Two new species of the Balkan genus *Paladilhiopsis* Pavlović, 1913 (Caenogastropoda, Moitessieriidae)

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Academic editor: Martin Haase | Received 16 February 2021 | Accepted 19 March 2021 | Published 21 June 2021

http://zoobank.org/9DC4BC40-58B3-4852-97AE-8DA0A7E4DD3A

Citation: Hofman S, Grego J, Rysiewska A, Osikowski A, Falniowski A (2021) Two new species of the Balkan genus *Paladilhiopsis* Pavlović, 1913 (Caenogastropoda, Moitessieriidae). ZooKeys 1046: 157–176. https://doi.org/10.3897/zookeys.1046.64489

Abstract
The Balkan Peninsula is inhabited by the worldwide most diverse subterranean gastropod fauna. This fauna is still poorly studied, since its habitats are not easily accessible, and its sampled populations are mostly not rich in specimens’ numbers. Often only empty shells are known, but the shell is hardly useful, not only in phylogeny reconstruction, but even in species determination. The exclusively obligatory subterranean family Moitessieriidae is especially poorly studied. Representatives of the genus *Paladilhiopsis* Pavlović, 1913 (Moitessieriidae) collected at three localities, distributed in Croatia and Bosnia & Herzegovina, were studied. The pigmentation of their shells and soft parts, as well as the female and male reproductive organs in one taxon, are presented. The partial sequences of the molecular markers mitochondrial cytochrome oxidase subunit I (COI) and nuclear histone 3 (H3) were used to infer their systematic status and phylogenetic relationships. Two species new to science are described. For one of them, also studied anatomically, 15 specimens were sequenced for COI, and all show the same haplotype.

Keywords
Anatomy, COI, H3, molecular systematic, mOTU, radula, shell, stygobiont
Introduction

Gastropods are an important component of the subterranean fauna (Culver 2012), but are still poorly studied (e.g., Culver and Pipan 2009, 2014). Of the approximately 20,000 worldwide species of subterranean animals (Culver and Pipan 2009), there are more than 350 described species of stygobiont (obligate subterranean aquatic) gastropods, 97% of them belong to the Hydrobiidae sensu lato (Bernasconi and Riedel 1994; Culver 2012), representing several families belonging to the Truncatelloidea (Criscione and Ponder 2013). The most diverse subterranean gastropod fauna worldwide inhabits the Balkan Peninsula, mainly the Dinaric karst. Sket et al. (2004) reported 169 obligate stygobiont gastropod species inhabiting this territory. As stressed by Falniowski (2018), variable shells as the only known structures, coupled with the widespread, dogmatic belief in geographic isolation and unavoidable, immediate speciation, resulted in descriptions of new species (nearly) in each cave or other subterranean habitat. Low densities of subterranean populations, coupled with not easily penetrable habitats, resulted in numerous nominal species known only as empty shells, washed out into springs at times of high flow, especially during spring (Haase 1995; Richling et al. 2016). Even the soft parts, if accessible, were not usually informative enough to resolve taxonomic questions, since the animals are very tiny and miniaturisation has resulted in simplification of their anatomy (e.g., Culver 2012; Falniowski 2018). Molecular data are helpful, but there are still only few studies applying them (e.g., Grego et al. 2019; Hofman et al. 2019).

All the restrictions of our knowledge outlined above are even more severe in the case of the family Moitessieriidae Bourguignat, 1863, whose monophyly has recently been proved (Falniowski et al. 2019). Its representatives are minute gastropods, all of them obligatory subterranean, exclusively inhabiting subterranean waters, including thermal ones (Skeč and Velkovrh 1981). High variability in shell morphology and the lack of diagnostic features in the morphology of the simplified soft parts, coupled with anticipated high levels of endemism has resulted in a long list of nominal moitessieriid species (see e.g., Glöer 2002 for *Bythiospeum* Bourguignat, 1882). The anatomy of the family is still poorly known and provided only for a few taxa. Detailed anatomy of *Bythiospeum* Bourguignat, 1882 was described and illustrated by Haase (1995) and Girardi and Rosello (2001). Some anatomical data on the Moitessieriidae were contributed also by Bole (1961, 1970), Giusti and Pezzoli (1980) (anatomy of *Iglica* Wagner, 1927 and *Paladilhiopsis* Pavlović, 1913), Radoman (1983), Bernasconi (1990, 1994), Boeters and Gittenberger (1990), Bodon and Giusti (1991), Boeters (1998), Szarowska (2006), Niero and Pezzoli (2016), and Hofman et al. (2018).

