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

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Abstract

Climatic changes associated with Pleistocene glacial cycles profoundly affected species distributions, patterns of interpopulation gene flow, and demography. In species restricted to montane habitats, ranges may expand and contract along an 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 narrow 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*, also emphasizes how responses can vary substantially even among closely related, similarly distributed taxa.

Keywords: biogeography, climate change, niche modelling, Ouachita Mountains, sky islands, statistical phylogeography

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Introduction

Climatic changes associated with Pleistocene glacial cycles profoundly affected species distributions and patterns of interpopulation gene flow (Hewitt 1996, 2000, 2004; Jansson & Dynesius 2002). These effects were perhaps most pronounced in temperate zones where glaciers displaced organisms, but species in many unglaciated regions were also

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impacted (Knowles 2000, 2001; Maddison & McMahon 2000; 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 an 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
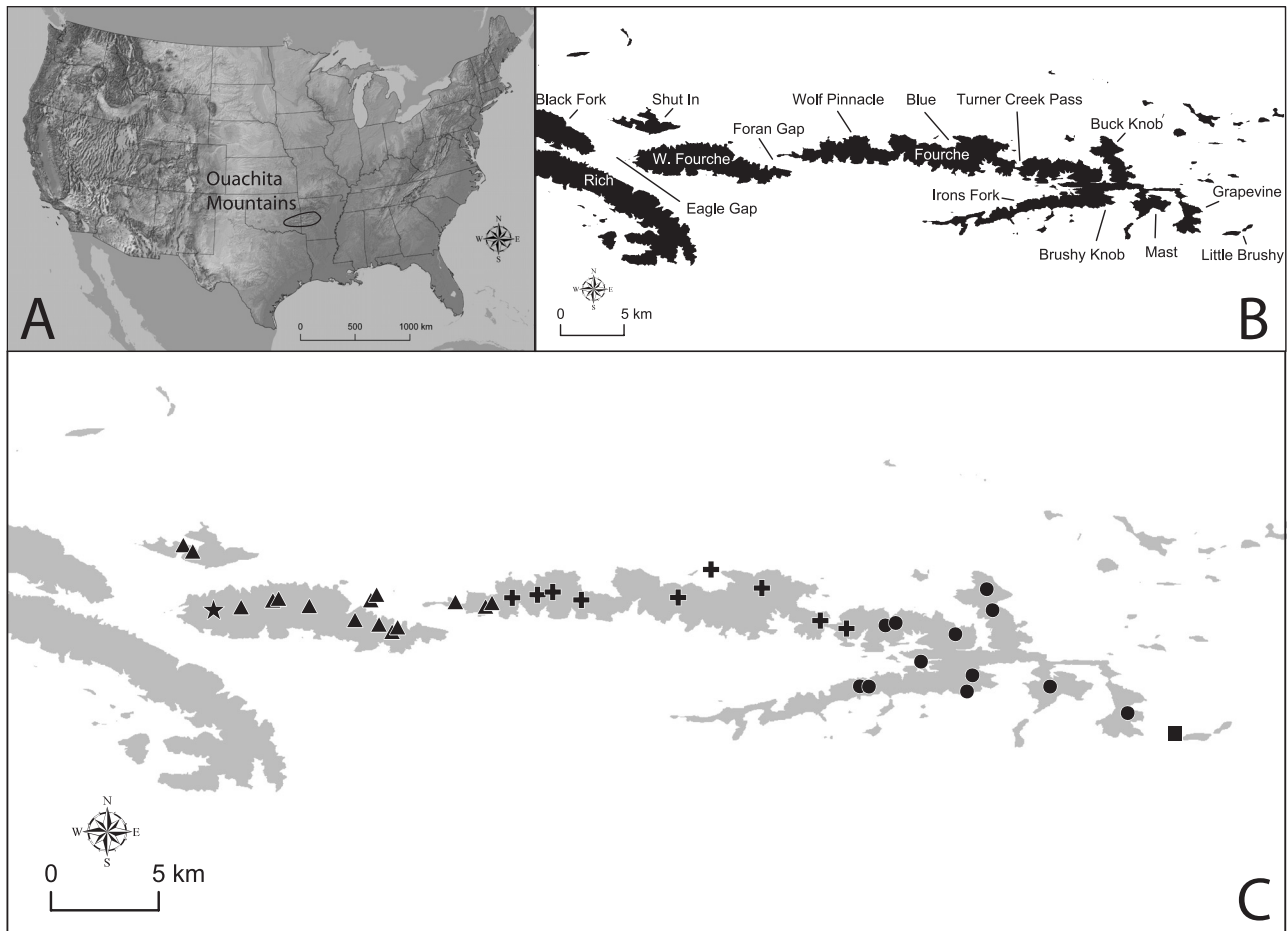
Fig. 1 Topographic map of the United States showing the location of the Ouachita Mountains (A), all elevations >500 m in the range of *Plethodon fourchensis* with names of major mountains and features labeled (B), and map of sampling localities for *P. fourchensis* coded by lineage (C). Lineages: (✚) Blue Mtn, (●) Buck Knob, (■) Little Brushy, (▲) W. Fourche, (★) Rich Mtn lineage of *Plethodon ouachitae*.

