



SPECIES DISTINCTNESS OF *LITHOGLYPHUS PRASINUS* (KÜSTER, 1852) (RISSOOIDEA: CAENOCASTROPODA)

ANDRZEJ FALNIOWSKI, MAGDALENA SZAROWSKA

Department of Malacology, Institute of Zoology, Jagiellonian University, Gronostajowa 9, 30-387 Kraków, Poland (email: andrzej.falniowski@uj.edu.pl)

ABSTRACT: *Lithoglyphus prasinus* (Küster, 1852) is the only representative of the genus *Lithoglyphus* which, instead of rivers, inhabits oligostenothermal waters of karst springs. The taxon is probably endemic to central and southeastern Slovenia and the adjacent territories of Croatia. Apart from the ecology and shell morphology, there are no differences between *L. prasinus* and the other *Lithoglyphus* species. The species distinctness of the taxon is doubtful; it has been postulated to be an oligostenothermal ecotype or race of *L. naticoides*. In the present paper partial sequences of cytochrome oxidase subunit I (COI) of mtDNA were used to check the species distinctness of *L. prasinus* from the Močilnik spring of Ljubljana river in Slovenia. For the COI sequences K2P distances between the two taxa were 0.03170–0.03347. This, coupled with small intraspecific differences in both taxa, suggests that *L. prasinus* is a distinct species.

KEY WORDS: lithoglyphid, oligostenothermal, COI

INTRODUCTION

The genus *Lithoglyphus* Hartmann, 1821, with its type species *L. naticoides* (Pfeiffer, 1828), is known from Europe (KABAT & HERSHLER 1993). There is a rather rich literature on the invasion and subsequent extinction of Ponto-Caspian *L. naticoides* in extensive areas of central and western Europe (FALNIOWSKI 1987, GLÖER 2002, IUCN 2010). In the Balkans there are a few other nominal species of *Lithoglyphus* (RADOMAN 1983, 1985, BANK et al. 2006, BABA 2007), but the species distinctness of those taxa remains doubtful (BOLE 1981, IUCN 2010, CUTTELOD et al. 2011). All the lithoglyphid taxa, with one exception, inhabit rivers or, less commonly, lakes. Only *L. prasinus* (Küster, 1852) inhabits oligostenothermal waters of karst springs. The water temperature in these habitats typically ranges between 7°C to 10°C throughout the year (BOLE 1981).

RADOMAN (1978) described *Lithoglyphus neofontinalis* n. sp. from the spring in Gabrovčec, the spring area of the Krka river in Slovenia, and considered *Paludina prasina* Küster, 1852 to be a synonym of *Sadleriana fluminensis* (Küster, 1852). However, as explained by BOLE (1981), the shells of the two species, *Paludina prasina* and *Sadleriana fluminensis*, although

similar in outline, are markedly different in size. RADOMAN (1978) probably misinterpreted “lines” (1 “line” equals 2.25 mm) in KÜSTER’s (1852) original description as millimetres. Thus, *L. neofontinalis* is a junior synonym of *L. prasinus* (BOLE 1981, BANK et al. 2001), and *L. prasinus* remains the only lithoglyphid species inhabiting springs.

The type locality of *L. prasinus* is the source of the Krka river in Slovenia (KÜSTER 1852). BOLE (1981) listed springs in the river systems of the Krka, Ljubljana and Kolpa/Kupa rivers as inhabited by *L. prasinus*, and the Močilnik spring among the localities his materials had been collected from. BABA (2007) recorded the species from the Ogulin, Leskovac and Stunjica creeks in the coastal areas of Croatia. Probably the taxon is endemic to central and south-east Slovenia, and the adjacent territories of Croatia (SKET’s opinion cited in IUCN 2010). The geographically and ecologically narrow range of *L. prasinus*, as well as possible threats to the springs/rivers situated close to the coast, make *L. prasinus* a candidate for Near Threatened or Threatened status (IUCN 2010, CUTTELOD et al. 2011, SLAPNIK 2011).

