DEVELOPING NEW MORPHOLOGICAL, GEOGRAPHIC, AND MOLECULAR TOOLS TO CIRCUMSCRIBE *HEXASTYLIS NANIFLORA*

A Thesis
by
JACQUELINE RENEE WAGNER

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APPROVED BY:

Dr. Zack E. Murrell
Chairperson, Thesis Committee

Dr. Matt C. Estep
Member, Thesis Committee

Dr. Howard S. Neufeld
Member, Thesis Committee

Dr. Sue L. Edwards
Member, Thesis Committee

Dr. Edelma D. Huntley
Dean, Research and Graduate Studies

Abstract

DEVELOPING NEW MORPHOLOGICAL, GEOGRAPHIC, AND MOLECULAR TOOLS TO CIRCUMSCRIBE *HEXASTYLIS NANIFLORA*

Jacqueline Renee Wagner

B.S., Appalachian State University

M.S., Appalachian State University

Chairperson: Zack Murrell

In conservation biology there is a need to determine the autecology of imperiled species in order to maintain their genetic diversity and range. *Hexastylis* Rafinesque is a genus of 11 species that is broken down into three groups: Arifolia, Speciosa, and Virginica. The Virginica group is further divided into three subgroups, one of which being the Hexastylis Heterophylla subgroup containing three closely related species: *H. heterophylla* (Ashe) Small, *H. minor* (Ashe) Blomquist, and *H. naniflora* Blomquist.

Hexastylis naniflora (Dwarf Flowered Heartleaf) is a perennial evergreen herb, native to the southeastern United States, with a range overlapping a region of rapidly expanding urban, residential, and industrial areas in 13 counties of the Carolinas. *Hexastylis naniflora* was listed by the US Fish and Wildlife Service (USFWS) as federally 'threatened' in 1989 and continues to face threats associated with habitat loss and global climate change. Due to increased monitoring, the number of populations recognized by USFWS has increased four-fold, resulting in the consideration to de-list

this species. Monitoring and identification of *H. naniflora* have often been problematic due to the overlap in flower and leaf morphology with other members of the Hexastylis Heterophylla subgroup. In order to best devise management strategies for this imperiled species, it is vital to understand the geographic scope of *H. naniflora* and be able to distinguish it from co-occurring congeners.

This study involved a significant sampling effort across the range of *H. naniflora* and relatives. Five habitat variables were used to generate a site suitability model to predict quality of habitat for *H. naniflora* on a continuous scale. Upon testing the model, it was found to accurately predict suitable habitat for 81% of test populations. These findings can be used to discover new populations of *H. naniflora* and locate the best habitat for transplanting projects. Morphological analyses of leaf shape, leaf venation, leaf tip, and calyx ridge height have generated new markers to distinguish species of the Hexastylis Heterophylla subgroup. A canonical variate analysis of 17 leaf landmarks illustrates which leaf characters are driving the differences between species. A chi-square analysis demonstrated non-independence between leaf tip type and species: retuse leaf tips were found to be more common in *H. naniflora* than *H. heterophylla* and *H. minor.* A series of one-way ANOVAs revealed significant differences in the mean, maximum, and range of calyx ridge heights across the Hexastylis Heterophylla subgroup. Lastly, 15 primer pairs (12 polymorphic, 3 monomorphic) have been developed that amplify microsatellite loci across all three species to help identify evolutionarily significant units, distinguish among species groups, and answer questions of hybridization within the Hexastylis Heterophylla subgroup. This new suite of tools is expected to aid in future decision making for the management of this species.

Acknowledgements

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I have several institutions to acknowledge for their support over the past two years. Appalachian State University Biology Department offered tools and expertise, the North Carolina Department of Transportation provided funding and field assistance, and the North Carolina Natural Heritage Program delivered the data sets used for field work and biogeographical analysis.

Dedication

This work is dedicated to my Mom and Dad for their patience with scraped knees, dirty clothes, and collections of strange critters. Not every 7-yr old girl gets sea monkeys *and* an ant farm. They always fostered a healthy curiosity for all things 'outdoors' without which I may never have pursued the environmental sciences.

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Foreword

The research detailed in this thesis will be submitted to the *American Journal of Botany*, a peer reviewed journal. The thesis has been prepared according to the style guide for the journal.

INTRODUCTION

Background

Defining a species is of immense biological and ecological concern, influencing the political, financial, and theoretical pressures that drive conservation efforts. There are many approaches to defining species (over 24 listed by Mayden, 1997) that are at least partially incompatible in the sense that they lead to different conclusions concerning the boundaries and number of species. Most species concepts agree that speciation is the result of evolutionary forces on individuals of populations until all members of the population acquire attributes which grant species status (Templeton, 1989). Defining which attributes award species status and demarcating the continuous stages of speciation (species, subspecies, hybrids) is where the debate begins (Bachmann, 1998; Rieseberg et al., 1989; Templeton, 2006; Will and Rubinoff, 2004) and is further complicated by the fact that no single process can be used to define all species (e.g., sexual vs. asexual, extinct vs. extant).

This critical need for defining a species is particularly relevant to maintaining the health of imperiled species and preparing for new conservation challenges in the face of global climate change. This study explores the species boundaries of three congeners within the Hexastylis Heterophylla subgroup including the federally threatened *H. naniflora.* Morphological, geographic, and molecular tools have been employed to highlight the differences among this subgroup to satisfy separate species

status under several different species concepts. These new datasets can help in the development of new management plans for the protection of *H. naniflora.*

Hexastylis naniflora is afforded protection under the Endangered Species Act (ESA) and was listed as a federally "threatened" species by the US Fish and Wildlife Service (USFWS) in 1989 when only 24 populations were known. By 2010, the USFWS recognized 108 populations and the number of populations containing more than 1,000 rosettes increased from 3 to 27 (USFWS, 2010). For the purpose of this study, the term population will refer to a population of *Hexastylis* at least one-half mile from any existing population, as defined by the USFWS. The increase in the number of known populations is thought to be primarily due to surveys completed in agreement with the ESA (USFWS, 2010) and has resulted in the consideration of *H. naniflora* for de-listing (North Carolina Department of Natural Resources (NCDNR), 2011). Due to morphological similarities between congeners, there is a need to verify all *H. naniflora* populations. Padgett (2004) surveyed 64 presumed *H. naniflora* sites and found that four sites were misidentified as *H. naniflora* (two *H. minor* and two *H. heterophylla)*. It is probable that other newly discovered populations of *H. naniflora* have been misidentified and are actually *H. heterophylla* or *H. minor.*

In addition to problems with plant identification, the de-listing process is complicated by the lack of a recovery plan for *H. naniflora.* Recovery plans as described in Section 4 of the ESA describe protocols for enhancing and protecting endangered species populations. A recovery plan requires the outline of site specific management actions, objective baseline data to measure recovery, and an estimation of funding and

resources required to achieve de-listing. The USFWS is responsible for administering the recovery plan. Though no time frame is defined in the ESA, on average it takes 6 years from the listing date for a recovery plan to be outlined (Greenwald et al., 2005), but *H. naniflora*, listed over two decades ago, still does not have a recovery plan.

Taxonomy

Hexastylis is a genus in the family Aristolochiaceae, commonly called the Birthwort family. It is a member of the order Piperales and has a global tropical and temperate distribution. This family of flowering herbs and woody vines is made up of about 600 species across 6-12 genera (Weakley, 2012). The leaves are cordate, simple, and alternate. The flowers are radially or bilaterally symmetrical and grow in the leaf axils. Aristolochiaceae is generally split into two subfamilies: Asaroideae and Aristolochioideae. Asaroideae represents the herbaceous portion of the family and generally has a northern temperate range (Kelly, 1998) while Aristolochioideae represents the vine/liana portion of the family and tends to have a more tropical-subtropical distribution (Ma, 1990). Asaroideae is the smaller subfamily containing *Hexastylis* and two other genera for a total of 85 species (Kelly, 1998).

Hexastylis is generally recognized as a segregate of the genus *Asarum* but its status as a separate genus has been disputed (Blomquist, 1957; Kelly, 1997, 1998; Niedenberger, 2010). Using morphological and molecular data, Kelly (1997, 1998) claimed that *Hexastylis* is not monophyletic and therefore should be grouped within *Asarum.* Conflictingly, *Hexastylis,* has been segregated on its entirely North American

distribution, karyotype (Soltis, 1984; Sugawara, 1982), pollen morphology (Niedenberger, 2010), differences of 3 chloroplast regions (Niedenberger, 2010), glabrous leaves (Rafinesque, 1825), and several characteristics of flower morphology (Gaddy, 1987; Rafinesque, 1825; Sugawara, 1982). Based on these findings, for the purposes of this study, I am recognizing *Hexastylis* as a monophyletic group; although there is a need to name the remaining clades within the broader *Asarum* clade (Kelly, 1998; Niedenberger, 2010).

