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Microsatellites primer development for *Ottelia acuminata* (Hydrocharitaceae), a submerged macrophyte endemic to southwestern China

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Abstract: *Ottelia acuminata* (Gagnepain) Dandy (Hydrocharitaceae), is a submerged monocot endemic to southwestern China. Using the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol, nine polymorphic microsatellite loci were identified by the genotyping of forty-five individuals from three natural populations. The number of alleles (N_A) per locus within populations varied from one to three. The observed and expected heterozygosities ranged from 0.000 to 0.933 and 0.000 to 0.605, respectively. These microsatellite primers can be used in future studies on the phylogeography and ecological genetics of *O. acuminata*.

Key words: Hydrocharitaceae; *Ottelia acuminata*; polymorphic; microsatellite

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中国西南地区特有水生植物海菜花微卫星引物开发

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摘 要: 水鳖科(Hydrocharitaceae)海菜花(*Ottelia acuminata*)是中国西南地区特有的水生单子叶植物。基于AFLP技术的磁珠富集快速分离技术(Fast Isolation by AFLP of Sequences Containing Repeats, FIASCO),共筛选出9对多态性引物并对3个居群45个个体进行分析。结果表明:三个居群的等位基因数目为1～3个,观测杂合度从0.000～0.933,期望杂合度从0.000～0.605。这些筛选出的微卫星引物将用于海菜花后续的谱系地理学和生态遗传学研究。

关键词: 水鳖科; 海菜花; 多态; 微卫星

Ottelia acuminata (Hydrocharitaceae), a submerged monocot endemic to southwestern China (Guizhou, Sichuan, Yunnan, and Guangxi provinces), is scattered in the plateau freshwater lakes, ponds, and streams among the drainage areas of the Upper Yangtze, Pearl, Mekong, and Salween Rivers (Li, 1981). This plant is dioecious, and uses hydrochory for seeds dispersal (Jiang *et al*., 2010). *O. acuminata* is an economically and ecologically important plant. The inflorescence is a famous traditional vegetable in Yunnan.

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Due to its extreme susceptivity to the water pollution, the plant has been used as an indicator to monitor water deterioration in plateau lakes (Li, 1981). For the past few years, because of the rapid loss of the natural populations, *O. acuminata* has become an endangered plant and been listed in the Chinese Plant Red Book (Fu, 1992). Moreover, the sex ratio of *O. acuminata* is not constantly equal 1 ∶ 1 in natural populations, with the variation from male-to female-biased, and an entirely male populations (propagation by bulbs within the male spathe) were also documented (Li, 1981). Therefore, this plant provides us an ideal system to investigate the impacts of drainage on the genetic divergence, and the ecological genetics of sex ratios in natural populations.

Recently, studies on classifying the populations, morphological varieties and the evolution process of *O. acuminata* were reported (He *et al.*, 1991; Zhai *et al.*, 2010; Long *et al.*, 2010), and all these will be good context for further investigation on its ecological genetics and phylogeography. Our objective here was to develop a set of new microsatellites for *O. acuminata*, in order to facilitate the further research on its pattern of genetic diversity.

1　Material and methods

1.1 Plant materials

The plant materials of *O. acuminata* were collected from Yunnan, Guizhou and Guangxi, respectively. These populations included each 15 individuals from the Heilongtan Spring (26° 35′ N, 100° 11′ E, Xinhua Village, Jianchuan County, Yunnan Province), the Caohai Lake (26° 51′ N, 104° 16′ E, Weining County, Guizhou Province), and the Jiangxiwan population (25° 06′ N, 109° 44′ E, Yongfu County, Guangxi Province) (Table 1)

1.2 DNA Extraction

Total genomic DNA was extracted from silica-gel-dried leaves following the CTAB protocol described by Doyle (1991).

1.3 Isolation of Microsatellite Loci

We used the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol (Zane *et al.*, 2002) to develop microsatellite markers for *O. acuminata*. Genomic DNA were digested with *Mse*-I restriction enzyme (New England Biolabs, Ipswich, Massachusetts, USA) at 37 ℃ for 3 h and then ligated the fragments to the *Mse*-I adaptor pairs (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) with T_4 DNA ligase (Fermentas, Burlington, Ontario, Canada) at 37 ℃ for 2 h. These products were amplified according to the following protocol: 3 min at 95 ℃, 20 cycles of 30 s at 94 ℃, 1 min at 53 ℃, 1 min at 72 ℃, and a final extension cycle of 7 min at 72 ℃. The PCR products were detected by agarose electrophoresis and those fragments ranged 200-800 bp were purified with an agarose gel extraction kit (Sangon, Shanghai, China). The purified DNA was enriched with $(AG)_{15}$ and $(AC)_{15}$ biotinylated microsatellite probes. Then we isolated the fragments which containing microsatellite repeats by magnesphere. These fragments were recovered and amplified with *Mse*-I-N primer (5′-GATGAGTCCTGAGTAAN-3′) with the same PCR program mentioned above, but with 30 cycles and a last extension cycle of 8 min at 72 ℃ here.

