

Cave-obligate Biodiversity on the Campus of Sewanee: 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 University of the South in Franklin County, TN is one of the largest private landholders on the southern Cumberland Plateau. Its 13,000-acre campus has more than 30 caves and is underlain by more than 14 km of horizontal passageways. We examined the biodiversity of cave animals on the campus at the species level and at the genetic level. Through a survey of seven caves on the campus, we identified 24 cave-obligate species, including two new county records. This total accounts for half of the cave-obligate species reported for Franklin County. For our genetic analysis, we selected six diverse taxa (two millipedes, 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 six 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 campus supports tremendous species diversity, and even more remarkable genetic diversity within those species on a small geographic scale (no studied caves were >7 km apart). The divergence between cave populations and lack of gene flow between them that we observed across a range of taxa highlight the importance of cave conservation on a regional scale.

Introduction

Obligate cave-dwelling animals, both terrestrial (troglobites) and aquatic (stygobites), represent a variety of taxa that have drawn the attention of scientists for centuries. They typically share a suite of convergent morphological characteristics, including eyelessness, pigment loss, appendage elongation, and increased extravisual senses, collectively termed “troglomorphy” (reviewed in Culver and Pipan 2009). Cave-obligate organisms generally have small ranges (Culver et al. 2000), and many are known from only a single cave (e.g., 45% of eastern United States cave species are single-cave endemics (Christman et al. 2005)). In addition to their small range sizes and high rates of endemism, cave-obligate animals are restricted to karst (cave-bearing) regions, with the result that cave-obligate biodiversity is concentrated within relatively few geographical areas. Across nine major karst regions of the United States, the Appalachians and the Interior Low Plateaus have the most cave

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biodiversity (Culver et al. 2003). The southern edges of these regions meet at the southern Cumberland Plateau in Tennessee and Alabama.

