PHYLOGENETICS OF THE SOUTHERN CAVEFISH (*TYPHLICHTHYS SUBTERRANEUS*): IMPLICATIONS FOR CONSERVATION AND MANAGEMENT

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Abstract

Like other cave organisms, amblyopsid cavefishes have been a subject of continued debate regarding “regressive” evolution of characters in the adaptation from epigean to subterranean habitats. With six described species, the eastern North American endemic Amblyopsidae exhibit morphologies that range from epigean to troglomorphic. The clade also includes the most widespread stygobitic fish in North America, the Southern cavefish (*Typhlichthys subterraneus*). The widespread distribution and limited genetic work hinted that the Southern cavefish is comprised of several genetically distinct species obfuscated by convergent morphology. However, the phylogenetic relationships within *T. subterraneus* and within the family remain poorly understood. We investigated the intraspecific phylogenetic and biogeographic relationships of the Southern cavefish throughout its range in the Interior Low Plateau using DNA sequence data from a mitochondrial and nuclear gene. Our sampling includes populations both east and west of the Mississippi River. Our results support a monophyletic Amblyopsidae dating to the early Miocene with substantial divergence among the described forms. Considerable cryptic variation was observed within a monophyletic *T. subterraneus* with genetic variation structured within watersheds. Divergence times up to 11 Mya were estimated between certain drainages, indicating that some populations have been on separate evolutionary trajectories since the mid- to late Miocene. These findings suggest greater diversity within *Typhlichthys* than previously recognized, and support the idea that convergent evolutionary patterns associated with subterranean life make it difficult to infer the biogeographic history of subterranean lineages. While more extensive population-level data are needed to designate taxonomic groups or ESUs, it is clear that southern cavefish inhabiting different watersheds are demographically inconnected and possess unique genetic attributes.

Key words: *Typhlichthys subterraneus*, southern cavefish, amblyopsidae, evolution, phylogenetics, Alabama, Georgia, Tennessee, Kentucky, Missouri, Arkansas, Oklahoma

Introduction

Groundwater is an essential component of the global hydrological cycle and is fundamental to human development and survival (Danielopol et al. 2003, Boulton 2005). Groundwater organisms serve as ecological indicators of groundwater pollution and habitat degradation (Malard 1996, Elliott 2000, Culver et al. 2000) and perform vital ecosystem services, such as nutrient cycling and transformation and biological filtration (Hancock 2002, Danielopol et al. 2003, Boulton 2005). Subterranean ecosystems harbor significant biodiversity, nearly 1,800 subterranean species occur in North America alone, 25–30% of which are aquatic (Elliott 2000). However, recent molecular studies suggest that there is an underestimate of groundwater biodiversity, as considerable genetic variation has been observed among morphologically similar populations that are widely distributed (Buhay and Crandall 2005, Lefebure et al. 2006, Finkston et al. 2007). Discovery and protection of this evolutionary diversity is a conservation goal that requires significant input from molecular phylogenetics and population genetics.

Many aquatic, subterranean species (stygobites) are confined to distinct karstic hydrological systems (Finston et al. 2007, Zaksek et al. 2007), often endemic to a single aquifer. Their distributions are defined by both geologic structure and hydrological processes (Finston et al. 2007). The discontinuous distributions of many subterranean species resulting from presumed, limited dispersal ability and habitat fragmentation has led many to treat subterranean habitats as underground "islands" (Culver *et al.* 1995), even though the barriers between isolated habitats are unclear. Molecular analyses of many groundwater fauna have revealed highly subdivided population structure consistent with island-like habitat fragmentation, and genetic differentiation is often associated with hydrological patterns (Verovnik et al. 2004, Finston et al. 2007). Further, several instances of cryptic speciation have been revealed (e.g. amphipods, Finkston et al. 2007, crayfish, Buhay and Crandall 2005).

