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AGRAFIA SZAROWSKA ET FALNIOWSKI, 2011 (CAENOGASTROPODA: HYDROBIIDAE) IN THE CAUCASUS

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ABSTRACT: Freshwater gastropods of the Caucasus are poorly known. A few minute *Belgrandiella*-like gastropods were found in three springs in Georgia. Molecular markers: mitochondrial cytochrome oxidase subunit I (COI) and nuclear histone (H3) were used to infer their phylogenetic relationships. The phylogenetic trees placed them most closely to *Agrafia* from continental Greece. The p-distances indicated that two species occurred in the three localities. Two specimens from Andros Island (Greece) were also assigned to the genus *Agrafia*. The p-distances between the four taxa, most probably each representing a distinct species, were within the range of 0.026–0.043 for H3, and 0.089–0.118 for COI.

KEY WORDS: spring snail, DNA, Georgia, Andros, phylogeny

INTRODUCTION

The freshwater gastropods of Georgia, as well as those of all the Caucasus, are still poorly studied; this concerns especially the minute representatives of the Truncatelloidea. About ten species inhabiting karst springs and caves have been found so far (SHADIN 1932, 1952, TZVETKOV 1940, STAROBOGATOV 1962, KANTOR et al. 2010, VINARSKI et al. 2014, BARJADZE et al. 2015, CHERTOPRUD et al. 2016, VINARSKI & KANTOR 2016, SITNIKOVA et al. 2017). Morphology and anatomy of the soft parts are known only for some of them, not to mention DNA sequences used in phylogeny reconstruction.

Belgrandiella A. J. Wagner, 1927 is a group of minute, dioecious, caenogastropod snails, stygobionts, known to inhabit caves and springs of southern Europe, from Spain across Austria and the southern Balkans to the Caucasus and Asia Minor. However, ‘*Belgrandiella*’ determined or described in this way include nearly all minute conical or turritiform-shelled caenogastropods with a moderately high spire. Anatomically, several genera were recognised within the European ‘*Belgrandiella*’: *Litthabittella* Boeters, 1970,

Graziana Radoman, 1975, *Pontobelgrandiella* Radoman, 1973, and *Alzoniella* Giusti et Bodon, 1984. They are morphologically distinguishable but only slightly different, and molecularly represent not closely related lineages. All the Bulgarian ‘*Belgrandiella*’ belong to the genus *Pontobelgrandiella* (RYSIEWSKA et al. 2016, GEORGIEV et al. 2017) and the Slovak ones represent *Alzoniella* (SZAROWSKA et al. 2011); no Greek ‘*Belgrandiella*’ listed by SCHÜTT (1980) belongs to this genus (RADOMAN 1985, SZAROWSKA 2006, FALNIOWSKI et al. 2012a). Thus, the ‘real’ *Belgrandiella* is distributed from southern Germany and Austria, across northern Italy, Slovenia and Croatia, to Bosnia and Herzegovina (GIUSTI & PEZZOLI 1980, RADOMAN 1983, 1985, KABAT & HERSHLER 1993, BOETERS 1998, GLÖER 2002, FALNIOWSKI & BERAN 2015). The anatomy of female reproductive organs as well as the penis morphology are necessary to identify the genus and often to distinguish between species (e.g. BOETERS 1970, 1998, GIUSTI & PEZZOLI 1980, BODON 1988, HAASE 1993, 1994, 1996, HAASE et al. 2000, GLÖER 2002). On the other hand, anatomical differences

between closely related congeners are not always to be expected (e.g. RADOMAN 1983). The 'lock-and-key' mechanism needs not to work, especially in molluscs, without any sclerotised structures, and the variation of the reproductive/copulatory organs is striking, although not always noted (FALNIOWSKI 1987, SZAROWSKA & FALNIOWSKI 2008, FALNIOWSKI & SZAROWSKA 2011, FALNIOWSKI et al. 2012a). On the other hand, miniaturisation coupled with adaptations to freshwater habitats (osmoregulation, internal fertilisation, embryonic development inside a capsule) has resulted in a simple and uniform anatomy. Thus, molecular data are crucial for reconstruction of phylogenetic relationships within the Truncatelloidea.

