Biodiversity of Cave-Obligate Animals on the Domain of the University of the South, Franklin County, Tennessee

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Abstract

The southern Cumberland Plateau in Tennessee and Alabama has the greatest diversity of cave obligate animals in the United States. The 13,000 acre campus (referred to as the 'Domain') of Sewanee: The University of the South is located on the southern Cumberland Plateau in Franklin County, Tennessee. There are more than 70 caves on the Domain, which, combined, have more than 15 km of horizontal passageway. We examined the biodiversity of cave animals on the Domain at the species level and at the genetic level. Through a survey of the seven largest horizontal caves on the Domain, we identified 21 cave-obligate species, including two new county records. This accounts for nearly half of the species reported for Franklin County. For our genetic analysis, we selected five diverse taxa (a millipede, a beetle, a fly, an aquatic isopod, and a spider) that were collected from multiple caves, and compared their mitochondrial cytochrome oxidase I gene sequences. Across the five taxa we found: (1) low genetic diversity within caves (mean nucleotide diversity within caves across all taxa: 0.25%), (2) high genetic divergence between caves (divergence between caves within taxa ranged from 2.5%-10.9%, with two exceptions), and (3) little evidence for gene flow between caves (F_{ST} between caves within taxa > 0.57, with one exception). Thus, the Domain supports tremendous species diversity, and an even more remarkable level of genetic diversity within those species across caves on a very small scale (no caves used in the genetic comparisons were >3 km apart). Our observation of high genetic divergence between caves on a small scale highlights the importance of cave conservation on a regional scale.

Introduction

Obligate cave dwelling fauna, both terrestrial (troglobites) and aquatic (stygobites) represent a variety of taxa that have drawn the attention of scientist for centuries. They share a suite of convergently evolved morphological adaptations, including eyelessness, pigment loss, appendage elongation, and increased extra visual senses, collectively termed as troglomorphy, and are known to show distinct patterns of geographical distribution (Christman *et al*. 2005). Compared with epigean (surface dwelling) organisms, cave-obligates show higher rates of endemism, lower dispersal ability, and smaller range sizes at all scales of measurement (Porter 2007). Hypothesized mechanisms behind these biogeographical characteristics include dispersal, (an organism's ability to move from one location to another), and vicariance (isolation due to geographic barriers) and combinations of the two. Among cave-obligates, stygobites are said to show generally higher rates of dispersal and gene flow than troglobites, presumably due to greater hydrological connectivity between subterranean habitats (Buhay *et al*. 2005; Snowman 2010).

In addition to their smaller range sizes and higher rates of endemism, cave-obligates are restricted to karst (cave bearing) landscape, with the result that cave-obligate biodiversity is highly concentrated within relatively few geographical areas. From all cave bearing area in the United Sates, there are six hot spots for cave-obligate diversity. Among these, the kart area of the interior lowlands and southern Cumberland plateau supports the greatest richness of troglobitic and stygobitic species (Culver *et al*. 2000). The land area owned by the University of the South (the Sewanee Domain) is located in the northeast corner of Franklin County, TN, and covers 13,000 acres of this

leading biodiversity hotspot (Figure 1). As such a massive privately owned land area in an exceptionally biodiverse region, the Sewanee Domain offers a unique opportunity for the study and conservation of cave-obligate species. Despite this, there has been no systematic survey of the Domain's cave-obligate biodiversity.

In recent years, scientist have begun applying molecular genetic techniques in order to answer biogeographical questions through variability of molecular markers between individuals and populations. Using sequence polymorphism and microsatellite (sequences of DNA containing polymorphic numbers of tandemly repeated nucleotides) data, researchers are able to estimate effective populations sizes, levels of gene flow, , the genetic diversity of populations, and genetic divergence of related populations (Porter 2007; Buhay and Crandall 2005).

There is growing recognition of DNA barcoding as a methodology for linage recognition, species discovery, and phylogeny construction (Hebert *et al*. 2004; Le Gall and Saunders 2010). The mitochondrial gene, cytochrome c oxidase I (COI) is conducive to barcoding research because of its ubiquitous expression in eukaryotic organisms, easy extraction from cells, and relatively high mutation rate (Hebert *et al*. 2004). Advantages of using COI barcodes for invertebrate identification are that individuals may be identified regardless of sex, state of development, or physical damage to the specimen, and often with comparatively less expertise than is required for identifications by morphology (Armstrong and Ball 2005). These advantages, however, are dependent upon the accuracy and comprehensiveness of online databases.

In order to build a more complete understanding of Sewanee's cave obligate biodiversity, we conducted a biological survey of the top seven largest horizontal caves

within the Domain, accompanied by a comparison of the COI gene from six taxonomically diverse focal species. A study of the population genetics of the troglobitic spider, *Nesticus barri*, illustrated that populations from different caves showed high levels of intraspecific diversity, and apparent genetic isolation, even over small distances (Snowman and Zigler 2010). By containing our study within the boundaries of the Sewanee Domain, we examined the patterns of cave-obligate distribution and genetic diversity at a very small geographic scale (all sample caves < 4km apart). There were two purposes of our genetic analyses. The first was to provide a clearer picture of Sewanee's cave-obligate biodiversity with data at both the species and molecular level. The second was to test whether the small scale distributional characteristics found for *Nesticus barri* are consistent for a variety of cave-obligate taxa, including two species of millipede, a beetle, a fly, an aquatic isopod. By including five morphologically disparate taxa, we tested whether genetic isolation and high levels of intraspecific diversity between caves are characteristic traits of cave-obligates as a whole, or unique trait of a single cave-obligate lifestyle, morphology, or taxon.

Materials and Methods:

We surveyed seven caves on the Domain, which we selected on the basis of location, size and accessibility, using data from the Tennessee Cave Survey. The sampled caves represent the seven largest horizontal caves found within Sewanee's Domain, with a total horizontal passageway of ~19 km. We included previously reported data from three additional caves, Dry Cave, and Lost Cove Cave, which are located just outside the northern and southern borders of the Domain respectively, and Wet Cave which runs beneath the Domain, though the entrance is located outside of it (Figure 2) (Culver *et al*. 2000; Lewis 2005).