The genus *Hexastylis*, commonly known as "little brown jugs," is endemic to the southeastern United States (NCNHP, 2012). The genus is made up of perennial, evergreen, herbaceous plants with leaves and flowers arising directly from the rhizomes. The leaves are heart-shaped, leathery, untoothed, and may be with or without variegation. The axillary flowers are fleshy, firm, and grow at the end of short pedicels, lacking petals but, instead, comprised of a three lobed calyx tube containing 12 stamens. As currently described, *Hexastylis* contains 11 species and five varieties: *H. arifolia* (var. *arifolia, callifolia, ruthii), H. contracta, H. heterophylla, H. lewisii, H. minor, H. naniflora, H. rhombiformis, H. shuttleworthii (*var. *shuttleworthii, harperi), H. speciosa, H. virginica* and, most recently added, *H. sorriei* (Weakley, 2012).

Hexastylis is divided into three groups: 1) Arifolia and 2) Speciosa, each consisting of a single species, and 3) Virginica made up of the remaining species (with the exclusion of *H. sorreii* which has not yet been placed in a group). The Virginica group is broken into three subgroups: Heterophylla, Virginica, and Shuttleworthii (Blomquist, 1957). The Hexastylis Heterophylla subgroup contains *H. contracta* as well

as the three species of *Hexastylis* that are the most difficult to distinguish taxonomically; *H. minor, H. naniflora,* and *H. heterophylla* (Gaddy, 1987).

The taxonomy of *Hexastylis* relies heavily on morphology to discriminate among species. Currently, the best way to differentiate *Hexastylis* species is by using flower and pollen characters (Gaddy, 1987; Niedenberger, 2010). Classifying traits for these plants in a vegetative state are insufficient due to the interspecific overlap and intraspecific plasticity of leaf morphology within *Hexastylis* (Gaddy, 1987). This limits the time of field identification to about six weeks, starting in late March, when plants are in bloom.

Intraspecific variations in floristic qualities are also problematic. *Hexastylis naniflora* flowers have a wide range of size (6-13 mm long) and color (brown to green to purple) (Gaddy, 1987), making them both poor markers. *Hexastylis heterophylla,* the species most difficult to distinguish from *H. naniflora,* is considered to be the most morphologically variable species in the entire genus and is reported to show a morphological gradient from north to south across its range (Gaddy, 1987). The leaves of *H. naniflora* are the same shape (cordate to orbicular cordate) and size (4-6 cm long) as the other members of the Hexastylis Heterophylla subgroup (Table 1). *Hexastylis minor, H. heterophylla,* and *H. naniflora* all have calyces with inner ridged reticulations that show distinct vertical ridges, but *H. heterophylla* occasionally has a more irregular network of ridges (Gaddy, 1987). The best morphological characters that can be used to differentiate *H. naniflora* from the other members of its subgroup are ovary position and calyx tube diameter (Table 1.)

Table 1. Comparison of three species in the Heterophylla subgroup that have been difficult to differentiate due to overlap in morphological characters (Summary of Gaddy 1987).

Species	Calyx tube length (mm)	Calyx tube diameter (mm)	Lobe length (mm)	Lobe width at base (mm)	Leaf length (cm)	Ovary Position
H. heterophylla	8-15	7-14	5-15	6-17	4-8	1/3 inferior
H. minor	9-16	8-16	6-10	8-12	4-8	Superior
H. naniflora	6-13	4-7	4-7	4-7	4-6	1/2 inferior

The majority of species within *Hexastylis* have pollen that is similar in size (30-45 μm), shape (spheroidal), and surface pattern (microreticulate with gemmae) (Niedenberger, 2010), but the pollen grains of *H. naniflora* lack gemmae, making them distinguishable from other species (Niedenberger, 2010; Padgett, 2004). A magnification of about 13,000X is required to clearly see surface features of *Hexastylis* pollen (Niedenberger, 2010), necessitating scanning electron microscope (SEM) technology and thus time intensive sample preparation. This unique pollen characteristic results in evidence supporting separate species status for *H. naniflora,* but does not provide a quick, inexpensive, or practical tool for species identification.

While most populations follow the morphological characters in Table 1, some populations display morphologies that do not allow for confident placement into any one species category (Fig. 1). In Virginia, plants appearing to be intermediate between *H. minor* and *H. heterophylla* have been reported (Gaddy, 1987) and in North Carolina field botanists have reported *H. naniflora* displaying intermediate floral characteristics with both *H. heterophylla* and *H. minor* (USFWS, 2010). This provides anecdotal evidence of hybridization within the genus but determination of putative hybrids on

morphological grounds alone is difficult because of the broad intraspecific variation within *Hexastylis* species and because these intermediacies may also arise from forces other than hybridization, such as convergent morphological evolution (Dobzhansky, 1937) and/or environmental factors.

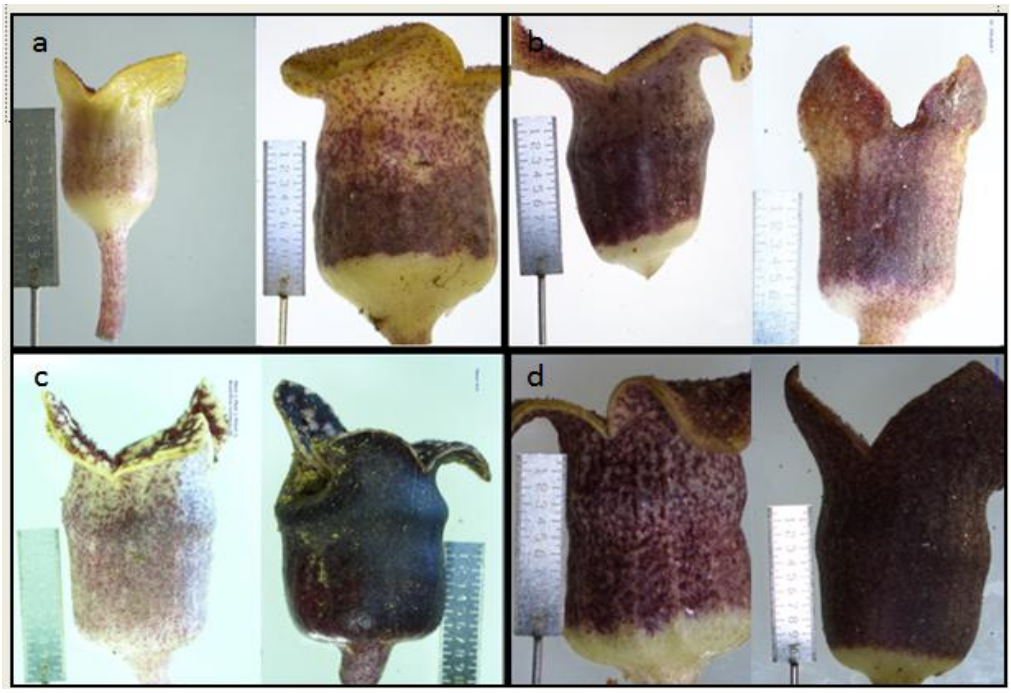

Fig. 1. Images of calyx morphology for (moving clockwise from top left): a) *H. naniflora,* b) H. spp.*,* c) *H. minor,* and d) *H. heterophylla.* Two images for each species group are shown to illustrate plasticity within the groups. The scale bar in each photo is 1 cm.

The Hexastylis Heterophylla subgroup displays characteristics associated with hybridizing genera including attributes that put the species at risk for introgression: perennial habitat, outcrossing breeding systems, taxa of the same ploidy levels, overlapping flowering times, small populations, unspecialized pollinators, and congeners that geographically co-occur (Levin et al., 1996). Other species of *Hexastylis*

(*H. arifolia and H. virginica*) have been shown to hybridize and produce viable offspring in lab conditions (Wyatt, 1954).

Ecology

The pollination of *Hexastylis* is not well studied but the genus is thought to be pollinated by insects including flies, wasps, and thrips (Otte, 1977). With stigmas located below the anthers, *Hexastylis* was reported by Gaddy (1987) to outcross about 95% of the time, but individuals are self-compatible and can self-fertilize with intrafloral movement of pollen by insects (Otte, 1977). Kelly (1997) summarized a suit of morphological traits presumably associated with outcrossing that are present in *Hexastylis* and include the physical separation of the anthers and stigmas, calyx surface ornamentation, glandular trichomes on the calyx, and synsepalous calyces.

Heterotropa tamaensis is closely related to *Hexastylis* and has similar flower structure with trichomes, internal ridges, and stigmas located above the anthers. Sugawara (1988) found that self-fertile flowers are primarily pollinated by insect vectors such as fungus gnats transferring pollen within an individual flower. Further research is needed to determine the relative rates of outcrossing within *Hexastylis,* but it is assumed that self-pollination is rare within this genus and that an insect vector is required for fertilization of these species.

Hexastylis relies on ant-mediated seed dispersal, a mutualism particularly common among understory herbs of temperate deciduous forests in the Northern Hemisphere (Beattie and Culver, 1981). The family Aristolochiaceae comprises a lineage of plants that are more than 50% myrmecochorous (Lengyel et al., 2010). Three

species within the Hexastylis Heterophylla subgroup, *H. heterophylla, H. naniflora,* and *H. minor*, have been reported to employ myrmecochory as a method for seed dispersal (Gaddy, 1986). Gaddy's 1986 study shows that out of 15 *H. heterophylla* and 20 *H. naniflora* encounters in a lab setting with the seed dispersing ant, *Aphaenogaster rudis,* the diaspores were removed 100% of the time. The diaspore consists of the seed and a fleshy, lipid and protein rich elaiosome which acts as the nutritional reward for the dispersing ants. The seasonal peak activity of *Aphaenogaster* (March-June) coincides with peak bloom time of members of the Hexastylis Heterophylla subgroup (Giladi, 2004).