SZAROWSKA & FALNIOWSKI (2011) described another 'Belgrandiella'-like minute gastropod, representing a new monotypic genus *Agrafia* with the type species *Agrafia wiktoria* Szarowska et Falniowski, 2011, from the Agrafa Mts in Thessalia, Northern Greece.

Recently a few shells of *Belgrandiella*-like gastropods were collected in springs in Georgia (Table 1, Fig. 1). In each locality only 1–2 live specimens (including juveniles) were found. Thus, no anatomical examination was possible. The aim of this study was to infer phylogenetic relationships of those snails by means of molecular markers. Additionally, we sequenced one of the two *Belgrandiella*-like gastropods collected in 2013 on Andros Island, Greece.

MATERIAL AND METHODS

The snails were collected in three localities in Georgia and one in Greece (Andros) (Table 1, Fig. 1). The Caucasian localities (1–3 in Table 1) were not typical karst springs: 1 – an outflow from a metal pipe well-paved with Lower and Middle Eocene marls and quartzite sandstones and conglomerates, situated in a forest; 2 – small spring at the contact of clastic-limestone with argillites at the bottom and clay shales on top, close to the river bank in a shaded forested place;

3 – two small springs on Upper Cretaceous clastic bedded limestone, artificial wall with metal pipe and a small sedimentary basin, inside a forested area below a walkway. Empty shells and live specimens were obtained from the springs by visual search and washing of vegetation and substratum with the use of either hand-net or metal sieve. The samples were preserved in 70–80 % ethanol and then transferred to 80% ethanol. The shells were photographed with

Table 1. Sampling localities with geographical coordinates

ID	Species	Site	Coordinates	Leg.
1	1117, <i>Agrafia</i> sp. 1	Adzharia Autonomous Republic, Batumi, Makhinjauri, spring near Shua, Georgia	41°40'32"N 41°42'25"E	J. GREGO
2	1118, <i>Agrafia</i> sp. 1	Samegrelo-Zemo, Svaneti Region, Mukhuri, right bank of Khobistskhali River, Georgia	42°39'17"N 42°13'27"E	J. GREGO
3	1123, <i>Agrafia</i> sp. 2	Imereti Region, Zeda Gordi, Dadiani Forest above Okatse Canyon, Georgia	42°27'20"N 42°31'47"E	J. GREGO
4	8N10; <i>Agrafia</i> sp. 3	Ammolochas, Vrysi Mourias, Andros Island, Greece	37°55'45"N 24°46'12"E	A. FALNIOWSKI
5	<i>Agrafia wiktoria</i>	Evrytania, Agrafa Mts, Greece	39°22'07"N 21°37'53"E	M. SZAROWSKA



Fig. 1. Localities of the studied *Agrafia* species: 1–2 – *Agrafia* sp. 1, Caucasus; 3 – *Agrafia* sp. 2, Caucasus; 4 – *Agrafia* sp. 3, Andros Island; 5 – *A. wiktoria*, Thessalia

a CANON EOS 50D digital camera, under a NIKON SMZ18 microscope with dark field.

DNA was extracted from foot tissue using a Sherlock extraction kit (A&A Biotechnology) and dissolved in 20 ml of tris-EDTA buffer. Details of PCR conditions, primers used and sequencing were given in SZAROWSKA et al. (2016). Sequences were initially aligned in MUSCLE (EDGAR 2004) pro-

gramme in MEGA 6 (TAMURA et al. 2013) and then checked in Bioedit 7.1.3.0 (HALL 1999). The saturation test (XIA 2000, XIA et al. 2003) was performed using DAMBE (XIA 2013). In the phylogenetic analysis additional sequences from GenBank were used as reference (Table 2). The data were analysed using approaches based on Bayesian inference and maximum likelihood (ML). We applied the GTR mod-

