Genes Genet. Syst. (2020) **95**, p. 1–5

Characterization of 30 microsatellite markers for distylous *Primula denticulata* (Primulaceae) using HiSeq sequencing

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(Received 20 January 2020, accepted 8 September 2020; J-STAGE Advance published date: 16 December 2020)

Primula denticulata exhibits considerable variation in floral morphology and flowering phenology along elevational gradients in SW China. We isolated 30 microsatellite markers from *P. denticulata* to facilitate further investigation of population genetics and floral evolution in this species. We used the HiSeq X-Ten sequencing system to develop a set of markers, and measured polymorphism and genetic diversity in a sample of 72 individuals from three natural populations of *P. denticulata* subsp. *denticulata*. The markers displayed relatively high polymorphism, with the number of alleles ranging from two to seven (mean = 3.567). The observed and expected heterozygosity ranged from 0 to 1.000 and 0.041 to 0.702, respectively. Twenty-eight of the loci were also successfully amplified in *P. denticulata* subsp. *sinodenticulata*. The microsatellite markers we have identified will provide valuable tools for investigations of the population genetic structure, mating systems and phylogeography of the *P. denticulata* complex, and will help to address questions concerning the ecological and genetic mechanisms responsible for the evolution of reproductive traits in the species.

Key words: distyly, HiSeq, microsatellites, *Primula denticulata*, Primulaceae

Primula denticulata Smith (Primulaceae) is an insect-pollinated, perennial herb belonging to *Primula* section *Denticulata*. The species is mainly restricted to China and is distributed from the southern margin of the Tibetan plateau to the Hengduan mountain region, commonly occurring in moist meadows, on open slopes, along roadsides and among shrubs (Hu and Kelso, 1996; Richards, 2003). Populations are typically distylous, comprising long-styled and short-styled floral morphs (Hu and Kelso, 1996). *Primula denticulata* is composed of two subspecies: subsp. *denticulata* is restricted to the Tibetan plateau at elevations between 2,800 and 4,600 m and subsp. *sinodenticulata* occurs in Yunnan, Sichuan and north-west Guizhou at lower elevations between 1,500 and 3,000 m. *Primula denticulata* subsp. *sinodenticulata* flowers from February to April whereas *P. denticulata* subsp. *denticulata* flowers from the middle of April to July. The flowering time of populations varies clinally along elevational gradients, along with a range of other reproductive characters including scape height, flower number and size, and herkogamy (stigma–anther separation). It is not yet known whether clinal variation is adaptive and maintained by natural selection, and evaluating this hypothesis is a major goal of our ongoing research on the ecological genetics of the species.

Distylous *Primula* species are well-known model systems for investigations of the pollination biology and mating systems of populations (Richards, 2003; de Vos et al., 2014, 2018), including several Chinese species (Yuan et al., 2017; Zhou et al., 2017; Zhong et al., 2019). In addition, *Primula* species have been the recent focus of detailed studies of the molecular genetic architecture of

Edited by Kenichi Nonomura
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DOI: http://doi.org/10.1266/ggs.20-00003

the distylous linkage group (Nowak et al., 2015; Huu et al., 2016, 2020; Li et al., 2016). The occurrence of conspicuous population differentiation of ecologically relevant traits along elevational gradients in *P. denticulata* provides opportunities for investigating a range of questions associated with the mechanisms that may be responsible for clinal variation in the species. The development of genetic markers to enable studies of the patterns of genetic diversity and the phylogeographic relationships of populations across elevational gradients is a necessary first step for any future microevolutionary studies of the *P. denticulata* complex.

Of the different types of PCR-based molecular markers, microsatellites [or simple sequence repeats (SSRs)] have been used most extensively in molecular population genetics because they can be readily amplified by PCR and, importantly, because of the large amount of allelic variation that is usually evident at each locus (Merritt et al., 2015). These markers are abundant, distributed throughout the genome, and are highly polymorphic compared with other genetic markers, as well as being species-specific and co-dominant (Vieira et al., 2016). For these reasons, they are widely used as genetic markers in population genetic studies in most non-model species. Although direct genotyping-by-sequencing methods, such as restriction site-associated DNA sequencing (Baird et al., 2008), are useful for addressing certain types of questions in evolution and ecology, SSR markers are still an efficient, less labor-intensive and relatively cheap approach for isolating genetic markers in non-model species for studies of population genetic structure and mating systems (e.g., Hodgins and Barrett, 2007, 2008; Zhou et al., 2012, 2015; Yakimowski and Barrett, 2014). Here, we use next-generation sequencing to develop a set of variable microsatellite markers in *P. denticulata* for our further molecular population genetic analysis of the complex.

