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Development of polymorphic microsatellite markers for distylous-homostylous *Primula secundiflora* (Primulaceae) using HiSeq sequencing

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Abstract

Primula secundiflora is an insect-pollinated, perennial herb belonging to section *Proliferae* (Primulaceae) and exhibits considerable variation in mating system with predominantly outcrossing populations comprising long-styled and short-styled floral morphs and selfing populations comprising only homostyles. To facilitate future investigations of the population genetics and mating patterns of this species, we developed 25 microsatellite markers from *P. secundiflora* using next-generation sequencing and measured polymorphism and genetic diversity in a sample of 30 individuals from three natural populations. The markers displayed relatively high polymorphism, with the number of observed alleles per locus ranging from three to 16 (mean = 8.36). The observed and expected heterozygosities ranged from 0.100 to 1.000 and 0.145 to 0.843, respectively. Twenty-one of the loci were also successfully amplified in *P. denticulata*. These microsatellite markers could provide powerful tools for investigating patterns of population genetic diversity and the evolutionary relationships between distyly and homostyly in this species.

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

Primula (Primulaceae) is an iconic taxon for the investigation of pollination and plant mating system evolution. Beginning with Darwin's classic work on heterostyly in *Primula* (Darwin, 1877), the genus has received over a century of sustained interest and today is the most intensively studied heterostylous taxon (Mast and Conti, 2006). The vast majority (92%) of *c*. 400-500 *Primula* species are distylous, with the remaining species monomorphic for stylar condition and homostylous (Richards, 2003; de Vos *et al.*, 2014). Homostyly is reliably reported from nearly half of the 38 sections of the genus, with phylogenetic analyses and ancestral state reconstructions indicating that a single origin of distyly in *Primula* followed by numerous independent transitions to homostyly (Mast *et al.*, 2006; de Vos *et al.*, 2014; Zhong *et al.*, 2019).

Primula secundiflora is an insect-pollinated, perennial herb belonging to section *Proliferae* (Primulaceae). The species is widely distributed throughout southwest China and eastern Tibet and Qinghai, commonly occurring in moist meadows, open slopes, and among shrubs (Hu and Kelso, 1996). As recorded, *P. secundiflora* exhibits distyly (Richards, 2003), with populations comprising long-styled and short-styled floral morphs enabling outcrossing mediated by pollinator. However, our recent field investigation found that the species also possesses monomorphic populations with a single floral phenotype, a condition known as homostyly. Therefore, because the intraspecific variation in population floral morph structure (as shown in Yuan et al., 2017; Zhou et al., 2017; Shao et al., 2019; Zhong et al., 2019; Wang et al., 2021; Zhang et al., 2021; Zeng et al., 2024), this species provides an outstanding opportunity for investigating the evolutionary relationships between distyly (outcrossing) and homostyly (selfing) and the ecological causes and genetic consequences of mating system transitions.

Of the different types of PCR-based molecular markers, microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats, have been widely used in molecular population genetics of non-model species because the large amount of allelic variation at each locus can be readily amplified by PCR. The markers are commonly abundant, distributed in both nuclear and plastome genome, and are highly polymorphic compared with other genetic markers, as well as being co-dominant and cross-species transferable. With technical developments such as next-generation sequencing and multiplex PCR, the costs and labor of identifying and genotyping large amount of microsatellite loci in non-model species have been greatly reduced. This enabled the marker still being a popular approach for addressing certain types of questions in evolution and ecology. Here, we employed next-generation sequencing to develop a set of variable microsatellite markers in *P. secundiflora* for our further molecular population genetic analysis of this distylous-homostylous species.

We isolated total genomic DNA from silica-dried leaf tissue of one *P. secundiflora* individual sampled from population CD (see Supplementary Table S1) following a modification of the cetyl trimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). The purified DNA was sheared into *c*. 700 bp fragments to construct a library according to the preparation procedures of NEBNext Ultral II DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). We then generated the paired-end (PE) reads (2 × 150 bp) using a HiSeq X-Ten sequencer (Illumina, San Diego, California, USA) at the Beijing Genomics Institute (Shenzhen, Guangdong, China). Approximately 2 Gb raw reads were obtained and deposited in the National Genomics Data Center (NGDC) of the China National Center for Bioinformation (CNCB) (BioProject: PRJCA021303; Genome Sequence Archive: CRA013486). We performed sequence quality screening using NGS QC TOOL KIT (Patel and Jain,

2012) with default parameters. The clean reads were trimmed from the raw sequences to removing adapter sequences, ambiguous ('N' > 10%) and low-quality reads (Phred score < 30) by using Trimmomatic v.0.39 (Bolger et al., 2014). Using the built-in Geneious assembler (Biomatters, Auckland, New Zealand), the cleaned reads were then assembled into 63,752 contigs with high sensitivity/medium for the sensitivity setting. We identified and excluded plastome contigs using BLASTX against GenBank.

