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Abstract

Genetic patterns were examined in five populations of North American *Cleistes* with three sources of molecular data: amplified fragment length polymorphisms (AFLP), DNA sequencing, and plastid microsatellites. Populations of *C. bifaria* were sampled in four areas of the south-eastern US: the coastal plains of Florida and North Carolina and the mountains of North Carolina and West Virginia. A population of *C. divaricata* sympatric with the North Carolina coastal plain *C. bifaria* was also sampled. Analysis of the three types of molecular data resulted in the same relationships among the five sampled populations. The coastal plain population of *C. divaricata* was consistently grouped with the *C. bifaria* populations from the mountains of West Virginia and North Carolina, and the two coastal plain populations of *C. bifaria* formed a separate group, results not supporting the existing concepts of species relationships. For future studies, greater sampling of *C. divaricata* populations and more detailed morphological and phenological studies are recommended for better characterization of the diversity within North American *Cleistes*.

Keywords: AFLP, floral fragrance, plastid microsatellites, phylogeography, *psaI-accD*, *rps16* intron, *trnL-F*, UPGMA

Introduction

According to Dodson & Escobar (1994), *Cleistes* L.C. Rich. ex Lindley comprises around 56 terrestrial orchid species distributed throughout the Americas, from eastern North America south to Brazil. Several features unite the species of *Cleistes*: erect slender stems, few leaves, a terminal inflorescence with one to three flowers, and a soft pollen mass with grains in tetrads (Ames, 1922; Luer, 1972). The genus can be divided geographically into two major groups: a large South American group centred in Brazil and a small North American group centred in the south-eastern United States. The North American group comprises two species, the large-flowered *C. divaricata* (L.) Ames and the smaller-flowered *C. bifaria* (Fernald) P. M. Catling & K. B. Gregg, which form a clade apart from their South American congeners (Cameron & Chase, 1999). As described by Catling & Gregg (1992), *C. bifaria* occurs in meadows, pine savannahs, and open oak-pine forests along the Gulf Coast from Louisiana to Florida and north along the Atlantic coast to North Carolina and inland to the mountains of Tennessee, Kentucky, and West Virginia (Figure 1A). *Cleistes* divaricata occurs mainly in pine savannahs along the Atlantic Coast from Florida to New Jersey (Figure 1B).Populations of each species grow sympatrically in the coastal plains of Florida, Georgia, North Carolina and South Carolina.

Previous taxonomic studies of North American *Cleistes* have relied primarily on morphological characters to delimit species. Fernald (1946: 187) examined several major herbarium collections of *Cleistes* and recognized that there were "two rather strongly defined varieties passing as *Cleistes divaricata*." The larger-flowered variety he designated as *C. divaricata* var. typica (= *C. divaricata* var. *divar-*

icata) and the smaller-flowered as *C. divaricata* var. *bifaria* Fernald.However, the latter was not recognized in most subsequent accounts of the orchids of the south-eastern United States (e.g. Correll, 1950). In an attempt to evaluate its distinctiveness, Catling & Gregg (1992) conducted a morphometric analysis of North American *Cleistes*. Based mainly on floral characters such as column height and lip length, but also on differences in floral fragrances and in flowering phenologies, especially of the North Carolina coastal plain sympatric pair, they concluded that *C. divaricata* and *C. bifaria* should be recognized as two species.

This study was undertaken to address two questions. First, we wanted to determine whether the use of molecular tools could clarify the relationship between *C. divaricata* and *C. bifaria*, particularly where they occur sympatrically. Second, we wanted to see whether molecular data could elucidate how the present distribution of *Cleistes* throughout the south-eastern United States might have arisen. For instance, we were interested in whether the northernmost population of *C. bifaria* was more likely a result of natural migration from the North Carolina mountains or an accidental introduction from coastal plain North Carolina, a possibility suggested by Gregg (1989).

Three molecular techniques were utilized: amplified fragment length polymorphisms (AFLP), DNA sequencing, and plastid microsatellites. Exploiting variation in the nuclear genome, AFLP has been demonstrated to be useful for analysing intra- and interspecific variability in plants (Beismann et al., 1997; Qamaruz-Zaman et al., 1998; Angiolillo, Mencuccini & Baldoni, 1999; Palacios, Kresovich & González-Candelas, 1999; Hedrén, Fay & Chase, 2001) and animals (Giannasi, Thorpe & Malhotra, 2001). AFLP was also chosen for this study because it requires no prior knowledge of the DNA sequence and provides large amounts of data with highly reproducible results.Plastid DNA sequences and microsatellite markers for *Cleistes* were expected to provide an additional independent data set for comparison with the AFLP results.

