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PRIMER NOTE

Characterization of 30 microsatellite markers in distylous *Primula sinolisteri* (Primulaceae) using HiSeq sequencing

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PREMISE OF THE STUDY: Microsatellite markers were developed for *Primula sinolisteri*, a perennial distylous herb belonging to section *Obconicolisteri* (Primulaceae), to facilitate future investigations of the population genetics and mating patterns of populations in this species.

METHODS AND RESULTS: We developed 30 microsatellite markers for *P. sinolisteri* using HiSeq X-Ten sequencing and measured 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 one to 19 (mean = 4.42). The observed and expected heterozygosity ranged from 0–1.000 and 0.083–0.882, respectively. Twenty-nine of the loci were also successfully amplified in homostylous *P. sinolisteri* var. *aspera*.

CONCLUSIONS: The microsatellite markers we have identified in *P. sinolisteri* provide powerful tools for investigating patterns of population genetic diversity and the evolutionary relationships between heterostyly and homostyly in this species.

KEY WORDS distyly; HiSeq; homostyly; microsatellites; *Primula sinolisteri*; Primulaceae.

Primula sinolisteri Balf. f. var. *sinolisteri* (Primulaceae) is an animal-pollinated, perennial, herbaceous species belonging to *Primula* L. sect. *Obconicolisteri*. It is restricted to northwestern Yunnan, from the Dali range to the Tibetan border (Hu and Kelso, 1996; Richards, 2003), and commonly occurs in dry rocky pastures at elevations between 2300 and 3000 m. *Primula sinolisteri* var. *sinolisteri* exhibits distyly, with populations comprising long-styled and short-styled floral morphs. However, *P. sinolisteri* var. *aspera* W. W. Sm. & H. R. Fletcher possesses a different floral phenotype in which stigmas and anthers occur at a similar height within flowers, a condition known as homostyly. Distyly and homostyly are widely reported in *Primula* (Richards, 2003). Phylogenetic reconstructions clearly indicate that the most recent common ancestor of *Primula* was distylous, and that homostyly has evolved repeatedly in the genus as a result of the evolutionary breakdown of heterostyly and transitions from outcrossing to selfing (Mast et al., 2006; Zhou et al., 2017).

Recent investigations of the molecular genetic architecture of the heterostyly linkage group in *Primula* provide an opportunity for comparative genetic analysis of the evolutionary events associated with the origin and breakdown of heterostyly (Huu et al., 2016; Li et al., 2016; Burrows and McCubbin, 2017). Therefore, because both intraspecific and interspecific variation in floral conditions occur in *P. sinolisteri* var. *sinolisteri* and closely related taxa within section *Obconicolisteri*, this group provides an outstanding opportunity for investigating the evolutionary relationships between distyly and homostyly and the ecological causes and population genetic consequences of mating system transitions.

Next-generation sequencing technology is now widely used in many areas of evolutionary biology, including the development of microsatellite markers for population genetic studies. Highly polymorphic microsatellite markers are useful tools for measuring the genetic diversity and structure of plant populations as well as patterns of mating (e.g., Matheny et al., 2013; Zhou et al., 2015, 2017; Yuan et al., 2017). Here, we used next-generation sequencing to develop a set of variable microsatellite markers in *P. sinolisteri* var. *sinolisteri*.

METHODS AND RESULTS

We isolated total genomic DNA from leaf tissue of one *P. sinolisteri* var. *sinolisteri* individual from the population SIN_QBX

TABLE 1. Characteristics of 30 microsatellite loci isolated from *Primula sinolisteri* var. *sinolisteri*.

