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DEVELOPMENT AND CHARACTERIZATION OF EST-SSR MARKERS FOR *OTTELIA ACUMINATA* VAR. *JINGXIENSIS* (HYDROCHARITACEAE)¹

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- *Premise of the study:* Simple sequence repeat (SSR) markers were derived from transcriptomic data for *Ottelia acuminata* (Hydrocharitaceae), a species comprising five endemic and highly endangered varieties in China.
- *Methods and Results:* Sixteen novel SSR markers were developed for *O. acuminata* var. *jingxiensis*. One to eight alleles per locus were found, with a mean of 2.896. The observed and expected heterozygosity ranged from 0.000 to 1.000 and 0.000 to 0.793, respectively. Interestingly, in cross-varietal amplification, 13 out of the 16 loci were successfully amplified in *O. acuminata* var. *acuminata*, and 12 amplified in each of the other three varieties of *O. acuminata*.
- *Conclusions:* These newly developed SSR markers will facilitate further study of genetic variation and provide important genetic data needed for appropriate conservation of natural populations of all varieties of *O. acuminata*.

Key words: expressed sequence tag–simple sequence repeat (EST-SSR); Hydrocharitaceae; microsatellite; *Ottelia acuminata* var. *jingxiensis*; transcriptome.

Ottelia acuminata (Gagnep.) Dandy (Hydrocharitaceae), a herbaceous perennial, is mainly distributed in the Yunnan–Guizhou Plateau (YGP) and adjacent areas (Chen et al., 2017). Currently, five varieties are recognized under this species based on morphological characters and phylogenetic analysis (Chen et al., 2017), including *O. acuminata* var. *acuminata*, *O. acuminata* var. *jingxiensis* H. Q. Wang & S. C. Sun, *O. acuminata* var. *lunanensis* H. Li, *O. acuminata* var. *crispa* (Hand.-Mazz.) H. Li, and *O. acuminata* var. *songmingensis* Z. T. Jiang, H. Li & Z. L. Dao. All of these varieties, with the exception of *O. acuminata* var. *acuminata*, are each restricted either to a lake, a stream, or a small river (Li, 1981).

Ottelia acuminata var. *jingxiensis*, which can be distinguished from the other varieties by its abundant flowers in the spathe, is distributed in a single river system in Guangxi Province (Wang et al., 1992). This variety is incredibly sensitive to water pollution, and as a result of habitat degradation and human disturbance, most of its previously known natural populations continue to decline and are gradually becoming extinct in many locations (Zhi-Zhong

Li, personal observation, 2017). The five varieties of *O. acuminata* have been recorded as endemic and highly endangered species in China (SEPA and IBCAS, 1987). Therefore, investigating the levels and distribution of genetic diversity will be vital for the conservation and management strategies of this species.

Xu et al. (2012) and Lu et al. (2014) reported eight and nine polymorphic microsatellite primers developed from two and three populations of *O. acuminata* var. *acuminata*, respectively. The two studies applied the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) technique. However, when we tested these primers, we found low (<50%) amplification efficiency from our tests, in which only eight primers were successfully amplified in *O. acuminata* var. *jingxiensis*. Hence, more effective simple sequence repeat (SSR) markers are needed for this group. Here, 16 novel SSR markers were developed from transcriptomic analysis of *O. acuminata* var. *jingxiensis*. Subsequently, marker validation tests were conducted on individuals from three populations of *O. acuminata* var. *jingxiensis*, and transferability tests were performed on five individuals from each of the other four varieties of *O. acuminata*.

