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Phylogeographical structure and temporal complexity in American sweetgum (*Liquidambar styraciflua*; Altingiaceae)

ASHLEY B. MORRIS,* STEFANIE M. ICKERT-BOND,† D. BURKE BRUNSON,* DOUGLAS E. SOLTIS‡ and PAMELA S. SOLTIS§

**Department of Biology, University of South Alabama, Mobile, AL 36688, USA, †Department of Biology and Wildlife, University of Alaska, Fairbanks, AK 99775, USA, ‡Department of Botany, University of Florida, Gainesville, FL 32611, USA, §Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA*

Abstract

Eastern North American plant biogeography has traditionally focused on two primary issues: (i) the location of temperate Pleistocene refugia and their proximity to the southern margin of the ice sheet during the last glacial maximum, and (ii) the origin of the temperate element of northern Latin America. While numerous population genetic and phylogeographical studies have focused on the first issue, few (if any) have considered the second. We addressed these issues by surveying 117 individuals from 24 populations of *Liquidambar styraciflua* (American sweetgum; Altingiaceae) across the southeastern USA, eastern Mexico, and Guatemala, using more than 2200 bp of chloroplast DNA sequence data. To specifically address the issue of timing, we estimated intraspecific divergence times on the basis of multiple fossil-based calibration points, using taxa from Altingiaceae (*Liquidambar* and *Altingia*) and Hammamelidaceae (*Hamamelis*) as outgroups. More than half of the sampled localities exhibited multiple haplotypes. Remarkably, the greatest variation was observed within the USA, with Mexico and Guatemala sharing widespread haplotypes with Texas, Mississippi, Kentucky, Ohio, and northern Virginia. This lack of differentiation suggests shared ancestral polymorphisms, and that the genetic signal we observed is older than the disjunction itself. Our data provide support for previously proposed hypotheses of Pleistocene refugia in peninsular Florida and along the eastern Atlantic, but also for deeper divergences (~8 million years ago) within the USA. These patterns reflect a dynamic biogeographical history for eastern North American trees, and emphasize the importance of the inclusion of a temporal component in any phylogeographical study.

Keywords: divergence time estimation, fossil calibration, Latin America, phylogeography, temperate disjunction

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A rapidly growing database of phylogeographical literature for eastern North American plants and animals indicates greater spatial and temporal complexity than previously suggested (Soltis *et al*. 2006). In the absence of divergence time estimates, most of these studies assume Pleistocene glaciation as the primary causal factor associated with observed phylogeographical breaks. Numerous researchers have indicated the potential for interpretative pitfalls in the absence of temporal data (Bermingham & Avise 1986; Cunningham & Collins 1994; Avise 2000; Donoghue *et al*. 2001; Xiang & Soltis 2001; Donoghue & Moore 2003) yet few phylogeographical studies have dealt with this issue (but see Church *et al*. 2003; Sota & Hayashi 2007). The possibility of convergent patterns being derived from different evolutionary processes at different times, often referred to as pseudocongruence (Cunningham & Collins 1994), has become an increasingly important consideration in phylogeographical studies. In fact, several recent studies suggest that observed phylogeographical breaks may predate the last glacial maximum (LGM), or even the Pleistocene (Klicka & Zink 1997; Austin *et al*. 2002; Church *et al*. 2003; Zamudio & Savage 2003; Near & Keck 2005; Howes *et al*. 2006). Divergence time estimation using fossil

Correspondence: Ashley B. Morris, Fax: 251-414-8220; Email: amorris@jaguar1.usouthal.edu

constraints of phylogenetic trees is becoming a standard analytical tool for many biogeographical studies of plants, and has provided a great deal of evidence for pseudocongruent patterns among codistributed plant genera in the Northern Hemisphere (Donoghue *et al*. 2001; Xiang & Soltis 2001). The same theory and methods used in higher-level taxa should be extended to intraspecific phylogeographical data to formally assess the commonly cited hypothesis of Pleistocene-driven divergence.

As many as 50 species of plants exhibit a disjunction between the temperate floras of the eastern USA and the mountains of eastern Mexico (Graham 1973, 1999). Oddly, this pattern is only seen in a few animal taxa (Martin & Harrell 1957), including the flying squirrel (*Glaucomys volans*, Arbogast 2007), the red-bellied snake (*Storeria occipitomaculata*), and certain Pselaphid beetle genera (Carlton 1990). Deevey (1949) suggested that this disjunction is the result of Pleistocene cooling in the southeastern USA, forcing temperate taxa into the southern reaches of the region, including peninsular Florida and the mountains of eastern Mexico. Braun (1950) countered that these elements arrived in Mexico as early as the mid-Cenozoic, and were later isolated by more arid conditions during the Pliocene. Fossil pollen data support Braun's hypothesis of early arrival, indicating the occurrence of 10 temperate woody genera (including *Liquidambar*) in the Mexican palynoflora as early as the mid-Pliocene, approximately 5.2 to 1.6 million years ago (Graham 1973, 1999). Graham (1999) further noted that the appearance of these temperate woody taxa is consistent with a major temperature decline in the mid-Miocene, before which very few of these elements are found in the northern Mexican palynofloras. He suggested that the absence of temperate pollen from earlier Mexican records, and the presence of temperate pollen in the Eocene record of the southeastern USA, provide evidence for a southeastern USA origin for the Mexican temperate element. However, Axelrod (1975) argued that the Mexican temperate element represents 'specialized outliers' of what was once a continentally extensive Paleogene temperate rainforest, and not the result of a southward migration from the southeastern USA.

For each of the taxa with a disjunct biogeographical distribution documented by Graham (1999), the extent of the disjunction varies (as does the degree of morphological variation). This might be expected given differences in life histories, habitat requirements, and responses to environmental change. The taxonomic rank of taxa involved varies as well, with some Mexican disjunct lineages considered conspecific to their US counterparts (e.g. *Carpinus caroliniana*, *Fagus grandifolia*, and *Nyssa sylvatica*), and others considered sister species to their US counterparts (e.g. *Illicium floridanum/mexicanum*, *Taxodium distichum/mucronatum*, and *Liquidambar styraciflua/macrophylla*; Graham 1973). While these differences are likely to result in some variability in phylogeographical structure, the overall expectation is one of phylogeographical convergence, given some common underlying biogeographical history. However, as mentioned above, shared phylogeographical patterns can also be the result of pseudocongruence, and it is essential that timing be taken into consideration. Divergence time estimation based on molecular sequences should provide some resolution to this issue.