Associating the shell with the spring habitat, neither being *Lithoglyphus*-typical, BOLE (1981) considered *L. prasinus* an oligostenothermal ecotype or race of *L. naticoides*. Based on morphological data alone, the spe-

cies distinctness of *L. prasinus* remains open (IUCN 2010, SLAPNIK 2011). The aim of our study was to validate *L. prasinus* species status using molecular data.

MATERIAL AND METHODS

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

The snails were washed twice in 80% ethanol and left to stand in it for ca. 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.

For the morphological study we cleaned the shells in an ultrasonic cleaner and photographed them with a CANON EOS 50D digital camera. Two males and two females were dissected, using a NIKON SMZ-U stereoscope microscope.

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 prod-

uct was dissolved in 20 µl TE buffer (the same concentration as used for hydration). The PCR reaction was performed with the following primers: LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') (FOLMER et al. 1994) and COR722b (5'-TAAACTCAGGGTGACCAAAAAATYA-3') (WILKE & DAVIS 2000) for the cytochrome oxidase subunit I (COI) mitochondrial gene. The PCR conditions were as follows: 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. 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 ran 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 (HILLIS et al. 1996) using BigDye Terminator v3.1 (Applied Biosystems), following the manufacturer's protocol and with the



Fig. 1. Močilnik spring

Table 1. Taxa used for phylogenetic inference, with GenBank Accession Numbers for cytochrome oxidase subunit I (COI) and references

Species	GB AccN	References
<i>Lithoglyphus prasinus</i> (Küster, 1852) A1	JX073651	present study
<i>Lithoglyphus prasinus</i> A2	JX073652	present study
<i>Lithoglyphus naticoides</i> (Pfeiffer, 1828) W	AF367642	WILKE et al. (2001)
<i>Lithoglyphus naticoides</i> L	AF354770	LIU et al. (2001)
<i>Lithoglyphus naticoides</i> H	AF445332	HAUSDORF et al. (2003)
<i>Bithynia tentaculata</i> (Linnaeus, 1758)	AF367643	WILKE et al. (2001)
<i>Sadleriana fluminensis</i> (Küster, 1853)	AF367643	WILKE et al. (2001)
<i>Marsoniopsis insubrica</i> (Küster, 1853)	AY027813	FALNIOWSKI & WILKE (2001)
<i>Bythinella austriaca</i> (Frauenfeld, 1857)	FJ545132	FALNIOWSKI et al. (2009)
<i>Rissoa labiosa</i> (Montagu, 1803)	AY676128	SZAROWSKA et al. (2005)

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).

The COI sequences were aligned by eye and edited using BioEdit 5.0.0 (HALL 1999). K2P and p genetic

distances (KIMURA 1980) were calculated with MEGA4 (KUMAR et al. 2004). MEGA4 was also applied to phylogeny reconstruction with minimum-evolution approach (SWOFFORD et al. 1996, NEI & KUMAR 2000) and K2P distances. Nodal support was estimated using the bootstrap approach (FELSENSTEIN 1985).

RESULTS AND DISCUSSION

The shells of *Lithoglyphus prasinus* from Močilnik (Fig. 2 A–B) looked like those of *L. prasinus* illustrated by BOLE (1981: fig. 2), and differed from the shell of *L. naticoides* (Fig. 2 C). The male and female genitalia, typically lithoglyphid, resembled the ones described for *L. naticoides* (RADOMAN 1983, FALNIOWSKI 1987, SZAROWSKA 2006) and were iden-

tical with the organs of *L. prasinus* portrayed in BOLE (1981: fig. 3).

Two COI sequences, each 654 bp long, were the same except for three positions. On the other hand, the two sequences of *L. prasinus* differed from *L. naticoides* in 13–14 positions. K2P distances (Table 2) were 0.00489 within *L. prasinus*, and 0.03170–0.03347

