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# Phylogeography of *Diadophis punctatus*: Extensive lineage diversity and repeated patterns of historical demography in a trans-continental snake

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**Abstract**

Dynamic climatic oscillations during the Pleistocene had profound effects on the distributions of species across North America. Although the role of historical climate change on speciation remains controversial, the impact on genetic variation within species has been well documented. We examined mtDNA sequences from the cytochrome *b* gene (1117 bp) and a portion of the *NADH*-4 gene (659 bp) for 286 individuals of *Diadophis punctatus* to infer phylogeographic patterns and population structure and to examine historical demographic patterns in both glaciated and unglaciated regions of North America. We inferred 14 lineages that replace each other geographically across the United States. Several of these lineages appear to be confined to specific habitats (floodplains, grasslands, montane environments) and traverse previously identified genetic barriers for terrestrial vertebrates including the Mississippi and Apalachicola Rivers, the Appalachian Mountains, and the western continental divide. We also observed overlapping ranges between some haplotype groups and several instances of secondary contact associated with ecological transition zones in eastern South Carolina, southern Oklahoma and central California. Within the US, diversification began during the late Miocene and continued into the mid-Pleistocene, suggesting these lineages pre-dated the last glacial maximum. Coalescent and non-coalescent demographic analyses indicate that independent lineages currently occupying previously glaciated or unsuitable areas in eastern, central and western US underwent post-glacial population expansion likely from southern refugia during the late Pleistocene/early Holocene. Conversely, southern lineages display patterns consistent with long-term population stability. Such long-term persistence of genetic structure may be due to the competitive effects between lineages or ecosystem stability in more southern latitudes.


*Keywords:* mtDNA; Trans-continental phylogeography; *Diadophis punctatus*; Population demography; Post-glacial expansion

## 1. Introduction

Historical processes such as population division due to isolation, long-distance dispersal or range expansion are expected to leave characteristic signals on the distribution and frequency of alleles (Hewitt, 1996). When analyzed using phylogenetic methods, the tree structure can be superimposed over the range of the population, revealing whether the population history has been one of isolation, panmixia or a combination of the two. Predictions based on population genetic theory can then be incorporated to reconstruct the demographic histories of these populations and to examine the genetic variation within and among populations. Combining these methods has allowed biologists to investigate how the distribution of taxa may have changed in response to climatic shifts in geologic history (Avise and Walker, 1998; Douglas et al., 2003; Zamudio and Savage, 2003; Mahoney, 2004).

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During the Pleistocene, at least six glacial advances affected the physical and biological environments of the Northern Hemisphere (Cox and Moore, 2000). The expansion and contraction of glacial ice sheets are thought to have played an important role in shaping the distribution of biodiversity throughout the Northern temperate regions (Hewitt, 1996). At the time of the last glacial maximum in North America, ~22–18 kya, ice sheets extended from southeastern Alaska throughout most of Canada and into the northeastern and northwestern United States (Mann and Hamilton, 1995). The presence of these glaciers combined with the climatic changes during the Pleistocene are hypothesized to have stimulated intra-specific diversification by separating populations through the formation of glacial barriers and by shifting the location of suitable habitat further south (Durand et al., 1999; Hewitt, 2000; Brunsfeld et al., 2001; Waltari et al., 2007). While phylogeographic patterns can vary across taxa, the concerted retreat into southern refugia has been proposed as an historical factor underlying the formation of major genetic lineages (Swenson and Howard, 2005). Thus, concordant patterns of population expansion from southern refugia into previously glaciated areas have been inferred across a variety of taxa with a broad range of life history strategies, indicating the overriding impact of glacial vicariant events on structuring genetic diversity at Northern latitudes. For many amphibian and reptile species, southern refugia have been proposed from across the conterminous United States including the Appalachian Mountains and Interior Highlands (Burbrink et al., 2000; Austin et al., 2002, 2004; Zamudio and Savage, 2003), the Atlantic and Gulf Coast coastal plains (Austin et al., 2004; Burbrink et al., 2000; Church et al., 2003; Zamudio and Savage, 2003), Northern Texas (Howes et al., 2006), the Clearwater drainage in Idaho (Carstens et al., 2004), the Sonoran and Chihauhuan deserts of the southwest (Castoe et al., 2007), the Columbia River Valley in Oregon and the Klamanth/Siskyou mountains of Northern California (Steele and Storfer, 2006). Although the role of glaciation events on speciation has been debated (Klicka and Zink, 1997, 1998, 1999; Avise and Walker, 1998; Avise et al., 1998), the formation of different geographic refugia allowed existing lineages to persist. Additionally, many of these species have retained geographic ranges separated by distinct geographic boundaries. However, in some cases phylogeographic breaks can occur that do not coincide with known geographic boundaries (Irwin, 2002). Examination of species distributions has revealed that non-climatic ecological factors such as breeding sites, host availability and especially species interactions can lead to genetic differentiation of local populations in unglaciated areas (Ehrlich, 1961; Hairston, 1987; Crespi et al., 2003; Kozak et al., 2006).

Widespread taxa occupying both historically glaciated and unglaciated regions provide an excellent opportunity to study the effects of climatic cycles on population fragmentation and historical demography. Such taxa also serve as excellent models because their geographic ranges likely span many previously identified phylogeographic barriers and provide an opportunity to uncover genetic breaks that are not associated with geographic boundaries. In addition to a broad geographic range, several features make certain taxa more amenable to phylogeographic study for the purposes of examining the influence of climatic shifts. Limited or restricted dispersal of a species facilitates the successful inference of historical patterns of migration by maintaining the genetic patterns created during the establishment of the current distribution (Crandall et al., 1996; Templeton, 1998). If a species becomes restricted to a particular area or colonizes an unoccupied region, the stability and maintenance of phylogeographic patterns will depend on longevity and vagility (Hewitt, 1996). Thus, taxa with poor dispersal capabilities and exhibiting strong philopatry should retain the genetic patterns that developed while occupying these regions. Because mutations occurring in cytoplasmic genomes are transmitted maternally, patterns of variation inferred from mtDNA should reflect the demographic history and historical processes responsible for the contemporary distribution (Avise, 2000).

The ringneck snake *Diadophis punctatus* possesses several of the aforementioned features making it an excellent organism to study phylogeography and to examine the effects of climatic cycles on demographic history. As one of only seven North American squamates with a transcontinental distribution, this snake exploits a variety of habitats and ecological niches ranging from the mixed hardwood forests of southern Canada to the deserts of the Southwestern US and Northern Mexico (Stebbins, 1985) (Fig. 1). This species is also characterized by morphological and behavioral variation (Blanchard, 1942; Gehlbach, 1965; Fitch, 1975; Blanchard et al., 1979; Connant and Collins, 1991; Stebbins, 1985) suggestive of extensive genetic variation. With the exception of the southwestern populations that feed exclusively on reptiles (Gehlbach, 1965), ringnecks have generalist diets, preying on salamanders, earthworms, reptiles and insects (Blanchard, 1942; Fitch, 1975; Blanchard et al., 1979; Stebbins, 1985; Connant and Collins, 1991). This species is relatively long-lived among small snakes, surviving to an average age of over ten years. Home ranges are estimated to have a diameter of a mere 70 m (Fitch, 1975; Blanchard et al., 1979) and Parker and Brown (1974) found that ringnecks in Utah returned to specific hibernacula over multiple years. The longevity, small home range and limited dispersal abilities of this species fulfill a number of the conditions beneficial to the inference of historical patterns of migration and fragmentation that have resulted in the current distribution of genetic diversity.

Despite the extensive variation in ecology, morphology, and life history of ringneck snakes, only two molecular studies have addressed lineage diversity within *D. punctatus*. However, neither was comprehensive; Pinou et al. (1995) examined immunological distances for a small sample of ringnecks while Feldman and Spicer (2007) restricted

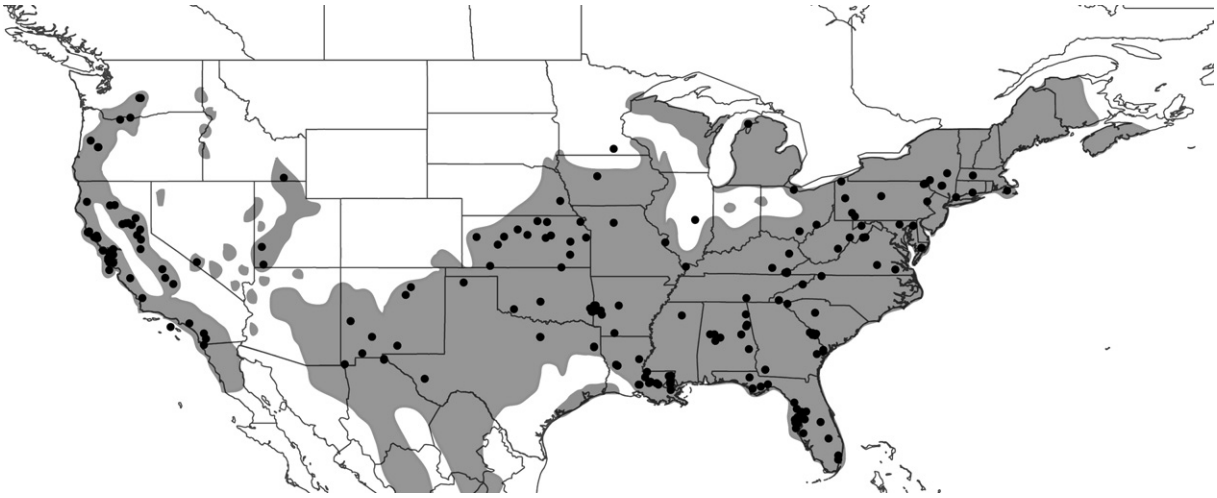

Fig. 1. Map of the United States showing the proposed range of *Diadophis punctatus* and location of samples used in this study. Locality and label designations are given in Appendix 1.

their phylogeographic analysis of mtDNA sequences to populations in California. Here we examine the historical patterns of *D. punctatus* with comprehensive transcontinental sampling using both phylogenetic and population genetic methods. Our goals are to: (1) describe the lineage diversity and phylogeographic patterns of the ringneck snake *Diadophis punctatus* and (2) to evaluate the effects of Pleistocene glaciation on population demography in glaciated and unglaciated regions.

## 2. Materials and methods

### 2.1. Molecular analyses

#### 2.1.1. Sampling and geographic distribution

Our geographic sampling ($n = 286$) covers most of the known the range of this species across the US (Fig 1; Appendix 1). Due to uncertainty of the sister group and to assess the monophly of *D. punctatus,* outgroup taxa included the other North American xenodontine snakes *Contia tenuis*, *Hypsiglena torquata, Rhadinaea flavilata, Francia abacura, Heterodon simus* and *Heterodon platyrhinos* (Pinou et al., 2004; Lawson et al., 2005).

#### 2.1.2. DNA extraction, amplification and sequencing

We extracted DNA from liver, muscle, blood or shed skin preserved in ethanol or frozen in liquid nitrogen using phenol chloroform and CTAB modified from Saghai-Maroof et al. (1984). We amplified the mitochondrial genes cytochrome *b* (1117 bp) and a portion of the *NADH* 4 subunit (659 bp) via PCR using previously published primers and protocols (Burbrink et al., 2000) and (Arevalo et al., 1994), respectively.

