Crenobiont, stygophile and stygobiont molluscs in the hydrographic area of the Trebišnjica River Basin

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Abstract

In the paper the crenobiont, stygophile and stygobiont malacoфаuna of the karst area of Popovo Polje around Trebinje (Eastern Herzegovina, BiH) is presented. The materials were collected from springs, caves and interstitial habitats (with a Bou-Rouch pump) at 23 localities. The following species were found: *Pisidium cf. personatum* A.W. Malm, 1855, *Theodoxus callosus* (Deshayes, 1833), *Sadleriana fluminensis* (Küster, 1852), *Radomaniola curta* (Küster, 1852), *Radomaniola cf. bosniaca* (Radoman, 1973), *Kerkia briani* Rysiewska & Osikowski, 2020, *Montenegrospeum bogici* (Pešić & Glöer, 2012), *Lithhabitella chilodia* (Westerlund, 1886), *Travunijana vruljakensis* Grego & Glöer, 2019, a new genus and species of the Sadlerianinae, *Emmericia ventricosa* Brusina, 1870, *Iglica cf. absoloni* (A.J. Wagner, 1914), *Plagigeyeria tribunicae* Schütt, 1963, *Paladilhiopsis arion* Rysiewska & Osikowski, 2021, *Valvata montenegrina* Glöer & Pešić, 2008, *Radix labiata* (Rossmässler, 1835), *Galba truncatula* (O. F. Müller, 1774), *Ancylus recurvus* Martens, 1783, *Ancylus sp.* and the amphibiotic *Succinea cf. putris* (Linnaeus, 1758). The redescription of the genus *Travunijana* Grego & Glöer, 2019, a new genus and species of the Sadlerianinae, *Emmericia ventricosa* Brusina, 1870, *Iglica cf. absoloni* (A.J. Wagner, 1914), *Plagigeyeria tribunicae* Schütt, 1963, *Paladilhiopsis arion* Rysiewska & Osikowski, 2021, *Valvata montenegrina* Glöer & Pešić, 2008, *Radix labiata* (Rossmässler, 1835), *Galba truncatula* (O. F. Müller, 1774), *Ancylus recurvus* Martens, 1783, *Ancylus sp.* and the amphibiotic *Succinea cf. putris* (Linnaeus, 1758). The redescription of the genus *Travunijana* Grego & Glöer, 2019, applying the characteristics of shell, female reproductive organs and penis, is also presented. The new genus and species are described, based on the shell, penis, radula and fragmentary data on the female reproductive organs. For all species, the mitochondrial cytochrome oxidase subunit I (COI) is applied to confirm the determination; in the case of *Travunijana* and the new genus, the nuclear histone H3 locus is also used, in order to infer both their distinctiveness and phylogenetic relationships.
Keywords
Balkans, Bosnia and Herzegovina, cave, COI, H3, karst area, meiofauna, molecular systematics, new genus, new species, spring

Introduction
The Dinaric Karst is a global hotspot for subterranean biodiversity. This is particularly true in the case of its stygobiont, stygophilic and crenobiont communities. The present paper focusses on providing further evidence of one generally under-reported aspect of freshwater aquatic biodiversity – namely the malacofauna of the Trebišnjica River Basin, predominantly in the hydrographically complex karst area of Eastern Herzegovina in Bosnia and Herzegovina.

The study reported below, was undertaken under the remit of the RS-Bosnia and Herzegovina Official Government Licence, which is granted annually to the “Proteus Project in Bosnia and Herzegovina” to undertake its objective of protecting and conserving endangered cave fauna and by extension, to protect and conservation-manage the natural karst conduit-aquifer hypogean ecosystems containing the endangered cave faunal species. One of the objectives of the Project is to fully characterise these ecosystems and in doing so, to provide an inventory of their biodiversity.

In this context, the contribution made by the visiting team of malacologists from the Department of Malacology of the Jagiellonian University’s Institute of Zoology and Biomedical Research and from Department of Animal Reproduction, Anatomy and Genomics of University of Agriculture in Krakow, both in Poland, has provided the “Proteus Project” with vital information on the biological characteristics and geographic distribution of a range of genera and species of malacofauna collected at 23 locations connected to 11 separate karst conduit-aquifer ecosystems across a wide area of the Trebišnjica River Basin. The 23 sampling locations were purposely selected by the Director of the “Proteus Project” to represent a typical range of karst hydrological features, such as cave resurgence springs (vrelo), ponors and estavelles, either underground or at surface outlets or inlets.

Speleomalacological research on this scale and in such an integrated form, has never been undertaken before now in Bosnia and Herzegovina. Not surprisingly, therefore, the Polish team has identified a new genus and species of meiofaunal gastropod (Mollusca). As a standalone account, these first results, containing verifiable genomic data are of great scientific importance in their own right, but when combined with the associated variety of environmental data being amassed by the “Proteus Project”, they assume a much greater value.

