ECOLOGICAL, MORPHOLOGICAL, MICROMORPHOLOGICAL AND MOLECULAR ANALYSES OF THE SPECIES IN THE *Hexastylis heterophylla* COMPLEX.

A Report to the NC Department of Transportation

By

ZACK E. MURRELL
JAMES E. PADGETT
EMILY L. GILLESPIE
FALINA WILLIAMS

Department of Biology
Appalachian State University
Boone, NC 28608

May 2007

NCDOT Project # 2002-04
Report # FHWA/NC/2004-01

Technical Report Documentation Page

1. Report No. **FHWA/NC/2004-01**	2. Government Accession No.	3. Recipient's Catalog No.
4. Title and Subtitle **Ecological, Morphological, Micromorphological, and Molecular Analyses of the Species in the *Hexastylis Heterophylla* Complex**		5. Report Date **May 2007** 6. Performing Organization Code
7. Author(s) **Zack E. Murrell, James E. Padgett, Emily L. Gillespie, Falina Williams**		8. Performing Organization Report No.
9. Performing Organization Name and Address **Department of Biology** **Appalachian State University** **572 Rivers Street** **Boone, NC 28608**		10. Work Unit No. (TRAIS) 11. Contract or Grant No.
12. Sponsoring Agency Name and Address **North Carolina Department of Transportation** **Research and Analysis Group** **1 South Wilmington Street** **Raleigh, North Carolina 27601**		13. Type of Report and Period Covered **Final Report** **July 2001 – June 2003** 14. Sponsoring Agency Code **2002-04**

Supplementary Notes:

This project was supported by a grant from the U.S. Department of Transportation and the North Carolina Department of Transportation, through the Center for Transportation and the Environment, NC State University.

16. Abstract

Both Blomquist and Gaddy recognized a group of eight *Hexastylis* (commonly known as "Wild Gingers" or "Little Brown Jugs") that are referred to as the Virginica Group. This group was further subdivided into the three Subgroups: Virginica, Shuttleworthii, and Heterophylla. Three species have been recognized in the Heterophylla complex. Field biologists have generally recognized considerable morphological overlap occurs in this group. The three species that are placed in the Heterophylla complex are *Hexastylis naniflora, H. heterophylla* and *H. minor*. *Hexastylis naniflora* is a federally threatened species that is found in the rapidly growing area of the western Piedmont of North and South Carolina. The range of *H. naniflora* is restricted by soil type, biogeography, and ecology. Herbarium specimens were borrowed from 17 herbaria and these 693 specimens were used to generate distribution maps for the three species in the *H. heterophylla* complex. Elemental occurrence data were obtained from the North Carolina Natural Heritage Program and the South Carolina Heritage Trust Program to augment the distribution map for *H. naniflora*. Based upon these maps, field investigations were conducted across the range of the three species in the complex. We conducted ecological, morphological, micromorphological, soil, pollen, and molecular analyses of the *H. heterophylla* complex. Using ecological and biogeographical information obtained from our study, we located 31 new populations of *H. naniflora*; one of the new populations was found to be unique to the Yadkin River drainage. This effort brings the total known populations of *H. naniflora* to 143. Eighty-five populations of the three species in the *H. heterophylla* complex were subjected to field investigations. Using Scanning Electron Microscopy (SEM), we found pollen characters that distinguish *H. naniflora* from other members within the subgroup. In a comparative analysis using Inter Simple Sequence Repeats, we were unable to find banding patterns that could be used to separate *H. naniflora* from the other members within the complex. Based upon biogeographical, ecological, molecular, morphological, as well as micromorphological work, our results show that *H. naniflora* Blomquist is a well-defined species, however, *Hexastylis minor* (Ashe) Blomquist and *Hexastylis heterophylla* (Ashe) Small exhibit considerable overlap that make species circumscription difficult. Our intraspecific analysis of *Hexastylis naniflora* was based on analysis of soil, ecology, molecular characters and morphology, where we compared populations in the Broad-Pacolet, Catawba, and Yadkin River drainages. This analysis provides information that can be used in future conservation and management efforts for *H. naniflora*.

17. Key Words	18. Distribution Statement
Hexastylis, Wild Gingers, Virginica Group, Virginica, Shuttleworthii, Heterophylla, *Hexastylis naniflora, H. heterophylla H. minor*	

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of Pages	22. Price
Unclassified	Unclassified	148	

ABSTRACT

Both Blomquist and Gaddy recognized a group of eight *Hexastylis* (commonly known as “Wild Gingers” or “Little Brown Jugs”) that are referred to as the Virginica Group. This group was further subdivided into the three Subgroups: Virginica, Shuttleworthii, and Heterophylla. Three species have been recognized in the Heterophylla complex. Field biologists have generally recognized considerable morphological overlap occurs in this group. The three species that are placed in the Heterophylla complex are *Hexastylis naniflora*, *H. heterophylla* and *H. minor*. *Hexastylis naniflora* is a federally threatened species that is found in the rapidly growing area of the western Piedmont of North and South Carolina. The range of *H. naniflora* is restricted by soil type, biogeography, and ecology. Herbarium specimens were borrowed from 17 herbaria and these 693 specimens were used to generate distribution maps for the three species in the *H. heterophylla* complex. Elemental occurrence data were obtained from the North Carolina Natural Heritage Program and the South Carolina Heritage Trust Program to augment the distribution map for *H. naniflora*. Based upon these maps, field investigations were conducted across the range of the three species in the complex. We conducted ecological, morphological, micromorphological, soil, pollen, and molecular analyses of the *H. heterophylla* complex. Using ecological and biogeographical information obtained from our study, we located 31 new

populations of *H. naniflora*; one of the new populations was found to be unique to the Yadkin River drainage. This effort brings the total known populations of *H. naniflora* to 143. Eighty-five populations of the three species in the *H. heterophylla* complex were subjected to field investigations. Using Scanning Electron Microscopy (SEM), we found pollen characters that distinguish *H. naniflora* from other members within the subgroup. In a comparative analysis using Inter Simple Sequence Repeats, we were unable to find banding patterns that could be used to separate *H. naniflora* from the other members within the complex. Based upon biogeographical, ecological, molecular, morphological, as well as micromorphological work, our results show that *H. naniflora* Blomquist is a well-defined species, however, *Hexastylis minor* (Ashe) Blomquist and *Hexastylis heterophylla* (Ashe) Small exhibit considerable overlap that make species circumscription difficult. Our intraspecific analysis of *Hexastylis naniflora* was based on analysis of soil, ecology, molecular characters and morphology, where we compared populations in the Broad-Pacolet, Catawba, and Yadkin River drainages. This analysis provides information that can be used in future conservation and management efforts for *H. naniflora*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Species recognition and delineation is a critical part of conservation biology. If we are to use species as our unit of conservation, we must be able to determine if the unit is a "good species." There is considerable literature addressing the question, "What is a species?" (Avise and Wollenburg 1997; Wu 2001; Noor 2002; Rundle et al. 2001; Orr 2001; Britton-Davidian 2001; Voger 2001; Bridle and Richie 2001; Shaw 2001; Rieseberg and Burke 2002). However, most studies are theoretical and very few address the issue of species in "real life" settings. We contend that there is a critical need to examine imperiled species to determine if they can be defended as biological units. This is necessary in order to show the public that the funds invested in species conservation are worth the costs as well as the effort.

There is also a critical need in conservation biology to examine and document the autecology of imperiled species. This effort is necessary in order to 1) maintain and/or extend the range of imperiled species in a time of ever-dwindling non-disturbed habitat, and 2) to prepare for the not-too-distant future need to reconstruct whole alliances of organisms in the face of climatic change and wholesale movement of appropriate microhabitat from current locations. If we fail to gain this information in the near future, we will not be prepared to adequately protect imperiled species through the 21st century.

This study examines the species boundaries and autecology of the federally threatened *Hexastylis naniflora* Blomquist in the family Aristolochiaceae. This species

appears to be closely related to two other species with sympatric distributions, *H. minor* and *H. heterophylla*. This study uses a variety of molecular, morphological, and ecological analyses to elucidate relationships among these three species, to examine the rarity of *H. naniflora*, and to develop a set of recommendations for management of this species that will ensure that *H. naniflora* is not in danger of extinction.

The family *Aristolochiaceae*, also known as the Birthwort family, consists of eight genera and more than five hundred species. The distribution of *Aristolochiaceae* is primarily pantropic with a few species found in temperate regions of Asia, Europe, and North America. The two major genera in the family are *Aristolochia* with 300-350 species and *Asarum* with about 70 species (80 if *Hexastylis* and *Heterotropa* are included) (Judd et al. 2002).

Most of the family consists of woody vines in the genus *Aristolochia*, which has a tropical distribution. *Asarum* occurs in North America and Asia and consists of herbaceous perennial and annual species. *Asarum* species often have aromatic stems or leaves, due in part to the ethereal oils many of them possess.

Depending on the authority used, the North American species of *Hexastylis* can be segregated as a separate genus or nested within *Asarum*. 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; Gonzalez 1972; Otte 1977; Kral 1983; Gaddy 1981, 1986, 1987, 1997; Wofford 1989; Rayner 1994). *Hexastylis* was segregated from *Asarum* primarily due to the persistent glabrous leaves and the unique ovary position (Rafinesque 1825).

Currently, *Hexastylis* is commonly used to describe a genus of nine species and four varieties that are endemic to the southeastern United States.

In spite of this general acceptance, several North American taxonomists refused to recognize *Hexastylis* as a genus and published their work using the genus *Asarum* (Peattie 1929,1940; Fernald 1943, 1950; Wyatt 1955; Gregory 1956). Barringer (1993) stated that new nomenclatural combinations were needed for North American species of *Asarum* to bring them in line with the current understanding of the genus and he revised *Asarum* and placed the *Hexastylis* names in synonymy. In transferring the species of *Hexastylis* to *Asarum*, Barringer (1993) expanded *Asarum* to eighty species. Barringer (1993) noted that all of the *Asarum* are linked together by similar vegetative and reproductive characteristics as well as having similar ethereal oils, as was first determined by Hayashi et al. (1982). Recent molecular work by Kelly (1997, 1998) has shown *Hexastylis* to be nested within the genus *Asarum*, further supporting the work of Barringer (1993).

The recent publication of the Flora of North America (FNA) again recognized *Hexastylis* as a separate genus apart from *Asarum*, but footnotes were added in both the *Hexastylis* and *Asarum* keys and descriptions to denote that some problems existed in our understanding of the phylogeny (Barringer 1997; Whittemore and Gaddy 1997).

One of the earliest descriptions and illustrations of *Asarum* was in an herbal by Dodoen (1574) who discussed the use of *Asarum* as a purgative. He noted the medicinal properties of the plant he called *Asaron* (*Asarum europaeum*). In the 17th century other herbals and botanical journals described several species of *Asarum* (Parkinson 1640; Tournefort, 1694, 1698). In 1789 de Jussieau recognized a relationship between *Asarum*,

a genus made up entirely of herbs, with *Aristolochia*, a large genus comprised almost entirely of woody vines. de Jussieau made these associations based on floral characteristics and plant morphology. In Species Plantarum Linnaeus (1753) described four species of *Asarum*, including two North American species, *Asarum canadense* L., and *Asarum virginicum* L. Andre Michaux (1803) published a description of a third North American *Asarum* he named *Asarum arifolium* Michx.

In 1825 when Rafinesque erected the genus *Hexastylis*, his circumscription was based on characters that were unique to the three or four known North American species of *Asarum*. Those characters used to delineate *Hexastylis* included glabrous persistent leaves, connate sepals, sessile anthers, and apical bifid styles. Based on those characters, Rafinesque (1825) segregated *Hexastylis virginica* (L.) Raf. and *Hexastylis arifolia* (Michx.) Raf., leaving *Asarum canadense* as a sole species in the genus *Asarum* in North America. Rafinesque (1825) description of *Hexastylis* in Neogenyton is as follows:

> "***Hexastylis.*** **Cal. Tubular, trifid, cor o. anthers twelve, sessile, bilobe adnate, epigyne; pistil half free, cylindrical, and concave; styles six, lateral erect; stigmas six, truncate, oblique, bicorne; caps. Six locul. Few central seeds. Type *Asarum arifolium*, Michx."**

Morren and Decaisne (1834) erected the genus *Heterotropa*, and described the Asian species *Heterotropa asarodies* Morr. & Dec. The characters used by Morren and Decaisne (1834) to describe *Heterotropa* were very similar to those used by Rafinesque (1825) to describe *Hexastylis* (1825). Braun (1861) was the first to recognize the similarity in the descriptions of the two genera and placed both *Hexastylis* and *Heterotropa* in synonymy within *Asarum*. Braun divided the genus *Asarum* into three

sections: *Ceratasarum*, *Heterotropa*, and *Euasarum* and placed *Hexastylis* within section *Ceratasarum*, which included *Asarum arifolium* and *Asarum virginicum* along with one Japanese species, *Asarum variegatum*. Duchartre (1864) closely followed Braun's treatment in A. P. de Candolle's publication Prodromus Systematis Naturalis Regni Vegetabilis.

In the late 19th century W. W. Ashe traveled extensively across much of the southeastern United States examining various plant communities. In his travels Ashe realized that separation of the species of *Asarum* was difficult and the genus exhibited considerable variation across its range. Seeing that many specimens did not fit within the circumscription for *Asarum virginicu*m, Ashe started collecting *Asarum* throughout the southeast United States. He also made numerous notes and sketches in regards to flower and leaf morphology. At the end of the 19th century, Ashe (1897) described several species of *Asarum*, including two species that would eventually be placed into the Virginica group of *Hexastylis*. Ashe's (1897) description of the three new species is as follows:

> "A***sarum minus*. Leaves solitary, glabrous, thick, round cordate at base, but rarely orbicular. Tube of calyx cylindro campanulate, about 1 cm wide, about the same length, the very short lobes spreading. Peduncle as long as flower, the large bract pointed. Projection of style very short; the seeds oblong."**
>
> ***"Asarum heterophyllum.* Leaf-blades orbicular, ovate or triangular in outline, cordate at base (or occasionally almost hastate), about the same size as in above. Calyx campanulate rounded at the base, the tube.7-1 cm long, the lobes nearly equaling in length .8-1 cm wide at the base, orange-purple or purple-brown without, bright within; the very stout notched style much prolonged the much minute round stigma; capsule short, cylinderous barely as long as the stamens, scarcely distending the calyx; seeds oval."**

> **"*Asarum heterophyllum ochranthum.* Calyx yellow or orange, oblong-campanulate, the spreading lobes as long as the 1cm tube. Calyx urceolate or somewhat contracted at the mouth, the oval stigma thicker than the slender deeply 2-parted projection of the style, and placed near the base of the style."**

Along with the descriptions quoted above, Ashe (1897) also described the distributions of the three new species. He described the distribution of *Asarum heterophyllum* (Ashe) (*Hexastylis heterophylla*) as being from North Carolina, Tennessee, and Virginia. Herbarium records and recent publications show *Hexastylis heterophylla* to be found in Alabama, Georgia, Kentucky, North Carolina, South Carolina, Tennessee, Virginia, and West Virginia, (Blomquist 1957; Radford et al, 1968; Gaddy 1987; Sutter 1983; Wofford 1989; Harvill et al 1994; Rayner 1994; McMillian 1995; Chester et al 1997; Amoroso 2002). *Asarum heterophyllum ochranthum* was never well accepted and today is included within *Hexastylis heterophylla*. *Asarum minus*, described by Ashe (1897) as being from North Carolina, South Carolina and Tennessee, is presently known from Georgia, North Carolina, South Carolina, and Virginia (Blomquist 1957; Radford et al, 1968; Gaddy 1987; Sutter 1983; Wofford 1989; McMillian 1995; Amoroso 2002).

The genus *Hexastylis* was not well recognized in publications until J. K. Small (1933) used *Hexastylis* instead of *Asarum* to denote the genus in his Manual of Southeastern Flora. This was the first major publication in the United States to recognize the genus *Hexastylis*. After Small's (1933) publication, the use of the name *Hexastylis* became accepted for the genus and the genus was recognized in most North American publications as being separate from *Asarum*. In fact, *Hexastylis* has for the most part replaced *Asarum* in North American publications.

In Southeast Asia the treatment of *Hexastylis* as a separate genus was not accepted and is still not referred to except when used as a synonym or as a common name to denote *Hexastylis* as a southeastern United States endemic. Schmidt (1937) recognized the same four sections within *Asarum* that were first erected by Duchartre (1864) and placed the four sections in two subgenera, *Asarum* subgenus *Heterotropa* and *Asarum* subgenus *Ceratasarum*. Schmidt (1937) also broadened the descriptions of the four sections to allow many other species described since Duchartre (1864) to fit within his taxonomic framework. Maekawa (1933, 1936) worked with Japanese flora and recognized two segregate genera for *Asarum*. He moved 45 species from *Asarum* to *Heterotropa* and described dozens of new species. Maekawa (1936) erected the genus *Asiasarum* and placed five new species and one variety into that genus. Included in one of those new genera was the newly described species *Asarum Japonasarum* Nakai (1936). Araki (1937, 1953) divided *Asarum* into two subgenera, *Asarum* section *Asarum*, and *Asarum* section *Asiasarum*. *Asarum* section *Asarum* was divided into three sections, *Euasarum*, *Calidasarum*, and *Japonasarum*. *Asarum* section *Asiasarum* consists of the three subsections *Asiasarum*, *Hexastylis*, and *Heterotropa*.

H. L. Blomquist (1957) made a complete revision of the genus *Hexastylis* in North America. Kelly (1998) suggested that Blomquist (1957) overlooked the work of Araki (1953), who lumped *Hexastylis* with the Japanese *Asarum*. In his treatment, Blomquist kept the genus name *Hexastylis*, divided the genus into three groups, and then recognized subgroups within these groups. Blomquist changed the specific epithet on the name of the species *Hexastylis minus* to *Hexastylis minor*. His work described one new species, which he placed in the subgroup *Heterophylla*. *Hexastylis naniflora* was described from

specimens found in three locations in North Carolina and South Carolina. The description of *Hexastylis naniflora* by Blomquist (1957) is as follows:

> ***Hexastylis naniflora* sp. nov. Leaf-blades cordate to orbicular-ovate, 4-5.76 cm long by 4-5.5 cm wide, the apices obtuse, the sinuses broad to narrow, the lobes rarely overlapping, usually variegated along the principal veins. Petioles averaging 10.5 cm long. Rhizomes short and moderately branching. Calyces relatively small, brown, thr tube cylinderic, slightly narrowing upwards, 7 mm long by 6.5 mm wide in diameter, sometimes pale brown above the base, the lobes relatively large, flaring at the base, 7 mm wide at the base by 5.5 mm long, moderately spreading, without colorless spots inside. Stamens essentially sessile, those opposite the styles conspicuously shorter than the alternating ones. 1.61 mm-1.84 mm long, the anther-connective not prolonged into three appendage. Ovary ca. ½ inferior. Styles ca. 2.5 mm long, extending 0.75mm above the stigma, only notched at apex. Mature seeds not seen.**

Recent work by Kelly (1997, 1998, 2000, and 2002) using morphology and molecular data support the previous Asian studies and show that *Hexastylis* should be recognized within *Asarum* under the section *Heterotropa.* Kelly (1997) conducted molecular analysis on the Internal Transcriber Space (ITS) region of a number of *Asarum* species from around the world as well as the southeastern species *Asarum canadense* and three species of *Hexastylis*. His work showed that *Hexastylis* is rooted within *Asarum* and should be treated as *Asarum* (Figure 1). However, due to the localized stigma associated with the use of the genus name *Asarum* for *Hexastylis*, the species in this paper will be called *Hexastylis* with the understanding that *Hexastylis* is rooted in *Asarum* and the proper treatment of the species is *Asarum*. Kelly (2002) advocated the use of *Asarum* and supported the monophyletic arrangement based on morphological and molecular data (Kelly 1997, 1998). His work supports the broad treatment of *Asarum* by Araki (1937,

1953) who recognized two subgenera, *Asarum* and *Heterotropa*. Under *Asarum* are the sections *Asarum* and *Geotaenium* and the sections *Asiasarum* and *Heterotropa* (which includes *Hexastylis*) are placed within the subgenus *Heterotropa*.

Hexastylis heterophylla Complex

By the late 1950's, *Hexastylis* was recognized as consisting of eight species that were endemic to the southeastern United States. Blomquist (1957) established the currently recognized grouping of *Hexastylis*, and it has become widely accepted, especially by botanists from the southeastern United States.

The genus, as recognized by Blomquist, consists of three groups: Arifolia, Speciosa, and Virginica (see Table 1). The group Arifolia has only one member, *Hexastylis arifolia*. There are also two varieties of *H. arifolia* in the group, *H. arifolia var. ruthii*, and *H. arifolia var. callifolia*. The second group, Speciosa, consists of a single species, *H. speciosa*. The third group, the Virginica group, is divided into three subgroups. Blomquist named the subgroups of the Virginica group Virginica, Shuttleworthii, and Heterophylla. The Virginica subgroup recognized by Blomquist contained only *H. virginic*a. Morphological analysis (Gaddy 1987) showed that *H. rhombiformis* is a close relative of *H. virginica* within the Virginica subgroup, and Gaddy (1987) placed *H. rhombiformis* in the Virginica subgroup. The Shuttleworthii subgroup, as recognized by Blomquist, had two species, *H. shuttleworthii* and *H. lewisii*. *Hexastylis shuttleworthii* has two varieties. The first is *H. shuttleworthii var. shuttleworthii* and the other is *H. shuttleworthii var. harperii*. The Heterophylla subgroup, as recognized by Blomquist, contains *H. heterophylla, H. minor,* and *H. naniflora*. Gaddy (1987) showed that *H. contracta* was allied with the Heterophylla subgroup and was subsequently placed into

this subgroup. The *Hexastylis heterophylla* subgroup has been thought to form an overlapping complex of species (Blomquist 1957; Gaddy 1987). One of the main concerns regarding this complex was the inability to distinguish between species without access to fresh flowers. Even with fresh flowers, Blomquist (1957) and Gaddy (1987) still recognized considerable overlap in flower morphology making species delineation difficult.

Through the 1980's, Gaddy examined the groups and subgroups of *Hexastylis* in closer detail. He retained the framework of groups and subgroups described by Blomquist (1957), and he added details to his keys to aid in the distinction of species within the genus. Along with characteristics known to exist, Gaddy looked at biogeography and soil types in an effort to resolve species level questions posed by Blomquist and himself as to the validity of species in the *H. heterophylla* complex.

Figure 1. Phylogeny showing *Hexastylis* nested within *Asarum* based on the molecular analysis preformed by Kelly (1997). *Hexastylis* is included in *Asarum* section *Heterotropa.*

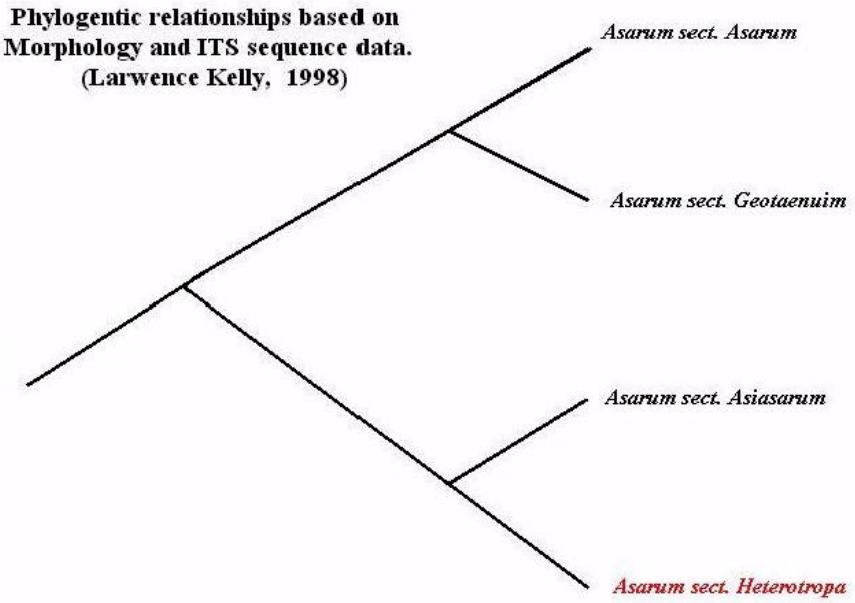

Phylogentic relationships based on
Morphology and ITS sequence data.
(Larwence Kelly, 1998)
Asarum sect. Asarum
Asarum sect. Geotaenuim
Asarum sect. Asiasarum
Asarum sect. Heterotropa

Table 1. *Hexastylis* groups and subgroups recognized by Blomquist (1957) and Gaddy (1987).

GROUPS	**SUBGROUPS**	**SPECIES**
ARIFOLIA		*Hexastylis arifolia* var. *arifolia* (Michx.) Small *Hexastylis arifolia* (Michx.) Small var. *callifolia* (Small) Blomquist *Hexastylis arifolia* (Michx.) Small var. *ruthii* (Ashe) Blomquist
SPECIOSA		*Hexastylis speciosa* Harper
VIRGINICA	VIRGINICA	*Hexastylis virginica* (L.) Small *Hexastylis rhombiformis* Gaddy
	SHUTTLEWORTHII	*Hexastylis shuttleworthii var. shuttleworthii* (B. & B.) Small *Hexastylis shuttleworthii var. harperii* (B. & B.) Gaddy *Hexastylis lewisii* (Fernald) Blomquist and Oosting
	HETEROPHYLLA	*Hexastylis heterophylla* (Ashe) Small *Hexastylis minor* (Ashe) Blomquist *Hexastylis naniflora* Blomquist *Hexastylis contracta* Blomquist

Research Questions

This study represents an attempt to resolve the taxonomic confusion in the *Hexastylis heterophylla* complex, comprised of *H. heterophylla*, *H. minor* and *H. naniflora*. One rationale for the study was to understand the relationship of *H. naniflora* to the other two species in the complex. *Hexastylis naniflora* (dwarf flowered heartleaf) is federally listed as threatened under the Endangered Species Act and state-listed as threatened by the North Carolina Plant Conservation Program (USFWS 1990; Amoroso 2001). *Hexastylis naniflora* is known from eight counties in North Carolina and three counties in South Carolina. It appears to be restricted to Pacolet sandy loam, Madison gravelly sandy loam, and Musella fine sandy loam soils (Gaddy 1981,1987). These soils are restricted to an area from near Charlotte, North Carolina west to the foot of the mountains near Rutherfordton, North Carolina, and from Hickory, North Carolina southward to just south of Spartanburg, South Carolina. This area is one of the fastest growing regions of North and South Carolina, and this plant has played a key role in several recent highway routing decisions in North Carolina. Given the rate of development within the region, it is likely that it will continue to impact highway construction projects. In order to assist the North Carolina Department of Transportation in their efforts to protect *H. naniflora*, we conducted a study to 1) utilize morphological, micromorphological and molecular methods to examine the species boundaries of *H. heterophylla, H. minor,* and *H. naniflora* and to use this information to generate distribution maps for the three taxa, 2) evaluate the ecology of known sites using 10 m^2 plots and test soil samples, 3) use the collected ecological and biosystematics information to search for new sites in Alexander, Iredell, Yadkin, and Gaston Counties, and 4) conduct ecological analyses at a transplant

site in Caldwell County, North Carolina to determine possible active management techniques that could be used to improve the reproductive capability of the species (Newberry 1996; Henderson 2001).

Molecular data have been used recently to explore species boundaries and to understand evolutionary relationships in enigmatic groups, such as *Hexastylis*. Numerous molecular techniques have recently been developed to analyze DNA by utilizing Polymerase Chain Reaction (PCR) methods. Sequencing of nuclear, mitochondrial and chloroplast genes have been extensively utilized to study relationship among species and populations. Inter Simple Sequence Repeats (ISSR) are highly reproducible, inexpensive, quick and easy, do not require sequence information and do not require any additional equipment outside of the basic PCR systems (Bornet and Branchard 2001; Mondal 2002; Wolfe and Liston 1998).

ISSRs have largely been utilized for studying relationships among cultivars (Wolfe and Liston, 1998). For example, this method has been used to distinguish varieties of grapes (*Vitis vinifera*), cotton (*Gossypium*), walnut (*Juglans regia*), and rice (*Oryza*) (Herrera et al. 2002; Liu and Wendel 2001; Potter et al. 2002; Joshi et al. 2000). These markers are now being used to determine relationships among non-cultivated native plants, such as *Tipularia discolor* and *Penstemon* (Smith et al. 2002; Wolfe et al. 1998). ISSR markers have been used to distinguish between populations, species and hybrids (Wolfe and Liston 1998; Wolfe et al. 1998). ISSR primers are designed with a two or three nucleotide repeat motif found within simple sequence repeat regions and a 1-3 nucleotide sequence to anchor the primer either at the 5’ or 3’ end to DNA (Liu and Wendel, 2001; Wolfe and Liston; 1998; Wolfe et al., 1998). Single or multiple ISSR

primers can be used during amplification by PCR, separated by electrophoresis on either an agarose or polyacrylamide gel and then stained with ethidium bromide to visualize under ultraviolet light (Liu and Wendel 2001; Wolfe and Liston 1998).

The Sanger Chain-Terminated Sequencing technique is used frequently today to examine the diversity and phylogenetic relationships of taxa. This type of sequencing uses double-stranded DNA that is separated into two single-stranded molecules (Weaver 1999). Oligonucleotide primers are annealed to the DNA strands by the site of interest, followed by amplification that generally occurs via PCR. Each reaction contains target DNA, primers, DNA polymerase, deoxynucleotides (dNTP) and dideoxynucleotides (ddNTP). Dideoxynucleotides are 2'-deoxy and lack the 3'-hydroxyl group causing termination of elongation when they are incorporated instead of dNTPs. The reaction is performed in four separate tubes where a different ddNTP is added. Deoxynucleotides are added in excess to ddNTPs to give a population of different length DNA fragments that are separated on a polyacrylamide gel by electrophoresis. Automated sequencing uses a ddNTP tagged with a molecule that fluoresces when encountered by a laser beam, which is interpreted by a detector.

