JOURNAL OF THE TENNESSEE ACADEMY OF SCIENCE 84(4):77–82 OCTOBER 2009

THE PHYLOGENETIC POSITION OF *EURYCEA LUCIFUGA*, THE CAVE SALAMANDER, AND THE EVOLUTION OF CAVE-ADAPTED SPECIES WITHIN *EURYCEA*

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ABSTRACT—Recent efforts have quantified the distribution of the cave fauna of the United States and Canada. These studies have identified hotspots of diversity and areas of endemism for cave-obligate species. Another crucial aspect to understanding the biodiversity of the North American cave fauna relates to its evolutionary history: how many times have cave-dwelling species evolved from surface-dwelling ancestors? In this study, we take a molecular phylogenetic approach to examine this question in the salamander genus *Eurycea*. *Eurycea* contains 26 species, eight of which are cave-obligates, and another (*E. lucifuga*, the cave salamander) that is a common cave inhabitant (a troglophile). We determined the phylogenetic position of *E. lucifuga* by sequencing two regions of the mitochondrial genome from multiple individuals and comparing those sequences with sequences previously obtained from 23 of the 26 members of the genus *Eurycea*. Using this approach, we identified a clade containing the cave salamander, the long-tailed salamander (*E. longicauda*), and the three-lined salamander (*E. guttolineata*). Our combined phylogenetic analysis identifies three lineages within *Eurycea* that have given rise to cave-adapted species: one cave-obligate species evolved from within the *E. multiplicata* species complex of the Ozark Mountains, seven cave-obligate species evolved within the perennibranchiate Texas *Eurycea* group of the Edwards Plateau, and the cave salamander-evolved troglophilism within the *E. lucifugal E. longicaudal E. guttolineata* clade identified here. Thus, we suggest that cave-adapted species have evolved at least three separate times within *Eurycea*.

Cave animals have long fascinated ecologists and evolutionary biologists. As surface-dwelling species invade underground habitats, they generally evolve a suite of characteristics related to living in the dark—loss or reduction of eyes, decreased pigmentation, elongated appendages, and enhanced non-visual sensory systems—collectively referred to as "troglomorphy" (Porter, 2007). Troglomorphy is an excellent example of convergent evolution among many distinct organismal groups. Subterranean ecosystems are generally species-poor and have been used as testing grounds for ecological theories (Culver, 1982). However, in spite of decades of interest in subterranean species and despite great potential value, molecular tools have been used only to a limited extent to study the evolutionary history of these species (Proudlove and Wood, 2003; Porter, 2007).

Cave-obligate species are generally referred to as troglobites (though aquatic cave-limited species may be more specifically referred to as stygobites). Common cave residents that are not limited solely to cave habitats are referred to as troglophiles (Barr and Holsinger, 1985). A recent tally of the troglobites and stygobites from the continental United States identified 927 species from more than 200 genera, representing a wide range of orders, classes, and phyla (Culver et al., 2000). Though we have a list of cave-limited species in the United States, we know little about how those species arose. Cave-limited species may originate in one of two ways: either from surface-dwelling ancestors by speciation after colonization of cave habitats or from cave-dwelling ancestors through the speciation of one previously cave-adapted species into two. It is unclear which of these two modes of speciation is more common in the evolution of cave faunas.

Without knowledge of the phylogenetic relationships among cave-dwelling species and their surface-dwelling relatives, it is impossible to determine how many independent evolutionary lineages have invaded cave habitats in North America. In this paper we consider this question for the salamander genus *Eurycea*, which contains more cave-obligate species (eight) than any other genus of vertebrates in North America (Culver et al., 2000).

Members of *Eurycea* range throughout eastern Canada and the United States. Twenty-six species have been described, and the life history and ecology of many of the species are well known (reviewed in Petranka, 1998; Ryan and Bruce, 2000). Major phylogenetic studies on *Eurycea* include those on the perennibranchiate *Eurycea* species of Texas (Chippindale et al., 2000; Hillis et al., 2001; Wiens et al., 2003), the *E. multiplicata* species complex (Bonett and Chippindale, 2004), and the *E. bislineata* species complex (Kozak et al., 2006). In all, DNA sequences have been used to examine phylogenetic relationships among 23 of the 26 described species. The three species of *Eurycea* that have not been studied using DNA sequences are the recently described *E. chamberlaini* (Harrison and Guttman, 2003), *E. robusta*, which has not been collected in fifty years (Potter and Sweet, 1981; Petranka, 1998), and *E. lucifuga*, the Cave Salamander.

