




## High levels of mitochondrial DNA sequence divergence among isolated populations of *Fontigens* (Truncatelloidea: Emmericiidae) in eastern USA

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### ABSTRACT

Populations of the minute emmericiid gastropod *Fontigens* inhabit isolated springs, caves and groundwaters widely scattered across eastern and midwestern USA. Nine species are currently recognized, with a tenth species, *Fontigens cryptica*, of uncertain status. Here, we survey intrapopulation, interpopulation and interspecific sequence variation of cytochrome *c* oxidase subunit I among six of the nine species (13 populations, 87 individuals), documenting up to 14.4% sequence divergence among conspecific populations. Interspecific divergence ranges from 8.7% to 21.9%. We also include in our analysis a single putative individual of *F. cryptica*, recently sampled from Kentucky, evaluating whether the population it represents may be genetically distinct. Fit within a larger phylogenetic context, the 14.4% sequence divergence demonstrated between this individual and its nearest neighbour, *F. bottimeri* sampled from Washington, DC, supports the specific status of *F. cryptica*.

### INTRODUCTION

Truncatelloidean gastropods of the genus *Fontigens* Pilsbry, 1933 (Emmericiidae) are an obscure and enigmatic element of the North American freshwater fauna. As their name (literally, “born of the wellspring”) implies, populations are obligately stygophilic, restricted to springs, spring runs, groundwaters and caves. Hershler, Holsinger & Hubricht (1990) recognized nine species, four of which [*Fontigens antroecetes* (Hubricht, 1940), *F. proserpina* (Hubricht, 1940), *F. tartarea* Hubricht, 1963 and *F. turritella* Hubricht, 1976] are apparently endemic to local spring or cave systems. The distributions of the other five species, however, are surprisingly broad. The most common species, *F. nickliniana* (Lea, 1838), ranges from Virginia to New York and west to Michigan. How an obligate stygophile might have become dispersed over a range so broad as to include ten US states is a fascinating question for evolutionary science.

Many populations of truncatelloidean gastropods from the American West have become the objects of significant conservation concern (Hershler, Liu & Howard, 2014b). A great deal of research effort has been directed towards the *Tryonia* (Cochliopidae) populations of the American Southwest (Hershler, Liu & Mulvey, 1999; Hershler, Liu & Landye, 2011), the *Fluminicola* (Lythoglyphidae) of the Pacific Northwest (Hershler, Liu & Hubbart, 2017) and the broadly western (but species-rich) *Pyrgulopsis* (Hydrobiidae)

(Hershler, Liu & Landye, 2002; Liu & Hershler, 2005; Hershler *et al.*, 2014c). These groups typically demonstrate isolated population structure, high levels of nominal endemism and 2% interspecific cytochrome *c* oxidase subunit I (COI) sequence divergence at minimum.

It is perhaps not surprising, therefore, that similar conservation concerns have been raised about endemic species of *Fontigens* inhabiting the American East. The Virginia Department of Conservation and Recreation, Division of Natural Heritage, for example, samples *Fontigens* populations in connection with a regular programme of cave monitoring. Almost nothing is known about the biology of *Fontigens*. As of January 2020, only two mitochondrial (mt) DNA sequences for *Fontigens* had been deposited in GenBank, partial sequences of the COI and 16S ribosomal RNA (16S rRNA) genes for a single individual *F. nickliniana* sampled from Michigan (Wilke *et al.*, 2013).

Recently, the popular press has carried reports of an obscure species of *Fontigens* rediscovered from a springhead on the property of the Bernheim Arboretum and Research Forest in Kentucky, c. 30 km south of Louisville. *Fontigens cryptica* was not included among the nine species reviewed in the monograph of Hershler *et al.* (1990) because no additional specimens had been found since its description from a small spring in Indiana c. 40 km north of Louisville (Hubricht, 1963).

