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Development and characterization of microsatellite markers for *Actaea racemosa* (black cohosh, Ranunculaceae)[1]

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- *Premise of the study:* Microsatellite markers were developed in *Actaea racemosa* to analyze population genetic structure, compare genetic diversity across the species' range, and provide a genetic context for studies of phytochemical variation.
- *Methods and Results:* A total of seven polymorphic loci were screened in 60 individuals from 12 localities. The number of alleles per locus ranged from three to six, and observed heterozygosity ranged from 0.133 to 0.900. Most of the loci tested cross-amplified in *A. pachypoda*, *A. podocarpa*, and *A. rubra*, indicating the utility of these markers for the genus.
- *Conclusions:* These new loci will provide tools for population genetics studies, including the characterization of genetic variation in *A. racemosa* and other eastern North American species of *Actaea*.

Key words: *Actaea racemosa*; black cohosh; medicinal plant; microsatellites; Ranunculaceae.

Actaea racemosa L. (Ranunculaceae, black cohosh) is a perennial forest herb primarily found in the Appalachian mountains of the eastern United States and Canada. It is strictly outcrossing and has a diploid chromosome number of $2n = 16$ (Ramsey, 1965). It is popular worldwide as an herbal remedy for menopausal symptoms (Upton, 2002). *Actaea racemosa* contains cycloartane triterpenoid glycosides (Shao et al., 2000), the concentrations of which are used as an industry standard of product quality. Due to extensive wild-harvesting of *A. racemosa* roots and rhizomes, the principal organs used for medicinal preparations, and projections of future demand, the sustainability of this species in the wild is of concern (Upton, 2002; Blumenthal et al., 2008). Thus, there is a need to develop a commercially feasible production system for *A. racemosa* using well-characterized germplasm material, which may provide significantly higher yields than wild-harvested material (McCoy et al., 2006).

The purpose of this study was to develop molecular markers for *A. racemosa*, using samples from a living collection, to screen for variability across the geographical range of the species. We designed microsatellite markers that could be used to measure genetic diversity across the species' range, provide a context for studies of variation in triterpenoid glycoside production, and potentially detect signs of wild population genetic depletion in the southeastern United States, where the highest quality plant material is reported to originate and where the largest suppliers of the wild-harvested rhizome material are based (Upton, 2002).

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METHODS AND RESULTS

Samples of *A. racemosa* were taken from the living collections at Bent Creek Germplasm Repository (BCGR) of the North Carolina Arboretum in Asheville, North Carolina, which maintains the U.S. Department of Agriculture's Agricultural Research Service collection of *A. racemosa* from throughout its native range in the United States. Voucher specimens from each accession are deposited at the herbarium of BCGR (Appendix 1). Exact localities of the wild source collections have been withheld due to concerns about illegal harvesting of *A. racemosa* for commercial sales. For the construction of the microsatellite library, total DNA was extracted from leaves of one individual of *A. racemosa* (voucher: North Carolina, Jackson Co., *KGM 427 with SJP*, WCUH) using the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). We followed the enrichment procedure of Glenn and Schable (2005), except as noted below. DNA was digested with the restriction enzyme *Rsa*I (New England Biolabs, Ipswich, Massachusetts, USA), ligated to double-stranded linkers (new linkers were used: SimpleX-6 Forward 5′-AAAAGCACGAGCGGAACT-3′ and SimpleX-6 Reverse 5′-pAGTTCCGCTCGTGC-3′), denatured and hybridized to biotinylated microsatellite oligonucleotide mixes (mix 2 = $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$; mix 3 = $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$; mix 4 = $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$), and then captured on magnetic streptavidin beads (Dynal, Life Technologies, Grand Island, New York, USA). Hybridized DNA was eluted from the beads and amplified in PCR using a new forward primer (SimpleX-1 Forward 5′-AAAACGTCGTGCGGAATC-3′). The enriched libraries were sequenced on a 454 FLX Genome Sequencer using titanium chemistry following standard Roche 454 library protocols (454 Life Sciences, Roche Company, Branford,

