



New Findings of the Little Known Crenobiotic Snail *Bythinella serborientalis* Radoman, 1978 (Gastropoda: Truncatelloidea, Bythinellidae) with Notes on its Morphological Variability

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Abstract: We report on two new sites for the little known crenobiotic snail *Bythinella serborientalis* Radoman, 1978, based on molecular and morphological analyses. A recent record of this species from western Serbia is discussed and corrected. In addition, we provide information about the intraspecific variability of *B. serborientalis*, both in terms of shell and penial morphology. In general, the morphological variability is observed as quite high and there are no stable characters which could distinguish this species from its congeners. Therefore, DNA analyses should be used in order to complement morphological descriptions.

Key words: shell morphology, penial morphology, distribution, intraspecific variability, molecular data

Introduction

The genus *Bythinella* Moquin-Tandon, 1856, is represented by minute dextral operculate snails inhabiting springs, brooks and groundwaters in hilly and mountainous regions. These snails are usually found under rocks, wood or fallen leaves in the water, as well as on aquatic plants (Falniowski et al. 2012). In rare cases, the snails of *Bythinella* have also been found in lakes (Boeters & Knebelsberger 2014). Currently, around 200 species/subspecies are considered valid (MolluscaBase eds. 2024). The genus is the most speciose in the Balkans, while Bulgaria is considered a hotspot of species diversity regarding *Bythinella* (Glöer & Georgiev 2011).

Most species within the genus have been described based on characters of the shell and male reproductive organs, although it has been shown that this is insufficient for distinguishing among species (Haase et al. 2007). Furthermore, the commonly used characters of the male genitalia are known to be highly variable, limiting their taxonomic importance (Falniowski et al. 2009).

In Serbia and the former Yugoslavia, these snails were mainly studied by Pavle Radoman (1976, 1983), who described numerous new species. According to the available data, eight species of the genus *Bythinella* are known from Serbia so far, while the presence of some species, such as *B. luteola* Radoman, 1976 is questionable (Marković

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et al. 2021). The presence of *B. austriaca* (Frauenfeld, 1857) in Serbia is also doubtful because it has been identified only based on the characters of the shell and possibly confused with *B. opaca* (M. von Gallenstein, 1848). Four species are considered Critically Endangered at a national level: *B. istoka*, *B. nonveilleri*, *B. pesterica* and *B. serborientalis* (Tomović et al. 2023).

In this article, we provide one additional locality from Serbia for the little-known crenobiotic, steno-endemic species of Stara Planina Mountains, *Bythinella serborientalis* Radoman, 1978, based on COI sequences. Moreover, we discuss the morphological variability of the shell and male genitalia, as well as their use for the identification of this species and confirm its presence in Bulgaria. Hitherto, *B. serborientalis* was known from the type locality (Spring Vrelo in the village of Visočka Ržana, Stara planina Mountains) and was described solely based on the characters of the shell (Radoman 1978), which are nowadays not sufficient to differentiate it from its congeners. The unexpected and dubious record of this species by Falniowski et al. (2023) from the vicinity of the village of Potpeće in western Serbia is discussed and corrected. Glöer (2022) provided the first data on the male genitalia of this species, based on the topotypic material collected by the first author of this paper. Intraspecific variability of this species has never been studied.

Materials and Methods

Sampling and processing

The sampling included two sites:

1. Stara planina Mountains, spring “Vrelo” ca. 3 km N of the village of Visočka Ržana (type locality of *B. serborientalis*, 43°11'00.08"N, 22°50'00.77"E) (20 specimens collected on 03.07.2021., lg. V. Gojšina).

2. Stara planina Mountains, spring of the Orlova River, Jabučko ravnište (43°21'44.0"N, 22°35'00.8"E) (794 specimens collected on 27.08.2021, lg. I. Živić, 389 specimens collected on 22.10.2021, lg. I. Živić, 250 specimens collected on 11.02.2022, lg. I. Živić and 135 specimens collected on 28.05.2022, lg. I. Živić).

