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Morphometrics and phylogeography of the cave-obligate land snail *Helicodiscus barri* (Gastropoda, Stylommatophora, Helicodiscidae)

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Abstract

Molecular studies have recently led to the detection of many cryptic species complexes within morphologically ambiguous species formerly undescribed by the scientific community. Organisms such as land snails are at a particularly higher risk of species misidentification and misinterpretation, in that gastropod systematics are based almost entirely on external shell morphology. Subterranean ecosystems are associated with especially high degrees of cryptic speciation, largely owing to the abiotic similarities of these systems. In this study, we attempt to diagnose the potential cryptic diversity in the troglobitic land snail *Helicodiscus barri*. Land snails are generally associated with having low vagility, and as such this species' broad, mosaic distribution indicates the misdiagnosis of this organism as a single species. We analyze both mitochondrial (16S, CO1) and nuclear (28S, H3) genetic data for 23 populations. Phylogeny for *H. barri* was reconstructed using both maximum-likelihood and Bayesian approaches to assess relationships among populations, and two species delimitation methods (mPTP and ABGD) were used to detect the presence of unique molecular operational taxonomic units (MOTUs). Species delimitation results revealed seven and sixteen MOTUs respectively, suggesting the presence of several cryptic lineages within *H. barri*. To assess how external shell morphology corresponds with patterns of genetic and environmental variation, two morphometric approaches were used incorporating 115 shells from 31 populations. Both morphometric approaches reveal a significant environmental influence on shell morphology, and one approach showed the significance of MOTU groups. We discuss the delimitation and morphometric results and additionally provide discussion on the taxonomic and conservation implications of this study.

Keywords
Helicodiscidae, subterranean ecology, morphometrics, MOTUs, cryptic species

Introduction

Caves provide a model system for studying the evolutionary processes and historical factors related to biogeography and speciation (Juan et al. 2010). Cave systems, characterized by geographic isolation and relatively simple biological communities, often are viewed as analogous to oceanic islands (Culver and Pipan 2009, Snowman et al. 2010). Strong selective pressures and the isolation of subterranean ecosystems can result in morphological stasis among otherwise genetically distinct species, largely due to the parallel or convergent evolution of these lineages (Lefébure et al. 2006, Finston et al. 2007, Niemiller et al. 2012). Further, many troglobites (i.e., terrestrial cave-obligates) exhibit broad, mosaic distribution patterns which, in conjunction with morphological stasis, often confound traditional approaches of delimitating species boundaries (Jochum et al. 2015). Consequently, troglobites are ideal models to address fundamental questions in ecology and evolution and provide a platform to approach a more modernized integration of taxonomic methods.

An increasing number of studies has examined population genetic and phylogeographic hypotheses of subterranean fauna (e.g., Moulds et al. 2007, Snowman et al. 2010, Weckstein et al. 2016), which have greatly increased our understanding of colonization history, speciation, dispersal, and biogeography of troglobitic taxa (Juan et al. 2010). Additional phylogeographic studies have uncovered considerable levels of cryptic diversity in subterranean species (Finston et al. 2007, Juan and Emerson 2010, Niemiller et al. 2012). Due to increasing advances in imaging technology, studies that incorporate morphometric analyses often complement such molecular findings (Jochum et al. 2015, Armbruster et al. 2016, Burress et al. 2017, Inäbnit et al. 2019). The misidentification of species can hinder assessments of biodiversity and conservation of cryptic species. Therefore, an integrative taxonomic evaluation of troglobitic taxa is needed to fully assess species richness within these systems, and to better inform their respective evolutionary histories. Moreover, cryptic species complexes may be comprised of groups already at significant risk of extinction (Niemiller et al. 2013).

Land snails (Phylum Mollusca, Class Gastropoda) are a species-rich group, with over 24,000 currently recognized species and over 35,000 species thought to exist globally (Barker 2001, Lydeard et al. 2004). The land snail fauna of eastern North America is exceptionally diverse, with over 500 documented species (Hubricht 1985, Nekola 2014). However, this likely represents an underestimate of total species richness in this region. Larger species are often associated with mesic forest ecosystems with high levels of moisture, leaf litter, and calcium (Goodfriend 1986, Pearce and Örstan 2006). Yet, land snails utilize a variety of microhabitats often neglected in sampling efforts within these areas (Cameron and Pokryszko 2005). Further, land snails occur at high density in karst-rich landscapes, and subterranean habitats are particularly under-sampled within the region (Clements et al. 2008, Niemiller and Zigler 2013).

Nearly 75% of all land snails in eastern North America are considered terrestrial micromolluscs (< 5 mm) and comprise a significant portion of all land snail diversity (Nekola 2005, Liew et al. 2008). Many of these species tend to be particularly undersampled and often require the collection of soil and leaf litter samples to discover them (Liew et al. 2008, Nekola and Coles 2010, Durkan et al. 2013). Regions hypothesized to have higher levels of snail biodiversity have had varying and potentially insufficient sampling effort, with many species remaining undescribed (Dourson 2007, Douglas et al. 2014, Dinkins and Dinkins 2018). Moreover, there is a paucity of studies examining intraspecific morphological variation in micromolluscs, obscuring accurate geographic ranges for these species (Nekola and Coles 2010). Thus, because most land snail species are delimited based on conchology (i.e., shell variation), a high incidence of misidentification of minute species occurs in many natural history collections (Hubricht 1985, Nekola and Coles 2010). The continued misidentification of species can have significant impacts on biodiversity assessments and conservation management (Bickford et al. 2007).

Strictly employing morphological data to delimit extant species in the genomic era is often met with criticism (Hermsen and Hendricks 2008, Duminil and Di Michele 2009, Carstens et al. 2013). An integrative taxonomy, i.e., a combination of morphological, ecological, and genetic data when considering phylogenetic relationships, is necessary to facilitate proper interpretations of biological patterns (Dayrat 2005, Weigand et al. 2012, Inäbnit et al. 2019). For gastropods, there are few discrete shell characters that can be used in phylogenetic hypotheses, and conchology is highly variable in response to environmental factors and other selective pressures (Goodfriend 1986, Smith and Hendricks 2013). However, morphometric analyses can contribute to species hypotheses when combined with genetic data (Hermsen and Hendricks 2008, Miller 2016, Inäbnit et al. 2019). Moreover, applying morphometric analyses can inform the causal mechanisms for shape variation between gastropod populations (Vergara et al. 2017).

Terrestrial micromolluscs of the genus *Helicodiscus* Morse, 1864 are found throughout the eastern United States (Hubricht 1985). This genus is known for its unique conchological sculpture, often exhibiting depigmented soft bodies and prominent spiraling striae on the shells of both surface and subterranean species. Many of these species are calciphiles, and two species – *H. barri* Hubricht, 1962 and *H. notius specus* Hubricht, 1962 – have even adopted a cave-obligate existence (Hubricht 1962). The distributions of these troglobites span both the Interior Low Plateau (ILP) and the Appalachians karst regions, covering multiple physiographic provinces within their ranges. The latter species is only known from six caves in Kentucky, Tennessee, and Virginia, whereas the former is known from 49 caves in Tennessee, Alabama, and Georgia. Two additional *Helicodiscus* species that were previously thought to be troglobitic – *H. hadenoecus* Hubricht, 1962 and *H. punctatellus* Morrison, 1942 – have been discovered at surface localities widely disjunct from their otherwise subterranean distribution (Coney et al. 1982; Hotopp et al. 2013). These distribution patterns suggest the potential for cryptic diversity among subterranean taxa within this genus. Morphological stasis is highly prevelant in troglo-

bites despite significant genetic divergence, and, therefore, the mosaic distributions of these snails warrant investigation (Juan and Emerson 2010, Weigand et al. 2012).

Here, we conduct the first study examining morphological variation and phylogeography of the cave-obligate land snail *Helicodiscus barri*. Recent cave bioinventory efforts within the ILP and Appalachians karst regions have yielded several additional specimens of this species for comparison across multiple physiographic provinces. The disjunct, mosaic distribution pattern of *H. barri* in conjunction with a lack of clear morphological variation is consistent with a high potential for cryptic diversity, as observed in other subterranean taxa (Snowman et al. 2010, Loria et al. 2011, Niemiller et al. 2012, Inäbnit et al. 2019). We examined museum accessions of *H. barri* while sampling caves within the states of Tennessee, Alabama, and Georgia for additional specimens. Using phylogeographic approaches, we (1) assessed patterns of genetic variation of *H. barri*; (2) employed two species delimitation methods (ABGD and mPTP) to infer the presence of cryptic lineages; and (3) tested if current species hypotheses based on conchology correspond with patterns of genetic variation. Further, we assessed morphological variation between *H. barri* populations using traditional morphometrics (TM) and landmark-based geometric morphometrics (GM).

