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Isolation and Characterization of Nine Microsatellite Markers for *Cymbidium sinense*

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Abstract. **Microsatellite-enhanced genomic library of *Cymbidium sinense* was constructed using repeat-enrichment method with biotin-labeled oligos and streptavidin magnetic beads. Twenty-five microsatellite loci were isolated from a microsatellite-enhanced genomic library of *Cymbidium sinense*. Nine of these loci displayed genetic diversity that ranged from one to 15 alleles per locus, and the observed heterozygosity of six polymorphic loci ranged from 0.18 to 0.90 with an average of 0.47 (0.29 SD) in a sample of 30 individuals. Eight of these loci were successfully amplified in at least one of the following species: *Paphiopedilum hissutissimum, P. wardii, P. armeniacum*, or *P. micranthum*. The study provides a base for molecular breeding and assessment of germplasm resources of the *Cymbidium sinense* and the results suggest that the microsatellite loci developed from *C. sinense* may have a broad applicability within the Orchidaceae family.**

Microsatellites, also known as single sequence repeats (SSRs), are a small array of one to six tandemly arranged bases spread throughout the genomes (Dietrick et al., 1992). The development of SSR-based markers has become increasingly accessible in recent years mainly as a result of novel library enrichment strategies and rapid fluorescence-based automatic sequencing technologies (Powell et al., 1996). SSRs have now been recommended as an effective genetics marker to be used in the construction of genetic linkage maps, molecular tagging, molecular marker-assisted selection, parentage test, fingerprint identification, population genetics, and the resources conservation and management (Dick and Hamilton, 1999; Dietrick et al., 1992; Jarne and Lagoda, 1996).

The genus *Cymbidium* is one of the favorite orchids in the Chinese culture (Hu, 1977). *Cymbidium sinense*, a terrestrial orchid whose native habitat spreads from India through Thailand and into South China, has been cultivated for several centuries in China and Japan (Du Puy and Cribb, 1988). The resources of this species decreased dramatically recently and it becomes one of the rare and endangered plants in the world. Despite their significance, many aspects of *C. sinense*'s life history and ecology are poorly understood. We report on novel microsatellite markers developed for *C. sinense* that can be used for population genetic analyses, detection of hybrids, and parentage studies in future, which in turn should help improve resource assessments and management strategies of this species.

An optimized affinity capture technique described by Fleischer and Loew (1995) and Xia et al. (2006) was used to construct a microsatellite-enhanced genomic library of *C. sinense*. Briefly, total genomic DNA was extracted following a hexadecyltrim–ethylammonium bromide-based procedure (Cullings, 1992) from leaf tissues of one individual of *C. sinense* sampled from South China Botanical Garden, Guangzhou, China. Genomic DNA (10 μg) was digested to completion with a 10-fold excess of restriction enzyme *Mbo*I (TaKaRa). Fragments of 300 to 1000 base pairs (bp) were selected. SAU linkers (SAULA: 5′-GCG GTA CCC GGG AAG CTT GG-3′; SAULB: 5-GAT CCC AAG CTT CCC GGG TAC CGC-3′) were then ligated to the fragments. The linker-ligated inserts were heat-denatured and allowed to hybridize to two single-stranded biotinylated microsatellite oligonucleotides [(CA)12 GCT TGA-biotin and biotin-(AG)12]. These hybrids were captured by streptavidin-coated magnetic beads (BioMag® Nuclease-Free Streptavidin; Qiagen, Germany). The magnetic beads were eluted, and the resulting DNA solution served as a template for polymerase chain reaction (PCR) using the SAULA oligonucleotide as the primer. The amplification product was directly ligated into PMD18-T Vector (TaKaRa) by TA cloning according to the manufacturer's protocol. The ligated vector fragments were transformed into competent *Escherichia coli* Top10 cells and plated onto LB agar medium. Recombinant plasmids were identified by means of blue–white screening, and a colony PCR for further identifying the clones containing microsatellites was performed with a primer combination of (CA)12 or (AG)12 and M13 universal primers.