Sequencing has been used in a number of studies to determine phylogeny. Ribosomal RNA (rRNA), low copy number genes and high copy number non-coding nucleotide sequences are nuclear DNA regions that have been sequenced (Judd et al. 2002). When trying to determine the relationships between populations, the best DNA regions to sequence are those that evolve rapidly. High copy number non-coding nucleotide sequences such as microsatellites and minisatellites are useful as well as short transcribed spacers (ITS or ETS) of rRNA. Mitochondiral DNA has also been sequenced. Due to its

slow evolution rate, it is most suitable to study ancient events. A number of chloroplast genes have been analyzed to show phylogenetic relationships at different levels. The genes *rbc*L, *ndh*F and *trn*L have been utilized (Judd et al. 2002; Taberlet et al. 1991). The non-coding chloroplast regions mutate at a high rate, and are thought to be useful for interspecific analyses. Sequencing of chloroplast *trn*L regions entails sequencing part of the gene with several hundred base pairs of intergenic spacers. This technique has been used to study populations of *Silene alba* (McCauley 1994).

The information presented here can be used to assist in management of NCDOT preserves, and well as in decisions concerning future highway development. The NCDOT natural resources staff can use the results to assist in Threatened and Endangered (T&E) surveys for the species. The information derived from this study can be used in future Section 7 consultations with the Federal Highway Administration, United States Fish and Wildlife Service, and the United States Army Corps of Engineers. The information has been provided to the US Fish and Wildlife Service and to the North Carolina Natural Heritage Program to assist in their efforts to determine the status and to develop a recovery plan for the imperiled species, *H. naniflora*.

The objectives of this study were twofold. The first objective was to evaluate species boundaries in the *Hexastylis heterophylla* complex using morphological, micromorphological, molecular and ecological analyses to determine if there were any gaps that could be used to delineate species in the group. Our focus was on the federally threatened *H. naniflora*, but we also gathered data on *H. heterophylla* and *H. minor*. Second, we wanted to determine what conditions are optimal to maintain a population of *Hexastylis naniflora* and to test whether we can use this information to search for new

populations and to relocate populations that are in danger of being eradicated by development projects in the region.

The hypotheses that address the first objective of this research are:

1. Null hypothesis. The variation in morphology, micromorphology, molecules and ecology of the three putative species in the *Hexastylis heterophylla* complex is continuous, and no species can be delineated within the group.

2. There is discontinuity in the variation among two, three or more groups of populations in the *Hexastylis heterophylla* complex, and two or more species can be recognized in this complex.

The hypotheses that address the second objective of this research are:

1. Null hypothesis. Habitat requirements for the establishment and maintenance of populations of *Hexastylis naniflora* are not unique to this species in the complex. There is no predictive value in the locating or transplanting of populations of *Hexastylis naniflora* based upon ecological data.

2. Habitat requirements for *Hexastylis naniflora* are unique within the complex. This information can be used to locate new populations and select sites to successfully transplant populations of *Hexastylis naniflora*.

The purpose of the molecular part of the study was to examine sequences chloroplast *trn*L region and Inter Simple Sequence Repeats (ISSR) to determine the relationships within the *H. heterophylla* complex. Three hypotheses were proposed that applied to the sequencing data and the ISSR data; (1) no variation was observed, (2) variation was

observed without a detectable pattern or (3) variation was observed with a detectable pattern.

METHODS

Biogeography

Six hundred and ninety-three herbarium specimens from the three species in the *Hexastylis heterophylla* complex were examined from seventeen herbaria (Table 2) in order to retrieve habitat, locality, and phenology label data (Appendix A). We also obtained and examined type specimens from the *Hexastylis heterophylla* complex. Living specimens were examined from collections made in the field and from samples that were sent to Appalachian State University for identification. Samples from Alabama, Georgia, eastern Kentucky, southwest Virginia, and North Carolina were examined. Locality data were compiled and used to create distribution maps for the *H. heterophylla* complex.

We obtained location information for most of the known sites of *Hexastylis naniflora,* which had been documented through the North Carolina Natural Heritage Program and South Carolina Heritage Trust Program. We compiled all the coordinates for known sites as well as those sites that were located in this study. Locality information was converted to Decimal Degree reading on a NAD-87 topography map projection for the area and maps were generated using ArcView (ESRI Inc.). We included river drainages in these maps to determine drainage information for each locality.

Table 2. Herbaria where *Hexastylis* specimens were examined and annotated.

Herbaria	**Location**	**Number of Specimens Examined**
BOON	Appalachian State University	77
CONV	Converse College	12
DUKE	Duke University	46
ETSU	East Tennessee State University	35
GH	Gray Herbarium	27
GWU	Gardner-Webb University	24
MOBOT	Missouri Botanical Gardens	3
NYBG	New York Botanical Gardens	26
UGA	University of Georgia	17
UNCCH	University of NC at Chapel Hill	144
US	Smithsonian Institute	5
USCH	University of SC at Columbia	106
USCS	University of SC at Spartanburg	44
TENN	University of TN at Knoxville	45
UWI	University of WI at Madison	6
VPI	Virginia Polytech Institute	60
WOFF	Wofford College	16
17		**693**

To develop a detailed map of the range of *H. naniflora*, we visited as many known populations as possible, obtained GPS data for all sites, and conducted field examinations of those populations (Appendix B). From early March to late June of 2001-2003 (three flowering/fruiting periods) we searched for new populations of *H. naniflora* throughout the eight counties currently known to contain this species, as well as adjacent counties.

United States Department of Agriculture (USDA) soil maps were consulted for Cleveland, Lincoln, and Rutherford Counties, North Carolina and Cherokee, Greenville and Spartanburg Counties, South Carolina where *H. naniflora* is known to exist, and all adjacent counties (USDA 1962, 1980, 1995, and 2000). We then used the soil and stream drainage data to predict where additional populations of *H. naniflora* might occur within counties of known occurrence and adjacent counties. We conducted field surveys using prediction data collected from distribution and soil maps. Field investigation sites in the first growing season (2001) were chosen based upon soil and stream drainage data. In this first year of the study we had two goals: 1) we attempted to locate new populations and 2) we began to develop strategies for use in field investigations conducted over the following two growing seasons.

In the first growing season we obtained soil data, preliminary distribution maps from herbarium and NC and SC Natural Heritage database information. Field investigations in the first growing season provided an understanding of the general ecological requirements of the species, by visiting known populations as well as searching for new localities. This baseline information allowed us to conduct more directed field investigations in the following two growing seasons. We were able to make more accurate assessments of ideal habitat and localities where new populations might exist.

Field examinations in the second and third growing seasons were more efficient, allowing more time for detailed examination at those sites where the species was more likely to exist.

Flower Morphometrics

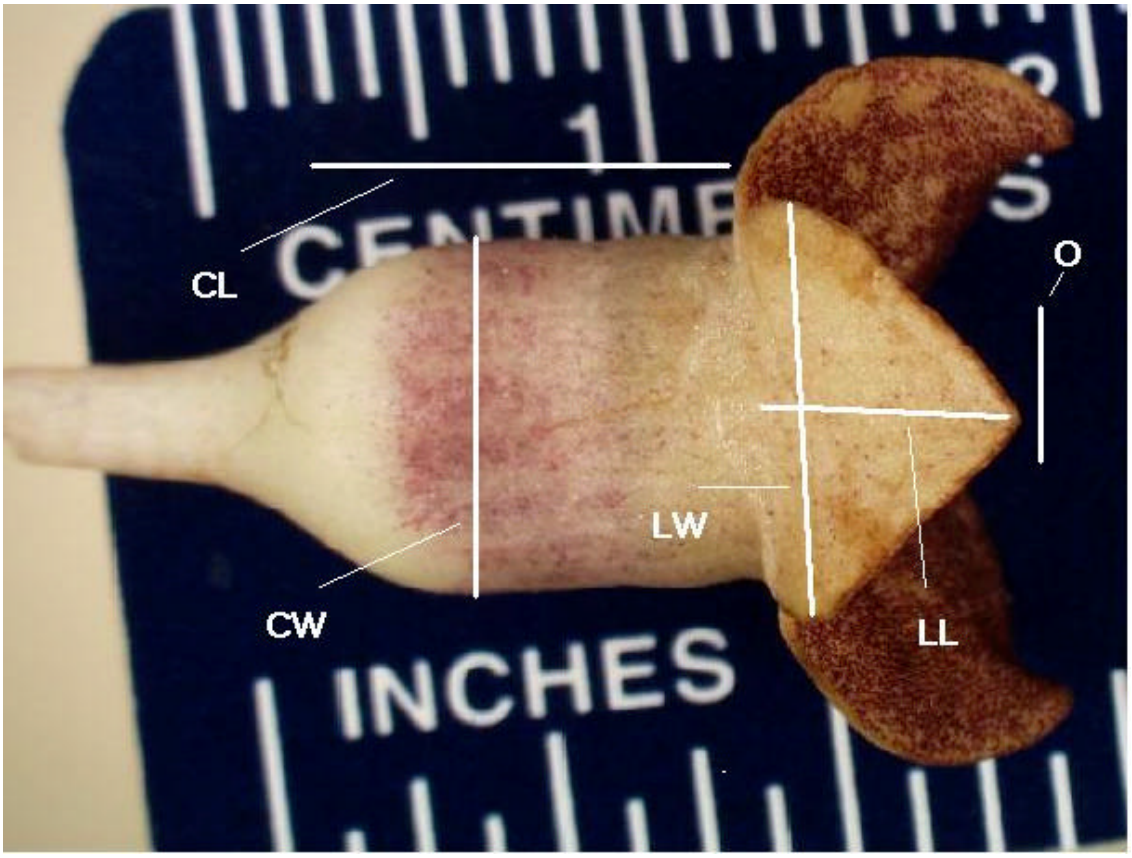
Federal, State and local (State and County Parks and Natural Areas) permits (Appendix E) were obtained to collect plants from localities across the range of *H. naniflora*. Flowers were collected from at least three individuals from eighty-five different *Hexastylis* populations in North Carolina, South Carolina, and Virginia (listed in Appendix B). Sixty-five of these populations were putative *H. naniflora* populations and 20 were *H. heterophylla* or *H. minor* populations. The collected flowers were placed into freezer bags with collection data placed inside the bag with the flowers as well as marked on the outside of the bag. The flowers were placed on ice until they could be stored in a refrigerator at ASU. The flowers were later removed from the refrigerator and five flower measurements were recorded for one flower from each individual. The measurements included calyx length (CL), calyx width (CW), calyx lobe length (LL), calyx lobe width (LW), and calyx opening (CO) (Figure 2). Measurements were recorded for 274 flowers from the eighty-five populations of the three species in the *H. heterophylla* complex. All the measurement data was compiled and the data were subjected to statistical analyses using Statistical Analysis Systems (SAS) (Delwiche and Slaughter 2000).

Materials collected for the morphological and micromorphological analyses consisted of flower materials collected from all three species in the *Hexastylis heterophylla*

complex. After being measured, they were either placed in a -80 freezer for future use or placed in a herbarium dryer at 29^{o} C (84.2^{o}F) to be used for pollen analysis.

Inner calyx reticulations have been examined in several studies and determined to be useful distinguishing characters to delineate some of the species of *Hexastylis*. These reticulations had been thought to be of taxonomic value in the *Hexastylis heterophylla* complex (Blomquist 1957; Gaddy 1987). Most *Hexastylis* species possess a series of ridges and reticulations, in the lower portions of the inner calyx tube of the flowers (*Hexastylis arifolia* does not possess them). Flowers taken from the eighty-five populations *H. heterophylla*, *H. minor*, and *H. naniflora* were examined using an Olympic SZX12 dissection scope and a DF PLFL 0.5X PF lens. Photographs were taken using an Olympic DP10 digital camera mounted on this dissecting scope. The inner calyx regions were photographed to compare the ridges and reticulations among the three species.

Figure 2. Flower measurement taken and used for morphological analysis in the *H. heterophylla* complex species. Measurements taken were Calyx length (CL), Calyx Width (CW), Calyx Lobe Length (LL), Calyx Lobe Width, (LW), and Calyx Opening (CO).


CL
O
LW
CW
LL
INCHES

Pollen Micromorphology

Pollen was obtained from fresh flower material, as well as from dried specimens. To compare pollen within the Heterophylla complex and within the genus, one to three flowers were collected from 24 individuals (Appendix B, populations indicated with asterisk) from the three species in the Heterophylla complex and 13 individuals from the other species in the genus (37 total specimens). Flowers were placed into separate plastic collection bags to avoid contamination from other flowers. The flowers were kept on ice while in the field and then transferred to refrigeration until they could be dried. The bags were placed into a plant dryer at 29^{o} C (84.2^{o} F) for three to five days to dry. After the flowers were dried, they were placed separately into paper envelopes with collection data recorded on the outside of each envelope. All 37 specimens were deposited at the Appalachian State University Herbarium (BOON).

The pollen was extracted and placed onto aluminum stubs, which were prepared by adding two-sided carbon tape to the top surface of each stub. The pollen was extracted by one of two methods. One was by the use of a miniature brush constructed from a toothpick with the bristles of a paintbrush attached to the end with scotch tape. The other method of pollen extraction was to remove one anther, and spread pollen from the anther over the stub and carbon tape.

Each stub was labeled separately by using a probe and etching an identification number into the carbon tape. They aluminum stubs were placed on a turntable mounted in the vacuum chamber of a FEI Philips Quanta 200 low/high vacuum SEM. Six stubs were loaded at one time for examination in the SEM.

A digital camera mounted inside the vacuum chamber of the SEM was used to acquire images of the pollen grains. Digital photos were taken of the pollen. We examined the specimens at low vacuum mode. We used a wide range of magnifications in order to obtain a variety of images. Magnifications ranged from 1000X to 5000X. Images captured between 2400X or 3000X were used to make size comparisons between pollen grains as well as to compare surface features of the pollen examined. The digital photos were collected on a computer hard drive linked to the digital camera and the images were transferred to a CD-ROM for analysis and examination.

Vegetation Survey

We examined thirteen population sites in North Carolina and South Carolina using the Carolina Vegetation Survey (CVS) methodology (Peet et al. 1998) to compare species richness between the three species of the *Hexastylis heterophylla* complex (Table 3). A total of seven *Hexastylis naniflora* population sites were examined from across the range of the species. Three population sites of *H. minor* and three population sites of *H. heterophylla* were also examined to compare species richness among the three species in the *H. heterophylla* complex. The 50 X 20 meter plots were established at each of these thirteen sites. Location data was recorded from the centerline of the 50 x 20 meter plot using GPS. Permanent markers were placed in eleven of the thirteen plots. In the two plots surveyed within the Pisgah National Forest, Madison County, North Carolina, no permanent markers were installed, but GPS plot locations were obtained (Table 3).

The 50 x 20 meter plots were constructed within the *Hexastylis* populations using five 50-meter measuring tapes. The corner of the plot was marked with a flag, as was the

centerline of the plot. Ten 10 x 10 meter plots in two rows of five plots each were nested within each 50 X 20 meter plot (Figure 3). Four of these ten plots were then used for intensive data collection (indicated as an I in Figure 3).

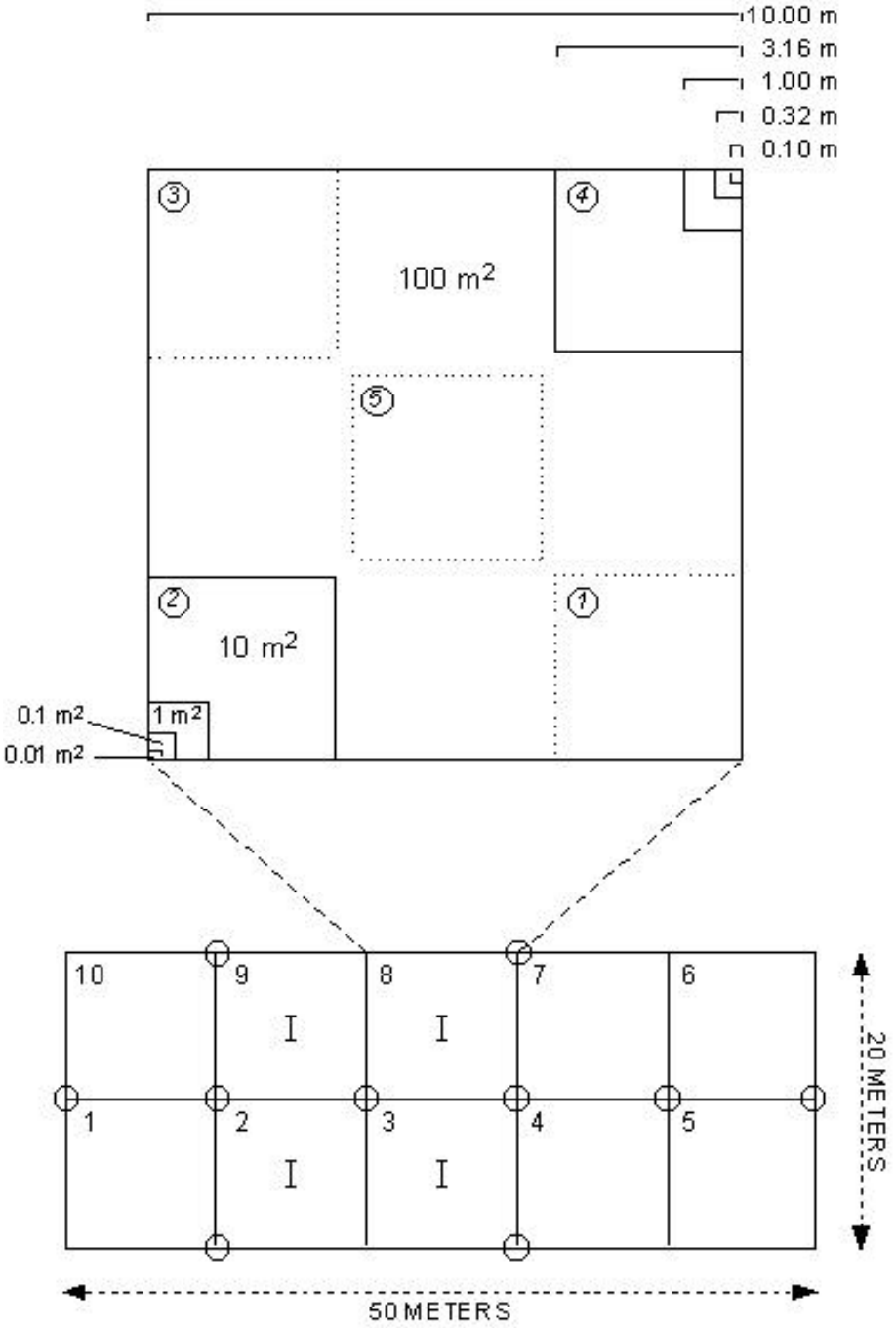
Two sites were sampled with a modified Carolina Vegetation Survey methodology where we established a single row of five 10 x 10 meter plots. These two sites had only two intense plots (indicated as I in Figure 3) for data collection. The reason for these two reduced plots was due to area constraints where the population sites were too small to fit a 50 x 20 meter plot, so a 50 x 10 meter plot was used instead. This methodological alteration is in line with the parameters set forth by Peet et al. (1998) to deal with smaller areas of analysis.

Within specified corners of the intense plots a series of nested plots were established, as indicated in Peet et al (1998). Vegetation data was collected from those nested plots. The nested plots were 0.10 meter, 0.32 meter, 1.0 meter, 3.16 meters, and 10.0 meters square. Species in these nested plots were assigned values from 5 in the smallest nested plot of 0.10 meter to 1 in the largest nested plot of 10.0 meters. Those values were assigned to a species when it was first observed in the series of nested plots (Figure 3). This is an importance value assigned to the species according to its first occurrence within the nested plots, and should not be confused with percent cover (also recorded as a second step in the sampling methodology). The CVS methodology requires identification of every species within each of the four intensively sampled 10 X 10 plots and within the larger 20 X 50 meter plots.

Table 3. Locations (with coordinates) where Carolina Vegetation Survey (CVS) analyses were conducted. Species are denoted as *H. heterophylla* (HH), *H. minor* (HM) and *H. naniflora* (HN).

Species	Location	Coordinates (D/M/S/)
HH	Appalachian Trail, Hot Springs Madison County, NC	35° 54' 01" N 82° 47' 40" W
HH	Hickey's Fork, Shelton Laurel Madison County, NC	35° 59' 31" N 82° 42' 10" W
HH	Bunker Hill Bridge, Claremont Catawba County, NC	35° 43' 12" N 81° 06' 57" W
HM	Broad River Greenway Cleveland County, NC	35° 12" 01" N 81° 39' 24" W
HM	Crowder's Mountain State Park Gaston County, NC	35° 13' 00" N 81° 17' 29" W
HM	Kings Mountain State Park York County, SC	35° 09' 01" N 81° 20' 26" W
HN	Henry Miller Bridge Road Alexander County, NC (HN 59)	35° 31' 34" N 81° 03' 22" W
HN	Little Gunpowder Creek Caldwell County, NC (HN 44)	35° 45' 09" N 81° 26' 21" W
HN	Kudzu Farm, Harris Rutherford County, NC (HN 10)	35° 14' 04" N 81° 53' 54" W
HN	Dan Rivers Property, Harris Rutherford County, NC (HN 63)	35° 13' 02" N 81° 52' 48" W
HN	Rhyne Preserve, Laboratory Lincoln County, NC	36° 26' 09" N 81° 14' 55" W
HN	Cowpens National Battlefield Cherokee County, SC	35° 07' 37" N 81° 48' 34" W
HN	Peters Creek Preserve Spartanburg County, SC	34° 59' 52" N 81° 52' 00" W

Figure 3. A typical ecological assessment plot (identified as a CVS site in this report) used with the Carolina Vegetation Survey (CVS). The large plot is 50 X 20 meters in overall size and is divided into ten 10 m^2 nested plots. Four of these 10 X 10 m^2 plots are intensively sampled in this methodology.



Peet, R.K. et al, 1998 (used with permission)

Percent cover data for each species found within each of the 10 X 10 meter plots was collected and assigned a number from 0-9 with 0 being the smallest cover class representing the smallest percent coverage and 9 representing the largest percent cover. Percent cover data was factored in to determine species richness. These data were recorded on a data sheet using the values obtained from the CVS. Species found outside the intense plots (but within the 20 X 50 meter plot) were recorded as residuals and entered into the datasheet.

The data were analyzed in a series of SAS statistical programs. The resulting data for species richness was then used in another series of SAS statistical programs along with other data in a Principle Components Analysis (PCA).

Species richness was used to obtain Sorenson index values, which were used to create a dendogram that showed the differences in the plots by species numbers. The following calculations were used to calculate Sorenson's Index of Community Similarity and Coefficient of community. The calculations were obtained from Communities and Ecosystems, second edition (Whittaker, 1975).

$$C_s = 2j / A+B \qquad C_n = 2N_j / N_A + N_B$$

C_S = Community Similarity **C_n= Coefficient of Community**
2j = Species Common To Both **$2N_j$= Number Species Common To Both**
A = Species In Community A **N_A= Number Species In Community A**
B = Species In Community B **N_B= Number Species In Community B**

Soil Analysis

Thirty-four soil samples were collected from North Carolina, South Carolina, and Virginia where *H. naniflora, H. heterophylla,* and *H. minor* localities between August 2001 and February 2003 (Table 4). Samples were collected using a standard 1" soil augur. The soil was collected from a mid-point within a population. Samples were collected to a depth of twelve inches and were placed into either a plastic 1" soil tube and sealed with a cap on each end, or the sample was placed into a new plastic storage bag, sealed and tagged.

Soil samples were taken to the ASU herbarium and placed into the plant dryer and allowed to dry slowly at around 29^{o}C (84.2^{o} F). Once the samples were dry, the tubes/bags were re-labeled on the outside and tags were placed inside the bags with collection information. They were then re-sealed and sent to the Clemson Soil Lab for analysis. Standard soil tests were performed on the collected samples. This analysis tested pH, Buffer pH, Phosphorous (P), Potassium (K), Magnesium (Mg), Zinc (Zn), Manganese (Mn), Copper (Cu), Boron (B), Sodium (Na), Cation Exchange Capacity (CEC), Acidity, Base Saturation for Magnesium (BSMg), Base Saturation for Potassium (BSK), Base Saturation for Sodium (BSNa), Total Base Saturation (TBS).

The results from the soil test were placed into a standardized form and entered into a SAS program, where statistical analyses were preformed. Soil data were analyzed using a General Linear Model (GLM) and Tukey's test.

Table 4. Soil samples collected and sent to Clemson Soil Lab for testing. *H. heterophylla* (HH) = 12, *H. minor* (HM) =7, and *H. naniflora* (HN) = 15.

COUNTY	STATE	SPECIES	LOCATION
CALDWELL	NC	HH	HWY 60/90 JUST ACROSS COUNTY LINE
CATAWBA	NC	HH	BUNKERHILL BRIDGE OFF US 70
IREDELL	NC	HH	HUNTER BRIDGE ROAD AT YADKIN RIVER
MADISON	NC	HH	HICKEY'S FORK ROAD IN NATIONAL FOREST
MADISON	NC	HH	OFF US 25 IN HOT SPRINGS ALONG AT
RUTHERFORD	NC	HH	LUCKADOO MT ROAD SITE 1
RUTHERFORD	NC	HH	LUCKADOO MT ROAD SITE 2
RUTHERFORD	NC	HH	PLEASANT MT CHURCH ROAD IN GOLDEN VALLEY
RUTHERFORD	NC	HH	CAMP McCALL ROAD SITE 1 OFF US HWY 226
RUTHERFORD	NC	HH	CAMP McCALL ROAD SITE 2 OFF US HWY 226
RUTHERFORD	NC	HH	OLD CC ROAD IN GOLDEN VALLEY
BUCHANNAN	VA	HH	OFF ROAD 628 ALONG CREEK
CLEVELAND	NC	HM	BROAD RIVER GREENWAY
CLEVELAND	NC	HM	BROAD RIVER GREENWAY SOUTH SIDE OF RIVER
GASTON	NC	HM	CROWDERS MOUNTAIN STATE PARK
MOORE	NC	HM	OFF US HWY 1 IN SOUTHER PART OF COUNTY
RANDOLPH	NC	HM	UHARRIE RIVER NEAR UWHARRIE GAME LANDS
RICHMOND	NC	HM	HWY 22 ALONG RIVER BANK
YORK	SC	HM	KINGS MOUNTIAN STATE PARK
ALEXANDER	NC	HN 59	OFF HWY 64 ON HENRY MILLER BRIDGE RD
BURKE	NC	HN 101	WILL HUDSON ROAD SR 1090 AT CREEK
CALDWELL	NC	HN 44	LITTLE GUNPOWDER CREEK OF HWY 321
CLEVELAND	NC	HN 100	HOUSLER PROPERTY, SANDY RUN CREEK
RUTHERFORD	NC	HN 10	KUDZU FARM SITE
RUTHERFORD	NC	HN 63	DAN RIVER PROPERTY NEAR POND HARRIS NC
RUTHERFORD	NC	HN 09	HENSON RAVINE OFF SR1106
RUTHERFORD	NC	HN 54	JEBB LAMB ROAD
RUTHERFORD	NC	HN 56	HENSON ROAD OFF HWY 221 AT FLOYDS CREEK
RUTHERFORD	NC	HN 52	HENSON RAVINE NORTH SIDE OF RIVER SR 1106
RUTHERFORD	NC	HN 62	DAN RIVER PROPERTY ACROSS CREEK
RUTHERFORD	NC	HN 181	DILLS CREEK TRIBUTARY
CHEROKEE	SC	HN	COWPENS NATIONAL BATTLEFIELD
SPARTANBURG	SC	HN	PETERS CREEK PRESERVE

Molecular analysis

DNA Extraction

DNA extractions were performed using quarter-sized samples of frozen leaf material that was macerated using liquid nitrogen with a cold mortar and pestle. The DNA was extracted using the CTAB micro-extraction protocol developed by Torsten Eriksson, 1994 (pers. comm.). The powdered samples were added to an 800 uL solution of 2X CTAB, 1 % PVP, 1 % sodium bisulfite, with 1.60 uL of 0.2 % BME added prior to mixing the solution in a 1.5 mL microcentrifuge tube. The samples were incubated in a 60°C water bath for 30 min. Then, 550 uL of a 24:1 chloroform: isoamyl alcohol solution was added, mixed by inverting 3-4 times followed by de-capping to allow ventilation, and centrifuged for 5 min at 20,800 x g in an Eppedorf Centrifuge (5810). The top (aqueous) layer was removed to another 1.5 mL microcentrifuge tube with 500 uL of a 24:1 chloroform: isoamyl alcohol solution, inverted to mix and de-capped for ventilation, then centrifuged for 5 min at 20,800 x g. The aqueous layer was removed, added to 400 uL of cold isopropanol, inverted to mix, and placed in a -20°C freezer overnight. The frozen samples were thawed and then centrifuged for 15 min at 20,800 x g. The supernatant was discarded, and the pellet was dissolved in 200 uL of 1X TE buffer, inverted, and incubated in a 37°C water bath for 30 min. Twenty uL of ammonia acetate and 600 uL of 100 % ethanol were added, the tubes inverted to mix, incubated in the -20°C freezer for 10 min and then centrifuged for 5 min at 20,800 x g. The supernatant

was discarded, and the pellet was dissolved with 200 uL of 1X TE buffer then incubated for 30 min in a 37°C water bath. Twenty- uL of 2.5 M sodium acetate and 440 uL of 100 % ethanol were added. The tubes were inverted to mix, incubated in the -20°C freezer for 10 min, and centrifuged for 5 min at 20,800 x g. The supernatant was discarded, and the pellet was covered with 500 uL of 70 % ethanol then centrifuged for 5 min at 20,800 x g. The supernatant was discarded and the pellet was allowed to air dry for three hrs, and then resuspended with 100 uL of 1X TE and incubated at 37°C for 30 min, then stored at -20°C. Five uL of each extracted DNA solution was electrophoresed on a 1.0 % agarose gel run at 100 V for one hour for verification of DNA isolation. The gel was soaked in an ethidium bromide solution and visualized by an Alpha Innotech Digital Imaging and Analysis System (Alpha Innotech Corp., San Leandro, CA).