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 levels of 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; Weisrock & Larson 2006; Wiens *et al.* 2006). This region has a history of climate-driven forest contraction, fragmentation, and expansion over the last 3 million years (Myr; King 1973; Davis 1983; Webb & Bartlein 1992), which is thought to have contributed greatly to diversification in *Plethodon* (Highton 1995; Kozak & Wiens 2006; Kozak *et al.* 2006a).

The Fourche Mountain Salamander *Plethodon fourchensis* is known only from Fourche and Irons Fork Mountains in the Ouachita Mountains of west-central Arkansas, USA. (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 largely restricted 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, offer 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 & Burbrink (2008) identified seven lineages within *Plethodon ouachitae* in the Ouachita Mountains corresponding to six major mountains. They found that 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.) to obtain template strength DNA/RNA ratios of 1.5–2.1:1 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.) 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: 94 °C for 2 min followed by 36 cycles of 94 °C for 10 s, 50 °C (cyt*b*) or 52 °C (ND4) for 30 s, and 72 °C for 90 s with a final 10 min extension period at 72 °C. We cleaned PCR products using 1 μL of ExoSap-it (USB Corp.) 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), 2 μL of 5-μM primer, 1–2 μL of DNA template, and 3–5 μL of H_2O. Sequencing products were purified following the ethanol-sodium-acetate protocol listed in the DTCS Kit and analysed on a Beckman CEQ 8000 sequencer (Beckman-Coulter). 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 Accession nos FJ611346–FJ611481 (cyt*b*) and FJ611482–FJ611617 (ND4; 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 version 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. 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 –ln *L* 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 version 1.4 (Rambaut & Drummond 2007). A BF > 10 was considered as strong evidence favouring 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 km²; 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° N latitude and 92.20–96.56° W 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 version 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 a grid map 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 & 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 ANOVA tests 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 version 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 (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 standard deviation (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 million years ago (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 an SD of 0.5 provides an upper 95% bound of 11 Myr (Middle Miocene).

The date estimate and associated error for the divergence of *P. fourchensis* from its sister taxon *P. ouachitae* generated by 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 MRCA. We used the mean divergence date of *P. fourchensis* and *P. ouachitae* as a prior for the origin of *P. fourchensis* with an SD to encompass

95% of the lognormal distribution of dates. 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 version 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 version 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, then we can reject a pre-Pleistocene divergence.

