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改良FIASCO方法筛选砷超富集植物蜈蚣草SSR分子标记

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摘 要: 蜈蚣草(*Pteris vittata* L.)是目前用于砷污染土壤修复最好的超富集植物, 但其分子水平上的研究数据较少。为了开发蜈蚣草特异性SSR遗传标记, 本文采用改良的FIASCO方法从蜈蚣草AG和AC微卫星富集文库中随机挑选100个克隆, 分离得到51个微卫星位点, 其中60%为完美型(Perfect)SSR。根据这些位点设计、合成了25对引物, 并对江西庐山及湖北恩施两地蜈蚣草种群各20个个体进行了遗传多样性检测, 结果发现: 其中8个完美型及1个间断型(intermittent)SSR位点的引物能够扩增出清晰、稳定且具有多态性的条带。9对引物共扩增出41个等位基因, 各位点等位基因数在2 ~ 7之间, 平均等位基因数为4.56个; 期望杂合度在0.0494 ~ 0.8169之间; 没有连锁不平衡现象发生。采用大叶井栏边草(*Pteris multifida* Poir.)进行跨种扩增, 结果发现其中6对引物能够进行种间扩增。这些SSR分子标记的开发有助于蜈蚣草生态适应性进化分析、揭示蜈蚣草地理分布格局以及探讨蜈蚣草遗传多样性, 还可用于品种鉴定及选育等。

关键词: 蜈蚣草; 微卫星; 多态性; FIASCO; *Cvi*QI接头; pig-tail *Cvi*QI-N引物

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Using Modified FIASCO Protocol to Isolate Polymorphic Microsatellite Loci in Chinese Brake Fern (*Pteris vittata* L.): an Arsenic-Hyperaccumulating Plant

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Abstract: Chinese brake fern (*Pteris vittata* L.) is the most important and well-known arsenic-hyperaccumulating plant used in phytoremediation of arsenic contaminated soils; however, little is known about its genetic diversity. In this study, 100 clones were randomly selected from the library enriched for AG and AC motifs using a modified FIASCO (Fast Isolation by AFLP of Sequences Containing Repeats) protocol and sequenced. Fifty-one microsatellite loci, of which 60% were pure repeats, were isolated. Twenty-five pairs of primers were designed and synthesized to evaluate their application and polymorphism in 20 individuals per sampling site obtained from Lushan and Enshi, respectively. Primers of eight loci of pure repeats and one locus of intermittent repeats were finally amplified successfully and yielded clear bands. A total of 41 alleles were detected. The allele number per locus of these microsatellites ranged from two to seven (mean 4.56). The expected heterozygosity (Exp-Het) ranged from 0.0494 to 0.8169. No linkage disequilibrium was found. Cross-species amplification demonstrated that six loci were amplified successfully in *P. multifida*. The markers helped to reveal the genetic variations of arsenic tolerant genotypes and understand the distribution pattern and ecological

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adaptation mechanism of *P. vittata*, and also assisted in breeding new varieties of fern for more effective remediation.

Key words: Chinese brake fern (*Pteris vittata* L.); Microsatellite; Polymorphism; FIASCO; *Cvi*QI-adaptor; Pig-tail *Cvi*QI-N primer

In recent years, arsenic pollution has become a worldwide problem affecting human health due to intense distortion caused by carcinogenic toxicity. However, the chemical form of arsenic in soil and groundwater varies widely, and governance is difficult. According to the World Health Organization, at least 50 million people worldwide have faced the threat of arsenic contamination. Arsenic pollution from manufacturing, mining, fertilizers and pesticides has resulted in China becoming one of the most polluted countries, with Inner Mongolia, Guizhou, Hunan, Sichuan and other provinces experiencing particularly serious contamination. Currently, the traditional methods of repairing arsenic-contaminated soil are technically difficult and costly, while phytoremediation based on hyperaccumulators, as first proposed by Brooks[1] in 1998, has attracted growing attention due to its low cost and environmental friendliness. A hyperaccumulator has the capacity to absorb heavy metal 100 times more than that of a typical plant. Chinese brake fern (*Pteris vittata* L.) was the first identified arsenic hyperaccumulator by Ma *et al.*[2] and Chen *et al.*[3] simultaneously. Currently, over 10 species of arsenic hyperaccumulators have been found in the world, which are capable of removing arsenic pollution from soil and groundwater.