Snails were collected either manually (for material from the type locality) or using standard Surber net with a catchment area of 300 cm² and mesh size of 250 mm (for specimens from the Orlova River). Collected specimens were subsequently preserved in 70% ethanol (specimens used for studying of the penial morphology) or 96% ethanol

(specimens used for molecular studies) and stored in 2 ml plastic vials. Shells were photographed using a Nikon SMZ800N stereomicroscope equipped with a Nikon DS-Fi2 camera. Scale bars were settled using a Nikon DS-L3 control unit. A total of 22 specimens were dissected and examined (12 females and 10 males). For the purpose of dissection, a Petri dish was filled with black candle wax. Last whorl was crushed using fine forceps, while minute needles were used for the removal of mantle and dissection of the male genitalia. Following the dissection, male genitalia were mounted on a microscope slide in glycerol, covered with a coverslip which was sealed with a nail polish. Genitalia were photographed using Leica DMLB light microscope (Leica Microsystems) equipped with Leica DFC295 camera. All non-processed specimens are stored in the Institute of Zoology, University of Belgrade – Faculty of Biology.

DNA extraction

Total genomic DNA was extracted from single individuals using the Quick-DNA MiniPrep Kit (Zymo Research Europe GmbH), following the manufacturers' protocol. A subset of the mitochondrial cytochrome c oxidase subunit I gene (COI, the DNA barcode region) was used. The primers used for amplification of COI were universal LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). The amplification was carried out in a total volume of 25 µl which contained 10 µl of extracted DNA, 12.50 µl of ready PCR mix (FastGene® Optima HotStart ReadyMix, Nippon Genetics Europe GmbH) and 1.25 µl of each primer (10 mM). PCR amplification was performed as follows: 3 min initial denaturation at 95°C followed by 35 cycles of 15 sec denaturation (95°C), 30 sec annealing (54 °C), 1 min extension (72 °C) and 1 min final extension (72 °C). The PCR product obtained was sent to Macrogen Inc. (Amsterdam, Netherlands) for sequencing. The final sequences were compared with those already deposited in the GenBank database under accession numbers KT381137, KT381139 and KT381140 (*Bythinella* sp. Bulgaria) as well as OR391929 and OR391928 (“*Bythinella serborientalis*” from the village of Potpeće near Sevojno). Sequences are deposited in BOLD Systems under Process IDs GASER001-24 and GASER002-24 (BIN: ADK1428). The sequences are also deposited in GenBank under accession numbers PV634317 (Orlova River) and PV634318 (type locality).

Results

Molecular analyses

We found that the COI sequences of GASER001-24 (specimens from Orlova River) differed in two base pairs from GASER002-24 (specimens from the type locality of *B. serborientalis*) and four base pairs from isolates KT381137, KT381139 and KT381140. COI sequences of GASER002-24 differ in three base pairs from the mentioned isolates KT381137, KT381139 and KT381140. This makes it clear that the “*Bythinella* sp.” from Vodni Pech Cave in Bulgaria (Stara Planina Mountains) mentioned in Osikowski et al. (2015) belongs to *B. serborientalis* as well. Very high differences were found between the specimens of *B. serborientalis* from the type locality and the one reported from the village of Potpeće by Falniowski et al. (2023) (OR391929 and OR391929).

Morphological analysis

Morphological analyses of specimens collected (n=10) from spring of the Orlova River revealed the following traits: shell height from 2.41 mm to 2.72 mm; shell width from 1.39 mm to 1.63 mm. Aperture height from 1 mm to 1.19 mm; width - from 0.84 mm to 0.98 mm. Approximately 4 convex whorls separated by a relatively deep suture. Protoconch sometimes sunken into first teleoconch whorl, making the initial whorls quite flat, sometimes almost forming a flat platform-like surface. Protoconch more regularly rounded in some specimens. Last two whorls much larger and usually contribute to 90% of the shell height. Peristome thin or moderately thickened at parietal side. Its colouration varies from white to brownish (as the rest of the shell). Umbilicus slit-like (Fig. 1). Tentacles short and pigmented in their middle part and around the eye. Mantle black, lighter towards the collar, where only isolated blackish pigmented spots could be visible. Mantle collar completely white. Penis and penial appendices of the analyzed male specimens showed three combinations: i) penis longer than penial appendix but only very slightly (Fig. 2B), ii) penis visibly longer than penial appendix (by almost one third) (Fig. 2C–E), iii) penis shorter than appendix (usually 2/3 of the appendix length) (Fig. 2A). Based on five measured male specimens, penis varied in length from 0.26 mm to 0.5 mm and the penial appendix varied from 0.32 mm to 0.5 mm. Tubular organ moderately long to long, in some specimens almost regularly broad along its entire length, and in some specimens attenuated proximally and broadened distally.

