Status, Life History, and Phylogenetics of Amblyopsid Cavefishes in Kentucky

Final Report

Prepared By

Matthew L. Niemiller, Ph.D.[1,2] and Benjamin M. Fitzpatrick, Ph.D.[1,3]

[1]Department of Ecology and Evolutionary Biology
University of Tennessee
Knoxville, TN 37996, USA

[2] cavemander17@gmail.com
[3] benfitz@utk.edu

Submitted to the Kentucky Department of Fish and Wildlife Resources
16 April 2013

Summary

The amblyopsid cavefishes are a small family of specialized fishes endemic to eastern North America. Although this family has been known to science since the 1840s, we still no little about the demography, life history, conservation, and genetics of most of the species in the family. Three species of amblyopsids occur in Kentucky: the Spring Cavefish (*Forbesichthys agassizii*), which inhabits caves, springs, and streams with the karst region of south-central Kentucky, the obligate cave-dwelling Southern Cavefish (*Typhlichthys subterraneus*) distributed is scattered localities with the Interior Plateau from the Mammoth Cave region to the Tennessee border and a disjunct population in Pulaksi County along the Cumberland Plateau, and the obligate cave-dwelling Northern Cavefish (*Amblyopsis spelaea*) found primarily in cave streams associated with the Crawford-Mammoth Cave Uplands and Mitchell Plain from the Mammoth Cave region northward to the Ohio River. Currently, the Spring Cavefish is listed as "Apparently Secure" (S4S5) in Kentucky; whereas the Southern Cavefish is listed as "Imperiled" to "Vulnerable" (S2S3) and Northern Cavefish as "Vulnerable" (S3) by NatureServe. All three species are included on the list of Species of Greatest Conservation Need in the KDFWR Wildlife Action Plan. The primary objectives of this study where to determine the distribution, population size, ecology, genetics, and threats to populations of each amblyopid species in Kentucky to provide a clearer understanding of the biology and conservation status of each species in the state. In addition, we examined the intra- and interspecific genetic relationships of each species.

We surveyed for Northern Cavefish, Southern Cavefish, and Spring Cavefish from May 2007 through September 2012 in caves, springs, and spring-fed streams throughout the Interior Plateau and the Cumberland Plateau of central Kentucky. In addition to acquiring data on ecology and life history, we collected tissue samples for phylogenetic analyses. Spring Cavefish have been reported from 53 localities in 17 counties in Kentucky; its distribution includes five ecoregions and nine watersheds in the state. Its distribution extends through much of the southern Interior Plateau in the central part of the state, including the Western Highland Rim, Eastern Highland Rim, Crawford-Mammoth Cave Uplands and Western Pennyroyal Karst from the Mammoth Cave region in Edmonson and Hart counties south to the Tennessee border and west to Trigg, Lyon, and Livingston counties along the Cumberland River. Southern Cavefish have been reported from 29 localities in eight counties and its distribution includes four ecoregions and five watersheds. Its distribution extends through much of the southern Interior Plateau in the central part of the state, including the Crawford-Mammoth Cave Uplands and Western Pennyroyal Karst from the Mammoth Cave region in Edmonson and Hart counties south to the Tennessee border and west to Trigg County. Newly discovered populations in Pulaski County represent a disjunct cluster of populations in Plateau Escarpment ecoregion of the Southwestern Appalachians. Northern Cavefish have been reported from 40 localities in five Kentucky counties. Its distribution extends throughout the Crawford-Mammoth Cave Uplands and Mitchell Plain from the Ohio River southward in an arc the the Mammoth Cave region and occurs in three ecoregions and

three watersheds. The population center for the species occurs in the Sinking Creek watershed of Breckinridge County.

Phylogentic analyses of amblyopsid cavefishes in Kentucky revealed several interesting results. We identified two distinct phylogenetic lineages in *Forbesichthys* that are on separate evolutionary trajectories that could be recognized as distinct species under several species concepts. Based on this evidence, we advocate resurrection of *F. papilliferus* (Northern Spring Cavefish) for populations in southern Illinois and south-central Kentucky. Tennessee populations on the Eastern Highland Rim represent *F. agassizii*. Because of the extensive distribution of Southern Cavefish and the results of molecular studies of other troglobites, several authors have speculated that this species represents several independent invasions and, therefore, distinct lineages. Our phylogenetic analyses revealed significant genetic divergence and both mitochondrial and nuclear DNA variation was structured among hydrological drainages. Uncorrected mtDNA sequence divergence ranged 3.6–12.2% among these lineages. Species delimitation analyses strongly support multiple cryptic lineages within *Typhlichthys* across its distribution, including two distinct lineages in Kentucky: one lineage consisting of populations from the Upper Green, Barren, and Red River watersheds, and the other that includes populations in the Upper Cumberland watershed in Pulaski County. We are currently examining acquiring and examining morphological data from these populations and intend to describe the Upper Cumberland lineage (*T. sp. nov. 1*; the Kentucky Cavefish) in the near future. Phylogenetic analyses on populations of the *Amblyopsis spelaea* revaled that the Ohio River is a significant isolating barrier separating populations north and south of the river. These lineages exhibited 3.1% mtDNA sequenc divergence and species delimitation analyses support the recognition of two distinct lineages with one located north of the Ohio River in Indiana and the other south of the river in Kentucky. The affinity of populations in the Mammoth Cave region still need to be assessed.

Amblyopsid cavefish populations in Kentucky face a number of threats, including habitat degradation and loss, hydrological manipulations, environmental pollution, overexploitation, and impacts of introduced aquatic animals. Despite significant scientific collections in the past, the Rich Pond population of Northern Spring Cavefish continues to thrive and illustrates how large populations can be for this species. Other significant populations likely exist in springs located on private properties. We do not recommend any change in conservation status at this time but recommend that efforts be made to survey springs located on private properties to identify additional populations, protect the most significant population in the state at Rich Pond, and study the connectivity of populations in the Western Pennyroyal Karst. The discovery of cryptic, distinct lineages and putative species within *T. subterraneus* has obvious conservation implications. We recommend the status of this undescribed species be listed as ‘Endangered’ (S1) because of the small number of occurrences (six), few individuals observed, and potential threats including hydrological changes associated with Lake Cumberland. Future research efforts should focus on locating additional populations in the Upper Cumberland River watershed and delineating recharge zones of cave systems containing significant populations. We recommend that the conservation status of Southern Cavefish (all other populations of *Typhlichthys* in Kentucky) remain the same. However, we recommend that cave cleanups be conducted at two localities affected by sinkhole dumping and that a public outreach program be initiated to warn of the problems associated with sinkhole dumping in Kentucky. Northern Cavefish currently are listed as Vulnerable. The species is known from at least 40 localities but several are associated with the Mammoth Cave system in Edmonson County and the Sinking Creek area in Breckinridge County and our genetic evidence suggests that individual caves in Sinking Creek likely do not represent distinct populations. This area supports several large and significant populations representing the main population center for the species but all cave systems occur on private property and are not afforded any protection. While we recommend that the conservation status of Northern Cavefish remain at Vulnerable, we strongly recommend that efforts be made to protect the cave systems containing significant numbers of individuals. Additionally, additional surveys are needed at caves in Hardin and Hart counties to determine in the two main clusters of populations in Breckinridge and Edmonson counties are continuous or separated from one another.

Introduction

The Appalachians and Interior Low Plateau support the highest aquatic subterranean biodiversity within the continental United States (Culver et al. 2003). Much of this diversity is the product of independent invasion and isolation of past surface-dwelling populations. Although endemism has resulted in high biodiversity, more than 95% of subterranean fauna in North America are considered vulnerable or imperiled (Culver et al. 2000) due, in large part, to habitat degradation (Elliott 2000; Danielopol et al. 2003; Boulton 2005) and restricted geographic ranges (Culver et al. 2000; Culver et al. 2003). Unfortunately, the distribution and status of local populations for many species is incomplete or lacking entirely, making conservation and management decisions by federal, state, and local agencies difficult. Here we investigate the distribution, phylogenetics, ecology, conservation status, and threats to populations of three cave-associated fish species in the family Amblyopsidae in Kentucky: the Northern Cavefish (*Amblyopsis spelaea*), Spring Cavefish (*Forbesichthys agassizii*) and Southern Cavefish (*Typhlichthys subterraneus*). Despite large distributions throughout the karst regions of central Kentucky, little is known regarding the life history and phlogenetics of these species, particularly of the two obligate taxa, *A. spelaea* and *T. subterraneus*.

The Amblyopsidae is a small family of specialized fishes endemic to the unglaciated regions of the eastern United States. The seven species (in six genera) in the family represent the transition from epigean to subterranean habitats and are an excellent system to investigate the evolution of troglomorphic characters and speciation in subterranean environments. Five species are obligate subterranean inhabitants that exhibit troglomorphic features (*Typhlichthys subterraneus*, *T. eigenmanni*, *Troglichthys rosae*, *A. spelaea*, and *Speoplatyrhinus poulsoni*), whereas *Forbesichthys agassizii* (facultative cavernicole) and *Chologaster cornuta* (epigean) do not. Although fishes from this family have been known to science since the early 1840s, little is known about the demography and persistence of local populations of the subterranean species, systematic relationships among species, or systematic relationships among populations within species.

Three species of amblyopsids occur in Kentucky: 1) the Spring Cavefish (*F. agassizii*, Fig. 1), which inhabits caves, springs, and spring-fed streams and swamps within the Interior Plateau of the Red, Lower Cumberland, Lower Tennessee, Lower Ohio-Bay, Tradewater, Pond, Barren, Middle Green, and Upper Green watersheds; 2) the cave-dwelling Southern Cavefish (*T. subterraneus*, Fig. 2) which has a discontinuous distribution in Kentucky with the majority of records occurring in the Interior Plateau of the Lower Cumberland, Red, Barren, and Upper Green watershed and another population located in the Upper Cumberland watershed of Pulaski County to the east (Fig. 3); and 3) the cave-dwelling Northern Cavefish (*A. spelaea*, Fig. 4), which inhabits caves within the Interior Plateau of the Upper Green, Rough, and Blue-Sinking watersheds. The overall distributions of each species is illustrated in Fig. 5.

The Spring Cavefish is listed as "Apparently Secure" (S4S5) in Kentucky by NatureServe (2013) and are locally abundant in ideal habitats (Niemiller & Poulson 2010). The Spring Cavefish is not listed in Kentucky at this time, but the species is included on Kentucky's list of "Species of Greatest Conservation Need" (Kentucky's Comprehensive Wildlife Conservation Strategy 2013). Clay (1975) listed five localities/areas where Spring Cavefish have been collected. Burr and Warren (1986) reported 29 localities for the Spring Cavefish, with a concentration of localities associated with the Western Highland Rim and western Crawford-Mammoth Cave Uplands of the Lower Cumberland watershed and another in the Mammoth Cave region associated with the Crawford-Mammoth Cave Uplands and Mitchell Plain of the Barren and Upper Green watersheds. The KDFWR has occurrence records for 24 localities distributed in 14 counties (KDFWR, unpublished data).

The Southern Cavefish is listed as "Imperiled" to "Vulnerable" (S2S3) in Kentucky by Natureserve (2013) and as "Vulnerable" by IUCN (World Conservation Monitoring Center 1996). Clay (1975) reported the species from only nine localities in four counties (Barren, Edmonson, Hart, and Warren). Only eleven localities for Southern Cavefish were reported by Burr and Warren (1986) with all but two occurring in the Mammoth Cave region associated with the Crawford-Mammoth Cave Uplands and Western Pennyroyal Karst of the Barren and Upper Green watersheds. Pearson and Jones (1998) reported on populations in the Mammoth Cave region. Additional localities were reported from the Upper Cumberland watershed in Pulaski County and the Lower Cumberland watershed in Trigg County.

Furthermore, because of its large distribution, several researchers have suggested that the Southern Cavefish may actually represent a species complex of morphologically cryptic, but related species. Preliminary genetic evidence supports this hypothesis. Although the species is not considered to be in any immediate danger (Etnier & Starnes 1993), local populations may be threatened by urbanization, groundwater pollution, and silviculture practices in the vicinity of recharge areas (Etnier and Starnes 1993; Aley & Aley 1997). All of the preceding perturbations of habitat are known or suspected to adversely affect populations of other amblyopsid species. Thus, there is a need to document the distribution of the species statewide and to assess demographic parameters of, and threats to, local populations, particularly if genetic and morphometric analyses reveal hidden diversity within the species.

The Northern Cavefish is listed as "Vulnerable" (S3) in Kentucky by both Natureserve (2013) and IUCN (Gimenez Dixon 1996). At the state level, this species is listed as "Special Concern." Clay (1975) reported Northern Cavefish from at least 17 localities. Burr and Warren (1986) reported ten localities for Northern Cavefish, which form two clusters: one cluster associated with the Crawford-Mammoth Cave Uplands and Mitchell Plain of the Blue-Sinking and Rough watersheds in Breckinridge, Hardin, and Meade counties and the other from the Mammoth Cave region in Edmonson and Hart counties. Keith (1988) listed 17 localities in Kentucky. Pearson and Boston (1995) listed 33 localities (32 caves and one spring) in Kentucky, 17 of which the authors observed cavefish during their surveys in 1993–1994. In total, Northern Cavefish have been reported from at least 38 localities in five counties in Kentucky: Breckinridge, Edmonson, Hardin, Hart, and Meade counties. Pearson and Boston (1998) also reported on populations in the Mammoth Cave region. The KDFWR has mappable occurrence records for 33 localities and unmappable records for another eight reports (KDFWR 2011).

Here we propose to investigate the status, distribution, population size, ecology, genetics, and threats to populations of the three amblyopsid species in Kentucky to provide a clearer understanding of the biology and conservation status of each species in the state. In addition, we will determine the genetic affinities of Kentucky populations. Pursuant with Kentucky's priority research and survey needs, the objectives of this study were to (1) conduct baseline surveys and status assessments of each amblyopsid species to determine their distribution and conservation status in the state, (2) obtain cavefish biology information, such as habitat requirements, ecology, and demography for each species, (3) identify potential threats to existing and significant populations of each species and develop recommendations for status evaluations and monitoring, and (4) conduct genetic analyses to resolve the intra- and interspecific relationships of each species and also assess the potential for cryptic diversity.

Methods

Sampling and surveys

We searched for Northern Cavefish, Southern Cavefish, and Spring Cavefish from May 2007 through September 2012 in caves, springs, and spring-fed streams throughout the Interior Plateau of central Kentucky, including many historic localities (Tables 1–3; Appendix 1). We conducted surveys throughout much of the year, but concentrated searches during periods of favorable conditions in subterranean streams (i.e., shallow, clear water with little flow) or during spring when water levels were higher and Spring Cavefish can be found in surface habitats. Surveys for cave species (i.e., Northern Cavefish and Southern Cavefish) were temporarily discontinued

To locate cavefish, we donned wetsuits and slowly walked along, waded through, or crawled in the cave stream channel and thoroughly scanned the streambed with the beams of our headlamps. We also carefully lifted flat rocks, small cobble, and detritus under which smaller individuals might seek refuge. Lifted rocks were returned to their original positions to minimize habitat disturbance. A similar approach was taken in surface springs, streams, and ponds while surveying for Spring Cavefish. We used large dipnets to search through aquatic vegetation and detritus where Spring Cavefish might seek refuge during the day. We also searched beneath rocks, logs, and other potential cover objects. A tally of each individual found was kept, and a concerted effort was made to capture, with small bait nets, each cavefish encountered. Captured fish were placed in clear plastic bags until standard length (SL) was measured to the nearest mm using a small metric rule or digital calipers. Other data were gathered from each captured fish if possible, including sex, condition (e.g., injuries, growths, or presence of parasites), habitat (aquatic: stream pool, stream riffle, rimstone pool; terrestrial: mud bank, bank-cut, crevice), substrate (mud, sand, cobble, gravel, bedrock, organic debris, artificial), cover type (rock, log, crevice, organic debris), and other aspects of life history (diet, behavior, community associates). Additionally, we excised a small tissue sample from the right pectoral fin or caudal fin of one or more cavefish captured at each locality (up to 15 at a given locality) for subsequent genetic analyses.

Phylogenetic analyses

Specimen sampling. We collected tissue samples (fin clips) from 70 individuals of nine populations of Spring Cavefish in Illinois, Kentucky, and Tennessee. We also collected specimens and tissue samples from 72 individuals of 16 populations of Northern Cavefish in Indiana and Kentucky and from 140 individuals of 62 populations throughout the distribution of Southern Cavefish in Alabama, Arkansas, Georgia, Kentucky, Missouri, and Tennessee. We included samples for all other amblyopsids as outgroups, including Swampfish (*Chologaster cornuta*), Alabama Cavefish (*Speoplatyrhinus poulsoni*), and Ozark Cavefish (*Amblyopsis rosae*). Because the fossil constraints used to estimate divergence times fall outside the amblyopsid clade, we included other related taxa that represent major lineages within the percopsiform fishes, including *Aphredoderus gibbosus*, *Aphredoderus sayanus*, *Percopsis omiscomaycus*, and *P. transmontana*. These outgroups include both surface and subterranean species.

DNA extraction, PCR and sequencing. Genomic DNA was extracted using the Qiagen DNEasy Kit (Qiagen Inc., Valencia, California). Polymerase chain reaction (PCR) was used to amplify one mitochondrial gene and up to six nuclear genes for each sample (Appendix 2). PCR primers and conditions followed protocols used in previous studies (Kocher et al. 1995; Holcroft 2004; Li et al. 2007; Niemiller et al. 2012, 2013a). Cleaned PCR products were sequenced at the Molecular Biology Resource Facility, Division of Biology, University of Tennessee, Knoxville, Tennessee, or at the High-Throughput Genomics Unit, University of Washington. The data set also was supplemented with available sequences on GenBank accessioned in related studies (Niemiller et al. 2012). Forward and reverse sequences for each template were aligned and edited using SEQUENCHER v4.5 (Gene Codes, Ann Arbor, Michigan) with ambiguous base calls verified manually by examining the electropherogram for each sequence. Resulting contigs were aligned using SEQUENCHER and MACCLADE v4.07 (Maddison & Maddison 2005). Some individuals contained heterozygous genotypes for the sampled nuclear loci. These positions were coded using standard degeneracy codes. Unique DNA sequences generated for this study were accessioned into GenBank.

Estimation of gene trees and haplotype networks. Gene trees for each locus were estimated using partitioned Bayesian analyses, with posterior probabilities estimated using Metropolis-coupled Markov chain Monte Carlo implemented in MRBAYES 3.1 (Ronquist & Huelsenbeck 2003). All loci are protein-coding and were partitioned by codon with the exception of the first intron of ribosomal protein *s7*. The best-fit models of molecular evolution for each partition were selected using the Akaike's Information Criterion (AIC) implemented in MODELTEST v3.7 (Posada & Crandall 1998). Each locus was partitioned accordingly and unlinked allowing values for transition/transversion ratio, proportion of invariable sites, and among-site rate heterogeneity to vary across codon partitions during analyses. Two independent runs using six Markov chains and temperature profiles at the default setting of 0.2 were conducted for 10 million generations, sampling every 1000th generation. Random trees were used to begin each Markov chain and a molecular clock was not enforced. We assessed convergence of runs by examining the standard deviation between the two independent runs until a value below 0.01 was obtained, indicating that the run had converged. Samples from the stationary distribution of trees were used to generate 50% majority-rule consensus trees for each locus. We also constructed unrooted statistical parsimony haplotype networks for all loci of *Amblyopsis spelaea* in the program TCS v1.21 (Clement et al. 2000) to visualize the number of mutations between groups of populations.

Tests for barriers to dispersal. Major contemporary river systems in North America have been proposed as significant barriers to dispersal and gene flow in surface (Kozak et al. 2006; Lemmon et al. 2007) and subterranean organisms (Rhoades 1962; Barr & Holsinger 1985; Niemiller & Poulson 2010; Niemiller et al. 2012). Because the distribution of *A. spelaea* occurs both north and south of the Ohio River, it may have had an influence on the genetic structure of this species. However, *A. spelaea* exhibits no differentiation across this potential barrier at several allozyme loci (Swofford 1982). To examine whether the Ohio River is a significant barrier to dispersal among populations, we conducted a partial Mantel test (Mantel 1967; Smouse et al. 1986). If the river is a barrier, then genetic distances between pairs of populations on the same side of the river should be lower than genetic distances between pairs of populations spanning the river. The partial Mantel test was performed to test for a correlation between genetic distance and position relative to the Ohio River while controlling for geographic distance. We first computed a matrix of pairwise uncorrected genetic distances from the *nd2* dataset, while a matrix of binary variables was calculated where the position of two populations relative to the Ohio River was coded as either occurring on the same side or on opposite sides of the river. Then we calculated a matrix of geographic distances as the great-circle distance between a pair of populations. All partial Mantel tests were calculated using ZT v1.1 (Bonnet & Van de Peer 2002) with 100,000 permutations.

As an additional test of the hypothesis that the Ohio River is a significant barrier to dispersal, we used hierarchical analyses of molecular variance (AMOVA, Excoffier et al. 1992) implemented in ARLEQUIN v3.5 (Excoffier & Lischer 2010). Hierarchical AMOVA partitions the total genetic variance into covariance components due to differences among *a priori* groups, among populations within groups, and within populations. We first grouped populations into regions north and south of the Ohio River to test if this river was a significant barrier to dispersal. Calculations were performed using uncorrected pairwise distances and significance of variance components was assessed by 10,000 permutations.

We also examined the partitioning of genetic variation by hydrological subbasins and ecoregions for both *T. subterraneus* and *A. spelaea*. Interconnectivity of drainage basins and ecoregions shape genetic structure in other aquatic, subterranean taxa in the Interior Highlands of eastern North America (Niemiller et al. 2008) and may also have an effect on patterns of genetic structure in both these subterranean species. We conducted AMOVAs as outlined above but grouping populations by hydrological subbasins and ecoregions for the *nd2* locus in *A. spelaea* and the *nd2*, *s7*, and *rag1* loci for *T. subterraneus*. First, we grouped populations by major hydrological basins. Additionally, we examined the effects of grouping on genetic variance by hydrological subbasins, as several subbasins may exist within a single major hydrological basin (e.g., Tennessee River basin). Lastly, we also grouped populations by ecoregion. Significance of variance components was assessed by 10,000 permutations.

Estimation of divergence times. To investigate timing of diversification, we estimated divergence times using the Bayesian, coalescence-based program BEAST v1.6.1 (Drummond & Rambaut 2007) in a species tree framework called *BEAST (Heled & Drummond 2010) that utilizes multilocus data to jointly estimate multiple gene trees embedded in a shared species tree under the multispecies coalescent. We used a dataset that was arbitrarily pruned to include two samples for each species (or clade within species) after examination of individual gene trees as well as samples for all other percopsiform species. Sequence data were partitioned by locus and by codon position for protein-coding loci. Partition-specific models of nucleotide substitution (Appendix 2) were implemented, all parameters were unlinked across loci (not across data partition), and an uncorrelated lognormal (UCLN) model of rate variation was assumed for each partition. A Yule process speciation prior was used for the branching rates.

Because no amblyopsid fossils exist, we used two fossil calibration age prior distributions from non-amblyopsid fossil taxa. †*Tricophanes foliarum* Cope (1872) is known from the Eocene and recovered as the sister taxon to *Aphredoderus* (Rosen 1962; Rosen & Patterson 1969). The age of the node containing the Aphredoderidae and Amblyopsidae was calibrated using the age of this fossil. We chose a lognormal distribution such that the minimum possible sampled age corresponded to 33.9 Ma. †*Lateopisciculus turrifumosus* (Murray & Wilson 1996) is known from the middle Paleocene and recovered as the sister taxon to Percopsidae (Murray & Wilson 1996). We calibrated the root using the age of this fossil, choosing a lognormal distribution such that the minimum possible sampled age corresponded to 58.7 Ma. Following McCormack et al. (2011), we hand-edited the XML file to incorporate fossil priors on the species tree. We conducted three independent MCMC runs for 100 million generations for each analysis, sampling every 2000 generations. All runs were examined in TRACER v1.5 to monitor convergence and likelihood stationarity and verify that an effective sample size (ESS) exceeded 200 for all parameters being estimated. A conservative burn-in of 40 million generations was excluded from each run. The tree and log files were combined using LOGCOMBINER (v. 1.6.1, distributed as part of the BEAST package). The maximum credibility tree with mean node heights was recovered in TREEANNOTATOR (v. 1.6, distributed as part of the BEAST package).

Reconstruction of demographic history. We also examined if climatic fluctuations during the Pleistocene significantly influenced patterns of genetic diversity in *A. spelaea*. If periglacial conditions were too harsh, even in subterranean habitats, then the present-day distribution of *A. spelaea* would reflect range expansion from areas further south. If this were the case, then we predict that populations north of the Ohio River would have lower genetic variation than populations south of the river and there would be a signal of recent population growth north of the Ohio River. We calculated measures of genetic diversity, including the number of unique haplotypes (*K*), the number of segregating sites (*S*), and nucleotide diversity (π), in ARLEQUIN for each group of populations north and south of the Ohio River and overall for the *nd2* locus.

To test for departures from neutrality or constant population size, we calculated the summary statistics *Fs* (Fu 1997), Tajima's *D* (Tajima 1989), and R_2 (Ramos-Onsins & Rozas 2002). Significant negative values of *Fs* and Tajima's *D* and small positive values of R_2 indicate population growth. Tests on the *nd2* locus were performed in DNASAM (Eckert et al. 2010) for each group of populations (north and south) and all populations combined. Significance was determined by 10,000 permutations.

We also reconstructed the demographic history of *A. spelaea* using GMRF skyride plots (Minin et al. 2008) implemented in BEAST. GMRF skyride plots are a nonparametric approach that incorporates the waiting time between coalescent events in a gene tree to estimate changes in effective population size over time. We constructed GMRF skyride plots for each group of populations north and south of the Ohio River using the nd2 dataset. The rate of molecular evolution for the *nd2* locus was determined from the divergence time analysis. We ran the GMRF skyride plot analysis twice for 20 million generations each, while sampling every 2,000 generations. All runs were visualized in TRACER and to verify that ESS values exceeded 200 for all parameters estimated. A conservative burn-in of 5 million generations was excluded from each run. Changes in effective population size over time were deemed significant if the upper and lower 95% confidence intervals at the root of the plot did not overlap those at the tips (Eytan & Hellberg 2010).

Detecting cryptic diversity. The documentation of cryptic diversity is an ever-increasing result of phylogeographic studies, and has become particularly prevalent in studies on subterranean taxa (Culver et al. 1995; Verovnik et al. 2003; Wiens et al. 2003; Finston et al. 2007; Zaksek et al. 2007; Trontelj et al. 2009; Niemiller et al. 2012). Several methodologies have recently been developed to delimit species and uncover cryptic diversity using molecular data (Knowles & Carstens 2007; Ence & Carstens 2010; O'Meara 2010; Yang & Rannala 2010). A consensus has yet to be reached as to which approach is most appropriate, however there is a general view that the use of multilocus datasets is warranted for accurate species delimitation (O'Meara 2010; Yang & Rannala 2010; Kubatko et al. 2011; Niemiller et al. 2012). Here we employ recently developed approaches to species delimitation that make use of multilocus data operating under the logic that consistent delimitation of sets of populations as distinct lineages across methods provides stronger support for species recognition than the results of a single approach alone. We conducted separate analyses to uncover hidden diversity for *Typhlichthys* and *Amblyopsis*.

First, we used the nonparametric heuristic method described in O'Meara (2010) to jointly delimit species and estimate the species tree using a multilocus dataset in the program BROWNIE v2.1 (O'Meara et al. 2006). This approach apportions individuals into putative species by attempting to minimize excess intraspecific structure while minimizing gene tree conflict among species. For the *Typhlichthys* analysis, we used a dataset of six genes (*nd2*, *s7*, *rag1*, *myh6*, *plagl2*, and *tbr1*) for 62 populations. For the *Amblyopsis* analyses, a subset samples of *A. spelaea* as well as *F. agassizii* and *F. papilliferus* were included. Because many individuals shared identical alleles we included seven individuals of each group in *Amblyopsis*, corresponding to the maximum number of alleles observed for a locus within a group. Heuristic searches were conducted with the number of random starting species trees (NReps) set to 100, all possible taxon reassignments on leaf splits were explored (Subsample=1), the minimum number of species (MinNumSpp) was set to 3, the maximum number of species (MaxNumSpp) was set to 6, and the minimum number of samples per species (MinSamp) was set to 2. The 50% majority-rule consensus gene trees generated from Bayesian analyses were used as input trees. We conducted 5,000 independent BROWNIE runs on the BulldogK cluster at Yale University.

As an additional measure to delimit species without *a priori* group assignments, we investigated genetic structure in *A. spelaea* using the MCMC clustering algorithm STRUCTURE 2.3.3 (Pritchard et al. 2000). Haplotypes for each locus were treated as alleles (e.g., Eytan & Hellberg 2010) and ten independent runs were conducted for each value of K=1 to K=10, with 100,000 generations of burn-in and 1 million post-burn-in replicates using the admixture model. Values of K were compared by the ΔK method (Evanno et al. 2005) to infer the best estimate of K.

Although species discovery methods are advantageous in systems that have not been well studied to develop taxonomic hypotheses within defining groups *a priori*, such as many subterranean organisms (Niemiller et al. 2012), it does not incorporate other sources of existing data available for more well-studied groups. Species validation methods (Cummings et al. 2008; Ence & Carstens 2010; Yang & Rannala 2010) quantify support for *a priori* groupings of samples that are based on other lines of evidence, such as morphological, geographical, and behavioral data. Geological and geographic evidence suggest that the Ohio River is a vicariant barrier for subterranean fauna and taxa distributed on opposite sides of the river, including *A. spelaea*, might constitute independent lineages. To test this hypothesis in *Amblyopsis*, we employed two species validation methods: Bayesian species delimitation (Yang & Rannala 2010) and genealogical tests of taxonomic distinctiveness (Cummings et al. 2008). We also conducted these species validation methods on the species delimited by O'Meara's (2010) method for *Typhlichthys*.

We conducted Bayesian species delimitation (Yang & Rannala 2010), a multilocus, coalescent-based method that includes prior information about population size and divergence times and uses reversible-jump Markov chain Monte Carlo (rjMCMC) to estimate the posterior distribution for different species delimitation models, in the program BPP version 2.0 (Rannala & Yang 2003; Yang & Rannala 2010). This method accommodates the species phylogeny as well as lineage sorting due to ancestral polymorphism. We used the species tree inferred from *BEAST analyses as the guide tree in each analysis. For the *Amblyopsis* analysis, we only included four species at the tips that included *F. agassizii*, *F. papilliferus*, and the two lineages within *A. spelaea*. The prior distributions on ancestral population size (ϑ) and root age (τ_0) were assigned gamma distributions of G(2,2000) and G(2,1000), respectively. Other divergence time parameters were assigned the Dirichlet prior (Yang & Rannala 2010: equation 2) and algorithm 0 was used with the fine-tuning parameter = 15.0. Each species delimitation model was assigned equal prior probability. Each rjMCMC analysis was run for 500,000 generations with a burn-in of 50,000 and run at least twice to confirm consistency between runs.

We also assessed the taxonomic distinctiveness of delimited species in *Typhlichthys* as well as *Amblyopsis* populations north and south of the Ohio River using the genealogical sorting index (*gsi*; Cummings et al. 2008) whereby a quantitative measure of the degree to which ancestry of delimited species is exclusive is generated for individual genes and for multilocus data combined. The relative degree of exclusive ancestry is on a scale from 0 to 1, where 1 indicates complete monophyly. Using this statistic, hypothesized species can be tested against a null hypothesis of no divergence. We calculated an ensemble *gsi* (*egsi*) and *gsi* for each locus using the Genealogical Sorting Index web server (http://www.genealogicalsorting.org). The 50% majority-rule consensus gene trees were used as input trees. The null hypothesis that the degree of exclusive ancestry is observed by chance alone (i.e., no divergence) was evaluated by estimating a *P* value using 10,000 permutations.

Results and Discussion

Morphology

All amblyopsids are characterized by possessing (i) a large, flat head and a tubular, non-streamlined body, (ii) an oblique mouth with the lower jaw protruding beyond the upper jaw, (iii) a segmented premaxilla, (iv) jugular position of the anus and urogenital pore, (v) reduced head lateral line canals on the head, and absence of the lateral line canal on the trunk, but the presence of superficial papillae (neuromasts) arranged in distinct rows on the head and body, and papillae of unknown function in 2–4 rows on the caudal fin, (vi) small, embedded cycloid scales except on the head, (vii) six branchiostegal rays, (viii) presence of a swim bladder, (ix) tubular anterior nostrils, (x) absence of pelvic fins (except rudimentary in *A. spelaea*). The troglobitic species also are characterized by (i) lack of externally visible eyes, (ii) reduced pigmentation, (iii) hypertrophy of the superficial lateral line system that includes an extensive system of elevated neuromasts arranged in distinct ridges, (iv) hypertrophy of the semicircular canals and otoliths, and (v) presence of highly-developed caudal sensory papillae. Amblyopsids are also characterized by having dorsal and anal fins that are similar in shape with the dorsal fin origin anterior to the anal fin origin. All fins lack spines, although the first ray in the dorsal fin of *Typhlichthys* has been reported as spinuous by some authors. Dorsal fin rays range 7–12, anal fins 7–11 rays, and pectoral fins 9–12 rays. Pelvic fins are absent except in *A. spelaea* that have 0–6 rays (Fig. 4). The caudal fin may be elliptical, lanceolate, or rounded with 9–22 branched rays. No fin rays are branched (except in Alabama Cavefish). The urogenital pore is positioned just anterior to the anal fin at hatching and migrates anteriad until it occupies a jugular position in adults (Woods & Inger 1957). Spring Cavefish have 9–11 dorsal fin rays, 9–11 anal fin rays, 9–11 pectoral fin rays, and 11–17 caudal fin rays (Fig 1). *Typhlichthys* have 7–10 dorsal fin rays, 7–10 anal fin rays, 9–12 pectoral fin rays, and 10–15 caudal fin rays (Figs. 2 and 3). Pelvic fins are absent in both of these species. Northern Cavefish have 9–11 dorsal fin rays, 8–11 anal fin rays, 9–11 pectoral fin rays, 11–13 caudal fin rays, and 0–6 pelvic fin rays (most have 4 rays) (Fig. 4). All three species cannot be sexed using external morphology. However, Spring Cavefish can be sexed by observing the gonads through the translucent body wall during the breeding season (Weise 1957).

Like many of the 170 species of cavefishes recongized worldwide (Soares and Niemiller 2013), troglobitic amblyopsids are known for their degenerate eyes and reduced pigmentation. However, Southern Cavefish have the least degenerate eyes of the cave amblyopsids but lack eye muscles, scleral cartilages, optic nerve connection, and pigment in the pigment epithelium, and either lack or cave undefined rods and cones. The eyes of Northern Cavefish are more degenerate with respect to parts of the eye lost or degenerate. Northern Cavefish lack eye muscles, have undefined rods and cones, have a closed pupil, and lack an optic nerve connection to the brain. Scleral cartilages are present. However, variation in eye degeneration among populations has yet to be examined for both species. The degenerate eyes of Northern Cavefish are slightly larger relative to body length than those in Southern Cavefish. Ontogenetically, the eyes of both Southern Cavefish and Northern Cavefish develop to a certain state of tissue differentiation and then become more simplified and degenerate with advancing age (Eigenmann 1897). Older cavefish also have more variable eye development and there is more right to left side variation of eyes in the same individual. Varying degrees of eye degeneration are exhibited in populations of Southern Cavefish and Northern Cavefish, including those from Kentucky. The eyes of Spring Cavefish examined by Eigenmann (1897) lack ciliary muscles. Both eye size, optic lobe length, and optic lobe volume is smaller in Spring Cavefish than in the Swampfish (Niemiller & Poulson 2010). The eyes and optic lobe of Southern Cavefish are about 80% and 30% smaller respectively than those of Spring Cavefish.

Contrary to popular belief, both Northern Cavefish and Southern Cavefish are not albinos; rather they have distinct, albeit small, melanophores. Live Southern Cavefish from across the range appear white to pearly opalescent and in high-resolution photographs outlines of the tiny embedded scales are visible as slightly grayer in color (Figs. 2 and 3). Several populations in Kentucky and Tennessee have visible blobs of fat around the vestigial eyes (Fig. 3); whereas others lack adipose tissue around the vestigial eyes (Fig. 2). Southern Cavefish examined have comparable densities of melanophores to Swampfish (*Chologaster cornuta*) but have much smaller surface areas of each pigmented melanophore, such that adjacent melanophores rarely overlap (Niemiller & Poulson 2010). In preserved specimens, the visible melanophores are concentrated along the dorsal myomere borders and scattered elsewhere on the body. Melanophores are not visible on the cheek. In the laboratory, Southern Cavefish exhibit increased pigmentation with exposure to light (Woods & Inger 1957; Niemiller & Poulson 2010). Northern Cavefish have fewer and smaller melanophores than those observed in Southern Cavefish examined. Spring Cavefish are dull brown dorsally to slightly lighter ventrally (Fig. 1) with only one kind of chromatophore–melanophores. Spring Cavefish have poorly defined stripes, one mid-dorsal and two lateral, and slightly more melanophores along the edges of the myomeres than elsewhere (Fig 1).

Distribution – Northern Spring Cavefish

Spring Cavefish are distributed from south-central Tennessee northward into south-central Kentucky then westward following the karst regions into southern Illinois and the Benton Hills west of the Mississippi River in southeastern Missouri (Fig. 5). This distribution can be divided into two main regions: 1) populations associated with the Eastern Highland Rim, particularly the Barrens region, of central Tennessee, and 2) a larger group of populations from north-central Tennessee and south-central Kentucky that extends across the Ohio River into southern Illinois and across the Missisippi River into southeastern Missouri. Populations of this second region are associated primarily with caves and springs in the Southern Shawnee Hills, Western Pennyroyal Karst, Crawford-Mammoth Cave Uplands, and Western Highland Rim of the Interior Plateau.

Within Kentucky, Spring Cavefish have been reported from at least 53 localities in 17 counties (Fig. 6; Table 1) based on literature and museum records, including at least eight records from caves. Spring Cavefish occur in four Level IV ecoregions in Kentucky (Fig. 7). This distribution extends through much of the southern Interior Plateau in the central part of the state, including the Western Highland Rim, Eastern Highland Rim, Crawford-Mammoth Cave Uplands and Western Pennyroyal Karst from the Mammoth Cave region in Edmonson and Hart counties south to the Tennessee border and west to Trigg, Lyon, and Livingston counties along the Cumberland River. At least six records exist within the Caseyville Hills of the Interior River Valleys and Hills. The number of Spring Cavefish localities averages 3.1 ± 2.8 localities per county with a maximum of nine localities in Edmonson County (Table 4). The highest density of Spring Cavefish localities occurs in the Land Between the Lake area in Lyon and Trigg counties, as well as south of the Bowling Green area in Warren County. The number of Spring Cavefish localities averages 10.6 ± 6.9 localities per ecoregion with a maximum of 20 localities in the Western Pennyroyal Karst (Table 5). Included in the distribution of the Spring Cavefish in Kentucky are nine HUC8 watersheds (Fig. 8), which includes the Cumberland, Green, Lower Ohio, and Lower Tennessee Basins. The number of Spring Cavefish localities averages 5.9 ± 5.8 localities per watershed, with a maximum of 15 localities within the Lower Cumberland subbasin of the Cumberland River basin and 14 localities in the Barren River subbasin of the Green River basin (Table 6). Below we discuss the distribution of Spring Cavefish in each HUC8 watershed.

Lower Cumberland Watershed.—The Lower Cumberland Watershed of the Cumberland River Basin drains portions of the Western Highland Rim, Western Pennyroyal Karst and Crawford-Mammoth Cave Uplands in Caldwell, Christian, Crittenden, Livingston, Lyon, Todd, and Trigg counties. Spring Cavefish have been reported from at least 15 localities in this watershed. Most records are from scattered records of one or just of few fish being caught during electrofishing surveys of streams, particularly in the Land Between the Lakes area in Lyon and Trigg counties. We sampled several surface streams at road crossings in this watershed via dipnetting and did not catch any Spring Cavefish, although this approach has yielded occurrence records historically.

Red River Watershed.—The Red River Watershed is a major tributary and part of the Cumberland River Basin, draining parts of Tennessee and Kentucky, including portions of Christian, Logan, Simpson, Todd, and Trigg counties in Kentucky. Spring Cavefish have been reported from at least seven localities in this watershed. As is the case for the majority of Spring Cavefish records in Kentucky, most involve one or a few fish collected during electrofishing surveys. We sampled several streams and discovered a new locality in Todd County in a spring-fed stream that has been channelized into an irrigation ditch off of Morton Road southeast of Fairview. We visited this locality on several occasions and captured up to 77 Spring Cavefish via dipnet at this site. Swofford (1982) reported collecting 22 Spring Cavefish at Little Whippoorwill Creek in Logan County.

Lower Ohio-Bay Watershed.—The Lower Ohio-Bay Watershed drains portions of southern Illinois and northwestern Kentucky, including parts of Crittenden, Livingston, and Union counties. Spring Cavefish have been reported from three localties within the Crawford-Mammoth Cave Uplands ecoregion of this watershed, including Deer Creek and a cave spring along the bluff line across from the city of Golconda, Illinois along the

Ohio River in Livingston County and Rush Creek in Crittenden County (Compton et al. 2004). We sampled several streams at road crossing in this area but not observe any Spring Cavefish.

Lower Tennessee-Kentucky Lake Watershed.—The Lower Tennessee-Kentucky Lake Watershed drains parts of Calloway, Livingston, Lyon, Marshall, and Trigg counties in Kentucky. Spring Cavefish have only been reported from a single locality in the Land Between the Lakes area of Trigg County. We did not search any streams in this watershed.

Tradewater Watershed.—The Tradewater Watershed of the Lower Ohio-Bay River Basin drains parts of Caldwell, Christian, Crittenden, Hopkins, Union, and Webster counties in Kentucky. Spring Cavefish have been reported from just a single locality in this watershed. A single fish was collected from Piney Creek in 2002 (Compton et al. 2004). We did not search any streams in this watershed.

Pond Watershed.—The Pond Watershed of the Green River Basin drains parts of Christian, Hopkins, McLean, Muhlenberg, Todd, and Webster counties in Kentucky. Spring Cavefish have been reported from just a single locality in this watershed. Burr (1980) collected a single fish from a small stream in this watershed in Muhlenburg County. We search several streams in the extreme southern section of this watershed but did not observe any Spring Cavefish.

Middle Green Watershed.—The Middle Green Watershed of the Green River Basin drains portions of Butler, Grayson, Logan, McLean, Muhlenberg, Ohio, Todd, and Warren counties in Kentucky. Spring Cavefish have been reported from a single locality in southern Butler County in this watershed. We search several streams in the extreme southern section of this watershed but did not observe any Spring Cavefish.

Barren Watershed.—The Barren Watershed of the Green River Basin drains portions of the Western Pennyroyal Karst, Eastern Highland Rim, and Crawford-Mammoth Cave Uplands in Allen, Barren, Butler, Edmonson, Logan, Metcalfe, Monroe, Simpson, and Warren counties in Kentucky. Spring Cavefish have been reported from 14 localities in this watershed, including at least three caves or cave springs. The most well-known and sampled locality is the spring resurgences in the Rich Pond area of Warren County. The water issuing from these springs forms a large (up to 340-acre) ephemeral lake in late winter and early spring. This temporary body of water slowly drains into a sink at its lower end and typically goes completely dry by July or August. Despite the ephemeral nature of this habitat, Spring Cavefish have been observed and collected here in great numbers over the last several decades. We visited this site on several occasions and observed up to 203 fish in a 50 m section of stream habitat from where KY 884 cross the stream upstream toward the springs. At least 1600 individuals have been collected from this area and accessioned into museums since the early 1950s. Despite great sampling intensity, the population still is robust and thriving, as we observed several age classes.

We samples several other surface localities in this watershed but did not observe any Spring Cavefish.

Upper Green Watershed.— The Upper Green Watershed of the Green River Basin drains portions of the Eastern Highland Rim, Western Pennyroyal Karst, Crawford-Mammoth Cave Uplands, Mitchell Plain, and Caseyville Hills in Adair, Barren, Butler, Casey, Cumberland, Edmonson, Grayson, Green, Hardin, Hart, Larue, Lincoln, Marion, Metcalfe, Pulaski, Russell, Taylor, and Warren counties. Spring Cavefish have been reported from the Mammoth Cave region in Edmonson County within the Caseyville Hills and Crawford-Mammoth Cave Uplands ecoregions, and from a tributary to Blue Spring Creek in Metcalfe County. Spring Cavefish have been sporadically observed in the subterranean streams of the Mammoth Cave system, including Buffalo Creek Cave, Cedar Sink, and the Echo River, Mystic River, and Roaring River sections of Mammoth Cave. During our surveys, we did not observe any Spring Cavefish in the Upper Green watershed.

Distribution – Southern Cavefish

The Southern Cavefish is the most widely distributed troglobitic fish in North America. Its range is discontinuous and divided into two main areas east and west of the Mississippi River: an eastern component that extends along the Cumberland Plateau and through the Interior Plateau from central Kentucky (Mammoth Cave region) southward into central Tennessee, northern Alabama, and northwestern Georgia, and a western component that includes the Ozark Plateau of central and southeastern Missouri and north-central Arkansas (Fig. 5). The distribution of the species was once thought to include southern Indiana and northeastern Oklahoma but these records are now thought to be erroneous.

Within Kentucky, Southern Cavefish have been reported from at least 29 localities, including 27 caves, one spring, and one well in eight counties (Fig. 9; Table 2). The number of Southern Cavefish localities averages 3.6 ± 3.6 localities per county with a maximum of 11 localities in Edmonson County associated with the Mammoth Cave system (Table 7). The highest density of Southern Cavefish localities occurs in Edmonson County and in Pulaski County. Southern Cavefish occur in four Level IV ecoregions in Kentucky (Table 8). This distribution extends through much of the southern Interior Plateau in the central part of the state, including the Crawford-Mammoth Cave Uplands and Western Pennyroyal Karst from the Mammoth Cave region in Edmonson and Hart counties south to the Tennessee border and west to Trigg County. A disjunct cluster of populations occurs in Plateau Escarpment ecoregion of the Southwestern Appalachians (Fig. 10). The number of Southern Cavefish localities averages 7.3 ± 4.9 localities per ecoregion with a maximum of 12 localities in the Western Pennyroyal Karst (Table 8). Included in the distribution of the Southern Cavefish in Kentucky are five HUC8 watersheds (Fig. 11). The number of Southern Cavefish localities averages 5.8 ± 4.8 localities per watershed, with a maximum of 13 localities within the Upper Green River subbasin of the Green River basin (Table 9). Below we discuss the distribution of Southern Cavefish in each HUC8 watershed.

