Development of 12 Polymorphic Microsatellite Loci in the High Alpine Perennial Primula halleri (Primulaceae)

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PRIMER NOTE

DEVELOPMENT OF 12 POLYMORPHIC MICROSATELLITE LOCI IN THE HIGH ALPINE PERENNIAL *PRIMULA HALLERI* (PRIMULACEAE)[1]

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- *Premise of the study: Primula halleri* is a high alpine endemic species with a patchy distribution in Central and Eastern European mountains. Little is known about the structure of genetic variation and mating system in populations of this species.
- *Methods and Results:* We report on the development of 12 novel, polymorphic microsatellite loci for *P. halleri*. Tests for amplification and polymorphism were performed on 45 individuals sampled from three populations in the Swiss Alps. Interspecific amplification of the loci was evaluated using samples of the closely related species *P. farinosa*, *P. frondosa*, and *P. scotica*.
- *Conclusions:* The results show that the new microsatellite loci will be useful to study the structure of genetic diversity and mating system of *P. halleri*, and possibly its closely related species.

Key words: genetic diversity; microsatellite; *Primula halleri*; *Primula* sect. *Aleuritia*.

Primula halleri J. F. Gmel. (Primulaceae) is a herbaceous perennial species with a distributional range limited to the Alps, Carpathians, and the Rila and Pirin mountains of the Balkan region. In these Central European mountains, *P. halleri* populations are relatively rare and restricted to high alpine habitats between 1500 and 2700 m (Richards, 2003; de Vos et al., 2012). Due to insufficient information for much of the geographic range, *P. halleri* is not formally listed as vulnerable or threatened in the IUCN Red List (Walter and Gillett, 1998), although it is defined as vulnerable or regionally extinct in Switzerland (Moser et al., 2002). Together, *P. halleri*, *P. farinosa* L., *P. frondosa* Janka, and *P. scotica* Hook. form a European clade within *Primula* sect. *Aleuritia* (Guggisberg et al., 2006). *Primula farinosa* has a wide but patchy distribution across Europe, *P. frondosa* is endemic to northeastern Bulgaria, and *P. scotica* is endemic to northern Scotland and the Orkney Islands (Richards, 2003). *Primula* sect. *Aleuritia* exhibits considerable variation in ploidy, but the species sampled here do not show evidence of intraspecific ploidy variation (Guggisberg et al., 2006). *Primula farinosa* and *P. frondosa* are both diploid ($2n = 2x = 18$), *P. scotica* is hexaploid ($2n = 6x = 54$), and *P. halleri* is tetraploid ($2n = 4x = 36$). These four species also exhibit different breeding systems; *P. farinosa* and *P. frondosa* are distylous, while *P. halleri* and *P. scotica* are homostylous (de Vos et al., 2012). Populations of distylous species have individuals of two distinct floral morphs, which differ in the reciprocal positioning of sexual organs and pollen mating type. Homostylous species are composed of individuals carrying only one floral morph and pollen mating type (Richards, 2003).

Here we report the development of 12 polymorphic novel microsatellite loci for *P. halleri*, which will be used in future studies to characterize the geographic structure of genetic diversity and mating system of this species. Additionally, we evaluate the amplification and polymorphism of these loci in the related species of the European clade of *Primula* sect. *Aleuritia*.

METHODS AND RESULTS

Genomic DNA was isolated from one *P. halleri* individual from the Binnergale population in Switzerland (voucher Z-88501; 46°22′53.1″N, 08°13′40.8″E) using the DNeasy Plant Mini Kit (QIAGEN, Hombrechtikon, Switzerland) according to the manufacturer's standard protocol. This DNA sample was sent to the Cornell Evolutionary Genetics Core Facility (https://cores.lifesciences.cornell.edu/brcinfo/) for the development of microsatellite loci. Briefly, genomic DNA was digested with two restriction enzymes, adapters were ligated, and the library was cloned into a pUC19 plasmid. The library was enriched for genomic fragments containing microsatellite loci using a standard pull-down method with streptavidin-coated magnetic beads and 3′-biotinylated oligonucleotides carrying repeat motifs $(TA)_{17}$, $(GA)_{17}$, $(TC)_{17}$, $(TTC)_7$, $(TACA)_7$, and $(TCAA)_7$. The enriched library was amplified and cloned, and 32 positive library colonies were selected for sequencing. From the sequence of these 32 colonies, 28 microsatellite loci were identified and primers were designed to amplify products ranging from 100 to 500 bp using Primer3 version 3.0 software (Rozen and Skaletsky, 2000).