We chose a single individual of *P. denticulata* subsp. *denticulata* from population LX to design markers (see Supplementary Table S1), and isolated total genomic DNA from the leaf tissue using a modification of the CTAB (cetyltrimethylammonium bromide) protocol (Doyle and Doyle, 1987). Library preparation was carried out using using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). We performed paired-end sequencing on a HiSeq X-Ten sequencer (Illumina, San Diego, CA, USA) with 2 × 150 bp read length. We obtained approximately 2 Gb of sequence data and deposited it in the National Center for Biotechnology Information Sequence Read Archive. The BioProject ID is PRJNA588183, and the accession number is SRR10419389. We performed a preliminary quality check of 3,122,591 raw reads with FastQC version 0.11.2. The clean reads were filtered from raw reads after trimming adapter sequences and removing both ambiguous ('N' > 10%) and low-quality reads (Phred score < 30) to ensure overall quality. We used the built-in Geneious assembler tool version 6.0 (Biomatters, Auckland, New Zealand) to assemble the cleaned reads into 16,109 contigs with high sensitivity/medium for the sensitivity setting. We identified and excluded plastome contigs using BLASTX against GenBank.

We used the MIcroSAtellite identification tool (Thiel et al., 2003) to identify unique reads containing microsatellites, and these satisfied the following criteria: more than five repeats for dinucleotides to hexanucleotides, and 100 bp for the maximal number of bases between two contiguous microsatellites. Minimal product size was set to 100 bp. In total, we isolated 2,939 contigs containing at least one microsatellite site. We randomly selected 150 SSR loci with di- or trinucleotide repeats for further characterization. We designed primers for these loci using PRIMER version 5.0 (Clarke and Gorley, 2001), in which we used the automatic search model to detect paired PCR primers. We used a Veriti 96-well Thermal Cycler gradient PCR machine (Applied Biosystems, Foster City, CA, USA) to initially test and optimize these primers.

We carried out the preliminary amplification tests with five individuals of *P. denticulata* subsp. *denticulata* from the LX 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 mM $MgCl_2$, 100 mM KCl, 0.5 mM of each dNTP, 20 mM Tris-HCl, pH 8.3 and 0.1 units *Taq* polymerase), 0.5 µl of each primer, 8.5 µl of deionized water and 30–40 ng of genome DNA. The PCR amplification was implemented under the following conditions: 95 °C for 3 min; 30 cycles at 95 °C for 30 s, at the annealing temperature (optimized for each locus in Table 1) for each specific primer for 30 s, and 72 °C for 30 s for extension; and a final extension step at 72 °C for 10 min. We separated PCR products on 8% polyacrylamide denaturing gels using a 100 bp ladder molecular size standard (Thermo Fisher Scientific; Fermentas, Shenzhen, China) and visualized them by silver staining. Out of the 150 primer pairs that we tested, 30 microsatellite loci amplified successfully with suitable fragment lengths and all exhibited polymorphism (Table 1). As evident from earlier studies of congeneric species of *Primula* (four loci in Yuan et al., 2017; 10 loci in Zhou et al., 2017; 15 loci in Zhong et al., 2019), 30 microsatellite loci with high polymorphism is sufficient for addressing most questions on the population genetic structure and mating systems of *Primula* populations.

We measured polymorphism for the above-mentioned 30 loci in 72 individuals sampled from three natural populations of *P. denticulata* subsp. *denticulata* located in Tibet and five individuals from one population of *P. denticulata* subsp. *sinodenticulata* from Yunnan (Supplementary Table S1). We calculated basic population

Table 1. Characteristics of 30 microsatellite loci isolated from *Primula denticulata* subsp. *denticulata*