Field methods

Sampling was conducted between January 30th, 2009 and February 2nd, 2010. We visited each of the caves on the Domain 4 to 6 times. We also made two visits to Dry Cave, before it was closed to the public midway through the study. Visits generally lasted 1 to 2 hours. Sampling methods included hand collecting, pitfall traps, and Berlese extraction from litter samples. Hand collected samples were stored temporarily in reagent alcohol, (90.25% ethanol, 4.75% methanol). For pitfall traps, we used 50 and 250mL plastic cups. We filled the bottoms of these with the same reagent alcohol, and covered the openings with 1.3cm wire mesh to prevent disruption by larger fauna, or their accidental capture. Pitfall traps were usually baited with Limburger cheese, smeared around the inner edge of the cup, though in several cases, cow dung proved an effective substitute. Traps were dug into banks, or wedged between rocks as to allow crawling access to the lip. They were

left two and five days before recovery on a return visit. Litter samples were transferred to the lab in plastic bags, and placed in Berlese funnels for invertebrate extraction.

Sample identification

In the lab, specimens were sorted and stored in 95% ethanol at -20°C. We identified as many species as possible using available literature. If a specimen could not be identified confidently, we deferred to recognized experts (See Acknowledgements).

Extraction, amplification, and sequencing of C01 *haplotypes*

From our total list of obligates within the Domain, we selected six species for genetic analysis. These were chosen for their abundance in our sample sites, their responsiveness to our sequencing methods, and their taxonomic and morphological variety. They included the Russel cave milliped, *Pseudotremia minos*, Barr's cave millipede, *Pseudotremia barri*, Hatch's cave fungus beetle, *Ptomaphagus hatchi*, the cave dung fly, *Spelobia tenebrarum*, the stygobitic two-toothed cave isopod *Caecidotea bicrenata*, and Barr's cave spider, *Nesticus barri*.

For DNA extraction, we used DNeasy Blood and Tissue kits (QIAGEN-69506) according to the protocol of the manufacturer. In order to balance our use of the buffers in the kits more appropriately with the amounts that each came in, we altered the protocol for a portion of our extractions, using half the measurement of buffers called for in steps 1 through 3: (90 μL ATL buffer, 10 μL Proteinase K solution, 100 μL AL, buffer and 100 μL ethanol). We found no apparent variation in extraction results due to these

modifications. 4 μL of each extraction was electrophoresed in 0.5% agarose gel to ensure success before amplification was attempted.

We amplified fragments of the mitochondrial cytochrome oxidase I (CO1) gene using the polymerase chain reaction (PCR). The cycles of our PCRs were conducted using an automated thermal cycler (MJ research, PTC-200). Our PCR reactions were 30 μL: 15 μL AmpliTaq Gold® PCR Master Mix (Applied Biosystems 4316753), 12 μL distilled water, 1 μL each of the selected 5' and 3' primers at 10 μm concentration, and 1 μL of extracted DNA. All amplifications were originally conducted using the universal CO1 primers HCO1-2198 and LCO1-1490 (Folmer *et al*. 1994). After a successful sequence was acquired for a species, we developed species specific primers in order to increase the efficiency of our amplification and sequencing reactions (Table 1). Our PCR cycle program was as follows: five minutes at 95°C, then 35 cycles of a 15 second denaturing step at 95°C, a 15 second primer annealing period at 45°C, and one minute at 72°C for replication. After the 35 cylces, the program completed with seven minutes 72°C. When using the species specific primers, the annealing temperature was increased to 50°C. 3 μL of each PCR were used in electrophoresis, to test the success of each reaction. Purified PCRs were sequenced on both strands by the DNA Analysis Facility at Yale University.

Sequence analysis

CO1 fragments were aligned automatically using Sequencher™ 4.9 (Gene Codes Corp., Ann Arbor, MI). Variable sites were verified manually from chromatograms and primers were excluded from all alignments.

In order to asses the level of genetic diversity of our species within individual caves, we calculated haplotype diversity (h), and nucleotide diversity (π) for every cave population for which we had 2 or more sequences, using DNA Sequence Polymorphism 5; (Librado and Rozas 2009). Transition, tranversion, silent, and replacement nucleotide substitutions for each species were counted using MEGA version 4 (Tamura, *et al*. 2007).

To examine variability between cave populations, we calculated pair-wise divergence, (D_{XY}) and population pair-wise F_{ST}. F_{ST} comparisons were only conducted between populations with 3 or more representative sequences. Divergence was calculated using DNA Sequence Polymorphism 5 (Librado and Rozas 2009). F_{ST} was calculated using Arlequin 3.11 (Excoffier 2007).

We also assessed variability and population structure for each species as a whole. To do this, we constructed phylogenetic networks using TCS 1.21 (Clement *et al.* 2000), and compared haplotype and nucleotide diversity over the entirety of our samples for each species.

Results:

Inventory

We observed 21 troglobites and stygobites within Sewanee's Domain. There were seven arachnids, five diplopods, four insects, three non-insect hexapods, two malacostracans, one turbellarian, and an amphibian (Table 2). Only *Ptomaphagus hatchi* was found in every cave sampled. Four species, the Tennessee cave salamander, *Gyrinophilus palleucus,* a cavernicolous pselaphid beetle, *Subterrochus steevesi*, the troglobitic Mammoth cave spider, *Anthrobia mamouthia,* and the cave flatworm, *Sphalloplana percoeca*, were found only in a single cave from the sample list.

Previous data from other surveys (Culver *et al.* 2000) and (Lewis, 2005) identified a total of 45 cave obligate species for the entirety of Franklin County, TN (Table 3). Our survey increased this total to 47, with the inclusion of two species previously unrecorded in Franklin County, *Subterrochus steevesi* and *Anthrobia mamouthia* (Lewis 2005). In our seven sample caves, we made 71 observations of species habitation (Table 2). There was an average of ten obligate species found in each cave on the Domain. The richest cave on the Domain was Walker Springs, with 13 cave-obligate species.

Genetic Results

We acquired COI sequences from 69 individuals, and found 29 unique haplotypes among our six species of interest (Table 4). Sequences ranged from 563 to 633bp of the CO1 coding region (primers not included). Comparisons within species revealed a total of 168 variable sites with zero stop codons. No gaps were found in any of the sequences. Of the 168 nucleotide substitutions, 129 were transitions, and 41 transversions, with two

variable sites showing both a transition and transversion (Table 5). 156 were silent substitutions, and twelve were replacement substitutions.