Due to the limited material of *B. serborientalis* from the type locality (20 specimens but only

three males could be examined), we could not study the intraspecific variability accordingly but we observed no significant differences in both shell and penial morphology when compared to specimens from the spring of the Orlova River.

Habitat of *B. serborientalis*

The type locality of *B. serborientalis* (Vrelo Spring) is located in the Stara Planina Mountains (south-eastern Serbia), ca. 3 km north from the village of Visočka Ržana. Snails do not actually live in the spring itself but in a smaller spring right next to Vrelo, which forms a small stream connected to the Jelovica River. Around the spring, there was a lot of vegetation and smaller rocks, even a large pipe that ran next to the Jelovica River, just a few meters away from the spring head. Since this locality is also known as a tourist spot, we consider it to be highly endangered. Snails were not numerous at the type locality, but also somehow difficult to search for because of the numerous plants growing around the spring. In contrast, the second known locality in Serbia reported herein (spring of Orlova River) was dominated by these snails and we collected hundreds of specimens on several occasions and in a short time. The spring is located in a forest and is overgrown with macrophytes. A small stream flows from the spring (ca. 0.35–0.4 m deep and 0.4–0.53 m wide). The spring is only ca. 2 meters away from the road and the nearby hotel which indicates higher anthropogenic pressure.

Discussion

After morphological examination, we found that the majority of the specimens examined shared traits such as the penis being longer than the appendix and sometimes quite flat initial whorls. The latter feature can also be observed in specimens found in the Vodni Pech Cave in Bulgaria (see Osikowski et al. 2015). The tubular organ attached to the penis was also variable, of moderate length or long. It also appeared to be attenuated to a different extent near the point where it joins the penis. However, since we have observed considerable variability in both penial and shell morphology, we can conclude that these morphological traits are not sufficient for reliable species identification. This is not surprising, as *Bythinella* are generally characterised by a lack of distinct morphological features and high variability even within a single population; this also applies to penial morphology variation, as seen in *B. hansboetersi* (Falniowski et al. 2009).

Falniowski et al. (2023) reported *B. serborientalis* from the village of Potpeće near Sevojno in