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METHODS AND RESULTS

Living individuals of *O. acuminata* var. *jingxiensis* were sampled from Jingxi, Guangxi Province, China, and transplanted in a greenhouse at Wuhan Botanical Garden, Chinese Academy of Sciences. Total RNA was extracted from fresh leaves using RNAsiso Plus (TaKaRa Biotechnology Co., Dalian, China) following the manufacturer's instructions. Quality control and library preparation were

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TABLE 1. Characteristics of 16 microsatellite loci developed for *Ottelia acuminata* var. *jingxiensis*.^a

Locus	Primer sequences (5'–3')	Repeat motif	Allele size range (bp)	GenBank accession no.
JX01	F: AACTTCTCTGCTGAGTGCCG R: GTCAAACCTGCTTTCTCGCCG	(TAT) ₅	231–260	MF580394
JX04	F: CCCCAATGGATAGCACCAACA R: CATAGTCCACAGCCTGACGG	(CAA) ₅	211–212	MF580395
JX06	F: TCCCTCTGTAAATCCACGCG R: CCGAGCTTTATCGCCAGAGT	(AG) ₇	263–289	MF580396
JX08	F: AGAGTGGATCCTCTGCTCTGT R: TCGATTCCAAAATCCAACCTTATGG	(T) ₁₀	335–346	MF580397
JX09	F: GAATACCTGTAGACCGCCGG R: GGATGACCAAGGGGATCGTC	(CCG) ₅	228–244	MF580398
JX10	F: TCGTCGCTTACCACTCTCTGC R: AGACGCGACCGAAGTTTCT	(TA) ₆	140–182	MF580399
JX12	F: ACATGAAGGACCTTGCAGCA R: GAAGATTGCAGGGACCGAGT	(A) ₁₀	227–261	MF580400
JX14	F: CGGGGACGCTGAAGTTAAA R: CGCTTCCATTTCGTACACGGA	(A) ₁₀	241–259	MF580401
JX15	F: TCGAAGCGGCTTCCTTCAAT R: GACCTTAACAGGCCCTGAC	(G) ₁₁	247–259	MF580402
JX16	F: GGAAGAGGGGTTAAGTGCC R: TGATGACAAGTAGAACATTAATCAAGA	(T) ₁₀	169–201	MF580403
JX19	F: CTAGGGTTTCGCGCACACT R: TCCTCTCTGTATTAATCGCCTTCA	(GA) ₈	257–280	MF580404
JX23	F: AAATGCGACCATTAGCACGC R: TTGCAGCACTTCCTCCGAAA	(T) ₁₀	238–249	MF580405
JX24	F: CTACGTCGACATCGGAGGTG R: CTTGCAACCTCAAAAACCGCG	(TG) ₆	263–264	MF580406
JX25	F: TACCGCAAGAAGAAGCCCTC R: ATCATCCTCATCCTCCGGGG	(AAG) ₅	181–216	MF580407
JX26	F: CTACTGGGCATTCAGGGTCG R: TTGCAGGTGAAGCTCTCCAG	(AGC) ₅	185–187	MF580408
JX29	F: TCGAACTTGACGGACTTGGG R: ACAATGCTAAAGGTGGCGGA	(TGC) ₅	215–220	MF580409

^aAnnealing temperature (T_a) was 60°C for all loci.

performed following Li et al. (2017). The cDNA library was constructed and sequenced using the Illumina HiSeq X Ten (Illumina, San Diego, California, USA) to produce 150-bp paired-end reads. The generated raw reads were subjected to the stringent filtering process, and a total of 53,865,436 clean reads were generated and assembled de novo into 77,758 contigs (N50 = 1473 bp) using Trinity (version trinityrnaseq_r2012-10-05; Grabherr et al., 2011) with its default parameters. Raw transcriptome data were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA; BioProject no. PRJNA404046, BioSample no. SAMN07629055).