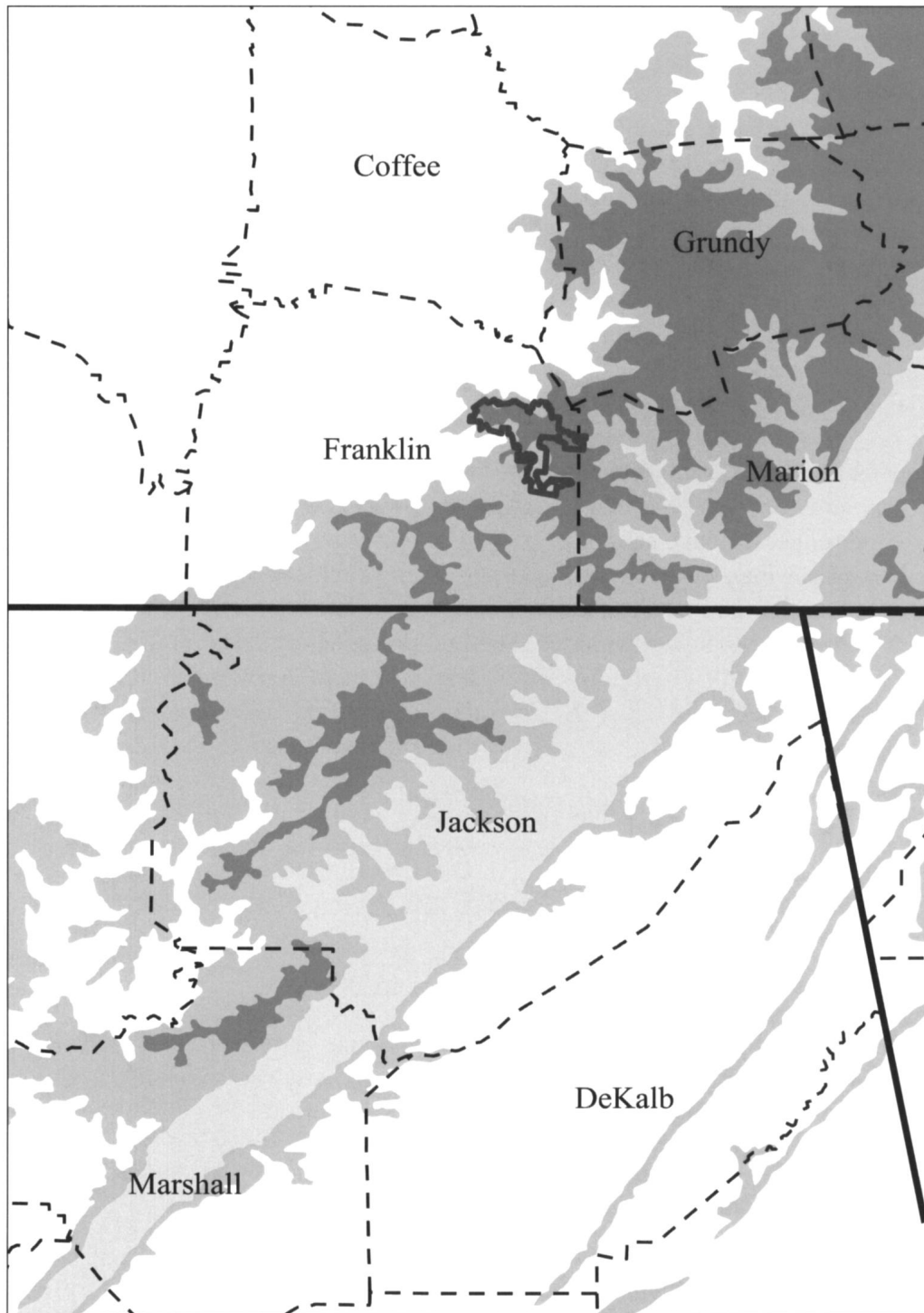


Figure 1. Extent of the southern Cumberland Plateau in Tennessee and Alabama. County borders and names are indicated, and the campus of The University of the South in Franklin County, TN is outlined in grey. Higher altitudes are designated by darker shades.

The southern Cumberland Plateau supports the greatest richness of cave-obligate species in North America (Culver et al. 2000), is a center of endemism for cave species (Christman et al. 2005), and is a biodiversity hotspot for cave fauna on a global scale (Culver et al. 2006). It is a region of high cave density and high temperate productivity, both of which likely contribute to high levels of cave biodiversity (Culver et al. 2006). The campus of The University of the South (known as “Sewanee”) is located in the northeast corner of Franklin County, TN, and covers 13,000 acres of this region (Fig. 1). As the privately held property of an educational institution, the Sewanee campus offers a unique opportunity for the study and conservation of cave biodiversity. Despite this, there has been no systematic survey of Sewanee’s cave-obligate biodiversity.

Most studies of cave biodiversity have focused solely on species diversity. On the southern Cumberland Plateau, a series of surveys (Lewis 2005; Peck 1989, 1995) and taxonomic revisions (Buhay and Crandall 2008, Lewis 2009, Shear 2010) have provided a well-developed picture of the species diversity of cave-obligate taxa for the region. Recently, several studies have examined the genetic diversity of specific cave-obligate taxa in this region. These include two studies on cave crayfish (Buhay and Crandall 2005, Buhay et al. 2007), one on Tennessee cave salamanders (Niemiller et al. 2008), and one on a cave spider (Snowman et al. 2010). These studies provide insights into the population genetics and evolutionary history of these cave-obligate taxa. Buhay and Crandall (2005) and Buhay et al. (2007) emphasized the large population sizes and extensive gene flow observed for the cave crayfish, Niemiller et al. (2008) highlighted evidence for speciation of the Tennessee cave salamanders in the presence of gene flow with surface salamander species, and Snowman et al. (2010) found significant divergence and little evidence for gene flow between cave spider populations. Undoubtedly, studies of genetic diversity are an important complement to studies of species diversity in caves (reviewed in Porter 2007).

In this study, we examined the biodiversity of cave-obligate animals on the Sewanee campus at the species level and at the genetic level. We first surveyed seven caves on the campus. We then selected six diverse taxa (two millipedes, 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. Although Sewanee has a large campus, all the caves we surveyed were within 7 km of one another, allowing us to examine gene flow and population divergence on a small scale. Previous genetic analyses on a troglotic spider from Sewanee’s campus found that cave populations were isolated and genetically divergent (Snowman et al. 2010). By studying diverse taxa, we tested whether cave populations are generally isolated from one another, and whether high levels of intraspecific diversity between caves are general characteristics of cave-obligate species, or if they are a unique trait of a single cave-obligate lifestyle, morphology, or taxon.