Eurycea lucifuga ranges from western Virginia to eastern Oklahoma and from central Indiana to central Alabama (Petranka, 1998). *Eurycea lucifuga* is found in limestone regions where individuals typically live in the twilight zones of caves (Briggler and Prather, 2006; Camp and Jensen, 2007), though they can be encountered deep within caves, as well as in a variety of non-cave habitats (Hutchinson, 1958; Petranka,

1998; Reichenbach et al., 2006). Given the range of habitats they occupy, individuals of *E. lucifuga* are troglophiles and not troglobites (Barr and Holsinger, 1985). No subspecies of *E. lucifuga* has been described, and an allozyme study found little differentiation across the range of the species (Merkle and Guttman, 1977).

In this study, we first surveyed the genetic information available about obligately cave-dwelling species. To do so, we used a published list of all the cave-obligate species found in the United States and Canada (Culver et al., 2000) and determined how much genetic information was available for these species. This allowed us to identify studies that addressed the question of how cave-obligate species originated. Next, we determined the phylogenetic position of *E. lucifuga*, and we then examined the evolution of cave-adapted species within *Eurycea*. Previous molecular studies on members of *Eurycea* utilized two different regions of the mitochondrial genome: cytochrome B sequences have been gathered from 18 *Eurycea* species (Chippindale et al., 2000; Bonett and Chippindale, 2004); and NADH subunit 2 (ND2) sequences have been gathered from eight *Eurycea* species (Kozak et al., 2006). Unfortunately, these two sets of data are largely non-overlapping in the taxa they cover; both gene regions have been sequenced in just three *Eurycea* species. As a result, we could not combine these studies to produce a single phylogenetic hypothesis for the genus, and this complicated determining the phylogenetic position of any individual *Eurycea* species with regard to all other members of the genus.

To overcome the problem of non-overlapping sets of molecular data, we sequenced both of the mitochondrial regions that had been used in previous studies of *Eurycea* species from multiple *E. lucifuga* specimens and compared these sequences with existing DNA sequences from other members of the genus. This allowed us to identify the phylogenetic position of *E. lucifuga* and helped to clarify how many times cave-adapted species have evolved within *Eurycea*.

Materials and Methods

The State of Genetic Studies of Cave-limited Species in North America and Canada—During February 2008 we downloaded the most recent list of cave-limited species in North America in Canada (http://www.karstwaters.org/trogslist.htm; Hobbs et al., 2003). We then searched GenBank (http://ncbi.nlm.nih.gov) for nucleotide data for each species on the list. We tallied the number of cave-limited species in each genus and the number of those species that were represented in Genbank by nucleotide data.

Eurycea *Samples*—One-cm long tail clips *Eurycea lucifuga* individuals (n = 10) were collected from three caves within 5 km of Sewanee, Tennessee, during June 2006. Tail clips were individually stored in 95% ethanol. The tail clips are stored in the collections of the Biology Department at the University of the South in Sewanee, Tennessee. Collections of *E. lucifuga* were permitted by the Tennessee Wildlife Resources Agency (Permit #2062).

DNA Amplification and Sequencing—DNA was extracted from each sample using the DNeasy Tissue Kit (Qiagen #69506) according to the manufacturer's protocol. A 982 bp fragment of the mitochondrial cytochrome B gene was amplified with the newly developed primers ELcybF1 (5′-AAGATTATTAATAACTCCTTTATTGA-3′) and ELcybR1 (5′-AAAATGCTTGTCCAATTTCAAT-3′). Our amplification protocol was 35 cycles of 1 min at 94°C, 2 min at 50°C, and 90 sec at 72°C; followed by 4 min at 72°C. Amplification products were cleaned using the QIAquick PCR Purification Kit (Qiagen #28106) according to the manufacturer's protocol. Cleaned products were then sequenced on both strands using an Applied Biosystems 3730 DNA sequencer with the primers ELcybF1, ELcybR1, as well as the internal primers ELcybF2 (5′-TTAGAGTTAAGTCCTGTTGGGTT-3′) and ELcybR2 (5′-GCAATCCCATATTTAGGAGACAC-3′). Sequences (n = 10) from *E. lucifuga* individuals were aligned and edited using Sequencher (v. 4.2.2; Gene Codes Corporation).