Each of these genes has been used successfully to examine intra-specific variation in snakes (Burbrink et al., 2000; Feldman and Spicer, 2002; Nagy et al., 2004). Amplifications were purified using either 2 μl of ExoSap-it (USB Corp.) per 10 μl of PCR product or AMPURE (Agencourt) following the manufacturer's instructions. Purified PCR products were sequenced in both directions for both genes using BigDye v.1.1 (Applied Biosystems, Perkin-Elmer, California, USA). For the cytochrome *b* gene, sequencing reactions were performed using internal sequencing primers designed specifically for *D. punctatus* (Dp-F CCTTCTGAGCAGCAACAGTAA) and (Dp-R GAAGAATCGTGTGAGGGTTGG). Occasionally extension of the Dp-R primer failed to cover the forward section of cytochrome *b*. When this occurred, samples were re-sequenced using the L14910 (de Queiroz et al., 2002) primer. Sequencing reactions for ND-4 were carried out using the amplification primers. Reactions were purified with the CleanSeq Dye-terminator removal kit (Agencourt) and analyzed on an ABI Prism 3730 sequencer (Applied Biosystems, Perkin-Elmer, California, USA). Sequences were assembled, edited, and aligned using SEQUENCHER 4.7 (Gene Codes, Corp.) and an open reading frame was verified for each gene. The alignments were unambiguous with no gaps present in either of the mitochondrial genes for any of the *D. punctatus* sequenced in this study. Although the ND4 primers amplify portions of the downstream Serine, Histidine, and Leucine trna genes, only the protein coding region was used in the analyses. The tRNA gene regions were deleted due to problems of alignment resulting from secondary structure and incomplete sequencing. Sequences were deposited in GenBank under the Accession Nos. EU193950–194234 and EU193663–193948.

### 2.2. Phylogenetic analyses

Phylogenetic relationships of *D. punctatus* samples were estimated for each gene separately and from the combined cytochrome *b* and *NADH*-4 data sets with maximum parsimony (MP) in TNT Goloboff et al., 2003) and Bayesian inference (BI) using MrBayes v3.1.2 (Heulsenbeck and Ronquist, 2001; Ronquist and Heulsenbeck, 2003).

Maximum parsimony analyses were conducted using a heuristic search method with equally weighted characters, 1000 random addition-sequence replicates and the tree-bisection-reconnection (TBR) branch-swapping algorithm. Support for internal nodes was assessed using non-parametric bootstrapping (BS) (Felsenstein, 1985) with 1000 pseudo-replicates and 100 random sequence-addition replicates.

ModelTest v3.7 (Posada and Crandall, 1998) was used to select the best-fit model of nucleotide change based on the Akaike Information Criteria (AIC) independently for each gene (Akaike, 1973). The GTR + Γ + I (general time reversible model with γ-distributed among-site rate variation and with a proportion of invariant sites) model was selected for each gene and implemented in MrBayes. In the separate analyses, two methods of model partitioning were explored for each gene. The first method implemented the GTR + Γ + I model without considering differences in codon position. The second method partitioned the model across the first, second and third codon positions 3(GTR + Γ + I). Additionally, a mixed model analysis was performed for the concatenated data to infer trees using the evolutionary information from both genes. Two differently partitioned analyses were conducted for the concatenated data set because how a data set is partitioned can have a greater influence on the mean −ln L and estimated posterior probabilities than the overall number of partitions (Brandley et al., 2005). In the first analyses, the appropriate model was partitioned across each gene 2(GTR + Γ + I). In the second, models were partitioned across the first, second and third codon positions of each gene 6(GTR + Γ + I). Each of the four analyses (implemented with two simultaneous runs) was conducted using default priors for parameters of the four markov chains with the model parameters unlinked among partitions for each run. Each run used a random starting tree and was run for $1 \times 10^7$ generations. Trees and their parameters were sampled once every 1000 generations. Stationarity of the likelihood scores was determined by examining the convergence in posterior probabilities between the simultaneous runs using the standard deviation of split frequencies based on Rubin and Gelmans's "r" statistic (Gelman et al., 1995). The partitioned models were evaluated using Bayes factors calculated from the harmonic mean of the posterior probability distribution for the models (Nylander et al., 2004). When comparing models using Bayes factors, a value greater than 10 is considered strong evidence for the selection of the more complex model (Kass and Raferty, 1995).

In order to conservatively delimit boundaries for each lineage, we followed the tree-based method of Wiens and Penkrot (2002), which incorporates geographic patterns of coalescence among DNA haplotypes to test for gene exchange between closely related lineages. This method assumes that discordance between haplotype clades and the geographic areas from which they are found or the failure of haplotypes from a given area to form a clade is evidence of potential gene flow with other populations (Slatkin and Maddison, 1989).

### 2.3. Population genetic analyses

#### 2.3.1. Diversity indices and population structure

We examined genetic structure and demographic patterns using both traditional population genetic and coalescent methods. All lineages were treated as separate units for tests of demographic history and genetic structure. The majority of the major clades inferred from the phylogenetic analyses occupied non-overlapping geographical areas. In regions where lineages overlapped there was no evidence of introgression of the mitochondria (see Section 3). To summarize the genetic variation among and within populations, we first conducted an analysis of molecular variation (AMOVA, Excoffier et al., 1992) in Arlequin v2.0 (Schneider et al., 2000).

#### 2.3.2. Historical demography

Haplotype (Hd) and nucleotide ($\theta$) diversity were calculated to measure DNA polymorphisms using DNAsp v3.0 (Roza and Roza, 1999). These measures are appropriate for this type of study because they do not depend on sequence length or sample size (Nei and Li, 1979; Nei, 1987). Nucleotide diversity can be calculated using the average number of pairwise nucleotide differences ($\pi$) or by calculating the number of segregating sites (S). Under the null hypothesis of population stability, the difference between these two values, Tajima's $D$ (Tajima, 1989) and Fu's $F_s$ (Fu, 1997), can be used to infer demographic history of a population. If populations have been stable over time both statistics are expected to be close to zero (Tajima, 1989; Fu, 1997). Significant deviation from zero (positive or negative) permits for the rejection of the null hypothesis of population stability. Under the assumption of neutrality, negative values are expected in populations that have undergone recent increases because rare alleles are more numerous than expected. Positive values occur if rare alleles are eliminated from populations following genetic bottlenecks (Tajima, 1989). The validity of the assumed stepwise expansion model and the significance of $D$ and $F_s$ were calculated by constructing 10,000 coalescent simulations in DnaSP.

Mismatch distributions were also calculated for each lineage to infer changes in population size. Assuming an infinite sites model, population expansion would be depicted as a unimodal distribution whereas the distribution expected for population stability is ragged and multimodal (Harpending et al., 1998). The fit of the observed data was compared using the sum of squares deviations between observed and expected data estimated from 10,000 coalescent simulations in DnaSP. Although the $R_2$ raggedness index (Ramos-Onsins and Rozas, 2002) is often used to assess significance of mismatch plots, non-unimodel models that fit sudden expansion models indicate structuring within populations or populations that are stable

or shrinking (Excoffier and Schneider, 1999; Rogers and Harpending, 1992; Rogers et al., 1996). Therefore, we do not consider the good fit of sudden expansion models to multimodal mismatch distributions strong evidence of population expansion. However, multimodal distributions can be inferred for populations that have undergone recent expansion but were recently sub-divided, subjected to substantial migration and/or have undergone historical contractions (Bertorelle and Slatkin, 1995; Marjoram and Donnelly, 1994; Ray et al., 2003; Castoe et al., 2007).

Although $D$, $F_s$ and mismatch distributions are able to provide insights into whether or not population growth has been expansive, they are not able to provide information about the shape of population growth over time. Non-significant negative values of $D$ and $F_s$ are indications that populations have not undergone expansive growth relative to a null hypothesis of population stability. However, such values are agnostic as to whether populations are expanding slowly, are contracting or remaining relatively constant in size. Therefore, to estimate the shape of population growth through time we constructed Bayesian skyline plots implemented in BEAST v1.4 (Drummond and Rambaut, 2006; Drummond et al., 2005). This method uses MCMC sampling procedures to estimate the posterior distribution for the effective population size given a model of nucleotide substitution and a set of aligned DNA sequences (Drummond et al., 2005). As above, the appropriate model of nucleotide substitution for each lineage was determined using ModelTest. Geneologies and model parameters for each lineage were sampled every 1000th iteration for $1 \times 10^7$ generations under a relaxed lognormal molecular clock with uniformly distributed priors and a pre-burnin of 100. Coalescent intervals ($m$) ranged from 3 to 15 depending on the total number of samples per lineage. We noticed that varying values of $m$ had little effect on the analyses and that the demographic function was highly consistent across a wide range of values for the $m$ hypervariable (Drummond et al., 2005). Demographic plots for each analysis were visualized using Tracer v1.0.1 (Rambaut and Drummond, 2003). If population sizes are constant through time then the slope of the skyline plot should not be significantly different than zero. We tested the slope of the skyline plot for each lineage against the null hypothesis of population stability (slope of zero) using GraphPAD Prism v.5.0 (GraphPad Software, Inc.)

#### 2.3.3. Divergence dating and dating demographic change

To infer the date of origin for each lineage without relying on a molecular clock and considering uncertainty in tree topology and branch length (i.e., 'the relaxed phylogenetics' method) we used BEAST v1.4.4 (Drummond and Rambaut, 2006). Phylogenetic estimates were constructed under the GTR + Γ + I model with an uncorrelated lognormal tree prior with a constant population size prior. Derived from fossil data (Holman, 1979, 2000), a mean calibration point of 7.5 my was placed at the root of *Diadophis* with a lognormal standard deviation of 0.29 producing a 95% credible sampling interval (CI) from 4.55 to 12.1 my. This time frame spans the North American Land Mammal Age (Hemphillian) into the Clarendonian Age; the latter age has not produced fossils for this species and is a reasonable cutoff point for the upper CI. Analyses were run for 20 million generations and sampled every 1000th iteration following a pre-burnin of 2000.

We estimated the date of expansion for each lineage using the formula $T = \tau/2u$, where $\tau$ is the relative time (in generations) since expansion obtained from 1000 replicates from Arlequin and $u$ is the product of the mutation rate, the generation time, and the sequence length. Females usually reach sexual maturity at approximately 3 years (Fitch, 1975; Blanchard et al., 1979) and the total sequence length was 1776 bp. To obtain mutation rates for each lineage we used the semiparametric approach utilizing penalized likelihood (PL) with the truncated Newton (TN) algorithm implemented in r8s v1.70 (Sanderson, 2003). Since large data sets with low sequence variation (such as population-level data) or phylogenies with very short branch lengths (phylogeographic studies) can lead to spurious results due to zero branch lengths, we followed the suggestions of Sanderson (2003) and constructed phylogeny using one exemplar from each lineage. This reduced data set was analyzed with the 6(GTR + Γ + I) partitioned model. Outgroup taxa were pruned prior to rate smoothing analyses with the root of *D. punctatus* set to 7.5 mya. An optimal smoothing value of 1000 was obtained using the cross-validation procedure implemented in r8s.

