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# Surface to subsurface freshwater connections: phylogeographic and habitat analyses of *Cambarus tenebrosus*, a facultative cave-dwelling crayfish

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## Abstract

This study examined the phylogeography and population demographics of *Cambarus tenebrosus*, which has an unusually large distribution for a freshwater crayfish species, encompassing the Interior Lowlands and Cumberland Plateau of the eastern United States. This facultative cave-dweller provides a unique perspective on the biologic connections between surface and subsurface freshwater ecosystems, which are considered to be highly imperiled due to pollution and habitat degradation. The 16S mitochondrial gene was sequenced for 233 individuals from 84 cave and 20 surface locations throughout the range, with most sampling concentrated around the Cumberland Plateau of the southern Appalachians, to assess conservation status of this species and examine the extent of gene flow between the two habitat types. Cave and surface populations formed a single monophyletic group relative to *Cambarus striatus*, and clades showed strong geographical associations, but lacked habitat structuring. Occupation of subterranean environments does not appear to be a recent event in the evolutionary history of the species. The large amount of genetic diversity within the species, coupled with its ability to inhabit surface and subsurface environments, suggests that this species may pose a threat as a possible invasive species in other karst-dominated landscapes.

## Introduction

The number of faunal extinctions occurring in North American freshwater environments has been steadily increasing (Master, 1990; Williams *et al*., 1993; Taylor *et al*., 1996; Schuster, 1997), and it has been estimated that the number of freshwater species in North America is decreasing at a rate of 4% per decade, which rivals extinction rates in tropical rainforests (Ricciardi & Rasmussen, 1999). Elevated extinction rates of freshwater fauna are typically associated with habitat destruction, organic pollution, stream regulation by dams and habitat fragmentation (Neves *et al*., 1997; Ricciardi, Neves & Rasmussen, 1998), yet current research also suggests that genetic factors play important roles in driving threatened and endangered species to extinction (Spielman, Brook & Frankham, 2004). Thus, it is important that the protection of freshwater environments be approached not only by reducing the impact of humans on the aquatic environment, but also by investigating the population structures and connectivity of its inhabitants using molecular assessments of conservation status.

The Nature Conservancy considers 95% of subterranean species in North America to be endangered or imperiled (Master, 1991; Culver *et al*., 2000). There is little doubt that subsurface groundwater fauna are threatened by surface pollution and habitat deterioration (Danielopol *et al*., 2003), but studies of aquatic cave organisms are sparse and often inconclusive, further adding to the enigmatic nature of the subterranean environment. Furthermore, information about the biological connections between surface and subsurface environments is lacking, and this study is the first species-specific genealogical investigation of any North American stygophilic (aquatic facultative cave-dwelling) species.

*Cambarus tenebrosus* (Hay, 1902) is unusual among freshwater crayfish species because it occupies both epigean (surface) and hypogean (subsurface) karst habitats. *Cambarus tenebrosus* also has a large range for a crayfish, extending from south-central Indiana southward to northern Alabama (Fig. 1). Because it is found in subterranean habitats typically occupied by obligate cave dwellers (stygobites), it was originally thought that *C. tenebrosus* was a transient member of the cave environment, perhaps being washed into the cave by accident. Hay (1902) refuted this hypothesis based partially on morphological characteristics indicative of stygobitic crayfishes, including the presence of reduced eyes and elongated limbs, which *C. tenebrosus*

possesses. These morphological characteristics, collectively referred to as troglomorphy, suggest that *C. tenebrosus* has partially adapted to subterranean life and, therefore, is not a passing member of the underground environment. A previous morphological study of *C. tenebrosus* showed no difference between individuals collected from surface and subsurface sites, but reflected overall intraspecific phenotypic plasticity (Taylor, 1997). This morphological plasticity might be caused by convergence due to similar environmental pressures in conjunction with active gene flow between the surface and cave habitats (Wiens, Chippindale & Hillis, 2003).

The objectives of this project were to (1) establish whether *C. tenebrosus* shows intraspecific geographic structuring of genetic variation, (2) test if there is a significant genetic association with the two habitats the crayfish occupies (cave vs. surface), and (3) provide molecular-based estimates of genetic diversity and effective population size for the species.

## Materials and methods

### Population sampling

Samples were collected at 104 sites (84 cave and 20 surface) throughout the range of *C. tenebrosus*, concentrating on areas of the Cumberland Plateau of the southern Appalachians and the Interior Lowlands which range from south-central Indiana to northern Alabama (Fig. 1; Table 1). A sample was considered subterranean or 'cave' if it was collected from an area not lit by natural light. Samples were included from the type locality at Mammoth Cave in Kentucky. In most cases, a non-destructive method of sampling was used, which involved collecting a leg from each individual and then returning the individual to the place of capture. Crayfish have the ability to regenerate lost limbs and, therefore, removing a limb during capture is not detrimental to the animal's survival (crayfish often lose their limbs in territorial battles). Tissue samples were stored in 95% ethanol, and each sample was given a unique identification number. Latitude and longitude coordinates were taken by a global positioning system (GPS) device at each sample site, including entrances to sampled caves. In a few cases, voucher specimens were taken and deposited at the Monte L. Bean Museum (BYU) and the North Carolina State Museum of Natural History. Additionally, *Cambarus striatus* (Hay, 1902), a closely related species (J. E. Buhay & K. A. Crandall, unpubl. data), was used as the outgroup to root the phylogenetic tree and haplotype network. The network analysis clearly shows *C. striatus* to be outside the 95% confidence interval for *C. tenebrosus* and phylogenetic analysis of the genus shows this species to be the sister taxon to *C. tenebrosus* (J. E. Buhay & K. A. Crandall, unpubl. data), making it an appropriate outgroup for this analysis.

### DNA sequencing

DNA was extracted from the samples using a cell lysis protocol (Crandall *et al.*, 1999). The protocol called for 5–15 mg of vacuum-dried tissue to be placed in a tube with 800 $\mu$L of cell lysis solution (1.21 g Tris, 37.1 g EDTA, 20 g SDS per liter, pH 8.0). Nine microliters of proteinase K (20 mg mL$^{-1}$) was added to this solution and the samples were incubated overnight at 55 °C while mixing on a shaker for tissue digestion. After 180 $\mu$L of 5 M NaCl was added, the mixture was vortexed and centrifuged to pellet out the salt. The supernatant was transferred to a clean cryotube. Immediately after, 420 $\mu$L of ice cold isopropanol was added and this mixture was centrifuged at 20 000 *g* for 10 min to pellet the DNA. After discarding the supernatant, the DNA pellet was washed with 500 $\mu$L of 70% ethanol using a cell

