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Range-wide phylogeography of a temperate lizard, the five-lined skink (*Eumeces fasciatus*)

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Abstract

We used mitochondrial DNA and microsatellite loci to examine the phylogeographic patterns of the most broadly distributed lizard in eastern North America, the five-lined skink (*Eumeces fasciatus*). We infer that longitudinal phylogeographic patterns in *E. fasciatus* are consistent with fragmentation due to refugial and post-glacial dynamics, but that deep divergences within the species imply historical fragmentation that predates the Pleistocene. The effect of multiple refugia is implied from our nested clade analyses, including a northern refugium in Wisconsin. Analysis of population structure using nuclear microsatellite data within the species suggests the importance of glacial dynamics in shaping more recent genetic structuring within one widely distributed lineage that ranges from the Mississippi River to the Atlantic Ocean in longitude and from southern Ontario to the Gulf of Mexico in latitude. Results shed light on the historical processes that have influenced current population structure of a temperate lizard, support the striking similarity of longitudinal phylogeographic structure across many herpetofaunal species in eastern North America, and illustrate the utility of employing multiple markers in phylogeographic studies.

Keywords: Phylogeography; mtDNA; Microsatellites; Pleistocene; Five-lined skink; *Eumeces fasciatus*

1. Introduction

The hypothesis that Pleistocene refugial dynamics have played a disproportionate role in generating current diversity in North America has proven controversial (Arbogast and Slowinski, 1998; Avise et al., 1998; Klicka and Zink, 1997; Zink and Slowinski, 1995). For example, dates of divergence of many species of birds appear to predate the latter half of the Pleistocene (Klicka and Zink, 1997), a period during which glacial expansion and recession was proposed to have fragmented the range of many ancestral species (Mengel, 1964). Recent evidence suggests that this debate over the importance of Pleistocene dynamics is somewhat misdirected because the majority of investigations of ice-age speciation have focused on taxa primarily distributed in areas that were not directly impacted by advancing glaciers (e.g., areas south of the boreal forest; Weir and Schluter, 2004). Moreover, phylogeographic studies of some North American species suggest deep divergences that significantly predate the late Pleistocene, and in some cases, the boundary between the Pleistocene and the Pliocene (e.g., spring peeper, *Pseudacris crucifer*—Austin et al., 2002; tiger salamander, *Ambystoma tigrinum tigrinum*—Church et al., 2003; yellow-spotted salamander, *Ambystoma maculatum*—Zamudio and Savage, 2003), implying here too that the Pleistocene glacial model is overly simplistic.

While phylogeographic patterns have proved to be variable across North American species, a sufficient number of studies have been done to derive some general statements regarding historical factors that may underlie major genetic disjunctions (see Swenson and Howard, 2005 for a review). Many amphibian and reptile species show striking longitudinal patterns in the distribution of major

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lineages (e.g., Austin et al., 2004; Burbrink et al., 2000; Walker et al., 1998) implying the action of similar long-standing barriers to dispersal (e.g., the Mississippi River and the Appalachians). Similarly, some studies have indeed suggested that refugial dynamics have played a significant role in generating phylogeographic structure (Hewitt, 1996). For example, the Appalachians have been proposed both as a barrier to gene flow and a glacial refugium for *A. maculatum* (Zamudio and Savage, 2003), the black ratsnake, *Elaphe obsoleta* (Burbrink et al., 2000), and *P. crucifer* (Austin et al., 2002; Austin et al., 2004). Other proposed areas of glacial refuge include the Central Highlands (including the Ozarks) (*P. crucifer*—Austin et al., 2004; highland fishes—Strange and Burr, 1997) and the Atlantic and Gulf coastal plains (bullfrog, *Rana catesbeiana*—Austin et al., 2004; northern short-tailed shrew, *Blarina brevicauda*—Brant and Ortí, 2003; *E. obsoleta*—Burbrink et al., 2000; *A. tigrinum tigrinum*—Church et al., 2003; *A. maculatum*—Zamudio and Savage, 2003). More surprisingly, recent work has suggested the existence of a northern refugium within present-day Wisconsin that impacted genealogical patterns in the eastern chipmunk, *Tamias striatus* (Rowe et al., 2004). This is further supported by the finding of high genetic differentiation of Wisconsin populations from neighboring northern populations in two species (Fassett's locoweed, *Oxytropis campestris* var. *chartacea*—Chung et al., 2004; northern prairie skink, *Eumeces septentrionalis*—Fuerst and Austin, 2004). If true, this challenges the notion that post-glacial expansion occurred entirely from southern refugia for many widespread, non-boreal species.

Researchers have primarily employed mitochondrial genes to investigate historical patterns of intraspecific phylogeography (Avise et al., 1987; Moore, 1995). Mitochondrial DNA continues to be the marker of choice in vertebrate phylogeographic research, but employing multiple markers with different modes of inheritance and rates of evolution can provide insight on the relative roles of historical versus contemporary factors in shaping range-wide population structure (e.g., Franck et al., 2001; Melnick and Hoelzer, 1992; Monsen and Blouin, 2003; Nyakaana and Arctander, 1999; Nyakaana et al., 2002). Moreover, given that several phylogeographic studies involving eastern North American herpetofauna have detected divergences that predate Pleistocene glaciation events, using a more rapidly mutating marker (e.g., microsatellites) may provide valuable insight into patterns of refugial dynamics and colonization patterns of a species (e.g., Hare, 2001). By employing molecular markers that reflect different temporal timescales, a more complete understanding of the impact of glacial dynamics on geographic genetic variation within a species.

We present a comprehensive range-wide genetic survey of an eastern North American lizard, the five-lined skink (*Eumeces fasciatus*, recently renamed *Plestiodon fasciatus*, Brandley et al., 2005), using both mitochondrial DNA sequence and nuclear DNA microsatellite data. *Eumeces fasciatus* is broadly distributed in eastern North America, from the Atlantic seaboard west to Texas in the south through Oklahoma, Kansas and Minnesota and extending into southern Ontario, Canada (Conant and Collins, 1998; see Fig. 1), thus providing us with an excellent opportunity to quantify and understand the processes that produce current geographical patterns in both previously glaciated and unglaciated areas. Its geographical range includes the locations of at least three previously identified glacial refugia and the aforementioned barriers to movement. Finally, this broadly distributed lizard species is sympatric with many other taxa that have been the focus of phylogeographic investigations, allowing us to comment on the congruence of phylogeographic patterns within eastern North America.

