

**Caves as Islands: Population Genetics of  
*Nesticus barri* (Araneae: Nesticidae)**

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## **Abstract**

Caves are abundantly found throughout the United States, especially in the southern Appalachian Mountains, and are home to a diverse fauna. This cave fauna, though diverse, often evolves in a convergent manner causing many obligate cave species to have small or absent eyes, to be light colored, and to have long appendages. The spider genus *Nesticus* is found throughout the southern Appalachian Mountains and Cumberland Plateau and most, though not all, are obligate cave species (troglobites). *Nesticus barri* are found in approximately sixty caves on the southern Cumberland Plateau in Tennessee and Alabama. This study examined the population genetics of *N. barri* to see how much gene flow occurs between populations by sequencing the mitochondrial DNA cytochrome oxidase I (COI) gene. We found that *N. barri* is a monophyletic species with six distinct clades that are largely geographically distinct. No haplotypes were shared between clades, indicating that there is little to no gene flow between populations of this species. This lack of gene flow between caves, and relatively deep divergence between clades, has implications for conservation of these spiders because a loss of any one cave would dramatically decrease the diversity of the population as a whole.

## **Introduction**

Caves are home to a unique and diverse community of species. There are thousands of caves throughout the contiguous 48 United States but there are a few areas in particular that have an abundance of caves and species diversity. As of 2000, a total of 973 obligate cave species had been described in the continental United States (Culver 2000). The dominant groups are arachnids, crustaceans, and hexapods. One area of particular cave abundance and species diversity is the southern edge of the Cumberland Plateau in Franklin and Marion Counties in Tennessee and in Jackson and Marshall Counties in Alabama. Jackson County Alabama has more described cave obligate species (52) and more endemic cave obligate species (24) than any other county in the country. Cave habitats are an extreme environment with species uniquely and evolutionarily suited to living in them. They usually have three relatively distinct habitats within them: aquatic communities, terrestrial communities, and communities living in dung located near the entrance of the cave. Another aspect of caves that lends itself to species diversity is that many species are endemic to a few caves in a single county or even to a single cave (Culver 2000).

Terrestrial obligate cave species (troglobites) and aquatic obligate cave species (stygbobites) often evolve in a convergent manner because of the extreme environment of the cave such that much of cave fauna have small to absent eyes, are light colored, and have long appendages. Because of this convergent evolution in cave species, phylogenetically distant species living in isolation may be morphologically very similar which can lead to the same name given to different species or a hypothesis of close evolutionary relatedness of two distantly related species (Hedin 1997a; Porter 2007). For

this reason, molecular evidence is often a more reliable means of classifying cave species than morphological comparisons alone. Cave habitats, as compared to surface habitats, are limiting in that cave faunas are often restricted in where they can live and their ability to migrate. This may lead to high levels of endemism in many cave species and to distinct populations and haplotypes within a species inhabiting several different caves (Porter 2007). Trogloniles, which are able to survive outside caves, though tend to complete their lifecycle in them, and troglonenes, which do not complete their lifecycle in caves but often use them for shelter, usually have higher levels of gene flow in their populations because of the greater continuity between habitats (Caccone 1985).

The only genetic studies of troglonites or styglonites on the southern Cumberland Plateau were conducted by Buhay and Crandall (2005) and Buhay *et al.* (2007) on two different genera of cave crayfish (*Orconectes* and *Cambarus*). They found that the crayfish have large population sizes, great genetic diversity, and that there is extensive gene flow between populations of these animals as evidenced by haplotypes that were found from more than one cave (Buhay and Crandall 2005; Buhay *et al.* 2007). One possible reason that this study shows that there is a great amount of gene flow in styglonitic species while the opposite results have been found for troglonitic species (Hedin 1997b) is that the crayfish are aquatic and may be able to travel through underwater aquifers to different caves while troglonites are restricted to terrestrial habitats and if there are no terrestrial connections between caves, gene flow would be nearly impossible.

