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Development of genome-wide microsatellites from *Primula tibetica* (Primulaceae) and their utility in congeneric species

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Running title: Polymorphic microsatellite markers for *Primula tibetica*

Key words: distyly, homostyly, microsatellites, *Primula tibetica*, Primulaceae

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Abstract

Primula tibetica is an insect-pollinated, herbaceous, perennial plant belonging to section Aleuritia (Primulaceae). The species exhibits the typical characteristics of heterostyly, with predominantly outcrossing populations comprising long-styled and short-styled floral morphs. Furthermore, the analysis demonstrates significant variation in floral morphology, categorised as homostyly, a phenomenon commonly associated with elevated selfing rates. Utilising next-generation sequencing, 25 microsatellite markers for P. tibetica were developed, with the objective of facilitating future investigations into the population genetics and mating patterns of the species. The characterisation of these markers was achieved through the measurement of polymorphism and genetic diversity in a sample of 36 individuals from three natural populations. The markers displayed relatively high polymorphism, with the number of observed alleles per locus ranging from two to 15 (mean = 7.26). The observed and expected heterozygosities ranged from 0.056 to 0.917 and 0.105 to 0.825, respectively. Furthermore, nineteen of these loci were also successfully amplified in P. pulchella. These microsatellite markers have the potential to serve as effective tools for investigating patterns of population genetic diversity and elucidating the evolutionary relationship between distyly and homostyly in P. tibetica.

Key words: distyly, homostyly, microsatellites, *Primula tibetica*, Primulaceae

Primula L. (Primulaceae) is an iconic taxon for studying pollination, floral morphology and mating system evolution. Since Darwin's classic work on heterostyly in *Primula* (Darwin, 1877), it has been the focus of sustained research for over a century and is now the most intensively studied heterostylous taxon (Mast and Conti, 2006). Approximately 92% of its 400-500 species are distylous, while the remaining species are monomorphic (homostylous) for stylar condition (Richards, 2003; de Vos *et al.*, 2014). Populations of distylous species consist of two floral morphs (distyly), which differ in the position of their stigmas and anthers. They usually possess heteromorphic self-incompatibility, which limits self- and intra-morph mating (Darwin, 1877; Ganders, 1979; Barrett, 2019). In contrast, homostylous individuals are self-compatible, with stigmas and anthers positioned close together within a flower, resulting in autonomous self-pollination and moderate-to-high selfing rates (Zhang et al., 2021; Wang et al., 2021; Yuan et al., 2023).Homostyly occurs in nearly half of the 38 sections of the genus, with phylogenetic analyses and ancestral state reconstructions indicating a single origin of distyly in *Primula,* followed by multiple independent transitions to homostyly (Mast *et al.*, 2006; de Vos *et al.*, 2014; Zhong *et al.*, 2019). Therefore, *Primula* provides a popular paradigm for studying how the breakdown of heterostyly to homostyly drives the mating system transition from outcrossing to selfing (Wu et al., 2023; Zeng et al., 2024).

Primula tibetica Watt is an insect-pollinated, perennial herb belonging to section *Aleuritia* (Primulaceae) (Hu and Kelso, 1996). The species is widely distributed throughout the Himalayas and Tibet Plateau, commonly occurring in diverse habitats at elevations ranging from 2700 to 4800 m (Fig. 1A) (Hu and Kelso, 1996). Historically, *P. tibetica* has been reported as distylous (Hu and Kelso, 1996; Richards, 2003), with populations comprising long- and short-styled morphs promoting

pollinator-mediated outcrossing (Fig. 1B, C). However, our recent field investigation identified a homostylous floral phenotype in this species, differing from both long- or short-styled morphs through significantly reduced herkogamy (Fig.1D). In contrast to other reported distylous-homostylous species (e.g. *P. chungensis, P. oreodoxa* and *P. secundiflora*) in the genus (as shown in Yuan et al., 2017; Zhou et al., 2017; Shao et al., 2019; Zhong et al., 2019; Wang et al., 2021; Zeng et al., 2024; Sun et al., 2024), homostyly of *P. tibetica* exclusively co-occurring with distylous morphs as trimorphic populations, with no monomorphic populations observed. Therefore, given the peculiar intraspecific variation in its population floral morph structure, this species provides an outstanding opportunity for investigating the evolutionary relationship between distyly (outcrossing) and homostyly (selfing), the ecological drivers, and the genetic outcomes of mating system transitions.

