

A NEW MONTENEGROSPEUM SPECIES FROM SOUTH CROATIA (MOLLUSCA: GASTROPODA: HYDROBIIDAE)

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ABSTRACT: The extremely rich stygobiont malacofauna of the Balkans is still poorly studied, and the systematics is based mostly on shells whose characters are often misleading. An interesting stygobiont gastropod species was found in several springs feeding the Cetina River in the SW. part of Sinj Basin in Croatia. Its shell resembled the ones of moitessieriid genera *Paladilhioopsis*, *Bythiospeum*, or *Iglica*. Analyses of COI and H3 markers placed it close to the hydrobiid *Montenegrospeum bogici* Pešić et Glöer, 2012 from central Montenegro. It is congeneric but molecularly and morphologically distinct. The new species is described herein as *Montenegrospeum sketi* n. sp.

KEY WORDS: cytochrome oxidase COI, histone H3, molecular phylogeny and species distinctness, shell, stygobiont, gastropod, Balkans

INTRODUCTION

The extremely rich stygobiont malacofauna of the Balkans is still poorly known, and the systematics is based mostly on shells whose characters are often misleading. *Bythiospeum bogici* was described by PEŠIĆ & GLÖER (2012) in the genus *Bythiospeum* Bourguignat, 1882 from subterranean waters of the spring Taban, in the central part of Montenegro near Podgorica. The description was based on empty shells only. Later PEŠIĆ & GLÖER (2013) obtained live specimens and described their soft parts: lack of eyes and pigment, and penis with a lobe at its medial part. They placed *B. bogici* in a new monotypic genus: *Montenegrospeum* Pešić et Glöer, 2013. The species is known from its type locality only (PEŠIĆ & GLÖER 2013). Later, applying molecular markers (mitochondrial cytochrome oxidase subunit I, COI and nuclear 18SrDNA), it was shown that *Montenegrospeum* did not belong to the Moitessieriidae, but to the Hydrobiidae

Troschel, 1857, subfamily Sadlerianinae Radoman, 1973, with *Dalmatinella* Radoman, 1973 as the sister taxon (FALNIOWSKI et al. 2014). This was also confirmed by morphological data (FALNIOWSKI et al. 2014): female reproductive organs with two seminal receptacles (rs_1 and rs_2 ; RADOMAN 1973, 1983), and penis with a lobe on the left side of its median part (PEŠIĆ & GLÖER 2013), also similar to the ones characteristic of several genera of the Sadlerianinae (SZAROWSKA 2006). Recently a few localities were found in Croatia with *Montenegrospeum*-like empty shells and a few live specimens became available for molecular studies. All those localities represented karst habitats. The aim of this paper is to establish the systematic position of the new species, and to describe it as *Montenegrospeum sketi* n. sp., applying shell morphology and molecular markers.

MATERIAL AND METHODS

The studied material was obtained by sieving sandy sediments of karstic springs using microhabitat preferences and sampling method according to GREGO et al. 2017a (Fig. 1).

Three live specimens were collected at two localities (Fig. 1):

1. (1G6) – Izvor Ruda-Beguša, Ruda, sand at the stream bottom below the spring lake, 13 km ESE of Sinj, Split district, Croatia, $43^{\circ}40'06.6''\text{N}$, $16^{\circ}47'45.6''\text{E}$, leg. JOZEF GREGO, 17.03.2017 (Figs 2, 3).
2. (1G7) – Izvor Grab, Grabska Mlinica, 12 km SE of Sinj, Split district, Croatia, $43^{\circ}38'27.4''\text{N}$, $16^{\circ}46'13''\text{E}$, leg. JOZEF GREGO, 16.03.2017 (Fig. 4).

Empty shells were found at another three localities (Fig. 1):

- 1a. Izvor Ruda-Beguša, cave just above the spring zone, sieved from muddy sediment at the cave bottom, 13 km ESE of Sinj, Split district, Croatia, $43^{\circ}40'6.6''\text{N}$, $16^{\circ}47'45.6''\text{E}$, leg. JOZEF GREGO, 17.03.2017 (the locality is very close to the spring zone of the type locality). Many aperture fragments and one empty intact shell were collected (Fig. 5).
3. Vrelo Kosinac on the left side of the road to Gala, sandy sediment at the spring zone. Sinjski Obrovac, Split district, Croatia $43^{\circ}43'48.4''\text{N}$, $16^{\circ}42'03.9''\text{E}$, leg. JOZEF GREGO, 17.03.2017 (only one empty adult shell).

