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Larval masquerade: a new species of paedomorphic salamander (Caudata: Plethodontidae: *Eurycea*) from the Ouachita Mountains of North America

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Abstract

Species with truncated developmental patterns may go undetected if they resemble the juveniles of their close relatives. Herein we present an example of this phenomenon with the description of a highly divergent, relict species of stream-dwelling plethodontid salamander from the Ouachita Mountains of North America. Both mitochondrial and nuclear sequence data show that this new species is most closely related to its syntopic relative, *Eurycea multiplicata*. Interestingly, *E. multiplicata* exhibits the ancestral biphasic (metamorphic) life cycle, whereas the new species maintains an aquatic larval form throughout life (paedomorphic) and superficially resembles larval *E. multiplicata*. The new species is the first known paedomorphic plethodontid from the Ouachita Mountains, and the most divergent paedomorphic salamander discovered in over seventy years. This species represents an independent instance of the evolution of paedomorphosis associated with a porous streambed, which may facilitate vertical seasonal movements. This new species currently has an extremely limited known distribution and is of immediate conservation concern.

Key words: endemic species, *Eurycea*, life history

Introduction

Developmental truncations can produce species that resemble the larvae or juveniles of close relatives (e.g. Camp *et al.* 2002). However, some ontogenetic stages, such as larval forms, can be difficult to identify morphologically and are often understudied (Thomas *et al.* 2005; Vences *et al.* 2005; Barber & Boyce 2006; Hubert *et al.* 2010; Ko *et al.* 2013). Therefore, without close examination of specific life history stages, species that arise from developmental truncations can go undetected, resulting in an underestimation of biodiversity.

The lungless salamanders, Family Plethodontidae, have diversified extensively since the Late Cretaceous (Vieites *et al.* 2007), and are the most species rich clade of caudates (currently 440 recognized species; AmphibiaWeb 2014). More than 80% of recognized plethodontids have been described since Dunn's (1926) seminal work on the family. This is in part due to increased survey efforts, but also due to the application of molecular techniques, which have revealed many cryptic species in morphologically conserved groups (e.g. Highton 1989; Chippindale *et al.* 2000; Garcia-Paris *et al.* 2000; Jockusch *et al.* 2002). Plethodontids are known to exhibit a wide diversity of life histories and developmental modes (Wake 1966; Chippindale *et al.* 2004; Mueller *et al.* 2004; Bonett *et al.* 2014), but ecologically and developmentally distinct new species are extremely rare, especially within North America, which has been extensively surveyed (Dunn 1926; Highton 1989; Camp *et al.* 2009).

The plethodontid tribe Spelerpini includes five genera (*Eurycea*, *Gyrinophilus*, *Pseudotriton*, *Stereochilus*, and *Urspelperpes*) and thirty-five recognized species. These primarily stream-dwelling salamanders display multiple areas of endemism in the southern Appalachian Mountains, Edwards Plateau, and Interior Highlands (Ouachita Mountains and Ozark Plateau; Fig. 1). While most spelerpines have a biphasic (metamorphic) life history, typical of amphibians, several members of this clade display paedomorphosis (Wake 1966; Ryan & Bruce 2000; Bonett *et*

al. 2014), the phenomenon where adults retain ancestral juvenile characteristics throughout life (e.g. gills, tailfin, larval throat structure; Duellman & Trueb 1986). Paedomorphosis in spelerpines has likely evolved independently in the Ozark Plateau, Edwards Plateau, Cumberland Plateau, and Gulf Coastal Plain (Wake 1966; Bonett & Chippindale 2004; Niemiller *et al.* 2008; Bonett *et al.* 2014). However, despite the Ouachita Mountains being a major biogeographic feature of eastern North America, the only spelerpine known to occur there is the widespread metamorphic *Eurycea multiplicata*.

The taxonomy of *E. multiplicata* and related species (*E. tynerensis* and *E. spelaea*) has been historically misconstrued by life history. Until recently, the alternative life histories of *E. tynerensis* within the Ozarks were considered to be two distinct species. Paedomorphic populations in the Ozarks were classified as *E. tynerensis*, and metamorphic populations were considered to be a subspecies of *E. multiplicata* (*E. m. griseogaster*; Petranka 1998). However, genetic analyses strongly support the monophyly of *E. tynerensis* and *E. m. griseogaster*, and *E. m. multiplicata* from the Ouachita Mountains as sister to the Ozarkian species: *E. tynerensis* (including *E. m. griseogaster*) and *E. spelaea* (Bonett & Chippindale 2004). Therefore, life history is variable within *E. tynerensis* in the Ozarks, but paedomorphosis has never been reported in *E. multiplicata*, in the Ouachita Mountains.

Here we describe a new species of paedomorphic salamander from the Ouachita Mountains. This new species is morphologically, genetically, ecologically, and developmentally distinct from syntopic *Eurycea multiplicata*, with phylogenetic reconstruction placing it as sister to *E. multiplicata*. This discovery represents the most divergent paedomorphic salamander described in the past 70 years (Moore & Hughes 1939; Carr 1939). This new species appears to have an extremely limited distribution and should be of immediate conservation concern.

Material and methods

The first specimen of the new species was discovered on 23-May 2011 (by MAS and KJI), while collecting *Eurycea multiplicata* at Lake Catherine State Park, Hot Spring County, Arkansas, USA (Fig. 1). This specimen was originally identified as highly distinct from *E. multiplicata*, based on sequence data, and the presence of very small immature egg follicles in a relatively large larval specimen. The original sampling area and nearby vicinity were searched several times in 2011 and 2012. It was not until February 2013 that additional specimens were found in the original location and an adjacent stream. These specimens allowed us to confirm that the new species is paedomorphic, and based on putative morphological characteristics was easily distinguishable from syntopic larvae of metamorphic *Eurycea multiplicata*. Our description is based upon genetic comparisons to representative samples of *E. multiplicata* from throughout the Ouachita Mountains (as well as outgroups; Table 1 & 2), and morphological comparisons to syntopic larvae of *E. multiplicata*. The species description is supported under the General Lineage, Phylogenetic, and Biological Species Concepts (Mayr 1942; de Queiroz 2007).

Morphological Analyses. Twenty-four specimens of the new species and 12 specimens of syntopic *E. multiplicata* larvae were collected from 25-March to 20-April 2013, and returned alive to the University of Tulsa. Specimens were anesthetized in a 0.1% solution of tricaine methanesulfonate (MS-222) for morphometric measurements. Specimens were handled in accordance with Institutional Animal Care and Use Committee (IACUC; TU-0029).

Dorsal and ventral images of the body and head were taken with a Pentax K-7 (14.6 megapixel), or Canon G9 (12.1 megapixel) camera. ImageJ (Abramoff *et al.* 2004) was used to obtain the following measurements: snout-vent length (SVL), trunk length (TL), head length (HL), head width (HW), interorbital distance (ID), eye to nostril distance (EN), eye diameter (ED), trunk width posterior to forelimbs (TRW) and tail width posterior to hindlimbs (TW). All standard morphological measurements were made to the nearest 0.1mm. Analysis of Covariance (ANCOVA) in SPSS v17, with SVL as the covariate, were used to test if any of the measurements were significantly different between the new species and syntopic larval *E. multiplicata*. Several specimens were fixed in 10% buffered formalin, including the holotype, and a subset were cleared and stained with alcian blue and alizarin red for cartilage and bone respectively (Hanken & Wassersug 1981). The osteology of seven specimens of the new species were compared to ten *E. multiplicata* larvae.

For geometric morphometrics, a series of 11 landmarks on body images, and eight landmarks and 35 semi-landmarks on head images were used to capture the shape of each specimen (Fig. 2). Images were rescaled and superimposed using procrustes superimposition on CoodGen7a (Sheets 2003). Head and body shape were then

analyzed using principle component analysis (PCA) with the program PCAGen7a (Sheets, 2003). We also tested for significant morphological shape variation between the two species, employing the Goodall's F-test in TwoGroup7 (Sheets 2003). To account for potential differences in ontogeny (size), we also compared eight of the smallest specimens of the new species to the *E. multiplicata* larvae. We realize that we are still comparing juvenile larvae to subadults (or small adults) of the new species, but to date, juvenile specimens of the new species have not been found.

