DOI: 10.3969/j.issn.1000-3142.2014.01.007
卢然然,杨振艳,陶程程,等. 中国西南地区特有水生植物海菜花微卫星引物开发[J]. 广西植物,2014,34(1):34-37
Lu RR, Yang ZY, Tao CC, *et al*. Microsatellites primer development for *Ottelia acuminata* (Hydrocharitaceae), a submerged macrophyte endemic to southwestern China[J]. Guihaia, 2014, 34(1):34-37

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

LU Ran-Ran[1,2], YANG Zhen-Yan[1], TAO Cheng-Cheng[1,2], CHEN Shao-Tian[1], JI Yun-Heng[1*]

(1. *Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences*, Kunming 650201, China; 2. *University of Chinese Academy of Sciences*, Beijing 100049, China)

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

GLC Number: Q949 **Document Code:** A **Article ID:** 1000-3142(2014)01-0034-04

中国西南地区特有水生植物海菜花微卫星引物开发

卢然然[1,2], 杨振艳[1], 陶程程[1,2], 陈绍田[1], 纪运恒[1*]

(1. 中国科学院昆明植物研究所 生物多样性与生物地理学重点实验室, 昆明 650201; 2. 中国科学院大学, 北京 100049)

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 eco-

收稿日期: 2013-05-09 修回日期: 2013-08-23
基金项目: 国家高技术研究发展计划项目(2010AA06Z301);国家自然科学基金(31070297,31070198)。
作者简介: 卢然然(1988-),女(蒙古族),内蒙古赤峰人,硕士研究生,植物学专业,(E-mail) luranran@mail.kib.ac.cn。
* 通讯作者: 纪运恒,副研究员,硕士生导师,从事植物地理学、植物分类学以及植物保护生物学等研究,(E-mail) jiyh@mail.kib.ac.cn。

nomically and ecologically important plant. The inflorescence is a famous traditional vegetable in Yunnan. 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)

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

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 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	(AG) 15	144	56	JN862971
	R-GGTAGCCCTTGCCTTTT				
oa03	F-GAGGACGGTCGGATATTGT	(CT) 8	185	56	JN862972
	R-AATGACCTCCAGTCTTTGC				
oa07*	F-GACCTCAGGGCCTTCACTTT	(GA) 10…(TCC) 4	190	57	JN862973
	R-TTGGAGGATTGGCACGA				
oa12*	F-CATCTGAGAATGGCTTGG	(CA) 3CC(CA) 5	279	57	JN862974
	R-CCGAATTGGAGCCTGTA				
oa13	F-GCGGTGAATAGAGGGTGAA	(GT) 6	256	56	JN862975
	R-GCTAGGATAATGACTGCCAAC				
oa15	F-AGTACACGGGACTCACAAA	(CA) 5	230	56	JN862976
	R-TAGCTTGGATTAGCAGGAG				
oa22*	F-GGCACCATAACTGGACTAAA	$(GT)_5$	150	59	JN862977
	R-TATCAGCGAGCGGGATT				
oa23*	F-TGGTGAATCGGGAGTTTGT	$(GT)_5$	157	59	JN862978
	R-AAGGAGGAGATGGATACGAGA				
oa25	F-TACAGCGGTCATCGTTTG	$(CA)_6$	142	57	JN862979
	R-AGCGTGAATTAGCAGGAG				
oa30	F-TTACATCTGTTGTCGCCTCG	$(TC)_9$	200	55	JN862980
	R-GAAATACGCCATTTGCTCCT				
oa35	F-CATGTGGACCATTGGATTTG	(TC) 7	245	60	JN862981
	R-AAGCACCGAAGAAGCGTAG				
oa36*	F-CCCTTGTCTTCGCTGGTTT	(GAG) 2(GA) 2(AGGAG) 2	260	53	JN862982
	R-CACCTCCATCATCCTCACTTC				
oa37*	F-TGAGTGCGTGAGTGAGTCGA	(TG) 3(GT) 3G(CT) 2(TG) 2	110	56	JN862983
	R-CACCTTCTCCGTTTCATTTC				
oa44	F-AGGTAGCCCTAGCATTTGA	$(CT)_5$	246	55	JN862984
	R-ATCTCCTGGTCTCGTCTCAC				
oa63*	F-GCCCCTTCCTGAGCATCTG	(CA) 3CC(CA) 5	104	50	JN862985
	R-CCCCGAATTGGAGCCTGTA				
oa66	F-TTGCTGGACCATGAAGACC	$(CA)_6$	266	52	JN862986
	R-GCGTGAATTAGCGGGAGAT				
oa70*	F-CGGTGAATAGAGGGTGAAG	$(GT)_6$	254	55	JN862987
	R-CTAGGATAATGACTGCCAAC				
oa72	F-GGACCATGAAGACCGAGGAT	$(CA)_6$	223	48	JN862988
	R-TGAATCGAGTGCGGAGCGT				
oa73*	F-GAATTTGAGGACGGATTTG	(TG) 10	134	55	JN862989
	R-TTCCAGCACTCACAATGTTT				
oa75	F-GAGATCGAGATAACCAAGTC	(GT) 5A(TG) 4	303	50	JN862990
	R-TACAAAGAAAGACGACCAT				

