Characterization of 24 microsatellite markers in *Primula chungensis* (Primulaceae), a distylous–homostyly species, using MiSeq sequencing

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A B S T R A C T

*Primula chungensis* is a species with considerable floral and mating-system variation, including distylous (outcrossing), homostylos (selfing) and mixed populations that contain both outcrossing and selfing forms. We isolated 24 microsatellite markers from *P. chungensis* using Illumina MiSeq sequencing. Polymorphism and genetic diversity were then measured based on a sample of 24 individuals from a natural population in southern Tibet. All loci were polymorphic with the number of alleles per locus ranging from 2 to 4. The observed and expected heterozygosity ranged from 0 to 1 and 0.219 to 0.708, respectively. The microsatellite markers we have identified will serve as valuable tools for the investigation of the population genetic structure and phylogeography of *P. chungensis* and will inform models of the evolutionary history of mating systems in the species.

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1. Introduction

*Primula chungensis* I. B. Balfour & Kingdon-Ward is a herbaeaceous, insect-pollinated, perennial species belonging to sect. *Pro-

lifera* (Primulaceae) and restricted to the mountainous regions of Yunnan, Sichuan and Tibet of China (Hu and Kelso, 1996; Richards, 2002). It commonly occurs in wet meadows, forest edges, open slopes, and roadsides at altitudes between 2900 and 3200 m. The species flowers from May to June and produces one to three in-

florescences composed of pale orange flowers (about 20 flowers per inflorescence) that last for up to 6 days. Our field investigations indicate that *P. chungensis* exhibits considerable variation in floral biology across its geographical range. Most populations are composed of a single self-pollinating floral phenotype with an-

thers and stigmas at equivalent height (homostyly). In contrast, other populations contain outcrossing long-styled and short-

styled floral morphs typical of the floral polymorphism distylly. Populations containing both homostylos and heterostylos morphs also occur, and finally some populations are monomorphic for heterostylos morphs, especially the long-styled morph. *Primula* is a well-known model system for studies of the evo-

lution, function and breakdown of heterostyly (Crosby, 1949; Ornduff, 1979; Piper et al., 1984; Richards, 2002; Mast and Conti, 2006; Mast et al., 2006; de Vos et al., 2014; Keller et al., 2014), and more recently for investigations of the molecular genetics of the heterostyly linkage group (McCubbin et al., 2006; Li et al., 2011; Nowak et al., 2015). The occurrence of polymorphism for floral morphology and mating system in *P. chungensis* provides outstanding opportunities for investigating a range of questions associated with the evolutionary maintenance and breakdown of heterostyly. Development of genetic markers to investigate the patterns of genetic diversity in populations of contrasting mating systems (outcrossing vs. selfing), and to determine the evolu-

tional relationships between populations containing different floral phenotypes is a necessary first step for evolutionary studies of *P. chungensis*. Here, we report the isolation and characterization

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of 24 polymorphic microsatellite markers from *P. chungensis*, which will be valuable for furthering our understanding of the evolutionary genetics of this species.

### 2. Materials and methods

We isolated total genomic DNA from leaf tissue of one *P. chungensis* individual from the Muli population (28°41′C14 54.604°N, 100°C14 47.268°E; 3489 m a.s.l.) in Sichuan using DNeasy Plant Mini Kit (QIAGEN, Irvine, USA) following the manufacturer’s protocol. Extracted DNA was used for a library preparation with a Nextera XT Library Prep Kit for Illumina. We performed sequencing on the MiSeq Benchtop sequencer (Illumina, Inc., San Diego, California, USA) using the 2 x 250 bp read length. Raw Illumina reads (579,708 reads) were analyzed and assembled using Geneious version 6.0 (Biomatters, Auckland, New Zealand) into 56,092 contigs. The contigs were BLASTed against NCBI GenBank using BLASTx to identify and exclude contigs with chloroplast genome hits. Microsatellites with at least 5 repeats were then detected using QDD version 2.1 Beta (Meglécz et al., 2010). A total of 2341 contigs contained at least one microsatellite, of which 127 loci were selected for primer design using the software Primer version 5.0 (Clarke and Gorley, 2001). These primers were initially tested and optimized using a Veriti 96-well Thermal Cycler Gradient PCR Machine (Applied Biosystems, Foster City, California, USA). A total of 24 primer pairs amplified consistently, and were used for further screening (Table 1). We assessed polymorphism at these loci on 24 individuals obtained from a distylous population located in southern Tibet (29°46′C14 46.616°N, 94°44.545°E; 3305 m a.s.l.).

