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Speciation, phylogeography and evolution of life history and morphology in plethodontid salamanders of the *Eurycea multiplicata* complex

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Abstract

Understanding the complex interactions among environment, genotype and ontogeny in determining organismal phenotypes is cental to many biological disciplines. The *Eurycea multiplicata* complex, endemic to the Interior Highlands (Ozark Plateau and Ouachita Mountains) of eastern North America, comprises a diverse radiation of paedomorphic surface-dwelling (*E. tynerensis*), metamorphic surface-dwelling (*E. multiplicata multiplicata* and *E. m. griseogaster*) and metamorphic subterranean (*Typhlotriton spelaeus*) hemidactyliine plethodontid salamanders. Portions of two mitochondrial genes, cytochrome-*b* and NADH dehydrogenase-4, totalling 1818 base pairs (bp) were sequenced for 70 ingroup individuals plus numerous outgroup taxa, to examine the biogeography and relationships among these morphologically disparate species. Results show the *E. multiplicata* complex to be monophyletic, with its two most divergent clades corresponding to geography, not morphology or life history. Transforming surface-dwelling populations from the Ouachitas (*E. m. multiplicata*) are sister to the Ozark taxa, including paedomorphic surface-dwelling (*E. tynerensis*), subterranean (*T. spelaeus*) and transforming surface-dwelling salamanders assigned to the 'subspecies' *E. m. griseogaster*. Among Ozark taxa *T. spelaeus* (deeply nested within *Eurycea*) is sister to a clade that includes *E. m. griseogaster* and *E. tynerensis*. Current taxonomy suggests that paedomorphic populations (*E. tynerensis*) from the western Ozarks are distinct from nearby transforming populations (*E. m. griseogaster*). However, paedomorphic and transforming salamanders do not form reciprocally monophyletic groups and many populations share almost identical haplotypes. Ancestral state reconstruction of life history traits shows that paedomorphosis arose independently from three to nine times. Most populations are either completely paedomorphic or completely transforming. This suggests that local habitat parameters strongly influence life history mode in this complex, either facultatively or by selection for particular genotypes.

Keywords: *Eurycea*, Interior Highlands, paedomorphosis, phylogeny, Plethodontidae, *Typhlotriton*

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Introduction

Habitat diversity can influence morphological and life history variation among organisms, and may promote speciation (e.g. Darwin 1859; Mayr 1942; Schluter 1998). However, when distinct lineages within a closely related group enter similar novel habitats independently, convergence in morphology and life history characters may confound phylogeny reconstruction (Hillis & Wiens 2000; Wiens *et al*. 2003) and obscure species boundaries. The Plethodontidae is the most speciose family of salamanders and exhibits tremendous diversity in ecology, life history and morphology.

Numerous instances of both convergence and morphological evolutionary stasis appear to have occurred in the group, complicating reconstruction of phylogenetic relationships and delimitation of species (Wake 1991, 1993; Larson & Chippindale 1993). However, plethodontids offer an ideal system for analysis of morphological evolution, adaptation and speciation, given a well-supported

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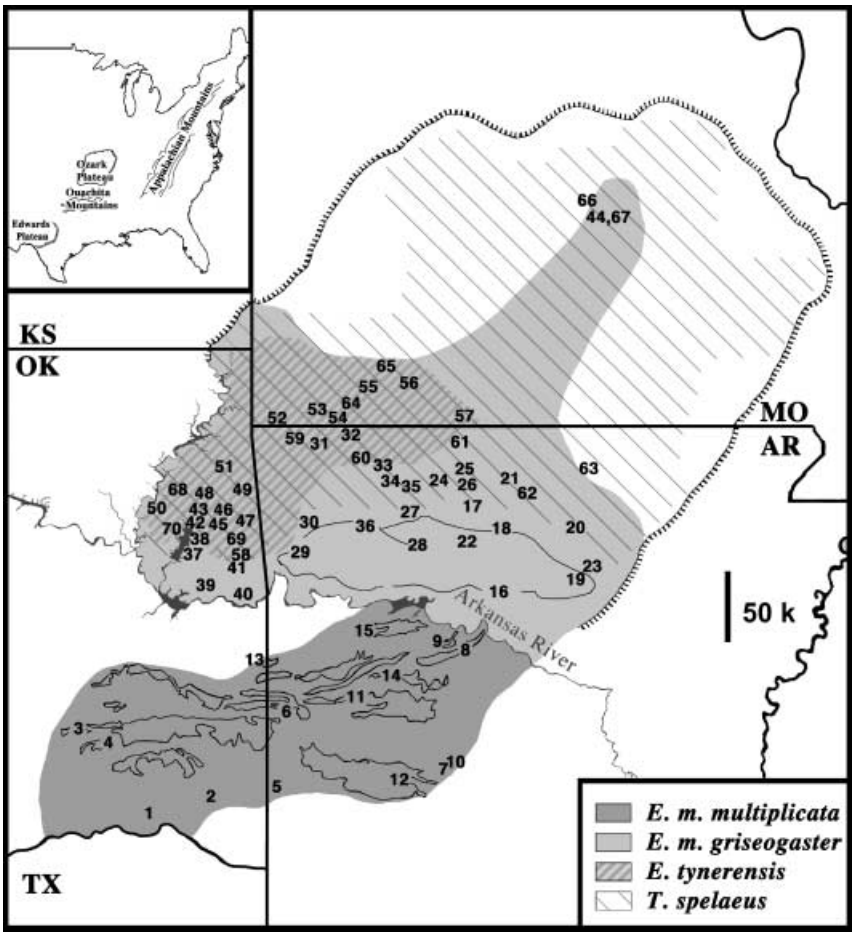

Fig. 1 Distribution of nominate taxa of the *E. multiplicata* complex, corresponding to the current taxonomy of the group. Numbers on map are localities of all ingroup taxa sampled, and correspond to those listed in Appendix I.

tree for the taxa of interest based on characters (especially molecular) that are unlikely to be affected by these sources of homoplasy.

Plethodontids of the tribe Hemidactyliini are endemic to eastern and central North America, and have a free-living aquatic larval form with external gills (Wake 1966; Petranka 1998; Frost 2002). In most species, just prior to sexual maturity larvae metamorphose into a typically more terrestrial adult form. Timing of life history events (e.g. duration of larval period) is variable across hemidactyliines and there are many paedomorphic taxa that become reproductively mature while retaining the aquatic larval morphology (e.g. Ryan & Bruce 2000). Several species of hemidactyliines inhabit subterranean systems, and exhibit some degree of cave-associated morphology (e.g. loss of pigment, reduction of eyes; Wake 1966; Petranka 1998; Chippindale 2000; Chippindale *et al*. 2000). *Eurycea* is the most speciose genus of hemidacyliines, with centres of diversity in the Interior Highlands, Appalachian Mountains and Edwards Plateau of Texas (Petranka 1998; Chippindale *et al*. 2000).