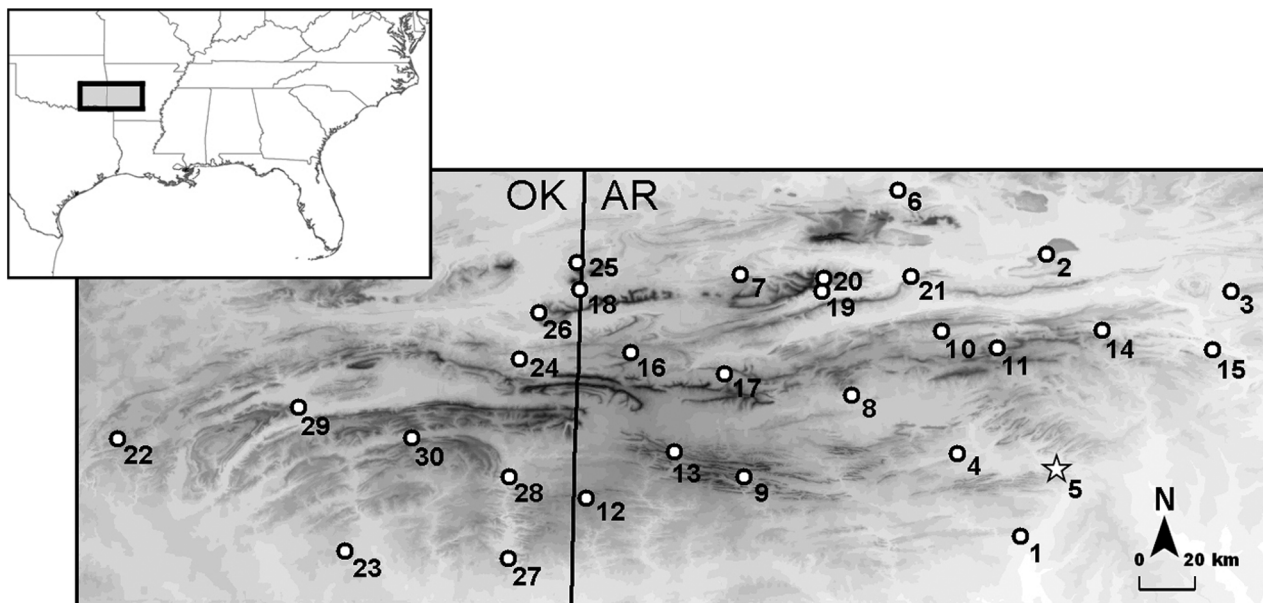


FIGURE 1. Distribution map of sampling localities across the Ouachita Mountains. The open star indicates the type locality and only known site of *E. subfluvicola*, where *E. multiplicata* were also sampled. Open circles indicate other genetic sampling localities for *E. multiplicata*. The numbers correspond with those in Table 1. Shaded box on inset map of the southeastern United States shows location of the Ouachita Mountains. AR=Arkansas and OK=Oklahoma.

DNA extraction, PCR, sequencing, and phylogenetic analyses. Genomic DNA was extracted from tail tips or liver tissue using QIAGEN DNeasy extraction kits. Portions of the mitochondrial gene *cytochrome b* (*Cytb*), and the nuclear recombination-activating gene 1 (*Rag1*), were amplified via polymerase chain reaction (PCR) using gene specific primers and standard conditions (Arévalo *et al.* 1994; Moritz *et al.* 1992; Timpe *et al.* 2009). Successful PCR products were cleaned using EXOSAP-IT (USB Corp.). Sequencing reactions were performed using BigDye v3.1 (Applied Biosystems Inc.), unincorporated dye terminators were removed with Sephadex G-50 (Sigma). Reaction products were sequenced in both directions on an ABI 3130xl capillary sequencer. For *Cytb*, we sequenced 19 individuals of the new species, and 40 individuals of *Eurycea multiplicata*, representing the breadth of genetic diversity of this taxon across the Ouachita Mountains (Bonett & Chippindale 2004; Steffen & Bonett in prep.). Thirteen *E. multiplicata* were from Lake Catherine State Park, 12 of which were collected syntopically with the new species (Table 1). For the more conserved gene *Rag1* we sequenced 11 specimens of the new species and 17 *E. multiplicata* from across the Ouachita Mountains (four of the *E. multiplicata Rag1* sequences were from Lake Catherine State Park).

Additional *E. multiplicata* and outgroup sequences were taken from Genbank (Table 1). Outgroups included other representatives from the “Interior Highland clade” (*E. tynerensis* and *E. spelaea*), all other major lineages of *Eurycea* (*E. bislineata*, *E. longicauda*, *E. neotenes*, and *E. quadridigitata*), and a distant spelerpine outgroup (*Pseudotriton ruber*; Bonett & Chippindale 2004; Bonett *et al.* 2014). Sequences were edited and aligned using Sequencher v4.8 (Gene Codes, Ann Arbor, MI). Both genes are protein coding and contained no stop codons or insertions/deletions. We trimmed the alignments to 880bp for *Cytb* and 1012bp for *Rag1*. The sequences for all taxa were complete, with no missing data.

The most appropriate model of nucleotide substitution for each codon position for each gene was chosen using jModelTest (Guindon & Gascuel 2003; Darriba *et al.* 2012; Table 3). Bayesian phylogenetic analyses were then performed using MrBayes v3.2 (Ronquist & Huelsenbeck 2003). We ran two replicate searches using four chains

each (three hot, one cold) for 15 million generations, sampling every 1000 generations, and assessed support with Bayesian posterior probabilities (BAPP). We assessed stationarity using Tracer v1.5 (Rambaut & Drummond 2007), and the first 3750 trees (25% of generations) were discarded as burn-in; well beyond stationarity. Uncorrected pairwise sequence divergences for *Cytb* and *Rag1* were determined using Mega v5.1 (Tamura *et al.* 2011).

TABLE 1. Voucher specimens, localities, and GenBank numbers for Bayesian analysis. Localities are listed as locality number: state abbreviation, county, other descriptive locality information. CG=Campground, Crk=Creek, E=East, N=North, S=South, SP=State Park, W=West, WMA=Wildlife Management Area.

Species		Locality #: Location	<i>Cytb</i>	<i>Rag1</i>
<i>Eurycea multiplicata</i>	MAS 0187	1: AR, Clark, AR, N of Caddo Valley	KJ372351	---
<i>Eurycea multiplicata</i>	RMB 2153	2: AR, Conway, AR, Rose Crk	KJ372393	KJ372328
<i>Eurycea multiplicata</i>	MAS 0226	3: AR, Faulkner, AR, Camp Robinson	KJ372352	KJ372319
<i>Eurycea multiplicata</i>	RMB 2607	4: AR, Garland, AR, Mazarn Crk	KJ372389	---
<i>Eurycea multiplicata</i>	MAS 0007	5: AR, Hot Spring, Lake Catherine SP	KJ372344	KJ372309
<i>Eurycea subfluvicola</i>	MAS 0232	5: AR, Hot Spring, Lake Catherine SP	KJ372376	KJ372314
<i>Eurycea multiplicata</i>	MAS 0233	5: AR, Hot Spring, Lake Catherine SP	KJ372353	KJ372308
<i>Eurycea multiplicata</i>	MAS 0234	5: AR, Hot Spring, Lake Catherine SP	KJ372354	---
<i>Eurycea multiplicata</i>	MAS 0447	5: AR, Hot Spring, Lake Catherine SP	KJ372359	---
<i>Eurycea multiplicata</i>	MAS 0448	5: AR, Hot Spring, Lake Catherine SP	KJ372360	---
<i>Eurycea multiplicata</i>	MAS 0449	5: AR, Hot Spring, Lake Catherine SP	KJ372361	---
<i>Eurycea multiplicata</i>	MAS 0450	5: AR, Hot Spring, Lake Catherine SP	KJ372362	---
<i>Eurycea multiplicata</i>	MAS 0451	5: AR, Hot Spring, Lake Catherine SP	KJ372363	---
<i>Eurycea multiplicata</i>	MAS 0452	5: AR, Hot Spring, Lake Catherine SP	KJ372364	---
<i>Eurycea multiplicata</i>	MAS 0819	5: AR, Hot Spring, Lake Catherine SP	KJ372366	KJ372317
<i>Eurycea multiplicata</i>	MAS 0820	5: AR, Hot Spring, Lake Catherine SP	KJ372367	---
<i>Eurycea multiplicata</i>	MAS 0821	5: AR, Hot Spring, Lake Catherine SP	KJ372368	---
<i>Eurycea subfluvicola</i>	MAS 0823	5: AR, Hot Spring, Lake Catherine SP	KJ372369	---
<i>Eurycea multiplicata</i>	MAS 0824	5: AR, Hot Spring, Lake Catherine SP	KJ372370	KJ372324
<i>Eurycea multiplicata</i>	MAS 0825	5: AR, Hot Spring, Lake Catherine SP	KJ372371	KJ372332
<i>Eurycea subfluvicola</i>	MAS 0826	5: AR, Hot Spring, Lake Catherine SP	KJ372372	KJ372331
<i>Eurycea subfluvicola</i>	MAS 0827	5: AR, Hot Spring, Lake Catherine SP	KJ372373	---
<i>Eurycea subfluvicola</i>	MAS 0828	5: AR, Hot Spring, Lake Catherine SP	KJ372374	KJ372313
<i>Eurycea subfluvicola</i>	MAS 0829	5: AR, Hot Spring, Lake Catherine SP	KJ372375	KJ372333
<i>Eurycea subfluvicola</i>	MAS 0830	5: AR, Hot Spring, Lake Catherine SP	KJ372377	KJ372334
<i>Eurycea subfluvicola</i>	MAS 0831	5: AR, Hot Spring, Lake Catherine SP	KJ372378	---
<i>Eurycea subfluvicola</i>	MAS 0832	5: AR, Hot Spring, Lake Catherine SP	KJ372379	KJ372330
<i>Eurycea subfluvicola</i>	MAS 0834	5: AR, Hot Spring, Lake Catherine SP	KJ372388	KJ372335
<i>Eurycea subfluvicola</i>	MAS 0835	5: AR, Hot Spring, Lake Catherine SP	KJ372380	---
<i>Eurycea subfluvicola</i>	MAS 0837	5: AR, Hot Spring, Lake Catherine SP	KJ372381	KJ372312
<i>Eurycea subfluvicola</i>	MAS 0838	5: AR, Hot Spring, Lake Catherine SP	KJ372382	KJ372322
<i>Eurycea subfluvicola</i>	MAS 0839	5: AR, Hot Spring, Lake Catherine SP	KJ372383	---
<i>Eurycea subfluvicola</i>	MAS 0841	5: AR, Hot Spring, Lake Catherine SP	KJ372384	---
<i>Eurycea subfluvicola</i>	MAS 0845	5: AR, Hot Spring, Lake Catherine SP	KJ372385	---
<i>Eurycea subfluvicola</i>	MAS 0846	5: AR, Hot Spring, Lake Catherine SP	KJ372386	---
<i>Eurycea subfluvicola</i>	MAS 0847	5: AR, Hot Spring, Lake Catherine SP	KJ372387	KJ372321
<i>Eurycea multiplicata</i>	MAS 0003	6 : AR, Logan, Shoal Bay CG	KJ372336	KJ372311
<i>Eurycea multiplicata</i>	MAS 0005	7 : AR, Logan, S of Golden City	KJ372337	KJ372310
<i>Eurycea multiplicata</i>	MAS 0420	8 : AR, Montgomery, W of Washita	KJ372358	---

