RESEARCH & DEVELOPMENT

Development of molecular and morphological tools to circumscribe and identify the Dwarf Flowered Heartleaf (*Hexastylis naniflora*)

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IDENTIFICATION PAGE

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16. Abstract

The Dwarf Flowered Heartleaf (DFH) is afforded protection under the Endangered Species Act as a "threatened" species. The 190 known populations are distributed across western Piedmont of North Carolina to the northwestern Piedmont of South Carolina. In the development of highway projects in North Carolina, there is a need to be able to locate populations of the DFH and provide conservation measures to offset impacts to the species. This means being able to 1) differentiate this species from co-occurring related species, and 2) differentiate this species from hybrid individuals. The project extensively sampled across the range of the DFH and less extensively from the five co-occurring species and the two species at the western periphery of the range. A microsatellite library was generated, and then used to screen for polymorphisms across the range of the DFH. We used the developed library to genotype individuals across the range of the DFH, as well as the putative hybrids and hybridizing taxa. Maps were generated to examine the morphological, anatomical and molecular variation within and between species across the landscape. These data were then be used to develop a robust set of characteristics (morphological, micromorphological and microsatellite markers) that can be used to identify the DFH and to differentiate members of the target species from congeners and hybrids. We included training of one graduate student, two post-graduates and ten undergraduate students in this project. We currently have one graduate student and two undergraduates using the data generated from this effort in further studies of the complex.

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Acknowledgments

The foundations of this study began through funding from NC DOT with the work of James Padgett, a MS student working with Murrell from 2001-2004, who focused his research on the species delineation in the *Hexastylis naniflora* complex. Joy Vanderwort-Sneed completed a MS degree in 2008 studying the systematics of the *Asarum rhombiformis* complex and Bryan Niedenberger completed his thesis in 2010 with a study of the systematics of the genus Hexastylis (Aristolochiaceae). All three of these efforts were critical first steps to the development of the current study. One graduate student (Jackie Wagner Hampstead), two post-graduates (Robyn Oaks and Alyssa Teat) and ten undergraduate students (Margaret Roberts, Taylor Jenson, George Godsmark, Brandon Snyder, Jason Woodward, Evan Fitzgerald, Carmen Vinson, Ben Banick, Nik Hay and Chris Bobby) made significant contributions during the course of this project. One Master's thesis was generated from this work (Jackie Wagner Hampstead) and one publication (microsatellite marker paper). We currently have one graduate student (Joe Mckenna) and two undergraduates (Ben Banick and Byron Burrell) that are using the data generated from this effort to use in further studies of the complex. Matt Estep, ASU faculty member and a co-author on this report, provided expert technical advice and leadership in development and testing of the molecular markers.

Support from NC DOT and Appalachian State University were critical to the success of this effort. The NC Natural Heritage Program and the US Fish and Wildlife Service offered support and advice regarding population and land owner information. NC DOT biologists Heather Wallace, Tim Bassett and Jared Grey provided insight into various problematic populations and helped with fieldwork. John Kirby (NC DOT) provided helpful and steady support throughout the effort. NC Natural Heritage Program biologists James Padgett and Laura Gadd provided baseline data for the project and assisted with site selection. NC. DOT, Appalachian State University (ASU) Department of Biology, ASU Vice Provost for Research, ASU College of Arts and Sciences and ASU's RIEEE provided financial support to enable the effort.

Executive Summary
Proposal ID: 2111

Proposal Title: Development of molecular and morphological tools to circumscribe and identify the Dwarf Flowered Heartleaf (*Hexastylis naniflora*)

The Dwarf Flowered Heartleaf (DFH) is afforded protection under the Endangered Species Act and is listed as a "Threatened" species by the United States Fish and Wildlife Service. The species is distributed across a range that extends from the western Piedmont of North Carolina to the northwestern Piedmont of South Carolina. There are approximately 190 known sites where this plant occurs in this region. Due to current U.S. Fish and Wildlife Service guidelines regarding the treatment of Federally Threatened plants, threats to populations of this species must be considered in any projects that involve federal funds. This is particularly problematic issue to address for this species, since it is distributed in one of the fastest growing areas of North Carolina.

In the development and construction of highway projects in North Carolina, there is a need to be able to locate populations of the DFH and provide conservation measures to offset impacts to the species. This means being able to 1) differentiate this species from co-occurring related species, and 2) differentiate this species from hybrid individuals. Although flower size and pollen surface features have been shown to be effective characters to identify DFH to species, no characters have been shown to be effective for species recognition when the plant is in a vegetative stage. This essentially shortens the period of field identification to, at best, six weeks.

The DFH distribution overlaps with five species across all of the DFH range and two species at the western periphery of the DFH range. Several botanists have described individuals in the field that appear to be intermediate between the DFH and one or more of these co-occurring species. Based upon conversations with NC Department of Transportation botanists, it appears that individuals with intermediate morphologies have made it difficult to identify some of populations to species, using information provided in peer-reviewed dichotomous keys (Gaddy 1987, FNA 2003), even with access to flowering materials.

The project extensively sampled across the range of the DFH and less extensively from the five co-occurring species (*H. arifolia* var. *ruthii, H. minor, H. heterophylla, H. virginica,* and *H. shuttleworthii*) and the two species at the western periphery of the range (*H. rhombiformis* and *H. contracta*). Photographs and scanning electron micrographs of flower and leaf were obtained. A microsatellite library was generated, and then used to screen for polymorphisms across the range of the DFH. We used the developed library to genotype individuals across the range of the DFH, as well as the putative hybrids and hybridizing taxa. Maps were generated to examine the morphological, anatomical and molecular variation within and between species across the landscape. These data were then be used to develop a robust set of characteristics (morphological, micromorphological and microsatellite markers) that can be used to identify the DFH and to differentiate members of the target species from congeners and hybrids.

Our dataset for the molecular work includes 184 *H. naniflora* from 45 sites, 80 *H. heterophylla* from 12 sites, 61 *H. minor* from 14 sites, 153 hybrids or unidentified from 18 sites and 168 other *Hexastylis* species from 49 sites for a total of 646 individuals from 138 sites. We have another approximately 150 individuals that were problematic in terms of DNA extraction that have been excluded from any analyses at this time, but we have materials that can be re-extracted, if needed.

We included training of one graduate student (Jackie Wagner), one post-graduate (Robyn Oaks) and ten undergraduate students (Margaret Roberts, Taylor Jenson, George Godsmark, Brandon Snyder, Jason Woodward, Evan Fitzgerald, Carmen Vinson, Ben Banick, Nik Hay and Chris Bobby) during the course of this project. One Master's thesis was generated from this work (Jackie Wagner) and one manuscript (microsatellite marker paper). We currently have one graduate student (Joe Mckenna) and two undergraduates (Ben Banick and Byron Burrell) that are using the data generated from this effort to use in further studies of the complex.

It is hoped that North Carolina DOT will use the information obtained from this study to be more efficient in planning for long-term highway corridors and to effectively identify mitigation sites in the region. Developed molecular markers can be used to identify DFH populations throughout the year instead of only during the flowering season. All of the generated data will enable NC DOT to be responsive to US Fish and Wildlife in their reassessment of the status of the DFH.

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Introduction including background of the research need

In the development and construction of highway projects in North Carolina, there is a need to be able to locate populations of the Dwarf Flowered Heartleaf (DFH) and provide conservation measures to offset impacts to the species. This means being able to 1) differentiate this species from co-occurring related species, and 2) differentiate this species from hybrid individuals. Although flower size and pollen surface features have been shown to be effective characters to identify DFH to species, no characters have been shown to be effective for species recognition when the plant is in a vegetative stage. This essentially shortens the period of field identification to, at best, six weeks. Other than overlap of flower morphology, no markers have been identified to differentiate this species from hybrids, and no molecular data exists to document the occurrence of hybridization in this group.

The DFH distribution overlaps with five species across all of the range (*H. arifolia* var. *ruthii, H. minor, H. heterophylla, H. virginica, and H. shuttleworthii*) and two species at the western periphery of the range (*H. rhombiformis* and *H. contracta*). Several botanists have described individuals in the field that appear to be intermediate between the DFH and one or more of these species. Based upon conversations with NC Department of Transportation biologists, it appears that individuals with intermediate morphologies have made it difficult to identify some of populations to species, based on information provided in peer-reviewed dichotomous keys (Gaddy 1987, FNA 2003), even with access to flowering materials.

Need Definition

1. No characters have been discovered (morphological or molecular) that can delineate *Hexastylis* species boundaries using vegetative materials. Flowers in this group are highly variable between species and can usually be a good indicator of species

boundaries, although anecdotal information suggests either 1) some hybridization or 2) some species with highly variable flower size and shape. There are known populations of *H. naniflora* that possess flowers that are larger than typical flowers for the species and their shape trends toward that of *H. minor, H. heterophylla, H. virginica* and *H. arifolia*.

2. NC DOT is seeking information regarding species variation and hybridization in *H. naniflora* in order to help with field identification of this species. Currently available dichotomous keys are not sufficient to correctly identify *Hexastylis* to species level in some areas of the state. Therefore, new methodologies are needed to identify *H. naniflora* in the field.
3. The need for high quality information regarding variation within the target species, including putative hybrids, and the lack of resolution generated in the three gene analysis conducted by Neidenberger (2010; see below) necessitate a rigorous field and lab analysis to reach robust conclusions regarding the biology of *H. naniflora*.

Research Objectives

1. Sample across the range of the DFH to collect vouchers and DNA for molecular analyses.
2. Develop a microsatellite library of molecular markers that can resolve population level variation in the target species.
3. Apply these markers to the target species, with an emphasis on intermediate populations identified by NC DOT biologists.

4. Obtain morphological and micromorphological information from each of the sampled populations (using standard tools).
5. Compare molecular results with morphological, micromorphological and distributional data to determine genetic structure of the DFH, biological boundaries of the DFH, and placement of putative hybrid or intermediate populations in relationship to the species boundaries of the target species.
6. Provide the analyzed data to NC DOT in a way that can used to make decisions regarding highway placement and mitigation site recognition. Also, to provide these data in a way that will assist NC DOT in being responsive to requests for information from US Fish and Wildlife Service.

Literature Review and the relevance of the proposed research vis-à-vis state of the art, science and practice.

Aristolochiaceae, the birthwort family, consists of eight genera and 500 species of herbs and vines. The two major genera in the family are *Aristolochia* with 300-350 species and *Asarum* with about 70 species (Stevens 2001). After the genus *Hexastylis* was first segregated from *Asarum* by Rafinesque (1825), it slowly gained general acceptance in the North American literature (Small 1933; Britton and Brown 1947; Radford et al. 1968; Blomquist 1957; Otte 1977; Kral 1983; Gaddy 1987; Wofford 1989; Rayner 1994). Currently, *Hexastylis* is commonly used to describe a genus of nine species and four varieties that are endemic to the southeastern United States.

Recent work by Neidenberger (2010), a graduate student in the Murrell lab, examined all nine species and four varieties included in *Hexastylis*. Pollen was examined and showed

similar surface features for most taxa, some variation in morphology became visible under increased magnification. Contrary to Kelly's (1997) study, *Hexastylis* was found to be monophyletic with all species in the genus possessing a 6 bp insertion. Neidenberger (2010) also reexamined distributions of species of *Hexastylis*. Based upon this work, *Hexastylis naniflora* overlaps in distribution with five species across all of the range (*H. arifolia* var. *ruthii, H. minor, H. heterophylla, H. virginica, and H. shuttleworthii*) and two species at the western periphery of the range (*H. rhombiformis* and *H. contracta*).

The DFH complex has been studied by several biologists without generating a solid understanding of the genetic structure of the DFH and hybrids to be able to differentiate at a usable level. This has mostly been due to a lack of resources and a lack of study breadth, in particular to determine the extent of hybridization and to determine the parent species of the putative hybrids. This study addressed the issues at a scale and level of detail that provide the needed knowledge to understand and recognize those individuals that are within the species circumscription and determine what individuals fall outside of that circumscription.

Body of Report

A. Methods

1. Working closely with NC DOT biologists, a sampling strategy was developed to adequately sample the diversity of the *H. naniflora* populations and the intermediate or hybrid populations known to occur throughout the range of the species. Appropriate collecting permits were obtained from state and federal agencies.
2. March/April 2012-August 2014. Flower and leaf material was collected from each of 162 localities across Alabama, North Carolina, South Carolina, Tennessee, and Virginia. Twenty-seven of these localities had two *Hexastylis* species present, so the 162

localities represent 135 sampling sites. Leaf tissue was obtained from 137 *H. naniflora* plants, 52 *H. minor* plants, 58 *H. heterophylla* plants, and 117 currently unidentified/putatively hybridized plants, as well as samples from other species in the genus. The North Carolina Department of Transportation (NCDOT) dictated the location of 29 of the field sites, prioritizing populations of *Hexastylis* that display intermediate morphologies and populations of *H. naniflora* expected to be threatened by proposed and on-going road construction projects. The NCDOT identified a total of 15 *H. naniflora* sites, 2 H. minor sites, and 12 sites displaying intermediate morphologies (henceforth referred to as H. spp.) that were to be included in this study. This site list was amended at ASU in order to cover the geographic range of *H. naniflora*, across all counties and watersheds where the species is known to occur (Fig. 1). Priority was given to sites with permission to access and those that contained more than one species of *Hexastylis* to address questions of hybridization. Sites for the other eight species of *Hexastylis* (excluding *H. sorriei*) were also included for sampling to be used in future studies (*H. arifolia* var. *ruthii, H. minor, H. heterophylla, H. virginica,* and *H. shuttleworthii*) and two species at the western periphery of the range (*H. rhombiformis* and *H. contracta*). Numbers of sites for each taxa were: *H. arifolia* = 20, *H. contracta* = 6, *H. heterophylla* = 14, *H. lewisii* = 1, *H. minor* = 19, *H. naniflora* = 51, *H. rhombiformis* = 8, *H. shuttleworthii* = 6, *H. speciosa* = 3, *Hexastylis* spp = 27, *H. virginica* = 5, Valdese spp = 2 (Fig. 2). We processed 526 individuals for microsatellite data. We gathered stream order information from 83 sites, estimates of disturbance from 112 sites, estimates of number of plants from 119 sites, land owner information from 111 sites, and an estimate of slope aspect from 117 sites.

Figure 1. Elemental Occurrence data for Hexastylis naniflora.