Table 2. Data obtained from the GenBank Nucleotide database. Names of taxa used for phylogenetic analyses with their accession numbers and references are provided

Species	COI	H3	References
<i>Agrafia wiktoriae</i> Szarowska et Falniowski, 2011	JF906762- JF906765	MG543157- MG543158	SZAROWSKA & FALNIOWSKI (2011: COI) This paper (H3)
<i>Agrafia</i> sp. 1.1	MG543150	MG543153	This paper
<i>Agrafia</i> sp. 1.2	MG543151	MG543154	This paper
<i>Agrafia</i> sp. 2	–	MG543155	This paper
<i>Agrafia</i> sp. 3	MG543152	MG543156	This paper
<i>Alzoniella finalina</i> Giusti & Bodon, 1984	AF367650		WILKE et al. (2001)
<i>Anagastina zetavalis</i> (Radoman, 1973)	EF070616		SZAROWSKA (2006)
<i>Avenionia brevis berengueri</i> (Draparnaud, 1805)	AF367638		WILKE et al. (2001)
<i>Belgrandiella zermanica</i> Radoman, 1973	KT218511		FALNIOWSKI & BERAN (2015)
<i>Bithynia tentaculata</i> (Linnaeus, 1758)	AF367643	MG551350	This paper
<i>Bythinella austriaca</i> (von Frauenfeld, 1857)	JQ639858		WILKE et al. (2001)
<i>Bythinella micherdzinskii</i> Falniowski, 1980	JQ639854		FALNIOWSKI et al. (2012b)
<i>Dalmatinella fluviatilis</i> Radoman, 1973	KC344541		FALNIOWSKI et al. (2012b)
<i>Daphniola lousi</i> Falniowski & Szarowska, 2000	KM887915		FALNIOWSKI & SZAROWSKA (2013)
<i>Ecrobia maritima</i> (Milaschewitsch, 1916)	KJ406200		SZAROWSKA et al. (2014a)
		MG551322	SZAROWSKA & FALNIOWSKI (2014b)
			This paper
<i>Emmericia expansilabris</i> Bourguignat, 1880	KC810060		SZAROWSKA & FALNIOWSKI (2013a)
<i>Fissuria boui</i> Boeters, 1981	AF367654		WILKE et al. (2001)
<i>Graziana alpestris</i> (Frauenfeld, 1863)	AF367641		WILKE et al. (2001)
<i>Grossuana codreanu</i> (Grossu, 1946)	EF061919		SZAROWSKA et al. (2007)
<i>Hauffenia michleri</i> Kuščer, 1932	–	KY087878	RYŚIEWSKA et al. (2017)
<i>Heleobia dobrogica</i> (Grossu & Negrea, 1989)	EU938131		FALNIOWSKI et al. (2008)
<i>Horatia klecakiana</i> Bourguignat 1887	KJ159128		SZAROWSKA & FALNIOWSKI (2014a)
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF278808		WILKE et al. (2000)
<i>Islamia zermanica</i> Radoman, 1973	KU662362		BERAN et al. (2016)
		MG551320	This paper
<i>Lithoglyphus prasinus</i> (Küster, 1852)	JX073651		FALNIOWSKI & SZAROWSKA (2012)
<i>Littorina littorea</i> (Linnaeus, 1758)	KF644330		LAYTON et al. (2014)
		KP113574	NERETINA (2014) unpublished
<i>Kerkia kusceri</i> (Bole, 1961)	–	KY087884	RYŚIEWSKA et al. (2017)
<i>Marstoniopsis insubrica</i> (Küster, 1853)	AF322408		FALNIOWSKI & WILKE (2001)
<i>Moitessieria</i> cf. <i>puteana</i>	AF367635		WILKE et al. (2001)
<i>Montenegrospeum bogici</i> (Pešić & Glöer, 2012)	KM875510		FALNIOWSKI et al. (2014)
<i>Onobops jacksoni</i> (Bartsch, 1953)	AF367645		WILKE et al. (2001)
<i>Peringia ulvae</i> (Pennant, 1777)	AF118302		WILKE & DAVIS (2000)
<i>Pontobelgrandiella</i> sp.	KU497013		RYŚIEWSKA et al. (2016)
		MG551321	This paper
<i>Pseudamnicola</i> sp.	–	KT710736	SZAROWSKA et al. (2016)
<i>Radomaniola curta</i> (Küster, 1853)	KC011814		FALNIOWSKI et al. (2012a)
<i>Sadleriana fluminensis</i> (Küster, 1853)	KF193067		SZAROWSKA & FALNIOWSKI (2013b)
<i>Salenthydrobia ferrerii</i> Wilke, 2003	AF449213		WILKE (2003)
<i>Semisalsa dalmatica</i> Radoman, 1974	AF367631		WILKE et al. (2001)
<i>Tanousia zermaniae</i> (Brusina, 1866)	KU041812		BERAN et al. (2015)
<i>Truncatella pulchella</i> Pfeiffer, 1839	AF253085		DAVIS et al. (1998)
<i>Truncatella scalaris</i> (Michaud, 1830)	JX970621		WILKE et al. (2013)

