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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS FOR *EURYA ACUMINATISSIMA* (THEACEAE)[1]

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- *Premise of the study:* Sixteen microsatellite markers were developed to study the fine-scale spatial genetic structure of *Eurya acuminatissima*, a dioecious tree species of Theaceae endemic to southern China.
- *Methods and Results:* A total of 30 primer pairs were initially designed and tested on the basis of the transcriptome data of *E. acuminatissima*, of which 16 were successfully amplified and showed clear polymorphism. For these microsatellites, one to 17 alleles per locus were identified. The observed and expected heterozygosities ranged from 0 to 1.000 and 0 to 0.903, respectively.
- *Conclusions*: The microsatellite markers described here can be used to study genetic diversity and spatial genetic structure of *E. acuminatissima*. Furthermore, all loci were successfully cross-amplified in a related species, *E. auriformis*.

Key words: *Eurya acuminatissima*; microsatellite marker; Theaceae; transcriptome.

Eurya Thunb., a genus in the family Theaceae, is mainly distributed in tropical and subtropical Asia, including the southern and western Pacific Islands (Ling, 1998; Ming and Bartholomew, 2007). There are about 83 species in China, of which 63 are endemic (Ming and Bartholomew, 2007). *Eurya* species are dioecious, insect-pollinated, and bird-dispersed small trees that constitute an important component in forests from low to middle elevations. To date, little is known about the genetic diversity, spatial genetic structure, reproductive biology, and ecological adaptations of species in the genus (Chung and Epperson, 2000; Wang et al., 2014; Mishio and Kawakubo, 2015). In particular, microsatellite markers for genetic analysis in the genus *Eurya* are not available.

Eurya acuminatissima Merr. & Chun, a species endemic to China, grows in forests on mountain slopes or in valleys from 200–1200 m and is a common component in the understory of old-growth and secondary evergreen broad-leaved forests in southern China. In this study, we developed 16 nuclear microsatellite markers for our ongoing research project regarding *E. acuminatissima*, in which we are investigating its genetic diversity and spatial genetic structure in a typical evergreen broad-leaved forest mountain area of southern China. We also tested the transferability of these markers in a congeneric species, *E. auriformis* H. T. Chang.

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METHODS AND RESULTS

Total RNA of *E. acuminatissima* was extracted from fresh leaves of one seedling using an improved cetyltrimethylammonium bromide (CTAB) method (Fu et al., 2005). The seedling was collected from Heishiding Nature Reserve, Guangdong Province, China (23°27′37.39″N, 111°54′9.78″E). Transcriptome sequencing of *E. acuminatissima* was conducted using the Illumina HiSeq 2500 system (Illumina, San Diego, California, USA). In total, 16,323,790 nucleotide paired-end reads were obtained and assembled into 143,640 nonredundant unigenes with an N50 length of 610 nucleotides using Trinity (Grabherr et al., 2011). The reads were then deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession no. SRR5512705). The redundant sequences were removed by CAP3 (Huang and Madan, 1999) with the criterion of a minimum identity of 99%.

All unigenes obtained in the study were used to screen for the presence of microsatellites using MISA (Thiel et al., 2003), with the criteria of a minimum of six, five, five, five, and five repeat units for di-, tri-, tetra-, penta-, and hexanucleotide motifs, respectively. Altogether, 23,872 simple sequence repeat (SSR) motifs were detected. Using Primer3 (Rozen and Skaletsky, 1999), 30 primer pairs were designed on the basis of randomly selected SSR motifs with the optimum conditions set at a length of 22–25 bp and a product size range of 100–500 bp.

Genomic DNA was isolated from silica-dried leaves of 83 individuals from three populations of *E. acuminatissima* and 27 individuals from one population of its congener *E. auriformis* using the DNA Extraction Kit (Magen, Guangzhou, China) following the manufacturer's protocol. All specimens are deposited at the Herbarium of Sun Yat-sen University (SYSU), Guangdong, China (Appendix 1). In the first PCR trial, five individuals were randomly selected from each population of *E. acuminatissima* to amplify the 30 primer pairs. PCR amplifications were performed according to Xie et al. (2015), except for the annealing temperatures as indicated in Table 1 for 45 s. PCR products were visualized in a 6% polyacrylamide gel with a 10-bp DNA ladder marker. Sixteen primer pairs produced PCR products with clear and polymorphic bands among the 15 individuals. The sequences of microsatellite loci were deposited into GenBank (Table 1).