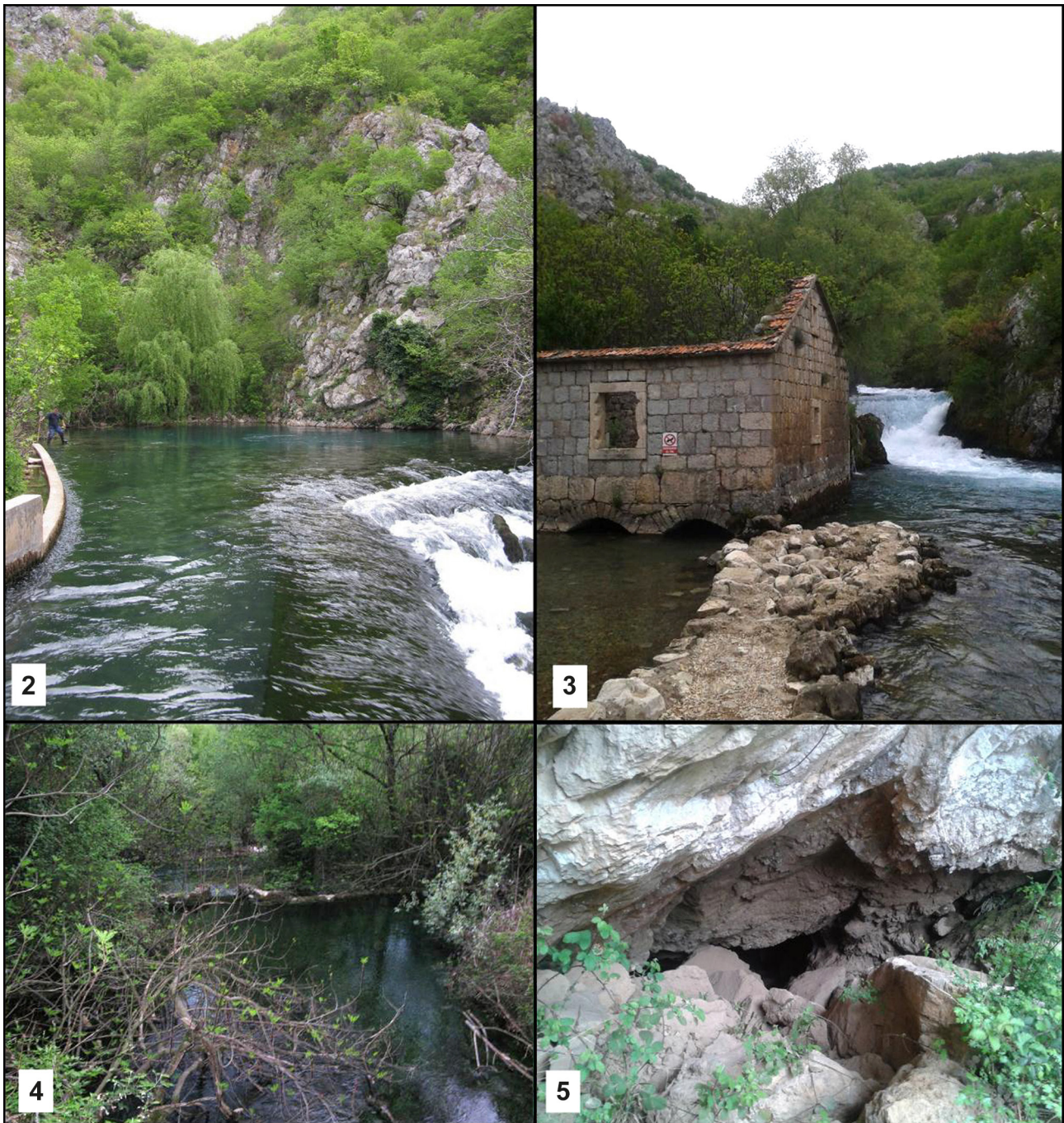
4. Spring Mali Rumin 100 m above the old water-mill, sandy sediment at the millstream, Rumin 6 km NW of Sinjski Obrovac, Split district, Croatia, $43^{\circ}46'49.8''\text{N}$, $16^{\circ}38'55.5''\text{E}$, leg. JOZEF GREGO, 17.03.2017 (only one empty juvenile shell probably representing the species).
5. Type locality of *Montenegrospeum bogici*, after PEŠIĆ & GLÖER (2012).