*trn*L Sequencing, Visualization and Analysis

PCR amplification was performed on the chloroplast *trn*L region using E and F (primer E – GGTTCAAGTCCCTCTATCCC and primer F - ATTTGAACTGGTGACACGAG) on 10 individuals. These primers, obtained from LI-COR, were tagged with an infrared dye (IRD). The automated sequencer detects IRD 700 and IRD 800 dyes, and the PCR reaction generated two sets of chain terminated fragments for simultaneous bi-directional sequencing. The PCR reaction was performed with one Ready-To-Go PCR bead, 7.5 uL of sterile water, 2.5 uL of 10 uM forward primer, 2.5 uL of 10 uM reverse primer, 12.5 uL of DNA template for each 25 uL

reaction (Amersham Pharmecia Biotech, Piscataway, NJ). Amplification was performed in a Perkin-Elmer Gene Amp 9700 PCR System using one hold at 94ºC for 5 min; 25 cycles of 94ºC for 1 min, 55ºC for 1 min, and 72ºC for 2 min; and 72ºC for 14 min then held at 4ºC (Perkin Elmer Biosystems, Foster City, CA). Five uL of each extracted DNA solution was run on a 1.0 % agarose gel in 1X TBE buffer at 100 V for 1.5 hrs stained in ethidium bromide and then visualized using the Alpha Innotech Digital Imaging and Analysis System.

Amplified DNA was cleaned using a YM-100 Microcon centrifugal filter device (Millipore Corp., Bedford, MA). Total PCR product with 450 uL of ultra pure water was added to the reservoir of the filter and centrifuged at 500 x g for 15 min. The filter was flipped then centrifuged at 1,000 x g for 3 min and stored at -20°C. The DNA concentration was determined by using a Hoefer TKO-100 fluorometer at 492 nm (Hoefer Scientific Instruments, San Fransico, CA). The equation pmoles/uL = (DNA concentration ug/uL)(10^6)/ (# of bases)(650) was used to determine the appropriate amount of DNA to add for sequencing (LI-COR Inc., Lincoln NE).

The amplified section of the chloroplast *trn*L gene was sequenced using the LI-COR Global Edition IR^2 System (LI-COR Inc., Lincoln NE). Amplification was performed using a Perkin-Elmer Gene Amp 960 Thermocycler and a USB thermosequenase kit (USB Corp., Cleveland, OH) following the procedure outlined by Estep (2002). Each reaction contained 2.5 mM of all four dNTP nucleotide mixes with 7 deaza-dGTP (Roche

Molecular Biochemicals, Indianapolis, IA), 2.0 uL of each of the 15 mM IR dye labeled primers, 2.0 uL of buffer, 2.0 uL of the USB thermo sequenase DNA polymerase, and the appropriate amounts of DNA template and water. Four uL of appropriate chain terminating dideoxy-nucleotide was also added. Cycle sequencing was performed in a Perkin-Elmer Gene Amp 9700 PCR System under the following conditions: one hold at 92ºC for 2 min; 30 cycles of 92ºC for 30 sec, 56ºC for 30 sec, and 70ºC for 1 min; and then held at 4ºC. Afterwards, 3 uL of LI-COR stop solution was added to each tube. Each reaction was heated to 92°C for 3 min to denature, then kept on ice until loaded on a sequencing gel.

Samples were run on a 5.5 % polyacrylamide gel using 41 cm glass plates and a 48-well sharks-tooth comb. The resulting sequences were examined using e-Seq DNA sequencing and analysis software (LI-COR Inc., Lincoln NE). AlignIR alignment software (LI-COR Inc., Lincoln, NE) was used to align the sequence information for the ten individuals.

ISSR PCR, Visualization, and Analysis

ISSR primers were obtained from The University of British Columbia Nucleic Acid - Protein Service (NAPS) Unit. To determine useful primers that produce variation, 62 primers were screened with 10 individuals from different populations. Ten variable primers were found and eight gave reproducible, scorable DNA fragments. Amplification was performed using a Sigma PCR Core Kit (CORE-T, Sigma Chemical Company). Forty-three individuals, from across the range of the species, were subjected

to PCR reactions for each of the eight variable systems. Each PCR reaction contained 3.4 uL of sterile water, 2.5 uL of 10X PCR buffer, 2.5 uL of 25 mM $MgCl_2$, 0.5 uL of dNTP mix, 0.2 uL of Taq DNA Polymerase, 3.4 uL of ISSR primer and 12.5 uL of target DNA. The 25 uL reaction was amplified using a Perkin-Elmer Gene Amp 9700 PCR System with the following protocol: 1.5 min at 94°C with 35 cycles of 40 sec at 94°C, 45 sec at 45°C, 1.5 min at 70°C followed by 4 holds at 94°C for 45 sec, 45°C for 45 sec, and 5 min at 72°C, and then held at 4°C.

Seven uL of the PCR product was mixed with 3 uL of tracking dye and separated by electrophoresis on a 1.5 % agarose gel in 1X TAE buffer at 30 V for 5 hrs, stained with ethidium bromide, and visualized by ultraviolet light using the Alpha Innotech Digital Imaging and Analysis System. Bands were compared to a 1 Kb ladder (D 0428, Sigma-Aldrich, Inc.).

Bands were scored manually based on presence or absence for each primer system. A data matrix was constructed using binary code for each primer system using Excel 2000 (1 for presence and 0 for absence with "?" for questionable bands). This matrix was entered into PAUP software using maximum parsimony and UPGMA algorithms to construct phenetic and phylogenetic trees (Phylogenetic Analysis Using Parsimony, version 3.1, Swofford).

Transplant analysis

In November 2000, representatives from Appalachian State University, the North Carolina Department of Agriculture, and the North Carolina Department of Transportation met at a NCDOT bridge construction site on Cedar Valley Road off of Hwy 321 near Saw Mills, in Caldwell County, North Carolina to transplant *H. naniflora* individuals from the bridge construction site onto an adjacent conservation easement established by the North Carolina Department of Transportation. The construction site included over two hundred *H. naniflora* plants that would have been destroyed during bridge construction if they were not moved.

The method used in transplanting individuals was developed by Dr. Gill Newberry (1996). The plants were dug up and placed into plastic one-gallon freezer bags. The freezer bags were used to avoid contamination from plant pathogens and to make sure that any beneficial bacterial components in the soil that might be associated with *H. naniflora* were transplanted along with the plants.

After the plants were extracted and placed into the freezer bags, they were transported to the easement site and placed into clusters for transplanting. The plants were then removed from the plastic freezer bags and placed into newly dug holes and replanted. Each transplanted individual was then marked with a flag for future reference. Once the freezer bag had been used once, it was discarded.

Over the next three-growing/flowering seasons, the site was revisited and data were collected and recorded on those individuals that had been transplanted, to determine

survival rate was for transplanted individuals. No data were recorded for non-transplanted individuals.

RESULTS

Biogeography

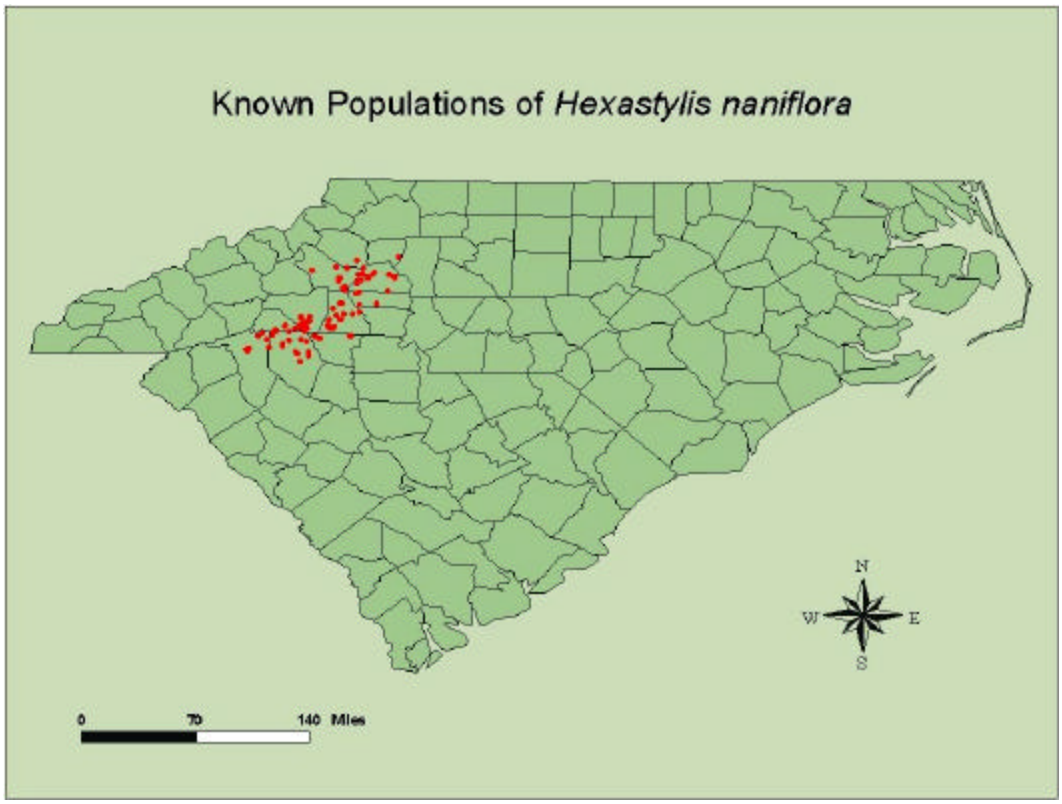
We examined and annotated a total of 693 specimens from seventeen herbaria. Ashe (1897) had reported that *Asarum minus* was located in Tennessee; however, from herbarium records of seventeen herbaria, no specimens of *Asarum minus* were found to be from Tennessee. We obtained information on *H. naniflora* that had been collected from Element of Occurrence (EOC) field sheets in North Carolina and South Carolina from the North Carolina Natural Heritage Program and the South Carolina Heritage Trust. We located thirty-one new *H. naniflora* populations over the three growing seasons (Figure 6), and this information was submitted to the Natural Heritage databases of North and South Carolina. We obtained GPS points for 123 existing *Hexastylis naniflora* populations. Some GPS points are recorded as two or more populations and the map represents a total of 143 populations. A map was generated from these data showing the known distribution of *H. naniflora* (Figure 4).

From the herbaria data collected we generated maps for the distributions of all three species in the *H. heterophylla* complex. Counties where considerable overlap occurs are denoted with color dots, which correspond to the species present in that county (Figure 5). Some of the information used to generate the distribution maps came from field collections, and these specimens were deposited in the Appalachian State University

herbarium (BOON) in Boone, North Carolina and Gardner Webb University herbarium (GWU) in Boiling Springs, North Carolina.

Over three flowering seasons, we located thirty-one new *H. naniflora* populations (Figure 6). A map generated using ArcView shows the localities of new *H. naniflora* population located over three growing seasons running from the spring of 2001 to the summer of 2003. We located one population of *H. naniflora* in the Yadkin River drainage. Previously, *H. naniflora* was only known to exist from the Broad-Pacolet and Catawba River drainages. After this initial discovery, we conducted numerous field surveys in the Yadkin River drainage in Iredell, Gaston, and Yadkin counties, but no other populations of *H. naniflora* were located. A map was generated that shows the distribution of populations of *H. naniflora* within the three river drainages (Figure 7).

Figure 4. Distribution maps showing the approximate locality of known or reported *H. naniflora* sites in North and South Carolina. All points were derived from GPS data.


Known Populations of *Hexastylis naniflora*
N
W
E
S
0
70
140 Miles

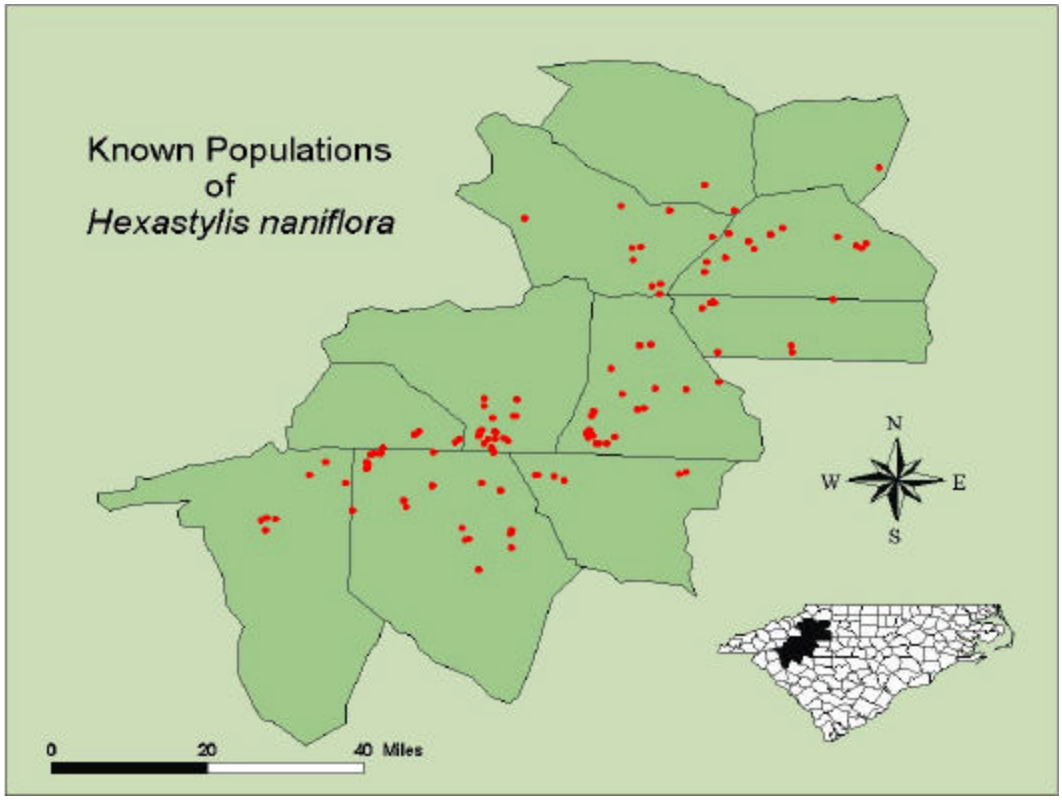

Known Populations
of
Hexastylis naniflora
N
W
E
S
0
20
40 Miles

Figure 5**.** Distribution map showing county records for the three species in the *H. heterophylla* complex. Data was gathered from herbarium specimens, Element Of Occurrence (EOC) sheets and field studies. Dots within *H. heterophylla* counties indicates co-occurrence with *H. minor*. Light dot within *H. naniflora* counties indicates co-occurrence with *H. heterophylla*, dark dot indicates co-occurrence with *H. minor*.

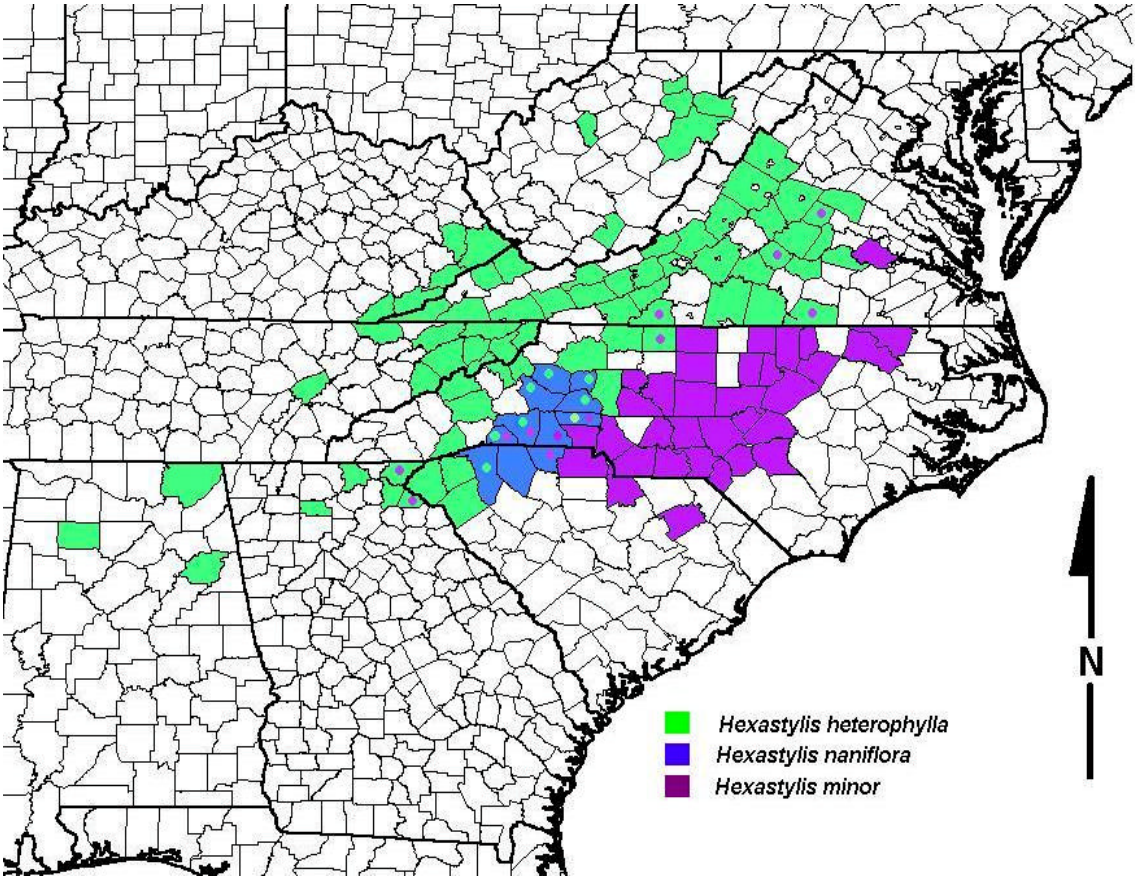

Hexastylis heterophylla
Hexastylis naniflora
Hexastylis minor
N

Figure 6. New populations of *Hexastylis naniflora* located during the field seasons of 2001-2003. Thirty-one new population and sub-populations were located between 2001-2003. Some populations are obscured due to scale of the map.

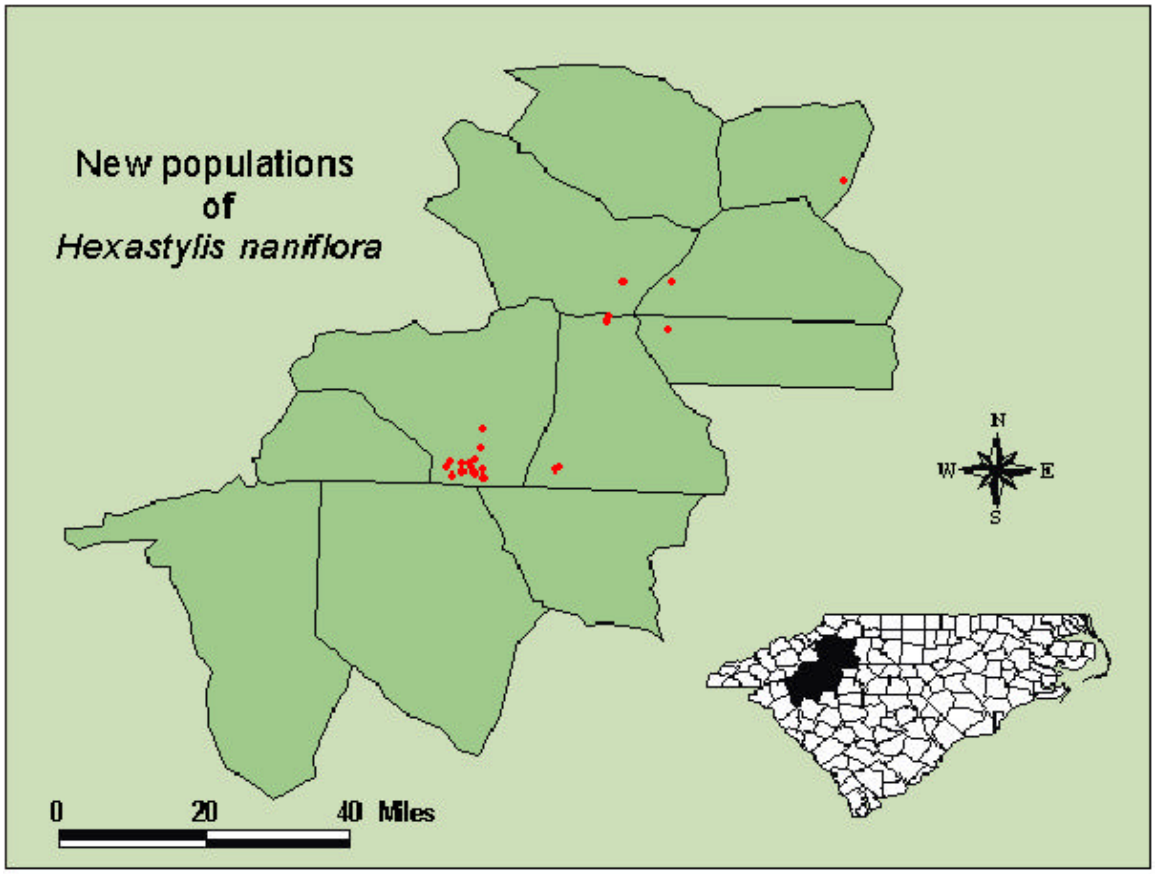
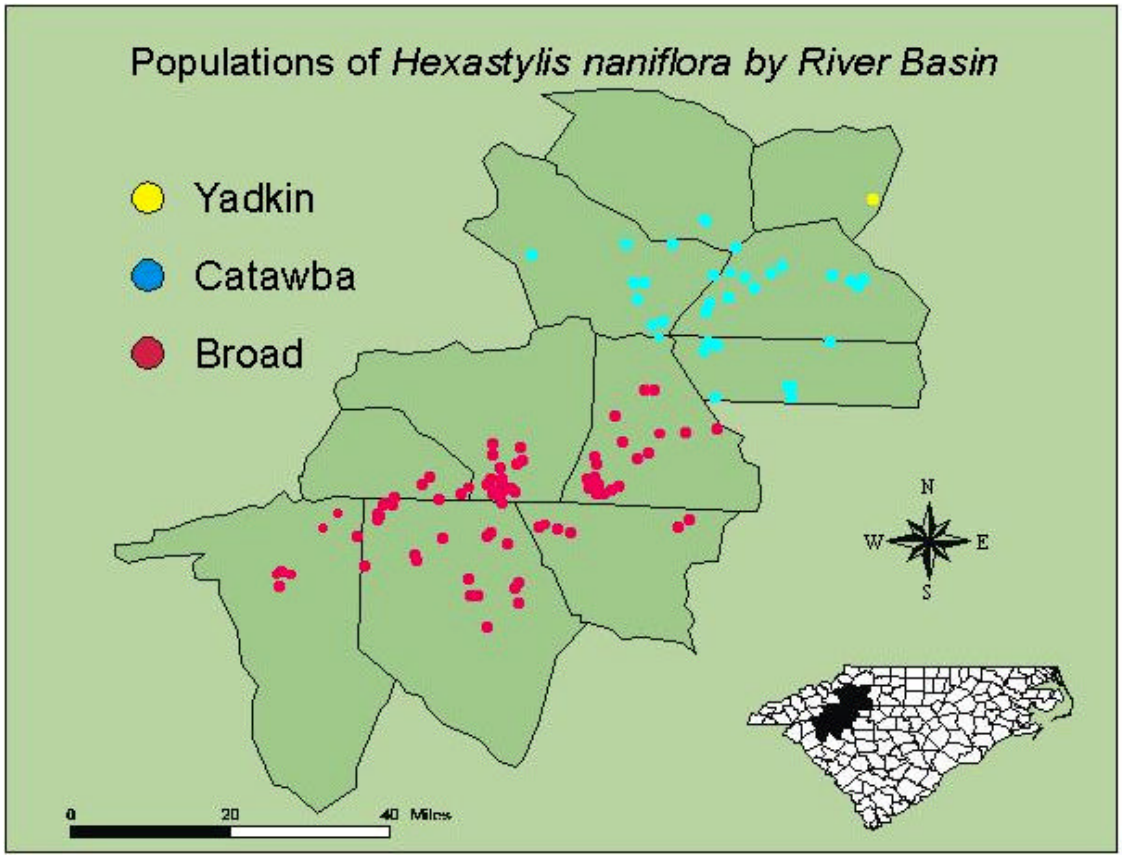

New populations
of
Hexastylis naniflora
N
W
E
S
0
20
40 Miles

Figure 7. Distribution map showing known sites of *H. naniflora* according to river drainage.


Populations of *Hexastylis naniflora* by River Basin
Yadkin
Catawba
Broad
N
W
E
S
0
20
40 Miles

Flower morphometrics

After the examination of fifty specimens representing the three species within the *Hexastylis heterophylla* complex, focusing on the inner calyx reticulations and ridges, it was determined that too many similarities existed among the three species of the *H. heterophylla* complex to accurately make species identification using inner calyx reticulations and ridges.

A univariate analysis of variance using flower measurements and compared across all three species in the complex was conducted. The results show that no statistical differences existed for a single species among the three species in the complex from either a GLM or a Tukey's test. The measurements generally followed those provided in The Flora of North America (Whittemore and Gaddy 1997).

A Principle Components Analysis (PCA) conducted using SAS showed that flower morphology can be used to separate *H. naniflora* from the other two species in the complex, but that separating *H. heterophylla* from *H. minor* was not possible due to the significant overlap that occurs in flower size measurement of the two species (Figures 8-11).

Figure 8. Principle Components Analysis (PCA) results comparing flower morphology measurements of *H. minor* (Circle), and *H. naniflora* (Cross).

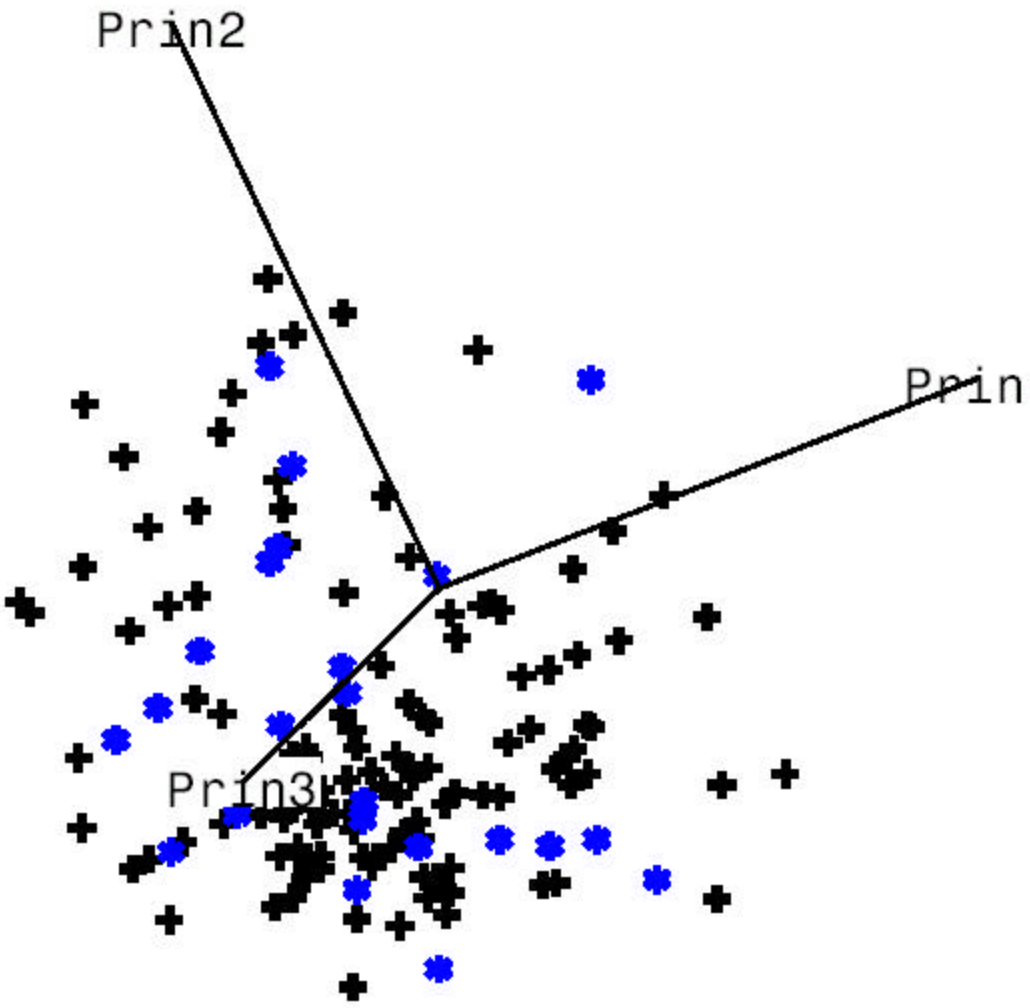

Prin2
Prin
Prin3

Figure 9. Principle Components Analysis (PCA) results comparing flower morphology measurements of *H. heterophylla* (Square), and *H. naniflora* (Cross).