Locus	Primer sequences (5′–3′)	Repeat motif	Fragment size range (bp)	*Ta* (°C)	DDBJ/EMBL/GenBank accession no.
QH006	F: GGGGATATAGGGCCTCAGTC R: TCGATGGAGAGAACTGACCTG	$(TC)_6$	110–140	62	MT415220
QH007	F: TGCTGCTAGGCTGATTTTGA R: CTACTGCGCATGTCCACACT	$(AG)_7$	95–153	55	MN694829
QH009	F: CCTGCTTGTGTTGCTTGTGT R: TTGCCGTTCAAATTGTGTGT	$(CA)_8$	125–135	53.9	MT415221
QH011	F: CGGAATCCTCTACCACCTCA R: CAAGGGTCCTATGGTGCAGT	$(ATAA)_5$	130–140	55	MN694830
QH016	F: CCAGCACTATGCCAAGAACA R: ATGCATTTTCACCTGTCCAA	$(AT)_6$	140–150	54.9	MT415222
QH018	F: AACTCTTGCTCTGCAGCCTC R: TGGTCTTTACTTTCCCCCAA	$(TA)_9$	160–175	54.9	MN694831
QH020	F: CGTTCGGTAGAAACGGTGAT R: ACCTCTATCAATTGCAGCCG	$(GTG)_5$	145–180	56.9	MN694832
QH022	F: ATCCCCTATCTCCATTGGCT R: GCAGGGAGGAGAAGGAGACT	$(TCA)_5$	120–170	57	MN694833
QH025	F: GAAGTATAAATTGGGGGTTTTGA R: AATCCCAGTCCTCCAACAAA	$(TG)_{10}$	160–180	54.4	MN694834
QH028	F: GGCGTTTACGTTTATGCGAT R: GTACACGTGTCACATCCCT	$(GA)_6$	133–152	59	MN694835
QH031	F: GTTGTGCCGAAGGAGATCAT R: AAGCTGCAGCAAACCAAACT	$(TTCT)_5$	147–182	55.9	MN694836
QH034	F: TCGTGATGACGACGAAAAAG R: AGAGAGCGTTGTGAGGTGGT	$(TC)_8$	130–150	55.9	MT458490
QH038	F: TCCCAGGAAAATCACCTGAA R: GGGACATGTCGATCTTTTCG	$(ATT)_9$	163–200	55.9	MN694837
QH037	F: TGCAACTCTCACCATGAAGC R: AGCAAGTGAAGTTGGCAGGT	$(AG)_6$	170–200	55.9	MT415223
QH040	F: GCGACAAATCAGCAACTCAA R: TGCGCCTCAGTGTATCTCAG	$(TG)_7$	175–200	55.9	MN694838
QH043	F: TAACTTTCCCATTTTCCCCC R: TCGTCGTAGCGAAGAGGAAT	$(TC)_7$	190–210	55.9	MT415224
QH045	F: TATTTGTCGCAAGTGGTTGG R: CCGAATCTTGTGAGAAAGGG	$(AG)_6$	180–200	53.9	MT415225
QH050	F: TGCTCGGTAGTCTGATCGTG R: GAGCCAGCTCATCATCAACA	$(TTG)_5$	180–225	54	MT415226
QH056	F: AGGGAAAGTTTTCACTGGCA R: CCTTTGCATTTTTCATGGCT	$(AG)_8$	205–208	56	MN694839
QH062	F: CCTCCTTTTTGCACTACCCA R: GGGCAATTTGGTCATTTCAG	$(GA)_6$	190–210	54.9	MT415227
QH078	F: CCACCAACACCTCACTCAGA R: CGTGAACCTCCAGTGTCCTT	$(TC)_7$	200–240	58.9	MN694840
QH079	F: AACTGCAAGCATTGCAACAG R: AACCCTTGTGGAGTTGCCTA	$(TC)_6$	200–215	55.9	MT415228
QH091	F: CAAGATGACACGTGGGATTG R: AGCTCAGGGAATCTTCCTCC	$(AG)_{10}$	200–220	55.9	MT458496
QH092	F: TGGACGGATGAGATGATGAA R: GCCATCTTAACAGCTGCCTC	$(AG)_6$	210–235	55.9	MT415229
QH101	F: TCCTTCCCACTCATCCAGTC R: GCTTGCACTCTTTCCACCTC	$(AG)_6$	190–217	54.9	MN694841
QH106	F: GGGTAAGTCTTAGCAGGGGG R: GCTGATCCACCACCACCTAT	$(ATT)_{10}$	190–220	56	MT415231
QH110	F: GCTCGACACCTAAGCCTCTG R: AGGCTTGCTAGGCTGTGAAC	$(AT)_6$	220–240	54.9	MT415232
QH111	F: CTCCTTCACCCACTCTTCCA R: AGGAAGACCCACCCAGTTTT	$(CA)_6$	220–225	54.9	MT415233
QH130	F: GCCTGAATGGAGATCGAGAG R: ACGACGTCACTGGTGTTTTC	$(GA)_6$	220–260	61	MN694842
QH146	F: TCGACGATCCATTCGTATCA R: AAATAAGCCACGGTCACAGG	$(AGA)_5$	240–260	55	MN694843

