Characterization of 30 microsatellite markers for distylos \textit{Primula denticulata} (Primulaceae) using HiSeq sequencing

Hao-Pu Cao\textsuperscript{1,2}, Yu-Ting He\textsuperscript{1,3}, Li Zhong\textsuperscript{3,4}, Xin-Jia Wang\textsuperscript{3,4}, Spencer C. H. Barrett\textsuperscript{5}, Hong Wang\textsuperscript{4}, De-Zhu Li\textsuperscript{1} and Wei Zhou\textsuperscript{1*}

\textsuperscript{1}Plant Germplasm and Genomics Center, Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650201, China
\textsuperscript{2}School of Life Science, Yunnan University, Kunming, Yunnan 650500, China
\textsuperscript{3}University of Chinese Academy of Sciences, Beijing 100049, China
\textsuperscript{4}Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650201, China
\textsuperscript{5}Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario M5S 3B2, Canada

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

\textit{Primula denticulata} exhibits considerable variation in floral morphology and flowering phenology along elevational gradients in SW China. We isolated 30 microsatellite markers from \textit{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 \textit{P. 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 \textit{P. denticulata} subsp. \textit{sinodenticulata}. The microsatellite markers we have identified will provide valuable tools for investigations of the population genetic structure, mating systems and phylogeography of the \textit{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, \textit{Primula denticulata}, Primulaceae

\textit{Primula denticulata} Smith (Primulaceae) is an insect-pollinated, perennial herb belonging to \textit{Primula} section \textit{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 distylos, comprising long-styled and short-styled floral morphs (Hu and Kelso, 1996). \textit{Primula denticulata} is composed of two subspecies: subsp. \textit{denticulata} is restricted to the Tibetan plateau at elevations between 2,800 and 4,600 m and subsp. \textit{sinodenticulata} occurs in Yunnan, Sichuan and north-west Guizhou at lower elevations between 1,500 and 3,000 m. \textit{Primula denticulata} subsp. \textit{sinodenticulata} flowers from February to April whereas \textit{P. denticulata} subsp. \textit{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.

Distylos \textit{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, \textit{Primula} species have been the recent focus of detailed studies of the molecular genetic architecture of

