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PRIMER NOTE

New microsatellite markers for the endemic Mediterranean seagrass *Posidonia oceanica*

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Abstract

The seagrass *Posidonia oceanica* is endemic to the Mediterranean Sea, where it plays an important role in coastal ecosystem dynamics. Because of this important role and concerns about the observed regression of some meadows, population genetic studies of this species have been promoted. However, the markers used until now were not polymorphic enough to efficiently assess the level and spatial pattern of genetic variability. Hypervariable molecular markers were obtained by screening a genomic library enriched for microsatellite dinucleotide repeats. Among 25 primer pairs defined, eight amplified polymorphic microsatellites with an encouraging level of variability at the two geographical scales sampled.

Keywords: clonal plant, dinucleotides, microsatellites, *Posidoniaceae*, seagrass

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The endemic seagrass *Posidonia oceanica* plays an important role in coastal ecosystem dynamics in the Mediterranean Sea. However, anthropogenic perturbations such as pollution, modifications of coastal areas or introduction of new species have led to widespread regression of the species (e.g. Duarte 2002). The threats to this species have led to an interest in genetic studies, aimed at elucidating the genetic variability and the level of gene flow within and between populations, as a basis for conserving and managing *P. oceanica* along Mediterranean coasts.

Posidonia oceanica is a clonal plant that can reproduce either sexually by seed production or asexually by vegetative propagation of rhizomes. The first studies based on allozymes and RAPD techniques revealed very low polymorphism, preventing the efficient study of genetic variability and gene flow (Capiomont *et al*. 1996; Procaccini & Mazella 1996). Six polymorphic microsatellites were isolated (Procaccini & Waycott 1998), mostly presenting trinucleotide (but sometimes irregular) motifs, which were limited in their capacity to resolve genetic structure relative to that expected from hypervariable markers (Procaccini & Mazzella 1998; Procaccini *et al*. 2001). In order to determine whether the low level of genetic variability of the species suggested by the use of these markers is unambiguously attributable to a biological feature of this clonal species, or, rather, reflects the limitations of the microsatellites isolated, we isolated new microsatellites with dinucleotide repeats.

Genomic DNA was isolated from a fresh meristematic portion of *P. oceanica* leaves, following standard cetyltrimethyl ammonium bromide (CTAB) extraction procedures, and was digested with *Afa*I (*Rsa*I) (Amersham Pharmacia Biotech). Total digested DNA was purified and ligated into annealed *Afa*I adaptors (AdapF: 5′-TCTTGCTTACGCGTGGACTA-3′ and AdapR: 5′-TAGTCCACGCGTAAGCAAGAGCACA-3′).

The enrichment procedure used streptavidin-coated magnetic particles (Magnesphere, Promega) and followed the protocol from Billote *et al*. (1999) based on modifications from the original (Kijas *et al*. 1994). A 5′-biotinylated with a probe 3′-dideoxyC was used to avoid production of chimeric clones (Koblízková *et al*. 1998). The enriched single-stranded DNA was amplified by polymerase chain reaction (PCR) using the AdaptF as a primer to recover double-stranded DNA. The double-stranded DNA was quantified and ligated into the pGEM-T Easy vector (Promega). The ligation was transformed into *Escherischia coli* cells (strain DH5α). About 730 colonies containing an insert of *P. oceanica* were transferred from the agar plates

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Table 1 Characterization of eight microsatellite loci, including locus name, repeated microsatellite motif, GenBank accession number, annealing temperature (T_a), approximate allele size (bp) and number of alleles detected on a sample of nine populations, number of alleles and expected (H_E) and observed (H_O) heterozygosity estimated in one natural population are given for each locus

