Diversity and Conservation of the Southern Cavefish, *Typhlichthys subterraneus*

by

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Abstract

The Southern Cavefish, *Typhlichthys subterraneus*, is one of the most fascinating stygobionts of the Amblyopsidae due to undescribed diversity within it. I investigated aspects of morphological and molecular diversity for their potential to describe diversity and how those aspects influenced the conservation status of the species.

I first quantified differences in shape within the Southern Cavefish utilizing Geometric Morphometrics. Firstly, the presence of ontogenetic allometry within the species was investigated. Relative Warps analysis was utilized to identify the axes of major shape variation. Specimens were then grouped into life history stages. Support for ontogenetic allometry was discovered by the significant prediction of shape (Relative Warps) by life history stage (standard length). Secondly, I performed an allometric correction to develop a size-independent morphospace. Principal Components Analysis indicated the size-independent major axes of shape variation occurred within the head length to predorsal length ratio and head size and shape in both lateral and dorsal views. Specimens were grouped by four categories: 1) aquifer association, 2) genetic lineage, 3) hydrological basin, and 4) ecoregion. Utilizing ANOVA and Tukey's Post-Hoc tests, I found shape differences among categories for some groups, but shape could not distinguish all groups from one another in any category. Poor agreement between morphology and multiple categories can be explained by convergent evolution of forms in caves, low genetic resolution, and possibly cryptic morphology (i.e., no morphological characters to define diversity).

Cryptic morphology coupled with collection difficulties and recent divergence of genetic lineages can inhibit the designation of species in stygobiont taxa. By designating Evolutionarily Significant Units (ESUs), the conservation community can create management plans to conserve the genetic diversity within a group. The lineages of *Typhlichthys subterraneus* were designated as ESUs and given conservation ranks; however, newly sampled populations had not been previously investigated as to their association with existing lineages. Genomic DNA was extracted from fin clips and three genes were amplified (ND2, S7, Rhod). Sequences were aligned and edited manually in Geneious, and Maximum Likelihood phylogenies were generated for each gene as well as a concatenated dataset. Newly sampled populations were recovered within existing lineages but unique positions of two previously designated caves were found. Conservation ranks utilizing both the NatureServe and IUCN Red List criteria were recalculated for each lineage. One lineage was downgraded to Vulnerable due to population sizes and another newly designated lineage was given a rank of Critically Imperiled (NatureServe) and Critically Endangered (IUCN Red List). Nonmonophyletic relationships within the phylogenies may be a result of gene flow between aquifers or low genetic resolution due to incomplete lineage sorting.

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List of Abbreviations

AUM	Auburn Museum of Natural History
GM	Geometric Morphometrics
RWA	Relative Warps Analysis
PCA	Principal Components Analysis

Chapter 1

Shape variation within the Southern Cavefish, *Typhlichthys subterraneus* (Percopsiformes: Amblyopsidae)

Introduction

The amblyopsid fishes (Actinopterygii: Percopsiformes) are one of few families of animals that include all three states associated with troglomorphy (morphological changes related to subterranean life). This family is restricted to North America and consists of species representing epigean (surface), stygophilic (facultative cave-dwelling), and stygobiotic (cave-obligate) forms (Woods and Inger 1957; Poulson 1963; Niemiller and Poulson 2010). Six genera comprise the family, four of which are stygobiotic. The described stygobionts include the Northern and Hoosier Cavefishes (*Amblyopsis spp.*), the Ozark Cavefish (*Troglichthys rosae*), the Alabama Cavefish (*Speoplatyrhinus poulsoni*), and the Southern Cavefish (*Typhlichthys spp.*). The Swampfish (*Chologaster cornuta*) and the Spring Cavefishes (*Forbesichthys spp.*) are epigean and stygophilic, respectively. Studies have detailed the higher phylogenetic relationships within Percopsiformes as well as within Amblyopsidae; the order and family have been recovered as monophyletic using both morphology and genetics (Dillman et al. 2011; Near et al. 2012; Niemiller et al. 2013b; Armbruster et al., in press; Figure 1).

The Southern Cavefish, *Typhlichthys subterraneus* Girard 1859, is one of the most fascinating stygobionts of the Amblyopsidae because of undescribed diversity contained within the species (Woods and Inger 1957; Poulson 1963; Swofford 1982; Niemiller and Poulson 2010; Niemiller et al. 2012). Dispute has lead to four species being raised and subsequently sunk

(Woods and Inger 1957); however, numerous lines of strong evidence indicate the existence of undescribed diversity.

Firstly, support for undescribed diversity develops from the observable patterns of extreme endemism exhibited by North American cave-obligate taxa. Within the contiguous United States, 25% of the cave-obligate aquatic species reside in just six counties (<0.14% total land area). 61% of stygobiotic and troglobiotic species and subspecies are found only in a single county (Culver et al. 2000). Low dispersal ability complimented by isolation of hydrological units can lead to restricted ranges and high endemism in stygobionts (Trontelj et al. 2009). As a single species, *T. subterraneus* ranges across three different karst regions: the Appalachians, the Interior Low Plateau, and the Ozarks. *Typhlichthys subterraneus* also has a distribution on both the east and west sides of the Mississippi River (Boschung and Mayden 2004). In the east, the cavefish range extends over Missouri, Arkansas, and possibly into the northeastern tip of Oklahoma (the Ozarks). The Ozark group of *T. subterraneus* has been recently referred to as *Typhlichthys eigenmanni* (Niemiller et al. 2012); however, this species has not been taxonomically redescribed. To the west of the Mississippi River, *T. subterraneus* ranges from Kentucky, into Tennessee, Alabama, and the Northwestern tip of Georgia. Compared to other North American stygobiont distributions, this extended range for single stygobiotic species is extremely unlikely. Due to the close association of stygobionts to isolated hydrological units, we examined the association of caves (and therefore the specimens collected in those caves) to aquifers in our data. Speleobiologists hypothesize that cavefish actually live in the groundwater and only occasionally venture above into cave streams where they can be encountered by cavers. Thus, we chose to examine shape variation in the context of aquifer association.

In addition to an unlikely single species distribution, multiple divergent genetic lineages have been recovered based on analyses of allozymes, and nuclear and mitochondrial genes (Swofford 1982; Niemiller and Fitzpatrick 2007; Niemiller et al. 2012). As many as 10 genetically distinct lineages may exist throughout the range of *T. subterraneus* (Niemiller et al. 2012); however, the number of delineated species differed with the alteration of three variables: 1) number of individuals, 2) number of populations, and 3) number of genes used. Niemiller et al. (2012) focused on O'Meara's (2010) species delimitation technique and particularly the changes in lineage designation of a population when the aforementioned variables were altered. The emphasis of this study was not to find an optimal lineage designation scheme; yet in the primary author's dissertation, a lineage designation arrangement was chosen and applied to the final chapter of the dissertation (Niemiller 2011, doctoral dissertation). This designation system included all populations, all individuals, and four genes (*nd2*, *s7*, *rag1*, and *rhod*). We mirrored this designation scheme to the populations that overlapped in our study and Niemiller et al.'s (2012) study (Figure 2). Though the lineage designation scheme provides a molecular hypothesis for the possible complex of species, the number of evolutionary lineages that could represent a species remains undefined. The genetics in Niemiller et al. (2012) were structured by surface hydrological basin and to a lesser extent surface ecoregion.

We noticed shape variation while visually comparing similarly sized museum specimens (Figure 3). Additionally, we noticed several differences when comparing dissimilarly sized specimens. Smaller individuals appeared to have a considerable degree of rostral flaring and dorsoventral compression, creating the appearance of a shovel-shaped snout (Figure 4a). Retention of neotenic characters (such as rostral flaring and larger head to body ratios) is apparent between genera within the Amblyopsidae (see Niemiller and Poulson 2010 and

Armbruster et al. 2016, in press); however, the shape changes between ontogenetic stages within the genera have not yet been quantified. We theorize that *T. subterraneus* has considerable ontogenetic allometry. Ontogenetic or growth allometry is the change in morphology and/or physiology of an organism through ontogenetic stages (Klingenberg and Zimmermann 1992; Klingenberg 1998). We believe that the degree of rostral flaring decreases as the fish ages and that the fish becomes less dorsoventrally compressed (Figure 4b). Poulson (1963) equated life history stages of *T. subterraneus* to size (i.e., standard length) by their scale and otolith annuli. We applied these life history categories to the specimens utilized in this study.

Our objective is to examine shape variation within *Typhlichthys subterraneus* in Kentucky, Tennessee, Alabama, and Georgia. To do this, we explore the effect of growth allometry by testing the ability of standard length to predict the geometric morphometric measurements. We also investigate if size-independent shape variation corresponds to four different categories used to support undescribed diversity including genetic variation and three different ecosystem associations.

Materials and Methods

Specimen Collection

Fieldwork began in the winter of 2012 and continued through the duration of this project. Fieldwork was concentrated in Alabama, Georgia, and Tennessee. Up to ten specimens were sacrificed at the time of collection. The fish were sacrificed by over-anesthetization using MS222 and fin clips stored in RNA Later. Whole specimens were treated with 10% formalin solution and are stored in 70% ethanol in the Auburn University Museum of Natural History Ichthyological Collection. We visited historical localities as well as new caves with the hope of finding new populations.

Shape Investigation

We photographed museum specimens in dorsal and lateral view with a Nikon D90 digital SLR camera attached to a copy stand. We then utilized Geometric Morphometrics (GM) to describe biologically relevant shape variation. Novel GM landmark schemes for both dorsal and lateral views were designed in loose association with Armbruster (2012). These schemes were digitized in tpsDig v. 2.16 (Rohlf 2010; Figure 5). 150 specimens were utilized for the dorsal view analysis and 154 were used for the lateral view.

Static landmarks were placed on homologous points across specimens. We utilized sliding landmarks to capture variation unable to be quantified by the static landmarks. Generalized Procrustes Analysis was performed to rotate, scale, and fit the specimen's shape onto a coordinate plane, which then provides coordinate points for each landmark. A covariance matrix was then constructed and Relative Warps Analysis (RWA) was performed. Both Procrustes Analysis and RWA were performed in tpsRelw v. 1.53 (Rohlf 2013). TpsRelw was utilized for its ability to account for sliding landmarks. RWA is identical to Principal Component Analysis (PCA); RWA is a variable reduction technique that quantifies individual variation (Birch 1997). The Unbend function was then utilized in tpsUtil v. 1.58 (Rohlf 2013). This particular function fits user-specified points to a quadratic curve, effectively removing the curvature of the specimen that may be associated with preservation effects.

Relative warps (RWs) were then plotted against standard length. We calculated correlation coefficients to determine the statistical degree to which standard length predicted shape variation. We performed these comparisons to investigate whether aspects of shape variation corresponded to size and age of the fish. Poulson (1963) studied the life history characteristics of the Amblyopsidae and determined life history stages for each genus. Age and

standard length were denoted for each of the life history stages. We used standard length to group the specimens in our study into three life history stages according to Poulson (1963): Juveniles with Vent Migration Complete (JVM) were 10-23 mm, Juveniles in First Annulus (JFA) were 21-30 mm, and Adults (A) were 33-62 mm.