The genus *Paladilhiopsis* Pavlović, 1913 (type species *Paladilhia robiciana* Clessin, 1882), inhabiting the Balkans (including Hungary) was considered as a subgenus of *Bythiospeum* by Slapnik (1995). Boeters (1998) synonymised *Paladilhiopsis* with *Bythiospeum*, based on the similarity of the general organisation of the female reproductive organs, i.e., a large bursa copulatrix situated at the proximal part of the albumen gland, which is markedly shortened. In our partial revision of the Balkan Moitessieriidae
Two new species of *Paladilhiopsis* (Hofman et al. 2018) we confirmed the anatomy of the female reproductive organs of *Paladilhiopsis*, but our molecular data definitely proved rather distant phylogenetic relationships between *Paladilhiopsis* and *Bythiospeum*, unequivocally classifying this morphological similarity (of very simple structures) as a homoplasy, certainly not a synapomorphy. Continued field collection, applying also the Bou-Rouch technique for collection of interstitial gastropods, resulted in some new *Paladilhiopsis*, which were checked for molecular markers. Their phylogenetic position, applying the shell, soft parts morphology (if the material was available) and molecular distinctness and relationships are the subject of the present paper.

**Materials and methods**

The snails were collected at three localities (Table 1), distributed in Bosnia and Herzegovina and Croatia (Figs 1, 2). They were either collected by hand and sieve in springs, or with a pump applying Bou-Rouch technique (Bou and Rouch 1967), to sample interstitial fauna below the bottom of streams, at the depth of ca. 50 cm. The tube was inserted in the bottom five times, and 20 litres were pumped each time. Samples were sieved through 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.

**Table 1.** Sample localities.

| Id # | Site name                                    | Coordinates            |
|------|----------------------------------------------|------------------------|
| 1    | Studena spring, left bank of Cetina River, Slime, Croatia, locality G25 | 43°25’43”N, 16°51’59”E |
| 2    | Spring Zvezda, above left bank of Cetina, Croatia, locality 26            | 43°26’13”N, 16°44’26”E |
| 3    | Vrelo „Lušac” (Gučina), BiH, locality 19-10a                               | 42°42’04”N, 18°21’27”E |

**Figure 1.** Localities map.
The shells were photographed with a Canon EOS 50D digital camera, under a Nikon SMZ18 microscope with a dark field. 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. The penes were photographed under Motic microscope with dark field. The shells were cleaned with an ultrasonic cleaner, 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. Morphometric parameters of the shell (following the scheme of Falniowski et al. 2007) were measured by one person using a Nikon DS-5 digital camera and ImageJ image analysis software (Rueden et al. 2017).
Snails for molecular analysis were fixed in 80% ethanol, changed twice, and later stored in 96% ethanol. 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 were given in Szarowska et al. (2016). Sequences were initially aligned in the MUSCLE (Edgar 2004) program in MEGA 7 (Kumar et al. 2016) and then checked in BI-OEDIT 7.1.3.0 (Hall 1999). Uncorrected p-distances were calculated in MEGA 7. In the phylogenetic analysis additional sequences from GenBank were used as reference (Table 2). The estimation of the proportion of invariant sites and the saturation test (Xia 2000; Xia et al. 2003) were performed using DAMBE (Xia 2013). The data were analysed using approaches based on Bayesian Inference (BI) and Maximum Likelihood (ML). We applied the GTR model whose parameters were estimated by RAxML (Stamatakis 2014). The General Time Reversible (GTR) model is the most complicated one, including all the simpler cases assumed by the other models. We agree with the arguments of Stamatakis that the simpler models are only computationally less expensive, which is of diminishing importance with modern computers.

