

# First definitive record of a stygobiotic fish (Percopsiformes, Amblyopsidae, *Typhlichthys*) from the Appalachians karst region in the eastern United States

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## Abstract

In the central and eastern United States, cavefishes have been known historically only from the Interior Low Plateau and Ozarks karst regions. Previously, cavefishes were unknown from the Appalachians karst region, which extends from southeastern New York southwestward into eastern Tennessee, northwestern Georgia, and northeastern Alabama. Here we report the discovery of a new population of the amblyopsid cavefish *Typhlichthys subterraneus* Girard, 1859 from a cave in Catoosa County, Georgia, that significantly extends the known distribution of the species. The cave is located in the Appalachian Valley and Ridge physiographic province and Appalachians karst region, and represents the first definitive report of a stygobiotic fish from the Appalachians karst region. Genetic analyses of one mitochondrial and one nuclear locus from the cavefish indicate this population is closely allied with populations that occur along the western margins of Lookout and Fox mountains in Dade County, Georgia, and populations to the northwest in southern Marion County, Tennessee. It is likely that these populations are also related to those from Wills Valley, DeKalb County, Alabama. The distribution of this new population of *T. subterraneus* and its close allies pre-dates the emergence of a Tennessee-Coosa River drainage divide in the Pliocene. The potential exists to discover additional populations in caves within the Appalachians karst region in Catoosa County and northward into Hamilton County, Tennessee.

**Keywords**

Appalachian Valley and Ridge, Catoosa County, cavefish, Cumberland Plateau, Georgia, range extension

**Introduction**

Of the more than 50,000 caves reported in the United States, about 30% occur in the states of Tennessee, Alabama, and Georgia (TAG). The two most biodiverse karst regions in the United States – the Interior Low Plateau (ILP) and Appalachians – occur in this region (Culver et al. 2000, Culver and Pipan 2009). The ILP is comprised of horizontal strata of Ordovician through Mississippian age that extend from southern Illinois and Indiana, southward through Tennessee and Kentucky and into northern Alabama. The escarpments of the Cumberland Plateau in Kentucky, Tennessee, Alabama, and Georgia are included in the ILP karst region (Culver et al. 2000). The ILP and Appalachians karst regions are proximal to each other near the junction of TAG state borders, although the boundary between the ILP region and the Appalachians karst region, and Appalachian Valley and Ridge (AVR) physiographic province, is somewhat arbitrary. Caves in the Appalachians karst are predominantly developed within Paleozoic rocks of an ancient fold-and-thrust belt associated with compression during Alleghenian orogenesis of the Appalachian Mountains (Hatcher et al. 2007, Hatcher 2010). The AVR physiographic province is comprised of parallel ridges of sandstones with intervening structural valleys of folded and faulted shales and carbonates that extend from southeastern New York to eastern Tennessee, northwestern Georgia, and northeastern Alabama between the Blue Ridge Mountains to the east and the Appalachian Plateau (specifically, the Cumberland Plateau) to the west.

The ILP and Appalachian karst regions contain the most caves and have the greatest richness of troglobiotic taxa in the United States (Culver et al. 2003, Hobbs 2012). In particular, a hotspot of subterranean biodiversity and endemism has been identified near the contact of the ILP and Appalachians karst regions along the escarpments of the Cumberland Plateau in northeastern Alabama and south-central Tennessee (Culver et al. 1999, 2000, 2006, Christman et al. 2005, Niemiller and Zigler 2013). Species richness in the Appalachians karst region (and AVR) is less than half that observed in the ILP in the TAG region, and AVR subterranean fauna are distinct from ILP fauna. Only 9% of the 200+ troglobionts in Tennessee occur in both karst regions (Niemiller and Zigler 2013).

Several factors may explain differences in species richness between these the ILP and Appalachians karst regions, such as differences in habitat availability, habitat connectivity, historical factors, and surface productivity (Christman and Culver 2001, Culver et al. 2006, Niemiller and Zigler 2013). Cave density has been viewed as a surrogate for habitat availability and connectivity because it positively correlates with regional species richness (Christman and Culver 2001, Culver et al. 2003, 2006). Cave density is considerably lower in the southern Appalachians karst region compared to the ILP in the TAG region. Moreover, the folded and faulted cave-bearing strata in the Appalachians karst region are dissected and discontinuous compared to horizontal



**Figure 1.** *Typhlichthys subterraneus* collected 25 November 2015 from Crane Cave (GCZ80), Catoosa County, Georgia. Photograph by B.R. Kuhajda.

strata of the ILP. A major zone of faulting along the eastern escarpment of the Cumberland Plateau in the Appalachians karst region has been hypothesized to act as a stratigraphic barrier to subterranean dispersal between the two karst regions (Barr and Holsinger 1985, Miller and Niemiller 2008, Niemiller et al. 2008, 2009), which may explain why so few species occur in both karst regions.