In regard to both ecosystem services and as a nutrient-rich food supply, the importance of the position of malacofauna near the bottom of the “foodchain” of the subterranean aquatic ecosystem, cannot be overstated. Without them being present in all their wonderful variety and population numbers, the diversity of many of the higher cave animals would certainly not be as great.
Material and methods

In June and September 2019, we collected aquatic gastropods from springs, interstitial habitats and caves at 23 localities (Table 1, Figs 1–3). They were either collected by hand and sieve in caves and springs, or with a pump applying the Bou-Rouch technique (Bou and Rouch 1967), to sample interstitial fauna below the sedimentoed floor.
of streams, at a depth of about 50 cm. The tube was inserted in the sediment five times, and 20 litres were pumped each time. Samples were sieved through a 500 μm sieve and fixed in 80% analytically pure ethanol, replaced twice, and later sorted. Next, the snails were put in fresh 80% analytically pure ethanol and kept at -20 °C temperature in a refrigerator. Percentages of each identified taxon in each locality are presented in Table 1, with division into samples collected on the surface and with a pump.

The shells were photographed with a Canon EOS 50D digital camera, under a Nikon SMZ18 microscope. The dissections were done under a Nikon SMZ18 microscope with dark field, equipped with Nikon DS-5 digital camera, whose captured images were used to draw anatomical structures with a graphic tablet. Morphometric parameters of the shell were measured by one person using a Nikon DS-5 digital camera and ImageJ image analysis software (Rueden et al. 2017). The radulae were extracted with Clorox, applying the techniques described by Falniowski (1990), and examined and photographed using a HITACHI S-4700 scanning electron microscope.

DNA was extracted from whole specimens; tissues were hydrated in TE buffer (3 × 10 min); then total genomic DNA was extracted with the SHERLOCK extraction kit (A&A Biotechnology), and the final product was dissolved in 20 μl of tris-EDTA (TE)
buffer. The extracted DNA was stored at -80 °C at the Department of Malacology, Institute of Zoology and Biomedical Research, Jagiellonian University in Kraków (Poland).

Mitochondrial cytochrome oxidase subunit I (COI) and nuclear histone 3 (H3) loci were sequenced. Details of PCR conditions, primers used and sequencing technique were as given in Szarowska et al. (2016a). Sequences were initially aligned in the MUSCLE (Edgar 2004) programme in MEGA 7 (Kumar et al. 2016) and then checked in BIOEDIT 7.1.3.0 (Hall 1999). Uncorrected p-distances were calculated in MEGA 7. Estimation of the proportion of invariant sites and the saturation test (Xia 2000; Xia et al. 2003) were performed using DAMBE (Xia 2018). In the phylogenetic analysis, additional sequences from GenBank were used (Table 2). The phylogenetic analysis was performed applying two approaches: Bayesian Inference (BI) and Maximum Likelihood (ML). The Bayesian analyses were run using MrBayes v. 3.2.3 (Ronquist et al. 2012) with defaults for most priors. Two simultaneous analyses were performed, each with 10,000,000 generations,
Table 1. The list of studied localities, with a short description of their characteristics, geographical co-