SSRs were detected using the perl script MISA (Thiel et al., 2003) with default settings. A total of 6376 SSRs were identified. Among them, trinucleotide repeats (2490, 39.05%) were the most common, followed by dinucleotide repeats (2306, 36.17%), and tetra-, penta-, and hexanucleotide repeats constituted 24.78%. Subsequently, all SSRs were selected for primer design using Primer3 software (Rozen and Skaletsky, 1999). Primer pairs were selected based on the optimum length of 20 bp (18–27 bp), annealing temperatures of 55–65°C (optimal temperature = 60°C), and a product size range of 100–300 bp. Finally, 3794 novel primer pairs were successfully designed for the same

TABLE 2. Polymorphism of the 16 EST-SSRs in populations of *Ottelia acuminata* var. *jingxiensis*.^a

Locus	Jingxi ($N = 15$)			Debao ($N = 15$)			Yongfu ($N = 22$)		
	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
JX01	1	0.000	0.000	2	0.067	0.064*	2	0.000	0.095*
JX04	2	0.067	0.420	2	0.067	0.180*	2	0.045	0.325*
JX06	4	0.467	0.540	1	0.000	0.000	3	0.864	0.571
JX08	1	0.000	0.000	2	0.000	0.124*	2	0.000	0.499*
JX09	3	0.400	0.540	3	0.133	0.127	1	0.000	0.000
JX10	3	0.067	0.184	2	0.000	0.498*	4	0.238	0.715*
JX12	2	0.000	0.498*	2	0.000	0.133*	3	0.048	0.398*
JX14	5	0.909	0.653*	3	0.533	0.480*	3	0.000	0.244*
JX15	8	0.933	0.713*	4	1.000	0.668*	6	0.909	0.793
JX16	2	0.000	0.408*	2	0.000	0.320*	4	0.136	0.462*
JX19	4	0.733	0.629*	5	0.214	0.724*	5	0.800	0.729*
JX23	3	0.133	0.127	3	0.000	0.427*	2	0.091	0.087
JX24	2	0.000	0.459*	2	0.133	0.498	2	0.048	0.046
JX25	2	0.000	0.498*	2	0.000	0.480*	4	0.350	0.581*
JX26	3	0.000	0.498*	1	0.000	0.000	2	0.000	0.363*
JX29	4	1.000	0.629	4	0.714	0.686*	5	0.773	0.735

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

^aLocality and voucher information are provided in Appendix 1.

*Significant deviation from Hardy–Weinberg equilibrium ($P < 0.001$).

TABLE 3. Cross-amplification results for the 16 microsatellite loci developed in two populations of *Ottelia acuminata* var. *jingxiensis* and four other varieties of *O. acuminata*.^{a,b}

Locus	<i>O. acuminata</i> var. <i>acuminata</i>	<i>O. acuminata</i> var. <i>crispa</i>	<i>O. acuminata</i> var. <i>jingxiensis</i>		<i>O. acuminata</i> var. <i>lunanensis</i>	<i>O. acuminata</i> var. <i>songmingensis</i>
			Luzhai	Du'an		
JX01	+	+	+	+	—	—
JX04	+	+	+	+	+	+
JX06	+	+	+	+	+	+
JX08	—	+	+	+	—	+
JX09	+	+	+	+	+	+
JX10	+	+	+	+	+	+
JX12	+	+	+	+	+	+
JX14	+	+	+	+	+	+
JX15	+	+	+	+	+	+
JX16	—	—	+	+	—	—
JX19	+	+	+	+	+	+
JX23	+	+	+	+	+	+
JX24	+	+	+	+	+	+
JX25	+	—	+	+	+	—
JX26	—	—	+	+	—	—
JX29	+	—	+	+	+	+

Note: + = successful amplification in all individuals; — = no amplification; *N* = number of samples.

^aLocality and voucher information are provided in Appendix 1.

^b*N* = 5 for all populations.

number of SSR loci, out of which 60 loci were randomly selected for further amplification tests, including the degree of polymorphism, in 52 samples from three populations of *O. acuminata* var. *jingxiensis* (Appendix 1). These samples were collected across the range of the species in China.

