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Development and characterization of polymorphic microsatellite loci in endangered fern *Adiantum reniforme* var. *sinense*

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Abstract

Fourteen polymorphic microsatellite loci in the endangered fern *Adiantum reniforme* var. *sinense* were developed and characterized using the Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) protocol. Polymorphism of each locus was assessed in a bulked sample of 30 individuals from 8 natural populations. The number of alleles per locus varied from 2 to 11, with an average value of 6.2. The ranges of observed and expected heterozygosity were 0.000–0.895 and 0.226–0.868, respectively. These microsatellite markers provide useful tools for the ongoing conservation genetic studies of *Adiantum reniforme* var. *sinense*.

Adiantum reniforme L. var. *sinense* Y. X. Lin (Adiantaceae) was first discovered in China in 1978 (Lin 1980). The discovery of this tertiary relict fern has attracted an immediate attention to botanists for its intercontinental disjunct distribution pattern of the species with *A. reniforme* previously found in the Azores, Atlantic Ocean and *A. reniforme* var. *asarifolium* in southern-central Africa (Fu and Jin 1992). This taxon is believed to be of great scientific importance in studies of evolution and phytogeography of ferns (Fu and Jin 1992). *A. reniforme* var. *sinense* occurs in only one small area in Wanxian county, Chongqing, Southwest China where it is restricted to a narrow strip (approximately 150 km^2) along the Yangtze river valley between 80 and 430 m above sea level. *A. reniforme* var.*sinense* has been listed as an endangered species in the Chinese Red Data Book shortly after its discovery due to its sparse distribution, limited population numbers with small population sizes and being severely disturbed by anthropological activities (Fu and Jin 1992). The ongoing construction of the Three Gorge Dam (TGD) in Yangtze river valley has being posing great threats and changes to natural habitat of this species (Shen and Xie, 2004), since the natural range of *A. reniforme* var. *sinense* will be partially submerged in 2009 when TGD is completed and the water level rises by 175 m. In addition, *A. reniforme* var. *sinense* is a traditional Chinese herbal medicine used by local residents for the treatment of fever, carbuncles, inflammation, dropsy, humidness and jaundice. Because of the illegal overharvesting of this medicinal herb, it is near the brink of extinction. The endangered status of the species prompted conservationists and government agencies to rescue *A. reniforme* var. *sinense*. Successful propagation by artificial spore sowing (Xu et al. 1998) and the establishment of a few *ex situ* conserved populations in several botanic gardens for at least 10 years has assisted in preventing the extinction of this species (Chen et al. 2004). Translocation of individuals that will be submerged into safe locations within the natural range has also been conducted. However, little is known about the level of genetic variation and population

structure of *A. reniforme* var. *sinense*. Here, we report development and characterization of a set of polymorphic microsatellite loci in *A. reniforme* var. *sinense* in order to obtain population genetic information in both wild and *ex situ* conserved populations. Such population genetic information is a prerequisite to understanding the species survival possibility in the short-term and so that an effective conservation strategy for long-term survival can be formulated and implemented.

Microsatellite markers were developed for *A. reniforme* var. *sinense* using a modified genomic enrichment protocol of FIASCO (Fast Isolation by AFLP of Sequences Containing repeats) (Zane et al. 2002). Total genomic DNA was extracted from leaf tissue of *A. reniforme* var. *sinense* using the CTAB method (Doyle and Doyle 1987). Genomic DNA (250 ng) was completely digested with 3 units of *Mse*I (BioLabs) in a 25 μl volume, and then 15 μl of digested DNA was ligated to *Mse*I AFLP adaptor (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) using 1 unit of T4 DNA ligase (Takara) in a volume of 30 μl. The digestion–ligation mixture was subsequently diluted 10 times, and directly amplified using AFLP adaptor-specific primers (5′-GATGAGTC CTGAGTAAN-3′, i.e. *Mse*I-N) in a 20 μl reaction containing: *Mse*I-N 0.5 μM, dNTPs 0.2 mM, $MgCl_2$ 1.5 mM, 1 unit of Taq DNA polymerase (Fermentas) and 5 μl diluted digestion-ligation DNA. The PCR was performed using a program of 94 °C 30 s, 53 °C 1 min, 72 °C 1 min for 19 cycles.

