MOLECULAR PHYLOGENY AND COMPARATIVE POLLEN MORPHOLOGY OF THE GENUS *HEXASTYLIS* (ARISTOLOCHIACEAE)

A Thesis
by
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FOREWORD

It is my intention to submit this thesis for publication in the journal *Systematic Botany*. Therefore, the contents and formatting of this thesis may deviate significantly from those required by the *Student Handbook for Dissertation and Thesis Preparation* to allow for publication in the aforementioned journal with minimal alterations.

ABSTRACT

Asarum, commonly known as wild ginger, heartleaves, or little brown jugs, is a genus belonging to the family Aristolochiaceae. *Asarum* exhibits a Laurasian distribution, with species in Europe, Asia, and North America. Of particular interest to this study is the genus *Hexastylis*. Recent studies showed that *Hexastylis* is rooted within *Asarum* and suggested to be paraphyletic, but failed to adequately examine relationships within this southeastern United States complex of species. The genus, as currently understood, consists of nine species and four varieties. Pollen was examined for all currently recognized *Hexastylis* taxa. Although pollen analysis showed similar surface features for most taxa, some variation in morphology became visible under increased magnification. One species, *Hexastylis naniflora* lacked some surface features present in the other taxa. There is currently no robust molecular analysis of relationships within the genus. Three chloroplast regions were sequenced to resolve the relationships. We have sequenced both genes for twenty taxa including ingroups and outgroups. Genetic information was analyzed using Bayesian, Parsimony, and Maximum Likelihood methods. Contrary to a prior study, *Hexastylis* was found to be monophyletic as indicated by a 6 bp insertion.

Keywords– *Asarum*, Bayesian, cpDNA, intergenic spacer, Maximum Likelihood, Maximum Parsimony, *matK*, phylogeography, SEM.

ACKNOWLEDGEMENTS

There many individuals who have influenced my interests and contributed to my research. In particular, I would like to acknowledge my undergraduate advisor, Dr. Gerald Smith at High Point University who inspired me to pursue research in botany.

During my time as a graduate student at Appalachian State University, my advisor Dr. Zack Murrell has been extremely helpful at every stage of my research from deciding on a topic to reporting my findings. I would also like to acknowledge my committee members, Dr. Eva Gonzales and Dr. John Walker. I would like to thank Emily Gillespie for sharing her extensive knowledge of molecular phylogenetics. I would also like to acknowledge Dr. Guichuan Hou and Jim Sobieraj for contributing their expertise. I would like to thank my predecessor in *Hexastylis* research at the Murrell lab, Joy Vandervort-Sneed for sharing *lfy* data and introducing me to pertinent techniques. Two undergraduates, Sam Marshall and Julia Mitchell assisted me in data entry and lab work.

Mark Rose (Greensboro, North Carolina) and James Padgett (Boiling Springs, North Carolina) provided me with fresh pollen and leaf specimens from their collections for my use. I would also like to acknowledge the Appalachian State University Biology Department and the Office of Student Research for funding.

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INTRODUCTION

Aristolochiaceae, the birthwort family, consists of eight genera and 500 species of herbs and vines. The two major genera in the family are *Aristolochia* with 300-350 species and *Asarum* with about 70 species (Judd et al. 2002). After *Hexastylis* was first segregated from *Asarum* by Rafinesque (1825), it slowly gained general acceptance in the North American literature (Britton and Brown 1913; Small 1933; Radford et al. 1968; Blomquist 1957; Gonzalez 1972; Otte 1977; Kral 1983; Gaddy 1981, 1986, 1987a, 1987b; Wofford 1989; Rayner 1994). Currently, *Hexastylis* is commonly used to describe a genus of nine species and four varieties that are endemic to the southeastern United States.

The genus *Asarum* has a Laurasian distribution. More than 75% of the species of *Asarum* are found in Asia, suggesting to some that it originated there. Fifteen to twenty percent of *Asarum* species occur in North America and only one species is found in Europe. The Laurasian distribution of *Asarum* is a result of the Eastern Asian-Eastern North American Floristic Disjunction (Kelly 1998a). Botanists since the time of Linnaeus have noticed a resemblance between the two floras. Asa Gray (1878) was aware of this distribution. Sixty-five genera are known to display the disjunction (Wen 1999). There are several possible explanations for this distribution. It is hypothesized that there was a widespread distribution of broad-leaved deciduous forest elements in the northern hemisphere in the past. The separation of the continents caused the loss of land bridges and led to fragmentation of the distribution (Wolfe 1975), leaving remnants in eastern Asia,

eastern North America, and the Pacific Northwest. Over the past several million years, there have been multiple periods of climatic cooling. Glaciers formed in northern North America forcing temperate forest elements (Delcourt and Delcourt 1991), and theoretically *Hexastylis*, to migrate southward. There is a lack of these forest elements in Europe due to the Caucus Mountains running east to west suggesting that there was no southward migration route during glacial periods (Gupta 1972).

Palynological techniques have been used to construct broad-brush maps of past vegetation. Pollen is extracted from cores taken from bogs and swamps, carbon dated, and compared with the taxa present in modern day pollen rain. Based upon the analysis of pollen cores from across eastern North America (Delcourt and Delcourt 1981), it is theorized that Eastern North America once consisted of spruce/fir, tundra, and oak/hickory forests at much greater latitudes than currently distributed. During the last glacial maximum (18,000 y.b.p), the Laurentide Ice Sheet covered northern North America (Delcourt and Delcourt 1981), forcing forests to migrate south. When the ice sheet retreated, the forests migrated north to their present locations. Since *Hexastylis* is not wind pollinated (Otte 1977), it is unknown where it migrated during glacial periods, although it is possible that it retreated towards the coast or into deep ravines and other areas protected from the extreme weather conditions (Padgett 2004).

Asarum is a genus first established by Linnaeus (1753). Rafinesque (1825) segregated *Hexastylis* from the genus Asarum. He separated *Hexastylis* from *Asarum* based on leaf morphology and texture. *Hexastylis* has thick leathery evergreen leaves, while *Asarum* has thinner deciduous leaves. There are other notable differences, including the fact that *Hexastylis* flowers and stems are glabrous on their outer surface, while stems and flowers of

Asarum are pubescent. Also the calyx lobes of *Asarum* tend to be much longer and come to a sharper point than those of *Hexastylis*. As a result of these morphological differences, most taxonomists recognize *Hexastylis* and *Asarum* as separate genera, as is evident in most modern floristic treatments.

Hexastylis is pollinated by insects, including wasps, flies, and thrips (Wyatt 1955; Otte 1977; Murrell and Carroll 1995; Libby et al. 1996). Studies indicate that individuals are self-compatible but outcross 95 percent of the time. Stigmas are located below the anthers, an arrangement that is thought to lead to low rates of self-fertilization (Gaddy 1987b). Pollination in *Hexastylis* is not well studied and subsequently knowledge is generally lacking in this area. Seeds of *Hexastylis* are dispersed by ants. The seeds have a fleshy and nutritious eliasome, which attracts ants as a food source. The ants carry the seeds back to their nests and consume the eliasome. Seed germination takes place at the nests. Thus, the dispersal of *Hexastylis* seeds is limited by the home range size of the ants (Gaddy 1987a).

Blomquist (1957) established the currently recognized grouping of *Hexastylis*, which gained wide acceptance. Blomquist's divisions of the genus were based upon morphological characters that have been shown to be very plastic, but the structure he erected does provide a framework for molecular analysis of the group. The genus, as recognized by Blomquist, consists of three groups: Arifolia, Speciosa, and Virginica. The group Arifolia has only one species, *Hexastylis arifolia*, with two varieties, var. *ruthii*, and var. *callifolia*. The second group, Speciosa, consists of a single species, *H. speciosa*. The third group, the Virginica group, is divided into three subgroups, Virginica, Shuttleworthii, and Heterophylla. The Virginica subgroup contained only *H. virginic*a. Morphological analysis by Gaddy (1987a) placed *H. rhombiformis* into the Virginica subgroup. The Shuttleworthii subgroup, as

recognized by Blomquist, had two species, *H. shuttleworthii* and *H. lewisii*. The Heterophylla subgroup contains *H. heterophylla, H. minor,* and *H. naniflora*. Gaddy (1987a) suggested that *H. contracta* was allied with the Heterophylla subgroup. The *Hexastylis heterophylla* subgroup was thought by both Blomquist (1957) and Gaddy (1987a) to form an overlapping complex of species.

Gaddy (1987a) also constructed distribution maps for *Hexastylis*. Since many more herbarium specimens had been collected since Blomquist's (1957) study, Gaddy had access to an extended supply of data, resulting in the mapping a greater number of populations. A new species, *Hexastylis naniflora* was included on Gaddy's maps. *Hexastylis shuttleworthii* var. *harperi* was segregated from *H. shuttleworthii* since Blomquist's publication and was therefore mapped by Gaddy (1987a).

Recent work by Kelly (1997, 1998a, 1998b, 2001) using morphology and molecular data supported that *Hexastylis* is not a genus distinct from Asarum, a treatment supported by the previous studies of Araki (1953) and Barringer (1993). Kelly (1998a) conducted molecular analysis on the Internal Transcriber Spacer (ITS) region from a number of *Asarum* species from Asia as well as the North American species *Asarum canadense*, but only included three species of *Hexastylis*. His work suggested that *Hexastylis* is rooted within *Asarum* and should be treated as *Asarum*, but sampling of taxa within this southeastern United States complex of species was limited. In addition, Kelly's results depicted *Hexastylis* with two separate lineages, and therefore not monophyletic (Fig. 1).