The objectives of this study were to use *L. styraciflua* (American sweetgum; Altingiaceae) to (i) assess the degree of genetic divergence between populations from eastern North America and northern Latin America, and (ii) test hypotheses related to the location of Pleistocene refugia for temperate taxa. The integration of chloroplast DNA (cpDNA) sequence data and fossil-based calibrations for the estimation of intraspecific divergence times should shed new light on this topic.

Materials and methods

Liquidambar styraciflua is considered a bottom-land species and is most abundant in the Lower Mississippi Valley (LMV). Its modern-day distribution is primarily in the southeastern USA, extending from Connecticut to central Florida and west to eastern Texas (Fig. 1). *Liquidambar styraciflua* (including *Liquidambar macrophylla* as a synonym of *L. styraciflua*) occurs frequently throughout eastern and central Mexico, and extends as far south as Nicaragua (Little 1971; Kormanik 1990). It is a relatively fast-growing pioneer species, has an average lifespan of 200 years, and reaches reproductive maturity at 20–30 years of age. It is a monoecious species, with wind-pollinated flower production in mid- to late spring and fruit production in late fall. Fruits, known colloquially as gumballs, open to release wind-dispersed seeds, which are also eaten (and ultimately dispersed) by birds, squirrels, and chipmunks. The relationship between North American *Liquidambar* and its Asian and European relatives is complex, but molecular data suggest that divergence between *L. styraciflua* and its sister, *Liquidambar orientalis*, occurred between the Oligocene and Miocene (Ickert-Bond & Wen 2006), during which time the North Atlantic Land Bridge is hypothesized to have been available for migration between these major areas (Donoghue *et al*. 2001). Post-glacial fossil pollen reconstructions for eastern North American *Liquidambar* support a western Gulf Coast refugium, with subsequent northeastern spread (Williams *et al*. 2004).

Data collection

ARCVIEW 3.2 (ESRI 1992–2000) was used to update the boundaries of Little's (1971) species distribution map for *L. styraciflua* using occurrence data acquired from North American herbarium records online (Fig. 1). Target sampling

Table 1 Collection locality information and cpDNA haplotypes recovered for *Liquidambar styraciflua*

Locality	State code	Latitude	Longitude	*N*	Haplotype(s)†
Hackneyville, AL	AL	33.07	–85.88	3	K Q R
Pinnacle Mountain State Park, AR	AR1	34.84	–92.48	12	K T V
Blaylock Mountain, AR	AR2	36.09	–94.37	1	S
Apalachicola Bluffs and Ravines Preserve, FL	FL1	30.49	–84.98	10	K L
San Felasco Hammock State Park, FL	FL2	29.70	–82.46	4	E
Wekiva Springs State Park, FL	FL3	28.71	–81.49	4	E G
George L. Smith State Park, GA	GA1	32.54	–82.12	4	E F
Tallulah Gorge State Park, GA	GA2	34.74	–83.39	2	A
Green River Lake State Park, KY	KY	37.25	–85.34	4	M P
Chemin-a-Haute State Park, LA	LA1	32.91	–91.85	4	H I T
Louisiana State Arboretum, LA	LA2	30.80	–92.28	4	K N
Ragland Hills, MS	MS1	31.20	–89.18	3	P
Starkeville, MS	MS2	33.46	–88.79	3	J T W
Ev-Henwood Nature Preserve, NC	NC	34.16	–78.12	9	A C D K
Hueston Woods, OH	OH	39.51	–84.75	2	P
Columbia, SC	SC	34.00	–81.04	4	T
Great Smoky Mountains National Park, TN	TN1	35.65	–83.51	8	T U
Paris Landing State Park, TN	TN2	36.43	–88.08	4	T
Sewanee, TN	TN3	35.20	–85.92	4	T
Big Thicket National Preserve, TX	TX	30.43	–94.10	4	P
Chesapeake, VA	VA1	36.58	–76.15	3	A B
Pace Estate, VA	VA2	37.92	–78.53	3	P
Mesa de la Yerba, Veracruz	MEX‡	19.56	–97.01	11	O P
San Pedro Carcha, Alta Verapaz	GUA§	15.54	–90.24	7	P

†Haplotype codes are from haplotype network in Fig. 1 and maximum likelihood topology in Fig. 2;
‡single collecting locality from Mexico; §single collecting locality from Guatemala.

localities were evenly distributed throughout the species range by superimposing a grid (cell size approximately 241 km^2) on the distribution map and identifying localities near the vertices of each cell. Actual sampled localities depended on the ability to identify suitable sites at or near those vertices, such that distances between sites varied (Fig. 1; Table 1). Leaf material was obtained from a maximum of 30 individuals at each locality and preserved in silica gel desiccant.

DNA extraction, polymerase chain reaction amplification, and DNA sequencing

Total genomic DNA was extracted from silica-dried material using a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle 1987). Four plastid regions were surveyed for genetic variation within and among populations of *L. styraciflua*: atpB-rbcL (Hodges & Arnold 1994), psbA-trnH (Sang *et al*. 1997; Tate & Simpson 2003), psbE-petL, and the trnL intron (Taberlet *et al*. 1991). For psbE-petL, primers used for initial amplification were taken from an unpublished source. Because those primers resulted in differential amplification success, *Liquidambar*-specific primers were designed for this study: PSBE1 5′-ATGCCGAGCTCCACATATTC-3′; PSBE2 5′-CGTTGTTCTC TTTCTTTCATCG-3′; PSBE3 5′-CGATGAAAGAAAGAGAA CAACG-3′; and PSBE4 5′-AGGCTGAAGGAACTAAATGAAA-3′. Final concentrations of polymerase chain reaction (PCR) components were as follows: 1× PCR buffer, 3 mM $MgCl_2$, 200 nM dNTPs, 200 nM forward primer, 200 nM reverse primer, 1 M Betaine, and 1.25 U *Taq* polymerase. The PCR profile followed that of Taberlet *et al*. (1991). PCR products were cleaned with Exo-Sap and were sequenced at a commercial sequencing facility (High-Throughput Genomics Unit, Department of Genome Sciences, University of Washington).

