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ORIGINAL ARTICLE

Ecological opportunity, historical biogeography and diversification in a major lineage of salamanders

Kenneth P. Wray* and Scott J. Steppan

Department of Biological Science, Florida State University, Tallahassee, FL 32306-4295, USA

ABSTRACT

Aim Spelerpini is a major radiation in the Plethodontidae, the largest family of salamanders. Seventy-five percent of its species richness occurs in *Eurycea*, one of its six genera. We hypothesized that this was the result of the transgression of the Western Interior Seaway that provided ecological opportunity via ancestral range expansion into novel geographical regions, leading to an adaptive radiation.

Location Eastern North America.

Methods We sampled all but one species and two subspecies of extant Spelerpini, including several putative species, for four genes using maximum likelihood and Bayesian inference approaches to generate a comprehensive, robust phylogeny. We used five fossil calibrations to generate a chronogram, a likelihood framework to estimate the ancestral ranges/splits of all nodes on the phylogeny, and a Bayesian inference method to estimate diversification rate shifts putting the evolution of this group into a historical biogeographical context.

Results A well-resolved, strongly supported phylogeny of the Spelerpini was recovered. *Eurycea* is among the oldest genera within the Spelerpini, originating *c.* 42 Ma with an ancestor occurring in four of five physiographical regions, each corresponding to a major *Eurycea* lineage. There is strong support for a rate shift in the Edwards Plateau neotenic *Eurycea*.

Main conclusions A pattern of niche lability was found in the Spelerpini, as opposed to a pattern of niche conservatism found in other major radiations of plethodontids in eastern North America. The genus *Eurycea* dispersed widely into novel regions experiencing ecological opportunity as the Western Interior Seaway transgressed. This represents the first fossil calibrated and the most thoroughly sampled phylogeny of the group to date.

Keywords

adaptive radiation, ancestral range reconstruction, ecological opportunity, *Eurycea*, historical biogeography, North America, Plethodontidae, salamander, Spelerpini, Western Interior Seaway

*Correspondence: Kenneth P. Wray, 319 Stadium Drive, Department of Biological Science, Florida State University, Tallahassee, FL 32306-4295, USA.
E-mail: kwray@bio.fsu.edu

INTRODUCTION

Adaptive radiation results when an ancestral population diverges into multiple descendent species due to natural selection on ecologically important traits (Dobzhansky, 1948; Simpson, 1953; Schluter, 2000; Gavrilets & Losos, 2009). A key model of adaptive radiations is ecological opportunity, whereby previously unavailable niches become accessible through one (or a combination) of three mechanisms that lead to morphological and species diversification (Yoder *et al.*, 2010): dispersal into a novel environment, evolution of key innovation(s), and extinction of competitors (Simpson, 1953; see Givnish, 1997; Losos, 2010; and Yoder *et al.*, 2010 for recent reviews of the role of ecological opportunity). Additionally, most definitions of adaptive radiation agree that ecological opportunity is the catalyst that sets in motion three key aspects of an adaptive radiation: (1) multiplication of species, (2) adaptation, and (3) extraordinary

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diversity; although the latter is controversial (Givnish, 1997; Schluter, 2000; Gavrilets & Losos, 2009; Glor, 2010). Ecological opportunity should be accompanied by an early burst of both speciation and phenotypic divergence as populations rapidly diverge from one another under selection and fill different parts of niche space. As niches rapidly fill, this burst of speciation and morphological diversification is expected to slow down compared to shortly after the ecological opportunity (Schluter, 2000; Glor, 2010; Losos & Mahler, 2010). One potential example of ecological opportunity lies in the complex and dynamic geological history of eastern North America. This history has played a major role in shaping the floral and faunal evolution of the region, particularly for one major radiation of vertebrates.

Salamanders (Order: Caudata) are one of the best-studied, major tetrapod lineages, having served as model systems for physiology, anatomy, community ecology and numerous studies of evolutionary processes (see Duellman & Trueb (1986) for detailed review). The *c.* 698 species (Frost, 2016) are found throughout most temperate regions of the Northern Hemisphere, with a single radiation (subfamily Hemidactyliinae) occurring in parts of the Neotropics. The largest salamander family is the Plethodontidae (455 species; 65%) occurring mostly in North America. Several major lineages within this family have received considerable attention from evolutionary biologists studying the patterns and processes associated with diversification (Wake, 1987, 2006; Moritz *et al.*, 1992; Kozak & Wiens, 2006, 2010; Kozak *et al.*, 2006b; Wiens *et al.*, 2006; Vieites *et al.*, 2007). In particular, several recent studies on plethodontids in eastern North America have argued that niche conservatism, wherein a group of organisms retain the ancestral niche and its associated ecological traits (Wiens & Graham, 2005), played a major role in the diversification of the species rich genera *Plethodon* and *Desmognathus* (Kozak & Wiens, 2006, 2010; Kozak *et al.*, 2006b). Despite this high species richness, *Plethodon* and *Desmognathus* show very little morphological disparity outside of body size divergence (Kozak *et al.*, 2005, 2006b, 2009), a pattern similar to that observed in the western North American *Batrachoseps* (Wake, 2006).

However, niche conservatism does not appear to explain the pattern of diversification in the tribe Spelerpini (Vieites *et al.*, 2011), a radiation of plethodontid salamanders in eastern North America comprised of 36 species in five genera: *Eurycea* (28 spp.; an underestimate because several species complexes consist of multiple, deep genetic lineages, at least some of which represent undescribed species (Kozak *et al.*, 2006a; Wray, unpublished data), *Gyrinophilus* (four spp.), *Pseudotriton* (two spp.), *Stereochilus* (one sp.), and *Urspelerpes* (one sp.) (Frost, 2016). Kozak & Wiens (2010) found a monotonic decline in species richness with increasing elevation in Spelerpini. They also found no correlation between the time an elevational band was occupied by the Spelerpini and species richness (i.e. no support for the time-for-speciation effect of Stephens & Wiens (2003)), a stark contrast to the direct relationship observed in *Plethodon* and *Desmognathus*. These patterns suggest that a different mechanism is responsible for the diversity observed in Spelerpini salamanders (particularly *Eurycea*), one that might be explained by the geological events that were taking place during the evolution of the group.