Locus	Primer sequences (5′–3′)	Repeat motif	Fragment size range (bp)	T_a (°C)	GenBank accession no.
PROB15	F: ATTGCCAGACAGAAAAAGGC R: CACAGTAAATTCATCACAGCAACA	$(AT)_7$	295–307	53.9	MH180228
PROB29	F: GCTTCCCAATCAAAACAATACC R: GACTCGTCGGATTTGTCCAT	$(AC)_{11}$	151–209	54.7	MH180229
PROB46	F: AGGCCATTACCCCCATAAAC R: TGGGCAAAGGAAGAAGAAGA	$(CT)_{11}$	152–166	54.4	MH180230
PROB48	F: CATTTGAATTTTGGACGCCT R: TACGGGTGAATTCGTCATTG	$(AC)_7$	256–304	52.3	MH180231
PROB54	F: CGACCAGGATTGATGTTGTG R: TGGTTCCGGAAACTACCATC	$(TG)_7$	146–156	55.4	MH180232
PROB55	F: TGTCTATCGTGGTGGGTTCA R: ATTCCGGGGTTTAATATCGG	$(GA)_{11}$	105–121	54.4	MH180233
PROB63	F: CCGCACCCAATCATATATCC R: GCTCAAAGATCTGCGAAACC	$(AG)_{15}$	140–154	55.4	MH180234
PROB70	F: TGAGGAAATTGATGGTGCAA R: GAAAGGTCAAGTGGAGCAGC	$(AG)_{14}$	111–125	54.4	MH180235
PROB72	F: TTTGGCCTTGCTTATTCACC R: AAATTTAGGGTGGTGGGGG	$(AG)_{12}$	168–190	54.3	MH180236
PROB73	F: ACCGATTTGACCTCTCATGC R: CATGCCTCTGTCATCCATTG	$(TC)_6$	95–111	55.4	MH180237
PROB83	F: TGCCAATTGCCATCCTTAAT R: TAAGTGGCAATGGTGGTGAA	$(TG)_8$	189–207	52.3	MH180238
PROB100	F: GCTTTTGTTGTTCCAGCCAT R: AGCCCAGCAGTTCTGGAGTA	$(TCT)_7$	106–176	55.4	MH180239
PB01	F: TCGTCATCATCCATTCACAA R: GATGAGATTGGGTTTGTGGC	$(AG)_{11}$	144–172	50.9	MH180240
PB02	F: AGCATGCTGAAGTAAGGCTTC R: GGATCGGTTTGAATGGAATG	$(AT)_6$	202–254	52.0	MH180241
PB18	F: GGGGAAATTGAGGACACAAA R: TGGATCGGTATCAGCATTGA	$(AG)_{10}$	228–238	50.9	MH180242
PB31	F: GCCATAAAGCAGGGTCCATA R: CTGTCGCTTGAGTAGCCGGT	$(CT)_{10}$	152–174	55.0	MH180243
PB34	F: TTTTTCTCCTGTGTGGGGAC R: AATCGTGCATTCGTTCCTTC	$(GT)_{14}$	193–205	51.9	MH180244
PB35	F: TCACCCTCTCAACAAAACCC R: GCTTTGATAAGCGGCATCAT	$(GT)_{10}$	182–208	51.9	MH180245
PB49	F: AAAGGGGAATGGATTGAACC R: ACCAGTGTTGGCGTTAGCTT	$(TC)_7$	167–205	51.9	MH180246
PB51	F: GAACTTCAAGGTGAGCTGCC R: GGTGGTGTTGGGTTCGTATC	$(GA)_7$	225–251	54.9	MH180247
PB56	F: GCACGAACGAGGAGTAGGAG R: AAAGCAACCAACTCCCCTCT	$(GA)_{10}$	234–262	55.0	MH180248
PB59	F: GGCCCCATGACAAACATATC R: GGTGAGAACCGTACTCCGAA	$(TA)_8$	224–248	53.9	MH180249
PB60	F: ATGTTTGGGAACCCATTGAA R: TCATTGAGACATGGCGAGTT	$(CA)_{15}$	221–233	49.9	MH180250
PB61	F: GAGACACCTGCTCACAACGA R: TCTTGCAGGCAGCTACAGAA	$(CT)_{15}$	211–221	53.9	MH180251
PB64	F: TATTGGATCGGAGTTGGAGC R: AGGCTTAAAAGATGCAGCCA	$(AC)_8$	151–171	51.9	MH180252
PB66	F: GAAAAGCAAAATGGAACGGA R: GCTGCCTTTCAGGTGTGTTT	$(GA)_7$	162–174	50.9	MH180253
PB72	F: CAAAGTCGATGACCGGAACT R: CCAGATCCCACGGTTAGTGT	$(TC)_8$	201–209	53.9	MH180254
PB84	F: CACTTTGGTGGGCTATGGAA R: AGCCAAGATTTGTGCAATCC	$(CT)_{14}$	141–171	51.9	MH180255
PB85	F: GGGCCAAAGCGAATAGACAT R: ATATACGCCGGTCTCCCTTT	$(TG)_{17}$	164–174	52.9	MH180256
PB95	F: TGGAGGTGAAACTGGAGGAG R: TTTGTTAATGAGAGCGCGTG	$(GTG)_6$	129–135	52.9	MH180257