One common troglonite of the southern Cumberland Plateau are spiders of the genus *Nesticus*. They are medium sized (2 to 7 mm long) and can be whitish, yellow, or

dusky in color. *Nesticus* species have been found in Europe, Mexico, Cuba, the Dominican Republic, and the United States. Their greatest species diversity, however, is found in the southeastern United States where there are at least 30 different species in the southern Appalachian Mountains and Cumberland Plateau. There is one species found in northeastern United States that was introduced from Europe and three species in California (Gertsch 1984; Hedin and Dellinger 2005). Southeastern *Nesticus* are limited to cool, moist microhabitats. Most, though not all, are obligate cave species (Hedin and Dellinger 2005). Few studies have been conducted about *Nesticus* spiders outside of describing new species. Hedin (1997a) studied the speciation history of the *Nesticus* species of the southern Appalachian Mountains. He also studied the population genetics and speciation mechanisms of the southern Appalachian Mountain *Nesticus tennesseensis* complex (1997b). This study examined the *N. tennesseensis* complex which is found in eastern Tennessee, western North Carolina, western Virginia, and southern West Virginia. This study found a small number of haplotypes in each population (at most four) and that haplotypes within a population were very closely related with at most three differences. Also, different haplotypes between populations were divergent, indicating that a common ancestor to these divergent haplotypes was present a relatively long time ago. This result also indicates that little to no gene flow occurs between populations of the *N. tennesseensis* complex because no haplotype was found in more than one population (Hedin 1997b).

*Nesticus barri* are troglobitic (pale, eyeless, and long-limbed) spiders that are known from more than 60 caves from the southern region of the Cumberland Plateau in Franklin and Marion Counties in Tennessee and Jackson and Marshal Counties in

Alabama (Gertsch 1984; Hedin and Dellinger 2005). They live in both cave entrances and deep cave habitats and spin webs that act as both a home and a means to catch prey. They hang upside down from their webs and do not stray far from them throughout their lives (Gertsch 1984; Hedin 1997a). Female spiders carry their egg sacs on their spinnerets until the offspring hatch (Reeves 1999). *N. barri* are habitat-limited because they are only able to live in cave environments. It has been reported that this species is morphologically diverse between populations (Hedin and Dellinger 2005). *Nesticus valentinei*, a species found only from one cave in Marion County, Tennessee (Heating Stove Cave) (Gertsch 1984) was considered morphologically distinct from comparative *N. barri* specimens from Tate Springs Cave, but within the range of diversity seen in the *N. barri* species. For this reason, Hedin and Dellinger (2005) predicted that DNA evidence will show that *N. valentinei* is not its own species, but another morphological variation of *N. barri*; therefore, they synonymized *N. valentinei* with *N. barri*. The object of this study was to research the population genetics of *N. barri* from caves across the range of the species to see how much gene flow occurs between the populations. Based on previous studies (Hedin 1997b), we hypothesize that there will be little to no gene flow between populations of *Nesticus barri*. This is the first study to examine the population genetics of a troglobite in the southern Cumberland Plateau.

## Methods

### *Samples*

Specimens were obtained from nine different caves (Table 1) which covered a representative sample of the entire *Nesticus barri* range (Figure 1). Individuals were preserved in the field in 95% ethanol and taken back to the lab.

### *DNA Extraction, Amplification, and Sequencing*

DNA was extracted using the tissue from one leg of small individuals or the femur of large individuals according the manufacturer's instructions for the DNeasy kit (Qiagen; P/N: 69506). Partial mtDNA cytochrome oxidase I (COI) sequences were amplified using the polymerase chain reaction (PCR) with the primers LCOI (5'-GGTCAACAAATCATAAAGATATTG-3') or LCOI-barri (5'-GGACTTTGTATTTTATTCTTGGGTC-3') and HCOI (5'-TAAACTTCAGGGTGACCAAAAATCA-3'). Two different enzymes were used, Amplitaq Gold PCR Master Mix (Applied Biosystems; P/N: 4318739) or Taq DNA Polymerase (Sigma; P/N: D5938). When the Taq DNA polymerase was used, the conditions for the PCR were 94°C for one minute, 50°C for two minutes, and 72°C for ninety seconds. The cycle was repeated a total of 35 times. When the Amplitaq Gold master mix was used, the conditions were 95°C for five minutes, followed by 35 cycles of fifteen seconds at 95°C, 50°C for fifteen seconds, and 72°C for one minute. The amplified DNA was purified according to the manufacturer's instructions for the DNeasy kit (Qiagen; P/N: 28106) for sequencing. Sequencing reactions were sent to the DNA Analysis Facility on Science Hill at Yale University and

sequences were returned via email. Sequences were edited using Sequencher (v. 4.9) (Gene Codes Corp., Ann Arbor, MI).

