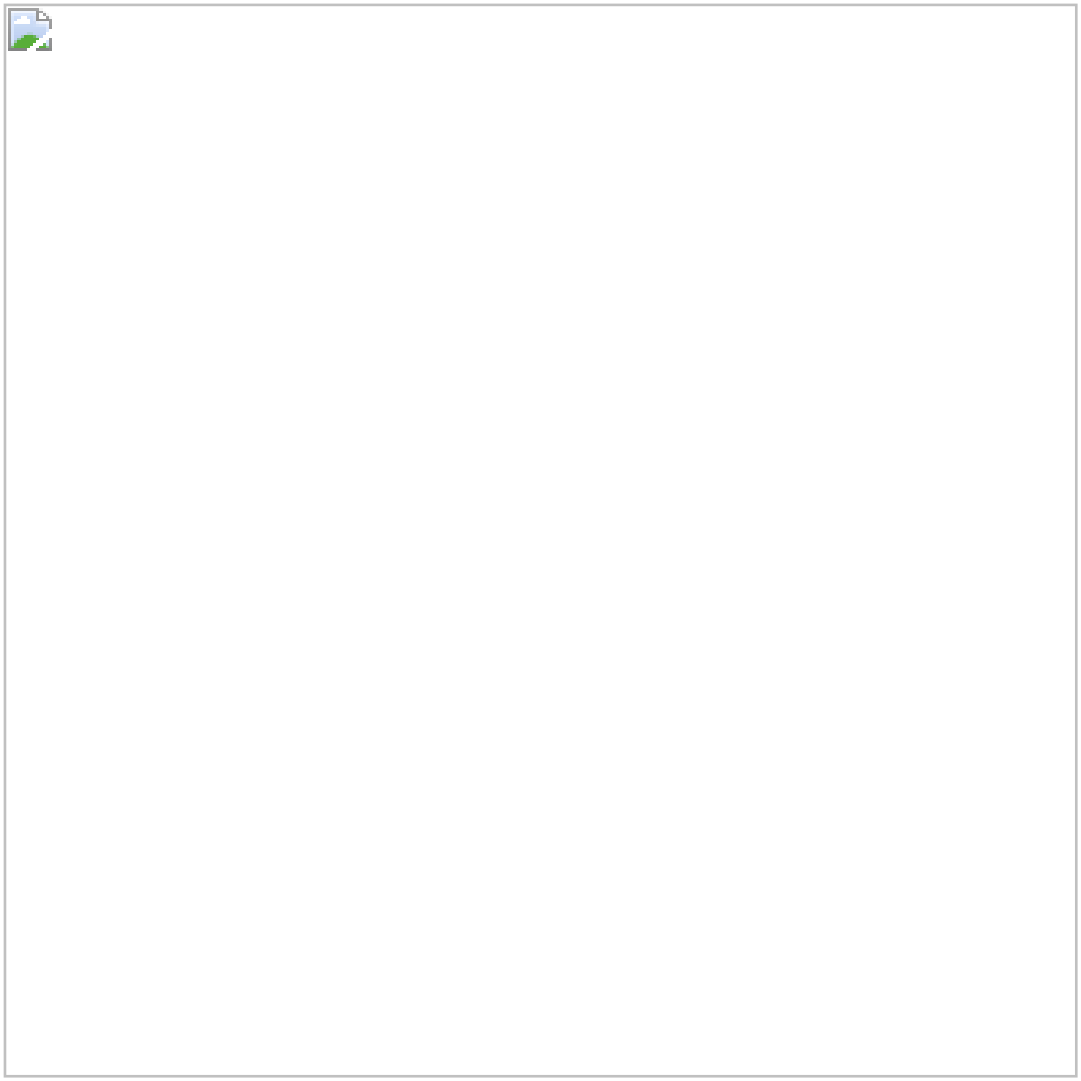

Development and Characterization of EST-SSR Markers for Ottelia acuminata var. jingxiensis (Hydrocharitaceae)

Authors: Li, Zhi-Zhong, Lu, Meng-Xue, Saina, Josphat K., Gichira, Andrew W., Wang, Qing-Feng, et al.

