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ORIGINAL ARTICLE

Phylogeography and climatic niche evolution in live oaks (*Quercus* series *Virentes*) from the tropics to the temperate zone

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ABSTRACT

Aim We investigated the phylogeography, geographical variation in leaf morphology, freezing tolerance and climatic niches of two widespread evergreen sister oak species (*Quercus*) in the series *Virentes*.

Location South-eastern USA, Mexico and Central America.

Methods Nuclear microsatellites and non-recombining nuclear and chloroplast DNA sequences were obtained from trees throughout the range of two sister lineages of live oaks, represented by *Quercus virginiana* in the temperate zone and *Q. oleoides* in the tropics. Divergence times were estimated for the two major geographical and genetic breaks. Differentiation in leaf morphology, analysed from field specimens, was compared with the molecular data. Freezing sensitivities of *Q. virginiana* and *Q. oleoides* populations were assessed in common garden experiments.

Results The geographical break between *Q. virginiana* and *Q. oleoides* was associated with strong genetic differentiation of possible early Pleistocene origin and with differentiation in freezing sensitivity, climatic envelopes and leaf morphology. A second important geographical and genetic break within *Q. oleoides* between Costa Rica and the rest of Central America showed a mid-Pleistocene divergence time and no differentiation in leaf morphology. Population genetic differentiation was greater but genetic diversity was lower within the temperate *Q. virginiana* than within the tropical *Q. oleoides*, and genetic breaks largely corresponded to breaks in leaf morphology.

Main conclusions Two major breaks, one between Mexico and the USA at the boundary of the two species, and a more recent one within *Q. oleoides* between Honduras and Costa Rica, implicate climatic changes as potential causes. The latter break may be associated with the formation of the Cordillera de Guanacaste, which was followed by seasonal changes in precipitation. In the former case, an 'out of the tropics' scenario is hypothesized, in which the acquisition of freezing tolerance in *Q. virginiana* permitted colonization of and expansion in the temperate zone, while differences in climatic tolerances between the species limited secondary contact. More pronounced Pleistocene changes in climate and sea level in the south-eastern USA relative to coastal Mexico and Central America may explain the greater population differentiation within temperate *Q. virginiana* and greater genetic diversity in tropical *Q. oleoides*. These patterns are predicted to hold for other taxa that span temperate and tropical zones of North and Central America.

Keywords

Central America, chloroplast DNA sequences, climatic niches, freezing tolerance, leaf morphology, nuclear DNA, Pleistocene glacial cycles, sea-level rise, south-eastern North America, species boundaries.

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INTRODUCTION

The temperate and tropical regions of south-eastern North America and Middle America (Mexico and Central America) have experienced very different historical processes, barriers to gene flow and climatic changes, with contrasting consequences for the phylogeographical structure of plant populations and their physiological adaptations to climate. Some common phylogeographical patterns are beginning to emerge in these regions, driven by topographical barriers, climatic changes, sea-level rise and volcanic activity (González-Rodríguez *et al.*, 2004; Soltis *et al.*, 2006; Jaramillo-Correa *et al.*, 2009). Differences in climatic regimes across the temperate–tropical divide in North and Middle America also influence species physiological adaptations to temperature, with consequences for range limits, population movement and gene flow.

Relatively few phylogeographical studies have been conducted in taxa spanning the temperate and tropical zones of eastern North and Middle America (cf. Morris *et al.*, 2008), and most studies do not consider the evolutionary shifts in climatic niches that may inform our understanding of historical population migration and gene flow. In this study we focus on the phylogeographic structure, variation in leaf morphology and climate adaptation in the tropical oak species *Quercus oleoides* Schlechtendal & Chamisso, and in its adjacent temperate sister lineage, represented by *Quercus virginiana* Miller. *Quercus oleoides* is widely distributed across the lowland, coastal regions of Middle America, and has its southern range limit in Costa Rica, while *Q. virginiana* is widely distributed across lowland and coastal regions throughout the south-eastern USA, and has its northern range limit in North Carolina (Fig. 1a).

We hypothesize that several geological and climatic events may have been important in causing vicariant speciation and in generating the major geographical, genetic and morphological breaks among populations of *Q. virginiana* and *Q. oleoides* (summarized in Fig. 1). First, the climatic history and the climatic factors known to circumscribe the Mexican floristic province may have played a role in the speciation of ancestral *Q. oleoides* and *Q. virginiana* populations, giving rise to the current geographical disjunction between them. A common taxonomic disjunction occurs at the species' break between northern Mexico and the southern USA that has been attributed to late Miocene to mid-Pleistocene climate change (Graham, 1999a). Differentiation in climatic distributions is likely to have been accompanied by adaptive shifts in freezing tolerance.

Second, several factors could have led to the geographical disjunction between the Costa Rican population of *Q. oleoides* and the rest of the range. These include: (1) the uplift of the Central American land bridge 20 Ma, which led to the formation of an expanding island archipelago before closure *c.* 3 Ma; (2) volcanic activity and the formation of volcanic mountain chains in southern Nicaragua and north-western Costa Rica, which was initiated *c.* 1.5 Ma, with a well-defined pulse of volcanic activity in Costa Rica beginning 0.6 Ma (Vogel *et al.*, 2004; Carr *et al.*, 2007; van Wyk de Vries *et al.*, 2007) creating physical barriers to gene flow and local climate alterations; and (3) anthropogenic land use changes (from *c.* 5 ka; Maquin, 1966; Bundschuh *et al.*, 2007).

Third, we hypothesize that, in unglaciated south-eastern North America, glacial climates during the Pleistocene are likely to have restricted the ranges of broadleaved evergreen tree species with limited cold tolerance, such as the live oaks, with consequences for genetic diversity and differentiation. Pleistocene glacial periods are hypothesized to have caused population declines for many temperate species, reducing genetic diversity (Demesure *et al.*, 1996; Hewitt, 1996, 2000, 2004; Petit *et al.*, 2008; Carnaval *et al.*, 2009) and resulting in isolation and genetic breaks among fragmented populations (Petit *et al.*, 2002; Hewitt, 2004; Schmitt *et al.*, 2006). In contrast, glacial climatic fluctuations in Middle America would have been less extreme and may have actually promoted range expansion for the live oaks (Maquin, 1966; Pennington *et al.*, 2000). In Middle America and in the Neotropics, in general, glacial periods are thought to have given rise to cooler, drier climates, and to have allowed savanna and tropical dry forests to expand where rain forests retracted (Graham, 1975; Aide & Rivera, 1998; Pennington *et al.*, 2000; Naciri *et al.*, 2006). In the temperate zone, fragmentation of the ranges of cold-sensitive species during glacial periods may have been exacerbated by sea-level rise in coastal regions during interglacial periods (McLachlan *et al.*, 2005; Soltis *et al.*, 2006). In the Gulf Coast of south-eastern North America, the Mississippi River and the Apalachicola Bay have been identified as significant biogeographical barriers that intersect the range of *Q. virginiana* (Soltis *et al.*, 2006). Interglacial sea-level rise would have expanded both river deltas and created or exacerbated potential barriers between the Florida peninsula and former barrier islands (Webb, 1990). Sea-level-rise barriers may also have been created along the Gulf Coast of Mexico within the range of *Q. oleoides* (Fig. 1).

Our goals were, first, to examine possible causes of the existing population structure and diversity across the latitudinal distribution of the live oaks from North Carolina to Costa Rica and to seek historical explanations for morphological differentiation. Second, we sought to examine genetically based variation within and between species in freezing tolerance in relation to species climatic distributions in order to shed light on the consequences of climatic niche evolution for species range limits and gene flow.

Specifically, we asked the following questions.

1. When and in what order did the major neutral genetic and morphological breaks among populations arise, and what are the plausible historical explanations?

2. How does freezing tolerance vary within and between species, and what implications do adaptive shifts in climatic tolerances have for limiting secondary contact between species?

3. Do populations of the temperate live oak *Q. virginiana* show reduced heterozygosity and allelic diversity compared with populations of the tropical live oak *Q. oleoides*, which would be consistent with range restriction during glaciations

in the temperate zone and greater climatic stability in the tropics?

4. Do populations of *Q. virginiana* show more pronounced genetic and morphological breaks than those of *Q. oleoides*, which would be consistent with hypothesized differences between the south-eastern USA and Middle America in patterns of glacial refugial isolation and sea-level variation during the Pleistocene?