### *Analysis Methods*

M. Hedin (San Diego State University) provided partially overlapping sequences for two *Eidmanella* spiders, another genus in the Nesticidae family, as well as a single sequence from each of the seven other *Nesticus* species found in the southern Cumberland Plateau. A neighbor joining phylogenetic tree was created in PAUP\* (v. 4.0b10) (Swofford 2001) using Kimura-2-parameter distances with the *Eidmanella* sequences as the root of the tree. A neighbor joining bootstrap analysis with 1000 replicates was conducted to determine support for the clades of the phylogenetic tree. Pairwise genetic distances were calculated between the most common haplotypes of each mtDNA clade within *N. barri* using Kimura-2-parameter distances. Mesquite (v. 2.5) was used to determine the number of synonymous and nonsynonymous changes observed, and the ratio of transitions to transversions.



## Results

### *Molecular Evolution*

There were 45 variable sites out of 634 bases within *N. barri*. Five of the 45 variable sites were transversions while the rest were transitions. When the amino acid sequence was analyzed, only nine out of 211 amino acids were changed and the rest of the 36 mutations were silent. No stop codons were observed within any translated amino acid sequence.

### *Population Structure*

A total of nine caves were sampled (Figure 1). We report the presence of *N. barri* in Grapeville cave and Sewanee Blowhole for the first time. These are the northernmost observations of this species (Table 2). Eleven different haplotypes were found (Table 3). These haplotypes were never shared between caves. In two of the caves (White Cricket Cave and Lost Cove Cave) two haplotypes were found. The haplotypes from White Cricket Cave differed by one base pair and the haplotypes from Lost Cove Cave differed by 14 base pairs or 2.3% (Table 2). This indicates that populations within caves are very similar to identical.

### *Phylogeny*

The phylogenetic tree constructed using sequences from 28 *N. barri* individuals, representations from seven *Nesticus* species, and two *Eidmanella* individuals provided strong support (bootstrap value of 97%; Figure 2) for the monophyly of *Nesticus barri*. It also showed that within *N. barri*, there are six distinct, well supported clades. The

bootstrap analysis calculated a 90% confidence for mtDNA clade C and a 100% confidence for the remaining five mtDNA clades (Figure 2). The clades are all at least 2.1% different and at most 4.1% different according to pairwise distances calculated with Kimura-2-parameters (Table 3). The average pairwise distance within *N. barri* is 2.7% and between the other *Nesticus* species is 16.1%. The average pairwise distance between *N. barri* and the other *Nesticus* species is 13.3%.

### *Geography*

The six clades were somewhat geographically distinct; all the caves with spiders from a specific clade were in the same area, at most 23 miles apart. The closest clades geographically were the two found in Lost Cove Cave (Figure 3). The two haplotypes found in this cave were different enough to be considered two separate mtDNA clades (Table 1). This is the exception, however, as all other caves have only one clade and usually have only one haplotype. Despite the close proximity of several of the caves, no two caves shared a haplotype (Table 1, Figure 2). The pairwise distances corroborate the clade groupings in that individuals geographically closer to each other were more related than individuals geographically more distant (Table 3).

