



## PHYLOGENETIC RELATIONSHIPS OF *ALZONIELLA SLOVENICA* (LOŽEK ET BRTEK, 1964) (CAENOGASTROPODA: HYDROBIIDAE)

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**ABSTRACT:** The shell, radula, penis and female reproductive organs of *Alzoniella slovenica* (Ložek et Brtek, 1964), from Slovakia, are described. The morphology of *A. slovenica* resembles the literature description of *A. hartwigschuetzi* Reischütz, 1983 from Austria, and is less similar to three *Alzoniella* species from Italy. Sequences of cytochrome oxidase subunit I (COI) were used for phylogenetic reconstruction. The closest relatives of *A. slovenica* are *Radomaniola* and *Anagastina*, not the Italian *Alzoniella finalina* Giusti et Bodon, 1984. *A. slovenica* is not congeneric with *A. finalina*, in which case morphological characters are misleading.

**KEY WORDS:** mtDNA, COI, Risssooidea, *Alzoniella*, morphology, phylogeny

### INTRODUCTION

Members of the genus *Alzoniella* Giusti et Bodon, 1984 are minute hydrobiid inhabitants of ground-water (KABAT & HERSHLER 1993). GIUSTI & BODON (1984) described the genus *Alzoniella*, with the type species *A. finalina* Giusti et Bodon, 1984, and two other new species of this genus (all of them found in Piedmont and Liguria, north Italy). LOŽEK & BRTEK (1964) described a new species of *Belgrandiella* A. J. Wagner, 1927: *B. slovenica* Ložek et Brtek, 1964, from Kláčno, NW Slovakia. Based upon shell characters alone, they distinguished three subspecies of the new *Belgrandiella* (*B. slovenica slovenica*, *B. slovenica bojnicensis*, and *B. slovenica alticola* Ložek et Brtek, 1964). HUDEC (1972) described another subspecies: *B. slovenica komenskyi* Hudec, 1972. The known localities of *B. slovenica* were scattered along the state bor-

der in the Czech Republic and Slovakia. LISICKÝ (1991) noted the occurrence of this species in the Inovec Mts., NW Slovakia. BODON (1988) revised the systematic position of *Belgrandiella hartwigschuetzi* Reischütz, 1983 from the North Kalkalpen (Niederösterreich). He transferred *B. hartwigschuetzi* to the genus *Alzoniella*, thus expanding the range of *Alzoniella* to East Austria (BODON 1988). BERAN & HORSÁK (2001) assigned *Belgrandiella slovenica* to the genus *Alzoniella*. In their shell morphometric study (BERAN & HORSÁK 2001) they rejected the distinctness of the four subspecies distinguished earlier.

The aim of the present study was to infer the phylogenetic relationships of *Alzoniella slovenica*, considering its morphology and molecular data on mtDNA (COI: cytochrome oxidase subunit I).

## MATERIAL AND METHODS

### MATERIAL COLLECTION AND FIXATION

All snails for the molecular study came from Kunova Teplica (Fig. 1: A). They were collected with a sieve, washed twice in 80% ethanol and left to stand in it for ca. 12 hours. Afterwards, the ethanol was changed twice in 24 hours and finally, after a few days, the 80% solution was replaced with a 96% one and the material was stored at  $-20^{\circ}\text{C}$ . For the morphological study, we used additional material – fixed in 4% formalin and stored it in 80% ethanol – coming from Nižna (Fig. 1: B).

### MORPHOLOGICAL TECHNIQUES

The snails were dissected using a NIKON SMZ-U stereoscope microscope with a NIKON drawing apparatus, and a NIKON DS-5 digital camera. We cleaned the shells in an ultrasonic cleaner and photographed them with a NIKON DS-5 or CANON EOS 50D digital camera. The protoconchs and radulae were examined using a JEOL JSM-5410 scanning electron microscope (SEM), applying the techniques described by FALNIOWSKI (1990).