We concluded that standard length and thus age of the specimen has a profound affect on the shape of the specimen (refer to Results: Pre-Allometric Correction). To investigate relevant shape variation not associated with size, we performed a residuals analysis similar to that employed in Sidlauskus et al. (2011). A standard linear regression using log centroid size on GM Procrustes Coordinates was performed in R Studio v. 0.99.473 (RStudio, Inc.). A PCA was then implemented on the residuals of the regression. We created four PC score scatterplots per orientation with each plot representing one of the following categories: 1) aquifer association (six aquifers; USGS 2003; Figure 6a); 2) the genetic lineage ascribed to the cave with which the specimen was associated based on the adapted molecular phylogeny from Niemiller et al. (2012; 10 lineages; Figure 6b); 3) HUC subregion association (six HUC subregions; Seaber et al. 1987 for USGS; Figure 6c); 4) surface ecoregion association (three ecoregions; Niemiller et al. 2012; Figure 6d). We chose these categories to examine if shape variation within the Southern Cavefish corresponded to the aforementioned lines of support for undescribed diversity. If a priori groups separate in morphospace within these categories, we may be able to use this information to distinguish diversity within the SE populations of the Southern Cavefish.

For each category, we performed an Analysis of Variance (ANOVA) on the PC's to determine if significant differences existed between the groups within the categories. If significant differences were found, we executed a Tukey's Honest Significant Difference (HSD) Post-Hoc test to extrapolate the pairwise groups with significantly different shapes.

Results

Pre-Allometric Correction

Correlation coefficients for the dorsal orientation were significant for RW1 (R^2= 0.27, p <0.001) and RW2 (R^2= 0.03, p <0.05), which explained 63.9% of shape variation (Figure 7a, c, and e). The first RW described variation in the head length and head width of the fish. The juveniles have more negative scores (narrow, elongate head) while the adults have more positive scores (wide, stout head). Thus, juveniles have longer, narrower heads than adults. Flaring of the rostral edges can also be seen in the RW demonstration plots. RW2 had an inverse relationship with standard length; as standard length increases, head width increases but predorsal length shortens. Adults have wider heads with shorter predorsal lengths than juveniles.

In the lateral view, the first RW had a significant correlation coefficient (R2= 0.36, p <0.001). The second RW was an artifact, thus we utilized the third RW for further analyses. RW3 was not significantly correlated with SL (R2= 0.01, p >0.05). The first and third RW explained 52% of shape variation (Figure 7b, d, and f). RW1 described variation in the head length to predorsal length ratio as well as head depth. Juveniles have an elongate and dorsoventrally flattened head with a high head to predorsal length ratio. Adults have a blunt head with a small ratio of head to predorsal length. We explored RW3 but do not report the results due to non-significance.

Allometric Correction

Two important PCs were returned explaining a total 62.5% of the variation in the dorsal orientation. The first PC explained 43.9% variation with the most variation in the ratio between predorsal length and head length. The second PC described the most variation in head width,

explaining 18.6% variation (Figure 8a, c, e, and g). Preservation effects prevent us from using more than the first two PCs.

The first and the third PC's in the lateral orientation explained a total of 47.6% variation. The first PC, explaining a ratio between head length and predorsal length, described 38.1% variation (Figure 8b, d, f, and h). PC3 explained 9.5% variation, with the most variation in head shape and depth. PC2 and the remaining PCs represented preservation effects and were not utilized in this study.

Aquifer Association Plots (Figure 8a, b)

Results of analysis of variance (ANOVA) for the dorsal orientation suggested significant differences in shape between aquifers utilizing only the first PC (F= 6.18, p <0.001) in the dorsal orientation (PC2: F= 2.35, p >0.05). Tukey's Honest Significant Difference (HSD) test yielded significant results (95% CI; p <0.05) for three comparisons using PC1 (Table 1). No structuring between a priori groups was distinguishable in the scatterplot of PC scores (Figure 8a).

The ANOVA results for the lateral orientation suggested significant differences between aquifer associations only with PC1 (PC1: F= 8.17, p <0.001; PC3: F= 1.66, p >0.05). Five comparisons returned significant results utilizing PC1 (Table 5). A priori groups were not identifiable from the single conglomerate of points in the scatterplot (Figure 8b).

In both the dorsal and the lateral view, the Mississippian Aquifer and Other Rocks Aquifer have wider distributions across morphospace within scatterplots than the remaining four aquifers (Figure 8a, b). The comparably larger range across morphospace for these two aquifers is most likely due to the geographic distribution of the caves in the Southeastern United States; there are more caves associated with the Mississippian and Other Rocks aquifers than the remaining aquifers. When comparing aquifers (such as the Mississippian aquifer to the Silurian-

Devonian aquifer), the aquifer with the greater geographic range will be associated with a greater range of caves (Figure 6a). Since there are more caves associated with the Mississippian aquifer than the Silurian-Devonian aquifer, there are a greater number of samples from the Mississippian aquifer. Subsequently, a larger sample group of specimens may possess more shape variation. More shape variation expands the distribution of the associated aquifer across morphospace in the PC scatterplot. Thus, the Mississippian aquifer has a wider distribution in morphospace when compared to another aquifer with less associated caves.

Genetic Lineages (Figure 8c, d)

ANOVA results indicated significant shape differences between genetic lineages in both PC1 (F= 10.56, p <0.001) and PC2 (F= 3.17, p <0.01). Ten comparisons from the Tukey's HSD Post-Hoc analysis yielded significant results (95% CI; p <0.05) for the first PC in the dorsal orientation (Table 2). For the second PC, three Post-Hoc comparisons indicated significant differences between genetic lineages. No significant comparisons were the same between the first and the second PC. No a priori groups were distinguishable from the conglomerate of points in the scatterplot (Figure 8c).

Lateral ANOVA indicated significant differences between lineages in PC1 but not PC3 (PC1: F= 13.4, p <0.001; PC3: F= 0.89, p >0.05). In the lateral view, 13 significantly different comparisons were made utilizing PC1 (Table 6). Scatterplots present a single cloud of points without separation of a priori groups (Figure 8d).

HUC Subregion (Figure 8e, f)

Results for both PC1 (F= 9.53, p <0.001) and PC2 (F= 2.54, p <0.05) were significant using ANOVA. Utilizing PC1, five comparisons yielded significant results from the Tukey's

HSD Post-Hoc test (Table 3). One significant comparison (LOW and GRE) was made with PC2; this comparison was found to be significant in both Post-Hoc analyses for PC1 and PC2.

ANOVA results suggested significant differences between groups using the first PC (F= 9.12, p <0.001) but not the third PC (F= 1.7, p >0.05). Significant differences were identified in three comparisons utilizing PC1 (Table 7). Previously structured groupings did not separate from the conglomerate of points in either dorsal or lateral view (Figure 8e, f).

Similarly to results for the other categories, no a priori groups were distinguishable in the scatterplots colored by HUC subregion. The Elk and the Cumberland subregions have wider distributions in morphospace when compared to the other four subregions (Figure 8e, f). The larger morphospace distribution of two of the subregions is a similar pattern to that observed in the Aquifer plot (Figure 8a, b). As with the Mississippian and Other Rocks aquifers, the subregions of the Elk and the Cumberland are associated with a wider geographic distribution of caves than the remaining four subregions. The number of specimens is greater with the larger number of caves. The larger sample size increases the chances of more shape variety. Consequently, the larger variety in shape is apparent in morphospace for both the Elk and Cumberland HUC subregions when compared to the remaining subregions.

Ecoregion (Figure 8g, h)

Significant shape variation was found between ecoregions using PC1 (F= 28.53, p <0.001) but not PC2 (F= 3.04, p >0.05) in the dorsal orientation. Differences in shape were significant between two comparisons utilizing the first PC (Table 4).

ANOVA results suggested significant shape variation utilizing PC1 (F= 24.7, p <0.001) in the lateral orientation, but not utilizing PC3 (F= 2.74, p > 0.05). Results from Tukey's HSD

Post-Hoc test indicated two comparisons with significant differences (Table 8). A priori groups did not separate from the cloud of points in either orientation (Figure 8g, h).

Just as with the aquifer and hydrological basin results, the distribution of an ecoregion across morphospace is dependent on the geographic size of the ecoregion itself (Figure 6d). The Interior Low Plateau spans the largest geographic area and concurrently, the largest morphological variation occurs within this ecoregion.

Discussion

Ontogenetic Allometry

Though allometry has been hypothesized for the genera of the Amblyopsidae, growth allometry had never before been quantified within the genus *Typhlichthys*. Our analyses revealed significant ontogenetic allometry in *Typhlichthys subterraneus* within its Southeastern range. We examined confounding affects growth allometry has on shape analyses. Standard length significantly predicted three of the four major axes of shape variation in the dorsal and the lateral orientations. Juveniles had elongate, dorsoventrally flattened heads, with higher ratios of head to predorsal length. Flaring of the rostral edges is also a noticeably juvenile trait. The shape of *T. subterraneus* juveniles is concordant with the shape of many vertebrate genera that retain neotenic characters (Niemiller and Poulson 2010; Christiansen 2012; Fenolio et al. 2013); the adaptive value for most of these neotenic characters is unknown and warrants exploration. Blunt, wide heads with deep bodies characterize adult *T. subterraneus* specimens.

Shape Variation Independent of Size

We discovered that the major axes of shape variation independent of size were head length to predorsal length ratio, head width, and head depth. Comparisons after allometric correction indicated that shape variation could distinguish some groups within categories;

significant shape diversity between groups was found within each category. However, not all shape variation can be explained by the categories.

A few possibilities exist as to why the variation within *Typhlichthys subterraneus* is visible but does not entirely correspond to either genetic or ecosystem categorizations. Firstly, convergent evolution is prevalent in cave environments. Similar harsh selective pressures (e.g., complete darkness and scarcity of resources) can cause morphological convergence on a phenotype that is advantageous, or at least less energy expensive. The phenotype of many cave-obligate vertebrate taxa converges on an eyeless, pigmentless form with neotenic characters (in salamanders- Fenolio et al. 2013; Christiansen 2012; Niemiller and Poulson 2010). Thus, both parallel and convergent evolution may confound shape analyses of *T. subterraneus*.

Furthermore, there may be functional sexual dimorphism within the Amblyopsidae. Eggs and yolk-sac fry were observed in the buccal cavity of the Northern Cavefish, *Amblyopsis spelaea* (Eigenmann 1909). The jugular positioning of the anus in the Amblyopsidae is also hypothesized for buccal brooding (Poulson 1963). If buccal brooding is family-wide, the head shape of the gender that broods the young may be different from that of the non-brooding parent due to physical and physiological demands from brooding the young. It is not unthinkable that the brooding parent might have a larger buccal cavity than the non-brooding parent, altering the head shape of the fish based on the gender; however, no *Typhlichthys* has ever been found with young, and it is unlikely that there is enough space in the buccal region to hold a clutch of eggs (Armbruster et al., in press). Future directions include dissection of reproductive organs to determine which specimens, and subsequently which shapes, are associated with each gender. Our inability to distinguish all groups within categories using only shape is not surprising when viewed in these contexts.

Although shape alone cannot distinguish all groups within categories, our results will assist investigations of discrete morphological characters that could. Our study provides further insight into the driving factors behind physical changes of troglomorphic fishes.

