Fourteen Polymorphic Microsatellite Markers for the Threatened Arnica montana (Asteraceae)

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PRIMER NOTE

FOURTEEN POLYMORPHIC MICROSATELLITE MARKERS FOR THE THREATENED *ARNICA MONTANA* (ASTERACEAE)[1]

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- *Premise of the study:* Microsatellite markers were developed to investigate population genetic structure in the threatened species *Arnica montana*.
- *Methods and Results:* Fourteen microsatellite markers with di-, tetra-, and hexanucleotide repeat motifs were developed for *A. montana* using 454 pyrosequencing without and with library-enrichment methods, resulting in 56,545 sequence reads and 14,467 sequence reads, respectively. All loci showed a high level of polymorphism, with allele numbers ranging from four to 11 in five individuals from five populations (25 samples) and an expected heterozygosity ranging from 0.192 to 0.648 across the loci.
- *Conclusions:* This set of microsatellite markers is the first one described for *A. montana* and will facilitate conservation genetic applications as well as the understanding of phylogeographic patterns in this species.

Key words: *Arnica montana*; microsatellites; population genetics; pyrosequencing.

Arnica montana L. (Asteraceae) is an important medicinal plant species (in traditional, classical, and homeopathic medicine) that is endemic to heath and grassland habitats of Europe. It is a predominantly self-incompatible, insect-pollinated species that is commonly visited by syrphid flies (Luijten et al., 1996, 2000). *Arnica montana* is still common in some mountain areas, but habitat fragmentation, eutrophication, acidification, abandonment of traditional agricultural practices, and flower picking for medicinal purposes has led to a rapid decrease in population sizes during the past decades, especially at lower altitudes (Dueck and Elderson, 1992; de Graaf et al., 1998; Fabiszewski and Wojtun, 2001; Falniowski et al., 2013). Although its threat category has been assessed as "least concern" in the 2014 IUCN Red List, the general trend is that population sizes are declining (Falniowski et al., 2013) and the species is classified as "endangered" in the national red list of vascular plants of Germany (Korneck et al., 1996).

Analysis of population genetic structure, gene flow, and levels of inbreeding will provide valuable insights for the effective conservation of declining populations. Here, we present a set of microsatellite markers for *A. montana* as a tool for population and conservation genetic studies.

METHODS AND RESULTS

***Plant material and DNA extraction*—**Plant material of *A. montana* was collected in five different geographic regions comprising the lowlands and midelevation mountain ranges of Germany (German states of Saxony, Mecklenburg-Western Pomerania, Brandenburg, and Baden-Württemberg) and the Italian Alps (South Tyrol). The populations were chosen to represent the species' spatial distribution in central Europe from the Southern Alps up to the Baltic. Of each population, five individuals were collected and genomic DNA was extracted from silica gel–dried leaves using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Purified DNA was eluted in a final volume of 100 µL, and final concentration was quantified using a NanoDrop ND-1000 Spectrophotometer (Peqlab, Erlangen, Germany) and gel electrophoresis.

***Microsatellite marker development*—**The GS FLX Titanium Rapid Library Preparation Kit and the GS Junior Titanium Sequencing Kit (Roche 454 Life Sciences, Branford, Connecticut, USA) were used to generate a shotgun library of genomic DNA and sequencing on the Roche GS Junior 454 System (Roche 454 Life Sciences). The 454 pyrosequencing yielded a total of 56,545 sequence reads ranging from 40 to 1195 bp with an average length of 476 bp.

To obtain a larger number of polymorphic markers, we additionally developed and sequenced a microsatellite-enriched library. Size-selected genomic DNA fragments were enriched for microsatellite content by using magnetic streptavidin beads and biotin-labeled GATA, GTAT, AAAC, and AAAG repeat oligonucleotides. The enriched library was prepared and analyzed on a Roche 454 platform using the GS FLX Titanium reagents according to the manufacturer's manual. This produced 14,467 sequence reads ranging from 80 to 550 bp with an average length of 459 bp.