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TABLE 1. (Continued)

Species		Locality #: Location	<i>Cytb</i>	<i>Rag1</i>
<i>Eurycea multiplicata</i>	RMB 2613	9 : AR, Montgomery, Long Crk	KJ372394	KJ372326
<i>Eurycea multiplicata</i>	MAS 0176	10 : AR, Perry, E of Steve	KJ372350	KJ372318
<i>Eurycea multiplicata</i>	MAS 0023	11 : AR, Perry, Garland county border	KJ372346	---
<i>Eurycea multiplicata</i>	UTA_56355	12 : AR, Polk, Bogg Springs	AY528334	---
<i>Eurycea multiplicata</i>	MAS 0067	13 : AR, Polk, NW of Wolf Pen Gap	KJ372349	---
<i>Eurycea multiplicata</i>	MAS 0252	14 : AR, Perry, S of Williams Junction	KJ372356	---
<i>Eurycea multiplicata</i>	RMB 3013	15 : AR, Pulaski, Little Rock	KJ372392	---
<i>Eurycea multiplicata</i>	MAS 0016	16 : AR, Scott, SW of Beauchamp	KJ372338	KJ372315
<i>Eurycea multiplicata</i>	MAS 0017	17 : AR, Scott, Turner Crk	KJ372339	KJ372320
<i>Eurycea multiplicata</i>	MAS 0414	18 : AR, Sebastian	KJ372357	---
<i>Eurycea multiplicata</i>	MAS 0019	19: AR, Yell, W of Waltreak	KJ372340	KJ372323
<i>Eurycea multiplicata</i>	MAS 0090	20: AR, Yell, NW of Waltreak	KJ372343	KJ372316
<i>Eurycea multiplicata</i>	MAS 0020	21: AR, Yell, SE of Danville	KJ372345	---
<i>Eurycea multiplicata</i>	UTA_56369	22: OK, Atoka, Atoka WMA	AY528332	---
<i>Eurycea multiplicata</i>	MAS 0086	23: OK, Choctow, E of Sobel	KJ372342	KJ372327
<i>Eurycea multiplicata</i>	MAS 0057	24: OK, Leflore, NW of Zoe	KJ372348	---
<i>Eurycea multiplicata</i>	RMB 2866	25: OK, Leflore, Gap Crk	KJ372390	---
<i>Eurycea multiplicata</i>	RMB 2867	26: OK, Leflore, Morris Crk	KJ372391	---
<i>Eurycea multiplicata</i>	MAS 0239	27: OK, McCurtain, Beavers Bend SP	KJ372355	---
<i>Eurycea multiplicata</i>	MAS 0463	28: OK, McCurtain, N Broken Bow Lake	KJ372365	---
<i>Eurycea multiplicata</i>	MAS 0037	29: OK, Pushmataha, Jack fork Crk	KJ372341	KJ372329
<i>Eurycea multiplicata</i>	MAS 0044	30: OK, Pushmataha, Honobia Crk WMA	KJ372347	KJ372325
Outgroups				
<i>Eurycea bislineata</i>	UTA_56411	NY	AY528402	EU275784
<i>Eurycea longicauda</i>	CM_147803	PA	AY528403	AY650121
<i>Eurycea neotones</i>	TNHC_60313	TX	AY528400	AY650122
<i>Eurycea spelaea</i>	UTA_56362	AR, Boone, Alcohol Springs	AY528390	KF562666
<i>Eurycea spelaea</i>	UTA_56364	MO, Barry, Rockhouse Cave	AY528393	---
<i>Eurycea spelaea</i>	UTA_56365	MO, Pulaski, Piquet Cave	AY528395	---
<i>Eurycea tynerensis</i>	UTA_56372	AR, Conway, Cypress Creek	AY528345	---
<i>Eurycea tynerensis</i>	UTA_56399	OK, Sequoyah, Near Cookson	AY528367	---
<i>Eurycea tynerensis</i>	UTA_56404	OK, Sequoyah, Little Lee Crk	AY528387	---
<i>Eurycea tynerensis</i>	UTA_53860	OK, Cherokee, Camp Egan	---	KF562675
<i>Eurycea quadradigita</i>	UTA_56412	TX	AY528401	---
<i>Pseudotriton ruber</i>	ASB 130	NY: Sullivan, Adams Road	AY528404	---
<i>Pseudotriton ruber</i>	RMB 3705	NC, Graham, Rattler Ford Campground	---	KF562700

To estimate relative divergence dates, we used the spelerpine dataset of Bonett *et al.* (2014), which used two unlinked genes (mtDNA and *Rag1*) for 75 taxa; including outgroups. To avoid missing data between taxa, we sequenced a portion of the mitochondrial gene NADH dehydrogenase subunit 4 (*Nd4*) for one specimen of the new species, and trimmed each gene alignment (*Cytb*=589, *Nd4*=630, and *Rag1*=1012; Table 2). The best-fitting models of nucleotide substitution were determined using jModelTest (Table 3). We followed the methods of Bonett *et al.* (2014) and the *BEAST function (Heled & Drummond 2010) in BEAST v1.7.5 (Drummond *et al.* 2012). *BEAST analyses were run three times independently for 20 million generations. Stationarity of likelihood values was evaluated using Tracer v1.5 (Rambaut & Drummond 2007) and the first 25% of generations were discarded from each run and remaining generations were combined using LogCombiner v1.7.5.

TABLE 2. Voucher specimens, localities, and GenBank numbers. Localities are listed as state abbreviation: county, other descriptive locality information. Cv.= Cave, Crk.=Creek, R.= River, Sprg.=Spring, SF=State Forest, SP=State Park, NF=National Forest.

Species	Location	Cytb	Nd4	Ragl
<i>Spelerpini</i>				
<i>Eurycea aquatica</i>	GA: Chattooga, Moseley Sprg.	KF562543	KF562594	KF562645
<i>Eurycea bislineata</i>	NY: Chenango, Geneganslet R.	AY528402	AY528327	AY691606
<i>Eurycea chamberlaini</i>	NC: Pitt, Greenville, Elm Street Park	KF562544	KF562595	KF562646
<i>Eurycea chisholmensis</i>	TX: Bell, Salado (main) Sprgs.	KF562545	KF562596	KF562647
<i>Eurycea cirrigera</i> 1	FL	KF562546	KF562597	KF562648
<i>Eurycea cirrigera</i> 2	GA: Polk	KF562547	KF562598	KF562649
<i>Eurycea cirrigera</i> 3	TN: Cannon, Woodbury	KF562548	KF562599	KF562650
<i>Eurycea guttolineata</i>	SC: Berkeley, Francis Marion NF	KF562549	KF562600	KF562651
<i>Eurycea junaluska</i>	TN: Sevier, Little R.	KF562550	KF562601	FJ750246
<i>Eurycea latitans</i>	TX: Kendall, Cascade Caverns	KF562551	KF562602	KF562652
<i>Eurycea l. longicauda</i>	PA: Washington	AY650121	AY528328	AY528403
<i>Eurycea l. melanopleura</i>	AR: Lawrence, Bubbling Sprg.	KF562552	KF562603	KF562653
<i>Eurycea lucifuga</i>	AR: Independence, Cushman Cv.	KF562553	KF562604	KF562654
<i>Eurycea multiplicata</i> 1	AR: Saline, Williams Crk.	AY528339	AY528264	AY691707
<i>Eurycea multiplicata</i> 2	OK: Choctaw, near Ft. Towson	AY528330	AY528255	KF562655
<i>Eurycea nana</i>	TX: Hays: San Marcos Sprg.	KF562554	KF562605	KF562656
<i>Eurycea naufragia</i>	TX: Williamson, Swinbank Sprg.	KF562555	KF562606	KF562657
<i>Eurycea neotenes</i>	TX: Bexar, Helotes Sprg.	AY528400	AY528325	AY650122
<i>Eurycea pteropila</i>	TX: Hays, Sycamore Sprg.	KF562556	KF562607	KF562658
<i>Eurycea quadridigitata</i> 1	AL	KF562557	KF562608	KF562659
<i>Eurycea quadridigitata</i> 2	AR: Calhoun, Hurricane Crk.	KF562558	KF562609	KF562660
<i>Eurycea quadridigitata</i> 3	LA: Washington, Stubbs Crk.	KF562559	KF562610	KF562661
<i>Eurycea quadridigitata</i> 4	NC	KF562560	KF562611	KF562662
<i>Eurycea quadridigitata</i> 5	TX: Tyler	AY528401	AY528326	AY691708
<i>Eurycea sosorum</i>	TX: Hays, Rattlesnake Cv..	KF562561	KF562612	KF562663
<i>Eurycea</i> sp. Devil's River	TX: Travis: Barton Sprg., Eliza outlet	KF562562	KF562613	KF562664
<i>Eurycea spelaea</i> 1	TX: Val Verde, Finnegan Sprg.	KF562563	KF562614	KF562665
<i>Eurycea spelaea</i> 2	AR: Boone, Alcohol Sprg.	AY528390	AY528315	KF562666
<i>Eurycea spelaea</i> 3	OK: Mayes, Pipe Sprg.	AY528397	AY528322	KF562667
<i>Eurycea subfluvicola</i>	AR: Hot Spring, Lake Catherine SP	KJ372376	KJ372307	KJ372314
<i>Eurycea tonkawae</i>	TX: Travis, Stillhouse Hollow Sprg.	KF562564	KF562615	KF562668
<i>Eurycea tridentifera</i>	TX: Comal, Honey Creek Cv.	KF562565	KF562616	KF562669
<i>Eurycea troglodytes</i> 1	TX: Bandera, Sutherland Hollow	KF562567	KF562618	KF562671
<i>Eurycea troglodytes</i> 2	TX: Bandera, Sabinal River Sprg.	KF562566	KF562617	KF562670
<i>Eurycea troglodytes</i> 3	TX: Kerr, Fessenden Sprg.	KF562568	KF562619	KF562672
<i>Eurycea tynerensis</i> 1	AR: White, Little Crk.	AY528348	AY528273	KF562673

