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ORIGINAL PAPER

Pleistocene climatic fluctuations explain the disjunct distribution and complex phylogeographic structure of the Southern Red-backed Salamander, *Plethodon serratus*

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Abstract The southeastern United States (U.S.) has experienced dynamic climatic changes over the past several million years that have impacted species distributions. In many cases, contiguous ranges were fragmented and a lack of gene flow between allopatric populations led to genetic divergence and speciation. The Southern Red-backed Salamander, *Plethodon serratus,* inhabits four widely disjunct regions of the southeastern U.S.: the southern Appalachian Mountains, the Ozark Plateau, the Ouachita Mountains, and the Southern Tertiary Uplands of central Louisiana. We integrated phylogenetic analysis of mitochondrial DNA sequences (1399 base pairs) with ecological niche modeling to test the hypothesis that climate fluctuations during the Pleistocene drove the isolation and divergence of disjunct populations of *P. serratus*. Appalachian, Ozark, and Louisiana populations each formed well-supported clades in our phylogeny. Ouachita Mountain populations sorted into two geographically distinct clades; one Ouachita clade was sister to the Louisiana clade whereas the other Ouachita clade grouped with the Appalachian and Ozark clades but relationships were unresolved. *Plethodon serratus* diverged from its sister taxon, *P. sherando*, ~5.4 million years ago (Ma), and lineage diversification within *P. serratus* occurred ~1.9–0.6 Ma (Pleistocene). Ecological niche models showed that the four geographic isolates of *P. serratus* are currently separated by unsuitable habitat, but the species was likely more continuously distributed during the colder climates of the Pleistocene. Our results support the hypothesis that climate-induced environmental changes during the Pleistocene played a dominant role in driving isolation and divergence of disjunct populations of *P. serratus*.

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Introduction

The geographic distribution of a species is determined by interactions among multiple biotic, abiotic, and historical factors (MacArthur 1984; Brown et al. 1996; Peterson et al. 2011). Biotic factors include interspecific ecological interactions such as competition, predation, and parasitism, whereas historical factors are related to dispersal opportunities and range dynamics of the species and its ancestors over evolutionary time (Chase and Leibold 2003; Gaston 2003; Morrone 2009). Abiotic factors include physical barriers to dispersal such as rivers and mountain ranges as well as climatic variables such as temperature, precipitation, and seasonality (Brown et al. 1996; Gaston 2003; Peterson et al. 2011). Many abiotic and biotic factors vary spatially and temporally, especially climate, which has changed considerably over the past 65 million years (Myr), greatly impacting the distributions and evolution of species (Webb and Bartlein 1992; Zachos et al. 2001; Jansson and Dynesius 2002).

The biological effects of climate fluctuations associated with Pleistocene glacial cycles [~2.58 million years ago (Ma)–11.7 thousand years ago (ka)] are well documented and are often evident at the level of populations and closely related species because of their recency (Bennett 1990; Hewitt 2000, 2004). These effects are pronounced in species in areas formerly covered by glaciers and recolonized after glacial retreat, but species in many unglaciated areas were also affected (Hewitt 1996, 2000, 2004). For example, the southeastern United States (U.S.) has experienced dynamic environmental changes over the past several million years that impacted species distributions, population connectivity, and speciation (Watts 1980; Soltis et al. 2006). In some cases, climatic changes facilitated range expansion and dispersal to new regions whereas in other cases range contraction and fragmentation resulted (Hewitt 1996; Soltis et al. 2006). In fact, the cyclical nature of Pleistocene climatic fluctuations means that many species experienced alternating episodes of range expansion and contraction (Bennett 1990; Hewitt 2004). In species whose ranges were fragmented, a lack of gene flow between allopatric populations sometimes led to genetic divergence and speciation (Hewitt 1996). Many responses to climatic fluctuations in southeastern U.S. species are related to the expansion and contraction of deciduous forest (Watts 1980; Davis 1983; Jackson et al. 2000). In addition, this region has physical barriers to dispersal such as the Mississippi and Apalachicola rivers as well as montane regions such as the Appalachian Mountains and Interior Highlands (Ozark Plateau and Ouachita Mountains), further complicating phylogeographic history of species in the region (Swenson and Howard 2005; Soltis et al. 2006; Rissler and Smith 2010).

One of the most prominent biotic components of southeastern U.S. forests is plethodontid salamanders, which often dominate vertebrate biomass (Petranka and Murray 2001; Crawford and Peterman 2013; Semlitsch et al. 2014). Salamanders of the family Plethodontidae are the largest and most diverse group of salamanders with >450 currently recognized species worldwide (Frost 2015). The genus *Plethodon* includes 55 recognized species, many of which can only be distinguished using molecular techniques due to conserved morphology (Highton 1995; Wake 2009; Frost 2015). These terrestrial salamanders require mesic environments for survival and reproduction so their distributions are strongly influenced by moisture and temperature (Spotila 1972; Feder 1983). Many closely related and similar-looking species of *Plethodon* are allopatric or parapatric, and many have restricted distributions (Highton 1995; Kozak and Wiens 2006). Pleistocene climatic fluctuations have been implicated in fragmenting distributions and driving lineage diversification in several species (Highton 1995; Weisrock and Larson 2006; Shepard and Burbrink 2008, 2009).