The Interior Highlands of central North America, composed of the Ouachita Mountains and the Ozark Plateau (Fig. 1 inset), support a high faunal diversity with many endemic species (Dowling 1956; Mayden 1988). This is attributable primarily to the region's disjunction from other eastern North American highlands, such as the Appalachian Mountains and the Edwards Plateau, and to its geological diversity. The Ouachitas are a series of folding mountains (Spearing 1991), with peaks as high as 790 m, and an abundance of streams and springs. The Ozark Plateau is a more complex geological system composed primarily of Mississippian karst limestone. Dissolution of the plateau has created a host of epigean and subterranean water channels throughout the region (Unklesbay & Vineyard 1992). Unique gravel-bottom habitats, composed of older and relatively insoluble Silurian and Ordovician chert, exist in the western Ozarks where waterways have completely worn through the Mississippian limestone (Fenneman 1938).

A major radiation of hemidactyliines (referred to herein as the *Eurycea multiplicata* complex) is endemic to the Interior Highlands, including some members of the genus *Eurycea* and the monotypic genus *Typhlotriton*. The taxonomic history of the *E. multiplicata* complex is complicated (see *Taxonomic implications*). In general, recent authors

recognize two relatively widespread and primarily metamorphic (transforming) surface-dwelling subspecies, *E. m. multiplicata* in the Ouachitas and *E. m. griseogaster* in the Ozarks (Petranka 1998; Fig. 1). *E. tynerensis* occurs in the chert gravel-bottom habitats of the western Ozarks, and is considered a distinct species because it is thought to be strictly paedomorphic (Petranka 1998; Tumlinson & Cline 2003). Curiously, the entire distribution of *E. tynerensis* is contained within that of *E. m. griseogaster*, suggesting that they may be broadly sympatric, but in nearly all localities only one 'species' is present. The enigmatic cave-dwelling grotto salamander, *Typhlotriton spelaeus*, which is distributed throughout the Ozark Plateau, was described as a distinct genus due to the extreme cave-associated morphology of adults, including loss of pigmentation and atrophied eyes (Stejneger 1892). However, based on morphology, Wake (1966) suggested a close relationship with members of the genus *Eurycea*, and mitochondrial (mt) and nuclear DNA sequences show a strong phylogenetic affinity to *E. multiplicata* and *E. tynerensis* (Chippindale *et al*. in prep.; this study).

Here we perform phylogenetic analyses on 70 ingroup (*E. multiplicata* complex) individuals from throughout the Interior Highlands plus numerous outgroup taxa, using portions of two mt genes, cytochrome *b* (1118 bp) and NADH dehydrogenase subunit 4 [700 base pairs (bp)] to elucidate the biogeographical and phylogeographical history of the major lineages of the complex. We gain insight into phenotypic evolution of the group by testing the monophyly of surface-dwelling transforming (*E. m. multiplicata* + *E. m. griseogaster*) and paedomorphic (*E. tynerensis*) species, and the genus *Eurycea* (excluding the cave-dwelling form *Typhlotriton*). We also examine the lability of occurrence of paedomorphosis in these salamanders and suggest possible determinants of life history mode. Finally, we revisit the taxonomy of this group inlight of our results.

Materials and methods

Specimens

Our sampling includes many individuals of each of the nominate taxa [*E. m. multiplicata* (Emm), *E. m. griseogaster* (Emg), *E. tynerensis* (Ety), and *T. spelaeus* (Tsp)] from throughout their distributions (Fig. 1). Tree codes, map numbers and locality information are listed in Appendix I. Outgroup taxa include representatives from the other four major lineages of *Eurycea* (*E. bislineata*, *E. longicauda*, *E. neotenes* and *E. quadridigitata*) and a member of the sister group to *Eurycea*, *Pseudotriton ruber* (Chippindale *et al*. in prep.). The majority of specimens were collected by R. M. B. and assistants from August 2000–March 2003. Additional specimens were obtained from colleagues.

Following euthanasia by submerging in a 10% solution of MS-222, tissues were dissected from each specimen and are currently kept at −80 °C at the University of Texas at Arlington (UTA). Vouchers are located in the Collection of Vertebrates at UTA, Texas Natural History Collection at the University of Texas at Austin (TNHC) or the Carnegie Museum of Natural History (CMNH). Here we initially follow the subspecific boundaries depicted in Petranka (1998) for *E. multiplicata*. *E. m. multiplicata*, as currently recognized, comprises populations from south of the Arkansas River, in the Ouachitas. Northern transforming populations in the Ozarks have been considered *E. m. griseogaster*. We initially treated Ozark surface-dwelling transforming individuals as *E. m. griseogaster* and paedomorphic individuals as *E. tynerensis*, according to currently accepted taxonomy. In order to properly identify life history mode of paedomorphs, sexual maturity of *E. tynerensis* was determined by the presence of well-developed pigmented testes in males and presence of oviducal eggs in females. It has been suspected that *E. m. griseogaster* may also be paedomorphic in the western Ozarks (Bishop 1944; Dundee 1947), so we included specimens from the type localities of *E. tynerensis* (specimen no. Ety 46) and *E. m. griseogaster* (Emg 37) to investigate this (see *Taxonomic implications*). All but two individuals, Emg 44 and Ety 57, of *E. m. griseogaster* and *E. tynerensis* were either completely transformed (*E. m. griseogaster*) or sexually mature paedomorphs (*E. tynerensis*). Emg 44 and Ety 57 are medium-sized larvae without well-developed reproductive organs, but are included in this study for distributional information.

DNA isolation, amplification and sequencing

Qiagen DNeasy extraction kits were used to isolate DNA from tissues. Portions of two mitochondrial genes, cytochrome *b* (cyt *b*, 1118 bp) and NADH dehydrogenase subunit 4 (ND4, 700 bp) were amplified via polymerase chain reaction (PCR), with primers listed in Table 1, for all 70 ingroup specimens, as well as for the five outgroup taxa. PCR products were run on 1% agarose gels. Bands of the expected molecular weight were excised, purified, ligated to a plasmid vector and transformed into *Escherichia coli* using Topo-TA kits (Invitrogen). Transformed cells were plated on kanamycin/X-gal plates for blue/white selection. White colonies were picked and cultured overnight in ~3 mL of Luria-Bertani (LB). Plasmids were purified using Qiagen Plasmid Miniprep kits, precipitated at –20 °C for at least 30 min in a solution of sodium acetate and 95% ethanol, washed with 70% ethanol and dried. Thermo Sequenase™ cycle sequencing kits (USB) and fluorescently labelled primers were used for sequencing reactions on plasmid DNA. Plasmids from at least two different clones of each fragment, for each specimen, were sequenced bidirectionally on a LiCor 4200 L long-read, dual-laser

Table 1 Amplification primers for cyt *b* and ND4

Primer	Gene	Primer sequence	Reference
MVZ15	cyt *b*	5′-GAA CTA ATG GCC CAC ACW WTA CGN AA-3′	Moritz *et al*. (1992)
ETCR	$tRNA^{Thr}$	5′-TTC TAA ACT ACA ACA GCA TC-3′	This study
HEMTHREV	$tRNA^{Thr}$	5′-CTT TGR CTT ACA AGG YCA ATG-3′	Hillis *et al*. (2001)
ND4F	ND4	5′-CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC-3′	Arévalo *et al*. (1994)
LEUR	$tRNA^{Leu}$	5′-CAT TAC TTT TAC TTG GAT TTG CAC CA-3′	Arévalo *et al*. (1994)
EML1R	$tRNA^{Leu}$	5′-CTT TCR TRT CTA GGG TCA CAG CCT AG-3′	This study

Ambiguity codes: N = G, A, T or C; R = A or G; W = A or T; Y = C or T.

sequencer. Both strands were sequenced completely for each sample.

