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PHYLOGENETIC POSITION OF *BOLEANA UMBILICATA* (KUŠČER, 1932) (CAENOGASTROPODA: RISSOOIDEA)

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ABSTRACT: Phylogenetic position of *Boleana umbilicata* (Kuščer, 1932) from Močilnik spring in Slovenia, the type locality of the species, was inferred with ML technique using nucleotide sequences of mitochondrial cytochrome oxidase subunit I (COI) and nuclear 18S rRNA genes. *Boleana* belongs to the Hydrobiidae, Sadlerianinae, and *Graziana* is its sister taxon.

KEY WORDS: Rissooidea, mtDNA, COI, 18S rRNA, phylogeny, spring, type locality

INTRODUCTION

Močilnik, the main source of the Ljubljanica river, Slovenia is inhabited by interesting representatives of the Hydrobiidae (BOLE 1967, 1985, RADOMAN 1983). It was from this locality that KUŠČER (1932) described *Belgrandiella umbilicata*. RADOMAN (1973) described a new monotypic genus *Boleana*, with the type species *Boleana umbilicalis* (Kuščer, 1932). SZAROWSKA (2006) confirmed RADOMAN's (1973) data concerning anatomy, and presented SEM photographs of the protoconch and radula. In the molecular phylogeny based on mitochondrial COI and nuclear 18S rRNA sequences (SZAROWSKA 2006: fig. 241) *Boleana* is placed close to *Daphniola* Radoman, 1973. Unfortunately, that phylogeny was based upon a very short (about 200 bp) COI sequence. The aim of this study is to correct the position of *Boleana* in the molecular phylogeny using a longer COI sequence, and to analyse the two loci together.

MATERIAL AND METHODS

Using a sieve, numerous specimens of *Boleana umbilicata* were collected from Močilnik (Fig. 1), the huge spring of the Ljubljanica river (45°57'15"N, 14°17'33"E, 313 m a.s.l.).

Snails were washed twice in 80% ethanol and left to stand in it for about 12 hours. Then the ethanol was changed twice more within 24 hours and finally, after a few days, the 80% solution was replaced with a 96% one, in which the samples were stored at –20°C.

The shells were cleaned in an ultrasonic cleaner and photographed with a CANON EOS 50D digital camera. Two males and two females were dissected, using a NIKON SMZ-U stereomicroscope.

DNA was extracted from foot tissue of each snail. The tissue was hydrated in TE buffer (10 mM TRIS-HCl pH 8.0, 1 mM EDTA) (3 × 10 min.); then total genomic DNA was extracted 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'-GGTCAACAAATCATAAAGATATTGG-3') (FOLMER et al. 1994) and COR722b (5'-TAAACTTCAGGGTGACCAAAAAATYA-3') (WILKE & DAVIS 2000) for the cytochrome oxidase subunit I (COI) mitochondrial gene, as well as SWAM18SF1 (5'-GAATGGCT CATTAAATCAGTCGAGGTTCCTTAGATGATCCAAATC-3') and SWAM18SR1 (5'-ATCCTCGTTAAAGGGTTTAAAGTGTACTCATTCCAATTACGGAGC-3') for the 18S ribosomal RNA gene (PALUMBI 1996). The PCR conditions were as follows: COI – ini-

Fig. 1. Močilnik spring

tial 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 – 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 was run on 1% agarose gel. The PCR products were purified using Clean-Up columns (A&A Biotechnology) and the purified PCR products were amplified in both directions 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. All the sequences were deposited in GenBank (Table 1).

In the phylogeny reconstruction, we used sequences of 27 rissooid taxa from GenBank (Table 1). Seven of them, used as outgroup, represented the main non-hydrobiid lineages within the Rissooidea (WILKE et al. 2001); another seven 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 2.012 (THOMPSON et al. 1997) and edited with MACCLADE 4.05 (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). Initially, we performed phylogeny reconstruction for 18S and COI data separately, using the maximum likelihood (ML) technique.

