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Microsatellite markers for the Chinese endangered and endemic orchid *Cymbidium tortisepalum* (Orchidaceae)[1]

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- *Premise of the study:* Microsatellite primers were developed in the Chinese endangered and endemic orchid, *Cymbidium tortisepalum*, to investigate its genetic diversity and population genetic structure, and to identify its varieties.
- *Methods and Results:* Using the Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) protocol, 15 primer sets were identified in two wild populations. The number of alleles per locus ranged from two to six, with a mean of 3.5. The observed and expected heterozygosities varied from 0.250 to 0.917 and from 0.228 to 0.841, respectively. All of these primers successfully amplified in the congener *C. goeringii*, and 12 were found useful in *C. faberi* and *C. sinense*.
- *Conclusions:* These markers will facilitate further studies on the population genetics and molecular identification of *C. tortisepalum*, its varieties, and other congener species.

Key words: *Cymbidium tortisepalum*; genetic structure; microsatellites; molecular identification; Orchidaceae.

Cymbidium tortisepalum Fukuy., called Lianban orchid by local people, is one of the most important and popular ornamental plants in Orchidaceae (DuPuy and Cribb, 1988). It is a narrowly endemic orchid of China, found only in the area of three parallel rivers (Jinsha River, Lancang River, and Nujiang River) in northwestern Yunnan Province and along the northern border of Sichuan Province between 98°00′–100°31′E and 25°30′–29°00′N. It usually grows on rocky and scrubby slopes, in forest margins, or in open places in forests at altitudes of 1500–2500 m (Liu et al., 2006). It has been cultivated for its excessively wide range of variation in shape, size, coloration, fragrance, and peloric flower structures for more than 10 centuries in China. As a result, it is highly valued in floritrade and holds enormous promise. For these reasons, it is under threat of extinction due to intense biotic pressures including habitat loss, indiscriminate wild collection, and illegal trade by the local people. Furthermore, there is considerable uncertainty concerning the identification of species and varieties. Cultivar identification is particularly difficult due to frequent intraspecific hybridization and selection of somatic mutation during vegetative propagation (Wu, 1993; Liu et al., 2006). Hence, 15 microsatellite markers have been developed and characterized for *C. tortisepalum*, which will be used for further studies of genetic diversity, population structure, and molecular identification of the cultivars.

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METHODS AND RESULTS

Genomic DNA was extracted from a single individual using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The microsatellite loci were isolated using the Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO) protocol developed by Zane et al. (2002). First, total genomic DNA (~500 ng) was completely digested with the *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA), and then ligated to an *Mse*I adapter pair (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) with T4 DNA ligase (Fermentas, Burlington, Ontario, Canada) in a 30 μL reaction mixture. A diluted digestion–ligation mixture (1:10) was amplified with the adapter-specific primers *Mse*I-N (5′GATGAGTCCTGAGTAAN-3′) (25 μM) in 20 μL reactions with the following conditions: 3 min denaturation at 95°C; followed by 26 cycles of 30 s denaturation at 94°C, 1 min annealing at 53°C, and 1 min extension at 72°C; with a final extension of 72°C for 5 min. Final concentrations were 10 ng of genomic DNA, 0.2 mM each dNTPs, 0.1 μM of each primer, 1× PCR buffer, 1.5 mM Mg^{2+}, and 0.4 U of DNA *Taq* polymerase (Sangon, Shanghai, China). Amplified DNA fragments, with a size range of 200–800 bp, were enriched for repeats by magnetic bead selection with a 5′-biotinylated $(AC)_{15}$, $(AG)_{15}$, and $(AAG)_{10}$ probe, respectively. Enriched fragments were amplified again with adapter-specific primers for 30 cycles as described above. PCR products were purified using an EZNA Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China). The purified DNA fragments were ligated into the pGEM-T vector (Promega, Madison, Wisconsin, USA), and transformed into DH5α cells (TaKaRa Biotechnology Co., Dalian, China). Positive clones were tested by PCR using $(AC)_{10}$/$(AG)_{10}$/$(AAG)_7$ and T7/Sp6 as primers, respectively. PCR was performed in a 15 μL final volume, which included approximately 10–20 ng of genomic DNA, 0.2 mM each dNTPs, 0.1 μM of each primer, 1× PCR buffer, 1.5 mM Mg^{2+}, and 0.4 U of DNA *Taq* polymerase (Sangon). Amplification was conducted under the following conditions: 3 min denaturation at 95°C; followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 55°C, 1 min extension at 72°C; and a final extension of 72°C for 8 min. In total, 150 clones with positive inserts were sequenced with an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Carlsbad, California, USA). Cycle sequencing conditions were as follows: 30 cycles of 10 s denaturation at 96°C, 5 s annealing at 50°C, and 4 min elongation at 60°C. A total of 120 (80%) sequences were found to contain microsatellite repeats, 50 of which presented sizes greater than 300 bp, and microsatellite sequences with at least five tandem repeats were suitable for designing locus-specific primers, using the PRIMER 5.0 program (Clarke and Gorley, 2001).