Phylogenetic analyses

SEQUENCHER™ 3.1 (Gene Codes Corp.) was used to align and edit sequences and to create contigs for both gene fragments. Only protein-coding sequences were analysed and no indels were observed. Alignment was unambiguous. Sequences were deposited in GenBank; Accession nos are provided in Appendix I.

A Nexus file containing a concatenated alignment of both genes was analysed using maximum parsimony (MP) and Bayesian inference (BI). A heuristic MP search using 100 random-taxon-addition replicates was performed in PAUP* 4.0b10 (Swofford 2001) to find the most parsimonious tree. *Pseudotriton ruber* was defined as the outgroup, so that the monophyly of the *E. multiplicata* complex, with respect to other *Eurycea*, could be tested. Nonparametric bootstrapping (Felsenstein 1985), based on 1000 pseudoreplicates with 10 random-taxon-addition replicates per pseudoreplicate, also implemented in PAUP*, was used to assess confidence at each node. MODELTEST version 3.06 (Posada & Crandall 1998) was used to calculate the most appropriate model of nucleotide substitution and its parameters. MODELTEST chose the same model (HKY + I + Γ) for each gene analysed separately, but a different model (TVM + I + Γ) for analyses of combined sequences. A partitioned BI analysis of both genes, using the HKY + I + Γ model but allowing different values for each parameter for each partition, was implemented via the program MRBAYES version 3.0 (Huelsenbeck & Ronquist 2001). The data were partitioned by gene (cyt *b* and ND4). Four simultaneous chains were run for 1 558 500 generations. The first 58 500 generations were discarded as 'burn-in'. The remaining 1.5×10^6 generations were used to reconstruct the topology and calculate posterior probabilities for each node. PAUP* was used to calculate uncorrected pairwise sequence divergences. Plethodontids are only sparsely represented in the fossil record (Duellman & Trueb 1986) and there are no unambiguous fossils of species from the *E. multiplicata* complex, making group-specific calibration of divergence times impossible. Here we employ the general vertebrate clock of 2% per million years as the highest rate estimate, and a general poikilotherm molecular clock of 0.5% per million years as the lowest rate estimate (Avise *et al*. 1998), to assess conservatively a range of dates for key nodes in the tree.

Shimodaira–Hasegawa (SH) tests (Shimodaira & Hasegawa 1999; Goldman *et al*. 2000), implemented in PAUP*, were used to test the monophyly of each of the morphologically based species, by comparing the likelihoods of constrained to unconstrained topologies. In test 1, all transforming surface-dwelling populations (*E. m. multiplicata* and *E. m. griseogaster*) were constrained to be monophyletic. In test 2, all paedomorphic populations (*E. tynerensis*) were constrained to be monophyletic. In test 3 the phylogenetic placement of *Typhlotriton* within *Eurycea* was tested, by constraining all *Eurycea* to be monophyletic exclusive of *Typhlotriton*. Heuristic MP searches with 100 random-taxon-addition replicates were performed in PAUP* to obtain the shortest constrained and unconstrained topologies. ML parameters from analyses of the combined sequences in MODELTEST were used in the SH test in the context of the MP topologies. We tested only topologies from MP searches because it is not yet possible to constrain topologies in MRBAYES; however, in almost all respects topologies from both sets of analyses were very similar.

MACCLADE version 4.0 (Maddison & Maddison 2001) was used to reconstruct evolution of life history mode (paedomorpic vs. metamorphic) via parsimony. We traced this character on the topology from both MP and BI analyses using the equivocal cycling option. We reduced the number of taxa used in the reconstructions to include only the relevant and necessary ones (see Results). We included all *E. m. griseogaster* and *E. tynerensis*, except for those of ambiguous life history mode (Emg 44 and Ety 57). Only one *T. spelaeus* and one *E. m. multiplicata* were included because all populations of these taxa are thought to be metamorphic (Brandon 1966; Ryan & Bruce 2000).

Table 2 Ranges of uncorrected pairwise sequence divergence and estimated divergence time between nominate taxa, between three major clades, and within three major clades

	Uncorrected *P*	Est. divergence time
Divergence among nominate taxa		
Emm – Emg	13.53–15.68%	6.8–31.4 Myr
Emm – Ety	13.37–15.40%	6.7–30.8 Myr
Emm – Tsp	14.52–15.13%	7.3–30.3 Myr
Emg – Ety	0.06–9.35%	0.03–18.7 Myr
Emg – Tsp	11.39–13.26%	5.7–26.5 Myr
Ety – Tsp	11.61–13.25%	5.8–26.5 Myr
Divergence among three major clades		
Emm – (Emg/Ety + Tsp)	13.37–15.68%	6.7–31.4 Myr
Tsp – (Emg/Ety)	11.39–13.26%	5.7–26.5 Myr
Emm – Tsp	14.52–15.13%	7.3–30.3 Myr
Emm – (Emg/Ety)	13.37–15. 68%	6.7–31.4 Myr
Divergence within three major clades		
Emm	0.88–11.28%	0.44–22.6 Myr
Emg/Ety	0.06–9.35%	0.03–18.7 Myr
Tsp	1.93–11.06%	0.97–22.1 Myr

Results

Sequence variation

The concatenated cyt *b* and ND4 sequences for all individuals formed an alignment of 1818 homologous base pairs. Eight hundred and fifty-nine sites were variable, and of those 681 were parsimony-informative. Each individual displayed a unique haplotype and uncorrected pairwise sequence divergence ranged from 0.06 to 15.68% among the 70 ingroup specimens (Table 2). For cyt *b*, transition/transversion (Ti/Tv) ratio = 6.0406, proportion of invariable sites (I) = 0.4849, the gamma distribution shape parameter (Γ) = 1.3626 and the mean base frequencies were A = 0.3795; C = 0.2303; G = 0.1053; T = 0.2849. For ND4, Ti/Tv ratio = 8.0552, I = 0.4327, Γ = 0.8199 and the mean base frequencies were A = 0.4150; C = 0.2383; G = 0.1010; T = 0.2457.

Biogeography and phylogeography

The Bayesian and MP trees had almost the same overall topology, with only a few discrepancies (Figs 2 and 3). Parsimony analysis yielded four equally parsimonious trees with conflicting resolution among only a few short, terminal branches. Both analyses recovered a relatively well-supported (100% BI posterior probability and 81% bootstrap) monophyletic *E. multiplicata* complex. The overall patterns within the *E. multiplicata* complex show phylogenetic patterns that are consistent with geography rather than morphology or development. There are three major clades, one in the Ouachitas (*E. m. multiplicata*) and the other two in the Ozarks (*E. m. griseogaster*/*E. tynerensis* and *T. spelaeus*). All populations of *E. m. multiplicata* from the Ouachitas are monophyletic and sister to Ozark taxa. Uncorrected pairwise sequence divergence between taxa from the Ouachitas and Ozarks is high (13.37–15.68%). Among the Ozark populations, the subterranean salamanders (*T. spelaeus*) are monophyletic and are sister to a clade of surface-dwelling salamanders that includes both *E. m. griseogaster* and *E. tynerensis.* Sequence divergence between Ozark surface-dwelling and subterranean lineages also is high (uncorrected *P* = 11.39–13.26%). There are also considerable levels of sequence divergence within all three major clades of the *E. multiplicata* complex (Table 2).