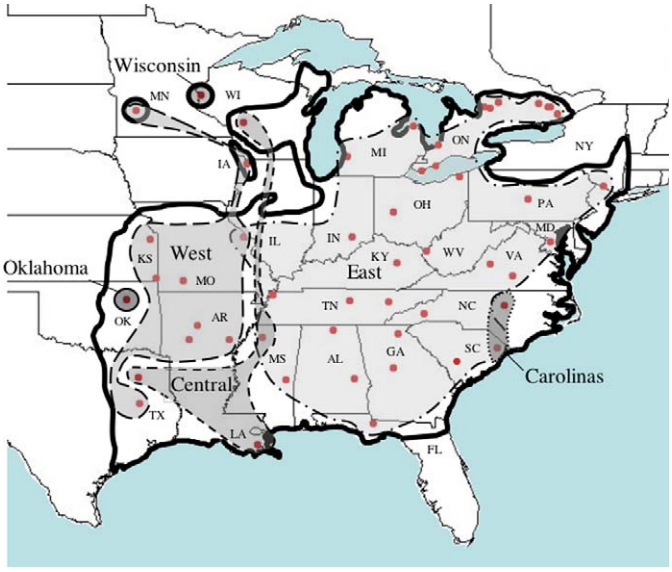

Fig. 1. Distribution and mitochondrial clade groupings of *Eumeces fasciatus* (range distribution based on Conant and Collins, 1998). States and provinces are indicated by abbreviations and sampling sites are marked with red circles. Species' range borders are marked with thick lines and include three disjunct series of populations (MN, WI, and IA). Clade groupings as determined by 769 bp of cytochrome *b* (mitochondrial DNA), nested clade analysis, and Bayesian phylogenetic analysis are indicated and include three main clades (East, Central, and West) and three geographically isolated clades (Carolinas, Oklahoma, and Wisconsin). Each major clade is outlined and shaded differently.

In the present study we test if genealogical patterns in mtDNA are spatially and temporally consistent with fragmentation due to refugial and post-glacial dynamics, and assess whether phylogeographic structure shows the geographically coincident longitudinal patterns evident in other herpetofaunal species implying common elements in their respective histories. We also contrast population structure based on mitochondrial and nuclear microsatellite markers to determine their utility in providing a more robust interpretation of post-glacial population history.

2. Materials and methods

2.1. Collection methods and sample preparation

Sampling throughout the species range (52 sites across southern Ontario and the eastern US) was conducted from

April to August during 2002, 2003, and 2004 (Fig. 1, Appendix I). Individuals were hand-captured, and the most distal 1 cm of tail tip was removed using a sterilized scalpel and stored in 95% ethanol. Tail ends were sprayed with antiseptic (Blu-Kote®), and individuals were released at site of capture. Total DNA was extracted using standard phenol–chloroform methods (Sambrook et al., 1989) or DNeasy Extraction kit (Qiagen) following manufacturer's instructions, and stored at −20 °C.

2.2. Mitochondrial DNA amplification and sequencing

Mitochondrial DNA (mtDNA) sequence data were obtained for 163 individuals of *E. fasciatus* sampled from all 52 sites (sample sizes ranging from one to five individuals per site; Fig. 1, Appendix 1), and for 10 individuals of *E. inexpectatus* and six individuals of *E. laticeps* (included as outgroup species because recent genetic work suggests they belong to the same species group (Richmond and Reeder, 2002), and are sympatric with the southern distribution of *E. fasciatus*, facilitating sample collection. An 1100 base pair (bp) segment of cytochrome *b* was amplified and sequenced for a subset of *E. fasciatus* individuals using CB1-5′ and CB6THR-3′ (Pearse and Pogson, 2000). Species-specific primers EUFAcytbF (5′-TATCGCGCAAGT AGCAACC-3′) and EUFAcytbR (5′-ACTGGACGAAA TGCGTTAGC-3′) were designed from these sequences to amplify a 769 bp segment of cytochrome *b* for all 163 *E. fasciatus* individuals,and 10 *E. inexpectatus* samples using polymerase chain reaction (PCR). Reaction cocktails were 50 μl in volume (approximately 20 ng of genomic DNA, 2.5 mM $MgCl_2$, 10 mM dNTPs, 0.3 μM of each primer, 10× Fermentas reaction buffer, and 0.5 U Fermentas *Taq* Polymerase), and were performed in a GeneAmp® PCR System 2700 (Applied Biosystems). Individuals of *E. laticeps* were amplified using CB1-5′ and CB6THR-3′ under similar conditions. PCR product was concentrated to approximately 20 μl using a Thermo Savant SPD SpeedVac and separated in a 0.5% agarose gel in 1× TBE buffer with ethidium bromide. Bands were visualized under UV light, excised from the gel, and cleaned using the Qiaquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions, resulting in a product that was approximately 20 ng/μl. Products were sequenced with EUFAcytbF using the ABI BigDye Terminator Kit (Applied Biosystems), on an ABIPRISM® 3100 automated sequencer (Mobix Lab, McMaster University, Hamilton). DNA sequences were edited and aligned using ClustalX (Thompson et al., 1997) and unique haplotypes were identified using MacClade 4.0 (Maddison and Maddison, 2000).

To ensure that sequences were authentic mitochondrial sequences, we purified genomic DNA for one sequenced individual from each of three distinct lineages (see Results). We isolated mtDNA using a CT Extractor Kit (Wako Chemicals), and serially diluted the extractions (1/10, 1/20, 1/50, and 1/100) until negligible nuclear amplification products (using a microsatellite locus, Eufa1 as described below) were observed when run on a 1% agarose gel (G. Ibarguchi, unpublished data). The concentration of DNA that showed negligible nuclear products was selected as the desired concentration for amplifying the mtDNA target gene as indicated above, and we compared these purified mtDNA reference sequences with those obtained using whole genomic DNA.