The gastropods were sieved and extracted from sandy sediments, and fixed in 70–80% ethanol. The live specimens were transferred to 80% analytical ethanol. The shells were photographed with a CANON EOS 50D digital camera, under a NIKON SMZ18 microscope with dark field and with a digital camera system Leica R8 (Leitz Photar 21 mm objective with Novoflex bellows), ImageJ scientific image analysing software (SCHNEIDER et al. 2012) was used for taking measurements together with direct measurement with eye-piece micrometer.

DNA was extracted from foot tissue using a Sherlock extraction kit (A&A Biotechnology) and dissolved in 20 ml of tris-EDTA buffer. For details of PCR conditions, primers used and sequencing see SZAROWSKA et al. (2016). The sequences were initially aligned in the MUSCLE (EDGAR 2004) programme 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



Fig. 1. Localities of the studied *Montenegrospeum* in Croatia (1–4) and Monenegro (5): 1 – Izvor Ruda-Beguša, Ruda, Split district, locus typicus of *M. sketi* n. sp.; 1A – cave near Izvor Beguša, Ruda, Split district; 2 – Izvor Grab, Grabska Mlinica, Split district; 3 – Izvor Kosinac, Sinjski Bobrova Split district; 4 – Izvor Mali Rumin, Rumin, Split district; 5 – Izvor Taban, Podgorica, locus typicus of *M. bogici* (Pešić et Glöer, 2012)



Figs 2–5. Studied localities: 2 – Izvor Ruda-Beguša, Ruda, Split district, locus typicus of *M. sketi* n. sp.; 3 – Izvor Ruda-Beguša, Ruda, old watermill bellow spring zone; 4 – Izvor Grab, Grabska Mlinica, one of the spring outlets where the specimens of *M. sketi* n. sp. were found; 5 – Izvor Ruda-Beguša Ruda, cave above the main spring (Photo: G. JAKAB and B. ŠMÍDA)

as a reference (Table 1). The data were analysed using approaches based on the Bayesian inference and maximum likelihood (ML). We applied the GTR model, whose parameters were estimated by RaxML (STAMATAKIS 2014).

The Bayesian analyses were run using MrBayes v. 3.2.3 (RONQUIST et al. 2012) with the default priors. The GTR model was best fitted to our data. Two simultaneous analyses were performed, each of which lasted 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 initiated with starting trees obtained using the randomized stepwise addition maximum parsimony method. The tree with the highest likelihood score was regarded as the best representation

