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A NEW SET OF MICROSATELLITE PRIMERS FOR *COELOGYNE FIMBRIATA* (ORCHIDACEAE) AND CROSS-AMPLIFICATION IN *C. OVALIS*¹

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- **Premise of the study:** Declining orchid populations have made it necessary to prioritize the study of population structure and genetic diversity for species including *Coelogyne fimbriata* (Orchidaceae).
- **Methods and Results:** A biotin-streptavidin capture method was used to construct a microsatellite library for *C. fimbriata*. A total of 15 polymorphic nuclear microsatellite loci were isolated and characterized using 47 *C. fimbriata* individuals from two natural populations in China. The number of alleles per locus for the two populations ranged from two to 17. The observed and expected heterozygosities ranged from 0.000 to 1.000 and from 0.000 to 0.867, respectively. Among these polymorphic primers, 11 loci were also successfully amplified in *C. ovalis*, and 10 loci showed moderate to high-level polymorphism. Cross-amplification of the 15 polymorphic loci was tested in five related species: *C. cumingii*, *C. eberhardtii*, *C. mayeriana*, *C. peltastes*, and *C. velutina*.
- **Conclusions:** Fifteen microsatellites in *C. fimbriata* and 10 in *C. ovalis* have moderate to high-level genetic variation, indicating their utility in population genetic studies, thus contributing to orchid conservation.

Key words: *Coelogyne fimbriata*; *Coelogyne ovalis*; medicinal orchid; microsatellites; Orchidaceae; polymorphic markers.

Coelogyne fimbriata Lindl. (Orchidaceae), a medicinal orchid, is mainly distributed in southern China, Cambodia, north-eastern Indonesia, Laos, Malaysia, Thailand, and Vietnam (Clayton and Beaman, 2002). Because southern China is the northernmost edge of its distribution region, Chinese *C. fimbriata* populations are of particular concern because populations on distribution margins are most vulnerable to disturbance (Channell and Lomolino, 2000). Furthermore, in consideration of global climate change and habitat fragmentation, it is urgent to design effective conservation strategies for endangered natural orchid populations (Swartz and Dixon, 2009). *Coelogyne fimbriata* is an epiphytic or lithophytic orchid, which requires a dormancy period in winter. This species grows on its substrate with creeping and slender rhizomes. It can reproduce both sexually via seed and vegetatively by rhizomatic growth. Usually blooming

in late summer, it produces one or two flowers on a scape. The flowers exhibit a type of pollinator deception in which the flower odor mimics food for foraging female wasps (Cheng et al., 2009).

Many studies have focused on the pollination syndromes of orchids (Tang et al., 2014); however, there is a lack of genetic information documented for this species. Because genetic information is important for the conservation and sustainable utilization of orchids (Gijbels et al., 2015), we developed microsatellite markers to allow studies of the genetic diversity, genetic structure, and mating system of *C. fimbriata*. In total, 15 polymorphic microsatellite loci were isolated and characterized to study genetic variation within this species clade. These highly polymorphic loci displayed high genetic variation and extensive usability in congeneric species, and may serve as a universal tool for orchid genetic studies.

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METHODS AND RESULTS

A biotin-streptavidin capture method was employed to construct a microsatellite-enriched DNA library (Jiang et al., 2011). First, we extracted genomic DNA from silica gel-dried leaves of one *C. fimbriata* individual using a Plant Genomic DNA Extraction Kit (Tiangen, Beijing, China). The enzyme *MseI* (New England Biolabs, Beverly, Massachusetts, USA) was used to

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digest approximately 300 ng of genomic DNA in a 25- μ L reaction volume for 2 h at 37°C. Fragments 200–1000 bp in length were then ligated to a *Mse*I-adaptor pair (F: 5'-TACTCAGGACTCAT-3' and R: 5'-GACGATGAGTCCT-GAG-3'). The ligation-digestion mixture was diluted with ultrapure water (1:4), and the diluted fragments were amplified using *Mse*I-N primer (5'-GATGAGTCCTGAGTAAN-3') in a 25- μ L PCR reaction volume at 95°C for 5 min, followed by 23 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min. Next, to obtain microsatellite-enriched DNA fragments, the PCR products were hybridized with 5'-biotinylated (AC)₁₅ probes. We used streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA) to capture single-stranded DNA fragments containing microsatellites. The enriched products were amplified using *Mse*I-N primers for 28 cycles. After the PCR products were purified using a multifunctional DNA Extraction Kit (Biotek Corporation, Beijing, China), they were ligated into *Escherichia coli* strain DH5 α with the pMD19-T vector (TaKaRa Biotechnology Co., Dalian, Liaoning, China).