TABLE 1. Characteristics of 16 microsatellite loci developed in *Eurya acuminatissima*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	Fluorescent label	GenBank accession no.	Putative function	*E*-value
Ea-1374	F: TGGTTTTCTTGGGTTGCTTACT R: CCTTTGCTTACTTCTGCTTTTG	$(CT)_{18}$	290	60	JOE	KY807054	Homeobox-leucine zipper protein ATHB-6 [*Vitis vinifera*]	7e-25
Ea-1519	F: TCTGAAAACCTTACAACGCTACC R: GGTAACCACTGAATCCTTCGAG	$(CA)_{17}$	261	60	FAM	KY807055	Zinc finger and BTB domain-containing protein 21 [*Poecilia mexicana*]	1e-11
Ea-1597	F: GAACACATTTCAAAGGGGGATA R: AGGAAGAAGAAACTGAAACCCC	$(TTC)_7$	289	60	FAM	KY807056	Probable nucleolar protein 5-2 [*Vitis vinifera*]	0.000
Ea-1853	F: CACACCATTCCTGAGGACTACA R: GGTCATTCCATTCCATTCTCTT	$(TCCC)_6$	453	61	JOE	KY807057	BZIP transcription factor [*Camellia sinensis*]	2e-111
Ea-46130	F: TTCAGGTGAAAACGACGATATG R: CCCCTGAAACCCTAATTTTCTC	$(AT)_8(AG)_{14}$	360	60	FAM	KY807058	Chromatin modification-related protein EAF7 [*Vitis vinifera*]	0.000
Ea-46976	F: AACGAAACCCTAGAATCGATCA R: GAGTTCGCTCTCTGTACGAAAAA	$(GA)_{23}$	267	60	JOE	KY807059	E3 ubiquitin-protein ligase SIS3 isoform X3 [*Vitis vinifera*]	0.000
Ea-47249	F: ATTGGTCATCGTTTCGGTATTC R: CCTCATTCCCTCACTCAATTTC	$(GAA)_6$	210	60	FAM	KY807060	Uncharacterized protein [*Juglans regia*]	4e-174
Ea-47797	F: CTTTCACACCCCTTCCATATTC R: CAATGACGAGGCAGAGTATCAG	$(ATCG)_5$	403	61	JOE	KY807061	Hypothetical protein [*Citrus clementina*]	0.000
Ea-35287	F: TTTTCATAGGGCTATTCTCGGA R: GTGTGAGCAAATCAGAAGGAAA	$(TTTC)_6$	160	60	JOE	KY807062	RalA-binding protein 1 [*Aedes albopictus*]	0.026
Ea-31862	F: CACATCGTTCGCACTCATAAAG R: TGTAGACGCGGGGAGTAGTAAT	$(TTCC)_5$	100	59	FAM	KY807063	3-ketoacyl-CoA synthase [*Ricinus communis*]	6e-11
Ea-33987	F: ACAAGATGGTGATGATGATTCG R: TCATTCGAAGGGTGTGGTTGTAGTT	$(CTCAT)_5$	205	60	FAM	KY807064	Uncharacterized protein LOC100246622 isoform X2 [*Vitis vinifera*]	2e-152
Ea-804	F: GCAAGTTGGGTAAAATCGATGAAG R: CTTCAATGCAACCTAGCCCTTACCT	$(TGTC)_5$	267	60	JOE	KY807065	Uncharacterized protein [*Theobroma cacao*]	2e-15
Ea-27742	F: CACTCAATTCGTCCAATGACAAACA R: CCAAGCTTTTGATGCCTGATATTGA	$(TTTA)_5$	135	59	FAM	KY807066	MDIS1-interacting receptor like kinase 2 [*Jatropha curcas*]	3e-08
Ea-24991	F: CAAAAGTGTTGACCACAGTGCTGAT R: CTAGGGACACCCTCGAGTAGTCACA	$(TCTT)_5$	339	60	FAM	KY807067	Oligosaccharide repeat unit polymerase Wzy [*Streptococcus pneumoniae*]	0.26
Ea-97085	F: CTGCTGTTGCTGTTGCTGTT R: CTGCATCAGTTGGTATCAGAGC	$(TGC)_8$	215	60	FAM	KY829135	Hypothetical protein [*Monoraphidium neglectum*]	2e-14
Ea-98287	F: GAAACACAGTCCCTGGAAGAAC R: CTGCATCAGTTGGTATCAGAGC	$(CT)_{23}$	161	60	JOE	KY829136	Uncharacterized protein, transcript variant X2 [*Macaca nemestrina*]	2e-16

Note: T_a = annealing temperature.

