DOI:10. 13471/j. cnki. acta. snus. 2017. 01. 018

Novel nuclear SSR markers in the large frond tree fern *Alsophila gigantea* and its congeneric species *Alsophila spinulosa**

RUAN Xiaoxian [1], *WANG Zhen* [2], *WANG Ting* [3], *SU Yingjuan* [1,4]
(1. School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China;
2. College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China;
3. College of Life Science, South China Agricultural University, Guangzhou 510642, China;
4. Research Institute of Sun Yat-sen University in Shenzhen, Shenzhen 518057, China)

Abstract: *Alsophila gigantea* is a large frond tree fern in family Cyatheaceae. The plant is a vulnerable species that prefers specific subtropical montane climate. Little work has been done so far to access the population genetic variation in *A. gigantea*. In this study, we developed 15 SSR markers using FIASCO protocol and evaluated these markers in four natural populations from Hainan Island, China. Seven loci proved to be polymorphic. The actual number of alleles ranged from one to nine, and the observed and expected heterozygosity varied from 0 to 0. 769, and from 0 to 0. 805, respectively. Loci *AG*-12 and *AG*-23 were found to significantly deviate from the Hardy-Weinberg equilibrium in populations HNjfl and HNbwl, respectively. Four null alleles were identified. Linkage disequilibrium was further detected at three pairs of loci. Moreover, four SSR markers were verified to be successfully transferred in *Alsophila spinulosa*. The novel polymorphic SSR markers characterized here will be used to survey population genetic variation and local adaptation in *A. gigantea*, which helps design effective conservation strategies.

Key words: *Alsophila gigantea*; SSR markers; cross-species amplification; genetic variation; local adaptation
中图分类号: Q347　　**文献标志码**: A　　**文章编号**: 0529－6579（2017）01－0115－06

大叶黑桫椤及桫椤 SSR 标记的开发

阮效先[1]，王桢[2]，王艇[3]，苏应娟[1,4]
（1. 中山大学生命科学学院，广东 广州 510275；
2. 南京农业大学生命科学学院，江苏 南京 210095；
3. 华南农业大学生命科学学院，广东 广州 510642；
4. 深圳市中山大学研究所，广东 深圳 518057）

摘　要：大叶黑桫椤是桫椤科的大型树蕨，多分布在亚热带山地，为渐危种。迄今为止，鲜有大叶黑桫椤种群遗传变异的报道。采用 FIASCO 法开发了大叶黑桫椤 15 个 SSR 标记，用来自海南岛的 4 个种群检测标记的多态性。其中，7 个标记具多态性，每个标记的实际等位基因数为 1～9，观察杂合度和期望杂合度范围分别为 0～0. 769 和 0～0. 805。位点 *AG*-12 和 *AG*-23 分别在 HNjfl 和 HNbwl 种群显著地偏离哈代温伯格平衡。鉴定出 4 个哑

---

* **收稿日期**：2016－06－29
基金项目：国家自然科学基金（31370364，31570652，31670200）；广东省自然科学基金（2016A030313320）；深圳市科技创新（JCYJ20160425165447211）

作者简介：阮效先、王桢并列第一作者；阮效先（1990 年生），女；**研究方向**：植物遗传适应性；E-mail：11011445012@ qq. con

通信作者：苏应娟（1965 年生），女；**研究方向**：植物遗传适应性；E-mail：suyj@ mail. sysu. edu. cn

等位基因。发现3对标记存在连锁不平衡现象。此外，4个SSR标记能成功地用于桫椤种群的遗传分析。这些新的多态性SSR标记将用于大叶黑桫椤的群体遗传变异、局部适应性的研究以及建立有效的保护策略。