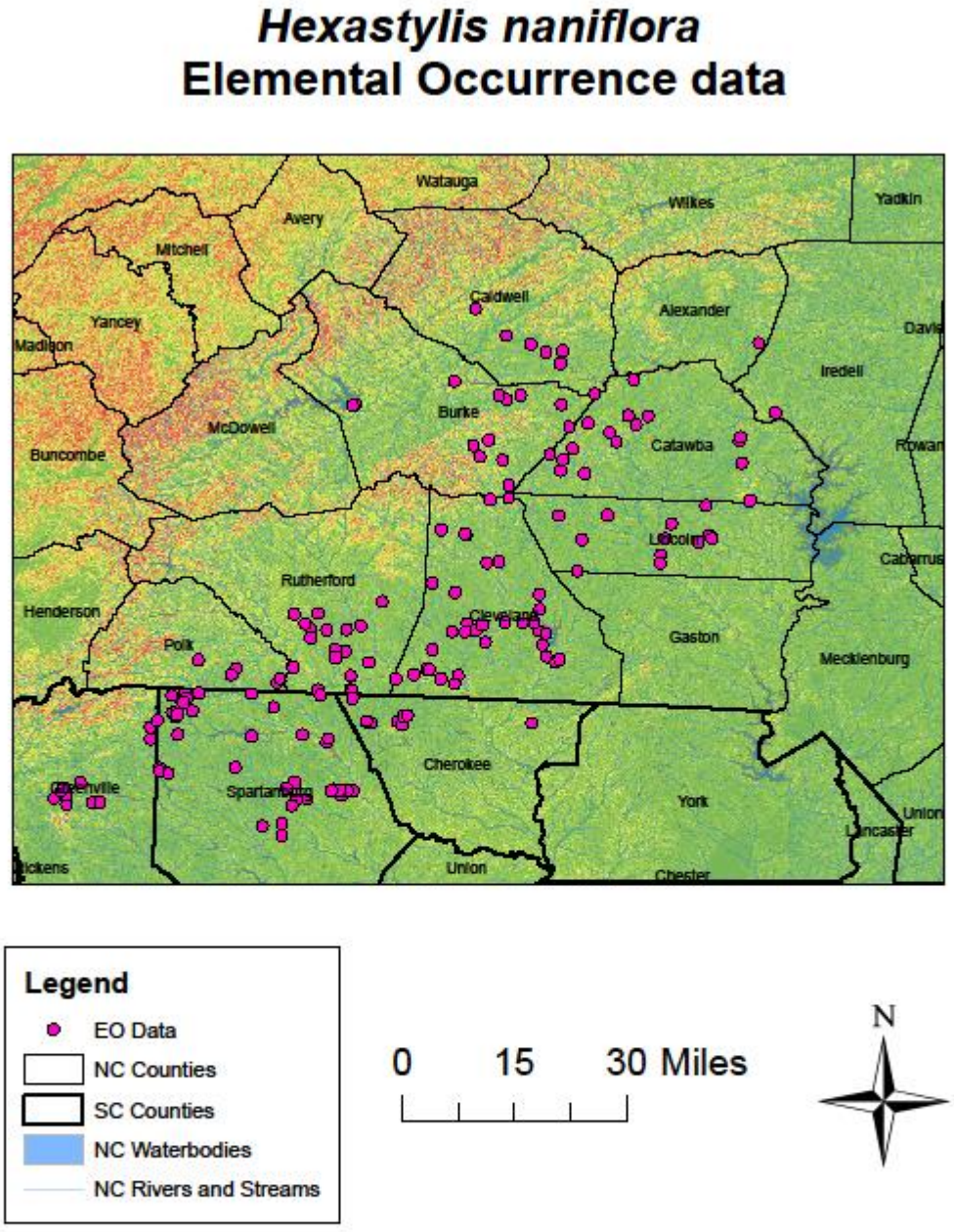
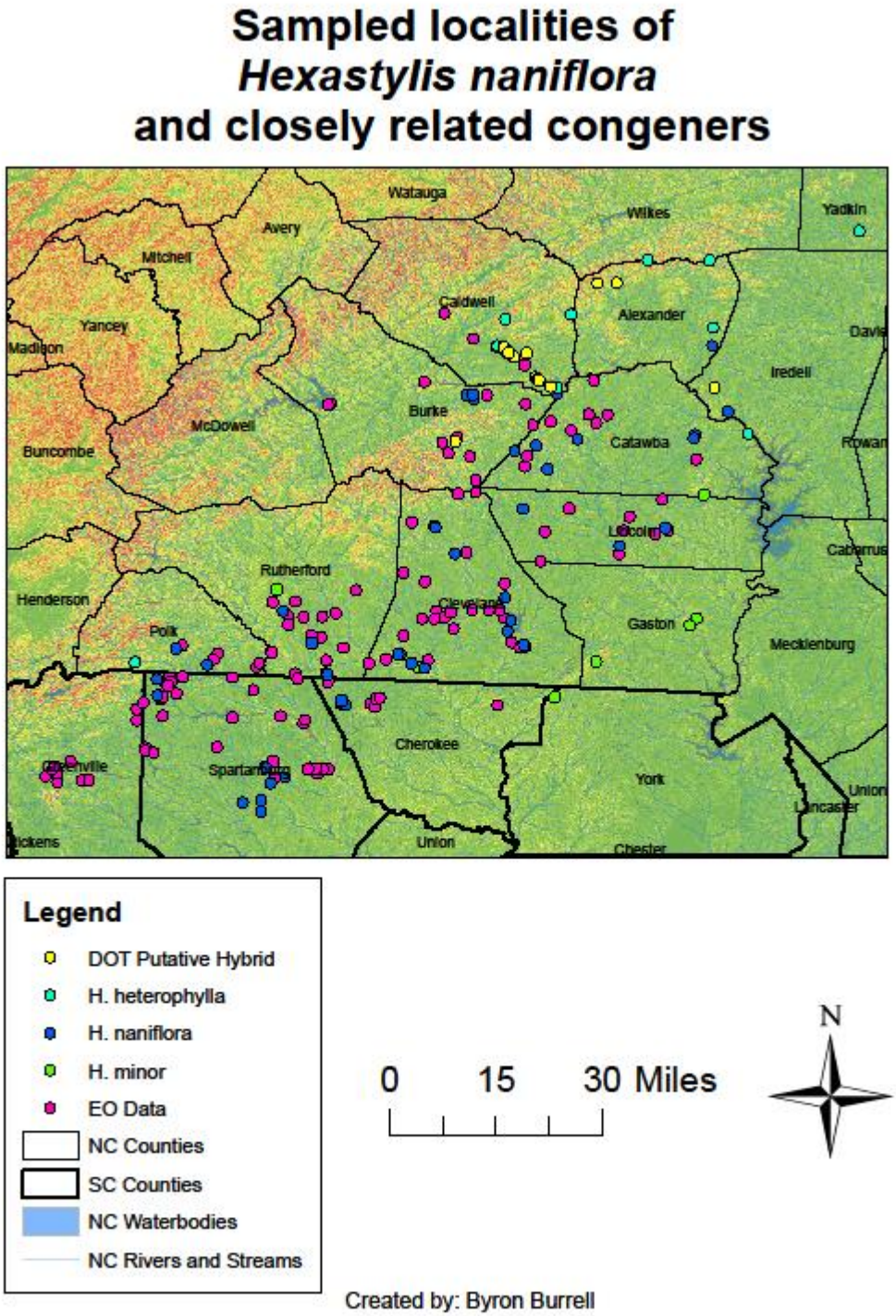


Figure 2. Collection sites for *H. naniflora*, *H. heterophylla*, *H. minor* and DOT Putative Hybrids.



3. At each site, one leaf and one flower were collected from each of 4-15 plants. Fewer leaf and flower samples were taken from sites with less than 20 individuals and those that did not show morphological or geographic evidence of hybridization. Leaves and flowers were transported on ice to ASU and processed within 24 hours. One voucher specimen from each site was photographed, dried, and pressed. These voucher specimens were included in the leaf morphology analysis but not in genetic or flower morphology investigations. We obtained some data (specimen, photo, microsatellite) for 646 individuals. From these collections, 84 herbarium specimens were prepared with at least one flower and one leaf from each population or site and these specimens have been deposited at the Appalachian State University Herbarium (BOON).
4. After collection and transportation back to ASU, the flowers from 414 individuals were photographed and immediately preserved in a 0.1M sodium phosphate buffer containing 2.5% gluteraldehyde. Immediately before imaging the flowers they were removed from the buffer, cut in half, and, using a razor blade, a one half centimeter square was cut from the center of the calyx starting from the first trichomes to standardize the cut (Fig. 3). The flower square was immediately placed under a Keyence VHX 1000 digital optical microscope (Itasca, Illinois, USA), photographed, and saved as a jpg file. The height data for each pixel could be extracted from the image into a 1236 X 1300 CVS file that was exported into a spreadsheet for statistical analysis. The first and last 200 rows and columns were not included in the analysis because the flowers did not lay completely flat during imaging, making the height of the edges erroneously high. The height data for the flower calyxes were then compared using a series of one-way ANOVAs, where n=15 and statistical significance was assumed for p

<0.05. The ranges of calyx heights were normalized prior to analysis by taking the square root of the raw heights.

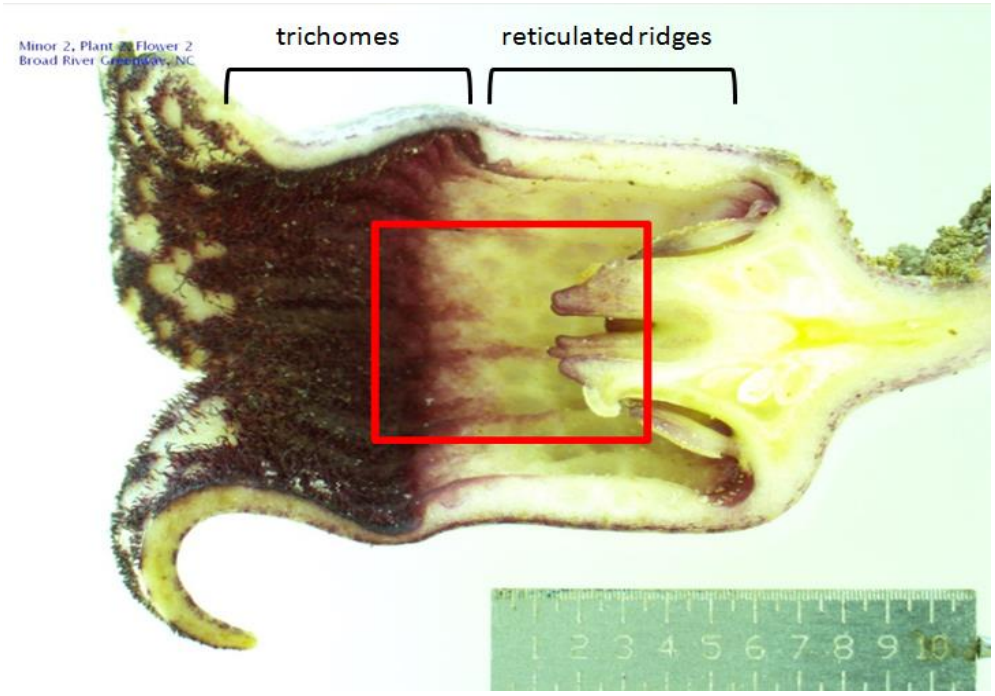


Fig. 3. An image of a *Hexastylis* flower. The red box indicates by the area cut out of the calyx tube for 3-D microscopy. The scale bar is 1 cm.

5. Leaf morphological analyses were performed using geometric morphometric analysis of leaf shape and leaf venation patterns defined by landmarks. We photographed fresh leaves using a Cannon Powershot camera. To ensure a comparable scale across all photographs, the camera was set to a standard zoom and a standard 48 cm away from the specimen when each photograph was taken.

These images were converted to jpgs and imported into TPSDig2 (Rohlf, 2004). In this freeware program, 17 landmarks were set following published protocols across one half of the leaf assuming bilateral leaf symmetry (Viscosi et al., 2009). The landmarks were set at the apex and base of the leaf and where leaf veins branched or intersected (Fig. 4). These points were also chosen based on their ease of replication

across all species. Within TPSDig the 17 landmarks had to be set in the same order on each leaf. Only one leaf per plant was landmarked and some plants could not be included because the quality of the photograph or the clarity of leaf venation did not allow for confident placement of each of the 17 landmarks.

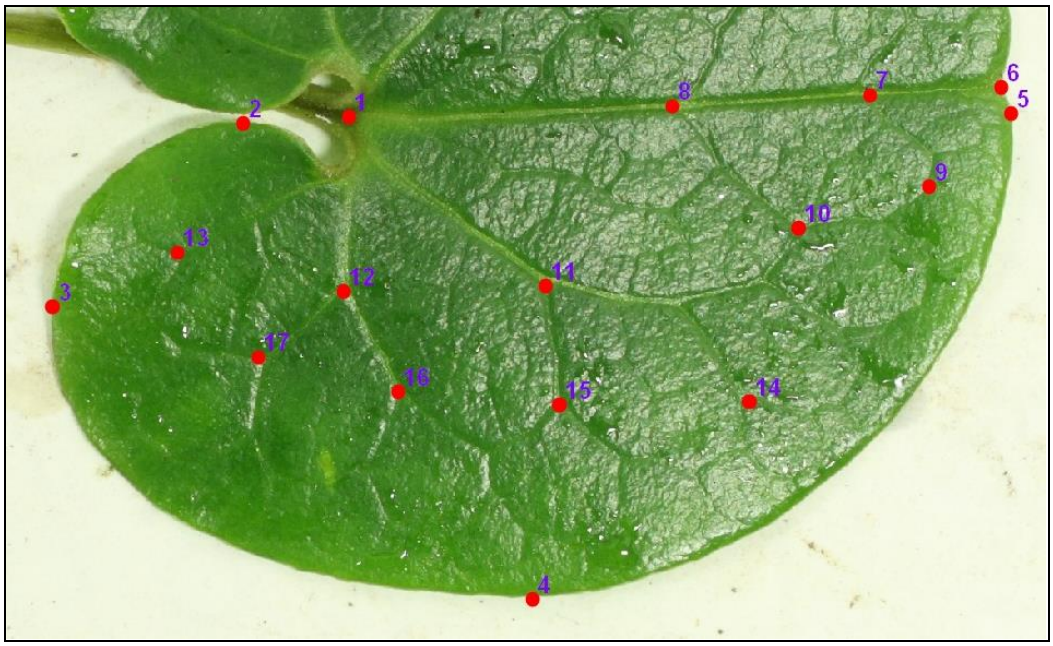


Fig. 4. Photograph of fresh *Hexastylis* leaf with the 17 landmarks used for geometric morphometric analysis. The landmarks were set in TPSDig and were replicated across all species.

The landmark data were moved into MorphoJ version 2.0 (Klingenberg, 2011), a freeware program designed to perform statistical and graphic analyses that quantify differences of form among groups. The first step in MorphoJ was to perform a Procrustes superimposition to separate form variation from size components by standardizing each leaf's landmarks to a unit centroid size (Viscosi et al., 2009). From there, we investigated differences between species using a canonical variate analysis (CVA) (performed in MorphoJ) which utilizes the two sets of variables (x and y at each landmark) to find the linear combinations of landmarks that maximize species differences (Cambell and Atchley, 1981).