We used the QDD v.3.1.2 (Meglécz et al., 2014) to identify unique reads containing microsatellites from the scaffolds generated by *de novo* assembly, and these satisfied the following criteria: more than four repeats for dinucleotides to hexanucleotides, and 100 bp for the maximal number of bases between two contiguous microsatellites. The Minimum product size was set to 120 bp. In total, we identified 4,762 contigs containing at least one microsatellite. One hundred simple sequence repeat loci with di- or tri-nucleotide repeats were randomly selected for further characterization. We designed primers for these loci using the automatic search model of PRIMER v.5.0 (Clarke and Gorley, 2001), taking into consideration the optimal PCR product size (100-300 bp), primer annealing temperature (57-61 ℃) and primer length (18-25 bp). We used a Veriti 96-well Thermal Cycler PCR Machine (Applied Biosystems, Foster City, California, USA) to optimize these primers initially.

Preliminary amplification tests were carried out with four individuals of *Primula secundiflora* from the CD population (Supplementary Table S1). We performed PCR amplification using the following protocol: 20 μL 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), 0.5 μmol/L of each primer, 8.5 μL of deionized water, and 30-50 ng of genomic DNA. We conducted PCR amplification under the following conditions: 95 ℃ for 3 min followed by 30 to 35 cycles at 95 ℃ for 30 s, at the annealing temperature for each specific primer (optimized for each locus; Table 1) for 30 s, 72 ℃ for 30 s for extension, and a final extension step at 72 ℃ for 10 min. We separated and visualized PCR products using a QIAxcel capillary gel electrophoresis system (QIAGEN, Valencia, California, USA) with an internal 10-300 bp size standard. We first determined allele sizes using GENEMAPPER v.3.7 software with GeneScan-500 ROX as an internal-lane size standard (Applied Biosystems) and then rechecked the data manually to reduce scoring errors. Out of the 100 primer pairs that we tested, 25 microsatellite loci amplified successfully with suitable fragment lengths and showed polymorphism (Table 1). This number of microsatellite loci with high polymorphism is sufficient for addressing most questions on population genetics and mating systems of *Primula* populations, as evidenced by recent studies of congeneric species (Zhou et al., 2017; Zhong et al., 2019).

For these 25 successful loci, we measured polymorphism in 30 individuals obtained from three natural distylous populations of *P. secundiflora* in Tibet and four individuals from one distylous population of *P. denticulata* in Tibet (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 16, with the mean ± SD = 8.36 ± 3.627 (Table 2). Among all the polymorphic loci, the observed and expected heterozygosity ranged from 0.100 to 1.000 (0.580 ± 0.269) and 0.145 to 0.843 (0.543 ± 0.205), respectively. The locus deviated from Hardy-Weinberg equilibrium in each population is indicated in Table 2, and there were no loci showing deviation from HWE in all three populations. Among the 25 microsatellite markers, 21 loci were successfully amplified in a population of *P. denticulata* sampled from Tibet, with allele numbers ranging from 1 to 5.

The microsatellite markers that we have isolated in *P. secundiflora* will provide a valuable resource for investigating mating system, population genetic structure, and phylogeography in *P. secundiflora* and its closely related species. It will be of particular interest to investigate the evolutionary relationships between distylous and homostylous populations and determine the direction of transitions between outcrossing and selfing and their genetic consequences. The high polymorphism of the microsatellite markers that we have identified will also be useful for parentage analysis and measures of mating patterns within the natural populations and should provide opportunities to assess the potential influence of demographic, ecological, and reproductive factors on mating system evolution.

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Botany, Chinese Academy of Sciences.

References

Bolger, A. M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics **30**, 2114–2120.

Clarke, K. R., and Gorley, R. N. (2001) Primer v5: User Manual/Tutorial. Primer-E Ltd., Plymouth, UK.

Darwin, C. (1877) The different forms of flowers on plants of the same species. John Murray Press, London, UK.

de Vos, J. M., Wüest, R. O., and Conti, E. (2014) Small and ugly? Phylogenetic analyses of the "selfing syndrome" reveal complex evolutionary fates of monomorphic primrose flowers. Evolution **68**, 1042–1057.