Material and Methods

Plant Samples

Leaf samples of individuals of *C. bifaria* and *C. divaricata* collected between 1996 and 1998 were used in this project. Populations of *C. bifaria* were sampled in Florida, North Carolina, and West Virginia, and a population of *C. divaricata* was sampled in the coastal plain of North Carolina (Table 1; Figure 1).It should be noted that populations of both species occur sympatrically in Bean Patch and Big Island Savannahs in the Green Swamp of Brunswick County, North Carolina. Leaves were collected from individuals at least 2 m and usually 4 m apart to avoid accidental sampling from the same clone. Collections from

Table 1. Sampling of *C. bifaria* and *C. divaricata*. Site descriptions from Gregg (1991) and C. Frye (pers. comm.). State abbreviations are as follows: FL, Florida; NC, North Carolina; WV, West Virginia

Species	Site (number sampled)	Location	Community	Dominant spp.
C. bifaria	Morningside Nature Center, Gainesville (16)	Alachua Co., FL	Savannah with poor sandy acidic soil	Slash pine (*Pinus elliotii* Engelm.), Long leaf pine (*Pinus palustris* Mill.), various shrubs (*Gaylussacia* spp., *Ilex glabra* A. Gray)
	Duke Powerline, Dot Perry Road (4)	Wilkes Co., NC	Occasionally mowed field	Virginia pine (*Pinus virginiana* Mill.), Shortleaf pine (*Pinus echinata* Mill.), various shrubs (*Rhododendron maximum* L., *Kalmia latifolia* L., *Rubus* spp.)
	Bean Patch Island and Big Island, 20 km from the coast (12)	Brunswick Co., NC	Savannah with poor sandy acidic soil	Longleaf pine (*Pinus palustris*), wiregrass (*Aristida stricta* Michx.)
	Beavers' Meadow (17)	Barbour Co., WV	Open meadow	Various grasses along with *Pteridium aquilinum* (L.) Kuhn, *Rubus hispidus* Marsh., and *Baptisia tinctoria* R. Br.
C. divaricata	Big Island, 20 km from the coast (16)	Brunswick Co., NC	Savannah with poor sandy acidic soil	Longleaf pine (*Pinus palustris*), wiregrass (*Aristida stricta*)