The leaf photographs used for geometric morphometric analysis and ASU herbarium specimens were both used to classify leaf tips by species looking at *H. naniflora*, *H. heterophylla*, and *H. minor*. Each leaf tip was classified as retuse, obtuse, or acute. The data were analyzed using a chi-square analysis to determine if species and leaf tip type were independently distributed.

6. Flower morphology was analyzed using geometric morphometrics across 91 different specimens. Fresh flower pictures were used that had already been assigned species labels and site information. Images had to be carefully selected for flowers that were suitable for the accurate and consistent placement of landmarks on flower homologies. Some images were not of high enough quality to be landmarked and therefore could not be included in this analysis. The usable images were imported into TPSdig2: a free software that allows for the digitization of landmarks for morphological analyses (Rohlf 2004).

   Landmarks were placed at the junction of the pedicel with the flower, the base of the ovaries, the convergence of the sepals, as well as on other prominent morphological homologies of the flower (Fig. 5). Thirteen landmarks were identified on each flower and they demonstrated bilateral object symmetry. This landmark data was imported into MorphoJ 2.0 (Klingenberg 2011) where a procrustes fit was performed to standardize flower size.

   A Principle Component Analysis (PCoA) and Canonical Variate Analysis (CVA) were performed in order to distinguish among pre-determined species classifiers and several revised morphologically determined classifiers. Certain landmarks and specimens could be removed from the analysis in order to isolate or emphasize different features, such as the ovary position to address superior versus inferior.

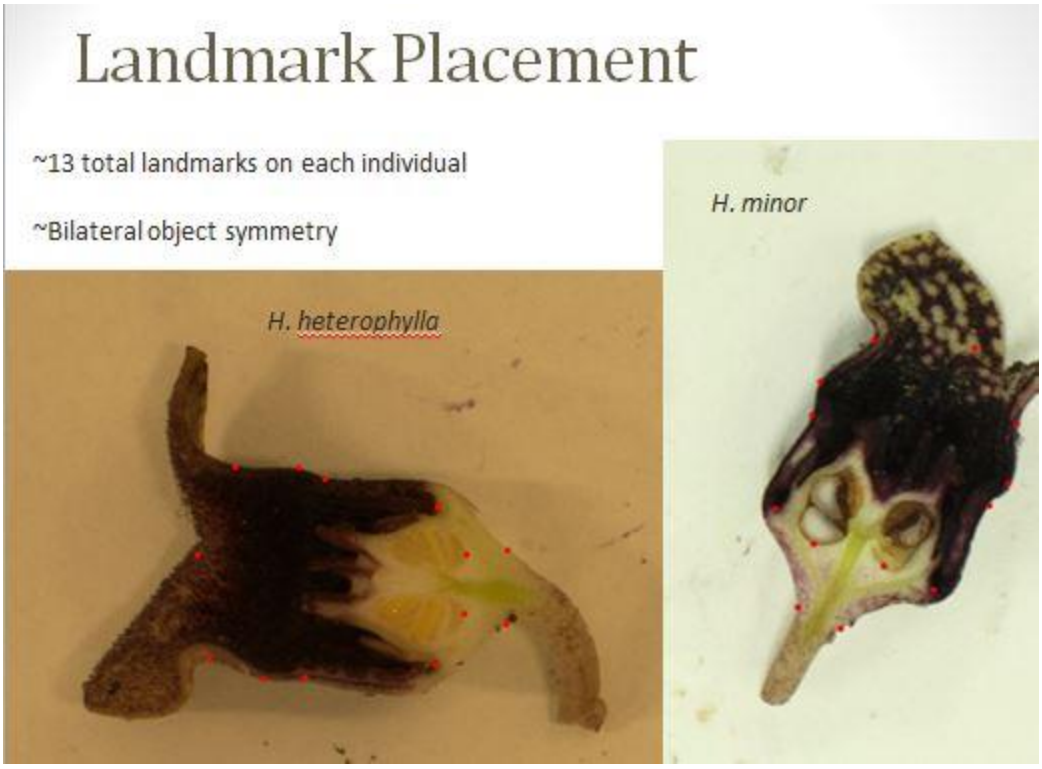


Figure 5. Landmark placement for flower shape analysis.

7. After leaf material was photographed for morphological analysis, leaves were dried in a mixture of high purity grade, pore size 22A silica gel and type III indicating silica gel. Leaves were dehydrated then hole-punched and the punches were weighed. A total of about 0.01 grams of leaf tissue were ground to a powder using liquid nitrogen to freeze the leaf and micropestals driven by a power drill to grind the sample. DNA extractions were then carried out according to protocol laid out using a Qiagen Plant Mini Kit (QIAGEN, Valencia, California, USA). The concentration and quality of the extracted DNA was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). All DNA samples were diluted to a standard 20 ng/µL for downstream applications..

8. Tissue samples from one plant of *H. naniflora* and one plant of *H. heterophylla* 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 AluI, Hpy166II, and RsaI (New England Biolabs, Ipswich, Massachusetts, USA) were used to digest the DNA, which was then ligated to Illumina Y-adapter 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, Ipswich, Massachusetts, USA) 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 300bp-600bp were recovered with Ampure beads (Beckman Coulter, Miami, Florida, 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, Madison, Wisconsin, USA). Contigs containing microsatellite repeats were identified using MSATCOMMANDER ver. 1.0.3 (Faircloth, 2008) and possible primer pairs were identified.

   One hundred and 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 10µL reaction consisting of GoTaq Flexi Buffer, 2.5 mM MgCl2, 800 µM dNTPs, 0.5 µM of each primer, 0.5 units of GoTaq Flexi DNA Polymerase, and ~20ng 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, Staffordshire, UK) encompassing a 13˚C span of annealing temperatures from 68 ˚C to 55 ˚C. Initial denaturation was at 94 ˚C for 5 minutes, 13 cycles at 94 ˚C for 45 seconds, touchdown for 2 minutes, and 72 ˚C for 1 minute, followed by 24 cycles at 94 ˚C for 45 seconds, 55˚C for 1 minute, and 72 ˚C for 1 minute followed by a final extension at 72 ˚C for 5 minutes. 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).

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

Locus	Primer sequence (5'– 3')	Fluorescent dyes	Sequence Motif	# of Repeats	Ta (°C)	Allele Size Range (basepairs)	GenBank accession number
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	AAAG	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	VIC	AAG	10	58.97	179-205	KM024990

	R: AATGTGGAGGAATCTGAGAACAC						
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: Ta = Annealing temperature, M = Monomorphic, All forward primers also contain an M13 tag (5'-CACGACGTTGTAAAACGAC-3') on their 5' end to allow fluorescent labeling of PCR products.

Polymorphism screening PCR reaction conditions were the same as above, except the forward primer concentration was reduced to 0.25µM and 0.25µM of an M13 primer (5'-CACGACGTTGTAAAACGAC-3'), labeled with FAM, VIC, NED, PET (Life Technologies, Grand Island, New York, USA), was added to the reaction. PCR products labeled with different fluorescent dyes were then pseudo-multiplexed and 2ul of the combined reactions were submitted for genotyping on an ABI3730 using a LIZ 500 ladder (Life Technologies). Resulting chromatograms were visualized and scored using the software package Geneious Version 7 (Biomatters Ltd., Auckland, New Zealand). The resulting genotypic data was then analyzed with GenAlEx Ver 6.5 (Peakall and

Smouse 2006, 2012) to obtain standard descriptive statistics and to test for deviations from Hardy-Weinberg equilibrium assumptions (Table 2).

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	Ho	He	HWE	A	Ho	He	HWE	A	Ho	He	HWE
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, Ho = Observed heterozygosity, He = Expected heterozygosity, HWE = Hardy-Weinberg Equilibrium test (*(P<0.05), **(P<0.01), ***(P<0.001), M = Monomorphic, n.s. = Not significant).

9. We have currently genotyped 280 individuals (245 in the species complex) from 138 separate populations for each of 16 loci. We expect to add 188 more individuals by May 2015. All individuals have been or will be genotyped at Georgia Genomics Facility for 17 previously identified microsatellite markers [published data] and the resulting chromatograms was scored using Geneious version 7 (Biomatters Ltd., Auckland, New Zealand). Five samples were removed from the analysis of the species complex due to a lack of reliable information on the plants' locality.
10. Genotypic data from the 280 individuals was then analyzed with GenAlEx Ver 6.5 (Peakall and Smouse 2006, 2012). Principal Coordinates Analysis (PCoA), a multivariate technique that allows one to find and plot the major patterns within a multivariate dataset, was used to initially explore the dataset. The Analysis option in

the PCoA menu was used to find the relationship between the distance matrix elements based on their first principal coordinates.

11. STRUCTURE analysis. A population assignment test was performed in Structure version 2.3.4 (1) utilizing the admixture model with a burn-in period of 15,000 and a Markov Chain Monte Carlo (MCMC) of 150,000 iterations. Three independent runs were performed each for K = 1-8. The number of populations was determined with the Evanno Method (2) utilizing STRUCTURE Harvester (3). Pie charts representing admixture levels of all individuals were created in PhyloGeoViz (4) and displayed in Google Earth.
12. Site suitability analysis. There are 273 Elemental Occurrence Records (EORs) for *H. naniflora*, with more than one EOR making up many of the 108 populations outlined by the USFWS. All of the habitat analyses for this project employed *H. naniflora* EOR data obtained from the North Carolina Natural Heritage Program (NCNHP). South Carolina EORs were not used because the precise boundaries are not currently available for most of them. Most SC sites were mapped out at point locations, depicting the centroid of the occurrence but not accurately displaying the shape of the boundary. The data are presented in North American Datum (NAD) 83, Universal Transverse Mercator (UTM) 17, meter projection. The EORs were created in July of 2011. Presumed accuracy for the data values are: seconds (+/- 3), minutes (+/- 1). Our dataset included 198 EORs to be used in the geographic analysis done in ArcMap 10.1, geographic information systems (GIS) software (Esri, Redlands, California) designed to process and analyze geospatial data.

Five categories of predictors were chosen for this study based on relevance to the species distribution and availability of high resolution datasets (10-50 m).

Shapefiles for elevation (USGS), soil type (Soil Data Mart), and landuse (USGS) were collected. The elevation and landuse datasets were already in a raster format but the soil type dataset had to be converted into a raster file from a vector file in ArcMap 10.1. Then, using the elevation dataset, raster files were created for both percent slope and slope aspect using "Surface Tools" within ArcMap10.1.

Where EOR polygons overlapped with each habitat variable shapefile, those values were recorded in a table ('zonal statistics as table'). This table enabled the classification of each EOR according to each habitat variable. The mean of every EOR polygon for each habitat variable was used to classify quantitative variables (percent slope, elevation), and the mode was used for qualitative variables (soil type, slope aspect, land use). Slope aspect was presented as both qualitative and quantative data. Categorical data were used to avoid complications arising from circular/ direction data. A histogram was created illustrate how many known *H. naniflora* populations fell within each category for each habitat variable. Ranks were assigned to each category, with the highest rank being applied to the category containing the highest frequency of *H. naniflora* populations. Each raster file was then reclassified according to these newly assigned ranks and added together using the 'map algebra' function within Arc Map 10.1. By adding the ranks for each variable together, it was possible to classify suitable habitat for *H. naniflora*. When assigning the ranks, 85% of the 198 EORs were used and 15% were reserved to be used to test the robustness of the model.

The site suitability model was assessed using 31 test populations, which were chosen using an online random number generator. These test populations were EORs not included when originally assigning ranks to habitat variables. We overlaid the test population polygon data on top of the site suitability model, assigning a habitat rank to

each test population to determine if the model could accurately discriminate between suitable and unsuitable habitat. The ranks of each test population were put into a histogram to illustrate graphically the distribution of ranks across those populations.

B. Results

1. Flower Internal Calyx Morphology

The mean heights of the flower calyx ridges across the three species and putative hybrid populations were compared using a one-way ANOVA (Fig. 6) and demonstrated significant differences between *H. naniflora* and *H. minor* with a *p*-value of <0.001. Comparisons between 1) *H. heterophylla* and *H. minor,* 2) *H. heterophylla* and *H. naniflora,* and 3) *H. minor* and H. spp. all have *p*-values of around 0.07 which is not statistically significant but may be ecologically important. *Hexastylis naniflora* had the lowest mean height while *H. minor* had the greatest. Hexastylis spp. grouped closest to *H. heterophylla* and between *H. heterophylla* and *H. naniflora.*

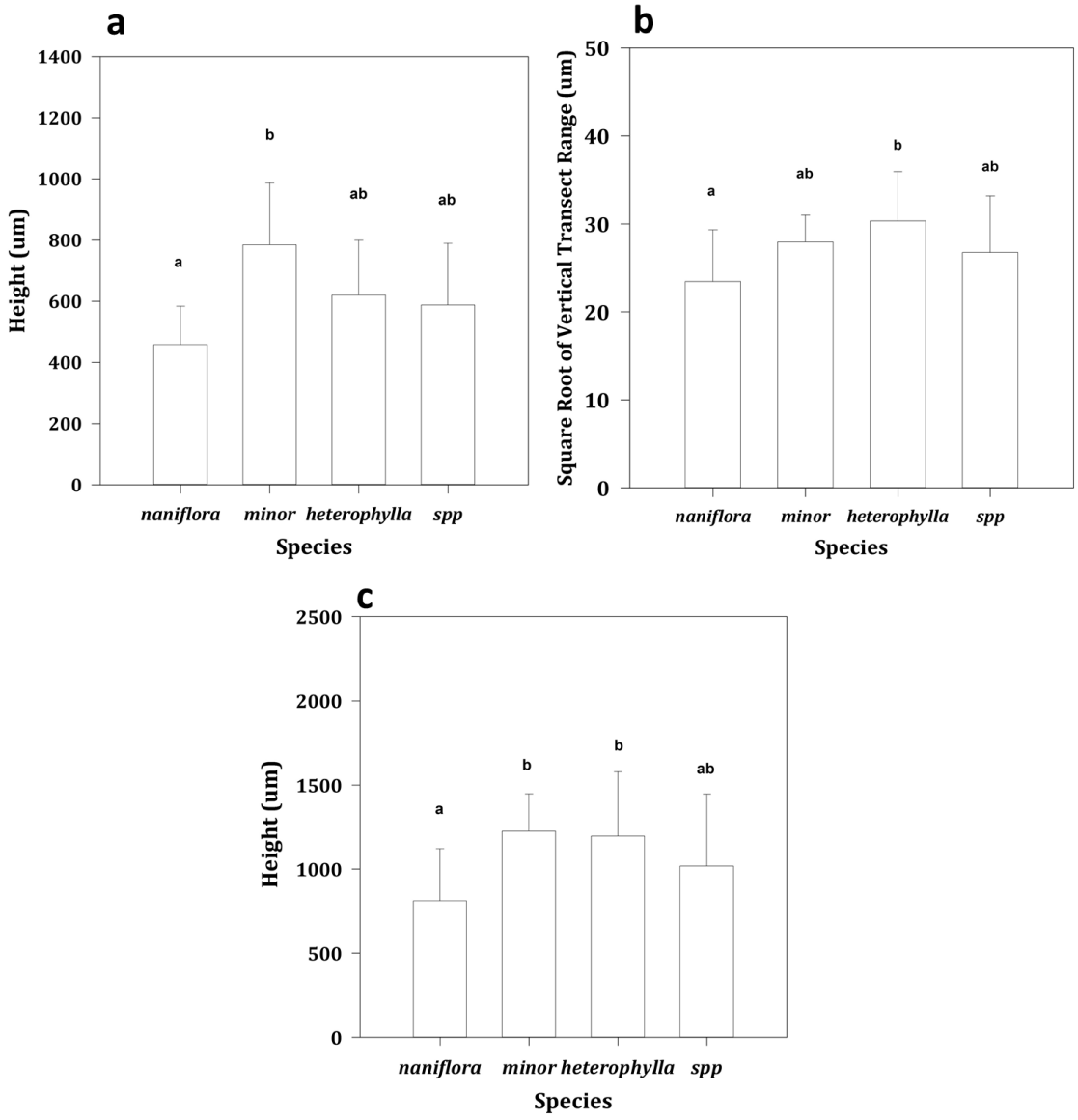


Fig. 6: a) mean height of flower calyx by species, b) range in heights across vertical calyx transects by species, and c) maximum calyx ridge heights per transect by species. Bars represent the means ± standard error. Differences of statistical significance (p<0.05) are indicated by the letters above each bar. For all three tests n=15.

