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PRIMER NOTE

HIGHLY POLYMORPHIC MICROSATELLITE MARKERS IN *PULSATILLA VULGARIS* (RANUNCULACEAE) USING NEXT-GENERATION SEQUENCING[1]

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- *Premise of the study:* We developed novel microsatellite markers for the perennial plant *Pulsatilla vulgaris* (Ranunculaceae) to investigate the effects of fragmentation on gene flow in this imperiled species.
- *Methods and Results:* We identified microsatellites and developed primers based on 454 shotgun sequences. We identified 14 markers that were polymorphic and produced clean bands. Of these, eight could be analyzed as diploids. Genotyping of 97 individuals across two populations revealed these markers to be highly polymorphic with seven to 17 alleles per locus and observed heterozygosity from 0.41 to 0.83.
- *Conclusions:* The markers are highly informative and will be used to test if the reintroduction of shepherding in southern Germany improves genetic connectivity among fragmented populations of *P. vulgaris.* The combination of diploid and tetraploid markers presented here will be useful in resolving the polyploidization history of this and related species.

Key words: 454 sequencing; microsatellites; *Pulsatilla vulgaris*; Ranunculaceae; simple sequence repeat (SSR) markers.

Pulsatilla vulgaris Mill. (Ranunculaceae) is an early-flowering perennial herb of conservation concern and a flagship and specialist species of calcareous grasslands across central Europe, ranging from France in the south to Sweden at its northern limit (Wells and Barling, 1971; Pfeifer et al., 2002). Over the last century, *P. vulgaris* has witnessed rapid decline and local extirpation across its range, and is listed as "near threatened" by the International Union for Conservation of Nature (IUCN, 2014). Its decline is linked to the abandonment of traditional grazing practices, which has resulted in the severe loss and fragmentation of calcareous grasslands following afforestation (Butaye et al., 2005), and increased above-ground competition from coarse grasses (Walker and Pinches, 2011). Knowledge of the landscape-scale distribution of genetic variation is required to create effective management plans for fragmented populations, and evaluations of genetic diversity and inbreeding will allow the identification of populations that are at highest risk of extirpation. This, however, requires genetic markers with high resolution such as microsatellites. No such markers are yet available for *P. vulgaris*, and any potential genetic analyses are complicated as this species is suspected to be an allotetraploid ($2n = 32$; Böcher, 1934). We therefore de novo developed microsatellite markers for this calcareous plant species, specifically selecting loci that can be analyzed as diploid or double-diploids in downstream genetic analyses.

METHODS AND RESULTS

We extracted genomic DNA from homogenized leaf tissue from two populations in the Franconian Alb, Germany (A25: 48°57′38.7″N, 10°56′32.2″E; A03: 48°59′51.7″N, 11°3′35.4″E), using the QIAGEN DNeasy Plant Mini Kit (QIAGEN, Mississauga, Ontario, Canada) following the manufacturer's protocol. A voucher from the study area is deposited at the Herbarium at the University of Toronto Mississauga (TRTE; voucher no.: BR-07052015; locality: Laubenthal, Landkreis Weissenburg, Germany; 48°58′48.7130″N, 11°03′14.2254″E). DNA from five individuals was mixed, and a 4-µL sample was sent to LGC Genomics (Berlin, Germany) for 454 shotgun sequencing on 1/8 of a plate using GS FLX Titanium (Roche, 454 Life Sciences, Branford, Connecticut, USA). In total, 92,833 reads were returned and assembled into 4088 contigs. We used MSATCOMMANDER (version 1.0.8; Faircloth, 2008) to identify di-, tri-, and tetranucleotide microsatellites to develop primers in regions flanking the identified microsatellites. A total of 457 microsatellites were identified, and 76 suitable primer pairs could be designed. We amplified 75 of these pairs by PCR in an initial screen of 10 individuals, and of these, 18 showed clear peaks and 14 of these were polymorphic (Table 1). Eight markers showed evidence of disomic inheritance (e.g., had a maximum of two alleles per individual) and were retained for further analysis (Table 2).

