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Phylogeography of the Brownback Salamander reveals patterns of local endemism in Southern Appalachian springs

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ABSTRACT

The Appalachian Mountains of eastern North America are characterized by high faunal diversity and many endemic species, especially in the unglaciated southern latitudes where lineages have been accumulating for tens of millions of years. The Brownback Salamander, *Eurycea aquatica*, is an enigmatic species that dwells in isolated springs in southeastern North America. *Eurycea aquatica* have often been dismissed as simply robust spring-adapted ecomorphs of the widespread and more gracile species *Eurycea cirrigera*. We sequenced the mitochondrial gene encoding NADH dehydrogenase subunit-2 (*ND2*; 753 bp) and the nuclear recombination activating gene-1 (*Rag1*; 1201 bp) for *E. aquatica* (*ND2* $n = 72$; *Rag1* $n = 17$) from across their presumed distribution and compared them to *E. cirrigera* (*ND2* $n = 23$; *Rag1* $n = 10$) from nearby populations. Using phylogenetic and morphological analyses we explicitly test if *E. aquatica* in the Southern Appalachians is simply a local spring-adapted ecomorph of *E. cirrigera* or a single lineage that resulted from fragmentation of (or dispersal to) spring habitats. We found that *E. aquatica* from isolated springs form a well-supported monophyletic group that is nested among *E. cirrigera*, *E. wilderae*, and *E. junaluska*. Furthermore, we uncovered three very divergent lineages of *E. aquatica* that we estimate have been isolated from each another since the early Pliocene to late Miocene (2.5–6.1 Myr) and may each represent distinct species. The distribution of these lineages is coincident with the distribution of other endemic spring-dwelling vertebrates, and suggests that this region may be a relictual habitat for an unexpected diversity of unrecognized endemics.

1. Introduction

The Appalachian Mountains are a region containing high levels of biodiversity and endemism, with many cryptic lineages that have only been recognized through genetic analyses (Highton, 1995; Tilley and Mahoney, 1996; Kozak and Wiens, 2006; Thomas and Hedin, 2008). Much of the species richness is restricted to high elevations, and these mountaintop communities have been afforded the greatest conservation protection (Stein et al., 2000). By comparison, the mid-elevation Appalachian fauna receives relatively little conservation attention, despite the fact that it includes many endemic taxa and divergent genetic lineages (Wynn et al., 1988; Anderson and Tilley, 2003; Kozak et al., 2005, 2006; Bonett et al., 2007). Furthermore, there have been relatively few studies that examine genetic diversity of extensive mid-elevation regions, such as the Ridge and Valley and Appalachian Plateau of northern Alabama and Georgia, making it difficult to estimate their actual faunal richness.

Lungless salamanders of the family Plethodontidae show high levels of lineage diversity (Garcia-Paris et al., 2000; Martinez-Solano et al., 2007), ecomorphological divergence (Parra-Olea and Wake, 2001; Bonett and Chippindale, 2004; Kozak et al., 2005; Niemiller et al., 2008) and convergence (Wiens et al., 2003) on very small geographic scales. These patterns, particularly among closely related species, can confound estimates of diversity, species boundaries, and relationships of plethodontid salamanders based on morphology alone (Larson and Chippindale, 1993; Wiens et al., 2003).

Members of the *Eurycea bislineata* complex are small plethodontid salamanders found in a variety of freshwater habitats throughout eastern North America. There are currently five nominate taxa recognized in this group: *E. aquatica*, *E. bislineata*, *E. cirrigera*, *E. junaluska*, and *E. wilderae*. *Eurycea bislineata*, *E. cirrigera* and E. *wilderae* are relatively slender (gracile) with narrow heads and long tails. Whereas, the remaining taxa, *E. aquatica* and *E. junaluska*, exhibit a robust morphology with large heads, thick trunks, and short tails relative to other members of this complex (Rose and Bush, 1963; Sever et al., 1976). However, some populations of the typically gracile species, *E. cirrigera* and *E. wilderae*, contain adult males that possess wide heads and stocky bodies

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(Mount, 1975; Sever, 1979, 2005; Jacobs, 1987; Smith, 2008). In some instances, gracile and robust individuals of *E. cirrigera* occur in sympatry, but in all cases examined thus far they are genetically identical (Wallace, 1975; Jacobs, 1987), indicating that robust body size is a relatively labile characteristic within these two species.

The Brownback Salamander (*E. aquatica*) was first described by Rose and Bush (1963) as a moderately sized, stocky *Eurycea* inhabiting spring habitats across the Ridge and Valley of north-central Alabama. Its range was later extended to include the Ridge and Valley and Appalachian Plateau Physiographic Provinces of Alabama and Georgia (Conant and Collins, 1998; this study; Fig. 1). Diagnosis of *E. aquatica* was based on its dark coloration, robust morphology (i.e., wide head, thick trunk, and short tail), increased egg number, and habitat preference (i.e., springs), which differentiated it from a more widespread member of the complex, *E. cirrigera* (Rose and Bush, 1963). Nonetheless, in the past *E. aquatica* was dismissed as a local spring-adapted ecomorph of the Southern Two-lined Salamander, *E. cirrigera* (Folkerts, 1968; Mount, 1975; Petranka, 1998). Subsequent genetic analyses have shown that *E. aquatica* is distinct from *E. cirrigera* (Jacobs, 1987; Kozak et al., 2006), and is now currently recognized as a full species (Kozak et al., 2006; Tilley et al., 2008). However, in past genetic studies sampling was limited to the type locality of *E. aquatica* and did not include nearby samples of *E. cirrigera* (Jacobs, 1987; Kozak et al., 2006). Given that populations resembling *E. aquatica* occur in isolated springs across northern Alabama and Georgia, there are two alternative hypotheses that may explain this pattern: (1) spring-adapted ecomorphs (i.e., *E. aquatica*) are from a single lineage that subsequently dispersed to springs across the region or (2) spring-adapted ecomorphs evolved independently multiple times when *E. cirrigera* colonized isolated springs across the region (e.g., Folkerts, 1968; Mount, 1975).

