Development and characterization of microsatellite markers for *Rhododendron purdomii* (Ericaceae) using next-generation sequencing

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Rhododendron purdomii (Ericaceae) is an endangered ornamental species endemic to the Qinling Mountains of China. Due to the impact of climate change and human disturbance, *R. purdomii* is threatened by habitat loss, and conservation of this species is urgently needed. In this study, we developed and characterized 13 novel microsatellite markers for *R. purdomii* based on next-generation sequencing data. For the 13 microsatellite markers in three *R. purdomii* populations, the number of alleles ranged from two to 12, the number of effective alleles was from 1.000 to 8.892, Shannon's information index was from 0.000 to 2.320, and the observed and expected heterozygosity were from 0.000 to 1.000 and from 0.000 to 0.888, respectively. Private alleles were found in all three populations. Moderate differentiation between population pairs was indicated by pairwise F_{ST} values. The microsatellite markers developed in this study will provide opportunities for examining the genetic diversity and population structure of *R. purdomii* and contribute to the effective conservation of this species.

Key words: microsatellites, *Rhododendron purdomii*, RAD-seq, Ericaceae, population genetics

Rhododendron is the largest genus of the family Ericaceae, containing more than 1,000 species worldwide (Ma et al., 2014). This genus includes many important ornamental plants. There are approximately 571 *Rhododendron* species in China, of which 409 are endemic (Fang et al., 2005). *Rhododendron* has been suggested as a good model system for evolutionary, ecological and horticultural studies (Ma et al., 2014), and there are many related studies on species located in hotspot areas (e.g., Hengduan Mountains) in South China (Yu et al., 2017;

Shrestha and Wang, 2018). However, little is known about the *Rhododendron* species that are restricted to the central region of China (Zhao et al., 2012).

Rhododendron purdomii Rehder & E. H. Wilson is an evergreen shrub or small tree species endemic to the Qinling Mountains of China, occurring in Henan, Shaanxi and Gansu provinces (Fig. 1). It was originally collected on Taibai Mountain of Shaanxi province by William Purdom in 1910, and is therefore named in honor of the collector (Fang et al., 2005). It is an important component of the montane ecosystem at 1,800–3,500 m altitude, and plays a vital role in erosion control and climate regulation (Yu et al., 2017). In addition, *R. purdomii* is a horticulturally valuable plant with colorful flowers. However, this species has been subject to some level of habitat disturbance due to human destruction and climate change (Ma et al., 2014; Yu et al., 2017). Based on a recent evaluation, *R. purdomii* is listed as vulnerable to extinction in the Red List of China Higher Plants (Qin et al., 2017). Therefore, understanding its genetic diversity and structural patterns is urgently required to effectively

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Fig. 1. Morphological illustrations and distribution map of *Rhododendron purdomii*. (A) A mature plant; (B) leaves; (C) flowers; (D) young fruits; (E) distribution map.

monitor and conserve this species.

Previous studies on *R. purdomii* are mainly about ornamental horticulture and resource development, such as evaluation of morphological traits (Si et al., 2012) and investigation of germplasm resources (Zhao et al., 2013). Despite its great value for horticulture research, the genetic diversity and population structure of *R. purdomii* have not been clarified, the only exception being that some populations in Shaanxi were assessed based on amplified fragment length polymorphism markers (Zhao et al., 2012). Due to the characteristics of codominant inheritance, high polymorphism and wide distribution in the genome, microsatellites are proven to be useful in population genetic studies (Feng et al., 2014; Qin et al., 2021). In this study, we identified microsatellite markers for *R. purdomii* based on restriction site-associated DNA sequencing (RAD-seq) data, and assessed the genetic diversity and differentiation of three *R. purdomii* populations using these novel markers.

To develop the microsatellite markers and evaluate their polymorphism, samples from three natural populations of *R. purdomii* were collected from Laojun Mountain (LJS, $n = 11$), Longyuwan (LYW, $n = 15$) and Laojieling (LJJ, $n = 17$) in Henan province, China. The locality information of the sampled populations is detailed in Supplementary Table S1. Fresh and healthy leaves of the investigated individuals were collected, and the individuals sampled within one site were at least 10 m apart. Permissions were obtained from the local nature reserve for collecting plant materials. Leaves were dried using silica gel and stored in plastic bags until DNA extraction, and voucher specimens were deposited in the Herbarium of Zhengzhou University.