For each ML analysis, we used the best fit model of sequence evolution found by Modeltest v3.06 (POSADA & CRANDALL 1998, POSADA 2003). Following the recommendations of POSADA & BUCKLEY (2004) and SOBER (2002), the best model for each dataset was chosen using the Akaike Information Criterion (AKAIKE 1974). We performed ML analyses in PAUP*4.0b10 (SWOFFORD 2002) 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). Nodal support was estimated using the bootstrap (BS) approach (FELSENSTEIN 1985). Bootstrap values for ML trees were calculated using 1000 bootstrap replicates, the "fast" heuristic

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

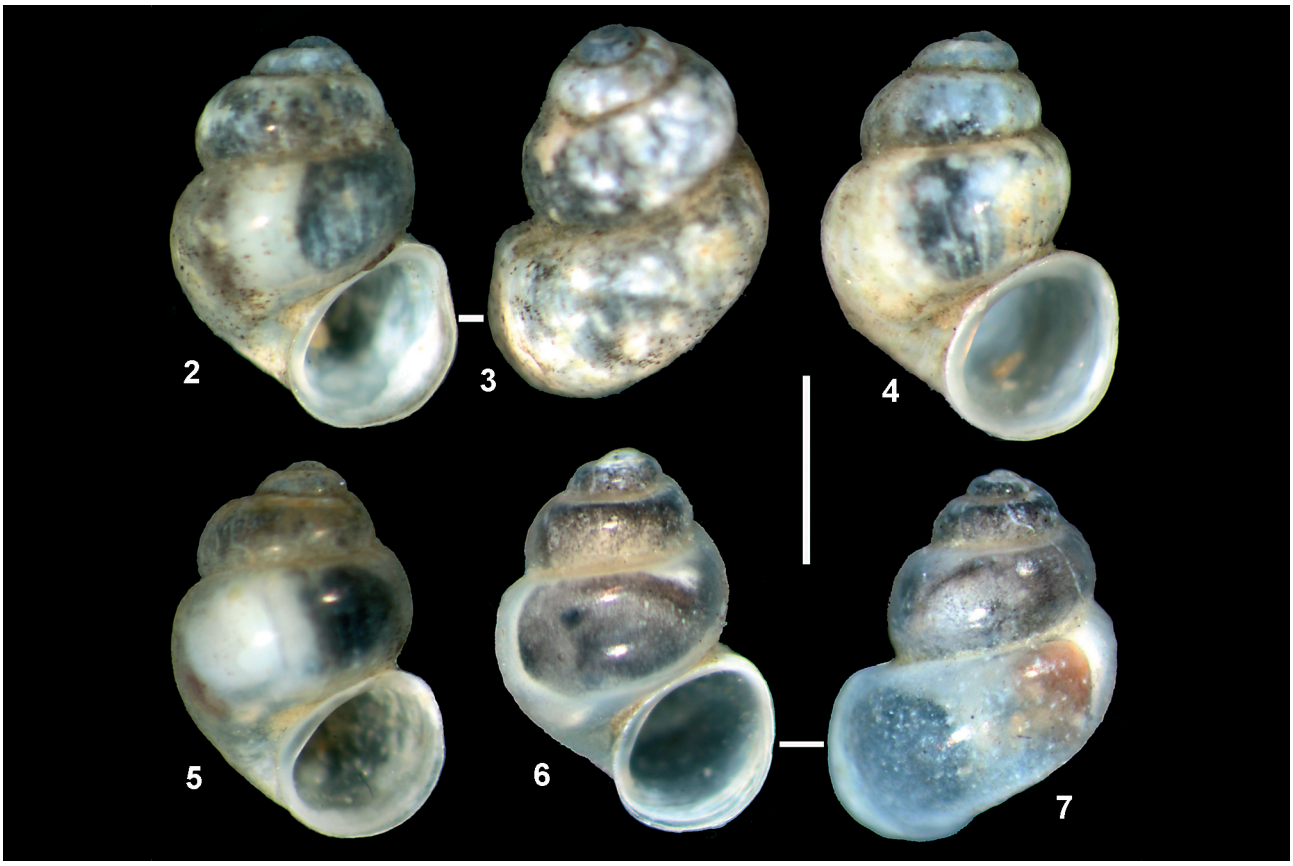
Species	18S GB#	COI GB#	References
Adriohydrobia gagatinella (Küster, 1852)	AF367657	AF317881	WILKE & FALNIOWSKI (2001)
Adrioinsulana conovula (Frauenfeld, 1863)	AF367656	AF367628	WILKE et al. (2001)
Agrafia wiktori Szarowska et Falniowski, 2011	JF906758	JF906762	SZAROWSKA & FALNIOWSKI (2011)
Alzoniella finalina Giusti et Bodon, 1984	AF367686	AF367650	WILKE et al. (2001)
Anagastina zetavalis (Radoman, 1973)	EF070622	EF070616	SZAROWSKA (2006)
Bithynia tentaculata (Linnaeus, 1758)	AF367675	AF367643	WILKE et al. (2001)
Boleana umbilicata (Kuščer, 1932)	JX982797	JX982795	present study
	JX982798	JX982796	present study
Bythinella austriaca (Frauenfeld, 1857)	AF212917	FJ545132	FALNIOWSKI et al. (2009)
Bythiospeum sp.	AF367664	AF367634	WILKE et al. (2001)
Daphniola graeca Radoman, 1973	EF070624	EF070618	SZAROWSKA (2006)
Dianella thiesseana (Kobelt, 1878)	AY676125	AY676127	SZAROWSKA et al. (2005)
Graecoarganiella parnassiana Falniowski et Szarowska, 2011	JN202341	JN202348	FALNIOWSKI & SZAROWSKA (2011)
Graziana alpestris (Frauenfeld, 1863)	AF367673	AF367641	WILKE et al. (2001)
Grossuana codreanui (Grossu, 1946)	EF061916	EF061919	SZAROWSKA et al. (2007)
Hauffenia tellinii (Pollonera, 1898)	AF367672	AF367640	WILKE et al. (2001)
Heleobia dalmatica (Radoman, 1974) 1	AF367661	AF367631	WILKE et al. (2001)
Hydrobia acuta (Draparnaud, 1805)	AF367680	AF278808	WILKE & DAVIS (2000)
Islamia piristoma Bodon et Cianfanelli, 2001	AF367671	AF367639	WILKE et al. (2001)
Lithoglyphus naticoides (C. Pfeiffer, 1828)	AF367674	AF367642	WILKE et al. (2001)
Marstoniopsis insubrica (Küster, 1853)	AF367676	AY027813	FALNIOWSKI & WILKE (2001)
Pseudamnicola lucensis (Issel, 1866)	AF367687	AF367651	WILKE et al. (2001)
Pyrgula annulata (Linnaeus, 1767)	AY676124	AY341258	SZAROWSKA et al. (2005)
Radomaniola callosa (Paulucci, 1881)	AF367685	AF367649	WILKE et al. (2001)
Rissoa labiosa (Montagu, 1803)	AY676126	AY676128	SZAROWSKA et al. (2005)
Sadleriana fluminensis (Küster, 1853)	AF367683	AY273996	WILKE et al. (2001)
Trichonia kephalovrissonia Radoman, 1973	EF070630	EF070619	SZAROWSKA (2006)
Ventrosia ventrosa (Montagu, 1803)	AF367681	AF118335	WILKE & DAVIS (2000)

