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Phylogeography, speciation, and cryptic diversity in the cave springtail Pseudosinella spinosa (Entomobryomorpha: Entomobryidae) from the Interior Low Plateau and Appalachian Valley & Ridge karst regions

Brendan Cramphorn

PHYLOGEOGRAPHY, SPECIATION, AND CRYPTIC DIVERSITY IN THE CAVE SPRINGTAIL *PSEUDOSINELLA SPINOSA* (ENTOMOBRYOMORPHA: ENTOMOBRYIDAE) FROM THE INTERIOR LOW PLATEAU AND APPALACHIAN VALLEY & RIDGE KARST REGIONS

Brendan Cramphorn

A THESIS

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Abstract

PHYLOGEOGRAPHY, SPECIATION, AND CRYPTIC DIVERSITY IN THE CAVE SPRINGTAIL *PSEUDOSINELLA SPINOSA* (ENTOMOBRYOMORPHA: ENTOMOBRYIDAE) FROM THE INTERIOR LOW PLATEAU AND APPALACHIAN VALLEY & RIDGE KARST REGIONS

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Caves offer a unique opportunity to study the ecology and evolution of life in extreme environments, particularly with respect to understanding patterns of diversity. Most cave-obligate species are dispersal limited and have restricted ranges, often endemic to a single or a few cave systems. However, a few species have particularly broad distributions suggesting that they either are not dispersal limited or perhaps represent a species complex of morphologically similar but genetically distinct species. In this thesis, I explore morphological variation, phylogeography, and possibly cryptic diversity in *Pseudosinella spinosa*, a cave collembolan (springtail) that inhabits the Interior Low Plateau and Appalachian Valley & Ridge karst regions of the eastern United States. I conducted morphological and molecular analyses on over 50 individuals from 22 caves. I examined molecular diversity and conducted species delimitation analyses using two mitochondrial loci (16S and COI). I found support for two primary genetically distinct clades loosely breaking up their range into a northern and a southern clade.

Various species delimitation approaches identified 3 to 28 potential unique lineages depending on the dataset (16S, COI, and concatenated 16S+COI). Moreover, morphological analysis revealed morphological variation in the species' labial triangle supporting two morphologically distinct groups. In total, evidence suggests that *P. spinosa* is a species complex; however, species boundaries are still not well understood requiring additional sampling as well as morphological and molecular investigation.

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This work would not be possible without the significant contribution from colleagues and lab members of the Niemiller laboratory. A special thank you to Matthew Niemiller, Aron Katz and Joseph Benito for guidance and assistance with species and molecular identification. Without contributions of cave training, collecting, and assisting in cave trips from cavers such as Amata Hinkle, Thany Mann, Brian Stoltz and many others this work would not have occurred. I want to specifically thank the SCCI, the owners and management of Raccoon Mountain Caverns and The Paint Rock Forest Research Center for letting us collect springtails and other organisms from their property. Lastly, I want to thank the National Cave and Karst Institute for funding this research.

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Chapter 1. Introduction

Caves offer a unique opportunity to study the ecology and evolution of life in extreme environments. Similarities among cave systems such as the lack of light and limited carbon input has led to evolution of organisms that possess similar morphological, physiological, and behavior adaptations (Juan *et al*., 2010). These similarities between caves offer the concept that caves are natural laboratories for studying diversification and biodiversity (Poulson & White, 1969). However, the same processes that drive diversification in subterranean fauna can also obscure true levels of biodiversity. For example, evolving to similar extreme environmental conditions can result in cryptic speciation *i.e*., morphologically similar organisms that also are genetically distant species (Juan *et al*., 2010; Niemiller *et al*., 2012; Katz *et al*., 2018). Examining how similar stressors, such as lack of light and low availability of food, among cave systems allow for the opportunity for an in-depth investigation into subterranean adaptation and speciation (Juan *et al*., 2010).

Different groups of invertebrates and vertebrates have evolved similar adaptations to living in habitats that lack light and generally have limited energy resources, including loss or reduction of eyes and pigmentation, enhancement of nonvisual sensory systems, and lower metabolisms (*e.g*., Poulson, 1963; Pipan & Culver, 2012; Retaux & Casane, 2013; Soares & Niemiller, 2018). These adaptations as well as other convergent adaptations associated with adapting to subterranean habitats are classified as troglomorphy (Christiansen, 1962; Pipan & Culver, 2012). Terrestrial troglomorphic

organisms are separated into two categories: troglophiles and troglobionts. Troglophiles are organisms that may or may not possess troglomorphic adaptations and can maintain populations or have populations within surface habitats (Pipan & Culver, 2012). Troglobionts are cave organisms that are adapted and restricted to terrestrial subterranean ecosystems (Sket, 2008; Pipan & Culver, 2012). Organisms without obvious troglomorphy are classified as facultative cave-dwelling organisms that use a cave for part of their life cycle but cannot establish a self-sustaining population (trogloxene) or organisms that enter subterranean habitats through accidental means (accidentals) (Sket, 2008). Aquatic organisms with troglomorphy follow the same trend with terminology where they're classified as stygobionts and stygophiles (Pipan & Culver, 2012).

Troglobionts adapted to caves and associated subterranean habitats typically have very restricted ranges, often known from just a few sites in a small geographical region and in some cases from a single cave system (Christman *et al*., 2016; Culver & Pipan, 2021). Genetic exchange among subterranean populations across broad distributions is thought to be rare (Barr & Holsinger, 1985). Some subterranean species appear to have large ranges that can span across multiple caves and or cave systems (*e.g*., Christiansen, 1960). For example, the southeastern cave pseudoscorpion (*Hesperochernes mirabilis*) has a distribution across much of the southern United States (Lewis, 2002; Stephens, 2022; Niemiller *et al*., 2023). Most cave pseudoscorpions, such as *Kleptochthonius* spp. and *Tyrannochthonius* spp., have restrictive ranges and high levels of endemism, making the range of the southeastern cave pseudoscorpion exceptional and unexpected for the order of chelicerates (Stephens, 2022). Another example is the subterranean sheetweb spider (*Phanetta subterranea*), which has a much larger distribution compared to *H. mirabilis* across karst regions ranging from Missouri to Indiana to Alabama (Holsinger, 1963;

Peck & Christiansen, 1990; Lewis & Lewis, 2008; Niemiller *et al*., 2023). One hypothesis to explain the distributions of these species is that they are not dispersal limited with minimal or limited barriers inhibiting gene flow (Trontelj, 2019). These species may use small crevices, flowing streams, or epikarst soil for initial spread and genetic exchange between established populations (Barr, 1967).

An alternative hypothesis for the large distributions of some troglobiotic species is that they, as currently recognized, are dispersal limited and the wide distribution reflects morphologically similar and genetically distinct species (cryptic species) (Juan *et al*., 2010; Niemiller *et al*., 2012; Zhang & Li, 2014; Katz *et al*., 2018). In recent years, uncovering cryptic diversity is a common finding of phylogeographic studies of subterranean fauna. For example, the cavefish *Typhlichthys subterraneus* had a documented range of over 140,000 km^2 in the Interior Low Plateau and Ozarks karst regions of the United States, the most expansive range for a subterranean fish species (Proudlove, 2010; Niemiller *et al*., 2012). However, phylogenetic and species delimitation analyses uncovered that this nominal species is a cryptic species complex consisting of up to 15 genetic lineages (Niemiller *et al*., 2012). Another example is with the cave spider *Telema cucurbitina*, a species whose range consists of over fifteen different caves within the karst region of southern China, after an extensive phylogenetic study, it was determined that the species was a species complex consisting of sixteen different cryptic species (Zhang & Li, 2014).

Several abiotic and biotic factors may influence cryptic speciation in troglobitic arthropods leading to considerable variation in levels of cryptic diversity among taxonomic groups (Zhang *et al*., 2014; Delić *et al*., 2017). One such factor is the amount

of light or lack thereof within the cave environment. Depending on what part of the cave that invertebrate inhabits, cave animals experience different levels of visible light depending on location within a cave system (*i.e.*, entrance vs twilight vs deep cave zones). Other factors such as seasonal variations of abiotic and biotic factors such as in facultative cave dwellers and moisture or water levels can also affect the degree to which similar organisms adapt to a cave system (Novak *et al.*, 2012; Mammola & Isaia, 2018; Balestra *et al.*, 2021). Due to potential greater connectivity in the saturated zone of subterranean habitats, aquatic invertebrates might experience fewer geographical barriers and consequently have larger ranges with less potential for cryptic speciation compared to their terrestrial counterparts (Lamoreux, 2004). For example, in European caves it has been found through phylogenetic studies that just 10% of stygobiont species have extensive ranges, where 90% of species are large species complexes of cryptic species or single site endemics (Trontelj *et al.*, 2009). Some species might be restricted by geographic and environmental barriers, such as temperature and moisture differences in surrounding areas, karst connectivity, and lack of large terrestrial corridors, can restrict small flightless troglobiotic species to individual cave systems (Snowman *et al.*, 2010; Niemiller & Zigler, 2013; Simoes *et al.*, 2015; Barlogh *et al.*, 2020; Souza-Silva *et al.*, 2021). Consequently, species with putative large geographical ranges often are found to be species complexes consisting of multiple morphologically cryptic species with significantly smaller, restricted distributions (Zhang & Li, 2014; Katz, 2018).

Cryptic diversity can result from multiple subterranean invasions of closely related surface species adapting to the same abiotic and biotic factors within cave systems (*i.e.*, Christensen, 1967; Niemiller *et al.*, 2008). This is a form of convergent evolution where different organisms develop similar morphological adaptations towards similar ecological pressures (Juan

et al., 2010). Alternatively, cryptic diversity may arise from multiple invasions of different cave systems from a single surface species that independently adapted to similar cave ecosystems (Niemiller *et al*., 2008; Trontelj, 2018). However, in some cases multiple invasions from the same or similar species can lead to cryptic speciation (Trontelj, 2019), or a single surface species initially colonizes a cave and then uses corridors to disperse out to other cave systems and adapt to the same environments developing cryptic species over time (Strecker *et al*., 2012). This could be considered a form of parallel evolution, where similar species adapt the same or similar morphology (Cerca, 2023). However, it has been noted that troglomorphic organisms contain both parallel and convergent characteristics (Christiansen, 1961).

Arthropods in the subclass Collembola (*i.e*., springtails) offer a fantastic opportunity to examine the factors driving speciation within subterranean ecosystems (Christiansen, 1960; Katz *et al*., 2018; Raschmanova *et al*., 2016). Springtails are a morphologically diverse group of hexapods that are joined by the shared appendage (furcula; Lubbock, 1873; Hopkins, 1997; Guzic *et al*., 2020), internal mouthparts (Hopkins, 1997), and lack of wings (Folsom, 1901; Hopkins, 1997). They are some of the most abundant animals in surface (Patapov, 2022; Cicconardi *et al*., 2013) as well as subterranean ecosystems (Marx & Weber, 2015). Subterranean springtails have been viewed as potential model organisms for studying the evolution of cave adaptation and speciation (Christiansen, 1961; Christiansen & Culver, 1969). Due to their small size, lack of wings, and physiological requirements, most troglobiotic springtails are thought to be dispersal-limited and to have restricted distributions (Niemiller & Zigler, 2013; Katz *et al*., 2018). *Pseudosinella* Schaeffer, 1897 is a polyphyletic genus (Wang *et al*.,

2004) of springtails in the family Entomobryidae that is characterized by an elongated fourth abdominal plate (Soto-Adames, 2010). The genus consists of more than 20 described species within North America and nearly 400 worldwide, with some diversity restricted to caves and associated subterranean environments (*i.e.*, Christiansen, 1960; Soto-Adames, 2010). Due to the varying degree of troglomorphic adaptations between individuals within the same species, *Pseudosinella* has been an interesting study system for research regarding evolutionary processes within subterranean ecosystems (*e.g.*, Christiansen *et al.*, 1967; Guzik *et al.*, 2021; Kováč *et al.*, 2023). In this study, I integrated morphological and molecular genetic approaches to determine levels of species and genetic diversity with *Pseudosinella spinosa* (Delmare Deboutteville, 1949) (Figure 1.1), a highly troglomorphic species with a clumped distribution across the Interior Low Plateau and Appalachians karst regions of the eastern United States and investigated factors that shape genetic structure and the species' distribution. *Pseudosinella spinosa* is the largest and most troglomorphic springtail within North America (Christiansen, 1960; Christiansen & Bellinger, 1998). They are a unique species with multiple special spine-like setae on their dens which separates them from all *Pseudosinella* in North America (Christensen & Bellinger, 1998). The unique setae on the dens is thought to be a cave dependent adaptation appearing in isolated clusters throughout the TAG (Tennessee, Alabama, and Georgia) region (Christiansen, 1960). Other studies have used this species in concurrence with *Pseudosinella hirsuta* (Delamare Deboutteville, 1949) to describe ecological and evolutionary concepts, such as convergence, macroevolution, and behavior within cave springtails (Christiansen, 1960; Christiansen & Culver, 1967; Christiansen, 1988). I hypothesize species diversity is underestimated in *Pseudosinella spinosa*. Multiple intrinsic and extrinsic factors, such as range size, habitat specialization, pockets of isolated karst and other geographic barriers, influence diversification

and shape species' distributions, with the expectation that delimited species will have smaller, restricted distributions like many other troglobiotic invertebrates.

Figure 1.1*: Pseudosinella spinosa* from Sneed Spring Cave, Madison County Alabama.

Chapter 2. Methods

2.1 Study Site and Specimen Collection

This study leveraged a large existing collection of *Pseudosinella* springtails collected from cave systems throughout the Interior Low Plateau (ILP) and Appalachian Valley (APV) from prior biological inventory studies by the Cave Bio Lab at UAH and colleagues collected from 2014 to 2021 with the intent to be evaluated. Additional cave bio-surveys were conducted from 2021 to 2023 from caves primarily in central Tennessee and northern Alabama. The specimens included in this study comprise individuals from across the entirety of the known *P. spinosa* range. The range is clumped with three distinct population clusters within the Appalachian Plateaus of northeastern Alabama and central Tennessee (Christiansen & Culver, 1967). In total, 368 *Pseudosinella* sp. from 128 caves across 56 counties in Alabama, Kentucky, Tennessee, and Virginia were evaluated (Figure 2.1).

