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PHYLOGEOGRAPHY AND DIVERSIFICATION OF THE OUACHITA MOUNTAIN ENDEMIC SALAMANDERS OF THE *PLETHODON OUACHITAE* COMPLEX

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PHYLOGEOGRAPHY AND DIVERSIFICATION OF THE OUACHITA MOUNTAIN ENDEMIC SALAMANDERS OF THE *PLETHODON OUACHITAE* COMPLEX

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF ZOOLOGY

BY

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Dedication

I dedicate this dissertation to my grandfathers, Wayne M. Aceto and Donald B. Shepard, both whom died during my graduate career. They were two of the most intelligent, hard-working, and loving people I've met. I strive to be more like them.

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Abstract

Climatic changes associated with Pleistocene glacial cycles profoundly affected species distributions, patterns of inter-population gene flow, and demography. In species restricted to montane habitats, ranges may expand and contract along elevational gradients in response to environmental fluctuations resulting in alternating periods of connection and isolation. Periods of isolation may result in population divergence whereas periods of connectivity allow for dispersal and gene flow among mountains. The salamanders *Plethodon ouachitae* and *Plethodon fourchensis* are endemic to the Ouachita Mountains and are largely restricted to high-elevation, mesic forest. Because the ranges of these species span several mountains which are separated by more xeric, low-elevation valleys, the salamanders appear to be isolated on sky islands where gene flow among populations on different mountains may be restricted. I used DNA sequence data along with ecological niche modelling and coalescent simulations to test several hypotheses related to responses of montane species to Pleistocene glacial cycle-induced climatic shifts. Further, I analyzed morphological variation between species and among lineages within species to assess the extent of morphological divergence. My results revealed that *P. ouachitae* is composed of seven well-supported lineages structured across six major mountains whereas *P. fourchensis* is composed of four lineages structured across five montane isolates. Geographic breaks between lineages occurred in the vicinity of major valleys or high-elevation passes. Environmental conditions in intervening valleys were warmer and drier than conditions at locations where salamanders occurred, but ecological niche modelling predicted that suitable conditions

were present between most mountains. Lineage diversification within each species occurred contemporaneously during the Middle Pleistocene, but diversification in *P. ouachitae* occurred in a stepping stone fashion compared to the fragmentation of a wide-ranging ancestor in *P. fourchensis*. Historical demographic analyses showed relatively stable population sizes over the last glacial cycle in *P. ouachitae*, but a gradual decrease in *P. fourchensis*. Both species, however, showed a slight to moderate amount of population growth in all lineages starting approximately 5 000–12 000 years ago, which coincides with the transition to the current interglacial period, the Holocene. Results from morphological analyses supported genetic evidence showing significant divergence between *P. ouachitae* and *P. fourchensis*, and among the different lineages within each species. My results not only demonstrate that climatic changes during the Pleistocene had profound effects on species restricted to montane habitats, but comparison of my results for *P. fourchensis* with its parapatric, sister taxon, *P. ouachitae*, emphasizes how responses can vary substantially even among closely related, similarly distributed taxa. The high level of diversity within *P. ouachitae* and *P. fourchensis* also has important implications for conservation because these endemic species have small ranges and lineages are usually restricted to single mountains.

Chapter I

Lineage diversification and historical demography of a sky island salamander, *Plethodon ouachitae*, from the Interior Highlands

(formatted for *Molecular Ecology*)

Abstract

Sky islands provide ideal opportunities for understanding how climatic changes associated with Pleistocene glacial cycles influenced species distributions, genetic diversification, and demography. The salamander *Plethodon ouachitae* is largely restricted to high-elevation, mesic forest on six major mountains in the Ouachita Mountains. Because these mountains are separated by more xeric, low-elevation valleys, the salamanders appear to be isolated on sky islands where gene flow among populations on different mountains may be restricted. We used DNA sequence data along with ecological niche modelling and coalescent simulations to test several hypotheses related to diversifications in sky island habitats. Our results revealed that *P. ouachitae* is composed of seven well-supported lineages structured across six major mountains. The species originated during the Late Pliocene, and lineage diversification occurred during the Middle Pleistocene in a stepping stone fashion with a cyclical pattern of dispersal to a new mountain followed by isolation and divergence. Diversification occurred primarily on an east–west axis, which is likely related to the east–west orientation of the Ouachita Mountains and the more favorable cooler and wetter environmental conditions on north slopes compared to south-facing slopes and valleys. All non-genealogical coalescent methods failed to detect significant population expansion in any lineages. Bayesian skyline plots showed relatively stable population sizes over time, but indicated a slight to moderate amount of population growth in all lineages starting approximately 10 000–12 000 years ago. Our results provide new insight into sky island diversifications from a previously unstudied region, and further demonstrate that climatic changes during the Pleistocene had profound effects on lineage diversification and demography, especially in

species from environmentally sensitive habitats in montane regions.

Introduction

Changing climatic conditions in montane regions can cause favorable environments for a species to shift, expand, or contract along elevational gradients (Hewitt 2000, 2004). In species that closely track the environmental conditions to which they have adapted over time, populations on different mountains may experience alternating periods of isolation and connectivity during climatic fluctuations (Hewitt 1996; Wiens 2004; Wiens & Graham 2005). Periods of isolation may result in genetic divergence among populations whereas periods of connectivity allow for dispersal and gene flow among mountains (Hewitt 1996, 2004; Avise 2000). Such range expansions and contractions are also predicted to result in changes in effective population size (N_e; Wakeley 2000; Jesus *et al.* 2006). The amount of time that populations are isolated and their N_e will determine whether they sort into independent evolutionary lineages whereas the level of divergence and ecological characteristics of the species often influence whether they remain distinct or merge back into a common gene pool upon secondary contact (Hewitt 1996; Maddison 1997; Avise 2000).

Clusters of mountains in which environmental conditions at higher elevations differ markedly from those in the intervening valleys are termed sky islands, and provide unique opportunities for understanding how climatic changes associated with Pleistocene glacial cycles influenced species distributions, genetic diversification, and demography. In the Madrean sky islands in the desert southwest of the United States, for example, the cool environmental conditions that predominated at low elevations during glacial periods contracted to higher elevations during interglacial periods and resulted in isolation and divergence of populations of jumping spiders (genus *Habronattus*) and longhorn beetles

(genus *Moneilema*) on different mountain tops (Maddison & McMahon 2000; Masta 2000; Smith & Farrell 2005). In contrast, grasshoppers (genus *Melanoplus*) on sky islands in the northern Rocky Mountains diverged while isolated in multiple refugia during glacial periods and underwent dispersal and range expansion during interglacial periods (Knowles 2000, 2001a,b; Carstens & Knowles 2007). Sky islands can generate high levels of inter-population genetic diversity, but because species responses to climatic changes are influenced by interacting factors such as ecology, landscape topography, latitude and longitude, the pattern and tempo of diversification will vary (Hewitt 1996, 2000, 2004). For example, the distribution of a species on multiple sky islands could result from the fragmentation of a wide-ranging common ancestor or may be due to inter-island dispersal. Thus far, our understanding of diversifications in sky islands is limited to a few examples, and new insights into how Pleistocene climatic changes affected diversification and demography may be provided by examining different species on sky islands in other regions.

The Interior Highlands, located in southern Missouri, northern and western Arkansas, and eastern Oklahoma, are composed of the Ozark Plateau and Ouachita Mountains (Fig. 1). Although this region was not glaciated during the Pleistocene, it experienced climatic fluctuations that likely impacted species distributions and demography (King 1973; Davis 1983; Hewitt 1996, 2000, 2004). The Interior Highlands occur at the eastern edge of a steep longitudinal environmental gradient in the central United States that transitions from mesic forest in the east to xeric grasslands in the west, and are inhabited by a large number of endemic species including several salamanders in the genus *Plethodon* (Dowling 1956; Mayden 1985, 1988; Costa *et al.* 2008). Species in

the genus *Plethodon* generally occupy moist terrestrial microhabitats in forested areas and reach their peak diversity in the mountainous regions of eastern North America (Appalachian and Interior Highlands) where many closely related taxa often occur on adjacent mountain tops (Highton 1995; Petranka 1998; Kozak *et al.* 2006a; Wiens *et al.* 2006). This region has a history of climate-driven forest contraction, fragmentation, and expansion over the last three million years (King 1973; Davis 1983; Webb & Bartlein 1992), which is thought to have contributed greatly to diversification in *Plethodon* (Highton 1995; Kozak *et al.* 2006a; Kozak & Wiens 2006). Although the conservative morphology of *Plethodon* makes diagnosis of species difficult, the boundaries of many morphologically cryptic species have been successfully investigated using genetic data (Highton *et al.* 1989; Highton 1995; Highton & Peabody 2000). Salamanders in the genus *Plethodon* are forest dwelling, lungless ectotherms that require mesic environments for cutaneous respiration, and their distributions are strongly influenced by moisture and temperature (Jaeger 1971; Spotila 1972). Consequently, among terrestrial vertebrates, they should be impacted the most by climatic changes.

Plethodon ouachitae is endemic to the Ouachita Mountains of southeastern Oklahoma and west-central Arkansas, and is mainly restricted to mesic forest (Petranka 1998). The Ouachita Mountains are unique among mountain ranges in North America because they trend east–west. This orientation results in mesic forest, and thus *P. ouachitae*, being primarily found on high-elevation, north-facing slopes (Blair & Lindsay 1965; Duncan & Highton 1979; Foti & Glenn 1991; Trauth & Wilhide 1999). This salamander occurs only at the higher elevations on six major mountains: Kiamichi, Round, Rich, Black Fork, Winding Stair, and Buffalo (Fig. 1). The area of suitable

environmental conditions for *P. ouachitae* on these mountain tops is predicted to have expanded and contracted in response to Pleistocene climatic fluctuations resulting in historic periods of connectivity and isolation. Given the potential for repeated geographic contact of populations on different mountains during Pleistocene climatic fluctuations, it is possible that populations merge into a single gene pool during periods of secondary contact. However, Duncan and Highton (1979) examined *P. ouachitae* from 10 localities and found large genetic differences, based on 23 allozyme loci, among the Kiamichi, Round, Winding Stair, Buffalo, Rich, and Black Fork Mountain populations, which suggests that gene flow among populations on different mountains is restricted and each mountain may comprise a distinct evolutionary lineage.

Here we sample *P. ouachitae* throughout its range and use DNA sequence data to evaluate several hypotheses related to sky island diversifications. First, we use statistical phylogenetic methods to test whether each geographically isolated mountain comprises a distinct evolutionary lineage (i.e., mountains are reciprocally monophyletic). Next, we use ecological niche modelling to test if identified lineages are separated by areas where environmental conditions are unsuitable and act as barriers to gene flow. Third, we use divergence dating to test whether the timing of diversification within *P. ouachitae* is consistent with Pleistocene glacial cycle-induced climatic shifts in montane habitats. Fourth, we use coalescent simulations to test whether populations on different mountains are descended from a single wide-ranging ancestor whose range became fragmented or alternatively, if the pattern of diversification is consistent with a colonization model involving dispersal from one mountain to another followed by isolation (i.e., a stepping stone model). Lastly, given that the area of suitable environmental conditions for *P.*

ouachitae on these sky islands is predicted to have expanded and contracted in response to Pleistocene climatic fluctuations, we examine historical demography to test for corresponding increases and decreases in N_e.

Materials and methods

Sampling and sequencing

We conducted extensive surveys throughout the Ouachita Mountains and intervening valleys to establish the distribution of *Plethodon ouachitae*, and collected 281 tissue samples from 55 unique localities throughout the range (Appendix I). We also collected samples of several closely related species, *Plethodon fourchensis* ($N = 2$), *Plethodon caddoensis* ($N = 2$), and *Plethodon kiamichi* ($N = 1$) for use as outgroups (Kozak *et al.* 2006a; Wiens *et al.* 2006; Appendix).

Whole genomic DNA was extracted from ethanol-preserved liver or muscle tissue using the DNeasy Kit (Qiagen Inc., USA) to obtain template strength DNA/RNA ratios of 1.5–2.1 and DNA concentrations from 10–200 ng/µl. We amplified two mitochondrial encoded genes, cytochrome *b* (cytb) and NADH dehydrogenase 4 (ND4), and a portion of tRNA-His using Polymerase Chain Reaction (PCR), with a negative control (water), following the specifications included with the AccuTaq Jumpstart Kit (USB Corp., USA) in a 10-µl reaction. For PCRs, we used the primers PGludg2 and PThrR1 for cytb, and Ephist and ND4(F) for ND4 (Wiens *et al.* 2006). Thermal cycling conditions used to amplify these genes were: 94º C for 2 min followed by 36 cycles of 94º C for 10 s, 50º C (cytb) or 52º C (ND4) for 30 s, and 72º C for 90 s with a final 10 min extension period at

72º C. We cleaned PCR products using 1 µl of ExoSap-it (USB Corp., USA) per 10 µl of PCR product.

We developed and used the following species-specific primers for sequencing: cytb – PouachCytbF (TTCTGAGGRGCCACAGTYATTACTAA) and PouachCytbR (GGGTTGTTTGAGCCKGTTTCATG), and ND4 – PouachND4F (GAACGAACACACAGCCGAACT) and PouachND4R (ATAAGCGGCYGTTAAGAGTGTGCC). Sequencing reactions consisted of 2–3 µl of DTCS (Beckman-Coulter, USA), 1 µl of 5-µM primer, 1–2 µl of DNA template, and 4–6 µl of H_2O. Sequencing products were purified following the ethanol-sodium-acetate protocol listed in the DTCS Kit and analyzed on a Beckman CEQ 8000 sequencer (Beckman-Coulter, USA). Nucleotide sequences were assembled, edited, and aligned by eye using the program Sequencher 4.1.2 (Genecodes 2000), and an open reading frame for these genes was verified. Alignments were unambiguous and no indels were found in these genes in *P. ouachitae*, *P. fourchensis*, and *P. caddoensis*. A two base pair insertion/deletion, however, was present in the tRNA-His flanking region of the ND4 gene when compared to the outgroup, *P. kiamichi*. Sequences were deposited in GenBank under the Accession Numbers FJ266739–FJ267024 (cytb) and FJ267025–FJ267299 (ND4; Appendix I).

Phylogeography

Phylogeographic relationships within *P. ouachitae* were estimated using the combined sequences from the cytb and ND4 genes, and tRNA-His. We used Maximum Likelihood

(ML) and Bayesian Inference (BI) with partitioned models incorporating evolutionary information specific to gene and codon position to infer trees and assess nodal support.

Prior to tree inference, three partitioning strategies were evaluated. The first model accounted for differences in evolutionary rate in each of the three codon positions of the cytb and ND4 genes and the sequences from tRNA-His using the GTR + Γ + I model with estimated base pair (bp) frequencies for each codon position in each gene and the tRNA. For this codon position-specific and tRNA-specific model, abbreviated 7(GTR + Γ + I), a single tree was estimated for all partitions simultaneously, but all other model parameters were unlinked among partitions. The second model simply applied the GTR + Γ + I model across all positions for each protein coding gene and the tRNA [3(GTR + Γ + I)] with no partitioning among codon positions. Finally, the last model simply applied one GTR + Γ + I model across both genes and the tRNA.

For each partitioning strategy, two independent searches were executed in MrBayes v.3.1.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) to ensure convergence of all parameters, which we assessed by comparing the variance across chains within a search to the chain variance among searches using Gelman and Rubin's "r" statistic (Gelman *et al.* 1995). Searches were considered burned-in when the values for "r" reached ~1.00. All searches consisted of three "heated" and one "cold" Markov chain estimated for 10 million generations with every 1000th sample being retained. Default priors were applied to all parameters except branch length, which was drawn from an exponential distribution. A split standard deviation less than 0.005 for -lnL tree values among chains indicated that parameter stationarity was achieved. Trees sampled prior to stationarity were discarded. The harmonic mean of the model

likelihood, $f(X|M_i)$, taken from the stationarity phase was compared among different partitioning strategies using Bayes Factors (BF) for the equation $2LnB_{10}$ in Tracer v.1.4 (Newton & Raftery 1994; Rambaut & Drummond 2007). A BF >10 was considered as strong evidence favoring the more partitioned model (Kass & Raftery 1995).

The ML tree and associated support were obtained from 1000 nonparametric bootstrap pseudoreplicates (Felsenstein 1985) under the preferred BF partitioning strategy using the GTRGAMMA model in the program RAxML-VI-HPC MPI v.2.0 (Stamatakis *et al.* 2005; Stamatakis 2006). Trees from BI were compared with the ML tree and the most credible inferences of relationship were confined to nodes where the Bayesian posterior probability was ≥95% and the nonparametric bootstrap value was ≥70% (Hillis & Bull 1993; Felsenstein 2004).

Ecological niche modelling

We downloaded raster coverages of 19 environmental-climatic variables from the WorldClim database (http://www.worldclim.org) at 30 arc-seconds resolution (~1 km^2; Hijmans *et al.* 2005) and clipped these coverages to a region that encompassed the entire Ouachita Mountain range and included most of eastern Oklahoma, western Arkansas, and parts of southern Missouri and northeastern Texas (92.20–96.56° longitude and 33.18–36.93° latitude). Next, we used the Principal Components tool in the Arc-GIS v.9.1 Spatial Analyst Tools (ESRI, Redlands, CA, USA) to construct a correlation matrix for the 19 variables, and examined the matrix to identify variables that were highly correlated. For variables where $r > 0.9$, we omitted one of the variables, preferentially choosing to omit variables that measured averages over those that measured extremes or

seasonality (Kozak & Wiens 2006; Rissler & Apodaca 2007). Ultimately, we retained 12 climatic variables for use in niche modelling (Table 1).

We constructed an ecological niche model for *P. ouachitae* using the 12 climatic variables and GPS coordinates of our 55 sampling localities with the program Maxent v.3.2.1 (Phillips *et al.* 2006; Appendix I). These points represent all known localities for *P. ouachitae*. Maxent uses environmental–climatic variables from localities in which a species has been documented previously to predict where else the species may occur because the environmental-climatic conditions are similar to the conditions at known localities. The output of Maxent consists of grid maps with each cell having an index of suitability between 0 and 1. Low values indicate conditions are unsuitable for the species to occur whereas high values indicate that conditions are suitable. To represent environmental suitability as a binary character, we used a threshold value of 0.442, as chosen using the 10 percentile training presence criteria calculated by Maxent. We then overlaid this niche model on a map of the Ouachita Mountains to examine visually if mountains/lineages were separated by areas of unsuitable environmental conditions.

To test if environmental conditions in areas where *P. ouachitae* is present are significantly different from conditions in the valleys separating mountains, where *P. ouachitae* is presumably absent, we first extracted values for each of the 12 climatic variables used in niche modelling from our 55 sampling localities. We then created a polygon using a portion of our sampling points that encompassed the main valley separating the Kiamichi Mountains and Round Mountain from Rich, Winding Stair, and Buffalo Mountains, the valley between Buffalo and Winding Stair Mountains, the valley between Winding Stair and Rich Mountains, and also the south slopes of Buffalo,

Winding Stair, and Rich Mountains. We generated 55 random points within this polygon and extracted values for each of the 12 climatic variables from these points. We cannot positively confirm that *P. ouachitae* does not occur at these locations, therefore these points are considered pseudoabsence locations (*sensu* Kozak & Wiens 2007). Because many of the 12 climatic variables are likely intercorrelated, we used Principal Components Analysis to reduce them to a smaller number of independent variables. We retained principal components with eigenvalues >1 and that explained >10% of the variation. We used the factor scores for these principal components as dependent variables in a MANOVA to test for differences between occurrence and pseudoabsence locations. We followed a significant multivariate effect with ANOVAs for each principal component, and examined loading factors for those principal components that were significantly different to determine the nature of the differences in environmental conditions between occurrence and pseudoabsence locations.

Divergence dating

To estimate the age of origin of *P. ouachitae*, we used a 'relaxed phylogenetics' method that does not rely on a molecular clock and incorporates uncertainty in the tree estimation process (Drummond *et al.* 2006). Using BEAST v.1.4.6 (Drummond & Rambaut 2007), we estimated the tree and divergence dates of the monophyletic Plethodontidae using all genes and individuals included in Wiens *et al.* (2006) employing the GTR + Γ + I model across all genes and codon positions. An uncorrelated lognormal tree prior with a constant population size prior and lognormal calibration dates (see below) were used to estimate the timing of divergences (Drummond *et al.* 2006). These analyses estimated

tree shape and divergence dates for all nodes and were sampled every 1000th iteration for 30 million generations with 10% of the initial samples discarded as burn-in.

To use this relaxed phylogenetics method, we provided calibration points and error estimates derived from a lognormal distribution (Drummond *et al.* 2006). Our calibration points for this tree came from three sources and were identical to those used in Wiens *et al.* (2006). The first two calibration points, the earliest fossils of *Plethodon* and *Aneides* (representing the MRCA of the genera *Aneides, Desmognathus* and *Phaeognathus*), were both from the Arikareean (Tihen & Wake 1981). Thus, both fossils are a minimum of 19 Myr. We used this age as the median for each of these calibration points and a SD of 0.3, which yields an upper 95% Credible Interval of 30 Myr, thereby encompassing the entire Arikareean. For the other calibration point, we used the fossil of *Aneides lugubris*, dating from the Late Miocene (~5 Ma; Clark 1985). As discussed in Wiens *et al.* (2006), this provides a minimum age for the MRCA between *A. lugubris* and *A. aeneus*. We used this date as the MRCA of these taxa, and a SD of 0.5 provides an upper 95% bound of 11 Myr (mid-Miocene).

The divergence date estimate and associated error for *P. ouachitae* from this tree were then applied to a tree of all 281 samples of *P. ouachitae* to estimate the age of each phylogeographic lineage. To test whether all diversifications within *P. ouachitae* occurred during the Pleistocene, we determined the probability that a pre-Pleistocene value (>1.8 Ma) could be found within the lognormal distribution of dates for the first divergence within the species. The dates for the MRCA of haplotypes within each lineage were used in the historical demographic analyses so that estimates of changing N_e

could be dated and related to geologic or climatic events in the past (e.g., glacial and interglacial periods).

Historical biogeography

To examine the area of origin for *P. ouachitae* and each clade and lineage, we used a maximum likelihood (ML) method of ancestral character estimation in Mesquite v.2.5 (Maddison & Maddison 2008). Each individual in the phylogeographic analysis was coded to one of seven major mountains in the Ouachita Mountains and the ancestral areas were estimated for each node using a ML model on the tree. We used an equal likelihood for the rate of change among different mountains for estimating ancestral areas because no prior knowledge of dispersal rate from one area to another exists. At each ancestral node, likelihoods for each area are summed and reported as proportional likelihoods. We chose this method to examine ancestral areas because the number of terminals in our tree is too large (>180) to be accommodated by the program DIVA (Ronquist 1996) and the number of distinct geographic areas within the Ouachita Mountains is too high (>5) for the program *lagrange* (Ree & Smith 2008).

We used coalescent simulations in Mesquite v.2.5 (Maddison & Maddison 2008) to test between a wide-ranging fragmented ancestor model of diversification and a structured colonization model (Knowles & Maddison 2002; Fig. 2). The Fragmented Ancestor model posits that all population divergences were in effect concurrent and the presence of any phylogeographic structure in our genetic marker is due to coalescent stochasticity (i.e., differential extinction of ancestral halplotypes among mountains; Knowles 2001a; Carstens *et al.* 2005). In contrast, the Colonization model posits a

history involving a series of dispersals from one mountain to another followed by isolation and divergence. The Colonization model was rooted based on the results for the most likely area of origin for *P. ouachitae* (see below) and then structured based on a hypothesized stepping stone pattern (Fig. 2).

For coalescent simulations, we first estimated N_e for *P. ouachitae* on each mountain using values for θ calculated in the program MIGRATE-N v.2.4 (Beerli 2008) under the following parameters: 20 small chains for 200 000 generations and three long chains for two million generations with four adaptive heating chains, chains sampled every 20 generations following a burn-in of 10 000 generations. Maximum likelihood estimates (MLE) were calculated three times to ensure convergence upon similar values for θ. We converted θ to N_e using the equation for maternally inherited mitochondrial DNA $\theta = N_e\mu$, where $\mu = 1.305 \times 10^{-7}$, which is based on an average substitution rate for *P. ouachitae* of 4.35×10^{-2} substitutions per site per million years calculated in BEAST v.1.4.6 and a generation time of three years (Pope & Pope 1951; Drummond & Rambaut 2007). We summed the estimates of N_e for all mountains to calculate Total N_e and scaled the branch widths of our hypothesized population trees using the proportion of Total N_e that each mountain comprised. Internal branches on the Colonization model were scaled such that all branch widths summed to Total N_e at any single point in time (Carstens *et al.* 2004a; Fig. 2). Because point estimates of θ calculated from a single locus may have large associated error (Edwards & Beerli 2000), we also used the lower and upper 95% CI's for our θ estimates to calculate lower and upper bound estimates of Total N_e to use in simulations in order to encompass a wide range of potential N_e values. For both the MLE and the lower and upper bounds of Total N_e, we simulated 1000 trees under a

neutral coalescent process on the Fragmented Ancestor model with a tree depth of 250k generations, which when based on a three year generation time (Pope & Pope 1951) is equivalent to 0.750 Myr (the approximate age estimated for the first divergence within *P. ouachitae* using the fossil calibrated relaxed phylogenetics method). We fit the simulated gene trees from the Fragmented Ancestor model to the Colonization model, calculated the number of deep gene coalescences (nDC), and built a distribution of nDC values (N = 1000 for each value of Total N_e). We then fit our reconstructed ML tree for *P. ouachitae* to the Colonization model and calculated the nDC value. If this observed nDC value falls below 95% of the distribution of nDC values calculated using the simulated gene trees (equivalent to one-tailed $P \leq 0.05$), then the Fragmented Ancestor hypothesis will be rejected in favor of the Colonization model. To calculate P values for the observed nDC in these analyses, we fit the distribution of simulated nDC values to a normal distribution with the given mean and standard deviation (SD).

We also used coalescent simulations in Mesquite v.2.5 (Maddison & Maddison 2008) to test, under a coalescent framework, whether the timing of diversification within *P. ouachitae* is consistent with the Pleistocene glacial cycle-induced climatic shift hypothesis. For this analysis, we simulated 1000 gene trees under a neutral coalescent process on the Colonization model at three different tree depths (250k, 400k, and 600k generations). Based on a three year generation time (Pope & Pope 1951), these depths are equivalent to 0.750 Myr (the approximate age estimated for the first divergence within *P. ouachitae* using the fossil calibrated relaxed phylogenetics method), 1.2 Myr (within the Early Pleistocene), and 1.8 Myr (the beginning of the Pleistocene), respectively. For these simulations, we used the MLE of N_e and scaled branch widths the

same as above (Fig. 2). We fit the simulated gene trees for each tree depth back into the Colonization model in which they were simulated, calculated the number of deep gene coalescences (nDC), and built a distribution of nDC values (N = 1000 for each tree depth). We then fit our reconstructed ML tree for *P. ouachitae* within each of these models and calculated the observed nDC value. If this observed nDC value falls below 95% of the distribution of nDC values calculated using the simulated gene trees, then the hypothesis that all diversification within *P. ouachitae* occurred after that point in time (0.750 Ma, 1.2 Ma, or 1.8 Ma) will be rejected. To calculate P values for our observed nDC in these analyses, we fit the distribution of simulated nDC values to a normal distribution with the given mean and standard deviation (SD).

Historical demography

We examined past population dynamics of all phylogeographic lineages of *P. ouachitae* using several methods including Bayesian skyline plots (BSP; Drummond *et al.* 2005). This technique permits the estimation of N_e through time and does not require a specified demographic model (e.g., constant size, exponential growth, logistic growth, or expansive growth) prior to the analysis. We used the HKY + Γ + I model with a relaxed lognormal clock to construct BSPs in BEAST v.1.4.6 (Drummond *et al.* 2005; Drummond & Rambaut 2007) for each lineage. We applied 10 grouped coalescent intervals (m), and priors for the phylogenetic model and population sizes were uniformly distributed. These analyses estimated genealogies and model parameters, and were sampled every 1000th iteration for 10 million generations with 10% of the initial samples discarded as burn-in. Additionally, to scale the time axis on BSPs, we used date estimates for the most recent

common ancestor (MRCA) of all haplotypes within a lineage (obtained from divergence dating described above) to place an estimate of time on these demographic analyses. We used a relaxed uncorrelated lognormal molecular clock with mean divergence dates and lognormal standard deviation values to reflect the median and 95% Credible Interval (CI) obtained from the dating estimates for each clade (see below) when inferring demographic changes using BSPs. Plots for each analysis were visualized using Tracer v.1.4 (Rambaut & Drummond 2007).

To provide other estimates of change in N_e, we also calculated Tajima's D^* (Tajima 1989) and Fu and Li's D^* (Fu 1997). Both Tajima's D^* and Fu and Li's D^* are expected to be near zero if population sizes have been stable. Significant negative values are expected in populations that have undergone recent population expansion whereas significant positive values are expected in populations that have recently experienced bottlenecks (Tajima 1989; Fu 1997). We tested for significant deviations from zero in Tajima's D^* and Fu and Li's D^* using 10 000 coalescent simulations in DnaSP v.4.20 (Rozas *et al.* 2003). Contrasting plots of observed versus theoretical distributions of site differences (mismatch) also yields insight into past population demographics. A unimodal mismatch distribution indicates a recent range expansion, a multimodal (including bimodal) mismatch distribution indicates diminishing population sizes or structured size, and a ragged distribution suggests that the lineage is widespread (Excoffier *et al.* 1992; Rogers & Harpending 1992; Rogers *et al.* 1996; Excoffier & Schneider 1999). A multimodal distribution may also indicate that the population is influenced by migration, is subdivided, and/or has undergone historical contraction (Marjoram & Donnelly 1994; Bertorelle & Slatkin 1995; Ray *et al.* 2003). The fit of the

observed data was tested against a null distribution of constant population size using the R_2 raggedness statistic of Ramos-Onsins and Rozas (2002) and 10 000 coalescent simulations in DnaSP v.4.20 (Rozas *et al.* 2003).

Results

Phylogeography

We sequenced 1052 bp of the cytb gene, 723 bp of the ND4 gene, and 41 bp of the tRNA-His for 281 *Plethodon ouachitae* and five outgroup taxa (1816 bp total). Bayes factors (BF) strongly favored the more parameter rich 7(GTR + Γ + I) model over the less parameterized 3(GTR + Γ + I) model (BF > 290). Therefore, we used the 7(GTR + Γ) model to run the ML analysis in RAxML-VI-HPC MPI v.2.0 (Stamatakis *et al.* 2005; Stamatakis 2006) and the 7(GTR + Γ + I) to run the BI analysis in MrBayes v.3.1.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). For the BI analysis, burn-in occurred at two million generations as determined by a Gelman and Rubin's "r" value near 1.0 for the -lnL tree likelihood. Both independent runs using the 7(GTR + Γ + I) model produced similar harmonic mean -lnL values for post burn-in trees with a difference in -lnL of 0.34. After discarding samples before burn-in and summing trees from the two independent runs, the posterior probability distribution contained 16 000 trees.

Tree topology was similar for ML and BI analyses, and both indicated that *P. ouachitae* is composed of seven well-supported, geographically structured lineages (Fig. 3). Two divergent sister lineages are found within the central part of the Kiamichi Mountains, one to the west (Kiamichi West) and one to the east (Kiamichi East), that are

sister to a clade composed of all other lineages (Figs. 1 & 3). Another lineage (Round Mtn) occurs in the eastern end of the Kiamichi Mountains and on Round Mountain, spanning a small valley that separates them (Figs. 1 & 3). Two non-sister lineages (Rich Mtn & Black Fork Mtn) occur on Rich and Black Fork Mountains, and the western edge of Fourche Mountain (Figs. 1 & 3). Winding Stair Mountain and Buffalo Mountain each form reciprocally monophyletic lineages (Winding Stair Mtn & Buffalo Mtn; Figs. 1 & 3). The Black Fork Mtn lineage is sister to a clade comprised by the Rich Mtn, Winding Stair Mtn, and Buffalo Mtn lineages, but relationships among the Rich Mtn, Winding Stair Mtn, and Buffalo Mtn lineages are unresolved (Fig. 3).

