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# Isolation and Characterization of Microsatellite Loci in the Tree Fern *Alsophila spinulosa*

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ABSTRACT.—*Alsophila spinulosa* is a famous relict tree fern. Three polymorphic microsatellite makers were developed and characterized using the Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) protocol. The polymorphism was quantified for a single population from Guizhou province, China. The number of alleles per locus varied from 2 to 4. The ranges of observed and expected heterozygosity were 0.000–0.750 and 0.218–0.651, respectively. These were the first microsatellites reported for the Cyatheaceae and will be useful for the ongoing population and conservation genetic studies of other remaining extant populations.

KEY WORDS.—*Alsophila spinulosa*, genetic variation, microsatellite

*Alsophila spinulosa* (Hook) Tryon (Cyatheaceae) is a famous relict fern with a tall, erect, arborescent rhizome. Historically, its distribution has been strongly influenced by Quaternary climatic changes (Tryon, 1970). During the last few decades, *A. spinulosa* has been experiencing a drastic decline caused by habitat loss and fragmentation, local economic exploitation and human activities. In China, wild populations and individuals of *A. spinulosa* are extremely rare and are restricted to tropical and subtropical montane regions, occupying warm, humid and shady niches at low latitudes (Fu, 1991). The species is now listed in the Chinese Red Book of endangered species (Fu, 1991). As a species with long evolutionary history, it is of great scientific importance for investigating pteridophyte phylogeography, speciation, and adaptive evolution (Su *et al.*, 2005).

Information about the level and partitioning of genetic variation within and among populations of threatened species is critical to determine appropriate management strategies (Dawson and Powell, 1999). In a previous study, the population genetic structure and variation of *A. spinulosa* were inferred using nuclear RAPD markers (Wang *et al.*, 2004). However, observed heterozygosity and population differentiation cannot be detected directly with dominant makers (Zhivotovsky, 1999), which prompted a search for codominant microsatellite markers. Microsatellite markers have already been developed in a range of plant taxa. However, to date there are only a few reports of microsatellite markers in pteridophytes (e.g. Pryor *et al.*, 2001; Vitalis *et al.*, 2001; Woodhead *et al.*, 2005; Kang *et al.*, 2006).

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Here we report the isolation and characterization of a set of polymorphic microsatellite loci from the genome of *A. spinulosa* and preliminarily assess genetic diversity using wild individuals from a population in Guizhou province, China.

## Materials and Methods

Total genomic DNA was extracted from leaf tissue following modified CTAB protocols (Su *et al.*, 1998). Microsatellite markers were isolated following the protocol of FIASCO (Fast Isolation by AFLP of Sequences Containing repeats) with minor modifications (Zane *et al.*, 2002). A total of 250 ng genomic DNA 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′-GACGATGAGTCCTGAG-3′/5′-TACTCAGGACTCAT-3′) using 1 unit of T4 DNA ligase (BioLabs) in a 30 μL volume at 20°C for 3 h. The digestion-ligation mixture was diluted (1:10), and directly amplified using *Mse*I adaptor-specific primers (5′-GATGAGTCCTGAGTAAN-3′) in 20 μL with 0.9 μM *Mse*I-N, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (Tiangen) 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 20 cycles. Approximately 1000 ng amplified DNA fragments were hybridized with 200 pmol of 5′-biotinylated $(AC)_{15}$ probe in a total volume of 250 μL of SSC 4.2× and 0.07% SDS, by denaturing DNA for 5 min at 95°C and incubating at 60°C for 2 h. The hybridized DNA was then mixed with 600 μL of Streptavidin MagneSphere Paramagnetic Particles (Promega) which had been treated 3 times with 150 μL of TEN100 (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5), allowing a selective binding at room temperature for 30 min. The beads-probe-DNA complex was separated by a magnetic field. After removing nonspecific DNA fragments by nonstringent washes (10 mM Tris-HCl, 1 mM EDTA, 1 mM NaCl, pH 7.5) and stringent washes (SSC 0.2× and 0.1% SDS) for 3 times each, the target DNA was released from the bead-probes with 50 μL TE (Tris–HCl 10 mM, EDTA 1.0 mM, pH 8.0) at 95°C for 5 min, and transferred as soon as possible.