Sequence alignment and haplotype network construction

All sequences were aligned using CLUSTAL_X version 1.83 (Thompson *et al*. 1997) and were manually checked for corrections using SE-AL Sequence Alignment Editor version 2.0a11, available for download from Andrew Rambaut (http://evolve.zoo.ox.ac.uk/software.html). Haplotype networks were constructed for *L. styraciflua* intraspecific

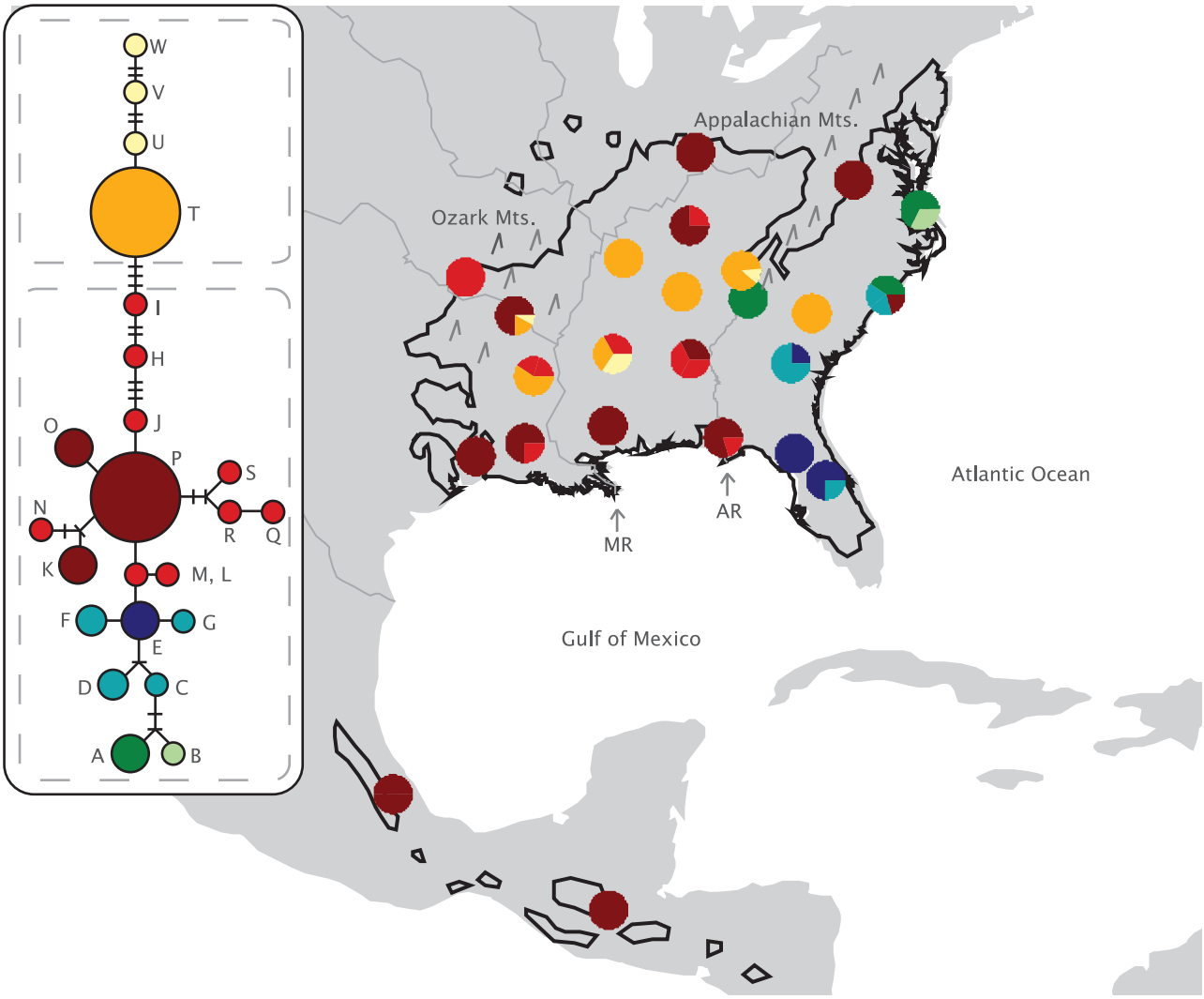

Fig. 1 Chloroplast haplotype distribution for *Liquidambar styraciflua* sampled in this study. The haplotype network constructed using statistical parsimony is given on the left side of the figure. Each circle represents a unique haplotype, with circle size reflecting frequency of that haplotype. Lines between circles indicate single mutational steps, while hash marks indicate unsampled or extinct haplotypes. Dotted boxes indicate the two major intraspecific clades recovered in phylogenetic analyses. Haplotype codes (A–W) correspond to those given in all other Tables and Figures. Colours correspond to major clades of interest as identified by maximum-likelihood analysis (Fig. 3), and are consistent throughout Figs 2 and 3. For the distribution map given on the right of the figure, the current species range is indicated by black outline. Pie charts provide haplotype frequency data for sampled localities, with colours being consistent with those given in the haplotype network. MR, Mississippi River; AR, Apalachicola/Chattahoochee River.

data only. A haplotype network was constructed using statistical parsimony as implemented in TCS 1.21 (Clement *et al*. 2000) with gaps treated as a fifth character state. While it is more desirable to treat indels as additional coded characters than as a fifth character state, TCS does not currently allow for the inclusion of symbols in the data matrix. All gaps were the result of length variation in mononucleotide repeat regions. While we acknowledge the potential for homoplasy in such regions (reviewed in Kelchner 2000), the observed patterns among individuals of *L. styraciflua* were largely congruent with those exhibited by substitution data, warranting the consideration of these repeats as potentially informative characters.