Ecological niche modelling

The niche model for *P. ouachitae* predicts that most lineages are separated from immediately adjacent lineages by areas where environmental conditions are suitable (Fig. 4). Buffalo Mountain is the exception and appears to be isolated from all other lineages by unsuitable conditions. The main valley separating the Kiamichi Mountains and Round Mountain from Rich, Winding Stair, and Buffalo Mountains is predicted to be suitable only on the eastern end where it is narrowest (~3.2 km) and highest in elevation (>350 m).

Principal components analysis (PCA) reduced the 12 climatic variables used in niche modelling to two principal components explaining 67.3% and 18.4% percent of the variation, respectively (Table 1). A MANOVA using the principal component scores revealed that environmental conditions at locations where *P. ouachitae* occurs were significantly different from random pseudoabsence locations in the intervening valleys

(Wilk's $\lambda = 0.72$, $F_{2,107} = 20.8$, $P < 0.001$). Specifically, locations differed along the first principal component axis ($F_{1,108} = 41.7$, $P < 0.001$), but did not significantly differ along the second axis ($F_{1,108} = 0.15$, $P = 0.70$). Based on the factor loadings (Table 1), the first principal component axis represented a gradient from cool and wet environmental conditions (low values) to dry and warm environmental conditions (high values), with locations in the valleys having significantly higher values (Fig. 4).

Divergence dates and historical biogeography

Our divergence dating analyses indicate that *P. ouachitae* diverged from its sister taxon, *P. fourchensis*, during the Late Pliocene ~2.192 Ma (95% CI: 0.732–3.851 Ma) and all divergences within *P. ouachitae* occurred at various times throughout the Middle Pleistocene (Fig. 5). From the combined ancestral area and divergence dating estimates, it appears that *P. ouachitae* originated in the Kiamichi Mountains, and divergence within this species began there ~0.756 Ma (95% CI: 0.197–1.549 Ma) with the basal node subtending the common ancestor of the Kiamichi West and Kiamichi East lineages and the common ancestor of all other lineages (Table 2; Fig. 5). Given this date and associated error, it is extremely improbable that divergence within *P. ouachitae* occurred prior to the Pleistocene (>1.8 Ma; $P = 5.48 \times 10^{-5}$). Following the initial divergence, dispersal from the eastern end of the Kiamichi Mountains or Round Mountain northward to Rich and Black Fork Mountains occurred ~0.667 Ma (95% CI: 0.195–1.430 Ma) and was followed by divergence of the Rich and Black Fork lineages ~0.524 Ma (95% CI: 0.138–1.109 Ma; Table 2; Fig. 5). Westward dispersal from Rich Mountain onto and along Winding Stair and Buffalo Mountains occurred after this, and then those three

lineages diverged ~0.412 Ma (95% CI: 0.094–0.853 Ma; Table 2; Fig. 5). The Kiamichi West and Kiamichi East lineages diverged ~0.442 Ma (95% CI: 0.061–1.033 Ma; Fig. 5).

Using Migrate-N (Beerli 2008), we calculated a maximum likelihood estimate (MLE) of $\theta_{Total} = 0.0738$ (95% CI: 0.0551–0.1032). The MLE estimate of θ_{Total} equates to a Total N_e of 565 517 with a lower and upper bound of 422 222 and 790 805, respectively. The number of deep coalescent events (nDC) for our ML tree fit into a population tree representing the Colonization model was 8. Results from coalescent simulations rejected the Fragmented Ancestor model of diversification in favor of the Colonization model at all three values of Total N_e (MLE N_e mean nDC = 21.76, SD = 3.72, $P = 0.0001$; lower N_e mean nDC = 16.64, SD = 3.29, $P = 0.004$; upper N_e mean nDC = 29.89, SD = 4.38, $P = 2.94 \times 10^{-7}$). Coalescent simulations on the Colonization model using the MLE of N_e rejected the null hypothesis that all diversification within *P. ouachitae* has occurred in the last 0.750 Myr (mean nDC = 19.80, SD = 3.91, $P = 0.001$). However, the null hypothesis could not be rejected at 1.2 Myr (mean nDC = 11.35, SD = 2.94, $P = 0.13$) and 1.8 Myr (mean nDC = 7.02, SD = 2.43, $P = 0.66$), thus supporting the Pleistocene glacial cycle-induced climatic shift hypothesis of diversification.

Historical demography

All non-genealogical coalescent methods (i.e., Tajima's D^*, Fu and Li's D^*, and the mismatch distribution) failed to detect significant population expansion in any lineage (Table 3). These methods, however, are weaker than genealogical coalescent methods (e.g., Bayesian skyline plots) because they fail to consider phylogenetic structure (Felsenstein 1992; Pybus *et al.* 2000). Bayesian skyline plots (BSP) show that all

lineages maintained relatively stable population sizes over the last 75 000–120 000 years; no extreme population crashes were present in lineages even considering the 95% HPD around the median BSP lines (Fig. 6). In contrast to the other less-sensitive historical demographic methods, BSPs indicated a slight to moderate amount of population growth in all lineages starting approximately 10 000–12 000 years ago (Fig. 6), which coincides with the beginning of the Holocene (i.e., the start of the current interglacial period).

Discussion

Sky islands are, by definition, separated by areas of disparate environmental conditions that act as barriers to gene flow for resident organisms. Consequently, species occupying sky islands commonly have high levels of inter-population genetic diversity (Knowles 2000; Masta 2000; Smith & Farrell 2005; Carstens & Knowles 2007). In the Ouachita Mountains, *Plethodon ouachitae* is restricted to mesic forest on the tops of six major mountains that are separated by warmer, more xeric valleys. Consistent with the hypothesis that these valleys are barriers to gene flow, we found that *P. ouachitae* is composed of seven divergent lineages structured across these mountains. Winding Stair and Buffalo Mountains were reciprocally monophyletic and with the exception of one individual, the three lineages in the Kiamichi Mountains and Round Mountain were also geographically distinct. These results indicate that all of those lineages have been isolated for an extended period, even over the last few glacial cycles. Two lineages occur on Rich and Black Fork Mountains, and although there is some mixing between these areas, each lineage is still strongly associated with one of the two mountains, suggesting historic isolation of these mountains. Whether gene flow occurs between lineages that

have come back into contact or if any lineages are reproductively isolated is unknown. Hybridization is common among closely related species of *Plethodon* and the frequency of occurrence is related to time since divergence (Weisrock *et al.* 2005; Weisrock & Larson 2006; Wiens *et al.* 2006). A small amount of hybridization in contact zones though is not enough to obscure the historic effects of prolonged isolation in *Plethodon* (Weisrock *et al.* 2005; Weisrock & Larson 2006). It is also possible that gene flow via male dispersal (Jockusch & Wake 2002; Keogh *et al.* 2007) occurs between *P. ouachitae* on different mountains, but we are unable test this with maternally inherited mtDNA.

The hypothesis that niche conservatism drives diversification in sky island species predicts that lineages should be separated by unsuitable conditions (Wiens 2004; Wiens & Graham 2005; Kozak & Wiens 2006). Predictions from our niche model for *P. ouachitae* concluded that most adjacent mountains/lineages are connected by areas where environmental conditions are suitable. Exceptions include Buffalo Mountain, which is disconnected from all other mountains, and Winding Stair Mountain, which is not directly connected to the Kiamichi Mountains. Although niche modelling did not show clear isolation of all mountains/lineages as we predicted, our PCA revealed that environmental conditions in the valleys separating mountains were significantly warmer and drier than conditions where *P. ouachitae* occurs at higher elevations. The valleys separating mountains are at most a few kilometers wide and the resolution of climatic data (~1 km^2) used to construct niche models may be too coarse to assess adequately the environmental factors that affect the distribution of *P. ouachitae*. Factors not included in the niche model such as the availability of rocky microhabitats or the presence of closely related species may also be important in determining the distributions of species of

Plethodon (Pope & Pope 1951; Petranka 1998; Kozak *et al.* 2008). Alternatively, interglacial periods like the present one are predicted to be a time of range expansion in *P. ouachitae* (see below), thus a niche model showing mountains connected by suitable environmental conditions would not be unexpected. Unfortunately, reconstructing the distribution of suitable conditions for *P. ouachitae* during glacial periods in the past (Carstens & Richards 2007; Richards *et al.* 2007; Kozak *et al.* 2008) is not possible with available Pleistocene paleoclimate models because their resolution is too coarse (10 arc-minutes or ~344 km^2) given the small geographic range of *P. ouachitae*.

Insight into the role that niche conservatism had in generating lineage diversity may also be gleaned by examining the spatial and temporal pattern of diversification. We found that diversification in *P. ouachitae* was more consistent with a colonization model involving a series of dispersals from one mountain to another followed by isolation and divergence rather than the fragmentation of a widely distributed common ancestor's range. The east–west orientation of the Ouachita Mountains creates contrasting environmental conditions on north- and south-facing slopes with north-facing slopes typically being mesic and dominated by oak-hickory forest whereas south-facing slopes are more xeric with an abundance of pines (Greller 1988; Foti & Glenn 1991). Because of these characteristics, north–south dispersals in *P. ouachitae* should be more difficult because they would require crossing both low-elevation valleys and south-facing slopes where environmental conditions are warmer and drier (Fig. 4). In support of this prediction, we found that diversification in *P. ouachitae* proceeded in a stepping stone fashion in which colonization occurred primarily on an east–west axis. Diversification in *P. ouachitae* began from west to east in the Kiamichi Mountains and Round Mountain,

and lastly east to west from Rich Mountain to Winding Stair and Buffalo Mountains. Dispersal across the valley that separates the Kiamichi Mountains and Round Mountain from Rich, Winding Stair, and Buffalo Mountains appears to have happened only once, occurring from south to north where the valley is narrowest and highest in elevation. North–south axis dispersals also occur between Rich and Black Fork Mountains; however, this valley is <600 m wide in most places and the elevation reaches 495 m near the eastern edge of Black Fork Mountain.

Climatic changes during Pleistocene glacial cycles are hypothesized to have induced environmental shifts in montane regions resulting in divergence of populations on different mountains (Hewitt 1996, 2004). Consistent with this hypothesis, divergence dates from both the fossil calibrated relaxed phylogenetic method and coalescent simulations indicated that lineage diversification in *P. ouachitae* occurred during the Pleistocene. Although coalescent simulations suggested that the mean date for the earliest divergence within *P. ouachitae* (~0.756 Ma; beginning of the Middle Pleistocene) estimated using the relaxed phylogenetic method may be too recent, they failed to reject the hypothesis that all diversification occurred in the last 1.2 Myr. This earlier date falls within the latter part of the Early Pleistocene rather than in the Middle Pleistocene, but is still within the 95% CI of the mean for the more recent estimate (0.197–1.549 Ma).

Between 1.2 and 0.8 Ma, a climatic change (termed the Middle Pleistocene Transition; MPT) occurred in which the 41-ky glacial cycles that characterized the Late Pliocene and Early Pleistocene shifted to 100-ky cycles with increased amplitude (i.e., greater extremes) in climatic fluctuations (Clark *et al.* 1999, 2006). In these 100-ky cycles, cold glacial periods lasted for most of the cycle and interglacials lasted only 10

000 – 20 000 years (Raymo 1997; Clark *et al.* 2006; Lisiecki & Raymo 2007). During interglacials, climatic conditions in unglaciated eastern North America were generally warmer and wetter, which led to expansion of deciduous forest (King 1973; Davis 1983; Denniston *et al.* 1999). Glacial periods in these same regions were generally colder and drier, leading to contraction of deciduous forest and a dominance of conifers (King 1973; Davis 1983; Jackson *et al.* 2000). The longer glacial and interglacial periods of the 100-ky cycles plus the increased amplitude of climatic fluctuations (i.e., greater temperature and precipitation extremes) after the MPT would have provided both more opportunity for *P. ouachitae* to disperse onto nearby unoccupied mountains (during interglacial periods) and more time for lineages isolated on different mountains to sort (during glacial periods). Diversification in other sky island species such as grasshoppers (genus *Melanoplus*) and butterflies (*Parnassius smintheus*) in the northern Rocky Mountains also occurred within a similar timeframe (Knowles 2000, 2001a,b; DeChaine & Martin 2004, 2006; Carstens & Knowles 2007).

Climatic changes that result in range expansion or contraction are expected to cause changes in N_e, leading to increases or decreases in levels of genetic variation and coalescence times (Wakeley 2000; Jesus *et al.* 2006). Because alternating periods of dispersal (i.e., range expansion) and isolation (i.e., range contraction) are evident in the evolutionary history of *P. ouachitae*, which appear to be linked to climatic fluctuations associated with Pleistocene glacial cycles, we expected to observe demographic changes over time in lineages on the different sky islands. However, population sizes in all lineages remained stable over the last 120 000 years and show only slight to moderate increases since approximately 10 000–12 000 years ago (i.e., the beginning of the

Holocene, the current interglacial period). The long-term stability in N_e in *P. ouachitae* suggests that population sizes were not negatively affected by climatic changes during glacial periods. Other North American ectothermic vertebrates have also exhibited a similar pattern of long-term population stability or growth only after the retreat of the last ice sheet (e.g., *Diadophis punctatus*: Fontanella *et al.* 2008; *Agkistrodon contortrix* & *Agkistrodon piscivorus*: Guiher & Burbrink 2008). Other studies on plethodontid salamanders have found evidence for recent population growth, but they were not able to place a timeframe on these changes (e.g., Carstens *et al.* 2004b; Mahoney 2004; Kozak *et al.* 2006b; Martínez-Solano *et al.* 2007). Population sizes of *P. ouachitae* may have declined during glacial periods, but this pattern could have been obscured by an increase in genetic variation and mean coalescence time due to population subdivision within a mountain (Wakeley 2000; Jesus *et al.* 2006). We found a high level of geographic structuring (i.e., population subdivision) within most lineages suggesting that this could be the case.

The high lineage diversity in *P. ouachitae* generated over a relatively short amount of time in a small area (approximately 1000 km^2) seems quite remarkable. However, these observations are not uncommon in *Plethodon* where several closely related species can occur within a small area on adjacent mountain tops, and some species may be restricted to single mountains (Highton 1995). The *glutinosus* group, of which *P. ouachitae* is a member, is the most speciose group of *Plethodon*, consisting of roughly 30 species distributed primarily throughout forests of the eastern United States, mostly in the Appalachian Mountains (Highton 1995; Kozak *et al.* 2006a; Wiens *et al.* 2006). Kozak *et al.* (2006a) found a rapid rate of diversification in the *glutinosus* group

early in its evolutionary history, but a slower rate toward the present. They proposed that the early rapid diversification was due to repeated bouts of dispersal to new unoccupied habitats followed by periods of isolation and divergence, and that the slower diversification rate toward the present occurred as available habitats became filled. Unfortunately, the large geographic area and high number of species, and thus the higher potential for historical patterns to be influenced by complex interspecies interactions (e.g., Crespi *et al.* 2003), make this hypothesis difficult to test in the Appalachian Mountains. In contrast, the Ouachita Mountains occupy a smaller geographic area and have a lower species richness of *Plethodon*, and thereby provide an opportunity to test this idea. Our results for *P. ouachitae* support a stepping stone colonization model and demonstrate that repeated bouts of dispersal to new areas followed by isolation resulted in a rapid accumulation of lineages within a small geographic area, and thus support the hypothesis of Kozak *et al.* (2006a). The restriction of *P. ouachitae* to higher elevations, and subsequent lineage isolation, could be influenced by competitive interactions with other members of the *glutinosus* group, which occur in the lower elevations of the Ouachita Mountains (e.g., *P. albagula* and *P. kiamichi*). However, this is unlikely because *P. ouachitae* in the Kiamichi Mountains and on Round Mountain are syntopic with *P. kiamichi* and they occur at nearly equal densities at several localities (Duncan & Highton 1979; D.B. Shepard, unpublished data). Further, behavioral experiments have shown that *P. ouachitae*, although smaller in size, is more aggressive and is able to exclude the larger *P. albagula* from cover objects (Anthony *et al.* 1997).

Our results provide further evidence that climatic changes during the Pleistocene had profound effects on lineage diversification and demography in species from

environmentally sensitive habitats such as sky islands. Whether a montane region is a sky island situation ultimately depends on the ecology of the organism under study. The contrast in environmental conditions at high and low elevations in the Madrean sky islands of the desert southwest U.S. is extreme (pine-oak forest vs. desert); however, environmental conditions at high and low elevations in the Ouachita Mountains are not as disparate. This disparity, however, appears to be enough to create a sky island situation given the temperature and moisture requirements of *P. ouachitae* (Spotila 1972). Our study is the first detailed phylogeographic study of a terrestrial organism inhabiting the Ouachita Mountains, thus it is unknown whether these mountains act as sky islands for other mesic-adapted organisms endemic to this region (e.g., plants, snails). This information is important for conservation because many currently recognized taxa may actually be composed of several morphologically cryptic species. Although most of the range of *P. ouachitae* is within the Ouachita National Forest, and thus theoretically protected, logging is a threat and management practices may need to be modified in order to conserve all evolutionary lineages.

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Table 1 Results from Principal Components Analysis on climatic variables used in niche modelling and comparison of climatic conditions between occurrence locations for *Plethodon ouachitae* and pseudoabsence locations in the intervening valleys.

Variable	PC1	PC2
BIO2 (Mean Diurnal Range)	0.9089	-0.3328
BIO3 (Isothermality)	0.6897	-0.6529
BIO4 (Temperature Seasonality)	0.9331	0.3178
BIO5 (Max Temperature of Warmest Month)	0.9925	0.0134
BIO6 (Min Temperature of Coldest Month)	-0.5780	0.1613
BIO8 (Mean Temperature of Wettest Quarter)	0.9728	0.1319
BIO9 (Mean Temperature of Driest Quarter)	0.8708	-0.1473
BIO13 (Precipitation of Wettest Month)	-0.0365	0.8918
BIO15 (Precipitation Seasonality)	0.7369	0.6479
BIO16 (Precipitation of Wettest Quarter)	-0.8320	0.3476
BIO17 (Precipitation of Driest Quarter)	-0.8083	-0.4105
BIO18 (Precipitation of Warmest Quarter)	-0.9935	-0.0488
Eigenvalue	8.0734	2.2102
% Variance Explained	67.2780	18.4185

Table 2 Proportional likelihoods for the area of origin for ancestral nodes and the most recent common ancestor of each extant lineage of *Plethodon ouachitae* (see Fig. 5 to reference node numbers). Numbers in bold represent the area with the highest likelihood for the given node.

Node	Kiamichi Mtns	Round Mtn	Rich Mtn	Black Fork Mtn	Winding Stair Mtn	Buffalo Mtn	W Fourche Mtn
1	NA	NA	NA	NA	NA	NA	NA
2	**0.402**	0.078	0.126	0.098	0.116	0.104	0.076
3	**0.334**	0.080	0.149	0.108	0.135	0.117	0.077
4	0.148	0.062	**0.234**	0.137	0.200	0.158	0.061
5	**0.622**	0.055	0.073	0.062	0.069	0.064	0.054
6	0.055	0.031	**0.330**	0.088	0.270	0.197	0.030
7	**0.999**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
8	**0.999**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
9	**0.869**	0.110	0.004	0.004	0.004	0.004	0.004
10	<0.001	0.001	<0.001	**0.996**	<0.001	<0.001	<0.001
11	0.017	0.014	0.055	0.022	**0.841**	0.037	0.014
12	<0.001	<0.001	<0.001	<0.001	<0.001	**0.999**	<0.001
13	0.033	0.024	**0.609**	0.103	0.118	0.089	0.023

Table 3 Nucleotide diversity (π), average number of pairwise differences (K), and results of Tajima's D^*, Fu and Li's D^*, and mismatch distribution analyses (R_2) for each lineage of *Plethodon ouachitae* calculated for all sites of the concatenated dataset. All results failed to reject the null hypothesis of constant population size (all P values > 0.10).

Lineage	π	K	Tajima's D^*	Fu & Li's D^*	R_2
Kiamichi West	0.0011	1.9636	-0.7238	-1.2307	0.1599
Kiamichi East	0.0013	2.3636	-0.5570	-0.4623	0.1764
Round Mtn	0.0040	6.6646	-1.1027	-1.0954	0.0857
Black Fork Mtn	0.0059	10.5751	-0.3566	-1.8639	0.1005
Rich Mtn	0.0112	9.9259	-0.6362	-1.5954	0.0748
Winding Stair Mtn	0.0084	10.4983	-0.2078	-1.6055	0.1060
Buffalo Mtn	0.0025	4.3333	-1.2135	-0.5526	0.0750

Figure legends

Fig. 1 Topographic map of the United States showing the location of the Ouachita Mountains (A), all elevations >450 m in the range of *Plethodon ouachitae* illustrating isolation of major mountains (B), and digital elevation map of the Ouachita Mountains with sampling localities for *Plethodon ouachitae* color-coded by lineage (C). Localities with more than one lineage present are colored half-and-half for the lineages that were present. Elevation within this region ranges from a low of 135 m (black) to a high of 818 m (white).

Fig. 2 Population trees representing the two biogeographic hypotheses for diversification within *Plethodon ouachitae* tested using coalescent simulations. The Fragmented Ancestor model posits that all population divergences were concurrent and resulted from the fragmentation of a widely distributed common ancestor's range. The Colonization model posits a history involving a series of dispersals from one mountain to another followed by isolation and divergence. The Colonization model was rooted based on results for the most likely area of origin for *P. ouachitae* and then structured based on a hypothesized stepping stone pattern. Branch lengths are time in generations based on a three year generation time in *P. ouachitae*. Branch widths (N_e) are scaled for each mountain based on the proportion of the Total N_e that each mountain comprised (listed below mountain name). Internal branches on the Colonization model were scaled such that all branch widths summed to Total N_e at any single point in time.

Fig. 3 Maximum likelihood (ML) tree produced using the 7(GTR + Γ) model for 281 individuals of *Plethodon ouachitae* and outgroups for 1816 bp of the mitochondrial cytb and ND4 genes, and tRNA-His. Values above branches indicate support from 1000 nonparametric bootstraps and values below branches are Bayesian posterior probabilities based on 16 000 post burn-in trees using the 7(GTR + Γ + I) model. Major lineages are indicated. Samples are labeled by mountain and voucher number (Appendix).

Fig. 4 Ecological niche model for *Plethodon ouachitae* constructed with 12 climatic variables at 30 arc-seconds resolution (~1 km^2) and our 55 sampling points in Maxent v.3.2.1 (Phillips *et al*. 2006). A map of all elevations >450 m (black) is overlaid by the niche model to show areas of predicted suitable (gray) and unsuitable (white) environmental conditions. Inset is the result from comparison of environmental conditions at sampling points for *P. ouachitae* versus random pseudoabsence points in the valleys showing conditions in the valleys are significantly warmer and drier than points at higher elevations where *P. ouachitae* occurs.

Fig. 5 Simplified tree showing mean divergence dates and 95% Credible Intervals (Ma) for major nodes and the time to the most recent common ancestor for major lineages within *Plethodon ouachitae*. Nodes are numbered for reference in Table 2.

Fig. 6 Bayesian skyline plots (Drummond *et al.* 2005) showing the demographic history of the seven lineages of *Plethodon ouachitae*. The 120 000 year-period on the X axis encompasses one complete glacial cycle from the latter part of the Sangamon interglacial,

through the entire Wisconsin glaciation, and to the present interglacial period, the Holocene. The central line represents the median value for the $\log_{10}$ of the population size ($N_e * \tau$) and the shaded area represents the 95% Highest Posterior Density. The MRCA of both the Kiamichi West and Kiamichi East lineages was <120 000 years ago, thus they are only shown for the last 75 000 years.

Fig. 1

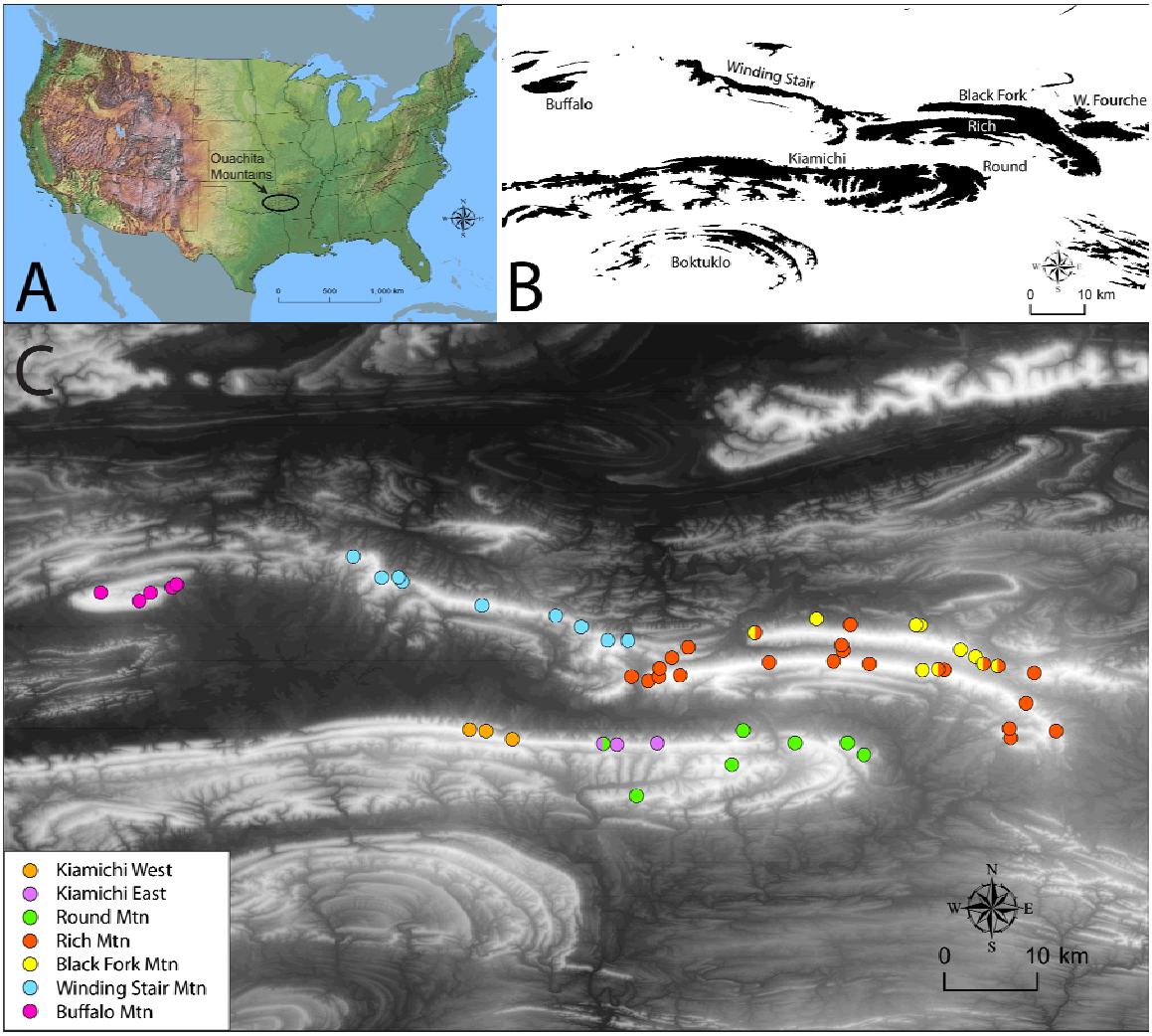

A
Ouachita Mountains
B
Buffalo
Winding Stair
Black Fork
W. Fourche
Rich
Kiamichi
Round
Boktuklo
0
10 km
C
Kiamichi West
Kiamichi East
Round Mtn
Rich Mtn
Black Fork Mtn
Winding Stair Mtn
Buffalo Mtn
N
W
E
S
0
10 km

Fig. 2

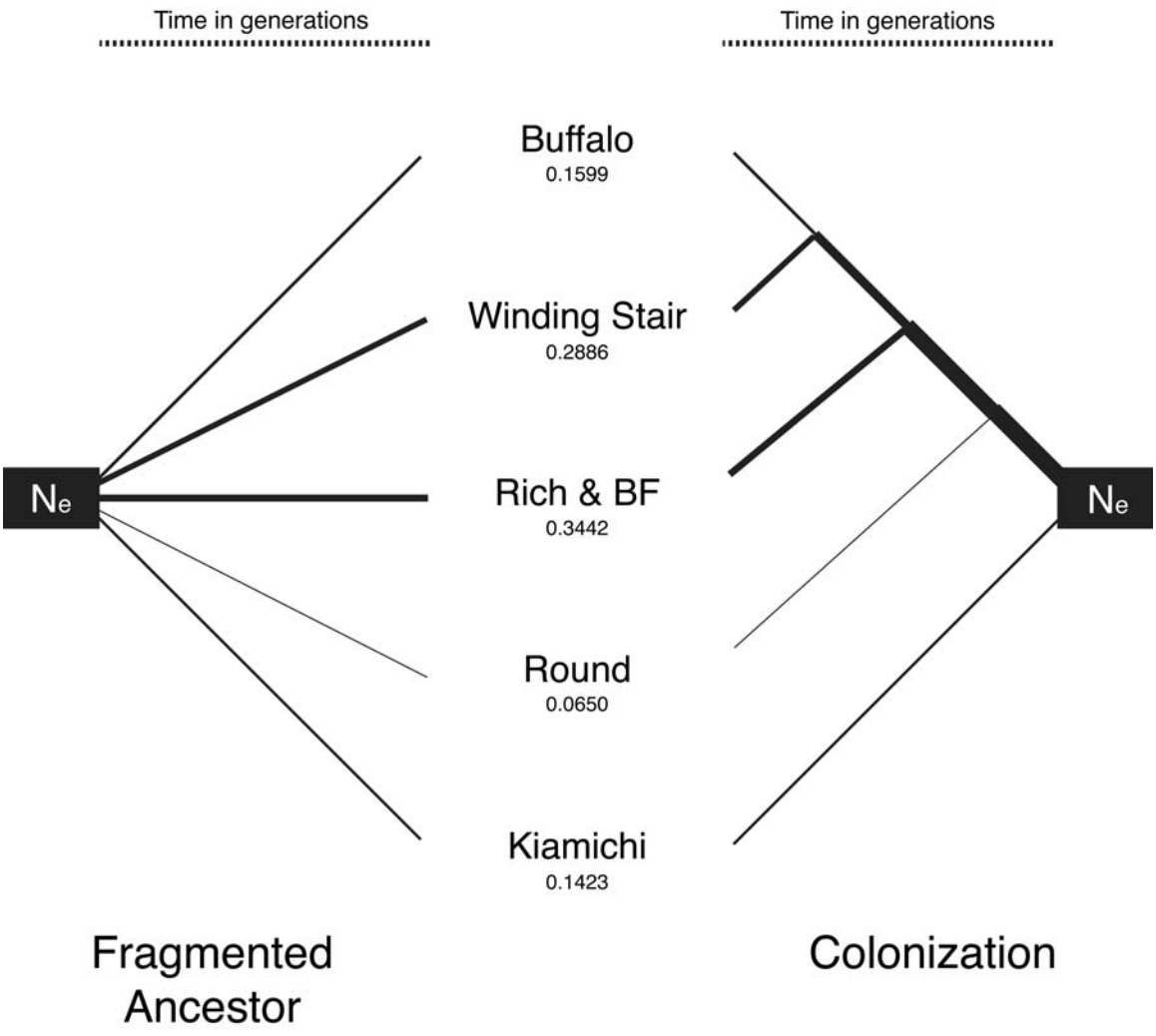

Time in generations
Time in generations
Buffalo
0.1599
Winding Stair
0.2886
Rich & BF
0.3442
Round
0.0650
Kiamichi
0.1423
Ne
Ne
Fragmented Ancestor
Colonization