The late Jurassic marked the beginning of the Zuni Sequence, a major cratonic event most likely caused by a large mantle plume that is thought to have led to increased seafloor spreading, resulting in sea level increases that eventually reached levels as high as 250 m above present (Stanley, 2008). In North America, this rise in sea levels was accompanied by the subduction of the Farallon tectonic plate under the North American plate during the mid-Cretaceous, initiating the formation of the Western Interior Seaway (WIS), as the Arctic Ocean transgressed from the north to join the transgressing waters of the Tethys Sea (present-day Gulf of Mexico) in the south (Mitrovica *et al.*, 1989; Stanley, 2008). By the late Cretaceous, the WIS had reached its maximum, reaching over 1000 km at its widest and completely separating the continent into Appalachia (east) and Laramidia (west). In the late Cretaceous, the WIS began to regress due to falling sea levels and uplifting of central portions of the North American continent. The early Palaeocene marked the almost complete regression of the WIS, although a shallow sea remained in the lower Mississippi River Valley (Stanley, 2008). The Zuni Sequence was the last, complete transgression of the North American Craton; however, during the Palaeocene and early Eocene, sea levels began to rise again during the Tejas Sequence (Stanley, 2008). During this sequence, the Atlantic coast of North America reached as far west as the Mississippi River Valley, but the interior highlands and plains of North America remained above sea level. By the end of the Eocene, sea levels fell once again, marking the end of the Tejas Sequence and the last transgressive event of North America.

These repeated transgression and regression events not only resulted in the splitting and isolation of numerous plant and animal clades in North America, they also changed the landscape of the central and southern portions of the continent. These large, relatively shallow seas teemed with life and, combined with the extensive deposition of silt and carbonate from the surrounding landmasses, ultimately formed layers of rich soils as they regressed (Stanley, 2008). These events opened up a variety of new habitats to the North American flora and fauna that were previously isolated on the landmasses of Appalachia and Laramidia.

Herein, we combine a Spelerpini phylogeny with divergence time estimation, ancestral geographical range estimation, and Bayesian analysis of evolutionary rates to test whether *Eurycea* constitutes an adaptive radiation. Specifically, we hypothesize that ancestral *Eurycea* would have colonized the newly exposed regions of the North America Craton coinciding with the regression of the WIS, leading to an increase in diversification rates.

MATERIALS AND METHODS

Taxon selection and sampling

We sampled 36 species representing all currently described Spelerpini salamanders and their subspecies, including the recently described *E. subfluvicola* (Steffen *et al.*, 2014), with the exception of *E. robusta*, which has not been seen since it was first discovered and for which no viable tissue exists (Chippindale *et al.*, 2000), and the subspecies *G. porphyriticus duryi* and *G. palleucus necturoides*. Additionally, our sampling included a number of putative species, primarily from the *E. bislineata*, *E. multiplicata* and *E. quadridigitata* complexes (Bonett & Chippindale, 2004; Kozak *et al.*, 2006a; Wray, unpublished data). Whenever possible, we included at least two samples of each taxon to alleviate confounding issues from misidentifications, hybridization, or mitochondrial introgression (Barraclough & Nee, 2001). Following Vieites *et al.* (2007), we chose five members of the genus *Batrachoseps* as the outgroup. In total, 100 operational taxonomic units (OTUs) were included in the analyses (see Appendix S1 in Supporting Information).

We generated 138 novel sequences for this study (Appendix S1), with the remaining sequences ($n = 215$) downloaded from GenBank. We attempted to generate or download all sequences for an OTU from the same individual. However, in five ingroup OTUs, and with all five outgroup OTUs, this was not possible. In the five ingroup OTUs, the species involved were restricted to small geographical ranges and did not overlap with a morphologically similar, close relative (*E. nana*, *E. naufragia*, *E. sosorum*, *E. tonkawae*, and *E. troglodytes*). All of these species are sufficiently rare and difficult to obtain tissue samples from (several are federally or state protected), so we deemed it important to combine these samples for analysis. Sequences generated in this study were obtained from tissue samples previously collected in the field or borrowed from the Texas Cooperative Wildlife Collection Division of Herpetology or from private collections. Tissue samples consisted of tail tips or liver samples and were preserved in 95% ethanol stored at −80°C.

DNA extraction, amplification and sequencing

Genomic DNA was extracted using the hot phenol-chloroform-isoamyl alcohol/chloroform-isoamyl alcohol method (Sambrook & Russell, 2001). Extracts were visualized on agarose gels and DNA concentration quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA). Polymerase chain reaction (PCR) was performed on the mitochondrial cytochrome b (CytB), NADH dehydrogenase subunit 2 (ND2) and NADH dehydrogenase subunit 4 (ND4) genes, as well as the first section of the nuclear recombination-activating gene 1 (RAG1). The following reagents and concentrations were used in all PCR runs: 13.3 μL distilled water, 5 μL 5X Colourless GoTaq Reaction Buffer, 1.5 μL $MgCl_2$ (25 mM), 1.5 μL dNTPs (2.5 mM), 0.2 μL GoTaq (5U μL^{-1}), 0.5 μL bovine serum albumin (10 mg mL^{-1}), 1.0 μL of each primer (10 ng μL^{-1}). Amplification was performed using the primers and thermal cycler programs listed in (Appendix S1) and negative and positive controls. Amplification products were cleaned enzymatically with Affymetrix-USB ExoSAP-IT PCR Product Clean-up kits (USB Corporation, Cleveland, OH, USA).

Sequencing reactions were performed at the Florida State University Sequencing Facility using an Applied Biosystems 3130xl Genetic Analyzer with capillary electrophoresis (Applied Biosystems Inc., Foster City, CA, USA) or at the DNA Analysis Facility at Yale University using an Applied Biosystems 3730xl Genetic Analyzer (Applied Biosystems Inc.). Sequencing was conducted using the amplifying primers and two internal sequencing primers designed for ND2 (Appendix S1). Sample sequence lengths varied within genes: CytB (493–1112 bp), ND2 (499–1039 bp), ND4 (638–725 bp), RAG1 (481–1467 bp). All novel sequences generated for this study were deposited in GenBank (Appendix S1).

Phylogenetic analyses and divergence time estimates

A total of 349 sequences, representing 41 plethodontid species and a number of putative species, were used in the alignments. Sequences were edited and aligned using the Geneious Alignment in GENEIOUS 5.5.7 (Kearse *et al.*, 2012) then adjusted by eye. Genes were translated to amino acids to ensure there were no premature stop codons and to verify the alignment. We used JMODELTEST 2.1.1 (Guindon & Gascuel, 2003; Darriba *et al.*, 2012) to fit 88 DNA-substitution models to the alignments and determined the best substitution model of nucleotide evolution for each of the four genes using the Akaike information criterion (AIC; Akaike, 1974).

