Sixteen Polymorphic Microsatellite Markers for a Federally Threatened Species, Hexastylis naniflora (Aristolochiaceae), and Co-Occurring Congeners

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PRIMER NOTE

SIXTEEN POLYMORPHIC MICROSATELLITE MARKERS FOR A FEDERALLY THREATENED SPECIES, *HEXASTYLIS NANIFLORA* (ARISTOLOCHIACEAE), AND CO-OCCURRING CONGENERS[1]

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- *Premise of the study:* Twenty microsatellite loci were developed for the federally threatened species *Hexastylis naniflora* (Aristolochiaceae) to examine genetic diversity and to distinguish this species from co-occurring congeners, *H. heterophylla* and *H. minor*.
- *Methods and Results:* Next-generation sequencing approaches were used to identify microsatellite loci and design primers. One hundred fifty-two primer pairs were screened for repeatability, and 20 of these were further characterized for polymorphism. In *H. naniflora*, the number of alleles identified for polymorphic loci ranged from two to 23 (mean ~8.8), with a mean heterozygosity of 0.39.
- *Conclusions:* These 16 polymorphic primers for *H. naniflora* will be useful tools in species identification and quantifying genetic diversity within the genus.

Key words: Aristolochiaceae; *Asarum*; *Hexastylis*; *Hexastylis naniflora*; hybrid; microsatellite markers.

The segregate genus *Hexastylis* Raf. (Aristolochiaceae), often included in *Asarum* L., is an enigmatic group of 12 species distributed in the southeastern United States (Blomquist, 1957; Niedenberger, 2010). *Hexastylis* has been segregated based upon its entirely North American distribution, karyotype (Sugawara, 1981; Soltis, 1984), pollen morphology (Niedenberger, 2010), and several characteristics of flower morphology (Gaddy, 1987). Multiple species complexes have been identified in this genus and this study focuses on the *H. heterophylla* complex, containing *H. heterophylla* (Ashe) Small, *H. minor* (Ashe) H. L. Blomq., and *H. naniflora* H. L. Blomq. The species in this complex are sympatric over portions of their ranges. Vegetative characters have limited taxonomic value, leaving ephemeral floral morphology as the only diagnosable field character for identification. Previous studies have recognized intermediate floral morphologies in some populations, leading some to question the validity of species circumscriptions. This is particularly problematic in *H. naniflora*, where land managers and conservation biologists are tasked with protection of this federally threatened species.

Through funding from the North Carolina Department of Transportation, 16 polymorphic microsatellite markers were developed to help distinguish *H. naniflora* from *H. minor* and *H. heterophylla*, to address questions of hybridization, and to identify evolutionarily significant units to aid in the management of these species. These markers have the potential to identify species and hybrids in their vegetative state, allowing land managers to evaluate population value and management strategies throughout the year, instead of only during the short flowering period.

METHODS AND RESULTS

Leaf tissue was collected and preserved on silica gel from plants at 15 sites in North and South Carolina (Appendix 1). Tissue samples from one plant of *H. naniflora* and one plant of *H. heterophylla* (selected from geographic ranges where the species do not overlap and confidently identified using flower material) were sent to the Cornell University Evolutionary Genetics Core Facility where total DNA was extracted using a QIAGEN Plant Mini Kit (QIAGEN, Valencia, California, USA). Restriction enzymes *Alu*I, *Hpy*166II, and *Rsa*I (New England Biolabs, Ipswich, Massachusetts, USA) were used to digest the DNA, which was then ligated to an Illumina Y-adapter (Illumina, San Diego, California, USA) using T4 DNA ligase. The DNA fragments were then hybridized to 3′ biotinylated oligonucleotide repeat probes: $(GT)_8$, $(TC)_{9.5}$, $(TTTTG)_{4.2}$, $(TTTTC)_{4.6}$, $(TTC)_7$, $(GTA)_{8.33}$, $(GTG)_{4.67}$, $(TCC)_5$, $(GTT)_{6.33}$, $(TTTC)_6$, $(GATA)_7$, $(TTAC)_{6.75}$, $(GATG)_{4.25}$, $(TTTG)_{5.25}$, $(TTTTG)_{4.2}$, $(TTTTC)_{4.6}$. Enriched fragments were then captured by streptavidin-coated magnetic beads (New England Biolabs) and PCR amplified. Agarose gel and a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, New York, USA) were used to analyze the PCR product, and fragments 300–600 bp were recovered with AMPure beads (Beckman Coulter, Brea, California, USA). Samples were then moved to Cornell Life Sciences Sequencing and Genotyping Facility for sequencing on an Illumina MiSeq. Raw sequence reads were then assembled using SeqMan NGen (v.11, Lasergene Genomics Suite; DNASTAR, Madison, Wisconsin, USA). Contigs containing microsatellite repeats were identified using MSATCOMMANDER version 1.0.3 (Faircloth, 2008), and possible primer pairs were identified.