Fig. 3

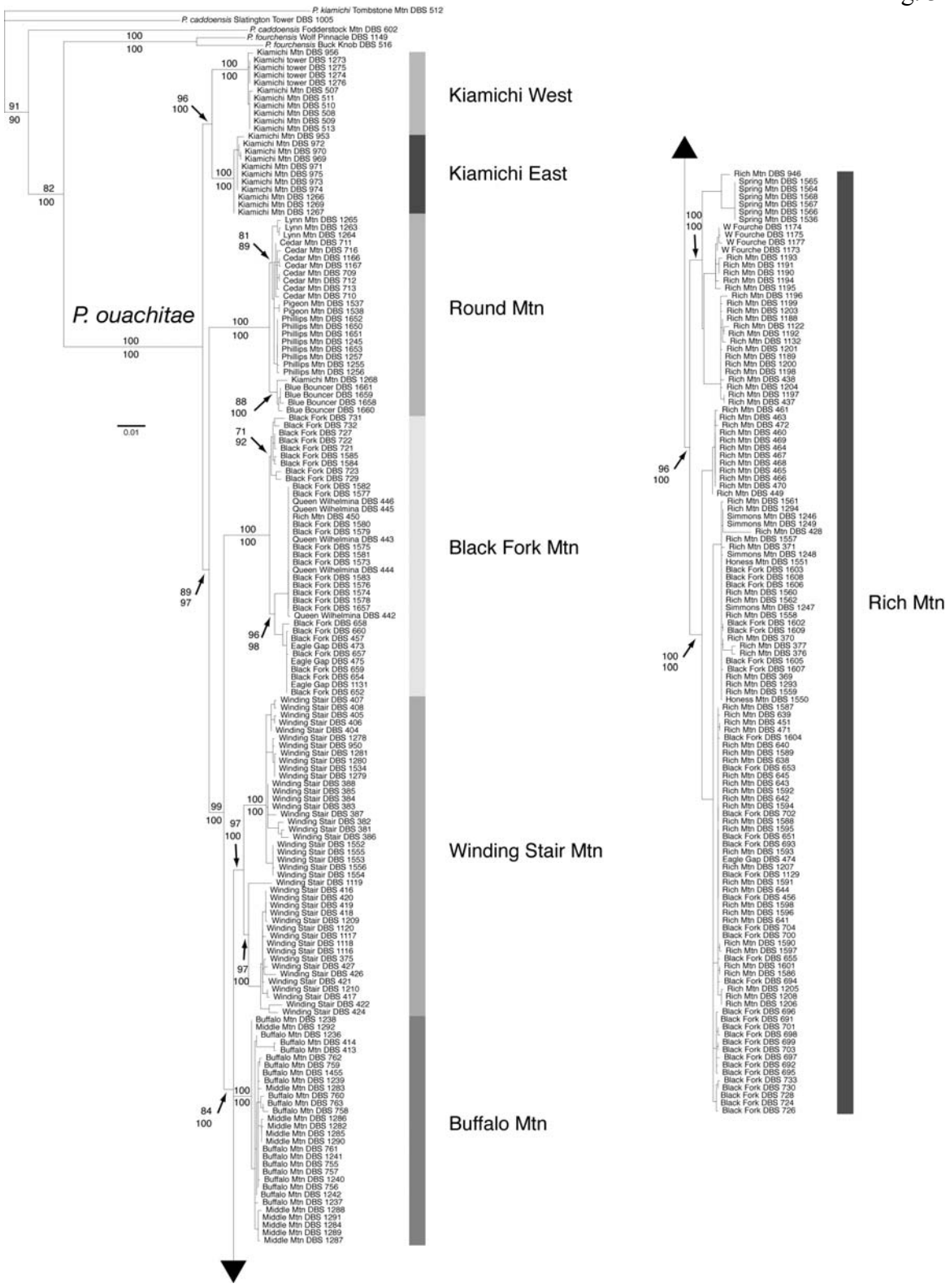

P. kiamichi Tombstone Mtn DBS 512
P. caddoensis Slatington Tower DBS 1005
P. caddoensis Fodderstock Mtn DBS 602
P. fourchensis Wolf Pinnacle DBS 1149
P. fourchensis Buck Knob DBS 516
P. ouachitae
0.01
Kiamichi West
Kiamichi East
Round Mtn
Black Fork Mtn
Winding Stair Mtn
Buffalo Mtn
Rich Mtn

Fig. 4

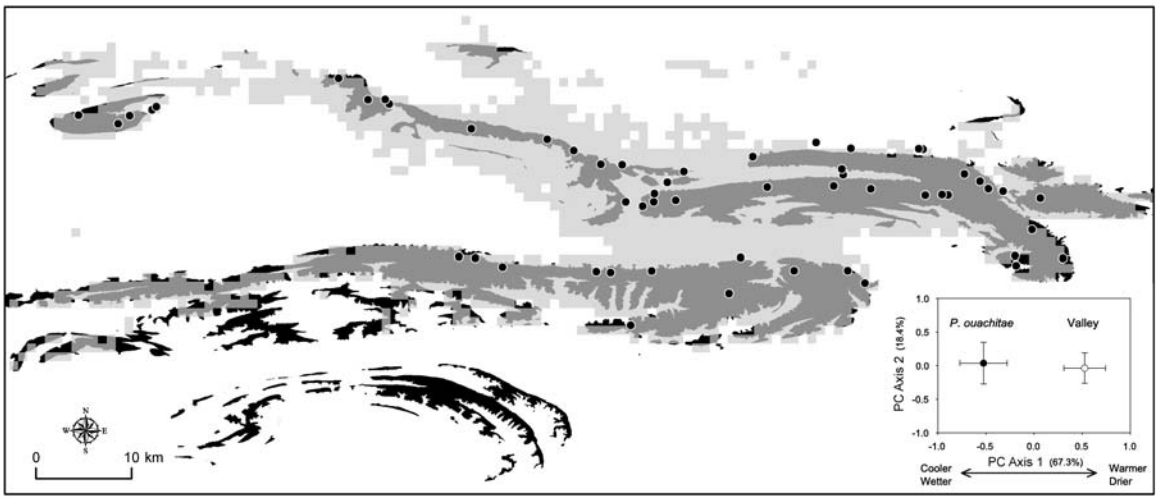

0
10 km
P. ouachitae
Valley
PC Axis 2 (18.4%)
PC Axis 1 (67.3%)
Cooler
Wetter
Warmer
Drier

Fig. 5

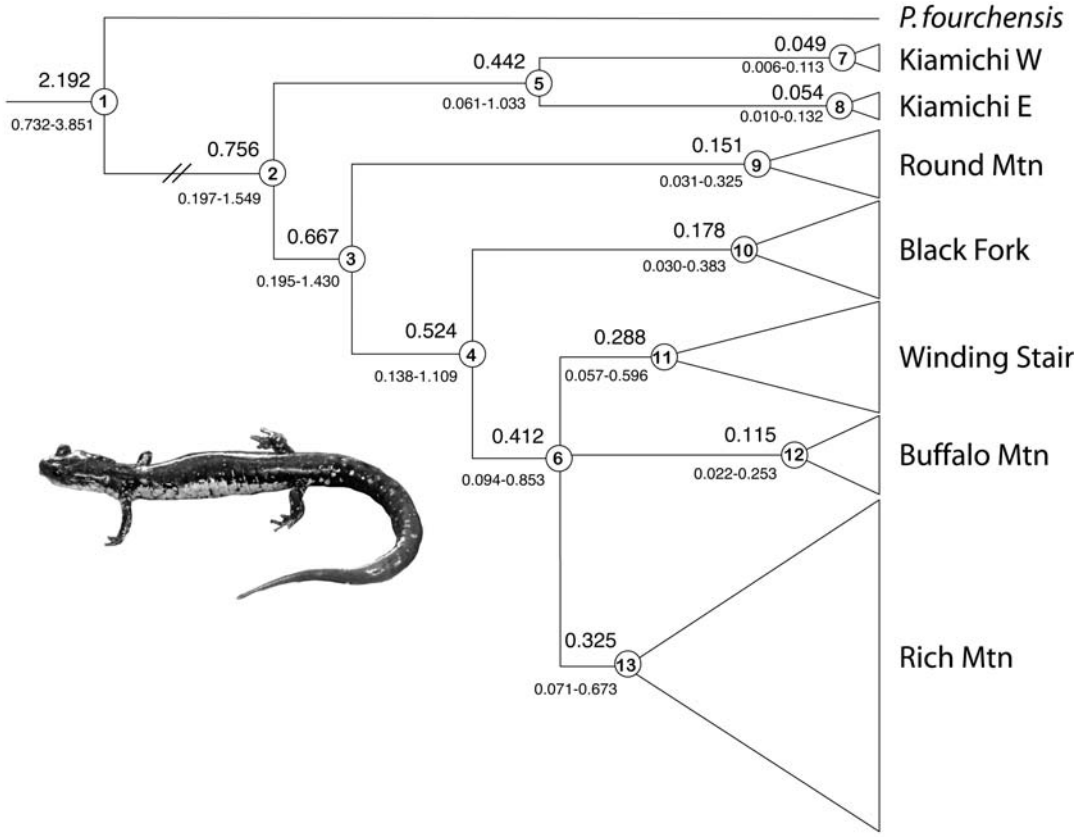
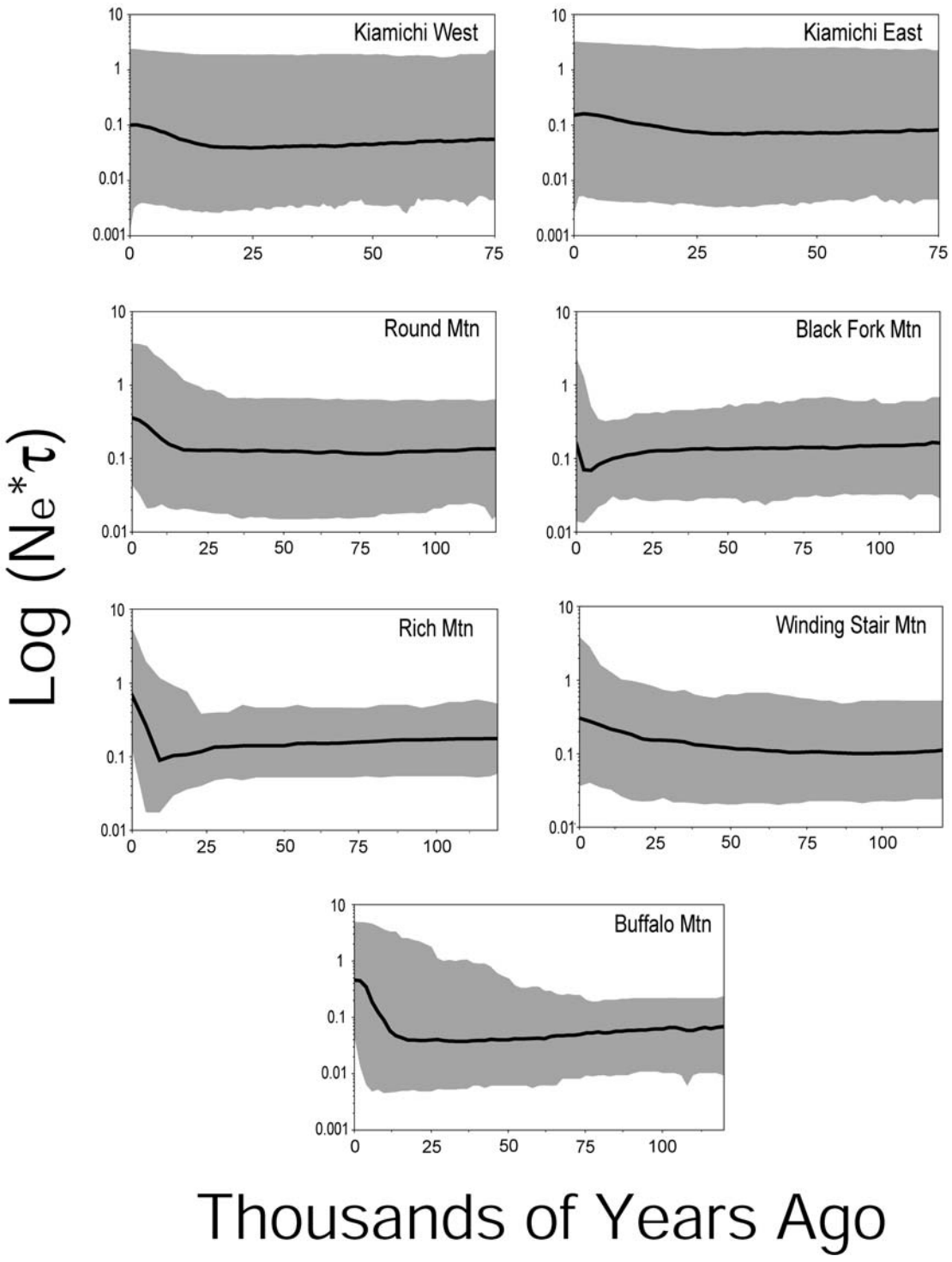

P. fourchensis
Kiamichi W
Kiamichi E
Round Mtn
Black Fork
Winding Stair
Buffalo Mtn
Rich Mtn
2.192
1
0.732-3.851
0.756
2
0.197-1.549
0.667
3
0.195-1.430
0.524
4
0.138-1.109
0.442
5
0.061-1.033
0.049
7
0.006-0.113
0.054
8
0.010-0.132
0.151
9
0.031-0.325
0.178
10
0.030-0.383
0.288
11
0.057-0.596
0.412
6
0.094-0.853
0.115
12
0.022-0.253
0.325
13
0.071-0.673

Fig. 6


Kiamichi West
Kiamichi East
Round Mtn
Black Fork Mtn
Rich Mtn
Winding Stair Mtn
Buffalo Mtn
Log (Ne*τ)
Thousands of Years Ago

Appendix. Voucher numbers, sample sizes (*N*), sample localities, geographic coordinates, and GenBank accession numbers for the sequences obtained for this study (DBS: Donald B. Shepard,).

Voucher Nos.	*N*	Lineage	Locality	Latitude	Longitude	Elev. (m)	GenBank No. cytb	GenBank No. ND4
DBS 657	1	Black Fork	Black Fork Mountain, along Black Fork Mtn Wilderness Trail, Polk County, Arkansas	34.69912	-94.32265	686	FJ266969	FJ267244
DBS 658–660	3	Black Fork	Black Fork Mountain, along Black Fork Mtn Wilderness Trail, Polk County, Arkansas	34.70601	-94.33724	758	FJ266970-72	FJ267245-47
DBS 1578–1583, 1657	7	Black Fork	Black Fork Mountain, S of FR 242 along Price Creek, Scott County, Arkansas	34.72940	-94.37607	363	FJ266852-57, FJ266886	FJ267138-43, FJ267172
DBS 1573–1577	5	Black Fork	Black Fork Mountain, S of FR 242 on N slope along Price Creek, Scott County, Arkansas	34.72989	-94.38028	376	FJ266847-51	FJ267133-37
DBS 1584–1585	2	Black Fork	Black Fork Mountain, S of FR 894 on N slope along Mitchell Creek, LeFlore County, Oklahoma	34.73567	-94.47664	296	FJ266858-59	FJ267144-45

DBS 442–446	5	Black Fork	Queen Wilhelmina State Park, Rich Mountain, Spring Trail below Wonder House, Polk County, Arkansas	34.68607	-94.37404	778	FJ266925-29	FJ267211-15
DBS 721–724, 726–733	7 5	Black Fork Rich Mtn	Black Fork Mountain, NE of Page, LeFlore County, Oklahoma	34.72229	-94.53616	401	FJ266993-7004	FJ267268-79
DBS 456–457, 473–475, 704, 1129, 1131	4 4	Black Fork Rich Mtn	Eagle Gap between E end of Black Fork Mtn and W end of Fourche Mtn along Ouachita Trail, Polk County, Arkansas	34.68991	-94.30086	427	FJ266933-34, FJ266947-49, FJ266986, FJ266750-51	FJ267219-20, FJ267222-24, FJ267261, FJ267036-37
DBS 413–414, 1236–1238	5	Buffalo Mtn	Buffalo Mountain, 5.26 mi W of Talihina, Latimer County, Oklahoma	34.75328	-95.13254	580	FJ266910-11, FJ266781-83	FJ267196-97, FJ267067-69
DBS 755–763	9	Buffalo Mtn	Buffalo Mountain, 6.6 km W of Talihina, Bear Den Hollow, Latimer County, Oklahoma	34.76108	-95.12178	481	FJ267005-13	FJ267280-88
DBS 1455	1	Buffalo Mtn	Buffalo Mountain, Devil's Hollow, Latimer County, Oklahoma	34.76949	-95.09667	277	FJ266824	FJ267110
DBS 1239–1242	4	Buffalo Mtn	Buffalo Mountain, E end, N slope above Devil's Hollow, Latimer County, Oklahoma	34.76658	-95.10081	395	FJ266784-87	FJ267070-73
DBS 1282–1292	11	Buffalo Mtn	Middle Mountain, W end, NW slope above tributary to Buffalo Creek, Latimer County, Oklahoma	34.76155	-95.16966	539	FJ266811-21	FJ267097-107

DBS 953	1	Kiamichi E	Kiamichi Mountains, 1.1 km W of Three Sticks Monument/Hwy 259, LeFlore County, Oklahoma	34.61364	-94.66984	646	FJ267016	FJ267291
DBS 969–975	7	Kiamichi E	Kiamichi Mountains, 3.8 km SSE of Big Cedar, ~135 m E of Hwy 259, LeFlore County, Oklahoma	34.61508	-94.63116	525	FJ267018-24	FJ267293-99
DBS 1266–1269	3 1	Kiamichi E Round Mtn	Kiamichi Mountains, 2.3 km W of Three Sticks Monument/Hwy 259, N of FR 6025 along FR 6024, LeFlore County, Oklahoma	34.61433	-94.68319	601	FJ266799-802	FJ267085-88
DBS 956	1	Kiamichi W	Kiamichi Mountains, 1.1 km E of CR291 on CR254/FR6025, LeFlore County, Oklahoma	34.61858	-94.77128	637	FJ267017	FJ267292
DBS 1273–1276	4	Kiamichi W	Kiamichi Mountains, N slope below Kiamichi Tower on FR 6025, LeFlore County, Oklahoma	34.62824	-94.81229	687	FJ266803-06	FJ267089-92
DBS 507–511, 513	6	Kiamichi W	Kiamichi Mountains, Tombstone Mountains Rd/FR252C near lookout, LeFlore County, Oklahoma	34.62697	-94.79690	533	FJ266950-55	FJ267225-30
DBS 437–438, 1196–1201	8	Rich Mtn	4.02 mi NW of Mena, FR 506 and Rock Creek between Round and Middle Mountains, Polk County, Arkansas	34.61987	-94.28862	384	FJ266923-24, FJ266767-72	FJ267209-10, FJ267053-58

DBS 1203–1204	2	Rich Mtn	4.02 mi NW of Mena, FR 506 and Rock Creek between Round and Middle Mountains, Polk County, Arkansas	34.62916	-94.28970	416	FJ266773-74	FJ267059-60
DBS 691–703	13	Rich Mtn	Black Fork Mountain, ~0.8 km E of state line and ~ 0.6 km S of FR 894 along tributary of Mitchell Creek, Scott County, Arkansas	34.73013	-94.44389	345	FJ266973-85	FJ267248-60
DBS 1602–1609	8	Rich Mtn	Black Fork Mountain, S slope above Hwy 270 ~0.25 km E of AR/OK stateline, Polk County, Arkansas	34.71060	-94.45238	418	FJ266874-81	FJ267160-67
DBS 1173–1175, 1177	4	Rich Mtn	Fourche Mountain, 3.33 km E of Eagle Gap, Polk County, Arkansas	34.68334	-94.26581	702	FJ266755-58	FJ267041-44
DBS 1550–1551	2	Rich Mtn	Honess Mountain, ~0.72 km SE of Kerr Nature Center parking lot off of nature trail, LeFlore County, Oklahoma	34.69812	-94.61645	442	FJ266829-30	FJ267115-16
DBS 1205–1208	4	Rich Mtn	Rich Mountain, 0.4 km W of stateline on N slope below Hwy 1/Talimena Drive, LeFlore County, Oklahoma	34.69469	-94.46003	771	FJ266775-78	FJ267061-64
DBS 1122, 1132, 1188–1193	8	Rich Mtn	Rich Mountain, Blue Haze Vista, Polk County, Arkansas	34.62677	-94.24461	596	FJ266749, FJ266752, FJ266759-64	FJ267035, FJ267038, FJ267045-50

DBS 1194–1195	2	Rich Mtn	Rich Mountain, Eagleton Vista, Polk County, Arkansas	34.65370	-94.27376	724	FJ266765-66	FJ267051-52
DBS 460–461, 463–472	12	Rich Mtn	Rich Mountain, Hwy 272 between Hwy 1 and Hwy 270, Polk County, Arkansas	34.68627	-94.35253	600	FJ266935-46	FJ267221
DBS 638–645	8	Rich Mtn	Rich Mountain, jct. of Hwy 88 (Talimena Drive) and FR 514, N slope, Polk County, Arkansas	34.69204	-94.42533	797	FJ266956-63	FJ267231-38
DBS 1586–1598, 1601	14	Rich Mtn	Rich Mountain, N slope above Hwy 270 ~0.4 km E of AR/OK state line, Polk County, Arkansas	34.70536	-94.45111	386	FJ266860-73	FJ267146-59
DBS 1293–1294	2	Rich Mtn	Rich Mountain, N slope across from Pine Mountain Vista, LeFlore County, Oklahoma	34.69370	-94.52264	714	FJ266822-23	FJ267108-09
DBS 946	1	Rich Mtn	Rich Mountain, Vertao Electronic Site off Talimena Drive/Hwy 1, LeFlore County, Oklahoma	34.68108	-94.60857	784	FJ267014	FJ267289
DBS 1557–1562	6	Rich Mtn	Rich Mountain, W end along FR 6068 at tributary to Big Cedar Creek, LeFlore County, Oklahoma	34.67554	-94.63972	432	FJ266836-41	FJ267122-27
DBS 376–377, 428	3	Rich Mtn	Rich Mountain, W end along Talimena Drive/Hwy 1, LeFlore County, Oklahoma	34.68750	-94.62863	499	FJ266895-96, FJ266922	FJ267181-82, FJ267208

DBS 369–371	3	Rich Mtn	Rich Mountain,Talimena Drive/Hwy 1 just E of Sunset Point Vista, LeFlore County, Oklahoma	34.67955	-94.62919	650	FJ266891-93	FJ267177-79
DBS 1246–1249	4	Rich Mtn	Simmons Mountain, 2.96 km N of Big Cedar along FR 6023, LeFlore County, Oklahoma	34.67974	-94.65552	329	FJ266789-92	FJ267075-78
DBS 1536, 1564–1568	6	Rich Mtn	Spring Mountain, 3.8 road km (2.35 mi) E of Hwy 1/Talimena Drive on Spring Mtn Rd/FR 6007, LeFlore County, Oklahoma	34.70839	-94.60093	454	FJ266826, FJ266842-46	FJ267112, FJ267128-32
DBS 651–655	3 2	Rich Mtn Black Fork	Black Fork Mountain, along Black Fork Mtn Wilderness Trail, Polk County, Arkansas	34.69226	-94.31475	595	FJ266964-68	FJ267239-43
DBS 449–451	2 1	Rich Mtn Black Fork	Rich Mountain, along Hwy 272 between Hwy 1 and Hwy 270, Polk County, Arkansas	34.68669	-94.35830	666	FJ266930-32	FJ267216-18
DBS 1658–1661	4	Round Mtn	Blue Bouncer Mountain, W end near Saddle Gap, N slope below road, LeFlore County, Oklahoma	34.56381	-94.65093	525	FJ266887-90	FJ267173-76
DBS 709–713, 716	6	Round Mtn	Cedar Mountain, 19.2 km W of Mena, S of FR 421 along Nichols Branch, Polk County, Arkansas	34.61528	-94.44688	482	FJ266987-92	FJ267262-67

DBS 1166–1167	2	Round Mtn	Cedar Mountain, 3.1 km S of Mountain Fork, 1.4 km SSW of FR 421 along stream, Polk County, Arkansas	34.60366	-94.43076	465	FJ266753-54	FJ267039-40
DBS 1537–1538	2	Round Mtn	Kiamichi Mountains, W of FR 6026/Pigeon Creek Rd at N base of Pigeon Mtn along FR K53A, LeFlore County, Oklahoma	34.62747	-94.54755	399	FJ266827-28	FJ267113-14
DBS 1263–1265	3	Round Mtn	Lynn Mountain, N slope below FR 6025 along tributary to Pigeon Creek, LeFlore County, Oklahoma	34.59387	-94.55838	691	FJ266796-98	FJ267082-84
DBS 1245, 1255–1257, 1650–1653	8	Round Mtn	Phillips Mountain, N slope below FR 6025, LeFlore County, Oklahoma	34.61537	-94.49725	655	FJ266788, FJ266793-95, FJ266882-85	FJ267074, FJ267079-81, FJ267168-71
DBS 381–388	8	Winding Stair	Winding Stair Mountain at Trailhead/Backpacker's Camp just W of Campground off Talimena Drive/Hwy 1, LeFlore County, Oklahoma	34.71504	-94.67884	583	FJ266897-904	FJ267183-90
DBS 404–408	5	Winding Stair	Winding Stair Mountain, 0.25 mi N of Hwy 1/Talimena Drive on Tall Cedar Rd, LeFlore County, Oklahoma	34.72803	-94.70453	483	FJ266905-08	FJ267191-95

DBS 420–422, 424	4	Winding Stair	Winding Stair Mountain, 0.4 mi N of Hwy 1/Talimena Drive on CR D4585/Deadman Trail Rd, LeFlore County, Oklahoma	34.77641	-94.88160	422	FJ266916-19	FJ267202-05
DBS 950, 1278–1281, 1534	6	Winding Stair	Winding Stair Mountain, 0.8 km W of Hwy 259 and ~200 m N of Hwy 1/Talimena Drive, LeFlore County, Oklahoma	34.71474	-94.65899	423	FJ267015, FJ266807-10, FJ266825	FJ267290, FJ267093-96, FJ267111
DBS 375, 426–427	3	Winding Stair	Winding Stair Mountain, 100 m S of Hwy 1/Talimena Drive on Lenox Ridge Rd, LeFlore County, Oklahoma	34.74874	-94.80066	670	FJ266894, FJ266920-21	FJ267180, FJ267206-07
DBS 416–419	4	Winding Stair	Winding Stair Mountain, 50 m N of Hwy 1/Talimena Drive on CR D4585/Deadman Trail Rd, LeFlore County, Oklahoma	34.77215	-94.87778	475	FJ266912-15	FJ267198-201
DBS 1552–1556	5	Winding Stair	Winding Stair Mountain, Horsethief Springs Picnic Area, ~0.25 km NW of lower parking area along Ouachita/Horsethief Springs trail, LeFlore County, Oklahoma	34.73882	-94.72930	521	FJ266831-35	FJ267117-21
DBS 1209–1210	2	Winding Stair	Winding Stair Mountain, Sycamore Tower, LeFlore County, Oklahoma	34.77620	-94.89785	708	FJ266779-80	FJ267065-66

DBS 1116–1120	5	Winding Stair	Winding Stair Mtn, N slope across from Potato Hills Vista on Hwy 1/Talimena Drive, LeFlore County, Oklahoma	34.79631	-94.92545	540	FJ266744-48	FJ267030-34
DBS 602	1	*Plethodon caddoensis*	CR79/FR140 near Fodderstock Mtn, Polk County, Arkansas	34.43742	-94.17537	345	FJ266742	FJ267028
DBS 1005	1	*Plethodon caddoensis*	Mosquito Gap, 1.0 km W of CR1 along road to Slatington Tower, Montgomery County, Arkansas	34.43402	-93.89666	661	FJ266743	FJ267029
DBS 516	1	*Plethodon fourchensis*	Fourche Mountain, Buck Knob, along FR 76A ~0.5 mi from top, Scott County, Arkansas	34.68304	-93.94145	599	FJ266740	FJ267026
DBS 1149	1	*Plethodon fourchensis*	Fourche Mountain, along Ouachita Trail ~3.2 km W of Wolf Pinnacle, Polk County, Arkansas	34.68457	-94.15258	602	FJ266741	FJ267027
DBS 512	1	*Plethodon kiamichi*	Kiamichi Mountains, Tombstone Mountains Rd/FR252C near lookout, LeFlore County, Oklahoma	34.62697	-94.79690	533	FJ266739	FJ267025

Chapter II

Phylogeographic and demographic effects of Pleistocene climatic fluctuations in a montane salamander, *Plethodon fourchensis*

(formatted for *Molecular Ecology*)

Abstract

Climatic changes associated with Pleistocene glacial cycles profoundly affected species distributions, patterns of inter-population gene flow, and demography. In species restricted to montane habitats, ranges may expand and contract along elevational gradients in response to environmental fluctuations and create high levels of genetic variation among populations on different mountains. The salamander *Plethodon fourchensis* is restricted to high-elevation, mesic forest on five montane isolates in the Ouachita Mountains. We used DNA sequence data along with ecological niche modelling and coalescent simulations to test several hypotheses related to the effects of Pleistocene climatic fluctuations on species in montane habitats. Our results revealed that *P. fourchensis* is composed of four well-supported, geographically structured lineages. Geographic breaks between lineages occurred in the vicinity of major valleys and a high-elevation pass. Ecological niche modelling predicted that environmental conditions in valleys separating most mountains are suitable; however, interglacial periods like the present are predicted to be times of range expansion in *P. fourchensis*. Divergence dating and coalescent simulations indicated that lineage diversification occurred during the Middle Pleistocene via the fragmentation of a wide-ranging ancestor. Bayesian skyline plots showed gradual decreases in population size in three of four lineages over the most recent glacial period and a slight to moderate amount of population growth during the Holocene. Our results not only demonstrate that climatic changes during the Pleistocene had profound effects on species restricted to montane habitats, but comparison of our results for *P. fourchensis* with its parapatric, sister taxon, *P. ouachitae*, emphasizes how responses can vary substantially even among closely

related, similarly distributed taxa.

Introduction

Climatic changes associated with Pleistocene glacial cycles profoundly affected species distributions and patterns of inter-population gene flow (Hewitt 1996, 2000, 2004; Jansson & Dynesius 2002). These effects were perhaps most pronounced in montane regions in temperate zones where glaciers displaced organisms, but species in many unglaciated montane regions were also impacted (Maddison & McMahon 2000; Knowles 2000, 2001; Masta 2000; Carstens *et al.* 2005a; Smith & Farrell 2005; Shepard & Burbrink 2008). Changing climatic conditions in montane regions can cause favorable environments for a species to shift, expand, or contract along elevational gradients (Hewitt 2000, 2004; DeChaine & Martin 2005). In species that closely track environmental conditions to which they have adapted over time (i.e., niche conservatism), populations on different mountains may experience alternating periods of isolation and connectivity during climatic fluctuations (Hewitt 1996; Wiens 2004; Wiens & Graham 2005). Periods of isolation can result in genetic divergence among populations whereas periods of connectivity allow for dispersal and gene flow among mountains (Hewitt 1996, 2004; Jansson & Dynesius 2002; Wiens 2004). Such range expansions and contractions are also predicted to result in changes in effective population size (N_e; Wakeley 2000; Jesus *et al.* 2006). The genetic consequences of historic periods of range contraction and isolation and range expansion and connectivity of populations are manifest in present-day patterns of phylogeographic structure and genetic variation (Avise 2000).

Genetic consequences of Pleistocene climatic fluctuations in montane habitats should be most evident in organisms that are highly sensitive to environmental change

(Hewitt 1996; Wiens 2004; Wiens & Graham 2005). Salamanders in the genus *Plethodon* are forest-dwelling, lungless ectotherms that require mesic environments for cutaneous respiration and egg deposition, and thus their distributions are strongly influenced by moisture and temperature (Jaeger 1971; Spotila 1972). Species diversity of *Plethodon* reaches its peak in the forested, montane regions of eastern North America (Appalachian and Interior Highlands) where many closely related taxa often occur on adjacent mountain tops (Highton 1995; Petranka 1998; Kozak *et al.* 2006a; Wiens *et al.* 2006). This region has a history of climate-driven forest contraction, fragmentation, and expansion over the last three million years (King 1973; Davis 1983; Webb & Bartlein 1992), which is thought to have contributed greatly to diversification in *Plethodon* (Highton 1995; Kozak *et al.* 2006a; Kozak & Wiens 2006).