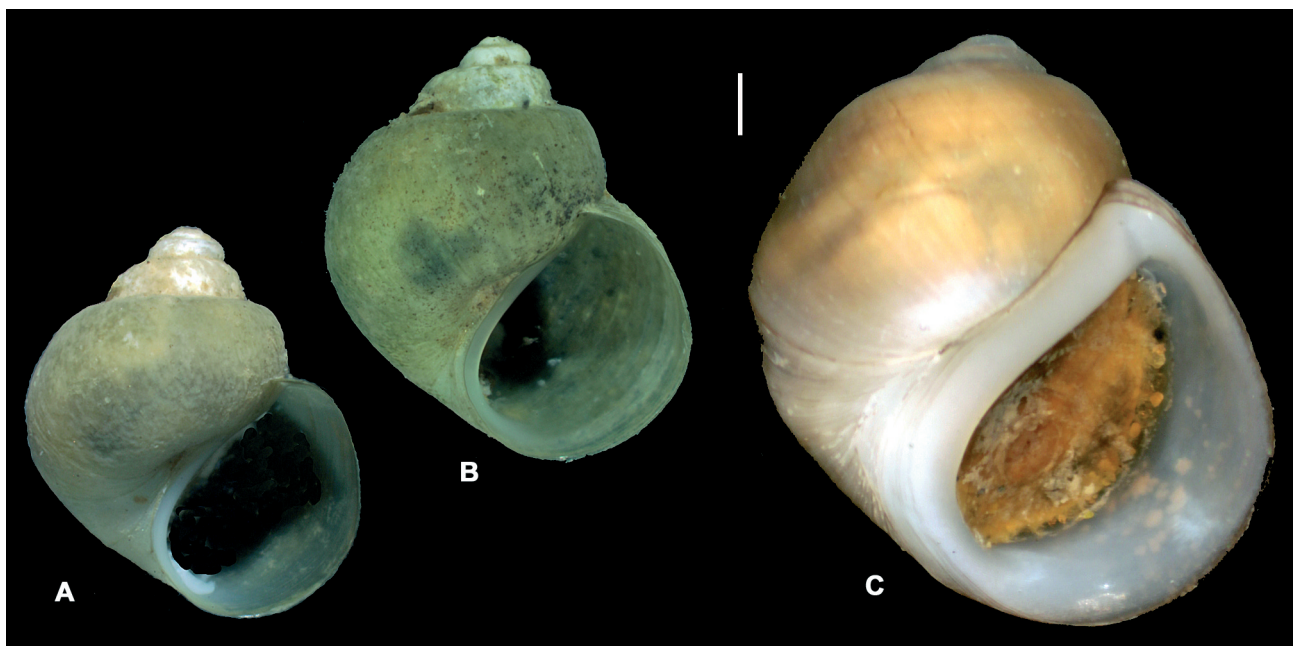


Fig. 2. Shells of *Lithoglyphus prasinus* (A–B) and *L. naticoides* (C)

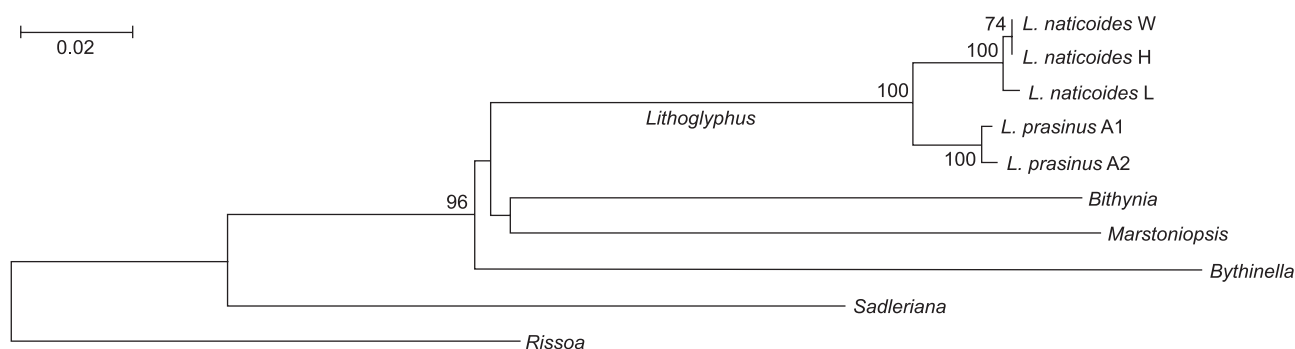


Fig. 3. Minimum evolution tree computed for K2P distances given in Table 2; bootstrap supports given where >50 (for A1, A2, H, L, W – see Table 1)