Upper Cumberland-Lake Cumberland Watershed.—The Upper Cumberland-Lake Cumberland watershed drains much of the Cumberland Plateau in southeastern Kentucky before flowing into the Upper Cumberland-Cordell Hull watershed downstream in Tennessee. Lake Cumberland is a large impoundment on the Cumberland River in this watershed, flooding over 265 km^2 at full pool and also flooding or partially flooding several cave systems in the area. Construction of Wolf River Dam occurred in 1952 resulting in a base level around 220 m. Beginning in 2007, the water level of Lake Cumberland was lowered up to 12 m to begin repairs to the dam caused by seepage of water due to karst geology under the dam. The drop in water level resulted in the exposure of formerly submerged cave entrances and passages. Southern Cavefish were reported from Sloans River Cave in Pulaski County by Cooper and Beiter (1972). Six specimens were collected in Ocotober and November 1969 in the major stream that flows through the Minton Hollow section, but only in a 300 m stretch that is normally affected by changes in water level caused by backflooding of Lake Cumberland. We visited this section and other sections of the cave on several occasions but failed to observe any cavefish. However, cavers have reported seeing cavefish in the Minton Hollow section of the cave several times during the 1990s and early 2000s before the water level of Lake Cumberland was lowered and subsequently lowered the water level in Sloans Valley Cave (B. Walden, pers. comm.). We surveyed several other caves in the vicinity of Sloans Valley Cave in Pulaski County after receiving unconfirmed reports of cavefish in several area caves (B. Walden and L. Simpson, pers. comm.). Southern Cavefish were discovered in Well’s Cave, Dave’s Cave, and Drowned Rat Cave along Buck Creek. The main entrance to Well’s Cave is owned by the National Speleological Society and a single fish was observed in the main stream channel a few hundred meters upstream from the Buck Creek entrance during the first survey. Cavefish were not observed during subsequent survey trips. Dave’s Cave is part of the Coral Cave system and cavefish have been unofficially reported from this system since the early 1970s. We observed nine fish in the sump pool at the end of Dave’s Cave in November 2007. Three cavefish were observed during a survey in May 2012. At least one cavefish was collected from Coral Cave in the early 1970s and sent to the Cincinnati Museum of Natural History (L. Simpson, pers. comm.), but apparently was not accessioned into a museum or reported to state biologists. Cavers observed two cavefish in 2009 and we believe this report is valid. Cavefish were also observed at Drowned Rat Cave located on Army Corps of Engineers land. At normal pool, the entrance to Drowned Rat Cave is inundated and cannot be accessed but construction on Wolf River Dam permitted entry when the lake level was lowered. Although having previously been partially flooded by Lake Cumberland, we observed several troglobitic species, including Southern Cavefish, cave crayfish (*Cambarus packardi*), millipedes, beetles, springtails, diplurans, and isopods. In total, 31 Southern Cavefish were observed during the first survey. We observed 17, 24, 14, and 5 cavefish during subsequent trips in January 2009, March 2010, July 2010 and April 2012. Cavers also reported cavefish from nearby Dyke’s Bridge Cave (L. Simpson, pers. comm.). We believe this report to be valid despite failing to discover Southern Cavefish during several surveys. Up to two cavefish have been observed in a sump pool at the back of the cave near the second entrance. Southern Cavefish have been confirmed in five caves in Pulaski County and another report is believed to be valid. All localities occur within the Plateau Escarpment of the Southwestern Appalachians and appear to be isolated from other populations in both Kentucky and Tennessee. We also surveyed Neely Creek Cave, Rabid Fish Cave, and Short Creek Cave in Pulaski County but did not observe any cavefish.

Red River Watershed.—The Red River Watershed is a major tributary and part of the Cumberland River Basin, draining parts of Tennessee and Kentucky, including portions of Christian, Logan, Simpson, Todd, and Trigg counties in Kentucky. Southern Cavefish have been reported from two localities in this watershed: Glover Cave in Christian County (one cavefish observed in 1978) and Webb Cave in Simpson County. We visited Webb Cave on two occasions in 2010 and 2011 observing as many as three cavefish during a single survey. We only surveyed the first 200 m of stream passage upstream from the sinkhole entrance before sediment deposits constricted the passage. The cave is reported to continue past this constriction for several hundred meters past this point and cavefish have reportedly been observed in that section of the cave. Cavefish were observed throughout the length of the stream searched including within 10 m of the entrance. We could not gain permission to enter Glover Cave in Christian County. Southern Cavefish are known from several caves within the Red River Watershed of Tennessee and it’s likely that additional localities exist within the watershed in Kentucky.

Lower Cumberland Watershed.—The Lower Cumberland Watershed of the Cumberland River drains portions of the Western Highland Rim, Western Pennyroyal Karst and Crawford-Mammoth Cave Uplands in Caldwell, Christian, Crittenden, Livingston, Lyon, Todd, and Trigg counties. Southern Cavefish have only been reported from Big Sulphur Spring Cave in Trigg County. A single cavefish was collected there in 1981 (MOSU 2041). Swofford (1982) reported collecting four cavefish from this locality. Southern Cavefish occur in several caves within the Lower Cumberland Watershed of Tennessee and it’s probable that other localities exist within this watershed in Kentucky. We did not survey any caves within this watershed during this study.

Barren Watershed.—The Barren Watershed of the Green River Basin drains portions of the Western Pennyroyal Karst, Eastern Highland Rim, and Crawford-Mammoth Cave Uplands in Allen, Barren, Butler, Edmonson, Logan, Metcalfe, Monroe, Simpson, and Warren counties in Kentucky. Southern Cavefish have been reported from five caves in this watershed, including Parker Cave and Mitchell Cave in Barren County, and Friendship Cave, Mill Cave, and Lost River Cave in Warren County. Southern Cavefish have also been reported from a well near Bowling Green (the type-locality of *T. subterraneus*) and from a spring near Rich Pond in Warren County. We observed up to two cavefish during surveys of Friendship Cave in 2010 and 2011. The spring at Rich

Pond was visited several times but Southern Cavefish were never observed. We could not acquire permission to enter Mitchell Cave or Mill Cave. Parker Cave is currently gated but the private landowner does not have in his possession a key to the lock; however, the landowner is amenable to allowing access for biological surveys in the future. Southern Cavefish have been reported from Parker Cave in the 1990s (Pearson & Jones 1998).

Upper Green Watershed.—The Upper Green Watershed of the Green River Basin drains portions of the Eastern Highland Rim, Western Pennyroyal Karst, Crawford-Mammoth Cave Uplands, Mitchell Plain, and Caseyville Hills in Adair, Barren, Butler, Casey, Cumberland, Edmonson, Grayson, Green, Hardin, Hart, Larue, Lincoln, Marion, Metcalfe, Pulaski, Russell, Taylor, and Warren counties. Southern Cavefish have been reported from at least 13 caves in this watershed, including L & N Railroad Cave in Barren County, Floyd Collins Crystal Cave, Colossal Cave, Buffalo Creek Cave, Sanders Cave, Cedar Sink-Turnhole Cave, Stillhouse Hollow Cave, and the Echo River, Hawkins River, Mystic River, Roaring River, and Eyeless Fish Trail sections of Mammoth Cave, and Hidden River Cave in Hart County. We observed 19 cavefish in the Hawkins River section (Proctor Cave) of Mammoth Cave in 2010. We also observed 22 cavefish in Sanders Cave, which lies north of the Green River in Mammoth Cave National Park. L & N Railroad Cave is located within the city limits of Cave City. We made five trips to this cave in 2010–2012 and observed 6–33 cavefish during each survey.

Distribution – Northern Cavefish

The Northern Cavefish is associated with caves and karst along a narrow arc from near Bedford in Lawrence Co., Indiana, just south of the East Fork White River southward to the Mammoth Cave area in central Kentucky (Fig. 5). The species has a nearly continuous distribution within the Crawford-Mammoth Cave Uplands and Mitchell Plain. Within Kentucky, Northern Cavefish have been reported from at least 40 localities, including 39 caves and one spring in five counties (Fig. 12; Table 3). The number of Northern Cavefish localities averages 8.0 ± 6.2 localities per county with a maximum of 18 localities in Breckinridge County with many associated with Sinking Creek (Table 10). Northern Cavefish occur in three Level IV ecoregions in Kentucky (Fig. 13; Table 11): the Crawford-Mammoth Cave Uplands and Mitchell Plain of the Interior Plateau and the Caseyville Hills of the Interior River Valley and Hills (two localities). The number of Northern Cavefish localities averages 13.3 ± 13.3 localities per ecoregion with a maximum of 28 localities in the Crawford-Mammoth Cave Uplands (Table 11). The highest density of Southern Cavefish localities occurs in the Sinking Creek valley in Breckinridge County and in the Mammoth Cave region in Edmonson County. Included in the distribution of the Northern Cavefish in Kentucky are three HUC8 watersheds (Fig. 14): the Rough and Upper Green watersheds of the Green River Basin and the Blue-Sinking Watershed of the Lower Ohio River Basin. The number of Northern Cavefish localities averages 13.3 ± 8.6 localities per watershed, with a maximum of 21 localities within the Blue-Sinking subbasin within the Lower Ohio River basin (Table 12). Below we discuss the distribution of Northern Cavefish in each HUC8 watershed.

Upper Green Watershed.— The Upper Green Watershed of the Green River Basin drains portions of the Eastern Highland Rim, Western Pennyroyal Karst, Crawford-Mammoth Cave Uplands, Mitchell Plain, and Caseyville Hills in Adair, Barren, Butler, Casey, Cumberland, Edmonson, Grayson, Green, Hardin, Hart, Larue, Lincoln, Marion, Metcalfe, Pulaski, Russell, Taylor, and Warren counties. Northern Cavefish occur primarily in the western part of the watershed from the Mammoth Cave System northward. Northern Cavefish have been reported from 13 caves and one spring in the watershed, including Salts Cave, Frenchman's Knob Cave, and Walker's Cave in Hart County, Bland's Cave and an unnamed cave on Nolin River in Hardin County, Colossal Cave, Florentine Cave, Cedar Sink, Running Branch Cave, and the Echo River, Hawkins River, Mystic River, Roaring River, and Eyeless Fish Trail sections of Mammoth Cave in Edmonson County. Northern Cavefish also have been reported from Echo Spring. We observed 1 cavefish in the Hawkins River section (Proctor Cave) of Mammoth Cave in 2010, but did actively search this section of the cave due to turbid water conditions. Mammoth Cave National Park personnel confirm that Northern Cavefish are regularly observed in the base level streams of the Mammoth Cave system (R. Toomey, pers. comm.). Pearson and Boston (1995) and Pearson and Jones (1998) reported on populations of several localities in the Mammoth Cave region.

Rough Watershed.—The Rough Watershed of the Green River Basin drains portions of the Mitchell Plain, Crawford-Mammoth Cave Uplands, and Caseyville Hills in Breckinridge, Butler, Daviess, Grayson, Hancock, Hardin, McLean, and Ohio counties. Northern Cavefish have been reported from just four caves in the watershed, including Big Bat Cave and Locust Hill Cave in Breckinridge County, and Belt Cave and Constantine Saltpeter Cave in Hardin County. Pearson and Boston (1995) reported two cavefish from Constantine Saltpeter Cave. We did not survey any caves in this watershed.

Blue-Sinking Watershed.—The Blue-Sinking Watershed of the Lower Ohio Basin drains portions of the Mitchell Plain and Crawford-Mammoth Cave Uplands in Indiana and Kentucky, including sections of Breckinridge, Hardin, Jefferson, and Meade counties. The core of the distribution of Northern Cavefish occurs in this watershed in both Indiana and Kentucky. In Kentucky, the species has been reported from 21 caves, with most localities occurring within the Sinking Creek drainage. Several localities support significant populations of Northern Cavefish (>20 cavefish observed during a single survey), including Rimstone Cave, Thornhill Cave, Amblyopsis Cave, Under the Road Cave, Webster's Cave, and Penitentiary Cave in Breckinridge County. Over 100 individuals have been observed in the latter four caves, including up to 515 cavefish during a single survey of Penitentiary Cave in 1994 by Pearson and Boston (1995) and 417 by Pearson and Jones at Amblyopsis Cave (W. Pearson, pers. comm. in McCandless 2005). We surveyed several caves in Breckinridge County, including Bandy Cave, Rimstone Cave, Under the Road Cave, and Webster's Cave and observed cavefish in all four caves. We surveyed the entire stream passage of Rimstone Cave, which is part of the Blissit's Cave system, on two occasions observing 21 and 26 cavefish, respectively. We could not gain access to Blissit's Cave or Amblyopsis Cave. We surveyed Bandy Cave three times and observed up to four cavefish. Webster's Cave was visited on four occasions and conducted surveys of the approximately the first 1,100 m of stream passage nearest the main entrance observing 29, 36, 41 and 27 cavefish. Under the Road Cave was visited on seven occasions. We only observed a single cavefish in the main trunk passage ca. 900 m from the Ammon's Entrance during the first two surveys due to elevated water levels and increased turbidity. However, we failed to search a left-hand side passage where the majority of cavefish have been observed during past surveys. We included this tributary in subsequent surveys under better searching conditions and found between 22 and 45 cavefish.

Cooccurrence of Amblyopsids

The Mammoth Cave area in Edmonson County, Kentucky, is one of the few locations where two or more species of stygobitic fishes cooccur within the same cave systems (Fig. 15). In the Mammoth Cave system, both *T. subterraneus* and *A. spelaea* can be found in six major streams, including Echor River, Roaring River, Eyeless Fish Trail, Hawkins River (Proctor Cave), Mystic River and the Cedar Sink area. All of these areas are within the Crawford-Mammoth Cave Uplands ecoregion (Fig. 16) in the Upper Green River subbasin (Fig. 17). Their cohabitation has been explained by competitive interactions (Woods & Inger 1957; Poulson 1992). Southern Cavefish are typically found in the master drains of vertical shafts, whereas Northern Cavefish inhabit deeper water with decreased food availability further downstream in base-level streams. It is unclear why Northern Cavefish are precluded from areas further upstream, although differences in body size and aggressive dominance by Southern Cavefish have been proposed as possible hypotheses (Poulson 1992). Alternatively, Southern Cavefish may be excluded from downstream habitats by decreased food availability or by competitive dominance by Northern Cavefish.

Northern Spring Cavefish also has been found with both Southern Cavefish and Northern Cavefish in Echo River, Mystic River, Roaring River, and the Cedar Sink area of the Mammoth Cave system. Additionally, Northern Spring Cavefish has been collected at Buffalo Creek Cave in Edmonson County and Lost River Cave, and Rich Pond in Warren County where Southern Cavefish also has been documented. In the Mammoth Cave system, it is thought that Northern Spring Cavefish are weshed into the cave from sinking streams during flood events but only survive as long as a year when the food supply in the cave stream is above average (Niemiller & Poulson 2010). Reproduction of Northern Spring Cavefish is unlikely for individuals that are permanent residents of cave streams, as not enough food can be consumed to support to the energetic demands associated with reproduction compared to the troglobitic species. However, in other areas, such as Rich Pond in Warren County, it is clear that Northern Spring Cavefish utilize subterranean habitats when surface conditions become hospitable (e.g., low stream flow and drying, increased water temperatures).

Habitat – Northern Spring Cavefish

Northern Spring Cavefish occur at the interface of epigean and subterranean habitats bridging the threshold toward a troglobitic life. Although the species have been reported from caves throughout much of its range, Northern Spring Cavefish are most abundant (based on sampling) in springs, spring runs, spring-fed ponds, and seeps. This is likely an artifact of sampling effort in surface habitats relative to subterranean habitats throughout the species' range. Both subterranean and surface habitats are used throughout much of the Northern Spring Cavefish's range; fish often emerge from subterranean haunts at dusk to feed and then later retreat back underground before dawn. Northern Spring Cavefish are particularly abundant in surface habitats near springs in late winter and early spring when the water table is high. It is during this time period that most collections in Kentucky have occurred. As surface water begins to retreat into subterranean courses in the karst landscapes of the Crawford-Mammoth Cave Uplands, Western Pennyroyal Karst, Western Highland Rim, and Eastern Highland Rim, Spring Cavefish also retreat underground. This annual pattern is clearly demonstrated by the population at Rich Pond in Warren County (Fig. 18), where cavefish can disperse into the spring-fed stream and ephemeral lake during spring but retreat back to the spring as the aquatic habitat dries into summer. Northern Spring Cavefish can be common in dense vegetation in spring runs and spring-fed stream course with low flow, such as that found in the spring-fed ditch at Morton Road in Todd County (Fig. 19). Northern Spring Cavefish also can be found underneath rocks in springs and spring runs during the day. They are negatively phototactic (Poulson 1963; Niemiller & Poulson 2010) and hide in vegetation or under objects even in low levels of ambient light. Northern Spring Cavefish are positively rheotactic and avoid strong currents. Although this species has been collected from streams, collections usually occur underneath cover or within dense vegetation in pools or at the margins of streams. During high water conditions in spring, Northern Spring Cavefish may use the vast ephermal waters in the Western Pennyroyal Karst as corridors for dispersal across local drainage boundaries (Fig. 20), although direct evidence to support this hypothesis is lacking at this time.

Northern Spring Cavefish might be only marginal troglophiles because there is no documentation of the species reproducing and living permanently in caves (Niemiller & Poulson 2010). The best-studied populations emerge nightly or seasonally from food-poor caves to feed in spring runs that have abundant live prey. Northern Spring Cavefish presumably spawn in caves, at least in southern Illinois and central Kentucky, as adults disappear from most surface habitats for a few months beginning in late summer (Weise 1957; Hill 1966). Both adults and small juveniles have been found on the surface in early spring, including during this study. However, gut content analyses reveal that cave populations of Spring Cavefish predominantly have empty guts and have poor condition factors (Niemiller & Poulson 2010).

Habitat – Southern Cavefish

Most Southern Cavefish habitat is at or near the water table (Niemiller & Poulson 2010). Southern Cavefish inhabit cool, 10–15°C subterranean waters, including vadose streams (those above the water table) as well as phreatic waters (those at the water table). However, most observations of Southern Cavefish during the current study occurred in large pools within major stream passages or in smaller, infeeder streams with little current. Southern Cavefish were often observed in the vicinity of breakdown and other rock piles, which offer protection from increased water flow and safety when alarmed. A notable exception were two cavefish found in Webb Cave in Simpson County that were observed actively swimming against the current when first discovered. Southern Cavefish are indifferent to light (Eigenmann 1909; Verrier 1929; Green & Romero 1997). Our observations are consistent with past researchers. Southern Cavefish do not respond directly to illumination by headlamps. Likewise, we observed Southern Cavefish in the twilight zones of caves, including Webb Cave and Sanders Cave in Edmonson County (Fig. 21). At Sanders Cave, Southern Cavefish were observed within 30 m of the entrance in a small stream with abundant rocky substrate (Fig. 22). Water velocity varies tremendously among seasons and years for most Southern Cavefish caves and habitats. This is clearly illustrated by the spring overflow entrance of Dykes Bridge Cave (Fig. 23) where the stream issues from the mouth of the cave with high flow during high water conditions. Southern Cavefish are positively rheotaxis and prefer habitats with little or no current. This species is sensitive to the slightest increase in water velocity, and is known to seek shelter hours before humans can detect an oncoming flood (W. Pearson, pers. comm. in Niemiller & Poulson 2010). We observed this behavior during moderate flow in L & N Railroad Cave in Barren County in the upstream section of the cave where the substrate is largely cobble and boulder. Cavefish were observed either underneath rocks in the stream or underneath overhanging ledges that provided shelter from increased water flow. The composition of substrates also vary a great deal within and among caves inhabited by Southern Cavefish, ranging from bedrock to mud to thick deposits of organic debris. However, most of our observations occurred in pools with sand to cobble to boulder substrates with some organic debris. Southern Cavefish also are found in pools with mud/silt substates, such as sections of the downstream passage at L & N Railroad Cave (Fig. 24). As mentioned above, Southern Cavefish also were frequently observed amongst larger breakdown piles. Moreover, Southern Cavefish are typically observed at depths less than one meter. We do not know to what depths Southern Cavefish occur, but anecdotal evidence from Missouri suggests this species may utilize deeper waters than currently recognized.

Habitat – Northern Cavefish

Northern Cavefish inhabit subterranean streams with low to moderate flow and cool temperatures (8–16°C). Many of the cave systems inhabited by Northern Cavefish, like those in Sinking Creek (Fig. 25) flood regularly and experience wide fluctuations in water flow and input of organic matter. In caves with the largest populations, Northern Cavefish are most abundant in deep pools or moderately deep shoals with ledges, overhangs, breakdown, and slackwater areas that serve as refugia during floods. These habitats often have an abundance of organic matter washed in from the surface with mud, silt, sand, to cobble substrates. Smaller individuals, particularly young-of-the-year are almost always found in smaller tributaries with very low flow. While these areas might serve as nursery areas, this observation could be the result of sampling bias, as small individuals are most easily detected in calm, shallow habitats. In the Mammoth Cave system where both Southern Cavefish and Northern Cavefish cooccur, the two species utilize different habitats. Southern Cavefish are found primarily in the master drains of vertical shafts, whereas Northern Cavefish are found in the deeper waters of base level streams further downstream.

Taxonomy, Systematics and Phylogenetics

The Amblyopsidae have been known to science since the 1840s. The family-group name (Amblyopsidae-Amblyopsini) was first used by Bonaparte (1846). Previously, the family names Heteropygii and Hypsaeidae were used by Tellkampf (1844, 1845) and Storer (1846) respectively, but these names are considered unavailable because they were not formed from the stem of an available genus-group name (Poly & Proudlove 2004). Aspects of taxonomy and systematics of the family have been examined by Cox (1905), Eigenmann (1909), Woods and Inger (1957), Swofford et al. (1980), Swofford (1982), Bergstrom et al. (1995), Bergstrom (1997), Niemiller and Fitzpatrick (2008), Dillman et al. (2011), and Niemiller et al. (2013a). The latter six studies were molecular in nature. Poly and Proudlove (2004) reviewed taxonomy and systematic relationships of the Amblyopsidae. Presently five genera and six species are recognized within the Amblyopsidae. These include the epigean Swampfish (*Chologaster cornuta* Agassiz), a troglophile, the Spring Cavefish (*Forbesichthys agassizii* Putnam), and five troglobitic species in order of presumed increasing time of isolation in caves: Southern Cavefish (*Typhlichthys subterraneus* Girard), Salem Plateau Cavefish (*T. eigenmanni*), Northern Cavefish (*Amblyopsis spelaea* DeKay), Ozark Cavefish (*Troglichthys rosae* Eigenmann), and the Alabama Cavefish (*Speoplatyrhinus poulsoni* Cooper and Kuehne). However, recent molecular work (Niemiller et al. 2012, 2013a; this study) has uncovered phylogenetic relationships among species that are discordant with past systematic hypotheses. Additionally, we have discovered evidence for cryptic diversity in all three species of amblyopsids that occur in Kentucky (Niemiller et al. 2013a,b). Here, we briefly discuss the higher-level relationships of the Amblyopsidae then discuss nomenclature, systematics, and phylogenetics for each species occurring in Kentucky.

Higher-level relationships

The Amblyopsidae have been considered the sister group to the pirate perches, family Aphredoderidae (Rosen 1962; Patterson 1981; Patterson & Rosen 1989) but also share a close affinity with the trout perches, family Percopsidae (Rosen 1962). Together, these three families have been included in the order Percopsiformes (Greenwood et al. 1966; Nelson 2006). However, some have questioned the monophyly of this order. Murray and Wilson (1999) suggested amblyopsids might be more closely related to the anacanthines and proposed recognition of amblyopsids as a distinct order, the Amblyopsiformes. Poly in Poly and Proudlove (2004) suggested that amblyopsids might be related to gobioids because of the distribution of amblyopsids in relation to the former Mississippi Embayment, similarities in patterns of neuromasts on the body, and similarities in morphology, particularly when compared with troglobitic gobioids. McAllister (1968) also suggested a possible relationship between gobioids and amblyopsids. Wiley et al. (2000) resolved a sister relationship between trout perches and pirate perches based on a combined analysis of morphological and molecular characters but did not include any amblyopsid species. More recently, a molecular study using both mitochondrial and nuclear markers and including members of all three families supports the inclusion of the Amblyopsidae in the order Percopsiformes with a sister relationship between cavefishes and pirate perches (Smith and Wheeler 2006). Immunological evidence also supports the inclusion of the Amblyopsidae in the order Percopsiformes (Kalayil & Clay 1976).

Southern Cavefish (*Typhlichthys subterraneus*)

The Southern Cavefish was described by Girard (1859) from a well near Bowling Green, Warren Co., Kentucky. Later, Eigenmann (1905) described both *T. osborni* and *T. wyandotte* based on differences in head width and eye diameter. *Typhlichthys osborni* was described from Horse Cave, Kentucky (most likely Hidden River Cave in Hart Co., Kentucky). *Typhlichthys wyandotte* was described from a well near Corydon, Indiana, that was later destroyed. Recently, a well-like entrance into a cave on the property of a car dealership in Corydon was discovered and is believed to represent the type locality (Black in Lewis 2002b). Regardless, this species is generally considered invalid and was not listed as a locality in Woods and Inger (1957). Recent surveys in the vicinity of Corydon have failed to document *T. subterraneus*, finding only *A. spelaea* (Lewis 1998; Lewis & Sollman 1999). *Typhlichthys eigenmanni* (*nomen nudeum*) was described as a fourth species in the genus from Camden Co., Missouri (likely River Cave). Recently, Parenti (2006) proposed that *T. eigenmanni* Charlton (1933) is a subjective synonym of *T. subterraneus*. Woods and Inger (1957) synonymized all species under *T. subterraneus* on the basis of lack of any clear geographic pattern in morphological variation. Since Woods and Inger (1957), all populations in Kentucky have been considered *T. subterraneus*; however, Cooper and Beiter (1972) noted that the Sloans Valley Cave population in Pulaski County differs in a number of ways from populations to the southwest in Tennessee along the Cumberland Plateau and might represent an undescribed taxon (J. Cooper, pers. comm. in Burr & Warren 1986).

Because of its large distribution across several major hydrological units and documentation of cryptic diversity in other wide-ranging subterranean taxa, several authors have hypothesized that the Southern Cavefish represents a species complex comprised of several morphologically cryptic species, possibly resulting from several parallel colonizations by a surface-dwelling common ancestor (Swofford 1982; Barr & Holsinger 1985; Holsinger 2000; Niemiller & Poulson 2010). The few studies that have examined genetic variation in Southern Cavefish have found considerable genetic differentiation among morphologically similar populations structured among hydrological units (Swofford 1982; Bergstrom et al. 1995; Bergstrom 1997; Niemiller & Fitzpatrick 2008). For instance, electrophoretic analyses by Swofford (1982) showed considerable differentiation among morphologically similar populations of *Typhlichthys*, indicative of multiple, independent lineages and limited gene flow. 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. The discovery of significant genetic variation across the range of *T. subterraneus* warrants further detailed investigations of species delimitation in these cavefish.

We constructed gene trees for six loci for 13 *Typhlichthys* lineages (identified below) from 62 populations throughout the distribution of the species (Table 13; Fig. 26) and these are presented in Fig. 27. For the mtDNA data (*nd2*), uncorrected sequence divergence among the major lineages ranged from 3.6% to 12.2%. Almost all internal nodes within *Typhlichthys* were well supported with high Bayesian posterior probabilities. Major lineages were split into two clades: (i) all lineages west of the Mississippi River (lineages I, J, and K) in the Ozark Highlands plus two lineages found in the Green River drainage (lineage L) in Kentucky and north-central Tennessee and Upper Cumberland River drainage (lineage M) in Kentucky; and (ii) all other lineages in the Interior Low Plateau, Cumberland Plateau, and Ridge and Valley of Alabama, Georgia, and Tennessee. For the nuclear data (*s7*, *rag1*, *plag*, *myh*, and *tbr*), Bayesian analyses showed moderate levels of incomplete lineage sorting (Fig. 27); many lineages observed in the mtDNA gene tree often grouped together in the nuclear gene phylogenies, while others were paraphyletic. The two major mtDNA clades were not recovered in all nuclear gene phylogenies, although lineages I, J, and K west of the Mississippi River almost always were recovered together.

Our multilocus approach using O'Meara's (2010) nonparametric method revealed the potential for as many as eleven or more species (based on the 6-gene, 62-individual dataset) within *Typhlichthys* (Table 13; Fig. 28), including two species occuring in Kentucky (species G and J in Fig. 28). One species (species G) is associated with Interior Plateau of south-central Kentucky and north-central Tennessee in the Upper Green River and Red River subbasins (populations 16–18, 53, and 61–62), while the other species (species J) consists of populations in the Upper Cumberland River subbasin in the Plateau Escarpment of the Southwestern Appalachians in Pulaski County (populations 19–21). These lineages are phylogenetic distinct and are not sister species

(Fig. 29). Rather, species J is sister to three delimited species distributed to the southwest along the Cumberland Plateau and Eastern Highland Rim in Tennessee (Fig. 28), whereas species G is sister to a lineage found west of the Mississippi River in the Ozark Highlands.

These delimited species were also supported by the results of Bayesian species delimitation and genealogical tests of taxonomic distinctiveness. Bayesian species delimitation supported the guide tree with speciation probabilities > 0.95 for all nodes but one (Fig. 29). Values of gsi and egsi indicated a high degree of exclusive ancestry within delimited species, including species G and J found in Kentucky (Table 14). However, no delimited species exhibited exclusive ancestry at all loci in the 6-gene, 62-individual dataset and some *gsi* values for delimited species were not significant for the *myh6*, *plagl2*, and *tbr1* loci. *Gsi* values were generally lower for these loci, which are consistent with lower overall genetic variation and shared ancestry across delimited species in these genes. Lack of genealogical exclusivity for some delimited species at nuclear loci can best be explained by recent divergence and incomplete lineage sorting.

Divergence time estimates derived from the multilocus species tree analysis, which used data from all *Typhlichthys* samples, support initial late Miocene/early Pliocene diversification of *Typhlichthys* from *Speoplatyrhinus* with the majority of divergence within *Typhlichthys* occurring throughout the late Pliocene and Pleistocene (Table 15; Fig. 30). There was strong support for a clade consisting of lineages G–M, which include Kentucky populations.

Hierarchical AMOVA of populations grouped by hydrological subbasin revealed that genetic structure at each locus (*nd2*, *s7*, and *rag1*) is highly correlated with hydrological boundaries, as the majority of variation was significantly partitioned among watersheds for each locus (66.7–79.5%; Table 16). Likewise, hierarchical AMOVA of populations grouped by hydrological basin also revealed genetic structure associated with hydrological boundaries, but to a much lesser degree (35.7–37.9%; Table 16). Genetic structure also is correlated with ecoregion but to a similar level observed for hydrological basins (27.2–30.9%; Table 16). These results indicate that dispersal is limited across hydrological boundaries and offers a hypothesis for a role of hydrological barriers in speciation of *Typhlichthys*, as has been suggested for other stygobitic fauna (Lefebure et al. 2006, 2007; Finston et al. 2007; Carlini et al. 2009).

Biogeographic reconstructions of hydrological drainages and ecoregions for major nodes identified in Fig. 30 within *Typhlichthys* are given in Table 15. The optimal reconstruction of hydrological drainages favored the MRCA of *Typhlichthys* and *Speoplatyrhinus* (node 1; Table 15; Fig. 30) and the ancestor of *Typhlichthys* (node 2; Table 15; Fig. 30) as having originated in the Tennessee River drainage. This analysis supported a single dispersal event across the Mississippi River into the White and Osage River drainages (node 4; Table 15; Fig. 30). The optimal reconstruction of ecoregions favored the MRCA of *Typhlichthys* and *Speoplatyrhinus* (node 1; Table 15; Fig. 30) and the ancestor of

Typhlichthys (node 2; Table 15; Fig. 30) as having originated in the Interior Plateau. As was the case in the biogeographic reconstruction of hydrological drainages, a single dispersal event across the Mississippi River from the Interior Plateau into the Ozark Highlands was suppported (node 4; Table 15; Fig. 30).

Our divergence time and ancestral area reconstruction analyses suggest that *Typhlichthys* originated in the Interior Plateau within the Tennessee River drainage during the late Miocene-early Pliocene (Fig. 30). However, the majority of dispersal and diversification events within *Typhlichthys* occurred primarily during the Pleistocene (Fig. 30), implicating climate change as the primary mechanism driving diversification. Divergence estimates among major lineages are considerably younger than those estimated using partial sequences of the mitochondrial *nd2* gene in Dillman et al. (2011), which inferred an early Miocene origin for *Typhlichthys* with the majority of diversification occurring in the middle to late Miocene. The traditional view of speciation in temperate cave fauna is that of an allopatric, climate-driven model. Under this so-called climate-relict hypothesis (Holsinger 1988, 2000; Ashmole 1993), surface ancestors adapted to cool habitats (e.g., springs and spring runs) in temperate areas retreated into subterranean habitats in response to climatic fluctuations, such as during the Pleistocene. Surface populations then were extirpated facilitating allopatric speciation as surface environmental conditions became inhospitable. Simultaneous, independent colonization events by a surface ancestral species that subsequently went extinct would be reflected as a polytomy on the inferred species tree. Conversely, successive independent colonization events of different groundwater basins by a surface ancestor or colonization of different groundwater basins via subterranean dispersal after an initial colonization event from the surface would be reflected as a series of dichotomous branching events on the inferred species tree. Rather than a strictly dichotomous pattern of branching, the multilocus species tree (Fig. 30) reflects more rapid divergence denoted by short branches at the base of the *Typhlichthys* clade (node 2 in Fig. 30) beginning around the onset of the Pleistocene. The phylogenetic pattern observed supports multiple subterranean colonizations over a short time period by a widespread surface ancestor rather a single colonization and subsequent subterranean dispersal and vicariance or successive colonization events by a surface ancestor.

This phylogenetic pattern could also result from a single colonization event followed by rapid subterranean dispersal and subsequent vicariance, such as dramatic groundwater level changes isolating populations in distinct hydrological basins. Although groundwater level changes likely occurred during the Pleistocene in concert with glaciation events, several lines of evidence suggest significant long distance dispersal in *Typhlichthys*, and aquatic cave organisms in general, is very unlikely. Although *Typhlichthys* has been observed rarely in surface streams (Mohr & Poulson 1966; Niemiller & Poulson 2010), dispersal via surface corridors is extremely unlikely (Woods & Inger 1957; Poulson 1963; Niemiller & Poulson 2010) given the hundreds of surface stream miles between some populations. Moreover, the ranges of most aquatic subterranean organisms are small compared to those of surface-dwelling species (Lamoreaux 2004), although the causes of such small distributions remain debated (see Christman et al*.* 2005; Culver et al. 2006).

Although little direct evidence exists, cavefish could move through solution channels in limestone bedrock located underneath surface rivers (reviewed in Niemiller & Poulson 2010) and some indirect evidence supports this hypothesis. Groundwater systems developed in the thick horizontal Ordovician and Mississippi limestone formations of the Interior Low Plateau and Ozark Plateau pass underneath major surface rivers, such as the Mississippi River. Genetic divergence is low within surface hydrological basins and subbasins, even among populations distributed on opposite sides of a river (Niemiller & Fitzpatrick 2008; Niemiller et al. 2012). However, the majority of genetic variation in *Typhlichthys* is partitioned among hydrological drainages, both surface basins and subbasins, and is indicative of significant dispersal barriers across hydrological boundaries (Table 16). Long distance subterranean dispersal to explain movement of *Typhlichthys* from the Interior Plateau into the Ozark Highlands underneath the current course of the Mississippi River followed by vicariance is very unlikely.

Based on the results of our and other studies on subterranean taxa in the region, we offer the following hypothesis describing the evolutionary and colonization history of *Typhlichthys*. As the climate became drier from the late Miocene and throughout the Pliocene, the surface ancestor of *Typhlichthys* diverged from *Speoplatyrhinus* in the Tennessee River basin. This ancestor dispersed northward via surface corridors throughout the Interior Low Plateau along the margin of Cumberland Plateau and across the Mississippi River into the Ozark Highlands during the late Pliocene and early Pleistocene. Dispersal from the Interior Low Plateau into the Ozark Highlands might have been facilitated by interconnected waterways along ice sheets or water level changes during Pleistocene glaciation events, as posited by the Pleistocene dispersal hypothesis (reviewed in Mayden 1988) to explain speciation and the presence of closely related species in fishes distributed among disjunct highland regions of the Central Highlands (i.e., Eastern Highlands and Ozark Highlands). However, genetic structure and levels of mtDNA divergence support that many lineages east of the Mississippi River in the Interior Low Plateau and Cumberland Plateau were already isolated in hydrological basins prior to glaciation events of the Pleistocene. Phylogeographical studies of other widely-distributed fishes that inhabit the Interior Highlands also show evidence that vicariance-dispersal events both before and during the Pleistocene have influenced the distribution and spatial patterns of genetic diversity within species or species complexes (e.g., Strange & Burr 1997). Dramatic climatic shifts in the late Pliocene and Pleistocene then facilitated multiple, independent subterranean colonization events, diversification, and extinction of the surface ancestor across the distribution of *Typhlichthys*. Colonizations did not occur simultaneously throughout the range of *Typhlichthys*, however.

The results of our analyses support several cryptic lineages within *Typhlichthys*, but the question remains as to how many distinct lineages to recognize taxonomically. This question, in part, depends on the species concept used to recognize species. The biological species concept (Mayr 1942) is difficult to use for many species, including most subterranean organisms, as it may be impossible to test for reproductive isolation because individuals are difficult to collect and rear or for conservation reasons. However, the Bayesian species delimitation method adopts the biological species concept, recognizing groups that have not experienced recent gene flow and where discordance among loci is due to lineage sorting only (Yang & Rannala 2010). Few species would be recognized under a phylogenetic species concept that allows only monophyletic groups to be considered species, as few lineages exhibit monophyly across all loci examined depending on the dataset. However, invoking a genealogical species concept (Baum & Shaw 1995) or metapopulation lineage species concept (de Queiroz 1998, 2007) would result in recognition of all delimited species of *Typhlichthys*. Our results suggest eleven or more lineages exist within *Typhlichthys* and that diversity is grossly underestimated. Uncorrected mtDNA sequence divergence ranged from 3.6 to 12.2% among these lineages. However, we refrain from describing these lineages with one exception below until additional work in an integrative framework (Dayrat 2005; Roe & Sperling 2007; Roe et al. 2010) incorporating information from different fields of study (e.g., morphology, genetics, behavior and geography) is conducted to assess validity of these putative lineages.

The discovery of cryptic, distinct lineages and putative species within the nominal *T. subterraneus* has obvious conservation implications. As currently recognized, *T. subterraneus* is considered secure, although the species is listed as 'Vulnerable' by IUCN (World Consevation Monitoring Center 1996) and afforded protection in several states, including Kentucky where it is listed as 'Special Concern'. Because *T. subterraneus* is already a species of conservation concern in many parts of its range, the recognition of multiple, cryptic species likely would result in several species more rare than previously supposed. These species would have a much more restricted distribution comprised of fewer populations and, consequently, fewer individuals. For example, the lineage found in Pulaski County is comprised of only six populations. Accordingly, the different species might require different conservation and management strategies.

Typhlichthys subterraneus was described from a well near Bowling Green, Warren Co., Kentucky, in the Green River drainage (Girard 1859). Eigenmann (1905) later described both *T. osborni* and *T. wyandotte* based on differences in head width and eye diameter. Although the type-locality of *T. subterraneus* is unknown, *T. subterraneus* and *T. osborni* likely are the same species, as *T. osborni* was described from nearby Horse Cave, Kentucky in the same hydrological basin. *Typhlichthys wyandotte* was described from a well near Corydon, far outside the known distribution of *Typhlichthys* but within the range of *Amblyopsis spelaea* and represents this species. *Typhlichthys eigenmanni* was described as a fourth species in the genus from Camden Co., Missouri, but synonomized along with all other species under *T. subterraneus* by Woods and Inger (1957). Recently, Parenti (2006) demonstrated that *T. eigenmanni* Charlton (1933) is an available name. In our analyses, populations west of the Mississippi River in the Ozark Highlands of Arkansas and Missouri showed strong support for phylogenetic distinctiveness with one to four lineages recognized depending on the dataset. These populations occur in a distinct ecoregion and are allopatric from populations east of the Mississippi River. Given biogeographical and phylogenetic evidence, we advocated resurrection of *T. eigenmanni* for Ozark Highland populations of *Typhlichthys* (Niemiller et al. 2012). Two lineages were delimited within Kentucky: one consisting of populations from the Upper Green, Barren, and Red River watersheds, and the other that includes populations in the Upper Cumberland watershed in Pulaski County. Our results support the hypothesis by Cooper and Beiter (1972) that Pulaski County *Typhlichthys* are a distinct taxon from populations to the west in south-central Kentucky and to the southwest in Tennessee. We are currently examining acquiring and examining morphological data from these populations and intend to describe this lineage (*T. sp. nov. 1*; the Kentucky Cavefish) in the near future.

Northern Cavefish (**Amblyopsis spelaea*****)***

The Northern Cavefish was the first stygobitic fish described in the scientific literature. The formal description is based on cavefish from Mammoth Cave, Edmonson County, Kentucky, by DeKay (1842). Originally described as *Amblyopsis spelaeus*, Woods and Inger (1957) changed the name to *A. spelaea* and provided the most significant definition of the species (Proudlove 2006).

The distribution of *A. spelaea* north of the modern Ohio River in Indiana lies within the area that remained ice free throughout the Pleistocene but occurs as close as 16 km from the glacial maxima during the Illinoian glaciation (300–130k years before present). Interspecific relationships among populations have not yet been investigated by molecular sequence data. However, Swofford (1982) found that populations north and south of the Ohio River are monomorphic for the same allozyme alleles and lacked heterozygosity. Given its distribution in close proximity to the glacial maximum and that it occurs both north and south of the Ohio River (Fig. 31), we expected that patterns of genetic variation in *A. spelaea* likely have been influenced by both geological and climatic processes that occurred during the Pleistocene. Additionally, because its distribution includes at least seven hydrological basins, *A. spelaea* might be comprised of several morphologically cryptic lineages, as has been documented in *Typhlichthys* (see above and Niemiller et al. 2012). We took an integrative hypothesis-testing approach to assess the roles of vicariance and dispersal and to examine cryptic diversity during the evolutionary history of *A. spelaea*.

Estimation of gene genealogies from 70 individuals of 16 populations (Table 17) resulted in nearly identical topologies for most of the five loci examined (Fig. 32), with two major clades in *Amblyopsis* corresponding to samples north and south of Ohio River, respectively. These clades were reciprocally monophyletic for the mitochondrial *nd2* and nuclear *rho* loci. Although not reciprocally monophyletic and exhibiting little differentiation, the two groups did not share *s7* alleles. Little differentiation was observed at *rag1* and *tbr1*. No alleles were shared on both sides of the river for three loci. The presence of shared alleles for *rag1* and *tbr1* likely reflects retention of ancestral polymorphism, recent divergence, and low mutation rates for these loci. Swofford (1982) hypothesized that populations north and south of the Ohio River had recently diverged and that insufficient time had elapsed for significant genetic differentiation at allozyme loci.

Haplotype networks also revealed division of *Amblyopsis* into two groups corresponding to alleles found north and south of the Ohio River (Fig. 33). No haplotypes were shared between groups for *nd2*, *s7*, and *rho*, whereas little variation was found for *rag1* and *tbr1*. Overall, few haplotypes were observed for nuclear loci (maximum of four in *rag1*) (Table 17; Fig. 33). Populations north and south of the Ohio River were separated by three mutations at the *rho* locus and just one mutation at the *s7* locus. Twenty-seven mutations steps separated these groups of populations at the *nd2* locus. The network for the *nd2* locus failed to connect at the 95% confidence interval between haplotypes north and south of the Ohio River, so the connect limit was set at 30 to connect populations for visualization purposes only. An insertion shared by all individuals was found in *rho* that is absent in all other amblyopsids. Additionally, individuals south of the Ohio River all shared a mutation that resulted in a premature stop codon in the open reading frame.

Although larger rivers are thought to be significant barriers to dispersal for terrestrial subterranean fauna, it has been hypothesized that major rivers have little to no influence on the dispersal of many aquatic species, particularly smaller invertebrates (Barr & Holsinger 1985). However, fluvial barriers may be less permeable to larger aquatic fauna, such as crayfishes and cavefishes (Rhoades 1962), which cannot travel through small solution or alluvial channels. Our results for *Amblyopsis* are consistent with this latter hypothesis. Phylogenetic analyses (Figs. 32 and 33) strongly suggest that the river is a genetic barrier and has limited dispersal between populations north and south of the Ohio River. Genetic distances between populations are significantly lower on the same side of the river compared to distances between populations located on different sides of the river (Table 18), even after controlling for geographic distance. Uncorrected pairwise sequence divergence between populations on opposite sides of the Ohio River average 0.031 ± 0.005, whereas sequence divergence averaged 0.0012 ± 0.0004 and 0.0006 ± 0.0003 between populations located on the north and south side of the Ohio River, respectively (Table 18). The results from the partial Mantel test suggest that the Ohio River is a significant barrier to dispersal and restricts gene flow between populations of *A. spelaea* north and south of the river (Table 19). The results of the hierarchical AMOVA revealed that genetic structure is highly correlated with regions north and south of the Ohio River, as 96.7% of variation was partitioned among regions (Table 20). These data strongly indicate that the Ohio River is a significant barrier for cavefish.