Among species of *Plethodon*, the Southern Red-backed Salamander, *Plethodon serratus* Grobman 1944, has one of the most intriguing distributions, consisting of four widely disjunct regions: (1) the southern Appalachian Mountains of western North Carolina, southeastern Tennessee, northwestern Georgia, and northeastern Alabama, (2) the Ozark Plateau of southeastern Missouri, (3) the Ouachita Mountains of southeastern Oklahoma and west-central Arkansas, and (4) the Southern Tertiary Uplands of central Louisiana (Fig. 1; Highton and Webster 1976; Petranka 1998). This small-bodied (<10 cm total length) woodland salamander was recognized as a subspecies of the widespread *P. cinereus* until 1976, when it was elevated to species based on allozyme data (Highton and Webster 1976). In addition to showing high genetic differentiation between *P. serratus* and *P. cinereus*, allele frequencies indicated "modest" differentiation between geographic isolates of *P. serratus* (Nei's $D = 0.01$–0.19; Highton and Webster 1976). Genus-level studies including samples of *P. serratus* from Oklahoma and Georgia also documented substantial mitochondrial DNA (mtDNA) divergence within the species (Sites et al. 2004).

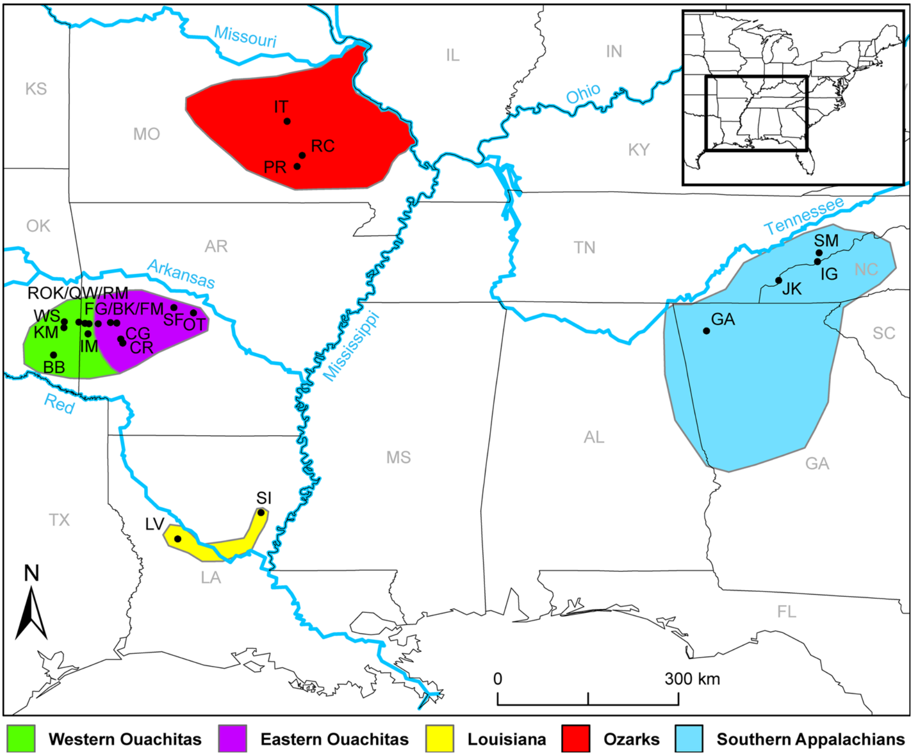

Fig. 1 Approximate distribution of *Plethodon serratus* showing sampling localities for this study. Areas are colored by phylogeographic lineage and major rivers are labeled. (Color figure online)

The disjunct distribution and documented genetic variation among geographic isolates suggests that Pleistocene climatic fluctuations may have impacted the distribution and lineage diversification of *P. serratus*.

Here we test the hypothesis that the disjunct distribution of *P. serratus* is the result of fragmentation driven by climatic changes during the Pleistocene. To evaluate this, we use mtDNA sequences from multiple populations within each of the four geographic isolates to test if populations within each isolate are genetically differentiated and form monophyletic groups, which would be indicative of long-term isolation. We also use divergence-dating methods to estimate the times that populations from the four isolates became isolated. We then use ecological niche modeling to predict the distribution of *P. serratus* to test if the availability of suitable environmental conditions limits the species' distribution and if the four isolates are separated by unsuitable areas. Lastly, we project the inferred ecological niche of *P. serratus* onto climate layers for the Last Glacial Maximum (LGM; ~21 ka) to test if the species was likely more widely distributed in the past with suitable environmental conditions connecting the four current isolates.