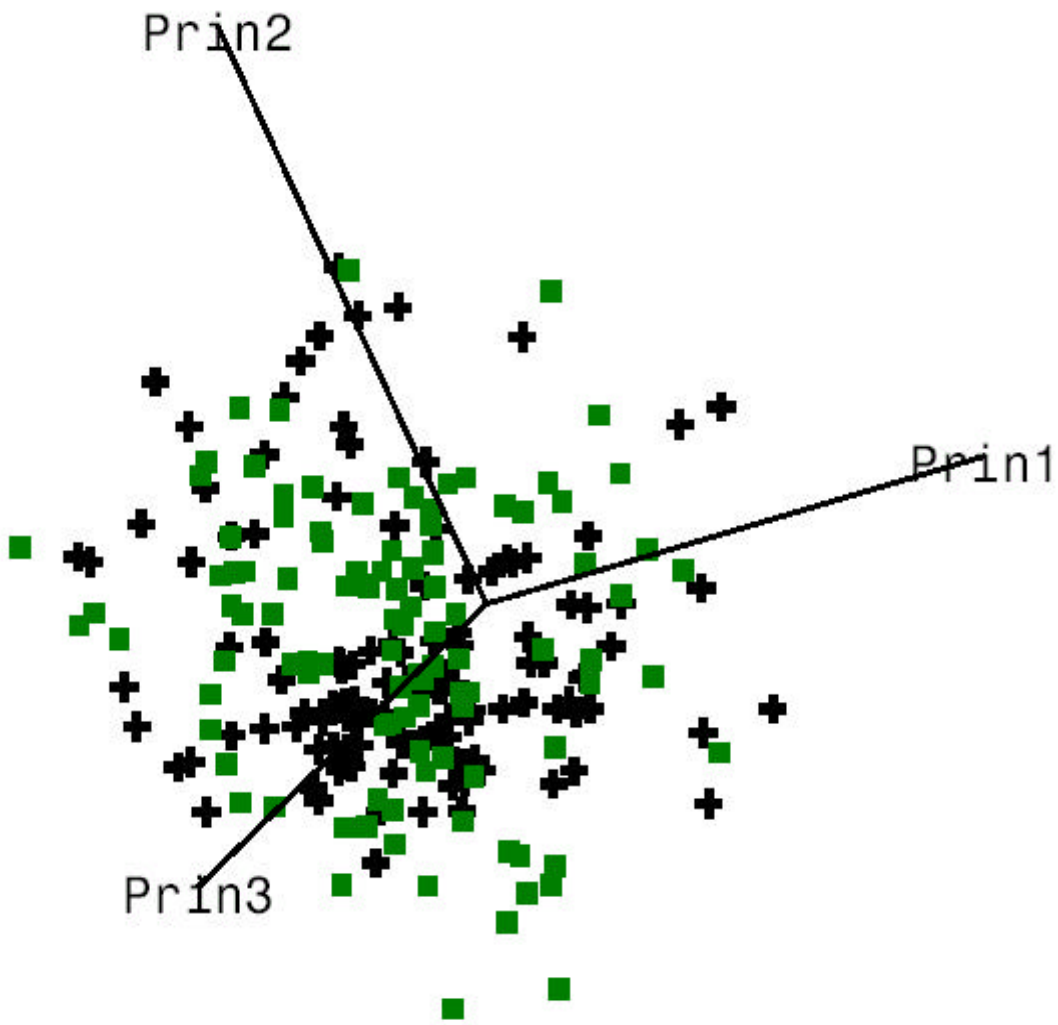

Prin2
Prin1
Prin3

Figure 10. Principle Components Analysis (PCA) results comparing flower morphology measurements of *H. heterophylla* (Square) *and H. minor* (Circle).

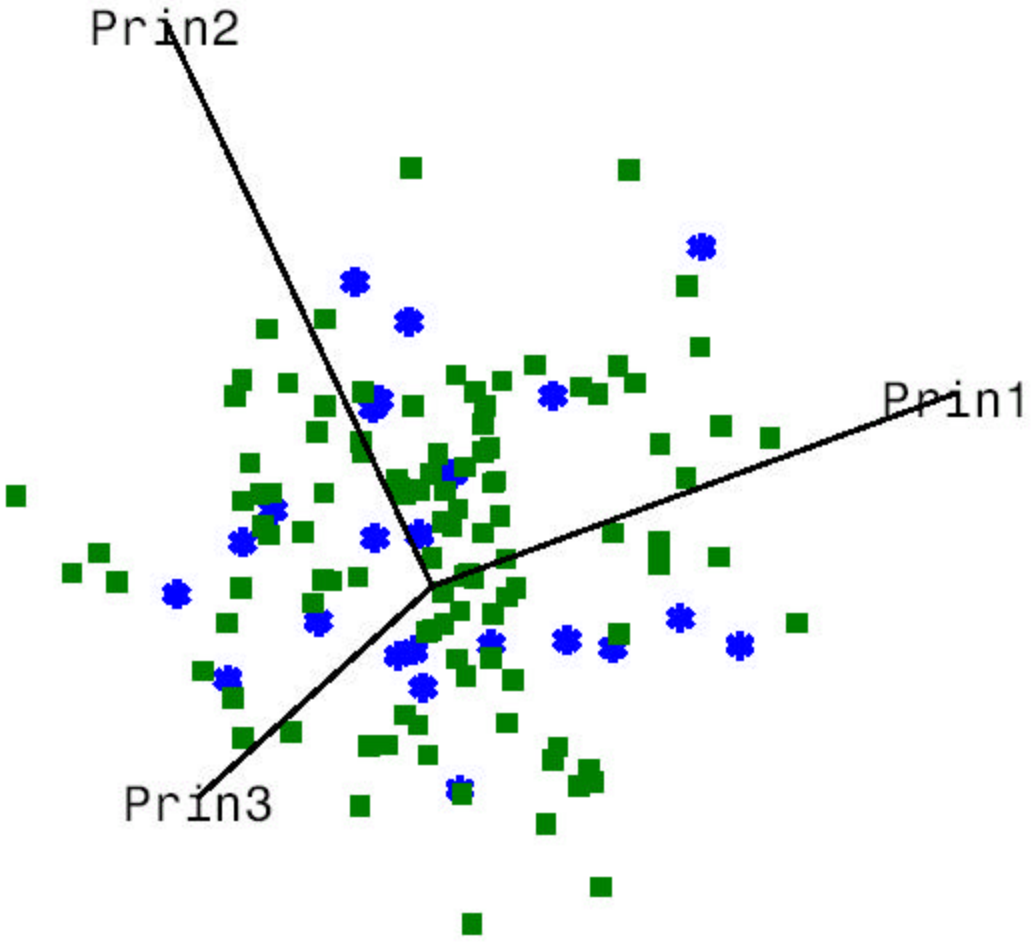
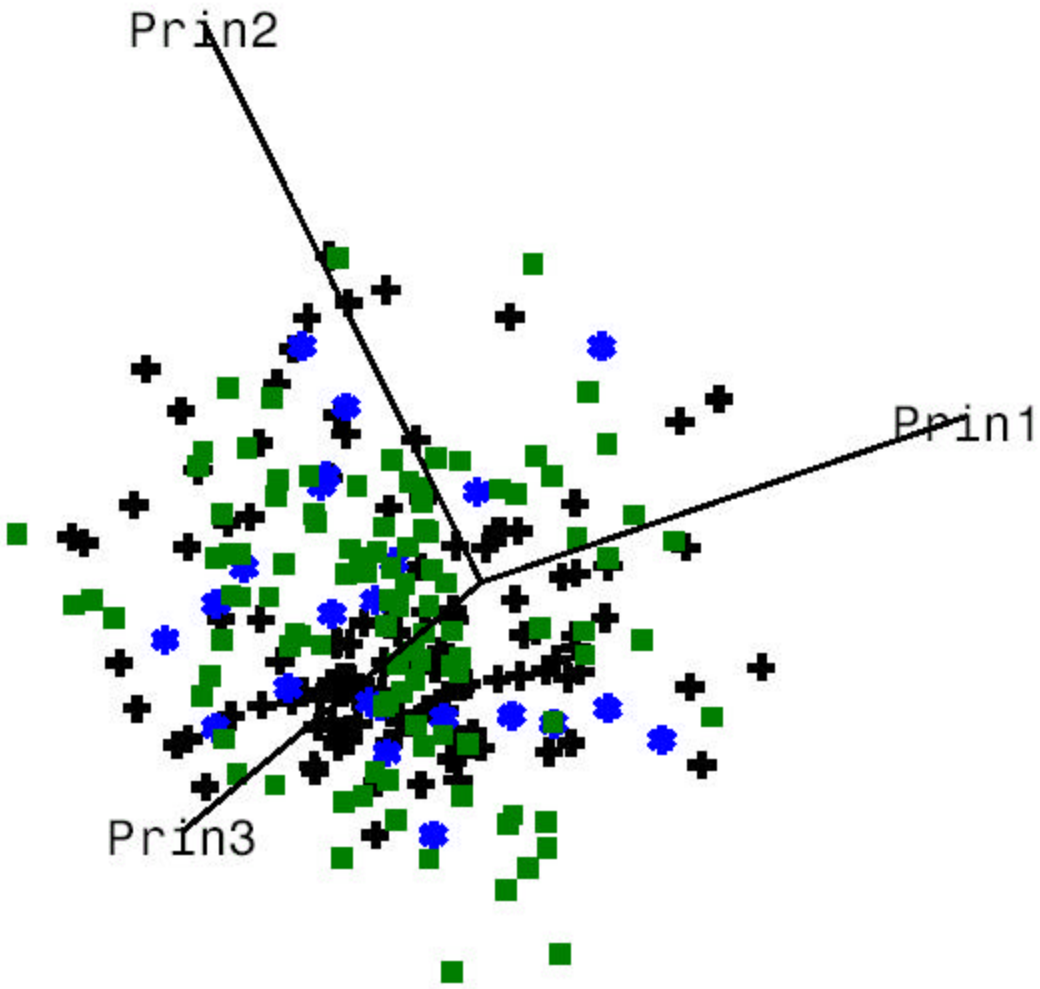

Prin2
Prin1
Prin3

Figure 11. Principle Components Analysis (PCA) results comparing flower morphology measurements of *H. heterophylla* (Square), *H. minor* (Circle), and *H. naniflora* (Cross).


Prin2
Prin1
Prin3

Pollen micromorphology

Results from digital images taken of pollen from the *H. heterophylla* complex using SEM showed differences in the surface features of pollen from the three species. *Hexastylis heterophylla* has an exine that contains both baculate and gemmate positive sculptural elements in high density where no flat or smooth surface area shows (Figures 12 and 13). *Hexastylis minor* has an exine of scattered gemmate sculptural elements. The remaining surface area visible on the exine appears smooth (Figure 12 and 14). *Hexastylis naniflora* has an exine that contains no positive surface elements and is rugulate in appearance along its surface (Figure 12 and 15).

Figure 12. Results from pollen analysis show that the exine from the three species in the *H. heterophylla* complex differs among the species. *Hexastylis heterophylla* (A), *Hexastylis minor* (B), and *Hexastylis naniflora* (C).

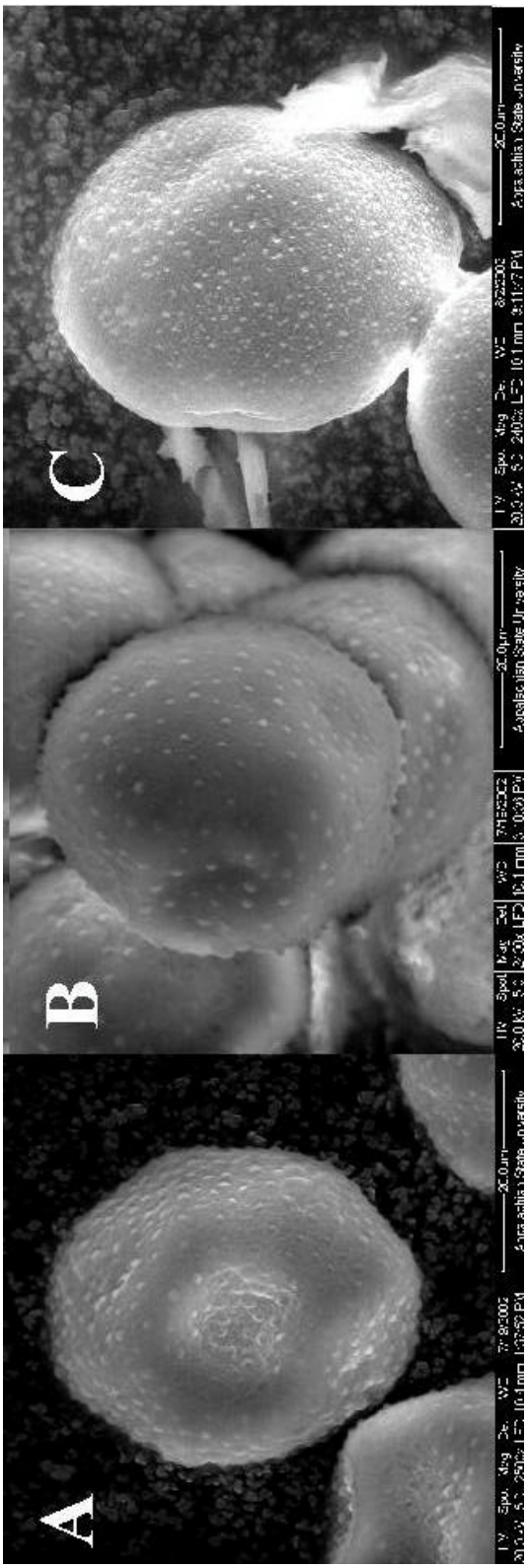

A
B
C

Figure 13. Close-up image of pollen from *H. heterophylla* showing it possesses many positive surface elements.

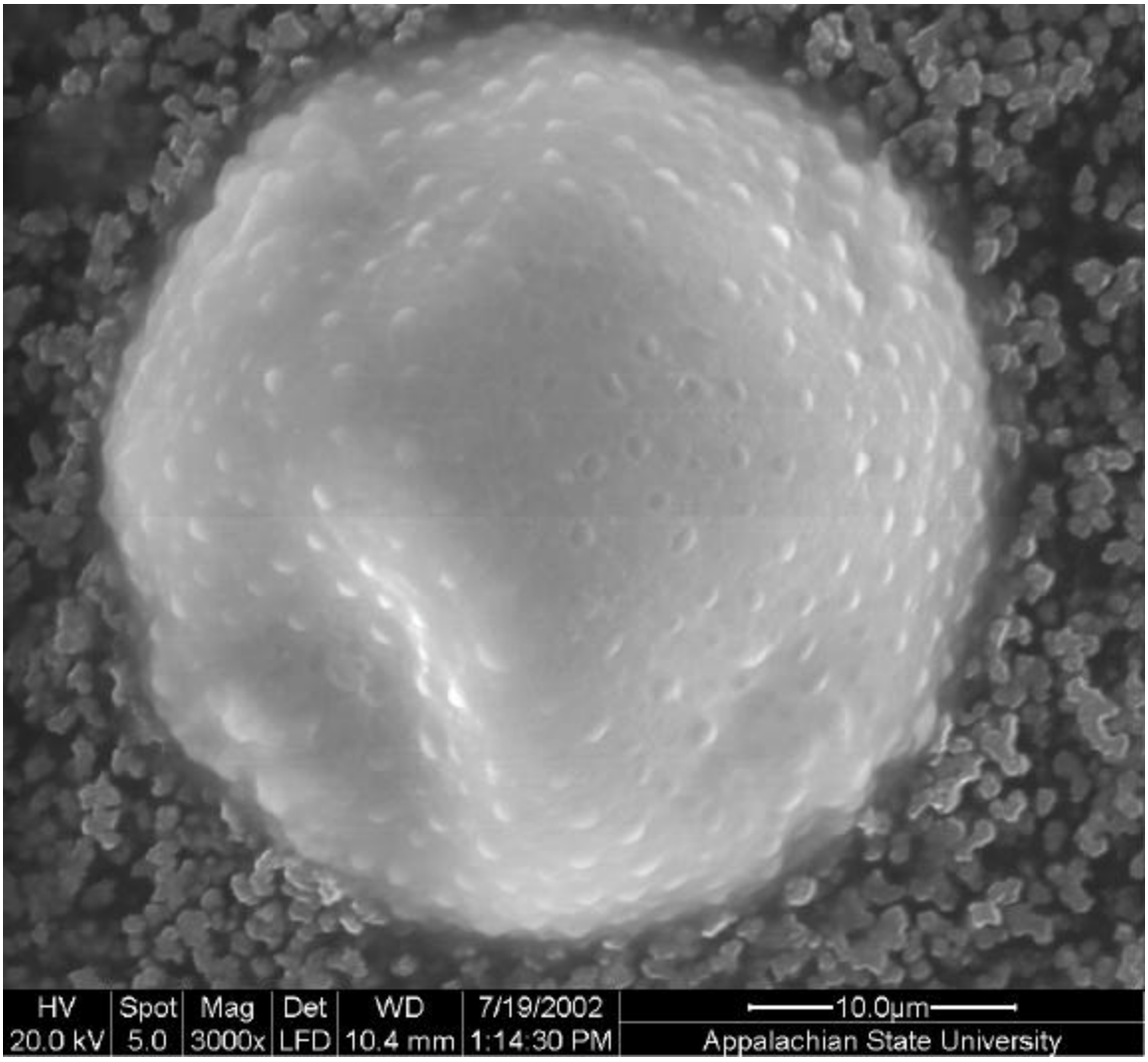

HV
20.0 kV
Spot
5.0
Mag
3000x
Det
LFD
WD
10.4 mm
7/19/2002
1:14:30 PM
10.0µm
Appalachian State University

Figure 14. Close-up of pollen from *H. minor* showing that it possesses positive surface elements, but fewer elements than *H. heterophylla*.

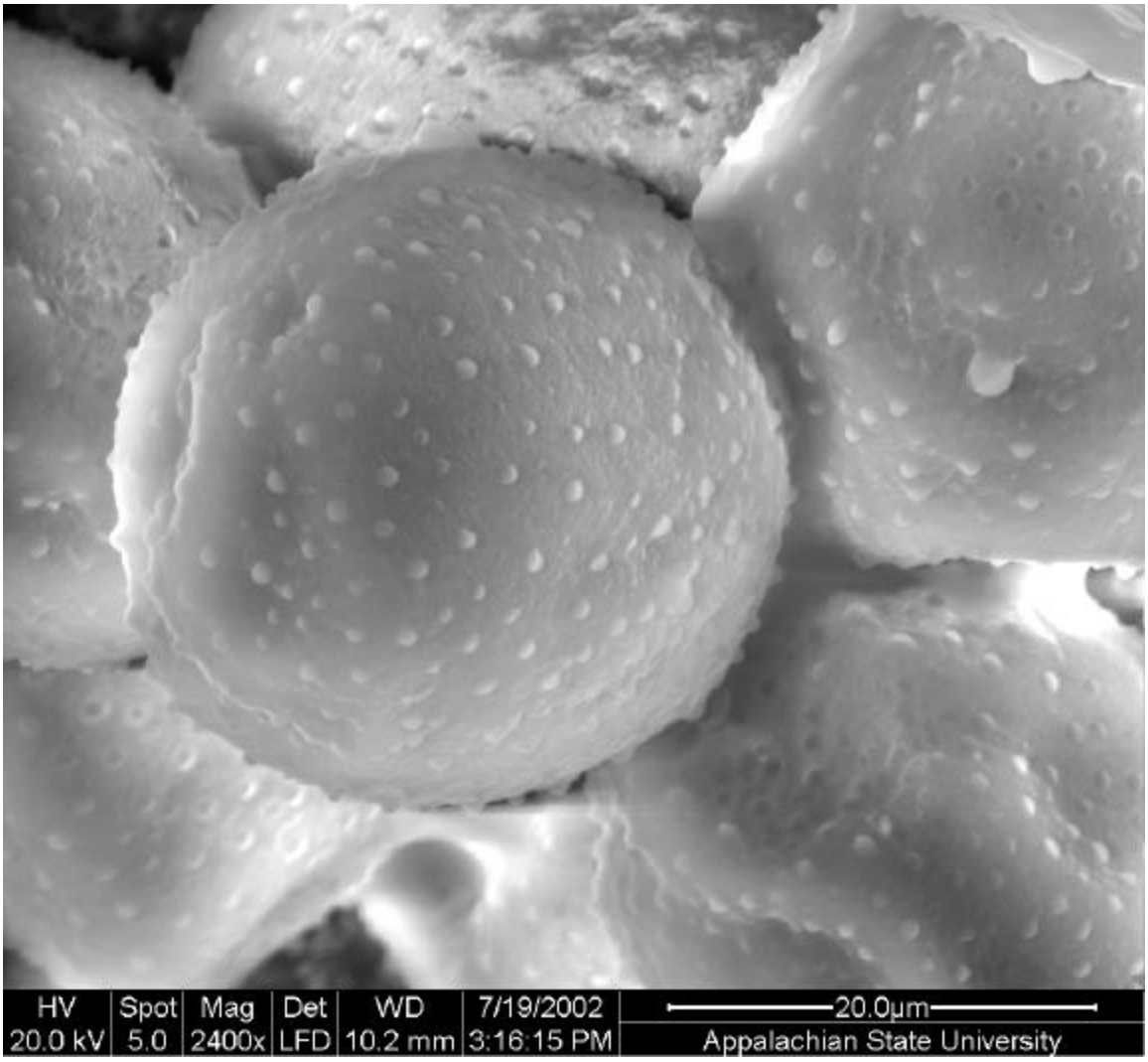
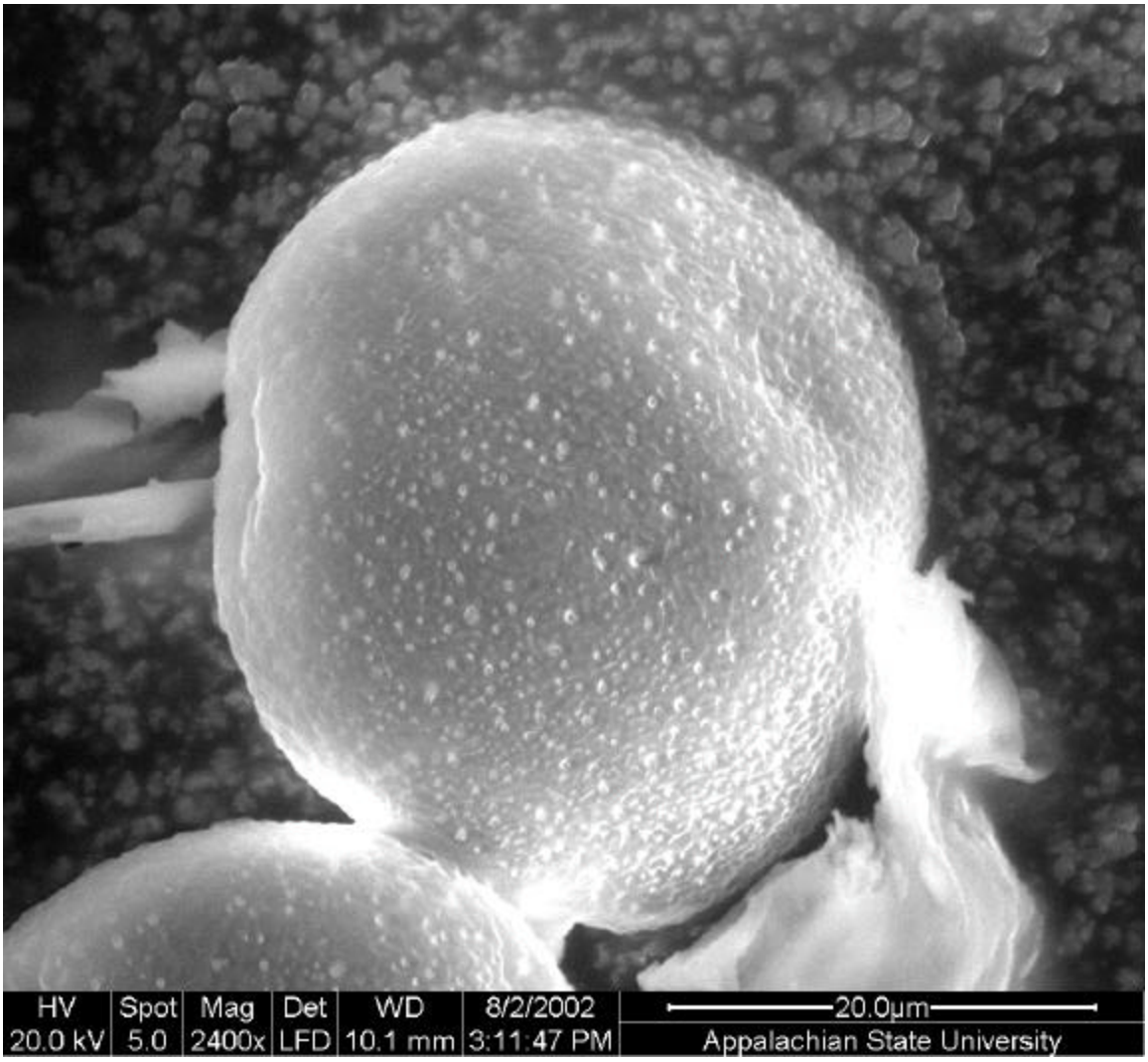

HV
20.0 kV
Spot
5.0
Mag
2400x
Det
LFD
WD
10.2 mm
7/19/2002
3:16:15 PM
20.0µm
Appalachian State University

Figure 15. Close-up of pollen from *H. naniflora*. No positive surface elements occur on the surface of the pollen, which is different from both *H. heterophylla* and *H. minor.*


HV
20.0 kV
Spot
5.0
Mag
2400x
Det
LFD
WD
10.1 mm
8/2/2002
3:11:47 PM
20.0µm
Appalachian State University

Vegetation Survey

Results from the species richness data collected using the CVS and the calculations from a series of SAS analyses are shown in Table 5. The Means at each Depth (DMS) (from 5 to 1; see Figure 3 for nested plots) of the four 10 X 10 meter plots sampled for each site are shown in Table 5. The DMS for each species at each nested plot (within the 10 X 10 meter plots) was subjected to a GLM analysis for each site surveyed using the CVS. The results showed no significant differences among the plots containing the three *Hexastylis heterophylla* complex species (Table 6).

The total number of associated species at the CVS sites for each species in the *H. heterophylla* complex was averaged and the results show that species richness is lowest for *H. heterophylla* at 46.3%, with an intermediate value for *H. naniflora* at 59.9%, and the highest value for *H. minor* at 62.3%. This part of the vegetation analysis is limited by the lack of inclusion of seasonal taxa, since the plots were only sampled once in the growing season. The results from the statistical analysis of species richness from the CVS showed no significance when comparing the nested plots between species (Figure 16).

Calculations were performed using the Sorenson's Index of Community Similarity and Coefficient of Community and the results are shown in Figure 17. With an index established we could then construct a dendrogram showing community similarity (Figure 18). This dendrogram shows that *H. heterophylla* and *H. minor* have more similar habitats and species richness. *Hexastylis heterophylla* is more variable in species richness across its range with the mountain populations exhibiting lower species richness,

whereas the Piedmont populations have a higher species richness. Therefore the Piedmont populations of *H. heterophylla* are found in habitats that are more similar to the habitats of *H. minor* and *H. naniflora*.

Species commonly associated with *H. naniflora*, *H. heterophylla*, and *H. minor* are shown in Figure 19. This information can be used to assess the potential for indicator species to locate new populations or to identify areas for transplants.

Table 5. Shows species richness Depth Means (DMS) at each level (5 - 1) of the nested plots and the total over-all species richness for the thirteen plots.

SPECIES	SITE	DMS 5	DMS 4	DMS 3	DMS 2	DMS 1	TOTAL SPECIES
HM	1	0.88	2.5	6.5	15.88	28.78	55
HN	2	1	2	3.88	7.5	16	50
HH	3	0.38	1.63	6.63	15.88	30.25	63
HN	4	0.25	0.5	2.23	5.13	11.5	41
HN	5	0.5	2.38	8	18.5	36	63
HN	6	1.13	3.38	9	18.38	39	69
HN	7	0.25	0.75	3.13	7.63	14.75	43
HN	8	0.38	2.13	6.38	13.5	28	48
HM	9	0.63	1.75	5.5	16.75	35.5	71
HH	10	0.5	1.63	5.63	12.75	21.75	41
HH	11	0.38	1.13	3.63	8.88	16.5	35
HN	12	0.5	2.13	6.75	16.5	32	55
HM	13	0.38	0.75	4.13	13.38	29.25	61

Table 6. **Comparison of the nested plots species richness with an alpha value at P< .05. None of the comparisons were significant.**

Plot	F Value	Pr > F
5	0.31	0.7397
4	0.12	0.8859
3	0.04	0.9588
2	0.63	0.5542
1	0.89	0.4392
Total	1.63	0.2437

Figure 16. Average species richness, based upon the thirteen CVS sites, for the three species in the *H. heterophylla* complex

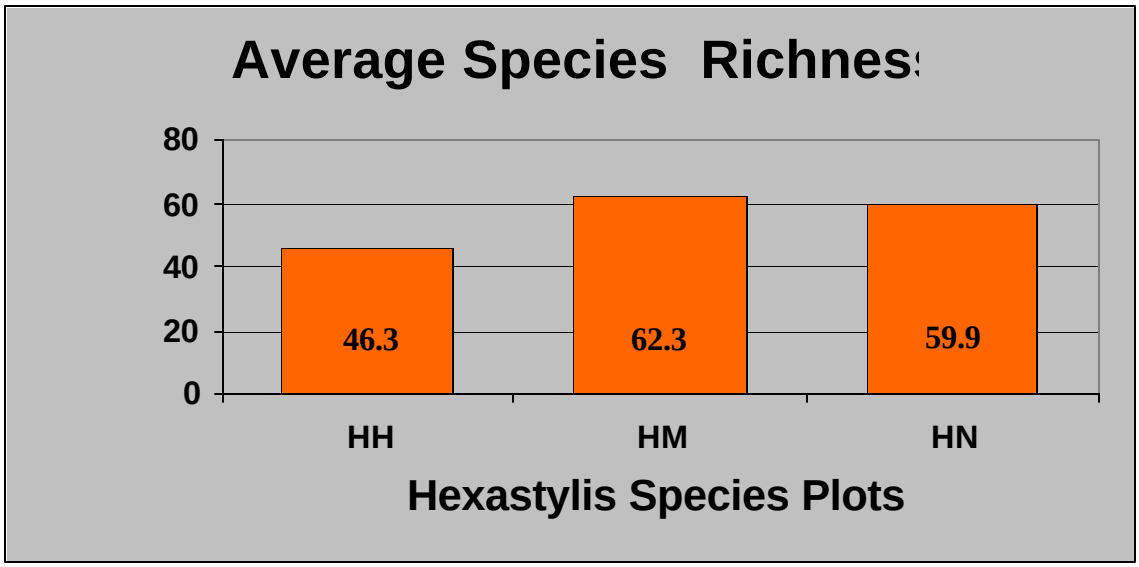

Average Species Richnes
80
60
40
20
0
46.3
62.3
59.9
HH
HM
HN
Hexastylis Species Plots

Figure 17. Results from calculations performed using Sorenson's Index of Community Similarity to compare the thirteen CVS sites. A-C = *Hexastylis heterophylla*, D-F = *Hexastylis minor*, and G-M = *Hexastylis naniflora*.