关键词：大叶黑桫椤；SSR标记；跨种扩增；遗传变异；局部适应性

Alsophila gigantea is a large tree fern belonging to family Cyatheaceae with fronds 2 to 3 meter long[1]. Its fronds are bright green and thinly textured. Unbranched trunk reaches 2 ~ 4 m in height and 12 ~ 13 cm in diameter. Long stipes are densely covered with shiny, dark brown scales. *Alsophila gigantea* forms a characteristic V-shaped soral arrangement in each pinnule lobe and margin of pinnae lobed 2 ~ 5 mm towards the costa[2]. The species is widely distributed in India, Sri Lanka, Burma, Thailand, Laos, Vietnam, and China. In China, it mainly occurs in Guangdong, Guangxi, Hainan, and Yunnan. *Alsophila gigantea* grows near streams, river banks, and on mountain slopes in dense forests at an altitude of 200 ~ 1 200 m[1]. Traditionally, this species has been used as an important medicinal plant to treat white discharges[3-4]. Its trunk is also exploited for starch and used for growing epiphytic orchids[4]. Overexploitation and human activities have greatly affected the populations of *A. gigantea*. Populations are drastically fragmented, and population sizes have diminished[5]. As a result, *Alsophila gigantea* has been declared as a vulnerable species and listed under IUCN Red data book and the Appendix-II of CITES (Convention of International Trade in Endangered Species of Wild Fauna and Flora)[6-7].

Simple sequence repeats (SSRs) are short, tandemly repeated sequences of 1 ~ 6 nucleotides[8]. SSRs are powerful PCR-based markers extensively used in assessing genetic variation of plants due to codominant inheritance, polymorphism, and abundant coverage. Currently, there are a number of methods used to isolate SSR markers. However, Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) represents an efficient technique to develop SSR markers[9]. The method is simple, fast, and economic[10]. Moreover, no genomic information is needed.

Alsophila gigantea is perceived to be more adapted to subtropical montane climate in comparison to other *Alsophila* species[11]. However, little work has been done on its adaptive population genetic variation and structure. To aid in this field, we have developed the SSR markers for *A. gigantea* using the FIASCO approach.

1 Materials and methods

1.1 Plant materials and DNA extraction

A total of 49 individuals of *A. gigantea* were collected from Diaoluoshan (N18°40′, E109°50′, N = 13), Wuzhishan (N18°54′, E109°54′, N = 11), Jianfengling (N18°44′, E108°52′, N = 13), and Bawangling (N19°05′, E109°11′, N = 12), Hainan Province, China. Two natural populations of the congeneric species *A. spinulosa* were sampled from Wuzhishan (Hainan, N18°54′, E109°40′, N = 5) and Wulaishan (Taiwan, N24°52′, E121°33′, N = 7) for cross-species amplification (Fig. 1). Vouchers have been deposited at the Herbarium of Sun Yat-sen University (*A. gigantea*, YZ Ying 200606, DLS1, WZS2, JFL3, and BWL4; *A. spinulosa*, YZ Ying 200605, WZS6, Q Fan 200808, WLS3). Fresh and young leaves were collected and preserved in Ziplock plastic bags with silica gel. Total genomic DNA was extracted using a modified CTAB (cetyltrimethylammonium bromide) method with -20 ℃ propanone pretreatment to eliminate polysaccharides[12]. The quality of the DNA was checked by 0.8% agarose gel electrophoresis. DNA concentration was quantified by spectrophotometry. DNA was stored at -20 ℃ for further study.

1.2 Construction of SSR-enriched genomic library

The FIASCO approach was applied to construct SSR-enriched genomic library from one random individual of *A. gigantea*. Approximately 250 ng DNA was digested with *Mse*I restriction enzyme at 37 ℃ for 3 hours (h), following by incubation at 65 ℃ for 15 min to inactivate the enzyme. Digested DNA was ligated to *Mse*I adaptors (F: 5′-TACTCAGGACTCAT-3′, R: 5′-GACGATGAGTCCTGAG-3′) with T4 DNA ligase. The adaptors-ligated DNA fragments were diluted ten folds and directly amplified in a total volume of 20 μL with *Mse*I-N primer. After denaturation, PCR products were