Table 2. Kimura 2-parameter (K2P) distances (below diagonal) and p-distances (above diagonal); for A1, A2, H, L, W – see Table 1

	1	2	3	4	5	6	7	8	9	10
<i>Lithoglyphus prasinus</i> A1	*.*****	0.00487	0.03084	0.03247	0.03084	0.16721	0.20455	0.17532	0.18669	0.22565
<i>Lithoglyphus prasinus</i> A2	0.00489	*.*****	0.03084	0.03247	0.03084	0.16883	0.20617	0.17532	0.18831	0.22565
<i>Lithoglyphus naticoides</i> W	0.03170	0.03179	*.*****	0.00487	0.00000	0.17370	0.20292	0.17532	0.19318	0.22727
<i>Lithoglyphus naticoides</i> L	0.03338	0.03347	0.00489	*.*****	0.00487	0.17532	0.20455	0.17370	0.19481	0.22890
<i>Lithoglyphus naticoides</i> H	0.03170	0.03179	0.00000	0.00489	*.*****	0.17370	0.20292	0.17532	0.19318	0.22727
<i>Bithynia tentaculata</i>	0.18970	0.19185	0.19826	0.20035	0.19826	*.*****	0.21266	0.17857	0.19805	0.24026
<i>Sadleriana fluminensis</i>	0.24076	0.24312	0.23857	0.24076	0.23857	0.25229	*.*****	0.22890	0.25162	0.20130
<i>Marsoniopsis insubrica</i>	0.20128	0.20128	0.20115	0.19881	0.20115	0.20575	0.27687	*.*****	0.20455	0.21753
<i>Bythinella austriaca</i>	0.21658	0.21887	0.22560	0.22775	0.22560	0.23206	0.31129	0.24372	*.*****	0.22403
<i>Rissoa labiosa</i>	0.27063	0.27101	0.27369	0.27597	0.27369	0.29666	0.23773	0.26185	0.27164	*.*****

between *L. prasinus* and *L. naticoides*. *L. prasinus* and *L. naticoides*, forming two distinct, highly supported monophyletic clades (bootstrap support 100), are sister to each other and form a well supported *Lithoglyphus* clade (Fig. 3).

There is no simple, “routine” way of interpretation of genetic distances while assessing species distinctness. Distance values are applicable only within a group of closely related species, and there are no such data for the Lithoglyphidae. In fact, the inferred values of K2P or p distances are in the range known for rissooids as interspecific in some cases and intraspecific in some others (PEREZ et al. 2005, FALNIOWSKI et al. 2007, SZAROWSKA et al. 2007, FALNIOWSKI & SZAROWSKA 2011). BICHAIN et al. (2007) reported the threshold value 0.015 in the west-European *Bythinella* species. Furthermore, the intraspecific K2P distances

between *L. naticoides* from GenBank (0.000–0.00489) are only one-tenth the distances between *L. naticoides* and *L. prasinus*. The geographic distance between the two GenBank localities of *L. naticoides*: the Narew river at Drozdowo, Poland (WILKE et al. 2001) and the Hron river at Zvolen, Slovakia (LIU et al. 2001) is 556 km. Moreover, the Narew river belongs to the Baltic Sea, and the Hron river to the Black Sea catchment area. On the other hand, such a low level of genotypic differentiation may be expected in the case of an invasive species, like *L. naticoides*. Obviously, more data on the genotypic differentiation within *Lithoglyphus* are needed, but with the present data it seems that *L. prasinus* is a distinct species, or, at least, a taxon genotypically different enough to deserve Near Threatened or Threatened status, and protection.

REFERENCES

- BÁBA K. 2007. Area-analytical zoogeographic classification for the members of the gastropod family Hydrobiidae of Southern Europe II. Malakológiai Tájékoztató. Malacol. Newslett. 25: 29–31.
- BANK R. A., BOUCHET P., FALKNER G., GITTENBERGER E., HAUSDORF B., PROSCHWITZ T. VON, RIPKEN T. E. J. 2001. Supraspecific classification of European non-marine Mollusca (CLECOM Sections I + II). *Heldia* 4: 77–128.



