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NOTE

DISCOVERY OF THE BLUE RIDGE SPRINGSNAIL, *FONTIGENS OROLIBAS*, HUBRICHT, 1957 (GASTROPODA: EMMERICIIDAE) IN EAST TENNESSEE AND ITS CONSERVATION IMPLICATIONS

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ABSTRACT

The study of spring- and subterranean-associated microsnail species in the Appalachian karst region has focused disproportionately on the northern Appalachian Valley and Ridge (AVR), leaving many areas in the southern Appalachians unexplored. Consequently, biological inventories of subterranean habitats have been initiated in the southern AVR, particularly in the state of Tennessee. In 2013 and 2018, several previously unknown populations of a microsnail species were discovered from caves in eastern Tennessee. Through both morphological and molecular analysis, we identified these populations as the Blue Ridge Springsnail, *Fontigens orolibas*. These newly discovered populations represent a significant range extension of *F. orolibas*. As such, we reassess the conservation status of *F. orolibas* under NatureServe criteria and emphasize the need for further sampling efforts in the southern AVR for microsnails.

KEY WORDS: freshwater snails, microsnails, *Fontigens orolibas*, southern Appalachian region

INTRODUCTION

Currently, the freshwater snail genus *Fontigens* Pilsbry, 1933 is the only recognized North American group among the family Emmericiidae (subfamily Fontigentinae; Hershler et al. 1990; Wilke et al. 2013). Compared with other hydrobioid microsnails (i.e., freshwater snails ranging from 2 to 5 mm of the family Hydrobiidae sensu lato; Davis 1979), most *Fontigens* species exhibit broad geographic distributions but are known primarily from karst landscapes of the central and eastern United States (Hershler and Holsinger 1990; Hershler et al. 1990; Culver et al. 2003). At present, there are 10 *Fontigens* species that are fully or partially restricted to springs and subterranean habitats (Hershler et al. 1990; Liu et al. in press). Six of these species are from the Appalachian karst region within the Appalachian Valley and Ridge (AVR) physiographic province (Hershler et al. 1990). Five of the six are endemic to the northern Appalachian karst in the states of Maryland, Pennsylvania, Virginia, and West Virginia (Fig. 1), where the majority of research efforts on aquatic subterranean-associated animals (i.e., stygofauna) has occurred (Holsinger et al. 1976; Holsinger and Culver 1988; Fong et al. 2007; Fong and Culver 2018). These five species are as follows: Appalachian Springsnail *Fontigens bottimeri*; Virginia Springsnail *Fontigens morrisoni*; Blue Ridge Springsnail *Fontigens orolibas*; Organ Cavesnail *Fontigens tartarea*; and Greenbrier Cavesnail *Fontigens turritella*. The other species known from the Appalachians—the Watercress Snail *Fontigens nickliniana* Lea, 1838—is the most geographically widespread of all *Fontigens,* occurring throughout the AVR, including in Tennessee, Alabama, and Georgia (TAG) karst, and into the northeastern and central USA. Comparatively, underground habitats in the southern extent of the Appalachian karst (or TAG) have been studied very