Historical biogeography

We used coalescent simulations in Mesquite version 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 haplotypes 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 version 2.4 (Beerli 2008) under the following parameters: 15 small chains for 200 000 generations and four long chains for 2 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 $θ = N_e μ$, where $μ = 1.365 \times 10^{-7}$,
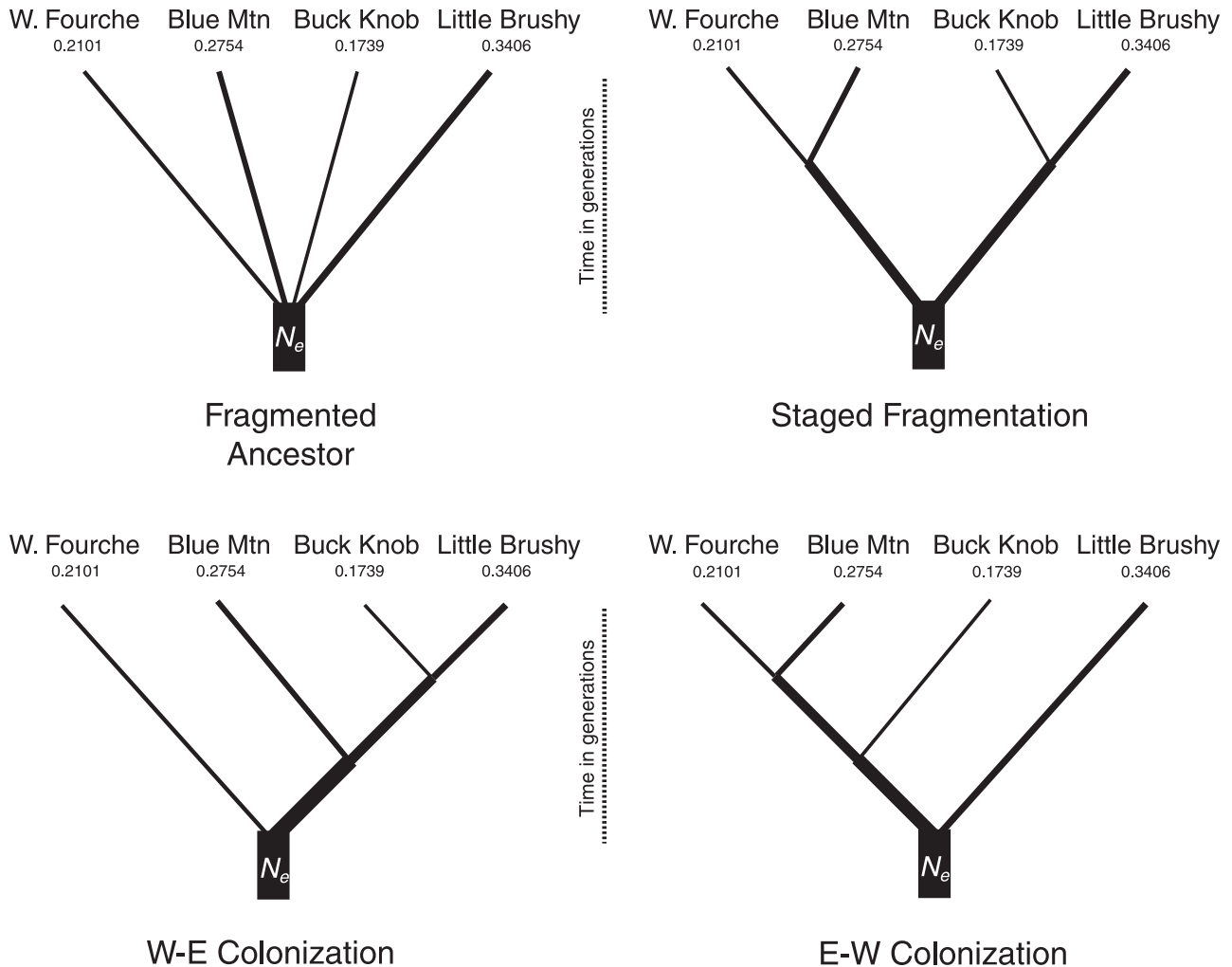
Fig. 2 Population trees representing the four biogeographic hypotheses of diversification within *Plethodon fourchensis* tested using coalescent simulations in Mesquite v.2.5 (Maddison & Maddison 2008). The Fragmented Ancestor model posits that all population divergences were concurrent and resulted 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 3-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.

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 version 1.4.7 and a generation time of 3 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 3-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 version 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 3-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 favour 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.

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 version 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 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 version 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 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 version 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 version 4.20 (Rozas *et al.* 2003). Contrasting plots of observed vs. 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 & Rozas (2002) and 10 000 coalescent simulations in DnaSP version 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 *Plethodon fourchensis* and nine outgroup taxa (1816 bp total). Bayes

factors (BF) strongly favoured 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 version 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 –ln *L* values for post burn-in trees with a difference in –ln *L* 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 Mountain lineage of *P. ouachitae* (Figs 1 and 3). The dorsal colour 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 Mountain 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 (Figs 1 and 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 Mountain 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 Mountain lineages abut approximately 4 km east of Foran Gap and 2.25 km west of Wolf Pinnacle (Fig. 1). The Blue Mountain 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 Mountain 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). Individuals from Shut In Mountain were monophyletic, but were nested within the W. Fourche lineage, suggesting that this mountain was only recently colonized from West Fourche Mountain (Fig. 3).