Of the different types of molecular markers, microsatellites (SSRs) and single nucleotide polymorphisms (SNPs) are widely employed in contemporary plant population genetics (Amom and Nongdam, 2017). While SNPs offer high genomic density and scalability, SSRs remain particularly valuable due to their hypervariable nature, allelic variation at each SSR locus is readily amplified via PCR and provides high-resolution genotyping (Putman and Carbone, 2014). These markers are typically abundant across nuclear and plastid genomes, exhibit codominance and cross-species transferability, and show greater polymorphism per locus than many alternatives. Advances like next-generation sequencing (NGS) and multiplex PCR have drastically reduced the cost and effort of developing SSR panels for non-model taxa (Šarhanová et al., 2018). Although SNP arrays are increasingly used for genome-wide studies, SSRs retain utility for targeted population analyses requiring high per-locus diversity. Here, we leverage NGS to develop polymorphic SSR markers for *P. tibetica*, enabling

detailed population genetic investigation of mating system transitions in this distylous-homostylous species.

We collected samples of *P. tibetica* in July 2019 and deposited the voucher specimens at the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN) under accession number 1710088. Total genomic DNA was isolated from silica-dried leaf tissue of a single *P. tibetica* individual sampled from population PT-1 (see Supplementary Table S1) using a modified cetyl trimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). Purified DNA was sheared to ~700 bp fragments, and a sequencing library was prepared using the NEBNext Ultral II DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). Paired-end (PE, 2 × 150 bp) sequencing was performed on an Illumina HiSeq X-Ten platform (San Diego, California, USA) at the Beijing Genomics Institute (Shenzhen, Guangdong, China). Approximately 3 Gb raw reads were generated and deposited in the National Genomics Data Center (NGDC) of the China National Center for Bioinformation (CNCB) under BioProject PRJCA041996 (Genome Sequence Archive: CRA027011). We performed sequence quality control using NGS QC TOOL KIT (Patel and Jain, 2012) with default parameters. Adapter trimming, removal of ambiguous reads (>10% 'N' bases), and quality filtering (Phred score <30) were conducted using Trimmomatic v.0.39 (Bolger et al., 2014). Cleaned reads were *de novo* assembled in Geneious Prime (Biomatters, Auckland, NZ) with high-sensitivity/medium settings, yielding 58,491 contigs. We identified and excluded plastid-derived contigs using BLASTX against the GenBank database.

We used the QDD v.3.1.2 (Meglécz et al., 2014) to identify unique microsatellite-containing sequences from the scaffolds generated by *de novo* assembly. The search parameters were configured to detect microsatellite repeats

meeting strict criteria: ≥ 4 repeat units for di- to hexanucleotide motifs, with ≤ 100 bp maximum spacing between two adjacent microsatellites. The minimum amplicon length was constrained to 120 bp to ensure compatibility with downstream fragment analysis. In total, we identified 3,218 contigs containing at least one microsatellite. From this candidate pool, 100 di- or tri-nucleotide SSR loci were randomly selected for experimental validation. We designed primer pairs for these loci using the automatic search model of PRIMER v.5.0 (Clarke and Gorley, 2001), taking into consideration the optimal PCR amplicon size (100-300 bp), primer annealing temperature (57-61 ℃) and primer length (18-23 bp). Initial PCR optimization was performed on an Applied Biosystems Veriti 96-well Thermal Cycler (Foster City, California, USA) using gradient PCR protocols to empirically validate primer functionality across the target annealing temperature range.

Preliminary amplification tests were carried out with five individuals of *P. tibetica* from the PT-1 population (Supplementary Table S1). The following protocol was utilised for the performance of PCR amplification: 20 μL of total reaction volume containing 10 μL of Master Mix (Tiangen Biotech, Beijing, China; including 3 mmol/L $MgCl_2$, 100 mmol/L KCl, 0.5 mmol/L of each dNTP, 20 mmol/L Tris-HCl [pH 8.3], and 0.1 units Taq polymerase), 0.6 μmol/L of each primer, 8.4 μL of deionized water, and 20-40 ng of genomic DNA. The following conditions were met during the execution of the PCR amplification process: The initial step is a denaturation process at 95°C for 3 minutes. This is followed by 30 to 35 cycles at 95°C for 30 seconds, at the annealing temperature for each specific primer (optimised for each locus; see Table 1) for 30 seconds, 72°C for 30 seconds for extension, and a final extension step at 72°C for 5 minutes. The separation and visualisation of PCR products was conducted using a QIAxcel capillary gel electrophoresis system

(QIAGEN, Valencia, California, USA) with an internal 10-300 bp size standard. Among the 100 primer pairs that were tested, 25 yielded successfully amplified polymorphic microsatellite loci with suitable fragment lengths (see Table 1 for details). This number of microsatellite loci with high polymorphism is sufficient for addressing most questions on population genetics and mating systems of Primula populations, as demonstrated in recent congeneric research (Zhong et al., 2019; Zeng et al., 2024).