Doyle, J. J., and Doyle, J. L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**, 11–15.

Hu, C. M., and Kelso, S. (1996) Primulaceae. *In* Flora of China. (eds.: Wu, Z. Y., and Raven, P. H.), pp. 156. Science Press, Beijing, China, and Missouri Botanical Garden Press, St. Louis, USA.

Mast, A. R., and Conti, E. (2006) The primrose path to heterostyly. New Phytol. **171**, 439–442.

Mast, A.R., Kelso, S., and Conti, E. (2006). Are any primroses (*Primula*) primitively monomorphic? New Phytol. **171**, 605–616.

Meglécz, E., Pech, N., Gilles, A., Dubut, V., Hingamp, P., Trilles, A., Grenier, R., and Martin, J. F. (2014) QDD version 3.1: a user-friendly computer program for microsatellite selection and primer design revisited: experimental validation of variables determining genotyping success rate. Mol. Ecol. Resour. **14**, 1302–1313.

Patel, R. K., and Jain, M. (2012) NGS QC Toolkit: a toolkit for quality control of next generation sequencing data. PLoS ONE **7**, e30619.

Peakall, P., and Smouse, P. (2012) GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research–an update. Bioinformatics **19**, 2537–2539.

Richards, J. (2003) *Primula*. Timber Press, Portland, USA.

Rousset, F. 2008. GENEPOP'007: a complete reimplementation of the GENEPOP

software for Windows and Linux. Mol. Ecol. Resour. **8**, 103–106.

Shao, J., Wang, H., Fang, S., Conti, E., Chen, Y., and Zhu, H. (2019) Intraspecific variation of self-incompatibility in the distylous plant *Primula merrilliana*. AoB Plants **11**, plz030.

van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M., and Shipley, P. (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes **4**, 535–538.

Wang, X., Barrett, S. C. H., Zhong, L., Wu, Z., Li, D., Wang, H., and Zhou, W. (2021) The genomic selfing syndrome accompanies the evolutionary breakdown of heterostyly. Mol. Biol. and Evol. **38**,168–180.

Yuan, S., Barrett, S. C. H., Duan, T., Qian, X., Shi, M., and Zhang, D. (2017) Ecological correlates and genetic consequences of evolutionary transitions from distyly to homostyly. Ann. Bot. **120**, 775–789.

Zeng, Z., Zhong, L., Sun, H., Wu, Z., Wang, X., Wang, H., Li, D., Barrett, S.C.H., and Zhou, W. (2024) Parallel evolution of morphological and genomic selfing syndromes accompany the breakdown of heterostyly. New Phytol. DOI: 10.1111/nph.19522.

Zhang, W., Hu, Y., He, X., Zhou, W., and Shao, J. (2021) Evolution of autonomous selfing in marginal habitats: spatiotemporal variation in the floral traits of the distylous *Primula wannanensis*. Front. Plant Sci. **12**, 781281.

Zhou, W., Barrett, S. C. H., Li, H., Wu, Z., Wang, X., Wang, H., and Li, D. (2017) Phylogeographic insights on the evolutionary breakdown of heterostyly. New Phytol. **214**, 1368–1380.

Zhong, L., Barrett, S. C. H., Wang, X., Wu, Z., Sun, H., Li, D., Wang, H., and Zhou, W. (2019) Phylogenomic analysis reveals multiple evolutionary origins of selfing from outcrossing in a lineage of heterostylous plants. New Phytol. **224**, 1290–1303.