Chinese brake fern is widely distributed in middle east and south China. It is traditionally cultivated in gardens, and is also used as an indicator of calcareous soil. Since arsenic pollution in soil has become a serious environmental and health problem worldwide, Chinese brake fern has attracted increasing interest not only because it is the first identified arsenic hyperaccumulator, but also because it is one of the most effective arsenic hyperaccumulators for use in phytoremediation of arsenic contaminated soils[2,3]. According to Chen *et al.*[3], Chinese brake fern in Hunan province efficiently extracted arsenic from soils and translocated it to its fronds, where arsenic concentrations reached 5070 mg/kg. Their experiment showed that *Pteris vittata* L. reduced total arsenic in the soil by 5% to 24% in one year, which was 200 times greater than that of a typical plant. As an arsenic hyperaccumulator, not only does *P. vittata* L. exhibit strong resistance to arsenic and high arsenic enrichment capability, but it also has the characteristics of fast growth, large biomass, and wide distribution, indicating that it is a very promising application plant. In the past few years, research on remediation efficiency[4] and the mechanisms of arsenic detoxification[5], distribution[6] and hyperaccumulation[7] have been developed. The morphology of Chinese brake fern varies greatly. Furthermore, findings from other studies have shown that arsenic accumulation level is positively correlated with frond and spore number as well as plant height[8], indicating that ecotype (or genotype) may be a key factor in arsenic accumulation. However, little is known about the genetic diversity of this species.

Microsatellites, or simple sequence repeats (SSR), has been proved to be a useful marker for studies on population genetics, molecular phylogeography and genetic resources assessment, conservation and management due to their co-dominance, high mutation rate and ease of scoring[9]. In this sense, SSR markers provide a

powerful tool for investigating genetic diversity, identifying genotypes or ecotypes, and breeding new varieties of Chinese brake fern for more effective remediation. Therefore, it is necessary to develop sufficient microsatellite markers with high polymorphism for this valuable species. In this study, we investigated the isolation of polymorphic microsatellite loci and their characterizations in Chinese brake fern, and examined the potential applicability of these DNA loci in *Pteris multifida* Poir., another potential phytoremediation plant[10] from China.

1 Materials and Methods

1.1 Sampling and DNA extraction

Specimens of Chinese brake fern were collected from Lushan National Park (29°34′21″N, 115°58′24″E) of Jiangxi province and Enshi city (30°16′13″N, 109°28′30″E) of Hubei province, China (20 individuals per sampling site). Genomic DNA was extracted using Cetyltrimethylammonium bromide (CTAB) combined with isopropanol precipitation[11].