**Table 1.** Specimen codes, localities and GenBank acc. no.

Identification	Code	Locality	<i>n</i>	GenBank acc. no.
<i>Fontigens antroecetes</i> <sup>1,*</sup>	ant22	Stemler Cave, St Clair County, IL	6	MT425002
<i>F. bottimeri</i> <sup>†</sup>	bot6	Wetzels Spring, Washington, DC (USNM; acc. no. 205943)	2	MT425003
<i>F. bottimeri</i> <sup>†2</sup>	bot11	Ogden's Cave (39.0451, -78.3175), Frederick County, VA	4	MT425004
<i>F. cryptica</i>	cry12	Spring in the Bernheim Cedar Grove Wildlife Corridor (37.9588, -85.6354), Bullitt County, KY	1	MT425005
<i>F. morrisoni</i> <sup>†</sup>	mor13	Spring 1.2 km southwest of Mustoe (38.3177, -79.6488), Highland County, VA	4	MT425006
<i>F. nickliniana</i>	nic3	Martin Fen, LaGrange County, IN (USNM; acc. no. 2043940)	1	MT425007
<i>F. nickliniana</i> <sup>*</sup>	nic14	Blowing Springs 10 km west of Warm Springs (38.0695, -79.8880), Bath County, VA	30	MT425008-14
<i>F. nickliniana</i>	nic15	Spring by Stoney Creek at Lantz Mills (38.8401, -78.5957), Shenandoah County, VA	10	MT425015-19
<i>F. nickliniana</i>	nic16	Fleenor Spring 11 km north of Bristol (36.6825, -82.1358), Washington County, VA	10	MT425020-27
<i>F. nickliniana</i>		Kalamazoo, MI	1	JX970609
<i>F. orolibas</i> <sup>*</sup>	oro17	Hawksbill Spring in Shenandoah National Park (38.5575, -78.3878), Page County, VA	6	MT425028
<i>F. orolibas</i>	oro18	Spring at the Humpback Visitor Center (37.9709, -78.8987), Augusta County, VA	6	MT425029
<i>F. orolibas</i>	oro19	Hugh Young Cave (37.0237, -81.6793), Tazewell County, VA	5	MT425030-31
<i>F. tartarea</i> <sup>*</sup>	tar1	Organ Cave, Greenbrier County, WV (USNM; acc. no. 2060292)	2	MT425032-33
<i>Bithynia tentaculata</i>				MK308073
<i>Bythinella austriaca</i>				FJ028979
<i>Bythinella pannonica</i>				HQ149623
<i>Bythinella viridis</i>				FJ029102
<i>Emmericia expansilabris</i>				KC810061

The number of sequenced specimens (*n*) is given for samples that are newly reported here. Abbreviations: USNM, National Museum of Natural History, Smithsonian Institution, Washington, DC, USA. Notes: 1, collected and sent to us by R. Weck; 2, collected and sent to us by W. Orndorff, T. Malabad and colleagues in the Virginia Department of Conservation and Recreation; \*, type localities or vicinity of type localities.

The primary purpose of the present study is to determine the species status of a single putative *F. cryptica* specimen recently recovered from Kentucky by surveying mt DNA sequence variation in a broad sample of *Fontigens* populations previously characterized by Hershler *et al.* (1990), comparing levels of intrapopulation, interpopulation and interspecific sequence divergence.

## MATERIAL AND METHODS

The 13 populations examined in the present work are listed in Table 1 and mapped in Figure 1, together with six sequences (one sequence of *Fontigens* and five outgroup sequences) retrieved from GenBank for comparative purposes. Six of our 13 samples were collected from type localities or their close vicinity, and are as follows: tar1, *Fontigens tartarea*; bot6, *F. bottimeri*; mor13, *F. morrisoni*; nic14, *F. nickliniana*; oro17, *F. orolibas*; and ant22, *F. antroecetes*. Samples from almost all the other localities we analysed were previously examined by Hershler *et al.* (1990), and our identifications were taken from that work. The only exception was our putative *F. cryptica* sample from Kentucky.

In an effort to assess intra- and interpopulation mt DNA variation, we sequenced 30 individuals from population nic14, which is from the type locality of *F. nickliniana*, and 10 individuals each for nic15 and nic16.