## 3. Results

### 3.1. Phylogenetic reconstruction

Two hundred ninety six sequences for 1776 aligned base pairs were obtained for *D. punctatus* and nine outgroup taxa. The absence of any internal stop codons in either protein coding gene and a bias against guanine on the light strand indicate that the sequences were from the mitochondrial genome and are not nuclear-integrated copies or pseudogenes (Zhang and Hewitt, 1996).

Of the total 1776 characters analyzed, 832 were constant and 758 variable characters were parsimony informative (considering both ingroup and outgroup taxa). Maximum parsimony analyses produced 14 trees of 3801 steps. Because both analyses produced highly congruent estimates of phylogenetic relationship for the major clades only the Bayesian consensus phylogram is presented with posterior probabilities and nonparametric bootstrap values from the MP analyses for the shared branches.

When genes were analyzed separately for the Bayesian analyses, 'burn in' occurred prior to one million generations both for the partitioned and un-partitioned analyses.

As such, results hereafter were based on harmonic means calculated from the remaining nine million generations. The data were combined and analyzed under a mixed model since each of the separate analyses inferred trees with similar topologies. Burnin for the mixed model analyses occurred between one and 2.5 million generations depending on how the data were partitioned. For each of the four analyses, Bayes factors always chose the most complex model with a value greater than 1000. Therefore, tree topology and posterior probabilities (PP) were inferred from the 6(GTR + Γ + I) model. The combined mixed-model analyses produced a 50% majority-rule consensus tree with a mean ln L of −24033.25 following a 'burn in' of 2500 generations.

The *Diadophis punctatus* complex formed a well-supported monophyletic group exclusive of the other North American xenodontines. This species complex was composed of four major clades consisting of 14 phylogenetically and geographically distinct, putatively independent, evolutionary lineages.

The basal node in the complex subtends a Gulf Coast clade (A) from the Mid-Atlantic and remaining clades of the continental United States (Figs. 2 and 3). The Gulf Coast clade extends from southeastern Louisiana to southern Florida and comprises three lineages. The first-diverging lineage is restricted to southeastern Louisiana and is the sister taxon to a group containing the Piedmont lineage ranging from southeastern South Carolina to eastern Alabama and a peninsular Florida lineage distributed from the Florida Keys north to the Suwannee River.

The second major division occurs between the Mid-Atlantic clade (B) and the more diverse continental clades west of the Appalachians. This clade consists of a single lineage that is confined to the eastern side of the Appalachian Mountains from costal southern Virginia to southeastern South Carolina.

The third major split occurs between the Appalachian (C) and Western clades (D), both of which comprise multiple lineages west and north of the Atlantic and Gulf Coast coastal plains. Within the Appalachian clade, the Northeast lineage ranges from central Tennessee north to Massachusetts and west to central Illinois above the Mississippi River Embayment. The Cumberland lineage includes populations confined to the mountains of northeastern Alabama and western North Carolina.

The Western clade is distributed from western Alabama to the coast of California and is divided further into two sub-clades. The Central sub-clade consists of two lineages, a Mississippi River Valley lineage ranging from southern Illinois south to central Arkansas and western Alabama and a Great Plains lineage extending throughout the prairies and grasslands of the central US from southern Minnesota to central Arkansas. Within the second sub-clade, a Western Louisiana lineage is distributed from west central Louisiana through eastern Texas and into southwestern Oklahoma. Further to the west, the North Texas lineage extends from east of the Guadalupe Mountains in New Mexico through north central Texas and into southern Kansas. Bound by the Guadalupe Mountains in the east and the Sierra Nevada Mountains in the West, the Great Basin lineage ranges from southern New Mexico north to southern Idaho. West of the Sierra Nevada Mountains three lineages were inferred ranging from southern California to central Washington. The Southern California lineage is composed entirely of populations south of the Transverse Mountains. North of the Transverse Mountains, the Coastal California lineage extends along the west coast of California into central Oregon. Haplotypes from this lineage were also present east of the Central Valley as far north as San Francisco Bay where they overlap with the southern range of the Eastern California lineage.

### 3.2. Divergence dating

Although the origin of *Diadophis* remains unknown due to the lack of sampling in Mexico, the initial divergence within the US occurred between the Gulf Coast clade and the continental clades during the late-Miocene (~6.7 mya Fig. 3; Table 1). The remaining major divisions (B–D; Fig. 2) and the divergences within the Gulf Coast clade and between the southwestern and California lineages occurred during the Pliocene. Each of the extant lineages shared a most recent common ancestor during the Pleistocene ranging from 0.238 to 0.985 mya, suggesting that the Laurentide ice sheet was not directly related to the formation of these lineages.

### 3.3. Population genetic analyses

#### 3.3.1. Diversity indices and population structure

Nucleotide diversity ($\pi$) across all *D. punctatus* haplotypes was 0.065 and the mean number of pairwise differences ($\kappa$) was 95.202. Fu's $F_s$ statistic was significantly negative for the entire species (−33.467) suggesting a recent range-wide expansion; however, Tajima's $D$ was not significant (−0.32475). The discrepancy between $D$ and $F$ is likely due to the decreased statistical power of $D$ in detecting significant changes in population size (Ramos-Onsins and Rozas, 2002). The molecular analysis of variance (AMOVA) suggests that genetic variation in the *D. punctatus* complex is apportioned non-randomly ($p < 0.01$) with 86% of the variance between lineages, 4% between populations within lineages, and only 10% within populations (Table 2).

#### 3.3.2. Historical demography and dating population growth

Tajima's $D$ and Fu's $F_s$ statistic were significant ($p = 0.05$) for the Northeast, Great Plains, Coastal California and Eastern California lineages. The Eastern Louisiana lineage was significant for Fu's $F_s$ and approached significance for Tajima's $D$ ($p = 0.057$). The remaining lineages, though not significant, were consistently negative for

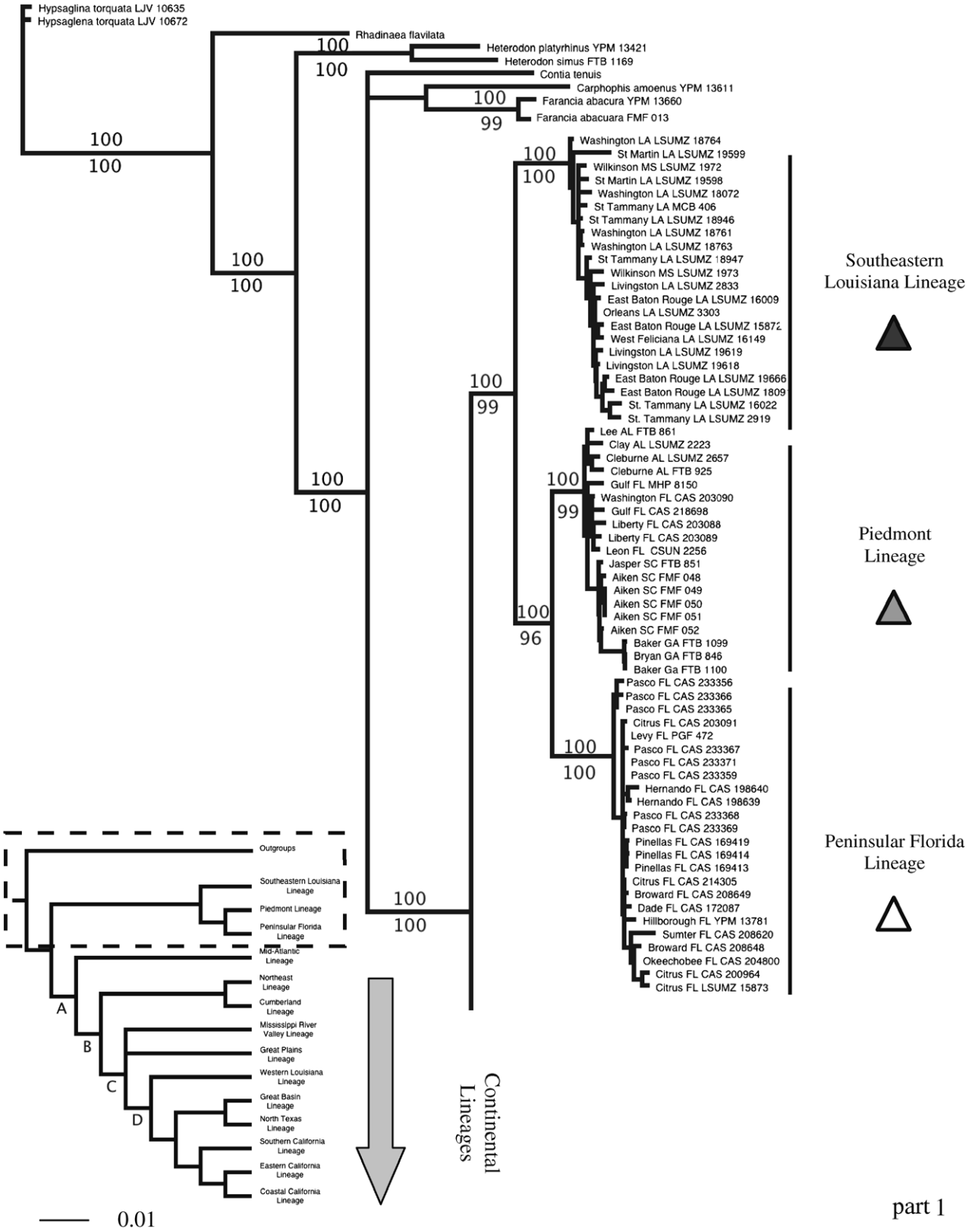


Fig. 2. Bayesian 50% majority-rule consensus tree for the 286 *Diadophis punctatus* samples and nine outgroup taxa. Posterior probabilities based on 7500 post burn-in trees are shown above the branches; nonparametric bootstrap proportions based on 1000 pseudo-replicates are listed below. Designations of the major population lineages follow the text. Symbols correspond to the geographic distribution of each lineage in Fig. 3. The dashed rectangle on the simplified tree depicts the position of the lineages in relation to entire phylogeny.