Table 1. 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 wiktoria</i> Szarowska et Falniowski, 2011	JF906762	-	SZAROWSKA & FALNIEWSKI (2011)
	-	MG543157	GREGO et al. (2017b)
<i>Alzoniella finalina</i> Giusti et 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	-	FALNIEWSKI & BERAN (2015)
	-	MG551350	GREGO et al. (2017b)
<i>Bithynia tentaculata</i> (Linnaeus, 1758)	AF367643	-	WILKE et al. (2001)
<i>Bythinella austriaca</i> (von Frauenfeld, 1857)	JQ639858	-	FALNIEWSKI et al. (2012b)
<i>Bythinella micherdzinskii</i> Falniowski, 1980	JQ639854	-	FALNIEWSKI et al. (2012b)
<i>Dalmanella fluviatilis</i> Radoman, 1973	KC344541	-	FALNIEWSKI & SZAROWSKA (2013)
<i>Daphniola louisii</i> Falniowski et Szarowska, 2000	KM887915	-	SZAROWSKA et al. (2014a)
<i>Emmericia expansilabris</i> Bourguignat, 1880	KC810060	-	SZAROWSKA & FALNIEWSKI (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 codreanui</i> (Grossu, 1946)	EF061919	-	SZAROWSKA et al. (2007)
<i>Hauffenia michleri</i> Kuščer, 1932	-	KY087878	RYSIEWSKA et al. (2017)
<i>Heleobia dobrogica</i> (Grossu et Negrea, 1989)	EU938131	-	FALNIEWSKI et al. (2008)
<i>Horatia klecakiana</i> Bourguignat 1887	KJ159128	-	SZAROWSKA & FALNIEWSKI (2014b)
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF278808	-	WILKE et al. (2000)
<i>Islamia zermanica</i> Radoman, 1973	KU662362	-	BERAN et al. (2016)
	-	MG551320	GREGO et al. (2017b)
<i>Lithoglyphus prasinus</i> (Küster, 1852)	JX073651	-	FALNIEWSKI & SZAROWSKA (2012)
<i>Littorina littorea</i> (Linnaeus, 1758)	KF644330	-	LAYTON et al. (2014)
	-	KP113574	NERETINA (2014) unpublished
<i>Kerkia kusceri</i> (Bole, 1961)	-	KY087884	RYSIEWSKA et al. (2017)
<i>Marstoniopsis insubrica</i> (Küster, 1853)	AF322408	-	FALNIEWSKI & WILKE (2001)
<i>Moitessieria cf. puteana</i>	AF367635	-	WILKE et al. (2001)
<i>Montenegrospeum bogici</i> (Pešić et Glöer, 2012)	KM875510	-	FALNIEWSKI et al. (2014)
	-	MG880218	This paper
<i>Montenegrospeum sketi</i> Grego et Glöer, 2018	MG880216- MG880217	MG880219- MG880220	This paper
<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	-	RYSIEWSKA et al. (2016)
	-	MG551321	GREGO et al. (2017b)
<i>Pseudamnicola</i> sp.	-	KT710736	SZAROWSKA et al. (2016)
<i>Radomaniola curta</i> (Küster, 1853)	KC011814	-	FALNIEWSKI et al. (2012a)
<i>Sadleriana fluminensis</i> (Küster, 1853)	KF193067	-	SZAROWSKA & FALNIEWSKI (2013b)
<i>Salenthydrobia ferrerii</i> Wilke, 2003	AF449213	-	WILKE (2003)
<i>Semisalsa dalmatica</i> Radoman, 1974	AF367631	-	WILKE et al. (2001)
<i>Tanousia zrmanjiae</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)
<i>Ecrobia maritima</i> (Milaschewitsch, 1916)	KJ406200	-	SZAROWSKA & FALNIEWSKI (2014a)
	-	MG551322	GREGO et al. (2017b)

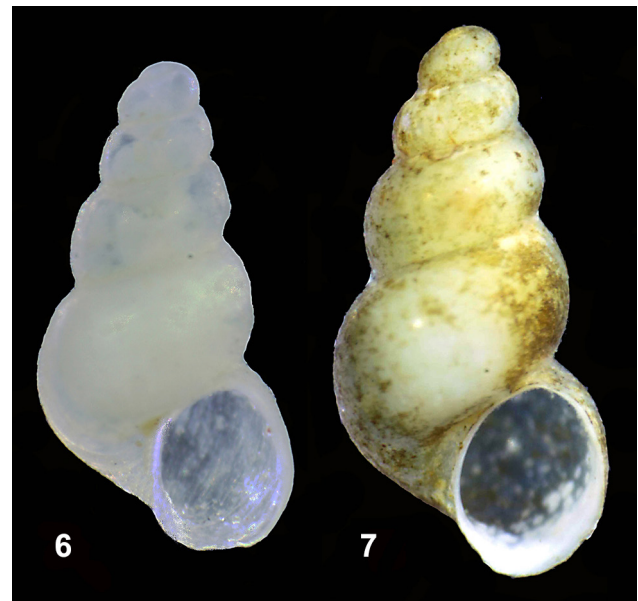
of the phylogeny. Bootstrap support was calculated with 1,000 replicates and summarised in the best ML tree. RAXML analyses were performed using the free computational resource CIPRES Science Gateway (MILLER et al. 2010).