Edited by Kenichi Nonomura
* Corresponding author. zhouwei@mail.kib.ac.cn
DOI: http://doi.org/10.1266/ggs.20-00003
the distylos 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 (Veira 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 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 MiCroSAAtellite 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 MgCl₂, 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  | (TC)_6       | 110–140                  | 62      | MT415220                      |
|       | R: TCGATGGAGAGAATCTGAGCTG|              |                          |         |                               |
| QH007 | F: TGCTGCTAGGCTAGTTTGTG  | (AG)_7       | 95–153                   | 55.9    | MN694829                      |
|       | R: CTACGGCCAGTCCACACT    |              |                          |         |                               |
| QH009 | F: CCTGCTGTTGTGGCTGGTG   | (CA)_8       | 125–135                  | 53.9    | MT415221                      |
|       | R: TTGGCTCAGTAACTGGCTAG  |              |                          |         |                               |
| QH011 | F: CCAGATCTCTCACTACCTCA  | (ATAA)_5     | 130–140                  | 55      | MN694830                      |
|       | R: CAAAGGTCTTGTGGTCAGT   |              |                          |         |                               |
| QH016 | F: CCAGCATTATGGCAAGACAA  | (AT)_6       | 140–150                  | 54.9    | MT415222                      |
|       | R: ATGCAATTTTCTACTGCCCAA |              |                          |         |                               |
| QH018 | F: AACCTTCGGCTCCAGCCTCC  | (TA)_9       | 160–175                  | 54.9    | MN694831                      |
|       | R: GTTGGCTTATCTACTCCTT   |              |                          |         |                               |
| QH020 | F: GTGCTGTTAGAAAAAGCTGAT | (TG)_10     | 145–180                  | 56.9    | MN694832                      |
|       | R: ACCTACATTTGTGCAGCC    |              |                          |         |                               |
| QH022 | F: ATCCCTATCTCACCAGGGCT  | (TCA)_5      | 120–170                  | 57      | MN694833                      |
|       | R: GCAGGGAGGAGAAGGAGACT  |              |                          |         |                               |
| QH025 | F: GAAGTATAAATTTGGGGTTTGAG  | (TG)_10   | 160–180                  | 54.4    | MN694834                      |
|       | R: AATCCCATCTCCTCAACAAA  |              |                          |         |                               |
| QH028 | F: GGCGATCTGTTTATTGCGAT  | (GA)_5      | 133–152                  | 59      | MN694835                      |
|       | R: GTACGGTGTCACATCTCC    |              |                          |         |                               |
| QH031 | F: GTTGGCTGCGAGAGAGATCAT | (TTCT)_5    | 147–182                  | 55.9    | MN694836                      |
|       | R: AAGCATGGCAAAACCAACT   |              |                          |         |                               |
| QH034 | F: TCCTGATGACGACGAAAAAG  | (TC)_8      | 130–150                  | 55.9    | MT458490                      |
|       | R: AGAACAGGGTGGTGGAGGCTG |              |                          |         |                               |
| QH038 | F: TCCAGAGAAAAACCTCTGAA | (ATT)_5     | 163–200                  | 55.9    | MN694837                      |
|       | R: GGGACATGCGATTCCTCTCG  |              |                          |         |                               |
| QH037 | F: TCGCAACTCTCACCAGGAAGCC| (AG)_7     | 170–200                  | 55.9    | MT415223                      |
|       | R: AGCAAGTGAAATGCTGAGGT  |              |                          |         |                               |
| QH040 | F: GCCACAAAATACGCAACTCAA | (TG)_7      | 175–200                  | 55.9    | MN694838                      |
|       | R: TGGCCCTAGCTGTCTCTCAG  |              |                          |         |                               |
| QH043 | F: TGCCCTAGCTGAGCTCTCCG  | (TC)_7      | 190–210                  | 55.9    | MT415224                      |
|       | R: TGCTCAGTTGCAAGAGGATAT |              |                          |         |                               |
| QH045 | F: TATTTTGTCGAGAATGTTGGG | (AG)_7      | 180–200                  | 53.9    | MT415225                      |
|       | R: CCAGAACTGGTGGAAAGGG   |              |                          |         |                               |
| QH050 | F: TGCTGGCTTAGCTGATCGTG  | (TTG)_5     | 180–225                  | 54      | MT415226                      |
|       | R: GAGGGACGGTCATCATCAACA |              |                          |         |                               |
| QH056 | F: AGGGGAAGTTTTCAGTGCCA  | (GA)_5      | 205–208                  | 56      | MN694839                      |
|       | R: CCTTTGGATTTTTTCATGCG  |              |                          |         |                               |
| QH062 | F: CTTCTTTTTGACATCCACA  | (GA)_5      | 190–210                  | 54.9    | MT415227                      |
|       | R: GGGCAATTTTGCTATTTCTCAG|              |                          |         |                               |
| QH078 | F: CCACACACACCTCTCAGA    | (TC)_7      | 200–240                  | 58.9    | MN694840                      |
|       | R: CGTTGACCTCCAGTGTCTT   |              |                          |         |                               |
| QH079 | F: AACGTGACAGCTATGGCAAACAG| (TC)_6    | 200–215                  | 55.9    | MT415228                      |
|       | R: AACCTTGTGAGGTGGCTCTA  |              |                          |         |                               |
| QH091 | F: CAAGATGACGGCTGGGATTG  | (AG)_10     | 200–220                  | 55.9    | MT458496                      |
|       | R: AGTCAGGGAATCTTCTCAG   |              |                          |         |                               |
| QH092 | F: TGAGCGAGATGAGATGAA    | (AG)_6      | 210–235                  | 55.9    | MT415229                      |
|       | R: GCCACTTAAAACAGCTGGCTCT|              |                          |         |                               |
| QH101 | F: TCCTTCTCCTTCATGCTC    | (AG)_6      | 190–217                  | 54.9    | MN694841                      |
|       | R: GCTGCGACTCTCTCTCCTC   |              |                          |         |                               |
| QH106 | F: GGGTACGCTCTCTAGGGCG   | (ATT)_10    | 190–220                  | 56      | MT415231                      |
|       | R: GCTGTGACCATGACACATG   |              |                          |         |                               |
| QH110 | F: GCTGACACCTAAGGCTCTG   | (AT)_6      | 220–240                  | 54.9    | MT415232                      |
|       | R: AGCTGCTAGGGCTGGAAG    |              |                          |         |                               |
| QH111 | F: CTCCTTCACCACCTCCTCAG  | (CA)_6      | 220–225                  | 54.9    | MT415233                      |
|       | R: AGGAAGGCCCACCCAGTTTTT|              |                          |         |                               |
| QH130 | F: GCTGGAATGGAGATGCGAG   | (GA)_6      | 220–260                  | 61      | MN694842                      |
|       | R: ACGAGCAGCTGTGGGTTTTTC |              |                          |         |                               |
| QH146 | F: TGAGAGACCTGCTGTATCA   | (AGA)_3     | 240–260                  | 55      | MN694843                      |
|       | R: AATTTAGGCAAGGCCTACAG  |              |                          |         |                               |