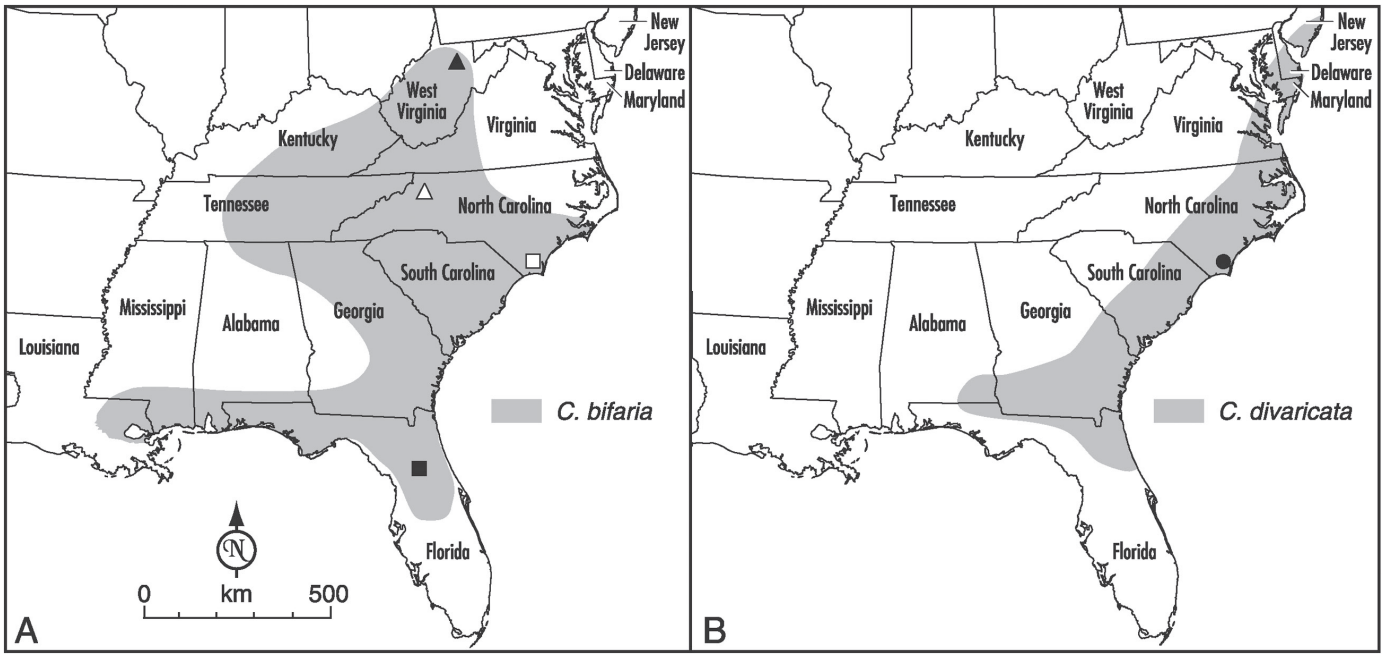

Figure 1. Distribution and sampling of North American *Cleistes*. A, *C. bifaria* (Barbour County, West Virginia; Wilkes County and Brunswick County, North Carolina; Alachua County, Florida). B, *C. divaricata* (Brunswick County, North Carolina).

the sympatric pair were made when plants were in flower to be certain of their identification.

DNA Extraction

Total genomic DNA was extracted from silica gel-dried leaf samples (Chase & Hills, 1991) of *C. bifaria* and *C. divaricata* using a modified 2XCTAB method (Doyle & Doyle, 1987). After precipitation in ethanol, DNA was resuspended in 100 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Extracted DNA was purified using QIAquick columns (Qiagen, Inc.) and quantified with a UV-1201 ultraviolet/visible spectrophotometer (according to the manufacturer's protocol; Shimadzu Europa, Milton Keynes, UK).

AFLP

AFLP analysis was conducted on individuals from each of the five sampled populations (Table 1; Figure 1).For *C. bifaria*, eight individuals were selected from West Virginia, eight from Florida, eight from coastal North Carolina, and three from the mountains of North Carolina; for *C. divaricata*, eight were selected from coastal North Carolina. An automated AFLP procedure using fluorescent dyes was carried out as described in the AFLP Plant Mapping Protocol (1996; Applied Biosystems Inc., ABI). The technique starts with digestion of genomic DNA with two restriction enzymes, EcoRI and MseI, and ligation of double-stranded DNA sequences (adaptors) to the ends of the restriction fragments. Two rounds of PCR amplification follow. The first amplifies a subset of the fragments using preselective primers that recognize the adaptors plus a single nucleotide in the original restriction fragment. The second PCR reaction uses more selective primers that amplify a yet smaller subset of the preselective products. In two selective primer trials, 27 primer combinations were tested with individuals of both *C. bifaria* and *C. divaricata*; two of these, B11 + C and B11 + G, were used to generate the final data set. B11 + C used the blue-labelled EcoRI primer with the selective bases -ACT and the MseI primer terminating in -CAGC.Combination B11 + G used the same EcoRI primer and an MseI primer with -CAGG.

Fluorescently labelled fragments from the selective amplification were separated by electrophoresis on a 5% denaturing polyacrylamide gel using an ABI 377 automated sequencer (according to the manufacturer's protocols; ABI). Gel analysis was carried out using GeneScan 3.1 and the bands were sized and scored in Genotyper 2.0 (Applied Biosystems, Inc.). A binary matrix listing each sample and the presence/absence (1/0) of each band was created from the AFLP data, and this matrix was exported to PAUP* (Swofford, 2000) for analysis with distance methods, e.g. UPGMA (Sneath & Sokal, 1973). Principal coordinates analysis (PCoA; Gower, 1966) was performed in the R program, version 4.0d0 (Casgrain & Legendre, 1998) using Jaccard's coefficient (Jaccard, 1908), which excludes similarity due to shared absences

(of AFLP bands here).The table of eigenvalues produced by the R program was exported to Microsoft Excel to produce XY scatter plots.