	A	B	C	D	E	F	G	H	I	J	K	L	M
A	-												
B	0.5	-											
C	0.4	0.2	-										
D	0.3	0.3	0.5	-									
E	0.3	0.2	0.6	0.5	-								
F	0.3	0.3	0.5	0.5	0.6	-							
G	0.3	0.3	0.6	0.4	0.6	0.5	-						
H	0.3	0.3	0.4	0.4	0.4	0.4	0.6	-					
I	0.3	0.3	0.6	0.5	0.4	0.5	0.4	0.4	-				
J	0.2	0.3	0.5	0.5	0.6	0.5	0.6	0.6	0.5	-			
K	0.3	0.3	0.5	0.6	0.6	0.7	0.6	0.5	0.6	0.5	-		
L	0.3	0.3	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.7	0.6	-	
M	0.3	0.3	0.4	0.4	0.4	0.5	0.4	0.6	0.6	0.5	0.5	0.5	-

Figure 18. Dendrogram showing community relationships between the three species in the *H. heterophylla* complex for the thirteen CVS sites. A-C = *Hexastylis heterophylla*, D-F = *Hexastylis minor*, and G-M = *Hexastylis naniflora*.

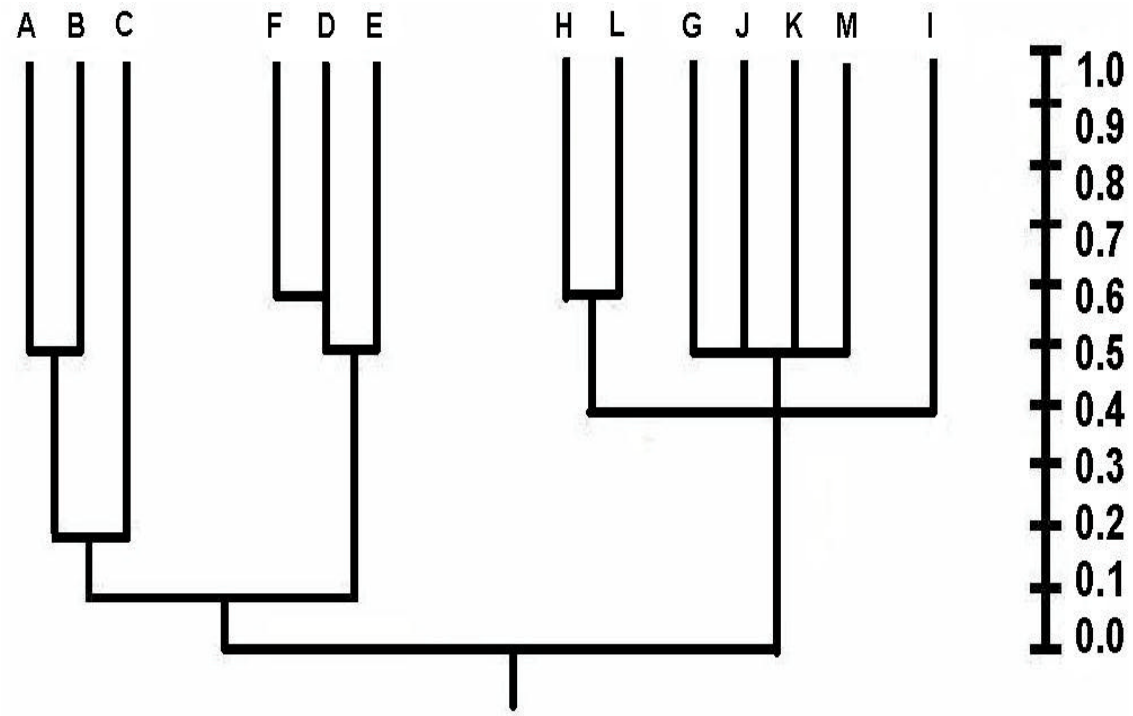

A B C F D E H L G J K M I
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

Figure 19. Graph showing species associates for the thirteen CVS sites. Abbreviations are as follows: ACERRUB = *Acer rubrum*, AMELARB = *Amelanchier arboreum*, BETUALE = *Betula allegheniensis*, CALAFLO = *Calycanthus floridus*, CAMPRAP = *Campsis radicans*, CARPCAR = *Carpinus caroliniana*, CARYGLA = *Carya glabra*, CARYTOM = *Carya tomentosa*, CORNFLO = *Cornus florida*, CORYCOR = *Corylus cornuta*, ILEXOPA = *Ilex opaca*, KALALAT = *Kalmia latifolia*, LECUAXI = *Leucothoe axillaris*, LIQUSTY = *Liquidamber styraciflua*, LIRETUL = *Lireodendron tulipifera*, METCREP = Mitchella repens, OXYOARB = *Oxydendrum arboreum*, PINEECH = *Pinus echinata*, PINESTR = *Pinus strobus*, QUERALB = *Quercus alba*, AUERCOC = *Quercus coccinea*, QUERPRI = *Quercus prinus*, QUERVEL = *Quercus velutina*, RHODMAX = *Rhododendron maximum*, SMIROT = *Smilax rotundifolia*, TSUGCAN = *Tsuga canadensis*, VITOROT = *Vitus rotundifolia*, QUERNIG = *Quercus nigra*, FAGUGRA = *Fagus grandifolia*, VACVAC = *Vaccinium vacillans*, POLYACR = *Polystichum acrostichoides*, HEXAHET = *Hexastylis heterophylla*, HEXNAN = *Hexastylis naniflora*, HEXAMIN = *Hexastylis minor*.

	HH	HH	HH	HN	HN	HN	HN	HN	HN	HN	HM	HM	HM
	AT	HYFK	BHB	ALEX	LGPC	KFS	DAN	RHY	COW	PCR	BRG	CMSP	KMSP
ACERRUB													
AMELARB													
BETUALE													
CALAFLO													
CAMPRAP													
CARPCAR													
CARYGLA													
CARYTOM													
CORNFLO													
CORYCOR													
ILEXOPA													
KALALAT													
LECUAXI													
LIQUSTY													
LIRETUL													
METCREP													
OXYOARB													
PINEECH													
PINESTR													
QUERALB													
QUERCOC													
QUERPRI													
QUERVEL													
RHODMAX													
SMIROT													
TSUGCAN													
VITOROT													
QUERNIG													
FAGUGRA													
VACCVAC													
POLYACR													
HEXAHET													
HEXNAN													
HEXAMIN													

Soils

The results of the soil samples analyzed at the Clemson soil lab suggest that some major differences in soil chemistry exist between the species in the *H. heterophylla* complex. GLM analysis of the soil samples showed that many of the basic elements were significantly different among the three species. Those significant differences occurred in Phosphorous (P), Potassium (K), Magnesium (Mg), Zinc (Zn), Manganese (Mn), (Na), Sodium, and Cation Exchange Capacity (CEC). Slightly significant differences were seen in Buffer pH (Bu pH), and Acidity (Table 7).

The Tukey's test results differed slightly from those obtained from the GLM. The major differences in soil chemistry occurred between soils collected from populations of *Hexastylis minor* and *Hexastylis naniflora*, with a few differences in soil chemistry between *Hexastylis heterophylla* and *Hexastylis naniflora* and between *Hexastylis heterophylla* and *Hexastylis minor* (Table 8).

All of the known populations of *H. naniflora* are found within the Pacolet sandy loam, Madison gravelly sandy loam, and Musella fine sandy loam soils, as had been indicated by Gaddy (1981,1987). The most significant difference to note is that the *H. naniflora* sites had significantly higher magnesium concentrations than did the sites for either of the other two species in the complex.

Table 7. Results from the soil analysis that shows significant differences between the soils collected from the localities of the three *Hexastylis heterophylla* complex species, from a GLM analysis using the results from soil chemistry. Those in red are significant and those in blue are slightly significant with a P> 0.05.

	GLM	
Test	**F Value**	**P > F**
Ph	**0.17**	**0.85**
Bu. pH	**3.18**	**0.06**
P	**4.39**	**0.02**
K	**5.66**	**0.01**
Ca	**1.97**	**0.16**
Mg	**3.32**	**0.05**
Zn	**6.66**	**0.004**
Mn	**4.6**	**0.02**
Cu	**1.47**	**0.25**
B	**0.75**	**0.48**
Na	**6.53**	**0.004**
CEC	**5.98**	**0.007**
Acidity	**3.18**	**0.06**
Base Sat	**2.34**	**0.11**

Table 8. The Tukeys test does a pair-wise comparison of the species in the complex using the same chemical analysis results. X indicates significant differences between the two species.

	HH/HM	HH/HN	HM/HN
Ph			
Bu. pH			
P			X
K			X
Ca			X
Mg			
Zn			X
Mn		X	X
Cu			X
B			
Na			
CEC	X		X
Acidity		X	X
Base Sat	X		

Molecular analysis

trnL sequencing

We sequenced approximately 350 bases of the E-F region of *trn*L. We found no variation among two *H. heterophylla*, two *H. minor*, and ten *H. naniflora* specimens. This gene did not provide any information about relationships in the group. We plan to sequence the C-D region of the same gene, but we do not know if it will provide any information.

ISSR analysis

We screened 50 ISSR primers for variation between species in the *H. heterophylla* complex and within *H. naniflora*. Eight primers (814, 824, 834, 835, 843, 844, 848, and 900) showed variation between *H. naniflora* and the other two species, *H. minor* and *H. heterophylla*. However, these patterns were equivocal and the bands were not always reproduced accurately. Based upon these results, it does not appear that this system is robust enough to be used to separate the three species in this complex from vegetative material only.

Although we found intraspecific variation in 22 primers, we were unable to detect any patterns associated with geography or drainages. Some of the banding patterns were not

clear and many were not repeatable. We are currently re-extracting the DNA from some specimens in an attempt to obtain greater resolution of genetic structure within *H. naniflora*.

Transplant analysis

The data collected from the transplant site located along Little Gunpowder Creek in Caldwell County, North Carolina was analyzed and the percent surviving was obtained from those data. In November 2000, 175 individuals, or 100% of the transplants were surviving. By April of 2002, 147 individuals, or 84% of the transplants were surviving. In April, 2002, 119 individuals, or 68% of the transplants were surviving. Although we did not gather survivorship information for individuals that were not transplanted, our collective field experiences have shown that this species is relatively long-lived (30-50 + years) and mature individuals are seldom lost from an undisturbed population.

DISCUSSION

Biogeography

One of the major goals of this project was to explore species boundaries between *H. naniflora* and the most closely related species, *H. heterophylla* and *H. minor*. Since the first description of *H. naniflora* by Blomquist in 1957, field identification of this species has been difficult without fresh flowering material. Locality data has helped in recent years, as biologists gained a better idea of the range of the species. With examination of herbaria specimens and visits to field sites during flowering times, we were able to eliminate four localities previously recognized as *H. naniflora* sites. All the questionable sites were visited during flowering times so that flower materials could be collected and those flowers examined. Four sites in the North Carolina Heritage database were found not to be *H. naniflora*. Two sites were found to be *H. heterophylla* (EO HN0041 and EO HN0042) and two sites were *H. minor* (EO HN0065 and EO HN0066). The locality map generated for this study provides an exact distribution map of all the known *H. naniflora* sites, and this should be of benefit to regional biologists and land managers.

Maps for the *H. heterophylla* complex were generated using presence or absence at the county level. These maps show the overlap among the three species along contact zones and should generate future analyses of speciation and hybridization.

Flower morphometrics

Flower data show that *H. naniflora* can be statistically differentiated from *H. heterophylla* or *H. minor*. The results from the PCA show the separation between *H. naniflora* and the other two species in the complex. While *H. naniflora* can be separated from the other two species in a PCA analysis, no clear separation can be made between *H. heterophylla* and *H. minor*. This species pair is clearly in need of further study.

In obtaining flower measurements for use in analyses of this group, it is clear that fresh flower materials must be used. Data obtained from dried or preserved flower materials is unreliable due to flower distortion that occurs when the flower is pressed, dried, or preserved.

Pollen

Our results from pollen grain analyses show clearly that *H. naniflora* pollen grain surface is unlike that of *H. heterophylla* or *H. minor*. The lack of surface features in *H. naniflora* pollen appears unique among all the *Hexastylis* species. Therefore, with scanning electron microscopy, species differentiation can be made of questionable populations of *Hexastylis* in the *H. heterophylla* complex. With new digital capabilities and low vacuum SEM, the cost of examination of pollen is now very inexpensive (less than $1.00 per sample).

Vegetation Survey

It has been intuitively known for years that certain plant assemblages are found in association with various species of *Hexastylis*. Many associated plant species have been identified in various publications (Blomquist 1957; Gaddy 1983 and 1987; Henderson

2001). The CVS analysis did not show statistical differences among the three species in the *H. heterophylla* complex. However, when those plots are compared with the Sorenson's Index. *Hexastylis naniflora* appears to have an association with three oak species which the other two species in the complex lack. *Hexastylis heterophylla* was the only species in the *H. heterophylla* complex found to occur with Canadian Hemlock (*Tsuga canadense*). *Hexastylis minor* is the only species in the complex that was found to grow in any aspect with respect to exposure to the sun, and was not restricted to a northern aspect, as are *H. naniflora* and *H. heterophylla.*

Soils

Soil chemistry showed marked differences between the species in the complex. The results indicated that soil chemistry is very different between *H. naniflora* and *H. minor* localities. The results also show that *H. heterophylla* and *H. naniflora* are found in soils where the chemistry is more similar, but still showed significant differences. It would appear that differentiation in soil types could be used as proxy for species delineation. The soil analysis indicates that soils must be considered when trying to select sites for relocation of imperiled populations of *H. naniflora*.

Molecular analysis

The trnL sequence data showed no variation within the *H. heterophylla* complex or within a limited sample (10 individuals) of *H. naniflora*. These results indicate that this group is very closely related, and the three species have not been reproductively isolated for a very long period of time. .

We were able to obtain ISSR banding patterns that can be used to separate *H. naniflora* from *H. minor* and *H. heterophylla*. Since this molecular method is relatively inexpensive, this provides land managers with a tool to identify species outside of the flowering season.

In spite of considerable effort, we have been unable to obtain information about genetic structure within *H. naniflora*. We have been able to ascertain that there is variation, but that variation does not coincide with any expected pattern. This may be the result of poor band resolution. We are currently extracting DNA with another method (Qiagen's DNeasy Plant kit mini protocol (i.e. OP1-27 and Eo6, Eo15-20) (69104, Qiagen Inc.)), that may provide a cleaner DNA product, that will possibly produce better band resolution.

Transplant analysis

The transplant at Gunpowder Creek was very successful, with 68% survival over a three year period. This was also a period of relative drought in the region, so this level of survivorship is probably lower than would occur in a more normal rain year. It should be noted that this relocation was to an adjacent site. The similar soil type and slope aspect should be recognized as conditions conducive to successful transplants of *H. naniflora*.

Hexastylis naniflora appears to have a restricted range due to its narrow habitat requirements and limited ability to disperse seeds. The habitat where *Hexastylis naniflora* exists is limited in size and scope due to a multitude of factors including soil

type, moisture availability, and slope aspect. This unique combination of factors limits not only the range of *Hexastylis naniflora*, but also the size of a particular population. With the limited range and size in populations, questions arise regarding gene flow between populations. How much is occurring and how often does it occur? It is due in part to extreme habitat requirements that conservation measures have been implemented for the protection of the species. Any efforts made to protect this species must consider giving protection to the available habitat.

According to U S Fish and Wildlife Service, The Natural Heritage Program, and The North Carolina Department of Agriculture, a definable and discernable population in *Hexastylis* is at least one-half mile from any existing population. If a new locality is found and it falls within the one half mile radius of another known population, that population then becomes a sub-population. A question that must be addressed in order to determine the value of this guideline is whether or not these plants can transfer seeds or pollen one-half mile. *Hexastylis naniflora* populations are all generally small, with less than a few hundred individuals. Very little work has been done with seed dispersal in the genus. The calyx disintegrates to release the seeds and it appears that the seeds are dispersed by gravity (downhill). Wyatt (1955) suggested that the seeds are ant dispersed, which would indicate short dispersal distances. Work on the pollination mechanism of various species of *Hexastylis* suggests that a variety of insects and other invertebrates visit the flowers (Wyatt 1955; Murrell and Carroll 1995), but there is no information available on pollen movement between populations. The lack of information concerning pollen and seed dispersal in this species would suggest that the one-half mile distance for

populations is speculation, at best, and it is likely that clusters of individuals that are no more than 100 m apart may be genetically isolated.

There are several populations of *Hexastylis* in the *H. heterophylla* complex that are either in close proximity or growing together in the same habitat. Of all the known populations that are overlapping, none have been found that produce hybrids. To date, no known hybrids have been found in nature. Past attempts at hybridization have failed due the inability to safely remove the anthers from the flower without causing flower death (Gonzalez 1972; Otte, 1977).

We have generated a suggested plan to assist in the management of *Hexastylis naniflora* (Appendix E). Included are recommendations for the number of populations which should be protected and measures that, based upon our knowledge of this species, should occur before delisting of the species from the Endangered Species List.

Species Conservation Recommendations.

The rarity of *Hexastylis naniflora* had prompted the protection of a number of sites. The Spartanburg County Water Works in South Carolina was among the first organizations to see the significance of protecting *Hexastylis naniflora* and they placed over 1,000 plants into protection in the late 1980's. Camp Mary Elizabeth, also in Spartanburg County, is another site where a number of *Hexastylis naniflora* have been protected. In the early 1990's *Hexastylis naniflora* was found to exist at the Cowpens National Battlefield in Cherokee County, South Carolina. This site protects about one thousand plants. The South Carolina Department of Natural Resources purchased and manages a 161-acre tract of land in Spartanburg County that was developed into the

Peters Creek Heritage Preserve. The Preserve contains over a thousand *H. naniflora* plants.

In North Carolina, the Henson's Ravine Site in Rutherford County was receiving some protection as a natural area as early as the mid 1980's and contains around 1,500 plants. Also in Rutherford County, the Sandy Mush Rock Outcrop supports around 300 *H. naniflora* plants, as well as other unique plant species. It was scheduled for development into a rock quarry until local citizens fought to have it stopped. In Cleveland County, North Carolina, The Broad River Greenway Council and the North Carolina Department of Transportation teamed together and obtained around 1500 acres along the Broad River, which contains over 5000 *H. naniflora* plants.

Recommendations for the future of species

While the number of known populations of *H. naniflora* has been greatly increased in the past few years, conservation efforts should continue for the unforeseeable future. Because the plant's distribution overlaps one of the fastest growing areas in the Southeast, it is imperative to make sure that enough populations are protected to maintain the genetic diversity of the species. The U.S. Fish and Wildlife Service suggested that de-listing of the species is possible if enough populations could be placed in protection. In Appendix D we have made recommendations for a set number of populations to be placed in protection and stabilized before moving towards de-listing of the species. Our evidence suggests that *H. naniflora* is not that rare, and a large number of populations still remain unknown, but habitat requirements severely limit the range of the species. When specific soil and moisture needs (based upon habitat slope and aspect) are

accounted for, the habitat limitations indicate that the plant must have some type of protection in order to assure its survival and genetic diversity. Recent easements and land mitigations by the Broad River Greenway, NCDOT, the Natural Heritage programs of North Carolina and South Carolina, and the Spartanburg County Water Works have paved the way for greater protection of the habitat and plant that could eventually lead to removal of *H. naniflora* from the Endangered Species list as a Threatened species.

BIBLIOGRAPHY

Amoroso, J. L. 2002. Natural Heritage Program List of Rare Plant Species of North Carolina. Raleigh, NC.

Araki Y. 1937. The species of *Asarum* in the Santan District. Acta Phytotaxonomica et Geobotanica 6:122-135.

Araki, Y. 1953. Systema generis Asari. Acta Phytotaxonomica et Geobotanica 15: 33-36.

Ashe, W. W. 1897. The Glabrous-Leaved Species of *Asarum* in the Southeastern United States. Elisha Mitchell Society 14: 31-36.

Avise, J. C. and K. Wollenberg. 1997. Phylogenetic and the origin of species. Proc. Natl. Acad. Sci. 94: 7748-7755.

Barringer, K. 1993. New Combinations in North American *Asarum* (Aristolochiaceae). Novon 3: 225-227.

Blomquist, H. L. 1957. A Revision of *Hexastylis* in North America. Brittonia 8: 255-281.

Bornet, B., and M. Branchard. 2001. Nonanchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. Plant Molecular Biology Reporter. 19: 209-215.

Braun, A. 1861. *Index seminum Hotri Botanici Berolinensis*. Appendix. Plantarum novarum et minus cognitarum, quae in Horto Regio Botanico, Berolinensi coluntum. Berlin.

Bridle, J. R. and M. G. Richie. 2001. Assortative mating and the genic view of speciation. Journal of Evolutionary Biology 14: 878-879.

Britton, N. L. and A. Brown. 1947. *Aristolochiaceae*. Pp. 641-645 in An Illustrated Flora of the Northern United States, Canada, and the British Possessions. New York Botanical Garden, New York, NY.

Britton-Davidian, J. 2001. How do chromosomal changes fit in? Journal of Evolutionary Biology 14: 872-873.

Chester, E. W., B. E. Wofford, and R. Kral. 1997. *Hexastylis*. Pp. 19-20 in Atlas of Tennessee Vascular Plants. The Center For Field Biology. Austin Peay State University, Clarksville, TN.

Cronquist, A. 1981 . *Magnolidae, Order Aristolochiales.* Chp. 4, Pp. 90-93 in An Intrigrated System of Classification of Flowering Plants. New York Botanical Garden, New York, NY.

Dodoens, R. 1574. Purgantium aliarumque eo facientum. Antverpiae: Offica C. Plantini.

Duchartre, P. 1864. *Aristolochiaceae*. Pp.421-498 in Prodromus systematis naturalis regnii vegtabilis, Vol. 15(1), ed. A. P. de Candolle. Paris: Truttel et Wurtz.

Fernald, M. L. 1943. Virginian botanizing under restrictions. Rhodora 45: 357-415.

Fernald, M. L. 1950. *Aristolochiaceae*. Pp. 562-566 in Gray's Manual of Botany. American Book Company, New York.

Gaddy, L. L. 1981. The Status of *Hexastylis naniflora* Blomquist in North Carolina. Unpublished Report. Plant Conservation Program, North Carolina Department of Agriculture, Raleigh, NC.

Gaddy, L. L. 1986. A New *Hexastylis* (Aristolochiaceae) From Transylvania County, North Carolina. Brittonia 38: 82-85.

Gaddy, L. L. 1986. Twelve new ant-dispersed species from the southern Appalachians. Bulletin of the Torrey Botanical Club 113: 247-251.

Gaddy, L. L. 1987. A Review of the Taxonomy and Biogeography of *Hexastylis*. Castanea 52: 186-196.

Gaddy, L. L. 2003. *Hexastylis heterophylla*. Electronic Mail.

Gonzalez, V. C. 1972. The Ecology of *Hexastylis arifolia*, An Evergreen Herb in the North Carolina Deciduous Forest. PhD Dissertation, Duke University, Durham, NC.

Gregory, M. P. 1956. A Phyletic Rearrangement in the Aristolochiaceae. American Journal of Botany 43: 110-122.

Harvill, A. M., T. R. Bradley, and C. E. Stevens. 1981. Atlas of the Virginia Flora. Virginia Botanical Associates, Farmville, VA.

Hayashi, N., K. Maeshima, and H. Komae. 1982. Phenol Ethers of Three North American *Hexastylis* Species. Phytochemistry 22: 299.

Henderson, A. 2001. Using ecological parameters to determine the location of *Hexastylis naniflora* (dwarf-flowered heartleaf) in the upper piedmont of North Carolina. Lenoir-Rhyne College, Hickory, NC.

Herrera, R., V. Cares, M. J. Wilkinson, and P. D. S. Caligari. 2002. Characterization of genetic variation between *Vitis vinifera* cultivars from central Chile using RAPD and Inter Simple Sequence Repeat markers. Euphytica. 124: 139-145.

Interior, Department of the. 1989. National Register Vol. 54: Pp.14964-14967 in the Federal Register, Washington, DC.

Joshi, S. P., V. S. Gupta, R. K. Aggarwal, P. K. Ranjekar, and D. S. Brar. 2000. Genetic diversity and Phylogenetic relationship as revealed by inter simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. Theor. Appl. Genet. 100: 1311-1320.

Judd, W. S., C. S. Campbell, E. A. Kellogg, P. F. Stevens, and M. J. Donoghue. 2002. Plant Systematics: A Phylogenetic approach. 2nd ed. Sinauer Associates, Inc.: Sunderland, MA.

Kelly, L. M. 1997. A Cladistic Analysis of *Asarum* (Aristolochiaceae) and Implications for the Evolution of Herkogamy. American Journal of Botany 84: 1752-1765.

Kelly, L. M. 1998. Phylogenetic Relationships in *Asarum* (Aristolochiaceae) Based on Morphology and ITS Sequences. American Journal of Botany 85: 1454-1467.

Kelly, L. M. 1998. Systematic in *Asarum* and Aristolochiaceae. PhD dissertation. Cornell University, Ithaca, NY.

Kelly, L. M. 2001. Taxonomy of *Asarum Section Asarum* (Aristolochiaceae). Systematic Botany 26: 17-53.

Kelly, L. M. 2002. *Asarum – Hexastylis* discussion. Electronic Mail.

Kral, R. 1983. Aristolochiaceae: *Hexastylis naniflora* Blomquist. Dwarf-flowered heartleaf. USFS, Region 8, Atlanta, GA. 318-321.

Linnaeus, C. 1753. *Species plantarum*. Stockholm: Impensis Laruentii Salvii.

Maekawa, F. 1936. *Asiasarum* Pp. 17-18 in *Flora sylvatica Koreana,* vol. 21, ed. T. Nakai. Keijyo: Government General of Chosen.

Maewawa, F. and M Ono. 1965. Karyotype analysis of the genus *Hexastylis* (Aristolochiaceae). Journal of the Faculty of Science. University of Toyko. Section III: Botany 9:151-159.

McCauley, D. E. 1994. Contrasting the distribution of chloroplast DNA and allozyme polymorphism among local populations of *Silene alba*: Implications for studies of gene flow in plants. Proc. Natl. Acad. Sci. USA. 91: 8127-8131.

McDonald, J. 2000. Management and Conservation Strategies for Seeps and Springs Habitat Associates. Chattahoochee-Oconee National Forest.

McMillian, P. D. 1995. The Ecological Phytogeography and Taxonomy of the *Asarum virginicum* Group. University of North Carolina-Chapel Hill. Honor's thesis. Department of Biology.

Merrill, E. D. 1949. Index Rafinesquianus. Jamaica Plain, The Arnold Arboretum of Harvard Univeristy.

Mondal, T. K., 2002. Assessment of genetic diversity of tea (*Camellia sinensis* (L.) O. Kuntze) by inter-simple sequence repeat polymerase chain reaction. Euphytica. 128: 307-315.

Morren C. and J. Decaisne. 1824. Observations sur la flore du japon. Annales des Sciences Naturalles 2: 308-320.

Nakai, T. 1936. *Japonasarum*. Pp. 17-18 in *Flora sylvatica Koreana*, vol. 21, ed. T. Nakai. Keijyo: Government General of Chosen.

Newberry, G. Undated. Notes on the Biology and Transplanting Techniques for the Federally Endangered Herb *Hexastylis naniflora* Blomquist (Aristolochiaceae). Unpublished.

Newberry, G. 1996. Technical and Agency Draft Recovery Plan for Dwarf-flowered Heartleaf (*Hexastylis naniflora*) Blomquist. U. S. Fish and Wildlife Service, Region 8, Atlanta, GA.

Noor, A. F. 2002. Is the biological species concept showing its age? Trends in Ecology and Evolution 17: 153-154.

Otte, D. S. 1977. The Pollination Ecology of *Hexastylis Arifolia* (Mich.) Small Var. *arifolia* and *Hexastylis minor* (Ashe) Blomquist (Aristolochiaceae) in the Area of Chapel Hill, North Carolina. Master's Thesis, University of North Carolina, Chapel Hill, NC.

Orr, H. A. 2001. Some doubts about (yes another) view of species. Journal of Evolutionary Biology 14: 870-871.

Peattie, D. C. 1929. Flora of the Tryon Region. Elisha Mitchell Scientific Society 44: 141-229.

Peattie, D. C. 1940. How is Asarum Pollinated. Castanea 5: 24-29.

Peet, R. K. et al. 1998. A Flexible, Multipurpose Method for Recording Vegetation Composition and Structure. Castanea 63: 262-274.

Potter, D., F. Gao, G. Aiello, C. Leslie, and G. McGranahan. 2002. Intersimple sequence repeat markers for fingerprinting and determining genetic relationships of walnut (*Juglans regia*) cultivars. J. Amer. Soc. Hort. Sci. 127: 75-81.

Radford, A. E., et al. 1968. Manual of the Vascular Flora of the Carolinas. University of North Carolina Press, Chapel Hill, NC.

Radford, A. E. et al. 1974. Palynological Evidence. Chp. 8, Pp. 211-222 in Vascular Plant Systematics. Harper & Row, New York, NY.

Rafinesque, C. S. 1825. *Neogenyton, or indication of sixty-six new genera of plants in North America.* 1-4.

Rayner, D. A. 1994. Inventory of the Natural Areas of the Pacolet Area. Heritage Program, N. C. Department of the Environment, Health and Natural Resources. Raleigh, NC.

Rieseberg, L. H. and J. M. Burke. 2002. A genic view of species integration. Journal of Evolutionary Biology 14: 883-886.

Rundle, H. D. et al. 2001. Hybridization without guilt: gene flow and the biological species concept. Journal of Evolutionary Biology 14: 868-869.

Schafale, M. P. and. A. S. Weakly. 1990. Classification of the Natural Communities of North Carolina, Third Approximation. Natural Hertiage Program, Division of Parks and Recreation, Department of Environment Health and Natural Resources. Raleigh, NC.

Schmidt, O. C. 1935. *Aristolochiaceae*. Pp 204-242 in Die Naturlichen Pflantzenfamilien ed. 2, Vol. 16b, eds. Engler & Prantl, Liepzig: Verlag Wilhelm Engelmann.

Shaw, K. L. 2001. The genealogical view of speciation. Journal of Evolutionary Biology 14:880-882.

