

Evidence for range stasis during the latter Pleistocene for the Atlantic Coastal Plain endemic genus, *Pyxidanthera* Michaux

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Abstract

The general phylogeographical paradigm for eastern North America (ENA) is that many plant and animal species retreated into southern refugia during the last glacial period, then expanded northward after the last glacial maximum (LGM). However, some taxa of the Gulf and Atlantic Coastal Plain (GACP) demonstrate complex yet recurrent distributional patterns that cannot be explained by this model. For example, eight co-occurring endemic plant taxa with ranges from New York to South Carolina exhibit a large disjunction separating northern and southern populations by >300 km. *Pyxidanthera* (Diapensiaceae), a plant genus that exhibits this pattern, consists of two taxa recognized as either species or varieties. We investigated the taxonomy and phylogeography of *Pyxidanthera* using morphological data, cpDNA sequences, and amplified fragment length polymorphism markers. Morphological characters thought to be important in distinguishing *Pyxidanthera barbulata* and *P. brevifolia* demonstrate substantial overlap with no clear discontinuities. Genetic differentiation is minimal and diversity estimates for northern and southern populations of *Pyxidanthera* are similar, with no decrease in rare alleles in northern populations. In addition, the northern populations harbour several unique cpDNA haplotypes. *Pyxidanthera* appears to consist of one morphologically variable species that persisted in or near its present range at least through the latter Pleistocene, while the vicariance of the northern and southern populations may be comparatively recent. This work demonstrates that the refugial paradigm is not always appropriate and GACP endemic plants, in particular, may exhibit phylogeographical patterns qualitatively different from those of other ENA plant species.

Keywords: amplified fragment length polymorphism, cpDNA, Diapensiaceae, phylogeography, Pleistocene, refugium

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Introduction

The alternating glacial and interglacial periods that characterized the Pleistocene had major impacts on the biogeography and genetic diversity of plant species in the Northern Hemisphere (Comes & Kadereit 1998; Hewitt 2000). The LGM, approximately 18 000 years BP, saw the Laurentide ice sheet reach its southern extent

in eastern North America (ENA) (Ehlers & Gibbard 2004). The primary scenario describing plant species' ranges during and following the LGM in ENA includes (i) range contraction to southern refugia (Delcourt & Delcourt 1981) and (ii) subsequent recolonization of northern habitats after the retreat of the glaciers (Dorken & Barrett 2004). Previous studies have identified the resulting phylogeographical patterns of plant species in ENA and made inferences about possible refugia during the glacial maxima (Soltis *et al.* 2006). Several such patterns have been identified in the ranges

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of ENA plant and animal species. These include east-west divisions between Gulf Coast and Atlantic Coast populations (Mylecraine *et al.* 2004), phylogeographical separation by river drainage systems (Church *et al.* 2003), and the identification of refugia closer to the glacial front, either in the southern Appalachians (Gonzales *et al.* 2008) or farther north (Magni *et al.* 2005; McLachlan *et al.* 2005).

Eastern North America has generally been divided into four physiogeographical regions: Interior Lowlands, Appalachian Highlands, Piedmont, and Gulf and Atlantic Coastal Plain (GACP) (Fenneman 1938). Compared to the other regions, the GACP is well defined geologically and floristically (Takhtajan 1986), but little is known about the phylogeography of the many widespread species that are endemic to the GACP. Previous phylogeographical studies of GACP plant species have generally focused on narrow endemics with small latitudinal ranges (Evans *et al.* 2000; Oliveira *et al.* 2007) or species with ranges that cross multiple physiographical regions (Morris *et al.* 2008; however, see Mylecraine *et al.* 2004). This is unfortunate; over 1300 species and 47 genera are endemic to the region (Sorrie & Weakley 2001), the second-highest concentration in the United States and only exceeded by the California Floristic Province (Flora of North America Editorial Committee 1993). Without taking into account the endemic species of the GACP and their distributional patterns, any attempt to understand the postglacial phylogeography of ENA is limited.

Sorrie & Weakley (2001) documented 27 different recurrent distributional patterns for endemic plant species of the GACP. One of the most interesting of these patterns is the disjunct distribution of eight taxa (*Calamovilfa brevipilis*, *Dichanthelium hirstii*, *Eupatorium resinosum*, *Gentiana autumnalis*, *Lobelia canbyi*, *Nartheceum americanum*, *Pyxidantha barbulata*, and *Rhynchospora pallida*) that occur in New York and New Jersey and eastern North and South Carolina, but not in the intervening areas of Maryland, Delaware, and most of Virginia. In addition to these eight taxa, numerous species exhibit the same disjunction between New Jersey and the southern GACP, but are more widespread in the southern part of the GACP. Common distributional patterns may be the result common biogeographical processes, but there is always the possibility of pseudocongruence (Hafner & Nadler 1990; Cunningham & Collins 1994).

We focus here on the genus *Pyxidantha* Michaux as a case study to investigate the refugial paradigm in the GACP. *Pyxidantha* is in Diapensiaceae, a small family with a circumboreal distribution, with some taxa extending southward into eastern Asia and ENA. The genus includes two recognized species; both are

woody, winter-flowering, evergreen cushion plants. Populations of the more widespread *P. barbulata* occur on Long Island in New York, the Pine Barrens of New Jersey, several locations in southeastern Virginia, and the coastal plain of North Carolina and South Carolina. *P. brevifolia* has a more limited range; it has only been documented in six counties in the Sandhills region of North Carolina and South Carolina. *P. brevifolia*, currently under intensive study as a species at risk by the US Department of Defense, is considered vulnerable to extinction in North Carolina, with over 80% of the North Carolina populations confined to Fort Bragg Military Reservation, NC (Buchanan & Finnegan 2008). *P. brevifolia* is nearly restricted to xeric sandhill scrub communities within the long-leaf pine ecosystem (Schafale & Weakley 1990; Sorrie *et al.* 2006), one of the most imperiled ecosystems in North America, with approximately 2% of the historical area currently extant (Frost 2006). In addition to containing most of the remaining *P. brevifolia* populations, Fort Bragg Military Reservation is also one of the few places where the two species of *Pyxidantha* co-occur. When sympatric, *P. barbulata* and *P. brevifolia* occupy nonoverlapping ecological habitats, with *P. barbulata* occupying wetter sites such as pocosin ecotones and *P. brevifolia* occurring on extremely xeric sand ridges.