We randomly selected and sequenced 249 positive clones using M13+/M13- primers on an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA). Of the 249 sequenced clones, 136 contained microsatellites. Twenty-four sequences were discarded because of short flanking regions for primer design. Finally, we designed 112 primer pairs using Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). We selected 28 individuals from Dawei Mountain, Yunnan Province, and 19 from Diaolu Mountain, Hainan Province, China (Appendix 1), for PCR using these 112 primers. Of the 112 primers, 47 produced an expected band on 1% agarose gel, 40 failed to obtain amplification products, and 25 others produced multiple bands that were difficult to discriminate. To test for polymorphism, we used the M13(-21)-tailed primer method to fluorescently label alleles and PCR products, which were electrophoretically resolved using an ABI 3730 DNA Sequence Analyzer (Applied Biosystems) with an internal lane standard (GeneScan 500[-250] LIZ) (Schuelke, 2000). Microsatellite loci were amplified under the following conditions: 5 min of denaturation at 94°C; 30 cycles of 30 s at 94°C, 30 s at 53–65°C, and 30 s at 72°C; eight cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C; and a final 10-min extension at 72°C. Allele binning and calling

were conducted using GeneMapper 4.0 (Applied Biosystems), revealing 15 polymorphic loci.

Characteristics of the 15 polymorphic microsatellite loci developed for *C. fimbriata* are shown in Table 1. An additional 32 monomorphic loci are described in Appendix 2. Each polymorphic locus had two to 17 alleles, with a mean of 5.1. At the population level, the observed and expected heterozygosities were calculated with GenAlEx 6.5 (Peakall and Smouse, 2012) and ranged from 0.000 to 1.000 and from 0.000 to 0.867, respectively (Table 2). FSTAT 2.9.2.3 (Goudet, 1995) was used to analyze linkage disequilibrium and Hardy–Weinberg equilibrium (HWE). No significant linkage disequilibrium at any locus was detected for either population. However, significant deviation from HWE was found for most loci in the two populations (Table 2), which may be indicative of strong clonality. Signs of null alleles were detected in the loci CF1-11, CF1-51, and CF2-126 with MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004).

Two wild *C. ovalis* Lindl. populations (Appendix 1) were used to test the cross-compatibility and polymorphism of the 15 microsatellite loci. This testing was performed because *C. ovalis* and *C. fimbriata* have been visually confused as the same species, with no clear differences shown in studies of pollinaria or other morphological characters (Pelsner et al., 2000). However, many researchers do recognize *C. fimbriata* and *C. ovalis* as separate species (Govaerts, 1999; Wu and Hong, 2009; George and George, 2011). In our results, 11 of 15 loci could obtain clear PCR products and 10 showed moderate to high levels of polymorphism, with the exception of one locus (CF2-147). No significant linkage disequilibrium was detected in *C. ovalis* populations; however, significant deviation from HWE was found in these populations at most loci (Table 2). Signs of null alleles were detected in the CF1-26, CF1-120, CF2-126, and CF2-172 loci. In addition, cross-amplification of 15 polymorphic loci was conducted on another five related species ($n = 5$ for each species): *C. cumingii* Lindl., *C. eberhardtii* Gagnep., *C. mayeriana* Rchb. f., *C. peltastes* Rchb. f., and *C. velutina* de Vogel; these samples were collected from living plants at Shanghai Chenshan Botanical Garden (Appendix 1). Four to eight loci were successfully amplified in all five *Coelogyne* species (Table 3).

TABLE 1. Characteristics of 15 polymorphic microsatellite markers developed for *Coelogyne fimbriata*.