Sequencing and Plastid Microsatellites

Three noncoding regions, *rps16* intron, *trnL-F* intergenic spacer (IGS), and *psaI-accD* IGS, were amplified for *C. bifaria* and *C. divaricata* using primer pairs from Oxelman, Lidén & Berglund (1997; rpsF and rpsR2), Taberlet et al. (1991; c and f), and Barkman & Simpson (2002; ACCD-769F and PSAI-75R). The PCR products were sequenced in both directions using modified dideoxy cycle sequencing with dye terminators according to manufacturer's protocols (Big Dye 2.0, ABI). The cycle sequencing products were separated by electrophoresis on an ABI 377 automated sequencer (according to the manufacturer's protocols; ABI). Sequences were edited in Sequence Navigator and assembled using AutoAssembler version 1.4.0 (ABI).

A microsatellite region showing variation between *C. bifaria* and *C. divaricata* was identified within each of the three plastid regions sequenced. Forward and reverse primers were designed for the sequences flanking the microsatellites (Table 2). The reverse primers for each microsatellite were labelled with one of three fluorescent dyes, green, yellow, or blue. Amplification products were diluted 1 : 40 after comparison with a previously run sample. For each sample, 0.4 μL blue-labelled fragments, 0.4 μL green-labelled fragments, 0.8 μL yellow-labelled fragments and 1.2 μL of loading buffer (including the ROX-labelled internal size standard) were combined and loaded into a 5% polyacrylamide gel. The gel was run on the ABI 377 automated sequencer using the same conditions as for AFLP gels. Gel analysis was carried out with GeneScan 3.1 and Genotyper 2.0 (ABI). In addition, an indel (insertion/deletion) in the *rps16* intron was scored by running the amplification products for each sample on a 0.5% agarose gel.

Results

AFLP

The primer combination B11 + C produced 38 interpretable bands, 17 of which were variable; B11 + G produced 42 interpretable bands, 19 of which were variable. Overall, 45% of the scored bands generated by the two primers were polymorphic. Of the 36 variable bands, 33 were variable between populations and four were variable only within one of the populations (one in CbifNCC, one in CbifWV and two in CdivNCC, following abbreviations in Figure 2). None of the variable bands supported a division between *C. divaricata* and *C. bifaria*, i.e. no bands were shared by all individuals of *C. divaricata* and absent in all individuals of *C. bifaria* or vice versa.

The UPGMA revealed two main clusters (Figure 2).One cluster is composed of coastal plain populations of *C. bifaria* from Florida and North Carolina. The Florida individual 97-9M falls outside this group, although it is clearly more related (i.e. more similar) to the two coastal plain populations than it is to the mountain populations of *C. bifaria* (Figure 2). The other cluster groups individuals of *C. bifaria* from the mountains of West Virginia and North Carolina with the coastal population of *C. divaricata* (Figure 2). Each of the three populations forms a distinct subcluster, and the three individuals from the mountains of North Carolina were indistinguishable.

The PCoA generally confirmed the relationships shown by UPGMA. The two XY scatterplots (Figure 3A, B) created with the first three coordinates showed the individuals divided into two groups: coastal plain *C. bifaria* (CbifNCC +CbifFL) and mountain *C. bifaria* with coastal plain *C. divaricata* (CbifWV + CbifNCMT + CdivNCC). In Figure 3A, CbifNCC was divided into two clusters, with some bifFL individuals grouping with each. Florida individual 97-9 m was again an outlier. The individuals of *C. divaricata* appear mixed with some of the *C. bifaria* mountain individuals.In Figure 3B, coordinates 1 and 3 sepa-

Table 2. Plastid microsatellite primers

Name	Direction	Sequence	Target microsatellite
MS.Cleistes.rps16F	Forward	5'-CCC AAC TTT AGC TAG GAG TAC-3'	Poly G (8 or 9
MS.Cleistes.rps162R	Reverse	5'-TCG GGA TCG AAC ATC AAT TGC AAC-3'	in *Cleistes*)
MS.Cleistes.trnLE	Forward	5'-GGA TAT ATA TGA TAC CTG-3'	Poly T (10 or 11
MS.Cleistes.trnLF	Reverse	5'-GCC CCG TAC TTC ATT TAT TA-3'	in *Cleistes*)
MS.Cleistes.accDF	Forward	5'-GGT GTT TTG AGT GAG TTA-3'	Poly A (10 or 11
MS.Cleistes.accDR	Reverse	5'-CGA ATA TGT ATG AGA ATC-3'	in *Cleistes*)