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.

Acknowledgements The authors are grateful to Drs Ying Yang, Yaowu Xing, Tao Su, and Zhiling Dao for their help in sample collection.

References:

Doyle J. 1991. DNA protocols for plants-CTAB total DNA isolation [M] // Hewitt GM and Johnston A, Molecular Techniques in Taxonomy. Berlin: Springer: 283-293

Fu LG. 1992. Chinese Plant Red Book, Vol. 1 [M]. Beijing: Science Press: 334-335

He JB, Sun XZ, Zhong Y, *et al.* 1991. The Infraspecific classification of the populations of *Ottelia acuminata* (Gagnep.) Dandy [J]. *J Wuhan Univ* (*Nat Sci Edi*), 3: 114-120

(下转第 83 页 Continue on page 83)

蕨属(5 种),共有 39 种,占本区蕨类植物总种数的 35.5%,这表示该地区蕨类植物的优势属表现明显。

4.3 特化程度高

太白山自然保护区蕨类植物种的中国特有种有 48 种,占本区蕨类植物总种数的 43.6%,以西北、华北、华中等地分布为主,这与该地区地处秦岭之峰以及秦岭的地史和地质构造密切相关。

4.4 温带性质显著,与热带植物区系有一定联系

从太白山蕨类植物区系分析可知,属、种的地理成分以温带成分为主,分别占属、种总数的 58.3%、48.2%,同时也有一定数量的热带分布类型,显示出热带、亚热带向温带过渡的特点。

4.5 与相邻地区的联系

本区蕨类植物与河南伏牛山自然保护区和陕西化龙山自然保护区的联系最为密切,与四川唐家河自然保护区的联系较为密切,与山西五鹿山自然保护区、北京松山自然保护区、河北茅荆坝自然保护区、湖北神农架自然保护区有一定的联系,与湖南壶瓶山自然保护区、甘肃祁连山自然保护区的关系较为疏远,与宁夏南华山自然保护区的的联系最为疏远。这些主要是与气候因素、地理位置、区系起源等因素有关,也可能是也因为蕨类植物在较大程度上要依赖于当地森林植被的发育,特别是树干附生及林下阴生的种类(许冬焱,2008)。

参考文献:

王玛丽,邢连喜,张国昌. 2004. 陕西化龙山自然保护区综合科学考察报告[M]. 西安: 西安地图出版社: 204-205

中国植物志编辑委员会. 2004. 中国植物志[M]. 北京: 科学出版社: 90

毕润成. 2004. 山西省五鹿山自然保护区科学考察报告[M]. 哈尔滨: 中国科学技术出版社: 391-392

任毅,刘明时,田联会,等. 2006. 太白山自然保护区生物多样性研究与管理[M]. 北京: 中国林业出版社

朱兆泉. 1999. 神农架自然保护区科学考察集[M]. 北京: 中国林业出版社: 124-138

刘健泉. 2008. 甘肃祁连山国家级自然保护区综合科学考察报告[M]. 兰州: 甘肃科学技术出版社: 232-233

李家俊. 1989. 太白山自然保护区综合考察论文集[M]. 西安: 陕西师范大学出版社: 196-201

张国珍,杨道德. 2004. 湖南壶瓶山国家级自然保护区科学考察报告集[M]. 长沙: 湖南科学技术出版社: 336-346

吴跃峰. 2006. 河北茅荆坝自然保护区科学考察集[M]. 北京: 科学出版社: 255-261

宋朝枢. 1994. 伏牛山自然保护区科学考察集[M]. 北京: 中国林业出版社: 106-112

陆树刚. 2004. 中国蕨类植物区系概论[M]. 北京: 植物科学进展: 29-41

南华山自然保护区科考组. 2005. 宁夏南华山自然保护区综合考察报告[M]. 银川: 宁夏人民出版: 82

胡锦矗. 2005. 四川唐家河自然保护区综合科学考察报告[M]. 成都: 四川科学技术出版社

Wu ZY(吴征镒). 1991. The areal-types of chinese genera of seed plants(中国种子植物属的分布区类型)[J]. *Acta Bot Yunnanica* Ⅳ(云南植物研究: 增刊Ⅳ): 1-139

Wang CJ(王诚吉), Li DW(李登武), Dang KL(党坤良). 2006. Pteridophyte flora in Tianhuashan Nature Reserve of Shaanxi Province(陕西天华山自然保护区蕨类植物区系研究)[J]. *Acta Bot Bor-Occ Sin*(西北植物学报), **26**(3): 592-597

Xu H(徐皓), Wang ML(王玛丽). 2008. Floristic analysis of pteridophyte in Taibaishan Nature Reserve(太白山自然保护区蕨类植物区系分析)[J]. *J Northwest Univ*(西北大学学报): 951-954

Xu DY(许冬焱). 2008. Analysis of pteridophyte flora of Dabashan Nature Reserve(大巴山自然保护区蕨类植物的区系分析)[J]. *J Anhui Agric Univ*(安徽农业大学学报), **35**(1): 93

Zhang FG(张富光), Shen XS(沈显生). 2008. Pteridophyte flora of Tiantangzhai Nature Reserve of Dabieshan Mountain(大别山自然保护区蕨类植物区系分析)[J]. *J Mount Sci*(山地学报), **18**(5): 468-473

(上接第 37 页 Continue from page 37)

Ji ang ZT, Li H, Dao ZL, *et al*. 2010. Ethnobotanical study on *Ottelia acuminata*, an aquatic edible plant occurring in Yunnan[J]. *J Inn Mongolia Norm Univ*: *Nat Sci Edi*, **39**: 163-168

Li H. 1981. Classification, distribution and phylogeny of the genus Ottelia[J]. *Acta Phytotax Sin*, **19**: 29-42

Long CL, Jiang ZT, Dao ZL. 2010. Research Genetic diversity of *Ottelia acuminata* (Hydrocharitaceae) from the Eastern Himalayas, revealed by ISSR markers[J]. *Bot Orientalis*: *J Plant Sci*, **7**: 56-63

Raymond M, Rousset F. 1995. GENEPOP(Ver. 1.2): Population genetics software for exact tests and ecumenicism[J]. *J Hered*, **86**: 248-249

Spencer C, Christopher G, Brian C. 1993. Evolutionary processes in aquatic plant populations[J]. *Aquat Bot*, **44**: 105-145

Zane L, Bargelloni L, Patarnello T. 2002. Strategies for microsatellite isolation: a review[J]. *Mol Ecol*, **11**: 1-16

Zhai SH, Wang B, Wang DK, *et al*. 2010. Caryotype of *Ottelia acuminata* var. *lunanensis* H. Li and evolution of *Ottelia* Pers.[J]. *J Lake Sci*, **22**: 735-738