Locus	Primer sequences (5'–3') ^a	T _a (°C)	Repeat motif	A	Allele size range (bp)	GenBank accession no.
CF1-11	F: <6-FAM>CAACATCCTCTCGGCATAT R: GACCACACTACACCTACAC	60	(GT) ₉	7	429–443	KP676048
CF1-26	F: <6-FAM>ATAACTCACGCCCGATTC R: CCTGTTGTTGCCTGCTGT	60	(CT) ₅	3	210–218	KP676049
CF1-30	F: <6-FAM>CACCTCTCCTCAATTACATCA R: AGTTGGCGTAAGGCTAATG	58	(ATC) ₆	2	103–109	KP676050
CF1-51	F: <HEX>TGAGAATGTCCGTAGGTT R: GGGATTGGAGTAAAGGGT	58	(AG) ₁₄	7	336–362	KP676052
CF1-60	F: <ROX>AAACCTTCGTTCCGCTCCT R: GTGCCTGCTAGGGTTCCA	60	(TC) ₅ ...(CT) ₅ ...(TC) ₅	2	344–346	KP676053
CF1-120	F: <6-FAM>GGAATCACTCTCAACTTCAC R: ATCATAGGATGGACTCTGTAG	60	(GT) ₆	5	362–372	KP676054
CF1-167	F: <ROX>CAAGCAAGCACTGAGCAA R: GAGACCATCACCGCATTC	58	(AG) ₆	8	259–293	KP676055
CF1-229	F: <6-FAM>AGGCTTACTCGCATACTCT R: ATCTCGCTTCTGGCTTCA	52	(CT) ₇ ...(TC) ₇	2	181–187	KP676056
CF1-231	F: <HEX>GGTGCTATGTATGTGAA R: CAGACCATCAAGAAGCATA	52	(AG) ₃₃ ...(GA) ₁₈	2	285–289	KP676057
CF2-26	F: <6-FAM>CTCCCATACCCACCATTT R: ATAGCCTACCTCAAGACG	55	(AG) ₁₆ ...(AG) ₆	4	153–171	KX237659
CF2-29	F: <ROX>TTGTAGTCTTCATCCTTT R: TCTAGTCTACCCATACTT	52	(TG) ₅	5	270–308	KX237660
CF2-57	F: <HEX>TGACTTAGGACCAGGAA R: GTCGCAAGCACAGATA	55	(CT) ₂₇	17	173–213	KX237661
CF2-126	F: <6-FAM>CTCCCGTGCCTATGTTTC R: ATCCGCTCTGATTTCCA	52	(CT) ₂₀	4	253–259	KX237663
CF2-147	F: <HEX>GGAGGCTTTGATTAGAT R: ATGGAGGATTATCAGTAT	52	(CT) ₆ CA(CT) ₅	4	254–260	KX237664
CF2-172	F: <ROX>CTTGATTTTCCCTTCTTG R: TGAGATAACTAAACCCAGA	52	(CT) ₉ (CA) ₆	4	257–271	KX237666

Note: A = number of alleles; T_a = annealing temperature.

^aFluorescent dyes (i.e., HEX, ROX, and 6-FAM) are presented with the forward primers.

TABLE 2. Characteristics of 15 polymorphic microsatellite loci in *Coelogyne fimbriata* and *C. ovalis* populations, respectively.^a

Locus	<i>Coelogyne fimbriata</i>						<i>Coelogyne ovalis</i>					
	DWS population (n = 28)			DLS population (n = 19)			MHX population (n = 21)			JGX population (n = 16)		
	A	H _o	H _e	A	H _o	H _e	A	H _o	H _e	A	H _o	H _e
CF1-11	7	0.259*	0.740	4	0.500	0.475	—	—	—	—	—	—
CF1-26	1	0.000	0.000	3	0.053*	0.101	9	0.333*	0.859	5	0.188*	0.756
CF1-30	2	1.000*	0.500	2	0.158	0.229	1	0.000	0.000	2	0.000*	0.469
CF1-51	7	0.286*	0.665	4	0.250	0.736	—	—	—	—	—	—
CF1-60	2	0.643	0.436	2	0.688	0.451	—	—	—	—	—	—
CF1-120	4	0.889*	0.658	3	0.316	0.277	4	0.095	0.255	5	0.500*	0.585
CF1-167	7	0.964*	0.766	5	0.579*	0.672	1	0.000	0.000	4	0.000	0.516
CF1-229	2	0.179	0.316	2	0.063	0.061	—	—	—	—	—	—
CF1-231	2	0.680	0.449	1	0.000	0.000	1	0.000	0.000	5	0.200*	0.391
CF2-26	2	0.750	0.469	3	0.368	0.597	6	0.667*	0.604	10	0.625*	0.813
CF2-29	3	0.769	0.500	4	0.333	0.474	4	0.900	0.546	3	0.929	0.554
CF2-57	5	0.926*	0.598	14	0.789*	0.867	4	0.952*	0.585	6	0.875*	0.809
CF2-126	2	0.111*	0.500	4	0.474*	0.669	2	0.000	0.472*	3	0.333*	0.611
CF2-147	1	0.000	0.000	3	0.842	0.554	1	0.000	0.000	1	0.000	0.000
CF2-172	2	0.036	0.035	3	0.421	0.639	10	0.529	0.877	7	0.063*	0.510

Note: — = failed to amplify; A = number of alleles; H_e = expected heterozygosity based on Hardy–Weinberg equilibrium; H_o = observed heterozygosity; n = number of individuals genotyped.

^aVoucher and locality information for the populations are shown in Appendix 1.