2.3. Phylogenetic analyses

Phylogenetic relationships among all haplotypes were estimated using the neighbor joining (NJ) criterion implemented by PAUP* 4.0 (Swofford, 2002) and Bayesian inference implemented by MRBAYES 3.1 (Huelsenbeck and Ronquist, 2001). NJ analysis was based on distances calculated based on a TIM + I + G model of evolution (see below), with *E. inexpectatus* and *E. laticeps* specified as outgroups. Support for the tree was assessed using nonparametric bootstrap analysis with 1000 replicates (Felsenstein, 1985). The best-fit model of evolution for our cytochrome *b* sequence data (TIM + I + G with Pinvar = 0.5385 and the Γ shape parameter = 1.0152) was selected using the Akaike Information Criterion (AIC) in MODELTEST, version 3.06 (Posada and Crandall, 1998). Two independent Bayesian analyses were run simultaneously with Metropolis-coupled MCMC using four incrementally heated Markov chains. For each analysis, we specified the TIM + I + G model of DNA evolution, with the parameter values estimated as part of the analysis. Beginning with random starting trees we ran the analyses for 2.2×10^6 generations at which point the standard deviation of the split frequencies was <0.01. Trees were sampled every 100 generations, with the first 550 of these discarded as burn-in. We confirmed that we had a sufficient sample from the posterior probability distribution in two ways. First, we examined the Potential Scale Reduction Factors (Gelman and Rubin, 1992) produced by MRBAYES for all parameters, and these were very close to one (to the second decimal), indicating that the runs had adequately converged. Second, we used TRACER (Rambaut and Drummond, 2003) to estimate effective sample size for all parameters and these were all >180, suggesting that we have effectively sampled from the posterior distribution of all parameters (Rambaut and Drummond, 2003).

We calculated pairwise, corrected sequence divergence among major clades identified by these phylogenetic analyses using the moment method of Nei and Li (1979).

2.4. Nested clade analysis

We used Nested Clade Analysis (NCA) to separate population structure from population history as a source of geographic genetic variation (Templeton, 1998; Templeton et al., 1992, 1995). A 95% statistical parsimony network was constructed with the program TCS version 1.18 (Clement et al., 2000). Network ambiguities (multiple loops) were resolved using predictions based on coalescent theory according to rules as outlined in Pfenninger and Posada (2002). Haplo-

types were nested into hierarchical clades using the nesting rules of Templeton and Sing (1993) and geographical and genetic associations of nested clades were tested for significance using the program GEODIS (Posada et al., 2000). The NCA inference key (Templeton and Posada, 2004) was applied to nested clades with significant associations to differentiate among equilibrium (isolation by distance) and historical, non-equilibrium patterns (range expansion and past fragmentation) (Templeton, 1998). We inferred ancestral haplotypes using the method of Castelloe and Templeton (1994) based on coalescent theory.

2.5. Microsatellite genotyping

Five of the six species-specific microsatellite primer pairs used in this study have been previously published (Eufa1, Eufa7, Eufa21, Eufa24, and Eufa27; Howes et al., 2004). The final primer pair of Eufa19F (5′-CCCTTGCTCA CCTGTTCATT-3′) and Eufa19R (5′-GCGAAGCAAAT AGCAGAAGG-3′) is composed of a $(GT)_n$ motif and was designed from the microsatellite-enriched genomic library described in Howes et al. (2004). Variation at each locus was assessed by PCR in 10 μl reactions containing approximately 10 ng of genomic DNA, TSG 10× Reaction (BioBasic), 3.0 mM $MgCl_2$, 0.1 mM of each nucleotide, 3 pmol IR-labeled M13-29 forward primer, 3 pmol reverse primer, 2.5 pmol of IR-labeled M13-29 primer, 4 μg bovine serum albumin, and 0.5 U of *Taq* DNA polymerase (Fermentas). PCR products were screened on a Li-cor DNA sequencer (IR^2 System), and alleles were scored using Gene ImagIR software with IRD 800 50–350 bp size standards. A total of 649 individuals from 30 populations ($n = 10$–28) were genotyped and used in the present study (Fig. 1, Appendix I).

2.6. Genetic structure using nuclear microsatellite data

We first tested for linkage disequilibrium between pairs of loci and departures from Hardy–Weinberg within each population and locus using a Markov Chain approximation of an exact test as implemented in GENEPOP web version 3.4 (Raymond and Rousset, 1995). We estimated the frequency of null alleles for each locus for each population using the program MICRO-CHECKER (Van Oosterhout et al., 2004).

Analysis of molecular variance (AMOVA) was used to examine partitioning of microsatellite genetic diversity within and among major populational lineages inferred from mitochondrial DNA variation identified by our phylogeographic analyses. The first AMOVA included all populations ($n = 30$) that had at least ten genotyped individuals for six identified lineages, and a second AMOVA included only the populations ($n = 27$) within the three major mtDNA lineages with the broadest geographic distribution. AMOVAs were hierarchical and used 1000 permutations as implemented in Arlequin 2.0 (Schneider et al., 2000) to test whether each of three levels of organization (within populations, and within and among lineages) explained a significant portion of overall microsatellite diversity.

2.7. Comparing patterns of population differentiation in mtDNA and microsatellites

To compare population-level relationships identified by mtDNA and microsatellite markers, we constructed neighbor-joining (NJ) dendrograms based on matrices of genetic distances between all pairs of populations for each class of marker. For each population pair, we calculated average Kimura 2 parameter distances between all cytochrome *b* haplotypes, weighted by the frequency of their occurrence in each population (Kimura, 1980) using the program MEGA (Kumar et al., 2004). A population mtDNA NJ dendrogram was then constructed using a matrix of pairwise distances in the program NEIGHBOR as implemented in PHYLIP version 3.6 (Felsenstein, 1993).

For microsatellites, we calculated Nei's standard genetic distance (Nei, 1979) between all pairs of populations using the program MICROSATELLITE ANALYZER version 4.0 (Dieringer and Schlötterer, 2002). A microsatellite NJ dendrogram was constructed in NEIGHBOR and PHYLIP version 3.6 (Felsenstein, 1993). Support for microsatellite Nei's distance dendrogram was based on 1000 replicate distance measures constructed in MICROSATELLITE ANALYZER version 4.0 by permuting genotypes among populations (Dieringer and Schlötterer, 2003). We used NEIGHBOR and CONSENSE (PHYLIP version 3.6; Felsenstein, 1993) to generate NJ dendrograms for all replicates and to calculate percentage support for the resulting consensus tree. Both the mitochondrial and microsatellite NJ dendrograms were viewed using TREEVIEW (Page, 1996). We also examined population structure based on microsatellites by performing Principal Components Analysis (PCA) based on Nei's standard genetic distance between all pairs of populations using the program GenAl Ex (version 6, Peakall and Smouse, 2005).