The Fourche Mountain Salamander *Plethodon fourchensis* is known only from Fourche and Irons Fork Mountains in the Ouachita Mountains of west-central Arkansas (Duncan & Highton 1979; Trauth & Wilhide 1999; Anthony 2005; Fig. 1). The Ouachita Mountains are part of the Interior Highlands, and are unique among mountain ranges in North America because they trend east–west (Foti & Bukenhofer 1998). This orientation results in mesic forest occurring primarily on high-elevation, north-facing slopes, and thus *P. fourchensis* is primarily confined to these cooler and wetter areas of habitat (Blair & Lindsay 1965; Duncan & Highton 1979; Greller 1988; Foti & Glenn 1991; Trauth & Wilhide 1999). Although this region was not glaciated during the Pleistocene, it experienced climatic fluctuations that impacted species distributions and demography (King 1973; Davis 1983; Shepard & Burbrink 2008). The area of suitable environmental conditions for *P. fourchensis* is predicted to have expanded and contracted along an

elevational gradient in response to Pleistocene climatic fluctuations resulting in historic periods of connectivity and isolation of populations on different mountains. Periods of isolation may have resulted in divergence of populations on different mountains whereas periods of connectivity might have allowed for dispersal to adjacent, unoccupied mountains and permitted secondary contact of previously isolated populations.

Studying phylogeographic patterns of similarly distributed, closely related taxa can reveal whether they are limited by similar factors and responded similarly to historic environmental changes (Zink 1996; Arbogast & Kenagy 2001; Lapointe & Rissler 2005). Furthermore, such studies provide multiple tests of hypotheses related to the processes involved in diversification and thus, provide information on the generality of conclusions about how diversifications occurred within a specific taxon or in organisms within a certain region (Sullivan *et al.* 2000; Carstens *et al.* 2005a; Feldman & Spicer 2006; Soltis *et al.* 2006). Shepard and Burbrink (2008) identified seven lineages within *Plethodon ouachitae* in the Ouachita Mountains corresponding to six major mountains. They found that all diversification occurred during the Middle Pleistocene in a stepping stone fashion consisting of several cycles of dispersal to a new mountain followed by divergence (Shepard & Burbrink 2008). Because *P. fourchensis* and *P. ouachitae* are sister taxa, and the two are geographically proximate and even hybridize within a narrow zone (~1.8 km) on the western end of Fourche Mountain (Duncan & Highton 1979; Kozak *et al.* 2006a; Wiens *et al.* 2006), *P. fourchensis* may have diversified in a similar manner across the mountains it currently occupies.

Here we sample *P. fourchensis* throughout its range and use DNA sequence data to evaluate several hypotheses related to the effects of Pleistocene climatic changes on

diversification in montane species. First, we use statistical phylogenetic methods to test whether each geographically isolated mountain comprises a distinct evolutionary lineage (i.e., montane isolates are reciprocally monophyletic). Next, we use ecological niche modelling to test whether identified lineages are separated by areas where environmental conditions are unsuitable, and thus act as barriers to gene flow. Third, we use divergence dating to test whether the timing of diversification within *P. fourchensis* is consistent with climatic shifts induced by Pleistocene glacial cycles. Fourth, we use coalescent simulations to test whether populations on different mountains are descended from a wide-ranging common ancestor whose range became fragmented or alternatively, if the pattern of diversification is consistent with a colonization model involving dispersal from one mountain to another followed by isolation. Lastly, given that the area of suitable environmental conditions for *P. fourchensis* on these mountains is predicted to have expanded and contracted in response to Pleistocene climatic fluctuations, we examine historical demography to test for corresponding increases and decreases in effective population size (N_e).

Materials and methods

Sampling and sequencing

We conducted extensive surveys throughout the Ouachita Mountains and intervening valleys to establish the distribution of *Plethodon fourchensis* and collected 142 tissue samples from 38 unique localities throughout its range (Appendix). We also collected samples of several closely related species, *Plethodon ouachitae* ($N = 7$; one of each lineage identified by Shepard & Burbrink 2008), *Plethodon caddoensis* ($N = 1$), and

Plethodon kiamichi ($N = 1$) for use as outgroups (Kozak *et al.* 2006a; Wiens *et al.* 2006; Appendix).

We extracted whole genomic DNA from ethanol-preserved liver or muscle tissue using the DNeasy Kit (Qiagen Inc., USA) to obtain template strength DNA/RNA ratios of 1.5–2.1 and DNA concentrations from 10–200 ng/µl. We amplified two mitochondrial encoded genes, cytochrome *b* (cyt*b*) and NADH dehydrogenase 4 (ND4), and a portion of tRNA-His using Polymerase Chain Reaction (PCR), with a negative control (water), following the specifications included with the AccuTaq Jumpstart Kit (USB Corp., USA) in a 10-µl reaction. For PCRs, we used the primers PGludg2 and PThrR1 for cyt*b*, and Ephist and ND4(F) for ND4 (Wiens *et al.* 2006). Thermal cycling conditions used to amplify these genes were: 94º C for 2 min followed by 36 cycles of 94º C for 10 s, 50º C (cyt*b*) or 52º C (ND4) for 30 s, and 72º C for 90 s with a final 10 min extension period at 72º C. We cleaned PCR products using 1 µl of ExoSap-it (USB Corp., USA) per 10 µl of PCR product.

For sequencing, we used the primers PouachCytbF and PouachCytbR for cyt*b* and PouachND4F and PouachND4R for ND4 (Shepard & Burbrink 2008). Sequencing reactions consisted of 2–3 µl of DTCS (Beckman-Coulter, USA), 1 µl of 5-µM primer, 1–2 µl of DNA template, and 4–6 µl of H_2O. Sequencing products were purified following the ethanol-sodium-acetate protocol listed in the DTCS Kit and analyzed on a Beckman CEQ 8000 sequencer (Beckman-Coulter, USA). Nucleotide sequences were assembled, edited, and aligned by eye using the program Sequencher 4.2 (Genecodes 2000), and an open reading frame for these genes was verified. Alignments were unambiguous and no indels were found in these genes in *P. fourchensis, P. ouachitae*,

and *P. caddoensis*. A two base pair insertion/deletion, however, was present in the tRNA-His flanking region of the ND4 gene when compared to the outgroup, *P. kiamichi*. Sequences were deposited in GenBank under the Accession Nos. xxxxxxx and xxxxxxx (Appendix).

Phylogeography

We estimated phylogeographic relationships within *P. fourchensis* using the combined sequences from the cyt*b* and ND4 genes and tRNA-His. We used Maximum Likelihood (ML) and Bayesian Inference (BI) with partitioned models incorporating evolutionary information specific to gene and codon position to infer trees and assess nodal support.

Prior to tree inference, three partitioning strategies were evaluated. The first model accounted for differences in evolutionary rates in each of the three codon positions of the cyt*b* and ND4 genes and the sequences from tRNA-His using the GTR + Γ + I model with estimated base pair (bp) frequencies for each codon position in each gene and the tRNA. For this codon position-specific and tRNA-specific model, abbreviated 7(GTR + Γ + I), a single tree was estimated for all partitions simultaneously, but all other model parameters were unlinked among partitions. The second model applied the GTR + Γ + I model across all positions for each protein-coding gene and the tRNA [3(GTR + Γ + I)] with no partitioning among codon positions. The last model applied one GTR + Γ + I model across both genes and the tRNA.

For each partitioning strategy, two independent searches were executed in MrBayes v.3.1.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) to ensure convergence of all parameters, which we assessed by comparing the variance

across chains within a search to the chain variance among searches using Gelman and Rubin's "*r*" statistic (Gelman *et al.* 1995). Searches were considered burned-in when the values for "*r*" reached ~1.00. All searches consisted of three "heated" and one "cold" Markov chain estimated for 10 million generations with every 1000th sample being retained. Default priors were applied to all parameters, except branch length, which was drawn from an exponential distribution. A split standard deviation less than 0.005 for -lnL tree values among chains indicated that parameter stationarity was achieved. Trees sampled prior to stationarity were discarded. The harmonic mean of the model likelihood, $f(X|M_i)$, taken from the stationarity phase was compared among different partitioning strategies using Bayes Factors (BF) for the equation $2LnB_{10}$ (Newton & Raftery 1994) in Tracer v.1.4 (Rambaut & Drummond 2007). A BF >10 was considered as strong evidence favoring the more partitioned model (Kass & Raftery 1995).

The ML tree and associated support were obtained from 1000 nonparametric bootstrap pseudoreplicates (Felsenstein 1985) under the preferred BF partitioning strategy using the GTRGAMMA model in the program RAxML v.7.0 (Stamatakis *et al.* 2005; Stamatakis 2006; Stamatakis *et al.* 2008). Trees from BI were compared with the ML tree and the most credible inferences of relationship were confined to nodes where the Bayesian posterior probability was ≥95% and the nonparametric bootstrap value was ≥70% (Hillis & Bull 1993; Felsenstein 2004).

Ecological niche modelling

We downloaded raster coverages of 19 environmental-climatic variables from the WorldClim database (http://www.worldclim.org) at 30 arc-seconds resolution (~1 km^2;

Hijmans *et al.* 2005) and clipped these coverages to a region that encompassed the entire Ouachita Mountain range and included most of eastern Oklahoma, western Arkansas, and parts of southern Missouri and northeastern Texas (33.18–36.93° latitude and 92.20–96.56° longitude). We constructed an ecological niche model for *P. fourchensis* using the 19 climatic variables and GPS coordinates of our 38 sampling localities using the default settings in the program Maxent v.3.2.1 (Phillips *et al.* 2006; Appendix). These points represent all known localities for *P. fourchensis*. Maxent uses environmental–climatic variables from localities in which a species has been documented previously to predict where else the species may occur because the environmental-climatic conditions are similar to the conditions at known localities. The output of Maxent consists of grid maps with each cell having an index of suitability between 0 and 1. Low values indicate conditions are unsuitable for the species to occur whereas high values indicate that conditions are suitable. To represent environmental suitability as a binary character, we used a threshold value of 0.496, as chosen using the 10 percentile training presence criteria calculated by Maxent. We then overlaid this niche model on a map of the Ouachita Mountains to examine visually if mountains/lineages were separated by areas of unsuitable environmental conditions.

Because *P. fourchensis* is parapatric with its sister taxon *P. ouachitae*, we wanted to determine whether the species occupied habitats with similar environmental conditions in order to examine potential factors limiting their distributions. To test if environmental conditions at locations occupied by *P. fourchensis* are different from conditions at locations where *P. ouachitae* occurs, we first extracted values for each of the 19 climatic variables used in niche modelling from our 38 sampling localities for *P. fourchensis* and

the 55 sampling localities for *P. ouachitae* reported by Shepard and Burbrink (2008). Because many of the 19 climatic variables are intercorrelated, we used Principal Components Analysis to reduce them to a smaller number of independent variables. We retained principal components with eigenvalues >1 and that explained >10% of the variation. We used the factor scores for these principal components as dependent variables in a MANOVA to test for differences between *P. fourchensis* and *P. ouachitae*. We followed a significant multivariate effect with ANOVAs for each principal component, and examined loading factors for those principal components that were significantly different to determine the nature of the differences in environmental conditions between species.

Divergence dating

To estimate the age of origin of *P. fourchensis*, we used a 'relaxed phylogenetics' method that does not rely on a molecular clock and incorporates uncertainty in the tree estimation process (Drummond *et al.* 2006). Using BEAST v.1.4.7 (Drummond & Rambaut 2007), we estimated the tree and divergence dates of the monophyletic Plethodontidae using all genes and individuals included in Wiens *et al.* (2006) employing the GTR + Γ + I model across all genes and codon positions. An uncorrelated lognormal tree prior with a constant population size prior and lognormal calibration dates (see below) were used to estimate the timing of divergences (Drummond *et al.* 2006). These analyses estimated tree shape and divergence dates for all nodes and were sampled every 1000th iteration for 30 million generations with 10% of the initial samples discarded as burn-in.

To use this relaxed phylogenetics method, we provided calibration points and error estimates derived from a lognormal distribution (Drummond *et al.* 2006). Our calibration points for this tree came from three sources and were identical to those used in Wiens *et al.* (2006). The first two calibration points, the earliest fossils of *Plethodon* and *Aneides* (representing the most recent common ancestor of the genera *Aneides, Desmognathus* and *Phaeognathus*), were both from the Arikareean (Tihen & Wake 1981). Thus, both fossils are a minimum of 19 Myr. We used this age as the median for each of these calibration points and a SD of 0.3, which yields an upper 95% Credible Interval of 30 Myr, thereby encompassing the entire Arikareean. For the other calibration point, we used the fossil of *Aneides lugubris*, dating from the Late Miocene (~5 Ma; Clark 1985). As discussed in Wiens *et al.* (2006), this provides a minimum age for the MRCA between *A. lugubris* and *A. aeneus*. We used this date as the MRCA of these taxa, and a SD of 0.5 provides an upper 95% bound of 11 Myr (Middle Miocene).

The divergence date estimate and associated error for *P. fourchensis* from the Plethodontidae tree above were applied to a tree of all samples of *P. fourchensis* to estimate the age of each phylogeographic lineage and their most recent common ancestors (MRCA). We applied the GTR + Γ + I model across all genes and codon positions and used an uncorrelated lognormal tree prior with a constant population size prior (Drummond *et al.* 2006). We ran two independent searches of 10 million generations in BEAST v.1.4.7 (Drummond & Rambaut 2007) sampling every 1000th iteration with 10% of the initial samples discarded as burn-in. We used Bayes Factors in Tracer v.1.4 (Rambaut & Drummond 2007) to determine whether runs had converged on similar values. The dates and associated error for the MRCA of haplotypes within

lineages were used in the historical demographic analyses so that estimates of changing N_e can be dated and related to geologic or climatic events in the past (e.g., glacial and interglacial periods).

To test whether all diversifications within *P. fourchensis* occurred during the Pleistocene, we determined the probability that a pre-Pleistocene value (>1.8 Ma) could be found within the lognormal distribution of dates for the first divergence within the species. If this probability is ≤0.05, we can reject a pre-Pleistocene divergence.

Historical biogeography

We used coalescent simulations in Mesquite v.2.5 (Maddison & Maddison 2008) to test between four biogeographic models of diversification (Knowles & Maddison 2002; Fig. 2). The Fragmented Ancestor model posits that all population divergences were in effect concurrent and resulted from the fragmentation of a widely distributed common ancestor's range. The presence of phylogeographic structure under this model would be due to differential extinction of ancestral halplotypes among mountains (Knowles 2001; Carstens *et al.* 2005b). The Staged Fragmentation model posits that a wide-ranging common ancestor was first fragmented into two ancestral populations (an eastern and a western), and then each of those was subsequently fragmented. We also tested two colonization models that posit a history involving a series of dispersals from one mountain to another followed by isolation and divergence. One model (E–W Colonization) hypothesizes an east to west stepping stone pattern whereas the other model (W–E Colonization) hypothesizes a west to east pattern (Fig. 2).

For coalescent simulations, we first estimated N_e for *P. fourchensis* on each mountain using values for θ calculated in the program MIGRATE-N v.2.4 (Beerli 2008) under the following parameters: 15 small chains for 200 000 generations and four long chains for two million generations with four adaptive heating chains, chains were sampled every 20 generations following a burn-in of 10 000 generations. Maximum likelihood estimates (MLE) were calculated three times to ensure convergence upon similar values for θ. We converted θ to N_e using the equation for maternally inherited mitochondrial DNA $\theta = N_e\mu$, where $\mu = 1.365 \times 10^{-7}$, which is based on an average substitution rate for *P. fourchensis* of 4.55×10^{-2} substitutions per site per million years calculated in BEAST v.1.4.6 and a generation time of three years (Pope & Pope 1951; Drummond & Rambaut 2007). We summed the estimates of N_e for all mountains to calculate Total N_e and scaled the branch widths of our hypothesized population trees using the proportion of Total N_e that each mountain comprised. Internal branches on the Staged Fragmentation and both colonization models were scaled such that all branch widths summed to Total N_e at any single point in time (Carstens *et al.* 2004a; Shepard & Burbrink 2008; Fig. 2).

The method of counting deep coalescences assumes that deep coalescent events are due to incomplete lineage sorting and not because of migration among populations (Maddison 1997; Knowles & Maddison 2002). In cases where the number of deep coalescences may be inflated by recent migration, it is important to account for migration in simulations to build null models that better reflect history under a given scenario. Using the MLE of Total N_e, we simulated 500 trees under a neutral coalescent process with migration on the Fragmented Ancestor model at a tree depth of 225 000 generations,

which when based on a three year generation time (Pope & Pope 1951) is equivalent to 0.675 Myr (the approximate age estimated for the first divergence within *P. fourchensis* using the fossil calibrated relaxed phylogenetics method). To calculate the probability of migration per individual per generation for these simulations, we first multiplied values of M among adjacent populations (mountains) calculated in MIGRATE-N v.2.4 (Beerli 2008) by the θ of the receiving population to derive the number of immigrants per generation among pairs of adjacent populations. We divided these values by the estimated N_e of the source population to calculate the probability of emigration per individual per generation in the source population, and then calculated the harmonic mean of all population pairs to derive the average probability of migration per individual per generation. We also set the timing of migration in simulations to be concentrated in the last 1667 generations (~5000 years based on a three year generation time), which was shown by our historical demography results to be a period of population expansion in major lineages of *P. fourchensis* (see below).

We fit the simulated gene trees from the Fragmented Ancestor model into each of the other models, calculated the number of deep gene coalescences (nDC), and built a distribution of nDC values ($N = 500$ for each model). We then fit our reconstructed ML tree for *P. fourchensis* to each of these models and calculated the nDC value. If this observed nDC falls below 95% of the distribution of nDC values calculated using the simulated gene trees (equivalent to one-tailed $P \leq 0.05$), then the Fragmented Ancestor model will be rejected in favor of the alternative model. To calculate P values for the observed nDC values in these analyses, we fit the distribution of simulated nDC values to a normal distribution with the given mean and standard deviation (SD).

We also examined the area of origin for *P. fourchensis* and each clade and lineage using a maximum likelihood (ML) method of ancestral character estimation in Mesquite v.2.5 (Maddison & Maddison 2008). Each individual *P. fourchensis* in the phylogeographic analysis was coded to one of five montane isolates and the ancestral areas were estimated for major nodes using a Markov model (Mk1) on the tree. We used an equal likelihood for the rate of change among different mountains for estimating ancestral areas because no prior knowledge of dispersal rate from one area to another exists. At each ancestral node, likelihoods for each area are summed and reported as proportional likelihoods.

Historical demography

We examined past population dynamics of all phylogeographic lineages of *P. fourchensis* using several methods including Bayesian skyline plots (BSP; Drummond *et al.* 2005). This genealogical method permits the estimation of N_e through time and does not require a specified demographic model (e.g., constant size, exponential growth, logistic growth, or expansive growth) prior to the analysis. We used the HKY + Γ + I model to construct BSPs in BEAST v.1.4.8 for each lineage (Drummond *et al.* 2005; Drummond & Rambaut 2007). We applied 10 grouped coalescent intervals (*m*), and priors for the phylogenetic model and population sizes were uniformly distributed. These analyses estimated genealogies and model parameters, and were sampled every 1000th iteration for 20 million generations with 10% of the initial samples discarded as burn-in. Additionally, to scale the time axis on BSPs, we used date estimates for the most recent common ancestor (MRCA) of all haplotypes in a lineage (obtained from divergence dating described

above). We used a relaxed uncorrelated lognormal molecular clock with mean divergence dates and lognormal standard deviation values to reflect the median and 95% Credible Interval (CI) obtained from the dating estimates for each clade (see below) when inferring demographic changes using BSPs. Plots for each analysis were visualized using Tracer v.1.4 (Rambaut & Drummond 2007).

To provide other estimates of change in N_e, we also calculated Tajima's D^* (Tajima 1989) and Fu and Li's D^* (Fu 1997). Both Tajima's D^* and Fu and Li's D^* are expected to be near zero if population sizes have been stable. Significant negative values are expected in populations that have undergone recent population expansion whereas significant positive values are expected in populations that have recently experienced bottlenecks (Tajima 1989; Fu 1997). We tested for significant deviations from zero in Tajima's D^* and Fu and Li's D^* using 10 000 coalescent simulations in DnaSP v.4.20 (Rozas *et al.* 2003). Contrasting plots of observed versus theoretical distributions of site differences (mismatch) also yields insight into past population demographics. A unimodal mismatch distribution indicates a recent range expansion, a multimodal (including bimodal) mismatch distribution indicates diminishing population sizes or structured size, and a ragged distribution suggests that the lineage is widespread (Excoffier *et al.* 1992; Rogers & Harpending 1992; Rogers *et al.* 1996; Excoffier & Schneider 1999). A multimodal distribution may also indicate that the population is influenced by migration, is subdivided, and/or has undergone historical contraction (Marjoram & Donnelly 1994; Bertorelle & Slatkin 1995; Ray *et al.* 2003). The fit of the observed data was tested against a null distribution of constant population size using the

R_2 raggedness statistic of Ramos-Onsins and Rozas (2002) and 10 000 coalescent simulations in DnaSP v.4.20 (Rozas *et al.* 2003).

Results

Phylogeography

We sequenced 1052 bp of the cyt*b* gene, 723 bp of the ND4 gene, and 41 bp of the tRNA-His for 142 putative *P. fourchensis* and nine outgroup taxa (1816 bp total). Bayes Factors (BF) strongly favored the more parameter rich 7(GTR + Γ + I) model over the less parameterized 3(GTR + Γ + I) model (BF > 115). Therefore, we used the 7(GTR + Γ + I) model to run the BI analysis in MrBayes v.3.1.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) and the 7(GTR + Γ) model to run the ML analysis in RAxML 7.0 (Stamatakis *et al.* 2005; Stamatakis 2006; Stamatakis *et al.* 2008). For the BI analysis, burn-in occurred at 2.5 million generations as determined by a Gelman and Rubin's "*r*" value near 1.0 for the -lnL tree likelihood. Both independent runs using the 7(GTR + Γ + I) model produced similar harmonic mean -lnL values for post burn-in trees with a difference in -lnL of 1.394. After discarding samples before burn-in and summing trees from the two independent runs, the posterior probability distribution contained 15 000 trees.

Monophyly of *P. fourchensis* was not strongly supported by either ML or BI results (Fig. 3). All individuals ($N = 12$) from the western-most sampling locality on West Fourche Mountain (the western edge of the hybrid zone of Duncan & Highton 1979) had mtDNA from the Rich Mtn lineage of *P. ouachitae* (Figs. 1 & 3). The dorsal color and pattern of these individuals were highly variable and not typical of either *P.*

ouachitae or *P. fourchensis* (D. Shepard, unpublished data). All other individuals from West Fourche Mountain had mtDNA of *P. fourchensis*, and all but one had the typical dorsal pattern of *P. fourchensis*. The high support for the monophyly of the Rich Mtn lineage of *P. ouachitae* and the 12 putative hybrids suggests that these individuals are not contributing to the low support for monophyly of *P. fourchensis*, as might occur if there had been mitochondrial recombination.

Tree topology was similar for BI and ML analyses, and both indicated that *P. fourchensis* is composed of four geographically structured lineages (Fig. 1 & 3). All lineages were strongly supported by Bayesian posterior probabilities, and all but one lineage were strongly supported by ML bootstrap values (Fig. 3). Two divergent lineages occur within the eastern part of the range. The Little Brushy lineage is restricted to Little Brushy Mountain, the eastern most montane isolate, whereas the Buck Knob lineage is more widespread, occurring on Grapevine Mountain, Mast Mountain, Brushy Knob, Buck Knob, and Irons Fork Mountain (Fig. 1). These two lineages form a clade that is sister to a clade in the western part of the range comprised by the other two lineages. One of these, the Blue Mtn lineage, occurs on Blue Mountain and Wolf Pinnacle whereas the other, the W. Fourche lineage, occurs primarily on West Fourche Mountain and Shut In Mountain (Fig. 1). Foran Gap which separates Fourche Mountain from West Fourche Mountain is spanned by the W. Fourche lineage. The W. Fourche and Blue Mtn lineages abut approximately 4 km east of Foran Gap and 2.25 km west of Wolf Pinnacle (Fig. 1). The Blue Mtn and Buck Knob lineages abut in the vicinity of Turner Creek Pass, which is the only high-elevation connection between the montane areas occupied by the two lineages. The Blue Mtn lineage spans the pass by at least 0.75 km, but no individuals

from the Buck Knob lineage were found on the opposite side of the pass (Fig. 1). Shut In Mountain was monophyletic, but was nested within the W. Fourche lineage suggesting that it was only recently colonized from West Fourche Mountain.

Ecological niche modelling

The niche model predicts that environmental conditions in the valleys between most montane isolates are suitable for *P. fourchensis* (AUC > 0.99; Fig. 4). The exception is Little Brushy Mountain, which appears to be isolated from adjacent mountains by unsuitable conditions. The niche model also predicts suitable environmental conditions for *P. fourchensis* exist on the eastern ends of Rich and Black Fork Mountains, which are occupied by its sister taxon, *P. ouachitae*.

Principal components analysis (PCA) reduced the 19 Bioclim variables to two principal components explaining 86.71% of the total variation (69.62% and 17.09%, respectively; Table 1). A MANOVA using the principal component scores for the two retained axes revealed that environmental conditions at locations where *P. fourchensis and P. ouachitae* occur are significantly different (Wilk's $\lambda = 0.486$, $F_{2,90} = 47.64$, $P < 0.001$). Locations significantly differed along both the first principal component axis ($F_{1,91} = 32.27$, $P < 0.001$) and the second axis ($F_{1,91} = 30.73$, $P < 0.001$). Based on the factor loadings (Table 1), the first principal component axis represented a gradient from cool and wet environmental conditions (low values) to dry and warm environmental conditions (high values) whereas the second axis represented a gradient from more variable environmental conditions (low values) to less variable conditions (high values).

Environmental conditions where *P. fourchensis* occurs were cooler, wetter, and less variable than conditions where *P. ouachitae* occurs (Fig. 5).

Divergence dates and historical biogeography

Plethodon fourchensis diverged from its sister taxon, *P. ouachitae*, during the Late Pliocene ~2.192 Ma (95% CI: 0.732–3.851 Ma) and all divergences within *P. fourchensis* occurred in the Middle Pleistocene (Fig. 6). The first divergence within *P. fourchensis* occurred ~0.674 Ma (95% CI: 0.235–1.247 Ma) and gave rise to the common ancestor of the two eastern lineages (Little Brushy & Buck Knob) and the common ancestor of the two western lineages (Blue Mtn & W. Fourche). The Blue Mtn and W. Fourche lineages diverged ~0.514 Ma (95% CI: 0.176–0.991), and then the Little Brushy and Buck Knob lineages diverged ~0.418 Ma (95% CI: 0.142–0.788 Ma). Given the date for the first divergence within *P. fourchensis* and the associated error, it is extremely improbable that divergence within *P. fourchensis* occurred prior to the Pleistocene (>1.8 Ma; $P = 4.51 \times 10^{-6}$).

Using Migrate-N v.2.4 (Beerli 2008), we calculated a maximum likelihood estimate (MLE) of $\theta_{Total} = 0.0276$ (95% CI: 0.0128–0.0672). The MLE of θ_{Total} equates to a Total N_e of 202 198. Based on values of M and θ from Migrate-N (Beerli 2008), we calculated a mean probability of migration per individual per generation of 5.842×10^{-6}, and used this value in coalescent simulations. The number of deep coalescent events (nDC) of our ML tree for *P. fourchensis* fit into a population tree representing each of the hypothesized biogeographic scenarios was 13 for the Fragmented Ancestor model, 22 for the Staged Fragmentation model, 17 for the W–E Colonization model, and 21 for the E–

W Colonization model. Results from coalescent simulations failed to reject the Fragmented Ancestor model in favor of any of the alternative models of diversification (Staged Fragmentation mean nDC = 26.45, SD = 9.63, $P = 0.32$; W–E Colonization nDC = 26.89, SD = 9.79, $P = 0.16$; E–W Colonization mean nDC = 25.62, SD = 8.87, $P = 0.30$).

Reconstruction of the ancestral area for *P. fourchensis* assigned the highest probability to West Fourche Mountain; however, the probabilities for Little Brushy Mountain and Blue Mountain were not considerably lower (Table 2). Relatively high probabilities for multiple areas being the ancestral area at the deeper nodes in the tree support results from coalescent simulations that diversification occurred via the fragmentation of a wide-ranging common ancestor. Ancestral areas for each lineage corresponded to the mountains after which they were named.

Historical demography

All non-genealogical coalescent methods (i.e., Tajima's D^*, Fu and Li's D^*, and the mismatch distribution) failed to reject the null hypothesis of population stability for all lineages (Table 3). These methods, however, are weaker than genealogical coalescent methods (e.g., Bayesian skyline plots) because they fail to consider phylogenetic structure (Felsenstein 1992; Pybus *et al.* 2000). Bayesian skyline plots (BSP), in contrast, show that population sizes in three of the four lineages gradually declined over the last 125 000 years and then began increasing sharply approximately 5000 years ago, which is after the beginning of the Holocene, the current interglacial period (all ESS

values > 300; Fig. 7). We were not able to construct a BSP for the Little Brushy lineage because it was represented in our sample by only four individuals.

Discussion

Species restricted to montane habitats commonly have high levels of inter-population genetic diversity because populations on different mountains are separated by low-elevation areas with disparate environmental conditions that act as barriers to gene flow, thereby creating a sky island situation (Knowles 2000; Masta 2000; DeChaine & Martin 2005; Smith & Farrell 2005; Carstens & Knowles 2007; Shepard & Burbrink 2008). In the Ouachita Mountains, *Plethodon fourchensis* is restricted to mesic forest on the tops of five major mountains separated by low-elevation valleys or connected only by narrow high-elevation passes. Consistent with the hypothesis that these valleys and narrow passes prevent or restrict gene flow, we found that *P. fourchensis* is composed of four divergent lineages structured across these mountains. Geographic breaks between lineages occurred in the vicinity of major valleys and at high-elevation passes; however, these barriers were usually spanned a short distance by one lineage. The Little Brushy lineage is an exception as it was reciprocally monophyletic with respect to its sister lineage (Buck Knob) and restricted to Little Brushy Mountain. The coincidence of genetic breaks in the vicinity of valleys and a narrow high-elevation pass combined with the observation that some lineages presently span these geographic features for a short distance supports the idea that *P. fourchensis* has expanded and contracted its range along an elevational gradient in the past. Individuals that were on the opposite side of hypothesized geographic barriers (e.g., Foran Gap and Turner Creek Pass) from the

primary area occupied by their lineage were from localities directly across the barrier and exhibited little sequence divergence, suggesting that the presence of these haplotypes in those areas was due to recent migration rather than incomplete lineage sorting.

The hypothesis that niche conservatism drives diversification in montane species predicts that lineages should be separated by unsuitable environmental conditions (Wiens 2004; Wiens & Graham 2005; Kozak & Wiens 2006). Predictions from our niche model for *P. fourchensis*, however, showed that most adjacent mountains/lineages are connected by areas where environmental conditions are suitable. Shepard and Burbrink (2008) found a similar result with niche modelling for *P. ouachitae*, but showed that environmental conditions in the valleys separating mountains were significantly warmer and drier than conditions where *P. ouachitae* occurred at higher elevations. The valleys separating mountains within the range of *P. fourchensis* are at most 1 km wide and the resolution of climatic data (1 km^2) used to construct niche models may be too coarse to assess the environmental factors that affect the distribution of *P. fourchensis*. Factors not included in the niche model such as the availability of rocky microhabitats or the presence of closely related species may also be important in determining the distributions of species of *Plethodon* (Pope & Pope 1951; Petranka 1998; Kozak *et al.* 2008). Alternatively, interglacial periods like the present one are predicted to be a time of range expansion in *P. fourchensis* (see below), thus a niche model showing mountains connected by suitable environmental conditions would not be unexpected. Unfortunately, reconstructing the distribution of suitable conditions for *P. fourchensis* during glacial periods in the past (Carstens & Richards 2007; Richards *et al.* 2007; Kozak *et al.* 2008) is not possible with available Pleistocene paleoclimate models because their resolution is

too coarse (10 arc-minutes or ~344 km^2) given the small geographic range of *P. fourchensis*.