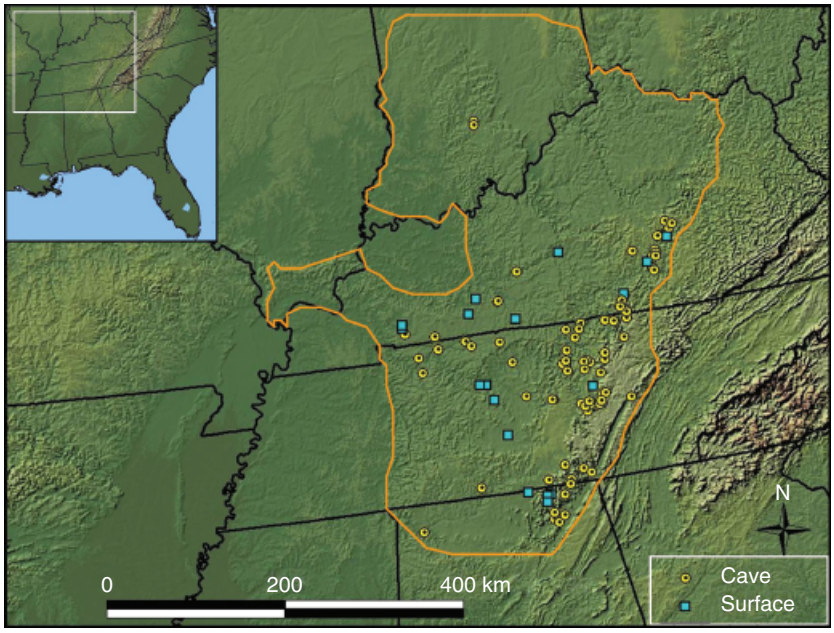


**Figure 1** Outlined range (in orange, adapted from Taylor, 1997) of *Cambarus tenebrosus* extending from central Indiana to northern Alabama. Blue dots represent surface collection sites whereas yellow dots represent cave collection sites.

**Table 1** Locations and sample sizes for all *Cambarus tenebrosus* used in this study

| Site | Location | Habitat | State | Country | Sample size | Haplotype (no. of individuals) |
|---|---|---|---|---|---|---|
| 1 | Arthur Singleton | Cave | KY | Rockcastle | 2 | 1(2) |
| 2 | Bakers | Cave | TN | Robertson | 1 | 1(1) |
| 3 | Bartlett | Cave | TN | Putnam | 4 | 1(3), 35(1) |
| 4 | Beaver Creek | Surface | TN | Wayne | 1 | 47(1) |
| 5 | Bellamy | Cave | TN | Montgomery | 3 | 1(3) |
| 6 | Bible Springs | Cave | TN | Marion | 2 | 31(1), 32(1) |
| 7 | Big Bush Creek | Surface | KY | Green | 2 | 1(1), 13(1) |
| 8 | Big Sink | Cave | KY | Pulaski | 2 | 1(2) |
| 9 | Big Sulphur | Cave | KY | Trigg | 2 | 3(2) |
| 10 | Blackpatch Hollow | Cave | TN | Robertson | 3 | 1(2), 4(1) |
| 11 | Blind Fish | Cave | TN | Putnam | 1 | 37(1) |
| 12 | Bluehole Resurgence | Cave | KY | Rockcastle | 4 | 1(4) |
| 13 | Bluff River | Cave | AL | Jackson | 2 | 49(1), 51(1) |
| 14 | Boone Hollow | Cave | TN | Clay | 2 | 1(2) |
| 15 | Browns Creek | Surface | TN | Davidson | 2 | 21(1), 22(1) |
| 16 | Bunkum | Cave | TN | Pickett | 2 | 9(1), 10(1) |
| 17 | Camps Gulf | Cave | TN | Van Buren | 2 | 37(1), 40(1) |
| 18 | Capshaw | Cave | TN | Putnam | 4 | 37(4) |
| 19 | Car Parts | Cave | KY | Rockcastle | 2 | 1(1), 5(1) |
| 20 | Cedar Creek | Cave | KY | Pulaski | 4 | 1(4) |
| 21 | Cherry | Cave | TN | Jackson | 1 | 11(1) |
| 22 | Climax | Cave | KY | Rockcastle | 1 | 14(1) |
| 23 | Clinton | Cave | TN | Pickett | 4 | 1(4) |
| 24 | Cornstarch | Cave | TN | Fentress | 1 | 5(1) |
| 25 | Cummings Cove | Surface | TN | Van Buren | 3 | 37(2), 58(1) |
| 26 | Dave's | Cave | KY | Pulaski | 1 | 5(1) |
| 27 | Dillions | Cave | IN | Orange | 2 | 56(1), 57(1) |
| 28 | Doug Green | Cave | AL | Jackson | 1 | 27(1) |
| 29 | Dripping Spring | Cave | TN | Franklin | 2 | 62(2) |
| 30 | Dumpling | Cave | KY | Pulaski | 2 | 1(1), 5(1) |
| 31 | Dunbar | Cave | TN | Montgomery | 1 | 5(1) |
| 32 | Duvalts | Cave | KY | Rockcastle | 1 | 6(1) |
| 33 | Edmonson Branch | Surface | TN | Davidson | 3 | 1(2), 46(1) |
| 34 | England Cove | Surface | TN | White | 5 | 17(2), 20(1), 37(1), 44(1) |
| 35 | Estill Fork | Surface | AL | Jackson | 3 | 33(1), 34(2) |
| 36 | Fancher | Cave | TN | Overton | 3 | 1(1), 38(2) |
| 37 | Fletcher Spring | Cave | KY | Rockcastle | 4 | 1(2), 7(2) |
| 38 | Flynn Creek | Cave | TN | Jackson | 1 | 1(1) |
| 39 | Gallatin Steam Plant | Cave | TN | Wilson | 1 | 1(1) |
| 40 | Garner Spring | Cave | TN | Franklin | 2 | 33(2) |
| 41 | Garretts Mill | Cave | TN | Overton | 3 | 40(3) |
| 42 | Grayson Gunner | Cave | KY | Wayne | 3 | 1(3) |
| 43 | Hail | Cave | KY | Pulaski | 3 | 1(2), 12(1) |
| 44 | Herring | Cave | TN | Rutherford | 4 | 1(3), 8(1) |
| 45 | Hester Creek | Surface | AL | Madison | 1 | 59(1) |
| 46 | Jared Hollow | Cave | TN | Putnam | 3 | 1(2), 24(1) |
| 47 | John Griffin | Cave | KY | Jackson | 1 | 14(1) |
| 48 | Kuykendall | Cave | TN | Putnam | 12 | 37(7), 43(5) |
| 49 | Larkin Fork | Surface | AL | Jackson | 1 | 33(1) |
| 50 | Larkin Spring | Cave | AL | Jackson | 3 | 49(3) |
| 51 | Laurel Creek | Cave | TN | Van Buren | 1 | 45(1) |
| 52 | Lick Fork | Surface | AL | Jackson | 1 | 61(1) |
| 53 | Limrock Blowing | Cave | AL | Jackson | 2 | 49(1), 50(1) |
| 54 | Lost Cove | Cave | TN | Franklin | 1 | 33(1) |
| 55 | Lost Cove | Cave | TN | Van Buren | 1 | 40(1) |
| 56 | Lost Creek | Cave | TN | White | 3 | 1(2), 9(1) |
| 57 | Lost River | Cave | KY | Warren | 2 | 14(2) |
| 58 | Mammoth | Cave | KY | Edmonson | 3 | 1(2), 3(1) |