Weakley's (2010) flora contains the most current treatment of *Hexastylis*, recognizing ten species. Citing a lack of sufficient evidence for combination of *Hexastylis* into *Asarum*, he segregated *Hexastylis* as a distinct genus. Included in Weakley's flora are *H.*

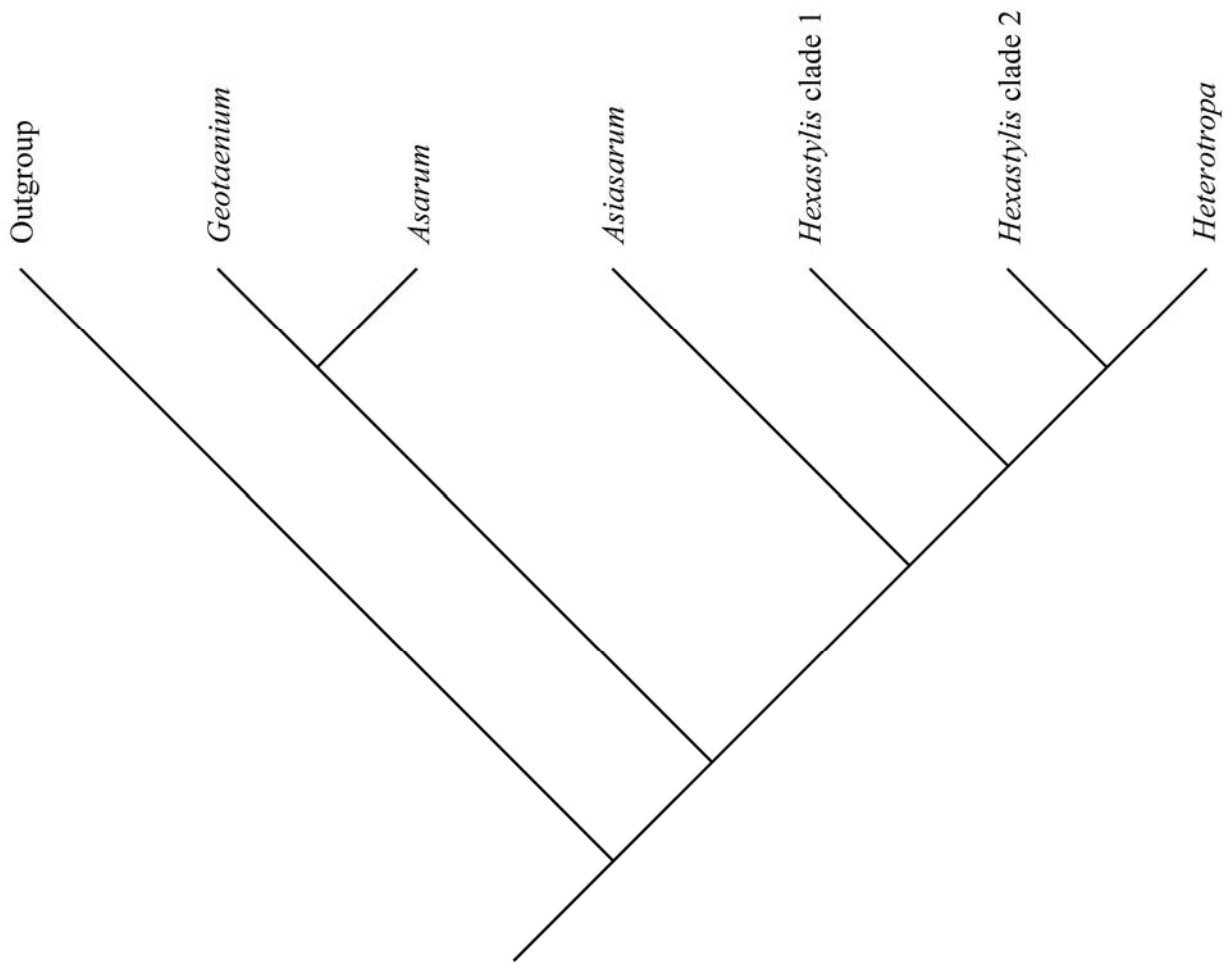

Fig. 1. A greatly simplified representation of Kelly's (1998a) phylogeny of *Asarum* sensu lato based on ITS sequences and morphology. *Hexastylis* clade 1 consists of *H. arifolia* and *H. speciosa*. *Hexastylis* clade 2 consists of *H. shuttleworthii*, *H. minor*, and *H. virginica*.

arifolia with three varieties: *H. arifolia* var. *arifolia, H. arifolia* var. *ruthii, H. arifolia* var. *callifolia*. *Hexastylis shuttleworthii* is recognized as two varieties: *H. shuttleworthii* var. *shuttleworthii* and *H. shuttleworthii* var. *harperi*. The other species are *H. lewisii, H. virginica, H. heterophylla, H. naniflora, H. minor, H. contracta, and H. rhombiformis*.

Objectives—My objectives were to estimate the phylogeny of *Hexastylis* using chloroplast genes, document any variations in pollen morphology for the genus, and produce updated distribution maps.

MOLECULAR—Noncoding chloroplast genes as well as sensitive fingerprinting techniques are commonly used to resolve plant phylogenies. The *matK* gene, a rapidly evolving chloroplast gene has been used extensively to resolve phylogenies (Soltis et al. 2000)**.** Shaw et al. (2007) described the relative usefulness of various chloroplast intergenic spacer regions with potential utility in low level phylogenetic studies. Due to the variation in sequences of *Hexastylis* species I found in preliminary studies for the *matK* gene and the *rpl32-trnL* and *trnQ-5-rps16* intergenic spacer regions, I used those regions for my analysis.

POLLEN**—**Walker (1974) examined the pollen of 230 genera of primitive angiosperms including that of *Asarum caudatum, Hexastylis virginica*, and *H. heterophylla*. He observed pollen that was inaperturate, but with some pollen grains having irregularly-shaped breaks or holes in the exine, or outer wall. Noting that these breaks develop from pollen hypothesized to be ancestrally inaperturate, he suggested that these represent "stages in the evolution of colpate and porate pollen within the Aristolochiaceae."

Padgett (2004) reported that the pollen of *Hexastylis naniflora* was unique in that it the lacked obvious raised surface structures, or gemmae, found in the other species of the genus. In the present study, I document the morphological plasticity of the pollen within the

genus *Hexastylis*. Such information could be useful in helping understand evolutionary relationships as well as in the identification of herbarium specimens. In my preliminary studies, I noted that pollen obtained from herbarium specimens was often considerably distorted. Therefore, to avoid these distortions, I only used freshly collected, critical-point dried pollen for data analysis. I examined the pollen grains of eleven of the thirteen taxa. The difficulty of obtaining fresh pollen grains in the short window of flower maturation prevented me from examining the pollen of all thirteen taxa.

DISTRIBUTION MAPS—Since Gaddy (1987a) published distribution maps for *Hexastylis*, new resources such as the Flora of the Southeast (2009) have become available online. Additionally, new populations of *Hexastylis naniflora* have been documented by Padgett (2004). To utilize the resources available and provide up to date distribution data, I felt it necessary to produce updated distribution maps.

METHODS

Molecular—Samples were obtained from all named taxa of *Hexastylis*. This includes *H. arifolia* var. *arifolia, H. arifolia* var. *ruthii, H. arifolia* var. *callifolia, H. lewisii*, *H. virginica, H. heterophylla, H. naniflora, H. minor*, *H. contracta, H. rhombiformis, H. shuttleworthii* var. *shuttleworthii* and *H. shuttleworthii* var. *harperi*. *Isotrema macrophylla* was used as an outgroup. *Asarum canadense, A. caudatum, Asiasarum sieboldii*, *Heterotropa hatsushimae, H. savatieri,* and *H. savatieri* were used as placeholders for their clades.

DNA was extracted from frozen leaf material with Qiagen DNeasy Plant Mini Kits (Qiagen, Valencia, CA, USA). Concentration and quality of the extracted DNA was assessed using a NanoDrop spectrophotometer (NanoDrop Technogies).

Methods for amplifying the *matK* gene (Tables 1-2) follow those used by Ooi (1995). The *matK* gene was then PCR amplified (GeneAmp PCR system 9700) using *matK*-AF (CTATATCCAATCTTTCAGGAGT) and *matK*-8R (AAAGTTCTAGCACAAGAAAGTCGA) primers and GoTaq Green Master Mix (Promega). Reactions contained 12.5µl GoTaq, 10.5µl Nuclease free water, 0.5µl *matK*-AF forward primer, 0.5µl *matK*-8R reverse primer and 1µl template DNA. PCR cycles consisted of 1 min at 94°C followed by 30 cycles of one minute at 94°C , 1 minute at 45°C, and 2 minutes at 72°C with a final extension of 15 minutes at 72°C. PCR product was purified using a Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). A solution of 80-90 ng of DNA, 0.8ul of *matK* forward or reverse primer and enough water to make the total volume

of 20 ul was sent to Cornell CLC (Ithaca, NY, USA) for sequencing. Methods for sequencing the intergenic spacer regions (Tables 1-2) follow those used by Shaw et al. (2007). The *rpl32-trnL* region was PCR amplified (GeneAmp PCR system 9700) using *rpl32-F* (CAGTTCCAAAAAAA CGTACTTC) and *trnL*$^{(UAG)}$ (CTGCTTCCTAAGAGCAGCGT) primers and GoTaq Green Master Mix (Promega). The *trnQ-5-rps16* region was ampified using *trnQ*$^{(UUG)}$ (GCGTGGCCAAGYGGTAAGGC) and *rpS16x1* (GTTGCTTTYTACCACATCGTTT). PCR cycles consisted of 5 min at 80°C once followed by 30 cycles of one minute at 95°C, 1 minute at 50°C, and 4 minutes at 65°C with a final extension of 5 minutes at 65°C. PCR product was purified using a Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). Sequencing was performed by Cornell CLC. A solution of 80-90 ng of DNA, 0.8ul of primer and enough water to make the total volume 20ul was sent to Cornell CLC (Ithaca, NY, USA) for sequencing. Sequences were obtained in two parts, one with the forward primer and the other with the reverse primer.

Sequence Alignment and Phylogenetic Analysis—Sequences were aligned using ClustalW (Larkin et al. 2007). Sequences were then visually examined using MacClade 4 (Maddison and Maddison 2005) and any necessary changes were made. Sequences were combined into a single data matrix for analysis. Sequences were analyzed with Bayesian methods using MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) parsimony using PAUP (Swofford 2002), and maximum likelihood using RAxML (Stamatakis et al. 2008).

TABLE 1. Chloroplast regions and primers used.

DNA region	Primer name	Primer sequence (5'-3')	Source
matK	*matK*-AF	CTATATCCAATCTTTCAGGAGT	Ooi et al. (1995)
	matK-8R	AAAGTTCTAGCACAAGAAAGTCGA	Ooi et al. (1995)
rpl32-trnL	$trnL^{(UAG)}$	CTGCTTCCTAAGAGCAGCGT	Shaw et al. (2007)
	rpl 32-F	CAGTTCCAAAAAAACGTACTTC	Shaw et al. (2007)
trnQ-rps16	$trnQ^{(UUG)}$	GCGTGGCCAAGYGGTAAGGC	Shaw et al. (2007)
	rpS16x1	GTTGCTTTYTACCACATCGTTT	Shaw et al. (2007)

TABLE 2. PCR cycling conditions.