Materials and Methods

Sample sites

We surveyed seven caves on the campus of The University of the South. The caves were selected on the basis of location, size, and accessibility. The sampled caves represent the six largest horizontal caves found within the campus, with a total of ≈ 6 km of horizontal passageways. In addition, we surveyed TD Cave, a small cave located near Solomon's Temple Cave. We also gathered previously reported data on Dry Cave and Lost Cove Cave, which are located just outside the northern and southern borders of the campus, respectively, and Wet Cave, which runs beneath the campus, although the entrance is located outside of it. With the exception of TD Cave, which is not listed by the Tennessee Cave Survey (TCS), we refer to all caves by their TCS names. To protect sensitive cave habitats, cave locations are not disclosed in this paper.

Field methods

Sampling was conducted between 30 January 2009 and 2 February 2010. We visited each cave 4 to 6 times. Visits generally lasted 1 to 2 hours. Our 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 250-mL plastic cups. We filled the bottoms of these with reagent alcohol, and covered the openings with 1.3-cm 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 for two to 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. Cave-obligate salamanders and crayfish were observed and recorded, but were not collected.

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 we could not identify a species confidently, we deferred to recognized experts (see Acknowledgments). Scientific nomenclature including authorities for all species collected in this study are listed in Table 2.

DNA extraction, amplification, and sequencing

We selected six cave-obligate taxa for genetic analysis: *Pseudotremia minor* (Russell Cave Millipede), *Pseudotremia barri* (Barr's Cave Millipede), *Ptomaphagus hatchi* (Hatch's Cave Fungus Beetle), *Spelobia tenebrarum* (Cave Dung Fly), *Caecidotea bicrenata* (Two-toothed Cave Isopod), and *Nesticus barri* (Barr's Cave Spider). These were chosen for their abundance in our sample sites, their responsiveness to our sequencing methods, and their taxonomic and

morphological variety. For Barr's Cave Spider, we supplemented sequences already collected from two caves on the campus by Snowman et al. (2010).

For DNA extractions, we used the DNeasy Blood and Tissue kit (Qiagen #69506) according to the manufacturer's protocol. DNA extractions were examined by electrophoresis to ensure success before amplification was attempted. We amplified fragments of the mitochondrial cytochrome oxidase I (COI) gene using the polymerase chain reaction (PCR). Our PCRs were 30 μ L in volume: 15 μ L AmpliTaq Gold PCR Master Mix (Applied Biosystems #4316753), 12 μ L distilled water, 1 μ L each of the selected 5' and 3' primers at 10 μ M concentration, and 1 μ L of extracted DNA. All amplifications were originally conducted using the universal COI primers HCOI-2198 and LCOI-1490 (Folmer et al. 1994). After a successful sequence was acquired for a species, we developed species-specific primers to increase the efficiency of our amplification and sequencing reactions (Table 1). Our PCRs ran as follows: 5 min at 95 °C; then 35 cycles of 15 sec at 95 °C, 15 sec at 45 °C, and 1 min at 72 °C; with a final 7 min at 72 °C. When using species-specific primers, we increased the annealing temperature to 50 °C. PCRs were examined by electrophoresis. Successful PCRs were purified (Qiagen #28106) and sequenced on both strands. All sequences have been submitted to GenBank (Accession #HM245857–HM245919).

Sequence analysis

COI fragments were aligned and edited using Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI). Variable sites were verified manually from chromatograms, and primers were excluded from all alignments. No indels were present. To assess the level of genetic diversity of populations within individual

Table 1. Focal taxa, their common names, and the primer combinations which gave the 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. The *Nesticus barri* primers are from Snowman et al. (2010).

Species	Primer	Sequence (5'-3')
<i>Pseudotremia barri</i> (Barr's Cave Millipede)	HC01-tremia	GTTGATATAAAATTGGGTCCCCTCC
	LC01-1490	GGTCAACAAATCATAAAGATATTG
<i>Pseudotremia minos</i> (Russell Cave Millipede)	HC01-tremia	GTTGATATAAAATTGGGTCCCCTCC
	LC01-1490	GGTCAACAAATCATAAAGATATTG
<i>Ptomaphagus hatchi</i> (Hatch's Cave Beetle)	HC01-ptom	GGGACATCCTTAAGACTTTTAATTC
	LC01-ptom	GCTGGTAAAGAATTGGATCCCC
<i>Spelobia tenebrarum</i> (Cave Dung Fly)	HC01-ten	GAACCTTATATTTTATATTTGGGGC
	LC01-ten	GTCTCCTCCACCAGCAGGGTC
<i>Caecidotea bicrenata</i> (Two-toothed Cave Isopod)	HC01-caec	AGGGTCCCTCCCCCTGGGG
	LC01-caec	GGGGCTTGAGCCGGAAGAGTCGG
<i>Nesticus barri</i> (Barr's Cave Spider)	HC01-2198	TAAACTTCAGGGTGACCAAAAAATCA
	LC01-barri	GGACTTTGTATTTTATTCTTGGGTC