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*

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
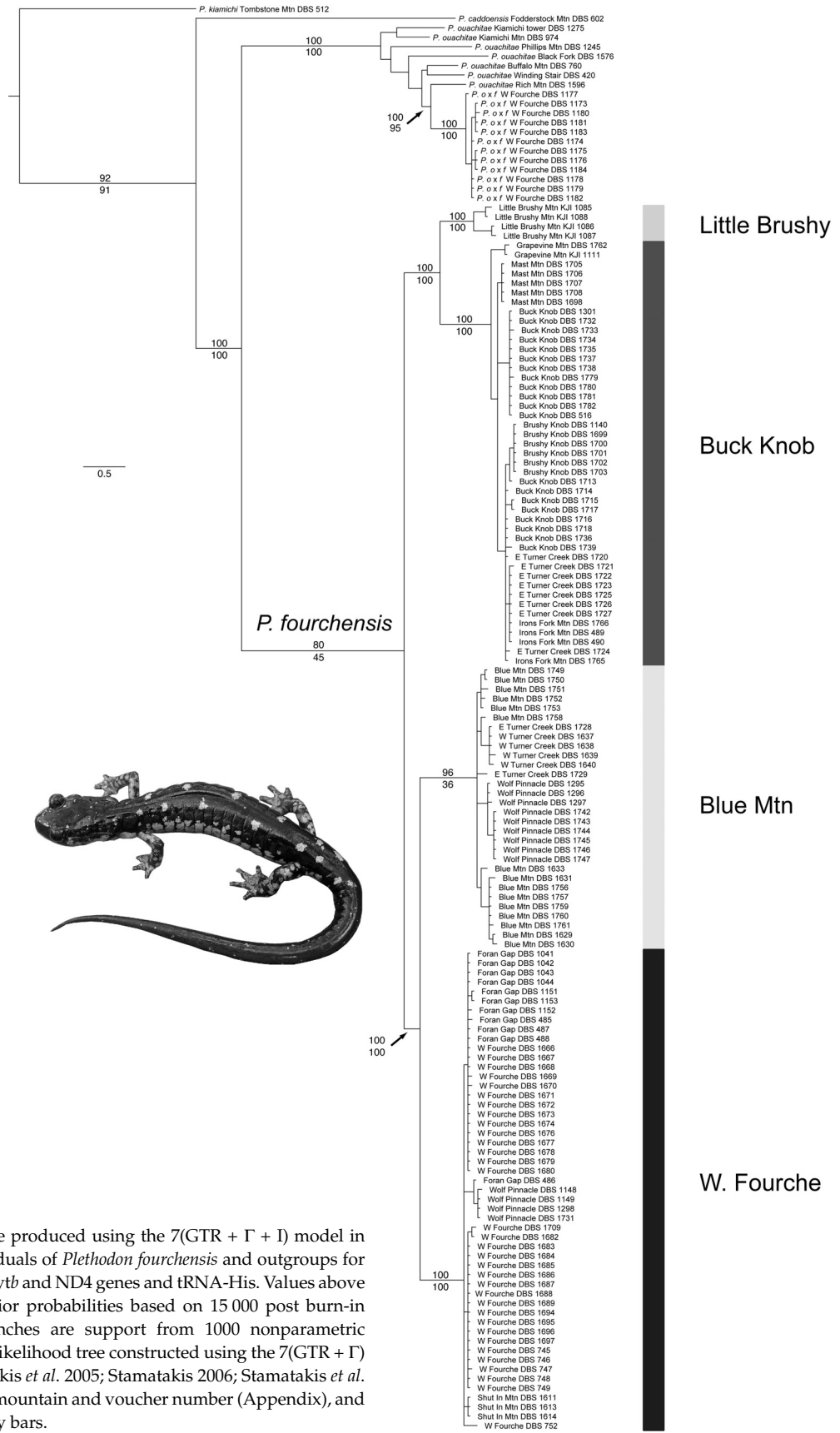
Fig. 3 Bayesian consensus tree produced using the 7(GTR + Γ + I) model in MrBayes v.3.1.1 for 130 individuals of *Plethodon fourchensis* and outgroups for 1816 bp of the mitochondrial cyt*b* and ND4 genes and tRNA-His. Values above branches are Bayesian posterior probabilities based on 15 000 post burn-in 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.
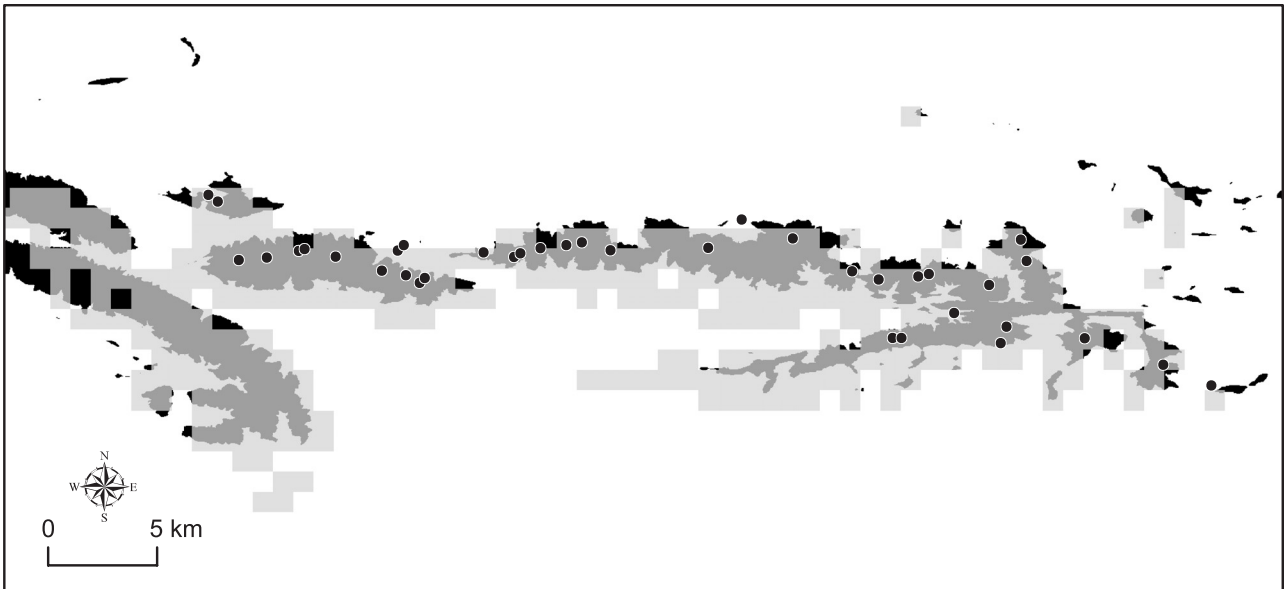
Fig. 4 Ecological niche model for *Plethodon fourchensis* constructed with Maxent v.3.2.1 (Phillips *et al.* 2006) using 19 climatic variables (Hijmans *et al.* 2005) at 30 arc-seconds resolution (~1 km²) and our 38 sampling points (AUC > 0.99). A map of all elevations >500 m (black) is overlaid by the niche model to show areas of predicted suitable (gray) and unsuitable (white) environmental conditions.