.....continued on the next page

TABLE 2. (Continued)

Species	Location	Cyrb	Nd4	Ragl
<i>Eurycea tynerensis</i> 2	MO: Christian, Buseik SF	AY528385	AY528310	KF562674
<i>Eurycea tynerensis</i> 3	OK: Cherokee, Rock Crk.	AY528374	AY528299	KF562675
<i>Eurycea tynerensis</i> 4	OK: Cherokee, Cookson	AY528367	AY528292	KF562676
<i>Eurycea tynerensis</i> 5	OK: Sequoyah, Tin Cup Crk.	AY528368	AY528293	KF562677
<i>Eurycea tynerensis</i> 6	OK: Sequoyah, Little Lee Crk.	AY528387	AY528312	KF562678
<i>Eurycea wallacei</i>	FL: Jackson	KF562583	KF562634	KF562693
<i>Eurycea waterloensis</i>	TX: Travis: Barton Sprng.	KF562569	KF562620	KF562679
<i>Eurycea wilderae</i>	GA: Union, Wolf Crk.	KF562570	KF562621	KF562680
<i>Gyrinophilus gulolineatus</i>	TN: Knox, Christian Cv.	KF562571	KF562622	KF562681
<i>Gyrinophilus p. pallidus</i>	TN: Franklin, Custard Hollow Cv.	KF562574	KF562625	KF562684
<i>Gyrinophilus p. necturoides</i> 1	TN: Coffee, Blowing Spr Cv.	KF562572	KF562623	KF562682
<i>Gyrinophilus p. necturoides</i> 2	TN: Coffee, Lust Cv.	KF562573	KF562624	KF562683
<i>Gyrinophilus p. danielsi</i> 1	NC: Yancey, Black Mt. Campground	KF562576	KF562627	KF562686
<i>Gyrinophilus p. danielsi</i> 2	NC: Wilkes	KF562577	KF562628	KF562687
<i>Gyrinophilus p. damni</i>	SC: Aconi, Winding Stairs Road	KF562578	KF562629	KF562688
<i>Gyrinophilus p. porphyriticus</i> 1	AL	KF562575	KF562626	KF562685
<i>Gyrinophilus p. porphyriticus</i> 2	TN: Knox, Cruze Cv.	KF562579	KF562630	KF562689
<i>Gyrinophilus p. porphyriticus</i> 3	TN: Knox, Mudflats Cv.	KF562580	KF562631	KF562690
<i>Gyrinophilus p. porphyriticus</i> 4	TN: DeKalb Co., Pauley Cv.	KF562581	KF562632	KF562691
<i>Gyrinophilus p. porphyriticus</i> 5	WV: Greenbrier, General Davis Cv.	KF562582	KF562633	KF562692
<i>Gyrinophilus p. porphyriticus</i> 6	KY: Lincoln	KF562584	KF562635	KF562694
<i>Pseudotriton m. diastictus</i>	AL: Macon, Tuskegee NF	KF562585	KF562636	KF562695
<i>Pseudotriton m. flavissimus</i>	NC: Wake, Falls of the Neuse	KF562586	KF562637	KF562696
<i>Pseudotriton m. montanus</i>	GA: Emmanuel	KF562587	KF562638	KF562697
<i>Pseudotriton r. nitidus</i> 1	NC: Watauga	KF562588	KF562639	KF562698
<i>Pseudotriton r. nitidus</i> 2	NC: Graham, Rattler Ford Campground	KF562590	KF562641	KF562700
<i>Pseudotriton r. schencki</i>	NY: Sullivan, Adams Road	AY528404	AY528329	AY650123
<i>Pseudotriton r. ruber</i> 1	TN: Cumberland, Staples Sprng. Breh.	KF562589	KF562640	KF562699
<i>Pseudotriton r. ruber</i> 2	AL	KF562591	KF562642	KF562701
<i>Pseudotriton r. vioscai</i> 1	MS: Clay, Shimburne Crk.	KF562592	KF562643	KF562702
<i>Pseudotriton r. vioscai</i> 2	SC: Charleston, Santee Coastal Res.	AY691753	AY691797	AY691713
<i>Stereochilus marginatus</i>	GA: Stephens, Panther Crk.	KF562593	KF562644	KF562703
<i>Ursperpes brucei</i>				
Outgroups				
<i>Aneides aeneus</i>	WV: Kanawha, Kanawha SF	AY691742	AY691786	AY691701
<i>Bolitoglossa helmrichi</i>	Guatemala	AY691755	AY691799	AY650124
<i>Desmognathus brimleyorum</i>	AR: Garland, Cearly Crk.	AY691737	AY691781	AY691697
<i>Ensatina eschscholtzii</i>	OR: Curry	AY691743	AY691787	AY691702
<i>Hemidactylium scutatum</i>	AR: Saline, Williams Crk.	AY691751	AY691795	AY691711
<i>Plethodon cinereus</i>	NC: Caldwell, Dixon Crk.	AY691745	AY691789	AY691703
<i>Plethodon vehicular</i>	WA: Lewis, Trib. of Tilton R.	AY691760	AY691804	AY691716
<i>Amphiuma means</i>	FL: Levy, Gulf Hammock	AY691722	AY691766	AY650127

TABLE 3. Models applied to each data partition for Bayesian phylogenetic analyses and divergence time estimation.

Gene/partition-MrBayes-Bayesian Inference	# of positions in partition	Model	rates
<i>Cytb</i> : alignment pos 1, codon pos 2	294	HKY	propinv
<i>Cytb</i> : alignment pos 2, codon pos 3	293	GTR	gamma
<i>Cytb</i> : alignment pos 3, codon pos 1	293	GTR	invgamma
<i>Rag1</i> : alignment pos 1, codon pos 1	338	HKY	propinv
<i>Rag1</i> : alignment pos 2, codon pos 3	337	HKY	gamma
<i>Rag1</i> : alignment pos 3, codon pos 2	337	HKY	propinv
Gene/partition-*BEAST-Divergence chronogram			
<i>Cytb</i> : alignment pos 1, codon pos 2	197	HKY	gamma
<i>Cytb</i> : alignment pos 2, codon pos 3	196	GTR	gamma
<i>Cytb</i> : alignment pos 3, codon pos 1	196	SYM	gamma
<i>Nd4</i> : alignment pos 1, codon pos 1	210	HKY	invgamma
<i>Nd4</i> : alignment pos 2, codon pos 2	210	GTR	invgamma
<i>Nd4</i> : alignment pos 3, codon pos 3	210	GTR	gamma
<i>Rag1</i> : alignment pos 1, codon pos 1	338	GTR	gamma
<i>Rag1</i> : alignment pos 2, codon pos 3	337	HKY	propinv
<i>Rag1</i> : alignment pos 3, codon pos 2	337	GTR	gamma

Fragment lengths for MrBayes analyses: *Cytb* = 880bp, *Rag1* = 1012bp

Fragment lengths for *BEAST analysis: *Cytb* = 589bp, *Nd4* = 630bp, *Rag1* = 1012bp; Total = 2,231