The 16 polymorphic primer pairs were tested for polymorphisms in 83 individuals from three populations of *E. acuminatissima*. In addition, 27 individuals from one population of *E. auriformis* were also used to detect the efficiency of these markers in cross-species amplification. PCR products were analyzed using the ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, California, USA), resolved with an internal size standard (GeneScan 500 LIZ; Applied Biosystems). The peaks of the loci were read by Peak Scanner Software version 1.0 (Applied Biosystems). PCR amplifications were performed in a final volume of 20 µL, containing 20 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 (5′ labeled with FAM or JOE; Life Technologies, Shanghai, China), and 1 unit EasyTaq DNA polymerase (TransGen Biotech Co.). The PCR reactions were carried out in a 2720 Thermal Cycler (Applied Biosystems) under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, then annealing for 45 s at the optimal temperature for each primer pair (Table 1). Number of alleles, observed heterozygosity, unbiased expected heterozygosity, and fixation index were obtained using GenAlEx version 6.5 (Peakall and Smouse, 2012). Deviations from Hardy–Weinberg equilibrium (HWE) at each locus in each population were analyzed with GENEPOP version 4.3 (Rousset, 2008). In *E. acuminatissima*, the number of alleles per locus ranged from one to 17, the observed heterozygosity ranged from 0 to 1.000, and the expected heterozygosity ranged from 0 to 0.903 (Table 2). Of the 16 polymorphic SSR loci, six, 11, and six loci showed significant deviations from HWE in the Zhaoqing, Huizhou, and Yingde populations, respectively (Table 2). In *E. auriformis*, the number of alleles per locus ranged from two to 12, observed heterozygosity ranged from 0 to 0.889, and expected heterozygosity ranged from 0.036 to 0.850 (Table 2). The genotype frequencies at four out of 16 polymorphic microsatellite loci were in HWE (Table 2).

CONCLUSIONS

The 16 microsatellites of *E. acuminatissima* reported here are useful to investigate the genetic diversity and population structure of this species. We are currently using these markers to investigate fine-scale spatial genetic structure and to estimate gene flow among populations of *E. acuminatissima* in a 50-ha plot in Heishiding Nature Reserve, Guangdong Province, China. The successful transferability of these markers in its congeneric species *E. auriformis* suggests that they may be useful in studies of other related species in *Eurya*.

LITERATURE CITED

CHUNG, M. G., AND B. K. EPPERSON. 2000. Clonal and spatial genetic structure in *Eurya emarginata* (Theaceae). *Heredity* 84: 170–177.

FU, X., Y. HUANG, S. DENG, R. ZHOU, G. YANG, X. NI, W. LI, AND S. SHI. 2005. Construction of a SSH library of *Aegiceras corniculatum* under salt stress and expression analysis of four transcripts. *Plant Science* 169: 147–154.

GRABHERR, M. G., B. J. HAAS, M. YASSOUR, J. Z. LEVIN, D. A. THOMPSON, I. AMIT, X. ADICONIS, ET AL. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology* 29: 644–652.

HUANG, X., AND A. MADAN. 1999. CAP3: A DNA sequence assembly program. *Genome Research* 9: 868–877.

TABLE 2. Results of initial primer screening of 16 microsatellite loci developed in *Eurya acuminatissima* in three populations of *E. acuminatissima* and one population of *E. auriformis*.[a]