Small, J. K. 1933. Manual of Southeastern Flora. Published by Author, New York, NY.

Smith, J. L., K. L. Hunter, and R. B. Hunter. 2002. Genetic variation in the terrestrial orchid *Tipularia discolor*. Southestern Naturalist. 1: 17-26.

Soltis, D. E. 1984. Karyotyping of Species of *Asarum* and *Hexastylis* (Aristolochiaceae). Systematic Botany 9: 490-493.

Sutter, K. D. et al. 1983. Endangered, threatened, and rare plant species of North Carolina: A revised list. Assocation of Southeastern Biologist. 30: 147-157.

Taberlet, P., L. Gielly, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Molecular Biology. 17: 1105-1109.

Traverse, A. 1988. *Spore and Pollen Morphology*. Chp. 5 Pp 60-116 in Paleopalynology. Unwin Hyman, Boston, MA.

USDA. 1962. Soil Survey of Cherokee County, South Carolina, series 1958, No. 19. U.S. Government Printing Office, Washington, DC.

USDA. 2000. Soil Survey of Cleveland County, North Carolina. U.S. Government Printing Office, Washington, DC.

USDA. 1980. Soil Survey of Henderson County, North Carolina. U.S. Government Printing Office, Washington, DC.

USDA. 1995. Soil Survey of Lincoln County, North Carolina. U.S. Government Printing Office, Washington, DC.

USDA. 1995. Soil Survey of McDowell County, North Carolina. U.S. Government Printing Office, Washington, DC.

USDA. 2000. Soil Survey of Rutherford County, North Carolina. U.S. Government Printing Office, Washington, DC.

USDA. 2000. Soil Survey of Spartanburg County, South Carolina. U.S. Government Printing Office, Washington, DC.

USFWS. 1990. Dwarf-Flowered Heartleaf. Department of the Interior, Washington, DC.
Vogler, A. P. 2001. The genic view: a useful model of the process of speciation? Journal of Evolutionary Biology 14:876-877.

Wofford, B. E. 1989. Guide to the Vascular Plants of the Blue Ridge. University of Georgia, Athens, GA..

Walker, J. W. 1974. Aperture Evolution in the Pollen of Primitive Angiosperms. American Journal of Botany 61: 1112-1136.

Whittemore, A. T., and L. L. Gaddy. 1997. *Hexastylis*. Pp. 54-58 in *Flora of North America*, eds. Flora of North America Editorial Committee. New York: Oxford University Press.

Wu, C. 2001. The genic view of the process of speciation. Journal of Evolutionary Biology 14: 851-865.

Wyatt, R. L. 1955. Floral morphology and phylogeny of the Aristolochiaceae. Master's Thesis, University of North Carolina, Chapel Hill, NC.

Wolfe, A. D. and A. Liston. 1998. Contributions of PCR-Based methods to plant systematics and evolutionary biology. In: Plant Molecular Systematics II. Soltis, Soltis, and Doy: Chapam Hall. p. 43-86.

Wolfe, A. D., Q.-Y. Xiang, and S. R. Kephart. 1998. Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable intersimple sequence repeat (ISSR) bands. Molecular Ecology. 7: 1107-1125.

APPENDIX A
Herbaria Specimens Annotated

HERBARIA	SPECIES	STATE	COUNTY	COLLECTOR	DATE	ASC #
BOON	HAA	NC	BURKE	B CROUCH	4/18/1976	10307
BOON	HAA	SC	ABBEVILLE	ELLIS	6/11/1971	5626
BOON	HAA	NC	CABARRUS	L C BARRINGER	4/15/1979	12735
BOON	HAA	GA	LINCOLN	M GEIMAN	3/24/1977	11241
BOON	HAA	TN	ANDERSON	M L HICKS	4/24/1963	9075
BOON	HAA	NC	WATAGUA	MILLER & BAUCOM	5/4/1967	4639
BOON	HAA	NC	CHEROKEE	S MORROW	10/17/1979	13458
BOON	HAA	NC	CABARRUS	T DAGGY	5/29/1967	4164
BOON	HAC	LA	PERRY MILES	E LICKEY	4/4/2001	16047
BOON	HAR	NC	McDOWELL	D BUFF	5/4/1967	4638
BOON	HAR	TN	KNOX	M L HICKS	5/1/1968	8998
BOON	HAR	NC	CLAY	S W LEONARD & K MOORE	6/6/1968	3168
BOON	HH	NC	AVERY	GROUP V	6/20/1960	2202
BOON	HH	NC	CATAWBA	J PADGETT & E GILLESPIE	4/18/2002	16383
BOON	HH	NC	CALDWELL	K OAKLEY	4/7/1980	13547
BOON	HH	VA	VA	NICK DROZDA	6/19/1905	16598
BOON	HH	VA	FLUVANNA	NICK DROZDA	N/A	16591
BOON	HH	TN	UNICOI	NICK DROZDA	N/A	16593
BOON	HH	VA	FLUVANNA	NICK DROZDA	N/A	16594
BOON	HH	AL	WINSTON	ROBERT F C NACZI	5/21/1996	16596
BOON	HH	AL	CLEBURNE	ROBERT F C NACZI	5/23/1996	16595
BOON	HH	NC	BURKE	T D TAYLOR	5/22/1973	9905
BOON	HM	NC	GASTON	J PADGETT	3/24/2002	16378
BOON	HM	NC	GASTON	J PADGETT	3/24/2002	16381
BOON	HM	NC	ORANGE	RADFORD & STEWERT	4/4/1940	1279
BOON	HM	NC	RANDOLPH	S SMITH & ALLEN	3/14/1976	10308
BOON	HN	NC	RUTHERFORD	J PADGETT	4/2/2001	15993
BOON	HN	NC	RUTHERFORD	J PADGETT	4/4/2001	15992
BOON	HN	NC	RUTHERFORD	J PADGETT	4/4/2001	16001
BOON	HN	NC	RUTHERFORD	J PADGETT	4/4/2001	15994
BOON	HN	NC	RUTHERFORD	J PADGETT	4/6/2001	15995
BOON	HN	NC	LINCOLN	J PADGETT	4/6/2001	15991
BOON	HN	NC	RUTHERFORD	J PADGETT	4/6/2001	15990
BOON	HN	NC	CLEVELAND	J PADGETT	4/10/2001	15996
BOON	HN	NC	RUTHERFORD	J PADGETT	4/21/2001	16384
BOON	HN	NC	RUTHERFORD	J PADGETT	5/13/2001	15997
BOON	HN	NC	RUTHERFORD	J PADGETT	5/13/2001	15998
BOON	HN	NC	RUTHERFORD	J PADGETT	5/17/2001	15999
BOON	HN	NC	RUTHERFORD	J PADGETT	5/18/2001	16000
BOON	HN	NC	ALEXANDER	J PADGETT	4/3/2002	16376
BOON	HN	NC	RUTHERFORD	J PADGETT	4/20/2002	16385
BOON	HN	NC	RUTHERFORD	J PADGETT	4/19/2003	16599
BOON	HN	NC	RUTHERFORD	J PADGETT	4/19/2003	16600
BOON	HN	NC	RUTHERFORD	J PADGETT	4/19/2003	16601
BOON	HN	NC	RUTHERFORD	J PADGETT	4/20/2002	16388

BOON	HN	NC	BURKE	J PADGETT & E GILLESPIE	4/18/2002	16399
BOON	HN	NC	BURKE	J PADGETT & E GILLESPIE	4/18/2002	16377
BOON	HN	NC	CATAWBA	J PADGETT & E GILLESPIE	4/18/2002	16379
BOON	HN	NC	BURKE	J PADGETT & E GILLESPIE	4/18/2002	16380
BOON	HN	NC	CALDWELL	J PADGETT & E GILLESPIE	4/18/2002	16382
BOON	HN	NC	CATAWBA	J PADGETT & E GILLESPIE	4/18/2002	16387
BOON	HN	NC	LINCOLN	J ROBINSON	4/26/1970	5121
BOON	HN	NC	BURKE	JACKSON ET AL	5/5/1956	1522
BOON	HN	NC	CLEVELAND	NICK DROZDA	3/21/1996	16592
BOON	HN	NC	RUTHERFORD	NICK DROZDA	N/A	16597
BOON	HN	NC	BURKE	R D HARBISON	4/10/1971	7295
BOON	HN	NC	BURKE	SMITH ET AL	5/4/1956	1556
BOON	HR	NC	HENDERSON	K BERRY	4/8/1978	12513
BOON	HS	NC	AVERY	D BUFF	5/4/1967	4640
BOON	HS	NC	McDOWELLl	J PADGETT	4/18/2002	16386
BOON	HS	NC	McDOWELL	J PADGETT	4/18/2002	16391
BOON	HS	NC	McDOWELL	J PADGETT	4/18/2002	16390
BOON	HS	NC	McDOWELL	J PADGETT	4/18/2002	16400
BOON	HS	NC	MACON	M L HICKS	8/8/1978	12346
BOON	HS	NC	BURKE	R B HARBISON	5/8/1971	7294
BOON	HS	NC	BURKE	R B HARBISON	6/13/1971	5634
BOON	HS	NC	BURKE	R B HARBISON	6/13/1971	5639
BOON	HS	NC	BURKE	R B HARBISON	6/13/1971	6015
BOON	HS	NC	CHEROKEE	S R MORROW	10/21/1979	13063
BOON	HSp	AL	AUTAUGA	F C NACZI	5/28/1997	16491
BOON	HV	NC	WATAGUA	D BOONE	4/26/1977	11306
BOON	HV	NC	WILKES	D S GOFORTH	6/10/1971	5539
BOON	HV	NC	WATAGUA	GREER	6/22/1964	4302
BOON	HV	NC	STONE MT NC	HOLBROOK & GREER	5/2/1964	4407
BOON	HV	NC	CALDWELL	JACKSON ET AL	4/23/1956	1523
BOON	HV	NC	HENDERSON	P MORRISON	4/8/1978	13752
BOON	HV	NC	WATAGUA	W DOBY	3/29/1976	10393
BOON	HV	NC	WATAGUA	W HESTER	4/25/1977	11217
CONV	HAA	SC	SPARTANBURG	D L RICHARDSON	5/4/1978	5251
CONV	HAA	NC	DAVISON	LISA WILLIAMSON	3/30/1974	5020
CONV	HAA	SC	SPARTANBURG	T. ATWATER & G. WOOD	4/12/1988	5432
CONV	HAA	NC		TOM DASSY	5/29/1967	4419
CONV	HAA	SC	SPARTANBURG	W. MOORE & W. JOLLEY	4/12/1988	5516
CONV	HAR	TN	BLOUNT	H M JENNISON	5/1/1936	3060
CONV	HAR	NC	CLAY	S W LEONARD & K MOORE	6/6/1968	4310
CONV	HM	SC	SPARTANBURG	K MATZENGA & R VALDES	3/3/1976	5094
CONV	HM	VA	BOTETOURT	S S MOORE & K SIMMONS	5/6/1998	5094
CONV	HN	SC	SPARTANBURG	J BOWMAN & S HALEY	4/1/1968	3946
CONV	HN	SC	SPARTANBURG	M G McMILLANn	4/5/1967	3837
CONV	HN	SC	SPARTANBURG	P VOYLES	4/5/1967	2056
DUKE	HH	NC	ALEXANDER	CATHERINE KEEVER	5/10/1941	73375
DUKE	HH	SC	OCONEE	H L BLOMQUIST	6/16/1940	61099

DUKE	HH	SC	PICKENS	H L BLOMQUIST	5/3/1954	160588
DUKE	HH	NC	STOKES	H L BLOMQUIST	6/17/1945	160571
DUKE	HH	NC	STOKES	H L BLOMQUIST ET AL	4/24/1950	160605
DUKE	HH	NC	STOKES	H L BLOMQUIST ET AL	4/24/1950	160612
DUKE	HH	SC	PICKENS	LELAND RODGERS	5/4/1942	90679
DUKE	HH	SC	OCONEE	M R CROSBY & W R ANDERSON	4/25/1964	162907
DUKE	HH	VA	BUCHANAN	R KRAL	5/2/1965	178171
DUKE	HH	SC	OCONEE	R L WILBUR	6/2/1947	160604
DUKE	HH	NC	ALEXANDER	TOM DAGGY ET AL	5/16/1958	143017
DUKE	HH	NC	ALEXANDER	TOM DAGGY ET AL	5/16/1958	143018
DUKE	HH	NC	ALEXANDER	TOM DAGGY ET AL	5/16/1958	143001
DUKE	HH	NC	ALEXANDER	TUCKER CLINE & JAMES McNAIR	4/25/1972	362163
DUKE	HH	SC	OCONEE	W T BATSON	6/6/1950	138358
DUKE	HH	GA	UNION	WILBUR H DUNCAN	6/10/1942	N/A
DUKE	HM	NC	GRANVILLE	AHLES & RADFORD	4/26/1956	139119
DUKE	HM	NC	DURHAM	BETTY G BLACK	4/30/1954	160561
DUKE	HM	NC	WAKE	CARL MONK	5/16/1954	160537
DUKE	HM	NC	ORANGE	D S CORRELL	5/6/1935	134721
DUKE	HM	NC	RICHMOND	D S CORRELL	6/15/1935	136523
DUKE	HM	NC	WAKE	DEXTER HESS	1951-1952	151438
DUKE	HM	NC	DURHAM	H L BLOMQUIST	4/17/1932	19904
DUKE	HM	NC	DURHAM	H L BLOMQUIST	5/26/1945	160560
DUKE	HM	NC	NASH	H L BLOMQUIST	5/28/1945	160539
DUKE	HM	NC	PERSON	H L BLOMQUIST	4/9/1950	160538
DUKE	HM	NC	MOORE	H L BLOMQUIST	3/28/1952	160563
DUKE	HM	NC	GRANVILLE	H L BLOMQUIST	4/18/1953	160547
DUKE	HM	NC	PERSON	H L BLOMQUIST	4/10/1955	160711
DUKE	HM	NC	DURHAM	H L BLOMQUIST	N/A	489
DUKE	HM	NC	WAKE	H L BLOMQUIST	N/A	160562
DUKE	HM	NC	WAKE	M F BUELL	4/15/1956	37957
DUKE	HM	NC	ORANGE	MICHAEL W PALMER	4/28/1986	351682
DUKE	HM	NC	RANDOLPH	R K GODFREY ET AL	4/25/1948	126247
DUKE	HM	NC	STANLEY	R L WILBER	5/10/1963	154974
DUKE	HM	NC	WAKE	R L WILBUR	5/11/1965	169844
DUKE	HM	NC	WAKE	R L WILBUR	5/28/1974	237935
DUKE	HM	NC	CUMBERLAND	ROBERT A CLARK	5/1/1937	94629
DUKE	HM	NC	CHATHAM	TOM DAGGY ET AL	5/3/1958	143000
DUKE	HM	NC	DURHAM	W B DAVIS	3/13/1932	493
DUKE	HM	NC	MOORE	WILLIAM B FOX	5/13/1950	128501
DUKE	HM	NC	LEE	WILLIAM B FOX	5/13/1950	128503
DUKE	HM	NC	RICHMOND	WILLIAM B FOX	6/8/1950	128505
DUKE	HM	NC	FRANKLIN	Z E MURRELL	5/15/1991	339820
DUKE	HN	NC	CLEVELAND	AHLES & BELL	4/19/1956	166157
DUKE	HN	SC	CHEROKEE	AHLES & BELL	4/22/1956	166156
ETSU	HH	TN	CARTER	J C WARDEN	1977	12887
ETSU	HH	TN	WASHINGTON	W G PLESS	4/28/59	11071
ETSU	HH	T	WASHINGTON	J PEARMAN	6/9/56	11075

ETSU	HH	TN		J PAYNE	5/22/66	154/488
ETSU	HH	TN		EE EASLY	4/30/28	1543/11077
ETSU	HH	TN	UNICOI	F DAVISON	6/25/61	1544/11094
ETSU	HH	TN	UNICOI	McGINLEIS	5/18/56	1545/11098
ETSU	HH	TN	CARTER	A B BIGGERSTAFF	4/12/56	1494/1081
ETSU	HH	TN	GREENE	R HOWE	5/15/77	13085
ETSU	HH	TN	WASHINGTON	C Y LAFFITE	4/10/57	1553/11068
ETSU	HH	TN	CARTER	C WILSON	5/18/56	1547/11088
ETSU	HH	TN		J PAYNE	5/22/66	486
ETSU	HH	TN	WASHINGTON	UNK	4/30/52	1557/11078
ETSU	HH	TN	WASHINGTON	H SPARKS	4/24/56	1556/11076
ETSU	HH	TN	WASHINGTON	G JOHNSON	4/24/56	1558/11084
ETSU	HH	TN	WASHINGTON	GILBREATH	4/24/56	1559/11086
ETSU	HH	TN	WASHINGTON	S CLINTON	4/24/56	1560/11087
ETSU	HH	TN	WASHINGTON	TORRES	4/24/56	1561/11089
ETSU	HH	TN	WASHINGTON	B J SAMS	4/15/56	1562/11090
ETSU	HH	TN	SULLIVAN	L HOWARD	4/17/72	1550/11081
ETSU	HH	TN	SULLIVAN	L HOWARD	5/5/72	1549/11083
ETSU	HH	TN	UNICOI	T WILDS	6/30/52	1570/11067
ETSU	HH	TN	UNICOI	E BAILCLIFF	5/21/61	1569/11072
ETSU	HH	TN	UNICOI	HOUCHERS	4/2/50	1568/11079
ETSU	HH	TN	WASHINGTON	J A WILLIAMS	4/24/56	1567/11097
ETSU	HH	TN	WASHINGTON	P A WHITEHEAD	4/24/56	1563/11091
ETSU	HH	TN	WASHINGTON	L LAWRENCE	4/24/56	1564/11092
ETSU	HH	TN	WASHINGTON	PA PAYNE	4/24/56	1565/11095
ETSU	HH	TN	WASHINGTON	M McCLELLAN	4/24/56	1566/11096
ETSU	HH	TN	UNICOI	DELASHNIT	6/30/52	1540/11065
ETSU	HH	TN		M MANNING	4/18/??	1539/11066
ETSU	HH	TN		J SEAL	6/22/58	1571/11085
ETSU	HH	TN	SULLIVAN	L KINKHEAD	3/7/31	1530/11099
ETSU	HH	TN	GREENE	R HOWE	5/1/77	16638
ETSU	HH	VA	SCOTT	R DAVIS	4/15/78	N/A
GHH	HH	VA	BEDFORD	A H CURTIS	5/25/1877	N/A
GHH	HH	VA	ROANOKE	C E WOOD	6/14/1956	5996
GHH	HH	NC	MADISON	D E BOUFFORD ET AL	5/17/1974	N/A
GHH	HH	VA	HALIFAX	D E BOUFFORD ET AL	4/28/1982	22779
GHH	HH	SC	OCONEE	D E BOUFFORD ET AL	5/10/1982	22827
GHH	HH	VA	ROCKBRIDGE	E B BARTRAM	5/28/2009	N/A
GHH	HH	VA	ROANOKE	ET WHERRY & J W ADAMS	4/13/1936	N/A
GHH	HH	VA	RONOKE	ET WHERRY & J W ADAMS	4/13/1936	2651
GHH	HH	NC	JACKSON	F RUGEL	5/1844	24
GHH	HH	VA	ROCKBRIDGE	G G KENNEDY	4/28/1886	N/A
GHH	HH	NC	POLK	J R CHURCHILL	5/29/1899	N/A
GHH	HH	VA	BOTETOURT	R S FREER	3/31/1947	1194
GHH	HH	VA	ROCKBRIDGE	R S FREER	4/10/1947	1260
GHH	HH	SC	OCONEE	S R HILL & C N HORN	5/11/1989	20540
GHH	HH	TN	ROANE	V E McNEILUS	5/24/1987	N/A

GHH	HH	NC	ALEXANDER	W C GREGORY ET AL	6/15/1963	2639
GHH	HH?	TN	FENTRESS	H K SVENSON	6/17/1938	9017
GHH	HM	NC	ORANGE	A S PEASE	4/4/1939	27,014
GHH	HM	NC	N/A	D BOUFFORD & S SPONGBERG	4/26/1982	22773
GHH	HM	SC	CHEROKEE	J W HARDIN & R HARPER	4/16/1953	15491
GHH	HM	NC	WAKE	R K GODFREY	4/11/1938	3420
GHH	HM	NC	RANDOLPH	R K GODFREY ET AL	4/25/1948	48074
GHH	HN	SC	CHEROKEE	S R HILL	4/6/1989	20406
GHH	HN	SC	CHEROKEE	S W LEONARD & A E RADFORD	4/7/1969	2325
GHH	HV	VA	JAMES CITY	J T BALDWIN JR	2/17/1939	9
GHH	HV	NC	WILKES	S R HILL	4/12/1989	20426
GHH	HM	NC	DURHAM	R K GODFREY	5/4/1938	3800
GWU	HH	NC	RUTHERFORD	J COLE	4/20/1997	4741
GWU	HH	NC		M WRIGHT	3/8/1992	3252
GWU	HH	NC		P PRICE	3/21/1992	3268
GWU	HM	NC		B WILSON	4/12/1995	4225
GWU	HM	NC	CLEVELAND	C BAILEY	4/9/1997	4943
GWU	HM	NC	CLEVELAND	J SILVER	4/11/1997	4792
GWU	HM	NC		L LEE	3/7/9/2	3247
GWU	HM	NC		R L WRIGHT	4/12/1995	4357
GWU	HM	NC	CLEVELAND	S WARE	4/15/1997	5012
GWU	HN	NC	LINCOLN	B SAIN	4/14/2001	5917
GWU	HN	NC	RUTHERFORD	J BIGGERS	4/18/1992	3769
GWU	HN	NC		J PADGETT	3/15/1997	4515
GWU	HN	NC		J PADGETT	5/15/1998	6045
GWU	HN	NC		J PADGETT	4/2/2001	6039
GWU	HN	NC		J PADGETT	4/2/2001	6043
GWU	HN	NC		J PADGETT	4/4/2001	6044
GWU	HN	NC		J PADGETT	4/10/2001	6040
GWU	HN	NC		J PADGETT	5/10/2001	6042
GWU	HN	NC		J PADGETT	5/13/2001	6041
GWU	HN	NC		K McNEILY	5/3/1983	216
GWU	HN	NC		L SMITH	4/26/1975	219
GWU	HN	NC		M HOUSER	4/15/1995	3979
GWU	HN	NC		M LAIL	4/24/1983	217
GWU	HN	NC		T VINSETTE	1/3/1985	215
MOBOT	HH	NC	STOKES	S LEONARD & K MOORE	5/31/1968	2377113
MOBOT	HM	GA	STEPHENS	D E BOUFFORD ET AL	5/12/1976	2468716
MOBOT	HM	NC	UNK	W W ASHE	N/A	1985266
NYBG	HH	VA	BEDFORD	A BROWN ET AL	6/6/1890	N/A
NYBG	HH	VA	BEDFORD	A H CURTIS	5/15/1871	N/A
NYBG	HH	VA	BEDFORD	A H CURTIS	6/1868	N/A
NYBG	HH	VA	BEDFORD	A H CURTIS	6/1868	N/A
NYBG	HH	TN	GREENE	A J SHARP ET AL	5/17/1970	45209
NYBG	HH	TN	COCKE	B E WOFFORD	4/17/1979	79-44
NYBG	HH	VA	BEDFORD	CURTIS AND GARNER	5/1868	N/A
NYBG	HH	NC	MADISON	J S NEWBERRY	3/23/1891	N/A

NYBG	HH	NC	MADISON	J S NEWBERRY	5/1891	N/A
NYBG	HH	VA	SMYTH	N L & E BRITTON & M VAIL	6/22/1892	N/A
NYBG	HH	VA	N/A	N L BRITTON	9/2/1885	N/A
NYBG	HH	NC	STOKES	R KRAL	4/9/1960	9803
NYBG	HH	KY	HARLAN	T S PATRICK	4/5/1985	5556
NYBG	HH	TN	ROANE	V E McNEILUS	5/24/1987	N/A
NYBG	HM	VA	BEDFORD	A H CURTIS	5/15/1873	N/A
NYBG	HM	NC	MOORE	B FOX & S G BOYCE	5/13/1950	3609
NYBG	HM	NC	GASTON	C R BELL	4/19/1956	N/A
NYBG	HM	NC	ORANGE	LARRY A BURASKI ?	3/18/1976	553
NYBG	HM	NC	DURHAM	MARGARET P GREGORY	6/1/1944	N/A
NYBG	HM	NC	ORANGE	N/A	N/A	N/A
NYBG	HM	NC	WAKE	R K GODFREY	4/3/1937	N/A
NYBG	HM	NC	WAKE	STEVENS	3/16/1905	N/A
NYBG	HM	NC	ORANGE	W W ASHE	N/A	N/A
NYBG	HM	NC	ORANGE	W W ASHE	N/A	285575
NYBG	HN	SC	CHEROKEE	LENORD & RADFORD	4/7/1969	N/A
NYBG	HV	NC	WILKES	STEVEN R HILL	4/12/1989	N/A
UGH	HH	SC	PICKENS	D S CAMPBELL	4/24/1991	216331
UGH	HH	GA	HABERSHAM	G W McDOWELL & W DUNCAN	5/7/1950	50248
UGH	HH	NC	MADISON	H E AHLES & J A DUKE	4/26/1958	64092
UGH	HH	NC	STOKES	H L BLOMQUIST ET AL	4/24/1950	59580
UGH	HH	GA	STEPHENS	H M McKAY	4/15/1931	50247
UGH	HH	GA	UNION	L FOOTE	5/9/1964	102756
UGH	HH	GA	STEPHENS	M A GARLAND	5/28/1983	157397
UGH	HH	NC	BUNCOME	R WYATT	4/7/1991	200524
UGH	HH	TN	UNICOI	R WYATT	4/18/1993	202500
UGH	HH	NC	ALEXANDER	W C GREGORY ET AL	6/15/1963	92032
UGH	HM	SC	ANDERSON	H E AHLES & H E RADFORD	5/31/1956	64094
UGH	HM	SC	CHEROKEE	J HARDIN & R HARPER	4/16/1953	67685
UGH	HM	NC	GUILFORD	R KRAL	4/17/1966	107163
UGH	HM	NC	ORANGE	R WYATT	4/4/1970	158751
UGH	HM	NC	ORANGE	R WYATT	4/10/1970	158592
UGH	HN	NC	CLEVELAND	W C GREGORY ET AL	6/16/1963	92034
UGH	HV	VA	PULASKI	G P FRANK ET AL	5/1/1981	157247
UNCCH	HH	NC	MADISON	O M FREEMAN	6/5/1956	89893
UNCCH	HH	NC	STOKES	A E RADFORD	5/10/1953	56880
UNCCH	HH	NC	CALDWELL	A E RADFORD	5/12/1956	176089
UNCCH	HH	NC	ALEXANDER	A E RADFORD	5/13/1956	86899
UNCCH	HH	NC	STOKES	A E RADFORD	5/4/1968	176093
UNCCH	HH	TN	WASHINGTON	C E BEAUMONT & W W ASHE	5/22/1926	186371
UNCCH	HH	SC	SPARTANBURG	C R BELL	4/13/1957	176470
UNCCH	HH	NC	BURKE	C R BELL	4/27/1957	176087
UNCCH	HH	NC	CATAWBA	C R BELL	4/29/1957	176088
UNCCH	HH	NC	CATAWBA	C R BELL	6/12/1957	176123
UNCCH	HH	NC	POLK	D C PEATTIE	4/6/1937	14879
UNCCH	HH	NC	POLK	D C PEATTIE	4/13/1937	14888

UNCCH	HH	NC	POLK	D C PEATTIE	4/16/1937	14765
UNCCH	HH	NC	POLK	D C PEATTIE	4/16/1937	14917
UNCCH	HH	NC	MADISON	D E BOUFFORD ET AL	5/17/1974	307417
UNCCH	HH	NC	MADISON	D SATHER	5/28/1981	516491
UNCCH	HH	NC	HENDERSON	E R MEMMINGER	N/A	49426
UNCCH	HH	GA	STEPHENS	E W WOOD & D E BOUFFORD	6/19/1975	469940
UNCCH	HH	GA	STEPHENS	E W WOOD & D E BOUFFORD	6/30/1975	493054
UNCCH	HH	NC	GREENE	F BOWERS	5/17/1970	400860
UNCCH	HH	SC	ANDERSON	H E AHLES & A E RADFORD	3/31/1956	176121
UNCCH	HH	NC	MADISON	H E AHLES & J A DUKE	4/26/1958	176091
UNCCH	HH	NC	MICTHELL	H E AHLES & J A DUKE	6/16/1958	176090
UNCCH	HH	NC	IREDELL	H E AHLES & J McNEELY	4/19/1960	225123
UNCCH	HH	NC	IREDELL	H E AHLES & R BRITT	5/18/1958	184111
UNCCH	HH	SC	PICKENS	J E FAIREY III	5/22/1984	542063
UNCCH	HH	NC	TRANSYLVANIA	O M FREEMAN	4/24/1957	202355
UNCCH	HH	NC	STOKES	R KRAL	4/9/1960	217489
UNCCH	HH	NC	STOKES	R KRAL	4/9/1960	217785
UNCCH	HH	VA	PATRICK	R KRAL	4/20/1960	165406
UNCCH	HH	VA	CARROLL	R KRAL	4/22/1960	165937
UNCCH	HH	VA	MONTGOMERY	R KRAL	5/3/1960	161627
UNCCH	HH	VA	MONTGOMERY	R. KRAL	4/16/1960	217862
UNCCH	HH	VA	MONTGOMERY	R. KRAL	4/16/1960	127853
UNCCH	HH	NC	STOKES	RADFORD & STEWERT	6/1/1940	20728
UNCCH	HH	NC	STOKES	S LEONARD & A E RADFORD	4/23/1970	378299
UNCCH	HH	VA	APPOMATTOX	T F WIEBOLDT	5/9/1983	523692
UNCCH	HH	VA	MECKLENBURG	W D SEAMAN	4/10/1967	388850
UNCCH	HH	NC	HALIFAX	W E WES??? III	4/21/1967	296090
UNCCH	HH	TN	TOWN OF ERWIN	W W ASHE	5/1/1926	256037
UNCCH	HH	NC	POLK	W W ASHE	N/A	72633
UNCCH	HH	TN	WASHINGTON	W W ASHE	N/A	72629
UNCCH	HH	NC	STOKES	Y McCURDY	4/22/1974	473555
UNCCH	HM	NC	ORANGE	A E RADFORD	4/27/1946	31035
UNCCH	HM	NC	PERSON	A E RADFORD	4/4/1954	226411
UNCCH	HM	NC	RANDOLPH	A E RADFORD	4/13/1955	86921
UNCCH	HM	NC	WAKE	A E RADFORD	3/17/1956	86918
UNCCH	HM	NC	RANDOLPH	A E RADFORD	3/30/1956	86943
UNCCH	HM	NC	RANDOLPH	A E RADFORD	3/30/1956	86945
UNCCH	HM	NC	DAVIE	A E RADFORD	4/21/1956	173091
UNCCH	HM	NC	RANDOLPH	A E RADFORD	4/21/1956	173094
UNCCH	HM	NC	RICHMOND	A E RADFORD	5/19/1956	176469
UNCCH	HM	NC	DAVIDSON	A E RADFORD	6/16/1956	86944
UNCCH	HM	NC	MARTIAN	A E RADFORD	4/26/1958	176463
UNCCH	HM	NC	MONTGOMERY	A E RADFORD	5/24/1960	198160
UNCCH	HM	NC	MONTGOMERY	A E RADFORD	3/29/1961	249015
UNCCH	HM	NC	ANSON	A E RADFORD	5/20/1961	249025
UNCCH	HM	NC	GRANVILLE	A E RADFORD & A E AHLES	4/26/1956	86936
UNCCH	HM	NC	RICHMOND	A E RADFORD ET AL	4/3/1954	57377