Variation within caves

Levels of CO1 nucleotide diversity (π) within caves were generally low but varied greatly between caves and species (mean $\pi_{\text{all populations}} = 0.0025 \pm 0.0049$) (Table 4). Seven of our sample populations were represented by a single haplotype, and therefore had nucleotide and haplotype diversities of zero. This was true for *P. barri* in Walker Springs, *P. minos* in Grapeville, *S. tenebrarum* in Grapeville and Sewanee Blowhole, *N. barri* in Sewanee Blowhole, and *C. bicrenata* in Buckets of Blood and Walker Springs. For those populations represented by multiple halplotypes, haplotype diversity (h) was generally large, ($h = 0.389 \pm 0.164$ to 0.844 ± 0.080). Thus haplotype diversity was also highly variable among taxa and caves (mean $h_{\text{all populations}} = 0.359 \pm 0.379$).

Variation between populations

Divergence between caves was typically about 3-4%, but varied both by species and cave (Table 6). Notably, the two populations of *C. bicrenata*, each represented by a single haplotype, showed a divergence of nearly 11%, whereas other populations, of *S. tenebrarum* and *P. minos*, showed divergences as low as 0.3% and 0.4% between caves. Interestingly, the lowest and highest divergence values came from comparisons of populations from the same two caves. Interspecific divergence between populations of *P. minos* and *P. barri* were relatively similar to most intraspecific comparisons (mean $D_{XY} = 3.7\% \pm 0.1\%$). Overall, pair-wise F_{ST} values between caves were also high, (mean $F_{ST} =$

0.68 ± 0.33)(Table 6). Of eight F_{ST} comparisons, only three were <0.900. The lowest of these was between the Buckets of Blood and Walker Springs populations of *S. tenebrarum* (F_{ST}=0.10).

Intraspecific variation and population structure

Nucleotide diversity was always highest when all the sequences for a species were included (Table 4). The two *Pseudotremia* species showed the lowest species wide nucleotide diversities (π_{barri} = 0.38% ± 0.16%) and (π_{minos} = 0.33% ± 0.04%), while *C. bicrenata* showed the highest, (π = 5.83%). Nucleotide diversity for the remaining species fell between 1.8% and 2.4%.

Of the 29 haplotypes observed across our six focal taxa, only three were shared between caves, one *S. tenebrarum* haplotype from Walker Springs and Buckets of Blood, one *Nesticus barri* haplotype from Grapeville and Buckets of Blood, and one *Pseudotremia barri* haplotype from Solomon's Temple and Thumping Dick (Figure 3). Haplotype diversities at the species level were high, (mean h = 0.681 ± 0.210), and five of our six species showing values >0.50.

Discussion:

Species and distribution

From our inventory data, it is clear that Sewanee's Domain supports an exceptionally rich collection of troglobitic and stygobitic fauna, representing 21 out of Franklin County's 47 known cave-obligates (Table caves' troglobites). According to Culver *et al*. (2000), over 50% of the ~930 obligate species in the continental United States can be found within just 23 counties, and less than 1% of its land area. Of this total continental diversity the Sewanee Domain alone can account for 2.48%, within just 13,000 acres. The success of this inventory, and the addition of two new species to the Franklin County list indicates the likelihood of still more unrecorded taxa remain to be discovered on the Domain, Franklin County, and the Cumberland karst area as a whole.

Sewanee appears be transected by local range borders of eight out of its 21 different obligate species (Table 2; Figure 2). This large proportion is congruous with small range sizes and high rates of endemism characteristic of troglobionts (Christman *et al*. 2005; Porter 2007; Culver *et al* 2000). Those with southern range borders include *Scoterpes ventus*, *Pseudotremia. barri*, *Pseudanopthalmus humeralis*, and *Pseudanopthalmus intermedius*. Those with northern range borders include *N. barri*, *Tetracion jonesi*, *Scoterpes stewartpecki,* and *Pseudotremia minos*. The diplopod genera, *Scoterpes* and *Pseudotremia*, represent the clearest range borders, with only one species from each genus found on the northern side of the Domain, and the second species found in caves on the southern side. Within Sewanee's borders, the distributions of the northern and southern species pairs from each genus are identical. Whether this correlation is coincidental, or the result of a shared evolutionary history, remains to answered.

Gene flow

Our molecular data indicate that troglobitic and stygobitic populations from a variety of taxa show high levels of genetic isolation between caves, even when they are incorporated within a relatively small area (no two sample caves > 4 km apart). This is evident from high levels of intraspecific divergence between caves, ($D_{XY} \geq 2.7\%$ for 12 out of 14 comparisons), comparatively low diversity within populations (mean $\pi_{\text{all taxa}} = 0.00249 \pm 0.00489$), high pair-wise F_{ST} values (≥ 0.55 for all but 1 comparison), and restrictive clustering of haplotypes by cave (Table 4, 5, Figure 3). Craft *et al*. (2010) deemed a population divergence greater than 2% as indicative of deep cryptic lineage diversity. According to this standard, 10 out of 14 intraspecific population comparisons showed deep cryptically distinct lineages. The exceptions were the populations of *Pseudotremia minos*, and one pair of *S. tenebrarum* populations, though these two were distinct from the other two populations (Table 6).

These two taxa also represent the only haplotypes that are shared between more than one cave (Figure 3). The causes for these lower instances of divergence appear to be unique to the taxa involved. In each case of lower divergence for which there is data from other taxa, the other taxa show contrastingly high levels divergence between the same two caves; for example: the divergence between the *Pseudotremia minos* populations from Grapeville and Buckets of Blood is 0.4%, whereas the divergence between *Ptomaphagus. hatchi* populations from Grapeville and Buckets of Blood is 3.7%. Thus, if connectivity between any two caves were to be inferred from lower divergence measurements, that connectivity would necessarily be unique only to the taxa that demonstrated the lower measurements.

The comparatively lower divergences between the *Pseudotremia* species may be the result of the comparatively lower genetic diversities of the two species taken as a whole ($\pi_{barri} = 0.0038 \pm 0.0016$) and ($\pi_{minos} = 0.0033 \pm 0.0004$); that there is actually similar isolation between the population, and they simply have not diverged to the extent of the other taxa. This possibility is supported by high F_{ST} value between *P. minos* in Grapeville and Buckets of Blood ($F_{ST} = 0.577$), and high haplotype diversities for the *P. minos* as a whole ($h_{minos} = 0.844 \pm 0.080$). This could be the result of differential mutation rates, or differences in timing of vicariant events.

