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PRIMER NOTE

DEVELOPMENT OF EST-SSR MARKERS IN *BARRINGTONIA RACEMOSA* (LECYTHIDACEAE) AND CROSS-AMPLIFICATION IN RELATED SPECIES[1]

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- *Premise of the study:* Microsatellite markers were identified and characterized to study the genetic diversity and structure of *Barringtonia racemosa* (Lecythidaceae).
- *Methods and Results:* Based on the transcriptome data of *B. racemosa*, 30 primer pairs were initially designed and tested, of which 15 were successfully amplified and displayed clear polymorphisms across the 43 individuals from three distant populations tested in the study. The results showed that the number of alleles per locus ranged from two to seven and the expected heterozygosity and observed heterozygosity per locus varied from 0 to 0.772 and from 0 to 0.933, respectively.
- *Conclusions:* The expressed sequence tag–simple sequence repeat (EST-SSR) markers described here will be useful for studying genetic diversity and structure of *B. racemosa*. Furthermore, all loci were successfully cross-amplified in *B. asiatica* and *B. acutangula* and will be of great value for genetic studies across this genus.

Key words: *Barringtonia racemosa*; cross-amplification; EST-SSR; Lecythidaceae; transcriptome.

Barringtonia racemosa (L.) Spreng. (Lecythidaceae), the most widespread species of *Barringtonia*, is characteristic of coastal communities in the Paleotropics (Tomlinson, 1986; Prance, 2012). The flowers open at night and are pollinated by night-flying animals. The bark and crushed fruit contain saponins and are used as a fish poison (Payens, 1967). This tree has a distribution from the Pacific to East Africa (Prance, 2012); it is variously recorded as a mangrove associate and is abundant along tidal rivers and in areas subject to tide and salinity. It also occurs inland on the edge of peat swamp forests and on hillsides to altitudes of 200 m (Tomlinson, 1986). The fruit are buoyant because of the spongy fibrous pericarp, and the wide distribution is on account of its dispersal by water (Prance, 2012). However, the actual level of interpopulation migration has never been studied. Recent advances in molecular techniques, such as microsatellite markers, have created new opportunities for population genetic research that can be used to estimate the genetic variation and clarify the level of migration among populations of *B. racemosa* across its range. Transcriptome sequencing is an efficient method for acquiring expressed sequence tag–simple sequence repeat (EST-SSR) markers (Bouck and Vision, 2007) based on its low cost and high reliability. Here, we describe the development of 15 nuclear microsatellite markers for ongoing population genetic research in *B. racemosa*. Additionally, we tested the transferability of these markers for studying two related species, *B. asiatica* (L.) Kurz and *B. acutangula* (L.) Gaertn.; these three species comprise all of the wide-ranging, water-dispersed species in *Barringtonia* (Prance, 2012).

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METHODS AND RESULTS

In this study, a three-month-old seedling of *B. racemosa* (from Dongzhai harbor, Hainan, China) was immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction. The total RNA of *B. racemosa* was extracted using an improved cetyltrimethylammonium bromide (CTAB) method (Fu et al., 2005). The RNA quality and quantity were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). The mRNA was isolated using Oligotex-dT30 (TaKaRa Biotechnology Co., Dalian, China) after extraction and then fragmented ultrasonically. The cDNA libraries were prepared for sequencing following the Illumina protocol, and paired-end short read sequencing was done using the Illumina Genome Analyzer II system (Illumina, San Diego, California, USA; sequencing performed by BGI, Shenzhen, Guangdong Province, China) (Huang et al., 2012; Qiang et al., 2015). A total of 13,510,000 90-nucleotide paired-end reads were obtained and assembled using Trinity (release 20140413) with the default parameters (Grabherr et al., 2011). CAP3 (Huang and Madan, 1999) was further used to cluster similar contigs and obtain a high-quality reference genome with nonredundant unigenes (minimum identity = 99%). The analysis yielded 63,932 contigs with an average length of 589 bp, an N50 length of 808, and an average depth of coverage of 18.14×. The software MISA (Thiel et al., 2003) was used to detect