Life history and morphology

Tests of the monophyly of the morphologically defined species, via the SH test, showed highly significant ($P < 0.001$ in both cases) differences in likelihood between the unconstrained MP tree and the tree with all transforming surface-dwelling individuals (Emm & Emg) constrained to be monophyletic (test 1), and also with all paedomorphic individuals (Ety) constrained as monophyletic (test 2) (Table 3). However, the tree that constrained all *Eurycea* to be monophyletic (in order to test for the exclusion of *Typhlotriton*) was not significantly less likely than the unconstrained tree ($P < 0.349$). This is probably because

Table 3 Results of Shimodaira–Hasegawa tests for topological comparisons of three constrained trees, to the unconstrained parsimony consensus

Test	Topology	Tree length (steps)	Length difference from unconstrained	Difference in –ln likelihood	*P*
	Unconstrained	3578	—	—	—
1	Emm and Emg monophyletic	3810	232	1205.11	< 0.001
2	Ety monophyletic	3773	195	1120.24	< 0.001
3	*Eurycea* monophyletic	3620	42	5.66	< 0.349
	Unconstrained (reduced)	1493	—	—	—
3 (reduced)	*Eurycea* monophyletic	1527	34	60.73	< 0.028

Emm = *E. m. multiplicata*, Emg = *E. m. griseogaster*, Ety = *E. tynerensis*.

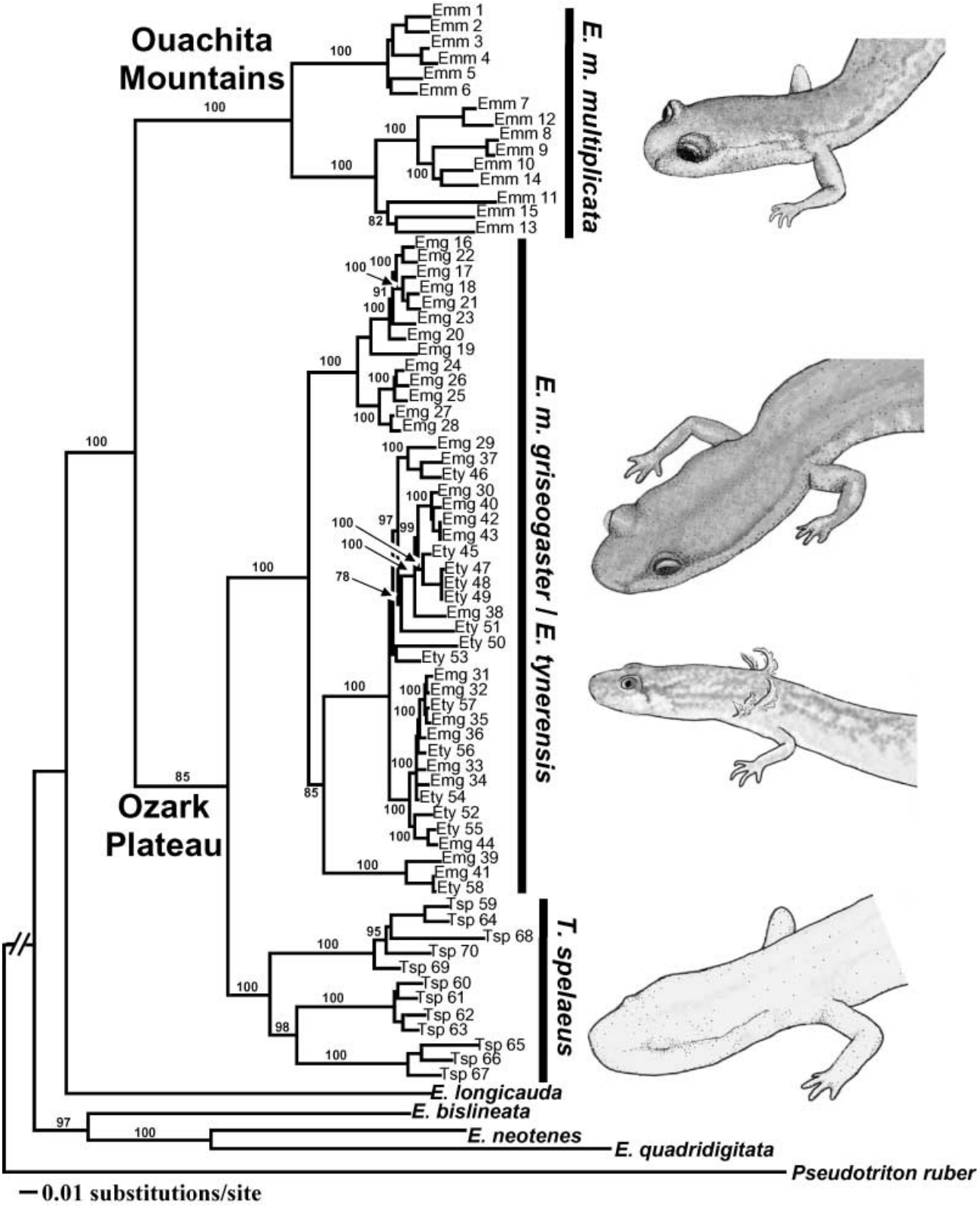

Fig. 2 Majority rule consensus based on Bayesian analysis of 1.5×10^6 generations. Posterior probabilities indicating node support are shown. Only posterior probabilities of 50% or higher are shown. Support for some recent nodes in densely sampled clades is not shown, due to space constraints.