Phylogeny and divergence time estimates were conducted on the four concatenated genes using maximum likelihood (ML) as implemented in PAUP* 4.0a126 (Swofford, 2003) and using Bayesian inference (BI) as implemented in BEAST 1.7.4 (Drummond & Rambaut, 2007). For the ML analysis, a heuristic search with 10 stepwise random-addition sequence replicates using the tree bisection-reconnection method was performed on the data set partitioned by codon. To assess support for the ML tree, we also performed a nonparametric bootstrap analysis using 100 pseudoreplicates with two stepwise random-addition sequence replicates. The ML tree was used as the starting tree for the BEAST analysis partitioned by codon position. For the BI analysis, we performed two independent runs of 2×10^8 generations, sampling every 1000 generations. We combined the resultant data sets using LOGCOMBINER 1.7.4 (Drummond & Rambaut, 2007) and checked for convergence using AWTY (Nylander *et al.*, 2008) and TRACER (Rambaut & Drummond, 2007). TRACER was used to check for stationarity and we conservatively discarded the first 4×10^4 trees as burn-in.

The divergence time estimations utilized an uncorrelated lognormal relaxed molecular clock (Drummond *et al.*, 2006),

a birth–death speciation prior (Gernhard, 2008), and five fossil-based node calibrations. We used a lognormal prior distribution on all fossil calibrations (Ho, 2007) with mean = 1 and SD = 2.0. For the offset of each prior, we used the lower boundary of the time period the fossil was confirmed from, making this a conservative estimate of the node age. The outgroup was constrained to be monophyletic and we calibrated the basal node of this group at 4.9 Ma using a fossil *Batrachoseps* sp. from the late Hemphillian North American land mammal age (NALMA; Clark, 1985; Holman, 2006). A fossil sample of *G. porphyriticus* and one of *P. ruber*, both from the Irvingtonian NALMA, were used to calibrate their respective nodes at 0.24 Ma (Holman, 1977, 1995, 2006; Holman & Grady, 1987). The final two fossil calibrations were of *E. lucifuga* and *E. cirrigera* and came from the same Rancholabrean NALMA deposit 0.011 Ma at Cheek Bend Cave, Maury County, Tennessee (Miller, 1992; Holman, 2006). The results of Kozak *et al.* (2006a,b) indicated that the *E. cirrigera* from this region of Tennessee are a distinct, divergent lineage that may represent a distinct species. Given these results, combined with the widespread present-day range of this species, we used this fossil to calibrate the node for our *E. cirrigera* A (clade C of Kozak *et al.*, 2006a).

Ancestral range estimation

We used the likelihood-based method that employs the dispersal-extinction-cladogenesis (DEC) model as implemented in LAGRANGE (Ree *et al.*, 2005; Ree & Smith, 2008) to estimate the ancestral ranges and descendant splitting events of the Spelerpini. This was done with the chronogram generated from the BEAST analysis and five physiographical regions (Fig. 1) encompassing the entire range of the extant Spelerpini: Appalachian Highlands (AH), Atlantic Plain (AP), Interior Highlands (IH), Interior Plains (IP) and Laurentian Uplands (LU). These regions are based on landforms (not vegetation or climate), having persisted in their current areas and geographical relationships for over 100 million years (Fenneman, 1916; Vigil *et al.*, 2000). In fact, four of the five are part of the North American (Laurentian) Craton, with only the AP occurring outside of this tectonically stable region. Nonetheless, even the AP has existed, at least in part, during much of the last 60–70 million years.

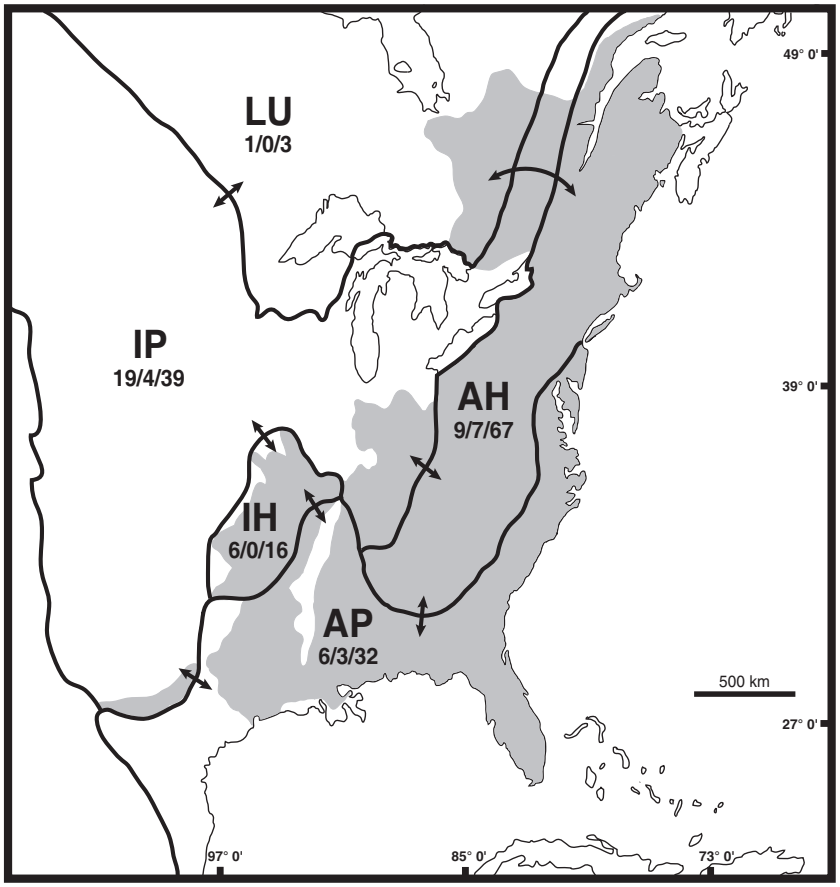


Figure 1 Physiographical regions of eastern North America: AH (Appalachian Highlands), AP (Atlantic Plain), IH (Interior Highlands), IP (Interior Plains), LU (Laurentian Uplands). Heavy black lines represent physiographical region boundaries. Arrows represent dispersal routes allowed in the ancestral estimation rate matrix. Numbers represent the number of species of *Eurycea*/other Spelerpini/other plethodontids in the physiographical region. Gray shaded area represents the modern range of the Spelerpini (= *Eurycea*, *Gyrinophilus*, *Pseudotriton*, *Stereochilus*, *Urspelerpes*).