### MOLECULAR TECHNIQUES

The snails were hydrated in TE buffer ( $3 \times 10$  min.), and DNA was extracted with the SHERLOCK extracting kit (A&A Biotechnology); the final product was dissolved in 20  $\mu\text{m}$  of TE buffer. The PCR reaction

(PALUMBI 1996) was performed with the following primers: LCOI490 (5'-GGTCAACAAATCATAAAGA TATTGG-3') and COR722b (5'-TAAACTTCAGGGT GACCAAAAAATYA-3') for the COI gene (FOLMER et al. 1994). The PCR conditions were as follows: initial denaturation step of 4 min. at  $94^{\circ}\text{C}$ , followed by 35 cycles at  $94^{\circ}\text{C}$  for of 1 min.,  $55^{\circ}\text{C}$  for 1 min., and  $72^{\circ}\text{C}$  for 2 min., and a final extension of 4 min. at  $72^{\circ}\text{C}$ . The total volume of each PCR reaction mixture was 50  $\mu\text{l}$ . To check the quality of the PCR products, 10  $\mu\text{l}$  of the PCR product was ran on 1% agarose gel. The PCR product was purified using Clean-Up columns (A&A Biotechnology) and the purified PCR product was 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); the sequences were read using the ABI Prism sequencer.

### DATA ANALYSIS

The sequences were aligned by eye, using BioEdit 5.0.0 (HALL 1999) and edited with MACCLADE 4.05 (MADDISON & MADDISON 2002). The phylogeny was inferred using maximum-likelihood (ML), maximum parsimony (MP), minimum evolution (ME), and neighbor-joining (NJ) techniques.

The maximum likelihood technique of phylogeny reconstruction has many shortcomings (SWOFFORD et

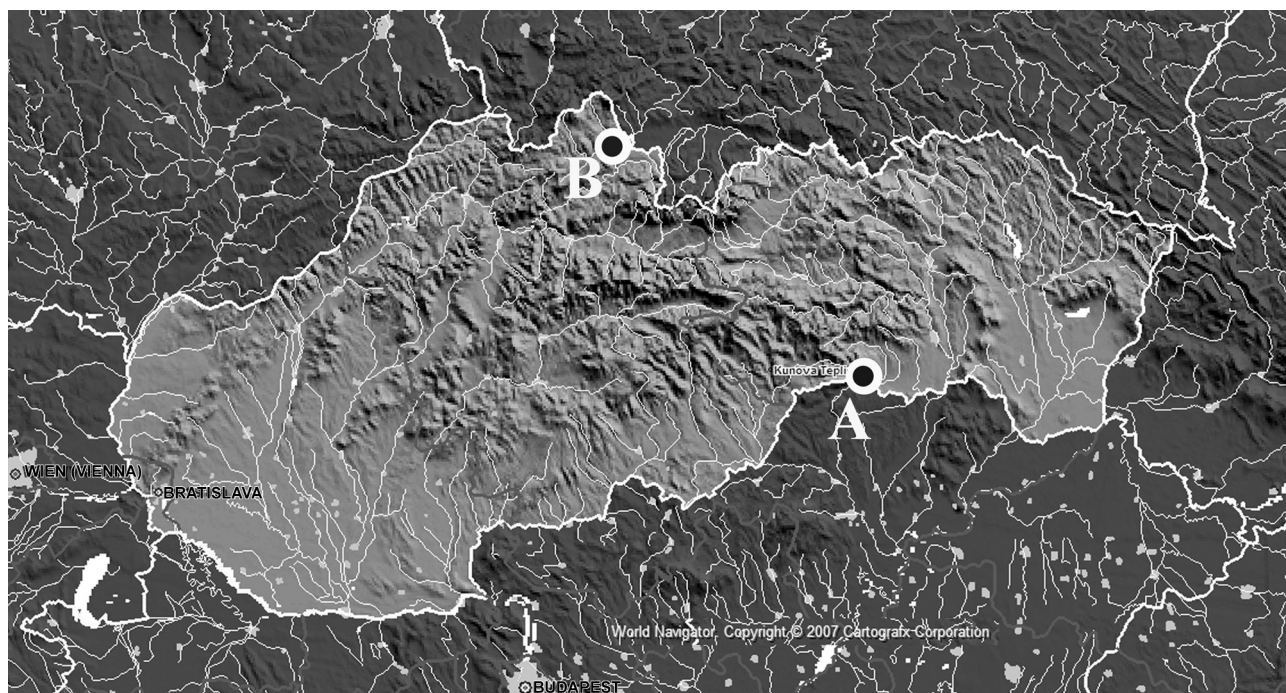


Fig. 1. Localities of the studied *Alzoniella slovenica*: A – Kunova Teplica, B – Nižna. Figure produced using Cartografx Professional Software