occur are significantly different (Wilk's λ = 0.486, $F_{2,90}$ = 47.64, P < 0.001). Locations were significantly different 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 and Buck Knob) and the common ancestor of the two western lineages (Blue Mountain and W. Fourche). The Blue Mountain and W. Fourche lineages diverged ~0.514 Ma (95% CI, 0.176–0.991 Ma), 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
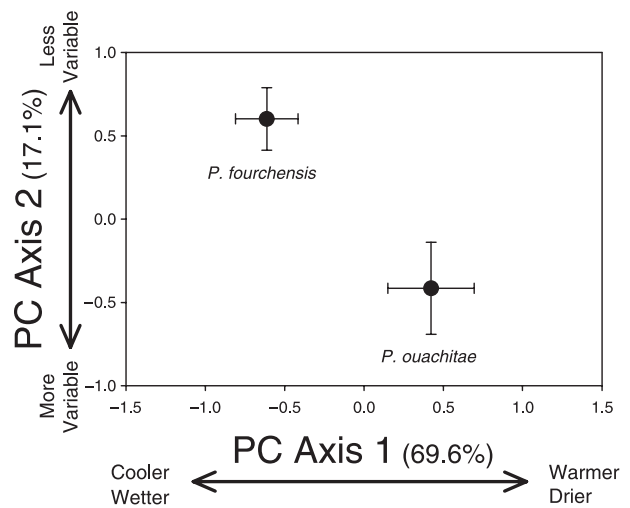

Fig. 5 Results from the comparison of environmental conditions at sampling points for *Plethodon fourchensis* versus *Plethodon ouachitae* (Shepard & Burbrink 2008) showing environmental conditions (means ± 95% CIs) occupied by *P. fourchensis* are significantly cooler, wetter, and less variable than environmental conditions where *P. ouachitae* occurs.

divergence within *P. fourchensis* occurred prior to the Pleistocene (> 1.8 Ma; P = 4.51 × 10⁻⁶).

