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"*HAUFFENIA*" POLLONERA, 1898 (CAENOGASTROPODA: HYDROBIIDAE) IN SLOVAKIA: A PRELIMINARY REPORT

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ABSTRACT: We studied the shell and penis morphology, and cytochrome oxidase subunit I (COI) gene sequences, in minute, valvatiform hydrobiid gastropods from Slovensky Kras, Slovakia. The morphology confirmed the assignment of the studied snails to the genus *Hauffenia*, while in the molecular tree they were placed within the Hydrobiidae, and not close to *Hauffenia*. The results indicate that the Slovak valvatiform hydrobiids are two taxa which presumably represent two genera: *Hauffenia* Pollonera, 1898 and *Lobaumia* Haase, 1993. More molecular data on the Austrian and Hungarian taxa are needed.

KEY WORDS: hydrobiid, valvatiform, morphology, COI

INTRODUCTION

The genus *Hauffenia* Pollonera, 1898 – with the type species *H. tellinii* (Pollonera, 1898) described from Italy – is widely distributed in Europe. It was reported from Italy and France to east Austria, and the northern part of the Balkans. There are several minute valvatiform hydrobiids assigned to this genus, but their soft part anatomy is known in few cases only (BOLE 1970, BERNASCONI 1985, HAASE 1992, 1993, KABAT & HERSHLER 1993, BODON et al. 2001, GLÖER 2002). The real range of the genus remains thus enigmatic. In east Austria there are three species: *H. kerschneri* (Zimmermann, 1930) (with two subspecies: *H. kerschneri kerschneri*, and *H. kerschneri loichiana* Haase, 1993), *H. danubialis* (Haase, 1993), and *H. wienerwaldensis* Haase, 1992. Based on anatomical evidence, HAASE (1993) demonstrated that *H. danubialis* was genus-level distinct, and described a new genus: *Lobaunia* Haase, 1993 with *L. danubialis* as type species. ERÖSS & PETRÓ (2008) described a new species of *Hauffenia* from Hungary: *H. kisdalmae* Eröss et Petró, 2008. In 2003–2005 valvatiform *Hauffenia*-like gastropods (most of them empty shells) were found in a few springs in Slovensky Kras, Slovakia (ŠTEFFEK & GREGO 2008). In this study we applied soft part morphology and molecular (mt COI) data to obtain more information on the systematic position of these hydrobiids.

MATERIAL AND METHODS

MATERIAL COLLECTION AND FIXATION

The studied snails came from four localities in Slovensky Kras: Kunova Teplica (Hučiaca Spring and Gemerska Hôrka), Patročnica Spring, and Vidová. 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 after a few days, the 80% solution was replaced with a 96% one and the material was stored at –20°C. The material comprised numerous empty shells and a few specimens (mostly juvenile) with soft parts.

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. The shells were cleaned in an ultrasonic cleaner and photographed with a NIKON DS-5 or CANON EOS 50D digital camera. Protoconchs 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 × 10 min.); their DNA was extracted with the SHERLOCK extracting kit (A&A Biotechnology); the final product was dissolved in 20 µm of TE buffer. The PCR reaction (PALUMBI 1996) was performed with the following primers: LCOI490 (5'-GGTCAACAAATCATAAA GATATTGG-3') and COR722b (5'-TAAACTTCA GGGTGACCAAAAAATYA-3') for the COI gene (FOLMER et al. 1994). The PCR conditions were as follows: initial denaturation step of 4 min at 94°C, followed by 35 cycles at 94°C for of 1 min, 55°C for 1 min, and 72°C for 2 min, and a final extension of 4 min at 72°C. The total volume of each PCR reaction mixture was 50 µl. To check the quality of the PCR products we ran 10 µl of the PCR product on 1% agarose gel. The PCR product was purified using Clean-Up columns (A&A Biotechnology) and 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