Table 1. GenBank Accession Numbers and references for COI sequences of species used as outgroup

Species	GenBankAN	References
<i>Adriohydrobia gagatinella</i> (Küster, 1852)	AF317881	WILKE & FALNIOWSKI (2001)
<i>Adrioinsulana conovula</i> (Frauenfeld, 1863)	AF367628	WILKE et al. (2001)
<i>Alzoniella finalina</i> Giusti et Bodon, 1984	AF367650	WILKE et al. (2001)
<i>Alzoniella slovenica</i> (Ložek et Brtek, 1964)	JF742656	present study
	JF742657	present study
<i>Anagastina zetavalis</i> (Radoman, 1973)	EF070616	SZAROWSKA (2006)
<i>Bithynia tentaculata</i> (Linnaeus, 1758)	AF367643	WILKE et al. (2001)
<i>Bythinella austriaca</i> (Frauenfeld, 1857)	FJ545132	FALNIOWSKI et al. (2009)
<i>Bythiospeum</i> sp.	AF367634	WILKE et al. (2001)
<i>Daphniola graeca</i> Radoman, 1973	EF070618	SZAROWSKA (2006)
<i>Dianella thiesseana</i> (Kobelt, 1878)	AY676127	SZAROWSKA et al. (2005)
<i>Graziana alpestris</i> (Frauenfeld, 1863)	AF367641	WILKE et al. (2001)
<i>Grossuana codreanui</i> (Grossu, 1946)	EF061919	SZAROWSKA et al. (2007)
<i>Hauffenia tellinii</i> (Pollonera, 1898)	AF367640	WILKE et al. (2001)
<i>Heleobia dalmatica</i> (Radoman, 1974)	AF367631	WILKE et al. (2001)
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF278808	WILKE & DAVIS (2000)
<i>Islamia piristoma</i> Bodon et Cianfanelli, 2001	AF367639	WILKE et al. (2001)
<i>Lithoglyphus naticoides</i> (C. Pfeiffer, 1828)	AF367642	WILKE et al. (2001)
<i>Marstoniopsis insubrica</i> (Küster, 1853)	AY027813	FALNIOWSKI & WILKE (2001)
<i>Pseudamnicola lucensis</i> (Issel, 1866)	AF367651	WILKE et al. (2001)
<i>Pseudobithynia</i> sp.	EF070620	SZAROWSKA (2006)
<i>Pyrgula annulata</i> (Linnaeus, 1767)	AY341258	SZAROWSKA et al. (2005)
<i>Radomaniola callosa</i> (Paulucci, 1881)	AF367649	WILKE et al. (2001)
<i>Rissoa labiosa</i> (Montagu, 1803)	AY676128	SZAROWSKA et al. (2005)
<i>Sadleriana fluminensis</i> (Küster, 1853)	AY273996	WILKE et al. (2001)
<i>Trichonia kephalovrissonia</i> Radoman, 1973	EF070619	SZAROWSKA (2006)
<i>Ventrosia ventrosa</i> (Montagu, 1803)	AF118335	WILKE & DAVIS (2000)

al. 1996, NEI & KUMAR 2000, TAKAHASHI & NEI 2000, FALNIOWSKI 2003). Nevertheless, it is widely used for molecular data and many authors regard it as the most reliable, thus we decided to apply the ML approach to each of the two data sets. For each maximum likelihood analysis, different models of sequence evolution were tested using 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). ML analyses were performed in PAUP\*4.0b10 (SWOFFORD 2002), using an heuristic search strategy with stepwise addition of taxa, 10 ran-

dom-sequence addition replicates, and tree-bisection-reconnection (TBR) branch swapping (SWOFFORD et al. 1996). Nodal support was estimated using the bootstrap approach (FELSENSTEIN 1985). Bootstrap values for ML trees were calculated using 1,000 bootstrap replicates, the "fast" heuristic search algorithm, and the same model parameters as for each ML analysis.

We ran minimum evolution and maximum parsimony on PAUP\*, and neighbour-joining on MEGA4 (TAMURA et al. 2007). Nodal support was estimated using the bootstrap approach (full heuristic search) with 1,000 replicates.