Source: Applications in Plant Sciences, 5(11)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1700083

Applications in Plant Sciences

Applications in Plant Sciences 2017 5(11): 1700083

Primer Note

Development and characterization of EST-SSR markers for *Ottelia acuminata* var. *jingxiensis* (Hydrocharitaceae)[1]

Zhi-Zhong Li[2,3], Meng-Xue Lu[4], Josphat K. Saina[2,3,5], Andrew W. Gichira[2,3,5], Qing-Feng Wang[2,5,6], and Jin-Ming Chen[2,6]

[2]Key Laboratory of Aquatic Botany and Watershed Ecology, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074, People's Republic of China; [3]University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China; [4]College of Life Science, South-Central University for Nationalities, Wuhan 430074, People's Republic of China; and [5]Sino-Africa Joint Research Center, Chinese Academy of Sciences, Wuhan 430074, People's Republic of China

- *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*.

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 RNAiso Plus (TaKaRa Biotechnology Co., Dalian, China) following the manufacturer's instructions. Quality control and library preparation were

[1]Manuscript received 2 August 2017; revision accepted 22 September 2017.

The authors thank Ying Zhang and Shi-Xu Huang for their assistance in the laboratory, and Wen Huang for his help in fieldwork. This work received financial support from the Strategic Priority Research Program of the Chinese Academy of Sciences (grant no. XDPB02) and the National Natural Science Foundation of China (grant no. 31570220).

[6]Authors for correspondence: qfwang@wbgcas.cn (Q.F.W.), jmchen@wbgcas.cn (J.M.C.)

doi:10.3732/apps.1700083

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	$(TAT)_5$	231–260	MF580394
	R: GTCAAACTGCTTTCTCGCCG			
JX04	F: CCCCAATGGATAGCACCAACA	$(CAA)_5$	211–212	MF580395
	R: CATAGTCCACAGCCTGACGG			
JX06	F: TCCCTCTGTAAATCCACGCG	$(AG)_7$	263–289	MF580396
	R: CCGAGCTTTATCGCCAGAGT			
JX08	F: AGAGTGGATCCTCTGCTCTGT	$(T)_{10}$	335–346	MF580397
	R: TCGATTCCAAAATCCAACCTTATGG			
JX09	F: GAATACCTGTAGACCGCCGG	$(CCG)_5$	228–244	MF580398
	R: GGATGACCAAGGGGATCGTC			
JX10	F: TCGTCGCTTACCATCTCTGC	$(TA)_6$	140–182	MF580399
	R: AGACGCGACCGAAGTTTTCT			
JX12	F: ACATGAAGGACCTTGCAGCA	$(A)_{10}$	227–261	MF580400
	R: GAAGATTGCAGGGACCGAGT			
JX14	F: CGGGGGACGCTGAAGTTAAA	$(A)_{10}$	241–259	MF580401
	R: CGCTTCCATTCGTACACGGA			
JX15	F: TCGAAGCGGCTTCCTTCAAT	$(G)_{11}$	247–259	MF580402
	R: GACCTTAACAGGCCCCTGAC			
JX16	F: GGAAGAGGGGGTTAAGTGCC	$(T)_{10}$	169–201	MF580403
	R: TGATGACAAGTAGAACATTAATCAAGA			
JX19	F: CTAGGGTTTCGCGACACACT	$(GA)_8$	257–280	MF580404
	R: TCCTCTCTGTATTTAATCGCCTTCA			
JX23	F: AAATGCGACCATTAGCACGC	$(T)_{10}$	238–249	MF580405
	R: TTGCAGCACTTCCTCCGAAA			
JX24	F: CTACGTCGACATCGGAGGTG	$(TG)_6$	263–264	MF580406
	R: CTTCGAACCTCAAAACCGCG			
JX25	F: TACCGCAAGAAGAAGCCCTC	$(AAG)_5$	181–216	MF580407
	R: ATCATCCTCATCCTCCGGGG			
JX26	F: CTACTGGGCATTCAGGGTCG	$(AGC)_5$	185–187	MF580408
	R: TTGCAGGTGAAGCTCTCCAG			
JX29	F: TCGAACTTGACGGACTTGGG	$(TGC)_5$	215–220	MF580409
	R: ACAATGCTAAAGGTGGCGGA			