search algorithm, and the same model parameters as for each ML analysis. Next, the partition homogeneity test (FARRIS et al. 1995) was performed (1000 replicates) with PAUP*, to check whether the two genes could be analysed together. The maximum likelihood heuristic search was then run for the combined molecular data. MEGA4 (KUMAR et al. 2004) was used to estimate nodal support with BS and minimum-evolution (ME) and neighbor-joining (NJ) approach (SWOFFORD et al. 1996, NEI & KUMAR 2000), applying composite-likelihood distances with gamma distribution shape parameter estimated with MODELTEST.

RESULTS AND DISCUSSION

The shell (Figs 2–7) and soft parts of *B. umbilicata* resembled the ones shown in RADOMAN (1983: fig. 53, pl. VI, fig. 108) and SZAROWSKA (2006: fig. 26).

Two sequences of COI (each 653 bp long) and two of 18S (each 419 bp long) of *B. umbilicata* were analysed (Table 1). DAMBE 5.2.9 saturation test revealed a significant degree of saturation in the third position of the sequences. In the rissooids, 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 but was used for the analysis. For the COI sequences the Akaike Information Criterion (AIC) with ModelTest found model TVM+I+Γ, with base frequencies: A=0.3500, C=0.1330, G=0.1097, T=0.4073; substitution rate matrix: [A–C]=0.2566, [A–G]=10.7137, [A–T]=0.0709, [C–G]=1.1198, [C–T]=10.7137, [G–T]=1.0000, proportion of invari-