The one-way ANOVA comparing maximum heights per transect between each species (Fig. 7) shows that *Hexastylis naniflora* was significantly lower in heights of the calyx ridges when compared to *H. heterophylla* or *H. minor.* The populations showing intermediate morphologies were not significantly different from any of the other species groups. The last

test performed on the calyx ridges looked at the range of heights across each transect. *Hexastylis naniflora* had a smaller range than *H. heterophylla* with statistical significance.

The correlations of mean calyx ridge height of *H. naniflora* versus latitude, longitude, and elevation show a significant relationship with both latitude (p=0.022) (r=0.57, and longitude (p=0.039) (r= -.052)(Fig. 7), but not for elevation (p=0.41). These correlations indicate that calyx ridge height generally increases moving from the southeast to the northwest extent of the *H. naniflora* range.

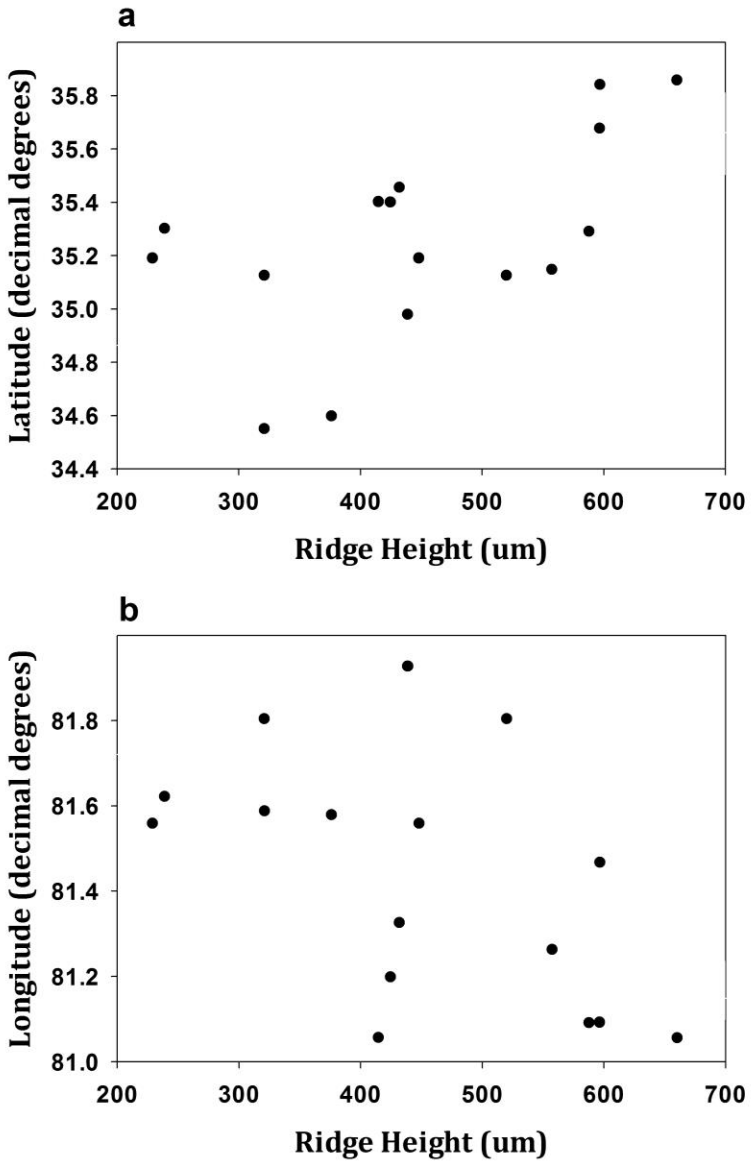


Fig. 7. Plot of mean calyx height versus a) latitude and b) longitude for 16 individuals of *H. naniflora* across 16 different populations across the range of the species. For a) Mean p=0.0216, Pearson's r= 0.57, and for b) p=0.0389, Pearson's r= -0.52 .

2. Leaf Morphology:

The first CVA compares leaf morphology of *H. naniflora* (25 observations), *H. minor* (15 observations), and *H. heterophylla* (48 observations). Two axes explained 100% of the total variance: 54% for CV1 and 46% for CV2. The variation among groups was scaled by the

inverse of the within-group variation. The scatterplot of CV1 and CV2 (Fig. 8) shows that along CV1 *H. naniflora* separates out from *H. minor* and that CV2 explains differences between *H. heterophylla* and the other two species. Landmarks y15, y7, and x2 were the strongest drivers of CV1 and landmarks y7, x1, and x4 where the strongest drivers of CV2 (Table 3).

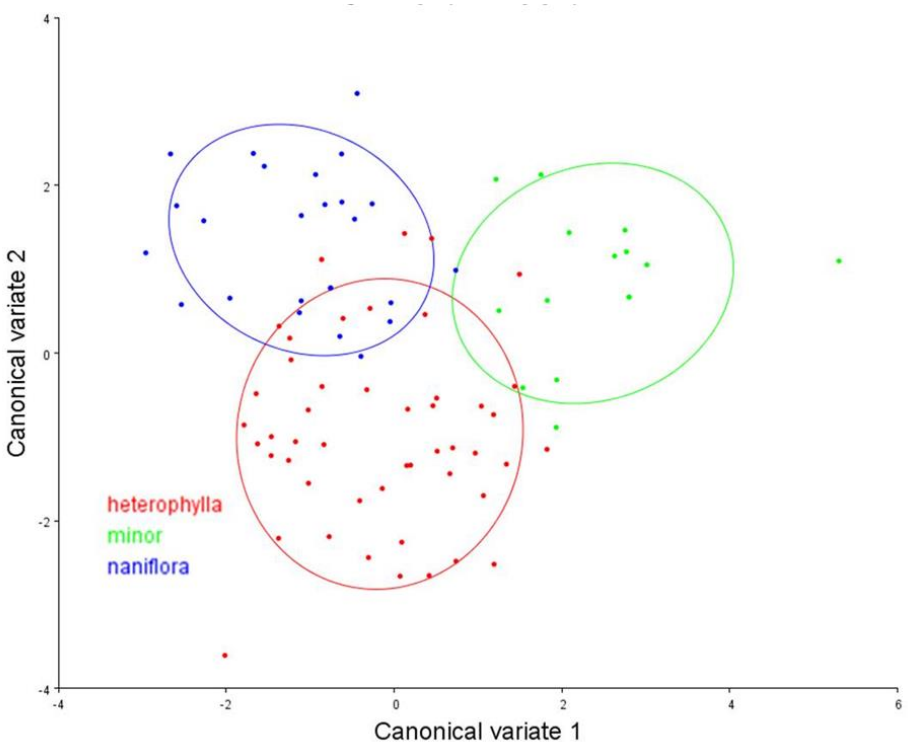


Fig. 8. Canonical variate analysis of leaf morphology for the Hexastylis Heterophylla subgroup not inlcuding populations displaying intermediate morphologies. Axes were defined by morphometric landmark data. Ellipses indicate a 90% confidence interval.

Table 3. The strength of each predictor variable (landmark) on the canonical variate analysis(CVA) axes (CV1, CV2, CV3) for each of two CVAs. The first CVA excludes the intermediate morphologies (H. spp.) while the second one includes it.

	CVA1:*h, m, n*		CVA2: *h, m, n,* spp.		
Landmark	CV1	CV2	CV1	CV2	CV3
x1	-13.57	5.43	7.15	13.31	-2.86
y1	58.96	3.78	0.45	-48.54	16.06
x2	-25.37	11.36	1.00	13.35	0.85
y2	-32.53	-16.99	-5.22	18.87	4.94
x3	2.77	-26.06	-14.92	5.45	13.32
y3	-16.65	-3.21	-8.88	-0.29	-24.49
x4	0.64	8.01	4.75	7.47	-5.18
y4	-3.14	5.21	7.17	-13.21	2.16
x5	-3.97	17.57	48.82	92.14	27.70
y5	-12.24	-25.69	-27.61	20.01	9.15
x6	-10.54	-26.91	-53.24	-70.35	-7.07
y6	12.42	31.53	36.18	-32.80	-13.97
x7	-47.57	-10.11	-11.18	8.13	-5.61
y7	68.38	14.03	-6.41	-21.89	-0.10
x8	26.15	-16.51	-23.11	-15.12	-0.68
y8	-105.04	-15.30	4.08	87.36	18.25
x9	19.77	26.47	16.35	-8.87	-24.18
y9	23.52	11.89	16.78	-5.13	-7.86
x10	1.24	11.79	23.25	4.60	7.52
y10	-4.02	0.24	-6.32	-21.30	-6.18
x11	40.13	-63.49	-40.48	-32.40	12.09
y11	-64.00	-9.15	-13.63	54.97	-12.48
x12	29.51	-24.03	-40.78	-20.04	26.93
y12	-6.02	-12.96	-31.66	20.05	-30.97
x13	30.12	33.18	31.64	-10.92	-4.96
y13	33.59	35.24	36.89	-7.37	18.75
x14	34.14	-1.45	4.06	-3.28	15.47
y14	18.95	-39.49	-24.42	-32.31	1.71
x15	6.01	46.68	18.46	-12.68	-9.23
y15	-13.70	31.87	33.70	4.54	-4.80
x16	-62.34	-24.09	19.06	54.38	-12.33
y16	13.57	16.28	12.01	9.69	11.60
x17	-27.13	32.18	9.16	-25.18	-31.78
y17	27.96	-27.27	-23.12	-32.66	18.22

The Mahalanobis distances among groups show the greatest differences between *H. minor* and the other two species while *H. heterophylla* and *H. naniflora* group more closely (Table 4). Permutation tests (1000 permutation rounds) for Mahalanobis distances among groups are all statistically significant, with *p*-values of <0.0001.

Table 4. Mahalanobis distances and the associated *p*-value for each species group comparison as reported from the canonical variate analysis. *h= H. heterophylla, m= H. minor, n= H. naniflora.*

Comparison	Mahalanobis distance	P-value
h-n	2.4966	<0.0001
h-m	3.1103	<0.0001
m-n	3.5175	<0.0001

The second CVA is the same as the first but with the inclusion of H. spp. (93 observations). Three axes explained 100% of the total variance: CV1: 43%, CV2: 36%, and CV3 21%. The scatterplot of CV1 and CV2 show *H.naniflora* separating out from *H. heterophylla* along CV1 while CV2 highlights differences between *H. minor* and the other three groups (Fig. 9). The strongest drivers for CV1 are landmarks x6, x5, and x12. The strongest drivers for CV2 are x5, y8, and y11 (Table 5).

Table 5: Mahalanobis distances and the associated *p*-value for each species group comparison as reported from the canonical variate analysis. *h= H. heterophylla, m= H. minor, n= H. naniflora,* spp..= populations displaying intermediate morphologies.

Comparison	Mahalanobis distance	*p*-value
h-n	2.2325	<0.0001
h-m	2.8049	<0.0001
m-n	2.8282	<0.0001
h-spp.	1.6989	<0.0001
m-spp.	2.5655	<0.0001
n-spp.	1.6022	0.0047

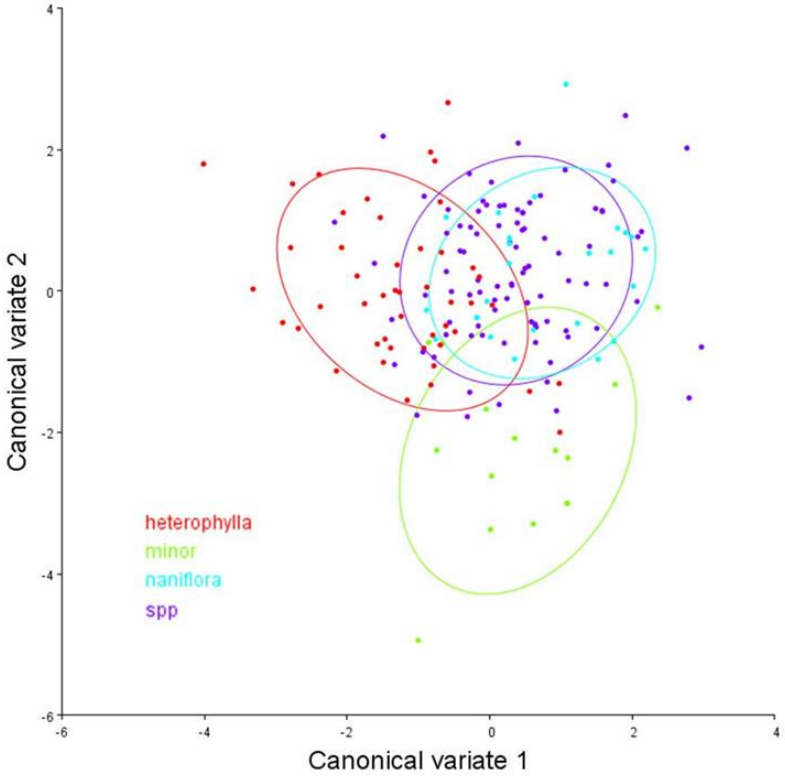


Fig. 9. Canonical variate analysis of leaf morphology for the Hexastylis heterophylla subgroup including populations displaying intermediate morphologies (H. spp.). The ellipses indicate 90% confidence. The axes were defined by morphometric landmark data.

In the second CVA the Mahalanobis distances are again greatest when comparing *H. minor* to the other groups while H. spp. groups closest with *H. naniflora* and *H. heterophylla* (Table 5). Again, the *p*-values from permutation tests for Mahalanobis distances among groups are all <0.0001 with the exceptions of *H. naniflora* versus H. spp. which has a *p*-value of 0.0047.