Genomic DNA of all the individuals investigated was extracted from dried leaf tissues using a modified CTAB method (Doyle and Doyle, 1987). An RAD library of one *R. purdomii* individual was constructed using the EcoRI

(5'-GAATTC-3') enzyme following Miller et al. (2007) and Baird et al. (2008). The library was sequenced at Novogene (Beijing, China) using the Illumina HiSeq 2000 platform with 150-bp paired-end reads. After filtering low-quality reads, *de novo* assembly of the clean reads was performed using Velvet (Zerbino and Birney, 2008) with default parameters. Perfect microsatellite motifs with a repeat unit of 2–6 bp and a minimum number of four repeats were detected using MISA (Beier et al., 2017) with default settings. The program Primer3 (Untergasser et al., 2012) was used to design microsatellite primers; the length of primers ranged from 20 to 28 bp, and the annealing temperature was 60–65 °C.

Microsatellite loci were validated through three steps as follows. First, we randomly selected three individuals from three different populations of *R. purdomii* to test the success of amplification for the designed primer pairs. Next, the forward primers of microsatellites successfully amplified in the three individuals were labeled with fluorescent dye, and fluorescent PCR products of six individuals selected from three different populations were analyzed for polymorphism. Third, microsatellites showing the expected size range on agarose gels, as well as clear peaks and polymorphism during capillary electrophoresis in six individuals, were further amplified in all the investigated individuals of *R. purdomii*. The PCR reaction mixture volume was 50 μ l, consisting of distilled water (22 μ l), 2 $\times$ PCR Mixture (25 μ l, Beibei Biotech, Henan, China), 10 μ M forward and reverse primers (1 μ l for each primer) and genomic DNA (1 μ l). The PCR reaction conditions were: an initial denaturation at 94 °C for 4 min; 35 cycles at 94 °C for 30 s, annealing temperature for 30 s and 72 °C for 30 s; a final extension at 72 °C for 7 min. The success of amplification was determined by electrophoresis on a 1% agarose gel. The fluorescent PCR products were analyzed through capillary electrophoresis on an ABI 3730XL DNA Sequencer (Applied

Biosystems, Foster City, CA, USA) at the Sangon Biotech Corporation (Shanghai, China), and the genotypes were obtained using GeneMarker (SoftGenetics, State College, PA, USA).

For population genetics analyses, the number of alleles (N_A), number of effective alleles (N_E), Shannon's information index (I), number of private alleles (NPA), observed heterozygosity (H_O) and expected heterozygosity (H_E) were estimated using GenAlEx (Peakall and Smouse, 2006). Pairwise F_{ST} values and number of migrants (Nm) were also calculated by GenAlEx. Polymorphism information content (PIC) was calculated using CERVUS (Kalinowski et al., 2007). Tests for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium between microsatellite loci were conducted using GENEPOP (Rousset, 2008). Frequencies of null alleles were estimated by Micro-Checker (van Oosterhout et al., 2004).

For the RAD-seq data of *R. purdomii*, a total of 16,152,280 paired-end reads were generated after quality filtering. After *de novo* assembly of the clean reads, 301,199 contigs with a mean size of 353 bp and a mean GC content of 40.17% were obtained. The software MISA detected 6,853 perfect microsatellite loci in the assembled contigs, 6,714 of which were suitable for primer

design. Finally, of the 60 primer pairs selected for the initial tests, 31 could be amplified with clear bands. In these 31 microsatellite loci, 13 loci presenting reproducible amplification, clear peaks and rich polymorphism were selected for *R. purdomii*.