| Id | Site names, characteristics and codes | Coordinates | Taxa confirmed | % of taxa in site (surface/pump) |
|----|-------------------------------------|-------------|----------------|----------------------------------|
| 1  | Vrelo „Vrijeka“ (Bijeljani), Dabarsko Polje; at the outlet (BiH19_08) A permanent cave resurgence spring whose water originates from ponors located in Lukavac Polje. | 43.07417, 18.23899 | Emmericia ventricula, Montenegrognatogobius Radomaniola cf. boniaca | 0/12.6 100/0 0/87.4 |
| 2  | Estavela „Ljeljena“ (Bijeljani); inside the cave (BiH19_14) When checked, this location was hydrologically inactive. | 43.05400, 18.24069 | – – | – – |
| 3  | Rijeka (river) „Vrijeka“ (Dabarsko Polje); on the surface near entrance of Ponor „Ponikva“ (BiH19_15) Samples taken under low-flow conditions. | 43.04535, 18.25217 | Radomaniola cf. boniaca | 100.0 |
| 4  | Estavela „Kapuša“ (Dračevo); inside the entrance (BiH19_24) Checked when the estavelle was hydrologically inactive. | 42.85692, 18.07665 | Emmericia ventricula | 0/100 |
| 5  | Vrelo „Pokrivenik“ (Muhareva Ljuti), Popovo Polje; spring at the cave entrance; high water level variation (BiH19_05) Samples taken when the location was hydrologically inactive. | 42.85166, 17.99838 | Radomaniola cf. boniaca | 0/100 |
| 6  | Vrelo „Lučavac“ (Zavala); outlet for Vjetrenica Pečina. Spring below the cave entrance; high water level variation (BiH19_06) Samples taken when the location was hydrologically inactive. | 42.84643, 17.9846 | Radomaniola cf. boniaca | 0/100 |
| 7  | Vrelo „Bitomilje“ (Golubina); in valley above Zavala, with Austro-Hungarian infrastructure (BiH19_07) Samples taken under extremely low-flow conditions. | 42.83799, 17.97161 | Listhlabitella chilodla, Montenegrognatogobius | 40.3/0 59.7/0 |
| 8  | Izvor „Kneginja“ (Trklja); a low-flow groundwater spring in Dolomite coming from a limestone blockhouse (BiH19_20) Samples taken under low-flow conditions. | 42.75729, 18.3693 | Ancylus sp., Listhlabitella chilodla | 0/2.7 0/97.3 |
| 9  | Izvor „Knez“ (Trklja); a low-flow groundwater spring in Dolomite coming from a limestone blockhouse (BiH19_21) Samples taken when the location was hydrologically inactive. | 42.75465, 18.37218 | Ancylus sp., Listhlabitella chilodla | 0/2.7 0/97.7 |
| 10 | Confluence of Trebišnjica River with the Potok (stream) Blace (Blace); surface stream from a cave spring group on the right bank of Trebišnjica River (BiH19_17) Samples taken when the location was hydrologically inactive. | 42.71556, 18.35077 | Radomaniola curta, Sadleriana fluminensis, Succinea cf. putris | 100/52.1 0/64.3 0/2.6 |
| 11 | Vrelo „Tučevac“ (Mostači); the spring inside the cave (BiH19_13) A high-level overflow spring from a locally complex estavelle cave system. When active, its water originates from ponors in Ljubomirsko Polje 14 km away. This was hydrologically inactive when sampled. | 42.71445, 18.30278 | Radomaniola cf. boniaca | 100.0 |
| 12 | Vrelo „Vruljak 1“ (Gorica), Trebinjsko Polje; This was sampled in the resurgence pool before which 2 cave rivers Rijeka „Gorčica“ and Rijeka „Vrulja“ have joined inside & emerge (BiH19_03) The cave resurgence spring is just one outlet from a locally very complex cave system, containing a very rich biodiversity. The water originates from ponors in Ljubomirsko Polje about 12 km away. | 42.71395, 18.36833 | Emmericia ventricula, Psidium cf. personatum, Radomaniola cf. boniaca, Travunijana vuljukensis | 0/7.8 0/50 0/92.2 50/0 |
| 13 | Vrelo „Pulički Studenac“ (Crkvina); a cave spring in the left bank of Trebišnjica River (BiH19_11) Samples taken when the estavelle was hydrologically inactive. | 42.71288, 18.36514 | Ancylus recurvus, Emmericia ventricula, Ilyocypris bunoi sp. nov., Kerkia brenn, Radomaniola curta, Radomaniola cf. boniaca, Travunijana vuljukensis | 3/7.0 0/44.3 27.8/0 38.9/0 10.27.6 0/48.1 19.4/0 |
| 14 | Vrelo „Oko“ (Zasadi); a spring in the entrance to the cave system; surrounded by ancient limestone-block housing; at the centre of Trebinje (BiH19_23) This location is permanently hydrologically active and its water originates from ponors in Ljubomirsko Polje 14 km away. Although it is locally regarded as a vrelo, it is actually an estavelle. This was once used as a public water supply. | 42.71274, 18.33697 | Radomaniola cf. boniaca, Travunijana vuljukensis | 0/5.9 0/94.1 |
| 15 | Estavela „Pečine“ (Mostači) (BiH19_12) | 42.71244, 18.30497 | Ancylus recurvus, Galba truncatula | 100/0 0/100 |
| 16 | Igorovo Jesero (lake) (Gorica); small lake in a collapsed cave passage with cave springs and containing many ponors; muddy bottom (BiH19_19) The water originates from ponors in both Ljubomirsko Polje and Cibijansko Polje. The ponors in and around the lake feed water underground downstream to Vrelo „Vruljak 2“ (Gorica). Samples taken when the estavelle was hydrologically inactive. | 42.71111, 18.38495 | Ancylus sp., Galba truncatula, Radix labiata, Sadleriana fluminensis | 0/9.1 0/36.4 0/9.1 0/45.4 |
### Aquatic molluscs of the Trebišnjica River Basin

| Id | Site names, characteristics and codes | Coordinates | Taxa confirmed | % of taxa in site (surface/ pump) |
|----|--------------------------------------|-------------|---------------|----------------------------------|
| 17 | Vrelo „Vruljak 2“ (Gorica), Trebinjsko Polje; this location was sampled at the resurgence spring outlet before which 2 cave rivers have joined inside: Rijeka “Peterčica” and Rijeka “Venator” (BiH19_02) This is a permanently hydrologically active outlet from a locally very complex cave system containing a very rich biodiversity. | 42.71062, 18.37618 | Keratia briani | 15.3/0 |
|    |                                      |             | Plagigeyeria tribunicae | 2.3/0 |
|    |                                      |             | Radomaniola curta | 0/3.0 |
|    |                                      |             | Salleriana fluminensis | 0/96.5 |
|    |                                      |             | Travunijana vruljakensis | 81.8/0 |
| 18 | The intermittently active cave spring, Vrelo „Vražiji Mlin“ (D. Grančarevo); Trebišnjica Canyon (BiH19_04) This is fed by ponors in Jasen Polje. The location is set in dolomitic limestone. | 42.70847, 18.44801 | Radomaniola cf. bomiaca | 0/100 |
| 19 | “Slomljen pecina“ (Mokri Dolovi); (BiH19_22) Since being sampled, this location has now been buried and made inaccessible by urban development. | 42.70844, 18.35419 | – | – |
| 20 | Confluence of Sušica River and Jazina River (Jazina) (BiH19_16) This was sampled under low-flow conditions. The source of the water is a giant estavelle situated in karstified dolomite with dolomitic limestone. | 42.70429, 18.50491 | Iglica cf. absoloni | 16.7/0 |
|    |                                      |             | Litthabitella chilodia | 83.3/0 |
|    |                                      |             | Radix labiata | 0/72.2 |
|    |                                      |             | Valvata montenegrina | 0/27.8 |
| 21 | Vrelo „Lušac“ (Gučina); at the surface outlet (BiH19_10) A permanently hydrologically active outlet from a complex karst conduit-aquifer, whose principal source is unproven. This was once a public water supply. | 42.70111, 18.3575 | Litthabitella chilodia | 14.6/0 |
|    |                                      |             | Montenegropeum bogici | 22.0/0 |
|    |                                      |             | Pisidium cf. personatum | 4.9/0 |
|    |                                      |             | Paladilhiopsis arton | 58.5/0 |
|    |                                      |             | Travunijana vruljakensis | 0/100 |
| 22 | Estavela „Mali Šumet“ (Bugovina), Mokro Polje; in the entrance shaft (BiH19_01) The entrance comprises a neo-circular stone wall leading down into the interior by more than 20 stone steps set into the natural stone floor of the karst conduit. The construction is of Austro-Hungarian origin and designed to give easy access to the potable water supply for local people. The location was hydrologically inactive when sampled. | 42.65665, 18.34458 | Emmericia ventricosa | 0/100 |
| 23 | River Konavoska Ljuta (Ljuta), Croatia; samples from the surface (Stones, plants) (BiH19_18) This karst river originates from Vrelo “Konavoska Ljuta” a few metres upstream from the sampling location. However, the water itself originates from a ponor 10 km away in Zubačko Polje near Trebinje in Eastern Herzegovina. This cave resurgence spring is used as a public water supply. The samples were collected under low-flow conditions. | 42.53408, 18.37647 | Pisidium cf. personatum | 15.6/0 |
|    |                                      |             | Radomaniola curta | 84.4/100 |

