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ISLAMIA ZERMANICA (RADOMAN, 1973) (CAENOGASTROPODA: HYDROBIIDAE): MORPHOLOGICAL AND MOLECULAR DISTINCTNESS

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ABSTRACT: Two living populations of *Islamia zermanica* (Radoman) were found in the mid section of the Zrmanja River in Croatia. The species is endemic to the freshwater part of this river and is regarded as Critically Endangered/Possibly Extinct. Specimens of *I. valvataeformis* (Möllendorf) were collected at Slime, Croatia. The shell habitus, soft parts' pigmentation and the terminal part of the penis confirmed their generic assignment and distinctness of the two *Islamia* species. Partial sequences of cytochrome oxidase subunit I (*COI*) confirmed the specific status of *I. zermanica* and *I. valvataeformis*, and their appurtenance to the same genus as the Italian *I. piristoma* Bodon et Cianfanelli.

KEY WORDS: protoconch, teleoconch, reproductive organs, DNA, cytochrome oxidase, phylogeny

INTRODUCTION

The rich fauna of Truncatelloidea of the former Yugoslavia has been extensively studied (e.g. RADOMAN 1976, 1983 and references therein, 1985, SZAROWSKA 2006 and references therein, FALNIOWSKI et al. 2012a). However, the taxonomy of the group has generally been based on morphological characters alone, often not sufficient even for generic assignment (FALNIOWSKI et al. 2012b).

Islamia Radoman, 1973 is a genus of minute freshwater snails of the family Hydrobiidae. A few nominal species have been described from Croatia, Bosnia and Herzegovina, Greece, Italy, Turkey (KABAT & HERSHLER 1993) and western Europe (ARCONADA & RAMOS 2006). The female reproductive organs of the genus are characteristic, with a pair of receptacula seminis situated close to each other, and no bursa copulatrix (RADOMAN 1983, BODON et al. 2001, SZAROWSKA 2006). However, species distinction within the genus has been based mostly on the shell, and the habitus of the penis in some cases (RADOMAN 1983). Molecular data (partial sequences of *COI* and *18SrDNA* genes) are known so far only for the Italian *Islamia piristoma* Bodon et Cianfanelli, 2001.

Islamia valvataeformis (Möllendorf, 1873) is the type species of *Islamia* (RADOMAN 1973, 1983), described from Vrelo Bosne near Sarajevo (Fig. 1). Another species, described from Bosnia and Herzegovina, is *I. bosniaca* Radoman, 1973 (type locality: Podgaj, springs along the road between Zenica and Doboj). The other two species of *Islamia* from the former Yugoslavia have been described from Croatia: *I. latina* Radoman, 1973 from the Miraca creek near the village of Islam Latinski 20 km south of Zadar, and *Islamia zermanica* Radoman, 1973 from the freshwater parts of the Zrmanja River.

The Zrmanja River in Croatia, 69 km long, with its catchment area covering 907 km², is one of the European diversity hot spots of freshwater gastropods. It is inhabited by 16 species, five of them endemic (STRONG et al. 2008; 22 species according to BERAN 2011). The Rissooidea are represented by three species found in the brackish-water part below the Jankovica Buk waterfalls, and by eight species in the freshwater part above Jankovica Buk. Four of the freshwater species: *Belgrandiella krupensis* Radoman, 1973, *B. zermanica* Radoman, 1973, *Islamia zermanica* Radoman, 1973, and *Tanousia zrmanjae* (Brusina, 1866) (= *Lithoglyphulus tedanicus* Schlickum et Schütt, 1971) have been described from this part including its tributary, the Krupa River (RADOMAN 1985). However, it has to be noted that species distinctness of *Belgrandiella krupensis* and *B. zermanica* is doubtful (FALNIOWSKI & BERAN 2015). In the IUCN Red List (CUTTELOD et al. 2011) *Islamia zermanica* is classified as Critically Endangered (Possibly Extinct), based on the habitat loss at the type locality. The river has been dammed in its lower part (dividing the site of *I. zermanica*), and its upper course, due to the karstic character of the river, represents a system of stagnant pools (like that at Kaštel Zegarski) interspersed with dry river bed (like that at Ervenik): only a few gastropod species live in the upper section.

The aim of this study was to check the distinctness of *Islamia zermanica*, compared with *I. valvataeformis*, using morphological and molecular data.