Pyxidantha was monotypic until 1929, at which time *P. brevifolia* was separated from sympatric populations of *P. barbulata* in the Sandhills region of North Carolina and upper South Carolina based on habitat differences, shorter leaves, and dense pubescence relative to the more widespread *P. barbulata* (Wells 1929). Differing ecological niches and morphological characters of *P. barbulata* and *P. brevifolia* led to a debate regarding the proper taxonomic status of the two taxa. In 1964, *P. brevifolia* was reduced to a variety of *P. barbulata* without comment (Ahles 1964). Afterwards, several studies investigated the appropriate taxonomic status of *P. brevifolia*. An embryological study (Reynolds 1966) concluded that both notable developmental similarities and differences existed between the two species and ultimately relied on the ecological and morphological differences to support the continued recognition of two species. Primack & Wyatt (1975) found correlation between leaf length and soil moisture of *P. brevifolia* and *P. barbulata* at a single site in South Carolina and concluded that the difference in leaf length between the two species is clinal, suggesting that *P. brevifolia* is simply a morphological variant of *P. barbulata*. More recently, an allozyme study – restricted to the populations from the southern range of the genus – found that the two species share similar levels of genetic diversity, with very little intertaxa genetic differentiation (Godt & Hamrick 1995). However, recent floras for the region

(Weakley 2008; Sorrie *et al.* 2009) have continued to recognize two species, emphasizing the morphological, ecological, and embryological differences between them.

In this study, we use cpDNA sequences, amplified fragment length polymorphism (AFLP) markers, and morphological measurements to investigate the taxonomy and phylogeography of both *P. barbulata* and *P. brevifolia* across the entire range of the genus. We attempt to determine whether clear morphological and genetic differences exist between the two species and whether the morphological, ecological, and embryological variation previously observed in the southern populations of *Pyxidanthera* (in the text, *Pyxidanthera* will refer to both *P. barbulata* and *P. brevifolia*) correlate with greater genetic diversity in the south. Using genetic data, we attempt to distinguish between two plausible phylogeographical scenarios. The first scenario represents typical refugial patterns described for numerous species in ENA; the genus *Pyxidanthera* was isolated in one or more southern refugia during the Pleistocene and subsequently recolonized northern areas after the LGM. Genetic patterns supporting this scenario would include reduced genetic diversity in northern populations (Hewitt 2000), recolonized areas containing only a subset of refugial population alleles (Broyles 1998), and putative refugia having a greater number of rare alleles, which may reflect historical processes better than genetic diversity estimates (Comps *et al.* 2001; Paun *et al.* 2008). Alternatively, the two species in *Pyxidanthera* could have persisted in their present ranges through the later Pleistocene rather than retreating into one or more glacial refugia. Genetic patterns that would suggest this second scenario include no reduction in genetic diversity or rare alleles in northern populations and the presence of alleles restricted to northern populations.

Materials and methods

Sampling and morphological measurements

We collected leaf tissue samples of 423 individuals from 29 *Pyxidanthera brevifolia* populations (defined as all *P. brevifolia* individuals that occurred within 0.75 km of each other) and 178 individuals from 14 *P. barbulata* populations, across the ranges of both species. A priori taxonomic identity was determined based on habitat differences, State Natural Heritage Program records, and geographical region (*P. brevifolia* is restricted to the Sandhills region of North and South Carolina). For each sample, we measured the longest leaf length and width and categorized the leaf pubescence into one of two categories: pubescence covering more than half of the leaf, and pubescence covering half or less of the leaf.

We evaluated differences in leaf length and leaf width means between *P. barbulata* and *P. brevifolia* using *t*-tests and evaluated differences in leaf pubescence categories using a chi-square test.

Molecular methods

DNA was extracted from 319 *P. brevifolia* individuals across 17 populations and 157 *P. barbulata* individuals across 14 populations using the CTAB method with minor modifications (Doyle & Doyle 1987). After an initial screening of 16 cpDNA regions known to be highly polymorphic (Shaw *et al.* 2007), we amplified two polymorphic regions – *atpI-atpH* and *psbD-trnT(GUU)* – of 63 and 42 samples from 14 and 10 populations of *P. barbulata* and *P. brevifolia*, respectively, using universal primer pairs (Shaw *et al.* 2007). PCR conditions followed Shaw *et al.* (2005) in 12.5 µL solutions using the following protocol: 1 hold (5 min per 80 °C), 30 cycles [(1 min per 95 °C), (1 min per 50 °C), (4 min per 65 °C)], 1 hold (5 min per 65 °C). PCR products were cleaned prior to sequencing using Antarctic Phosphatase (0.5 Units), Exonuclease I (0.2 Units), and 1 µL 10× Antarctic Phosphatase buffer (New England BioLabs, Ipswich, MA, USA) at 37 °C (15 min) and 80 °C (15 min). We sequenced in the forward direction for the *atpH* and *psbD* regions using the Big Dye 3.1 kit (Applied Biosystems, USA) and analysed the products using an ABI 3730 DNA sequencer (Applied Biosystems, USA). We edited and aligned sequences using Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI, USA) and MEGA version 4 (Tamura *et al.* 2007). Sequences were submitted to GenBank under accession numbers nos. HM564379–HM56491 (Table S1).

