

# A New Set of Microsatellite Primers for Coelogyne fimbriata (Orchidaceae) and Cross-Amplification in C. ovalis

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PRIMER NOTE

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

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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, northeastern 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 (Swarts 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

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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.

## 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-µL reaction volume for 2 h at 37°C. Fragments 200-1000 bp in length were then ligated to an MseIadapter 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 MseI-N primer (5'-GATGAGTCCTGAGTAAN-3') in a 25-µL 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 streptavidincoated magnetic beads (Promega Corporation, Madison, Wisconsin, USA) to capture single-stranded DNA fragments containing microsatellites. The enriched products were amplified using MseI-N primers for 28 cycles. After the PCR products were purified using a multifunctional DNA Extraction Kit (Bioteke Corporation, Beijing, China), they were ligated into Escherichia coli strain DH5a with the pMD19-T vector (TaKaRa Biotechnology Co., Dalian, Liaoning, China).

We randomly selected and sequenced 249 positive clones using M13+/M13primers 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 Diaoluo 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 GenAIEx 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 (Pelser 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	of 15 pc	olymorphic	microsatellite	markers	developed fo	r Coelogyne fimbriata.
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Locus		Primer sequences (5'–3') <sup>a</sup>	$T_{\rm a}$ (°C)	Repeat motif	Α	Allele size range (bp)	GenBank accession no.
CF1-11	F:	<6-FAM>CAACATCCTCTCGGCATAT	60	(GT) <sub>9</sub>	7	429–443	KP676048
	R:	GACCACACTACACCTACAC					
CF1-26	F:	<6-FAM>ATAACTCACGCCCGATTC	60	(CT) <sub>5</sub>	3	210-218	KP676049
	R:	CCTGTTGTTGCCTGCTGT					
CF1-30	F:	<6-FAM>CACCTCTCCTCAATTACATCA	58	$(ATC)_6$	2	103-109	KP676050
	R:	AGTTGGCGTAAGGCTAATG					
CF1-51	F:	<hex>TGAGAATGTCCGTAGGTT</hex>	58	$(AG)_{14}$	7	336–362	KP676052
GE1 (0	R:	GGGATTGGAGTAAAGGGT	~~~			211.216	
CF1-60	F:	<rox>AAACCTTCGTTCGCTCCT</rox>	60	$(1C)_5(CT)_5(1C)_5$	2	344-346	KP6/6053
OF1 100	R:	GTGCCTGCTAGGGTTCCA	(0)		_	262, 272	VD(7(054
CF1-120	F.:		60	$(G1)_{6}$	5	362-372	KP6/6054
CE1 1(7	R:	ATCATAGGATGGACTCTGTAG	50		0	250, 202	VD(7(055
CF1-10/	E:		38	$(AG)_6$	ð	259–295	KP0/0055
CE1 220	K:		52	(CT) $(TC)$	2	101 107	VD676056
CF1-229	r: D.		52	$(C1)_7(1C)_7$	2	101-107	KF0/0030
CE1-231	г. г.		52	$(AG)_{ij}$ $(GA)_{ij}$	2	285_289	KP676057
CI 1-231	P.		52	(AG)33(GA)18	2	205-207	KI 070057
CF2-26	F.		55	$(AG)_{i}$ $(AG)_{i}$	4	153-171	KX237659
012 20	R:	ATAGCCTACCTCAAGACG	00	(110)16.11(110)6		100 171	111207007
CF2-29	F:	<rox>TTGTAGTCTTCATCCTTT</rox>	52	(TG)5	5	270-308	KX237660
	R:	TCTAGTCTACCCATACTT		( -75			
CF2-57	F:	<hex>TGACTTAGGACCAGGAA</hex>	55	(CT) <sub>27</sub>	17	173-213	KX237661
	R:	GTCGCAAGCACAGATA					
CF2-126	F:	<6-FAM>CTCCCGTGCCTATGTTTC	52	$(CT)_{20}$	4	253-259	KX237663
	R:	ATTCCGCTCTGATTTCCA					
CF2-147	F:	<hex>GGAGGTCTTTGATTAGAT</hex>	52	$(CT)_6CA(CT)_5$	4	254-260	KX237664
	R:	ATGGAGGATTATCAGTAT					
CF2-172	F:	<rox>CTTGTATTTCCCTTTCTTG</rox>	52	$(CT)_9(CA)_6$	4	257-271	KX237666
	R:	TGAGATAACTAAACCCAGA					

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

<sup>a</sup>Fluorescent 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 an	nd C. ovalis populations, respectively. <sup>a</sup>
	1 7 1		

		Coelogyne fimbriata						Coelogyne ovalis					
	DV	VS population	(n = 28)	DL	S population (	n = 19)	MH	IX population	( <i>n</i> = 21)	JG	X population (	n = 16)	
Locus	A	$H_{\rm o}$	H <sub>e</sub>	A	$H_{\rm o}$	H <sub>e</sub>	Α	$H_{\rm o}$	H <sub>e</sub>	Α	$H_{\rm o}$	$H_{\rm e}$	
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.

<sup>a</sup>Voucher 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	C. cumingii $(n = 5)$	<i>C. mayeriana</i> $(n = 5)$	C. eberhardtii ( $n = 5$ )	C. peltastes $(n = 5)$	<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.<sup>a</sup>

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

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

<sup>a</sup>All voucher specimens were deposited in Shanghai Chenshan Herbarium (CSH), Shanghai, China.