[a]Annealing 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 trnityrnaseq_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]

	Jingxi (N = 15)			Debao (N = 15)			Yongfu (N = 22)		
Locus	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.
[a]Locality 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	*O. acuminata* var. *acuminata*	*O. acuminata* var. *crispa*	*O. acuminata* var. *jingxiensis*		*O. acuminata* var. *lunanensis*	*O. acuminata* var. *songmingensis*
			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.
[a] Locality 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.

LITERATURE CITED

CHEN, J. M., Z. Y. DU, Z. C. LONG, A. W. GICHIRA, AND Q. F. WANG. 2017. Molecular divergence among varieties of *Ottelia acuminata* (Hydrocharitaceae) in the Yunnan-Guizhou Plateau. *Aquatic Botany* 140: 62–68.

GICHIRA, A. W., Z. Z. LI, J. K. SAINA, G. W. HU, R. W. GITURU, Q. F. WANG, AND J. M. CHEN. 2017. Demographic history and population genetic structure of *Hagenia abyssinica* (Rosaceae), a tropical tree endemic to the Ethiopian highlands and eastern African mountains. *Tree Genetics & Genomes* 13: 72.

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.

LI, H. 1981. Classification, distribution and phylogeny of the genus *Ottelia. Acta Phytotaxonomica Sinica* 19: 29–42.

LI, Z., S. SUN, Q. WANG, AND J. CHEN. 2017. RNA-Seq analysis of the distylous plant *Nymphoides peltata* identified ortholog genes between long- and short-styled flowers. *Frontiers in Ecology and Evolution* 5: 59.

LU, R. R., Z. Y. YANG, C. C. TAO, S. T. CHEN, AND Y. H. JI. 2014. Microsatellites primer development for *Ottelia acuminata* (Hydrocharitaceae), a submerged macrophyte endemic to southwestern China. *Guihaia* 34: 34–37.

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.

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.

SEPA (STATE ENVIRONMENT PROTECTION ADMINISTRATION) AND IBCAS (INSTITUTE OF BOTANY, CHINESE ACADEMY OF SCIENCES). 1987. List of rare and endangered plants of China, vol. 1. Science Press, Beijing, China.

THIEL, T., W. MICHALEK, R. 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, H. Q., L. Y. ZHOU, AND Q. Y. LI. 1992. New data of flora from China. *Journal of Wuhan Botanical Research* 10: 12–14.

XU, C., Z. Y. DU, J. M. CHEN, AND Q. F. WANG. 2012. Microsatellite primers for the endangered aquatic herb, *Ottelia acuminata* (Hydrocharitaceae). *American Journal of Botany* 99: e262–e264.

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

Species	Population code	*N*	Locality	Geographic coordinates	Voucher no.[a]
O. acuminata (Gagnep.) Dandy var. *jingxiensis* 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
O. acuminata var. *acuminata*	HQ	5	Heqing, Yunan	26°24′N, 100°09′E	HIB-Otte003
O. acuminata var. *crispa* (Hand.-Mazz.) H. Li	LGH	5	Luguhu, Yunan	27°40′N, 100°46′E	HIB-Otte011
O. acuminata var. *lunanensis* H. Li	LN	5	Shilin, Yunnan	24°50′N, 103°27′E	HIB-Otte008
O. acuminata var. *songmingensis* 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.

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