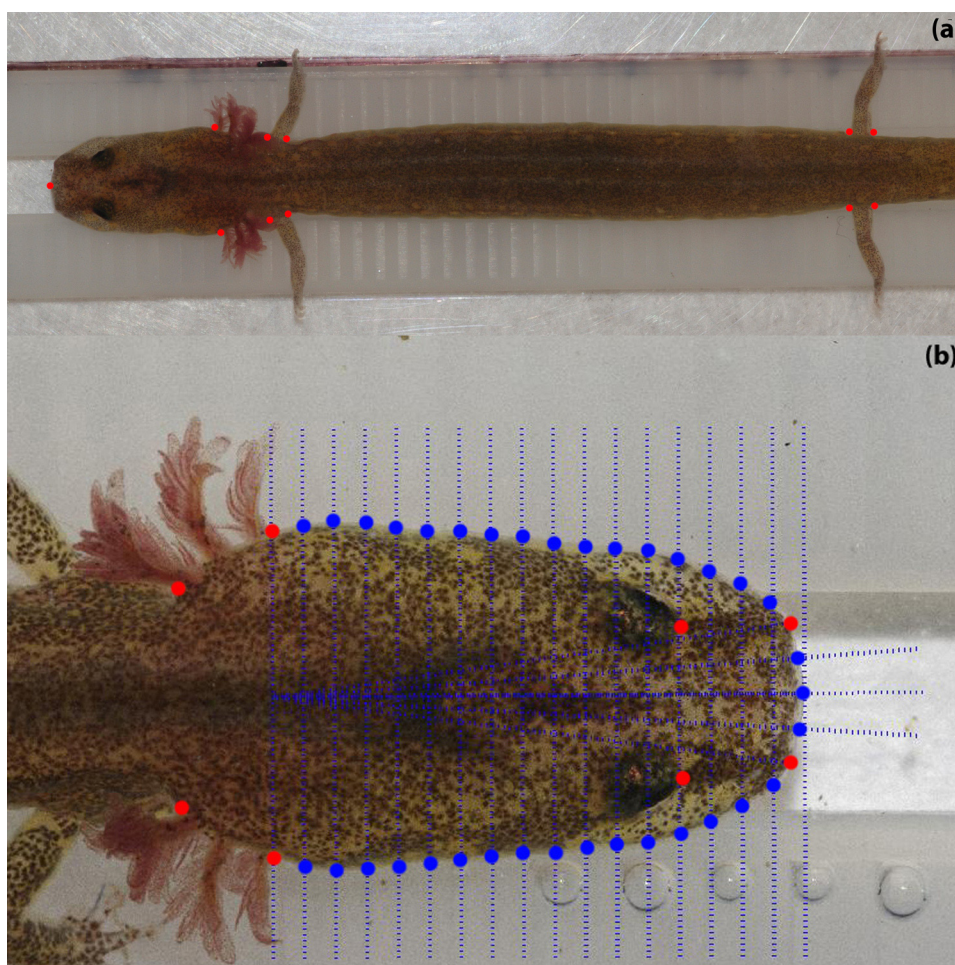


FIGURE 2. Position of landmarks (red) and semilandmarks (blue) used in Principle Component Analysis (PCA) and the Goodall's F test for body (a) and head (b) data. The fan and comb feature in MakeFan7 are also shown to demonstrate how semilandmarks were selected.

Results

Eurycea subfluvicola sp. nov.

Ouachita Streambed Salamander

Holotype. The holotype is an adult female collected by MAS and KJI on 25-March 2013 and deposited at the Museum of Vertebrate Zoology, University of California, Berkeley (MVZ 269485; Fig. 3). Detailed measurements (mm) post preservation is listed in Table 4. Though slightly faded in preservation, the holotype is similar in coloration and pattern as described in the species diagnosis. The holotype contains nine enlarged oviductal eggs that are visible through the venter.

TABLE 4. Detailed measurements (mm) of *Eurycea subfluvicola* holotype.

Total Length	67.9
Standard Length (SVL)	42.0
Head length (snout to gular fold)	5.1
Head depth at posterior angle of jaw	1.8
Head width	5.0
Eye to nostril (left)	1.5
Eye diameter (horizontal)	0.8
Interorbital distance	1.4
Snout to forelimb insertion	9.5
Trunk Length	27.5
Width at mid-body	5.5
Depth at mid-body	4.2
Tail length	26.0
Tail width at base	3.4
Tail depth at base	3.0
Forelimb length to tip of longest digit (left)	4.6
Hindlimb length to tip of longest digit (left)	5.4

Paratypes. Paratypes are one formalin fixed specimen (AMNH A191438) and seven cleared and stained specimens deposited at the American Museum of Natural History in New York City, New York (AMNH A191439-A191441) and the Museum of Vertebrate Zoology (MVZ 269485-269489).

Type locality. A unnamed first order tributary of Slunger Creek, located in the Trap Mountains, a subdivision in the southeastern portion of the Ouachita Mountain physiographic province, Hot Spring County, Arkansas, USA. This locality is within the confines of Lake Catherine State Park.

Etymology. The specific name, *subfluvicola*, is an adjective derived from the Latin prefix, *sub-* meaning “below”, *fluvius* which is “a stream”, and *colo* meaning “to dwell.” The translation then is “dwells below the stream”, in reference to its existence below the streambed during xeric conditions.

Diagnosis. This species is assigned to the genus *Eurycea* based on strong molecular phylogenetic evidence, and overall similarity to other larval *Eurycea*. The genus *Eurycea* is highly heteromorphic, with no single character to diagnosis it from other spelerpine genera (Wake 1966; Camp et al. 2009; Adams et al. 2009). However, members are consistently monophyletic in molecular phylogenetic studies (Camp et al. 2009; Bonett et al. 2014).

The dorsum of *Eurycea subfluvicola* is primarily uniform amber/yellow background color, pigmented with numerous dark brown melanophores, which create irregularly shaped blotches throughout the dorsum and flanks (Fig. 3). In most individuals irregularly spaced spots are formed by the absence of melanophores along the dorso-lateral region of the trunk, possibly indicative of the lateral line. The semi-transparent venter is unpigmented, except for a few widely dispersed melanophores beneath the tail. Dorsal and ventral coloration is separated by a sharply defined ventral-lateral boundary along the trunk.



FIGURE 3. Dorsal and ventral images of the female holotype (MVZ 269485) of *Eurycea subfluvicola*. Preserved October 2013.

Morphological characteristics that distinguish *Eurycea subfluvicola* from syntopic *E. multiplicata* larvae are: 1) *Eurycea subfluvicola* is paedomorphic (i.e. presence of mature testes and ova, and deposition of fertilized eggs while in the larval form) and, based on our samples, is sexually mature by at least 32mm SVL. *Eurycea multiplicata* are never known to be paedomorphic and typically metamorphose between 24 to 41mm SVL. 2) Head and body shape differ significantly between the two species. PCAs show only minimal overlap in morphospace between body shape, and no overlap in head shape (Fig. 4). Additionally, the Goodall's F-test on geometric morphometric data was highly significant for heads, and bodies ($F_{82,2706} = 35.85$, $p < 0.001$ and $F_{18,630} = 13.60$, $p < 0.001$). The same pattern was also seen when comparing only small *E. subfluvicola* (<40mm SVL) to *E. multiplicata* larvae ($F_{82,1558} = 25.53$, $p < 0.001$, and $F_{18,324} = 4.94$, $p < 0.001$). 3) Relative to SVL, *E. subfluvicola* has a longer trunk, and a shorter and narrower head compared to *E. multiplicata* larvae (Table 5). This may be driven by the fact that *E. subfluvicola* has a very long trunk relative to its head, but these differences are not significant. 4) In profile, *Eurycea subfluvicola* has a flat head and longer snout (ANCOVA, $p < 0.001$; Table 5) with relatively smaller in diameter and depressed eyes, compared to *E. multiplicata* larvae (ANCOVA, $p < 0.05$; Fig. 5, Table 5). 5) Individuals of *E. subfluvicola* are more attenuate in body form than *E. multiplicata* larvae, having smaller diameter trunks and tail widths, with the latter being highly significant (ANCOVA, $p < 0.001$; Table 5). 6) *E. multiplicata* have many more iridophores in their irises, whereas *E. subfluvicola* has reduced coloration in its iris making the eye primarily black in color. 7) A prominent black stripe of pigmentation is present along the lateral side of the snout and head in *E. multiplicata*, this stripe is absent in *E. subfluvicola* (Fig. 5).

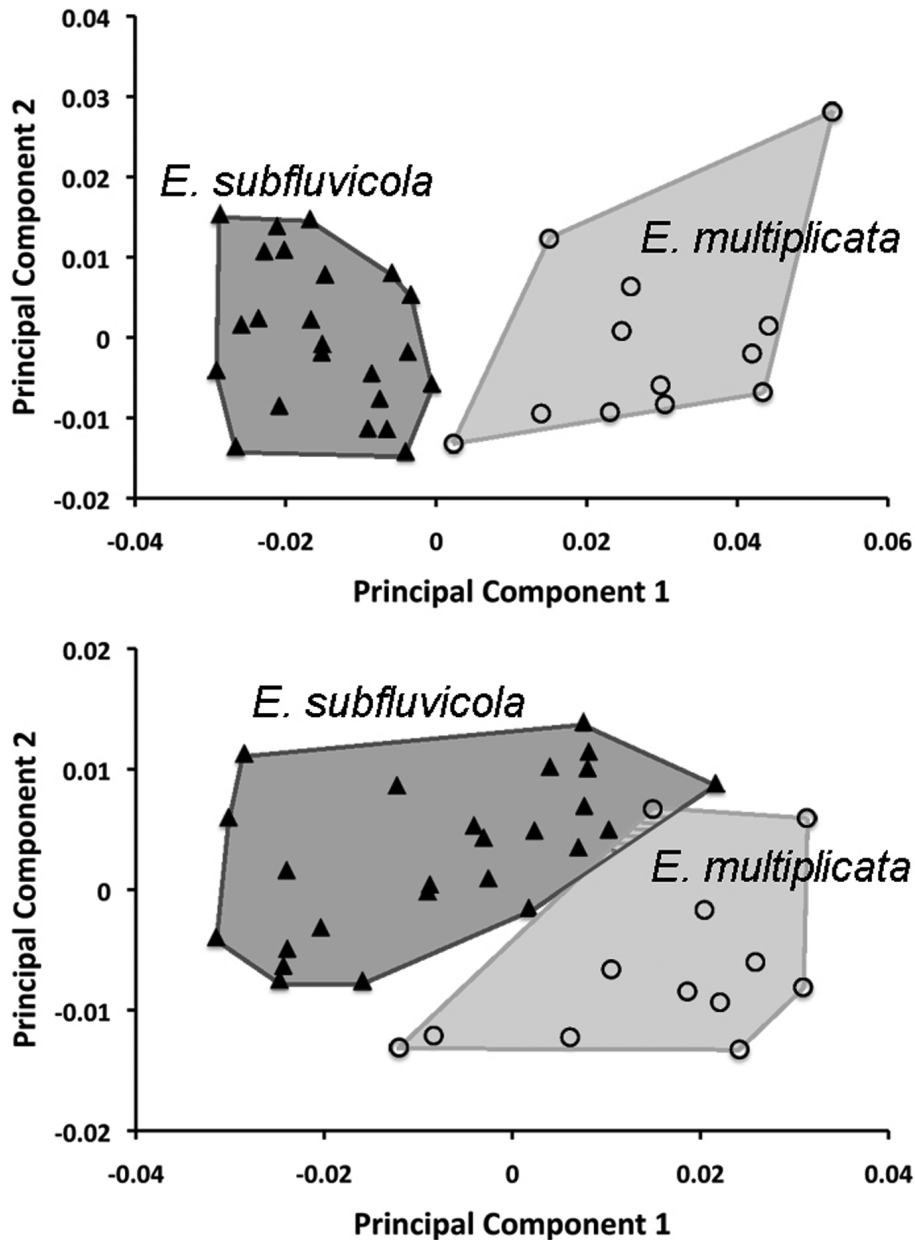


FIGURE 4. Principle Component Analysis (PCA) of adult *E. subfluvicola* (dark grey) and larval *E. multiplicata* (light grey), demonstrating minimal overlap in head (top) and body (bottom) morphospace. Landmarks and semilandmarks are shown in Figure 2.