We amplified these eight microsatellites in two multiplex reactions in 97 individuals with the QIAGEN Multiplex PCR Kit (QIAGEN) in 10-µL final reaction volumes with 0.2 µM of each primer, 4.6 µL of Multiplex Mix, 1.2 µL of DNase-free water, and 5–30 ng of template DNA. Thermocycling conditions for Multiplex A (Table 1) strictly followed the manufacturer's protocol. Multiplex B was amplified using a touchdown procedure with an initial denaturation at 95°C for 15 min; 10 cycles of denaturation at 95°C for 60 s, annealing at

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TABLE 1. Characteristics of 18 de novo–developed microsatellite markers for *Pulsatilla vulgaris.*

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Multiplex	Fluorescent dye[a]	Allele size range (bp)	*A*	Marker ploidy	GenBank accession no.
PV2	F: GTTTGCGATGATCATGAAGTGC	$(AAAT)_6$	55	A	HEX	410–426	4	diploid	KP885677
	R: AGAACTTCCACGAAACAAGGC								
PV7	F: ACCGCCAACATAGCAAACAC	$(AG)_{10}$	TD 65–55	B	6-FAM	326–362	8	diploid	KP885678
	R: ACCCACCACAACTTGAGAGG								
PV9	F: GAACTAACCTGCTTCCGTGC	$(AG)_{11}$	55	single	HEX	283–309	12	tetraploid	KP885679
	R: GCAAGCCAAAGTCCACTCTG								
PV16	F: TTGTTGGGTCGCTTGAGAAG	$(AC)_{12}$	55	single	6-FAM	330–362	11	tetraploid	KP885680
	R: ATCACTTGTAGCCTCCGGTC								
PV18	F: CCATGAACCCTTGTCACGAG	$(AC)_{10}$	55	single	6-FAM	415–451	11	tetraploid	KP885681
	R: AGGACCCAAGATCTCAAGCC								
PV27	F: AACCCTTGCACACCAACTTG	$(AG)_8$	55	A	6-FAM	392–454	5	diploid	KP885682
	R: AATCTTATCTGGGCGGGAGG								
PV32	F: CATGCCTTTGTACCCTGCTG	$(AAG)_9$	55	single	6-FAM	356–422	8	tetraploid	KP885683
	R: ACGACCTTTGTTTGACCGTC								
PV33	F: AGCCTTGGTGTTATTTGGGC	$(AC)_9$	TD 65–55	B	6-FAM	442–452	8	diploid	KP885684
	R: GCTCACTTTGACCAACTCCC								
PV44	F: GTATGTGTGTGCCAAGGGTC	$(ATC)_{10}$	55	single	HEX	429	1	—	KR109213
	R: TGCTTAAGAGTAGCATGCCG								
PV48	F: CGGGCTGTAACTGATGCTTC	$(AG)_{10}$	55	single	HEX	235	1	—	KR109214
	R: GCAAGTGAGCCAGTTCCATC								
PV50	F: GATGGTGATGAGGGTTTGGG	$(AAT)_{12}$	55	single	HEX	406–466	10	tetraploid	KP885685
	R: TGCCACCTACTTTCCACACC								
PV52	F: TTGGTCAAATGGTCGCAACG	$(AG)_{10}$	55	A	6-FAM	262–280	7	diploid	KP885686
	R: GGTGCTCAAGATTATCGGGC								
PV54	F: TACTCGCGACTGACAAGCTC	$(AT)_{10}$	55	single	6-FAM	204	1	—	KR109215
	F: TTGTGGGAGTGGAAGGAACC								
PV56	F: CGTCACGTGTGGGCTAAATC	$(AC)_{11}$	TD 65–55	B	6-FAM	236–270	9	tetraploid	KP885687
	R: TCCTTTGTACTCTTCCAACGG								
PV57	F: GTGCAAATTACTCACACTGCAG	$(AC)_{10}$	55	single	6-FAM	419–429	5	tetraploid	KP885688
	R: TGCTCGAAACCATAAGTCTGC								
PV59	F: TGGACAACGTACCTTACACAG	$(AG)_{10}$	55	single	HEX	171	1	—	KR109216
	R: AGCTTCCACACCTCAACTGG								
PV64	F: AGCAATCTCTGTCCGGCTAG	$(AC)_{13}$	TD 65–55	B	HEX	368–404	7	diploid	KP885689
	R: GTGACTGCAGATGTTGGTGG								
PV65	F: ACGGACGCAAATCTTCTGAC	$(AG)_{10}$	55	A	6-FAM	a: 114–138	6	diploid	KP885690
	R: GAGAACGAACGCCATGACAG					b: 150–174	6	diploid	

Note: *A* = total number of alleles based on initial screen of 10 individuals; T_a = annealing temperature.
[a]Fluorescent dye on forward primers used for fragment analysis.