Amplified fragment length polymorphism markers are appropriate for examining low levels of genetic divergence within and between closely related taxa (Coart *et al.* 2002; McKinnon *et al.* 2008) and have been successfully used in phylogeographical studies (Meudt & Bayly 2008; Perez-Collazos *et al.* 2009). AFLP genotyping followed the multiplexing protocol described by Trybush *et al.* (2006), with the minor modification that the restriction and ligation steps were combined in a single reaction at a total volume of 10 µL. For the pre-amplification reaction, we used EcoRI+A and MseI+C primers. Three selective primer pairs were chosen after a trial based on the number of reproducible polymorphic markers produced: Eco-ACC/MseI-CAT (Hex), Eco-ATG/MseI-CAT (FAM), and Eco-AGG/MseI-CAT (NED). Selective amplification products were separated and analysed using an ABI 3730 DNA sequencer (Applied Biosystems, USA) and automatically scored with Genemarker version 1.8 (Softgenetics LCC, State College, PA, USA) using the default settings, with the

exception that we normalized the FAM-dyed markers and set the allele evaluation peak score to 'pass' if it was ≥ 1 (Holland *et al.* 2008). The reproducibility of the AFLP profiles was evaluated by running eight duplicate samples for each 96-well plate. Error rates between duplicates were calculated using a Euclidean distance measure (Bonin *et al.* 2004). To reduce the error rate, we removed bands with 10 or more errors when comparing duplicate samples (Zhang *et al.* 2010).

cpDNA data analysis

Because the chloroplast represents a single nonrecombining locus, sequences of the two sampled regions were concatenated. We recoded insertions or deletions (indels) that did not violate the assumptions of the infinite sites model (Kimura 1969) as identified by SNAP Workbench (Price & Carbone 2005). We performed three separate tests of neutrality to test for evidence of population expansion or selection in the cpDNA – Fu and Li's D^* and F^* (Fu & Li 1993) and Fu's F_s (Fu 1997) – using SNAP Workbench. Fu and Li's D^* and F^* neutrality tests are more powerful for detecting background selection, while Fu's F_s is more powerful for detecting population growth (Ramos-Onsins & Rozas 2002). We estimated an unrooted haplotype network using the haploNet function as implemented in the pegas package (Paradis 2009) in R (R Development Core Team 2009). This package implements the statistical parsimony method for network reconstruction (Templeton *et al.* 1992). We performed two analyses of molecular variance (AMOVA) using Arlequin version 3.01 (Excoffier *et al.* 2005) with the data set hierarchically partitioned by region and individual populations within regions (with two regions defined as New Jersey and New York, hereafter referred to as northern populations, and Virginia, North Carolina, and South Carolina, hereafter referred to as southern populations) and by species (*P. barbulata* and *P. brevifolia*) and populations within species. We estimated the nucleotide genetic diversity (π) (Nei 1987) for each population using DnaSP version 5 (Librado & Rozas 2009).

To test for phylogeographical structure in the data set, we compared two measures of genetic differentiation between populations – G_{ST} , based on haplotype frequency, and N_{ST} , by similarities between haplotype sequences – using PERMUT 2.0 (Pons & Petit 1996) with 1000 permutations. If N_{ST} is significantly greater than G_{ST} , it is taken as evidence of a phylogeographical signal in the data set. To test for isolation by distance (IBD), we performed Mantel tests using the R package vegan (Oksanen *et al.* 2009) between the log-transformed geographical distance matrix and the pairwise population N_{ST} matrix as calculated in DnaSP version 5

(Librado & Rozas 2009) for all populations and for the southern and northern populations separately.

We reconstructed the gene genealogy for the sampled chloroplast regions using Genetree version 9.0 (Bahlo & Griffiths 2000) as implemented in SNAP Workbench (Price & Carbone 2005). We estimated the population mutation rate (θ), using Watterson's method (1975) as calculated in Genetree for both geographical regions and used the average between the two regions as the starting θ . Because of the larger geographical area covered by the two species of *Pyxidantha* in the southern populations, we assumed a model of unequal population sizes, with the southern population twice as large as the northern population, and nonexponential population growth. We performed ten independent simulations with different starting values of 10^6 iterations, selecting the rooted genealogy and mutation age estimates with the highest probability.

To simultaneously analyse the effects of incomplete lineage sorting and gene flow on the genetic structure of the northern and southern *Pyxidantha*, we employed an isolation with migration model of population divergence (Nielsen & Wakeley 2001) implemented in the program IMA2 (Hey & Nielsen 2007). IMA2 estimates the following parameters based on the genetic data: θ for all populations (extant and ancestral), migration parameters (m) for gene flow between populations, and t , time in coalescent units since divergence of the extant populations. We performed three independent runs with ten chains each under an infinite sites model with a burn-in period of 150 000 steps. We sampled 500 000 genealogies, saving one genealogy every 100 steps. We evaluated proper mixing based on the absence of trends in plotted parameter estimates and congruence of parameter estimates between runs. 100 000 of the 500 000 saved genealogies were combined to evaluate 24 models that were either nested within the full model or that constrained select parameters by setting them equal to each other (e.g. equal migration between populations). We compared the different model posterior probabilities using an information-theoretic approach recently extended to phylogeographical data (Carstens *et al.* 2009). Information theory statistics were calculated according to Burnham and Anderson (2002).