Larval morphology of plethodontids is highly conserved, and *E. subfluvicola* is very similar to the larvae of other species of *Eurycea* (Wake 1966). Trunk vertebral counts (between atlas and sacrum) are 21–22 ($n=7$, mode=21), whereas syntopic and nearby populations of *E. multiplicata* ($n=17$) have 21 vertebrae. The trunk vertebrae of *E. subfluvicola* also appears to be longer and narrower than *E. multiplicata* (Fig. 6). The phalanges are well ossified with cartilaginous caps, exhibiting the standard plethodontid phalangeal formulae: 1–2–3–2 for the forelimbs, and 1–2–3–3–2 for the hindlimbs (Shubin & Wake 2003). Carpal counts are eight, and tarsal counts are nine.

The quadrate and squamosal, which are part of the mandibular suspensorium, are collectively more robust in *E. subfluvicola* than in *E. multiplicata* (Fig. 6). As in most spelerpine larval forms, *E. subfluvicola* contain 3 cartilaginous epibranchials, and in some individuals these structures are partially ossified (two of five specimens), whereas no ossification is apparent in *E. multiplicata* larvae ($n=10$; Fig. 6).

TABLE 5. Standard measurements of *E. subfluvicola* and *E. multiplicata*. All standard morphological measurements were made to the nearest 0.1mm. TL=Trunk Length, SVL=Snout vent length, HL=head length, HW=head width, EN=eye to nostril distance, ED=eye diameter, ID=interorbital distance. TRW=trunk width anterior to forelimbs, TW=tail width.

Measurement Stats	<i>E. subfluvicola</i> (n=24)(n=8 pairs for ED)	<i>E. multiplicata</i> larvae (n=12)(n=8 pairs for ED)
Mean TL/SVL ± SD	0.632 ± 0.019	0.601 ± 0.020
Range TL/SVL	0.581–0.667	0.558–0.634
Range	19.1–31.7	10.5–26.0
Mean HL/SVL ± SD	0.160 ± 0.009	0.182 ± 0.014
Range HL/SVL	0.146–0.186	0.159–0.199
Range	5.6–7.5	3.7–6.7
Mean HW/SVL ± SD	0.128 ± 0.007	0.151 ± 0.012
Range HW/SVL	0.117–0.143	0.127–0.175
Range	4.5–6.1	3.2–5.7
Mean TRW/SVL ± SD	0.090 ± 0.004	0.097 ± 0.007
Range TRW/SVL	0.085–0.095	0.088–0.115
Range	2.9–4.2	2.1–3.6
Mean TW/SVL ± SD	0.081 ± 0.004	0.089 ± 0.005
Range TW/SVL	0.074–0.085	0.080–0.097
Range	2.6–4.0	1.6–3.5
Mean EN/SVL ± SD	0.042 ± 0.002	0.039 ± 0.003
Range EN/SVL	0.037–0.045	0.035–0.046
Range	1.3–2.0	0.8–1.5
Mean ED/SVL ± SD	0.022 ± 0.002	0.032 ± 0.004
Range ED/SVL	0.019–0.025	0.027–0.040
Range	0.7–1.0	0.7–1.0
Mean ID/SVL ± SD	0.035 ± 0.003	0.040 ± 0.003
Range ID/SVL	0.031–0.040	0.035–0.046
Range	1.1–2.0	0.7–1.4

Phylogenetic Reconstruction and Divergence Time Estimation. As in previous phylogenetic analyses our *Cytb* phylogeny strongly supports the monophyly of the *Eurycea multiplicata* complex, which includes *E. multiplicata*, *E. tynerensis*, *E. spelaea* (Bonett & Chippindale 2004; Bonett *et al.* 2014), and now *E. subfluvicola* (Fig. 7a). *Eurycea subfluvicola* is sister to the clade that includes all representative *E. multiplicata* populations from throughout the Ouachita Mountains (BAPP=0.99). Uncorrected pairwise sequence divergence between *E. subfluvicola* and *E. multiplicata* ranges from 13.5–16.6% (mean=15%) for *Cytb*. This is more than two to five times the divergence of many recognized sister species of plethodontid salamanders (Chippindale *et al.* 2000; Hillis *et al.* 2001; Jockusch *et al.* 2002; Garcia-Paris *et al.* 2000; Vences & Wake 2007).

The phylogeny of *Rag1* (Fig. 7b) also shows *E. subfluvicola* as sister to all *E. multiplicata* (BAPP=0.77), with relatively high sequence divergence between these taxa for *Rag1*, uncorrected p=0.9–2.3% (mean=1.4%), which is considerable given that it is a much more conserved nuclear gene (Hoegg *et al.* 2004). What is most informative about the DNA sequence data is that it shows *E. subfluvicola* is highly divergent from syntopic *E. multiplicata* for both a mitochondrial (n=32 specimens) and a nuclear (n=15 specimens) gene (Fig. 7).

Bayesian species-tree and divergence time estimation based on mitochondrial (*Cytb* and *Nd4*) and nuclear (*Rag1*) gene trees shows strong support for the sister relationship of *E. subfluvicola* and *E. multiplicata* (Fig. 8). If we constrain the basal node of all spelERPINE plethodontids to be 49 million years old (Bonett *et al.* 2014), then the divergence between *E. subfluvicola* and *E. multiplicata* dates to approximately 18 million years ago.

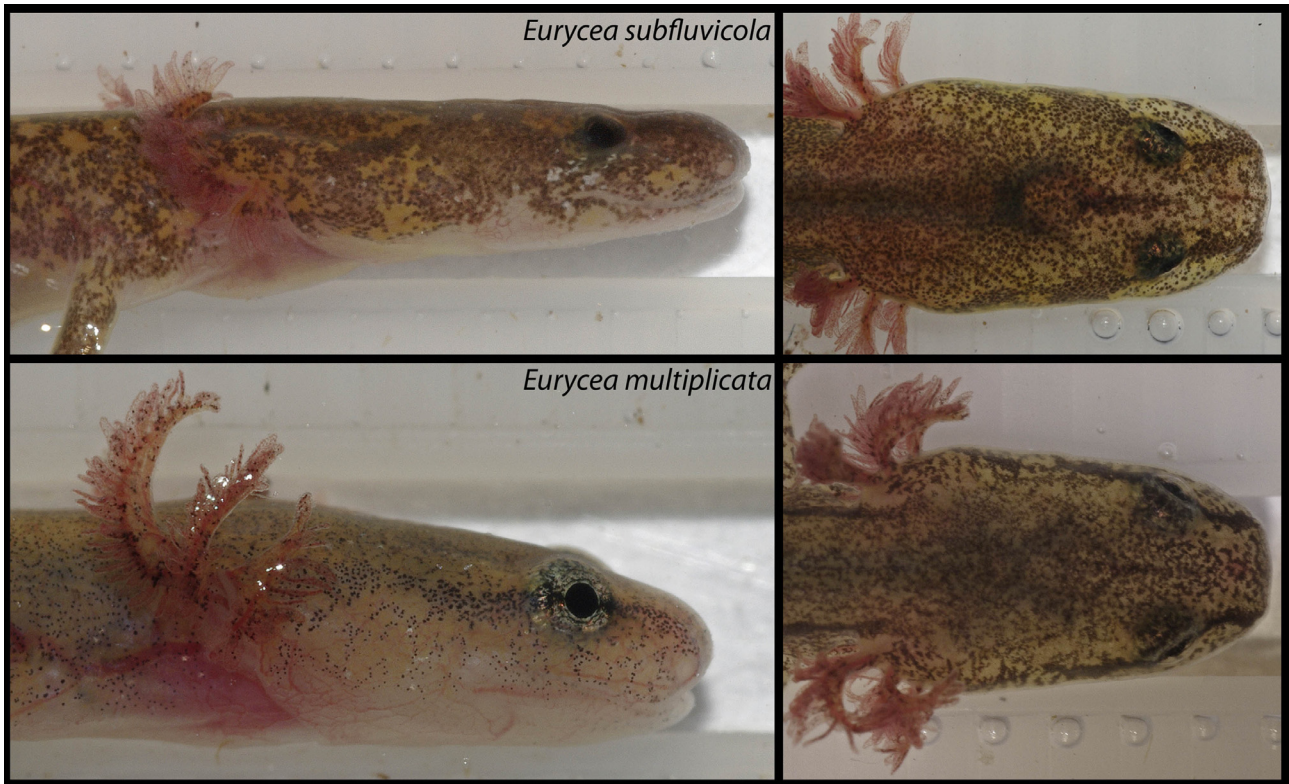


FIGURE 5. Side by side lateral and dorsal images of adult *E. subfluvicola* (top) and larval *E. multiplicata* (bottom) illustrating head shape differences between syntopic species.