**Table 2.** Taxa used for phylogenetic analyses with their GenBank accession numbers and references.

| Species                          | COI/H3 GB numbers | References                  |
|----------------------------------|-------------------|-----------------------------|
| Bythiospeum acicula (Hartmann, 1821) | KU341350/MK609534 | Richling et al. 2016/Falniowski et al. 2019 |
| Bythiospeum alzenre Boeters, 2001 | KU341354/–       | Richling et al. 2016        |
| Ecrobia maritima (Milaschewitsch, 1916) | KK355835/MG551322 | Osikowski et al. 2016/Grego et al. 2017 |
| Iglica cf. gracilis (Clessin, 1882) | MH720985–MH720986/| Hofman et al. 2018          |
| Iglica cf. gracilis (Clessin, 1882) | MH721002–MH721003 |                             |
| Iglica hellenica Falniowski & Sarbu, 2015 | KT825581/MH721007 | Falniowski and Sarbu 2015/Hofman et al. 2018 |
| Lanzaioptis sasinica Bole, 1989   | MN272428–MN272429/| Prevorčnik et al. 2019      |
|                                  | MN272430–MN272431 |                             |
| Moitesieria cf. puteana Coutagne, 1883 | AF367635/MH721012 | Wilke et al. 2001/Hofman et al. 2018 |
| Paladilhiopsis cf. abaloni (A. J. Wagner, 1914) | –/MH721021 | Hofman et al. 2018          |
| Paladilhiopsis bibrnesis (Glöer & Grego, 2015) | –/MH721015 | Hofman et al. 2018          |
| Paladilhiopsis bonatica (Clessin, 1910) | –/MH721020 | Hofman et al. 2018          |
| Paladilhiopsis bonica Bole, 1970   | –/MH721021 | Hofman et al. 2018          |
| Paladilhiopsis grobbeni Kučer, 1928 | MH720991/MH721014 | Hofman et al. 2018          |
| Paladilhiopsis turrita (Kučer, 1933) | MH720992/MH721015 | Hofman et al. 2018          |
| Paladilhiopsis gittenbergeri (A. Reischutz & P. L. Reischutz, 2008) | MH720993/MH721025 | Hofman et al. 2018          |
| Paladilhiopsis matejkoi Glöer & Grego, 2019 | MK632245/MK632246 | Grego et al. 2019          |
| Paladilhiopsis marnoki (Glöer & Grego, 2015) | –/MH721017 | Hofman et al. 2018          |
| Paladilhiopsis montenegrinus       | MW452318–MW452319/| Rysiewska et al. unpub.     |
|                                  | MW452604–MW452605 |                             |
| Pandaninicola pieperi (Westerlund, 1886) | KT710668/KT710740 | Szarowska et al. 2016      |
At the same time, as pointed out already by Nei and Kumar (2000), the criteria like AIC often collapse in choosing the proper model of DNA evolution. With several data sets, MODELTEST and MEGA totally collapsed in attempting to find the proper model (pers. obs. AF).

The Bayesian analyses were run using MrBayes v. 3.2.3 (Ronquist et al. 2012) with defaults of most priors. Two simultaneous analyses were performed, each with 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 the trees were discarded as burn-in. The analyses were summarised as a 50% majority-rule tree. Convergence was checked in Tracer v. 1.5 (Rambaut and Drummond 2009). 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). Two species delimitation methods were performed: Poisson Tree Processes (PTP) (Zhang et al. 2013) and Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2011). The PTP approach was run using the web server https://species.h-its.org/ptp/, with 100 000 MCMC generations, 100 thinning and 0.1 burn-in. We used RAxML output phylogenetic tree. The ABGD approach using the web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) and the default parameters.

**Results**

We obtained 17 new sequences of COI (457 bp, GenBank accession numbers MW741724–MW741740) and nine of H3 (310 bp, GenBank accession numbers MW776417–MW776425). The tests by Xia et al. (2003) revealed no saturation. In all analyses, the topologies of the resulting phylograms were identical in both the Maximum Likelihood (ML) and Bayesian Inference (BI).

All newly sequenced specimens belonged to the Moitessieriidae at the COI (Fig. 3) as well as H3 (Fig. 4) trees. In the maximum likelihood tree computed for nine new concatenated sequences of the both studied loci, together with all the Balkan species of the Moitessieriidae whose COI and H3 sequences were available (Fig. 5), PTP and ABGD methods inferred twelve mOTUs (A-L), two of them (A, C) new, most probably of the species rank. The p-distances between mOTUs (Table 3) ranged from 0.059 to 0.298 for COI and from 0.016 to 0.142 for H3. All the new taxa belong to the genus *Paladilbiopsis*, as defined by Hofman et al. (2018). The levels of divergence for these new taxa were comparable to other *Paladilbiopsis* taxa of species rank (Table 3). At locality 2, as many as 13 specimens were sequenced for the cytochrome oxidase subunit I (COI), and no polymorphism was found in this variable locus; thus, only seven specimens were sequenced also for COI and the much more conservative histon 3 (H3) locus, showing no infrapopulation polymorphism.
Two new species of Paladilhiopsis

Figure 3. The maximum-likelihood phylogram for the COI gene. Bootstrap supports given if ≥ 60%.