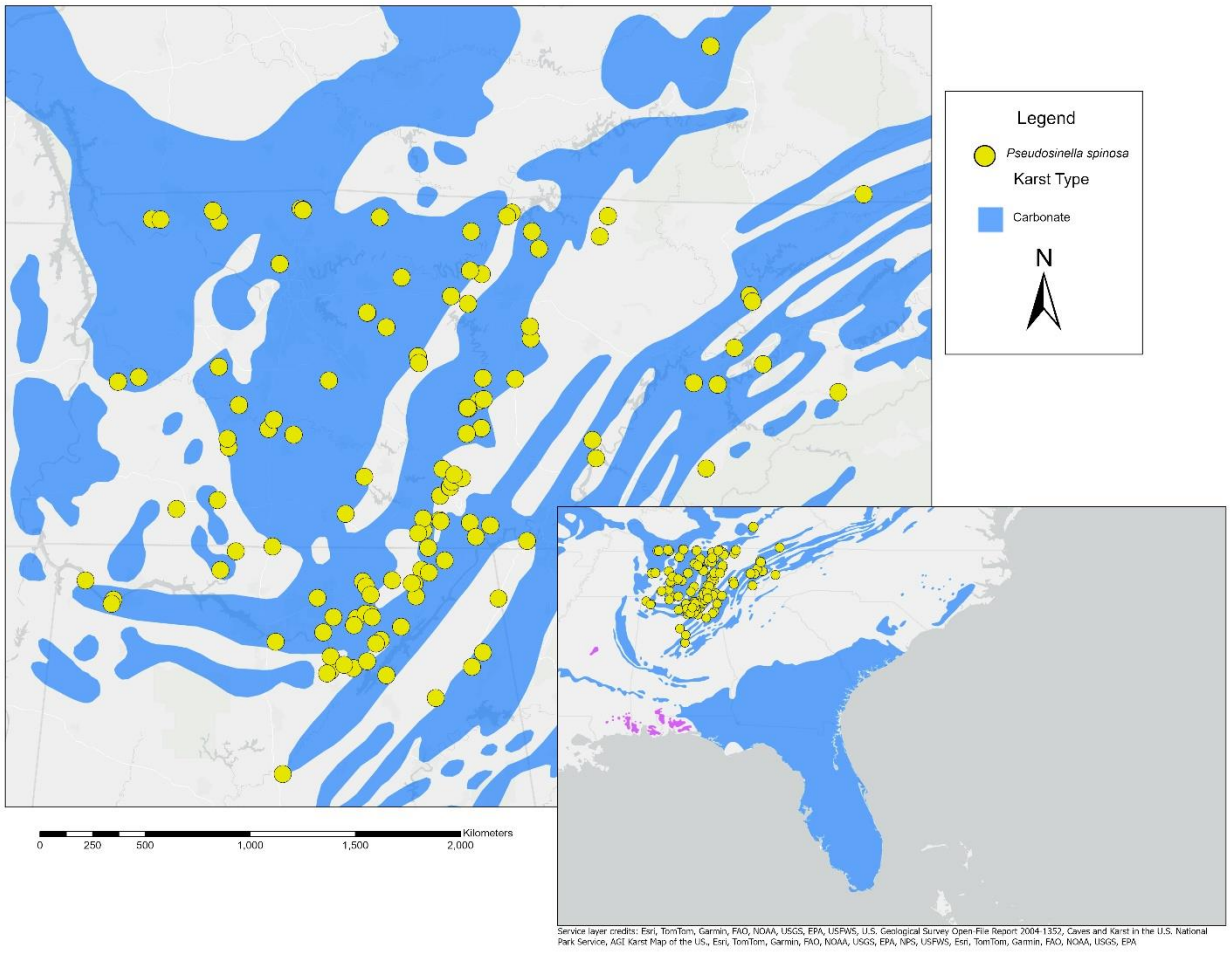

Figure 2.1: Map showing the locations and study sites of *Pseudosinella* initially evaluated within the study. Yellow dots indicate a cave where a cave *Pseudosinella* was collected, and blue is the carbonate karst the cave(s) was located. With a scale representing distance on larger map and source credits on smaller map.

Specimens were collected indiscriminately from the entrance to the dark zone of the cave. Except for one site, specimens were collected using multiple active collection methods. Most specimens were collected using hard and soft tipped watercolor paintbrushes. I utilized a method of touching the springtail's abdomen to influence it to jump into a vial of 100% ethanol (EtOH). To avoid destruction of setae on the furcula and "collar" of specimens, aspirators also were used. Specimens were collected with other organisms from the same locality in a vial of 100% EtOH.

Pseudosinella sp. were later sorted into vials based on the length of antenna and color of their scaleless abdomen and thorax with a Celsion dissecting scope.

2.2 Morphological Analysis

To obtain specimen macro-morphological data, a representative of each morphotype per site was photographed using a Macropod Pro Imaging System (Macroscopic Solutions, LLC), which consisted of a Canon EOS 6D camera and a Cognisys Stackshot 3x controller. Prior to photography, specimens were left to soak in a 50:50 DI water to EtOH solution for two minutes to avoid potential moving during the photographic process. Soaked specimens were placed with a drop of DI water and positioned to emphasize the legs, head, and furcula morphology on a pre-cleaned glass slide. Once settled on slide, a set of 50 to 100 photographs were taken and then stacked with the program Zerene Stacker Pro (Version 1.04). Final editing was conducted using Adobe Photoshop (Version 2024:25.6, Adobe Inc.).

To obtain micro-identification features, the photographed specimens along with voucher specimens were slide mounted after DNA extraction. To avoid potentially losing all morphological data, the head was separated from the body prior to DNA extraction (see next section). The head was placed in a small one-use petri-dish with either DELICLEAR® Clearing Agent (76% Lactic Acid, 18% DI Water, 3% Chlorobutanol, 2.5% Sodium Hydroxide, 0.05% Polyethylene glycol) or BioQuip™ Clearing solution (Lactophenol sol W/glacial acetic acid) for one to three days. After the clearing period, specimens were mounted on a slide using a Synthetic Hoyer's mounting medium (60% chloral, 20% water, 13% glycerol, 7% gum arabic) by lightly spreading the mounting medium onto the center of the glass slide and carefully placing the specimen on the slide. A square coverslip of #1 thickness was placed directly on top of the

specimen to reduce the potential for air bubbles obstructing morphological features. Specimens were also slide mounted using smaller circular 12 mm #1 thickness coverslips, where head and body were slide mounted on the same slide. Prepared slides were left on a CNAXH-2004 slide warmer set at 47.1 °C for a minimum of 48 hours and then microscope-slide ringing sealant (89% collodion, 8% petroleum-based stabilizers, 3% ethyl centralite) or nitrocellulose was used to seal the coverslip before placing the slide back on the slide warmer for an additional 24 to 48 hours.

Morphological analyses relied on existing keys in "The Collembola of North America" (Christensen & Bellinger, 1998). I used an Amscope T670Q-PL-NL04 with an infinity-corrected phase-contrast kit attachment, 10X ocular lenses, and a Digital Camera 18MP APTIMA COLOR CMOS (MU1803) attachment. Specimens were identified and drawn under a PH achromatic 40X or 100X objective lens. To draw key morphological features of each specimen, I projected the specimen onto a piece of drawing paper using the AmScope microscope camera software (Version: x64.4.11.19757.20211031, AmScope™) and a table-top projector. Drawings were then sharpened and digitalized using GIMP (Version: 2.10.36). To separate *Pseudsinella* from other Entobryomorpha, I used the following characters: four segmented non-scaled antennal segments, 4th abdominal segment to be at least 2 ½ the size of the 3rd segment, mucro with subequal teeth with a basal spine that extends to the top of the tooth and lack of eyes. The presence of spine-like setae on the dens and the labial triangle configuration of (M_1)M_1M_2r(R)EL_1L_2 (Chen & Christiansen, 1993) was used to separate *P. spinosa* from other *Pseudosinella*. In addition, *P. spinosa* has an elongated unguis with three small

inner teeth and lacks an external tooth, a mucro with an anteapical tooth that is displaced dorsally, a lack of coloration, and an extended 4th antennal segment.

2.3 DNA Extraction, PCR, and Sequencing

I used a modified non-destructive DNA extraction approach where the specimen's whole body except for the head was used and slide-mounted after extraction (*e.g.*, Aoyama *et al.*, 2015; Katz *et al.*, 2018). I extracted DNA from at least one specimen from each morphospecies at a site, for a maximum of twelve extracted individuals per trip. Individual specimens were isolated and moved to a UV treated glass Petri dish with 100% ethanol. They were decapitated with the head placed in clearing solution to be slide-mounted and the body was placed in an empty microcentrifuge tube and left to dry following Katz *et al.* (2018). Using a modified Qiagen Dneasy Blood & Tissue kit protocol, 180 µL ATL buffer with 20 µL proteinase-k was added to the microcentrifuge tube and left in a thermal cycler at 56 °C overnight (Katz *et al.*, 2018). The specimen was left in the initial microcentrifuge tube while transferring the lysis buffer containing DNA from the microcentrifuge tube to a spin column. The solution was carefully transferred to reduce the potential for accidental removal of specimen to the spin column. Ethanol (100%) was added to the microcentrifuge tubes containing specimens to preserve their cleared exoskeletons. The other modification to the protocol was the reduction of AE buffer during the elution step to 50 µl.

I amplified the mitochondrial cytochrome c oxidase 1 (COI) and 16S ribosomal RNA mitochondrial genes. These loci have been employed previously in evaluating population-level phylogenetics within Collembola (Hogg & Herbert, 2004; Katz *et al.*, 2018). A 685-bp fragment of the COI locus was amplified using primer sets jgLCOI490/igHCO2198 and

LCOI490/HCO219 primers (Folmer *et al.*, 1994; Geller *et al.*, 2013; Katz, 2018). A 496–528 bp fragment of the 16S locus was amplified using primers sets 16Sar/16Sbr, 16Sacoll/16Sbcoll, LR-J-12887M/LR-N-13398M (France & Kocher, 1996; Zang *et al.*, 2013). I used the Promega GoTaq PCR system (Promega catalogue # M3001) in 25-ul volume reactions and using 2µl of DNA template. PCR products were visualized using gel electrophoresis and then purified using ExoSAP-IT (Thermofisher). Sanger sequencing in both directions was conducted using BigDye chemistry at Eurofins Inc (Louisville, Kentucky). The resulting sequences were quality trimmed and then aligned using De Novo Assemble in Geneious Prime (Dotmatics, 2024.0.1) and reviewed using MEGA 11 (Mega Software,11.0.13).

2.4 Phylogenetic Inference

To infer individual relatedness and phylogenetic structure I performed a phylogenetic analysis using both a maximum likelihood and Bayesian approach. I looked at 50 *Pseudosinella spinosa* individuals with 2 *Pseudosinella hirsuta* as outgroups. I determined the optimal models of nucleotide substitution for each mitochondrial locus, including first, second, and third codon positions for COI and as its own character set for 16S using PartitionFinder v2.1.1 (Lanfear *et al.*, 2017) implementing the 'greedy' search algorithm (Lanfear *et al.*, 2012) to select for the best partitioning strategy for the data under the General Time Reversible + Gamma (GTRGAMMA) site rate substitution model using the AICc metric (Burnham & Anderson 2002). I then conducted 20 maximum-likelihood (ML) searches in RaxML-HPC2 Workflow on XSEDE (Towns *et al.*, 2014). I also performed non-parametric bootstrap replicates under GTRGAMMA

using the autoMRE option to optimize the number of bootstrap replicates for this large dataset. I reconciled the bootstrap replicates with the best fitting ML tree. To confirm the reliability of the tree topology, the dataset was also analyzed using MrBayes (Version: 3.2.7a) for Bayesian phylogenetic reconstruction. I used the same partitioning strategy described above and estimated the most appropriate site rate substitution model for MrBayes using PartitionFinder. I conducted two independent runs of one cold chain and three heated chains (default settings) for 5,000,000 Markov chain Monte Carlo (MCMC) generations sampling every 100 generations in MrBayes. After dropping the first 25% 'burn-in' trees to ensure stationarity and examining the log-likelihood values for each Bayesian run using Tracer v1.7 (Rambaut *et al*., 2018), the remaining 37,500 sampled trees were used to estimate the consensus tree and the associated Bayesian posterior probabilities. A midpoint rooted ML and Bayesian trees with support values were generated using FigTree v1.4.4 (Rambaut, 2010). In addition to analyzing each locus separately, I also conducted the above-mentioned analyses on the concatenated COI+16S dataset. For outgroups, I used two *Pseudosinella hirsuta* from the type locality Raccoon Mountain Caverns in Hamilton County, Tennessee and Stewart Spring Cave in DeKalb County, Alabama.

2.5 Species Delimitation and Molecular Diversity

To look at potential speciation between populations and individuals, I performed species delimitation analysis looking at the 50 *Pseudosinella spinosa* individuals. I implemented two single-locus species delimitation approaches on the mitochondrial COI, 16S, and concatenated COI+16S datasets to define molecular operational taxonomic units (MOTUs): Automatic Barcode Gap Discovery (ABGD; Puillandre *et al*., 2012) and Multi-rate Poisson Tree Processes

(mPTP; Kapli *et al*., 2017). ABGD partitions sequences into candidate species based on a statistically inferred barcode gap defined as a significant disparity between pair-wise genetic distances, presumably between intraspecific and interspecific distances. This process is applied recursively to newly obtained groupings of sequences to assess the potential of internal division. This method was employed excluding outgroup taxa using the ABGD web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) with the Kimura two-parameter (Kimura, 1980) model and a standard X (relative gap width) = 1.5. The initial development of the multispecies coalescent PTP model assumed one exponential distribution for speciation events and one for all coalescent events (Zhang *et al*., 2013). The mPTP approach fits speciation events for candidate species to a unique exponential distribution (Kapli *et al*., 2017) rather than assuming one exponential distribution for speciation events and one for all coalescent events in PTP models (Zhang *et al*., 2013). The mPTP method was employed using rooted ML trees for each dataset for 10 million generations, with a burn-in discarding the first 20% in mPTP (Kapli *et al*., 2017).

I estimated molecular diversity statistics for delimited lineages in each morphospecies and overall, including number of haplotypes, number of segregating sites, haplotype diversity, and nucleotide diversity. I also tested for departures from neutrality or constant population size using Tajima's D (Tajima, 1989) and R_2 (Ramos-Onsins and Rozas, 2002) with the COI and 16S datasets in DNaSP v6 (Rozas *et al*., 2017) and in the package pegas v1.0.1 (Paradis, 2010) in R v4.1.0 (R Core Team, 2021). Significant negative values of Tajima's D and small positive values of R_2 indicate population growth.

Chapter 3. Results

3.1 Morphological Analyses

I sorted through 1,116 cave-dwelling springtail specimens across the southern Interior Low Plateau and Appalachians karst regions, of which 410 were *Pseudosinella*. I made 557 singular glass slides from 386 genetically extracted *Pseudosinella* specimens from 134 caves located in 49 counties distributed across Alabama, Tennessee, Kentucky, and Virginia (Figure 2.1, Table A.1). Of the 386 specimens, 137 were genetically or morphologically identified as *Pseudosinella spinosa* from 61 caves in 24 counties across Alabama and Tennessee (Figure 3.1A). Morphologically identified *Pseudosinella spinosa* were not included in the study if they did not have both a successful 16S and COI sequence run. The final working dataset included 50 specimens from 22 caves, across six counties in Alabama and Tennessee (Figure 3.1B, Table A.1).