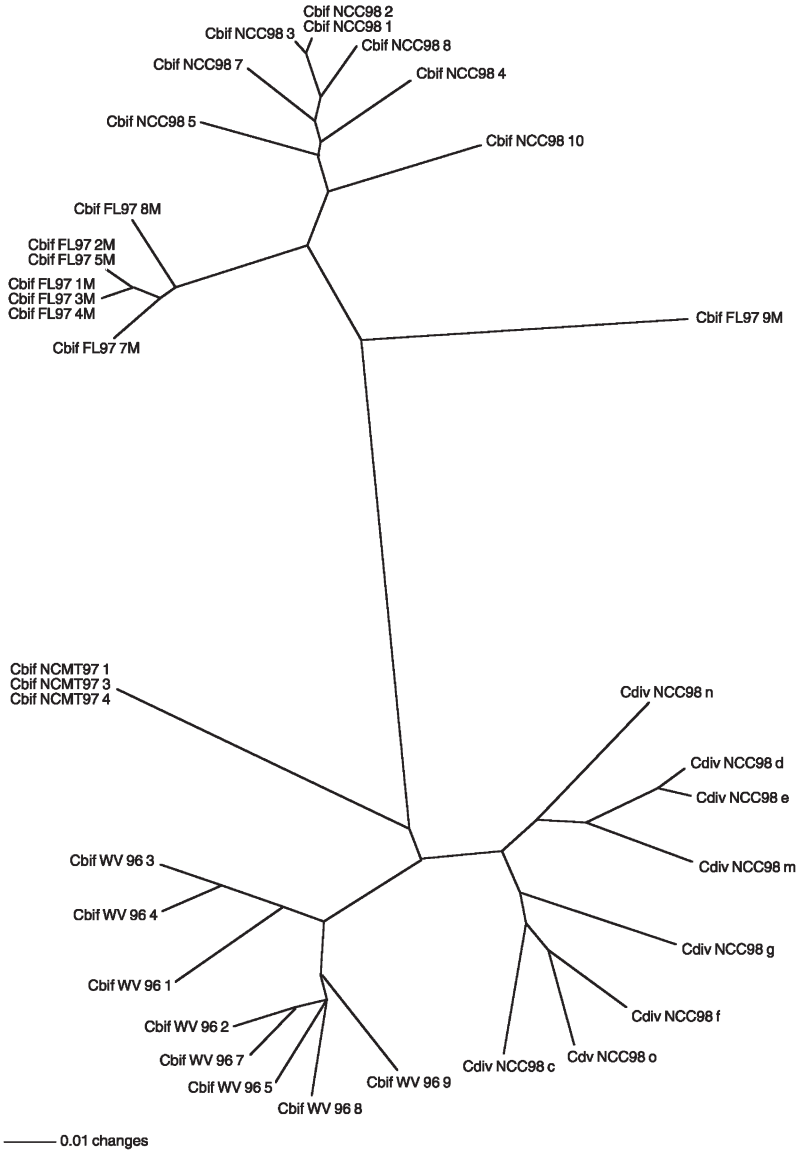

Figure 2. UPGMA tree showing relationships between the *Cleistes* populations sampled. *Abbreviations*: Cbif NCC, North Carolina coastal *C. bifaria*; Cdiv NCC, North Carolina coastal *C. divaricata*; Cbif WV, West Virginia *C. bifaria*; Cbif FL, Florida coastal *C. bifaria*; and Cbif NCMT, North Carolina mountain *C. bifaria*.

rated coastal plain *C. bifaria* populations from Florida and North Carolina from each other. There was, again, overlap between *C. divaricata* and the mountain *C. bifaria*.

