Short Communication

Polymorphic microsatellite markers in the traditional Chinese medicinal plant *Paris polyphylla* var. *yunnanensis*

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ABSTRACT. *Paris polyphylla* var. *yunnanensis* is a traditional Chinese medicinal plant and is listed as vulnerable by the IUCN. This medicinal herb is well known for its analgesic and anti-inflammatory properties, most notably as an ingredient of the hemostatic compound "Yunnan Baiyao". However, over-exploitation of the plant for economic purposes is pushing the species to the brink of extinction. Therefore, there is an urgent need to develop conservation strategies for this endangered species, for example, through assessment of its genetic structure and diversity. Here, we developed ten novel polymorphic microsatellite loci. The characteristics of these markers were assessed in 60 individuals from South China. The number of alleles per locus ranged from 4 to 12, Observed and expected heterozygosity ranged from 0.303 to 0.969

and from 0.790 to 0.976, respectively. These markers will therefore be useful tools for future population genetic studies and for the conservation of genetic resources of *P. polyphylla* var. *yunnanensis*.

Key words: *Paris polyphylla* var. *yunnanensis*; Genetic diversity; Microsatellites markers

INTRODUCTION

Paris polyphylla var. *yunnanensis* is a traditional Chinese medicinal plant that is listed as vulnerable by the IUCN. The herb is well-known for its analgesic and anti-inflammatory properties, most notably as an ingredient of the hemostatic compound "Yunnan Baiyao" (Wu et al., 2004). However, over-exploitation for economic purposes is pushing this species to the brink of extinction. Therefore, there is an urgent need to develop conservation strategies for this endangered species through the characterization of its genetic structure and diversity.

Microsatellite markers (SSRs) have been successfully used for population genetic diversity analysis because of their co-dominant inheritance and high levels of polymorphism (Varshney et al., 2005; Kalia et al., 2011) and used in investigations of many species of medicinal plants (Verma et al., 2013). To date, 12 polymorphic microsatellite loci have been reported in *P. polyphylla* var. *yunnanensis* (Zheng et al., 2012). Here, we report 10 new polymorphic microsatellite markers for this species and expect them to facilitate the further development of genetic conservation strategies.

MATERIAL AND METHODS

Sixty-two individuals were collected from Yunnan in South China and their identity confirmed by morphology. Genomic DNA was extracted from leaves using the cetyltrimethyl-ammonium bromide method (Doyle and Doyle, 1987). A magnetic bead enrichment strategy was used to isolate the microsatellites (Nunome et al., 2006). Approximately 50 μg genomic DNA was digested with *Sau*3AI (New England Biolabs, Beijing, China) for 4 h at 37°C. The digested DNA was separated with centrifugal concentrators. Fragments 300-1000 bp in length were excised and recovered using the AxyPrep DNA Gel Extraction Kit (Axygen, Shanghai, China). The purified fragments were then ligated to two adaptor oligonucleotides (adaptor A: 5'-GATCGTCGACGGTACCGAATTCT-3'; adaptor B: 5'-GTCAAGAATTCGGTACCGTCG AC-3') with T4 DNA ligase (Takara, Dalian, China) overnight at 16°C. The PCR products were then hybridized to biotinylated probes (AC)$_{15}$ or (AG)$_{15}$ to select fragments with microsatellites. Hybridization products were captured with streptavidin-coated magnetic beads (Promega, Beijing, China), and enriched by PCR. The PCR products were ligated into pEASY-T1 using a Cloning Kit (TransGene Biotech, China) and then transformed into DH5α competent cells (TaKaRa). Ninety-eight positive clones were identified, 45 of which had microsatellite repeats. Thirty-seven clones were suitable for designing locus-specific primers using Primer Premier 3.0 (Rozen and Skaletsky, 2000).

Ten SSR primers were tested using the 62 Yunnan province individuals. Amplification was performed in a 20-μL volume containing 75 ng template DNA, 2.5 X multiplex buffer, 1 U Taq DNA polymerase (Microread, Beijing, China) and 5 μM of each primer (Sangon, Shanghai, China), one of which was labelled with a fluorescent dye. Amplification conditions

were 95°C for 5 min, followed by 30 cycles at 94°C for 30 s, 56°C for 45 s, and 72°C for 45 s; 10 cycles at 94°C for 30 s, 53°C for 45 s, 72°C for 45 s and a final extension at 72°C for 12 min. Fluorescently labeled fragments were visualized on an ABI Prism 377 DNA Sequencer with a GeneScan-500 ROX size standard (Applied Biosystems). Allele size was called with the GeneScan software ver. 3.7 (Applied Biosystems).