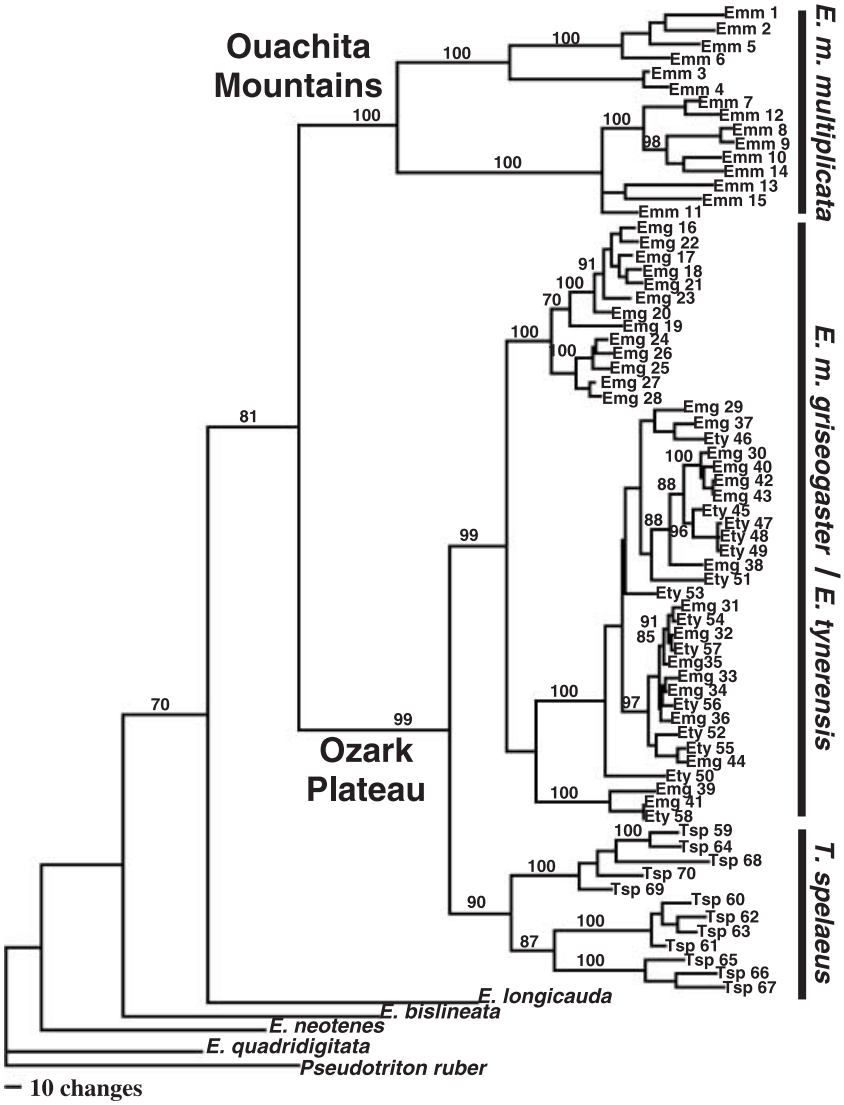

Fig. 3 Parsimony phylogram from a heuristic search using 100 random-taxon-addition replicates. Topology depicts the strict consensus of four equally parsimonious trees. Node support from bootstrap analysis based on 1000 pseudoreplicates is shown. Only bootstrap values of 50% or higher are shown. Support for some recent nodes in densely sampled clades is not shown, due to space constraints.

constraining *Eurycea* to be monophyletic only affects a few deeper nodes, and does not affect any lower level relationships, where most of the tree length among our 75 samples exists. In a subsequent test we made the same topological comparison as in test 3, but reduced the number of taxa, in both constrained and unconstrained trees, to include only relevant ones (one of each nominate taxon). In the reduced data set the unconstrained tree is significantly less likely to be monophyletic ($P < 0.028$) than the tree with all *Eurycea* constrained. In addition, more comprehensive analyses of hemidactyliine phylogeny show *Typhlotriton* to be nested consistently within *Eurycea* (Chippindale *et al.* in prep.).

The Ozark surface-dwelling clade is a paraphyletic mixture of transforming *E. m. grioseogaster* and paedomorphic *E. tynerensis* populations (Fig. 4). There is notably low mt sequence divergence between some populations of *E. m. griseogaster* (transforming) and *E. tynerensis* (paedomorphic) in the western Ozarks (uncorrected *P* as low as 0.06%; Table 2). Tracing life history mode on the BI tree via parsimony shows that paedomorphosis has arisen independently eight times within this clade. The MP tree analysis shows that paedomorphosis arose independently from three to nine times. This ambiguity in reconstruction on the MP tree primarily is due to the phylogenetic position of one paedomorphic individual (Ety 50), which renders a few key branches equivocal. Although this is the most parsimonious topology, there is less than 50% bootstrap support for the placement of specimen Ety 50.

Discussion

Biogeography and phylogeography

Biogeography of the Interior Highlands has received considerable attention due to the high level of regional

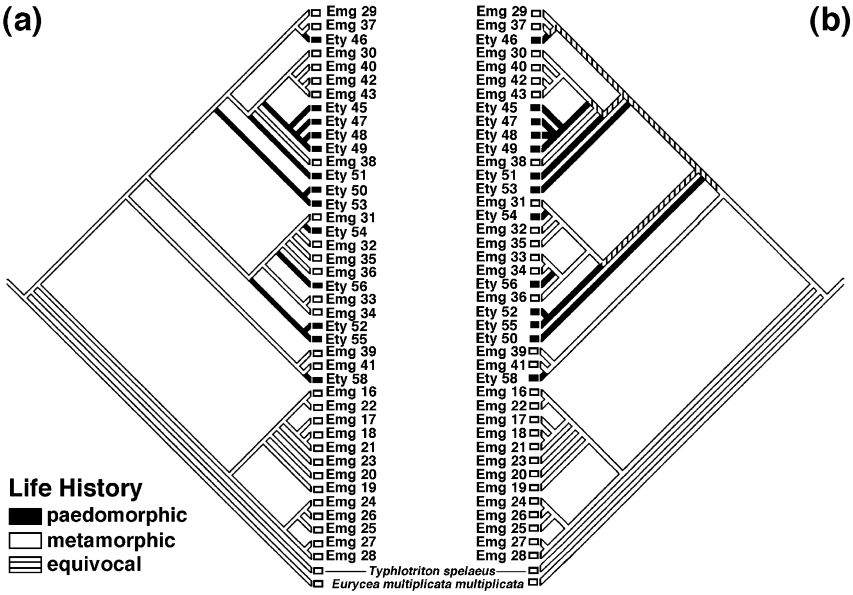

Fig. 4 Parsimony-based reconstruction of evolution of life history mode (paedomorphic vs. metamorphic) in the *E. multiplicata* complex on the Bayesian topology (a) and parsimony topology (b).

endemism (Mayden 1988; Routman *et al*. 1994; Crandall & Templeton 1999; Hardy *et al*. 2002). However, most studies to date have focused on faunal exchanges between the Interior Highlands and adjacent regions, such as the Appalachians, and surprisingly there has been little phylogeographical concordance among the organisms studied (Hardy *et al*. 2002). The *E. multiplicata* complex is a well-supported monophyletic group, and probably represents a single invasion of the Interior Highlands. The relationship of the *E. multiplicata* complex to other lineages of *Eurycea* is not well established, so we restrict our biogeographical interpretations to those within the Interior Highlands.

The deepest divergence within the *E. multiplicata* complex corresponds to the major physiographical features of the region, the Ouachita Mountains and the Ozark Plateau. The highlands were once (pre-Pleistocene) continuous (Quinn 1958), but are divided currently by the Arkansas River Valley. The precise time of origin of the Arkansas River is not clear. The river itself may be an ancient feature of the region, but has only recently (postglaciation) attained its present-day size, by capturing several midwestern rivers (Quinn 1958). Divergence time estimates between the Ouachita and Ozark clades, at 6.8–31.4 Myr depending on the clock used, suggest that this split may even predate the ancestral Arkansas River.

Our samples from the Ozark Plateau form a well-supported monophyletic group, and represent a radiation of all three morphological types. The Ozark populations can be divided into two distinct, and highly divergent, clades, one surface-dwelling and the other subterranean. The surface-dwelling clade includes transforming *E. m. griseogaster* and paedomorphic *E. tynerensis* and the subterranean clade contains the enigmatic cave-dweller, *Typhlotriton spelaeus*. The split between the surface-dwelling and subterranean lineages is relatively old (5.7–26.5 Myr). Interestingly, in the Edwards Plateau of central Texas a subterranean clade of *Eurycea* (formerly *Typhlomolge*) has similar levels of divergence from its nearby surface-dwelling relatives (Chippindale 2000; Chippindale *et al*. 2000; Hillis *et al*. 2001), paralleling what we report for surface-dwelling and subterranean lineages in the Ozarks.