MATERIAL AND METHODS

SAMPLE COLLECTION AND PRESERVATION

We collected specimens of *Islamia* from three localities in Croatia (Fig. 1): *I. zermanica* from Jankovica Buk waterfalls, Zrmanja River (44°12'09.8"N, 15°43'16.9"E; Fig. 2) and from a small spring at Berberi (44°11'27.3"N, 15°45'58.8"E), and *I. valvataeformis* from a rather large spring at Slime (43°25'45.6"N, 16°51'59.5"E).

The snails were collected by hand or with a sieve. Specimens for molecular analyses were washed in 80% ethanol, in which they were left to stand for approximately 12 h. The ethanol was subsequently changed twice over 48 h and the specimens were finally transferred to 96% ethanol after a few days. The samples were stored at –20°C prior to DNA extraction. Dissections were done under a Nikon SMZ18 stereomicroscope with dark field and phase contrast illumination. The shells were cleaned in an ultrasonic cleaner. The protoconchs and teleoconchs were examined using a JEOL JSM-5410 scanning electron microscope, applying the techniques described by FALNIOWSKI (1990).

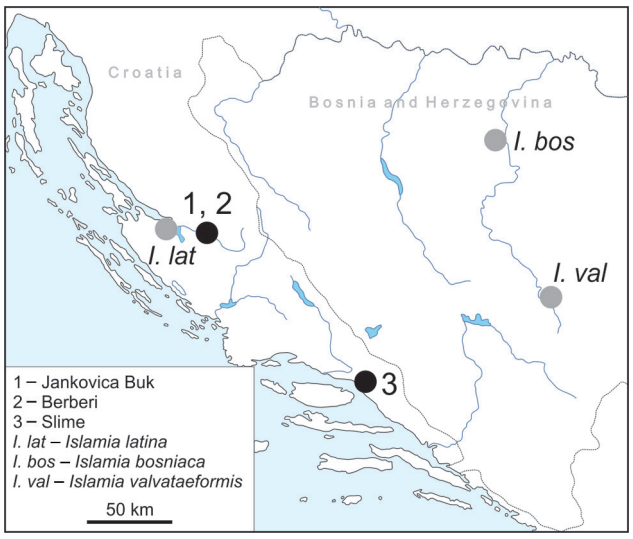


Fig. 1. Sampling sites used in the present study. Three type localities of *Islamia* species from Croatia and Bosnia and Herzegovina are also marked

DNA EXTRACTION AND SEQUENCING

DNA was extracted from foot tissue using a Sherlock extraction kit (A&A Biotechnology) and dissolved in 20 μl of TE buffer. PCR was performed in a reaction mixture with a total volume of 50 μl using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') (FOLMER et al. 1994) and COR722b (5'-TAAACT TCAGGGTGACCAAAAAATYADSA-3') (WILKE & DAVIS 2000) for *COI*. The PCR conditions were as follows: initial denaturation step of 4 min at 94°C, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, with final extension of 4 min at 72°C. A 10 μl sample of the PCR product was run on a 1% agarose gel to check the product's quality. The PCR product was purified using Clean-Up columns (A&A Biotechnology). The purified PCR product was then sequenced in both directions using BigDye Terminator v. 3.1 (Applied Biosystems), following the manufacturer's protocol and using the primers indicated above. The products of the sequencing reaction were purified using ExTerminator Columns (A&A Biotechnology), and the sequences were read using an ABI Prism sequencer.

DATA ANALYSIS

Sequences were aligned and edited in Bioedit v. 7.1.3.0 (HALL 1999). Basic sequence statistics, including haplotype polymorphism and nucleotide di-

Fig. 2. Jankovica Buk waterfall

Table 1. Reference sequences used in phylogenetic analyses

Species	GB number	References
Agrafia wiktori	JF906762	Szarowska & Falniowski 2011
Alzoniella finalina	AF367650	Wilke et al. 2001
Avenionia brevis	AF367638	Wilke et al. 2001
Fissuria boui	AF367654	Wilke et al. 2001
Islamia piristoma	AF367639	Wilke et al. 2001
Pseudamnicola brachia	KT710659	Szarowska et al. 2015

vergence, were calculated in DnaSP v. 5.10 (Librado & Rozas 2009). The saturation test was performed using DAMBE (Xia 2013).

In the phylogenetic analysis, six other sequences from GenBank were used as reference and outgroup (Table 1). The data were analysed using approaches based on Bayesian inference (BI) and maximum likelihood (ML). We applied the GTR model, which is the only nucleotide substitution model implemented in RaxML (Stamatakis 2014), with the option + I + Γ.