Table 3. P-distances between main COI mOTUs of Paladilhiopsis. For details, see Fig. 5.

|   | A    | B    | C    | D    | E    | F    | G    | H    | I    | J    | K    | L    |
|---|------|------|------|------|------|------|------|------|------|------|------|------|
| A | 0.035| 0.045| 0.035| 0.042| 0.042| 0.071| 0.135| 0.126| 0.113| 0.100| 0.113| 0.110|
| B | 0.113| 0.035| 0.026| 0.035| 0.039| 0.071| 0.132| 0.126| 0.123| 0.103| 0.113| 0.113|
| C | 0.145| 0.140| 0.016| 0.023| 0.035| 0.068| 0.132| 0.110| 0.019| 0.106| 0.110| 0.110|
| D | 0.124| 0.116| 0.059| 0.016| 0.026| 0.061| 0.129| 0.116| 0.113| 0.110| 0.110| 0.110|
| E | 0.132| 0.140| 0.091| 0.081| 0.029| 0.061| 0.129| 0.116| 0.113| 0.110| 0.110| 0.110|
| F | 0.116| 0.094| 0.118| 0.105| 0.118| 0.061| 0.142| 0.116| 0.113| 0.106| 0.123| 0.116|
| G | 0.137| 0.134| 0.164| 0.134| 0.181| 0.137| 0.126| 0.113| 0.119| 0.123| 0.116| 0.116|
| H | 0.167| 0.177| 0.177| 0.164| 0.194| 0.164| 0.156| 0.135| 0.126| 0.116| 0.100| 0.100|
| I | 0.156| 0.173| 0.196| 0.172| 0.183| 0.157| 0.176| 0.198| 0.103| 0.119| 0.119| 0.110|
| J | 0.220| 0.226| 0.204| 0.194| 0.192| 0.202| 0.204| 0.215| 0.185| 0.087| 0.061| 0.061|
| K | 0.231| 0.242| 0.199| 0.212| 0.212| 0.215| 0.247| 0.253| 0.203| 0.185| 0.065| 0.065|
| L | 0.298| 0.282| 0.245| 0.250| 0.237| 0.263| 0.288| 0.277| 0.273| 0.255| 0.226| 0.226|
Figure 4. The maximum-likelihood phylogram for the H3 gene. Bootstrap supports given if ≥ 60%.

Systematic part

Family Moitessieriidae Bourguignat, 1863
Genus *Paladilhiopsis* Pavlović, 1913

*Paladilhiopsis stellatus* Grego & Hofman, sp. nov.
http://zoobank.org/01ff1956-336a-4942-b634-1a5d036b4cdd
Figures 6A–P, 7B–D, 8, 9

**Note.** mOTU A (Fig. 5); localities 1 and 2; Fig. 6A–P; GenBank numbers: COI: MW741724–MW741738; H3: MW776417–MW776423
Two new species of *Paladilhiopsis*

**Figure 5.** Maximum likelihood tree computed for concatenated partial sequences of COI and H3 sequences. Bootstrap supports given if ≥ 60%.

**Type locality.** Spring Zvezda, above left bank of Cetina River, Slime, Omiš district, Croatia (43°26'13"N, 16°44'26"E) (Fig. 2B, locality 2).

**Holotype.** Ethanol-fixed specimen (Fig. 6C), interstitially in the gravel below the bottom of the spring; J. Grego, A. Falniowski, R. Ozimec, M. Ošavský, J. Ošavská leg.; 08 August 2020; NHMW113632.

**Paratypes.** Type locality; J. Grego, A. Falniowski, R. Ozimec, M. Ošavský, J. Ošavská leg.; 08 August 2020; ten ethanol-fixed paratypes in the collection of the Department of Malacology of Jagiellonian University, dry specimens: ZMUJ-M.2633-2642, HNHM/2 specimens, NHMW-MO 113627/2 specimens, SMF362990/2 specimens, PG/2 specimens, JG-F1628 /158 specimens.

**Other material.** Studena spring, concrete well ca. 80 m from road at hillside, Slime, Omiš district Croatia; 43°25’43"N, 16°51’59.74"E; J. Grego, A. Falniowski, R. Ozimec, M. Ošavský, J. Ošavská leg.; 08 August 2020; JG F1626/20 specimens (Fig. 2A, locality 1); Studena spring, stony catchment, left bank of Cetina River, Slime, Omiš district Croatia; 43°25’45.48"N, 16°51’59.57"E; J. Grego, A. Falniowski, R. Ozimec, M. Ošavský, J. Ošavská leg.; 08 August 2020; JG-F1621/3 fragmented specimens (ca. 80 m uphill from locality 1).
Figure 6. Shells of *Paladilhiopsis* A–P: *P. stellatus* A, B locality 1, Studena spring (2H58, 2H59) C–P locality 2, Zvezda spring (holotype, 2H60, 2H61, 2I19-2I28, respectively) Q–S *P. arion*, locality 3, Vrelo „Lušac” (Gučina), BiH (holotype, 2G19, 2G22).

