SIXTEEN POLYMORPHIC MICROSATellite MARKERS FOR A FEDERALLY THREATENED SPECIES, HEXASTYLiS NANIFLORA (ARISTOLOCHiACEAE), AND CO-OCCurring CONGENERs

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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 entire North American distribution, karyotype (Sugawara, 1981; Solts, 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 Adal, Hpy166I, and Rsal (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)6, (TC)6.5, (TTTGG)1.2, (TTTTC)6.8, (TTTTC)6, (TTC)5.5, (GTA)6.5, (GTA)6.5, (TTCC)6, (GTTA)6.5, (GTTA)6, (TTAC)6, (GATTG)6.2, (TTTGGAT)6, (TTTGGAT)6, (TTTGGAT)6.2, (TTTGGAT)6.8, (TTTGGAT)6.4. 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 NGene (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 MgCl2, 800 μM dNTPs, 0.5 μM of each primer, 0.5 units

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Sixteen of the primer pairs tested were polymorphic, with the number of alleles ranging from two to 23 (mean = 8.8) in \textit{H. naniflora}, two to nine (mean = 4.9) in \textit{H. minor}, and one to 14 (mean = 6.1) in \textit{H. heterophylla} (Table 2). Excise homozygosity was identified at several of the loci in all three species, and locus Hn00567 was monomorphic in \textit{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 \textit{H. naniflora} (Hn7116 [422 bp], Hn01135 [300 bp], and Hn00304 [179 bp]), one in \textit{H. minor} (Hn00252 [224 bp]), and one in \textit{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 \textit{H. naniflora}, and these primers also amplify in two other species of Hexastylis (\textit{H. heterophylla} and \textit{H. minor}). These markers provide a means to assess genetic diversity and to assist in circumscription of the three species in the \textit{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′) | Fluorescent dye | Repeat motif | T<sub>a</sub> (°C) | Allele size range (bp) | GenBank accession no. |
|-------------|--------------------------|-----------------|--------------|---------------------|------------------------|-----------------------|
| Hn00002     | F: AGGCTTCTTACACAAATACACCGC<br>R: ATGCTTTGACACATGCTTTTG | FAM | (AAC)<sub>5</sub> | 60.51 | 297–306 | KM242087 |
| Hn00011     | F: CACGGCTTAGTACAAAGATGCCG<br>R: TGATACCTGTCATGACCAAGGA | PET | (AAG)<sub>6</sub> | 59.73 | 229–269 | KM242088 |
| Hn00014     | F: GAGATCTTGCAGGCTGAGAC<br>R: GGCACTAGCTATGCTATCTCC | NED | (ACC)<sub>2</sub> | 59.37 | 265–274 | KM242089 |
| Hn00147     | F: GGTAAAGTCAATACCGCTGTCG<br>R: AAGGATAGTCTAAGGTGCCG | VIC | (AGAT)<sub>3</sub> | 59.69 | 217–241 | KJ619759 |
| Hn00167     | F: ATGATGAGTTGACATGTTGAGAC<br>R: GTATTCTACAACTACTGCGC | FAM | (AGA)<sub>4</sub> | 59.81 | 160 (M) | KM242090 |
| Hn00193     | F: ATGAGTAGCTAGTGGAGAC<br>R: TTTGGTTGTGATTGCTTTCTG | PET | (AAG)<sub>14</sub> | 59.82 | 337–369 | KM242091 |
| Hn00197     | F: CGGTCACCAAAGACATGATAC<br>R: CTCGGCTATCTAAGATGGATAG | VIC | (ACT)<sub>12</sub> | 60.74 | 242–272 | KM024991 |
| Hn00236     | F: AGGGGTTGGGACATTTATAG<br>R: GCCGCTCAACACTCTGACCTC | FAM | (ACC)<sub>3</sub> | 59.82 | 219 (M) | KM242094 |
| Hn00252     | F: AGGGATAGTCTAAGGTGCCG<br>R: AAGGATAGTCTAAGGTGCCG | NED | (ACC)<sub>2</sub> | 59.58 | 221–241 | KM242095 |
| Hn00304     | F: AAGATGTTAGGCTAGAAGTG<br>R: AAGAATGTTAGGCTAGAAGTG | VIC | (AAG)<sub>10</sub> | 59.87 | 179–205 | KM024990 |
| Hn00366     | F: AGGATATCAACACCTGAC<br>R: CGATTTCTTCCATGTCATGTC | VIC | (AC)<sub>6</sub> | 59.39 | 162 (M) | KM242098 |
| Hn00567     | F: ACTATCTCTCTCCTTTCACT | FAM | (ACC)<sub>3</sub> | 59.64 | 213–239 | KM242100 |
| Hn00855     | F: GAGAACGAGAAGTCCGAC<br>R: ATGGCCGATCCGCTACCAAC | NED | (AGAT)<sub>4</sub> | 61.52 | 276–346 | KJ619760 |
| Hn00955     | F: CTGGTAGGTTGGAGG<br>R: GAGATAGTCTAAGGTGCCG | VIC | (AAT)<sub>3</sub> | 59.77 | 366–429 | KJ619751 |
| Hn01096     | F: CTGGATAGCTACCTGGTTAG<br>R: TTGGGCTTTCATGCTTCTTTC | FAM | (AAG)<sub>21</sub> | 58.76 | 252 (M) | KM242103 |
| Hn01135     | F: TCGAGCTGTCAC<br>R: TCTGGATAGCTACCTGGTTAG | PET | (ACC)<sub>11</sub> | 59.3 | 238–312 | KM024992 |
| Hn1825      | F: TAGAATGAAATCTCCATCTAC<br>R: ATGGCCGATCCGCTACCAAC | FAM | (AAG)<sub>22</sub> | 60.42 | 236–284 | KM024993 |
| Hn4600      | F: AGGAGCTGGAG<br>R: AAAGATGTTAGGCTAGAAGTG | FAM | (AAAG)<sub>5</sub> | 60.36 | 304–370 | KM024994 |
| Hn7116      | F: GTGATACATGTTGAC<br>R: GTGATACATGTTGAC | NED | (AAGGA)<sub>5</sub> | 59.7 | 422–451 | KM024995 |
| Hn12441     | F: TCACTGACAGAG<br>R: GAGATGTTACCAAGTG | PET | (AGG)<sub>9</sub> | 60.14 | 164–183 | KM024989 |