Each primer pair was tested for amplification using a test panel of four *P. halleri* individuals from each of three populations in Switzerland (see Table 1). Genomic DNA was isolated as described above, and PCR was performed in 25-μL reactions containing: 10–30 ng of DNA, 2.5 μL of 10× reaction buffer, 2 μL of $MgCl_2$ (50 mM), 1.25 μL of each dNTP (10 mM), 0.5 μL of both forward and reverse primer (10 μM), and 0.25 μL of *Taq* DNA polymerase (5 U/μL; Bioline GmbH, Luckenwalde, Germany). PCR was performed in a Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) under the following conditions: initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 1 min, 55–57°C for 1 min (see Table 1), and 72°C for

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TABLE 1. Primer sequences and characteristics of microsatellite loci developed in *Primula halleri*.

Locus	GenBank accession no.	Primer sequences (5′–3′)	Repeat motif	Size range (bp)[a]	T_a (°C)
H1	KC928405	F: M13-TCGTTCAGTACTCTATTCTCCC R: ACCACGTCAAAATCATAAAACCG	$(CT)_{10}(AT)_7$	268–274	55
H4-1[b]	KC928407	F: M13-CGCAAAGACATCTCCGCTC R: AGAGCAAATCGAAGTTGGCG	$(AG)_{10}$	110–126	55
H4-2[b]	KC928407	F: M13-CGCAAAGACATCTCCGCTC R: AGAGCAAATCGAAGTTGGCG	$(AG)_{10}$	130–142	55
H5	KC928408	F: M13-CCTGGGTTTGTTGGCTTCC R: TTCCACCTTCGAATTGGCG	$(AG)_9(GT)_7$	204–222	57
H8	KC928409	F: M13-AGTCTCCAACCCAAACCCC R: GAGTTCCTCCTCTAGCAGCC	$(CTTT)_7$	200–216	55
H9	KC928410	F: M13-GAGTTCCTCCTCTAGCAGCC R: CCAACCCAAACCCCATCC	$(AAAG)_7$	190–210	55
H10	KC928411	F: M13-GGAATGCCTTTGACTAATGGG R: GTCTGGTATCTCATCATCTCGC	$(ATGT)_6$	168–176	55
H11[c]	KC928412	F: M13-GGAATGCCTTTGACTAATGGG R: GTCTGGTATCTCATCATCTCGC	$(AGTT)_6$	227	55
H12	KC928413	F: M13-CGAACTTTCAGGTCACCCC R: ACTCCAGTTTGATGCCATTTCTC	$(ATGT)_5$	190–195	55
H13[c]	KC928414	F: M13-GGAATGCCTTTGACTAATGGG R: GTCTGGTATCTCATCATCTCGC	$(CT)_9$	253	60
H15	KC928415	F: M13-CTTCCTTGCCGTCTGCTTG R: GATACCGATCCGCGTCCTC	$(AG)_9$	248–260	55
H20	KC928418	F: M13-CCCGACACAAAGTTAAAACACG R: CGTAGATTGAAATGAACGTAAACAG	$(CT)_{13}$	202–212	55
H21	KC928419	F: M13-GCCTTGGAAAGGAGAATGAGG R: GCCAAGGTTCTTGCGTACAG	$(AG)_{11}$	197–201	55
H22	KC928420	F: M13-TCCTGATGGATAAAACACGAGC R: GGCCTCACCACTGGATTTC	$(CT)_{10}$	178–186	55

Note: M13 = TGTAAAACGGCCAGT; T_a = annealing temperature.

[a]Fragment size range based on 45 samples collected from three populations in Switzerland. Geographical coordinates and voucher information: Binnergale = 46°22′53.1″N, 08°13′40.8″E, voucher Z-88501; Saflischmatta = 46°20′53.6″N, 08°09′17.8″E, voucher Z-88502; Schmidigewyssi = 46°23′11.9″N, 08°14′57.95″E, voucher Z-88503.

[b]Locus H4 has two distinct allelic size ranges (110–126 bp and 130–142 bp), and is treated as two distinct diploid loci (H4-1 and H4-2) here.

[c]H11 and H13 were not polymorphic and thus were not used to analyze genetic diversity.

1 min, with a final extension at 72°C for 5 min. Successful amplification and quantification of fragment length was determined on a QIAxcel Advanced electrophoresis system using the DNA High Resolution kit and ScreenGel software (QIAGEN).

Thirteen of the 28 primer pairs tested consistently amplified a single locus and were thus selected to evaluate polymorphism in the 12 individuals of the test panel used above. PCR was performed in 10-μL reactions containing 10–30 ng of genomic DNA, 2.0 μL of 5× GoTaq buffer, 0.5 μL of each dNTP (10 mM), 0.8 μL of $MgCl_2$ (50 mM), 0.2 μL of the forward primer with an M13(–21) tail at the 5′ end (10 μM), 0.5 μL of the reverse primer (10 μM), 0.5 μL of fluorescently labeled M13(–21) primer (FAM, NED, PET; 10 μM), and 0.1 μL of GoTaq DNA polymerase. Amplifications were performed in a BioRad C1000 Thermal Cycler (Bio-Rad Laboratories) following the two-step method described by Schuelke (2000) with cycling conditions as described above and an

TABLE 2. Summary statistics of microsatellite polymorphism determined by screening 45 *Primula halleri* samples from three populations in Switzerland.[a]