Note: T_a = annealing temperature.

(Appendix 1) following a modified version of the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). We prepared a library using a NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA). We performed sequencing on a HiSeq X-Ten sequencer (Illumina, San Diego, California, USA) using 2 × 150 bp read length. Raw reads were obtained and deposited in the National Center for Biotechnology Information Sequence Read Archive (BioProject ID PRJNA485859; accession no. SRP157868). Using Geneious version 6.0 (Biomatters, Auckland, New Zealand), the resulting 10,687,169 raw reads were quality filtered by trimming adapter sequences and by removing reads with quality scores <10. Using the built-in Geneious assembler, the cleaned reads were then assembled into 55,878 contigs with high sensitivity/medium for the sensitivity setting. Plastome contigs were identified using BLASTX against GenBank and were excluded.

We used the MIcroSAtellite identification tool (MISA; Thiel et al., 2003) to identify unique reads containing microsatellites based on the following criteria: more than five repeats for dinucleotides to hexanucleotides and 100 bp for the maximal number of bases between two adjacent microsatellites. Minimum product size was set to 100 bp. A total of 3264 contigs contained at least one microsatellite. Two hundred simple sequence repeat (SSR) loci with di- or trinucleotide repeats were randomly selected for further characterization. Primers were designed for these loci using PRIMER version 5.0 (Clarke and Gorley, 2001) using the automatic search model to detect paired PCR primers of 24 bp in length. We used a Veriti 96-well Thermal Cycler Gradient PCR Machine (Applied Biosystems, Foster City, California, USA) to test and optimize these primers initially.

Preliminary amplification tests were carried out with four individuals of *P. sinolisteri* var. *sinolisteri* from the SIN_QBX population (Appendix 1). 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^{-1} $MgCl_2$, 100 mmol·L^{-1} KCl, 0.5 mmol·L^{-1} of each dNTP, 20 mmol·L^{-1} Tris-HCl [pH 8.3], and 0.1 units *Taq* polymerase), 0.6 μmol·L^{-1} of each primer, 8.4 μL of deionized water, and 30–50 ng of genomic DNA. We conducted PCR amplification under the following conditions: 95°C for 3 min followed by 30 to 35 cycles at 95°C for 30 s, at the annealing temperature for each specific primer (optimized for each locus; Table 1) for 30 s, 72°C for 30 s for extension, and a

TABLE 2. Population genetic parameters in three populations of *Primula sinolisteri* var. *sinolisteri* and amplification tests in *P. sinolisteri* var. *aspera*.[a]