3. Results

3.1. Mitochondrial DNA phylogeography

Within the ingroup, a total of 82 haplotypes were identified in 163 sequenced individuals of *E. fasciatus* (GenBank Accession Nos. DQ241592–DQ241673). There were a total of 137 variable sites of which 91 were parsimony informative. Two lines of evidence suggested that we have authentic mtDNA sequence. First, the characteristics of the amplified sequences are consistent with a protein-coding gene. We found neither indels nor stop codons. We also observed a third position codon bias with 67.9% of all changes in the third position, 19.7% in the first codon position, and 12.4% in the second codon position. Second, sequences obtained using isolated mitochondrial template DNA and whole genomic template DNA were identical, suggesting that our results are based on authentic mitochondrial DNA and not pseudogenes that have been transported to the nucleus.

The NJ (not shown) and Bayesian method of tree construction resulted in highly concordant tree topologies

(Fig. 2), both supporting *E. fasciatus*, *E. inexpectatus*, and *E. laticeps* as monophyletic groups and indicating the presence of six main lineages in *E. fasciatus*: East, Central, West, Wisconsin, Oklahoma, and Carolinas. All major lineages were supported by >58% bootstrap support and >0.62 support from posterior probabilities (Fig. 2). Corrected sequence divergence within *E. fasciatus* ranged from 1.66% between the Oklahoma and West lineages and 3.79% between the Carolinas lineage and all other lineages (see Table 1).

Table 1
Pairwise point estimates of genetic divergences between all reciprocally monophyletic groups of *Eumeces fasciatus* based on 769 bp of cytochrome *b*

Monophyletic group comparison	Divergence (%)
Carolinas vs. all other clades	3.79
Wisconsin vs. East + Central + West + Oklahoma	3.72
East + Central vs. West + Oklahoma	2.37
East vs. Central	1.79
West vs. Oklahoma	1.66

3.2. Nested clade analysis

Together, Bayesian posterior support, NJ bootstrap analysis and the statistical parsimony network (95% parsimony limit of 12 steps) suggested six distinct lineages within *E. fasciatus*. (Figs. 2 and 3).

Only the three broadly distributed lineages (East, Central, and West, Fig. 1) had significant geographic genetic structuring based on the permutation analysis. The East lineage showed evidence of contiguous range expansion and fragmentation or isolation by distance in the north (East 1-1, East 2-1), and restricted gene flow or long-distance dispersal across the

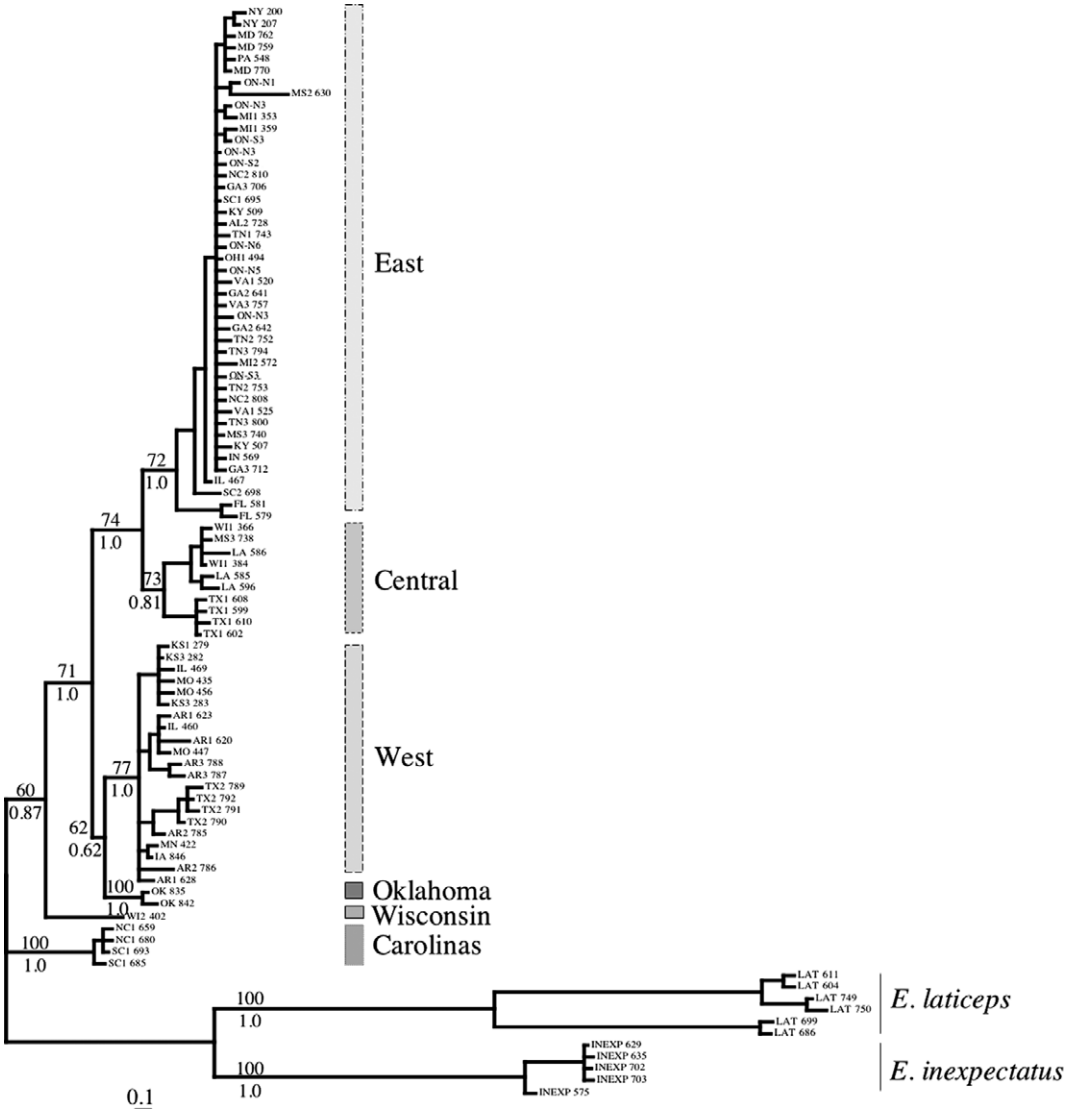

Fig. 2. Bayesian 50% majority rule consensus tree for *Eumeces fasciatus* based on 769 bp of cytochrome *b* from 163 individuals sampled from 52 sites across the species' range and rooted with *E. inexpectatus* and *E. laticeps*. Numbers at each node supporting a major clade indicate bootstrap support (above) and Bayesian posterior probabilities (below). Major clades are indicated and correspond with Fig. 1.