	Binnergale (N = 15)			Saflischmatta (N = 15)			Schmidigewyssi (N = 15)			
Locus	A	H_o	H_e[b]	A	H_o	H_e[b]	A	H_o	H_e[b]	A_T (N = 45)
H1	5	0.667	0.724	2	0.583	0.485	5	0.500	0.794	5
H4-1	4	0.667	0.644	1	0.000	0.000	3	0.130	0.126	6
H4-2	2	0.286	0.375	4	0.667	0.493	3	0.391	0.414	4
H5	4	0.417	0.735*	6	0.533	0.736*	6	0.533	0.817	6
H8	2	0.083	0.083	1	0.000	0.000	2	0.067	0.067	3
H9	2	0.067	0.067	1	0.000	0.000	3	0.071	0.071	4
H10	3	0.467	0.581	3	0.273	0.446	3	0.615	0.683	3
H12	1	0.000	0.000	2	0.200	0.443	2	0.071	0.071	2
H15	5	0.467	0.469	3	0.273	0.400	3	0.308	0.500	5
H20	5	0.733	0.721	8	0.667	0.867	4	0.714	0.703	8
H21	2	0.385	0.410	2	0.200	0.186	3	0.214	0.528*	3
H22	3	0.357	0.604	2	0.083	0.538*	3	0.462	0.567	4

Note: A = number of alleles; A_T = total number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size for each population.

[a]Geographical coordinates and voucher information: Binnergale = 46°22′53.1″N, 08°13′40.8″E, voucher Z-88501; Saflischmatta = 46°20′53.6″N, 08°09′17.8″E, voucher Z-88502; Schmidigewyssi = 46°23′11.9″N, 08°14′57.95″E, voucher Z-88503.

[b]Statistically significant departure from Hardy–Weinberg equilibrium is indicated with $*P < 0.05$.

TABLE 3. Cross-amplification of 11 novel microsatellite loci in *Primula farinosa*, *P. frondosa*, and *P. scotica.*

Species[a]	H1	H4	H5	H8	H9	H10	H12	H15	H20	H21	H22
P. farinosa (16)	–	P [9]	+	M	–	P [6]	–	+	M	P [8]	P [5]
P. frondosa (8)	–	M	+	+	–	–	–	+	M	M	M
P. scotica (12)	–	M	P [2]	M	P [2]	M	M	+	+	+	M

Note: + = successful amplification but no information polymorphism; – = failed amplification; M = successful amplification but locus is monomorphic; P = polymorphic locus showing the number of alleles identified in brackets.

[a]The number of individuals genotyped for each species is shown in parentheses.

additional 10 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min prior to the final extension. Amplified products from as many as three loci were multiplexed for capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA) using GeneScan 500 LIZ as internal standard. Fragment lengths were determined using GeneMapper software version 4.1 (Applied Biosystems).

Amplicons from 11 of the 13 microsatellite loci showed clear evidence of polymorphism, while two loci were monomorphic in the 12 individuals tested. Only one (H4) of the 11 polymorphic loci was found to carry more than two alleles per individual. In all of the samples genotyped at this locus, no more than two alleles were observed within two distinct allelic size ranges (110–126 bp and 130–142 bp). Thus, following Saltonstall (2003), we treated this locus as if it were two distinct diploid loci (H4-1 and H4-2; see Table 1). The remaining 10 loci appear to be amplifying only one of the two diploid copies of the tetraploid genome, suggesting that homeologous loci in this species may be quite divergent at the sequence level (Soltis et al., 2010).

The 12 polymorphic loci were used to estimate genetic diversity parameters and deviation from Hardy–Weinberg equilibrium in 45 individuals chosen randomly from the three Swiss populations described in Table 1. Genetic diversity statistics (e.g., observed [H_o] and expected heterozygosity [H_e], respectively) were calculated in GENEPOP version 4.0 (Rousset, 2008; Table 2). The total number of observed alleles per locus in all populations ranged from two to eight, per locus H_o ranged from 0 to 0.733, and H_e ranged from 0 to 0.867.

Cross-amplification was tested in closely related species using 16 individuals of *P. farinosa* (voucher no. Z-88504), eight individuals of *P. frondosa* (voucher no. Z-88505), and 12 individuals of *P. scotica* (voucher no. Z-88506). Because locus H4 does not show more than two alleles per individual in these three species (unlike the results for *P. halleri*), it is treated as one locus in the cross-amplification results. The results presented in Table 3 show that of the 11 loci tested, only eight consistently amplify across species boundaries.

CONCLUSIONS

We describe here the development of 12 polymorphic microsatellite loci for the high alpine endemic species *P. halleri*. These loci make possible future studies focused on estimating parameters of the mating system and genetic structure of *P. halleri* populations. Successful cross-amplification suggests that some of these loci could be applied to estimate the genetic diversity in other species of *Primula* sect. *Aleuritia*.

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