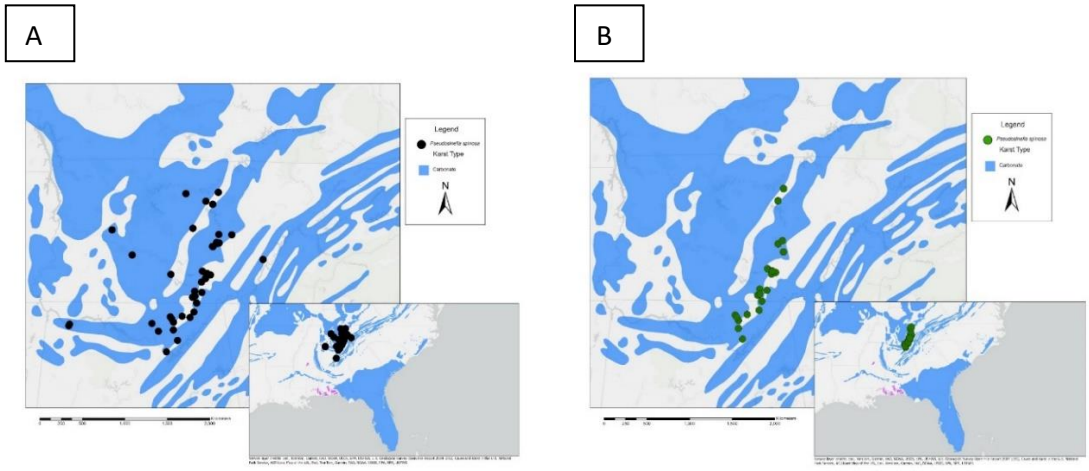

Figure 3.1: A. Map showing the localities of the specimens identified as *Pseudosinella spinosa*. B. Map showing the locations of *Pseudosinella spinosa* individuals that successfully sequenced (both COI and 16S) and therefore, included in the final working dataset. I COI16S. With a scale representing distance on larger map and source credits on smaller map.

All but a single individual had their furcula. The furcular of this individual was missing due to collection or slide mounting damage. Forty-nine of the 50 (98%) of specimens with a furcula had at least one spine-like seta on the dorsal 2/3rds area of the dens. The amount and configuration of setae on the dens varied from five similar sized spine-like setae clumped together (Figure 3.2) to varied types of setae (Figure 3.3) to multiple variable-sized spine-like setae forming multiple rows on the dens (Figure 3.4). All individuals with a mucro had an anteapical tooth displaced dorsally. The length of the mucro varied among individuals from normal (5 of 50; 10%), to extended (43 of 50; 88%), to one individual (2%) without a mucro (Table A.1). All specimens had a hindfoot complex with both an unguis and a lanceolate unguiculus that had a basal inner swelling that was weakly developed. Forty-eight of 50 (96%) had 3 small inner teeth and a lack of external teeth on the unguis (Table A.1). The unguis of the specimen from Fern Cave in Jackson County, Alabama, had 3 internal and 1 external tooth (Table A.1). A single specimen from Bluff River Cave in Jackson County, Alabama, had no external and internal teeth on the unguis (Table A.1; Figure 3.5).

Labial triangles of all specimens had normal smooth chaetae with a minute r chaeta. Six individuals (12%) did not have labial triangles with chaetae due to destruction in slide mounting or collecting procedures or lacked a head slide mounted. Eleven individuals (22%) of specimens lacked the labial chaetae configuration of $(M_{1s})M_1M_2r(R)EL_1L_2$ on the left or right labial triangle. Four specimens with the $(M_{1s})M_1M_2r(R)EL_1L_2$ configuration had a socket in place of a missing M, r, or E chaetae. One specimen from Sneed Spring Cave in Madison County, Alabama, had an extra

chaeta above the M_2 with a configuration of $(M_{1s})M_1M_2r(R)EL_1L_2$. Six specimens had a chaetae configuration of $(M_{1s})M_1M_2r(R)EL_1$, lacking a L_2 chaetae. The configuration of $(M_{1s})M_1M_2r(R)EL_1$ appeared in four caves: three individuals from Trench Cave in Jackson County, Alabama, (Figure 3.2), two individuals from Lomond Stoompway Cave and a single individual from Wise Buzzard Cave in Warren County, Tennessee. A single specimen from Bluff River Cave in Jackson County, Alabama, had the $(M_{1s})M_1?r(R)EL_1$ chaetae configuration with a socket replacing the M_2 chaetae (Figure 3.5).

Forty-four (88%) of the specimens did not have coloration. Of the six specimens with color, three had yellow patches and were from Bluff River Cave in Jackson County, Alabama. A single specimen each from three caves had red speckling and a red eyepatch: Fern Cave in Jackson County, Alabama, Custard Hollow Cave in Franklin County, Tennessee, and Red Trillium Cave in Grundy County, Tennessee. The median size of specimens was 2.46 mm with the smallest specimen being 1.0 mm and largest at 3.9 mm. Forty individuals had an extended 4th antennal segment greater than the cephalic diagonal.

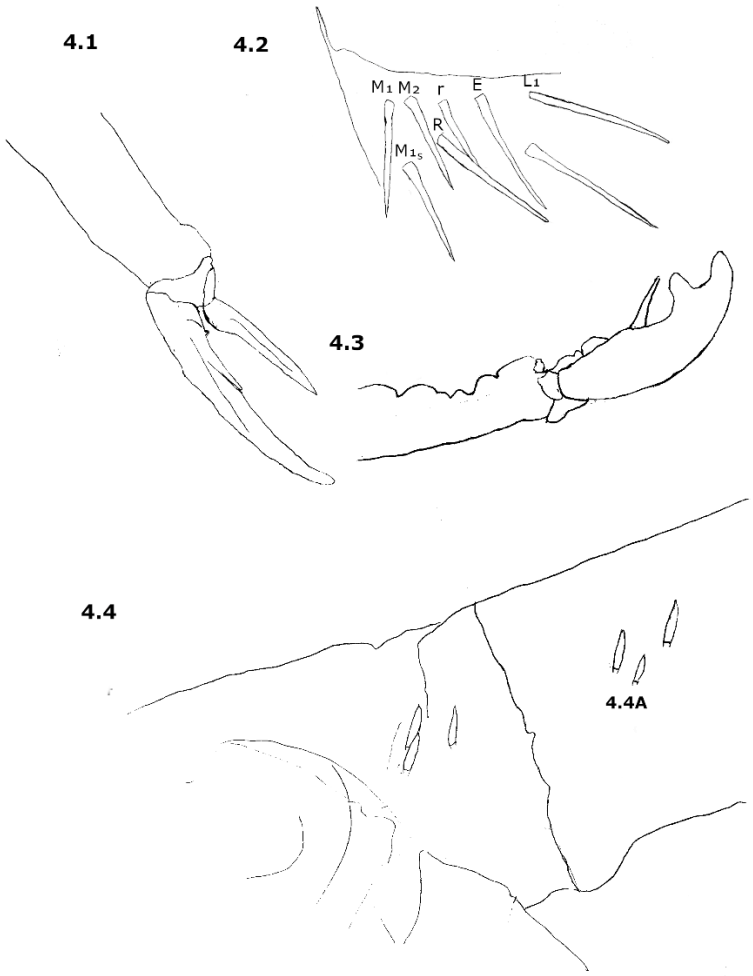

Figure 3.2: *Pseudosinella spinosa* (specimen no. 485) from Trench Cave in Jackson County, Alabama. 4.1 showing claw morphology with an unguis with 3 internal and no external teeth. The unguiculus has a basal weakly developed inner swelling, 4.2 labial with a (M_{1s})M_1M_2r(R)EL_1 chaetae configuration, 4.3 extended mucral with anteapical tooth, 4.4 furcula dens with 4.4A spine-like setae.

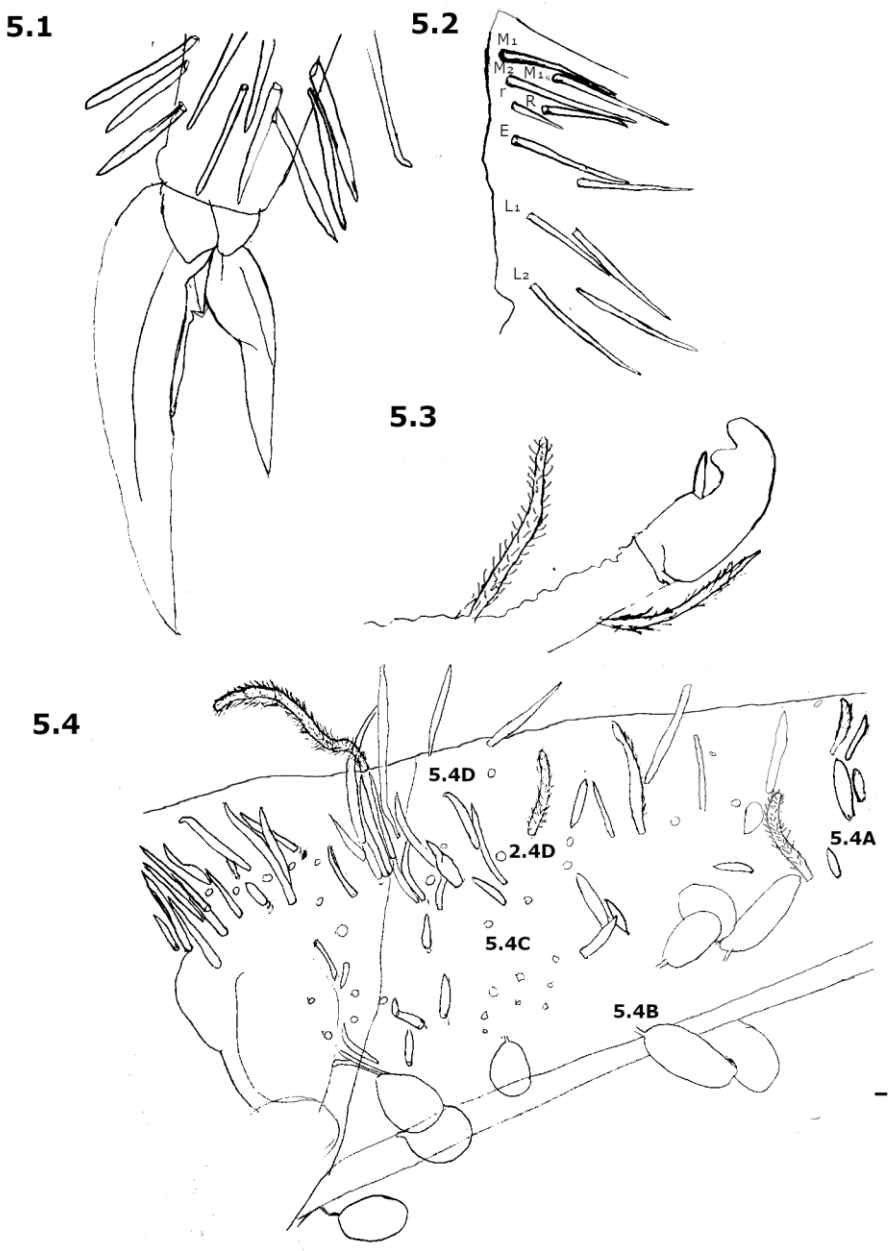

Figure 3.3: *Pseudosinella spinosa* (specimen no. 69) from Salt River Cave in Jackson County, Alabama ., 5.1 showing claw morphology with an unguis with 3 internal and no external teeth. The unguiculus has a basal weakly developed inner swelling, 6.2 labial with a $(M_{1s})M_1M_2r(R)EL_1L_2$ chaetae configuration, 6.3 extended mucral with anteapical tooth, 6.4 furcula dens with 6.4A typical setae. 6.4B scale, 5.C spine-like setae.

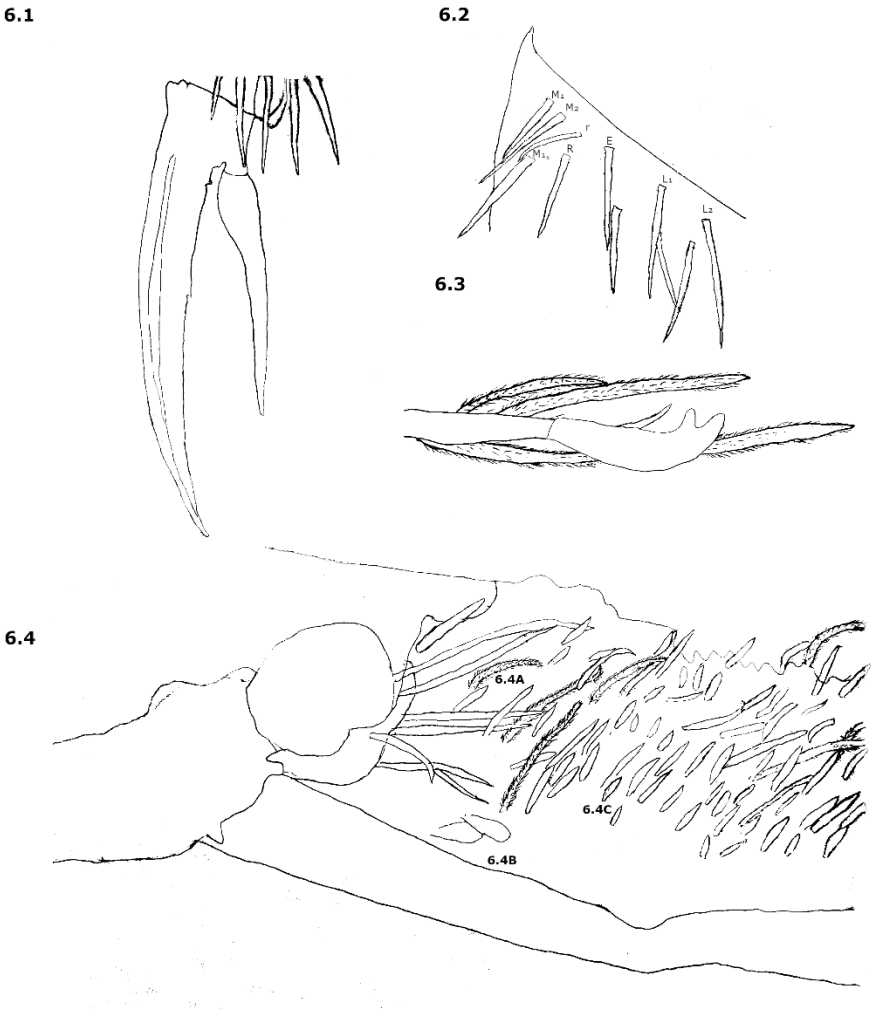

Figure 3.4: *Pseudosinella spinosa* (specimen no. 805) from Sneed Spring Cave in Madison County, Alabama. 6.1 showing claw morphology with an unguis with 3 internal and no external teeth. The unguiculus has a basal weakly developed inner swelling, 6.2 labial with a $(M_{1s})M_1M_2r(R)EL_1L_2$ chaetae configuration, 6.3 extended mucral with anteapical tooth, 6.4 furcula dens with 6.4A typical setae. 6.4B scale, 5.C spine-like setae.