The divergence dating analysis also supports the Ohio River as a significant isolating barrier. Estimation of the species tree and mean divergence times based on the multilocus dataset revealed a sister relationship between *Amblyopsis* and *Forbesicthys*, which diverged 4.96 mya (95% HPD: 2.64–7.17 mya) during the early Pliocene (Fig. 34). Diversification within these genera occurred during the Pleistocene. A middle Pleistocene date 0.53 mya (95% HPD: 0.12–1.06 mya) was inferred for the split between *A. spelaea* populations north and south of the Ohio River (Fig. 34). The 95% highest posterior density for the divergence date of this split includes the date of the formation of the modern course of the Ohio River ca. 0.8 mya. Prior to the river's formation, the distribution of *Amblyopsis* was likely continuous throughout the cave and karst-bearing geological formations of the Crawford-Mammoth Cave Uplands and Mitchell Plain, as ancient drainages (e.g., the Old Ohio River) were considerably smaller and not as deeply entrenched as the modern Ohio River is today. Once the modern course of the Ohio River was formed after the damming and overflow of headwater drainages of the Teays River (Teller & Goldthwait 1991), it began to cut into the soluble, cave-bearing limestone formations. Entrenchment of the modern Ohio River is attributed to episodic glacial meltwater discharge and probably occurred fairly rapidly during its early development (Teller & Goldthwait 1991). The modern Ohio River is now 75 m below the bottom of the Teays-aged valleys and has completely bisected the cave-bearing St. Genevieve and St. Louis limestones in parts of its course through the Crawford-Mammoth Cave Uplands and Mitchell Plain effectively isolating cave faunas on either side of the river. However, even if the cave-bearing strata has not been completely bisected, it is very likely that caverns or solution channel networks below the Ohio River were quickly filled by sediment or glacial outwash (Rhoades 1962; Poulson & White 1969), as water velocities continually decreased as the Ohio River became larger with less of a gradient.

Interestingly, we found little evidence for an influence of hydrological drainage or ecoregion boundaries on genetic structure in *Amblyopsis*. Results of partial Mantel tests revealed that hydrological boundaries and ecoregions do not significantly affect genetic structure in *A. spelaea* (Tables 19 and 20). The partial Mantel test for a partial correlation between genetic distance and hydrological subbasins showed a significant negative relationship. Populations that occur within the Blue-Sinking River subbasin, which spans both sides of the Ohio River, drove this negative relationship. When these populations were partitioned into groups north and south of the river, the correlation disappeared and was not significant. Although AMOVAs showed that genetic variation is significantly partitioned among hydrological basins as well as ecoregions, the percentage of variation explained by partitioning by hydrological basin (45.5%) or ecoregion (61.8%) was substantially less than that when partitioning by region north and south of the Ohio River (Table 20). Phylogeographic studies of other aquatic subterranean fauna often have documented structuring of genetic variation with hydrological drainage boundaries (Lefebure et al. 2006, 2007; Finston et al. 2007; Carlini et al. 2009), including *Typhlichthys* (Niemiller et al. 2012). Lack of genetic structure associated with hydrological drainages and ecoregions and little differentiation among populations suggests there is greater connectivity of cave systems in the subbasins and geological strata inhabited by *Amblyopsis* compared to other areas of the Interior Highlands where related amblyopsid cavefishes occur (Niemiller & Poulson 2010).

Our results also suggest that subterranean colonization and diversification in *Amblyopsis* was also driven by changing climate during the Pleistocene. Although determining the exact timing of subterranean colonization is especially difficult for most taxa, several lines of evidence indicate that divergence was facilitated by climatic changes during the Pleistocene. First, the geographic distribution of *Amblyopsis* in close proximity to the glacial maximum implicates a significant influence of Pleistocene climatic fluctuations. It is very unlikely that *Amblyopsis* could have persisted in surface refugia so close to the southern glacial extent due to the harsh periglacial conditions. The onset of glacial advances during the Pleistocene are thought to have occurred rapidly (Adams et al. 1999). Consequently, the ancestor to *A. spelaea* likely was already facultatively living underground but might not have yet become troglomorphic, perhaps much like members of the sister lineage to *Amblyopsis* today (*Forbesichthys agassizii* and *F. papilliferus*).

Phylogenetic, divergence dating, and demographic reconstruction analyses support the existence of two refugia during the middle to late Pleistocene located on opposite sides of the Ohio River. Tests of population expansion combining both groups did not show a significant signature of expansion. In addition, levels of genetic diversity (Table 21) were greater north of the Ohio River than to the south, in contrast to the expectation if there was a single southern refugia and then colonization northward. Levels of genetic diversity were low for both lineages and suggest that both groups suffered through significant reductions in population size. Recovery from this event has been slow, as illustrated by tests of population expansion and Bayesian GMRF skyride plots (Table 21 and Fig. 35), which show evidence of population expansion north of the Ohio River but not in the southern group. An alternative explanation for reduced genetic variation is a selective sweep; however, we observed low levels of genetic variation across multiple unlinked loci.

Patterns of molecular evolution in the eye photoreceptor gene *rhodopsin* also provide insight into the evolutionary history of *Amblyopsis*. Protein-coding genes are predicted to evolve like pseudogenes once freed of selective constraint, accumulating higher rates of nonsynonymous mutations compared to synonymous mutations as well as loss-of-function mutations (Yokoyama et al. 1995). Niemiller et al. (2013a) demonstrated that selection is relaxed in subterranean lineages in amblyopsids including *Amblyopsis*. We found that all individuals sampled possess an amino acid insertion not found in *rhodopsin* gene copies in surface taxa. In addition, all individuals sampled south of the Ohio River possess a mutation that results in a premature stop codon not found in individuals north of the river. The presence of different fixed mutations, including loss-of-function mutations, between populations north and south of the river also indicates that they were isolated in separate refugia throughout the middle to late Pleistocene. The accumulation of nonsynonymous mutations in *Amblyopsis* is low compared to some other subterranean lineages (Niemiller et al. 2013a) and also suggests that subterranean colonization has been relatively recent.

Based on our results, we propose the following hypothesis to explain the evolutionary history of *Amblyopsis* throughout the Pleistocene. Prior to the Pleistocene, the surface ancestor had already migrated into karst regions of the Crawford-Mammoth Cave Uplands and Mitchell Plain inhabiting cool springs, spring runs, and streams, and may also have been present further north than the present-day distribution of *Amblyopsis*. It is also likely that this ancestor was facultatively utilizing caves prior to the Pleistocene. Dramatic climatic shifts and inhospitable surface conditions in the early Pleistocene facilitated further subterranean colonization and extinction of surface populations; however, gene flow between populations continued via subterranean corridors until the overflow of Lake Tight and formation of the modern course of the Ohio River ca. 0.8 Mya, which effectively isolated populations to the north and south of this barrier as the river cut through the cave-bearing geological formations. During glacial advances, populations were isolated in refugia and subsequently expanded in geographic extent during warmer interglacial periods.

All species delimitation approaches recognized populations north and south of the Ohio River into distinct lineages. O'Meara's (2010) nonparametric heuristic approach delimited two species within *A. spelaea* with all samples north of the Ohio River in Indiana as a species separate from samples south of the river (Fig. 36). The STRUCTURE analysis also returned K = 2 as the optimal vale for K, and resulted in complete separation into two groups (Fig. 37): a group corresponding to individuals from north of the Ohio River and a group from south of the Ohio River. Bayesian species delimitation supported the guide tree when assuming four species (two *Amblyopsis* plus *F. agassizii* and *F. papilliferus*) with speciation probabilities of 1.0 on all nodes (Fig. 36) and this was the lone species delimitation model supported (P = 1.0). Values of *gsi* and *egsi* indicate a high degree of exclusive ancestry in *A. spelaea* north and south of the Ohio River (Table 22). *Gsi* values were greater than 0.9 for both groups for all loci except *rag1* and *tbr1*, which exhibited shared ancestry of alleles and low levels of variation compared to other loci (Figs. 32 and 33). *Egsi* values were also significant for both groups with moderately high values of exclusive ancestry, reflecting lack of differentiation at the *rag1* and *tbr1* loci. These results also confirm that the Ohio River is a major barrier to dispersal separating *Amblyopsis* into two distinct groups.

It is important to note that the southern clade may be further isolated by the Hart County Ridge (also known as the Rough Creek Fault Zone) that runs east-west from Hart County to Webseter and Union counties in Kentucky. The fault has resulted in up to 800 m of uplift at several locations. *Amblyopsis* have been reported both north and south of this potential barrier to dispersal but occurrences are lacking in the vicinity of the fault in Grayson County and western Hart County despite some field surveys (McCandless

2005). This potential barrier to dispersal for aquatic subterranean fauna is supported by the biogeographic patterns of subterranean crayfishes: *Orconectes inermis* inhabits cave systems to the north of the Hart County Ridge, whereas *O. pellucidus* inhabits caves to the south. Regrettably, we did not acquire samples for this study from the Mammoth Cave area to the south of the Hart County Ridge to test if this geological formation also is a significant barrier to dispersal in *Amblyopsis*. If we find that this is the case, *A. spelaea* would be comprised of three distinct lineages.

We identified two distinct phylogenetic lineages in *A. spelaea* that are separated by a major barrier to gene flow (i.e., the Ohio River) and are on independent evolutionary trajectories, in contrast to previous genetic investigations (Swofford 1982). These lineages are reciprocally monophyletic at three loci (*nd2*, *s7*, and *rho*) and could be recognized as distinct species under the genealogical species concept (Baum & Shaw 1995) and metapopulation lineage species concept (de Queiroz 1998, 2007). Both lineages also would be recognized under the biological species concept (Mayr 1942), where both groups have not experienced recent gene flow and incongruence among loci (i.e., *rag1* and *tbr1*) is the result of lineage sorting only. Some morphological data support recognition of two lineages in *Amblyopsis* (Poulson 1960), including differences in eye size and pigmentation. Based on these lines of evidence, we believe that populations north of the Ohio River should be recognized as an entity distinct from populations south of the river, which would retain the name *A. spelaea*. However, we refrain from formally describing a new species for the northern lineage until a further examination of morphological differentiation is conducted to help elucidate the taxonomic status of these groups.

The identification of cryptic diversity in *Amblyopsis* has immediate conservation implications. *Amblyopsis spelaea* is listed as ‘Vulnerable’ by IUCN (Gimenez Dixon 1996), as regional species of concern by the U.S. Fish & Wildlife Service, and as ‘Endangered’ and ‘Special Concern’ in Indiana and Kentucky, respectively (Niemiller & Poulson 2010). It has been reported from 76 localities in Indiana and 40 localities in Kentucky; however, relatively few localities are known to support large populations (>50 individuals). Several populations, particularly those south of the Ohio River, have been significantly impacted by habitat alteration and degradation, groundwater pollution, disease, and over-collection (Niemiller & Poulson 2010). The conservation status of *A. spelaea* in both Kentucky and Indiana may need to be elevated in the future, given evidence of low census population sizes (Pearson & Boston 1995), low genetic diversity, and ever increasing anthropogenic threats.

***Spring Cavefish* (*Forbesichthys agassizii*)**

The Spring Cavefish was described as *Chologaster agassizi* by Putnam (1872) from a well near Lebanon in Wilson County, Tennessee. Later, Forbes (1882) described *C. papilliferus* from a spring in western Union Co., Illinois, on the basis of coloration differences between the Tennessee and Illinois populations. Jordan and Evermann

(1927) erected a new genus, *Forbesella*, citing that the subterranean nature of spring cavefish warrants separate recognition from the surface-dwelling *Chologaster cornuta* found in the Atlantic Coastal Plain. Jordan (1929) later replaced *Forbesella* with *Forbesichthys*, as the former was preoccupied in tunicates. *Forbesichthys* is still considered a junior synonym of *Chologaster* by some authors, however. Woods and Inger (1957) noted that the slight differences among populations of Spring Cavefish from southern Illinois, central Kentucky, and central Tennessee did not warrant specific or subspecific designation. Therefore, the authors synonymized *C. papilliferus* with *C. agassizi*, a revision that has been followed by most subsequent authors. Exceptionally, Clay (1975) suggested that *C. agassizi* and *C. papilliferus* are specifically distinct. More recently, allozyme analyses by Swofford (1982) revealed considerable differentiation between populations and also justified resurrection of the genus *Forbesichthys* by Page and Burr (1991).

Spring Cavefish have not been the subject of phylogenetic studies since Swofford (1982). Swofford found that Spring Cavefish show slightly less allozyme differentiation than Swampfish (*C. cornuta*) with an average heterozygosity of 0.028 compared to 0.040. Swofford's data imply that Spring Cavefish utilize surface corridors for dispersal, at least in central Kentucky and the Eastern Highland Rim in Tennessee. We sought to begin a preliminary genetic investigation of populations throughout the range of *F. agassizii*, including populations in Kentucky.

Estimation of gene genealogies from 70 individuals of nine populations sampled across the distribution of *Forbesichthys* resulted in nearly identical topologies for the five loci examined (Fig. 32), with two major clades in *Forbesichthys* corresponding to populations sampled from the Eastern Highland Rim in central Tennessee and populations in south-central Kentucky and southern Illinois. These clades were reciprocally monophyletic at all loci. Divergence dating analysis show that this lineages diverged 1.6 Mya (95% HPD: 0.8–2.4) during the early to middle Pleistocene based on the multilocus dataset (Fig. 34). Within clades, little differentiation was discovered even at the mitochondrial *nd2* locus. Within the two Kentucky populations sampled (Rich Pond in Warren County and Morton Road in Todd County) three mitochondrial haplotypes were shared between these two populations from the 26 combined samples (n=15 at Rich Pond and n=11 at Morton Road).

We conducted several species delimitation approaches (see above) and all recognized populations in Illinois and Kentucky distinct from populations in the Eastern Highland Rim of central Tennessee. O'Meara's (2010) nonparametric heuristic approach delimited two species within *Forbesichthys* (Fig. 36) while the STRUCTURE analysis also returned *K* = 2 as the optimal vale for *K*, and resulted in complete separation into two groups: a group corresponding to individuals from the Eastern Highland Rim of Tennesee and another group consisting of populations in the Western Pennyroyal Karst of Kentucky and the Shawnee Hills area of southern Illinois. Bayesian species delimitation supported the guide tree when assuming four species (two *Amblyopsis* plus two *Forbesichthys*) with speciation probabilities of 1.0 on all nodes (Fig. 36) and this was the lone species delimitation model supported (P = 1.0). Values of *gsi* and *egsi* indicate a high degree of exclusive ancestry in *Forbesichthys* (Table 22). *Gsi* values were all 1.0 for both groups for all loci. *Egsi* values were also significant for both groups with high values of exclusive ancestry. These results also confirm that these two lineages in *Forbesichthys* are genetically distinct.

We identified two distinct phylogenetic lineages in *Forbesichthys* that are on separate evolutionary trajectories. These lineages are reciprocally monophyletic at all loci examined and could be recognized as distinct species under several species concepts, including the genealogical species concept (Baum & Shaw 1995) and metapopulation lineage species concept (de Queiroz 1998, 2007). Both lineages also would be recognized under the biological species concept (Mayr 1942), where both groups have not experienced recent gene flow. Some morphological data also support recognition of two lineages (Forbes 1882). Based on this evidence, we advocate resurrection of *F. papilliferus* for populations in southern Illinois and south-central Kentucky. Tennessee populations on the Eastern Highland Rim represent *F. agassizii*.

Population Size and Relative Abundance

Few studies have attempted to quantify population sizes and relative abundance of amblyopsids, including Spring Cavefish, Southern Cavefish and Northern Cavefish. The few studies that have attempted to quantify population sizes via techniques such as mark-recapture or survey removal have focused on caves that are known to contain relatively large populations. Other studies for which the most reliable estimates of abundance have been obtained have focused on the species of conservation concern. Additional demographic studies, including long-term censuses, are needed for both epigean and subterranean populations.

Northern Spring Cavefish. Historically, Northern Spring Cavefish has been considered rare to uncommon throughout much of its range. In Kentucky, *F. papilliferus* has been widely reported but most localities yield fewer than ten fish during a single survey (Fig. 38). To our knowledge, Northern Spring Cavefish have only been observed in excess of 25 fish at two localities: a ditch off of Morton Road in Todd County and Rich Pond in Warren County. Smith and Welch (1978) estimated less than a thousand individuals from eight springs in Union County, Illinois. We did not observe Northern Spring Cavefish during any cave surveys. However, Northern Spring Cavefish have been reported from a few caves in the Western Pennyroyal Karst of Kentucky. Estimates of population densities in caves range from as many as 8.0 fish per m^2 in spring runs in the Pine Hills of Illinois (Weise 1957) to 0–0.01 fish per m^2 in Mammoth Cave (Poulson 1969). Most surveys yield just a few fish; however, this likely is an artifact of habitats sampled, as many ichthyological surveys focus on streams and other larger bodies of water rather than spring runs and springs. Moreover, most springs are located on private property and the vast majority have been poorly sampled. Because Northern

Spring Cavefish return and persist in spring heads and underground waters when their surface habitats dry in late summer and autumn, the best chance of detecting this species occurs when water levels are high in late winter and early spring.

We found only a single new locality, a spring-fed ditch off of Morton Road in Todd County (Table 1). This stream has been channelized for irrigation and averages ca. 2 m wide. It is full of aquatic vegetation, which provide ample cover for Northern Spring Cavefish. During our first visit on 31 Mar 2010, we captured 77 fish in the 30 m stretch upstream of the road crossing and we estimated a population density of 12,833 fish per hectare at this locality. However, the number of fish dramatically decreased in subsequent weeks as water levels began to decrease and fish presumably moved upstream. By mid-June in both 2010 and 2011, we were unable to capture a single Northern Spring Cavefish at this site. A histogram of body size shows that most individuals captured are young first-year fish in May (Fig. 39) with fewer older and reproductive. This pattern is not surprising considering the short life span of *Forbesichthys* (3–4 years) and timing of sampling occuring a couple of months after reproduction.

A similar phenomenon was observed at Rich Pond in Warren County. We surveyed a 50 m section of stream upstream of the road crossing on several occassions throughout the year. The stream at Rich Pond issues from a sevies of small springs then flows for a few hundred meters through an agricultural field before issuing into a large depression in an agricultural field (Fig. 18). In the spring during high water levels, the water from the stream isssues into this depression forming a large pond (up to 340 acres in size). However, as the season progresses, water levels drop and flow is usually reduced to a small stream that eventually goes completely dry by July or August. During our surveys, we observed as few as zero and as many of 203 Northern Spring Cavefish in this 50 m section amidst flooded vegetation. We estimated a population density up to 27,067 fish per hectare in the spring but dropping to 0 fish per hectare in the autumn when fish move underground and the stream dries. A histogram of body size also shows that most individuals captured are young first-year fish (Fig. 40) with fewer indviduals in second and 3–4 year size cohorts. Again, this pattern is not surprising considering the short life span of the species and timing of sampling.

Southern Cavefish. Fifty-six percent of reported *T. subterraneus* localities yield fewer than ten cavefish during a single survey (Fig. 41). Only Hawkins River in Mammoth Cave, Hidden River Cave in Hart County, and L & N Railroad Cave in Barren County have historically produced in excess of 25 fish during a single survey. We discuss the significant populations of *Typhlichthys* in Kentucky below.

Although Southern Cavefish have been found in many portions of the Mammoth Cave system, the vast majority of cavefish observed are from the Proctor Cave section of the system, and more specifically Hawkins River. Pearson and Boston (1995) observed up to 104 *Typhlichthys* during several surveys in 1993 and 1994. We visited the Logsdon River section and observed 19 cavefish in 2010 even though water levels were slightly elevated from recent rainfall. Southern Cavefish are routinely observed in this section of Mammoth Cave (Rick Toomey, personal communication). Pearson and Jones (1998) reported up to 5 Southern Cavefish in Echo River, 7 cavefish in Roaring River, 10 cavefish in the Eyeless Fish Trail, 1 cavefish in the Golden Triangle area, 2 cavefish in Mystic River, and 103 cavefish in Hawkins River in 1995.

Pearson and Boston (1995) observed up to 45 cavefish during several surveys of L & N Railroad Cave in 1993 and 1994. Pearson and Jones (1998) observed 13 cavefish in August 1995. We visited the cave on five occasions and observed 6–33 cavefish, respectively, in a ca. 300 m section of the stream. This population is unusual in that cavefish are found in the cave stream with considerable flow, often underneath rocks in the middle of the channel or under undercut ledges around bends and meanders. During two surveys, water levels were elevated with low visibility and we observed few cavefish. We estimate a population density of 450 cavefish per hectare in this section of stream.

Hidden River Cave in Hart County was significant impacted by groundwater pollution from domestic sewage, industrial waste, and chemical spills during the 1980s, which decimated subterranean organisms living in the groundwater associated with the cave system. However, by the mid-1990s populations of organisms were increasing, including Southern Cavefish. Pearson and Boston (1995) reported up to 29 cavefish during several surveys in 1993 and 1994. Pearson and Jones (1998) observed 19 cavefish in 1995. We were unable to make arrangements to survey the cave during this study.

Historically, Southern Cavefish have only been reported from Sloans Valley Cave in Pulaski Co, Kentucky. Cooper and Beiter (1972) collected four and five individuals from the Minton Hollow section of the cave during two trips. We searched this section of the cave on five occasions and failed to find a single cavefish. However, we discovered a new significant population of *Typhlichthys* nearby at Drowned Rat Cave. We searched ca. 400 m of steram passage on six occasions and observed 0–31 cavefish, including young-of-the-year fish. We estimate a population density of 258 cavefish per hectare in this section of stream. New populations were also discovered at Dave's Cave (part of the Coral Cave system) and Well's Cave but fewer than ten cavefish were observed during a single survey of these localities.

***Northern Cavefish*.** Like *T. subterraneus*, the majority of localities for *A. spelaea* yield few cavefish, as ten or fewer cavefish have been observed from 66% of localities in Kentucky (Fig. 42). The largest populations exist in Breckinridge County, including Webster's Cave, Penitentiary Cave, Amblyopsis Cave, and Under the Road Cave where over 100 individuals have been observed during a single survey. This area and the Mammoth Cave system have been identified as population centers for *Amblyopsis* in Kentucky (Pearson & Boston 1995). Our surveys focused primarily on the northern population center in Breckinridge County where we observed significant numbers in several caves, including Under the Road Cave, which may have experienced a population decline (Pearson & Boston 1995). We discuss the significant populations of *Amblyopsis* in Kentucky below.

Webster's Cave in Breckinridge County also supports a large population of *Amblyopsis*. Louis (1999) estimated a population size of 211 ± 37 individuals in a 2530 m section of stream passage at Webster's Cave using mark-recapture with visual implant elastomers. However, this estimate likely is conservative given that Pearson and Boston (1995) observed 162 individuals during a single survey and estimated a population size of 456 cavefish. We observed as many as 41 individuals during our surveys of the first 1200 m of this passage. Based on Pearson and Boston's (1995) data, we estimate a population density of 64 cavefish per hectare in the surveyed portion of Webster's Cave, but it should be noted that cavefish have been observed all throughout the cave system, including areas not subject to survey (Chris Anderson, personal communication).

Although we did not survey Penitentiary Cave in Breckinridge County, it contains the largest *Amblyopsis* population known to date. Pearson and Boston (1995) observed 515 cavefish during a single survey of 1234 m of stream passage. Niemiller and Poulson (2010) estimated a population density of 2643 cavefish per hectare based on their data.

Pearson and Jones (pers. comm. in McCandless 2005) observed 417 Northern Cavefish in Amblyopsis Cave in 1995, which is upstream of Blissit's Cave, Rimstone Cave, and Under the Road Cave in Breckinridge County. Only 11 individuals were observed at this cave by Pearson and Boston (1995), illustrating the stochastic nature of cave surveys. We could not gain permission to access this cave.

Under the Road Cave in Breckinridge County historically supported a large population of *Amblyopsis spelaea*. Tom Poulson (personal communication) observed as many as 111 individuals during his surveys in the late 1950s and early 1960s, with the majority of individuals found in a side passage that contains a tributary to the main cave stream. Pearson and Boston (1995) did not observe a single invidiual during their surveys. They attributed the lack of cavefish to (1) potential population decline or (2) poor hydrological conditions (i.e., high flow and low visibility). During our first two surveys, we only observed a single cavefish in the main stream passage; however, water conditions were also marginal with low visibility and high water. However, we observed up to 45 individuals, including many first-year cavefish, during our last five visits during ideal survey conditions (except the January 2011 survey). Almost all of these individuals were found in a side tributary to the main passage that we presume is the same passage Poulson had mentioned. William Pearson (personal communication) also has observed cavefish in this passage in subsequent surveys conducted after 1994, confirming that the population likely has not declined. McCandless (2005) observed 82 individuals during a survey in 2002. All but seven individuals were observed in this side passage. Niemiller and Poulson (2010) estimated an incredible population density of 4199 cavefish per hectare in this side passage based on Poulson's survey data.

Rimstone Cave is part of the Blissit's Cave system. Pearson and Boston (1995) surveyed 812 m of stream passage and observed only eight *Amblyopsis*. They estimated a population size of only 15 cavefish. McCandless (2005) observed 17 individuals in 2003. We surveyed this same stretch of stream passage twice, observing up to 26 individuals, including first-year fish. Following Pearson and Boston's (1995) methodology to estimate population size, we estimate at least 49 cavefish in this stretch of stream passage at Rimstone Cave and a population density of 107 cavefish per hectare.

Although we did not actively survey the base level streams because of poor visibility during scheduled trips in Mammoth Cave, *Amblyopsis* are still observed regularly (Rick Toomey, pers. comm.). We observed a single individual in the Hawkins River section (Proctor Cave). Based on Pearson and Boston's (1995) survey results and estimates, the largest population occurs in Roaring River where they estimated 316 cavefish. Other areas of the Mammoth Cave system have significantly less cavefish with estimates ranging from 6 to 34 individuals. McCandless (2005) reported 34 *Amblyopsis* in Mystic River in 2003, but noted that surveys were conducted in areas not surveyed by Pearson and Boston (1995) who observed a maximum of 11 individuals during their surveys. Pearson and Jones (1998) observed 14 Northern Cavefish in Echo/Styx River, 41 cavefish in Roaring River, 3 cavefish in the Eyeless Fish Trail, 8 cavefish in the Golden Triangle area, 8 cavefish in Mystic River, and 4 cavefish in Hawkins River in 1995. *Amblyopsis* may never have been in high abundance in Mammoth Cave (see Pearson & Boston 1995); however, the lack of large museum collections from the system and low numbers of individuals sighted during surveys might also reflect the difficulty associated with deep-water subterranean habitats that are subject to low visibility because of siltation.

Conclusions on abundance in cave species. Although these results might be a reflection of actual abundance in some instances, the distribution and abundance of troglobitic species likely is greater than currently realized. Localities for which Southern Cavefish and Northern Cavefish have been reported likely represent but a fraction of total available habitat accessible to cavefish. This was clearly illustrated during a fertilizer pipeline break within the recharge zone of Maramec Spring that resulted in the death of at least 1,000 Southern Cavefish and likely many more (see discussion in Noltie & Wicks 2001). This unfortunate kill is informative because the drainage basin had few records documented previously. The problem with inferring population densities from such fish kills is that we do not know the volume or extent of habitat impacted.

Most observations of Southern Cavefish and Northern Cavefish are restricted to caves near the surface and there is some controversy as to whether even the best cavefish caves are sources or sinks (Niemiller & Poulson 2010). Habitats where few or no cavefish are observed likely represent population sinks and not sources. Examples include Friendship Cave in Warren County for *T. subterraneus*, Wells Cave for *Typhlichthys sp. nov.* 1, and Bandy Cave in Breckinridge County for *A. spelaea*. Wells and short stream segments encountered in an otherwise dry cave may not be representative of the habitat that most cavefish inhabit. Cavefish can disperse through and occupy submerged passages inaccessible to humans but these habitats are probably neither usual for the fish nor optimal. These habitats likely act as corridors for dispersal. Given their longevity, low metabolic rates, and foraging efficiency, cavefish likely can move relatively long distances. Regardless, size distributions of *Typhlichthys* and *Amblyopsis* at significant populations (Figs. 43–45) show unimodal distributions of body size (standard length), which is indicative of stable populations.

Ecology, Life History, and Behavior

Reproduction

Troglobitic species in many systems are subject to pronounced seasonality in food availability and water levels (flooding). Accordingly, cave amblyopsids have peaks in reproduction just after spring floods (Poulson & Smith 1969; reviewed in Niemiller & Poulson 2010). Synchronization of reproduction with spring flooding is adaptive because offspring survival is maximized. Young are produced shortly after spring floods when food availability is still high, yet mortality due to extreme flow is reduced (Poulson & Smith 1969). However, timing of these cues is unpredictable and may occur from late fall into spring. Rises in water level and alkalinity, coupled with subtle drops in water temperature, may be triggers to reproduction and synchronization of circannian rhythms of reproductive readiness (Poulson 1963; Jegla & Poulson 1970).

Breeding in Southern Cavefish likely occurs during spring, associated with higher water levels from later winter and early spring rains. However, data is lacking regarding reproduction in this species despite its broad distribution. Hatchlings have never been observed or collected. Poulson (1960) observed 15–20 mm TL fish in Shelta Cave in Madison Co., Alabama. We captured two and observed six other Southern Cavefish in the 15–25 mm TL size class at Jacques Cave in Putnam County, Tennessee, in October 2007. Likewise, we have observed similar-sized fish in small tributaries to the main stream in Big Mouth Cave, Grundy County, Tennessee, during several trips in autumn and winter. These individuals likely represent first-year fish and are consistent with reproduction occurring in spring. We did not observed very small fish that likely represent first-year fish at L & N Railroad Cave in Barren County, Kentucky, or at Drowned Rat Cave in Pulaski County, Kentucky. However, we did observed several smaller individuals 25–35 mm TL at both of these localities, which we believe represent juveniles. In the Hawkins River area of Mammoth Cave, we did observe but did not capture two Southern Cavefish <25 mm TL. More data are needed to better understand the reproductive biology of this species.

Breeding in Northern Cavefish occurs during high water conditions from February through April. This is estimated from data on the seasonal occurrence of gravid females, females incubating eggs in various stages of development, and observations of newly hatched and young-of-the-year fish (Eigenmann 1899; Poulson & Smith 1969; Niemiller

& Poulson 2010). Females are known to brood eggs in their gill cavities until hatching and retain young until yolk reserves are depleted. Fry begin to appear in late summer and early autumn (Poulson 1960; Niemiller & Poulson 2010). Brooding females have been observed at both Under the Road Cave (Niemiller & Poulson 2010) and Webster's Cave (Bill Pearson, pers. comm.) in Breckinidge County, Kentucky. Although we observed presumed females in the vicinity of young at both Webster's Cave and Under the Road Cave, we did not observe branchial brooding during the current study at these and any other *A. spelaea* localities.

The timing of reproduction in Northern Spring Cavefish is fairly clear. In the Illinois populations studied by Weise (1957) and Smith and Welch (1978), most adults presumably spawn underground during late winter. This assumption is based on the nearly complete disappearance of adults from springs during this season. Populations of Northern Spring Cavefish in Kentucky apparently also breed underground; although some populations might breed and oviposit in springs or in other surface habitats. Ova begin to enlarge in autumn reaching mature size in January when adults move underground (Weise 1957; Poulson 1963). Subterranean spawning is believed to occur from January through April and peaks in February when water levels typically are at their maximum. Fry appear and adults return to the surface by April (Niemiller & Poulson 2010).

Cave amblyopsids, including Southern Cavefish and Northern Cavefish, have fewer, larger, and potentially more nutrient-rich eggs than their surface counterparts (Poulson 1985). Compared to smaller eggs, larger eggs contain more yolk and produce larger larvae with greater starvation tolerance, greater swimming ability when foraging and when avoiding predation, and can accommodate a wider range of prey sizes when all yolk reserves are depleted. All of these are adaptations that increase survival in a food-poor environment (Niemiller & Poulson 2010).

Egg diameter in Southern Cavefish and Northern Cavefish averages 2.3 mm (Niemiller & Poulson 2010). Clutch size is reportedly low for Southern Cavefish, perhaps fewer than 50 eggs. In Northern Cavefish, clutch sizes average 65 eggs. Ovarian eggs of Northern Spring Cavefish range from 1.5–2.0 mm in diameter (Niemiller & Poulson 2010) and clutch sizes average about 100 eggs. Hatchling fry after yolk sac absorption are about 6.0 mm long (Hill 1966).

The jugular position of the urogenital pore in all amblyopsids is circumstantial evidence for branchial brooding of eggs. Some have speculated that branchial brooding reduces egg predation in cave habitats (Noltie & Wicks 2001). Indeed, this has been observed in the Northern Cavefish. However, Niemiller and Poulson (2010) argue against branchial brooding in most amblyopsid species, citing that, except for the Northern Cavefish, total egg volume exceeds branchial volume.

Growth and Development

Generally, cave organisms exhibit reduced growth rates and delayed development and maturity compared to related surface species. Reduced growth rates represent an adaptive response to low food supplies in cave environments because less energy over a given amount of time is needed (Hüppop 2000). Within the Amblyopsidae, growth and developmental rates decrease with increasing cave adaptation (Poulson 1963). First-year Northern Spring Cavefish grow 10–20 mm SL per year on average and also exhibit variable growth rates from season to season (Smith & Welch 1978; Hill 1966). Hill (1971) studied squamation and pigmentation development in Spring Cavefish. Scale primordia first appear on the caudal peduncle at around six weeks. By 12 weeks, both squamation and pigmentation pattern are well developed. Vent migration is characteristic of the Amblyopsidae and Aphredoderidae (Poulson 1963). Hill (1966) found that vent migration to the jugular position was complete in 16–18 mm SL Northern Spring Cavefish. Although we did not individually mark Northern Spring Cavefish at Morton Road in Todd County or Rich Pond in Warren County, body size data throughout the spring when fish are abundant on the surface indicate that Northern Spring Cavefish grow rapidly with first-year fish reaching 20 mm SL by late June or early July at Rich Pond (Fig. 40). Vent migration in Northern Cavefish takes longer, as the vent is only just posterior to the pectoral fins by 35 mm SL (Eigenmann 1909).

Growth rates for troglobitic amblyopsids are substantially slower with estimates of 1.0–1.25 mm month^{-1} for Southern Cavefish and 1.0 mm month^{-1} for Northern Cavefish. Sexual maturity is also delayed in cave amblyopsids. Northern Spring Cavefish reach sexual maturity around 12 months of age and may reproduce in their second year, whereas Southern Cavefish may take four years or longer and Northern Cavefish 10 years or longer to reach sexual maturity (Niemiller & Poulson 2010).

Longevity

Increased longevity of cave organisms compared to their surface relatives is one of several life history adaptations toward a low *r* strategy by which cave organisms cope with limited food resources. Prolonged life spans, coupled with a trend from semelparity to iteroparity, increases the chance of population persistence over time, as a population is less likely to be extirpated during times of extremely low food supplies that result in little to no recruitment (Hüppop 2000). This pattern is evident in the Amblyopsidae as longevity inferred from scale marks increases with increased adaptation to cave environments (Poulson 1963).

Northern Spring Cavefish are known to live up to three years (Hill 1966; Smith & Welch 1978). It is likely that, as in many short-lived species, death occurs after a single reproductive attempt (semelparity) in this species. Therefore, older individuals are those that simply did not acquire enough resources to reproduce at a younger age. Size data collected during this study is consistent with a 3–4 year lifespan (Figs. 39 and 40) and is particularly apparent at Rich Pond (Fig. 40). Troglobitic species live considerably longer.

Conservatively based on scale marks, Southern Cavefish were estimated to live 3–4 years and Northern Cavefish 5–7 years (Poulson 1963); however, individuals have been maintained in captivity for over a decade and likely live considerably longer than initial estimates in nature (Noltie & Wicks 2001). Poulson (2001) later questioned his original longevity estimates of the troglobitic species stating they may be off by a factor of 3–4, partly because of the difficulty in determining scale annuli in larger individuals, but primarily because of observed growth rates of marked individuals in nature (see above Growth Rates). Accordingly, Southern Cavefish may live 16–24 years or longer and Northern Cavefish 24–28 years (Niemiller & Poulson 2010). Interestingly, Pearson and Boston (1995) conducted mark-recapture studies at Webster Cave for Northern Cavefish and at L & N Railroad Cave for Southern Cavefish (Bill Pearson, pers. comm.), yet we did not capture a single individual with an elastomer tag. While this might reflect tag loss over the past 15 years, it could also reflect shorter life spans that predicted by Poulson. Further study is warranted to better assess growth rates and life span.

Diet

All amblyopsids eat live, moving prey, with invertebrates comprising most of the diet. However, cannibalism has been documented and small amounts of nonliving food, such as bat guano and detritus have been observed in the stomach contents of some species. Indeed, three Southern Cavefish in a Tennessee cave located in a pool beneath a Gray Bat (*Myotis grisescens*) roost had bat guano visible in their guts (M.L. Niemiller, unpublished data). These are probably ingested along with live prey and would provide much lower nutritional benefit per volume than live prey.

The diet of Northern Spring Cavefish varies geographically and between cave and surface populations. Illinois populations feed almost exclusively on *Gammarus* amphipods (Forbes & Richardson 1908; Layne & Thompson 1952; Gunning & Lewis 1955; Weise 1957), although amphipods (Weise 1957), insect remnants (Gunning & Lewis 1955), and detritus (Gunning & Lewis 1955) also have been found in stomachs. In Kentucky, Northern Spring Cavefish living in surface habitats feed primarily on chironomids, but also copepods, oligochaetes, nematodes, and ostracods (Hill 1969). Small worms and chironomids were regurgitated by captured Northern Spring Cavefish during this study. On the contrary, individuals of the same population at Rich Pond are strongly cannibalistic on younger individuals when underground in the cave spring during the dry season. In caves, cannibalism may represent an alternative feeding strategy in response to competition for more typical but extremely rare invertebrate food sources.

The diet of Southern Cavefish consists largely of copepods and isopods, but rare larger food items, such as young crayfish, salamander larvae, or conspecifics may result in high growth efficiency and a burst in growth rate (Poulson 2001). A variety of prey have been reported in stomach contents of *T. subterraneus*, including copepods, amphipods, isopods, trichopteran and tendepedid larvae, cladocerans, isopods, and crayfish

(Poulson 1960, 1963; Cooper & Beiter 1972); however, copepods are the primary food source accounting for 60–90% of the diet by volume (Poulson 1963).

Smaller Northern Cavefish primarily eat copepods but larger individuals consume a greater proportion of other crustaceans, including amphipods, isopods and small crayfish (Poulson 1963; Niemiller & Poulson 2010). Both amphipods and isopods have been confirmed in the gut contents of Northern Cavefish (J. Ballowe, pers. comm. in McCandless 2005). Like Northern Spring Cavefish, *A. spelaea* also are known to cannibalize on smaller individuals. A 78 mm SL individual at Under the Road Cave regurgitated a small cave crayfish (*Orconectes inermis inermis*).

Predators

Southern Cavefish and Northern Cavefish are at the top of the food chain in most cave systems that they inhabit, and, therefore, have few natural predators. Epigean fishes potentially prey on both species, as well as larger surface and troglobitic crayfish; however, direct evidence is lacking. A large population of Banded Sculpin (*Cottus carolinae*) exists in the main stream of Under the Road Cave in Breckinridge County. Northern Cavefish densities are very low in the main cave stream but are much greater in side tributaries. McCandless (2005) also spectulated that Banded Sculpin may be significant predators of Northern Cavefish based on observations of relative abundance of the two species at Lamon's Ladder Cave in Crawford Co., Indiana. Additionally, ictalurids might be predators of both Southern and Northern Cavefish. Yellow Bullhead were observed in several caves with cavefish populations, including Under the Road Cave, Webster's Cave, and Rimstone Cave in Breckinridge County, and Dyke's Bridge Cave, Well's Cave, and Sloans Valley Cave in Pulaski County. Young Southern Cavefish and Northern Cavefish likely are susceptible to cannibalism by larger adults (Poulson 1963; Niemiller & Poulson 2010). Cannibalism may serve as one means to regulate population densities in a food-limited environment (Poulson 1969).

Cannibalism has been reported in at least one cave population of the Northern Spring Cavefish at Rich Pond in Warren County. Surface populations of Northern Spring Cavefish likely are occasional prey for other surface fish, snakes, birds, and mammals (Smith & Welch 1978). At Rich Pond in Kentucky, natural predation is seasonally heavy (J.E. Cooper in Smith & Welch 1978) but is primarily from terrestrial predators. However, at the newly discovered locality in Todd County, predation from aquatic predators likely is very significant. Several potential predators were captured along with Northern Spring Cavefish, including Redfin Pickerel (*Esox americanus*), Bluegill (*Lepomis macrochirus*), Snapping Turtles (*Chelydra serpentina*), predaceous diving beetles, water scorpions, and dragonfly nymphs.

Parasites

Like most obligate cave-dwelling vertebrates, few parasites have been reported afflicting Southern Cavefish, Northern Cavefish, and their surface relative, the Northern Spring Cavefish. Proteocephalan cestodes have been collected from the pyloric caeca of Northern Spring Cavefish and Northern Cavefish. Whittaker & Hill (1968) described *Proteocephalus chologasteri* from the Northern Spring Cavefish. Three *Proteocephalus poulsoni* were collected from two Northern Cavefish at Under the Road Cave in Breckinridge County (Whittaker & Zober 1978). In southern Illinois, 71 percent of Northern Spring Cavefish examined were parasitized by cestodes and other internal parasites (G. Garoian in Smith & Welch 1978). The acanthocephalan *Neoechinorhynchus cylindratus* has been reported from the intestines of *A. spelaea* (Nickol & Whittaker 1978).

A species of copepod, *Cauloxenus stygius*, is an obligate ectoparasite found on the upper lip of Northern Cavefish but little is known about this parasite. Few records exist (Cope 1872; Blatchley 1897; Giovannoli 1933; Lewis 2002a,b; Niemiller & Poulson 2010) but the distbribution of these species is believed to be that of its host. Small, unidentified leeches also have been reported on Northern Spring Cavefish in Illinois (Smith & Welch 1978). We did not observed any ectoparasites on cavefish captured during this study.

Diseases

Few diseased amblyopsid cavefishes have been reported in nature. Fournie and Overstreet (1985) reported on an adult Northern Spring Cavefish from Union County, Illinois, with a retinoblastoma on the right side of the head. This condition may be related to chromosomal abnormalities. At least one other individual collected at the same spring had a similar tumor in appearance and eventually died after the tumor involved the entire head. However, this specimen was not available for histological examination (D. Bechler, pers. comm. in Fournie & Overstreet 1985).

Gas bubble disease has been documented in recently collected Southern Cavefish at a spring site in Missouri (Schubert et al. 1993). Southern Cavefish from Missouri may be particularly susceptible to this disease because of the depths at which individuals reside (Schubert et al. 1993; Noltie & Wicks 2001). No data is available from Kentucky populations of *Typhlichthys*.

A bacterial infection resulting in rotting and shortening of fins, particularly pectoral and caudal fins, and small, red spots scattered on the body, has been documented in Northern Cavefish at Donaldson Cave in Lawrence County, Indiana (Pearson & Boston 1995). Broken back syndrome also has been documented at this locality and potentially is linked to pesticide and other chemical contamination (Keith & Gray 1979; Keith & Poulson 1981). We did not observe any Northern Cavefish with these and other conditions in Kentucky populations.

Community Associates

Northern Spring Cavefish are associated with several fish species in surface habitats in Kentucky, including Western Mosquitofish (*Gambusia affinis*), Black-Spotted Topminnows (*Fundulus olivaceus*), Fantail Darter (*Etheostoma flabellare*), Southern Redbelly Dace (*Phoxinus erythrogaster*), Creek Chub (*Semotilus atromaculatus*), Central Stoneroller (*Campostoma anomalum*) Bluntnose Minnows (*Pimephales notatus*), Banded Sculpin (*Cottus carolinae*), Longear Sunfish (*Lepomis megalotis*), Bluegill (*Lepomis macrochirus*), Green Sunfish (*Lepomis cyanellus*), and Redfin Pickeral (*Esox americanus*) (Whalen et al. 2002; current study). Other nonavian and nonmammalian vertebrate associates include the Green Frog (*Rana clamitans*), Bullfrog (*Rana catesbeiana*), Southern Leopard Frog (*R. sphenocephala*) and Snapping Turtle (*Chelydra serpentina*).

Southern Cavefish are commonly found in the same cave systems with other cave macrofauna, such as cave crayfish (*Orconectes packardi* and *O. pellucidus*). Epigean fish species commonly found in the same cave systems with Southern Cavefish include Banded Sculpin (*Cottus carolinae*) and Yellow Bullhead (*Ameirus natalis*). Northern cavefish are associated with the cave crayfish *Orconectes inermis* and the epigean fish *C. carolinae* and *A. natalis*. McCandless (2005) found a positive assocation between abundance of *A. spelaea* and *O. inermis* in cave systems where the two species cooccur.

Agonistic Behavior

Agonistic behavior of amblyopsids has been investigated by Bechler (1980, 1981, 1983). Northern Spring Cavefish, Southern Cavefish, and Northern Cavefish exhibit two submissive acts, “freeze” and “escape.” Adult Northern Spring Cavefish and Northern Cavefish cannibalize smaller conspecifics (Hill 1966; Niemiller & Poulson 2010) and freezing should be an excellent defense because amblyopsids use only their lateral line to detect other fish and prey. Thus, it is not surprising that fish that perceive that they are losing in an agonistic encounter ‘freeze’ more often than escaping by fleeing. This allows avoidance of the most intense kinds of acts. Recently hatched Northern Cavefish will freeze when under threat of cannibalism (Niemiller & Poulson 2010). Both Northern Spring Cavefish and Southern Cavefish always initiated agonistic acts from under or next to rock shelter. This is consonant with the high importance of thigmotaxis to both species. In contrast, Northern Cavefish are more active and do not regularly establish “stations.”

Bechler (1983) hypothesized degenerate evolution as the most probable mechanism for reduced agonistic behavior in amblyopsid cavefishes. Retention of some agonistic behavior in Northern Cavefish might reflect either lower degree of subterranean specialization or potential for defense of prime foraging areas. However, agonistic behavior has not been observed in nature in Northern Cavefish.

Territoriality and Social Groups

Although epigean species are often territorial or form social groups, troglobitic species generally are found in low population densities and are usually solitary with a large home range (Langecker 2000). In general, populations Southern Cavefish and Northern Cavefish are low in density (but see Poulson 1969; Niemiller & Poulson 2010) and individuals are irregularly distributed over suitable habitat (Poulson 1963). Individual cavefish have large home ranges, cover long distances in search of food, and never defend areas (Poulson 1963; Mohr & Poulson 1966). However, during aggression trials by Bechler (1983), both Northern Spring Cavefish and Southern Cavefish established distinct territories in aquaria with rocks ("stations"). All amblyopsids do not exhibit schooling behavior, although individuals are sometimes observed in close proximity to conspecifics. These loose aggregations are typically around food sources, such as underneath a bat roost in Southern Cavefish or aggregated in dense vegetation in Northern Spring Cavefish. In contrast, Northern Cavefish are more active and do not establish “stations” in laboratory trials.