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Figures and Tables

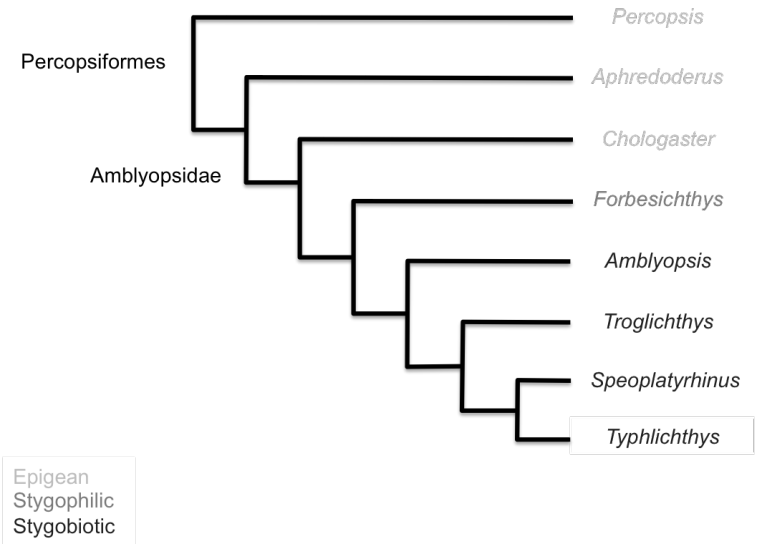

Figure 1. Morphological phylogeny of the Percopsiformes; adapted from Armbruster et al. 2016 (in press).

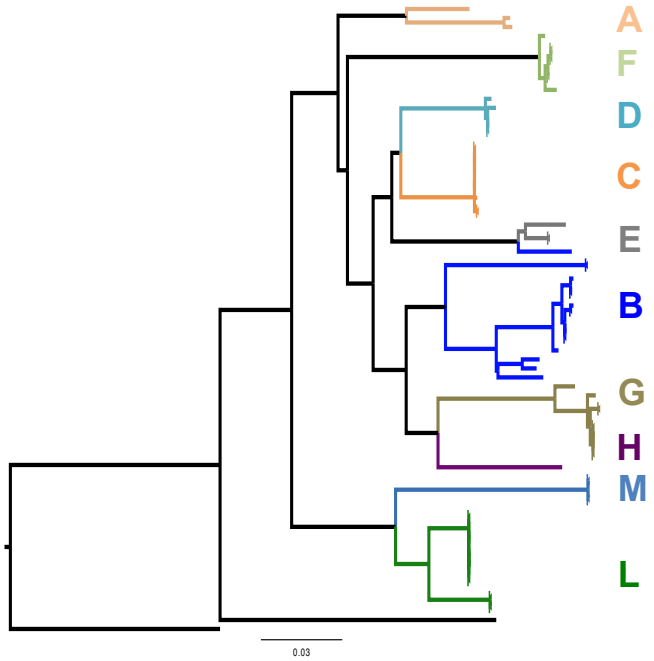

Figure 2. Maximum likelihood multilocus molecular phylogeny of the Southeastern T. subterraneus populations colored by genetic lineage association from Niemiller et al. (2012). Outgroup taxa include Speoplatyrhinus poulsoni and Amblyopsis spelaea. Scale bar unit: expected substitutions per site.

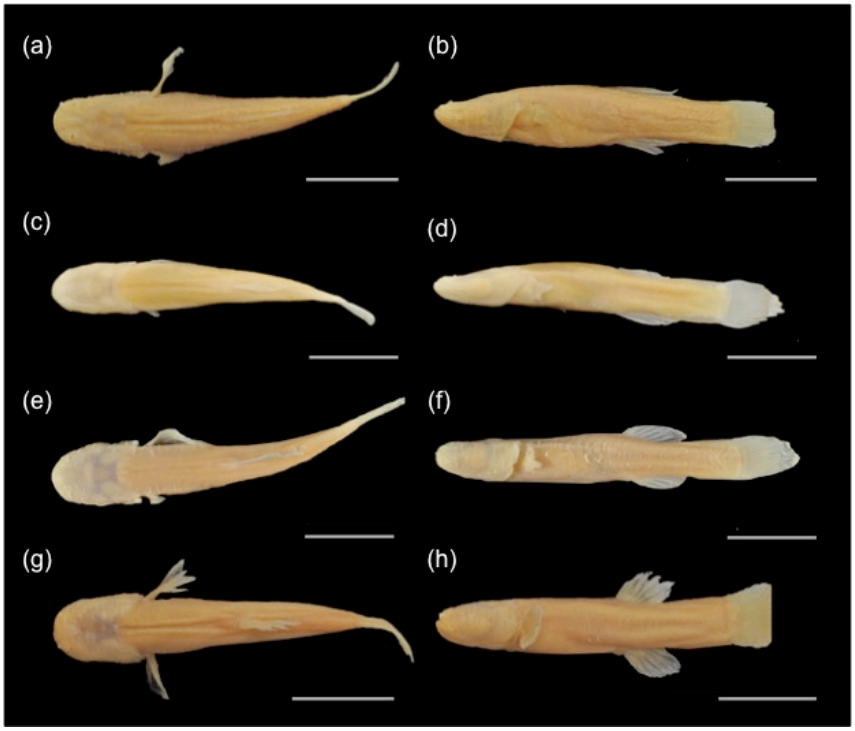

Figure 3. Shape variation between adult Typhlichthys subterraneus specimens. Scale bars are 1 cm. Cave locality, museum accession [number in jar; SL]. (a,b) Tally Ditch Cave, AUM 63190 [2; 32.73] (c,d) Baugus Cave, AUM 57001 [3; 32.31] (e,f) Camps Gulf Cave No.2, AUM 56982 [2; 34.09] (g,h) L & N Railroad Cave, UF 35665 [1; 39.12].

Figure 4. An example of shape differences between juvenile and adult Typhlichthys subterraneus. Both specimens are from a single cave. (a) Juvenile, Vent Migration Complete [20.07 mm] (b) Adult, [55.78 mm].

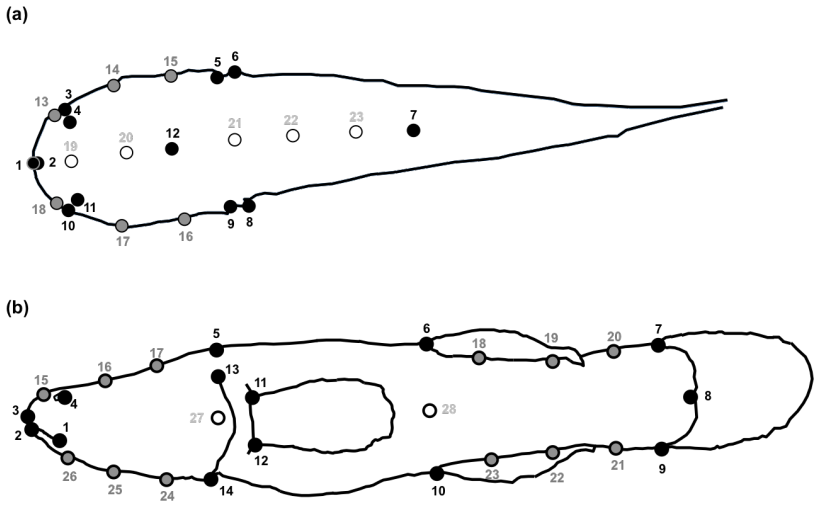

Figure 5. Landmark schemes used for Geometric Morphometrics analysis. (a) Dorsal view and (b) lateral view. Static landmarks are indicated by black circles; sliding landmarks are indicated by grey circles; landmarks used strictly for the Unbend function are indicated as open circles.

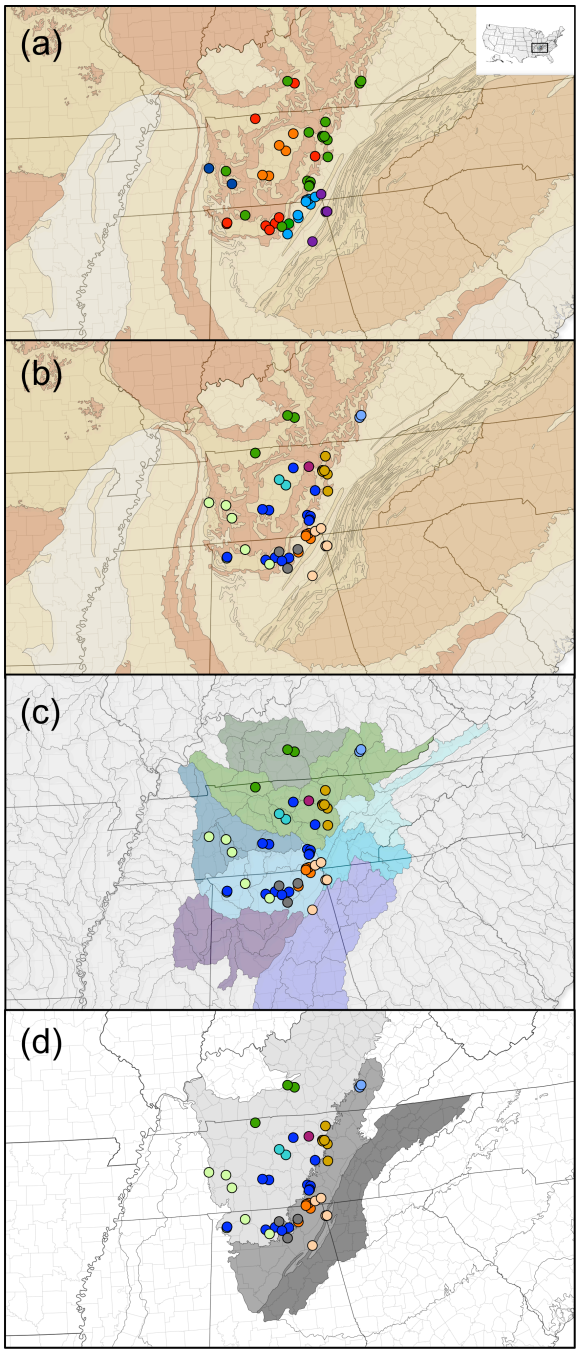

Figure 6. Maps of T. subterraneus populations utilized for this study. (a) Points are colored to signify aquifer association; aquifer rock types are shown in browns, (b) points are colored by genetic lineage; aquifer rock types are shown in browns, (c) points are colored by genetic lineage; HUC subregions are shown in cool colors, (d) points are colored by genetic lineage; ecoregions are shown in greys. Refer to Figure 8 for color keys.