This explanation does not hold for shared haplotypes and low divergences of the *S. tenebrarum* populations from Walker Springs and Buckets of Blood (Figure 3; Table 6). On a whole, *S. tenebrarum* shows higher species wide diversity, comparable to that of the other three taxa ($\pi_{tenebrarum} = 0.023$; mean $\pi_{all\ taxa} = 0.025$) and high divergence between each of its other population pairs (Table 6). It is tempting to attribute the apparent gene flow to a greater dispersal ability over other obligate taxa, due to *S. tenebrarum's* unique capability of flight. However, if this were the case, there is no explanation for the high genetic distinction between the populations in Buckets of Blood, Grapeville and Sewanee Blowhole, which are all in closer proximity to one another than any of them are to Walker Springs (Figure 2). There being no evidence for a species specific corridor for *S. tenebrarum* between Walker Springs and Buckets of Blood, we believe the best explanation for the shared haplotypes between Walker Springs and Buckets of Blood is a singular introduction event, rather than continued gene flow between the populations.

Hydrological interconnection

Our molecular results for the aquatic isopod, *C. bicrenata*, were contrary to general assumption that aquatic subterranean habitats are better connected than terrestrial ones, facilitating greater dispersal ability for stygobites over troglobites (Lamoreaux 2004; Culver *et al*. 2007; Snowman *et al* 2010). Studies have shown that some stygobites are capable of both wide ranging dispersal (Danielopol *et al*. 1994; Buhay and Crandall 2005), and correlation of population differential with hydrology (Porter 2007; Carlini *et al*. 2009). Given the documentation for this phenomenon, it is curious that our two populations of the stygobitic *C. bicrenata* showed the greatest divergence of any in our sample group (D_{XY} > 10%; Table 6). This high divergence between such nearby populations (< 4 km apart) is even more striking given the species' exceptionally large range, spanning from northern Alabama through Tennessee, and into southern Illinois and Kentucky (Lewis and Bowman 1981). As we only sampled 2 populations of the species, no substantial conclusions can be drawn. However, if future studies rendered comparable results, this broad ranging species may turn out to be a massive mosaic of independent lineages.

Conservation Implications

Of Sewanee's 21 cave obligates, 13 of these species have a global rank of rarity of G3 or higher, (G3=vulnerable, G2=imperiled, G1=critically imperiled) (Lewis 2005). Three of the species, *Kleptochthonius tantalus, K. magnus,* and *Pseudotremia minos,* are considered critically imperiled (Lewis 2005). The extent of the obligate diversity

encompassed within this privately owned Domain makes Sewanee an excellent opportunity for conservation.

Conclusions:

Sewanee is home to an immensity of hypogean diversity, both in terms of species richness and genetic diversity. Taking into account that Sewanee's Domain supports at least 21 obligate species, and over 100 caves, one can only guess at the level of diversity encompassed within its borders.

There is a high level of genetic isolation between cave obligate populations, even on a very small geographic scale. This trait holds true for a variety of taxa, with varied mechanism of locomotion, ecological roles, and preferred habitat, supporting it as a characteristic trait of cave-obligates as a whole.

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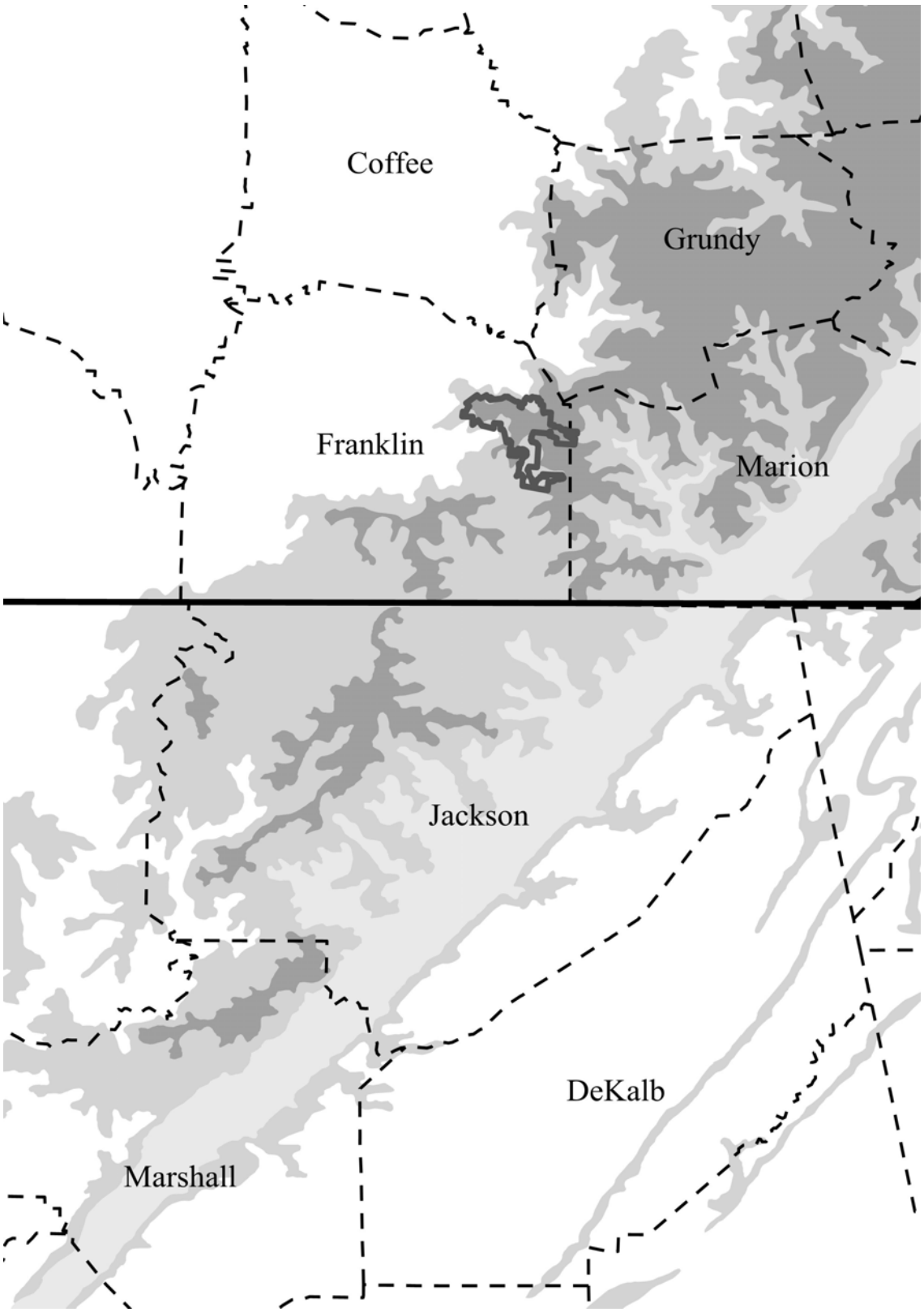


Figure 1: Geographic extent of the Southern Cumberland Plateau in Tennessee and Alabama and the location of the Domain of the University of the South in Franklin County, TN. Higher altitudes are designated by darker shades.