	E. acuminatissima												*E. auriformis*			
	Zhaoqing (*N* = 30)				Huizhou (*N* = 28)				Yingde (*N* = 25)				Huizhou (*N* = 27)			
Locus	*A*	H_o	H_e	*F*[b]	*A*	H_o	H_e	*F*[b]	*A*	H_o	H_e	*F*[b]	*A*	H_o	H_e	*F*[b]
Ea-1374	10	0.733	0.863	0.150	14	0.571	0.893	0.360***	9	0.600	0.802	0.252***	10	0.630	0.850	0.259**
Ea-1519	14	0.900	0.884	−0.018	4	0.321	0.544	0.409***	7	0.600	0.729	0.177	4	0.074	0.173	0.571***
Ea-1597	4	0.167	0.214	0.221	2	0.000	0.137	1.000***	2	0.000	0.077	1.000***	3	0.077	0.500	0.846**
Ea-1853	8	0.483	0.773	0.375**	5	0.000	0.621	1.000***	4	0.040	0.252	0.841	7	0.038	0.752	0.949***
Ea-46130	14	0.600	0.898	0.332***	8	0.036	0.842	0.958***	10	0.200	0.794	0.748	10	0.296	0.842	0.648***
Ea-46976	14	0.767	0.845	0.093***	10	0.571	0.767	0.255***	8	0.560	0.745	0.248	8	0.704	0.675	−0.043**
Ea-47249	4	0.400	0.638	0.373**	3	0.036	0.409	0.913***	3	0.000	0.538	1.000***	2	0.000	0.071	1.000***
Ea-47797	4	0.276	0.392	0.296	3	0.464	0.420	−0.105	3	0.320	0.422	0.242	5	0.148	0.267	0.445**
Ea-35287	4	0.333	0.407	0.181	4	0.393	0.457	0.140	4	0.400	0.425	0.058	4	0.296	0.680	0.564***
Ea-31862	2	0.133	0.124	−0.071	3	0.393	0.493	0.203**	2	0.320	0.269	−0.190	3	0.037	0.204	0.818***
Ea-33987	3	0.107	0.427	0.749***	3	0.179	0.166	−0.073	5	0.080	0.288	0.722***	3	0.148	0.261	0.432***
Ea-804	5	0.600	0.554	−0.083	4	0.286	0.559	0.489***	2	0.080	0.211	0.621**	5	0.481	0.501	0.038
Ea-27742	1	0.000	0.000	—	1	0.000	0.000	—	2	0.080	0.077	−0.042	2	0.037	0.036	−0.019
Ea-24991	3	0.333	0.335	0.005	4	0.214	0.197	−0.087	4	0.320	0.368	0.130	2	0.259	0.226	−0.149
Ea-97085	2	1.000	0.500	−1.000**	6	1.000	0.586	−0.707***	2	1.000	0.500	−1.000***	7	0.889	0.701	−0.268**
Ea-98287	17	0.893	0.878	−0.017	15	0.963	0.903	−0.067*	14	0.952	0.880	−0.082	12	0.778	0.807	0.037

Note: *A* = number of alleles; *F* = fixation index; 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.

LING, L. K. 1998. Theaceae (Ternstroemioideae). *In* L. K. Ling [ed.], Flora Reipublicae Popularis Sinicae, vol. 50(1), 70–176. Science Press, Beijing, China.

MING, T. L., AND B. BARTHOLOMEW. 2007. Theaceae. *In* Z. Y. Wu and P. H. Raven [eds.], Flora of China, vol. 12, 447–478. Science Press, Beijing, China, and Missouri Botanical Garden Press, St. Louis, Missouri, USA.

MISHIO, M., AND N. KAWAKUBO. 2015. Variations in leaf morpho-anatomy and photosynthetic traits between sun and shade populations of *Eurya japonica* (Pentaphylacaceae) whose seeds are dispersed by birds across habitats. *Plant Species Biology* 30: 147–158.

PEAKALL, R., AND P. E. SMOUSE. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research–An update. *Bioinformatics (Oxford, England)* 28: 2537–2539.

ROUSSET, F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

ROZEN, S., AND H. SKALETSKY. 1999. Primer3 on the WWW for general users and for biologist programmers. *In* S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

THIEL, T., W. MICHALEK, R. K. VARSHNEY, AND A. GRANER. 2003. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* 106: 411–422.

WANG, Q., H. P. DENG, B. DING, AND G. L. ZHOU. 2014. Flower structure and sex expression of leaky dioecy in *Eurya obtusifolia. Nordic Journal of Botany* 32: 314–319.

XIE, H., Y. YUAN, X. FANG, Y. LIU, C. YANG, J. JIN, F. TAN, AND Y. HUANG. 2015. Development of EST-SSR markers in *Barringtonia racemosa* (Lecythidaceae) and cross-amplification in related species. *Applications in Plant Sciences* 3: 1500080.

APPENDIX 1. Voucher and location information for the species and populations used in this study.[a]

Species	Population code	Voucher no.	Collection locality	Geographic coordinates	*N*
Eurya acuminatissima Merr. & Chun	Zhaoqing	Shixg150801	Heishiding Nature Reserve, Zhaoqing, Guangdong, China	23°27′37.39″N, 111°54′9.78″E	30
	Huizhou	Shixg161203	Nankunshan Nature Reserve, Huizhou, Guangdong, China	23°38′17.60″N, 113°50′47.79″E	28
	Yingde	Shixg161210	Shimentai Nature Reserve, Yingde, Guangdong, China	24°23′38.18″N, 113°09′4.66″E	25
E. auriformis H. T. Chang	Huizhou	Shixg161205	Nankunshan Nature Reserve, Huizhou, Guangdong, China	23°38′17.60″N, 113°50′47.79″E	27

Note: *N* = number of individuals sampled.

[a] Voucher specimens are deposited at the herbarium of Sun Yat-sen University (SYSU), Guangzhou, China.