DNA containing repeats were amplified for 30 cycles with *Mse*I-N primers and the same program mentioned above was used. Fragments ranging from 400 to 1000 bp were isolated and purified (Omega Biotek). They were then ligated into the pMD19-T plasmid vector (TaKaRa) and were transformed into competent *Escherichia coli* cells DH-5α. Positive clones were identified by blue/white selection, then were amplified using M13 universal primers and visualized by agarose gel electrophoresis. Eighty clones with different insert fragments were sequenced, 85% of which contained simple sequence repeats. Subsequently, 33 primer pairs were developed from simple sequence repeats containing ten or more repeats with suitable flanking sequences.

All of the 33 pairs of primers were tested using 74 *A. spinulosa* individuals sampled from a population located in Guizhou province, southern China. PCR reactions were performed in a 10 μL volume containing approximately 20 ng DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM each primer, 1×*Taq* buffer and 0.5 unit *Taq* polymerase (Tiangen). The PCR profiles included an initial

TABLE 1. Characterization of 3 polymorphic microsatellite loci in *Alsophila spinulosa*. $T_a$, annealing temperature; $N_A$, number of alleles; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity.

| Locus | GenBank Accession no. | Repeat motif | Primer sequence (5′–3′) | $T_a$ (°C) | Size range (bp) | $N_A$ | $H_O$ | $H_E$ |
|---|---|---|---|---|---|---|---|---|
| As1 | EU036670 | $(GA)_{5...}(Ac)_5$ $(AG)_{10...}$ $(AG)_{13}$ | F:GCACGGGTAGCCCAGATG R:ATGGGTTCGCCCCTTCTT | 54 | 215–219 | 3 | 0.000 | 0.493 |
| As2 | EU036671 | $(GA)_{19}$ | F:TCAAACATTCTACCAC-GAAGC R:TCATTTCATACCATTCC TCCC | 52 | 225–228 | 2 | 0.000 | 0.218 |
| As3 | EU036672 | $(GT)_{10}(GA)_{7....}$ $(TG)_6(AG)_6$ | F: TATTTGGTGGAAAGT-GAA R: ATCTTGGTTTGCGTCTAA | 48 | 177–201 | 4 | 0.750 | 0.651 |

denaturation at 94°C for 5 min, followed by 35 cycles of 50 s at 94°C, 50 s at annealing temperature, 90 s at 72°C and then 10 min at 72°C. Amplified products were electrophoresized in 6% denaturation polyacrylamide gel and visualized by silver staining. A 25 bp DNA ladder (Promega) was used to identify alleles.

## RESULTS AND DISCUSSION

Fourteen of the 33 primer pairs successfully amplified DNA fragments, but only three yielded polymorphic loci. Preliminary population genetics analyses were performed using GENEPOP (Raymond and Rousset, 1995).The number of alleles, the observed and expected heterozygosities, Hardy-Weinberg equilibrium and linkage disequilibrium were examined. The markers reveal relatively low level of variation within the population. The number of alleles per locus ranged from 2 to 4, $H_O$ ranged from 0 to 0.750 and $H_E$ from 0.218 to 0.651, respectively (Table 1). All three polymorphic loci deviated significantly from Hardy-Weinberg equilibrium and showed notable deficits of heterozygotes ($P < 0.001$). This deviation from Hardy-Weinberg suggests high levels of inbreeding within this population. No significant linkage disequilibrium was observed among the three pairs of loci.

The three polymorphic loci presented here provide the first set of codominant markers for population genetic study of *A. spinulosa*. Although these markers revealed very low genetic diversity, it is not surprising for a potential self-fertilization fern. We believe that these makers will be useful for the ongoing population and conservation genetic studies of other remaining extant populations of *A. spinulosa*. Moreover, since limited population genetic studies exist on pteridophytes using SSRs, further analyses of *A. spinulosa* may provide data of more general relevance.

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