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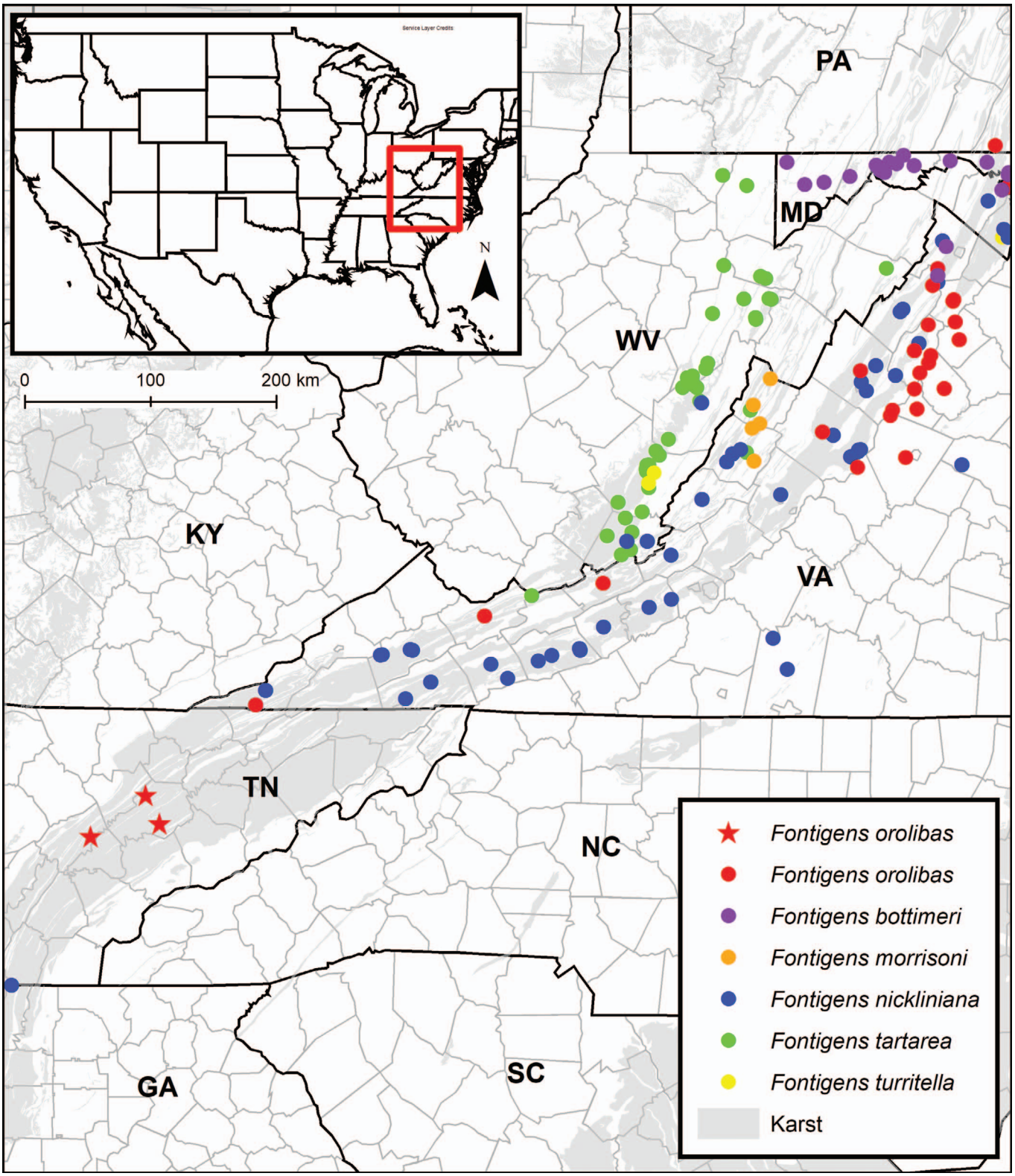

Figure 1. Geographic distributions of all *Fontigens* species occurring in the Appalachians. Newly discovered *Fontigens orolibas* populations are denoted with a star.

Table 1. Detailed site descriptions of Tennessee caves sampled in this study.