*Indicates significant deviation from Hardy–Weinberg equilibrium (P < 0.001).

CONCLUSIONS

In the current study, although a majority of the developed loci showed monomorphism (68.1%), 15 polymorphic loci were identified in *C. fimbriata*. These polymorphic loci are valuable for orchid population genetic studies. For example, these markers can be used to characterize the clonal structure of *C. fimbriata* to estimate seed and pollen flow at a fine scale. Furthermore, these polymorphic loci can provide more information, such as genetic diversity indices, which are important for the conservation and management of the species.

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TABLE 3. Amplification of 15 microsatellite loci developed for *Coelogyne fimbriata* in five other *Coelogyne* species.

Locus	<i>C. cumingii</i> (n = 5)	<i>C. mayeriana</i> (n = 5)	<i>C. eberhardtii</i> (n = 5)	<i>C. peltastes</i> (n = 5)	<i>C. velutina</i> (n = 5)
CF1-11	—	—	—	—	—
CF1-26	+	+	+	+	+
CF1-30	+	+	+	+	+
CF1-51	—	—	—	—	—
CF1-60	—	—	—	—	—
CF1-120	—	+	+	+	—
CF1-167	+	+	+	+	+
CF1-229	—	—	—	—	—
CF1-231	—	—	—	—	+
CF2-26	—	+	—	+	+
CF2-29	—	—	+	—	—
CF2-57	—	—	+	+	+
CF2-126	—	—	—	—	—
CF2-147	—	—	+	—	—
CF2-172	+	+	+	+	+

Note: + = primer successfully amplified; — = primer failed to amplify; n = number of individuals.

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APPENDIX 1. Locality information for the *Coelogyne fimbriata* and *C. ovalis* samples used in this study.^a

Species	Locality ID	Collection locality	Geographic coordinates	Collector	Collection no.	<i>n</i>
<i>Coelogyne fimbriata</i> Lindl.	DWS	Yunnan, China	22.931°N, 103.685°E	Wei-Chang Huang	CS-HWC201606-2	28
<i>Coelogyne fimbriata</i>	DLS	Hainan, China	18.659°N, 109.916°E	Ming-Zhong Huang	CS-HMZ201610-6	19
<i>Coelogyne ovalis</i> Lindl.	MHX	Yunnan, China	23.051°N, 103.356°E	Wei-Chang Huang	CS-HWC201606-5	21
<i>Coelogyne ovalis</i>	JGX	Yunnan, China	23.523°N, 100.646°E	Wei-Chang Huang	CS-HWC201509-8	16
<i>Coelogyne cumingii</i> Lindl. ^b	—	Taiwan	—	Wei-Chang Huang	—	5
<i>Coelogyne eberhardtii</i> Gagnep. ^b	—	Thailand	—	Wei-Chang Huang	—	5
<i>Coelogyne mayeriana</i> Rchb. f. ^b	—	Taiwan	—	Wei-Chang Huang	—	5
<i>Coelogyne peltastes</i> Rchb. f. ^b	—	Taiwan	—	Wei-Chang Huang	—	5
<i>Coelogyne velutina</i> de Vogel ^b	—	Taiwan	—	Wei-Chang Huang	—	5

Note: — = no detailed information available; *n* = number of individuals sampled.

^aAll voucher specimens were deposited in Shanghai Chenshan Herbarium (CSH), Shanghai, China.

^bSamples of *Coelogyne cumingii*, *C. eberhardtii*, *C. mayeriana*, *C. peltastes*, and *C. velutina* were collected from living plants at Shanghai Chenshan Botanical Garden (introduced from Taiwan and Thailand according to the record).

APPENDIX 2. Characteristics of 32 monomorphic microsatellite markers developed for *Coelogyne fimbriata*.