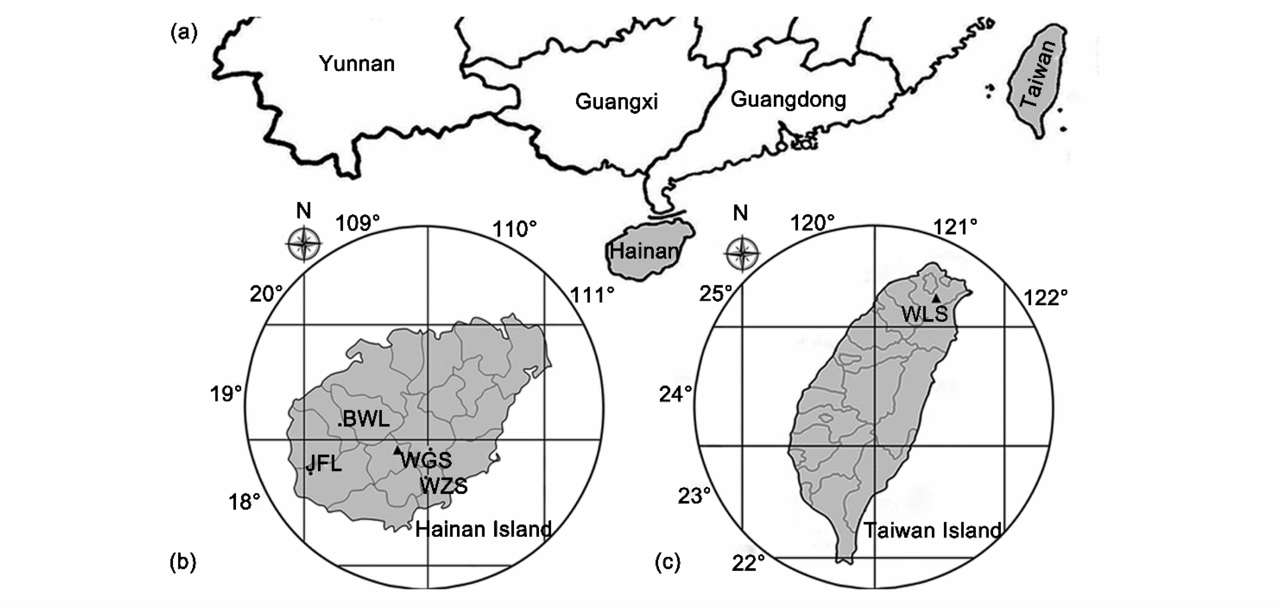

Fig. 1　The sampling location of *Alsophila gigantea* (solid dots) and *Alsophila spinulosa* (solid triangle)

(a) The map shows a brief part of China; (b) Hainan; (c) Taiwan.

WZS: Wuzhishan; DLS: Diaoluoshan; JFL: Jianfengling; BWL: Bawangling; WLS: Wulaishan

hybridized with 5′-biotinylated (AC)$_{15}$ probe at 95 ℃ for 3 minutes (min), then captured by Streptavidin-coated beads. To eliminate non-specific DNA, the captured products were treated by three non-stringent washes (1 mol/L Tris-HCl, 0.5 mol/L EDTA, 2 mol/L NaCl) and three stringent washes (20 × SSC, w = 10% SDS, ddH$_2$O). The enriched products were eluted, precipitated, dissolved, and recovered by PCR with *Mse*I-N primer. The PCR reaction program included 30 cycles of 30 seconds (s) at 94 ℃, 1 min at 53 ℃, and 1 min at 72 ℃. Amplified products purified by the E. Z. N. A. Cycle-Pure kit (Omega Bio-Tek, Guangzhou, China) were ligated into pMD-18T vector. The plasmids were transformed into *Escherichia coli* DH5α competent cells. The recombinant clones were screened on LB agar medium containing ampicillin and IPTG/X-galactosidase by blue-white selection.

1.3　Screening of positive clones and primer design

The recombinant clones were identified using PCR with M13 forward and reverse universal primers. Positive clones were sequenced using ABIPRISM 3730 Automated Sequencer. After the vector sequences were removed, sequences including SSRs and sufficient flanking region were screened for SSRs with SSRHunter. SSR primer pairs were designed using Primer Premier 5.0 software[13].

1.4　Polymorphism and cross-species amplification

PCR amplification for SSR loci were performed in 20 μL reaction system including 2 μL 10 × PCR buffer, 0.4 μL 10 mmol/L dNTPs, 1.2 μL 1.5 mmol/L Mg^{2+}, 1.25 U *Taq* polymerase, 0.5 μL 10 mmol/L each of primers, and 20 ng DNA template. It was conducted under the following program: initial denaturation at 94 ℃ for 5 min; followed by 35 cycles of 94 ℃ for 50 s, annealing at optimal annealing temperature for 50 s, 72 ℃ for 90 s; and final extension at 72 ℃ for 10 min[14]. Amplified products were separated using 6% denaturing polyacrylamide gel with 50 bp marker and visualized with silver staining. Cross-species amplification was performed as described above.