**Table 1** Continued

| Site | Location | Habitat | State | Country | Sample size | Haplotype (no. of individuals) |
|---|---|---|---|---|---|---|
| 59 | Manning Spring | Cave | TN | Cumberland | 1 | 60(1) |
| 60 | Markham | Cave | TN | Clay | 1 | 1(1) |
| 61 | Martin Creek | Surface | TN | Putnam | 3 | 9(2), 36(1) |
| 62 | McBrides | Cave | AL | Jackson | 5 | 33(3), 49(2) |
| 63 | McKinney Pit | Cave | AL | Colbert | 1 | 28(1) |
| 64 | Merrybranch | Cave | TN | White | 2 | 1(1), 9(1) |
| 65 | Miller | Cave | TN | Warren | 3 | 29(1), 30(1), 52(1) |
| 66 | Moore's Spring | Cave | TN | Giles | 1 | 27(1) |
| 67 | Mud River | Surface | KY | Logan | 2 | 1(2) |
| 68 | Muddy Creek | Surface | KY | Logan | 1 | 1(1) |
| 69 | Natural Bridge | Cave | TN | Pickett | 1 | 1(1) |
| 70 | North Fork Creek | Surface | TN | Bedford | 2 | 23(2) |
| 71 | Norton Spring | Cave | TN | Warren | 2 | 33 (1), 53(1) |
| 72 | Pearson Spring | Cave | TN | Franklin | 3 | 26(1), 33(2) |
| 73 | Pennywinkle Spring | Cave | TN | Van Buren | 1 | 40(1) |
| 74 | Pitman Creek | Surface | KY | Pulaski | 1 | 48(1) |
| 75 | Pless | Cave | IN | Lawrence | 1 | 56(1) |
| 76 | Pond Cave | Cave | TN | Cannon | 1 | 1(1) |
| 77 | Price Valley | Cave | KY | Pulaski | 2 | 1(2) |
| 78 | Redmond Creek | Cave | KY | Wayne | 3 | 1(2), 5(1) |
| 79 | Richland Creek | Surface | TN | Davidson | 2 | 18(1), 19(1) |
| 80 | Roundstone Creek | Surface | KY | Rockcastle | 3 | 1(1), 7(1), 15(1) |
| 81 | Rumbling Falls | Cave | TN | Van Buren | 2 | 37(2) |
| 82 | Sauta | Cave | AL | Jackson | 2 | 49(2) |
| 83 | Sheldon | Cave | AL | Jackson | 2 | 49(2) |
| 84 | Short Creek | Cave | KY | Pulaski | 2 | 7(2) |
| 85 | Sinking Fork | Surface | KY | Trigg | 1 | 2(1) |
| 86 | Skillmans Mark | Cave | TN | Fentress | 1 | 5(1) |
| 87 | Spring at Fahey | Cave | TN | Putnam | 1 | 25(1) |
| 88 | Spring off Little Creek | Cave | TN | Putnam | 3 | 37(1), 41(1), 42(1) |
| 89 | State Trooper | Cave | KY | Warren | 3 | 14(3) |
| 90 | Steele Branch | Surface | KY | Trigg | 8 | 1(8) |
| 91 | Stout | Cave | TN | Putnam | 2 | 35(1), 39(1) |
| 92 | Stream | Cave | KY | Wayne | 4 | 1(2), 5(1), 16(1) |
| 93 | Sump Jump | Cave | TN | Robertson | 6 | 1(6) |
| 94 | Thorp | Cave | TN | Clay | 2 | 1(2) |
| 95 | Tonyas | Cave | KY | Wayne | 2 | 1(2) |
| 96 | Trammel Creek | Surface | KY | Allen | 1 | 1(1) |
| 97 | Trick or Treat | Cave | TN | Putnam | 2 | 1(2) |
| 98 | Turkeyscratch | Cave | TN | Warren | 2 | 33(1), 40(1) |
| 99 | Turner | Cave | TN | Houston | 1 | 1(1) |
| 100 | Twin Levels | Cave | KY | Christian | 1 | 5(1) |
| 101 | Upper Sheep | Cave | TN | White | 1 | 1(1) |
| 102 | Waterfall Hollow | Cave | TN | Van Buren | 1 | 37(1) |
| 103 | West Cemetery | Cave | TN | Putnam | 1 | 37(1) |
| 104 | Winching Hollow Water | Cave | TN | Van Buren | 3 | 37(1), 43(1), 44(1) |

rotator for 1 h. The supernatant was removed and the DNA pellet was vacuum dried for 15 min at 55 °C, and then the pellet was re-suspended in 200 $\mu$L of double-distilled water.

The 16S mitochondrial gene was sequenced for all samples because it is highly variable and appropriate for population genetic or intraspecific studies (Fetzner & Crandall, 2003; Buhay & Crandall, 2005). The following reactants were used in each of the 50 $\mu$L reactions: 5 $\mu$L 10 × buffer, 8 $\mu$L dNTPs, 8 $\mu$L 25 mM magnesium chloride, 5 $\mu$L of each 10 mM primer, 0.3 $\mu$L Taq Polymerase and 1.5 $\mu$L DNA with water added to total 50 $\mu$L. The primers used were 16SF (5′ GAC CGT GCK AAG GTA GCA TAA TC 3′) and 1472 (5′ AGA TAG AAA CCA ACC TGG 3′) (Crandall & Fitzpatrick, 1996). Polymerase chain reaction (PCR) was performed on a Peltier Thermal Cycler machine (AB9800, Foster City, CA, USA) or a GeneAmp PCR System 9700 (AB9700, Foster City, CA, USA) using the following program: 96 °C for 3 min, followed by 45 cycles of 94 °C for 1 min, annealing between 45 and 47 °C for 1 min, and 72 °C for 1 min, followed by a final elongation at 72 °C

for 5 min. PCR products were examined on a 1.5% agarose gel using an ethidium bromide stain. The PCR products were purified using a Montage $PCR_{96}$ plate (Millipore, Billerica, MA, USA). The PCR products were cycle sequenced using the ABI Big-dye Ready-Reaction kit with 1/4 or 1/8 of the normal reaction size, and sequences were generated on an Applied Biosystems (Foster City, CA, USA) 3730 XL Automated Sequencer at the BYU DNA Sequencing Center. Resulting sequences were edited using Sequencher 4.2 OS X (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned by eye using MacClade 4.05 OS X (Madison & Madison, 2000).

## Delimiting species

Although methods of diagnosing species remain a controversial issue in systematic biology (Sites & Marshall, 2003, 2004), they are highly relevant to conservation studies (Sites & Crandall, 1997) because the method of delimitation can have a significant impact on the number of species diagnosed (Agapow *et al*., 2004). We prefer a statistically testable method developed for use with molecular data for our study. Templeton's test of cohesion (Templeton, 1989) uses both historical and current processes to statistically delimit species boundaries through a suite of nested null hypotheses. The hypotheses are then used to determine correlations between genotype and geographic location, habitat or other ecological variables [nested clade analysis (NCA); Templeton, Routman & Phillips, 1995]. Under this definition, two organisms would be considered a single species if they are genetically and/or ecologically exchangeable (Templeton, 2001; Rader *et al*., 2005).

