

Hydrologic and geologic history of the Ozark Plateau drive phylogenomic patterns in a cave-obligate salamander

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Funding information

Theodore Roosevelt Memorial Grant - American Museum of Natural History; University of Tulsa, Grant/Award Number: 20-2-1211607-53600; Oklahoma Department of Wildlife Conservation, Grant/Award Number: E-22-18 and E22-20; National Science Foundation, Grant/Award Number: DEB 1050322; Oklahoma NSF-EPSCoR Program, Grant/Award Number: IIA-1301789

Editor: Aristeidis Parmakelis

Abstract

Aim: Habitat specialization can constrain patterns of dispersal and drive allopatric speciation in organisms with limited dispersal ability. Herein, we tested biogeographic patterns and dispersal in a salamander with surface-dwelling larvae and obligate cave-dwelling adults.

Location: Ozark Plateau, eastern North America.

Methods: A population-level phylogeny of grotto salamanders (*Eurycea spelaea* complex) was reconstructed using mitochondrial (mtDNA) and multi-locus nuclear DNA (nucDNA), primarily derived from anchored hybrid enrichment (AHE). We tested patterns of molecular variance among populations and associations between genetic distance and geographic features.

Results: Divergence time estimates suggest rapid formation of three major lineages in the Middle Miocene. Contemporary gene flow among divergent lineages appears negligible, and mtDNA suggests that most populations are isolated. There is a significant association between phylogenetic distance and palaeodrainages, contemporary drainages and sub-plateaus of the Ozarks, as all features explain a proportion of genetic variation. However, the greatest proportion of genetic variation is explained by the combined effects of palaeodrainages and sub-plateaus.

Main conclusions: The geologic and hydrologic history of the Ozark Plateau has influenced lineage diversification in the grotto salamander, leading to genetic isolation among populations. Limited gene flow and strong phylogeographic structure in this complex may result from the restriction of highly specialized adults to caves.

KEYWORDS

amphibians, anchored hybrid enrichment, conservation genetics, cryptic speciation, db-RDA, dispersal, interior highlands, phylogeography, SAMOVA, subterranean biology

1 | INTRODUCTION

Some widespread species are generalists that successfully occupy a diversity of habitats within landscapes, while many specialists experience greater success in more exclusive habitats (e.g. Williams, 1972). One possible consequence of habitat specialization is the inhibition of dispersal (e.g. Pandit, Kolasa & Cottenie, 2009), particularly when a heterogeneous landscape impedes movement of individuals (e.g. Goodwin & Fahrig, 2002). In addition, dispersal capabilities can vary

across ontogeny (e.g. Clobert, Ims & Rousset, 2004; Didham et al., 2012), so specific life history stages may drive phylogeographic structure.

Subterranean aquifers and caves are relatively stable, yet extreme habitats that typically require significant specialization for permanent occupation. Many groundwater and cave-obligate species have limited ranges (e.g. Christman, Culver, Madden & White, 2005; Culver & Holsinger, 1992; Gibert & Deharveng, 2002), and exhibit adaptations for exploiting subterranean environments (e.g.

Christiansen, 1962, 1992). Most cave-adapted species complete their life cycle underground, and have limited overland dispersal (e.g. Bonett, Steffen, Lambert, Wiens & Chippindale, 2014; Buhay & Crandall, 2005; Christiansen, 1962, 1992). Therefore, fragmented subterranean habitats are effectively islands in the vein of MacArthur and Wilson's (1967) Theory of Island Biogeography (e.g. Culver, 1970, 1971; Culver, Holsinger & Baroody, 1973; Vuilleumier, 1973). We might expect geologic structure to influence biogeographic distributions of cave species, especially in topologically diverse systems (e.g. karst). Due to high levels of fragmentation and limited gene flow, cave populations may contain highly differentiated lineages even within small geographic areas (e.g. Barr, 1967). Recent phylogeographic studies suggest high genetic isolation among populations of obligate cave-dwelling taxa, consistent with fragmentation in karst topography (e.g. Buhay & Crandall, 2005; Crandall & Templeton, 1999; Niemiller, Near & Fitzpatrick, 2012; Niemiller et al., 2013a,b). Additionally, evidence of cryptic speciation has been found in many subterranean organisms (e.g. Buhay & Crandall, 2005, 2008; Finston, Johnson, Humphreys, Eberhard & Halse, 2007; Niemiller et al., 2012, 2013a; Verovnik, Sket, Prevorcnik & Trontelj, 2003).

In North America, there are several karst regions replete with caves and associated fauna (e.g. Barr & Holsinger, 1985). One such area is the Ozark Plateau of the south-central United States, a dissected limestone uplift of Palaeozoic bedrock containing thousands of caves and springs (Graening, Fenolio & Slay, 2011; Howe & Koenig, 1961; Steyermark, 1963; Thornbury, 1965). The Ozarks have high levels of diversity for many taxonomic groups such as insects (Poulton, 1988; Stark, Stewart & Feminella, 1983), fishes (Mayden, 1988; Robison, Hocutt & Wiley, 1986), crayfish (Crandall, 1998; Crandall & Templeton, 1999), and salamanders (Martin, Shepard, Steffen, Phillips & Bonett, 2016) including over 160 described endemic species (Buhay & Crandall, 2008; Crandall, 1998; Crandall & Templeton, 1999; Dowling, 1956; Mayden, 1988). However, relatively few studies have analysed phylogeographic patterns of Ozark taxa, and most were with surface-dwelling species (primarily fishes; Mayden, 1988; Berendzen, Simons, Wood, Dowling & Secor, 2008; Mayden, 2010; Nagle & Simons, 2012; Echelle et al., 2014, 2015; but see Crandall & Templeton, 1999 and Emel & Bonett 2011). In fact, despite a wealth of distribution data (Graening et al., 2011) only one broad-ranging molecular-based biogeographic study includes fine-scale sampling of an Ozark Plateau cave species: a cavefish, *Typhlichthys eigenmanni* (Niemiller et al., 2012). As a consequence, there is limited knowledge on how the geographic features of the Ozark Plateau shape evolutionary patterns of cave-dwelling species.