Here, we test these competing hypotheses using mitochondrial (*mt*) and nuclear (*nuc*) DNA sequences of a thorough sampling of salamanders from springs and streams throughout the Ridge and Valley and Appalachian Plateau (i.e., Cumberland Plateau and Highland Rim) of Alabama and Georgia. We find that *E. aquatica* diverged from other members of the *E. bislineata* complex during the Miocene and spread to springs across the region. These populations have since experienced substantial isolation that has generated three divergent genetic lineages. We discuss our results in the context of the largely unrecognized biodiversity and endemism of spring-dwelling species in this region, which are currently threatened by multi-causal habitat destruction.

2. Methods

2.1. Sampling

Tissues of 158 *E. aquatica* and *E. cirrigera* were collected from 38 localities in Alabama, Georgia, and Tennessee (Fig. 1, Appendix 1). Fourteen of the springs were within the presumed range of *E. aquatica*, including most of the historical localities sampled by Jones (1980) in the Ridge and Valley of Alabama and Georgia. Addition-

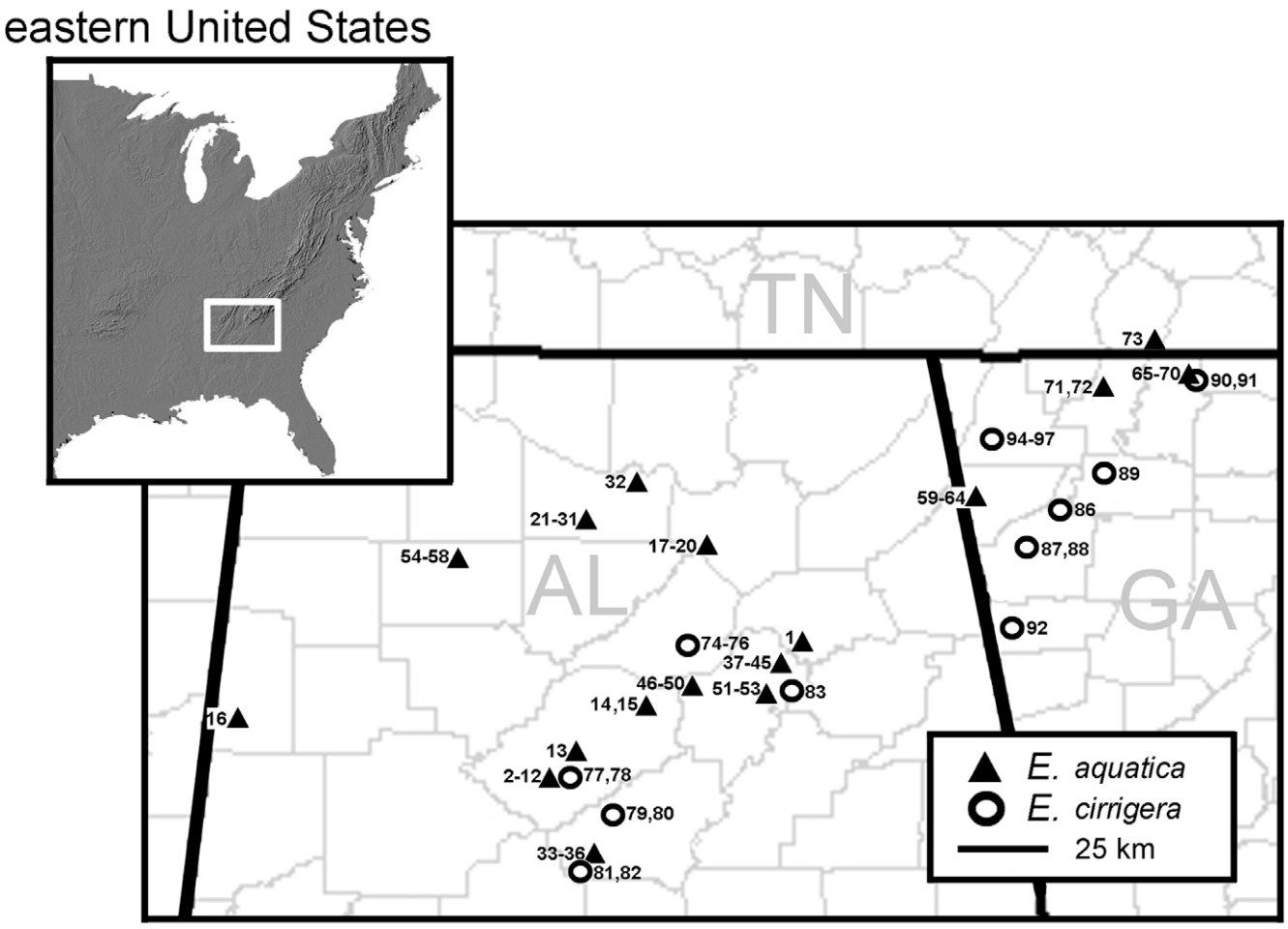

Fig. 1. Distribution of sampling localities of *E. aquatica* (filled triangles) and *E. cirrigera* (open circles) in northern Alabama (AL), northern Georgia (GA), and Tennessee (TN). Inset map is of the eastern United States with the main map highlighted with a white box.

ally, we expanded our sampling to include eight localities in the Cumberland Plateau ($n = 4$), Highland Rim ($n = 3$), and Fall Line Hills ($n = 1$) of northern Alabama where we and other authors (Mount, 1975; Jones, 1980) have found populations of robust *Eurycea* that resemble *E. aquatica* and others that were of ambiguous assignment. We also collected samples that morphologically resemble *E. cirrigera* from 12 nearby populations (3 to 20 km away). Tail tips were collected in the field and stored in 95% ethanol (EtOH), and approximately 75 total individuals were collected for morphological analyses. These animals were euthanized by cutaneous administration of Orajel®, and liver tissues were collected and stored in 95% EtOH.