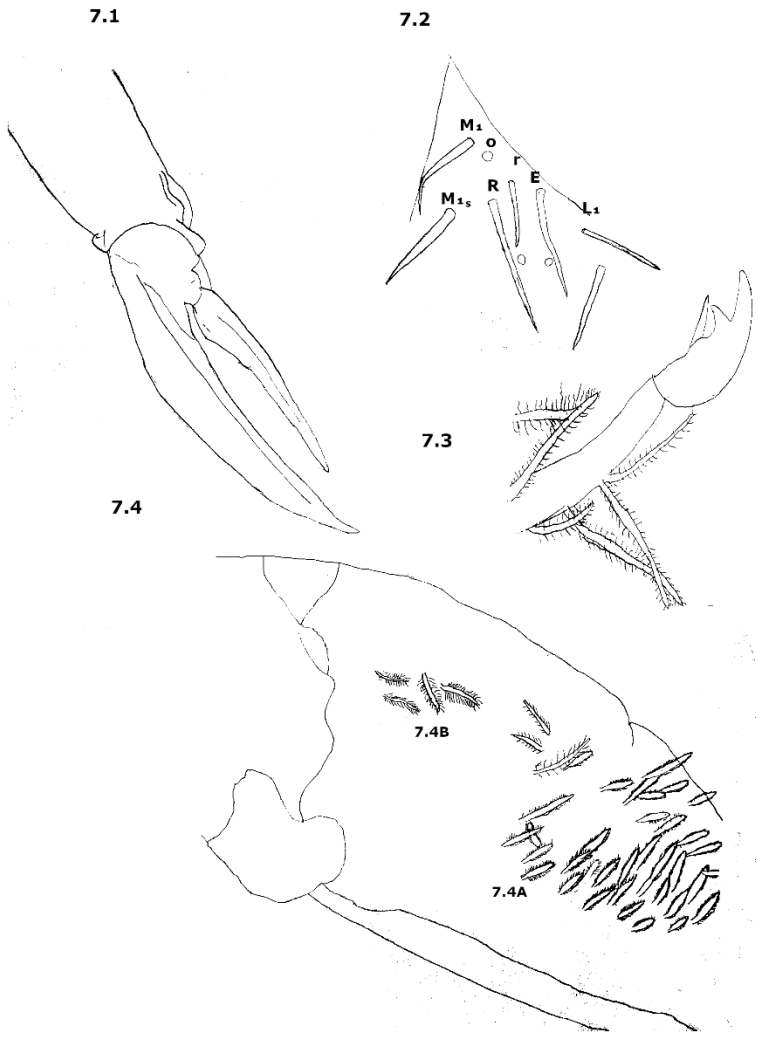

Figure 3.5: *Pseudosinella spinosa* (specimen no. 319) from Bluff River Cave in Jackson County Alabama. 7.1 showing claw morphology with an unguis with no internal and no external teeth. The unguiculus has a basal weakly developed inner swelling, 7.2 labial with a (M_{1s})M_1or(R)EL_1 chaetae configuration, 7.3 slightly extended mucral with anteapical tooth, 7.4 furcula dens with 7.4A Spine-like setae, 7.4B typical setae.

3.2 Phylogenic Analyses

We generated novel DNA sequences from 50 *P. spinosa* or the mitochondrial 16S and COI loci. The best model for nucleotide substitution for each data partition was as follows: first codon position COI – GTR+G, second codon position COI – F81+1, third codon position COI – GTR+I+G, and 16S – GTR+I+G. Maximum likelihood and Bayesian phylogenetic analyses for

all datasets (*i.e.*, COI, 16S, and concatenated COI+16S) revealed two main clades with high support (Figures 3.6–3.11). Clade 1 consisted of 30 individuals from 8 caves along the Cumberland Plateau in Jackson and Madison counties in Alabama, and Franklin County in Tennessee (Figure 3.12). Clade 2 consisted of 20 individuals from 11 caves along the Cumberland Plateau and Eastern Highland Rim in Grundy, Warren, Coffee, Putnam, and Jackson counties in Tennessee, and Jackson County, Alabama. A contact zone between the two clades occurs within Jackson County, Alabama, and Franklin County, Tennessee. A single individual from Trench Cave (specimen no. 495) in Jackson County, Alabama, did not group with other individuals from the same cave in all phylogenetic analyses (Figure 3.12).

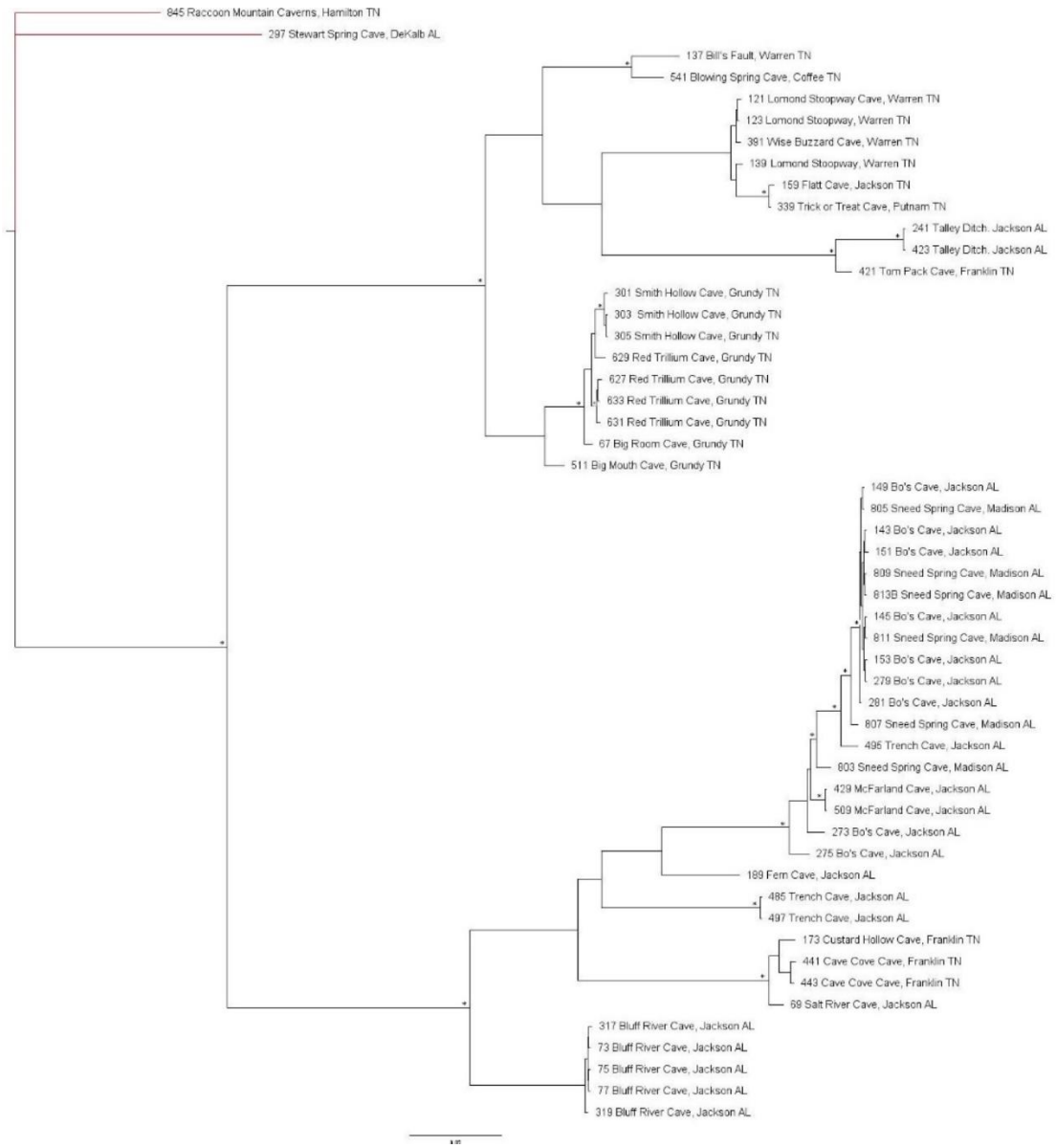

Figure 3.6: Bayesian phylogram for the mitochondrial COI locus for *P. spinosa*. Nodes with posterior probabilities >0.95 are indicated with an asterisk. *Pseudosinella hirsuta* from two different caves were used as outgroups. Tip labels refer to the specimen code number, cave, county, and state.

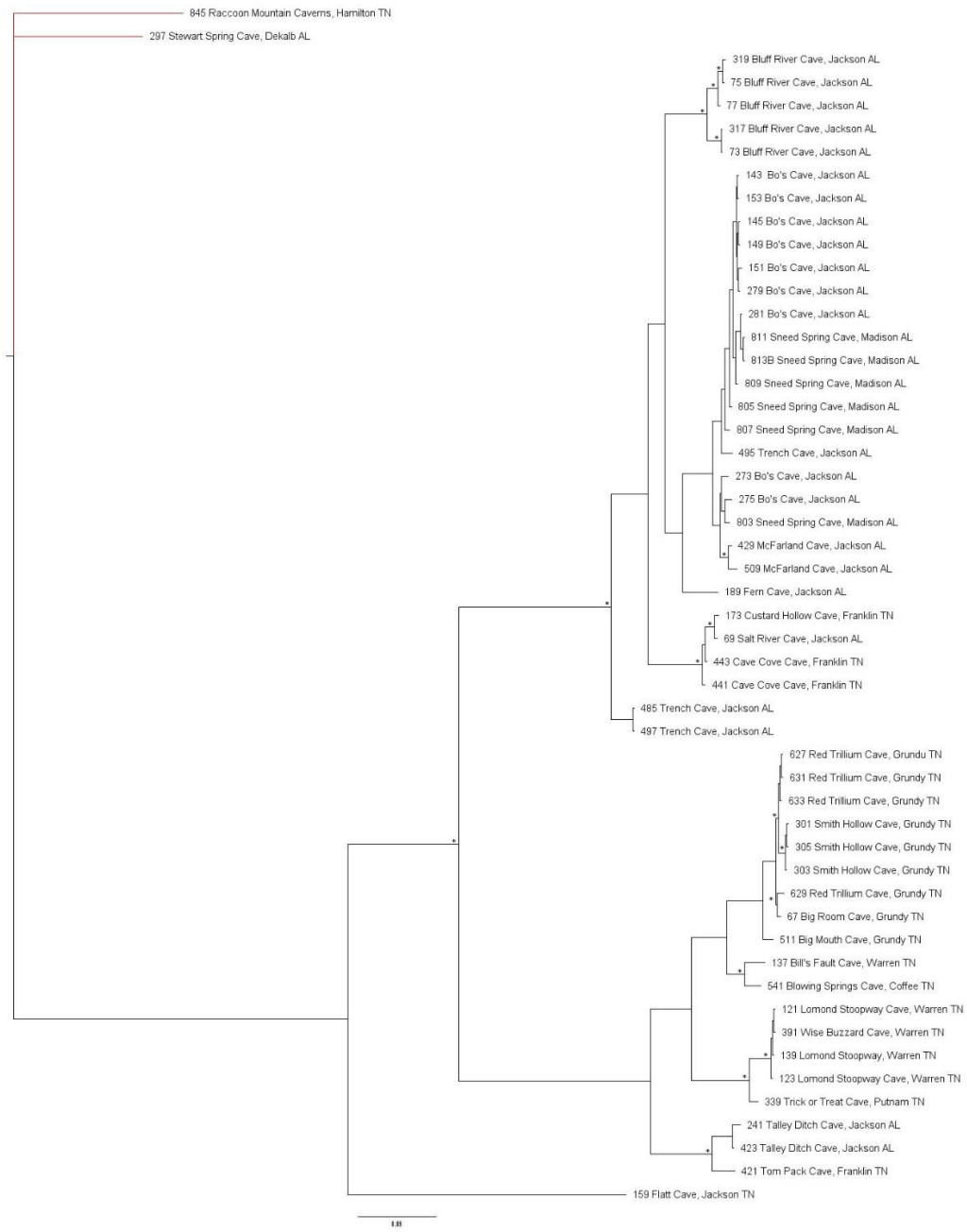

Figure 3.7: Bayesian phylogram for the mitochondrial 16S locus for *P. spinosa*. Nodes with posterior probabilities >0.95 are indicated with an asterisk. *Pseudosinella hirsuta* from two different caves were used as outgroups. Tip labels refer to the specimen code number, cave, county, and state.