Regions	Initial denaturing step, time	reps	Denaturing temp., time	Primer annealing temp., time	Chain extension temp., time	Final extension temp., time	Primer source
matK	94°C, 1min	30	94°C, 1min	45°C, 1min	72°C, 2min	72°C, 15min	Ooi et al. (1995)
rpl32-trnL	80°C, 5min	30	95°C, 1min	50°C, 1min	65°C, 4min	65°C, 5min	Shaw et al. (2007)
trnQ-rps16	80°C, 5min	30	95°C, 1min	50°C, 1min	65°C, 4min	65°C, 5min	Shaw et al. (2007)

MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck 2003) is a Bayesian method for phylogenetic analysis that requires the use of a model to run. Evolutionary models were chosen with MrAIC.pl 1.4.3 (Nylander 2004) using AICc criterion for *matK, rpl32-trnL,* and *trnQ-5-rps16* regions. For *matK* the GTR+G model was chosen, for *rpl32-trnL* the GTR model, and for *trnq-rps16* the GTR+G model. Sequences were then partitioned for analysis in MrBayes using MCMC. To access the relative informativeness of each gene, each was analyzed with a Bayesian MCMC as implemented in MrBayes of 10 million generations sampled every 100 generations and a burn-in of 25%. Each sequence was run individually with MCMC implemented in MrBayes to examine the relative informativeness of each gene. *MatK, rpl32-trnL,* and *trnQ-5-rps16* sequences were then combined and analyzed using a mixed model in MrBayes with an MCMC of 30 million generations sampled every 100 generations and a burn-in of 25%.

PAUP (Swofford 2002) was used to conduct Maximum Parsimony (MP) analyses on the combined dataset of *matK, rpl32-trnL,* and *trnQ-5-rps16* regions. A full heuristic search with gaps treated as missing data was performed with TBR branch swapping and 1000 random stepwise addition replicates. Bootstrap analysis with 500 replicates (Felsenstein 1985, 1988) was used to assess relative support for clades.

Maximum Likelihood (ML) analysis was undertaken using RAxML (Stamatakis et al. 2008) as implemented on an online server. Combined data of *matK, rpl32-trnL,* and *trnQ-5-rps16* sequences was uploaded to a server and analyzed as a partitioned dataset. Support for each clade was assessed with bootstrap analysis.

A tree was constructed showing support of Bayesian, MP, and ML methods. The topology of the tree was based on the Bayesian analysis due to high degree of resolution

obtained using the method.

Pollen—Samples of pollen for this study was either obtained from Mark Rose (Greensboro, North Carolina), James Padgett (Boiling Springs, North Carolina), or collected in the field. Fresh anthers were fixed for 24h minimum in vials containing a solution of 3% formalin in a 0.1M sodium phosphate buffer. Vials were vortexed to dislodge the pollen, anthers were removed, and the vials centrifuged. The supernatant was removed and replaced with H_20 to remove traces of formalin. The pollen was dehydrated with a series of ethanol dilutions at concentrations of 50%, 75%, 85%, and 100%. Pollen was than critical-point dried, mounted on SEM stubs with carbon tape and sputter coated with gold for 2 minutes. Pollen was observed using a FEI Quanta 200 scanning electron microscope at magnifications of 4000x and 13454x. Measurements were taken and pollen was described using Hesse et al. (2009) as a guide.

Distribution maps—Using the Flora of the Southeast (2008), sources listed as "Documented Occurrences" were examined for each taxon and the county of occurrence recorded. Padgett (2004) was used to determine the counties in which *Hexastylis naniflora* occurs. Herbarium specimens, totaling 301 sheets, from Duke University (DUKE) and Appalachian State University (BOON) were examined and their county-level locality information recorded. ArcMap GIS software (ESRI 2006) was used to make the final distribution maps (Fig. 9-12).

RESULTS

***Molecular phylogenetics*—**(see Table 3) The aligned *matK* dataset included 1172 characters, 867 (74.0%) of which were constant, 252 (21.5%) characters that were variable but parsimony uninformative and 53 (4.5%) characters that were parsimony informative. The *matK* sequence for *Asarum canadense* was missing from the matrix due to amplification difficulties.

The aligned *rpl32-trnL* dataset included 570 characters, 545 (95.6%) of which were constant. The dataset contained 17 (3.0%) characters were variable but parsimony uninformative and 8 (1.4%) characters that were parsimony informative.

The aligned *trnQ-rps16* dataset includes 984 characters, 942 (96.7%) of which were constant. The dataset contains 25 (2.5%) characters that were variable but parsimony uninformative and 17 (1.7%) characters that were parsimony informative (PI). The *trnQ-rps16* sequences for *Isotrema macrophylla* and *Asarum candense* were missing from the matrix due to amplification difficulties

The length of the aligned sequences for the combined chloroplast regions was 2726. Sequences had 2354 bases (86.3%) that were constant. Variable characters totaled 294 (10.8%) and 78 (2.9%) of the characters were parsimony informative.

The combined Bayesian phylogeny (Fig. 2) suggests that *Hexastylis* is a monophyletic clade with 99% support. Within *Hexastylis*, there is a well-supported clade (100%) containing the three varieties of *H. arifolia* plus *H. lewisii* and *H. speciosa*. There is a

TABLE 3. Statistics for chloroplast regions.

Statistic	All	*matK*	*rpl32-trnL*	*trnQ-rps16*
Range of raw length		1122-1178	1147-1175	1060-1128
Aligned length	2726	1172	570	984
Variable sites (percent)	372 (13.6)	305 (26.0)	25 (4.4)	42 (4.3)
PI sites (percent)	78 (2.9)	53 (4.5)	8(1.4)	17(1.7)

TABLE 4. Shared indels. Insertion 1, found in the *trnQ-5-rps16* intergenic spacer region is shared by all *Hexastylis* taxa. Insertion 2, also found in the *trnQ-5-rps16* intergenic spacer region is shared by *H. contracta, H. heterophylla, H. minor, H. naniflora, H. rhombiformis,* the varieties of *H. shuttleworthii,* and *H. virginica*. Question marks indicate missing data and dashes indicate gaps in the alignment.

Taxon	Insertion 1	Insertion 2
Isotrema macrophylla	??????	??????
Asarum canadense	------	------
A. caudatum	------	------
Asiasarum sieboldii	------	------
Heterotropa asaroides	------	------
H. hatsushimae	------	------
H. savatieri	------	------
Hexastylis arifolia var. arifolia	ATTTGT	------
H. arifolia var. callifolia	ATTTGT	------
H. arifolia var. ruthii	ATTTGT	------
H. lewisii	ATTTGT	------
H. speciosa	ATTTGT	------
H. contracta	ATTTGT	GATCGT
H. heterophylla	ATTTGT	GATCGT
H. minor	ATTTGT	GATCGT
H. naniflora	ATTTGT	GATCGT
H. rhombiformis	ATTTGT	GATCGT
H. shuttleworthii var. *shuttleworthii*	ATTTGT	GATCGT

H. shuttleworthii var. harperi	ATTTGT	GATCGT
H. virginica	ATTTGT	GATCGT

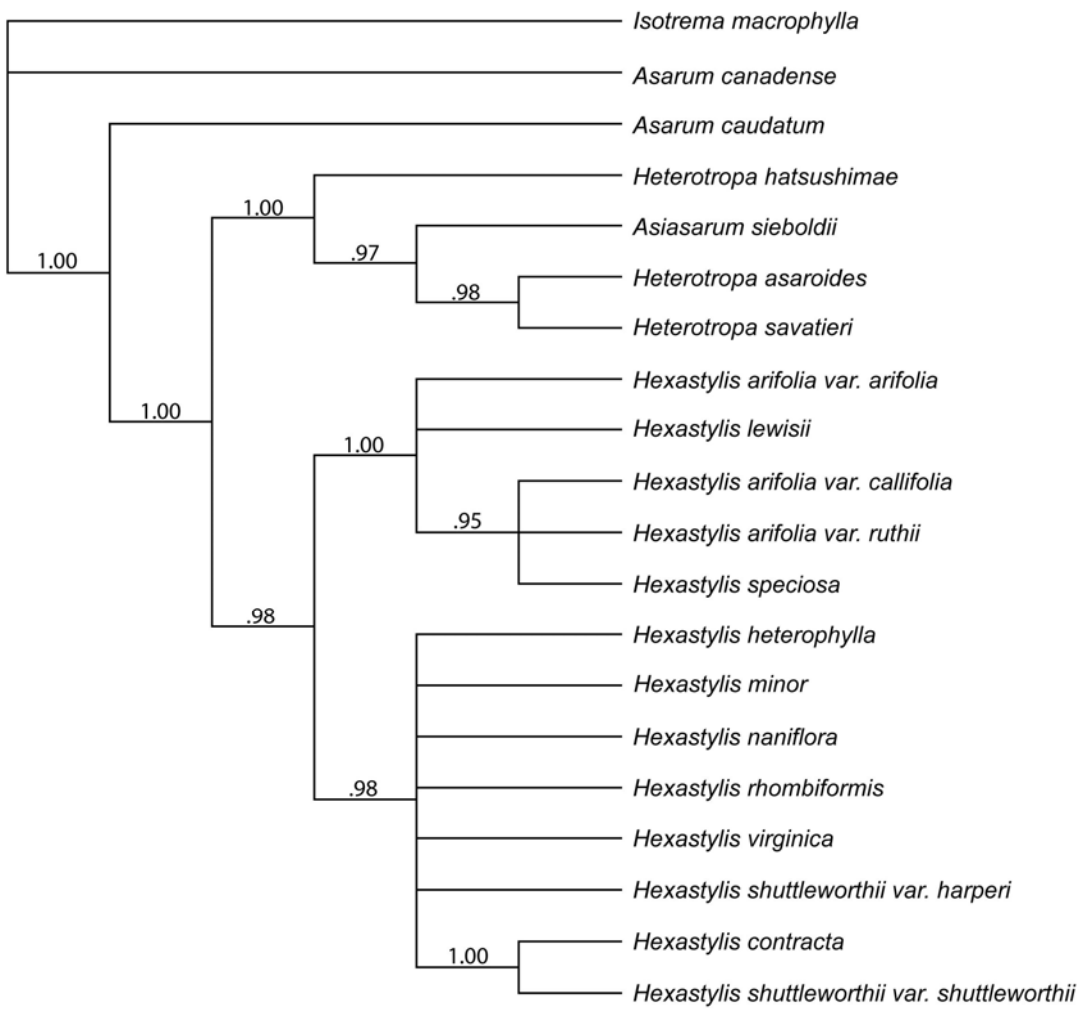

Fig. 2: Chloroplast phylogeny inferred by Bayesian methods. Posterior probabilities are shown.