1.5　Data analysis

GenAlEx software[15] was applied to assess genetic parameters including the effective number of alleles (*N*e), actual number of alleles (*N*a), expected heterozygosity (*H*e), and observed heterozygosity (*H*o). The deviation from the Hardy-Weinberg equilibrium was evaluated using the same software. The null alleles and linkage disequilibrium were estimated using MICRO-CHECKER version 2.2.3[16] and GENEPOP version 4.0.10[17], respectively.

2 Results and discussion

2.1 Identification of the SSR markers

From 129 recombinant clones sequenced, 96 sequences were acquired, in which 89 sequences contained SSR motifs with enough fianking region for primer design. We designed 29 sets of SSR primers. Fifteen of these primer pairs amplified successfully in four populations of *A. gigantea*. Of them, seven were polymorphic, whereas eight were monomorphic (Table 1).

2.2 Population genetic analysis

A total of 127 alleles were detected across the seven polymorphic SSR loci in *A. gigantea* (Table 2). The actual and effective number of alleles ranged from 1 to 9 and 1 to 5.121, respectively. The observed heterozygosity was from 0 to 0.769, with a mean of 0.489, and expected heterozygosity varied from 0 to 0.805, with the average of 0.594. Significant different levels of genetic diversity were observed in the four populations (Table 2). Population HNdls was found to possess the highest level of genetic variation, whereas Population HNjfl the lowest (Table 2). The high discriminatory power showed that these SSR loci are suitable for investigation of genetic variation in *A. gigantea*. All loci except *AG*-12 locus in HNjfl and *AG*-23 locus in HNbwl were in Hardy-Weinberg equilibrium ($P < 0.001$) (Table 2). Four null alleles (*AG*-12, *AG*-40, *AG*-41, and *AG*-133) were detected. Three pairs of loci (*AG*-23 and *AG*-24; *AG*-23 and *AG*-105; and *AG*-24 and *AG*-105) were found to be in linkage disequilibrium ($P < 0.05$).

Table 1 Characterization of 15 novel nuclear SSR markers developed in *Alsophila gigantea*1)

Locus	Primer sequence (5′–3′)	Repeat motif	Size/bp	T_a/℃	GenBank accession No.
AG-1	F: GAAAAGATTGAGGGGAGGGAAG R: GTGCGTTTGGTAGCCGACAT	(CA)$_6$	314	57	KT833359
AG-6	F: GTGTTGTTCTACTACGCCTTC R: GTGTGCTGGATACTCTATTTGT	(CA)$_5$	124	57	KT833360
AG-12 *	F: ATGTCATTCCCAGAGTCCTAT R: CCACACGCTATCTATCTACCA	(CA)$_5$	160	55	KT833361
AG-23 *	F: AGAGAGCCTTTACCTTTGTGC R: GCCCCGAGTGTGGAGAT	(CA)$_5$TGGGA (AC)$_6$	176	57	KT833362
AG-24 *	F: TTTTGGAATGCGATGTG R: GCCTTTACCTTTGTGCC	(TG)$_5$TTCCCA (TG)$_5$	142	51	KT833363
AG-40 *	F: CTCCCCCACATTCTTGAT R: GACTCCATTCCATTAGATAGGT	(AG)$_5$	158	51	KT833364
AG-41 *	F: GTGTTTGGGTTTGGATGTGTG R: GATGGCATAGTTAGTGGTGGAGAG	(CT)$_7$	114	58	KT833365
AG-61	F: TCTTCACCACACAAACCTGGCTCT R: CGGATTCACACTATGGACCGACCT	(CA)$_5$	255	57	KT833366
AG-78	F: GGCAAGAACCGATTACCGATG R: TTTGGGGTGTGTTTGTCTATGATG	(AG)$_5$	111	62	KT833367
AG-79	F: GGAGAGGGAGAGAGTCACGATTC R: CACTATGGACCGACCTTTGTATG	(AC)$_8$AATGAACTTGA CAAGCACA (AG)$_6$	206	58	KT833368
AG-81	F: GAAGAGAACACGCAAGATGAG R: AGTCCACACCCACTACTTTATG	(TTC)$_5$	158	57	KT833369
AG-100	F: TTAGGGTTAGGTTTTGGGGTGTG R: GTGAAGCGTGGCAAGAACTGAT	(CT)$_5$	133	58	KT833370
AG-105 *	F: TGCCTTTACCTTTGTGCC R: GCCCCGAGTGTGGAGAT	(CA)$_{12}$	154	59	KT833371
AG-118	F: CAAGGCGTAGTGGGAAAAGG R: AACAAGCATCAATGGCAAAGC	(CT)$_7$	189	59	KT833372
AG-133 *	F: AAAATCCACATCCGTCG R: CGCAGAGTAGAGGGCAT	(TC)$_{11}$	123	57	KT833373