Table 2. Cave-obligate species found in and just outside Sewanee's campus and the caves they inhabit. X designates observations made in this study, L designates records from Lewis (2005), and S designates records from Shear (2010). Unconfirmed records are indicated with a '?'. Caves are listed from south to north and abbreviated as follows: LC = Lost Cove Cave, GV = Grapeville, BB = Buckets of Blood, BH = Sewanee Blowhole, MC = Miller Cave, TD = TD Cave, ST = Solomon's Temple, WC = Wet Cave, WS = Walker Springs, DC = Dry Cave.

Class	Order	Species	Cave													
			LC	GV	BB	BH	MC	TD	ST	WC	WS	DC				
Arachnida	Araneae	<i>Anthobia monmouthia</i> Tellkamp		X	X		X					X				
		<i>Liocranoides archeri</i> Platnick		X	X	X	X									L
		<i>Nesticus barri</i> Gertsch	L	X	X	X	X									L
		<i>Phanetta subterranea</i> (Emerton)			X	X	X									L
		Pseudoscorpionida														
		<i>Hesperocheernes mirabilis</i> (Banks)		X			X							X		L
		<i>Kleptochthonius magnus</i> Muchmore														L
		<i>Kleptochthonius tantalus</i> Muchmore														L
		Opiliones														
		<i>Tolus appalachius</i> Goodnight & Goodnight												L	X	X
Diplopoda	Callipodida	<i>Tetracion jonesi</i> Hoffman		X	X		X									
	Chordeumatida	<i>Pseudotremia barri</i> Lewis		X	X					X	X		L	X		L
		<i>Pseudotremia minos</i> Shear		X	X											
		<i>Scoterpes stewartpecki</i> Shear	S	X	X		?									S
		<i>Scoterpes ventus</i> Shear								S			S			S
Entognatha	Collembola	<i>Pseudosinella pecki</i> Christiansen & Belling		X	X		X								X	L
		<i>Pseudosinella hirsuta</i> (Delamare)		X	X		X			X	X					X
		<i>Pseudosinella spinosa</i> (Delamare)		X	X		X			X	X					X

Table 2, continued.

Class	Order	Species	Cave												
			LC	GV	BB	BH	MC	TD	ST	WC	WS	DC			
	Diptera	<i>Litocampa cookei</i> (Packard)								L					
Insecta		<i>Litocampa valentini</i> (Conde)		X		X				X			X		L
	Coleoptera	<i>Pseudanophthalmus humeralis</i> Valentine													L
		<i>Pseudanophthalmus intermedius</i> Valentine											X		L
		<i>Plomaphagus hatchi</i> Jeannel		X	X	X		X	X	L			X		L
		<i>Subterrochus ferus</i> (Park)													L
		<i>Subterrochus steevesi</i> Park			X										
	Diptera	<i>Spelobia tenebrarum</i> (Aldrich)		X	X	X				L			X		L
Malacostraca															
	Isopoda														
	Decapoda	<i>Caecidotea bicrenata bicrenata</i> (Steeves)	L	X	X	X				L		X	X		L
		<i>Orconectes australis australis</i> (Rhoades)											L		
	Amphipoda	<i>Stygobromus</i> sp.										X			L
Turbellaria															
	Tricladida	<i>Sphalloplana percoeca</i> (Packard)												X	L
Amphibia															
	Caudata	<i>Gyrinophilus palleucus</i> McCrady	L											X	
Total per cave:			4	12	11	11	11	7	6	8	10	11	11	11	17

caves, we calculated haplotype diversity (h), and nucleotide diversity (π) for every cave population for which we had two or more sequences, using DnaSP v.5 (Librado and Rozas 2009). Transition, transversion, silent, and replacement nucleotide substitutions for each species were counted using MEGA v.4 (Tamura et al. 2007).