*Typhlichthys subterraneus* s.l. Girard, 1859 is one of the most wide-ranging cavefishes in the world (Proudlove 2006, Niemiller and Poulson 2010). In the TAG region, this cavefish is known from >180 caves in the ILP, with the greatest concentration of occurrences in central Tennessee and northern Alabama (Niemiller et al. 2013b,c). In Georgia, *T. subterraneus* is known only from four caves developed in Mississippian Bangor Limestone along the western margins of Lookout Mountain and Fox Mountain in Dade County, Georgia (Cooper and Iles 1971, Freeman and Niemiller 2009, Niemiller et al. 2012a, 2013b,c). Here we report the discovery of a population of the Southern Cavefish (*Typhlichthys subterraneus*) from Crane Cave in Catoosa County, northwestern Georgia (Fig. 1), located in the center of the AVR physiographic province and the Appalachians karst region. Not only does this record represent a significant range extension for this species, but it also represents the first definitive report of a stygobiotic fish from the Appalachians karst region.

## Materials and methods

### Study site

Crane Cave (Georgia Speleological Survey cave no. GCZ80) is located ca. 7 km SSE of Fort Oglethorpe, Georgia, in the South Chickamauga Creek watershed. Crane Cave formed in the Ordovician Newala Limestone, and has 292 m of mapped length with 11 m of vertical extent and three entrances. A small stream runs through the cave and emerges at the spring entrance. The stream begins in a large pool at the back of the

cave called “The Found Sea.” The pool is ca. 10 m in length and ca. 6 m in width, and has a mud/silt substrate bottom. The full extent of the pool is unknown, as it extends underneath a ledge at the back of the cave. At base level, water depth is ca. 2 m deep in the deepest portion of the pool.

### **Cavefish survey**

Crane Cave was visited on four occasions: 10 August 2015, 18 August 2015, 29 October 2015, and 25 November 2015. The Found Sea and other aquatic habitats were sampled using time-constrained visual surveys with headlamps and handheld dive lights. Richness and abundance data for aquatic fauna were recorded, and a concerted effort was made to capture fish with handheld dipnets. A voucher specimen and tissue sample (fin clip) was obtained for morphological and genetic analyses.

### **Molecular methods and analyses**

Genomic DNA was extracted from fin clips using the EZNA DNA Extraction Kit (Omega Biotek). Two gene loci were chosen from six previously used by Niemiller et al. (2012b) to determine the genetic identity and relationships of the Crane Cave population to other *Typhlichthys* populations. The protein-coding mitochondrial NADH dehydrogenase 2 (ND2) gene was amplified by PCR with primers TyCon1F (5'-TGAACCCTTTCATCCTAATAGCC-3') and TyCon1R (5'-GGTTGTGAG-GAGGGTCAGG-3'). Each PCR reaction contained 8.5  $\mu$ L of purified water, 12.5  $\mu$ L Master Mix (Promega Corporation), 2.0  $\mu$ L DNA template, 1.0  $\mu$ L each of 10  $\mu$ M forward and reverse primers. Amplification began with an initial denaturation of 94 °C for 30 seconds, followed by 30 cycles of 94 °C denaturing for 30 seconds, annealing at 51.2 °C for 30 seconds, elongation at 72 °C for 75 seconds, then a final elongation step of 10 minutes. The gene sequence was 957 base pairs (bp) long. A 774-bp section of the first intron of the ribosomal nuclear encoded S7 gene was amplified with the primers S7Con1F (5'-TCTGCAGGATGGAAGATTTTGT-3') and S7Con1R (5'-GCTTGTACTIONGAACATGGCCC-3'). The PCR reactions contained the same amount and concentration of reagents as the ND2 reaction. The initial denaturation for amplification began at 95 °C for 60 seconds, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 for 60 seconds, elongation at 72 °C for 2 minutes, followed by final elongation at 72 °C for 10 minutes. PCR products were cleaned using ExoSAP-IT (Affymetrix) and bidirectionally sequenced at Genewiz, Inc. (Cambridge, Massachusetts, USA). Unique sequences generated for the Crane Cave sample were accessioned into GenBank (ND2: KX173801 and S7: KX246929).