Cervus ver. 2.0 (Marshall et al., 1998) was used to calculate observed (H_O) and expected heterozygosity (H_E), to evaluate deviation from the Hardy-Weinberg equilibrium (HWE), and to obtain polymorphic information content (PIC) values. Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested using GenePop 3.4 (Raymond and Rousset, 1995).

RESULTS AND DISCUSSION

Forty-five sequences were identified that contained microsatellites with perfect repeat motifs, and 37 of these microsatellites were suitable for designing locus-specific primers. In total, 10 loci were successfully amplified and showed high levels of polymorphism (Table 1). The number of alleles per locus ranged from 4 to 12, with an average of 7. H_O ranged from 0.303 to 0.969 and H_E from 0.790 to 0.976. PIC values ranged from 0.766 to 0.976 (mean = 0.930). All ten loci were highly polymorphic (PIC > 0.5). Six loci (*CL35*, *CL38*, *CL1*, *CL13*, *CL3*, and *CL32*) showed significant deviation from HWE (P < 0.05), probably due to an excess of homozygotes. Analyses using GenePop showed that significant LD was observed between two locus pairs, *CL11* and *CL37*, and *CL34* and *CL47*.

The microsatellite markers reported here will be of value for genetic diversity studies in *P. polyphylla* var. *yunnanensis* and will facilitate the future investigation of the population genetic structure in this species. Furthermore, we expect that these 10 markers will aid the development of efficient strategies for the conservation and management of the germplasm of this medicinal plant.

Table 1. Characteristics of 10 microsatellite loci in *Paris polyphylla* var. *yunnanensis*.

GenBank accession No.	Primer sequence (5'-3')	Repeat motif	Size range (bp)	N_A	H_O	H_E	PIC
CL11 KR047182	F: GCTGGGCTAGGATTTTGGTA						
	R: GCAATTTTGAGCTGCTGACTC	$(GA)_7…(GA)_9…(GA)_9$	285-335	12	0.969	0.968	0.967
CL35 KR047186	F: CCTCGACCGGAGTCCATAT						
	R: CGTTGTGCGGGTGATGA	$(AG)_{26}$	200-300	7	0.818	0.96*	0.963
CL37 KR047183	F: CCCGATCCCACCAGAGAAG						
	R: ACCCCGGTTTGGTAGGCTA	$(AG)_8…(AG)_{20}$	200-300	7	0.788	0.963	0.962
CL38 KR047187	F: CGAACATGATCGGAGTACGG						
	R: CCGACAACATCGGACCTCAT	$(GA)_{15}$	200-300	7	0.787	0.927*	0.922
CL1 KR047184	F: GACGACGGCTATTGCTCC						
	R: AGCCCGTCGCCATTAGCT	$(AG)_7…(AGC)_6…(AG)_{18}…(AG)_5…$	300-480	7	0.758	0.962*	0.961
CL13 KR047188	F: GAAGCACCAGTTGGTGGAG	$(AG)_{30}…(AG)_{12}$					
	R: GGGTCACCCTACCTTTCAGC	$(TC)_6…(TC)_{23}…(CT)_6$	150-340	10	0.818	0.976*	0.976
CL3 KR047185	F: AGCCATCTTCTTCGTTTGGC						
	R: CGAATCAACGGCGAGTGTC	$(CT)_{20}$	120-220	6	0.485	0.953*	0.950
CL34 KR047189	F: GGTGACGCCACAAAGAAGAC						
	R: CGTCGTGACCGCCTACTACT	$(AG)_6…(AG)_{22}$	90-190	7	0.697	0.950	0.945
CL32 KR047190	F: TAATCCCTATCCACCCTCACC						
	R: GCACTCCATCATGACGAGAGT	$(TC)_7$	120-220	7	0.879	0.904*	0.897
CL47 KR047191	F: CGACGACGATTGCCTCC						
	R: TTCCCTCTCAACTGCCTGG	$(C)_{14}…(CA)_9…(T)_7$	100-130	4	0.303	0.790	0.766

N_A = number of observed alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphism information content. *significant deviation from HWE after Bonferroni correction (P < 0.05).

Conflicts of interest

The authors declare no conflict of interest.

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