To examine variability between cave populations, we calculated mean pair-wise divergence (D_{XY}) and population pair-wise F_{ST} using DnaSP. F_{ST} comparisons were only conducted between populations with three or more representative sequences. We also assessed variability and population structure for each species as a whole. For these assessments, we constructed phylogenetic networks using TCS 1.21 (Clement et al. 2000), and compared haplotype and nucleotide diversity calculated for the entirety of our samples for each species.

Results

Species diversity

We observed 24 cave-obligate species within the campus of The University of the South. There were six arachnids, five diplopods, four insects, four non-insect hexapods, three malacostracans, one turbellarian, and an amphibian (Table 2). A total of 19 troglobites and 5 stygobites were observed. Only *Ptomaphagus hatchi* (Hatch's Cave Beetle) was found in every cave sampled. Five species were found only in a single cave: *Gyrinophilus palleucus* (Tennessee Cave Salamander); a cavernicolous pselaphid beetle, *Subterrochus steevesi*; the troglobitic *Anthrobia monmouthia* (Mammoth Cave Spider); the cave flatworm, *Sphalloplana percoeca*; and an unidentified amphipod, *Stygobromus* sp.

Previous surveys and taxonomic revisions identified 45 described cave-obligate species (and several undescribed species) for the entirety of Franklin County, TN (Culver et al. 2000; Lewis 2005, 2009; Shear 2010). Our survey increased this total to 47 with the observation of two previously unrecorded species (*Subterrochus steevesi* and *Anthrobia monmouthia*). *Subterrochus steevesi* was previously known only from Marshall County, AL (Chandler 1997, Park 1960). *Anthrobia monmouthia* is known from scattered collections from Kentucky, Tennessee, Virginia, and Alabama (Miller 2005).

An average of ten cave-obligate species were found in each cave on the campus (Table 2). Two species identifications from the Sewanee Blowhole are unconfirmed. We collected only immature *Scoterpes* individuals from this cave and so are unable to confirm the species. Other caves on the southern side of campus have *S. stewartpecki*, but it is also possible that these individuals represent the southernmost record of *S. ventus* (Table 2; Shear 2010). We also observed, but did not collect, a troglobitic crayfish in this cave. This specimen was probably *Orconectes australis australis* (Rhoades) (known from caves on the north side of campus), but as it was found on the southern side of campus, it may be *Cambarus hamulatus* (Cope) (known from caves in southern Franklin County) (Buhay and Crandall 2005, Buhay et al. 2007). We also collected an unidentified amphipod of the genus *Stygobromus* in Solomon's Temple cave.

Five species of *Stygobromus* are reported from Franklin County, three of which are undescribed (Lewis 2005).

Intraspecific genetic variation

We acquired COI sequences from 69 individuals, and found 29 unique haplotypes among our six species of interest. Sequences ranged from 563 to 633 bp of the COI coding region. No stop codons or indels were observed (Table 3). Comparisons within species revealed a total of 168 variable sites: 156 were silent substitutions, and 12 were replacement substitutions. Across these sites we observed 129 transition mutations and 41 transversion mutations, with 2 variable sites showing both a transition and transversion (Table 3).

Nucleotide diversity (π) of populations within caves across all species was generally low (mean $\pi_{\text{all populations}} = 0.25\%$) but varied across caves and taxa (Table 4). Seven populations were represented by a single haplotype, with nucleotide and haplotype diversities of zero. For populations represented by multiple haplotypes, haplotype diversity (h) ranged from 0.39 to 0.84 (Table 4). Haplotype diversities across all caves were high (mean $h = 0.681$), with five of our six focal species showing values >0.50 .

Population structure

Divergence between populations averaged 3.7% and varied widely across species and the caves compared (Table 5). Notably, the sampled populations of *C. bicrenata* (from Walker Springs and Buckets of Blood), showed a divergence of 10.9%, whereas populations of other species, such as those of *S. tenebrarum* and *P. minos*, showed divergences of 0.3% and 0.4% between caves (Table 5). Interspecific divergence between *P. minos* and *P. barri* was 3.7%, which was similar to most intraspecific population comparisons.