Phylogenetic analyses

Maximum parsimony (MP), maximum-likelihood (ML), and Bayesian analyses were conducted using PAUP* (Swofford 2002) and BEAST version 1.43 (Drummond & Rambaut 2007). Analyses were performed on the combined data,

collapsing all individuals with the same haplotype to a single representative (see Supplementary material). To allow for fossil-based calibrations of our intraspecific data, we included accessions of *Liquidambar acalycina* (EU595851, EU595860, EU595869, DQ352216), *Liquidambar formosana* (EU595852, EU595861, EU595870, DQ352221), *L. orientalis* (EU595846, EU595855, EU595864, DQ352222), *Altingia chinensis* (EU595847, EU595856, EU595865, DQ352203), *Altingia excelsa* (EU595850, EU595859, EU595868, DQ352226), *Altingia obovata* (EU595853, EU595862, EU595871, DQ352208), *Altingia poilanei* (EU595849, EU595858, EU595867, DQ352210), and *Altingia yunnanensis* (EU595848, EU595857, EU595866, DQ352211), as well as *Hamamelis virginiana* (Hamamelidaceae; EU595854, EU595863, EU595872, DQ352196), the latter serving as an outgroup to Altingiaceae.

MP analyses were performed both with gaps treated as missing and with gaps coded as additional characters following the simple indel-coding method of Simmons & Ochoterena (2000). For each analysis, we performed a heuristic search with 1000 random addition replicates (holding one tree each step), tree-bisection–reconnection (TBR) branch swapping, and MULTREES in effect. All characters were treated as unordered and equally weighted. Support for recovered nodes was inferred by bootstrap analysis (1000 replicates, search parameters same as above).

Recent work by Posada & Buckley (2004) suggests that while the hierarchical likelihood-ratio tests (HLRT) are the most commonly employed model selection strategy, the Akaike information criterion (AIC) is a superior approach. We therefore used the AIC as implemented in MODELTEST version 3.6 (Posada & Crandall 1998) to determine an appropriate model of evolution for use in ML analyses. The best-fit model for the combined cpDNA data was TVM + G, with base frequencies of A = 0.3096, C = 0.1533, G = 0.1512, and T = 0.3858, and a gamma distribution shape parameter equal to 0.1785. A heuristic search was performed using 1000 random addition sequence replicates, holding one tree at each step, with MULTREES in effect, and saving all trees, and TBR branch swapping. Internal support was inferred from 100 bootstrap replicates, using the full heuristic search option and the same search strategy as above.

Estimating divergence times using fossil calibrations

The ML topology recovered above was used to test for rate constancy. Log-likelihood scores were generated with (+cl) and without (–cl) a molecular clock enforced. A likelihood-ratio test (LRT) was performed to determine if there was a significant difference in evolutionary rates among lineages (Felsenstein 1988). The hypothesis of a molecular clock was rejected (P value = 0.015). Divergence time estimation was performed under a Bayesian approach as implemented in BEAST version 1.43 (Drummond & Rambaut 2007). For readers unfamiliar with BEAST version 1.43, two substitution models [HKY (Hasegawa–Kishino–Yano) or GTR (general time reversible)], and three site heterogeneity models (gamma, invariant sites, or gamma + invariant sites) are available. Based on the results of AIC model selection above, we used a GTR + G model of sequence evolution, under an uncorrelated lognormal relaxed clock model. We constrained several groups to be monophyletic: *L. styraciflua*; *L. styraciflua* + *L. orientalis*; and *A. obovata* + *L. acalycina* + *L. formosana* (see below for details). Because the tree priors available in the current version of BEAST are not designed to model mixed inter- and intraspecific data (A. Drummond, personal communication.), we performed a sensitivity analysis comparing two approaches to modelling the tree prior: (i) the Yule speciation process, and (ii) a coalescent model assuming logistic population growth. Fossil calibration points were used to determine specific node priors (see discussion in Ickert-Bond & Wen 2006), and lognormal distributions were used for all priors to approximate minimum ages while allowing nodes to be slightly younger or considerably older. *Microaltingia* Zhou, Crepet, and Nixon from the Late Cretaceous of New Jersey was used to calibrate the root node, approximating a median age of 90 million years [lognormal mean 4.5, SD 0.2, zero offset 0; range of 60–133 million years (Myr)]. In a morphological study comparing *Liquidambar changii* Pigg, Ickert-Bond, and Wen from the Middle Miocene of eastern Washington to all other recognized species of *Liquidambar*, *L. changii* was found to most closely resemble *L. acalycina* (Pigg *et al*. 2004; Ickert-Bond & Wen 2006). The original survey of *L. changii* recovered the fossil as sister to all other *Liquidambar* species, which have since been shown by molecular data to be nested within *Altingia* (Shi *et al*. 2001; Ickert-Bond & Wen 2006). However, Ickert-Bond *et al*. (2007) recently published a more extensive morphological survey of Altingiaceae and found strong support for *Altingia* and *Liquidambar* as mutually exclusive sister clades, citing a complex relationship between morphological convergence and evolutionary diversification rates.

Previous molecular work recovered a clade containing *L. formosana*, *A. obovata*, and *L. acalycina*, the latter of which (as described above) is most similar to *L. changii*, leading Ickert-Bond & Wen (2006) to use *L. changii* to constrain the age of this clade, which we do here as well (approximate median age of 15.6 Myr; lognormal mean 2.71, SD 0.5, zero offset 0; range of 15–40 Myr). For the *L. styraciflua* crown group, we set a median age of 3 Myr (lognormal mean 1.1, SD 1.0, zero offset 0; range of 3–21 Myr) on the basis of fossil material from the Citronelle formation of southern Alabama, which appears to be identical to modern *L. styraciflua* (B. Axsmith, personal communication, unpublished data). We compared the estimated mean ages and 95% confidence intervals for individual fossil calibrations (i.e. just *Microaltingia*; *Microaltingia* and *L. changii*; *Microaltingia* and *L. styraciflua*) as well as for the three calibration points combined.