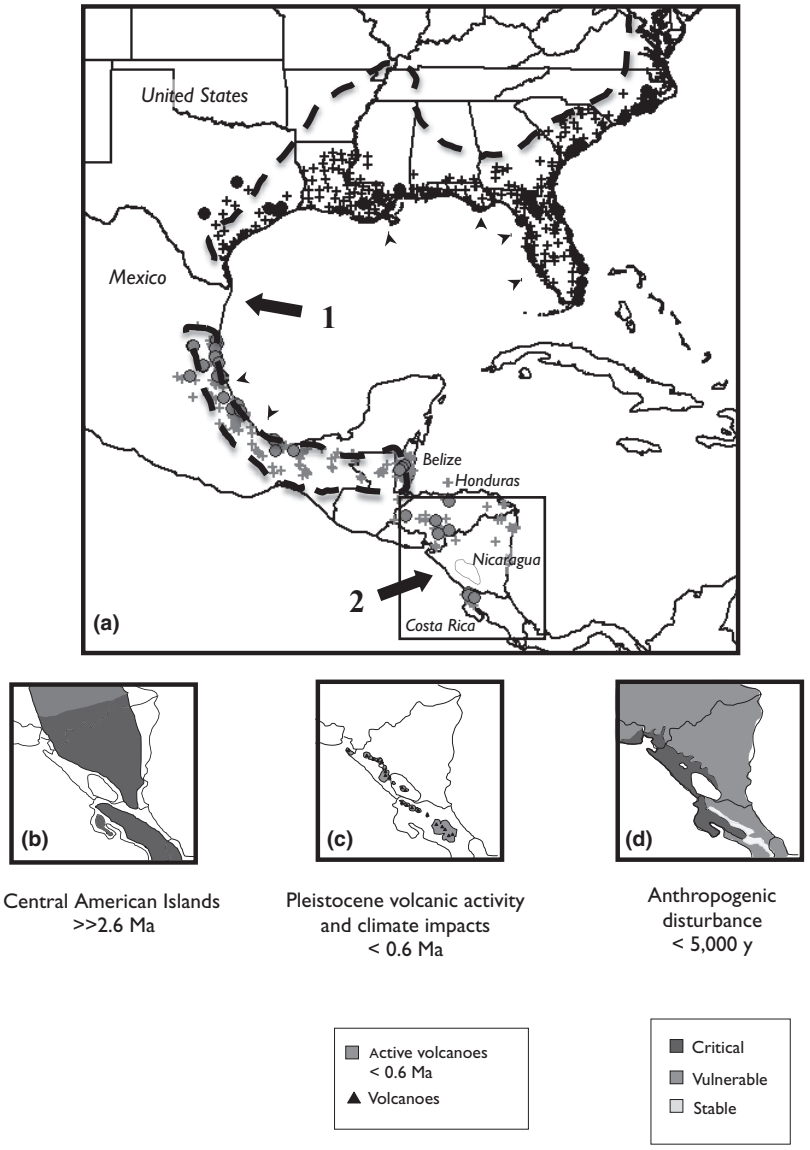

Figure 1 (a) Ranges of *Quercus virginiana* (black crosses) and *Q. oleoides* (grey crosses) from herbarium records accessed through the GBIF Data Portal (http://www.gbif.org/, 7 July 2008) and the USDA PLANTS Database. Circles indicate collection locations. Thick numbered arrows indicate the two major geographic breaks in the distributions. The first occurs at the species boundary. The second occurs between southern Honduras and north-western Costa Rica. Thick dashed lines indicate important taxonomic disjunctions of tree taxa summarized in Graham (1999a) and Jaramillo-Correa *et al.* (2009). Small arrows indicate coastal locations where sea-level rise would have created or exacerbated geographical discontinuities, including: across the Mississippi River and Apalachicola River deltas; the western coast of the Florida peninsula; and two possible sites on the east coast of Mexico. These locations are based on simulations of a 25-m sea-level rise. (b)–(d) Three hypotheses for the timing of population breaks between Honduras and Costa Rica. (b) The Pliocene island hypothesis posits that ancient colonization and isolation on island landmasses before their connection *c.* 3 Ma would account for current population structure. (c) A pulse of volcanic activity in north-western Costa Rica was initiated 0.6 Ma, leading to the formation of a volcanic mountain chain that would have altered climatic conditions and created suitable conditions for dry forest species to colonize north-western Costa Rica (Carr *et al.*, 2007; Hoernle *et al.*, 2008). (d) Anthropogenic activity dating from early colonization *c.* 5000 years ago (5 ka) to the present could have caused land use change that isolated the Honduran and Costa Rican populations (adapted from Bundschuh *et al.*, 2007).

MATERIALS AND METHODS

Study species and sites

Live oaks (*Quercus* series *Virentes* Nixon) form a small monophyletic lineage of interfertile oaks (seven putative species) that spans a range of climatic regimes from the seasonal dry tropics to the temperate zone, forming a nearly continuous distribution in lowland and coastal areas (Muller, 1961; Nixon, 1985; Nixon & Muller, 1997). *Quercus oleoides* and *Q. virginiana* together span the entire latitudinal range of the *Virentes* from North Carolina to Costa Rica. Both species are wind-pollinated and interfertile (Nixon, 1985; Nixon & Muller, 1997) and occur in well-drained sandy soils or volcanic tuff (particularly in Costa Rica). According to recent phylogenetic analyses, the group is basal to the white oak clade (section *Quercus* s.s.) (Pearse & Hipp, 2009), indicating that the *Virentes* represent a fairly old clade within the New World oaks.

Quercus oleoides is a common element of the tropical dry forest but maintains an evergreen canopy (Janzen, 1983), suggesting that the species did not evolve in highly seasonal climates. From a conservation perspective, live oak forests are critical ecosystems that provide shade and shelter during the dry season (Boucher, 1981, 1983; Janzen, 1983) and influence water balance, carbon storage, temperature and seasonal productivity (Powers *et al.*, 2009; Kissing & Powers, 2010; Klemens *et al.*, 2010). *Quercus oleoides* is thought to have covered vast regions of the tropical dry forest of north-western Costa Rica in previous centuries, forming monodominant forests (Maquin, 1966; Boucher, 1983), but over the last 50 years these live oak forests have been subject to large-scale land use change with the conversion of forest to pasture for cattle grazing (Boucher, 1983). Understanding the history of Costa Rican live oak forests and the extent to which they are genetically different from other live oak populations is of significant conservation interest.

Quercus virginiana is the most widespread species within the *Q. virginiana* clade, which also includes *Q. geminata* and *Q. minima* (Manos *et al.*, 1999; Cavender-Bares *et al.*, 2004; Pearse & Hipp, 2009). It maintains an evergreen canopy where winter temperatures are mild, but appears brevi-deciduous where freezing temperatures occur (Kurz & Godfrey, 1962) because annual leaf drop is accelerated by freezing events (Cavender-Bares & Holbrook, 2001). Population-level variation has been found in the timing of winter leaf drop in response to cold stress (Cavender-Bares, 2007). Live oaks are coveted trees, used historically for a variety of economic purposes, and are legally protected in many cities and counties in the south-eastern USA. In recent decades, their population sizes have been reduced as a result of overharvesting (Wood, 1981; Spector & Putz, 2006), conversion of forested land to agriculture (Boucher, 1983), canopy crowding owing to fire suppression (Putz *et al.*, 1984), and pathogen outbreaks (Appel, 1995).

Common garden experiment to test for freezing sensitivity

Acorns were collected from multiple sites in the autumn of 2005 and grouped within three populations for *Q. oleoides* and within four populations for *Q. virginiana*. Populations for *Q. oleoides* were from Mexico, Belize and Costa Rica; populations for *Q. virginiana* were from North Carolina, Florida, Louisiana and Texas (see Appendix S1 in the Supporting Information for sampling details). Seeds were stored at 6 °C for approximately 1 month and synchronously planted in greenhouse facilities at the University of Minnesota. All the necessary collection permits, export permits, phytosanitary certificates and import permits were obtained (available upon request). Each population included five to ten maternal families spanning a 50–100 km^2 region. The experimental design included two temperature treatments: (1) a tropical treatment, in which daytime temperature was maintained between 30 ° and 35 °C, and night-time temperature between 22 ° and 26 °C; and (2) a temperate treatment, in which winter growth temperature reached a minimum night-time temperature of 4 °C with a daytime temperature of 15 °C, simulating the monthly average temperatures in Liberia (Costa Rica) and Wilmington (North Carolina), respectively. Plants in the temperate treatment were acclimatized to the winter temperature regime approximately 3 months prior to measurement. Each temperature regime was replicated in two independently controlled greenhouse rooms. Overnight freeze–thaw events were simulated for shoots excised under water, using a custom-designed freezer box (Geneva Scientific LLC, Fontana, WI, USA), where temperature was regulated at 1-min intervals to within 0.5 °C for freezing sensitivity measurements following previously described methods (Cavender-Bares & Holbrook, 2001; Cavender-Bares *et al.*, 2005; Cavender-Bares, 2007).

In mid-February, approximately 3 months after the onset of cold temperatures in the temperate climate treatment in the second year of growth, freezing manipulations were conducted to determine differences in freezing sensitivity within and between the two species grown in both climate treatments. Freezing cycles were conducted in the dark to minimum temperatures of −5, −7 and −10 °C, based on a previous assessment of temperature sensitivities of live oaks (Cavender-Bares, 2007). Freezing temperatures were maintained for 2 h prior to thawing at 0.1 °C per minute and recovery in a dark room at 22 °C. The maximum quantum yield of photosynthesis was measured from dark-acclimatized chlorophyll fluorescence (Fv/Fm, the ratio of variable to maximum chlorophyll fluorescence) on an individual fully expanded leaf at growth temperatures prior to the freezing cycle and at multiple time intervals after recovery from freezing (following Cavender-Bares, 2007). For brevity, we report only the quantum yield measured 12 h after freezing. One individual per maternal family was measured per population in each growth temperature treatment. Leaves of control branchlets placed in the freezer box maintained at room temperature (22 °C) demonstrated no effect of excising, as in Cavender-

Bares (2007). For each maternal line, a quadratic curve was fitted to the mean Fv/Fm after freezing at each temperature to predict the critical temperature at which quantum yield would reach an Fv/Fm of 0.40 (50% of the dark-acclimated Fv/Fm of a healthy plant; Schreiber *et al.*, 1994).

Climatic distribution models

To investigate climatic niches across the species' ranges we used the WorldClim climate data (Hijmans *et al.*, 2005), which consist of 19 bioclimatic variables derived from climate data over the last 50 years. Herbarium information, including latitude and longitude of each occurrence locality, was compiled across the entire range for the two species. Occurrence localities for *Q. oleoides* were provided by Tropicos® Missouri Botanical Garden (http://www.tropicos.org) and the Instituto Nacional de Biodiversidad, Costa Rica (accessed through the GBIF Data Portal, http://www.gbif.org/, 7 July 2008). Occurrence localities for *Q. virginiana* were provided by Tropicos® and University of Alabama Biodiversity and Systematics (accessed through the GBIF Data Portal, http://www.gbif.org/, 7 July 2008) and also by the United States Department of Agriculture (USDA) PLANTS Database (http://plants.usda.gov/). There were 224 occurrence localities for *Q. oleoides* and 245 for *Q. virginiana*.

Climate models were generated using the program Maxent 3.3.1, which utilizes the maximum entropy method for modelling species geographical distributions (Phillips, 2006). The model was run separately for each species with all 19 climatic variables from WorldClim at each locality. Fifty per cent of the occurrence localities were used for training the data to fit a model and the other fifty per cent for testing the fit of the model. The AUC (area under the curve of a receiver operating characteristic plot) values were examined to measure model performance. An AUC value of 1.0 is optimal, with the model predicting each occurrence of a species.

The minimum temperature of the coldest month for each locality and the percentage of occurrences at each temperature were used to test for differences between *Q. oleoides* and *Q. virginiana* in their climatic distributions. Record low temperatures, which would be more predictive of freezing tolerances, are not available in the WorldClim data.