Materials and methods

Sampling and DNA sequencing

Between September 2010 and April 2012, we collected tissue samples of *P. serratus* Grobman 1944 throughout the species' distribution, including multiple locations within each of the four geographic isolates. We also obtained tissue samples of *P. serratus* through loans from the Louisiana Museum of Natural History at Louisiana State University (LSUMZ) and Bell Museum of Natural History at the University of Minnesota (JFBM). In total, we obtained 43 samples of *P. serratus*: 25 from the Ouachita Mountains, five from the Ozark Plateau, seven from the southern Appalachian Mountains, and six from central Louisiana (see Appendix S1 in Supporting Information for specific locality data). Specimens and tissues from our fieldwork were deposited at LSUMZ (Appendix S1).

We extracted genomic DNA from tissues using commercial DNA extraction kits (Qiagen DNeasy; Viogene Blood & Tissue Miniprep) and used polymerase chain reaction (PCR) to amplify portions of the mitochondrial cytochrome *b* (*cytb*) and NADH dehydrogenase subunit 4 (*ND4*) genes. For *cytb*, we used the primers Pcytb419F and PThrR1 from Wiens et al. (2006) and we used the primers ND4(F) and Ephist from Arévalo et al. (1994) and Wiens et al. (2006) for *ND4*. Each PCR contained 10 µl ddH$_2$0, 6 µl GoTaq® Green Master Mix (Promega), 2.0 µl DNA template (10 ng/µl), and 1 µl of each primer (10 µM). For both loci, PCRs consisted of an initial denaturing stage of 94 °C for 2 min, followed by 34 cycles of 94 °C for 1 min, 55 °C for 45 s, and 72 °C for 2 min, with a final extension of 72 °C for 5 min. We verified PCR products by gel electrophoresis and cleaned PCR reactions using an exonuclease/shrimp alkaline phosphatase protocol (Werle et al. 1994). We prepared sequencing reactions using 2 µl of purified PCR product and 5 pmol of either the same forward or reverse primer used in PCR; PCR products were sequenced in both directions. Sequencing reactions and automated sequencing were performed by the University of Missouri DNA Core Facility using an ABI3730xl 96-capillary sequencer. We edited chromatograms by eye to verify base calls and assembled contigs from forward and reverse sequences using Sequencher v.5.0 (Gene Codes Corp.). We aligned sequences for each gene using the MUSCLE algorithm in MEGA v.5 (Tamura et al. 2011) and visually inspected alignments to verify an open reading frame for these protein-coding genes.

In total, we sequenced 1399 base pairs (bp) of mtDNA for 43 *P. serratus*. The *cytb* alignment comprised 698 bp of which 112 sites were variable and 96 were parsimony informative within *P. serratus*. The *ND4* alignment was 701 bp with 106 variable sites of which 91 were parsimony informative. We deposited sequences generated in this study on GenBank (see Appendix S1 in Supporting Information for GenBank accession numbers). We obtained *cytb* and *ND4* sequences for *P. cinereus* (NC_006343) from GenBank to use as an outgroup (Mueller et al. 2004; Kozak et al. 2009) and concatenated alignments for phylogenetic inference.

Phylogenetic and divergence dating analyses

We assessed phylogeographic relationships and estimated divergence times within *P. serratus* using BEAST v.1.8.0 (Drummond and Rambaut 2007), which employs a Bayesian approach to simultaneously infer topology and divergence times, thus accounting for phylogenetic uncertainty. To do this, we first estimated the phylogeny and divergence times of Plethodontidae using the taxa and sequences from Kozak et al. (2009). This aligned data set comprises 5514 bp of DNA sequence from three mitochondrial genes (*cytb*, *ND2*, *ND4*) and three nuclear genes (*BDNF*, *POMC*, *RAG1*) for 184 plethodontid taxa and the outgroup, *Amphiuma tridactylum*. We used PartitionFinder (Lanfear et al. 2012) to evaluate different data-partitioning schemes and choose the best substitution model for each partition. Based on the Bayesian Information Criterion (BIC), the best partitioning scheme consisted of eight partitions with the following substitution models: (1) *cytb* first codon positions: SYM + Γ + I, (2) *cytb* and *ND4* second codon positions: HKY + Γ + I, (3) *cytb* and *ND4* third codon positions: GTR + Γ + I, (4) *ND2* and *ND4* first codon positions: GTR + Γ + I, (5) *ND2* second codon positions: GTR + Γ + I, (6) *ND2* third codon positions: TrN + Γ, (7) *POMC* and *RAG1*: SYM + Γ + I, and (8) *BDNF*: K80 + Γ + I. We unlinked substitution model parameters among partitions, linked clock models of mitochondrial DNA partitions (1–6 above), and linked clock models of nuclear DNA partitions (7–8 above). We used uncorrelated lognormal relaxed-clock models and a birth–death tree model. For this analysis, we inferred a single tree from the concatenated data set and provided six fossil-based calibration points (see Appendix S2 in Supporting Information for details). We estimated tree topology and divergence times using three independent searches of 30 million generations each, sampling every 1000th iteration with 10 % of the initial samples discarded as burn-in. We viewed log files in Tracer v.1.6 (Rambaut and Drummond 2009) to verify that parameter estimates had reached stationarity and that ESS values in the combined post-burn-in samples were >200.