1.2 Isolation of microsatellite markers

One *Pteris vittata* L. sample collected from Enshi city (30°16′13″N, 109°28′30″E) was selected to isolate polymorphic microsatellite loci based on the modified FIASCO[9]. The DNA was digested with *Cvi*QI (NEB) and ligated to *Cvi*QI-adaptors (5′-TAGTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAC-3′) simultaneously in a total volume of 25 μL containing 250 ng of genomic DNA, 1 × OnePhorAll buffer, 5.0 mmol/L DTT, 50 μg/mL BSA, 1.0 μmol/L adaptor, 200 μmol/L ATP, 2.5 U of *Cvi*QI, and 1.0 U of T4 DNA ligase (Promega). The reaction was then incubated at 25℃ for 16 h. The digestion-ligation mixture was diluted (1∶10) and the digested-ligated DNA was further amplified using a pig-tail[12] *Cvi*QI-N primer (5′-GTTTATGAGTCCTGACTACN-3′) on a Veriti® Thermal Cycler (Applied Biosystems). This PCR reaction was performed in a total volume of 20 μL containing 1 × *Taq* reaction buffer (Sangon Biotech Company, Shanghai), 1.5 mmol/L $MgCl_2$, 2.5 μmol/L primer, 200 μmol/L of each dNTPs, 0.5 U of *Taq* DNA polymerase (Sangon), and 5 μL of digested-ligated DNA dilution. The PCR cycling conditions were: an initial denaturation at 95℃ for 3 min, followed by 14–26 cycles of 94℃ for 30 s, 53℃ for 30 s, 72℃ for 30 s, and a final extension at 72℃ for 10 min. The PCR conditions producing a clearly visible smear were considered optimal and were selected for further use. The purified PCR products (500 ng) were hybridized to Biotin-labeled probes containing repeat motifs $(AC)_{12}$ and $(AG)_{12}$ (100 μmol each) in 100 μL hybridization solution (6 × SSC + 0.1% SDS) at 48℃ for 90 min. Probe-bound DNA fragments were then captured by streptavidin-coated magnetic beads (Promega) at room temperature for 30 min. Nonstringency washes and stringency washes were performed as described in the FIASCO protocol, except the stringency eluent was 0.5 × SSC + 0.1% SDS and the last stringency wash was incubated for 5 min at 45℃ to remove nonspecifically bound and unbound DNA. Enriched DNA fragments were amplified with a pig-tail *Cvi*QI-N primer under the conditions described above. The purified PCR products were ligated to pMD18-T vector (TaKaRa) according to the manufacturer's instructions, and then transformed into *Escherichia coli* DH5α competent cells. The positive clones were confirmed by colony PCR using $(AG/AC)_{12}$ and $M13^+/M13^-$ as primers[13], and sequenced by the Sangon Biotech Company (Shanghai) using an ABI PRISM 3730 sequencer (Applied Biosystems). Microsatellite sequences were screened using the software SSRHunter 1.3[14]. Primer pairs for each microsatellite locus were designed with PrimerPre-

mier 5.0 software (http://www.premierbiosoft.com/primerdesign/) and synthesized by Sangon Biotech Company (Shanghai).

1.3 PCR amplification and genotyping

The microsatellite primers were then optimized for annealing temperature and screened for consistent amplification with 10 individuals. PCR reactions were carried out in 10 μL total volume, which included 10 ng of genomic DNA, 0.2 mmol/L of each dNTPs, 0.2 μmol/L of each primer, 1 × PCR buffer (Sangon), 2 mmol/L Mg^{2+}, and 0.25 U of DNA *Taq* polymerase (Sangon). Amplification conditions were: 4 min denaturation at 94℃, 35 cycles of 30 s at 94℃, 30 s at Ta (℃), 30 s at 72℃, and a final extension of 72℃ for 10 min. Touch Down PCR was performed for WGC-1, WGC-7 and WGC-14: an initial denaturation at 94℃ for 5 min; 10 cycles of 30 s at 94℃, 30 s at (Ta + 4)℃, and 20 s at 72℃; 30 cycles of 30 s at 92℃, 30 s at Ta (℃) temperature, and 20 s at 72℃, and a final extension at 72℃ for 10 min. The amplification products were detected on 3% agarose gel.

Primers of eight loci of pure repeats and one locus of near pure repeats (Table 1) were selected to examine polymorphism in the 40 individuals collected from Lushan National Park of Jiangxi province and Enshi city of Hubei province, China (20 individuals from each site). Amplified products were separated on a 10% non-denaturing polyacrylamide gel using silver staining. A 20 bp DNA ladder (TaKaRa) was used to identify alleles.

Table 1 Characteristics of nine microsatellite loci for *Pteris vittata* tested on 40 individuals (each population had 20 individuals)