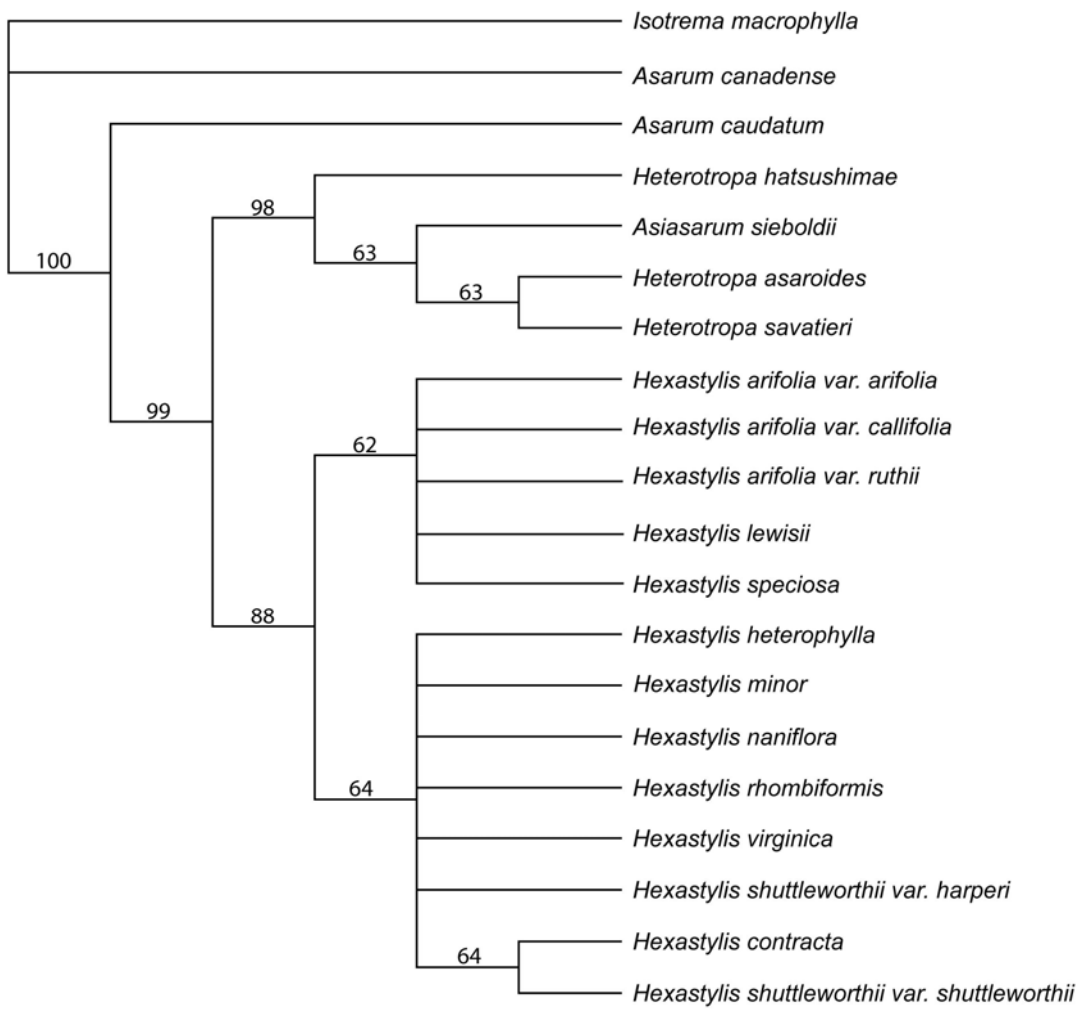

Fig. 3: Chloroplast phylogeny inferred by Maximum Parsimony (MP). Bootstrap support is indicated.

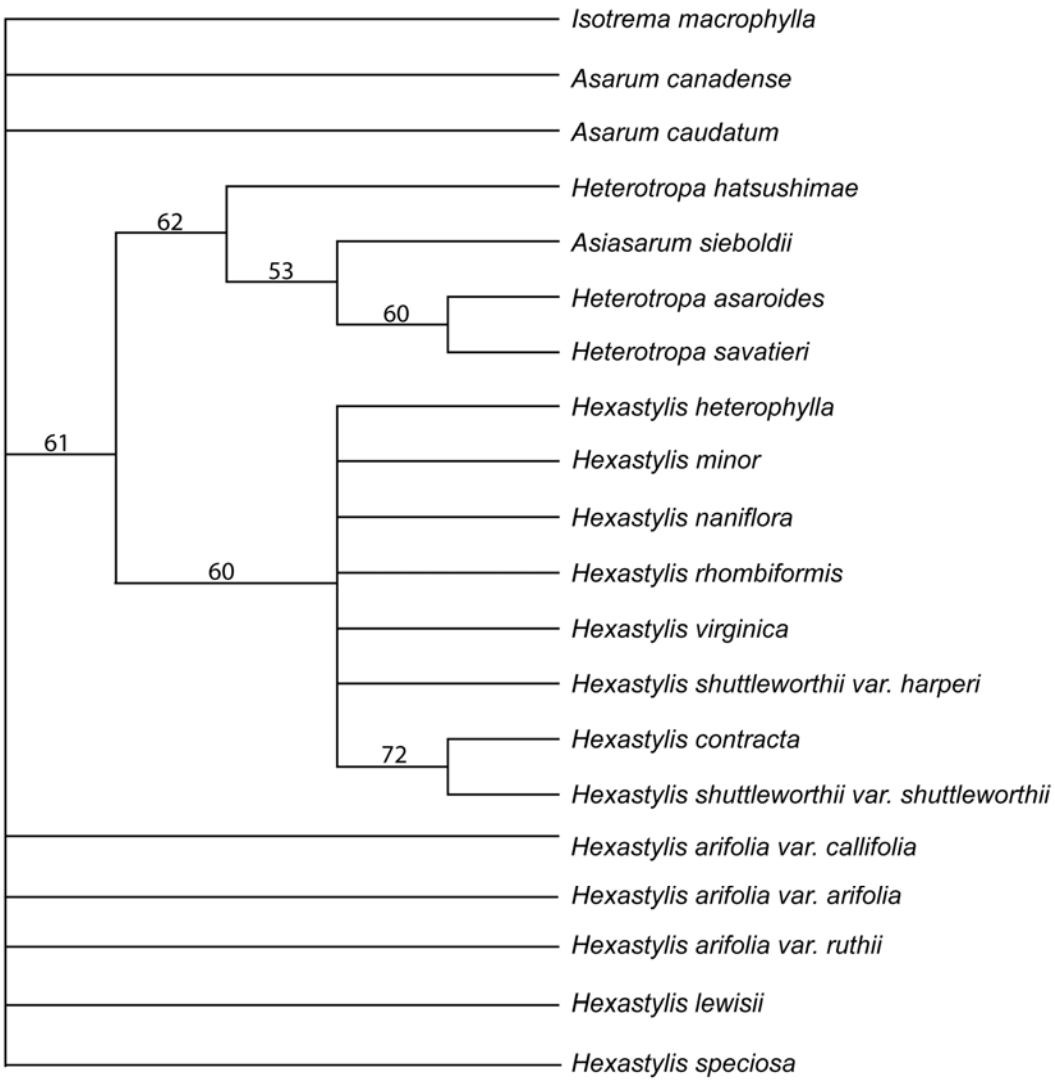

Fig. 4: Chloroplast phylogeny inferred by Maximum Likelihood (ML). Bootstrap support is indicated.

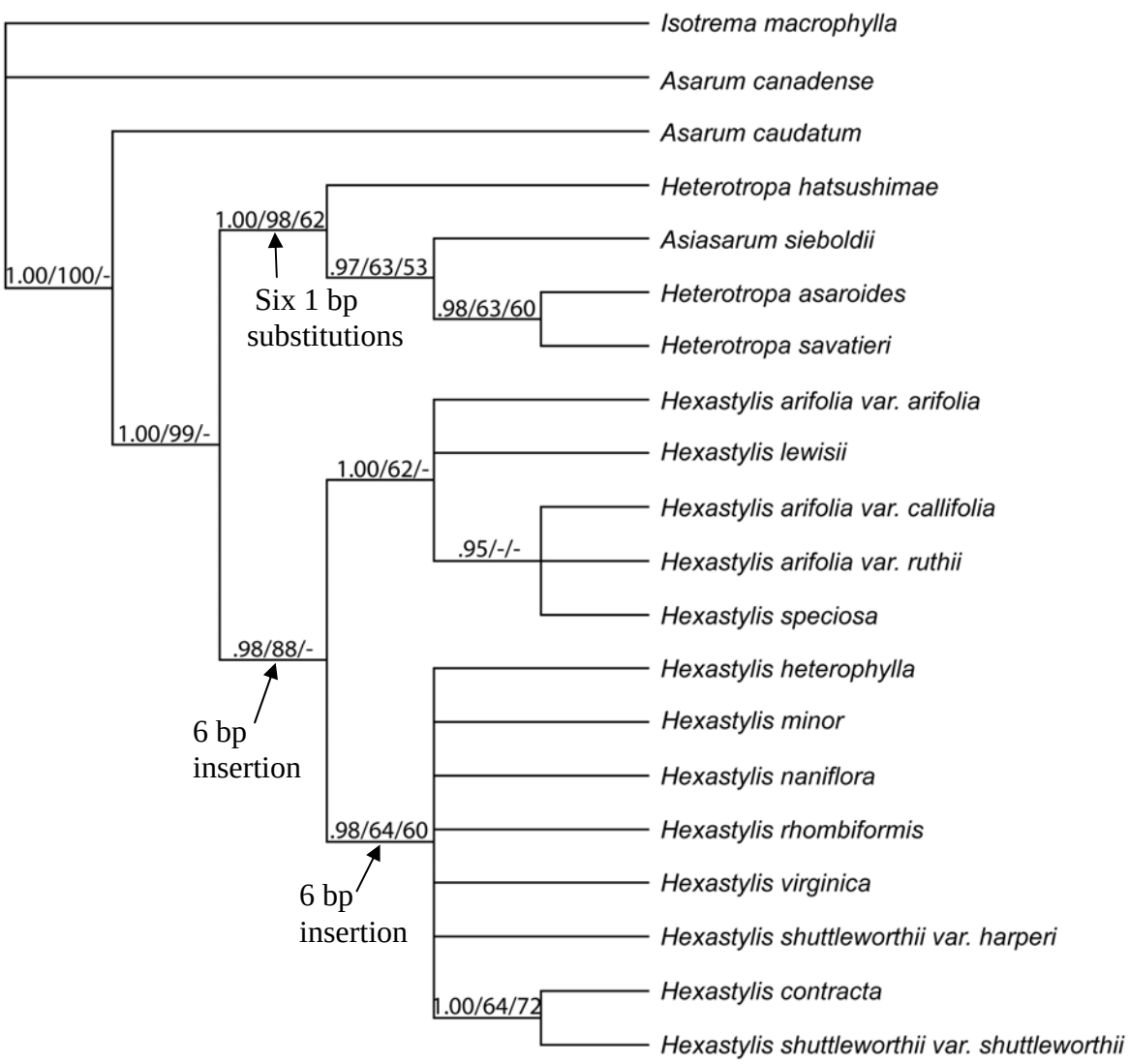

Fig. 5: Chloroplast phylogeny with support values for Bayesian/Parsimony/Maximum Likelihood methods respectively. Dashes indicate weak support. Major indels and substitutions are mapped.

second well-supported clade (98%) made up of all other *Hexastylis* taxa. A third monophyletic clade consists of *Heterotropa* plus *Asiasarum*. Support for this clade is 100%.