Due to low dispersal rates and strong geographic genetic structure, lungless salamanders (Plethodontidae) are an excellent model for testing biogeographic patterns (e.g. Dowling, 1956; Kozak, Blaine & Larson, 2006; Kuchta, Haughey, Wynn, Jacobs & Highton, 2016; Martin et al., 2016). One plethodontid, the grotto salamander (*Eurycea spelaea* Stejneger, 1892), is widespread across the Ozark Plateau and may contain several cryptic species (Bonett & Chippindale, 2004). The grotto salamander is an intriguing system for testing biogeographic patterns

due to an ontogenetic shift resulting in drastic morphological and ecological changes, which potentially influences dispersal patterns. Adult grotto salamanders are highly specialized to cave environments, where they play an influential role in subterranean ecosystems (Fenolio, Graening, Collier & Stout, 2006). However, grotto salamander larvae possess functional eyes and pigments like other species of surface-dwelling *Eurycea* and are common in both surface and subterranean habitats. After an extended larval period of 2–6 years, larvae are believed to return to caves and underground aquifers to metamorphose, whereupon their eyes degenerate and pigments become non-functional (Brandon, 1971; Fenolio et al., 2014). Adult grotto salamanders have rarely been documented outside of caves (but see Mohr & Poulson, 1966) and mating and oviposition are likely exclusive to sub-surface habitats (Fenolio & Trauth, 2005; Smith, 1960). The retention of surface-dwelling larvae may allow dispersal opportunities unavailable to other cave-obligate species, while the subterranean specialization of post-metamorphic grotto salamanders may lead to significant constraints on dispersal.

In this study, we reconstruct a time-calibrated phylogeny of grotto salamanders using 13 mitochondrial protein-coding genes and 251 nuclear loci derived from anchored hybrid enrichment (AHE) and high-throughput sequencing. Next, we evaluate isolation among grotto salamander lineages by examining spatial genetic variance and population structure. Finally, we test for associations between phylogenetic structure and palaeodrainages, contemporary drainages and sub-plateau structure of the Ozarks. This study has implications for understanding broader biogeographic patterns of obligate subterranean species.

2 | MATERIALS AND METHODS

2.1 | Specimens

We sampled 194 grotto salamanders across the Ozark Plateau (Figure 1). Individuals were tail clipped in the field (specimens released unharmed) or live specimens were returned to the University of Tulsa and euthanized with 0.1% tricaine methanesulfonate (MS-222). Voucher specimens were deposited at the Sam Noble Museum (University of Oklahoma) and the American Museum of Natural History (Appendix S1). This study was conducted following the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Institutional Animal Care and Use Committee (IACUC) at the University of Tulsa approved this protocol (TU-0029).

2.2 | Anchored Hybrid Enrichment Sequence Capture/High Throughput Sequencing

Despite recent advances in next-generation sequencing (NGS), the application of genome-scale data to phylogenetic questions is lacking for many taxa, especially organisms with large genomes like salamanders (Jockusch, 1997; Keinath et al., 2015; Sun & Mueller, 2014; Sun et al., 2012). Notable exceptions include recent studies on tiger salamanders and European newts using high-throughput

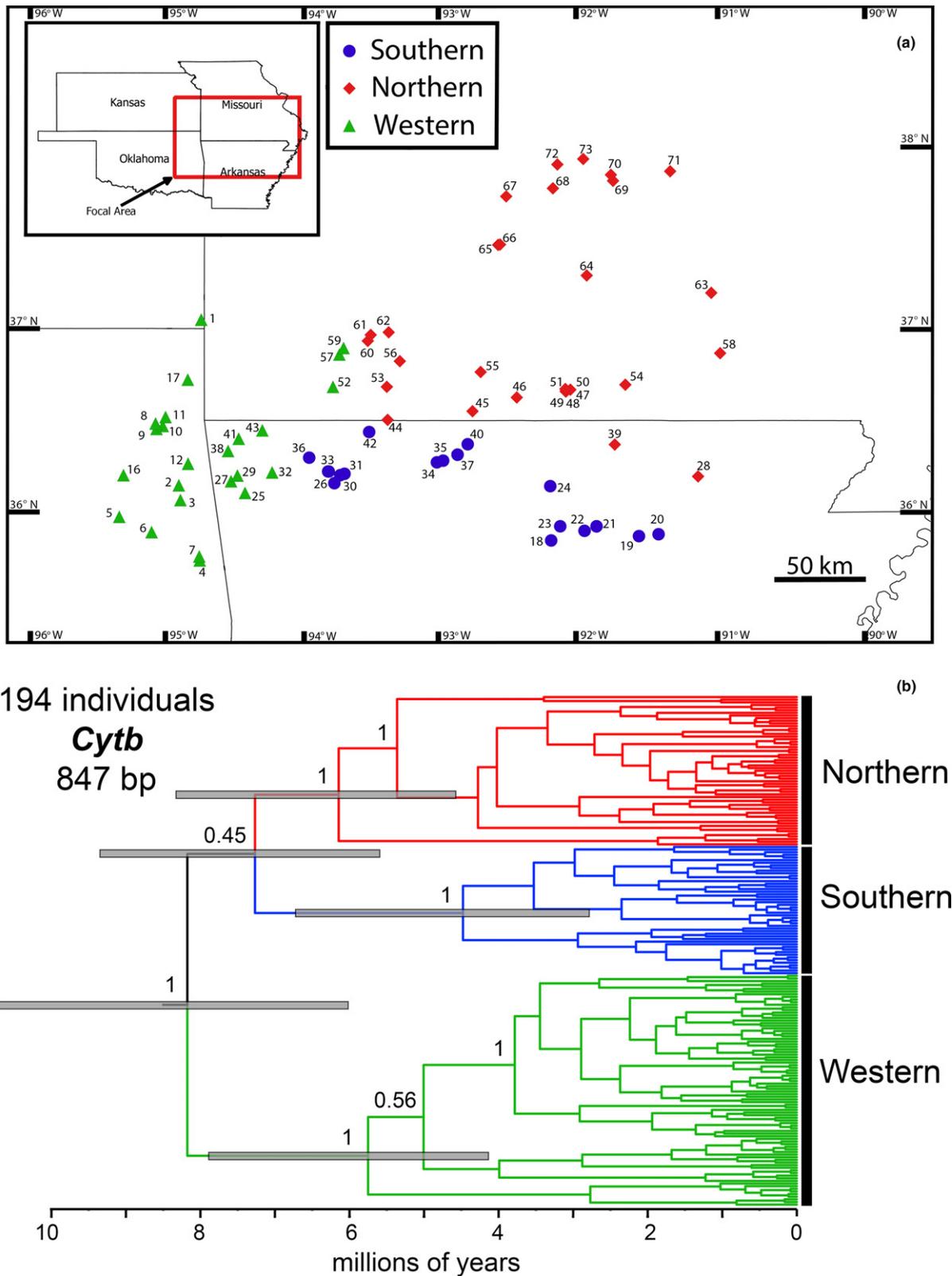


FIGURE 1 (a) Map of grotto salamander localities used in this study. Site numbers correspond to Appendix S1. (b) Bayesian chronogram constructed from *cytb* for 194 Grotto Salamanders. Posterior probabilities and node bars representing 95% HPD are shown on major nodes. Clades are coloured for online-only version: Southern Clade (blue), Western Clade, (green) and Northern Clade, (red). The fully annotated *cytb* tree can be found in Appendix S6