Note: *Ta* = annealing temperature.

genetic parameters of diversity, including the number of alleles and observed/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), and detected null alleles using MICRO-CHECKER (van Oosterhout et al., 2004).

The number of alleles of each locus ranged from 2 to 7, with the mean ± SD = 3.567 ± 1.278 (Table 2). Among all the polymorphic loci, the observed and expected heterozygosity ranged from 0 to 1.000 (0.394 ± 0.267) and 0.041 to 0.702 (0.436 ± 0.165), respectively. The inbreeding coefficients ranged from −1.000 to 1.000. The locus deviation from Hardy–Weinberg equilibrium in each population is indicated in Table 2. Among the 30 SSR markers, 28 loci were successfully amplified in a population of *P. denticulata* subsp. *sinodenticulata* sampled from Yunnan (Table 2).

Our results for *P. denticulata* are consistent with the distylous and outbreeding mating system of populations,

Table 2. Population genetic parameters in three populations of *P. denticulata* subsp. *denticulata* and amplification tests in *P. denticulata* subsp. *sinodenticulata*[a]

	Primula denticulata subsp. *denticulata*													*Primula denticulata* subsp. *sinodenticulata*
	LX (N = 24)				LL (N = 24)				CY (N = 24)					DH (N = 5)
Locus	A	H_O	H_E	F_{IS}	A	H_O	H_E	F_{IS}	A	H_O	H_E	F_{IS}	Total A	○ or ×[d]
QH006	3	0.542	0.468	−0.158	3[b]	0.565	0.644[c]	0.122	2	0.273	0.236	−0.158	3	○
QH007	3[b]	0.375	0.633	0.407	5	0.591	0.655[c]	0.098	2	0.125	0.499	0.750	7	○
QH009	4	0.316	0.580[c]	0.456	5	0.667	0.683	0.024	2	0.632	0.511	−0.236	5	○
QH011	2	0.458	0.353[c]	−0.297	2[b]	0.125	0.395	0.684	2	0.696	0.454	−0.533	2	×
QH016	2	0.792	0.531[c]	−0.490	3	0.625	0.478	−0.307	3	0.583	0.452	−0.290	3	○
QH018	2	0.958	0.499	−0.920	2	1.000	0.500	−1.000	3	0.435	0.405	−0.075	3	×
QH020	2	0.750	0.469	−0.600	2	0.708	0.457	−0.548	2	0.708	0.457	−0.548	4	○
QH022	2[b]	0.167	0.278[c]	0.400	2	0.583	0.469[c]	−0.244	3	0.292	0.379	0.231	5	○
QH025	3	0.667	0.497[c]	−0.343	3	0.773	0.584	−0.324	2	0.652	0.485[c]	−0.345	3	○
QH028	2[b]	0.500	0.444[c]	−0.125	2	0.208	0.187[c]	−0.116	2	0.042	0.041	−0.021	5	○
QH031	2	0.125	0.187[c]	0.330	3	0.571	0.563[c]	−0.014	1	NA	NA	NA	5	○
QH034	2	0.042	0.041	−0.021	1	NA	NA	NA	2	0.167	0.153	−0.091	2	○
QH037	2	0.375	0.305	−0.231	3	0.200	0.524[c]	0.619	2	0.375	0.305	−0.231	3	○
QH038	5[b]	0.667	0.702[c]	0.051	3	0.167	0.190[c]	0.123	2	0.095	0.091[c]	−0.050	6	○
QH040	3[b]	0.167	0.403	0.586	4[b]	0.348	0.590	0.410	2	0.250	0.330[c]	0.242	5	○
QH043	3	0.542	0.588	0.078	3	0.917	0.582[c]	−0.574	3	0.083	0.484[c]	0.828	3	○
QH045	2	0.077	0.204	0.623	2	0.600	0.500	−0.200	3	0.467	0.611	0.236	3	○
QH050	4[b]	0.292	0.689[c]	0.577	4[b]	0.375	0.695[c]	0.461	3[b]	0.000	0.625[c]	1.000	4	○
QH056	2[b]	0.083	0.080[c]	−0.043	2[b]	0.087	0.227	0.617	1	NA	NA	NA	4	○
QH062	3[b]	0.167	0.614[c]	0.728	2	0.353	0.415	0.150	2	0.357	0.436	0.181	3	○
QH078	2	0.478	0.364[c]	−0.314	2[b]	0.048	0.427	0.889	2	0.556	0.401[c]	−0.385	5	○
QH079	3[b]	0.375	0.629[c]	0.404	3[b]	0.292	0.586[c]	0.502	2	0.792	0.492[c]	−0.608	3	○
QH091	1	NA	NA	NA	2	0.087	0.454[c]	0.808	3	0.625	0.650[c]	0.039	3	○
QH092	3	0.636	0.582[c]	−0.094	3	0.458	0.525	0.127	2	0.522	0.386	−0.353	3	○
QH101	3[b]	0.333	0.612	0.455	2	0.708	0.457	−0.548	2	0.000	0.080	1.000	3	○
QH106	2	0.250	0.375	0.333	3	0.263	0.355	0.258	2	0.467	0.420	−0.111	3	○
QH110	2	0.875	0.531[c]	−0.647	3	0.917	0.588[c]	−0.560	2	0.292	0.249	−0.171	3	○
QH111	2	0.000	0.469[c]	1.000	2	0.043	0.043	−0.022	2	0.455	0.351	−0.294	2	○
QH130	2	0.958	0.499	−0.920	2	0.958	0.499	−0.920	2	0.542	0.492[c]	−0.101	2	○
QH146	2	0.333	0.278[c]	−0.200	2	0.458	0.395[c]	−0.160	2	0.375	0.492	0.238	2	○
Mean	2.5	0.424	0.445	0.05	2.667	0.472	0.458	0.012	2.167	0.388	0.391	0.005	3.567	—