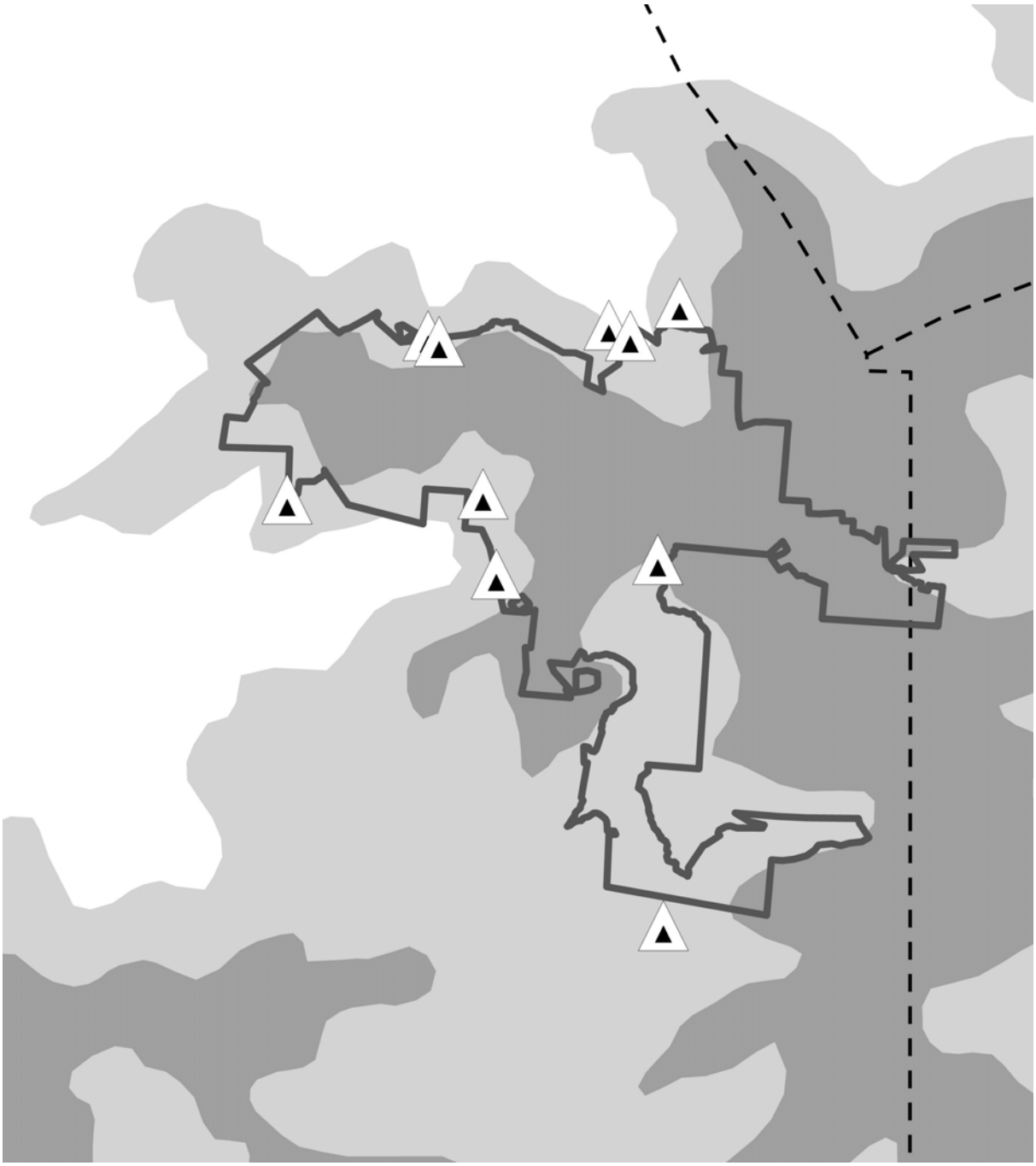


Figure 2: Relative location of caves discussed in this paper with the boundary of the Domain of the University of the South.

Table 1:
Focal taxa with their common names, and the primer combinations which rendered most consistent results for each. All sequences were first amplified using the universal primers HC01-2198 and LC01-1490 (Folmer *et al*. 1994) before development of species specific primers. Primers used for *Nesticus barri* taken from (Snowman and Zigler, 2010).

Species	Primer	Sequence (5'-3')
Pseudotremia barri	HC01-tremia	GTTGATATAAAATTGGGTCCCCTCC
(Barr's cave millipede)	LC01-1490	GGTCAACAAATCATAAAGATATTG
Pseudotremia minos	HC01-tremia	GTTGATATAAAATTGGGTCCCCTCC
(Russel cave millipede)	LC01-1490	GGTCAACAAATCATAAAGATATTG
Ptomaphagus hatchi	HC01-ptom	GGGACATCCTTAAGACTTTTAATTC
(Hatch's cave beetle)	LC01-ptom	GCTGGTAAAGAATTGGATCCCC
Spelobia tenebrarum	HC01-ten	GAACTTTATATTTTATATTTGGGGC
(Cave dung fly)	LC01-ten	GTCTCCTCCACCAGCAGGGTC
Caecidotea bicrenata	HC01-caec	AGGGTCCCTCCCCCCTGGGG
(Twotoothed cave isopod)	LC01-Caec	GGGGCTTGAGCCGGAAGAGTCGG
Nesticus barri	HC01-2198	TAAACTTCAGGGTGACCAAAAAATCA
(Barr's cave spider)	LC01-barri	GGACTTTGTATTTTATTCTTGGGTC

Table 2:
List of species found in and just outside Sewanee's Domain, the caves they inhabit, and their global ranks of rarity as given in (Lewis 2005). X designates observations made in this study, L designates records from Lewis (2005), S designates records from Shear (2010). Caves are listed from south to north, with blue cells indicating species with apparent northern range border, and pink indicating species with apparent southern range borders. Cave abbreviations are as follows: LC: Lost Cove cave, GV: Grapeville, BB: Buckets of Blood, BH: Sewanee Blowhole, MC: Miller Cave, TD: Thumping Dick, ST: Solomon's Temple, WC: Wet Cave, WS:Walker Springs,