el whose parameters were estimated by RaxML (STAMATAKIS 2014).

The Bayesian analyses were run using MrBayes v. 3.2.3 (RONQUIST et al. 2012) with default priors. Two simultaneous analyses were performed, each with 10,000,000 generations, with one cold chain and three heated chains, starting from random trees and sampling the trees every 1,000 generations. The first 25% of the trees were discarded as burn-in. The analyses were summarised as a 50% majority-rule tree. The ML approach was applied with RAXML v.

8.0.24 (STAMATAKIS 2014). One thousand searches were started with initial trees obtained using the randomised stepwise addition maximum parsimony method. The tree with the highest likelihood score was considered as the best representation of phylogeny. Bootstrap support was calculated with 1,000 replicates and summarised on the best ML tree. RAXML analyses were performed using the free computational resource CIPRES Science Gateway (MILLER et al. 2010).

RESULTS

All shells from locality 1 (Figs 2–5), with still incomplete peristomes, were juvenile. Two (Figs 6–7) of the three (Figs 6–8) shells in locality 2 were adult. The single specimen from locality 3 (Fig. 9) was also juvenile, but the shell habitus differed from one of the other presented shells (narrow spire, broad body whorl) more than could be explained by ontogeny. The shells from Andros Island (Figs 10–11) resembled the ones from locality 2 in Georgia (Figs 6–7), as well as the ones of *Agrafia wiktoria* from northern Greece (Figs 12–13).

We sequenced histone H3 in six specimens (314 bp, GB Accession Numbers MG543153–MG543158) and cytochrome oxidase subunit I (COI; 442 bp; GB Accession Numbers MG543150–MG543152) in three specimens. Both H3 and COI sequences were identical in localities 1 and 2 (Table 3). The p-distances between the four taxa were within the range of 0.026–0.043 for H3, and 0.089–0.118 for COI (Table 3). For coding of COI, XIA's (2013) saturation test revealed a significant degree of saturation in the third position of the sequences. In the truncatelloids, COI approaches saturation of ca. 18.6% or 120 nucleotide differences (DAVIS et al. 1998, WILKE et al.

2000), which seems to happen after approximately 10 million years. However, to avoid a substantial loss of information in the case of closely related species, this position was not excluded from the dataset and was used for the analysis.