To infer phylogeographic relationships and estimate divergence times within *P. serratus*, we performed a second analysis in BEAST in which we used the mean age estimate (12.7 Myr) and 95 % Highest Posterior Density (HPD; 9.3–16.8 Myr) for the MRCA of *P. serratus* and *P. cinereus* from the Plethodontidae analysis above to place a lognormally distributed prior (Mean = 12.7, SD = 0.15) on the root age of a tree containing all samples of *P. serratus* and the outgroup, *P. cinereus*. Additionally, we included a *cytb* sequence from GenBank (JF731320) for *P. sherando*, a recently described, narrowly distributed species from the Appalachian Mountains that was not included in the Kozak et al. (2009) study but was shown to be the sister taxon of *P. serratus* in recent analyses (Bayer et al. 2012; Highton et al. 2012). For our analysis of *P. serratus*, we used PartitionFinder (Lanfear et al. 2012) to evaluate different data-partitioning schemes and choose the best substitution model for each partition. Based on BIC, the best scheme consisted of three partitions that combined like codon positions of the two genes. The best substitution models were: HKY + Γ for first codon positions, HKY + I for second codon positions, and TrN + Γ for third codon positions. We unlinked substitution models among partitions, linked the clock models and tree among partitions, and used a constant population size tree prior. We ran analyses under a strict-clock and a relaxed-clock model (Drummond et al. 2006) and then compared them using path sampling and stepping-stone sampling in BEAST following the methods of Baele et al. (2012, 2013). In both cases, we simultaneously estimated topology and divergence times in a single search of 20 million generations sampling every 1000th iteration with 10 % of the initial samples discarded as burn-in. We viewed log files in Tracer v.1.6 to verify that parameter estimates had reached stationarity and that all ESS values were >200. For both path sampling and stepping-stone sampling, the difference in marginal likelihoods between strict-clock and relaxed-clock analyses, termed the Bayes factor (lnBF), was <1. Kass and Raftery (1995) state that 2lnBF values between 2 and 6 are positive evidence favoring one model over another, thus for our data set the more parameterized relaxed-clock model was not positively favored over the simpler strict-clock model (2lnBF = 0.9 for path sampling and −0.25 for stepping-stone sampling). We therefore used TreeAnnotator (Drummond and Rambaut 2007) to summarize the post-burn-in sample of trees from the strict-clock analysis and generate a maximum clade credibility tree with mean node heights. Nodes were considered to have high support when the Bayesian posterior probability (Bpp) was ≥0.95. We used the chronogram and estimates of node ages (means and 95 % HPDs) from this analysis to determine the approximate time that *P. serratus* diverged from its sister taxon, *P. sherando*, and also the timing of divergences within *P. serratus*. We calculated mean uncorrected pairwise sequence divergence between clades using MEGA v.5 (Tamura et al. 2011).

Ecological niche modeling

To test if the availability of suitable environmental conditions limits the distribution of *P. serratus* to the four current isolates and whether these populations were likely connected under past climatic conditions, we used geographic information system (GIS)-based ecological niche modeling (Peterson 2001; Kozak et al. 2008; Elith and Leathwick 2009). We obtained 678 unique georeferenced localities for *P. serratus* from several sources including the Global Biodiversity Information Facility (www.gbif.org), HerpNET (www.herpnet2.org), the Arkansas Natural Heritage Commission, and from our own collections and fieldwork (see Appendix S3 in Supporting Information for locality data). Next, we acquired GIS layers for 19 environmental variables from the Worldclim project (www.worldclim.org). These data comprise 19 bioclimatic variables (BIO1–BIO19) related to temperature, precipitation, and seasonality (Hijmans et al. 2005). We downloaded layers for both current conditions and the Last Glacial Maximum (LGM; ~21 ka) at 2.5 arc-min (~5 km^2) resolution. For building the model, we drew a polygon around all locality points and used the geometry of that polygon to clip layers for current conditions to a spatial extent that provided adequate information to train the model while also restricting analysis to a geographic area in which the species could possibly occur (Barve et al. 2011). For projecting the model, we clipped layers for current conditions and the LGM to the southeastern U.S.

We constructed an ecological niche model (ENM) for *P. serratus* using a maximum entropy method implemented in Maxent v.3.3.3 (Phillips et al. 2006; Phillips and Dudík

2008; Elith et al. 2011). The output of Maxent consists of a threshold-independent measure of the overall performance of the model (Area Under the Receiver Operating Curve or AUC) and a grid map with each cell having an index of suitability between 0 and 1 (logistic output); higher values indicate higher predicted suitability. An AUC value of 0.5 indicates the predictive model is no better than random, whereas higher AUC values indicate better predictive ability with a value of 1 indicating perfect prediction. We used auto-features and the default regularization multiplier parameter (1.0). Additionally, we removed duplicate presence points (i.e., points mapping to the same grid cell) and increased the number of iterations to 5000.