The same methods were used to amplify and to sequence the complete mitochondrial ND2 gene (totaling 1041 bp) from *E. lucifuga* individuals (n = 5). We used primers L4437 (5′-AAGCTTTCGGGCCCATACC-3′) and H5617a (5′-AAAATRTCTGRGTTGCATTCAG-3′) from Macey et al. (1997) for both amplification and sequencing. Sequences from the cytochrome B and the ND2 genes have been deposited in NCBI (Accession numbers EF044239–EF044248 and EF043386–EF043390, respectively).

Phylogenetic Analyses—We gathered cytochrome B sequences from 18 species of *Eurycea* from the NCBI database. When more than one sequence per species was available, we used the longest sequence. If more than one sequence of the same length was available, we randomly chose one. With the exception of *E. bislineata*, cytochrome B sequences were not available for members of the *E. bislineata* species complex (*E. cirrigera*, *E. junaluska*, *E. wilderae*, and *E. aquatica*). Cytochrome B sequences were also not available for *E. guttolineata*, *E. robusta* and *E. chamberlaini*. We rooted the cytochrome B tree with a sequence from *Pseudotriton ruber*, another member of the Tribe Hemidactyliini known to represent an outgroup to *Eurycea* (Chippindale et al., 2004; Mueller et al., 2004).

No gaps were present and sequences were aligned by eye. The final alignment for *E. lucifuga* was 982 bp, with 25 sequences of this length. Four sequences were shorter at the 3′ end by up to 33 bp. Mean sequence length was 979 bp. We used MrBayes (v. 3.1.2, Ronquist and Huelsenbeck, 2003) to conduct Bayesian phylogenetic analyses. We partitioned the data by codon and used a model with six substitution types, estimated nucleotide frequencies, invariable sites, and gamma-distributed rates. We calculated clade credibility values from 4000 trees by sampling every 1000th tree from two runs of 5,000,000 trees after discarding the first 3001 sampled trees of each run. We also used Modeltest (v. 3.7; Posada and Crandall, 1998) to identify the model that best described the evolution of the sequences. We then used the parameters identified in Modeltest to conduct a distance-based neighbor-joining bootstrap analysis (1000 replicates) in PAUP* (v. 4.0b10; Swofford, 2001).

We gathered ND2 sequences from five members of the *E. bislineata* species complex (*E. bislineata*, *E. cirrigera*, *E. wilderae*, *E. junaluska*, and *E. aquatica*), as well as *E. longicauda*, *E. guttolineata*, and *E. quadridigitata* from the NCBI database, and combined them with our new sequences from *E. lucifuga*. ND2 sequences were not available for the other 18 members of the genus. We chose sequences scattered throughout the various clades of the *E. bislineata* species complex as identified by Kozak et al. (2006). As for the cytochrome B analysis, we used sequences from *Pseudotriton*

ruber as the outgroup. All sequences were 1041 bp in length with the exception of one sequence that had a 3 bp deletion with respect to all the other sequences. Sequences were aligned by eye. We used MrBayes, Modeltest, and PAUP* as described for the cytochrome B sequences to examine the phylogenetic relationships between species.

RESULTS

Availability of Genetic Data for Cave-limited Species in North America and Canada—The most recent list of cave-limited species included 1170 species in 262 genera. Genetic data are present in GenBank for 63 of these species (5.4%) representing 25 genera. Mitochondrial genes (including cytochrome oxidase I and the 12S and 16S rRNAs) were most commonly sequenced, with some nuclear genes (including histone genes and the 5.8S and 28S rRNAs) also sequenced. Eight genera had genetic data present for more than one cave-limited species. Only three genera—*Nesticus* (spiders), *Orconectes* (crayfish), and *Eurycea*—that contain more than six cave-limited species had genetic data for a majority of those species.