Note: A = number of alleles per locus; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = inbreeding coefficient; N = number of individuals; NA = not applicable; [a] Voucher and locality information are provided in Supplementary Table S1; [b] Significant frequency of null alleles ($P < 0.05$); [c] Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$); [d] ○ indicates that fragments can be amplified for this locus, × indicates fragments cannot be amplified for this locus.

and are broadly similar to those we have previously reported in distylous populations of *P. sinolisteri* (Wang et al., 2019). All loci were polymorphic across three populations of *P. denticulata* subsp. *denticulata* sampled from Tibet. In addition, we confirmed cross-amplification of these microsatellite loci in *P. denticulata* subsp. *sinodenticulata* from Yunnan. The markers will be useful tools for future genotyping and studies of population genetic structure in the *P. denticulata* complex. The high discriminatory power of the markers will also be useful for parentage analysis in dimorphic populations and should provide opportunities to investigate the potential influence of ecological and demographic factors on mating patterns.

This research was funded by the National Natural Science Foundation of China (31770417 and 31971394), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB31000000), the Yunling Scholarship of Yunnan Province (YLXL20170001) and the Light of West China Program of the Chinese Academy of Sciences. Laboratory work was performed at the Laboratory of Molecular Biology at the Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences.

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Supplementary Table S1. Locality and voucher information for populations of *Primula denticulata* investigated in this study

Species	Population code	Voucher number	Location	Geographic coordinates	Elevation (m)	*N*
Primula denticulata subsp. *denticulata*	LX	C. HP 078	China, Nyingchi, Langxian	28.74823° N, 93.43852° E	4231	24
Primula denticulata subsp. *denticulata*	LL	C. HP 083	China, Nyingchi, Lulang	29.63213° N, 94.62472° E	4316	24
Primula denticulata subsp. *denticulata*	CY	C. HP 112	China, Nyingchi, Chayu	29.33732° N, 97.05625° E	4388	24
Primula denticulata subsp. *sinodenticulata*	DH	C. HP 056	China, Dehong, Yingjiang	25.29461° N, 98.02300° E	2651	5

Notes: Voucher specimens are deposited in the herbarium of the Kunming Institute of Botany, Kunming, Yunnan, China. *N* = number of individuals sampled.