Sampling and leaf morphological analysis

Quercus virginiana and *Q. oleoides* were sampled throughout their ranges (Fig. 1a; Appendix S1), from North Carolina to Texas (*Q. virginiana*), and from lowland coastal regions of north-eastern Mexico to Costa Rica (*Q. oleoides*). Each sampled tree was marked and its location was recorded with a hand-held geographic positioning system (GPS) instrument. Wherever possible, sites were chosen from parks and protected areas to prevent sampling of planted trees. Trees sampled at each site were at least 100 m apart. Sampling intensity varied among sites owing to the accessibility of trees and other factors. Sunlit branches were sampled for herbarium specimens and used for leaf morphological analysis. One herbarium specimen from each site is held in a permanent collection at the University of Minnesota Herbarium in the Bell Museum of Natural History (Appendix S1). Three dried, pressed leaves from a subsample of individuals from each site were scanned and analysed for laminar leaf area and leaf shape using the leaf imaging software Shape 1.2 (Iwata & Ukai, 2002). A total of 184 individuals of *Q. oleoides* and 103 of *Q. virginiana* were analysed for leaf morphology.

Molecular data

For 54 sites and 424 trees (Appendix S1), we extracted DNA (Appendix S2) and amplified 11 previously published nuclear simple sequence repeat (SSR) microsatellite loci located on seven chromosomes: *QpZAG 1/2*, *QpZAG 1/5*, *QpZAG 9*, *QpZAG 15*, *QpZAG 16*, *QpZAG 36*, *QpZAG 46*, *QpZAG 102*, *QpZAG 110* (Steinkellner *et al.*, 1997); *QrZAG 11*, *QrZAG 30* (Kampfer *et al.*, 1998). Nuclear microsatellites were chosen because of their high variability and their ability to give genome-wide information that reflects both pollen and seed dispersal. Forward primers were labelled with a fluorescent tag (HEX™, NED™, 6-FAM™). Polymerase chain reactions (PCRs), fragment analysis and allele scoring were performed following Cavender-Bares & Pahlich (2009; Appendix S2).

A 569-bp chloroplast sequence in the *trn*T–*trn*D region was amplified using a primer pair that we designed (Appendix S2). The chloroplast reflects a different evolutionary history from nuclear DNA because of its lower effective population size and because dispersal is only through seeds in oaks. A total of 220 individuals were sequenced from 44 sites (Appendix S1). Nuclear ribosomal DNA in the internal transcribed spacer region (ITS and ITS2) and 5.8S coding sequence (Coleman & Mai, 1997) (hereafter referred to collectively as 'ITS'), and in an intron region of the low-copy nuclear gene nitrate reductase (*NIA*-i3) (Howarth & Baum, 2002) were sequenced for a subsample of individuals (57 trees from 27 sites for ITS and 79 trees from 35 sites for *NIA*-i3; Appendix S1). The ITS region was amplified with flanking primers ITS.LEU and ITS.4 plus internal primers ITS.2 and ITS.3B, following previously described methods (Baum *et al.*, 1994; Cavender-Bares *et al.*, 2004). *NIA*-i3 was amplified using *NIA*-i3F and *NIA*-i3R (Howarth & Baum, 2002). Procedures for PCR and DNA sequencing are provided in Appendix S2. All sequences were submitted to GenBank (accession numbers HQ173340–HQ173700).

Data analysis

We tested for evidence of recombination within the sequenced nuclear regions using IMgc (Woerner *et al.*, 2007). For *NIA*-i3, the sequence region of 449 base pairs was returned as a 389-bp non-recombining region. For ITS, the 543-bp sequence was returned as a 356-bp non-recombining region. These regions were used for haplotype network analysis and divergence time estimates. Sequences were tested for deviance from neutrality

using Tajima's *D* in DnaSP 5.0 (Librado & Rozas, 2009), but none showed significant deviance.

Haplotype networks for the chloroplast sequence region, ITS and *NIA*-i3 were constructed in TCS 1.21 using an algorithm for cladograms estimated by maximum parsimony, with insertions–deletions coded as a fifth state (Clement *et al.*, 2000).

To test for underlying genetic structure and admixture among the populations and to determine how well the genetic structure in the molecular data corresponds to species designations, we used a Bayesian clustering algorithm implemented in the program STRUCTURE 2.2 (Pritchard *et al.*, 2000). We restricted this analysis to the nuclear microsatellites, for which we had large sample sizes (424 individuals). We tested the microsatellites for neutrality using the Ewens–Watterson test in POPGENE 1.32 (Yeh *et al.*, 1997). Two loci, ZAG 1/2 and ZAG 16, exhibited non-neutral behaviour (observed *F*-values were higher or lower than the 95% confidence intervals from a simulated null distribution) and were removed from the analyses. STRUCTURE assigns individuals to admixtures of ancestral populations, where the number of populations is unknown. Briefly, we used the admixture model and the correlated model (as in Cavender-Bares & Pahlich, 2009), with a burn-in period of 50,000 and 1,000,000 Markov chain Monte Carlo (MCMC) replicates. We set *K* (the number of ancestral populations) to range from 1 to 12 and ran 10 iterations for each. For each value of *K*, we averaged the log probabilities ($\ln P$) of the data (*D*) [$\ln P(D)$]. We used the method of Evanno *et al.* (2005) for detecting the *K*-value from the point of diminishing returns where there is a large-magnitude second derivative of the log-likelihood (ΔK). This provides criteria for determining the uppermost level of structure in the data.

Several parameters of genetic diversity for each population, including the number of alleles (*A*), effective number of alleles (A_e), number of private alleles (A_p), information index (*I*) and expected heterozygosity (*He*), were calculated using GenAlEx 6.0 (Peakall & Smouse, 2006). Values of these parameters were then compared among the three population groups analysed (*Q. virginiana*, *Q. oleoides* excluding Costa Rica, and *Q. oleoides* from Costa Rica) using Wilcoxon nonparametric tests in JMP 7 (SAS Institute Inc., Cary, NC, USA).

Analysis of molecular variance (AMOVA) was used to investigate the partitioning of genetic variation among groups of populations, among populations within groups and within populations. Population assignments are shown in Appendix S1. Groupings of populations were based on the results of the Bayesian clustering analysis and were: (1) *Q. virginiana* (VI); (2) *Q. oleoides* excluding Costa Rica (OL); and (3) *Q. oleoides* from Costa Rica (CR). Distributions generated from 10,000 random permutations were used to estimate the significances of the various variance components. These analyses were performed with Arlequin 3.0 (Excoffier *et al.*, 2005).

The geographic locations of genetic discontinuities among populations across the entire range covered by the two oak species were assessed with Monmonier's maximum difference algorithm implemented in Barrier 2.2 (Manni *et al.*, 2004) using the microsatellite loci. This program first creates a map of the sampling locations from geographical coordinates. From a matrix of pairwise genetic (or other) distances between populations, barriers are then represented on the map by identifying the edges of polygons where the maximum distances occur. We calculated a matrix of Slatkin's linearized F_{ST} (Slatkin, 1995) values from microsatellites. A similar analysis was conducted using a morphological distance matrix of leaf size (laminar area) and shape (the first principal component axis of a series of elliptic Fourier descriptors) based on multivariate Euclidean distances between populations. To obtain statistical confidence values for the barriers, 100 replicates of both distance matrices were calculated by resampling individuals within populations.

We used the uppermost hierarchical levels of structure indicated by the Bayesian clustering analysis to identify the major historical population splits. These also correspond to the major geographical splits (Fig. 1a). High levels of migration can confound estimates of population divergence times by reducing the degree of genetic differentiation between populations (Slatkin, 1993). We thus used the isolation with migration model in IMa (February 2008 version; Hey & Nielsen, 2007) to estimate the timing of population divergence (*t*), migration rates (*m*) between populations, and the effective populations sizes (*q*) before and after (1) the split between the two species (indicated by '1' in Fig. 1a) and (2) the split between the *Q. oleoides* population in Costa Rica and all remaining *Q. oleoides* populations (indicated by '2' in Fig. 1a). Initially, we ran trials with low burn-in periods (10,000 steps) and low MCMC genealogies (10,000) to determine the upper bounds for the priors. Owing to the high autocorrelation of parameters with low burn-in periods, we then ran trials with burn-in periods of 1,000,000 and 10,000,000 MCMC genealogies, recording after every 10. Three trials were run with different random number seeds to ensure the stability of results. Hey & Nielsen (2007) recommend a minimum effective sample size (ESS) score (a measure of the number of independent data points sampled for each parameter) of 50 to determine convergence. With long burn-in periods, the three final runs for the first split between *Q. virginiana* and *Q. oleoides* had ESS values for *t* of 103, 92 and 88; for the split between Costa Rica and Honduras, ESS values for *t* were 81, 72, and 62. We show averages of the parameter estimates across the three runs.

The divergence time estimate is partly dependent on the mutation rate. Mutation rates for ITS, *NIA*-i3 and chloroplast loci were calculated from Jukes & Cantor (1969) corrected substitution rates from sequence data for oaks as explained in Appendix S2. Data are from the present study, from a previous study using ITS (Cavender-Bares *et al.*, 2004), from P. Gugger and J. Cavender-Bares (unpublished chloroplast data) and from P. Manos (unpublished *NIA*-i3 data). Mean mutation rates per site per year were 8.0×10^{-11} ($\pm 0.4 \times 10^{-11}$) for the chloroplast; 3.4×10^{-10} ($\pm 0.4 \times 10^{-10}$) for the entire ITS region [comparable to low rates found for *Hamamelis* (Wen &

Shi, 1999) and lower than the arboreal clock reported in Dick *et al.* (2003)]; 1.9×10^{-10} ($\pm 0.3 \times 10^{-10}$) for the non-recombining ITS region used in the IMa analyses; and 1.8×10^{-9} ($\pm 0.5 \times 10^{-9}$) for the non-recombining *NIA*-i3 region. For the nuclear microsatellites, no estimates of mutation rates are available for oaks. We therefore used published nuclear dinucleotide microsatellite mutation rates per locus per generation for tomato (*Lycopersicum esculentum*), 1.76×10^{-4} (Azaiez *et al.*, 2006), reported as one-fifth the rate of *Arabidopsis* (8.8×10^{-4}; Marriage *et al.*, 2009), and for wheat (*Triticum turgidum*), 2.4×10^{-4} (Thuillet *et al.*, 2002), for the low and high rate locus^{-1} generation^{-1} estimates, respectively. Mutation rates for nuclear microsatellites in *Arabidopsis* (Marriage *et al.*, 2009) are unusually high (Azaiez *et al.*, 2006) and unlikely to be representative of oak microsatellite mutation rates. A generation time of either 120 or 220 years was used for live oak. An approximate average generation time (T) can be calculated according to $T = \alpha + [s/(1 - s)]$ (Lande *et al.*, 2003; Spellman & Klicka, 2006), where α is the time to maturity and s is the adult annual survival rate. If time to maturity is 20 years (Loftis & McGee, 1992) and adult annual survival is high (0.99) or very high (0.995), typical values for long-lived tree species (Franklin & Debell, 1988), T is calculated as 120 or 220 years, respectively. To convert the IMa time since divergence parameter to years, we calculated the geometric mean of the high, mean and low mutation rate estimates for all loci.