Locus	Repeat motif	Primer sequences (5′–3′)	Size range (bp)	Ta (℃)	Na	Ne	Obs-Het	Exp-Het	*Fis*	*Fst*	GB Acc. No.
WGC-1	$(TC)_{17}$	F: TTTTGTATTGGACACTGACACG R: CGGACGGCATGGAGAAG	296–348	54*	6	4.4024	0.0000	0.7832	1.0000	0.0164	KC698878
WGC-2	$(AG)_{13}$	F: AGCCGCTTGAACAAAGTAAA R: TACAAATTCCATTGAATGTGCT	136–144	45	3	1.6134	0.2432	0.3854	0.3670	0.0040	KC698879
WGC-7	$(TC)_{26}$	F: TCTCGTAGACCGATTTCCT R: ACAGCCCACCTCTTTCC	102–108	52*	4	2.2668	0.0000	0.5687	1.0000	0.4729	KC698884
WGC-10	$(GA)_{18}$	F: CTCCCCTATAAGCGTTCAAGC R: GCCCACATGGTCACCTCAA	100–140	53	7	5.1429	0.2500	0.8169	0.6789	0.0339	KC698887
WGC-11	$(CA)_{13}$	F: TCACCCTTGAACTCCCTC R: TGAAGCCCTGCTATTGG	124–126	55	2	1.0512	0.0000	0.0494	1.0000	0.0256	KC698888
WGC-13	$(GA)_{14}$	F: TGCGTAAGCCAGCTCCACATC R: GCCTCTATCTTCAGCGGGTTCAT	147–167	55	6	4.1570	0.1333	0.7723	0.7849	0.1172	KC698890
WGC-14	$(GA)_{23}$	F: AGTTGAAGGCTAAGGATGAT R: GCACTATTCTAAATGGGTGA	179–185	48*	4	3.5266	0.0323	0.7282	0.9532	0.0064	KC698891
WGC-22	$(CT)_8CC(CT)_{14}$	F: ATGGCAGTAATGTTGCTATCTCCG R: ATGGTGCATGTAAGGCTATTGGTT	226–238	54	4	1.7455	0.2222	0.4331	0.4764	0.0063	KC698899
WGC-25	$(CT)_{13}$	F: TCTATTGCTCTTACGCCCCTGAA R: TTTGTGCCCATCCAACCTGA	176–190	54	5	2.8851	0.3103	0.6649	0.5055	0.0407	KC698902

Notes: Ta = Optimal annealing temperatures (℃); Na = Observed number of alleles; Ne = Effective number of alleles; Observed heterozygosity (Obs-Het) and expected heterozygosity (Exp-Het) were computed using Levene (1949); *Fis* = Wright's (1978) fixation index (*Fis*) as a measure of heterozygote deficiency or excess; *Fst* = F-statistics (*Fst*) as a measure of genetic differentiation coefficient; Accession number of GenBank (GB Acc. No.). *: Touch Down PCR with 10 cycles of (Ta + 4)℃, followed by 30 cycles at Ta (℃).

1.4 Cross-species amplification

Pteris multifida is another plant that could be potentially used in phytoremediation of arsenic contaminated soils[10]. Specimens of *P. multifida* Poir. were collected from the campus of the China University of Geosciences (Wuhan). Genomic DNA was extracted using the CTAB method and cross-species amplification was done under the same PCR conditions used above.

1.5 Data analysis

The genotyping data were assessed for scoring errors using MICRO-CHECKER 2.2.3[15]. All diversity characteristics were calculated using POPGENE V1.31 software[16]. Tests for genotypic linkage disequilibrium and Hardy-Weinberg equilibrium (HWE) were also conducted using the same software package.

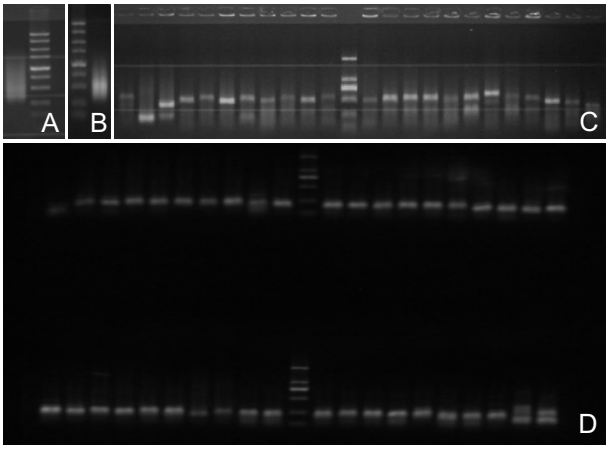
2 Results

2.1 Digestion, ligation and adaptor-mediated amplification of genomic DNA

The genomic DNA was efficiently digested by *Cvi*QI endonuclease and ligated to the *Cvi*QI-adaptor simultaneously. The digested-ligated DNA was successfully amplified using a pig-tail *Cvi*QI-N primer, forming a clear smear of fragments centered at approximately 500 bp (Fig. 1: A). The enriched DNA was also recovered by the primer (Fig. 1: B) successfully.