To gauge the sensitivity of the ENM to the samples used in training and test the predictive ability of the model, we performed 10 replicate runs using a different random seed and subsampling with 70 % of samples allotted for training and 30 % for testing. We examined the mean AUC for test data across the 10 replicates and considered a mean AUC $\geq$ 0.9 as very good support for the model (Swets 1988). To construct the final ENM for *P. serratus*, we used all locality points. We projected this ENM onto layers for current conditions and the LGM for the southeastern U.S., and used the Minimum Training

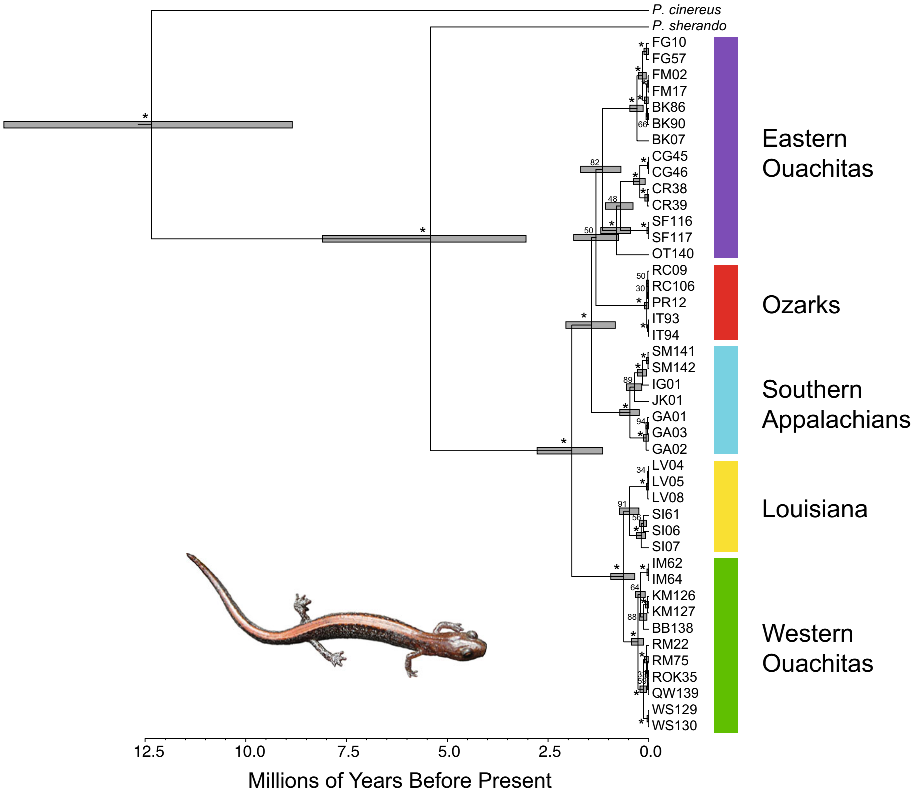

Fig. 2 Bayesian maximum clade credibility chronogram for *Plethodon serratus* based on concatenated analysis of *cytb* and *ND4* mitochondrial DNA sequences in BEAST. Nodes are labeled with Bayesian posterior probabilities (%); *asterisks* indicates 100 %. Node ages are presented as means with bars representing 95 % Highest Posterior Densities around age estimates. Phylogeographic lineages are labeled and *bars* next to labels are *colored* to match distributions in Fig. 1. (Color figure online)

Presence threshold to classify grid cells as unsuitable if they fell below this value (Liu et al. 2005). We retained values above this threshold to group cells into classes representing different levels of predicted suitability.

Results

Phylogeny and divergence times

The Bayesian phylogeny from BEAST revealed five geographically distinct lineages within *P. serratus* distributed across the four geographic isolates (Fig. 2). The Appalachian, Ozark, and Louisiana populations each formed well-supported clades (Bpp = 0.91–1.0). In contrast, Ouachita Mountain populations sorted into two clades, an eastern (Bpp = 0.82) and a western (Bpp = 1.0), that do not form a monophyletic group. The western Ouachita clade was sister to the Louisiana clade (Bpp = 1.0) whereas the eastern Ouachita clade was sister to the Ozark clade (Bpp = 0.50); however, support for the monophyly of the eastern Ouachita clade and its relationship to the Ozark clade was too low to have sufficient confidence about this relationship. The Appalachian clade grouped with Ozark and eastern Ouachita populations (Bpp = 1.0), but relationships among these three clades were unresolved. The greatest sequence divergence (6.5 %) was between the eastern and western Ouachita clades whereas the lowest (2.1 %) was between the Louisiana and western Ouachita clades. Sequence divergence between other clades ranged from 4.4 to 6.4 % (Table 1). Divergence times indicated that *P. serratus* diverged from its sister taxon, *P. sherando*, ~5.4 Ma (95 % HPD: 3.1–8.1 Ma; Fig. 2). The MRCA of all *P. serratus* was ~1.9 Ma (95 % HPD: 1.1–2.8 Ma). Divergences among the Appalachian, Ozark, and eastern Ouachita mtDNA lineages dated to ~1.3–1.4 Ma (95 % HPD: 0.8–2.1 Ma) whereas the divergence between the Louisiana and western Ouachita mtDNA lineages was more recent, dating to ~0.6 Ma (95 % HPD: 0.4–0.9 Ma).