## RESULTS

### MORPHOLOGY

The shells (Figs 2–4) are within the variability range presented by LOŽEK & BRIEK (1964). The protoconch (Figs 5–6) has about 1½ whorls growing slowly in breadth; the border between the protoconch and the teleoconch is inconspicuous. The sculpture on the protoconch (Figs 7–10) is fine but without a regular pattern (Figs 7–8); under high magnification it reveals unevenly scattered, irregular pits (Fig. 9), and grainy microsculpture (Fig. 10).

The radula, shown in Figs. 11–12, has a rhachis (Fig. 12) described by the formula:

$$\frac{4-1-4}{1-1}$$

Its cusps are moderately slender, the biggest median cusp moderately prominent. The lateral teeth (Fig. 11) have a narrow but moderately prominent, biggest cusp lying in the centre, and about four narrow cusps on each side; the inner marginal tooth has about 15 narrow cusps, the outer marginal tooth – a few short and blunt cusps (Fig. 11).

The penis (Fig. 13) has a prominent, broad, glandular outgrowth on the left side, its position corresponding to “AG<sub>1</sub>” position illustrated by GIUSTI & BODON (1984), and another outgrowth (“AG<sub>2</sub>”) on the right side. The well visible vas deferens runs in a zigzag in the long axis of the penis.

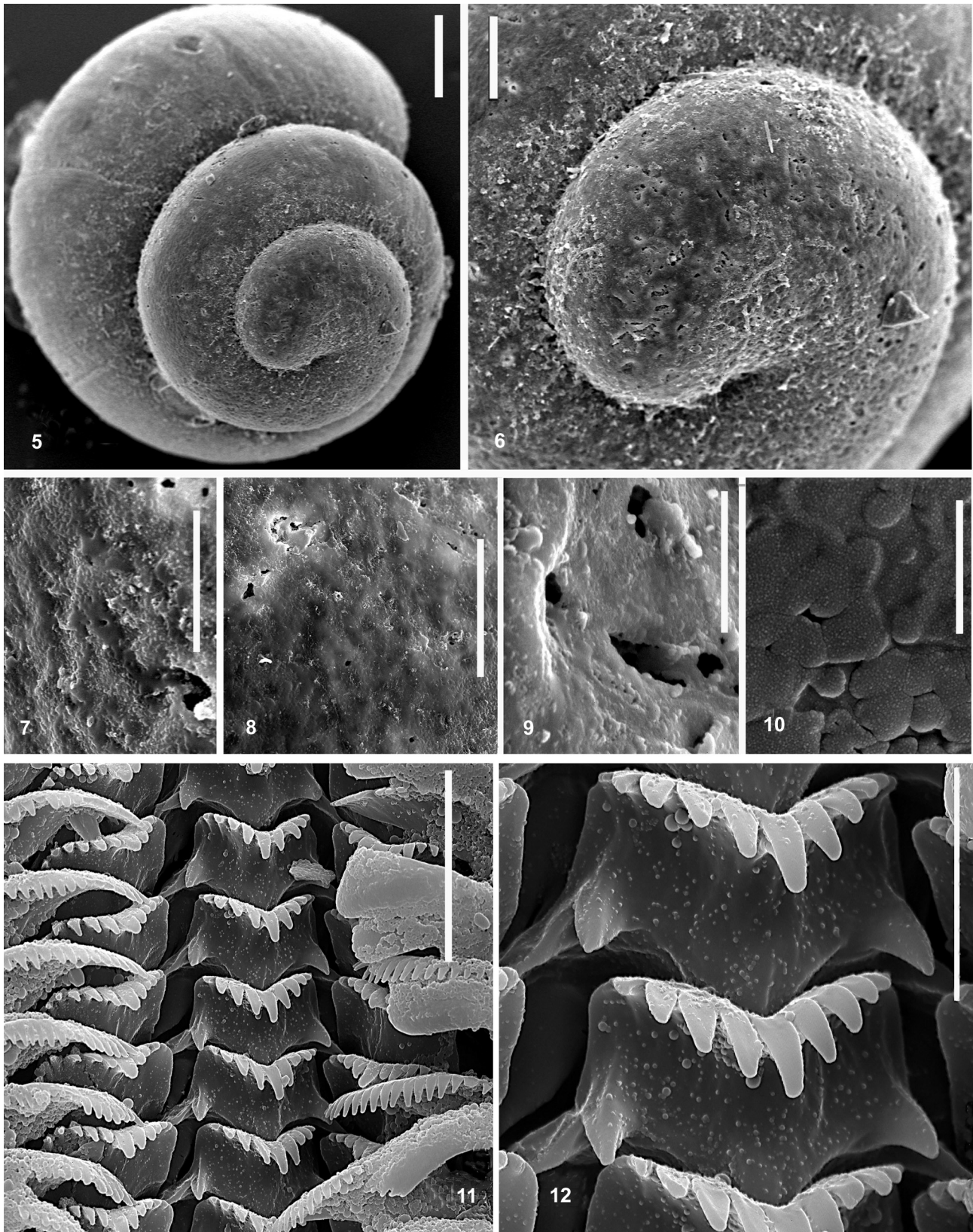
The rectum is curved in a characteristic, sharp bend (Fig. 14). The female reproductive organs (Fig. 14) with a big and bulky bursa copulatrix, with a rather short and well discernible duct, and two seminal receptacles of a similar size: rs<sub>2</sub> (relatively big for an rs<sub>2</sub>) and rs<sub>1</sub> (relatively small for an rs<sub>1</sub>) (Fig. 14).