<sup>b</sup>Samples 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 mo	nomorphic microsate	llite markers develop	ped for Coelogyn	e fimbriata

Locus		Primer sequences (5'-3')	$T_{\rm a}$ (°C)	Repeat motif	Allele size (bp)	GenBank accession no.
CF1-33	F:	TAAGTAATTCAGCCTCCC	55	(CT) <sub>8</sub>	197	KP676050
CF2-2	R: F:	CAGACCATCAAGAAGCATA CAAGTCCAAATCAGCGAAGG	56	(AG) <sub>15</sub>	152	KY744706
CF2-3	R: F:	TCCAGAATACATCCAGGCAC GAAGAAATCAAGGACCAATG	64	(TCT) <sub>5</sub>	198	KX237656
CF2-8	R: F:	TCTGAGAACGAAGGAGGC TAGGAGGTGAGGAGGAA	56	(AG) <sub>15</sub>	402	KX237657
CF2-15	R: F:	CCAGATGCCAAGATAAA CGACTTTCTCCGGTATCTC	56	(AG) <sub>14</sub>	248	KY744707
CF2-20	R: F:	CACTCACTCAGCCTCTTCC GGAAAATAGTAAAAGCCAT	56	(TC) <sub>18</sub>	181	KY744708
CF2-23	R: F:	TCCCAAACTTCAAACC CTCCCCGTTGTAATCCAATCAT	64	(CT) <sub>12</sub>	178	KY744709
CF2-24	R: F:	GTTCCTCCTTCGGCTACGTTAG ACCCTTCCTATCGCTGTATT	62	(CT) <sub>7</sub>	186	KX237658
CF2-27	R: F:	CTCTTCCCACCAAGTCTTTT GAGAGTGGAGGGTAGGAGAA	62	(AG) <sub>6</sub>	111	KY766112
CF2-59	R: F:	GGAGGAGGCTATGGAGAA GAAGCAGAAAATAACATA	56	(TC) <sub>22</sub>	90	KY744710
CF2-101	R: F:	TCTCACTCCACTCTATCT TGGTCAGTCGGAGGAG	64	(TC) <sub>27</sub>	355	KY744711
CF2-112	R: F:	ATGGAGGTGGTAGTGTTGG GGGATTCGGACTGAGATT	64	(GA) <sub>39</sub>	228	KX237663
CF2-127	R: F:	TTAGTAGGGATGCGAGGAG TCAAGTCCCATCAATC	53	(CT) <sub>23</sub>	153	KY744712
CF2-129	R: F:	TTTAGTGCTCCACATT TTGGCATTTCGCTTCT	59	(CT) <sub>12</sub> (TC) <sub>16</sub>	235	KY744713
CF2-136	R: F:	TCGACCCGTAGTACGCAACA	64	(TC) <sub>8</sub>	285	KY744714
CF2-137	R: F:	ATGGACACCAGGGCAAGG GGAAGGCTATGGAGAAAT	64	$(TC)_{10}(CT)_7$	126	KY744715
CF2-140	R: F:	ATGGGATGACCAGAGGAT GAAGATGGGAAGAAAGAA	62	(GA) <sub>10</sub>	108	KY744716
CF2-146	R: F:	TGAAAGGAGGAGTAGGAG TATGCAAATGATATGA	53	(TC) <sub>15</sub> (AC) <sub>10</sub>	347	KY744717
CF2-148	R: F:	GAGAATGTGAGAAAGT TGAATAAGATATTCGGATCA	56	(AG) <sub>20</sub>	227	KY744718
CF2-149	R: F:	AAATCGGTGTATGGAGAC GTCAAACAGAAAGCCAAG	56	(CT) <sub>18</sub>	350	KX237665
CF2-155	R: F:	AAAGATCCGCTCCACTAT TCTCGTCTTTTCCTCTTACC	56	(CT) <sub>20</sub>	104	KY744719
CF2-160	R: F:	CCATTACCTCCTCACCATAC GAATCCTCGCTCCCATTT	53	$(TG)_{11}(GA)_{15}$	109	KY744720
CF2-171	R: F:	GTTGGTTTAGAGTTTGCAGGTA TCCTTGTTCGCGTGAAAC	59	(GA) <sub>43</sub>	232	KY744721
CF2-177	R: F:	GAGATCCCTCGACCATAC AAGAGTTAGAAGTGGGGAGG	59	(AG) <sub>19</sub> AA(AG) <sub>15</sub>	405	KY744722
CF2-192	R: F:	GGGGAAGTGCCTTATGAT ACGGGTGAGTATCTTGGC	64	(CA) <sub>16</sub>	274	KY744723
CF2-213	R: F:	GAGGTGGTGAACTCCATTTA ACCAATAGGAAGTGAGGAGGAA	64	(CT) <sub>16</sub>	151	KY744724
CF2-217	R: F:	ATGGCGGAGCAAGAAAGG CTTGTTTCATAAAGCGAAGT	64	(CT) <sub>23</sub> (CA) <sub>13</sub>	181	KX237667
CF2-222	R: F:	CTITTTATCACAGTCACCCAT TAGGGGAAACTATGGACAAA	63	(CT) <sub>46</sub>	181	KY744725
CF2-232	R: F:	CGAGTTAGGGATTAGAGGGT AATAAGATAATGGAAGGA	62	(GA) <sub>9</sub>	108	KY744726
CF2-234	R: F:	ACTCCAGTTTGTCTTTTA ATCAAAGCCTATTATTCCC	64	(TC) <sub>8</sub>	292	KY744727
CF2-238	K: F:	ACCCCCACCACCAAGTA	64	(GA) <sub>11</sub>	379	KY744728
CF2-240	F: R:	TACACGCCCTAATACCCA CGAGGAGGAGGTTGAGAT	60	(TC) <sub>13</sub>	488	KY744729

*Note:*  $T_a$  = annealing temperature.