Abbreviations: CNHM – Croatian Natural History Museum, Zagreb; HNHM – Hungarian Natural History Museum, Budapest; H – shell height; W – shell width; BH – height of body whorl; BW – width of body whorl; AH – aperture height; AW – aperture width; LT – locus typicus.

MOLECULAR PART – RESULTS AND DISCUSSION

The sequences were obtained from two specimens (Figs 6, 7). The saturation tests revealed no saturation. The ML tree (Fig. 8) computed for the COI (442 bp, GenBank Accession numbers MG880216-MG880217) clearly showed close relationships of both specimens with *M. bogici* (bootstrap support 100%), and the position of the genus within the Hydrobiidae Troschel, 1857, subfamily Sadlerianinae. However, the bootstrap supports of deeper nodes were small which is typical of COI (e.g. SZAROWSKA 2006). In the tree computed for COI but including only the genera of the Sadlerianinae (Fig. 9) bootstrap 68% (very close to 70% usually accepted as significant enough, although there are no strict rules) supported *Tanousia* Bourguignat in Servain, 1881, as potential sister clade of *Montenegrospeum*, and 90% bootstrap supported (Bayesian probability 0.98) the *Montenegrospeum/Tanousia* and *Dalmatinella* clade.

The p-distance between *M. sketi* and *M. bogici* calculated for COI was 0.011. The relative range of genotypic differentiation, measured as simple p-distance (or, often, K2P distance which is not justified, but the values are only somewhat higher than the ones of p-distance), most often calculated for mitochondrial COI (commonly used in phylogenetic studies: DAVIS et al. 1998), was used (e.g. BICHAIN et al. 2007). It must be stressed, however, that the given values of the distances between closely related but still distinct species may characterise a group of rather closely related species, but may be too low in another group, since there is no general rule defining the threshold value: in some genera the interspecies distances are higher, in some other they are smaller (e.g. WILKE & DAVIS 2000, WILKE 2003, FALNIOWSKI et al. 2007, 2009, SZAROWSKA et al. 2007, FALNIOWSKI & SZAROWSKA 2012, 2013, 2015, SZAROWSKA & FALNIOWSKI 2013a, b, 2014a, b, SZAROWSKA et al. 2014b). The p-distance between



Figs 6–7. Sequenced specimens: 6 – locality 1, 1G6; 7 – locality 2, 1G7

M. bogici and *M. sketi* was close to the threshold value between the intra- and interspecies distances in most of the Truncatelloidea.

In the ML tree computed for histone H3 (308 bp, GenBank Accession numbers MG880218-MG880220) (Fig. 10) *Belgrandiella* Wagner, 1927 was the sister clade of *Montenegrospeum*, bootstrap-support 78%, clearly within the Sadlerianinae. The sister-clade relationship between *M. bogici* and *M. sketi* was 100% supported. The p-distance was 0.010, which is relatively high for this locus (e.g. SZAROWSKA et al. 2016). For each of the presented sets of taxa the Bayesian trees presented identical topologies as in the ML trees, only the Bayesian probabilities, shown in the trees, were not always correlated with the bootstrap supports.

SYSTEMATIC PART (BY J. GREGO & P. GLÖER)

Family: Hydrobiidae Stimpson, 1865

Genus: *Montenegrospeum* Pešić et Glöer, 2013

Type species: *Montenegrospeum bogici* (Pešić et Glöer, 2012)

***Montenegrospeum sketi* n. sp.** (Figs 11, 12)

Diagnosis: The new species differs from the only other known representative of the genus, *Montenegrospeum bogici* Pešić et Glöer, 2012 (Figs 13, 14), in its more elongated conical shell shape, slightly more inflated whorls, more prominent umbilicus as well as in the

different aperture situated more to the right from the columellar axis. The lateral profile of the lip is more forward protruding at its lower end and more sinuated in *M. bogici*. The proportionally smaller aperture of the new species is less produced at the lower part of the shell outline and its margin is less reflected.

Type locality: Croatia, Split district, Ruda, Izvor Ruda-Beguša 13 km ESE of Sinj, sand at the stream bottom below the spring lake, 43°40'06.6"N, 16°47'45.6"E.