Tennessee Cave Survey no.	County	Cave name	Visitation	Personnel involved	Lithology	Cave description	Water depth	Benthic habitat	Watershed
TKN24	Knox	Cruze Cave	One trip May 2013, one monthly trip from March–October 2014, one trip June 2018, one trip February 2019	A.S. Engel, S. Engel, D. Fong, M.L. Niemiller, M.E. Slay, M.L. Porter, S. Keenan, S.J. Taylor, K.S. Zigler, A. Paterson, K. Brennan, T. Brown, A. England, N.S. Gladstone, E.B. Pieper	Ordovician Knox Dolomite	Main passage traversable for about 300 m with cave stream throughout	~0.1 to <0.25 m depth	Small to large rocks in sections of passage, small cobble and fine silt/sand in other sections	Melton Hill Lake watershed of the Clinch River, which flows into Watts Bar Lake and the Tennessee River
TKN103	Knox	Pedigo Cave	Two trips July 2018, one trip December 2018	N.S. Gladstone, E.B. Pieper, M.L. Niemiller	Cambrian Maynardville Limestone	Ca. 35 m of traversable passage, with stream flow in small room near cave terminus	2-m-deep pool	Fine silt, sand, and gravel mixed with larger cobble and smooth-faced rocks	Melton Hill Lake watershed of the Clinch River, which flows into Watts Bar Lake and the Tennessee River
TRN6	Roane	Eblen Cave	One trip May 2013, one trip December 2018	A.S. Engel, S Engel, D. Fong, M.L. Niemiller, M.E. Slay, M.L. Porter, S. Keenan, S.J. Taylor, K.S. Zigler, A. Paterson, K. Brennan, T. Brown, A. England, N.S. Gladstone, E.B. Pieper, E.T. Carter, L.E. Hayter	Copper Ridge Dolomite	1,020 m of traversable passage, with 200 m of cave stream	<0.3 m deep	Large rocks, mixed with cobble/fine silt/sand throughout passage	Cave stream flows into Mill Creek on the surface, which is in the Clinch River watershed of the Tennessee River

Figure 2. *Fontigens orolibas* specimens. Top: Cave specimens collected from Cruze Cave (TKN24), Knox Co., Tennessee (1, 2); Pedigo Cave (TKN103), Knox Co., Tennessee (3); and Eblen Cave (TRN6), Roane Co., Tennessee (4). Photo credit: N.S. Gladstone. Bottom: Live *F. orobilas* from TKN103. Photo credit: M.L. Niemiller.

little (Niemiller and Zigler 2013; Niemiller et al. 2019). Only *F. nickliniana* has been described previously within TAG, from two spring sites.

Since 2012, ongoing biological inventories of cave systems in the AVR of eastern Tennessee have uncovered several new populations of freshwater snails from cave streams. These include recently described stygobiotic species of the genus *Antrorbis* from two caves (Gladstone et al. 2019). The others were of a *Fontigens*-like snail, with two populations being found within the Melton Hill Lake watershed of the Clinch River, which flows into Watts Bar Lake and the Tennessee River, and a third within the Clinch River watershed of the Tennessee River. Using morphological and molecular data, we diagnose these three new *Fontigens*-like snail populations as the Blue Ridge Springsnail *F. orolibas*. In light of confirming these new populations as *F. orolibas*, we also reassess the conservation ranks of this species using NatureServe criteria (Master et al. 2009).

METHODS

Field Sampling

All biological surveys involved at least two, and as many as 12, researchers. Scientific research and collection in the caves were permitted, with renewals, by the Tennessee Department of Environment and Conservation (TDEC) and the Tennessee Wildlife Resource Agency (TWRA) (TDEC number 2013-026 and TWRA number 1605). Table 1 presents the timeline of population discovery and site summaries. A single population was first discovered in 2013 from a cave system in Knox Co., Tennessee (TKN24), and other populations were found in a different cave in Knox Co. (TKN103) and in a cave in Roane Co. (TRN6), Tennessee (Fig. 1). Monthly surveys were performed at the original locality (TKN24; physicochemical measurements, abundance data, and microhabitat descriptions can be found in Keenan et al. [2017]). We report only individual Tennessee Cave Survey

Table 2. Mean shell measurements (mm) for specimens examined from each locality. Standard deviations in parentheses. * represents populations with measurements from Hershler et al. (1990).

