

MONTENEGROSPEUM PEŠIĆ ET GLÖER, 2013: A REPRESENTATIVE OF MOITESSIERIIDAE?

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The paper is dedicated to the memory of JOZEF ŠTEFFEK (1952–2013), the famous Slovak zoologist and malacologist, partner in our joint research.

ABSTRACT: The paper deals with phylogenetic relationships of recently described *Montenegrospeum bogici* Pešić et Glöer, 2012 from central Bosnia. The female reproductive system was found to bear two receptacula seminis. Mitochondrial cytochrome oxidase subunit I and nuclear 18S rRNA gene partial sequences were used for the maximum likelihood phylogenetic inference. Morphological and molecular data are congruent: *Montenegrospeum* does not belong to the Moitessieriidae, and is phylogenetically remote from *Bythiospeum*; it belongs to the Hydrobiidae, subfamily Sadlerianinae, and its sister taxon is *Dalmatinella* Radoman, 1973 (bootstrap support 87%).

KEY WORDS: Truncatelloidea, Moitessieriidae, Sadlerianinae, female reproductive organs, COI, 18S rRNA, molecular phylogeny

INTRODUCTION

PEŠIĆ & GLÖER (2012) described a new species of *Bythiospeum* Bourguignat, 1882: *B. bogici* Pešić et Glöer, 2012 from underground waters of the spring Taban, in the central part of Montenegro. Its description was based on empty shells. Later they (PEŠIĆ & GLÖER 2013) collected live specimens, and described the lack of eyes and pigment, and the penis with a lobe at its medial part. They considered *B. bogici* as

belonging to a new genus: *Montenegrospeum* Pešić et Glöer, 2013, still within the family Moitessieriidae Bourguignat, 1863. *M. bogici* is known from its type locality only (PEŠIĆ & GLÖER 2013).

The aim of the present paper is to establish phylogenetic relationships of *Montenegrospeum*, applying two DNA fragments, and reconsidering morphological data.

MATERIAL AND METHODS

Four specimens of *Montenegrospeum bogici* from the spring Taban at Montenegro were used: two for morphology, and the other two for molecular work.

Gastropods were dissected using a NIKON SMZ18 stereoscope microscope with dark field.

For DNA, foot tissue was hydrated in TE buffer (3 × 10 min.); then total genomic DNA was ex-

tracted with the SHERLOCK extracting kit (A&A Biotechnology), and the final product was dissolved in 20 µl TE buffer. The PCR reaction was performed with the following primers: LCO1490 (5'-GGTCAA-CAAATCATAAAGATATTGG-3') (FOLMER et al. 1994) and COR722b (5'-TAAACTT CAGGGTGAC-CAAAAATYA-3') (WILKE & DAVIS 2000) for the

cytochrome oxidase subunit I (COI) mitochondrial gene and SWAM18SF1 (5'-GAATGGCTCATTAAAT-CAGTCGAGGTCCTT AGATGATCCAAATC-3'), and SWAM18SR1 (5'-ATCC TCGTTAAAGGGT-TTAAAGTGACTCATTCAATTACGGAGC-3') for the 18S ribosomal RNA gene (ATTWOOD et al. 2003). The PCR conditions were as follows: COI – initial denaturation step of 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and a final extension of 4 min at 72°C; 18S rRNA – initial denaturation step of 4 min at 94°C, followed by 40 cycles of 45 s at 94°C, 45 s at 51°C, 2 min at 72°C and, after all cycles were completed, an additional elongation step of 4 min at 72°C was performed. The total volume of each PCR reaction

mixture was 50 µl. To check the quality of the PCR products 10 µl of the PCR product were ran on 1% agarose gel. The PCR products were purified using Clean-Up columns (A&A Biotechnology) and were then amplified in both directions (HILLIS et al. 1996) using BigDye Terminator v3.1 (Applied Biosystems), following the manufacturer's protocol and with the primers described above. The sequencing reaction products were purified using ExTerminator Columns (A&A Biotechnology); DNA sequences then underwent electrophoresis on an ABI Prism sequencer. The two sequences were deposited in GenBank (Table 1).