A χ^2 test found non-independence between leaf tip type and species, $\chi^2 = 13.25$, df= 4, *p*=0.010. *Hexastylis naniflora* had the greatest frequency of leaves with retuse leaf tips and the least amount of leaves in the acute category while the inverse was true for both *H. heterophylla* and *H. minor* (Fig. 10).

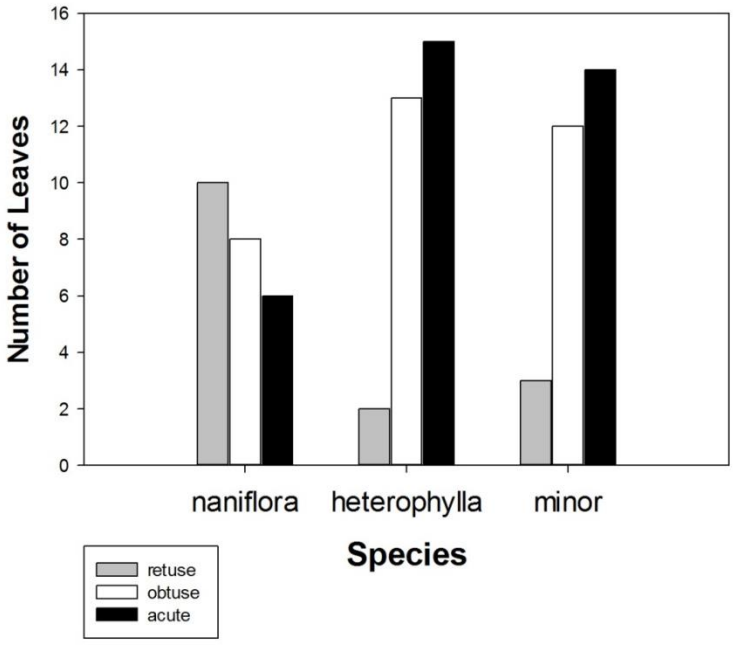


Fig. 10: Raw values for leaf tip types by species. *Hexastylis naniflora* has more retuse tips while the other species have fewer, showing non-independence among species and leaf tip type. N=83, χ^2= 13.25, df= 4, p= 0.010.

3. Flower shape

Thirteen landmarks were identified on each flower and they demonstrated bilateral object symmetry (Fig. 11). This landmark data was imported into MorphoJ 2.0 (Klingenberg 2011) where a procrustes fit was performed to standardize flower size. Principle Component Analysis (PCoA) and Canonical Variate Analysis (CVA) were performed in order to distinguish among pre-determined species classifiers and several revised morphologically determined classifiers and results are shown in Fig. 12, comparing the a priori species assignments, the two cluster STRUCTURE assignments (Fig. 13) and the four cluster STRUCTURE assignments (Fig. 14).

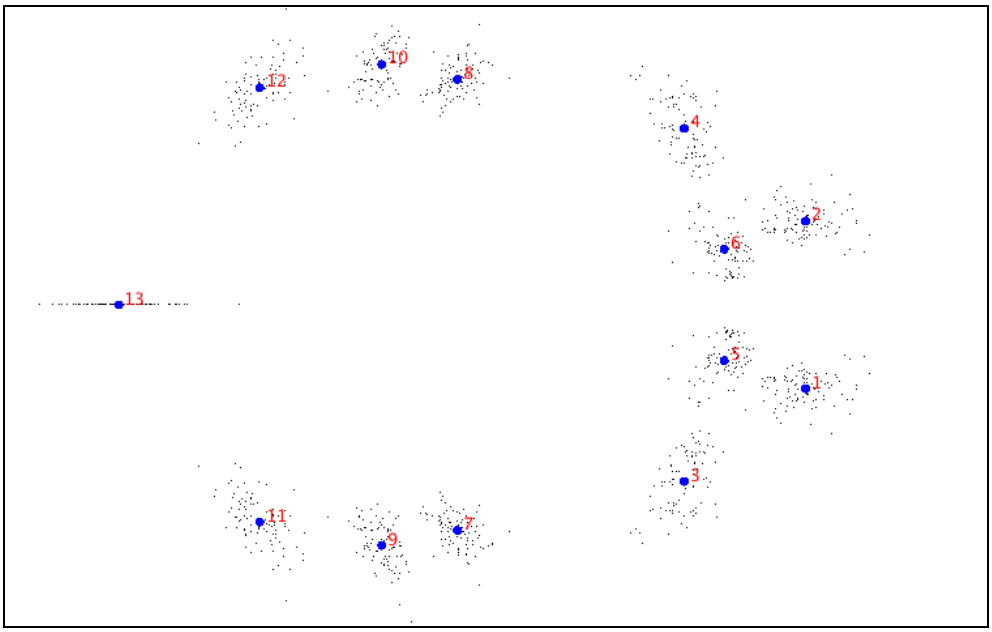


Figure 11. Mean landmark positions of flower analysis.

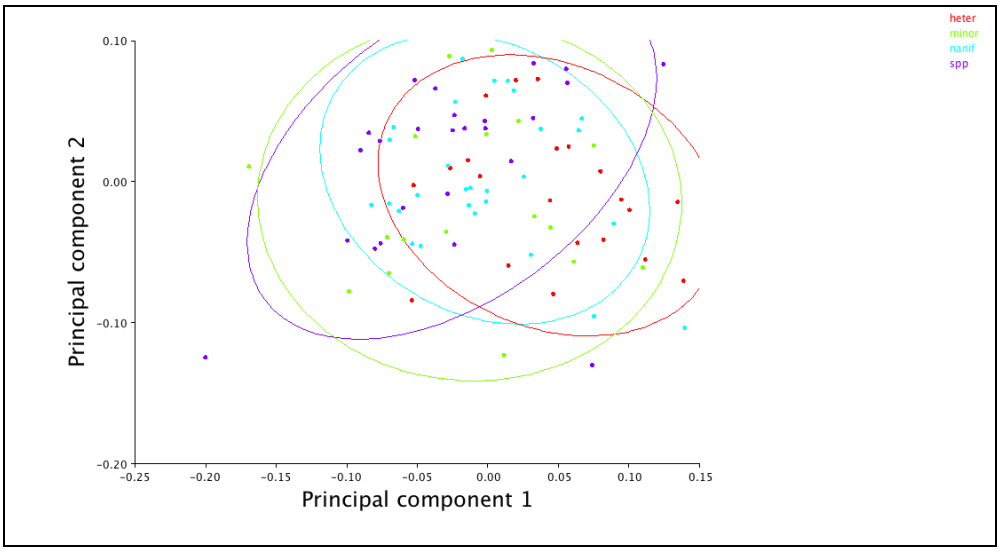


Figure 12. PCoA using four a priori species assignments.

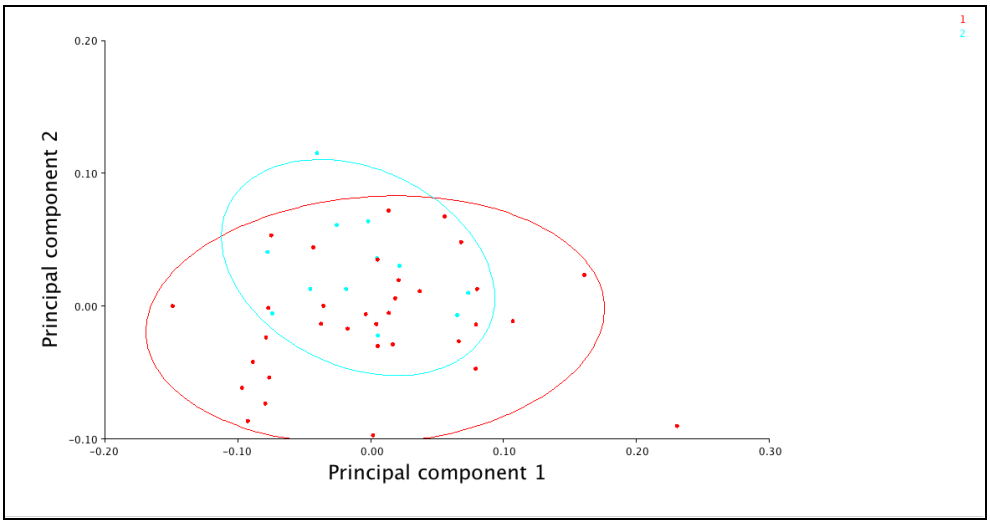


Figure 13. PCoA of flower shape using the two cluster STRUCTURE assignments.

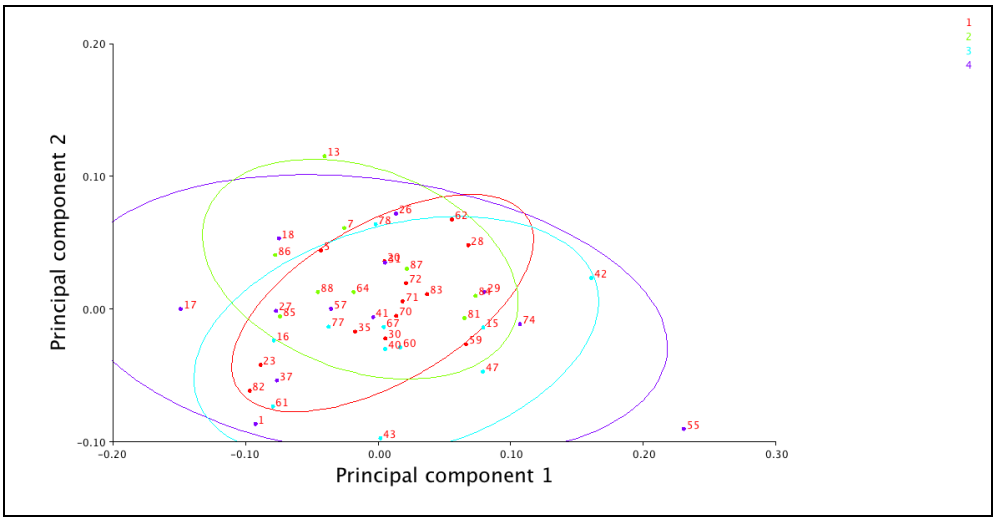


Figure 14. PCoA of flower shape using the four cluster STRUCTURE assignments.

4. Molecular Analyses

4A. Microsatellite development

Sixteen of the primer pairs tested were polymorphic with the number of alleles ranging from 2 to 23 (Mean ~8.8) in *H. naniflora*, 1 to 9 (Mean ~4.9) in *H. minor*, and 1 to 14 (Mean ~6.1) in

H. heterophylla (Table 6). 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 – 422bp; Hn01135 – 300bp; and Hn00304 – 179bp), one in H. minor (Hn00252 - 224bp), and one in H. heterophylla (Hn00002 – 297bp) were identified with a frequency greater than 10% and these can be diagnostic in species identification when morphological characters are unavailable.

Table 6. 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	Ho	He	HWE	A	Ho	He	HWE	A	Ho	He	HWE
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.
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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.
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Hn4600	2	0.385	0.393	n.s.	2	0.250	0.219	n.s.	2	0.500	0.486	n.s.
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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, Ho = Observed heterozygosity, He = Expected heterozygosity, HWE = Hardy-Weinberg Equilibrium test (*(P<0.05), **(P<0.01), ***(P<0.001), M = Monomorphic, n.s. = Not significant).

4B. Microsatellite analysis

Genotypic data from the 280 individuals analyzed with GenAlEx Ver 6.5 (Peakall and Smouse 2006, 2012) using the Analysis option in the PCoA menu generated one PCoA using only the three species assignments (H. naniflora, H. heterophylla and H. minor) and omitting the putative intermediates (Fig. 15) and a second PCoA using all 280 individuals, including putative intermediates (Fig. 16).

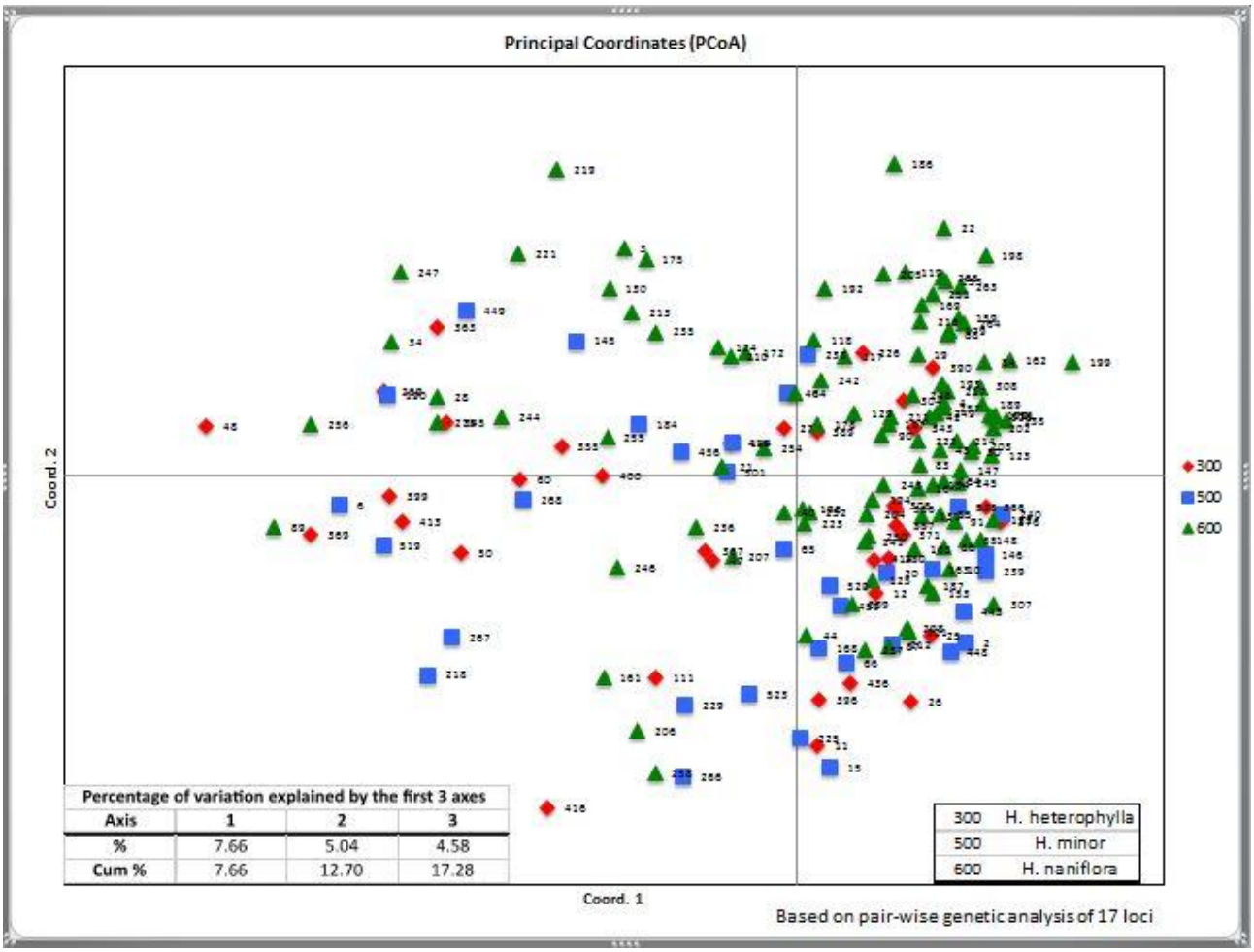

Figure 15. PCoA of three species assignments (*H. naniflora* [green triangle], *H. heterophylla* [red diamond] and *H. minor* [blue square]) and omitting the putative intermediates.