Methods

Specimen collection

Shell specimens were collected from 31 populations of cave-dwelling *Helicodiscus barri* from the dark zone of caves within both the ILP and Appalachian karst regions in Tennessee and Alabama (115 total individual specimens collected). Each survey typically involved two to four researchers (maximum 12), with a search effort of two to 36 person-hours per cave visit. In total, 74 caves were visited from 13 March 2013 to 19 June 2018 by NSG, totaling ca. 300 person-hours. Snail specimens were preserved in 100% ethanol and identified using published keys and species descriptions (Pilsbry 1948, Hubricht 1962, Dourson 2010), as well as examination by taxonomic specialists (Dan and Judy Dourson). Specimens from ten additional populations were provided by the Field Museum of Natural History (FMNH), Florida Museum of Natural History (FLMNH), and the Auburn Museum of Natural History (AUM). In total, 154 shells were examined (see Table 1). The geographic distribution of populations utilized within this study can be found in Figure 1.

DNA extraction, amplification, and sequencing

Genomic DNA was obtained from soft tissue of each live specimen collected. The shells of smaller individuals were removed prior to DNA extraction. Tissue was removed from larger shells by breaking a small opening into the abapertural side of the

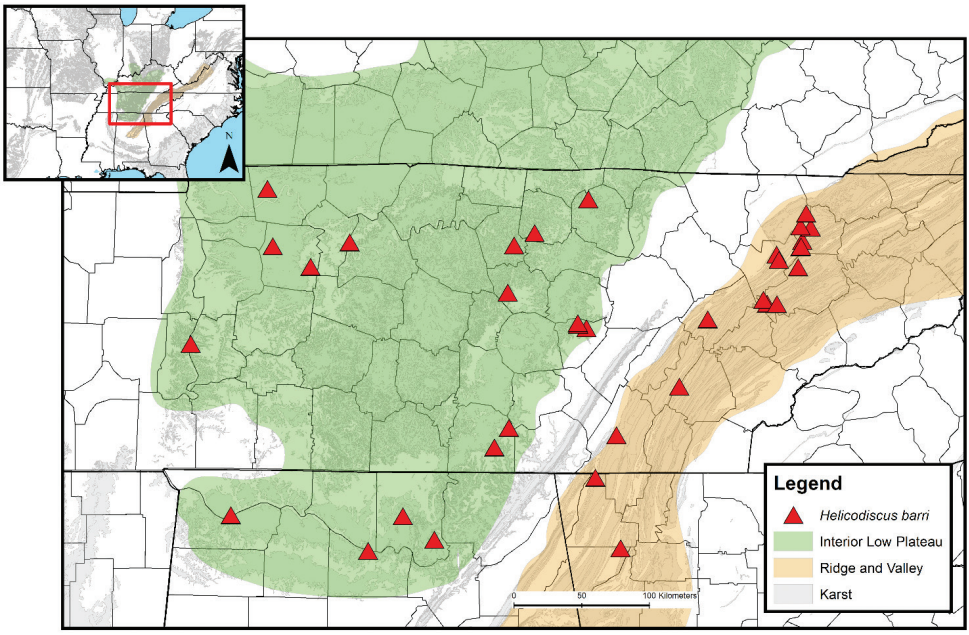

Figure 1. Geographic distribution of *Helicodiscus barri* from this study in relation to karst adapted from Weary and Doctor (2014). Triangles represent cave populations.

shell or the shell base, so that the shell was not completely destroyed and remained identifiable. Each DNA extraction was performed using the Qiagen® DNeasy Blood and Tissue kit following the manufacturer's protocol (Qiagen Sciences, Louisville, KY). Polymerase chain reaction (PCR) was used to amplify fragments of the mitochondrial (mt) 16S ribosomal RNA locus using the primer pair 16Sa/16Sb (Palumbi et al. 1991), mt cytochrome oxidase subunit 1 (COI) locus using the primer pair LCOI490/ HCO2198 (Folmer et al. 1994), nuclear 28S ribosomal RNA locus using the primer pair 28Sna1/28Sna2 (Kano et al. 2002), and nuclear histone 3 (H3) locus using the primer pair H3F/H3R (Colgan et al. 2000). PCR products were purified using ExoSAP-IT (Affymetrix) and sequenced in both directions with BigDye chemistry at Eurofins MWG Operon (Louisville, KY).

Genetic analyses

Forward and reverse sequences were assembled into contigs and edited in Sequencher v.5.1 (Gene Codes Corporation, Ann Arbor, MI). Alignments were modified by the manual trimming of the 3' and 5' primer ends. Ambiguous base calls and double peaks within heterozygotes were assessed visually with the chromatograms. Sequences were then aligned using MUSCLE under default parameters implemented in MEGA X v.10.0.5 (Kumar et al. 2018). All sequence data generated from this study was accessioned into

Table 1. *Helicodiscus barri* populations incorporated in this study, including 17 new populations. Cave names, Tennessee Cave Survey (TCS) cave number, county and state are provided, as well as information regarding which populations were considered in morphometric and genetic analyses.

Sample	Cave	TCS No.	County	State	References	n	Morphology	Genetic
NSG-DI3	Bowman Cave	TDI3	Dickson	TN	This study	2	X	X
NSG-KN112	Brents Cave	TKN112	Knox	TN	This study	3	X	X
AUM28348	Bull Run Cave	TDA4	Davidson	TN	Hubricht 1964	2	X	X
MLN 14-054.12; NSG-JK3	Carter Cave	TJK3	Jackson	TN	Gladstone et al. 2018	5	X	X
NSG-VB547	Cave Between the Caves	TVB547	Van Buren	TN	Lewis 2005	6	X	
NSG-RN5	Cave Creek Cave	TRN5	Roane	TN	This study	1		X
MLN 14-007	Christmas Cave	TDK72	DeKalb	TN	Gladstone et al. 2018	1	X	X
MLN 13-056	Clarksville Lake Cave	TMY11	Montgomery	TN	Gladstone et al. 2018	2	X	X
FMNH239117	Collier Cave	ALD100	Lauderdale	AL	Peck 1989	4	X	
FMNH239122; NSG-DI6	Columbia Caverns	TDI6	Dickson	TN	Hubricht 1962	7	X	X
NSG-KN50	Conner Creek Cave	TKN50	Knox	TN	This study	5	X	X
FMNH239121	Culbertson Cave	TUN22	Union	TN	Hubricht 1985	1	X	
NSG-AN5	Demarcus Cave	TAN5	Anderson	TN	This study	3		X
MLN 14-015.3	Dry Cave	TFR9	Franklin	TN	Gladstone et al. 2018	2		X
AUM27534-T2	Frazier Hollow Cave	DK11	DeKalb	TN	This study	1	X	X
NSG-DI27	East Fork Cave	TDI27	Dickson	TN	This study	2	X	
AUM28173	Hering Cave		Madison	AL	This study	1	X	X
NSG-FR14	Keith Cave	TFR14	Franklin	TN	Lewis 2005	10	X	X
UF 405128	Lady Finger Bluff Trail		Perry	TN	Gladstone et al. 2018	1	X	
WC13-165	Lovelady Cave	THM56	Hamilton	TN	This study	1		
NSG-MM10	McCorkle Cave	TMM10	McMinn	TN	This study	1		
NSG-VB9	McCoy Cave	TVB9	Van Buren	TN	This study	2	X	X
AUM27855	New Salem Cave Nr1	TSM10	Smith	TN	This study	2	X	X
MLN 15-007.9	Oaks Cave	TUN5	Union	TN	Gladstone et al. 2018	1	X	X
FMNH305126; NSG-AN12	Offut Cave	TAN12	Anderson	TN	Hubricht 1985	8	X	X
MLN 15-006.19; NSG-CM8	Panther Cave No. 1	TCM8	Campbell	TN	Gladstone et al. 2018	7	X	X
FMNH239120	Parkers Cave	GKH119	Chattooga	GA	Holsinger and Peck (1971)	2	X	
NSG-KN108	Pedigo Cave Nr. 2	TKN108	Knox	TN	This study	1		X