Just as distinct evolutionarily significant units (ESUs) of salmonids inhabiting different drainages merit individual management attention (Waples 1991, 1995), groups of cavefish in different hydrological systems may be genetically isolated and each represent an important component of the evolutionary legacy of the group, i.e., they should be recognized as ESUs or full taxonomic species. The presence of genetic diagnosable lineages that are morphologically indistinguishable or contradict patterns predicted by morphology suggest that defining subterranean species solely on the basis of morphology may be misleading and underestimate subterranean diversity. It is estimated that fewer than half of the obligate subterranean fauna of the United States have been described (Elliott 2000). Cryptic diversity obscured by convergent morphologies resulting from similar responses

Figure 1 *An adult southern cavefish, Typhlichthys subterraneus, from Marion County, Tennessee.*

to selection likely accounts for a large proportion of undescribed species. Molecular genetic data may be particularly appropriate for identifying cryptic species or ESUs with similar phenotypes (Allendorf and Luikart 2007). Given the potential decoupling of morphological and molecular evolution in subterranean environments resulting in gross underestimates of biodiversity based on morphological taxonomy alone, examining cryptic diversity in widely distributed groundwater taxa should be a priority for subterranean biologists and management agencies. Determining the genetic distinctiveness of subterranean populations, particularly those that are morphologically conservative, is critical to the conservation and management of subterranean biodiversity.

The amblyopsid cavefishes have intrigued students of ichthyology and evolutionary biology since the 1840s. The southern cavefish, *Typhlichthys subterraneus* (Figure 1), is an obligate cave-dwelling fish within the Amblyopsidae, a small family endemic to the unglaciated regions of the eastern United States. The family includes surface, stygophilic (facultative cave-inhabiting), and stygobitic (obligate cave-inhabiting) species that represent a graded series from surface to subterranean inhabitation, and are viewed as an excellent system to investigate evolutionary trends and speciation in subterranean environments (Eigenmann 1909, Poulson 1963, 1985). Although the family has been known to science since the early 1840s, the systematic relationships among and within species remain poorly understood. The southern cavefish has one of the largest distributions of any North American aquatic, cave-dwelling vertebrate with two major centers of distribution: the Ozark Plateau of central and southeastern Missouri, northeastern Oklahoma and northwestern Arkansas, and the Cumberland and Interior Low Plateau of northern Alabama, northwest Georgia, central Tennessee and Kentucky. Because of its large distribution and presumed limited dispersal, the potential is high for both subsurface and surface hydrologic patterns to contribute to the genetic structuring of populations and potentially facilitate cryptic speciation within southern cavefish. Electrophoretic analyses by Swofford (1982) showed considerable differentiation among populations of *Typhlichthys*, and suggested that the group may represent multiple, independent invasions of subterranean waters. However, owing to small sample size, Swofford's study was limited in its ability to distinguish modular or hierarchical subdivision from a continuous relationship between genetic and geographic distance.

This study examines the genetic structure of populations of *Typhlichthys* testing for an association of genetic divergence with hydrological patterns. If hydrological boundaries, either surface or subsurface, are barriers to dispersal and gene flow, genetic structure is expected to be associated with hydrological structure. Specifically, we examine sequence variation in both mitochondrial and nuclear markers to: 1) examine genetic diversity and structure, 2) determine the relationships among species within the Amblyopsidae, 3) examine the potential for cryptic diversity, and (iv) identify ESUs for management and conservation agencies. For the purposes of this paper, we focus on preliminary data obtained for populations of *Typhlichthys* in Tennessee, Alabama, and Georgia, but we also address interspecific relationships within the family.

Materials and Methods

Tissue samples (fin clips) or voucher specimens were collected from 66 southern cavefish from 32 localities throughout the range of the species in Tennessee, Alabama, Georgia, and from Arkansas (Figure 2). Tissue samples or DNA for the other amblyopsid species (except *S. poulsoni*) and eight *T. subterraneus* localities were provided by T. Near (Yale University), D. Neely and B. Kuhajda (University of Alabama), and Aldemaro Romero and Ron Johnson (Arkansas State University). Voucher specimens will be deposited into the University of Tennessee Ichthyological Collection.