Of the 29 haplotypes observed across our six focal taxa, 25 were restricted to a single cave (Fig. 2). In three of the four cases in which haplotypes were shared between caves, the caves are within 3.5 km of one another (between TD

Table 3. Focal taxa and their sequencing data. Number of individuals sequenced (n), the number of base pairs sequenced (bp), the number of conserved and variable sites, the number of transition (Transi.), transversion (Transv.), silent, and replacement (Repl.) mutations observed, and the number of stop codons present. There were two instances (marked with an asterisk) when a transition and a transversion were found at a single variable site.

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

and Solomon's Temple in *Pseudotremia barri*; between TD, Solomon's Temple, and Walker Springs in *Ptomaphagus hatchi*; and between Grapeville and Buckets of Blood in *Nesticus barri*). In one case, the caves are on opposite sides of the campus (between Walker Springs and Buckets of Blood in *Spelobia tenebrarum*) (Fig. 2). F_{ST} values between caves were high (mean $F_{ST} = 0.68$), and only three of eight comparisons had F_{ST} less than 0.90 (Table 5). The lowest of these was between the Buckets of Blood and Walker Springs populations of *S. tenebrarum* ($F_{ST} = 0.10$; Table 5).

Discussion

Species diversity and distributions

The campus of The University of the South supports an exceptionally rich collection of troglotic and stygotic species. As noted by Culver et al. (2000), the southern Cumberland Plateau is particularly rich in troglotic

Table 4. Summary of genetic diversity data for focal taxa. For caves for which there were at least two representative sequences, the number of individuals sequenced (n), number of haplotypes per cave population, haplotype diversity and its standard deviation (h), and nucleotide diversity and its standard deviation (π) are reported. 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	# haplotypes	h	π
<i>Pseudotremia barri</i>					
	Walker Springs	7	1	0	0
	All	9	2	0.389 ± 0.164	0.0038 ± 0.0016
<i>Pseudotremia minos</i>					
	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
<i>Ptomaphagus hatchi</i>					
	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
<i>Spelobia tenebrarum</i>					
	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
<i>Caecidotea bicrenata</i>					
	Buckets of Blood	4	1	0	0
	Walker Springs	2	1	0	0
	All	6	2	0.533 ± 0.019	0.0583 ± 0.0188
<i>Nesticus barri</i>					
	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

species and is less rich in stygobitic species. This description correlates with our findings of 19 troglobitic species and 5 stygobitic species. Culver et al. (2000) noted that only 8 out of 3112 counties in the United States had more than 20 troglobitic species, yet here, on only 13,000 acres, we identified 19. The 24 total species found represent half of the 47 total cave-obligate species known from Franklin County. Also notable is the exceptionally species-rich Dry Cave, located just to the north of the campus. Seventeen cave-obligate species have been found in Dry Cave (Table 2), which is an impressive number given that only 36 caves worldwide are known to have 20 or more cave-obligate species (Culver and Pipan 2009).

The Sewanee campus is transected by species range borders for eight of the 24 cave-obligate species we observed (Table 2). This large proportion reflects the small range sizes characteristic of troglobionts (Christman et al. 2005, Culver et al. 2000). The campus covers the southern range borders for *Scoterpes ventus*, *Pseudotremia barri*, *Pseudanophthalmus humeralis*, and *Pseudanophthalmus intermedius*, and the northern range borders for *Scoterpes stewartpecki*, *Pseudotremia minos*, *Nesticus barri*, and *Tetracion jonesi* (Table 2). The diplopod genera *Scoterpes* and *Pseudotremia* represent the clearest range borders, with one species from each genus found in caves on the northern side of the campus, and a second species from each genus found in caves on the southern side of the campus. For these genera, the distributions of the northern and southern species pairs on the campus are identical (Table 2). Whether this correlation is coincidental or the result of a shared evolutionary history remains to be determined.

Table 5. Genetic divergence between populations for the focal taxa. Pair-wise distances (below diagonal) between cave populations represented by at least two sequences, and pair-wise F_{ST} (above diagonal, in bold) between caves represented by at least three sequences for *Pseudotremia minos* and *P. barri* (*Ps*), *Ptomaphagus hatchi* (*Pt*), *Spelobia tenebrarum* (*Sp*), *Caecidotea bicrenata* (*Ca*), and *Nesticus barri* (*Nb*).