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TABLE 1. Characteristics of 15 microsatellite loci isolated from *Barringtonia racemosa*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.	BLAST top hit description [organism]	BLAST top hit accession no.	*E*-value
br-39616	F: TTGGCTCTGCTCTGGTATCC R: AGTTGCGCTCATCTCTCTCC	$(TG)_8$	112–126	56	KR822410	CASP-like protein 4 [*Zea mays*]	NM_001137348	5e-07
br-60918	F: ATCTACATGGTGGCTGCACA R: CCAAACTCAGCTCCCCATAA	$(TC)_8$	106–131	58	KR822411	Hypothetical protein partial [*Morus notabilis*]	XM_010103213	3e-20
br-6303	F: GTTTTGGAGGACAGAGAGCG R: ACTCTGAATCCCAAACGCAC	$(GA)_7$	233–266	58	KR822412	DNA photolyase, putative [*Ricinus communis*]	XM_002523046	0.000
br-39818	F: TCCGATACTTTGGTGGTTCC R: AAAAGAGCACTCCGACCTCA	$(ATC)_5$	193–200	60	KR822413	Uncharacterized protein [*Arabidopsis thaliana*]	NM_121752	2e-68
br-39641	F: TAGTCTCCAAACGACGGCTT R: AAGGAGGAGATGACGCAACA	$(GCT)_5$	113–128	56	KR822414	Transcription factor E2F isoform 1 [*Theobroma cacao*]	XM_007015654	0.000
br-7338	F: GTGATGGCTTTGCTGCTGTA R: AGCACCATTACCAGCAATCC	$(TGG)_5$	244–259	58	KR822415	E2F transcription factor-1 family protein [*Populus trichocarpa*]	XM_006384725	0.000
br-6800	F: ACTGTCACTGAACCGTGCAA R: ACACAGCTTGCTGAACGATG	$(AG)_8$	184–206	60	KR822409	Major facilitator protein isoform 1 [*Theobroma cacao*]	XM_007031842	0.000
br-7130	F: AGTCACGCATTAACCTTCCG R: GGAAGAGACGGATTGCTGAG	$(CCG)_5$	154–170	60	KR822416	Major facilitator superfamily protein isoform 1 [*Theobroma cacao*]	XM_007031841	0.000
br-38256	F: ATTACTCCTCGCCACCATTG R: TGGTGATCACTGCCTCGTAG	$(AGG)_5$	124–133	60	KR822418	Hypothetical protein [*Morus notabilis*]	XM_010114075	0.000
br-39436	F: CAGCCAGATGAACAAACCCT R: GGGGCTTGTTGTGTCAGAAT	$(TA)_6$	154–178	58	KR822417	Class III peroxidase [*Populus trichocarpa*]	KJ_201853	4e-50
br-32408	F: TGAGCAAGCAGAGAAGCTCC R: GTTCAAACTTTGGGCATGCCA	$(TG)_9$	218–248	58	KR822404	Rhodanese/cell cycle control phosphatase superfamily protein [*Francoa sonchifolia*]	KM_461132	2e-142
br-38577	F: CTCCTCTCCCCTGTCTCCAA R: AACCTGAAAGTCCGGCGAAA	$(GAA)_7$	244–268	58	KR822405	Leucine-rich repeat extension-like protein 4 [*Morus notabilis*]	XM010090551	2e-110
br-11107	F: AGACAACCGGGTCCTTCATT R: GCGTCAACAAAAGCTGCAGA	$(AGG)_7$	262–274	60	KR822406	Hypothetical protein [*Phaseolus vulgaris*]	XM_007161158	1e-165
br-54330	F: ACGCCTTGTTCCTGGATAGC R: AACGCTCGCAGAAAACAACC	$(AT)_9$	240–270	56	KR822407	Hypothetical protein [*Morus notabilis*]	XM_010092747	1e-180
br-23885	F: GGTTTGGCAGTTGGTTCTGG R: TGCTGCCACATTGAATCCCT	$(TGC)_7$	262–280	58	KR822408	Auxin response factor 5 [*Morus notabilis*]	XM_010108646	0.000

Note: T_a = annealing temperature.

microsatellites from all unigenes obtained in the study. We screened for SSR motifs containing two to six nucleotides with the minimum number of repeats as follows: six for dinucleotide and five for trinucleotide, tetranucleotide, pentanucleotide, and hexanucleotide. Altogether, 6524 SSR motifs were found, and 30 of them were selected at random to design primers using Primer3 (Rozen and Skaletsky, 1999), with the optimum conditions set at a length of 20 bp (18–22 bp), a temperature of 60.0°C (56–62°C), and a product size range of 120–250 bp.