Drainage history has been used primarily to explain biogeographical patterns observed among many aquatic organisms of the Central Highlands (Interior Highlands and southern Appalachians). Our results are not consistent with previous hypotheses regarding drainage evolution, because populations of *Eurycea* from a given drainage rarely form a monophyletic group. Most of the salamanders in the *E. multiplicata* complex, with the exception of paedomorphic ones, which have relatively localized distributions, require only an aquatic medium for larval development, and these salamanders are restricted typically to seeps and springs of headwater streams. Due to the (presumably) extremely low vagility of these salamanders, even small rivers may pose considerable barriers to dispersal rather than acting as corridors that facilitate it. Previous studies of the effects of drainage evolution on local fauna show that even habitat-specialized aquatic organisms have closely related populations in tributaries that flow into opposite sides of major rivers. For example, divergences as low as 0.91% occur between populations of the slender madtom catfish, *Noturus exilis*, from opposite sides of the Arkansas River (Hardy *et al*. 2002), whereas the Arkansas River marks the separation of the two most divergent lineages in the *E. multiplicata* complex.

There are considerable levels of divergence within each of the three major clades (Table 2), which may correspond to further subdivision of the Ouachitas and Ozarks. We are currently conducting studies of fine-scale haplotype diversity, including multiple individuals per population, to explore historical patterns of gene flow and vicariance within each clade.

Evolution of life history and morphology

Salamanders of the *E. multiplicata* complex, like all hemidactyliine plethodontids, have a free-living aquatic larval stage. Young larvae of all species in the complex appear almost identical, with external gills, pigmentation and functional eyes, which reflects their larval life in spring-fed streams. However, ontogenetic changes in morphology have led to disparate adult forms and exemplify the varying habitats utilized by adults. These changes include: (i) those associated with metamorphosis into a surface-dwelling terrestrial adult form; (ii) bypassing metamorphosis and becoming sexually mature while maintaining the aquatic larval form; and (iii) developing subterranean characteristics such as loss of pigmentation and eyes becoming covered with skin while metamorphosing into a subterranean adult. Despite equivalent levels of divergence between the Ouachita Mountain and Ozark Plateau clades, only surface-dwelling transforming salamanders occur in the Ouachitas, whereas all three morphological/life history types are present in the Ozarks. This disparity between these lineages appears to be driven by the geological complexity in the karst-based Ozark Plateau, which provides a diverse array of surface and subterranean aquatic habitats.

The surface-dwelling metamorphosing salamanders (all *E. m. multiplicata* and *E. m. griseogaster*) clearly do not represent a monophyletic assemblage. This is supported by results from SH test 1, as well as our phylogenetic analyses which show all populations of *E. m. griseogaster* to be related more closely to other Ozark salamanders (*E. tynerensis* and *T. spelaeus*) than to any *E. m. multiplicata* (endemic to the Ouachitas). The similarities in morphology and life history among surface-dwelling, transforming salamanders from the Ouachitas and Ozarks are probably the result of similar adaptations to the stream habitats where they occur or may reflect morphological evolutionary stasis, a well-documented phenomenon in plethodontid salamanders (e.g. Wake 1991). Eggs of *E. m. multiplicata* and *E. m. griseogaster* are laid from autumn to spring and larval development is relatively rapid, from 5 to 8 months (Petranka 1998). This reduction in the length of larval period may have allowed epigean *Eurycea* to colonize the Interior Highlands, and metamorphosis may occur due to the necessity to exit seasonally ephemeral aquatic habitats (Ryan & Bruce 2000).

Among the 10 recognized families of salamanders, nine demonstrate paedomorphosis, defined in part by the retention of gills in reproductive adults. Paedomorphosis apparently has independently evolved in many families, and in five of these (Ambystomatidae, Dicamptodontidae, Hynobiidae, Salamandridae and Plethodontidae) it is polymorphic within species or variable among genera (Wilbur & Collins 1973; Petranka 1998). It is also probable that paedomorphosis has evolved multiple times within hemidactyliine plethodontids (e.g. Wake 1966; Chippindale *et al*. 2000).

Our results indicate that most of the paedomorphic populations in our study do not form a monophyletic group. Several populations of *E. tynerensis* are related more closely to *E. m. griseogaster* than to other *E. tynerensis*. It appears that life history (paedomorphosis vs. metamorphosis) is highly variable in the western Ozark populations of *E. m. griseogaster*/*E. tynerensis* (Fig. 4). *E. m. multiplicata* and *T. spelaeus* are believed to be completely metamorphic (Brandon 1966; Ryan & Bruce 2000) and under this premise the ancestral state for the *E. m. griseogaster*/*E. tynerensis* clade is metamorphic. Parsimony-based ancestral state reconstruction of life history mode in this clade shows that paedomorphosis has arisen independently eight times given the Bayesian topology (Fig. 4a), and from three to nine times on the parsimony topology (Fig. 4b). The close relationships among populations suggest that this is a result of phenotypic plasticity (facultative paedomorphosis) or local selection on alleles that control life history mode.

Many workers have studied the mechanism and evolution of paedomorphosis in salamanders, but most have focused on ambystomatids (e.g. Shaffer 1984; Semlitsch *et al*. 1990; Ryan & Semlitsch 1998; Voss *et al*. 2003) or salamandrids (Harris 1987; Denöel & Joly 2000). Very little attention has been paid to plethodontids in this regard (reviewed in Ryan & Bruce 2000), probably because most paedomorphic species have limited distributions and/or are endangered, making them difficult subjects of study. To date, work on paedomorphosis in the *Eurycea multiplicata* complex has been limited to a single demonstration of thyroxin-induced transformation in *E. tynerensis* (Kezer 1952).

Life history mode can be a facultative process in which ecological conditions are the deciding factors, as opposed to it being strictly a genotypic effect (Whiteman 1994). In the *E. tynerensis*/*E. m. griseogaster* group most populations are either completely paedomorphic or completely transforming. Local habitat conditions may provide cues that determine life history mode. Preliminary ecological data suggest that there is a strong correlation between various habitat parameters (especially substrate type) and life history mode (R.M. Bonett unpubl.). Other studies have also shown that the presence of '*E. tynerensis*' is significantly associated with streams containing Silurian and

Ordivician chert gravel (see especially Tumlinson & Cline 2003). There also is evidence of genetic variability for metamorphosis in closely related lineages of salamanders (e.g. Harris *et al*. 1990; Voss *et al*. 2003). Recent candidate gene analysis has identified specific genes (thyroxin hormone receptors) that may be responsible for variation in the timing of metamorphosis in *Ambystoma* (Voss *et al*. 2003). In plethodontids the ecological or genetic basis of metamorphosis is unknown and, to date, unexplored. Our discovery that life history is highly polymorphic in these relatively common salamanders provides a possible model for the further study of evolution of paedomorphosis in plethodontids.

Morphological characteristics associated with a subterranean existence are seen in members of diverse animal phyla (Culver 1982). In salamanders these characteristics include the loss of pigmentation, reduction of eyes, elongation of limbs, increased numbers of teeth and usually paedomorphosis (Brandon 1971). These character states have evolved independently in two distantly related families of salamanders, the Proteidae and the Plethodontidae, and multiple times within the hemidactyliine plethodontids (Petranka 1998; Wiens *et al*. 2003; Chippindale *et al*. in prep.). There is some debate over whether these characteristics are true adaptations or the result of mutations accumulating from relaxed selection, and in some cases cave-associated morphology may simply result from development in the absence of light (reviewed in Brandon 1971).