For these 25 successful loci, we measured polymorphism in 36 individuals obtained from three natural populations of *P. tibetica* in Tibet and four individuals from one population (PP-12) of *P. pulchella* in Sichuan (Supplementary Table S1). We calculated basic population genetic parameters of diversity, including the number of alleles (N_A), observed (H_O) and expected heterozygosity (H_E), and inbreeding coefficient (F_{IS}) by using GenAlEx v.6.5 (Peakall and Smouse, 2012). We tested for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium between microsatellite loci using GENEPOP v.4.7.2 (Rousset, 2008). Frequencies of null alleles were detected by MICRO-CHECKER (van Oosterhout et al., 2004).

The number of alleles per locus ranged from 2 to 15, with the mean ± SE = 7.26 ± 0.25 (Table 2). Among all the polymorphic loci, the observed and expected heterozygosity ranged from 0.056 to 0.917 (0.0.368 ± 0.033) and 0.105 to 0.825 (0.492 ± 0.029), respectively. The inbreeding coefficient ranged from -0.309 to 0.920 (0.218 ± 0.049). Deviations from Hardy-Weinberg equilibrium due to heterozygote deficiency in each population are indicated for each locus in Table 2. However, no single locus deviated from HWE across all populations. We did not find loci pairs exhibiting consistent linkage disequilibrium across all three populations. The potential presence of null alleles was assessed with results for each locus provided in Table 2.

Among the 25 microsatellite markers, 19 loci were successfully amplified in a population of *P. pulchella* sampled from Sichuan with alleles number ranged from 1 to 4.

The microsatellite markers we isolated for *P. tibetica* will provide a valuable resource for advancing research on this species and its closely related species. These highly polymorphic loci will enable detailed investigations in mating system dynamics, contemporary population genetics structure and gene flow patterns, and historical phylogeographic relationships across its range. It will be of particular interest to use these markers to investigate the evolutionary relationships between distylous and distylous-homostylous populations and to determine the process by which homostyly spread into outcrossing populations and the associated genetic consequences, including changes in genetic diversity, effective population size, or the accumulation of deleterious mutations. Furthermore, the high polymorphism of these markers will also be useful for fine-scale parentage analysis within natural populations and for measuring mating patterns and individual reproductive success. This research will provide opportunities to assess the potential influence of demographic, ecological, and reproductive factors on mating system evolution.