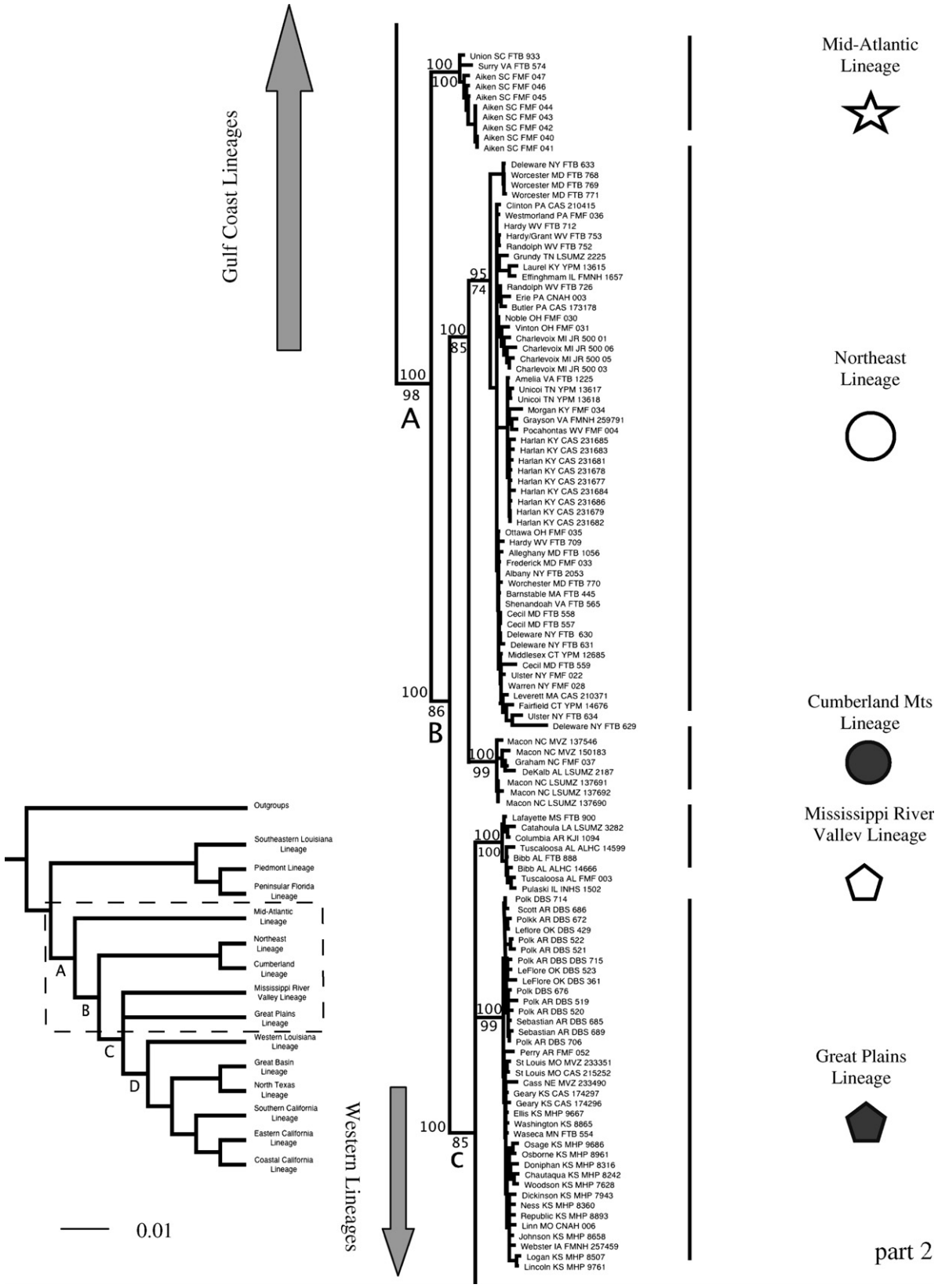


Fig. 2 (*continued*)

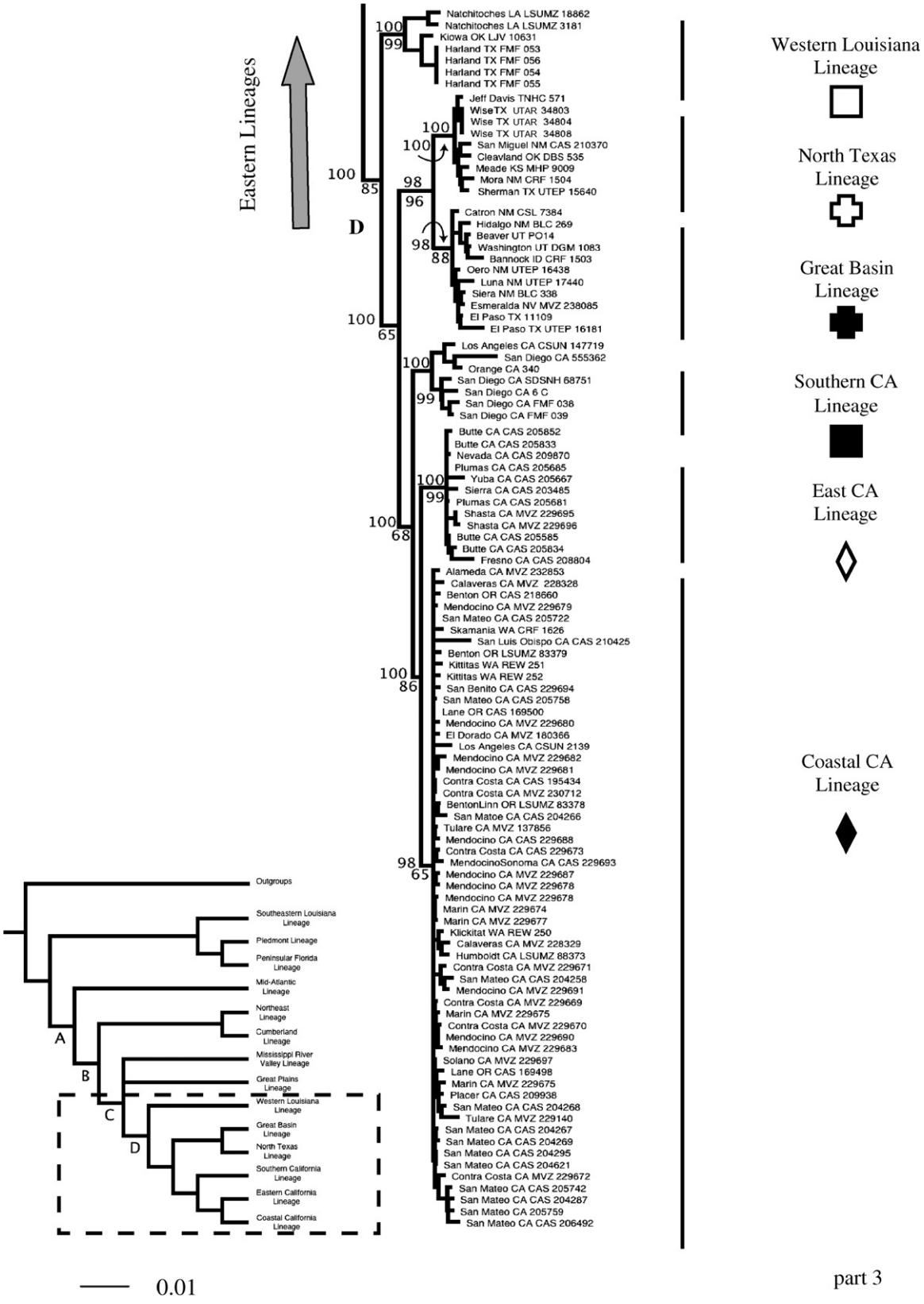


Fig. 2 (*continued*)

Tajima's *D* (Table 3) indicating an excess of low frequency haplotypes and putatively gradual population growth. The mismatch distributions of pairwise nucleotide differences were multimodal for most lineages with the exception of the Great Plains, Coastal California, Eastern California and Northeast lineages.

The effective sample size (ESS) for each of the Bayesian skyline analyses was greater than 200, suggesting that the 10 million generations were sufficient to determine the demographic history for each lineage (Fig. 4). None of the plots show any evidence of genetic bottlenecks, recent sub-divisions or historical population contractions. Furthermore, the Mid-Atlantic and West Louisiana lineages had slopes that were not significantly different than zero, suggesting that these populations have maintained a relatively stable size.

Unimodal distributions with low raggedness values, significantly negative $D$ and $F_s$ values and the Bayesian skyline plots depicting growth provide strong support that the Coastal California, Eastern California, Great Plains

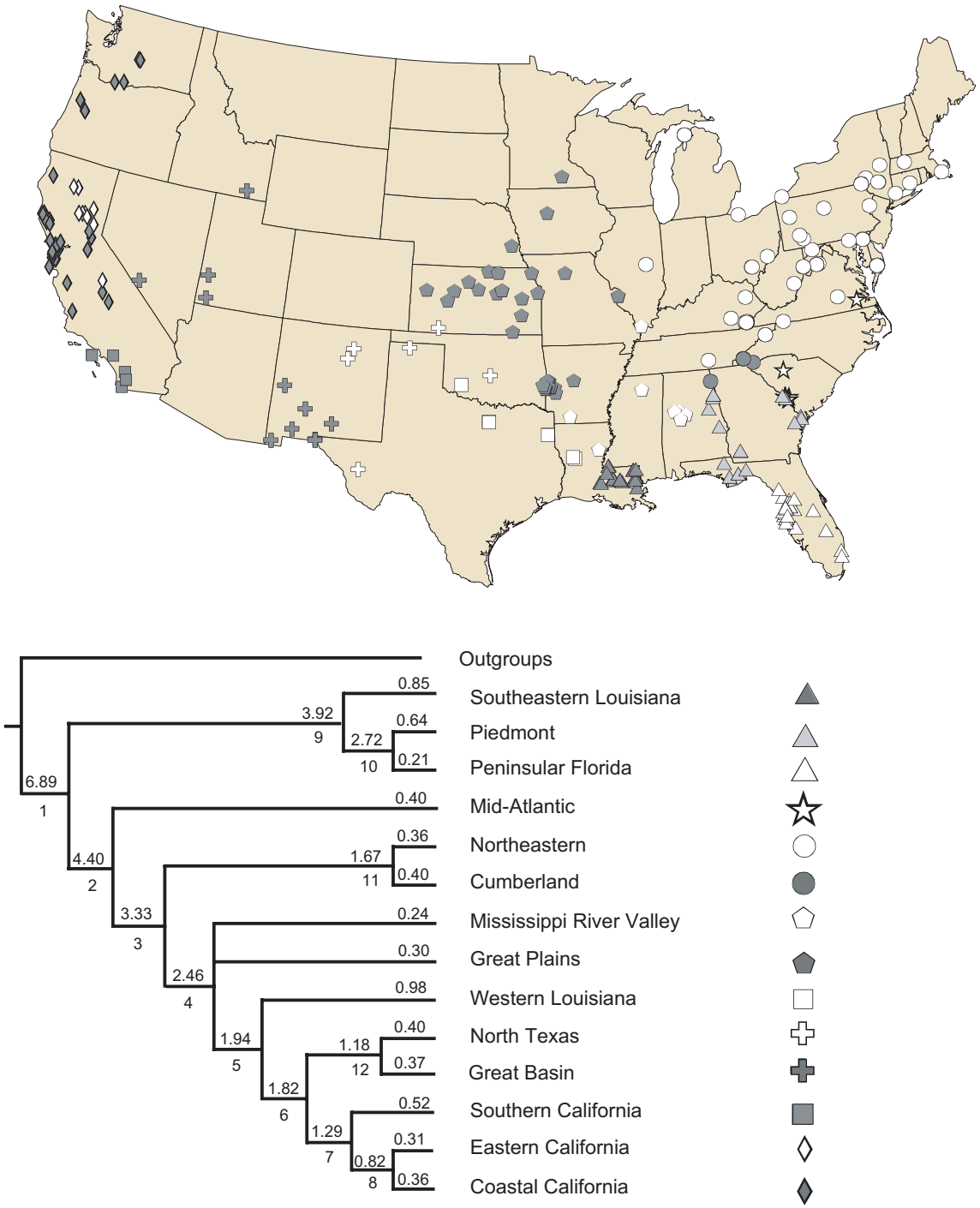


Fig. 3. Map showing the distribution of each *D. punctatus* population lineage diagnosed by mtDNA variation. Numbers above branches on the simplified tree are the mean dates of origin for each of the major lineages and haplogroups. Numbers below branches refer to Table 1.