with one cold chain and three heated chains, starting from random trees and sampling the trees every 1000 generations. The first 25% of the trees were discarded as burn-in. The analyses were summarised as a 50% majority-rule tree. The Maximum Likelihood analysis was conducted in RAxML v. 8.2.12 (Stamatakis 2014) using the RAxML-HPC v.8 on XSEDE (8.2.12) tool via the CIPRES Science Gateway (Miller et al. 2010). We applied the GTR model whose parameters were estimated by RAxML (Stamatakis 2014).

### Systematic part

**Bivalvia**

**Pisidiidae**

**Pisidium cf. personatum** A.W. Malm, 1855

**Remarks.** Specimens of this common, widely distributed, Holarctic and eurybiotic species were found in many springs. It was also collected from interstitial habitats (with a Bou-Rouch pump) at the localities 12, 21 and 23 (Fig. 4).
### Table 2. Taxa used for phylogenetic analyses with their GenBank accession numbers and references.

| Species | COI/H3 GB numbers | References |
|---------|-------------------|------------|
| Agrafia wiktori Szarowska & Falniowski, 2011 | JF906762/MG543158 | Szarowska and Falniowski 2011/Grego et al. 2017 |
| Alonziella finalina Giusti & Bodon, 1984 | AF183149 | Szarowska and Falniowski 2011 |
| Anagastina zetavalis (Radoman, 1973) | DQ301830 DQ301838 | Falniowski et al. 2006 |
| Ancylus sp. C4 | KY012232 KY012233 | Falniowski et al. 2016 |
| Ancylus sp. – clade 3 | AY350516 AY350519 | Falniowski et al. 2003 |
| Ancylus sp. – clade 4 | AY350520 AY350521 | Falniowski et al. 2003 |
| Avenionia brevis berenguieri (Bourguignat, 1882) | AF367638 | Wilke et al. 2001 |
| Belgrandia thermalis (Linnaeus, 1767) | AF367648 | Wilke et al. 2001 |
| Belgrandiella kuesteri (Boeters, 1970) | MG551325 | Osikowski et al. 2018 |
| Belgrandiella kusceri (A. J. Wagner, 1914) | -/MG551366 | Osikowski et al. 2018 |
| Bithynia tentaculata (Linnaeus, 1758) | AF367643 | Wilke et al. 2001 |
| Bracenica gloeri Grego, Fehér & Erőss, 2020 | MT396209 | Hofman et al. 2020a |
| Bythinella cretensis Schütt, 1980 | KT353689 | Szarowska et al. 2016b |
| Bythiospeum acicula (Hartmann, 1821) | KU341350/MK690536 | Richling et al. 2016/Falniowski et al. 2019 |
| Daphniola louisi Falniowski & Szarowska, 2000 | KM887915 | Szarowska and Falniowski 2014a |
| Dalmatinnella flavialitii Radoman, 1973 | KV344541 | Falniowski and Szarowska 2013 |
| Dalmatinnella simonei Beran & Rysiewska, 2021 | MT773271 | Beran et al. 2021 |
| Ecrobia maritima (Milaschewitsch, 1916) | KX355835/MG551322 | Osikowski et al. 2016/Grego et al. 2017 |
| Emmericia expansilabris Bourguignat, 1880 | KC810060 | Szarowska and Falniowski 2013a |
| Fissuria boui Boeters, 1981 | AF367654 | Wilke et al. 2001 |
| Graecoarganiella parnassiana Falniowski & Szarowska, 2011 | JN202352 | Falniowski and Szarowska 2011 |
| Graecoarganiella sp. | JN202353/MN03140 | Falniowski and Szarowska 2011/Hofman et al. 2019 |
| Graziana alpestris (Frauenfeld, 1863) | AF367641 | Wilke et al. 2001 |
| Grossuana hohenackeri (Kuščer, 1932) | KC011794 | Falniowski et al. 2012 |
| Hauffenia michleri (Küster, 1853) | KT236167/KY087878 | Szarowska and Falniowski 2015/Rysiewska et al. 2017 |
| Helobia molatzi (Westerlund, 1886) | KM213723/MK690534 | Szarowska et al. 2014b/Falniowski et al. 2019 |
| Horatia klecakiana Bourguignat, 1887 | KJ159128 | Szarowska and Falniowski 2014 |
| Iglica gracilis (Clessin, 1882) | MH729085/MH721003 | Hofman et al. 2018 |
| Islamia zermonica (Radoman, 1973) | KU662362/MG551320 | Beran et al. 2016/Grego et al. 2017 |
| Littorina littorea (Linnaeus, 1758) | KF644330/KP113574 | Layton et al. 2014/unpub. |
| Littorinopsis praetexta (Küster, 1852) | JX073951 | Falniowski and Szarowska 2012 |
| Marstoniopsis insubrica (Küster, 1853) | AF322408 | Falniowski and Wilke 2001 |
| Moitessieria cf. puteana Couttagne, 1883 | AF367635/MH721012 | Wilke et al. 2001/Hofman et al. 2018 |
| Montenegropeum bogici (Pelíč & Glöer, 2012) | KM875510/MG880218 | Falniowski et al. 2014/Grego et al. 2018 |
| Montenegropeum iderti G. & G. Glöer, 2018 | MG880216 | Grego et al. 2018 |
| Paludilimnophilia grobbeni Kuščer, 1928 | MH720991/MH721004 | Hofman et al. 2018 |
| Pontobelgrandiella sp. Radoman, 1978 | KU407024/MG551321 | Rysiewska et al. 2016/Grego et al. 2017 |
| Pseudamnicola pieperi (Schütt, 1980) | KT721067 | Szarowska et al. 2016a |
| Pseudorientalia sp. | KJ920477 | Szarowska et al. 2014c |
| Radomaniola curta (Küster, 1853) | KC011814 | Falniowski et al. 2012 |
| Radomaniola curta curta (Küster, 1853) | KC011778 KC011784 KC011787 KC011788 KC011791 KC011792 | Falniowski et al. 2012 |
| Radomaniola sp. | KC011772 KC011745 KC011747 KC011764 KC011765 | Falniowski et al. 2012 |
| Sadleriana fluminensis (Kuščer, 1928) | KF193067 | Szarowska and Falniowski 2013b |
| Sargiana affinis (Brancsik, 1888) | MN031431/MN031438 | Hofman et al. 2019 |
| Sargiana cf. affinis | MN031431 | Hofman et al. 2019 |
| Tanousia zrmanjae (Brusina, 1866) | KU041812 | Beran et al. 2015 |