Locality	NW	SH	SW	W	D	T	AS
Cruze Cave, Knox Co., Tennessee ($n = 24$)	4.31 (0.13)	1.97 (0.09)	1.44 (0.07)	1.25 (0.09)	0.55 (0.06)	6.03 (0.93)	1.22 (0.05)
Pedigo Cave, Knox Co., Tennessee ($n = 4$)	4.56 (0.19)	1.83 (0.11)	1.32 (0.07)	1.3 (0.07)	0.58 (0.05)	6.71 (0.76)	1.38 (0.07)
Eblen Cave, Roane Co., Tennessee ($n = 3$)	4.42 (0.24)	2.08 (0.14)	1.51 (0.11)	1.35 (0.16)	0.61 (0.02)	7.14 (1.01)	1.26 (0.05)
*Hawksbill Shelter, Madison Co., Virginia ($n = 10$)	4.58 (0.36)	2.53 (0.15)	1.46 (0.10)	1.27 (0.08)	0.56 (0.04)	5.57 (0.85)	1.24 (0.05)
*Blue Ridge Parkway, Augusta Co., Virginia ($n = 9$)	5.03 (0.08)	3.27 (0.17)	1.69 (0.08)	1.23 (0.07)	0.50 (0.08)	7.61 (1.10)	1.39 (0.13)
*Witheros Cave, Bath Co., Virginia ($n = 13$)	4.23 (0.07)	1.63 (0.10)	0.99 (0.05)	1.39 (0.08)	0.55 (0.03)	5.41 (0.78)	1.22 (0.08)
*Tawneys Cave, Giles Co., Virginia ($n = 10$)	4.40 (0.18)	2.07 (0.12)	1.20 (0.06)	1.44 (0.35)	0.57 (0.03)	6.04 (0.80)	1.21 (0.04)
*Harveys Cave, Giles Co., Virginia ($n = 9$)	4.22 (0.23)	1.98 (0.14)	1.14 (0.10)	1.40 (0.12)	0.65 (0.08)	7.44 (1.20)	1.23 (0.08)
*Indian Run Shelter, Rappahannock Co., Virginia ($n = 12$)	4.25 (0.00)	2.45 (0.10)	1.38 (0.06)	1.35 (0.09)	0.62 (0.05)	6.22 (0.74)	1.18 (0.05)
*Browntown Valley Overlook, Warren Co., Virginia ($n = 10$)	5.03 (0.08)	2.76 (0.22)	1.42 (0.09)	1.23 (0.06)	0.62 (0.08)	6.40 (0.77)	1.26 (0.10)

NW = number of whorls; SH = shell height; SW = shell width; W = whorl expansion rate; D = distance of generating curve; T = translation rate; AS = aperture shape.

(TCS) inventory numbers rather than exact geographic coordinates, but cave system descriptions and location data are maintained by the TCS (http://www.subworks.com/tcs/) and are available from the authors upon request.

Morphological Analysis

Specimens collected from each site were preserved in 100% ethanol and transferred to the laboratory for morphological analysis and imaging. Upon completing the re-evaluation of these materials, the specimens were deposited in the Auburn Museum of Natural History. We used a Jenoptik SUBRA full high-definition microscope camera to photograph and evaluate shells (Fig. 2). Standard shell measurements included in Hershler et al. (1990) were recorded for specimens, including number of whorls, shell height, shell width, whorl expansion rate, distance of generating curve, translation rate, and aperture shape (Table 2). We also compared the shell

Table 3. Sequence and locality information. All *Fontigens* sequences without a GenBank accession number were generated and provided by Liu et al. (in press). Numeric code next to locality information represents population identification shown in phylogeny.

