Plant Diversity 38/2 (2016) 110–113

Contents lists available at ScienceDirect

Plant Diversity

journal homepage: http://journal.kib.ac.cn

Original article

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

Wei Zhou[1], Haidong Li[2,3], Zhikun Wu[4], Spencer C.H. Barrett[5], Dezhu Li[1], Hong Wang[2*]

1. *Plant Germplasm and Genomics Center, Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China*
2. *Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China*
3. *Kunming College of Life Sciences, University of Chinese Academy of Sciences, Kunming 650201, China*
4. *Kunming Botanical Garden, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China*
5. *Department of Ecology and Evolutionary Biology, University of Toronto, Toronto Ontario M5S 3B2, Canada*

ARTICLE INFO

Article history:
Received 5 December 2015
Received in revised form 24 December 2015
Accepted 24 December 2015
Available online 25 April 2016

Keywords:
Distyly
Homostyly
Microsatellites
Polymorphism
Primula chungensis

ABSTRACT

Primula chungensis is a species with considerable floral and mating-system variation, including distylous (outcrossing), homostylous (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.

Primula chungensis I.B. Balfour & Kingdon-Ward is a herbaceous, insect-pollinated, perennial species belonging to sect. *Prolifera* (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 2,900 and 3,200 m. The species flowers from May to June and produces one to three inflorescences 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 anthers and stigmas at equivalent height (homostyly). In contrast, other populations contain outcrossing long-styled and short-styled floral morphs typical of the floral polymorphism distyly. Populations containing both homostylous and heterostylous morphs also occur, and finally some populations are monomorphic for heterostylous morphs, especially the long-styled morph.

Primula is a well-known model system for studies of the evolution, 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 evolutionary relationships between populations containing different floral phenotypes is a necessary first step for evolutionary

* Corresponding author.
E-mail address: wanghong@mail.kib.ac.cn

studies of *P. chungensis*. Here, we report the isolation and characterization of 24 polymorphic microsatellite markers from *P. chungensis*, which will be valuable for furthering our understanding of the evolutionary genetics of this species.

1. Materials and methods

We isolated total genomic DNA from leaf tissue of one *P. chungensis* individual from the Muli population (28°54.604′N, 100°47.268′E; 3,489 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 × 250 bp read length. Raw Illumina reads (679,708 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 2,341 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°46.616′N, 94°44.545′E; 3,305 m a.s.l.).

Table 1 – Primer sequences and characterization of 24 microsatellite loci isolated from *Primula chungensis*.

Locus name	Primer sequence (5′–3′)	Repeat motif	Size/bp	T_a/℃	GenBank accession no.
PC109	F: ACGGGTCATTGCGTTTAAGTCG R: TTGTCGTTTGTCGGAGTGTTCG	$(CGA)_5$	190	54	KT033877
PC4007	F: ATGGTGCTAAACGCCTTGTG R: TTTGAAGTAGGGGTGAGGTG	$(CT)_{10}$	308	54	KT033878
PC4198	F: CTACACCTCTCCTCCTCTTC R: CCCTAGCCGTCCAACTTA	$(TC)_9$	153	56	KT033879
PC9123	F: CAAAAGAGCGTTAGGAGTGA R: GATGGTTGTCGTTGTCGGT	$(TG)_7$	338	48	KT033880
PC11592	F: TACCCTAACCTATTTTTCCC R: CGCTACCTTACCTCTCTTCT	$(AG)_{15}$	165	56	KT033881
PC14297	F: ACTTACTGGTCTTTCGGTGA R: CTCTCGCTTTATGTGCCTG	$(AG)_{16}$	246	52	KT033882
PC15519	F: ACTCGTGTTCCGTTTGTTCTAA R: AGTAACTGTTTGTTTGGCTATTGA	$(GTTTT)_8$	240	50	KT033883
PC15877	F: CGGTTTATTTGGTTGAAGTC R: CTCCTTGTTCTTTGTTTGAC	$(CT)_6$	228	50	KT033884
PC20540	F: CTCATCGTCTTTCCTATTTC R: AAGGCAATCTGTTCTGAC	$(TTTTTG)_5$	213	48	KT033885
PC21731	F: ATGTGTCCTTGTTTTATGTG R: ATGGTGTTTATCAATGTAGG	$(GA)_6$	173	48	KT033886
PC29976	F: CAATGATTTCTCTCTCGTCC R: AGATGGTTGAAGATGGTGAT	$(TC)_6$	184	50	KT033887
PC30591	F: GGAAATCGCAGCCTCATAAC R: GAGAGCGGAGTGTTCACCTA	$(TG)_7$	220	52	KT033888
PC30882	F: GCACAAACCCTAACTAAAGT R: GCACAATCAAGCCAATG	$(AG)_8$	245	48	KT033889
PC32026	F: CTTTTCACCAGACGCTACCC R: GCGGAACATCATTGAACCA	$(CT)_6$	221	56	KT033890