The maximum likelihood (ML) tree for COI (Fig. 14) placed all the Caucasian and Greek populations within the subfamily Sadlerianinae in one, monophyletic and well supported clade (bootstrap value 77%), whose sister clade grouped *Pontobelgrandiella*, *Fissuria* Boeters, 1981, *Avenionia* Nicolas, 1882, *Alzoniella* and *Islamia* Radoman, 1973, but neither *Belgrandiella* nor *Graziana*. The ML tree for H3 (Fig. 15) clearly showed distinctness of the two Caucasian and two Greek (Agrafa and Andros Island) taxa, the mean p-distance between these two subclades being 0.099 for COI and 0.035 for H3. The divergence level between the Caucasian/Greek populations and *Belgrandiella* was higher, the p-distance was 0.124 for COI and 0.054 for H3. In both COI and H3 trees the snails from the five studied populations formed a monophyletic group, and in both the population from Andros Island clustered between the ones from Georgia and continental Greece.

DISCUSSION

Considering the inferred H3 tree, localities 1 and 2 hold the same taxon, while the taxa at each of the other localities (3–5) are distinct. Most probably, the two Georgian taxa belong to the genus *Agrafia*, like the snails from Andros Island, and most probably each taxon represents a distinct species. Anyway, they form a distinct clade, not close to *Belgrandiella*.

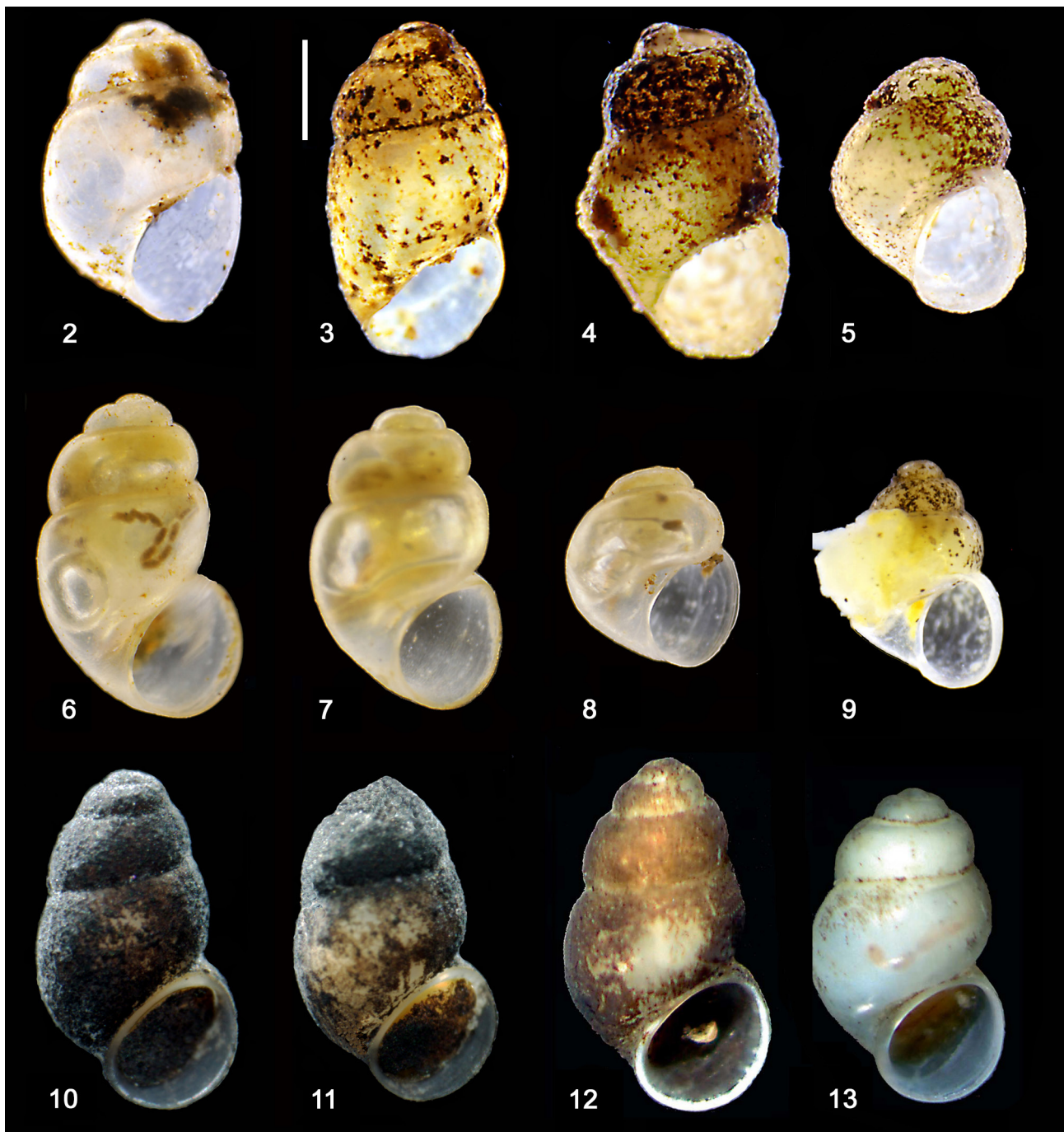
The shells from locality 1 (Figs 2–5) are juvenile, making species identification impossible. Two of the shells from locality 2 (Figs 6–7) are adult and resemble *Belgrandiella caucasica* Starobogatov, 1962 (type specimens shown by SITNIKOVA et al. 2017). Thus, the proper name of the taxon should be *Agrafia caucasica* (Starobogatov, 1962). The specimen from locality