## Phylogenetic analysis

The model of evolution that best fits the sequence data was determined using the program ModelTest 3.06 (Posada & Crandall, 1998), with the unique 16S haplotypes determined by TCS 1.18 (Clement, Posada & Crandall, 2000). A Bayesian phylogeny was obtained using MrBayes v3.0b4 (Huelsenbeck & Ronquist, 2001; Huelsenbeck *et al*., 2001) with over 20 Markov chains run simultaneously using only unique haplotypes, with each chain initiating at a random tree and parameters nst = 6 and rates = adgamma provided by ModelTest. This analysis was run for 20 million generations on 20 processors on a 64-node RackSaver computing cluster, taking samples from the chain every 1000th tree, totaling 20 001 trees. Using the sampled trees minus the burn-in determined by Tracer (http://evolve.zoo.ox.ac.uk/software.html), a majority-rule consensus tree was constructed. A posterior probability of 95% or greater is considered to be strong Bayesian support for a node (Huelsenbeck & Ronquist, 2001).

## Genetic–geographic associations

NCA allows the partitioning of current population parameters (e.g. recent gene flow) from historical events (e.g. range expansion). NCA is a statistical approach that distinguishes among alternative hypotheses to explain contemporary and historical genetic patterns using haplotype diversity information coupled with geographic location information (Templeton *et al*., 1995; Templeton, 1998; Avise, 2000). Inferences about genetic patterns can be made by testing a null hypothesis of no association between the collecting locale and the genetic variability (Templeton *et al*., 1995).

To perform an NCA, a haplotype network was constructed using TCS 1.18 set at a 95% confidence level. The original haplotype network contained several loops, which would be ambiguous in the NCA. These loops were broken using the protocol of Crandall & Templeton (1993) and Templeton & Sing (1993), where the number of sequences in a haplotype and geographic location were most heavily considered. The network was then converted into a series of nesting groups (Templeton, Boerwinkle & Sing, 1987), with the haplotypes exhibiting the highest sequence frequency and most connections being ancestral to the others. According to coalescent theory, haplotypes found at the tips are more recently evolved than those in the interior of the network (Crandall & Templeton, 1993; Templeton, 2004).

To test the null hypothesis of no geographical association, two measurements were calculated by the program GeoDis 2.2 (Posada, Crandall & Templeton, 2000). The first is 'clade distance' ($D_c$), which measures the geographical range of a clade at each nested level. Distances were determined by GeoDis using the longitude and latitude coordinates taken at each sample site. Fetzner & Crandall (2003) suggested, for aquatic species, a 'river' distance (measuring the distance between two points following only linear water bodies) rather than great circle distance (which uses latitude–longitude coordinates). This approach was not taken for this project because aquatic distances are not known for subterranean basins due to unknown and inaccessible connections. Although the approach used in this study could have some effect on the lower (newer) nesting levels, the higher (older) nesting levels would presumably remain unaffected (Fetzner & Crandall, 2003). The second measurement calculated by GeoDis is 'nested clade distance' ($D_n$), which estimates the evolutionary distance between two haplotypes or clades from the center (oldest) nested clade. The output of GeoDis was used to answer a series of dichotomous questions in the NCA inference key (Templeton, 2004). These inferences help explain what type of event [such as contiguous range expansion (CRE) or restricted gene flow (RGF)] led to the current haplotype diversity of a species. The most recent version of the GeoDis inference key can be found at http://darwin.uvigo.es/software/geodis.html.

## Genetic–habitat associations

GeoDis was used to test for significant associations between genetic and habitat (cave or surface) patterns for clades that include both habitat types. This was done by reducing the number of 'locations' in the GeoDis input file to two (cave and surface). These two new 'locations' were assigned different coordinates and the test of habitat association ($\chi^2$)

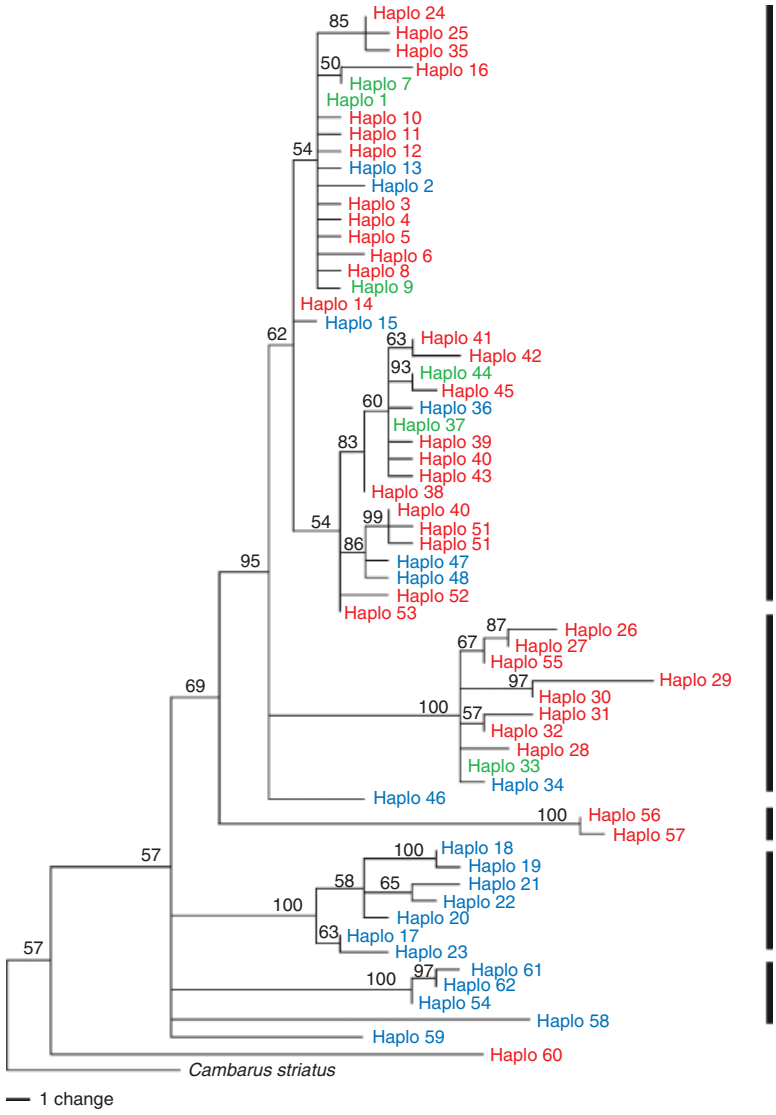


**Figure 2** Phylogenetic relationships of 62 *Cambarus tenebrosus* haplotypes of 16S mtDNA sequences. The Bayesian analysis was run using the GTR + I + G (general time reversible plus proportional invariant plus gamma) model of evolution determined by ModelTest. The numbers above the branches indicate posterior probabilities. Haplotypes were colored according to habitat (red = cave, blue = surface and green = both cave and surface). The five main clades are labeled with roman numerals as I, eastern Cumberland Plateau; II, central Tennessee; III, Indiana; IV, western Cumberland Plateau; V, entire sampled range except Indiana. *Cambarus striatus* was used as an outgroup.

was performed over 5000 permutations. This effectively results in a permutation $\chi^2$ test as described by Roff & Bentzen (1989).