While species with ant dispersed seeds have been shown to have slower migration rates than species with seeds that are adhesive or ingested (Brunet and Von Oheimb, 1998), the multiple benefits myrmecochory provides for plants is evidence for the adaptive advantages of this strategy. Ants can disperse seeds to sites that might be nutrient enhanced or where plant fitness will be higher. For example, most species of *Formica* have nutrient-rich, long-term nesting sites that may increase seed survivorship and seedling growth (Gorb and Gorb, 2003). Burial of seeds by some species of ants reduces the ability of predators, such as rodents, to feed on the seeds or the plant itself (Heithaus, 1981). Predation can also be reduced when seeds are re-dispersed from the nest due to decreasing seed densities (Canner et al., 2012). Burying seeds has been shown to protect them from fire and drought (Boyd, 2001). Elaiosomes may be used over other dispersal mechanisms such as fruit because they are much lower in potassium and less expensive for the plant to produce (Hughes et al., 1994). Finally, scattered seeds may escape parent or sibling competition for space and resources as

increases in local seed densities have been shown to decrease growth and survival rates in *H. arifolia* (Gonzalez, 1972).

Threats

Hexastylis naniflora occurs in a rapidly growing region of the country bordered by Hickory, NC to the north, the expanding suburbs of Charlotte, NC to the east, and Greenville, SC and Spartanburg, SC to the south (Fig. 2). Habitat loss due to land use change is the biggest threat to populations (NCDNR 2011)(Fig. 2). Forty percent of all known *H. naniflora* populations have already been affected or are considered to be in imminent danger from threatening disturbance events (NCDNR, 2011). A large number of *H. naniflora* populations occur near rapidly growing urban areas, facing threats of residential and commercial development, road improvement, damming, sedimentation, and erosion (Fig. 2) (NCDNR, 2011; USFWS, 1988). In more rural areas, this species faces threats from agriculture, deforestation, and invasive species. All the populations face risks associated with climate change, hybridization, and subsequent loss of mutualisms (Fig. 2)(Giladi, 2004; NCDNR, 2011; USFWS, 2010).

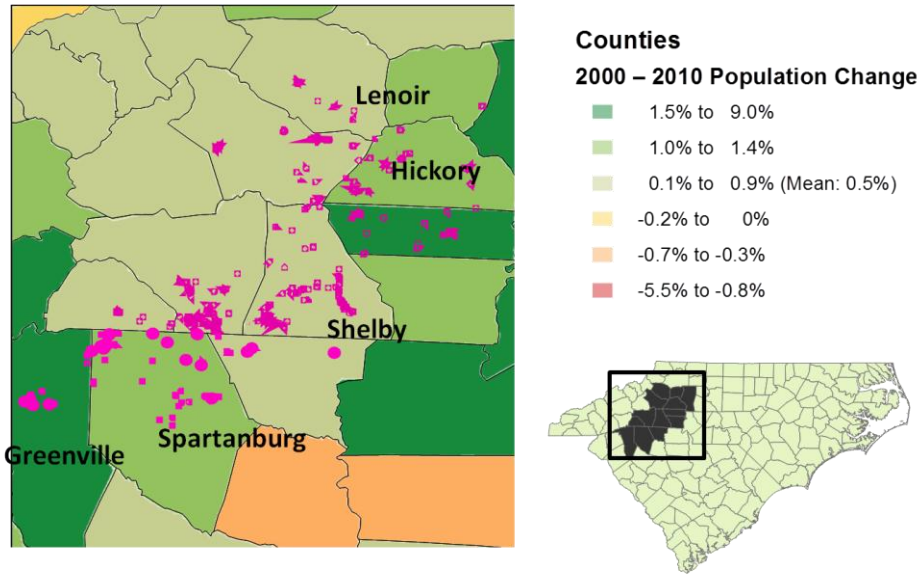

Fig. 2. Range map of *H. naniflora* showing human population growth from 2000-2010. Element occurrence records are in pink.

While there is little information on this species' response to fire, evidence indicates that moderate burns do not adversly impact *H. naniflora;* however fire suppression could be a hazard to *H. naniflora* by allowing pyrophobic, non-native, invasive plants to thrive, as well as the build up of a thick litter layer that may shade low growing species. Cowpens National Battlefield (Cherokee County, SC) conducted burns within portions of *H. naniflora* habitat and preliminary data suggests no adverse effects upon growth or flowering (Walker, 2009). Anecdotal evidence reported by USFWS (2010) suggests that *H. naniflora* populations were not negatively impacted by a dormant season wildfire in Caldwell County. Congeners (in particular *Hexastylis sorriei*) have been shown to prosper in habitats exposed to frequent burns and may even be fire dependant (Gaddy, 2011).

Invasive species have been shown to compete with native plants for space, sunlight, nutrients, water, and pollinators as well as affect fire patterns by altering forest floor fuel loads (Brooks et al., 2004). Non-native invasive species including, among others, *Hedera helix, Ligustrum* spp., *Lonicera japonica,* and *Microstegium vimineum,* are spreading across the range of *H.naniflora*, especially throughout riparian corridors (USFWS, 2010). Active management, including technical expertise, funding, and personnel are required to sucessfully abate the threat of these invasive species. These resources are currently unavailable for the majority of populations that are protected from habitat conversion (USFWS, 2010).

Non-native, often invasive, ornamental plants commonly stem from landscaping urban areas. Across the United States urbanization threatens more species and is more geographically pervasive than any other human activity (Czech et al., 2000). The USFWS (1988) has reported that populations of *H. naniflora* surrounding rapidly expanding urban areas are being threatened by habitat loss due to residential and commercial development. Conversions of woodlands to pasture and creeks to small ponds for agriculture are practices that have been reported to be threatening *H. naniflora* populations in Greenville and Spartanburg Counties in South Carolina (USFWS, 1988). The creation of additional edge habitat may increase the effects from invasive species up to 120 m from mechanized clearing (Forman and Deblinger, 2000).

Road and bridge improvement projects are one of the most recurrent sources of habitat disturbance for *H. naniflora* (USFWS, 2010). Recent NCDOT projects have impacted or are expected to impact 10 of the 27 largest populations of *H. naniflora*,

affecting an estimated 22,135 rosettes (NCDNR, 2011). Road improvements have both direct (excavation, fill, construction footprint, mechanized clearing) and indirect (biological pollution, altered hydrological regime) effects that occur on different time scales from immediate to those spread over several years. The proposed Shelby bypass exemplifies the negative impacts of highway development on *H. naniflora* survival. The direct effects are expected to result in the loss of 3,060 plants while indirect effects will take a toll on another 2,267 plants (Bassett, 2012).

The Blalock Reservoir population in Spartanburg County SC, once the largest known population of *H. naniflora,* has twice been impacted by illegal timber harvest by neighboring landowners resulting in the loss of over 600 plants (Newberry, 2009). Timber harvests have also negatively affected populations in Lincoln and Cleveland County, NC (USFWS, 1988). One third of the Blalock Reservoir population was directly threatened when the elevation of the county water supply storage was raised. The initial reservior construction destroyed over 2500 plants in SC (USFWS, 1988).

The lack of timely responsiveness of plants to current and ongoing climate change suggests that these trends could lead to species extinctions (Davis and Shaw, 2001). Under moderate emisson scenarios, temperatures will increase by 3^{o}C and drought conditions will increase across the range of *H. naniflora* by the end of this century (Pachauri, 2008). Future changes in precipitation are more difficult to predict than temperature (Pachauri, 2008). Annual averge precipitation has decreased by 5-10% across the range of *H. naniflora* from 1958-2008 (Pachauri, 2008). Precipiation is projected to decrease in the spring and summer and increase in the fall and winter in

the peidmont uplands of North and South Carolina (Pachauri, 2008). Also, the number of days a year with heavy rains (the top 1% of rainfall events for 1958-2007) and the average number of dry days between rainfall events are expected to increase across the southeastern United States leading to increases in both flood and drought conditions (Pachauri, 2008).

Habitat fragmentation and slow migration rates of *H. nanilfora* will impede the ability of the species to reach climatically suitable habitats in response to hotter and increased drought conditions. A model based on mid-range climate change scenarios has projected that 24% of species in temperate deciduous forest systems (habitat typical for *H. naniflora*) will be extinct by 2050 (Thomas et al., 2004). The most severe threats from climate change will likely occur from an interaction between hazards. Increased solar irradiation can lead to increasing drought conditions which, in recent years in the southeastern US, are correlated with declines in *H. naniflora* populations and declines in transplant survivorship (NCDNR, 2011; Padgett, 2004).