RESULTS

Common garden experiment to test for freezing tolerance

In the tropical treatment, where plants were maintained at warm temperatures all year, there was little differentiation between species and populations regarding dark-acclimatized quantum yield values of leaves after freezing (Fig. 2a). Only at −10 °C was there a significant difference between species (d.f. = 1, sum of squares, SS = 0.20, *F*-ratio = 13.17, P = 0.0006), but populations within species did not differ. At −7 °C, there was no species effect, and populations within species differed little, except that the Texas population showed significantly higher values than populations from Florida, Mexico and Costa Rica. At −5 °C species did not differ, nor did populations within species.

In contrast, in the temperate treatment, where plants were allowed to acclimatize to cold temperatures for 3 months prior to freezing, the species differed at all freezing temperatures: all *Q. virginiana* populations showed an increase in freezing tolerance, but *Q. oleoides* populations did not (Fig. 2b). For example, at −10 °C in the temperate treatment, there was a highly significant species effect (d.f. = 1, SS = 0.66, *F*-ratio = 31.39, $P < 0.0001$), and all populations of *Q. virginiana* were different from all populations of *Q. oleoides*, based on Student's t of least square means differences for $P < 0.05$. However, populations within the same species were not significantly different. An analysis of variance for Fv/Fm after freezing at −10 °C showed a significant species effect (d.f. = 1, SS = 0.83, *F*-ratio = 49.2, $P < 0.0001$) and a significant species by treatment interaction (d.f. = 1, SS = 0.098, *F*-ratio = 5.78, P = 0.018). Even prior to freezing, there were slight differences between the two species in their pre-dawn dark-acclimatized quantum yields under the winter treatment conditions in the greenhouse (d.f. = 1, SS = 0.0393, *F*-ratio = 11.511, P = 0.0014), with *Q. oleoides* populations showing lower values than *Q. virginiana* populations, and all populations showing lower values in the temperate treatment than in the tropical treatment (Fig. 2b).

Predictions of critical freezing temperatures for each population indicate a *c.* 3 °C (±SE) difference in critical freezing temperature between the two species. For populations of *Q. oleoides* from Mexico, Belize and Costa Rica, the critical freezing temperature at which Fv/Fm reached 0.4 was *c.* −7 °C (±SE) (Fig. 2d). For populations of *Q. virginiana* from North Carolina, Florida, Louisiana and Texas, the critical temperature was just below −10 °C.

In the Maxent climate models, both species had high AUC values, indicating that the climate model predicted occurrence well [*Q. virginiana* (VI) test data AUC = 0.983, SD = 0.002 and *Q. oleoides* (OL + CR) test data AUC = 0.989, SD = 0.002]. A jackknife test of the AUC values for both species indicated the most effective climate variables for predicting occurrence were mean temperature of the coldest quarter (*Q. virginiana* AUC = 0.94, *Q. oleoides* AUC = 0.955), minimum temperature of the coldest month (*Q. virginiana* AUC = 0.935, *Q. oleoides* AUC = 0.945), and temperature seasonality (*Q. virginiana* AUC = 0.92, *Q. oleoides* AUC = 0.96). The climate model indicates that low temperatures are important in predicting species distributions. Clear climatic niche differentiation is indicated between *Q. virginiana* and *Q. oleoides*, as shown for the minimum temperature of the coldest month (d.f. = 1, SS = 39111.5, *F*-ratio = 5125.9, $P < 0.0001$; Fig. 2c), corresponding to significant differences in freezing tolerance between the two species (Fig. 2d).

Leaf size and shape

Foliar area differed significantly between *Q. oleoides* (mean ± SE = 16.04 ± 0.39 cm^2) and *Q. virginiana* (7.7 ± 0.29 cm^2) based on a nested ANOVA with populations nested within species (Fig. 2e). Within *Q. oleoides*, there were also significant differences among populations: the Costa Rican population differed significantly from the Honduran population based on Student's t ($\alpha = 0.05$), but neither population differed from Belizean or Mexican populations. Of the total variation in leaf size, 60% was explained at the species level, only 2% at the population level, and 38% within populations. The first principal component (PC1) derived from the elliptical Fourier descriptors (Iwata & Ukai, 2002) explained 74% of the total variation in leaf shape. Leaf shape differed significantly between the two species (Fig. 2f), reflecting the characteristically more rounded leaves of *Q. oleoides* compared with the

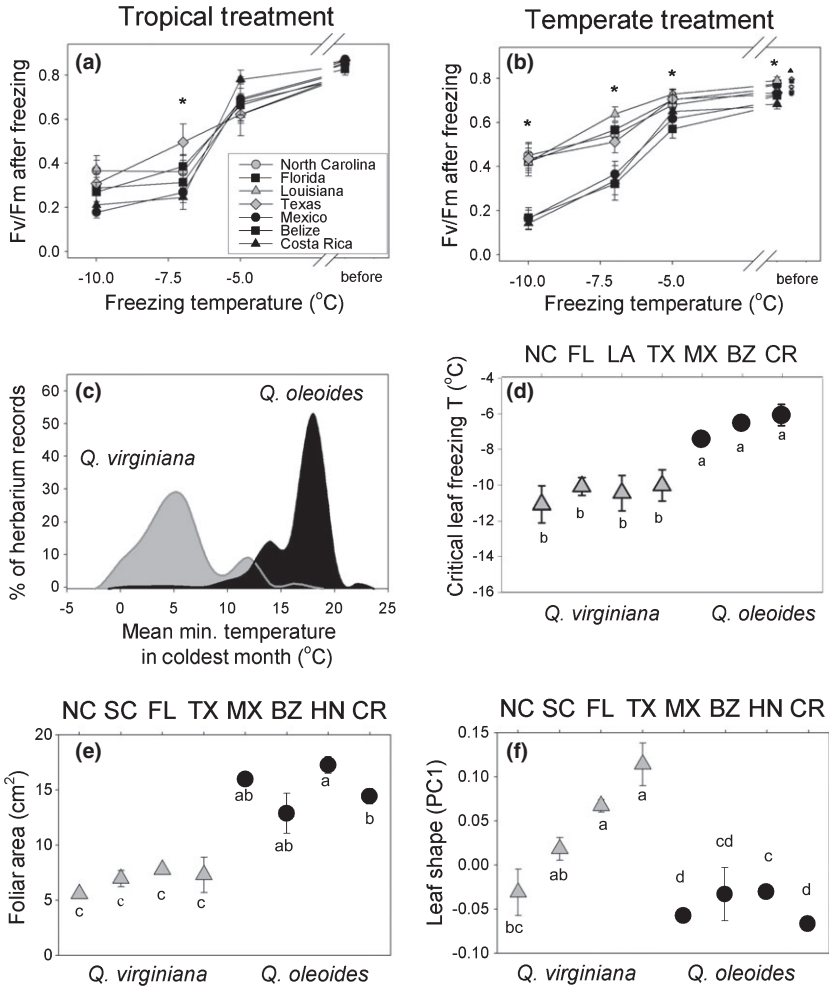

Figure 2 Genetically based differences in freezing sensitivity between *Quercus virginiana* and *Q. oleoides*. The dark-acclimatized quantum yield of photosynthesis (ratio of variable to maximum chlorophyll fluorescence, Fv/Fm) was measured in a common garden experiment under (a) tropical conditions and (b) winter-treated temperate conditions, before freezing and 12 h after freezing at −5 °C, −7 °C and −10 °C. Populations within species did not differ. Asterisks indicate temperatures and treatments for which the two species were significantly different ($\alpha = 0.05$). Small symbols in (b) indicate dark-acclimatized Fv/Fm values of leaves warmed at 22 °C for 48 h. (c) Climatic distributions showing the percentage of herbarium record occurrences at each temperature for the mean minimum temperature in the coldest month. These occurrence localities were used in the MAXENT model to predict climatic distributions for *Q. oleoides* and *Q. virginiana*, which showed that minimum temperature was an effective climate variable for predicting species occurrence and was significantly different between the species ($P < 0.0001$). (d) A *c.* 3 °C difference in critical freezing temperatures between the two species is indicated. Quadratic curves fitted to Fv/Fm responses of individuals to three freezing temperatures (not shown) allowed the prediction of critical freezing temperatures (the freezing temperature at which Fv/Fm = 0.4) for each population (see text for details). (e) Population means for foliar leaf area ± SE. (f) Population means for leaf shape based on elliptical Fourier descriptors using the first principal component (PC1) ± SE. Different letters in panels (d), (e) and (f) indicate that population means are significantly different ($\alpha = 0.05$) based on Student's *t* least square means differences. Region codes are as follows: BZ, Belize; CR, Costa Rica; FL, Florida, USA; HN, Honduras; LA, Louisiana, USA; MX, Mexico; NC, North Carolina; SC, South Carolina, USA; TX, Texas, USA.

more elliptical shape of *Q. virginiana* leaves. Interestingly, within *Q. virginiana*, leaf shape shifted from elliptical to orbicular with increasing latitude, with significant differences among populations. Within *Q. oleoides*, the Costa Rican population had significantly rounder leaves than the Honduran population but did not differ from the Belizean or Mexican populations. Of the total variation in leaf shape, 43.4% was explained at the species level, 13.3% at the population level, and 43.2% within populations.

Bayesian clustering analysis

Results of the Bayesian clustering analysis of microsatellites revealed that the largest ΔK was found for three clusters (mean $\Delta K = 0.6621 \pm 0.011$ SD), followed by two clusters (mean $\Delta K = 0.4295 \pm 0.011$ SD) (Table 1), with much lower ΔK values for larger numbers of clusters. The two genetic breaks identified by STRUCTURE fall at the species boundary between *Q. virginiana* and *Q. oleoides*, corresponding to the geographic

Table 1 Results of Bayesian clustering analysis for all sampled *Quercus virginiana* and *Q. oleoides* populations in North and Central America, using STRUCTURE 2.2 (Pritchard *et al.*, 2000). Number of clusters (K), log-likelihood [ln $P(D)$] and ΔK values for increasing numbers of clusters, based on nine nuclear microsatellites. Mean standard deviations (SDs) are shown to the right of ln $P(D)$. SDs of ΔK values are shown based on 10 runs at each level of K. Three clusters have the highest ΔK.