Table 1
The mean date of origin for each lineage of *Diadophis punctatus* and the time to the most recent common ancestor (tmrca) of each haplogroup

| Node | Origin (Mya) | MtDNA lineage | tmrca of Haplogroups (Mya) |
|---|---|---|---|
| 1 | 6.689 (SD = 0.0207; 95% CI = 3.320–10.630) | SE LA | 0.845 (SD = 0.0074; 95% CI = 0.237–1.630) |
| 2 | 4.396 (SD = 0.0213; 95% CI = 1.840–7.388) | Piedmont | 0.638 (SD = 0.0056; 95% CI = 1.590–1.291) |
| 3 | 3.328 (SD = 0.0195; 95% CI = 1.353–5.638) | Pen FL | 0.211 (SD = 0.0018; 95% CI = 0.035–0.455) |
| 4 | 2.461 (SD = 0.0155; 95% CI = 0.998–4.172) | Mid-Atlantic | 0.402 (SD = 0.0039; 95% CI = 0.007–0.830) |
| 5 | 1.943 (SD = 0.0130; 95% CI = 0.769–3.297) | Northeast | 0.364 (SD = 0.0034; 95% CI = 0.081–0.768) |
| 6 | 1.829 (SD = 0.0121; 95% CI = 0.738–3.315) | Cumberland | 0.399 (SD = 0.0036; 95% CI = 0.083–0.834) |
| 7 | 1.288 (SD = 0.0148; 95% CI = 0.562–2.014) | MRV | 0.238 (SD = 0.0022; 95% CI = 0.038–0.515) |
| 8 | 0.819 (SD = 0.0056; 95% CI = 0.312–1.483) | Great Plains | 0.304 (SD = 0.0027; 95% CI = 0.054–0.657) |
| 9 | 3.920 (SD = 0.0225; 95% CI = 1.477–6.683) | Western LA | 0.984 (SD = 0.0081; 95% CI = 0.320–1.855) |
| 10 | 2.721 (SD = 0.0198; 95% CI = 0.953–4.88) | Great Basin | 0.377 (SD = 0.0030; 95% CI = 0.087–0.761) |
| 11 | 1.674 (SD = 0.0144; 95% CI = 0.511–3.178) | North Texas | 0.396 (SD = 0.0033; 95% CI = 0.079–0.801) |
| 12 | 1.178 (SD = 0.0081; 95% CI = 0.411–2.071) | South CA | 0.523 (SD = 0.0032; 95% CI = 0.122–0.940) |
| | | Eastern CA | 0.310 (SD = 0.0023; 95% CI = 0.075–0.611) |
| | | Coastal CA | 0.361 (SD = 0.0028; 95% CI = 0.084–0.706) |

Values in parentheses represent standard deviation (SD) and the 95% credible interval (CI) estimated using the uncorrelated lognormal Bayesian relaxed molecular clock in BEAST v. 1.4.4. Numbers for nodes follow Fig. 3.

Table 2
Results of the hierarchical analysis of molecular variance (AMOVA) testing the geographic structure for the 14 inferred lineages

| Source of variation | df | Sum of squares | Percentage of variation |
|---|---|---|---|
| Among groups | 13 | 14130.29 | 85.72 |
| Among populations within groups | 29 | 581.282 | 4.20 |
| Within Populations | 248 | 16285.32 | 10.07 |

Table 3
Hapotype diversity (Hd), nucleotide diversity ($\pi$), average number of pairwise differences (k) and results of Tajima's $D$ and Fu's $F_s$ statistic for each lineage of *Diadophis punctatus* calculated for all sites of the concatenated data set

| Lineage | Tajima's $D$ | Fu's $F_s$ | Hd | $\pi$ | $K$ | $r$ | $R_2$ | $\tau$ | Rate of substitution / million years | Expansion time |
|---|---|---|---|---|---|---|---|---|---|---|
| SE LA | −1.405 | −10.050 | 1.000 | 0.0099 | 16.810 | 0.0042 | 0.074 | 14.23 (4.60–39.44) | 6.58E−08 | 10,137 (3274–28,108) |
| Piedmont | −1.118 | −1.800 | 0.977 | 0.0126 | 19.430 | 0.0219 | 0.085 | 23.33 (8.36–37.76) | 6.51E−08 | 15,365 (6021–27,201) |
| Pen. FL | −1.447 | −2.736 | 0.972 | 0.0109 | 18.680 | 0.0124 | 0.072 | 8.32 (0–31.31) | 6.46E−08 | 6043 (0–22,753) |
| Mid-Atlantic | −0.292 | −1.612 | 0.911 | 0.0077 | 13.622 | 0.0810 | 0.151 | 16.61 (2.51–38.61) | 6.32E−08 | 12,326 (1,861–28,657) |
| Northeast | −2.073* | −15.760* | 0.989 | 0.0133 | 19.966 | 0.0036 | 0.054 | 22.04 (8.07–42.47) | 6.41E−08 | 16,145 (5911–31,104) |
| Cumberland | −0.873 | −1.280 | 1.000 | 0.0067 | 11.762 | 0.1180 | 0.113 | 12.36 (7.18–18.48) | 6.48E−08 | 8943 (5194–13,374) |
| MRV | −0.768 | −1.333 | 1.000 | 0.0098 | 15.536 | 0.0740 | 0.103 | 10.8 (4.50–20.41) | 6.95E−08 | 7293 (3032–13,783) |
| Great Plains | −1.690* | −21.996* | 0.986 | 0.0044 | 7.287 | 0.0054 | 0.056 | 9.79 (4.41–17.17) | 7.27E−08 | 6318 (2846–11,080) |
| West LA | 0.119 | 6.968 | 0.714 | 0.0181 | 31.238 | 0.2404 | 0.174 | 25.01 (4.33–64.40) | 7.86E−08 | 14,927 (2586–38,445) |
| North TX | −0.879 | 1.027 | 0.917 | 0.0084 | 14.306 | 0.1150 | 0.104 | 16.98 (11.80–22.08) | 7.87E−08 | 10,114 (7026–13,154) |
| Great Basin | −1.111 | −1.200 | 0.982 | 0.0090 | 15.164 | 0.0380 | 0.109 | 14.20 (6.48–25.08) | 7.83E−08 | 8,500 (3879–15,020) |
| South CA | −0.378 | −0.016 | 1.000 | 0.0170 | 27.857 | 0.0988 | 0.150 | 35.27 (21.11–51.73) | 7.74E−08 | 15,320 (6741–25,299) |
| East CA | −1.754* | −1.249* | 0.974 | 0.0078 | 13.205 | 0.0250 | 0.095 | 15.69 (1.22–38.15) | 7.66E−08 | 9609 (748–23,368) |
| Coastal CA | −2.410* | −25.750* | 0.940 | 0.0044 | 7.112 | 0.0095 | 0.039 | 11.86 (2.40–26.31) | 7.59E−08 | 7326 (1482–16,250) |

Tests that were significantly different at a $p = 0.05$ are labeled with an *. The raggedness statistic, $r_g$, and the Ramos–Orsins and Rozas $R_2$ statistic are reported for the mismatch distributions. Substitution rates/million years were obtained from r8's (Sanderson, 2003) using a calibration point of 7.5 my. The average tau ($\tau$) and expansion time are listed with the upper and lower bounds in parentheses.

and Northeast lineages have undergone sudden expansion. However, the lack of evidence for population declines and significantly positive slopes from skyline plot suggests that the other lineages have also undergone population growth, albeit very slowly. Therefore, for each lineage we estimated the years since expansion based on the estimate of $\tau$ from the mismatch distribution. Results suggest that expansion ages of the lineages range from 5 kya (Peninsular FL) to 16 kya (Northeast US) (Table 3), all of which follow the recession of the Laurentide ice sheets ~18 kya.