In the phylogeny reconstruction, we used 29 truncatelloid taxa sequences, and one of *Rissoa*, from GenBank (Table 1). Seven of them, used as an out-

Table 1. Taxa used for phylogenetic analyses, with their GenBank Accession Numbers and references

Species	18S GB#	COI GB#	references
<i>Adrioinzulana conovula</i> (Frauenfeld, 1863)	AF367656	AF367628	WILKE et al. (2001)
<i>Agrafia wiktora</i> Szarowska et Falniowski, 2011	JF906758	JF906762	SZAROWSKA & FALNIOWSKI (2011)
<i>Alzoniella finalina</i> Giusti et Bodon, 1984	AF367686	AF367650	WILKE et al. (2001)
<i>Anagastina zetavalis</i> (Radoman, 1973)	EF070622	EF070616	SZAROWSKA (2006)
<i>Avenionia brevis</i> (Draparnaud, 1805)	AF367670	AF367638	WILKE et al. (2001)
<i>Bithymia tentaculata</i> (Linnaeus, 1758)	AF367675	AF367643	WILKE et al. (2001)
<i>Boleana umbilicata</i> (Kuščer, 1932)	JX982797	JX982795	FALNIOWSKI & SZAROWSKA (2012)
<i>Bythinella austriaca</i> (Frauenfeld, 1857)	AF212917	FJ545132	FALNIOWSKI et al. (2009)
<i>Bythiospeum</i> sp.	AF367664	AF367634	WILKE et al. (2001)
<i>Dalmatinella fluviatilis</i> Radoman, 1973	KC344539	KC344541	FALNIOWSKI & SZAROWSKA (2013)
<i>Daphniola graeca</i> Radoman, 1973	EF070624	EF070618	SZAROWSKA (2006)
<i>Dianella thiesseana</i> (Kobelt, 1878)	AY676125	AY676127	SZAROWSKA et al. (2005)
<i>Graecoarganiella parnassiana</i> Falniowski et Szarowska, 2011	JN202341	JN202348	FALNIOWSKI & SZAROWSKA (2011)
<i>Graziana alpestris</i> (Frauenfeld, 1863)	AF367673	AF367641	WILKE et al. (2001)
<i>Grossuana codreanui</i> (Grossu, 1946)	EF061916	EF061919	SZAROWSKA et al. (2007)
<i>Hauffenia tellinii</i> (Pollonera, 1898)	AF367672	AF367640	WILKE et al. (2001)
<i>Heleobia dalmatica</i> (Radoman, 1974)	AF367661	AF367631	WILKE et al. (2001)
<i>Horatia klecakiana</i> Bourguignat, 1887	KJ159127	KJ159128	SZAROWSKA & FALNIOWSKI (2014)
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF367680	AF278808	WILKE & DAVIS (2000)
<i>Islamia piristoma</i> Bodon et Cianfanelli, 2001	AF367671	AF367639	WILKE et al. (2001)
<i>Lithoglyphus naticoides</i> (C. Pfeiffer, 1828)	AF367674	AF367642	WILKE et al. (2001)
<i>Marstoniopsis insubrica</i> (Küster, 1853)	AF367676	AY027813	FALNIOWSKI & WILKE (2001)
<i>Montenegrospeum bogici</i> (Pešić et Glöer, 2012)	KM875509	KM875510	present study
<i>Pseudamnicola lucensis</i> (Issel, 1866)	AF367687	AF367651	WILKE et al. (2001)
<i>Pyrgula annulata</i> (Linnaeus, 1767)	AY676124	AY341258	SZAROWSKA et al. (2005)
<i>Radomaniola callosa</i> (Paulucci, 1881)	AF367685	AF367649	WILKE et al. (2001)
<i>Rissoa labiosa</i> (Montagu, 1803)	AY676126	AY676128	SZAROWSKA et al. (2005)
<i>Sadleriana fluminensis</i> (Küster, 1853)	AF367683	AY273996	WILKE et al. (2001)
<i>Trichonia kephalovrissonia</i> Radoman, 1973	EF070630	EF070619	SZAROWSKA (2006)
<i>Ventrosia ventrosa</i> (Montagu, 1803)	AF367681	AF118335	WILKE & DAVIS (2000)
<i>Vinodolia fumana</i> Radoman, 1973	KF359899	KF359900	SZAROWSKA et al. (2013)

group, represented the main non-hydrobiid lineages within the Rissooidea (WILKE et al. 2001); the other six taxa represented the Hydrobiinae (including “Pyrgulinae”: SZAROWSKA et al. 2005). The remaining taxa were chosen to represent all the main lineages within the European Sadlerianinae (SZAROWSKA 2006).