Type material: Holotype: CNHM11394 from type locality, leg. JOZEF GREGO, 17.03.2017, Paratypes:

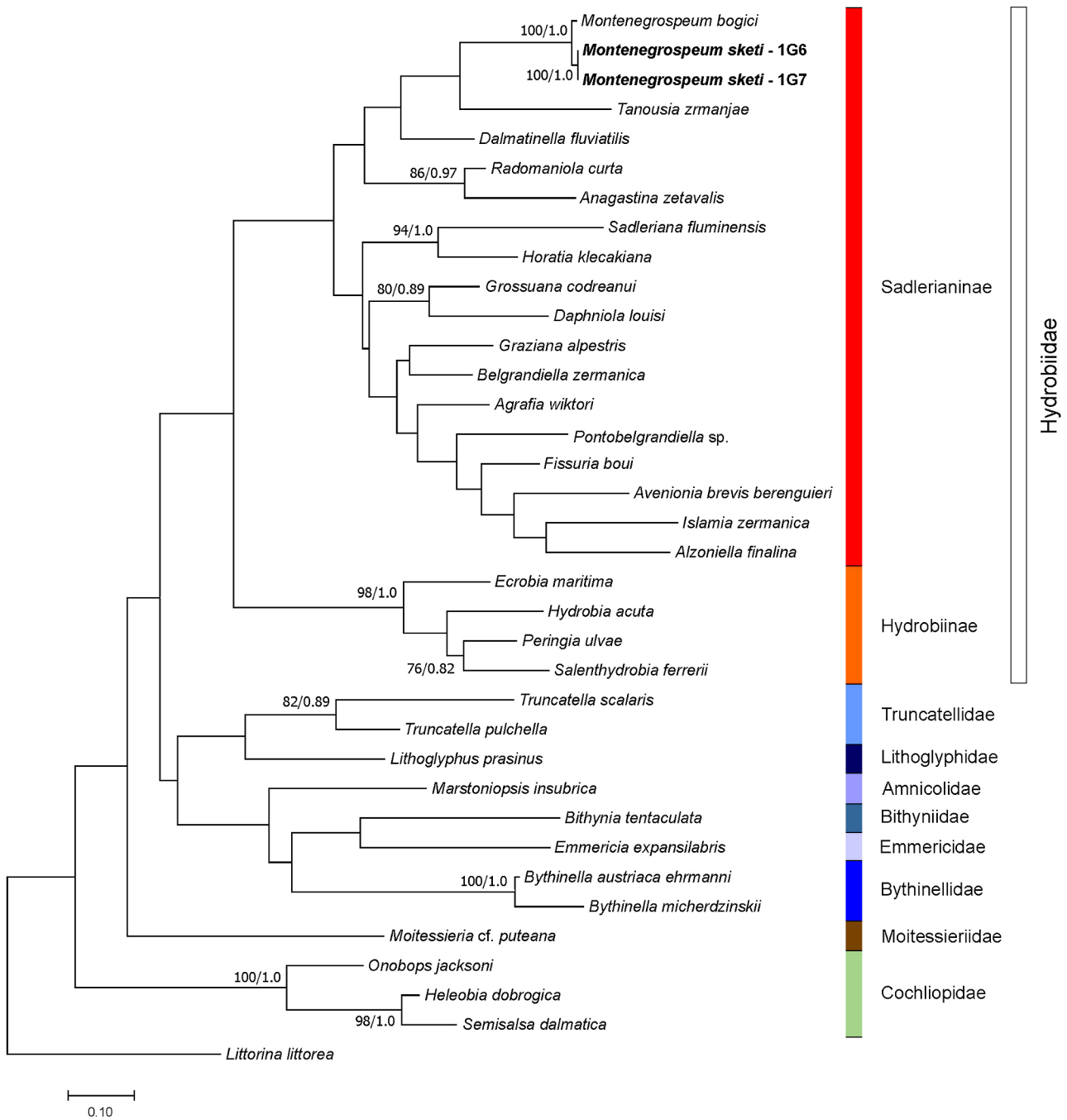


Fig. 8. Maximum likelihood tree of selected Truncatelloidea, computed for COI; bootstrap supports given if >50%, and Bayesian probabilities if >0.80

