Development and Characterization of EST-SSR Markers for Artocarpus hypargyreus (Moraceae)

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Primer Note

Development and characterization of EST-SSR markers for *Artocarpus hypargyreus* (Moraceae)[1]

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- *Premise of the study:* Polymorphic microsatellite markers were developed for *Artocarpus hypargyreus* (Moraceae), a threatened species endemic to China, to investigate the genetic diversity and structure of the species.
- *Methods and Results:* Based on the transcriptome data of *A. hypargyreus*, 63 primer pairs were preliminarily designed and tested, of which 34 were successfully amplified and 10 displayed clear polymorphisms across the 67 individuals from four populations of *A. hypargyreus*. The results showed the number of alleles per locus ranged from three to 10, and the observed heterozygosity and expected heterozygosity per locus varied from 0.000 to 0.706 and from 0.328 to 0.807, respectively.
- *Conclusions:* These microsatellite markers will be useful in exploring genetic diversity and structure of *A. hypargyreus.* Furthermore, most loci were successfully cross-amplified in *A. nitidus* and *A. heterophyllus*, indicating that they will be of great value for genetic study across this genus.

Key words: *Artocarpus hypargyreus*; microsatellite marker; Moraceae; transcriptome.

Artocarpus hypargyreus Hance (Moraceae), a tall evergreen tree endemic to southern China, is valued for its milky latex for making stiff rubber and for its wood for making furniture (Zhou and Gilbert, 2003). Its natural populations have declined because of overexploitation and habitat loss, and it was listed as a vulnerable species in the IUCN Red List of Threatened Species in 1997 (IUCN, 2015). Therefore, genetic information, such as genetic diversity and population structure, will be important for the conservation of this species.

Here, we developed 34 novel simple sequence repeat (SSR) markers for *A. hypargyreus*, of which 10 were polymorphic in *A. hypargyreus* and the additional 24 successfully amplified loci were monomorphic. These 10 polymorphic markers were tested on 67 individuals from four populations of *A. hypargyreus*, and their transferability was tested in two other *Artocarpus* species.

METHODS AND RESULTS

The transcriptome of *A. hypargyreus* was sequenced with Illumina paired-end sequencing for the development of expressed sequence tag (EST)–SSR markers. The total RNA was extracted from the fresh leaves of *A. hypargyreus* (Appendix 1) using the modified cetyltrimethylammonium bromide (CTAB) method (Fu et al., 2004). Normalized cDNA libraries were constructed and sequenced using the HiSeq 2000 system (Illumina, San Diego, California, USA). The raw reads were cleaned by removing reads containing unknown "N" bases or more than 10% bases with a Q value < 20 using custom Perl scripts. A total of 25.34 million cleaned 100-bp paired-end reads were de novo assembled into 121,556 contigs (N50 = 906 bp) using Trinity version 2.1.1 (Grabherr et al., 2011) with default parameters.

The software QDD version 3.1 (Meglécz et al., 2014) was used to search 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. A total of 14,143 SSR loci were detected in 12,013 contigs. Among them, dinucleotide repeats account for the largest proportion for 54.7%, trinucleotide repeats account for 40.5%, and tetranucleotide repeats account for 1.1%. Subsequently, using Primer3 (Rozen and Skaletsky, 1999) implemented in the QDD program, primer pairs were successfully designed for 3693 SSR loci, which were further subjected to an "all-against-all" BLAST with an *E*-value of $1E^{-40}$ to remove redundancy. Finally, we obtained 2084 unique SSR loci based on which primer pairs were successfully designed.

Field investigations indicated that the individuals of *A. hypargyreus* showed a scattered distribution in their natural environments, causing difficulties in collecting large samples for each population. A total of 67 individuals were collected from four populations of *A. hypargyreus* (16–18 individuals for each population, see Appendix 1) to evaluate the polymorphisms of these SSR loci. In addition, five individuals of *A. nitidus* Trécul and nine individuals of *A. heterophyllus* Lam. were sampled to test the transferability of these primers. Genomic DNA was extracted from silica gel–dried leaves with the CTAB method (Doyle and Doyle, 1986).