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***Microsatellite screening*—**The screening of the generated DNA sequences from the nonenriched shotgun library for microsatellite loci and the design of oligonucleotide PCR primers was conducted with QDD software version 2.1 (Meglecz et al., 2010). A total of 1197 microsatellite loci, containing di-, tri-, tetra-, penta-, and hexanucleotide repeat motifs, with a GC content of 35–60% and a melting temperature (T_m) ranging from 57°C to 60°C were identified. A total of 60 microsatellite loci with at least five repeats, including six loci with hexanucleotide repeats, two loci with pentanucleotide repeats, three loci with tetranucleotide repeats, 35 loci with trinucleotide repeats, and 14 loci with dinucleotide repeats, were tested for proper PCR amplification using genomic DNA.

The sequences obtained with the enriched library contained 676 sequences with microsatellite inserts. Using a Primer3-based (Koressaar and Remm, 2007; Untergasser et al., 2012) custom-made software (property of Ecogenics, Zurich, Switzerland), 361 reads were suitable for designing primers with a GC content of 20–80% and a T_m ranging from 57°C to 63°C. Of these loci, 24 were selected for screening. All of the selected loci had at least six repeat units in the case of tri- and tetranucleotide repeat motifs or at least 10 repeat units in the case of dinucleotide repeat motifs.

For assessing optimal annealing temperatures, a gradient PCR with annealing temperatures varying between 53°C and 63°C was carried out for each primer pair in a 15.6-µL reaction volume containing 20–40 ng DNA, 0.16 µM of each forward and reverse primer (Eurofins MWG Operon, Ebersberg, Germany), 1× TaqBuffer S (PeqLab), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, and 0.03 units Hot *Taq* polymerase (PeqLab). For all primer pairs, the temperature profile of the PCR was as follows: 95°C for 1 min; 30 cycles of 94°C for 1 min, 58°C ± 5°C for 1 min, and 72°C for 1 min; plus a final extension of 72°C for 7 min. Amplification of PCR was evaluated by visual inspection of gel electrophoresis.

For assessing polymorphism of the microsatellite markers, genomic DNA templates from 25 *A. montana* specimens of five distinct populations were used (five specimens per population; Appendix 1). PCR amplification was performed using forward primers labeled with fluorescent dyes (FAM, YakimaYellow, ATTO 565, and ATTO 550) and reverse primers (Armo01–Armo03) with a 7-bp (GTTTCTT) extension at the 5′-end to reduce stutter bands ("PIG-tailing"; Brownstein et al., 1996). The PCR products were sent to Macrogen Europe (Amsterdam, The Netherlands) for fragment analysis on an ABI 3730 sequencer.

Of 60 markers that were tested, only three markers turned out to be polymorphic: Armo01, Armo02, and Armo03 (Table 1). The remaining microsatellite markers were either monomorphic, failed to amplify consistently, or were difficult to score unambiguously.

Of 24 selected candidate primer pairs from the microsatellite-enriched library, 11 (Arm01–Arm11) were found to be polymorphic, with at least four alleles per locus (Table 1).

***Microsatellite marker data analysis*—**Genotypes were scored by analyzing the electropherograms from the fragment analysis using GeneMarker version 1.95 (SoftGenetics, State College, Pennsylvania, USA). The fragments were scored relative to a GeneScan 500 LIZ Size Standard (Applied Biosystems, Carlsbad, California, USA). Descriptive statistics (number of alleles, observed and expected heterozygosities) were calculated with GenAlEx 6.5 (Peakall and Smouse, 2006). All 14 loci were polymorphic with four to 11 alleles across 25 individuals from five different populations with a total number of 102 alleles. Observed heterozygosity values ranged from 0.200 to 0.880 and expected heterozygosity from 0.192 to 0.648. Linkage disequilibrium was tested using GENEPOP (Raymond and Rousset, 1995), and the test revealed no significant association between the loci. CERVUS 3.0 (Marshall et al., 1998) was used to calculate the polymorphism information content (PIC) (Table 2) and deviations from Hardy–Weinberg equilibrium. Three loci (Arm04, Arm07, and Arm10) significantly deviated from Hardy–Weinberg equilibrium after Bonferroni correction.