K	ln $P(D)$	Mean SD	ΔK	SD
1	−16304.6	99.4		
2	−15615	358.2	0.4295	0.011
3	−15079.2	481.4	0.6621	0.011
4	−14862.1	645.6	0.1228	0.039
5	−14724.3	805.8	0.0743	0.036
6	−14646.3	991.1	0.2943	0.175
7	−14860	1666.6	−0.2962	0.127
8	−14580.1	1442.5	0.2503	0.154
9	−14661.2	1816.7	−0.0987	0.098
10	−14563	1869.1	0.1453	0.141
11	−14736.4	2401.6	−0.1609	0.037
12	−14523.5	2138.1		

break between Texas and northern Mexico (indicated by '1' in Fig. 1a), and at a geographic break in the distribution of *Q. oleoides*, namely at the Nicaraguan depression separating Honduras and Costa Rica (indicated by '2' in Fig. 1a). Few individuals show evidence for admixed ancestry across the species boundary (first genetic break), except in one site in Texas (Fig. 3b). However, substantial admixture among ancestral groups is apparent across the second break between populations from Costa Rica and Honduras.

Genetic diversity and differentiation within and among populations and species

The Wilcoxon test performed on genetic diversity parameters detected significant differences among the three population groups (Table 2). According to these results, *Q. oleoides* (excluding the Costa Rican population) showed a higher number of alleles per locus, a higher effective number of alleles, a higher information index value and higher expected heterozygosity than *Q. virginiana* and Costa Rican population groups. The latter two groups had similar values for all these parameters. The number of private alleles did not differ significantly among the three groups but tended to be higher for the Costa Rican population.

Estimates of genetic differentiation among the population groups (Table 3) were low but significant. The comparison between *Q. virginiana* and *Q. oleoides* (excluding the Costa Rican population) indicated that only 6% of the total variation is explained at this level, while another 6% is between populations within species and about 89% is within populations. The comparison between the Costa Rican population and the rest of *Q. oleoides* populations gave very similar differentiation estimates (Table 3). Within population groups, genetic diversity was partitioned differently. The highest differentiation was among the populations from Costa Rica (F_{ST} = 0.11), even though they encompass a comparatively small geographic area. Variation among *Q. virginiana* populations was 8.3%. Finally, even though they range from Honduras to north-eastern Mexico, *Q. oleoides* populations were differentiated by only 4.6%.

Genetic discontinuities

In agreement with genetic differentiation values obtained through AMOVA, Monmonier's maximum difference algorithm also significantly separated *Q. oleoides* from *Q. virginiana* at the species boundary; recognized the Costa Rican population of *Q. oleoides* (CR) as differentiated from the rest of *Q. oleoides*; and suggested much more fragmented population structure within *Q. virginiana* (VI) and within the Costa Rican population of *Q. oleoides* (CR) than within *Q. oleoides*, excluding Costa Rica (OL) (Fig. 4). The fragmentation was particularly evident in Florida, with four barriers separating populations, and with the highest differentiation corresponding to the southernmost one, Key Largo. Another important subdivision within the *Q. virginiana* range was a barrier that separated populations from either side of the Mississippi River delta. In Costa Rica, two barriers isolated the El Hacha and Bagaces populations from the others. Multiple leaf morphological barriers corresponded strongly to the genetic barriers along the Gulf coast, including across the Mississippi River delta, in Florida, and at the *Q. virginiana*–*Q. oleoides* species boundary between Texas and Mexico.

Haplotype networks

Chloroplast DNA

There was limited variation within the chloroplast marker, despite the fact that the sequence region analysed was the most variable region within *trn*D–*trn*T. Two common and widespread haplotypes (Cp1 and Cp2) were found in both species crossing the tropical–temperate divide (Fig. 3c). These were separated by five mutations. Four other rare haplotypes were found in Honduras, Mexico, Texas and southern Florida. These were separated by one to three mutations from the common haplotypes. The rare haplotypes were in all cases only found in single sites or in two geographically proximal sites. A unique haplotype (Cp9) was found throughout the entire Costa Rican population, separated from the most common haplotype (Cp1) by a 10-bp indel region and three single base-pair mutations. No other haplotypes were present in Costa Rica.

ITS

Thirteen homologous ITS haplotypes were identified (Fig. 3d). The three most common haplotypes (IT1, IT6 and IT9) were found in both species. Two relatively common haplotypes (IT2 and IT3) were found in multiple populations of *Q. virginiana*

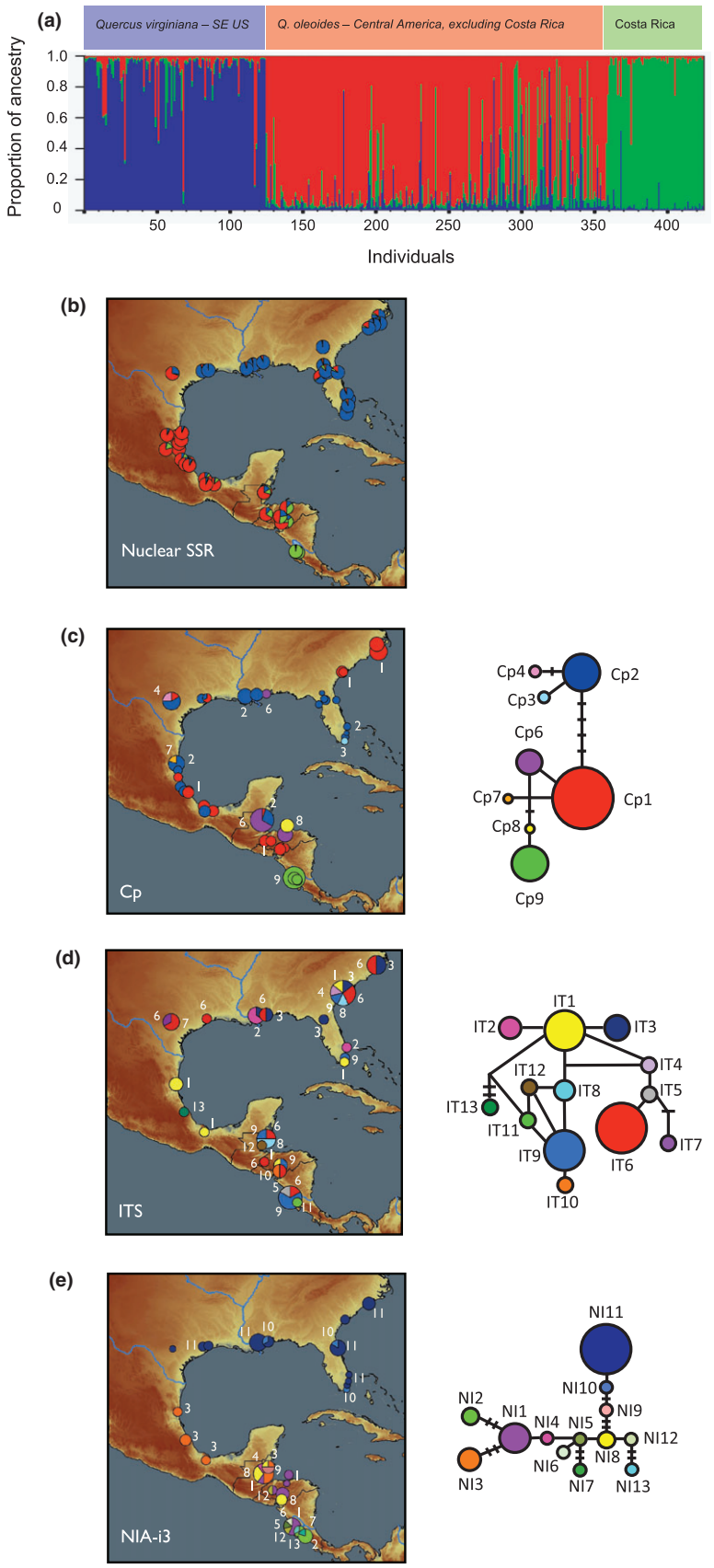

Figure 3 (a) Proportion of ancestry assigned to individuals of *Quercus virginiana* and *Q. oleoides* based on nine nuclear simple sequence repeat microsatellite loci. The *x*-axis represents the 424 individuals in the analysis. Three populations gave the highest ΔK. Larger numbers of clusters were much lower; see Table 2. (b) Ancestral groups based on the STRUCTURE analysis are colour-coded, and the mean proportion of ancestry for the three groups at each sampling location is shown. Pie charts showing groups are not proportional to scale (n = 424). (c) Haplotype maps and networks for chloroplast sequences in the *trn*D–*trn*T region. Pie charts on the maps are proportional to the number of samples in each region (n = 220). (d) Internal transcribed spacer haplotypes. Pie charts on the map are proportional to the number of samples in each region (n = 57). (e) The third intron of the nitrate reductase gene (*NIA*-i3). Pie charts are proportional to the number of samples in each region (n = 79). Numbers on maps (c)–(e) correspond to haplotypes in the adjacent networks.

Table 2 Mean (±SE) number of alleles (A), number of effective alleles (A_e), information index of alleles (I), number of private alleles (A_p) and expected heterozygosity (He) based on nine nuclear microsatellites for the major live oak clusters identified in Table 1 in North and Central America: *Quercus virginiana*, *Q. oleoides* (excluding the Costa Rican population) and the Costa Rican population of *Q. oleoides*. Significant differences are indicated by different letters with a P cut-off of 0.05 using Wilcoxon nonparametric tests.

Wilcoxon test-score means						
Level	No. populations	A	A_e	I	A_p	He
Q. virginiana	22	4.32 (0.397)[a]	3.33 (0.254)[a]	1.15 (0.085)[a]	0.091 (0.028)[a]	0.595 (0.030)[a]
Q. oleoides (excl. Costa Rica)	25	6.24 (0.290)[b]	4.29 (0.139)[b]	1.51 (0.038)[b]	0.093 (0.025)[a]	0.712 (0.009)[b]
Costa Rican population of *Q. oleoides*	7	4.41 (0.467)[a]	2.83 (0.183)[a]	1.11 (0.081)[a]	0.095 (0.045)[a]	0.576 (0.029)[a]

Table 3 Analysis of molecular variance (AMOVA) of nine nuclear microsatellites using $F_{ST:}$ (1) within and among populations of *Q. virginiana* (VI); (2) within and among populations of *Q. oleoides*, excluding the Costa Rican population (OL); (3) within and among the Costa Rican population of *Q. oleoides* (CR); (4) hierarchically between the Costa Rican population (CR) and the rest of *Q. oleoides* (OL); and (5) hierarchically between VI and OL population groups. Similar results were obtained using R_{ST} (Appendix S3).