TABLE 1. Characteristics of seven microsatellite regions developed in *Actaea racemosa.*

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	TD (°C)	GenBank accession no.
2Ar	F: *CAAAGGGACAATAAGAGAGG	$(ATC)_7$	187–208	65	JN848456
	R: GTTTCTCGAATTACAGCCCTAATC				
10Ar	F: *GTTTGGTCCTTCACACAATAC	$(AC)_{16}$	185–199	65	JN848457
	R: GTTTGACGCATAGCTCAACATAG				
15Ar	F: *CATCCTTTAGTCTACCAACATG	$(AAG)_7$	231–246	65	JN848458
	R: GTTTAGTGGGTGAGAGGAGAGTTC				
24Ar	F: *ATTCTGCCAACATAGCTCAC	$(AAC)_7$	350–368	65	JN848459
	R: GTTTACTCAATGGAATTTCGAGTG				
35Ar	F: GTTTAGTAGGCCAACAGTGTGAAG	$(AAG)_{12}...(AAG)_7$	182–203	55	JN848460
	R: *GAACTAAGCGCCGTTATTAG				
36Ar	F: GTTTATCAAACGTTGCTTCAAAC	$(AAC)_9$	137–143	55	JN848461
	R: *ACTTCTGCAGCTGGATTG				
47Ar	F: GTTTGGTTCAACAAATCCATTCAC	$(AAG)_8$	342–375	58	JN848462
	R: *TATTTCGGACGGTATTTCAC				

Note: TD = touchdown high annealing temperature used for PCR.
* Indicates CAG (5′-CAGTCGGGCGTCATCA-3′) labels.

Connecticut, USA). Sequences were subjected to a 3′ quality trim where only one base in the last 25 bases of the sequence contains a quality score less than 20 or alternatively contains one ambiguous base. CAP3 (Huang and Madan, 1999) was used to assemble sequences at 98% sequence identity using a minimal overlap of 75 bp. Along with singlets, contigs of two or three sequences were searched for the presence of microsatellite DNA loci using MSATCOMMANDER version 0.8.1 (Faircloth, 2008) and primers designed with Primer3 (Rozen and Skaletsky, 2000). One primer from each pair was modified on the 5′ end with an engineered sequence (CAG tag 5′-CAGTCGGGCGTCATCA-3′) to enable use of a third primer in the PCR (identical to the CAG tag) that was fluorescently labeled. The sequence GTTT was added to primers without the universal CAG tag addition.

Forty-eight primer pairs were tested for amplification and polymorphism using DNA from seven *A. racemosa* individuals from throughout the species' range. Frozen leaves were ground using a plastic homogenizer attached to a handheld electric drill placed inside a BioMasher straining column inside a 1.5 mL tube. DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's protocol. PCR amplifications were performed in a 12.5 μL volume (25.0 μg/mL BSA, 0.4 μM unlabeled primer, 0.04 μM CAG-tag labeled primer, 0.36 μM universal dye-labeled primer, 3.0 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 U TaKaRa Ex Taq Polymerase (TaKaRa Bio Inc., Otsu, Shigu, Japan), 1× Ex Taq reaction buffer, and ≤10 ng DNA template) using an Eppendorf Mastercycler pro thermal cycler (Eppendorf, Hamburg, Germany). A touchdown thermal cycling program (Don et al., 1991) encompassing a 10°C span of annealing temperatures ranging between 65°C and 45°C was used for all loci. Amplification parameters consisted of an initial denaturation step of 5 min at 96°C followed by 20 cycles of 96°C for 30 s, highest annealing temperature (decreased 0.5°C per cycle) for 30 s, and 72°C for 30 s; and 30 cycles of 96°C for 30 s, lowest annealing temperature for 30 s, and 72°C for 30 s. PCR products were run on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) and sized with ROX size standard (Applied Biosystems). Results were analyzed using GeneMapper version 4.1 (Applied Biosystems). Any loci exhibiting null alleles or potential locus duplication were excluded from further screening. Seven of the 48 tested primer pairs that reliably amplified fragments exhibiting polymorphism were selected for further screening.