A climate change simulation model shows that an increase of atmospheric CO_2 may increase the leaf area index and mean leaf life span in deciduous canopy trees (Holmgren et al., 2006). These predictions mean a longer-lasting, fuller canopy which may prove problematic for understory evergreens dependent upon brief periods of high solar irradiance in spring and fall (Warren, 2008). It is during these times that, *Tiarella cordifolia* which has a similar leaf lifespan as *Hexastylis*, achieves 75% of its annual growth (Rothstein and Zak, 2001). When the canopy is leafless, in the spring and fall, many evergreen understory herbs have increased photosynthetic rates (Skillman et al.,

1996) and *H. arifolia* has been reported to upregulate photosynthetic capacity directly after canopy leaf drop (Skillman et al., 1996). According to two separate climate change models, leaf lifespan of evergreen species would be reduced at higher temperatures (Holmgren et al., 2006; Wright et al., 2004). The increased expenditure of carbon in order to maintain and producing leaves (Sabate et al., 2002) and the potential decrease in withdrawal of nutrients from the leaf back into the plant may ultimately lead to net carbon losses.

Environmental drivers influence flowering and fruiting time of myrmecochorous plants as well as foraging behavior of seed-dispersing ants (Warren et al., 2011). Warming temperatures often act as the primary cue for plant-fruiting and ant foraging but photoperiod and moisture levels also generate impacts on these life history traits (Warren et al., 2011). Factors influencing phenology are species specific and those engaged in seasonal mutualisms might respond differently to environmental drivers, thus losing the benefits of the mutualism. Given that ants can shift their range faster than woodland herbs, and do not change seasonal behavior due to moisture levels (Warren et al., 2011), it is likely that climate change will lead to declines in myrmecochorous mutualisms.

Another potential threat to *H. naniflora* is hybridization. The melding of gene pools introduces the hazard of genotypically indistinct populations, even leading to extinction of rare species in extreme cases (Rhymer and Simberloff, 1996). The evolutionary outcomes of most ancient cases of hybridization appear to be introgression, not hybrid speciation (Rieseberg et al., 1996), which is why hybridization

poses a greater threat to rare species (*H. naniflora)* that cross with more abundant species (*H. heterophylla, H. minor)* (Allendorf et al., 2001). Even if hybrid populations are weak or sterile, and introgression isn't a direct threat to a rare species, the wasted reproductive effort can pose additional threats to the population (Robinson et al., 1991). Hybrid seeds are produced at the expense of conspecific seed, reducing the potential for population growth and generating a reproductive impediment.

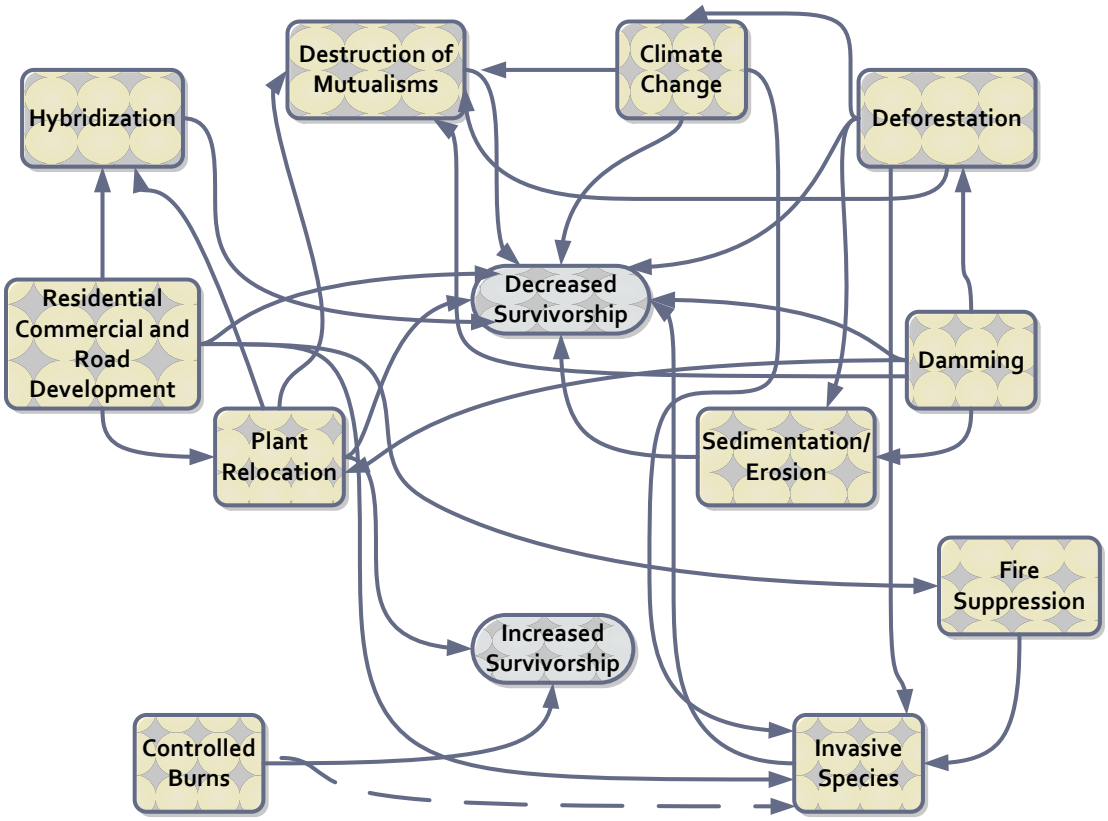

Fig. 2: A flow chart illustrating the impacts of different disturbance events on members of *Hexastylis* reported by USFWS (2010). Solid lines represent positive relationships and the dotted line represents a negative relationship.

All of the threats discussed above must be addressed to encourage survival of *H. naniflora*. Section 7 of the ESA requires that actions of state and federal agencies do not

jeopardize the existence of an endangered species without a granted exemption. Exemptions to Section 7 may require mitigation and habitat enhancement measures including: on-site protections, relocation of plants, or the purchase of off-site populations to attenuate the adverse effects of construction (USFWS, 2010). As a result of Section 7 the North Carolina Department of Transportation (NCDOT) currently protects more populations of *H. naniflora* than any other conservation partner (USFWS, 2010). A 2012 report from the NCDOT estimates 86,870-87,575 plants are currently protected or considered for protection through various easements, state heritage preserves, restrictive covenants, state/national park ownership, or other conservation strategies (Bassett, 2012).

Biogeography

The 108 populations of *H. naniflora* currently recognized by the USFWS collectively total about 250,000 rosettes (NCDNR, 2011). Although its range is very restricted, it is not as rare as was once thought (Blomquist, 1957). In North Carolina, *H. naniflora* is found in: Alexander, Burke, Caldwell, Catawba, Cleveland, Gaston, Iredell, Lincoln, Polk, and Rutherford Counties. In South Carolina, its range includes Cherokee, Greenville, and Spartanburg counties. The distribution of *H. naniflora* overlaps with five congener species, including the species within the Hexastylis Heterophylla subgroup. It is thought that within the subgroups of *Hexastylis*, species barriers are primarily maintained by geographic isolation (Gaddy, 1987).

Habitat variables may serves as geographic barriers to the spread and establishment of *H. naniflora*. Aspect impacts habitat temperature and moisture and is the best indicator of evergreen understory communities (Warren, 2008). North facing slopes throughout the northern hemisphere receive less solar irradiation and retain moisture better, making them cooler and wetter (Auslander et al., 2003). Slope and elevation best discriminate between species within an aspect (Warren, 2008) by influencing erosion, water drainage, and temperature and for *H. naniflora* in particular, soil type has been shown to be the strongest predictor of suitable habitat (Padgett, 2004).

The sandy-loam soils typical of *H. naniflora* habitat (Gaddy, 1981, 1987) are very deep, moderately permeable, acidic, and restricted to an area along the piedmont upland in North and South Carolina. *Hexastylis naniflora* is also often restricted to deciduous forests, frequently associated with *Kalmia latifolia* (Padgett, 2004) along rivers, lakes, and streams, often within the floodplain where this plant has been shown to grow larger and have more frequent flowering (Newberry, 1993).

Goals of this thesis

This study focused on strategies to 1) minimize adverse effects on *H. naniflora* due to environmental changes, and 2) aid state and federal agencies by providing tools that can help manage *H. naniflora* in compliance with the ESA. To achieve these goals it is vital to understand the geographic scope of *H. naniflora,* be able to

distinguish it from co-occurring congeners and hybrid populations, and to collect and organize data, plant material, and genetic material to be used in future studies.

This project takes a multidisciplinary approach to achieve these goals. To delineate where *H.naniflora* is most likely to occur, where new populations are likely to be found, and where threatened populations can be relocated, a site suitability model was created. To distinguish among *Hexastylis* congeners, morphological analyses were conducted on both leaf and flower morphology, and a set of microsatellite loci were developed into genetic markers that will be used in future studies to generate an understanding of the genetic structure of the species. Lastly, dried and pressed voucher specimens, liquid preserved flowers, and extracted leaf tissue DNA will be collected and kept at the Appalachian State University (ASU) herbarium in Boone, North Carolina to be used in future studies.