Conservation

The conservation status of subterranean fishes has received increasing attention in the past few years. According to Proudlove (2006), 63 of the 104 known species of subterranean fishes at the time of publication are listed by IUCN. The Southern Cavefish and Northern Cavefish are included on this list. In this section we review the conservation status of the three species of amblyopsids that occur in Kentucky, examine the major threats facing each species, and conservation measures that have either been implemented or proposed. A more in depth review of threats to all cave amblyopsids is found in Niemiller and Poulson (2010).

Geographic distribution. Northern Spring Cavefish have the largest geographic extent of all amblyopsids in Kentucky with an extent of occurrence of 14,786.2 km^2, an area of occupany of 720.0 km^2 (based on 4 x 4 km grid cells) and has been documented from 53 localities. Southern Cavefish have an extent of occurrence of 4,547.9 km^2, an area of occupancy of 320.0 km^2, and have been recorded from 23 localities in Kentucky. The newly discovered lineage of *Typhlichthys* in Pulaski County only has an extent of occurrence of 38.3 km^2, an area of occupancy of 80.0 km^2, and occurs at only six localities. Northern Cavefish have an extent of occurrence of 2700.6 km^2, an area of occupancy of 432.0 km^2, and have been observed at 40 localities.

Population size. Determining the actual population sizes of amblyopsid cavefishes is extremely difficult because of the difficulty and inaccessibility of the habitats that each species inhabits. Only a fraction of the actual census population likely is sampled during a given survey; however, estimating that fraction sampled is not trivial. Here we apply an order of magnitude scaling factor for estimating population size, but recognize that actual population sizes could be lesser or greater than our coarse estimates. We estimate a minimal population size of over 12,000 individuals for Northern Spring Cavefish, 3,200 individuals for Southern Cavefish, 500 individuals for the new lineage of *Typhlichthys*, and 14,900 individuals for Northern Cavefish in Kentucky.

Short-term and long-term trends. Trends refer to directional change over the short-term (within three generations) and long-term (within 100 years) in population size, extent of occurrence, area of occupancy, or number of ocurrences. There is no current evidence to suggest that there have been substantial changes in any of these factors over the short-term or long-term for amblyopsid cavefishes in Kentucky, although these factors should be reassessed in the future (e.g., every 5 years). Southern Cavefish have not been observed at Sloans Valley Cave since the late 1960s, but cavers have reported seeing white, blind fish in the same pools where Cooper and Beiter (1972) collected cavefish more recently in the last 10 years. If this population is extirpated, a significant reduction in extent of occurrence and area of occupancy would occur for this lineage. Northern Cavefish may have experienced a population decline after excessive collections in the late 1800s, but there is no evidence to suggest that current population densities are any less than those in the mid 1800s when the species was first discovered in the Mammoth Cave system.

Threats. Proudlove (2006) listed five general threats faced by subterranean fishes. This includes: (1) habitat degradation, (2) hydrological manipulations, (3) environmental pollution, (4) overexploitation, and (5) impacts of introduced aquatic animals. Many of the threats discussed below are interrelated because of their wide range of potential effects. For example, dam construction can result in direct destruction and degradation of cavefish habitat, alter hydrological patterns, and allow surface species to colonize and either compete or prey on existing cavefish populations. Here we generally follow the broad classification of threats listed by Proudlove (2006) and review the threats to populations of Northern Spring Cavefish, Southern Cavefish and Northern Cavefish in Kentucky. We focus on the first four of Proudlove's list as little work has investigated the effects of introduced species on cavefish populations.

Habitat degradation and alteration

Habitat degradation and alteration can result from the direct destruction or manipulation of habitat during quarrying and mining operations, highway construction, and urban development. The majority of habitat degradation and alteration threats are indirect, resulting in loss of habitat because of siltation, sedimentation, and alteration of hydrological flow patterns and levels. Many caves in the eastern Interior Plateau have massive silt banks along streams that are likely associated with farming that began in the 1800s (Poulson, pers. comm.). However, some caves contain cavefish populations that are found entirely on silt substrates and have high population sizes and frequent reproduction. Likewise, on a much longer time scale, huge changes in habitat composition and food availability must have occurred with glacial cycles during the Pleistocene. Studies are needed to assess the actual rather than speculative impacts on cavefish populations from increased siltation and sedimentation.

Land development within cave recharge zones can alter surface runoff patterns or even block or destroy major recharge points. This can result in dramatic habitat alteration because of increased or decreased water volume, water velocity, sedimentation, or stream scouring depending on local hydrological patterns. In forested areas, increased erosion and production of sediment because of logging can result in increased siltation and sedimentation or the complete blockage of a cave passage. For Northern Spring Cavefish, loss of forested areas associated with agriculture can cause the decline or loss of local populations. Removal of the surrounding forest causes increased insolation and drying of aquatic habitat. Indeed, many spring habitats in the Barrens of the Eastern Highland Rim in Tennessee have been altered during the development of nurseries and farmlands. However, the surface stream at Rich Pond in Warren County, Kentucky, flows through an agricultural field with little tree cover and limited vegetation yet the population of Northern Spring Cavefish still thrives here. The newly discovered population in Todd County exists in a channelized stream adjacent to agricultural fields as well. Although loss of surrounding forest habitats might result in degraded aquatic habitat for Northern Spring Cavefish for some populations, effects appear minimal for others.

Reduced input of surface runoff in recharge zones could potentially have impacts on Southern Cavefish and Northern Cavefish reproduction. Both species are thought to rely on increased flow and small temperature changes associated with cave flood events during winter and spring to coordinate reproduction and spawning (Poulson 1963, 1969). Reductions in surface runoff may disrupt the environmental cues necessary for successful reproduction leading to greater susceptibility to extirpation. In addition, most cave ecosystems in Kentucky are driven by allochthonous organic input from the surface. A decrease in organic input likely would have dramatic inputs on many subterranean species, including cavefishes.

Impoundments are another serious threat for cavefish populations. A primary example is the construction of Lock and Dam #6 constructed on the Green River below Mammoth Cave in 1906. Although the Green River naturally back-floods into the cave system, flood levels have increased since dam construction (Lisowski & Poulson 1981). The Styx and Echo River areas in Mammoth Cave experienced an apparent decline in cave biota, including cavefish, from the late 1800s to the 1920s (Elliott 2000). Northern Spring Cavefish populations likely have been significantly impacted from the construction of Lake Barkley and Kentucky Lake but data on distribution and abundance in the Land Between the Lakes area are lacking to assess direct impacts. Impoundments also change flow regimes and local groundwater levels within cave systems. At least two *Typhlichthys* populations in Pulaski County, Kentucky have been impacted by backflooding caused by construction of Lake Cumberland: Sloans Valley Cave and Drowned Rat Cave. The lower 300 m reaches of Sloans Valley Cave are back flooded by Lake Cumberland but it is in this area that cavefish have been observed (Cooper & Beiter

1972); however, we failed to observe cavefish in this area of the cave but cavers (Bill Walden, pers. comm.) have observed *Typhlichthys* in this area on several occasions during the last 10 years. At Drowned Rat Cave, the entrance usually is completely flooded when Lake Cumberland is at normal pool with lake water extending at least 200 m into the cave. However, a large population of *Typhlichthys* exists in the cave as well as several other terrestrial and aquatic troglobitic species, suggesting that impacts from backflooding by Lake Cumberland are not as deterimental as previously thought.

Hydrological manipulations

Hydrological manipulations can include underground water removal for human consumption, irrigation, or industry. However, some hydrological manipulations, such as impoundments or increased surface runoff, can raise water tables and alter habitats (see above). Lowering of the water table resulting from direct human consumption, irrigation, or industrial use may threaten cavefish populations. Unfortunately, data are lacking on how Southern Cavefish and Northern Cavefish respond to hydrological manipulations and the impacts of such manipulations on local populations.

Groundwater pollution

Groundwater pollution has been listed as factor negatively affecting populations for all cave amblyopsids, including Southern Cavefish and Northern Cavefish. This threat includes eutrophication and contamination from agricultural and industrial runoff containing pesticides, fertilizers, and heavy metals, sewage effluent, spills and illegal dumping of hazard materials, and thermally altered runoff. Although few studies have examined the direct effects of groundwater pollution on cavefish populations in detail, several studies implicate this threat in population declines. Certainly, Northern Spring Cavefish populations are likely to be exposed to runoff from agricultural fields, but data are lacking that link chemical runoff and any documented Northern Spring Cavefish population declines.

Groundwater pollution may be acute in nature, such as a toxic spill resulting in a large impulse of contaminants, or chronic occurring over several months to years (Proudlove 2001). Both forms have been attributed to cavefish declines or extirpations from cave systems. Nearly 1,000 dead or dying Southern Cavefish were expelled from Meramec Spring in Missouri after a fertilizer pipeline rupture in November 1981 caused acute, catastrophic deoxygenation of groundwater (Vandike 1984; Crunkilton 1985). In contrast, several decades worth of gross pollution by decomposable organic matter (creamery waste) and heavy metal contamination (electroplating waste) is the suspected cause of the apparent extirpation of Southern Cavefish along with other cave life at Hidden River Cave in Kentucky (Lewis 1996). However, cavefish and other cave life have re-colonized areas in Hidden River Cave previously affected from far upstream refuges.

Heavy metal and hazardous chemical contamination of groundwater also are threats to Southern Cavefish populations. Heavy metal runoff from a local landfill may threaten populations of *T. subterraneus* in Pulaski Co., Kentucky (Tercafs 1992). At least three stressors (industrial effluents, underground storage tank leaks and sinkhole dumping) have been connected to the decline of Southern Cavefish and other cave life from Hidden River Cave (Pearson & Boston 1995; Lewis 1996). Organic enrichment from sewage treatment plant effluents and septic tank leaks also have been implicated at Hidden River Cave and other caves with amblyopsids. Organic enrichment can increase nutrients in an otherwise low-nutrient environment and drastically alter food web dynamics, increase risk of disease, and dramatically decrease dissolved oxygen levels.

A petroleum-based chemical is leaching into the cave stream at Friendship Cave in Warren County. The point of input appears to come a small tributary ca. 200 m into the cave likely connected to a nearby sink. A strong petroleum-like odor was detected from the entrance upstream to the junction with the small tribuary. Upstream of this point, air quality improves. Southern Cavefish were never observed downstream of this input point. Cave crayfish (*Oroconectes pellucidus*) were observed downstream of this input point but in less numbers compared to upstream. Friendship Cave is known to serve as a summer colony roost for bats; however, we did not observe a single bat during our surveys.

Sinkhole dumping is a problem in the vicinity of Under the Road Cave in Breckinridge County. Numerous tires, railroad ties, treated lumber, and other large debris were observed in the main stream of the cave, likely washed in from a series of larger sinkholes located upstream on private property. A similar phenomenon is occur at L & N Railroad Cave in Barren County where creosote-treated wood from the delapidated pumping house is washing into the cave where it collects in pools inhabited by Southern Cavefish (Fig. 46). The long-term effects on this population are unknown.

McCandless (2005) reported high fecal coliform counts at Rimstone Cave in Breckinridge County. He attributed the high bacterial counts to the large number of surface ponds used for watering livestock and the scattered homes in the recharge area that likely are on septic systems but recognized that because only a single time sample was taken, the results might not represent prevailing water quality at Rimstone Cave.

Quarrying and oil and natural gas development

The loss and degradation of habitat as well as disturbance associated with blasting during quarrying operations may negatively impact cavefish populations. At least two Northern Cavefish populations in Indiana have been lost or suffered population declines because of limestone quarrying.

Exploration and development of oil and natural gas reserves in Kentucky may have a deterimental impact on cavefish populations in Kentucky. In addition to direct habitat degradation associated with drilling operations, brine produced by wells and contaminate groundwater or waste brine ma be injected into existing wells (Keith 1988). Brine is highly toxic to aquatic organisms and contains high levels of heavy metals, halides, and dissolved hydrogen sulfide. At least one *Typhlichthys* locality is thought to have been negatively impacted by drilling operations and brine contamination. Parker Cave in Barren County is a suspected site of illegal injection of wast brine in nearby wells where well casings eventually leaked and brine contaminated portions of the cave system.

Over-collection and cave visitation

The collection of cavefish, illegal or otherwise, for the aquarium trade or scientific purposes may pose a threat to Southern Cavefish and Northern Cavefish. Because of their uniqueness to hobbyists and the ease at which individuals can be captured, both species can be easily exploited. Over-collection of fish can potentially reduce or even eliminate local populations. The rarity of Northern Cavefish in the Echo River and River Styx sections of Mammoth Cave system and its presumed absence from adjacent caves to the north have led some to speculate that the species was either introduced or decimated during the 1800s when it was sold as a novelty (Poulson 1968; Elliott 2000). Several museum accessions of all three species are known from Kentucky (Table 24), including several collections of Northern Spring Cavefish from Rich Pond in Warren County, and both Southern Cavefish and Northern Cavefish from the Mammoth Cave system. However, this threat appears to be minimal for most other populations in the state. Amazingly, over 1500 individual Northern Spring Cavefish have been collected from the Rich Pond area in Warren County and certainly this number is much greater if the number of fish not accessioned into museum collections is considered. Despite this intense collection pressure, the population at Rich Pond continues to thrive. While over-collection is real threat for many species, the threat of over-collection due to scientific research appears minimal, at least in Kentucky.

Commercial exploitation of caves can alter or even destroy considerable amounts of cavefish habitat. Commercial caves increase human traffic and disturbance in addition to increased light levels. At least three populations of Southern Cavefish are indirectly affected by commercial cave tours in Kentucky (historically or currently) including Mammoth Cave in Edmonson County, Hidden River Cave in Barren County and Lost River Cave in Warren County. Cave tours at Mammoth Cave may indirect affect populations of Northern Cavefish in the system as well. However, the exact impacts and long-term effects of commercial cave operations remains to be examined.

Human disturbance caused by increased traffic is more of a concern than commercial exploitation. The activities of even the most cautious caver may have serious impacts on cave organisms in shallow, silt-bottomed streams. Disturbance caused by substantial cave visitation may alter breeding of cavefish populations, disturb food sources, and unknowingly stress individual fish by increasing fish activity. However, no evidence has been obtained for any of the above potential threats. Cave vandalism is a major problem at Wells Cave in Pulaksi County where unscrupulous visitors have sprayed graffiti and strewn broken bottles and other refuse in the upper level passages that are inhabited by several bat species (Fig. 47).

Introduction of exotic species

Few studies have assessed the impacts of introduced, exotic species on subterranean fauna. There is concern that the introduction of Rainbow Trout (*Oncorhynchus mykiss*) in Sinking Creek, Breckinridge County, negatively impact Northern Cavefish populations (Pearson & Boston 1995). Rainbow Trout have been reported to feed in darkness and low-light levels but no data exist as to whether their introduction pose a significant threat to cavefish populations. We did not observed any Rainbow Trout during our cave surveys in Breckinridge County but did observe other native surface fish, including Yellow Bullhead and Banded Sculpin.

Conservation measures. Several conservation measures have been proposed or implemented for populations of cave amblyopsids, including Southern Cavefish and Northern Cavefish. Fencing or gating of cave entrances have been proposed or implemented to reduce and control human visitation to sensitive cave ecosystems, such as the many entrances to the Mammoth Cave system in Edmonson County, Thornhill Cave in Breckinridge County and Parker Cave in Barren County. Special bat gates are needed to allow entry and exit by bats but stop human entry. Bat Conservation International and The National Speleological Society have been leaders in the improvement and installation of such gates on an increasing number of bat caves. At other cave, such as Wells Cave in Pulaski County, signs have been posted to help reduce illegal visitation (Fig. 48).

Protection of cave surface and subsurface watersheds is probably the most important intervention for cavefish localities. Watershed protection has included establishing preserves as well as institution of best land management practices around sinkholes and sinking creeks. This includes reforestation. Indeed, a number of cave systems receive some protection by occurring on state or federally owned land or are owned or leased by conversation agencies. In other cases, water tracing has identified the source of pollutants and so allowed legal action that remedied the situation. Hidden River Cave in Hart County is one example. We suggest that demographic source caves deserve complete protection of their watersheds, such as Northern Cavefish localities those located in Sinking Creek. Only a few caves have the vast majority of all Northern Cavefish ever censused. Attention to protecting these caves is a number one priority for the near future. Likewise, source populations of Northern Spring Cavefish, such as Rich Pond, should be identified and protected.

To this end, several management policies should be implemented in the immediate recharge basins of significant cavefish populations to protect the health and integrity of source populations: (1) alter land use practices and implement runoff control measures to reduce the input of sediments and runoff into cave systems, (2) reduce or eliminate the use toxic pesticides and herbicides known to negatively impact the fragile subterranean ecosystem, (3) identify and protect critical input points (sinkholes and sinking streams) into cave systems, and (4) limit access to areas within cave systems that support large cavefish subpopulations,.

In the future, the introduction of all amblyopsid species in Kentucky to new localities or to caves that were historic localities is worth considering in the event of dramatic large-scale population declines. However, until we learn to breed amblyopsids, the only source for introductions is caves with thriving populations.

Conservation status. Northern Spring Cavefish are thought to be secure throughout their range, although disjunct populations in southeast Missouri are listed as endangered (Missouri Natural Heritage Program 2008). In Kentucky, Northern Spring Cavefish are not listed and have an S4S5 Natureserve status designation. Northern Spring Cavefish have been reported from over 50 localities in the state, although few are known to produce over 30 fish in a single survey. This likely is an artifact of habitats sampled, as many ichthyological surveys focus on streams and other larger bodies of water rather than spring runs and springs. Moreover, most springs are located on private property and the vast majority have been poorly sampled in the state. However, the incredible numbers of fish collected and observed at Rich Pond in Warren County illustrate how abundant this species can be, even in presumably suboptimal habitats. Consequently, we do not recommend any status change for Northern Spring Cavefish at this time but recommend that efforts be made to survey small springs and spring runs on private lands.

Troglobitic amblyopsids are considered vulnerable or endangered across their respective distributions, including the Southern Cavefish and Northern Cavefish. Southern Cavefish (as currently recognized) is the most widely distributed and least cave-adapted (Poulson 1963) of the troglobitic amblyopsids. As such, it is considered the most secure (although it is afforded protection in several states) and is considered endangered only in Georgia where it ranges only into the extreme northwest corner of the state. In Kentucky, the Southern Cavefish is currently a “species of special concern” and has an S2S3 Natureserve status designation (Imperiled to Vulnerable). The discovery of cryptic, distinct lineages and putative species within *T. subterraneus* has obvious conservation implications. Two lineages exist in Kentucky: *T. subterraneus* in south-central Kentucky and *Typhlichthys sp. nov. 1* in Pulaski County. Our results support the hypothesis by Cooper and Beiter (1972) that Pulaski County *Typhlichthys* are a distinct taxon from populations to the west in south-central Kentucky and to the southwest in Tennessee. This new species (Kentucky Cavefish) consist of only six reported populations within a small geographic area, with the largest population at Drowned Rat Cave on Army Corps of Engineers land. Another locality, Wells Cave, occurs on National Speleological Society property and is offered some protection. We recommend the status of this undescribed species be listed as ‘Endangered’ with an S1 Natureserve status designation. All other 23 populations in Kentucky are assigned to the more widely distributed Southern Cavefish (*T. subterraneus*), with its population center in the Mammoth Cave area of Barren, Edmonson, and Warren counties. Several populations in this area are offered some protection from human visitation and exploitation. These include localities occuring within the boundaries of Mammoth Cave National Park, L & N Railroad Cave owned by the city of Cave City, and commerical caves (Lost River Cave and Hidden River Cave). We recommend that the conservation status of Southern Cavefish remain the same with an S2S3 status designation.

The Northern Cavefish is a species of “special concern” in Kentucky and also by the USFWS (USFWS 1996). It is also designated as a Regional Forester Sensitive Species within the Hoosier National Forest in Indiana (Lewis 2002b). In Kentucky, Northern Cavefish currently have an S3 Natureserve status designation (Vulnerable). The species is known from at least 40 localities; however, several localities are associated with the Mammoth Cave system in Edmonson County and the Sinking Creek area in Breckinridge County and evidence suggests that individual localities in these areas likely do not represent distinct populations. Future work is needed to define distinct population segements within the Northern Cavefish. Some cave systems in Breckinridge County support large populations, including Amblyopsis Cave, Webster Cave, Pentitentiary Cave, and Under the Road Cave; however, all these localities are located within a small geographic area of the Sinking Creek system. Localities in the Mammoth Cave area are afforded protection, as the occur within the boundaries of Mammoth Cave National Park. All other localities with significant populations of Northern Cavefish occur on private land, although Thornhill Cave in Breckinridge County is gated and managed by the Louisville Grotto. We recommend that the conservation status of Northern Cavefish remain at S3 (Vulnerable).

Recommendations

In light of the current state of knowledge regarding amblyopsid populations in Kentucky, we offer the following recommendations for future research and conservation management:

Northern Spring Cavefish

1. Identify and survey springs located on private property located within the suspected distribution of the species to discover additonal significant populations.

2. Work to protect the Rich Pond population through purchase of the spring and surrounding area, implementing habitat protection strategies, or by obtaining a conservation agreement with the private landowner.

3. Additional population genetic analyses and long-term mark-recaptured are warranted to determine connectivity of populations and dispersal ability of the species in the Western Pennyroyal Karst. Although dispersal ability in amblyopsids is generally thought to be low, major flood events, such as the event during May 2010, may be important for long distance dispersal in this species.

4. Establish a yearly census at the two most significant localities (Morton Road in Todd County and Rich Pond in Warren County) during April or May to monitor population and demographic trends over time.

5. Delineate the recharge zone and conduct annual monitoring water quality at Rich Pond.

Southern Cavefish

1. Delineate the recharge zones of known localities of the undescribed species in Pulaski County, particularly the Coral Cave system and Hail Cave system.

2. Additional surveys are needed to document additional sites for the undescribed species in Pulaski and determine if the distribution extends to the southwest along the escarpment of the Cumberland Plateau in Wayne County.

3. Determine the point source of groundwater contamination at Friendship Cave in Warren County and initiate a chemical cleanup of the cave if possible.

4. Implement a public awareness program to inform landowners and others of the harmful impacts of dumping into sinkholes on groundwater and life it contains.

5. Remove the delapidated pump house and other debris at the entrance of L & N Railroad Cave in Barren County to improve terrestrial and aquatic habitat in the cave.

Northern Cavefish

1. Surveys of cave systems are needed that occur between the main centers of distribution for *Amblyopsis spealea* in portions of Grayson, Hardin and Hart counties to determine if the two main population centers in Kentucky are continuous or isolated by the Hart County Ridge. Additionally, future genetic work should focus on determining with relationships of southern populations of *Amblyopsis* in the Mammoth Cave area with those to the north in the the Sinking Creek area of Breckinridge County. This latter recommendation is currently underway.

2. Because the populations in Sinking Creek in Breckinridge County represent the most significant population center of the species, efforts should be made to protect these populations through landowner agreements, the purchase of cave entrances and surrounding land within recharge zones, and measures to reduce development and construction activities in the area.

3. Implement a public awareness program to inform landowners and others of the harmful impacts of dumping into sinkholes on groundwater and life it contains.

4. Conduct *in situ* studies to determine if Rainbow Trout and Banded Sculpin significantly prey on subterranean fauna, including Northern Cavefish, in subterranean habitats and determine their influence on subterranean faunal abundance and behavior.

Acknowledgements

We are grateful to Tom Barr, Tom Poulson, John Cooper, Bill Walden, Larry Simpson, Daphne Soares, Dante Fenolio, Rick Toomey, Rick Olsen, Bill Pearson, Rob McCandless, Graham Reynolds, Christen Bossu, Phillip Hollingsworth, James Caddle, John Bills, Lee Florea, Premal Shah, Jen Buhay, Zach Marion, Megan Todd-Thompson, Trent Niemiller, and Heather Hensley for assistance in the field or for providing data. We thank Dante Fenolio for providing photographs and with assistance in the field. We thank Thomas Near, Rob McCandless, Christopher Tillquist, Graham Reynolds, James Caddle, David Neely, Al Romero, Ron Johnson, and Bernie Kuhjada for assistance with molecular aspects of this project. We also thank Tom Poulson, Bill Pearson, and Rick Toomey for discussions about amblyopsids in Kentucky. We thank the Kentucky Department of Fish and Wildlife Resources for providing locality data and permits and the state agencies of Alabama, Georgia, Missouri, and Tennessee for also providing permits for the collection of tissue samples for genetic analyses. We also thank the National Speleological Society, City of Cave City, and private landowners for allowing access to their property.

Literature Cited

Adams J, Maslin M, Thomas E. 1999. Sudden climatic transitions during the Quaternary. Progress in Physical Geography 23: 1–36.

Aley T, Aley C. 1997. Groundwater recharge area delineation, hydrobiological assessment, and vulnerability mapping of four Ozark cavefish (*Amblyopsis rosae*) populations in Missouri. Report to the Missiouri Department of Conservation.

Ashmole NP. 1993. Colonization of the underground environment in volcanic islands. Mémoires de Biospéologie 20: 1–11.

Barr TC, Kuehne RA. 1962. The cavefish, Amblyopsis spelaea, in northern Kentucky. Copeia 1962. 662.

Barr TC, Kuehne RA. 1971. Ecological studies in the Mammoth Cave System of Kentucky. II. The ecosystem. Annals de Speleologie 26: 47–96.

Barr TC, Holsinger J. 1985. Speciation in cave faunas. Annual Review of Ecology and Systematics 16: 313–337.

Baum DA, Shaw KL. 1995. Genealogical perspectives on the species problem. Pp. 289–303 *in* Hoch PC, Stephenson AG, eds. Experimental and Molecular Approaches to Plant Biosystematics. Missouri Botanical Garden, St. Louis.

Bechler DL. 1980. The evolution of agonistic behavior in Amblyopsid fishes. Ph.D. dissertation. Saint Louis University, Saint Louis, Missouri. 160pp.

Bechler DL. 1981. Agonistic behavior in the Amblyopsidae, the spring, cave, and swamp fishes. Proceedings of the International Congress of Speleology 8: 68–69.

Bechler DL. 1983. The evolution of agonistic behavior in Amblyopsid fishes. Behavioral Ecology and Sociobiology 12: 35–42.

Bergstrom DE. 1997. The phylogeny and historical biology of Missouri's *Amblyopsis rosae* (Ozark cavefish) and *Typhlichthys subterraneus* (southern cavefish). Master's Thesis, Univ. of Missouri, Columbia, Missouri.

Bergstrom DE, Noltie DB, Holtsford TP. 1995. Ozark cavefish genetics: the phylogeny of Missouri's Ozark cavefish (*Amblyopsis rosae*) and southern cavefish (*Typhlichthys subterraneus*). Final Report, Endangered Species Project SE-01-27: Improving the status of endangered species in Missouri Ozark cavefish genetics. Missouri Dept. of Conservation, Jefferson City, Missouri.

Blatchley WS. 1897. Indiana caves and their fauna. Report of the Indiana State Geologist for 1896.

Bonaparte CL. 1846. Catalogo metodico dei pesci europei. Atti Scienz. Ital. Settima Adunanza (1845): 1–95.

Bonnet E, Van de Peer Y. 2002. ZT: a software tool for simple and partial Mantel tests. Journal of Statistical Software 7: 1–12.

Boulton AJ. 2005. Chances and challenges in the conservation of groundwaters and their dependent ecosystems. Aquatic Conservation: Marine and Freshwater Ecosystems 15: 319–323.

Burr BM. 1980. A distributional checklist of the fishes of Kentucky. Brimleyana 3: 53–84.

Burr BM, Warren Jr ML. 1986. A distributional atlas of Kentucky fishes. Volume 4. Kentucky State Nature Preserves Commission Scientific and Technical Series, Frankfort, Kentucky.

Carlini DB, Manning J, Sullivan PJ, Fong DW. 2009. Molecular genetic variation and population structure in morphologically differentiated cave and surface populations of the freshwater amphipod *Gammarus minus*. Molecular Ecology 18: 1932–1945.

Charlton HH. 1933. The optic tectum and its related fiber tracts in blind fishes. A. *Troglichthys rosae* and *Typhlichthys eigenmanni*. Journal of Comparative Neurology 57: 285–325.

Christman MC, Culver DC, Madden M, White D. 2005. Patterns of endemism of the eastern North American cave fauna. Journal of Biogeography 32: 1441–1452.

Clay W. 1962. A Field Manual of Kentucky Fishes. Kentucky Department of Fish and Wildlife Resources, Frankfort, Kentucky.

Clay WM. 1975. The Fishes of Kentucky. Kentucky Department of Fish and Wildlife Resources, Frankfort, Kentucky.

Clement M, Posada D, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology 9: 1657–1659.

Compton MC, Eisenhour DJ, Cicerello RR, Kornman LE, Surmont A, Laudermilk EL. 2004. Distributional records of selected Kentucky fishes. J. Kentucky Academy of Science 65: 76–84.

Cooper JE, Beiter DP. 1972. The southern cavefish, *Typhlichthys subterraneus* (Pisces, Amblyopsidae), in the eastern Mississippian Plateau of Kentucky. Copeia 1972: 879–881.

Cope ED. 1872. Bulletin of the US Geological and Geographical Survey of the Territories*,* Government Printing Office, Washington, DC, p. 641.

Cox UO. 1905. A revision of the cave fishes of North America. Appen. Rept. Com. Fish for 1904: 377–393.

Crunkilton R. 1985. Subterranean contamination of Maramec Spring by ammonium nitrate and urea fertilizer and its implication on rare cave biota. Missouri Speleologist 25:151–158.

Culver DC, Christman MC, Elliott WR, Hobbs HH, III, Reddell JR. 2003. The North American obligate cave fauna: Regional patterns. Biodiversity and Conservation 12: 441–468.

Culver DC, Deharveng L, Bedos A, Lewis JJ, Madden M, Reddell JR, Sket B, Trontelj P, White D. 2006. The mid-latitude biodiversity ridge in terrestrial cave fauna. Ecography 29: 120–128.

Culver DC, Kane TC, Fong DW. 1995. Adaptation and natural selection in caves: the evolution of *Gammarus minus*. Harvard University Press, Cambridge.

Culver DC, Master LL, Christman MC, Hobbs III HH. 2000. Obligate cave fauna of the 48 contiguous United States. Conservation Biology 14: 386–401.

Cummings MP, Neel MC, Shaw KL. 2008. A genealogical approach to quantifying lineage divergence. Evolution 62: 2411–2422.

Danielopol DL, Griebler C, Gunatilaka A, Notenboom J. 2003. Present state and future prospects for groundwater ecosystems. Environmental Conservation 30: 104–130.

Dayrat B. 2005. Toward integrative taxonomy. Biological Journal of Linnean Society 85: 407–415.

DeKay JE. 1842. Zoology of New York or the New York fauna; comprising detailed descriptions of all the animals hitherto observed within the state of New York, with brief notices of those occasionally found near its borders, and accompanied by appropriate illustrations. Part IV, Fishes. Printed by W. and A. White and J. Visscher, Albany, New York.

de Queiroz K. 1998. The general lineage concept of species, species criteria, and the process of speciation: a conceptual unification and terminological recommendations. Pp. 57–75 *in* Howard DJ, Berlocher SH, eds. Endless Forms: Species and Speciation*,* Oxford Univ. Press, Oxford.

de Queiroz K. 2007. Species concepts and species delimitation. Systematic Biology 56: 879–886.

Dillman CB, Bergstrom DE, Noltie DB, Holtsford TP, Mayden RL. 2011. Regressive progression, progressive regression or neither? Phylogeny and evolution of the Percopsiformes (Teleostei, Paracanthopterygii). Zoologica Scripta 40: 45–60.

Drummond A, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7: 214.

Eckert A.J, Liechty JD, Tearse BR, Pande B, Neale DB. 2010. DnaSAM: software to perform neutrality testing for large datasets with complex null models. Molecular Ecology Resources 10**:** 542–545.

Eigenmann CH. 1897. The Amblyopsidae, the blind fish of America. Report of the British Association for the Advancement of Science 1897: 685–686.

Eigenmann CH. 1899. The blind fishes of North America. Popular Science Monthly 56: 473-486.

Eigenmann CH. 1905. Divergence and convergence in fishes. Biological Lectures from the Marine Biological Laboratory of Woods Hole 8: 59–66.

Eigenmann CH. 1909. Cave Vertebrates of America. A Study in Degenerative Evolution. Carnegie Institution of Washington, Washington, D.C. 241pp.

Elliott WR. 2000. Conservation of the North American cave and karst biota. Pp. 665–689 *in* Wilkens H, Culver DC, Humphreys WF, eds. Ecosystems of the Word. Subterranean Ecosystems. Volume 30. Elsevier, Amsterdam.

Ence DD, Carstens BC. 2011. SpedeSTEM: a rapid and accurate method for species delimitation. Molecular Ecology Resources 11: 473–480.

Etnier DA, Starnes WC. 1993. The Fishes of Tennessee. University of Tennessee Press, Knoxville, Tennessee.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software Structure: a simulation study. Molecular Ecology 14: 2611–2620*.*

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10: 564–567.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479–491.

Eytan RI, Hellberg ME. 2010. Nuclear and mitochondrial sequence data reveal and conceal different demographic histories and population genetic processes in Caribbean reef fishes. Evolution 64: 3380–3397.

Finston TL, Johnson MS, Humphreys WF, Eberhard SM, Halse SA. 2007. Cryptic speciation in two widespread subterranean amphipod genera reflects historical drainage patterns in an ancient landscape. Molecular Ecology 16:355–365.

Forbes SA. 1882. The blind cave fishes and their allies. American Naturalist 16: 1–5.

Forbes SA, Richardson RE. 1908. The Fishes of Illinois. Illinois State Laboratory of Natural History, Urbana, Illinois.

Fournie JW, Overstreet RM. 1985. Retinoblastoma in the spring cavefish, *Chologaster agassizi* Putnam. Journal of Fish Diseases 8: 377–381.

Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147: 915–925.

Gimenez Dixon M. 1996. *Amblyopsis spelaea*. In IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. Available at http://www.iucn.redlist.org. Accessed 13 April 2013.

Giovannoli, L. 1933. Invertebrate life of Mammoth and other neighboring caves. American Midland Naturalist 14: 600–623.

Girard CF. 1859. Ichthyological notices. Proceedings of the Academy of Natural Sciences of Philadelphia 1859: 56–68.

Green SM, Romero A. 1997. Responses to light in two blind cave fishes (*Amblyopsis spelaea* and *Typhlichthys subterraneus*) (Pisces: Amblyopsidae). Environmental Biology of Fishes 50: 167–174.

Greenwood PH, Rosen DE, Weitzman SH, Myers GS. 1966. Phyletic studies of teleostean fishes, with a provisional classification of living forms. Bulletin of the American Museum of Natural History 131: 339–456.

Gunning GE, Lewis WM. 1955. The fish population of a spring-fed swamp in the Mississippi bottoms of southern Illinois. Ecology 36: 552–558.

Heled J*,* Drummond A. 2010. Bayesian inference of species trees from multilocus data. Molecular Biology and Evolution 27: 570–580.

Hill LG. 1966. Studies on the biology of the spring cavefish, *Chologaster agassizi* Putnam. Ph.D. Dissertation, Department of Biology, University of Louisville. 104 pp.

Hill LG. 1968. Oxygen preference in the spring cavefish, *Chologaster agassizi*. American Fisheries Society Transaction 97: 448–454.

Hill LG. 1969. Feeding and food habits of the spring cavefish, *Chologaster agassizi*. American Midland Naturalist 82: 110–116.

Hill LG. 1971. Scale development and patterns of squamation on the spring cavefish, *Chologaster agassizi* (Amblyopsidae). Proceedings of the Oklahoma Academy of Science 51: 13–14.

Holcroft NI. 2004. A molecular test of alternative hypotheses of tetraodontiform (Acanthomorpha: Tetraodontiformes) sister group relationships using data from the RAG1 gene. Molecular Phylogenetics and Evolution 32: 749–760.

Holsinger JR. 1988. Troglobites: the evolution of cave-dwelling organisms. American Scientist 76: 146–153.

Holsinger JR. 2000. Ecological derivation, colonization, and speciation. Pp. 399–415 in H. Wilkens, D. Culver, and W. Humphreys, eds. Ecosystems of the World. Subterranean Ecosystems. Volume 30. Elsevier, Amsterdam.

Hüppop K. 2000. How do cave animals cope with the food scarcity in caves? Pp. 159–188 in H. Wilkens, D. Culver, and W. Humphreys, eds. Ecosystems of the World. Subterranean Ecosystems. Volume 30. Elsevier, Amsterdam.

Jegla TC, Poulson TL. 1970. Circadian rhythms, I: Reproduction in cave crayfish *Orconectes pellucidus inermis*. Comparative Biochemical Physiology 33: 347–355.

Jordan DS. 1929. *Forbesichthys* for *Forbesella*. Science 70: 68.

Jordan DS, Evermann BW. 1927. New genera and species of North American fishes. Proceedings of the California Academy of Science 16: 501–507.

Kalayil PK, Clay WM. 1976. Immunological characteristics and relationships of tissue antigens in Amblyopsid fishes. Federation Proceedings 35: 751.

Keith JH. 1988. Distribution of northern cavefish, *Amblyopsis spelaea* DeKay, in Indiana and Kentucky and recommendations for its protection. Natural Areas Journal 8: 69–79.

Keith JH, Gray LM. 1979. A preliminary study of the occurrence of brokenback syndrome in the northern cavefish (*Amblyopsis spelaea*) at Spring Mill State Park, Mitchell, Indiana. Proceedings of the Indiana Academy of Sciences 88: 163.

Keith JH, Poulson TL. 1981. Broken-back syndrome in Amblyopsis spelaea, Donaldson-Twin Caves, Indiana. Cave Research Foundation 1979 Annual Report: 45–48.

Kentucky's Comprehensive Wildlife Conservation Strategy. 2013. Kentucky Department of Fish and Wildlife Resources, Frankfort, Kentucky 40601. Available at http://fw.ky.gov/kfwis/stwg/. Accessed on 12 April 2013.

Knowles LL, Carstens BC. 2007. Delimiting species without monophyletic gene trees. Systematic Biology 56: 887–895.

Kocher TD, Conroy JA, McKaye KR, Stauffer JR, Lockwood SF. 1995. Evolution of the ND2 gene in East African cichlids. Molecular Phylogenetics and Evolution 4: 420–432.

Kozak KH, Blaine RA, Larson A. 2006. Gene lineages and eastern North American paleodrainage basins: phylogeography and speciation in salamanders of the *Eurycea bislineata* species complex. Molecular Ecology 15: 191–207.

Kubatko L, Gibbs HL, Bloomquist EW. 2011. Inferring species-level phylogenies and taxonomic distinctiveness using multi-locus data in *Sistrurus* rattlesnakes. Systematic Biology 60: 393–409.

Kuehne RA. 1966. Depauperate fish faunas of sinking creeks near Mammoth Cave, Kentucky. Copeia 1966: 306–311.

Lamoreux J. 2004. Stygobites are more wide-ranging than troglobites. Journal of Cave and Karst Studies 66: 8–19.

Langecker TG. 2000. The effects of continuous darkness on cave ecology and cavernicolous evolution. Pp. 135–138 in Ecosystems of the World. Subterranean Ecosystems. Volume 30. Wilkens H, Culver DC, Humphreys WF, eds. Elsevier, Amsterdam.

Layne JN, Thompson DH. 1952. Recent collections of the Amblyopsid fish *Chologaster papillifera* in Illinois. Copeia 1952: 39–40.

Lefebure T, Douady CJ, Gouy M, Trontelj P, Briolay J, Gibert J. 2006. Phylogeography of a subterranean amphipod reveals cryptic diversity and dynamic evolution in extreme environments. Molecular Ecology 15: 1797–1806.

Lefebure T, Douady CJ, Malard F, Gibert J. 2007. Testing dispersal and cryptic diversity in a widely distributed groundwater amphipod. Molecular Phylogenetics and Evolution 42: 676–686.

Lemmon EM, Lemmon AR, Cannatella DC. 2007. Geological and climatic forces driving speciation in the continentally distributed trilling chorus frogs (*Pseudacris*). Evolution 61: 2086–2103.

Lewis JJ. 1996. The devastation and recovery of caves affected by industrialization. Proceedings of the 1995 National Cave Management Symposium: 214–227.

Lewis JJ. 1998. The subterranean fauna of the Blue River area. Final Report, The Natural Conservancy, 266p.

Lewis JJ. 2002a. Conservation assessment for northern cavefish copepod (*Cauloxenus stygius*). USDA Forest Service, Eastern Region, Report. 10 pp.

Lewis JJ. 2002b. Conservation assessment for southern cavefish (*Typhlichthys subterraneus*). USDA Forest Service, Eastern Region, Report. 9p.

Lewis JJ, Sollman T. 1998. Groundwater monitoring in significant aquatic caves that lie beneath impending residential developments in the Blue River basin of southern Indiana. Final Report, U.S. Fish and Wildlife Service. 89p.

Li C, Orti G, Zhang G, Lu G. 2007. A practical approach to phylogenomics: the phylogeny of ray-finned fish (Actinopterygii) as a case study. BMC Evolutionary Biology 7:44.

Lisowski EA, Poulson TL. 1981. Impacts of Lock and Dam Six on base level ecosystems in Mammoth Cave. Cave Research Foundation 1981 Annual Report: 48–54.

Maddison WP, Maddison DR. 2005. MacClade: analysis of phylogeny and character evolution. Version 3.0. Sinauer Associates, Inc, Sunderland, Massachusetts.

Mantel N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Research 27: 209–220.

Mayden RL. 1988. Vicariance biogeography, parsimony, and evolution in North American freshwater fishes. Systematic Zoology 37: 329–355.

Mayr E. 1942. Systematics and the origin of species. Columbia Univ. Press, New York.

McAllister DE. 1968. The evolution of branchiostegals and associated opercular, gular, and hyoid bones and the classification of teleostome fishes, living and fossil. Bulletin of the National Museum of Canada 221: 1–239.

McCandless JR. 2005. Field surveys and use of a spatially realistic stochastic patch occupancy model to evaluate the conservation status of the northern cavefish, *Amblyopsis spelaea* DeKay. Ph.D. Dissertation, University of Louisville, Louisville, Kentucky. 192p.

McCormack JE, Heled J, Delaney KS, Peterson AT, Knowles LL. 2011. Calibrating divergence times on species tree versus gene trees: implications for speciation history of *Aphelocoma* jays. Evolution 65: 184–202.

Minin V, Bloomquist E, Suchard M. 2008. Smooth skyride through a rough skyline: Bayesian coalescent-based inference of population dynamics. Molecular Biology and Evolution 25: 1459.

Missouri Natural Heritage Program. 2008. Missouri species and communities of conservation concern checklist. Missouri Department of Conservation, Jefferson City, Missouri. 55pp.

Mohr CE, Poulson TL. 1966. The Life of the Cave. McGraw-Hill Book Co., New York.

Murray AM, Wilson MVH. 1996. A new Paleocene genus and species of percopsiform (Teleostei: Paracanthopterygii) from the Paskapoo Formation, Smoky Tower, Alberta. Can. Journal of Earth Science 33: 429–438.

NatureServe. 2013. NatureServe Explorer: An online encyclopedia of life [web application], Version 7.1, NatureServe, Arlington, Virginia. http://www.natureserve.org/explorer. Accessed on 12 April 2013.

Nelson JS. 2006. Fishes of the World. 4th Edition. John Wiley Sons, New York.

Nickol BB, Whittaker FB. 1978. *Neoechinorhynchus cylindratus* (Acanthocephala) from the troglodytic fish, *Amblyopsis spelaea*, in Kentucky. Proceedings of the Helminthological Society of Washington 45: 136–137.

Niemiller ML, Fitzpatrick BM. 2008. Phylogenetics of the southern cavefish, *Typhlichthys subterraneus*: implications for conservation and management. Proceedings of the 18th National Cave and Karst Management Symposium 18: 79–88.

Niemiller ML, Poulson TL. 2010. Subterranean fishes of North America: Amblyopsidae. Pp. 169–280 *in* The Biology of Subterranean fishes. Trajano E, Bichuette ME, Kapoor BG, eds. Science Publishers, Enfield, New Hampshire.

Niemiller ML, Fitzpatrick BM, Miller BT. 2008. Recent divergence with gene flow in Tennessee cave salamanders (Plethodontidae: *Gyrinophilus*) inferred from gene genealogies. Molecular Ecology 17: 2258–2275.

Niemiller ML, Fitzpatrick BM, Shah P, Schmitz L, and Near TJ. 2013a. Evidence for repeated loss of selective constraint in rhodopsin of amblyopsid cavefishes (Teleostei: Amblyopsidae). *Evolution* 67: 732–748.

Niemiller ML, McCandless JR, Reynolds RG, Caddle J, Tillquist CR, Near TJ, Pearson WD, and Fitzpatrick BM. 2013b. Effects of climatic and geological processes during the Pleistocene on the evolutionary history of the northern cavefish, *Amblyopsis spelaea* (Teleostei: Amblyopsidae). Evolution 67: 1011–1025.

Niemiller ML, Near TJ, Fitzpatrick BM. 2012. Delimiting species using multilocus data: diagnosing cryptic diversity in the southern cavefish, *Typhlichthys subterraneus* (Teleostei: Amblyopsidae). Evolution 66: 846–866.

Noltie DB, Wicks CB. 2001. How hydrogeology has shaped the ecology of Missouri's Ozark cavefish, *Amblyopsis rosae*, and southern cavefish, *Typhlichthys subterraneus*: insights on the sightless from understanding the underground. Environmental Biology of Fishes 62: 171–194.

O'Meara BC. 2010. New heuristic methods for joint species delimitation and species tree inference. Systematic Biology 59: 59–73.

O'Meara BC, Ane C, Sanderson MJ, Wainwright PC. 2006. Testing for different rates of continuous trait evolution using likelihood. Evolution 60: 922–933.

Orr JM. 1934. Studies on a cave fish of uncertain classification. Master's thesis. Western Kentucky State College, Bowling Green, Kentucky.

Page LM, Burr BM. 1991. A Field Guide to Freshwater Fishes of North America North of Mexico. Houghton Mifflin, Boston, 432p.

Parenti LR. 2006. *Typhlichthys eigenmanni* Charlton, 1933, an available name for a blind cavefish (Teleostei: Amblyopsidae), differentiated on the basis of characters of the central nervous system. Zootaxa 1374: 55–59.

Pearson WD, Boston CH. 1995. Distribution and status of the northern cavefish, *Amblyopsis spelaea*. Final report, Nongame and Endangered Wildlife Program, Indiana Department of Natural Resources, Indianapolis, Indiana.