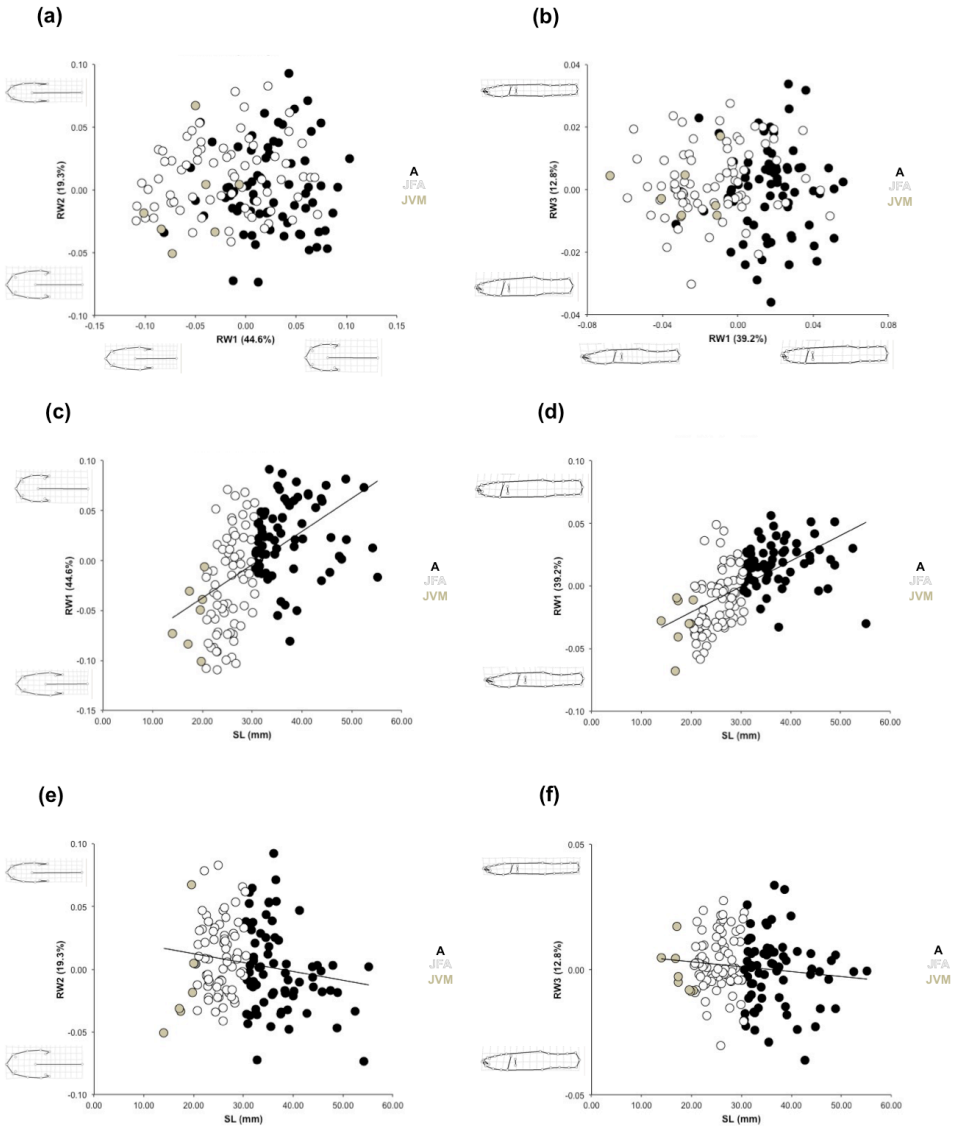

Figure 7. Scatterplots of Relative Warps before allometric correction coded by life history stage. The trendline indicates the linear relationship between Relative Warp and Standard Length. Dorsal (a,c,e) and lateral (b,d,f).

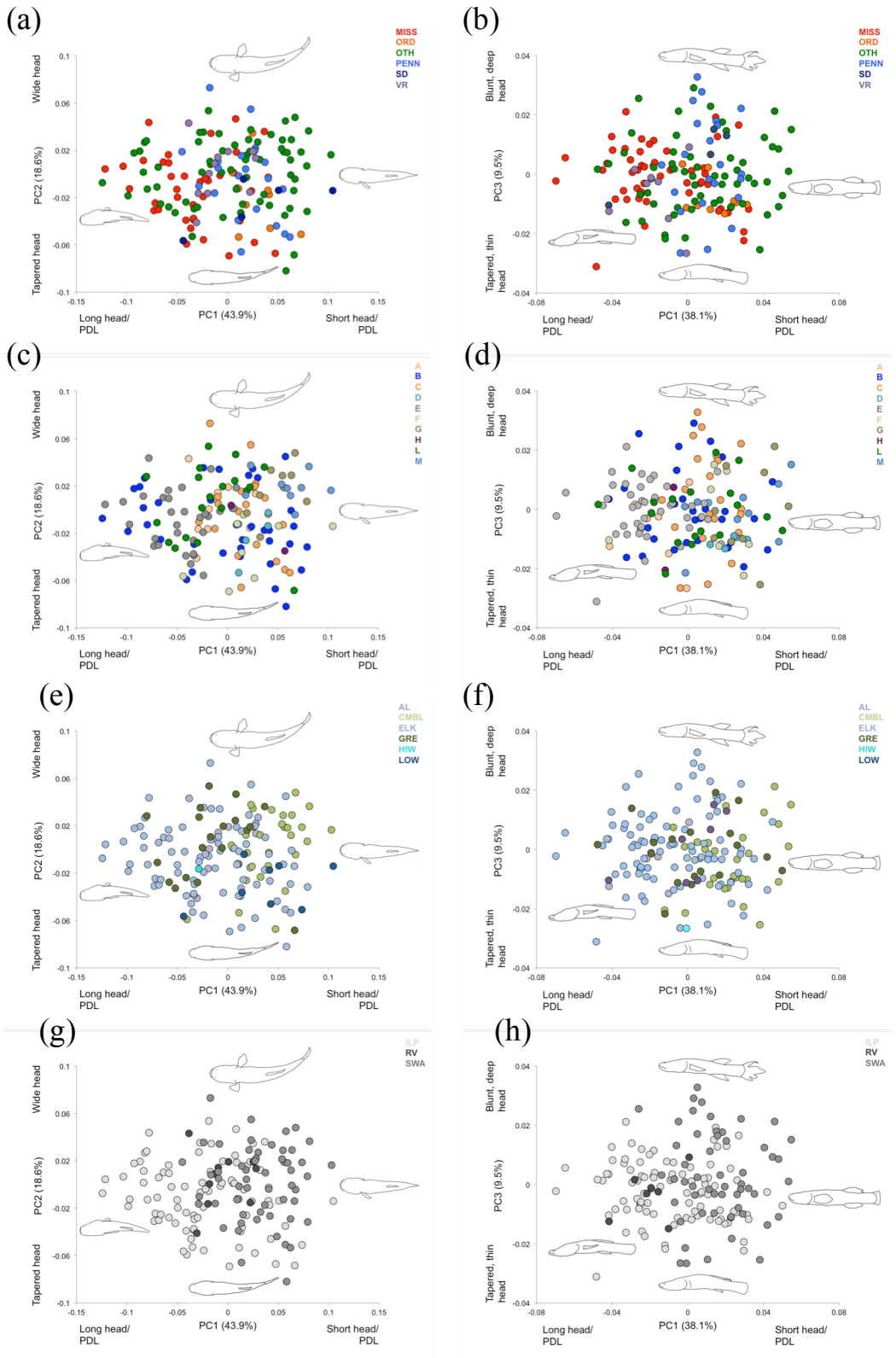

Figure 8. Scatterplots of allometrically corrected PCA results. Color coded by aquifer (a&b), genetic lineage (c&d), HUC subregion (e&f), and ecoregion (g&h).

Table 1. Tukey's Honest Significant Difference analysis adjusted p-values for aquifer comparisons in the dorsal orientation. PC1 below the diagonal; PC2 above the diagonal. Significant values are bolded.

	Mississippian	Ordovician	Other Rocks	Pennsylvanian	Silurian Devonian	Valley and Ridge
Mississippian		0.9945067	0.1302632	0.8889649	0.9557108	0.6242064
Ordovician	**0.0015371**		0.3104008	0.8053595	0.9987196	0.5588781
Other Rocks	**0.0007533**	0.5641632		0.9537606	0.358605	0.9999997
Pennsylvanian	**0.0011459**	0.9657685	0.9163649		0.7210283	0.9869313
Silurian Devonian	0.0551425	0.9999985	0.8840818	0.9956372		0.5020962
Valley and Ridge	0.5674232	0.3827153	0.9518001	0.700643	0.6848607	

Table 2. Tukey's Honest Significant Difference analysis adjusted p-values for genetic lineage comparisons in the dorsal orientation. PC1 below the diagonal; PC2 above the diagonal. Significant values are bolded.

	Lineage A	Lineage B	Lineage C	Lineage D	Lineage E	Lineage F	Lineage G	Lineage H	Lineage L	Lineage M
Lineage A		0.8422935	0.9987602	0.9161471	0.9867084	0.1738526	0.9825757	0.9976148	0.9999969	0.9588119
Lineage B	0.9999997		0.9949797	0.9999964	0.9993921	0.7195827	0.1368816	1	0.2973485	0.0858419
Lineage C	0.6890991	0.4947589		0.9952666	0.9999997	0.3783055	0.6166665	0.9999759	0.9354116	0.4954627
Lineage D	0.912644	0.9172564	1		0.9987958	0.9917054	0.3794222	1	0.6972147	0.3005217
Lineage E	0.1119365	**0.0004503**	**0.0000008**	0.0050726		0.4389028	0.3890342	0.9999973	0.7457268	0.2807789
Lineage F	0.8193014	0.7911316	1	1	**0.0005607**		**0.0137602**	0.9987992	**0.033947**	**0.0085033**
Lineage G	**0.0437449**	0.0126952	0.6663239	0.9265852	**0**	0.9134666		0.8999015	0.9968646	1
Lineage H	0.9607559	0.9724178	0.9999982	0.9999997	0.1715778	0.9999999	0.9997588		0.9866181	0.8629277
Lineage L	0.9999152	0.9829821	0.1285884	0.5905545	0.1553564	0.3781699	**0.0022695**	0.8514526		0.9882865
Lineage M	**0.0065531**	**0.0009503**	0.2424918	0.6673828	**0**	0.6102803	0.9999098	0.9930393	**0.0001639**	

Table 3. Tukey's Honest Significant Difference analysis adjusted p-values for HUC subregion comparisons in the dorsal orientation. PC1 below the diagonal; PC2 above the diagonal. Significant values are bolded.

	Alabama	Cumberland	Elk	Green	Hiwasse	Low
Alabama		0.999541	0.7593804	0.9999651	0.9833213	0.1345284
Cumberland	**0.0494718**		0.6114328	0.9863983	0.9913806	0.0851706
Elk	0.9712689	**0.0000001**		0.3143602	0.9998147	0.3657821
Green	0.9894626	**0.0002531**	0.9999998		0.9716295	**0.0393022**
Hiwassee	0.9952609	0.6173112	0.9997417	0.999684		0.997993
Low	0.3014615	0.9999853	0.0122183	**0.045949**	0.6979223	

Table 4. Tukey's Honest Significant Difference analysis adjusted p-values for ecoregion comparisons in the dorsal orientation. PC1 below the diagonal; PC2 above the diagonal. Significant values are bolded.

	Interior Low Plateau	Ridge and Valley	Southwestern Appalachians
Interior Low Plateau		0.2978325	0.0693587
Ridge and Valley	0.4309499		0.9409052
Southwestern Appalachians	**0**	**0.0288556**	

Table 5. Tukey's Honest Significant Difference analysis adjusted p-values for aquifer comparisons in the lateral orientation. PC1 below the diagonal; PC2 above the diagonal. Significant values are bolded.

	Mississippian	Ordovician	Other Rocks	Pennsylvanian	Silurian Devonian	Valley and Ridge
Mississippian		0.5931961	0.9999977	0.8013743	0.8885248	0.8375202
Ordovician	**0.0101235**		0.5435635	0.2050799	0.4049423	0.9998327
Other Rocks	**0.0000074**	0.986777		0.8305977	0.9017935	0.8038471
Pennsylvanian	**0.0005321**	0.9988023	0.9995865		0.9979585	0.4143504
Silurian Devonian	0.7746199	0.940183	0.9892597	0.9772002		0.560635
Valley and Ridge	0.9997266	0.0672632	**0.0409635**	**0.0494644**	0.7864254	

Table 6. Tukey's Honest Significant Difference analysis adjusted p-values for genetic lineage comparisons in the lateral orientation. PC1 below the diagonal; PC2 above the diagonal. Significant values are bolded.