DNA was extracted using standard methods and polymerase chain reaction (PCR) was used to amplify portions of one mitochondrial gene, ~1218bp of NADH dehydrogenase subunit 2 (ND2) including the entire coding region and portions of flanking tRNAs, and one nuclear gene, 820bp of ribosomal protein S7. Sequencing reactions were performed using original PCR primers and run on an ABI Prism 3730 at the Molecular Biology Resource Facility at the University of Tennessee. The trout-perches (*Percopsis omiscomaycus* and *P. transmontana*) and pirate perch (*Aphredoderus sayanus*) served as outgroups because of their alliance with the amblyopsids within the order

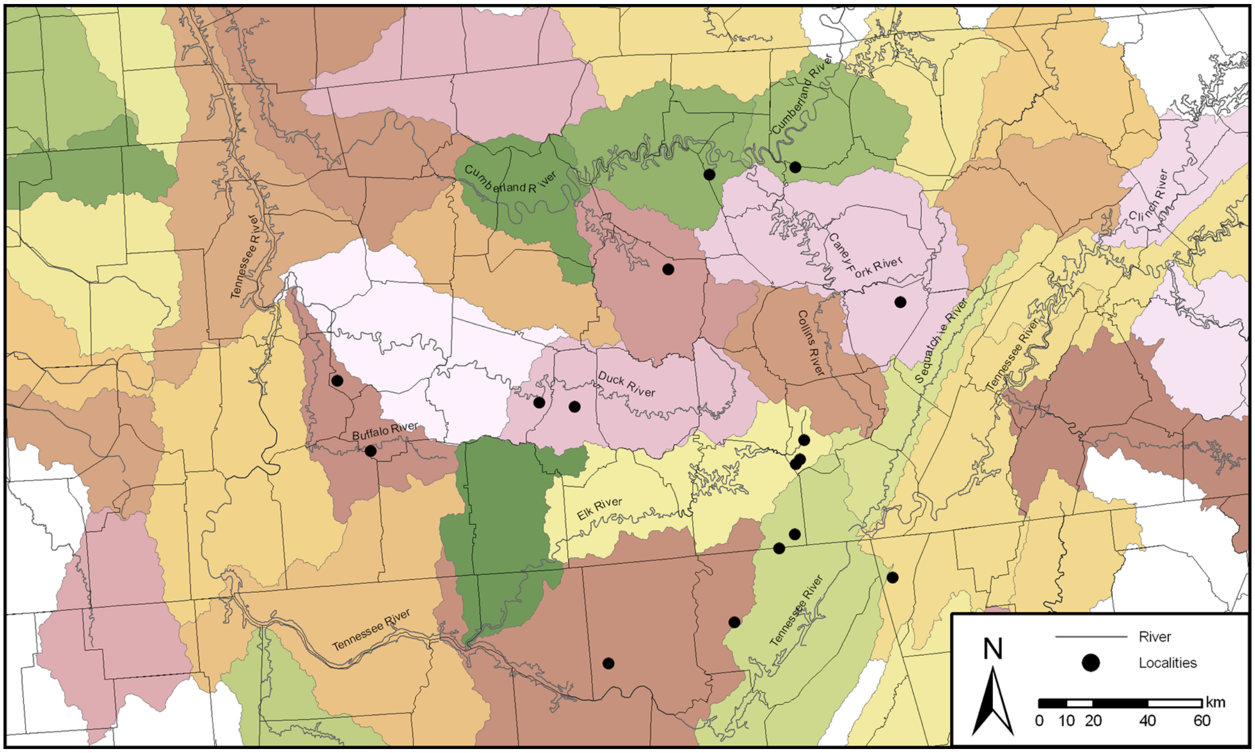

Figure 2 *Sampling localities and distribution of the southern cavefish,* Typhlichthys subterraneus. *Localities are color-coded to match clades in ND2 phylogeny. Present-day drainages are also highlighted. Sampling localities from Arkansas are not shown.*

Percopsiformes (Nelson 2006). Sequences were aligned to each other and to outgroup sequences for each locus.