Climatic changes during Pleistocene glacial cycles are hypothesized to have induced environmental shifts in montane regions resulting in divergence of populations on different mountains (Hewitt 1996, 2004; Jansson & Dynesius 2002). Consistent with this hypothesis, divergence dates indicated that lineage diversification in *P. fourchensis* occurred during the Middle Pleistocene. Between 1.2 and 0.8 Ma, a climatic change (termed the Middle Pleistocene Transition; MPT) occurred in which the 41-ky glacial cycles that characterized the Late Pliocene and Early Pleistocene shifted to 100-ky cycles with increased amplitude (i.e., greater extremes) in climatic fluctuations (Bennett 1990; Clark *et al.* 1999, 2006). In these 100-ky cycles, cold glacial periods lasted for most of the cycle and interglacials lasted only 10 000–20 000 years (Raymo 1997; Clark *et al.* 2006; Lisiecki & Raymo 2007). Glacial periods were slow to build whereas transitions from glacial to interglacial periods were relatively rapid (Clark *et al.* 1999). Climatic conditions during interglacial periods in unglaciated eastern North America were generally warmer and wetter, which led to expansion of deciduous forest (King 1973; Davis 1983; Denniston *et al.* 1999). Glacial periods in these same regions were generally colder and drier, leading to contraction of deciduous forest and a dominance of conifers (King 1973; Davis 1983; Jackson *et al.* 2000). The longer glacial and interglacial periods of the 100-ky cycles plus the increased amplitude of climatic fluctuations (i.e., greater temperature and precipitation extremes) after the MPT would have provided both more opportunity for *P. fourchensis* to disperse onto adjacent mountains (during interglacial periods) and more time for lineages isolated on different mountains to sort (during glacial

periods). Diversification in other montane species such as grasshoppers (*Melanoplus*) and butterflies (*Parnassius smintheus*) in the northern Rocky Mountains and *P. ouachitae* in the Ouachita Mountains also occurred within a similar timeframe (Knowles 2000, 2001; DeChaine & Martin 2004, 2005, 2006; Carstens & Knowles 2007; Shepard & Burbrink 2008).

Diversification in *Plethodon* is hypothesized to have occurred primarily through repeated cycles of colonization of available, unoccupied habitats followed by isolation and divergence (Kozak *et al.* 2006a). Consistent with this hypothesis, diversification in *P. ouachitae* was found to have occurred in a stepping stone fashion (Shepard & Burbrink 2008). Our analyses on *P. fourchensis*, however, favored a model of diversification involving the fragmentation of a wide-ranging ancestor, and results from the ancestral area reconstruction are suggestive of this scenario as well. Distribution patterns in *Plethodon* are often strongly influenced by competitive interactions with closely related species, and thus there is the potential for historical patterns to be influenced by competitive interactions (Hairston 1951; Crespi *et al.* 2003). Shepard and Burbink (2008) estimated that *P. ouachitae* colonized Rich and Black Fork Mountains ~0.667 Ma (95% CI: 0.195–1.430 Ma). If, as the fragmentation hypothesis predicts, the common ancestor of *P. fourchensis* was already present on West Fourche Mountain before *P. ouachitae* had colonized the adjacent Rich and Black Fork Mountains, then why did *P. fourchensis* not colonize those mountains first?

The Ouachita Mountains occur on the edge of a steep longitudinal environmental gradient that transitions from more mesic conditions in the east to more xeric conditions in the west (Costa *et al.* 2008). One possibility is that *P. fourchensis* is more restricted by

environmental conditions than *P. ouachitae*. This has been suggested previously (Trauth & Wilhide 1999), and our results showed *P. fourchensis* occupies cooler, wetter, and more stable environmental conditions compared to *P. ouachitae*. Results from niche modelling, however, indicate a zone on the western part of West Fourche Mountain and the eastern ends of Rich and Black Fork Mountains where suitable conditions for *P. fourchensis* and *P. ouachitae* overlap (Shepard & Burbrink 2008; this study). Thus, it remains unclear why *P. fourchensis* did not expand farther west, and suggests that other factors may have been involved. Competition with a now extinct congener or with the highly aggressive *P. ouachitae* after it colonized may have also restricted the distribution of *P. fourchensis* (Anthony *et al.* 1997).

Climatic changes that result in range expansion or contraction are expected to cause changes in N_e, leading to increases or decreases in levels of genetic variation and coalescence times (Wakeley 2000; Jesus *et al.* 2006). Because alternating periods of dispersal (i.e., range expansion) and isolation (i.e., range contraction) are evident in the evolutionary history of *P. fourchensis*, which appear to be linked to climatic fluctuations associated with Pleistocene glacial cycles, we expected to observe demographic changes over time in lineages on the different montane isolates. As predicted, population sizes in three of the four lineages analyzed declined gradually over the last 125 000 years and increased only after the beginning of the Holocene, the current interglacial period. Population growth during the Holocene was also observed in all seven lineages in *P. ouachitae*; however, population sizes since 120 000 years ago showed no declines and instead indicated stable population sizes. The decline in population size in *P. fourchensis* in comparison to the long-term stability in *P. ouachitae* suggests that *P. fourchensis* was

affected more severely by climatic changes during glacial periods. Comparison of environmental conditions between *P. ouachitae* and *P. fourchensis* indicated that *P. fourchensis* occupies more mesic habitats, and thus supports the idea that they would be more negatively affected by the drier climatic conditions of glacial periods. Other studies on plethodontid salamanders have also found evidence for recent population growth, but they were not able to place a time on these changes (e.g., Carstens *et al.* 2004b; Mahoney 2004; Kozak *et al.* 2006b; Martínez-Solano *et al.* 2007).

The phylogeographic structure observed in *P. fourchensis* indicates that lineages have been isolated on their respective montane isolates for an extended period. Furthermore, they have remained distinct over the last several glacial cycles. Whether gene flow occurs between lineages that have come back into contact or if any lineages are reproductively isolated is unknown. Duncan and Highton (1979) examined *P. fourchensis* from three localities using allozymes and found only a small amount of differentiation among the three montane isolates examined. Within *Plethodon*, hybridization is common among closely related species, and the frequency of occurrence is related to time since divergence (Weisrock *et al.* 2005; Weisrock & Larson 2006; Wiens *et al.* 2006). Because *P. fourchensis* and *P. ouachitae* hybridize within a narrow zone on West Fourche Mountain (Duncan & Highton 1979), it is likely that lineages within *P. fourchensis* also hybridize within the areas where they come into secondary contact. A small amount of hybridization in contact zones, however, is not enough to obscure historic effects of prolonged isolation in *Plethodon* (Weisrock *et al.* 2005; Weisrock & Larson 2006). More extensive gene flow between *P. fourchensis* on

different mountains may occur via male dispersal (Jockusch & Wake 2002; Keogh *et al.* 2007), but we were unable test this with maternally inherited mtDNA.

Plethodon fourchensis and *P. ouachitae* are sister taxa, have parapatric distributions within a small geographic area in the Ouachita Mountains, and are restricted to high-elevation mesic forest on multiple montane isolates. Because of these characteristics, both species would have experienced the same climatic changes during the Pleistocene, and would be predicted to exhibit similar responses. In support of this prediction, both species appear to have diversified within the same time period in the Pleistocene and exhibit a phylogeographic structure consistent with a sky island diversification model (DeChaine & Martin 2005; Shepard & Burbrink 2008; this study). However, despite these similarities, species responses show several important differences. First, diversification in *P. fourchensis* appears to have occurred through the fragmentation of a wide-ranging common ancestor whereas diversification in *P. ouachitae* occurred in a stepping stone fashion (Shepard & Burbrink 2008; this study). Second, historical demographic analyses indicated a gradual decrease in N_e in *P. fourchensis* over the most recent glacial period whereas N_e in *P. ouachitae* remained stable (Shepard & Burbrink 2008; this study). These differences illustrate how responses to historic environmental changes can vary considerably even between closely related, similarly distributed taxa, and emphasize the importance of comparative phylogeographic approaches to understanding the processes involved in diversification. Although multiple species may show similar phylogeographic patterns within a region, the route by which they arrived at those patterns may differ due to species-specific demographic and ecological characteristics (DeChaine & Martin 2005; Hickerson & Cunningham 2005;

Feldman & Spicer 2006). Only through rigorous testing of hypotheses across multiple taxa and regions can we be confident about conclusions regarding the processes that ultimately lead to the origin of new species.

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Table 1 Results from Principal Components Analysis on climatic variables (Hijmans *et al.* 2005) used in comparison of climatic conditions between occurrence locations for *Plethodon fourchensis* and *Plethodon ouachitae*.

Variable	PC1	PC2
BIO1 (Annual Mean Temperature)	0.974	0.009
BIO2 (Mean Diurnal Range)	0.775	0.596
BIO3 (Isothermality)	0.422	0.863
BIO4 (Temperature Seasonality)	0.910	-0.193
BIO5 (Max Temperature of Warmest Month)	0.968	0.162
BIO6 (Min Temperature of Coldest Month)	-0.480	-0.324
BIO7 (Temperature Annual Range)	0.929	0.214
BIO8 (Mean Temperature of Wettest Quarter)	0.973	-0.006
BIO9 (Mean Temperature of Driest Quarter)	0.846	0.315
BIO10 (Mean Temperature of Warmest Quarter)	0.976	-0.014
BIO11 (Mean Temperature of Coldest Quarter)	0.846	0.315
BIO12 (Annual Precipitation)	-0.931	0.022
BIO13 (Precipitation of Wettest Month)	0.055	-0.841
BIO14 (Precipitation of Driest Month)	-0.875	0.445
BIO15 (Precipitation Seasonality)	0.756	-0.631
BIO16 (Precipitation of Wettest Quarter)	-0.843	-0.138
BIO17 (Precipitation of Driest Quarter)	-0.850	0.447
BIO18 (Precipitation of Warmest Quarter)	-0.975	0.110
BIO19 (Precipitation of Coldest Quarter)	-0.850	0.447
Eigenvalue	13.23	3.25
% Variance Explained	69.62	17.09

Table 2 Proportional likelihoods for the area of origin for ancestral nodes and the most recent common ancestor of each extant lineage of *Plethodon fourchensis* (see Fig. 6 to reference node numbers). Numbers in bold represent the area with the highest likelihood for the given node.

Node	Little Brushy Mtn	Buck Knob	Blue Mtn	West Fourche Mtn	Shut In Mtn
1	NA	NA	NA	NA	NA
2	0.225	0.170	0.210	**0.270**	0.125
3	**0.413**	0.236	0.119	0.134	0.098
4	0.170	0.139	0.244	**0.335**	0.112
5	**0.971**	0.009	0.007	0.007	0.006
6	0.003	**0.991**	0.002	0.002	0.002
7	0.002	0.011	**0.984**	0.002	0.001
8	<0.001	<0.001	<0.001	**0.997**	<0.001

Table 3 Nucleotide diversity (π), average number of pairwise differences (K), and results of Tajima's D^*, Fu and Li's D^*, and mismatch distribution analyses (R_2) for each lineage of *Plethodon fourchensis* calculated for all sites of the concatenated dataset. All results failed to reject the null hypothesis of constant population size (all P values > 0.05).

Lineage	π	K	Tajima's D^*	Fu & Li's D^*	R_2
Little Brushy	0.00566	10.1667	1.6804	1.6804	0.9093
Buck Knob	0.00323	3.4647	-0.3458	0.3965	0.1076
Blue Mtn	0.00382	5.3839	-0.7526	-1.4398	0.1189
W. Fourche	0.00139	2.0580	-1.3001	-2.3686	0.6662

Figure legends

Fig. 1 Topographic map of the United States showing the location of the Ouachita Mountains (A), all elevations >500 m in the range of *Plethodon fourchensis* with names of major mountains and features labeled (B), and map of sampling localities for *P. fourchensis* coded by lineage (C). Lineages: (✚) Blue Mtn, (●) Buck Knob, (■) Little Brushy, (▲) W. Fourche, (★) Rich Mtn lineage of *Plethodon ouachitae*.

Fig. 2 Population trees representing the four biogeographic hypotheses of diversification within *Plethodon fourchensis* tested using coalescent simulations in Mesquite v.2.5 (Maddison & Maddison 2008). The Fragmented Ancestor model posits that all population divergences were concurrent and resulted from the fragmentation of a widely distributed common ancestor's range. The Staged Fragmentation model posits that a wide-ranging common ancestor was first fragmented into two ancestral populations (an eastern and a western), and then each of those was subsequently fragmented. The W–E Colonization model posits a history involving a series of west to east dispersals from one mountain to another followed by isolation and divergence whereas the E–W Colonization model posits that the sequence of colonization occurred from east to west. Branch lengths are time in generations based on a three year generation time. Branch widths (N_e) are scaled based on the proportion of the Total N_e that each mountain comprised (listed below mountain name). Internal branches on models were scaled such that all branch widths summed to Total N_e at any single point in time.

Fig. 3 Bayesian consensus tree produced using the 7(GTR + Γ + I) model for 130 individuals of *Plethodon fourchensis* and outgroups for 1816 bp of the mitochondrial cyt*b* and ND4 genes and tRNA-His. Values above branches are Bayesian posterior probabilities based on 15 000 post burn-in trees and values below branches are support from 1000 nonparametric bootstraps on the Maximum Likelihood tree constructed using the 7(GTR + Γ) model in RAxML 7.0 (Stamatakis *et al.* 2005; Stamatakis 2006; Stamatakis *et al.* 2008). Samples are labeled by mountain and voucher number (Appendix), and major lineages are indicated by bars.

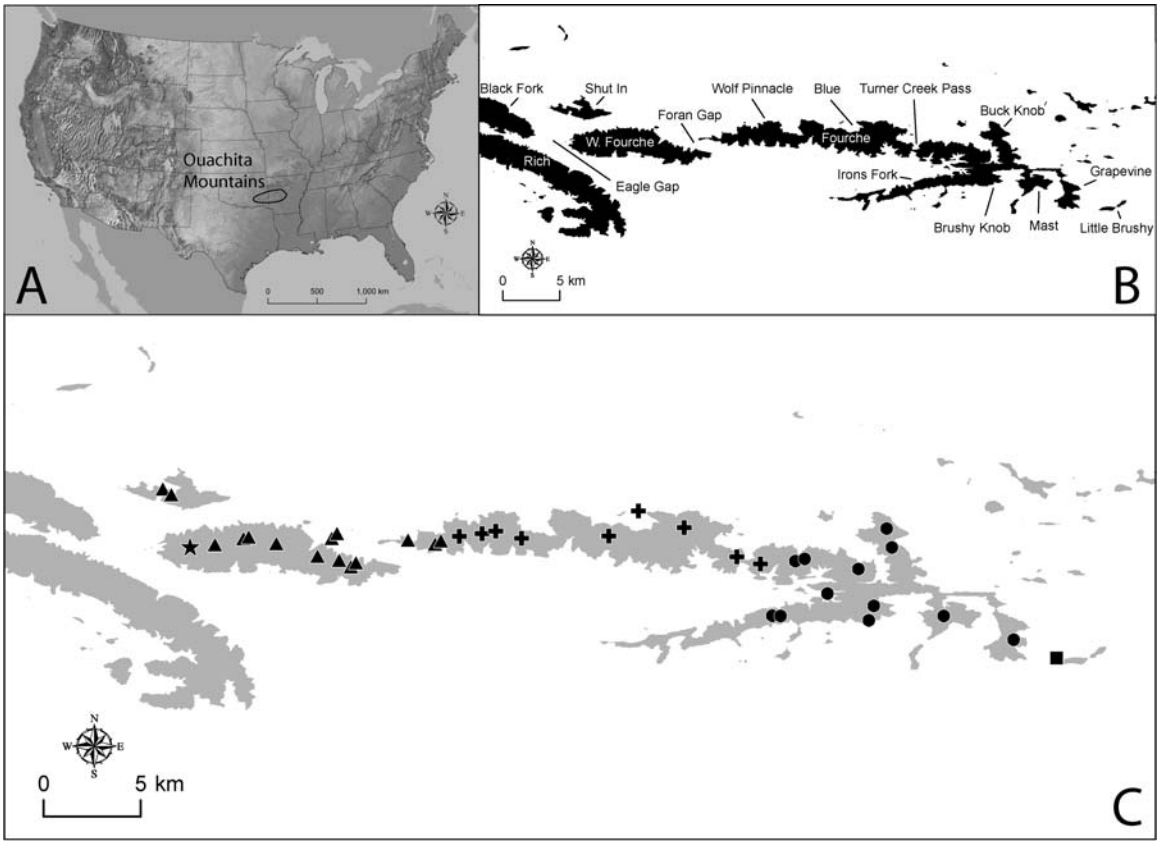
Fig. 4 Ecological niche model for *Plethodon fourchensis* constructed with Maxent v.3.2.1 (Phillips *et al.* 2006) using 19 climatic variables (Hijmans *et al.* 2005) at 30 arc-seconds resolution (~1 km^2) and our 38 sampling points (AUC > 0.99). A map of all elevations >500 m (black) is overlaid by the niche model to show areas of predicted suitable (gray) and unsuitable (white) environmental conditions.

Fig. 5 Results from the comparison of environmental conditions at sampling points for *Plethodon fourchensis* versus *Plethodon ouachitae* (Shepard & Burbrink 2008) showing environmental conditions occupied by *P. fourchensis* are significantly cooler, wetter, and less variable than environmental conditions where *P. ouachitae* occurs.

Fig. 6 Simplified tree showing mean divergence dates and 95% Credible Intervals (Ma) for major nodes and the time to the most recent common ancestor (MRCA) for major lineages within *Plethodon fourchensis*. Nodes are numbered for reference in Table 2.

Fig. 7 Bayesian skyline plots (Drummond *et al.* 2005) showing the demographic history of three of the four lineages of *Plethodon fourchensis*. The 125 000 year-period on the X axis encompasses one complete glacial cycle from the latter part of the Sangamon interglacial, through the entire Wisconsin glaciation, and to the present interglacial period, the Holocene. The central line represents the median value for the $\log_{10}$ of the population size ($N_e * \tau$) and the shaded area represents the 95% Highest Posterior Density. The MRCA of the W. Fourche lineage was <125 000 years ago so it appears truncated.

Fig. 1


A
Ouachita
Mountains
0
500
1,000 km
B
Black Fork
Shut In
Wolf Pinnacle
Blue
Turner Creek Pass
Buck Knob
Foran Gap
W. Fourche
Fourche
Rich
Eagle Gap
Irons Fork
Grapevine
Brushy Knob
Mast
Little Brushy
0
5 km
C
N
W
E
S
0
5 km

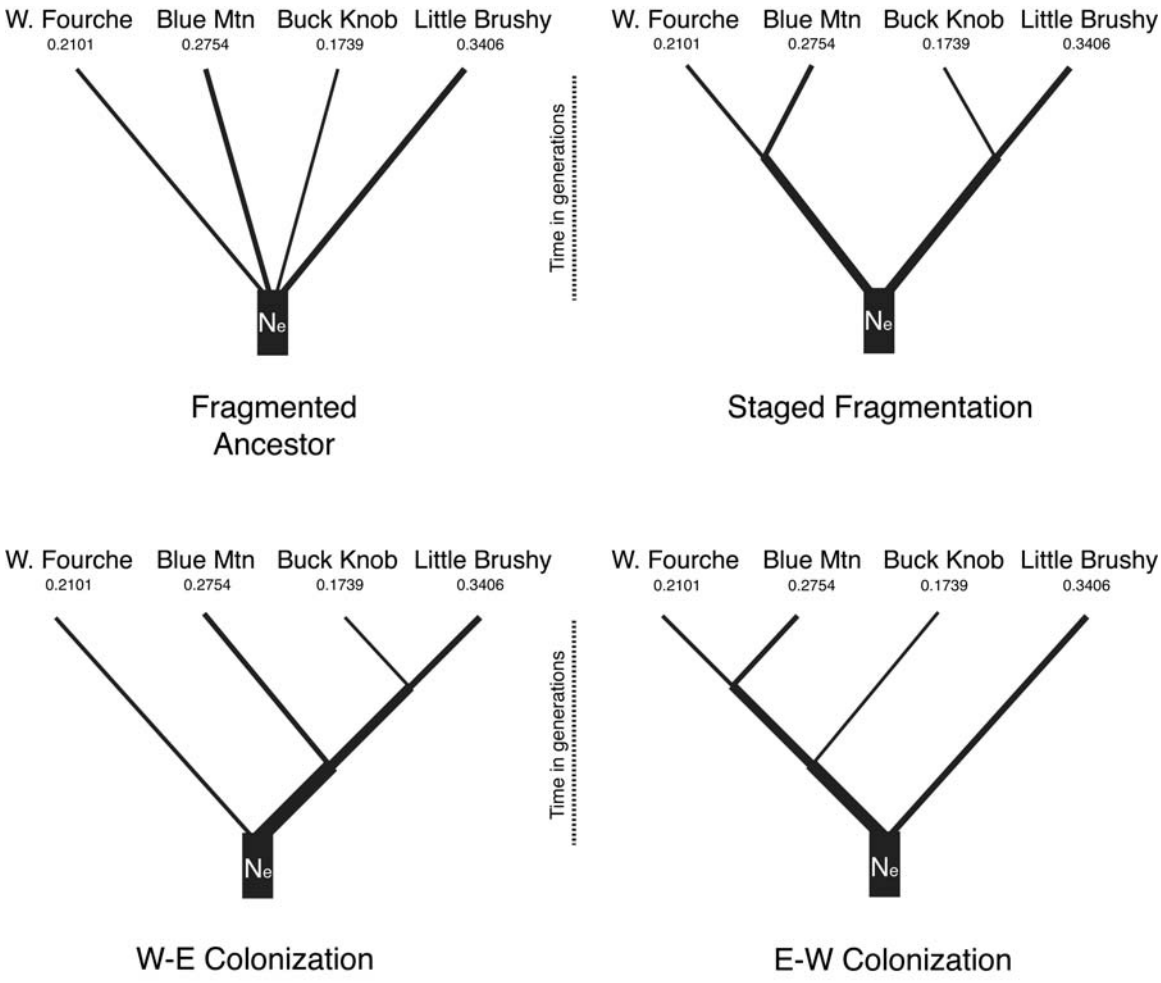

W. Fourche
0.2101
Blue Mtn
0.2754
Buck Knob
0.1739
Little Brushy
0.3406
Time in generations
Ne
Fragmented Ancestor
Staged Fragmentation
W-E Colonization
E-W Colonization

Fig. 3

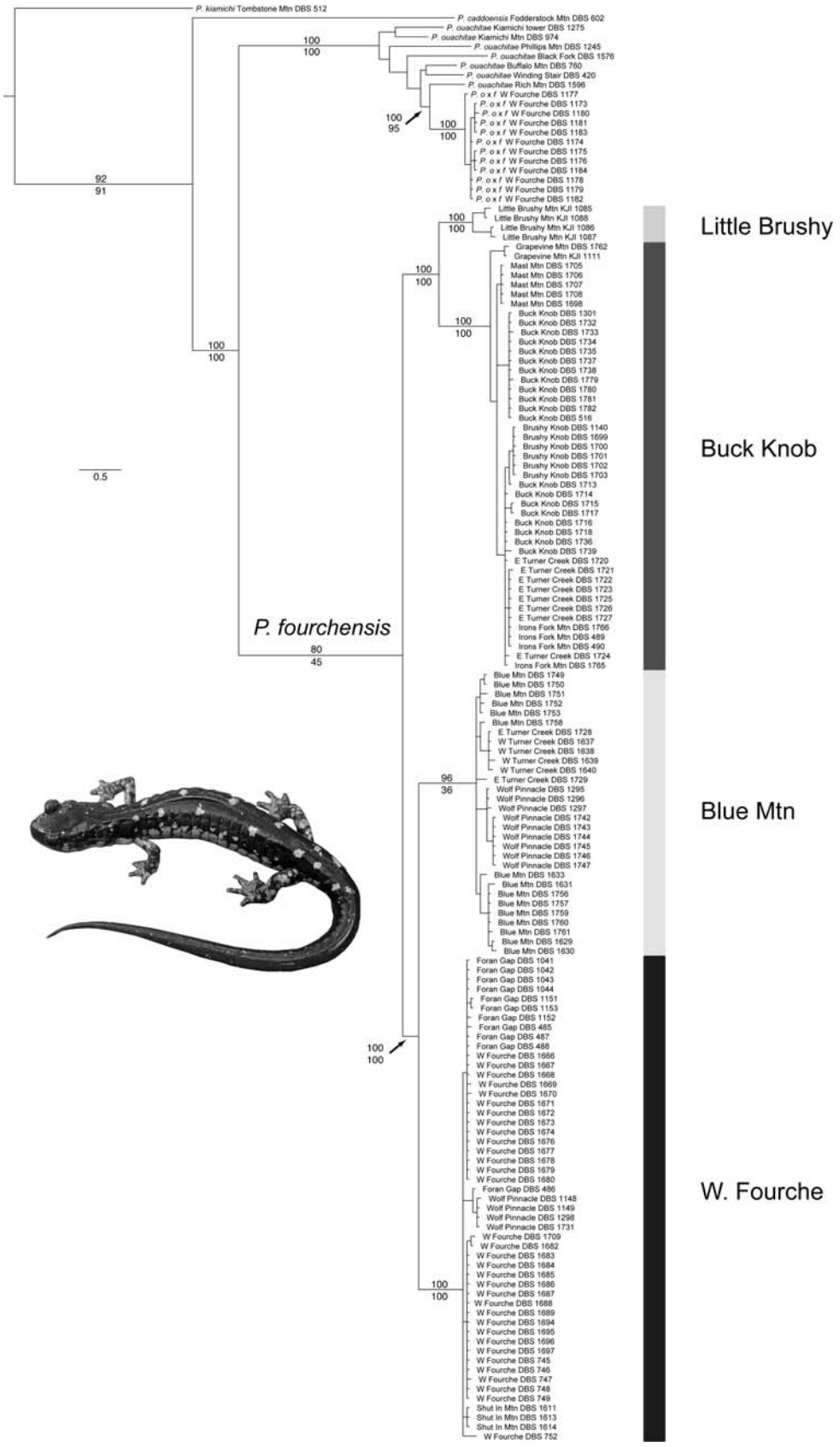

P. kiamichi Tombstone Mtn DBS 512
P. caddoensis Fodderstock Mtn DBS 602
P. ouachitae Kiamichi tower DBS 1275
P. ouachitae Kiamichi Mtn DBS 974
P. ouachitae Phillips Mtn DBS 1245
P. ouachitae Black Fork DBS 1576
P. ouachitae Buffalo Mtn DBS 760
P. ouachitae Winding Stair DBS 420
P. ouachitae Rich Mtn DBS 1596
P. o x f W Fourche DBS 1177
P. o x f W Fourche DBS 1173
P. o x f W Fourche DBS 1180
P. o x f W Fourche DBS 1181
P. o x f W Fourche DBS 1183
P. o x f W Fourche DBS 1174
P. o x f W Fourche DBS 1175
P. o x f W Fourche DBS 1176
P. o x f W Fourche DBS 1184
P. o x f W Fourche DBS 1178
P. o x f W Fourche DBS 1179
P. o x f W Fourche DBS 1182
Little Brushy Mtn KJI 1085
Little Brushy Mtn KJI 1088
Little Brushy Mtn KJI 1086
Little Brushy Mtn KJI 1087
Grapevine Mtn DBS 1762
Grapevine Mtn KJI 1111
Mast Mtn DBS 1705
Mast Mtn DBS 1706
Mast Mtn DBS 1707
Mast Mtn DBS 1708
Mast Mtn DBS 1698
Buck Knob DBS 1301
Buck Knob DBS 1732
Buck Knob DBS 1733
Buck Knob DBS 1734
Buck Knob DBS 1735
Buck Knob DBS 1737
Buck Knob DBS 1738
Buck Knob DBS 1779
Buck Knob DBS 1780
Buck Knob DBS 1781
Buck Knob DBS 1782
Buck Knob DBS 516
Brushy Knob DBS 1140
Brushy Knob DBS 1699
Brushy Knob DBS 1700
Brushy Knob DBS 1701
Brushy Knob DBS 1702
Brushy Knob DBS 1703
Buck Knob DBS 1713
Buck Knob DBS 1714
Buck Knob DBS 1715
Buck Knob DBS 1717
Buck Knob DBS 1716
Buck Knob DBS 1718
Buck Knob DBS 1736
Buck Knob DBS 1739
E Turner Creek DBS 1720
E Turner Creek DBS 1721
E Turner Creek DBS 1722
E Turner Creek DBS 1723
E Turner Creek DBS 1725
E Turner Creek DBS 1726
E Turner Creek DBS 1727
Irons Fork Mtn DBS 1766
Irons Fork Mtn DBS 489
Irons Fork Mtn DBS 490
E Turner Creek DBS 1724
Irons Fork Mtn DBS 1765
Blue Mtn DBS 1749
Blue Mtn DBS 1750
Blue Mtn DBS 1751
Blue Mtn DBS 1752
Blue Mtn DBS 1753
Blue Mtn DBS 1758
E Turner Creek DBS 1728
W Turner Creek DBS 1637
W Turner Creek DBS 1638
W Turner Creek DBS 1639
W Turner Creek DBS 1640
E Turner Creek DBS 1729
Wolf Pinnacle DBS 1295
Wolf Pinnacle DBS 1296
Wolf Pinnacle DBS 1297
Wolf Pinnacle DBS 1742
Wolf Pinnacle DBS 1743
Wolf Pinnacle DBS 1744
Wolf Pinnacle DBS 1745
Wolf Pinnacle DBS 1746
Wolf Pinnacle DBS 1747
Blue Mtn DBS 1633
Blue Mtn DBS 1631
Blue Mtn DBS 1756
Blue Mtn DBS 1757
Blue Mtn DBS 1759
Blue Mtn DBS 1760
Blue Mtn DBS 1761
Blue Mtn DBS 1629
Blue Mtn DBS 1630
Foran Gap DBS 1041
Foran Gap DBS 1042
Foran Gap DBS 1043
Foran Gap DBS 1044
Foran Gap DBS 1151
Foran Gap DBS 1153
Foran Gap DBS 1152
Foran Gap DBS 485
Foran Gap DBS 487
Foran Gap DBS 488
W Fourche DBS 1666
W Fourche DBS 1667
W Fourche DBS 1668
W Fourche DBS 1669
W Fourche DBS 1670
W Fourche DBS 1671
W Fourche DBS 1672
W Fourche DBS 1673
W Fourche DBS 1674
W Fourche DBS 1676
W Fourche DBS 1677
W Fourche DBS 1678
W Fourche DBS 1679
W Fourche DBS 1680
Foran Gap DBS 486
Wolf Pinnacle DBS 1148
Wolf Pinnacle DBS 1149
Wolf Pinnacle DBS 1298
Wolf Pinnacle DBS 1731
W Fourche DBS 1709
W Fourche DBS 1682
W Fourche DBS 1683
W Fourche DBS 1684
W Fourche DBS 1685
W Fourche DBS 1686
W Fourche DBS 1687
W Fourche DBS 1688
W Fourche DBS 1689
W Fourche DBS 1694
W Fourche DBS 1695
W Fourche DBS 1696
W Fourche DBS 1697
W Fourche DBS 745
W Fourche DBS 746
W Fourche DBS 747
W Fourche DBS 748
W Fourche DBS 749
Shut In Mtn DBS 1611
Shut In Mtn DBS 1613
Shut In Mtn DBS 1614
W Fourche DBS 752
100
100
100
95
100
100
92
91
100
100
100
100
100
100
100
100
100
100
0.5
P. fourchensis
80
45
96
36
100
100
100
100
Little Brushy
Buck Knob
Blue Mtn
W. Fourche