LAGRANGE allows taxa to occur across multiple regions, so we scored all 95 ingroup OTUs for presence/absence in each of the five regions in the matrix. Given the small size and low vagility of plethodontid salamanders, we used an unequal rates model of dispersal between the five areas. In order for dispersal to take place from one area to the next, areas had to be in direct contact with one another (Fig. 1). In order to test the robustness of these predictions, we also performed a more conservative, equal rates analysis in which dispersal was allowed between any two areas with equal probability and compared the –ln likelihoods of each model. In LAGRANGE, statistical significance between two models is assessed using the method of Edwards (1992), wherein a score of two log-likelihood units or more is considered significantly different.

Diversification rates

We used BAMM version 2.0 (Bayesian Analysis of Macroevolutionary Mixtures: (Rabosky *et al.*, 2013; Rabosky, 2014) to assess diversification rate shifts and rate regimes (Rabosky *et al.*, 2014a) on our time calibrated chronogram. BAMM uses a Metropolis-coupled reversible-jump Markov chain Monte Carlo (MCMCMC; MC3) to detect and quantify rate heterogeneity in lineage diversification under a vast number of evolutionary models. We ran two independent analyses each for 10 million generations sampling from the posterior every 1000 generations. Our MC3 utilized four chains with swaps proposed every 1000 generations. We used an exponential hyperprior for the number of distinct rate shifts of 1.0 (considered a conservative hyperprior and one that minimizes type I errors; Rabosky, 2014) and estimated other priors using 'BAMMTools 2.0' (Rabosky *et al.*, 2014b). We checked for convergence using the output from the two independent runs ensuring that the effective sample sizes were above at least 10% of our sampled generations and by plotting our likelihood scores versus the number of sampled generations.

RESULTS

Phylogenetic analyses and divergence time estimates

No premature stop codons were detected in any of the four protein-coding genes used, suggesting that the amplicons were orthologous (Zhang & Hewitt, 1996). The resulting alignments were unambiguous. The AIC scores determined that GTR + I + Γ was the best-fit model of evolution for each gene.

The ML and BI trees were almost completely congruent and most major nodes were strongly supported with few exceptions (Fig. 2). The ML analysis had poor support for the relationships among the southern Edwards Plateau neotenes clade (M, Fig. 2), whereas in the BI analysis this clade was well supported. In addition, the two samples of *E. tridentifera* were sister in the ML analysis, but not in the BI tree. The other discrepancy between the two analyses was in the placement of the enigmatic *E. wallacei* clade. The ML analysis placed this clade in a well-supported polytomy with *E. aquatica, E. junaluska* and members of the southern *E. cirrigera*/*E. wilderae* clade. In the BI tree, there was weak support for the *E. wallacei* clade being sister to a clade containing *E. aquatica*, *E. junaluska*, and the southern *E. cirrigera*/*E. wilderae* clade.

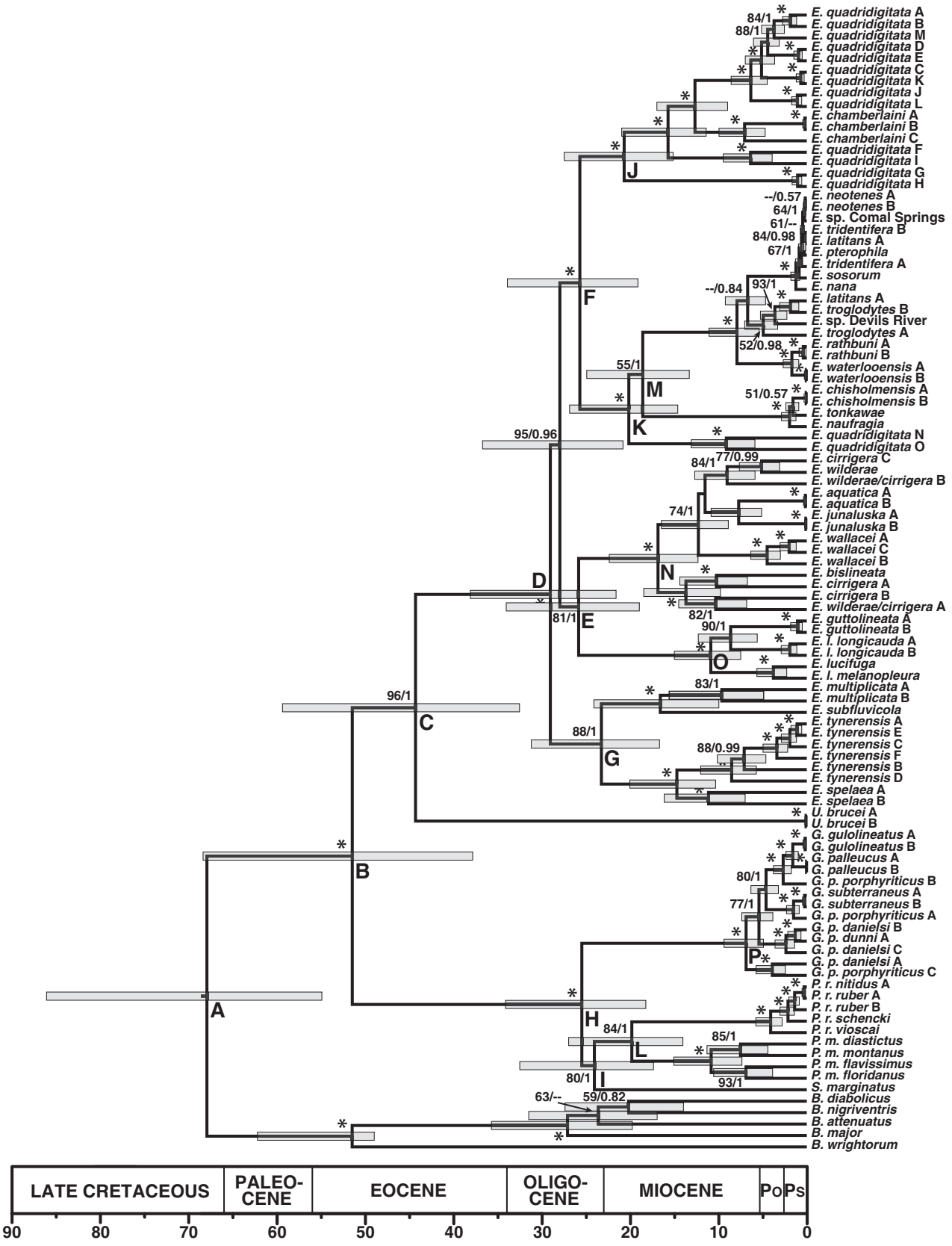
The analyses revealed two major lineages within the Spelerpini (B, Fig. 2): one clade representing the genera *Gyrinophilus*, *Pseudotriton* and *Stereochilus* (H, Fig. 2) and the other the genera *Eurycea* and *Urspelerpes* (C, Fig. 2). *Pseudotriton* was the sister taxon to *Stereochilus* (I, Fig. 2), with a deep divergence between the two species of *Pseudotriton* (L, Fig. 2). In addition, there was a deep divergence between the two subspecies of *P. montanus*. *Urspelerpes* formed the deeply divergent sister taxon to *Eurycea* (C, Fig. 2).