We performed PCR amplification using the following protocol: 20 µL reaction volume containing 25–50 ng of genomic DNA, 0.6 µM of each primer, 10 µL × Taq PCR MasterMix (Tiangen (Tiangen Biotech, Beijing, China)); 3 mM MgCl₂, 100 mM KCl, 0.5 mM of each dNTP, 20 mM Tris–HCl (PH 8.3), 0.1 U Taq polymerase] and 10 × PCR buffer. We conducted PCR amplifications under the following conditions: 95 °C for 4 min followed by 30–35 cycles at 94 °C for 45 s, at the annealing temperature for each specific primer (optimized for each locus; Table 1) for 45 s, 72 °C

### Table 1

| Locus name | Primer sequence (5′–3′) | Repeat motif | Size (bp) | Tₐ (°C) | GenBank accession no. |
|------------|------------------------|--------------|-----------|---------|----------------------|
| PC109      | F:ACGGGTCATTGCTTAACTGC | (CGA)₅       | 190       | 54      | KT033877             |
|            | R:TGTCCCTTGGTCGGCTTCCG |             |           |         |                      |
| PC4007     | F:ATGGGTCATACGGGATGCGT | (CT)₁₀      | 308       | 54      | KT033878             |
|            | R:TGGGAGGCTGAGGCTGGTG  |             |           |         |                      |
| PC4198     | F:TCATACCTCCTCCTCTCTC | (TC)₉       | 153       | 56      | KT033879             |
|            | R:CCTAGCCTCCCAACATTCA |             |           |         |                      |
| PC9123     | F:AAAAGGCCTAGGAGCTGA  | (TG)₇       | 338       | 48      | KT033880             |
|            | R:GGTTGGTGGTGTGGTGGT  |             |           |         |                      |
| PC11592    | F:CACCTACCAATTTTCTTCC | (AG)₁₅      | 165       | 56      | KT033881             |
|            | R:GCTGACATTGCTTCTCTCT |             |           |         |                      |
| PC14297    | F:ACTACTGTTGCTTCTGTCGA | (AG)₁₆      | 246       | 52      | KT033882             |
|            | R:CTTCCTCGTATTGATGCTC |             |           |         |                      |
| PC15519    | F:ACTGCTGTGCTGCTGCTT | (GTTT)₆     | 240       | 50      | KT033883             |
|            | R:AGTAACCTTCTTGTTGCTGTA |             |           |         |                      |
| PC15877    | F:CTTCCTGCTTCTCTCCTCTC | (CT)₆      | 228       | 50      | KT033884             |
|            | R:AGTAAGCTAAGTGGTGTGAT |             |           |         |                      |
| PC20540    | F:CTACCTGTTGCTTCTTCT | (TTC)₃      | 213       | 48      | KT033885             |
|            | R:AGACGAGCTGACCTCTGAC |             |           |         |                      |
| PC21731    | F:ATGGTGCTTTGTTTATTGCC | (GA)₆     | 173       | 56      | KT033886             |
|            | R:ATGGCTTTTCTTATCTGAG |             |           |         |                      |
| PC29976    | F:CAATTGCATCTCTCTCCCT | (TC)₆      | 184       | 50      | KT033887             |
|            | R:GAGGAGGGTATGATGATGCT |             |           |         |                      |
| PC30591    | F:GCAATGCTGTCGCTGCTGCT | (TG)₇     | 221       | 56      | KT033890             |
|            | R:GGGAGACATTGGAAAGACT |             |           |         |                      |
| PC30882    | F:GCCAACCACGTATGAGTC | (AG)₁₅      | 245       | 48      | KT033889             |
|            | R:GCAAGTAAAGCTACACGAT |             |           |         |                      |
| PC31302    | F:CTTCTTAGAAGCCCCCTAC | (CT)₆     | 221       | 56      | KT033890             |
|            | R:GCCAAGCTTGCTGCTGCTG |             |           |         |                      |
| PC33802    | F:ACTAAAGCTATGCTGACT  | (AG)₉      | 134       | 52      | KT033891             |
|            | R:CAATGCTAAGCTGACCTG |             |           |         |                      |
| PC33837    | F:GTACACCTGATGCTATTGAA | (GAT)₆   | 266       | 52      | KT033892             |
|            | R:TTGAAACATGCTACATGGTAC |             |           |         |                      |
| PC34870    | F:ACATCACATAGGTCCTTCAA | (AG)₆     | 113       | 52      | KT033893             |
|            | R:CTTCCTTCCTCCTCCTTC |             |           |         |                      |
| PC37139    | F:CTCCTCCTCCTACCTCTCTC | (TC)₆    | 126       | 50      | KT033894             |
|            | R:TGACAGCAGAAACATACATTG |             |           |         |                      |
| PC39361    | F:GCATTGCAGTTTTTCGGTG | (TG)₇     | 134       | 52      | KT033895             |
|            | R:GTTAATCCTGGCCGACCTG |             |           |         |                      |
| PC39450    | F:CGTGGCAATGTGCTGCTGAG | (AGA)₉ | 177       | 52      | KT033896             |
|            | R:GACAAAGAATCTCCGCAAAT |             |           |         |                      |
| PC46911    | F:CGAATAGGGCAGGACATTGCGAAT | (AG)₈  | 255       | 52      | KT033897             |
|            | R:CTGACGACCAATGCTTGGGCA |             |           |         |                      |
| PC47381    | F:GACGGGAGACCGAAGAGAAGGGA | (CTC)₇ | 189       | 54      | KT033898             |
|            | R:GCAAGTACAGGCAGGAACTA |             |           |         |                      |
| PC50689    | F:GCGGTTTGCATCTGCTGAG | (AAC)₁₀ | 183       | 48      | KT033899             |
|            | R:CTTCGGATATGCTGCTGCTG |             |           |         |                      |
| PC52377    | F:CTTCCTCTCTTTTTCTCTGTC | (TC)₉   | 111       | 48      | KT033900             |
|            | R:TACAGAAAATGAGCAGAAACG |             |           |         |                      |

