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Development of 11 Polymorphic Microsatellite Loci from *Primula amthystina* Franchet. (Primulaceae)

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***Abstract. Primula amethystina* Franchet. is a beautiful perennial herbaceous plant locally endemic to the alpine area in southwest China. We isolated and characterized 11 polymorphic microsatellite primer pairs from this species. The number of alleles ranged from two to five. The observed and expected heterozygosities (H_O and H_E) were 0.25 to 0.875 and 0.223 to 0.691, respectively. Six loci were significantly deviated from Hardy-Weinberg equilibrium as a result of the heterozygote deficiency. These markers will have great potential to reveal the genetic population structure and genetic diversity of *P. amethystina*.**

Primula amethystina Franchet. is a beautiful perennial herbaceous plant locally endemic to the alpine area in southwest China. It is widely distributed in alpine meadows at altitudes of 3400 to 5000 m in west and northwest Yunnan, southwest Sichuan, over the border into Tibet (Hu and Kelso, 1996; Richards, 2002). *P. amethystina* subsp. *amethystina* is confined to the Cangshan Mountains, Dali, west Yunnan, whereas *P. amethystina* subsp. *brevifolia* widely spreads through the range. Frequently, *P. amethystina* subsp. *brevifolia* is more robust than *P. amethystine* subsp. *amethystina* with up to 20 flowers borne on stems to 25 cm. To classify these two species and study the speciation mode of them, their genetic structures need to be understood. Here, 11 polymorphic microsatellite loci of *P. amethystina* were developed as potential tools to investigate the genetic structures of these species.

Genomic DNA was extracted from leaf tissues using the cetyltrimethyl ammonium bromide (CTAB) method (Milligan, 1992). The isolation of microsatellite loci was performed according to the fast isolation of microsatellite by amplified fragment length polymorphism of sequences containing repeats (FIASCO) (Zane et al., 2002). Approximately 300 ng genomic DNA was completely digested with *Mse*I restriction enzyme (Fermantas). The digested DNA was ligated to *Mse*I adaptor pair (Vos et al., 1995), then 5 μL of the adapter-ligated fragments acted as templates to perform polymerase chain reaction (PCR) in a volume of 20 μL using *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) as a primer following the program: 95 °C for 3 min, 30 cycles of 94 °C for 30 s, 53 °C for 60 s, 72 °C for 60 s followed by an elongation step of 5 min at 72 °C.

For enrichment, the PCR products were denatured at 95 °C for 5 min and then hybridized with a 5′-biotinylated probe $(AG)_{15}$ in 250 μL hybridization solution (20 × SSC, 10% SDS, 100 pmol/μL probe) at 48 °C for 2 h. The DNA hybridized to the probe was separated and captured by streptavidin-coated magnetic beads at room temperature for 20 min followed by two washing steps, including three times in TEN_{100} for 15 min and three times in TEN_{1000} for 24 min. The separated single-stranded DNA was subjected to a second round of PCR according to the same procedure as the first round of PCR. The PCR products, after being purified with the E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, Atlanta, GA), were ligated into $_{pMD}$18-T vector (Takara, Otsu, Shiga, Japan) according to the manufacturer's instructions and then transformed into *Escherichia coli* strain JM109 (Sangon, Shanghai, China). The positive clones were picked out by blue–white screening and tested by PCR using $(AG)_{10}$ and $M13^+/M13^-$ as primers, respectively. Of the 91 clones sequenced, 79 contained potential microsatellite motifs and 42 with unique microsatellites were selected for designing primers using Primer 5.0 (Clarke and Gorley, 2001).

A total of 12 primer sets were abandoned as a result of amplification of multiple bands or unsuccessful amplification of target fragments, and the remaining 30 primer pairs were tested for polymorphisms across 24 individuals from a population of *P. amethystina* subsp. *amethystina* and three populations of *P. amethystina* subsp. *brevifolia* (Table 1). PCR reaction was done in a 20-μL volume using a PTC0200 thermal cycler (MJ Research, Ashland, OR). Each reaction was performed using 20 ng of genomic DNA, 1 μM of each dNTP, 1 μM of each primer, 1× *Taq* buffer [100 mM Tris-HCl, pH 8.8, 2.0 mM $MgCl_2$, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], and 1 U of *Taq* polymerase (Takara). The PCR programs are as follows: initial denaturing step at 95 °C for 5 min, 30 cycles of 94 °C for 30 s, primer-specific annealing temperature 55 to 62 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 8 min. The PCR products were electrophoresed in denaturing 6% polyacrylamide gels using a 25-bp DNA ladder molecular size standard (Fermatas, Ontario, Canada) to estimate allele sizes by silver staining.