MATERIALS AND METHODS

Site Selection/ Collection of Plant Material

Flower and leaf material was collected from each of 136 field sites across Alabama, North Carolina, South Carolina, Tennessee, and Virginia. 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. 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.

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.

Biogeography

An Element Occurrence Record (EOR) is a data management tool defined as an area of land or water where a species or community with conservation value is or was present. There are 273 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). My 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. I 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.

Flower Morphology

After collection and transportation back to ASU, the flowers 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.

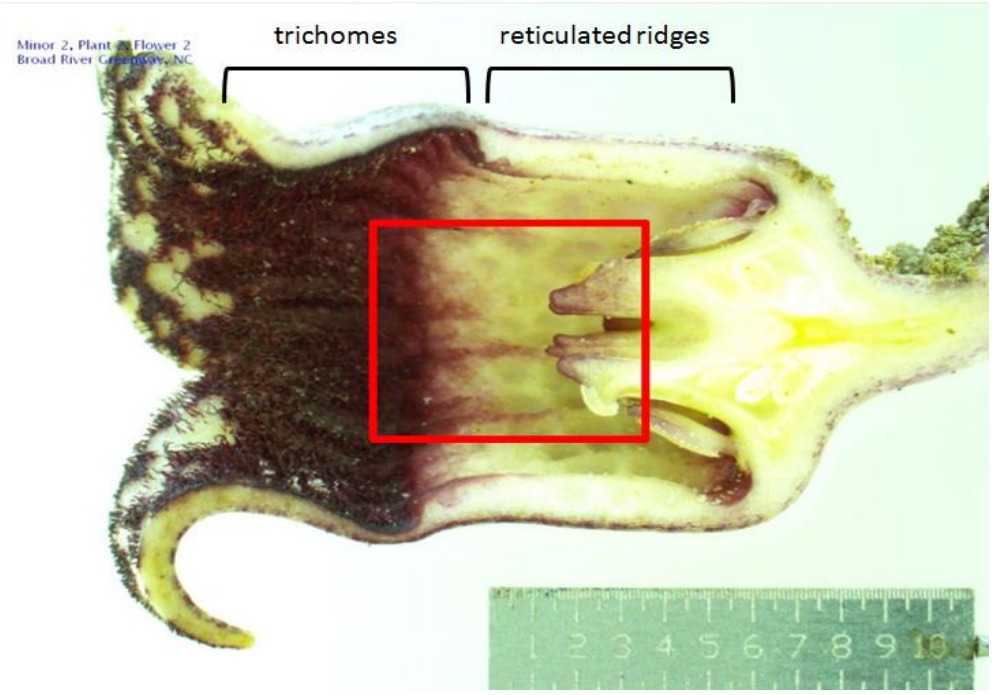

Fig. 3. An image of a *Hexastylis* flower. The red box indicates by the area cut out of the calyx tube for 3-D microscopy. The scale bar is 1 cm.

Flower morphology was then compared to geographic variables to determine if ridge height changed across a landscape gradient. Mean calyx height for 16 individuals of *H. naniflora* across 16 different populations was compared to the latitude, longitude, and elevation for that site using Pearson's correlations.

Leaf Morphology

Leaf morphological analyses were performed using geometric morphometric analysis of leaf shape and leaf venation patterns defined by landmarks. I 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.

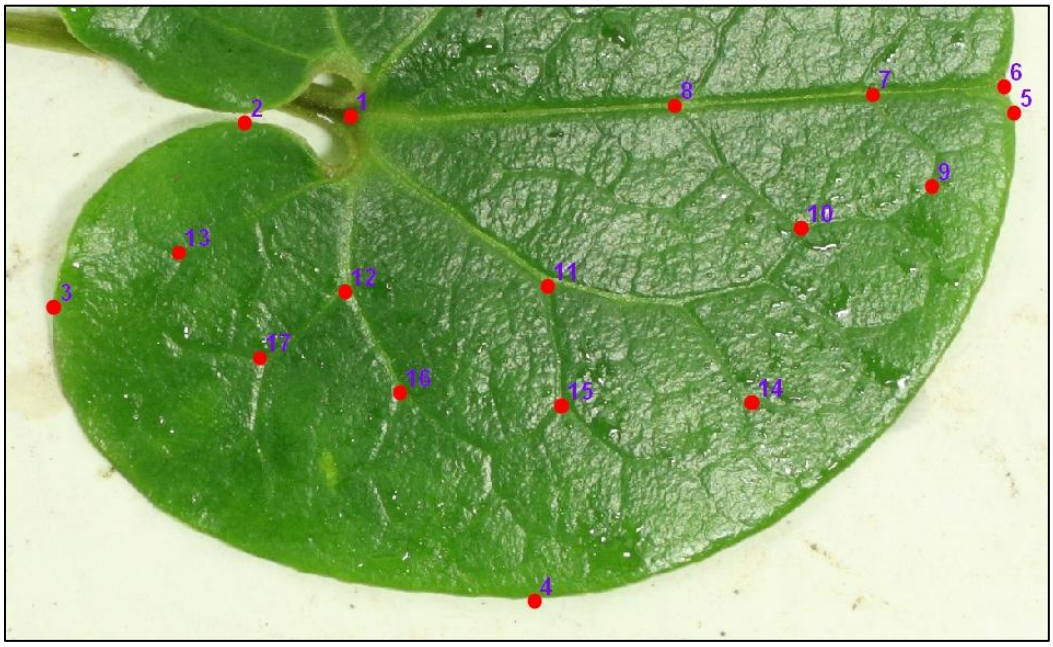

Fig. 4. Photograph of fresh *Hexastylis* leaf with the 17 landmarks used for geometric morphometric analysis. The landmarks were set in TPSDig and were replicated across all species.

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, I 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.

Molecular

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.

One tissue sample each for *H. naniflora, H. heterophylla,* and *H. arifolia* was sent off to the Cornell University Evolutionary Genetics Core Facility to generate a comprehensive list of primer pairs for microsatellite loci. Total DNA was extracted from each tissue sample using a QIAGEN Plant Mini Kit. 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-adapters with T4 DNA ligase. The DNA fragments were 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 captured by streptavidin-coated magnetic beads (New England Biolabs, Ipswich, Massachusetts, USA) and PCR amplified. Agarose gel and a Qubit 2.0 fluorometer (Invitrogen, Grand Island, New York, USA) were used to analyze the PCR product and 100bp-600bp fragments were recovered with magnetic Ampure beads (Beckman Coulter, Miami, Florida, USA). Samples were then sent to the Cornell Life Sciences Sequencing and Genotyping Facility for sequencing on an Illumina MiSeq. Spreadsheets containing over 5,000 primer pairs for microsatellite loci for each of the three species were reported to ASU within five weeks of leaf tissue submission.

From over 5,000 primer pairs 102 *H. naniflora* primer pairs were selected to screen for amplification in eight individuals: six *H. naniflora* (covering the geographic range of this species), one *H. heterophylla,* and one *H. minor.* Priory was given to loci that: 1) were represented across more than one species while displaying polymorphism, 2) had a greater number of repeats, thus a greater potential for proofreading errors resulting in mutations, and 3) that provided a variety of microsatellite motifs. Polymerase chain reaction amplifications were conducted in a 10µL reaction consisting of 5X Green GoTaq Flexi Buffer without $MgCl_2$, 2.5 mM $MgCl_2$, 800 µM dNTP Mix, 0.5 µM of the unlabeled primer, 0.25 µM of the M13 labeled primer, 0.25 µM of FAM (Sigma-Aldrich, St. Louis, MO) fluorescent dye, 0.5 units of GoTaq Flexi DNA Polymerase, and 20 ng of DNA. Polymerase chain reaction amplification was done 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, Touch down for 2 minutes, and 72 for 1 minute. Then, there were 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 checked for amplification on a 1% agarose gel.

The primer pairs yielding PCR products that cleanly amplified across all species were sent to the Georgia Genomics Facility (GGF) for genotyping using an ABI 3730 sequencer (Applied Biosystems, Foster City, California, USA). PCR product was loaded into an ABI compatible PCR plate with 1 µL of the size standard (600 LIZ, Invitrogen, Carlsbad, California, USA) and 10 µL of highly deionized formamide (HiDi, Applied

Biosystems) to ensure sample preservation and resistance to evaporation. Primer pairs were analyzed 12 at a time against 8 DNA samples in three 96 well plates for a total of 36 tested loci. Data from GGF were returned to ASU in 2-10 days as fragment (.fsa) files which could then be scored and screened for polymorphism in PeakScanner Version 1.0 (Applied Biosystems). The alleles for each DNA sample across all primer pairs were recorded and observed and expected heterzygosity was tested in GenAlEx 6.5 (Peakall and Smouse, 2006, 2012) to determine if each locus was in Hardy-Weinberg equilibrium (HWE).

RESULTS

Biogeography

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. 5 & 6). 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. 7). 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 2).