**Gastropoda**

**Neritopsina: Neritidae**

**Theodoxus callosus** (Deshayes, 1833)

**Remarks.** This species, described from Greece and reported from Greece and Albania, was found at some larger springs, but never in subterranean waters.


Caenogastropoda
Hydrobiidae: Sadlerianinae

Sadleriana fluminensis (Küster, 1852)
Fig. 5A

GenBank no. COI: MZ027620–MZ027622
Remarks. The most widely distributed species of Sadleriana. Found at the localities 10, 16 and 17 (Fig. 4).

Radomaniola Szarowska, 2006

Remarks. Replacement name for Orientalina Radoman, 1978. The genus is widely spread in the former Yugoslavia, but recorded also from Italy. Radoman (1983) distinguished six species of Radomaniola, and in one of them – R. curta – eight subspecies. It has to be noted that in modern phylogenetics, the only acceptable meaning of a subspecies is a geographic race, which was hardly the case in Radoman’s classification; also, far from being acceptable is that all his species-level taxonomy was based on the shell alone, strikingly variable in this genus (e.g., Falniowski et al. 2012; see also Fig. 5B–M). Molecular and anatomical data (Falniowski et al. 2012) did not confirm the classification of Radoman (1983), but demonstrated high genetic diversity, suggesting a flock of distinct species. The phylogeography as well as molecularly-based
species discrimination in *Radomaniola* should be studied with more extensive material, which we are proposing to do. At the moment, considering only *Radomaniola* from the area sampled in this study, one can distinguish two main clades (Fig. 6), representing at least two distinct species. For the one including the sequences of the snails from the spring at Vranjicke Njive, type locality of *Radomaniola curta curta* (sequences KC011781 and KC011784), we used a provisional assignment to this species; for the second clade we provisionally used the name *R. cf. bosniaca*. In general, the representatives of *Radomaniola* were the most common snails at the studied localities, and were found at the surface, as well as in the pumped interstitial samples and could also be found in caves. *Radomaniola*, pigmented and with eyes, is a stygophile gastropod.
Figure 6. Maximum likelihood (ML) phylogram of the studied *Radomaniola*, based on the partial cytochrome oxidase subunit I (COI) sequences, bootstrap supports given if >60%, together with Bayesian probabilities; toponyms of *R. curta curta* marked with asterisks.
**Radomaniola curta (Küster, 1852)**

Fig. 5B–H

**GenBank no.** COI: MW879241–MW879250

**Remarks.** Found at the localities 10, 13 and 23 (Fig. 4) on the surface and also interstitially and at the locality 17 only on the surface. At the locality 13 in the spring Polički Studenac, in sympatry with *R. cf. bosniaca*.