After denaturation at 95 °C for 5 min, 20 μl amplified DNA fragments in the size range of 200–1000 bp were hybridized with 0.15 μM of 5′-biotinylated $(AAG)_8$ probe in 250 μl hybridization buffer containing SSC 4.2× and SDS 0.07%. The mixture was annealed at 48 °C for 2 h. Hybridization products were selectively captured with 300 μl Streptavidin MagneSphere® Paramegnetic Particles (Promega), which were prepared by washing in 150 μl TEN100 (10 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5) for three times. The mixture was incubated at room temperature for 30 min with constant gentle agitation. The beads–probe–DNA complex was separated by a magnetic field. Three low stringency washes in 500 μl of TEN1000 (10 mM Tris–HCl, 1 mM EDTA, 1 M NaCl, pH 7.5) were performed followed by three washes in 500 μl high stringency buffer (SSC 0.2× and 0.1% SDS) at room temperature. Captured DNA fragments were released from the complex by incubating for 5 min at 95 °C in 50 μl of TE (Tris–HCl 10 mM, EDTA 1.0 mM, pH 8.0).

Recovered DNA fragments were amplified using *Mse*I-N primers for 23 cycles as described above. PCR products were purified using E.Z.N.A® Gel Extraction Kit (Omega). The purified fragments were ligated into pMD 18-T plasmid vector (Takara) and transformed into *E. coli* strain (JM109, Promega) following the manufacturer's instructions. Recombinant clones were identified using blue/white screening on Luria–Bertani agar plates containing ampicillin, X-gal and IPTG. Insert-positive bacterial clones were amplified using M13 forward and reverse primers and visualized by agarose gel electrophoresis. Identified positive clones were sequenced with ABI BigDyeTM Terminators Cycle Sequencing Kit (Applied Biosystems) in an ABI PRISM3100 automated sequencer.

Of the 126 clones sequenced, 86 (68%) were found to contain simple sequence repeats. Forty-one primer pairs flanking microsatellite regions were designed using the software Primer3.0 program (Rozen and Skaletsky 2000) and used for preliminary assessment. The PCR amplifications were performed in a final volume of 10 μl containing 10 mM Tris–HCl (pH 8.4), 50 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each primer, 1 unit *Taq* polymerase (Fermentas), and 20 ng of genomic DNA. The amplification profiles included an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 45 s at 94 °C, 1 min at 56–58 °C depending on the primer pair (Table 1) and 1 min at 72 °C, followed by a final extension step for 8 min at 72 °C. Amplified products were separated on a 6% denaturation polyacrylamide gel using silver staining. A 25 bp DNA ladder (Promega) was used to identify alleles.

Fourteen primer pairs were characterized by screening in a bulked sample including 30 individuals of *A. reniforme* var. *sinense* randomly sampled from eight populations, with three or four samples per population. The number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) were calculated using the program Genepop version 3.4 (Raymond and Rousset 1995). The 14 polymorphic loci revealed a total of 87 alleles

Table 1. Characterization of 14 microsatellites in *Adiantum reniforme* var. *sinense*

