCACA	62	124	9	0.01	0.16	AF317638
IIIB8	(AG) <sub>24</sub>	F: GTTGAACCACTTCACATTTTCCC R: ATAGCTTCCTAGAATCCCAGGC	56	135	8	0.04	0.18	AF317639
IIIC8	(CT) <sub>21</sub>	F: GCGTTGTGTACTGTTCAAGACC R: TGCATGTAAATTGCCCTGAA	64	111	6	0.01	0.20	AF317640
IVH5	(AC) <sub>24</sub> AA(AC) <sub>10</sub>	F: GATCACATAGACGAGACAC R: AACATGTTGTGAAGAATTC	50	173	7	0.00	0.13	AF317641
XIIE3	(AG) <sub>40</sub>	F: CGAGATCTCTCACATCTACACA R: AGCAATCATTGTGTAAGTCA	58	192	16	0.03	0.28	AF317642

900 bp were isolated, purified (GeneClean Kit, Bio101) and ligated into pUC19 using a 3:1 insert:vector molar ratio. The ligation was performed in 30 µL with 115 ng vector, approximately 90 ng purified PCR product and 3 units T4 DNA ligase (Biolabs). Approximately 50 ng of the ligation was transformed into 100 µL of ultracompetent cells (Epicurian Coli XL1-Blue, Stratagene). A total of 368 recombinant clones (from approximately 1500 obtained) were screened for (CT)<sub>n</sub> and (GT)<sub>n</sub> motifs, according to the PCR-based method described by Waldbieser (1995).

Thirty-one positive clones were sequenced. Among those, nine contained long (CT)<sub>n</sub> or (GT)<sub>n</sub> motifs ( $n \geq 15$ ), 19 contained short or degenerated tandem repeats and three appeared to be false positives. Nine pairs of primers were defined. Six of them gave clear amplification products (loci IA10 to IVH5) with the PCR conditions given in Table 1. A partial genomic library was also constructed with standard protocols (see, e.g. Estoup *et al.* 1993) and screened for (CT)<sub>n'</sub>, (GT)<sub>n'</sub>, (CAC)<sub>n'</sub>, (CTC)<sub>n'</sub>, (CTAT)<sub>n'</sub>, (TGTA)<sub>n</sub> and (GACA)<sub>n</sub> motifs. Among 1692 recombinant clones, a single (CT)<sub>n</sub> microsatellite locus was found (locus XIIE3).

PCR reactions were performed in a 10-µL volume containing approximately 10 ng DNA, 0.3 pmoles of [<sup>33</sup>P]-dATP labelled forward primer, 4 pmoles of (unlabelled) reverse primer, 0.2 mM dNTP, 1.5 mM MgCl<sub>2</sub>, 0.5 units of *Taq* polymerase (Goldstar, Eurogentec), 75 mM Tris-HCl pH 9.0, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% (w/v) Tween 20, using a PTC-100 thermocycler (MJ Research Inc.): initial denaturation was 4 min at 94 °C followed by 30 cycles (1 min at 94 °C, 1 min at annealing temperature, 1 min at 72 °C) and 4 min at 72 °C. PCR products were electrophoresized in 6% acryl-bisacrylamide and 8 M urea sequencing gels.

DNA from 138 individuals sampled in seven populations in Spain and two populations in France was extracted (CTAB 2%, Tris-HCl 100 mM, EDTA 20 mM, NaCl 1.4 M, β-mercapto-ethanol 0.2% v/v) and scored for polymorphism at the seven microsatellite loci. Highly significant differentiation was detected within populations (Fisher exact tests,  $P < 0.0001$ ) for all loci but IE3, for which the test could not be performed (GENEPOP 3.2a; Raymond & Rousset 1995). These departures from Hardy-Weinberg equilibrium are interpreted as being the consequence of high selfing rates. Finally, it is noteworthy that enrichment techniques may be a valuable approach in those species where microsatellites are scarce in the genome.

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