\textit{Note}: M = monomorphic; \( T_a \) = annealing temperature.

*All forward primers also contain an M13 tag (5′-CAGCAGTTGTTAAACGAC-3′) on their 5′ end to allow fluorescent labeling of PCR products.
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.

| Species                  | Geographic coordinates | Elevations (m) | State (Country) | County | N |
|--------------------------|------------------------|----------------|-----------------|--------|---|
| H. heterophylla          | 36.00152, −81.01013    | 270            | NC (USA)        | Alexander | 3 |
| H. heterophylla          | 35.85079, −81.47797    | 337            | NC (USA)        | Caldwell | 7 |
| H. minor                 | 35.21389, −82.23407    | 385            | NC (USA)        | Polk | 2 |
| H. naniflora             | 36.03405, −81.06168    | 385            | NC (USA)        | Wilkes | 2 |
| H. minor                 | 35.24580, −81.43860    | 273            | NC (USA)        | Cleveland | 5 |
| H. heterophylla          | 36.05922, −78.96552    | 144            | NC (USA)        | Durham | 5 |
| H. naniflora             | N/A, N/A              | 293            | NC (USA)        | Alexander | 3 |
| H. heterophylla          | N/A, N/A              | 337            | NC (USA)        | Burke | 5 |
| H. naniflora             | N/A, N/A              | 279            | NC (USA)        | Catawba | 11 |
| H. naniflora             | N/A, N/A              | 219            | NC (USA)        | Cleveland | 3 |
| H. minor                 | N/A, N/A              | 237            | NC (USA)        | Iredell | 3 |
| H. naniflora             | N/A, N/A              | 336            | NC (USA)        | Polk | 3 |
| H. heterophylla          | N/A, N/A              | 282            | NC (USA)        | Rutherford | 9 |
| H. naniflora             | N/A, N/A              | 287            | SC (USA)        | Cherokee | 3 |
| H. naniflora             | N/A, N/A              | 244            | SC (USA)        | Spartanburg | 4 |

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

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

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