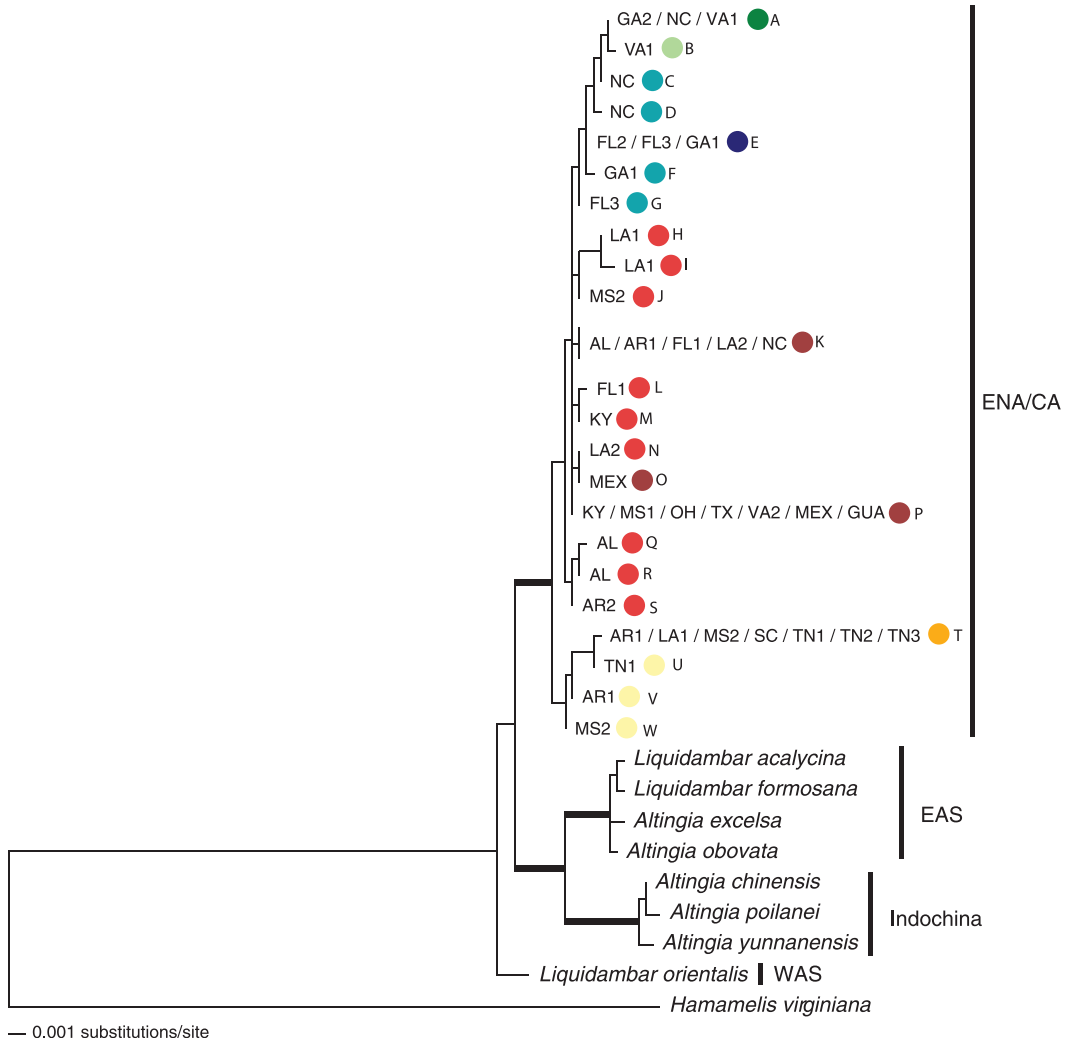

Fig. 2 Maximum-likelihood topology for all inter- and intraspecific cpDNA sequences collected for Altingiaceae. Thicker branches correspond to bootstrap values of 93% or greater from all maximum-likelihood and maximum parsimony analyses. Additional limited support (55–74%) was recovered within each of the major clades (data not shown). State codes within the ENA/CA clade (*Liquidambar styraciflua*) correspond to those given in Table 1; haplotype codes (A–W) correspond to those in Table 1 and Fig. 1. Colour-coding indicates major clusters of interest and is used throughout all figures. ENA/CA, Eastern North America/Central America; EAS, Eastern Asia; WAS, Western Asia.

Results

Haplotype network

The aligned haplotype data set for *Liquidambar styraciflua* (excluding all other taxa) was 2165 bp and included two variable mononucleotide repeat regions. We initially sampled a maximum of four individuals from each of 24 populations for a total of 96 individuals (Fig. 1). Twenty-three haplotypes were recovered with these samples, with 13 of 25 localities exhibiting more than one haplotype (including five populations with three haplotypes each). Samples from Guatemala and Mexico shared one of the most frequently recovered haplotypes (P, shown in dark red, Figs 1–3), present in samples from Texas, southern Mississippi, Kentucky, Ohio, and northern Virginia. Given the higher than expected haplotype diversity recovered from our initial sampling, we increased the sample sizes for six populations: Pinnacle Mountain State Park (AR2); Apalachicola Bluffs and Ravines Preserve (FL1); Ev-Henwood Nature Preserve (NC); Great Smoky Mountains National Park (TN1); Mesa de la Yerba, Mexico (MEX); and San Pedro Carcha, Guatemala (GUA). For each of these populations, we sequenced an additional six to eight individuals, bringing the total number sampled to 117. No additional unique haplotypes were recovered (within or among populations), but one haplotype (K, shown in dark red, Figs 1–3) was recovered in a new locality (North