AFLP data analysis

We calculated the percentage of polymorphic loci (P%) and Nei's expected heterozygosity (Nei 1987) using AFLPsurv version 1.0 (Vekemans *et al.* 2002) and the 'frequency down weighted marker score' (DW) (Schönwetter & Tribsch 2005) using the R script AFLPdat (Ehrich 2006); several population genetic diversity

measures were included to ensure consistency between methods. DW is calculated by summing each occurrence of a particular marker in a population and dividing that value by the sum of the marker across all populations. For each population, these values are then averaged across all markers. Populations that have been isolated are expected to accumulate rare markers and thus their DW scores should be higher. We first removed populations that contained fewer than seven samples to minimize effects of low sample size (Bonin *et al.* 2007), leaving a total of 437 samples from 25 populations. We tested for effects of sample size on all the calculated genetic diversity estimates by regressing estimated diversity on sample size, and we also tested for correlation between all possible pairings of the included diversity measures. Populations were grouped according to taxonomic identity and region, and we tested for significant differences between diversity estimates using *t*-tests in R.

Population differentiation and structure were explored by first running an ordination using nonmetric multidimensional scaling (NMDS) to graphically display population pairwise genetic distances (D) (Nei 1972) in a reduced dimensional space using the R package *labdsv* (Roberts 2010). We included all populations regardless of sample size for the analysis. In addition, we explored population genetic structure using STRUCTURE 2.3.2.1 (Pritchard *et al.* 2000; Falush *et al.* 2007). For K1 through 9, we performed three runs with a burn-in length of 10 000 and post burn-in length of 25 000, assuming admixture and correlated allele frequencies. We determined the most likely number of populations by graphically analysing the model log likelihoods for each K. Because $\ln P(D)$ did not increase monotonically to the optimal K (Herrera & Bazaga 2008), we did not use the methods of Evanno *et al.* (2005). Three analyses of molecular variance (AMOVA) were performed using Arlequin version 3.01 (Excoffier *et al.* 2005), with partitioning of the data following the chloroplast AMOVAS. To test for IBD, we performed a Mantel test between the population genetic distance matrix and the log-transformed geographical distance matrix using the R package *vegan* (Oksanen *et al.* 2009).

Results

Morphology

Pyxidantha barbulate has significantly longer leaf lengths (6.3 mm vs. 4.5 mm, respectively, $P < 0.001$) and widths (1.9 mm vs. 1.3 mm, $P < 0.001$) compared to *P. brevifolia*, but there is considerable overlap between the two species in both traits (Fig. 1). The variation in

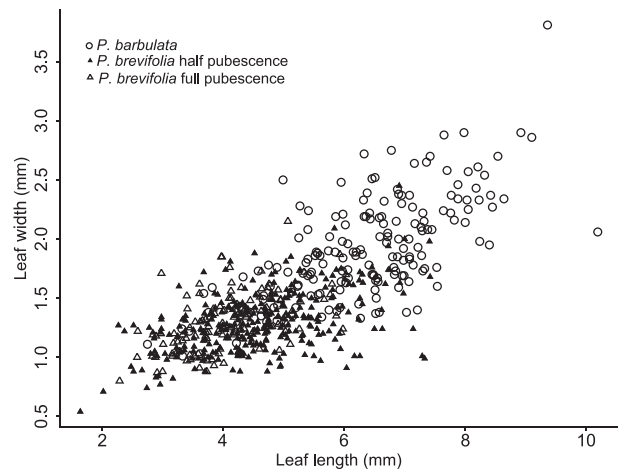


Fig. 1 Morphological variation in leaf length, leaf width, and pubescence of *Pyxidantha barbulate* (circles) and *P. brevifolia* (triangles). Solid triangles represent *P. brevifolia* specimens that had pubescence for half or less than half of the leaf; open triangles represent *P. brevifolia* specimens with pubescence greater than half of the leaf. Although there are statistically significant differences between the two species for mean leaf length, leaf width, and pubescence, there is considerable overlap in the ranges of these traits between the two species.

leaf length is continuous between *P. barbulate* and *P. brevifolia* with no obvious break, certainly not at the 3.5–4 mm size suggested in taxonomic keys (Sorrie *et al.* 2009). There is a significant difference in leaf pubescence between the two species ($P < 0.001$). All *P. barbulate* had pubescence covering less than half of their leaves, but 49% of *P. brevifolia* also had pubescence covering more than half of their leaves. As with leaf length and width, there is considerable variation within taxa.

cpDNA

The two sampled cpDNA regions for 105 individuals yielded 975 characters, of which 14 were polymorphic (Table S2). The data set included 12 substitutions and two indels that did not violate the infinite sites model. None of the three neutrality tests (Fu and Li's D^* and F^* and Fu's F_s) were significant ($P > 0.05$), indicating that there is no evidence of either population growth or background selection. A statistical parsimony haplotype tree identified 12 haplotypes (Fig. 2). The interior haplotypes of the network (H7, H11) are geographically widespread compared to the derived haplotypes, which tend to be both less frequent and geographically restricted (Fig. 2; Table S2). Results from Genetree indicate that H11 is the haplotype with the highest probability of being ancestral (average relative likelihood 69.5%); however, both of the interior haplotypes were