Of the 13 polymorphic microsatellite loci, five were dinucleotide repeats, three were trinucleotides, three were tetranucleotides and two were hexanucleotides (Table 1). All 13 sequences were submitted to GenBank (MW736532–MW736544). The number of alleles per locus ranged from four (P24, P60) to 18 (P23) over all investigated *R. purdomii* individuals, while polymorphism information content values were from 0.240 (P58) to 0.915 (P23). In three investigated populations of *R. purdomii*, N_A ranged from two to 12, N_E ranged from 1.000 to 8.892, I ranged from 0.000 to 2.320, the H_O ranged from 0.000 to 1.000 and the H_E ranged from 0.000 to 0.888 (Table 2). Of all the loci, locus P23 had the highest NPA, while loci P57, P58 and P60 contained no private alleles. Private alleles were found in all three populations and the LYW population possessed the highest number of private alleles (12). Null alleles were found in the locus P23 in three populations, P28 in the LJS population, P33 in the LYW population, P57 and P58 in the LJJL population, and P59

Table 1. Characterization of microsatellite markers for *Rhododendron purdomii*

Locus	Primer sequences (5'–3')	Repeat motif	Size range (bp)	N_A	PIC	T_a (°C)	GenBank accession no.
P3	F:AAGAATGCTGAAAAATGTCTTCCA R:ACCATCTGGCTTCTTTAGTTTCC	(TC) ₁₆	133–167	13	0.813	52	MW736532
P16	F:GTTCTAAGATCCAAGCCTTCTGG R:AGAACAAATCAAGGACATAAGCG	(TC) ₁₅	97–133	15	0.910	53	MW736533
P23	F:TTGGGTGTCAAAATAAAACCAAG R:GAAGCCAGGAACAGGATAGAATC	(AG) ₁₆	135–207	18	0.915	52	MW736534
P24	F:GTCCTAGGAAAAAGATGCCCTTACA R:CGCTACGAGTGGATTTCAGCTA	(TTC) ₇	118–127	4	0.460	54	MW736535
P28	F:GCTATTTACCTTCTATTGCACGC R:TCTGGACAGAGAGAATATGGACC	(CT) ₁₅	113–157	13	0.802	53	MW736536
P33	F:GTAATACGATGCTATCGCTCCAC R:TCAATTGAATTTCAACACACAC	(CT) ₁₅	121–157	15	0.898	52	MW736537
P41	F:ATGAAATTGAGAGGAACGATTGA R:AAGTCAATCCACAGAGATTCCAA	(AGA) ₈	100–127	7	0.687	51	MW736538
P42	F:GCACGCAAATAATCAAAACATT R:ATAGATTGAAAAACCATCGGACA	(TAA) ₈	101–188	13	0.789	50	MW736539
P46	F:GGGCTTCCAATAGATTTAAGGGT R:CCATACGAGACCTTACCCTGATT	(TTTA) ₅	136–156	6	0.625	54	MW736540
P57	F:TGGGTCTACTTATTCCCAATTT R:AACGTACGACGACCAAGATTTC	(TGTA) ₅	121–141	5	0.647	53	MW736541
P58	F:GGTACACATCGACAAGCTCTCTT R:TCCTTTTCGGCTCTCCTTACTTAT	(CGGGAG) ₅	131–179	6	0.240	54	MW736542
P59	F:TGTTTCGGACAACAAAGAGTATG R:ACCAAATAAAATACAACCTGCGG	(TTTTAT) ₆	125–149	5	0.572	52	MW736543
P60	F:GTAATAGGGTTGGTATGGGGAAG R:ATAATCGAAATGAACGTAAGCCA	(TTCT) ₅	145–157	4	0.596	52	MW736544

N_A , number of alleles; PIC, polymorphism information content; T_a , annealing temperature.

