MICROSATELLITE LOCI FOR *AVICENNIA ALBA* (ACANTHACEAE), *SONNERATIA ALBA* (LYTHRACEAE) AND *RHIZOPHORA MUCRONATA* (RHIZOPHORACEAE)

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WEE AKS, TAKAYAMA K, KAJITA T & WEBB EL. 2013. Microsatellite loci for *Avicennia alba* (Acanthaceae), *Sonneratia alba* (Lythraceae) and *Rhizophora mucronata* (Rhizophoraceae). Microsatellite markers were developed for three major mangrove species *Avicennia alba, Sonneratia alba* and *Rhizophora mucronata* to investigate the genetic diversity and spatial genetic structure of these species. The numbers of polymorphic compound microsatellite markers isolated were four, four and three for *A. alba, S. alba* and *R. mucronata* respectively. Genetic parameters of these markers were evaluated with three populations from the Malay peninsula. The numbers of alleles per locus within each population ranged from 1–13 for *A. alba*, 1–6 for *S. alba* and 1–3 for *R. mucronata*. Expected heterozygosity per population ranged from 0.000–0.877 for *A. alba*, 0.000–0.647 for *S. alba* and 0.000–0.558 for *R. mucronata*. All 11 microsatellite markers displayed polymorphism within and between populations. Most markers were also amplified in closely-related species: *Avicennia rumphiana, Sonneratia caseolaris, S. ovata, Rhizophora stylosa* and *R. apiculata*. This demonstrates the usefulness and robustness of the described markers in population genetic studies and potential forestry research in mangroves.

Keywords: Compound microsatellites, conservation, gene flow, genetic diversity, mangrove, SSR

WEE AKS, TAKAYAMA K, KAJITA T & WEBB EL. 2013. Lokus mikrosatelit bagi *Avicennia alba* (Acanthaceae), *Sonneratia alba* (Lythraceae) dan *Rhizophora mucronata* (Rhizophoraceae). Penanda mikrosatelit dibangunkan untuk tiga spesies pokok bakau utama iaitu *Avicennia alba, Sonneratia alba* dan *Rhizophora mucronata* untuk menyiasat kepelbagaian genetik dan struktur genetik ruang spesies-spesies tersebut. Bilangan penanda mikrosatelit majmuk polimorfik yang diasingkan ialah empat untuk *A. alba,* empat untuk *S. alba* dan tiga untuk *R. mucronata*. Tiga populasi dari Semenanjung Tanah Melayu digunakan dalam penilaian parameter genetik penanda-penanda ini. Bilangan alel setiap lokus dalam setiap populasi berjulat antara 1–13 untuk *A. alba*, 1–6 untuk *S. alba* dan 1–3 untuk *R. mucronata*. Keheterozigotan yang dijangkakan bagi setiap populasi adalah dalam lingkungan 0.000–0.877 untuk *A. alba*, 0.000–0.647 untuk *S. alba* dan 0.000–0.558 untuk *R. mucronata*. Kesemua 11 penanda mikrosatelit adalah polimorfik dalam dan di antara populasi. Kebanyakan penanda ini boleh digunakan untuk spesies bakau yang berkait rapat: *Avicennia rumphiana, Sonneratia caseolaris, S. ovata, Rhizophora stylosa* dan *R. apiculata*. Ini menunjukkan kegunaan penanda-penanda ini dalam kajian genetik populasi dan penyelidikan tentang hutan bakau.

INTRODUCTION

Mangroves are intertidal plants occurring in tropical and subtropical coasts. They have widespread distribution (Tomlinson 1986) owing largely to their high potential for water-mediated long distance dispersal. Inclusion of population genetics in conservation and sustainable management of mangroves is becoming increasingly important as mangrove forests are threatened by landward encroachment and habitat conversion (Duke et al. 2007). To understand the historical and contemporary genetic connectivity in such high gene flow plants, it is necessary to employ highly polymorphic molecular markers. Microsatellite markers are highly useful as genetic tools that can be applied to forest

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conservation and management (Tsumura et al. 2005).

We isolated and characterised microsatellite markers for three of the most common mangrove species in the Indo West Pacific region: *Avicennia alba* (Acanthaceae), *Sonneratia alba* (Lythraceae) and *Rhizophora mucronata* (Rhizophoraceae) (Polidoro et al. 2010). Six nuclear microsatellite markers have been previously developed for *A. alba* (Teixeira et al. 2003). However, additional loci are necessary to study its genetic structure in detail within the Indo West Pacific region. No microsatellite markers have been developed for *S. alba* and *R. mucronata*.