### MOLECULAR PHYLOGENY

The Akaike Information Criterion (AIC) with ModelTest selected the model TVM+I+Γ, with base frequencies: A=0.3388, C=0.1371, G=0.1128, T=0.4112; substitution rate matrix: [A-C]=0.3165, [A-G]=5.7819, [A-T]=0.0481, [C-G]=0.7576, [C-T]=5.7819, [G-T]=1.0000, proportion of invariable sites: (I)=0.4541, and Γ distribution with the shape parameter =0.4110. Fig. 15 shows the inferred maxi-



Figs. 2–4. Shell of *Alzoniella slovenica*, bar equals 1 mm



Figs. 5–12. Protoconch and radula of *Alzoniella slovenica*: 5–6 – protoconch habitus, 7–10 – protoconch surface, 11 – radula, 12 – central teeth of the radula; bars equal: 5 – 100  $\mu\text{m}$ , 6 – 230  $\mu\text{m}$ , 7–8 – 20  $\mu\text{m}$ , 9 – 6  $\mu\text{m}$ , 10 – 1  $\mu\text{m}$ , 11 – 30  $\mu\text{m}$ , 12 – 10  $\mu\text{m}$

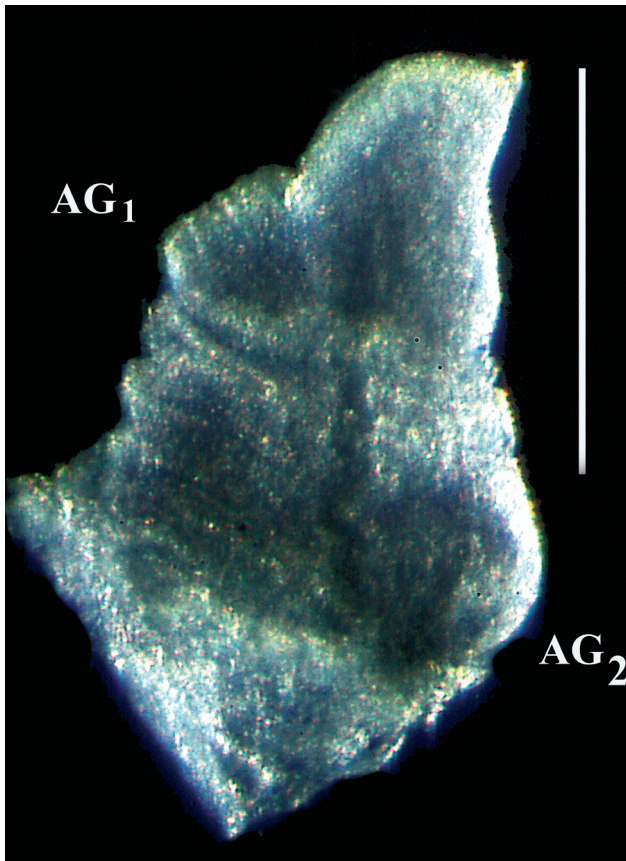


Fig. 13. Penis of *Alzoniella slovenica*, dorsal view, AG<sub>1</sub> and AG<sub>2</sub> – glandular outgrowths distinguished after GIUSTI & BODON (1984), bar equals 0.25 mm

mum likelihood tree placing *Alzoniella slovenica* within the Hydrobiidae, but not close to *A. finalina*. The latter species forms a clade with *Islamia*, but *A. slovenica* clusters close to the clade that represents *Radomaniola* and *Anagastina*. In the ML tree *A. slovenica* did not

## DISCUSSION

Our localities in Nižna (central part of north Slovakia) and Kunova Teplica (southeastern Slovakia) are outside the formerly known range of the genus (LISICKÝ 1991, BERAN & HORSÁK 2001, GLÖER 2002).