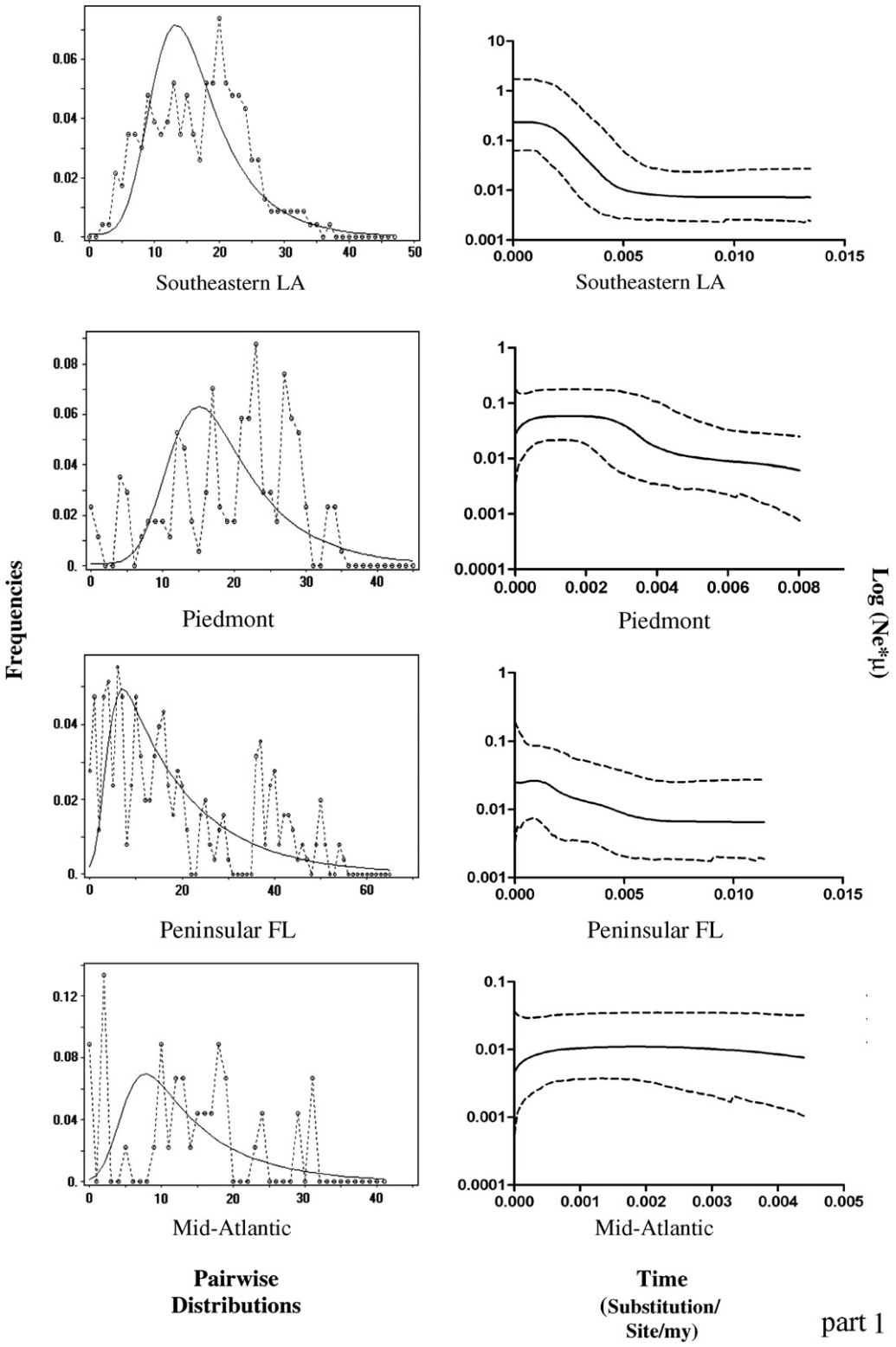


Fig. 4. Mismatch distributions (left) and Bayesian skyline plots (right) depicting the demographic history for each lineage of *D. punctatus*. For the mismatch distributions, open circles represent the observed distribution of pairwise differences and the solid line represents the expected distribution assuming population expansion. For the skyline plots, data were analyzed for 10 million generations in the program BEAST v1.4 (Drummond and Rambaut, 2006; Drummond et al., 2005) using the appropriate model of evolution determined from ModelTest 3.7 (Posada and Crandall, 1998). The solid line represents the median value for the log of the population size ($\log N_e$) and the dashed lines represent the upper and lower 95% credible intervals. Graphs labeled with an asterisk represent expanding populations.

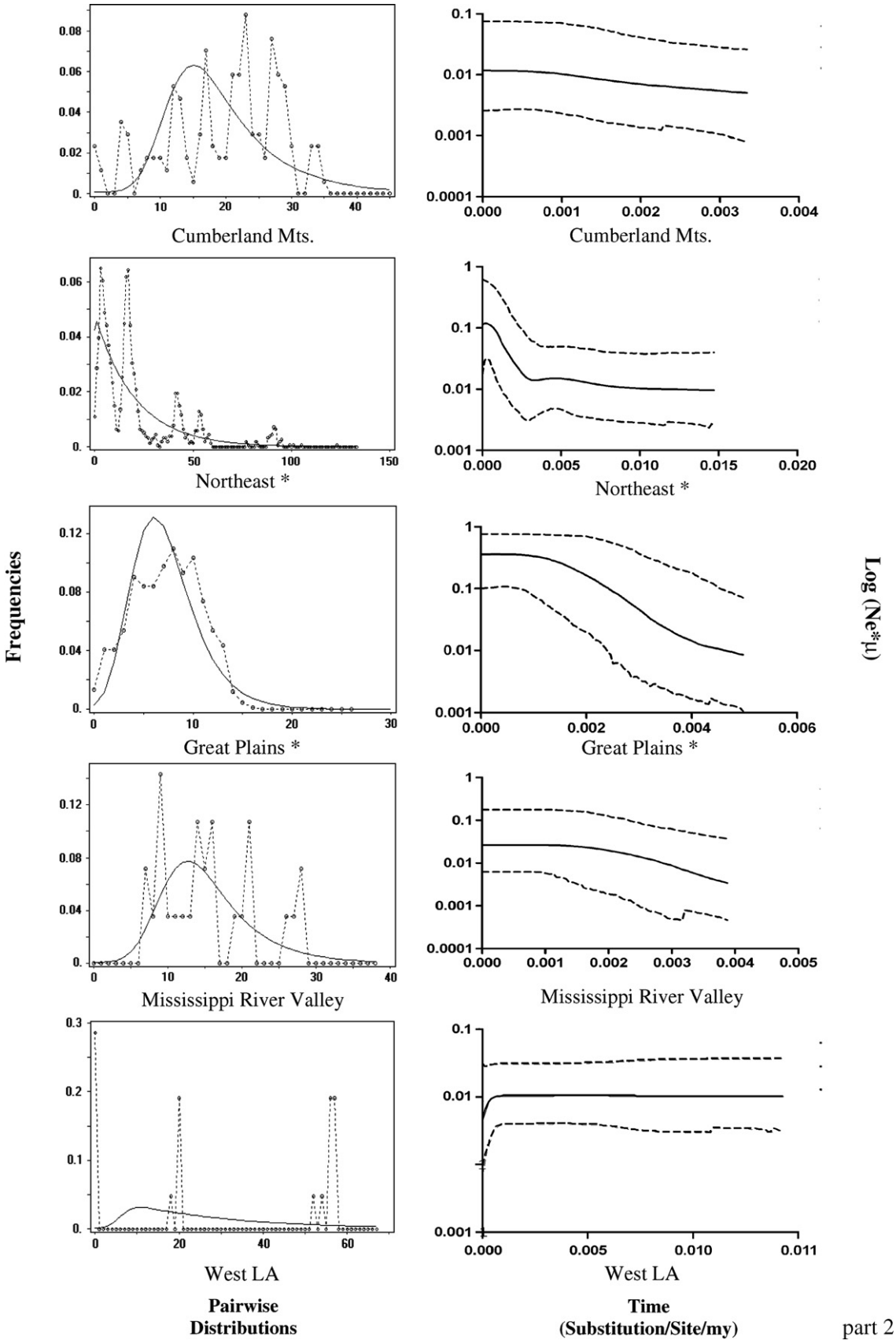


Fig. 4 (*continued*)

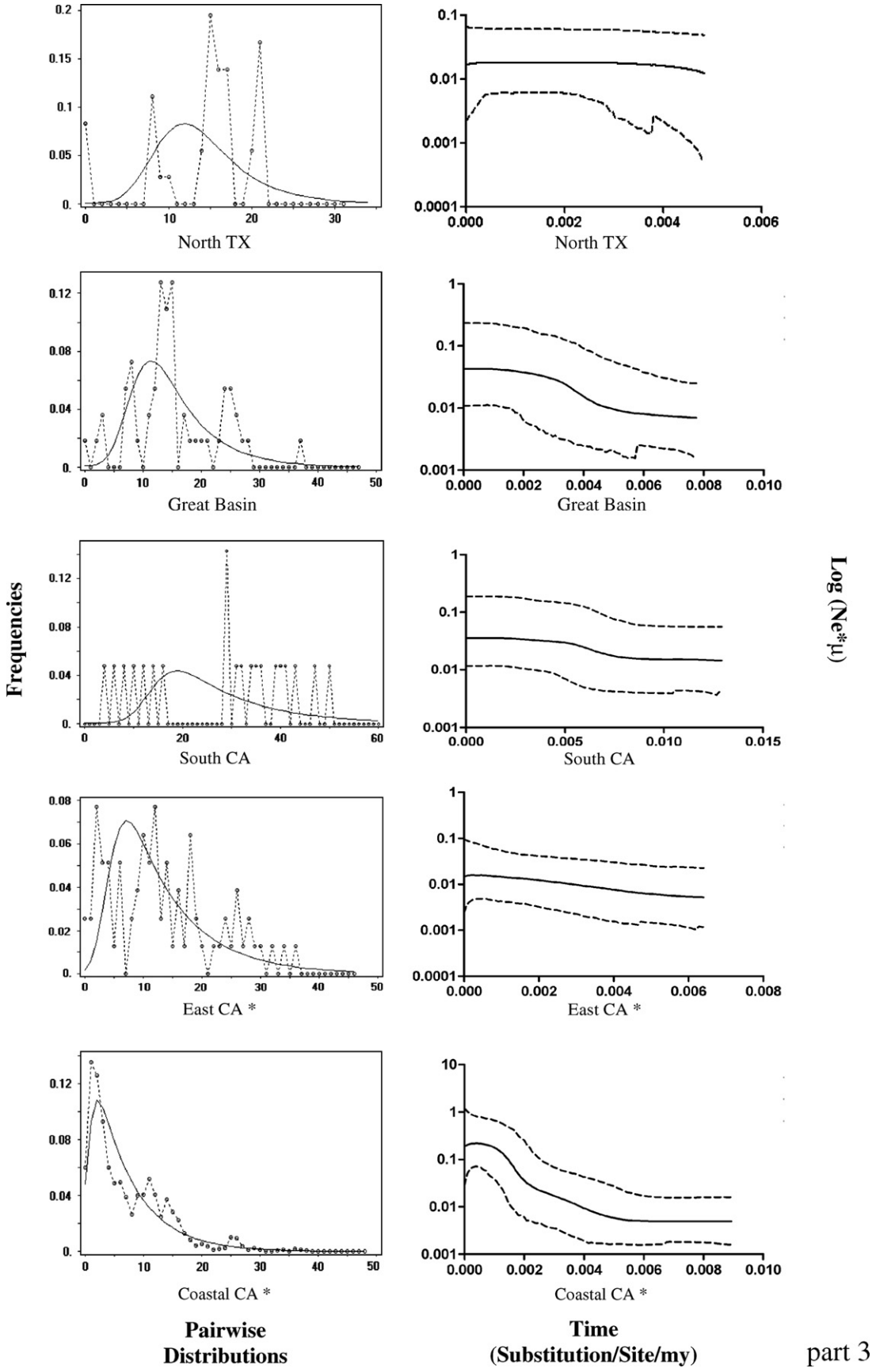


Fig. 4 (*continued*)

## 4. Discussion

### 4.1. Phylogeographic patterns across North America

Our analyses revealed four major clades composed of 14 geographical lineages within *D. punctatus* (Fig. 2). Within the US, the pattern of genetics breaks originate in the southeastern US and spread along the eastern coastal plain into the northeast, then east across the central US and into the west throughout California. The phylogeographic patterns inferred from west of the Mississippi River are broadly concordant with those of previous studies of North American taxa (Calsbeek et al., 2003; Pook et al., 2000; Castoe et al., 2007). However, the lineage diversity and phylogeographic patterns east of the Mississippi River are not concordant with most other vertebrates studied (Hayes and Harrison, 1992; Burbrink et al., 2000; Church et al., 2003).