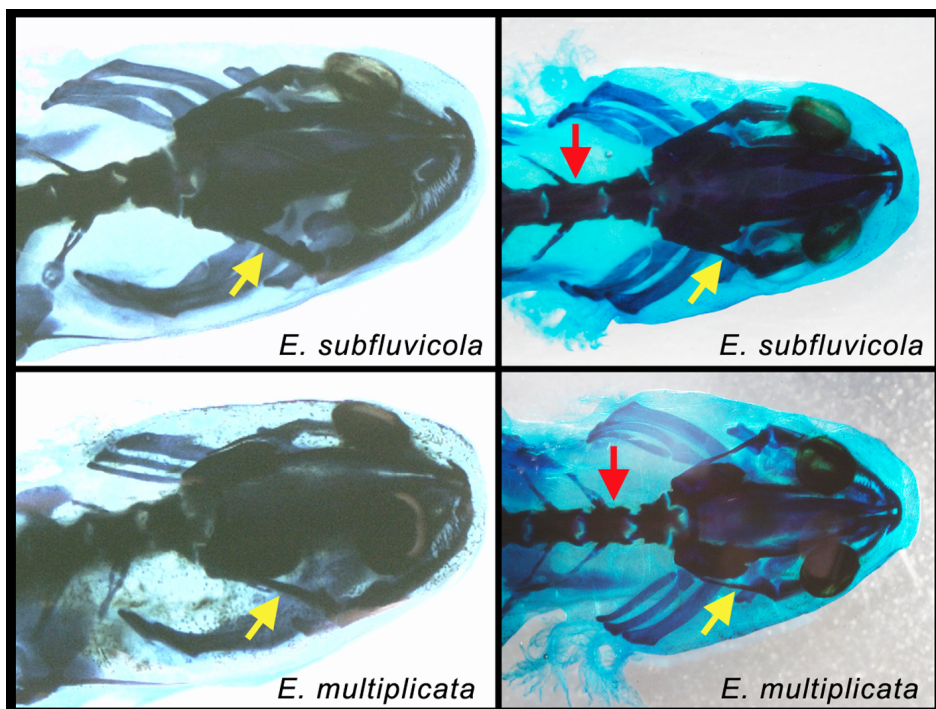


FIGURE 6. Cleared and stained images of *E. subfluvicola* (top) and *E. multiplicata* (bottom) skulls. Yellow arrows indicate quadrate and squamosal bones which are collectively more robust in *E. subfluvicola* than *E. multiplicata*. Red arrows point to trunk vertebrae which appear more elongate in *E. subfluvicola* than *E. multiplicata*.

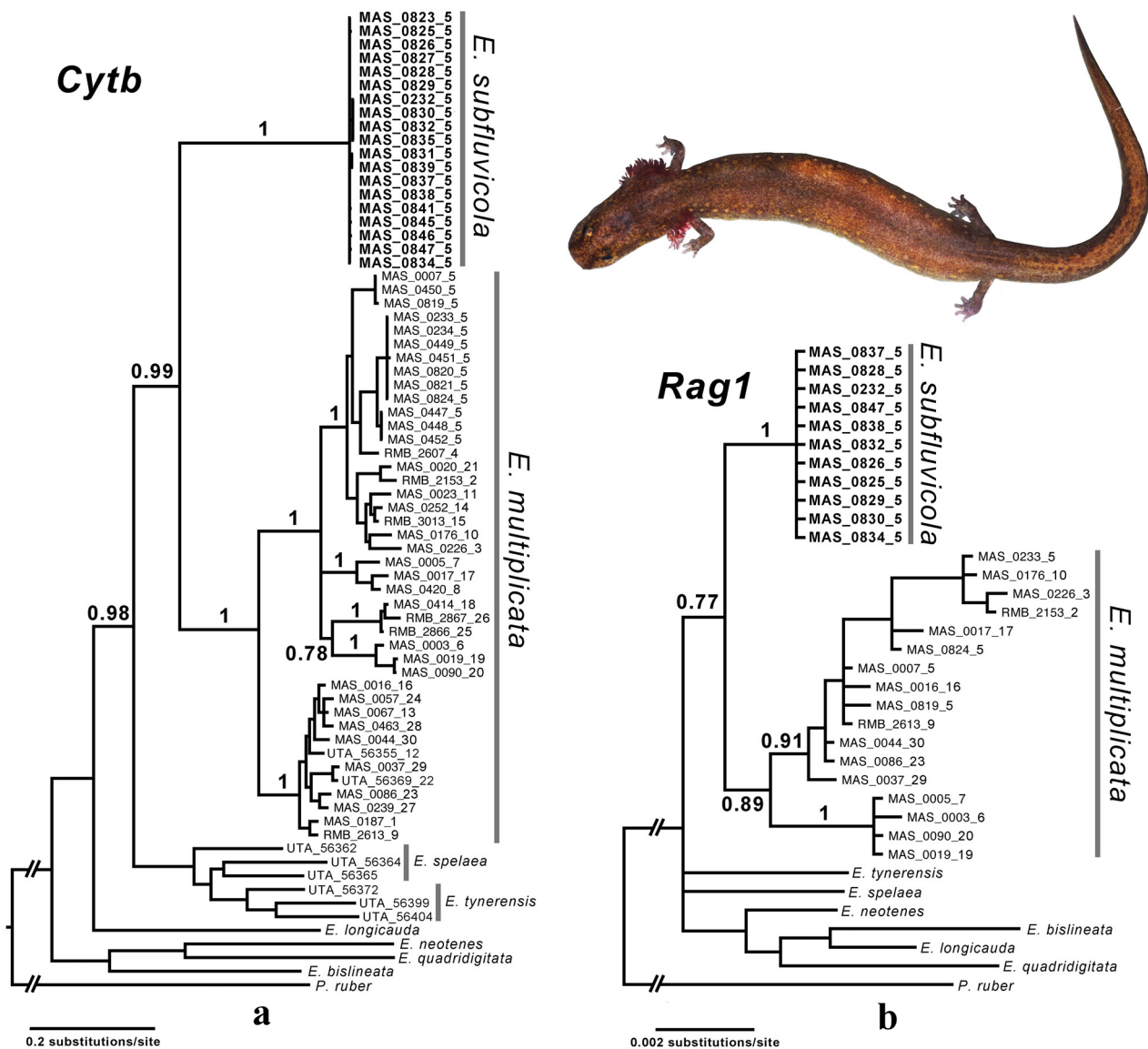


FIGURE 7. The 50% majority rule phylogram from a Bayesian inference analysis of mitochondrial and nuclear genes. Partitioned Bayesian analyses of *Cytb* (a) and *Rag1* (b), based on 15 million generations, showing *E. subfluviicola* (bold) and its sister taxon *E. multiplicata*. Posterior probabilities indicating node support are shown for major nodes of ingroup clades. For ingroup taxa, the label is the field number followed by the locality number (1 through 30; Figure 1; Table 1). Note that locality number 5 includes *E. subfluviicola* and sympatric *E. multiplicata*. *E*=*Eurycea* and *P*=*Pseudotriton*.

Variation. Variation is based on 24 specimens of *E. subfluviicola* (8 males, 16 females). All 24 individuals were adults, based on presence of mature or maturing egg follicles, or enlarged testes (Figs. 3 and 9). Male SVL ranged in size from 34.9–45.7mm (mean=40.8) and between 31.4–48.0mm (mean=41.1) for females. There are no somatic differences between the sexes, and only minor differences in coloration among individuals. We found 0.1–0.3% nucleotide divergence (uncorrected p) in *Cytb* and 18 sites with heterozygous positions across all individuals of *E. subfluviicola* for *Rag1*.

Distribution and Habitat. The known distribution of *Eurycea subfluviicola* is currently limited to the Trap Mountains, a range of generally east-west trending ridges in the southeastern portion of the Ouachita Mountain physiographic province. These uplands are composed of Paleozoic marine sedimentary rocks; principally the Devonian/Mississippian Arkansas Novaculite and younger Mississippian Stanley Shale formations. Stream valley alluvium deposits overlie the Stanley Shale and predominantly consist of Arkansas Novaculite gravels and cobbles. *Eurycea subfluviicola* is only known from one locality where it can be found in two nearby sites. The two sites are a 15m section of Slunger Creek and a 50m section of an unnamed tributary within the Slunger Creek alluvial valley;

