Isolation and Characterization of 10 Polymorphic Microsatellite Loci in *Paphiopedilum concolor* (Batem.) Pfitzer (Orchidaceae) and Cross-species Amplification

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Abstract. Ten polymorphic microsatellite loci were isolated and characterized from an enriched genomic library of *Paphiopedilum concolor* (Batem.) Pfitzer. The number of alleles per microsatellite locus ranged from three to 11 with an average of 6.4 in a sample of 30 individuals from three populations. The observed and expected heterozygosity ranged from 0.200 to 0.800 and from 0.544 to 0.827, respectively. These microsatellites can be used as tools to investigate the genetic structure of *P. concolor* populations and relationship patterns with closely related taxa.

The genus *Paphiopedilum* Pfitzer, popularly known as slipper orchids because of the resemblance of the pouch-shaped lip to a lady's slipper (Cribb, 1998; Sheehan and

Sheehan, 1994), have captured the interest of many orchid growers and hobbyists, and *Paphiopedilum* are one of the most popular and rare orchid genera being sold and exhibited today. Their unique shape, variety of colors, and extended flowering period make them distinctive to the orchid family. The genus is comprised of ≈ 77 species found in southern India, Nepal, Bhutan, northeast India, and Burma across to southern China, Southeast Asia, the Malay Archipelago, the Philippines, New Guinea, and the Solomon Islands. A total of 27 species of *Paphiopedilum* have been reported to be present in China (Cribb, 1998; Liu et al., 2009a, 2009b). Wild populations are under increasing pressure from overcollection and habitat destruction, and all species are listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora

(CITES), Appendix I, thus restricting trade of these plants.

Like many other slipper orchids, *P. concolor* (Batem.) Pfitzer, a native of southeast Burma, southwest China (Yunnan, Guizhou, and Guangxi provinces), Thailand, Laos, Cambodia, and Vietnam, has become a popular ornamental plant since its discovery in 1865 (Cribb, 1998; Liu et al., 2009a). This popularity as well as general habitat destruction and overcollection, has contributed to a drastic decline in the number and range of the wild populations. Determining the genetic diversity of the remaining wild plants is a critical step in developing effective conservation strategies for *P. concolor* as well as many other species. Microsatellites are ideal markers for characterizing of population structure (Schlotterer, 1998) and have previously been used for the study of endangered orchids (Rodrigues and Kumar, 2009). In the current investigation, we focused on microsatellite isolation in *P. concolor* to obtain polymorphic markers for investigation of population genetics.

A microsatellite-enhanced genomic library was constructed using an optimized affinity capture technique described by Fleischer and Loew (1995) and Xia et al. (2006). A *P. concolor* plant was sampled from Yunnan province, China. Genomic DNA was extracted from fresh leaf tissue by using the cetyltrimethyl ammonium bromide method (Doyle, 1991). We constructed an enriched partial genomic library following a modified protocol of fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO) (Zane et al., 2002). Approximately 250 ng of genomic DNA was digested into 200 to 1000 bp by a restriction enzyme *Mbo*I (TaKaRa, Japan) and the resulting fragments were ligated to *Mbo*I linkers (SAULA: 5'G CCGTACCCGGGAAGCTTGG3'; SAULB: 5'GATCCCAAGCTTCCCGGGTACCGC3') with T4 DNA ligase (TaKaRa) and incubated overnight at 16 °C. The digestion–ligation mixture was subsequently diluted 10 times, and 2 μ L was used for polymerase chain reaction (PCR) amplification with SAULA oligonucleotide as the primer. PCR products were hybridized to two single-strand biotinylated microsatellite oligonucleotides [(CA)₁₂-biotin and (GA)₁₂-biotin]. These hybrids were captured by streptavidin-coated magnetic beads (BioMag Nuclease-Free Streptavidin; Qiagen). The magnetic beads were eluted and the resulting DNA solution served as a template for PCR using SAULA oligonucleotide as the primer. The amplification product was directly ligated into the pGEM-T plasmid vector (Promega, Madison, WI) and transformed into *Escherichia coli* DH5 α competent cells (TaKaRa) were plated onto LB agar medium (Bacto-Tryptone 10 g·L⁻¹, Bactoyeast extract 5 g·L⁻¹, NaCl 5 g·L⁻¹, agar 6 g·L⁻¹). Recombinant plasmids were identified through blue–white screening. For further identification of the clones containing microsatellites, colony PCR was performed with a primer combination of (CA)₁₂ or (GA)₁₂ and M13 universal primers. The insert fragments

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Table 1. Characteristics of 10 microsatellite loci for *P. concolor*