	Lineage A	Lineage B	Lineage C	Lineage D	Lineage E	Lineage F	Lineage G	Lineage H	Lineage L	Lineage M
Lineage A		0.9665679	0.5985342	0.9999983	0.9506494	0.9999759	0.9959067	1	0.9862384	0.9951314
Lineage B	0.348429		0.9702743	0.8804833	1	0.9999089	1	0.9994745	1	1
Lineage C	**0.024263**	0.7790174		0.452384	0.9653092	0.9445904	0.9891436	0.9776479	0.9762231	0.9943479
Lineage D	0.0172723	0.4678866	0.9880009		0.8477846	0.9985335	0.9697161	1	0.931734	0.9671521
Lineage E	0.9998251	**0.0000819**	**0.0000002**	**0.0000844**		0.9998103	1	0.999291	1	1
Lineage F	0.1283643	0.9589122	1	0.9984366	**0.0018173**		0.9999978	0.9999988	0.9999835	0.9999953
Lineage G	**0.0000155**	**0.0010473**	0.128669	0.9746857	**0**	0.5020554		0.9998769	1	1
Lineage H	0.9999829	0.9986305	0.9112127	0.6644827	0.9978929	0.930263	0.1481732		0.9997346	0.9998341
Lineage L	0.256124	0.999987	0.9830024	0.7385291	**0.0002676**	0.9964582	**0.0116484**	0.994196		1
Lineage M	**0.0003733**	**0.0287379**	0.5916022	0.9998651	**0**	0.8867136	0.9997446	0.3383813	0.1340057	

Table 7. Tukey's Honest Significant Difference analysis adjusted p-values for HUC subregion comparisons in the lateral orientation. PC1 below the diagonal; PC2 above the diagonal. Significant values are bolded.

	Alabama	Cumberland	Elk	Green	Hiwassee	Low
Alabama		0.9999993	0.9491664	0.9961149	0.4715093	0.9873022
Cumberland	**0.0002407**		0.4528078	0.9529796	0.4356473	0.9435561
Elk	0.744132	**0**		0.9966155	0.2193077	1
Green	0.1920171	0.0636472	0.3931951		0.3006083	0.9997855
Hiwassee	0.9718211	0.9086241	0.9997396	0.9999512		0.2834994
Low	0.5508591	0.1935472	0.9435047	0.9995952	0.9999998	

Table 8. Tukey's Honest Significant Difference analysis adjusted p-values for ecoregion comparisons in the lateral orientation. PC1 below the diagonal; PC2 above the diagonal. Significant values are bolded.

	Interior Low Plateau	Ridge and Valley	Southwestern Appalachians
Interior Low Plateau		0.9698041	0.065134
Ridge and Valley	0.4394994		0.4602143
Southwestern Appalachians	**0**	**0.000271**	

Materials examined

Institutional abbreviations:
AUM: Auburn University Museum; UMMZ: University of Michigan Museum of Zoology; UF: University of Florida; UAIC: University of Alabama Ichthyology Collection; YPMICH: Yale Peabody Museum Ichthyology Collection; CU: Cornell University; SIUC: Southern Illinois University Collection; USNM: United States National Museum; INHS: Illinois Natural History Survey; TU: Tulane University

Cave, County, State, Country. Museum Code. (# specimens) [range of SL in mm]

Allens Creek Cave, Lewis Co., TN, USA. AUM56986. (1) [30.14];
Anderson Spring Cave, Putnam Co., TN, USA. AUM56997. (1) [29.81];
Austin Peay Pit Cave, Montgomery Co., TN, USA. UMMZ196194. (1) [39.85];
Bartlett Cave, Putnam Co., TN, USA. AUM56984. (2) [20.39-28.96];
Baugus Cave, Decatur Co., TN, USA. AUM57001. (3) [32.31-36.31];
Beech Spring Cave, Marshall Co., AL, USA. AUM58749. (1) [25.04];
Big Mouth Cave, Grundy Co., TN, USA. AUM57010. (4) [19.99-30.43];
Big Mouth Cave, Grundy Co., TN, USA. AUM56985. (2) [37.07-41.24];
Big Mouth Cave, Grundy Co., TN, USA. UF697. (2) [33.42-40.13];
Blind Fish Cave, Putnam Co., TN, USA. AUM57012. (2) [26.18-52.46];
Blowing Cave, Warren Co., TN, USA. AUM56994. (1) [30.65];
Camps Gulf Cave No. 2, Van Buren Co., TN, USA. AUM56982. (2) [26.6-34.09];
Cave Branch Cave, Hickman Co., TN, USA. AUM56987. (1) [23.01];
Crystal Cave, Grundy Co., TN, USA. AUM56978. (3) [35.25-36.06];
Crystal Cave, Grundy Co., TN, USA. UAIC1977. (2) [25.14-26.67];
Crystal Cave, Grundy Co., TN, USA. UAIC 3958.01. (4) [25.91-35.49];
Daves Cave, Pulaski Co., KY, USA. YPMICH25338. (3) [22.67-32.65];
Drowned Rat Cave, Pulaski Co., KY, USA. AUM57011. (4) [25.07-36.01];
Drowned Rate Cave, Pulaski Co., KY, USA. YPMICH25293. (2) [25.89-39.97];
Flat Rock Cave, Smith Co., TN, USA. AUM56983. (1) [31.31];
Gallagher Cave, Marshall Co., TN, USA. AUM56979. (2) [25.99-35.1];
Garner Spring Cave, Franklin Co., TN, USA. AUM56988. (3) [22.07-29.84];
Herring Cave, Rutherford Co., TN, USA. AUM56980. (3) [31.32-36.04];
Jaco Spring Cave, Warren Co., TN, USA. AUM56995. (4) [24.09-36.59];
Jacques Cave, Putnam Co., TN, USA. AUM56996. (1) [38.64];
L&N Railroad Cave, Barren Co., KY, USA. UF35665. (2) [30.86-39.12];
Limrock Blowing Cave, Jackson Co., AL, USA. AUM63167. (3) [41.21-44.81];
Limrock Blowing Cave, Jackson Co., AL, USA. UAIC14801.01. (1) [31.99];
Little Crow Creek Cave, Franklin Co., TN, USA. AUM57014. (2) [28.82-33.10];

Mammoth Cave National Park, Edmonson Co., KY, USA. CU21726. (2) [29.94-35.76];
Mammoth Cave National Park, Edmonson Co., KY, USA. SIUC18917. (2) [25.40-27.01];
Mammoth Cave National Park, Edmonson Co., KY, USA. SIUC63818. (7) [35.12-55.13];
Mammoth Cave National Park, Edmonson Co., KY, USA. UF32994. (1) [54.15];
Mammoth Cave National Park, Edmonson Co., KY, USA. USNM36632. (4) [26.59-36.54];
Mammoth Cave National Park, Edmonson Co., KY, USA. USNM101172. (3) [17.38-38.31];
McKinney Pit, Colbert Co., AL, USA. AUM56992. (3) [27.94-31.21];
Muddy Cave, Madison Co., AL, USA. AUM58754. (8) [17.14-28.46];
Muddy Cave, Madison Co., AL, USA. UAIC14843.01 (3) [19.76-25.08];
Pattons Cave, Rutherford Co., TN, USA. AUM56991. (3) [31.92-43.82];
Pompie Cave, Maury Co., TN, USA. AUM56981. (1) [35.14];
Pryor Cave Spring, Marion Co., TN, USA. AUM57009. (1) [26.23];
Salt River Cave, Franklin Co., TN, USA. AUM56993. (6) [26.39-31.29];
Sells Cave, Dekalb Co., AL, USA. AUM62589. (2) [22.50-24.41];
Sells Cave, Dekalb Co., AL, USA. UAIC656. (7) [24.67-37.49];
Shelta Cave, Madison Co., AL, USA. INHS60576. (4) [21.13-22.57];
Shelta Cave, Madison Co., AL, USA. TU22765. (30) [16.85-45.71];
Shelta Cave, Madison Co., AL, USA. TU22766. (9) [20.5-47.45];
Shelta Cave, Madison Co., AL, USA. UMMZ146990. (3) [13.96-38.4];
Stamps Cave, Putnam Co., TN, USA. AUM56999. (3) [38.85-48.83];
Tally Ditch Cave, Jackson Co., AL, USA. AUM58746. (1) [31.48];
Tally Ditch Cave, Jackson Co., AL, USA. AUM63190. (3) [30.71-32.73];
Trussell Cave, Grundy Co., TN, USA. AUM56989. (1) [31.63];
Wells Cave, Pulaski Co., KY, USA. AUM57000. (1) [24.17].

Chapter 2

Molecular relationships within *Typhlichthys subterraneus* (Percopsiformes: Amblyopsidae) and conservation implications

Introduction

Characteristics of subterranean life associated with troglomorphy have captured the attention of scientists for centuries; however, biodiversity within the clandestine ecosystem has not drawn the same amount of consideration. Subterranean ecosystems, particularly aquatic habitats, have been largely overlooked in terms of diversity. Around 7,000 aquatic, subterranean species have been described while the total of aquatic species worldwide is over one million (Gibert and Daharveng 2002). With ~94% of the world's freshwater being subsurface, it is extremely likely that groundwater fauna has been vastly underestimated (Gibert and Deharveng 2002, Culver and Pipan 2009, Gibert et al. 2009).

Although biodiversity of aquatic subterranean habitats seems to be an untapped and exciting territory, difficulties plague investigations into the topic. Specimens are difficult to collect, species lack many morphological features typically used in taxonomy, and many lineages have recent divergence times (Verovnik et al. 2003, Trontelj et al. 2009, Niemiller et al. 2012, Niemiller et al 2013a, Cruz-Lopez et al. 2016). In addition to these problems, the dwindling number of taxonomists causes a backlog of diversity being discovered via molecular methods (Pearson et al. 2011, Wagele et al. 2011). When taxonomy cannot keep up with the diversity being discovered, another way must be found to protect the variation within an organismal group.

Current taxonomy may not encompass all of the diversity of many groups, thus Evolutionarily Significant Units (ESUs) are often utilized in an attempt to recognize underlying

diversity (Ryder 1986, Moritz 1994). ESUs as well as Management Units (MUs) allow the conservation community to move forward with management plans even while the taxonomy of a group remains uncertain.

One group that epitomizes troubles plaguing subterranean biodiversity studies is the Southern Cavefish (*Typhlichthys subterraneus* Girard 1859) complex. *Typhlichthys subterraneus* is a cave-obligate (stygobiotic) member of the amblyopsid fishes. Amblyopsidae is an endemic North American fish family, with four of the six recognized genera stygobiotic and one stygophilic. As a stygobiont, *T. subterraneus* is troglomorphic (morphological traits associated with cave adaptation); it is virtually eyeless and pigmentless with enhanced mechanoreception (Eigenmann 1909; Poulson 1963). Throughout its taxonomic history, *Typhlichthys* has encompassed varied numbers of species due to debate over a variety of characters (Eigenmann 1905, Eigenmann 1909, Woods and Inger 1957, Niemiller et al. 2013a). Currently, two species are recognized within *Typhlichthys*: 1) *Typhlichthys eigenmanni* residing west of the Mississippi River in Missouri and Arkansas and 2) the *Typhlichthys subterraneus* complex to the east, with a range from Kentucky, Tennessee, Alabama, and Georgia (Figure 1). Although *T. eigenmanni* has not been formally redescribed, the species is supported by robust molecular phylogenetics and is being used by speleobiologists (Niemiller et al. 2013a).

Multiple molecular analyses support the existence of genetically distinct lineages within *T. subterraneus* (Swofford et al. 1982, Dillman et al. 2011, Niemiller et al. 2012, Niemiller et al. 2013a). One of the molecular investigations into *Typhlichthys subterraneus* uncovered at least ten distinct genetic lineages within the eastern United States (Niemiller et al. 2012). We have attempted to categorize the lineages through morphometrics, but were unable to resolve these lineages into diagnosable units (Chapter 1). Genetic lineages are ESUs, however, and

NatureServe rankings and IUCN Red List classifications were assigned to lineages within Niemiller et al. (2013a). Additionally, several hypotheses were made for some historical populations for which genetic samples were not available.