Genomic DNA was extracted from entire snails using a CTAB protocol (Bucklin, 1992). A partial (709 bp) segment of mt COI corresponding to 'Folmer's fragment' (Folmer *et al.*, 1994) was amplified and sequenced with primers LCO1490 and HCOI2198, following the protocols of Liu, Hershler & Clift (2003). Folmer's COI primers did not work consistently with *F. antroecetes* and *F. orolibas*. We designed two reverse primers COHFantroecetes (5'-AAATGTTGGTACAAAATAGG-3') and COHForolibas (5'-AAATGYTGGTATAAAATTGG-3') specifically for *F. antroecetes* and *F. orolibas*, respectively, to amplify (with LCO1490 as forward primer) a 705-bp fragment of COI. Sequences were determined

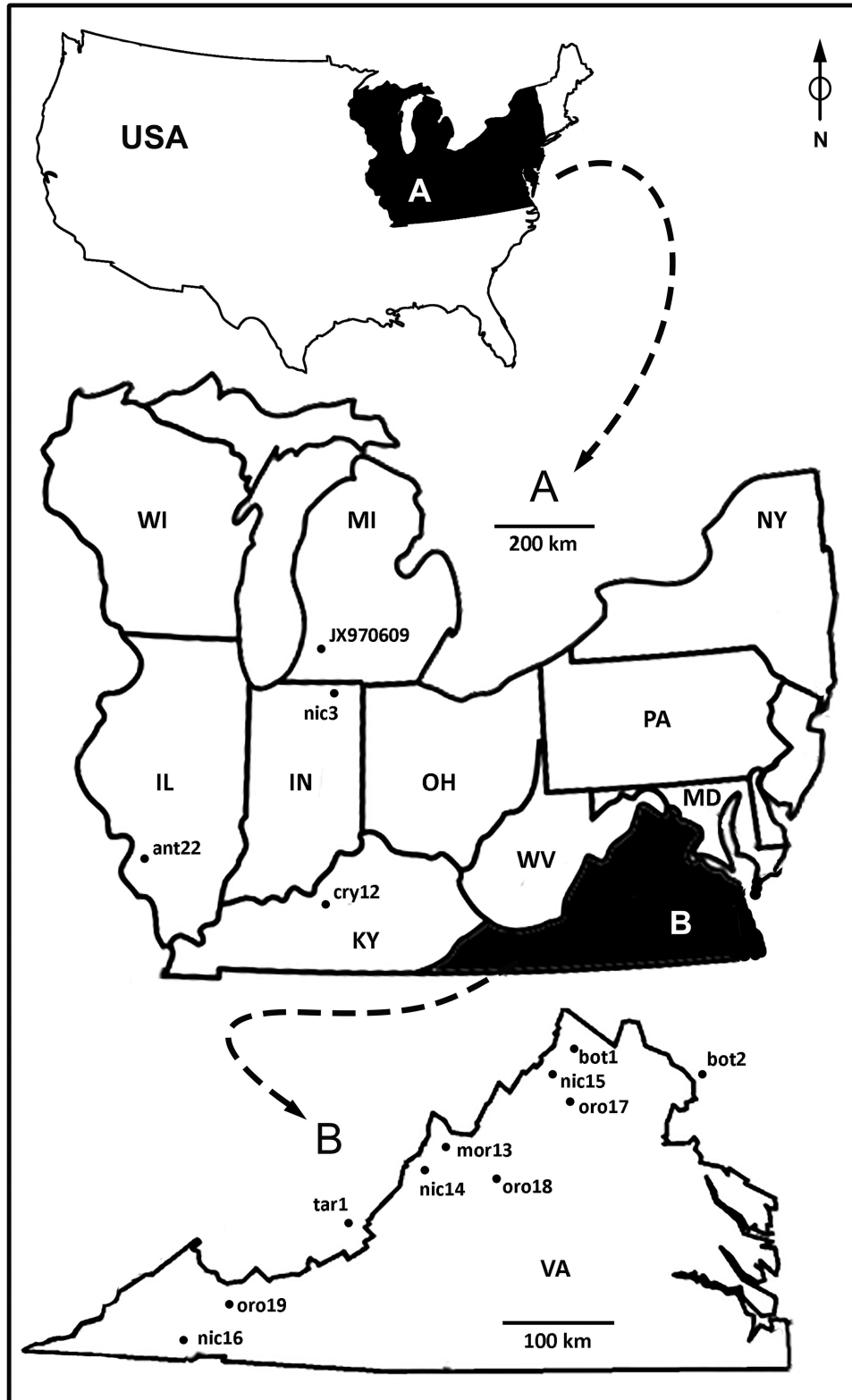
for both strands and then edited and aligned using Sequencher v. 5.4.1 (Gene Codes Corp.; <http://genecodes.com>).