2.2. DNA extraction, amplification, and sequencing

DNA was extracted from EtOH-preserved *E. aquatica* and *E. cirrigera* tissues using a Qiagen DNEasy kit. We sequenced a portion of the mitochondrial gene encoding NADH dehydrogenase subunit-2 (*ND2*) because of its high level of variation between species and previous use in a phylogeographic study of the *E. bislineata* complex (Kozak et al., 2006). A 753 base pairs (bp) portion of *ND2* was amplified with the primers EaqND2Fc 5′-CCATATGCCTTT TCAATAYTAATTT CTAG-3′ and EaqND2Ra 5′-GCTTTGAAGGCCY TTGGTCT-3′ for 96 putative *E. aquatic* and *E. cirrigera* and one *E. junaluska* via polymerase chain reaction (PCR). Amplifications were performed with standard reagents and the thermocycler regime of Macey et al. (1998). In addition we amplified a 1201 bp portion of the independent, but evolutionarily more conserved nuclear recombination activating gene-1 (*Rag1*) for 27 representative individuals using the primers EuryceaRag1F 5′-GGTAYGATGTTGCA TTGGTTGCCA-3′, Rag1midElongFb 5′-TGCACTGTGAYATNGGGAA TGCTG-3′, ElongRag1R 5′-TTGACTGCCATCGCTTCCTCT CTT-3′, and Rag1endElongRb 5′-AACTTGGACTGCCTGGCGTTCATT-3′. PCR products were sized on 1% agarose gels and successful products were cleaned using EXOSAPIT (USB Corp). Sequencing reactions were performed using BigDye v3.1 (Applied Biosystem Inc.). Unincorporated dye terminators were cleaned with Sephadex G-50 (Invitrogen), and reaction products were run on an ABI 3130xl capillary sequencer in both directions.

2.3. Phylogenetic analyses and hypothesis testing

Sequences were aligned and edited using Sequencher 4.8 (Gene Code, Ann Arbor, MI). Alignments of both *ND2* and *Rag1* were unambiguous and had no length variation or stop codons. Additionally, we obtained *ND2* sequences for nine individuals from the *Eurycea bislineata* complex and two outgroups, *E. longicauda* (Kozak et al., 2006) and *Pseudotriton ruber* (Mueller et al., 2004), as well as an *E. longicauda* (Wiens et al., 2005), *E. quadridigitata* (Chippindale et al., 2004), and *P. ruber* (Wiens et al., 2005) *Rag1* sequence from Genbank. The final alignments included 753 bp of *ND2* for 107 individuals and 1201 bp of *Rag1* for 31 individuals. Sequences were deposited in Genbank (Appendix 1).

We individually analyzed *ND2* and *Rag1* with Bayesian Analysis (BA) and Maximum Parsimony (MP). Employing PAUP* v. 4.0b10 (Swofford, 2001), we performed MP analyses using heuristic searches of 100 random-taxon-addition replicates. To assess confidence at each node we employed non-parametric bootstrapping (Felsenstein, 1985) based on 1000 pseudoreplicates and 10 random taxon-addition-replicates per pseudoreplicate. We partitioned each gene by codon position and used MrModeltest (Nylander, 2005) to determine the most appropriate model of nucleotide substitution for each data partition. We ran partitioned Bayesian analyses (all partitions unlinked) implemented via MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) using four chains and uniform priors for five million generations and saved a tree every 1000 generations. We conservatively discarded the first 1000 trees (one million generations) as burn-in. We used PAUP* (Swofford, 2001) to calculate a 50% majority-rule consensus tree of the 4001 post burn-in BA trees. Both BA and MP analyses were rooted with *Pseudotriton ruber*, which is a member of the lineage that appears to be the sister taxon to the Genus *Eurycea* (Chippindale et al., 2004; Mueller et al., 2004).

We used the Shimodaira–Hasegawa test (SH-test; Shimodaira and Hasegawa, 1999) implemented in PAUP* (Swofford, 2001) to compare the −ln likelihoods of alternative phylogenetic hypotheses that could explain the distribution of *E. aquatica* in isolated springs across the Ridge and Valley, Cumberland Plateau, and Highland Rim Provinces of northern Alabama and Georgia. If a single lineage of robust spring-adapted salamanders (i.e., *E. aquatica*) diverged from other spring members of the *E. bislineata* complex and subsequently spread across the Ridge and Valley, Cumberland Plateau, and Highland Rim, then these populations should form a monophyletic group. However, if *E. aquatica* is simply a local spring-adapted ecomorph of *E. cirrigera*, isolated populations of *E. aquatica* should be genetically most similar to nearby populations of *E. cirrigera*. The SH-test was performed in a likelihood framework using the *ND2* alignment with the HKY + Γ model and parameters determined by MrModeltest 2.2 (Nylander, 2005). We reduced sampling to include only the most divergent representatives of *E. aquatica* and *E. cirrigera* from the Ridge and Valley, and Cumberland Plateau/Highland Rim as well as appropriate outgroups. *Eurycea aquatica* was monophyletic in all of our phylogenetic analyses (see Section 3), and therefore this was our unconstrained topology, which represents hypothesis 1. To test this against the alternative hypothesis we constrained three pairs of *E. aquatica* and *E. cirrigera* from nearby populations in the same drainage to be monophyletic. The drainages are as follows: (1) Cahaba Drainage (Birmingham Valley; Eaqu34 and Ecir81); (2) Coosa Drainage (Coosa Valley; Eaqu69 and Ecir91); (3) Warrior Drainage (Cumberland Plateau; Eaqu57 and Ecir75). We also performed the SH-test using trees with only a pair of *E. aquatica* and *E. cirrigera* from a single drainage constrained to be monophyletic.