Taxon	Locality	GenBank accession
Fontigens antroecetes	Stemler Cave, St. Clair Co., Illinois (22)	MT425002
Fontigens bottimeri	Wetzels Spring, Washington, District of Columbia (6)	MT425003
	Ogden's Cave, Frederick Co., Virginia (11)	MT425004
Fontigens cryptica	Spring in the Bernheim Cedar Grove Wildlife Corridor, Bullitt Co., Kentucky (12)	MT425005
Fontigens morrisoni	Spring at Mustoe, Highland Co., Virginia (13)	-
	Martin Fen, LaGrange Co., Indiana (3)	MT425007
	Blowing Springs, Bath Co., Virginia (14)	MT325008
Fontigens nickliniana	Spring at Lantz Mills, Shenandoah Co., Virginia (15)	MT425015
	Fleenor Spring, Washington Co., Virginia (16)	MT425020
	Kalamazoo, Michigan	JX970609
	Cruze Cave, Knox Co., Tennessee	TBD
	Hawksbill Spring, Page Co., Virginia (17)	MT425028
Fontigens orolibas	Spring at the Humpback Visitor Center, Augusta Co., Virginia (18)	MT425029
	Hugh Young Cave, Tazewell Co., Virginia (19)	MT425030, MT425031
Fontigens tartarea	Organ Cave, Greenbrier Co., West Virginia (1)	MT425032, MT425033
Bithynia tentaculata		MK308073
Bythinella austriaca		FJ028979
Bythinella pannonica		HQ149623
Bythinella viridis		FJ029102
Emmericia expansilabris		KC810061