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

Locus	Primer sequence (5'- 3')	Repeat motif	Fragment size (bp)	T_a (°C)	GenBase accession no.
PS647	F:GAGTAAATGCTGCTCCAGCTC R: CAAAACCCTCGTATGGTGCT	$(AAT)_{10}$	262	59.7	C_AA051754
PS216	F:AGCAACCTAACGGATCAACTG R: CCGACAACGTTAAGGTAGGG	$(AAT)_5$	197	59.5	C_AA051743
PS020	F: CATACATCAGCCATCGCTTC R: GCCTATGACGCCTTCTACCA	$(CGC)_6$	214	60.2	C_AA051738
PS620	F:CGGTGTCTTCTATACTTTACGCC R: AGGTATAGAAAAGGGCCAACA	$(TTA)_5$	286	57.6	C_AA051753
PS117	F: TGGGTTTCTGTTCTTGTTTCAG R: CCACCTCCACTACCACAACC	$(AGC)_8$	256	60.1	C_AA051740
PS375	F: CAAAGCCTAAATCAGCACCA R: GGGGTTTGTGGTATTGGATG	$(CT)_7$	116	58.8	C_AA051747
PS554	F: TGTTCGCCAAAGAACATAGC R: AATCAACTCAAAAGGGGGCT	$(AG)_6$	227	58.4	C_AA051750
PS013	F: CCTTAAACACGGAGCTCACA R: GGTCGGTGTCTATGCATGTTT	$(AC)_7$	150	59.8	C_AA051737
PS765	F: CTTTTCCTCAACATCAGAGGC R: CCTGACGTGTACAGAGGGGT	$(AT)_{10}$	126	60.0	C_AA051756
PS897	F: AGATGAAGGCTTTTGTGAGGA R: TTTCGCGAATTGCCTTAAAC	$(CT)_8$	234	59.8	C_AA051758
PS982	F: GACAACCCTAGGGAGGGAA R: TCCAAAATCGATAAGCGTCC	$(AG)_8$	284	60.4	C_AA051761
PS223	F: TTTTCCCTTACATGCAAATCC R: CCTCTTTCTTTCGGCCTCTC	$(AG)_{10}$	125	59.7	C_AA051744
PS413	F: AGGAGTGACGCCTCCTTTTA R: AATGAAGCGGTGGTGCTTAC	$(TA)_7$	229	60.5	C_AA051748
PS433	F: TGCGTTACATCAACGCTACA R: CGTTGGAGATACAAAACGACG	$(AG)_{10}$	145	57.0	C_AA051749
PS560	F:TGAAAGAGAAAGGACGGAAAA R: GCATTCCTCTTTTGGGTTCA	$(AG)_6$	269	59.4	C_AA051751
PS768	F:CTTTCAACTGACATGAAACTTCG R: GGTGGGTGAAAAAGAAATGC	$(TG)_{12}$	161	59.5	C_AA051757
PS282	F: TTCTTGCGAGTTTTTGTTGG R: ACTGGAATGCAATTGACCGT	$(TA)_6$	165	60.0	C_AA051746
PS684	F: TTGGGGACATGTGTCACTCT R: AATAGCCAACCCGTTCTCCT	$(AG)_8$	276	58.8	C_AA051755
PS082	F: TGATGCACGTGTGATTATGG R:CTCTACAACGAAAAACACTGGG	$(TA)_6$	199	60.3	C_AA051739
PS940	F: CCGTTGGATTAAATCAAGATGA R: TTGGAACAGATCCACAGGGT	$(TA)_6$	265	59.5	C_AA051759
PS957	F: CTACCGTACCCTTTTCCCAA R: TGCTGCAAGAAATCAAGCAT	$(TC)_6$	168	60.1	C_AA051760
PS247	F:GAATTAAGGTTTCATCTCCATTTAC R: GGAGGAGAGAGAAAGTGGGG	$(CT)_6$	266	59.6	C_AA051745
PS607	F: CGTTGTCGGGGAGTAATTTT R: CGGATCTCAAGAATCAGATGG	$(TG)_7$	314	59.9	C_AA051752
PS212	F: GCACCAATGACAATTTAACAAAA R: TATGTGTCGATGACCGGAAA	$(TA)_6$	132	60.1	C_AA051742
PS136	F: ACCAGAGGATGGACACTTCC R: TTGCTTCCATGATCCCTTTC	$(AT)_6$	260	58.4	C_AA051741