sequencing of parallel-tagged amplicons (O'Neill et al., 2013; Wielstra et al., 2014; Zielinski, Stuglik, Dudek, Konczal & Babik, 2014), and even more recently with AHE for *Plethodon* phylogeography (Newman & Austin, 2016) and ddRADseq for testing *Ambystoma* population dynamics (Nunziata, Lance, Scott, Lemmon & Weisrock, 2017). Here, we report on our development of a tool to collect diverse multi-locus nuclear data in *Eurycea* and other salamanders based on AHE (see also Faircloth et al., 2012; Lemmon, Emme & Lemmon, 2012; McCormack et al., 2012). We demonstrate the utility of AHE and high throughput sequencing for obtaining a large panel of nuclear loci (and mitochondrial genomes) in salamanders for testing fine-scale phylogeographic questions. Most vertebrate exons are relatively conserved and only 100–200 bp in length. In AHE, RNA-baits designed for anchoring to conserved exons should enrich for the exon and flanking/intervening introns, which include a higher number of variable sites. We used plethodontid transcriptomes to develop RNA-baits to target diverse portions of salamander genomes in order to answer a variety of phylogenomic and population-genomic questions in this and future studies. Specifics of AHE methods and NGS data analysis can be found in Appendix S2. We also sequenced the mitochondrial gene Cytochrome-b (*cytb*) for 194 individuals (methods for single-gene sequencing can be found in Appendix S3).

2.3 | Phylogenetic Analyses

Bayesian analyses for *cytb*, mtDNA (13 protein-coding genes) and nucDNA (251 orthologous loci) datasets were conducted using BEAST 2.3 (Bouckaert et al., 2014) for 100 million generations, sampling every 1000 generations with node support evaluated via posterior probabilities (BAPP). Methods for single-gene sequencing and models of evolution are presented in Appendix S3. Divergence times were also estimated using BEAST 2.3 (Bouckaert et al., 2014). For nuclear DNA ($N = 39$), divergence times were estimated based on a basal calibration date of 16 Ma, which is an estimated divergence date from *E. tynerensis* (Bonett et al., 2014; scaled according to Shen et al., 2016). For the mitochondrial genome ($N = 37$), grotto salamander genes were aligned to five other *Eurycea* species plus two additional species from the same subfamily (Spelerpinae; *Gyrinophilus* and *Pseudotriton*). The basal node was dated at 40.0 Ma (95% credible interval: 31.3–49.6) as estimated by Shen et al. (2016). We also analyzed *cytb* separately ($N = 194$), using the rate of molecular evolution (0.62 substitutions/100 bp/million years) calculated by Mueller (2006). This rate has been used for dating mitochondrial phylogenies of plethodontids (e.g. Kuchta et al., 2016) since there are no pre-Pleistocene *Eurycea* fossils that can be used to reliably calibrate the phylogeny.

Due to concerns regarding concatenated datasets (Edwards, Liu & Pearl, 2007; Kubatko & Degnan, 2007), we also used a species tree approach to test relationships among grotto salamander populations (hereafter referred to as the 'species tree'). Unfortunately, allele phasing can be difficult when short sequence reads are collected through AHE (McCormack, Hird, Zellmer, Carstens & Brumfield,

2013). Therefore, we selected the 30 genes with the highest percentage of parsimony-informative SNPs (24,094 total bp) and phased alleles manually. Two alleles for each individual ($n = 24$, i.e. 48 alleles per alignment) were input into *BEAST (Heled & Drummond, 2010). 15 individuals from our nuclear dataset were omitted as they were lacking sufficient data (<50% coverage) for the 30 loci chosen. This analysis was run for 200 million generations with a coalescent constant population speciation prior and a lognormal relaxed clock under a HKY model of evolution (selected due to computational feasibility similar to the concatenated nuclear tree). We evaluated stationarity of variables for all analyses by examining our output via TRACER 1.5 (Rambaut & Drummond, 2011). The first 20% of trees (pre-stationarity) were discarded as burning and a majority rule consensus tree was used to summarize the results.

2.4 | Population Level Analysis

A spatial analysis of molecular variance (SAMOVA, Dupanloup, Schneider & Excoffier, 2002) was used to test for genetic differentiation among 17 populations of grotto salamanders (each with ≥ 5 specimens; Appendix S4) into a number of genetically partitioned clusters (K). STRUCTURE 2.3.4 (Pritchard, Stephens & Donnelly, 2000) was used to evaluate genetic structuring in the nuclear data among grotto salamander populations. Linkage among the 251 nuclear regions was unknown, but these genes are widely dispersed in other vertebrate genomes. We selected one parsimony-informative SNP from each nuclear region, and treated each as an independent locus to incorporate haplotypes. Parameters of SAMOVA and STRUCTURE can be found in Appendix S5.