The markers for these three species are expected to be used widely in studies across the Indo West Pacific because as true mangrove species they are important components of the mangrove ecosystems in terms of structure, composition and ecology. These species differ in pollination syndrome as well as potential dispersal distances (Tomlinson 1986). These ecological differences would be expected to lead to interspecific variation in gene flow and genetic structuring.

MATERIALS AND METHODS

Genomic DNA of *A. alba, S. alba* and *R. mucronata* was extracted from dried leaf tissue with a modified version of the cetyl trimethylammonium bromide (CTAB) extraction method. For each species, a microsatellite-enriched genomic library of an individual from Singapore was constructed using a recently developed technique for isolation of compound microsatellite markers (Lian et al. 2006). Genomic DNA was digested using six blunt-end restriction enzymes—*Hae*III, *Pvu*II, *Alu*I, *Ssp*I, *Eco*RV and ScaI—followed by ligation to a specific blunt adapter (Lian et al. 2001) using a T4 DNA ligation kit. Polymerase chain reaction (PCR) amplification of the ligation products was carried out in 25 µL reaction volume containing the solution of ligated fragments, 4 µL; 1 × *Ex*Taq buffer with 2 mM $MgCl_2$; dNTPs, 250 µM each; compound microsatellite primer $(AC)_6(AG)_8$ and adapter primer AP2 (5'-CTATAGGGCACGCGTGGT-3'), 0.5 µM each; and *Ex*Taq (TaKaRa, Shiga, Japan), 1 unit. The PCR conditions were: initial denaturation (95°C for 1 min), followed by 35 cycles of denaturation (95 °C for 45 s), annealing (55 °C for 45 s), extension (72 °C for 1 min) and a final extension step (72 °C for 15 min). Amplified fragments were cloned using TOPO TA cloning kit. The plasmid DNA obtained was then amplified with illustra TempliPhi DNA amplification kit and sequenced using M13R primer on an ABI 3130xl automated DNA sequencer with the BigDye terminator v3.1 cycle sequencing kit under standard conditions.

Unique fragments containing the $(AC)_6(AG)_n$ compound microsatellite sequence at one end were selected for amplification test. Specific primers were designed using the Primer3 software (Rozen & Skaletsky 1999). At the 5' end of the primers, seven nucleotides (GTTTCTT) were added to promote adenylation in the 3' end of PCR amplified products. A set of universal primers $(AC)_6(AG)_5$ were labelled with one of the four fluorescent dyes, 6-FAM, VIC, NED and PET. Each universal primer was then used in PCR amplification for polymorphism screening together with a specific primer designed in the previous step. PCR amplification was carried out in 10 µL reaction volume containing template DNA, 40 ng; 1×PCR buffer; dNTPs, 400 µM each, Mg^{2+}, 2.5 mM; fluorescently-labelled primer, 0.2 µM; specifically-designed primer, 0.2 µM and iTaq DNA polymerase, 0.25 U. The PCR conditions were initial denaturation (94 °C for 5 min), followed by 35 cycles of denaturation (94 °C for 45 s), annealing (55 °C for 45 s), and extension (72 °C for 45 s), followed by a final extension step (72 °C for 10 min). Genotyping of PCR products were conducted with an ABI 3130xl automated DNA sequencer under standard conditions. Results were analysed with GeneMapper v4.1.

Amplified loci were used in polymorphism tests with individuals collected from the east, west and south of the Malay peninsula, as represented by one population from Trang (7.3° N, 99.5° E), Nakhon Si Tammarat (8.5° N, 100.1° E) and Singapore (1.4° N, 103.7° E). To ensure time and cost efficiency, the polymorphism test was first carried out with a subsample of three individuals from each of the three populations. Monomorphic loci with strong and specific amplification (i.e.

identified by clear peak with high intensity) were compiled and reported. Only loci that showed polymorphism among these nine individuals were tested with the rest of the samples from three populations.