Parsimony analysis (Fig. 3) for the combined three genes resulted in the best tree having a length of 420. The topology of this tree was largely the same as the tree obtained with Bayesian methodology. *Hexastylis* is indicated to be monophyletic with bootstrap support of 88%. *H. contracta, H. heterophylla, H. minor, H. naniflora, H. rhombiformis, H. virginica,* and the varieties of *H. shuttleworthii* form a clade within the *Hexastylis* with 64% support. The varieties of *H. arifolia, H. lewisii,* and *H. speciosa* form a second monophyletic clade within *Hexastylis* with 62% support, and *Heterotropa* and *Asiasarum* form another clade, well supported by a 98% bootstrap.

Maximum Likelihood (ML) analysis (Fig. 4) for the three chloroplast regions does not support the monophyly of *Hexastylis*. However, *H. contracta, H. heterophylla, H. minor, H. naniflora, H. rhombiformis, H. virginica,* and the varieties of *H. shuttleworthii* form a clade with 60% bootstap support, a clade also supported by Bayesian and MP methods. In contrast to Bayesian and MP methods, ML shows this clade of *Hexastylis* to be sister to *Heterotropa* and *Asiasarum* with 61% support.

A tree showing support for all three methods with mapping of major indels and substitutions is shown in Fig. 5.

***Pollen*—**Pollen grains of *Hexastylis* measure between 24 and 51 micrometers in diameter and are spheroidal. *Hexastylis. shuttleworthii* has pollen grains that are significantly larger than the other species (Table 5). Under high magnification (13454x) surface features are clearly visible. Most species have a microreticulate surface with some micropores visible. Pollen grains within the same species can be inaperturate, porate, or colpate. There are

basically four morphological types of *Hexastylis* pollen grains that are explained in the following section. Pollen types are described in Table 5, the general form of an air-dried pollen grain is shown in Fig. 6, and pollen images can be found in Figs. 7 and 8.

POLLEN TYPE 1—The shape is spheroidal when critical-point dried and irregularly infolded when air-dried. The surface is reticulate with gemmae. These pollen grains may be inaperturate, aperturate, or sulcate within the same species. *Hexastylis arifolia var. arifolia, H. arifolia var. ruthii, H. lewisii, H. heterophylla, H. minor, H. rhombiformis,* and *H. speciosa* have this type of pollen.

POLLEN TYPE 2 —The shape is spheroidal when critical-point dried and irregularly infolded when air-dried. The surface is microporate with gemmae. These pollen grains may be inaperturate, aperturate, or sulcate. *Hexastylis virginica* has this type of pollen.

POLLEN TYPE 3 — The shape is spheroidal when critical-point dried and irregularly infolded when air-dried. The surface is reticulate with verrucae. These pollen grains may be inaperturate, aperturate, or sulcate. *Hexastylis shuttleworthii* has this type of pollen.

POLLEN TYPE 4 — The shape is spheroidal when critical-point dried and irregularly infolded when air-dried. The surface ornamentation is reticulate. Gemmae and verrucae are absent. These pollen grains may be inaperturate, aperturate, or sulcate within the same species. *Hexastylis naniflora* has this type of pollen.

TABLE 5. Morphometric data for *Hexastylis* pollen. P-length of polar axis ± standard deviation (µm), E-length of equatorial axis ± standard deviation (µm), P/E-ratio between polar and equatorial axis, Pt- pollen type.

Taxon	P	E	P/E	Pt
Hexastylis arifolia var.				
arifolia	33.7±5.4	33.1±4.7	0.98	1
H. arifolia var. ruthii	40.9±4.4	39.0±4.1	0.95	1
H. contracta	35.7±1.6	32.1±1.2	0.9	1
H. heterophylla	42.2±3.9	40.5±3.4	0.96	1
H. lewisii	36.0±1.8	31.8±0.8	0.88	1
H. minor	35.8±3.2	34.1±3.7	0.95	1
H. naniflora	34.3±2.3	33.9±2.3	0.99	4
H. rhombiformis	38.2±3.7	33.5±4.0	0.88	1
H. shuttleworthii	44.5±0.5	41.8±2.2	0.94	3
H. speciosa	38.2±3.0	35.9±1.2	0.94	1
H. virginica	41.2±2.5	39.3±2.4	0.95	2

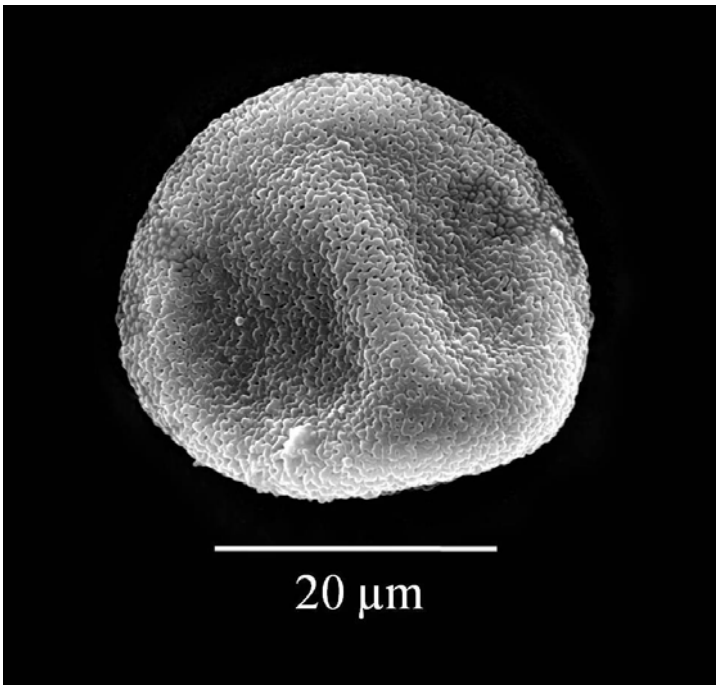

Fig. 6: Whole pollen grain of *Hexastylis naniflora*, air-dried, SEM. Note the irregular folding of the exine when air-dried.

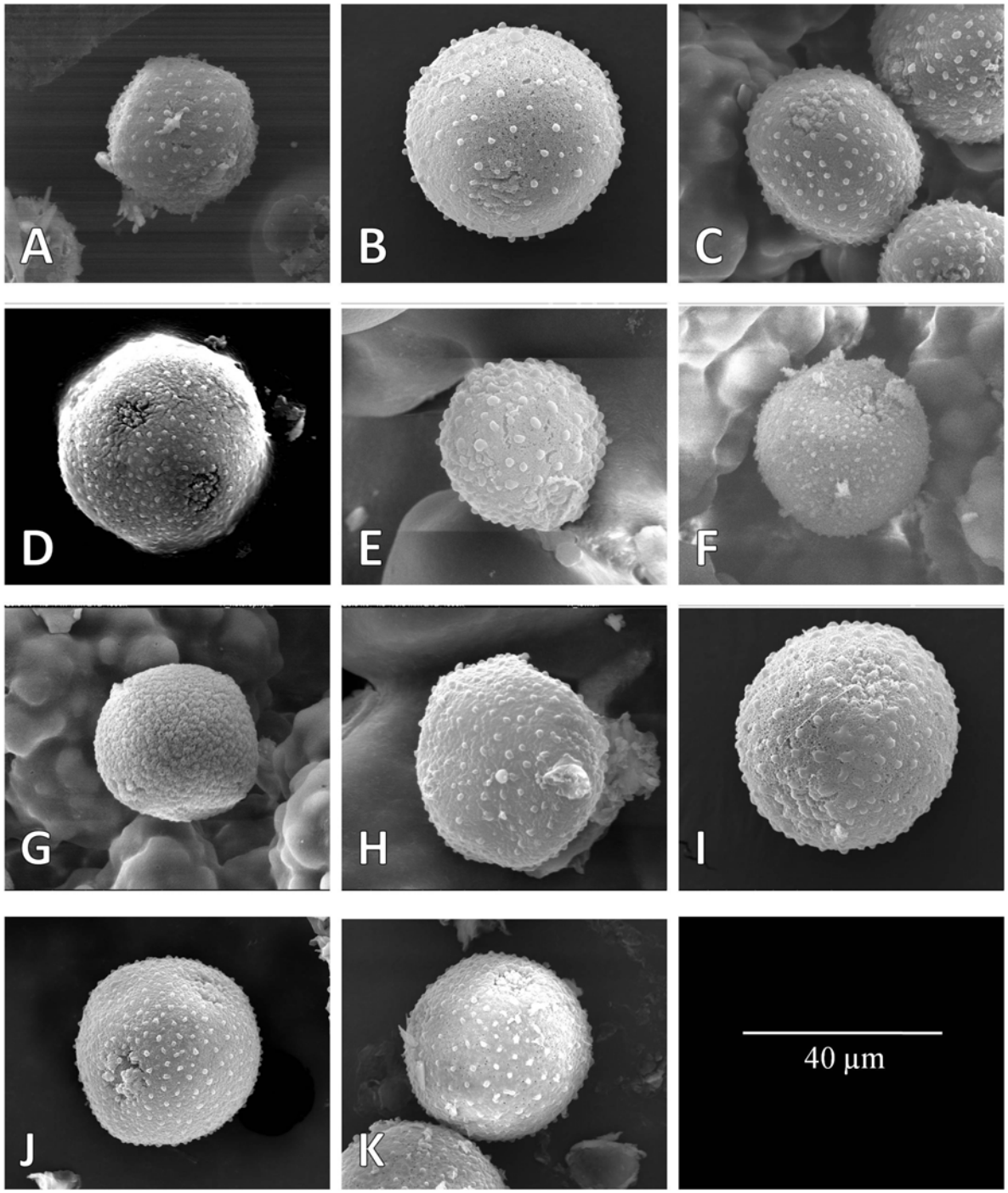

Fig. 7: Whole pollen grains of *Hexastylis*, critical-point dried, SEM: (A) *H. arifolia* var. *arifolia*, (B) *H. arifolia* var. *ruthii*, (C) *H. contracta*, (D) *H. heterophylla*, (E) *H. lewisii*, (F) *H. minor*, (G) *H. naniflora*, (H) *H. rhombiformis*, (I) *H. shuttleworthii*, (J) *H. speciosa*, and (K) *H. virginica*.