One hundred fifty-two primer pairs were selected to screen for amplification in eight individuals: six *H. naniflora*, one *H. heterophylla*, and one *H. minor*. PCR amplifications were prepared in a 10-μL reaction consisting of GoTaq Flexi Buffer, 2.5 mM $MgCl_2$, 800 μM dNTPs, 0.5 μM of each primer, 0.5 units

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of GoTaq Flexi DNA Polymerase, and ~20 ng of DNA (Promega, Madison, Wisconsin, USA). PCR was completed using a touchdown thermal cycling program on a Techne TC-5000 Thermal Cycler (Bibby Scientific Limited, Stone, Staffordshire, United Kingdom) encompassing a 13°C span of annealing temperatures from 68°C to 55°C. Initial denaturation was at 94°C for 5 min, 13 cycles at 94°C for 45 s, touchdown for 2 min, and 72°C for 1 min; followed by 24 cycles at 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min; followed by a final extension at 72°C for 5 min. The PCR products were examined on a 1% agarose gel and scored for presence or absence of an appropriately sized PCR product. Twenty primer pairs produced repeatable results across all three species (Table 1). These were further screened for polymorphism on a total of 68 individuals, including 44 *H. naniflora*, 10 *H. minor*, and 14 *H. heterophylla* (Appendix 1).

Polymorphism screening PCR reaction conditions were the same as above, except the forward primer concentration was reduced to 0.25 µM, and 0.25 µM of an M13 primer (5′-CACGACGTTGTAAAACGAC-3′), labeled with FAM, VIC, NED, or PET (Life Technologies), was added to the reaction. PCR products labeled with different fluorescent dyes were then pseudo-multiplexed, and 2 µL of the combined reactions were submitted for genotyping on an ABI 3730 DNA sequencer using a GeneScan 500 LIZ Size Standard (Life Technologies). Resulting chromatograms were visualized and scored using the software package Geneious Version 7 (Biomatters Ltd., Auckland, New Zealand). The resulting genotypic data were then analyzed with GenAlEx version 6.5 (Peakall and Smouse, 2006, 2012) to obtain standard descriptive statistics and to test for deviations from Hardy–Weinberg equilibrium assumptions (Table 2).

Sixteen of the primer pairs tested were polymorphic, with the number of alleles ranging from two to 23 (mean ~8.8) in *H. naniflora*, two to nine (mean ~4.9) in *H. minor*, and one to 14 (mean ~6.1) in *H. heterophylla* (Table 2). Excessive homozygosity was identified at several of the loci in all three species, and locus Hn00567 was monomorphic in *H. heterophylla*. A total of 52 private alleles were identified in one of the three species, mostly at low frequencies (<0.05). Three of these private alleles in *H. naniflora* (Hn7116 [422 bp], Hn01135 [300 bp], and Hn00304 [179 bp]), one in *H. minor* (Hn00252 [224 bp]), and one in *H. heterophylla* (Hn00002 [297 bp]) were identified with a frequency greater than 10%, and these can be diagnostic in species identification when morphological characters are unavailable.

CONCLUSIONS

Sixteen polymorphic microsatellite markers were developed for *H. naniflora*, and these primers also amplify in two other species of *Hexastylis* (*H. heterophylla* and *H. minor*). These markers provide a means to assess genetic diversity and to assist in circumscription of the three species in the *H. heterophylla* complex. This provides the first opportunity to examine species boundaries and hybrids in the complex with molecular tools; application of these tools should lead to a

Table 1. Characteristics of 20 microsatellite primer pairs developed for *Hexastylis*.