Forward and reverse sequences were aligned into contigs and edited with manual verification using Geneious v. 6.0.6 (Biomatters Ltd.). Maximum likelihood gene

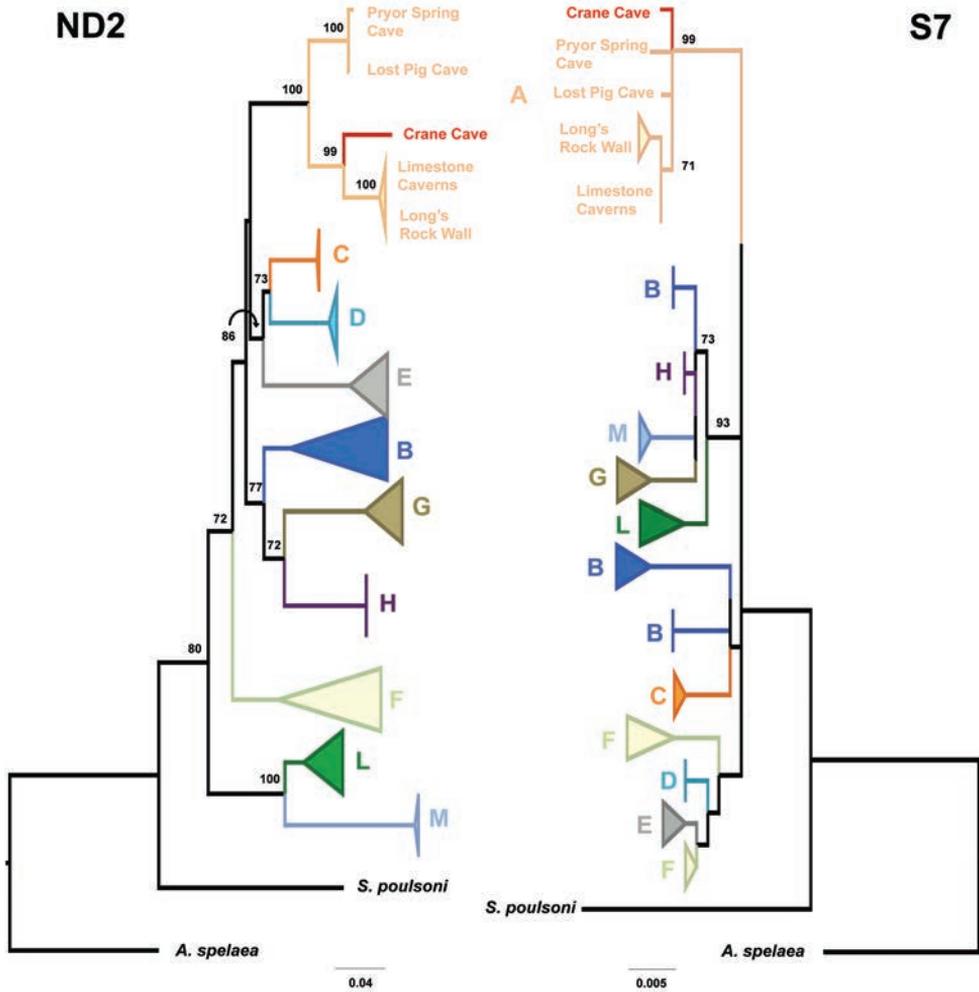
trees were generated for both ND2 and S7 loci with raxmlGUI v.1.31 (Silvestro and Michalak 2012). Codon partitioning according to Niemiller et al. (2012b) was utilized for ND2. For both loci, a maximum likelihood + thorough bootstrap analysis was conducted with 10 replicates of 100 runs utilizing the cavefishes *Speoplatyrhinus poulsoni* Cooper & Kuehne, 1974 and *Amblyopsis spelaea* DeKay, 1842 as outgroup taxa.