3 is similar to *Plagigeyeria horatieformis* (Starobogatov, 1962), or *Pontohoratia* spp. (SITNIKOVA et al. 2017).

Undoubtedly, all four species form one clade. Considering the p-distances, each taxon should represent a distinct species (e.g. SZAROWSKA et al. 2007, 2016, FALNIOWSKI & SZAROWSKA 2011, 2012, 2013b, BERAN et al. 2016), and for congeneric species the distances are relatively high. However, it still seems justified to assign the two Caucasian and the two Greek species to the genus *Agrafia*. The geographical distance between the two areas exceeds 1,500 km. *Agrafia* must thus occur also in Asia Minor, but – with its minute shell resembling those of *Belgrandiella* – it may be easily overlooked.

Table 3. p-distances for COI (above diagonal) and H3 (below diagonal); 1.1 and 1.2 – *Agrafia* sp. 1 from localities 1 and 2 (Fig. 1), respectively

	<i>Agrafia</i> sp. 1.1	<i>Agrafia</i> sp. 1.2	<i>Agrafia</i> sp. 2	<i>Agrafia</i> sp. 3	<i>Agrafia wiktori</i>
<i>Agrafia</i> sp. 1.1		0.000	–	0.118	0.089
<i>Agrafia</i> sp. 1.2	0.000		–	0.118	0.089
<i>Agrafia</i> sp. 2	0.026	0.026		–	–
<i>Agrafia</i> sp. 3	0.032	0.032	0.026		0.095
<i>Agrafia wiktori</i>	0.043	0.043	0.037	0.024	



Figs 2–13. Shells of *Agrafia*: 2–5 – *Agrafia* sp. 1, locality 1 (Makhinjauri, spring, Georgia); 6–8 – *Agrafia* sp. 1, locality 2 (Khobistskhali River, Mukhuri, Georgia); 9 – *Agrafia* sp. 2, locality 3 (Dadiani Forest, Zeda Gordi, Georgia), body whorl punctured, right tentacle and proboscis extruded through the puncture; 10–11 – *Agrafia* sp. 3, locality 4 (Ammolochas, Vrysi Mourias, Andros Island, Greece); 12–13 – *Agrafia wiktori*, paratypes, locality 5 (Evrytania, Agrafa Mts, Greece), after SZAROWSKA & FALNIOWSKI (2011); bar represents 0.5 mm