2.2 Distribution and characteristics of the microsatellites

We randomly selected 100 clones. Among them, 61 clones were confirmed positive by colony PCR (Fig. 1: C) and sequenced. A total of 51 clones contained the target repeating sequences, indicating that enrichment efficiency was as high as 51% (51/100). Among the repeating sequences, approximately 80% contained the AG/CT motif, only 4% contained the AC/GT motif, and the other sequences contained AG/CT and AC/GT compound motifs, indicating that the AG repeating sequence was much more abundant in the genome of Chinese brake fern than that of AC. Furthermore, 60% of the SSR-containing sequences had perfect repeats. Because perfect repeats[17] are more suitable for population analysis, these results revealed that our modified FIASCO method worked very well in Chinese brake fern genome and exhibited very high specificity.



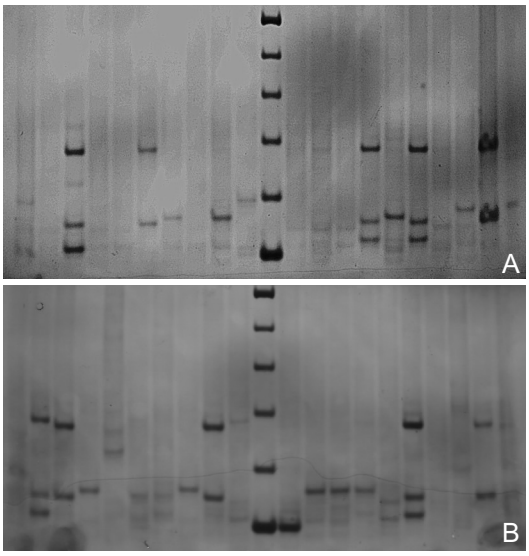
A: Amplification after digestion-ligation (1.5% agarose gel, Marker: TaKaRa DL5000); B: PCR recovery of enriched DNA (1.5% agarose gel, Marker: TaKaRa DL5000); C: Identification of positive clones in the enriched library by colony PCR (3% agarose gel, Marker: TaKaRa DL2000); D: Amplification of primer WGC-22 in 40 individuals (3% agarose gel, Marker: TaKaRa DL2000).

Fig. 1　Results of the modified FIASCO protocol

2.3 Primer design and polymorphism detection

Among the 51 microsatellite loci, PrimerPremier created putative primer sets for 25 loci (GB Acc. No. KC698878-KC698902) with scores higher than 90 points. These primer sets were considered as candidate pairs and synthesized. Primers of eight loci of pure repeats and one locus of intermittent repeats (Table 1) yielded successful amplicons of the expected size consistently (Fig. 1: D), and were further examined for polymorphism.

Polymorphisms were detected on 10% non-denaturing polyacrylamide gel (Fig. 2). The number of alleles of the primers ranged from two to seven. Furthermore, seven loci possessed more



A: Amplification of primer WGC-10 (100–120 bp) in LuShan population (20 individuals) (10% PAGE gel, Marker: TaKaRa 20 bp DNA ladder. From top to bottom: 200, 180, 160, 140, 120, 100 bp); B: Amplification of primer WGC-10 in EnShi population (20 individuals) (10% PAGE gel, Marker: TaKaRa 20 bp DNA ladder. From top to bottom: 200, 180, 160, 140, 120, 100 bp).

Fig. 2 Amplified results of primer WGC-10

than three alleles per locus. The effective numbers of alleles were between 1.0512 and 5.1429. Obs-Het were 0 to 0.3103, while Exp-Het were between 0.0494 and 0.8169. We also tested the coefficients for heterozygote deficiency (*Fis*) and genetic differentiation coefficient (*Fst*). The *Fis* were between 0.3670 and 1.0000, while the *Fst* were between 0.0040 and 0.4729 (Table 1). No significant linkage disequilibria were detected. All loci deviated from the Hardy-Weinberg equilibrium ($P < 0.05$).

2.4 Cross-species amplification

To examine the potential utility of these microsatellite markers developed for Chinese brake fern in other *Pteris* species, cross-species amplification was also conducted using genomic DNA from 10 individuals of *P. multifida* (Table 2). Primer sets of WGC-1 and WGC-7 amplified an unstable band with a size similar to Chinese brake fern, while primer set of WGC-25 amplified a very clear band (750 bp) that was much larger than expected. Six of the nine loci amplified in Chinese brake fern also amplified a band of similar size in *P. multifida* (Table 2). Therefore, these loci might facilitate evolutionary and population genetic studies as well as breeding and cultivar development in *P. multifida*.