Note: Tₐ = annealing temperature.
for 15 min for extension, and a final extension step at 72 °C for 10 min. PCR products were separated and visualized using a QIAxcel capillary gel electrophoresis system (QIAGEN, Irvine, USA) with an internal 10–300 bp size standard.

3. Results and discussion

Of 127 primer pairs tested, 24 primer pairs amplified microsatellite loci displaying polymorphism, whereas the remaining 103 pairs were monomorphic. All sequences were deposited in GenBank (Table 1). We calculated basic population genetic parameters of diversity, including the number of alleles (Nₐ), observed and expected heterozygosities (H₀, Hₑ), Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between pairs of loci using the package GENEPOP version 4.0 (Raymond and Rousset, 1995). The number of alleles per locus (Nₐ) ranged from 2 to 4, with a mean of 2.458. In the population investigated, the observed (H₀) and expected (Hₑ) heterozygosities ranged from 0 to 1.000 and from 0.219 to 0.708, with averages of 0.516 and 0.469, respectively (Table 2). Nine loci (PC9123, PC11592, PC20540, PC30882, PC33837, PC37139, PC39361, PC46911, PC52377) deviated significantly from Hardy–Weinberg equilibrium indicating the possibility of null alleles, the Wahlund effect and disassortative mating in this distylous population. After Bonferroni correction, no significant pairwise linkage disequilibrium was observed for any pair of loci.

In this study, we isolated 2341 microsatellite loci from *P. chungensis*. Primer pairs were designed to test 127 of these loci for polymorphism in 24 individual plants from a distylous population. Among the loci we tested, 24 microsatellite markers were polymorphic. These 24 polymorphic microsatellite markers will be powerful molecular tools for analyzing population structure and mating systems of *P. chungensis*, particularly the evolutionary relationship between distylous and homostylous populations. The high discriminatory power of these microsatellite loci will also be useful for parentage analysis in floral polymorphic populations of this species, which may provide an opportunity to evaluate the potential influence of ecological and reproductive factors on mating patterns (Zhou et al., 2015).

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