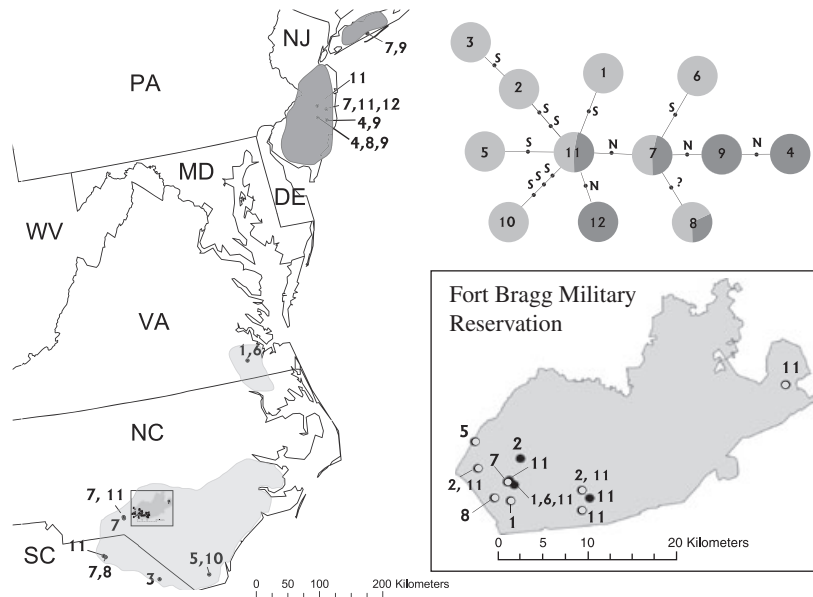


Fig. 2 Geographical distribution (shaded in grey) and statistical parsimony network for 12 haplotypes from 2 cpDNA regions of *Pyxidantha*. State names are in bold abbreviations and numbers represent haplotypes from Table S2. Black dots in the haplotype network represent mutational steps; associated letters (S for South, N for North) represent the most likely (>95% probability) geographical origin of mutations inferred using Genetree 9.0. Light grey shading of haplotype network represents proportion of the associated haplotype comprised of southern individuals and darker grey shading represents proportion comprised of northern individuals. Inset map: Sampling of *Pyxidantha* populations on Fort Bragg Military Reservation. *Pyxidantha barbulate* populations are represented by closed circles and *P. brevifolia* populations are represented by open circles.

almost equally common in the northern and southern populations (Fig. 2). Two of the four haplotypes derived from H7 (H4 and H9) only occur in New Jersey and New York and had the highest probability of a northern origin, while the other two derived haplotypes (H6 and H8) occur in both northern and southern population. Five of the six haplotypes derived from the second interior haplotype, H11, only occur in southern populations and most likely are of southern origin. Only H12 has a higher probability of a northern origin;

it is a private haplotype restricted to one northern population.

Region explains a small but statistically significant percentage of the genetic variation when used as the highest grouping variable in a hierarchical AMOVA (17.27%, $P < 0.05$), revealing that genetic variation is not evenly spread across the northern and southern populations (Table 1). Populations within regions explain most of the variation (56.58%, $P < 0.001$); within-population genetic differences and region

Table 1 Analyses of molecular variance (AMOVA) results for *Pyxidantha barbulate* and *P. brevifolia* using cpDNA sequences and amplified fragment length polymorphism (AFLP) markers

Source of variation	AFLP			cpDNA		
	d.f.	Variance	% of variation	d.f.	Variance	% of variation
Grouped by species						
Between species	1	0.37	1.60**	1	-0.01	-1.71 NS
Among populations within species	23	1.85	7.87***	22	0.63	72.22***
Within populations	412	21.30	90.53***	81	0.26	29.49***
Grouped by region (North vs. South)						
Between regions	1	0.76	3.20**	1	0.17	17.27*
Among populations within regions	23	1.86	7.79***	22	0.56	56.58***
Within populations	412	21.30	89.01***	81	0.26	26.15***

***Indicates P -value < 0.001 , ** P -value < 0.01 , * P -value < 0.05 , and NS indicates nonsignificance of variation.

account for a smaller but still significant percentage of the variation (26.15%, $P < 0.001$). When species is used as the highest grouping variable, AMOVA results demonstrate significant genetic differences among populations (72.22%, $P < 0.001$), but not significant differences between the two species (0%, $P > 0.05$). The nucleotide genetic diversity (π) averages 0.0004 across all specimens with no significant differences between means for either regions or species ($P > 0.05$) (Table 2).

Geographically distant populations are not more differentiated from each other than populations in closer geographical proximity (Fig. 3). N_{ST} is significantly greater than G_{ST} (0.788 vs. 0.695, $P < 0.01$), indicating

that there is a phylogeographical signal in the chloroplast data; in other words, haplotypes within populations are more similar to each other than expected. However, Mantel tests for IBD find no significant signal across the range of *Pyxidanthera* populations (Fig. 3, $R = 0.01$, $P = 0.39$). This pattern generally arises when genetic drift exerts more influence than gene flow at the regional scale (Hutchison & Templeton 1999). When northern and southern populations are analysed separately, there is no significant IBD in the northern populations ($R = -0.05$, $P = 0.47$) but there is marginally significant IBD in the southern populations, although the effect is weak ($R = 0.13$, $P = 0.049$).

Table 2 Genetic diversity indices for *Pyxidanthera barbulata* and *P. brevifolia* based on cpDNA sequences and AFLP markers