The products were purified and then cloned into the pGEM-T vector (Promega, Madison, Wisconsin, USA), and transformed into Trans1-T1 Phage Resistant Chemically Competent Cells (Quanshijin, Beijing, China). Clones containing the insert were selected according to a method of blue-white screening, and culti vated in an incubator at 37 ℃ for 12 h, then detected by PCR with the primer pairs $(AG)_{10}$ / $(AC)_{10}$ and

Table 1　Sampling locations for *Ottelia acuminata* (Hydrocharitaceae)

Population	Voucher No.	Collection date	Longitude and latitude of sampling location	Elevation (m)	Habitat
Heilongtan Spring	LYJ 004	Sep 17, 2010	26° 35′ 9″ N, 100° 11′ 22″ E	2198	Spring
Caohai	JH 009	Aug 6, 2009	26° 51′ 7″ N, 104° 16′ 3″ E	2160	Plateau freshwater lake
Jiangxiwan	LYW 005	Mar 13, 2011	25° 06′ N, 109° 44′ E	233	Stream

Table 2 Characterization of twenty microsatellite primers for *Ottelia acuminata*

Locus	Primer sequence (5′-3′)	Repeat motif	Expected length (bp)	Ta(℃)	GenBank accession No.
oa02	F-ATTCGACCGTACTGTACTCTG R-GGTAGCCCTTGCCTTTT	(AG)15	144	56	JN862971
oa03	F-GAGGACGGTCGGATATTGT R-AATGACCTCCAGTCTTTGC	(CT)8	185	56	JN862972
oa07 *	F-GACCTCAGGGCCTTCACTTT R-TTGGAGGATTGGCACGA	(GA)10…(TCC)4	190	57	JN862973
oa12 *	F-CATCTGAGAATGGCTTGG R-CCGAATTGGAGCCTGTA	(CA)3CC(CA)5	279	57	JN862974
oa13	F-GCGGTGAATAGAGGGTGAA R-GCTAGGATAATGACTGCCAAC	(GT)6	256	56	JN862975
oa15	F-AGTACACGGGACTCACAAA R-TAGCTTGGATTAGCAGGAG	(CA)5	230	56	JN862976
oa22 *	F-GGCACCATAACTGGACTAAA R-TATCAGCGAGCGGGATT	$(GT)_5$	150	59	JN862977
oa23 *	F-TGGTGAATCGGGAGTTTGT R-AAGGAGGAGATGGATACGAGA	$(GT)_5$	157	59	JN862978
oa25	F-TACAGCGGTCATCGTTTG R-AGCGTGAATTAGCAGGAG	$(CA)_6$	142	57	JN862979
oa30	F-TTACATCTGTTGTCGCCTCG R-GAAATACGCCATTTGCTCCT	$(TC)_9$	200	55	JN862980
oa35	F-CATGTGGACCATTGGATTTG R-AAGCACCGAAGAAGCGTAG	(TC)7	245	60	JN862981
oa36 *	F-CCCTTGTCTTCGCTGGTTT R-CACCTCCATCATCCTCACTTC	(GAG)2(GA)2(AGGAG)2	260	53	JN862982
oa37 *	F-TGAGTGCGTGAGTGAGTCGA R-CACCTTCTCCGTTTCATTTC	(TG)3(GT)3G(CT)2(TG)2	110	56	JN862983
oa44	F-AGGTAGCCCTAGCATTTGA R-ATCTCCTGGTCTCGTCTCAC	$(CT)_5$	246	55	JN862984
oa63 *	F-GCCCCTTCCTGAGCATCTG R-CCCCGAATTGGAGCCTGTA	(CA)3CC(CA)5	104	50	JN862985
oa66	F-TTGCTGGACCATGAAGACC R-GCGTGAATTAGCGGGAGAT	$(CA)_6$	266	52	JN862986
oa70 *	F-CGGTGAATAGAGGGTGAAG R-CTAGGATAATGACTGCCAAC	$(GT)_6$	254	55	JN862987
oa72	F-GGACCATGAAGACCGAGGAT R-TGAATCGAGTGCGGAGCGT	$(CA)_6$	223	48	JN862988
oa73 *	F-GAATTTGAGGACGGATTTG R-TTCCAGCACTCACAATGTTT	(TG)10	134	55	JN862989
oa75	F-GAGATCGAGATAACCAAGTC R-TACAAAGAAAGACGACCAT	(GT)5A(TG)4	303	50	JN862990

Ta: PCR annealing temperature; * indicating polymorphisms.