same data (HNHM102778/1; coll. GREGO/10; coll. GLÖER/1); Croatia, Split district, Grabska Mlinica, Izvor Grab, 12 km SE of Sinj, $43^{\circ}38'27.4''\text{N}$, $16^{\circ}46'13''\text{E}$, leg. JOZEF GREGO, 16.03.2017 (CNHM11395/1, coll. GREGO/7); Croatia, Split district, Sinjski Obrovac, Vrelo Kosinac, on the left side of road to Gala, sandy sediment at the spring zone, $43^{\circ}43'48.4''\text{N}$, $16^{\circ}42'03.9''\text{E}$, leg. JOZEF GREGO, 17.03.2017 (coll. GREGO/1).

Other material: Ruda, Izvor Ruda-Beguša, 13 km ESE of Sinj, Split district, Croatia, Cave right above the spring zone, sieved from muddy sediment at the cave bottom, $43^{\circ}40'06.6''\text{N}$, $16^{\circ}47'45.6''\text{E}$, leg. JOZEF

GREGO, 17.03.2017 (1 whole specimen, and several aperture fragments in coll. GREGO); Sinjski Obrovac, spring Mali Rumin 100 m above the old watermill, sandy sediment at the millstream, Rumin 6 km NW of Sinjski Obrovac, Split district, Croatia, $43^{\circ}46'49.8''\text{N}$, $16^{\circ}38'55.5''\text{E}$, leg. JOZEF GREGO, 17.03.2017 (1 juvenile shell in coll. GREGO).

Etymology: Named in honour of prof. Boris Sket, University of Ljubljana, Slovenia, a well-known expert in biospeleology who contributed much to the knowledge of subterranean invertebrates of the Alps and Dinarides.

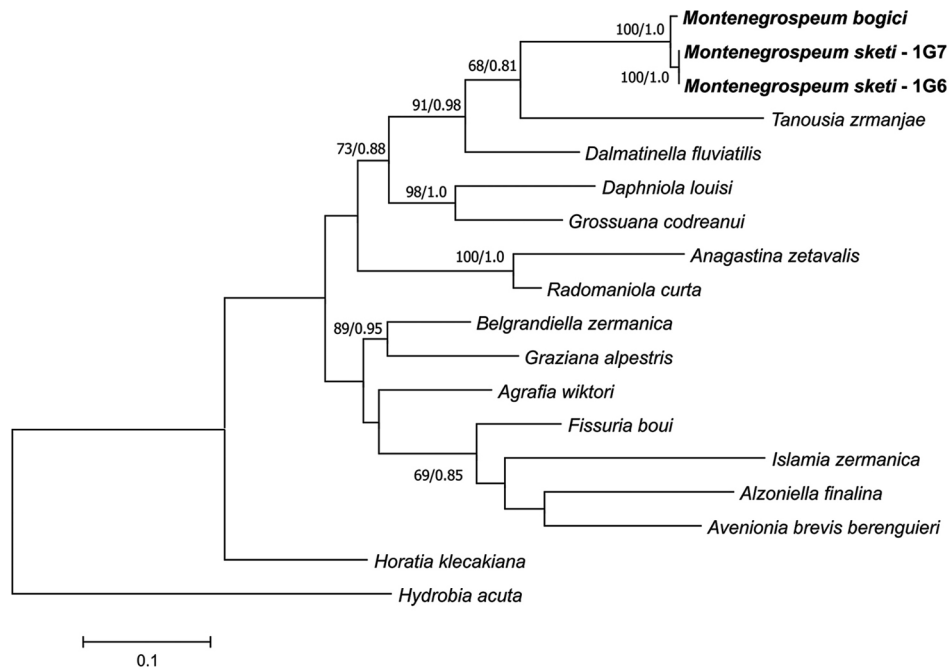


Fig. 9. Maximum likelihood tree of selected Sadlerianinae, computed for COI; bootstrap supports given if >50% and Bayesian probabilities if >0.80

Description: The milky white semi-translucent shell has five convex inflated whorls separated by a deep suture. It is elongated-conical with smooth surface and aperture slightly protruding against the body whorl and its left margin not protruding beyond the columellar axis. The aperture is oval, vertically elongated and attached to the body whorl by a tiny furrow; the peristome slightly outward expanded in some specimens. The umbilicus is tiny and opened. The lateral profile of the lip margin very slightly sinuated and almost parallel with the columellar axis.