Sample	Cave	TCS No.	County	State	References	n	Morphology	Genetic
NSG-AN6	Robert Smith Cave	TAN6	Anderson	TN	This study	2		X
MLN 13-000	Rockhouse Cave	ALM312	Limestone	AL	Gladstone et al. 2018	1	X	
AUM27652	Rogers Hollow Cave	TUN23	Union	TN	This study	2	X	
FMNH239118	Shelta Cave	AMD4	Madison	AL	Peck 1989	3	X	
NSG-OV440; GC1	Slippery Slit Cave	TOV440	Overton	TN	Lewis 2005	3	X	X
KSZ15-313	Smartt Farm Cave	GWK124	Walker	GA	This study	1		
NSG-VB657	Swamp River Cave	TVB657	Van Buren	TN	This study	1	X	
MLN-16.0228	Weavers Cave	TAN22	Anderson	TN	Gladstone et al. 2018	2	X	X
NSG-KN80	Wilke Waller Cave	TKN80	Knox	TN	This study	1		

GenBank (see Suppl. material 1). PartitionFinder v.2.1.1 (Lanfear et al. 2012) was used to determine the best model of sequence evolution for each partition based on the Bayesian information criterion (BIC). A general time-reversible model of sequence evolution with corrections for a discrete gamma distribution and a proportion of invariant sites (GTR+Γ+I) was chosen for 16S. The Hasegawa et al. (1985) model (HKY) with corrections for a discrete gamma distribution was chosen for the first and second codon positions of both CO1 and H3 as well as for 28S. A symmetrical model with corrections for a discrete gamma distribution (SYM+ Γ) was chosen for the third codon position of CO1 and H3 (Zharkikh 1994). Due to uneven coverage of genetic data across specimens, three unique *H. barri* datasets were assessed: CO1, *mtDNA* (CO1 + 16S), and *mtDNA* + *nDNA* (CO1 + 16S + 28S + H3). *Discus rotundatus* was used as an outgroup for all phylogenetic analyses. Summary statistics of the *H. barri* molecular dataset including haplotype and nucleotide diversity, number of segregating sites, haplotypes, and mutations were calculated in DnaSP v.6.12.01 (Librado and Rozas 2009). Uncorrected p-distances within and between cave populations were used as a metric of genetic divergence and calculated in MEGA X v.10.0.5 (Kumar et al. 2018). A haplotype network for all specimens for which genetic data were available was created in SplitsTree v.4.14.8 (Huson and Bryant 2005) using the NeighborNet network method with uncorrected p-distances.

Phylogenetic analyses and species delimitation

Phylogenetic trees were inferred utilizing both a Maximum-Likelihood (ML) and Bayesian-inference (BI) approach. ML analyses were conducted using RAxML v.8.0 (Stamatakis 2014) as implemented through the T-REX web server (Boc et al. 2012). A consensus tree was generated from the CO1, *mtDNA*, and *mtDNA* + *nDNA* datasets using rapid bootstraps for 100,000 replicates under a GTR+Γ+I model of evolution.

The BI analyses were conducted in MrBayes v.3.2.6 (Ronquist et al. 2012) using a random start tree with three heated and one cold chain (default temperature of 0.1). This was run twice for 50,000,000 generations and sampled every 1,000 generations under the models of evolution determined by PartitionFinder. The first 25% of samples (12,500,000) were discarded as burn-in. Convergence of runs was assessed utilizing Tracer v. 1.4 (Rambaut and Drummond 2007).

The generation of molecular barcodes is often utilized in species delimitation in understudied groups (or in this case, those that are morphologically ambiguous; Pons et al. 2006; Rubinoff 2006; Weigand et al. 2012, 2014). As such, two species delimitation approaches were subsequently used in the identification of Molecular Operational Taxonomic Units (MOTUs; Floyd et al. 2002): 1) Automatic Barcode Gap Recovery (ABGD; Puillandre et al. 2012), and 2) Multi-rate Poisson Tree Processes (mPTP; Kapli et al. 2017). ABGD partitions samples into candidate species based on a statistically inferred barcode gap. The barcoding gap is defined as a notable disparity between pairwise genetic distances, presumably between intraspecific and interspecific distances. This process is applied recursively to newly obtained groupings of sequences, to assess the possibility of internal division. This method was employed on the CO1 dataset excluding the outgroup (n = 24) via the ABGD web server (http://wwwabi.snv.jusieu.fr/public/abgd/abgdweb.html) using the Kimura two-parameter (K2P; Kimura 1980) model with a standard X (relative gap width) = 1.5.

The initial development of PTP models assumed one exponential distribution for speciation events and one for all coalescent events (Zhang et al. 2013). In contrast, the mPTP approach fits speciation events for each candidate species to a unique exponential distribution, greatly improving the quality of results (Kapli et al. 2017). This method requires a rooted phylogenetic tree and partitions samples into candidate species based upon the number of substitutions under assumed Poisson processes. Intraspecific substitution rates should be notably smaller than interspecific rates. This method does not require an ultrametric tree, which is ideal given little reliable fossil data for Helicodiscidae and the variability of molecular clock rates in Stylommatophoran gastropods (Thomaz et al. 1996, Chiba 1999, Van Riel et al. 2005). A rooted tree was generated for the CO1 dataset using the methods previously outlined for RAxML under the models of evolution determined by PartitionFinder. Analysis was carried out on the mPTP webserver (http://mptp.h-its.org) for the maximum 100,000 MCMC generations, with 25% of samples (25,000) conservatively discarded as burn-in.

Morphometric analyses

Specimens were photographed using a Canon 6D digital SLR camera mounted on the Macropod PRO Micro Kit (Macroscopic Solutions, Tolland, CT). Each shell was photographed using a Canon MP-E 65mm f/2.8 1–5× macro lens in three views: ventral, dorsal and apertural. MacroMagnification settings were extracted from the images using ExifTool v.5.16.0.0. Images were imported to Adobe Photoshop CS5 Extended

v.12.1 and were subsequently scaled. Reproductive anatomy was not evaluated, given that most specimens were taken from museum collections with no soft tissue available. Two morphometrics methods were employed: geometric morphometrics (GM) and traditional morphometrics (TM).

GM techniques allow for the quantification and assessment of morphological variation. Biologically-meaningful landmarks (LMs) and semilandmarks (SLMs) of the *Helicodiscus* shells were digitized using tpsDig2 v. 2.32 (Rohlf 2015, available at http://life.bio.sunysb.edu/morph/). Nine homologous static LM were placed across each specimen. LM 1 is Type 1, characterizing the discrete juxtaposition of the homologous shell structure. LM 4 and LM 5 are Type 2, characterizing geometric maxima of curvature. All remaining LMs were Type 3, characterizing more than one region of each shell (Bookstein 1997). These nine LMs were combined with two manually traced curves of three equidistant SLMs anchored on LM 4–5 and LM 5–6 (see Figure 2A). Appending tps curves to SLM was achieved using tpsUtil v. 1.76 (Rohlf 2015). This results in a total of nine fixed and six semi-landmarks.

To eliminate variation due to orientation of the shell or size, a Procrustes superimposition was performed using the *geomorph* package v.2.0. in RStudio v 1.1.456 with R v. 3.5 (Adams and Otarola-Castillo 2013, Adams et al. 2014, RC Team 2014). These data were subjected to principal component analysis (PCA) to evaluate the distribution of populations in morphospace. Several alternative landmark schemes were tested but provided no notable differences in PCA results. The most conservative approach was employed to reduce the number of variables introduced into the downstream analyses. Correlation coefficients (PC loadings) of individual variables were assessed visually to determine which specific variables are significant to each PC as to interpret what shell characteristics account for variability of the dataset. To quantify error associated with landmark placement and shell placement during photography, a set of replicate images with digitized landmarks was used to calculate the disparity using the *morphol.disparity* function in *geomorph* (Adams et al. 2013, 2014).

Specimens were grouped by MOTUs to identify detectable differences in shell variation in concordance with genetic variation. Reduced datasets of those specimens with obtained genetic data were used for these groups. However, in the absence of genetic data, all specimens for which data are available were grouped by the physiographic province associated with the collection locality (i.e., Cumberland Plateau, Eastern Highland Rim, Valley and Ridge, and Western Highland Rim). These regions possess unique environmental characteristics (e.g., soil physiochemistry, rock type, vegetation; Fenneman 1917) and were utilized as broad-scale categories to test for the effect of environmental variation on conchology. Before assessing the significance of these groups in explaining morphological variation, the first and second PCs were subjected to a test of spatial autocorrelation (SAC) to prevent the increase of Type 1 errors introduced to the analyses (Perez et al. 2010). SAC was determined using the Moran's I statistic (Sokal and Oden 1978) and was found to be non-significant (PC 1=0.2184, PC 2=0.0832). These groups were subjected to Procrustes Analysis of Variance (ANOVA) following a randomized residual permutation procedure (RRPP) for 10,000 iterations.

