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PRIMER NOTE

Polymorphic microsatellite DNA markers in the mangrove tree *Avicennia alba*

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Abstract

Like most species of mangrove trees of the genus *Avicennia*, *A. alba* is widely distributed among tropical and subtropical coasts around the world. Mangroves play an essential role in ecosystem dynamics but are reported to be regressing as human pressure increases on coastal zones. Hypervariable genetic markers are useful for population genetics studies, to estimate the level of impact and the populations potential for recovery. Microsatellite markers for *A. alba* were obtained by screening a partial genomic library enriched for microsatellite dinucleotide repeats. Among 20 primer pairs defined, six amplified polymorphic microsatellites with a satisfying level of variability.

Keywords: *Avicennia alba*, dinucleotides, mangrove, microsatellites

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Mangroves form the dominant intertidal ecosystems throughout the tropical regions of the world and range from Japan (Northern limit) to Victoria, Australia (Southern limit). The total area of mangroves worldwide is estimated at about 182 000 Km^2. However, due to increasing human pressure in coastal zones, accompanied by the conversion of mangrove forests to cultivated areas, nonsustainable harvesting and pollution, those ecosystems are seriously affected (Dodd *et al*. 1998). The conservation and sustainable management of mangroves is thus a major priority in coastal areas of many countries (Maguire *et al*. 2000). *Avicennia alba* is one of the key species in the Southeast Asia mangrove ecosystems (Tomlinson 1986) and some of its populations in Vietnam have undergone strong perturbations. In order to assess the consequences of these perturbations we isolated and characterized polymorphic microsatellite loci in the *Avicennia alba* genome.

Genomic DNA was extracted from leaf tissue of two individuals from the Mekong Delta, Vietnam, using the CTAB method (Doyle & Doyle 1988). 5 µg of DNA was digested with *Afa*I and an RSA adaptor (consisting of one 21-mer and one 25-mer primer) was ligated to the digested fragments. This was followed by an enrichment for microsatellites, carried out with streptavidine-labelled magnetic particles (Biocontec) with the $(CT)_{15}$ motif, following the protocol from Billote *et al*. (1999). The DNA fragments bound to the spheres were eluted and ligated to a pGem-T easy plasmid (Promega). Plasmids were then transformed into competent cells (DH5αF′, GibcoBRL) and plated. The positive colonies were transferred to plaques and replicated onto Hybond N^+ membranes, using a Bio-Dot micro filtration apparatus (Bio-Rad). The probe $(CT)_{15}$ was labelled with [γ^{32}P]-dATP and hybridized with the membranes. We selected 85 colonies that were strongly positive from a total of 384 probed, isolated the DNA using the Wizard plus SV miniprep DNA purification kit (Promega), and the DNA was sequenced commercially (Macrogen). Four samples could not be sequenced, and upon the remaining 81, 56 contained microsatellites, 20 of which were largely uninterrupted and had sufficient flanking regions for primer design.

We tested those 20 primer pairs for polymorphism on 16 *Avicennia alba* individuals from the Mekong Delta, Vietnam. Six primers pairs did not yield satisfying amplification products, seven of the locus amplified appeared to be monomorphic, and the remaining six primer pairs allowed the amplification of polymorphic loci (Table 1). Amplifications were performed in 10 µL volumes containing 20–50 ng of template DNA, 1 µL of reaction buffer 200 mM Tris-HCL (pH 8.4), 500 mM KCl and 0.5 units of *Taq* DNA polimerase (Invitrogen, Life technologies), 0.5 µL of 1%

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Table 1 Characterization of the six microsatellite loci identified in *Avicennia alba*. The name, motif, primer sequence, size of cloned allele and GenBank accession number are detailed for the six loci isolated

Locus	Repeat sequence	Primer sequence (5′–3′)	Size of (bp) cloned allele	Accession numbers
Aa 13	$(TG)_{12}(AG)_5T(GA)_7$	F: CCGTTTCCATTTTCCTTTATTC R: GCACTCCTACTCTCATCCC	246	AY281858
Aa 22	$(AC)_{11}$	F: TCCCATTTGCATTACAGTCTG R: CGAGCGTGTGCTAATCTTCC	269	AY281859
Aa 23	$(CT)_{11}$	F: ACTGGATGATTGGTGTTTTTTA R: AGGTGCGTGGGTATGTTG	207	AY281862
Aa 26	$(TC)_{22}$	F: GGATTAAGAATGAAGAAAGGGG R: CCAAGTGTGGAATGTTGTATCTT	178	AY281857
Aa 28	$(AG)_{11}$	F: CTCGTGGACACCTCATTATCC R: TAACCACTGGCACAAACTCC	175	AY281860
Aa 67	$(GA)_{11}$	F: AACTCAAGAGAAGCGATGGC R: TAAGCGAAGATCCTGATTCG	171	AY281861

Table 2 Characterization of microsatellite loci isolated from the *Avicennia alba* genome. Number of alleles n, observed and expected heterozygosities, H_O and H_E, heterozygote deficiency F_{IS}, the percentage of simulated values (obtained on the basis of a 1000 permutation test) superior or equal to the estimated value are detailed

Locus	n° alleles	Allele size range	H_E	H_O	F_{IS}	% values
Aa 13	5	242–258	0.3288	0.2571	0.220	0.08
Aa 22	3	268–272	0.4808	0.5000	−0.040	0.54
Aa 23	2	205–207	0.3803	0.3333	0.125	0.12
Aa 26	9	154–184	0.7617	0.8611	−0.133	0.93
Aa 28	9	176–210	0.6264	0.5882	0.062	0.16
Aa 67	6	152–164	0.6894	0.7778	−0.130	0.84

W-1 solution (Invitrogen), 2 mM of $MgCl_2$, 1 μM of each primer, 60 μM of dCGTP mix, 10 μM of dATP and 1 μCi of [α^{35}S]-dATP. Reactions were performed in a Gene Amp PCR system 9700 (PE Applied Biosystems) with the following program: 5 min denaturation at 95 °C, then 30 cycles of 45 s at each temperature 95 °C, 50 °C, and 72 °C, and a final extension of 7 min at 72 °C. PCR products were separated in 6% denaturing polyacrylamide gels and visualized by autoradiography.

We estimated the level of genetic variability and tested for Hardy–Weinberg equilibrium by genotyping a sample of 36 individuals from the Mekong Delta, Vietnam (Table 2). Number of alleles, heterozygosity levels and heterozygote deficiencies were computed using the program GENETIX 4.0 (Belkhir *et al*. 1996–2001). The number of alleles was found to range between two and nine, the observed heterozygosity (H_O) ranged between 0.26 and 0.86. No significant heterozygote deficiency was observed, suggesting the loci studied segregate in a Mendelian fashion, the population sampled is in Hardy–Weinberg equilibrium, and no high frequency null alleles exist. We tested for linkage disequilibrium according to the Black & Krafsur (1985) procedure, and the significance of the results were tested with a 1000 permutation test. The values corresponding to Aa26-Aa67 and Aa28-Aa67 were significant (respectively 0.02 and 0.04).

These results make those microsatellites useful markers for studying intraspecific variability in *A. alba* populations. We also tested cross-amplification in 7 individuals of *A. marina* from Northern Vietnam, these appeared to be monomorphic for three loci (Aa 13, Aa 26 and Aa 67) and it was not possible to obtain amplification products from the remaining loci.

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