Ecological niche modeling

Across the ten subsampling replicates, the ENM for *P. serratus* had a mean test AUC of 0.949 (SD = 0.009), indicating a very good ability to discriminate between presence and absence locations (Swets 1988). The Minimum Training Presence threshold was 0.02; thus, areas with values lower than this were classified as unsuitable. The predicted

Table 1 Mean pairwise uncorrected sequence divergence between phylogeographic lineages of *Plethodon serratus* based on analysis of 1399 base pairs of *cytb* and *ND4*

Clade	Appalachian (%)	Ozark (%)	Eastern Ouachita (%)	Western Ouachita (%)	Louisiana (%)
Appalachian	–				
Ozark	4.4	–			
Eastern Ouachita	4.6	4.6	–		
Western Ouachita	5.4	5.8	6.5	–	
Louisiana	5.8	5.9	6.4	2.1	–

distribution of *P. serratus* fits well with its actual present-day distribution restricted to four geographic isolates (Fig. 3a). All four isolates of *P. serratus* are separated from each other by large expanses of unsuitable habitat. A few areas within the circumscribed range of *P. serratus* as well as a few areas outside the current range are predicted to be suitable, but are not currently occupied by the species. The ENM also predicted suitable conditions along the Gulf Coast and the Kansas/Missouri border, but predictions in these regions should be treated with caution because climatic variables were outside the range of training data.

When we project the ENM onto climate layers for the LGM, the distribution of *P. serratus* is predicted to have been considerably expanded in the past compared to present-day (Fig. 3b). The model predicts suitable environmental conditions existed at the LGM everywhere within the current circumscribed range of *P. serratus*, although some areas

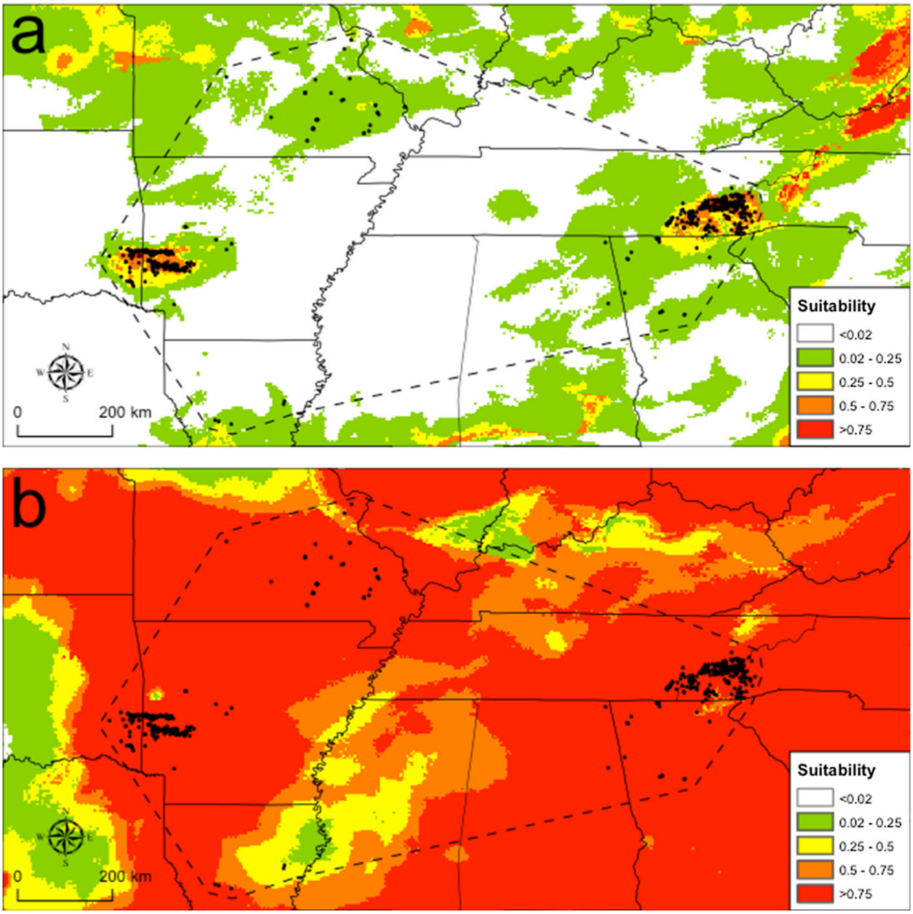

Fig. 3 Predicted distribution of *Plethodon serratus* under **a** current climate conditions and **b** at the Last Glacial Maximum ($\sim$21,000 years ago). Models were constructed in Maxent using 19 bioclimatic variables at 2.5 arc-min resolution ($\sim$5 km^2). Locality points for *P. serratus* ($n = 678$) used in modeling are shown as *black points* and the *dashed polygon* is the spatial extent used to train the model. Suitability values range from 0 to 1 with higher values indicating higher predicted suitability. (Color figure online)

along the Mississippi River in northeastern Louisiana and west-central Mississippi are marginal. Thus, this ENM predicts that the four current isolates of *P. serratus* were connected by suitable habitat during the LGM.