**Radomaniola cf. bosniaca (Radoman, 1973)**

Fig. 5I–M

**GenBank no.** COI: MW879222–MW879240

**Remarks.** Collected at the localities 1, 6, 12, 13, 14 and 18 (Fig. 4) on the surface, but only at the localities 3 and 11 interstitially. At the locality 13 in sympatry with *R. curta*.

**Kerkia briani Rysiewska & Osikowski, 2020**

Fig. 7A–C

**GenBank no.** COI: MT780191–MT780196; H3: MT786730–MT786735; Hofman et al. 2020b

**Remarks.** Found at the locality 13 (Fig. 4), its type locality, and at locality 17 (about 1 km away), where it is an element of the meiofauna; pumped with a Bou-Rouch pump (Hofman et al. 2020b).

**Montenegrospeum bogici (Pešić & Glöer, 2012)**

Fig. 7D–K

**GenBank no.** COI: MZ266648–MZ266650

**Remarks.** Pešić and Glöer (2012) described a new species of *Bythiospeum* Bourguignat, 1882: *B. bogici* Pešić & Glöer, 2012 from underground waters of Vrelo “Taban”, in central Montenegro. Their description was based on empty shells. Later they (Pešić and Glöer 2013) collected live specimens, and described the lack of eyes and pigment and the penis with a lobe at its medial part. They considered *B. bogici* as belonging to a new genus: *Montenegrospeum* Pešić & Glöer, 2013. Later, Falniowski et al. (2014) demonstrated with molecular data that *Montenegrospeum* belongs to the Hydrobiidae, not Moitessieriidae, despite striking similarity of the shell between this snail and e.g., *Iglica* Wagner, 1927. Numerous live specimens of this species were pumped from interstitial habitats at the localities 1, 7 and 21 (Fig. 4).
Figure 7. Shells of the studied gastropods: A–C *Kerkia briani* D–K *Montenegrospeum bogici* (localities: D–F – 1, G, H – 7, I – 13, J – 14, K – 21) L–O *Litthabitella chilodia* (localities: L, M – 17, N–O – 8). Scale bars: 1 mm.
*Litthabitella chilodia* (Westerlund, 1886)
Fig. 7L–O

**GenBank no.** H3: MZ285059–MZ285063

**Remarks.** This species was found at the localities 7, 8, 9, 20 and 21 (Fig. 4). It was numerous and was also found in a cave and sometimes interstitially; pumped.

*Travunijana vrljakensis* Grego & Glöer, 2019
Fig. 9

**GenBank no.** COI: MW879256–MW879272; H3: MW865737–MW865748

**Remarks.** Grego and Glöer (2019) described a new monotypic genus *Travunijana* from Vrelo “Goricki Studenac” (Gorica), a spring at the right bank of the Trebišnjica River, this being its type locality. They found it also in two other springs: Vrelo Vruljak 1 (Gorica; our locality 12), and Vrelo Vruljak 3 (Gorica). Their diagnosis of the genus was based on a single “unique” character – the strange morphology of the penis – which was based on artefactual appearance, caused by fixation: a nonglandular outgrowth on the left side, located distally (Grego and Glöer 2019). The penis photographed by them presents a bulbous, drastically contracted distal section, making copulation impossible.

Our molecular data (Fig. 8) confirmed the distinctiveness of the genus *Travunijana* Grego & Glöer, 2019. The phylograms based on H3, as well as on both concatenated loci placed *Travunijana* as the sister species with *Graecoarganiella* Falniowski & Szarowska, 2011, and *Sarajana* Radoman, 1975 (bootstrap 85%). The shell habitus is different (conic in *Travunijana*, ovate-conic in *Sarajana*), and the penial morphology differs (Hofman et al. 2019): the outgrowth on the left side is simple and filamentous in *Sarajana*, and short and bi-lobed in *Travunijana*. The phylogram based on COI showed a more complicated pattern, but bootstrap supports were too low for any more certain placement in the phylogeny.

**Redescription of the genus* Travunijana* Grego & Glöer, 2019**

**Diagnosis.** Shell conic with moderately convex whorls, big sphaerical bursa copulatrix and two nearly vestigial receptacula seminis, penis long and slender, distally forming a slightly bent filament, at the base of the filament an outgrowth on the left side of the penis, formed of two finger-like lobes.

**Description.** The shell (Fig. 9) as described by Grego and Glöer (2019). The female reproductive organs (Fig. 10) with bulbous loop of (renal) oviduct, big and spherical bursa copulatrix and two nearly vestigial receptacula seminis: proximal (rș₂ of Radoman 1973) and distal (rș₁ of Radoman 1973) one. The penis
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(Fig. 11) long and slender, slightly bent at its medial section, at the base of the long filamentous distal section and an outgrowth on the left side, consisted of two finger-like lobes.

*Travunijana vruljakensis* was common at the studied territory, found at the localities 12, 13, 14, 17 and 21. At 12, 13 and 17 (Fig. 4) interstitially pumped.

**Iglicopsis Falniowski & Hofman, gen. nov.**

http://zoobank.org/77758877-EEF4-448E-B727-D5632F9E5F51

**Type species.** *Iglicopsis butoti* by original designation

**Diagnosis.** Shell ovate-conic with broad and flat apex, transparent, operculum smooth, no pigment, eyes absent, ctenidium present, penis long, tapering, with bilobed outgrowth on the left side and flat outgrowth at the right side, unpigmented renal oviduct, bursa copulatrix and two small receptacula seminis.

