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Applications in Plant Sciences

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Primer Note

Development of microsatellite markers for *Cypripedium tibeticum* (Orchidaceae) and their applicability to two related species[1]

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- *Premise of the study:* To investigate genetic diversity and structure, microsatellite markers were developed and characterized for *Cypripedium tibeticum*, an endangered orchid species, and their utility tested in two relatives: *C. flavum* and *C. bardolphianum*.
- *Methods and Results:* Candidate microsatellite loci were isolated based on next-generation sequencing. We identified 20 polymorphic loci with di- or trinucleotide repeats in three populations of *C. tibeticum*. Genotyping results showed the total number of alleles per locus varied from two to 21, and the observed and expected heterozygosity ranged from 0.261 to 0.967 and 0.441 to 0.960, respectively. Cross-species amplification showed most of the markers were applicable to *C. flavum* and *C. bardolphianum*.
- *Conclusions:* The 20 polymorphic microsatellite markers developed in this study are useful tools for studying the conservation genetics and phylogeography of *C. tibeticum* and its related species.

Key words: *Cypripedium tibeticum*; genetic diversity; microsatellites; Orchidaceae.

Cypripedium tibeticum King ex Rolfe (Orchidaceae), known as Chinese lady slipper, is a unique orchid species with high ornamental and medicinal value. It is endemic to southwestern China, including Tibet, Gansu, Yunnan, Guizhou, and Sichuan provinces. *Cypripedium tibeticum* mainly grows in alpine meadows, scrub forests, and forest margins at high altitudes (2800–4200 m a.s.l.). The natural population size of this orchid is usually limited by habitat fragmentation and overharvesting (Chen and Tsi, 1998; Fay and Chase, 2009; Swarts and Dixon, 2009; Bronstein et al., 2014). Due to habitat destruction and human activities, the survival of *C. tibeticum* in its native range is threatened, and it is currently listed as a national protected plant (Luo et al., 2003; Qian et al., 2014).

Microsatellites (simple sequence repeats [SSRs]) are widely used molecular markers that are applied in population genetics to investigate genetic diversity, population structure, and evolutionary history (Hodel et al., 2016; Vieira et al., 2016). To date, microsatellite markers have been developed for several *Cypripedium* L. species; however, scarcely any markers are applicable to *C. tibeticum* (Fay and Cowan, 2001; Pandey and Sharma, 2013; Yamashita et al., 2016). In this study, we developed and characterized 20 polymorphic SSR markers in *C. tibeticum* and tested their applicability in two related species: *C. flavum* P. F. Hunt & Summerh. and *C. bardolphianum* W. W. Sm. & Farrer. These microsatellites will be valuable tools for ecological, phylogeographic, and conservation studies of *C. tibeticum* and other related *Cypripedium* species.

METHODS AND RESULTS

Plant sample collection and DNA extraction—Leaf samples of *C. tibeticum* were collected from three locations in Sichuan Province, including Huanglong, Kangding, and Xiaojinxian in China (population codes: HL, KD, and XJX, respectively [Appendix 1]). Genomic DNA was extracted from fresh leaf tissue of each individual using the DNeasy Plant Mini Kit according to manufacturer instructions (QIAGEN, Valencia, California, USA).

Development and screening of microsatellite markers—Total genomic DNA was digested with the restriction enzyme *Hae*III, and products were 5′ phosphorylated and a single A was added at the 3′ end. DNA was ligated to a Solexa adapter and sequenced on a single HiSeq 2500 flow cell (Illumina, San Diego, California, USA). A total of 7,210,538 reads longer than 100 bp