Discussion

Significant levels of genetic differentiation among the four isolates of *P. serratus* was not surprising given the geographic distances among them and results of previous work (Highton and Webster 1976; Sites et al. 2004). The Appalachian, Ozark, and Louisiana populations each formed monophyletic groups, indicating long-term isolation. What was surprising is that the Ouachita Mountains contain two non-sister parapatric lineages that show the highest level of sequence divergence within the species (6.5 %). In their allozyme study, Highton and Webster (1976) sampled only two populations of *P. serratus* from the Ouachitas, but it appears that they sampled both lineages. Our study included samples from the same two localities, FG and BB, which belong to the eastern and western lineages, respectively. Furthermore, their BB population was more similar genetically to their Louisiana population than it was to the FG population, which is consistent with our results showing a sister-taxon relationship between the western Ouachita and Louisiana lineages. Highton and Webster (1976) could not resolve relationships among Appalachian, Ozark, and Ouachita populations of *P. serratus* with 20 nuclearly encoded allozyme loci. Similarly, we could not resolve relationships among Appalachian, Ozark, and eastern Ouachita clades. Data from multiple nuclear DNA sequence loci combined with more extensive population sampling might provide better resolution. We also note that our results are based on mtDNA, which provides only a single genealogical estimate of evolutionary history that may be different from the true species history (Edwards 2009).

The eastern and western Ouachita clades abut along a roughly north–south line that is coincident with where the ranges of the Ouachita endemics *P. ouachitae* and *P. fourchensis* meet. These sister taxa were estimated to have diverged at approximately the same time as we inferred for the MRCA of eastern and western Ouachita lineages of *P. serratus* (~2.2 vs. ~1.9 Ma, respectively; Shepard and Burbrink 2008, 2009). There are no physical barriers to dispersal that would explain the distinct genetic break between eastern and western lineages of *P. serratus*; however, the contact zone appears to correspond to an extended divide separating major eastward- and westward-draining river systems (Arkansas and Ouachita rivers from the Red River). Studies on plethodontid species with aquatic larval phases have found a relationship between phylogeographic structure and river drainages (Jones et al. 2006; Kozak et al. 2006a); however, *Plethodon* are terrestrial, direct-developers and thus such a relationship would not be predicted. The congener, *P. caddoensis,* is sympatric with *P. serratus* in one region of the Ouachita Mountains, and also shows genetic structure associated with stream drainages, which was posited to be due to the unique geology of the region and flash flood events transporting individuals downstream (Shepard and Burbrink 2011). The process by which river drainage patterns might contribute to genetic differentiation of populations of *P. serratus* is unknown, but more detailed sampling of the contact zone and estimating gene flow between eastern and western lineages would be informative.

Although the biogeographic history of *P. serratus* would be better addressed within a broader taxonomic context including all species of *Plethodon* or all North American plethodontids, we can still make some inferences about the roles of dispersal and vicariance in shaping the species' distribution. Given that most species of *Plethodon* occur in the eastern U.S. (Kozak et al. 2009; Kozak and Wiens 2012), including *P. serratus* and its sister taxon, *P. sherando*, we can infer at least one dispersal from the Appalachians westward across the Mississippi River to the Interior Highlands. Dowling (1956) hypothesized that plethodontid salamanders colonized the Interior Highlands multiple times from different sources, including the Appalachians and southern Coastal Plain. The Appalachians are also considered the source of other Interior Highlands fauna, including some darters (*Nothonotus*; Near and Keck 2005), crayfishes (*Orconectes*; Crandall and Templeton 1999), and insects (Allen 1990). The lower Mississippi River appears to have been a major barrier to east–west dispersal between the Appalachians and Interior Highlands since well before the Pleistocene (>2.5 Ma), but these two regions are thought to have been connected via a large upland north of the present-day Ohio River until the onset of Pleistocene glaciations and subsequent vicariance (Mayden 1985, 1988; Soltis et al. 2006; Pyron and Burbrink 2010). Studies on eastern North American fishes and crayfishes have found that both dispersal and vicariance have been important in shaping species distributions and biogeographic patterns, but species vary in how they responded to historical events (Strange and Burr 1997; Crandall and Templeton 1999; Near et al. 2001).