Our objective was to determine the conservation rank for important populations not originally included in Niemiller et al. (2013a). Of particular importance are populations in the Big Wills Valley of the Coosa River Drainage. With the exception of Big Wills Valley, all localities in Alabama of *Typhlichthys* are in the Tennessee River Drainage, making the identification of Coosa fishes of special importance. To do this, we expanded the molecular dataset from Niemiller (2013a) by sampling populations from which genetic samples were not obtained previously with a particular emphasis on extralimital caves for several of the previously proposed lineages.

Materials and Methods

Collection Methods

Collection of samples began in 2012 and is ongoing. Fish were captured using hand nets and were then anesthetized in buffered MS 222 (Tricaine Methanesulfonate) at 500 mg/l. Fin clips were taken for genetic analyses and placed either in 95% or RNALater. Newly sampled populations came from eight different caves in three different states (Figure 1 and Table 1).

Molecular Methods

Genomic DNA was extracted from fin clips using the EZNA DNA Extraction Kit (Omega Biotek). We amplified the protein-coding mitochondrial NADH dehydrogenase subunit 2 (ND2), the first intron of the ribosomal nuclear encoded S7 gene (S7), and Rhodopsin, a visual photoreceptor gene (Rhod), three genes chosen from six previously used by Niemiller et al. (2012) because they held the greatest amount of phylogenetic information. Two sets of primers

were utilized for ND2, one set from Kocher et al. (1995) and the other designed by the primer design software Primer3 in Geneious v.6.0.6 (called TyCon; Biomatters Ltd.). Primers for S7 (S7Con) and Rhod (RhodCon) were designed using Primer3 (Table 2).

All three genes were amplified using Polymerase Chain Reaction (PCR) with the following amounts and concentrations of reagents: 8.5 μL of purified water, 12.5 μL Master Mix (Promega Corporation), 2.0 μL DNA template, 1.0 μL each of 10 μM forward and reverse primers. Amplification for ND2 with TyCon primers was optimized from Kocher et al. (1995; Table 3). S7 amplification followed Chow and Hazama (1998) and Rhod amplification was performed according to Sevilla et al. (2007; Table 3). PCR products were cleaned using ExoSAP-IT (Affymetrix) and bidirectionally sequenced at Genewiz, Inc. (Cambridge, Massachusetts, USA). All Unique sequences generated will be accessioned into GenBank.

We aligned forward and reverse sequences into contigs and edited these with manual verification using Geneious v. 6.0.6 (Biomatters Ltd.). Sequences were aligned and the three genes were concatenated. We utilized raxmlGUI v.1.31 (Silvestro and Michalak 2012) to generate a maximum likelihood (ML) phylogeny. Codon partitioning according to Niemiller et al. (2012) was utilized for ND2, but a GTR+Γ evolutionary model was applied for the concatenated dataset. A maximum likelihood + thorough bootstrap analysis was conducted with 10 runs of 1000 replicates utilizing the amblyopsid cavefishes *Speoplatyrhinus poulsoni* and *Amblyopsis spelaea* as outgroup taxa.

Results

We recovered lineages in accordance with those in Niemiller et al. (2013a) and adopt the same alphabetical lineage scheme (Figure 2). All new populations were recovered within lineages A, B, C, and L. Lineages D, E, F, and M included no new populations. The only major

difference was that Niemiller et al. (2013a) found that specimens from Key Cave were part of Lineage F, but we found them to be sister to Lineage G (Figure 2).

Most notably, Sells Cave (Wills Valley, Coosa River drainage) and Crane Creek (a recently discovered, extralimital population found in the Appalachian Ridge and Valley ecoprovince) were found to be members of lineage A. The Crane Creek population is the subject of a recently submitted paper (Niemiller et al. submitted), who note the biogeographic implications for this new population, which is the first population firmly entrenched in the Appalachian Karst (all other populations are in the Interior Low Plateau karst). Sell's Cave samples were not monophyletic (Figure 2). The Crane Cave sample is in a polytomy with other specimens from Georgia and one of the Sells Cave samples.

The new samples from Hering Cave are nonmonophyletic within lineage B with some as sister to another northern Alabama population, Muddy Cave, and some in a clade with Big Mouth Cave in Tennessee (Figure 2). Both clades including Hering Cave samples are nested within clades of Tennessee caves. Muddy Cave is now included in lineage B by both molecular and geographic data. Both Muddy Cave and Hering Cave occur in the same aquifer (Other Rocks Aquifer, Figure 4) as Blowing Spring Cave, Big Mouth Cave, and Crystal Cave, with which they form a clade (TN+AL clade; Figure 2). Within lineage B, the TN+AL clade is sister to an all Tennessee population (TNonly) clade. The two samples of Jaco Spring Cave are nonmonophyletic within lineage B; one sample is recovered with the TN+AL clade while the other is part of the TNonly clade. Jaco Spring Cave is located in an aquifer between the TN+AL clade and the TNonly clade. This relationship indicates possible gene flow within and between aquifers and/or that there is incomplete lineage sorting.

Four of the newly sampled caves are recovered within lineage C (Figure 2). Crow Creek Cave is nonmonophyeltic within the lineage. Each of the three Crow Creek Cave samples is recovered sister to different caves, namely Limrock Blowing Cave, Geiger Cave, and Tally Ditch Cave. Geiger Cave and Limrock Blowing caves are each paraphyletic with a Crow Creek Cave sample and Tally Ditch Cave is sister to a Crow Creek Cave specimen.

Kentucky lineages (L and M) were referred to as *Typhlichthys subterraneus*, as these lineages encompass the type locality of the species (Niemiller et al. 2013a; Figure 2). Hidden River Cave samples are nonmonophyletic within *T. subterraneus*. Samples for Hidden River Cave are recovered within a clade of Sanders Cave specimens and within a clade that included a Sanders Cave specimen, Mammoth Cave specimens and L&N Railroad Cave specimens.

Discussion

Molecular Phylogenetics and Biogeography

Aquatic cave diversity investigations are increasingly important as aquifer exploitation and groundwater pollution continue at unsustainable levels (Foster and Chilton 2003, Bichuette and Trajano 2010, Niemiller et al. 2013a). Difficulties ranging from cave access, to specimen collection, to description of cryptic species, prove to be immense setbacks in cave biodiversity studies. By the continuation of or addition of data to previous projects, we create a comprehensive knowledge of groundwater biodiversity.

We have added newly sampled populations to previous molecular investigations of the *Typhlichthys subterraneus* complex. Understanding the relationships at population levels may yield insight into aquifer connectivity, as the complex extends across many hydrological basins. The maximum likelihood phylogeny suggested the newly sampled populations are within the previously denoted lineages, and none represent new lineages (Figure 3). One cave (Muddy

Cave, Madison Co., AL) has been re-designated from the lineage assigned in Niemiller et al. (2013a) and another cave (Key Cave, Lauderdale Co., AL) is suggested as a new lineage. Though most of the new samples were attained from historical localities, one population (Crane Cave, Catoosa Co., Georgia) was very recently discovered and extends the range of the *T. subterraneus* complex into the heart of the Appalachian Ridge and Valley ecoregion (Figure 1).

Lineage A is the lineage furthest to the southeast. As was hypothesized in Niemiller et al. (submitted), we found Sells Cave to be part of Lineage A. Sells Cave is one of only two known localities within the Coosa River drainage where *Typhlichthys* is known. One specimen examined from Sells Cave was sister to Pryor Spring Cave in Tennessee and one was in a clade with all of the Georgia specimens.

Lineage B includes specimens from caves in both Tennessee and Alabama. Utilizing both molecular data and aquifer association, we include Muddy Cave in lineage B. The entire lineage B clade actually includes two subclades. In the first subclade, the Alabama caves Muddy and Hering are recovered with Blowing Spring, Big Mouth, and Crystal caves from Tennessee. All of these caves are associated with the same aquifer (Figure 4). The second clade includes Gallagher Cave and Flat Rock Cave, both within one aquifer. Included in both of the lineage B subclades are samples from Jaco Spring Cave. Jaco Spring Cave is located in an aquifer between that of the two lineage B subclades. The recovery of Jaco Spring Cave samples in both subclades suggests either incomplete lineage sorting of loci or gene flow within and between aquifers.

It is pertinent to note that the Key Cave population in Lauderdale County, Alabama, is recovered as sister to lineage G, found solely in the northeastern-most range of *Typhlichthys* in Tennessee (Figure 4). Key Cave is also the only known locality of the Alabama cavefish, *Speoplatyrhinus poulsoni* (Kuhajda and Mayden 2001). The recovery of this relationship

indicates that the Key Cave population of *Typhlichthys* may have diverged earlier than many of the other lineages and should be recognized as a unique lineage.

Interestingly, lineage F is found only in caves in the floor of the Tennessee River Valley (Figure 4). The furthest cave downstream (Cave Spring Cave) is sister to upstream populations, and the two most upstream populations (Davis Bat Cave and Baugus Cave) form a monophyletic group. These relationships may indicate that extinct epigean ancestors could have traveled via the Tennessee River and colonized caves alongside the river, or that cavefish may be able to access the Tennessee River. Davis Bat Cave, for example, is located along Second Creek a short ways up from the creek's mouth with the Tennessee River; Wheeler Dam currently floods Second Creek. The mouth of Davis Bat Cave is currently located just above the waterline and the cave passage is very likely flooded by river water. Divers mapped a nearby cave (Watkins Lake Cave) and found the cave to be connected to the Tennessee River (landowner pers. comm.). We observed *Typhlichthys* in Watkins Lake Cave, but were unable to capture any.

The prevalence of multiple caves per genetic lineage may either indicate recent divergence of the lineages from a common ancestor and/or movement of fish between caves. Divergence of the entire *T. subterraneus* group began and occurred primarily in the Pleistocene around 3.5 MYA, which is relatively recent in geologic time (Niemiller et al 2013b). With such a recent divergence, there may not have been enough time for gene families to completely diverge from polyphyletic ancestral gene copies to a monophyletic allele, thus ancestral polymorphisms are still recovered within clades (i.e., incomplete lineage sorting). In addition to recent divergence, genetic variation is partitioned among hydrological subbasins (Niemiller et al. 2012). This particular genetic structuring indicates vicariance rather than dispersal across hydrological barriers.

We feel, however, that there may be more movement between caves than previously expected. Although *Amblyopsis* is known to brood eggs in its gill chambers, it is unlikely that other cavefishes do so (Adams and Johnson 2001; Armbruster et al. in press). Gravid females of *Troglichthys* (Adams and Johnson 2001) and *Forbesichthys* (Hill 1969; JWA pers. obs.) have been examined, but thus far, no gravid female *Typhlichthys* have been collected. The average standard length of museum specimens is 30.3 mm SL, putting the majority of specimens within the life history stage before reproductive maturity (Juvenile, First Annulus life history stage- 20.5 to 30.5 SL; Poulson 1963). The overall age and size of the collected fishes suggests that almost all *Typhlichthys* we see are not mature. Additionally, five or fewer fish have been observed in 65.4% of caves with *Typhlichthys* occurrence (Niemiller et al. 2013a). This low abundance of fish coupled with a lack of gravid females indicates that there may not be a viable population of fishes within the cave itself; in turn, this suggests that the primary environment of the fishes may not be a cave with surface access. In addition, in the Coosa Drainage, only two populations are known. One, Browder Cave, has only had fish discovered after it was filled from the aquifer (Cooper, unpublished data), and the other, Sells Cave, may be an opening directly into the aquifer. These observations suggest that *Typhlichthys* may be found deeper in aquifers (similar to the stygobiont catfishes *Satan* and *Trogloglanis* in the Edwards Aquifer of Texas; Lundberg 1982). If they are located in aquifers, the wide range of *Typhlichthys* as a whole and of some of its clades could be explained by the large extent of the aquifers.