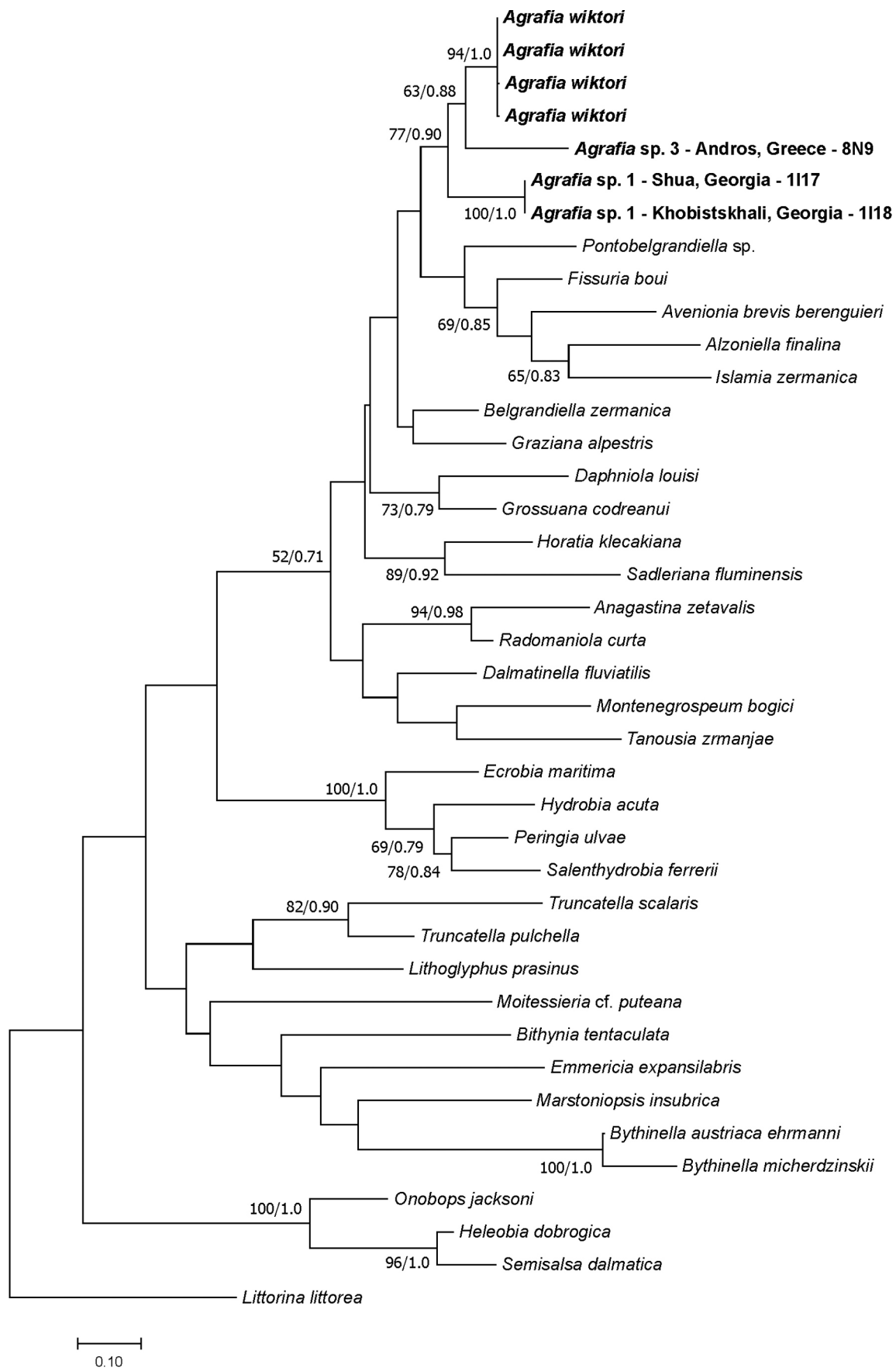


Fig. 14. Maximum likelihood (ML) tree presenting phylogenetic relationships of *Agrafia*, inferred with COI; bootstrap values (>50%) and Bayesian probabilities given