## Discussion

### *Overview*

Genetic evidence suggests that *Nesticus barri* can be separated into six geographically isolated clades (Figure 3). These clades are all genetically diverse (Table 3). Haplotypes within clades were unique to a single cave with no shared haplotypes between caves (Table 1) which strongly suggests that there is no migration between populations of *N. barri*. Two clades were found in one cave (Lost Cove Cave) because the two haplotypes found in that cave were genetically distinct to a degree comparable to the difference between the other clades (Table 3, Figure 2). Despite this great amount of genetic diversity within the species, the phylogenetic tree provided strong support for the monophyly of *N. barri*. While the six clades were genetically different from each other, the seven other *Nesticus* species were much more distinct both from each other and from *N. barri* (Figure 2). The average distance between the other *Nesticus* species was 16.1%, while the average distance within *N. barri* was 2.7%, supporting the conclusion that the distinct groups within *N. barri* are clades within the same species, rather than six separate species.

### *Status of N. valentinei*

Gertsch (1984) described *Nesticus valentinei* as a new species from Heating Stove Cave on the northeastern edge of *N. barri*'s range. While the cave entrance is now destroyed, it is connected to Tate Spring Cave. Hedin and Dellinger (2005) were able to collect specimens by using this connection. These individuals were compared, morphologically, to other *N. barri* individuals and the *N. valentinei* holotype. They

concluded that *N. valentinei* is not actually a separate species, but part of *N. barri*. They hypothesized that DNA evidence would also support this theory (Hedin and Dellinger 2005). Results from this study do support the synonymization of *N. valentinei* with *N. barri* because we do not find individuals from Tate Spring Cave to be distinct from the rest of the species.

#### *Gene Flow in N. barri*

The genetic evidence from *N. barri*, specifically the high percent differences between clades, up to 4.1% (Table 3), and the fact that haplotypes were not found to be shared between caves strongly suggests that there is little to no gene flow between populations in different caves, and that for this troglobite, there is no evidence of migration between populations. This is consistent with what Hedin (1997b) concluded about the *Nesticus tennesseensis* complex, that there was little to no gene flow between their populations. The results in *N. barri* contrast with the two studies on stygobites in the region which indicate a much higher rate of gene flow in the population. Stygobites may have a higher rate of gene flow between populations because they may be able to migrate through underground aquifers that are inaccessible to troglobites. (Buhay and Crandall 2005; Buhay *et al.* 2007).

#### *Conservation Implications*

The great amount of genetic diversity in *Nesticus barri* and uniqueness of separate cave populations have implications when considering the conservation of these spiders. *N. barri* shows significant genetic diversity on a small, subpopulation scale. However,

the diversity is isolated to specific clades and haplotypes within the clades that are not widespread, indicating that *N. barri* is not one large interbreeding population, but several smaller populations defined by the caves in which they live. If the genetic diversity of the species is to be preserved, then several individual caves in which *N. barri* are found would need to be protected specifically, rather than the entire *N. barri* range in general.

### *Summary*

The monophyletic species *Nesticus barri* can be divided into six distinct clades based on mitochondrial DNA evidence. The similar haplotypes within these clades are not shared among any caves. In Lost Cove Cave there are two haplotypes that belong to two separate clades. *Nesticus valentinei* as described first by Gertsch (1984) was synonymized with *N. barri* based on morphological evidence (Hedin and Dellinger 2005). This study supports this synonymization based on mtDNA evidence because the specimens from Tate Springs Cave, which is connected to Heating Stove Cave where *N. valentinei* were found, are not distinct from the rest of the species. The isolation and diversity of different populations of *N. barri* has conservation implications because the loss of just one population would mean the loss of at least one haplotype and possibly an entire clade. Further studies on other troglobites in this biodiversity region will clarify whether the patterns observed in *N. barri* are common for a variety of troglobite species.