Gene trees were constructed using Bayesian analyses with the ND2 and S7 datasets analyzed separately. The optimal model of sequence evolution for each dataset was determined using Akaike's information criterion (AIC) implemented in Modeltest 3.7 (Posada and Crandall 1998). Bayesian posterior probabilities were estimated in MrBayes 3.1 (Ronquist and Huelsenbeck 2003). Two independent runs using four Markov chains and temperature profiles at the default setting of 0.2 were conducted for 8 million generations, sampling every 100th generation. Modeltest selected different models of sequence evolution for first, second, and third position codons of ND2. Therefore, the ND2 dataset was partitioned accordingly and unlinked allowing values for transition/transversion ratio, proportion of invariable sites and among-site rate heterogeneity to vary across codon positions during analysis. Random trees were used to begin each Markov chain and a molecular clock was not enforced. The first 1.5 million generations were discarded as "burn-in" to ensure stationarity after examination of the posterior probability. Samples from the stationary distribution of trees were used to generate 50% majority-rule consensus trees for each locus. Divergence times for uncalibrated nodes in the ND2 dataset were derived by using the program r8s (M.J. Sanderson). Two fossils were used to date key nodes representing the most recent common ancestor (MRCA) of all percopsids and the MRCA of the Aphredoderidae and Amblyopsidae (Rosen 1962, Rosen and Patterson 1969, Murray and Wilson 1996). Fossil dates were treated as a fixed minimal age.

To test whether genetic population structure is best described as isolation by distance or as hierarchical subdivision, distance-based redundancy analysis (dbRDA, Legendre and Anderson 1999, McArdle and Anderson 2001, Geffen et al. 2004) was used to investigate the joint effects of distance and watershed boundaries on genetic structure in *T. subterraneus*. Understanding geographic population structure can yield important information regarding whether gene flow is sufficiently restricted across a species range to allow substantial differences to accumulate via genetic drift, and if so, whether the genetic population structure is best described as isolation by distance or as hierarchical subdivision. The program DISTLM (Anderson 2004) was used to perform dbRDA using a second-order polynomial function of latitude and longitude as our distance variable set (Borcard et al. 1992). First, the relationship between the DNA distance matrices and the distance variable set was analyzed alone using dbRDA with p-values estimated from 9999 permutations of the distance matrix. Then a set of dummy variables indicating the watershed containing each site was analyzed as a predictor variable set with the distance variable set fitted as covariates. We used 9999 permutations of the residual distance matrix to estimate p-values.

Results

Phylogenetic analyses. Bayesian analyses of both the mitochondrial ND2 and nuclear S7 datasets support the monophyly of *T. subterraneus*. Likewise, the Amblyopsidae was monophyletic with *C. cornuta* most basal, however, monophyly of the genus *Amblyopsis* was not supported by both the ND2 and S7 datasets. Solutions to this problem in classification include lumping all four troglomorphic species into the oldest genus, *Amblyopsis*, or splitting the existing *Amblyopsis* into *A. spelaea* and *Troglichthys rosae* (Eigenmann 1899).

Within *Typhlichthys*, 41 haplotypes were recovered for the ND2 dataset. There was a clear pattern of correspondence between mtDNA lineages and surface hydrological boundaries (Figure 2) with sequence divergence up to 11.6% among lineages. Almost all haplotypes from a given hydrological unit grouped within the same lineage (Figures 2 and 3). Exceptions included haplotype TsubAE from Marion County, Tennessee, that grouped with haplotypes from northwest Geor-