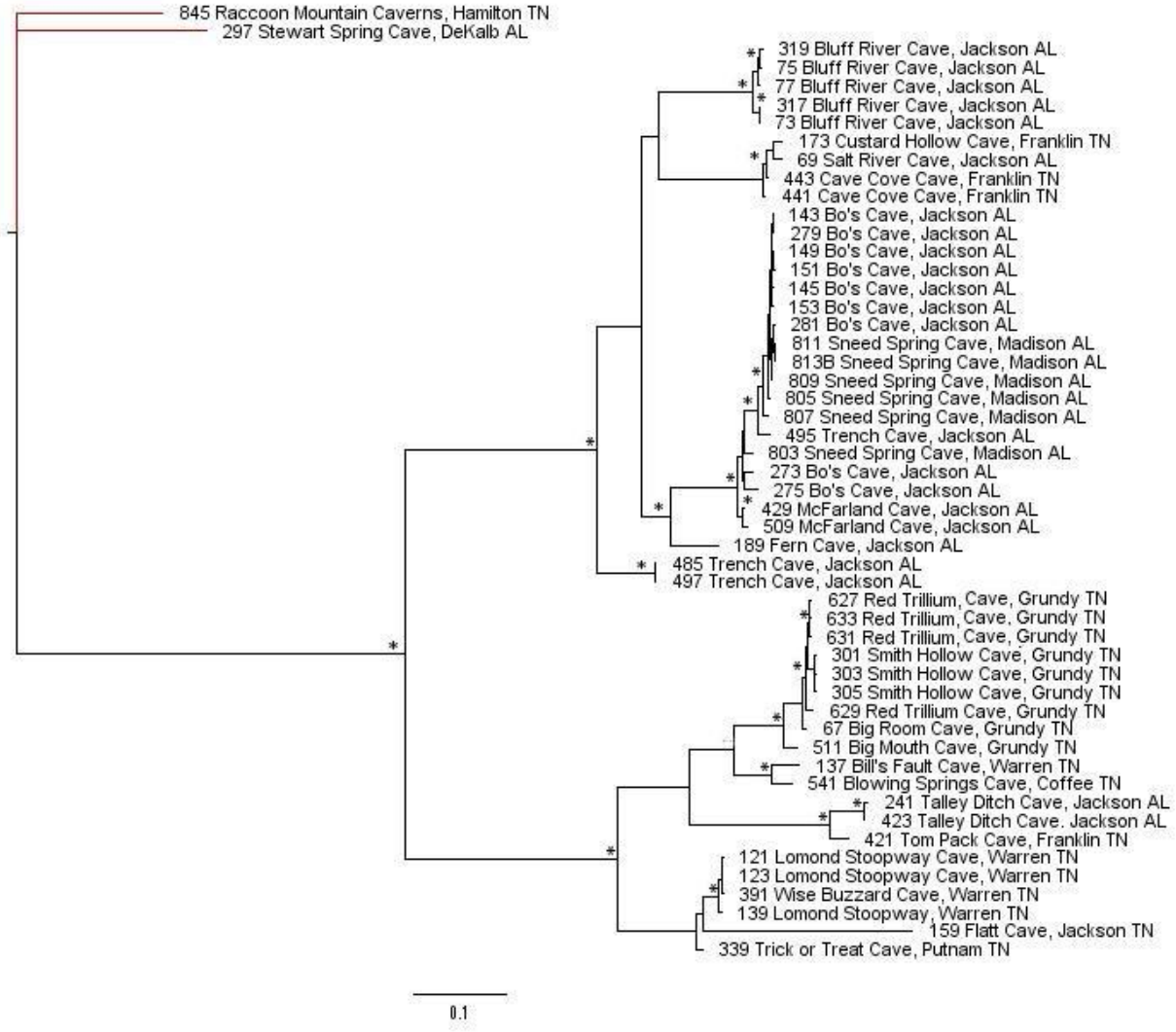

Figure 3.8: Bayesian phylogram for the concatenated COI+16S dataset for *P. spinosa*. Nodes with posterior probabilities >0.95 are indicated with an asterisk. *Pseudosinella hirsuta* from two different caves were used as outgroups. Tip labels refer to the specimen code number, cave, county, and state.

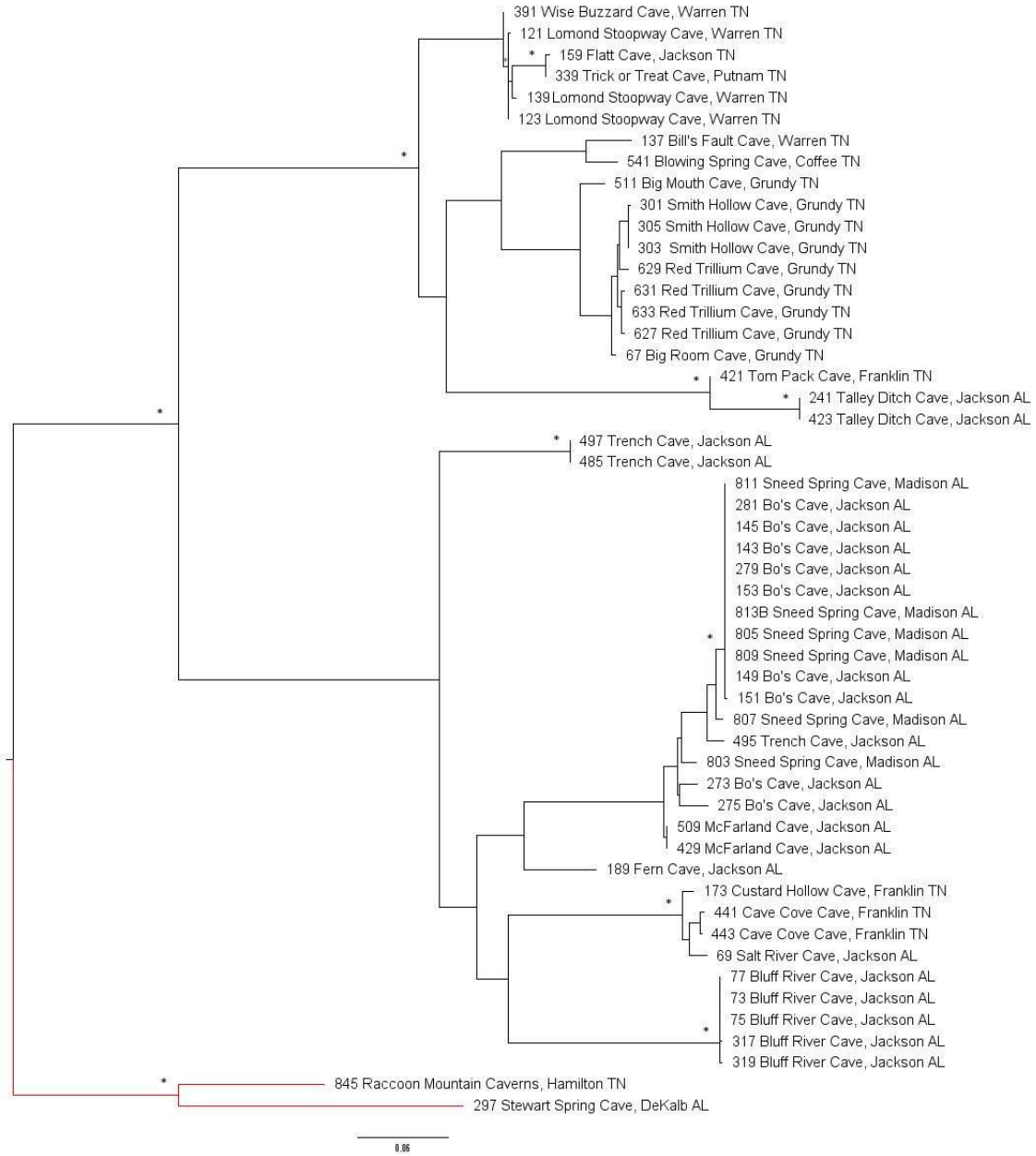

Figure 3.9: Maximum-likelihood phylogram for the mitochondrial COI locus for *P. spinosa*. Nodes with >0.95 are indicated with an asterisk. *Pseudosinella hirsuta* from two different caves were used as outgroups. Tip labels refer to the specimen code number, cave, county, and state.

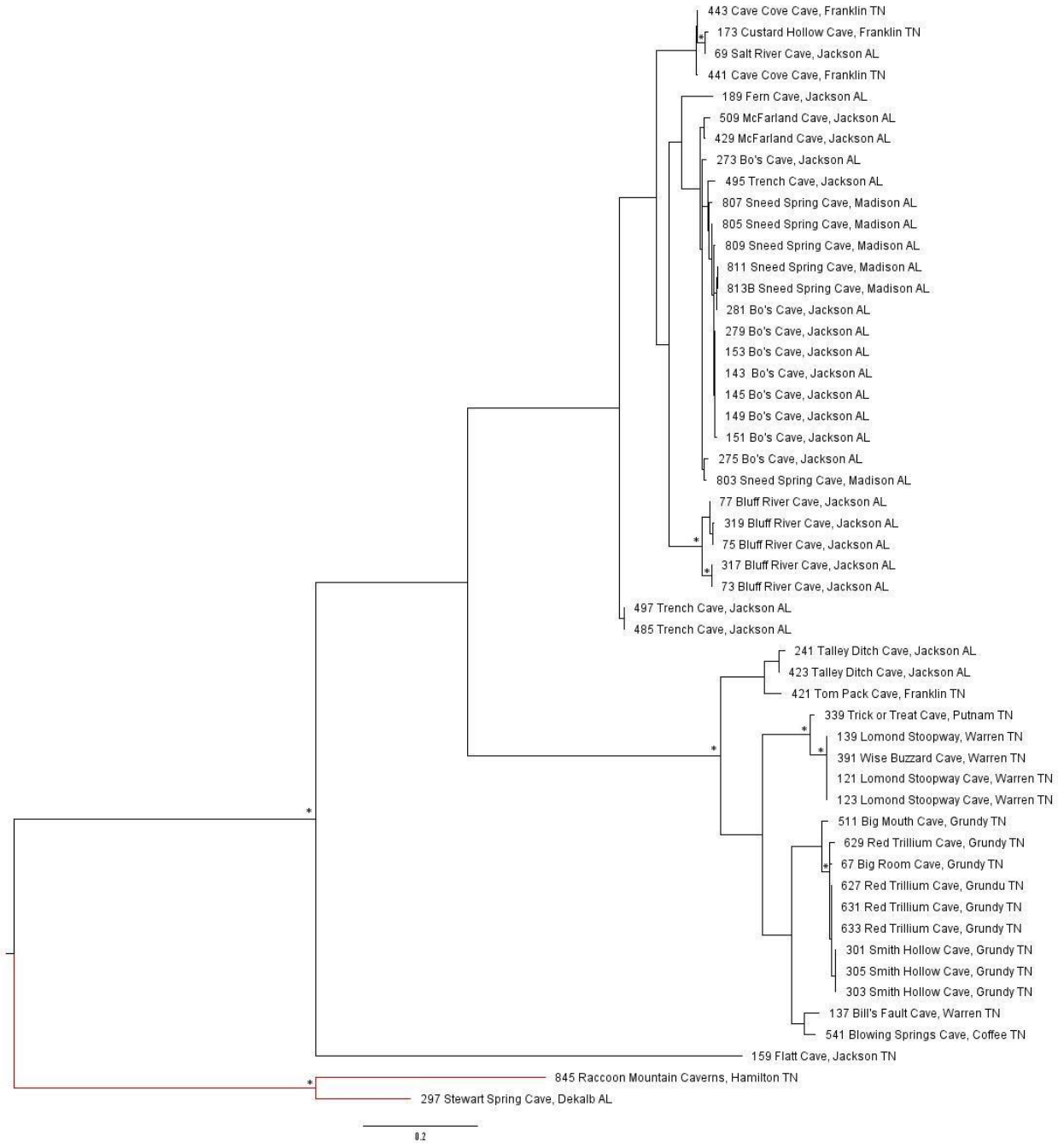

Figure 3.10: Maximum-likelihood phylogram for the mitochondrial 16S locus for *P. spinosa*. Nodes with >0.95 are indicated with an asterisk. *Pseudosinella hirsuta* from two different caves were used as outgroups. Tip labels refer to the specimen code number, cave, county, and state.

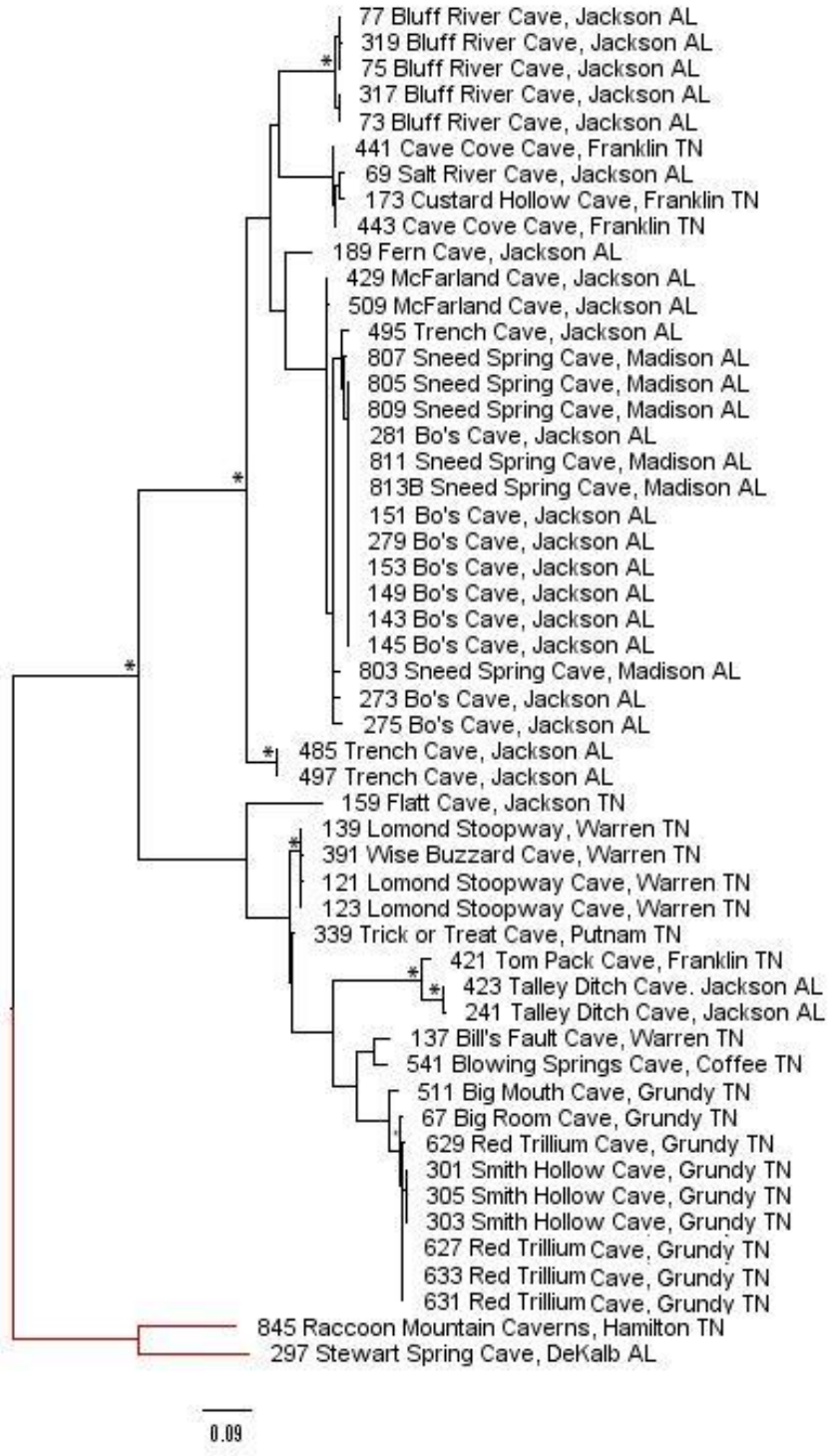

Figure 3.11: Maximum-likelihood phylogram for the concatenated COI+16S dataset for *P. spinosa*. Nodes with >0.95 are indicated with an asterisk. *Pseudosinella hirsuta* from two different caves were used as outgroups. Tip labels refer to the specimen code number, cave, county, and state.