Sequence divergences (uncorrected *p*-distance) within and between phylogenetic lineages were calculated using MEGA7 (Kumar, Stecher & Tamura, 2016), with pairwise deletion for missing data. Phylogenetic analyses were performed using Bayesian inference (BI). In order to provide a readable tree, only one sequence of each haplotype per population sample was used in the phylogenetic analyses. Our molecular phylogenetic analysis included the newly sequenced specimens and six sequences retrieved from GenBank for comparative purposes (see Table 1). Trees were rooted with *Bythinella viridis* (Bythinellidae) (following Wilke *et al.*, 2013). MrModeltest2 (Nylander, 2004) was used to obtain an appropriate substitution model (using the Akaike information criterion) and parameter values for the analyses. BI was performed using MrBayes v. 3.2.7a (Ronquist *et al.*, 2012). In the Bayesian analysis, Metropolis-coupled MCMC simulations were performed with four chains for 5,000,000 generations and Markov chains were sampled at intervals of 10 generations to obtain 500,000 sample points. We used the default settings for the priors on topologies and the HKY + I + G model parameters selected by MrModeltest2 as the best-fit model. At the end of the analysis, the average standard deviation of split frequencies was 0.001 and the potential scale reduction factor was 1, indicating that the runs had reached convergence. The sampled trees with branch lengths were used to generate a 50% majority rule consensus tree, with the first 25% of the samples removed to ensure that the chain sampled a stationary portion.

## RESULTS

The 87 individuals from 13 *Fontigens* populations newly sequenced here returned 32 unique haplotypes (GenBank acc. nos MT425002–MT425033; Table 1).

Genetic diversity within the three populations of *Fontigens nickliniana* we studied intensively was not exceptional (Table 2). The



**Figure 1.** Sampling sites for *Fontigens* in the northeastern quarter of the USA highlighted in black (A), and in Virginia (B). For site/population codes, see Table 1. State abbreviations are as follows: IL, Illinois; IN, Indiana; KY, Kentucky; MD, Maryland; MI, Michigan; NY, New York; OH, Ohio; PA, Pennsylvania; VA, Virginia; WI, Wisconsin; WV, West Virginia.

**Table 2.** Mean (uncorrected) COI sequence divergence within and among populations of *Fontigens nickliniana*.

	nic3	nic14	nic15	nic16
nic3 (LaGrange County, IN)	N/C			
nic14 (Bath County, VA)	0.109	0.003		
nic15 (Shenandoah County, VA)	0.010	0.110	0.009	
nic16 (Washington County, VA)	0.101	0.108	0.104	0.009
<i>F. nickliniana</i> (JX970609; Kalamazoo, MI)	0.003	0.113	0.010	0.107

30 individuals sequenced from the type population of *F. nickliniana* (nic14) returned six haplotypes, with 24 individuals displaying the modal haplotype, and the maximum divergence from that mode just two nucleotides (0.3%). Greater sequence diversity was apparent in the two other *F. nickliniana* populations, the ten individuals sequenced from nic15 returning five distinct haplotypes differing by up to 10 nucleotides (1.5%) and the ten individuals sequenced from nic16 returning eight distinct haplotypes differing by up to 14 nucleotides (2.0%). Similarly, no variation was detected in the six individuals sequenced from two populations of *F. orolibas* (oro17 and oro18) and only two haplotypes that differed by one nucleotide were discovered in the third population of *F. orolibas* (oro19).

A phylogenetic analysis depicting these 13 *Fontigens* populations, together with the singleton *F. nickliniana* sequence previously deposited in GenBank (JX970609) and outgroups, is shown in Figure 2. All conspecific populations formed well-supported clusters, except *F. bottimeri*. All *Fontigens* sequences were resolved as a strongly supported clade. Within that clade, our singleton sample of *F. cryptica* was depicted as the most divergent.