A total of 82, 58 and 38 individuals of *A. alba, S. alba* and *R. mucronata* respectively were included to test for polymorphism. Linkage disequilibrium was tested using FSTAT 2.9.3 (Goudet 2001). The test for departure from Hardy–Weinberg equilibrium (HWE) was performed with GENEPOP 3.4V (Raymond & Rousset 1995). Micro-checker (Van Oosterhout et al. 2004) was used to test for null alleles in each locus for each population. Cross-amplifications were also carried out to test for the transferability of developed microsatellite markers to closely-related species. Microsatellite markers for *A. alba* were cross-amplified to *Avicennia rumphiana* and *A. officinalis*; markers for *S. alba* were cross-amplified to *Sonneratia ovata* and *S. caseolaris*; markers for *R. mucronata* were cross-amplified to *Rhizophora stylosa* and *R. apiculata*. Two individuals from each species were tested using the experimental protocols described above. The homology of the microsatellite marker was confirmed with nucleotide sequences of the flanking regions. Cross-amplified markers were not tested for polymorphism.

RESULTS AND DISCUSSION

A total of 192 clones were sequenced for each species, of which 102, 155 and 159 were obtained for *A. alba, S. alba* and *R. mucronata* respectively. Among these sequences, 65, 63 and 113 primers were designed and tested for *A. alba, S. alba* and *R. mucronata* respectively. Excluding monomorphic loci or those with excessive stutter peaks and low amplification intensity, four, four and three loci for *A. alba, S. alba* and *R. mucronata* respectively showed clearly interpretable amplicons (Table 1). The values for expected heterozygosity per population varied from 0.000 to 0.877 for *A. alba*, from 0.000 to 0.647 for *S. alba* and from 0.000 to 0.558 for *R. mucronata* (Table 1). No significant linkage disequilibrium was found in all loci. The test for departure from HWE showed significant ($p \leq 0.01$) departure from HWE in locus AA18 for the *A. alba* populations from east and south of the Malay peninsula, locus RMu35 for *R. mucronata* population from east of the Malay peninsula and locus AA29 for *A. alba* population from west of the Malay peninsula. Departure from HWE could be due to high levels of inbreeding or the presence of null alleles. Only AA18 from east of the Malay peninsula showed evidence of null alleles in the micro-checker analysis.

Seven of the eleven developed markers were transferable to at least one other closely-related species (Table 2). Eight monomorphic microsatellite loci—one for *A. alba*, two for *S. alba* and five for *R. mucronata*—that were isolated together with the 11 microsatellite markers are presented in Table 3. These loci were tested with nine individuals from three populations during the initial polymorphism test. These monomorphic loci may be useful for studies on populations beyond the Malay peninsula and other related taxa.

As a conclusion, this study characterised four, four and three polymorphic microsatellite loci for *A. alba, S. alba* and *R. mucronata* respectively. The markers can be used effectively in studies with regard to genetic diversity, spatial genetic structure and mating systems of these three mangrove species. Cross-amplification test revealed that 7 out of 11 developed markers were transferable to at least one other closely-related species, indicating that these markers could be used in genetic studies of other mangrove species.

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Table 1 Characteristics of 11 dinucleotide microsatellite loci developed in this study