Pearson WD, Jones TG. 1998. A final report based on a faunal inventory of subterranean streams and development of a cave aquatic biological monitoring program using a modified index of biotic integrity. Unpublished report submitted to the National Park Service, Mammoth Cave National Park. 139 pp.

Poly WJ, Proudlove GS. 2004. Family Amblyopsidae Bonaparte 1846—cavefishes. California Academy of Sciences Annotated Checklists of Fishes 26. 7 pp.

Posada D, Crandall KA. 1998. MODELTEST: Testing the model of DNA substitution. Bioinformatics 14:817–818.

Poulson TL. 1960. Cave adaptation in Amblyopsid fishes. Ph.D. Dissertation, Department of Zoology, University of Michigan, Ann Arbor. University Microfilms 61-2787.

Poulson TL. 1963. Cave adaptation in Amblyopsid fishes. American Midland Naturalist 70: 257–290.

Poulson TL. 1968. Aquatic cave communities. Cave Research Foundation Annual Report 1968: 16–18.

Poulson TL. 1969. Population size, density, and regulation in cave fishes. Actes of the 4th International Congress of Speleology, Ljubljana, Yugoslavia 4–5: 189–192.

Poulson TL. 1985. Evolutionary reduction by neutral mutations: Plausibility arguments and data from amblyopsid fishes and linyphiid spiders. Bulletin of the National Speleological Society 47: 109–117.

Poulson TL. 1992. The Mammoth Cave Ecosystem. Pp. 1–52 *in* The Natural History of Biospeleology. Camacho AI, ed. Monografias Museo Nacional De Ciencias Naturales. Madrid, Spain. ISBN 84-00-01280-4.

Poulson TL. 2001. Adaptations of cave fishes with some comparisons to deep-sea fishes. Environmental Biology of Fishes 62: 345–364.

Poulson TL, Smith PM. 1969. The basis for seasonal growth and reproduction in aquatic cave organisms. Actes of the 4th International Congress of Speleology, Ljubljana, Yugoslavia 4–5: 197–201.

Poulson TL, White WB. 1969. The cave environment. Science 165: 971–981.

Pritchard JK, Stephen M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945–959.

Proudlove GS. 2001. The conservation status of hypogean fishes. Environmental Biology of Fishes 62: 201–213.

Proudlove GS. 2006. Subterranean Fishes of the World. International Society for Subterranean Biology, Moulis.

Putnam FW. 1872. The blind fishes of the Mammoth Cave and their allies. American Naturalist 6: 6–30.

Ramos-Onsins S, Rozas J. 2002. Statistical properties of new neutrality tests against population growth. Molecular Biology and Evolution 19: 2092.

Rannala B, Yang Z. 2003. Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. Genetics 164: 1645–1656.

Retzer ME, Burr BM, Warren ML. 1983. Fishes of the lower Green River drainage, Kentucky. Kentucky Natural Preserces Committee Scientific Technical Series 3: 1–48.

Rhoades R. 1962. The evolution of crayfishes of the genus *Orconectes* section *limosus*. Ohio Journal of Science 62: 65.

Rice SP, Macgregor JR, Davis WL. 1983. Distributional records for 14 fishes in Kentucky. Transactions of the Kentucky Academy of Science 44: 125–128.

Robison WA. 1981. A note on the occurrence of *Chologaster agassizi* (Amblyopsidae) in Kentucky. Transactions of the Kentucky Academy of Science 42: 106–107.

Roe AD, Rice AV, Bromilow SE, Cooke JEK, Sperling FAH. 2010. Multilocus species identification and fungal DNA barcoding: insights from blue stain fungal symbionts of the mountain pine beetle. Molecular Ecology Resources 6: 946–959.

Roe AD, Sperling FAH. 2007. Population structure and species boundary delimitation of cryptic *Dioryctria* moths: an integrative approach. Molecular Ecology 16: 3617–3633.

Ronquist F, Huelsenbeck JP. 2003. MRBAYES3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Rosen DE, Patterson C. 1969. The structure and relationships of the paracanthopterygian fishes. Bulletin of the American Museum of Natural History 141: 357–474.

Rosen DE. 1962. Comments on the relationships of the North American cavefishes of the family Amblyopsidae. American Museum Novitates. 35 pp.

Schubert ALS, Nielsen CD, Noltie DB. 1993. Habitat use and gas bubble disease in southern cavefish (*Typhlichthys subterraneus*). International Journal of Speleology 22: 131–143.

Smith PW, Welch NM. 1978. A summary of the life history and distribution of the spring cavefish, *Chologaster agassizi*, Putnam, with population estimates for the species in southern Illinois. Illinois Natural History Survey Biological Notes 104: 1–8.

Smouse PE, Long JC, Sokal RR. 1986. Multiple regression and correlation extensions of the Mantel test of matrix correspondence. Systematic Zoology 35: 627–632.

Soares D, and Niemiller ML. 2013. Sensory adaptations of fishes to subterranean environments. BioScience 63: 274–283

Storer DH. 1846. Synopsis of the fishes of North America. Memoirs of the American Academy 2: 436.

Strange RM, Burr BM. 1997. Intraspecific phylogeography of North American highland fishes: a test of the Pleistocene vicariance hypothesis. Evolution 51: 885–897.

Swofford DL. 1982. Genetic variability, population differentiation, and biochemical relationships in the family Amblyopsidae. M.S. Thesis. Eastern Kentucky University, Richmond, Kentucky.

Swofford DL, Branson BA, Sievert G. 1980. Genetic differentiation of cavefish populations (Amblyopsidae). Isozyme Bulletin 13: 109–110.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123: 585.

Teller JT, Goldthwait RP. 1991. The Old Kentucky River; A major tributary to the Teays River. Pp. 29–41 *in* Geology and hydrogeology of the Teays-Mahomet Bedrock Valley System, Special Paper 258. Melhorn WN, Kempton JP, eds. Geological Society of America, Boulder, Colorado.

Tellkampf TG. 1844. Ueber den blinden Fisch der Mammuth-Hohle in Kentucky, mit Bemerkungen ueber einige undere in dieser Hohle lebenden Thiere. Archiv fur Anatomie, Physiologie und wissenschaftliche Medicin 4: 381–394.

Tellkampf TG. 1845. Memoirs on the blind-fishes and some other animals living in the Mammoth Cave in Kentucky. New York Journal of Medical and Collateral Sciences 5: 84–93.

Tercafs R. 1992. The protection of the subterranean environment. Conservation principles and management tools. Pp. 481–524 *in* The Natural History of Biospeleology. Camacho AI, ed. Monografias del Museo Nacional de Ciencias Naturales, Madrid, Spain.

Trontelj P, Douady CJ, Fiser C, Gibert J, Goricki S, LeFebure T, Sket B, Zakšek V. 2009. A molecular test for cryptic diversity in ground water: how large are the ranges of macro-stygobionts? Freshwater Biology 54: 727–744.

Vandike JE. 1984. Hydrogeologic aspects of the November 1981 liquid fertilizer pipeline break on groundwater in the Meramec Spring recharge area, Phelps County, Missouri. Proceedings of the 1984 National Cave Management Symposium, Missouri Speleology 25: 93–101.

Verovnik R, Sket B, Trontelj P. 2004. Phylogeography of subterranean and surface populations of water lice *Asellus aquaticus* (Crustacea: Isopoda). Molecular Ecology 13: 1519–1532.

Verrier ML. 1929. Observations sur le comportement d'un poisson cavernicole *Typhlichthys osborni* Eigenmann. Bulletin of the Museum of Natn. Hist. Nat. Paris (Ser. 2) 1: 82–84.

Weise JG. 1957. The spring cavefish, *Chologaster papilliferus*, in Illinois. Ecology 38: 195–204.

Whittaker FH, Hill LG. 1968. *Proteocephalus chologaster* sp. n. (Cestoda: Proteocephalidae) from the spring cavefish *Chologaster agassizi* Putnam, 1782 [error = Putnam 1872] (Pisces: Amblopsidae) of Kentucky. Proceedings of the Helminthological Society of Washington 35: 15–18.

Whittaker FH, Zober SJ. 1978. *Proteocephalus poulsoni* sp. n. (Cestoda: Proteocephalidae) from the northern cavefish *Amblyopsis spelaea* DeKay, 1842 (Pisces: Amblyopsidae) of Kentucky. Folia Parasitologica 25: 277–280.

Wiens JJ, Chippindale PT, Hillis DM. 2003. When are phylogenetic analyses misled by convergence? A case study in Texas cave salamanders. Systematic Biology 52: 501–514.

Wiley EO, Johnson GD, Dimmick WW. 2000. The interrelationships of acanthomorph fishes: A total evidence approach using molecular and morphological data. Biochemical Systematics and Ecology 28: 319–350.

Woods LP, Inger RF. 1957. The cave, spring, and swamp fishes of the family Amblypsidae of central and eastern United States. American Midland Naturalist 58: 232–256.

World Conservation Monitoring Center. 1996. Typhlichthys subterraneus. In IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. Available at http://www.iucnredlist.org (Accessed 13 April 2013).

Yang Z, Rannala B. 2010. Bayesian species delimitation using multilocus sequence data. Proceedings of the National Academy of Science USA 107: 9264–9269.

Yokoyama S, Meany A, Wilkens H, Yokoyama R. 1995. Initial mutational steps toward loss of opsin gene function in cavefish. Molecular Biology and Evolution 12: 527–532.

Zakšek V, Sket B, Trontelj P. 2007. Phylogeny of the cave shrimp *Troglocaris*: evidence of a young connection between Balkans and Caucasus. Molecular Phylogenetics and Evoluton. 42: 223–235.

Table 1. Localities of the Northern Spring Cavefish (*Forbesichthys papilliferus*) in Kentucky compiled from literature sources, museum records, and the current study. [1] – Locality information estimated.

Data available from the authors or KDFWR upon request.

Table 2. Localities of the Southern Cavefish (*Typhlichthys subterraneus*) and Kentucky Cavefish (*Typhlichthys sp. nov. 1*) in Kentucky compiled from literature sources, museum records, and the current study. Kentucky Cavefish localities are highlighted in gray.

Data available from the authors or KDFWR upon request.

Table 3. Localities of the Northern Cavefish (*Amblyopsis spelaea*) in Kentucky compiled from literature sources, museum records, and the current study.

Data available from the authors or KDFWR upon request.

Table 4. Distribution of Northern Spring Cavefish (*Forbesichthys papilliferus*) localities (n = 53) and maximum number of cavefish observed during a single survey by county.

County	Localities	Maximum Fish Observed
Allen	2	2
Barren	1	2
Butler	1	na
Caldwell	1	1
Christian	1	na
Crittenden	2	1
Edmonson	9	12
Livingston	5	4
Logan	3	22
Lyon	4	22
Metcalfe	1	na
Monroe	1	1
Muhlenburg	1	1
Simpson	2	1
Todd	3	77
Trigg	8	3
Warren	8	1006

Table 5. Distribution of Northern Spring Cavefish (*Forbesichthys papilliferus*) localities (n = 53) and maximum number of cavefish observed during a single survey by IV Ecoregion.

Ecoregion	Localities	Maximum Fish Observed
Interior Plateau: Crawford-Mammoth Cave Uplands	9	12
Interior Plateau: Western Pennyroyal Karst	20	1006
Interior Plateau: Eastern Highland Rim	3	2
Interior Plateau: Western Highland Rim	15	22
Interior River Valleys and Hills: Caseyville Hills	6	9

Table 6. Distribution of Northern Spring Cavefish (*Forbesichthys papilliferus*) localities (n = 53) and maximum number of cavefish observed during a single survey by HUC8 watershed.

HUC8 Watershed	Localities	Maximum Fish Observed
Cumberland: Lower Cumberland	15	22
Cumberland: Red	7	77
Green: Barren	14	1006
Green: Middle Green	1	na
Green: Pond	1	1
Green: Upper Green	10	12
Lower Ohio: Bay	3	4
Lower Ohio: Tradewater	1	1
Lower Tennessee: Kentucky Lake	1	1

Table 7. Distribution of Southern Cavefish (*Typhlichthys subterraneus*) localities (n = 29) and maximum number of cavefish observed during a single survey by county. The Kentucky Cavefish (*Typhlichthys sp. nov. 1*) is known from six localities in Pulaski County only and is highlighted in gray.

County	Localities	Maximum Fish Observed
Barren	3	45
Christian	1	1
Edmonson	11	104
Hart	1	29
Pulaski	6	31
Simpson	1	3
Trigg	1	4
Warren	5	16

Table 8. Distribution of Southern Cavefish (*Typhlichthys subterraneus*) localities (n = 29) and maximum number of cavefish observed during a single survey by IV Ecoregion. The Kentucky Cavefish (*Typhlichthys sp. nov. 1*) is found only in the Plateau Escarpment of the Southwestern Appalachians only and is highlighted in gray.

Ecoregion	Localities	Maximum Fish Observed
Interior Plateau: Crawford-Mammoth Cave Uplands	10	104
Interior River Valley and Hills: Caseyville Hills	1	12
Interior Plateau: Western Pennyroyal Karst	12	45
Southwestern Appalachians: Plateau Escarpment	6	31

Table 9. Distribution of Southern Cavefish (*Typhlichthys subterraneus*) localities (n = 29) and maximum number of cavefish observed during a single survey by HUC8 watershed. The Kentucky Cavefish (*Typhlichthys sp. nov.* 1) is found in the Upper Cumberland-Lake Cumberland subbasin of the Cumberland River basin only and is highlighted in gray.

HUC8 Watershed	Localities	Maximum Fish Observed
Cumberland: Lower Cumberland	1	4
Cumberland: Red	2	3
Cumberland: Upper Cumberland-Lake Cumberland	6	31
Green: Barren	7	20
Green: Upper Green	13	104

Table 10. Distribution of Northern Cavefish (*Amblyopsis spelaea*) localities (n = 40) and maximum number of cavefish observed during a single survey by county.

County	Localities	Maximum Fish Observed
Breckinridge	18	515
Edmonson	10	75
Hardin	5	2
Hart	3	na
Meade	4	6

Table 11. Distribution of Northern Cavefish (*Amblyopsis spelaea*) localities (n = 40) and maximum number of cavefish observed during a single survey by IV Ecoregion.

Ecoregion	Localities	Maximum Fish Observed
Interior Plateau: Crawford-Mammoth Cave Uplands	28	515
Interior Plateau: Mitchell Plain	10	6
Interior River Valley and Hills: Caseyville Hills	2	8

Table 12. Distribution of Northern Cavefish (*Amblyopsis spelaea*) localities (n = 40) and maximum number of cavefish observed during a single survey by HUC8 watershed.

HUC8 Watershed	Localities	Maximum Fish Observed
Green: Rough	4	5
Green: Upper Green	15	75
Lower Ohio: Blue-Sinking	21	515

Table 13. Locality information, including county, state, sample size, major hydrological basin, subbasin (in parentheses), and ecoregion, delimited species and lineage assignments for 62 populations of *Typhlichthys*. Populations from Kentucky are highlighted in gray.

No.	Locality	County	State	*n*	Basin (Subbasin)	Ecoregion	Delimited Species	Lineage in Div. Time Analyses
1	McKinney Pit	Colbert	AL	4	Tennessee (TN-Pickwick)	Interior Low Plateau	I	F
2	Guess Creek Cave	Jackson	AL	1	Tennessee (TN-Wheeler)	Southwestern Appalachians	F	E
3	Davis Bat Cave	Lauderdale	AL	1	Tennessee (TN-Pickwick)	Interior Low Plateau	I	F
4	Key Cave	Lauderdale	AL	2	Tennessee (TN-Pickwick)	Interior Low Plateau	J	B
5	White Spring Cave	Limestone	AL	1	Tennessee (TN-Wheeler)	Interior Low Plateau	F	B
6	Bobcat Cave	Madison	AL	1	Tennessee (TN-Wheeler)	Interior Low Plateau	I	B
7	Muddy Cave	Madison	AL	1	Tennessee (TN-Wheeler)	Interior Low Plateau	C	B
8	Shelta Cave	Madison	AL	3	Tennessee (TN-Wheeler)	Interior Low Plateau	F	E
9	Beech Spring Cave	Marshall	AL	1	Tennessee (TN-Wheeler)	Southwestern Appalachians	F	E
10	Cave Spring Cave	Morgan	AL	1	Tennessee (TN-Wheeler)	Interior Low Plateau	I	F
11	Norfolk Lake	Baxter	AR	1	White (North Fork White)	Ozark Highlands	B	K
12	Alexander Cave	Stone	AR	2	White (Middle White)	Ozark Highlands	B	I
13	Ennis Cave	Stone	AR	1	White (Middle White)	Ozark Highlands	B	I
14	Limestone Caverns	Dade	GA	2	Tennessee (TN-Chickamauga)	Ridge and Valley	E	A
15	Long's Rock Wall Cave	Dade	GA	3	Tennessee (TN-Chickamauga)	Ridge and Valley	E	A
16	L and N Railroad Cave	Barren	KY	4	Green (Upper Green)	Interior Low Plateau	G	L
17	Mammoth Cave	Edmonson	KY	4	Green (Upper Green)	Interior Low Plateau	G	L
18	Sander's Cave	Edmonson	KY	4	Green (Upper Green)	Interior Low Plateau	G	L
19	Dave's Cave	Pulaski	KY	3	Cumberland (Cumberland-Lake Cumberland)	Southwestern Appalachians	J	M
20	Drowned Rat Cave	Pulaski	KY	3	Cumberland (Cumberland-Lake	Southwestern Appalachians	J	M

					Cumberland)			
21	Well's Cave	Pulaski	KY	1	Cumberland (Cumberland-Lake Cumberland)	Southwestern Appalachians	J	M
22	Carroll Cave	Camden	MO	4	Osage (Osage-Lake of the Ozarks)	Ozark Highlands	B	J
23	Coalbank Cave	Carter	MO	3	White (Current)	Ozark Highlands	B	K
24	Concolor Cave	Howell	MO	3	White (Current)	Ozark Highlands	B	K
25	Bliss Camp Cave	Oregon	MO	2	White (Eleven Point)	Ozark Highlands	B	K
26	Falling Spring Cave	Oregon	MO	2	White (Eleven Point)	Ozark Highlands	B	K
27	Posy Spring Cave	Oregon	MO	4	White (Eleven Point)	Ozark Highlands	B	K
28	Roaring Spring Cave	Oregon	MO	3	White (Eleven Point)	Ozark Highlands	B	K
29	Turner Spring Cave	Oregon	MO	1	White (Eleven Point)	Ozark Highlands	B	K
30	Panther Cave	Ripley	MO	2	White (Current)	Ozark Highlands	B	K
31	Brawley Cave	Shannon	MO	1	White (Eleven Point)	Ozark Highlands	B	K
32	Flying W Cave	Shannon	MO	2	White (Current)	Ozark Highlands	B	K
33	Blowing Springs Cave	Coffee	TN	4	Tennessee (Upper Elk)	Southwestern Appalachians	C	B
34	Baugus Cave	Decatur	TN	4	Tennessee (TN-Beech)	Interior Low Plateau	F	F
35	Garner Spring Cave	Franklin	TN	4	Tennessee (TN-Guntersville)	Southwestern Appalachians	K	C
36	Little Crow Creek Cave	Franklin	TN	2	Tennessee (TN-Guntersville)	Southwestern Appalachians	K	C
37	Salt River Cave	Franklin	TN	5	Tennessee (TN-Guntersville)	Southwestern Appalachians	K	C
38	Big Mouth Cave	Grundy	TN	4	Tennessee (Upper Elk)	Southwestern Appalachians	C	B
39	Crystal Cave	Grundy	TN	3	Tennessee (Upper Elk)	Southwestern Appalachians	C	B
40	Trussell Cave	Grundy	TN	1	Tennessee (Upper Elk)	Southwestern Appalachians	C	B
41	Cave Branch Cave	Hickman	TN	1	Tennessee (Buffalo)	Interior Low Plateau	F	F
42	Allens Creek Cave	Lewis	TN	1	Tennessee (Buffalo)	Interior Low Plateau	F	F
43	Lost Pig Cave	Marion	TN	1	Tennessee (TN-Guntersville)	Southwestern Appalachians	H	A

44	Pryor Cave Spring	Marion	TN	1	Tennessee (Sequatchie)	Southwestern Appalachians	H	A
45	Gallagher Cave South	Marshall	TN	2	Tennessee (Upper Duck)	Interior Low Plateau	D	B
46	Pompie Cave	Maury	TN	1	Tennessee (Upper Duck)	Interior Low Plateau	D	B
47	East Water Supply Cave	Overton	TN	1	Cumberland (Cumberland-Cordell Hull)	Interior Low Plateau	A	G
48	Anderson Spring Cave	Putnam	TN	2	Cumberland (Caney Fork)	Interior Low Plateau	A	G
49	Bartlett Cave	Putnam	TN	2	Cumberland (Cumberland-Cordell Hull)	Interior Low Plateau	A	H
50	Blind Fish Cave	Putnam	TN	2	Cumberland (Caney Fork)	Southwestern Appalachians	A	G
51	Jacque's Cave	Putnam	TN	3	Cumberland (Caney Fork)	Southwestern Appalachians	A	G
52	Stamp's Cave	Putnam	TN	2	Cumberland (Caney Fork)	Southwestern Appalachians	A	G
53	Sinking Ridge Cave	Robertson	TN	2	Cumberland (Red)	Interior Low Plateau	G	L
54	Herring Cave	Rutherford	TN	3	Cumberland (Stones)	Interior Low Plateau	F	D
55	Patton's Cave	Rutherford	TN	4	Cumberland (Stones)	Interior Low Plateau	F	D
56	Flat Rock Cave	Smith	TN	2	Cumberland (Cumberland-Old Hickory Lake)	Interior Low Plateau	D	B
57	Camps Gulf Cave	Van Buren	TN	1	Cumberland (Caney Fork)	Southwestern Appalachians	A	G
58	Camps Gulf Cave No. 2	Van Buren	TN	2	Cumberland (Caney Fork)	Southwestern Appalachians	A	G
59	Blowing Cave	Warren	TN	1	Cumberland (Collins)	Interior Low Plateau	E	G
60	Jaco Spring Cave	Warren	TN	3	Cumberland Collins)	Interior Low Plateau	F	B
61	Webb Cave	Simpson	KY	3	Cumberland (Red)	Interior Low Plateau	G	L
62	Friendship Cave	Warren	KY	2	Green (Upper Green)	Interior Low Plateau	G	L

Table 14. Genealogical sorting index (*gsi*) and *P* values of 11 delimited *Typhlichthys* species for gene trees based on the 60-individual, 6-gene dataset. *P* values are based on 10,000 permutations and are given in parentheses. Species highlight in gray occur in Kentucky.

Species	*nd2*	*s7*	*rag1*	*myh6*	*plagl2*	*tbr1*	All combined
S. poulsoni	1.0000 (0.0115)	1.0000 (0.0106)	1.0000 (0.0106)	1.0000 (0.0097)	1.0000 (0.0117)	1.0000 (0.0081)	1.0000 (0.0004)
A	0.7490 (<0.0001)	0.7490 (<0.0001)	0.7490 (<0.0001)	0.5293 (<0.0001)	0.5892 (<0.0001)	0.8588 (<0.0001)	0.7040 (0.0001)
B	1.0000 (<0.0001)	1.0000 (<0.0001)	1.0000 (<0.0001)	0.7617 (<0.0001)	0.5159 (<0.0001)	0.4801 (<0.0001)	0.7930 (0.0001)
C	0.2152 (0.0463)	0.7860 (<0.0001)	0.6433 (<0.0001)	0.4649 (0.0002)	1.0000 (<0.0001)	0.1676 (0.1233)	0.5462 (0.0001)
D	1.0000 (0.0005)	0.6554 (0.0014)	0.4831 (0.0037)	0.4831 (0.0038)	1.0000 (0.0003)	0.2246 (0.0462)	0.6410 (0.0004)
E	0.2615 (0.0193)	1.0000 (<0.0001)	0.1384 (0.2464)	0.1729 (0.0906)	0.1959 (0.0617)	0.0601 (0.9079)	0.3048 (0.0038)
F	0.3826 (0.0003)	0.3826 (0.0002)	0.4135 (<0.0001)	0.2668 (0.0062)	0.4134 (<0.0001)	0.3548 (0.0003)	0.3690 (0.0001)
G	1.0000 (<0.0001)	1.0000 (<0.0001)	1.0000 (<0.0001)	0.2638 (0.0136)	1.0000 (<0.0001)	0.4741 (0.0007)	0.7897 (0.0001)
H	1.0000 (0.0126)	1.0000 (0.0098)	1.0000 (0.0116)	0.1867 (0.1110)	0.3222 (0.0476)	0.4917 (0.0292)	0.6668 (0.0058)
I	0.2638 (0.0114)	0.3427 (0.0040)	0.2638 (0.0111)	0.1736 (0.0938)	0.2989 (0.0066)	0.1455 (0.1947)	0.2480 (0.0057)
J	0.2989 (0.0071)	0.5793 (0.0002)	0.3990 (0.0020)	0.3990 (0.0028)	0.3427 (0.0034)	1.0000 (<0.0001)	0.5031 (0.0002)
K	1.0000 (0.0003)	1.0000 (0.0004)	1.0000 (0.0002)	0.2246 (0.0377)	0.6554 (0.0018)	0.6554 (0.0011)	0.7559 (0.0002)

Table 15. Divergence times and probabilities of reconstructed ancestral areas for selected nodes labeled in Fig. 30, for the multilocus species tree phylogenies. Mean divergence times (Mya) are reported with 95% confidence intervals. Marginal probabilities of reconstructed ancestral areas are reported based on analyses in S-DIVA.

Analysis	1 Split between *Typhlichthys* and *Speoplatyrhinus*	2 MRCA of *Typhlichthys*	3 MRCA of *Typhlichthys* A–F [a]	4 MRCA of *Typhlichthys* I–M
Divergence Time (Mya)	6.69 (3.89–9.76)	2.32 (1.27–3.31)	1.49 (0.72–2.20)	2.08 (1.15–2.98)
S-DIVA: Watershed	96.0 Tenn 2.0 Tenn + Cumb 1.0 Tenn + White 1.0 Cumb + White	66.6 Tenn 23.7 Tenn + Cumb 6.7 Tenn + White 3.0 Cumb + White	83.2 Tenn 16.8 Tenn + Cumb	89.3 Cumb + White 7.7 Cumb + Osage 3.0 White + Osage
S-DIVA: Ecoregion	99.2 ILP 0.8 SWA + ILP	63.2 ILP 19.8 SWA + ILP 14.8 ILP + RV 2.3 SWA + RV	86.3 ILP 13.7 SWA + ILP	91.8 ILP + OZH 5.2 SWA + ILP 3.0 SWA + OZH

Table 16. Hierarchical analysis of molecular variance for three loci (*nd2*, *s7*, and *rag1*) sequenced for 142 individuals grouped according to a) hydrological basin, b) hydrological subbasin, and ecoregion (see Table 13). Significance is based on 10,000 permutations: *- <0.05, **- <0.01, *- < 0.001.**

A)

Locus	Source of variation	d.f.	SS	VC	V%	ϕ-statistics
nd2	Among basins	4	2313.828	19.803	37.94	ϕ_{CT}=0.379***
	Among populations within basins	55	3800.636	31.513	60.38	ϕ_{SC}=0.973***
	Within populations	75	65.571	0.874	1.68	ϕ_{ST}=0.983***
	Total	134	6180.034	50.286		
s7	Among basins	4	637.482	2.711	36.77	ϕ_{CT}=0.368***
	Among populations within basins	55	1076.152	4.476	60.70	ϕ_{SC}=0.960***
	Within populations	210	39.198	0.187	2.53	ϕ_{ST}=0.975***
	Total	269	1752.831	7.373		
rag1	Among basins	4	487.905	2.067	35.74	ϕ_{CT}=0.357***
	Among populations within basins	55	838.867	3.464	59.88	ϕ_{SC}=0.932***
	Within populations	210	53.214	0.253	4.38	ϕ_{ST}=0.956***
	Total	269	1379.986	5.784		

B)

Locus	Source of variation	d.f.	SS	VC	V%	ϕ-statistics
nd2	Among subbasins	21	5352.530	38.520	79.45	ϕ_{CT}=0.794***
	Among populations within subbasins	38	761.934	9.091	18.75	ϕ_{SC}=0.912***
	Within populations	75	65.571	0.874	1.90	ϕ_{ST}=0.982***
	Total	134	6180.034	48.485		
s7	Among subbasins	21	1485.164	5.299	77.17	ϕ_{CT}=0.772***
	Among populations within subbasins	38	228.470	1.381	20.11	ϕ_{SC}=0.881***
	Within populations	210	39.198	0.187	2.72	ϕ_{ST}=0.973***
	Total	269	1752.831	7.373		
rag1	Among subbasins	21	1070.860	3.584	66.71	ϕ_{CT}=0.667***
	Among populations within subbasins	38	255.912	1.535	28.58	ϕ_{SC}=0.858***
	Within populations	210	53.214	0.253	4.72	ϕ_{ST}=0.953***
	Total	269	1379.986	5.373		

C)

Locus	Source of variation	d.f.	SS	VC	V%	ϕ-statistics
nd2	Among ecoregions	3	1743.117	15.960	30.85	ϕ_{CT}=0.309***
	Among populations within ecoregions	56	4371.346	34.895	67.46	ϕ_{SC}=0.976***
	Within populations	75	65.571	0.874	1.69	ϕ_{ST}=0.983***
	Total	134	6180.034	48.485		
s7	Among ecoregions	3	442.676	1.967	27.17	ϕ_{CT}=0.272***
	Among populations within ecoregions	56	1270.957	5.088	70.26	ϕ_{SC}=0.965***
	Within populations	210	39.198	0.187	2.58	ϕ_{ST}=0.974***
	Total	269	1752.831	7.242		
rag1	Among ecoregions	3	377.813	1.729	30.04	ϕ_{CT}=0.300***
	Among populations within ecoregions	56	948.959	3.773	65.55	ϕ_{SC}=0.937***

Within populations	210	53.214	0.253	4.40	ϕ_{ST}=0.956***
Total	269	1379.986	5.531		

Table 17. Locality information, including cave, county, state, sample, hydrological basin and subbasin, and ecoregion, and haplotype numbers observed at each locality for 16 populations of *Amblyopsis spelaea*. Eleven populations (n = 36) were sampled north (N) and five populations (n = 36) south (S) of the Ohio River.

No.	Group	Locality	Abbrev.	County	State	n	Basin: Subbasin	Ecoregion	*nd2*	*s7*	*rag1*	*tbr*	*rho*
1	N	BB Hole	BBH	Crawford	IN	2	Lower Ohio: Blue-Sinking	Crawford-Mammoth Cave Uplands	1	1	1	1	1
2	N	Eric's River Cave	ERI	Crawford	IN	4	Lower Ohio: Blue-Sinking	Crawford-Mammoth Cave Uplands	3, 6	1	1	1	1
3	N	Marengo New Discovery	MND	Crawford	IN	3	Lower Ohio: Blue-Sinking	Mitchell Plain	1	1	1	1	1
4	N	Black Medusa Cave	BLM	Harrison	IN	5	Lower Ohio: Blue-Sinking	Mitchell Plain	1	1	1	1	1
5	N	Blue Springs Caverns	BLS	Lawrence	IN	11	Wabash: Lower East Fork White	Mitchell Plain	1	1	1	1	1
6	N	Donaldson Cave	DON	Lawrence	IN	3	Wabash: Lower East Fork White	Mitchell Plain	2	1	1	1	1
7	N	Henshaw Bend Cave	HSB	Lawrence	IN	1	Wabash: Lower East Fork White	Crawford-Mammoth Cave Uplands	5	1	1	1	1
8	N	Upper Twin Cave	UPT	Lawrence	IN	1	Wabash: Lower East Fork White	Mitchell Plain	7	1	1	1	1
9	N	Elrod Cave	ELR	Orange	IN	1	Wabash: Lower East Fork White	Mitchell Plain	1	2	1	1	1
10	N	Murray Spring Cave	MUR	Orange	IN	1	Wabash: Lower East Fork White	Mitchell Plain	1	1	2	1	1
11	N	Spring's Spring Cave	SPR	Orange	IN	4	Wabash: Patoka	Crawford-Mammoth Cave Uplands	1, 4	1	1, 3	1	1
12	S	Bandy Cave	BND	Breckinridge	KY	3	Lower Ohio: Blue-Sinking	Crawford-Mammoth Cave Uplands	10	2	1	2	2
13	S	Penitentiary Cave	PEN	Breckinridge	KY	11	Lower Ohio: Blue-Sinking	Crawford-Mammoth Cave Uplands	8	2	1, 4	1	2
14	S	Rimstone Cave	RIM	Breckinridge	KY	10	Lower Ohio: Blue-Sinking	Crawford-Mammoth Cave Uplands	8	2	1	1	2
15	S	Under the Road Cave	UTC	Breckinridge	KY	4	Lower Ohio: Blue-Sinking	Crawford-Mammoth Cave Uplands	8, 11	2	1, 4	1	2
16	S	Webster Cave	WEB	Breckinridge	KY	8	Lower Ohio: Blue-Sinking	Crawford-Mammoth Cave Uplands	8, 9	2	1	1, 3	2

Table 18. Uncorrected pairwise distances for the mtDNA *nd2* locus among 16 populations of *A. spelaea* sampled. Population abbreviations are those listed in Table 1. Region (north or south of the Ohio River) is indicated in parentheses. Distances between populations on opposite sides of the Ohio River are highlighted in grey.

	WEB (S)	RIM (S)	BND (S)	UTR (S)	PEN (S)	BBH (N)	MND (N)	BLM (N)	BLS (N)	UPT (N)	DON (N)	ELR (N)	MSP (N)	SPR (N)	HSB (N)
RIM (S)	0.001														
BND (S)	0.002	0.001													
UTR (S)	0.001	0.000	0.001												
PEN (S)	0.001	0.000	0.001	0.000											
BBH (N)	0.031	0.030	0.031	0.030	0.030										
MND (N)	0.031	0.030	0.031	0.030	0.030	0.000									
BLM (N)	0.031	0.030	0.031	0.030	0.030	0.000	0.000								
BLS (N)	0.031	0.030	0.031	0.030	0.030	0.000	0.000	0.000							
UPT (N)	0.034	0.033	0.034	0.033	0.033	0.003	0.003	0.003	0.003						
DON (N)	0.032	0.031	0.032	0.031	0.031	0.001	0.001	0.001	0.001	0.002					
ELR (N)	0.031	0.030	0.031	0.030	0.030	0.000	0.000	0.000	0.000	0.003	0.001				
MSP (N)	0.031	0.030	0.031	0.030	0.030	0.000	0.000	0.000	0.000	0.003	0.001	0.000			
SPR (N)	0.031	0.030	0.031	0.030	0.030	0.000	0.000	0.000	0.000	0.003	0.001	0.000	0.000		
HSB (N)	0.030	0.029	0.030	0.029	0.029	0.001	0.001	0.001	0.001	0.004	0.002	0.001	0.001	0.001	
ERI (N)	0.034	0.033	0.034	0.033	0.033	0.003	0.003	0.003	0.003	0.006	0.004	0.003	0.003	0.004	0.004

Table 19. Results of partial Mantel tests to test the partial correlation between genetic distance and potential barriers to dispersal after controlling for geographic distance (*r*). Significant *P*-values are denoted by an asterisk and indicate that a barrier restricts gene flow between populations of *A. spelaea*.

Barrier	*r*	*P*
Ohio River	0.99	<0.001
Hydrological subbasins	-0.67	<0.001
Hydrological subbasins (Blue-Sinking split)	0.04	0.38
Ecoregions	-0.11	0.09

Table 20. Hierarchical analysis of molecular variance for the mtDNA *nd2* locus grouped according to a) region (north and south of Ohio River), b) hydrological subbasin, and c) ecoregion (see Table 1). Significance is based on 10,000 permutations: *- *P* <0.05, **- *P* <0.01, *- *P* < 0.001.**

A)

Source of variation	d.f.	SS	VC	V%	ϕ-statistics
Among regions	1	558.514	15.438	96.74	ϕ_{CT}=0.967***
Among populations within regions	14	23.222	0.364	2.28	ϕ_{SC}=0.700***
Within populations	56	8.750	0.156	0.98	ϕ_{ST}=0.990***
Total	71	590.486	15.958		

B)

Source of variation	d.f.	SS	VC	V%	ϕ-statistics
Among basins	2	246.782	5.347	45.50	ϕ_{CT}=0.455*
Among populations within basins	13	334.954	6.249	53.17	ϕ_{SC}=0.976***
Within populations	56	8.750	0.156	1.33	ϕ_{ST}=0.987***
Total	71	590.486	11.752		

C)

Source of variation	d.f.	SS	VC	V%	ϕ-statistics
Among ecoregions	1	298.483	8.111	61.83	ϕ_{CT}=0.618*
Among populations within ecoregions	14	283.253	4.851	36.98	ϕ_{SC}=0.969***
Within populations	56	8.750	0.156	1.19	ϕ_{ST}=0.988***
Total	71	590.486			

Table 21. Genetic diversity and test statistics of selective neutrality within regions of *A. spelaea* populations north and south of the Ohio River for the mitochondrial *nd2* locus. Statistics were based on 36 individuals sampled for each group (72 individuals total). *K*: number of unique haplotypes, *S*: number of segregating sites, π: nucleotide diversity. Significance for neutrality tests were based on 10,000 permutations. *-*P* < 0.05.

	North	South	All
K	7	4	11
S	10	4	44
π	0.001157	0.000593	0.015932
Tajima's *D*	-1.52*	-0.88	2.72
Fu's *Fs*	-1.79	-0.58	15.56
R_2	0.07*	0.08	0.19

Table 22. The genealogical sorting index (*gsi*) for each group of *Amblyopsis spelaea* and each species of *Forbesichthys* for the five loci examined and for all loci combined. Significance is based on 10,000 permutations: * *P* < 0.05, ** *P* < 0.01, * *P* < 0.001.**

Species	*nd2*	*s7*	*rag1*	*rho*	*tbr1*	All combined
Forbesichthys agassizii	1.000**	1.000**	1.000**	1.000**	1.000***	1.000**
Forbesichthys papilliferus	1.000**	1.000**	1.000**	1.000***	1.000***	1.000***
Amblyopsis spelaea North	1.000***	1.000***	0.090	1.000***	0.948***	0.808***
Amblyopsis spelaea South	1.000***	0.948***	0.135*	1.000**	0.036	0.624***

Table 23. Locality information, including county, state, sample size, major hydrological basin, subbasin (in parentheses), ecoregion, and delimited species for nine populations of *Forbesichthys*. Populations from Kentucky are highlighted in gray.

No.	Locality	County	State	*n*	Basin (Subbasin)	Ecoregion	Delimited Species
1	Fults Pond	Coffee	TN	3	Tennessee (Upper Duck)	Eastern Highland Rim	*agassizii*
2	Jarrell's Spring	Coffee	TN	10	Tennessee (Upper Duck)	Eastern Highland Rim	*agassizii*
3	Rigsby Pond	Coffee	TN	21	Cumberland (Collins)	Eastern Highland Rim	*agassizii*
4	Blue Springs	DeKalb	TN	5	Cumberland (Caney Fork)	Eastern Highland Rim	*agassizii*
5	Mountain Creek	Warren	TN	1	Cumberland (Collins)	Eastern Highland Rim	*agassizii*
6	Pine Hills Swamp	Union	IL	1	Mississippi (Upper Mississippi-Cape Girardeau)	Southern Ozarkian River Bluffs	*papilliferus*
7	Cave Springs Cave	Union	IL	3	Mississippi (Upper Mississippi-Cape Girardeau)	Southern Ozarkian River Bluffs	*papilliferus*
8	Ditch off of Morton Road	Todd	KY	11	Cumberland (Red)	Western Pennyroyal Karst	*papilliferus*
9	Rich Pond	Warren	KY	15	Green (Barren)	Western Pennyroyal Karst	*papilliferus*

Table 24. Museum accessions for amblyopsid cavefishes (*Forbesichthys papilliferus*, *Amblyopsis spelaea* and *Typhlichthys subterraneus*) collected in Kentucky. Museums queried are listed in Appendix 3.