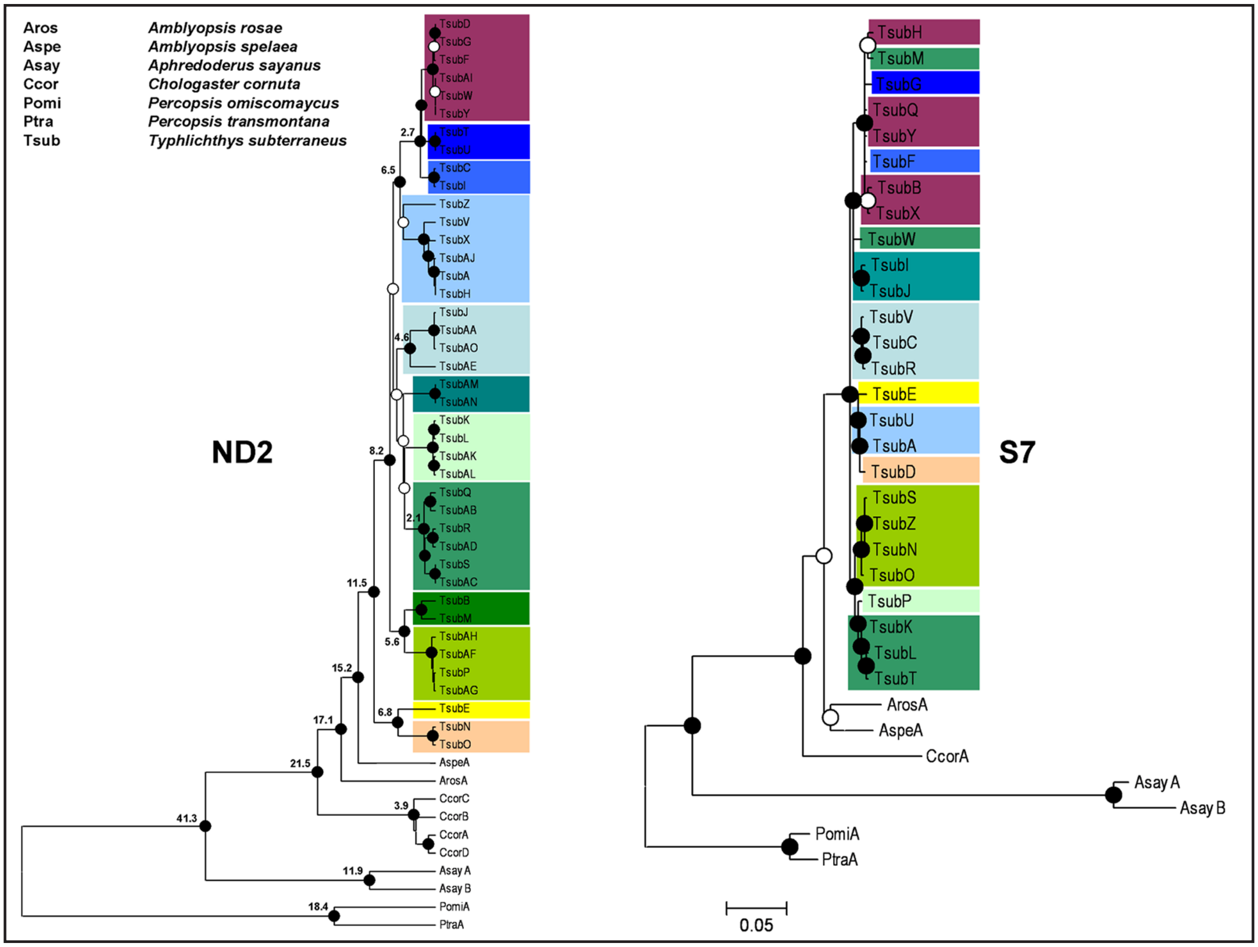

Figure 3 Bayesian chronogram of ND2 (left) and phylogram of S7 (right) datasets. Solid circles at nodes indicate posterior probabilities > 0.95. Numbers above nodes indicate divergence time estimates in Mya. Outgroups used were Asay, Pomi, and Ptra.

gia and haplotypes from caves in Overton and Putnam counties in the Upper Cumberland River drainage of Tennessee that grouped with haplotypes from the Upper Caney Fork River drainage in Van Buren County rather than other Cumberland River haplotypes downstream. Little evidence of contemporary gene flow was found, particularly among drainages. Only two localities located in Franklin County, Tennessee, and separated by 2.5 km shared ND2 haplotypes. The S7 dataset also supported a monophyletic *Typhlichthys* but relationships among drainages were not nearly as resolved (Figure 2). Twenty-six S7 haplotypes were recovered with uncorrected sequence divergence up to 2.6% observed among drainages.