To estimate divergence times of *E. aquatica* from other members of the *E. bislineata* complex, as well as among the major lineages of *E. aquatica*, we employed a molecular clock approach based on uncorrected pairwise sequence divergence of *ND2* calculated in PAUP* (Swofford, 2001) at a rate of 1.28% change per million years (Weisrock et al., 2001).

2.4. Morphological comparisons

Previous attempts to morphologically distinguish *E. aquatica* from *E. cirrigera* led to equivocal results since individuals were assigned to groups more-or-less arbitrarily (Jones, 1980). For this study, the initial assignment to species was based on identification in the field then confirmed by phylogenetic position based on *ND2* sequences (see Section 3). Morphological measurements were taken for adult *E. aquatica* ($n = 48$) from 17 populations and *E. cirrigera* ($n = 28$) from 11 nearby populations.

Our measurements, following those of Jones (1980), included snout-vent length, tail length, total length, trunk length, trunk width at the midpoint of trunk, average hind limb length (AHLL), average front limb length, head width (HW) at its widest point, head length (HL), and costal groove number. Additional measurements were calculated based on the above morphological data, including relative tail length (RTL), which is the difference between the total length and snout-vent length, divided by the snout-vent length (Rose and Bush, 1963), trunk length divided by trunk width, excess trunk length, which is trunk length minus the average length of the front and hind limbs (Sever, 1972; Jones, 1980), and head length divided by head width (HL/HW). Individuals with bro-

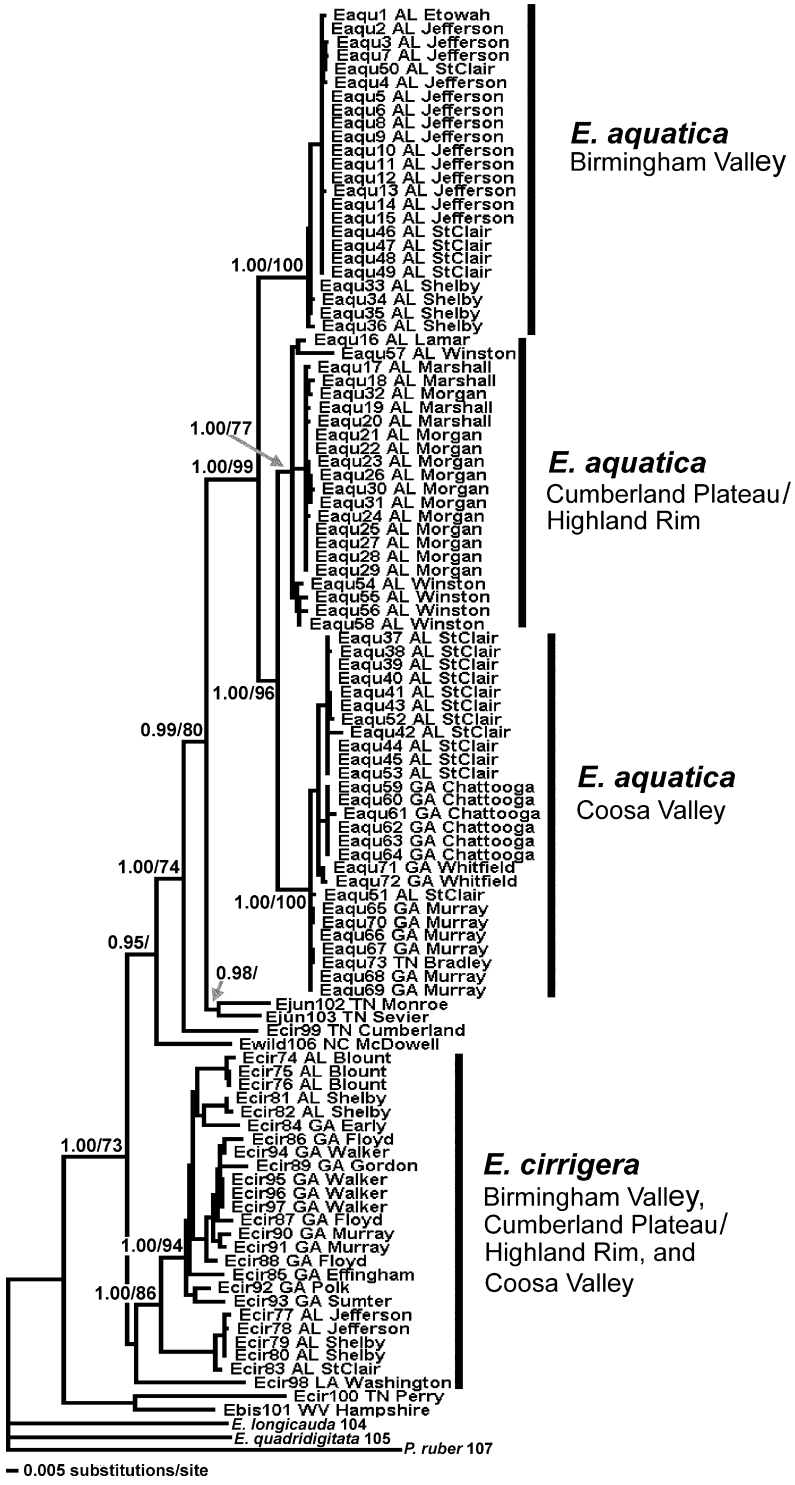

Fig. 2. Bayesian phylogram of *ND2* for *E. aquatica* and *E. cirrigera*. Numbers subtending each branch represent Bayesian posterior probabilities (before slash) and Maximum Parsimony bootstrap values (after the slash) for the major clades.

ken or clearly regenerated tails were excluded from all analyses involving tail measurements. Animals were sexed based on the presence of enlarge premaxillary teeth and large cirri in adult males and eggs in adult females. Independent sample *T*-tests were run for all morphological characters using SPSS 14.0 statistical software, and all analyses were considered significant if $p < 0.05$. Additionally, we used principle components analysis (PCA) to extract a limited number of independent variables or factors from the measured characters in SPSS 14.0. Only factors with Eigen values >1.0 were considered because factors <1.0 accounted for less of the variation among individuals than the original variables (Norusis, 1994).