Class	Order		LC	GV	BB	BH	MC	TD	ST	WC	WS	DC	Threat
Arachnida	**Araneae**	*Anthrobia mammouthia*									x		G3
		Liocranoides archeri		x	x		x	x				x ,L	G2
		Nesticus barri	L	x	x	x	x	x	x				G3
		Phanetta subterranea			x	x	x	x	x	L		L	G5
	Pseudoscorpionida	*Hesperochernes mirabilis*		x			x				x		G5
		Kleptochthonius magnus										L	G1
		Kleptochthonius tantalus										L	G1
	Opiliones	*Tolus appalachius*					x			L	x,L	x	G2
Diplopoda	**Callipodida**	*Tetracion jonesi*		x	x	x							G4
	Chordeumatida	*Pseudotremia barri*						x	x	L	x	L	G2
		Pseudotremia minos		x	x								G1
		Scoterpes stewartpecki	L	x	x	x?							G2/G3
		Scoterpes ventus						S		S		x, L	G3
Entognatha	**Collembola**	*Pseudosinella pecki*										L	G2
		Pseudosinella sp.		x	x	x	x	x	x		x	x	G2/G3
		Pseudosinella spinosa		x			x						G2
	Dipluran	*Litocampa cookei*								L			G3
		Litocampa valentinei		x		x				x	x	L	G3
Insecta	**Coleoptera**	*Pseudanopthalmus humeralis*										L	G1
		Pseudanopthalmus intermedius									x	L	G2
		Ptomaphagus hatchi		x	x	x	x	x	x	L	x	L	G3
		Subterrochus ferus										L	G1/2
		Subterrochus steevesi			x								G1/2
	Diptera	*Spelobia tenebrarum*		x	x	x				L	x	L	G5
Amphipoda	**Isopoda**	*Caecidotea bicrenata*	L	x	x	x			x	L	x	L	G4
	Decapoda	*Orconectes australis*	x			x				x ,L	x		G3
	Malacostraca	*Stygobromus sp.*										L	G3
Tubellaria	**Tricladida**	*Sphalloplana percoeca*							x			L	G3
Amphibia	**Caudata**	*Gyrinophilus palleucus*	L			x							G2/3
		Total per cave:	5	12	11	11	8	7	7	10	13	18	

DC: Dry Cave.

Table 3:
List of cave-obligates identified for Franklin County, TN, with sources: Culver *et al*. (2000), Lewis (2005), and this study. Species marked with X were observed in this study, within the Domain of the University of the South.

Class	Order	Family	Genus	Species	Source	
Amphibia	Urodela	Plethodontidae	*Gyrinophilus*	*palleucus*	Culver, Lewis	X
Arachnida	Pseudoscorpiones	Chthoniidae	*Kleptochthonius*	*magnus*	Culver, Lewis	X
Arachnida	Pseudoscorpiones	Chthoniidae	*Kleptochthonius*	*tantalus*	Culver, Lewis	X
Arachnida	Araneae	Nesticidae	*Nesticus*	*barri*	Culver, Lewis	X
Arachnida	Opiliones	Phalangodidae	*Tolus*	*appalachius*	Culver, Lewis	X
Arachnida	Pseudoscorpiones	Chthoniidae	*Tyrannochthonius*	*fiskei*	Culver 2000	
Arachnida	Araneae	Linyphidae	*Porhomma*	*cavernicola*	Lewis 2005	
Arachnida	Araneae	Linyphidae	*Phanetta*	*subterranean*	Lewis 2005	X
Arachnida	Araneae	Linyphidae	*Anthrobia*	*mammouthia*	This Study	X
Copepod	Cyclopidae	Diacyclops	*Diacyclops*	*indianensis*	Lewis 2005	
Crustacea	Isopoda	Asellidae	*Caecidotea*	*alabamensis*	Culver 2000	
Crustacea	Isopoda	Asellidae	*Caecidotea*	*bicrenata*	Culver, Lewis	X
Crustacea	Decapoda	Cambaridae	*Cambarus*	*hamulatus*	Culver 2000	
Crustacea	Amphipoda	Crangonyctidae	*Crangonyx*	*antennatus*	Culver 2000	
Crustacea	Podocopida	Entocytheridae	*Dactylocythere*	*steevesi*	Culver 2000	
Crustacea	Decapoda	Cambaridae	*Orconectes*	*australis*	Culver, Lewis	X
Crustacea	Amphipoda	Crangonyctidae	*Stygobromus*	*exilis*	Culver, Lewis	
Crustacea	Amphipoda	Crangonyctidae	*Stygobromus*	*vitreus*	Culver, Lewis	
Diplopoda	Callipodida	Lysiopetalidae	*Tetracion*	*jonesi*	Culver, Lewis	X
Diplopoda	Chordeumatida	Trichopetalidae	*Scoterpes*	*ventus*	Lewis 2005	X
Diplopoda	Chordeumatida	Trichopetalidae	*Scoterpes*	*stewartpecki*	Lewis 2005	X
Diplopoda	Chordeumatida	Cleidogonidae	*Pseudotremia*	*barri*	Lewis 2005	X
Diplopoda	Chordeumatida	Cleidogonidae	*Pseudotremia*	*minos*	Lewis 2005	X
Gastropod	Basommatophora	Noctuoidea	*Carychium*	*stygium*	Lewis 2005	
Hexapoda	Diplura	Campodeidae	*Litocampa*	*valentinei*	Culver, Lewis	X
Hexapoda	Collembola	Entomobryidae	*Pseudosinella*	*hirsuta*	Culver 2000	
Hexapoda	Collembola	Entomobryidae	*Pseudosinella*	*spinosa*	Culver 2000	X
Hexapoda	Collembola	Entomobryidae	*Pseudosinella*	*christianseni*	Lewis 2005	
Hexapoda	Collembola	Entomobryidae	*Pseudosinella*	*pecki*	Lewis 2005	
Hexapoda	Diplura	Campodeidae	*Litocampa*	*cookei*	Lewis 2005	
Insecta	Coleoptera	Carabidae	*Pseudanophthalmus*	*humeralis*	Culver, Lewis	
Insecta	Coleoptera	Leptodiridae	*Ptomaphagus*	*chromolithus*	Culver 2000	
Insecta	Coleoptera	Leptodiridae	*Ptomaphagus*	*fecundus*	Culver, Lewis	
Insecta	Coleoptera	Leptodiridae	*Ptomaphagus*	*hatchi*	Culver, Lewis	X
Insecta	Coleoptera	Carabidae	*Pseudanophthalmus*	*intermedius*	Lewis 2005	X
Insecta	Diptera	Sphaeroceridae	*Spelobia*	*tenebrarum*	Lewis 2005	X
Insecta	Coleoptera	Pselaphinae	*Subterrochus*	*ferus*	Lewis 2005	
Insecta	Coleoptera	Carabidae	*Anillinius*	*sp.*	Lewis 2005	
Insecta	Coleptera	Pselaphinae	*Subterrochus*	*steevesi*	This Study	X
Oligochaeta	Branchiobdellida	Branchiobdellidae	*Cambarinicola*	*alienus*	Culver 2000	
Oligochaeta	Lumbriculida	Lumbriculidae	*Trichodrilus*	*allegheniensis*	Culver 2000	
Tubellaria	Tricladida	Planariidae	*Sphalloplana*	*percoeca*	Lewis 2005	X
Arachnida	Pseudoscorpiones	Chernetidae	*Hesperochernes*	*miribilis*	Lewis 2005	X
Arachnida	Pseudoscorpiones	Chthoniidae	*Apochthonius*	*sp*	Lewis 2005	
Osteichthyes	Percopsiformes	Amblyopsidae	*Typhlichthys*	*subterraneus*	Lewis 2005	
Arachnida	Pseudoscorpiones	Chthoniidae	*Tyrannochthonius*	*fiski*	Lewis 2005	
Arachnida	Pseudoscorpiones	Chthoniidae	*Tyrannochthonius*	*halopotamus*	Lewis 2005	