TABLE 1. Characteristics of 15 microsatellite loci in *Cymbidium tortisepalum*.

Locus	Primer sequence (5′–3′)	Repeat motif	Size (bp)	T_a (°C)	GenBank Accession No.
CTSSR01[a]	F: CATATCTGTATGGCTTCC R: CCAATGGTTACTTTCCTA	$(CT)_5(CA)_{12}$	176	53	JN054638
CTSSR02[a]	F: TAATGGAATGATGCGTAG R: CAGTAATTGGACCCTAAC	$(GA)_{10}(CA)_7(AC)_6$	214	53	JN054639
CTSSR03[a]	F: AGTAACCCAATCCCAAAT R: GCTCCCTAATATCCTCTT	$(AG)_{15}$	231	53	JN054640
CTSSR04[a]	F: GGGATTTACATACATACC R: GAGAAACGACGACGAAGT	$(AC)_{17}$	221	54	JN054641
CTSSR05	F: GCTCTGCTCATTCCCTCT R: GTAACCAAGAATTTCAACAT	$(AC)_{10}$	185	54	JN054642
CTSSR06[a]	F: ACTCTGCGATGCTGAAAT R: GATAAGGGATCGAGGGAG	$(TC)_4TA(TC)_4$	127	55	JN054643
CTSSR07[a,b]	F: TAAGTACCAGTGTATTGGCG R: CTGCTCAGCGTCTTCAAC	$(AG)_5\ldots(AG)_{13}$	137	56	JN054644
CTSSR08[a]	F: AACTTGCCGATGATAGAA R: AGTCCTGAGTAACCACCT	$(CT)_{19}$	219	58	JN054645
CTSSR09[a]	F: GAGTTAGCAGAACCCACG R: CTGTCGCTTGCCTACTTT	$(AG)_{22}$	173	58	JN054646
CTSSR10[a]	F: GTCTTCTCCTTCTCCCTC R: CTTCTGGCTCTTCTTCACT	$(CT)_{14}$	152	58	JN054647
CTSSR11	F: GTGTTCCATGTTAGCTGGTC R: TGCTACAATCCCACTCCC	$(AG)_{16}$	197	60	JN054648
CTSSR12[a]	F: TTCTACCTGGCCCTATGC R: GACGACCTTCCTTTCTCC	$(AG)_7(GA)_{15}$	176	60	JN054649
CTSSR13[a]	F: GGAACAAGTGGGCTTCAC R: CCTTGCCAGCTTAGTCTCC	$(AG)_4AA(AG)_{13}$	167	63	JN054650
CTSSR15	F: GGTGGTTCATTTGGTGAT R: TGATTCGTTATTGCTCCT	$(CT)_{16}T(TC)_5(AC)_7$	281	54	JN054651
CTSSR16[a]	F: AGGGTGAAGTTCTCATTT R: AATAGGGTGAGTGGAGTG	$(TC)_{18}$	326	53	JN054652

Note: T_a = annealing temperature.
[a] Successfully amplified in *C. faberi* and *C. sinense.*
[b] Significant departure from Hardy–Weinberg equilibrium.

Polymorphisms of all 50 microsatellite loci were assessed in 24 individuals of *C. tortisepalum* from two natural populations Gongshan, Yunnan (27°45′48″N, 98°39′05″E) and Baoshan, Yunnan (25°11′19″N, 99°03′23″E) (Appendix 1). PCR reactions were performed in a 15 μL reaction containing 30–50 ng genomic DNA, 0.6 μM of each primer, and 7.5 μL 2× *Taq* PCR MasterMix (0.1 U *Taq* polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl [pH 8.3], 100 mM KCl, 3 mM $MgCl_2$ [Tiangen Biotech, Beijing, China]). PCR amplifications were conducted under the following conditions: 95°C for 3 min; followed by 30–36 cycles at 94°C for 30 s, at the optimized annealing temperature (Table 1, each primer pair was tested separately) for 30 s, 72°C for 1 min; and a final extension step at 72°C for 7 min. PCR products were separated and visualized using QIAxcel capillary gel electrophoresis system (QIAGEN, Irvine, California, USA).