1) *: Polymorphic loci; T_a: annealing temperature

Table 2　Genetic parameters of seven polymorphic SSR markers in four *Alsophila gigantea* populations[1)]

Locus	HNdls（$N=13$）				HNwzs（$N=11$）				HNjfl（$N=13$）				HNbwl（$N=12$）			
	*N*a	*N*e	*H*o	*H*e	*N*a	*N*e	*H*o	*H*e	*N*a	*N*e	*H*o	*H*e	*N*a	*N*e	*H*o	*H*e
AG-12	5.000	2.704	0.462	0.630	6.000	4.745	0.455	0.789	4.000	2.467	0.077 *	0.595	4.000	3.512	0.333	0.715
AG-23	7.000	5.121	0.615	0.805	6.000	4.654	0.727	0.785	3.000	2.153	0.769	0.536	6.000	3.165	0.750 *	0.684
AG-24	3.000	2.770	0.462	0.639	5.000	3.457	0.727	0.711	3.000	2.432	0.769	0.589	6.000	3.512	0.667	0.715
AG-40	7.000	2.965	0.692	0.663	5.000	2.327	0.273	0.570	5.000	2.153	0.231	0.536	3.000	1.185	0.167	0.156
AG-41	3.000	2.048	0.385	0.512	3.000	1.582	0.091	0.368	1.000	1.000	0.000	0.000	6.000	2.796	0.500	0.642
AG-105	3.000	2.074	0.615	0.518	4.000	3.723	0.727	0.731	2.000	1.899	0.769	0.473	5.000	2.692	0.750	0.628
AG-133	9.000	4.507	0.692	0.778	5.000	2.602	0.364	0.616	4.000	2.504	0.385	0.601	4.000	2.796	0.250	0.642
Mean	5.286	3.170	0.560	0.649	4.857	3.299	0.481	0.653	3.143	2.087	0.429	0.475	4.857	2.808	0.488	0.598

1) *N*: The sample size for each population; *N*a: The actual number of alleles; *N*e: The effective number of alleles; *H*o: observed heterozygosity; *H*e: expected heterozygosity;

*: Significant deviation from Hardy-Weinberg equilibrium at $P<0.001$ level

2.3　SSR markers cross-amplification

Cross-amplification in *A. spinulosa* successfully yielded PCR products, indicating transferability of the markers (Table 3). Two loci (*AG*-12 and *AG*-61) were polymorphic, which compensated for the pitfall that only three polymorphic loci were developed in *A. spinulosa*[14].

Table 3　Genetic estimation of cross-species amplification for two polymorphic SSR loci in the congeneric species *Alsophila spinulosa*[1)]

Locus	WLS（$N=7$）				WZS（$N=5$）			
	*N*a	*N*e	*H*o	*H*e	*N*a	*N*e	*H*o	*H*e
AG-12	3.000	2.800	0.857	0.643	3.000	2.632	0.600	0.620
AG-61	4.000	2.649	1.000	0.622	2.000	2.000	1.000	0.500
Mean	3.500	2.724	0.929	0.633	2.500	2.316	0.800	0.560

1) *N*: Population sample size; *N*a: The actual number of alleles; *N*e: The effective number of alleles; *H*o: observed heterozygosity; *H*e: expected heterozygosity

3　Conclusions

It is the first to develop seven polymorphic SSR loci in *A. gigantea* and to examine transferability in its congeneric species *A. spinulosa*. The markers will be used to explore the population genetic adaptation. In addition, the high discriminatory power of SSR loci also allows for an accurate estimation of genetic variation. All of these are important factors for developing effective conservation strategies.