Accession No.	Collection	Species	Date	Collector	Locality	County	State	Count	Type	Notes
50129	INHS	*A. spelaea*	1999		Tributary to Sinking Creek	Breckinridge	KY		EtOH	
179789	USNM	*A. spelaea*	21-Sep-57	Clay, et al.	Near Rosetta	Breckinridge	KY	1	EtOH and Radiograph	
7961	ANSP	*A. spelaea*		Mitchill, J.E.	Mammoth Cave	Edmonson	KY	1	EtOH	
7962	ANSP	*A. spelaea*		Darley, J.	Mammoth Cave	Edmonson	KY	1	EtOH	
7963	ANSP	*A. spelaea*		Graff, C.H. et al.	Mammoth Cave	Edmonson	KY	1	EtOH	
20737	ANSP	*A. spelaea*		Frederick, Mrs.	Mammoth Cave	Edmonson	KY	1	EtOH	
7964	ANSP	*A. spelaea*		Craig, W.T.	Mammoth Cave	Edmonson	KY	2	EtOH	
7966	ANSP	*A. spelaea*		Lambert, J.	Mammoth Cave	Edmonson	KY	2	EtOH	
1322	CUMV	*A. spelaea*	27 Dec 1875	Sloan, J.	Mammoth Cave	Edmonson	KY	1	EtOH	
1636	CUMV	*A. spelaea*			Mammoth Cave	Edmonson	KY	1	EtOH	
60573	INHS	*A. spelaea*	1872			Edmonson	KY		EtOH	
779	MCZ	*A. spelaea*			Mammoth Cave	Edmonson	KY	1	EtOH	
6595	MCZ	*A. spelaea*			Mammoth Cave	Edmonson	KY	1	EtOH	
30678	MCZ	*A. spelaea*	07 Nov 1874	Putnam, F.W.	Mammoth Cave	Edmonson	KY	1	EtOH	
37173	MCZ	*A. spelaea*	1949		Mammoth Cave	Edmonson	KY	1	EtOH	
31080	MCZ	*A. spelaea*		Loring, David L	Mammoth Cave	Edmonson	KY	3	EtOH	
35056	MCZ	*A. spelaea*	07 Nov 1874	Putnam, F.W.	Mammoth Cave	Edmonson	KY	5	EtOH	
31078	MCZ	*A. spelaea*		Wyman collection	Mammoth Cave	Edmonson	KY	6	EtOH	
778	MCZ	*A. spelaea*			Mammoth Cave	Edmonson	KY	8	EtOH	
8046	ROM	*A. spelaea*		Sloan, J.	Mammoth Cave	Edmonson	KY	1	EtOH	
NRM 8000	SMNH	*A. spelaea*	24 Dec 1876	Lindahl, J.	Mammoth Cave	Edmonson	KY	1	EtOH	
NRM 8001	SMNH	*A. spelaea*		Fr. Bromer	Mammoth Cave	Edmonson	KY	1	EtOH	
NRM 8380	SMNH	*A. spelaea*	18 Jun 1853		Mammoth Cave	Edmonson	KY	2	EtOH	
8913	TU	*A. spelaea*	30 Dec 1899		Mammoth Cave	Edmonson	KY	1	EtOH	
F.7255	UCZM	*A. spelaea*			Mammoth Cave	Edmonson	KY	1	EtOH	
146991	UMMZ	*A. spelaea*	31-Aug-39	Hubricht, L.	Stream in Cedar Sink Cave, near Mammoth Cave	Edmonson	KY	1	EtOH	
44435	USNM	*A. spelaea*	1893	Ganter, H.	Mammoth Cave	Edmonson	KY	1	EtOH	
48867	USNM	*A. spelaea*			Mammoth Cave	Edmonson	KY	1	EtOH	
127056	USNM	*A. spelaea*	1 Sep 1901	Hay, W.	Mammoth Cave, Roaring River	Edmonson	KY	1	EtOH	

5863	USNM	*A. spelaea*			Mammoth Cave	Edmonson	KY	2	EtOH	
127055	USNM	*A. spelaea*	15-May-05	Eigenmann, Carl H.	Mammoth Cave	Edmonson	KY	3	EtOH	
237004	USNM	*A. spelaea*			Mammoth Cave, Echo River	Edmonson	KY	4	EtOH	
237001	USNM	*A. spelaea*			Mammoth Cave, Echo River	Edmonson	KY	7	EtOH	
4147	KU	*A. spelaea*		Minckley, W.L. et al.	Cave 5 mi E of Big Springs	Meade	KY	3	EtOH	37.7995, -86.0623
2040	MOSU	*A. spelaea*	16-Jan-81		Unnamed cave 2000 ft N of Grahampton on Fort Knox Military Reservation	Meade	KY	1	EtOH	
180644	UMMZ	*A. spelaea*	2-Dec-61	Kuehne, R.A.	Sig Shacklett's Cave, 3 mi due N of Big Spring	Meade	KY	6	EtOH	
109164	ANSP	*A. spelaea*		Hyrtl Collection			KY	1	Skeleton	
12844	KU	*F. papilliferus*		Hibbard, C.W.		Barren	KY	2	EtOH	
51051	SIUC	*F. papilliferus*	16-Apr-02		Piney Creek, 0.15 km above an unnamed tributary near Haile Road ford	Caldwell	KY	1	EtOH	
2232	MOSU	*F. papilliferus*	8-Jun-04		Rush Creek (Crooked Creek), 0.4 km below US 60 and KY 641	Crittenden	KY	1	EtOH	
57170	CAS	*F. papilliferus*	Nov 1898	Eigenmann, Carl H.	Mammoth Cave	Edmonson	KY	3	EtOH	
43978	FMNH	*F. papilliferus*	23-Oct-47	Winn, J.W., Winn, M.E.K.	Mammoth Cave National Park, Wet Prong, Buffalo Creek	Edmonson	KY	6	EtOH and CS	5 in EtOH, 1 CS
60745	INHS	*F. papilliferus*	1991		Tributary to Beaverdam Creek	Edmonson	KY		EtOH	
27624	MCZ	*F. papilliferus*			Dismal Creek, near Bee Spring	Edmonson	KY	1	EtOH	
31079	MCZ	*F. papilliferus*	07 Nov 1874	Putnam, F.W.	Mammoth Cave	Edmonson	KY	1	EtOH	
31082	MCZ	*F. papilliferus*	07 Feb 1897	Eigenmann, Carl H.		Jackson	KY	1	EtOH	No Jackson Co. in Kentucky; most likely Jackson Co., IL
174210	FLMNH	*F. papilliferus*	17-Apr-09	Thomas, M.R., Page, L.M.		Livingston	KY	1	EtOH	
128567	FLMNH	*F. papilliferus*	20-Apr-03	Page, L.M.	Hazel Creek - Route 917 bridge 5 miles north Iuka	Livingston	KY	3	EtOH	
62043	FMNH	*F. papilliferus*	13-Oct-51	Woods, L.P.	Cave spring across river (Ohio) from Golconda	Livingston	KY	1	CS	
62042	FMNH	*F. papilliferus*	13-Oct-51	Woods, L.P.	Cave spring mouth	Livingston	KY	4	EtOH	
32127	INHS	*F. papilliferus*	1964		Richland Creek	Livingston	KY		EtOH	
51614	INHS	*F. papilliferus*	1999		Hazel Creek	Livingston	KY		EtOH	
76939	INHS	*F. papilliferus*	1977		Deer Creek	Livingston	KY		EtOH	

1345	MOSU	*F. papilliferus*	12-May-99		Ferguson Creek at KY 70	Livingston	KY	2	EtOH	
10706	AUM	*F. papilliferus*	1-Sep-72	Bauer, Bruce	Whippoorwill Creek	Logan	KY	2	EtOH	
60771	INHS	*F. papilliferus*	1991		Tributary to Blue Spring Creek	Metcalfe	KY		EtOH	
58174	INHS	*F. papilliferus*	1990		Sharps Branch	Simpson	KY		EtOH	
51625	INHS	*F. papilliferus*	1999		Tributary to West Fork Red River	Todd	KY		EtOH	
76940	INHS	*F. papilliferus*	1977		Spring Creek	Todd	KY		EtOH	
55874	INHS	*F. papilliferus*	2000		Donaldson Creek	Trigg	KY		EtOH	
64763	INHS	*F. papilliferus*	1989		Donaldson Creek	Trigg	KY		EtOH	
1959-0082.1	CMN	*F. papilliferus*	31-Mar-57			Warren	KY	1	EtOH	36.75,-86.5
1982-0399.1	CMN	*F. papilliferus*	24-May-82	Pearson, W.D., Clay, W.M.		Warren	KY	10	EtOH	36.833, -86.5
30975	CUMV	*F. papilliferus*	18-Feb-56	Clay, W.M., Montgomery, J.	2 mi S of Rich Pond, 7 mi S & 2 mi W of Bowling Green	Warren	KY	12	EtOH	
14627	FLMNH	*F. papilliferus*	15-May-56	Bailey, R.M., Brudon, W.L.	Sinks of stream 2.4 miles N of Woodburn.	Warren	KY	22	EtOH	
2156	MOSU	*F. papilliferus*	11-Mar-80		Ditch under KY 884	Warren	KY	2	EtOH	
38041	OKMNH	*F. papilliferus*	27-Feb-69	Prins		Warren	KY	7	EtOH	
40658	OKMNH	*F. papilliferus*	6-Mar-65	Hill	3 mi E, 1 mi S of Rich Pond	Warren	KY	537	EtOH	
83867	TU	*F. papilliferus*	8-Apr-71	Baker et al.	Rich Pond, 5 mi SW of Bowling Green, KY	Warren	KY	1	EtOH	
11613	TU	*F. papilliferus*	4-Jun-55	Clay, B.	Rich Pond, 5 mi SW of Bowling Green, KY	Warren	KY	26	EtOH	
F1635	UAMZ	*F. papilliferus*	Apr-67	Nelson, J.S.	Bowling Green	Warren	KY	3	EtOH	
177561	UMMZ	*F. papilliferus*	15-May-56	Bailey, R.M., Brudon, W.L.	Sinks and casual sink stream, 2.4 mi N of Woodburne, Barren River	Warren	KY	1006	EtOH	
66255	UMMZ	*F. papilliferus*		Lancaster, L.Y.	Near Bowling Green		KY	3	EtOH	
198929	USNM	*F. papilliferus*	7-Aug-64	Puckett & H. Shadowen	Lost River Cave, 3 mi S of Bowling Green		KY	2	EtOH	
35665	FLMNH	*T. subterraneus*	29-Jun-82	Leitheuser, A.T.	L and N Cave, Old Bardstown Road, 0.2 miles S of junction with US 31 W, Cave City.	Barren	KY	2	EtOH	
ICH.020461	YPM	*T. subterraneus*	24-Aug-65	Norton, R.M.	Cave City	Barren	KY	1	EtOH	
SU25283	CAS	*T. subterraneus*	Sep-05		Mammoth Cave-River Styx	Edmonson	KY	1	EtOH	
1958-0005.1	CMN	*T. subterraneus*	Aug 1863	Stevenson, F.J.		Edmonson	KY	2	EtOH	37.187, -86.097
21726	CUMV	*T. subterraneus*	1952		Mammoth Cave	Edmonson	KY	2	EtOH	37.1883, -86.1053
32994	FLMNH	*T. subterraneus*	2-Feb-82	Leitheuser, A.T.	Mammoth Cave National Park	Edmonson	KY	1	EtOH	

					; River Styx at confluence of run from Charon's Cascade.					
62050	FMNH	*T. subterraneus*	14-Nov-50	Woods, L.P., Inger, R.F.	Mammoth Cave	Edmonson	KY	1	EtOH	
62051	FMNH	*T. subterraneus*	15-Nov-50	Woods, L.P., Inger, R.F.	Mammoth Cave National Park, Stillhouse Hollow Cave	Edmonson	KY	6	EtOH	
12853	KU	*T. subterraneus*				Edmonson	KY	6	EtOH and Radiograph	5 EtOH, 1 Radiograph
35058	MCZ	*T. subterraneus*	Sep 1859	Hyatt, Alpheus	Mammoth Cave	Edmonson	KY	1	EtOH	
780	MCZ	*T. subterraneus*	Sep 1859	Hyatt, Alpheus	Mammoth Cave	Edmonson	KY	7	EtOH	
6268	TU	*T. subterraneus*	20-Aug-38	Viosca, P.	Mammoth Cave	Edmonson	KY	1	EtOH	
36806	USNM	*T. subterraneus*	1884		Mammoth Cave	Edmonson	KY	1	EtOH	
101172	USNM	*T. subterraneus*		Hay, W.	Mammoth Cave, Roaring River	Edmonson	KY	3	EtOH	
45490	USNM	*T. subterraneus*	1894	Owsley, E.	Mammoth Cave	Edmonson	KY	6	EtOH	
78370	CAS	*T. subterraneus*	1 Dec 1902	Eigenmann, Carl H.	Horse Cave	Hart	KY	1	EtOH	Holotype of T. osborni
91981	CAS	*T. subterraneus*	2 Dec 1902	Eigenmann, Carl H.	Horse Cave	Hart	KY	1	EtOH	Syntype of T. osborni
91982	CAS	*T. subterraneus*		Eigenmann, Carl H.	Horse Cave	Hart	KY	2	EtOH	Syntype of T. osborni
91980	CAS	*T. subterraneus*	2 Dec 1902	Eigenmann, Carl H.	Horse Cave	Hart	KY	4	EtOH	Syntype of T. osborni
696	FLMNH	*T. subterraneus*	1938		Horse Cave	Hart	KY	1	EtOH	Listed as T. osborni
3871	FMNH	*T. subterraneus*	1900	Farrington, O.C.	Horse Cave	Hart	KY	2	EtOH	
88027	UMMZ	*T. subterraneus*	28-Aug-29	Creaser & Becker	Hidden River Cave at Horse Cave	Hart	KY	1	EtOH	
2041	MOSU	*T. subterraneus*	1-May-81		Big Sulphur Spring Cave, 2.5 mi NNE of Roaring Spring	Trigg	KY	1	EtOH	
148699	ANSP	*T. subterraneus*	1937	Merrill, G.P.	Mitchell's Cave			1	EtOH	
91983	CAS	*T. subterraneus*	Dec 1902	Eigenmann, Carl H.	Mitchell's Cave, Glasgow		KY	1	EtOH	
91984	CAS	*T. subterraneus*	Dec 1902	Eigenmann, Carl H.	Mitchell's Cave, Glasgow		KY	1	EtOH	
91985	CAS	*T. subterraneus*	1 May 1900	Eigenmann, Carl H.	Cave City		KY	1	EtOH	
91986	CAS	*T. subterraneus*			Cave City, Glasgow, or Mammoth Cave		KY	1	EtOH	
91987	CAS	*T. subterraneus*	1873	Putnam, F.W.	Small cave near Mammoth Cave		KY	1	EtOH	
62047	FMNH	*T. subterraneus*		Smith, P., Huston	Floyd Collins Crystal Cave		KY	2	EtOH	

62046	FMNH	*T. subterraneus*	13-Nov-50	Woods, L.P., Inger, R.F.	Hidden River Cave, Horse Cave	KY	14	EtOH and CS	10 in EtOH, 4 CS
157008	UMMZ	*T. subterraneus*		Eigenmann, Carl H.	Mitchell's Cave, Glasgow	KY	2	EtOH	
8563	USNM	*T. subterraneus*			Well near Bowling Green	KY	3	EtOH	

Figure 1. The Northern Spring Cavefish (*F. papilliferus*) from Todd County, Kentucky. Photo by Dante Fenolio.

Figure 2. The Southern Cavefish (*Typhlichthys subterraneus*) from L & N Railroad Cave, Barren County, Kentucky. Photo by Dante Fenolio.

Figure 3. The Kentucky Cavefish (*Typhlichthys sp. nov. 1*) from Drowned Rat Cave, Pulaski County, Kentucky. Photo by Dante Fenolio.

Figure 4. The Northern Cavefish (*Amblyopsis spelaea*) from Webster Cave, Breckinridge County, Kentucky. Photo by Dante Fenolio.

Figure 5. Distribution by county of the Amblyopsidae in the eastern United States. Southern Cavefish (*Typhlichthys sp*.), Northern Cavefish (*Amblyopsis spelaea*) and Northern Spring Cavefish (*Forbesicthys papilliferus*) occur in Kentucky (from Niemiller and Poulson 2010).

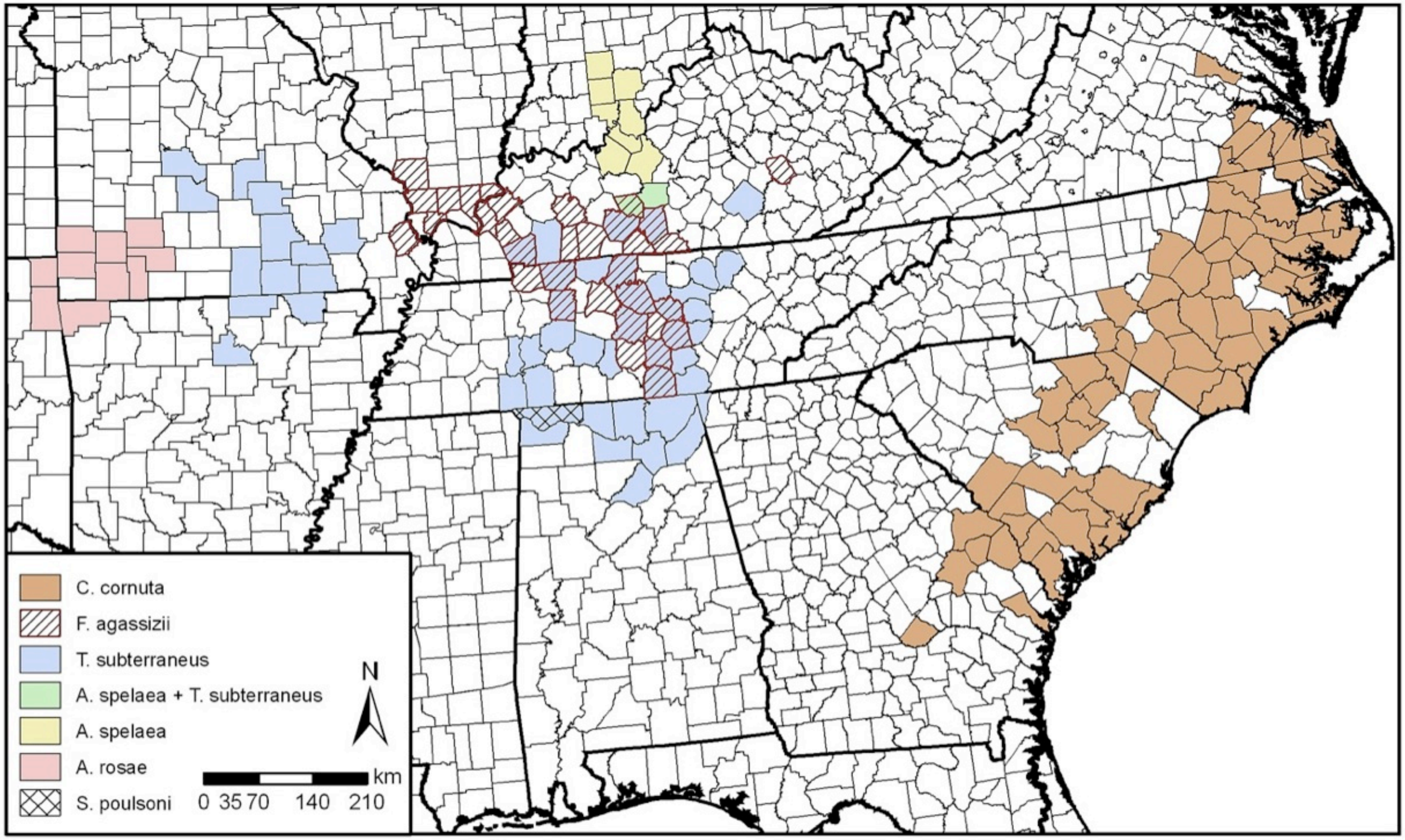

Figure 6. The distribution of the Northern Spring Cavefish (*Forbesichthys papilliferus*) in Kentucky. Northern Spring Cavefish have been reported from at least 48 localities, including at least five cave streams, in 17 counties (denoted by white circles). Counties in darker gray have confirmed locality records.

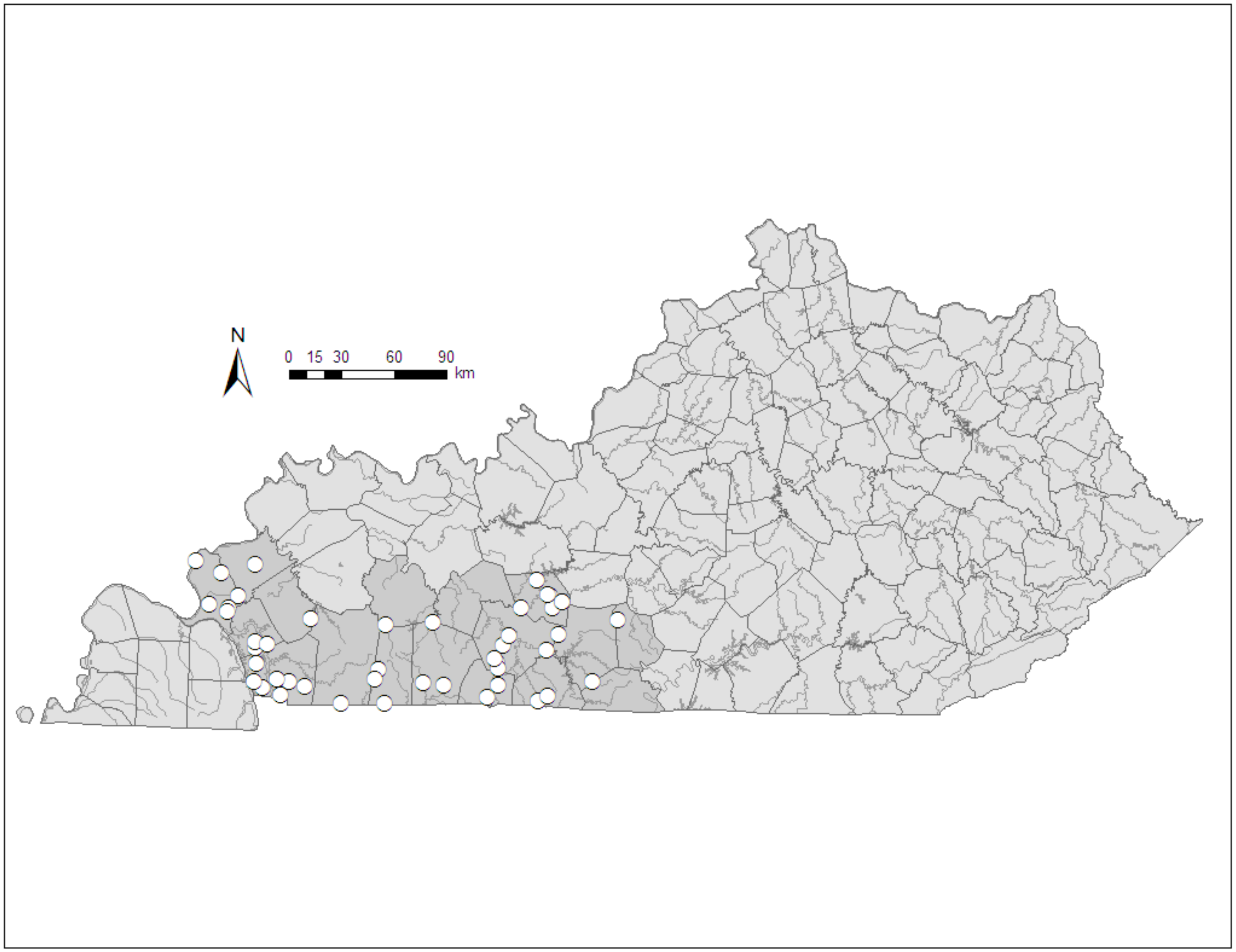

Figure 7. The distribution of the Northern Spring Cavefish (*Forbesichthys papilliferus*) in ecoregions of Kentucky. Northern Spring Cavefish have been reported from five ecoregions, but primarily have been reported from the Western Pennyroyal Karst and Western Highland Rim.

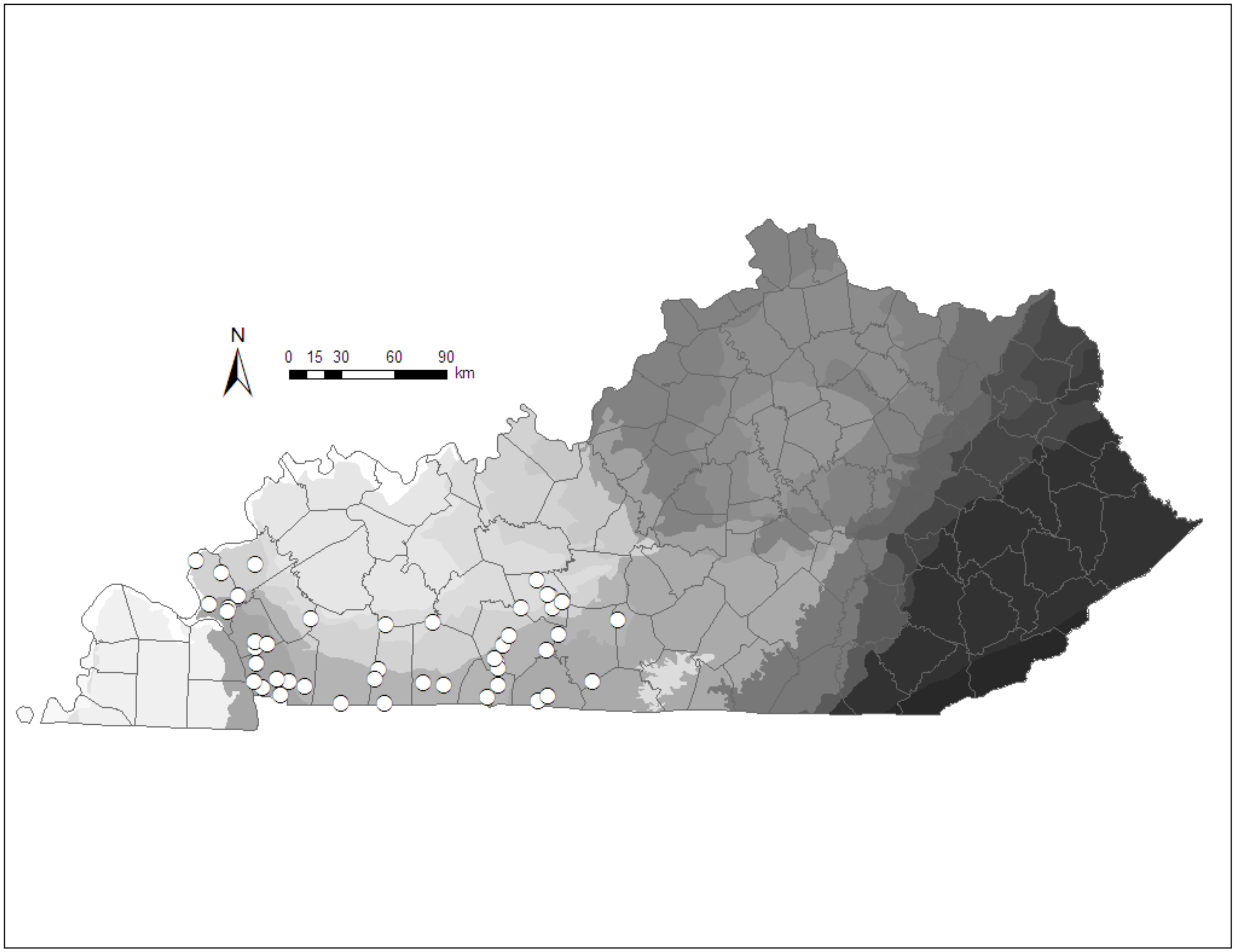

Figure 8. The distribution of the Northern Spring Cavefish (*Forbesichthys papilliferus*) in HUC8 watersheds of Kentucky. Northern Spring Cavefish have been reported from nine watersheds, but primarily have been reported from the Lower Cumberland River subbasin of the Cumberland River basin and the Barren River subbasin of the Green River basin.

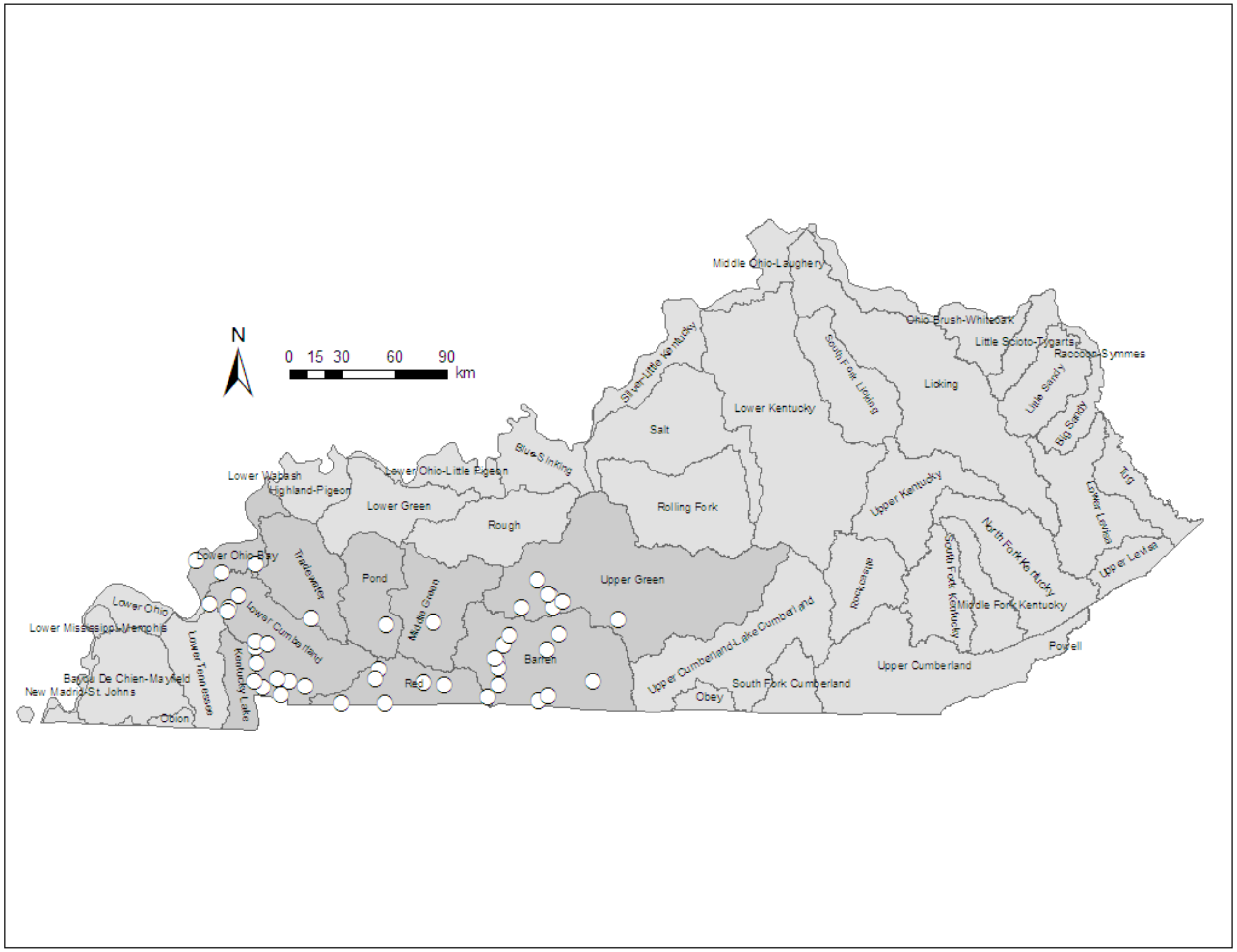

Figure 9. The distribution of the Southern Cavefish (*Typhlichthys sp.*) in Kentucky. Southern Cavefish have been reported from 27 caves, one spring, and one well in eight counties (denoted by white circles). Counties in darker gray have confirmed locality records.

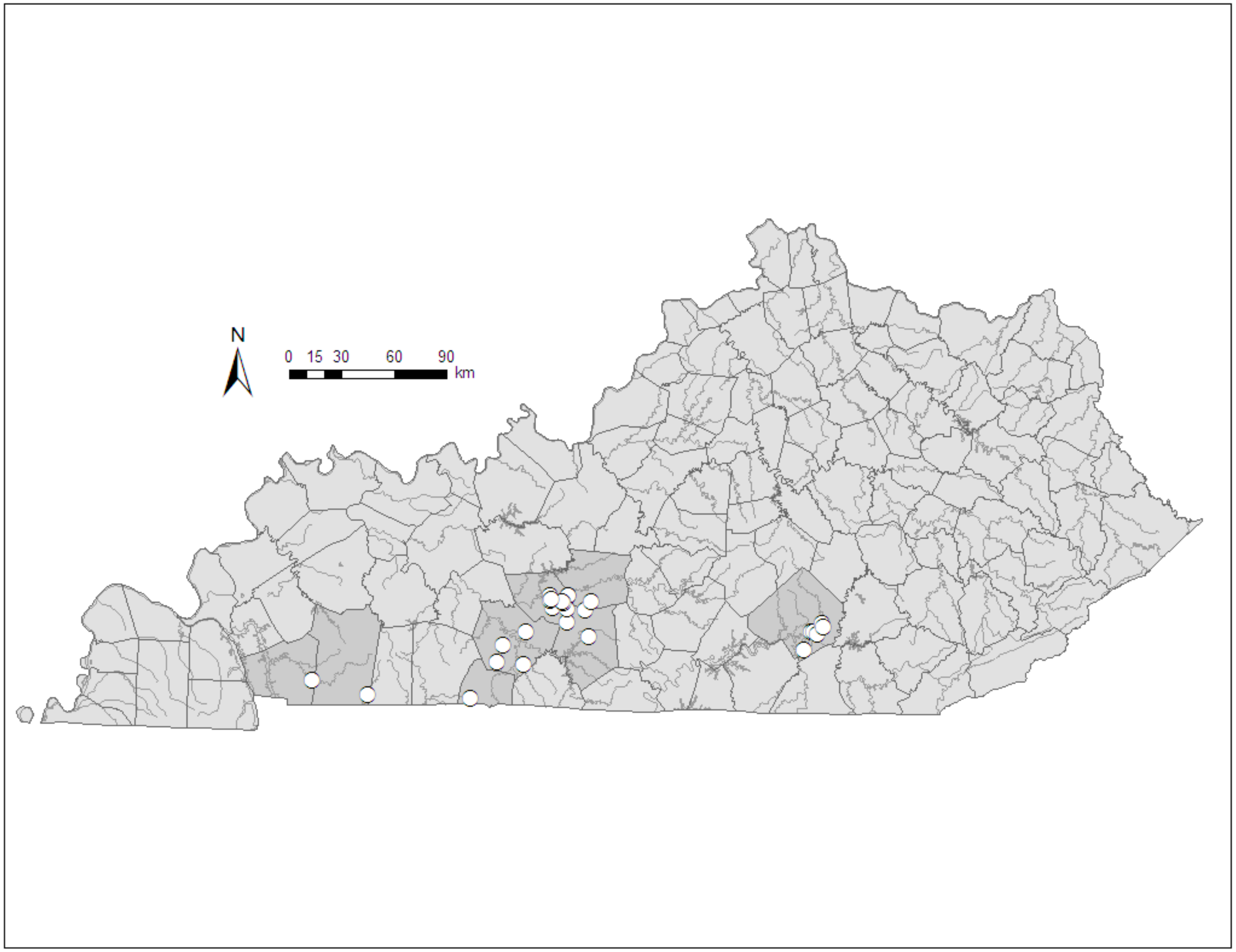

Figure 10. The distribution of the Southern Cavefish (*Typhlichthys sp.*) in ecoregions of Kentucky. Southern Cavefish have been reported from four ecorgions, but primarly have been reported from the Western Pennyroyal Karst and Crawford-Mammoth Cave Uplands. The Kentucky Cavefish (*Typhlichthys sp. nov. 1*) is known from six localities in the Plateau Escarpment of the Southwestern Appalachians.

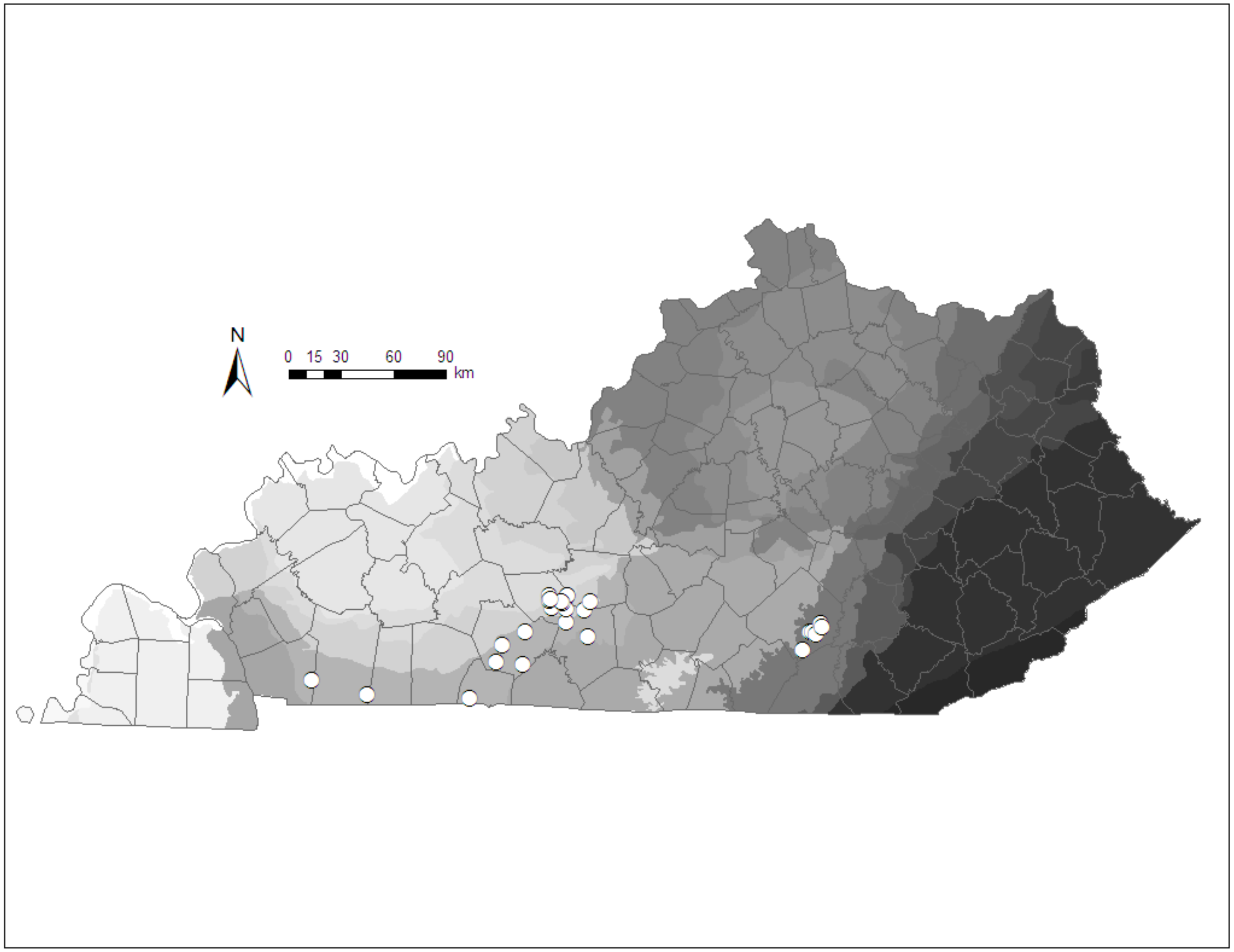

Figure 11. The distribution of the Southern Cavefish (*Typhlichthys sp.*) in HUC8 watersheds of Kentucky. Southern Cavefish have been reported from five watersheds, but primarily have been reported from the Upper Green River and Barren River subbasins of the Green River basin. The Kentucky Cavefish (*Typhlichthys sp. nov. 1*) is known from six localities in the Upper Cumberland-Lake Cumberland subbasin of the Cumberland River basin.

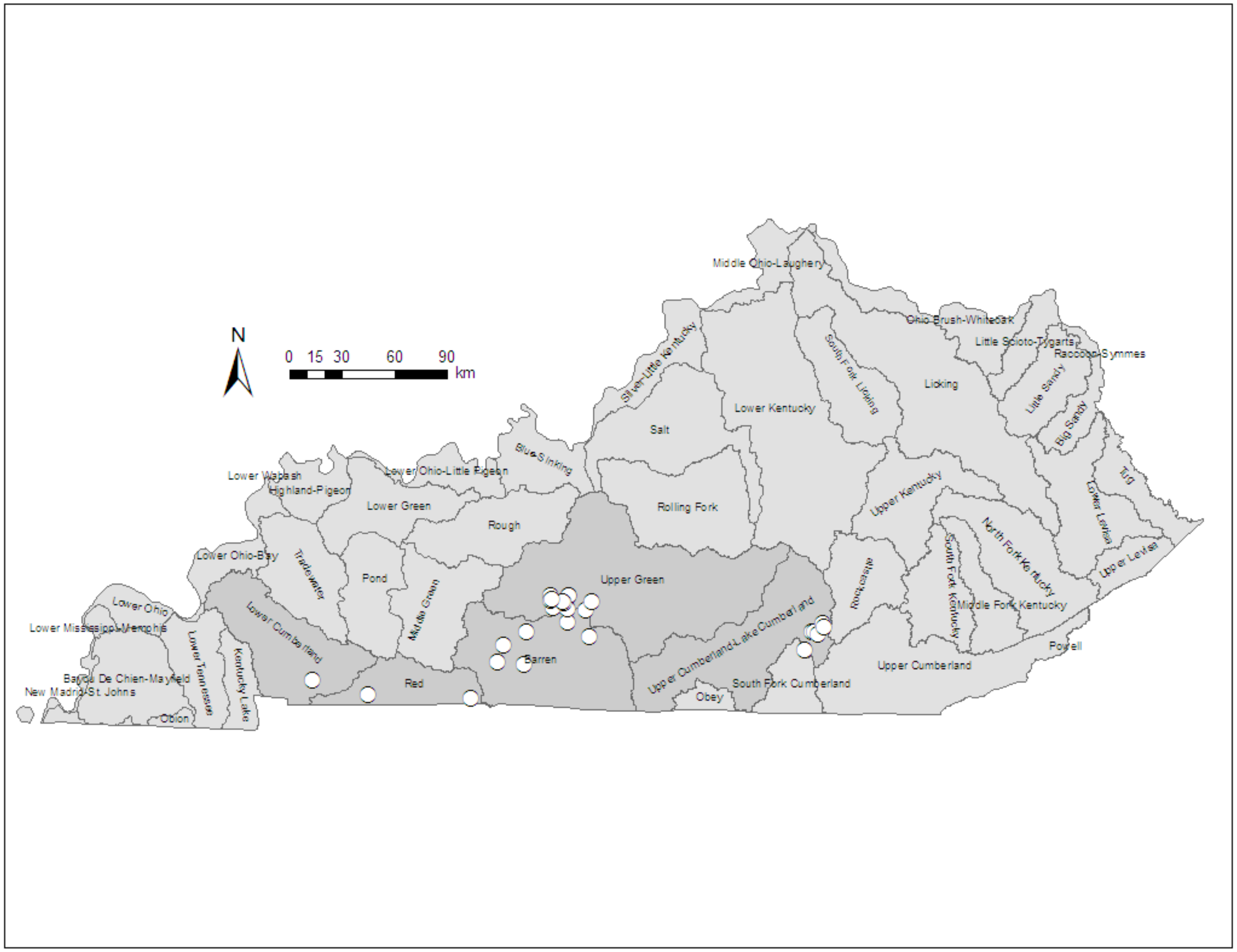

Figure 12. The distribution of the Northern Cavefish (*Amblyopsis spelaea*) in Kentucky. Northern Cavefish have been reported from 38 caves and one spring in five counties (denoted by white circles). Counties in darker gray have confirmed locality records.

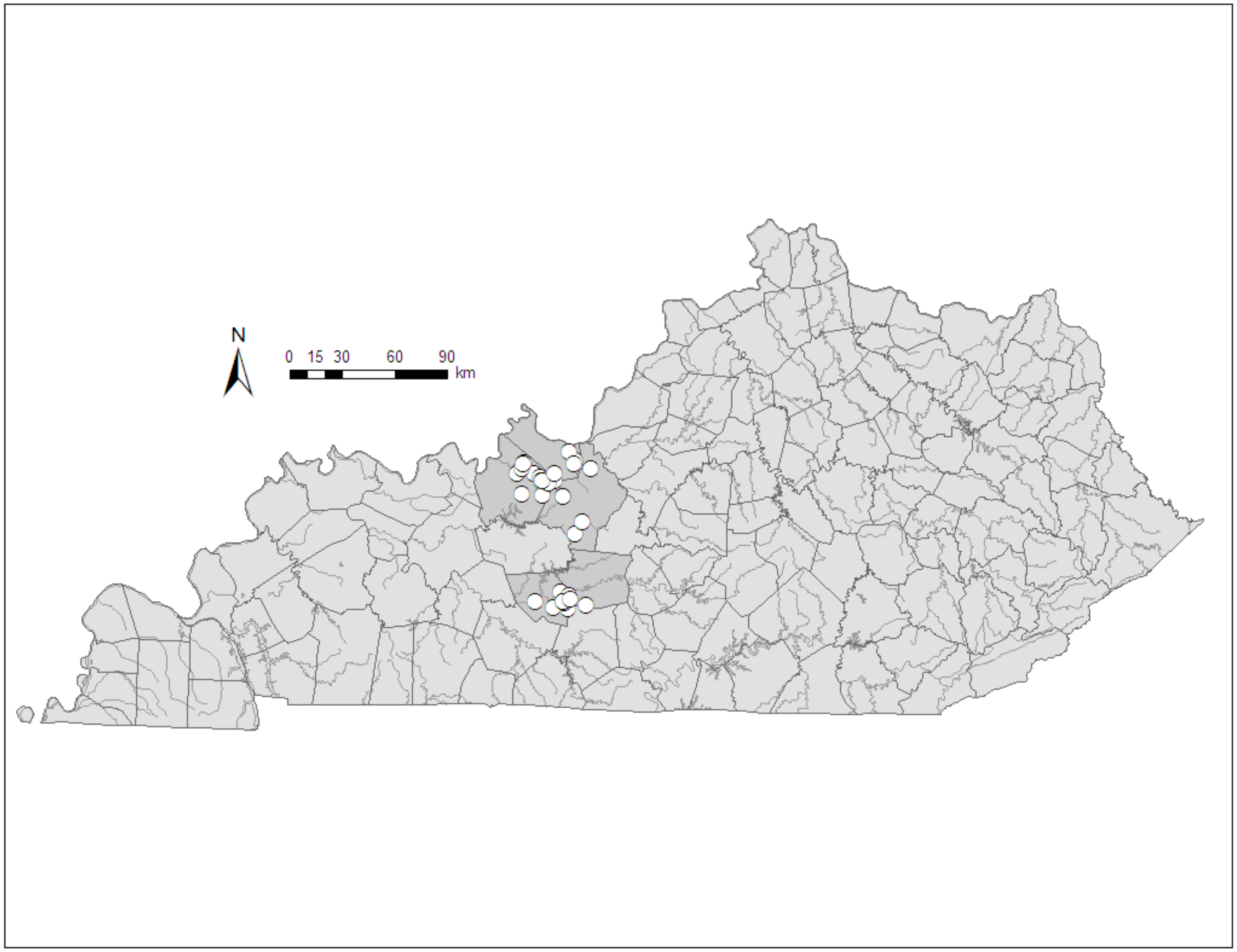

Figure 13. The distribution of the Northern Cavefish (*Amblyopsis spelaea*) in ecoregions of Kentucky. Northern Cavefish have been reported from three ecorgions, but primarily have been reported from the Crawford-Mammoth Cave Uplands and Mitchell Plain.

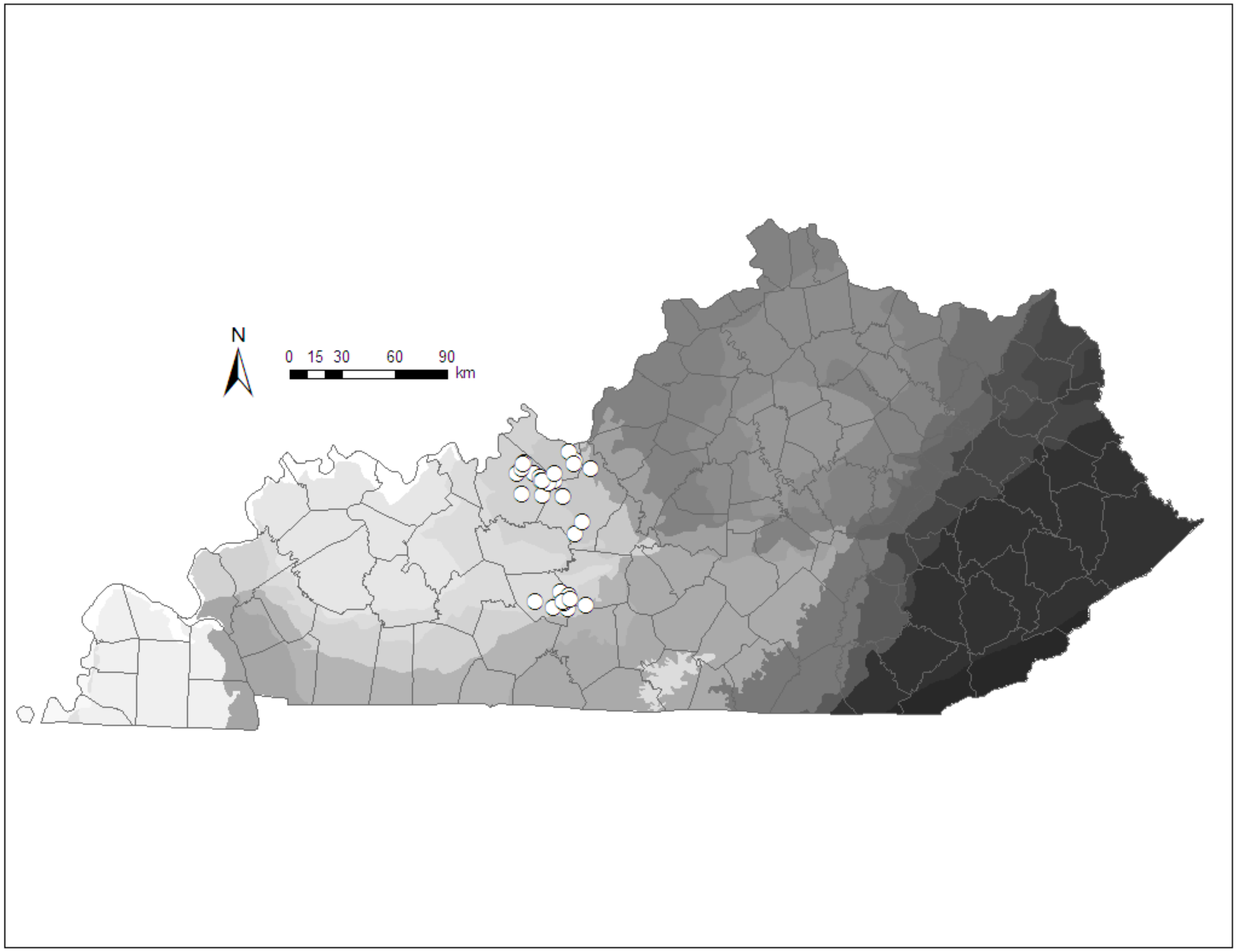

Figure 14. The distribution of the Northern Cavefish (*Amblyopsis spelaea*) in HUC8 watersheds of Kentucky. Northern Cavefish have been reported from three watersheds, but primarily have been reported from the Blue-Sinking subbasin of the Lower Ohio River basin and the Upper Green River subbasin of the Green River basin in the Mammoth Cave area.