Levels of sequence divergence were high, both among conspecific populations and among species (Tables 2–4). In *F. nickliniana* (Table 2), the mean divergence from population nic16, sampled from the Clinch/Tennessee drainage of southwest Virginia, and nic14, the James drainage population from the type locality, was 10.8% (range 10.3–11.2%). Mean divergences from nic16 and nic14 to nic15, sampled from Virginia's Shenandoah drainage, were 10.4% (range 10.2–11.1%) and 11.0% (range 10.8–11.2%), respectively. Additionally, our singleton samples of *F. nickliniana* from Michigan (JX970609) and Indiana (nic3) nested within the nic15 cluster (Fig. 2).

The mean sequence divergence values recorded among our three populations of *F. orolibas* were even higher than those observed among our three *F. nickliniana* populations (Table 3): 14.3% between oro19 (sampled from a cave in the Tennessee/Clinch drainage) and oro17 (from a spring in the Shenandoah drainage 250 km northeast of oro19), 13.1% between oro19 and oro18 (also from a spring in the Shenandoah drainage about 200 km northeast of oro19) and 8.7% between oro17 and oro18 (separated by *c.* 80 km). The mean sequence divergence (2.3%) we observed between our two populations of *F. bottimeri*, sampled from springs about 100 km apart, was much lower.

The present analysis included six species previously monographed by Hershler *et al.* (1990): *F. nickliniana* (five populations), *F. orolibas* (three populations), *F. bottimeri* (two populations), *F. antroecetes*, *F. morrisoni* and *F. tartarea* (the last three taxa represented by one population each). The mean sequence divergence among the six species ranged from 8.7% to 21% (Table 4). From these six recognized species, the minimum COI sequence distance to our singleton *F. cryptica* sample was 14.5%, and the maximum 19.5%.

In two cases, the minimum divergence (8.7%) observed among well-characterized species of *Fontigens* overlapped the maximum interpopulation divergence (14.3%) observed within well-characterized species. The lowest interspecific value (8.7%) was

recorded between *F. antroecetes* of Missouri (ant22) and *F. bottimeri*. The 9.0% sequence divergence observed between *Fontigens morrisoni* of Virginia (mor13) and *F. tartarea* of West Virginia (tar1) was also relatively low.