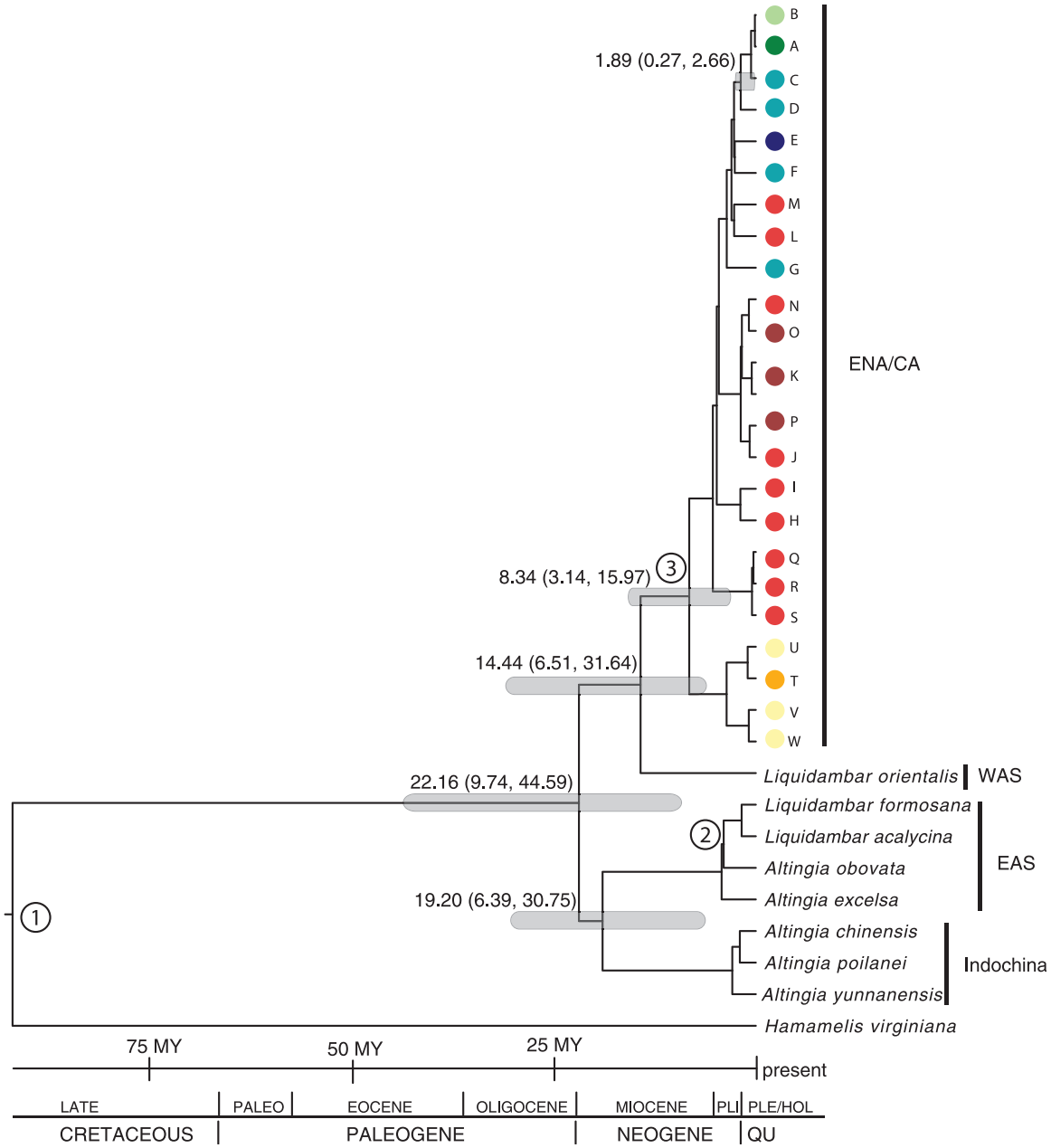

Fig. 3 Chronogram for Altingiaceae based on a coalescent-based Bayesian approach assuming logistic population growth. Calibrated nodes are indicated by numbers (1–3): (1) *Microaltingia* was used to calibrate the root; (2) *Liquidambar changii* was used to calibrate the clade including *Liquidambar formosana, Liquidambar acalycina,* and *Altingia obovata*. All calibration points were calibrated using lognormal priors to approximate minimal ages (see methods for details). Gray bars indicate 95% HPD intervals for nodes of particular interest, with ages and 95% HPD given (in millions of years) above the bars. These nodes all have posterior probabilities of 0.99–1.00 (Table 2). Haplotype letter codes (A–W) and colour codes correspond to those in all other Tables and Figures. Divergence time estimates are further summarized in Table 2. ENA/CA, Eastern North America/Central America; EAS, Eastern Asia; WAS, Western Asia.

Carolina). Haplotype distribution and GenBank Accession nos are provided in Appendix S1, Supplementary material. Descriptions of unique haplotypes defined by polymorphic sites are provided in Appendix S2, Supplementary material.

Phylogenetic analyses

The aligned *Liquidambar/Altingia* data matrix included 33 taxa and 2297 bp of data. Several indels, ranging in length from 5 to 20 bp, were required to align the data. Eight

Node	Yule speciation prior		Coalescent prior	
	PP	Age (95% CI)	*PP*	Age (95% CI)
Root	1.00	101.78 (50.35, 107.50)	1.00	93.11 (64.81, 149.61)
EAS/ENA*	0.90	43.55 (19.45, 72.45)	0.99	22.16 (9.74, 44.59)
IND/EAS†	0.98	28.29 (10.41, 51.36)	0.99	19.20 (6.39, 30.75)
LORIE/LS‡	1.00	35.85 (13.18, 58.80)	1.00	14.44 (6.51, 31.64)
LS major clades§	1.00	19.21 (8.52, 39.31)	1.00	8.34 (3.14, 15.97)
LS Carolina clade¶	0.99	5.48 (0.85, 9.34)	0.99	1.89 (0.27, 2.66)

Table 2 Summary of divergence time estimation results using two alternative Bayesian tree priors using multiple fossil calibration points. Estimates are given as mean ages (in millions of years) with 95% confidence intervals in parentheses. See methods for explanation of fossil calibrations and a discussion of models. Posterior probabilities (*PP*) are given for each model

*Split between the Eastern Asian + Indochina and Eastern North American clades of Altingiaceae.
†Split between the Eastern Asian and Indochina clades of Altingiaceae.
‡Split between *Liquidambar orientalis* and *Liquidambar styraciflua*.
§Split between the two major clades within *L. styraciflua*.
¶Split between *L. styraciflua* clade containing haplotypes A–D and all other clades.

synapomorphic indels were coded for parsimony analyses. MP analyses with gaps treated as missing recovered 26 most parsimonious trees (MPT) with a shortest length of 187 (48 parsimony-informative characters; CI = 0.7761; HI = 0.2239; RC = 0.8515), while analyses with gaps coded as additional characters recovered 52 MPTs with a shortest length of 197 (56 parsimony-informative characters; CI = 0.7792; HI = 0.2208; RC = 0.8476). While all gaps were phylogenetically informative, their inclusion decreased bootstrap support for some nodes (data not shown). Relationships among the species of *Liquidambar* and *Altingia* are largely congruent with previously published data (Ickert-Bond & Wen 2006), with the exception of the position of *Liquidambar orientalis*. This species has been shown to be sister to *L. styraciflua* in almost every other study of the group (Hoey & Parks 1994; Li *et al*. 1997; Li & Donoghue 1999; Shi *et al*. 2001; Ickert-Bond & Wen 2006), but its position is somewhat unresolved here. The strict consensus constructed from MPTs with gaps treated as missing recovered *L. orientalis* as sister to all other members of the family, while the strict consensus constructed from MPTs with gaps coded as additional characters recovered the *L. styraciflua* sister relationship, as previously published. However, neither position is strongly supported (< 50% and 67% bootstrap support, respectively). Short branch lengths across the tree indicate limited divergence within *L. styraciflua* and among members of otherwise well-supported clades (i.e. the Indochina and Eastern Asian clades). ML analyses recovered one tree with limited intraspecific resolution; major clades were congruent with those recovered using parsimony (Fig. 3).