Within *Eurycea*, five strongly supported lineages are recovered (D, Fig. 2), nearly identical to those reported by Kozak *et al.* (2009) and Bonett *et al.* (2014). The first lineage contained the IH endemics (G, Fig. 2), which formed the sister clade to all other *Eurycea*. Within the remaining *Eurycea*, two clades are represented (E and F, Fig. 2). One of these (E, Fig. 2) is composed of the *E. longicauda* and *E. bislineata* complex clades (O and N, respectively, Fig. 2), with the troglobitic *E. wallacei* contained within the *E. bislineata* complex clade. The other clade (F, Fig. 2) consisted of the Edwards Plateau neotenes (M, Fig. 2) and two samples of *E. quadridigitata* originating from Mississippi and Texas (K, Fig. 2), sister to a group (J, Fig. 2) with *E. chamberlaini* nested within several, deeply divergent lineages of *E. quadridigitata*.

The origin of the Spelerpini (Fig. 2, Table 1) occurred *c.* 51 Ma with a 95% highest posterior density confidence interval of 68–38 Ma [herein represented as 51 Ma (68–38)], most likely in the Palaeocene or Eocene. Clades C and H diversified at *c.* 44 Ma (59–33) and 25 Ma (34–18) respectively. Within the latter, the first origin of *Pseudotriton* [20 Ma (27–14)] and *Stereochilus* [24 Ma (33–17)] occurred much earlier than *Gyrinophilus* [7 Ma (9–5)]. The genus *Eurycea* first appeared around 29 Ma (38–22), most likely in the late Eocene or the Oligocene. In contrast to the genera in clade H, the major lineages within *Eurycea* split temporally close. Clade E originated around 26 Ma (34–19) during the Oligocene or early Miocene, followed almost simultaneously by the appearance of Clade F [26 Ma (34–19)] and clade G [23 Ma (31–17)]. The former then diversified into eastern [J: 21 Ma (27–15)] and western [K: 20 Ma (27–15)] lineages. Numerous additional diversifications have occurred within these major Spelerpini lineages, ranging from the very shallow Pliocene and Pleistocene (e.g. some Edwards Plateau neotenes and the genus *Gyrinophilus*) to the Miocene (e.g. *E. quadridigitata* and *E. bislineata* complexes).

Ancestral range estimation and diversification rates

The unequal rates of dispersal model had a significantly better –ln likelihood (−138.90) than the equal rates of dispersal



model (−147.93), although the two dispersal models yielded largely congruent results, with 90 of 94 nodes estimated with the same ancestral range and 89 of 94 splits estimated with the same ancestral split. In all nine disagreements the highest likelihood of the ancestral range or split in one model was swapped with the second best likelihood in the other model

Figure 2 Bayesian inference chronogram of the Spelerpini (*Eurycea*, *Gyrinophilus*, *Pseudotriton*, *Stereochilus* and *Urspelerpes*) based on the mitochondrial CytB, ND2 and ND4 genes and the nuclear RAG1. The 95% highest posterior density credible intervals are reported as grey bars at nodes. Nodal support values are reported as ML bootstrap (BS)/Bayesian inference posterior probabilities (PP). Asterisks represent nodes supported by BS/PP that are ≥ 95/0.95. Geological time-scale at bottom of figure is in millions of years. Pliocene = P_O, Pleistocene = P_S, A = *Batrachoseps*+Spelerpini, B = Spelerpini, C = *Eurycea* + *Urspelerpes*, D = *Eurycea*, E = *E. bislineata* + *E. longicauda* complexes, F = *E. quadridigitata* + Edwards Plateau neotenes, G = Interior Highlands *Eurycea*, H = *Gyrinophilus* + *Pseudotriton* + *Stereochilus*, I = *Pseudotriton* + *Stereochilus*, J = *E. quadridigitata* + *E. chamberlaini*, K = Western *E. quadridigitata* + Edwards Plateau neotenes, L = *Pseudotriton*, M = Edwards Plateau neotenes, N = *E. bislineata* complex, O = *E. longicauda* complex, P = *Gyrinophilus*.

Table 1 Estimated ages of key Spelerpini nodes from Bayesian inference chronogram in Fig. 2 (95% highest posterior density credible interval in millions of years). Node letters correspond to labelled nodes in Fig. 2.

Clade	Name	Node age (Ma)	95% HPD CI (Ma)	Bonett *et al.* (2014)
A	*Batrachoseps* + Spelerpini	68	86–55	n/a
B	Spelerpini	51	68–38	60–38
C	*Eurycea* + *Urspelerpes*	44	59–33	55–31
D	*Eurycea*	29	38–22	44–27
E	*E. bislineata* + *E. longicauda* complexes	26	34–19	33–15
F	*E. quadridigitata* + Edwards Plateau neotenes	26	34–19	37–19
G	Interior Highlands *Eurycea*	23	31–17	37–17
H	*Gyrinophilus* + *Pseudotriton* + *Stereochilus*	25	34–18	40–18
I	*Pseudotriton* + *Stereochilus*	24	33–17	n/a
J	*E. quadridigitata* + *E. chamberlaini*	21	27–15	25–7
K	Western *E. quadridigitata*+Edwards Plateau neotenes	20	27–15	31–8
L	*Pseudotriton*	20	27–14	10–1
M	Edwards Plateau neotenes	19	25–13	31–13
N	*E. bislineata* complex	17	22–12	20–10
O	*E. longicauda* complex	11	15–7	20–4
P	*Gyrinophilus*	7	9–5	8–2

and vice versa. Since it significantly fit the data better (Ree *et al.*, 2005), herein, we refer to the results from the unequal rates of dispersal model and its most likely reconstructions (Fig. 3; for alternative estimates see the LAGRANGE output file in Appendix S3).