The radula is similar to the ones figured and described by GIUSTI & BODON (1984) for the Italian representatives of *Alzoniella*.

The penis with the outgrowths is situated as in the Italian *A. feneriensis* Giusti et Bodon, 1984, and unlike in the other two Italian species (GIUSTI & BODON 1984). It bears an overall resemblance to the penes drawn schematically by LOŽEK & BRTEK (1964) and BERAN & HORSÁK (2001), as well as to *A. hartwigschuetzi* drawn by BODON (1988). The position of lobe “AG<sub>2</sub>” in these drawings is, however, unclear. In our *A. slovenica* this lobe is also hardly distinguishable. The

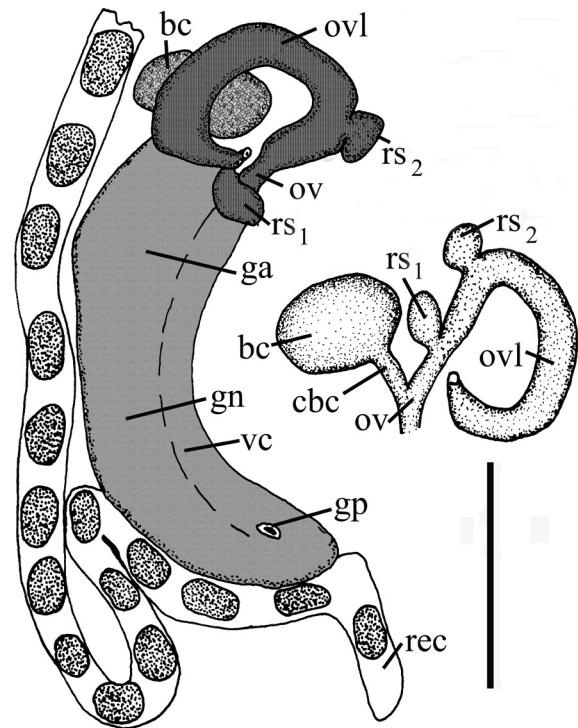


Fig. 14. Female reproductive organs of *Alzoniella slovenica*. Abbreviations: bc, bursa copulatrix; cbc, canal of bursa copulatrix; ga, albumen gland; gn, capsule gland; gp, gonoporus; ov, pallial oviduct; ovl, coil of “renal” oviduct; rec, rectum; rs, seminal receptacle (1 and 2 after RADOMAN 1983); vc, ventral channel; bar equals 0.25 mm

form a clade with *Radomaniola* and *Anagastina* but such clade (a vertical bar in Fig. 15) was supported by 62% of the ML trees; the supports for this clade computed with MP, ME and NJ trees were: 93, 91 and 96, respectively.

penis of our *Alzoniella* resembles, in general, the penes of *A. slovenica* and *A. hartwigschuetzi* from Austria, illustrated in the literature, and is somewhat different from the penes of the Italian *Alzoniella*.

The female reproductive organs and the characteristic bend of the rectum are similar to the corresponding organs described and drawn by GIUSTI & BODON (1984) and BODON (1988). The bursa copulatrix in *A. slovenica* is as big and bulky as in *A. hartwigschuetzi* (BODON 1988). In the Italian species the bursa copulatrix is either elongated with a less distinct duct (*A. finalina*), or smaller (GIUSTI & BODON 1984). The two seminal receptacles resemble, in their relative dimensions, the receptacles of *A. hartwigschuetzi* (BODON 1984).