2.5 | Geographic Factors

A partial mantel test (Smouse, Long & Sokal, 1986) was implemented using the R package 'ecodist' (Goslee & Urban, 2007) to test for relationships between pairwise genetic distances (both mtDNA and nucDNA) and geographic distances plus three other matrices of spatial variables: the three major drainages of the Ozark Plateau that comprise the range of grotto salamanders (Lower Missouri, Middle Arkansas and White River drainages, defined in Cross, Mayden & Stewart, 1986), the sub-plateau structure of the Ozarks (Salem, eastern Springfield and western Springfield Plateaus), and Ozark palaeodrainages. Some studies have shown that pre-glacial "palaeodrainages" can be important for understanding phylogeographic patterns in North American salamanders (e.g. Kozak et al., 2006; Kuchta et al., 2016) and drainage boundaries have significantly impacted isolation and gene flow in populations of aquatic subterranean organisms (e.g. Finston et al., 2007; Niemiller et al., 2012, 2013b; Verovnik et al., 2003). Therefore, we tested the effects of palaeodrainages on grotto salamander phylogeography since there is geological evidence that the Gasconade and Osage Rivers (major components of the Lower Missouri drainage) flowed south into the White River drainage during the early Pleistocene, but currently flow north into the Lower Missouri drainage (Bretz, 1965). Our palaeodrainage model contains two



hydrologic units: the White and Middle Arkansas palaeodrainages (only differing from the contemporary drainage model by combining the Lower Missouri and White River drainages into a single unit). All analyses used a single individual per locality to ensure independence of observations as little diversity in *cytb* was detected within a locality (Appendix S6). A significant association between geographic and genetic distance would indicate isolation-by-distance as a factor for phylogeographic structure in grotto salamanders. Likewise, a significant partial correlation coefficient for different spatial variables would indicate hydrology and/or geology as a driver of lineage divergence. Although this method has been used in other species, including cave-fish and salamanders (e.g. Kozak et al., 2006; Niemiller et al., 2012), there are concerns with the ability of Mantel tests to evaluate the effect of spatial variables on genetic structures due to autocorrelation with isolation-by-distance (Legendre & Fortin, 2010). Therefore, following Kuchta et al. (2016), we conducted a distance-based redundancy analysis (db-RDA) to test the impact of landscape on genetic variation. Specifically, we tested whether contemporary drainages, palaeodrainages or the sub-plateaus had a greater impact on shaping the phylogeographic structure of grotto salamanders. The proportion of variance explained by each variable was calculated in R using the 'vegan' package with the function *varpart* (Oksanen et al., 2016). Geographical distances were calculated using GEOGRAPHIC DISTANCE MATRIX GENERATOR 1.2.3 (Ersts, 2010).

3 | RESULTS

All phylogenies recovered three deeply divergent clades (Figures 1–3), corresponding to northern, southern and western parts of the grotto salamander's distribution. No individuals switched among the three clades in any analyses and each clade was highly supported (BAPP=1 for all gene trees). The nuclear species tree analysis recovered a similar topology, with high support values for each of the three main clades, but with poor support for the relationship among these clades (Figure 3). The concatenated nuclear reconstruction estimated coalescence of all clades around 10 Ma (Figure 2b, Table 1), with Northern and Southern clades splitting ~9 Ma, indicating rapid divergence among the three major lineages. In *cytb*, the *E. spelaea* complex lineages diverged from one another 7–8 Ma (Figure 1b, Appendix S6, Table 1), while mtDNA suggests diversification around 13–15 Ma (Figure 2a, Table 1). The range in our dates suggests divergence among the major lineages of grotto salamanders in the Middle Miocene.

SAMOVA designated the maximum number of sites as distinct mitochondrial groups ($K = 16$), indicating limited gene flow among sites. Rodríguez-Robles et al. (2010) showed that F_{CT} peaks where $F_{SC} = 0$, so we used F_{SC} (0.07 at $K = 16$, Figure 4) as the determining factor in our analysis. Combined with a lack of shared haplotypes among localities, this suggests limited gene flow, at least in *cytb*. For the STRUCTURE analysis, estimated $\log(\ln)$ probabilities plateaued at $K = 3$ (Figure 5a,d), while ΔK peaked at $K = 2$ (Figure 5b,c). In both cases STRUCTURE placed all individuals in clusters identical to their

major clades in nuclear and mitochondrial phylogenies. ΔK 's identification of a lower K is likely identifying hierarchical structure in the data at a basal level (Evanno, Regnaut & Goudet, 2005) as has been found in past studies (e.g. Weisrock et al., 2010). The use of ΔK as a measure of population structure focus on basal divergences (Evanno et al., 2005), and as a result can recommend the lowest K even when $\log(\ln)$ likelihood values suggest a higher number of clusters with geographic partitioning (Kalkvik, Stout & Parkinson, 2012; Weisrock et al., 2010). Since our analysis suggests minimal gene flow among lineages and the $\log(\ln)$ likelihood values plateauing at a $K = 3$ with high individual membership coefficients (posterior probabilities >0.9) for 34 of 39 individuals to each of their clusters (Appendix S7), we favour the measure of $\log(\ln)$ probability over ΔK in estimating K in our case, but present the results for both measures (Figure 5).

The partial mantel test found a significant association between all spatial variables (palaeodrainages, contemporary drainages, sub-plateaus: Figure 6a,b) and genetic distance for mitochondrial and nuclear datasets ($p < 0.001$). No significant support was found for isolation-by-distance ($p = 0.53$ nucDNA, 0.27 mtDNA). Geographic distance was also tested with the db-RDA, but explained 0% variation in all cases and is omitted from the analyses presented. The db-RDA found 73% of nuclear variation explained by the spatial variables tested (Figure 6c), with palaeodrainages+sub-plateaus explaining the greatest amount of variation (28%), followed by the combination of all three (24%). No variable independently explained more than 9% variation. For the mitochondrial dataset 65% of variation was explained by the three spatial variables (Figure 6d) with palaeodrainages+sub-plateaus also explaining the greatest amount of variation (32%).

4 | DISCUSSION

Despite the high diversity and endemism of cave-associated species in the Ozark Plateau, empirical tests of phylogeographic patterns in subterranean fauna are notably lacking. Through phylogenomic analyses, we found three divergent lineages of grotto salamanders across the Ozark Plateau (Figures 1–3). The estimated coalescence of these lineages occurred in the Middle Miocene (~10–15 Ma, based on 251 nuclear loci or 13 mitochondrial protein-coding genes, respectively), with the deepest divergence separating populations from the western and eastern portions of the distribution. These two clades follow the palaeodrainage divide between the Middle Arkansas and ancestral White River palaeodrainages. The south-flowing White River drainages previously included the Gasconade and Osage Rivers, but both rivers were captured by the Lower Missouri River drainage during the Pleistocene. A biogeographic link between White and Lower Missouri drainages has also been hypothesized for several species of fishes showing similarities across drainages (Cross et al., 1986; Miller, 1968). Geologic substructure of the Ozark Plateau impacted early phylogeographic patterns of grotto salamanders as eastern populations (White River Palaeodrainage) also contain an early divergence