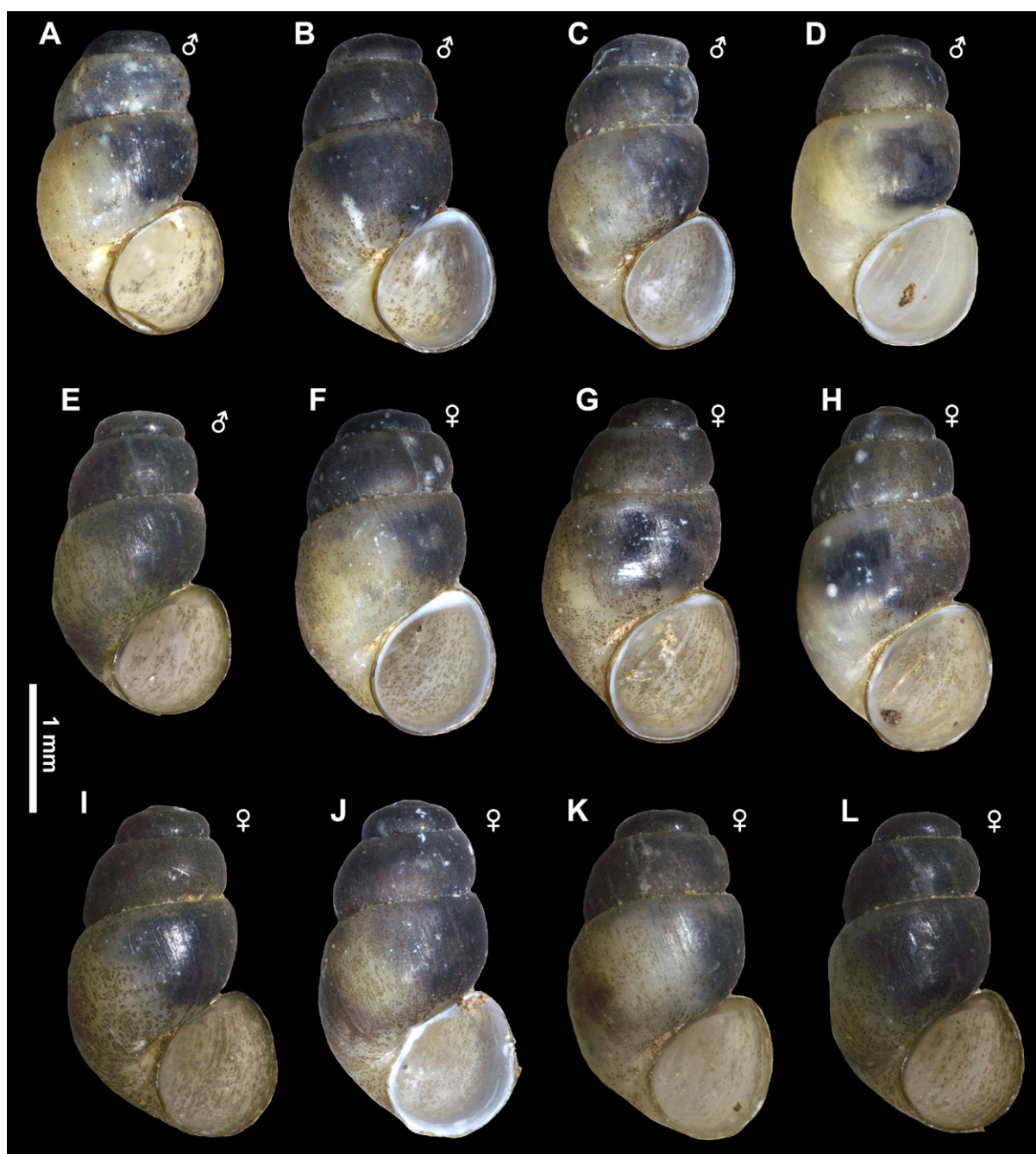


Fig. 1. *Bythinella serborientalis* from the spring of Orlova River, shells.

western Serbia (43°47'51.70" N, 19°56'8.90" E). This locality is ca. 240 km west of the type locality of *B. serborientalis* and these two regions of Serbia do not normally share the same crenobiotic fauna (Marković et al. 2021). It is unclear why Falniowski et al. (2023) identified these specimens as *B. serborientalis*, as specimens from the type locality were apparently not analysed and compared. We compared our sequence of the specimen from the type locality with the one for "*B. serborientalis*" provided by Falniowski et al. (2023) and found significant differences: well above the suggested threshold of

1.5% for *Bythinella* by Bichain et al. (2007), which is why we consider this record erroneous.