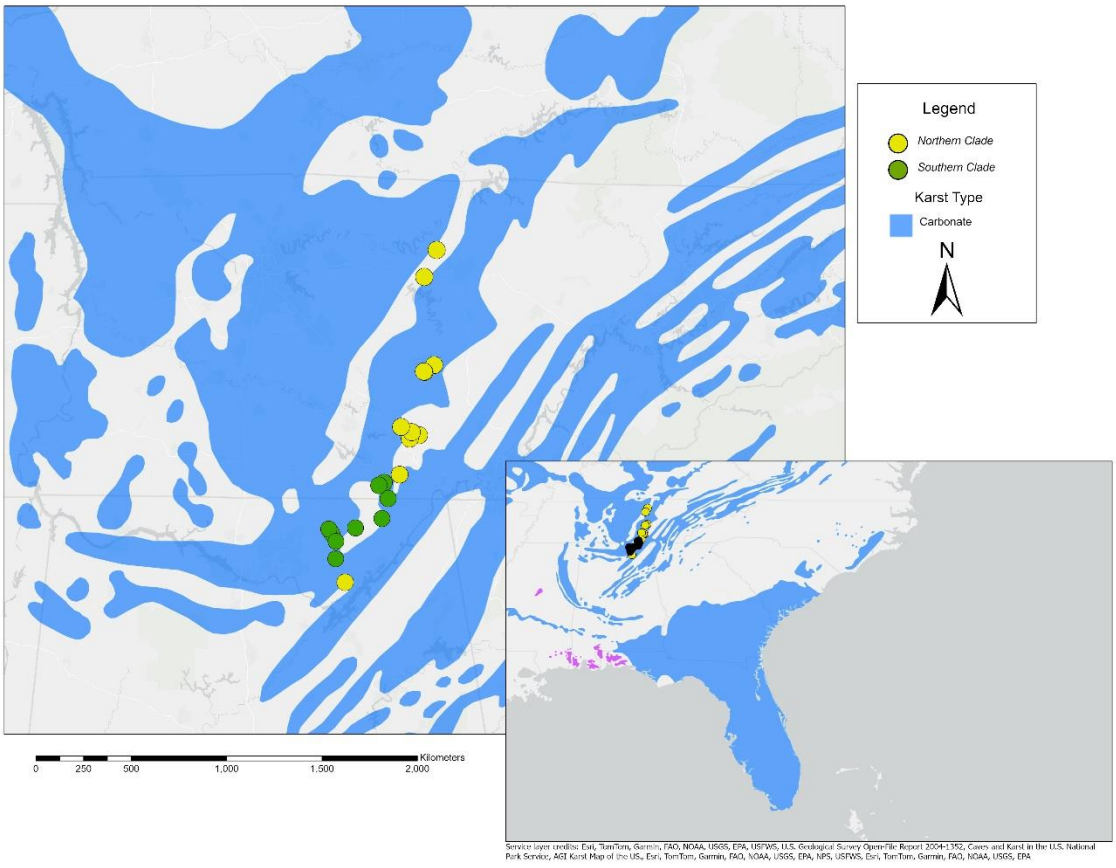

Figure 3.12: Map highlighting caves associated with the northern clade (Yellow) and southern clade (Green) of *Pseudosinella spinosa*. With carbonate karst highlighted blue. With a scale representing distance on larger map and source credits on smaller map.

3.3 Species Delimitation Analyses

For the COI dataset, the ABGD analysis yielded 21 MOTUs with initial and recursive partitions before the intraspecific divergence of (P)=0.001. Initial partitions stabilized at (P)=0.001; the recursive partitions did not stabilize. bPTP analysis identified 22 MOTUs, 8 unique from the ABGD analysis. This approach isolated specimen 139 from Lomond Stoopway in Warren County, Tennessee, specimen 173 from Custard Hollow Cave in Franklin County, Tennessee, and specimen 69 from Salt River Cave in Jackson County, Alabama, into distinct

MOTUs (Figure 3.13). Coupled specimens 443 and 441 from Cave Cove Cave in Franklin County, Tennessee, specimens 241 and 243 from Talley Ditch Cave in Jackson, Alabama, specimen 137 from Bill's Fault Cave in Warren County, Tennessee, and specimen 541 from Blowing Springs Cave in Coffee County, Tennessee, specimens 121 and 123 from Lomond Stoopway Cave in Warren County, Tennessee, and specimen 391 from Wise Buzzard Cave in Warren County, Tennessee, into distinct MOTUs (Figure 3.13). The ASAP analysis identified 9 MOTUs, 4 of which were unique from the ABGD and bPTP analyses. This approach isolated specimens from Bo's Cave in Jackson County, Alabama, Sneed Spring Cave in Madison County, Alabama, and McFarland Cave in Jackson County, Alabama, into a distinct MOTU (Figure 3.13). Isolated specimens 137 from Bill's Fault Cave in Warren County, Tennessee, and 541 from Blowing Springs Cave in Coffee County, Tennessee, specimens 421 from Tom Pack Cave in Franklin County, Tennessee, and 241 and 423 from Talley Ditch Cave in Jackson County into unique MOTUs (Figure 3.13). The mPTP analysis identified 11 MOTUs with no unique MOTUs (Figure 3.13).

For the 16S dataset, the ABGD analysis yielded 28 MOTUs with an initial and recursive partition before intraspecific divergence of (P)=0.001. Initial partitions and the recursive did not stabilize. The ASAP analysis identified 5 MOTUs, all 5 are unique. This approach isolated specimen 159 from Flatt Cave in Jackson County, Tennessee, specimens 485, 495, and 497 from Trench Cave in Jackson County, Alabama, specimen 189 from Fern Cave in Jackson County, Alabama, specimens 429 and 509 from McFarland Cave in Jackson County, Alabama, specimens from Bo's Cave in Jackson County, Alabama, specimens from Sneed Spring Cave in Madison County, Alabama,

specimens from Bluff River Cave in Jackson County, Alabama, specimen 69 from Salt River Cave in Jackson County, Alabama, specimens 441 and 443 from Cave Cove Cave in Franklin County, Tennessee, specimen 173 from Custard Hollow Cave in Franklin County, Tennessee, into a single unique MOTUs (Figure 3.14). Isolated specimens 241 and 423 from Talley Ditch Cave in Jackson County, Alabama, and 421 from Tom Pack Cave in Franklin County, Tennessee, specimens 121,123 and 139 from Lomonds Stoopway in Warren County, Tennessee, 339 from Trick or Treat Cave in Putnam County, Tennessee, and 391 from Wise Buzzard Cave in Warren, Tennessee, Specimens 627,629,631 and 633 from Red Trillium Cave in Grundy County, Alabama, 67 from Big Room Cave, Grundy County, Tennessee, 301,303, and 305 from Smith Hollow Cave in Grundy County, Tennessee, 137 from Bill's Fault Cave in Warren County, Tennessee, 511 from Big Mouth Cave in Grundy, Tennessee into distinct MOTUs (Figure 3.14). The mPTP analysis identified 15 MOTUs, 5 are unique. This approach identified specimen 137 from Bill's Fault Cave in Warren County, Tennessee, specimen 541 from Blowing Springs Cave in Coffee County, Tennessee, specimens 241 and 423 from Talley Ditch Cave in Jackson County, Alabama, specimens 627, 629, 631, and 633 from Red Trillium Cave in Grundy County, Tennessee, 301, 303 and 305 from Smith Hollow Cave from Grundy County, Tennessee, 67 from Big Room Cave in Grundy County, Tennessee, specimens from Sneed Cave in Madison County, specimens from Bo's Cave in Jackson County, Alabama, and 429 and 509 from McFarland Cave in Jackson County, Alabama, distinct MOTUs (Figure 3.14).The bPTP analysis yielded 15 MOTUs, none of which are unique (Figure 3.14).

For the concatenated COI+16S dataset, the ABGD analysis yielded 22 MOTUs with initial and recursive partitions before intraspecific divergence of (P)=0.001. Initial partitions stabilized at (P) =0.001; the recursive partitions did not stabilize. The ASAP analysis yielded 11

MOTUs, with 3 unique MOTUs. This approach isolated specimens 301,303, and 305 from Smith Hollow Cave in Grundy County, Tennessee, specimens 627,629,631, and 633 from Red Trillium Cave in Grundy County, Tennessee, specimen 67 from Big Room Cave in Grundy County, Tennessee, and specimen 511 from Big Mouth Cave in Grundy County, Tennessee into a distinct MOTU (Figure 3.15). This approach also isolated specimens from Bluff River Cave in Jackson County, Alabama, from Sneed Spring Cave in Madison County, Alabama, from Bo's Cave in Jackson County, Alabama, specimens 429 and 509 from McFarland Cave in Jackson County, Alabama, and specimen 495 from Trench Cave in Jackson County, Alabama. The bPTP analysis identified 19 MOTUs, all 3 are unique. Specimen 421 from Tom Pack Cave in Franklin County, Tennessee, specimens 241 and 423 from Talley Ditch Cave in Jackson County, Alabama, specimens 67 from Big Room Cave in Grundy County, Tennessee, 301, 303, 305 from Smith Hollow Cave in Grundy County, Tennessee, 627, 629, 631, and 633 from Red Trillium Cave in Grundy County, Tennessee into independent MOTUs (Figure 3.15). The mPTP analysis identified 14 MOTUs with 1 unique. Specimens 429 and 509 from McFarland Cave in Jackson, Alabama, specimens from Bo's Cave in Jackson County, Alabama, specimens from Sneed Spring Cave in Madison County, Alabama, and 495 from Trench Cave in Jackson County, Alabama, were included into a single MOTU (Figure 3.15).

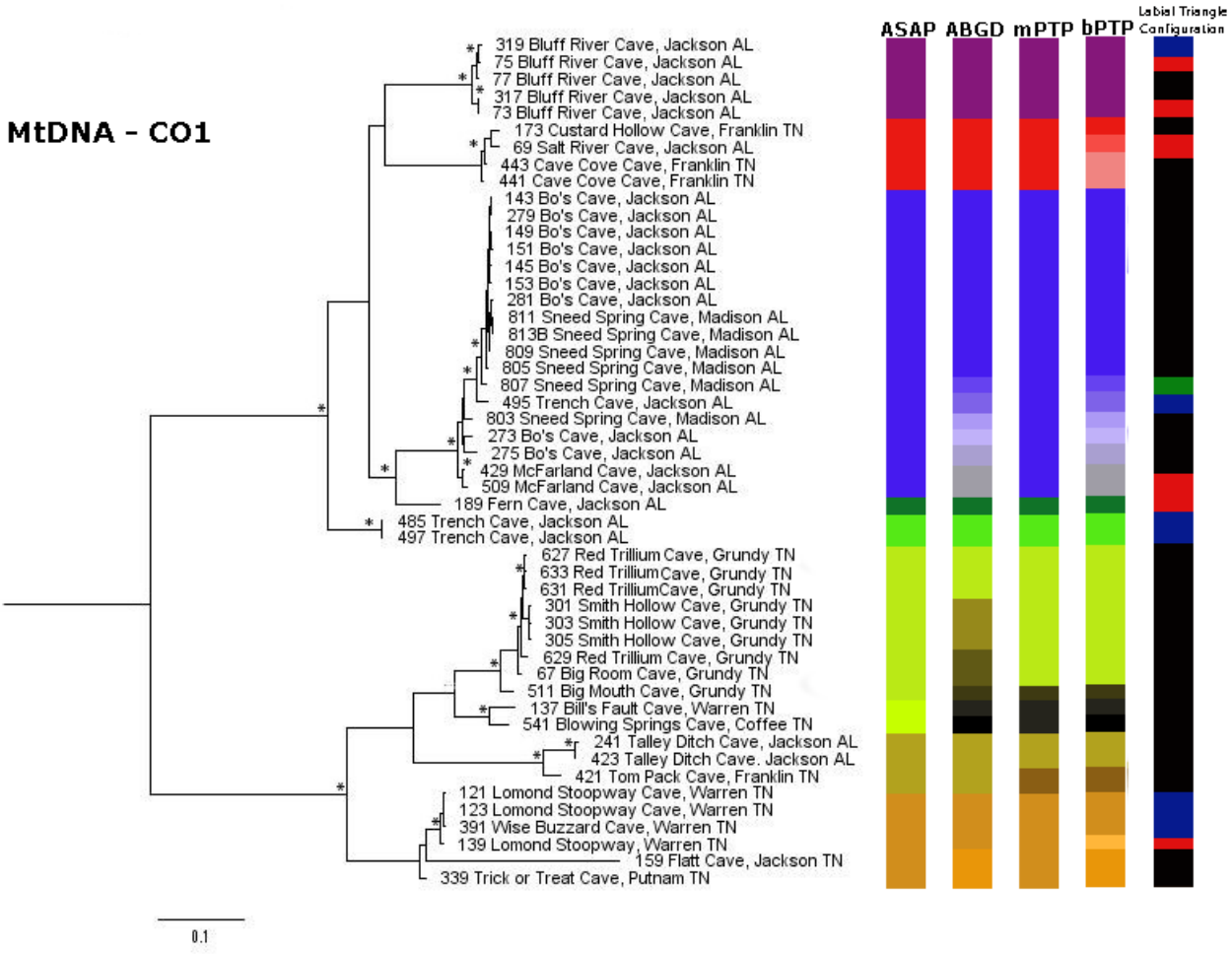

Figure 3.13: Bayesian phylogram for the mitochondrial COI dataset with results of species delimitation analysis (ASAP, ABGD, mPTP, and bPTP), with labial triangle configuration (no labial in red, loss of L2 chaetae in blue and additional chaetae in green). Nodes with posterior probabilities >0.95 are indicated with an asterisk. *Pseudosinella hirsuta* from two different caves were used as outgroups. Tip labels refer to the specimen code number, cave, county, and state.