Using Migrate-N version 2.4 (Beerli 2008), we calculated an MLE of θ_{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

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 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.
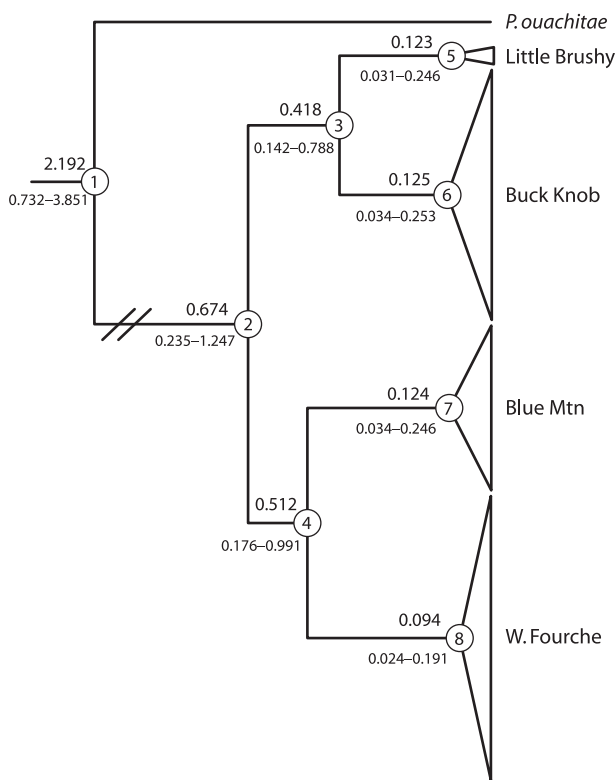

Fig. 6 Simplified tree showing mean divergence dates and 95% Credible Intervals (Ma) for major nodes and the time to the most recent common ancestor (MRCA) for major lineages within *Plethodon fourchensis*. Nodes are numbered for reference in Table 2.

5.842×10^{-6}, and used this value in coalescent simulations. The number of deep coalescent events (nDC) for 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. BSPs) because they fail to consider phylogenetic structure (Felsenstein 1992; Pybus *et al*. 2000). BSPs, 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 interpopulation genetic divergence 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

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

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).
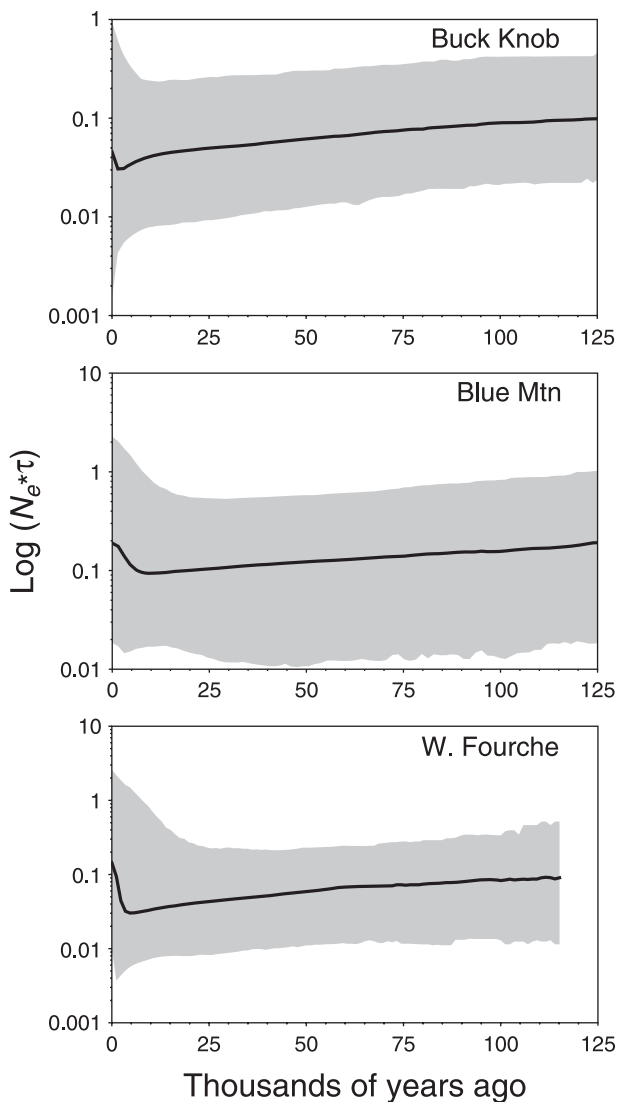

Fig. 7 Bayesian skyline plots (Drummond *et al.* 2005) showing the demographic history of three of the four lineages of *Plethodon fourchensis*. The 125 000 year-period on the X axis encompasses one complete glacial cycle from the latter part of the Sangamon interglacial, through the entire Wisconsin 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.