Sequencing

DNA sequences were generated for the forward and reverse strands of three plastid regions: *rps16* intron, *trnL-F* intergenic spacer, and *psaI-accD* spacer. Amplification of *rps16* intron resulted in the largest fragment, 888 bp. The *psaI-accD* product was 656 bp long and the *trnL-F* fragment 430 bp. The *rps16* region was the most variable of the three; it had several point mutations and a 24 bp indel event. The mountain populations of *C. bifaria* and the population of *C. divaricata* shared a small *rps16* amplification product whereas the coastal plain populations of *C. bifaria* produced a larger product. One individual from each population was sequenced to ensure that the size difference corresponded to the same indel. This split between coastal plain and mountain *C. bifaria* plus *C. divaricata* was the same result produced by the AFLPs.

Plastid Microsatellites

Sequencing of the three plastid regions (*rps16* intron, *trnL-F*, and *accD-psaI*) revealed three variable microsatellites. Using primers designed for the flanking sequences, these microsatellites were amplified for the 65 available samples

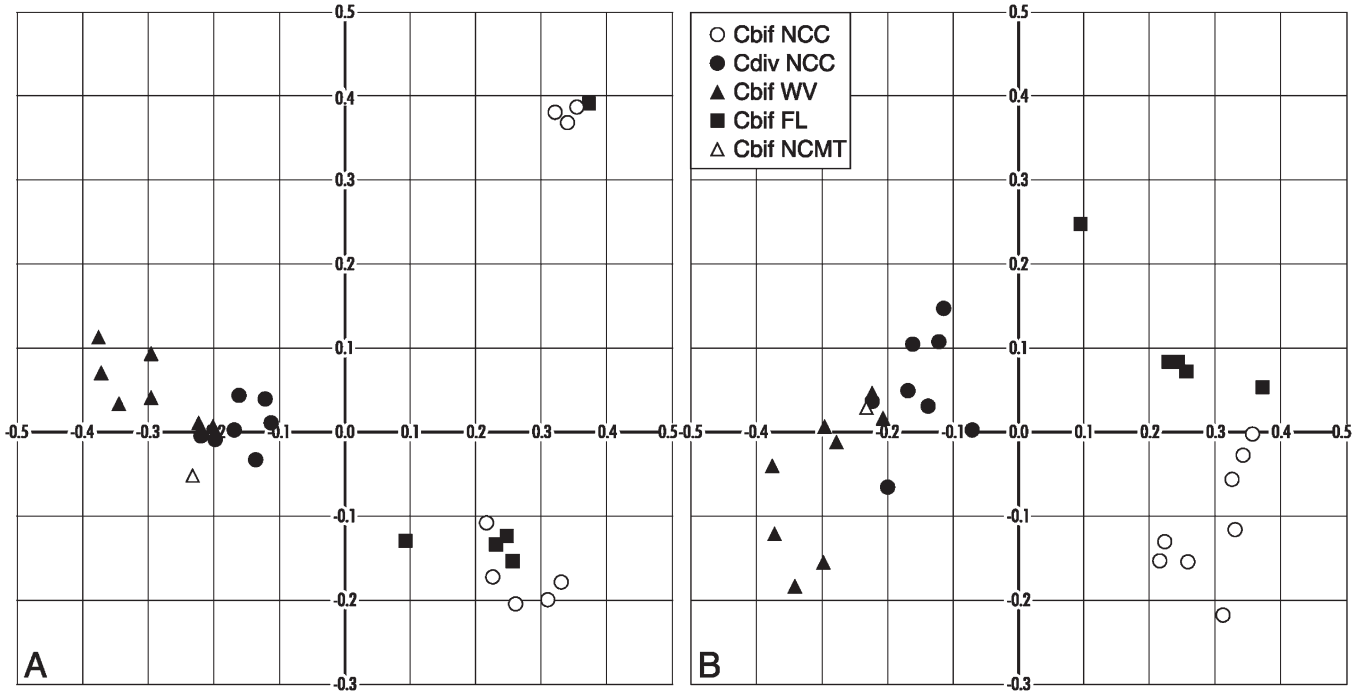

Figure 3. Principal coordinates analysis (PCoA). A, coordinates one by two. Coordinate one accounts for 31.97% of the AFLP variation and coordinate two for 13.39%. B, coordinates one by three. Coordinate three accounts for 6.26% of the AFLP variation. Abbreviations for populations follow Figure 2. Note: individuals with similar values may appear superimposed.

of *C. divaricata* and *C. bifaria*.Length variation was observed at each of the regions.The green-labelled M*SaccD* primers amplified fragments of 156 or 157 bp, named alleles A and B, respectively.The fragments produced by the blue-labelled MS*rps16* primers were 195 or 196 bp, termed alleles C and D. The yellow-labelled MS*trnL* primers amplified fragments that were 132 or 133 bp, alleles E and F, respectively.