**Remarks.** *Iglicopsis* shell resembles that of *Montenegrospeum*, but is more oval, with broader spire and broader flat apex, sometimes showing scalarity at the body

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**Figure 8.** Phylogenetic relationships of *Travunijana* and *Iglicopsis* based on COI, H3 and concatenated loci; bootstrap supports given if over 60%, their values together with Bayesian probabilities.
whorl; the penis with the left-side outgrowth located more proximally and bi-lobed and additional flat outgrowth on the right side; the molecular divergence ($p = 0.186$ for mitochondrial COI and $p = 0.114$ for nuclear H3) at the genus-level.

Figure 9. Shells: A–L Travunijana vruljakensis M–P Iglicopsis butoti M holotype N 2F61 O 2F68 P 2F69 (extraction numbers, see Table 3). Scale bars: 1 mm.
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Iglicopsis butoti Falniowski & Hofman, sp. nov.
http://zoobank.org/C1A9D0B0-4B10-4977-B69B-7C4C42BB19D3
Fig. 9M–P

Type materials. Holotype. Ethanol-fixed specimen (Fig. 9M), Vrelo „Polički Studenac” (Crkvina); a cave spring in the left bank of and adjacent to the Trebišnjica River (N 42.71288, E 18.36514) (our locality 13, Fig. 4) close to Trebinje (Bosnia and Herzegovina), interstitially, 50 cm below the gravel floor of the spring; in the collection of the Department of Malacology of Jagiellonian University, voucher number ZMUJ-M.2651.

Paratypes. Three paratypes destroyed to extract DNA, one specimen ethanol-fixed, in the collection of the Department of Malacology of Jagiellonian University, ZMUJ-M.2652.

Diagnosis. Shell minute, ovate-conic, distinguishable from Montenegrospeum by a more oval habitus, broader spire and broader flat apex, sometimes showing scalarity at the body whorl; the penis with the left-side outgrowth located more proximally and bi-lobed, and additional flat outgrowth on the right side.

GenBank no. COI: MW879273–MW879275; H3: MW865749–MW865751

Figure 10. Female reproductive organs of Travunijana vruljakensis (bc – bursa copulatrix, cbc – duct of bursa, ga – albuminoid gland, gn – nidamental gland, gp – gonoporus, ov – oviduct, ovl – loop of (renal) oviduct, rs₁ – distal seminal receptacle, rs₂ – proximal seminal receptacle). Scale bar: 0.25 mm.
Description. Shell (Fig. 9M–P) up to 1.49 mm high and 0.55 mm broad, ovate-conic, whitish, translucent, thin-walled, and consisting of about five whorls, growing regularly and separated by moderately deep suture. Spire high and broad, apex broad and flat, body whorl less than 0.5 of the shell height, Aperture small, prosocline, oval in shape, peristome complete and thin, somewhat swollen, in contact with the wall of the body whorl, in some specimens showing scalarity close to the aperture, umbilicus slit-like. Shell surface smooth, with growth lines hardly visible.

Measurements of holotype and sequenced and illustrated shells: Table 3. Shell variability slight; scalarity and much bigger dimensions of one specimen (Fig. 9P) most probably caused by the larval Trematoda (parasite gigantism).

Soft parts morphology and anatomy. Body white, pigmentless, with no eyes. Ctenidium with nine short lamellae, osphradium elongated. Tectum forming a characteristic broad loop (Fig. 9N). Female reproductive organs with unpigmented renal oviduct, bursa copulatrix and two small receptacula seminis; details unknown.

The radula (Fig. 12) with the central tooth cusp formula:

\[
\frac{(4)3-1-3(4)}{1-1} \quad \text{or} \quad \frac{(5)4-1-4(5)}{1-1}
\]
Figure 12. Radula of *Iglicopsis butoti*, scale bars: 10 μm.
Rather long and slender cusps grow regularly to central one. Lateral cusp with 5 – 1 – 5(6) long and massive cusps. Inner marginal tooth with ca 23 slender cusps of nearly invariable length along the tooth edge, outer marginal tooth with 26 broadly triangular cusps.

Penis (Fig. 13A) long, tapering, below the half of its length, proximally, bi-lobed outgrowth on the left side and flat outgrowth at the right side, at the distal part and the vas deferens well visible inside, running in zigzags.

**Derivatio nominis.** The genus name refers to the similarity of the shell to the moitessieriid genus *Iglica* Wagner, 1927. The specific epithet *butoti* refers to the memory of Dr Louis J. M. Butot, a Dutch malacologist devoted mostly to the Greek malacofauna, good friend and the mentor of AF.

**Distribution and habitat.** Known from the type locality only.

**Molecular relationships.** despite its shell morphology, *Iglicopsis* clearly belongs to the Hydrobiidae Stimpson, 1865, Sadlerianinae Szarowska, 2006, and not to the
Moitessieridae Bourguignat, 1863 (Fig. 8). Its sister species is *Montenegrospeum bogici* in the H3 tree (Fig. 8, bootstrap 95%), and on the tree based on both concatenated loci (but with bootstrap 63% only); in the COI tree the bootstrap does not support its phylogenetic position.