Larval *Typhlotriton* can be found in surface springs and streams and have typical epigean morphology, but lose pigment and functionality of eyes during ontogeny as adults shift to a subterranean existence. *Typhlotriton* raised in light maintain some pigmentation and the eyelids will not fuse (Noble & Pope 1928), which suggests that for this species, degree of subterranean morphology is the result of genetic and environmental interactions. Among the cave-dwelling aquatic salamanders *T. spelaeus* is the only one that is not paedomorphic, demonstrating that paedomorphosis is not a prerequisite for subterranean existence (Ryan & Bruce 2000). It has been proposed that some populations of *T. spelaeus* (formerly considered *T. nereus*) are paedomorphic (Bishop 1944); however, this has been strongly refuted (Brandon 1966).

Taxonomic implications

Mitochondrial divergences within currently recognized species in the *E. multiplicata* complex are as high as 15.7%, with divergences as high as 11.3% within the 'subspecies' *E. m. multiplicata*. Recognition of species based solely on patterns of mt sequence divergence is controversial (e.g. see Wiens & Penkrot 2002, who generally favour this approach). However, the relationships revealed by mtDNA are highly consistent with geography and suggest that the current taxonomy of the group is inconsistent with evolutionary history. Furthermore, the levels of divergence within 'species' in the *E. multiplicata* complex are equivalent to or greater than those separating other recognized salamander species (e.g. Chippindale *et al*. 2000; Jockusch *et al*. 2001). We are currently investigating additional, independently evolving nuclear loci, to further delimit species boundaries within this group. None the less, phylogenetic patterns uncovered by our mt sequence data reveal some obvious taxonomic issues that can be addressed here.

E. multiplicata was described by Cope (1869) from specimens collected near Ft Towson in southeastern Oklahoma, and subsequently reported from several localities from throughout the Interior Highlands (Dunn 1926). *E. tynerensis* was described from the western Ozarks based upon its paedomorphic 'nature' (Moore & Hughes 1939). Shortly thereafter, Moore & Hughes (1941) described *E. griseogaster* from a series of transformed salamanders collected from a tributary of the Illinois River, < 50 km from the type locality of *E. tynerensis*. Since then there has been much confusion over the number and identification of species of paedomorphic and surface-dwelling transforming salamanders within the Interior Highlands (Bishop 1944; Dundee 1947, 1965; Ireland 1971, 1976; Thornhill 1990; Tumlison *et al*. 1990). Our populations from south of the Arkansas River (Emm 1–Emm 15), including specimens from near (< 5 km) the type locality of *E. m. multiplicata* (Emm 1), form a well-supported monophyletic group and are highly divergent from *E. m. griseogaster* (uncorrected *P* from 13.53 to 15.68%). We suggest that only these populations should carry the specific epithet *multiplicata*. We expect to divide the Ouachita group into additional species given the high levels of regional mt divergence. Our sampling of *E. m. griseogaster* and *E. tynerensis* includes individuals from 41 populations (28 transforming and 13 paedomorphic). Although it is likely that more than one species may be present among these populations, individuals from the type localities of *griseogaster* (Emg 37) and *tynerensis* (Ety 46) share haplotypes that are very similar (maximum 2% divergent, similar to the levels of haplotype divergence seen within some populations), providing little evidence that they are different species. The most reasonable explanation is that there is a single species in the western Ozarks that contains transforming and paedomorphic populations. This is supported further by our finding that many populations of paedomorphic salamanders are related more closely to tranforming salamanders than to other paedomorphs (Figs 2 and 3). The description of *E. tynerensis* (Moore & Hughes 1939) predates that of *E. griseogaster* (Moore & Hughes 1941), so we recommend that all Ozark populations in this clade be referred tentatively to *E. tynerensis*. Additional species will almost certainly need

to be recognized and we currently are preparing formal descriptions of these.

The sister relationship between *T. spelaeus* and the *E. tynerensis* clade is very well supported, and combined with results of the reduced-taxon SH test (plus other molecular data, Chippindale *et al*. in prep.) this shows the placement of *Typhlotriton* outside *Eurycea* to be improbable. Thus, we suggest that *Typhlotriton* should be synonomized under *Eurycea* according to the Linnean system of classification. However, the clade name *Typhlotriton* could still be retained under Phylocode (e.g. Cantino *et al*. 1999; Hillis *et al*. 2001). Mitochondrial sequence divergence among populations of *Typhlotriton* also are high (up to 10.8%). The previously described species *T. nereus* (Bishop 1944) and *T. braggi* (Smith 1968) have been synonymized under *T. spelaeus* (Brandon 1966; Brandon & Black 1970), although additional molecular data may demonstrate that some of these taxa should be resurrected, and additional new species may need to be described.

Conclusions

The *Eurycea multiplicata* complex represents a single ancient invasion of the Interior Highlands, and relationships among the morphologically diverse members of the group based on mitochondrial sequences are consistent with geography. The two most highly divergent lineages in the complex correspond to the Ouachita Mountains and the Ozark Plateau. All populations from the Ouachita Mountains are surface-dwelling and metamorphose, while the more diverse habitats of the Ozark Plateau harbour paedomorphic surface-dwelling, metamorphic surface-dwelling and metamorphic subterranean salamanders. The Ozark lineage contains two highly divergent clades, one surface-dwelling and the other subterranean. Transformation occurs in most members of the complex, but is highly labile among surface-dwelling populations in the western Ozarks, providing a novel system for studying the evolution of paedomorphosis in plethodontid salamanders. We recommend that the Ouachita clade be referred to as *E. multiplicata*, the Ozark surface-dwelling clade (including transforming and paedomorphic populations) as *E. tynerensis* and the Ozark subterranean clade as *E. spelaeus*. High levels of sequence divergence occur within each of these clades, suggesting that additional species should be recognized in the Interior Highlands.