	Cave			
	Grapeville	Buckets of Blood	Sewanee Blowhole	Walker Springs
Grapeville		0.577 (<i>Ps</i>) 0.901 (<i>Pt</i>)	1.000 (<i>Ne</i>)	1.000 (<i>Ps</i>) 0.901 (<i>Pt</i>)
Buckets of Blood	0.004 (<i>Ps</i>) 0.037 (<i>Pt</i>) 0.044 (<i>Sp</i>)			0.957 (<i>Ps</i>) 0.597 (<i>Pt</i>) 0.104 (<i>Sp</i>)
Sewanee Blowhole	0.071 (<i>Sp</i>) 0.032 (<i>Ne</i>)	0.046 (<i>Sp</i>)		
Walker Springs	0.036 (<i>Ps</i>) 0.030 (<i>Pt</i>) 0.045 (<i>Sp</i>)	0.038 (<i>Ps</i>) 0.027 (<i>Pt</i>) 0.003 (<i>Sp</i>) 0.109 (<i>Ca</i>)	0.046 (<i>Sp</i>)	

Gene flow between caves

Troglobitic and stygobitic populations from a variety of taxa show high levels of genetic isolation between caves, even when they are found within a relatively small area (no caves sampled in this study are more than 7 km apart). This isolation is evident from the high proportion of haplotypes that are not shared between caves (25 of 29), the high pair-wise F_{ST} values (>0.55 , with one exception) between caves (Table 5), and the restrictive clustering of haplotypes by cave (Table 4, Fig. 2).

Even in cases where haplotypes are shared between caves, there is evidence that gene flow is limited by distance. In three of the four cases where haplotypes are shared between caves, the caves are adjacent to one another. The sole exception is in *Spelobia tenebrarum*, where a haplotype is shared between caves on opposite sides of the campus. As the only cave-obligate fly in North America, *S. tenebrarum* is clearly exceptional. It has a large range (Marshall and Peck 1985) and is one of only nine cave-obligate species recorded from more than 30 counties in the eastern United States (Christman

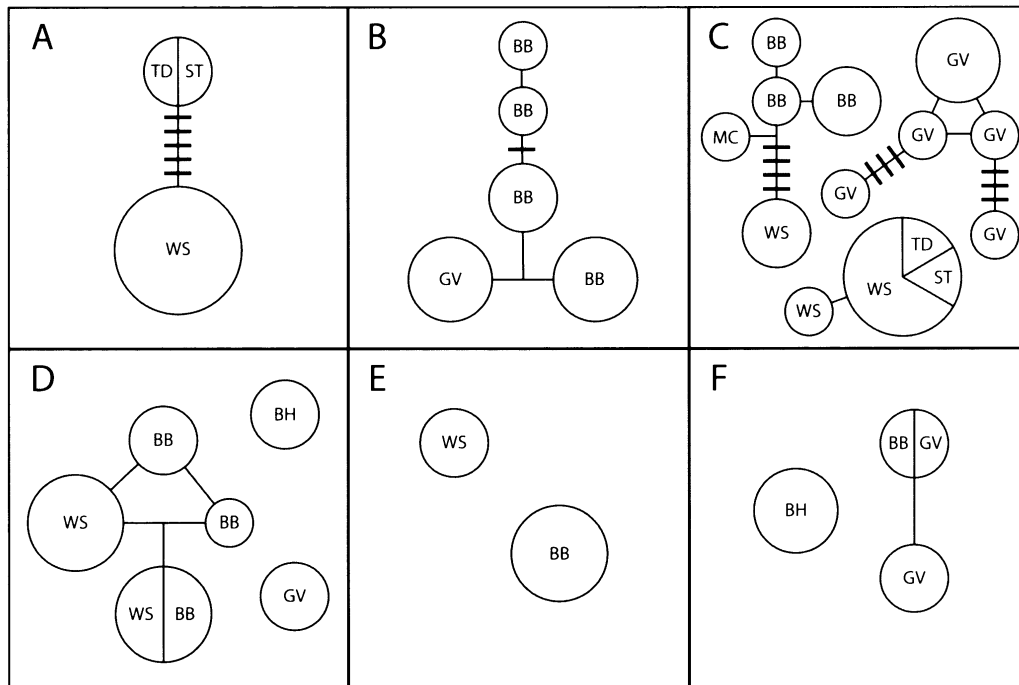


Figure 2. Haplotype networks for six focal species: (A) *Pseudotremia barri*, (B) *Pseudotremia minos*, (C) *Ptomaphagus hatchi*, (D) *Spelobia tenebrarum*, (E) *Caecidotea bicrenata*, and (F) *Nesticus barri*. Circles represent haplotypes, and lines connecting them represent single nucleotide differences. Black bars and junctions between lines indicate unsampled and/or extinct haplotypes. Circle area is proportional to the number of sampled individuals with that haplotype (ranging from 1 to 7). Labels within circles indicate the cave (or caves) where that haplotype was found (abbreviated as in Table 1). Haplotypes found in more than one cave are indicated by the presence of more than one label within a circle. Unconnected networks or haplotypes differ by ten or more nucleotide differences from all others.