## Results

A single cavefish was observed in The Found Sea of Crane Cave but evaded capture during an initial bioinventory on 10 August 2015. No cavefish were observed during two subsequent trips on 18 August 2015 and 29 October 2015. Two cavefishes were observed on 25 November 2015. One specimen was collected and retained as a voucher specimen (Fig. 1). The specimen was identified as *Typhlichthys subterraneus* by the lack of external eyes (vs. presence in *Chologaster* and *Forbesichthys*), presence of one row of exposed neuromasts on each half of the caudal fin (*Amblyopsis*, *Speoplatyrhinus*, and *Troglichthys* have four to six rows, two to three on each half of the caudal fin), the presence of branched rays in the pectoral fins (vs. unbranched in *Speoplatyrhinus*), the lack of pelvic fins (vs. presence in *Amblyopsis*), and nine dorsal-fin rays (vs. 7–8 in *Troglichthys*). In addition, only *Typhlichthys*, among stygobiotic amblyopisids, is known to have an extensive pigment response when exposed to light (Eigenmann 1909; Poulson 1963). The Crane Cave specimen has extensive melanophore development particularly along the edges of myomeres, on the head, and at the bases of the median fins (*Amblyopsis*, *Speoplatyrhinus*, and *Troglichthys* have far fewer melanophores with less melanin, and color is not generally noticeable in preserved specimens). The specimen was cataloged into the Auburn University Museum of Natural History (AUM 67212) and a tissue sample (fin clip) was accessed into the Auburn University Fish Tissue Collection (AUFT 2651).

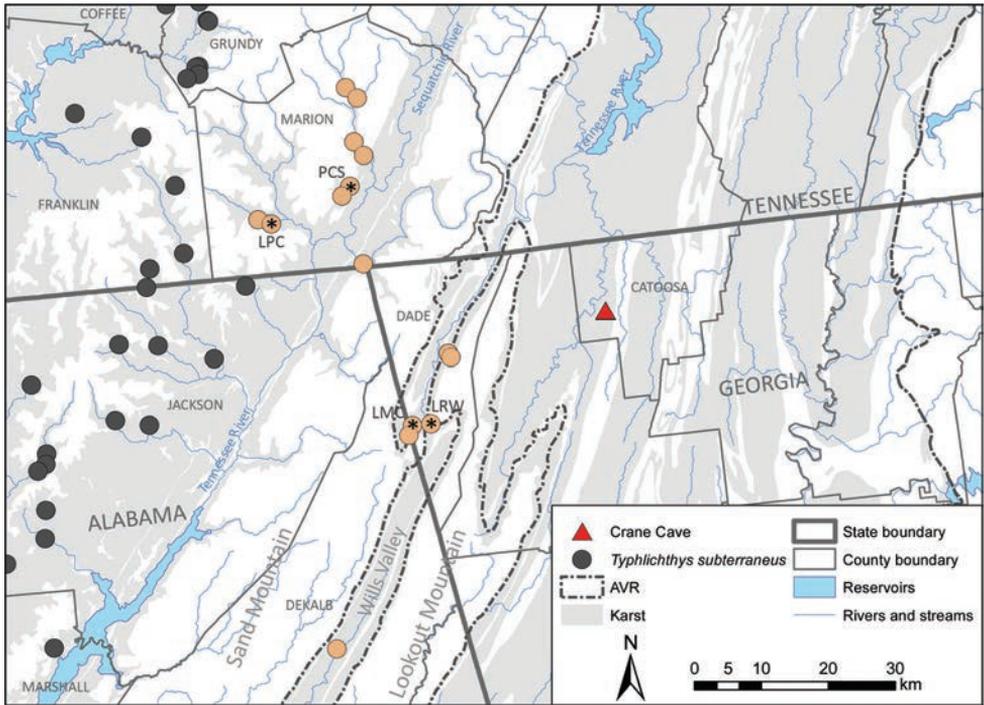
Other notable fauna observed during the four biological surveys at Crane Cave included aquatic species *Crangonyx antennatus* Cope & Packard, 1881 (Amphipoda: Crangonyctidae), *Caecidotea richardsonae* Hay, 1901 (Isopoda: Asellidae), and *Cottus* sp. (Scorpaeniformes: Cottidae), and terrestrial species *Hesperochnes mirabilis* (Banks, 1895) (Pseudoscorpiones: Chernetidae), *Bishopella* sp. (Opiliones: Phalangodidae), *Amoebalaria* sp. (Diptera: Heleomyzidae), and *Eidmanella pallida* (Emerton, 1875) (Araneae: Nesticidae).