Locus Accession no.	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	Size range (bp)	A	H_E	H_O	F_{IS}
Ars03 DQ205524	$(AC)_{20}$	F: GCTTCCTTTTGTGCAACTCC R: AAGAAGCCAAAGGGGCTAAG	56	248–250	2	0.266	0.000	1.000
Ars07 DQ205525	$(AG)_{24}$	F: AGGCTCACCTGTCCTCATTG R: CGTGCGTTTAGGGTTCAATC	56	175–220	7	0.751	0.895	−0.166
Ars10 DQ205526	$(CT)_{13}\cdots(CT)_9\cdots(CA)_9$	F: GACTCTTGCCCTCCATCAAA R: GGAGCTAGAGCTTGCCAAAA	57	199–203	3	0.406	0.167	0.608
Ars17 DQ205527	$(AG)_{19}$	F: ACCCATGCATGCACAATTT R: GTACGTCTCCAACCGCAAAC	56	220–240	5	0.690	0.250	0.656
Ars18 DQ205528	$(CT)_{27}\cdots(CT)_{17}$	F: TGTCATCCCTGTGATTTGCT R: GCACACTTGTGAAACTCATGC	58	200–250	8	0.770	0.421	0.474
Ars20 DQ205529	$(GT)_{19}(GA)_{21}$	F: GACTGGTCAAAGAGGCATCC R: TTTTGAGCCCATTTTGAGGT	58	225–275	8	0.824	0.400	0.533
Ars21 DQ205530	$(CT)_{22}\cdots(AC)_9$	F: TCCCTTCCTCTTCCTTTCTG R: TCAAATGGGTGTGATTGTGTG	57	175–220	7	0.596	0.421	0.318
Ars29 DQ205531	$(CT)_8TT(CT)_{15}$	F: TTCAAGGGAAGCCTACGAAA R: GCACCCAATTTCTCAAGGAG	57	170–176	4	0.666	0.350	0.494
Ars31 DQ205532	$(GA)_{23}$	F: AAAAGTGAATCAAGAGTCCAAGC R: TGTGTTTTGCAAGAGGGAAA	57	225–250	8	0.810	0.474	0.438
Ars32 DQ205533	$(CT)_{29}$	F: CACACTCATGCATGGGAAAA R: AATGAGAGGTGGGCATTGAC	57	145–170	8	0.852	0.611	0.309
Ars33 DQ205534	$(CT)_{16}\cdots(CACT)_7$	F: GGGCTTTTACCTACACCCACA R: GCACATTGTTTCGATTCTTATTG	56	184–190	4	0.676	0.650	0.064
Ars36 DQ205535	$(CT)_{25}$	F: CCCTCAAACTTTGGAGTGGA R: TTTCCCCTTTGAGATGCTTG	56	140–186	11	0.868	0.526	0.417
Ars37 DQ205536	$(TG)_{16}(GA)_{16}$	F: TGCGTTTACTAATTGCAGGTG R: ACTTTGGCCAATAGCGTCAC	57	200–226	6	0.745	0.579	0.249
Ars41 DQ205537	$(TC)_{42}$	F: GGCTTGTGTCTACGTGCAAA R: CGGTTACTTCAGGGCAACAT	56	210–260	6	0.796	0.500	0.403

T_a, annealing temperature; A, number of alleles revealed; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient. Nucleotide sequences from which these primers were designed had been deposited into GenBank under accession numbers DQ205524–DQ205537.

across the *A. reniforme* var. *sinense* individuals tested, ranging from 2 to 11 alleles per locus (Table 1). The observed heterozygosity and expected heterozygosity ranged from 0.000 to 0.895 and from 0.266 to 0.868, respectively.

The loci were further tested for both Hardy–Weinberg equilibrium and linkage disequilibrium using the software Genepop version 3.4 (Raymond and Rousset 1995) and significance levels were determined after 100 batches of 5000 iterations each. The analysis revealed significant ($P < 0.05$) heterozygote deficiency for all loci, with the exception of locus Ars07. The generality of observed heterozygote deficiencies is likely the result of small population size, genetic drift and the presence of null alleles. No evidence of linkage disequilibrium between pairs of loci was found in any combination of loci pairs.

These 14 polymorphic microsatellite loci should furnish the first benchmark for population genetic studies aimed at both *in situ* and *ex situ* conservation of *A. reniforme* var. *sinense* and may also be useful for other closely related taxa in the *Adiantum* genus.

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