Table 2. 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								

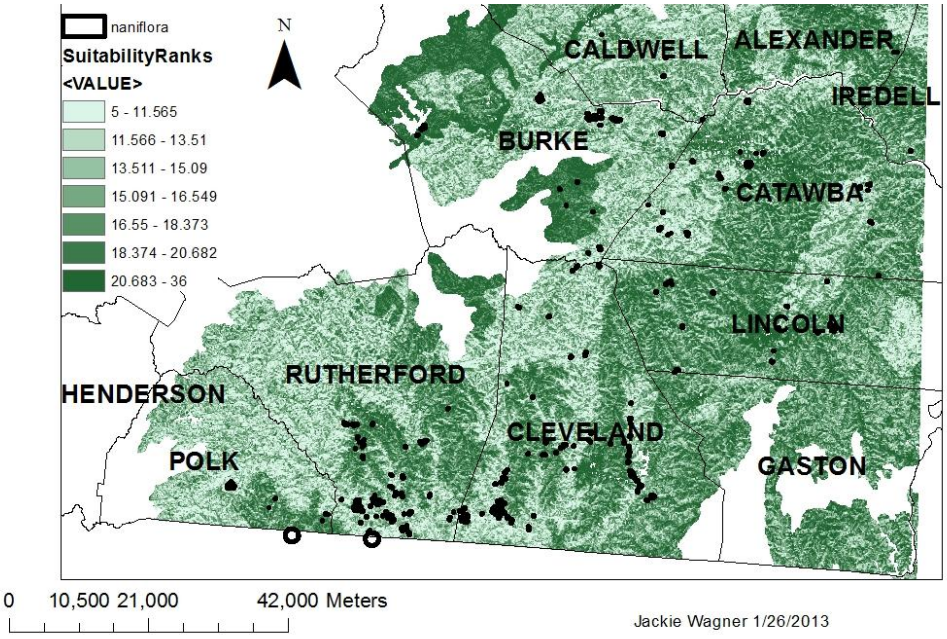

Fig. 5: The site suitability ranks based on five habitat variables across the counties where *H. naniflora* is known to exist in NC. The smaller numbers indicate a higher rank and lighter green areas denote more suitable habitat. Known populations of *H. naniflora* are outlined in black.

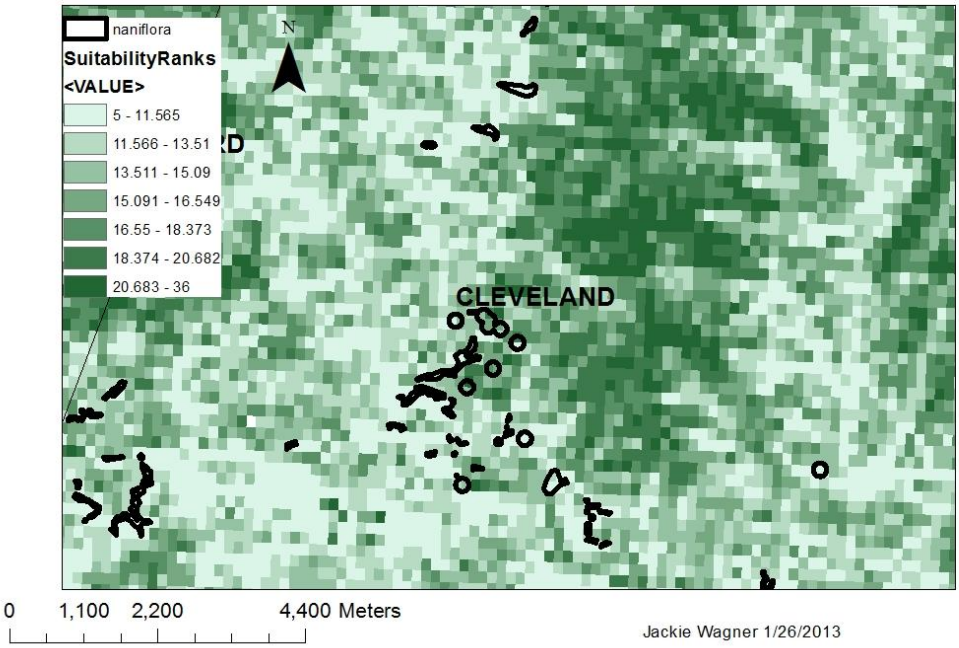

Fig. 6: A close-up of Cleveland County, NC displaying site suitability ranks. The smaller numbers indicate a higher rank and lighter green areas denote more suitable habitat. Known populations of *H. naniflora* are outlined in black.

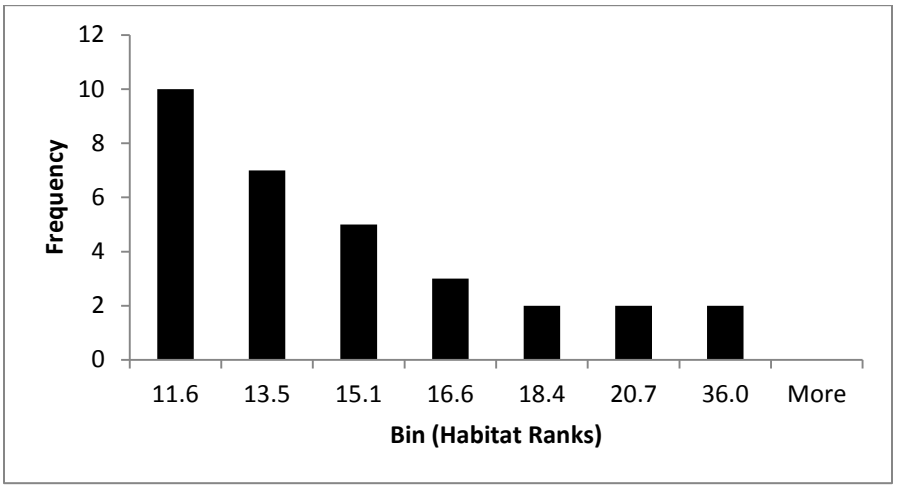

Fig. 7: A histogram displaying the frequency of 31 test populations in each habitat rank bin. The bins decrease in habitat suitability moving from left to right across the graph. The values along the x-axis indicate the bottom of the bin range. Bins were set using quantile class breaks so that total area (km^2) was equal across all bins.

Flower 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. 8) 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.*

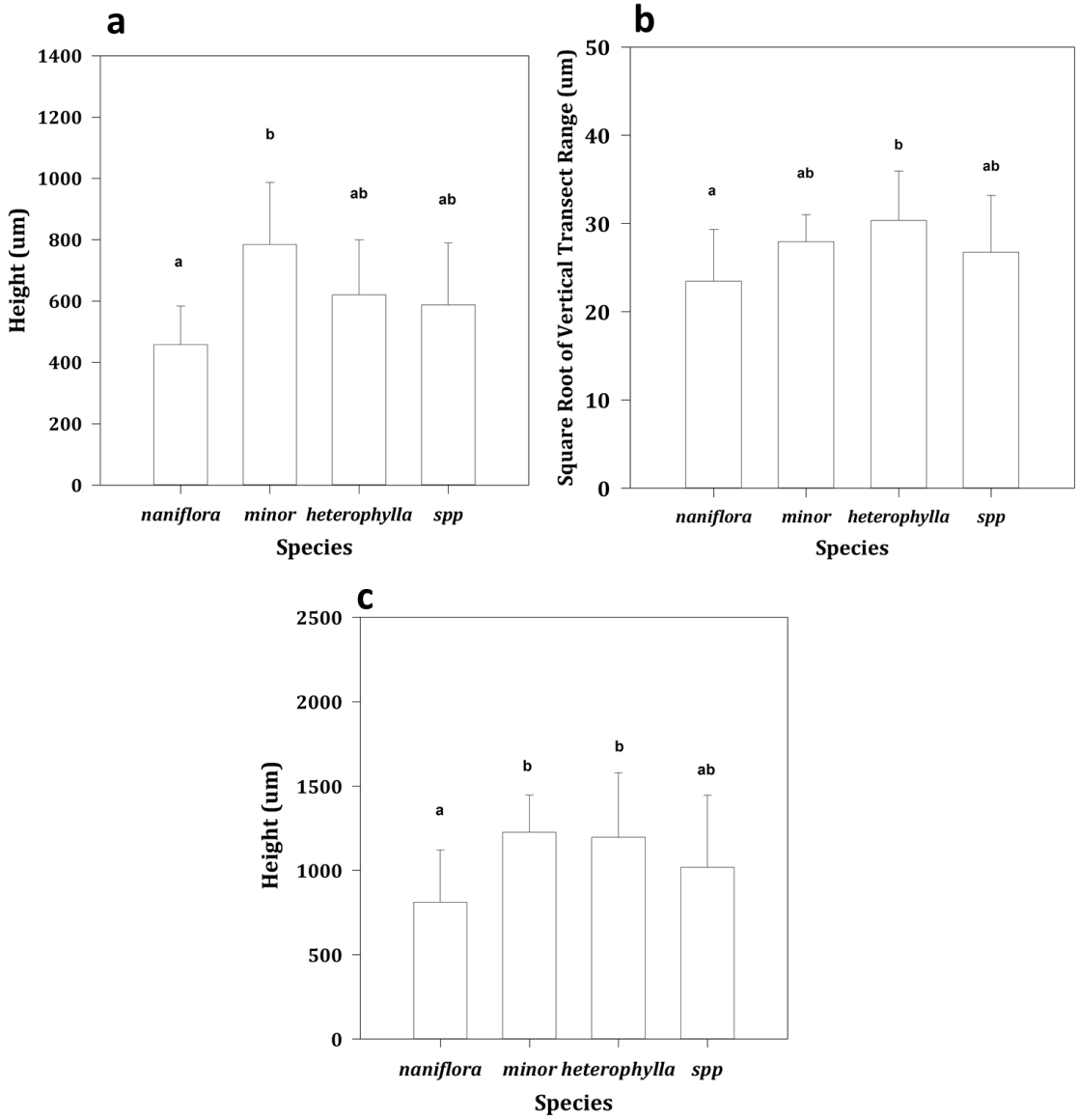

Fig. 8: a) mean height of flower calyx by species, b) range in heights across vertical calyx transects by species, and c) maximum calyx ridge heights per transect by species. Bars represent the means $\pm$ standard error. Differences of statistical significance (p<0.05) are indicated by the letters above each bar. For all three tests n=15.