The model supported a Spelerpini ancestor occurring in the AH and AP, with both daughter lineages going extinct in the AP but remaining in AH (Fig. 3). Clade H (Fig. 3) is reconstructed as having occurred in the AH and AP, but with the subsequent *Pseudotriton* + *Stereochilus* lineage (I, Fig. 3) going extinct in the AH and diversifying in the AP where they underwent further diversification, while the lineage leading to *Gyrinophilus* (P, Fig. 3) remained in the AH where it underwent further diversification. Lineage C shows a much more dynamic history (Fig. 3), with the ancestral range reconstructed as having occurred in the AH. Although the monotypic *Urspelerpes* remained in this region, the ancestral range of the sister genus *Eurycea* is reconstructed as occurring in the AH, AP, IH and IP. Subsequently, all four of these regions also serve as the ancestral range of four major lineages within the genus: clade E (AH), clade F (AP), clade G (IH) and clade M (IP). There was a significant range expansion at the base of *Eurycea*, followed by several vicariant events, wherein further diversification took place largely *in situ*, although with little subsequent dispersal in clade E (Fig. 3).

The BAMM analysis on our BI chronogram supported a single diversification rate shift (posterior probability = 0.42) at the Eastern Edwards Plateau neotenes (Clade M; Fig. 4). Additional models of shift configurations were supported with varying probabilities (two shifts, pp = 0.28; zero shifts, pp = 0.14; three shifts, pp = 0.12; five shifts, pp = 0.008; six shifts, pp = 0.002). Although we used a conservative prior on the expected number of rate shifts, the posterior distribution of models is not independent of the prior. To address this, we computed Bayes factors (BF) to test these models of rate shifts against a null of zero rate shifts. The model of three shifts had the highest BF support (5.69), but this was only marginally strong support and other models had near similar support (two shifts, BF = 5.64; four shifts, BF = 4.81; one shift, BF = 4.38). We generated a mean phylorate plot, which displays model-averaged diversification rates at every point along our BI chronogram (Fig. 4a). Additionally, we generated the 95% credible set of distinct shift configurations to recognize core shifts (shifts with marginal probabilities that occur more frequently than expected) and plotted them on (Fig. 4a), although a shift configuration with zero core shifts cannot be plotted, but occurred with a marginal

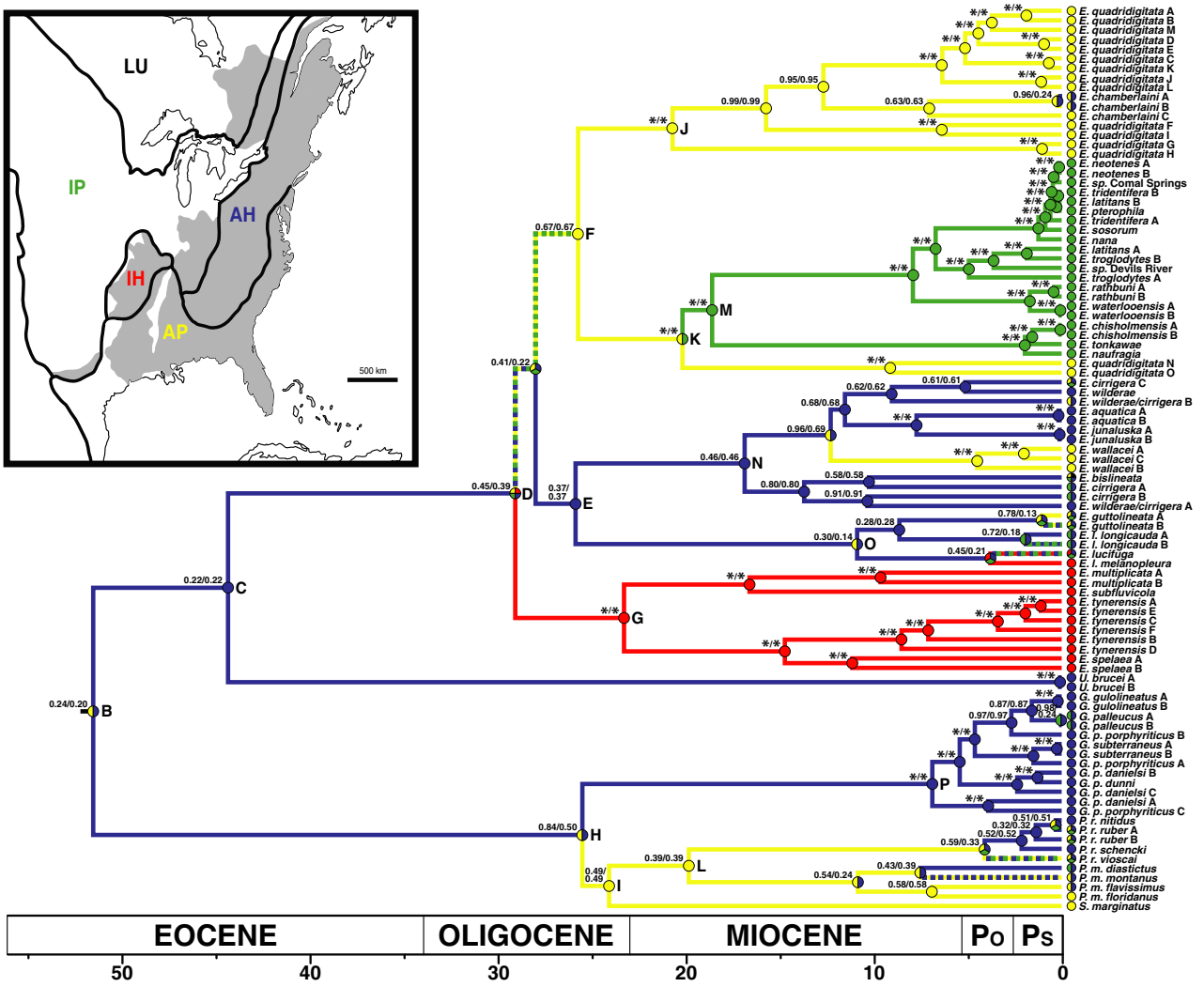


Figure 3 Dispersal-extinction-cladogenesis reconstruction of historical distribution in the Spelerpini under the unequal rates dispersal model using the chronogram from Fig. 2. Map inset from Fig. 1 with physiographical ranges colour-coded. Circles at nodes are coloured based on the most likely ancestral range estimation, not proportions of particular models (e.g. a circle at a node that is half yellow and half red reflects the ancestral range estimation as being AP and IH). Coloured circles in front of tip names represent the modern range of that taxon. Coloured branches reflect the most likely ancestral splits. Values at nodes represent the marginal likelihoods of ancestral state/ancestral splits. Asterisks represent marginal likelihoods of 1.0. Abbreviations on geological time-scale at bottom stand for Pliocene (P_O) and Pleistocene (P_S).

probability of 0.3. All other configurations contain core shifts for various clades that contain clade M.