Divergence time estimation

Results from divergence time estimation using the two tree prior alternatives (Yule speciation process and coalescent assuming logistic population growth) are summarized in Table 2. Implementation of individual fossil calibration points relative to an approach with three calibration points result in considerably older nodes with much wider 95% higher posterior density intervals (HPD; data not shown). For the combined calibration approach, the coalescent model assuming logistic population growth yielded considerably younger mean ages and narrower 95% HPD intervals than did the Yule speciation prior. Of particular interest are those nodes within *L. styraciflua.* Under the coalescent model, the two major intraspecific clades diverged approximately 8.34 million years ago (95% HPD = 3.14–15.97) relative to 19.21 million years ago (95% HPD = 8.52–39.31) under the Yule speciation model. Additionally, the Carolina coastal clade (haplotypes A–D, shown in greens and blues, Figs 1–3) diverged during the Pleistocene (mean 1.89, 95% HPD = 0.27–2.66) under the coalescent model, relative to the Miocene age estimated under the Yule speciation model (mean 5.48, 95% HPD = 0.85–9.34). Given the differences in the two approaches, and the predominantly intraspecific nature of the data, the coalescent tree prior appears to be a better fit to the data.

Discussion

The patterns revealed in *Liquidambar styraciflua* reflect the dynamic phylogeographical history of eastern North American taxa, and emphasize the importance of the integration of a temporal component into any phylogeographical study. The greatest divergence observed within the species was not between the disjunct US and eastern Mexican populations, as may have been expected due to the great geographical distance between extant populations, but was instead within the US populations (Figs 1–3). Our estimates attribute this break to a pre-Pleistocene event, roughly 8 million years ago, with

admittedly broad confidence intervals (Table 2; Fig. 3). The family Altingiaceae has a long and diverse fossil record in the Northern Hemisphere that spans the Late Cretaceous (*Microaltingia*) into the Recent in North America, Europe, and Asia. There are North American Miocene reports of *Liquidambar* from Alaska (Seldovian point), Idaho (Clarkia), Georgia, Kansas, and eastern Mexico, but the relationships of these fossil taxa to extant *L. styraciflua* are poorly known. Given this information, the two clades of *L. styraciflua* recovered here may represent ancient relicts of a once widespread *Liquidambar* species complex. A lack of differentiation among Latin American and North American populations may actually reflect the retention of ancestral haplotypes, as these widespread types are found in the interior of the haplotype network (Fig. 1).

Phylogeographical structure in L. styraciflua

Previous allozyme work by Hoey & Parks (1994) uncovered lower than average genetic diversity in *L. styraciflua* when compared to other wind-pollinated taxa. From their data, they inferred two closely allied population centres (i.e. the southeastern US and eastern Mexico) representing one species. Hoey & Parks (1994) further suggested that the Mexican populations must be relatively old, consistent with fossil data supporting a Miocene arrival of the temperate element into Mexico, while the disjunction itself is relatively recent. They found greater structure among Mexican populations than among US populations, and suggested that any observable structure in the US would have been erased by persistent glacial expansion and contraction during the Pleistocene, whereas Mexican populations would have remained relatively unaffected. The data presented here are consistent with this hypothesis, in that only ancestral, widespread haplotypes were recovered in Latin American populations sampled for this study (Fig. 1). Unlike Hoey & Parks (1994), we recovered limited genetic differentiation within and among Latin American populations. Differences in our results and those of the previous study could be attributed to variation in pollen- and seed-mediated gene flow. Nuclear markers, such as allozymes, are bi-parentally inherited, reflecting a combination of both pollen and seed movement. Chloroplast markers, which are maternally inherited in most plants, reflect only seed movement. *Liquidambar styraciflua* is wind-pollinated and its seeds are largely wind-dispersed, although they are also known to be eaten by birds, squirrels, and chipmunks (Kormanik 1990). In a study of seed dispersal in bottomland hardwood forests, Nuttle & Haefner (2005) found that the majority of *L. styraciflua* seeds fell within 50 to 100 m of the parent tree, with many gumballs containing as many as 50 seeds falling directly below the tree. Such limited dispersal would likely result in greater localized structure for chloroplast markers, while nuclear markers should exhibit a more homogeneous signal, due to wind pollination, which is the inverse of the patterns seen here. It may be more likely that the cpDNA regions used here evolve more slowly than the allozyme markers used by Hoey & Parks (1994), such that the resulting patterns reflect different timescales.

In the present study, we recovered only two haplotypes from Mexico and Guatemala (haplotypes O and P, shown in dark red on Figs 1–3). Furthermore, haplotype O, which was shared by both Latin American localities, was one of the two most common types recovered within and among US populations. Graham's (1999) palynological data indicate the presence of *Liquidambar* in eastern Mexico as early as the Pliocene, and there are additional macrofossil reports of *Liquidambar* from this region as early as the late Miocene (Berry 1923). Graham (1999) suggested that the arrival of temperate taxa such as *Liquidambar* into eastern Mexico was the result of range expansion from the southeastern USA in response to climatic cooling, such that the observed modern disjunction would be a consequence of repeated range expansion and contraction in response to dynamic climate change over the last 3–6 Myr. From an evolutionary perspective, it is possible that frequent population extinction and recolonization of ephemeral sinks from a stable source (within Mexico) could have prevented population subdivision by drift, and resulted in relatively short coalescence times among populations (Avise 2000). Alternatively, the genetic signal we observed may be older than the disjunction itself, reflecting a time of admixture among continuously distributed stands of *Liquidambar*. Additional genetic markers (e.g. nuclear DNA sequences and microsatellites) will be needed to provide additional resolution to this question.