Forty-three individuals of *B. racemosa* from three natural populations (Appendix 1) were used to evaluate the polymorphisms of the target microsatellite loci. In addition, individuals from one population of *B. asiatica* and one population of *B. acutangula* were also sampled to detect the efficiency of these markers in cross-species amplification (Appendix 1). Genomic DNA was extracted from silica gel–dried leaves using the DNA Extraction Kit (Magen, Guangzhou, China) following the manufacturer's protocol. PCR amplifications were performed in a final volume of 30 µL, containing 60 ng of genomic DNA, 1× PCR buffer (10 mM Tris-HCl [pH 8.4] and 1.5 mM $MgCl_2$; TransGen Biotech Co., Beijing, China), 0.2 mM dNTPs (TransGen Biotech Co.), 0.5 µM of each primer (Life Technologies, Shanghai, China), and 1 unit *EasyTaq* DNA polymerase (TransGen Biotech Co.). The PCR reactions were carried out under standard conditions for all primers in a Bio-Rad PTC-200 thermocycler (Bio-Rad Laboratories, Hercules, California, USA) under the following conditions: initial denaturation was at 94°C for 4 min, followed by 35 cycles at 94°C for 40 s, then annealing for 45 s at the optimal temperature for each primer pair (from 56–60°C, see Table 1), and 72°C for 1 min, with a final extension of 10 min at 72°C. To test the utility of the primers, PCR products were detected on 1% agarose gels. Finally, a total of 15 of the 30 primer pairs were successfully amplified. The sequences of microsatellite loci were deposited into GenBank (accession no. KR822404–KR822418; Table 1). The SSR genotyping was performed using a Fragment Analyzer Automated CE System (Advanced Analytical Technologies [AATI], Ames, Iowa, USA) using the Quant-iT PicoGreen dsDNA Reagent Kit, 35–500 bp (Invitrogen, Carlsbad, California, USA). Raw data were analyzed and allele sizes and number of alleles per locus were called using PROSize version 2.0 software (AATI). GenAlEx version 6.5 software (Peakall and Smouse, 2012) was used to calculate the average number of alleles per locus (A), the observed heterozygosity (H_o), and the expected heterozygosity (H_e) of each microsatellite locus. Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were tested using GENEPOP 4.3 (Rousset, 2008). MICRO-CHECKER (van Oosterhout et al., 2004) was employed for testing scoring errors and null alleles.

In total, 15 out of 30 primer pairs amplified the expected products and displayed clear polymorphisms. In *B. racemosa*, the number of alleles per locus ranged from two to seven, the observed heterozygosity ranged from 0 to 0.933, and the expected heterozygosity ranged from 0 to 0.772 (Table 2). All 15 primer sets also successfully amplified SSR loci in *B. asiatica* and *B. acutangula.*

In *B. racemosa*, five out of the 15 polymorphic microsatellite loci showed significant deviations from HWE in population BRSH and BRKL, respectively (Table 2). Only one locus showed significant deviation from HWE in population BRDR (Table 2). Deviations from HWE may have been related to the presence of null alleles, although we found no null homozygotes. There was also no significant linkage equilibrium ($P < 0.05$) between locus pairs (Table 2). In *B. asiatica*, two out of the 15 polymorphic microsatellite loci showed significant deviations from HWE (Table 2), and no linkage disequilibrium was detected between any pair of loci. In *B. acutangula*, no loci deviated from HWE, and no linkage disequilibrium was detected between any pair of loci.