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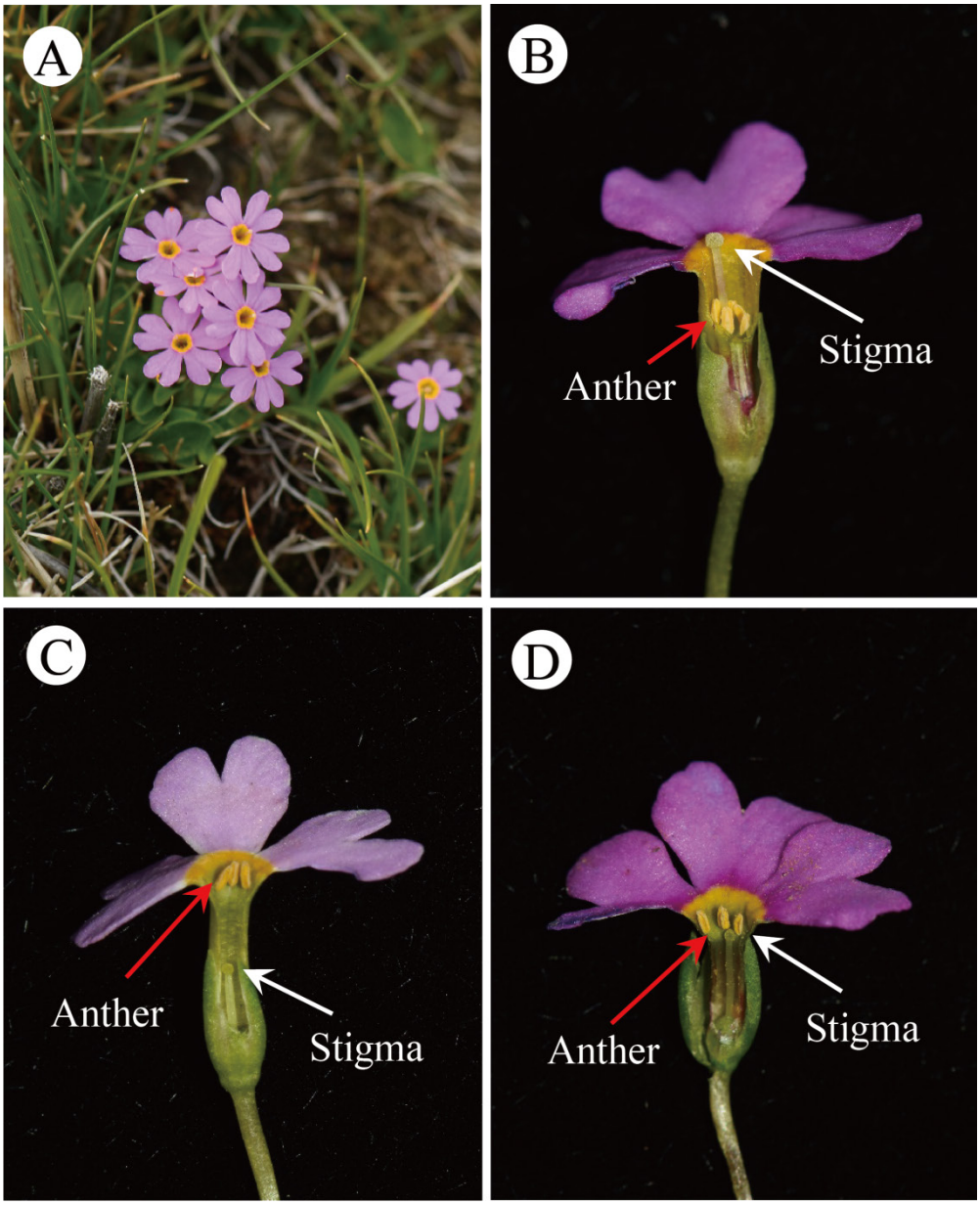

Figure 1 Heterostyly and homostyly in *Primula tibetica*. (A) Field photograph of a plant in its natural habitat. Dissected flowers of (B) long-styled morph, (C) short-styled morph and (D) homostyly show reproductive organ positions. White arrows indicate stigma positions; red arrows indicate anther positions.

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296 **Table 1** Characteristics of 25 microsatellite loci isolated from *Primula tibetica.*