Table 1 continued

Locus name	Primer sequence (5′–3′)	Repeat motif	Size/bp	T_a/℃	GenBank accession no.
PC33802	F: ACTAAACATAACAAGCATCG R: CAACTGTAATCTGAGCCAAT	$(AG)_9$	134	52	KT033891
PC33837	F: GTAGACACCTTGAATCATAACAAGTAAC R: TTGAAACCAAATTGCCACCT	$(GAT)_5$	266	52	KT033892
PC34870	F: AATCAGCATAGGGTTCTCAA R: CCTCTCTTCTCCTCCCTTTC	$(AG)_8$	113	52	KT033893
PC37139	F: GATGGCTCTCGTTTTTTTAC R: TCAGAGTAGAACCACATCAG	$(TC)_6$	126	50	KT033894
PC39361	F: GCATTCCACGTCTTTTGTTG R: GGTAATCCCCTCGCATGACT	$(TG)_7$	134	52	KT033895
PC39450	F: CGTGGCAATTTCTCGTAG R: ATGGCATTTGTCCTGTCTT	$(AAGA)_9$	177	52	KT033896
PC46911	F: GCAAAAGAAACTCCCGCAAT R: CTAAGCACAATGTTGAGGCA	$(AG)_8$	255	52	KT033897
PC47381	F: GAGGGAGACCGAAAGAGGA R: GCGGTAGGCACAGGAGAGTA	$(CTC)_7$	189	54	KT033898
PC50689	F: GGGTTTTTGTTCATCTTAGC R: TCTTCGGTATGTGTCTCGTG	$(AAC)_{10}$	183	48	KT033899
PC52377	F: TCTCTCTCTTTTCTTTGTTC R: TAGAGGAAATGGAGGAAG	$(TC)_9$	111	48	KT033900

Note: T_a = annealing temperature.

We performed PCR amplification using the following protocol: 20 μL reaction volume containing 25 to 50 ng of genomic DNA, 0.6 μmol·L^{-1} of each primer, 10 μL 2 × Taq PCR MasterMix [Tiangen (Tiangen Biotech, Beijing, China); 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), 0.1 U Taq polymerase] and 10 × PCR buffer. We conducted PCR amplifications under the following conditions: 95 ℃ for 4 min followed by 30 to 35 cycles at 94 ℃ for 45 s, at the annealing temperature for each specific primer (optimized for each locus; Table 1) for 45 s, 72 ℃ for 1.5 min for extension, and a final extension step at 72 ℃ 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.

2. 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 (N_a), observed and expected heterozygosities (H_O, H_E), 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 (N_a) ranged from 2 to 4, with a mean of 2.458. In the population investigated, the observed (H_O) and expected (H_E) 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, PC47381, PC50689; see Table 2) 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 2,341 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

Table 2 – Genetic diversity parameters of the 24 microsatellite loci developed for *Primula chungensis* based on 24 individuals sampled from a distylous population located in southern Tibet.

