Molecular Ecology Notes (2003) **3**, 397–399 doi: 10.1046/j.1471-8286.2003.00463.x

PRIMER NOTE

Isolation and characterization of microsatellite markers for the seagrass *Cymodocea nodosa*

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Abstract

In order to study the spatial patterns of genetic diversity of a clonal marine angiosperm, the seagrass *Cymodocea nodosa*, microsatellite markers were obtained by screening a genomic library enriched for the (CT) dinucleotide motif. Of 38 primer pairs defined, 15 amplified polymorphic microsatellites and are described. These loci identified a number of alleles ranging from two to seven, and showed expected heterozygosity ranging from 0.35 to 0.76, when a group of 40 individuals from Cadiz Bay in Spain was analysed. Additionally, we describe here the multiplexing conditions for 12 of these loci.

Keywords: clonal plant, *Cymodocea nodosa*, microsatellites, seagrass

Received 27 February 2003; revision accepted 2 April 2003

Seagrasses are marine angiosperms that reproduce sexually by means of hydrophilous pollination (all except one species) and asexually by rhizomatous vegetative growth. They form intertidal and subtidal meadows that are essential components of the biological and physical structures and processes of coastal areas (Hemminga & Duarte 2000). *Cymodocea nodosa* is a dioecious seagrass widely distributed in the Mediterranean, and also occurring along the Southern Portuguese and Northwest African coasts (den Hartog 1970). It exhibits an extensive morphological plasticity, growing on both sand and mud substrates, and it can be found from the upper subtidal limit to depths of more than 30 m. The vegetative growth habit of *C. nodosa* renders it the species of choice for seagrass demography research (Duarte *et al*. 1994; Vidondo *et al*. 1997). Random amplified polymorphic markers (RAPD) have been used to study the genetic diversity in the Mediterranean island of Ischia, Italy (Procaccini & Mazzela 1996) and in the Ria Formosa, Portugal, an Atlantic biogeographically-isolated coastal lagoon (Alberto *et al*. 2001). Theses studies showed contrasting patterns, from high genetic diversity in the former to almost complete monomorphism in the latter. However, due to the dominant nature of RAPD markers, it is not possible to calculate the probability of a given multilocus genotype occurring by chance as a consequence of sexual recombination. Only with hyper-variable molecular markers, such as microsatellites, can these probabilities be assessed and the genetic individuality in clonal plants elucidated. The development of microsatellite markers in *C. nodosa* therefore opens the possibility of assessing patch dynamics, clonal structure, biogeographical patterns and gene dispersion of this species.

Genomic DNA was isolated from a fresh meristematic portion of *C. nodosa* leaves following standard cetyltrimethyl ammonium bromide (CTAB) extraction procedures, and digested with *Afa*I(*Rsa*I) (Amershan Pharmacia Biotech). Total digested DNA was purified and ligated to annealed *Afa*I adaptors (AdapF: 5′-TCTTGCTTACGCGTGGACTA-3′ and AdapR: 5′-TAGTCCACGCGTAAGCAAGAGCACA-3′). The enrichment procedure followed the protocol of Billote *et al*. (1999), which uses streptavidin-coated magnetic particles and biotinylated probes (Magnesphere, Promega), and is based on modifications of the original method of Kijas *et al*. (1991). We used a 5′-biotinylated $(CT)_{15}$ probe, with a 3′-dideoxyC end, following suggestions made by Koblízková *et al*. (1998), to avoid producing chimeric clones. The enriched ssDNA was amplified by polymerase chain reaction (PCR) using the AdaptF as a

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Table 1 Fifteen *Cymodocea nodosa* microsatellite loci. Locus name, primers, motif repetitions, multiplex reaction code and dye (five multiplexes are described using a total of 12 loci; the three loci left were screened using autoradiography methods), and annealing temperature (TD: touchdown PCR), $MgCl_2$, number of alleles detected in Cadiz (and total alleles when extra samples were analysed), approximate allele size range (bp), observed (H_O) and expected (H_E) heterozygosity in the Cadiz bay seedling population. The GenBank accession numbers for the cloned sequences are provided