During the Miocene (23–5.3 Ma), central North America experienced a transition from warm and humid climates to cool and dry climates with increased seasonality (Zachos et al. 2001; Kohn and Fremd 2008; Kürschner et al. 2008). This change in climate led to a contraction of forest and an expansion of grasslands accompanied by major faunal changes (Axelrod 1985; Kohn and Fremd 2008; Kürschner et al. 2008). Since the Late Miocene, a steep longitudinal moisture gradient has characterized this region with mesic conditions in the east and xeric conditions in the west (Axelrod 1985; Costa et al. 2008). The Ouachitas and Ozarks occur at the eastern edge of this gradient and are thought to have served as refugia for mesic-adapted organisms (e.g., salamanders) whenever xeric conditions expanded eastward in the past (Dowling 1956; Costa et al. 2008). The edges of this gradient fluctuated along an east–west axis during the climatic oscillations of the Pleistocene, which would have provided multiple opportunities for dispersal among regions when mesic conditions expanded westward and also multiple instances of vicariance when they contracted eastward (Axelrod 1985; Webb and Bartlein 1992; Grundstein 2009).

The divergence of *P. serratus* and *P. sherando* near the Miocene/Pliocene boundary (~5.4 Ma) corresponds to a period of rapid speciation in eastern North American *Plethodon* that has been attributed to widespread aridification (Highton 1995; Kozak et al. 2006b; Wiens et al. 2006; Highton et al. 2012). Using different methods, Bayer et al. (2012) estimated the MRCA of *P. serratus* and *P. sherando* to be 6.0 Ma (95 % HPD: 4.4–7.6 Ma), which is similar to our estimate (~5.4 Ma; 95 % HPD: 3.1–8.1 Ma). The divergence of mtDNA lineages within *P. serratus* ~1.9–0.6 Ma is consistent with the hypothesis that Pleistocene climatic fluctuations facilitated multiple instances of dispersal and vicariance. We note that our divergence time estimates are based gene tree inference and that gene coalescence will predate species/population divergence (Edwards and Beerli 2000; Edwards 2009). Thus, divergences among geographically isolated populations of *P. serratus* would likely be more recent than our mtDNA-based estimates, but still well within the Pleistocene.

Ecological niche models indicated that abiotic factors primarily limit the distribution of *P. serratus*. Our present-day ENM for *P. serratus* predicted that suitable habitat is largely restricted to the four geographic isolates and that intervening areas are unsuitable. In contrast, the ENM for the LGM predicted that *P. serratus* was widely distributed during colder climates of the Pleistocene and the four current isolates were connected by suitable habitat. Although divergences among the four isolates of *P. serratus* are older than the time periods of available climate layers, the current climate is similar to earlier interglacial climates of the Pleistocene and the LGM climate is similar to climates of earlier Pleistocene glacial periods (Petit et al. 1999). Therefore, ENMs based on these climate data likely capture the extent of broad-scale impacts of climatic fluctuations throughout the Pleistocene. A few areas within the current circumscribed range of *P. serratus* as well as some areas outside the species' current range were predicted to be suitable but are not currently occupied by the species. These areas are, however, occupied by other species of small-bodied *Plethodon* (Petranka 1998), which may preclude *P. serratus* from occurring in those areas. Species of *Plethodon* of similar body size tend not to coexist due to competitive interactions (Hairston 1951; Jaeger 1971; Adams 2007; Kozak et al. 2009). Thus, although abiotic factors appear to have been the major driver of the disjunct distribution of *P. serratus*, biotic interactions have also likely had an influence on where the species occurs.

Niche conservatism is considered to be the primary mechanism by which speciation in temperate-zone plethodontids has occurred (Kozak and Wiens 2006, 2010). Under this model, the distribution of a wide-ranging species is fragmented into multiple isolated populations as individuals track their ecological niche when climate changes. Over time, these isolated populations diverge and become new species (Wiens 2004; Wiens and Graham 2005). The five lineages within *P. serratus* are geographically distinct and reciprocally monophyletic for mtDNA. Nuclear loci also indicate significant genetic differentiation (Highton and Webster 1976). Furthermore, the four geographic isolates of *P. serratus* are separated by both physical barriers (e.g., Mississippi River, Arkansas River) and environmental barriers (e.g., unsuitable habitat, closely related species). Given these factors and current climate trends, it is unlikely that these disjunct populations will become connected again in the near future and they will likely continue to diverge genetically. The amount of sequence divergence found between most lineages of *P. serratus* (4.4–6.5 %) is comparable to the divergence between some currently recognized species of *Plethodon*. For example, comparing a similar region of cytochrome *b*, *P. virginia* and *P. hoffmani* had an average divergence of 4.1 % and *P. nettingi* and *P. hubrichti* were on average 5.4 % divergent (Sites et al. 2004). Given our hypothesis of phylogenetic relationships, whether eastern and western Ouachita lineages interbreed where they come into contact is central to determining whether lineages should be recognized as species. Because of a longitudinal climatic gradient in the Ouachita Mountains, the eastern and western Ouachita lineages likely occupy different climatic niches (Shepard and Burbrink 2009). Whether this niche variation is of an extent that would impact ecological exchangeability is unknown, but would be important for delimiting species (Rader et al. 2005; Stockman and Bond 2007; Bond and Stockman 2008). Furthermore, such information would be important for defining and managing conservation units to preserve diversity within what is currently considered a single, broadly distributed species (Moritz 1994; Crandall et al. 2000; Fraser and Bernatchez 2001).

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