3. Results

Three hundred and eighty-four of the *ND2* characters were variable and 262 were parsimony informative out of 753 bp. Bayesian and Maximum Parsimony analyses of *ND2* show individuals morphologically assignable to *E. aquatica* from the Ridge and Valley

Table 1
Results of Shimodaira–Hasegawa tests for four topological comparisons between the unconstrained ML analysis and ML analyses with *E. aquatica* and *E. cirrigera* from the same drainage constrained to be monophyletic (alone and in combination).

Test	Topology	−ln likelihood	Difference in −ln likelihood	*p*
1	Unconstrained analysis — *E. aquatica* monophyletic	3242.33	—	—
2	All pairs of *E. aquatica* and *E. cirrigera* from the same drainage constrained	3435.05	192.72	<0.001
3	*E. aquatica* and *E. cirrigera* from the Cahaba Drainage (Birmingham Valley) constrained	3318.69	76.37	<0.001
4	*E. aquatica* and *E. cirrigera* from the Coosa Drainage (Coosa Valley) constrained	3364.45	122.12	<0.001
5	*E. aquatica* and *E. cirrigera* from the Warrior Drainage (Cumberland Plateau/Highland Rim) constrained	3358.43	116.09	<0.001

Table 2
Ranges of uncorrected pairwise sequence divergence and estimated divergence time between nominate taxa and between major *E. aquatica* clades. Eaqu, *E. aquatica*; Ecir, *E. cirrigera*; Ejun, *E. junaluska*; BV, Birmingham Valley; CV, Coosa Valley; CP, Cumberland Plateau; HR, Highland Rim; Myr, million years.

	Uncorrected *p* (%)	Estimated divergence time (Myr)
Divergence among nominate taxa		
Eaqu–Ejun	7.70–9.70	6.0–7.6
(Eaqu/Ejun)–Ecir (most closely related)	7.97–10.49	6.2–8.2
Eaqu–Ecir (sympatric)	10.24–13.55	8.0–10.6
Ejun–Ecir	10.37–11.29	8.1–8.8
Divergence among major E. aquatica clades		
BV Eaqu–CP/HR Eaqu	5.18–5.58	4.0–4.4
BV Eaqu–CV Eaqu	5.99–7.84	4.7–6.1
CP/HR Eaqu–CV Eaqu	3.19–5.31	2.5–4.1
BV Eaqu–(CP/HR Eaqu/CV Eaqu)	5.18–7.84	4.0–6.1

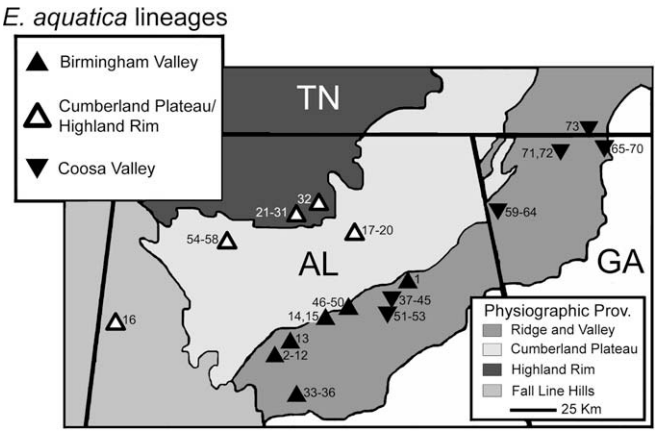

Fig. 3. Distribution of the three most divergent mitochondrial clades of *E. aquatica* in the Birmingham (closed triangles) and Coosa Valleys (upside down closed triangles), and the Cumberland Plateau/Highland Rim (open triangles) of northern Alabama (AL), northern Georgia (GA), and Tennessee (TN) (Fig. 1, Appendix 1).

(specifically the Birmingham and Coosa Valleys) and the Cumberland Plateau/Highland Rim to form a well-supported monophyletic group (Bayesian posterior probability [BAPP] = 1.00, maximum parsimony bootstrap [MPBS] = 99; Fig. 2). This clade also includes individuals from a few populations in the Cumberland Plateau (Eaq54-58) and the Coosa Valley (Eaq37) that were of ambiguous assignment. In both BA and MP analyses of *ND2 E. aquatica* forms the sister taxon to *E. junaluska* (BAPP = 0.99, MPBS = 80), consistent with Jacobs' (1987) allozyme study and Kozak et al's. (2006) analysis of *ND2*. By comparison, populations of *E. cirrigera* from the Ridge and Valley and the Cumberland Plateau/Highland Rim of Alabama and Georgia form a well-supported monophyletic group exclusive of *E. aquatica* (BAPP = 1.00, MPBS = 86). The SH-test further shows that the unconstrained topology is significantly more likely than when *E. cirrigera* and *E. aquatica* from the same drainage and close geographic proximity are constrained to be monophyletic (Table 1).