Locus	Primer Sequences (5′–3′)	Repeat motif	Multiplex/dye anneal. temp (°C)	$MgCl_2$ (mM)	No. of alleles in Cadiz (total no. alleles)	Approx. size range of alleles (bp)	H_O	H_E	GenBank accession number
Cn2–14	CTCGTGTTTTATTTCTTGCT CAAGATTCATTTAAGAACGG	$(GA)_{13}$	M1/6-FAM/59	1.5	2[4]	223–238	0.40	0.46	AY162190
Cn2–16	AGGCATGGCGGAGTTG ATGTTTCATTTAGGGTTTTAACTTTTT	$(GA)_{19}$	M2/HEX/TD[55–50]	2	5[5]	96–104	0.78	0.69	AY162191
Cn2–18	TTATTCTGCCTCCCC GTACGATGGGATTCAATA	$(GA)_{14}.(AG)_{11}(TG)_9$	M2/6-FAM/TD[55–50]	2	2[4]	108–114	0.35	0.35	AY162192
Cn2–24	AGGATGGTAGTCTGGG TTGAGATGGGTAGTCTGT	$(GA)_{29}$	–/–/TD[55–50]	2	7[7]	169–203	0.69	0.73	AY162193
Cn2–38	CCAAGACAAAAAGGCAAA AACGAGTGATTAGAAGAAAGGA	$(GA)_{21}$	M3/HEX/TD[55–50]	2	4[5]	182–202	0.60	0.67	AY162194
Cn2–44	CGAGAAAGAGGAGCAG CATCACCCGAGAAGAG	$(GA)_{16}.(GA)_{14}$	M1/HEX/59	1.5	3[4]	234–264	0.50	0.58	AY162195
Cn2–45	CGTGTTTCTTTCTTCTTTC GCTTTGAACCAAATCCT	$(GA)_{25}$	M2/6-FAM/TD[55–50)	2	3[5]	212–228	0.62	0.66	AY162196
Cn2–86	GACGATGCTTACATA TACGATCAACCCAACC	$(GA)_{23}$	M3/6-FAM/TD[55–50]	2	5[6]	125–155	0.45	0.56	AY162197
Cn3–22	CAGTAATAATCTCTTTGGAGGA TTACTACCACCACAACAACA	$(GAA)_5(GCA)_5$ … $(GAA)_{10}(TAG)_5$	M3/NED/TD[55–50]	2	3[3]	138–144	0.38	0.46	AY162198
Cn4–5	ATGGGAGGGAGGAAGACG GTGTCATGCACGCCATCC	$(GA)_{15}$	–/–/58	2	5[5]	90–106	0.83	0.71	AY173121
Cn4–6	TACCTTCTCGCTTTCTTT TTTTGAAGCCCATCC	$(AG)_{14}$	M4/HEX/TD[55–50]	2	5[5]	273–279	0.35	0.52	AY162206
Cn4–19	AGAGGAGGATTTTCGG TTTTCCACTTCTCTCGTT	$(GA)_{20}$	M4/NED/TD[55–50]	2	7[7]	226–258	0.85	0.76	AY162200
Cn4–27	GTTCTCCCTGGCCTTGTT TTGTTTAGACTTTTCTTCATCGT	$(GA)_{14}$	m5/6-fam/57	1.5	3[3]	345–351	0.50	0.49	AY162201
Cn4–29	TTTGGCTGAGGGAGACCG TCTCGCCTCGCTTTATTTCT	$(GA)_{13}$	m5/ned/57	1.5	3[5]	193–203	0.50	0.49	AY162202
Cn4–35	TTTCACTTGTTACTGCCACC CTTTCCTTCCTTCCCAGA	$(GA)_{12}$	–/–/TD[55–50]	2	2[2]	158–162	0.34	0.38	AY162203

primer to recover double-strand DNA. This was ligated into pGEM-T Easy vector (Promega) and transformed into DH5α competent cells. A total of 736 positive clones were transferred to microplates containing 150 μL LB/Ampicillin solution, incubated (4 h, 37 °C), diluted 5× in ultrapure water (Sigma) and heated (10 min) to provoke cell lysis. Insert sizes were estimated by agarose gel electrophoresis of PCR products using standard M13 primers. Gels were alkaline Southern transferred to Hybond N+ nylon membranes (Amersham) and hybridized with a ^{32}P radiolabelled $(CT)_{15}$ probe. About 27% of the clones contained a microsatellite region. From these, 95 were sequenced (MWG-Biotech AG) and 38 primer pairs were designed.

In order to test the obtained markers and to avoid sampling redundancy (expected when sampling clonal species), we used 40 *C. nodosa* seedlings collected haphazardly at Cadiz Bay, southwest Spain. PCR reactions in 15 μL contained approximately 20 ng DNA, 1 μM each primer (Table 1), 60 μM each of dCTP, dGTP and dTTP, 10 μM dATP and 0.08 μL [α^{35}S]-dATP (12.5 mCi/mL, 1250 Ci/nM), 2.5 mM $MgCl_2$ (see Table 1 for locus optimizations), 1 μL 10× PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl) and 0.5 U *Taq* DNA polymerase (Invitrogen, Life Technologies). Cycling conditions consisted of an initial denaturing step of 4 min at 94 °C, followed by 24 cycles of 'touchdown' PCR 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, 10 additional cycles consisting of 30 s at 94 °C, 30 s at 52 °C and 40 s at 72 °C, and a final elongation step at 72 °C for 10 min. All PCR reactions were performed on a GeneAmp 9700 thermocycler (PE Applied Biosystems). PCR products were separated on a denaturing 6% acrylamide-bisacrylamide gel and visualized using autoradiography. Fifteen loci produced clear amplification products and were polymorphic. Twelve loci were then optimized for multiplexing in an ABI 377 sequencing system (PE, Applied Biosystems) (Table 1). No linkage disequilibrium was detected among these loci (option 2.1 in GENEPOP). The *C. nodosa* seedlings exhibit significant multiloci heterozygous deficit ($P = 0.0021$) U-test in GENEPOP (Raymond & Rousset 1995). These seedlings were isolated without any adult meadow in the immediate surroundings, which indicates that they are emigrants colonizing a new site. It is possible that admixture from more than one source population caused the observed heterozygote deficit.

Acknowledgements

This work was supported by ESF/FCT (Portugal) fellowships to F.A. (PhD) and C.B. (postdoc), the EU/project EVK3-CT-2000-00044-Monitoring and Management of European Seagrass Beds (M&MS) and the FCT (Portugal) project PNAT/1999/BIA/15003/C. We thank Lucas Llorens and Fernando Brun for their help in Cadiz. We thank Vicent Laizé for technical advice and Gareth Pearson for comments on the manuscript.

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