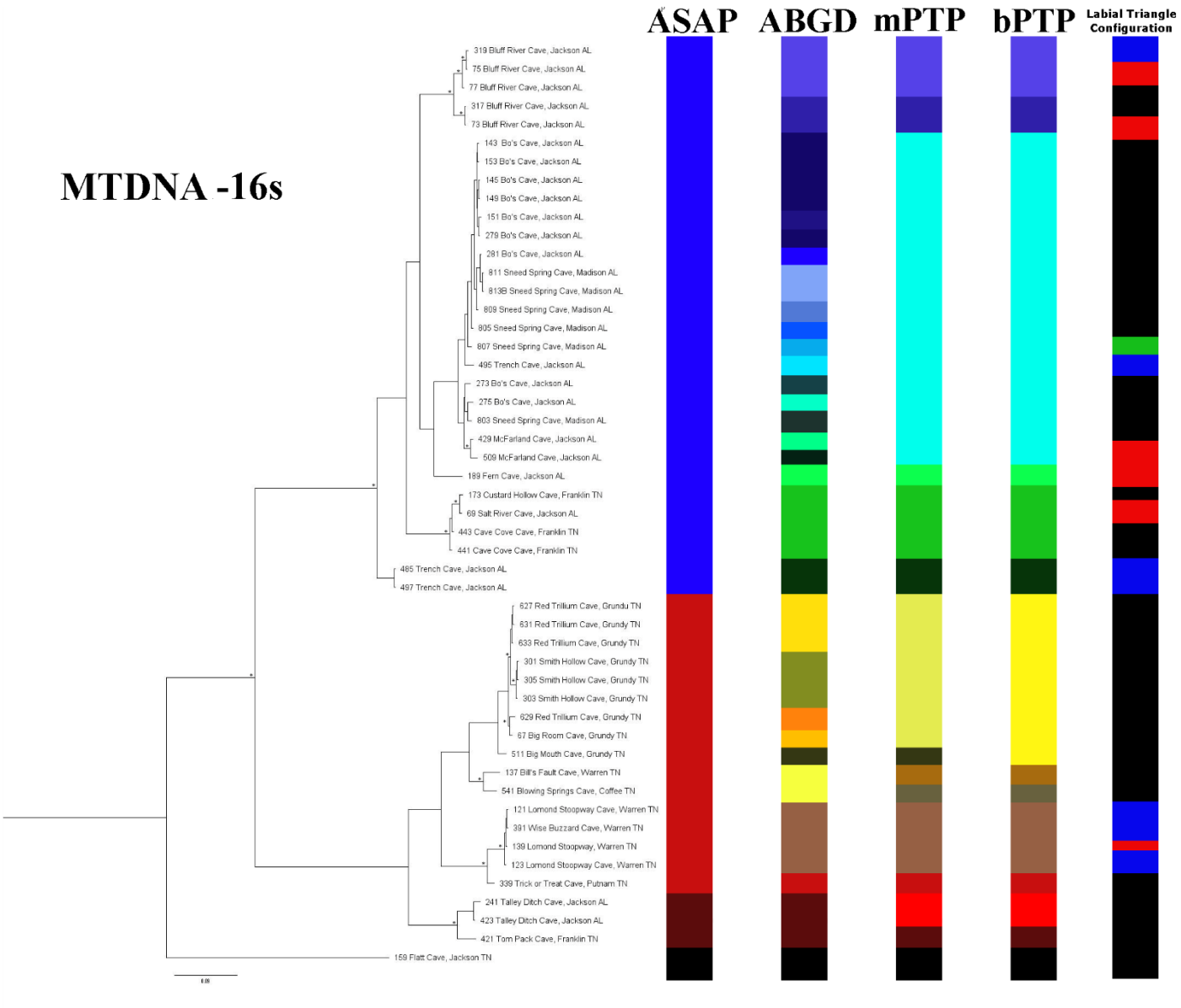

Figure 3.14: Bayesian phylogram for the mitochondrial 16S dataset with results of species delimitation analysis (ASAP, ABGD, mPTP, and bPTP), with labial triangle configuration (no labial in red, loss of L2 chaetae in blue and additional chaetae in green). Nodes with posterior probabilities >0.95 are indicated with an asterisk. *Pseudosinella hirsuta* from two different caves were used as outgroups. Tip labels refer to the specimen code number, cave, county, and state.

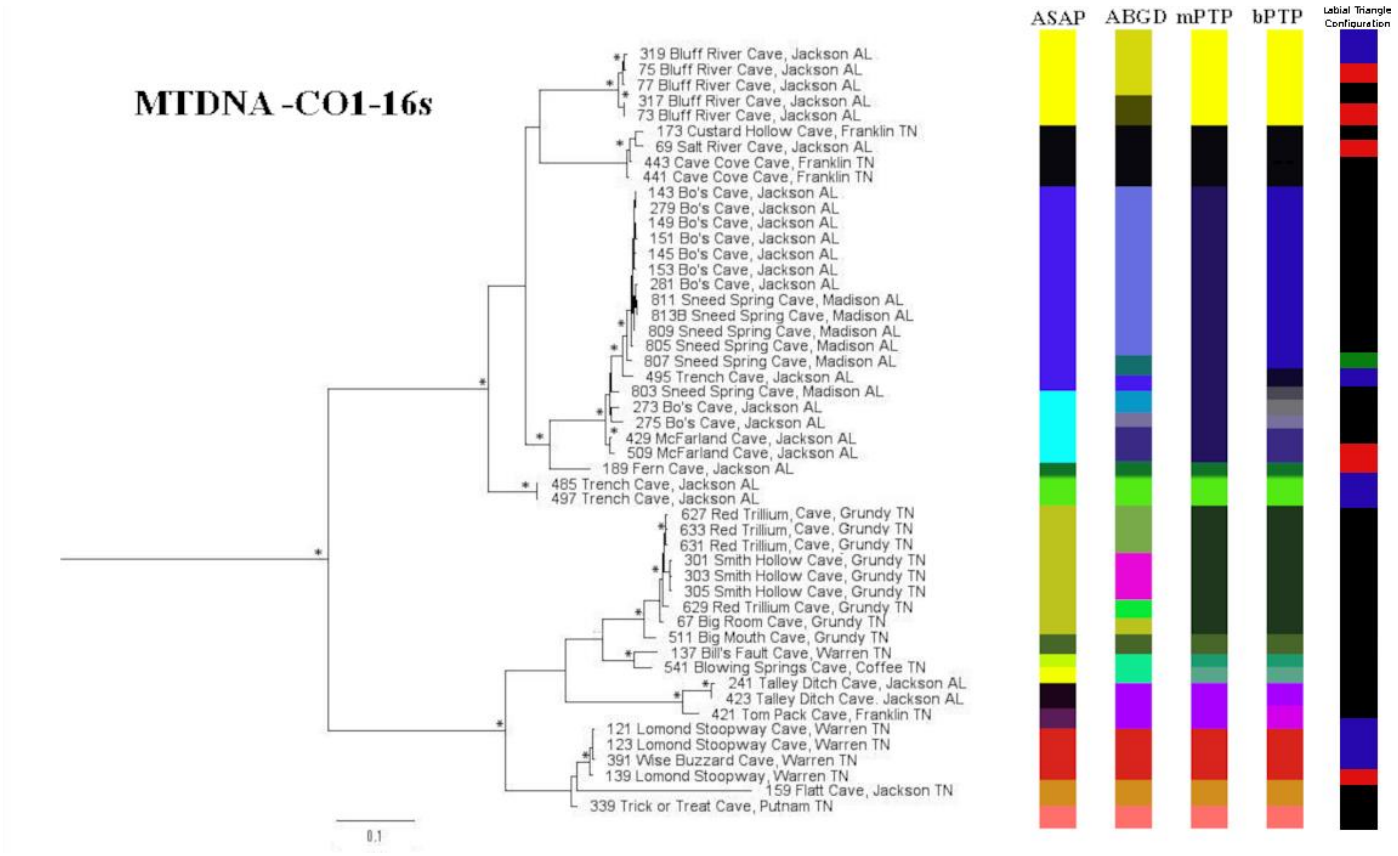

Figure 3.15: Bayesian phylogram for the concatenated COI and 16S dataset with results of species delimitation analysis (ASAP, ABGD, mPTP, and bPTP), with labial triangle configuration (no labial in red, loss of L2 chaetae in blue and additional chaetae in green). Nodes with posterior probabilities >0.95 are indicated with an asterisk. *Pseudosinella hirsuta* from two different caves were used as outgroups. Tip labels refer to the specimen code number, cave, county, and state.

3.4 Molecular Diversity

The COI locus had higher genetic diversity than that of 16S (Table 3.1). Genetic diversity was greater in the greater in the Northern Clade compared to the Southern Clade. Tajima's D was positive for the COI locus (Table 3.1), indicating a possible decrease in population size and/or balancing selection (Tajima, 1989).

Table 3.1: Molecular diversity metrics for the Northern Clade, Southern Clade of *Pseudosinella spinosa*, and overall for the COI and 16S loci.

Locus	K	S	Hd	π	Tajima's D	R_2
Northern Clade						
COI	18	168	0.99	0.11	1.79	0.02
16S	13	166	9.94	0.10	0.01	0.03
Southern Clade						
COI	16	183	0.86	0.10	1.59	0.06
16S	17	160	0.87	0.05	-1.01	0.06
Overall						
COI	34	231	0.958	0.153	3.399***	0.214
16S	30	251	0.941	0.130	1.994	0.171

K, number of unique haplotypes; S, number of segregating sites; Hd, haplotype diversity; π, nucleotide diversity, *P<0.05, **P<0.01, ***P<0.001

Chapter 4. Discussion

4.1 Morphology

According to Soto-Adames (2010) and Kováč *et al*. (2023), the labial chaetae configuration should be static and a critical morphological feature for *Pseudosinella* identification. However, I found that cave-dwelling *Pseudosinella* from the TAG Region have a labial triangle that is more morphologically diverse than was previously recorded in Christiansen (1988) and Christiansen (1960). The only reliable morphological character that coincided with a clear species separation among *P. spinosa*, *P. hirsuta*, and *Pseudosinella christianseni* Salmon, 1964 was the spine-like setae on the dens of the furcula. However, according to Christiansen (1960), these structures might be challenging to locate without proper magnification. With the 40x and 100x PH objective lens, there was no issue seeing the setae, including the spine-like setae on the dens. Suggesting they may be an important diagnostic character for differentiating *P. spinosa* from *P. hirsuta* and *P. christianseni* compared to using labial triangle chaetotaxy alone.

Morphologically identified *Pseudosinella spinosa* all shared spine-like setae on the dens; however, other keyable features varied among individuals. In North America, the spine-like setae should and did separate *P. spinosa* from all other *Pseudosinella* within North America. Christiansen & Bellinger (1998) noted that *P. spinosa* is a highly troglobitic organism that generally lacks pigment, has an extended abdomen, and extended antennas. However, six individuals in this study either had color or did not have extended appendages. Specimens from Bluff River Cave in Jackson County, Alabama, were the most unique with all but two individuals (specimens 317 and 319) having yellow coloration on the body and head. The coloration of the individuals did not follow species delamination analysis' and generally grouped with their

respective caves. Six out of the fifty individuals did not have the labial triangle formation of (M_{1s})M_1M_2r(R)EL_1L_2 highlighted in Christensen & Bellinger (1998). Specimens with the labial triangle configuration of (M_{1s})M_1M_2r(R)EL_1 was locality specific and with the exception of two individuals and corresponded with the potential species analyses. Individual 495 from Trench Cave in Jackson County, Alabama, did not fallout with others from the respective cave, but the other individuals from Trench Cave corresponded to a distinct MOTU. Individual 319 from Bluff River Cave of Jackosn County, Alabama, was the only individual to lack a chaetae, but was closely lumped with other individuals within this population. I did not look at the exposed anal plate of the individuals to separate life stages. This diversity in the configuration of the labial triangle could be due to the different life stages. There was also a variation within the degree of normalness (basal) of the mucronal (mucro) teeth length and position. Which was further explained in, Christiansen (1960) where it was noted that the only striking morphological variation within *P. spinosa* should be with their mucro being more basal in some populations.

4.2 Phylogenetics and Species Delimitation

Phylogenetic analyses of all three mitochondrial datasets (COI, 16S, and concatenated COI+16S) revealed two primary clades with high support as well as additional genetic structure within these main clades that generally correspond to geography. It is not uncommon for a high degree of genetic divergence among cave springtail species with historically large geographic distributions (*e.g.*, Katz *et al.*, 2018; Kováč *et al.*, 2023; Paromuchova *et al.*, 2023). This can be attributed to factors that

hinder or promote the dispersal of troglobiont springtails, such as dispersal through subterranean routes (Katz *et al*., 2018), multiple cave invasions (Christiansen & Culver, 1987), environmental and ecological differences of the caves (Fiera *et al*., 2021). In *P. spinosa*, the most probable factor that influences genetic diversity and structure are multiple cave invasions and dispersal within subterranean routes (*e.g*., Christiansen & Culver 1967; Katz *et al*., 2018). I only looked at the mitochondrial locus COI and 16S, the results might differ if I was able to successfully amplify nucleic loci 28s with the *Pseudosinella spinosa* individuals. I had significant inconsistencies with successful Sanger sequencing with the 28S locus. Other studies such as in Katz *et al*. (2018) also had inconsistencies in cave springtail rDNA 28S amplifications. Springtails are known to have a high level of divergence and genetic differences with the mtDNA locus (Katz *et al*., 2015; Katz *et al*., 2018). Due to the slow evolving nature of nucleic DNA, the 28S locus could help explain the odd placement of some of the individuals used. Such as individual 495 from Trench Cave in Jackson County, Alabama, that groups with individuals from Sneed Spring Cave in Madison County, Alabama, over other individuals from Trench Cave.

Species delimitation approaches based on the mitochondrial 16S and/or COI loci have been used in other springtail studies (*e.g*., Porco *et al*., 2012; Zhang *et al*., 2014; Katz *et al*., 2015; Zhang *et al*., 2018; Yu *et al*., 2018; Guzik *et al*., 2020; Kováč *et al*., 2023). Zhang *et al*. (2018) used such approaches with these loci to explore cryptic speciation and justify morphological differences among a springtail species complex of *Coecobrya sp.* Yu *et al*. (2018) used species delimitation approaches to support justification for using previously ignored morphological structures in a cryptic species complex. With molecular species delimitation, I was able to identify between 4 and 28 MOTUs depending on the dataset and species delimitation

approach. Such a wide range in delimited MOTUs is not uncommon in species delimitation studies using single locus approaches (Ranasinghe *et al*., 2023). The lack of congruence of MOTUs among species delimitation approaches may be because of over-lumping or splitting from the delimitation tools (Dellicour & Flott, 2018; Ranasinghe *et al*., 2023) due to varying age of the tree nodes (Miralles & Vences, 2013), potential gene exchange among populations (Ranasinghe *et al*., 2023), analysis ignoring barcoding gaps (Ranasinghe *et al*., 2022), the size of sampled populations (Dellicour & Flot, 2018) and the number of mutations (Dellicour & Flot, 2018), among other factors.