Genetic Diversity in Eurycea lucifuga—Among ten cytochrome B sequences, we identified nine haplotypes and 21 variable sites. At each of these sites all of the observed variation was silent; no replacement mutations were observed. The mean uncorrected pairwise difference between *E. lucifuga* cytochrome B sequences was 0.5% (range = 0.0–2.0%). From complete mitochondrial ND2 gene sequences from five individuals, we identified five haplotypes and 25 variable sites. Variation at nineteen of these sites was silent, whereas six sites contained replacement mutations. The observed changes were concentrated on a single lineage (*E. lucifuga* 2), which was the only haplotype with variation at sixteen of the silent sites and two of the replacement sites. Mean uncorrected pairwise difference between *E. lucifuga* sequences was 1.0% (range = 0.0%–2.1%).

Phylogenetic position of Eurycea lucifuga—The cytochrome B phylogenetic tree showed strong support (100% values for both the Bayesian clade credibility and distance bootstrap analyses) for a clade including *E. lucifuga* and *E. longicauda* (Fig. 1). The mean cytochrome B pairwise difference between *E. lucifuga* and *E. longicauda* sequences was 10.6% (range = 10.4–10.8%). The ND2 tree showed support (71% clade credibility, 100% bootstrap support) for a clade containing *E. lucifuga*, *E. longicauda*, and *E. guttolineata* (Fig. 2). The mean ND2 uncorrected distance between *E. lucifuga* sequences and *E. longicauda* sequences was 10.2% (range = 9.6–11.0%), and the mean uncorrected distance between *E. lucifuga* and *E. guttolineata* was 9.6% (range = 8.9–9.8%).

DISCUSSION

Nearly 95% of all cave-obligate species in the United States and Canada have never been examined at a genetic level. Only in a few cases have molecular analyses provided enough information to determine how many times troglobitism and troglophilism has evolved. Buhay and Crandall (2005) identified a clade of four stygobitic crayfish species within the genus *Orconectes*. Given that phylogenetic information, in combination with morphological and geographical data, the most parsimonious explanation is that these four species arose after a single colonization of cave habitats by a surface-dwelling ancestor followed by subsequent speciation underground. Similarly, Buhay et al. (2007) showed that at least four stygobitic species of crayfish (genus *Cambarus*) have arisen following a single invasion of cave habitats. Both *Orconectes* and *Cambarus* contain other, as yet unstudied, stygobitic species, so it remains to be seen how many times stygobitism has evolved within these genera. Hedin (1997a; 1997b) studied the spider genus *Nesticus* containing surface-dwelling, troglophilic, and troglobitic species. The phylogenetic results for *Nesticus* suggest that troglophilism and troglobitism have evolved on multiple occasions within the genus, though lack of complete taxonomic coverage precludes determining exactly how many times this has occurred. Within these genera there are examples of multiple invasions of cave habitats within a single genus and examples of speciation in lineages that have already invaded cave habitats. Outside of the United States and Canada, there are a few examples of both of these patterns as well (reviewed in Porter, 2007).

We have herein considered this question for *Eurycea*. We found support for a clade containing *Eurycea lucifuga*, *E. longicauda*, and *E. guttolineata*. These species are morphologically similar, and *E. guttolineata* was treated as a subspecies of *E. longicauda* until Carlin (1997) showed fixed allozyme differences supporting their separation into distinct species. *E. guttolineata* and *E. longicauda* have largely allopatric ranges in eastern North America, with *E. guttolineata* found to the east and south of the Appalachian Mountains and *E. longicauda* to the west and north. The range of *E. lucifuga* largely overlaps that of *E. longicauda*. Of the three species, only *E. longicauda* is divided into subspecies, with one (*E. l. longicauda*) in the eastern part of its range and another (*E. l. melanopleura*) in the west. Further genetic surveys over the ranges of the three species would be valuable, though an allozyme study over the range of *E. lucifuga* did not identify any major genetic differentiation (Merkle and Guttman, 1977). Future studies of *E. lucifuga* life history, behavior, and adaptations to a troglophilic lifestyle should be interpreted with comparison to *E. longicauda* and *E. guttolineata*.