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 & 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 km²) 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 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 times 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 palaeoclimate models because their resolution is too coarse (10 arc-minutes or ~344 km²) 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. Other montane species such as grasshoppers (*Melanoplus*) and butterflies (*Parnassius smintheus*) in the northern Rocky Mountains and *P. ouachitae* in the Ouachita Mountains also diversified within a similar time frame (Knowles 2000, 2001; DeChaine & Martin 2004, 2005, 2006; Carstens & Knowles 2007; Shepard & Burbrink 2008). Between 1.2 and 0.8 Ma, a climatic change (termed the Middle Pleistocene Transition; MPT) occurred in which the 41 000-year glacial cycles that characterized the Late Pliocene and Early Pleistocene shifted to 100 000-year cycles with increased amplitude (i.e. greater extremes) in climatic fluctuations (Bennett 1990; Clark *et al*. 1999, 2006). In these 100 000-year 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). Because *P. fourchensis* is strongly associated with mesic forest, range expansion and contraction in this species should mirror the expansion and contraction of deciduous forest. The longer glacial and interglacial periods of the 100 000-year 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). This pattern of range expansion during interglacial periods and range contraction during glacial periods is in contrast to the pattern observed in the Madrean sky islands in the desert

of the southwestern USA and adjacent Northern Mexico. In that situation, environmental conditions that predominated during glacial periods have contracted to the higher elevations during the present interglacial period resulting in dramatically contrasting habitats at high (pine-oak forest) and low elevations (desert) and the isolation and divergence of populations of some organisms on adjacent mountain tops (Maddison & McMahon 2000; Masta 2000; Smith & Farrell 2005). Responses to Pleistocene glacial cycles are expected to vary among species and geographic regions (Hewitt 1996, 2004) and the difference between these two systems is a prime example.

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, suggested a model of diversification involving the fragmentation of a wide-ranging ancestor. In our historical biogeography analysis for *P. fourchensis* using coalescent simulations, we failed to reject the hypothesis of simultaneous fragmentation, meaning that none of the alternative models (e.g. staged fragmentation, colonization) were significantly better representations of the biogeographic history of the species. Somewhat contrasting to this, our results from phylogenetic and divergence dating analyses showed an initial divergence into eastern and western clades followed by divergences within each of those clades that gave rise to the four extant lineages. Failing to reject the null hypothesis in the historical biogeography analysis is clearly not as strong of support for a conclusion as rejecting the null hypothesis would have been. Given the short internodes between the first divergence in *P. fourchensis* that gave rise to eastern and western clades and the subsequent divergence within each of those clades, it may be too difficult to discriminate between a simultaneous fragmentation and staged fragmentation model due to uncertainty in the coalescent process, especially when employing a single genetic marker (Edwards & Beerli 2000). However, fragmentation, whether it was simultaneous or staged, rather than colonization still appears to be the most likely biogeographic scenario, given the tree topology, the high support for major nodes, and the results from the ancestral area estimation.

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 & 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 that *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 analysed 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 (King 1973; Davis 1983; Denniston *et al.* 1999; Jackson *et al.* 2000). 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; Weisrock & Larson 2006; 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 & 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). If lineages within *P. fourchensis* have undergone hybridization, it is evident that mitochondrial introgression does not extend much beyond these contact zones. 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 (Hewitt 1996, 2004; 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 lead to divergence of populations and which may ultimately lead to the origin of new species.

Acknowledgements

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Donald Shepard is interested in the evolution of behavioural, ecological and morphological variation in amphibians and reptiles, and the mechanisms that generate and maintain biodiversity. Frank Burbrink is interested in phylogenetic theory and phylogeographic and divergence dating methods, and applies these techniques to better understand speciation and biogeographic patterns in reptiles and amphibians.

Appendix

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

Species	Voucher Nos.	Lineage	Locality	Latitude	Longitude	Elev.	GenBank Nos. cyt*b*	GenBank Nos. 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	FJ611368	FJ611504
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	FJ611369–70	FJ611505–06
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	FJ611476	FJ611612
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	FJ611471–75, FJ611393–400	FJ611607–11, FJ611529–36
P. fourchensis	DBS 1694–1697, 1709	W. Fourche	Fourche Mountain, ~6.0 km E of Eagle Gap on Ouachita Trail, Polk County, Arkansas	34.68789	−94.23879	736	FJ611401–04, FJ611415	FJ611537–40, FJ611551
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	FJ611388–92	FJ611524–528
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	FJ611382–87	FJ611518–23
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	FJ611346–49	FJ611482–85
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	FJ611352–54	FJ611488–90
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	FJ611379–81	FJ611515–17
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	FJ611467–68	FJ611603–04
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	FJ611465–66	FJ611601–02
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	FJ611432	FJ611568
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	FJ611351, FJ266741	FJ611487, FJ267027
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	FJ611366	FJ611502
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	FJ611365	FJ611501
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	FJ611363	FJ611499