The basal split within *D. punctatus* took placed during the late Miocene and subtends the Gulf Coast clade from the three continental clades, which originated during the Pliocene. Within the Gulf Coast clade, the Southeastern Louisiana lineage is limited to lowland areas of southern Louisiana and southwestern Mississippi, confined by the Red River to the north. During the Pleistocene, the mouth of the Mississippi River was shifted to the west towards the present day Atchafalaya River (Mayden, 1988). Glacial melt waters carried increased sediment loads causing an eastward shift of the river basin (Brown and Kennett, 1998). The occurrence of Southeastern Louisiana haplotypes west of the current Mississippi River could be a result of these recent shifts. The Piedmont lineage is confined to the southeastern coastal plain where the Southeastern Fall Line separates the eastern coastal plain from the Appalachian Mountains. Throughout the southeastern United States, the east–west genetic discontinuities observed in freshwater and terrestrial species have largely been explained by two topographic features, the Tombigbee River in eastern Alabama and the Apalachicola River that transects the panhandle of Florida (Avise, 2000; Burbrink et al., 2000; Near et al., 2001; Hoffman and Blouin, 2004; Soltis et al., 2006; Pauly et al., 2007). This lineage represents one of the few squamate distributions that does not have an east-west genetic break associated with the Apalachicola/Tombigbee Rivers, raising questions as to why and how this low-vagility, coastal-plain endemic has been able to disperse across barriers that have been important in shaping phylogeographic patterns of this region. A second factor thought to have influenced the genetic patterns of Florida taxa has been fluctuations in sea level during the late Miocene/early Pliocene. As Florida became inundated, the north-south central ridges persisted as a series of offshore islands separating populations from the mainland (Webb, 1990; Clark et al., 1999). Although further sampling is needed to determine the Northern extent of the Peninsular Florida lineage, several taxa are endemic to this region of Florida including squamates (Connant and Collins, 1991), birds (Peterson, 1980), mammals (Burt and Grossenheider, 1976), turtles (Walker and Avise, 1998), butterflies (Kimball, 1965) and plants (Elias, 1987).

The second major division (B) corresponds to a southern Appalachian Mountain discontinuity, separating the Mid-Atlantic clade from the Appalachian and Western clades. This lineage is restricted to mesic forests and floodplains of the Atlantic Coastal Plain from southeastern Virginia to western South Carolina. Similar east–west genetic breaks have been found in the unglaciated regions of the Appalachian Mountains for salamanders (Donovan et al., 2000; Zamudio and Savage, 2003; Church et al., 2003), turtles (Walker and Avise, 1998), Atlantic white cedar (Mylecraine et al., 2004) and the groundnut (Joly and Bruneau, 2004). In western South Carolina, a secondary contact zone between the Mid-Atlantic and Piedmont lineages is present along the Fall Line separating the upland Piedmont of the Appalachians from the lowland Coastal Plain.

North of the Coastal Plain, the Northeast lineage of the Appalachian clade spans the Appalachian Mountains and is confined to the east by the Mississippi River. A genetic discontinuity associated with the Mississippi River has been well documented both for plants and animals (Near et al., 2001; Al-Rabab'as and Williams, 2002). However, several animal species show additional sub-structuring associated with the Apalachicola and Tombigbee Rivers (Burbrink et al., 2000; Brant and Orti, 2003; Soltis et al., 2006). These rivers appear to have had little effect on the genetic structure of the Northeast lineage, which occupies mostly previously glaciated regions. Similar patterns observed in the leopard frog (*Rana pipiens*) are thought to be due to expansion into previously glaciated areas from Northern refugia (Hoffman and Blouin, 2004). At the southern extent of the Appalachian Mountains, the Cumberland lineage is confined to the montane environments of Western North Carolina and the Cumberland Plateau of northeastern Alabama. Elevational changes across the range of a species can influence dispersal patterns, causing genetic divergence between populations that have become adapted to different elevations (Slatkin, 1987; Manel et al., 2003; Palo et al., 2003). The preference for high-elevation habitat may be a factor in limiting the dispersal of these snakes into the lowland areas currently occupied by other lineages of *D. punctatus*. Although uncommon in North American squamates, genetic differentiation has been shown between low-altitude and high-altitude populations in the Pacific jumping mouse (Vignieri, 2005), the tiger salamander (Spear et al., 2005) and the long-toed salamander (Giordano et al., 2007).

Within the Western clade, the Central sub-clade consists of the Mississippi River Valley (MRV) and the Great Plains lineages. Although the relationship between these two groups remains unresolved, each lineage was well supported (100 PP and 95 BS) in both analyses. The distribution of the MRV lineage is restricted to low elevation (<200 m) regions associated with the Mississippi Alluvial

Plain (MAP). This flood plain extends through seven states (southern Illinois, southeastern Missouri, western Kentucky, eastern Arkansas, western Mississippi and Louisiana) and formed as a result of an influx of water into the Mississippi River due to the melting of the North American ice sheets (Perlmutter, 1985). Although outside what is normally considered the MAP, western Alabama consists of lowland areas (<200 m) associated with the western extent of the Gulf Coast coastal plain. The apparent association with flood plain and lowland forest habitat may be a factor inhibiting dispersal into the surrounding higher-elevation areas while expansion into southern Louisiana appears to be restricted by the Red River. For several species of plants and animals, the Mississippi River represents a major biogeographical barrier with distinct mtDNA haplotype groups occurring east and west of the river (Brown et al., 1996; Burbrink et al., 2000; Near et al., 2001; Berendzen et al., 2003; Brant and Orti, 2003). Our study indicates that *D. punctatus* may be the only squamate that does not correspond to the east–west genetic break typical of other taxa. The distribution spanning the Mississippi River below the Embayment of southern Illinois again raises questions as to how this low-vagility snake remains unimpeded by barriers that have shaped the phylogeographic patterns of other taxa within this region. North of the embayment, the influence of the Mississippi River becomes evident separating the Northeast and Great Plains lineages. West of the Mississippi River, the Great Plains lineage extends throughout the grasslands and prairies of the central US, from southern Minnesota to the Central Highlands of western Arkansas. These snakes occupy areas with elevations above 200 m, which may restrict dispersal into the lower-elevation eastern flood plain occupied by the MRV lineage.

Restricted in the east by the Mississippi River and the Red River to the north, the Western Louisiana lineage extends through the Piney Woods forests of western Louisiana, eastern Texas and southern Oklahoma. A preference for these high-density coniferous forests may be a factor limiting dispersal into the adjacent hardwood forests of the East Central Texas Plains. Throughout the North American Deserts, *D. punctatus* becomes less abundant occurring in several small disjunct populations (Fig. 1). Two lineages reciprocally monophyletic for mtDNA haplotypes were inferred within the southwest, corresponding to a Southern Rocky Mountain genetic discontinuity. East of the Guadalupe Mountains, the North Texas lineage occupies the semi-arid highland regions associated with the eastern Chihuahuan Desert and the Southern Plains of the southwestern and central US. Haplotypes from both the Western Louisiana and North Texas lineages occur in central Oklahoma, where the habitat shifts from the semi-arid steppe of the southwest to the grassland prairies of the central US. West of the Rocky Mountains, the Great Basin lineage is confined to the cooler, high elevations of the Sonoran and Mojave Deserts extending north throughout the Great Basin. The Colorado Plateau may be a genetic barrier isolating the Great Basin and North Texas lineages. Ringneck snakes are largely absent from the high elevation xeric scrublands typical of this region and are limited to small disjunct populations across the Great Basin and Mojave Deserts (Fig. 1). Their limited dispersal abilities combined with the unfavorable habitat and smaller population sizes have likely contributed to maintaining a stable or non-expanding demographic signal. The east–west split inferred across the North American Deserts is broadly concordant with the vicariant phylogeographic patterns inferred for several other desert species, including fence lizards (Leaché and Reeder, 2002), western rattlesnakes (Pook et al., 2000; Ashton and deQueiroz, 2001), diamondback rattlesnakes (Castoe et al., 2007), horned lizards (Reeder and Montanucci, 2001; Leaché and McGuire, 2006) and scaled quails (Zink and Blackwell, 1998). However, the distribution of the genetic break points is not shared across taxa, suggesting that the patterns represent different underlying causal factors. It would be of interest to expand upon the sampling within these studies and to evaluate the geographical structure and timing of molecular diversification across this region.

The phylogeographic patterns of *D. punctatus* in California and along the West Coast have been detailed elsewhere (Feldman and Spicer, 2007). However, our increased mitochondrial sampling has delimited a third haplo-group restricted to the Sierra Nevada and Cascade Ranges. This Eastern California lineage may come into contact with the Coastal California lineage at several points along the central Sierra Nevada and again in the Cascade Range. Whether the presence of Coastal haplotypes in the interior ranges of California represents dispersal or simply the retention of ancestral haplotypes that have otherwise gone extinct in eastern California is unknown. Dispersal events across the Central Valley or through the Transverse Ranges into the Sierra Nevada have been proposed for a number of species (Moritz et al., 1992; Tan and Wake, 1995; Rodriguez-Robles et al., 1999; Feldman and Spicer, 2002; Spinks and Shaffer, 2005). The apparent absence of a geographical barrier between the Coastal and Eastern California lineages provides an additional opportunity to test the genetic exclusivity of these minimally diverged mtDNA lineages.

### 4.2. Demographic history

During the peak of the last continental glaciation, approximately 18,000 years ago, the Laurentide Ice Sheet extended south to about 39°N in eastern North America (Delcourt and Delcourt, 1987). Glaciers and associated climatic changes drove high latitude populations into more southern habitats (Hewitt, 1996). The leading edge model of population expansion predicts that lineages at the glacial margins would have undergone rapid population expansion as previously unsuitable habitat became colonized. Such rapid or step-wise colonizations would be characterized by low levels of genetic diversity as each new founding

population represented only a fraction of the ancestral population's genetic diversity (Nichols and Hewitt, 1994; Hewitt, 2000).

#### 4.2.1. Eastern US

For the six lineages inferred east of the Mississippi River, only the Northeast had significantly negative values for Tajima's $D$ (−2.07) and Fu's $F_s$ (−15.76), unimodal mismatch distributions and Bayesian skyline plots consistent with rapid population expansion (Fig. 4). The average estimates of $\tau$ suggest that expansion began during late Pleistocene (~16 kya) coinciding with retreat of the ice sheets. Additionally, the distribution of haplotypes throughout shows extensive panmixia between Northern and Southern populations with little to no sub-structuring across great distances. These results, when combined with the high haplotype and low nucleotide diversity, are consistent with patterns of population expansion out of more southern refugia. Given the complex lineage diversity inferred throughout the southeast, populations of the Northeast lineage likely survived glacial cooling periods further north in the Appalachian Mountains or interior refugia near the edge of the glacial ice sheets. Several lines of evidence have emerged suggesting that re-colonization of glaciated landscapes in the eastern US may have occurred from refugia located further north than previously proposed (McLachlan et al., 2005).

In contrast to the Northeast lineage, demographic analyses for the three Gulf Coast lineages as well as the Mid-Atlantic, Cumberland and Mississippi River Valley lineages revealed multimodal mismatch distributions and non-significant $D$ and $F_s$ statistics (Fig. 4, Table 3). Bayesian skyline plots for each group also showed little to no growth over time, with the exception of the Southeastern Louisiana lineage (Fig. 4 part 1). Further sampling across southern Mississippi and western Alabama may help to clarify the demographic history of this lineage. Thus, the other eastern lineages do not appear to have undergone a significant population expansion or contraction but have remained relatively stable through time.