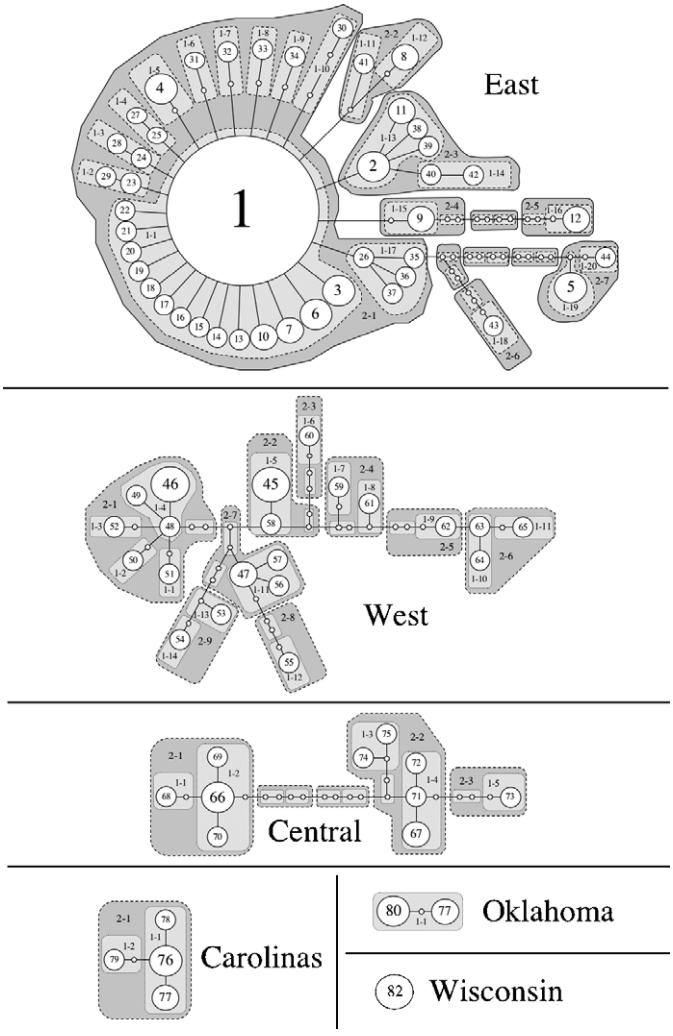

Fig. 3. One- and two-step 95% haplotype networks for 163 individuals of *Eumeces fasciatus* based on 769 bp of cytochrome *b*. Lines separate networks that are separated from each other by more than 12 mutational steps (East, Central, West, Carolinas, Oklahoma, and Wisconsin).

Table 2
Chain of inference for nested clade analysis of *Eumeces fasciatus* based on Templeton (1998)

Clade	Chain of inference	Inferred historic event
East 1-1	1-2-11-12-N	Contiguous range expansion
East 2-1	1-2-3-5-15-16-18-Y	Fragmentation/isolation by distance
East 3-1	1-2-3-5-6-7-Y	Restricted gene flow/dispersal but with some long distance dispersal
East	1-2-3-5-6-7-Y	Restricted gene flow/dispersal but with some long distance dispersal
West 3-2	1-2-11-12-N	Contiguous range expansion
West	1-2-3-5-6-13-21-N	Long-distance movement/gradual movement during a past range expansion and fragmentation
Central	1-19-20-2-3-4-9-N	Allopatric fragmentation

Only clades for which a statistically significant and conclusive geographical pattern was found are listed.

entire clade (see Table 2 for NCA inferences). The Central lineage included populations in northeast Texas, southeast Louisiana, and northwest Mississippi, but also included a population in central Wisconsin separated by sampling locales where we found East and West haplotypes only. NCA results for this lineage implied allopatric fragmentation stemming from the large geographic span between the Wisconsin and southern sampling locales. NCA results for the West lineage implied the action of contiguous range expansion and long-distance movement or gradual movement during a past range-expansion and fragmentation across the entire geographic range of the Central haplotype clade. Secondary contact between lineages was suggested in two populations; Site MS-N in Mississippi contains both East and Central haplotypes and IL (Illinois) consisted of both East and West haplotypes (cf. Fig. 4).

3.3. Genetic structure using nuclear microsatellite data

After sequential Bonferroni correction (Rice, 1989), only 2 of 615 pairwise tests for linkage disequilibrium were significant, suggesting that loci used in this study evolved