The insert fragments of 70 positive clones were finally sequenced using an ABI 3730 Genetic Analyzer (Applied Biosystems, USA) by United Gene Holdings, Ltd., China. Sixty-eight sequences with 25 unique loci were positive for microsatellites, 13 of which with eight to 63 repeat units and appropriate flanking regions were selected for primer development. Primers to amplify the microsatellites were designed using the program OLIGO 6 primer analysis software (Molecular Biology Insights, USA) according to its primer and probe search protocol. Synthesized primers (Sangon, Ltd., China) were optimized for amplification on the source plant's DNA. All primer pairs were tested. Nine primer pairs (Cym 9, Cym 12, Cym 15, Cym 18, Cym 344, Cym 371, Cym 8, Cym 35, and Cym 41) yielded products of the expected size, two primer pairs (Cym 59 and Cym 311) generated weak and nonreproducible bands, and two (Cym 19 and Cym 369) did not amplify any product. The PCR amplifications were carried out in 20 μL PCR reaction volume containing 50 ng genomic DNA, 1× PCR reaction buffer, 200 μM of each dNTPs, 1 μM of each primer, and 1 unit of Taq DNA polymerase (BBST) on a gradient thermal cycler (Biometra, Germany) with the following cycling profile: one denaturation step for 2 min at 95 °C was followed by 35 cycles of 1 min at 94 °C, 40 s at primer-specific annealing temperature (Table 1), and 40 s at 72 °C. The final step was a prolonged extension of 7 min at 72 °C. PCR products were analyzed on the Sequi-Gen® GT Sequencing Cell (Bio-Rad, USA). Microsatellite alleles were sized (base) relative to an internal DNA size standard (100-bp DNA ladder) using the software Labimage (version 2.6 by Kapelan, http://www.labimage.de).

To screen for polymorphism and test performance of the nine loci, DNA from 30 individuals of an ex situ conservation population of *C. sinense* in South China Botanical Garden was tested using the optimized PCR conditions. Variability of the microsatellite loci was analyzed using the program POPGENE (version 1.32; Yeh et al., 1997). Primer sequences, allele size range. and number of alleles per locus are summarized in Table 1. The observed number of alleles per locus ranged from one to 15. Six (Cym 9, Cym 12, Cym 15, Cym 18, Cym 344, and Cym 371) of these 9 loci showed polymorphism. The observed heterozygosity ranged from 0.18 to 0.90 with an average of 0.47 (0.29 SD), and the expected heterozygosity ranged from 0.19 to 0.93 with an average of 0.57 (0.30 SD) for the six polymorphic loci. Deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested with the software GENEPOP 3.4 (Raymond and Rousset, 1995) using the probability tests with default values. Significance levels for all tests were corrected for multiple simultaneous tests using the sequential

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Table 1. Summary of the developed microsatellite markers from *Cymbidium sinense*[z].