Molecular results indicated that the Crane Cave specimen was most closely related to the *T. subterraneus* populations designated lineage A in both the ND2 and S7 phylogenies (Niemiller et al. 2012b). In the ND2 phylogeny (Fig. 2), the Crane Cave specimen was sister to a clade containing populations from Long's Rock Wall (GDD101) and Limestone Caverns (GDD140) from Dade County, Georgia, in the Lookout Creek watershed, and the closest populations in geographical proximity to



**Figure 2.** Maximum likelihood gene trees for mitochondrial ND2 (left) and nuclear S7 (right) loci. Colors correspond to genetic lineages for *Typlichthys subterraenus* designated in Niemiller et al. (2012b). Bootstrap values are to the left (ND2) or right (S7) of the corresponding node with >0.70 support. Outgroup taxa include *Speoplatyrhinus poulsoni* and *Amblyopsis hoosieri*. Scale bar unit: expected substitutions per site.

Crane Cave (Fig. 3). The clade comprised of Crane Cave, Long’s Rock Wall, and Limestone Caverns was sister to a population from Pryor Cave Spring (Tennessee Cave Survey no. TMN129) and Lost Pig Cave (TMN20) located in the Little Sequatchie River Valley and Sweetens Cove of southern Marion County, Tennessee, respectively. In contrast, the base of lineage A in the S7 phylogeny was a strongly supported polytomy that consisted of Crane Cave, Pryor Cave Spring, and a Long’s Rock Wall + Limestone Caverns clade (Fig. 2). The ND2 and the S7 phylogenies both presented strong support for the monophyly of lineage A.



**Figure 3.** Distribution of *Typhlichthys subterraneus* (solid circles) in southeastern Tennessee, northeastern Alabama, and northwestern Georgia. The new record at Crane Cave is denoted with a red triangle and lineage A localities are highlighted in peach. Lineage A populations that have been genetically examined are marked with an asterisk and labeled as follows: LMC – Limestone Caverns, LPC – Lost Pig Cave, LRW – Long’s Rock Wall, and PCS – Pryor Cave Spring. Counties with *Typhlichthys* records are labeled. Karst and cave-bearing strata are shaded gray based on the U.S. karst map (Weary and Doctor 2014). The border of the Appalachian Valley and Ridge (AVR) physiographic province is denoted by the dot and dashed line.

## Discussion

The range of *Typhlichthys subterraneus s.l.* extends throughout the ILP of Kentucky, Tennessee, Alabama, and Georgia, which makes it one of the largest distributions of any cavefish in the world (Proudlove 2006, Niemiller and Poulson 2010). Because of the widespread distribution, even from distinct hydrological basins, several authors hypothesize that *T. subterraenus* represents a complex of morphologically cryptic, but genetically distinct, species (Swofford 1982; Barr and Holsinger 1985; Holsinger 2000; Niemiller and Fitzpatrick 2008; Niemiller and Poulson 2010). Niemiller et al. (2012b) identify at least ten cryptic lineages from a species delimitation analysis based on six loci and samples from 60 populations across the range. The most recent common ancestor of these lineages dates to the Late Pliocene to Early Pleistocene, about 2.8 million years ago (Mya) (95% confidence interval: 2.1–3.5 Mya; Niemiller et al. 2013a). Populations from Dade County, Georgia (Limestone Caverns and Long’s Rock Wall), and at least two populations from the Little Sequatchie River Valley in Tennessee, form

a distinct genetic *Typhlichthys* lineage, referred to as lineage A. Populations that occur in Wills Valley in DeKalb County, Alabama, also are thought to belong to lineage A (Niemiller et al. 2013b), but have not been genetically examined to date. This lineage diverged from others in the ILP about 2.2 Mya (1.6–2.9 Mya based on 95% confidence intervals; Niemiller et al. 2012b, 2013a).

Analyses of the mitochondrial ND2 and the nuclear S7 loci from Crane Cave *T. subterraneus* strongly support affinity to lineage A (as defined by Niemiller et al. 2012b). However, the new Crane Cave record is ca. 24.2 km straight-line distance to the east from the next closest populations in Georgia and Alabama. Specifically, the *T. subterraneus* populations in Dade County are from caves formed in the Mississippian-age Bangor Limestone on the escarpments of Lookout Mountain and Fox Mountain, clearly within the Cumberland Plateau physiographic province. Despite the arbitrary boundary between the ILP and AVR, the distribution of lineage A now extends from the ILP into the Appalachians karst region because Crane Cave is well within the AVR and is from a hydrologically distinct watershed compared to the previously described *T. subterraneus* populations in the TAG region (Fig. 3).