65–55°C for 60 s, and extension at 72°C for 90 s; 25 cycles of 95°C for 60 s, 55°C for 60 s, and 72°C for 90 s; and a final extension at 72°C for 10 min. PCR products were diluted 20× and 1–2 µL of product was loaded to an ABI 3730xl Capillary Sequencer (Applied Biosystems, Burlington, Ontario, Canada) using GeneScan 500 LIZ (Life Technologies, Burlington, Ontario, Canada) size standard for fragment analysis at the Centre for Applied Genomics (The Hospital for Sick Children, Toronto, Canada). Genotyping was performed in GeneMarker 2.4.0 (SoftGenetics, State College, Pennsylvania, USA).

TABLE 2. Results from HWE exact tests for diploid markers developed for *Pulsatilla vulgaris* in two populations.[a]

		A03 (*n* = 41)			A25 (*n* = 56)		
Locus	*A*	H_o	H_e	P[b]	H_o	H_e	P[b]
PV2	8	0.67	0.75	0.87	0.69	0.75	1.00
PV7	16	0.59	0.64	0.87	0.76	0.86	0.43
PV27	17	0.83	0.87	0.86	0.79	0.86	0.88
PV33	7	0.59	0.58	0.32	0.71	0.70	0.66
PV52	13	0.75	0.78	0.86	0.74	0.84	0.30
PV56	13	0.55	0.69	0.24	0.65	0.87	<0.001
PV64	11	0.41	0.77	<0.001	0.52	0.79	0.003
PV65a	13	0.41	0.79	<0.001	0.64	0.64	0.62
PV65b	13	0.68	0.67	0.54	0.75	0.77	0.62

Note: *A* = total number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals sampled.
[a]Geographic coordinates for the populations are A03: 48°59′51.7″N, 11°3′35.4″E; A25: 48°57′38.7″N, 10°56′32.2″E.
[b]*P* values are corrected for multiple testing.

All diploid markers except for PV65 amplified a maximum of two alleles per individual. PV65 amplified a maximum of four alleles per individual in two sets of size-separated fragments (PV65a and PV65b, Table 1). Loci showed high levels of polymorphism with seven to 17 alleles per locus, and observed heterozygosity from 0.41 to 0.83 per locus (Table 2). Exact tests performed in GENEPOP 4.2 (Rousset, 2008) revealed three loci with significant departures from Hardy–Weinberg equilibrium (HWE), but departures were not consistent across populations, except for locus PV64. It is possible that selfing in one or both populations, or the presence of population substructure (i.e., the Wahlund effect; Wahlund, 1928), led to this homozygote excess. Test for departures from linkage equilibrium expectations in GENEPOP showed that all pairs of loci were unlinked (results not shown).

We used open-pollinated progeny arrays to confirm that the identified "diploid" loci followed a disomic pattern of inheritance by amplifying the eight loci in 10 seeds collected from each of 15 mother plants. For PV65, all offspring genotypes had maternal contributions from both of the size-separated amplicons, suggesting that PV65a and PV65b represent independent genomic complements with disomic inheritance. For the remaining loci, disomic inheritance was confirmed by the observation of a maximum of two alleles for all offspring, with each offspring sharing an allele with the maternal genotype.

CONCLUSIONS

These new microsatellite markers are highly informative and will be used to quantify gene flow across fragmented

populations of *P. vulgaris* in southern Germany. We will test whether the reintroduction of shepherding is a suitable conservation measure to improve genetic connectivity among populations of this species. The combination of diploid and tetraploid markers presented here will be useful in clarifying the polyploidization history of this and related species.

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