Locus	Primer sequence (5'- 3')	Repeat motif	Fragment size (bp)	T_a (°C)	GenBase accession no.
PT595	F: TTTCGTTCATCTATCGCCAA R: ACTTCCTTCCGGTACGTGG	$(GA)_9$	147	60.5	C_AA111057
PT647	F: AAGGGGATCACTTACCACCA R: ATAAGAAATTAGCCGCGAAGG	$(CT)_6$	100	59.1	C_AA111060
PT190	F: CCCATACCCTTATAACCCCC R: GTTTTGGCGGTACGAGAGAG	$(TC)_7$	198	59.9	C_AA111043
PT400	F: ATGTTGTGTCCATTGAACCG R: ATGCGATGAAATCACGAACA	$(AT)_9$	212	57.8	C_AA111048
PT797	F: TGGAACTGAAATCTCACCTTTCT R: GTTAACGAACACGACGGGAT	$(TC)_{11}$	141	60.3	C_AA111064
PT748	F: TGTAGGTGAAAGCGATGGAA R: CAGAACAGCTACAACGCCAA	$(CT)_7$	189	58.0	C_AA111062
PT678	F: TCCCAAATTCACCTCATTATCA R: TTTGGTTTCTAGGTGTTTCCG	$(AT)_6$	132	59.4	C_AA111061
PT279	F: GTTATGACGGAAAATTGGGC R: TGATGCATTGTGTACTGGGAA	$(AT)_6$	225	59.8	C_AA111045
PT372	F: TGCTCCTCTCTTCTCCTCGT R: CCCTCCTCCCTCTCTCACTT	$(TC)_7$	149	59.9	C_AA111047
PT528	F: GCTACAACCAAACAGGCCTTA R: TCATTCATACACAACACTGGCA	$(AT)_6$	240	50.1	C_AA111053
PT980	F: CCGTTAGCTTTTGTTTTTGGA R: TGCCATGTAATCAAAACCTTCA	$(CA)_7$	129	59.0	C_AA111066
PT59	F: TGTTGTGTGGAAATTTGATGG R: TGTGTATCATGCCGAGGAAA	$(TG)_6$	166	59.2	C_AA111055
PT593	F: TGGCACTAGTGACTACCCTTAA R: TCATTGTCGGTGTTGGTGTT	$(AG)_8$	160	56.5	C_AA111056
PT507	F: AACTCCACCAGAATTCGAGC R: TGAAATTTGACGTATGGGCA	$(AC)_6$	132	58.0	C_AA111051
PT618	F: GACCAAAATACCCTCAACGG R: TGCTGAGTGATTTAGGGTTGG	$(TC)_6$	100	57.6	C_AA111059
PT575	F: CTCTATCTCGCCGGAAATGT R: AATCTTCCCCCTTTTAGGCA	$(TG)_6$	104	58.5	C_AA111054
PT771	F: CTCATGGCTGAAGGGCTATT R: AAATTACACGGCTCGTTTCG	$(AG)_6$	167	60.3	C_AA111063
PT296	F: GAGCCCACATTTTGTAACCC R: GCGAAGGGCCATAAGAAAAT	$(CT)_7$	148	59.8	C_AA111046
PT447	F: TTTCTCGTCGGTGAAACAAA R: AAACCCACCCAAACAATGAA	$(CT)_6$	246	57.3	C_AA111049
PT119	F: TTTTGGTTCTGAAGGTGCAA R: CGAGAATAATGACTCCAACGAG	$(TC)_6$	161	58.2	C_AA111042
PT251	F: GCAATGCCTTTTTAGGAGGA R: CCATGATTTCCTTTTGCACC	$(AG)_8$	195	60.5	C_AA111044
PT500	F: TTACTGTATCGCCCCCAAAT R: CGAGGAGAAGGAGCATGAAA	$(AG)_8$	145	57.6	C_AA111050
PT520	F: CCTAAACACACTTAACCCTAACC R: GGAGTACCTGGAGTGGGTGA	$(AC)_6$	131	58.8	C_AA111052
PT604	F: GGTGCAACCACCATAAACAA R: AGGATGAGAGAGAGGGGGAA	$(AC)_7$	145	60.3	C_AA111058
PT957	F: AACTTCCCCTCACCCTAACC R: TGACGAGCAACGACCTAATG	$(AG)_7$	242	57.8	C_AA111065

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Table 2 Genetic diversity statistics of 25 polymorphic microsatellite loci in three populations of *Primula tibetica*.