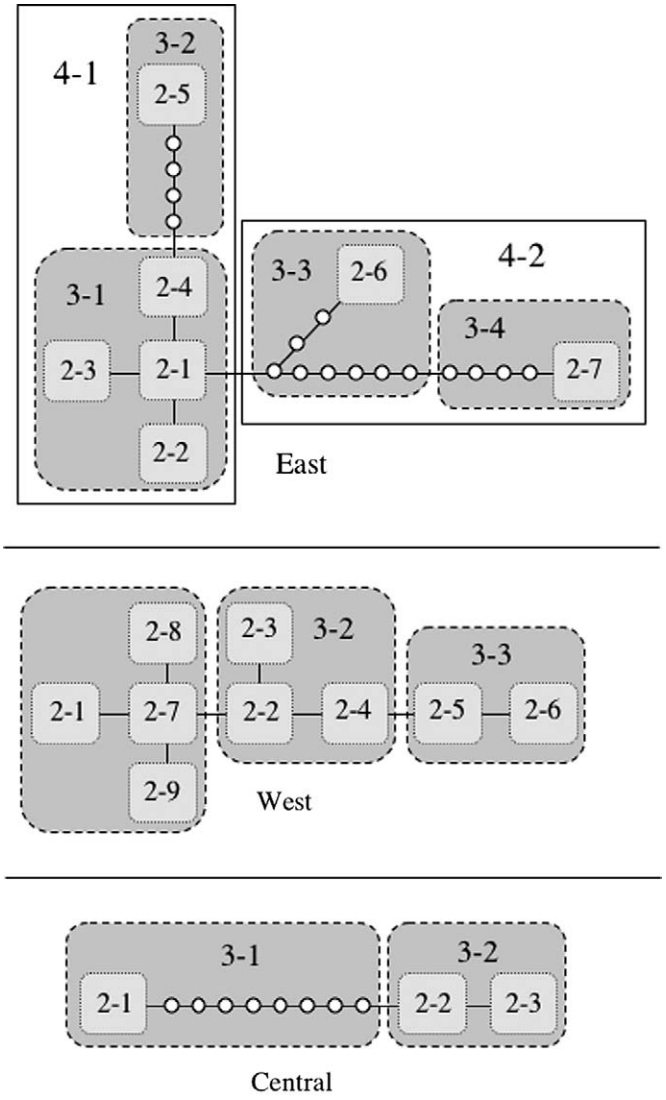

Fig. 4. Two-, three- and four-step 95% haplotype networks for the East, Central, and West networks.

independently. Nineteen of 228 tests for Hardy–Weinberg departures were statistically significant following Bonferroni correction: 9 for Eufa27, 4 for Eufa1, 4 for Eufa 21, and 1 for Eufa 19. Seventeen of 228 tests for heterozygote deficiencies were statistically significant after Bonferroni correction: 8 for Eufa 27, 3 for Eufa 1, 3 for Eufa 7, and 3 for Eufa 21. Null alleles were detected at "moderate frequencies" ($0.2 < p < 0.4$) in a small number of populations at each locus (ranging from 2.6% of populations in Eufa24 to 12.8% of populations in Eufa27). The presence of null alleles was mostly concordant with departures from Hardy–Weinberg and heterozygote deficiencies. Eufa27 showed the highest number of departures from Hardy–Weinberg and significant heterozygote deficiencies, along with the highest number of populations having a significant presence of null alleles. However, the mean population frequency of null alleles was still uncommon to rare for all loci ($p < 0.2$) (Eufa1 $p = 0.05$, Eufa7 $p = 0.04$, Eufa 19 $p = 0.06$, Eufa21 $p = 0.05$, Eufa24 $p = 0.03$, and Eufa27 $p = 0.09$) and unlikely to introduce serious bias in our analyses (Dakin and Avise, 2004).

Results of both AMOVAs indicated that a small but significant ($p < 0.0001$) proportion of the total variation in the microsatellite data were attributable to differences among clades (8.52% for the AMOVA with six clades, 7.26% for the AMOVA based on the three large mtDNA clades). A significant ($p < 0.0001$) proportion of variation also occurred among populations within clades (16.63% for the AMOVA with all clades and 16.69% for the AMOVA with three clades) and within populations (74.86% for the AMOVA with all clades and 76.05% for the AMOVA with three clades).

3.4. Comparing patterns of population differentiation in mtDNA and microsatellites

The mtDNA NJ dendrogram based on the matrix of average pairwise Kimura 2 parameter distances was very similar to that resolved in the phylogenetic analyses; clusters of populations that corresponded to the six main clades of the mtDNA haplotype phylogeny were identified (Fig. 5). The microsatellite NJ and mtDNA NJ dendrograms were discordant in that the main clusters of the latter were not coincident with those of the mtDNA NJ or Bayesian phylogenies. Rather, the microsatellite dendrogram contained three main clusters of populations. The first cluster was composed of southern populations from the East mitochondrial clade and populations belonging to the Central and Carolinas clades. The second cluster consisted of populations belonging to the West, Oklahoma, and Wisconsin clades. The final cluster was composed of northern populations of the East clade (Fig. 5). Our PCA also showed three discrete clusters corresponding to the same grouping as in our microsatellite dendogram (results not shown).

4. Discussion

Our results provide specific insight into the history of *E. fasciatus* and can also be embedded within a larger comparative phylogeographic framework to reveal the temporal and spatial biogeographical influences that have shaped the biodiversity of eastern North America. Similar to other eastern North American herpetofauna, phylogenetic groups within *E. fasciatus* are structured longitudinally and may have diverged from each other prior to the Pleistocene