Measurements:

Holotype of *M. sketi* sp. n.: H 2.53 mm; W 1.12 mm; BH 1.38 mm; BW 0.95 mm; AH 0.78 mm; AW 0.67 mm; H/W 2.26; AH/AW 1.16; W/BW 1.18; H/BH 1.83; H/AH 3.24; W/AW 1.67.

Holotype of *M. bogici*: H 2.33 mm; W 1.10 mm; BH 1.26 mm; BW 0.88 mm; AH 0.82 mm; AW 0.75 mm; H/W 2.12; AH/AW 1.09; W/BW 1.25; H/BH 1.85; H/AH 2.84; W/AW 1.47.

Habitat: The species is a true subterranean freshwater gastropod inhabiting the cave systems unstudied

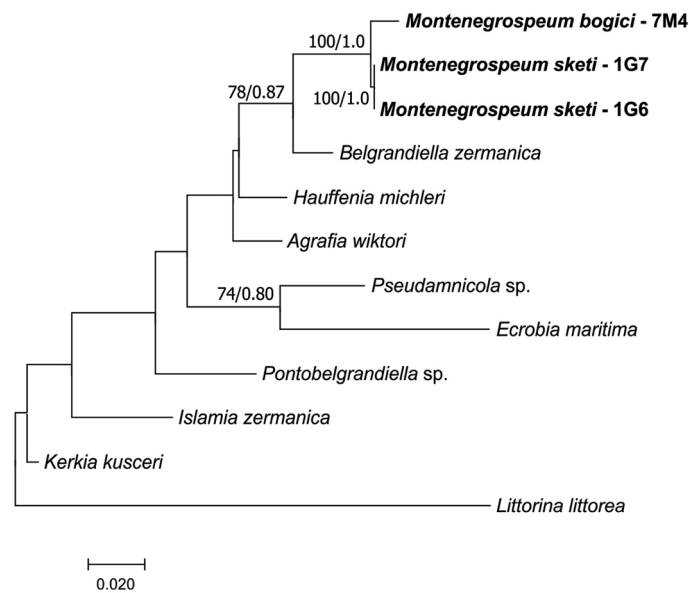


Fig. 10. Maximum likelihood tree of selected Sadlerianinae, computed for H3; bootstrap supports given if >50% and Bayesian probabilities if >0.80



Figs 11–14. Species of *Montenegrospeum*: 11–12 – *M. sketi* n. sp., (holotype); 13–14 – *M. bogici* (holotype)

so far, and adjacent submerged spring debris in the NE part of the Sinj Basin. Empty shells and a few live specimens were found washed out in the sand of the spring zone close to the spring outlets. An empty shell and many fragments were found in the muddy sediment inside the spring in the cave above the Ruda-Beguša spring zone, which indicates its troglobiont origin. All the known localities are remarkable and large karstic springs at the left tributary of the Cetina River draining SW edge of Livansko Polje (Basin) and Buško Jezero (Dam) in Bosnia, towards the Cetina River in Croatia, through unexplored cave passages under the Dinaric Alps (Kamešnica Massif) consisting of Triassic and Jurassic limestones. As most of the source sinkholes in Livansko Polje are under high anthropogenic pressure: agriculture, farming, waste disposal and drainage channels of Buško Jezero Dam with the power plant in Rumin, we consider the new species to be threatened by groundwater pollution and possible habitat alternations.

Distribution: So far known from four large karst spring at the SE border of the Sinj Basin at the left tributary of the Cetina River in Croatia (Fig. 1).

Remarks: As stated above, the molecular markers confirm a very close relationship of the new species to *M. bogici* from Taban Spring near Podgorica (Montenegro). The different shell morphology confirms its position as an independent taxon separated from the type species by the distance of over 230 km. Finding the new species indicates that the genus *Montenegrospeum* may be more widespread over the Dinarides than originally supposed.

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