Appendix *Continued*

Species	Voucher Nos.	Lineage	Locality	Latitude	Longitude	Elev.	GenBank Nos. cyt*b*	GenBank Nos. ND4
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	FJ611364	FJ611500
P. fourchensis	DBS 1742–1747	Blue Mtn	Fourche Mountain, ~1.0 km SE of Wolf Pinnacle on Ouachita Trail, Polk County, Arkansas	34.68738	−94.11278	655	FJ611441–46	FJ611577–82
P. fourchensis	DBS 1749–1753	Blue Mtn	Blue Mountain, along Ouachita Trail, Polk County, Arkansas	34.68834	−94.07250	668	FJ611447–51	FJ611583–87
P. fourchensis	DBS 1629–1631, 1633	Blue Mtn	Blue Mountain, N slope along FR 54, Scott County, Arkansas	34.70001	−94.05876	374	FJ611371–74	FJ611507–10
P. fourchensis	DBS 1756–1761	Blue Mtn	Blue Mountain, E end along Ouachita Trail, Polk County, Arkansas	34.69233	−94.03766	719	FJ611452–57	FJ611588–93
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	FJ611375–78	FJ611511–14
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	FJ611430–31	FJ611566–67
P. fourchensis	DBS 1765	Buck Knob	Irons Fork Mountain, S of CR 70/FR 76, Polk County, Arkansas	34.65131	−93.99664	606	FJ611459	FJ611595
P. fourchensis	DBS 1766	Buck Knob	Irons Fork Mountain, S of CR 70/FR 76, Polk County, Arkansas	34.65126	−93.99290	622	FJ611460	FJ611596
P. fourchensis	DBS 1722–1727	Buck Knob	Fourche Mountain, ~2.7 km E of Turner Creek Rd (FR 7172) along Ouachita Trail, Polk County, Arkansas	34.67663	−93.98601	620	FJ611424–29	FJ611560–65
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	FJ611422–23	FJ611558–59
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	FJ611469–70	FJ611605–06
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	FJ611416–21	FJ611552–57
P. fourchensis	DBS 1140	Buck Knob	Brushy Knob, SE side along CR 70/FR 216, Polk County, Arkansas	34.64920	−93.95208	525	FJ611350	FJ611486
P. fourchensis	DBS 1699–1703	Buck Knob	Brushy Knob, N slope above CR 70, Polk County, Arkansas	34.65593	−93.94975	627	FJ611406–10	FJ611542–46
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	FJ611435–40, FJ611461–64	FJ611571–76, FJ611597–600
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	FJ266740, FJ611367, FJ611433–34	FJ267026, FJ611503, FJ611569–70
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	FJ611405, FJ611411–14	FJ611541, FJ611547–50
P. fourchensis	DBS 1762, KJI 1111	Buck Knob	Grapevine Mountain, NE slope below FR 774, Montgomery County, Arkansas	34.64019	−93.88511	568	FJ611458, FJ611481	FJ611594, FJ611617

Appendix *Continued*

Species	Voucher Nos.	Lineage	Locality	Latitude	Longitude	Elev.	GenBank Nos. cyt*b*	GenBank Nos. ND4
P. fourchensis	KJI 1085–1088	Little Brushy	Little Brushy Mountain, Montgomery County, Arkansas	34.63174	–93.86533	453	FJ611477–80	FJ611613–16
P. oxf	DBS 1173–1184	Rich Mtn	Fourche Mountain, 3.33 km E of Eagle Gap, Polk County, Arkansas	34.68334	–94.26581	702	FJ266755–58, FJ611355–62	FJ267041–44, FJ611491–98
P. ouachitae	DBS 1275	Kiamichi W	Kiamichi Mountains, N slope below Kiamichi Tower on FR 6025, LeFlore County, Oklahoma	34.62824	–94.81229	687	FJ266805	FJ267091
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	FJ267023	FJ267298
P. ouachitae	DBS 1245	Round Mtn	Phillips Mountain, N slope below FR 6025, LeFlore County, Oklahoma	34.61537	–94.49725	655	FJ266788	FJ267074
P. ouachitae	DBS 1596	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	FJ266870	FJ267156
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	FJ266850	FJ267136
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	FJ266916	FJ267202
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	FJ267010	FJ267285
P. caddoensis	DBS 602	NA	CR79/FR140 near Fodderstock Mtn, Polk County, Arkansas	34.43742	–94.17537	345	FJ266742	FJ267028
P. kiamichi	DBS 512	NA	Kiamichi Mountains, Tombstone Mountain Rd/FR252C near lookout, LeFlore County, Oklahoma	34.62697	–94.79690	533	FJ266739	FJ267025