**Diagnosis.** Shell minute, elongate-conic (turriform), distinguishable from the geographically close *Paladilhiopsis elongata* (Kuščer, 1933) from spring Jadro near Split (HR) by less sinuated lateral labral profile, more inflated and more prominent body whorls, and a more elongate pyramidal shape. From *P. solida* Kuščer, 1933 from Vrelo Buna in Blagaj (BiH) differs by its smaller less conical shell with less prominent body whorl and slightly sinuate lateral labral margin adapically preceding. Can be distinguished from *P. pretneri* Bole & Velkovrh, 1987 from Antunovići near Kozice, Makarska district (HR) by its longer, more elongate shell and proportionally smaller body whorl. The receptaculum seminis long and tubular in shape, similar to that of *P. bosniaca* (Clessin, 1910), and different from the bulbous one with a long duct as in *P. grobeni* Kuščer, 1928.

**Description.** Shell (Fig. 6A–P) up to 2.94 mm high and 1.02 mm broad, ovate-conic (turriform), white or whitish, translucent, thin-walled, consisted of ca. six
whorls, growing slowly and regularly, and separated by moderately deep but sharply marked suture. Spire high and conic, apex narrow, body whorl height less than 0.5 of the shell height. Aperture small, proscoline, oval, or elongated oval in shape, peristome complete and thin, umbilicus slit-like. Shell surface smooth, glossy, with growth lines hardly visible.

**Measurements** of holotype and sequenced and illustrated shells presented in Table 4. Shell variability slight, marked mostly in breadth: height proportion of the shell and the aperture (Fig. 6A–P).

**Radula** (Fig. 7B–D) taenioglossate, typical of *Paladilhiopsis*, with numerous, long, and cusps. Rhachis formula:

\[
\frac{(5)4-1-4(5)}{1-1} \quad \text{or} \quad \frac{4-1-4}{1-1}
\]

Basal cusps widely triangular and massive, median cusp at the cutting edge 2 × longer than the adjacent ones, lateral tooth formula: 3 – 1 – 4, the largest cusp prominent, nearly 2 × longer than the adjacent ones, on the inner marginal tooth ca. 18 large cusps similar to the ones on the rhachis, ca. 20 smaller and more slender cusps on the outer marginal tooth.

**Soft parts morphology and anatomy.** Body white, with no pigment, with no eyes. Female reproductive organs (Fig. 8) typical of the genus *Paladilhiopsis* (Hofman et al. 2018), with unpigmented, long, and narrow renal oviduct, large bursa copulatrix, although less elongated than in *P. grobbeni*, and with its duct (characteristically for *Paladilhiopsis*) lying proximally, single small distal receptaculum seminis (in the position of rs, after Radoman 1973), with the outlet to the oviduct close to the outlet of the duct of the bursa copulatrix, and shortened accessory gland complex. Long narrow loop of the renal oviduct and small short seminal receptacle are characteristic of the species. The simple penis (Fig. 9) typical of the genus, without any outgrowth, tapering, in the form of an elongated triangle.

**Derivatio nominis.** The specific epithet *stellatus* refers to *stella*, the Latin word for star for the name of the type locality, Zvezda Spring, zvezda which means star in Croatian.

**Known distribution.** Besides the type locality (locality 2: Spring Zvezda, above left bank of Cetina, Croatia), found also at locality 1: Studena spring, left bank of Cetina River, Slime, Croatia, 43°25’45.48”N, 16°51’59.57”E.

**Remarks.** Molecularly this mOTU is the sister clade of the mOTU B (*Paladilhiopsis gittenbergeri*), but genetic distance is high, 11.3% for COI 3.5% for H3.

**Paladilhiopsis arion** Rysiewska et Osikowski, sp. nov.
http://zoobank.org/7901fa54-583b-4825-b482-d82413291808
Figures 6Q–S, 7A

**Note.** mOTU C (Fig. 5); locality 3; Fig. 6Q–S; GenBank numbers: COI: MW741739–MW741740; H3: MW776424–MW776425
Figure 7. A teleoconch sculpture of *Paladilbiopsis arion* B–D radula of *P. stellatus*. Scale bars 50 μm (A); 10 μm (B–D).

Figure 8. Renal and pallial section of the female reproductive organs of *Paladilbiopsis stellatus* X. Abbreviations: bc – bursa copulatrix, cbc – duct of bursa, ga – albuminoid gland, gn – nidamental gland, gp – gonoporus, ov – oviduct, ovl – loop of (renal) oviduct, rs – seminal receptacle (in the position of Radoman’s (1983) rs₁ – distal seminal receptacle). Scale bar: 500 μm.
Two new species of *Paladilhiopsis*

Type locality. Vrelo “Lušac” (Gučina), BiH (42°42’04”N, 18°21’27”E) (locality 3).