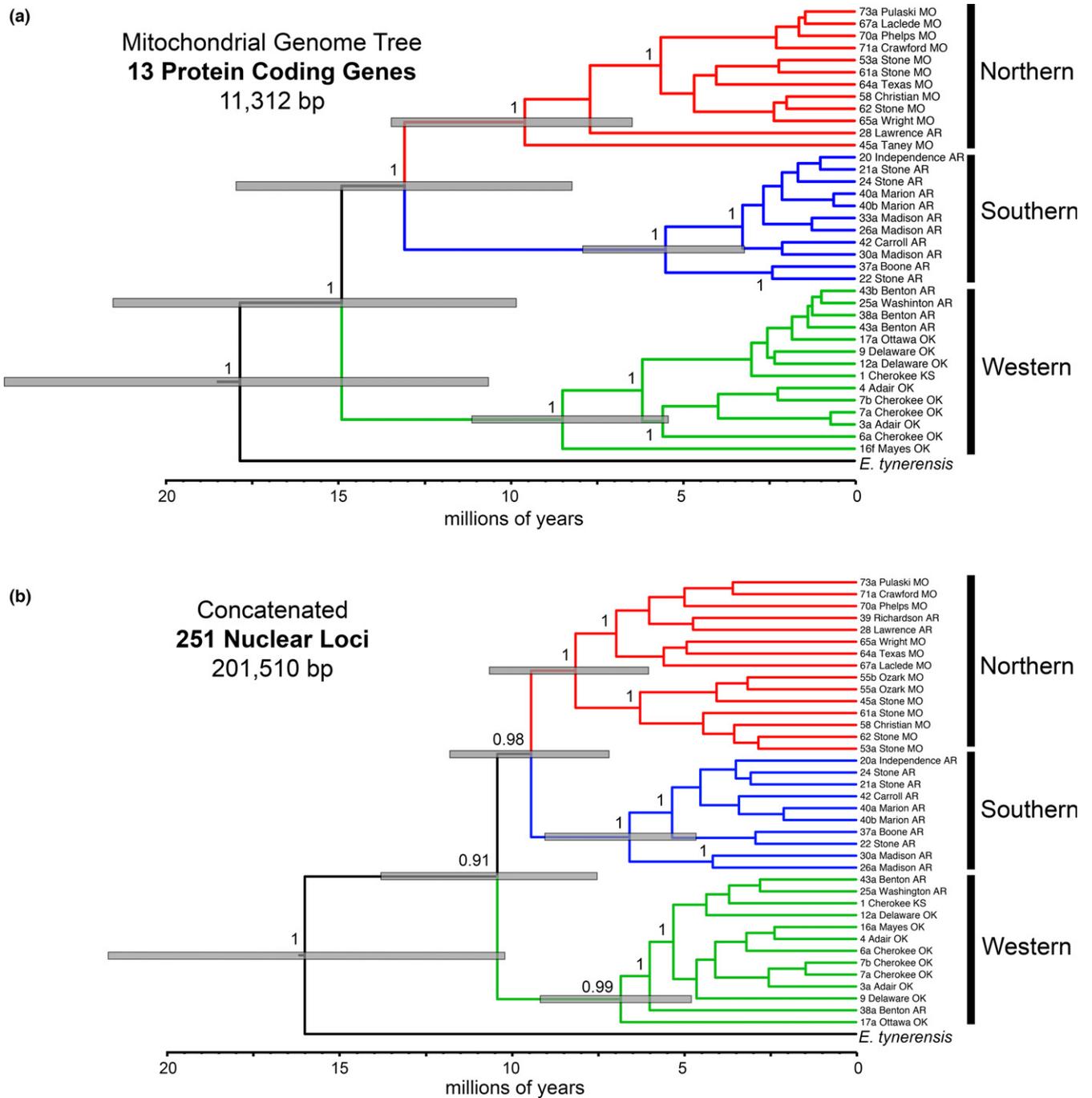


FIGURE 2 Bayesian chronogram constructed from (a) mitochondrial genomes from 37 grotto salamanders and (b) concatenated nuclear tree comprised of 251 loci for 39 grotto salamanders. Posterior probabilities and node bars representing 95% HPD are shown on major nodes. Taxon names include the population number corresponding to Figure 1 and Appendix S1, with County and State given. Clades are coloured for online-only version: Southern Clade (blue), Western Clade, (green) and Northern Clade, (red). For visualization purposes, outgroups were pruned in R using the ‘ape’ package 3.2.0 (Paradis et al., 2004)

(9–13 Ma) corresponding to the Northern and Southern clades, predominantly overlapping with the Salem and eastern Springfield Plateaus. db-RDA suggests that the phylogenetic patterns of grotto salamanders are best explained by a combination of palaeodrainage and sub-plateau boundaries, lending further support to the phylogenetic-based interpretations and suggesting that both hydrology and karst geology can influence the biogeography of stream-associated subterranean species.

The Middle Miocene divergence among grotto salamander lineages provides a minimum estimate for colonization of subterranean environments and subsequent evolution of cave-adaptations. By comparison, other colonizations by cave-obligate vertebrates have occurred more recently, within the last five million years (e.g. Niemiller, Fitzpatrick & Miller, 2008; Strecker, Faúndez & Wilkens, 2004), with subterranean taxa closely related to surface-dwelling congeners (e.g. Culver, Kane & Fong, 1995). The only other Ozark cave species

FIGURE 3 Species tree using the alleles of 30 phased genes for 24 grotto salamanders. Posterior probabilities are shown on major nodes. Taxon names include the population number corresponding to Figure 1 and Appendix S1, with County and State given. Clades are coloured for online-only version: Southern Clade (blue), Western Clade, (green) and Northern Clade, (red)