To test the hypothesis that *C. tenebrosus* is a recent invader of the cave habitat versus a long-standing resident, we used Fisher's exact test to identify significant associations between tip haplotypes (more recent events) and interior haplotypes (older events) for cave and surface habitats in clades with both habitats represented. If the species was a recent invader into subsurface waters, a significant association would be expected between the cave habitats and the tip locations of the tree. Likewise, if the species was historically located in the cave, but recently invaded surface waters, a significant association would be observed between the cave and interior clades (or surface and tip clades). If no significant association was found, this would provide evidence for long-term residence in both cave and surface waters.

## Demographic parameters

Current genetic diversity ($\theta_\pi$; Tajima, 1983) and historical-based genetic diversity ($\theta_w$; Watterson, 1975) were obtained using the computer program DnaSP 4.0 (Rozas *et al.*, 2003). Current genetic diversity was computed by pairwise differences between sequences whereas historical-based Watterson's $\theta$ was determined by the number of segregating sites. These two methods together provide a diversity comparison between current and recent historical diversity of a species for a conservation perspective (Templeton, 1993; Yu *et al.*, 2003; Buhay & Crandall, 2005). Recent losses of diversity (e.g. through selective sweeps or population bottlenecks) would

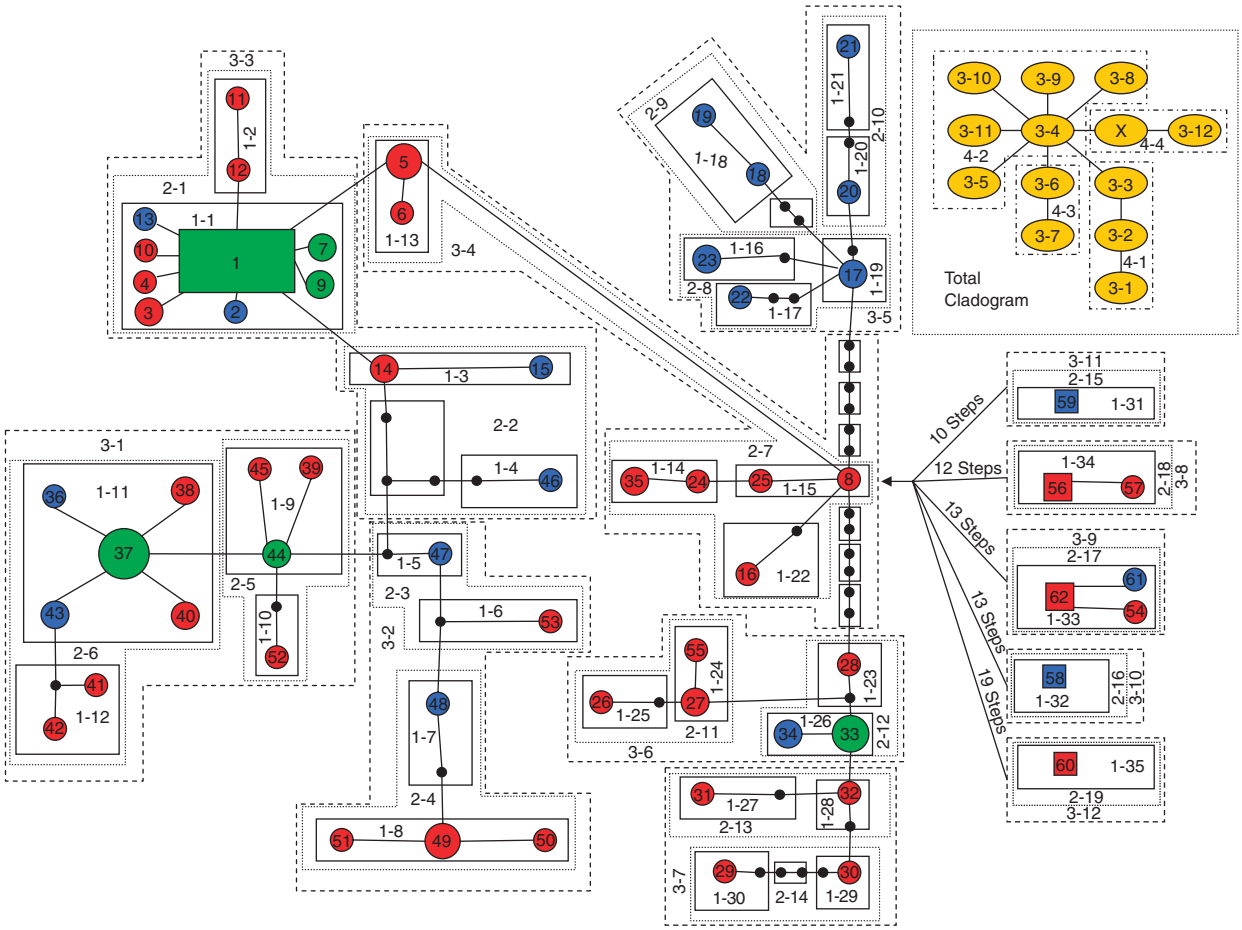


**Figure 3** Haplotype network showing the nesting levels used to infer historical processes. Numbers indicate haplotypes (62 total) with black dots representing unsampled or possible extinct haplotypes. The rectangular shape designates the ancestral haplotype for that network. Haplotypes represented by larger numbers of individuals (frequency) are depicted as larger shapes, but the size is not proportional to frequency. Colors correspond to habitat (red = cave, blue = surface and green = both cave and surface). The total cladogram is shown in orange.

typically show $\theta_\pi < \theta_w$, whereas recent increases in genetic diversity (e.g. through population growth) would show $\theta_\pi > \theta_w$.

# Results

## Phylogenetic analyses

A total of 233 partial 16S (485 base pairs) mitochondrial DNA sequences from 104 collection sites was gathered for *C. tenebrosus*, which included 62 unique haplotypes (Table 1). These haplotypes are accessioned into GenBank as DQ087332–DQ087393. Bayesian analysis (Fig. 2) revealed that *C. tenebrosus* from both cave and surface habitats formed a monophyletic group relative to *C. striatus* (GenBank DQ087394). The cave and surface populations did not form separate monophyletic groups, indicating that there is ongoing gene flow between these two habitats. Additionally, the same haplotype was found in both surface and subsurface habitat types in six instances.