With our sequencing of mitochondrial regions from *E. lucifuga*, only two members of the genus *Eurycea* remain without any published genetic information—*E. chamberlaini* and *E. robusta*. Although we lack molecular information, we can hypothesize as to their likely phylogenetic positions based on their distribution and morphology. It is likely that *E. robusta*, if still extant, is a member of the perennibranchiate group of Texas *Eurycea* species, given that it has been collected only in that area and resembles members of that group (Potter and Sweet, 1981; Petranka, 1998). *Eurycea chamberlaini* is a likely close relative of *E. quadridigitata*, with which it was confused until recently (Harrison and Guttman, 2003). Given the probable phylogenetic positions of *E. robusta* and *E. chamberlaini*, we are confident that our identification of *E. lucifuga*, *E. longicauda*, and *E. guttolineata* as a clade will not be affected by further genetic studies of those species.

Though we cannot combine all of the largely non-overlapping molecular studies on *Eurycea* to construct a single phylogenetic hypothesis for the genus, we can divide the genus into five subgroups based on current phylogenetic information. First, as identified here, is a clade containing *E. lucifuga*,

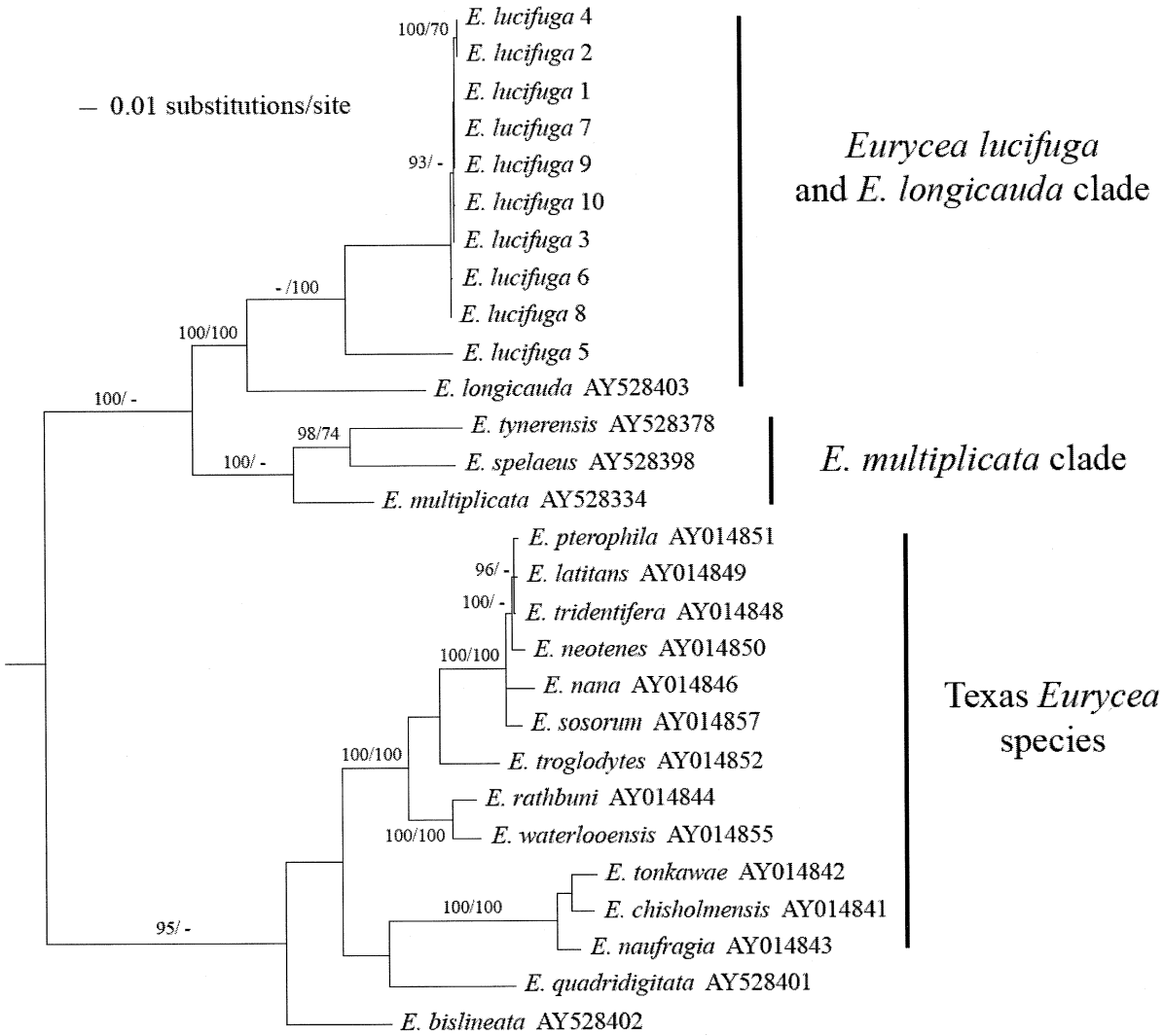

FIG. 1. Bayesian majority-rule consensus phylogram of *Eurycea* based on cytochrome B sequences. Branch lengths are the mean from 4000 post burn-in trees. Branch support is indicated by Bayesian clade credibility values followed by neighbor-joining distance (calculated using parameters chosen by Modeltest (Posada and Crandall, 1998)) bootstrap values (from 1000 replicates). Clade credibility values less than 90% and bootstrap values less than 70% are either left off or indicated by a '-'. The tree is rooted with a sequence from *Pseudotriton ruber* (not shown). Accession numbers are indicated for sequences from GenBank.