Locus	Primer sequences (5′–3′)[a]	Fluorescent dye	Repeat motif	T_a (°C)	Allele size range (bp)	GenBank accession no.
Hn00002	F: AAGTCTTTCCACCAATAACACCG R: ATGCCTTGAGTCAACATGCTTTG	FAM	$(AAC)_5$	60.51	297–306	KM242087
Hn00011	F: CCAGTCTTAGTTACAAGATGCCG R: TCGATACTGTGATCAAAGCCAAG	PET	$(AAC)_8$	59.73	229–269	KM242088
Hn00014	F: GAGATTTGATCAGCGGTTTGAAC R: GGGCAGTCAGAGTCATTTATCTC	NED	$(ACC)_7$	59.37	265–274	KM242089
Hn00147	F: GGTAAAGCTAACATCCGACTGTG R: AAGGGTAGCTATAAGTTGGTTGC	VIC	$(AGAT)_5$	59.69	217–241	KJ619759
Hn00167	F: AGATGAGATTGTACATGTGAAACG R: GTATTCTAACAACTACTGCTCCCG	FAM	$(AAG)_5$	58.91	160 (M)	KM242090
Hn00193	F: ATGTGAGATCAGTAGGAGACGAG R: TTTGGGTGGATAATGGCTTTCTG	PET	$(AAG)_{14}$	59.82	337–369	KM242091
Hn00197	F: CGGTCACACAGGACCATAGTAC R: CTCGGCGTCTAGACAGGTTATAG	VIC	$(ACT)_{12}$	60.74	242–272	KM024991
Hn00236	F: AGGAGGTTTGGGAGCATTATTTG R: GCCTGTCAAACATCCTATGACTC	FAM	$(ACC)_5$	59.82	219 (M)	KM242094
Hn00252	F: AGGCATACAGAGGGCACATATAG R: AAGAATGTTGAGAAGCTGCTTTG	NED	$(AAC)_7$	59.58	221–241	KM242095
Hn00304	F: CCACTCCACTCCTTAATATAGAGC R: AATGTGGAGGAATCTGAGAACAC	VIC	$(AAG)_{10}$	58.97	179–205	KM024990
Hn00366	F: TGAATATACCAGTGCACAAACCC R: CGATTCCTCTTCCGATCATAGTC	VIC	$(AC)_6$	59.39	162 (M)	KM242098
Hn00567	F: ACTCTACCTCTCAATTCCACTCC R: GCGTGAAATAATATGGCCAATGG	FAM	$(ACC)_5$	59.64	213–239	KM242100
Hn00855	F: GAGAACGAGAGAGTACCGCAAC R: ATGCCATATCAGCCGTCTACAAC	NED	$(AGAT)_8$	61.52	276–346	KJ619760
Hn00955	F: CTTAGAGGTGGTAGGAAGGAGTC R: GCAATGAACTCTAAATGGAATGGC	VIC	$(AAT)_{13}$	59.77	366–429	KJ619751
Hn01096	F: CATGATAGCTACCTGGGATGATG R: TTCGCTAATTTCATGCTTTCCTC	FAM	$(AAG)_{21}$	58.76	252 (M)	KM242103
Hn01135	F: TTCAGGCTGCAAACTATCTGAAC R: TTCAGCAACCAACACTCATTTAC	PET	$(ACC)_{11}$	59.3	278–312	KM024992
Hn1825	F: TGATGATGAAATGCTCCACTCAC R: AGACAAGACTGGATGGAGGTTTG	FAM	$(AAC)_{22}$	60.42	236–284	KM024993
Hn4600	F: GAGAGAACCGGTGAATCAAGTTG R: AAAGTAGCAATCAGAATTCGGGC	FAM	$(AAAG)_5$	60.36	304–370	KM024994
Hn7116	F: CTGATACCATGTGACAATGGAGG R: GTCATGATATTGGGCCTTCGTAG	NED	$(AAGGAG)_5$	59.7	422–451	KM024995
Hn12441	F: TCCATCGTACAAGGTCGTCTATG R: GAAGTCGAACCAAGGTCAATAGG	PET	$(AGGG)_5$	60.14	164–183	KM024989

Note: M = monomorphic; T_a = annealing temperature.
[a]All forward primers also contain an M13 tag (5′-CACGACGTTGTAAAACGAC-3′) on their 5′ end to allow fluorescent labeling of PCR products.

TABLE 2. Standard descriptive statistics for 16 polymorphic microsatellite loci in three species of *Hexastylis*.