The one-way ANOVA comparing maximum heights per transect between each species (Fig. 8) 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. 9), 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.

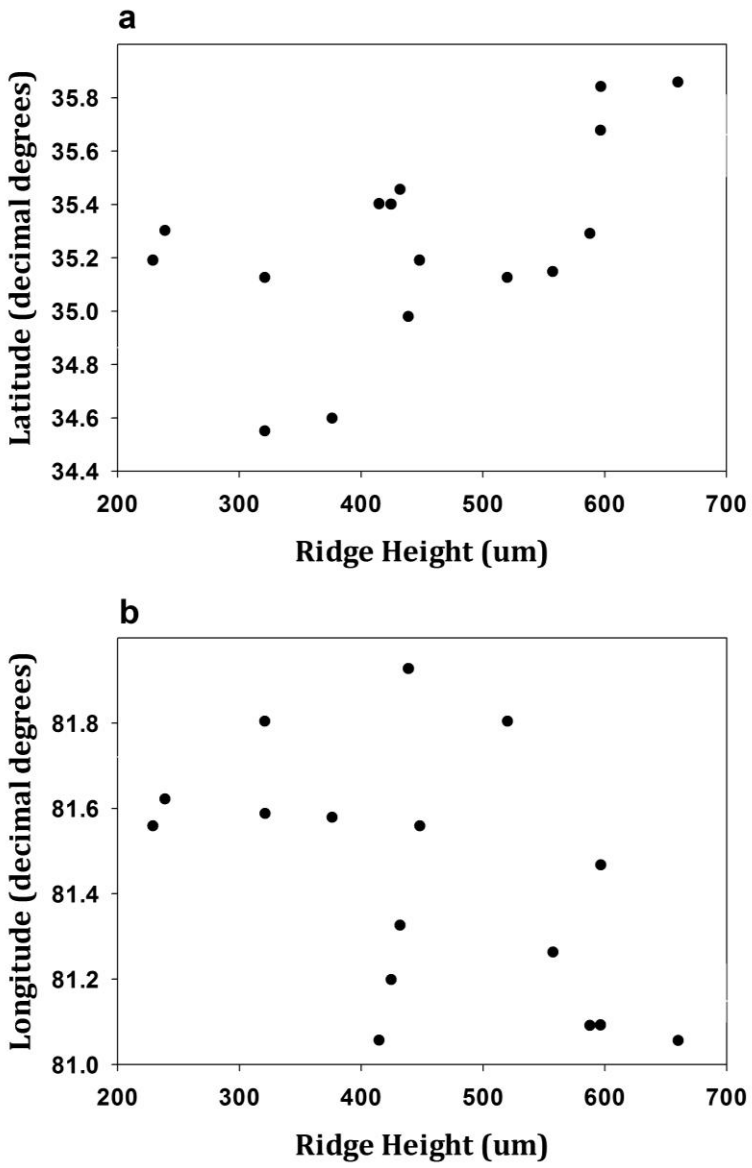

Fig. 9. Plot of mean calyx height versus a) latitude and b) longitude for 16 individuals of *H. naniflora* across 16 different populations across the range of the species. For a) Mean p=0.0216, Pearson's r= 0.57, and for b) p=0.0389, Pearson's r= -0.52 .

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. 10) 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).

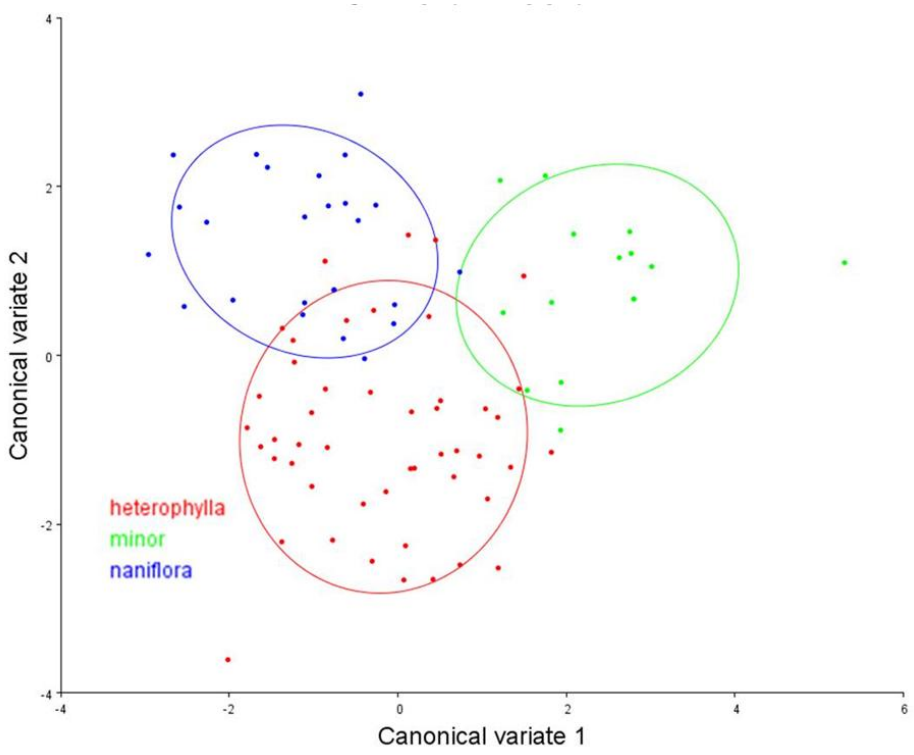

Fig. 10. Canonical variate analysis of leaf morphology for the Hexastylis Heterophylla subgroup not inlcuding populations displaying intermediate morphologies. Axes were defined by morphometric landmark data. Ellipses indicate a 90% confidence interval.

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. 11). The strongest drivers for CV1 are landmarks x6, x5, and x12. The strongest drivers for CV2 are x5, y8, and y11 (Table 3).

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

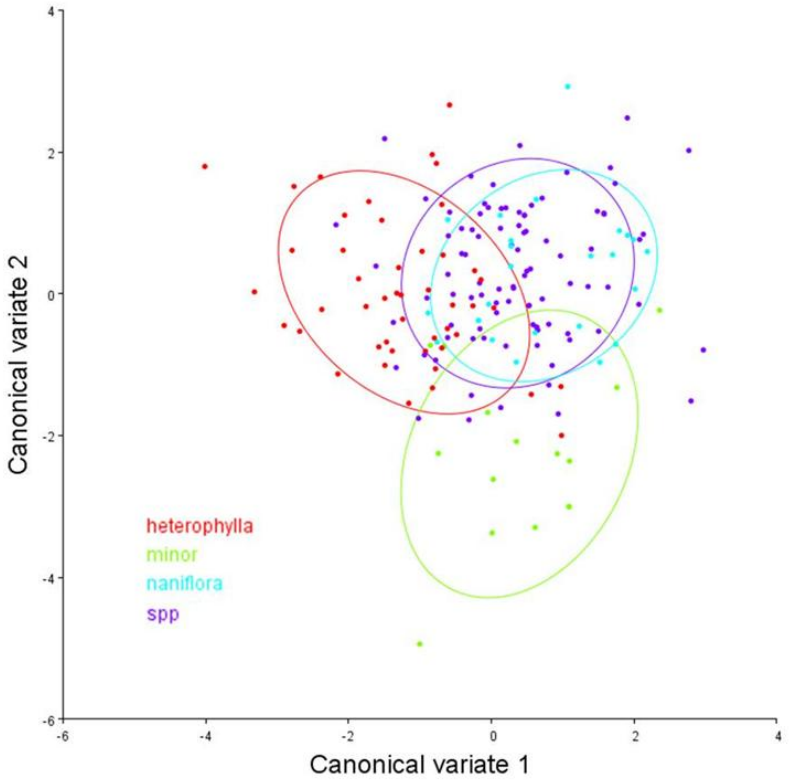

Fig. 11. Canonical variate analysis of leaf morphology for the Hexastylis heterophylla subgroup including populations displaying intermediate morphologies (H. spp.). The ellipses indicate 90% confidence. The axes were defined by morphometric landmark data.