UNCCH	HM	NC	N/A	B IVEY	3/31/1947	29582
UNCCH	HM	NC	GRANVILLE	B R DAYTON	4/23/1964	324377
UNCCH	HM	NC	ORANGE	B W WELLS	N/A	73902
UNCCH	HM	NC	GASTON	C R BELL	4/19/1956	176458
UNCCH	HM	SC	CHEROKEE	C R BELL	4/22/1956	174323
UNCCH	HM	SC	OCONEE	C R BELL	6/4/1956	176464
UNCCH	HM	NC	PERSON	C R BELL	4/22/1958	176365
UNCCH	HM	NC	RANDOLPH	C R BELL	5/27/1958	176468
UNCCH	HM	NC	POLK	D C PEATTIE	4/12/1937	14768
UNCCH	HM	NC	CUMBERLAND	D P JENSEN	3/30/1990	560748
UNCCH	HM	NC	ORANGE	E T BROWNE JR	4/10/1949	33776
UNCCH	HM	NC	ORANGE	G CHRISTENBERRY	3/22/1939	10651
UNCCH	HM	VA	FLUVANNA	G M DIGGS JR	4/20/1975	489867
UNCCH	HM	VA	APPOMATTOX	G W RAMSEY ET AL	6/20/1967	368214
UNCCH	HM	NC	CHATHAM	H E AHLES & M SEARS	3/19/1964	269593
UNCCH	HM	NC	VANCE	H E AHLES & C R BELL	4/15/1956	86920
UNCCH	HM	NC	NORTHAMPTON	H E AHLES & J A DUKE	3/31/1958	176169
UNCCH	HM	SC	CHEROKEE	H E AHLES & J G HEASLOOP	4/13/1957	174324
UNCCH	HM	NC	MONTGOMERY	H E AHLES & J G HEASLOOP	3/13/1965	271546
UNCCH	HM	NC	ORANGE	H HURLEY	4/15/XX	269563
UNCCH	HM	NC	HARNETT	H LAING	3/31/1957	176495
UNCCH	HM	NC	HARNETT	H LAING	3/31/1957	176103
UNCCH	HM	NC	HARNETT	H LAING	5/8/1957	176104
UNCCH	HM	NC	ORANGE	H SHERWIN	3/9/1943	73914
UNCCH	HM	NC	GUILFORD	J CAUSEY	N/A	12835
UNCCH	HM	NC	ORANGE	J G ULERIFRLY	4/9/1897	73907
UNCCH	HM	NC	ORANGE	J GLENN	4/11/1931	73913
UNCCH	HM	NC	MOORE	J H CARTER	4/8/1973	261808
UNCCH	HM	NC	ROWAN	J H HORTON	4/7/1957	198151
UNCCH	HM	NC	ORANGE	J LARKE	3/5/1990	558143
UNCCH	HM	NC	ORANGE	J R RAPER	4/15/1932	73903
UNCCH	HM	SC	LANCASTER	J W HARDIN & W H DUNCAN	4/21/1953	259271
UNCCH	HM	NC	GUILFORD	L MELVIN	3/24/1956	174800
UNCCH	HM	NC	GUILFORD	L MELVIN	3/29/1956	174799
UNCCH	HM	NC	MOORE	L MELVIN	5/13/1956	174802
UNCCH	HM	NC	ORANGE	L RUSH JR	5/6/1959	234625
UNCCH	HM	NC	LEE	L S BEARD	3/26/1955	176461
UNCCH	HM	NC	ORANGE	L W LYNCH	N/A	73911
UNCCH	HM	NC	ORANGE	L W OLSON	4/5/1964	255684
UNCCH	HM	NC	ORANGE	M MUNVH	3/13/1938	73912
UNCCH	HM	NC	ORANGE	N A BOATWRIGHT	4/11/1959	234617
UNCCH	HM	NC	ORANGE	N/A	4/3/1905	73905
UNCCH	HM	NC	ORANGE	N/A	N/A	73904
UNCCH	HM	NC	ORANGE	O WO HYMAN	4/5/1890	73910
UNCCH	HM	NC	ORANGE	P A KESSLER	2/21/1954	198120
UNCCH	HM	NC	CHATHAM	P A KESSLER	3/12/1956	86937
UNCCH	HM	NC	MOORE	P A KESSLER	5/16/1960	176462

UNCCH	HM	NC	ORANGE	P A WHITLOCK	2/27/1959	234630
UNCCH	HM	NC	CHATHAM	P J CRUTCHFIELD	5/30/1958	176122
UNCCH	HM	NC	MOORE	P KESSLER	4/2/1955	86932
UNCCH	HM	NC	MOORE	P KESSLER	4/17/1955	86938
UNCCH	HM	NC	ORANGE	R F BRITT	5/27/1957	178461
UNCCH	HM	NC	WAKE	R K GODFREY	4/4/1938	12036
UNCCH	HM	NC	ORANGE	RADFORD & STEWERT	4/4/1940	11467
UNCCH	HM	NC	STOKES	S W LEONARD & K MOORE	3/31/1968	323466
UNCCH	HM	NC	RICHMOND	T D NIFONG	4/17/1979	545244
UNCCH	HM	NC	N/A	TURRECTION ?	3/30/2001	73909
UNCCH	HM	NC	ORANGE	W C CONNER	4/11/1910	73908
UNCCH	HM	NC	ORANGE	W J KOCH	3/20/1943	32713
UNCCH	HM	NC	ROCKINGHAM	W MARTAIN	3/19/1966	275789
UNCCH	HM	NC	ORANGE	W W ASHE	4/1897	356927
UNCCH	HN	NC	LINCOLN	C R BELL	5/28/1957	176118
UNCCH	HN	NC	LINCOLN	C R BELL	9/9/1958	176119
UNCCH	HN	SC	GREENVILLE	O M FREEMAN	3/17/1956	86894
UNCCH	HR	NC	POLK	D C PEATTIE	N/A	73906
UNCCH	HR?	NC	POLK	D C PEATTIE	4/12/1937	14877
UNCCH	HV	NC	ROCKINGHAM	A E RADFORD	4/13/1956	86903
UNCCH	HV	NC	DAVIE	A E RADFORD	4/14/1956	86916
UNCCH	HV	NC	DAVIE	A E RADFORD	4/14/1956	86917
UNCCH	HV	NC	SURRY	A E RADFORD	4/16/1956	176092
UNCCH	HV	NC	SURRY	A E RADFORD	4/16/1956	87061
UNCCH	HV	NC	ALLEGHANY	A E RADFORD	5/2/1958	176094
UNCCH	HV	NC	STOKES	A E RADFORD	6/4/1958	176113
UNCCH	HV	NC	STOKES	A E RADFORD	6/4/1958	176112
UNCCH	HV	NC	ROCKINGHAM	A E RADFORD & H E AHLES	4/13/1956	176111
UNCCH	HV	NC	ROCKINGHAM	A E RADFORD & H E AHLES	4/13/1956	86907
UNCCH	HV	NC	FORSYTH	H E AHLES & R BRITT	5/17/1958	176097
UNCCH	HV	NC	WATAGUA	H E AHLES & R P ASHWORTH	5/4/1958	176114
UNCCH	HV	NC	WATAGUA	H E AHLES & R P ASHWORTH	5/4/1958	176504
UNCCH	HV	NC	PASQUOTANK	J W CHICKERING JR	4/1878	30108
UNCCH	HV	NC	GUILFORD	L MELVIN	4/12/1956	174804
UNCCH	HV	NC	GUILFORD	L MELVIN	4/25/1956	174801
UNCCH	HV	NC	ROBESON	R F BRITT	4/5/1958	184587
UNCCH	HV	VA	PATRICK	R KRAL	4/9/1960	217784
UNCCH	HV	VA	PATRICK	R KRAL	4/9/1960	217488
US	HH	VA	RONOKE	C E WOOD	6/14/1946	2051013
US	HH	VA	ALBEMARLE	E S RAWLINSON	5/10/1934	1622981
US	HH	NC	*ROAN MT*	J W CHICKERING JR	7/1/1880	796995
US	HH	TN	*RICH MT*	T H KEARNEY JR	4/25/1893	250060
US	HH	VA	AUGUSTA	W W EGGLESTON	N/A	1220664
USCH	HAA	SC	KERSHAW	A HOLLEY ET AL	4/10/1984	25742
USCH	HAA	SC	AIKEN	A M NIESEMANN	4/20/1969	7350
USCH	HAA	SC	KERSHAW	A T HOLLAND	7/16/1959	7362
USCH	HAA	SC	BAMBERG	B B BRANTLEY	3/31/1984	41691

USCH	HAA	SC	DALRINGTON	B E SMITH	5/28/1940	36349
USCH	HAA	SC	RICHLAND	BARTON & Kelley	3/8/1955	7386
USCH	HAA	SC	LEXINGTON	C A AULBACH-SMITH	3/31/1981	21698
USCH	HAA	SC	SALUDA	C A AULBACH-SMITH	4/17/1981	21687
USCH	HAA	SC	RICHLAND	C A AULBACH-SMITH	4/22/1982	25008
USCH	HAA	SC	JASPER	C A AULBACH-SMITH	3/16/1984	25451
USCH	HAA	SC	NEWBERRY	C H HORN	4/17/1987	40022
USCH	HAA	SC	BARNWELL	C L PORTER	8/17/1956	7357
USCH	HAA	SC	RICHLAND	C McCUTCHEN	4/23/1966	7388
USCH	HAA	SC	CALHOUN	C N HORN	4/18/1987	40021
USCH	HAA	AL	TUSCALOOSA	C N HORN	4/11/1992	64128
USCH	HAA	NC	COLUMBUS	C R BELL	4/25/1958	7393
USCH	HAA	SC	RICHLAND	COLUMBIA COLLEGE	2/12/1993	60966
USCH	HAA	SC	RICHLAND	COLUMBIA COLLEGE	4/16/1993	60665
USCH	HAA	SC	AIKEN	D A JOHNSON & J NELSON	4/6/1995	68678
USCH	HAA	NC	LEE	D CHEN	4/8/1965	36347
USCH	HAA	SC	LEXINGTON	D D DWEENEY	4/13/1992	59761
USCH	HAA	SC	RICHLAND	D H RENBERD?	4/26/1958	7371
USCH	HAA	NC	CLAY	D PITTILLO	6/12/1977	26220
USCH	HAA	SC	AIKEN	D SOBLO	3/14/1990	50506
USCH	HAA	SC	AIKEN	D SOBLO	3/14/1990	50507
USCH	HAA	SC	RICHLAND	ECOLOGY CLASS USCC	10/1/1927	7382
USCH	HAA	SC	KERSHAW	F McELVEEN	5/1/1964	7363
USCH	HAA	SC	PICKENS	G DOWNS	5/1/1975	2978
USCH	HAA	GA	DEKALB	G KEAFT	4/20/1965	7398
USCH	HAA	NC	ORANGE	G P SAWYER	4/3/1964	36348
USCH	HAA	SC	DARLINGTON	G P SAWYER	8/8/1975	2755
USCH	HAA	SC	RICHLAND	H HECHENBLEIKNER	3/7/1939	7375
USCH	HAA	SC	BERKELY	H TRAIT	4/30/1953	7358
USCH	HAA	SC	RICHLAND	J B NELSON	4/24/1984	32424
USCH	HAA	SC	LEE	J B NELSON	3/24/1989	49832
USCH	HAA	SC	GEORGETOWN	J B NELSON	3/28/1991	53913
USCH	HAA	SC	CLARENDON	J B NELSON ET AL	3/19/1986	33194
USCH	HAA	NC	ANSON	J B NELSON ET AL	3/28/1988	46722
USCH	HAA	SC	FAIRFIELD	J BASS	4/25/1965	7360
USCH	HAA	SC	SALUDA	J CROUCH	4/25/1965	7391
USCH	HAA	SC	CALHOUN	J E FAIREY ET AL	4/1/1961	7359
USCH	HAA	NC	GASTON	J E WARD & H J RICHARDS	4/13/1968	7392
USCH	HAA	SC	SUMTER	J F LUGUE	5/21/1982	40474
USCH	HAA	GA	PUTNAM	J H PYRON & R McVAUGH	4/2/1938	7394
USCH	HAA	SC	RICHLAND	J M BARRY	3/10/1967	7372
USCH	HAA	SC	LEXINGTON	J M BARRY	4/6/1967	7368
USCH	HAA	SC	RICHLAND	K R TREPANIER	4/21/1996	68851
USCH	HAA	SC	ALLENDALE	KELLEY & BATSON	3/30/1953	7352
USCH	HAA	SC	ALLENDALE	KELLEY & BATSON	4/6/1953	7351
USCH	HAA	SC	ALLENDALE	KELLEY & BATSON	4/6/1953	7356
USCH	HAA	SC	ALLENDALE	KELLEY & BATSON	5/11/1953	7353

USCH	HAA	SC	AIKEN	KELLEY & BATSON	4/1/1964	7349
USCH	HAA	SC	NEWBERRY	L H BUFF	8/12/1971	7370
USCH	HAA	SC	RICHLAND	L H ROBINSON	5/10/1966	7373
USCH	HAA	SC	LEXINGTON	L L SMITH ET AL	4/27/1966	7367
USCH	HAA	SC	SALUDA	L L SMITH ET AL	5/6/1966	7390
USCH	HAA	SC	LEXINGTON	L LOWENSTEIN	5/5/1960	7365
USCH	HAA	SC	RICHLAND	M J ROBINSON	4/23/1966	7383
USCH	HAA	SC	RICHLAND	M SAMPSON	4/15/1937	7377
USCH	HAA	SC	LEXINGTON	N/A	N/A	7387
USCH	HAA	SC	RICHLAND	N/A	N/A	7381
USCH	HAA	SC	RICHLAND	P J PHILSON	4/11/1936	7376
USCH	HAA	SC	RICHLAND	P J PHILSON	4/14/1936	7383
USCH	HAA	SC	RICHLAND	P J PHILSON	4/26/1940	7374
USCH	HAA	SC	RICHLAND	P J PHILSON	4/26/1940	7378
USCH	HAA	TN	COCKE	R D THOMAS	10/14/1989	50160
USCH	HAA	SC	DORCHESTER	R S HILL & D SOBLO	5/15/1988	50650
USCH	HAA	SC	AIKEN	R STICH	3/30/1992	57774
USCH	HAA	SC	LANCASTER	S CLYBURN	4/26/1958	7364
USCH	HAA	SC	FAIRFIELD	S GUERRY	4/19/1983	23366
USCH	HAA	SC	LEXINGTON	S L SMITH	5/8/1966	7366
USCH	HAA	SC	RICHLAND	T SMITH	N/A	7379
USCH	HAA	SC	RICHLAND	V UTSEY	N/A	7380
USCH	HAA	SC	AIKEN	W R KELLEY & W T BATSON	10/29/1951	7347
USCH	HAA	SC	AIKEN	W R KELLEY & W T BATSON	3/1/1952	7348
USCH	HAA	SC	McCORMICK	W T BATSON	4/16/1961	7369
USCH	HAA	SC	HAMPTON	W T BATSON	4/27/1988	45998
USCH	HAR	NC	CLAY	S W LEONARD & K MOORE	6/6/1968	7395
USCH	HH	SC	GREENVILLE	A E CRANDELL	9/18/1976	9119
USCH	HH	SC	GREENVILLE	A E CRANDELL	4/9/1977	9110
USCH	HH	SC	OCONEE	C H HORN	5/11/1989	52754
USCH	HH	SC	OCONEE	D MADSEN	3/3/1993	66017
USCH	HH	SC	OCONEE	D SOBLO	1/10/1991	52900
USCH	HH	SC	OCONEE	G P SAWYER ET AL	3/20/1965	36350
USCH	HH	SC	GREENVILLE	J B NELSON & S GREETER	3/30/1988	46718
USCH	HH	SC	OCONEE	J B NELSON & S MOFFAT	4/11/1989	48925
USCH	HH	SC	PICKENS	J E FAIREY III	5/22/1984	26472
USCH	HH	SC	PICKENS	J R CLONTS	8/21/1975	5643
USCH	HH	SC	PICKENS	W T BATSON	5/1/1954	7400
USCH	HM	SC	CHEROKEE	D A RAYNER	4/3/1986	48498
USCH	HM	NC	ORANGE	D CHEN	3/21/1965	36351
USCH	HM	SC	YORK	D E KENNEMORE & J B NELSON	5/18/1993	66287
USCH	HM	SC	YORK	D E KENNEMORE & K JSCKSON	3/28/1993	62648
USCH	HM	SC	YORK	D E KENNEMORE JR	4/19/1994	68475
USCH	HM	NC	RICHMOND	G P SAWYER & H AHLES	4/17/1964	36354
USCH	HM	SC	CHEROKEE	J B NELSON	3/17/1987	34754
USCH	HN	SC	GREENVILLE	D A RAYNER	4/21/1977	20729
USCH	HN	SC	GREENVILLE	D A RAYNER	5/19/1981	21514

USCH	HN	SC	GREENVILLE	D A RAYNER	5/21/1981	21511
USCH	HN	SC	GREENVILLE	D A RAYNER	6/1/1981	21516
USCH	HN	SC	SPARTANBURG	D A RAYNER	5/21/1985	47824
USCH	HN	SC	GREENVILLE	E A VERNON	5/10/1964	7361
USCH	HS	SC	PICKENS	C H HORN	5/9/1988	45627
USCH	HS	NC	BURKE	R HARBISON	6/13/1971	7397
USCH	HS	NC	TRANSYLVANIA	R JOHNSON	6/6/1993	62489
USCH	HS	SC	OCONEE	W T BATSON	5/29/1954	7401
UT	HH	TN	CARTER	A B GRINDSTAFF	4/12/1956	N/A
UT	HH	TN	COCKE	A J & EVELYN SHARP	10/12/1963	2170
UT	HH	TN	CARTER	A J & EVELYN SHARP	10/27/1963	2170
UT	HH	SC	PICKENS	A J SHARP	4/24/1955	2170
UT	HH	TN	CARTER	A J SHARP	5/5/1963	2170
UT	HH	TN	CARTER	A J SHARP	4/9/1967	2170A
UT	HH	TN	GREENE	A J SHARP	5/17/1970	2170A
UT	HH	TN	SULLIVAN	A J SHARP & C ELLIS	4/23/1979	2170A
UT	HH	TN	GREENE	A J SHARP & D K SMITH	9/23/1973	2170A
UT	HH	TN	CLAIBORNE	A J SHARP ET AL	6/10/1962	2170
UT	HH	TN	GREENE	A J SHARP ET AL	5/22/1986	2170A
UT	HH	TN	UNICOI	C LYLE	4/2/1955	2170
UT	HH	TN	UNICOI	E WOFFORD ET AL	7/13/1973	2170A
UT	HH	TN	COCKE	E WOFFORD ET AL	4/17/1979	2170A
UT	HH	TN	COCKE	E WOFFORD ET AL	4/17/1979	2170A
UT	HH	TN	COCKE	E WOFFORD ET AL	4/17/1979	2170A
UT	HH	NC	STOKES	H L BLOMQUIST ET AL	4/24/1950	2170A
UT	HH	TN	CARTER	J PEARMAN	6/9/1956	2170
UT	HH	NC	POLK	J R CHURCHILL	5/29/1899	2170A
UT	HH	TN	HAWKINS	J WOLFE	4/16/1955	N/A
UT	HH	TN	UNICOI	L L GADDY	10/7/1951	N/A
UT	HH	TN	CARTER	L L GADDY	6/12/1986	2170A
UT	HH	TN	UNICOI	L L GADDY	6/12/1986	2170A
UT	HH	TN	UNICOI	L L GADDY	6/12/1986	2170A
UT	HH	TN	UNICOI	L L GADDY	6/12/1986	2170A
UT	HH	TN	SULLIVAN	L L GADDY	6/12/1986	2170A
UT	HH	TN	SULLIVAN	L L GADDY	6/12/1986	2170A
UT	HH	TN	UNICOI	L L GADDY	6/13/1986	2170A
UT	HH	NC	MADISON	L L GADDY	6/30/1986	2170A
UT	HH	KY	BELL	L POUNDS	4/24/1985	2170A
UT	HH	TN	CARTER	R E SHANKS	9/11/1954	N/A
UT	HH	TN	CARTER	R E SHANKS & A J SHARP	7/24/1949	N/A
UT	HH	TN	UNICOI	R E SHANKS ET AL	9/7/1949	N/A
UT	HH	VA	LEE	R HINKLE	3/30/1974	2170A
UT	HH	KY	BELL	R HINKLE	4/12/1974	2170A
UT	HH	TN	UNICOI	R L JAMES	7/21/1952	2170
UT	HH	KY	BELL	T S PATRICK	5/4/1985	2170A
UT	HH	VA	SCOTT	T S PATRICK & B E PERKINS	4/18/1982	2170A
UT	HH	TN	ROANE	V E McNEILUS	4/7/1987	2170A

UT	HH	TN	ROANE	V E McNEILUS	4/9/1987	2170A
UT	HH	TN	ROANE	V E McNEILUS	5/24/1987	2170A
UT	HH	TN	ROANE	V E McNEILUS	4/8/1991	2170A
UT	HM	SC	LANCASTER	J HARDIN	4/21/1953	2170A
UT	HM	NC	ORANGE	W E KIRKLAND	3/2/1965	2170A
UT	HN	SC	CHEROKEE	S LEONARD & A E RADFORD	4/7/1969	2170A
UWI	HH	NC	MADISON	W W ASHE	3/11/1905	192
UWI	HH	NC	Boutes park CH	W W ASHE	4/5/1896	1372
UWI	HH	NC	POINT CREEK	W W ASHE	5/30/1898	192
UWI	HH?	TN	COCKE	A J & EVELYN SHARP	10/12/1963	32484
UWI	HM	NC	WINSTON-SALEM	W W ASHE	6/1897	192
UWI / US	HH	NC	CALDWELL	J K SMALL	6/20/1891	18262
VPI	HH	VA	RONOKE	L J UTTAL	4/13/1969	17715
VPI	HH	VA	BOTETOURT	A B MASSY	5/4/1940	36,873
VPI	HH	VA	MONTGOMERY	A B MASSY	5/16/1940	36876
VPI	HH	VA	MONTGOMERY	A B MASSY	4/22/1953	35,637
VPI	HH	VA	MONTGOMERY	A B MASSY	N/A	405
VPI	HH	VA	ROCKBRIDGE	B LONG	N/A	56722
VPI	HH	VA	MONTGOMERY	E A SMYTH	6/1893	35,635
VPI	HH	SC	ANDERSON	F EARLE	2/26/1905	35,640
VPI	HH	VA	ROCKBRIDGE	FREER	4/5/1966	44.637
VPI	HH	VA	BOTETOURT	FREER	4/21/1966	44,648
VPI	HH	VA	AMHERST	FREER ET AL	4/6/1966	44,532
VPI	HH	TN	UNICOI	G GONSOULIN	4/6/1974	N/A
VPI	HH	VA	PULASKI	G P FRANK ET AL	5/1/1981	68445
VPI	HH	VA	AMELIA	J B LEWIS	4/19/1905	3349
VPI	HH	VA	AMELIA	J B LEWIS	4/19/1905	36,875
VPI	HH	VA	AMELIA	J B LEWIS	4/12/1938	N/A
VPI	HH	VA	WASHINGTON	J C LUDWIG	5/7/1993	88425
VPI	HH	??	*BRUSH*MT	JSC	5-10-XX	8586
VPI	HH	VA	RONOKE	L J UTTAL	4/13/1969	39626
VPI	HH	VA	PULASKI	L J UTTAL	5/22/1969	27,048
VPI	HH	VA	RONOKE	L J UTTAL	5/5/1970	50158
VPI	HH	VA	PULASKI	L J UTTAL	5/7/1970	50127
VPI	HH	VA	PULASKI	L J UTTAL	5/7/1970	50145
VPI	HH	VA	LEE	L J UTTAL	6/5/1970	16,146
VPI	HH	VA	PATRICK	L J UTTAL	5/4/1971	51620
VPI	HH	VA	PATRICK	L J UTTAL	5/4/1971	25227
VPI	HH	VA	PULASKI	L J UTTAL	4/24/1975	60458
VPI	HH	TN	UNICOI	L J UTTAL	5/7/1985	77710
VPI	HH	NC	STOKES	R KRAL	4/9/1960	35,629
VPI	HH	VA	MONTGOMERY	R KRAL	4/16/1960	35,628
VPI	HH	VA	PATRICK	R KRAL	4/20/1960	35,636
VPI	HH	VA	CARROLL	R KRAL	4/22/1960	35,626
VPI	HH	VA	MONTGOMERY	R KRAL	5/1/1960	35,630
VPI	HH	VA	MONTGOMERY	R KRAL	5/3/1960	35,622
VPI	HH	VA	MONTGOMERY	R KRAL	5/23/1960	35,627

VPI	HH	VA	MONTGOMERY	R KRAL	4/26/1961	18,380
VPI	HH	VA	CAMPBELL	T F WIEBOLDT ET AL	5/13/1979	69977
VPI	HH	VA	SMYTH	T F WIEBOLDT ET AL	6/14/1980	79025
VPI	HH	VA	ALBEMARLE	T F WIEBOLDT ET AL	5/15/1982	72101
VPI	HH	VA	NELSON	T F WIEBOLDT ET AL	5/17/1982	72098
VPI	HH	VA	ROCKINGHAM	T F WIEBOLDT ET AL	5/17/1982	72099
VPI	HH	VA	PULASKI	T F WIEBOLDT ET AL	6/3/1982	72220
VPI	HH	VA	PULASKI	T F WIEBOLDT ET AL	6/3/1982	72219
VPI	HH	VA	FLOYD	T F WIEBOLDT ET AL	6/3/1982	72221
VPI	HH	VA	APPOMATTOX	T F WIEBOLDT ET AL	5/9/1983	73548
VPI	HH	VA	PRINCE EDWARD	T F WIEBOLDT ET AL	5/17/1984	76520
VPI	HH	VA	BUCKINGHAM	T F WIEBOLDT ET AL	5/17/1984	76519
VPI	HH	VA	MONTGOMERY	T F WIEBOLDT ET AL	5/20/1984	86380
VPI	HH	VA	PITTSYLVANIA	T F WIEBOLDT ET AL	4/23/1985	77929
VPI	HH	VA	ROCKBRIDGE	T F WIEBOLDT ET AL	6/9/1986	79273
VPI	HH	VA	CUMBERLAND	T F WIEBOLDT ET AL	5/11/1990	88026
VPI	HH	VA	WASHINGTON	W F RUSKA & S BENTLEY	5/10/1981	68512
VPI	HM	GA	RABUN	A E LANGLEY	4/27/1973	76099
VPI	HM	VA	APPOMATTOX	G W RAMSEY ET AL	6/20/1967	44,695
VPI	HM	VA	CHESTERFIELD	J C LUDWIG	4/9/1989	88397
VPI	HM	NC	MONTGOMERY	L J UTTAL	4/6/1976	62456
VPI	HM	NC	CHATHAM	P KESSLER	3/12/1956	17,323
VPI	HM	VA	MECKLENBURG	T F WIEBOLDT ET AL	5/12/1990	88071
VPI	HM	VA	MECKLENBURG	T F WIEBOLDT ET AL	5/12/1990	88072
VPI	HN	SC	CHEROKEE	S LEONARD & A E RADFORD	4/7/1969	60715
WOFF	HAA	SC	RICHLAND	DANNY HOLLIFIELD	4/1/1989	N/A
WOFF	HAA	SC	GREENVILLE	JAMAS GARDIN	4/16/1992	N/A
WOFF	HAA	SC	RICHLAND	SUZIE CHRISTOS	4/1/1989	N/A
WOFF	HH	SC	GREENVILLE	BRENDA WICHMANN	4/3/1998	N/A
WOFF	HH	SC	GREENVILLE	E E ELKINS	4/3/1992	N/A
WOFF	HH	SC	GREENVILLE	I B PARNELL	4/30/1998	N/A
WOFF	HH	SC	GREENVILLE	REGINA AYRES	5/14/1992	N/A
WOFF	HM	SC	YORK	SHAUNA D. CANNON	4/24/1990	N/A
WOFF	HM	SC	YORK	ZENOBIA L. COLLINS	5/10/1990	N/A
WOFF	HN	SC	SPARTANBURG	BRENDA WICHMANN	3/19/1998	N/A
WOFF	HN	SC	SPARTANBURG	D A RAYNER	4/15/1991	N/A
WOFF	HR	NC	POLK	GEORGE HUIZINGA	N/A	N/A
WOFF	HR	NC	POLK	HUGH BRADBURN	5/1/1993	N/A
WOFF	HR	NC	POLK	J E DOMBROSKI	4/4/1992	N/A
WOFF	HR	NC	POLK	MELISSA SHOULE	5/3/1990	N/A
WOFF	HS	NC	POLK	D A RAYNER	6/10/1991	N/A

APPENDIX B

Sites where flower and leaf materials were collected

NOTE: Asterisk indicates those populations that were examined in the pollen analysis using Scanning Electron Microscopy.