The Bayesian analyses were run using MrBayes v. 3.2.3 (Ronquist et al. 2012) with the default priors. Two simultaneous analyses were performed, each of which lasted 10,000,000 generations, with one cold chain and three heated chains, starting from random trees and sampling the trees every 1,000 generations. The first 25% of trees were discarded as burn-in. The analyses were summarised as a 50% majority-rule tree.

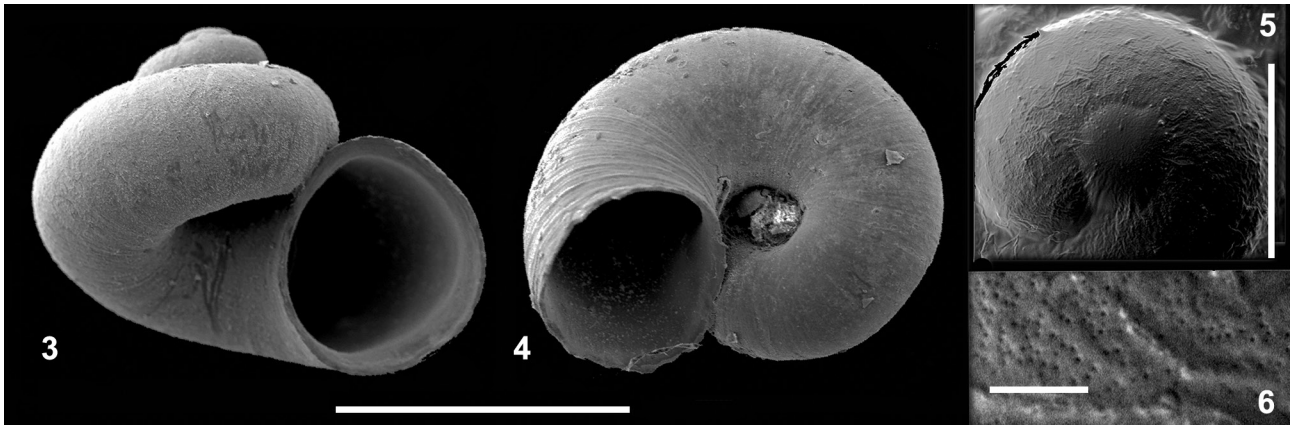
An ML approach was applied in RAxML v. 8.0.24. One thousand searches were initiated with starting trees obtained through the randomized stepwise addition maximum parsimony method. The tree with the highest likelihood score was selected as the best representation of the phylogeny. Bootstrap support was calculated with 1,000 replicates and summarised on the best ML tree. RAxML analyses were performed using the free computational resource CIPRES Science Gateway (Miller et al. 2010).

RESULTS

The shell of *Islamia zermanica* (Figs 3–4), broad, with wide umbilicus and low spire, resembles the one illustrated by RADOMAN (1983) and SZAROWSKA (2006). The shell of *I. valvataeformis* from Slime (not shown) is higher, with much higher spire and relatively narrower umbilicus. The protoconch of *I. zermanica* has a slowly broadening whorl (Fig. 5), and a microsculpture composed of delicate riblets and fine pores (Fig. 6). The soft parts in *I. valvataeformis* from Slime are slightly pigmented, the eyes present (as in the figure in BODON et al. 2001). The soft parts of *I. zermanica* are completely devoid of pigment, and the eyes are absent. The female reproductive organs in both species are identical, and correspond with the figures and descriptions in RADOMAN (1973), BODON et al. (2001), and SZAROWSKA (2006). The two approximately symmetrically arranged lobes forming the termination of the penis are, proportionally to the penis, smaller in *I. valvataeformis* from Slime (although the penis as such is bigger) than in *I. zermanica*.

In the present study we obtained five *COI* gene (552 bp, GenBank Accession numbers KU662358-KU662362) sequences for *Islamia*. The saturation test according to XIA et al. (2003) showed no saturation. The topology of the trees obtained from BI and ML analyses was identical.