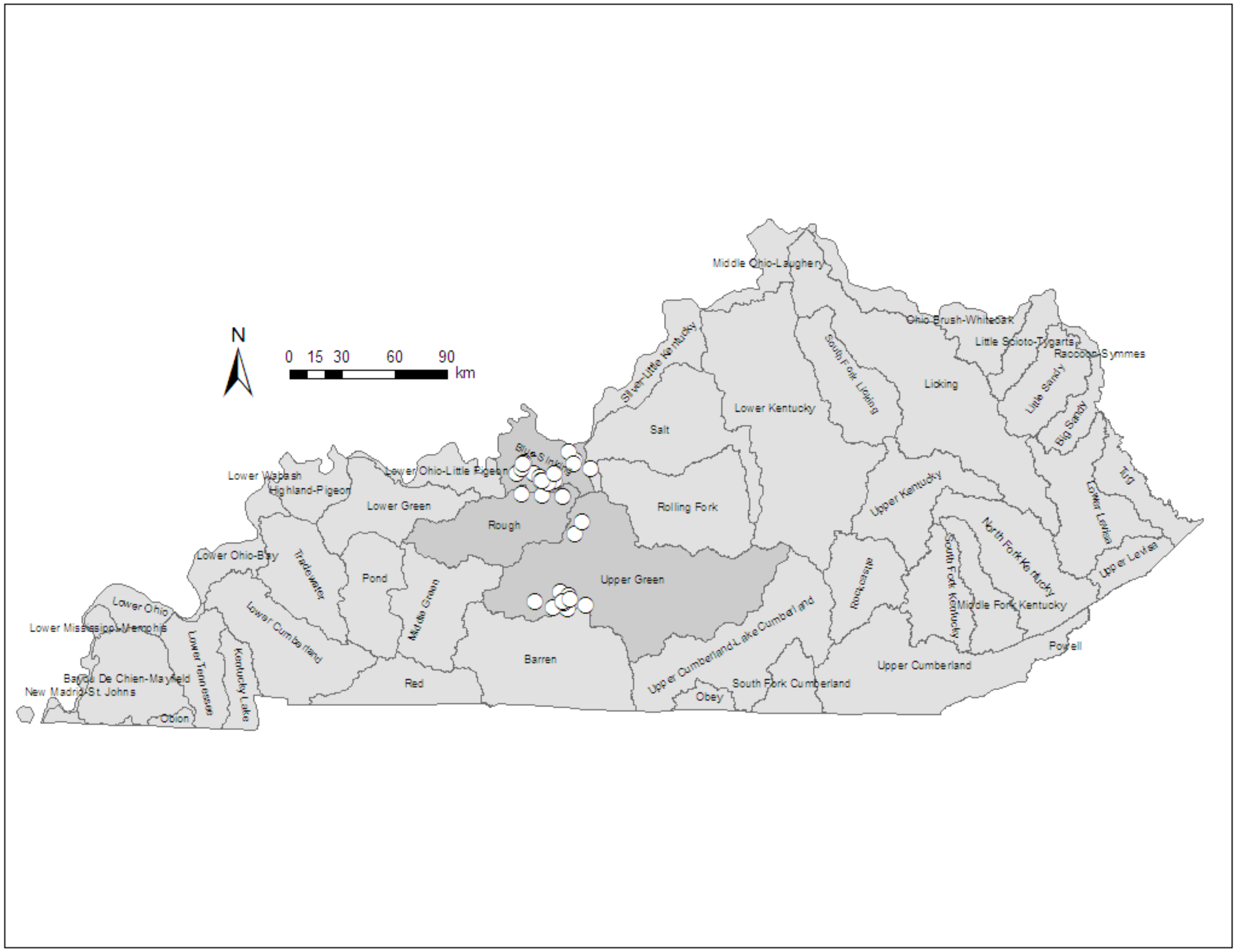

Figure 15. The distributions of the Southern Cavefish (*Typhlichthys sp.*) and Northern Cavefish (*Amblyopsis spelaea*) in Kentucky. Southern Cavefish localities are represented by black circles, Northern Cavefish localities by white circles, and localities were both species occur are represented by gray circles. Counties in darker gray have confirmed locality records.

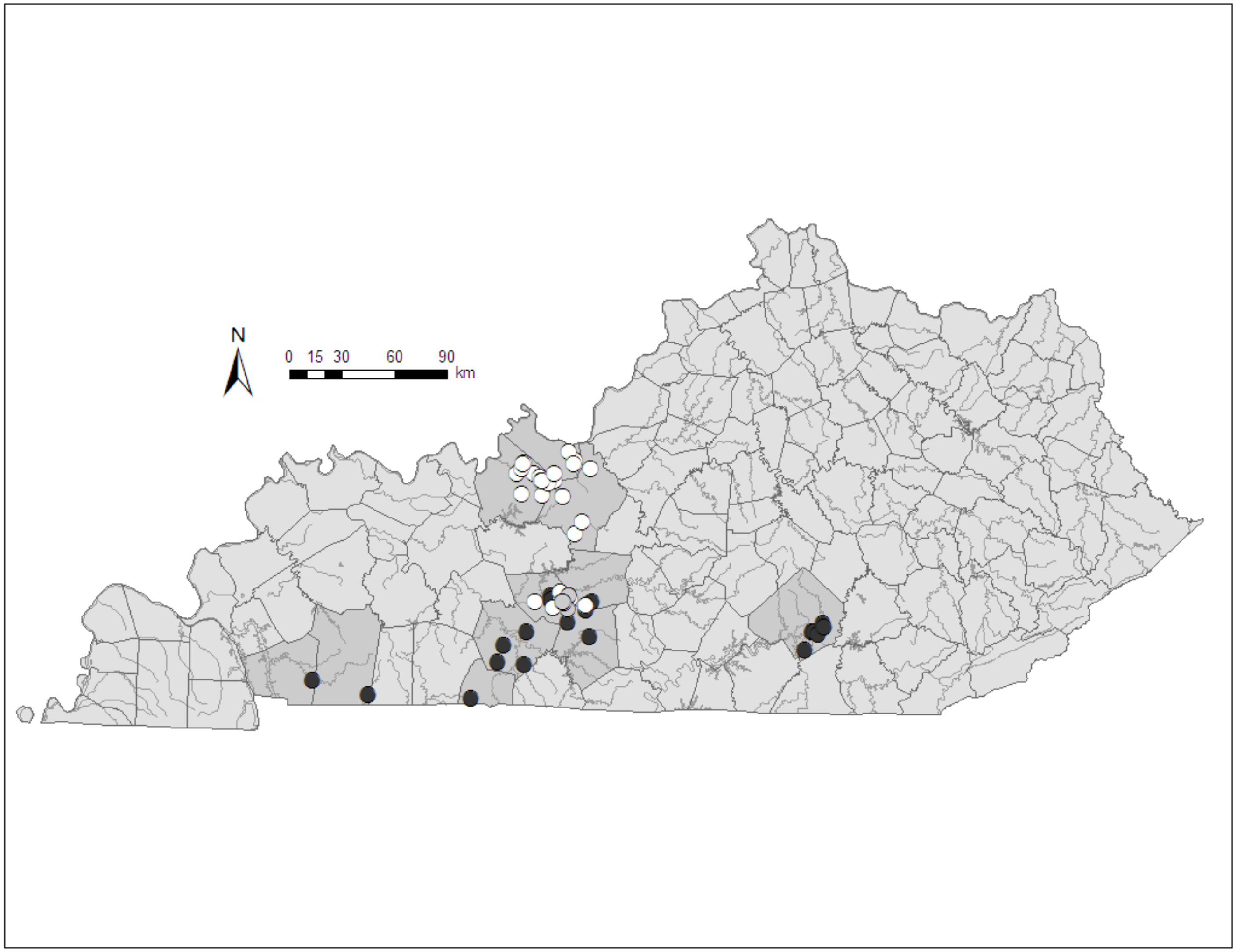

Figure 16. The distributions of the Southern Cavefish (*Typhlichthys sp.*) and Northern Cavefish (*Amblyopsis spelaea*) in ecoregions of Kentucky. Southern Cavefish localities are represented by black circles, Northern Cavefish localities by white circles, and localities were both species occur are represented by gray circles.

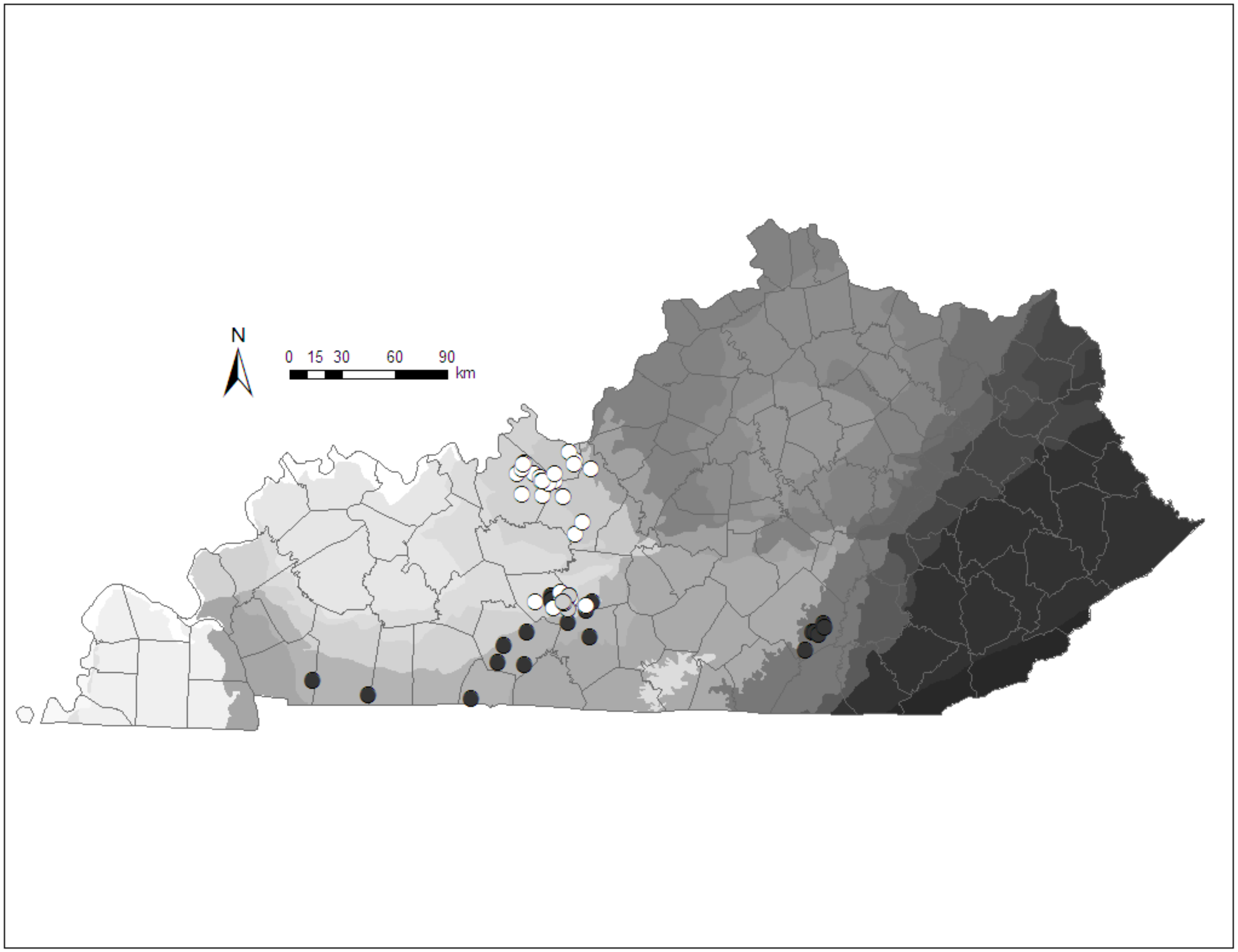

Figure 17. The distributions of the Southern Cavefish (*Typhlichthys sp.*) and Northern Cavefish (*Amblyopsis spelaea*) in HUC8 watersheds of Kentucky. Southern Cavefish localities are represented by black circles, Northern Cavefish localities by white circles, and localities were both species occur are represented by gray circles.

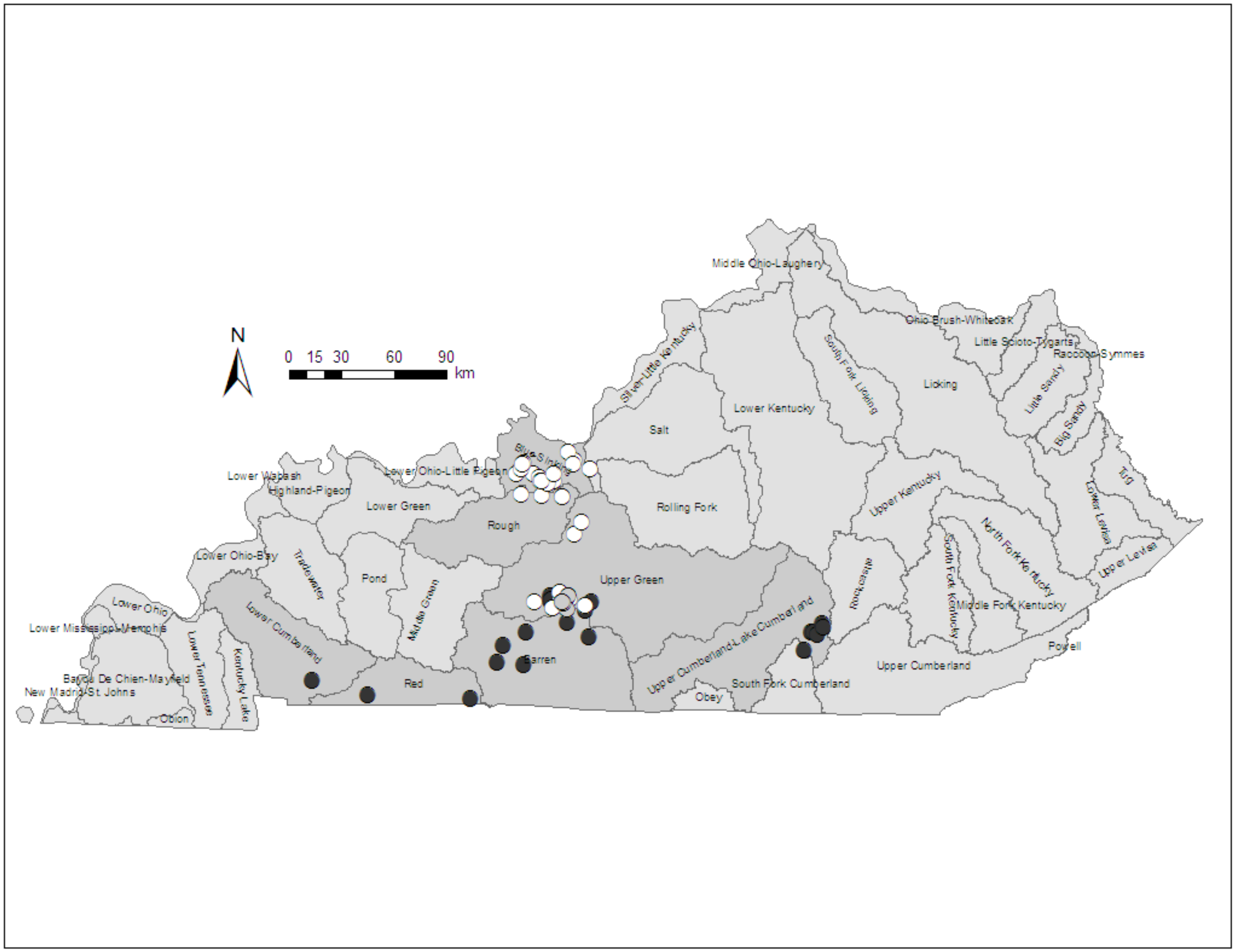

Figure 18. Aerial view of the Rich Pond area showing the location of the main spring and low-lying area that becomes a small lake every spring. Image from Google Earth (DigitalGlobe, GeoEye, USDA Farm Service Agency).

Figure 19. The site of a newly discovered population of Northern Spring Cavefish (*Forbesichthys papilliferus*), a spring-fed ditch off of Morton Road in Todd Co., Kentucky. Photo by Matthew L. Niemiller.

Figure 20. Flooded depression in an agricultural field located in Todd Co., Kentucky. During high water, Northern Spring Cavefish may disperse through these aquatic corridors. Photo by Matthew L. Niemiller.

Figure 21. Entrance to Sanders Cave located within Mammoth Cave National Park, Edmonson Co., Kentucky. Photo by Matthew L. Niemiller.

Figure 22. Stream passage in Sanders Cave, Edmonson Co., Kentucky. Southern Cavefish and Mammoth Cave Crayfish (*Orconectes pellucidus*) can be found in this habitat. Photo by Matthew L. Niemiller.

Figure 23. Entrance to Dykes Bridge Cave, Pulaski Co., Kentucky. Southern Cavefish have been reported from this cave. Photo by Matthew L. Niemiller.

Figure 24. Searching for Southern Cavefish in the downstream passage at L & N Railroad Cave, Barren Co., Kentucky. Photo by Daphne Soares.

Figure 25. An entrance to Under the Road Cave, Breckinridge Co., Kentucky. Photo by Matthew L. Niemiller.

Figure 26. Maps illustrating the distribution and sampling localities of *Typhlichthys*. Numbered localities correspond to sampled populations listed in Table 13 and are color-coded according to the major lineages with which populations are affiliated. Major river hydrological basins are colored in A and ecoregions are colored in B. State and county borders also are outlined.

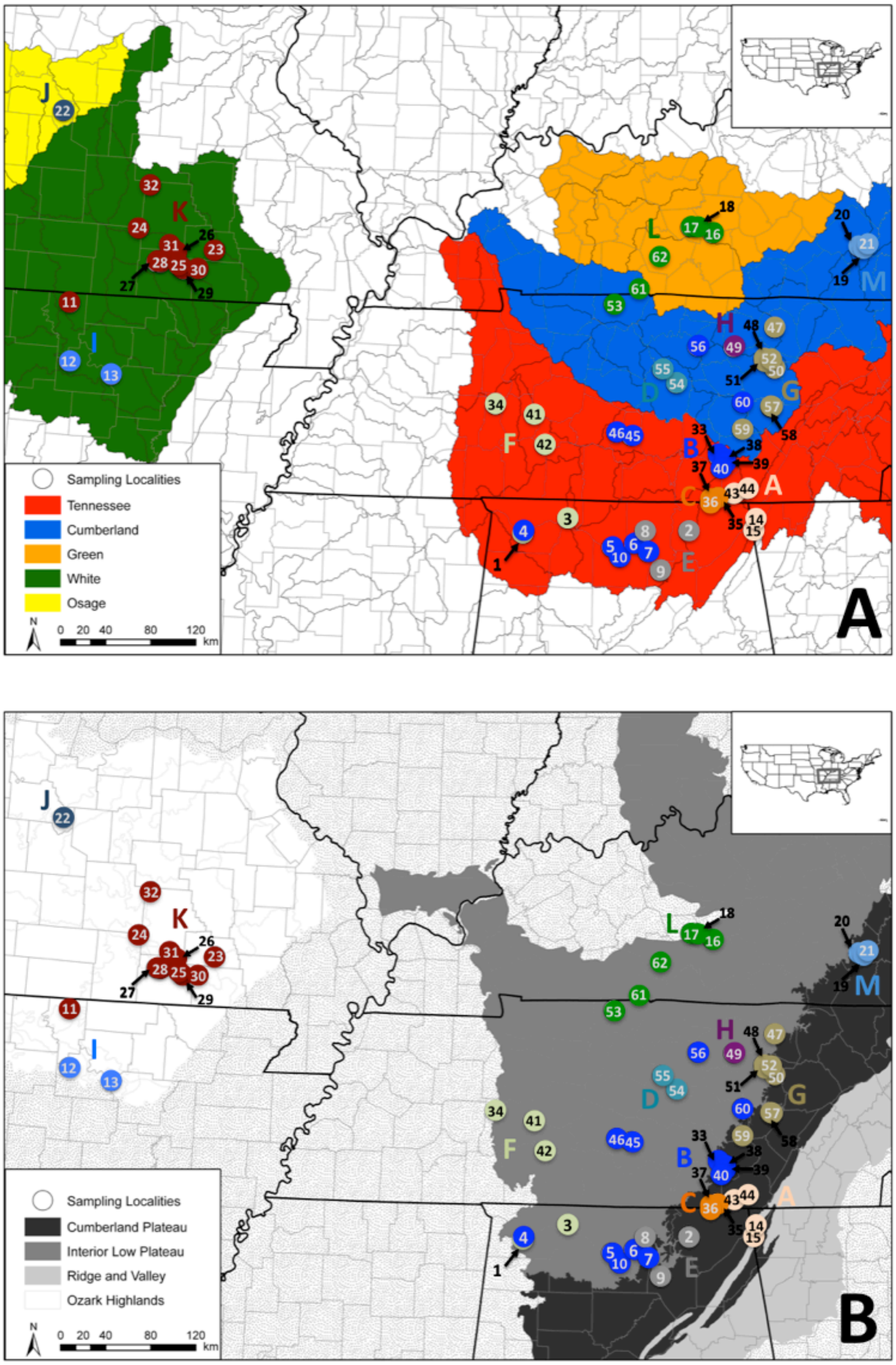

Figure 27. Gene trees estimated from partitioned Bayesian analyses of 137 *Typhlichthys* samples for six genes: *nd2*, *s7*, *rag1*, *tbr*, *plag*, and *myh*. Bayesian posterior probabilities ≥ 0.95 are indicated on the branch. Branches are color coded to indicate the major lineage with which they are affiliated (upper left and Table 13).

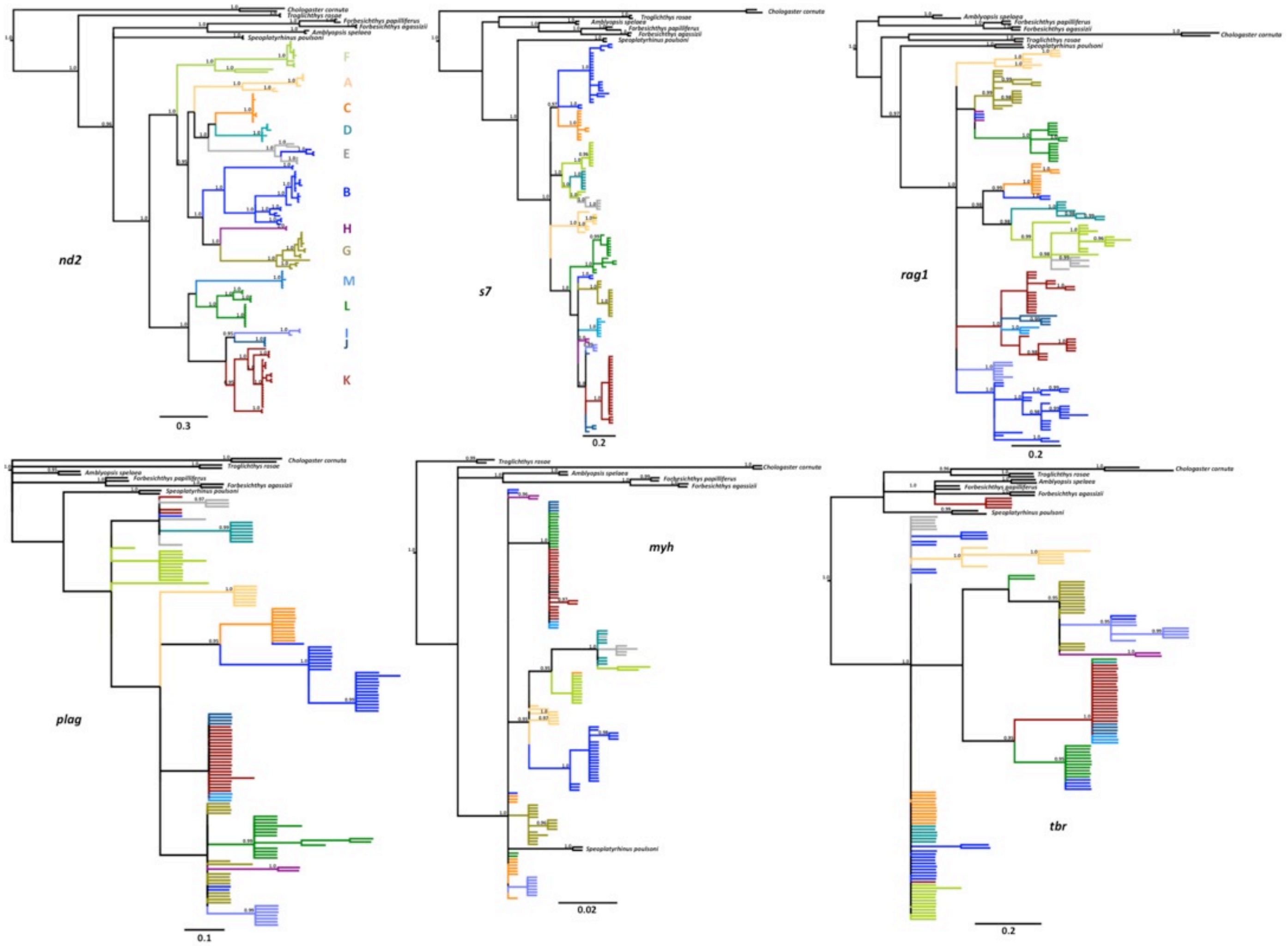

Figure 28. Geographic distribution of delimited species for the 62-individual, 6-gene dataset. Numbered localities and delimited species correspond to populations listed in Table 13. Species G and J occur in Kentucky.

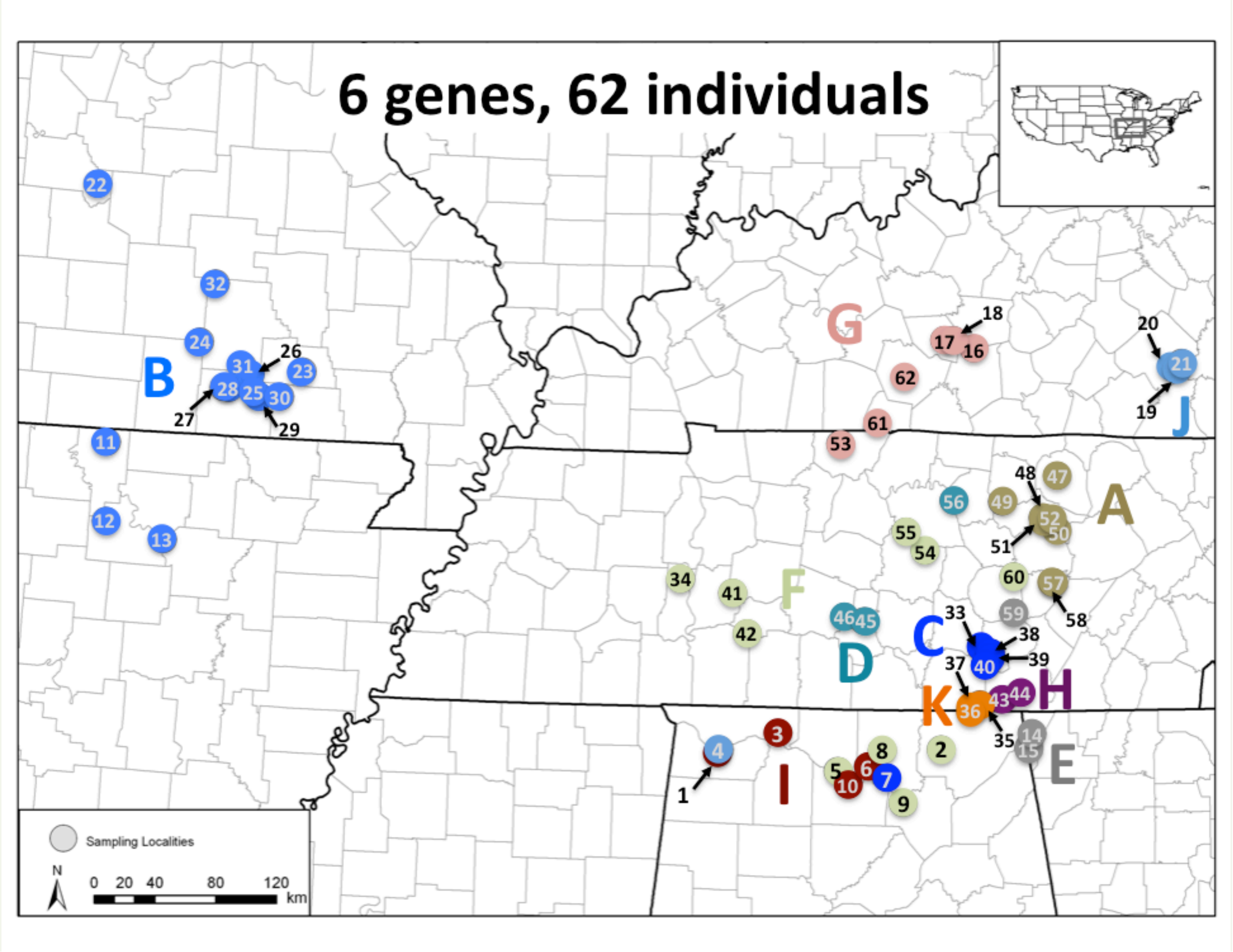

Figure 29. Species tree phylogenies based on the 135- and 60-individual datasets and delimited species assignments inferred using *BEAST: 135-individual, 3-gene (top), 60-individual, 3-gene (middle), and 60-individual, 6-gene (bottom). Clade posterior probabilities > 0.95 are indicated above the branch with an asterisk in black and uncertainty in the relative divergence times are shown by bars on nodes with the length corresponding to the 95% highest posterior density (HPD) of the node ages. Nodes with speciation probabilities > 0.95 under Bayesian species delimitation are denoted with an asterisk in blue. Scale bars represent substitutions per site. Delimited species in red boxes were collapsed into a single species under Bayesian species delimitation. Delimited species correspond to those labeled in Fig. 29. Species G and J occur in Kentucky.

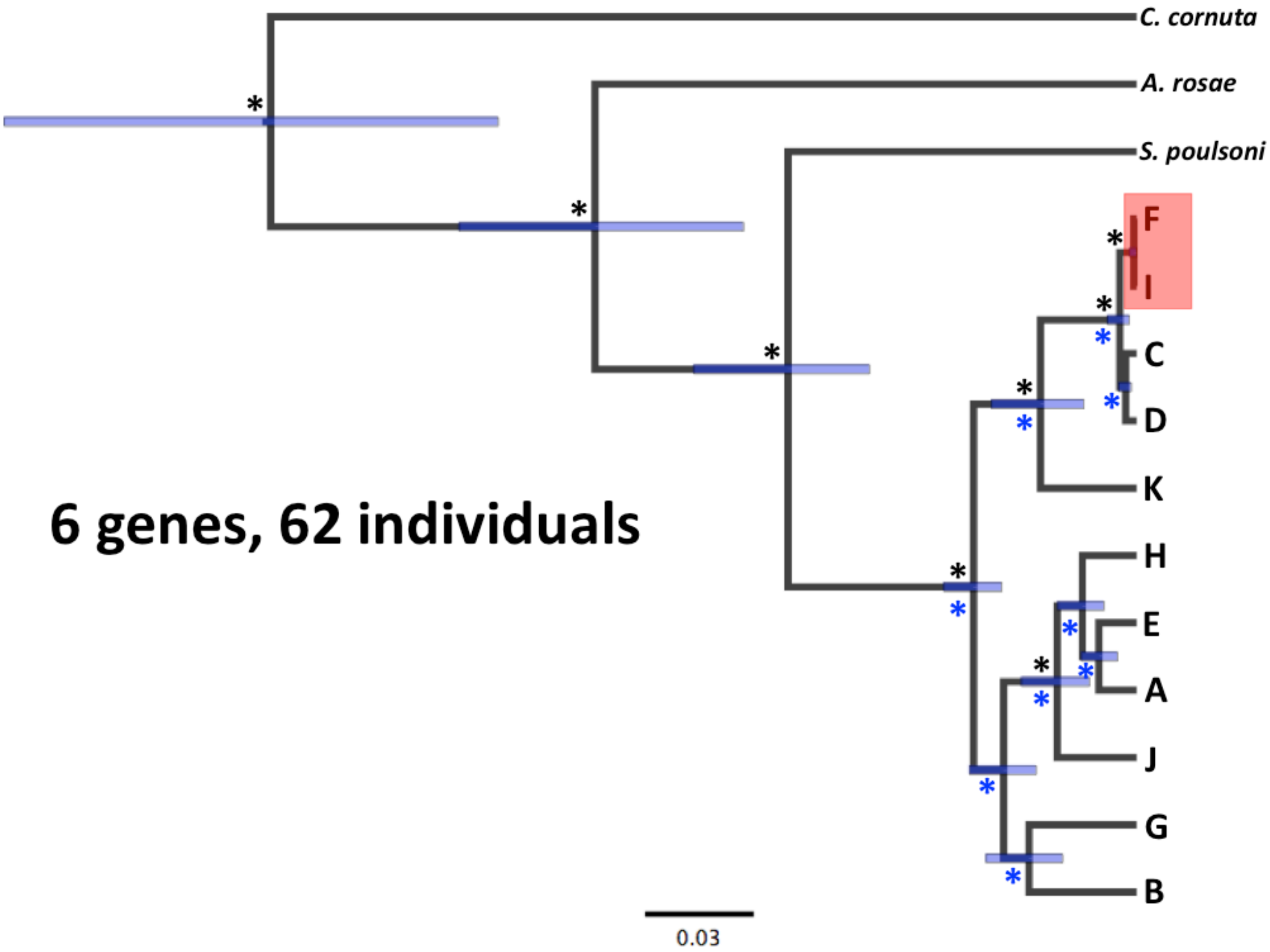

Figure 30. Time-calibrated phylogeny for *Typhlichthys* lineages inferred from multi-locus species tree analysis conducted in *BEAST. Clade posterior probabilities are indicated next to nodes. Labeled nodes (1–4) are the same as those listed in Table 15. The pie diagrams at each node reflect marginal probabilities for each alternative ancestral area derived from S-DIVA for major watershed (hydrological drainage basin; left) and ecoregion (right). Lineages L and M occur in Kentucky.

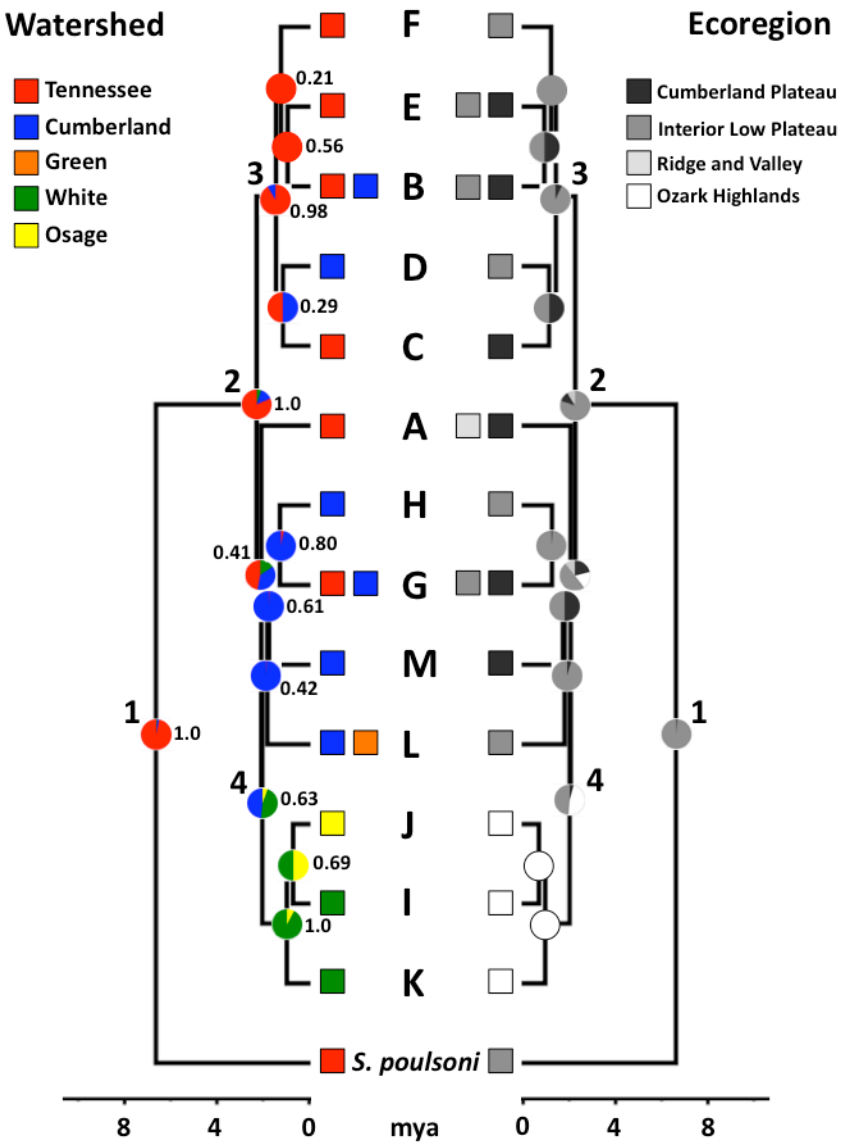

Figure 31. Map showing the distribution and sampling localities of *Amblyopsis spelaea* with respect to the Ohio River (bold dark grey line). Numbered localities correspond to populations listed in Table 17. Black circles represent populations belonging to the northern group whereas white circles represent populations belong to the southern group. Populations exist within the Crawford-Mammoth Cave Uplands (lighter grey) and Mitchell Plain (darker grey) ecoregions in an area that remained unglaciated throughout the Pleistocene. The southern extent of Pleistocene glaciations is indicated by a dashed black line.

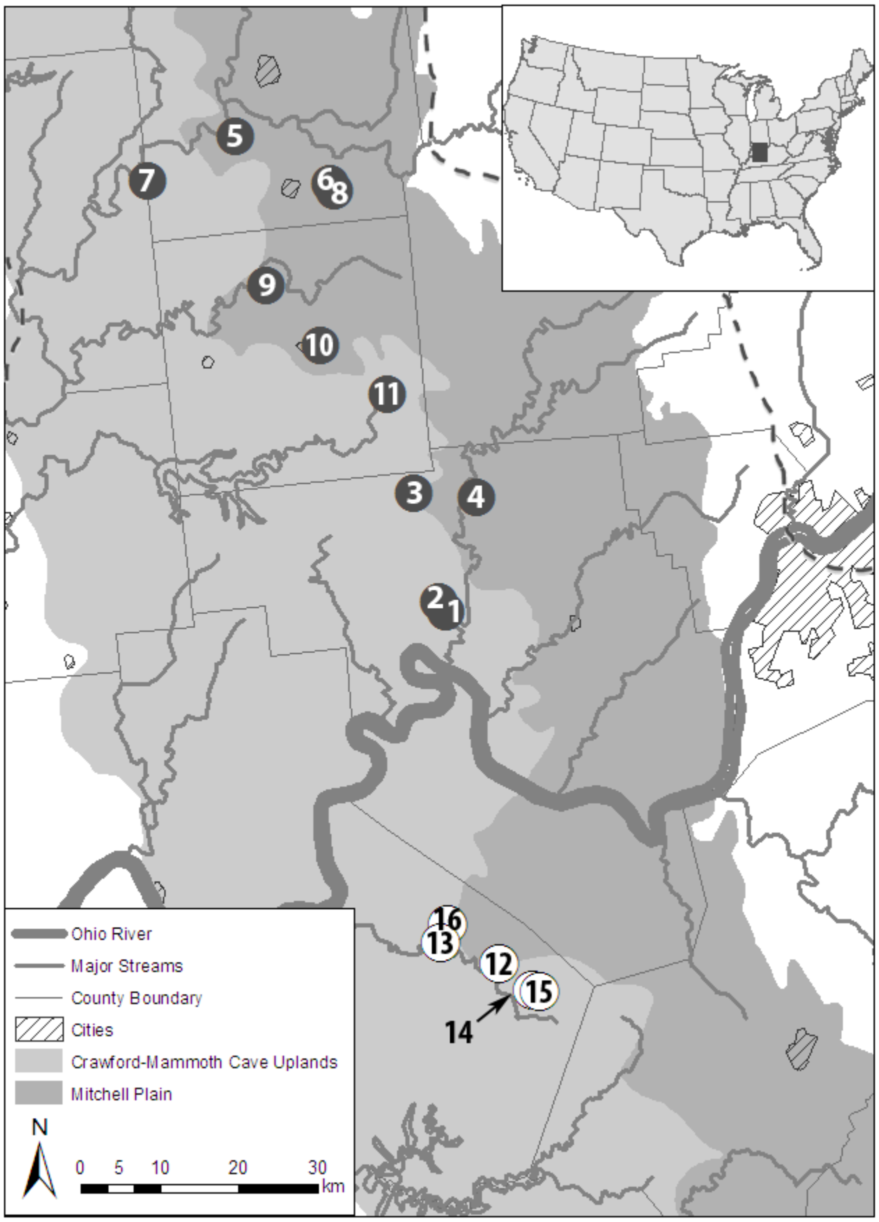

Figure 32. Gene genealogies estimated from partitioned Bayesian analyses of 72 *Amblyopsis* plus outgroup samples for five genes: *nd2*, *s7*, *rag1*, *rho*, and *tbr1*. Species and groups (north and south of the Ohio River) are highlighted. Alleles shared between groups north and south of the Ohio River are highlighted in purple.

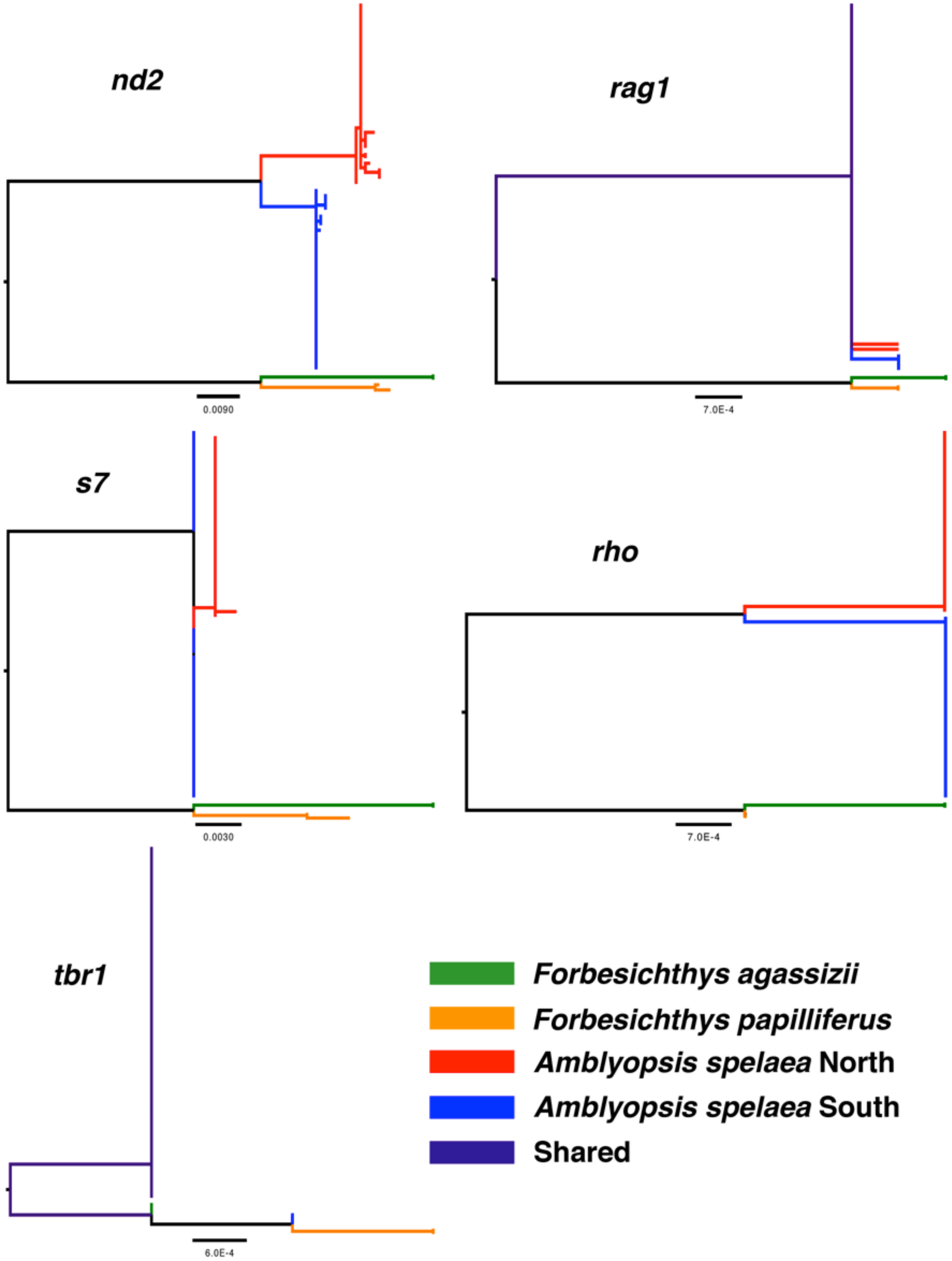

Figure 33. Haplotype networks for *Amblyopsis* for each locus. Circle color indicates the group (black for populations north of the Ohio River and white for populations south of the Ohio River) and size is proportional to the number of individuals sharing that haplotype. Small black squares on branches are inferred mutations not sampled. Number haplotypes correspond to those listed in Table 17.