Locus	Microsatellite motif	Primer sequences (5′–3′)	GenBank accession no.	T_a (°C)	$MgCl_2$ (μM)	Approx. size range of alleles (bp)	No. of alleles in 9 meadows of *P. oceanica*	No. of alleles in Formentera meadows	H_E	H_O
Po–5	$(GA)_{20}$	CACAATGGCCCGGTAGCA GTGGTTGCGTGCCTTCGGTTG	AF547650	64–52	2.5	164–188	6	4	0.43	0.48
Po5–10	$(GA)_{13}$	ATGAGACTCCCACAATAACA CATGGGAAGGTATAGAAGC	AF547651	55	1.5	151–179	8	4	0.60	0.94
Po5–39	$(GA)_{13}$	CATTTGGCTGAGTCCCTTTC GTCCAAGGCTTCCGTGATGG	AF547652	54	1.5	170–182	6	5	0.57	0.37
Po5–40	$(GA)_{24}$	AAAACCAACCCCCACGATAAG AATCCAAAGGAACGACACTCA	AF547653	55	1.5	186–258	21	8	0.75	0.80
Po5–49	$(GA)_{16}$	GGCTCGATGGTGCATTTCAGC GCCATTCTCCGCTCTGCTCC	AF547654	55	1.5	218–242	8	2	0.11	0.11
Po15	$(GA)_{20}$	AAGCACGCCGCTTAAACCATA CATGTTAGTAGGCAATATACTAGGC	AY152814	55	1.5	137–171	13	3	0.57	0.77
Po4–3	$(GA)_{10}$	ACAGAAACTACGAACCATCAG TAAGGAGAAGGAGAAGGAAA	AF547656	51	1.5	158–178	7	7	0.69	0.74
Po4–36	$(AG)_7N(AG)_{22}$	ATGAAATAGAGAGCAAGTCA TAAGCAGACATAAAGAGAGG	AF547657	55–50	1.5	185–197	4	3	0.21	0.17

into microplates containing 150 μL LB-Ampicilin solution, and were incubated for 4 h at 37 °C. Subsequently, the grown colonies were diluted 5× in Sigma Water on new microplates and heated for 10 min to induce cell lysis. The size of inserts was estimated by agarose gel electrophoresis of PCR products of standard M13 and reverse primers flanking pGEM-T easy polylinker. Gels were photographed and Alkaline Southern transferred onto nylon membranes (Hybond-N; Amersham Pharmacia Biotech). Hybridization was carried out with a ^{32}P-radiolabelled $(CT)_{15}$ probe (Cregan *et al*. 1994). About 20% of the clones sequenced contained microsatellites. Plasmids from the positive clones were extracted using the Wizard *Plus* miniprepDNA (Promega) purification system. From these, 73 were sequenced commercially (MWG-Biotech AG) and 25 primer pairs were designed using PRIMER 3 software (Rozen & Skaletsky 2000).

Microsatellite polymorphism was analysed by specific PCR at two population levels. Forty individuals randomly collected inside an apparently homogeneous 800 m^2 area of a meadow located at 10 m depth in the island of Formentera (Balearic Islands, Spain) were first analysed to assess the level of intrapopulation variability (number of alleles, heterozygosity and clonal resolution), and then five samples from nine geographically separated sites (from Greece to Spain) were screened to estimate allelic diversity across a broader geographical range.

Primers were first tested using touchdown PCR (described below), and the same PCR conditions as those described above were used for amplifying samples from natural populations, except specific T_a optimization for some of those nine loci (Table 1). The 10 μL PCR reaction mixture contained 10–50 ng of template DNA, 1 μM of each locus specific primer (Table 1), 60 μM of each dCTP, dGTP and dTTP, 10 μM of dATP and 0.08 μL of [α^{35}S]-dATP (12.5 mCi/mL, 1250 Ci/nM), 2.5 mM of $MgCl_2$ (see Table 1 for locus optimizations), 1 μL of 10× *Taq* DNA polymerase PCR buffer (200 mM Tris-HCl pH 8.4), 500 mM KCl and 0.5 units of *Taq* DNA polymerase (Invitrogen, Life technologies). After a denaturing step of 4 min at 94 °C, samples were processed through 24 cycles consisting of 30 s at 94 °C, 30 s at 64 °C (reduced by 0.5 °C each subsequent cycle) and 30 s at 72 °C, and 10 additional cycles consisting of 30 s at 94 °C, 30 s at 52 °C and 40 s at 72 °C. A final elongation step was performed at 72 °C for 10 min. All PCRs were performed on a GeneAmp thermocycler 9700 (PE Applied Biosystems). PCR products were fractioned over a denaturing 6% acrylamide-bisacrylamide gel and visualized using autoradiography.

Of the 25 loci isolated, eight were polymorphic, exhibiting from two to 12 alleles (Table 1). The number of alleles and the level of heterozygosity were relatively high for most of the markers (Table 1) and allowed detection of 24 genets among the 35 samples analysed from the natural population of Formentera. No deviation from Hardy–Weinberg equilibrium and no linkage disequilibrium were detected when excluding from the sample the replicates of the same clone (i.e. ramets issued of the same genet).

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