The *Islamia* sequences formed one well supported (99%) clade (Fig. 7), which was divided into two distinct subclades with p-distance of 0.033. The first group included three identical sequences from individuals of *I. zermanica* from the Zrmanja River (Jankovica Buk and Berberi). The second group com-



Figs 3–6. Shell of *Islamia zermanica*: 3–4 – habitus, bar equals 1 mm; 5 – protoconch habitus, bar equals 100 μm; 6 – protoconch surface, bar equals 5 μm

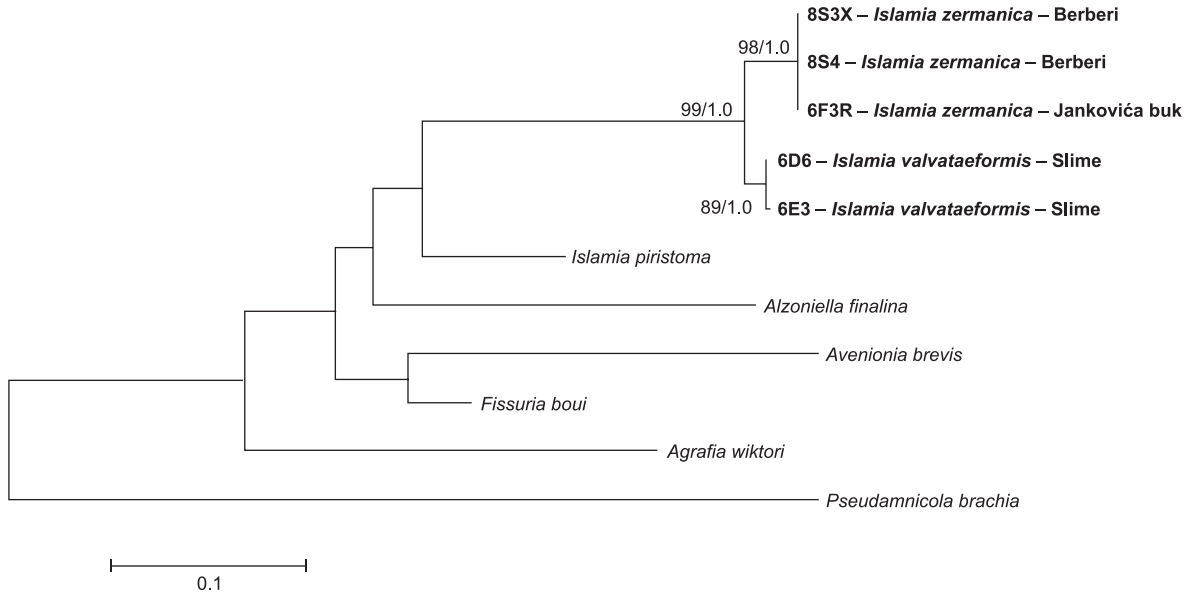


Fig. 7. Maximum-likelihood phylogram for *COI* gene

prised two sequences from specimens from Slime, which differed in one nucleotide position. *I. piristoma* with p-distance of 0.119 was the closest to the Balkan *Islamia* clade. The differences between *I. piristoma* and the two Balkan *Islamia* subclades (Zrmanja River and Slime) were 0.119 and 0.120, respectively.

DISCUSSION

All our new sequences belonged to one distinct and well supported clade, most similar to *I. piristoma*, the only *COI* sequence of *Islamia* available in the GenBank (Wilke et al. 2001). They represented two divergent subclades. The first, *I. zermanica*, was restricted to the Zrmanja River in Croatia, and corresponded to a well described species with distinctive morphology (Radoman 1983). The second *Islamia* subclade came from Slime in southern Croatia. This is the first *Islamia* site in this region. The closest *Islamia* localities are: the Zrmanja River (*I. zermanica*, about 130 km away) and Vrelo Bosne near Sarajevo (about 120 km away) in Bosnia and Herzegovina, the type locality of *I. valvataeformis*. The higher-spired shell of *Islamia* from Slime, as well as the relatively weaker lobes at the terminal part of the penis, and the presence of eyes and pigment, confirm that the population from Slime represents *I. valvataeformis*. It is noteworthy that in the French *Islamia* high- and low-spired shells may occur sympatrically and are most probably conspecific (Bichain & Prié 2005).

Islamia inhabit non-polluted springs, rich in water and aquatic vegetation. Many species of *Islamia* have a very narrow distribution, are threatened by human activities, and are only known from their type localities or are extinct (e.g. Arconada & Ramos 2006). *I. zrmanica*, according to the IUCN Red List (Cuttelod et al. 2011), is Critically Endangered (Possibly Extinct), based on the change of the habitat quality at the type locality due to the damming for hydro-power purposes. Our finding of *I. zermanica* in two sites confirms that the species is still extant, however, more field data are necessary.

I. valvataeformis was only known from the type locality, but its status was unclear. It may have been regarded as threatened, as there are data suggesting that the site lies in a tourist area. Our records suggest a more extensive distribution of the species.

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