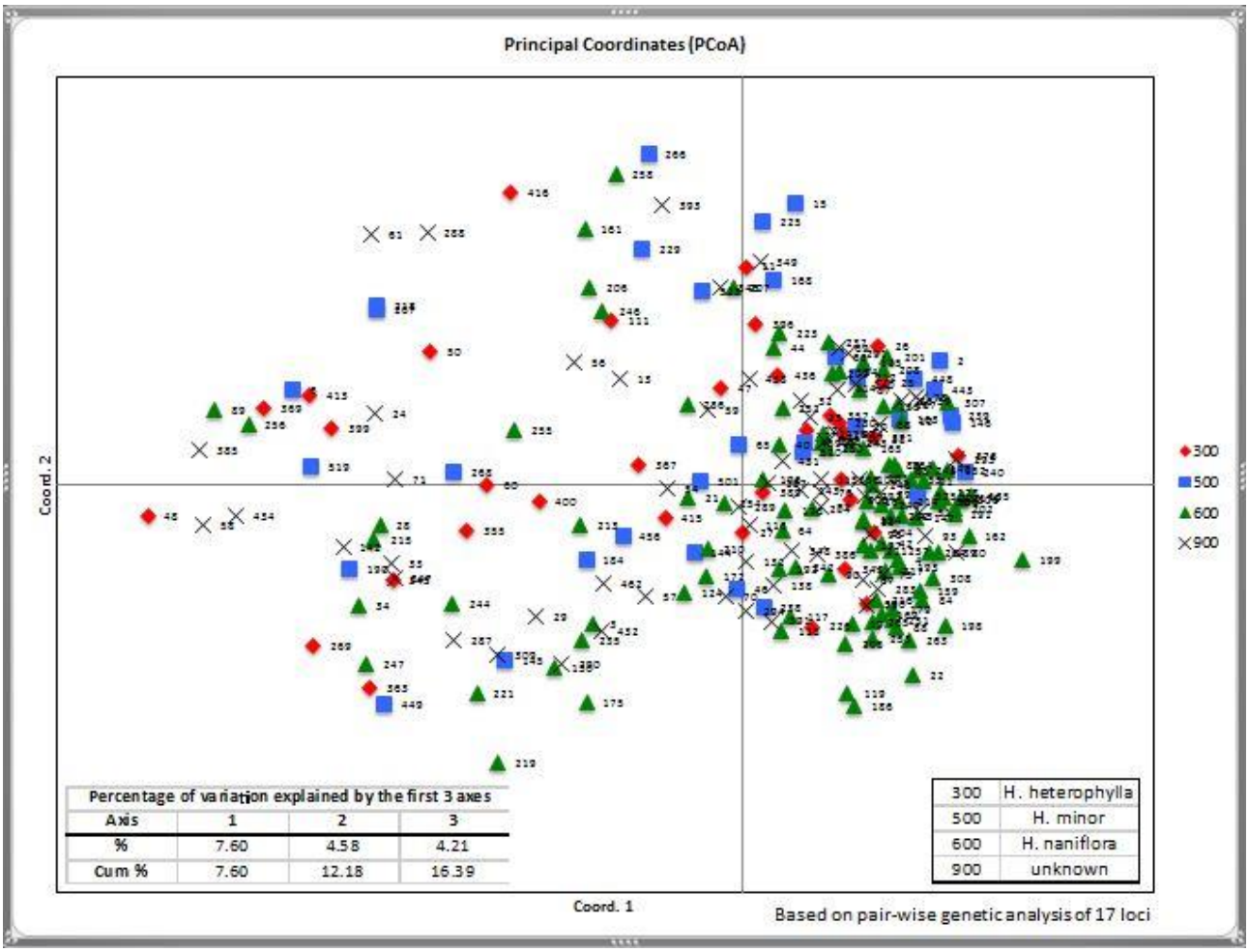

Figure 16. PCoA of three species assignments (*H. naniflora* [green triangle], *H. heterophylla* [red diamond] and *H. minor* [blue square]) and including the putative intermediates [symbolized with X].

4C. STRUCTURE analysis of microsatellite data

Three independent run performed for each of the K = 1-8 options for the population assignment test performed in Structure version 2.3.4 (1) determined best fit number of populations with the Evanno Method (2) utilizing STRUCTURE Harvester (3). The results (Figure 17) indicate that the data can be best represented with two clusters, but the four cluster option received some support.

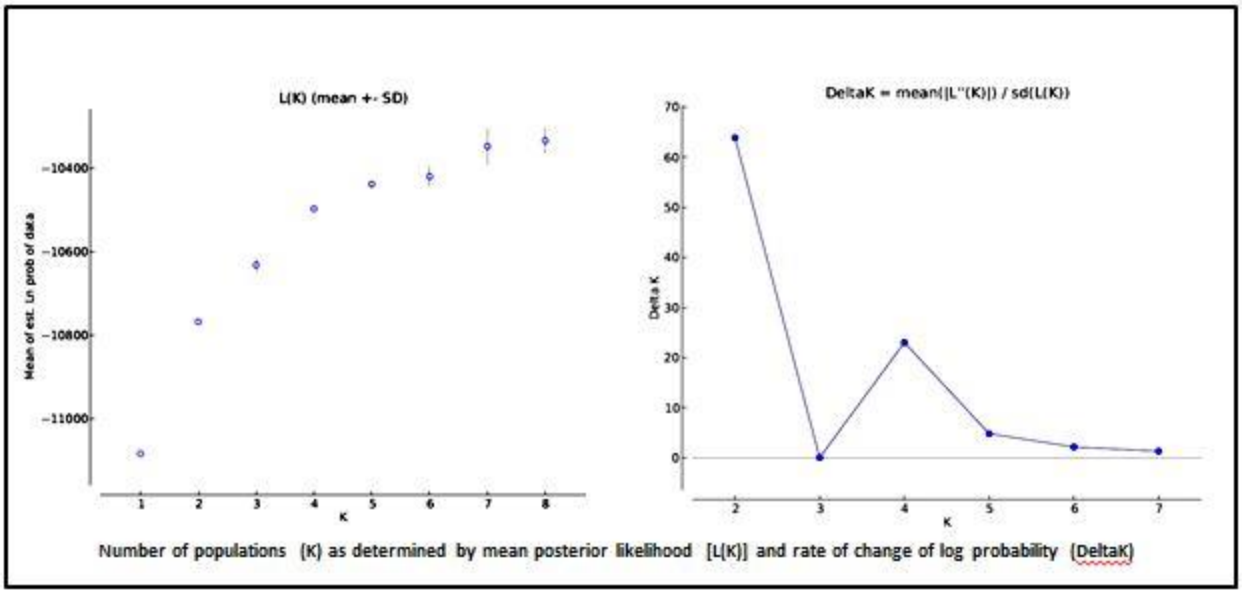

Figure 17. Results for population assignment analyses, showing support for the two and four cluster depictions of the genetic variation.

The results of the STRUCTURE analysis was represented as a bar chart showing the assignment of individuals, from our a priori identifications to species or hybrid/intermediate groups, into either the two cluster (Fig. 18) and the related statistics (Fig. 19) or the four cluster (Fig. 20) configurations and related statistics (Fig. 21).

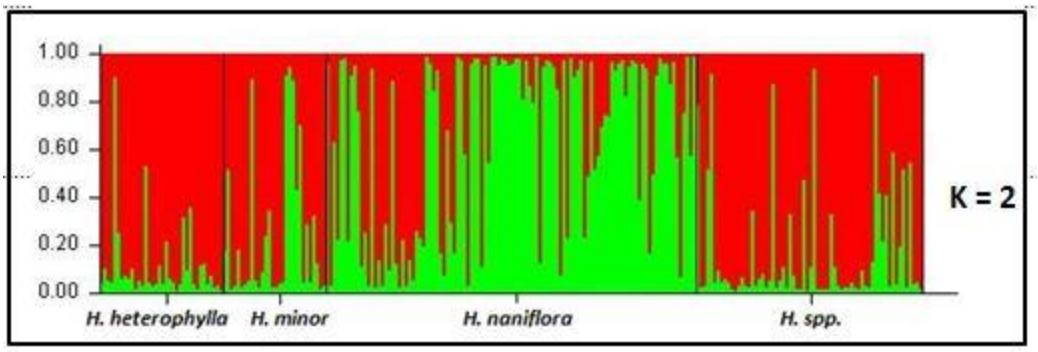

Figure 18. Results of STRUCTURE analysis for K = 2 represented as a barchart. Each vertical line represents an individual, broken up into K colored segments, representing the proportion of genes that seem to originate from a given cluster. Individuals are organized according to species identification assigned in the field based on morphology.

	heterophylla	minor	naniflora	spp	Total	% heterophylla	% minor	% naniflora	% spp.
Cluster 1	34	24	34	57	150	22.67	16.00	23.33	38.00
Cluster 2	2	6	74	9	90	2.202	6.67	81.11	10.00
Total	36	30	108	66	240	15.00	12.50	45.00	27.50
% Cluster 1	94.44	80.00	32.41	86.36	62.50				
% Cluster 2	5.56	20.00	67.59	13.64	37.50				

alpha	Fst1	Fst2
0.0965	0.0308	0.0426

Figure 19. Statistics for the STRUCTURE analysis for K = 2.

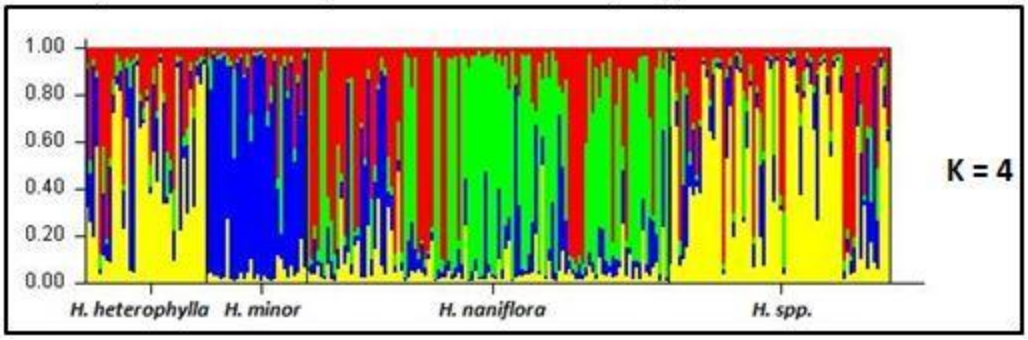

Figure 20. Results of STRUCTURE analysis for K = 4 represented as a barchart. Each vertical line represents an individual, broken up into K colored segments, representing the proportion of genes that seem to originate from a given cluster. Individuals are organized according to species identification assigned in the field based on morphology.

	H. heterophylla	H. minor	H. naniflora	H. spp	Total	% H. heterophylla	% H. minor	% H. naniflora	% H. spp.
Cluster 1	11	1	32	15	59	18.64	1.69	54.24	25.42
Cluster 2	1	1	62	3	67	1.49	1.49	92.54	4.48
Cluster 3	6	28	12	9	55	10.91	50.91	21.82	16.36
Cluster 4	18	0	2	39	59	30.51	0.00	3.39	66.10
Total	36	30	108	66	240	15.00	12.50	45.00	27.50

% Cluster 1	30.56	3.33	29.63	22.73	24.58
% Cluster 2	2.78	3.33	57.41	4.55	27.92
% Cluster 3	16.67	93.33	11.11	13.64	22.92
% Cluster 4	50.00	0.00	1.85	59.09	24.58

alpha	Fst1	Fst2	Fst3	Fst4
0.1008	0.0490	0.0850	0.0467	0.0804

Figure 21. Statistics for the STRUCTURE analysis for K = 4.

Pie charts representing admixture levels of all individuals were created in PhyloGeoViz (4) and displayed in Google Earth. The geographic map of the admixture pie charts for the two cluster analysis are shown in Fig. 22 and for the four cluster analysis in Fig. 23.

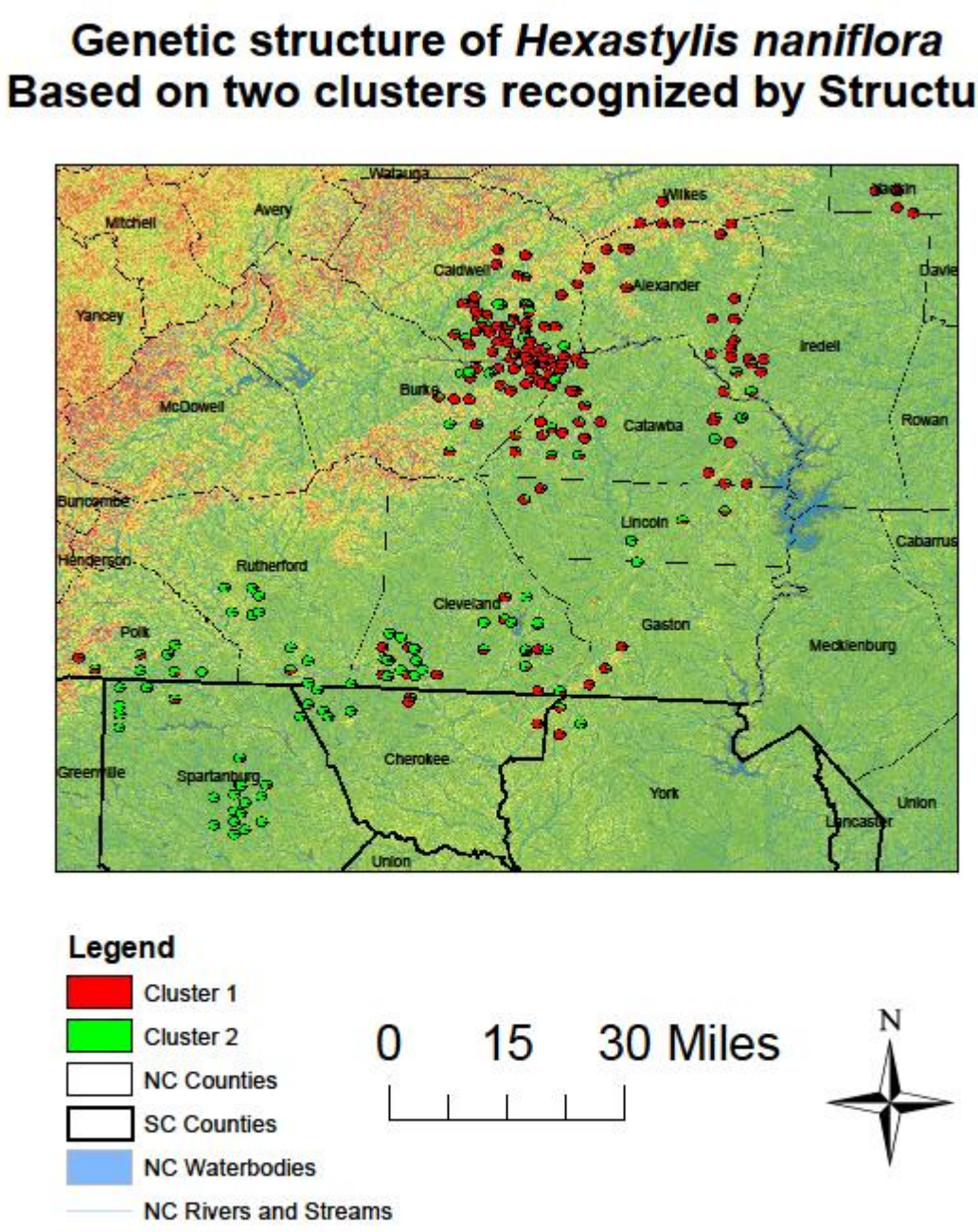

Figure 22. The geographic map of the admixture pie charts for the two cluster analysis.