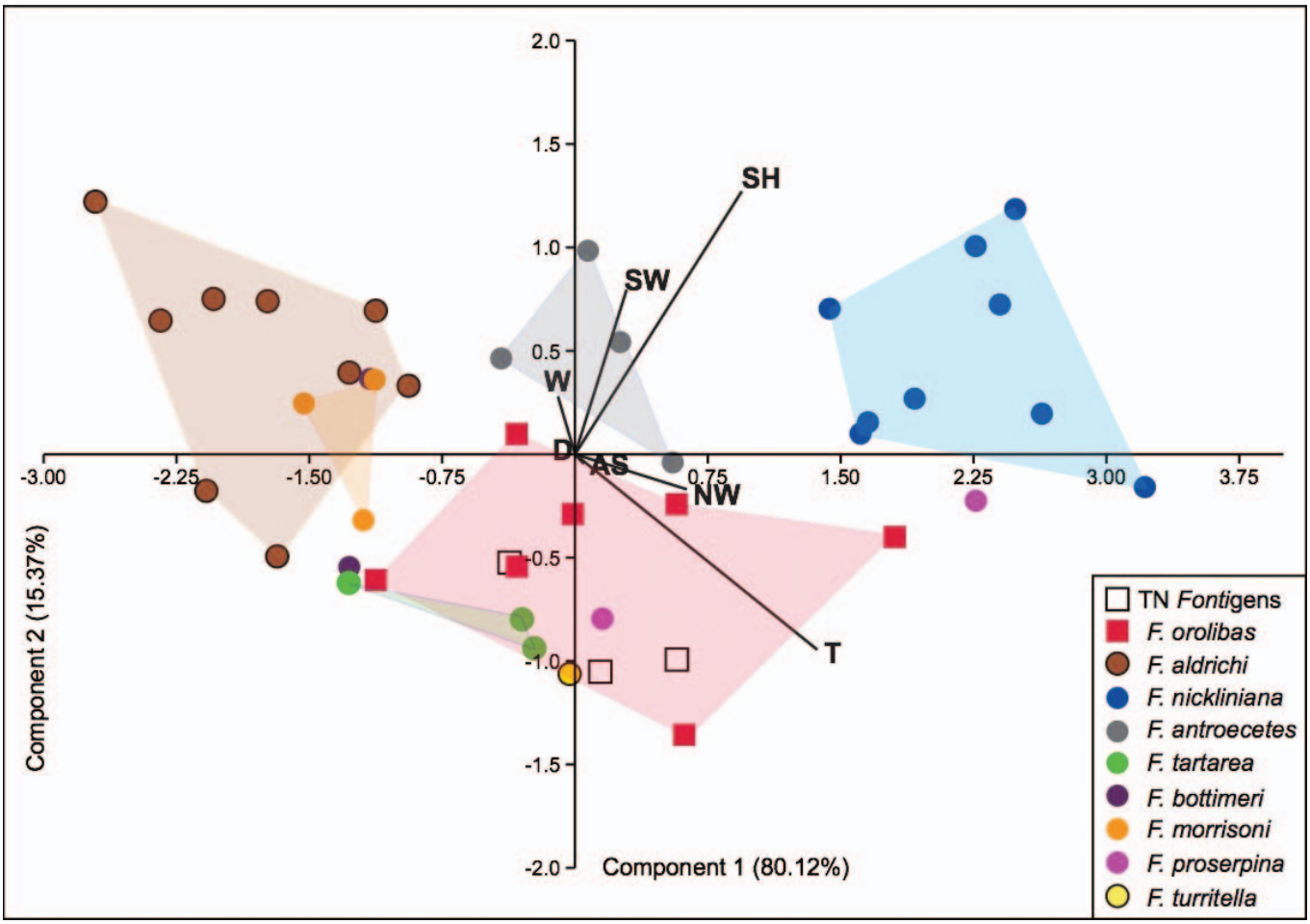

Figure 3. Principal components analysis of *Fontigens* spp. morphological data from Hershler et al. (1990) and this study. The Tennessee *F. orolibas* specimens are shown with an open square. Each point represents an individual specimen. Component 1 accounts for 80.12% of variability and component 2 accounts for 15.3% of variability. Other abbreviations defined in the text.

measurements of all individual *Fontigens* species included in Hershler et al. (1990) with three specimens from the TKN24 site via principal components analysis (PCA) with paleontological statistics software (Hammer et al. 2001). All seven shell measurements were included as components in the analysis and each individual specimen presented by Hershler et al. (1990) is included separately.

Molecular Methods and Phylogenetic Analysis

Genomic DNA was isolated from three specimens from the TKN24 population using the Qiagen DNeasy blood and tissue kit, following the manufacturer's protocol. We amplified a 638-base-pair fragment of mitochondrial cytochrome oxidase subunit 1 (CO1) locus using LC01490 and HC02198 primers (Folmer et al. 1994). PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and sequenced in both directions with BigDye chemistry at Eurofins MWG Operon (Louisville, KY, USA). Forward and reverse sequences were quality trimmed at the ends and assembled into contigs in DNA Baser v4.36 (Heracle BioSoft) and aligned using MAFFT (Katoh and Standley 2013). These sequences were then compared with CO1 sequences from several other *Fontigens* species generated by Liu et al. (in press) (Table 3).

The CO1 phylogeny was generated using a maximum-likelihood approach in IQ-TREE 2 (Minh et al. 2020) with the model-testing function to infer the best-fit substitution model for each codon partition under the corrected Akaike's information criterion. We implemented a general time-reversible model with corrections for a discrete gamma distribution (GTR + Γ) for the first and second codon positions, and the Hasegawa, Kishino, and Yano model with a discrete gamma distribution and a proportion of invariant sites (HKY + Γ + I) for the third codon position. Branch support was assessed with 10,000 ultrafast bootstrap replicates (Hoang et al. 2017).

Conservation Status Reassessment

On the basis of the taxonomic identity of these populations as *F. orolibas*, we reassessed the conservation statuses of the