- BANK R., PROSCHWITZ T. VON, FALKNER G. 2006. Unpublished manuscript of the mollusca section of the Fauna Europea web-site (<http://www.faunaeur.org>).
- BICHAIN J.-M., GAUBERT P., SAMADI S., BOISSELIER-DUBAYLE M.-C. 2007. A gleam in the dark: Phylogenetic species delimitation in the confusing spring-snail genus *Bythinella* Moquin-Tandon, 1856 (Gastropoda: Rissooidea: Amnicolidae). *Mol. Phyl. Evol.* 45: 927–941. doi: 10.1016/j.ympev.2007.07.018
- BOLE J. 1981. Zur Problematik der Gattung *Lithoglyphus* Hartmann 1821 (Gastropoda: Lithoglyphidae). *Academia Scientiarum et Artium Slovenica, Dissertationes Classis IV: Historia Naturalis XXIII/5*: 151–169.
- CUTTELOD A., SEDDON M., NEUBERT E. 2011. European Red List of Non-marine Molluscs. Publications Office of the European Union, Luxembourg.
- FALNIOWSKI A. 1987. Hydrobioidea of Poland (Prosobranchia: Gastropoda). *Folia Malacol.* 1: 1–122.
- FALNIOWSKI A., SZAROWSKA M. 2011. Radiation and phylogeography in a spring snail *Bythinella* (Mollusca: Gastropoda: Rissooidea) in continental Greece. *Ann. Zool. Fenn.* 48: 67–90.
- FALNIOWSKI A., SZAROWSKA M., GRZMIL P. 2007. *Daphniola* Radoman, 1973 (Gastropoda: Hydrobiidae): shell biometry, mtDNA, and the Pliocene flooding. *J. Nat. Hist.* 41: 2301–2311. doi: 10.1080/00222930701630733
- FALNIOWSKI A., SZAROWSKA M., SIRBU I. 2009. *Bythinella* Moquin-Tandon, 1856 (Gastropoda: Rissooidea: Bythinellidae) in Romania: species richness in a glacial refugium. *J. Nat. Hist.* 43: 2955–2973. doi: 10.1080/00222930903359636
- FALNIOWSKI A., WILKE T. 2001. The genus *Marstoniopsis* (Gastropoda: Rissooidea): intra- and intergeneric phylogenetic relationships. *J. Moll. Stud.* 67: 483–488. doi: 10.1093/mollus/67.4.483
- FELSENSTEIN J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791. doi: 10.2307/2408678
- FOLMER O., BLACK M., HOEH W., LUTZ R. A., VRIJENHOEK R. C. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* 3: 294–299.
- GLÖER P. 2002. Die Süßwassergastropoden Nord- und Mitteleuropas, Bestimmungsschlüssel, Lebensweise, Verbreitung. *Die Tierwelt Deutschlands* 73. Conchbooks, Hackenheim.
- HALL T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. [Computer software and manual] *Nucl. Acids Symp. Ser.* 41: 95–98.
- HAUSDORF B., RÖPSTORF P., RIEDEL F. 2003. Relationships and origin of endemic Lake Baikal gastropods (Caenogastropoda: Rissooidea) based on mitochondrial DNA sequences. *Mol. Phyl. Evol.* 26: 435–443. doi: 10.1016/S1055-7903(02)00365-2
- HILLIS D. M., MABLE B. K., LARSON A., DAVIS S. K., ZIMMER E. A. 1996. Nucleic acids IV: sequencing and cloning. In: HILLIS D. M., MORITZ C., MABLE B. K. (eds). *Molecular systematics*, 2 ed., Sinauer Associates, Inc., Sunderland, pp. 321–381.
- IUCN 2010. IUCN Red list of threatened species (ver. 2010.4). Available at: <http://www.iucnredlist.org>.
- KABAT A. R., HERSHLER R. 1993. The prosobranch snail family Hydrobiidae (Gastropoda: Rissooidea): review of classification and supraspecific taxa. *Smith. Contr. Zool.* 547: 1–94. doi: 10.5479/si.00810282.547
- KIMURA M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111–120. doi: 10.1007/BF01731581