Acknowledgments: We gratefully thank Zhanming Ying, Qiang Fan, Lu Huang, Qi Deng, Ning Li, and Limin Cui of School of Life Science, Sun Yat-sen University for assistance and guidance in material collection, experiments, and data analysis. This work was supported by the National Natural Science Foundation of China (31370364, 31570652, and 31670200), the National Natural Science Foundation of Guangdong Province, China (2016A030313320), and Project of Department of Science and Technology of Shenzhen City, China (JCYJ20160425165447211).

References:

[1] ZHANG X C, NISHIDA H. Cyatheaceae[M] //WU Z Y, RAVEN P H, HONG D Y. Flora of China. Beijing: Science Press, 2013: 134 – 138.

[2] DUDANI S N, MAHESH M K, CHANDRAN M D S, et al. *Cyathea nilgirensis* Holttum (Cyatheaceae: Pteridophyta): a threatened tree fern from central Western Ghats, India[J]. Journal of Threatened Taxa, 2014, 6 (1): 5413 – 5416.

[3] TALUKDAR A D, CHOUDHURY M D, CHAKRABORTY M, et al. Phytochemical screening and TLC profiling of plant extracts of *Cyathea gigantea* (Wall. ex. Hook.) Haltt. and *Cyathea brunoniana*. Wall. ex. Hook. (Cl. & Bak.)[J]. Assam University Journal of Science & Technology, 2010, 5 (1): 70 – 74.

［4］ KIRAN P M, RAJU A V, RAO B G. Investigation of hepatoprotective activity of *Cyathea gigantea* (Wall. ex. Hook.) leaves against paracetamol-induced hepatotoxicity in rats［J］. Asian Pacific Journal of Tropical Biomedicine, 2012, 2 (5): 352 – 356.

［5］ AO G H. Research advance in Cyatheaceae［J］. Journal of Neijiang Teachers College, 2004, 19 (6): 79 – 82.

［6］ FU L G R. List of China-rare and endangered plants［M］. Beijing: Science Press, 1992.

［7］ KHOLIA B S, JOSHI R, PUNETHA R. Extended distribution of *Cyathea spinulosa* Wall ex Hook in Uttarakhand Himalaya with a note on distribution and diversification of Himalayan ferns in relation to recent climate change［J］. NeBio, 2013, 4 (2): 40 – 45.

［8］ HOU X G, GUO D L, CHENG S P, et al. Development of thirty new polymorphic microsatellite primers for *Paeonia suffruticosa*［J］. Biologia Plantarum, 2011, 55 (4): 708 – 710.

［9］ ZHANG Y, HE J, ZHAO P X, et al. Genome-wide identification of microsatellites in white clover (*Trifolium repens* L.) using FIASCO and phpSSRMiner［J］. Plant Methods, 2008, 4 (1): 331 – 339.

［10］ ZANE L, BARGELLONI L, PATARNELLO T. Strategies for microsatellite isolation: a review［J］. Molecular Ecology, 2002, 11 (1): 1 – 16.

［11］ SINGH S, SAHU T R. Tree ferns of Pachmarhi Biosphere Reserve, Madhya Pradesh, India: taxonomy, ethnobotany and conservation［J］. International Journal of Advanced Research, 2015, 3 (8): 566 – 577.

［12］ SU Y J, WANG T, ZHENG B, et al. Genetic differentiation of relictual populations of *Alsophila spinulosa* in southern China inferred from cpDNA *trn*L-F noncoding sequences［J］. Molecular Phylogenetics & Evolution, 2005, 34 (2): 323 – 333.

［13］ CLARKE K R, GORLEY R N. PRIMER Version 5: User Manual/Tutorial［J］. Plymouth, United Kingdom: Primer-E Ltd, 2001.

［14］ ZHOU Y, CHEN G P, WANG T. Isolation and characterization of microsatellite loci in the tree fern *Alsophila spinulosa*［J］. American Fern Journal, 2008, 98 (1): 42 – 45.

［15］ PEAKALL R O D, SMOUSE P E. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research［J］. Molecular Ecology Notes, 2006, 6 (1): 288 – 295.

［16］ OOSTERHOUT C V, HUTCHINSON W F, WILLS D P M, et al. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data［J］. Molecular Ecology Notes, 2004, 4 (3): 535 – 538.

［17］ RAYMOND M, ROUSSET F. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism［J］. Journal of Heredity, 1995, 86 (3): 248 – 249.