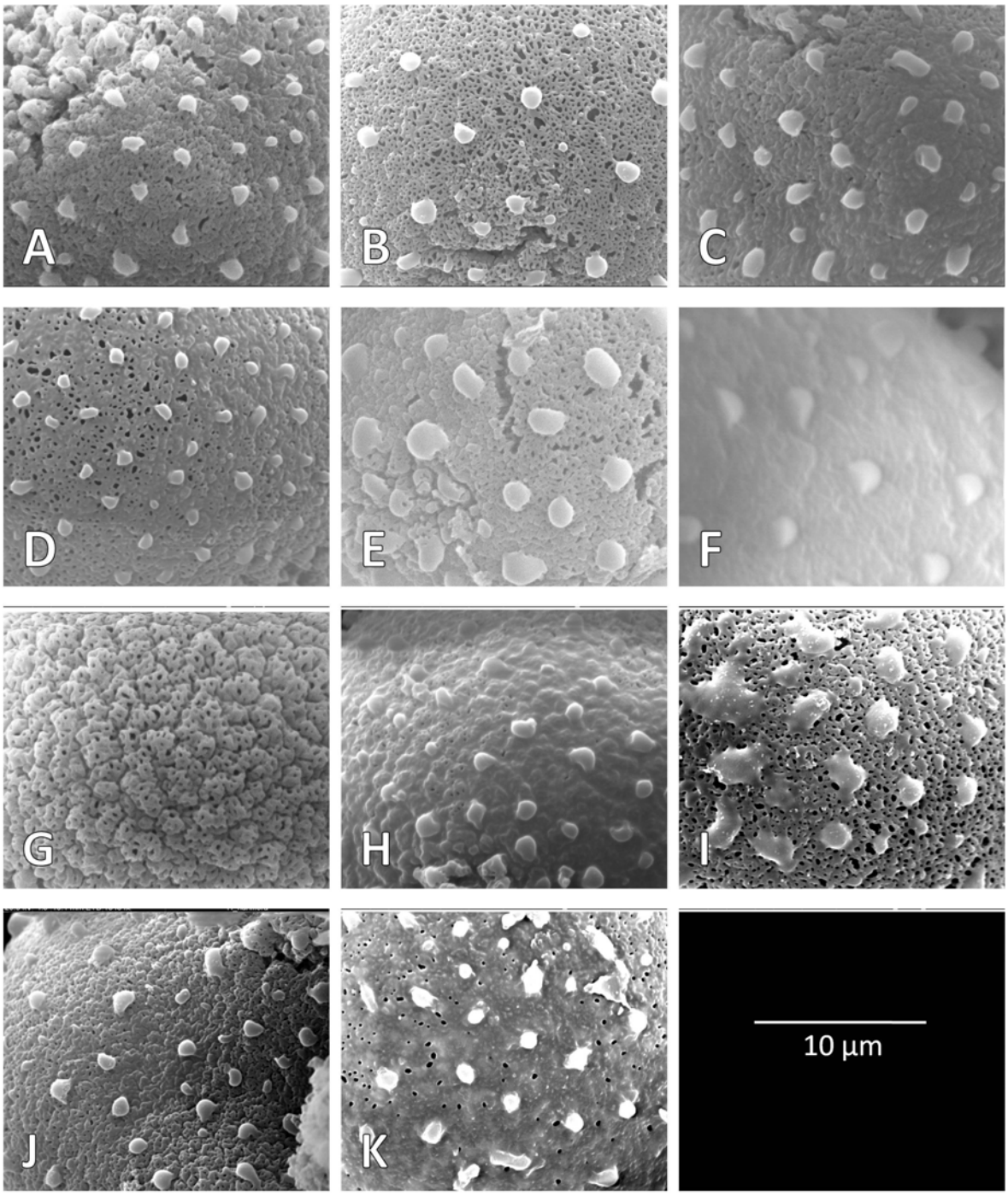

Fig. 8: Ornamentation of pollen grains of *Hexastylis*, critical-point dried*, SEM: (A) *H. arifolia* var. *arifolia*, (B) *H. arifolia* var. *ruthii*, (C) *H. contracta*, (D) *H. heterophylla*, (E) *H. lewisii*, (F) *H. minor* (from Padgett (2004)*air dried, (G) *H. naniflora*, (H) *H. rhombiformis*, (I) *H. shuttleworthii*, (J) *H. speciosa*, and (K) *H. virginica*.

TABLE 6. Comparative pollen morphology in *Hexastylis*.

Taxon	Shape	Aperture	Surface	Ornamentation
Hexastylis arifolia var. arifolia	spheroidal	monosulcate	microreticulate	gemmate
Hexastylis arifolia var. ruthii	spheroidal	monosulcate	microreticulate	gemmate
Hexastylis arifolia var. callifolia	spheroidal	monosulcate	microreticulate	verrucate
Hexastylis lewisii	spheroidal	monosulcate	microreticulate	gemmate
Hexastylis shuttleworthii	spheroidal	monosulcate	microreticulate	verrucate
Hexastylis heterophylla	spheroidal	monosulcate	microporate	gemmate
Hexastylis minor	spheroidal	monosulcate	microreticulate	gemmate
Hexastylis contracta	spheroidal	monosulcate	microreticulate	gemmate
Hexastylis rhombiformis	spheroidal	monosulcate	microreticulate	gemmate
Hexastylis virginica	spheroidal	monosulcate	microreticulate	gemmate
Hexastylis naniflora	spheroidal	monosulcate	microporate	none
Hexastylis speciosa	spheroidal	monosulcate	microreticulate	gemmate

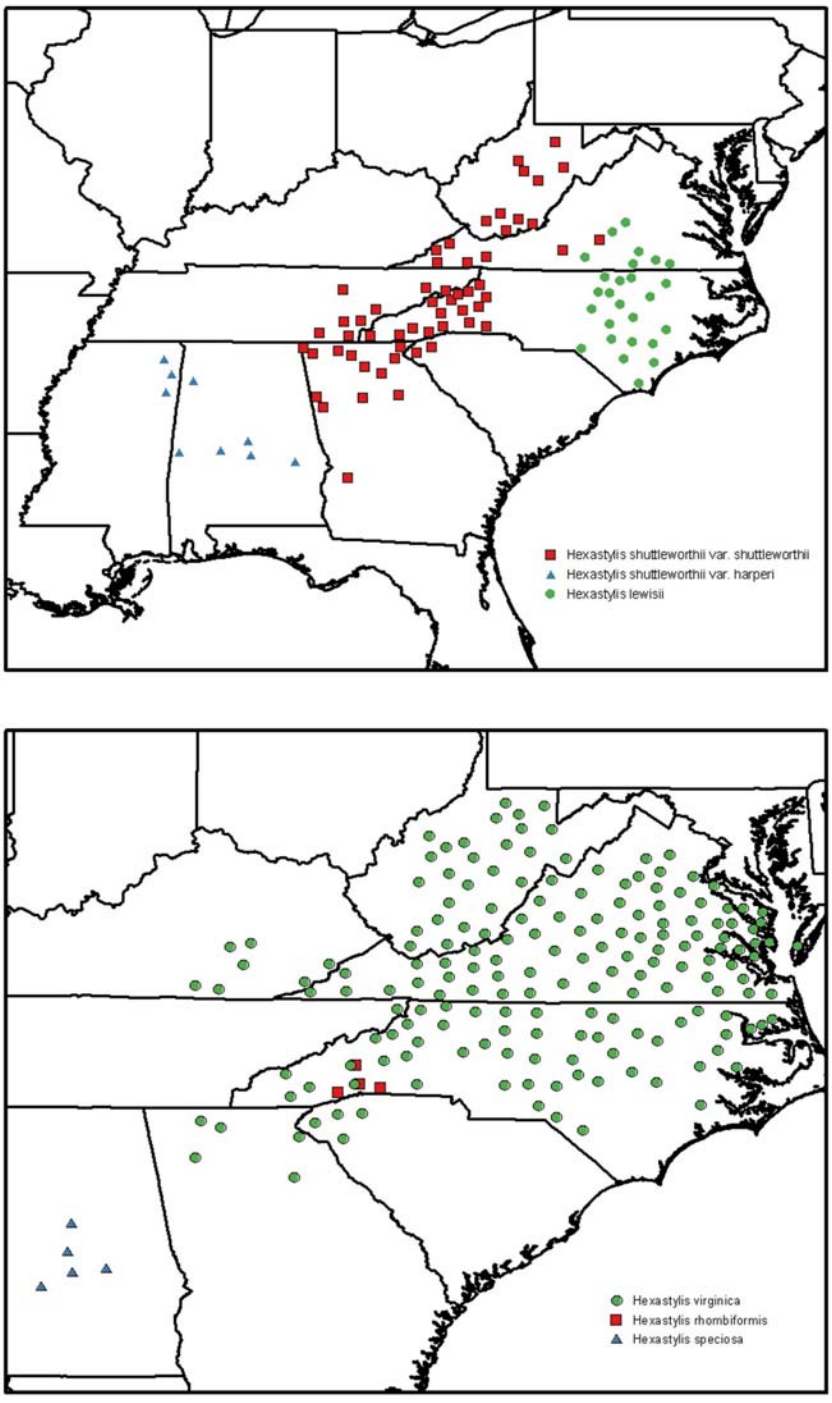

Fig. 9-10. Known distributions of *Hexastylis shuttleworthii*, *H. lewisii*, *H. virginica*, *H. rhombiformis*, and *H. speciosa*. Each marker represents a separate county of occurrence.