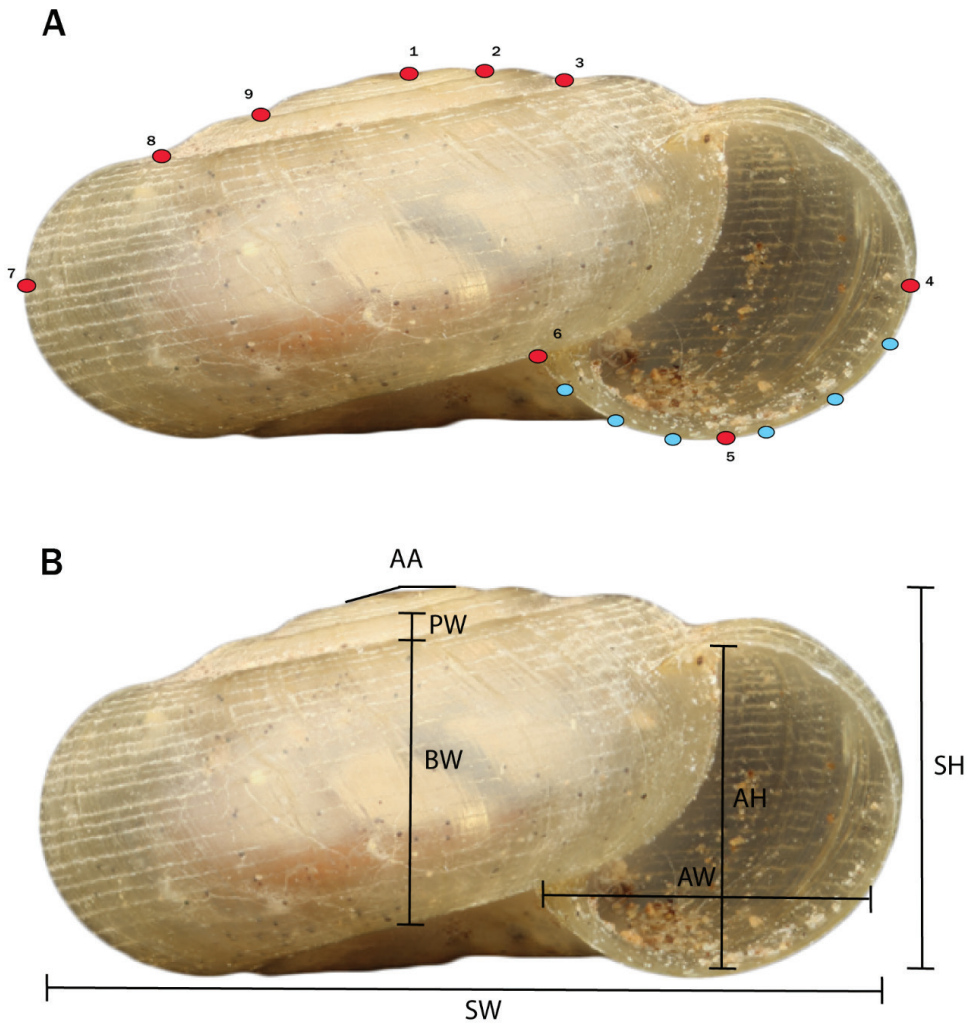

Figure 2. A Landmark scheme for geomorphometric analyses. Red circles represented landmarks (LM), blue circles represent semi-landmarks (SLM). **B** Shell measurements utilized for the traditional morphometric (TM) analyses.

Seven unique shell measurements from Burch (1962) were utilized in the TM approach (see Figure 2B): Shell width (SW), shell height (SH), aperture width (AW), aperture height (AH), body whorl height (BW), penultimate whorl height (PW), and angle of apex (AA). These shell characteristics are often utilized in morphometric analyses and are readily utilized in land snail species identification guides (Pearce and Örstan 2006, Dourson 2010). Scaled data were converted to a Euclidean distance matrix and subject to Permutational Multivariate Analysis of Variance (PERMANOVA). This test was performed in the *vegan* package in R for 10,000 permutations (Oksanen 2018). *P*-values extracted from pairwise comparisons were corrected using a Bonferroni test.

Results

Genetic analyses

Molecular sequence data were obtained from 32 specimens of 23 populations. Tissue samples were scarce, and the saturation of the land snail soft body with mucopolysaccharides inhibited the success of standard extraction procedures and subsequent sequencing. Thus, we were unable to obtain full genetic coverage (i.e., all four target genes sequenced) for all specimens. Summary statistics generated for the genetic data is presented in Table 2. The *mtDNA* dataset used for the gene tree estimation was unambiguously aligned (1316 base pairs; bp). A concatenated alignment of all specimens in which all four genes were amplified was also unambiguously aligned and assessed (n = 16; 3040 bp). The CO1 dataset was also assessed independently, as it was later utilized for the downstream species delimitation approaches. The CO1 dataset was unambiguously aligned (n=24; 704 bp). No shared haplotypes were observed between cave populations at CO1 (see Table 3), even in cases where caves were less than 15 m apart from one another (e.g., Demarcus Cave (AN5) and Robert Smith Cave (AN6) in Anderson County, Tennessee). The generated haplotype network strongly resembles MOTU delimitation results (see Figure 3), with the two most diverse MOTUs identified possessing five haplotypes each. Mean uncorrected p-distances between cave populations at CO1 was 16.14% (range 2.6–23.2%), indicating significant geographic isolation. For the concatenated genetic dataset, mean uncorrected p-distances between cave populations was 6% (range 1.3–10.3%). Due to the rarity of this species, there were only four instances of obtaining sequences of more than one individual per cave (Columbia Caverns (DI6), Keith Cave (FR14), Offut Cave (AN12), Panther Cave No. 1 (CM8)). Of these, two populations exhibited two haplotypes at CO1. Intrapopulation variation of these four populations was low, with a mean CO1 uncorrected p-distance of 1.48±0.3%.

Phylogenetic analyses

Both ML and BI approaches resulted in highly similar tree topologies for each unique concatenated genetic dataset (CO1, *mtDNA, mtDNA* + *nDNA*). The outstanding difference between the ML and BI phylograms generated from the *mtDNA* dataset was a resolution of polytomy from the ML approach in the Bowman Cave (DI3), Carter Cave (JK3), Keith Cave (FR14), McCoy Cave (VB9), and Slippery Slit Cave (OV440) clade. The *mtDNA* + *nDNA* phylograms also differed with the Hering Cave (AMD6) population representing a monotypic group in the ML approach, and grouping with the Brent's Cave (KN112), Columbia Caverns (DI6), and Weavers Cave (AN22) clade as it does in all other phylograms assessed. Additionally, there were several notable distinctions in the CO1 phylograms produced between BI and ML approaches (see Suppl. material 2). Despite these differences, only the representative phylogenies utiliz-

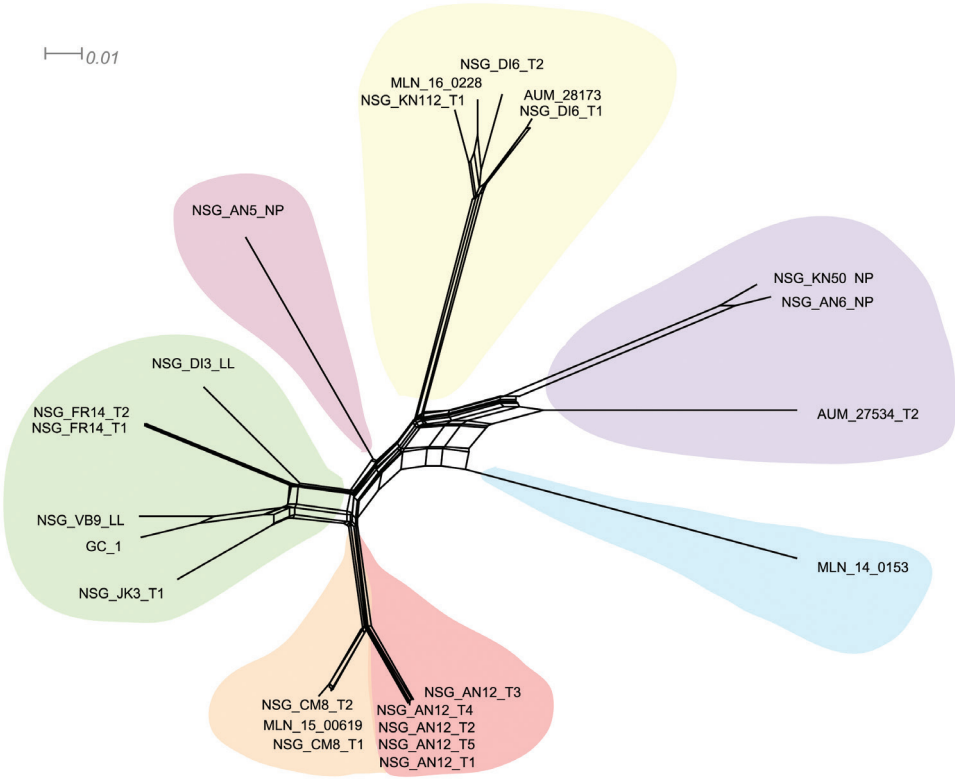

Figure 3. Haplotype network generated using the NeighborNet network method with uncorrected p-distances with the CO1 dataset. Species delimitation results are depicted using major color groups for the mPTP results, and subcolor groups for the ABGD results.