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Table 1. Characteristics of 20 microsatellite loci developed in *Cypripedium tibeticum*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent label	T_a (°C)	GenBank accession no.
M150	F: GGATGAGGAAACCCTAGCGT R: GATTCTCCTTGATGGCAATGT	$(GTA)_5(AT)_7$	153–164	HEX	60	KY490005
M164	F: GTTAGGTGAAGGCTAGAAGGT R: AGCTGAGAGTGGAAGATCATAT	$(AAG)_8$	137–188	FAM	60	KY490006
M172	F: CACCTCTATTCCACTTTGCCT R: GTAAAGGGATAGCACATGCAC	$(AG)_8$	153–161	HEX	62	KY490007
M182	F: GTGGTGGACCGATCATACCT R: GGAGAATTTAGTGGAAGAGAGAT	$(TTC)_{16}$	148–163	FAM	62	KY490008
M209	F: GGGTCCTTTTGTTGACCTACT R: AACCCTAATTGGTCAATTCGAT	$(CT)_8$	170–182	HEX	57	KY490009
M289	F: ATCACCTTTCCACAATCATGT R: TGCTGTCACCATTGAACTCT	$(AAG)_8$	177–201	HEX	58	KY490010
M294	F: ATACAGAGCCAGCCTACACT R: AGTAACTAGTGGAGCACAGCT	$(AT)_9$	161–169	FAM	63	KY490011
M372	F: TCCTAGTTCGAAGATGATGGT R: TAGCTACAGCCAAATTCGGT	$(TTC)_5$	154–160	HEX	60	KY490012
M401	F: TCAGGATTACAGCCCTAAACT R: GGGAGATTCAGTTACTAGAGCT	$(AG)_8$	161–175	FAM	59	KY490013
M576	F: AATCATCAAAGGGAGAGCAAT R: TTTGTCACCACCATGATAGAGT	$(AAG)_9$	301–310	FAM	62	KY490014
M112	F: AAGACTGGAAGTAGCGGAGAT R: GAAGTCTACATCCACTGTTCCT	$(CT)_{10}$	232–304	FAM	53	MF398589
M130	F: TTCAGCAGATTCAGATTTAGCT R: CTTCTGACAGATCTTCTCCAAT	$(CAT)_5(CTT)_3$	182–218	HEX	51	MF398590
M136	F: TCCAACCTCTCTATCCTCGAT R: GATCTGAGGAGGATGAAGGT	$(TTC)_9$	185–191	HEX	53	MF398591
M139	F: GAGGGAGGATTCTGACAACT R: GTGGTTCGACTTCGTGAAAT	$(TC)_{11}$	213–231	FAM	53	MF398592
M142	F: TTGTGCATCGTCATCAAGGT R: GATCACAAACATGCTGGGAT	$(TC)_7$	153–155	HEX	53	MF398593
M233	F: ACACGCTACAGACCGAGCT R: CTTGTTTGCAAGATGAATGCT	$(CAT)_6$	169–184	FAM	51	MF398594
M370	F: GGAGATTCAGTTATCAGAGCTG R: AGTCTACATCCACTATTCCTTGT	$(TC)_{24}$	271–339	FAM	52	MF398595
M681	F: ATGCCAGACGGCAGAGGT R: GGCTTTGAGCTAATGGCTCT	$(TG)_{10}$	150–160	HEX	55	MF398596
M880	F: GGAGAACAGGAAAACTGTTCAT R: AGCCGATTACAAAGTCACTGT	$(TC)_{10}$	137–147	FAM	52	MF398597
M886	F: CGAAATTCACTGGGATTCAGT R: TCTCAGGATTACAACAGTAGCT	$(CT)_9$	156–164	HEX	52	MF398598

Note: T_a = annealing temperature.

were obtained, and raw reads with quality scores less than 25 and lengths shorter than 25 bp after stripping the adapters were filtered using SeqPrep (https://github.com/jstjohn/seqprep). De novo assembly was performed using CLC Genomics Workbench (QIAGEN) and produced 1,920,476 contigs, where parameters were set as: average coverage value of 25× and minimum contig length of 299 bp. MSATCOMMANDER 1.0.8 software was used to identify contigs carrying di-, tri-, and tetranucleotide repeats with a minimum of five repeats and a minimum tract length of 100 bp (other parameters were set to default settings) (Faircloth, 2008). Ninety-four primer pairs were designed for microsatellite loci candidates using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, California, USA) and tested with 15 *C. tibeticum* samples (five individuals from each population) for amplification efficiency and polymorphism. Each 15-μL PCR reaction contained ~15 ng of genomic DNA, 1.5 μL of 10× PCR buffer, 0.8 μL of fluorescently labeled TP-M13 (5 mM), 1.0 μL of each primer (10 mM), 1.5 μL of dNTP (10 mM), and 0.1 μL of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, California, USA). All candidate primer pairs were tested by a touchdown PCR protocol as follows: 94°C for 2 min; five cycles of 94°C for 30 s, 60–56°C (Δ1°C touchdown per cycle) for 30 s, 72°C for 30 s; followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; and a final extension at 60°C for 30 min.