	Primula sinolisteri var. *sinolisteri*												*P. sinolisteri* var. *aspera*
	QBX (*n* = 12)				JZ (*n* = 12)				MRS (*n* = 12)				TCA (*n* = 6)
Locus	*A*	H_o	H_e	F_{IS}	*A*	H_o	H_e	F_{IS}	*A*	H_o	H_e	F_{IS}	Total *A*	Size range (bp)
PROB15	3	0.250	0.351[c]	0.287	2	0.545	0.496	−0.100	3	0.250	0.601[c]	0.584	5	305
PROB29	7	0.900	0.790	−0.139	7	0.818	0.661	−0.238	11	0.917	0.882	−0.040	19	203
PROB46	3	0.583	0.469	−0.244	5	0.833	0.712[c]	−0.171	5[b]	0.417	0.601[c]	0.306	6	154
PROB48	3	0.667	0.497	−0.343	1	NA	NA	NA	1	NA	NA	NA	3	256
PROB54	3	0.500	0.403	−0.241	2	0.545	0.397	−0.375	3	0.500	0.663[c]	0.246	4	154–158
PROB55	7[b]	0.500	0.799[c]	0.374	5	0.750	0.781[c]	0.040	7	0.750	0.851	0.118	9	105–107
PROB63	4	0.545	0.550[c]	0.008	5	0.750	0.726	−0.033	5[b]	0.333	0.590[c]	0.435	7	168
PROB70	7[b]	0.500	0.802[c]	0.377	6	0.833	0.767	−0.086	6[b]	0.500	0.788	0.366	8	117
PROB72	8	0.909	0.826	−0.100	6[b]	0.417	0.715	0.417	5	0.667	0.750	0.111	9	186
PROB73	3	0.455	0.483	0.060	3	0.333	0.538[c]	0.381	3	0.333	0.611[c]	0.455	5	97–101
PROB83	6	0.667	0.740	0.099	5	0.833	0.736[c]	−0.132	6[b]	0.455	0.727	0.375	8	205
PROB100	3	0.583	0.594	0.019	1	NA	NA	NA	7	0.917	0.813	−0.128	9	110–112
PB01	6[b]	0.455	0.769	0.408	4[b]	0.417	0.719[c]	0.420	4	0.500	0.705	0.291	10	146
PB02	5[b]	0.167	0.632[c]	0.736	6	0.917	0.767[c]	−0.196	5	0.667	0.681	0.020	8	204
PB18	5	0.750	0.712	−0.054	4	0.750	0.656	−0.143	1	NA	NA	NA	6	232
PB31	b	0.750	0.833[c]	0.100	7	1.000	0.844	−0.185	7	0.667	0.819	0.186	9	162
PB34	1	NA	NA	NA	6	0.833	0.781[c]	−0.067	1	NA	NA	NA	6	—
PB35	7	0.917	0.795	−0.153	4[b]	0.083	0.358[c]	0.768	5	0.750	0.701	−0.069	10	196–198
PB49	6	0.750	0.767	0.022	5	1.000	0.722[c]	−0.385	5	0.583	0.642[c]	0.092	9	171–173
PB51	2	0.000	0.153[c]	1.000	2	0.080	0.083	0.036	2	0.250	0.330	0.242	3	227
PB56	4	0.417	0.413	−0.010	4	0.583	0.660	0.117	8[b]	0.417	0.674[c]	0.381	8	238
PB59	3[b]	0.250	0.455[c]	0.451	3	0.750	0.594	−0.263	3	0.167	0.156	−0.067	4	264–266
PB60	5	0.750	0.681	−0.101	4	0.667	0.694[c]	0.039	2	0.091	0.087	−0.048	7	223
PB61	4[b]	0.083	0.615[c]	0.865	1	NA	NA	NA	5[b]	0.364	0.702	0.482	5	235
PB64	6	0.583	0.771[c]	0.244	4	0.250	0.295[c]	0.153	6	0.750	0.792	0.053	9	151–153
PB66	3	0.333	0.288	−0.156	4	0.333	0.545	0.389	4[b]	0.167	0.573	0.709	6	168
PB72	5	0.917	0.788	−0.164	4	0.636	0.680	0.065	1	NA	NA	NA	5	205
PB84	5	0.667	0.767	0.130	3	0.083	0.226[c]	0.633	6[b]	0.500	0.743	0.327	6	161–163
PB85	5	0.583	0.757	0.239	2	0.512	0.417	−0.228	4	0.417	0.663[c]	0.372	6	172
PB95	2	0.750	0.469[c]	−0.599	2[b]	0.000	0.500[c]	1.000	2	0.333	0.278	−0.200	3	129–132
Mean	4.633	0.558	0.620	0.107	3.900	0.576	0.595	0.069	4.433	0.416	0.547	0.218	7.067	—

Note: — = unsuccessful PCR amplification; *A* = number of alleles per locus; F_{IS} = inbreeding coefficient; H_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals; NA = not applicable.