Table 4:
Species, sample caves for which there were at least two representative sequences, the number of individuals sequenced (n), number of haplotypes per cave population (N), haplotype diversity and its standard deviation (*h*), and nucleotide diversity and its standard deviation (π). The 'All' rows include all sequences for the species, including those from caves with fewer than two representative sequences not listed in the table.

Species	**Cave**	n	N	*h*	π
Pseudotremia minos	Grapeville	3	1	0	0
	Buckets of Blood	7	4	0.810 ± 0.130	0.0032 ± 0.0006
	All	10	5	0.844 ± 0.080	0.0033 ± 0.0004
Pseudotremia barri	Walker Springs	7	1	0	0
	All	9	2	0.389 ± 0.164	0.0038 ± 0.0016
Ptomaphagus hatchi	Grapeville	7	4	0.714 ± 0.181	0.0047 ± 0.0016
	Buckets of Blood	4	3	0.833 ± 0.222	0.0021 ± 0.0007
	Walker Springs	7	3	0.667 ± 0.160	0.0186 ± 0.0064
	All	21	11	0.890 ± 0.049	0.0239 ± 0.0017
Spelobia tenebrarum	Grapeville	2	1	0	0
	Buckets of Blood	5	3	0.800 ± 0.164	0.0032 ± 0.0008
	Walker Springs	6	2	0.533 ± 0.030	0.0019 ± 0.0006
	Sewanee Blowhole	2	1	0	0
	All	15	6	0.857 ± 0.003	0.0231 ± 0.0067
Caecidotea bicrenata	Buckets of Blood	4	1	0	0
	Walker Springs	2	1	0	0
	All	6	2	0.533 ± 0.019	0.0583 ± 0.0188
Nesticus barri	Grapeville	3	2	0.667 ± 0.314	0.0011 ± 0.0005
	Sewanee Blowhole	4	1	0	0
	All	8	3	0.571 ± 0.094	0.0182 ± 0.003

Table 5:
Focal taxa, and their sequencing data: Number of indiciduals sequenced (*n*), the number of base pairs sequenced (bp), the number of conserved and variable sites, number of transitions and transversions, silent, and replacement mutations. There were two instances (marked*) when both a transition and transversion were found on a single variable site.

Species	*n*	bp	Conserved sites	Variable sites	Transitions	Transversions	Silent	Replacement	Stop Codons
Pseudotremia barri	9	626	620	6	4	2	6	0	0
Pseudotremia minos	10	626	620	6	5	1	6	0	0
Spelobia tenebrarum	15	589	543	46	26*	21*	39	7	0
Ptomaphagus hatchi	21	563	529	34	30*	5*	34	0	0
Caecidotea bicrenata	6	573	518	55	45	10	53	2	0
Nesticus barri	8	633	612	21	19	2	18	3	0
Total	69	3610	3442	168	129	41	156	12	0

Table 6:
Pair-wise distances (below diagonal) between cave populations represented by at least two sequences, and pair-wise F_{ST} (above diagonal) between caves represented by at least three sequences. A. Both *Pseudotremia* species included together, B. *Ptomaphagus hatchi*, C. *Spelobia tenebrarum*, D. *Ceacidotea bicrenata*, E. *Nesticus barri*. Cave abbreviations: GV: Grapeville, BB: Buckets of Blood, BH: Sewanee Blowhole, TD: Thumping Dick, ST: Solomon's Temple, WS: Walker Springs. Divergences marked* indicate of deep cryptic lineages (DXY > 0.020) (Craft *et al*. 2010)

A. *Pseudotremia* populations

	GV-minos	BB-minos	WS-barri
GV-*minos*		**0.577**	**1.000**
BB-*minos*	0.004		**0.957**
WS-*barri*	0.036	0.038	