Locus	GenBank accession no.	Repeat motif	Primer sequences (5′ → 3′)	Species	R (bp)	A	H_E/H_O	T_a(°C)	P	Fis
Cym 9	DQ501379	$(GA)_{33}$	F: CGCTACTGAAATAGAATAA	C.s.	105–172	12	0.93/0.71	53	0.0000	0.2135
			R: GATCAACTGATACTAAAGCTC	P.h.	—					
Cym 12	DQ494846	$(CAGA)_9$	F: TTAAGCAGGAGCCGTCACAG	C.s.	174–215	3	0.19/0.20	48	1.0000	–0.0843
			R: AGTTGCGGGTCAGTGTAAC	P.h.	—					
Cym 15	DQ501380	$(CT)_{36}$	F: TTCAAAGATAAATATGCTCC	C.s.	129–180	15	0.91/0.90	48	0.0000	–0.0020
			R: GTTGTGAAATACTTTTAAAGGTG	P.h.	—					
Cym 18	DQ501381	$(GT)_8$	F: GATCAAGTTGTCTTCAGACTG	C.s.	124–126	2	0.40/0.18	53	0.0063	0.5447
			R: CAAACTCGAAGAATTACC	P.h.	124–126	2	0.18/0.12	53	0.1976	0.3333
Cym 344	DQ494847	$(CA)_{63}$	F: TAGTGCCATCTAATCTAATG	C.s.	216–222	3	0.36/0.32	48	0.0061	0.0919
			R: TTTTCTTGTGCTCGAAG	P.h.	—					
Cym 371	DQ501382	$(GT)_{35}$	F: GTGAAAGCCACCTCCATG	C.s.	171–178	4	0.62/0.48	48	0.0059	0.2059
			R: GATGGATACCTCGCACTGG	P.h.	—					
Cym 8	DQ501383	$(GTCT)_8$	F: AGTTGCGGGTCAGTGTAAC	C.s.	283	1	0	50		
			R: TTAAGCAGGAGCCGTCACAG	P.h.	283	1	0	50		
Cym 35	DQ501384	$(CA)_{22}$	F: CAGATGGATACCTCGCACTG	C.s.	234	1	0	47		
			R: TAACTTCCCCAGGTTCAC	P.h.	234	1	0	47		
Cym 41	DQ501385	$(CA)_{18}$	F: CAGAGCAGCGGACATCA	C.s.	235	1	0	53		
			R: CCGCATACATGTTACAAGTC	P.h.	235	1	0	53		
Cym 59	DQ501386	$(GT)_{16}$	F: CATTCTTTCTTTATCCTCGC	C.s.	—					
			R: AGATTCCTCCCCACTATTC	P.h.	—					
Cym 311	DQ501387	$(CA)_{37}$	F: CGCCAGCCCTGTTAGGA	C.s.	—					
			R: TAGACTGGTGAGGCGTCAAG	P.h.	—					

[z]Repeat motif, primer sequences, species, range of observed alleles (*R*), number of alleles (*A*), observed and expected heterozygosities (H_E and H_O), annealing temperature (T_a), probability value (*P*) for χ^2 test of HWE and GenBank accession no. were provided. Wright's (1978) fixation index (Fis) as a measure of heterozygote deficiency or excess. C.s. and P.h. = *C. sinense* and *P. hissutissimum*, respectively. A dash indicates no amplification or nonreproductive amplification in the species.

Bonferroni procedure (Rice, 1989). Five loci (Cym 9, Cym 15, Cym 18, Cym 344, and Cym 371) showed significant deviations from the HWE (adjusted $P = 0.0083$; Table 1) in the screened samples, and the tested population was not within HWE when combined over the six loci (Fis = 0.18, $P < 0.001$). The observed heterozygote deficiencies and deviations from HWE in this study may be the result of sample mixture (Wahlund effect) or the presence of null alleles and genetic drift and/or inbreeding in an isolated ex situ population (Caughley, 1994; Mitton and Grant, 1984). Highly significant ($P < 0.001$) linkage disequilibrium was exhibited in the sampled population between locus Cym 9 and Cym 15 in the 15 tests for linkage disequilibrium performed for all possible pairwise comparisons of the sampled loci.

The 11 loci were also screened in 30 individual genomic DNAs of an ex situ conservation population of *Paphiopedilum hissutissimum* in South China Botanical Garden using the PCR conditions optimized for *C. sinense*. The results of cross-amplification are summarized in Table 1. Four of these loci were successfully amplified in the species tested, and one locus (Cym 18) showed polymorphism within the tested population with an observed heterozygosity of 0.12 and an expected heterozygosity of 0.18. Additionally, all these loci were also screened in two individuals of the species *P. wardii*, *P. armeniacum*, and *P. micranthum*, respectively, using the optimal PCR conditions for *C. sinense*. Seven of these loci were successfully amplified in at least one of the following species: *P. wardii* (Cym 9, Cym 12, Cym 344, Cym 371, and Cym 8), *P. armeniacum*, (Cym 12, Cym 344, Cym 371, and Cym 41), and *P. micranthum* (Cym 18, Cym 344, Cym 371, and Cym 41). The results presented here suggest that microsatellite loci developed for *C. sinense* may have a broad applicability within the Orchidaceae family as previous allozyme (Chung and Nason, 2007) and random amplified polymorphic DNA markers used in *Cymbidium* genus (Wen et al., 2001).

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