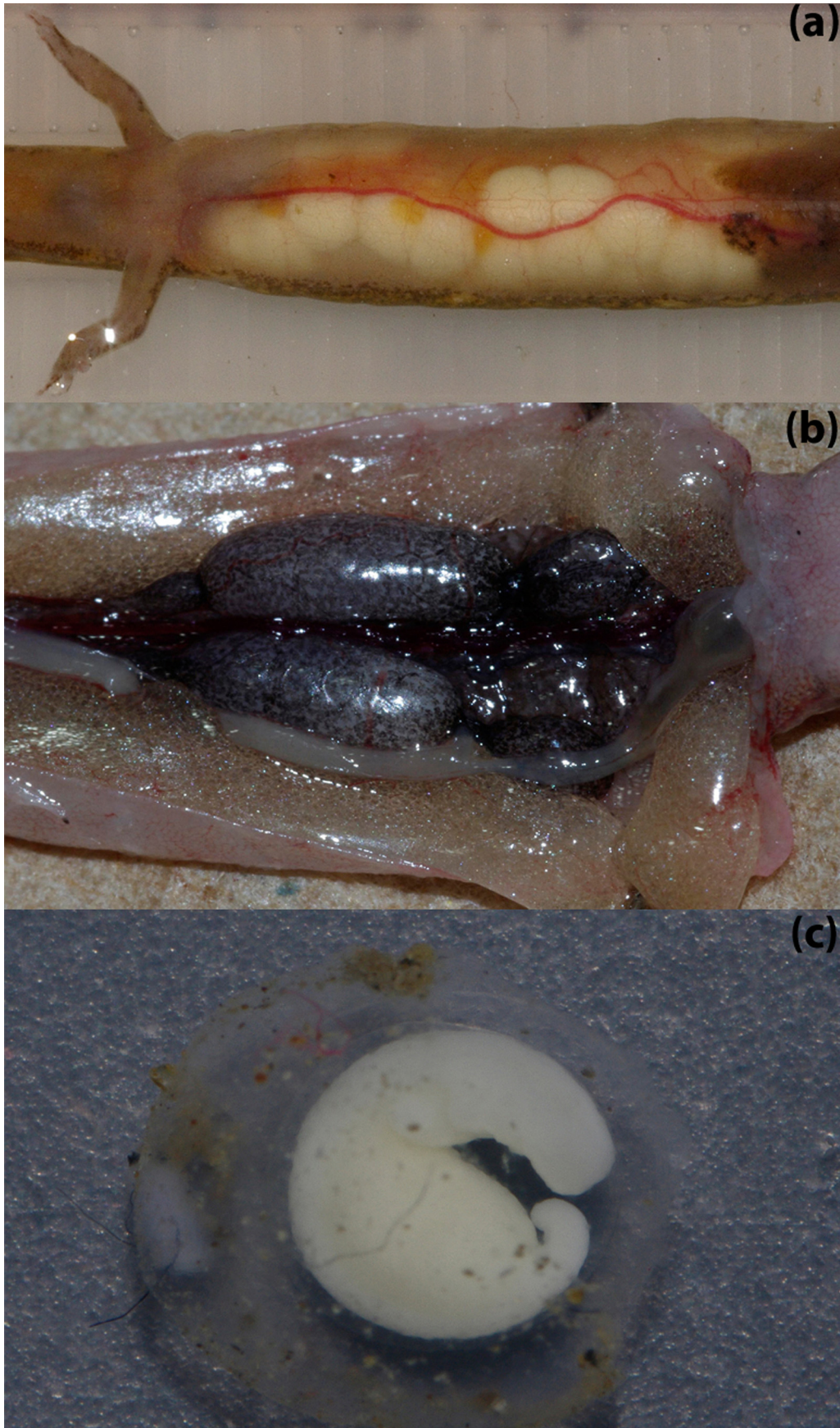


FIGURE 9. Images of reproductive status in *E. subfluvicola*. (a) Female with developing ova in ovaries. (b) Male with enlarged testicular lobes, and smaller lobules in development. (c) Developing embryo of *E. subfluvicola* (16 days old).

approximately 135m apart from one another. During our 2013 surveys, we found more individuals of *E. subfluvicola* (approximately 50 observations) in the longer stream section, while fewer individuals were found in the smaller stream section (<10 observations). Surface stream flows are seasonally ephemeral in these two stream reaches. However, surface flow persists, both upstream and downstream, when the streambed is completely dry at these two sites. This suggests a subsurface flow in the stream channels or hyporheic zone connectivity between these points, which may provide habitat for *E. subfluvicola* during periods of low rainfall. Our ability to observe *E. subfluvicola* is dependent upon the rise of the water table during winter and spring, which is similar to the seasonal observability of paedomorphic populations of *E. tynerensis* in the western Ozark Plateau (Bonett & Chippindale 2006). The Slunger Creek valley supports two metamorphic, stream-dwelling plethodontids (*E. multiplicata* and *Desmognathus brimleyorum*). Dominant vegetation at the type locality is second growth mixed pine-hardwood forest composed of *Pinus echinata* and several hardwoods (*Acer*, *Celtis*, *Cornus*, *Liquidambar*, *Ulmus*, and *Quercus*).

Life history and ecology. Most aspects of *E. subfluvicola* life history are derived from observations in captivity, and reproductive status has been determined by examining the gonads through the transparent venter. Enlargement of testes was first observed in the field in February, and reached maximum size in the lab by late June and July. Like related *E. multiplicata* and *E. tynerensis*, *E. subfluvicola* typically has single lobed testes (Sever 1974; MAS pers. obs.), but one individual possessed multiple lobes (Fig. 9b). Mature or maturing egg follicles have only once been observed in the field (late November), however, follicles started maturing in the lab (at 20°C) in June and were robust by August. We have not yet found definitive juveniles of *E. subfluvicola* in the field, which suggests that early development (<32mm SVL) may be primarily subterranean.

Coinciding with peak testicular size, the five largest captive males began to show signs of metamorphosis (i.e. gill reduction, changes in head morphology and coloration). For four individuals, the process took over 2.5 months, and metamorphosis was never completed before the animals died. *Eurycea multiplicata* can complete metamorphosis in less than one month after initiation under similar conditions (MAS pers. obs.). Two of the partially metamorphosed *E. subfluvicola* showed remodeling of the hyobranchial apparatus towards the adult form (Alberch *et al.* 1985). This includes the loss of larval epibranchials, and formation of the adult epibranchial, and the reshaping of the ceratohyal, basibranchials, and ceratobranchials. Adult maxillary morphology (Wilder 1925; Wake 1966) started to form in three specimens. The small external gills were also never completely absorbed. These individuals demonstrate that facultative metamorphosis might be possible in this species, however, given that four of the five large males died during this process (with the 5th individual still undergoing metamorphosis 5-February 2014), indicates that metamorphosis appears to be a grave event, as seen in some other paedomorphic salamanders that have naturally or experimentally metamorphosed (Dundee 1957, 1962; Brandon 1976). It is notable that partial metamorphosis only occurred in the largest, most reproductive males (based on testicular examination), while females showed no signs of metamorphosis (n=13). We hypothesize that high levels of endogenous androgens may induce metamorphosis of male *E. subfluvicola* in captivity. Studies have shown that testosterone, but not estradiol, can increase the rate of metamorphosis in other salamander species (Norris *et al.* 1973). Surface activity of *Eurycea subfluvicola* is primarily nocturnal, with individuals leaving the cover of rocks or leaf litter during the day. During periods without surface flow, individuals of *E. subfluvicola* presumably follow the water table using interstitial spaces in the streambed gravel. The subterranean ecology of *E. subfluvicola* in the hyporheic zone is completely unknown.

Discussion

We present life history, genetic and morphological evidence for the description of the first paedomorphic plethodontid from the Ouachita Mountains. *Eurycea subfluvicola* is the most divergent paedomorphic salamander described in the past 70 years; since the descriptions of *E. tynerensis* (compared to *E. multiplicata*; Moore & Hughes 1939) and *E. wallacei* (compared to *E. bislineata*; Carr 1939). Other more recently described paedomorphic salamanders are much more closely related to their sister taxa/clades, which in almost all cases are also paedomorphic (e.g. Netting & Goin 1942; Neill 1964; Chippindale *et al.* 2000; Hillis *et al.* 2001; for divergence times see Bonett *et al.* 2013, 2014). *Eurycea subfluvicola* also represents an additional independent instance of paedomorphosis within Spelerpini (now with 18 paedomorphic species; AmphibiaWeb 2014). Interestingly, *E. subfluvicola* appears to share similar ecology with paedomorphic populations of *E. tynerensis* from

the western Ozark Plateau (Bonett & Chippindale 2006). Like *E. tynerensis*, *E. subfluvicola* appear to undergo seasonal movements into the streambed when the surface streams recede. Paedomorphosis in *E. subfluvicola* seems to have enabled access to an otherwise unexploited subterranean niche, and may be important in maintaining reproductive isolation with syntopic *E. multiplicata*.

Over the last decade we sampled *E. multiplicata* from over 100 locations throughout the Ouachita Mountains, but often only metamorphic adults were collected and sequenced. Our fortuitous discovery of a highly divergent larval form in 2011 prompted intensive examination and sequencing of both larvae and adults in and around Lake Catherine State Park, which allowed us to delineate the new species. *Eurycea subfluvicola* represents an example of how species that primarily differ by an ontogenetic shifts can mask species diversity. This phenomenon may be more likely in organisms which display complex life histories, where deviant adult forms can masquerade as different developmental stages of related species. This discovery highlights how much biodiversity may still remain hidden, even in well studied areas.

Eurycea subfluvicola currently has one of the smallest, if not the smallest, known distribution of any North American salamander (AmphibiaWeb 2014). Surveys up and downstream of these sites, and several nearby streams, have yet to produce additional specimens, despite the presence of adult and larval *E. multiplicata*. It is possible that previously sampled *E. multiplicata* localities across the Ouachita Mountains also contain *E. subfluvicola*, since collection of larval forms was not emphasized. Regardless, *E. subfluvicola* is of immediate conservation concern, given this species' current known distribution. Fortunately, the known distribution of *E. subfluvicola* is within a protected site (Lake Catherine State Park). To ensure the long-term conservation of this unique species we recommend the following actions: 1) conduct additional surveys to further delineate the distribution; 2) establish conservation measures (as applicable) to preserve known sites; and 3) captive propagation efforts to ensure reserve stock in case of wild population extirpation.

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