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

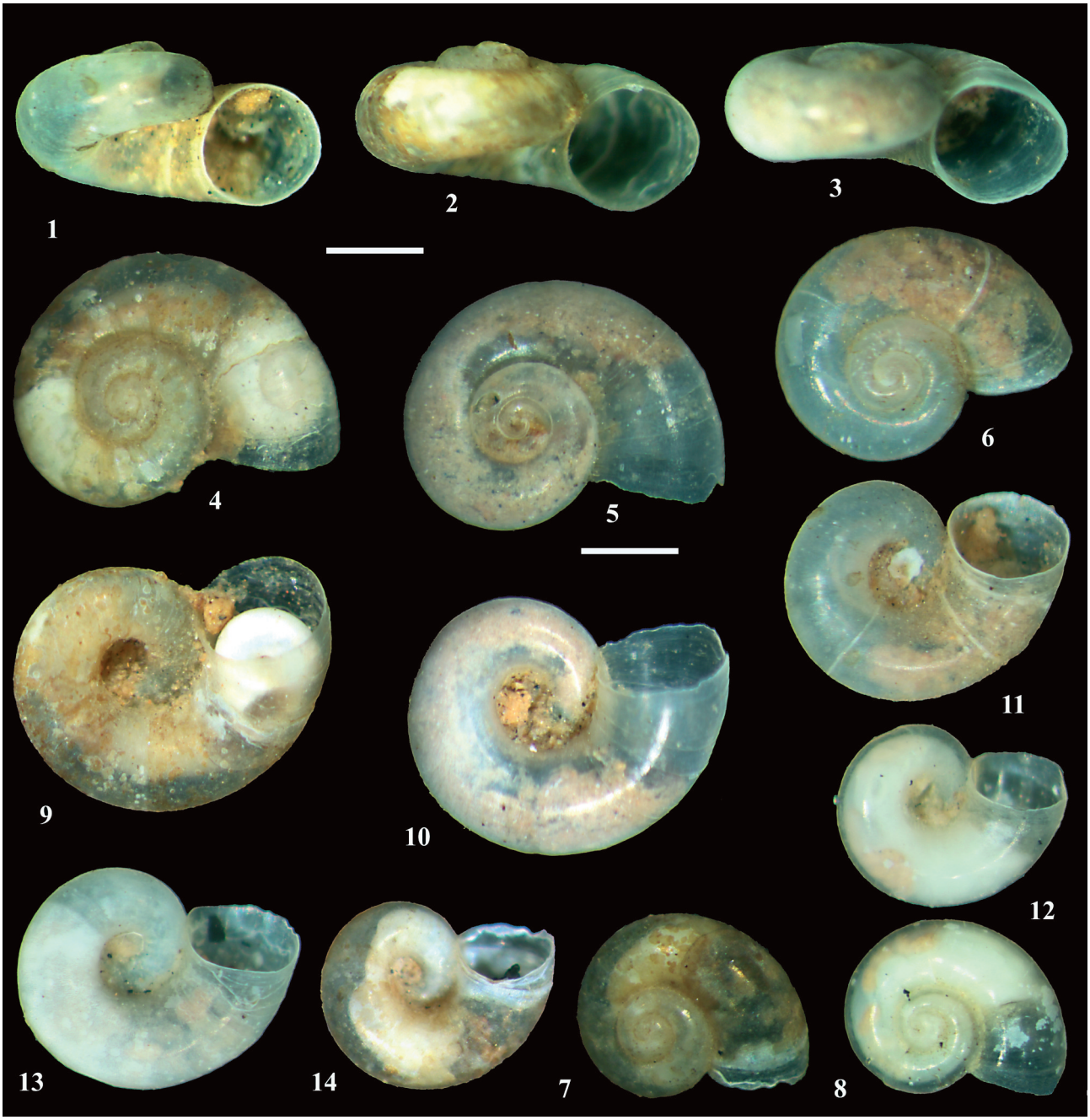
Species	GenBankAN	References
Adriohydrobia gagatinella (Küster, 1852)	AF317881	WILKE & FALNIOWSKI (2001)
Adrioinsulana conovula (Frauenfeld, 1863)	AF367628	WILKE et al. (2001)
Alzoniella finalina Giusti et Bodon, 1984	AF367650	WILKE et al. (2001)
Anagastina zetavalis (Radoman, 1973)	EF070616	SZAROWSKA (2006)
Bithynia tentaculata (Linnaeus, 1758)	AF367643	WILKE et al. (2001)
Bythinella austriaca (Frauenfeld, 1857)	FJ545132	FALNIOWSKI et al. (2009)
Bythiospeum sp.	AF367634	WILKE et al. (2001)
Daphniola graeca Radoman, 1973	EF070618	SZAROWSKA (2006)
Dianella thiesseana (Kobelt, 1878)	AY676127	SZAROWSKA et al. (2005)
Graziana alpestris (Frauenfeld, 1863)	AF367641	WILKE et al. (2001)
Grossuana codreanui (Grossu, 1946)	EF061919	SZAROWSKA et al. (2007)
Hauffenia tellinii (Pollonera, 1898)	AF367640	WILKE et al. (2001)
Hauffenia sp. 1 3O1	JF313940	present study