	H. naniflora (*N* = 44)				*H. minor* (*N* = 10)				*H. heterophylla* (*N* = 14)			
Locus	*A*	H_o	H_e	HWE[a]	*A*	H_o	H_e	HWE[a]	*A*	H_o	H_e	HWE[a]
Hn00002	2	0.421	0.494	n.s.	3	0.167	0.292	**	3	0.200	0.540	**
Hn00011	10	0.541	0.701	***	6	0.333	0.750	n.s.	4	0.727	0.533	n.s.
Hn00014	4	0.400	0.469	n.s.	3	0.429	0.663	n.s.	2	0.182	0.165	n.s.
Hn00147	13	0.649	0.781	**	7	0.714	0.786	n.s.	11	0.583	0.872	***
Hn00193	11	0.658	0.803	n.s.	5	0.400	0.760	n.s.	7	0.889	0.741	n.s.
Hn00197	10	0.216	0.843	***	9	0.700	0.860	n.s.	9	0.471	0.875	**
Hn00252	3	0.289	0.440	n.s.	2	0.000	0.278	*	2	0.364	0.397	n.s.
Hn00304	9	0.659	0.779	n.s.	6	0.556	0.765	n.s.	7	0.571	0.801	n.s.
Hn00567	2	0.049	0.048	n.s.	2	0.222	0.198	n.s.	1	0.000	0.000	M
Hn00855	23	0.674	0.930	***	6	0.556	0.765	n.s.	14	0.750	0.906	n.s.
Hn00955	17	0.537	0.897	***	7	0.375	0.750	**	11	0.571	0.865	n.s.
Hn01135	9	0.585	0.747	n.s.	6	0.429	0.776	n.s.	6	0.385	0.627	n.s.
Hn1825	13	0.818	0.871	n.s.	8	0.750	0.820	n.s.	10	0.786	0.878	n.s.
Hn4600	2	0.385	0.393	n.s.	2	0.250	0.219	n.s.	2	0.500	0.486	n.s.
Hn7116	9	0.585	0.717	**	4	0.625	0.680	n.s.	5	0.462	0.533	n.s.
Hn12441	4	0.159	0.290	*	3	0.571	0.503	n.s.	3	0.556	0.426	n.s.
Mean	8.81	0.388	0.523		4.937	0.346	0.501		6.062	0.410	0.492	

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium.

[a]Asterisks indicate significant deviation from Hardy–Weinberg equilibrium (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); M = monomorphic; n.s. = not significant.

reassessment of distributions and hybrid zones. These markers will also be valuable tools for vegetative identification of new *Hexastylis* populations when flowers are unavailable. These primers may also be useful in other species of *Hexastylis* and *Asarum*.

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APPENDIX 1. Location and sampling information for *Hexastylis* individuals used in this study.

		Geographic coordinates[b]					
Species	Herbarium accession no.[a]	Latitude (°N)	Longitude (°W)	Elevation (m)	State (Country)	County	*N*
H. heterophylla	28952	36.00152	−81.01013	270	NC (USA)	Alexander	3
H. heterophylla	28954	35.85079	−81.47797	337	NC (USA)	Caldwell	7
H. heterophylla	28947	35.21389	−82.23407	N/A	NC (USA)	Polk	2
H. heterophylla	28950	36.03405	−81.06168	385	NC (USA)	Wilkes	2
H. minor	28963	35.24580	−81.43860	273	NC (USA)	Cleveland	5
H. minor	28965	36.05922	−78.96552	144	NC (USA)	Durham	5
H. naniflora	28964	N/A	N/A	293	NC (USA)	Alexander	3
H. naniflora	28978	N/A	N/A	337	NC (USA)	Burke	5
H. naniflora	28973	N/A	N/A	279	NC (USA)	Catawba	11
H. naniflora	28975	N/A	N/A	219	NC (USA)	Cleveland	3
H. naniflora	29019	N/A	N/A	237	NC (USA)	Iredell	3
H. naniflora	28974	N/A	N/A	336	NC (USA)	Polk	3
H. naniflora	28988	N/A	N/A	282	NC (USA)	Rutherford	9
H. naniflora	28972	N/A	N/A	287	SC (USA)	Cherokee	3
H. naniflora	28987	N/A	N/A	244	SC (USA)	Spartanburg	4

Note: *N* = number of individuals; N/A = not available; NC = North Carolina; SC = South Carolina.

[a]All herbarium accession numbers refer to voucher specimens deposited in the Appalachian State University Herbarium (BOON).

[b]Geographic coordinates for federally listed species are not included.