Fig. 4

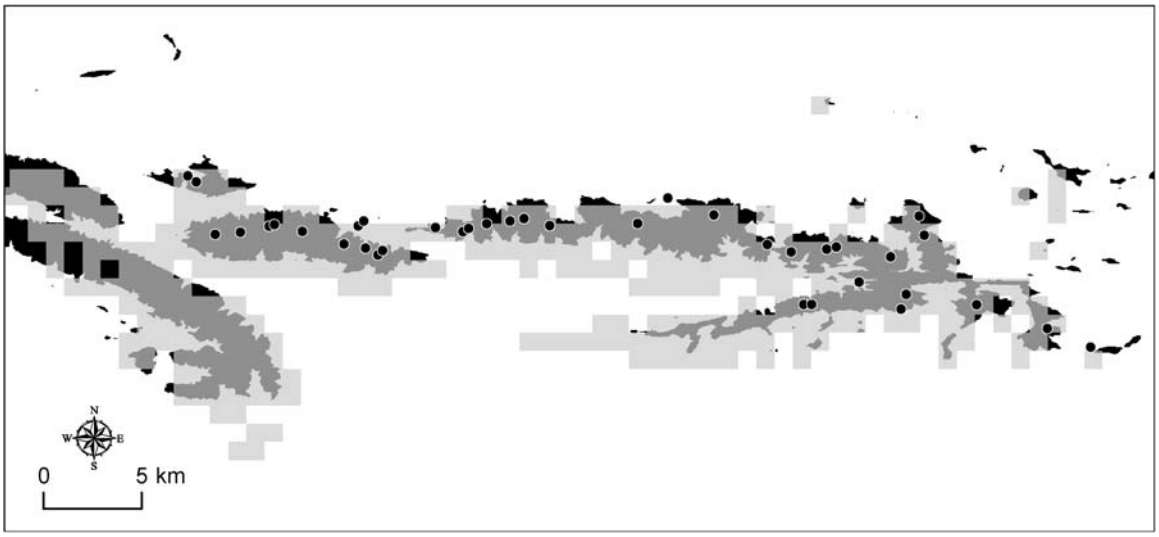


Fig. 5

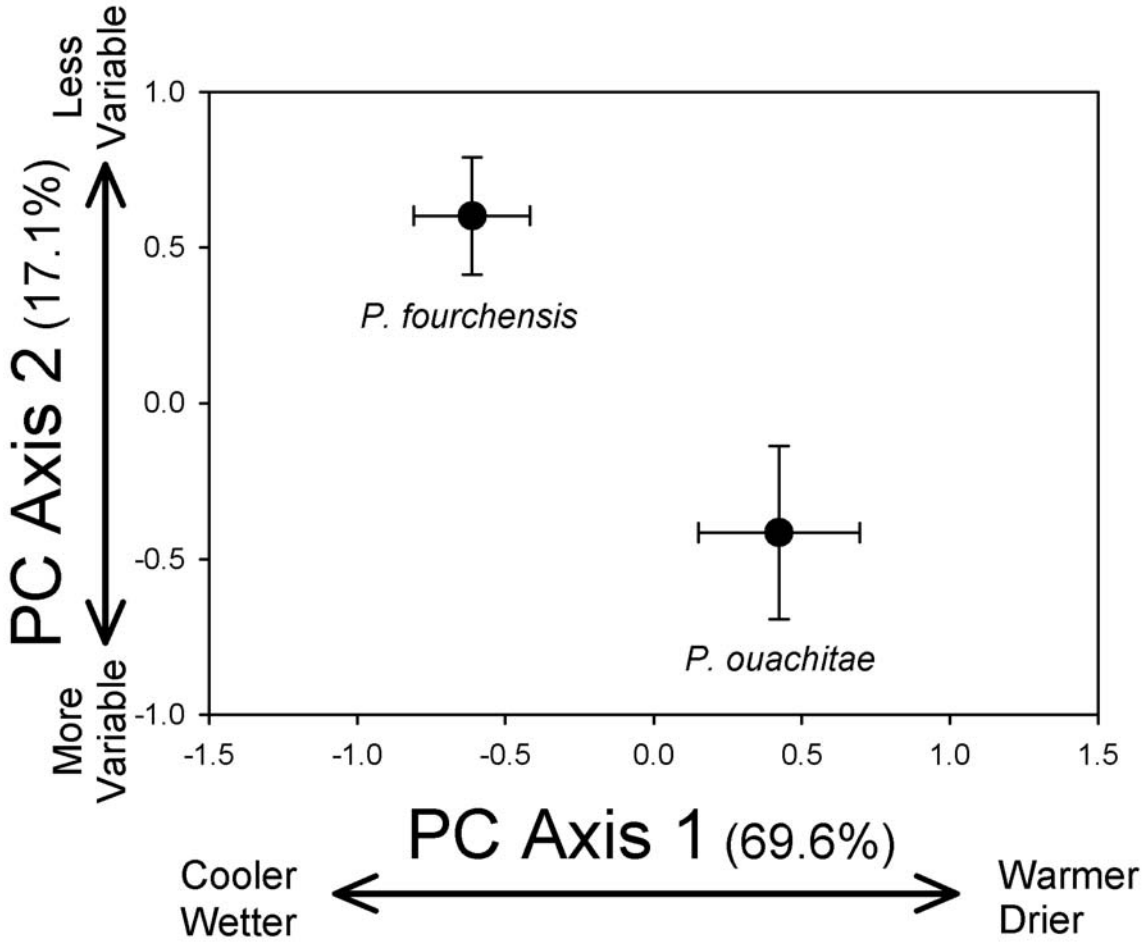

PC Axis 2 (17.1%)
Less Variable
More Variable
1.0
0.5
0.0
-0.5
-1.0
P. fourchensis
P. ouachitae
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
PC Axis 1 (69.6%)
Cooler Wetter
Warmer Drier

Fig. 6

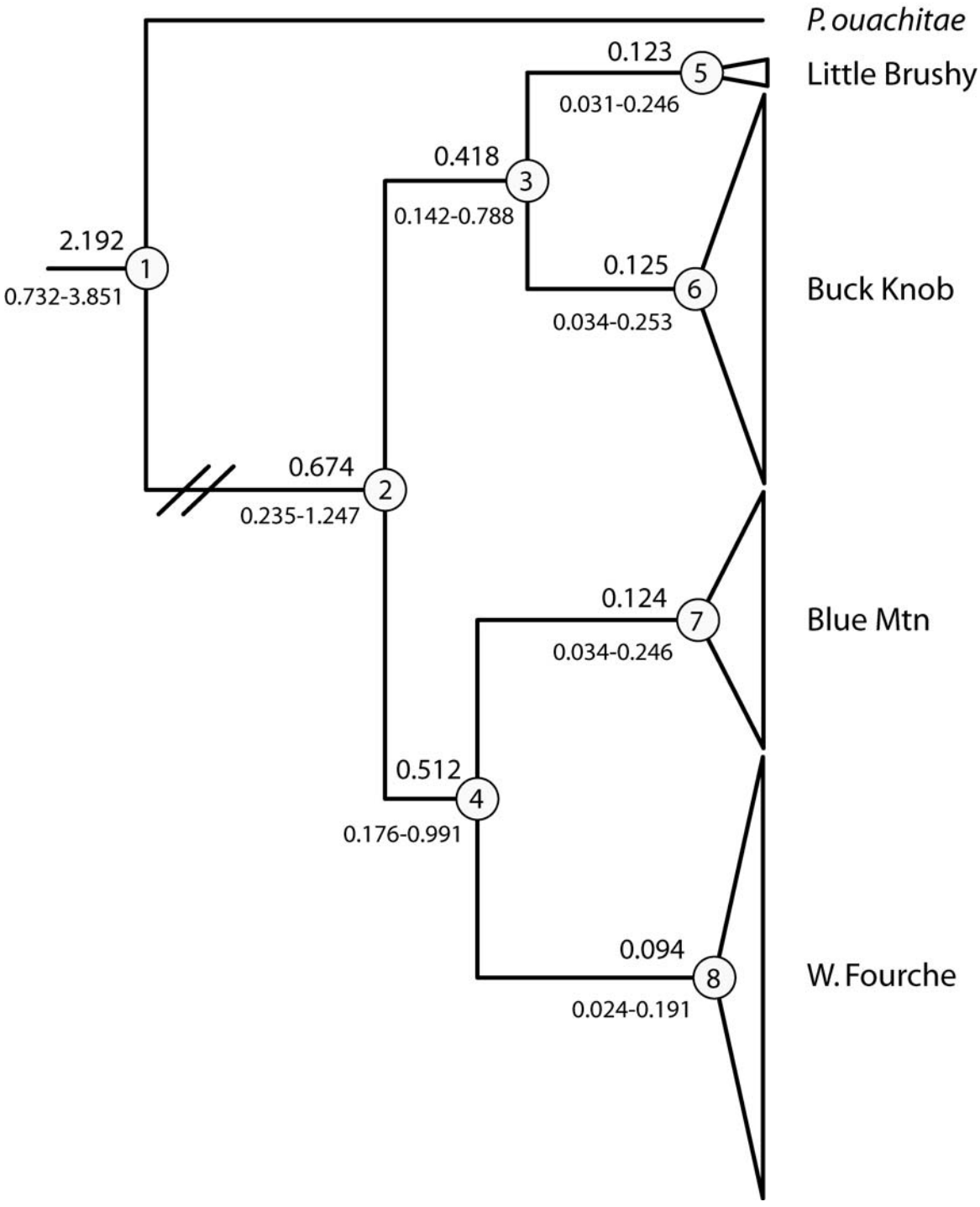
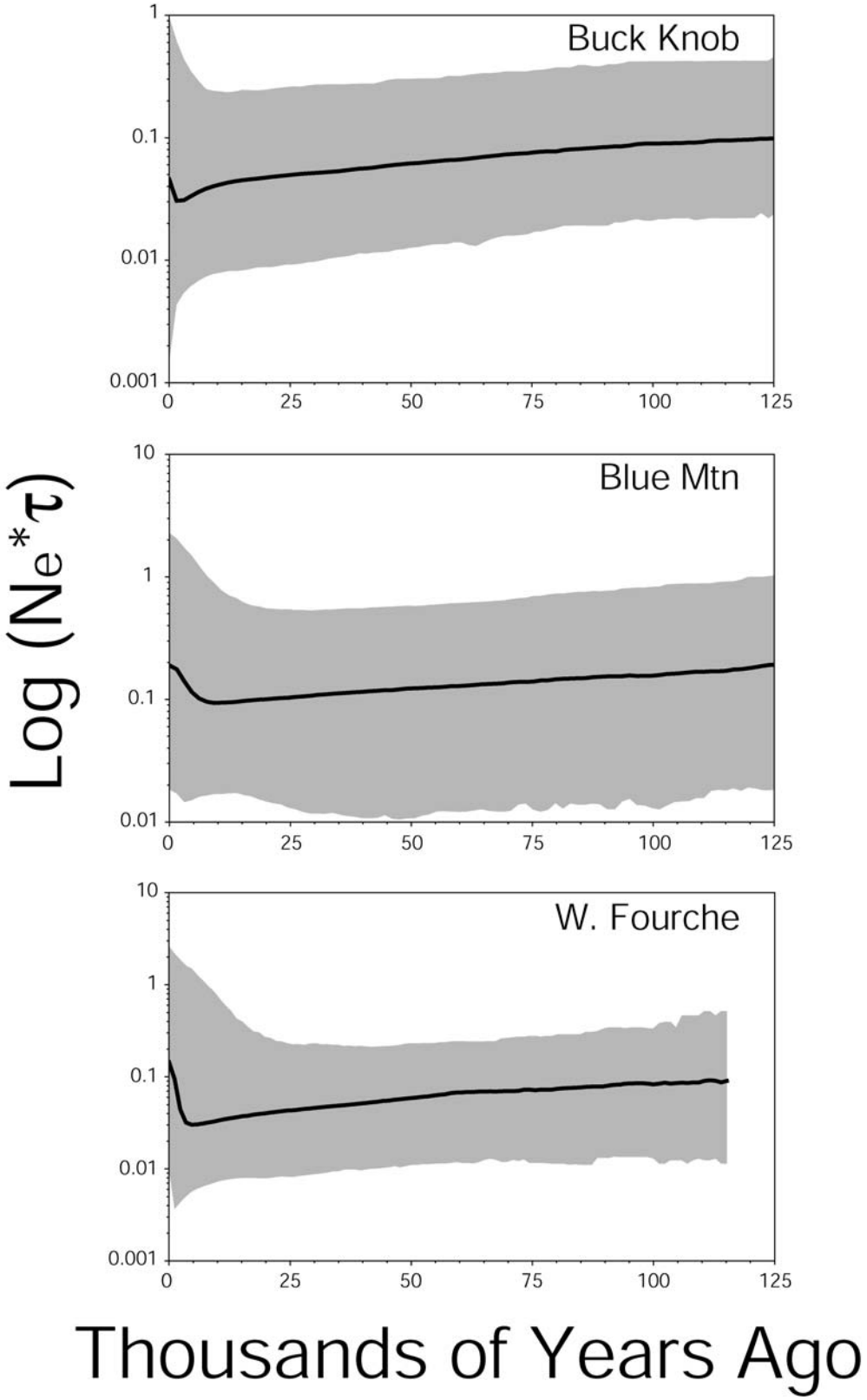

P. ouachitae
0.123
5
Little Brushy
0.031-0.246
0.418
3
0.142-0.788
2.192
1
0.732-3.851
0.125
6
Buck Knob
0.034-0.253
0.674
2
0.235-1.247
0.124
7
Blue Mtn
0.034-0.246
0.512
4
0.176-0.991
0.094
8
W. Fourche
0.024-0.191

Fig. 7


Buck Knob
Blue Mtn
W. Fourche
Log (Ne*τ)
Thousands of Years Ago
1
0.1
0.01
0.001
10
0
25
50
75
100
125

Appendix. Species, voucher numbers, lineage membership, sample localities, geographic coordinates, elevation (m), and GenBank accession numbers for the sequences used in this study (DBS: Donald B. Shepard, KJI: Kelly J. Irwin).

Species	Voucher #	Lineage	Locality	Latitude	Longitude	Elev. (m)	GenBank No. cyt*b*	GenBank No. ND4
P. fourchensis	DBS 1611	W Fourche	Shut In Mountain, SE of the end of FR M24 along tributary to Clear Fork Creek, Scott County, Arkansas	34.71018	-94.27843	529		
P. fourchensis	DBS 1613-1614	W Fourche	Shut In Mountain, SE of the end of FR M24 along tributary to Clear Fork Creek, Scott County, Arkansas	34.70744	-94.27448	540		
P. fourchensis	DBS 752	W Fourche	Fourche Mountain, ~4.34 km E of Eagle Gap on Ouachita Trail, Polk County, Arkansas	34.68434	-94.25429	711		
P. fourchensis	DBS 745-749, 1682-1689	W Fourche	Fourche Mountain, ~5.5 km E of Eagle Gap on Ouachita Trail, Polk County, Arkansas	34.68710	-94.24122	702		
P. fourchensis	DBS 1694-1697, 1709	W Fourche	Fourche Mountain, ~6.0 km E of Eagle Gap on	34.68789	-94.23879	736		

			Ouachita Trail, Polk County, Arkansas			
P. fourchensis	DBS 1676-1680	W Fourche	Fourche Mountain, ~5.5 km W of Foran Gap on Ouachita Trail, Polk County, Arkansas	34.68480	-94.22601	731
P. fourchensis	DBS 1669-1674	W Fourche	Fourche Mountain, ~3.7 km W of Foran Gap on Ouachita Trail, Polk County, Arkansas	34.67893	-94.20690	672
P. fourchensis	DBS 1041-1044	W Fourche	Fourche Mountain, along stream 1.9 km NW of Foran Gap/Ouachita Trail crossing on Hwy 71, Polk County, Arkansas	34.68726	-94.20039	395
P. fourchensis	DBS 1151-1153	W Fourche	~1.9 km NW of Foran Gap, on E side of Hwy 71/270 along Cedar Creek, Polk County, Arkansas	34.68949	-94.19797	370
P. fourchensis	DBS 1666-1668	W Fourche	Fourche Mountain, ~2.5 km W of Foran Gap on Ouachita Trail, Polk County, Arkansas	34.67709	-94.19698	597
P. fourchensis	DBS 487-488	W Fourche	Fourche Mountain, Foran Gap, 0.81 mi SW of Hwy 71/270 along Ouachita Trail, Polk County, Arkansas	34.67400	-94.19142	559

P. fourchensis	DBS 485-486	W Fourche	Fourche Mountain, Foran Gap, 0.62 mi SW of Hwy 71/270 along Ouachita Trail, Polk County, Arkansas	34.67593	-94.18924	543
P. fourchensis	DBS 1731	W Fourche	Fourche Mountain, ~4.0 km W of Wolf Pinnacle on Ouachita Trail/FR 278, Polk County, Arkansas	34.68650	-94.16506	517
P. fourchensis	DBS 1148-1149	W Fourche	Fourche Mountain, along Ouachita Trail ~3.2 km W of Wolf Pinnacle, Polk County, Arkansas	34.68457	-94.15258	602
P. fourchensis	DBS 1298	W Fourche	Fourche Mountain, 2.9 km W of Wolf Pinnacle on FR 278/Ouachita Trail, Polk County, Arkansas	34.68607	-94.14990	603
P. fourchensis	DBS 1297	Blue Mtn	Fourche Mountain, 2.0 km W of Wolf Pinnacle on FR 278/Ouachita Trail, Polk County, Arkansas	34.68820	-94.14156	640
P. fourchensis	DBS 1295	Blue Mtn	Fourche Mountain, 1.1 km W of Wolf Pinnacle on FR 278/Ouachita Trail, Polk County, Arkansas	34.68946	-94.13101	643
P. fourchensis	DBS 1296	Blue Mtn	Fourche Mountain, 0.8 km W of Wolf Pinnacle on FR 278/Ouachita Trail, Polk County, Arkansas	34.69059	-94.12463	656
P. fourchensis	DBS 1742-1747	Blue Mtn	Fourche Mountain, ~1.0 km SE of Wolf Pinnacle	34.68738	-94.11278	655

			on Ouachita Trail, Polk County, Arkansas			
P. fourchensis	DBS 1749-1753	Blue Mtn	Blue Mountain, along Ouachita Trail, Polk County, Arkansas	34.68834	-94.07250	668
P. fourchensis	DBS 1629-1631, 1633	Blue Mtn	Blue Mountain, N slope along FR 54, Scott County, Arkansas	34.70001	-94.05876	374
P. fourchensis	DBS 1756-1761	Blue Mtn	Blue Mountain, E end along Ouachita Trail, Polk County, Arkansas	34.69233	-94.03766	719
P. fourchensis	DBS 1637-1640	Blue Mtn	Fourche Mountain, ~0.25 km W of FR7172/Turner Creek Rd on Ouachita Trail, Polk County, Arkansas	34.67875	-94.01320	565
P. fourchensis	DBS 1728-1729	Blue Mtn	Fourche Mountain, ~0.8 km E of Turner Creek Rd (FR 7172) along Ouachita Trail, Polk County, Arkansas	34.67536	-94.00231	573
P. fourchensis	DBS 1765	Buck Knob	Irons Fork Mountain, S of CR 70/FR 76, Polk County, Arkansas	34.65131	-93.99664	606
P. fourchensis	DBS 1766	Buck Knob	Irons Fork Mountain, S of CR 70/FR 76, Polk County, Arkansas	34.65126	-93.99290	622
P. fourchensis	DBS 1722-1727	Buck Knob	Fourche Mountain, ~2.7 km E of Turner Creek Rd (FR 7172) along Ouachita Trail, Polk	34.67663	-93.98601	620

			County, Arkansas			
P. fourchensis	DBS 1720-1721	Buck Knob	Fourche Mountain, ~3.1 km E of Turner Creek Rd (FR 7172) along Ouachita Trail, Polk County, Arkansas	34.67764	-93.98171	598
P. fourchensis	DBS 489-490	Buck Knob	16.22 mi ENE of Mena along Irons Fork Creek on CR70/FR76 near Brushy Knob, Polk County, Arkansas	34.66161	-93.97120	564
P. fourchensis	DBS 1713-1718	Buck Knob	2.25 km SSW of Buck Knob off Ouachita Trail, Polk County, Arkansas	34.67303	-93.95681	601
P. fourchensis	DBS 1140	Buck Knob	Brushy Knob, SE side along CR 70/FR 216, Polk County, Arkansas	34.64920	-93.95208	525
P. fourchensis	DBS 1699-1703	Buck Knob	Brushy Knob, N slope above CR 70, Polk County, Arkansas	34.65593	-93.94975	627
P. fourchensis	DBS 1734-1739, 1779-1782	Buck Knob	Fourche Mountain, Buck Knob, N slope below FR 76A, Scott County, Arkansas	34.69172	-93.94386	655
P. fourchensis	DBS 516, 1301, 1732-1733	Buck Knob	Fourche Mountain, Buck Knob, along FR 76A ~0.5 mi from top, Scott County, Arkansas	34.68304	-93.94145	599

P. fourchensis	DBS 1698, 1705-1708	Buck Knob	Mast Mountain, 1.2 km S of CR 375/FR 76, Montgomery County, Arkansas	34.65121	-93.91746	613
P. fourchensis	DBS 1762, KJI 1111	Buck Knob	Grapevine Mountain, NE slope below FR 774, Montgomery County, Arkansas	34.64019	-93.88511	568
P. fourchensis	KJI 1085-1088	Little Brushy	Little Brushy Mountain, Montgomery County, Arkansas	34.63174	-93.86533	453
P. o x f	DBS 1173-1184	Rich Mtn	Fourche Mountain, 3.33 km E of Eagle Gap, Polk County, Arkansas	34.68334	-94.26581	702
P. ouachitae	DBS 1275	Kiamichi W	Kiamichi Mountains, N slope below Kiamichi Tower on FR 6025, LeFlore County, Oklahoma	34.62824	-94.81229	687
P. ouachitae	DBS 974	Kiamichi E	Kiamichi Mountains, 3.8 km SSE of Big Cedar, ~135 m E of Hwy 259, LeFlore County, Oklahoma	34.61508	-94.63116	525
P. ouachitae	DBS 1245	Round Mtn	Phillips Mountain, N slope below FR 6025, LeFlore County, Oklahoma	34.61537	-94.49725	655
P. ouachitae	DBS 1596	Rich Mtn	Rich Mountain, N slope above Hwy 270 ~0.4 km E of AR/OK state line,	34.70536	-94.45111	386

			Polk County, Arkansas			
P. ouachitae	DBS 1576	Black Fork	Black Fork Mountain, S of FR 242 on N slope along Price Creek, Scott County, Arkansas	34.72989	-94.38028	376
P. ouachitae	DBS 420	Winding Stair	Winding Stair Mountain, 0.4 mi N of Hwy 1/Talimena Drive on Deadman Trail Rd, LeFlore County, Oklahoma	34.77641	-94.88160	422
P. ouachitae	DBS 760	Buffalo Mtn	Buffalo Mountain, 6.6 km W of Talihina, Bear Den Hollow, Latimer County, Oklahoma	34.76108	-95.12178	481
P. caddoensis	DBS 602	NA	CR79/FR140 near Fodderstock Mtn, Polk County, Arkansas	34.43742	-94.17537	345
P. kiamichi	DBS 512	NA	Kiamichi Mountains, Tombstone Mountain Rd/FR252C near lookout, LeFlore County, Oklahoma	34.62697	-94.79690	533

Chapter III

Morphological variation in the Ouachita Mountain endemic salamanders *Plethodon fourchensis* and *Plethodon ouachitae*

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ABSTRACT.— Morphology of some groups of organisms has been highly conserved over evolutionary time, resulting in genetically divergent taxa with relatively little morphological variation. Salamanders of the genus *Plethodon* have a conserved morphology that makes distinguishing among closely related species difficult without genetic data. Significant morphological variation due to environmental factors and evolutionary history has been demonstrated in some *Plethodon* and suggests that morphology may still be informative in some contexts. We examined morphological variation among the Ouachita Mountain endemic salamanders *P. fourchensis*, *P. ouachitae*, and their hybrids, and tested whether recently identified phylogeographic lineages within each species exhibit morphological differences. We found that *P. fourchensis* and *P. ouachitae* significantly differed morphologically and could be classified correctly with approximately 90% accuracy. As predicted, hybrid individuals were morphologically intermediate to the two parental species. Phylogeographic lineages within each species also exhibited significant morphological differences. Our results support genetic evidence showing significant divergence between *P. fourchensis* and *P. ouachitae*, and among the different lineages within each species. The high level of diversity within *P. fourchensis* and *P. ouachitae* has important implications for conservation because these endemic species have small ranges and lineages are usually restricted to single mountains.

Key words: Character displacement; Cryptic species; Hybridization; Morphology; Niche conservatism; Plethodontidae

INTRODUCTION

Morphology is the product of environmental pressures (biotic and abiotic factors) and evolutionary history (Foote, 1997; Wainwright and Reilly, 1994). The evolution or loss of a particular trait in an ancestral species is likely to constrain the range of potential morphological variation in its evolutionary descendents (Gould and Lewontin, 1979). Because of such phylogenetic constraints, morphology of some groups of organisms has been highly conserved over evolutionary time (e.g., turtles; Rieppel and Reisz, 1999). Salamanders in the genus *Plethodon* are lungless ectotherms that require mesic environments for cutaneous respiration and egg deposition, and thus their distributions are strongly influenced by moisture and temperature (Jaeger, 1971; Petranka, 1998; Spotila, 1972). This narrow range of microhabitat conditions that allows survival in *Plethodon* is thought to constrain morphology while promoting isolation of populations (i.e., niche conservatism), resulting in high species diversity but little morphological variation among species (Kozak and Wiens, 2006; Kozak et al., 2006; Larson, 1989; Wake et al., 1983).

The *glutinosus* group is the most speciose clade of *Plethodon* and many species are considered to be undifferentiated morphologically, having been described based solely on genetic data (Highton, 1995; Highton et al., 1989; Highton and Peabody, 2000; Kozak et al., 2006; Wiens et al., 2006). Carr (1996), however, found significant morphological variation among many species and reported a positive correlation between morphological and genetic distance. In addition, several recent studies on *Plethodon* have found subtle variations in morphology associated with climatic variation, the presence of co-occurring closely related species (i.e., character displacement), and

genetic drift following significant time in isolation (Adams, 2004; Adams and Rohlf, 2000; Adams et al., 2007; Arif et al., 2007; Myers and Adams, 2008; Wilson and Larsen, 1999). These findings suggest that despite a relatively high level of conservatism, morphological studies on *Plethodon* may still be informative in some contexts and could help support or clarify some taxonomic issues.

Species in the *P. ouachitae* complex are endemic to the Ouachita Mountains (Fig. 1) of southeastern Oklahoma and west-central Arkansas and are largely restricted to high-elevation, mesic forest (Duncan and Highton, 1979; Shepard and Burbrink, 2008, 2009; Trauth and Wilhide, 1999). The Ouachita Mountains are part of the Interior Highlands, which share a close geologic and biogeographic relationship with the Appalachian Mountains further to the east (Hatcher et al., 1989; Mayden, 1985, 1988; Thomas, 1985). Dunn and Heinze (1933) first described *P. ouachitae* from Rich Mountain, Polk County, Arkansas, as a white-throated salamander with chestnut dorsal markings. Pope and Pope (1951) described a second species, *P. caddoensis*, within this complex from the Caddo Mountains, Montgomery County, Arkansas, as having an appearance similar to *P. ouachitae*, but lacking the chestnut markings. In a more extensive survey, Blair and Lindsay (1965) extended the range of *P. ouachitae* to include the higher elevations of Black Fork, Winding Stair, Buffalo, Kiamichi, Round, and Fourche Mountains, and noted marked differences in dorsal color pattern among mountains, which led them to name four variants. The Rich Mountain variant (Rich and Black Fork Mountains) had chestnut pigment over most of the dorsum, the Winding Stair variant (Winding Stair and Buffalo Mountains) had a noticeable reduction in chestnut pigment, and the Kiamichi Mountain variant (Kiamichi and Round Mountains) lacked the chestnut pigment altogether. The

fourth variant of *P. ouachitae* described by Blair and Lindsay (1965) was the Buck Knob variant occurring on Fourche Mountain. This variant was characterized by the presence of two longitudinal rows of large white or lichen-colored dorsal spots and no chestnut pigment.

Using allozymes, Duncan and Highton (1979) found that the Buck Knob variant was significantly differentiated genetically from the other variants of *P. ouachitae* and described it as *Plethodon fourchensis*. They also found large genetic differences among the Kiamichi, Winding Stair, Round, Buffalo, Rich, and Black Fork Mountain populations of *P. ouachitae,* but did not recognize these as distinct species. Duncan and Highton (1979) found a narrow zone (~1.8 km) on the western end of Fourche Mountain where *P. fourchensis* and *P. ouachitae* hybridize. Within *Plethodon*, hybridization in contact zones is not uncommon among closely related species (Weisrock et al., 2005; Weisrock and Larson, 2006; Wiens et al., 2006), and its occurrence has led some authors not to recognize many species including *P. fourchensis* (e.g., Petranka, 1998). Recent molecular phylogenetic studies, however, have supported the recognition of all three currently recognized species in the *P. ouachitae* complex (Kozak et al., 2006; Shepard and Burbrink, 2008, 2009; Wiens et al., 2006). Additionally, Shepard and Burbrink (2008, 2009) found that *P. fourchensis* is composed of four geographically distinct lineages and *P. ouachitae* is composed of seven lineages structured across six major mountains. Because each of these defined lineages corresponded to a particular mountain and mountains were separated by warmer, more xeric valleys that appeared to act as barriers to gene flow, Shepard and Burbrink (2008, 2009) suggested that each lineage may constitute an evolutionary distinct species.

In light of past taxonomic issues concerning *P. fourchensis* and new phylogeographic data for both *P. fourchensis* and *P. ouachitae*, we undertook the present morphological study to examine whether the species and lineages defined by genetic data can be discriminated using morphology. Such a correspondence of genetic and morphological data would provide support for the recognition of these as distinct taxa. Our objectives were first to determine whether *P. fourchensis* and *P. ouachitae* differ morphologically, second to examine whether individuals from the hybrid zone, as defined by Duncan and Highton (1979), were morphologically intermediate to the two parental species, and third to test if the lineages within *P. fourchensis* and *P. ouachitae*, as identified by Shepard and Burbrink (2008, 2009), can be discriminated morphologically.

Materials and Methods

Data Collection

We used specimens of *P. fourchensis* and *P. ouachitae* analyzed in the phylogeographic studies of Shepard and Burbrink (2008, 2009). Using these specimens allowed us to know the evolutionary lineage to which each individual belongs with certainty. Additionally, we used 10 individuals of *P. fourchensis* and two individuals of *P. ouachitae* that were not included in Shepard and Burbrink (2008, 2009), but were sequenced, analyzed, and assigned to one of their described lineages (Appendix). We defined hybrids as those individuals collected from the zone delineated by Duncan and Highton (1979). Although we do not know with certainty that all of these individuals were in fact hybrids, classifying individuals from this zone as such is the most conservative approach. We measured only individuals >43 mm snout-vent length (SVL),

which gave us sample sizes of 111 for *P. fourchensis*, 221 for *P. ouachitae*, and 25 for their hybrids. Size at sexual maturity for *P. ouachitae* is reported as 45–49 mm SVL for males and 52–54 mm SVL for females (Highton 1962; Pope and Pope 1951). No specific information on size at maturity for *P. fourchensis* is available, but we assumed it to be similar to *P. ouachitae* because they are sister taxa and similarly sized (Duncan and Highton, 1979; Kozak et al., 2006; Wiens et al., 2006). We determined the sex of individuals via dissection and examination of their gonads. Adult male *Plethodon* have a well-developed mental gland during the breeding season so it was not necessary to dissect individuals with an obvious mental gland.

For each individual, we used digital calipers to measure the following variables to the nearest 0.01 mm: snout-vent length (SVL) – distance from the tip of the snout to the posterior margin of the cloaca, head width (HW) – width of the head at its widest point, head length (HL) – distance from the midline of the gular fold to the tip of the snout, head height (HH) – height of the head at its tallest point, canthus rostralis length (CR) – distance from the anterior edge of the eye to the posterior edge of the nostril, interorbital distance (IOD) – shortest distance between the eyes taken dorsally, body width (BW) – width of the trunk at its widest point, body height (BH) – height of the trunk at its tallest point, axilla–groin length (AGL) – distance from the anterior edge of the hind limb insertion to the posterior edge of the front limb insertion, humerus length (HUM) – distance from the insertion of the forelimb to the tip of the elbow, and femur length (FEM) – distance from the insertion of the hindlimb to the tip of the knee. These measurements are commonly used in studies of morphological variation in salamanders of the genus *Plethodon*, and characters on the feet and digits are also common (e.g., Carr,

1996; Wilson and Larsen, 1999). However, species in the *P. ouachitae* complex suffer from mite infestations, unlike their syntopic congeners, which often deform their feet and digits making them unreliable for use as characters in morphological studies (Duncan and Highton, 1979; Pope and Pope, 1951; Winter et al., 1986).

Statistical Analyses

We tested for differences in SVL between *P. fourchensis* and *P. ouachitae* and between males and females using a two-way ANOVA with species and sex as classes and SVL as the dependent variable. Because all morphological measurements are related to overall body size and covary with one another, we created size-adjusted, independent morphological variables to determine whether species differed in other morphological attributes. To do this, we first pooled all data, regressed each variable against SVL, and calculated the standardized residuals to remove the effect of body size variation. We then used a principal components analysis (PCA) on the covariance matrix of these new size-adjusted variables to reduce them to a smaller number of independent variables. We retained components with eigenvalues greater than the mean eigenvalue, and used the scores for each retained component in a MANOVA to test for morphological differences between *P. fourchensis* and *P. ouachitae*, and between males and females. We excluded hybrids from these analyses and $\log_{10}$-transformed all measurements before analysis.