Amplification and polymorphism tests were performed for 63 randomly selected primer pairs using two individuals from each population of *A. hypargyreus*. PCR amplification was performed according to Fan et al. (2013) with an appropriate annealing temperature, and PCR products were detected on 1% agarose gels. A total of 34 primer pairs were successfully amplified, generating legible products of the expected fragment size. Sequences of these SSR loci have been deposited in GenBank (Table 1, Appendix 2). The products were inspected with the Fragment Analyzer Automated CE system (Advanced Analytical Technologies [AATI], Ames, Iowa, USA) with the Quant-iT PicoGreen dsDNA reagent kit, 35–500 bp (Invitrogen, Carlsbad, California, USA). The raw data were analyzed by using PROSize version 2.0 software (AATI). Ten loci were polymorphic among the populations, and 24 loci were monomorphic.

The allelic polymorphisms of the 10 loci were further tested in 67 individuals from four populations of *A. hypargyreus*, and the efficiency of these markers in cross-species amplification was detected in *A. nitidus* and *A. heterophyllus*. GenAlEx version 6.5 (Peakall and Smouse, 2012) was used to calculate the average

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TABLE 1. Characteristics of the 10 polymorphic microsatellite loci developed for *Artocarpus hypargyreus*.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Product size (bp)	Allele size range (bp)	*A*	GenBank accession no.	Putative function	Organism	*E*-value
AH1	F: GCAGCCGCCGTTGTTTCTCTTC R: ACGCACCGAAAACCCCACAAAC	$(GTG)_5$	225	204–231	8	KX495095	Hypothetical protein L484_018850	*Morus notabilis*	1.65E^{-70}
AH11	F: GGCTGAATCACCCACTTGAGTT R: TGAACCCTCGGCCACCAAAGAA	$(AT)_8$	185	170–182	4	KX495101	Ultraviolet-B receptor UVR8-like isoform X3 [*Nicotiana sylvestris*]		1E^{-85}
AH14	F: GCTTGTGGGTTCTGGGATCTAT R: CAGACACTAGTTTGGATGTACT	$(AAG)_5$	249	240–261	8	KX495103	Transcription factor MYB5	*Fragaria ×ananassa*	1.21E^{-27}
AH31	F: TCCTCTAACGTGCGCCCCTAAG R: AAACCCAGCGTGCCACCATTG	$(ACC)_8$	204	264–282	7	KX495107	Hypothetical protein L484_012061	*Morus notabilis*	9.80E^{-79}
AH33	F: TCGTCTTTGCAGGGCGATAAGT R: AGAATCGGAGCCATGTAGAAAT	$(TTA)_6$	242	242–251	4	KX495109	Xylosyltransferase 1	*Morus notabilis*	5.72E^{-132}
AH46	F: GGAGAGGCGGTGCAGTAGAA R: GCAGAGCAGACACTACAGTAGC	$(TTC)_5$	249	246–261	5	KX495114	Polyribonucleotide nucleotidyltransferase	*Morus notabilis*	1.83E^{-36}
AH59	F: TCTCCTCCACCTCCTCCATTGT R: GACCTTGGGACCCGCACTTCTT	$(GTG)_6$	238	220–244	9	KX495120	E3 ubiquitin-protein ligase RING1	*Morus notabilis*	4.19E^{-66}
AH76	F: GAACGGCAGATTTCACCATTTT R: AGGATCAACTTAGCCCACTATA	$(ATT)_5$	207	192–207	5	KX495125	Beta-glucosidase 42	*Morus notabilis*	0
AH77	F: CGAGAAGGTTCCGAGCCAGATT R: CCCGACCAAGACCCGGAGTATA	$(TTA)_7$	237	231–249	7	KX495126	Hypothetical protein PRUPE_ppa010130mg	*Prunus persica*	1.52E^{-50}
AH80	F: GACGTTTGAGGTGCGCGAAAAG R: GGCCTACCTCCTACGAACTCTA	$(TCA)_6$	203	200–206	3	KX495127	PHD finger protein 3	*Morus notabilis*	4.39E^{-134}

Note: *A* = number of alleles.
[a]Annealing temperature for all loci was 55°C.

number of alleles per locus, the observed heterozygosity, the expected heterozygosity, and deviation from Hardy–Weinberg equilibrium (HWE). The results showed that the number of alleles per locus ranged from three to 10 (Table 1). The observed and expected heterozygosity ranged from 0.00 to 0.706 and from 0.328 to 0.807, respectively, and all loci showed significant deviation from HWE (Table 2). The scattered distribution of *A. hypargyreus* may cause difficulties in the long-distance dispersal of pollen and eventually lead to a decrease in the observed heterozygosity values and the significant deviations from HWE. Of the 10 SSR markers tested, all successfully amplified in *A. nitidus* and nine successfully amplified in *A. heterophyllus* (Table 3).