There were associations between morphological variation and delimited MOTUs. For example, specimen 189 from Fern Cave in Jackson County, Alabama, was delimited as an isolated MOTU in all but the 16S ASAP analysis. This specimen also possessed a unique claw morphology distinct from other *P. spinosa* analyzed. Likewise, individuals that had a labial triangle configuration that differed from the formation of $(M_{1s})M_1M_2r(R)EL_1L_2$ tended to group separately. For example, specimens 485 and 497 from Trench Cave in Jackson County, Alabama, were delimited as a unique MOTU in all analyses but 16S ASAP analysis. However, the phylogenetic placements of some individuals were unexpected. For example, specimen 807 from Sneed Spring Cave in Madison County, Alabama, had an extra chaetae on the left labial triangle $[(M_{1s})M_1M_{2s}M_2r(R)EL_1L_2]$ and was grouped with other individuals from Sneed Spring Cave. Other studies also have found correlations between delimited MOTUs using species delimitation approaches and morphology in invertebrates. For example, when analyzing the phylogeography of *Pseudosinella paclti and P. aggtelekiensis* in caves from the Western Carpathians of Europe, Kováč *et al.* (2023) used both an ASAP and

bPTP approach with morphological and geographical information. They found that the established MOTUs from both the analysis tools corresponded to not only the morphologically established species, but also a new morphologically distinct species in *P. muranensis,* and two non-described cryptic species'. Likewise, species delimitation techniques allowed Nantarat *et al.* (2019) to split the land snail *C. volvulus* into three major clades, two new morphologically distinctive and up to an additional 11 cryptic species.

With the exception for the 16S ASAP analysis, MOTUs tended to correspond with geographical boundaries (*i.e.*, individuals from the same cave or geographic area grouping together). However, some specimens grouped with specimens from other caves in different exposures of karst. For example, specimen 159 from Flatt Cave in Jackson County, Tennessee and specimen 339 from Trick or Treat Cave in Putnam County Tennessee tended to group together in most species delimitation analyses. This was unexpected due to both being isolated from one another (different physiographic provinces and separated by 16.78 km linear distance). Other studies of cave-dwelling invertebrates have found relationships between delimited MOTUs, geography, and karst exposures (*e.g.*, Paromuchova *et al.*, 2020; Guzik *et al.*, 2021; Kováč *et al.*, 2023; Paromuchova *et al.*, 2023). For example, in Kováč *et al.* (2023), discrepancies with splitting of bPTP results were attributed to extreme distances of 16 km (10 miles). They determined that for the region this would be considered an extreme distance, where these distances could have led to reduced gene flow between cave springtail populations and genetic isolation. Likewise, Paromuchova *et al.* (2023) looked at the cave onychuirid *Deureraphorura kratochvili*. They used phylogeography as a tool with the addition of morphology to establish the validity of the new species *Deuteraphorura muranensis*. The species *D. kratochvili* was split into distinctive MOTUs that corresponded to populations separated by

geographical boundaries. It can be argued that using species delimination analysis is not useful without both geographical distance and morphological parameters to confirm species parameters (Paromuchova *et al*., 2023).

In contrast, Guzik *et al*. (2020) found that the MOTUs for *Pseudosinella* from Australian concretions revealed both isolated cryptic speciation and haplotypes that unexpectedly were in multiple previously predicted isolated concretions.

4.3 Phylogeography

Christiansen (1960) and Christiansen & Bellinger (1998) reported that the range of *P. spinosa* spans the Cumberland Plateau of northern Alabama and central Tennessee. Individuals identified morphologically as *P. spinosa* in this study also support this range extent. The total distribution of the locations sampled spanned over 250 km, with an apparent clumped distribution model. A more plausible explanation of this clumped distribution in Christensen & Culver (1967) and this study may be due to a sampling bias of accessible caves over the notion that *P. spinosa* populations are fully isolated. For example, Boyd *et al*. (2020) noticed similar population distributions within the cave beetle *Darlingtonea kentuckensis*. However, Boyd *et al*. (2020) concluded that the clumped distribution could be due to a lack of cave sampling in the region, over the populations being geographically isolated.

Genetically, the individuals sampled are split into two genetically distinct groupings identified herein as the Northern and Southern clades. The Southern Clade is centralized around western Jackson County, Alabama, Madison County, Alabama, and southern Franklin County, Tennessee, with an estimated linear extent of 74.5 km. The

Northern Clade consists of individuals from northern Tennessee and a single location (Talley Ditch Cave) in Jackson County, Alabama, with an estimated linear extent of 192.0 km. The two closely related sister clades highlight the potential for at least 2 distinct cryptic species with a potential of up to three additional morphologically distinctive species.

4.4 Conservation and Taxonomic Implications

Currently *P. spinosa* has not been assessed by IUCN and secure by NatureServe and has no protection within Alabama or Tennessee. The discovery of morphologically distinct populations, cryptic and distinct lineages within *P. spinosa* highlights a need for this species to be assessed. With the limited specimens used in the study, I was able to isolate over 20 cryptic MOTUs with up to 21 MOTUs being isolated to a single cave based on species delimitation approaches. With the high degree of potential cryptic speciation within this species and the two distinct clades, the degree of endemism could be considered higher than previously predicted. Highlighting a strong need for this species to be assessed for potential protection.

Pseudosinella spinosa was first described from Alladin Cave in Madison County, Alabama, by Delamare (1949). In the same manuscript, *P. hirsuta* was described, where the two were separated based on *P. spinosa*'s labial triangle formation, "thoracic hump" and spines on the dens of the furcula. Salmon (1964), separated *P. christianseni* from *P. hirsuta* based on the labial triangle formation being unique from the parent species and the high "degree" of troglomorphism within the species. Much like in *P. christianseni*, my study uncovered a high degree of troglomorphism in *P. spinosa* and supports at least three morphologically distinct species based on the labial triangle from the structures I analyzed. The three morphologically distinct species are also phylogenetically distinct across the three species delamination analyses.

In addition, I was also able to differentiate three potentially morphologically distinct species based on the lack of or addition of a chaetae on the labial triangle. Phylogenetic and species delimitation analyses support between 3 and 28 MOTUs, depending on the dataset and model employed. Four of these MOTUs coincide with the morphologically diagnosable individuals. At least three to ten of these MOTUs represent genetically and biogeographically distinctive populations. With the total combined analysis with morphology, phylogeography and species delamination there is an estimated of two to five distinct populations that differ from individuals collected from Sneed Spring Cave. The populations may represent distinctive species, both cryptic and morphologically distinctive individuals. This study is a start to help understand the true levels of cryptic speciation within this morphospecies. As such, further sampling is needed with an addition of nuclear markers to extend my findings.

Chapter 5. Conclusion and Future Work

Pseudosinella spinosa is a highly troglomorphic entomobryid springtail that inhabits the Interior Low Plateau and Appalachians karst regions. I incorporated a morphological and molecular approach to better understand this species complex's true levels of diversity. Previous morphological studies have established that *P. spinosa* is highly troglomorphic characterized by a greatly expanded thoracic hump, colorless appearance, labial triangle of $(M_{1s})M_1M_2r(R)EL_1L_2$ and spine-like setae on their dens (Christiansen, 1960; Christiansen & Bellinger, 1998). However, multiple individuals from caves such as Fern Cave in Jackson County, Alabama, Custard Hollow Cave in Franklin County, Tennessee, and Red Trillium Cave in Grundy County, Tennessee, did not follow this description. Likewise, this study supports that the species is morphologically more diverse than previously reported. Moreover, phylogenetic and species delimitation approaches suggest that *P. spinosa* is a species complex with at least two main mitochondrial clades and 2 to 28 cryptic species with at least 2 to 3 morphologically distinct species. I have established that *P.spinosa* is a species complex split by a Northern and a Southern Clade. These clades follow geographic barriers with the Northern Clade being restricted to karst regions of the Cumberland Plateau and the Southern Clade spanning across both the Cumberland Plateau and Highland Rim. Most the MOTUs developed by the analyses were cave specific, indicating high levels of endemism. Individuals from Fern Cave, Trench Cave and Lomond Stoopway /Wise Buzzard caves represent 3 morphologically and genetically distinct populations that need to be analyzed further. This study was a start in the process to help determine the true levels of diversity in this complex. Likewise, the true distributions of these clades are not fully understood due to inadequate sampling. Likewise, a more extensive study is warranted to better

understand true levels of diversity, distributions, and evolutionary history with this species and genus in the TAG Region.

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Appendix A. Morphological Data for *Pseudosinella spinosa* Specimens Examined

Table A.1: Compiled specimen morphological configuration table.

State	County	Cave	Cave Code	Labial	Hind-Claw Unguis	Mucro	Dens	Antennal 4th 2X Cephalic Diagonal	Color	Size (mm)	Specimen #
AL	Jackson	Tumbling Rock Cave	AJK171	(M_{1s})M_1M_2r(R)EL_1L_2	3 Internal	Extended	Spine-Like Setae	Yes	None	1	277
AL	Jackson	Salt River Cave	AJK221	X	3 Internal	Extended	Spine-Like Setae	Yes	None	3	69
AL	Jackson	Talley Ditch Cave	AJK248	(M_{1s})M_1M_2r(R)EL_1L_2	3 Internal	Extended	Spine-Like Setae	Yes	None	2.6	241
AL	Jackson	Talley Ditch Cave	AJK248	(M_{1s})M_1M_2r(R)EL_1L_2	3 Internal	Extended	Spine-Like Setae	X	None	2.4	423
AL	Jackson	Bluff River Cave	AJK2800	(M_{1s})M_1M_2r(R)EL_1L_2	3 Internal	Extended	Spine-Like Setae	Yes	Yellow Patches	3	77
AL	Jackson	Bluff River Cave	AJK2800	(M_{1s})M_1M_2r(R)EL_1L_2	3 Internal	Extended	Spine-Like Setae	Yes	None	2.1	317
AL	Jackson	Bluff River Cave	AJK2800	(M_{1s})M_1or(R)EL_1	None	Slightly Extended	Spine-Like Setae	X	None	3.2	319
AL	Jackson	Bluff River Cave	AJK2800	X	3 Internal	Extended	Spine-Like Setae	Yes	Yellow Patches	2	073
AL	Jackson	Bluff River Cave	AJK2800	X	3 Internal	X	X	Yes	yellow	3	075
AL	Jackson	Bo's Cave	AJK4273	(M_{1s})M_1M_2r(R)EL_1L_2	3 Internal	Extended	Spine-Like Setae	Yes	None	1.5	143
AL	Jackson	Bo's Cave	AJK4273	(M_{1s})M_1M_2r(R)EL_1L_2	3 Internal	Extended	Spine-Like Setae	Yes	None	2.5	145
AL	Jackson	Bo's Cave	AJK4273	(M_{1s})M_1M_2r(R)EL_1L_2	3 Internal	Extended	Spine-Like Setae	Yes	None	3.75	149
AL	Jackson	Bo's Cave	AJK4273	(M_{1s})M_1M_2r(R)EL_1L_2	3 Internal	Extended	Spine-Like Setae	Yes	None	2.75	151
AL	Jackson	Bo's Cave	AJK4273	(M_{1s})M_1M_2r(R)EL_1L_2	3 Internal	Extended	Spine-Like Setae	Yes	None	2	153

AL	Jackson	Bo's Cave	AJK4273	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	3	273
AL	Jackson	Bo's Cave	AJK4273	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2.25	275
AL	Jackson	Bo's Cave	AJK4273	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2	279
AL	Jackson	Bo's Cave	AJK4273	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2.9	281
AL	Jackson	Fern Cave	AJK597	X	3 Internal, 1 External	Extended	Spine-Like Setae	Yes	Red Speckling With Eyepatch	2.75	189
AL	Jackson	Mcfarland Cave	AJK65	$(M_{1s})M_1M_2r(R)oL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2.7	429
AL	Jackosn	Mcfarland Cave	AJK65	X	3 Internal	Extended	Spine-Like Setae	Yes	None	3	509
AL	Jackson	Trench Cave	AL1070	$(M_{1s})M_1M_2r(R)EL_1$	3 Internal	Extended	Spine-Like Setae	X	None	3	485
AL	Jackson	Trench Cave	AL1070	$(M_{1s})M_1M_2r(R)EL_1$	3 Internal	Extended	Spine-Like Setae	X	None	2.5	495
AL	Jackson	Trench Cave	AL1070	$(M_{1s})M_1M_2r(R)EL_1$	3 Internal	Extended	Spine-Like Setae	X	None	3.9	497
AL	Madison	Sneed Spring Cave	AL554	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2	803
AL	Madison	Sneed Spring Cave	AL554	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2	805
AL	Madison	Sneed Spring Cave	AL554	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2	809
AL	Madison	Sneed Spring Cave	AL554	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	1.1	811
AL	Madison	Sneed Spring Cave	AL554	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2	813B
AL	Madison	Sneed Spring Cave	AL554	$(M_{1s})M_1M_{2s}M_2r(R)EL_1L_2$	3 Internal	Normal	Spine-Like Setae	Yes	None	1.75	807
TN	Coffee	Blowing Springs Cave	TCF18	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	X	None	2.9	541

TN	Franklin	Tom Pack Cave	TDR87	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	X	None	3	421
TN	Franklin	Cave Cove Cave	TFR33	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Normal	Spine-Like Setae	Yes	None	3.001	441
TN	Franklin	Cave Cove Cave	TFR33	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Normal	Spine-Like Setae	Yes	None	2.6	443
TN	Franklin	Custard Hollow Cave	TFR7	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Normal	Spine-Like Setae	Yes	Red Speckling With Eyepatch	2	173
TN	Grundy	Big Mouth Cave	TGD2	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2.9	511
TN	Grundy	Red Trillium Cave	TGD292	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2.2	629
TN	Grundy	Red Trillium Cave	TGD292	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	3	631
TN	Grundy	Red Trillium Cave	TGD292	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2.5	633
TN	Grundy	Red Trillium Cave	TGD292	$(M_{1s})oM_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	No	Red With Red Eyepatch	2.05	627
TN	Grundy	Big Room Cave	TGD3	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	3	67
TN	Grundy	Smith Hollow Cave	TGD64	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2.75	301
TN	Grundy	Smith Hollow Cave	TGD64	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	Yes	None	2	303
AL	Jackson	Flatt Cave	TJK11	$(M_{1s})M_1M_2o(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	X	None	2.5	159
TN	Warren	Trick or Treat Cave	TPU285	$(M_{1s})M_1M_2o(R)EL_1L_2$	3 Internal	Extended	Spine-Like Setae	X	None	3.1	339
TN	Warren	Lomond Stoopway Cave	TWR340	$(M_{1s})M_1M_2r(R)EL_1$	3 Internal	Extended	Spine-Like Setae	Yes	None	2	121
TN	Warren	Lomond Stoopway Cave	TWR340	$(M_{1s})M_1M_2r(R)EL_1$	3 Internal	Extended	Spine-Like Setae	Yes	None	2	123
TN	Warren	Wise Buzzard Cave	TWR456	$(M_{1s})M_1M_2r(R)EL_1$	3 Internal	Extended	Spine-Like Setae	Yes	None	3.1	391

TN	Warren	Bill's Fault Cave	TWR505	$(M_{1s})M_1M_2r(R)EL_1L_2$	3 Internal	Normal	Spine-Like Setae	Yes	None	1.2	137