and Culver 2001). Exactly how *S. tenebrarum* moves around is unclear, but it must have more ability to do so than most cave-obligate species. Despite this, our sample of *S. tenebrarum* shows evidence of isolation and divergence, as two of the four caves we sampled have unshared haplotypes that do not connect to the network that contains the haplotype shared between Buckets of Blood and Walker Springs (Fig. 2).

Divergence between caves

Despite low genetic diversity within caves (mean $\pi_{\text{all populations}} = 0.25\%$; Table 4) there are high levels of intraspecific divergence between caves ($D_{XY} \geq 2.7\%$ for 12 out of 14 comparisons, Table 5). Craft et al. (2010), using COI sequences to study lepidoptera from New Guinea, deemed intraspecific divergence greater than 2% as indicative of “cryptic lineage diversity”. According to this standard, 12 of our 14 intraspecific population comparisons identified cryptically distinct lineages. High levels of genetic diversity within species are also evident in the haplotype networks. For only two of the six focal taxa did all of the observed haplotypes connect into a single network (Fig. 2).

It is generally assumed that aquatic subterranean habitats are better connected than terrestrial ones, facilitating greater dispersal ability for stygobites over troglobites (Culver et al. 2007, Lamoreaux 2004), and studies have shown that some stygobites are capable of wide-ranging dispersal (Buhay and Crandall 2005, Danielopol et al. 1994). Our limited molecular results for the aquatic isopod, *C. bicrenata*, were contrary to this pattern as they showed the greatest divergence of any in our sample group ($D_{XY} = 10.9\%$; Table 5). This divergence suggests that the two caves we sampled are not hydrologically connected, which may be the case as the stream from one of the caves drains to the south and the Tennessee River, whereas the other drains to the north and the Elk River. Further examination of this species would be interesting, particularly in light of its large range, which spans from northern Alabama through Tennessee, Kentucky, and into southern Illinois (Lewis 1982).

Two other recent studies have examined COI diversity and divergence of cave animals on a small geographic scale. Carlini et al. (2009) studied the amphipod *Gammarus minus* Say, surveying COI diversity and divergence for five cave populations, including four that are no more than 20 km apart. They found low nucleotide diversity within populations (mean $\pi = 0.70\%$), high divergence between cave populations ($\approx 5\%$ for most comparisons between caves), and isolation between caves. Snowman et al. (2010) studied *Nesticus barri* across its four-county range on the southern Cumberland Plateau, and found low COI diversity within caves, high divergence between caves (2–5%), and isolation between cave populations. Thus, the findings of these studies are consistent with our results, although we found these patterns on a smaller geographic scale, and for a variety of taxa. These results from small stygobitic and troglobitic invertebrates contrast with those from larger stygobites studied in this area (crayfish and cave salamanders), which found mitochondrial

haplotypes shared across counties (Buhay and Crandall 2005, Buhay et al. 2007, Niemiller et al. 2008). These large stygobites may be more mobile than troglobites or smaller stygobites, which may explain the patterns observed.

Conclusion

We identified 19 troglobites and 5 stygobites from the campus of The University of the South. For at least eight of these species, the campus is a northern or southern range boundary. Across six diverse taxa we found: (1) low genetic diversity within caves, (2) high genetic divergence between caves, and (3) little evidence for gene flow between caves. These patterns were generally consistent across the taxa studied, regardless of their mechanism of locomotion, ecological role, and habitat, suggesting these characteristics are common for cave-obligate species from this region. Particularly notable is that we observed these patterns within a small geographic area, where no surveyed cave was more than 7 km from another. Thus, the campus supports tremendous species diversity, and even more remarkable genetic diversity within those species on a very small scale. Our observation of high genetic divergence between caves, and little gene flow between them, highlights the importance of cave conservation on a regional scale, in order to conserve as much genetic diversity of cave-obligate species as possible.

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