Conduits within the karst aquifers and fluctuating water tables may facilitate the dispersal of cavefishes within the subterranean habitat (Hawes 1939). Dissolution of karstic rocks is a rapid process, needing only a few thousands of years to modify or create flow paths (White 2002). These relatively rapid flow changes may transport cavefishes from one area of an aquifer

to another. Groundwater basin divides are not firmly fixed and, depending on the rates of recharge and discharge (i.e., flooding), it can be common for ground water divides to shift (Hawes 1939, White 2002). With widespread flooding events, perhaps aquifer divides can be overcome and organisms from different groundwater systems may be exchanged.

Conservation and Future Directions

Conservation ranks were denoted for the newly sampled populations. Since all but one of the populations was hypothesized and thus included in the calculations in Niemiller et al. (2013a), it was not necessary to recalculate conservation ranks with the exception of Lineage C and the Key Cave lineage. We found around 40 fish in Geiger Cave during two visits and more than 10 in Crow Creek Cave during one visit. These are considered large populations, which suggests that lineage C may be more robust than originally thought. We recommend that the lineage be downgraded to G3 (NatureServe) and vulnerable (IUCN). Each newly sequenced population was recovered within an existing lineage and given the conservation rank of that lineage (Table 6). Both NatureServe and IUCN Red List conservation ranks (NatureServe 2015, IUCN Standards and Petitions Subcommittee 2014) were denoted for each lineage as both assessment systems have different criteria and can give different estimates (Gaston and Fuller 2009, Niemiller et al. 2013a).

The Key Cave lineage was found to be genetically distinct from any of the previously identified lineages, and we believe that it deserves separate conservation status as Critically Endangered under the IUCN and G1 (Critically Imperiled) under NatureServe because it is known from a single cave (Table 4 and 5). The closest population to Key Cave is McKinney Pit (located directly across the Tennessee River), but we found these populations are not closely

related. Given that Key Cave is already protected because of the presence of *Speoplatyrhinus*, no further action is necessary.

Though North-Central Alabama caves were pooled together into lineage E in Niemiller et al. (2013a), we recovered Hering Cave nested within lineage B, the Central-Tennessee lineage (Figure 3). Due to its position within the phylogeny and its aquifer association, we apply the conservation rank for lineage B to Hering Cave.

The lineages of *Typhlichthys subterraneus* have extremely truncated ranges when compared to that of *Typhlichthys subterraneus sensu lato*, increasing their extinction risk and increasing their endemism (Niemiller et al. 2013a). Four lineages are Endangered according to the IUCN Red List Rank and two are Critically Endangered (Table 6). The rest of the lineages are considered Vulnerable. Though they are not yet designated taxonomically, each lineage is an ESU and the diversity therein is unique and worth protecting.

If *Typhlichthys* are more abundant deep within aquifers, populations may be robust enough to be sustainable. However, this widespread nature may actually be disadvantageous, as a single contamination event of the aquifer could have widespread consequences. Regardless, it is near impossible to truly establish population sizes for *Typhlichthys*, thus, the lineages need to be protected.

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Figures and Tables

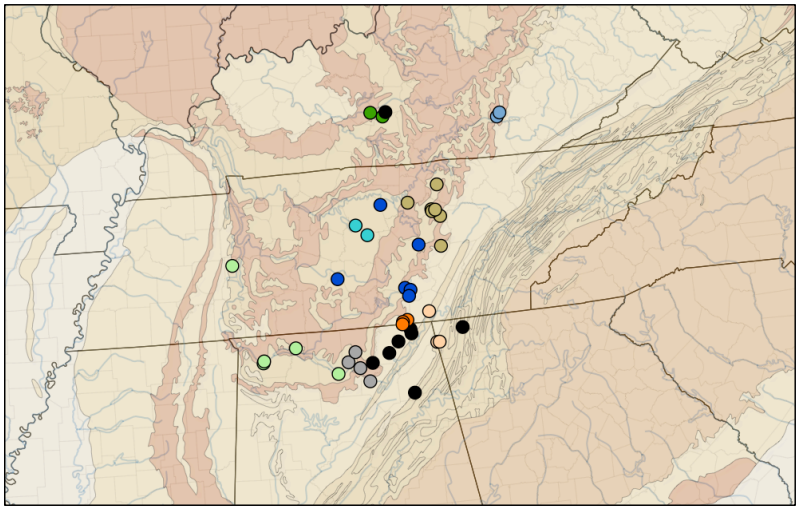

Figure 1. Map of Typhlichthys subterraneus populations genetically sampled for this study. Points are colored by genetic lineage. Newly sampled populations are indicated by black points. Aquifers are shown in shades of brown in the background.

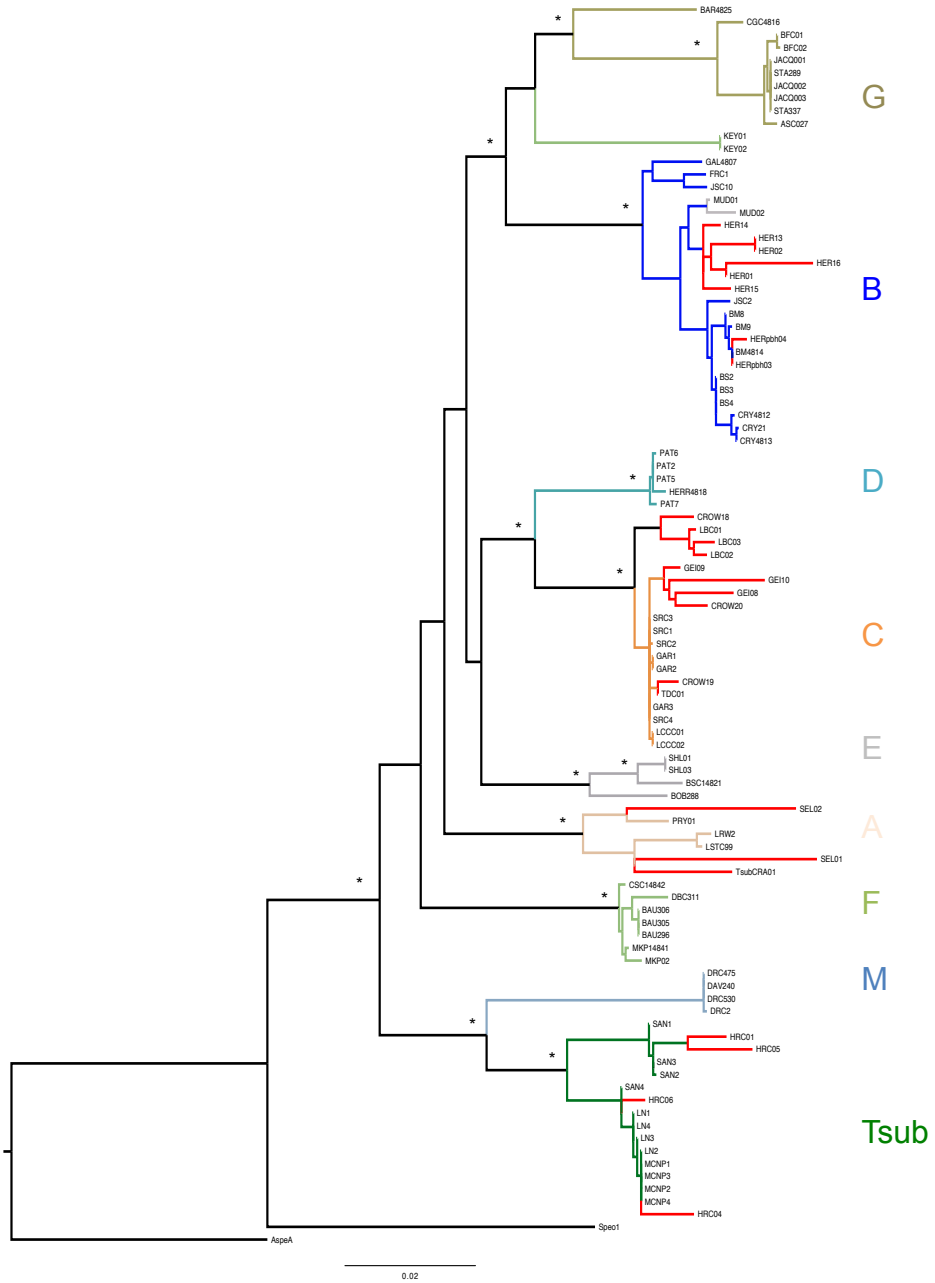

Figure 2. Maximum likelihood phylogeny of concatenated ND2, S7, and Rhod sequences for the Typhlichthys subterraneus complex. Red branches indicate new samples. Scale bar unit: expected substitutions per site. Asterisks represent >80 bootstrap support.