Comparisons with other ENA taxa

Some phylogeographical structuring in *L. styraciflua* is consistent with previously published biogeographical patterns for eastern North America (reviewed in Soltis *et al*. 2006). While phylogenetic support was limited, there appears to be a trend towards an 'out of Florida' track, with haplotypes from peninsular Florida, eastern and northern Georgia, and coastal North Carolina and Virginia clustering together in the haplotype network (haplotypes A–G, shown in blues and greens, Figs 1–3). Based on our estimates, the age of this group corresponds with the Pleistocene (Table 2; Fig. 3), which is consistent with hypotheses that peninsular Florida served as an ice-age refugium to many temperate plants and animals. Alternatively, some authors have suggested that the coastal areas of the Carolinas may have also played a role as temperate refugia, based in part on modern species diversity and endemism (Estill & Cruzan 2001; Sorrie & Weakley 2001), and in part on the presence of early Pleistocene fossils for temperate species (including *Liquidambar*; Whitehead 1983). Most recently, in a phylogeographical survey of the eastern tiger salamander

(*Ambystoma tigrinum*), Church *et al.* (2003) found evidence for two independent refugia along the mid-Atlantic Coastal Plain, one of which is centred on the Carolina coast, which is consistent with what we have found here. Given this information, it is possible that this primarily coastal clade recovered for *L. styraciflua* may represent multiple refugia (Florida refugium in blue, Carolina refugium in green), although additional data are needed to assess this hypothesis.

Caveats to divergence time estimation

While our study represents one of only a few examples of intraspecific dating in plants, there are numerous caveats to the analyses presented here. First, limited phylogenetic resolution within *L. styraciflua* likely dramatically increased confidence intervals associated with estimated mean ages. Future work including more rapidly evolving sequence data may provide a solution to this issue. Second, generally speaking, fossil position is somewhat subjective when not resolved phylogenetically through inclusion in a morphological matrix. In this case, previously published morphological studies on *Liquidambar* (Pigg *et al*. 2004; Ickert-Bond *et al*. 2005), *Altingia* (Ickert-Bond *et al*. 2007), and Altingioid relatives (Zhou *et al*. 2001; Ickert-Bond & Wen 2006) provided strong rationale for our choices for fossil placement. Third, theoretical models for divergence time estimation currently do not explicitly allow for the analysis of mixed inter- and intraspecific data. Recent studies have indicated the potential for decreasing substitution rates with increasing calibration depth, which may ultimately overestimate intraspecific divergence times (Ho *et al*. 2005, 2007, 2008). Approaches to rectify this issue are in the early stages of development, and will ultimately serve as powerful tools for estimating divergence times in such cases. Finally, divergence time estimates are based on gene trees, and may not reflect species trees. Particularly in the case of more recently diverged taxa (both intra- and interspecific), gene divergence times are likely to precede species divergence, such that the latter may be overestimated in attempts to estimate divergences within phylogeographical studies (Jennings & Edwards 2005). Comparison of multiple gene trees from independent loci coupled with coalescent approaches my aid in the resolution of this issue (Jennings & Edwards 2005).

Given these caveats, the work presented here still provides an important lesson. In the absence of any divergence time estimates, a standard phylogeographical approach to data interpretation for *L. styraciflua* would be to assume two primary Pleistocene refugia on the basis of two intraspecific clades, one potentially originating along the Gulf Coast (shown in red, blue, and green, Figs 1–3), and one from the highlands of the Cumberland Plateau and the Southern Appalachians (shown in orange and yellow, Figs 1–3). Our divergence time estimates indicate that such inferences would be misleading, given that those two primary clades appear to have diverged at least 8 million years ago. Regardless of the potential challenges associated with intraspecific divergence time estimation, the importance of such attempts is obvious, as has been seen in other tree species (Dick *et al*. 2003; Magri *et al*. 2007).

Importance of recognizing pseudocongruence

Pleistocene glaciation is often presumed to be the primary factor resulting in observed phylogeographical breaks, but studies rarely include data that provide support for temporal associations. It is obvious from comparing molecular topologies of codistributed organisms that while major clades may be congruent, there tends to be great heterogeneity among mutation rates of these codistributed species (reviewed in Avise 2000). Such heterogeneity does not preclude the assumption of a shared vicariant event, but it does suggest the potential for other possibilities (Avise 2000). In particular, relatively frequent climatic oscillations during the course of the last three million years likely resulted in numerous cycles of species range expansions and contractions, overlaying multiple evolutionary signals on the landscape of the genome. To better evaluate the extent to which this occurred, it will be necessary to approach phylogeographical studies much the same way phylogeneticists are approaching the reconstruction of relationships among species and genera, which is with the integration of fossil data (e.g. Manos *et al*. 2007). While this will not be a viable option for many population-level studies due to lack of appropriate fossils, a 'hybrid phylogeographical' approach aimed at both inter- and intraspecific resolution (e.g. Liston *et al*. 2007) will allow for the integration of a temporal element in groups with good fossil histories. Additionally, such an approach would better allow for the inclusion of fossil DNA, providing potentially more accurate divergence time estimates as the relative position of the fossil in the topology becomes more certain. Another approach that is gaining attention for testing phylogeographical hypotheses is that of niche modelling of paleodistributions (Hugall *et al*. 2002; Carstens & Richards 2007). Because these models are entirely dependent on modern collection records and historical climate reconstructions, they may provide an additional line of evidence towards the resolution of biogeographical histories.

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Ashley Morris is interested in phylogeography of disjunct populations. This work was part of her PhD at the University of Florida. Steffi Ickert-Bond is an expert on biography and character evolution of Altingiaceae. Burke Brunson is a Master's student in the Morris lab. Pam and Doug Soltis are interested in phylogeography, systematics, and polyploidy in angiosperms.

Supplementary material

The following supplementary material is available for this article:

Appendix S1 Haplotype distribution and GenBank Accession nos for *Liquidambar styraciflua*. Haplotype codes correspond to those in Figs 2–4 and Table 1. Sample ID, GenBank isolate name; localities, all sampling localities in which a given haplotype was recovered

Appendix S2 Polymorphic sites among cpDNA haplotypes recovered within *Liquidambar styraciflua*. Numbers indicate base position; '.', same as previous sample; and '–', an indel

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