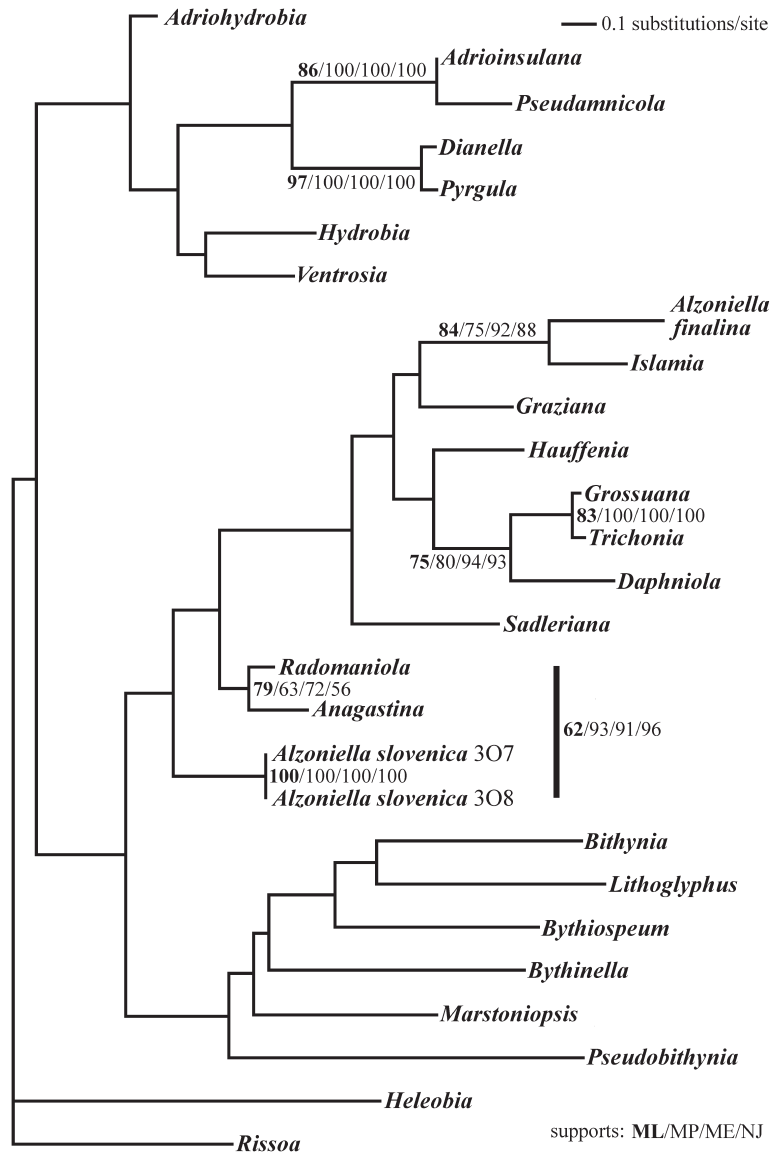


Fig. 15. Maximum likelihood phylogram, bootstrap supports (1,000 replicates given for each branch if >50: **ML/MP/ME/NJ**)

The molecular phylogeny placed *A. slovenica* close to *Radomaniola* and *Anagastina*, but far from *A. finalina*. This, however, clustered both species within the Hydrobiidae Troschel, 1857, among the bearers of two seminal receptacles, a bursa copulatrix, and an outgrowth (usually bi-lobed) on the penis (SZAROWSKA 2006): the subfamily Sadlerianidae Radoman, 1973 (SZAROWSKA 2006). In the light of the molecular data it is evident that *A. finalina* and *A. slovenica* are not to be placed in the same genus. *A. finalina* is the type species of *Alzoniella* (GIUSTI & BODON 1984), thus *A. slovenica* should be placed in another genus, perhaps together with the Austrian *A. hartwigschuetzi*. The known distribution of the two species – close to each other, and far from the Italian *Alzoniella* – supports this hypothesis. Before any taxonomic decision is made, we are, however, badly in need of molecular data on *A. hartwigschuetzi*. Some differences in soft

part morphology support the distinctness of *A. slovenica* and *A. hartwigschuetzi* from the Italian *Alzoniella* (see above), but they are poorly marked: the soft parts in all the species are, in general, almost identical. As shown by, e.g., SZAROWSKA (2006) and SZAROWSKA & FALNIOWSKI (2008), common parallelisms, reversals and convergences, coupled with the structure simplification caused by miniaturization and functional factors, result in limited reliability of morphology-based classification within the Risssooidea, of which *A. slovenica* is one more example.

#### ACKNOWLEDGEMENTS

The study was supported by a grant from the Polish Ministry of Science and Higher Education (PB 2443/P01/2006/31) to Andrzej Falniowski.

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*Received: June 24th, 2010*

*Accepted: September 27th, 2010*