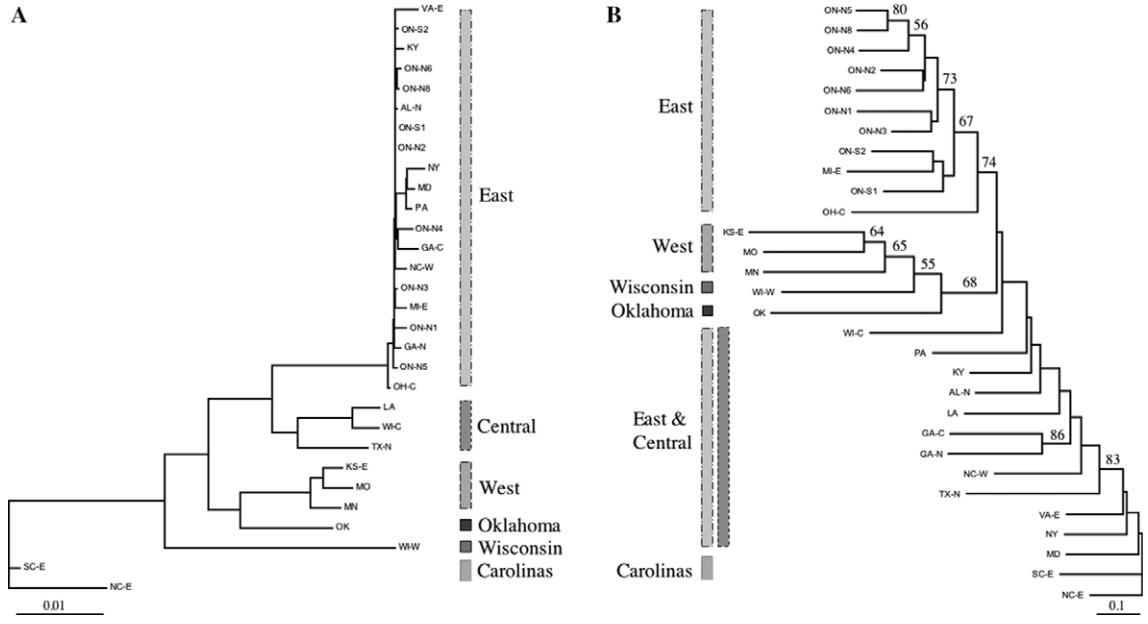

Fig. 5. Neighbor-joining tree describing different population-level patterns based on 769 bp of cytochrome *b* in the mitochondrial DNA (A) and neighbor-joining dendogram based on six microsatellite loci (B). The mitochondrial DNA tree is based on Kimura-2 distance among populations, and the microstallite dendogram is based on Nei's distance among populations with bootstrap values from 1000 replicates. Bootstrap support (>50%) for the microsatellite dendogram is shown, and the recovery of the major phylogeographic clades is indicated for the mtDNA tree and microsatellite dendogram.

based on the depth of divergence among major mtDNA lineages. We find support for the influence of at least one biogeographic barrier and several areas of glacial refugia including a northern refugium in Wisconsin. Our mitochondrial phylogenies and NCA show strongly concordant phylogeographic patterns, revealing three main lineages and three geographically restricted lineages. Population structure derived by analysis of our microsatellite data detected differentiation between populations lying within previously glaciated and unglaciated regions of a lineage. We elaborate on these findings below.

4.1. Genealogical history and biogeographic barriers

Similar to other eastern North American herpetofauna, *E. fasciatus* is structured in a manner that reflects divergence from east to west (longitudinal phylogeographic structure) (e.g., Burbrink et al., 2000; Zamudio and Savage, 2003). The species has three broadly distributed lineages (East, Central, and West), and three geographically restricted clades (Oklahoma, Wisconsin, and Carolinas). If the parsimony limit of the statistical parsimony network is reduced to 90% (17 steps), the Central clade is subsumed in the larger East clade, and the Oklahoma clade is included in the larger West clade mirroring the topology of our NJ and Bayesian trees (Fig. 2).

Populations of *E. fasciatus* appear to have been influenced by at least one biogeographic barrier to gene flow. The bulk of the broad distributions of the East and West lineages lie east and west of the Mississippi River, respectively, with only a single population containing haplotypes from both, suggesting its importance as a historical barrier to gene flow within *E. fasciatus*. This conclusion is consistent with other studies of eastern North American species (e.g., Austin et al., 2002; Brant and Ortí, 2003; Hoffman and Blouin, 2004; Templeton et al., 1995). The Mississippi River was a primary outlet for glacial meltwater throughout the Pleistocene (e.g., Schumm and Brakenridge, 1987). Since the late Pleistocene, it has undergone considerable change including reduction in width, as a result of many factors, most importantly the loss of meltwater flow from the Wisconsinan glacier (Royall et al., 1991; Smith, 1996). By the end of the Pleistocene approximately 8000 years before present, the Mississippi River had evolved from a broad, braided outwash channel to a narrower, meandering channel (Smith, 1996) that would have allowed migration between populations within the ranges of previously isolated lineages. The Appalachian Mountains do not appear to be an important barrier to gene exchange among populations of *E. fasciatus* because haplotypes belonging to the East lineage are distributed on both sides of the mountain range throughout most of its geographic span.

4.2. Refugial dynamics of E. fasciatus

The existence of several longitudinally distributed lineages within *E. fasciatus* coupled with evidence of range expansion from our NCA supports the hypothesis of multiple glacial refugia in eastern North America. We attempted to identify ancestral haplotypes within each of the major lineages (East, Central, and West) based on assumptions of coalescence theory described in Castelloe and Templeton (1994). The widespread and common haplotype of the East lineage makes it difficult to discriminate among a variety of potential refugia including southern Appalachian, Atlantic coastal plain, or Gulf coastal plain (east of the Mississippi). The Central lineage is composed of a geographically disjunct northern population and three southern populations that contain ancestral haplotypes, suggesting a Gulf coastal plain refugium west of the Mississippi. Based on our TCS analyses, haplotypes from the northern Texas population had the largest outgroup probability in this lineage, further supporting this claim. The geographic distribution of the West lineage and its more ancestral haplotypes in eastern Kansas, southern Illinois, and southern Missouri suggest a refugium in the Central Highlands west of the Mississippi River. The presence of the Oklahoma lineage may suggest another interior refugium west of the Mississippi River, although given that this lineage is so geographically restricted and that it is included in the West lineage when the statistical parsimony limit is reduced to 90%, its presence may also reflect historical range contraction and fragmentation in the West. The geographically restricted and strongly supported Carolinas lineage implies an Atlantic coastal plain refugium. This is congruent with other studies that have shown that the Coastal Plain is an important glacial refugial area from which other taxa have expanded into more interior habitats and subsequently diversified (Austin et al., 2002; Austin et al., 2004; Church et al., 2003; Zamudio and Savage, 2003).

The Wisconsin lineage is of special interest because its northern, disjunct location overlaps with an unglaciated region located in a gap of the Laurentide Ice Sheet during the last glacial maximum. It is plausible that this disjunct Wisconsin population was colonized prior to the last glacial advance, and that it persisted in a northern refugium throughout the most recent glacial advance and recession where it has remained isolated. Evidence suggests that this "driftless region" in Wisconsin remained unglaciated (Holliday et al., 2002) and may have even harbored deciduous forests during the last glacial maximum (Jackson et al., 2000). Recent support for such an ice-free refuge comes from Rowe et al. (2004), who suggested that populations of the eastern chipmunk (*T. striatus*) have expanded southward from a northern refugium located in Wisconsin. Other studies have also found intriguing genetic patterns within Wisconsin populations that may be explained by an isolated northern glacial refuge. For instance, a Wisconsin population of *E. septentrionalis* located in the unglaciated region contains a relatively high amount of haplotypic diversity and is genetically distinct from other northern populations (Fuerst and Austin, 2004). A perennial variety endemic to the unglaciated region of Wisconsin (*O. campestris* var. *chartacea*) contains relatively high levels of intrapopulation genetic diversity and low among population divergence, suggesting its persistence in the unglaciated

region during the most recent glacial advance and retreat as a relict population (Chung et al., 2004).