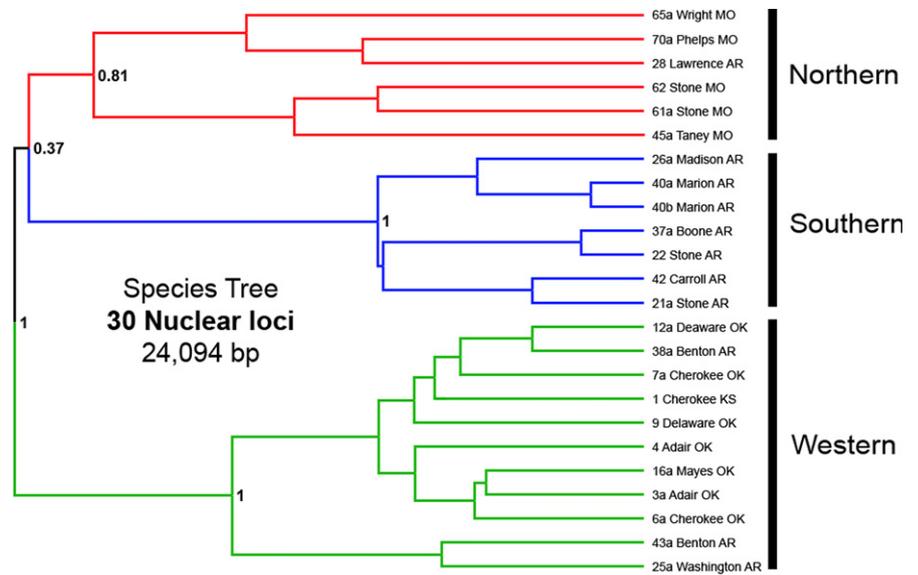


TABLE 1 Estimated dates of major nodes of grotto salamanders among Bayesian phylogenetic reconstructions using nuclear, mitochondrial and *cytb* datasets (95% HPD)

Clade	Cytb (N = 194)	Mitochondrial (13 loci)	Nuclear (251 loci)
Northern (<i>E. nerea</i>)	6.2 (4.5–8.3)	9.8 (6.0–13.9)	8.1 (5.5–10.4)
Southern (<i>E. braggi</i>)	4.5 (2.7–6.7)	5.6 (3.2–8.1)	6.6 (4.6–8.6)
Western (<i>E. spelaea</i>)	5.8 (4.0–7.9)	8.7 (5.2–12.5)	6.8 (4.7–8.7)
Northern + Southern	7.3 (5.3–9.5)	13.4 (8.5–18.8)	9.4 (6.4–11.9)
Entire <i>E. spelaea</i> complex	8.2 (6.0–11.0)	15.3 (9.6–21.2)	10.4 (7.1–12.7)

to which we can compare this biogeographic pattern (*Typhlichthys eigenmanni*) displays a similar geographic-genetic break, but as a result of a much more recent invasion of the Ozark Plateau (~2.5 Ma, Niemiller et al., 2012, 2013a). The *T. eigenmanni* populations appear to form two main clades divided by the Salem and eastern Springfield Plateaus in eastern Arkansas, concordant to a break between northern and southern grotto salamander lineages. The biogeography of these two cave-obligate species may have been shaped by similar factors, but at different time periods.

A profound constraint of habitat specialization is reduced dispersal, which can facilitate population divergence and produce strong phylogeographic structure. Degree of specialization and dispersal ability can vary across ontogeny, especially for species that exhibit morphologically diverse life history stages (e.g. Clobert et al., 2004; Didham et al., 2012). Therefore, phylogeographic structure can be highly contingent on the ecology of a given life stage. We find grotto salamander populations to be highly fragmented as evidenced by limited mitochondrial gene flow among localities (Figure 5). If caves are highly fragmented, we might attribute dispersal to the maintenance of a more generalized, ancestral larval form that have retained the ability to disperse through surface streams, unlike the subterranean-obligate adults. Hydrologic boundaries have had a significant impact

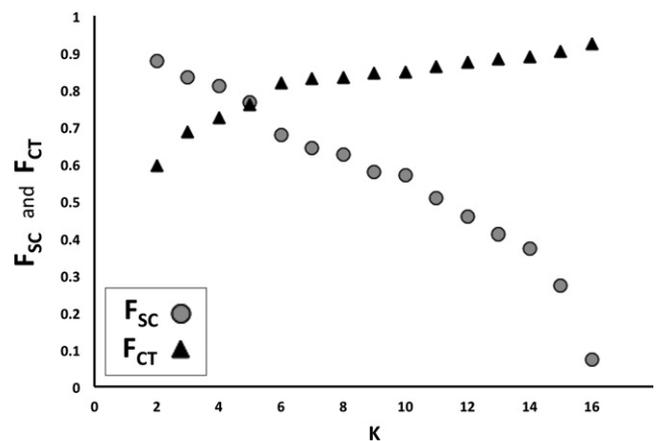


FIGURE 4 Spatial analysis of molecular variance (SAMOVA) based on 17 grotto salamander populations (K), with F_{SC} and F_{CT} values presented for each putative K to evaluate optimal genetic partitioning among sampling localities

on phylogeographic structure and dispersal patterns of surface stream-dwelling spelerpine salamanders, characterized by an extended aquatic larval phase and semi-terrestrial adults (Kozak et al., 2006; Lowe et al. 2008; Kuchta et al., 2016). Direct measures through mark and recapture have found no significant difference in movement distances between larvae and adults in one species (*Gyrinophilus porphyriticus*; Lowe 2003; Lowe et al. 2006; Lowe 2010). Grotto salamanders and *G. porphyriticus* both have long-lived larvae (>2 years; e.g. Fenolio et al. 2014). Many cave-obligate organisms have small ranges (e.g. Christman et al., 2005; Culver & Holsinger, 1992; Gibert & Deharveng, 2002), often indicative of poor dispersal. This is exemplified by cave-obligate spelerpine salamanders, which have limited ranges and no evidence of historical dispersal between major physiographic regions (Bonett et al., 2014). However, grotto salamanders have comparatively large ranges and their hydrologically structured phylogeographic patterns may be attributed to the maintenance an ancestral surface-dwelling larval form.