We assessed the variability of the seven polymorphic loci in 60 *A. racemosa* individuals (using leaves or rhizomes), five each originating from 12 geographic localities, as well as in three additional North American species of *Actaea*: *A. pachypoda* Elliott, *A. podocarpa* DC., and *A. rubra* (Aiton) Willd. Conditions and characteristics of the loci are provided in Tables 1 and 2 and in Appendix S1. All primer pairs amplified microsatellite regions in *A. racemosa* from across its range, and most of them amplified in the related species (Appendix 2). We estimated the number of alleles per locus (*A*) and observed and expected heterozygosity (H_o and H_e) using GenAlEx version 6.41 (Peakall and Smouse, 2006). Tests for deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium were conducted using GENEPOP version 4.1 (Rousset, 2008). Because natural population boundaries have not been assessed, population genetics measures are reported for all samples together (Table 2), and for each of the 12 geographical accessions separately (Appendix S1). Altogether, the number of alleles ranged from three to six per locus (average: 4.4). H_e ranged from 0.115 to 0.698 while H_o ranged from 0.133 to 0.900. After Bonferroni correction for multiple comparisons (adjusted $P < 0.007$), one locus (15Ar) showed a significant deviation from expectations under HWE, but no linkage disequilibrium was detected for the 21 paired loci comparisons.

TABLE 2. Results of initial primer screening in *Actaea racemosa*.

Locus	*n*	*A*	H_o	H_e
2Ar	60	6	0.433	0.360
10Ar	60	4	0.700	0.578
15Ar	60	4	0.833*	0.613
24Ar	60	6	0.900	0.698
35Ar	60	3	0.483	0.447
36Ar	60	3	0.133	0.115
47Ar	60	5	0.367	0.457

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals screened.
* Indicates significant deviation from Hardy–Weinberg equilibrium.

CONCLUSIONS

The seven microsatellite loci described here were found to amplify reliably and were sufficiently variable to enable researchers to assess population structure and genetic relatedness among populations of *A. racemosa* and, potentially, related species. They may also allow us to examine variability in the production of triterpenoid glycosides and other active compounds in this medicinal plant in the context of population genetic structure.

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Appendix 1. State of origin and accession numbers of *Actaea racemosa* voucher specimens for the living collections at Bent Creek Germplasm Repository, Asheville, North Carolina, used in this study.

Arkansas (JM200712017), Delaware (JM200712001, JM200712002), Indiana (JM200712003), Kentucky (JM200712004), Maryland (JM200712005), Missouri (JM200712016), New York (JM200712009, JM200712018), North Carolina (JM200712006, JM200712007, JM200712008, JM200712021), Pennsylvania (JM200712010, JM200712019), Tennessee (JM200712011), Virginia (JM200712012, JM200712020), West Virginia (JM200712013, JM200712014).

Appendix 2. Cross-species amplification of *Actaea racemosa* microsatellite primers in other North American *Actaea* species.[a]

Species	*n*	2Ar	10Ar	15Ar[b]	24Ar	35Ar	36Ar	47Ar	Voucher[c]
A. pachypoda	3	2	2	6	1	—	1	—	USA, North Carolina (JM201009019)
A. podocarpa	3	1	4	7	1	3	1	—	USA, North Carolina (JM200808159)
A. rubra	1	1	1	2	2	1	1	—	USA, Vermont, *KGM 416*, WCUH

Note: *n* = number of individuals screened.

[a] Successful amplifications are indicated by the number of alleles observed, and failed amplifications are indicated by —.

[b] Up to six alleles were observed within individuals of *A. pachypoda* and *A. podocarpa* for this locus, suggesting locus duplication in these species.

[c] Vouchers include Bent Creek Germplasm Repository accession number or collection number and herbarium.