4.3. Timing of divergence

Although the application of a molecular clock remains controversial (e.g., Hillis et al., 1996; Klicka and Zink, 1997), it can be a valuable heuristic tool (Avise, 1994). Divergence rates of cytochrome *b* between lineages for a variety of small-bodied lizards generally range from 1 to 2% (e.g., Creer et al., 2001; Malhorta and Thorpe, 2000; Thorpe and Stenson, 2003; Thorpe et al., 2005). Using this range, we calculated a lower and upper estimate of divergence times for lineages within *E. fasciatus*. The most recent divergence in *E. fasciatus* (between the Oklahoma and West lineages) occurred between ca. 830,000 and 1.7 million years ago (mya), while the deepest divergence (between the Carolinas lineage and all other lineages) occurred ca. 1.9 and 3.8 mya. These divergence estimates predate the most recent Pleistocene glacial maxima, and may indeed predate the Pleistocene. Other eastern North American herpetofauna have showed similar or greater intraspecific divergences in cytochrome *b* ranging from 2.76% for *R. catesbeiana* (Austin et al., 2004), 6.62% for *P. crucifer*, and up to 16.9% for *E. obsoleta* (Burbrink et al., 2000). These findings cannot refute the potential importance of the Pleistocene in shaping contemporary patterns of biodiversity, and we note that such divergence dates should remain in geographical context of the distribution of *E. fasciatus* (i.e., it is not a boreal species that would have been maximally impacted by glacial cycles; see Weir and Schluter, 2004). However, our analyses clearly support the notion that lineages of many northern temperate amphibian and reptile species originated prior to the late Pleistocene, and that historical range fragmentation, whatever the cause, is a common element in the history of these species (Austin et al., 2004; Church et al., 2003; Zamudio and Savage, 2003). Thus, present-day geographic distributions of mitochondrial lineages in *E. fasciatus* imply that glacial refugia were used throughout periods of glacial retreat and advance in the Pleistocene, and indeed may have been used throughout glacial cycles that occurred prior to the Pleistocene (see Kozak et al., 2006).

4.4. Comparisons of patterns derived from mtDNA and microsatellite markers

Molecular markers with distinct modes of inheritance and rates of evolution can provide different insights into the factors that have shaped the structure and distribution of populations within a species. Here, we have employed both mitochondrial DNA sequence and nuclear microsatellites. Mitochondrial DNA is typically the marker of choice for phylogeographic studies (Avise, 1994). The mitochondrial genome is haploid, and is almost exclusively inherited maternally, meaning that recombination is extremely rare (Moritz et al., 1987). It is suggested to evolve 5–10 times faster than nuclear DNA: with average sequence divergence from 0.5% per million years in *Drosophila* and sea urchins to 2% per million years in primates and ungulates (Brown et al., 1979; Harrison, 1989). In contrast, microsatellites are inherited biparentally, and generally have highly variable mutation rates ranging from 10^{-6} to 10^{-2} per generation (Jarne and Lagoda, 1996; Schlötterer, 2000). This marker reflects the action of more contemporary processes with the possibility that historical signatures of separation will have been overwritten because of allele size constraints and homoplasy in repeat numbers that define alleles (Garza et al., 1995; Goldstein and Pollock, 1997; Nauta and Weissing, 1996). Because analyses using mitochondrial and nuclear markers convey genetic patterns at different temporal scales, their combined use may provide insight into the relative importance of historical and contemporary forces influencing the genetic relationships among populations of a species (e.g., Lemaire et al., 2005; Monsen and Blouin, 2003; Phillips et al., 2004).

Our mitochondrial NJ dendrogram based on average pairwise Kimura 2 parameter distances was largely concordant with results from our Bayesian analysis and NCA. Our microsatellite NJ dendrogram and PCA analysis resolved three genetic groupings; the first cluster was composed of the Central lineage, the Carolinas lineage, and southern populations of the East lineage. The second cluster was composed of the West, Oklahoma, and Wisconsin lineages, while northern populations of the East lineage form the final cluster. The fact that the microsatellite NJ dendogram does not mirror the mitochondrial phylogenies nor the mitochondrial NJ dendrogram is not unexpected and is likely due to the elevated mutation rate of microsatellites and homoplasy (e.g., Garza et al., 1995; Nauta and Weissing, 1996; Paetkau et al., 1997), and possibly contemporary mixing of populations and lineages.

We employed microsatellites to gain insight into more recent historical events that may have influenced the structuring of populations in *E. fasciatus*. In this light, our microsatellite analyses support the importance of glacial dynamics in the genetic structuring of populations within the most broadly distributed East lineage. Populations of the East lineage that were most directly impacted by glaciation form their own cluster in the microsatellite NJ dendrogram. We interpret this pattern as the signature of sequential founder events that likely occurred throughout northward post-glacial colonization. This finding emphasizes that present-day population structure was shaped both by deep historical events such as range fragmentation that may have occurred millions of years before present, but also by more recent processes such as the re-colonization of previously glaciated regions that occurred within the last 10–20,000 years before present.

5. Conclusions

Phylogeographic patterns evident in *E. fasciatus* are generally concordant with those based on mtDNA from other eastern North American herpetofauna. Our mitochondrial analyses show that the species is structured from east to

west and that genealogical patterns are spatially congruent with fragmentation due to refugial isolation and post-glacial colonization, while estimates of divergence dates among some mtDNA lineages predate the most recent Pleistocene glacial maximum and perhaps even the Pleistocene. Mitochondrial analyses suggest that the Mississippi River has acted as an historical barrier to gene flow for populations of *E. fasciatus*. A genetic signal of northern expansion, based on patterns of differentiation for our six microsatellite loci, is evident in the most broadly distributed East mitochondrial lineage. Our results highlight the potential utility of employing both mitochondrial and microsatellite markers in phylogeographic studies; this combined approach emphasized the importance of glacial dynamics in the genetic structuring of northern populations of *E. fasciatus*.

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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.2006.03.008.

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