## DISCUSSION

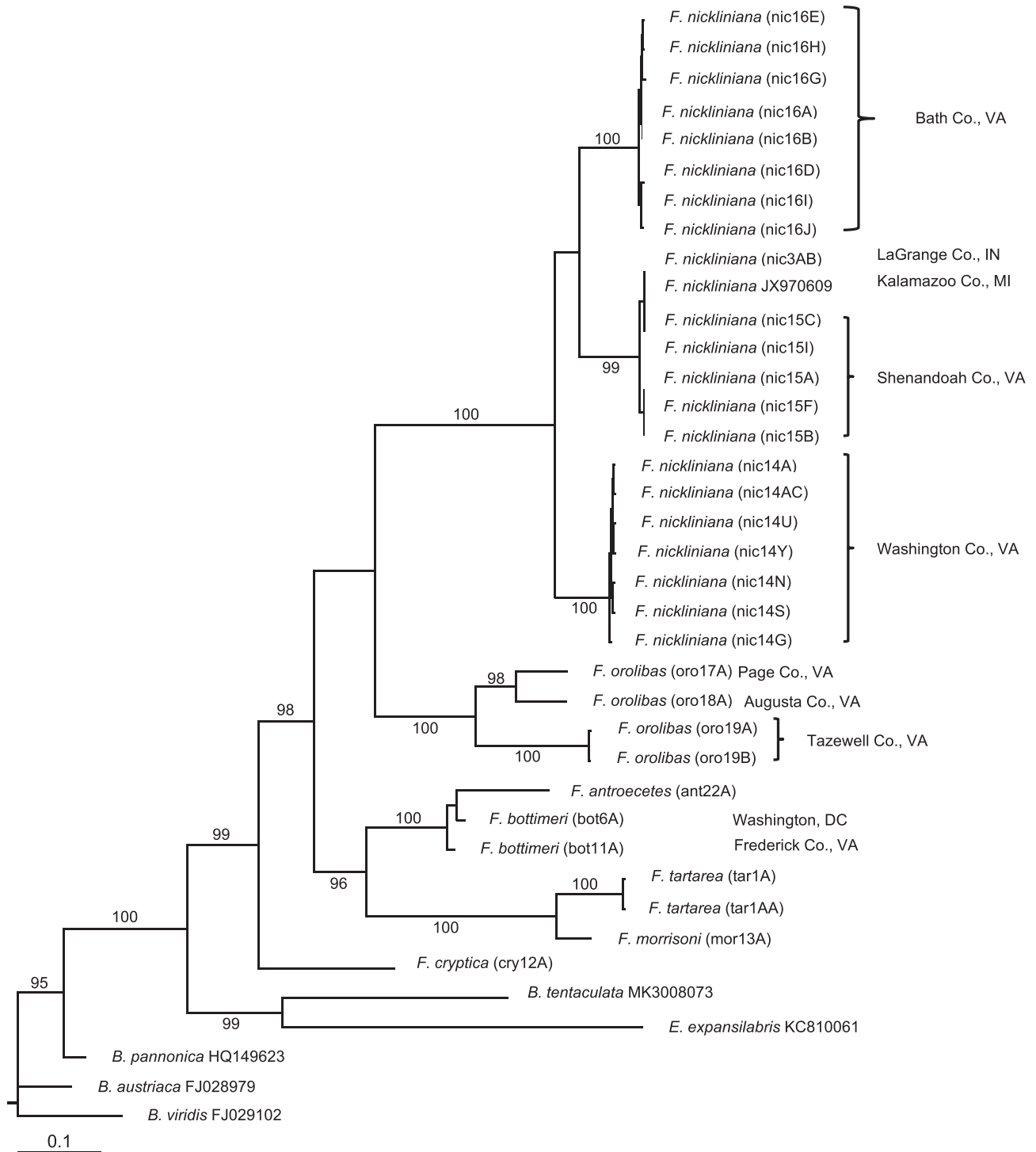
The levels of intrapopulation sequence variation reported here (Tables 2 and 3) do not differ much from values previously reported in truncatelloidean populations worldwide. Surveys in the American West typically return maximum intrapopulation sequence divergences of no more than a few bp (e.g. Hershler & Liu, 2012). Wilke *et al.* (2000) sequenced ten individuals from each of ten populations of the Chinese pomatiopsid *Oncomelania hupensis*, reporting mean intrapopulation divergences of up to 1.96%. Wilke & Falniowski (2001) reported that the maximum sequence divergence across four Croatian populations of the hydrobiid *Adriohydrobia gagatinella* (ten individuals per population) was 1.82%.

Wilke *et al.* (2006) reported that a single population of *Oncomelania* sampled from China's Anning River contained individuals with COI haplotypes differing at as many as 8.5% of their nucleotide positions. This phenomenon, termed “mitochondrial superheterogeneity” by Dillon (2019: 145–170), has been documented in a variety of other freshwater gastropod populations, including North American pleurocerids (Dillon & Frankis, 2004; Dillon & Robinson, 2009; Whelan & Strong, 2016), Old World cerithiaceans (Lee *et al.*, 2007; Miura *et al.*, 2013) and the pulmonate limpet *Laevapex* (Walther *et al.*, 2006). Although we did not observe any cases of mitochondrial superheterogeneity in our sample of *Fontigens*, given the levels of sequence divergence documented here among conspecific populations, the phenomenon does not seem unlikely.