Next, we used a stepwise discriminant function analysis (DFA) on the size-adjusted morphological variables to calculate the probability of correctly classifying the two species based on morphology. We performed separate analyses for males and females because the previous analysis indicated significant sexual dimorphism in both

species. We excluded the hybrids from the discriminant analyses, but used the resulting functions to classify them to one of the two parental species. We followed this analysis by conducting a canonical correspondence analysis (CCA) in CANOCO v.4.5 to determine which variables had the most power to discriminate among species (Ter Braak, 1986; Ter Braak and Šmilauer, 1998). CCA is a constrained ordination technique that relates two data matrices and maximizes their correlation (Ter Braak, 1986). For this analysis, we constructed three matrices with each individual represented as a row: 1) species/hybrid, 2) log-transformed morphometric data excluding SVL, and 3) log-transformed SVL (as a covariate). We analyzed females and males separately. We performed a stepwise manual selection of variables using 9999 Monte Carlo unrestricted permutations and added variables until the addition of variables no longer contributed significantly to the model (i.e., $P > 0.05$). We used the CANOCO options: focus on inter-species distances and Hill's scaling (Lepš and Šmilauer, 2003).

We tested for differences in SVL among lineages within *P. fourchensis* ($n = 4$) and *P. ouachitae* ($n = 7$) using ANOVA with lineage as the class and $\log_{10}$-SVL as the dependent variable. A significant overall ANOVA was followed by post-hoc Tukey HSD tests to determine which lineages were significantly different from each other. To test whether lineages within species differed in other morphological aspects, we recalculated size-adjusted variables as above on each species separately and used these in a PCA for each species. We used the scores for each retained component of these PCAs in a MANOVA with lineage and sex as classes. Next, we performed a DFA for each species using their size-adjusted morphological variables to calculate the probability of correctly classifying the different lineages based on morphology. We conducted separate

DFAs for males and females, and excluded hybrids from these analyses. We followed these analyses with a CCA in CANOCO v.4.5 to determine which variables had the most power to discriminate among lineages within each species. For CCAs, we constructed three matrices with each individual represented as a row: 1) lineage, 2) morphometric data, and 3) SVL (as a covariate). We analyzed females and males separately. The process of selecting variables and options in CANOCO were the same as above.

RESULTS

Interspecies Variation

Snout-vent length significantly differed between *P. fourchensis* and *P. ouachitae* ($F_{1,328} = 36.11$, $P < 0.001$) with *P. fourchensis* being larger on average (Table 1). Males and females did not significantly differ in SVL ($F_{1,328} = 0.11$, $P = 0.74$), and this was true for both species (i.e., non-significant interaction: $F_{1,328} = 0.04$, $P = 0.85$; Table 1). Principal components analysis reduced the size-adjusted morphological variables to three components that explained 66.94% of the variation. A MANOVA using the scores of the three principal components as dependent variables showed significant differences between *P. fourchensis* and *P. ouachitae* (Wilk's $\lambda = 0.52$, $F_{3,326} = 100.06$, $P < 0.001$). Males and females also significantly differed (Wilk's $\lambda = 0.75$, $F_{3,326} = 35.88$, $P < 0.001$), and this was true for both species (i.e., non-significant interaction: Wilk's $\lambda = 0.99$, $F_{3,326} = 0.94$, $P = 0.42$). Discriminant function analysis using the size-adjusted morphological variables correctly classified 88.1% of females and 92.3% of males correctly to species (null expectation = 50%). Of the 12 female hybrids, seven (58.3%) were classified as *P. fourchensis* and five (41.7%) were classified as *P. ouachitae*. Of the 13 male hybrids,

eight (61.5%) were classified as *P. fourchensis* and five (38.5%) were classified as *P. ouachitae*.

Results from CCA showed a significant association between species and morphology for females ($F_{2,188} = 7.50$, $P = 0.0001$) and males ($F_{2,167} = 8.60$, $P = 0.0001$). For both females and males, Monte Carlo permutation tests indicated that five variables contributed significantly to the total amount of variation between species, but which variables were important differed slightly between the sexes (Table 2). For females, IOD and HUM explained the most variation between species whereas CR and HL explained the most variation between species for males (Table 2; Fig. 2). Relatively speaking, *P. fourchensis* had wider heads (HW) and longer forelimbs (HUM) whereas *P. ouachitae* had a wider distance between the eyes (IOD) and a longer canthus rostralis in males (CR). Plots of individual scores along the first and second canonical axes showed that species separated primarily along the first axis, but still overlapped a moderate amount (Fig. 2). Hybrid individuals appeared intermediate to the parental species in both females and males; however, overlap with *P. fourchensis* was greater than overlap with *P. ouachitae* (Fig. 2)

Intraspecies Variation

Lineages within *P. fourchensis* did not differ significantly in SVL ($F_{3,107} = 0.80$, $P = 0.50$). A PCA reduced size-adjusted morphological variables to four components that explained 76.78% of the variation. A MANOVA using the scores of the four retained principal components as dependent variables showed significant differences between lineages of *P. fourchensis* (Wilk's $\lambda = 0.59$, $F_{12,265} = 4.89$, $P < 0.001$). Males and females

also significantly differed (Wilk's $\lambda = 0.56$, $F_{4,100} = 20.02$, $P < 0.001$), but this was not consistent across all lineages (i.e., a significant interaction: Wilk's $\lambda = 0.76$, $F_{12,265} = 2.23$, $P = 0.01$). Discriminant function analysis using the size-adjusted morphological variables correctly classified 72.2% of females and 63.2% of males correctly to one of the four lineages (null expectation = 25%). Results from CCA showed a significant association between lineage and morphology for females ($F_{3,53} = 2.15$, $P = 0.002$), but male results were marginally non-significant ($F_{3,56} = 1.42$, $P = 0.09$). Monte Carlo permutation tests indicated that HL, HH, and AGL contributed significantly to the total amount of variation between lineages in females, but only CR was significant in males (Table 3; Fig. 3). In females, the Little Brushy lineage had relatively deeper heads (HH), the Blue Mountain and Little Brushy lineages had relatively longer trunks (AGL), and the W. Fourche and Buck Knob lineages had relatively longer heads (HL). In males, the W. Fourche and Blue Mountain lineages had relatively longer canthus rostralis lengths (CR). Plots of individual scores along the first and second canonical axes showed that lineages within *P. fourchensis* overlapped considerably (Fig. 3).

Lineages within *P. ouachitae* significantly differed in SVL ($F_{6,214} = 5.26$, $P < 0.001$). Post-hoc Tukey HSD tests showed that individuals from Kiamichi E, Kiamichi W, and Round Mountain lineages were significantly smaller on average ($P < 0.05$) than individuals from the Black Fork and Buffalo Mountain lineages (Fig. 4). A PCA reduced the size-adjusted morphological variables to three components that explained 65.02% of the variation. A MANOVA using the scores of the three retained principal components as dependent variables showed significant differences between lineages of *P. ouachitae* (Wilk's $\lambda = 0.75$, $F_{18,580} = 3.48$, $P < 0.001$). Males and females also significantly differed

(Wilk's $\lambda = 0.76$, $F_{3,205} = 21.41$, $P < 0.001$), and this was consistent across all lineages (i.e., non-significant interaction: Wilk's $\lambda = 0.74$, $F_{18,580} = 0.94$, $P = 0.77$). Discriminant function analysis using size-adjusted morphological variables correctly classified 56.9% of females and 64.3% of males to one of the seven lineages (null expectation = 14.3%). Results from CCA showed a significant association between lineage and morphology for females ($F_{6,122} = 1.62$, $P = 0.003$) and males ($F_{6,97} = 1.76$, $P = 0.0006$). Monte Carlo permutation tests indicated that CR, BH, HL, and HH contributed significantly to the total amount of variation between lineages in females, whereas HH and BH were significant in males (Table 3; Fig. 5). In females, the Kiamichi E and Kiamichi W lineages had relatively shorter and flatter heads (HL and HH), the Rich Mountain and Black Fork lineages had a relatively longer canthus rostralis (CR), and the Buffalo, Winding Stair, and Round Mountain lineages had higher trunks (BH). In males, the Kiamichi E and Kiamichi W lineages had relatively flatter heads (HH). Plots of individual scores along the first and second canonical axes showed that lineages within *P. ouachitae* overlapped considerably (Fig. 5).

DISCUSSION

Strong niche conservatism in *Plethodon* has helped produce high species diversity, but at the same time appears to have constrained morphological variation (Kozak and Wiens, 2006; Larson, 1989; Wake et al., 1983). Consequently, distinguishing among closely related species of *Plethodon* is difficult without genetic data (Highton, 1995; Highton et al., 1989; Highton and Peabody, 2000). The degree of morphological variation among species in the *glutinosus* group is positively related to

genetic distance (Carr, 1996); therefore, morphological differences are predicted to be smallest for recently diverged taxa. Genetic data indicate that *P. fourchensis* and *P. ouachitae* diverged approximately 2.2 mya and lineage diversification within each species occurred during the Middle Pleistocene (~0.750–0.400 mya; Shepard and Burbrink, 2008, 2009). Our results here showed that *P. fourchensis* and *P. ouachitae* significantly differ morphologically and can be discriminated with about 90% accuracy. Therefore, the recognition of two species as suggested by genetic studies is well-supported by our morphological data (Duncan and Highton, 1979; Kozak et al., 2006; Shepard and Burbrink, 2008, 2009; Wiens et al., 2006).

Individuals from the hybrid zone were morphologically intermediate to the two parental species, but the majority (60%) was more similar to *P. fourchensis*. Shepard and Burbrink (2009) found that individuals in the western-most locality in the hybrid zone had mitochondrial DNA of *P. ouachitae*, but individuals from other localities further east in the hybrid zone had mitochondria of *P. fourchensis*. Further, they noted that individuals in the eastern part of the hybrid zone had the typical dorsal color pattern of *P. fourchensis* whereas the dorsal color pattern of individuals from the western-most locality was highly variable and not typical of either parental species (Shepard and Burbrink, 2009). Hybridization is common among closely related species of *Plethodon* that diverged in allopatry and then come back into secondary contact (Weisrock et al., 2005; Weisrock and Larson, 2006; Wiens et al., 2006). A small amount of hybridization in contact zones is not enough to obscure the historic effects of prolonged isolation in *Plethodon* because gene flow between species does not typically penetrate beyond these narrow contact zones (Weisrock et al., 2005; Weisrock and Larson, 2006). Further,

hybridization in contact zones is likely a crucial step in the evolution of reproductive isolation through reinforcement, and therefore the occurrence of hybridization should not necessarily preclude recognition of taxa as distinct species (Coyne and Orr, 2004; Wake, 2006). Female hybrids in our study differed from parental species most notably in having smaller body widths and head widths, and several of these individuals appeared emaciated when collected (D. Shepard, personal observation). Poorer body condition in hybrid individuals could indicate hybrid inviability; however, our sample sizes were too small to draw any conclusions.

Lineages within *P. fourchensis* showed no difference in overall body size (SVL); however, lineages within *P. ouachitae* exhibited significant differences. Lineages within *P. ouachitae* from the Kiamichi Mountains (Kiamichi W, Kiamichi E, Round Mountain lineages) were significantly smaller in SVL than some lineages on other mountains. The smaller size of *P. ouachitae* in the Kiamichi Mountains (including Round Mountain) has been reported previously (Blair and Lindsay, 1965; Duncan and Highton, 1979). In the Kiamichi Mountains, *P. ouachitae* is syntopic with *P. kiamichi*, another member of the *glutinosus* group, and they occur in near equal numbers (Duncan and Highton, 1979; D. Shepard, unpublished data). Other sympatric members of the *glutinosus* group (e.g., *P. albagula*) are largely absent from the higher elevations on Rich, Black Fork, Winding Stair, and Buffalo Mountains, and behavioral experiments have shown that *P. ouachitae*, although smaller in size, is able to exclude the larger *P. albagula* by being highly aggressive (Anthony et al., 1997; Duncan and Highton, 1979; Trauth and Wilhide, 1999; D. Shepard, unpublished data). Because of intense inter-specific competition, morphological character displacement is a common phenomenon in *Plethodon* where

closely related species co-occur (Adams, 2004; Adams and Rohlf, 2000; Adams et al., 2007; Hairston, 1951). Smaller body size of *P. ouachitae* in the Kiamichi Mountains may have evolved to reduce competitive interactions with the larger, syntopic *P. kiamichi*. Because the Kiamichi Mountains were the most probable ancestral area for *P. ouachitae* and dispersal northward to the other mountains occurred more recently in its evolutionary history (Shepard and Burbrink, 2008), it suggests that the evolution of larger body size in lineages on other mountains may be the result of competitive release.

Despite a large amount of similarity, significant differences in morphology independent of body size were found among lineages within each species. This subtle variation allowed lineages to be discriminated with a moderately high level of accuracy given their recent origins (Shepard and Burbrink, 2008, 2009). Lineages within *P. ouachitae* also vary in dorsal color pattern; however, similar color patterns are shared by some lineages (i.e., the so-called variants of *P. ouachitae*; Blair and Lindsay, 1965; Duncan and Highton, 1979; D. Shepard, unpublished data). No geographic variation in dorsal color pattern has been reported in *P. fourchensis*.

Much of the diversity in *Plethodon* is morphologically cryptic, which has important implications for conservation because many genetically distinct taxa have small ranges or are restricted to single mountain tops (Highton, 1995; Highton et al., 1989; Shepard and Burbrink, 2008, 2009). Identification and recognition of evolutionary unique entities is imperative to conserve biodiversity. The Appalachian Mountains have the highest diversity of *Plethodon* and most genetic work to identify morphologically cryptic species has been conducted there (Highton, 1995; Highton et al., 1989; Highton and Peabody, 2000). The Ouachita Mountains have a smaller number of species;

however, little work has been conducted to examine patterns of genetic diversity within these species until recently. The Ouachita Mountain endemic salamanders *P. fourchensis* and *P. ouachitae* have been shown to be genetically and morphologically differentiated (Blair and Lindsay, 1965; Duncan and Highton, 1979; Shepard and Burbrink, 2008, 2009; this study). Further, each of these species is composed of multiple geographically distinct, and genetically and morphologically divergent lineages (Shepard and Burbrink, 2008, 2009; this study). Although most of the ranges of *P. fourchensis* and *P. ouachitae* are within the Ouachita National Forest, and thus somewhat protected, logging is a threat and management practices may need to be modified to conserve all evolutionary lineages.

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TABLE 1.— Means (± SD) of morphological variables measured for *P. fourchensis*, *P. ouachitae*, and their hybrids. F: female, M: male, *n*: sample size, SVL: snout-vent length, HW: head width, HL: head length, HH: head height, CR: canthus rostralis length, IOD: interorbital distance, BW: body width, BH: body height, AGL: axilla–groin length, HUM: humerus length, FEM: femur length.

	P. fourchensis			*P. ouachitae*			Hybrids		
Sex	F	M	Total	F	M	Total	F	M	Total
n	54	57	111	123	98	221	12	13	25
SVL	62.05 ± 8.16	62.10 ± 7.52	62.07 ± 7.80	56.94 ± 6.18	57.22 ± 4.93	57.06 ± 5.65	60.15 ± 3.19	59.37 ± 6.53	59.75 ± 5.12
HW	9.28 ± 1.06	9.43 ± 0.94	9.35 ±1.00	8.44 ± 0.83	8.56 ± 0.75	8.50 ± 0.79	8.84 ± 0.49	9.04 ± 0.92	8.95 ± 0.74
HL	13.76 ± 1.55	14.20 ± 1.52	13.98 ± 1.55	12.63 ± 1.21	13.02 ± 1.09	12.80 ± 1.17	13.47 ± 0.64	13.72 ± 1.27	13.60 ± 1.00
HH	4.40 ± 0.60	4.54 ± 0.51	4.47 ± 0.56	3.93 ± 0.55	3.97 ± 0.50	3.95 ± 0.53	4.01 ± 0.55	4.22 ± 0.60	4.12 ± 0.57
CR	2.29 ± 0.33	2.47 ± 0.40	2.38 ± 0.38	2.31 ± 0.33	2.57 ± 0.34	2.43 ± 0.35	2.33 ± 0.27	2.43 ± 0.46	2.38 ± 0.38
IOD	2.61 ± 0.31	2.72 ± 0.36	2.67 ± 0.34	2.64 ± 0.31	2.81 ± 0.26	2.71 ± 0.30	2.58 ± 0.20	2.62 ± 0.38	2.60 ± 0.30
BW	8.16 ± 1.25	8.22 ± 1.03	8.19 ± 1.14	7.74 ± 1.18	7.63 ± 0.95	7.69 ± 1.08	7.32 ± 1.01	7.72 ± 1.27	7.53 ± 1.15
BH	5.22 ± 0.87	5.29 ± 0.66	5.25 ± 0.77	4.88 ± 0.86	4.98 ± 0.65	4.93 ± 0.78	5.14 ± 0.61	5.10 ± 0.78	5.12 ± 0.69
AGL	33.19 ± 4.63	32.39 ± 4.17	32.77 ± 4.40	30.86 ± 3.68	30.31 ± 2.73	30.61 ± 3.30	32.19 ± 1.99	31.01 ± 4.12	31.58 ± 3.27
HUM	6.27 ± 0.59	6.34 ± 0.64	6.30 ± 0.61	5.65 ± 0.47	5.85 ± 0.43	5.74 ± 0.46	6.11 ± 0.37	6.01 ± 0.51	6.06 ± 0.44
FEM	6.40 ± 0.60	6.47 ± 0.61	6.44 ± 0.60	5.83 ± 0.52	5.98 ± 0.47	5.89 ± 0.50	6.19 ± 0.43	6.08 ± 0.48	6.13 ± 0.45

TABLE 2.—Results from canonical correspondence analysis on morphology of female and male *P. fourchensis*, *P. ouachitae*, and their hybrids showing which variables explained a significant amount of the morphological variation between species (%VarExp). Significance was tested using 9999 Monte Carlo permutations in CANOCO v.4.5.

Females				Males			
Variable	*F*	*P*	%VarExp	Variable	*F*	*P*	%VarExp
IOD	26.06	0.0001	41.27	CR	44.26	0.0001	59.56
HUM	20.49	0.0001	29.45	HL	12.98	0.0001	16.30
HW	7.38	0.0012	10.23	HW	4.38	0.0209	5.33
BW	7.08	0.0030	9.52	IOD	3.83	0.0289	4.59
BH	4.20	0.0225	5.47	HUM	3.84	0.0292	4.59

TABLE 3.—Results from canonical correspondence analysis on morphology of lineages within *P. fourchensis* and *P. ouachitae* showing which variables explained a significant amount of the morphological variation among lineages for females and males (%VarExp). Significance was tested using 9999 Monte Carlo permutations in CANOCO v.4.5. .

P. fourchensis

Females

Variable	*F*	*P*	%VarExp
HL	5.77	0.0006	30.04
HH	3.23	0.0206	16.13
AGL	2.82	0.0400	13.61

Males

Variable	*F*	*P*	%VarExp
CR	6.33	0.0002	43.85
AGL	1.87	0.1333	12.71

P. ouachitae

Females

Variable	*F*	*P*	%VarExp
CR	3.48	0.0013	22.19
BH	2.64	0.0154	16.58
HL	2.38	0.0258	14.71
HH	2.18	0.0382	13.37

Males

Variable	*F*	*P*	%VarExp
HH	3.48	0.0011	20.88
BH	2.70	0.0112	15.86

FIGURE LEGENDS

FIG. 1.—Map of the United States showing the location of the Ouachita Mountains (A), major mountains within the range of *P. fourchensis* and *P. ouachitae* showing elevations >500 m, (B), and a digital elevation map showing the localities of specimens of *P. fourchensis* (●) and *P. ouachitae* (○) used in Shepard and Burbrink (2008, 2009) and this study (C). Localities within the hybrid zone of Duncan and Highton (1979) are shown as ×. Elevation within this region ranges from a low of 135 m (black) to a high of 818 m (white).

FIG. 2.—Canonical correspondence analysis biplots for female (A) and male (C) *P. fourchensis*, *P. ouachitae*, and their hybrids. Canonical axes represent linear combinations of morphological variables. The angle and length of arrows indicate the direction and magnitude of the correlation of the variable with each axis and show how species differ with respect to the variables. Plots of individual scores along the first and second canonical axes show *P. fourchensis* (●), *P. ouachitae* (○), and their hybrids (×) for females (B) and males (D). In A and B, the first canonical axis accounts for 85.9% of the total explained variation and second canonical axis 14.1%. In C and D, the first canonical axis accounts for 93.2% and second canonical axis 6.8%.

FIG. 3.—Canonical correspondence analysis biplots for lineages within *P. fourchensis* for females (A) and males (C). Canonical axes represent linear combinations of morphological variables. The angle and length of arrows indicate the direction and magnitude of the correlation of the variable with each axis and show how lineages differ

with respect to the variables. Plots of individual scores along the first and second canonical axes show the lineages for females (B) and males (D). Lineages are: Blue Mountain (○), Buck Knob (●), Little Brushy (×), and W. Fourche (✦). In A and B, the first canonical axis accounts for 74.2% of the total explained variation and second canonical axis 17.2%. In C and D, the first canonical axis accounts for 98.7% and second canonical axis 1.3%.

FIG. 4.—Average snout-vent length (SVL) for the seven lineages within *P. ouachitae*. Error bars are 95% Confidence Intervals. Means with the same letter above (a,b) are not significantly different from each other ($P > 0.05$). Sample sizes are listed below.

FIG. 5.—Canonical correspondence analysis biplots for lineages within *P. ouachitae* for females (A) and males (C). Canonical axes represent linear combinations of morphological variables. The angle and length of arrows indicate the direction and magnitude of the correlation of the variable with each axis and show how lineages differ with respect to the variables. Plots of individual scores along the first and second canonical axes show the lineages for females (B) and males (D). Lineages are: Black Fork (○), Buffalo Mountain (△), Rich Mountain (●), Round Mountain (▲), Winding Stair (×), Kiamchi E (✦), and Kiamichi W (□). In A and B, the first canonical axis accounts for 61.9% of the total explained variation and second canonical axis 21.6%. In C and D, the first canonical axis accounts for 70.8% and second canonical axis 29.2%.

Fig. 1

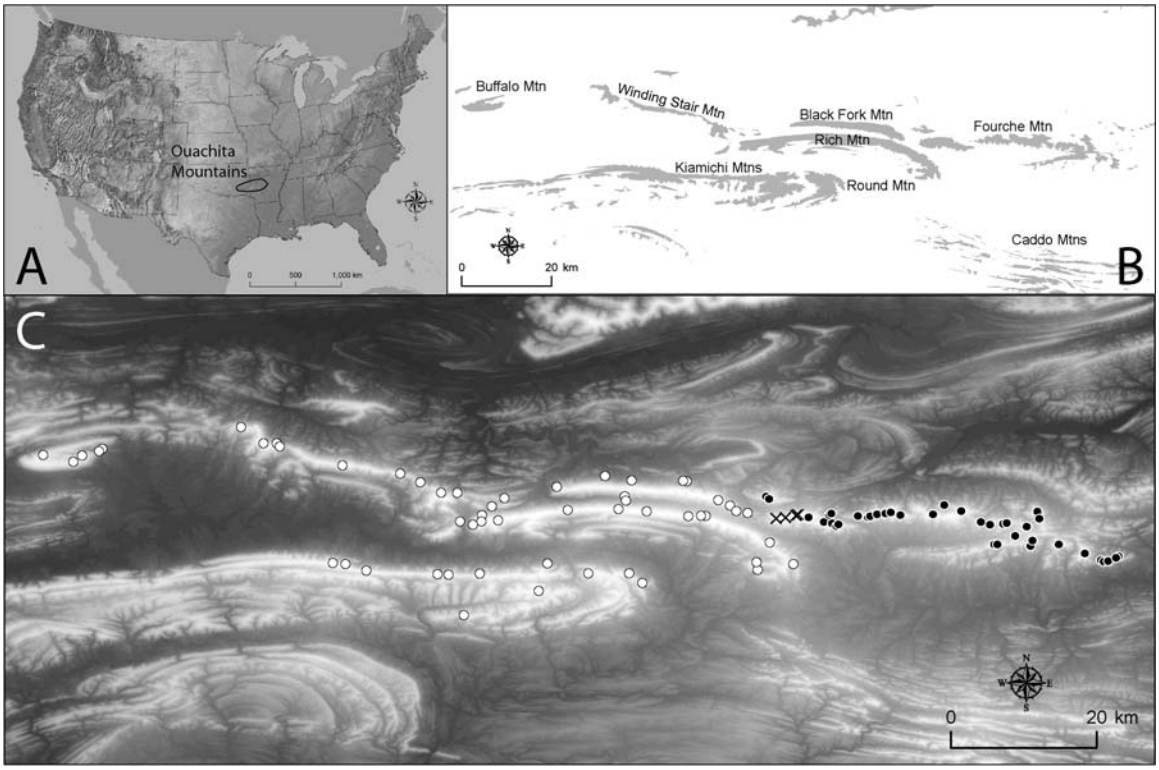

A
Ouachita
Mountains
0
500
1,000 km
B
Buffalo Mtn
Winding Stair Mtn
Black Fork Mtn
Rich Mtn
Fourche Mtn
Kiamichi Mtns
Round Mtn
Caddo Mtns
0
20 km
C
N
W
E
S
0
20 km

Fig. 2

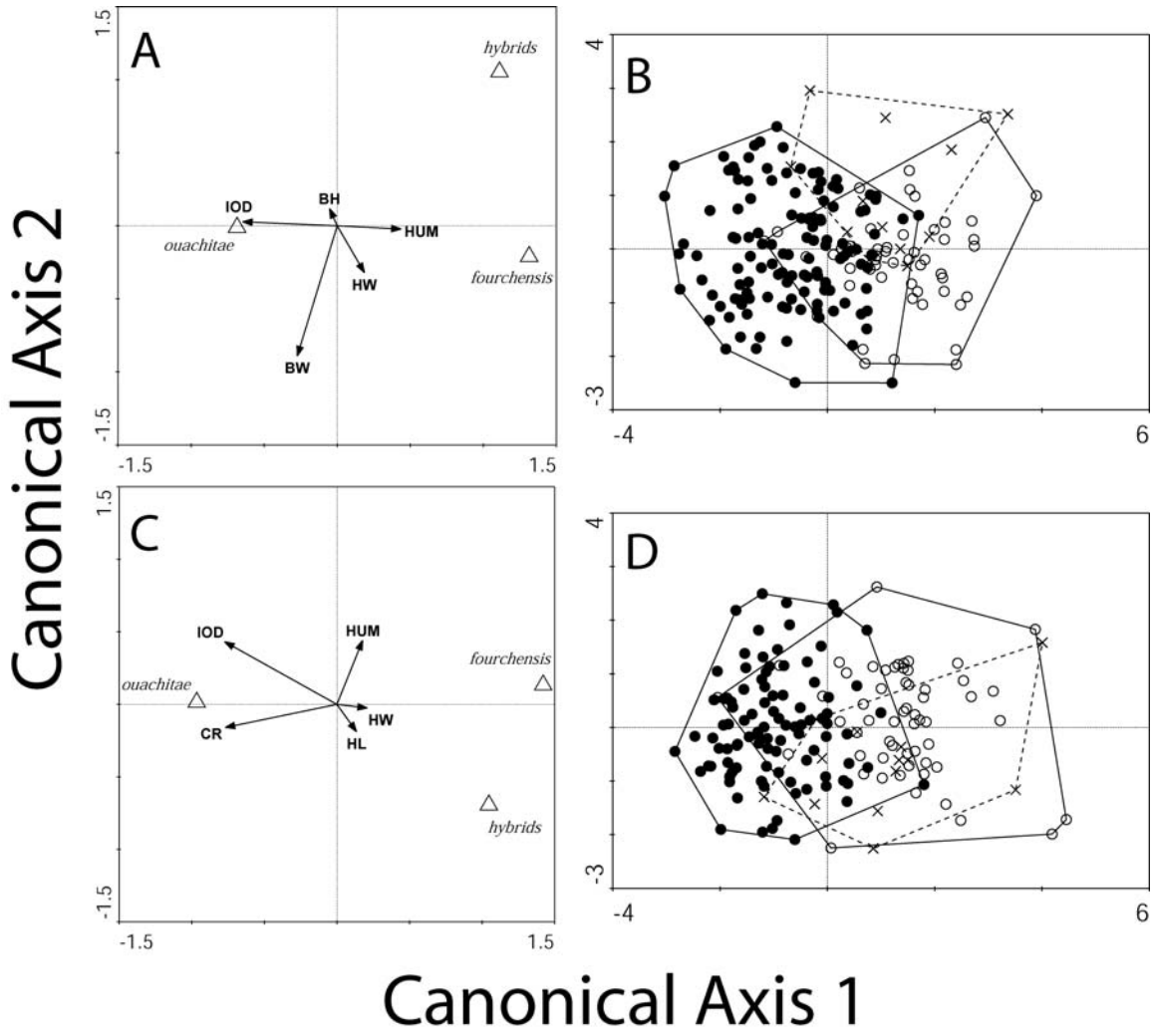

A
hybrids
IOD
BH
HUM
ouachitae
fourchensis
HW
BW
1.5
-1.5
-1.5
1.5
B
4
-3
-4
6
C
IOD
HUM
fourchensis
ouachitae
HW
CR
HL
hybrids
D
Canonical Axis 2
Canonical Axis 1

Fig. 3

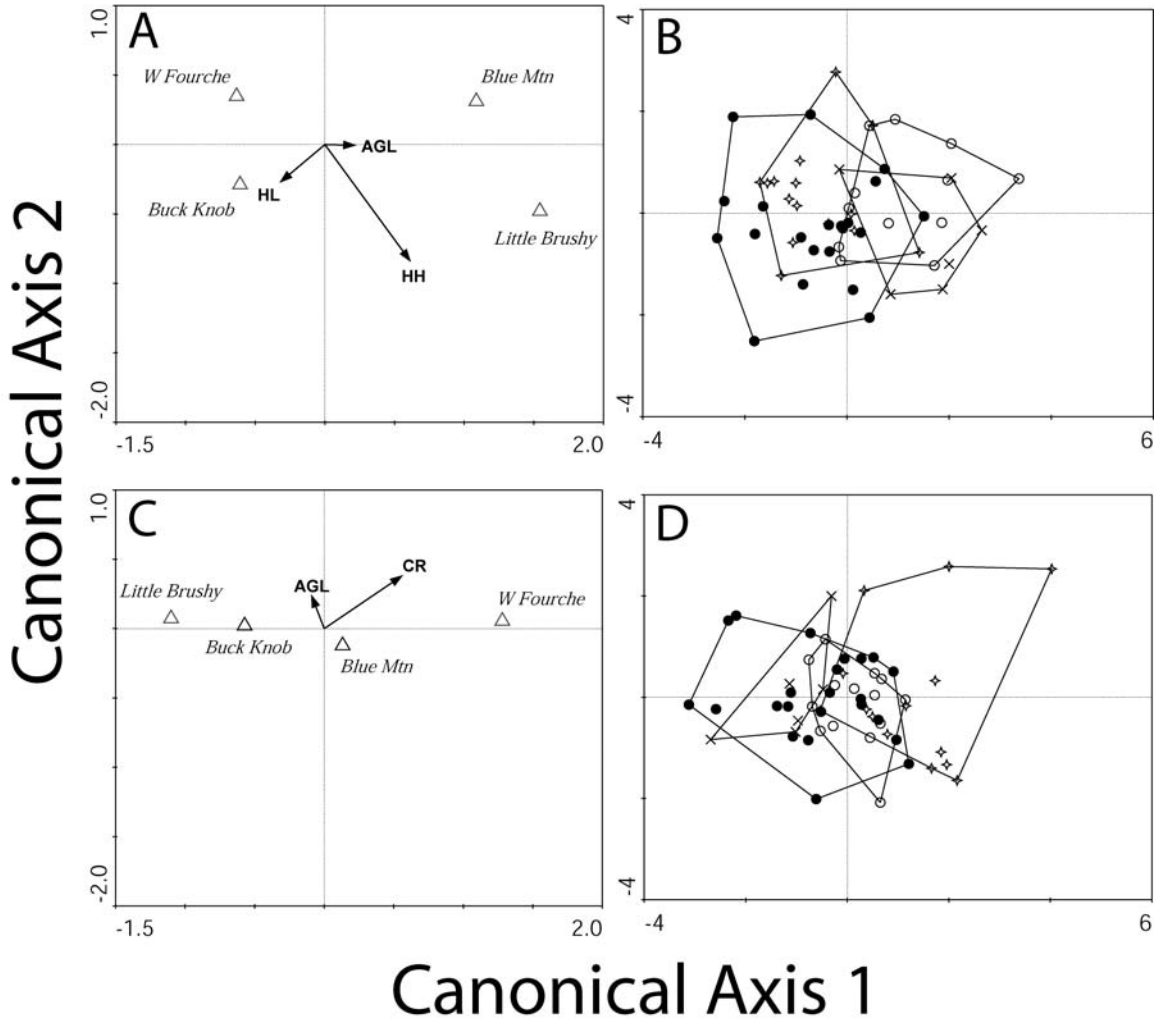

A
W Fourche
Blue Mtn
AGL
HL
Buck Knob
Little Brushy
HH
1.0
-2.0
-1.5
2.0
B
4
-4
-4
6
C
Little Brushy
AGL
CR
W Fourche
Buck Knob
Blue Mtn
1.0
-2.0
-1.5
2.0
D
4
-4
-4
6
Canonical Axis 2
Canonical Axis 1