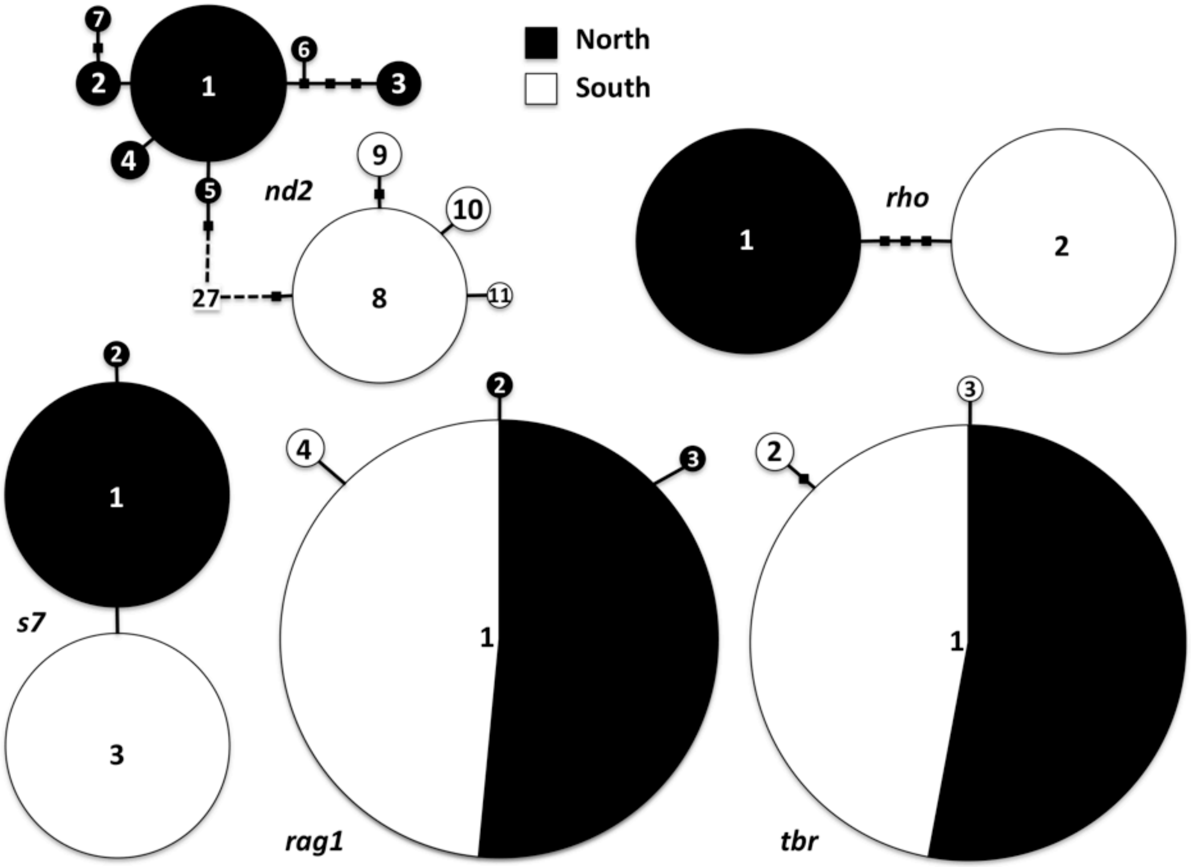

Figure 34. Fossil-calibrated phylogeny for amblyopsid lineages including populations north and south of the Ohio River as separate lineages inferred from the multilocus species tree analysis. Clade posterior probabilities are indicated next to nodes, and uncertainty in divergence time estimates are shown by blue bars on nodes with the length corresponding to the 95% highest posterior density of node ages.

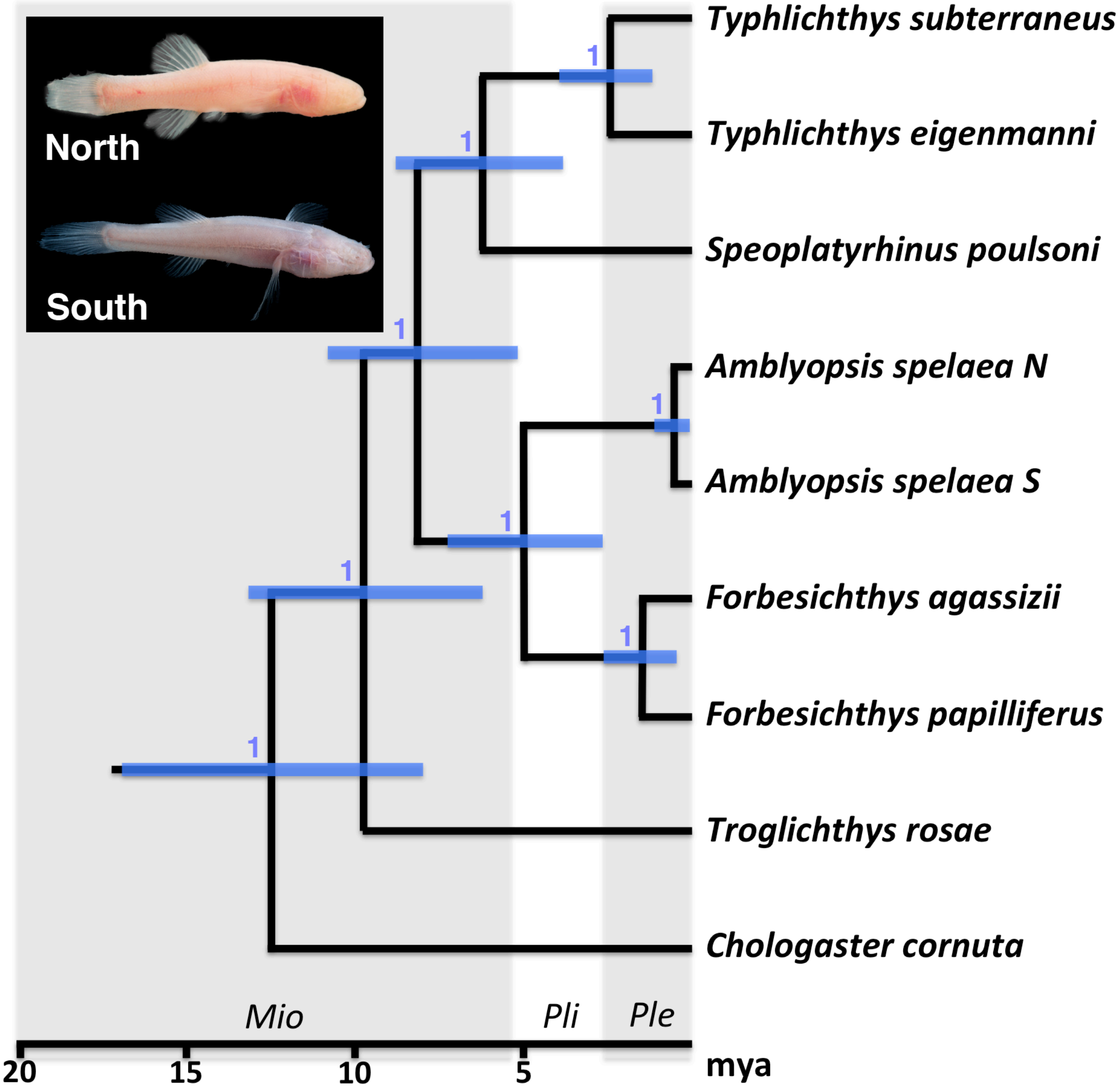

Figure 35. GMRF skyride plots for the *nd2* locus for groups of populations north and south of the Ohio River in *Amblyopsis*. Time (in years) is shown on the x-axis and the effective population size (number of individuals) is shown on the y-axis. The central dark horizontal line in the plot is the median value for effective population size and the shaded area represents the 95% HPD interval for those estimates. The vertical dashed line represents the median TMRCA. The upper 95% HPD for the TMRCA is at the right edge of the plot, whereas the lower 95% HPD is the vertical line to the left of the median.

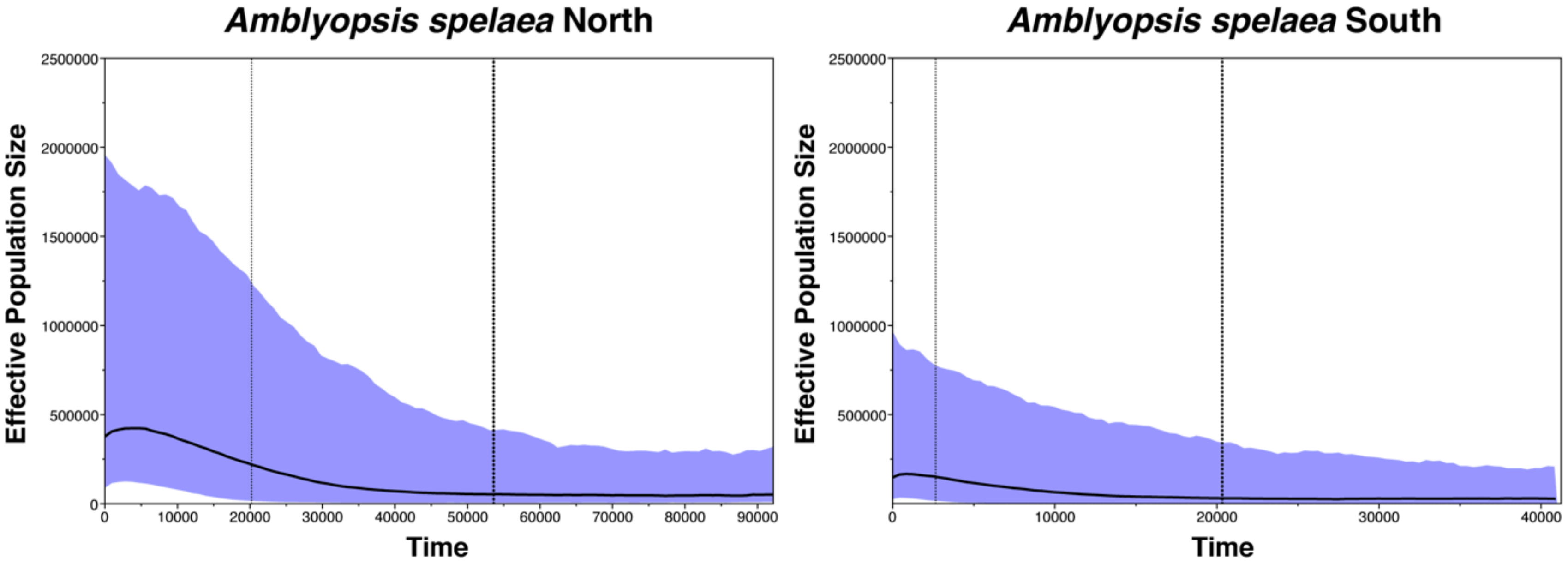

Figure 36. Results from both O'Meara's (2010) and Bayesian species delimitation support two species within *Amblyopsis spelaea*. The percentage of best trees recovering a node in the nonparametric heuristic approach (top) and Bayesian speciation probabilities (bottom) are provided for each node. The Bayesian posterior estimates for θ and τ are also provided on the specie tree.

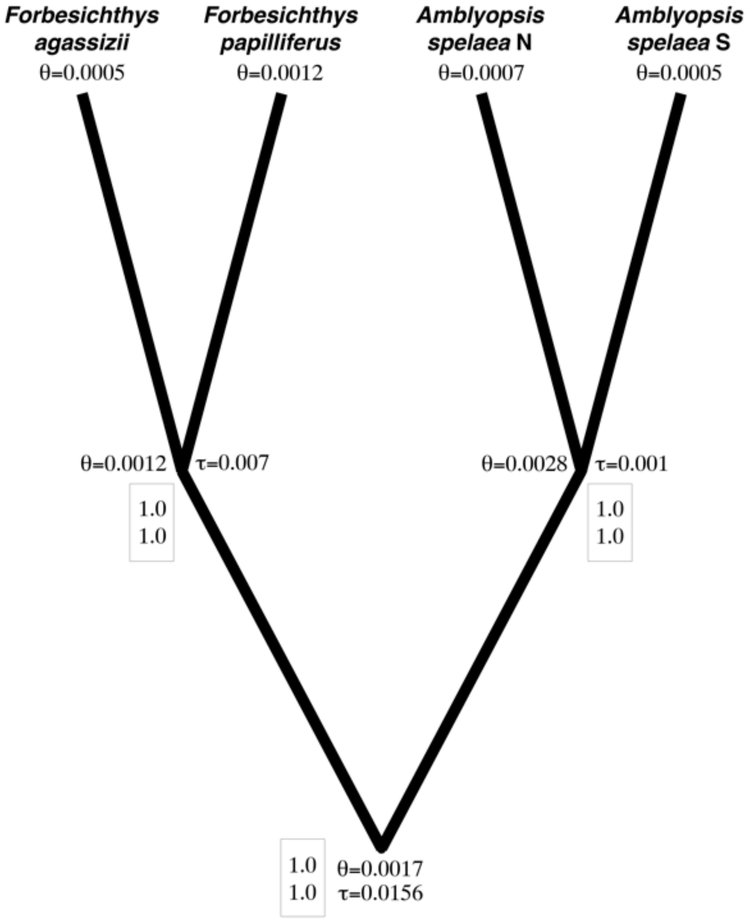

Figure 37. A graphical representation of the results from the Structure analysis for *K*=2 for *Amblyopsis spelaea*. Each individual is represented by a vertical line broken into two colored segments to represent the estimated proportions of the individual's genome originating from each of the two inferred clusters.

Figure 38. Proportion of Northern Spring Cavefish (*Forbesichthys papilliferus*) localities with survey data (n = 27) in Kentucky with the maximum number of cavefish observed during a single survey. Eight-four percent of localities have yielded ten or fewer cavefish during a given survey.

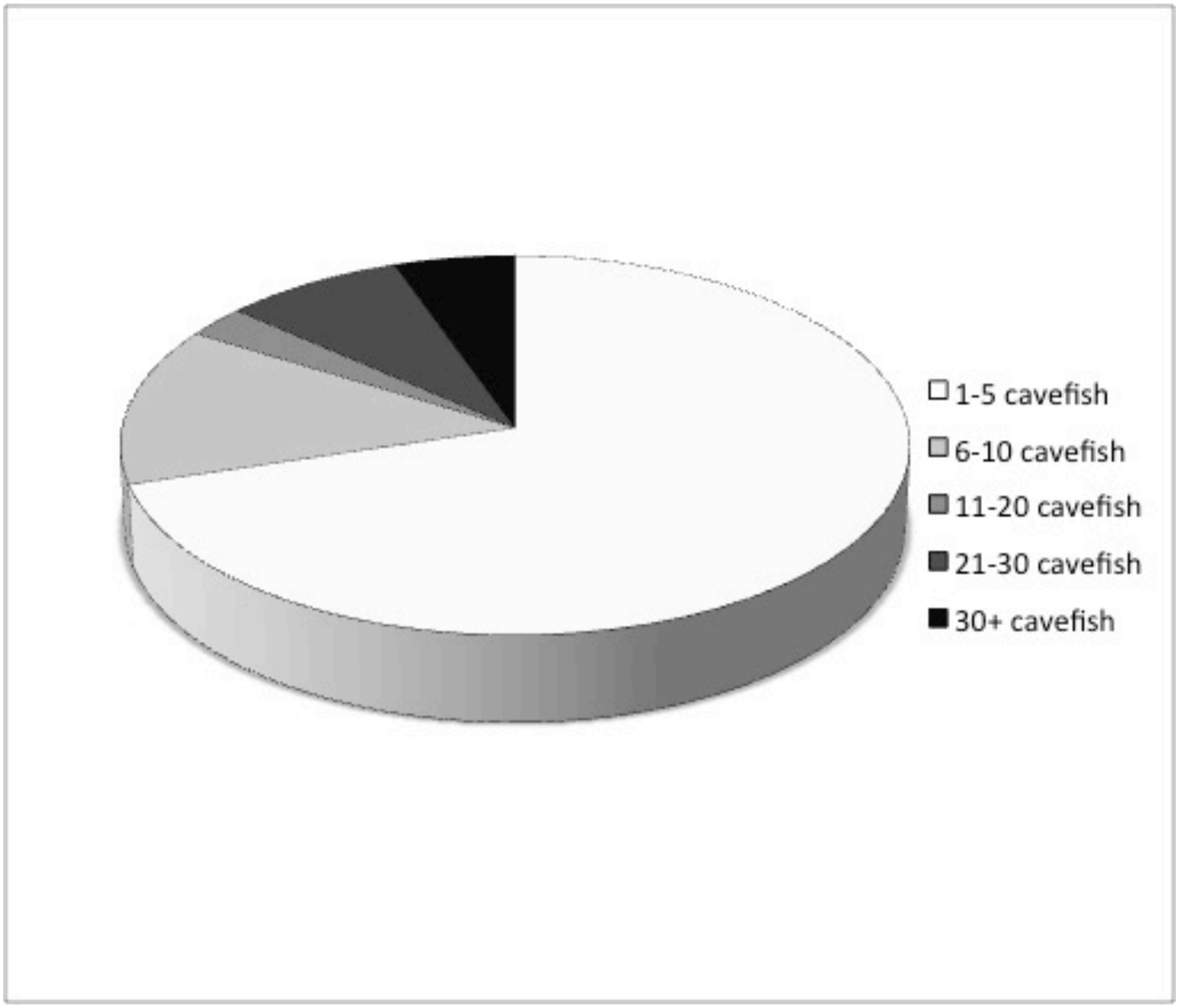

Figure 39. Histogram of standard length for 59 Northern Spring Cavefish (*Forbesichthys papilliferus*) measured on 31 March 2010 at the ditch at Morton Rd, Todd Co., Kentucky.

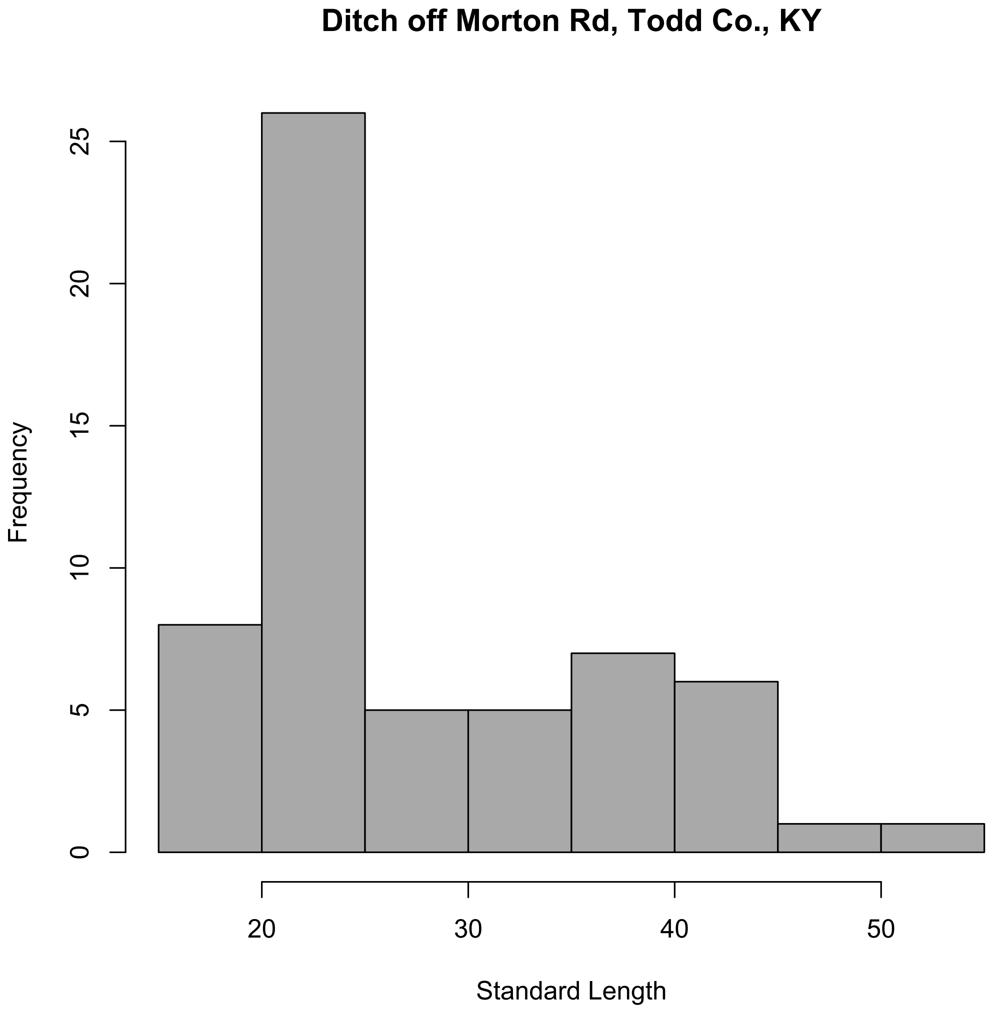

Figure 40. Histogram of standard length for 203 Northern Spring Cavefish (*Forbesichthys papilliferus*) measured on 01 April 2010 at Rich Pond, Warren Co., Kentucky.

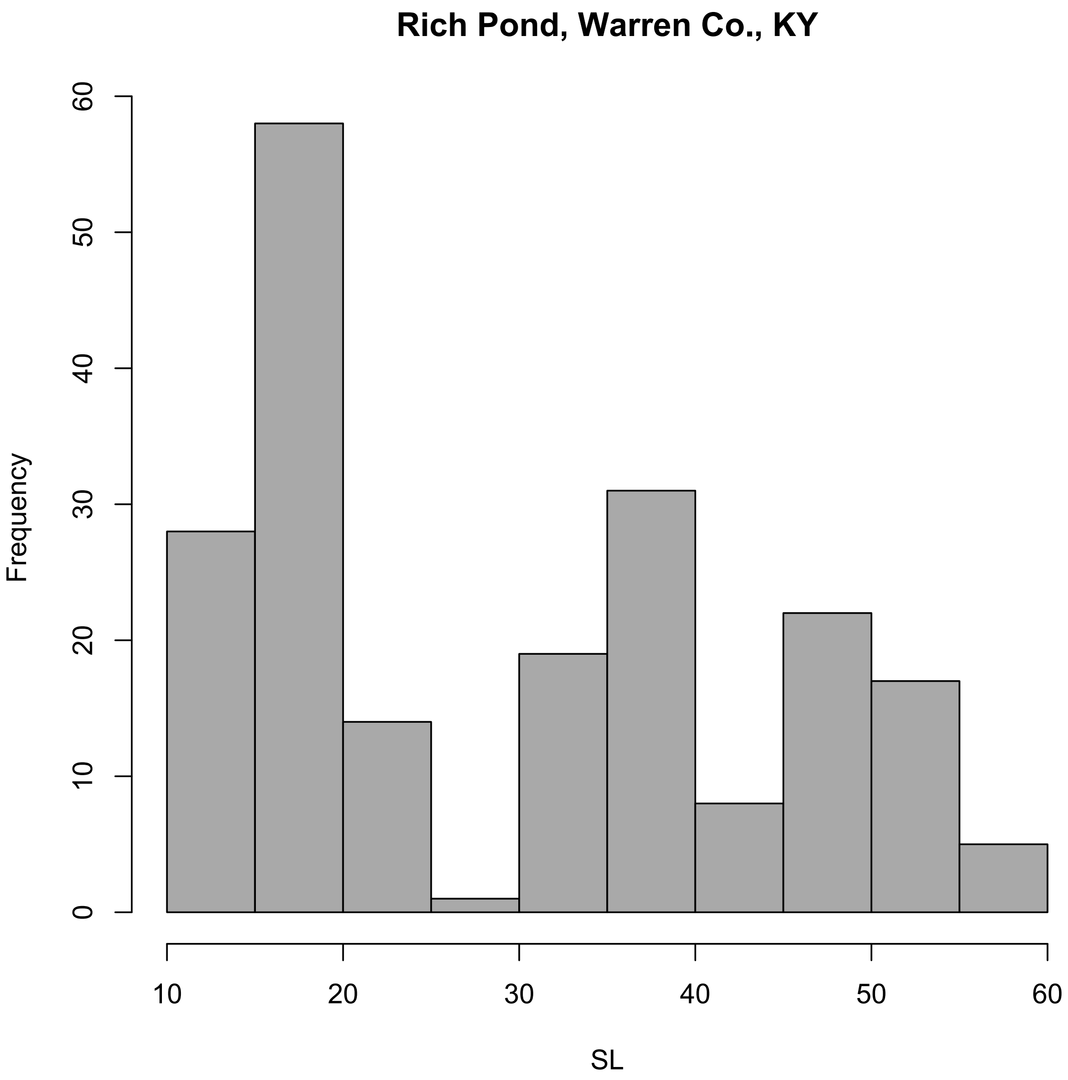

Figure 41. Proportion of Southern Cavefish (*Typhlichthys subterraneus*) localities with survey data (n = 27) in Kentucky with the maximum number of cavefish observed during a single survey. Nearly 56% of localities have yielded ten or fewer cavefish during a given survey.

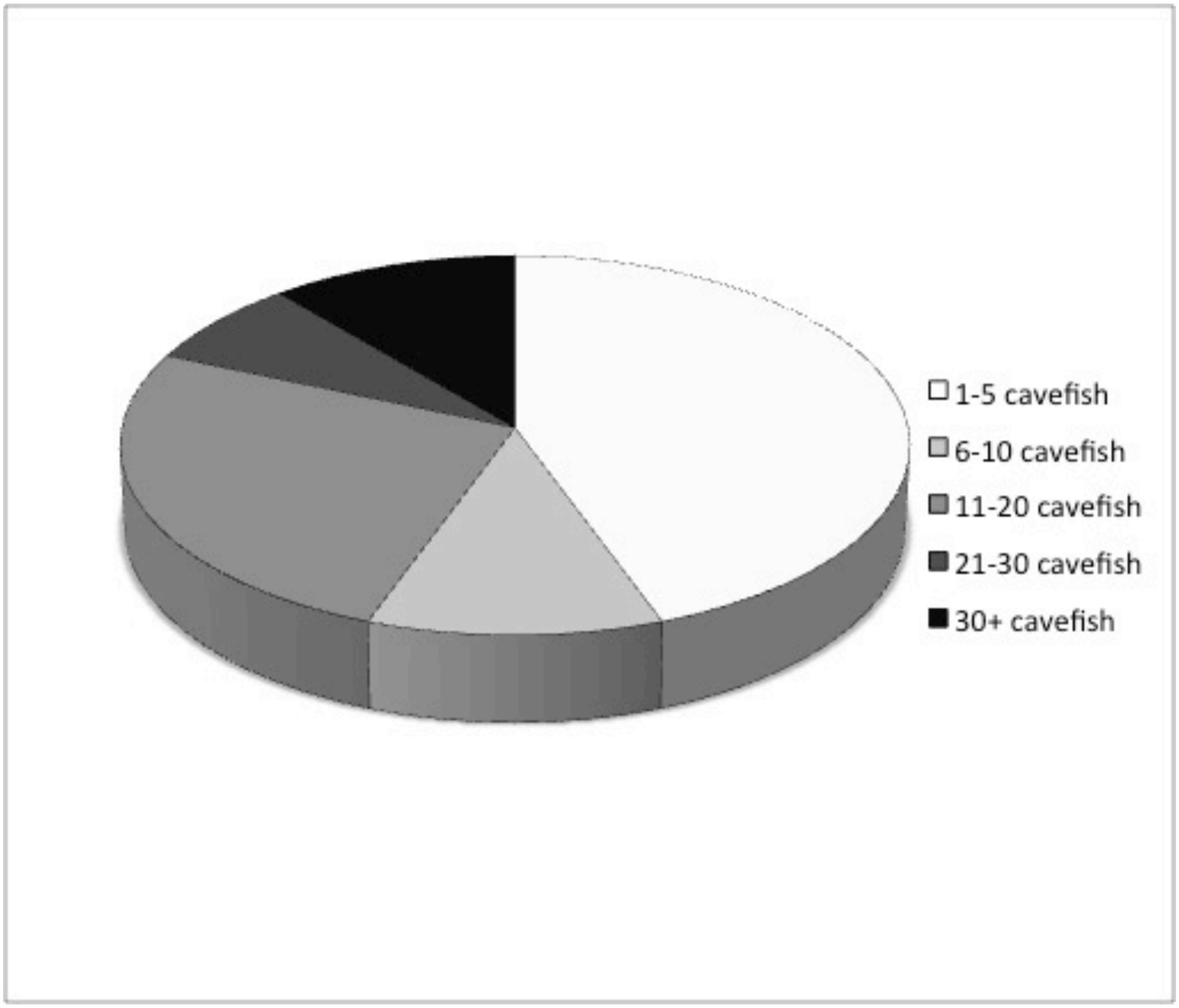

Figure 42. Proportion of Northern Cavefish (*Amblyopsis spelaea*) localities with survey data (n = 29) in Kentucky with the maximum number of cavefish observed during a single survey. Sixty-six percent of localities have yielded ten or fewer cavefish during a given survey.

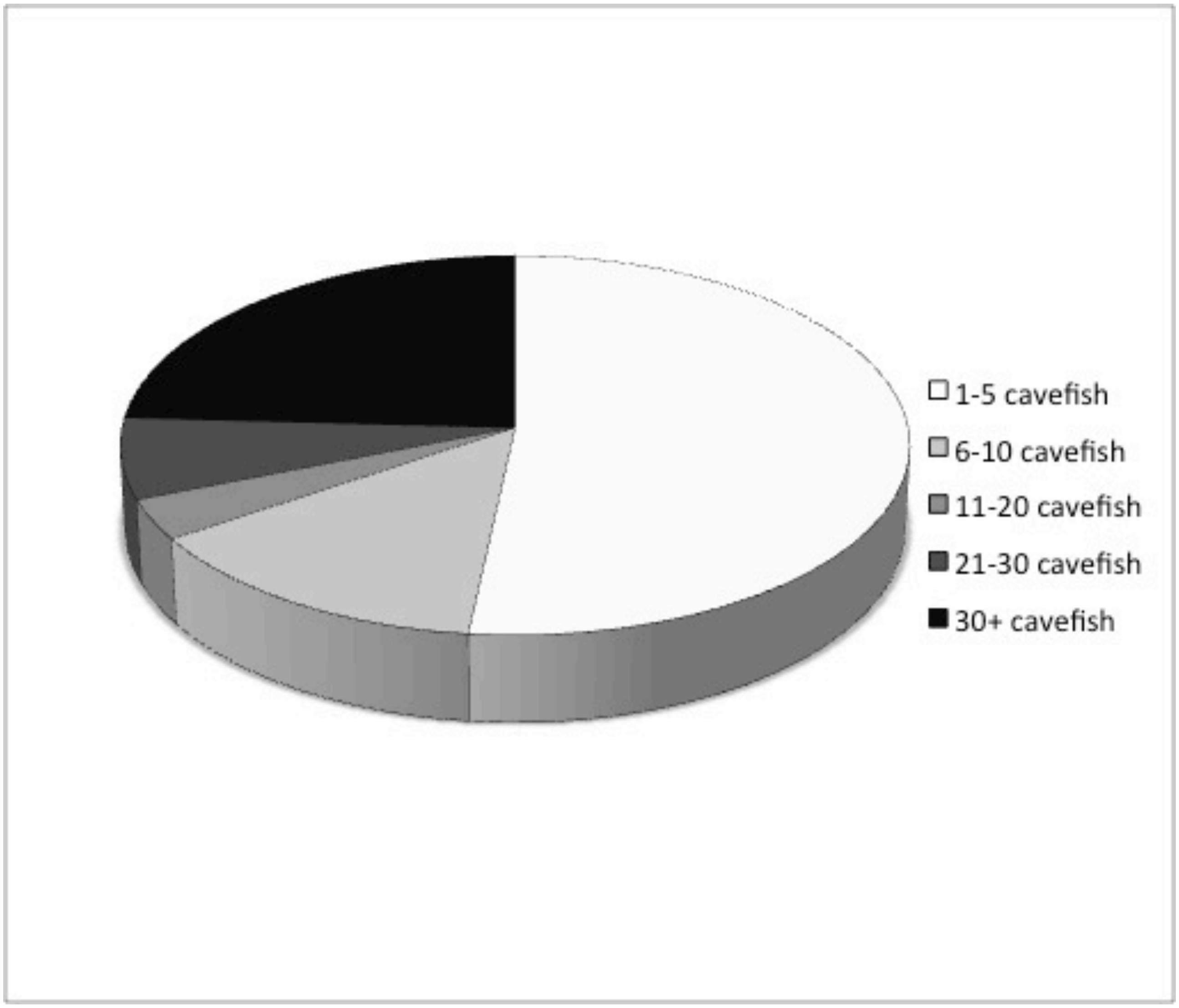

Figure 43. Histogram of standard length for 25 Southern Cavefish (*Typhlichthys subterraneus*) measured on 16 August 2010 at L & N Railroad Cave, Barren Co., Kentucky.

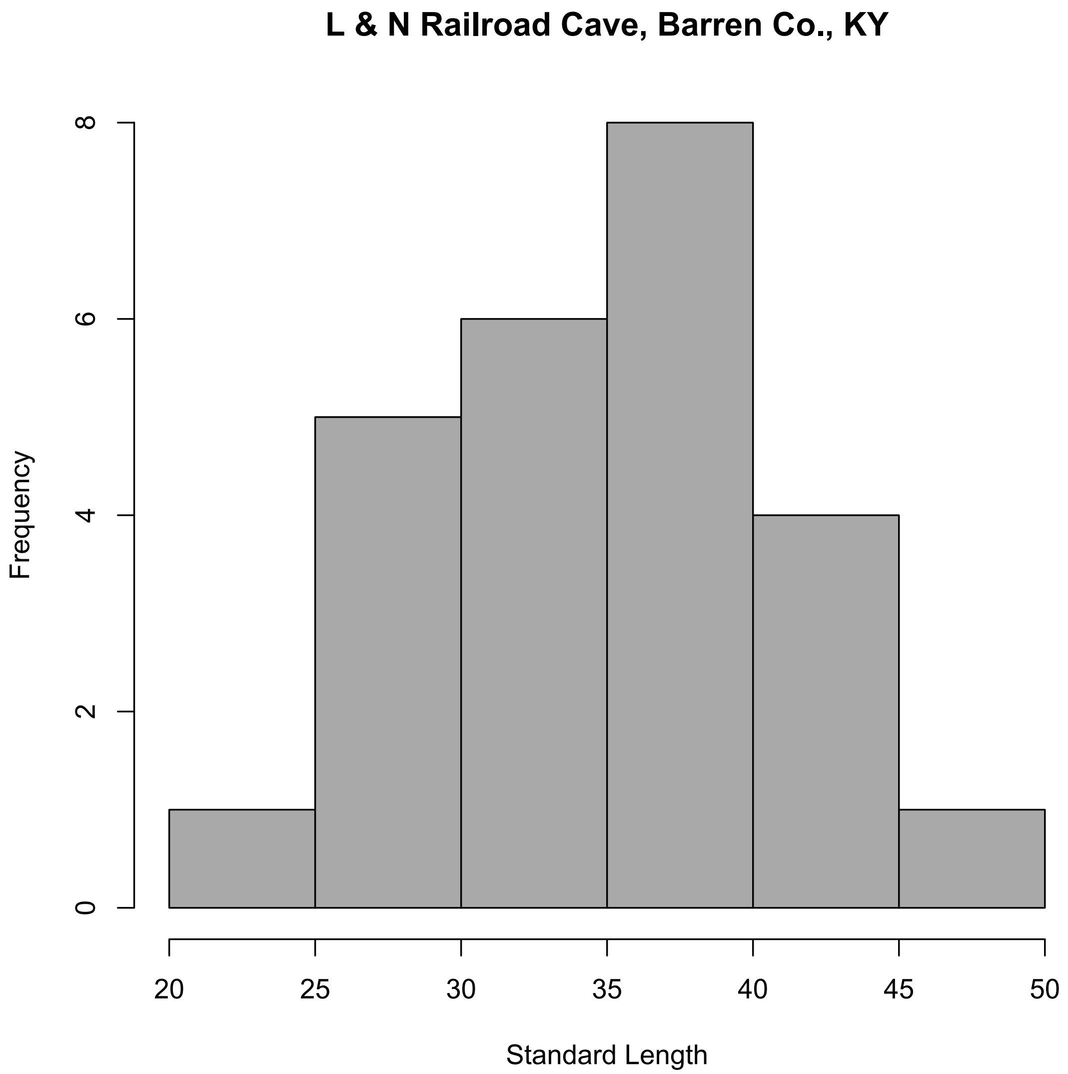

Figure 44. Histogram of standard length for 18 Kentucky Cavefish (*Typhlichthys sp. nov. 1*) measured on 02 August 2008 at Drowned Rat Cave, Pulaski Co., Kentucky.

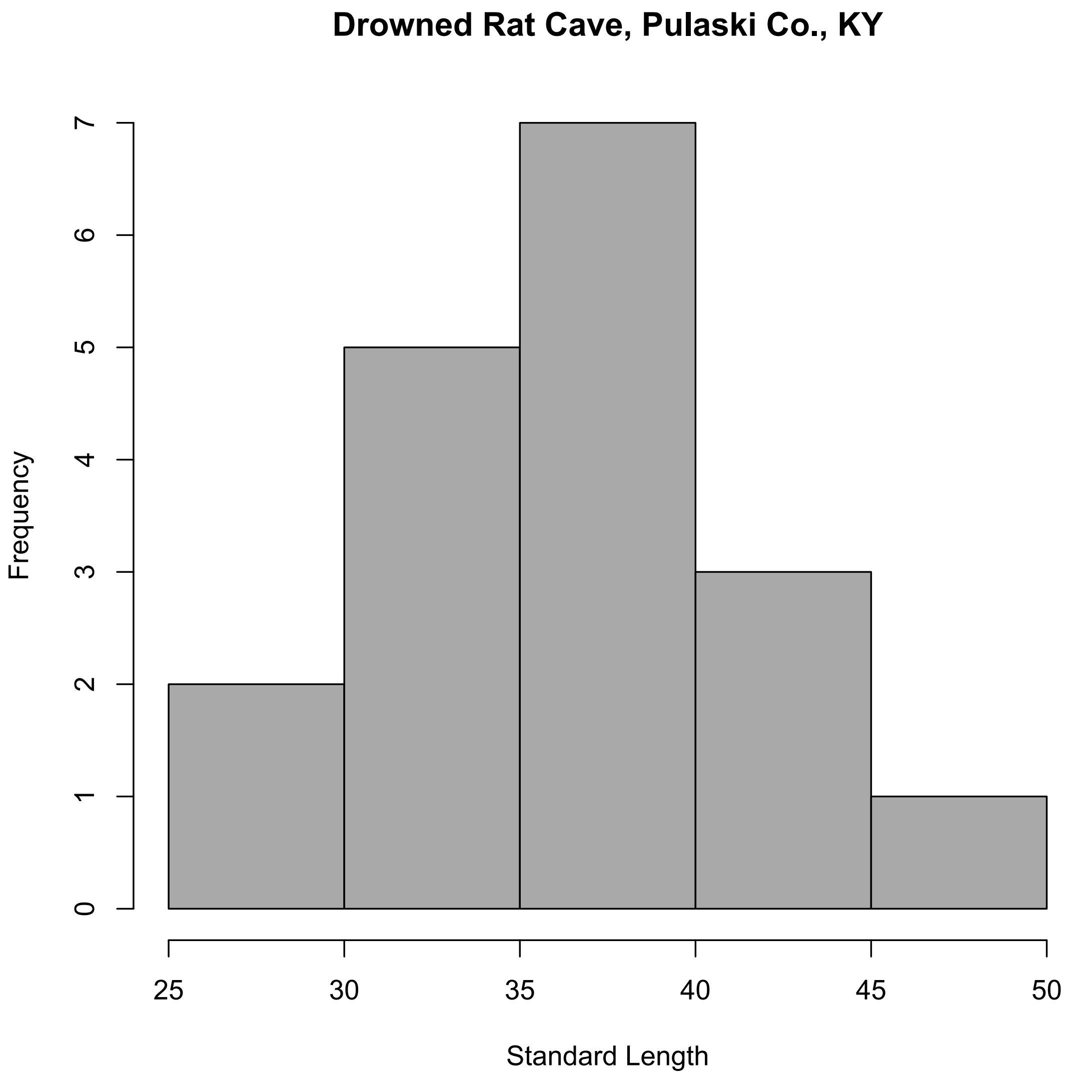

Figure 45. Histogram of standard length for 39 Northern Cavefish (*Amblyopsis spelaea*) measured at Under the Road Cave, Breckinridge Co., Kentucky.

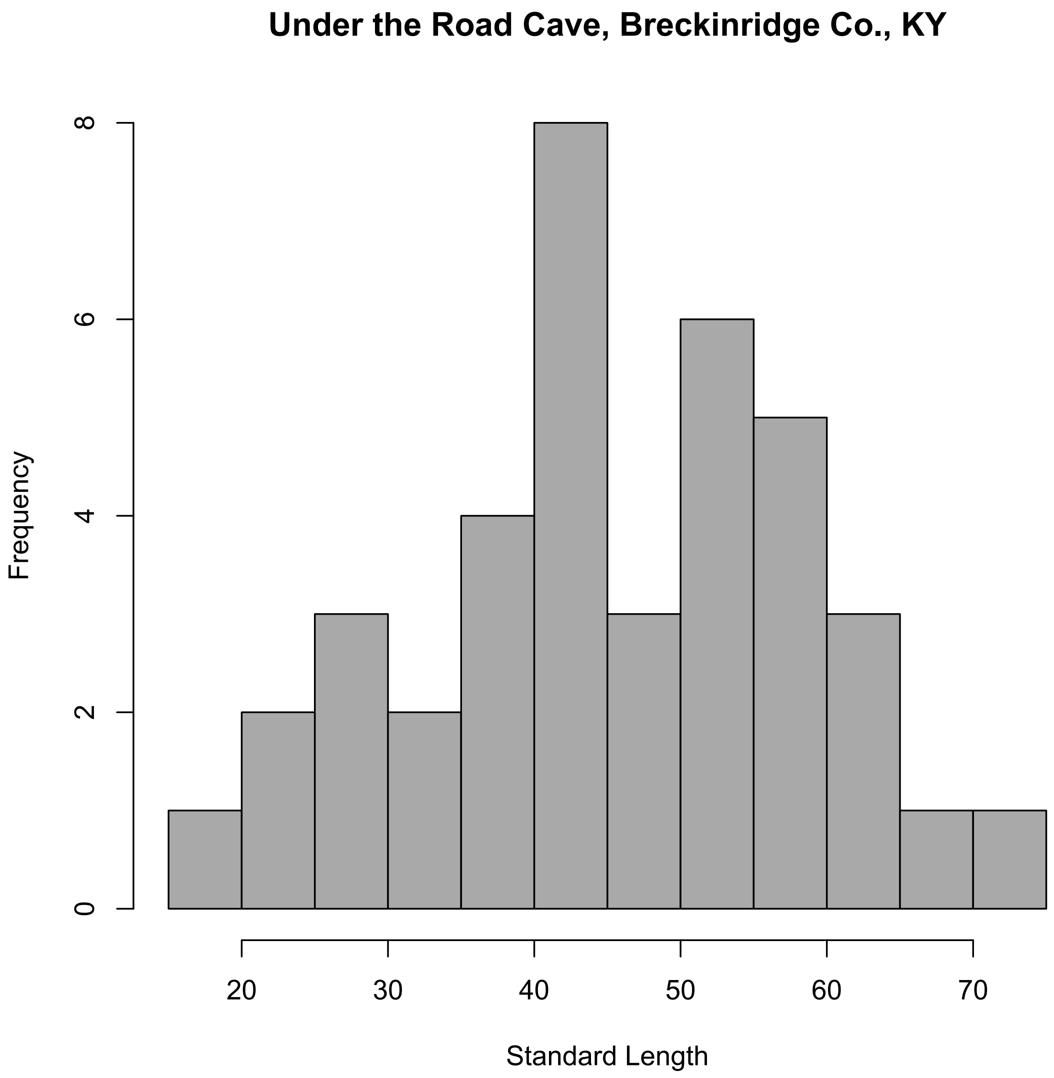

Figure 46. Failing support structure for a formerly operational water pump located at the entrance to L & N Railroad Cave in Barren Co., Kentucky. This wood has been treated with creosote and several have been washed into the cave. Photo by Matthew L. Niemiller.

Figure 47. An upper-level passage in Wells Cave, Pulaski Co., Kentucky, where vandals have sprayed graffiti, broken bottles, and have done other damage. Several species of bats, including endangered Indiana Bats, use this cave as a roost. The lower levels of this cave are home to Southern Cavefish and other aquatic subterranean fauna. Photo by Matthew L. Niemiller.

Figure 48. A sign hung at the main entrance to Wells Cave warning that the cave is closed to visitation in response to the USFWS White-Nose Syndrome Cave Advisory on March 26, 2009 and recent vandalism. Photo by Matthew L. Niemiller.

Appendix 1. Localities where amblyopsid cavefishes have been reported in Kentucky and surveyed during the current study.

Data available from the authors or KDFWR upon request.

Appendix 2. Loci and selected best-fit molecular evolutionary models for data partitions implemented in phylogenetic analyses.

Locus	Abbreviation	Length (bp)	Ploidy	Model of first codon	Model of second codon	Model of third codon	Model of intron
NADH dehydrogenase 2	*nd2*	1044	n	TVM+I+G	GTR+I+G	GTR+I+G	NA
intron 1 of ribosomal protein S7	*s7*	841	2n	NA	NA	NA	HKY+G
exon 3 of recombination activating gene 1	*rag1*	1446	2n	HKY+I	TVM+I	TVM+G	NA
rhodopsin	*rh1*	798	2n	HKY+G	TIM+I+G	HKY+G	NA
myosin heavy polypeptide 6	*myh6*	786	2n	HKY+I	HKY	TVM+I	NA
T-box brain 1	*tbr1*	705	2n	HKY	F81	HKY+I	NA
pleiomorphic adenoma gene-like 2	*plagl2*	603	2n	GTR	TVM	TVM	NA

NA - the gene does not contain the specified partition.

Appendix 3. Museum collections queried for lots of amblyopsid cavefishes from Kentucky (see Table 24).

Collection	Abbreviation
Academy of Natural Sciences, Natural History Museum in Philadelphia	ANSP
Arctos-WNMU	WNMU
Auburn University Natural History Museum	AUM
Bernice Pauahi Bishop Museum	BPBM
California Academy of Sciences	CAS
Canadian Museum of Nature Fish Collection	CMN
Cornell University Museum of Natural History	CUMV
Field Museum of Natural History	FMNH
Florida Museum of Natural History	FLMNH
GBIF-MNHN (Paris)	MNHN
Illinois Natural History Survey	INHS
Los Angeles County Museum of Natural History	LACM
Massachusetts Museum of Natural History	MMNH
Michigan State University Museum	MSUM
Mississippi Museum of Natural Science	MMNS
Museum of Comparative Zoology, Harvard University	MCZ
National Museum of Natural History, Smithsonian Institution	USNM
North Carolina State Museum of Natural Sciences	NCSM
Royal Ontario Museum	ROM
Sam Noble Oklahoma Museum of Natural History	OKMNH
Santa Barbara Museum of Natural History	SBMNH
Sternberg Museum of Natural History	MHP
Swedish Museum of Natural History	SMNH
Texas Cooperative Wildlife Collection	TCWC
Tulane University Museum of Natural History	TU
Universidad Nacional Autonoma de Mexico-Ibiologia	IBUNAM
University Museum of Zoology Cambridge	UCZM
University of Alberta Museums	UAMZ
University of Arizona Museum of Natural History	AZMNH
University of Colorado Museum of Natural History	UCM
University of Kansas Biodiversity Institute	KU
University of Michigan Museum of Zoology	UMMZ
University of Nebraska State Museum	UNSM
University of Washington Fish Collection	UWFC
Yale University Peabody Museum	YPM