- KUMAR S., TAMURA K., NEI M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* 5: 150–163. doi: 10.1093/bib/5.2.150
- KÜSTER H. C. 1852. Die Gattung *Paludina*, *Hydrocaena* und *Valvata*. *Mart.-Chemn. Syst. Conch. Cab.* 1: 1–96.
- LIU H. P., HERSHLER R., THOMPSON F. G. 2001. Phylogenetic relationships of the Cochliopinae (Rissooidea: Hydrobiidae): an enigmatic group of aquatic gastropods. *Mol. Phyl. Evol.* 21: 17–25. doi: 10.1006/mpev.2001.0988
- NEI M., KUMAR S. 2000. *Molecular evolution and phylogenetics*. Oxford University Press, Oxford.
- PEREZ K. E., PONDER W. F., COLGAN D. J., CLARK S. A., LYDEARD C. 2005. Molecular phylogeny and biogeography of spring-associated hydrobiid snails of the Great Artesian Basin, Australia. *Mol. Phyl. Evol.* 34: 545–556. doi: 10.1016/j.ympev.2004.11.020
- RADOMAN P. 1978. Neue Vertreter der Gruppe Hydrobioidea von Balkanhalbinsel. *Arch. Moll.* 109: 27–44.
- RADOMAN P. 1983. Hydrobioidea a superfamilie of Prosobranchia (Gastropoda). I. Systematics. *Serbian Academy of Sciences and Arts, Monographs* 547, Department of Sciences 57: 1–256.
- RADOMAN P. 1985. Hydrobioidea, a superfamily of prosobranchia (Gastropoda). II. Origin, zoogeography, evolution in the Balkans and Asia Minor. *Faculty of Science Department of Biology, Monographs* 1, Institute of Zoology Beograd 1: 1–173.
- SLAPNIK R. 2011. *Lithoglyphus prasinus*. In: IUCN 2011. IUCN Red List of threatened species (ver. 2011.2), Available at: <http://www.iucnredlist.org>.
- SWOFFORD D. L., OLSEN G. J., WADDELL P. J., HILLIS D. M. 1996. Phylogenetic inference. In: HILLIS D. M., MORITZ C., MABLE B. K. (eds), *Molecular systematics*, 2 ed., Sinauer Associates, Inc., Sunderland, pp. 407–514.
- SZAROWSKA M. 2006. Molecular phylogeny, systematics and morphological character evolution in the Balkan Rissooidea (Caenogastropoda). *Folia Malacol.* 14: 99–168.
- SZAROWSKA M., FALNIOWSKI A., RIEDEL F., WILKE T. 2005. Phylogenetic relationships of the subfamily Pyrgulinae (Gastropoda: Caenogastropoda: Hydrobiidae) with emphasis on the genus *Dianella* Gude, 1913. *Zootaxa* 891: 1–32.
- SZAROWSKA M., GRZMIL P., FALNIOWSKI A., SIRBU I. 2007. *Grossuana codreanui* (Grossu, 1946) and the phylogenetic relationships of the East Balkan genus *Grossuana*



- (Radoman, 1973) (Gastropoda: Rissooidea). *Hydrobiologia* 579: 379–391. doi: 10.1007/s10750-006-0530-4
- WILKE T., DAVIS G. M. 2000. Intraspecific mitochondrial sequence diversity in *Hydrobia ulvae* and *Hydrobia ventrosa* (Hydrobiidae: Rissoacea: Gastropoda): Do their different life histories affect biogeographic patterns and gene flow? *Biol. J. Linn. Soc. London* 70: 89–105. doi: 10.1111/j.1095-8312.2000.tb00202.x
- WILKE T., DAVIS G. M., FALNIOWSKI A., GIUSTI F., BODON M., SZAROWSKA M. 2001. Molecular systematics of Hydrobiidae (Gastropoda: Rissooidea): testing monophyly and phylogenetic relationships. *Proc. Acad. Nat. Sci. Philadelphia* 151: 1–21. doi: 10.1635/0097-3157(2001)151[0001:MSOHMG]2.0.CO;2

Received: January 2nd, 2012

Revised: April 2nd, 2012

Accepted: April 5th, 2012