In the American West, the levels of COI sequence divergence among conspecific truncatelloidean populations are often only slightly greater than the levels recorded within populations. Hershler *et al.* (2003a) reported maximum divergences of 0.50% among four populations of *Colligyrus convexus* (Amnicolidae) and 0.17% among seven populations of *Pyrgulopsis archimedis*. In Idaho, 12 populations of *Taylorconcha serpenticola* (Lithoglyphidae) demonstrated a mean sequence divergence of  $0.15 \pm 0.05\%$ , and 4 populations of *Taylorconcha insperata*  $0.18 \pm 0.09\%$  (Hershler *et al.*, 2006). Hershler *et al.* (2011) reported that two populations of *Tryonia peregriana* differed by 0.17–0.50%, two populations of *Tryonia contrerasi* by 0.60%, and two populations of *Tryonia taylori* by 0.3–1.7%. A maximum of just 0.8% sequence difference was recorded from nine populations of *Pyrgulopsis robusta* sampled across three western states in the USA (Hershler & Liu, 2004).

Elsewhere the observed levels of interpopulation COI sequence divergence seem somewhat higher. Wilke *et al.* (2000) reported that the mean nucleotide divergence between ten populations of *O. hupensis* sampled from East China (ten individuals per population) ranged from 0.4% to 2.2%. Wilke & Pfenniger (2002) reported similar results (0–1.98%) among conspecific populations in three species of the estuarine *Hydrobia sensu stricto*. The mean COI sequence divergence between four populations (ten individuals each) of *A. gagatinella* (Hydrobiidae) was lower, ranging from 0.18% to 0.51% (Wilke & Falniowski, 2001).

Observed levels of COI sequence divergence among congeneric species of truncatelloideans worldwide generally average around 3–4%, ranging to minima of *c.* 2% (Wilke *et al.*, 2000; Wilke & Pfenniger, 2002). The survey of Hershler, Liu & Thompson (2003b) returned ranges of 0.5–6.1% among 5 species of *Floridobia*, 1.0–8.5% among 7 species of *Marstonia* and 2.8–11.2% among 17 species of *Pyrgulopsis*. Hershler *et al.* (2007) reported COI sequence



**Figure 2.** Bayesian tree based on the COI dataset. Posterior probabilities values  $\geq 95\%$  are shown for the relevant branches. Scale bar shows number of substitutions per site.

divergences ranging from 4.1% to 18.8% in 9 previously recognized species of *Fluminicola* from California and Oregon, setting a cut point of  $>2\%$  for the recognition of 13 new species. Hershler *et al.* (2014a) set a genetic distance  $\geq 1.9\%$  for the description of 13 new species of *Tryonia* in the Chihuahuan Desert.

Liu, Hershler & Hovingh (2018) extracted a set of 666 *Pyrgulopsis* COI sequences from GenBank, representing 122 currently

recognized species. Their analysis showed 95% of all intraspecific comparisons had uncorrected pairwise distances of  $<3.2\%$ , and the maximum sequence divergence among any pair of *Pyrgulopsis* species in the database was *c.* 12%.

In comparison with the published truncatelloidean studies reviewed above, the interpopulation COI sequence divergence reported here seems atypically high. The overall mean COI



**Table 3.** Mean (uncorrected) COI sequence divergence within and among populations of *Fontigens orolibas*.

	oro17	oro18	oro19
oro17 (Page County, VA)	0.000		
oro18 (Augusta County, VA)	0.087	0.000	
oro19 (Tazewell County, VA)	0.143	0.131	0.002

sequence divergence is 7.7% (range: 0.3–11.3%; Table 2) for five conspecific populations of *F. nickliniana* and 10.6% (range: 8.7–14.3%; Table 3) for three conspecific populations of *F. orolibas*. However, in comparison with other invertebrate groups, high interpopulation sequence divergence is not uncommon. Boyer, Baker & Giribet (2007) reported the average pairwise differences between populations for COI ranged from 0% to 19.2% in the widespread mite harvestmen *Aoraki denticulata* endemic to New Zealand. Juan, Oromi & Hewitt (1996) found interpopulation COI divergences of 5–12% in the darkling beetle *Heteroglyphus politus*.

Most previously published studies have focused on truncatelloidean populations of the American West, however, which are believed to be relatively young. Molecular clock analyses have generally suggested Neogene origins for the genus-level clades, with subsequent Pleistocene dispersal (Hershler *et al.*, 1999, 2006). We do not have any hypothesis regarding the dates for the evolution and dispersal of *Fontigens* in the East but suggest that these events must be much older.