CONCLUSIONS

Ten novel polymorphic SSR markers were developed for *A. hypargyreus*, which are likely to be useful for evaluating the genetic diversity and population structure of *A. hypargyreus*, and for facilitating the development of a conservation strategy for this species. The cross-amplification of these microsatellite loci in *A. nitidus* and *A. heterophyllus* suggests that they will also be useful in studies of other species within *Artocarpus*.

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TABLE 2. Polymorphism of the 10 EST-SSRs in four populations of *Artocarpus hypargyreus*.[a]

	DGD (N = 16)			NLD (N = 17)			XG (N = 16)			HSD (N = 18)		
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]
AH1	5	0.615	0.523	6	0.529	0.704**	5	0.375	0.635	2	0.167	0.674
AH11	4	0.688	0.537	4	0.353	0.593	4	0.375	0.561**	3	0.111	0.623
AH14	5	0.125	0.773**	5	0.235	0.633**	4	0.063	0.588**	4	0.333	0.656*
AH31	5	0.188	0.717**	5	0.118	0.740**	5	0.000	0.758**	5	0.278	0.778*
AH33	4	0.438	0.646*	4	0.235	0.670**	4	0.188	0.725**	2	0.056	0.495**
AH46	4	0.125	0.328**	3	0.471	0.567**	4	0.438	0.717**	3	0.278	0.415*
AH59	7	0.563	0.758	7	0.706	0.751	7	0.375	0.807**	5	0.333	0.765
AH76	5	0.313	0.604**	4	0.353	0.471**	4	0.250	0.537*	4	0.222	0.634**
AH77	5	0.500	0.684*	6	0.706	0.740	7	0.438	0.762**	6	0.611	0.759
AH80	3	0.250	0.531	3	0.000	0.602**	2	0.063	0.404**	2	0.056	0.526**

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sampled individuals from each population.

[a] Voucher and locality information are provided in Appendix 1.

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

TABLE 3. Cross-amplification of the 10 polymorphic EST-SSR markers developed for *Artocarpus hypargyreus* in *A. nitidus* and *A. heterophyllus.*

Species	N	AH1	AH11	AH14	AH31	AH33	AH46	AH59	AH76	AH77	AH80
A. nitidus	5	+	+	+	+	+	+	+	+	+	+
A. heterophyllus	9	+	+	+	+	+	+	+	+	—	+

Note: + = primers could be successfully amplified in all individuals; — = primers could not be amplified in any individual; N = number of individuals.

Appendix 1. Voucher specimen information for *Artocarpus* populations used in this study. Specimens are deposited at the Herbarium of Sun Yat-sen University (SYSU), China.

Species	Population	Voucher no.	Collection locality	Geographic coordinates	*N*
A. hypargyreus Hance[a]		NLD20151219051	Neilingding Island, Shenzhen, China	22°24′29.85″N, 113°49′00.46″E	1
A. hypargyreus[b]	DGD	DGD20160112003	Zhuhai, Guangdong, China	22°02′28.51″N, 114°16′19.84″E	16
A. hypargyreus[b]	NLD	NLD20151219054	Shenzhen, Guangdong, China	22°24′36.23″N, 113°48′20.35″E	17
A. hypargyreus[b]	XG	JSGY20160119087	Hong Kong, China	22°21′19.29″N, 111°53′25.96″E	16
A. hypargyreus[b]	HSD	HSD20151223124	Fengkai, Zhaoqing, Guangdong, China	23°27′25.60″N, 113°48′20.35″E	18
A. nitidus Trécul[c]	Cultivated	SYSU2015060501	SYSU, Guanghzhou, China	23°5′51.88524″N, 113°18′4.34″E	5
A. heterophyllus Lam.[c]	Cultivated	SYSU2015060502	SYSU, Guanghzhou, China	23°5′51.88524″N, 113°18′4.34″E	9

Note: *N* = number of individuals sampled.
[a] Samples used for cDNA library construction.
[b] Samples used for initial PCR amplification trials and detailed evaluation for polymorphisms.
[c] Samples used for transferability test.