Locus	N_a	H_O	H_E	Locus	N_a	H_O	H_E
PC109	2	0.667	0.444	PC30882*	2	0.111	0.494
PC4007	2	0.250	0.219	PC32026	2	0.250	0.219
PC4198	2	0.333	0.278	PC33802	2	0.500	0.375
PC9123*	2	0.917	0.497	PC33837*	2	0.000	0.444
PC11592*	2	0.750	0.469	PC34870	3	0.875	0.539
PC14297	2	0.250	0.219	PC37139*	2	0.000	0.500
PC15519	4	1.000	0.653	PC39361*	3	0.833	0.559
PC15877	3	1.000	0.611	PC39450	4	0.833	0.708
PC20540*	2	0.000	0.500	PC46911	3	0.750	0.531
PC21731	3	0.792	0.598	PC47381*	2	0.000	0.375
PC29976	3	1.000	0.625	PC50689*	2	0.000	0.500
PC30591	3	0.778	0.512	PC52377	2	0.500	0.375

Note: N_a = the number of allele; H_O = observed heterozygosity; H_E = expected heterozygosity; Geographic coordinate of the population: 29°46.616′N, 94°44.545′E. * indicates significant departure from Hardy-Weinberg equilibrium ($P < 0.01$).

provide an opportunity to evaluate the potential influence of ecological and reproductive factors on mating patterns (Zhou et al., 2015).

Acknowledgments

This research was funded by the National Key Basic Research Program of China (2014CB954100), the Key Research Program of the Chinese Academic of Sciences (KJZD-EW-L07), the National Natural Science Foundation of China (31200289, 31570384), and the Natural Science Foundation of Yunnan Province (2012FB182). We appreciate the help of Zhibin Tao, Minlu Liu, Mingying Zhang and Yanhui Zhao (all at the Kunming Institute of Botany, KIB) during fieldwork. Junbo Yang and Zhirong Zhang provided technical assistance in the lab work and data analysis. 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

Clarke, K.R., Gorley, R.N., 2001. Primer v5: user manual/tutorial. PRIMER-E Ltd., Plymouth, 91.

Crosby, J.L., 1949. Selection of an unfavorable gene-complex. Evolution. 3, 212–230.

de Vos, J.M., Hughes, C.E., Schneeweiss, G.M., et al., 2014. Heterostyly accelerates diversification via reduced extinction in primroses. Proc. R. Soc. B-Biol. Sci. 281, 20140075.

Hu CM, Kelso S, 1996. Primulaceae. Wu, Z.Y., Raven, P.H. (eds.), Flora of China. Beijing: Science Press; St. Louis: Missouri Botanical Garden Press. 15, 39–189.

Keller, B., Thomson, J.D., Conti, E., 2014. Heterostyly promotes disassortative pollination and reduces sexual interference in Darwin's primroses: evidence from experimental studies. Funct. Ecol. 28, 1413–1425.

Li, J., Webster, M.A., Smith, M.C., et al., 2011. Floral heteromorphy in *Primula vulgaris*: progress towards isolation and characterization of the *S* locus. Ann. Bot. 108, 715–726.

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

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

McCubbin, A.G., Lee, C., Hetrick, A., 2006. Identification of genes showing differential expression between morphs in developing flowers of *Primula vulgaris*. Sex. Plant Reprod. 19, 63–72.

Meglécz, E., Costedoat, C., Dubut, V., et al., 2010. QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. Bioinformatics. 26, 403–404.

Nowak, M.D., Russo, G., Schlapbach, R., et al., 2015. The draft genome of *Primula veris* yields insights into the molecular basis of heterostyly. Genome Biol. 16, 12.

Ornduff, R., 1979. Pollen flow in a population of *Primula vulgaris* Huds. Bot. J. Linn. Soc. 78, 1–10.

Piper, J.G., Charlesworth, B., Charlesworth, D., 1984. A high rate of self-fertilization and increased seed fertility of homostyle primroses. Nature. 310, 50–51.

Raymond, M., Rousset, F., 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. J. Hered. 86, 248–249.

Richards, A.J., 2002. Primula, 2nd ed. London: Batsford.

Zhou, W., Barrett, S.C.H., Wang, H., et al., 2015. Reciprocal herkogamy promotes disassortative mating in a distylous species with intramorph compatibility. New Phytol. 206, 1503–1512.

(Editor: Sergei Volis)