Population	Species	State	N	%P	DW	He	Π	Haplotypes
NC_1	<i>barbulata</i>	NC	14 (5)	41.0	12.99	0.15	0.0019	H1,H6,H11
NC_2	<i>barbulata</i>	NC	0 (5)	–	–	–	0.0000	H11
NC_3	<i>barbulata</i>	NC	0 (4)	–	–	–	0.0000	H2
NC_4	<i>barbulata</i>	NC	9 (4)	50.0	7.89	0.14	0.0000	H11
NC_6	<i>barbulata</i>	NC	19 (1)	26.5	7.40	0.08	0.0000	H7
NC_8	<i>barbulata</i>	NC	12 (4)	23.2	4.09	0.09	0.0005	H7,H11
NC_9	<i>barbulata</i>	NC	19 (6)	44.2	18.91	0.15	0.0013	H5,H10
NJ_CB	<i>barbulata</i>	NJ	0 (5)	–	–	–	0.0010	H7,H11,H12
NJ_CW	<i>barbulata</i>	NJ	17 (5)	34.2	14.41	0.11	0.0012	H4,H8,H9
NJ_WB	<i>barbulata</i>	NJ	15 (6)	27.1	9.48	0.10	0.0000	H11
NJ_WG	<i>barbulata</i>	NJ	18 (5)	38.7	18.69	0.12	0.0004	H4,H9
NY_1	<i>barbulata</i>	NY	0 (5)	–	–	–	0.0004	H7,H9
SC_1	<i>barbulata</i>	SC	8 (3)	50.3	12.67	0.16	0.0000	H3
VA_1	<i>barbulata</i>	VA	13 (5)	32.6	10.08	0.12	0.0019	H1,H6
SC_HP	<i>brevifolia</i>	SC	8 (5)	25.2	1.83	0.08	0.0004	H7,H8
SC_SL	<i>brevifolia</i>	SC	26 (5)	26.1	8.33	0.08	0.0000	H11
002A	<i>brevifolia</i>	NC	19 (5)	41.0	16.25	0.12	0.0000	H11
10	<i>brevifolia</i>	NC	17 (0)	47.4	23.21	0.15	–	–
20	<i>brevifolia</i>	NC	21 (5)	51.3	23.17	0.14	0.0008	H2,H11
24	<i>brevifolia</i>	NC	19 (0)	46.1	17.26	0.14	–	–
026D	<i>brevifolia</i>	NC	19 (1)	39.0	9.66	0.12	0.0000	H1
03_25	<i>brevifolia</i>	NC	30 (5)	36.1	15.18	0.11	0.0008	H2,H11
028E	<i>brevifolia</i>	NC	0 (4)	–	–	–	0.0000	H7
33	<i>brevifolia</i>	NC	33 (0)	31.3	22.40	0.11	–	–
038D	<i>brevifolia</i>	NC	15 (0)	41.9	13.65	0.14	–	–
057Y	<i>brevifolia</i>	NC	24 (3)	41.9	25.43	0.13	0.0000	H8
058B	<i>brevifolia</i>	NC	22 (0)	40.6	18.61	0.14	–	–
065N	<i>brevifolia</i>	NC	15 (5)	31.6	7.24	0.11	0.0000	H11
066A	<i>brevifolia</i>	NC	0 (4)	–	–	–	0.0000	H11
092B	<i>brevifolia</i>	NC	8 (0)	34.2	3.26	0.11	–	–
93_115	<i>brevifolia</i>	NC	17 (0)	43.2	20.41	0.14	–	–
Overall mean				37.8	13.70	0.12	0.0004	
<i>barbulata</i>				36.8	11.66	0.12	0.0006	
<i>brevifolia</i>				38.5	15.06	0.12	0.0002	
Northern				33.3	14.20	0.11	0.0006	
Southern				38.5	13.63	0.12	0.0004	

Amplified fragment length polymorphism (AFLP) genetic diversity indices were only calculated for populations with more than seven genotyped individuals (437 total specimens). %P represents the number of polymorphic loci, DW is a measure of rare alleles per population, and He is a measure of expected heterozygosity based on the AFLP markers. π is a measure of cpDNA nucleotide diversity. N represents the number of specimens for each population for AFLP markers and cpDNA sequences (in parentheses).

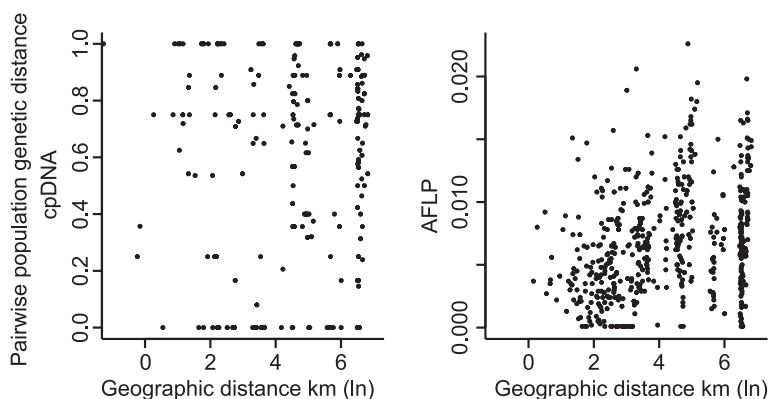


Fig. 3 Isolation by distance for cpDNA (left side) and amplified fragment length polymorphism (AFLP) (right side) markers for *Pyxidantha barbulata* and *P. brevifolia* populations across the range of the genus *Pyxidantha*. cpDNA data demonstrates no genetic isolation by geographical distance ($R = 0.01$, P -value = 0.39), while AFLP markers demonstrate weak but significant ($R = 0.27$, P -value = 0.02) isolation by distance at shorter distances with effects of genetic drift more evident at greater distances.

The highest posterior density for θ_{South} was higher than both $\theta_{\text{Ancestral}}$ and θ_{North} , although there is significant overlap between the 95% confidence intervals (Fig. S1, Supporting Information). The highest posterior density parameter estimate for migration from south to north is 2.09, while the estimate for migration from northern populations into southern populations is 0.01, indicating there has been gene flow between the two regions, with possibly greater migration from the southern populations into the northern. Past gene flow between the two populations is also supported by the model selection exercise; the worst-performing models constrained both migration parameters to 0 (Table S3). Time since divergence, t , was poorly estimated and failed to converge; this typically reflects a lack of a signal available in analyses that incorporate only a single locus with limited informative characters (J. Hey, personal communication).

AFLP

Three hundred and ten polymorphic bands were scored based on the three primer pairs. Each individual produced a unique AFLP profile, and the Euclidean error rate (based on 47 replicate pairs) was 4.2%, within the margin of acceptable error rates (Bonin *et al.* 2004). All genetic diversity indices were highly correlated, and sample size was not significantly correlated with any of the genetic diversity values (all $P > 0.05$). The population genetic diversity estimates for *P. barbulata* and *P. brevifolia* populations do not differ significantly for %P, DW, or H_e (all $P > 0.05$). In addition, there are no significant differences between regional genetic diversity estimates for percentage %P, H_e , or DW (all $P > 0.05$) (Table 1). The percentage of polymorphic loci (%P) ranges from 23.2% to 51.3%, with a mean of 37.8%, while Nei's population genetic diversity (H_e) ranges from 0.08 to 0.16 with a mean of 0.12 (Table 1).