AMOVA results using F_{ST} Source of variation	d.f.	SS	Variance components	Percentage of variation	F-statistics†
(1) *Q. virginiana*					
Among populations of *Q. virginiana*	21	150.4	0.32	8.32	F_{ST} = 0.09***
Within populations	226	806.3	3.57	91.68	
Total	247	956.7	3.89		
(2) *Q. oleoides* – Central America (excluding Costa Rica)					
Among populations of *Q. oleoides*	24	165.9	0.17	4.56	F_{ST} = 0.05***
Within populations	445	1625.7	3.65	95.44	
Total	469	1791.7	3.83		
(3) *Q. oleoides* – Costa Rica					
Among populations of *Q. oleoides* – Costa Rica	6	59.9	0.39	11.10	F_{ST} = 0.11***
Within populations	123	386.7	3.14	88.9	
Total	129	446.7	3.54		
(4) *Q. oleoides* – Costa Rica and Central America					
Between Costa Rica and Central America	1	66.1	0.28	6.96	F_{CT} = 0.07***
Among populations within groups	30	225.9	0.22	5.35	F_{SC} = 0.06***
Within populations	568	2012.5	3.54	87.69	F_{ST} = 0.13***
Total	599	2304.5	4.04		
(5) *Q. virginiana* and *Q. oleoides* (excluding Costa Rica)					
Between *Q. virginiana* and *Q. oleoides*	1	85.5	0.24	5.87	F_{CT} = 0.06***
Among populations within groups	45	316.36	0.22	5.50	F_{SC} = 0.06***
Within populations	671	2432	3.62	88.63	F_{ST} = 0.12
Total	717	2833.9	4.09		

†F_{CT} = the proportion of variation among groups; F_{SC} = the proportion of variation among populations within groups; and $F_{ST} = F_{CT} + F_{SC}$.
***$P < 0.001$.

but not in *Q. oleoides*. One haplotype (IT13) was found only in Mexico in one location; three haplotypes (IT8, IT10, IT12), were found exclusively in Belize and Honduras; and two (IT11, IT5) were unique to Costa Rica.

NIA-*i3*

There was no overlap in *NIA*-i3 haplotypes across the species boundary between *Q. virginiana* and *Q. oleoides* (Fig. 3e). *Quercus virginiana* showed low haplotype diversity, with one widespread haplotype (NI1) found in all sites and a less common one (NI2) found in three sites east of the Mississippi River. Neither haplotype was found in *Q. oleoides*. Much higher haplotype diversity was found in *Q. oleoides*, particularly in the Belize/Honduras region, as well as in Costa Rica. A single haplotype (NI10) was found throughout Mexico; it also occurred in northern and southern Belize. A total of six haplotypes were found in Belize (NI1, NI3, NI4, NI8, NI9) and Honduras (NI1, NI8, NI12), with two shared between them. Only two of these (NI1, NI12) were also found in Costa Rica. Five additional haplotypes were unique to Costa Rica (NI2, NI5, NI6, NI7, NI13).

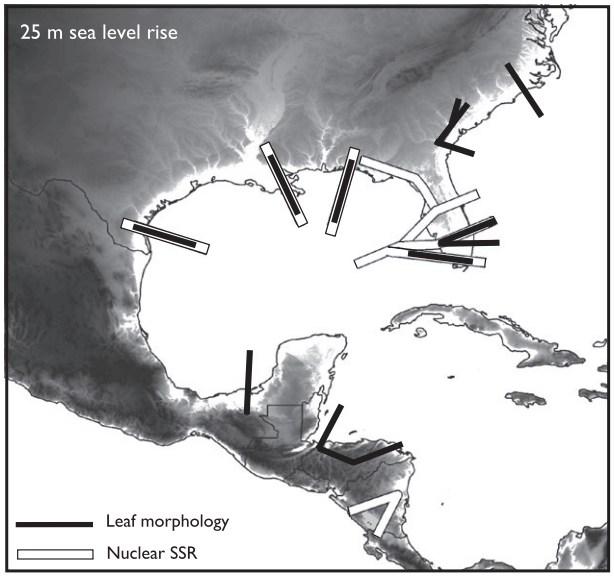

Figure 4 Genetic (white) and morphological (black) boundaries among populations of *Quercus virginiana* and *Q. oleoides*, obtained with Monmonier's maximum difference algorithm for genetic distances derived from nuclear simple sequence repeat microsatellite frequencies, and Euclidean distances calculated from leaf area and shape. Coast contours for the present-day sea level and a 25-m sea-level rise are shown. These sea levels would have occurred during interglacial periods of the Pleistocene (Miller *et al.*, 2005). The simulation of sea-level rise in the base map was created using Earth elevational data from the NASA Satellite Radar Topography Mission (Farr *et al.*, 2007) using the program OmniGlyph 5.2 (Lehman, 2007).

Divergence time estimates and population demographics using the isolation with migration model

Texas–Mexico disjunction at the species boundary

Assuming a generation time of 120 years, we estimate the divergence time between *Q. oleoides* and *Q. virginiana* to be 1.48 Ma for the low mutation rate, 0.99 Ma for the high mutation rate, and 1.2 Ma for the mean mutation rate (Table 4). The ancestral population size (q_A) is estimated to be high ($\approx$ 143,000 to 214,000 individuals), as are the derived population sizes (q_{VI} and q_{OL}) for both species (*Q. virginiana* $\approx$ 9000 to 14,000; *Q. oleoides* $\approx$ 12,000 to 19,000; for high and low mutation rates, respectively). Extremely low migration rates following the split are indicated (*Q. virginiana:* 9.82×10^{-7} to 1.47×10^{-6}; *Q. oleoides*: 4.9×10^{-7} to 7.34×10^{-7} individuals year^{-1}), with higher migration from *Q. virginiana* to *Q. oleoides* than in the reverse direction. Increasing the generation time to 220 years increases the divergence time to range between 1.5 and 2.3 Ma; it slightly decreases the ancestral and derived population sizes and lowers the migration rates (Table 4).

Costa Rica–Honduras split

At the population-level split between Costa Rica and Honduras, the IMa model estimated a divergence time of 231 to 347 ka for the low and high mutation rates, respectively (mean mutation rate: 231 ka), for a 120-year generation time (Table 4). The ancestral population size (q_A) is estimated to be moderate ($\approx$ 62,000 to 93,000 individuals) and the derived Honduran and Costa Rican population sizes asymmetrical (Honduras: $\approx$ 7100 to 11,000; Costa Rica: $\approx$ 1800 to 2600, for high and low mutation rates, respectively). Low migration following the split is indicated (Honduras: 2.5×10^{-6} to 3.7×10^{-6}; Costa Rica: 2.2×10^{-6} to 3.3×10^{-6} individuals year^{-1}), with slightly higher migration from Honduras to Costa Rica than in the reverse direction. Increasing the generation time to 220 years increases the divergence time from 365 to 548 ka (mean mutation rate: 436 ka), and slightly lowers the estimated population sizes before and after the split, as well as the migration rates.

DISCUSSION

Two major genetic breaks that correspond to major geographic disjunctions (Fig. 1a) occur within the distributions of *Q. oleoides* and its temperate sister taxon, *Q. virginiana*. The first is between Mexico and Texas, corresponding to the species boundary, with divergence times estimated in the late Miocene to mid-Pleistocene (Fig. 3; Table 4). Range restriction as a result of differentiation in climatic tolerance may be important in preventing secondary contact between the species. The current climatic niches of the two species differ and correspond to a 3 °C evolutionary shift in critical freezing temperature, with minimal population-level variation (Fig. 2). The second major genetic break is more recent and occurs within *Q. oleoides* between Costa Rica and the rest of Middle America (Fig. 3). Divergence time estimates fall within the mid- to late Pleistocene (Table 4) and may correspond to orographic effects and local climate changes generated by the rise of the volcanic mountain belt in northwestern Costa Rica. Genetic diversity is lower in *Q. virginiana* than in *Q. oleoides* (Table 2), which may reflect population declines in the temperate zone during glacial periods when cold-sensitive, broadleaved evergreen trees would have been forced into refugia further south than their current distributions. The greater differentiation among populations of *Q. virginiana* (Table 3) may reflect fragmentation during glacial cycles as well as barriers created or exacerbated by interglacial sea-level rise. The higher diversity and gene flow in the tropical species is consistent with a more stable historical climate in the tropics. Genetic breaks throughout the distributions of these taxa were matched by differentiation in leaf morphology, indicating that morphological variation shows an historical signature similar to that of neutral markers.

Table 4 Results of the isolation with migration model (IMa) for the two major genetic breaks, one between the two species, *Quercus oleoides* and *Q. virginiana*, and the other between the *Q. oleoides* populations from Costa Rica and Honduras, based on nine nuclear microsatellites, the internal transcribed spacer region (ITS), the third intron of the nitrate reductase gene (*NIA*-i3) and chloroplast sequences. The parameters are as follows: t, time since divergence (years); q_{VI} and q_{OL}, size of derived populations of *Q. virginiana* (VI) and *Q. oleoides* excluding Costa Rica (OL), respectively; q_A, size of ancestral population before the split; $m_{VI\rightarrow OL}$ and $m_{OL\rightarrow VI}$, migration rates in individuals per year of *Q. virginiana* (VI) to *Q. oleoides* (OL) and vice versa, respectively. For the second split: q_{CR} and q_{HN}, size of derived populations of *Q. oleoides* from Costa Rica (CR) and from Honduras (HN), respectively; q_A: size of ancestral population before the split; $m_{CR\rightarrow HN}$ and $m_{HN\rightarrow CR}$, migration rates in individuals per year from Costa Rica to Honduras and vice versa, respectively. Values for estimated parameters are calculated using geometric means of mutation rates for all loci using low, high and mean mutation rate estimates calculated with generation times (G) of 120 years or 220 years. The 90% highest posterior density (HPD) + and – range around the parameter estimate is given on the right.