Locus	Population												Total (n = 36)				
	PT-1 (n = 12)				PT-5 (n = 12)				PT-7 (n = 12)								
	N_A	H_O	H_E	F_{IS}	N_A	H_O	H_E	F_{IS}	N_A	H_O	H_E	F_{IS}	N_A	Size range (bp)	H_O	H_E	F_{IS}
PT595	6[a]	0.250	0.743	0.66	8[ab]	0.500	0.771	0.351	5[ab]	0.083	0.628	0.867	8	136-150	0.278	0.714	0.627
PT647	3	0.250	0.226	-0.108	4	0.417	0.358	-0.165	3	0.167	0.156	-0.067	4	104-116	0.278	0.247	-0.113
PT190	2	0.083	0.219	0.619	3[a]	0.167	0.292	0.429	3[ab]	0.000	0.667	1.000	5	178-185	0.083	0.392	0.683
PT400	5[ab]	0.083	0.642	0.870	7[ab]	0.500	0.806	0.379	6[b]	0.417	0.788	0.471	11	212-227	0.333	0.745	0.574
PT797	7[ab]	0.333	0.667	0.500	3 [a]	1.000	0.538	-0.858	2	0.500	0.375	-0.333	7	144-156	0.611	0.527	-0.230
PT748	2[ab]	0.000	0.444	1.000	4[ab]	0.167	0.354	0.529	4	0.500	0.538	0.071	7	178-296	0.222	0.446	0.533
PT678	2	0.083	0.080	-0.043	2	0.083	0.080	-0.043	3	0.333	0.403	0.172	3	125-147	0.167	0.188	0.028
PT279	3[ab]	0.000	0.569	1.000	4[ab]	0.167	0.698	0.761	4[ab]	0.000	0.681	1.000	6	220-240	0.056	0.649	0.920
PT372	6	0.667	0.681	0.020	6	0.667	0.559	-0.193	6	0.833	0.691	-0.206	6	135-172	0.722	0.644	-0.126
PT528	2	0.250	0.219	-0.143	2	0.417	0.330	-0.263	2	0.333	0.278	-0.200	2	224-230	0.333	0.275	-0.202
PT980	6	0.500	0.573	0.127	2	0.083	0.219	0.619	11[ab]	0.583	0.847	0.311	13	125-169	0.289	0.546	0.353
PT59	4	0.583	0.490	-0.191	2	0.083	0.080	-0.043	2 [a]	0.000	0.153	1.000	5	157-205	0.222	0.241	0.255
PT593	2	0.083	0.080	-0.043	2	0.083	0.219	0.619	5[b]	0.333	0.715	0.534	6	162-182	0.083	0.338	0.370
PT507	9	0.667	0.840	0.207	8[b]	0.500	0.806	0.379	9[b]	0.500	0.830	0.397	15	70-132	0.556	0.825	0.328
PT618	3[a]	0.083	0.226	0.631	5[b]	0.417	0.691	0.397	6[ab]	0.333	0.774	0.570	8	74-116	0.278	0.564	0.532
PT575	2	0.167	0.153	-0.091	1	0.000	0.000	–	2	0.417	0.330	-0.263	3	85-110	0.194	0.161	-0.177
PT771	3[a]	0.333	0.569	0.415	4[ab]	0.250	0.573	0.564	6[ab]	0.250	0.569	0.561	6	140-235	0.278	0.571	0.513
PT296	4	0.333	0.413	0.193	6	0.667	0.531	-0.255	6	0.750	0.708	-0.059	8	126-158	0.583	0.551	-0.040
PT447	4	0.833	0.649	-0.283	4	1.00	0.684	-0.462	4	0.750	0.635	-0.180	4	230-250	0.861	0.656	-0.309
PT119	4	0.417	0.462	0.098	2	0.333	0.278	-0.200	3	0.167	0.156	-0.067	4	184-195	0.306	0.299	-0.056
PT251	4	1.000	0.733	-0.365	8[b]	0.417	0.795	0.476	8[b]	0.583	0.816	0.285	14	184-324	0.667	0.781	0.132
PT500	3[ab]	0.083	0.434	0.808	5[ab]	0.250	0.420	0.405	4[ab]	0.083	0.642	0.870	8	141-166	0.139	0.499	0.694
PT520	2	0.083	0.080	-0.043	3	0.167	0.156	-0.067	2	0.083	0.080	-0.043	3	116-130	0.111	0.105	-0.051
PT604	3[a]	0.083	0.226	0.631	7	0.667	0.792	0.158	7	0.583	0.764	0.236	11	128-156	0.444	0.594	0.342
PT957	4	1.000	0.642	-0.557	6[a]	0.833	0.736	-0.132	10	0.917	0.858	-0.069	14	157-237	0.917	0.745	-0.235
Mean	3.8	0.330	0.442	0.237	4.3	0.393	0.471	0.141	4.9	0.380	0.563	0.274	7.2	–	0.368	0.492	0.218

Note: Bold loci were successfully amplified in *Primula pulchella*; N_A, number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; *n*, number of individuals.

[a] Significant deviation from Hardy–Weinberg equilibrium: $P < 0.01$.

[b] Null allele present.

Supplementary Table S1. Locality information for three populations of *Primula tibetica* and one population of *P. pulchella* used in this study.

Species	Population code	Location (county, province, country)	Latitude (°N)	Longitude (°E)	Altitude (m)
P. tibetica	PT-1	Changdu, Tibet, China	31.698	94.984	4091
P. tibetica	PT-5	Naqu, Tibet, China	31.271	91.810	4545
P. tibetica	PT-7	Shannan, Tibet, China	28.696	92.194	4821
P. pulchella	PP-12	Xiangcheng, Sichuan, China	28.623	99.752	4101