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 distylosous and outbreeding mating system of populations,

| Locus  | LX (N = 24) | LL (N = 24) | CY (N = 24) | DH (N = 5) |
|--------|-------------|-------------|-------------|------------|
|        | A  | H0  | Hk  | Fis       | A  | H0  | Hk  | Fis  | Total A |
| QH006  | 3  | 0.542 0.468 −0.158 | 3b  | 0.565 0.644 0.122 | 2  | 0.273 0.236 −0.158 | 3  | ○ |
| QH007  | 3b | 0.375 0.633 0.407 | 5  | 0.591 0.655 0.098 | 2  | 0.125 0.499 0.750 | 7  | ○ |
| QH009  | 4  | 0.316 0.550 0.456 | 5  | 0.667 0.683 0.024 | 2  | 0.632 0.511 −0.236 | 5  | ○ |
| QH011  | 2  | 0.458 0.353 −0.297 | 2b  | 0.125 0.395 0.684 | 2  | 0.696 0.454 −0.533 | 2  | × |
| QH016  | 2  | 0.792 0.531 −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  | 2b | 0.167 0.276 0.400 | 2  | 0.583 0.469 −0.244 | 3  | 0.292 0.379 0.231 | 5  | ○ |
| QH025  | 3  | 0.667 0.497 −0.343 | 3  | 0.773 0.584 −0.324 | 2  | 0.652 0.485 −0.345 | 3  | ○ |
| QH028  | 2b | 0.500 0.444 −0.125 | 2  | 0.208 0.187 −0.116 | 2  | 0.042 0.041 −0.021 | 5  | ○ |
| QH031  | 2  | 0.125 0.187 0.330 | 3  | 0.571 0.563 −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 0.619 | 2  | 0.375 0.305 −0.231 | 3  | ○ |
| QH038  | 5b | 0.667 0.702 0.051 | 3  | 0.167 0.190 0.123 | 2  | 0.095 0.091 −0.050 | 6  | ○ |
| QH040  | 3b | 0.167 0.403 0.586 | 4b  | 0.348 0.590 0.410 | 2  | 0.250 0.330 0.242 | 5  | ○ |
| QH043  | 3  | 0.542 0.588 0.078 | 3  | 0.917 0.582 −0.574 | 3  | 0.083 0.484 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  | 4b | 0.292 0.689 0.577 | 4b  | 0.375 0.695 0.461 | 3b  | 0.000 0.625 1.000 | 4  | ○ |
| QH056  | 2b | 0.083 0.080 −0.043 | 2b  | 0.087 0.227 0.617 | 1  | NA NA NA | 4  | ○ |
| QH062  | 3b | 0.167 0.614 0.728 | 2  | 0.353 0.415 0.150 | 2  | 0.357 0.436 0.181 | 3  | ○ |
| QH078  | 2b | 0.478 0.364 −0.314 | 2b  | 0.048 0.427 0.889 | 2  | 0.556 0.401 −0.385 | 5  | ○ |
| QH079  | 3b | 0.375 0.629 0.404 | 3b  | 0.292 0.586 0.502 | 2  | 0.792 0.492 −0.608 | 3  | ○ |
| QH091  | 1  | NA NA NA | 2  | 0.087 0.454 0.808 | 3  | 0.625 0.650 0.039 | 3  | ○ |
| QH092  | 3  | 0.636 0.582 −0.094 | 3  | 0.458 0.525 0.127 | 2  | 0.522 0.386 −0.353 | 3  | ○ |
| QH101  | 3b | 0.333 0.612 0.455 | 2  | 0.708 0.457 −0.548 | 2  | 0.000 0.080 1.000 | 3  | ○ |
| QH110  | 2  | 0.250 0.375 0.333 | 3  | 0.263 0.355 0.258 | 2  | 0.467 0.420 −0.111 | 3  | ○ |
| QH111  | 2  | 0.000 0.469 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 −0.101 | 2  | ○ |
| QH146  | 2  | 0.333 0.278 −0.200 | 2  | 0.458 0.395 −0.160 | 2  | 0.375 0.492 0.238 | 2  | ○ |

Note: A = number of alleles per locus; H0 = observed heterozygosity; Hk = expected heterozygosity; Fis = 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.