CONCLUSIONS

The 15 microsatellite markers developed here have been proven to be useful in the evaluation of the genetic diversity of *B. racemosa*. Cross-amplification of these microsatellite loci in *B. asiatica* and *B. acutangula* suggests that they will also be useful in studies of other species within *Barringtonia*. We are currently using these markers for inferring genetic diversity and spatial genetic structure in these species. In particular, we are using these markers to assess the impact of seed dispersal by water and to estimate seed dispersal distances for these species in coastal tropical landscapes of the Indo-West Pacific.

TABLE 2. Results of initial primer screening in populations of *Barringtonia racemosa*, *B. asiatica*, and *B. acutangula*.[a]

	B. racemosa									*B. asiatica*			*B. acutangula*		
	BRSH ($N = 19$)			BRKL ($N = 15$)			BRDR ($N = 9$)			BASB ($N = 7$)			BACC ($N = 7$)		
Locus	A	H_o	H_e[b]	A	H_o	H_e[b]	A	H_o	H_e[b]	A	H_o	H_e[b]	A	H_o	H_e
br-39616	5	0.842	0.661	5	0.800	0.696	4	0.778	0.660	4	0.143	0.643	3	0.143	0.357
br-60918	2	0.421	0.332	2	0.533	0.391	2	0.556	0.401	3	0.714	0.520	8	0.667	0.847
br-6303	4	0.105	0.593***	3	0.267	0.587**	3	0.125	0.461	4	0.400	0.480	4	0.500	0.563
br-39818	2	0.158	0.229	2	0.133	0.124	3	0.111	0.290	2	0.333	0.278	2	0.714	0.459
br-39641	3	0.263	0.273	3	0.267	0.238	2	0.000	0.219	4	0.571	0.735*	2	0.286	0.245
br-7338	2	0.053	0.051	2	0.200	0.180	1	0.000	0.000	5	0.429	0.612	3	0.286	0.255
br-6800	2	0.211	0.188	4	0.400	0.429	4	0.222	0.673	3	0.400	0.560	2	0.000	0.320
br-7130	3	0.526	0.410	3	0.933	0.620*	3	0.500	0.531	1	0.000	0.000***	3	0.143	0.520
br-38256	3	0.706	0.581	3	0.571	0.538	1	0.000	0.000	1	0.000	0.000	2	0.167	0.375
br-39436	2	0.375	0.430	3	0.667	0.540	3	0.667	0.537	3	0.800	0.540	4	0.833	0.681
br-32408	3	0.421	0.609***	1	0.000	0.000	1	0.000	0.000	2	0.000	0.500	3	0.167	0.403
br-38577	4	0.737	0.569	3	0.200	0.184	7	0.625	0.750	1	0.000	0.000	2	0.000	0.245
br-11107	4	0.474	0.572*	5	0.667	0.720***	4	0.875	0.711	1	0.000	0.000	4	0.857	0.663
br-54330	2	0.000	0.457***	4	0.267	0.684***	6	0.556	0.772	2	0.200	0.180	7	0.667	0.764
br-23885	2	0.000	0.100***	3	0.000	0.338***	4	0.111	0.623*	1	0.000	0.000	3	0.000	0.500

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals analyzed.

[a]Locality and voucher information are provided in Appendix 1.

[b]Significant deviations from Hardy–Weinberg equilibrium after sequential Bonferroni corrections: *** represents significance at the 0.1% nominal level; ** represents significance at the 1% nominal level; * represents significance at the 5% nominal level.

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Appendix 1. Voucher and location information for the species and populations used in this study. All voucher specimens are deposited at the herbarium of Sun Yat-sen University (SYSU), Guangzhou, China.

Species	Population code	Voucher no.	Collection locality	Geographic coordinates	*N*
Barringtonia racemosa	BRSH	ZCR2013081	Hainan, China	19°32′58.2″N, 110°34′22″E	19
	BRKL	HYL2014154	Sabah, Malaysia	5°27′1.44″N, 115°36′47.88″E	15
	BRDR	SSH2012112	Daintree River, Australia	12°23′0.6″S, 130°51′58.68″E	9
B. asiatica	BASB	HSN2014026	Palawan, Philippines	10°11′48.48″N, 118°54′16.92″E	7
B. acutangula	BACC	LY2013216	Kampong Pluck, Cambodia	13°12′33.12″N, 103°58′25.68″E	7

Note: *N* = number of individuals.

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