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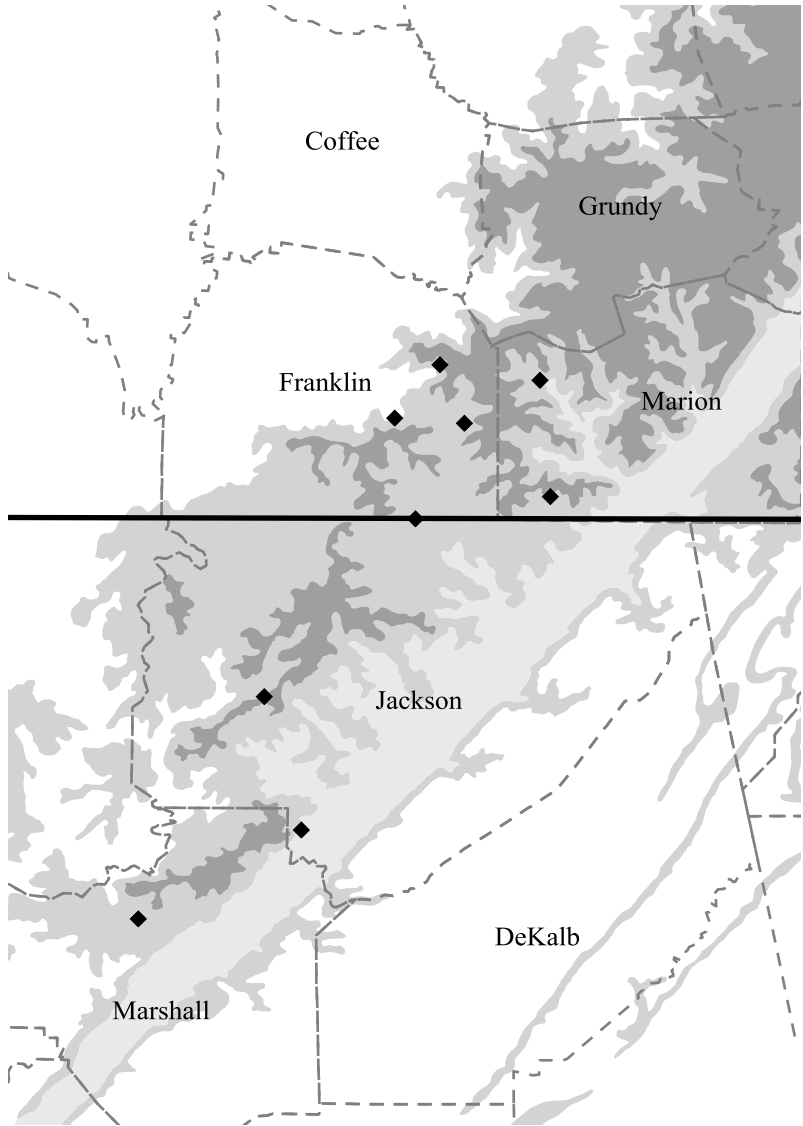


Figure 1: Distribution of caves sampled on the Cumberland Plateau in Tennessee and Alabama.