Table 2. Genetic diversity parameters of 13 polymorphic microsatellite loci in three populations of *Rhododendron purdomii*

Locus	LJS (n = 11)							LYW (n = 15)							LJL (n = 17)						
	N_A	N_E	I	H_O	H_E	NPA	Null present	N_A	N_E	I	H_O	H_E	NPA	Null present	N_A	N_E	I	H_O	H_E	NPA	Null present
P3	9	6.205	1.981	1.000	0.839	2	no	4	2.018	0.936	0.467	0.504	0	no	10	5.780	1.994	0.824	0.827	3	no
P16	11	8.345	2.248	0.909	0.880	2	no	9	7.895	2.119	0.933	0.873	0	no	9	6.721	2.035	0.941	0.851	2	no
P23	11	7.118	2.177	0.636	0.860	2	yes	10	7.500	2.147	0.600	0.867	2	yes	12	8.892	2.320	0.706	0.888	3	yes
P24	2	1.198	0.305	0.182	0.165	0	no	2	1.385	0.451	0.333	0.278	0	no	3	2.117	0.805	0.706	0.528	1	no
P28	6	2.916	1.397	0.455	0.657	0	yes	9	5.844	1.948	0.667	0.829	2	no	8	4.699	1.752	0.882	0.787	0	no
P33	9	6.205	1.981	0.636	0.839	0	no	11	7.627	2.199	0.600	0.869	4	yes	8	5.207	1.833	0.941	0.808	0	no
P41	5	3.408	1.384	1.000	0.707	0	no	6	3.516	1.434	1.000	0.716	1	no	6	3.753	1.470	1.000	0.734	1	no
P42	4	2.444	1.053	0.455	0.591	0	no	6	3.214	1.372	0.533	0.689	1	no	7	4.188	1.596	0.706	0.761	0	no
P46	4	2.814	1.162	0.909	0.645	1	no	5	3.147	1.320	0.933	0.682	2	no	3	2.206	0.869	0.588	0.547	0	no
P57	5	2.916	1.277	0.727	0.657	0	no	3	1.613	0.683	0.333	0.380	0	no	3	2.847	1.073	0.412	0.649	0	yes
P58	3	1.322	0.485	0.273	0.244	0	no	1	1.000	0.000	0.000	0.000	0	no	3	1.197	0.355	0.059	0.164	0	yes
P59	5	4.172	1.520	0.364	0.760	1	yes	4	3.285	1.282	0.400	0.696	0	yes	4	2.460	1.114	0.471	0.593	0	no
P60	3	2.142	0.837	0.636	0.533	0	no	4	2.778	1.139	0.600	0.640	0	no	3	2.042	0.829	0.412	0.510	0	no

n , number of sampled individuals; N_A , number of alleles; N_E , number of effective alleles; I , Shannon's information index; H_O , observed heterozygosity; H_E , expected heterozygosity; NPA, number of private alleles.

Table 3. Pairwise F_{ST} values (below diagonal) and number of migrants (Nm) (above diagonal) between three *Rhododendron purdomii* populations

	LJS	LYW	LJL
LJS	*	5.902	4.091
LYW	0.041	*	4.180
LJL	0.058	0.056	*

in the LJS and LYW populations. After a Bonferroni correction for multiple comparisons, no significant deviations from HWE or linkage disequilibrium were found for any loci. Three populations showed moderate differentiation as indicated by pairwise F_{ST} values (Table 3), among which the LJS and LJL populations indicated the highest level of differentiation (0.058) while the LJS and LYW populations showed the lowest (0.041). Nm values between population pairs ranged from 4.091 to 5.902.

In this study, we developed and characterized 13 microsatellite markers for *R. purdomii* based on next-generation sequencing data and successfully validated them in three *R. purdomii* populations. These markers will be useful for future population genetic and evolutionary history studies of *R. purdomii*, which are necessary for the efficient management and conservation of this species.

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Supplementary Table S1. Locality and voucher information of *Rhododendron purdomii*

Population code	n	Locality	Latitude (°N)	Longitude (°E)	Elevation (m)	Voucher accession no.
LJS	11	Laojun Mountain, Luoyang, Henan	33°43'5.66"N	111°38'23.59"E	2170	LY2020051601
LYW	15	Longyuwan, Luoyang, Henan	33°39'37.41"N	111°47'13.31"E	2120	LY2020051701
LJL	17	Laojieling, Nanyang, Henan	33°39'51.48"N	111°46'22.66"E	1800	LY2020082601

n, number of sampled individuals.