Hauffenia sp. 2 3O5	JF313941	present study
Hauffenia sp. 2 3O6	JF313942	present study
Hauffenia sp., Patročnica	EF070614	SZAROWSKA (2006)
Heleobia dalmatica (Radoman, 1974)	AF367631	WILKE et al. (2001)
Hydrobia acuta (Draparnaud, 1805)	AF278808	WILKE & DAVIS (2000)
Islamia piristoma Bodon et Cianfanelli, 2001	AF367639	WILKE et al. (2001)
Lithoglyphus naticoides (C. Pfeiffer, 1828)	AF367642	WILKE et al. (2001)
Marstoniopsis insubrica (Küster, 1853)	AY027813	FALNIOWSKI & WILKE (2001)
Pseudamnicola lucensis (Issel, 1866)	AF367651	WILKE et al. (2001)
Pseudobithynia sp.	EF070620	SZAROWSKA (2006)
Pyrgula annulata (Linnaeus, 1767)	AY341258	SZAROWSKA et al. (2005)
Radomaniola callosa (Paulucci, 1881)	AF367649	WILKE et al. (2001)
Rissoa labiosa (Montagu, 1803)	AY676128	SZAROWSKA et al. (2005)
Sadleriana fluminensis (Küster, 1853)	AY273996	WILKE et al. (2001)
Trichonia kephalovrissonia Radoman, 1973	EF070619	SZAROWSKA (2006)
Ventrosia ventrosa (Montagu, 1803)	AF118335	WILKE & DAVIS (2000)