**Holotype.** Ethanol-fixed specimen (Fig. 6Q), interstitially (pumped with Bou-Rouch pump), in the gravel below the bottom of the spring; 10 Sept 2019; A. Falniewski, A. Rysiewska and A. Osikowski leg., voucher number: ZMUJ-M.2643.

**Paratypes.** Five empty shells, in the collection of the Department of Malacology of Jagiellonian University, voucher numbers: ZMUJ-M.2644-2648.

**Diagnosis.** Shell minute, elongate-conic (turriform), with relatively narrow spire, whose breadth grows rapidly, acute narrow apex, and narrow but long aperture with prominent lip, distinguishable from the geographically close *Paladilhiopsis matejkoi* Grego & Glöer, 2019 from Nemila Spring, Herceg Novi (MNE) and *P. montenegrinus* (Schütt, 1959, described as *Saxurinator*) from Bileća (BiH) by its more conical shell shape with slightly pagoda-shaped whorls, sharper apex, and deeper suture. From *P. matejkoi* it can additionally be differentiated by its more declined aperture.

**Description.** Shell (Fig. 6Q–S) 2.51 mm high and 0.91 mm broad, elongate-conic (turriform), white, translucent, thin-walled, consisted of ca. six and half whorls, growing slowly and regularly in their height, but growing rapidly in breadth, separated by moderately deep but sharply marked suture. Spire high and slim, apex acute, body whorl height ca. 0.38 of the shell height. Aperture narrow but long, peristome com-

![Figure 9. Penes of *Paladilhiopsis stellatus*. Scale bars: 200 μm.](image-url)
complete, forming a prominent lip, umbilicus slit-like. Shell surface smooth, glossy, with clearly visible broad growth lines, forming ribs (Fig. 7A).

**Measurements** of holotype and sequenced and illustrated shells provided in Table 4.

**Soft parts morphology and anatomy.** The body is white, without pigment, with no eyes. The arrangement of pellets in the rectum characteristic for the Moitessieriidae (Boeters and Gittenberger 1990). The anatomy is unknown.

**Derivatio nominis.** The specific epithet *arion* refers to the ancient name of River Trebišnjica, which in classical antiquity was known as the *Arion*, rising and sinking through its course before resurfacing at various places from the Neretva to the coast; Trebišnjica is adjacent to the type locality.

**Known distribution.** Only the type locality.

**Remarks.** Molecularly this mOTU is the sister clade of the mOTU D (*Paladilhiospis matejkoi*), with genetic distances 5.9% for COI 1.6% for H3.

**Discussion**

Molecularly, the strict monomorphism in the mitochondrial locus in the mOTU A is striking: the same haplotype occurs at two localities. The monomorphism in the stygobiont organism is often recorded, but usually only a single or few specimens are available, thus one cannot deny the possibility that the picture is biased by too many
samples. In our materials as many as 13 species from locality 2 (and two from locality 1) were identical, confirming the real lack of genetic polymorphism. The presented photographs show the shell morphology in one population, which is also slightly variable.

As demonstrated by Falniowski (1987, 1990, 2018) the details of the reproductive organs, such as the shape of the receptaculum and/or bursa copulatrix, are hardly useful in species-level taxonomy of the Truncatelloidea: they are either uniform above species level or variable within a species. This is clearly visible in the moitessieriid gastropods, whose anatomy is even more simplified, due to miniaturisation, than in most of the truncatelloidean families. The female reproductive organs of Paladilhiopsis stellatus are practically identical as in P. bosniaca, and only slightly different from that of P. grobbeni.

Progress in molecular taxonomy methods has led to the development of many tools for species delimitation. Among the most widely used, the automatic barcode gap discovery (ABGD), Poisson tree processes (PTP), as well as the general mixed Yule coalescent (GMYC) were proposed (Pons et al. 2006). All of them have many limitations, like sensitivity to gene flow and the ratio of the population size to the divergence time (PTP, GMYC), tendency to under- or over-split species (ABGD, GMYC), and many others (e.g., Pentinsaari et al. 2017; Luo et al. 2018). We have not been able to use the GMYC methods because we have no strictly ultrametric input tree. For this reason, we have used the alternative PTP technique and, for comparison, also the ABGD delimitation. Low numbers of DNA fragments also possibly weakened our results. On the other hand, increasing the number of loci as well as the sample size per species results only in a modest benefit for species delimitation methods (Luo et al. 2018). This, together with identical results obtained with two methods based on different assumptions, supports the reliability of our inference of species distinction. This is also enhanced by comparisons of the genetic distances. They are quite high, especially for P. stellatus. The p-distance between different Paladilhiopsis species varied from 0.013 to 0.125 (Hofman et al. 2018), and our values are higher than or close to the mean. Moreover, for other snails closely related to the Moitessieriidae, the estimated threshold p-distances for species delimitation applying COI were strikingly low: ca. 0.015 in the genus Bythinella (Bichain et al. 2007) and 0.023 (Hurt 2004) for Pyrgulopsis. All the above, coupled with the differences in morphology, supports the descriptions of these two new species.