The distribution of these six alleles divided the *C. bifaria* individuals into two major groups, corresponding to the mountain and coastal plain populations.The microsatellites grouped together the *C. bifaria* populations from Wilkes County, North Carolina, and Barbour County, West Virginia, the two mountainous areas. Individuals from these two populations share three alleles, A, C and E, hereafter referred to as the ACE haplotype (Figure 4). The plastid data also grouped the coastal plain *C. bifaria* populations from North Carolina and Florida. These populations share the B, C and F alleles, hereafter referred to as the BCF haplotype (Figure 4).

The *C. divaricata* population was not found to pos-sess a separate haplotype, but in fact shared alleles with the mountain populations of *C. bifaria*. Most of its individuals possessed the ACE haplotype, indicating a close relationship with the *C. bifaria* populations in the mountains of West Virginia and North Carolina.Of the 16 *C. divaricata* individuals sampled, three were found to have a different haplotype, ADE. This D allele was not present in any other population examined.

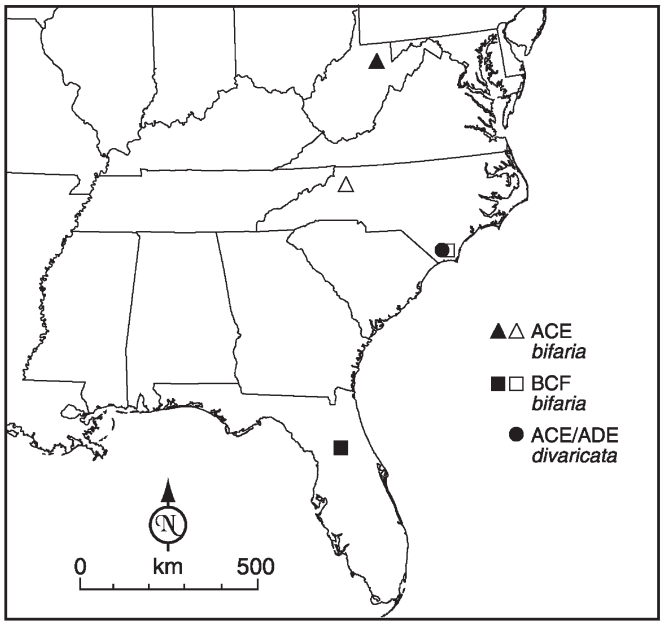

Figure 4. Distribution of plastid microsatellite haplotypes.

Discussion

Genetic Relationships

In all analyses, the data from AFLP, plastid sequences and microsatellites produced two distinct groupings: (1) the two coastal plain populations of *C. bifaria* and (2) the *C. bifaria* populations from the mountains of West Virginia and North Carolina together with the coastal plain population of *C. divaricata*. These groupings do not support the current delimitation of *C. bifaria* since the individuals identified as *C. bifaria* do not form a single cohesive group and are split into a mountain cluster and a coastal plain cluster. These molecular data appear to contradict morphological and other evidence, which supports the cohesiveness of *C. bifaria* (Fernald, 1946; Catling & Gregg, 1992).

Phylogeography

The distinct geographical distribution of these two taxa has spawned much speculation about their evolutionary history. Noting that *C. bifaria* is more widespread and tolerates higher elevations (Figure 1), Fernald (1946) proposed that it is the ancestral taxon or 'biological type' for the North American clade. He suggested that when the sea levels fell after the Tertiary Period and exposed the coastal plain, some individuals of bifaria dispersed to the south and east, giving rise to the larger-flowered *C. divaricata*. Relating the migration of *Cleistes* to events of the Tertiary Period some 2.5 million years ago, however, seems improbable (i.e. recent events are more likely to be the major factors). Fernald's proposal retains merit if instead one hypothesizes that ice sheets formed during the last glaciation c. 10,000 years ago could have caused bifaria to spread south and east. On the other hand, Luer (1972) thought it was equally possible that *C. divaricata* was the ancestral species and that *C. bifaria* might represent "a depauperate race straggling inland from the coast." He cited the fact that the rest of the genus is tropical, and presumably more suited to warm climates, as support for this second hypothesis. Luer's (1972) argument is contested by the work of Cameron & Chase (1999), who showed that the temperate *Cleistes* are more closely related to the temperate genus *Isotria* than to tropical species of *Cleistes*. Obviously, due to the sparse sampling of populations and the lack of a complete phylogenetic framework, the data sets presented here do not allow us to distinguish between these two hypotheses.However, they clearly lay to rest an earlier suggestion (Gregg, 1989) that the Barbour County, West Virginia, population arose by accidental importation from coastal North Carolina. Natural migration northward from the North Carolina mountains (Strausbaugh & Core, 1977) now appears the more likely source.