Figs 2–7. Shells of *Boleana umbilicata*, bar represents 1 mm

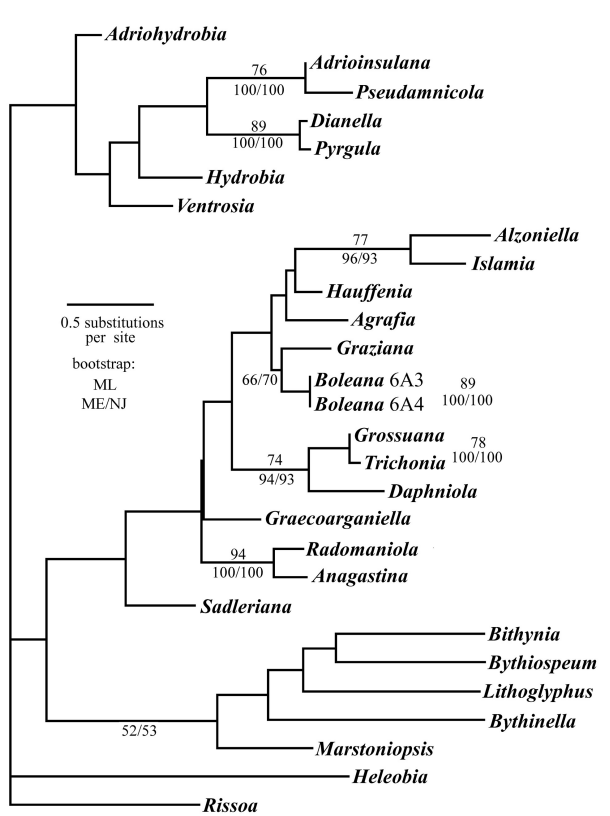


Fig. 8. ML phylogram based on COI, bootstrap supports given where >50

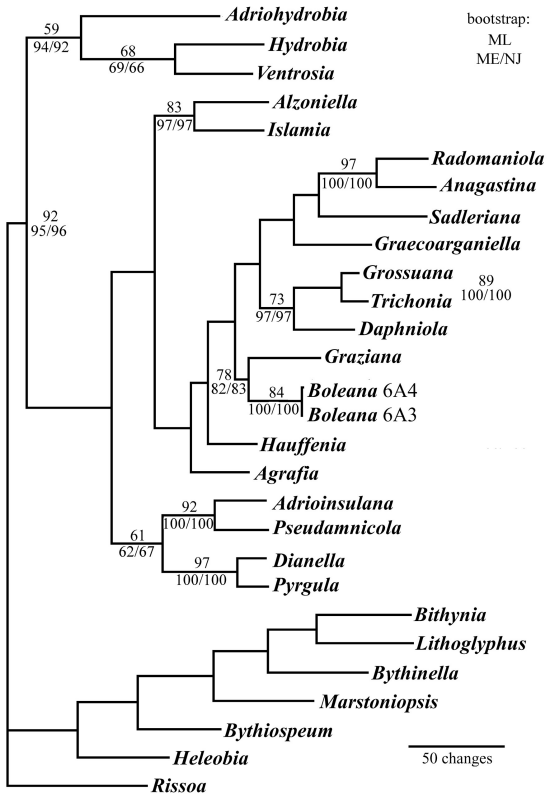


Fig. 9. ML phylogram based on COI and 18S, bootstrap supports given where >50

able sites: (I)=0.4054, and Γ distribution with the shape parameter = 0.2528. For the combined data set the Akaike Information Criterion (AIC) with ModelTest found model GTR+I+Γ, with base frequencies: A=0.3384, C=0.1506, G=0.1406, T=0.3704; substitution rate matrix: [A–C]=0.8317, [A–G]=7.6497, [A–T]=0.2018, [C–G]=2.7010, [C–T]=12.9307, [G–T]=1.0000, proportion of invariable sites: (I)=0.6017, and Γ distribution with the shape parameter =0.3486.

The ML tree computed for the COI sequences (Fig. 8), like the corresponding tree in SZAROWSKA (2006: fig. 239), shows *Boleana* in the Hydrobiidae, Sadlerianinae. However, in Fig. 8, unlike in SZAROWSKA (2006: fig. 239), the sister taxon of *Boleana* is not *Daphniola* Radoman, 1973, but *Graziana* Radoman, 1975. Moreover, in Fig. 8 the *Boleana*/*Graziana* clade is close to *Agrafia* Szarowska et Falniowski, 2011, *Hauffenia* Pollonera, 1898, *Islamia* Radoman, 1973, and *Alzoniella* Giusti et Bodon, 1984, not to *Grossuana* Radoman, 1973 and *Trichonia* Radoman, 1973 (SZAROWSKA 2006: fig. 239). Interestingly, in the tree based on 18S in SZAROWSKA (2006: fig. 237), *Boleana* forms a trichotomy with *Graziana* and *Trichonia*, and we found the same in the present study (the tree not shown). In the ML tree computed for the two sequences together (Fig. 9), as in the COI tree (Fig. 8), *Graziana* is the sister taxon of *Boleana* while *Daphniola* is not close to the latter. Unlike in the COI tree, only the genera *Hauffenia* and *Agrafia* (but not *Alzoniella* nor *Islamia*) are closely related to *Boleana* and *Graziana* (Fig. 9).

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