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 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, we tested different models of sequence evolution 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). We performed ML analyses in PAUP*4.0b10 (SWOFFORD 2002) and used an heuristic search strategy with stepwise addition of taxa, 10 random- sequence addition replicates, and tree-bisec-



Figs 1–14. Shells of *Hauffenia*: 1–3 – front view, 4–8 – dorsal view, 9–14 – ventral view; bars equal 0.5 mm

tion-reconnection (TBR) branch swapping (SWOFFORD et al. 1996). We estimated nodal support 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.

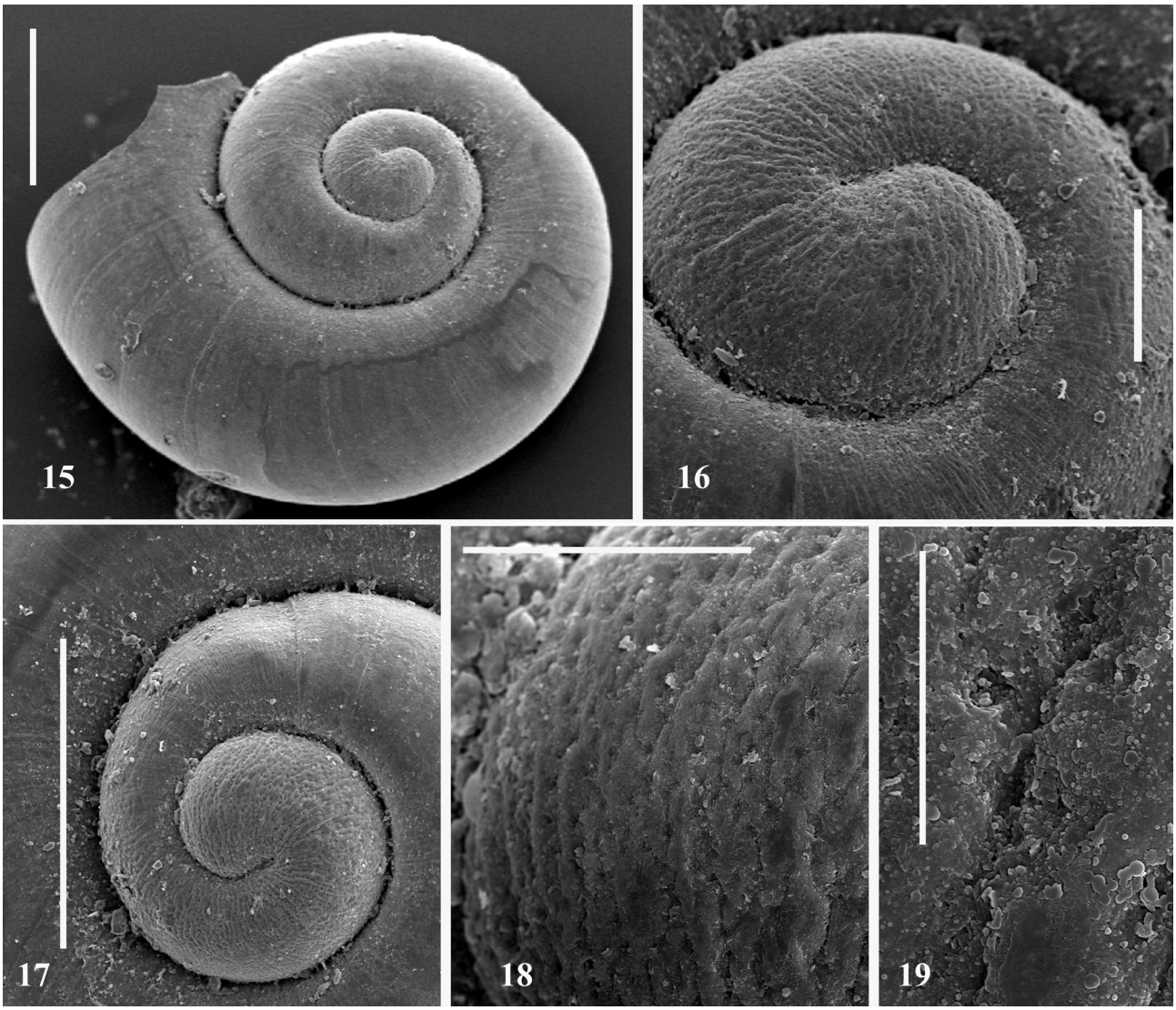
We ran minimum evolution and maximum parsimony on PAUP*, and neighbour-joining on MEGA4 (TAMURA et al. 2007). Nodal support was estimated using the bootstrap approach (full heuristic search) with 1,000 replicates.