The Truncatelloidea are one of the great examples of the Balkan biodiversity (Bănărescu 2004). New species from this region, including the ones whose distinctiveness is supported by molecular data, are still being described from this area (e.g., Hofman et al. 2019, 2020; Falniowski et al. 2021). The Mediterranean Basin Hotspot is one of the 34 biodiversity hotspots in the world (Nyers et al. 2000. Many factors are responsible for the high level of the biological diversity in this region, e.g., location at the intersection of two major landmasses (Eurasia and Africa), a complex geological history, huge topographical diversity and altitudinal differences, unique climate with cool, wet winters and hot, dry summers. Unfortunately, human activity has drastically affected aquatic habitats decreasing this biodiversity (e.g., Szarowska and Falniowski 2011). Rivers and their floodplains, lakes, wetlands, and especially springs, exploited as sources of water and contaminated, offer increasingly bad conditions for the aquatic fauna. In this context, studies of this diminishing biodiversity are important and urgent.
Acknowledgements

The study was supported by a grant from the National Science Centre 2017/25/B/NZ8/01372 to Andrzej Falniowski. We are grateful for the assistance of Anna Łatkiewicz (Laboratory of FE Scanning Microscopy and Microanalysis, Institute of Geological Sciences, Jagiellonian University, Krakow, Poland) for her help with the SEM. Our gratitude is to Roman Ozimec (Zagreb, Croatia) and Márió Olšavský and Jolana Olšavská (Banská Bystrica, Slovakia) for supporting the 2020 field trip as well as to Brian Lewarne (Devonshire UK) for supporting our field research around Trebinje.

References

Bânărescu PM (2004) Distribution Pattern of the Aquatic Fauna of the Balkan Peninsula. In: Griffiths HI, Krystufek BR, Jane M (Eds) Balkan Biodiversity, Pattern and Process in the European Hotspot, 203–217. https://doi.org/10.1007/978-1-4020-2854-0_12

Bernasconi R (1990) Revision of the genus *Bythiospeum* (Mollusca Prosobranchia Hydrobiidae) of France, Switzerland and Germany. Lägass-Druck, Bern, 44 pp.

Bernasconi R (1994) Le genre *Moitessieria* BGT, 1863 en France. Revision, inventaire et description de *M. corsica* n. sp. (Mollusca Gasteropoda Prosobranchia Hydrobiidae). Memoires de Biospeologie 21: 7–20.

Bernasconi R, Riedel A (1994) Mollusca. In: Juberthie C, Decu V (Eds) Encyclopedia Biospeologica I. Société Biospéologie, Moulis, France, 54–61.

Bichain JM, Gaubert P, Samadi S, Boisselier-Dubayle MC (2007) A gleam in the dark: Phylogenetic species delimitation in the confusing spring-snail genus *Bythinella* Moquin-Tandon, 1856 (Gastropoda: Rissooidea: Amnicolidae). Molecular Phylogenetics and Evolution 45: 927–941. https://doi.org/10.1016/j.ympev.2007.07.018

Bodon M, Giusti F (1991) The genus *Moitessieria* in the island of Sardinia and in Italy. New data on the systematics of *Moitessieria* and *Paladilhia* (Prosobranchia: Hydrobiidae) (Studies on the Sardinian and Corsican malacofauna, IX). Malacologia 33: 1–30.

Boeters HD (1998) Mollusca: Gastropoda: Supérfamille Rissooidea. In: Schwoerbel J, Zwick P (Eds) Süsswasserfauna von Mitteleuropa. Begründet von A. Brauer. 5/1–2. Gustav Fischer Verlag, Jena/Lübeck/Ulm, 76 pp.

Boeters HD, Gittenberger E (1990) Once more on the Moitessieriidae (Gastropoda Prosobranchia), with the description of *Clameia brooki* gen. et spec. nov. Basteria 54: 123–129.

Bole J (1961) Nove Hidrobide (Gastropoda) iz podzemeljskih voda zahodnega Balkana. Biološki Vestnik 9: 59–69.

Bole J (1970) Prispevek k poznavanju anatomije in taksonomije podzemeljskih hidrobiid (Gastropoda, Prosobranchia). Razprave SAZU, Ljubljana, IV, 10: 73–108.

Bou C, Rouch R (1967) Un nouveau champ de recherches sur la faune aquatique souterraine. Comptes Rendus de l’Académie des Sciences Series III Sciences 265: 369–370.