Fig. 4

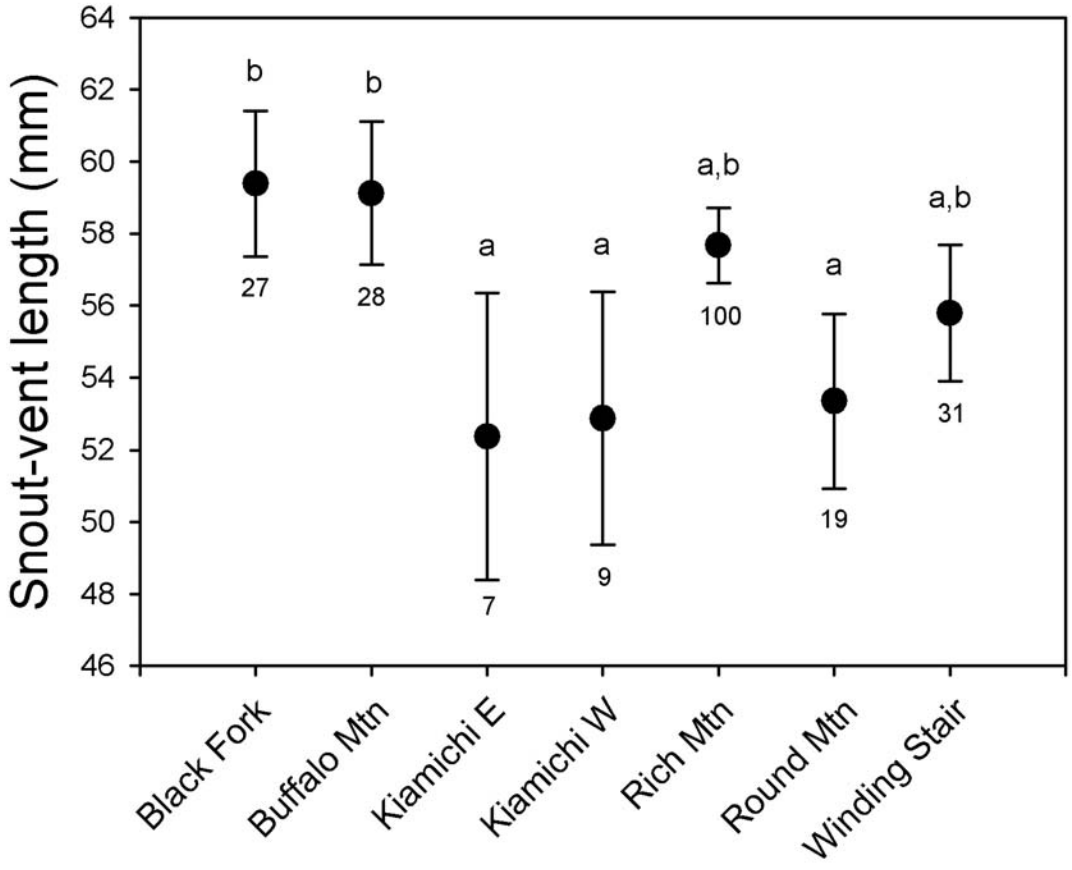
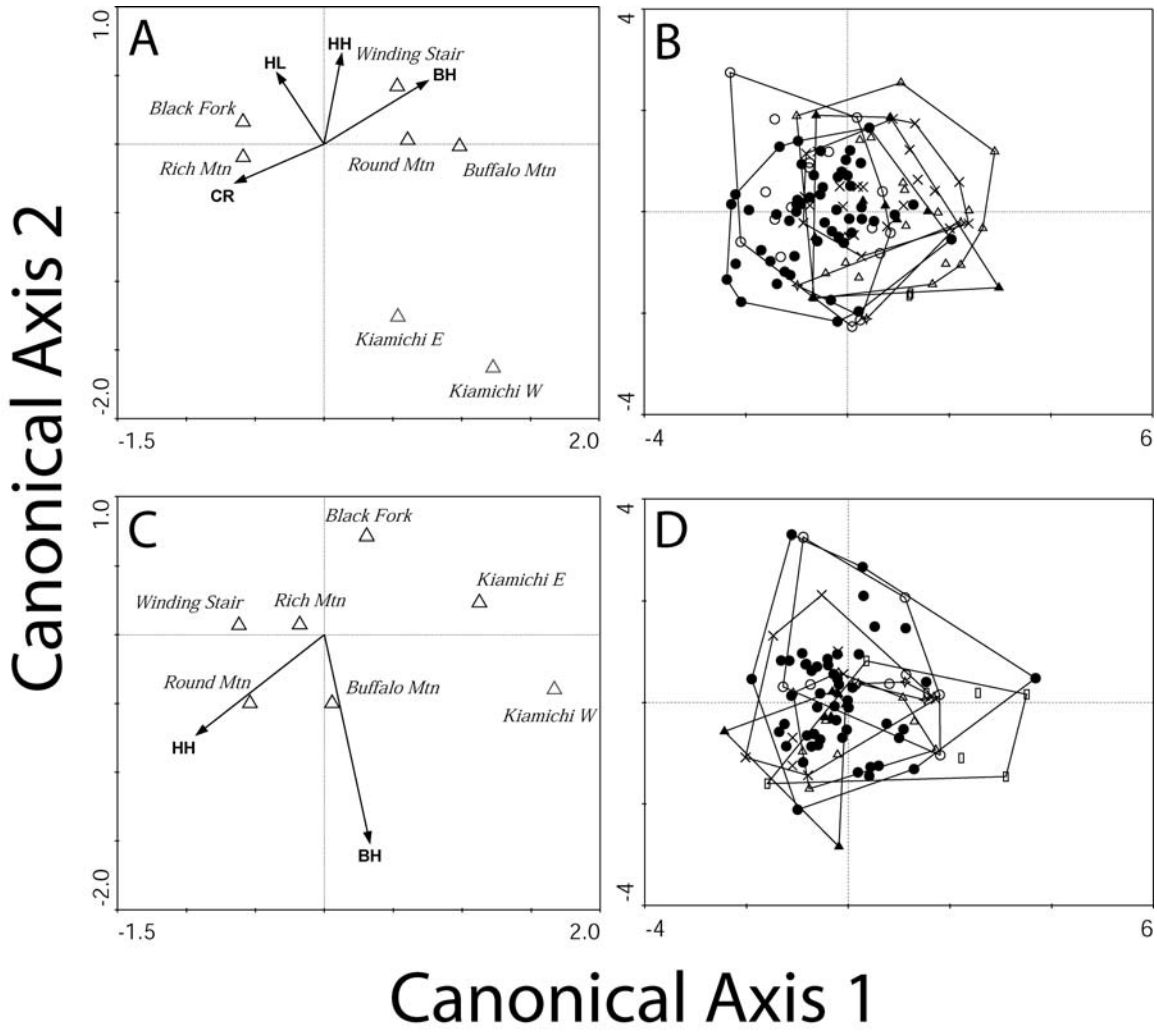

Snout-vent length (mm)
64
62
60
58
56
54
52
50
48
46
b
b
a
a
a,b
a
a,b
27
28
7
9
100
19
31
Black Fork
Buffalo Mtn
Kiamichi E
Kiamichi W
Rich Mtn
Round Mtn
Winding Stair

Fig. 5


A
HL
HH
Winding Stair
BH
Black Fork
Rich Mtn
CR
Round Mtn
Buffalo Mtn
Kiamichi E
Kiamichi W
1.0
-2.0
-1.5
2.0
B
4
-4
-4
6
C
Black Fork
Kiamichi E
Winding Stair
Rich Mtn
Round Mtn
Buffalo Mtn
Kiamichi W
HH
BH
1.0
-2.0
-1.5
2.0
D
4
-4
-4
6
Canonical Axis 2
Canonical Axis 1

APPENDIX.—Specimens used in this study in addition to those used in Shepard and Burbrink (2008, 2009) with voucher numbers (DBS: Donald B. Shepard; KJI: Kelly J. Irwin) and locality data. Individuals were sequenced and analyzed to place them into their respective lineage (D. Shepard, unpublished data). Elevation (Elev.) is in meters a.s.l.

ID#	Species	Lineage	Locality	Latitude	Longitude	Elev.	Sex
DBS 1632	*Plethodon fourchensis*	Blue Mtn	Blue Mountain, N slope along FR 54, Scott County, Arkansas	34.70001	-94.05876	374	Male
KJI 1120	*Plethodon fourchensis*	Little Brushy	Little Brushy Mtn, Montgomery County, Arkansas	34.63063	-93.85651	557	Female
KJI 1121	*Plethodon fourchensis*	Little Brushy	Little Brushy Mtn, Montgomery County, Arkansas	34.63063	-93.85651	557	Male
KJI 1122	*Plethodon fourchensis*	Little Brushy	Little Brushy Mtn, Montgomery County, Arkansas	34.62991	-93.86179	531	Juvenile
KJI 1123	*Plethodon fourchensis*	Little Brushy	Little Brushy Mtn, Montgomery County, Arkansas	34.62991	-93.86179	531	Female
KJI 1124	*Plethodon fourchensis*	Little Brushy	Little Brushy Mtn, Montgomery County, Arkansas	34.63063	-93.85651	557	Male
DBS 2045	*Plethodon fourchensis*	Little Brushy	Little Brushy Mtn, Montgomery County, Arkansas	34.63622	-93.84354	534	Female
DBS 2046	*Plethodon fourchensis*	Little Brushy	Little Brushy Mtn, Montgomery County, Arkansas	34.63622	-93.84354	534	Male
DBS 2047	*Plethodon fourchensis*	Little Brushy	Little Brushy Mtn, Montgomery County, Arkansas	34.63451	-93.84664	535	Male
DBS 2048	*Plethodon fourchensis*	Little Brushy	Little Brushy Mtn, Montgomery County, Arkansas	34.63451	-93.84664	535	Female

DBS 1262	*Plethodon ouachitae*	Round Mtn	Lynn Mountain, N slope below FR 6025 along tributary to Pigeon Creek, LeFlore County, Oklahoma	34.59387	-94.55838	691	Male
DBS 1563	*Plethodon ouachitae*	Rich Mtn	Spring Mountain, 3.8 km E of Hwy 1/Talimena Drive on Spring Mtn Rd/FR 6007, LeFlore County, Oklahoma	34.70839	-94.60093	454	Male

CURRICULUM VITAE

DONALD B. SHEPARD

Department of Zoology and
Sam Noble Oklahoma Museum of Natural History
University of Oklahoma
2401 Chautauqua Ave
Norman, OK 73072, USA

BIOGRAPHICAL INFORMATION

Date and Place of Birth: 10 May 1975, Mattoon, IL, USA
Home Address: 810 Hardin Drive, Norman, OK 73072, USA
Email: dshepard@ou.edu
Webpage: http://students.ou.edu/S/Donald.B.Shepard-1/
Telephone: Office 405-325-7771
Mobile 405-255-5617

EDUCATION

Ph.D. Zoology, University of Oklahoma, Norman, Oklahoma, 2008.
Advisor: Janalee P. Caldwell
Dissertation Title: Phylogeography and Diversification of the Ouachita Mountain Endemic Salamanders of the *Plethodon ouachitae* complex

M.S. Biological Sciences, Illinois State University, Normal, Illinois, 2000.
Major Professor: Lauren E. Brown
Thesis Title: Aggressive Behavior and Potential of Territoriality in the Green Frog *Rana clamitans*

B.S. Biology, University of Illinois, Urbana-Champaign, Illinois, 1997.

RESEARCH INTERESTS

- Evolutionary Ecology
- Herpetology
- Phylogeography
- Speciation
- Biogeography
- Amphibian and Reptile Conservation

RESEARCH EXPERIENCE

- **Graduate Research Assistant**, Oklahoma Museum of Natural History, University of Oklahoma, Norman, OK. Supervisors: Drs. Jan Caldwell and Laurie Vitt – August 2004 to June 2008
  As part of an NSF funded project "Survey and Inventory of the Brazilian Cerrado Herpetofauna", I assist with field surveys and making collections of amphibians and reptiles throughout the Cerrado region of Brazil. Additionally, I conduct and assist with research on the behavior and ecology of Cerrado amphibians and reptiles.
- **Graduate Research Assistant,** Oklahoma Museum of Natural History, University of Oklahoma, Norman, OK. Supervisors: Drs. Jan Caldwell and Laurie Vitt – June to August 2006, May to August 2007, and May to August 2008
  Conduct field surveys and make collections of amphibians and reptiles on State Wildlife Management Areas in Oklahoma.
- **Graduate Research Assistant,** Oklahoma Museum of Natural History, University of Oklahoma, Norman, OK. Supervisors: Drs. Jan Caldwell and Laurie Vitt – May to August 2005
  Conduct field surveys and make collections of amphibians and reptiles on BLM lands in the region of Billings, Montana.
- **Graduate Research Assistant,** Oklahoma Museum of Natural History, University of Oklahoma, Norman, OK. Supervisors: Drs. Jan Caldwell and Laurie Vitt – October to December 2003
  Assist with research and surveys on the amphibians and reptiles in the Vale do Paranã, Goias, Brazil.
- **Graduate Research Assistant**, Oklahoma Museum of Natural History, University of Oklahoma, Norman, OK. Supervisors: Drs. Jan Caldwell and Laurie Vitt – May to August 2003
  Conduct field surveys and make collections of amphibians and reptiles at Camp Gruber, U.S. Army Training Grounds, Muskogee County, Oklahoma.
- **Field Ecologist**, Illinois Natural History Survey, 607 East Peabody Drive, Champaign, IL 61820. Supervisor: Dr. Chris Phillips – August 2000 to July 2002
  Conduct field surveys for the state-endangered eastern massasauga rattlesnake (*Sistrurus catenatus*) at Carlyle Lake, IL, including tracking snakes using radio-telemetry and collecting life history and ecological data on snakes in the field. Respond to requests to relocate massasaugas from developed areas, present educational programs concerning the massasauga, data management, data analysis, and report, manuscript and grant writing.
- **Field Assistant,** Illinois Natural History Survey, 607 East Peabody Drive, Champaign, IL 61820. Supervisors: Drs. Scott Robinson and Jim Herkert – May to August 1997
  Searching for and monitoring of nests of grassland bird species at the Midewin National Tallgrass Prairie (former Joliet Arsenal), Will Co., IL. Census birds by song using point counts.
- **Undergraduate Research,** University of Illinois, Urbana-Champaign, IL. Supervisor: Dr. Lowell Getz January to May 1997
  Trapping of small mammals in tallgrass prairie, bluegrass, and alfalfa fields in central Illinois as part of a 25-year population study of the prairie vole (*Microtus orchrogaster*) and meadow vole (*Microtus pennsylvanicus*).

TEACHING EXPERIENCE

- **Ecology**, Teaching Assistant, Department of Zoology, University of Oklahoma – Fall 2008
- **Herpetology,** Teaching Assistant, Department of Zoology, University of Oklahoma – Spring 2005
- **Human Anatomy,** Teaching Assistant, Department of Zoology, University of Oklahoma – Spring 2003
- **Introduction to Zoology,** Teaching Assistant, Department of Zoology, University of Oklahoma – Fall 2002
- **Comparative Vertebrate Anatomy,** Teaching Assistant, Department of Biological Sciences, Illinois State University – Spring 2000
- **Biology of the Lower Vertebrates,** Teaching Assistant, Department of Biological Sciences, Illinois State University – Fall 1998 and 1999
- **Fundamental Concepts of Biology,** Teaching Assistant, Department of Biological Sciences, Illinois State University – Spring and Fall 1998 and 1999
- **Human Biology,** Instructor, Department of Biological Sciences, Illinois State University – Summer 1999 and last ¼ of Fall 1999

COLLECTIONS EXPERIENCE

- Graduate Student, Department of Herpetology, Sam Noble Oklahoma Museum of Natural History, University of Oklahoma – August 2002 to present.
- Graduate Research Curatorial Assistant, Division of Herpetology, Sam Noble Oklahoma Museum of Natural History, University of Oklahoma – August 2003 to July 2004.

  Assist with general maintenance of herpetology collection (50,000+ specimens). Collect and catalogue specimens and assist curators on collecting trips to Brazil. Organize, check identifications and update taxonomy of snakes in the collection.
- Graduate Student, Illinois State University Museum, Illinois State University – January 1998 to May 2000.

  Assist curator with maintenance of amphibian and reptile museum and teaching collections.

PROFESSIONAL MEMBERSHIPS (PRESENT)

American Society of Ichthyologists and Herpetologists
Herpetologists' League
Partners in Amphibians and Reptile Conservation
Society for the Study of Amphibians and Reptiles
Society for the Study of Evolution
Society of Systematic Biologists

PROFESSIONAL SERVICE

- Assistant Editor, *Herpetological Conservation and Biology* – 2006 to present
- Assistant to the Corresponding Editor, *Alytes-International Journal of Batrachology* – 2004 to present
- Journal Referee for: *Copeia, Herpetologica, Journal of Herpetology, Austral Ecology, Oecologia, Alytes-International Journal of Batrachology,*

Herpetological Review, Acta Ethologica, Western North American Naturalist, and *University of Chittagong Journal of Zoology*
- Member of IUCN/SSC Amphibian Specialist Group 2005–2008
- Local Committee for the 2004 Joint Meeting of Ichthyologists and Herpetologists, Norman, OK
- Contributing Scientist for IUCN Red List Assessment of *Bufo houstonensis* for Global Amphibian Assessment Project – 2003

PUBLICATIONS

2009 Goldberg, S.R., C.R. Bursey, J.P. Caldwell, and **D.B. Shepard**. Gastrointestinal helminths of six sympatric species of *Leptodactylus* from Tocantins State, Brazil. *Comparative Parasitology*, in press

2009 Aldridge, R.D., B.C. Jellen, M.C. Allender, M.J. Dreslik, **D.B. Shepard**, J.M. Cox, and C.A. Phillips. Reproductive biology of the massasauga (*Sistrurus catenatus*) from south-central Illinois. In *Biology of Rattlesnakes*. W.K. Hayes, K.R. Beaman, M.D. Cardwell, and S.P. Bush, eds. Loma Linda University Press, Loma Linda, California, in press.

2008 **Shepard, D.B.**, and F.T. Burbrink**.** Lineage diversification and historical demography of a sky island salamander, *Plethodon ouachitae*, from the Interior Highlands. *Molecular Ecology* 17:5315–5335.

2008 **Shepard, D.B.**, A.R. Kuhns, M.J. Dreslik, and C.A. Phillips. Roads as barriers to animal movement in fragmented landscapes. *Animal Conservation* 11:288–296.

2008 Costa, G.C., C.A. Wolfe, **D.B. Shepard**, J.P. Caldwell, and L.J. Vitt. Detecting the influence of climatic variables on species' distributions: a test using GIS niche-based models along a steep longitudinal environmental gradient. *Journal of Biogeography* 35:637–646.

2008 **Shepard, D.B.**, M.J. Dreslik, B.C. Jellen, and C.A. Phillips. Reptile road mortality around an oasis in the Illinois corn desert with emphasis on the endangered eastern massasauga. *Copeia* 2008:350–359.

2008 Brown, L.E., A. Dubois, and **D.B. Shepard**. Inefficiency and bias of search engines in retrieving references containing scientific names of fossil amphibians. *Bulletin of Science, Technology & Society* 28:279–288.

2008 Vitt, L.J., **D.B. Shepard**, G.H.C. Vieira, J.P. Caldwell, G.R. Colli, and D.O. Mesquita. Ecology of *Anolis nitens brasiliensis* in cerrado woodlands of Cantão. *Copeia* 2008:144–153.

2007 **Shepard, D.B.** 2007. Habitat but not body shape affects predator attack frequency on lizard models in the Brazilian Cerrado. *Herpetologica* 63:193–202.

2007 Vitt, L.J., **D.B. Shepard**, J.P. Caldwell, G.H.C. Vieira, F.G.R. França, and G.R. Colli. Living with your food: Geckos (*Gymnodactylus carvalhoi*) in termitaria of Cantão. *Journal of Zoology* 272:321–328.

2007 Caldwell, J.P., and **D.B. Shepard**. Calling site fidelity and call structure of a Neotropical toad, *Rhinella ocellata* (Anura: Bufonidae). *Journal of Herpetology* 41:611–621.

2007 Jellen, B.C., **D.B. Shepard,** M.J. Dreslik, and C.A. Phillips. Male movement and body size affect mate acquisition in the Eastern Massasauga (*Sistrurus catenatus*). *Journal of Herpetology* 41:451–457.

2005 **Shepard, D.B.**, and J.P. Caldwell. From foam to free-living: Ecology of larval *Leptodactylus labyrinthicus*. *Copeia* 2005:803–811.

2005 Leary, C.J., D.J. Fox, **D.B. Shepard**, and A.M. Garcia. Body size, age, growth and alternative mating tactics in toads: satellite males are smaller but not younger than calling males. *Animal Behaviour* 70:663–671.

2005 Vitt, L.J., J.P. Caldwell, G.R. Colli, A.A. Garda, D.O. Mesquita, F.G.R. França, **D.B. Shepard**, G.C. Costa, M.M. Vasconcellos, and V. De Novaes e Silva. Uma atualização do guia fotográfico dos répteis e anfíbios da região do Jalapão no Cerrado Brasileiro. *Special Publications in Herpetology, Sam Noble Oklahoma Museum of Natural History* 2:1–24.

2005 **Shepard, D.B.**, and L.E. Brown. *Bufo houstonensis* Houston toad. Pp. 415–417. In *Amphibian Declines: The Conservation Status of United States Species*. M. Lannoo (ed.). University of California Press, Berkeley.

2005 **Shepard, D.B.**, L.E. Brown, and B.P. Butterfield. *Pseudacris streckeri* Strecker's Chorus Frog. Pp. 484–485. In *Amphibian Declines: The Conservation Status of United States Species*. M. Lannoo (ed.). University of California Press, Berkeley.

2004 **Shepard, D.B.**, C.A. Phillips, M.J. Dreslik, and B.C. Jellen. Prey preference and diet of neonate eastern massasaugas (*Sistrurus c. catenatus*). *American Midland Naturalist* 152:360–368.

2004 **Shepard, D.B.** Seasonal differences in aggression and site tenacity in male green frogs, *Rana clamitans*. *Copeia* 2004:159–164.

2002 **Shepard, D.B.** Spatial relationships of male green frogs (*Rana clamitans*) throughout the activity season. *American Midland Naturalist* 148:394–400.

Shorter Contributions

2007 Petzing, J.E., J.M. Mui, M.J. Dreslik, A.R. Kuhns, **D.B. Shepard**, C.A. Phillips, J.K. Tucker, J.K. Warner, D. Mauger, T.G. Anton, E.J. Gittinger, T.R. Hunkapiller, B.C. Jellen, and R.J. Cosgriff. Filling in the gaps II: New Illinois amphibian and reptile county records from 2000–2005. *Herpetological Review* 38:240–243.

2007 Jellen, B.C., C.A. Phillips, M.J. Dreslik, and **D.B. Shepard**. *Sistrurus catenatus*. Reproduction. *Herpetological Review* 38:343–344.

2005 **Shepard, D.B.** *Eumeces fasciatus*. Predation. Herpetological Review 36:177.

2004 Richter, S.C., and **D.B. Shepard**. *Rana areolata*. Geographic distribution. *Herpetological Review* 35:283.

2003 **Shepard, D.B.**, M.J. Dreslik, C.A. Phillips, and B.C. Jellen. *Sistrurus catenatus catenatus*. Male-male aggression. *Herpetological Review* 34:155–156.

2002 Petzing, J.E., J.M. Mui, M.J. Dreslik, C.A. Phillips, **D.B. Shepard**, J.A. Crawford, A.R. Kuhns, M.J. Mayer, T.G. Anton, E.O. Moll, J.G. Palis, and D. Mauger. Filling in the gaps I: New county records for amphibians and reptiles in Illinois. *Herpetological Review* 33:327–330.

2000 **Shepard, D.B.**, and A.R. Kuhns. *Pseudacris triseriata*. Calling sites after drought. *Herpetological Review* 31:235–236.

2000 Petzing, J.E., M.J. Dreslik, C.A. Phillips, C.D. Smith, A.R. Kuhns, **D.B. Shepard**, J.G. Palis, E.O. Moll, D.J. Olson, T.G. Anton, D. Mauger, and B.A. Kingsbury. New amphibian and reptile county records in Illinois. H*erpetological Review* 31:189–194.

2000 **Shepard, D.B.,** and H.M. Burdett. *Pseudacris clarkii*. Geographic distribution. *Herpetological Review* 31:50.

In Review

Shepard, D.B.. and F.T. Burbrink. Phylogeographic and demographic effects of Pleistocene climatic fluctuations in a montane salamander, *Plethodon fourchensis*. Submitted to: *Molecular Ecology*.

Shepard, D.B.. K.J. Irwin, and F.T. Burbrink. Morphological variation in the Ouachita Mountain endemic salamanders *Plethodon fourchensis* and *Plethodon ouachitae*. Submitted to: *Herpetologica*

Crawford, J.A., **D.B. Shepard**, and C.A. Conner. Diet composition and overlap between recently metamorphosed *Lithobates areolatus* and *Lithobates sphenocephalus*: Implications for a frog of conservation concern. Submitted to: *Copeia*.

PRESENTATIONS

Invited Speaker

2008 Lineage Diversification in Sky Island Salamanders. Department of Zoology, University of Oklahoma, USA.

2008 Life without Legs: Evolution and Diversity of Snakes, Oklahoma City Audubon Society, Oklahoma City, Oklahoma, USA.

2001 Ecology of the eastern massasauga rattlesnake at Carlyle Lake, Illinois: A case study. Co-presented with C. Phillips, M. Dreslik, and B. Jellen at Herpetology Group, Washington University, St. Louis, Missouri, USA.

Contributed Papers

2008 Reassessment of species boundaries in salamanders of the *Plethodon ouachitae* complex. Arkansas Wildlife Action Plan Conference, Mt. Magazine State Park, Arkansas, USA.

2008 Lineage diversification and historical demography in a sky island salamander. Joint Annual Meeting of the American Society of Naturalists, Society of the Study of Evolution, and the Society of Systematic Biologists. Minneapolis, Minnesota, USA

2007 A new perspective on the morphological and genetic variation among *Plethodon ouachitae* from different mountains in the Ouachita Range. Joint Meeting of American Society of Ichthyologists and Herpetologists, Society for the Study of Amphibians and Reptiles, and Herpetologists' League. St. Louis, Missouri, USA.

2006 Habitat but not body shape affects predator attack frequency on lizards in the Brazilian Cerrado. Joint Meeting of American Society of Ichthyologists and

Herpetologists, Society for the Study of Amphibians and Reptiles, and Herpetologists' League. New Orleans, Louisiana, USA.

2005 Reptile road mortality around an oasis in the Illinois corn desert, with emphasis on the endangered eastern massasauga (*Sistrurus c. catenatus*). 19th Annual Meeting of the Society for Conservation Biology. Universidade de Brasilia, Brasília, DF, Brazil.

2005 Reptile road mortality around an oasis in the Illinois corn desert, with emphasis on the endangered Eastern Massasauga. Joint Meeting of American Society of Ichthyologists and Herpetologists, Society for the Study of Amphibians and Reptiles, and Herpetologists' League. University of South Florida, Tampa, Florida, USA.

2003 Foraging ecology of neonate eastern massasaugas (*Sistrurus c. catenatus*). 50th Annual Meeting of the Southwestern Association of Naturalists, Norman, Oklahoma, USA.

2002 Ecology of neonate eastern massasauga rattlesnakes (*Sistrurus c. catenatus*). Joint Annual Meeting of the American Society of Ichthyologists and Herpetologists, the Society for the Study of Amphibians and Reptiles, and the Herpetologists' League, Kansas City, Missouri, USA.

2001 Population ecology of the eastern massasauga rattlesnake, *Sistrurus catenatus catenatus*. Joint Annual Meeting of the Society for the Study of Amphibians and Reptiles and the Herpetologists' League, Indianapolis, Indiana, USA.

2001 Population ecology of the eastern massasauga rattlesnake, *Sistrurus catenatus catenatus*. 93rd Annual Meeting of the Illinois State Academy of Sciences, Macomb, Illinois, USA.

2000 Absence of territoriality in the green frog *Rana clamitans*. Joint Meeting of the American Society of Ichthyologists and Herpetologists, American Elasmobranch Society, Neotropical Ichthyological Association, Herpetologists' League, Canadian Association of Herpetologists, and Society for the Study of Amphibians and Reptiles, La Paz, B.C.S., Mexico.

2000 Aggressive behavior and the potential of territoriality in the green frog *Rana clamitans*. 1st Annual Phi-Sigma Biological Sciences Research Symposium, Illinois State University, Normal, Illinois, USA.

GRANTS

2007 Graduate Student Senate Research and Activity Grant, University of Oklahoma. $218.40

2006 State of Arkansas, Game and Fish Commission SWG grant. "Reassessment of species boundaries in the endemic Arkansas salamanders of the *Plethodon ouachitae* complex using molecular phylogeographic techniques." $95,181 (3 yrs. with F. Burbrink and K. Irwin)

2006 Graduate Student Senate Research and Activity Grant, University of Oklahoma. $250

2006 Graduate Student Senate Conference and Creative Exhibition Grant, University of Oklahoma. $250

2005 Graduate Student Senate Research and Activity Grant, University of Oklahoma. $209.25

2005 Graduate Student Senate Conference and Creative Exhibition Grant, University of Oklahoma. $232.50
2004 Graduate Student Senate Research and Activity Grant, University of Oklahoma. $182.50
2004 Graduate Student Senate Conference and Creative Exhibition Grant, University of Oklahoma. $124
2003 Graduate Student Senate Research and Activity Grant, University of Oklahoma. $208
2002 U.S. Fish and Wildlife Service Candidate Species Fund. "Ecology and Conservation of the Eastern Massasauga Rattlesnake at Carlyle Lake, Illinois." $43,000 (with C. Phillips and M. Dreslik)
2000 Graduate Student Association Research Grant, Illinois State University. $125
1999 Illinois Wildlife Preservation Fund Small Project Grant, Illinois Department of Natural Resources. "Herpetofaunal Survey along the Illinois River in Woodford and Marshall Counties." $742.90

HONORS, SCHOLARSHIPS, AND AWARDS

2007 Adams Scholarship, Department of Zoology, University of Oklahoma
2004 George Miksch Sutton Scholarship in Ornithology, University of Oklahoma.
2000 Graduate Student Association Professional Advancement Award, Illinois State University.
2000 Omar Rilett Scholarship Award, Department of Biological Sciences, Illinois State University.
2000 Outstanding Oral Presentation Award, First Annual Phi-Sigma Biological Sciences Research Symposium, Illinois State University.
2000 Nominated for Outstanding University Graduate Student Teaching Award, Illinois State University.
1999 Outstanding Teaching Assistant Award, Department of Biological Sciences, Illinois State University.
1997 Dean's List, University of Illinois.
1996 Dean's List, University of Illinois.