Finally, we generated a macroevolutionary cohort matrix from our BI chronogram (Fig. 4b). The results suggest moderately strong support for the eastern Edwards Plateau neotenes belonging to a separate evolutionary rate regime, while the western Edwards Plateau neotenes and the highly specialized troglobitic Edwards Plateau neotenes share a regime that is moderately supported. A third, weakly supported regime is shared among the genus *Gyrinophilus* (clade P, Figs 2–3).

DISCUSSION

Our phylogenetic results provide robust evidence that *Eurycea* split into three to four geographically distinct clades (see also Bonett *et al.*, 2014), suggesting a causal relationship between geographical and habitat expansion with diversification. The topological results are consistent with those of other studies (Bonett & Chippindale, 2004; Chippindale *et al.*, 2004; Kozak *et al.*, 2006a, 2009; Bonett *et al.*, 2014), despite some differences in analytical approaches. Bonett *et al.* (2014) used a species tree approach with two mitochondrial genes and one nuclear gene, which requires a complete data matrix (i.e. sequences of the same length and no missing sequences for all taxa). In contrast, by concatenating data, we could include additional OTUs that might lack one or more genes, as well as longer sequences for some taxa, resulting in 95 vs. 67 Spelerpini OTUs, 4 vs. 3 genes, and 4338 vs. 2397 bp compared to Bonett *et al.* (2014). The few, minor topological differences consisted of nodes with

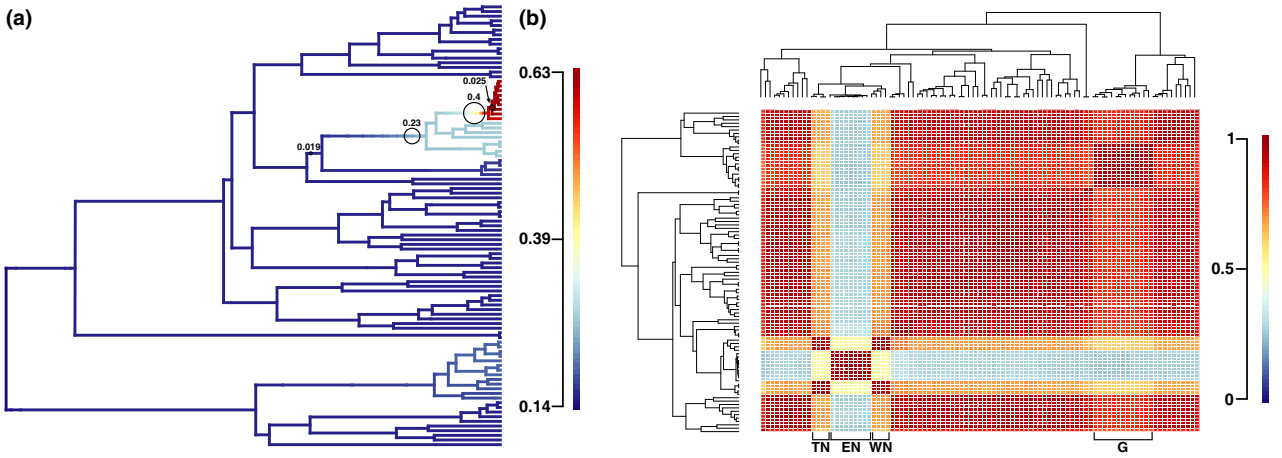


Figure 4 (a). Phylorate plot from BAMM analysis of the Spelerpini showing the 95% credible set of distinct shift configurations. Circles represent core shifts in the credible set, with the size of the circle relative to its posterior probability, which is denoted above (Note: a distinct shift configuration of zero shifts occurred with a pp = 0.3). Scale on right represents speciation rates with warm colours indicating faster rates. (b). Macroevolutionary cohort matrix from BAMM analysis of Spelerpini (EN = Eastern Edwards Plateau clade; G = *Gyrinophilus* clade; TN = Troglobitic Edwards Plateau clade; WN = Western Edwards Plateau clade). Scale on right represents the probability that any pairwise comparison of OTUs on the tree share the same rate regime. There is a strong probability that the EN clade shares a different rate regime than the rest of the Spelerpini, whereas the TN and WN have a moderate probability of having a different rate regime. There is weak support for the Gyrinophilus clade (G) having a different rate regime.

increased support in this study (and other studies, e.g. Kozak *et al.*, 2006a, 2009; e.g. monophyly of *Pseudotriton*) or for nodes that likely represent hard polytomies (e.g. the placement of *E. wallacei* within the *E. bislineata* complex). Interestingly, despite very different approaches to calibrating the chronogram, this study and Bonett *et al.* (2014) estimated nearly identical dates for most nodes, but with 11 of 14 nodes here having narrower 95% HPD CIs (Table 1). The greatest discrepancies tended to be in more recent nodes (J-P), but the means were still well within both study's HPDs.

Although there was not a significant increase in diversification rate at the base of *Eurycea*, there was evidence that *Eurycea* experienced ecological opportunity via expansion into three recently opened physiographical regions, a stark contrast to other Spelerpini genera, that largely remained in the ancestral regions (AH and AP; Fig. 3). The most-recent common ancestor (MRCA) of Spelerpini existed in the AH and AP *c.* 51 Ma (68–38) during the Late Cretaceous/Palaeocene, a time when sea levels were high and only a small portion of the AP along the Mississippi River was exposed (Miller *et al.*, 2005; Appendix S2). Sometime during the Palaeocene/Eocene, an ancestor to *Eurycea* + *Urspelerpes* existed solely in the AH. Beginning in the Palaeocene, sea levels dropped extensively, exposing much of eastern North America (Miller *et al.*, 2005; Appendix S2). These newly exposed regions were lower in elevation and warmer than the ancestral uplands in the AH, providing numerous novel habitats and unoccupied niches. By *c.* 29 Ma (38-22), the MRCA of *Eurycea* was widespread in the AH, AP, IH and IP. In contrast, the other four Spelerpini genera remained in the AH and AP with few exceptions (Fig. 3). Then, the four major lineages of *Eurycea* diversified *in situ*, with only one of these lineages (E, Fig. 3) secondarily colonizing other regions (e.g. *E. lucifuga* and *E. longicauda melanopleura* colonizing IH between 12–5 Ma).