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

	Population												Total (n = 30)		Mean		
	CD (n = 10)				MK (n = 10)				XLS (n = 10)								
Locus	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}
PS647	4	1.000	0.715	-0.399	9	0.900	0.810	-0.111	5	1.000	0.735	-0.361	13	247–287	0.967	0.753	-0.290
PS216	5	0.800	0.630	-0.270	3^{ab}	0.200	0.340	0.412	4	0.600	0.510	-0.176	9	175–199	0.533	0.493	-0.011
PS020	3^{abc}	0.100	0.515	0.806	7^{b}	0.800	0.760	-0.053	4^{ac}	0.111	0.735	0.849	11	192–222	0.337	0.670	0.534
PS620	3^{a}	1.000	0.605	-0.653	2^{a}	1.000	0.500	-1.000	7	1.000	0.780	-0.282	9	264–292	1.000	0.628	-0.645
PS117	5^{b}	0.400	0.485	0.175	2^{a}	1.000	0.500	-1.000	1	0.000	0.000	0.000	7	200–252	0.467	0.328	-0.275
PS375	3	0.300	0.265	-0.132	3^{b}	0.300	0.265	-0.132	2	0.300	0.255	-0.176	5	106–124	0.300	0.262	-0.147
PS554	6^{b}	0.800	0.650	-0.231	5^{b}	1.000	0.715	-0.399	3^{b}	0.300	0.535	0.439	11	184–250	0.700	0.633	-0.064
PS013	3^{a}	1.000	0.545	-0.835	2^{b}	0.700	0.455	-0.538	3^{b}	0.600	0.535	-0.121	5	156–180	0.767	0.512	-0.498
PS765	4^{ab}	0.400	0.510	0.216	2^{b}	0.500	0.495	-0.010	1	0.000	0.000	0.000	6	142–160	0.300	0.335	0.069
PS897	8	0.700	0.750	0.067	2^{b}	0.200	0.480	0.583	4^{abc}	0.300	0.565	0.469	12	227–284	0.400	0.598	0.373
PS982	3^{b}	0.700	0.535	-0.308	4^{abc}	0.200	0.480	0.583	2^{a}	0.000	0.180	1.000	5	270–286	0.300	0.398	0.425
PS223	7	0.800	0.795	-0.006	9	1.000	0.850	-0.176	10	1.000	0.885	-0.130	15	141–171	0.933	0.843	-0.104
PS413	7^{b}	0.700	0.775	0.097	10^{a}	0.900	0.845	-0.065	9	0.800	0.840	0.048	16	204–246	0.800	0.820	0.027
PS433	2	0.100	0.095	-0.053	4^{b}	0.500	0.625	0.200	1	0.000	0.000	0.000	6	147–165	0.200	0.240	0.049
PS560	1	0.000	0.000	0.000	2^{b}	0.600	0.480	-0.250	1	0.000	0.000	0.000	2	264–267	0.200	0.160	-0.125
PS768	4	0.500	0.615	0.187	2^{a}	1.000	0.500	-1.000	3	0.900	0.580	-0.552	6	146–173	0.800	0.565	-0.455
PS282	7^{b}	0.800	0.790	-0.013	8	0.900	0.785	-0.146	10	0.900	0.845	-0.065	14	150–180	0.867	0.807	-0.075
PS684	7	0.700	0.765	0.085	5^{ac}	0.400	0.760	0.474	6	0.500	0.730	0.315	10	260–298	0.533	0.752	0.291
PS082	5^{a}	0.900	0.685	-0.314	4	0.600	0.565	-0.062	4	0.700	0.525	-0.333	8	187–220	0.733	0.592	-0.236
PS940	4^{b}	0.200	0.345	0.420	4	0.500	0.575	0.130	4	0.600	0.575	-0.043	5	227–294	0.433	0.498	0.169
PS957	7^{b}	1.000	0.760	-0.316	3^{b}	0.800	0.640	-0.250	4	1.000	0.715	-0.399	7	156–204	0.933	0.705	-0.322
PS247	6^{b}	0.700	0.600	-0.167	4	0.900	0.635	-0.417	3	0.600	0.620	0.032	7	262–286	0.733	0.618	-0.184
PS607	1	0.000	0.000	0.000	2	0.100	0.095	-0.053	3^{a}	0.200	0.340	0.412	4	309–321	0.100	0.145	0.180
PS212	5	0.300	0.420	0.286	3^{b}	0.700	0.565	-0.239	4	0.600	0.525	-0.143	7	139–160	0.533	0.503	-0.032
PS136	4	0.600	0.685	0.124	6^{b}	0.500	0.700	0.286	7^{b}	0.800	0.730	-0.096	9	264–294	0.633	0.705	0.105
Mean	4.56	0.580	0.541	-0.054	4.28	0.648	0.577	-0.129	4.20	0.512	0.510	0.033	8.36	–	0.580	0.543	-0.050

Note: Bold loci were successfully amplified in *Primula denticulate*; 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 ($p < 0.05$).

[b] Significant linkage disequilibrium ($p < 0.05$).

[c] Null allele present.

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

Species	Population code	Location (county, province, country)	Latitude (°N)	Longitude (°E)
P. secundiflora	CD	Changdu, Tibet, China	29.6918	98.1452
P. secundiflora	MK	Mangkang, Tibet, China	29.2430	98.6901
P. secundiflora	XLS	Changdu, Tibet, China	31.6985	94.9550
P. denticulata	LL	Nyingchi, Tibet, China	29.3373	97.0652