Table 2. Summary statistics generated for all four genes assessed (*mtDNA*: CO1, 16S; *nDNA*: 28S, H3).

	n	**bp**	***h***	***Hd***	***Π***	***S***	***Eta***
mtDNA							
CO1	24	668	18	0.957 ± 0.031	0.14346 ± 0.012	164	245
16S	28	605	20	0.968 ± 0.019	0.09019 ± 0.011	71	99
nDNA							
28S	29	1305	6	0.424 ± 0.111	0.00327 ± 0.001	20	20
H3	25	337	6	0.427 ± 0.122	0.00322 ± 0.001	7	7

n – number of sequences, ***bp*** – alignment size, ***h*** – number of haplotypes, ***Hd*** – haplotype diversity, ***Π*** – nucleotide diversity, ***S*** – number of polymorphic sites, ***Eta*** – number of mutations

ing the BI approach for the CO1, *mtDNA*, and *mtDNA* + *nDNA* datasets are shown (Figures 4, 5). All other trees are placed within Suppl. materials 2, 3.

Due to an inability to amplify all genes per specimen, some specimens are not represented in all phylogenies. However, among the representatives included in all three datasets, there is a consistent topology. The only differences between the *mtDNA* tree

Table 3. Species delimitation results from both ABGD and mPTP analyses. Haplotype diversity, specimen ID, state, karst region, and physiographic province also included.

mPTP	ABGD	Haplotype ID	Specimen ID	State	Karst	Physiographic
PG1	AG1	H1, H2	NSG-CM8-T1, NSG-CM8-T2, MLN-15-006.19	TN	APP	VR
PG2	AG2	H3	NSG-AN12-T1, NSG-AN12-T2, NSG-AN12-T3, NSG-AN12-T4, NSG-AN12-T5	TN	APP	VR
PG3	AG3, AG4, AG5, AG6, AG7	H4, H5, H6, H7, H8	NSG-JK3-T1, NSG-VB9-LL, GC1, NSG-DI3-LL, NSG-FR14-T1, NSG-FR14-T2	TN	ILP	CP, EHR, WHR
PG4	AG8	H9	NSG-AN5-NP	TN	APP	VR
PG5	AG9	H10	MLN-14-0153	TN	ILP	CP, EHR
PG6	AG10, AG11, AG12	H11, H12, H13	AUM27534-T2, NSG-KN50-NP, NSG-AN6-NP	TN	APP, ILP	VR, WHR
PG7	AG13, AG14, AG15, AG16	H14, H15, H16, H17, H18	MLN-16.0228, NSG-KN112-T1, NSG-DI6-T1, NSG-DI6-T2, AUM28173	AL, TN	APP, ILP	CP, VR, WHR

and the *mtDNA* + *nDNA* BI trees are 1.) the resolution of polytomy and varied topology in the Bowman Cave (DI3), Carter Cave (JK3), Keith Cave (FR14), McCoy Cave (VB9), Slippery Slit Cave (OV440) clade, and 2.) the relative placement of the Frazier Hollow Cave (DK11), Robert Smith Cave (AN6), and Conner's Creek Cave (KN50) clade. All other clades remain consistent. Bootstrap support for the ML approach were notably lower at deeper nodes in each phylogram, and the same occurred with posterior probabilities generated from the BI approach. Node posterior probabilities and confidence values increased overall after the addition of the *nDNA* data. Comparison of both *mtDNA* and *nDNA* phylograms show the existence of at least seven monophyletic clades across the Appalachians and ILP karst regions (Figure 5). The monotypic Dry Cave (FR9) and Demarcus Cave (AN5) samples seem to be considerably divergent from other groups. While the former is known from the southern extent of the Eastern Highland Rim, the latter monotypic clade is in immediate proximity to Robert Smith Cave (less than 15 m) yet both are significantly delineated in the CO1 phylogram and the subsequent delimitation approaches.

Species delimitation

The ABGD method generated two partition strategies. At prior intraspecific divergence (*P*) values between 0.0010 and 0.0215, sixteen MOTUs were recognized in initial and recursive partitions. Both partition schemes remained stable at these values until reaching congruency at $P = 0.0359$, grouping all populations together into a single MOTU. The barcode gap was discovered at 0.14–0.16 K2P distance. The PTP results generated seven MOTUs for both single and multi-coalescent rate models (see Suppl. material 4). Both delimitation approaches show highly similar MOTU designations, with most identified groups being known from individual caves (Figure 4).

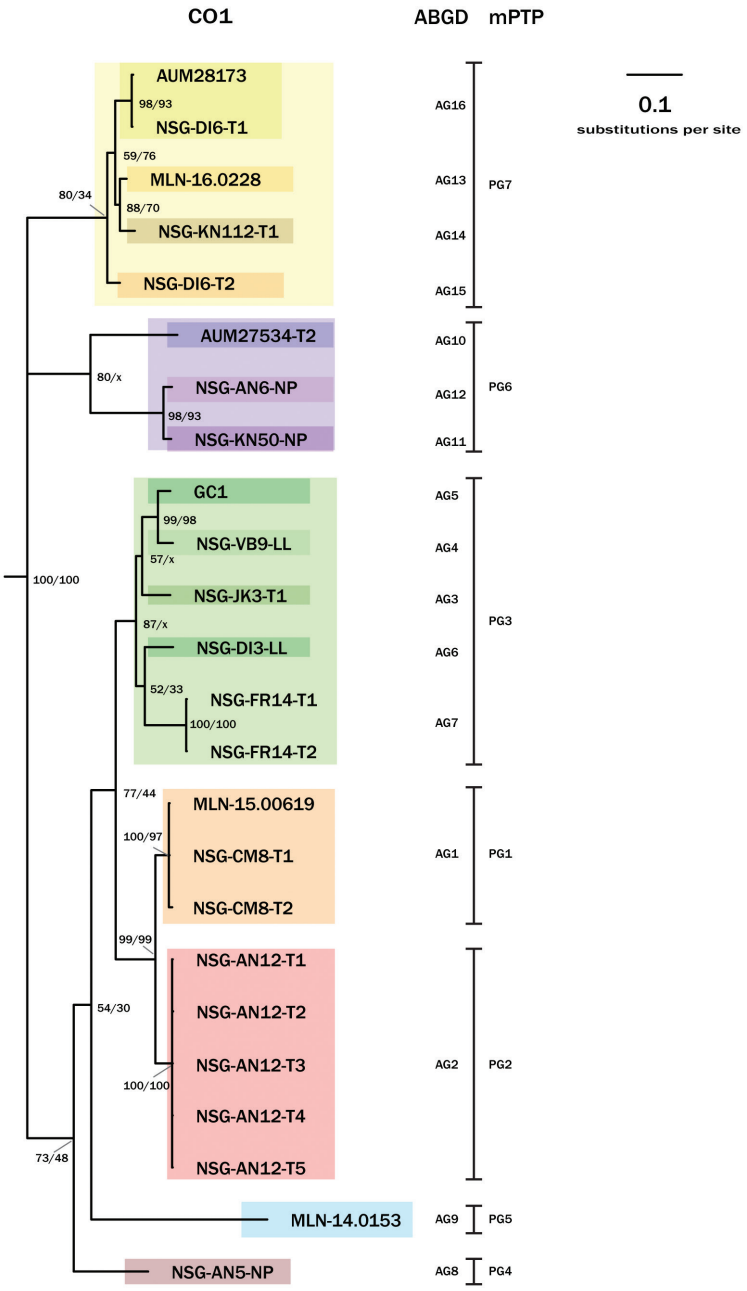

Figure 4. Phylogenetic tree of the CO1 dataset (808 bp) using the BI methodology. Posterior probabilities generated from the analysis are shown for each clade with the top numbers. Confidence values given from the bootstrapped ML method are shown for each clade with the bottom numbers. The 'x' symbols indicate varying topology between the BI and ML analyses. ML trees are reported in the Appendix for cross-reference. Species delimitation results are depicted using major color groups for the mPTP results, and subcolor groups for the ABGD results.