## NCA

Haplotype connections $\leq$ nine substitutions for the 485 bp of the 16S mitochondrial gene were determined to be part of the 95% confidence set of network connections. All haplotypes were included in a single network created by TCS with the exception of haplotypes 54 and 56–62 (Fig. 3). Although these haplotypes were determined to be outside the 95% confidence level (by 13 or fewer mutational steps for every haplotype, except 60 which was 19 steps), they were still included in the analysis. Haplotypes 56 and 57 (Indiana cave sites) may have connected to the network had more sampling taken place in north-western Kentucky and southern Indiana (Fig. 1). *Cambarus striatus* was also outside the 95% confidence level, being 10 mutational steps from haplotype 8. The network mostly centered around a single ancestral haplotype (haplotype 1 in Fig. 3) that contained 88 sequences from 42 locations (both cave and surface) found throughout the range of *C. tenebrosus*, excluding Indiana. Nesting of the haplotype network resulted in 35 one-step clades, 19 two-step clades, 12 three-

**Table 2** Results of the nested clade analysis of *Cambarus tenebrosus* 16S mtDNA haplotypes based on 5000 permutations

| 0-step clades | | | 1-step clades | | | 2-step clades | | | 3-step clades | | | 4-step clades | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | $D_c$ | $D_n$ | Clade | $D_c$ | $D_n$ | Clade | $D_c$ | $D_n$ | Clade | $D_c$ | $D_n$ | Clade | $D_c$ | $D_n$ |
| 1 | 93 | 93.62 | 1-1 | 97.36L | 97.24 | 2-1 | 95.67 | 96.23 | 3-3 | 98.37 | 100.14 | 4-1 | 95.81S | 96.38S |
| 2 | 0.00 | 177.66 | | | | | | | | | | | | |
| 3 | 53.67 | 151.04L | | | | | | | | | | | | |
| 4 | 0.00 | 105.75 | | | | | | | | | | | | |
| 7 | 11.84S | 135.42 | | | | | | | | | | | | |
| 9 | 36.95S | 67.72 | | | | | | | | | | | | |
| 10 | 0.00 | 47.63 | | | | | | | | | | | | |
| 13 | 0.00 | 77.22 | | | | | | | | | | | | |
| I-T | 69.18L | −16.79 | | | | | | | | | | | | |
| 11 | 0.00 | 29.19S | 1-2 | 43.73 | 45.88 | | | | | | | | | |
| 12 | 0.00 | 87.35L | | | | | | | | | | | | |
| I-T | 0.00 | 58.16L | I-T | 53.63 | 51.36 | | | | | | | | | |
| 14 | 105.03L | 105.04 | 1-3 | 103.93 | 105.09S | 2-2 | 105.03 | 116.12 | | | | | | |
| 15 | 0.00 | 90.61 | | | | | | | | | | | | |
| I-T | 105.03L | 14.44 | | | | | | | | | | | | |
| 46 | | | 1-4 | 0.00 | 175.99 | | | | | | | | | |
| | | | I-T | 103.93 | −70.90S | | | | | | | | | |
| 40 | 23.27 | 23.36 | 1-9 | 24.23 | 23.90 | 2-5 | 23.56 | 27.81 | 3-1 | 24.62S | 52.25S | | | |
| 45 | 0.00 | 16.66 | | | | | | | | | | | | |
| 39 | 0.00 | 46.28 | | | | | | | | | | | | |
| I-T | 23.27 | −8.11 | | | | | | | | | | | | |
| 52 | | | 1-10 | 0.00 | 17.99 | | | | | | | | | |
| | | | I-T | 24.23 | 5.91 | | | | | | | | | |
| 41 | | | 1-12 | 0.00 | 23.04 | 2-6 | 22.41S | 22.85S | | | | | | |
| 42 | | | | | | | | | | | | | | |
| 36 | 0.00 | 37.36L | 1-11 | 22.37 | 22.36 | | | | | | | | | |
| 37 | 21.35 | 21.40 | | | | | | | | | | | | |
| 38 | 0.00 | 28.24 | | | | | | | | | | | | |
| 43 | 23.51 | 24.68 | | | | | | | | | | | | |
| 44 | 8.98 | 16.16 | | | | | | | | | | | | |
| I-T | 18.22 | −3.12 | I-T | 22.37 | −0.68 | I-T | 1.15 | 4.96 | | | | | | |
| 47 | | | 1-5 | 0.00 | 47.76 | 2-3 | 63.74 | 131.49 | 3-2 | 105.62 | 159.68L | | | |
| 58 | | | 1-6 | 0.00 | 95.70 | | | | | | | | | |
| 48 | | | 1-7 | 0.00 | 250.57 | 2-4 | 78.58 | 99.56 | | | | | | |
| 49 | 12.36 | 13.02 | 1-8 | 12.99S | 46.72S | | | | | | | | | |
| 50 | 0.00 | 6.90 | | | | | | | | | | | | |
| 51 | 0.00 | 18.84 | | | | | | | | | | | | |
| I-T | 12-36 | 0.15 | I-T | −12.99L | 203.84L | I-T | −14.83 | 31.93 | I-T | 74.63L | 55.11L | | | |
| 5 | 103.49 | 100.27 | 1-13 | 79.01 | 86.06 | 2-7 | | | 3-4 | 93.77S | 102.43S | 4-2 | 128.07L | 125.60L |
| 6 | 0.00 | 108.48 | | | | | | | | | | | | |
| I-T | 103.49 | −8.21 | | | | | | | | | | | | |
| 35 | 0.64S | 1.81 | 1-14 | 2.44S | 61.05 | | | | | | | | | |
| 24 | 0.00 | 3.86L | | | | | | | | | | | | |
| I-T | −0.64 | 2.05 | | | | | | | | | | | | |
| 25 | 0.00 | 11.04S | 1-15 | 17.68 | 74.72 | | | | | | | | | |
| 8 | 0.00 | 44.22 | | | | | | | | | | | | |
| 16 | | | 1-22 | 0.00 | 37.37 | | | | | | | | | |
| | | | I-T | 64.91 | 28.66 | | | | | | | | | |
| 23 | | | 1-16 | 0.00 | 29.83 | 2-8 | 46.67 | 49.35 | 3-5 | 45.42S | 90.31S | | | |
| 22 | | | 1-17 | 0.00 | 48.65 | | | | | | | | | |
| 17 | | | 1-19 | 0.00 | 86.28 | | | | | | | | | |
| | | | I-T | 0.00 | 50.17 | | | | | | | | | |
| 18 | | | 1-18 | | | 2-9 | 0.00 | 35.86 | | | | | | |
| 19 | | | | | | | | | | | | | | |
| 20 | | | 1-20 | 0.00 | 85.24L | 2-10 | 48.68 | 48.42 | | | | | | |