B. *Ptomaphagus hatchi* populations

	GV	BB	WS
GV		**0.901**	**0.901**
BB	0.037*		**0.597**
WS	0.030*	0.027*	

C. *Spelobia tenebrarum* populations

	GV	BB	WS	BH
GV				
BB	0.044*		**0.104**	
WS	0.045*	0.003		
BH	0.071*	0.046	0.046	

D. *Caecidotea bicrenata* populations

	BB	WS
BB		
WS	0.109*	

E. *Nesticus barri* populations

	GV	BH
GV		**1.000**
SB	0.032*	

A. *Pseudotremia barri*

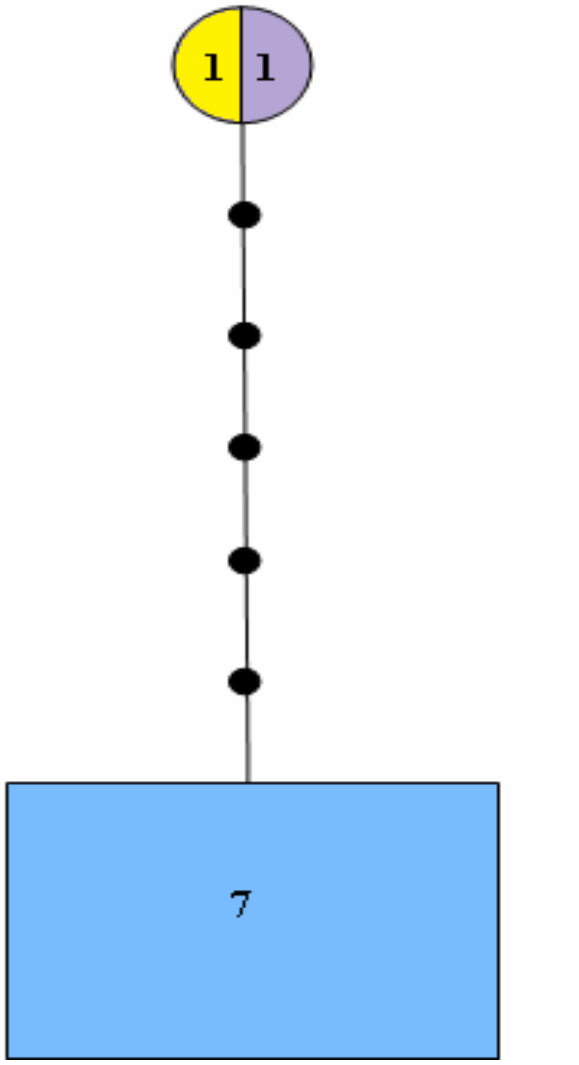
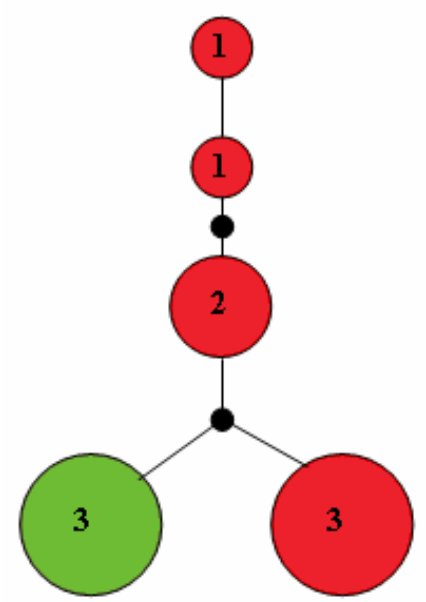
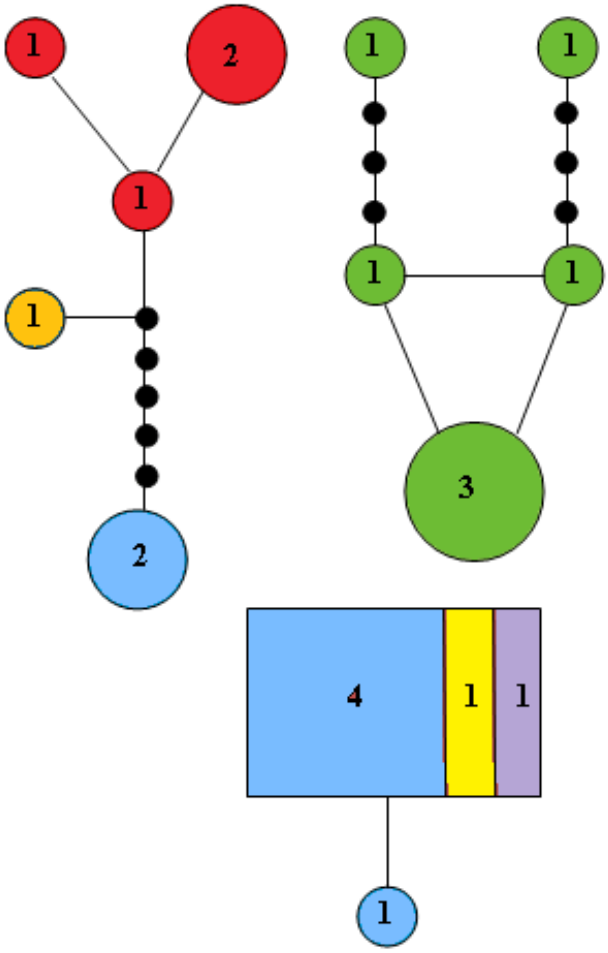


Figure 3: Phylogenetic networks for five focal species, *Pseudotremia barri* (A), *Pseudotremia minos* (B), *Ptomaphagus hatchi* (C), *Spelobia tenebrarum* (D), *Nesticus barri* (E). Colored ovals and rectangles represent haplotypes, and lines connecting them represent single nucleotide polymorphisms. A line separated by a node indicates two lines, and thus two nucleotide polymorphisms. The numbers in the ovals and rectangles indicate the number of individuals that matched the haplotype. The colors indicate the cave the individuals came from: Grapeville: Green, Buckets of Blood: Red, Walker Springs: light blue, Thumping Dick: Yellow, Solomon's Temple: purple, Miller Cave: orange, The Sewanee Blowhole: dark blue. Unconnected networks differ by more than ten nucleotide substitutions.

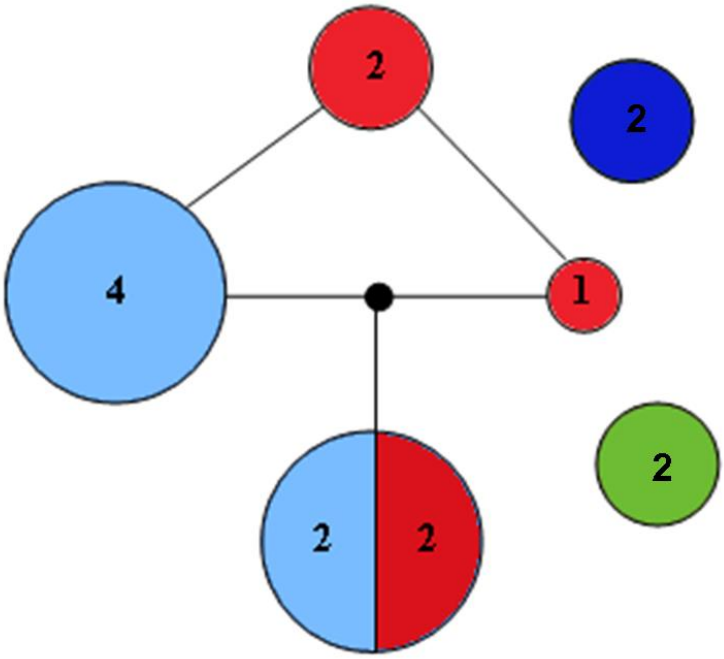
B. ***Pseudotremia minos***



C. ***Ptomaphagus hatchi***



D. ***Spelobia tenebrarum***



E. ***Nesticus barri***