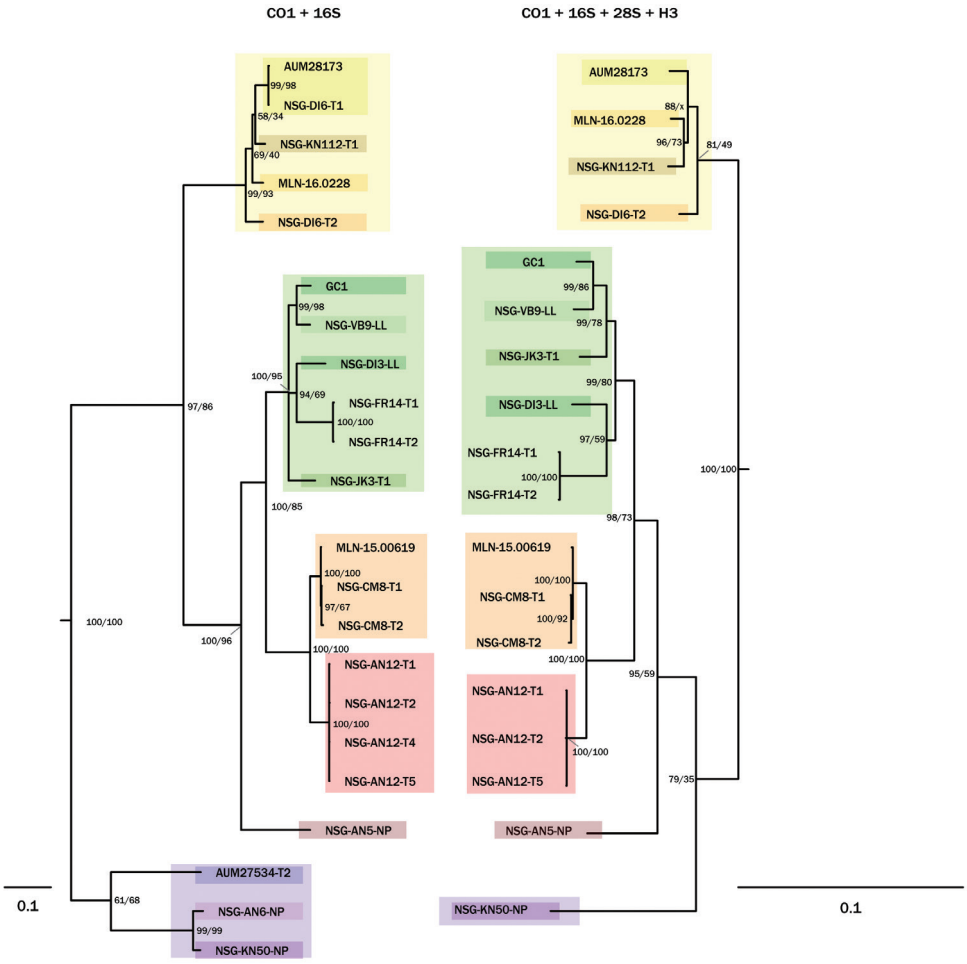

Figure 5. Phylogenetic trees of the concatenated *mtDNA* (CO1 + 16S; 1316 bp) and the full *mtDNA* + *nDNA* (CO1 + 16S + 28S + H3; 3040 bp) datasets. Posterior probabilities generated from the analyses are shown for each clade with the top numbers. Confidence values given from the bootstrapped ML method are shown for each clade with the bottom numbers. The 'x' symbols indicate varying topology between the BI and ML analyses. ML trees are reported in the Appendix for cross-reference. Species delimitation results are depicted using major color groups for the mPTP results, and subcolor groups for the ABGD results.

There were four cases of both delimitations methods producing the same results (PG1, PG2, PG4, PG5). Three groups of five, four, and three MOTUs generated by ABGD were consolidated into three MOTUs generated by mPTP (PG3, PG6, PG7), respectively. The consolidated PG3 MOTU group is largely clustered within the Eastern Highland Rim (AG3, AG4, AG5, AG7), with only one disjunct representative being found in a fragmented karst formation on the eastern extent of the Western Highland Rim (AG6). The PG6 and PG7 MOTU groups exhibit an irregular geographic struc-

ture, with both possessing representatives from both karst regions (Appalachians and ILP). Further, the ABGD results suggest two MOTU groups (AG15, AG16) within a single cave population at Columbia Caverns, with AG16 comprising this cave on the eastern extent of the Western Highland Rim and another in the southern extent of the Cumberland Plateau in the state of Alabama.

Morphometric analyses

In total, 65 specimens were incorporated into both the GM and TM datasets from 28 cave populations. The disparity test used to indicate possible error introduced from shell and landmark placement (2.28%) was negligible. PC plots for each grouping are displayed in Figure 6A–D. For the GM PCA, the first three principal components account for 69.52% of the total variance. PC 1 (31.24%) was interpreted as the curvature of the shell, with higher PC scores exhibiting a higher angle of apex and larger shell height. The X-coordinates of LM 3, LM 2, and LM 6 all had the highest PC loadings associated with PC 1 (0.418, 0.312, 0.243 respectively). PC 2 (25.18%) was interpreted as the size of the secondary body whorl in relation to the aperture, with higher PC scores exhibiting significantly wider secondary body whorls with an annular apertural structure. The Y-coordinate of LM 8 and the X-coordinates of LM 9 and LM 1 had the highest PC loadings associated with PC 2 (0.281, 0.189, 0.159 respectively). PC 3 (13.10%) was interpreted as the size of the aperture, with higher PC scores exhibiting larger apertures and higher shell width. The X-coordinates of LM 6 and SLM 15 and the Y-coordinate of LM 4 had the highest PC loadings associated with PC 3 (0.354, 0.333, 0.301 respectively). A smaller morphometric dataset (n = 39) was assessed for those individuals for which molecular data was available. Only the MOTU groups from the mPTP analysis were considered, as these were the larger groups. The first three principal components for this smaller dataset account for 74.90% of the total variance (PC 1 = 31.03%; PC 2 = 28.76%; PC 3 = 15.11%).

For the TM PCA, the first three principal components accounted for 79.36% of the total variance. PC 1 (66.03%) was interpreted as the overall size of the shell, with high PC scores exhibiting larger shell height and shell width (PC loadings = 0.4553, 0.4370 respectively). PC 2 (13.23%) was interpreted as the height of the shell, with higher PC scores exhibiting much larger penultimate whorls and shell height (PC loadings = 0.6336, 0.6011 respectively). PC 3 (9.85%) was interpreted as the curvature of the shell, with higher PC scores having higher angles of apex and smaller shell width (PC loadings = 0.6815). For the smaller mPTP dataset, the first three principal components accounted for 85.98% of the total variance. Procrustes ANOVA and PERMANOVA tested the influence of environmental variation (i.e., respective physiographic province) on external shell morphology, indicating significance for both morphometric approaches. MOTU groups did not significantly explain shell variation with the GM approach, but it was significant for the TM approach.