Divergence time estimates. Fossil-calibrated divergence estimates place the MRCA of *Typhlichthys* at 11.5 Mya with the MRCA of all amblyopsids dating to 21.5 Mya. Divergence estimates among some eastern lineages dated to 8.2 Mya. Interestingly, the population sampled from the Red River drainage in the Highland Rim in Robertson County, Tennessee, grouped more closely with western populations in Arkansas than other eastern populations. This split is estimated to have occurred around 6.8 Mya suggesting that the biogeographic history of populations east and west of the Mississippi River is more complex than a single vicariant event.

Distance-based redundancy analyses. Distance-based redundancy analyses further emphasized the hierarchical genetic subdivision of populations (Table 1). Significant association between genetic variation and geographic distance was detected for both datasets. Moreover, conditional tests revealed a significant association between genetic variation and drainages for both the ND2 and S7 datasets accounting for 42.6 and 47.6% of the variation above and beyond geographic distance alone.

Discussion

Cryptic diversity and conservation. The identification of cryptic species and ESUs has important implications for conservation and management. The occurrence of cryptic species in endangered nominal species requires special consideration in conservation planning (Bickford et al. 2007). First, species already having a conservation listing might be comprised of multiple species that may be more rare than previously thought. Second, these species might require different conservation strategies (Schonrogge et al. 2002). Here we examined genetic variation and structure in the widely-distributed southern cavefish, *T. subterraneus*, a species of conservation concern in all states throughout its distribution. Our analyses reveal a diversity of deeply divergent lineages within *Typhlichthys* and support provisional recognition of ESUs and even new species with more restricted geographic distributions than *T. subterraneus sensu lato*.

A definite pattern of correspondence existed between mitochondrial lineages and surface hydrological drainages within *Typhlichthys*. This pattern also has been observed for several other stygobitic species (Verovnik *et al.* 2004, Finston *et al.* 2007). No haplotypes were shared among drainages and pairwise sequence divergence between some drainages was as high as 11.6%. Lower levels of sequence divergence between some surface drainages east of the Mississippi River indicate a more recent connection. However, we found little evidence

Table 1 *Tests for the relationships between DNA variation of* Typhlichthys *populations and the predictor variables distance and hydrologic drainage using dbRDA. On the left are the results of marginal tests of the distance variable set where a second-order polynomial function of latitude and longitude was fitted. On the right are the results of conditional tests evaluating drainage connections as predictors of DNA variation with the distance variable set included as covariables. All P-values were <0.05.*

Dataset	Distance			Drainage		
	F	*P*	%var	F	*P*	%var
ND2	5.770	0.0001	54.59	21.234	0.0001	42.60
S7	2.347	0.0027	38.18	3.726	0.0035	47.62

of contemporary gene flow among drainages and among populations within drainages. In addition to lack of haplotype sharing, distance-based redundancy analyses further emphasized the hierarchical genetic structure of mtDNA and S7 haplotypes according to surface hydrological drainages. Haplotype sharing was observed only between two caves separated by 2.5 km in southern Franklin County, Tennessee. However, sampling for this preliminary dataset is sparse within localities and within some drainages. Therefore, more thorough collections are needed to elucidate contemporary gene flow among *Typhlichthys* populations.