Several closely related species have been found in western, south-western Serbia and the adjacent regions of Montenegro (Falniowski et al. 2012), which usually share the following morphological features: colourless shell, blackish body (which provokes a blue hue through the translucent shell), well thickened peristome at the columellar side of the aperture and a wide black stripe which spreads across the head and tentacles (Glöer 2008; Glöer & Pešić 2014). These species could

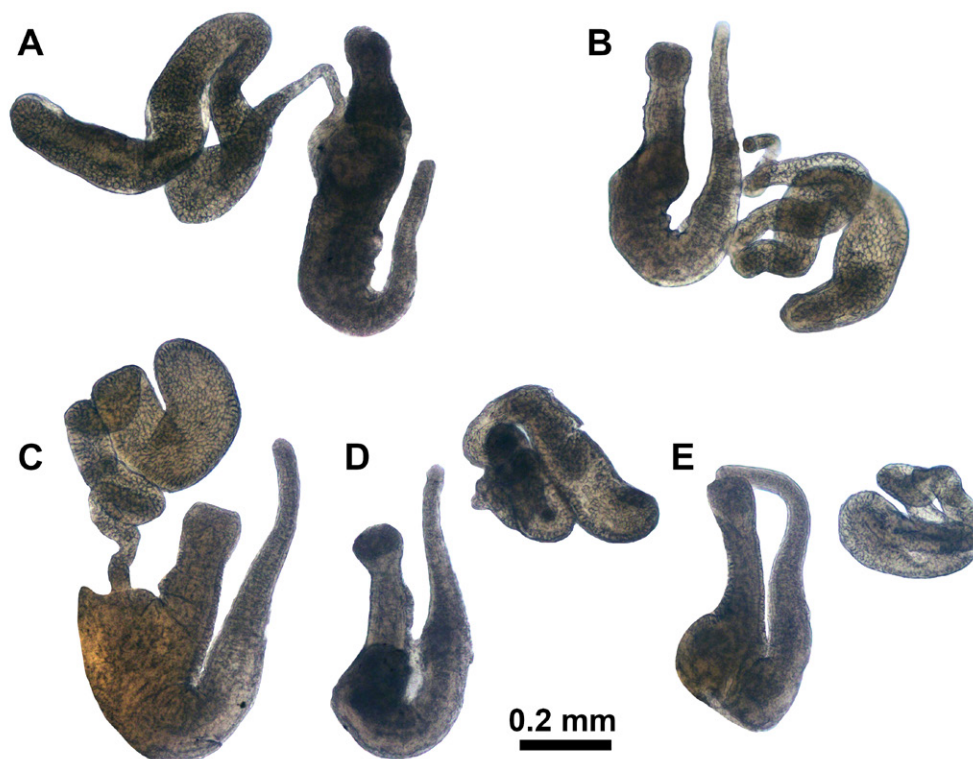


Fig. 2. *Bythinella serborientalis* from the spring of Orlova River, male genitalia.

be grouped in the „*Bythinella dispersa* group“ and include: *B. dispersa*, *B. istoka*, *B. pesterica* and *B. taraensis*. The molecular distinctness of *B. dispersa*, *B. pesterica* and *B. taraensis* has already been proven (Falniowski et al. 2012) but nothing is known on *B. istoka*. The above-mentioned characters are all shared among these taxa and they can be considered as not clearly morphologically distinct among each other, assuming that the male genitalia are also variable. However, this group is well delimited from *B. serborientalis* and related species, since the morphology of the shell and body colouration is largely different (i.e. *B. serborientalis* is more brownish, lacks the black stripe across the head and the columellar side of the aperture is usually less thickened). This is an example of two different „species groups“ that could be morphologically separated based on some „secondary“ characters (body and shell colouration, as well as some morphological characters of the shell), while the penial morphology is much more similar among them and showing a clear continuum (and overlap) of characters. The two groups are also well delimited geographically since they occur in different karst areas in Serbia (eastern vs. western parts), which usually do not share the same fauna.

Conclusion

The case of morphological indistinctness of *B. serborientalis* is one additional example that species within this genus cannot be identified or accordingly described based on shell and male genitalia only. Its comparison with some western and south-western Serbian populations showed that sometimes, colouration of the body or appearance of some other shell characters can be used to separate distant species groups. In these cases, shell morphology and body colouration can be even more informative than the male genitalia. However, it seems that none of these characters can be used to separate closely related species which is why the analyses must be coupled with molecular data. *Bythinella serborientalis* remains restricted to Stara Planina Mountains (both Serbian and Bulgarian part) and do not occur in western Serbia as reported by Falniowski et al. (2023).

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