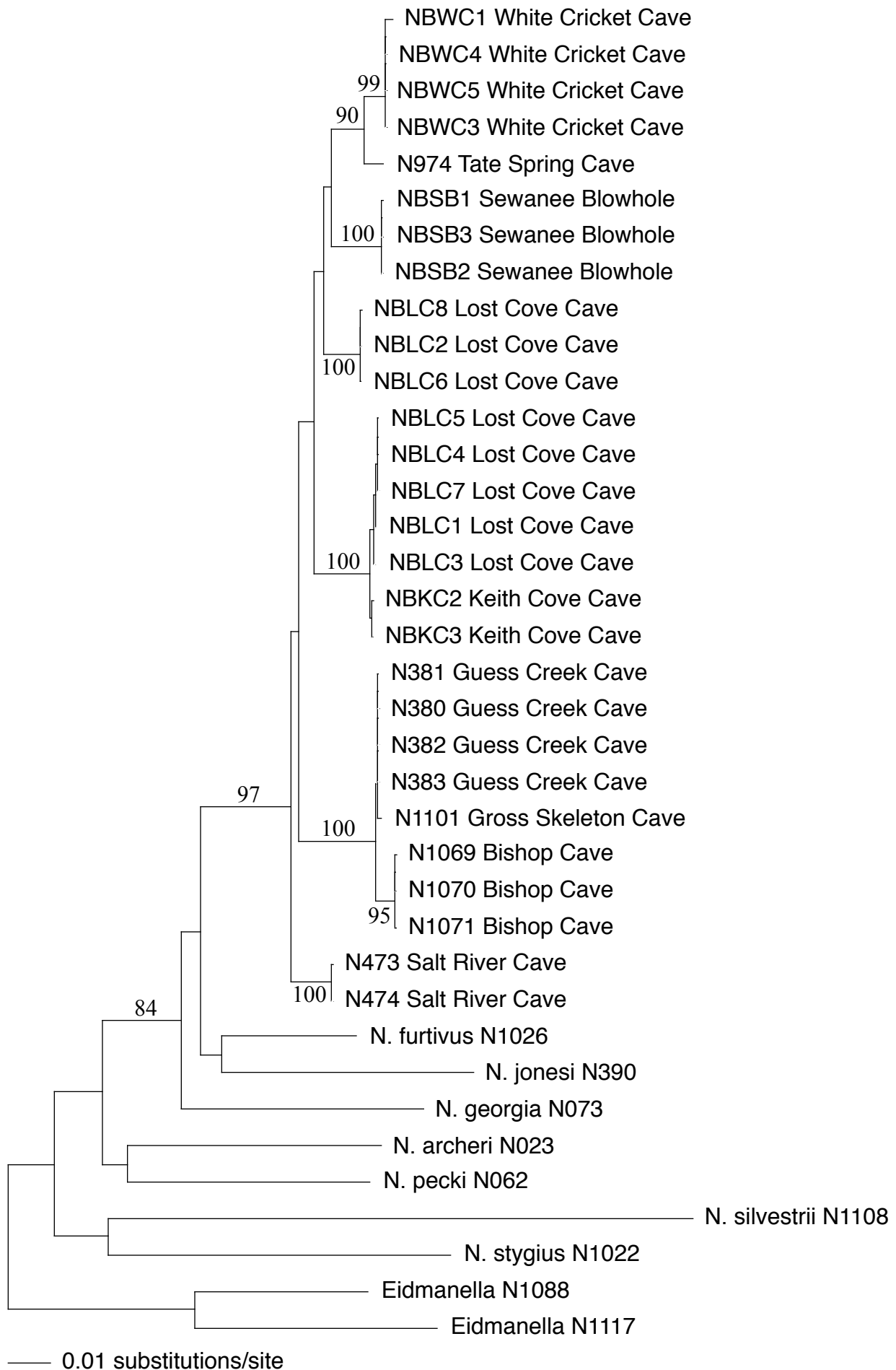


Figure 2: Neighbor joining phylogenetic tree based on Kimura-2-distance calculations. Bootstrap calculations were done with 1000 replicates. Only branches with a 70% confidence or greater were reported.