CONCLUSIONS

The 14 microsatellite markers reported in this study, developed using both enriched and nonenriched DNA libraries, are

TABLE 1. Characteristics of the 14 microsatellite loci for *Arnica montana*.

Locus	Primer sequences (5′–3′)	Fluorescent label	Repeat motif	T_a (°C)	Allele size range (bp)	GenBank accession no.
Arm01[e]	F: TTACAATGTGTGTGCCTGCG*	6-FAM	$(CATA)_{13}$	58	112–160	KM516768
	R: AGGAGTTCTCATTTGATCCTTTCC					
Arm02[e]	F: AACACACATCCACGTTTGGC*	Yakima Yellow	$(TACA)_8$	58	177–249	KM516769
	R: AACCGTGCATCATTCTGTGG					
Arm03[e]	F: TCAGGAAGTTTGCCCCTCTC*	6-FAM	$(TACA)_7$	58	155–175	KM516770
	R: TTGCTGCGCAATGGGTTTAC					
Arm04[e]	F: ACTTGCATGTAGAGACGGATG*	ATTO 565	$(CATA)_{18}$	58	161–237	KM516771
	R: GTGGAGTCGGAACTTAACCG					
Arm05[e]	F: ACTGTCACCTAGGGGTGTTC*	ATTO 565	$(AACA)_7$	58	174–186	KM516772
	R: TAAGCGGGGAGTCTTTCTGG					
Arm06[e]	F: TGTCGCCTCAATCCTTGGTG*	Yakima Yellow	$(ACAT)_8$	58	182–234	KM516773
	R: GCTGAAGTCCTTCCTTGGAC					
Arm07[e]	F: ACATGACGCAAAAAGCGTAG*	Yakima Yellow	$(TATG)_{10}$	58	197–229	KM516774
	R: CCATGTTACCACCATGTCGC					
Arm08[e]	F: AGATGAGGTTCTTGCAGCATC*	ATTO 550	$(TGTA)_9$	58	131–155	KM516775
	R: TGCTTGCAGTTGAAGTAAAGGG					
Arm09[e]	F: TAGGCGTGAGTTTGTACTCG*	6-FAM	$(TATG)_{10}$	58	223–239	KM516776
	R: AAGCGTGTTAACTTCGTGAG					
Arm10[e]	F: ACCAGCTGACTCTCTTTCCG*	ATTO 565	$(CATA)_9$	58	125–181	KM516777
	R: CAAGGATGAACATCGGCCTC					
Arm11[e]	F: TGGCACAAGGTATGTGTTGC*	ATTO 550	$(GT)_{12}$	58	154–162	KM516778
	R: TCTTCGACCGAATGTTTTCACC					
Armo01[n]	F: TTCCTTGCTCATTTCAAGATTC*	6-FAM	$(AG)_9$	58	131–159	KM516779
	R: GTTTCACACTCAAAGGAGCG					
Armo02[n]	F: GGTTTGAACACGAGATAGCG*	ATTO 550	$(AT)_8$	58	207–215	KM516780
	R: ACAAACTTCCTGTTGTCCCG					
Armo03[n]	F: TCAAACAGTCACCAGCAACC*	ATTO 565	$(ACCTGG)_5$	58	205–223	KM516781
	R: CAGAGGCTGCAACCCTAATG					

Note: F = forward primer sequence; R = reverse primer sequence; T_a = optimal annealing temperature.
*Labeled primer.
[e]Enriched library.
[n]Nonenriched library.

TABLE 2. Genetic properties of the developed microsatellites of specimens from five populations of *Arnica montana*.