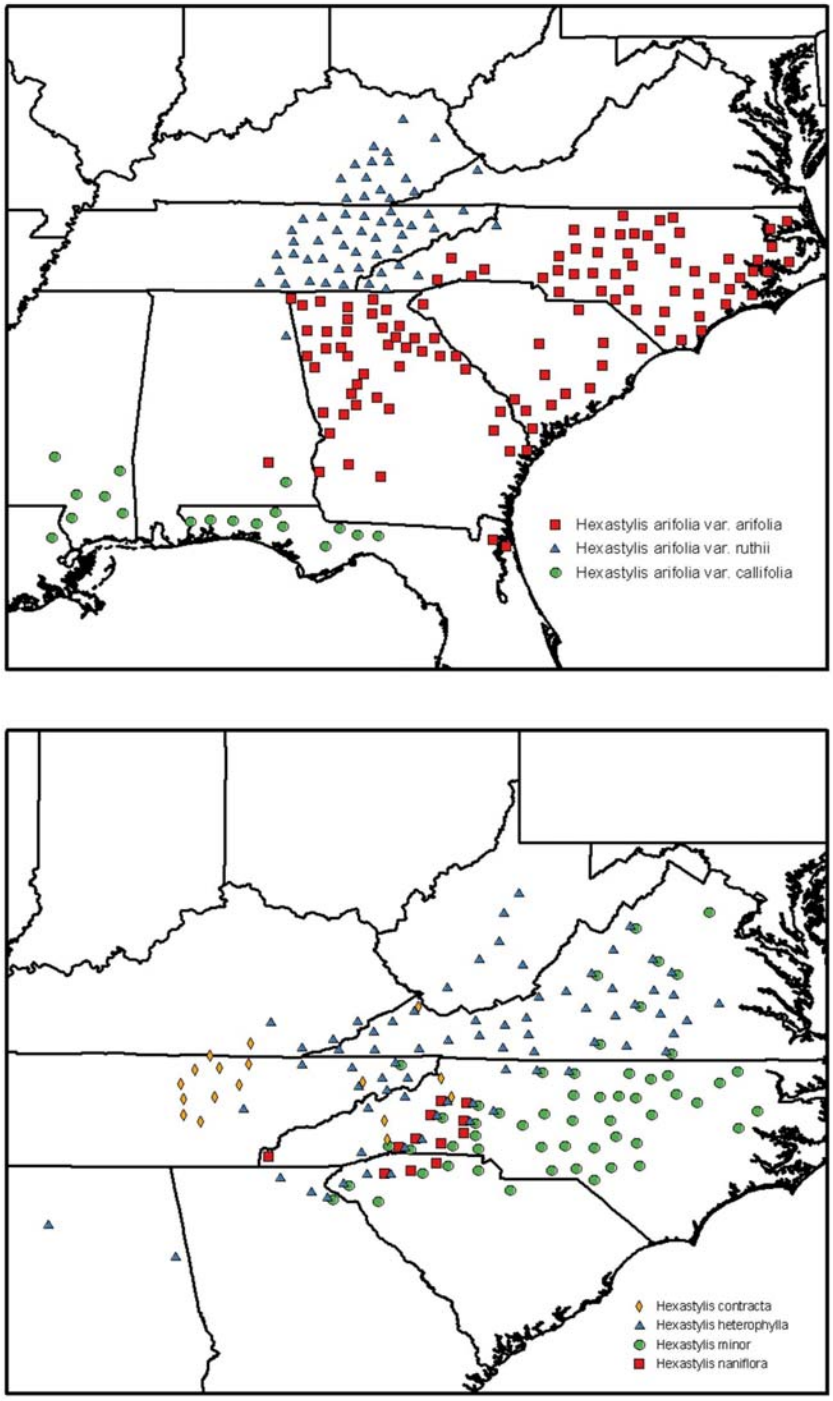

Fig. 11-12. Known distributions of *Hexastylis arifolia*, *H. contracta*, *H. heterophylla*, *H. minor*, and *H. naniflora*. Each marker represents a separate county of occurrence.

DISCUSSION

***Phylogeny*—**Combined chloroplast data has helped to elucidate relationships in *Asarum* sensu lato. Although the phylogeny was not resolved in its entirety, relationships recovered with these data are generally well-supported. Bayesian and MP methods indicate that *Hexastylis* forms a monophyletic clade. This contrasts with Kelly (1998a) who found *Hexastylis* to be paraphyletic. Maximum Likelihood methods as implemented by RAxML did not show *Hexastylis* to be monophyletic. Also of interest is the strong support for a *Heterotropa* + *Asiasarum* clade, supported by all three methods, which also contradicts Kelly's findings.

HEXASTYLIS—This group consists of nine species and 13 total taxa. All taxa were sampled for this study. A 6 bp synapomorphic insertion (Table 4, Fig. 5) unites *Hexastylis* as a monophyletic group. Bayesian analysis indicates the presence of two main clades within the group. The first clade consists of *Hexastylis arifolia* with its three varieties as well as *H. speciosa* and *H. lewisii*. This supports the relationship found by Kelly (1998a) for *H. arifolia* + *H. speciosa* clade, although Kelly did not sample *H. lewisii*. Blomquist (1957) and Gaddy (1987a) also grouped *H. arifolia* and *H. speciosa* together based on morphological similarities, but found no such characters suggesting either is allied with *H. lewisii*, which instead was thought by both to be allied with *H. shuttleworthii*. *Hexastylis arifolia* and *H. speciosa*, members of the first clade, have deltoid leaves and a notched style extension which are characteristics absent from all other taxa. Another clade is formed by the remaining

species of *Hexastylis*, all of which have cordate leaves and an unnotched style extension. A six bp insertion is shared by every taxon in the second clade (Table 4, Fig. 5).

Maximum Likelihood (ML) methods, as implemented RAxML did not support the monophyly of *Hexastylis* and supported a clade of *H. contracta*, *H. heterophylla*, *H. minor*, *H. naniflora*, *H. rhombiformis*, *H. virginica*, and the varieties of *H. shuttleworthii* to be sister to *Heterotropa* and *Asiasarum*. This discrepancy is possibly due to the way ML methods are implemented in RAxML. The result may have been a model that was a poor fit to the data, causing *Hexastylis* to not be recognized as monophyletic even with its 6 bp shared insertion.

HEXASTYLIS TAXA WITH UNIQUE INDELS—The concept of DNA barcoding has been proposed as a method for identifying taxa (Blaxter 2004). Four *Hexastylis* were found to have indels unique to that particular taxon. These unique indels may have the practical application of enabling identification of *Hexastylis* taxa to the species level based on vegitative material alone, which is often difficult due to morphologically similar leaves (Gaddy 1987a). Unique indels are listed as follows: *Hexastylis lewisii* contains a 5 bp deletion in the *matK* region. *Hexastylis speciosa* has a 6 bp deletion in the *matK* region and a 7 bp deletion in the *trnQ-5-rps16* region. *Hexastylis minor* has a 5 bp insertion in the *trnQ-5-rps16* region, and *Hexastylis virginica* has an 11 bp insertion in the *rpl32-trnL* region.

HETEROTROPA—This group consists of 15 species, three of which were sampled for the purposes of this study. All species sampled for this study were from Asia. The phylogeny suggests that *Asiasarum sieboldii*, another Asian taxon, should be considered part this group. This contradicts Kelly's (1997, 1998a) treatment based on morphological characters suggesting *Asiasarum* to be basal to *Hexastylis* and *Heterotropa*.

Pollen—*Hexastylis* pollen grains are spheroidal, medium-size (30-45µm), with the majority of taxa having a microreticulate surface and gemmae. However, there are a few taxa that can be easily distinguished from the remaining species. *Hexastylis shuttleworthii* has a microreticulate surface but has flattened wart-like verrucae on the exine instead of globose gemmae. *Hexastylis virginica* lacks microreticulations on the exine, but has micropores. One taxon, *H. naniflora* lacks gemmae entirely, as reported by Padgett (2004), but has a microreticulate exine.

As reported by Walker (1974) pollen grains of *Hexastylis* taxa can be inaperturate, colpate, or porate within the same species. Any breaks or pores in the exine appear to be irregularly shaped with no apparent pattern to their arrangement.

Air-dried pollen of *Hexastylis* species folds irregularly. This is possibly due to the pollen grains having thin walls that would allow the pollen tube to break through the exine in the absence of any apertures. Due to the irregular and unpredictable folding patterns, it is recommended that *Hexastylis* pollen grains be critical-point dried for high-vacuum SEM examination.

Distribution maps—Distributions were similar to those produced by Gaddy (1987a). However there were new datapoints for *Hexastylis naniflora*, reflecting populations found after Gaddy's results were published.

Biogeographical implications—When considering why a plant is distributed where it is, it is important to take into account present distribution patterns, associations with other species, as well as other related characteristics. *Hexastylis* species occupy temperate forests that require moderate rainfall, typically co-occurring with species such as *Quercus* and *Tsuga* (Padgett 2004). When considering where *Hexastylis* occurred in the past, it can be assumed

that it would have occupied localities with similar climates, and therefore would have associated with similar taxa.

Although a few fossilized leaves have been found for *Asarum*, the fossil record of as *Asarum* and *Hexastylis* is generally lacking. The pollen is not present in the fossil record, as is typical for most plants that are not wind pollinated. Even without a clear fossil record, past distributions of *Hexastylis* and *Asiasarum* can be inferred by comparing the taxa currently associated with these genera with those of the fossil record. The distribution of the so-called Arcto-Tertiary geoflora can provide clues about these taxa. From the Cretaceous (105-65 m.y.a.) until the early Tertiary or Paleocene (65-47 m.y.a) as it is called in the modern interpretation, temperate forests would have ranged across much of North America and Asia including Alaska and Siberia (Sveshnikova and Budantsev 1969; Creber and Chaloner 1984; Axelrod et al. 1991). *Hexastylis* and *Heterotropa* would have occurred in those places, migrating across the Bering Strait land bridge. Assuming that the range was once broad, one can speculate on what caused those taxa not occupying the present range to become extinct.

During the middle Eocene (47-43 m.y.a) North America began to experience cooler drier climates and the buildup of the Rocky Mountains (Lipman et al. 1972). This would likely lead to the loss of *Hexastylis* and *Heterotropa* ancestors in the region of west central to central North America.. The vegetation in northern North America at this time consisted of montane coniferous forests above 1200 m (Axelrod et al. 1991), which would be inhospitable for *Hexastylis* and *Asarum*.