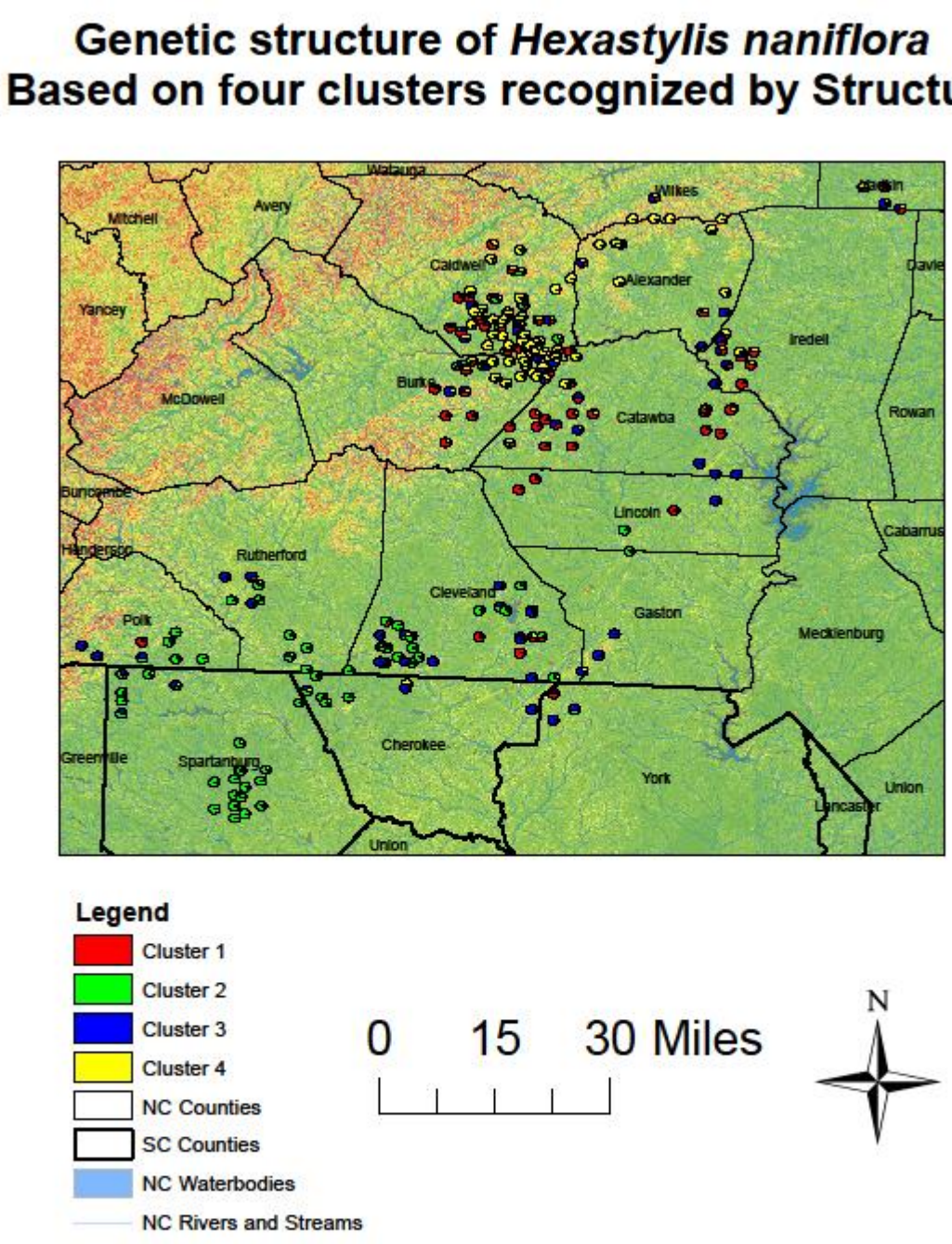

Figure 23. The geographic map of the admixture pie charts for the four cluster analysis.

5. Biogeography and site suitability analysis

A site suitability model was generated with habitat ranking from 5 (the most suitable habitat) to 36 (least suitable habitat) across 7 rank classes (Fig. 24 & 25). The site suitability model showed that 81% of the test populations were found in habitat that was considered fair to excellent. Only 19% of the test populations fell within habitat that was classified by our model as poor to very poor (Fig. 26). The histogram bins were designated using quantile class breaks so that the area of habitat is equal in each bin. The most common classification within each habitat variable was found: slope aspect: north, soil type: Pacolet sandy loam, elevation: 230 m-260 m, percent slope: 8-10.5%, landuse: deciduous forest (Table 7).

Table 7. Frequency of element occurrence records (EORs) for each habitat variable. For continuous variables (slope and elevation) data were grouped into classes with the value shown being the top end of the range. * indicates most common classification for that habitat variable.

slope		landuse (LU)		soil		elevation		aspect	
% slope	ROs	LU type	EROs	soil code	EORs	Elev. (m)	EORs	aspect	EOR
5.5	20	open water	1	6*	123	<199	0	N*	57
8	50	Low intensity residential	14	7	59	229.8571	34	NE	32
10.5*	54	High intensity residential	3	10	1	260.7143*	65	NW	32
13	35	Commercial/ Industrial	1	11	8	291.5714	29	E	13
15.5	22	Deciduous Forest*	153	other	0	322.4286	34	W	10
18	10	Evergreen Forest	13			353.2857	24	SE	22
20.5	3	Mixed Forest	1			384.1429	10	SW	13
23	3	Grassland	3			415	4	S	20
25.5	0	Pasture	11			More	0		
28	2								
More	0								

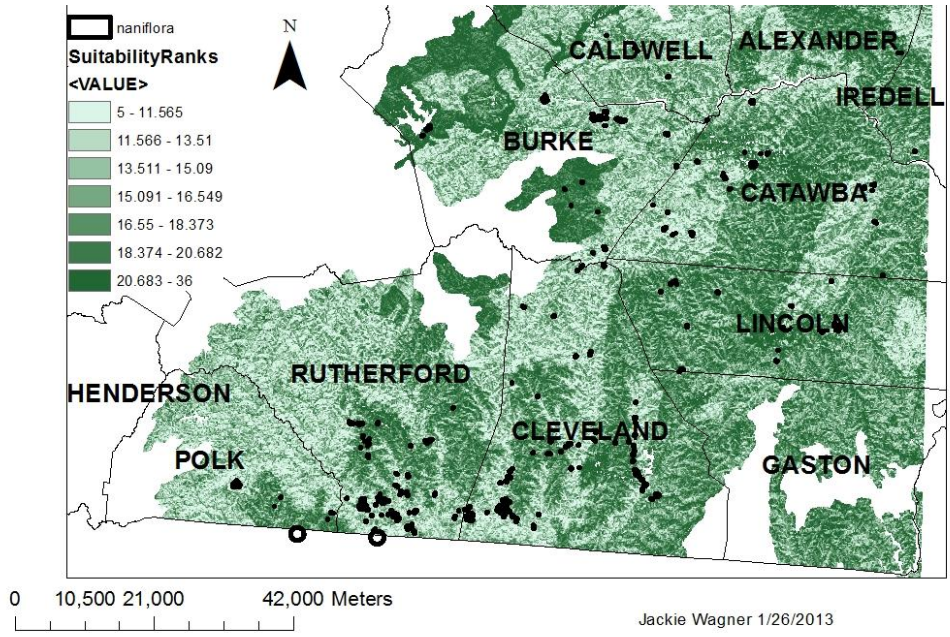

Fig. 24: The site suitability ranks based on five habitat variables across the counties where *H. naniflora* is known to exist in NC. The smaller numbers indicate a higher rank and lighter green areas denote more suitable habitat. Known populations of *H. naniflora* are outlined in black.

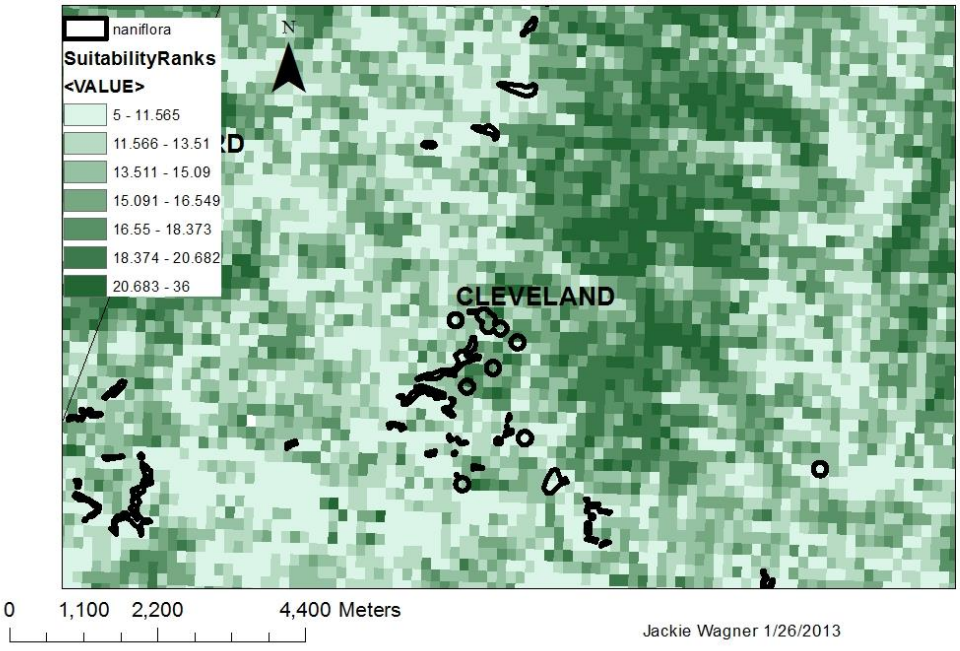

Fig. 25: A close-up of Cleveland County, NC displaying site suitability ranks. The smaller numbers indicate a higher rank and lighter green areas denote more suitable habitat. Known populations of *H. naniflora* are outlined in black.

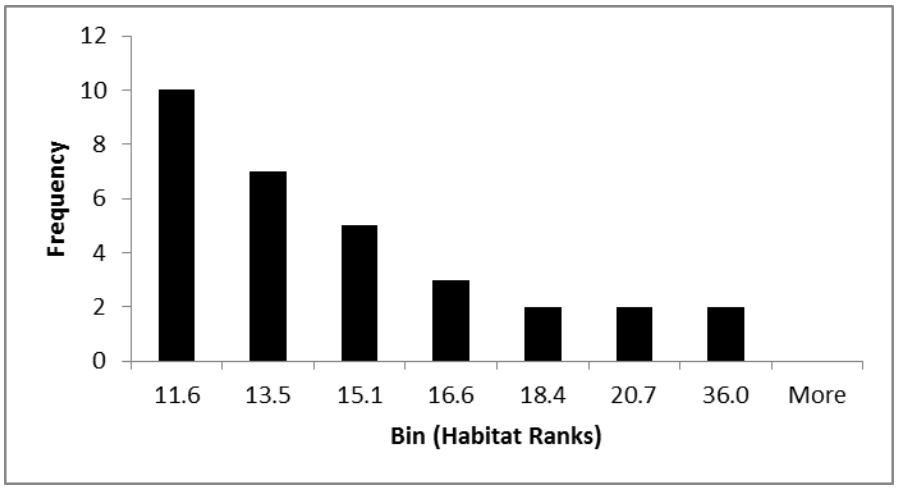

Fig. 26: A histogram displaying the frequency of 31 test populations in each habitat rank bin. The bins decrease in habitat suitability moving from left to right across the graph. The values along the x-axis indicate the bottom of the bin range. Bins were set using quantile class breaks so that total area (km^2) was equal across all bins.

C. Findings and Conclusions

- *Hexastylis naniflora* is listed as Threatened bu the US Fish and Wildlife Service and is known to be difficult to identify to species in the field, especially in areas where the distribution is sympatric with *H. minor* and *H. heterophylla*.

- Leaf tip is helpful in distinguishing populations in a vegetative state, although this is complicated by apparent hybridization.

- Calyx ridge analysis using direct microscopy shows promise as an additional tool for floristic classification.

- A model developed as part of this study, to delineate suitable *H.naniflora* habitat, appears robust and can be used as a search aid in seeking new populations, as well as a means to identify potential sites for relocation projects.

- Molecular data indicates microsatellites are an efficient tool to differentiate species and hybrids.

- *Hexastylis naniflora* exhibits a uniform genetic pattern in the southern portion of the range, with some hybridization with *H. minor*.

- In the northern portion of the range, *H. naniflora* appears to have hybridized with both *H. heterophylla* and *H. minor*, although most of the hybridization is with *H. heterophylla*.

- Populations in the northern part of the range do contain individuals with "pure" *H. naniflora* genotypes, although most individuals at these sites are hybrids.

- The morphology is, in general, in agreement with the microsatellite data in providing tools to recognize putative hybrids with reproductive samples. Vegetative materials are more difficult to confidently assign to species or hybrid groups.

1. Flower internal Calyx Morphology

Flower size and shape have been the foundation for identification of *Hexastylis* species due to the perceived similarities in leaf structure. Calyx ridges have not previously been quantitatively compared across the three closely related species: *H. heterophylla, H. minor,* and *H. naniflora.* Results from the three ANOVAs indicate that these three species can be statistically differentiated by calyx ridge height characters, which may provide a new morphological tool for this genus at the population level. These calyx height characters are not perfect differentiators and contain interspecific overlap and therefore can only be used to identify populations, not individual plants. The subtleties of these markers are difficult to resolve without a 3-D microscope rendering them ineffective in the field.