The maintenance of population stability across the complex ecosystems characterizing the southeastern US is likely due to a combination of extrinsic and intrinsic barriers to dispersal. Even in the absence of geological barriers, habitat requirements alone can limit the ability of individuals to disperse across inhospitable landscapes or into novel communities (Crespi et al., 2003). Furthermore, the precedence of established lineages, competitive interactions between lineages or the presence of narrow hybrid zones may restrict gene flow and limit dispersal (Hewitt, 1996). These factors should have had little effect on the expansion of temperate biota from long-term southern refugia into previously unsuitable unoccupied areas. As populations confined in Northern refugia expanded following retreat of the glaciers, competitive effects between neighboring southern lineages may have restricted dispersal, maintaining the genetic structure of each lineage (Hewitt, 1996). Inter-lineage interactions affecting population expansion have been proposed for the distribution of other Southeastern taxa with limited dispersal capabilities (Zamudio and Savage, 2003; Crespi et al., 2003; Kozak et al., 2006).

#### 4.2.2. Central US

Even though the Laurentide Ice Sheets did not extend as far south in the center of the continent as they did in the East, the effects on regional biota were similar (Brant and Orti, 2003). Genetic variation in the Great Plains lineage conforms to predictions of population expansion models with unimodal mismatch distributions, significantly negative values of $D$ (−1.690) and $F_s$ (−21.99), and Bayesian skyline plot depicting rapid growth. Although the average time since expansion was relatively recent (<7 kya), the high haplotype diversity and low nucleotide diversity are consistent with population expansion following colonization of previously glaciated regions. The relatively homogenous topography and climatic conditions of the Midwest region likely have contributed to a lack of local differentiation among Great Plains ringnecks as well as other species (Stein et al., 2000). Tree topologies with shallow branches, little internal resolution (Fig. 2 part 2) and low sequence divergence further suggest recent population expansion from refugial areas. Throughout the Midwest, glacial refugia have been proposed in Oklahoma, Northern Texas and the Interior Highlands, the latter of which includes the Ozark and Ouachita Uplands of Missouri and Arkansas (Wiley and Mayden, 1985; Mayden, 1988; Brant and Orti, 2003; Zamudio and Savage, 2003). The lack of Great Plains haplotypes within western Oklahoma and Northern Texas suggests that the Interior Highlands acted as a southern refugium for ringnecks during times of glacial expansion (Fig. 3).

The historical demographic patterns inferred between northern and southern lineages of the central US were similar to those in the eastern US. Neither the Western Louisiana nor the North Texas lineage showed signatures of population expansion in the demographic analyses, suggesting long-term population stability (Table 3 Fig. 4). These results are consistent with the prediction that Northern lineages nearest the receding glacial edge underwent exponential population growth, while ecosystem stability or competitive interactions between southern lineages restricted geographic expansion (Hewitt, 1996). Additional factors affecting expansion of the North Texas lineage may be the unsuitable habitat of the Rocky Mountains and Intermontane Plateaus to the north and the Guadalupe Mountains to the west.

#### 4.2.3. Western US

In the arid West, *Diadophis* appears restricted to patches of suitable mesic environments surrounded by less hospitable xeric habitats. Thus widespread movements or colonization events seem unlikely. Indeed, genetic variation in the Great Basin lineage conforms to predictions regarding long-term population stability (Table 3 Fig. 4). The specific

habitat requirements of these snakes may be inhibiting population expansion into the surrounding areas. Increased sampling and the incorporation of microsatellite loci would be appropriate for testing the localized effects of altitude, geography and habitat shifts on the genetic diversity and connectivity between fragmented southwestern populations.

With respect to the California clade, Feldman and Spicer (2007) have recently provided a detailed explanation of the evolutionary and demographic history of California woodland reptiles. Our analyses of the combined data set inferred an additional Northern California lineage confined to the Sierra Nevada Mountains (Fig. 3). The separate demographic analyses of the two Northern California lineages are consistent with the findings of Feldman and Spicer (2007), regarding Coastal and East California populations experiencing rapid population expansion during the Holocene approximately 7 (kya) and 10 (kya) respectively (Table 3 Fig. 4). This expansion would have been exacerbated by Holocene warming explaining a spread of suitable woodland habitat in the central and Northern regions of California (Van Devender and Spaulding, 1979; Smith et al., 2000). This warming trend simultaneously decreased the extent of suitable habitat south of the Transverse Mountains. The demographic results for the Southern California lineage (Table 3 Fig. 4) lend further support to the conclusions of Feldman and Spicer (2007) that the loss of suitable habitat throughout southern California had a stabilizing effect on the populations.

## 5. Conclusions

This study is the first comprehensive phylogeographic analysis of the trans-continental snake *Diadophis punctatus*. Separate and combined analyses of *cytochrome b* and *NADH* 4 data sets inferred 14 well-supported mtDNA lineages occupying specific habitats separated by putative physical, biotic or ecological barriers. Previous studies of such broad ranging species have revealed considerable cryptic diversity and a more complex evolutionary history than postulated by previous workers, however the distribution and diversity of lineages in *D. punctatus* is unlike any other North American squamate. Whereas the patterns inferred throughout the western US were broadly concordant with previously identified genetic barriers, several lineages in the eastern US exhibited distinct patterns not shared by any other species. We attribute these patterns to the presence of Pleistocene glaciers and the associated climatic shifts. With the exception of the fragmented populations throughout the southwest, coalescent and non-coalescent measures of demographic history suggest post-glacial expansion into previously unsuitable habitats for each of the four northernmost lineages. However, the estimates of time since expansion suggest that factors leading

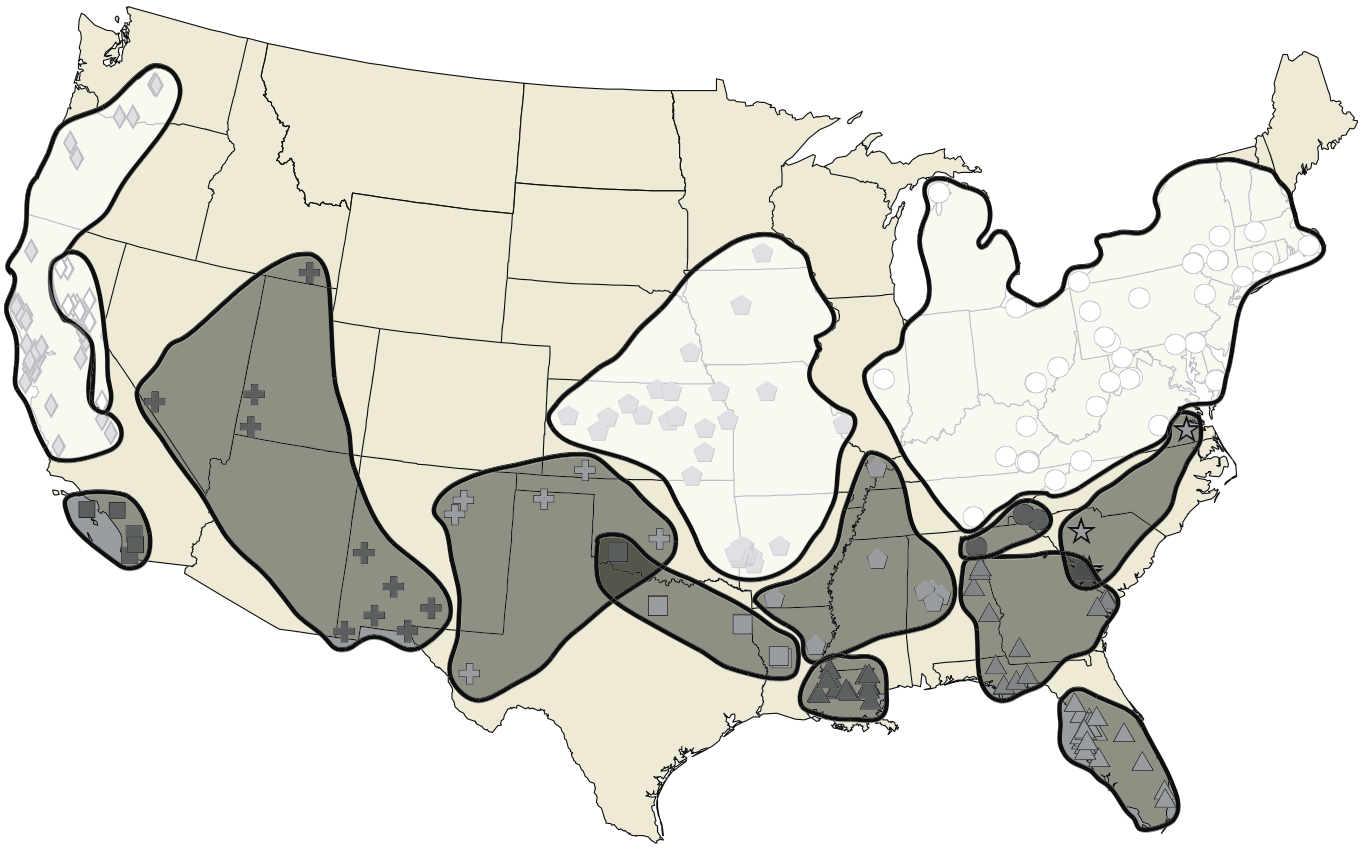

Fig. 5. Map showing the distribution of the lineages depicting rapid expansion of the northern most lineages (light gray) and long-term population stability of the southern lineages (dark gray). Dark lines represent the proposed geographic range for each group. Symbols correspond to Fig. 2.

to population growth differed between regions. In the Eastern and Central US, advancing Pleistocene glaciers drove Northern populations into southern refugia promoting intra-specific lineage diversity. As glaciers receded, lineages in relatively Northern refugia expanded into previously glaciated areas, while poor dispersal abilities and possibly increased inter-lineage competition restricted southern lineages resulting in the current phylogeographic patterns (Fig. 5). The general warming trends that led to the recession of glaciers caused fundamental ecosystem-wide changes across North America. In accordance with these changes, demographic patterns suggest that the Northern California lineages expanded as suitable habitat spread further north as temperatures increased during the Holocene.

An additional striking outcome of our study is the identification of three putative areas of secondary contact. Such zones are of particular interest because they can provide important information about the interaction between lineages, how populations merge or diverge, and even how adaptive or maladaptive variation can be transferred through introgressive hybridization (Wake, 1997). The contact zones within Oklahoma and South Carolina are especially intriguing because they appear to be associated with ecological transition zones. Secondary contact zones that are situated along ecotones provide an opportunity to test the Bounded Hybrid Superiority model (Moore, 1977). Under this model ecological requirements are viewed as the causal factors in determining variation in fitness among individuals, making hybrids that are more fit in transitional habitats than either of the parental lineages and less fit in the parental habitats (Anderson, 1949; Moore, 1977). Finer scale sampling incorporating nuclear loci may reveal details of the genetic properties (cline shape, width, fitness) within these putative contact zones.

Finally, it seems apparent from these results that the species level diversity is currently underestimated and that a full taxonomic review is warranted. However given the extensive lineage diversity and multiple secondary contact zones, a taxonomic revision should not be undertaken without further sampling, particularly throughout Mexico, and the addition of nuclear data to examine gene flow across the putative barriers.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.10.017.

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