**Emmericiidae**

*Emmericia ventricosa* Brusina, 1870

Fig. 14A–C

GenBank no. COI: MZ027623–MZ027627

Remarks. The species was found at the localities 1, 5, 12, 13, 22 (estavelle) (Fig. 4), at the surface. Molecular data rather confirms its distinctiveness ($p = 0.038$) from *E. expansilabris* (Bourguignat, 1870), described from Vrelo “Ombla” on the Dalmatian coast in nearby Croatia.

Moitessieriidae

*Iglica* cf. *absoloni* (A.J. Wagner, 1914)

Remark. Empty shell was found interstitially at the locality 20 (Fig. 4).

*Plagigeyeria tribunicae* Schütt, 1963

Remark. Empty and incomplete shell was found interstitially at the locality 17 (Fig. 4).

*Paladilbiopsis arion* Rysiewska & Osikowski, 2021

Fig. 14D, E

GenBank no. COI: MW741739–MW741740; H3: MW776424–MW776425

Remarks. Live specimens were pumped from an interstitial habitat at the locality 21 (Fig. 4). They were recently described as new to science (Hofman et al. 2021). Morphologically and molecularly, they were distinct from the moitessieriid species discussed in Hofman et al. (2018). Rysiewska et al. (2021) demonstrated that at least some of the species assigned to the genus *Plagigeyeria* Tomlin, 1930 belong to the genus *Paladilbiopsis* Pavlović, 1913. Our specimens from Gučina in Trebinje molecularly formed the sister clade with *Plagigeyeria montenegrina* Bole, 1961 from Obodskaja Pečina in Montenegro. Also, the outline and orientation of the long axis of the aperture was characteristic of *Plagigeyeria*. The similarly shaped shell and geographic range may suggest assignment to *P. nitida* Schütt, 1963, but the number of whorls of our specimens is much higher than presented by Schütt (1972).
Heterobranchia  
Heterostropha: Valvatidae  

**Valvata montenegrina** Glöer & Pešić, 2008  
Fig. 14F  

**GenBank no.** COI: MZ027632–MZ027633  
**Remark.** Some specimens found at the locality 20 (Fig. 4); in the surface waters.

Pulmonata  
Lymnaeidae  

**Radix labiata** (Rossmässler, 1835)  
Fig. 14G  

**GenBank no.** COI: MZ027630  
**Remarks.** This common Central-European and Mediterranean species was found at the localities 16 and 20 (Fig. 4). Inhabits slowly running or stagnant small water bodies (e.g., Glöer 2019), preferably close to ground waters, but not found in subterranean habitats.

**Galba truncatula** (O. F. Müller, 1774)  
Fig. 14H, I  

**GenBank no.** COI: MZ027628–MZ027629  
**Remarks.** Common Palaearctic gastropod, inhabiting nearly all of Europe. This amphibious and calcifilous (e.g., Glöer 2019) species inhabits small water bodies, rich in vegetation, such as at our locality 16 – a small lake in a collapsed cave, rather than subterranean habitats, but at the locality 15 it was found in an estavelle, a kind of vast subterranean tunnel transporting water either down, as outlet of surface waters, or up, forming temporary active springs. Shells of our specimens (Fig. 14H, I) were somewhat untypical, with low and broad spire, but the variation of the shell in the Lymnaeidae has been long known (e.g., Roszkowski 1914; Falniowski 1980, 1981), as being wider than in any other gastropod group.

Ancylidae  

**Ancylus recurvus** Martens, 1783  
Fig. 14J, K  

**GenBank no.** COI: MW879251–MW879253  
**Remarks.** *Ancylus* is known as a stygophile gastropod (e.g., Culver and Pipan 2009; Macher et al. 2016; personal observations); also inhabiting caves. *Ancylus recurvus* at
the locality 13 was also found interstitially, pumped, and at the locality 15 (Fig. 4) it inhabited an estavelle. Our *A. recurvus* molecularly belonged to the clade “*Ancylus* sp. B” of Albrecht et al. (2006), Clade 3 of Pfenninger et al. (2003) (Fig. 15). It is molecularly different from *A. fluviatilis* by 9%.
**Ancylus sp.**

Fig. 14L, M

**GenBank no.** COI: MW879254–MW879255

**Remarks.** Considering the shell morphology, it should be determined as *A. fluviatilis* O. F. Müller, 1774, a species reported from this region. However, Pfenninger et al. (2003) demonstrated that *A. fluviatilis* inhabits a wide range throughout Europe, but in the southern regions there are a few cryptic, molecularly defined species of *Ancylus*. Our *Ancylus sp.* molecularly belonged to the Clade 4 of Pfenninger et al. (2003) and “Ancylus sp. C4” of Albrecht et al. (2006) (Fig. 15). It was found as a crenobiont in the cave springs at the localities 8, 9 and 16 (Fig. 4). Molecular divergence between this Ancylus sp. and *Ancylus recurvus* is 7%, and similar value (7.5%) is observed between this Ancylus sp. and *A. fluviatilis*.

**Stylommatophora: Succineidae**

**Succinea cf. putris** (Linnaeus, 1758)

Fig. 14N

**GenBank no.** COI: MZ027631

**Remarks.** Our specimen differed by 12 substitutions (97.55% of identity) from *Succinea* sp. GenBank number KF412772 from “Egypt: Fayoum Governorate”. For the closest European *Succinea, S. putris* the identity was only 86.73%. In fact, this
value is close to the threshold one to distinguish species in the Pulmonata, thus our specimen may represent some still unsequenced species of *Succinea*. This amphibious snail was found at locality 10 (Fig. 4).

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