Populations with a mean calyx ridge height greater than 600 µm (one standard error away from the mean) can be eliminated as *H. naniflora* and populations with an average calyx ridge height greater than 800 µm can be considered *H. minor* with moderate confidence. The results from the calyx height data indicate that *H. minor* (785 µm) has a greater mean height than *H. heterophylla* (620 µm) but a lower mean height range for each vertical transect, further supporting the observation that the ridges of *H. heterophylla* are more randomly reticulated, as first noted by Gaddy (1987). Differences in reticulation pattern could also be used to distinguish species at a population level.

Populations displaying intermediate flower morphologies did not separate out from any of the species groups for any of the tests on calyx reticulation height. This indicates that intermediacies in calyx ridge traits are consistent with other flower traits (calyx length, diameter of calyx opening) for which these populations were classified as H. spp.

Trends in calyx ridge height of *H. naniflora* across geographical gradients likely have complex causes. Variations across landscapes in plant morphology have been seen in other plants including *Arabidopsis thaliana* (Li et al., 1998), *Carex aquatilis* (Chapin and Chapin, 1981), and *Verbascum thapsus* (Reinartz, 1984) that are caused by both adaptive and non-adaptive (genetic drift) genetic shifts as well as environmental variables. Our results show an increase in ridge height from the southeastern to the northwestern end of the range associating higher calyx ridges with colder temperatures. One possibility for these morphological shifts in *H. naniflora* could be an adaptive trait associated with attracting pollinators. *Drosophila,* a potential pollinator of *Hexastylis* (Otte, 1977), has been shown to produce larger eggs at lower temperatures associated with higher latitudes (Azevedo et al., 1996). This phenomenon may also apply to fungus gnats, which have been shown to lay their

eggs in the calyx ridges of a related genus (Sugawara, 1988). Thus, deeper calyx ridges could be an adaptive trait associated with the larger eggs of potential pollinators. The drivers for these shifts in morphology could also be environmental, caused by shifts in temperature and length of growing season (Olsson and Agren, 2002). Understanding geographic gradients in morphology can aid in the identification of species at their latitudinal and longitudinal extremes.

Shifts across latitude and longitude are generated by complex mixes of environmental (temperature and precipitation), and ecological (soil type and pollinators) factors. Interpretations of the findings in this study are speculative since they are limited to a correlation framework as opposed to an experimental one. Future experiments of environmental factors should be done to determine the contribution of each factor to the geographic variation in morphology.

2. Leaf Morphology

This comparative study examining leaf venation and leaf shape highlights some of the variation across *H. minor, H. heterophylla,* and *H. naniflora* and illustrates how closely populations displaying intermediate flower morphologies group with each species. While further investigation is required to determine what is driving the differences in leaf morphology across *Hexastylis,* these differences still provide new tools for identification of these species. Again, these markers are not perfect and can only be used at the population level due to interspecific overlap.

Leaf tip type is a quick and realistic tool for field identification for populations that are not in bloom. Populations containing more than 30% of leaves that are retuse can be classified as *H. naniflora,* while populations containing more than 40% acute leaves can be eliminated as

H. naniflora with high confidence. Geometric morphometric analysis of leaf venation requires laboratory analysis and may not be a time-efficient tool for species delineation due to broad intraspecific variation, overlap across species, and the subtleties of the differences. The lack of, and demand for, vegetative markers in this genus indicate the value of this tool despite its impracticality. The report of which landmarks drive the differences between species (Table 4) increases the utility of leaf venation markers. The greater Mahalanobis distance separating *H. minor* from H. spp. supports that these putative hybrid populations are more likely to be a cross between *H. naniflora* and *H. heterophylla.*

Hexastylis spp. populations were classified as intermediate primarily based on external calyx features. The consistent placement of these populations in between *H. heterophylla* and *H. naniflora* when considering leaf shape, leaf venation, and internal calyx features could be further evidence of hybridization within the genus or could be explained as individuals of species that are at the extremes of their morphological boundaries. Determining which of the above scenarios is driving the morphological intermediacies requires future molecular work.

3. Flower shape

The landmark placement was developed to explore two factors, flower shape and degree of calyx tissue surrounding the androgynecium. Flower shape and ovary position (half inferior in *H. naniflora* and superior in *H. heterophylla* and *H. minor*) have been the major characteristics used to differentiate species in this complex. Results presented here indicate that neither of this traits can be conclusive and both appear in intermediate forms in the hybrid individuals.

4. Molecular

The identification of reliable and polymorphic primers across closely related species of *Hexastylis* will prove valuable in a variety of investigations including identification of true species in a vegetative state, detection of hybrid individuals or populations, genetic diversity, and patterns of gene flow (Selkoe and Toonen, 2006). These investigations can be used in conservation by contributing to the identification of evolutionarily significant units for *H. naniflora* and (dis)confirming threats of introgression. Microsatellites can also determine if morphological variance is being driven by genetics.

The variation in the allelic diversity of the loci reported can be used in several questions of interest. The monomorphic loci that amplified across all three species represent markers with lower mutation rates. Slower mutations allow evidence of events in the distant past to persist longer while microsatellites with higher mutation rates and therefore higher allelic diversity can be used to detect changes in the past 10-100 generations (Selkoe and Toonen, 2006). Providing data on the size and annealing temperature of these microsatellite loci allows for them to be easily integrated into future studies.

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 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 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*.

The STRUCTURE analysis provides strong evidence that populations in the southern part of the range of *H. naniflora* are not subject to significant hybridization pressures, whereas populations in the southeast portion of the range appear to by hybridizing with *H. minor* and populations in the northern portion of the range appear to form a hybrid swarm with *H. heterophylla* and *H. minor*. The four cluster grouping, although with less support than the two cluster grouping, shows more support for *H. mi*nor, and this mirrors the morphological analyses that tended to show greater separation for H. minor than *H. heterophylla* from the hybrids. This finding suggests that *H. heterophylla* is hybridizing more freely with *H. naniflora* than is *H. minor*.

5. Biogeography and site suitability analysis

The question of why a species is present is equally as important as where that species is present and one of the major goals of this research was to investigate how habitat affects the geographic range of *H. naniflora.* While the geographic boundaries of *H. naniflora* have been known, until now the habitat requirements have not been quantitatively assessed. The model created in this study accurately predicts habitat suitability at a local scale 81% of the time and the high resolution of the model (10m x 10m) increases its utility. This biogeographic assessment describes the micro-scale habitats which promote survival as well as those that limit migration and population size. These geographic variables may serve as a proxy for species delineation as it is unlikely that newly discovered populations of *H. naniflora* will inhabit areas geographically dissimilar to those already known. Populations found in areas with a percent slope of greater than 28, soil codes other than 6, 7, or 11, or elevation less than 199 m or greater than 415 m are unlikely to be *H. naniflora* (Table 2). These models can be used in the identification of new populations, assessment of sites in consideration for

relocation projects, and in the prioritization of habitat for conservation. Similar methodology could be used to develop habitat suitability models for other rare species but environmental variables must be selected based on their predictive utility.

It is important to understand the limitations of these models to prevent misapplication of these data in the process of conservation planning. This model may classify habitat as 'highly suitable' for *H. naniflora* but the probability of a population actually existing there may be very low due to issues of plant migration to, and establishment in, areas isolated from other populations. For future habitat assessments it might be beneficial to include climatic variables (temperature, soil moisture, for example) and apply weighted values to plant populations based on size and to habitat variables based on predictive utility. Also, experimental research involving transplanting and the manipulation of environments would further clarify the niche requirements of *H. naniflora*.

Changing climate has a profound influence on species range expansion and contraction (reviewed in Walther et al., 2002). Results of this study indicate that there are suitable soils, slopes, landuse types, and aspects at adjacent higher elevations where *H. naniflora* could potentially retreat to avoid the increasing temperatures predicted for the southeastern USA over the next century (Pachauri, 2008). On the other hand, slope aspect analysis shows that *H. naniflora* has already adapted to the cooler, wetter conditions of north facing slopes suggesting that this species would fare poorly under climate change scenarios predicting warmer and drier environments (mimicking south facing slopes) throughout their range, supporting a similar claim from Warren (2008). There is potential to couple dispersal simulations with climate change models (Peterson et al., 2001) suggesting that habitat suitability models generated in this study could also be analyzed with simulated global

climate change models and estimations of migration rates to predict future risks for these species. Although the results would be speculative, the high resolution of habitat variables used in our model are an appropriate spatial scale for this type of predictive modeling and may be the best available guide for policy makers at this time.

D. Discussion

The results presented here are in general support of the field observations and measurements that have been conducted by the NC DOT biologists over the past five years. The northern portions of the range of H. naniflora offer a challenge to field biologists due to the level of hybridization that appears to have occurred in this region, particularly in the Hgwy 321 corridor south of Lenoir. The morphological and molecular studies presented here are generally showing similar results, giving us confidence that the microsatellite markers are effective tools to identify members of the Hexastylis Heterophylla complex to species or hybrid with vegetative materials only. This offers DOT biologists with a means of identifying new populations outside of the flowering season. Based on the correlation of morphological and molecular marks demonstrated here, it appears that flower shape is a reasonable proxy for identification of hybrid or introgressed individuals, with the limitation that flower shape cannot be used to differentiate whether the source of the variation was from hybridization of *H. naniflora* with *H. heterophylla* or *H. minor*, only that *H. naniflora* has hybridized with one or the other, or both.

These results bring to question the US Fish and Wildlife position on recognition of hybrids involving parent or parent species that are listed as endangered or threatened. A draft report from US Fish and Wildlife Service (USFWS 1996) details many issues associated with hybrids, but this draft report was never finalized. Based upon our reading and

discussion, it appears that this will need to be addressed on a case-by-case basis. The choice would appear to be either 1) continue to use morphology to categorize *H. naniflora*, 2) reduce the current circumscription to include only the southern portion of the range, or 3) expand the circumscription to include any *Hexastylis* populations that "contain" *H. naniflora* genes. Data are currently available to use options 1 and 2. Our sampling of *H. minor* and *H. heterophylla* would need to be expanded to non-sympatric portions of the range in order to address option 3. We are actively seeking to expand our sampling to get a better understanding of the extent of the zone of introgression between *H. naniflora* and related species.

E. Recommendations

The area of western North Carolina where the DFH occurs is, according to US Census figures, one of the fastest growing regions in the nation. There is a strong need to be able to respond to this increase in the human population with appropriate highway development, yet this is often hampered in this region by the discovery of new populations of *Hexastylis* that may or may not be the Federally Threatened DFH. The development of tools to 1) understand the genetic structure of this species and 2) to be able to identify this species without flowering materials will allow the NC DOT to work throughout the year without having to stop efforts in order to determine the identity of newfound *Hexastylis* populations. In addition, the US Fish and Wildlife Service, by mandate of the US Endangered Species Act, needs to be able to understand the impacts of development and mitigation on Endangered and Threatened species and this project provides information to NC DOT that will enable NC DOT to be responsive to the US Fish and Wildlife Service in their reassessment of the status of the DFH.

In general, there is strong morphological and molecular data suggesting the three species of *H. naniflora*, *H. heterophylla* and *H. minor* have either 1) hybridized over portions of

their overlapping ranges, or 2) they are in the process of speciation and we are looking at a case of incomplete speciation. As we continue to analyze the dataset and add the remaining individuals into the molecular analysis, it is likely that we can provide a more definitive explanation of the variation seen in the complex. At present, we can confidently use the 17 microsatellite markers to determine if an individual falls within the traditional species boundaries of these three taxa, or if the individual demonstrates an intermediate genotype, and therefore likely to be of hybrid origins.

Future needs: 1) More extensive sampling of *H. heterophylla* and *H. minor* across their distributions are needed to fully understand the presence and extent of hybrid zones between these two species and *H. naniflora*, 2) Pollination studies are needed to understand the causes of the genetic and morphological patterns revealed by this effort, 3) Seed dispersal studies are also needed to provide better understanding of the current relationships in the complex.

F. Implementation and Technology Transfer Plan

Products:

1. Updated floral and vegetative morphological markers of Heterophylla subgroup.
2. New molecular markers to identify species and hybrids in the Heterophylla subgroup.
3. Much better understanding of the genetic structure of Hexastylis naniflora and congeners.
4. Improved comprehension of H. naniflora habitat requirements.
5. Voucher collections of plant material and DNA that can be used in future studies

F1. Anticipated Research Products

1. Understanding of the genetic structure of DFH, both within the species, and in relationship to possible hybrids and sympatric species.

2. Range map of DFH depicting this genetic structure. This may be of significant value if we can find structure within species that indicates location of rare alleles.
3. Molecular markers to inexpensively identify leaf material when plants are not in flower.
4. Range map of DFH with clear indications of known hybrid populations and known populations that fall within the circumscription of the DFH.
5. Morphological/anatomical key to recognize DFH, hybrids, and co-occurring *Hexastylis* species during reproductive periods.
6. Possible recognition of vegetative characters that can be used to delineate DFH and hybrids (based upon the presence or absence of characteristics, such as leaf mottling and trichome (hair) shapes and distributions on the leaf) and can be used for comparisons with genetic data.
7. Voucher collections of plant material and DNA (housed in appropriate facilities at the Appalachian State University Herbarium [BOON]) that document range and variation that can be used for future studies and to support veracity of analysis. These collections will be available to NC DOT biologists for any needed use or further analyses.

F2. How DOT will use the Research Products

1. Range map can be used in long term planning for highway corridors and identification of possible mitigation sites.
2. Molecular markers can be used to IT leaf materials throughout the growing season, overcoming the impediment of flower only identification.
3. Range map of hybrids will help with identification of newly discovered sites found during future explorations.

4. Morphological and anatomical keys will assist DOT biologists in assessment of newly found populations. This will help determine what is not a DFH.
5. Recognition of new characters will also aid the DOT biologists in field assessments of newly discovered populations.
6. Voucher collections can be used to support findings and to use for comparative materials in identification.
7. All of the generated data will be of value to US Fish and Wildlife in their re-assessment of the status of the DFH.

F3. Plan for Implementation and Technology Transfer

Field and lab notebooks will be stored at ASU, along with voucher collections. Copies of notebooks can be transferred to NC DOT. Maps and genetic data will be transferred in appropriate formats. Presentations will be made for NC DOT and will also be conducted at regional meetings, such as the Association of Southeastern Biologists. Interactive keys will be made available to the NC DOT biologists. Keys, maps and data will be made available in a Web-based format and posted on the ASU Herbarium Website.

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Appendix 1: Location and sampling information for individuals used in the initial development of microsatellites.

Species	Herbarium Accession no.	Geographic coordinates		Elevation	State /country	County	No. of individuals
		Latitude (N)	Longitude (W)				
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/A = Not Available – Geographic coordinates for federally listed species not included. All Herbarium Accession numbers refer to voucher specimens deposited in the Appalachian State University Herbarium (BOON).