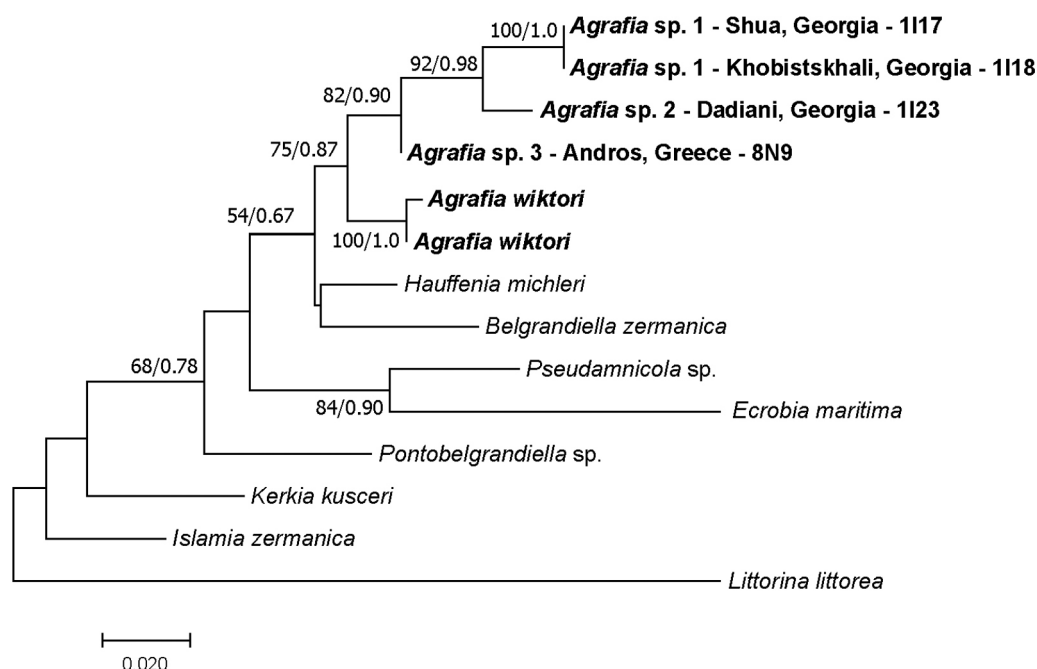


Fig. 15. ML tree presenting relationships between the studied *Agrafia*, inferred with H3; bootstrap values (>50%) and Bayesian probabilities given

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APPENDIX 1

Empty adult shells of most probably *Agrafia* sp. 1 were found also in localities 1 (Figs 16–17) and 2 (Figs 18–19). They resemble the adult sequenced specimens of *Agrafia* sp. 1 (Figs 6–7). Empty adult shells of *Agrafia* sp. 2 (Figs 20–21) and of *Agrafia*-like gastropod, probably representing *Agrafia* cf. sp. 1 (Figs 22–23) were found at locality 3. An *Agrafia*-like empty shell (Fig. 24) was also found in another locality: Imereti Region, Kinchkaperdi, ca. 1 km NE of Kinchkha, 42°30'10"N, 42°33'28"E.



Figs 16–24. Empty shells of adult *Agrafia* from Georgia: 16–17 – *Agrafia* sp. 1, locality 1 (Makhinjauri, spring); 18–19 – *Agrafia* sp. 1, locality 2 (Khobistskhali River, Mukhuri); 20–21 – *Agrafia* sp. 2, locality 3 (Dadiani Forest, Zeda Gordi); 22–23 – *Agrafia* cf. sp. 1, locality 3 (Dadiani Forest, Zeda Gordi); 24 – putative *Agrafia* from the locality not given in Table 1 (Imereti Region, Kinchkaperdi, about 1 km NE of the Kinchkha, 42°30'10"N, 42°33'28"E); bar represents 1 mm. Photo: PETER GLÖER and JOZEF GREGO