E. longicauda, and *E. guttolineata*. Second, the *E. multiplicata* complex consists of three species: *E. tynerensis*, *E. spelaeus*, and *E. multiplicata* (Bonett and Chippindale, 2004). Third, the *E. bislineata* complex consists of five named species—*E. bislineata*, *E. cirrigera*, *E. wilderae*, *E. junaluska*, and *E. aquatica*—and a number of other phylogentically independent lineages (Kozak et al., 2006). Fourth is a group of twelve Texas *Eurycea* species (Chippindale et al., 2000; Hillis et al., 2001; Wiens et al., 2003), which likely also includes *E. robusta*. Finally, *E. quadridigitata* (likely with *E. chamberlaini*) forms a fifth group, whose phylogenetic position is unclear. Though identifying subgroups may aid in understanding this large genus, the relationships between subgroups remain to be determined, and their monophyly remains to be confirmed.

Our identification of the *E. lucifuga*/*E. longicauda*/*E. guttolineata* clade highlights a remarkable pattern within the genus *Eurycea*: three of the five groups described above contain a mix of surface- and cave-adapted species. Culver et al. (2000) classified eight *Eurycea* species as "cave-obligates," including six species in the well-supported clade containing the Texas *Eurycea* species found south of the Colorado River (Fig. 1) (Chippindale et al., 2000). These species are *E. latitans*, *E. tridentifera*, *E. neotenes*, *E. troglodytes*, *E. rathbuni*, and *E. waterlooensis*. No genetic data are available for a seventh cave-obligate species, *E. robusta*, which is likely a member of this clade as well. We tentatively consider the other species in this clade to be troglophilic, but note that some could be considered troglobites, in particular *E. chisholmensis*, which has reduced eyes (Chippindale et al., 2000). Given the incompletely resolved phylogenetic relationships within this clade, which is more extensively discussed in Chippindale et al. (2000), and the morphological diversity among its species, it is unclear how many times troglobitism has evolved in this group; at a minimum, it has happened once. The eighth cave-obligate *Eurycea* species (*E. spelaeus*) is found within the *E. multiplicata* clade (Fig. 1) and represents a separate lineage in which sytgobitism has evolved. Within the *E. lucifuga*/*E. longicauda*/*E. guttolineata* clade (Fig. 2), *E. lucifuga* has