Locus	GenBank accession no.	Restriction enzyme	Primer sequence (5'–3')	Repeat motif	Size range (bp)	East of the Malay peninsula				West of the Malay peninsula				South of the Malay peninsula			
						A	Size range (bp)	N_A	H_O	H_E	Size range (bp)	N_A	H_O	H_E	Size range (bp)	N_A	H_O	H_E
Avicennia alba																	
AA18	JQ290307	PvuII	atttgaaattgcgcataca	$(AC)_6(AG)_{10}$	166–172	3	166–168	2	0.000	0.403*†	168	1	0.000	0.000	168–172	2	0.147	0.136*
AA29	JQ290308	AluI	ggccttgtacagaatgaaga	$(AC)_6(AG)_{10}$	154–164	5	160–164	2	0.320	0.403	156–160	3	0.130	0.124*	154–160	2	0.176	0.208
AA53	JQ290309	SspI	cacatattcatacctccatga	$(AC)_6(AG)_{10}$	131–139	3	131–139	3	0.640	0.607	131–135	2	0.043	0.043	135–139	2	0.029	0.084
AA54	JQ290310	SspI	ctcaaaatgcatgatggatag	$(AC)_6(AG)_{14}$	219–259	18	221–255	10	0.760	0.776	223–259	13	0.783	0.877	219–231	4	0.588	0.667
Sonneratia alba																		
SA08	JQ290311	SspI	tattttactaattaacttcatccac	$(AC)_6(AG)_{12}$	247–253	4	253	1	0.000	0.000	247–253	3	0.565	0.647	247–253	4	0.619	0.630
SA11	JQ290312	PvuII	cttgtaatcttcgcttcgt	$(AC)_6(AG)_8$	286–292	3	292	1	0.000	0.000	286–292	2	0.043	0.043	286–292	3	0.476	0.414
SA15	JQ290313	AluI	gaactcgaactcgaggaact	$(AC)_6(AG)_{17}$	161–181	6	171	1	0.000	0.000	161–181	6	0.261	0.342	169–177	3	0.429	0.534
SA16	JQ290314	PvuII	aggcaccaactctccatac	$(AC)_6(AG)_{12}$	154–166	2	166	1	0.000	0.000	154–166	2	0.348	0.340	166	1	0.000	0.000
Rhizophora mucronata																		
RMu21	JQ290315	EcoRV	tcctctttcctataatctgacc	$(AC)_6(AG)_{18}$	113–127	3	113–127	3	0.500	0.518	113–127	3	0.333	0.558	113–127	2	0.111	0.105
RMu35	JQ290316	HaeIII	ccttatttgttggcttgct	$(AC)_6(AG)_{19}$	216–220	3	218–220	2	0.500	0.375*	218	1	0.000	0.000	216–220	3	0.333	0.438
RMu54	JQ290317	PvuII	aagtcattgctgtccagttc	$(AC)_6(AG)_{12}$	312–316	3	312–316	3	0.357	0.426	312–314	2	0.067	0.064	312–314	2	0.111	0.475

Repeat motif is given for the cloned allele; A = total number of alleles in the three populations; N_A= number of alleles; H_O= observed heterozygosity; H_E= expected heterozygosity; * significant deviations from Hardy–Weinberg equilibrium ($p \leq 0.01$); † possible presence of null allele (as detected by Micro-checker); sample sizes for east, west and south of the Malay peninsula were 25, 23 and 34 respectively for *A. alba*; 14, 23 and 21 respectively for *S. alba*; and 14, 15 and 9 respectively for *R. mucronata*

Table 2 Cross-amplification of microsatellite loci from *Avicennia alba* to *Avicennia rumphiana* and *Avicennia officinalis*; from *Sonneration alba* to *Sonneratia ovata* and *Sonneratia caseolaris*; and from *Rhizophora mucronata* to *Rhizophora stylosa* and *Rhizophora apiculata*

Avicennia alba

Locus	*A. rumphiana*	*A. officinalis*
AA18	–	–
AA29	+	–
AA53	–	–
AA54	+	–

Sonneratia alba

Locus	*S. ovata*	*S. caseolaris*
SA08	–	–
SA11	–	–
SA15	+	+
SA16	+	–

Rhizophora mucronata

Locus	*R. stylosa*	*R. apiculata*
RMu21	+	+
RMu35	+	–
RMu54	+	+

+ = successful amplification with confirmed homology, – = no amplification

Table 3 Monomorphic microsatellite isolated in the study

Locus	GenBank accession no.	Restriction enzyme	Primer sequence (5'–3')	Repeat motif	Size range (bp)
Avicennia alba					
AA48	JX644076	SspI	catgtaggcatacgtgtttc	$(AC)_6(AG)_{29}$	194
Sonneratia alba					
SA12	JX644077	ScaI	ccctacttcggtcgatac	$(AC)_6(AG)_8CC(AG)_5$	293
SA29	JX644078	PvuII	atcccattatctccatcca	$(AC)_6(AG)_8$	104
Rhizophora mucronata					
RMu18	JX644079	ScaI	tggtcattcattctgcttc	$(AC)_6(AG)_{12}$	136
RMu29	JX644080	SspI	gcttccttattcgtcttcag	$(AC)_6(AG)_{15}$	252
RMu76	JX644081	AluI	attcagtgccttgatctgac	$(AC)_6(AG)_8$	331
RMu86	JX644082	PvuII	aaaagtgcatgatttacatgg	$(AC)_6(AG)_8$	284
RMu105	JX644083	HaeIII	ccacacaaatcaatatgcac	$(AC)_6(AG)_8$	138

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