During the Neocene (25-2 m.y.a), tectonic shifts caused Australia and South America to drift northward, opening up passages between each continent and Antarctica. Cold water began to flow from Antarctica to the western coast of North America, contributing to less

humidity and rainfall as well as strengthened high-pressure systems in western North America (Axelrod et al. 1991). This would lead to drier climates in the area, as indicated by the presence of taxa suited to drier climates such as conifers and grasslands (Smith 1941; Matthews and Rouse 1963) in the fossil record.

During glacial periods, *Hexastylis* would have been forced south due to the colder climate. It has been hypothesized (Delcourt and Delcourt 1981) and others that *Hexastylis* migrated into ravines and river drainages. Padgett (2004) proposed that the north-south orientation of the Appalachian Mountains would help facilitate such migrations and that populations in different drainages would undergo allopatric speciation. Noting that some species, such as *Hexastylis naniflora* have narrow distributions and others such as *H. arifolia* have very broad distributions, Padgett proposed that each could have migrated in different patterns during ice ages. Taxa with narrow distributions would have migrated south along ravines, river drainages, and mountain corridors, while taxa with broad distributions, such as *Hexastylis arifolia* would not be confined to such places most likely migrating eastward to coastal areas, encountering milder climates.

One of the implications of the monophyly of both *Hexastylis* and *Heterotropa* within *Asarum* sensu lato is the discussion of which genus originated first. *Asarum* was thought to have originated in Asia by past authors mostly due to Asia being the center of diversity for the genus. Kelly's (1998a) study supported Asia as the origin of *Asarum* due to Asian *Asarum* sensu stricto taxa forming basal clades within the lineage. The phylogeny presented here differs from Kelly's in several aspects, the first being that it samples *Hexastylis* taxa exhaustively but only includes *Asarum*, *Heterotropa*, and *Asiasarum* as placeholders. *Asarum* is the basal group within this study clade, but the results shown here do not provide evidence

as to whether *Asarum* originated in North America or Asia because of a lack of sampling of the Asian *Asarum*. Secondly, the topology of the tree differs from Kelly's in that *Heterotropa* forms a monophyletic clade within *Asarum*, as supported by six shared 1 bp substitutions. *Hexastylis* forms a monophyletic clade that is sister to *Heterotropa*, not basal or derived. Assuming from Kelly's (1998a) phylogeny that *Asarum* originated in Asia and later migrated into North America and Europe, giving rise to *Heterotropa* and *Hexastylis* and taking into account that these are sister clades, it is not possible to determine whether *Heterotropa* or *Hexastylis* originated earliest from sequence data presented here.

Taxonomic conclusions— *Hexastylis* should be recognized as a genus, as it is a monophyletic group. My chloroplast phylogeny suggests that *Asiasarum* can be combined with *Heterotropa*. This combination should be approached with caution because it contradicts significant morphological evidence presented by Kelly (1997, 1998a) suggesting *Asiasarum* to be basal to *Hexastylis* and *Heterotropa*.

LITERATURE CITED

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

Axelrod, D. I., M. T. Kalin Arroyo, and P. R. Raven, 1991. Historical development of temperate vegetation in the Americas. *Revista Chilena de Historia Natural* 64: 413-446.

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

Blaxter, M. L. 2004. The promise of a DNA taxonomy. *Philosophical Transactions of the Royal Society B: Biological Sciences* 359: 669-679.

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

Britton, N. L. and A. Brown. 1913. Aristolochiaceae. Pp. 641–645 in *An Illustrated Flora of the Northern United States, Canada, and the British Possessions* vol. 3. New York: Charles Scribner's Sons.

Creber G. T. and W. G. Chaloner. 1984. Climatic indications from growth rings in fossil woods. Pp. 49–74 in *Fossils and Climate*, ed. P. J. Benchley. New York: John Wiley and Sons.

Delcourt, P. A. and H. R. Delcourt. 1981. Vegetation maps for eastern North America: 40,000 years B.P. to the present. Pp. 123–165 in *Geobotany II*, ed. R. C. Romans. New York: Plenum.

ESRI. 2006. ArcMap GIS version 9.2. Environmental Systems Research Institute.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.

Felsenstein, J. 1988. Phylogenies from molecular sequences: inference and reliability. *Annual Review of Genetics* 22: 521-565.

Flora of the Southeast. 2009. http://www.herbarium.unc.edu/seflora/firstviewer.htm.

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

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

Gaddy, L. L. 1987b. Hexastylis shuttleworthii var. harperi (Aristolochiaceae), a new variety of heartleaf from Alabama and Georgia. *Sida* 12: 51-56.

Gonzalez, V. C. 1972. *The ecology of* Hexastylis arifolia, *an evergreen herb in the North Carolina deciduous forest*. Ph.D. dissertation. Durham, North Carolina: Duke University.

Gray, A. 1878. Forest geography and archaeology, a lecture delivered before the Harvard University Natural History Society. *American Journal of Science* 16: 85–94, 183–96.

Gupta, R. K. 1972. Boreal and arcto-boreal elements in the flora of Western Himalaya. *Vegetatio* 24: 159-175.

Hesse, M., H. Halbritter, R. Zetter, M. Weber, R. Buchner, A. Frosch-Radivo, and S. Ulrich. 2009. *Pollen Terminology: An illustrated Handbook*. Vienna: Springer Verlag.

Huelsenbeck, J. P. and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17: 754-755.

Judd, W., Campbell, C. S, Kellogg, E. A, Stevens, P. F., and M. J. Donoghue. 2002. *Plant Systematics: A Phylogenetic Approach*. Ed. 2. Sunderland, Massachusetts: Sinauer Associates.

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

Kelly, L. M. 1998b. *Systematic in* Asarum *and Aristolochiaceae*. Ph.D. dissertation. Ithaca, New York: Cornell University.

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

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

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947-2948.

Libby, G. W., Z. E. Murrell, and P. E. Carroll.1996. Inventory for *Hexastylis contracta* Blomquist (Southern Heartleaf) in Kentucky and Tennessee. Technical report to the U. S. Fish and Wildlife Department National Biological Survey.

Linnaeus, C. 1753. *Species Plantarum*. Stockholm: Impensis Laurentii Salvii.

Lipman, P. W., H. J. Protska, and R. L. Christiansen. 1972. Cenozoic volcanism and plate techtonic evolution of the western United States. I. Early and Middle Cenozoic. *Philosophical Transactions of the Royal Society of London. Series A* 271: 217-248.

Maddison, D. R. and W. P. Maddison. 2005. MacClade. Sinauer Associates, Sunderland, Massachusetts.

Matthews, W. H. and G. E. Rouse. 1963. Late Tertiary volcanic rocks and plant-bearing deposits in British Columbia. *Bulletin of the Geological society of America* 74: 55-60.

Murrell, Z. E. and P. E. Carroll. 1995. Status survey of *Hexastylis contracta* in Tennessee. Technical report to the Kentucky Nature Preserves Commission.

Nylander, J. A. A. 2004. MrAIC.pl. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Ooi, K., Y. Endo, J. Yokoyama, and N. Murakami. 1995. Useful primer designs to amplify DNA fragments of the plastid gene *matK* from angiosperm plants. *Journal of Japanese Botany* 70: 328-331.

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*. M.S. thesis. Chapel Hill, North Carolina: University of North Carolina.

Padgett, J. E. 2004. *Biogeographical, ecological, morphological, and micromorphological analyses of the species in the* Hexastylis heterophylla *complex.* M.S. thesis. Boone, North Carolina: Appalachian State University.

Radford, A. E., H. Ahles, and R. Bell. 1968. *Manual of the Vascular Flora of the Carolinas.* Chapel Hill, NC: University of North Carolina Press.

Rafinesque, C. S. 1825. *Neogenyton, or indication of sixty-six new genera of plants in North America*. Lexington, Kentucky: Published by Author.

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

Ronquist, F. and J. P. Huelsenbeck. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572-1574.

Shaw, J., E. B. Lickey, E. E. Schilling, and R. L. Small. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetics studies in angiosperms: the tortoise and the hare III. *American Journal of Botany* 94: 275-288.

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

Smith, H. V. 1941. A Miocene flora from Thorn Creek, Idaho. *American Midland Naturalist* 25: 473-522.

Soltis, D. E., P. S. Soltis, M. W. Chase, M. E. Mort, D. C. Albach, M. Zanis, V. Savolainen, W. H. Hahn, S. B. Hoot, M. F. Fay, M. Axtell, S. M. Swensen, L. M. Prince, W. J. Kress, K. C. Nixon, and J. S. Farris. 2000. Angiosperm phylogeny inferred from 18S rDNA, *rbcL,* and *atpB* sequences. *Botanical Journal of the Linnean Society* 133: 381–461.

Stamatakis, A., P. Hoover, and J. Rougemont. 2008. A Rapid Bootstrap Algorithm for the RAxML Web-Servers. *Systematic Biology* 75: 758-771.

Sveshnikova, I. N. and L. Y. Budantsev. 1969. *Florulae fossils arcticae I. Nauka Filia Leningradensis*. Leningrad: Komarov Botanical institute.

Swofford D. 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Walker, J. W. 1974. Aperture Evolution in the Pollen of Primitive Angiosperms. *American Journal of Botany* 61: 1112-1137.

Weakley, A. S. Working Draft of 8 March 2010. *Flora of the Southern and Mid-Atlantic States*. Chapel Hill, North Carolina: University of North Carolina Herbarium.

Wen, J. 1999. Evolution of Eastern Asian and Eastern North American Disjunct Distributions in Flowering Plants. *Annual Review of Ecology and Systematics* 30: 421-455.

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

Wolfe, J. A. 1975. Some aspects of plant geography of the northern hemisphere during the late Cretaceous and Tertiary. *Annals of the Missouri Botanical Garden* 62: 264-279.

Wyatt, R. L. 1955. *Floral morphology and phylogeny of the Aristolochiaceae*. M.S. thesis. Chapel Hill, North Carolina: University of North Carolina.

BIOGRAPHICAL SKETCH

Bryan Alan Niedenberger was born on September 22, 1983 in Newport News, Virginia to Alan and Gina Niedenberger. In 2002, he graduated from York High School in Yorktown, Virginia. He entered High Point University in the fall of 2002. He graduated from High Point University in 2006 and enrolled in Appalachian State University. He graduated from Appalachian State University in May 2010 with a Master of Science degree in Biology.