Appendix 2. Characteristics of 24 monomorphic EST-SSR markers in *Artocarpus hypargyreus.*

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Expected allele size (bp)	GenBank accession no.
Art_SSR2	F: CACACAAAATTCGTGCCCATTA	$(GGA)_8$	207	KX495096
	R: TCCTGAGGTTTTGGCTGCTGTT			
Art_SSR3	F: CCAACAAACAGGGCCAACTCAA	$(CCA)_5$	164	KX495097
	R: ATGTCGCCAAGGGAGCTGTATC			
Art_SSR4	F: TGGTGGTGGATGATGCACAATT	$(GTG)_6$	248	KX495098
	R: CGTCTCAATCTACCTTCGCATA			
Art_SSR6	F: TTGAGGCAGGGTGGATGTAATC	$(AG)_6$	188	KX495099
	R: TGTTTCTTTTGCATCCTTCTTC			
Art_SSR8	F: TGGCATCAACGCGGAAGGATAT	$(GAA)_5$	248	KX495100
	R: CCCCTCATCCTTCACCCTTCC			
Art_SSR12	F: TGACAACCATGGGCACGATCAT	$(CAT)_5$	215	KX495102
	R: TGTCAGCCAGAACATGCAAGAA			
Art_SSR24	F: AGGCTCAAAAGGGTGGCAATAA	$(GCA)_5$	204	KX495104
	R: GGGTGTTGGAGTTGGGCATCAT			
Art_SSR25	F: GGTACACAGCCGCGAAGAATAA	$(TC)_6$	227	KX495105
	R: CACTTTTCACCACCACCAAACA			
Art_SSR27	F: AAGGTGGCAGAGCAGAGGAGTC	$(AT)_8$	218	KX495106
	R: ACCAAGCAAGAACCAGGTCAGC			
Art_SSR32	F: GGCACGGTTCTCAAGCCTGAA	$(GAA)_5$	234	KX495108
	R: TCATGATCAATCCAGGCACAAA			
Art_SSR36	F: GGGCCTCGACGAGCACTCTATC	$(CCG)_5$	124	KX495110
	R: GGCAGCGTGGAACCGATCTG			
Art_SSR39	F: CAACAGCCCATACGTCGGATCT	$(TAG)_5$	174	KX495111
	R: CTCTCTGAGGGTCCCACTATTC			
Art_SSR40	F: TCGCCGCTGTCCTCGTCTTC	$(ACC)_5$	145	KX495112
	R: CCCTCTACCGTACGATCCTCAT			
Art_SSR43	F: GCAAGCAGACAGTGGGGAGATA	$(AAG)_6$	232	KX495113
	R: GGTTGAGCCTTCTTCGCGTACA			
Art_SSR49	F: AACAGCACCGTCAATGGAACTT	$(GA)_6$	202	KX495115
	R: CCTTGCAGCCTCCCGAGCTATC			
Art_SSR52	F: TCCCCTGGAGCCTGATGAGTTT	$(TC)_{10}$	225	KX495116
	R: CGCGAACTGAAAAGGGGTTATG			
Art_SSR55	F: GAGTTCAGCCCAGCCTGCA	$(TA)_8$	137	KX495117
	R: TTGCCAAAACACATGAAACAGT			
Art_SSR56	F: AAGACCCGGAAGAAAGGAAAGA	$(GA)_7$	173	KX495118
	R: CCCTCTGTAACGACTGTGATTT			
Art_SSR58	F: GCAAGGGGAAGCTGAGGGTATA	$(GA)_6$	239	KX495119
	R: AGGCCTTTTCTCGCTCCTCAAA			
Art_SSR61	F: TTACCCTAATAGCCGCCGATTT	$(TTA)_5$	199	KX495121
	R: AGTAGCGCTCCAATGCCATTCA			
Art_SSR62	F: GAAGGGACGGAGGGGAGAGTTT	$(GCG)_5$	208	KX495122
	R: CCGGCATGAGGGTCCAAATCAA			
Art_SSR67	F: TCTTCTTCGGAGCTGGCATAGA	$(CGC)_5$	179	KX495123
	R: AGGAGGTTCGCTTGTCCTTGTT			
Art_SSR72	F: ATGGGTGAGGAGAGCGTGATGA	$(AG)_7$	146	KX495124
	R: TTCTCCTTTCGCTTCCCCTTCT			
Art_SSR84	F: TGACCACCATCACCACCAAAAC	$(ACA)_7$	165	KX495128
	R: GCAGCCAAGAGACGGTGGTAAT			

[a] Annealing temperature for all loci was 55°C.