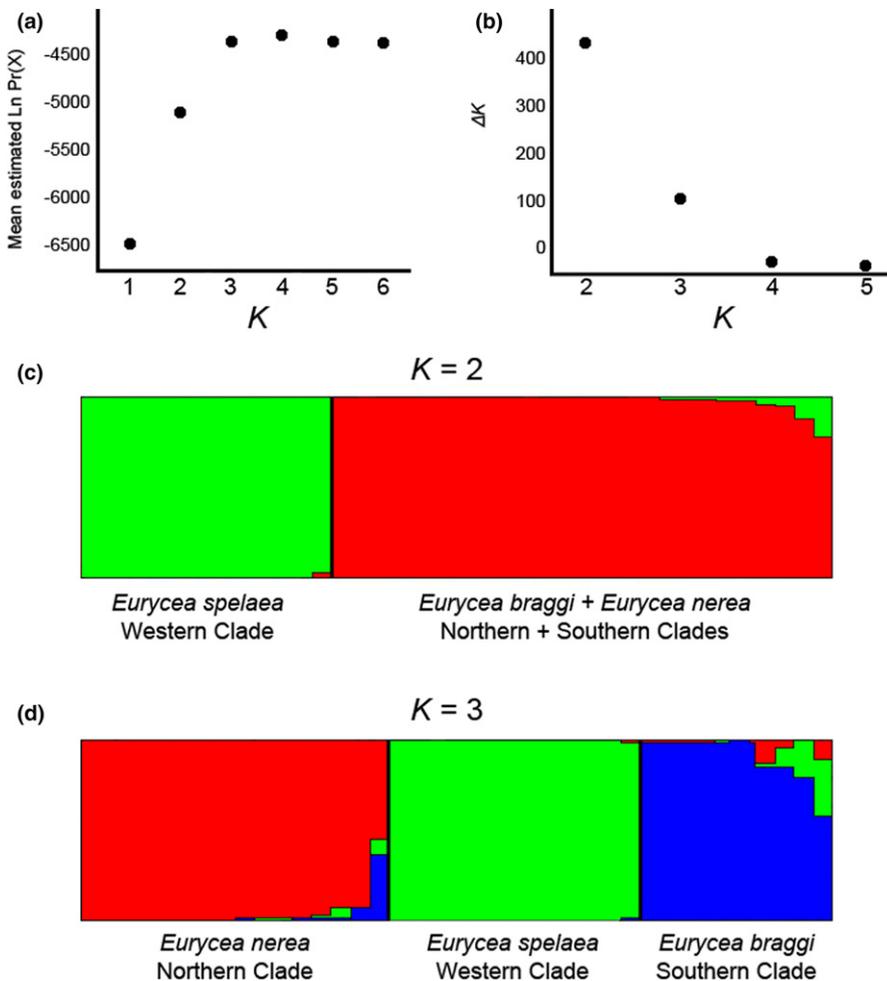


FIGURE 5 STRUCTURE analysis of 39 grotto salamanders (*Eurycea spelaea* complex) from 36 populations across the Ozark Plateau, to test for an optimal number of genetic partitions. (a) Log(ln) probabilities of the data for $K = 1$ through $K = 6$. (b) ΔK calculated for $K = 2$ through $K = 5$. (c) Cluster bar plot for the two partition model as recommended using ΔK . (d) Cluster bar plot for the three partition model as recommended using log(ln) probabilities of the data

4.1 | Conservation Status and Taxonomic Implications

The STRUCTURE analysis was used to designate genetic groupings, and recommended three distinct geographically partitioned clusters at the population level, concordant with the phylogenies of both nuclear and mitochondrial genomes. Furthermore, genetic distances among clades (10.6% mtDNA, 1.1% nucDNA) are greater than other salamanders recognized as distinct species (e.g. Baird et al., 2006; Martínez-Solano et al., 2012; Parra-Olea, García-París & Wake, 2004). Caution is needed when delimiting species based on the multispecies coalescent (Sukumaran & Knowles, 2017), however, support for strong levels of genetic isolation, mito-nuclear concordance and deeper divergences than other plethodontids recognized as distinct species. We conclude that our results diagnose species-level divergence, not just population structure. Therefore, we recommend dividing *Eurycea spelaea* into three species based on genomic and geographic partitioning identified in this study.

Initially described as *Typhlotriton spelaeus* (Stejneger 1892), the grotto salamander remained a distinct genus based on morphological characters until recently (Bonett & Chippindale, 2004). There were two other described species of *Typhlotriton*: *T. nereus* (Bishop, 1944) and *T. braggi* (Smith, 1968). Both were described using erroneous characteristics and quickly synonymized with *E. spelaea* (Brandon,

1966; Brandon & Black, 1970). The range of each of the three divergent lineages identified in this study corresponds to the type localities for the three synonyms (*E. braggi*, *E. nerea*, and *E. spelaea*), but not necessarily to the morphological features referenced therein. In accordance with ICZN protocol, we recommend that the name *Eurycea braggi* be resurrected for the Southern Clade and *Eurycea nerea* be resurrected for the Northern Clade, while the Western Clade should retain the name of *Eurycea spelaea*. These three cryptic species warrant evaluation of conservation status, as do the watershed and sub-plateau elements within the Ozarks. *Eurycea braggi* and *E. spelaea* both have relatively small geographic distributions, which may increase conservation concern for these species. There is no evidence of sympatry among the three molecular lineages, so we suggest using geographic range to differentiate among the three species in the field.

5 | CONCLUSIONS

Deep phylogenomic divergences suggest that grotto salamanders have been cave-adapted since the Middle Miocene, older than many cave-obligate lineages. Grotto salamander phylogeography is associated with major contemporary and historical drainage boundaries, as well as sub-plateau structure of the Ozarks. Isolation among populations highlights the importance of conservation of separate hydrologic units