RESULTS

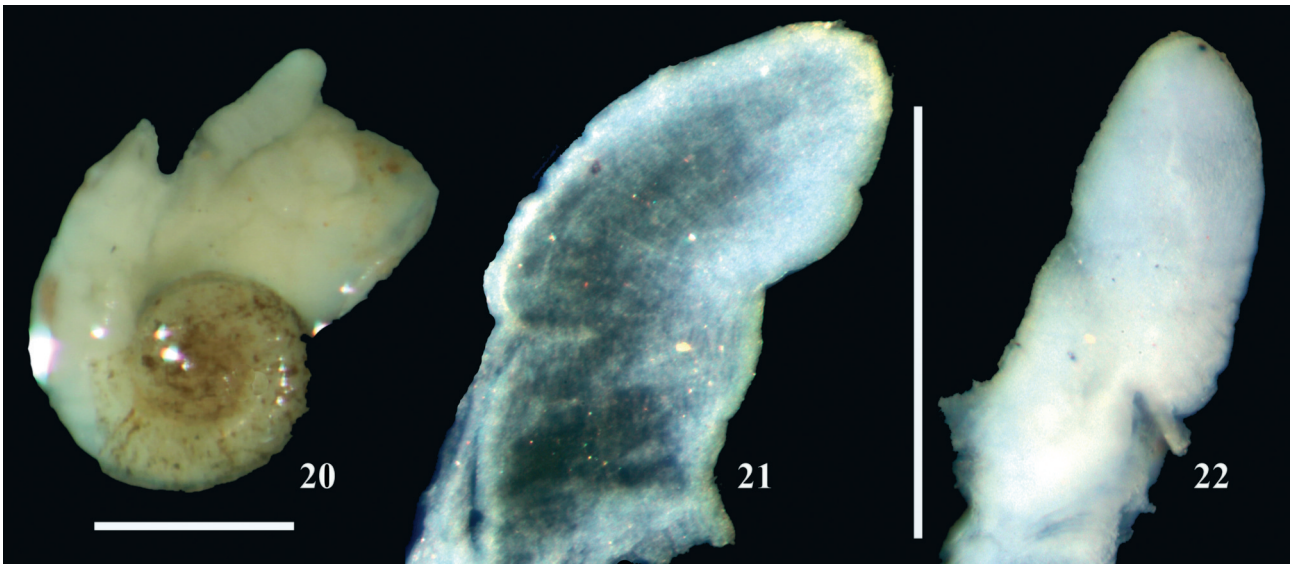
MORPHOLOGY

The shell (Figs 1–14) – up to 1.8 mm broad and 0.9 mm high – has 2–2.5 rapidly but regularly growing whorls (Figs 4–8). The spire is low (Figs 1–2) or very low (the specimen in Fig. 3 resembles a planispiral shell). The umbilicus (Figs 9–14) is very wide, with the earlier whorls visible inside. The shell is thin-walled and glossy. The teleoconch (Fig. 15) is very finely sculptured with weakly marked growth lines. The protoconch (Figs 16–17) has about 1¼ whorls growing slowly; the border between the proto- and teleoconch is indistinct (Figs 16–17); the protoconch surface is nodular (Figs 18–19).

There is neither body pigment nor eyes (Fig. 20). The penis (Figs 21–22), broad and blunt, has a weakly marked lateral lobe on its left side near the apex, a very small stylet, a penial duct running in a zigzag,



Figs 15–19. Shell of *Hauffenia*: 15 – dorsal view of whole shell, 16–17 – protoconch habitus, 18–19 – protoconch microsculpture; bars equal: 300 μm, 75 μm, 300 μm, 50 μm and 10 μm, respectively



Figs 20–22. Soft parts external morphology of *Hauffenia* male: 20 – whole specimen, 21–22 – penis (21 – under cover slip); bar equals 0.5 mm

and no visible trace of an ejaculatory duct (Fig. 21). The female reproductive organs are of *Hauffenia*-type. Unfortunately, because of the shortage of adult and well fixed females, we could not study the details of the female organs.

MOLECULAR PHYLOGENY

The Akaike Information Criterion (AIC) with ModelTest selected the model TVM+I+Γ, with base frequencies: A=0.3336, C=0.1553, G=0.1258, T=0.3854; substitution rate matrix: [A-C]=0.5823, [A-G]=5.3092, [A-T]=0.6297, [C-G]=1.4460, [C-T]=5.3092, [G-T]=1.0000, proportion of invariable sites: (I)0.3369, and Γ distribution with the shape parameter =0.5928. In Fig. 23 the resulting ML tree undoubtedly places the studied Slovak valvatoid-shelled snails within the Hydrobiidae. Haplotypes 3O5 and 3O6, close to each other and forming a clade (support 57/90/100/100), are distinct from both haplotype 3O1 and the haplotype of *Hauffenia* sp. from Patročnica, published by SZAROWSKA (2006). The latter two haplotypes do not form a clade in this ML tree, but such a clade appeared in 62 ML bootstrap trees (Fig. 23). Therefore, there are two molecularly distinct taxa, none of them close to *H. tellinii* (Fig. 23). Only a few clades within the tree are significantly supported.