**Table 2** Continued

| 0-step clades | | | 1-step clades | | | 2-step clades | | | 3-step clades | | | 4-step clades | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | $D_c$ | $D_n$ | Clade | $D_c$ | $D_n$ | Clade | $D_c$ | $D_n$ | Clade | $D_c$ | $D_n$ | Clade | $D_c$ | $D_n$ |
| 21 | | | 1-21 | 0.00 | 34.06 | | | | | | | | | |
| | | | I-T | 0.00 | 51.18 | I-T | 22.33 | 7.21 | | | | | | |
| 56 | 1.99 | 2.24L | 1-34 | | | 2-18 | | | 3-8 | 2.24S | 269.13 | | | |
| 57 | 0.00 | 2.24 | | | | | | | | | | | | |
| I-T | 1.99 | 0.00L | | | | | | | | | | | | |
| 54 | 0.00 | 35.10 | 1-33 | | | 2-17 | | | 3-9 | 25.67S | 158.72 | | | |
| 61 | 0.00 | 25.55 | | | | | | | | | | | | |
| 62 | 0.00 | 16.37 | | | | | | | | | | | | |
| I-T | 0.00 | −13.96 | | | | | | | | | | | | |
| 58 | | | 1-32 | | | 2-16 | | | 3-10 | 0.00 | 78.77 | | | |
| 59 | | | 1-31 | | | 2-15 | | | 3-11 | 0.00 | 166.96 | | | |
| | | | | | | | | | I-T | 64.98 | −36.50 | | | |
| 26 | | | 1-25 | 0.00 | 37.69 | 2-11 | 58.30 | 57.31 | 3-6 | 54.68 | 53.87 | 4-3 | 54.40S | 131.65L |
| 27 | 37.65S | 52.99S | 1-24 | 60.53 | 60.59 | | | | | | | | | |
| 55 | 0.00S | 75.61 | | | | | | | | | | | | |
| I-T | 37.65 | −22.61S | I-T | 60.53 | 22.90 | | | | | | | | | |
| 28 | | | 1-23 | 0.00 | 147.89 | 2-12 | 50.12 | 53.47 | | | | | | |
| 33 | 26.76 | 26.32 | 1-26 | 26.77 | 34.51 | | | | | | | | | |
| 34 | 0.00 | 30.51 | | | | | | | | | | | | |
| I-T | 26.76 | −4.19 | | | | I-T | −8.18 | −3.84 | | | | | | |
| 31 | | | 1-27 | | | 2-13 | 0.00 | 29.02 | 3-7 | 34.83 | 57.79 | | | |
| 32 | | | 1-28 | | | | | | | | | | | |
| 30 | | | 1-29 | | | 2-14 | 0.00 | 43.53 | | | | | | |
| 29 | | | 1-30 | | | | | | | | | | | |
| | | | | | | I-T | 0.00 | −14.51 | I-T | 19.85 | −3.92 | | | |
| 60 | | | 1-35 | | | 2-19 | | | 3-12 | | | 4-4 | 0.00 | 84.02 |
| | | | | | | | | | | | | I-T | 37.47L | 25.25L |

Clade ($D_c$) and nested clade ($D_n$) distances are given. S indicates that the distance is significantly small at the 5% level and L indicates that the distance is significantly large. In clades with both tip and interior nested clades, the average distance I-T is given. Shaded regions indicate interior grouping.

step clades, four four-step clades and the total cladogram (Fig. 3). The NCA returned 23 significantly large and 27 significantly small associations between genetic variance and geographic location (Table 2).

The NCA revealed significant genetic associations of clades and sampling locations at all clade levels except level two (Table 3). The null hypothesis of no geographic association was rejected at two 1-step clades (1-1 and 1-11), three 3-step clades (3-1, 3-2, and 3-3), all 4-step clades (4-1, 4-2 and 4-3) and the total cladogram. RGF with isolation by distance (IBD) was inferred for four of the nine significant clades (at 3- and 4-step levels; Table 3). An inference of RGF with long-distance dispersal (LDD) was determined for the total cladogram.

## Habitat association

The habitat association $\chi^2$ test revealed no significant association between current genetic patterning and habitat type (cave and surface), except for clades 1-26, 3-2 and 4-2 (Table 4). For Fisher's exact test, we counted 23 cave haplotypes occurring on the tips with 12 interior and 13 surface tip haplotypes with five interior. This resulted in no significant association between habitat (cave and surface) and relative age (recent and historical) of the tested haplotypes ($P = 0.76$).

## Demographic parameters

Estimates of the current ($\theta_\pi$) and recent historical ($\theta_w$) genetic diversity for *C. tenebrosus* are extremely high (Nei, 1987) and independent of habitat type (Table 5). These diversity estimates are proportional to the effective population sizes ($\theta = 2N_e\mu$), suggesting that the number of breeding individuals is large in both cave and surface populations. The estimate of effective population size should not be considered a census of the total population of the species, as it only estimates the number of breeding individuals contributing to the gene pool. Interestingly, the recent historical diversity estimates ($\theta_w$) are almost double those of the current diversity estimates ($\theta_\pi$) (Table 5), showing a sharp decline, nearly 50% loss, in the recent history of the species (Sinclair *et al.*, 2002; Yu *et al.*, 2003; Buhay & Crandall, 2005).

**Table 3** Nested contingency results based on 5000 permutations in GeoDis

| Clade | $\chi^2$ | Probability | Inference chain | Inferred pattern |
|---|---|---|---|---|
| 1-1 | 464.80 | 0.01* | 1-2-3-5-6-7-8-No | IS |
| 1-2 | 2.00 | 1.00 | 1-19-20-2-11-17-4-No | RGF with some IBD |
| 1-3 | 8.00 | 0.37 | 1-2-3-4-No | RGF with some IBD |
| 1-8 | 11.82 | 0.45 | Nothing significant | NA |
| 1-9 | 17.00 | 0.43 | Nothing significant | NA |
| 1-11 | 86.92 | 0.007* | 1-2-11-17-No | IO |
| 1-13 | 10.00 | 0.30 | Nothing significant | NA |
| 1-14 | 3.00 | 1.00 | 1-2-3-4-No | RGF with some IBD |
| 1-15 | 2.00 | 1.00 | 1-19-20-2-11-17-4-No | RGF with some IBD |
| 1-24 | 3.00 | 1.00 | 1-19-20-2-11-12-No | CRE |
| 1-26 | 8.56 | 0.60 | Nothing significant | NA |
| 1-33 | 8.00 | 0.16 | Nothing significant | NA |
| 1-34 | 0.75 | 1.00 | 1-2-11-17-4-No | RGF with some IBD |
| 2-1 | 70.65 | 0.14 | 1-2-3-4-No | RGF with some IBD |
| 2-2 | 8.00 | 0.34 | 1-19-20-2-3-5-6-7-Yes | RGF/D with LDD |
| 2-3 | 2.00 | 1.00 | Nothing significant | NA |
| 2-4 | 13.00 | 0.07 | 1-19-20-2-3-5-6-7-8-No | IS |
| 2-5 | 10.00 | 0.30 | Nothing significant | NA |
| 2-6 | 22.63 | 0.10 | Nothing significant | NA |
| 2-7 | 34.13 | 0.88 | 1-2-3-4-No | RGF with some IBD |
| 2-8 | 10.00 | 0.06 | Nothing significant | NA |
| 2-10 | 2.00 | 1.00 | 1-2-11-17-4-No | RGF with some IBD |
| 2-11 | 4.00 | 1.00 | Nothing significant | NA |
| 2-12 | 14.00 | 0.34 | Nothing significant | NA |
| 3-1 | 42.11 | 0.00* | 1-2-3-4-No | RGF with some IBD |
| 3-2 | 16.00 | 0.03* | Nothing significant | NA |
| 3-3 | 114.00 | 0.0002* | Nothing significant | NA |
| 3-5 | 10.65 | 0.17 | Nothing significant | NA |
| 3-6 | 14.99 | 0.11 | Nothing significant | NA |
| 3-7 | 4.00 | 0.35 | Nothing significant | NA |
| 4-1 | 346.72 | 0.00* | 1-2-3-4-No | RGF with some IBD |
| 4-2 | 170.00 | 0.00* | 1-2-11-12-No | CRE |
| 4-3 | 23.00 | 0.02* | Nothing significant | NA |
| Total | 612.58 | 0.00* | 1-2-3-5-6-7-Yes | RGF/D with LDD |

*indicates significance with a probability of 0.05 or less. Inferences were made using Templeton's (2004) revised key. RGF/D, restricted gene flow/dispersal; IBD, isolation by distance; CRE, contiguous range expansion; IS, inadequate sampling; IO, inconclusive outcome; LDD, long-distance dispersal.