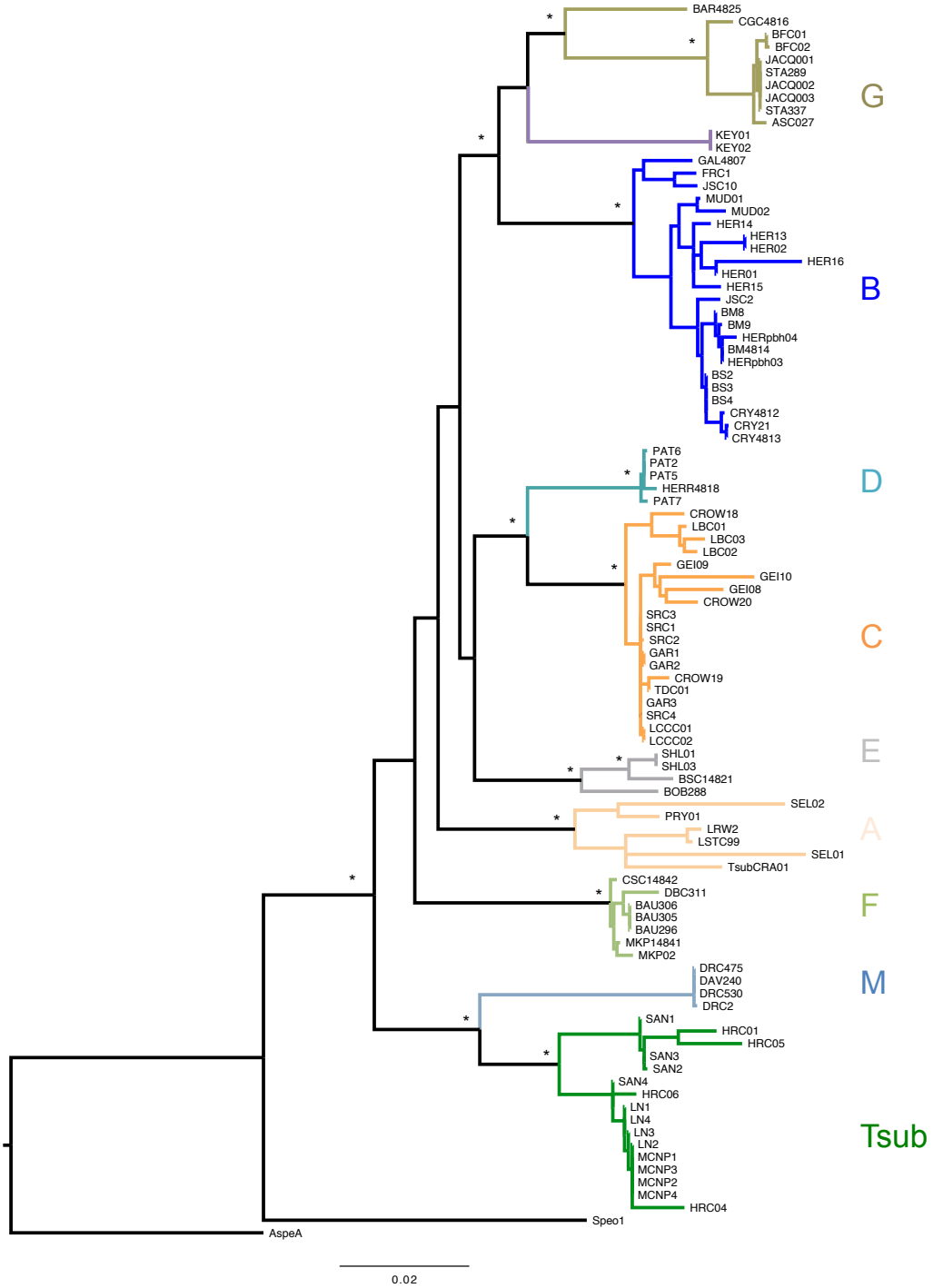

Figure 3. Maximum likelihood phylogeny of concatenated ND2, S7, and Rhod sequences for the Typhlichthys subterraneus complex after applying lineage designations to newly sampled populations. Scale bar unit: expected substitutions per site. Asterisks represent >80 bootstrap support.

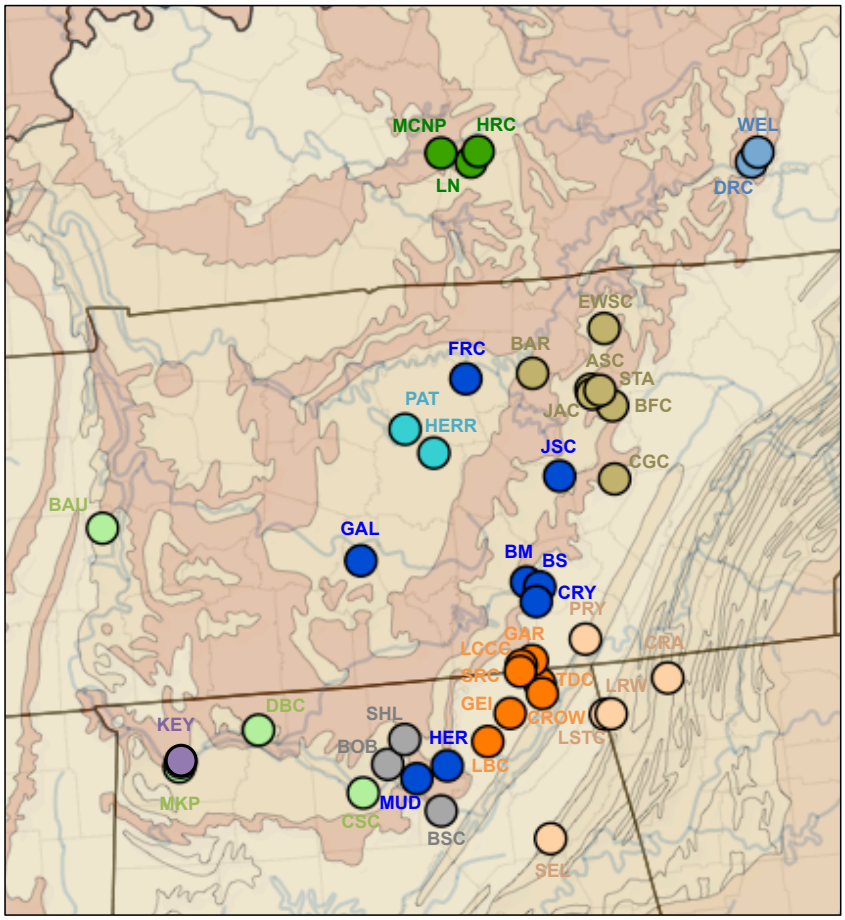

Figure 4. Enlarged map of caves with new Typhlichthys subterraneus lineage designations.

Table 1. List of cave populations that were sampled for this investigation.

Cave Name	ID	County	State
Sells Cave	SEL	Dekalb	Alabama
Crow Creek Cave	CROW	Jackson	Alabama
Geiger Cave	GEI	Jackson	Alabama
Limrock Blowing Cave	LBC	Jackson	Alabama
Tally Ditch Cave	TDC	Jackson	Alabama
Hering Cave	HER	Madison	Alabama
Crane Cave	CRA	Catoosa	Georgia
Hidden River Cave	HRC	Hart	Kentucky

Table 2. Primer sequences utilized for molecular work in this study.

Gene	Forward (5'-3')		Reverse (5'-3')	
ND2	TyCon1F	TGAACCCTTTCATCCTAATAGCC	TyCon1R	GGTTGTGAGGAGGGTCAGG
ND2	GLN1F	CTACCTGAAGAGATCAAAAC	ASN6R	CGCGTTTAGCTGTTAACTAA
S7	S7Con1F	TCTGCAGGATGGAAGATTTTGT	S7Con1R	GCTTGTACTGAACATGGCCC
Rhod	RhodCon1F	GCCTGGCTGCCTACATGT	RhodCon1R	ACAGCGTTGTGATCATGCAG

Table 3. Thermocycler protocol for all primer sets and each of the three genes sequenced.

ND2: TyCon

°C	Time	Process
94	0:30	Initial Denaturation
94	0:30	Denaturation
51.2	0:30	Annealing
72	1:15	Elongation
72	10:00	Final Elongation
10	Forever	Hold
#2-4 x 30 cycles		

ND2: GLN1F and ASN6R (Kocher et al. 1995)

°C	Time	Process
94	0:30	Initial Denaturation
94	0:30	Denaturation
41	0:30	Annealing
72	1:15	Elongation
72	10:00	Final Elongation
10	Forever	Hold
#2-4 x 30 cycles		

Rhod

°C	Time	Process
95	7:00	Initial Denaturation
94	0:30	Denaturation
62	0:30	Annealing
72	0:30	Elongation
72	7:00	Final Elongation
10	Forever	Hold
#2-4 x 40 cycles		

S7

°C	Time	Process
95	1:00	Initial Denaturation
95	0:30	Denaturation
60	1:00	Annealing
72	2:00	Elongation
72	10:00	Final Elongation
10	Forever	Hold
#2-4 x 30 cycles		

Table 4. Reanalyzed NatureServe conservation ranks. For comparison purposes, we include *Typhlichthys subterraneus sensu lato*.

	Tsub s.l.	C	Key
States	6	2 (AL, TN)	1 (AL)
Counties	57	2	1
HUC8 watersheds	32	1	1
Level IV ecoregions	19	2	1
NatureServe criteria			
Rarity factors			
EOO (km2)	106,668	382	3.4
AOO (km2)	900	44	3.4
Occurrences	242	11	1
Protected occurrences (%)	81 (33.5)	3 (27.2%)	1
Population size	4,952-41,265	121-1,100	Unknown
Threat factors			
Threat impact	M	ML	VHH
Calculated rank	G4	G3	G1

Table 5. Reanalyzed IUCN Red List conservation ranks. For comparison purposes, we include *Typhlichthys subterraneus sensu lato*.

IUCN Red List criteria	*Tsub s.l.*	C	Key
A. Population reduction	DD	DD	DD
B. Geographic range			
B1. EOO		EN	CR
B2. AOO	VU	EN	EN
(a) Severly fragmented	N	Y	Y
(b) No. of Locations		Y	VU
(c) Continuing decline	Y(iii)	Y(iii)	Y (iii)
(c) Extreme fluctuations	N	N	N
C. Small population size and decline	DD	DD	DD
D. Very small or restricted population			
No. of mature individuals		DD	
E. Quantitative analysis	DD	DD	DD
Red List category	NT	VU	CR
Applicable criteria		B1ab(iii)	B1ab(iii)

Table 6. Conservation ranks for all caves utilized in this study.

Cave	Cave ID	County	State	Lineage	NatureServe Rank	IUCN Red List Rank
Sell's Cave	SEL	DeKalb	AL	A	G3	VU
Crane Cave	CRA	Catoosa	GA	A	G3	VU
Long's Rock Wall	LRW	Dade	GA	A	G3	VU
Limestone Caverns	LSTC	Dade	GA	A	G3	VU
Pryor Cave Spring	PRY	Marion	TN	A	G3	VU
Key Cave	KEY	Lauderdale	AL	KEY	G1	CR
Hering Cave	HER	Madison	AL	B	G3G4	VU
Muddy Cave	MUD	Madison	AL	B	G3G4	VU
Blowing Spring Cave	BS	Coffee	TN	B	G3G4	VU
Big Mouth Cave	BM	Grundy	TN	B	G3G4	VU
Crystal Cave	CRY	Grundy	TN	B	G3G4	VU
Gallagher Cave	GAL	Marshall	TN	B	G3G4	VU
Flat Rock Cave	FRC	Smith	TN	B	G3G4	VU
Jaco Spring Cave	JSC	Warren	TN	B	G3G4	VU
Crow Creek Cave	CROW	Jackson	AL	C	G3	VU
Geiger Cave	GEI	Jackson	AL	C	G3	VU
Tally Ditch Cave	TDC	Jackson	AL	C	G3	VU
Limrock Blowing Cave	LBC	Jackson	AL	C	G3	VU
Garner Spring Cave	GAR	Franklin	TN	C	G3	VU
Little Crow Creek Cave	LCCC	Franklin	TN	C	G3	VU
Salt River Cave	SRC	Franklin	TN	C	G3	VU
Herring Cave	HERR	Rutherford	TN	D	G3	EN
Patton's Cave	PAT	Rutherford	TN	D	G3	EN
Bobcat Cave	BOB	Madison	AL	E	G3	EN
Shelta Cave	SHL	Madison	AL	E	G3	EN
Beech Spring Cave	BSC	Marshall	AL	E	G3	EN
McKinney Pit	MKP	Colbert	AL	F	G3	VU
Davis Bat Cave	DBC	Lauderdale	AL	F	G3	VU
Cave Spring Cave	CSC	Morgan	AL	F	G3	VU
Baugus Cave	BAU	Decatur	TN	F	G3	VU
Anderson Spring Cave	ASC	Putnam	TN	G	G3G4	EN
Bartlett Cave	BAR	Putnam	TN	G	G3G4	EN
Blind Fish Cave	BFC	Putnam	TN	G	G3G4	EN
Jacque's Cave	JAC	Putnam	TN	G	G3G4	EN
Stamp's Cave	STA	Putnam	TN	G	G3G4	EN
Camps Gulf Cave No.2	CGC	Van Buren	TN	G	G3G4	EN
Dave's Cave	DAV	Pulaski	KY	M	G1G2	CR
Drowned Rat Cave	DRC	Pulaski	KY	M	G1G2	CR
L&N Railroad Cave	LN	Barren	KY	Tsub	G3	VU
Mammoth Cave National Park	MCNP	Edmonson	KY	Tsub	G3	VU
Sander's Cave	SAN	Edmonson	KY	Tsub	G3	VU
Hidden River Cave	HRC	Hart	KY	Tsub	G3	VU