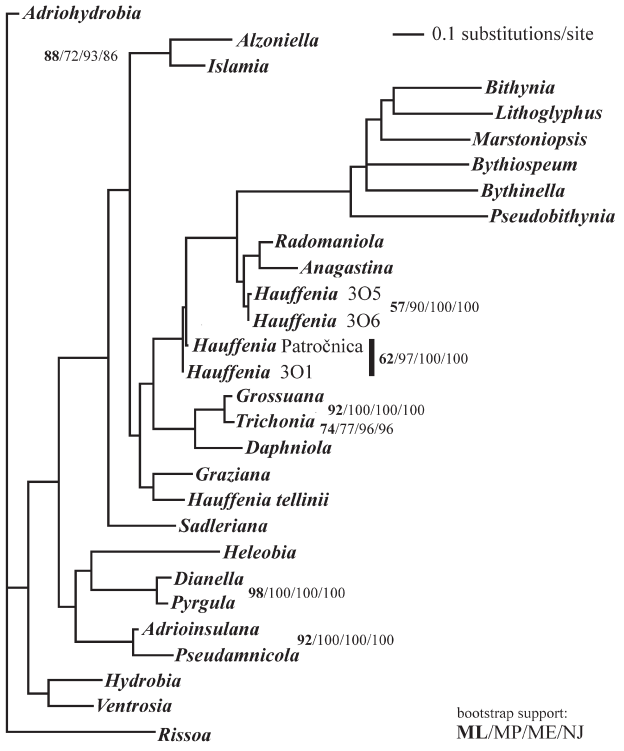


Fig. 23. Maximum likelihood phylogram, bootstrap supports (1,000 replicates given for each branch if >50: **ML**/MP/ME/NJ

DISCUSSION

The studied material consisted of a few living specimens, most of them juvenile. Because of the collection technique applied many specimens were poorly fixed. The valvatiform hydrobiids are very rare in the study area, so there is little chance of collecting more material soon. Therefore, the study is based upon scarce and insufficient data, and its results are preliminary. Nevertheless, for the above reasons we decided to publish the results, taking into account that all conclusions we could make would be provisional only.

The penis of the studied snails resembles the penes of *Hauffenia* known from the literature (BOLE 1970, BERNASCONI 1985, HAASE 1992, 1993, BODON et al. 2001, GLÖER 2002). We did not find any trace of an ejaculatory duct, thus the studied specimens cannot be assigned to the genus *Lobaunia* (HAASE 1993), but only a few adult males were collected and examined. The penial characters indicate that the Slovak specimens belong to *Hauffenia* Pollonera, 1898, but morphological characters may be misleading in phylogeny reconstruction within the Rissooidea (SZAROWSKA 2006).

The molecular data do not confirm that the Slovak valvatiform hydrobiids belong to *Hauffenia*. The type species, *H. tellinii*, is not close to the Slovak specimens. The latter undoubtedly are not conspecific. Haplotypes 3O5 and 3O6 represent one taxon while haplotype 3O1 and the haplotype published by SZAROWSKA (2006) represent another. The K2P distances between the two taxa, reflected in the length of the branches of the phylogram (Fig. 23) indicate that the two taxa probably do not belong to one genus. One of them may thus represent *Lobaunia* Haase, 1993, and the other may belong to *Hauffenia* in Haase's sense (HAASE 1992, 1993). If this is the case, however, the Austrian *Hauffenia* will not be congeneric with the Italian *H. tellinii*, that is the type species of *Hauffenia*. Perhaps our Slovak species are close to the Hungarian (ERÖSS & PETRÓ 2008), and Austrian (HAASE 1992, 1993, GLÖER 2002) species, but not to the Italian *Hauffenia*. To be able to make the final taxonomic decisions, we are badly in need of molecular data on all the Austrian and Hungarian species.

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