In all, of the 50 primers, 12 primers were not successfully amplified in all samples. The other 38 primers could be amplified, with 23 primers showing monomorphisms and 15 primers displaying polymorphisms. Standard genetic diversity parameters, departure from Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium (LD) between pairs of loci were estimated in POPGENE version 1.32 (Yeh et al., 1997). The number of alleles per locus was two to six with an average of 3.5, values for observed and expected heterozygosities ranged from 0.250 to 0.917 and from 0.228 to 0.841, with an averages of 0.621 and 0.593, respectively (Table 2). One locus (CTSSR07) significantly deviated from HWE proportions in both the Gongshan and Baoshan populations. No significant LD was detected between the loci ($P < 0.001$) in our analysis. For cross-species application, these 15 primer pairs were tested in three individuals of other *Cymbidium* species, *C. goeringii* (Rchb. f.) Rchb. f. (Xingyi, Guizhou: 25°12′37″N, 104°54′25″E), *C. faberi* Rolfe (Leye, Guangxi: 24°49′44″N, 106°12′22″E), and *C. sinense* (Jacks. ex Andrews) Willd. (Shenzhen, Guangdong: 22°46′58″N, 114°13′29″E) (Appendix 1), using the same PCR conditions as previously described. All positive amplifications occurred in *C. goeringii*. Twelve of the 15 primer pairs (Table 1) were successfully amplified in *C. faberi* and *C. sinense*.

CONCLUSIONS

These polymorphic simple sequence repeat markers will be useful tools in population genetics studies and assessing genetic variations to establish conservation strategies and molecular identification. Their highly promising cross-taxa applicability

TABLE 2. Results of initial primer screening in *Cymbidium tortisepalum.*

	Gongshan (N = 12)			Baoshan (N = 12)		
Locus	N_a	H_e	H_o	N_a	H_e	H_o
CTSSR01	4	0.545	0.500	4	0.540	0.507
CTSSR02	4	0.649	0.921	4	0.653	0.917
CTSSR03	4	0.654	0.917	4	0.649	0.924
CTSSR04	3	0.301	0.339	3	0.310	0.333
CTSSR05	3	0.372	0.250	3	0.366	0.254
CTSSR06	2	0.437	0.250	2	0.431	0.248
CTSSR07	4	0.667	0.837	4	0.671	0.833
CTSSR08	2	0.231	0.250	2	0.228	0.245
CTSSR09	5	0.772	0.917	5	0.772	0.917
CTSSR10	4	0.750	0.579	4	0.745	0.583
CTSSR11	4	0.698	0.583	4	0.692	0.583
CTSSR12	3	0.366	0.417	3	0.366	0.420
CTSSR13	4	0.641	0.750	4	0.641	0.750
CTSSR15	3	0.605	0.917	3	0.605	0.917
CTSSR16	6	0.841	0.669	6	0.841	0.665

Note: H_e = expected heterozygosity; H_o = observed heterozygosity; N_a = number of alleles.

indicates their potential usefulness to other congeneric orchid species.

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Appendix 1. Locality and voucher information of the *Cymbidium* species sampled in this study. All vouchers are deposited at Kunming Institute of Botany, Chinese Academy of Sciences.

Taxon	Locality	GPS coordinates	Voucher information
C. tortisepalum	Gongshan, Yunnan	27°45′48″N, 98°39′05″E	HJL091027-1; HJL091027-2; HJL091027-3
C. tortisepalum	Baoshan, Yunnan	25°11′19″N, 99°03′23″E	HJL091035-1; HJL091035-2; HJL091035-3
C. goeringii	Xingyi, Guizhou	25°12′37″N, 104°54′25″E	HJL091211
C. faberi	Leye, Guangxi	24°49′44″N, 106°12′22″E	HJL090971
C. sinense	Shenzhen, Guangdong	22°46′58″N, 114°13′29″E	HJL090706