	Low mutation rate	90% HPD	High mutation rate	90% HPD	Mean mutation rate	90% HPD
Split 1: *Quercus virginiana* versus *Quercus oleoides*						
$G = 120$						
t	1,479,577	$+4.2 \times 10^5$, -3.1×10^5	986,620	$+2.8 \times 10^5$, -2.1×10^5	1,176,858	$+3.3 \times 10^5$, -2.5×10^5
q_{VI}	13,546	$+3.7 \times 10^3$, -3.2×10^3	9033	$+2.4 \times 10^3$, -2.2×10^3	10,775	$+2.9 \times 10^3$, -2.6×10^3
q_{OL}	18,576	$+4.7 \times 10^3$, -3.9×10^3	12,387	$+3.2 \times 10^3$, -2.6×10^3	14,775	$+3.1 \times 10^3$, -3.3×10^3
q_A	213,841	$+9.8 \times 10^4$, -6.3×10^3	142,595	$+6.5 \times 10^4$, -4.2×10^4	170,090	$+7.8 \times 10^4$, -5.0×10^4
$m_{VI\rightarrow OL}$	9.82×10^{-7}	$+5.7 \times 10^{-7}$, -4.3×10^{-7}	1.47×10^{-6}	$+8.6 \times 10^{-7}$, -6.5×10^{-7}	1.23×10^{-6}	$+7.2 \times 10^{-7}$, -5.4×10^{-7}
$m_{OL\rightarrow VI}$	4.9×10^{-7}	$+7.1 \times 10^{-7}$, -2.5×10^{-7}	7.34×10^{-7}	$+4.7 \times 10^{-7}$, -3.8×10^{-7}	6.16×10^{-7}	$+3.9 \times 10^{-7}$, -3.2×10^{-7}
$G = 220$						
t	2,331,142	$+6.6 \times 10^5$, -4.9×10^5	1,554,465	$+4.4 \times 10^5$, -3.3×10^5	1,854,194	$+5.2 \times 10^5$, -3.3×10^5
q_{VI}	11,643	$+3.1 \times 10^3$, -2.8×10^3	7764	$+2.1 \times 10^3$, -1.9×10^3	9261	$+2.5 \times 10^3$, -2.2×10^3
q_{OL}	15,964	$+4.1 \times 10^3$, -3.4×10^3	10,645	$+2.7 \times 10^3$, -2.2×10^3	12,698	$+2.6 \times 10^3$, -2.9×10^3
q_A	183,773	$+8.4 \times 10^4$, -5.4×10^4	122,544	$+5.6 \times 10^4$, -3.6×10^4	146,173	$+6.7 \times 10^4$, -4.3×10^4
$m_{VI\rightarrow OL}$	6.23×10^{-7}	$+3.6 \times 10^{-7}$, -2.7×10^{-7}	9.34×10^{-7}	$+5.5 \times 10^{-7}$, -4.1×10^{-7}	7.83×10^{-7}	$+4.6 \times 10^{-7}$, -3.4×10^{-7}
$m_{OL\rightarrow VI}$	3.11×10^{-7}	$+4.5 \times 10^{-7}$, -1.6×10^{-7}	4.66×10^{-7}	$+3.0 \times 10^{-7}$, -2.4×10^{-7}	3.91×10^{-7}	$+2.5 \times 10^{-7}$, -2.0×10^{-7}
Split 2: Costa Rica versus Honduras						
$G = 120$						
t	347,591	$+6.6 \times 10^5$, -1.7×10^5	231,782	$+4.9 \times 10^5$, -1.2×10^2	276,474	$+5.8 \times 10^5$, -1.4×10^5
q_{CR}	2645	$+1.9 \times 10^3$, -1.2×10^3	1764	$+1.3 \times 10^3$, -7.8×10^2	2104	$+1.5 \times 10^3$, -9.3×10^2
q_{HN}	10,705	$+6.5 \times 10^3$, -4.0×10^3	7138	$+4.4 \times 10^3$, -2.7×10^3	8515	$+5.2 \times 10^3$, -3.2×10^3
q_A	92,717	$+6.7 \times 10^4$, -3.3×10^4	61,826	$+4.4 \times 10^4$, -2.2×10^4	73,747	$+5.3 \times 10^4$, -2.6×10^4
$m_{CR\rightarrow HN}$	2.18×10^{-6}	$+4.0 \times 10^{-6}$, -1.7×10^{-6}	3.28×10^{-6}	$+6.0 \times 10^{-6}$, -2.6×10^{-6}	2.75×10^{-6}	$+5.0 \times 10^{-6}$, -2.1×10^{-6}
$m_{HN\rightarrow CR}$	2.47×10^{-6}	$+5.1 \times 10^{-6}$, -1.7×10^{-6}	3.70×10^{-6}	$+4.9 \times 10^{-6}$, -2.6×10^{-6}	3.10×10^{-6}	$+4.1 \times 10^{-6}$, -2.2×10^{-6}
$G = 220$						
t	547,645	$+1.0 \times 10^6$, -2.7×10^5	365,184	$+7.7 \times 10^5$, -1.8×10^5	435,598	$+9.2 \times 10^5$, -2.2×10^5
q_{CR}	2273	$+1.7 \times 10^3$, -1.0×10^3	1516	$+1.1 \times 10^3$, -6.7×10^2	1808	$+1.3 \times 10^3$, -8.0×10^2
q_{HN}	9200	$+5.6 \times 10^3$, -3.5×10^3	6135	$+3.7 \times 10^3$, -2.3×10^3	7317	$+4.5 \times 10^3$, -2.8×10^3
q_A	79,680	$+5.7 \times 10^4$, -2.8×10^4	53,133	$+3.8 \times 10^4$, -1.9×10^4	63,378	$+4.6 \times 10^4$, -2.3×10^4
$m_{CR\rightarrow HN}$	1.39×10^{-6}	$+2.5 \times 10^{-6}$, -1.1×10^{-6}	2.08×10^{-6}	$+3.8 \times 10^{-6}$, -1.6×10^{-6}	1.74×10^{-6}	$+3.2 \times 10^{-6}$, -1.4×10^{-6}
$m_{HN\rightarrow CR}$	1.57×10^{-6}	$+1.0 \times 10^{-6}$, -2.7×10^{-5}	2.35×10^{-6}	$+7.7 \times 10^{-5}$, -1.8×10^{-5}	1.97×10^{-6}	$+9.2 \times 10^{-5}$, -2.2×10^{-5}

The *Quercus oleoides* and *Q. virginiana* genetic, climatic and adaptive split

Our results show clear genetic differentiation between *Q. oleoides* and *Q. virginiana* between south-eastern North America and north-eastern Mexico, indicating a distinct species boundary (Muller, 1961; Nixon & Muller, 1997) rather than a series of clinal populations. The Bayesian clustering analysis of nuclear microsatellites indicates a definitive break between the species (Table 1; Fig. 3a), with possibly some admixture only in Texas. The *NIA*-i3 network supports this break. The two temperate haplotypes (NI10 and NI1) are found only in *Q. virginiana*, while all other haplotypes are found only in *Q. oleoides* (Fig. 3e). Graham (1999b) summarized floristic affinities across this disjunction, noted earlier by others (Fernald, 1931; McVaugh, 1943; Miranda & Sharp, 1950; Graham, 1975). These disjunctions have been supported with molecular data (Hoey & Parks, 1991, 1994; Morris *et al.*, 2008), and reviewed by Jaramillo-Correa *et al.* (2009), showing that a common break occurs between north-eastern Texas and north-eastern Mexico at the tropical–temperate divide around the Tropic of Capricorn (Fig. 1a). Here, a number of temperate genera reach their southern limits. Among the plant taxa that show disjunctions across this gap are species of *Acer*, *Alnus*, *Carpinus*, *Carya*, *Cercis*, *Cornus*, *Fagus*, *Fraxinus*, *Juglans*, *Liquidambar*, *Magnolia*, *Myrica*, *Ostrya*, *Platanus*, *Prunus*, *Rhus*, *Smilax*, *Tilia* and *Ulmus* (Martin & Harrell, 1957; Graham, 1999a). The cause of this gap has not been resolved,

but is hypothesized to result from climatic warming and drying during the late Miocene to early Pleistocene (Graham, 1999a). The biotic affinities across this region are largely deeper in history than the species level, implying a substantial period of geographic isolation. The current distribution of *Q. oleoides* matches nearly perfectly the floristic Province of the Gulf of Mexico (Fig. 1a), suggesting that climate plays an important role in circumscribing its range. Our estimate of the divergence time between *Q. oleoides* and *Q. virginiana* of 1.5 to 2.3 Ma for the low mutation rate, depending on generation time, and of 0.99 to 1.6 Ma for the high mutation rate (Table 4) suggests that speciation in the live oaks may have been driven by the same forces that caused disjunctions in other taxa.

The pattern of haplotype variation for the chloroplast suggests an 'out of the tropics' colonization hypothesis for *Q. virginiana* (Fig. 3c), although phylogenetic relationships that would clarify this remain unresolved. The common and widespread Cp1 and Cp2 haplotypes are found both in the tropics and further north. Cp1, abundant at its southern extent in Honduras and extending northwards, is the more central and potentially the most ancestral haplotype in the network. Cp2 extends northwards from its southern extent in Belize. All of the external haplotypes are only found in specific regions and are therefore likely to be more recent. The *NIA*-i3 haplotype network is consistent with a tropical origin for the live oaks. High haplotype diversity in *Q. oleoides* in the Honduras/Belize region contrasts with the relatively depauperate and potentially more recent haplotypes of *Q. virginiana* (NI10 and NI11) that extend from the tropical haplotypes NI9 and NI8 (Fig. 3e).

If an 'out of the tropics' scenario is correct, the lower critical leaf-freezing temperatures in *Q. virginiana* populations compared with *Q. oleoides* populations (Fig. 2c) can be interpreted as an adaptive shift in the physiological tolerance of winter that would have been critical to colonization of the temperate zone. *Quercus virginiana* populations demonstrated a greater ability to acclimatize to cold and to increase freezing tolerance after exposure to chilling temperatures than *Q. oleoides* populations, resulting in a 3 °C greater leaf-freezing tolerance (Fig 2a and b). The MAXENT models indicate that *Q. virginiana* has a climatic envelope with significantly lower minimum temperatures than *Q. oleoides*, and both species distributions are well predicted by minimum temperatures.

Freezing temperatures are strongly implicated in limiting the ranges of species (Burke *et al.*, 1976; Körner & Larcher, 1988; Tyree & Cochard, 1996; Guy, 2003; Cavender-Bares, 2005; Cavender-Bares *et al.*, 2005), and freezing tolerance has been hypothesized to be an important barrier to range expansion (Wiens, 2004; Wiens *et al.*, 2006; Donoghue, 2008). The differentiation in freezing tolerance and in climatic envelopes between the two species may thus serve as a barrier to colonization of *Q. oleoides* north of the Mexican lowlands and may be critical in preventing secondary contact. This is consistent with the lower migration rates of *Q. oleoides* northwards than of *Q. virginiana* southwards (Table 4). Climate adaptation may thus explain the very limited gene exchange between *Q. oleoides* and *Q. virginiana*.