Based on the uncorrected pairwise sequence divergence of *ND2*, *E. aquatica* are 7.7–9.7% divergent from their nearest heterospecific relative *E. junaluska* (Table 2). Applying the molecular clock rate of 1.28% change per million years (Weisrock et al., 2001), we calculate that these taxa diverged 6.0–7.6 million years (Myr) ago. The clade of *E. aquatica* + *E. junaluska* is 7.97–10.49% (6.2–8.2 Myr) divergent from the nearest *E. cirrigera* (Cumberland Co., TN; Ecir99). Furthermore, we estimate *E. aquatica* is 10.24–13.55% (8.0–10.6 Myr) divergent from *E. cirrigera* collected from sympatric populations in the Ridge and Valley and Cumberland Plateau/Highland Rim of Alabama and Georgia.

The *E. aquatica* clade contains three very well-supported divergent lineages, which correspond to the Birmingham Valley, Coosa Valley, and Cumberland Plateau/Highland Rim (Figs. 2 and 3). The Coosa Valley and Cumberland Plateau/Highland Rim lineages form a well-supported monophyletic group (BAPP = 1.00, MPBS = 96) and are well-supported as the sister taxon to populations from the Birmingham Valley (BAPP = 1.00, MPBS = 100). The Birmingham Valley lineage is 5.18–7.84% divergent from the Coosa Valley + Cumberland Plateau/Highland Rim clade and corresponds to an estimated divergence time of 4.0–6.1 Myr ago (Table 2). The divergence between the Coosa Valley and Cumberland Plateau/Highland Rim lineages ranges from 3.19 to 5.13% (2.5–4.1 Myr). In the Ridge and Valley of Alabama, we found several localities in Etowah and St. Clair Counties where individuals from the Birmingham Valley clade (Eaqu1) and Coosa Valley clade (Eaqu37-45; Eaqu51-53) were less than 10 km apart (Fig. 3).

As expected, the nuclear locus *Rag1* was substantially less variable than *ND2*, with only 101 variable and 35 parsimony informative characters in a 1201 bp sequence. Nonetheless, consistent with our *ND2* phylogeny, all putative *E. aquatica* samples collected from the Ridge and Valley, Cumberland Plateau, and Highland Rim of Alabama and Georgia form a well-supported clade (BAPP = 0.96) independent from *E. cirrigera* (Fig. 4). Three distinct *E. aquatica* clades were recovered in both BA and MP analyses, each assignable to the Birmingham Valley (BAPP = 0.99), the Coosa Valley (BAPP = 0.99), and the Cumberland Plateau/Highland Rim (BAPP = 0.93). Concordant with *ND2*, *E. aquatica* from the Birmingham Valley are monophyletic and form the sister lineage to a clade consisting of individuals collected from the Coosa Valley and the Cumberland Plateau/Highland Rim (BAPP = 90).

The only major difference between the *ND2* and *Rag1* phylogenies is that the *Rag1* phylogeny shows *Eurycea junaluska* (Ejun103, Sevier Co., TN) nested among *E. aquatica* from the Coosa Valley and genetically similar to a nearby population of *E. aquatica* (Eaqu73, Bradley Co., TN). Whereas, the *ND2* phylogeny shows all *E. aquatica* forming the sister taxon to *E. junaluska* from Monroe Co. and Sevier Co., Tennessee (Ejun102-103). Given that *E. junaluska* contains unique divergent mitochondrial haplotypes, and there is strong concordance between the *ND2* and *Rag1* phylogenies for populations of *E. aquatica*, we suspect that *Rag1* alleles of *E. aquatica* have introgressed into nearby populations of *E. junaluska*.

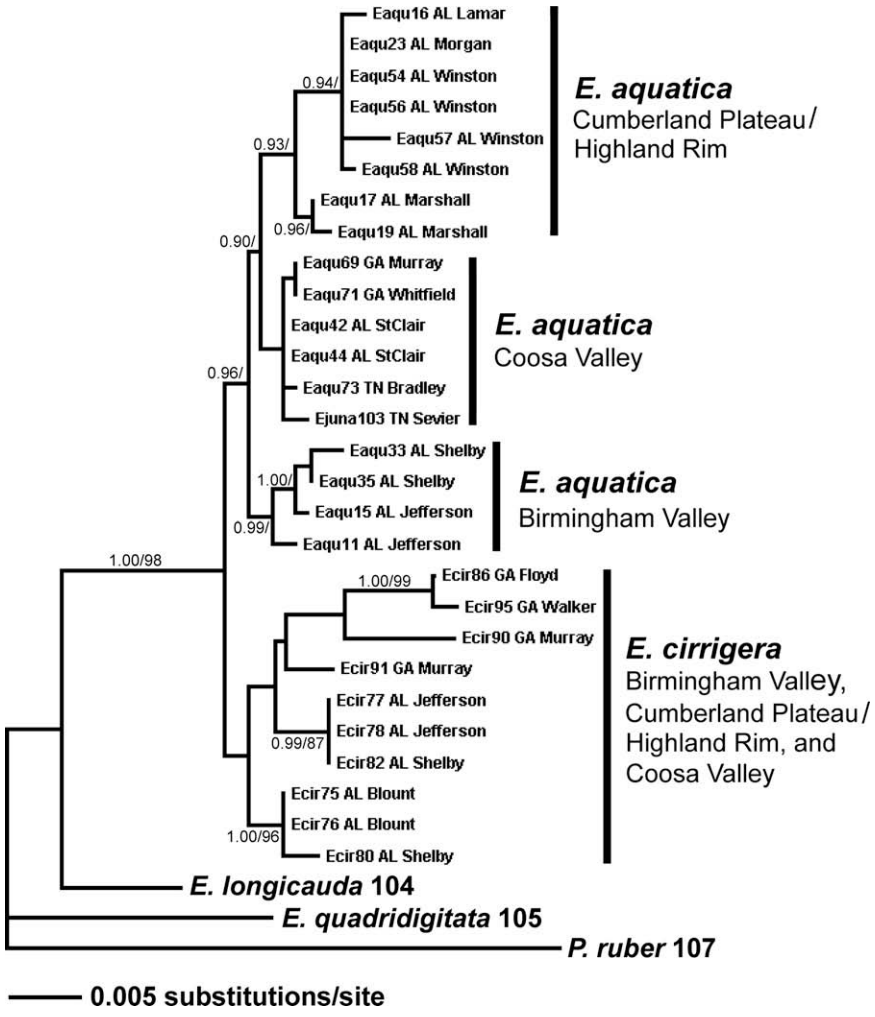

Fig. 4. Bayesian phylogram of *Rag1* for a subset of our *E. aquatica* and *E. cirrigera* samples. Numbers subtending each branch represent Bayesian posterior probabilities (before slash) and Maximum Parsimony bootstrap values (after the slash).