The COI sequences were aligned by eye using BioEdit 5.0.0 (HALL 1999). For 18S, an initial alignment was performed using CLUSTALX 1.82 (THOMPSON et al. 1997) and edited with MACCLADE (MADDISON & MADDISON 2002). Mutational saturation for the COI dataset was examined by plotting the numbers of transitions and transversions for all the codon positions together, and for the 3rd position separately, against the percentage sequence divergence, using DAMBE 5.2.9 (XIA 2000). We also used DAMBE 5.2.9 to perform the saturation test (XIA et al. 2003). It revealed a significant degree of saturation in the third position of the sequences. In truncatelloids, COI approaches saturation with about 18.6% or 120 nucleotide differences (DAVIS et al. 1998), 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 it was used for the analysis. In fact, the analysis carried out on the 2nd and 3rd position only resulted in similar deep phylogeny, but with several polyto-

mies within more terminal nodes. It should be noted that the saturation test of XIA et al. (2003), for symmetrical tree, and the proportion of invariable sites $I = 0.49$, resulted in $I_{ss} = 0.4606$, $I_{ss.c} = 0.7060$, $p = 0.000$, thus showing little saturation.

Initially, we performed phylogeny reconstruction for 18S and COI data separately, using the maximum likelihood (ML) technique. Next, the partition homogeneity test (FARRIS et al. 1995) was performed (1,000 replicates) with PAUP*4.0b10 (SWOFFORD 2002), to check whether the two genes could be analysed together. Since $p > 0.792$, the maximum likelihood heuristic search was then run for the combined molecular data. Following the recommendations of POSADA & BUCKLEY (2004) and SOBER (2002), the best model for each dataset was chosen using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC); both chose the same model: Tamura-Nei + Γ + I (NEI & KUMAR 2000). We performed ML analyses in PAUP* and used a heuristic search strategy with stepwise addition of taxa, 10 random-sequence addition replicates, and tree-bisection-reconnection (TBR) branch swapping (SWOFFORD et al. 1996), and with MEGA5.10 (TAMURA et al. 2011). Nodal support was estimated using the bootstrap (BS) approach (FELSENSTEIN 1985). Bootstrap values for ML trees were calculated using 10,000 bootstrap replicates, with MEGA5.10 and the same model parameters as for ML analysis.

RESULTS AND DISCUSSION

The female reproductive organs of the two studied specimens, with two seminal receptacles (rs_1 and rs_2 ; RADOMAN 1973, 1983) were characteristic of numerous genera of the Sadlerianinae Radoman, 1973, certainly not of the Moitessieriidae Bourguignat, 1863 (SZAROWSKA 2006). The penis with a lobe on the left side of its median part (PEŠIĆ & GLÖER 2013) was also similar to the ones characteristic of several genera of the Sadlerianinae. In fact, its appearance is simple, not unique. A similar penis could be found in *Dalmatinella fluviatilis* Radoman, 1973 (FALNIOWSKI & SZAROWSKA 2012).

Two sequences of COI were identical, the same concerns two sequences of 18S. The molecular tree based on the two loci (Fig. 1) confirmed general conclusions based on the morphology: *Montenegrospeum* does not belong to the family Moitessieriidae, thus being phylogenetically remote from *Bythiospeum*, and belongs to the family Hydrobiidae Troschel, 1857 (bootstrap support 97%), and to the subfamily Sadlerianinae Radoman, 1973 (bootstrap support 73%). In the tree, *Dalmatinella* is the sister taxon of *Montenegrospeum* (bootstrap support 87%). Considering the genetic distance between the two

taxa, within the range typical of inter-genus relationships within the Truncatelloidea, *Montenegrospeum* could be considered a distinct genus, as proposed by PEŠIĆ & GLÖER (2013).