Of the 30 new primers, 11 of them displayed polymorphism across populations. The number of alleles per locus, observed (H_O) and expected heterozygosity (H_E), and deviation form Hardy-Weinberg equilibrium (HWE) were assessed using GENEPOP Version 3.4 (http://wbiomed.curtin.edu.au/genepop/) (Raymond and Rousset, 1995). The number of alleles per locus ranged from two to five with an average of 2.8 (Table 2). The observed and expected heterozygosities (H_O and H_E) ranged from 0.25 to 0.875 and from 0.223 to 0.691 with averages of 0.508 and 0.442, respectively (Table 2). Among the 11 microsatellite markers, six loci showed significant deviation from HWE ($P < 0.01$) (Table 2), which was the result of deficiency of heterozygotes or the limitation of sample size. Tests for linkage disequilibrium were run in FSTAT Version 2.9.3.2 (Goudet, 1995). Significance levels were adjusted using sequential Bonferroni corrections (Rice, 1989). No loci showed significant linkage disequilibrium after Bonferroni correction. These polymorphic microsatellite loci presented here would provide a useful tool for studying the pop-

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Table 1. Sample information of *P. amethystina* subsp *amethystina* and *P. amethystine* subsp. *brevifolia.*

Population	Species	Location	Position	Altitude (m)	Accession no.
Z1	*P. amethystine* subsp. *brevifolia*	Laojun Shan, Lijiang, Yunnan province	N26°54′10.51″ E 99°37′07.62″	3500	Wu20080612
Z2	*P. amethystine* subsp. *brevifolia*	Tianchi, Xianggelila, Yunnan province	N27°37′14.3″ E99°38′10.5″	3800	Huang20080529
Z3	*P. amethystina* subsp *amethystina*	Chang shan, Dali, Yunnan province	N25°40′37.48″ E100°05′33.80″	3900	Wu20090621
Z4	*P. amethystine* subsp. *brevifolia*	Changhaizi, Muli, Sichuan province	N27°56′32.41″ E101°11′17.32″	3400	Wu20090609

Table 2. Characteristics of 11 polymorphic microsatellite loci for *Primula amethystine* Franchet.

Locus (GeneBank no.)	Repeat motif	Primer sequence (5′–3′)	Allele size range (bp)	A	$T_{a\ (°C)}$	H_O	H_E	HWE (P value)
GQ397487	$(AG)_8$	R: CTACAATCAACGCTAAAACG F: ACTTCACGGTGAAGAAAGAA	291	3	53	0.75	0.606	0.0017*
GQ397488	$(AT)_4(AC)_3(AC)_4(AC)_2$	R: CGGAAACCCGAAACTCTTAC F: CCCATACTTGCACTCCACTC	285	2	53	0.292	0.254	1
GQ397489	$(GA)_5(GA)_5(AG)_5$	R: TAGATGGGTAGGTATTGACG F: ATATGCGGTTACAGTAGAAA	261	2	57	0.875	0.503	0.0002*
GQ397490	$(AG)_3(AG)_3(AG)_{11}$	R: ATATTCACCCCTACCTTTTG F: ACACGTTACTTTTCTCAGAG	253	3	54	0.75	0.494	0.0070*
GQ397491	$(CT)_3(TCT)_3(CT)_3(CT)_7$	R: CTAGTCCCCATATTCATTTT F: ACACCCCTACCTCATAAGTC	251	3	54	0.792	0.558	0.0180
GQ397492	$(CT)_{11}$	R: ACCTAGAGTGCATTTGAGCC F: GTTAGCCCCTCTATCACCTC	236	3	60	0.333	0.439	0.0001*
GQ397493	$(CTT)_3(CT)_3(CT)_3(CT)_3$	R: TACAACAAACCCTAGCCTC F: AGCAAGTGATCTCACCAAT	223	2	57	0.333	0.284	1
GQ397494	$(GA)_8$	R: TTGAGTTGTAAGCTCTTGAG F: GGTAAGGGATTAGGAGTGTA	208	2	56	0.25	0.223	1
GQ397495	$(AG)_8$	R: AAACTGATACCAATAGGACC F: CATAGATAGTGTACGGTGAG	190	4	53	0.625	0.691	0.0072*
GQ397496	$(CT)_3(CT)_7$	R: TTTGTTCCTTGTGAAGAGTA F: TGGACCAAGTCAGTGATTAT	186	2	58	0.25	0.223	1
GQ397497	$(GA)_5(AG)_{11}(GA)_3(GA)_3$	R: AACAAACAAACCAGCGAACT F: TTTCAGTCGCATATCTACAC	186	5	53	0.333	0.590	0.0001*

Ta = annealing temperature of primer pair; A = number of alleles; H_o = observed heterozygosity; H_E = expected heterozygosity. Statistically significant deviation from Hardy-Weinberg expectation (HWE) is indicated by * ($P < 0.01$).

ulation genetic structure and genetic diversity of *P. amethystina*.

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