Dillon & Robinson (2009) have hypothesized that the freshwater snail populations of the family Pleuroceridae, which is widespread in streams of the Southern Appalachians today, may have arisen during or, possibly even, prior to the Appalachian orogeny. The regions of Virginia and West Virginia, which comprise the centre of *Fontigens* diversity today and broadly overlap the Dillon & Robinson study area, are in general underlain by Ordovician limestones. Our samples of *F. orolibas* oro17 and oro18 were collected on the Blue Ridge, from springs in rock of Proterozoic age.

Given the elevated genetic distance demonstrated among our three Virginia populations of *F. nickliniana*, the relatively negligible COI sequence divergence we uncovered among our samples from Indiana, Michigan and Lantz Mills, Virginia was most unexpected. This phenomenon suggests to us that the COI sequences we obtained from these three populations approximate an ancestral sequence, common when *F. nickliniana* colonized a vast region of eastern North America, under some conditions very different from today. Other sequences, such as those recovered from nic14 and nic16, seem to have evolved subsequently, under the present conditions where dispersal is extremely rare.

The smallest COI sequence divergence observed between the single putative specimens of *F. cryptica* sampled from the Bernheim property in Kentucky and other *Fontigens* species analysed in this

study was with *F. bottimeri* at 14.5%. *Fontigens bottimeri* is a distinctly different snail, bearing a stubby shell and with the apertural margin often thickened. Populations of *F. bottimeri* primarily inhabit spring runs, those ranging from the vicinity of Washington, DC to northern Virginia and western Maryland. Also, in this species, the eyes are pigmented, unlike our single putative *F. cryptica* from Kentucky.

Indeed, the individual snail we sampled from the springhead on the property of the Bernheim Arboretum and Research Forest, c. 30 km south of Louisville, is the first *Fontigens* of any species to have ever been reported from the Commonwealth of Kentucky. Hershler *et al.* (1990) monographed three species that we did not analyse here, none of which bear shells similar to our putative *F. cryptica*. Of these, *F. turritella* is endemic to a few neighbouring caves in West Virginia, *F. proserpina* is endemic to a few neighbouring caves in Missouri and *F. aldrichi* is restricted to Missouri and Illinois.

The shell morphology of our single Kentucky individual does match that of *F. cryptica*, as described by Hubricht (1963) from a spring on the Indiana side of the Ohio River just 40 km north of Louisville. We still lack anatomical observations on the Kentucky population, as indeed no anatomical observations were originally reported by Hubricht for *F. cryptica*. However, the level of COI sequence divergence from six other *Fontigens* species we have documented here, together with the distinctiveness of the shell morphology, habitat and range of *F. cryptica*, suggests strongly that *F. cryptica* is a valid biological species and that a springhead on the property of the Bernheim Arboretum and Research Forest, south of Louisville, is inhabited by a population of it. Nevertheless, our conclusion is based only on a single partial mt gene sequence.

Questions remain regarding the population status, natural history and distribution of *Fontigens* in the Ohio River Valley, broadly. Clearly, a larger sample of *F. cryptica* populations will be essential to document genetic divergence and anatomical detail, and surveys should extend beyond the knob lands of central Kentucky to the southern Indiana type locality of the species. Broader and more complete studies of the entire regional groundwater fauna would be a service to the cause of conservation biology generally.

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**Table 4.** Mean (uncorrected) COI sequence divergence within and among species of *Fontigens*.

	<i>F. antroecetes</i>	<i>F. bottimeri</i>	<i>F. cryptica</i>	<i>F. morrisoni</i>	<i>F. nickliniana</i>	<i>F. orolibas</i>	<i>F. tartarea</i>
<i>F. antroecetes</i>	N/C						
<i>F. bottimeri</i>	0.087	0.023					
<i>F. cryptica</i>	0.173	0.145	N/C				
<i>F. morrisoni</i>	0.178	0.156	0.185	N/C			
<i>F. nickliniana</i>	0.191	0.191	0.189	0.210	0.077		
<i>F. orolibas</i>	0.180	0.169	0.179	0.189	0.191	0.106	
<i>F. tartarea</i>	0.180	0.173	0.195	0.090	0.201	0.192	0.003

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