Independent sample *T*-tests reveal significant morphological differences between *E. aquatica* and *E. cirrigera* (Table 3) in regards to relative tail length (RTL; $F = 14.25$; $p < 0.001$), head width (HW; $F = 3.64$; $p = 0.004$), and head length/head width (HL/HW; $F = 9.74$; $p = 0.003$). *Eurycea aquatica* have shorter tails relative to their snout-vent length (mean = 0.93 mm), wider heads (mean = 5.65 mm), and smaller head length to head width ratios (mean = 1.46 mm) than *E. cirrigera* (means = 1.21 mm; 1.60 mm; 4.85 mm). The principle components analysis indicated a significant effect of species (i.e., *E. aquatica*, *E. cirrigera*) on morphology with two components (i.e., factors) explaining approximately 80.0% of all variance (Table 4). Factor 1 accounted for 47.42% of variance and relative tail length (RTL), head width (HW), and head length/head width (HL/HW) loaded heavily ($r = 0.578$; -0.876; 0.854). Factor 2 explained 32.59% of variance with RTL and average hind limb length (AHLL) loading heavily ($r = 0.691$; 0.892). Overall, the PCA demonstrates strong morphological differences between the more robust *E. aquatica* and the more gracile *E. cirrigera* (Fig. 5).

Table 3
Results of independent sample *T*-tests between morphological characteristics of *E. aquatica* and *E. cirrigera*. * denotes a significance of $p < 0.05$. Bold signifies morphological characters used in PCA.

Morphological Character	*F*	*p*
Snout-vent length	0.06	0.806
Tail length	1.47	0.231
Total length	0.21	0.651
Relative tail length (RTL)	**14.25**	**<0.001***
Trunk length	0.19	0.664
Trunk width	1.41	0.239
Trunk length/trunk width	0.01	0.926
Average hind limb length (AHLL)	**3.64**	**0.060**
Average front limb length	0.00	0.985
Excess trunk length	1.13	0.292
Head width (HW)	**8.65**	**0.004***
Head length	0.62	0.433
Head length/head width (HL/HW)	**9.74**	**0.003***
Costal groove number	0.10	0.757

Table 4
Eigen values and % of variance for each of the four principle components of the PCA. RTL, relative tail length; HL/HW, head length divided by head width; AHLL, average hind limb length; HW, head width. Bold denotes factors with Eigen values > 1.00.

Component	Eigen values	% of variance
1 (RTL, HW, HL/HW)	**1.90**	**47.42**
2 (RTL, AHLL)	**1.30**	**32.59**
3	0.46	11.42
4	0.34	8.57

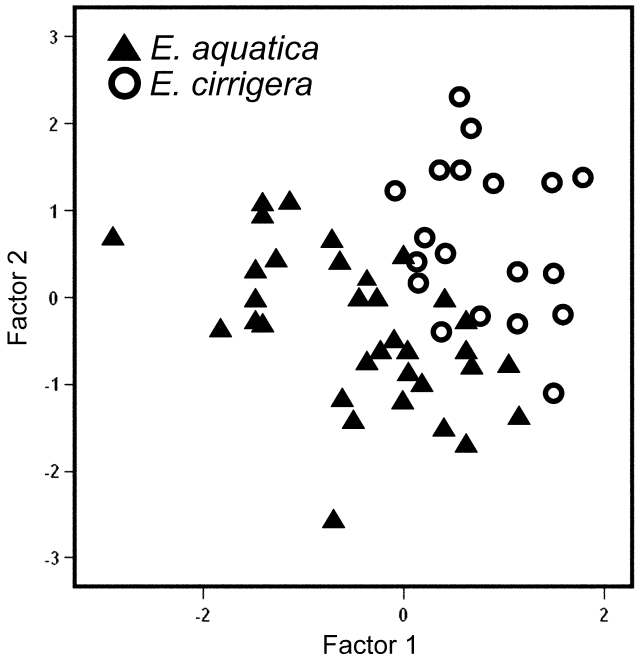

Fig. 5. Plotted results of Factor 1 (RTL, HW, HL/HW) and Factor 2 (RTL, AHLL) of the principle component analysis for *E. aquatica* (closed triangles) and *E. cirrigera* (open circles). RTL, relative tail length; HW, head width; HL/HW, head length/head width; AHLL, average hind limb length.

4. Discussion

4.1. Phylogeny and biogeography of Eurycea aquatica

Our phylogenetic analyses of both *ND2* and *Rag1* provide substantial support for a widespread clade of primarily robust spring-dwelling salamanders within the *E. bislineata* complex from the Ridge and Valley, Cumberland Plateau, and Highland Rim of Alabama and Georgia (Figs. 2–4). Herein we refer to this clade as *Eurycea aquatica*, which includes individuals from its type locality and is the sister lineage to *E. junaluska*, consistent with Kozak et al. (2006). Our divergence time estimates based on *ND2* suggest that *E. aquatica* is approximately eight to ten million years divergent from nearby populations of *E. cirrigera*. The distributions of *E. aquatica* and *E. cirrigera* broadly overlap in the Ridge and Valley. However, *E. aquatica* are largely restricted to headwater springs and spring runs whereas *E. cirrigera* are found along streams. Phylogenetic analyses based on mitochondrial and nuclear DNA indicate that *E. aquatica* is genetically distinct from *E. cirrigera* in this region. Morphological analyses show that individuals in the *E. aquatica* clade typically possess a robust morphotype with a wide head and short tail, while *E. cirrigera* are generally more gracile with narrower heads and longer tails. Taken together, this shows that in the Ridge and Valley, robust spring-dwelling *E. aquatica* are a distinct species and not simply a local spring dwelling ecomorph of nearby populations of *E. cirrigera*.

