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 Islamia 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 Islamia zermanica and Islamia valvataeformis, and their appurtenance to the same genus as the Italian Islamia 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 Islamia bosniae 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: Islamia latina Radoman, 1973 from the Miraca creek near the village of Islan 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).

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’-TAAACTTCAGGGTGACCAAAAAATYADSA-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-
Distinctness of *Islamia zermanica*

 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).

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**Table 1. Reference sequences used in phylogenetic analyses**

<table>
<thead>
<tr>
<th>Species</th>
<th>GB number</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agrafia wiktori</em></td>
<td>JF906762</td>
<td>Szarowska &amp; Falniowski 2011</td>
</tr>
<tr>
<td><em>Alzoniella finalina</em></td>
<td>AF367650</td>
<td>Wilke et al. 2001</td>
</tr>
<tr>
<td><em>Avenionia brevis</em></td>
<td>AF367638</td>
<td>Wilke et al. 2001</td>
</tr>
<tr>
<td><em>Fissuria boui</em></td>
<td>AF367654</td>
<td>Wilke et al. 2001</td>
</tr>
<tr>
<td><em>Islamia piristoma</em></td>
<td>AF367639</td>
<td>Wilke et al. 2001</td>
</tr>
<tr>
<td><em>Pseudamnicola brachia</em></td>
<td>KT710659</td>
<td>Szarowska et al. 2015</td>
</tr>
</tbody>
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Fig. 2. Jankovica Buk waterfall
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
DISCUSSION

All our new sequences belonged to one distinct and well supported clade, most similar to I. piristo-
ma, the only COI sequence of Islamia available in the GenBank (Wilke et al. 2001). They represented two divergent subclades. The first, I. zermanica, was re-
stricted to the Zrmanja River in Croatia, and corre-
sponded 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
(aboud 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 wa-
ter 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. zermanica, according to the IUCN Red List
(Cutteled et al. 2011), is Critically Endangered
(possibly Extinct), based on the change of the hab-
itat 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 lo-
cality, 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 sug-
gest a more extensive distribution of the species.

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family Hydrobiidae (Gastropoda: Rissooidea): review

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


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