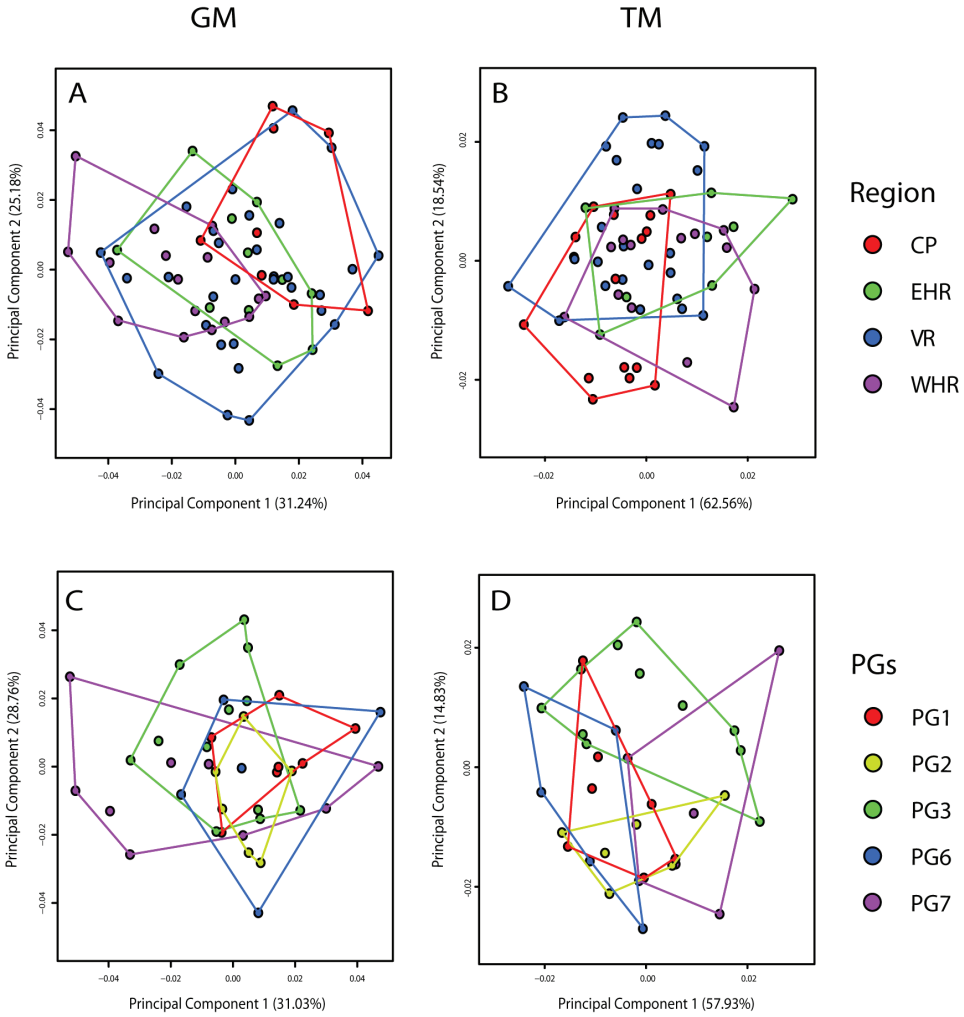

Figure 6. PCA results from both geometric morphometric (left) and traditional morphometric (right) analyses. **A, B** Total morphometric dataset (n=65) grouped by physiographic province. **C, D** Morphometric dataset with complimentary molecular data (n=39) grouped by MOTUs from the mPTP analysis.

Discussion

Many molecular studies of troglobitic taxa have revealed previously unknown cryptic lineages in North America (Buhay and Crandall 2009, Snowman et al. 2010, Niemiller et al. 2012, Weckstein et al. 2016). Troglobites are hypothesized to have fewer opportunities for dispersal than obligately-subterranean aquatic species (i.e., stygobites), due to limited connectivity of terrestrial subterranean passages (Culver et al. 2009). This may promote isolation and short-range endemism in troglobites (Culver et al. 2009,

Niemiller and Zigler 2013). No phylogeographic study of troglobitic snails has been conducted in North America, and all other molecular studies of troglobites in the Appalachians and Interior Low Plateau have focused on organisms with comparatively higher vagility and dispersal potential (e.g., Buhay et al. 2007, Niemiller et al. 2008, Niemiller et al. 2012, Snowman et al. 2010, Loria et al. 2011). Using both a multilocus molecular and a morphometrics approach, we investigated genetic diversity within *H. barri* to identify potential cryptic populations within the species' range, and to further determine whether external shell morphology was a useful indicator of differing patterns of genetic variation.

Genetic diversity of *Helicodiscus barri*

Despite limited sampling success of this rare species, our study revealed high genetic diversity in *H. barri*. Haplotypic diversity is strongly dictated by individual caves, and there appears to be little to no dispersal between cave systems regardless of proximity. Mitochondrial genetic divergence among *H. barri* populations is significantly higher (16.14%) compared to other troglobitic invertebrate taxa studied in the region (e.g. 3.1% for *Nesticus* spiders (Snowman et al. 2010); 0.06% for *Tetracion* millipedes (Loria et al. 2011); 2.1% for *Ptomaphagus* beetles (Leray et al. 2019)), suggesting that the low vagility of land snails accentuates the isolation caused by subsurface habitat fragmentation. Rates of mitochondrial gene evolution for land snails vary considerably, with estimates of 1.6–12.9% per million years for ribosomal genes and 2.8–13% for CO1 (Thomaz et al. 1996, Chiba 1999, Van Riel et al. 2005). Further, land snails often exhibit high levels of intraspecific genetic divergence and population structure (Guiller et al. 1994, Davison et al. 2009, Perez et al. 2014). An estimated 1.6% divergence per million years has been a proposed standard for other gastropods (Liu and Hershler 2007, Murphy et al. 2012, Harris et al. 2013). With this conservative estimate, CO1 sequence divergence suggests the average timing of isolation between *H. barri* populations is 10.1 million years, and up to 14.5 million years. In this scenario, not only do these results indicate the evolutionary independence of these cave populations, they suggest that the subterranean colonization of this species predates Pleistocene glaciation. The Climatic Relict Hypothesis suggests that environmental stress (such as the often implicated Holsinger (1988) "Pleistocene-effect" model) drives the colonization of organisms into subterranean environments (Leys et al. 2003, Culver and Pipan 2009). Using this gastropod CO1 molecular clock, the Climate-relict hypothesis is not supported. Rather, a scenario in which a geographically widespread proto-troglobitic (i.e., troglophilic) species colonized different subterranean systems independent of obvious environmental stress is favored instead.

Mitochondrial divergence estimates of ≥10% per million years are, however, often associated with terrestrial gastropods on island systems (Chiba 1996, Thacker and Hadfield 2000, Van Riel et al. 2005), which are highly comparable to cave systems due

to the isolation of subterranean environments and the discontinuity of these habitats across karst landscapes (Culver 1970, Snowman et al. 2010). In this latter scenario with a high rate of mitochondrial gene evolution (10% per million years), average timing of isolation is 1.6 million years. This would suggest a climatically-driven subterranean colonization during the mid-Pleistocene, failing to reject the Climatic Relict Hypothesis. Thus, it is difficult to differentiate between varying biogeographic scenarios without the application of a *Helicodiscus*-specific molecular clock model. However, the development of an accurate molecular clock is problematic due to a notable gap in fossil material for the genus in North America. There are several occurrences of fossil material in surface and cave habitats across central and eastern North America during the Pleistocene (Rinker 1949, Wetmore 1962, Slaughter 1966, Schultz and Cheatum 1970, Guilday et al. 1977, Dalquist and Stangl 1984, Eshelman and Hager 1984), and one fossil record in central North America in the upper Miocene (Liggert 1997). Moreover, dating based on biogeographic barrier formation is also problematic, as timing estimates of cave formation across the distribution of *H. barri* are highly variable. The formation of some caves in the eastern Appalachians and Cumberland Plateau have been estimated to occur in the late Pliocene to middle Pleistocene (Davies 1953, Anthony and Granger 2004, White 2009), while other caves along the Tennessee River Valley and the Highland Rim have been estimated to form in the late Mesozoic to the early Tertiary (Moneymaker 1948, Barr 1961). Therefore, assessing the timing of colonization is currently beyond the scope of this study.

Delimitation analyses revealed up to sixteen unique MOTUs within *H. barri*, largely organized by geographic and geological similarity (see Figure 7). Most MOTUs belong to similar rock groups, arranged largely in association with each respective physiographic province. There were two unique cases of MOTUs being distributed across both the Appalachians and ILP karst, each exhibiting irregular geographic structure. PG7 is distributed across four cave populations from the northeastern Valley and Ridge, the southernmost contact zone of the Cumberland Plateau and the Eastern Highland Rim, and the westernmost extent of the Western Highland Rim. PG6 is distributed across three cave populations in the eastern Central Basin and the northeastern Valley and Ridge. Further, the ABGD results reveal two distinct MOTUs (AG15, AG16) within a single cave population at Columbia Caverns (DI6). AG15 is comprised of a single individual from Columbia Caverns, whereas AG16 is comprised of one individual from Columbia Caverns and another from the Hering Cave population in northern Alabama. This pattern may be the product of multiple cave colonization events in Columbia Caverns, or perhaps this demonstrates a case of sympatric speciation because of niche partitioning (e.g., Cooper et al. 2002, Niemiller et al. 2008), as these individuals were found in two separate areas of this large cave system. However, due to a low sample size, the aforementioned limited fossil data, and the uncertainty in estimating biogeographic barrier formation, it is difficult to determine the evolutionary history of this species and the geologic context whereby these unique MOTU groups may have developed.