M13F (5′-GTAAACGACGGCCAG-3′), and $(AG)_{10}$/$(AC)_{10}$ and M13R (5′-GATGAGTCCTGAGTAAN-3′), respectively.

A total of 287 positive colonies were selected to be sequenced. In those colonies, 111 sequences containing the repeat region were identified by the SSRIT software (http://www.gramene.org/db/searches/ssrtool/). The software Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA) was used to design primers, and 83 pairs of primers were designed and synthetized by BGI (Shenzhen, Guangdong, China).

1.4 Detection of Polymorphism

Polymorphismof microsatellites was detected among 45 individuals of *O. acuminata* from three natural populations. PCR amplification was performed in a reaction volume of 25 μL, containing 1 μL of genomic DNA, 2.5 μL of 10× PCR buffer, 0.5 μL of dNTP(2.5 μmol each), 0.5 μL of each primer(10 μmol/L), and 0.3 U of Taq DNA polymerase(Takara, Dalian, Liaoning, China). PCR amplifications were performed by 3 min at 93 ℃; followed by 35 cycles of 30 s at 93 ℃, 30 s at the optimized annealing temperature(Table 2), and 30 s at 72 ℃; then a final step for 7 min at 72 ℃. The PCR products were separated by the 8% sodium dodecyl sulfate polyacrylamide gel(SDS-PAGE). And the fragment sizes were determined by a standard 25-bp DNA ladder(25—500 bp).

1.5 Data Analysis

Genetics statistics were analyzed using GENEPOP Version 3.4 software(Raymond and Rousset, 1995) to compute allele numbers(N_A), expected heterozygosities(H_E), observed heterozygosities(H_O), and deviations from the Hardy-Weinberg equilibrium(HWE).

2 Results and discussion

For a total of 20 microsatellites isolated, nine displayed polymorphism. Details of these polymorphic microsatellites were listed in Table 2. The number of alleles per locus varied from one to three within populations. And the values of H_O and H_E for Heilongtan Spring population ranged from 0.000 to 0.400 and from 0.000 to 0.605, respectively. The values of H_O and H_E for Caohai population ranged from 0.000 to 0.933 and 0.000 to 0.605, respectively. And the values of H_O and H_E for Jiangxiwan population ranged from 0.000 to 0.733 and 0.000 to 0.515, respectively (Table 3). HWE tests revealed that four loci(oa12, oa22, oa 23 and oa70)had significantly deviated from the equilibrium($P<0.001$).

This result showed much low polymorphism within these three populations and relatively high polymorphism among them, likely owing to the homozygotes accounting for a large proportion of individuals of these three populations, which seems to be resulted from the geographic isolation. Each of these three populations comes from a different river system. Jian Lake locates in the Mekong River ; Caohai locates in the Chin-sha River, which is the upper reaches of the Yangtze River; and Jiangxiwan locates in the Pearl Rivers. The distribution of aquatic habitats may influence the patterns of genetic differentiation among populations (Spencer, 1993). These lakes are discontinuous and long-term isolated, and *O. acuminata* has been adapted to the respective lake habitats and produced some endemic variations (Li, 1981), which were fixed in each population owing to the lack of hydrochory to facilitate seed flow among populations. Therefore, our results seem to indicate genetic isolation by water system in *O. acuminata*.

Table 3　Genetic diversity of nine polymorphic microsatellites

Locus	Heilongtan spring			Caohai			Jiangxiwan		
	N_A	H_o	H_E	N_A	H_o	H_E	N_A	H_o	H_E
oa07	2	0.067	0.186	2	0.200	0.481	1	0.000	0.000
oa12	2	0.000	0.331 *	2	0.067	0.067	1	0.000	0.000
oa22	2	0.400	0.460	3	0.267	0.605	2	0.000	0.129 *
oa23	3	0.200	0.605 *	2	0.933	0.515	2	0.733	0.508
oa36	1	0.000	0.000	2	0.200	0.481	1	0.000	0.000
oa37	1	0.000	0.000	1	0.000	0.000	2	0.133	0.239
oa63	1	0.000	0.000	2	0.067	0.186	2	0.067	0.287
oa70	2	0.000	0.129 *	2	0.067	0.435 *	2	0.133	0.515
oa73	2	0.133	0.129	2	0.133	0.497	1	0.000	0.000

N_A: the number of alleles; Ho: observed heterozygosity; H_E: expected heterozygosity; * statistical deviation from Hardy-Weinberg equilibrium(HWE)($P<0.001$).

3 Conclusions

We reported the nine polymorphic microsatellite loci developed in *O. acuminata*. They will facilitate the studies on the phylogeography of the species, which will improve knowledge on the impacts of drainage on genetic differentiation of aquatic plants. Furthermore, they will be useful tools in further studies on ecological genetics of sex ratios of its natural population.

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