Results from testing primer pairs showed that 26 pairs produced bands within the expected size range on agarose gels in all the samples tested. The size of amplified fragments from these loci was further analyzed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA), and allele sizes were scored by GeneMapper version 3.2 software (Applied Biosystems). Twenty loci were polymorphic and showed clear peaks. The authenticity of these amplified loci was confirmed by Sanger sequencing of representative PCR products, and sequences have been deposited in GenBank (Table 1). Sequence library data of this study were deposited to the Sequence Read Archive of the National Center for Biotechnology Information (NCBI; BioProject ID: PRJNA393499).

Data analysis and results—These 20 microsatellite loci were PCR-amplified in an additional 74 individuals from three *C. tibeticum* populations collected in Sichuan Province. For each locus, the observed number of alleles, effective number of alleles, observed heterozygosity, and expected heterozygosity were calculated using PopGene32 version 1.32 (Yeh et al., 1999). Tests for Hardy–Weinberg equilibrium and linkage disequilibrium were performed by GENEPOP Web version 4.2 (Rousset, 2008). The total number of alleles per locus ranged from two to 21 (mean ± SD: 6.350 ± 4.320). The observed heterozygosity and expected heterozygosity ranged from 0.261 to 0.967 (0.664 ± 0.143) and from 0.441 to 0.960 (0.745 ± 0.119), respectively (Table 2). Of the 20 polymorphic loci, nine loci in the Huanglong population, six loci in the Kangding population, and five loci in the Xiaojinxian population deviated significantly from Hardy–Weinberg equilibrium, respectively ($P < 0.05$; Table 2). Linkage disequilibrium was not detected at any locus.

The utility of these 20 microsatellite loci developed for *C. tibeticum* was also detected in two other *Cypripedium* species: *C. flavum* and *C. bardolphianum*.

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TABLE 2. Genetic variation of the 20 polymorphic microsatellite loci in three populations of *Cypripedium tibeticum*.[a]

	Huanglong (n = 30)				Kangding (n = 23)				Xiaojinxian (n = 21)			
Locus	A	A_e	H_o	H_e	A	A_e	H_o	H_e	A	A_e	H_o	H_e
M150	5	3.141	0.533*	0.693	5	3.752	0.696*	0.750	5	4.027	0.619	0.770
M164	9	4.369	0.667	0.784	9	3.586	0.522*	0.737	10	7.230	0.620*	0.883
M172	4	3.352	0.700	0.714	4	3.765	0.826	0.751	4	3.303	0.667	0.714
M182	4	3.834	0.607	0.753	6	5.042	0.636	0.820	5	4.376	0.737	0.792
M209	5	4.380	0.700*	0.785	6	4.038	0.609	0.769	5	3.920	0.619	0.763
M289	5	4.478	0.967*	0.790	5	4.640	0.957	0.802	6	4.624	0.800	0.804
M294	5	3.939	0.633	0.759	4	3.174	0.636	0.701	5	4.302	0.714*	0.786
M372	3	2.410	0.833*	0.595	3	2.489	0.870	0.612	3	2.513	0.619	0.617
M401	5	3.516	0.933*	0.728	5	2.315	0.696	0.581	7	4.432	0.667	0.793
M576	3	2.925	0.379*	0.670	4	3.010	0.619	0.684	3	2.882	0.714	0.669
M112	20	10.852	0.724*	0.924	21	16.133	0.636*	0.960	20	14.700	0.714*	0.955
M130	7	5.202	0.700*	0.822	7	5.426	0.696	0.834	6	5.313	0.667*	0.832
M136	3	2.062	0.467	0.524	3	1.796	0.435	0.453	3	1.757	0.286	0.441
M139	9	4.485	0.690	0.791	9	5.728	0.682	0.845	7	5.714	0.700	0.846
M142	2	2.000	0.310	0.509	2	1.910	0.261	0.487	2	1.930	0.429	0.494
M233	5	3.666	0.667*	0.740	6	4.172	0.591	0.778	5	3.571	0.667	0.738
M370	17	8.476	0.786	0.898	15	10.377	0.810*	0.926	12	8.112	0.632*	0.900
M681	5	3.303	0.733	0.709	5	4.445	0.739*	0.792	6	3.571	0.762	0.738
M880	5	4.000	0.767	0.763	6	4.944	0.783*	0.816	6	4.846	0.619	0.813
M886	5	4.854	0.700	0.753	5	4.165	0.739	0.777	5	4.027	0.667	0.770
Mean	6.300	4.212	0.675	0.735	6.500	4.745	0.672	0.744	6.250	4.758	0.646	0.756

Note: A = number of alleles; A_e = effective number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled for each population.

* Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

[a] Voucher and locality information are provided in Appendix 1.

Eighteen loci were successfully PCR-amplified in *C. flavum*, and 17 loci were amplified in *C. bardolphianum* (locus M294 did not amplify in *C. bardolphianum*, M370 and M886 did not amplify in either species). Genotyping results showed locus M372 was monomorphic in both species, and M136 was polymorphic in *C. bardolphianum* but monomorphic in *C. flavum*. All the other markers were polymorphic in both species (Table 3).

CONCLUSIONS

In this study, we developed and validated 20 polymorphic microsatellite markers for the orchid *C. tibeticum*, most of which showed applicability in two related *Cypripedium* species:

TABLE 3. Characteristics and polymorphism of 20 microsatellite loci developed for *Cypripedium tibeticum* in *C. flavum* and *C. bardolphianum*.[a]

	C. flavum (n = 16)				*C. bardolphianum* (n = 15)			
Locus	Allele size range (bp)	A	H_o	H_e	Allele size range (bp)	A	H_o	H_e
M150	155–217	6	0.750	0.807	157–217	4	0.733	0.687
M164	143–188	6	0.625	0.786	140–185	6	0.667	0.752
M172	155–159	3	0.625*	0.647	147–169	5	0.733	0.736
M182	136–163	4	0.688	0.659	142–163	4	0.667	0.720
M209	156–173	6	0.813	0.730	151–181	6	0.800	0.786
M289	180–197	4	0.875	0.724	183–192	3	0.867	0.674
M294	161–163	2	0.563	0.466	—	—	—	—
M372	157	1	0.000	0.000	157	1	0.000	0.000
M401	161–167	4	0.813	0.684	151–173	7	0.867	0.777
M576	301–307	3	0.750	0.679	301–304	2	0.400	0.497
M112	234–286	13	0.733*	0.906	238–280	11	0.769	0.905
M130	189–198	4	0.667*	0.687	174–186	4	0.750	0.736
M136	188	1	0.000	0.000	182–185	2	0.308	0.492
M139	215–221	3	0.600	0.662	215–225	5	0.643	0.743
M142	147–151	2	0.154*	0.492	155–159	2	0.333	0.391
M233	173–182	3	0.500	0.623	171–177	2	0.200	0.186
M370	—	—	—	—	—	—	—	—
M681	146–152	3	0.563	0.684	150–152	2	0.400	0.515
M880	140–146	3	0.571	0.659	144–150	3	0.539	0.655
M886	—	—	—	—	—	—	—	—
Mean		3.944	0.572	0.605		4.059	0.569	0.603

Note: — = unsuccessful amplification; A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled for each population.

* Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

[a] Voucher and locality information are provided in Appendix 1.

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C. flavum and *C. bardolphianum*. These markers will be useful for population genetic investigation and species conservation in natural habitats of *C. tibeticum* and other closely related species.

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Appendix 1. Voucher and locality information for *Cypripedium* species used in this study.[a]

Species	Voucher specimen accession no.[b]	Collection locality	Locality ID	Geographic coordinates	*n*
C. tibeticum King ex Rolfe	Cti-HLa05-XL	Huanglong, Sichuan Province, China	HL	32.750367°N, 103.823067°E	30
C. tibeticum	Cti-KDa03-XL	Kangding, Sichuan Province, China	KD	30.007833°N, 101.947433°E	23
C. tibeticum	Cti-XJXa06-XL	Xiaojinxian, Sichuan Province, China	XJX	31.053192°N, 102.786408°E	21
C. flavum P. F. Hunt & Summerh.	Cfl-HLb03-XL	Huanglong, Sichuan Province, China	HL	32.750367°N, 103.823067°E	16
C. bardolphianum W. W. Sm. & Farrer	Cba-HLc01-XL	Huanglong, Sichuan Province, China	HL	32.750367°N, 103.823067°E	15

Note: n = number of individuals sampled.
[a] Vouchers are deposited in Xi'an University, Xi'an, Shaanxi Province, China.
[b] XL = Lingling Xu.

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