There is the issue of whether the other *T. subterraneus* populations in lineage A, specifically those in Wills Valley formed in Cambrian-Ordovician Knox group dolomites in AVR-style structural valleys, are also considered AVR distributions or ILP distributions. The physiographic distinction of Wills Valley has been a matter of debate in the literature. Wills Valley is an anticlinal valley flanked by Sand Mountain to the west and Lookout Mountain to the east. Both ridges are considered parts of the Cumberland Plateau (Johnson 1930, Harkins et al. 1982, Raymond et al. 1988). As such, previous studies comparing subterranean biodiversity among karst regions have considered Wills Valley to be associated with the Cumberland Plateau and ILP karst region rather than the AVR within the Appalachians karst region (Peck 1989, 1995, Culver et al. 2003, Hobbs 2012). However, others have placed Wills Valley as part of the Ridge and Valley Level III ecoregion (Griffith et al. 2001) based on ecosystem similarity according to land use, geology, physiography, hydrology, climate, natural vegetation, and soils (Omernik 1987). Regardless, the distribution of *T. subterraneus* in Wills Valley caves and the evolution of the karst in the valley warrant further study. The transitional location of Wills Valley between the ILP and Appalachians karst region and its length (100+ km) may have been critical in the movement of *T. subterraneus* between the two larger karst regions.

Another important aspect of *T. subterraneus* in Wills Valley is that these populations are in the Coosa River watershed, which flows into the Alabama River and then Mobile Bay. Crane Cave occurs in the South Chickamauga Creek watershed, which flows into the Tennessee River. Moreover, all four documented populations in Dade County, Georgia, occur in the Lookout Creek watershed, and the caves in Marion County, Tennessee, are part of the Sequatchie River watershed. Both Lookout Creek and the Sequatchie River empty into the Tennessee River, which eventually flows into the Ohio River and then the Mississippi River. River drainages in the southern region of North America and the Appalachian Mountains became established at least by the Eocene, 55 Mya (Galloway et al. 2011, Hoagstrom et al. 2013). At this time, the ancestral Tennessee River and the Coosa River formed the Appalachian River that

flowed to Mobile Bay (Johnson 1905, Milici 1968). By the mid-Miocene through the Pliocene, uplift in the Southern Appalachians (Gallen et al. 2013) or of the Nashville Dome (Clark 1989), as well as potential regional base-level lowering, initiated downcutting by the ancestral Tennessee River through Walden Ridge and westward flow into the Sequatchie Valley, then around the Nashville Dome before being captured by the Ohio River (Milici 1968, Clark 1989, Self 2000). Some suggest that stream capture may have been facilitated by karst as “cavern capture” (*s.s.* Johnson 1905) in Walden Ridge and the Sequatchie Valley.

Today, the Tennessee and Coosa rivers are separated by a divide, whereby the southern part of Wills Valley flows to the Coosa River and the northern section flows to the Tennessee River. The genetic affiliation of the Crane Cave *T. subterraneus* population to lineage A (Niemiller et al. 2012b) suggests that this lineage has a shared evolutionary history, whereby a common ancestor must pre-date the emergence of the Tennessee-Coosa drainage divide and subsequent isolation of the Tennessee River from the Coosa River. The drainage divide likely formed in the late Pliocene based on evidence from changes in deltaic sedimentation (Galloway et al. 2011) and age dates from cave sediment records (Anthony and Granger 2007). This timeframe corresponds with the estimated divergence of lineage A from 2.9 to 1.6 Mya from other lineages in the ILP (Niemiller et al. 2012b, 2013a). Continued uplift and stream incision further isolated lineage A populations throughout the TAG region in the early Pleistocene, which resulted in genetically distinct populations in Crane Cave, Dade County, and Alabama/Tennessee.

In conclusion, although no additional cavefish populations have been discovered in the past several years (Freeman and Niemiller 2009), with the exception of Crane Cave, and despite several cave bioinventories and other studies in northwestern Georgia (Reeves et al. 2000, Buhlmann 2001, Miller and Niemiller 2008), the potential exists for additional *T. subterraneus* populations to be discovered. Caves to be targeted for exploration would be those within the South Chickamauga Creek watershed and formed in the Newala Limestone throughout Catoosa County, as well as extending southward into Walker County and northward into Hamilton County, Tennessee. Lastly, these *T. subterraneus* populations may provide insight into the geologic history of the Tennessee and Coosa rivers, as well as aid in the understanding of other endemic cave fauna in the TAG region. In particular, the boundary between the ILP and Appalachians karst regions (and Interior Plateau and AVR physiographic provinces) may not be as strong a barrier to dispersal for stygobiotic taxa as previously thought.

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