Nonmetric multidimensional scaling ordination based on the population genetic distances (D) reveals no

discrete grouping of populations based on either region or species (Fig. 4). Results from STRUCTURE also demonstrate little population genetic structure based on either geographical location or taxonomic identification (Fig. S2), and there was no graphical evidence for an optimal number of K distinct genetic groups (Fig. S3). The hierarchical AMOVAS grouped according to species (*P. barbulata* vs. *P. brevifolia*) and geographical region (North vs. South) found small but significant variation was explained by species (1.60%, $P < 0.01$) and region (3.20%, $P < 0.01$) (Table 1), while within-population variation remained high (90.53% and 89.01%, respectively, $P < 0.001$). There is evidence for a weak but significant effect of IBD in the AFLP data (Fig. 3; $R = 0.27$, $P = 0.02$). Genetic differentiation between populations increases with geographical distance, indicating low to moderate levels of short distance gene flow but little evidence of long-distance

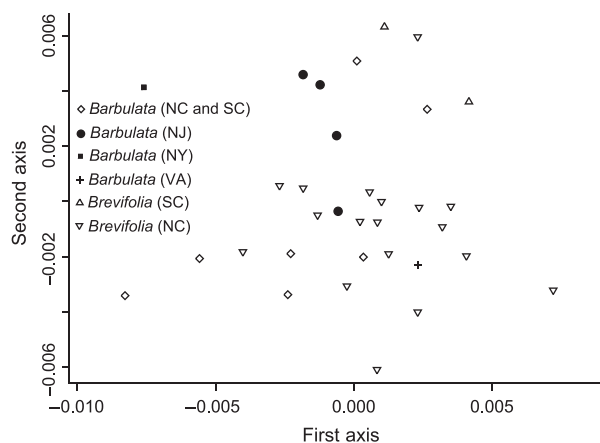


Fig. 4 Nonmetric multidimensional scaling ordination of *Pyxidantha barbulata* and *P. brevifolia* population genetic distances (Nei's D) based on amplified fragment length polymorphism markers. In the legend, letters in parentheses represent US states. Little separation is evident among populations defined according to either taxonomic identity or geographical location.

gene flow; both gene flow and genetic drift influence the pattern depending on the geographical scale (Hutchison & Templeton 1999). At shorter distances, gene flow is dominant, increasing the correlation between genetic and geographical distances, while at greater distances genetic drift predominates.

Discussion

Taxonomy

There does not appear to be clear separation between *Pyxidanthera barbulata* and *P. brevifolia* based on either the morphological or genetic data (Figs 1 and 4). Although *P. brevifolia* in general has shorter and narrower leaves than the more widespread *P. barbulata*, there is significant overlap between the two species for both leaf length and width. Previously published work on the leaf morphology indicated that the differentiation between the two species was because of hydrological differences between the habitats that *P. barbulata* and *P. brevifolia* occupy (Primack & Wyatt 1975), with leaf length increasing continuously with increasing soil moisture. Although *P. brevifolia* individuals tend to be more pubescent than *P. barbulata* individuals (Fig. 1), there is significant variation in the pubescence of *P. brevifolia* both at the taxonomic level and within-populations (data not shown), with both glabrous and pubescent individuals represented in most populations. Interestingly, there are herbarium specimens of *P. barbulata* from xeric habitats of the Outer Coastal Plain of North Carolina that exhibit the shorter leaves of *P. brevifolia* specimens, but that are not pubescent; the extreme pubescence appears to be restricted to *P. brevifolia*.

Although several authors have suggested that *P. brevifolia* may represent a preadapted *P. barbulata* ecotype that moved into the Sandhills region from the Outer Coastal Plain (Wells & Shunk 1931; Primack & Wyatt 1975), the current study using cpDNA sequences and AFLP markers and a previous study using allozymes (Godt & Hamrick 1995) do not support this hypothesis. *P. barbulata* and *P. brevifolia* populations in the Sandhills are not genetically distinct from each other, with *P. barbulata* populations on Fort Bragg sharing cpDNA haplotypes with nearby *P. brevifolia* populations (Fig. 2 inset). In addition, there is no separation between *P. barbulata* and *P. brevifolia* populations in their AFLP profiles (Fig. 4). We cannot rule out the possibility that *P. brevifolia* is a recently derived ecotype of *P. barbulata*, restricted to the Sandhills, and that a few mutations have led to local adaptation, but this would need to have been recent enough that genetic differentiation is not apparent in

AFLP profiles. Even though *P. brevifolia* appears to be an extreme morphological variant of *P. barbulata* associated with sandy, xeric sites, in our estimation, it warrants continued active management – specifically the regular prescribed fire schedule that Fort Bragg Military Reservation maintains – and further study because of its potentially critical role as an early season pollen and nectar provider and as a system for studying physiological adaptation to drought stress and phenotypic plasticity.