There was little evidence for an increase in low-temperature tolerance with latitude within each species (Fig. 2c). A previous study with only two populations per species found greater freezing tolerance in the North Carolina population at the northern range limit than in the Florida population, although a much higher proportion of variation was explained at the species level (Cavender-Bares, 2007). The extent of local adaptation within species is a subject of ongoing investigation, but the lack of intraspecific variation found here suggests that the evolution of low-temperature tolerance may be constrained and that adaptation to freezing stress may have occurred early in the colonization of the temperate zone.

Geographic break between populations of *Quercus oleoides* in north-western Costa Rica and Honduras

The second important geographic break found in the live oak distribution occurs between southern Honduras and north-western Costa Rica within *Q. oleoides*, and is considerably more recent (Table 4). This break is evident in the Bayesian clustering analysis (Fig. 3a and b), which indicates a distinct ancestral group in Costa Rica, with some mixed ancestry in Honduras. In addition, five *NIA*-i3 haplotypes are unique to Costa Rica. The differentiation of Costa Rica from the rest of the species is most strongly supported by a fixed, unique chloroplast haplotype throughout the Costa Rican population (Fig. 3c), indicating a long period of geographic isolation. Given that the chloroplast is maternally inherited in oaks and dispersed through seeds (Dumolin *et al.*, 1995), the clearer differentiation in the chloroplast between Honduras and Costa Rica (Fig. 3c) compared with in the nuclear markers (Fig. 3b, 3d, 3e) may reflect longer-distance dispersal of wind-transported pollen relative to seeds. Greater differentiation in the chloroplast also reflects the fact that chloroplasts are more sensitive to genetic drift than are nuclear markers because of their haploid status and, hence, lower effective population sizes. Chloroplast patterns are thus more likely to reflect colonization history, while nuclear markers reflect gene exchange.

There are three major alternative explanations for the disjunction and genetic break between *Q. oleoides* from Costa Rica and Honduras (Fig. 1b–d), which also appears in pitvipers, and other vertebrates (Castoe *et al.*, 2009), and is emerging as a pattern in tropical tree species (M. Poelchau, University of Georgia, pers. comm.). First, uplift of the Central American land bridge led to the formation of an expanding island archipelago in Central America during the Miocene and Pliocene (23–2.6 Ma), which closed near the end of the Pliocene (Fig. 1b, Coates & Obando, 1996; Alvarado *et al.*, 2007). These islands could have been colonized through rare dispersal events leading to long-term isolation and population divergence; however, this explanation is not supported by the divergence time estimates that post-date the closure of these islands.

At the other temporal extreme, anthropogenic land use change in coastal Central America over the last 5000 years

could have contributed to the disjunction. Coastal Nicaragua was one of the earliest regions to be developed, in conjunction with European settlement. Anthropogenic influences may have reduced genetic diversity and/or led to local extinction. For example, Maquin (1966) noted that humans occupied the regions in which *Q. oleoides* occurs for centuries, practising milpa agriculture, a land use that would have caused periodic burning and forest destruction. Some forest species may have been eradicated in the Pacific region by extensive clearing or overharvesting for firewood (Bundschuh *et al.*, 2007). An anthropogenic explanation seems unlikely, however, given our divergence time estimates that pre-date human impacts.

The most likely explanation for the Costa Rica–Honduras disjunction, given the mid-Pleistocene divergence time estimates (Table 4), is that Pleistocene volcanic activity and the formation of the Cordillera de Guanacaste mountain chain in Costa Rica created a physical barrier and fundamentally altered the climate (Coates & Obando, 1996; van Wyk de Vries *et al.*, 2007). The Cordillera de Guanacaste mountain range bounds the northern and eastern limits of the Costa Rican *Q. oleoides* population. Peak volcanic activity, both within the Nicaraguan depression that parallels the Pacific coast and within the Cordillera de Guanacaste, is estimated to have initiated *c.* 0.6 Ma (Vogel *et al.*, 2004; Carr *et al.*, 2007). Earlier volcanoes would have erupted *c.* 1.5 Ma at the Nicaraguan–Costa Rican border (Carr *et al.*, 2007), covering the entire current distribution of the Costa Rican population with volcanic ash.

In addition to the habitat destruction caused by large regions of volcanic ash and the potential dispersal barrier created by the emerging mountain chain, mountain formation would also have caused orographic effects that altered climatic conditions. The strong seasonality and severe dry season to the west of the Cordillera de Guanacaste in Costa Rica, where *Q. oleoides* currently occurs, can be attributed to these effects. A seasonally dry climate would have provided suitable conditions for oaks pre-adapted to drought, allowing them to replace rain forest species over the last several hundred thousand years. Wet tropical forest is thought to have existed in this region prior to the rise of this mountain chain.

The divergence time estimates (Table 4) that post-date the initiation of the Cordillera de Guanacaste suggest that *Q. oleoides* colonized north-western Costa Rica via long-distance dispersal after the rise of these mountains. More extreme long-distance dispersal has been recognized as a critical factor in explaining range disjunctions of Neotropical forest taxa (Pennington, 2006; Schmitt *et al.*, 2006; Dick *et al.*, 2007). Nixon (1985) hypothesized that long-distance dispersal via birds (e.g. passenger pigeons) may have been responsible for the long-distance movement of acorns over land.

Range retraction rather than colonization may also be consistent with the observed molecular patterns. Graham (1975) hypothesized that intervals of warming and increased precipitation caused both an expansion of the rain forest and range retraction of a previously broader distribution of *Q. oleoides*-dominated communities; this general scenario is supported by more recent evidence (Pennington *et al.*, 2000). The distinct chloroplast haplotype in Costa Rica, however, makes range retraction a less likely scenario than colonization. If colonization was successful only after the initiation of seasonally dry conditions caused by mountain formation, an important role for climate in driving species colonization history is again implicated.

While the mid- to late Pleistocene estimates for the divergence between Costa Rican and Honduran populations are most consistent with the volcanic activity and local climate change hypothesis, the history of *Q. oleoides* in Costa Rica was probably complex and driven by multiple processes at different time periods.

Consequences of climate change and sea-level fluctuations for historical gene flow and genetic diversity

Contrasting population histories for the tropical and temperate live oaks may be linked to contrasting patterns of climate and sea-level fluctuations. In temperate regions, colder temperatures during glacial periods are likely to have caused population decline in the live oaks, which do not undergo winter dormancy and have limited cold tolerance, leading to a loss of allelic diversity. Fragmentation and isolation of populations, limiting gene flow, is another predicted outcome. In contrast, less drastic changes in climate may have minimized these impacts in Central America. Genetic diversity, based on nuclear microsatellites, is higher in the tropical *Q. oleoides* than in the temperate *Q. virginiana* (Table 2), but neutral genetic differentiation among populations is higher in *Q. virginiana* (Table 3). Other nuclear markers (Fig. 3c–e) support patterns of genetic diversity shown by the nuclear microsatellites (Table 2). These results are consistent with impacts of glacial cycles in the temperate zone and a more stable historical climate in the tropics. Fluctuations in precipitation levels in Middle America, however, would have followed glacial cycles and influenced the population history and distributions of alternating dry forest and wet forest species. Novick *et al.* (2003) found strong regional differentiation in Central America in the economically important wet forest species *Swietenia macrophylla* (big leaf mahogany), attributable to climate-related population fluctuations.

Historical sea-level rise and fall may also have caused barriers and passages that influenced the population genetic structure of low-elevation and coastal species. During the Pleistocene, $\delta^{18}O$ signatures of foraminifera indicate that the global sea level rose *c.* 25 m above current levels during the interglacial periods (Fig. 4) and fell by a maximum of 125 m (Miller *et al.*, 2005). In Florida and northwards along the Atlantic coastal plain, six successive shorelines above present sea level are confidently recognized (Webb, 1990), and are thought to represent successively higher sea levels with older interglacial periods (Cooke, 1945; MacNeil, 1950; Jones *et al.*,

1973). Sea-level rise would have created or exacerbated potential barriers to gene flow between peninsular Florida and former barrier islands and across river deltas along coastal regions of the Gulf of Mexico. Simulations of a 25-m sea-level rise (Fig. 4) indicate a larger number of obvious sea-level barriers in the Gulf Coast of the USA than in coastal Middle America. In the south-eastern USA, sea-level rise is a potential cause of the break across the Mississippi River delta and the Apalachicola River, and of multiple barriers in Florida. These are commonly observed discontinuities in many North American plant and animal taxa (Soltis *et al.*, 2006; Jaramillo-Correa *et al.*, 2009). The two potential sea-level barriers on the Gulf Coast of Mexico are in regions where *Q. oleoides* does not currently occur.

A striking correspondence is apparent between neutral genetic and morphological breaks, several of which correspond to geographic discontinuities attributable to sea-level rise (Fig. 4). In concert, the molecular and morphological data (Fig. 4) indicate that there was sufficient isolation across these barriers for drift and/or selection to dominate over gene flow and cause phenotypic differentiation. Without quantitative genetic analyses in reciprocal common garden experiments, however, we cannot distinguish between these alternatives.

CONCLUSIONS

We show an initial split between *Q. virginiana* and *Q. oleoides* (possibly during the early Pleistocene) accompanied by significant differences in freezing tolerance and climate adaptation that may have prevented significant gene flow between the species. A more recent split between Costa Rica and Honduras is most consistent with Pleistocene volcanic activity initiated *c.* 0.6 Ma, which gave rise to the Cordillera de Guanacaste, producing orographic effects that would have caused seasonally dry climate conditions suitable for the colonization of *Q. oleoides*. We find higher genetic diversity but lower genetic differentiation among populations of the tropical species than of the temperate species – effects that may be explained by differential impacts of climatic fluctuations during glacial and interglacial cycles. Sea-level rise is likely to have increased genetic differentiation in *Q. virginiana*. Several important barriers resulting from sea-level rise appear likely, particularly across the Mississippi River delta, the Apalachicola River and in the Florida peninsula, where morphological and neutral genetic differentiation is strong. We predict other coastal taxa to follow similar patterns across the temperate–tropical regions of North and Middle America.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Sampling locations, geographical coordinates and sample sizes.
Appendix S2 Molecular methods.
Appendix S3 Analysis of molecular variance (AMOVA) results using R_{ST}.

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