**REFERENCES**

Baird, N. A., Etter, P. D., Atwood, T. S., Currey, M. C., Shiver, A. L., Lewis, Z. A., Selker, E. U., Cresko, W. A., and Johnson, E. A. (2008) Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS One* 3, e3376.

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

de Vos, J. M., Keller, B., Zhang, L.-R., Nowak, M. D., and Conti, E. (2018) Mixed mating in homostyly species: genetic and experimental evidence from an alpine plant with variable herkogamy, *Primula halleri*. Int. J. Plant Sci. 179, 87–99.

de Vos, J. M., Wüst, R. O., and Conti, E. (2014) Small and ugly? Phylogenetic analyses of the "selling 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.

Hodgins, K. A., and Barrett, S. C. H. (2007) Population structure and genetic diversity in tristylos *Narcissus triandrus*: insights from microsatellite and chloroplast DNA variation. *Mol. Ecol.* 16, 2317–2332.

Hodgins, K. A., and Barrett, S. C. H. (2008) Natural selection on floral traits through male and female function in wild populations of the heterostylous daffodil *Narcissus triandrus*. *Evolution* 62, 1751–1763.

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.

Huu, C. N., Kappel, C., Keller, B., Sicard, A., Takebayashi, Y., Breuninger, H., Nowak, M. D., Bäurle, I., Himmelbach, A., Burkart, M., et al. (2016) Presence versus absence of *CYP734A50* underlies the style-length dimorphism in primroses. *eLife* 5, e17956.

Huu, C. N., Keller, B., Conti, E., Kappel, C., and Lenhard, M. (2020) Supergene evolution via stepwise duplications and neofunctionalization of a floral-organ identity gene. *Proc. Natl. Acad. Sci. USA* 117, 23148–23157.

Li, J., Cocker, J. M., Wright, J., Webster, M. A., McMullan, M., Dyer, S., Swarbreck, D., Caccamo, M., van Oosterhout, C., and Gilmartin, P. M. (2016) Genetic architecture and evolution of the *S* locus supergene in *Primula vulgaris*. *Nat. Plants* 2, 16188.

Merritt, B. J., Culley, T. M., Avanesyan, A., Stokes, R., and Brzyksi, J. (2015) An empirical review: characteristics of plant microsatellite markers that confer higher levels of genetic variation. *Appl. Plant Sci.* 3, 1500025.

Nowak, M. D., Russo, G., Schlaphach, R., Huu, C. N., Lenhard, M., and Conti, E. (2015) The draft genome of *Primula veris* yields insights into the molecular basis of heterostyly. *Genome Biol.* 16, 12.

Peakall, R., and Smouse, P. E. (2012) *GENALEX* 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics* 28, 2537–2539.

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

Rousset, F. (2008) *GENEPOP*’007: a complete re-implementation of the *GENEPOP* software for Windows and Linux. *Mol. Ecol. Resour.* 8, 103–106.

Thiel, T., Michalek, W., Varshney, R. K., and Grauer, A. (2003) Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.* 106, 411–422.

van Oosterhout, C., Hutchison, 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.

Vieira, M. L. C., Santini, L., Diniz, A. L., and Munhoz, C. de F. (2016) Microsatellite markers: what they mean and why they are so useful. *Genet. Mol. Biol.* 39, 312–328.

Wang, X., Zhong, L., Wu, Z., Sun, H., Wang, H., Li, D., Barrett, S. C. H., and Zhou, W. (2019) Characterization of 30 microsatellite markers in distylous *Primula sinolisteri* (*Primulaceae*) using HiSeq sequencing. *Appl. Plant Sci.* 7, e01208.

Yakimowski, S. B., and Barrett, S. C. H. (2014) Clonal genetic structure and diversity in populations of an aquatic plant with combined vs. separate sexes. *Mol. Ecol.* 23, 2914–2928.

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

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.

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

Zhou, W., Barrett, S. C. H., Wang, H., and Li, D. (2012) Loss of floral polymorphism in heterostylous *Luculia pinceana* (*Rubiaceae*): a molecular phylogeographic perspective. *Mol. Ecol.* 21, 4631–4645.

Zhou, W., Barrett, S. C. H., Wang, H., and Li, D. (2015) Reciprocal herkogamy promotes disassortative mating in a distylous species with intramorph compatibility. *New Phytol.* 206, 1503–1512.
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.