	AM01 ($N = 5$)			AM07 ($N = 5$)			AM15 ($N = 5$)			AM20 ($N = 5$)			AM21 ($N = 5$)			Total ($N = 25$)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	PIC
Arm01	4	0.400	0.660	3	1.000	0.580	3	0.600	0.660	6	0.600	0.800	3	0.600	0.540	10	0.640	0.648	0.826
Arm02	4	0.800	0.580	2	1.000	0.500	3	0.800	0.620	7	1.000	0.780	3	0.600	0.460	11	0.840	0.588	0.782
Arm03	2	1.000	0.500	2	0.200	0.180	2	0.200	0.180	3	0.600	0.620	1	0.000	0.000	6	0.400	0.296	0.388
Arm04	4	0.800	0.640	3	1.000	0.580	1	0.000	0.000	7	0.800	0.820	3	0.400	0.540	11	0.600	0.516	0.815
Arm05	2	0.600	0.420	2	1.000	0.500	2	0.800	0.480	2	0.200	0.180	4	0.600	0.580	4	0.640	0.432	0.564
Arm06	5	0.600	0.780	2	0.800	0.480	2	0.600	0.420	7	1.000	0.820	3	0.600	0.580	9	0.720	0.616	0.774
Arm07	2	0.600	0.420	1	0.000	0.000	3	0.400	0.340	2	0.200	0.500	5	0.800	0.740	7	0.400	0.400	0.770
Arm08	3	0.600	0.580	2	1.000	0.500	4	1.000	0.660	4	1.000	0.640	5	0.800	0.720	6	0.880	0.620	0.780
Arm09	2	0.600	0.420	2	1.000	0.500	2	0.200	0.420	2	0.600	0.420	3	0.800	0.560	4	0.640	0.464	0.581
Arm10	4	0.600	0.580	1	0.000	0.000	3	0.400	0.660	6	0.800	0.760	2	0.200	0.180	10	0.400	0.436	0.830
Arm11	2	0.800	0.480	2	0.200	0.180	2	0.200	0.180	4	0.600	0.480	2	0.200	0.180	4	0.400	0.300	0.550
Armo01	3	0.600	0.460	2	0.800	0.480	2	0.600	0.420	5	0.800	0.680	5	0.800	0.780	11	0.720	0.564	0.760
Armo02	4	1.000	0.720	2	0.800	0.480	2	0.200	0.180	4	0.400	0.660	3	0.400	0.580	5	0.560	0.524	0.616
Armo03	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	4	0.600	0.480	2	0.400	0.480	4	0.200	0.192	0.280

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size; PIC = polymorphism information content.

highly polymorphic with allele numbers ranging between four and 11. These markers will provide a valuable tool for genetic studies on large-scale and fine-scale population genetic structure, as well as on levels of inbreeding in *A. montana*. This study also demonstrates that the use of microsatellite-enriched DNA libraries allows a more efficient development of microsatellite loci as shown before (Lepais and Bacles, 2011).

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Appendix 1. Locations and voucher information for populations of *Arnica montana* used in this study.[a]

Population	Locality	Altitude	Geographic coordinates	Collection no.
AM01	Oelsen, Osterzgebirge, Saxony, Germany	632 m a.s.l.	50°47′N, 13°56′E	E. Sossai 01
AM07	Zarrendorf, Stralsund, Mecklenburg-Western Pomerania, Germany	0 m a.s.l.	54°14′N, 13°05′E	E. Sossai 22
AM15	Naturpark Niederlausitzer Heidelandschaft, Brandenburg, Germany	97 m a.s.l.	51°30′N, 13°46′E	E. Sossai 04
AM20	Alps, South Tyrol, Italy	2062 m a.s.l.	46°37′N, 11°56′E	E. Zippel 13214
AM21	Black Forest, Baden-Württemberg, Germany	1424 m a.s.l.	47°52′N, 8°01′E	E. Sossai 14

[a]Vouchers deposited at the herbarium of the Botanischer Garten und Botanisches Museum Berlin-Dahlem, Berlin, Germany.