Locus	Repeat motif	Primer sequence (5'-3')	T_m (°C)	Size range (bp)	N_a	H_E	H_O	F_{IS}	P-HWE	GenBank accession no.
PCCA25	(CA) ₂₉	TGGATAATCTGTCTGGTAA GTGTAAAGAACTGGAGGG	54	170–182	3	0.623	0.767	-0.278	0.1101	GU994029
PCCA88	(CA) ₁₄	GGCGGTCCCCCTCAGATAT TGTGGTACCCGGGAAGCT	48	142–153	6	0.739	0.600	+0.193	0.0564	GU994030
PCCA100	(CA) ₁₀ (GA) ₆	GGGGTGATTACAGGAAGCT TCTGTCTCCTTTCACGGT	50	182–216	9	0.808	0.414	+0.460	0.0000 ^z	GU994031
PCCA120	(CA) ₂₀	ACACGGTCGTACACTTTCA CAGGCATCTACTGGGATTA	58	170–183	4	0.567	0.423	+0.258	0.0278	GU994032
PCCA176	(TG) ₃₀	TACCAGCACCTTAGTTTC ATTGTAAGCCCCACCTCT	48	168–179	3	0.544	0.800	-0.408	0.0011	GU994033
PCGA2	(GA) ₁₁	TCATCGGAGATGGCTACTA CTGGAGTGTGTTGGAGTTC	58	132–145	6	0.720	0.200	+0.724	0.0000 ^z	GU994034
PCGA29	(CT) ₁₇	TGAGAATGGAAAAGGAGGAT ACGCGAACATGACAAGAA	50	114–143	11	0.792	0.786	+0.008	0.6846	GU994035
PCGA40	(GA) ₁₂	TCTTCATCGCCTAGAGTTG TTGATTCCCCTGGTTGTT	54	185–218	6	0.827	0.200	+0.721	0.0000 ^z	GU994036
PCGA49	(CT) ₁₀	TGACATCATCGGAGAACAA TCAGAGGTGAGCCAGAAT	50	217–241	5	0.754	0.500	+0.341	0.0002 ^z	GU994037
PCGA52	(CT) ₁₄	CGTAGTGGTTGGGATAA TAGGAGTGGAGGTCATCG	48	197–224	11	0.809	0.700	+0.136	0.0053	GU994038

^zSignificant deviation from Hardy-Weinberg equilibrium corrected for multiple comparisons using the Bonferroni correction ($P < 0.001$).

T_m = annealing temperature; N_a = number of alleles; H_E = expected heterozygosity; H_O = observed heterozygosity; F_{IS} = inbreeding coefficient; P-HWE = probability of Hardy-Weinberg equilibrium.

of 124 positive clones were sequenced. Among the 97 (78.23%) positive clones containing microsatellites with five or more repeat units, 63 were selected for primer development. Amplification primers were designed using PRIMER 5.0. To control for polymorphism of the identified microsatellite loci, 30 individual *P. concolor* were used from three natural populations in Baoshan and Wenshan in Yunnan province and Pengxiang in Guangxi province and planted in a greenhouse in South China Botanical Garden, Chinese Academy of Sciences. PCR amplification was performed in 10-μL reaction mixtures consisting of ≈50 ng of template DNA, 50 mM of KCl, 20 mM of Tris-HCl (8.0), 1.5 mM of MgCl₂, 0.2 mM of each dNTP, 0.2 μM of each primer, and 1 U of Taq DNA polymerase (TaKaRa). The reaction mixture was subjected to PCR amplification in a PTC-100 (MJ) with the following cycling profile: 5 min at 95 °C followed by 35 cycles of 40 s at 94 °C, 50 s at the primer specific annealing temperature (Table 1), 1 min at 72 °C, and a final prolonged extension of 10 min at 72 °C. PCR products were separated on 5% denaturing polyacrylamide gels using a 100-bp DNA ladder standard (Promega) as the reference and visualized by silver staining. The microsatellite alleles were sized by using LABIMAGE Version 2.7.1.

Approximately 47 of the 63 primer pairs were successfully amplified containing 10 polymorphic loci and 37 monomorphic loci. Variability of the microsatellite loci was evaluated using POPGENE32 (Yeh et al., 1997). Deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested with GENEPop Version 3.4 (Raymond and Rousset, 1995) and Rice's method of sequential Bonferroni correction (Rice, 1989).

Genotyping of all individuals revealed moderately high polymorphism with three

to 12 alleles per locus and an across-loci average of 6.4 alleles per locus. The expected heterozygosity ranged from 0.200 to 0.800, and the observed heterozygosity varied from 0.544 to 0.827 (Table 1), respectively. No evidence of linkage disequilibrium was found between any loci pairs, indicating physical independence of the 10 loci. Four loci, PCCA100, PCGA2, PCGA40, and PCGA49, exhibited significant deviation from HWE after Bonferroni correction (adjusted P value = 0.001; Table 1) as a result of heterozygote deficiency (positive F_{IS} values), which could be caused by inbreeding in this species.

We also tested for cross-species amplification of the 10 *P. concolor* microsatellite primers in two closely related species, *P. bellatulum* (Rchb.f.) Pfitzer and *P. wenshanense* Z.J.Liu & J.Yong Zhang (Liu et al., 2009a, 2009b) using the same PCR conditions as previously described. Seven positive amplifications occurred across all tested species; the remaining three (PCCA88, PCCA100, and PCCA176) resulted in weak amplifications.

Data generated in this study strongly indicate that these newly developed microsatellite loci are potentially useful markers for investigating both population genetics and conservation biology of the congenetic *Paphiopedilum* species. The results have also great potential value for identification of hybrids of this slipper orchid, for analyzing genetic structure of native populations, and for learning the genetic background of the artificial growing individuals for restoring wild populations of this slipper orchid.

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