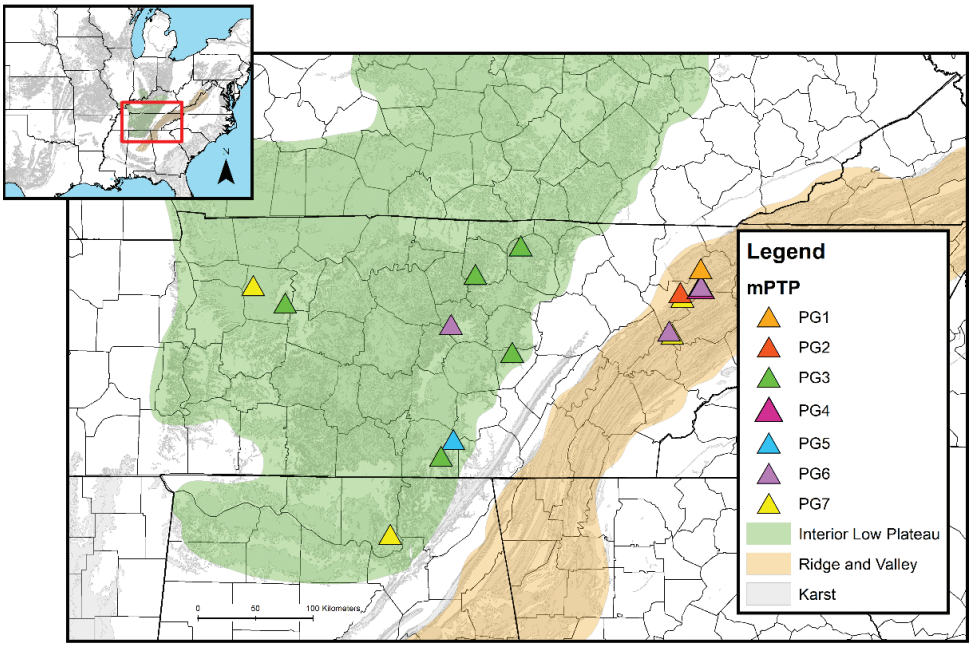

Figure 7. Geographic distribution of MOTUs generated from the mPTP delimitation method in relation to karst adapted from Weary and Doctor (2014). Triangles represent cave populations. The numbers associated with each unique color corresponds to the associated mPTP MOTUs found in Table 3.

Utility of shell morphometrics in species delimitation of cryptic terrestrial micromolluscs

There has been much debate regarding the use of gastropod shell morphology in phylogenetic analyses (Emberton 1995, Wagner 2001, Uit de Weerd et al. 2004, Smith and Hendricks 2013, Miller 2016). Shell variation, while informative at lower taxonomic resolutions (e.g., Smith and Hendricks 2013), may not be useful in accurate delimitation of cryptic lineages, owing to the high responsiveness of shell structure to environmental factors and commonality of local adaptations in land snails (Goodfriend 1986, Fiorentino et al. 2008, Stankowski 2011, Razkin et al. 2017). Moreover, though many subterranean taxa (including *Helicodiscus barri*) exhibit disjunct, fragmented distributions, the ecological similarity of subterranean environments can lead to the protraction of morphological distinguishability between distinct genetic lineages (Losos and Mahler 2010, Eme et al. 2018, Inäbnit et al. 2019). Terrestrial micromolluscs pose additional difficulty in morphological delimitation due to their small size and similarities in external shell morphology, and molecular approaches have been favored (e.g., Weigand et al. 2012). Recent study of troglobitic *Zospeum* snails show that external shell morphology shows high variability both within and between cave populations, further obscuring the taxonomic identity of these cryptic groups without molecular data and intensive study of internal shell structure and soft tissue histology (Jochum et al. 2015).

Table 4. Results from both TM and GM analyses. Asterisk (*) denotes significant p-values.

Group	Degrees of Freedom	Sums of Squares	R^2	*F*	*P*
Procrustes ANOVA					
MOTUs (mPTP)	4	0.00763	0.14037	1.388	0.1311
Physiographic Province	3	0.01291	0.13503	3.1742	2.00E-04*
Permutational MANOVA					
MOTUs (mPTP)	4	80.084	0.30107	3.6614	3.00E-04*
Physiographic Province	3	69.84	0.15589	3.7553	0.0021*

Results herein indicate geographic variation of shell morphology as shown by the distinction of physiographic province groups, although intensive study of habitat variation was not performed. Both GM and TM methods resulted in significant differences among physiographic provinces. These findings further suggest an environmental influence on overall external shell morphology, agreeing with previous studies (Goodfriend 1986, Fiorentino et al. 2008, Vergara et al. 2017). The smaller MOTU dataset, comparatively, exhibited large morphological overlap between MOTU groups. However, results were significant for distinction from the TM groupings (see Table 4). This significance is most likely the result of population-based similarity in shell size, rather than the respective MOTU, of which many consist of multiple populations. Further, the GM groups were not significantly different for the MOTU dataset. The small sample size is a potential drawback to the utilization of these morphometric approaches. Terrestrial micromolluscs are notoriously difficult to sample (Boag 1982, Durkan et al. 2013), and sampling in cave environments significantly increases this difficulty. Moreover, as many of these populations remain understudied, morphometric methods may negatively impact populations subject to high amounts of collection and disturbance. This said, application of molecular barcodes may be most useful in the identification of these terrestrial micromolluscs (Weigand et al. 2011, 2014).

Taxonomic and conservation implications

The discovery of cryptic evolutionary lineages within *H. barri* has significant conservation implications. Recent reassessment of the conservation status of *H. barri* listed this species as Vulnerable (G3) under NatureServe criteria and Least Concern (LC) under the IUCN Red List criteria (Gladstone et al. 2018). Though our study suggests that this species is more geographically wide-spread than previously known, the distribution of individual MOTUs is greatly reduced, sometimes being restricted to a single cave. However, this species' presence in both karst regions despite separation by a considerable amount of non-karst strata, and the discovery of a single specimen from surface habitat (see Table 1) suggests that it may not be limited to cave systems. Rather, like other *Helicodiscus* species, *H. barri* could be highly calciphilic, dwelling in rock talus piles or potentially interstitial habitats (Gladstone et al. 2018, Dr. Jeff Nekola, personal comm.). Few studies have investigated the significance of epikarst and

other subsurface habitats to troglobitic fauna in North America (Culver et al. 2012), and a more intensive sampling effort may be necessary to assess the importance of these habitats in facilitating the dispersal of such snail fauna.

This study offers an important first step in outlining the presence of cryptic lineages within *H. barri*. However, many aspects of this species' ecology and life history remain unknown, and the subsequent assessment of distinguishing ecology or habitat requirements for these cryptic groups is essential for their conservation and management. As with other recently discovered cryptic species, additional study of MOTU distribution, ecology, and conservation status are all necessary (Niemiller et al. 2013, Schlesinger et al. 2018).

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Supplementary material 1

GenBank accession numbers for all sequence data

Authors: Nicholas S. Gladstone, Matthew L. Niemiller, Evelyn B. Pieper, Katherine E. Dooley, Michael L. McKinney

Data type: list

Link: https://doi.org/10.3897/subtbiol.30.35321.suppl1

Supplementary material 2

Maximum-Likelihood (ML) CO1 Phylogram

Authors: Nicholas S. Gladstone, Matthew L. Niemiller, Evelyn B. Pieper, Katherine E. Dooley, Michael L. McKinney

Data type: multimedia

Explanation note: CO1 (704 bp) phylogram of *H. barri* generated from RAxML. Outgroup not shown due to long branch length.

Link: https://doi.org/10.3897/subtbiol.30.35321.suppl2

Supplementary material 3

Maximum-Likelihood (ML) mtDNA + nDNA Phylogram

Authors: Nicholas S. Gladstone, Matthew L. Niemiller, Evelyn B. Pieper, Katherine E. Dooley, Michael L. McKinney

Data type: multimedia

Explanation note: mtDNA + nDNA (CO1 + 16S + 28S + H3; 3040 bp) phylogram generated of *H. barri* from RAxML. Outgroup not shown due to long branch length.

Link: https://doi.org/10.3897/subtbiol.30.35321.suppl3

Supplementary material 4

ABGD Delimitation Results

Authors: Nicholas S. Gladstone, Matthew L. Niemiller, Evelyn B. Pieper, Katherine E. Dooley, Michael L. McKinney

Data type: multimedia

Explanation note: ABGD species delimitation results. A: Recursive and initial partitions under varying prior intraspecific divergences. B: Frequency histogram of K2P pairwise divergences.

Link: https://doi.org/10.3897/subtbiol.30.35321.suppl4