It is important not to employ a single source of data, even molecular, when identifying units for conservation and management. Some sources, such as morphology for many wide-ranging cave organisms like *T. subterraneus*, may not offer much valuable information when discerning taxonomic or conservation units. Therefore, multiple sources of data, if available, should be utilized when identifying units for conservation and management. For many cave organisms, sources may be limited to geography, geology, morphology and a few molecular markers. The monophyly of several lineages within *Typhlichthys* that correspond to distinct drainages provides evidence that these lineages have evolved independently for considerable amounts of time. Some lineages have been separated since the late Miocene (longer, for example, than humans and chimpanzees Kumar et al. 2005). Relying on genetic evidence alone, many of these lineages would be considered distinct species despite lack of morphological differences. Many of these lineages also inhabit different geological units and physiographic regions. However, can we demonstrate that genetic variation among lineages corresponds to speciation? Life history and behavioral evidence for reproductive isolation among lineages are lacking and remain to be domonstrated. However, several lineages can be defined as "genealogical species" under the genealogical species concept (Avise and Ball 1990, Baum and Shaw 1995) based on concordance of genetic, geographic, and geologic datasets. Likewise, these lineages can be considered "diagnosable species" under the criteria of the phylogenetic species concept (de Queiroz and Donoghue 1990) and as ESUs (*sensu* Moritz 1994) for conservation and management.

At this time, we offer three provisional recommendations. First, the *Typhlichthys* found on the Ozark Plateau west of the Mississippi are geographically and genetically distinct and should be designated an ESU or potentially a separate species. Second, *Typhlichthys* north of Tennessee must be studied further, as our single sample from the Red River drainage appears to be sister to the Ozark group and deeply divergent from all other eastern samples. Third, each of the other watersheds in Tennessee and Alabama should be considered ESUs or at least as demographically separate management units (Palsboll et al. 2007) because the lack of haplotype sharing and deep divergences among haplotypes suggest that each drainage harbors a unique and historically significant portion of the evolutionary diversity of *Typhlichthys* and dispersal among drainages is insignificant.

Systematic relationships in the Amblyopsidae. Although amblyopsid fishes have been known to science since the early 1840s, the systematic relationships among species within the family remain poorly understood. Previous systematic investigations are limited to the morphological study of Eigenmann (1909) and Woods and Inger (1957) and genetic studies by Swofford (1982), Swofford *et al.* (1980), Bergstrom *et al.* (1995) and Bergstrom (1997). Woods and Inger (1957) synonymized all four species of *Typhlichthys* recognized prior to their study on the basis of lack of any clear geographic pattern in morphological variation. Likewise, *Troglichthys rosae* was synonymized under *Amblyopsis* and *Forbesichthys* was synonymized under *Chologaster* (Woods and Inger 1957). Electrophoretic analyses by Swofford (1982) showed considerable differentiation among populations of *Typhlichthys* suggestive of multiple, independent invasions of subterranean waters. Likewise, substantial differentiation was detected between the synonymized species of *Chologaster*, warranting resurrection of the genus *Forbesichthys*. However, the relationships among amblyopsid species were equivocal. More recently, Figg and Bessken (1995) have questioned the monophyly of *Amblyopsis*. Likewise, Bergstrom et al. (1995) and Bergstrom (1997) examined variation at the mitochondrial ND2 locus and resolved *Amblyopsis* as polyphyletic and *Typhlichthys* as paraphyletic. Regrettably, incomplete sampling and inadequate sample sizes limited past studies.

Our study also resolved *Amblyopsis* as non-monophyletic but supported the monophyly of *T. subterraneus* despite several, highly genetically-differentiated lineages. *Amblyopsis rosae* is the sister lineage to *T. subterraneus* and *A. spelaea*. The MRCA of these two lineages is estimated at 15.2 Mya based on the ND2 phylogeny. *Amblyopsis* also is unsupported in the S7 phylogeny, however, the branching order of *A. rosae*, *A. spelaea*, and *T. subterraneus* are equivocal. Although preliminary, our results support the nonmonophyly of *Amblyopsis*. If other genetic markers reveal a similar topology, the genus *Troglichthys* (Eigenmann 1898) may need to be resurrected for *A. rosae*.

Summary

The deep genetic divergence in Southern cavefish highlights discordance between molecular and morphological evolution, a finding that is becoming more prevalent in studies investigating genetic divergence in cave organisms. These results demonstrate that current morphological taxonomy may greatly underestimate genetic diversity, and, in turn, biodiversity in subterranean ichthyofauna. Future studies of subterranean fauna should incorporate multiple datasets, including morphological, genetic, geographic and geological, when identifying cryptic species or ESUs with similar phenotypes for conservation and management.

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