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. 13).

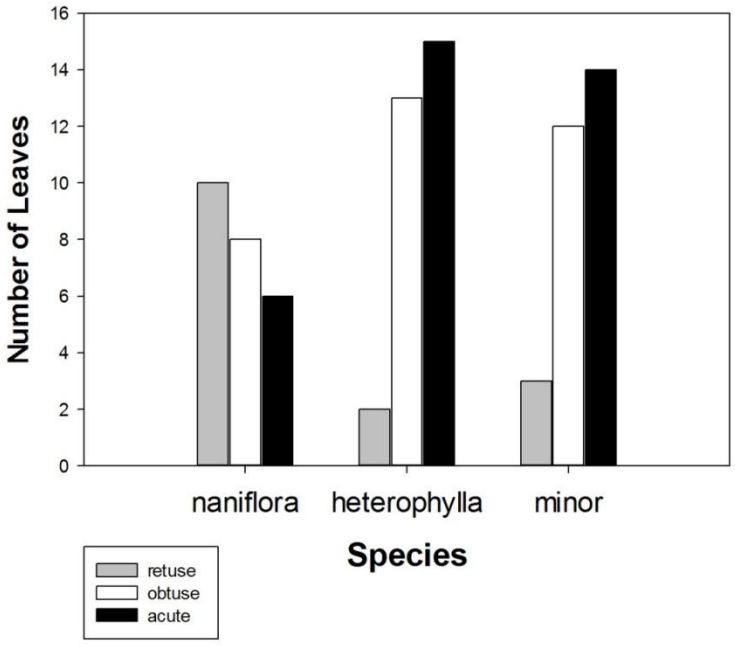

Fig. 13: Raw values for leaf tip types by species. *Hexastylis naniflora* has more retuse tips while the other species have fewer, showing non-independence among species and leaf tip type. *N*=83, χ^2= 13.25, df= 4, *p*= 0.010.

Molecular:

Of the 102 primer pairs tested, we found 36 cleanly amplified fragments across all three species and 15 of those were confidently scorable. Twelve loci were polymorphic and three were monomorphic across all populations (Table 6). Eleven different di, tri, tetra, and penta nucleotide repeat motifs were represented across 15 loci. No loci were found to be polymorphic across species and monomorphic within each species therefore out of all 12 loci displaying polymorphism, no alleles were specific to one species across our 8 individuals. The number of alleles at each locus ranged from 1-11 and we detected five or more alleles at 9 loci (Table 7). All but two of the loci were found to be in HWE. Observed heterozygosity ranged from 0.63-0.88 across all loci.

Table 6. Characterization of 12 polymorphic and 3 monomorphic microsatellite loci for *Hexastylis.* T_a: annealing temperature, F: forward primer, R: reverse primer. Each forward primer has an M-13 (5'-CACGACGTTGTAAAACGAC-3') tag on the 5' end.

Locus		sequence	motif	repeat Count	Size (bp)	T_a (°C)
Hh6251	F:	ATAGAGGTAGCAGCCCAAAGAAG	AAAG	6	241-252	60
	R:	AACGTCCCAGGTGAACTACTATC				
Hn187	F:	TCATCACCCAAGAAGAATAGCAG	AAG	18	94	60
	R:	CCGAACTCTTCCTCTGCTATTTG				
Hn955	F:	CTTAGAGGTGGTAGGAAGGAGTC	AAT	13	374-417	60
	R:	GCAATGAACTCTAAATGGAATGGC				
Hn304	F:	CCACTCCACTCCTTAATATAGAGC	AAG	10	179-206	59
	R:	AATGTGGAGGAATCTGAGAACAC				
Hn419	F:	CGGTCACACAGGACCATAGTAC	ACT	16	282-323	61
	R:	CTCGGCGTCTAGACAGGTTATAG				
Hn1135	F:	TTCAGGCTGCAAACTATCTGAAC	ACC	11	282-302	59
	R:	TTCAGCAACCAACACTCATTTAC				
Hn1825	F:	TGATGATGAAATGCTCCACTCAC	AAC	22	243-266	61
	R:	AGACAAGACTGGATGGAGGTTTG				
Hn4600	F:	GAGAGAACCGGTGAATCAAGTTG	AAAG	5	264-355	60
	R:	AAAGTAGCAATCAGAATTCGGGC				
Hn4816	F:	AGCCAATCAACAATTACCCATGG	AAAAG	5	274	58
	R:	GGATAAAGGTATGCGAAGTGTATC				
Hn6236	F:	GCACACCCTAACTCTTACTTGTG	AG	17	424-444	60
	R:	ACCATCAATTCTCTGTGTCGTTG				
Hn147	F:	GGTAAAGCTAACATCCGACTGTG	AGAT	5	220-230	59
	R:	AAGGGTAGCTATAAGTTGGTTGC				
Hn855	F:	GAGAACGAGAGAGTACCGCAAC	AGAT	8	278-302	62
	R:	ATGCCATATCAGCCGTCTACAAC				
Hn12441	F:	TCCATCGTACAAGGTCGTCTATG	AGGG	5	168	60
	R:	GAAGTCGAACCAAGGTCAATAGG				
Hn575	F:	AAAGATGGTGAGAGTGGAAGTGG	AAAG	6	336-347	60
	R:	GTACATATGACTCTCCACTTGTGC				
Hn1024	F:	ATTAATGACTGCAACCACCCTTC	ATCC	5	295-299	60
	R:	CGTTTAGAATTTGCTTGCCCTTG				

Table 7. Levels of diversity for microsatellite across 8 individuals of *Hexastylis.* A= number of alleles observed; Ho= observed heterozygosity, He= expected heterzygosity; * indicates significant deviation from Hardy-Weinberg expectations,† indicates monomorphic loci.

Locus	**A**	**H_o**	**H_e**
Hn187†	1	0.00	0.00
Hn4816†	1	0.00	0.00
Hn575†	1	0.00	0.00
Hn4600*	2	0.00	0.47
Hn1024	2	0.63	0.43
Hh6251	4	0.50	0.60
Hn6236	5	0.50	0.69
Hn147	5	0.63	0.72
Hn12441*	6	0.60	0.82
Hn304	7	0.75	0.74
Hn1135	8	0.75	0.84
Hn419	9	0.57	0.87
Hn1825	10	0.88	0.87
Hn855	10	0.75	0.87
Hn955	11	0.88	0.88

DISCUSSION

Biogeography

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.

Flower Morphology

Flower size and shape have been the foundation for identification of *Hexastylis* species due to the 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. My 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.

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.

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 (1-11) 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. The two loci not in HWE were still reported because the disequilibrium may be due to isolation or inbreeding of plants caused by anthropogenically modified habitat and the test is highly sensitive due to the small sample size of individual plants.

Changes to species status

In order to de-list *H. naniflora*, one of the following reasons for doing so must become evident to USFWS: 1) elimination or control of threats to the species, 2) stability of habitat quality and quantity, and 3) inaccuracy of data requiring the species to be listed in the first place (USFWS, 1973). The most likely cause for de-listing will be the last as there has been more than a four-fold increase in the number of known populations since *H. naniflora* was listed while habitat loss is undoubtedly a continuing threat. There has been an approximate 5,000% increase in the number of documented

H. naniflora plants from 1989 to 2012 (Bassett, 2012). The removal of *H. naniflora* from the endangered species list would save hundreds of thousands of taxpayer dollars currently going toward mitigation projects for the NCDOT. Resources currently provided to *H. naniflora* could be re-allocated to other endangered species facing more imminent threats of extinction. Easements and mitigation sites will continue to provide protection for several populations of *H. naniflora* even after it is de-listed. There has been a 725% increase (currently ~33% of all known plants) in the number of sites receiving or having the potential to afford long-term protection but additional safeguards such as propagation of plants in a greenhouse and the creation of a seed bank could ensure the preservation of genetic diversity if one or several protected populations experience losses (Padgett 2004).

If *H. naniflora* loses its 'threatened' status, it would result in the loss of protection from the species' largest threats. This species relies heavily on federal protection for survival because *H. naniflora* is distributed in a region of rapid development. Evidence from this study indicates that habitat requirements like soil and slope aspect severely limit the range of this species indicating that some type of protection is vital to the persistence of this species. The loss of protection would ultimately mean the loss of populations that may provide sources of genetic variability to surrounding populations. This study indicates that populations displaying intermediate external flower morphologies also display intermediate leaf and flower morphologies. This warrants future investigation of hybridization within the genus which could be a major threat to *H. naniflora* and discourage the de-listing of the species. The microsatellites provided in this work can serve as the tool to investigate

hybridization within this subgroup and it is the recommendation that this investigation take place before the de-listing of *H. naniflora* is considered further.

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Vita

Jacqueline Renee Wagner was born to Tom and Kathi Wagner in 1985 in Herndon, VA. She grew up in Raleigh, NC and graduated from Leesville Road High School in 2004. Jackie received her Bachelor of Arts degree in Interdisciplinary Studies: Sustainable Development at Appalachian State University in 2008 and started a Master of Science program in the Department of Biology in August of 2011 at Appalachian State, completing her degree in August 2013.