Phylogeography of the genus *Pyxidanthera*

Contrary to the well-documented trends of range contraction observed in many temperate plant species during the last glacial period in ENA, we found little evidence for either a southern refugium or range expansion following the LGM in the genus *Pyxidanthera*. Genetic diversity estimates for both the AFLP and cpDNA markers were not significantly different for northern and southern *P. barbulata* populations (Table 1), and northern populations contained several cpDNA haplotypes that did not occur in the southern populations (Fig. 2). More pointedly, estimates of the number of rare AFLP markers (DW), which may be more helpful in identifying refugial phylogeographical patterns (Paun *et al.* 2008), did not demonstrate significant differences between northern and southern populations. Finally, the two interior haplotypes – H7 and H11 – were widespread in both northern and southern populations with comparable frequencies (Fig. 2). These genetic patterns are contrary to what would be expected if there was a southern refugium for *Pyxidanthera* (Comps *et al.* 2001; Ikeda *et al.* 2008; Paun *et al.* 2008). Thus, it appears that the most likely scenario includes range stasis through the later Pleistocene. Furthermore, evidence of gene flow between geographically close populations suggests a possible explanation for low levels of genetic differentiation between northern and southern populations; these populations may not have been as geographically isolated in the recent past, with populations in the intervening area facilitating gene flow.

Several studies of tree species have also demonstrated the absence of typical refugial patterns (Palme *et al.* 2003; Maliouchenko *et al.* 2007), indicating that some species may have persisted closer to the ice sheet than previously thought. There is increasing evidence for 'cryptic refugia' in more northern latitudes for a number of mammal and plant species (Stewart & Lister 2001). Although mid-latitude refugia are possible, several alternatives have also been put forth. In the case of *Salix caprea*, which demonstrates little phylogeographical patterning, Palme *et al.* (2003) posit

high rates of dispersal, hybridization with other *Salix* species, and high mutation rates as possible reasons. These explanations are not very probable in the case of *Pyxidanthera*. *Pyxidanthera* seeds lack obvious morphological adaptations for dispersal, although ants have been observed transporting seeds (W. Wall, personal observation). Hybridization with other species is implausible, because *Pyxidanthera* is well differentiated from all other taxa within Diapensiaceae (Ronblom & Anderberg 2002). Although we have not estimated mutation rates, this alone would not generate the observed patterns.

That *P. barbulata* would persist, rather than retreat, during the climatic oscillations of the Pleistocene is consistent not only with the genetic data but also with our knowledge of Pleistocene habitats and the species' natural history. The GACP physiographical region, relative to more interior physiographical regions, may have been climatically buffered during the Pleistocene because of the moderating influence of the Atlantic Ocean (Rahmstorf 2002); moderation of climatic extremes could have allowed persistence closer to the ice sheet during glacial periods for some GACP species. Still, *P. barbulata* populations in New Jersey and New York would have experienced much colder conditions through much of the last glacial period (Jacobson *et al.* 1987; French *et al.* 2003, 2007). The vegetation community of the late Pleistocene in some of the areas of ENA does not have a modern analogue; most likely, it would have been a relatively open spruce (*Picea* spp.) forest with an herbaceous understory dominated by *Carex* spp. (Overpeck *et al.* 1992). The most important factors in determining the ecological niche of *P. barbulata* may be high light levels and an absence of competition, rather than temperature or moisture. The frequently burned habitats of the Sandhills of North and South Carolina and the Pine Barrens of New Jersey provide this habitat; it is conceivable that environments near the glacial boundary that lacked a dominant canopy cover during the last glacial period did as well. Finally, lower sea levels during glacial periods may have periodically increased available habitats for Atlantic Coastal Plain species such as *P. barbulata* on the exposed continental shelf (Hobbs 2004).

The present-day disjunction in the range of *P. barbulata* may be related to regional geomorphology. The Atlantic Coastal Plain is characterized by a series of alternating arches and embayments (Ward 1992); *Pyxidanthera* populations occur on the Cape Fear, Norfolk, and South New Jersey Arches, but are absent in the intervening Salisbury Embayment. The current disjunction in the range of the genus *Pyxidanthera* may be the result of oscillating sea levels that inundate embayment areas while arches remain above sea level (Bloom 1983;

Sorrie & Weakley 2001). It is unlikely that long-distance gene flow between the northern and southern populations without intermediate populations would be high enough to prevent genetic differentiation. This suggests that the current vicariance between northern and southern populations may be recent and that during periods of relatively low sea levels, suitable habitat was exposed on the continental shelf, connecting northern and southern populations and allowing gene flow to minimize genetic differentiation.

The GACP floristic province contains the second-highest level of endemism in North America north of Mexico, yet the endemic plant species have been relatively understudied. Despite subtle topographic variation across the region, complex vegetation patterns exist and the biogeographical processes involved elude simple characterization. Although more phylogeographical studies of GACP endemic plant species are needed to determine whether the recent phylogeographical history of the genus *Pyxidanthera* is representative of multiple taxa or is simply an isolated case, it is apparent that the simple refugial model cannot account for the phylogeographical pattern in the genus *Pyxidanthera*. If similar phylogeographical patterns are found in similarly distributed GACP endemics, it would suggest a common mechanism was responsible and the remaining challenge would be to explain why only these taxa were thusly affected. Refugia are generally thought of as existing in the past; it could be the case that contemporary distributional patterns represent modern-day refugia for many Atlantic Coastal Plain endemic plant species.

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Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Parameter estimates for θ (southern, northern, and ancestral populations), time since divergence, and migration (gene flow) between northern and southern populations of the genus *Pyxidanthera* based on results from IMA2.

Fig. S2 Population genetic structure for the genus *Pyxidanthera* as inferred from the program STRUCTURE for K2 through 9.

Fig. S3 Log likelihood [$\ln P(D)$] and standard deviation results from program STRUCTURE for K1 through 9.

Table S1 Chloroplast haplotype accession numbers as archived in Genbank for the *atpI-atpH* intergenic spacer region (partial sequence) and the *psbD-trnT* intergenic spacer region (partial sequence)

Table S2 Polymorphisms of the 12 cpDNA haplotypes based on the cpDNA regions *atpI-atpH* and *psbD-trnT* in the genus *Pyxidanthera*

Table S3 Summary of model statistics for the 24 IMA2 models.

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