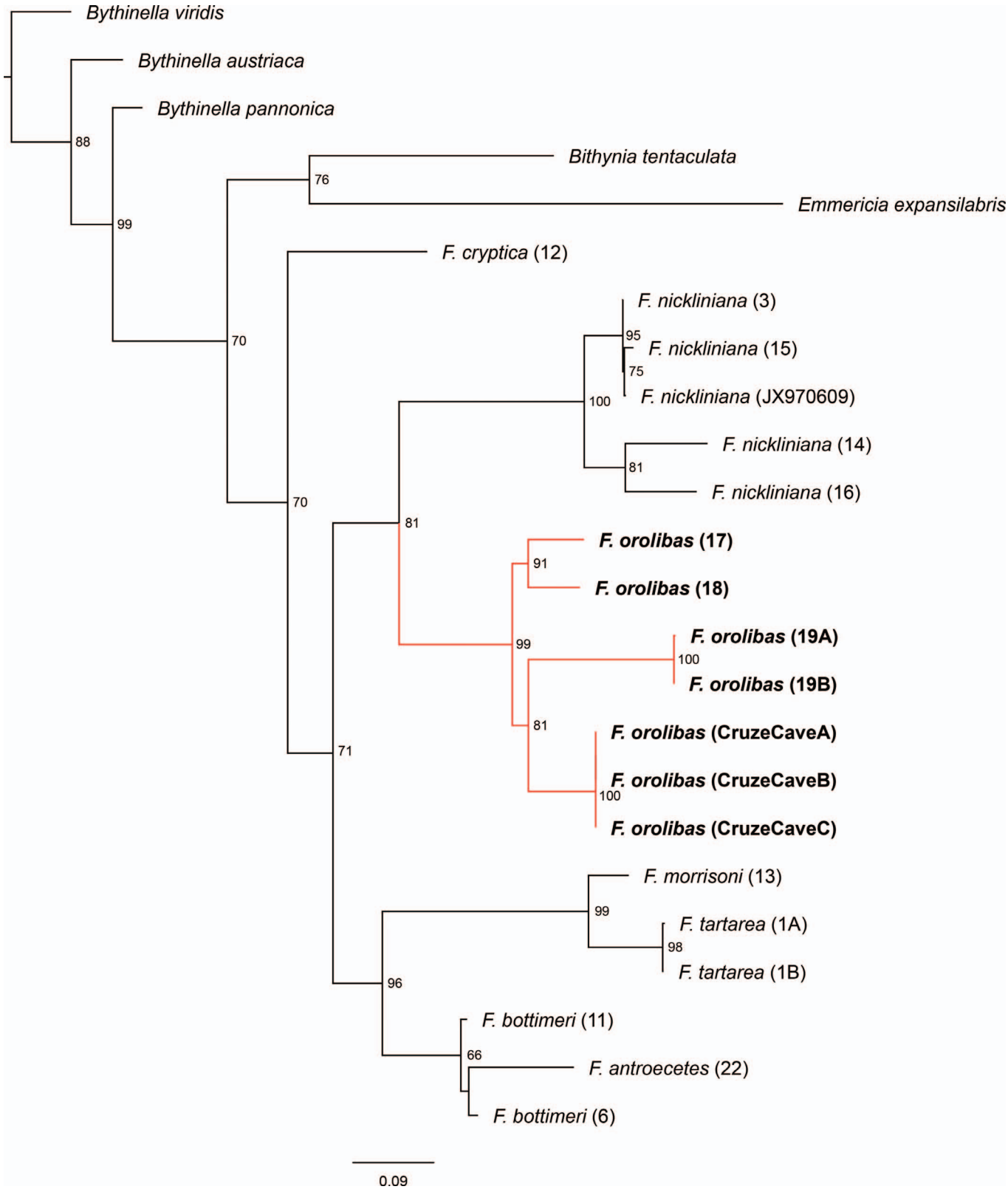

Figure 4. Mitochondrial cytochrome oxidase subunit 1 (CO1) phylogeny of *Fontigens* species with sequence data generated from this study and by Liu et al. (in press). Internal values represent bootstrap support and the scale bar represents branch length in units of sequence divergence. The species *Bythinella viridis* was rooted as the outgroup. Location identification numbers are shown to the right of the species name and listed in Table 3.