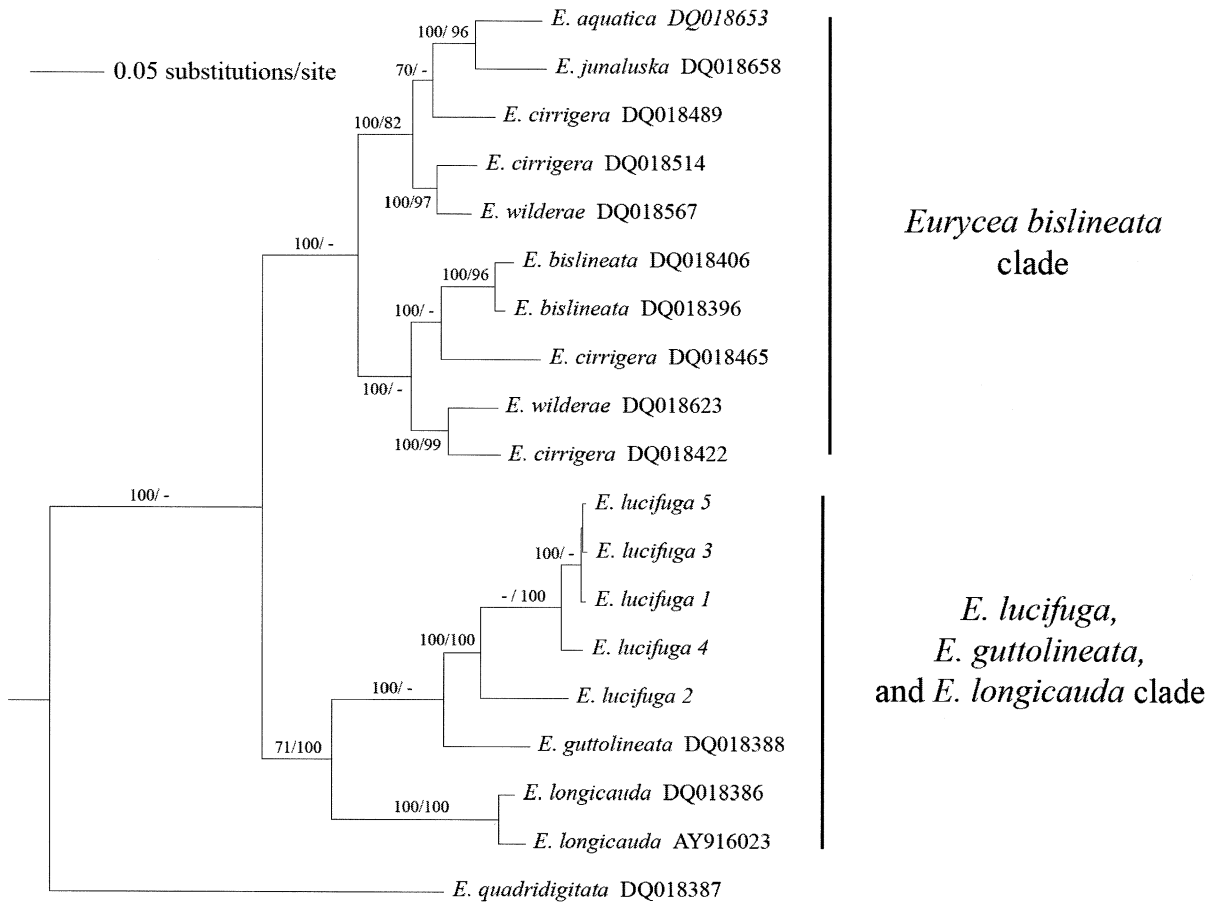

FIG. 2. Bayesian majority-rule consensus phylogram of *Eurycea* based on NADH subunit 2 sequences. Branch lengths are the mean from 4000 post burn-in trees. Branch support is indicated by Bayesian clade credibility values followed by neighbor-joining distance (calculated using parameters chosen by Modeltest (Posada and Crandall, 1998)) bootstrap values (from 1000 replicates). Clade credibility values less than 90% and bootstrap values less than 70% are either left off or indicated by a '-'. The tree is rooted with two sequences from *Pseudotriton ruber* (not shown). Accession numbers are indicated for sequences from GenBank.

evolved troglophilism from surface-dwelling ancestors. Thus, at a minimum, stygobitism has evolved twice in *Eurycea*, and troglophilism has evolved once.

ACKNOWLEDGMENTS

This work was supported by a Yeatman Fellowship (AJH) from the Biology Department of the University of the South and by research funds from the University of the South. We thank M. Lemon, C. Snowman, H. Yeatman, and J. Benson for advice and assistance with this project, and J. Lively for lab space. Comments from two anonymous reviewers substantially improved the manuscript.

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Submitted 18 Jun 2008; accepted 16 March 2009.