3 Discussion

The morphology of Chinese brake fern varies greatly. The height ranges from 0.2 to 2 m. It also has a great change in biomass ranging in 5–36 t/hm^2 (fresh weight)[3]. Wei[18] suggested that the uptake of arsenic had no relationship with the ecotype of *Pteris vittata* L., while other studies[8] showed that arsenic accumulation was positively correlated with frond number, spore number and plant height, which provides a realistic basis as well as theoretical significance for the development of SSR markers. At present, few studies have been conducted on the genetic diversity of ferns[19]. Furthermore, genetic research on species such as *Pteris vittata* L. is particularly limited. Zhou[20] found a high degree of genetic diversity in limestone areas in Guangxi *Pteris vittata* L. populations using allozyme analysis, but the degree of difference was not proportional to distance and space. Yang[21] collected *Pteris vittata* L. samples from Guangzhou and Hunan province and developed eight pairs of SSR primers. A preliminary analysis of genetic diversity of the two populations was conducted; however, the eight

Table 2 Amplification of nine microsatellite primers developed for *Pteris vittata* in *P. multifida*

Locus	WGC-1	WGC-2	WGC-7	WGC-10	WGC-11	WGC-13	WGC-14	WGC-22	WGC-25
Result	±	+	±	+	+	+	+	+	–

Notes: '+' denotes amplification of a band in the same size range as *P. vittata*, '±' denotes unstable amplification, and '–' denotes no amplification in the same size range as *P. vittata*.

pairs of primers were not particularly perfect.

In the present study, the construction of the library enriched for $(AG)_n/(AC)_n$ microsatellite repeat sequences and primer exploitation of *Pteris vittata* was based on a modified FIASCO protocol. Our data showed that the modifications were successful, and both enrichment efficiency and specificity were improved.

The GTTT "pig-tail" was first used in microsatellite isolation by Glenn and Schable[12] to ensure efficient A-tailing of each PCR product, which would yield good results from TA cloning. We also found that the pig-tail *Cvi*QI-N primer could improve the amplification efficiency compared to that of normal *Cvi*QI-N primer (5′-GATGAGTCCTGACTACN-3′) (data not shown). We also found that the stringent eluent 0.5 × SSC + 0.1% SDS used to remove nonspecifically bound and unbound DNA performed better than that of stringent eluent 0.2 × SSC + 0.1% SDS, and the last stringent washing incubated at 45℃, which was about 10℃ lower than the melting temperature of the probes, was an appropriate stringent washing strength to get the best elution efficiency.

The high percentage (60%) of pure repeating sequences indicated that the loci we enriched were more ideal than those enriched by Yang[21] and also demonstrated that our modifications worked well with the FIASCO protocol. The AG repeating sequence was much more abundant in the Chinese brake fern genome than that of AC, which is consistent with the findings of Yang[21].

However, during the evaluation of these SSR polymorphisms, we found that all loci deviated from the Hardy-Weinberg equilibrium ($P < 0.05$). Although limited sample size, null alleles and geographical sites of the samples might result in the deviation, the more reasonable explanation for the deviation is that most ferns, including Chinese brake fern, reproduce by selfing. This is also proved by the positive coefficients for heterozygote deficiency (*Fis*), which illustrated that the loci were dominantly homozygous. We also tested the degree of genetic differentiation between Enshi and Lushan populations by *Fst*, which ranged from 0.0040 to 0.4729. This indicated that no gene flow barrier existed between the two populations.

The highly conserved flanking sequences of the SSR ensured the potential utility of these microsatellite markers developed for Chinese brake fern in *P. multifida* Poir., except that different species had different amplification conditions. Six of the nine loci amplified in Chinese brake fern also amplified similar band(s) in *P. multifida*, revealing that these loci can facilitate evolutionary and population genetic studies as well as breeding and cultivar development in *P. multifida*.

The new microsatellite markers developed in this study will be useful for investigating genetic diversity, population structure, and mating patterns, for identifying varieties and ecotypes, and for hybrid selection in breeding program of Chinese brake fern.

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