The strongest evidence for a diversification rate increase occurred in *Eurycea* among the Edwards Plateau neotenes. Within our 95% credible set of shift configurations, 67% of the shifts occurred along the lineage from ancestor M to the eastern Edwards Plateau neotenes (Fig. 4a). The cohort analysis revealed a high probability that this eastern Edwards Plateau clade had a distinctive rate regime, separate from the rate regime governing all other Spelerpini, although this probability is slightly lower when compared to the other Edwards Plateau neotene clades (Fig. 4b). There was moderate support for all neotenes having a separate regime from the other Spelerpini. Interestingly, *Gyrinophilus* also was weakly supported for a separate rate regime (Fig. 4b). The Edwards Plateau clade is composed entirely of paedomorphic (neotenic; obtaining sexual maturity while retaining the larval body form) species and the majority of species in *Gyrinophilus* (75%) are also paedomorphic. Bonett *et al.* (2014) showed strong support for the evolution of paedomorphosis in combination with inhospitable environmental conditions. The recent divergence of most of these species coincides with increased aridification of their environment in the Pliocene and Pleistocene, which could have driven the evolution of paedomorphosis and consequently isolation and speciation in these clades, resulting in increased speciation rates. Recent work has called into question the use of BAMM as a viable method to estimate diversification rates (Moore *et al.*, 2016; but see http://bamm-project.org/replication.html for rebuttal). The debate surrounding the use of BAMM is far from resolved, however, given that we used a conservative hyperprior for the number of distinct rate shifts in our analysis

and we failed to detect an increase in diversification rates in the genus *Eurycea* compared to other Spelerpini, we are more likely to have committed a type II error rather than a critical type I error.

The pattern in *Eurycea* is consistent with early shifts in ecology, perhaps associated with a shift in physiological tolerances, which then led to range expansion and subsequent vicariance with *in situ* diversification. An additional expectation of this hypothesis is that ecological shifts within *Eurycea* should correlate with changes in morphology and vary greatly from other Spelerpini. Adams *et al.* (2009) used seven morphological traits to show that among 15 major plethodontid clades, *Eurycea* had the highest rate variation in body size, second highest rate variation in shape and second highest level of morphological disparity. However, there is a need to test whether morphological variation is correlated with habitat differences. Blankers *et al.* (2012) found a significant relationship between microhabitat and morphological variation among plethodontids (including most Spelerpini), although they concluded this might be driven primarily by the Mesoamerican Hemidactyliinae. We agree with the criticism of Blankers *et al.* (2012) that there may be other aspects of microhabitat that are important, but not captured in their categorizations. Nonetheless, the overall pattern suggests that the high level of morphological variation seen in *Eurycea* is due to adaptation to a wide range of habitats.

The Spelerpini fall into two broad categories: fully aquatic and semi-aquatic species. Other major lineages of eastern plethodontids are either terrestrial (*Plethodon*) or aquatic/semi-aquatic (*Desmognathus*), although only five of the 21 species of *Desmognathus* occur outside the AH. Modern *Eurycea* occur in a wide variety of aquatic/semi-aquatic habitats. Many are classic streamside salamanders (e.g. *E. bislineata* and *E. longicauda* complexes), known to utilize different microhabitats within a stream system. For instance, in the south-eastern Coastal Plain, *E. cirrigera* occurs more often in the first and second order streams, while *E. guttolineata* occupies third order streams and floodplains of rivers (Means, 2000). The *E. quadridigitata* complex breeds mostly in lentic environments, such as ponds, swamps and ephemeral wetlands, rather than the lotic systems most other *Eurycea* utilize and are often found far from any body of water outside of the breeding season. Although many species can be found at the mouth of caves and even in the twilight zone, three species (representing two different major lineages) have become cave specialists. The dorsal-ventrally flattened *Eurycea l. melanopleura* and *E. lucifuga* are both troglophiles, often encountered in caves or surrounding rocky outcrops and other broken terrain (Petranka, 1998). *Eurycea spelaea* is a troglobyte with a unique life cycle in which larvae live in surface systems that usually exit from caves, but migrate upstream into the cave systems where they transform into a cave adapted adult (Petranka, 1998).

Three of the four major lineages (Fig. 3) have independently evolved permanently aquatic, neotenic species (Bonett *et al.*, 2014). The Edwards Plateau clade is composed entirely of neotenes, but neotenic forms exist in the IH clade (*E. tynerensis*) and in the *E. bislineata* complex (*E. wallacei*). Several of these neotenic forms (e.g. *E. wallacei* and *E. rathbuni*) represent some of the most extreme examples of cave adapted animals known (Wiens *et al.*, 2003). Such varied ecologies in a single genus represent most of the extremes in the entire family, suggesting that increased evolutionary flexibility in habitat early in the evolution of *Eurycea* led to range expansion and allopatric diversification as the WIS regressed and exposed a variety of novel habitats.

CONCLUSION

Species diversity in *Eurycea* does not fit the classic model of adaptive radiation or the recently popularized pattern of niche conservatism. Instead, it appears that a direct ancestor to *Eurycea* dispersed out of the Appalachian Highlands, the cradle of eastern plethodontid diversity, and into surrounding physiographical regions during the Eocene, *c.* 42 Ma, as sea levels fell and exposed previously uninhabitable lowlands. This early range expansion was most likely the result of a shift in ecology, which facilitated dispersal and colonization. This ecological shift and associated greater geographical range facilitated subsequent ecological and morphological diversification beyond that of their plethodontid relatives.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Samples and primers used
Appendix S2 Sea fluctuations of North America
Appendix S3 LAGRANGE output file.

BIOSKETCHES

Kenneth P. Wray is broadly interested in evolution and the patterns and processes it generates. He is particularly interested in the process of speciation and mechanisms that generate biological diversity. This study was part of his doctoral dissertation research in the Steppan lab at Florida State University.

Scott J. Steppan studies the evolutionary processes that promote biological diversity. His research attempts to bridge micro- and macro-evolutionary scales and apply process-based models to understand and explain large-scale patterns.

Author contributions: K.P.W. designed the study and performed the laboratory work and data analyses. K.P.W. drafted the manuscript, which was approved by S.J.S.

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