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Appendix I

Specimen information

Tree code/ map number	Locality	Museum Accession no.	GenBank Accession no. ND4	GenBank Accession no. CYTB
Emm 1	OK: Choctow Co., near Ft Towson	UTA 56367	AY 528255	AY 528330
Emm 2	OK: McCurtain Co., Cedar Creek	UTA 56368	AY 528256	AY 528331
Emm 3	OK: Atoka Co., Trib. to Chicksaw Creek	UTA 56369	AY 528257	AY 528332
Emm 4	OK: Pushmataha Co., Crumb Creek	No voucher	AY 528258	AY 528333
Emm 5	AR: Polk Co., Bogg Springs	UTA 56355	AY 528259	AY 528334
Emm 6	AR: Polk Co., Queen Creek	UTA 56356	AY 528260	AY 528335
Emm 7	AR: Saline Co., Owensville	UTA 56350	AY 528261	AY 528336
Emm 8	AR: Perry Co., Haydon Brook	UTA 56351	AY 528262	AY 528337
Emm 9	AR: Conway Co., Rose Creek	UTA 56352	AY 528263	AY 528338
Emm 10	AR: Saline Co., Williams Creek	UTA 56353	AY 528264	AY 528339
Emm 11	AR: Montgomery Co., Brushy Creek	UTA 56354	AY 528265	AY 528340
Emm 12	AR: Garland Co., Mazarn Creek	UTA 56357	AY 528266	AY 528341
Emm 13	OK: Leflore Co., Gap Creek	UTA 56370	AY 528267	AY 528342
Emm 14	AR: Yell Co., Turner Brook	UTA 56358	AY 528268	AY 528343
Emm 15	AR: Logon., Co., Mount Magazine	UTA 56359	AY 528269	AY 528344
Emg 16	AR: Conway Co., Cypress Creek	UTA 56372	AY 528270	AY 528345
Emg 17	AR: Searcy Co., near Noahs	UTA 56373	AY 528271	AY 528346
Emg 18	AR: Van Buren Co., Weaver Creek	UTA 56374	AY 528272	AY 528347
Emg 19	AR: White Co., Little Creek	UTA 56375	AY 528273	AY 528348
Emg 20	AR: Clerburne Co., near Ida	UTA 56376	AY 528274	AY 528349
Emg 21	AR: Baxter Co., Farris Spring	UTA 56377	AY 528275	AY 528350
Emg 22	AR: Van Buren Co., near Scotland	UTA 56378	AY 528276	AY 528351
Emg 23	AR: White Co., near Clay	UTA 56379	AY 528277	AY 528352
Emg 24	AR: Searcy Co., Glenco Spring	UTA 56380	AY 528278	AY 528353
Emg 25	AR: Marion Co., Gray Spring	UTA 56381	AY 528279	AY 528354
Emg 26	AR: Marion Co., Caney Cave	UTA 56382	AY 528280	AY 528355
Emg 27	AR: Newton Co, near Norton Gap	UTA 56383	AY 528281	AY 528356
Emg 28	AR: Pope Co., Trib. to Big Piney Creek	No voucher	AY 528282	AY 528357
Emg 29	AR: Crawford Co., Cedar Creek	UTA 56384	AY 528283	AY 528358
Emg 30	AR: Washington Co., Iron Bridge	UTA 56385	AY 528284	AY 528359
Emg 31	AR: Benton Co, Spakner Creek	UTA 56386	AY 528285	AY 528360
Emg 32	AR: Benton Co., Ashmore Creek	UTA 56387	AY 528286	AY 528361
Emg 33	AR: Madison Co., near Rockhouse	UTA 56388	AY 528287	AY 528362
Emg 34	AR: Newton Co., Low Gap Springs	UTA 56389	AY 528288	AY 528363
Emg 35	AR: Newton Co., near Jasper	UTA 56390	AY 528289	AY 528364
Emg 36	AR: Johnson Co., Ozone Rec. Area	UTA 56391	AY 528290	AY 528365
Emg 37	OK: Sequoyah Co., 10 mi NE of Gore	UTA 56398	AY 528291	AY 528366
Emg 38	OK: Sequoyah Co., near Cookson	UTA 56399	AY 528292	AY 528367
Emg 39	OK: Sequoyah Co., Tin Cup Creek	UTA 56402	AY 528293	AY 528368
Emg 40	OK: Sequoyah Co., near Muldrow	UTA 56403	AY 528294	AY 528369
Emg 41	OK: Sequoyah Co., Pole Cat Creek	UTA 56400	AY 528295	AY 528370
Emg 42	OK: Cherokee Co., McLeeland Spring	UTA 56408	AY 528296	AY 528371
Emg 43	OK: Cherokee Co., N. McLeeland Spring	UTA 56409	AY 528297	AY 528372
Emg 44	MO: Pulaski Co., Mud Cave	UT 56512	AY 528298	AY 528373
Ety 45	OK: Cherokee Co., Rock Creek	UTA 53860	AY 528299	AY 528374
Ety 46	OK: Cherokee Co., Trib. of Tyner Creek	UTA 56397	AY 528300	AY 528375
Ety 47	OK: Adir Co, near Strawberry Springs	UTA 56407	AY 528301	AY 528376
Ety 48	OK: Adir Co., Peavine Creek	UTA 56405	AY 528302	AY 528377
Ety 49	OK: Adir Co., Ballard Creek	UTA 56401	AY 528303	AY 528378
Ety 50	OK: Mayes Co., Snake Creek	UTA 56406	AY 528304	AY 528379
Ety 51	OK: Delaware Co., Spring near Colcord	UTA 56410	AY 528305	AY 528380
Ety 52	MO: McDonald Co., Mill Creek	UTA 56392	AY 528306	AY 528381
Ety 53	MO: McDonald Co., Mike's Creek	UTA 56393	AY 528307	AY 528382

Appendix I *Continued*

Tree code/ map number	Locality	Museum Accession no.	GenBank Accession no. ND4	GenBank Accession no. CYTB
Ety 54	MO: McDonald Co., Big Sugar Creek	UTA 56394	AY 528308	AY 528383
Ety 55	MO: Stone Co., Pine Run	UTA 56395	AY 528309	AY 528384
Ety 56	MO: Christian Co., Buseik S.F.	Not catalogued*	AY 528310	AY 528385
Ety 57	MO: Taney Co., Protem	UTA 56396	AY 528311	AY 528386
Ety 58	OK: Sequoyah Co., Little Lee Creek	UTA 56404	AY 528312	AY 528387
Tsp 59	AR: Benton Co., Blowing Springs	UTA 56360	AY 528313	AY 528388
Tsp 60	AR: Madison Co., near Clifty	UTA 56361	AY 528314	AY 528389
Tsp 61	AR: Boone Co., Alcohol Springs	UTA 56362	AY 528315	AY 528390
Tsp 62	AR: Stone Co., Salt and Peter Cave	UTA 56363	AY 528316	AY 528391
Tsp 63	AR: Independence Co., Cushman Cave	UTA 53865	AY 528317	AY 528392
Tsp 64	MO: Barry Co., Rockhouse Cave	UTA 56364	AY 528318	AY 528393
Tsp 65	MO: Stone Co., Hooten Cave	TNHC 53853	AY 528319	AY 528394
Tsp 66	MO: Pulaski Co., Piquet Cave	UTA 56365	AY 528320	AY 528395
Tsp 67	MO: Pulaski Co., Mud Cave	UTA 56366	AY 528321	AY 528396
Tsp 68	OK: Mayes, Pipe Sring	UTA 53846	AY 528322	AY 528397
Tsp 69	OK: Adir Co., Cherry Tree	UTA 56371	AY 528323	AY 528398
Tsp 70	OK: Cherokee Co., Talequah	No voucher	AY 528324	AY 528399
E. neotenes	TX: Bexar Co., Helotes Creek	TNHC 60313	AY 528325	AY 528400
E. quadridigitata	TX: Smith Co., Tyler	UTA 56412	AY 528326	AY 528401
E. bislineata	NY: Chenango Co., Genegantslet R.	UTA 56411	AY 528327	AY 528402
E. longicauda	PA: Washington Co.	CMNH 147803	AY 528328	AY 528403
P. ruber	NY: Sullivan Co.	No voucher	AY 528329	AY 528404

*Ety 56 is not catalogued, but TNHC 54006–54010 are from the same field series.
Emm = *Eurycea multiplicata multiplicata*.
Emg = *E. m. griseogaster*.
Ety = *E. tynerensis*.
Tsp = *Typhlotriton spelaeus*.