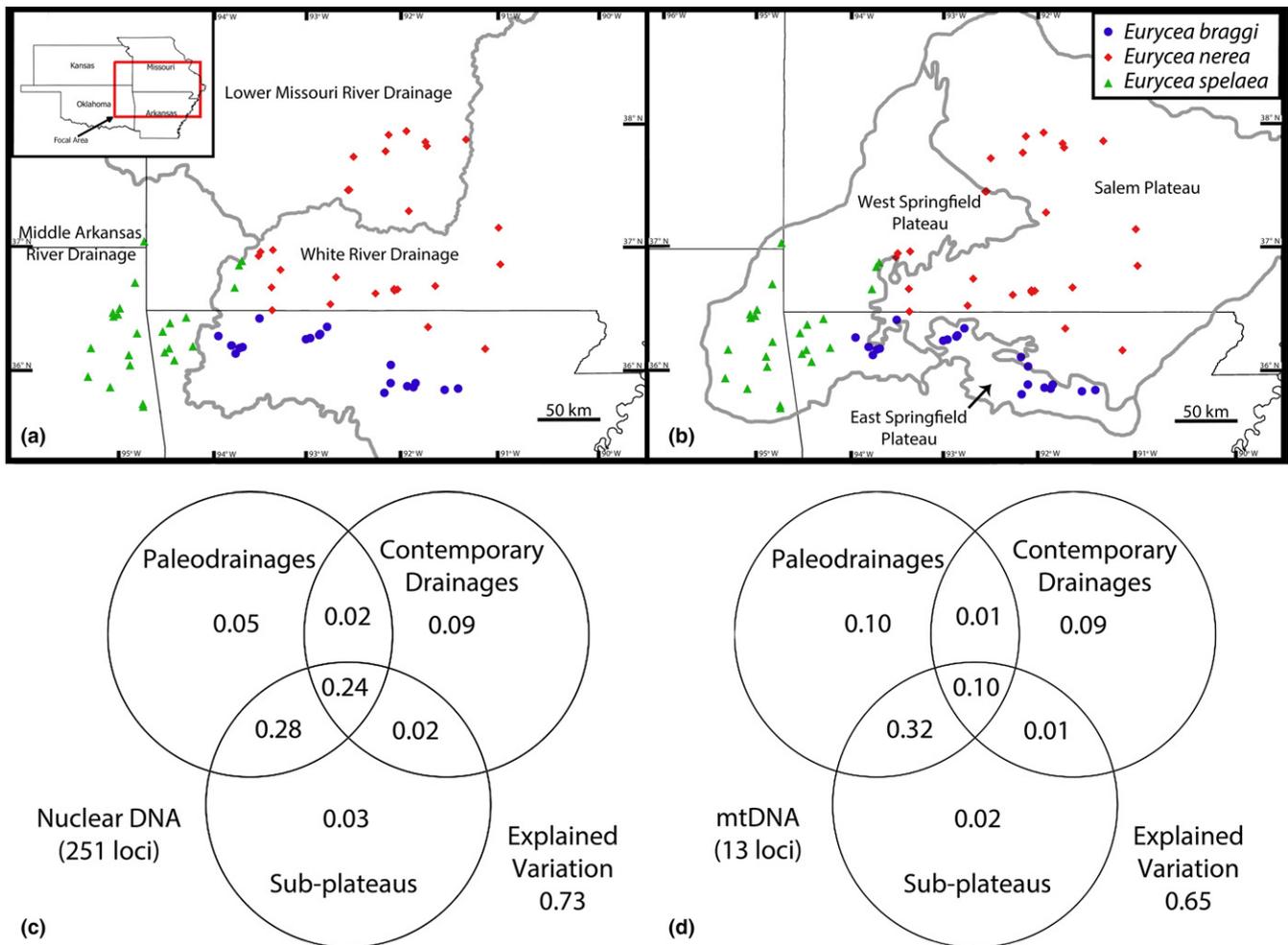


FIGURE 6 Association of the three major lineages of grotto salamanders with drainages and sub-plateaus of the Ozarks. (a) Drainages with grotto salamander sites. (b) Sub-plateaus with grotto salamander sites. (c) db-RDA results with nuclear DNA. (d) db-RDA results with mitochondrial DNA. The palaeodrainage model is not pictured, but is identical to the drainage model except that all localities from White and Lower Missouri drainages are incorporated as a single unit

in the Ozark Plateau. Concordance between highly divergent mtDNA and nucDNA lineages suggests that Grotto Salamanders are comprised of multiple cryptic species and therefore we recommend elevating two synonyms of *E. spelaea* to specific status. Much of our work was accomplished using anchored hybrid enrichment of exons and introns, a method that has only recently been applied to phylogeography. This fine-scale analysis can serve as a phylogeographic model to test whether hydrologic and geologic boundaries had community level impacts on biogeographic patterns across the Ozark Plateau.

ACKNOWLEDGEMENTS

The authors thank K. Irwin from the Arkansas Fish and Game Commission, J. Briggler from the Missouri Department of Conservation, and M. Howery from the Oklahoma Department of Wildlife Conservation for assistance with obtaining permits and locating sampling sites. We thank T. Aley, J. Beard, A. Blair, S. Burmeister, P. Chippindale, J. Cooley, B. Goodnight, J. Holt, J. Holt, K. Irwin, A. Linzey, O. Linzey, S. Martin, E. Robinson, M. Slay, M. Steffen, A. Trujano, R. Van

Devender, J. Wiens and M. Wine for assistance in field collections. We are indebted to the many government agencies, private landowners and commercial caves that granted us permission to collect salamanders on their property, including the Ozark Plateau National Wildlife Refuge Mark Twain National Forest, Ozark-St. Francis State Forest, War Eagle Cavern, Cosmic Caverns, Tumbling Creek Cave, Marvel Cave and Cloud 9 Ranch. W. Booth, C. Brown, M. Buchheim, M. Dawson, C. Siler and anonymous reviewers provided invaluable comments on an earlier draft of this manuscript. S. Kuchta and W. Booth provided assistance with analyses and M. Treglia advised bioinformatic sorting of AHE data. Many phylogenetic analyses were run on the Tandy Supercomputer in Tulsa, OK. This work was funded by grants from the Theodore Roosevelt Memorial Grant (American Museum of Natural History to JGP), the University of Tulsa Office of Research and Sponsored Programs (20-2-1211607-53600 to JGP), Oklahoma Department of Wildlife Conservation (ODWC Grant: E-22-18 and E22-20 to RMB and DBF), U.S. Fish and Wildlife Service, National Science Foundation (DEB 1050322 to RMB) and the Oklahoma NSF-EPSCoR Program (IIA-1301789 to RMB).

DATA ACCESSIBILITY

All mtDNA sequences are available on Genbank (Appendix S1). Nuclear alignments can be found on Dryad (doi:10.5061/dryad.7d1sn). Voucher specimens are deposited at the American Museum of Natural History and the Sam Noble Museum at the University of Oklahoma. Locality data are provided to county level (Appendix S1). Exact coordinates are sensitive, but further information is available from the authors upon request.

AUTHOR CONTRIBUTIONS

RMB conceived the project. All authors collected specimens. JGP, SLE, and RMB conducted labwork. JGP conducted analyses. JGP and RMB led the writing with contributions from all other authors.

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BIOSKETCHES

John G. Phillips recently finished his Ph.D. at the University of Tulsa in Oklahoma. Research interests include causes and consequences of reproductive isolation and speciation, particularly those driven by biogeography, ecology and molecular evolution.

Ronald Bonett's Lab has broad research interests including understanding the influence of life history and developmental variation on patterns of biogeography, reproductive isolation and evolution. We use phylogenomics and comparative methods to analyse these patterns across salamanders, our primary model system (<http://ronbonett.weebly.com/index.html>).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Phillips JG, Fenolio DB, Emel SL, Bonett RM. Hydrologic and geologic history of the Ozark Plateau drive phylogenomic patterns in a cave-obligate salamander. *J Biogeogr*. 2017;00:1–12. <https://doi.org/10.1111/jbi.13047>