The results presented above may be a good illustration of the already well known fact that neither shell nor penis character states (especially if only the habitus of the latter is considered) could be used as the only basis of classification within the Truncatelloidea. Considering that most of the numerous nominal species of *Bythiospeum* recorded from Europe are known as empty shells only – since the snails inhabit strictly subterranean, running or interstitial waters and only their shells could be found on the surface – one could expect that phylogenetic relationships within this group (including *Paladilhiopsis* Pavlovic, 1913 as well) may be complex, and the genus is polyphyletic.

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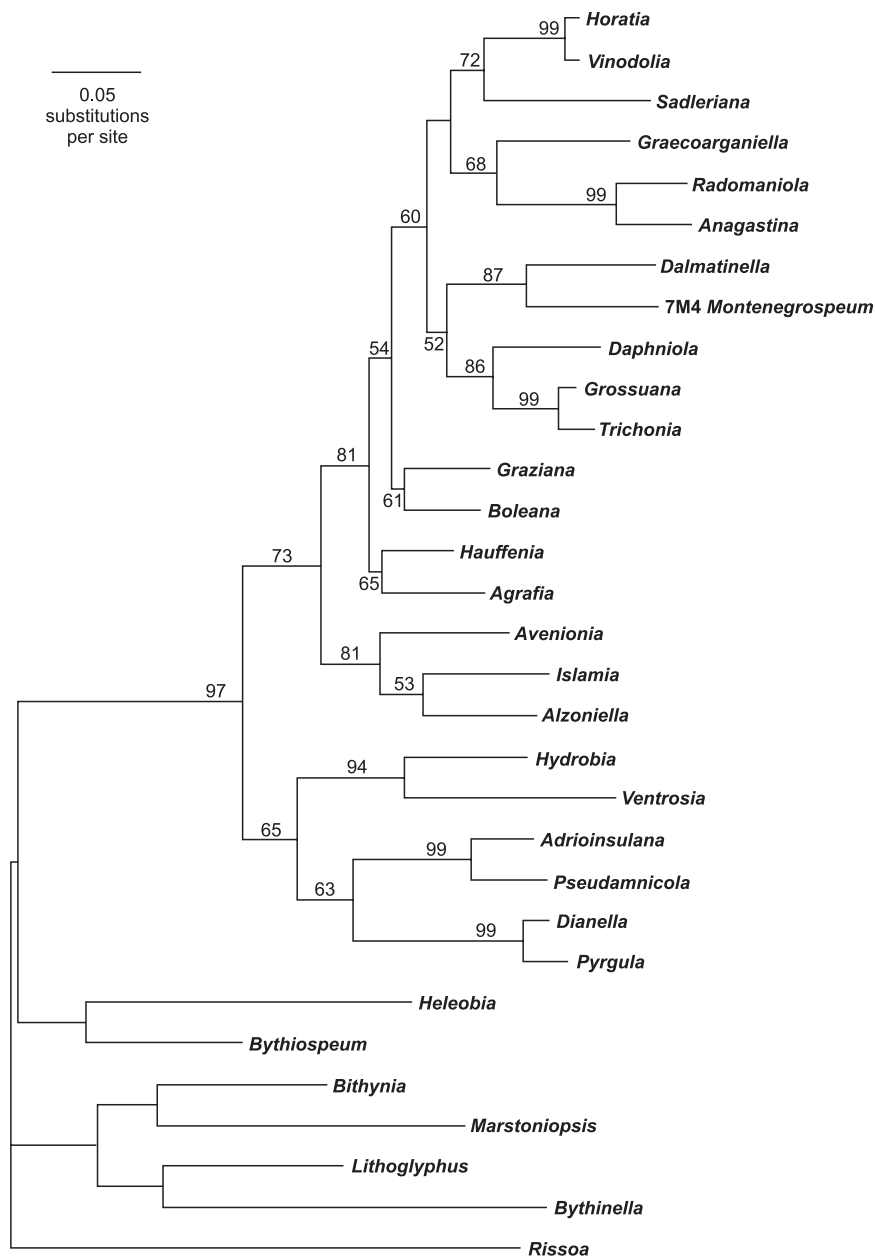


Fig. 1. Maximum likelihood tree of the two concatenated sequences (18S and COI), bootstrap supports (10,000 replicates) given if >50%

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