A total of 62 individuals of *O. acuminata* var. *jingxiensis* were sampled, including 10 samples that were used to check cross-varietal amplification, while only five individuals were sampled from each of the other remaining varieties. Total genomic DNA was extracted from the dry leaves using the Plant Genomic DNA Isolation Kit (Tiangen, Beijing, China). To filter the SSR primers, preliminary tests were conducted on three individuals from each population using all 60 primer pairs. PCR amplifications were carried out following Gichira et al. (2017), with an annealing temperature of 55°C. The PCR products were detected by 2% agarose gel electrophoresis to assess if the expected size was produced for each primer. Sixteen of the 60 tested primer pairs were selected based on PCR amplification success rate and band length difference. The forward primer of each pair was labeled with FAM fluorescent dye (Table 1). The modified PCR reactions were similarly conducted following the same protocol, and the products were analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA) with an internal size standard (GeneScan 500 LIZ; Applied Biosystems). The allele sizes were assessed by GeneMapper 3.0 (Applied Biosystems). GenAlEx 6.5 (Peakall and Smouse, 2012) was used to calculate the deviation from Hardy–Weinberg equilibrium, number of alleles, and observed and expected heterozygosity.

The results showed that the number of alleles ranged from one to eight with a mean of 2.896. The observed and expected heterozygosity ranged from 0.000 to 1.000 and 0.000 to 0.793, respectively (Table 2). Fourteen loci showed significant deviations from Hardy–Weinberg equilibrium ($P < 0.001$). Twenty individuals from four varieties (*O. acuminata* var. *acuminata*, *O. acuminata* var. *crispa*, *O. acuminata* var. *lunanensis*, and *O. acuminata* var. *songmingensis*) and 10 samples of *O. acuminata* var. *jingxiensis* from different populations were used to check the transferability of the 16 markers. Out of the 16 loci, 13 were successfully amplified in *O. acuminata* var. *acuminata*, and 12 amplified in each of the other three varieties of *O. acuminata* (Table 3).

CONCLUSIONS

The 16 SSR markers developed here will facilitate further study on genetic variation in *O. acuminata*. Successful cross-transferability tests of the newly developed markers were conducted among varieties of *O. acuminata*. Therefore, these primers are crucial for further molecular research on *O. acuminata* to provide appropriate genetic information for their effective conservation.

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APPENDIX 1. Sampling information of *Ottelia acuminata* used in this study.

Species	Population code	<i>N</i>	Locality	Geographic coordinates	Voucher no. ^a
<i>O. acuminata</i> (Gagnep.) Dandy var. <i>jingxiensis</i> H. Q. Wang & S. C. Sun	JX	15	Jingxi, Guangxi	23°12'N, 106°18'E	HIB-Otte010
	DB	15	Debao, Guangxi	22°35'N, 106°16'E	HIB-Otte009
	BS	22	Yongfu, Guangxi	25°07'N, 109°44'E	HIB-Otte012
	LZ	5	Luzhai, Guangxi	24°42'N, 109°41'E	HIB-Otte013
	DA	5	Du'an, Guangxi	24°04'N, 108°03'E	HIB-Otte014
<i>O. acuminata</i> var. <i>acuminata</i>	HQ	5	Heqing, Yunan	26°24'N, 100°09'E	HIB-Otte003
<i>O. acuminata</i> var. <i>crispa</i> (Hand.-Mazz.) H. Li	LGH	5	Luguhu, Yunan	27°40'N, 100°46'E	HIB-Otte011
<i>O. acuminata</i> var. <i>lunanensis</i> H. Li	LN	5	Shilin, Yunnan	24°50'N, 103°27'E	HIB-Otte008
<i>O. acuminata</i> var. <i>songmingensis</i> Z. T. Jiang, H. Li & Z. L. Dao	SM	5	Songming, Yunnan	25°16'N, 102°52'E	HIB-Otte007

Note: *N* = number of individuals.

^aVoucher specimens from all populations were deposited in the herbarium of the Wuhan Botanical Garden (HIB), Chinese Academy of Sciences, Wuhan, Hubei Province, China.