species using NatureServe criteria (Master et al. 2009). Conservation status, as designated by NatureServe, is calculated on the basis of several risk categories, including range/distribution, abundance/population condition, threat impacts, and population trends (for details on calculation procedure, see Faber-Langendoen et al. 2012). Each risk category as assessed through NatureServe was calculated with the NatureServe Rank Calculator v3.1932 (Faber-Langendoen et al. 2012). Abundance data for *F. orolibas* are virtually nonexistent, but the earlier efforts of Keenan et al. (2017) represent the first systematic survey of a single population to date. Consequently, population trends and viability information could not be completely assessed for the species. Geographic range size (as assessed by the geographic coordinates associated with each population) was calculated as extent of occurrence (EOO) and area of occupancy (AOO) using the web-based program GeoCAT (Bachman et al. 2011). To determine potential threats to *F. orolibas* throughout its range, we identified whether populations occurred on state or federally protected lands using the U.S. Geological Survey Protected Areas Database v1.3 (shapefile available at http://gapanalysis.usgs.gov/padus/). For each known population, we also examined history of disturbance of each site (if known), the adjacent human population density according to the 2018 U.S. Census from the U.S. Census Bureau (TIGER/Line®), and land cover associations according to the 2016 National Land Cover Database (Yang et al. 2018).

RESULTS AND DISCUSSION

Shell measurements from all new populations in Tennessee showed considerable similarity with *F. orolibas* shells measured in Hershler et al. (1990). Results of the PCA also showed that our three *Fontigens* specimens from the TKN24 populations are within the range of morphological variability of *F. orolibas*. Principal component 1 accounts for 80.12% of variability and is highly influenced by translation rate, shell height, and number of whorls. Separation of taxa along principal component 2, which accounts for 15.3% of observed variability, is influenced by changes to shell height and shell width (Fig. 3). The resulting phylogeny shows 99% bootstrap support at the internal node for all *F. orolibas* individuals including the TKN24 population (Fig. 4), further supporting the species identification. These combined results confirm that the population at TKN24 is indeed *F. orolibas*, and as such we infer that the other two newly discovered populations are also *F. orolibas* on the basis of geographic proximity and near morphologic indistinguishability (Figs. 1, 2).

The population discoveries in eastern Tennessee extend the known geographic range of *F. orolibas* to 26 localities from 19 counties in four states and increase the EOO to 55,631 km^2 and the AOO to 124 km^2. The current NatureServe rank of *F. orolibas* is Vulnerable (G3), and our reassessment does not change this status. Despite a significant increase to its known range, there persists a near complete lack of information regarding threats, baseline monitoring of population trends, and population viability for this species. This information deficiency is likely due to the difficulty of surveying for and studying *F. orolibas* populations, making it problematic to identify both overarching and population-specific threats that could be mitigated by land conservation or management efforts. However, although no direct threat assessments have been reported for *F. orolibas*, freshwater snails restricted to such subterranean or spring habitats are broadly considered at elevated risk of local extirpation or extinction owing to habitat degradation such as groundwater pollution or extraction (Lysne et al. 2008; Hershler et al. 2014). Currently, eight of the 26 known localities of *F. orolibas* are within federally protected lands (i.e., Shenandoah National Park in Virginia), and these populations are most likely secure. Though almost all previous collection events of *F. orolibas* in its northern range are not accompanied by formal reports of abundance, the findings of Keenan et al. (2017) suggest that high population densities can occur within cave ecosystems, with the TKN24 site continuing to have hundreds of individuals throughout the cave stream since it was last visited in 2019 (N.S. Gladstone personal observation).

Some localities occur in areas of high human population density and increased urbanization, with ~27% of *F. orolibas* populations occurring within 5 km^2 of urban land cover, including two of the three new localities reported here. Both newly discovered cave populations in Knox Co., Tennessee are immediately adjacent to suburban neighborhoods, and both have increased levels of pollution and other forms of habitat degradation (Keenan et al. 2017). Given the lack of sampling within the AVR in eastern Tennessee, it is likely that other *F. orolibas* populations exist within the updated range extent and into the southern AVR. We hope the discovery of these previously unreported populations promotes further study of this species.

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