Hexastylis naniflora sites visited 2001-2003

County	State	EO #	Location
*Alexander	NC	NA	US 64 and Hunter Bridge Road
*Burke	NC	NC-005	Will Hudson Rd, SR 1910
Burke	NC	NC-011	Pleasant Grove Site, SR 1924
Burke	NC	NA	Corn Hill Rd off of Sugarloaf Rd
Caldwell	NC	NC-044	Little Gunpowder Creek, SR 1108
Catawba	NC	NC-021	Catawba River at US 321
Catawba	NC	NC-022	Murray's Mill at Balls Creek, SR 1003
Catawba	NC	NC-030	W of Tate Blvd., SR 1476
Catawba	NC	NC-031	Between I-40 and US 70 near Fairgrove
Catawba	NC	NC-039	Shiloh Church, Murray's Mill Lake, SR1824
Catawba	NC	NC-042	Bunker Hill Bridge
*Catawba	NC	NA	Greedy Hwy and Hudson Road
Catawba	NC	NA	SR 1692 Fairgrove
Catawba	NC	NA	Conally Springs
Cleveland	NC	NC-001	Brushy Creek Bluff
*Cleveland	NC	NC-008	Poundingmill Creek
*Cleveland	NC	NC-014	Sandy Run Bluff Site, College Farm Road
Cleveland	NC	NC-017	Sandy Run Creek 1 miles west fo Boiling Springs
Cleveland	NC	NC-018	Sandy Run Creek, SR 1164
Cleveland	NC	NC-028	Cleveland County Landfill
Cleveland	NC	NC-046	Buffalo Creek, SR 1908
Cleveland	NC	NC-049	IP Tract (Now DOT-Greenway)
Cleveland	NC	NC-050	IP Tract (Now DOT-Greenway)
Cleveland	NC	NC-051	IP Tract (Now DOT-Greenway)
Cleveland	NC	NA	Along Leaman Gap Road just inside county
Cleveland	NC	NA	Dirty Ankle Road from Leaman Gap Road
Lincoln	NC	NC-002	Cat Square, Exerpated
Lincoln	NC	NC-015	Off US 274 3 miles N of Cherryville
*Lincoln	NC	NA	SR 1104 Near new bridge
*Polk	NC	NC-023	E of Kross Keys, N of NC 9 and E of SR1338
*Rutherford	NC	NC-009	Henson's Creek Ravine
*Rutherford	NC	NC-010	Kudzu Cow Farm Site
*Rutherford	NC	NC-013	Sandy Mush Rock Outcrop
Rutherford	NC	NC-016	Off US 221 near Danieltown, Exerpated
Rutherford	NC	NC-037	Hunter Road, SR 1124, behind trailer
Rutherford	NC	NC-040	Jonas Rd. SR-1109
Rutherford	NC	NC-041	Pot Branch
Rutherford	NC	NC-052	Dills Creek Tributary
Rutherford	NC	NC-053	Broad River near SR 1111 from Bridge go North
Rutherford	NC	NC-054	Jebb Lamb Road, SR 1108 at McKinney Creek
Rutherford	NC	NC-055	Off SR 1111 below house on Dan River Prop.
Rutherford	NC	NC-056	Danieltown south to Henson Rd. to Floyd's Creek
Rutherford	NC	NC-057	Alexander Mills off 221A along RXR right of way
*Rutherford	NC	NA	Dan River Property off SR 1111 at pond
Rutherford	NC	NA	Dan River Property across Richardson Creek
Rutherford	NC	NA	Duke Power-Crescent Industries along Broad River
Rutherford	NC	NA	Duke Power-Crescent Industries along Broad River

Rutherford	NC	NA	Broad River near Railroad Trestle
Rutherford	NC	NA	Harris NC off of Road along Floyds Creek
Rutherford	NC	NA	Harris NC off of Hogan's Road along Floyds Creek
*Cherokee	SC	SC-016	Cowpens National Battlefield
Cherokee	SC	SC-017	Cowpens National Battlefield
Cherokee	SC	SC-018	Cowpens National Battlefield
Greenville	SC	SC-015	Bunched Arrowhead Preserve
Spartanburg	SC	SC-039	Landrum
Spartanburg	SC	SC-043	Landrum, back of 184 McKee Dr.
Spartanburg	SC	SC-027	Peters Creek Preserve
Spartanburg	SC	SC-028	Peters Creek Preserve
Spartanburg	SC	SC-032	Page Creek
Spartanburg	SC	SC-034	Arrowood Branch
*Spartanburg	SC	SC-026	Peters Creek Preserve
Spartanburg	SC	SC-019	USCS Campus
Spartanburg	SC	SC-011	Peters Creek Preserve
Spartanburg	SC	SC-014	Peters Creek Preserve

***Hexastylis heterophylla* and *Hexastylis minor* sites visited 2001-2003**

Species	County	State	Location
*HH	Caldwell	NC	HWY64/90
*HH	Catawba	NC	Bunkerhill Bridge,
HH	Iredell	NC	Harris Bridge Rd.
*HH	Madison	NC	Hickey's Fork
*HH	Madison	NC	AT Trail near Hot Springs
*HH	Polk	NC	Green River Cove
HH	Rutherford	NC	Luckadoo Mt 1
*HH	Rutherford	NC	Camp McCall Road 2
*HH	Rutherford	NC	Jonestown Road x Mt. Pleasant Church Rd.
*HH	Wilkes	NC	Brocktown Rd 1
HH	Wilkes	NC	Brocktown Rd 2
HH	Wilkes	NC	Wilkes Community College
HH	Wilkes	NC	Brocktown Rd 3
HH	Buchanan	VA	Rd. 628
*HM	Cleveland	NC	Broad River Greenway in plot
*HM	Gaston	NC	Crowders Mt. St. Park.
HM	Moore	NC	HWY 22 on Deer River
HM	Randolph	NC	Randolph Co.
HM	Richmond	NC	Marshland off Hwy 1 near Masrton
*HM	York	SC	Kings Mountain State Park.

APPENDIX C

Coordinates for *Hexastylis naniflora* populations

DD.DDN	DD.DDW
35.1914	81.9069
35.2028	81.9219
35.3067	81.9206
35.2081	81.8736
35.2089	81.6950
35.2103	81.8758
35.2108	81.9125
35.2114	81.8983
35.2119	81.8969
35.2125	81.8656
35.2136	81.8736
35.2150	81.6794
35.2153	81.6944
35.2161	81.6792
35.2167	81.8806
35.2192	81.8830
35.2217	81.6844
35.2222	81.9333
35.2247	81.6922
35.2253	81.0561
35.2267	81.6992
35.2289	81.8942
35.2292	81.6981
35.2292	81.9314
35.2317	81.0639
35.2317	81.9000
35.2319	81.9000
35.2333	81.9264
35.3333	81.9314
35.2620	81.9056
35.2667	81.8544
35.2686	81.8590
35.2800	81.6820
35.2825	81.5847
35.2847	81.5703
35.3075	81.8520
35.3086	81.9208
35.3189	81.6194
35.1264	81.3056
35.1816	81.9013
35.0503	82.0921

DD.DDN	DD.DDW
36.6125	81.4380
35.6347	81.4364
35.6403	81.5958
35.6447	81.3928
35.6686	81.3317
35.6697	81.5972
35.6700	81.0944
35.6728	81.5789
35.6742	81.1083
35.6786	81.0861
35.6836	81.3428
35.6972	81.1481
35.6975	81.4228
35.7022	81.2944
35.7047	81.3878
35.7175	81.2694
35.7194	81.1158
35.7408	81.8342
35.7597	81.5181
35.7611	81.3731
35.7711	81.6214
35.8189	81.4386
35.2043	81.9841
35.2124	81.9765
35.1571	82.2702
35.1443	82.1805
35.1818	82.0338
35.2243	82.0756
35.1269	81.8094
35.4353	81.2480
35.1230	81.7677
35.1075	82.2265
35.1766	82.1477
35.0195	82.4104
35.0227	82.3988
35.1063	81.9256
35.0221	82.3808
34.9882	81.8650
35.1572	82.1815
35.5120	82.1776
35.1809	82.1622

DD.DDN	DD.DDW
35.3306	81.4794
34.9709	81.9627
35.3514	81.4086
35.3792	81.6431
35.4203	81.4108
35.9952	81.8635
34.9718	81.9562
34.9952	82.4029
35.4203	81.2463
35.5375	81.4278
35.5375	81.4167
35.5406	81.4200
35.5464	81.1597
35.5519	81.7094
35.5594	81.5386
35.5764	81.5572
35.5819	81.5375
35.1267	81.8052
34.9991	81.9708
34.9002	81.9350
34.0406	82.2116
35.1004	82.0367
35.1017	82.0367
35.0907	81.8869
35.0681	81.0963
35.1067	81.9256
35.1742	81.1714
35.1264	81.3056
35.1816	81.9013
35.0503	82.0921
35.1572	82.1815
35.5120	82.1776
35.1809	82.1622
35.1279	81.4940
35.1139	81.7469
35.1328	81.4805
35.1279	81.4940
35.1139	81.7469
35.1328	81.4805
35.1909	82.1428

APPENDIX D
Recommendations for Conservation of *Hexastylis naniflora*

Recommendations for Conservation of *Hexastylis naniflora*:

These recommendations were written using the US Fish and Wildlife Service (USFWS) recovery plan for *Liatris helleri* Porter as a template. Taxonomy and ecology in *Hexastylis naniflora* are not addressed here, since they are addressed in other parts of the report.

It is worth mentioning here a few notable people who have contributed to the conservation efforts of *Hexastylis naniflora.* H. L. Blomquist (1957) described *Hexastylis naniflora* and stated that it was rare and restricted to a small area of North and South Carolina. It would be another twenty years before L. L. Gaddy (1980, 1981, and 1987) would address the conservation issues regarding *Hexastylis naniflora.*

In April of 1989 the Department of the Interior formally listed *Hexastylis naniflora* as a federally Threatened species and afforded it some protection. In the late eighties and early nineties, Dr. Gillian Newberry (1995, 1996) made progress in developing techniques for moving and transplanting *Hexastylis naniflora* populations that were in danger of being destroyed. Her techniques have been used in recent moves of the plant from North Carolina Department of Transportation (NCDOT) construction sites. Dr. Newberry was instrumental in the location of a large number of new populations in South Carolina and a few new sites in North Carolina. With the number of sites increasing over time and with a few sites already receiving some protection, conservation efforts have greatly improved the outlook for *Hexastylis naniflora.* This situation affords the USFWS with a rare opportunity to move towards delisting *Hexastylis naniflora*.

Current Status :

*Hexastylis naniflor*a is listed as a federally Threatened plant species. It is currently known from approximately 150 populations and sub-populations in an eleven county area of North and South Carolina. Declines in known populations have occurred in Lincoln and Rutherford Counties in North Carolina as well as Spartanburg and Greenville Counties in South Carolina. The reasons for those declines range from highway construction and lake construction to urban sprawl and logging. Also, habitat destruction from pasture and small pond development has eradicated a number of populations.

Habitat Requirements and Limiting Factors:

Hexastylis naniflora is a very restricted species. Even with the seemingly high number of populations present, the actual numbers of individual plants vary greatly. Some populations have as few as twenty individuals while others may have upwards of 2000. The reason for this varying fluctuation in population sizes is due mainly to the soil which *Hexastylis naniflora* is found in. *Hexastylis naniflora* prefers acidic soils that are sandy-loam such as Pacolet, Madison, and Museulla soils. Recent soil analyses show that soil chemistry is very important to the location of *Hexastylis naniflora* (Padgett et al. 2003). Topography also seems to play a part in *Hexastylis naniflora* location in any given habitat. It generally grows on the north facing side of slopes hills and ravines.

Recovery Objective:

Delisting of the species from the Endangered Species List.

Recovery criteria:

Hexastylis naniflora will be considered recovered when ten healthy populations are self-sustaining within its historical distribution and the locality of each of those populations contains substantial genetic variability. A population that reproduces and is large enough to maintain genetic variability to survive and respond to natural changes in the habitat and environment will meet the criteria as a population healthy enough to receive protection. *Hexastylis naniflora* should be considered for delisting when the following criteria are met.

1. Of the 150 plus known populations and sub-populations of *Hexastylis naniflora* which are known to exist, at least twenty should be offered some sort of protection with at least ten populations receiving greater protection.

2. Management of those protected populations should be done in cooperation with the landowners and the necessary government agencies, and any and all management actions should be well documented to ensure that future protection of those sites is not an issue.

3. With the location of new sites over time, at least one site per ten new sites found should be set aside and protected especially if they fall into locations where genetic variability might be of concern.

4. With the original ten sites placed under protection, ensure than any future human encroachments or natural threats are dealt with and that the survival of those sites is ensured.

Actions needed:

1. Survey of suitable habitats without *Hexastylis naniflora* present as possible transplant locations.

2. Monitor sites already under some protection.

3. Pollination studies.

4. Conduct research into threats on *Hexastylis naniflora* and its habitat, both biotic and abiotic.

5. Implement management practices at all key sites.

6. Involve the public though media and educational efforts.

7. Genetic analysis of intra-specific variation.

Date of Recovery:

The delisting date is not known at this time.

Management and Recovery Plan for *Hexastylis naniflora*

With *Hexastylis naniflora* being a Federally Threatened and State Threatened plant species in both North Carolina and South Carolina, efforts should be made to protect a set number of populations across the natural range to ensure its survival. The ultimate goal is to have *H. naniflora* delisted, but to do that a substantial number of viable populations with intact plant communities must be set aside and given protection. Another consideration when setting aside protected site should be the plant's ability to transfer genetic material in order to maintain a self-sustaining population. With the pollination mechanisms not well known, a study into pollination vectors might be required before any recovery plan can be successful. In order for the delisting and recovery of *Hexastylis naniflora* to be successful, the following criteria must be met.

1. Of the 150 plus known populations and sub-populations of *Hexastylis naniflora* which are known to exist, at least twenty should be offered some sort of protection with ten populations receiving greater protection.

2. Management of those protected populations should be done in cooperation with the landowners and the necessary government agencies, and that any and all such actions should be well documented to ensure that future protection of those sites is not an issue.

3. With the location of new sites over time, at least one site per ten new sites found

should be set aside and protected especially if they fall into locations where genetic variability might be of concern.

4. With the original ten sites placed under protection, ensure than any future human encroachments or natural threats are dealt with and that the survival of those sites is ensured.

The timetable for a recovery and management plan of *Hexastylis naniflora* could proceed quickly if all the agencies and individuals involved can work towards getting critical habitat under protection either by outright purchase of property or by mitigation for sites. After ten good sites are protected, the USFWS could start the proceedings for a delisting of *Hexastylis naniflora* from the Endangered Species List.

Narrative Outline:

Hexastylis naniflora is an herbaceous evergreen perennial found in the western piedmont and foothills of North and South Carolina. It is limited in range due to its need for acidic sandy-loam soils and topographic locality. It is also generally restricted to stream heads and the moist ridges and hills adjacent to those streams, provided they are north facing and have suitable habitat. It is associated with a number of species that are found to be frequent in those same habitats, so when locating new populations, associate species information is very useful in locating favorable habitat. Of the 150 plus *Hexastylis naniflora* sites located in North Carolina and South Carolina, only a few are

under any sort of protection. In the past, suitable habitat for *Hexastylis naniflora* was destroyed for use as pasture- land, ponds, lakes, and peach orchards, which are all found frequently around the stream head habitats where *Hexastylis naniflora* is generally located. Only one site falls under Federal protection (Cowpens National Battlefield) and a few others fall under some sort of State and Local protection. Spartanburg Waterworks currently has one of the largest populations of *Hexastylis naniflora* with some formal local protection. Other sites of interest with large populations of *Hexastylis naniflora*, and some protection with the Natural Heritage Program are Henson's Ravine in southern Rutherford County, NC and Peter's Creek Heritage Preserve in northern Spartanburg County, SC.

Management Issues:

1.1 The first step will be setting aside ten well-protected viable *Hexastylis naniflora* populations. There are currently five *Hexastylis naniflora* sites that are receiving some sort of protection at the Federal, State, or Local level. With more sites protected across its historical range, a delisting of *Hexastylis naniflora* can proceed with minimum concern about long-term survival of the species.

1.2 Search for additional population should be encouraged and documented with the proper agencies. In recent years the number of known *Hexastylis naniflora*

populations has increased dramatically, but those sites generally harbor small numbers of individuals due to the habitat restrictions of this plant. The historical range of *Hexastylis naniflora* has changed over time as well with several counties found to have small populations located in them. Additional populations might give rise in an increased number of protected sites over time, which further aids in the recovery of the species.

1.3 Habitat protection should be considered when setting aside *H. naniflora* populations for protection. Well-maintained habitats offer a higher species diversity and provide a more stable environment for *Hexastylis naniflora.*

1.4 North Carolina Department of Transportation (NCDOT) has to mitigate for *Hexastylis naniflora* when Highway right-of-way comes in contact with populations of *Hexastylis naniflora.* The process of mitigation cost the taxpayers millions of dollars each year when mitigation takes place. With mitigation dollars, NCDOT could help to place a number of *Hexastylis naniflora* sites into protection, which should allow the US Fish and Wildlife Service to consider the process of delisting.

1.5 The USFWS would benefit from a delisting by focusing their attention on other more important issues at hand. The legal issues that USFWS faces from outside groups, which are in contest with them over their actions regarding the Endangered Species Act of 1973, would be reduced through delisting of *Hexastylis naniflora*.

1.6 Develop management plans and research programs at protected sites, which include, USFWS, NCDOT and the landowners. A working partnership between those agencies directly associated with *Hexastylis naniflora* and the landowners where protected sites may fall will be crucial for the future conservation of those sites. With this type of management practice now well developed, a close working relationship between those involved in management and protection should be maintained in order to promote the survival of *Hexastylis naniflora.*

1.7 Look at protection alternatives for *Hexastylis naniflora*. There are two areas of major interest here. The first would be to find suitable sites that are currently protected which might have no *Hexastylis naniflora* located on them, but might be used for re-location of populations in danger of being destroyed. Re-establishment or establishment of *Hexastylis naniflora* into an area must be looked at in further detail. Seed collection and propagation should be studied in order to have success in any such attempts. Another alternative to protecting sites is through transplanting. In the fall of 2000, 175 H*exastylis naniflora* plants were transplanted onto an adjacent site along Little Gunpowder Creek in Caldwell County, North Carolina using a technique developed by Dr. Gill Newberry (1996) at the University of South Carolina. After three years and harsh drought conditions, 68% of the initial transplants were still alive. With this site, the conditions for *Hexastylis naniflora* were pre-existing because of plants growing adjacent to site which was to be destroyed. If a suitable site is not adjacent to a proposed site to be destroyed, then special attention must be paid to the soils and topography of any site thought to be favorable. The second initiative would entail cultivating a number of *Hexastylis*

naniflora plants in greenhouse(s) for the purpose of providing a seed bank. This would ensure that genetic variability is maintained and if a protected site is destroyed by some natural occurrence, that replacement plants for that site would be available.

1.8 Populations that are protected or otherwise should be give a rating for size and habitat quality. Each existing known population of *Hexastylis naniflora* should be examined and a rate given for the number of individuals in that population and a score given for the quality of that habitat. Once each population has been scored, they can then be monitored for short and long term changes. The following are examples of scoring systems that might be used.

Table 1. Class scoring that might be used for data collection regarding population size of *H. naniflora.*

Population Size	**Class for Population Size**
< 50	**1**
50-100	**2**
100-300	**3**
300- 500	**4**
500-1000	**5**
> 1000	**6**

Table 2. Habitat scoring that might be used in data collection regarding habitat quality of *Hexastylis naniflora* populations

Grades for Habitat
A - Excellent habitat. Mature forest with all the elements of the forest community present. Low percentage of invasive species present.
B - Very good habitat with maturing trees and all elements of the forest community present. Low percentage of invasive species present.
C - Above average habitat. Most of the elements of the forest community still in present. Moderate percentage of invasive species present.
D - Average habitat. Logging in the past 50 years evident by tree size and some elements of the forest community missing. Moderate percentage of invasive species present.
E - Below average habitat. Recent logging, erosion or urban sprawl apparent with a lot of the element of the forest community missing. High percentage ofinvasive species present.
F – Poor habitat. Clear cut, or recent logging. Erosion massive and urban sprawl eminent. Most of the elements of the forest community are missing. Existing plants are imperiled. High percentage of invasive species present.

Monitoring of *Hexastylis naniflora* populations can give information regarding abiotic and biotic factors within those sites. The effects of weather such as periods of drought and excess moisture can be examined. The effects of human impact on sites can be examined as well by looking at foot trample, the effects of logging, and burning (prescribed or natural) on existing sites. This information would be very valuable to individuals and agencies that are trying to develop and set up management plans on existing sites under protection or those proposed to be protected.

1.9 Designate and enforce laws to protect *Hexastylis naniflora* and its habitat. With protection comes enforcement of those protected site. North Carolina prohibits the taking of this species without a permit and the landowner's permission and regulates trade in the species. Signs should be placed in high-risk areas where collection might occur. Unwanted attention should not be given to the species in any location where it might be collected or removed. Law enforcement agents whose jurisdiction includes protected sites should be made aware of the status of *Hexastylis naniflora* and should be taught how to identify the species. Anyone caught digging; cutting, removing or destroying plants in knowing violation without a permit should be subject to any State law or regulation, including criminal trespass laws.

2.0 Information released through various media is important in the education of the public with regards to *Hexastylis naniflora*. In recent years, the public has become more aware of conservation issues, and many of them are willing to help, but they lack the knowledge or information to do so. Though news releases and informational

brochures, the public can be made aware of the efforts being made to protect a species, which is federally and state endangered or threatened. Publications in popular magazines and science journals, regarding research being done with *Hexastylis naniflora*, the public as well as the scientific community can be made aware of conservation and protection efforts ongoing. A periodic review of recovery efforts should be given stating the current status of the managements implications and evaluations should be made regarding ongoing actions or ant re-directional changes which might be called for in assuring that the plans goals are being achieved is a quick and successful manner.

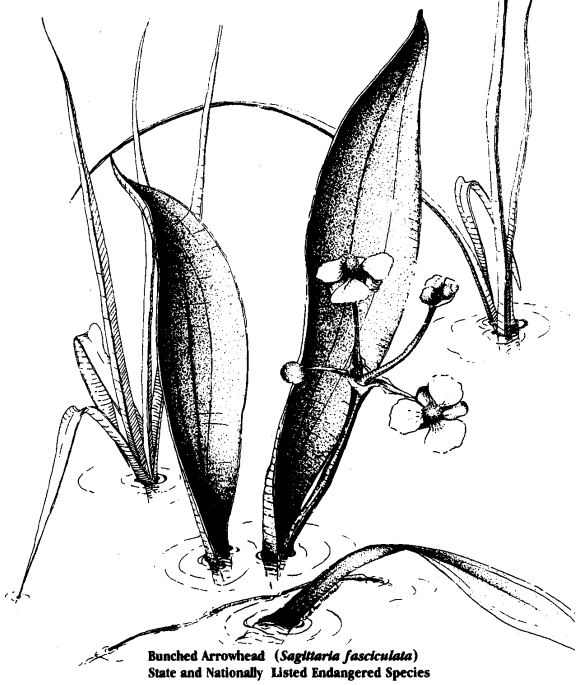
Appendix E. Collecting and transplanting permits from North and South Carolina.

rotected Plant Conservation Permit and Record

C

North Carolina Department of Agriculture **Plant Industry Division**

Conservation Permits are issued to individuals who have legally obtained state listed ndangered and threatened plant species: either propagated material already under ermit, or plants collected under appropriate approval. Permits can be obtained for all ndangered and threatened plant species for purposes that will enhance the survival of ıe species, including scientific research for the purpose of studying the biology and ropagation of the species, rescue operations to save plants from unavoidable destruc- ion, and growing of plants by home gardeners.
State regulations do not allow the sale or exchange of plants covered by this permit.



Bunched Arrowhead (*Sagittaria fasciculata*)
State and Nationally Listed Endangered Species

James E. Padgett
(nursery or individual)

ıas been approved for the possession of the plants listed below for the purposes of :onservation.

Address Box 11349 Appalachian St. U.
Boone, NC 28608

Phone (828) 245-6762

I certify that this record of state listed Endangered and Threatened plants is accurate and true; that I understand the regulations concerning the conservation of state listed Endangered and Threatened plants; that these plants, their propagules and offspring cannot be legally sold, exchanged, or given away; and that this permit must accompany these plants wherever they are held or whenever they are moved.

Signature Jaun E. Padgett Date 3-9-01 Nursery

The plants recorded below have been inspected and found to be obtained and held in accordance with the Plant Protection and Conservation Act, GS 19b, 106-202.12 to 106-202.19.

Inspector [signature] Date 3/15/01

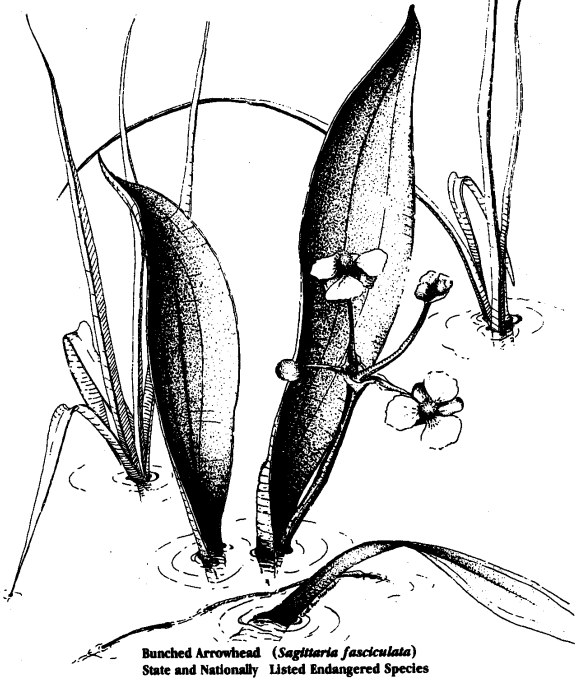
Plant Pest Administrator [signature]

Species:	Number/Unit of plants in possession:	Original source of plants:	Notes:
Hexastylis ~~Species~~ naniflora	3 lvs + 3 fls per pop. plus voucher for new pops	All NC sites	Sp research - see application. Not to exceed 10% of lvs or fls in any one pop. NHP field survey form completed for ea new pop.

~~otected~~ Plant Conservation Permit and Record

North Carolina Department of Agriculture **Plant Industry Division**

C



Bunched Arrowhead (*Sagittaria fasciculata*)
State and Nationally Listed Endangered Species

Conservation Permits are issued to individuals who have legally obtained state listed
langered and threatened plant species: either propagated material already under
mit, or plants collected under appropriate approval. Permits can be obtained for all
langered and threatened plant species for purposes that will enhance the survival of
species, including scientific research for the purpose of studying the biology and
pagation of the species, rescue operations to save plants from unavoidable destruc-
n, and growing of plants by home gardeners.
tate regulations do not allow the sale or exchange of plants covered by this permit.

James E. Padgett
(nursery or individual)

s been approved for the possession of the plants listed below for the purposes of
nservation.

ldress ASU Box 11349
Boone, NC 28608

one (828) 262-2674 (Zack Murrell's office)

I certify that this record of state listed Endangered and Threatened plants is accurate and true; that I understand the regulations concerning the conservation of state listed Endangered and Threatened plants; that these plants, their propagules and offspring cannot be legally sold, exchanged, or given away; and that this permit must accompany these plants wherever they are held or whenever they are moved.

Signature James E. Padgett Date 12-7-00 Nursery _____

The plants recorded below have been inspected and found to be obtained and held in accordance with the Plant Protection and Conservation Act, GS 19b, 106-202.12 to 106-202.19.

Inspector _____ Date 12-7-2000

Plant Pest Administrator Gene B. Crow

Species:	Number/Unit of plants in possession:	Original source of plants:	Notes:
Hexaslylis naniflora	15 plants & cuttings	Rescue 12/7/2000 at Little Gunpowder Creek, Caldwell County	For research at ASU

SPECIAL USE PERMIT APPLICATION

SC DEPARTMENT OF NATURAL RESOURCES
WILDLIFE DIVERSITY SECTION
PO BOX 167
COLUMBIA, SC 29202

Site Name/Description: Bunched Arrowhead Preserve

Duration and Times for Use/Study: My current study/research runs through December 2002

Purpose: I would like to obtain leaf material (3 leaves per population) of Hexastylis naniflora. I would also like to obtain permission to do an ecological survey if possible.

Personnel: 1. James E. Padgett – Grad student, ASU
2. Dr Zack E. Murrell - Advisor/Professor, ASU
3. Yet to be named undergrad assistant

Special Conditions: No digging or removal of artifacts.

Approved:

SCDNR _____ Archaeologist

SCDNR _____ Preserve Manager, Mary Bunch

7/26/01

B- Pls mail this to Jim Sorrow

Jim

SPECIAL USE PERMIT APPLICATION

SC DEPARTMENT OF NATURAL RESOURCES
WILDLIFE DIVERSITY SECTION
PO BOX 167
COLUMBIA, SC 29202

Site Name/Description: Peters Creek Heritage Preserve

Duration and Times for Use/Study: My current study / research runs through December 2002

Purpose: I would like to obtain leaf material (3 leaves per population) of Hexastylis naniflora for molecular work. I would also like to obtain permission to do an ecological survey of the population.

Personnel: 1. James E. Padgett Grad Student, ASU
2. Dr Zack E. Murrell Advisor/Professor, ASU
3. Yet un-named undergrad assistant

Special Conditions: no ARTIFACT collection Allowed

Approved:
SCDNR _____ Archaeologist
SCDNR
James A. Sorrow Preserve Manager, ~~Mary Bunch~~