Pollination Biology and Gene Flow

Two different pollination strategies are known for North American *Cleistes*. Its flowers can act as 'foodfraud' mimics (Ackerman, 1986; Ackerman, Meléndez-Ackerman & Salguero-Faria, 1997), which is the primary strategy in the West Virginia population, where the yellow labellar crest of the nectarless and scentless flower probably mimics pollen, thus attracting naive bees seeking food (Gregg, 1989, 1991). Plants from the North Carolina mountain population of *C. bifaria* share these three characteristics (K. B.Gregg, unpubl. data) and may also be food frauds. On the other hand, at the Brunswick County savannah in coastal North Carolina, where a substantial proportion of bumblebee pollinators collect pollen, a reward strategy appears more important (Gregg, 1991). Here, flowers of *C. bifaria* emit a strong vanilla scent whereas those of *C. divaricata* produce a daffodil-like scent. Floral fragrance is thus associated with pollen reward and may encourage bees to visit the flowers. A vanilla fragrance is also produced by flowers in the Florida coastal plain population (K. B. Gregg, unpublished data). Presence or absence of vanilla scent in these populations corresponds with the separation of *C. bifaria* found in our analyses.

The development of different fragrances and peak flowering times one week apart (Catling & Gregg, 1992) where the two taxa grow sympatrically is a possible instance of character displacement and may be evidence for selection against hybrid formation. For example, in preferring one fragrance over another, individual bumblebees may help maintain reproductive isolation. Our analyses corroborate the absence of gene flow where *C. bifaria* and *C. divaricata* occur sympatrically, as in Brunswick County, North Carolina.

On the other hand, the clear genetic link between the coastal plain *C. divaricata* and the two mountain populations of *C. bifaria* raises the question of whether gene flow may be occurring among these groups. This is improbable, however, for two reasons.First, the bee pollinators of *Cleistes* are unlikely to carry the pollen over such long distances. Second, although long distance seed dispersal via air currents is possible, it is highly unlikely that seeds of coastal plain *C. divaricata* would germinate or their seedlings survive the much more severe winters of the North Carolina or West Virginia mountains. It is remotely possible but still not likely that seeds from a mountain population of *C. bifaria* might be viable in the coastal plain of North Carolina. Thus, the shared ACE haplotype, AFLP markers, and indel character between the coastal plain *C.*

divaricata and the mountain *C. bifaria* probably indicate a recent common ancestor as opposed to contemporary gene flow. The geographical pattern of haplotypes (Figure 4) may have been produced by the existence of two refugia during the last glaciation as in *Liriodendron* (Sewell et al., 1996), in which there was a distinct border between northern and southern US races.

Conclusions

Molecular data from the nuclear and plastid genomes were used to assess genetic relationships among five populations of North American *Cleistes*.This study has shown that *C. bifaria* as currently described does not form a genetically cohesive group but rather consists of two clearly distinct groupings, one represented by populations from the mountains of North Carolina and West Virginia and the other by populations from the coastal plains of North Carolina and Florida. Because this division corresponds to known differences in floral fragrance (i.e. two scentless mountain populations and two vanilla-scented coastal plain populations), splitting *C. bifaria* into two species could be a viable taxonomic solution. Renewed investigation of the morphology and pollination biology in light of the molecular data might, in fact, uncover greater differences between coastal plain and mountain populations of *C. bifaria* which would support their genetic distinctiveness. However, with these data we are unable to make any clear species distinctions. A wider sampling of both *C. divaricata* and *C. bifaria* throughout their ranges will be necessary to fully understand the complex relationships of North American *Cleistes*.

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