In the Cumberland Plateau and Highland Rim of northern Alabama, *E. cirrigera* appear to be much less common, and *E. aquatica* inhabit both springs and streams throughout this region. Additionally, there appears to be more morphological diversity within the Cumberland Plateau/Highland Rim clade compared to the other two *E. aquatica* lineages, with several less robust individuals collected at stream habitat in one location (Eaqu54-58, Winston Co., AL). Despite differences in morphology and habitat, these individuals are deeply nested among robust individuals (i.e., all other populations in the *E. aquatica* + *E. junaluska* clade) in both our *mt* and *nuc* DNA analyses. Therefore, in the Cumberland Plateau and Highland Rim gracile stream-dwelling members of the *E. bislineata* complex may be less robust *E. aquatica* rather than *E. cirrigera*.

4.2. Biodiversity and conservation of Southern Appalachian springs

We recovered three very divergent lineages within *E. aquatica* with distributions that correspond to the Birmingham Valley, Coosa Valley, and Cumberland Plateau/Highland Rim, each potentially representing a distinct species that diverged approximately four million years ago. The close geographical proximity (<10 km) of individuals from the Birmingham Valley and the Coosa Valley clades provides additional support for the genetic distinction between these *E. aquatica* lineages. Taxonomically, the Birmingham Valley clade should remain *E. aquatica* as it includes populations from the type locality in Jefferson Co., Alabama, while the other two clades potentially represent undescribed species. Within each of these lineages (especially within the Birmingham and Coosa Valleys), genetic divergence and diversity are very low, indicating that they have only recently spread across their respective regions or are currently experiencing a high level of gene flow among populations. We speculate that the divergence of these lineages was facilitated by the complex topography of this region, which is characterized by narrow ridges separating flat-bottomed valleys of limestone and dolomite that contain isolated, ancient aquifers (ADEM, 2006; Mills and Kaye, 2001).

Similar to other studies of eastern North American salamanders (Kozak et al., 2006) and Southern Appalachian cave crayfish (Buhay et al., 2007), the major clades of *E. aquatica* do not correspond to modern river drainages. For example, the clade of *E. aquatica* from the Birmingham Valley includes populations from the modern Warrior, Cahaba, and Coosa River drainages, and the Cumberland Plateau/Highland Rim clade includes individuals collected from the Tennessee and Warrior River drainages. Similarly, populations within the same modern drainage also do not represent a distinct evolutionary lineage. Samples collected from populations in the Coosa River watershed and in close geographic proximity to one another (<10 km) are phylogenetically nested in two divergent *E. aquatica* clades (the Birmingham Valley, Eaqu1; the Coosa Valley clade, Eaqu37-45, Eaqu51-53). Kozak et al. (2006) found that the genetic structure of the widespread *Eurycea bislineata* complex is a historical signature of paleodrainage patterns. There is little information available on paleodrainage patterns in northern Alabama and Georgia at a fine enough scale needed to test this hypothesis specifically for *E. aquatica*. Therefore, our results could be attributable to paleodrainage patterns, current land formations, or modern gene flow across drainages.

The distribution of these three divergent *E. aquatica* lineages is concordant with other spring endemics in this region. For instance, two of the clades correspond geographically to the ranges of spring-endemic fishes, the Watercress Darter (*Etheostoma nuchale*) in the Birmingham Valley, and the Coldwater Darter (*E. ditrema*) from the Coosa Valley (Mayden et al., 2005). A third *E. aquatica* lineage comprises populations from the Cumberland Plateau and Highland Rim of northern Alabama and includes the "Cole Springs" morph referenced in Mount (1975). Several fish species in northern Alabama mirror this trend (e.g., the Spring Pygmy Sunfish, *Elassoma alabamae*, Quattro et al., 2001; the Bankhead Darter, *Percina sipsi*, Williams et al., 2007). Overall, the evolutionary patterns of all three spring-associated *E. aquatica* clades exhibit marked similarities with those of spring-dwelling fishes in the same region.

The large number of high-elevation endemics in the Appalachian Mountains is well recognized, and many mountain-top habitats receive protection from state parks and national forests. We find that mid-elevation regions also contain a high number of endemic lineages, especially karst areas rich in spring and spring-run

habitats. Here unique biota exist in narrow and restricted distributions, which are further challenged by the threat of agriculture, urban development (e.g., Birmingham metropolitan area), dewatering of aquifers, and contamination (Hubbs, 1995).

In September 2008, one of our *E. aquatica* sampling localities (Roebuck Spring in Roebuck-Hawkins Park, Jefferson Co., AL) completely dried when a dam was intentionally removed (Bouma, Birmingham News, September 23, 2008). As a result, more than 1000 federally endangered watercress darters (*Etheostoma nuchale*) from this location, once the largest of four known populations of *E. nuchale*, were found dead. The impact the dam removal and subsequent pond drying had on Roebuck Spring's *E. aquatica* population is unknown. Continued anthropogenic pressures on vulnerable spring habitats across the Ridge and Valley, Cumberland Plateau, and Highland Rim of the Southern Appalachian Mountains could potentially eliminate a large number of very divergent endemic lineages before they are even recognized.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.03.023.

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