[a]Voucher and locality information are provided in Appendix 1.

[b]Significant frequency of null alleles ($P < 0.05$).

[c]Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

final extension step at 72°C for 5 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. Out of the 200 primer pairs that we tested, 30 microsatellite loci amplified successfully with suitable fragment lengths and showed polymorphism (Table 1).

For these 30 successful loci, we measured polymorphism in 36 individuals obtained from three natural populations of distylous *P. sinolisteri* var. *sinolisteri* and six individuals from one population of homostylous *P. sinolisteri* var. *aspera* (Appendix 1). We calculated basic population genetic parameters of diversity, including the number of alleles and observed and unbiased expected heterozygosity, using GenAlEx version 6.5 (Peakall and Smouse, 2012). We tested for deviations from Hardy–Weinberg equilibrium at each locus using GENEPOP version 4.0.7 (Rousset, 2008). Null alleles were detected by MICRO-CHECKER (van Oosterhout et al., 2004).

The number of alleles per locus ranged from one to 19, with a mean (±SD) = 4.42 ± 1.977 (Table 2). Among polymorphic loci, the observed heterozygosity and expected heterozygosity ranged from 0–1.000 (mean ± SD = 0.564 ± 0.260) and 0.083–0.882 (0.626 ± 0.180), respectively. The inbreeding coefficient ranged from −0.599 to 1.000. Some loci deviated significantly from Hardy–Weinberg equilibrium in each population (Table 2), as a result of heterozygote deficiency. This can likely be attributed to the presence of null alleles as detected by MICRO-CHECKER (Table 2). Among the 30 SSR markers, 29 loci were successfully amplified in *P. sinolisteri* var. *aspera* (Table 2).

CONCLUSIONS

The microsatellite markers that we have isolated in *P. sinolisteri* var. *sinolisteri* will provide a valuable resource for investigating mating systems, population genetic structure, and phylogeography in *P. sinolisteri* and its varieties. It will be of particular interest to investigate the evolutionary relationships between distylous and homostylous populations and determine the number of transitions from outcrossing to selfing and their genetic consequences. The high discriminatory power of the microsatellite markers that we have identified will also be useful for parentage analysis and measures of disassortative mating in populations and should provide opportunities to evaluate the potential influence of ecological, demographic, and reproductive factors on mating patterns.

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DATA ACCESSIBILITY

Raw reads were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (BioProject ID PRJNA485859; accession no. SRP157868). Sequence information for the developed primers has been deposited to NCBI; GenBank accession numbers are provided in Table 1.

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APPENDIX 1. Locality and voucher information for populations of *Primula sinolisteri* var. *sinolisteri* and *P. sinolisteri* var. *aspera* used in this study. Voucher specimens have been deposited at the herbarium of the Kunming Institute of Botany (KUN), Kunming, Yunnan, China.

Species	Population code	Floral morph structure	Voucher no.	Location	Geographic coordinates	Elevation (m)	*n*
Primula sinolisteri Balf. f. var. *sinolisteri*	SIN_QBX	Distyly	Z. Wei 123	Dali, China	25°64.386′N, 100°13.554′E	2743	12
Primula sinolisteri var. *sinolisteri*	SIN_JZ	Distyly	Z. Wei 112	Jinzhan, China	25°80.183′N, 99°99.014′E	2736	12
Primula sinolisteri var. *sinolisteri*	SIN_MRS	Distyly	Z. Wei 118	Heqing, China	25°25.829′N, 100°128.13′E	2934	12
Primula sinolisteri var. *aspera* W. W. Sm. & H. R. Fletcher	ASP_TCA	Homostyly	Z. Wei 203	Gaoligong Mountain, China	25°17.27′N, 98°43.50′E	2963	6

Note: n = number of individuals sampled.