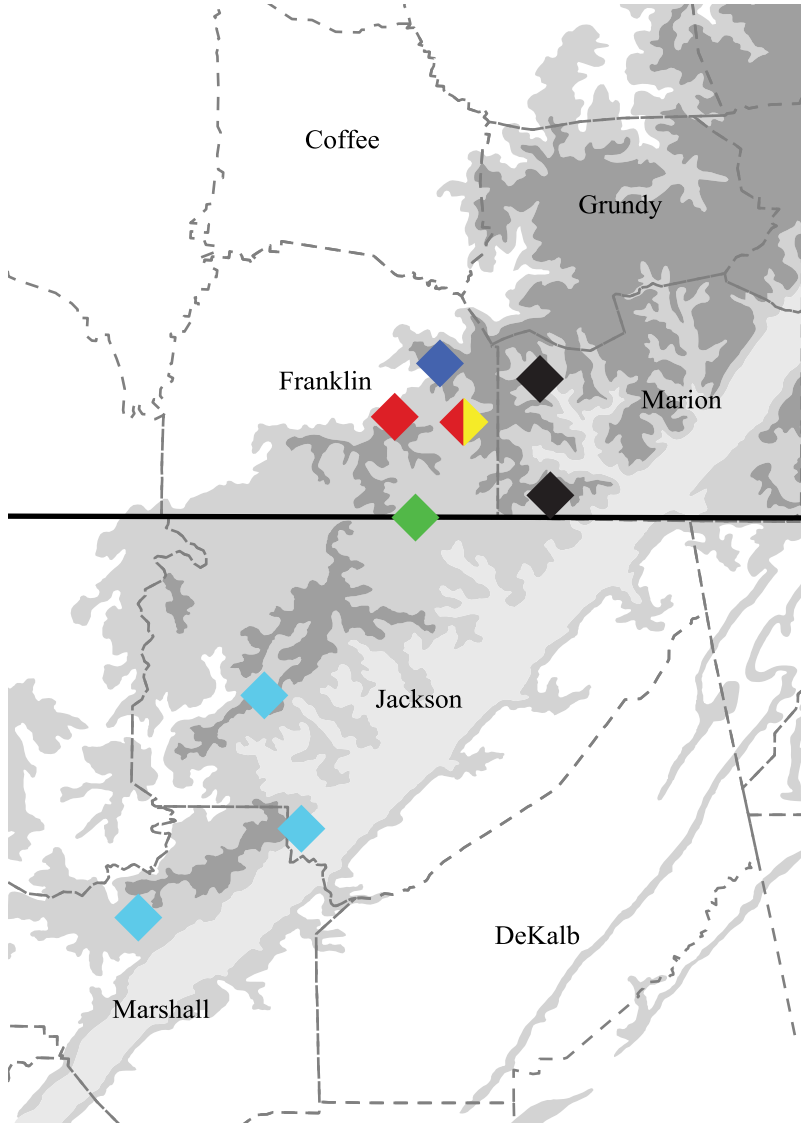


Figure 3: Caves from which samples were collected, color-coded according to mtDNA clade.

Table 1: Caves from which samples were collected, the number of individuals sequenced there, and haplotype found, separated by mitochondrial DNA clades.

<b>mtDNA Clade A</b>	<b>County</b>	<b>State</b>	<b>Individuals</b>	<b>Haplotype</b>
Sewanee Blowhole	Franklin	Tennessee	3	A
<b>mtDNA Clade B</b>	<b>County</b>	<b>State</b>	<b>Individuals</b>	<b>Haplotype</b>
Salt River Cave	Franklin	Tennessee	2	B
<b>mtDNA Clade C</b>	<b>County</b>	<b>State</b>	<b>Individuals</b>	<b>Haplotype</b>
White Cricket Cave	Marion	Tennessee	4	C1 (x3), C2 (x1)
Tate Spring Cave	Marion	Tennessee	1	C3
<b>mtDNA Clade D</b>	<b>County</b>	<b>State</b>	<b>Individuals</b>	<b>Haplotype</b>
Lost Cove Cave	Franklin	Tennessee	3	D
<b>mtDNA Clade E</b>	<b>County</b>	<b>State</b>	<b>Individuals</b>	<b>Haplotype</b>
Lost Cove Cave	Franklin	Tennessee	5	E1
Keith Cove Cave	Franklin	Tennessee	2	E2
<b>mtDNA Clade F</b>	<b>County</b>	<b>State</b>	<b>Individuals</b>	<b>Haplotype</b>
Guess Creek Cave	Jackson	Alabama	4	F1
Gross Skeleton Cave	Jackson	Alabama	1	F2
Bishop Cave	Marshall	Alabama	3	F3

Table 2: Caves by county and state in which *Nesticus barri* has been reported.