Locus	Primer sequences (5'–3')	T_a (°C)	Repeat motif	Allele size (bp)	GenBank accession no.
CF1-33	F: TAAGTAATTCAGCCTCCC R: CAGACCATCAAGAAGCATA	55	(CT) ₈	197	KP676050
CF2-2	F: CAAGTCCAAATCAGCGAAGG R: TCCAGAATACATCCAGGCAC	56	(AG) ₁₅	152	KY744706
CF2-3	F: GAAGAAATCAAGGACCAATG R: TCTGAGAACGAAGGAGGC	64	(TCT) ₅	198	KX237656
CF2-8	F: TAGGAGGTGAGGAGGAA R: CCAGATGCCAAGATAAA	56	(AG) ₁₅	402	KX237657
CF2-15	F: CGACTTTCCTCCGGTATCTC R: CACTCACTCAGCCTCTTCC	56	(AG) ₁₄	248	KY744707
CF2-20	F: GGAAAATAGTAAAAGCCAT R: TCCCAAACCTCAAACC	56	(TC) ₁₈	181	KY744708
CF2-23	F: CTCCTCCGTTGTAATCCAATCAT R: GTTCTCTCTTCGGCTACGTTAG	64	(CT) ₁₂	178	KY744709
CF2-24	F: ACCCTTCCCTATCGCTGTATT R: CTCTTCCCACCAAGTCTTTT	62	(CT) ₇	186	KX237658
CF2-27	F: GAGAGTGGAGGTAGGAGAA R: GGAGGAGGCTATGGAGAA	62	(AG) ₆	111	KY766112
CF2-59	F: GAAGCAGAAAATAACATA R: TCTCACTCCACTCTATCT	56	(TC) ₂₂	90	KY744710
CF2-101	F: TGGTCAGTCGGAGGAG R: ATGGAGGTGGTAGTGTGG	64	(TC) ₂₇	355	KY744711
CF2-112	F: GGGATTCGGACTGAGATT R: TTAGTAGGGATGCGAGGAG	64	(GA) ₃₉	228	KX237663
CF2-127	F: TCAAGTCCCATCAATC R: TTTAGTGCCTCCACATT	53	(CT) ₂₃	153	KY744712
CF2-129	F: TTGGCATTTCGCTTCT R: CGTGTCTTTGTCGGTTT	59	(CT) ₁₂ ...(TC) ₁₆	235	KY744713
CF2-136	F: TCGACCCGTAGTACGCAACA R: ATGGACACCAGGGCAAGG	64	(TC) ₈	285	KY744714
CF2-137	F: GGAAGGCTATGGAGAAAT R: ATGGGATGACCAGAGGAT	64	(TC) ₁₀ ...(CT) ₇	126	KY744715
CF2-140	F: GAAGATGGGAAGAAAGAA R: TGAAGGAGGAGTAGGAG	62	(GA) ₁₀	108	KY744716
CF2-146	F: TATGCCAAATGATATGA R: GAGAATGTGAGAAAAGT	53	(TC) ₁₅ ...(AC) ₁₀	347	KY744717
CF2-148	F: TGAATAAGATATTCGGATCA R: AAATCGGTGTATGGAGAC	56	(AG) ₂₀	227	KY744718
CF2-149	F: GTCAAACAGAAAAGCCAAG R: AAAGATCCGCTCCACTAT	56	(CT) ₁₈	350	KX237665
CF2-155	F: TCTCGTCTTTTCTCTTACC R: CCATTACCTCCTCACCATAC	56	(CT) ₂₀	104	KY744719
CF2-160	F: GAATCCTCGCTCCCATTT R: GTTGGTTTAGAGTTTGCAGGTA	53	(TG) ₁₁ ...(GA) ₁₅	109	KY744720
CF2-171	F: TCCTTGTTTCGCGTGAAAC R: GAGATCCCCTCGACCATAC	59	(GA) ₄₃	232	KY744721
CF2-177	F: AAGAGTTAGAAGTGGGGAGG R: GGGGAAGTGCCTTATGAT	59	(AG) ₁₉ AA(AG) ₁₅	405	KY744722
CF2-192	F: ACGGGTGAAGTATCTTGGC R: GAGGTGGTGAACCTCATTTA	64	(CA) ₁₆	274	KY744723
CF2-213	F: ACCAAATAGGAAGTGAAGGAGAA R: ATGGCGGAGCAAGAAAAGG	64	(CT) ₁₆	151	KY744724
CF2-217	F: CTTGTTTCATAAAGCGAAGT R: CTTTTATCACAGTCACCCAT	64	(CT) ₂₃ ...(CA) ₁₃	181	KX237667
CF2-222	F: TAGGGGAAACTATGGACAAA R: CGAGTTAGGGATTAGAGGGT	63	(CT) ₄₆	181	KY744725
CF2-232	F: AATAAGATAATGGAAGGA R: ACTCCAGTTTGTCTTTTA	62	(GA) ₉	108	KY744726
CF2-234	F: ATCAAAGCCTATTATTCCC R: AGATTTACCGTCGTCAGC	64	(TC) ₈	292	KY744727
CF2-238	F: AACGCCACCACCAAGTA R: CGGCCCTATTCCCTCACA	64	(GA) ₁₁	379	KY744728
CF2-240	F: TACACGCCCTAATACCCA R: CGAGGAGGAGGTTGAGAT	60	(TC) ₁₃	488	KY744729

Note: T_a = annealing temperature.