## Discussion

The support values for most nodes in Bayesian topology are markedly low. The polytomies in the tree are not a result of low overall genetic diversity, but rather are caused by small mutations in the 16S gene that cannot be resolved at the intraspecific level using a phylogenetic approach. However, some deep structure exists in the tree, showing four well-supported clades, which mainly cluster according to geography (Fig. 2). Clade I is localized near the eastern border between Alabama and Tennessee along the Cumberland Plateau. All the haplotypes in clade II are from the surface sites in central Tennessee. Clade III is localized to south-central Indiana and could represent a distinct evolutionarily significant unit (ESU), but more sampling is required in this area to support this conclusion. The haplotypes in clade IV are concentrated along the border separating Alabama and Tennessee to the west of those haplotypes found in clade I. Clade V is a mixture of both surface and cave populations and spans the entire sampled distribution of *C. tenebrosus*, except for Indiana.

RGF and contiguous range expansion were inferred for most of the significant phylogeographic patterns within *C. tenebrosus*, particularly in clade 4-2 (Fig. 3), which includes six (of 12 total) of the 3-step clades. This may explain why *C. tenebrosus* is found across such a large distribution for a freshwater crayfish species. The network was less informative at some nesting levels because of possible short isolation periods, insufficient geographic sampling or panmixia.

Samples of *C. tenebrosus* from Indiana (haplotypes 56 and 57) were separated by 12 steps and haplotype 60 from central Tennessee was 19 steps from the 95% network. With such extensive geographic overlap of the clades, particularly in Tennessee and Alabama, it becomes difficult to define boundaries for ESU designation within *C. tenebrosus*. Additional sampling in northern Kentucky and

**Table 4** $\chi^2$ test of habitat association executed in GeoDis. This test includes only clades with both cave and surface locales

| Clade | $\chi^2$ | Probability |
|---|---|---|
| 1-1 | 10.682 | 0.162 |
| 1-3 | 8.000 | 0.126 |
| 1-11 | 8.957 | 0.086 |
| 1-26 | 8.556 | 0.036* |
| 1-33 | 4.000 | 0.497 |
| 2-1 | 0.498 | 1.000 |
| 2-2 | 3.938 | 0.226 |
| 2-4 | 14.000 | 0.074 |
| 2-6 | 0.354 | 1.000 |
| 2-12 | 0.268 | 1.000 |
| 3-1 | 1.607 | 0.332 |
| 3-2 | 9.905 | 0.026* |
| 3-3 | 0.035 | 1.000 |
| 3-6 | 0.950 | 0.569 |
| 4-1 | 1.724 | 0.452 |
| 4-2 | 30.716 | 0.000* |
| 4-3 | 0.726 | 0.617 |
| Total | 6.681 | 0.074 |

*indicates significance with a probability of 0.05 or less.

**Table 5** Current ($\theta_\pi$) and historical-based ($\theta_w$) estimates of genetic diversity and corresponding effective population size estimates for *Cambarus tenebrosus* (collectively and segregated based on habitat)

| | Current | | Historical | |
|---|---|---|---|---|
| *Cambarus tenebrosus* | $\theta_\pi$ | $N_e$ | $\theta_w$ | $N_e$ |
| All samples ($n$=233) | 0.02359 | 428 910 | 0.04394 | 798 910 |
| Cave ($n$=187) | 0.02142 | 389 450 | 0.04007 | 728 550 |
| Surface ($n$=46) | 0.02677 | 486 730 | 0.03501 | 636 550 |

Effective population sizes were determined using a substitution rate of 2.2% per million years with a generation time of 5 years (Buhay & Crandall, 2005).

southern Indiana may support the recognition of ESUs or even distinct species which do not overlap geographically with other clades. Our outgroup species, *C. striatus*, fell just outside the 95% confidence limit in the haplotype network at 10 mutational steps. Further sampling of *C. striatus* and other closely related species may provide additional insight into phylogenetic relationships with *C. tenebrosus*.

*Cambarus tenebrosus* appears to have occupied both cave and surface habitats throughout its evolutionary history. This is supported by the presence of haplotypes from both cave and surface habitats situated in the interior of the network. Therefore, rather than an incipient cave species, it appears that *C. tenebrosus* is a long-term inhabitant of caves and associated streams, despite the morphological changes typically associated with the obligate cave-dwelling species.

Despite having a relatively abrupt decrease in genetic diversity in recent history, *C. tenebrosus* still maintains an extremely high level of diversity. This high level of diversity is not surprising considering its unusually large range, its ability to survive in above-ground and below-ground aquatic habitats, and a certain degree of population subdivision among the major clades. *Cambarus tenebrosus* is an opportunistic crayfish, occupying almost any freshwater karst area, including subterranean areas with and without obligate cave-dwelling crayfish species. For the subterranean populations, open habitat increases as the limestone erodes, which creates new subterranean spaces and corridors (i.e. connections between two previously separated karst areas). These newly formed groundwater connections provide new habitat over time as well as access to other neighboring gene pools.

*Cambarus tenebrosus* is a robust species of freshwater crayfish in that it has attained an extremely high level of genetic diversity because it can thrive in two very different yet connected habitats. Important factors in shaping the genetic patterns of aquatic species are climatic fluctuations and glacial events (Graham & Grimm, 1990; Vrba, 1992; Roy *et al*., 1996). It appears that the cave populations of *C. tenebrosus* have slightly higher historical genetic diversity than surface populations. A higher genetic diversity in the caves may suggest that the underground environment possibly acted as refugia during glacial/interglacial periods when surface waters were in flux between drought during glacial periods and flooding periods during interglacials of south-flowing meltwaters.

Personal observations regarding the troglomorphisms of *C. tenebrosus* indicate that populations in the northern portion of the species' distribution were notably less sensitive to artificial light (e.g. flashlights) in the caves, whereas *C. tenebrosus* in northern Alabama caves were often startled by light and retreated. Moreover, *C. tenebrosus* in the more northern areas were mostly gray or light brown in body color, whereas the southern populations possessed more coloration such as light orange, green and pink. This might indicate that the crayfish expanded into the southern regions more recently, and have not had time to accumulate fixed troglomorphisms (such as loss of body pigmentation) in the southern populations.

Invasive species are acknowledged as a major economical threat as well as a threat to indigenous species (Vitousek *et al*., 1996; Pimentel *et al*., 2000; Mooney & Cleland, 2001) throughout the world. Invasive species are typically genetically diverse (Lee, 2002), thus providing a rich pool to draw from to adapt to new surroundings and to out-compete species that occupy a similar niche. *Cambarus tenebrosus* would certainly fall into this description of a potential invasive species because of its high levels of genetic variability and its capacity to thrive in cave and surface environments, particularly karst-dominated areas. Identifying possible invasive species is necessary to protect the overall biological diversity of freshwater systems (Lodge *et al*., 1998). By identifying potential invasive species, precautions can be taken to help avoid their introduction into new areas. Crayfishes are particularly troublesome because they are often used as fish bait and, therefore, are easily transferred artificially from one location to another. If this form of unnatural range expansion were to happen with *C. tenebrosus*, it would be especially problematic in both surface and cave environments.

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