Cave Name	State	County	Source
Black Hole of Calcutta	Tennessee	Franklin	Lewis 2005
Crownover Saltpeter Cave	Tennessee	Franklin	Gertsch 1984
Custard Hollow Cave	Tennessee	Franklin	Gertsch 1984
Keith Cave	Tennessee	Franklin	Hedin and Dellinger 2005
Little Crow Creek Cave	Tennessee	Franklin	Lewis 2005
Lost Cove Cave	Tennessee	Franklin	Gertsch 1984
Ranie Willis Cave	Tennessee	Franklin	Gertsch 1984
Round Cove Cave	Tennessee	Franklin	Lewis 2005
Stubblefield Pit	Tennessee	Franklin	Lewis 2005
Grapeville Cave	Tennessee	Franklin	this study
Sewanee Blowhole	Tennessee	Franklin	this study
Fishtrap Spring	Tennessee	Marion	Gertsch 1984
Excited Cow Cave	Tennessee	Marion	Lewis 2005
Honeycutt Cave	Tennessee	Marion	Gertsch 1984
Nickajack Cave	Tennessee	Marion	Gertsch 1984
Tate Spring Cave	Tennessee	Marion	Hedin and Dellinger 2005
White Cricket Cave	Tennessee	Marion	Lewis 2005
Bat Cave	Alabama	Jackson	Gertsch 1984
Boxes Cove Cave	Alabama	Jackson	Gertsch 1984
Cornelson Cave 2	Alabama	Jackson	Gertsch 1984
Driftwood Cave	Alabama	Jackson	Gertsch 1984
Fern Cave	Alabama	Jackson	Gertsch 1984
Gross Cave	Alabama	Jackson	Gertsch 1984
Guess Creek Cave	Alabama	Jackson	Gertsch 1984
Hall Cave	Alabama	Jackson	Gertsch 1984
Happy Hollow Cave	Alabama	Jackson	Gertsch 1984
Horseskull Cave	Alabama	Jackson	Gertsch 1984
Indian Rocks Cave	Alabama	Jackson	Gertsch 1984
Isbell Spring Cave	Alabama	Jackson	Gertsch 1984
Jess Elliott Cave	Alabama	Jackson	Gertsch 1984
Kenamer Cave	Alabama	Jackson	Gertsch 1984
Kyle Cave	Alabama	Jackson	Gertsch 1984
Larkin Cave	Alabama	Jackson	Gertsch 1984
Limrock Blowing Cave	Alabama	Jackson	Gertsch 1984
Montague Cave	Alabama	Jackson	Gertsch 1984
Moody Cave	Alabama	Jackson	Gertsch 1984
Out Cave	Alabama	Jackson	Gertsch 1984
Pigpen Cave	Alabama	Jackson	Gertsch 1984
Rainbow Cave	Alabama	Jackson	Gertsch 1984
Ridley Cave	Alabama	Jackson	Gertsch 1984
Russell Cave	Alabama	Jackson	Gertsch 1984
Salt River Cave	Alabama	Jackson	Gertsch 1984
Sheldon's Cave	Alabama	Jackson	Gertsch 1984
Shiffman Cave	Alabama	Jackson	Gertsch 1984
Steele Saltpeter Cave	Alabama	Jackson	Gertsch 1984
Talley Ditch Cave	Alabama	Jackson	Gertsch 1984
Tate Cave	Alabama	Jackson	Gertsch 1984
Tumbling Rock Cave	Alabama	Jackson	Gertsch 1984
Wynne Cave	Alabama	Jackson	Gertsch 1984
Bishop Cave	Alabama	Marshall	Gertsch 1984
Cathedral Caverns	Alabama	Marshall	Gertsch 1984
Devil's Dungeon Cave	Alabama	Marshall	Gertsch 1984
Guffey Cave	Alabama	Marshall	Gertsch 1984
Honeycomb cave	Alabama	Marshall	Gertsch 1984
Keller Cave	Alabama	Marshall	Gertsch 1984
Kristy's Cave	Alabama	Marshall	Gertsch 1984
McHardin Cave	Alabama	Marshall	Gertsch 1984
Porch's spring Cave	Alabama	Marshall	Gertsch 1984
Quarry Cave	Alabama	Marshall	Gertsch 1984
Royal Shaft	Alabama	Marshall	Gertsch 1984

Table 3: Pairwise (Kimura-2-parameter) distances between mtDNA clades using a representative individual with the most common haplotypes in each mtDNA clade.

	NBLC6	N380	NBWC4	N474	NBLC1
NBLC6 Lost Cove Cave					
N380 Guess Creek Cave	0.033				
NBWC4 White Cricket Cave	0.024	0.039			
N474 Salt River Cave	0.026	0.033	0.031		
NBLC1 Lost Cove Cave	0.023	0.033	0.031	0.029	
NBSB3 Sewanee Blowhole	0.021	0.041	0.026	0.027	0.031