Bourguignat J-R (1863) Monographie du nouveau genre français *Moitessieria*. Revue et Magazine de Zoologie, Pure et appliquée, Series 2, 15: 432–445.
Two new species of *Paladilhiopsis* 173

Clessin S (1882) Monographie des Gen. *Vitrella* Cless. Malakozoologische Blätter, New Series, 5: 110–129.

Criscione F, Ponder W (2013) A phylogenetic analysis of rissooidean and cingulopsoidoidean families (Gastropoda: Caenogastropoda). Molecular Phylogenetics and Evolution 66: 1075–1082. https://doi.org/10.1016/j.ympev.2012.11.026

Culver DC (2012) Mollusks. In: White WB, Culver DC (Eds) Encyclopedia of Caves (2nd edn.). Academic Press, New York, 512–517. https://doi.org/10.1016/B978-0-12-383832-2.00074-8

Culver DC, Pipan T (2009) The Biology of Caves and Other Subterranean Habitats. Oxford University Press, Oxford, 254 pp.

Culver DC, Pipan T (2014) Shallow Subterranean Habitats. Ecology, Evolution and Conservation. Oxford University Press, Oxford, 258 pp. https://doi.org/10.1093/acprof:oso/9780199646173.001.0001

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32: 1792–1797. https://doi.org/10.1093/nar/gkh340

Falniowski A (1987) Hydrobioidea of Poland (Prosobranchia: Gastropoda). Folia Malacologica 1: 1–122. https://doi.org/10.12657/folmal.001.001

Falniowski A (1990) Anatomical characters and SEM structure of radula and shell in the species-level taxonomy of freshwater prosobranchs (Mollusca: Gastropoda: Prosobranchia): a comparative usefulness study. Folia Malacologica 4: 53–142. https://doi.org/10.12657/folmal.004.005

Falniowski A (2018) Species Distinction and Speciation in Hydrobioid Gastropods (Mollusca: Caenogastropoda: Truncatelloidea). Journal of Archive Zoology 1: 003. https://doi.org/10.24966/AZS-7779/100003

Falniowski A, Grego J, Rysiewska A, Osikowski A, Hofman S (2021) Two new stygobiotic species of *Horatia* Bourguignat, 1887 (Hydrobiidae) from Croatia. Subterranean Biology 37: 89–104. https://doi.org/10.3897/subtbiol.37.61573

Falniowski A, Prevorčnik S, Delić T, Alther R, Altermatt F, Hofman S (2019) Monophyly of the Moitessieriidae Bourguignat, 1863 (Caenogastropoda: Truncatelloidea). Folia Malacologica 27: 61–70. https://doi.org/10.12657/folmal.027.005

Falniowski A, Sarbu S (2015) Two new Truncatelloidea species from Melissotrypa Cave in Greece (Caenogastropoda). ZooKeys 530: 1–14. https://doi.org/10.3897/zookeys.530.6137

Falniowski A, Szarowska M, Grzmil P (2007) *Daphniola* Radoman, 1973 (Gastropoda: Hydrobiidae): shell biometry, mtDNA, and the Pliocene flooding. Journal of Natural History 41: 2301–2311. https://doi.org/10.1080/00222930701630733

Girardi H, Rosello M (2001) Notes sur *Bythiospeum klemmi* (Boeters, 1969) (Mollusca: Prosobranchia: Hydrobiidae) dans un des émergence karstique de la Gardonnenque (Gard, France). Documents Malacologique 2: 13–29.

Giusti F, Pezzoli E (1980) Guide per il riconoscimento delle specie animali delle acque interne Italiane 8. Gasteropodi 2 (Gastropoda: Prosobranchia; Hydrobioidea, Pyrguloidea) [Guide for the recognition of animal species in inland waters of Italy 8. Gastropods 2 (Gastropoda: Prosobranchia; Hydrobioidea, Pyrguloidea)). Consiglio Nazionale delle Ricerche, Verona, 67 pp. [AQ/1/47]
Glöer P (2002) Die Süßwassergastropoden Nord- und Mitteleuropas, Bestimmungsschlüssel, Lebensweise, Verbreitung. Die Tierwelt Deutschlands (Vol. 73). [Freshwater Gastropods of North and Middle Europe, Identification, Biology, Distribution. Fauna of Germany (Vol. 73.)] ConchBooks, Hackenheim, 327 pp.

Grego J, Hofman S, Mumladze L, Falniowski A (2017) Aagraia Szarowska et Falniowski, 2011 (Caenogastropoda: Hydrobiidae) in the Caucasus. Folia Malacologica 25: 237–247. https://doi.org/10.12657/folmal.025.025

Grego J, Glöer P, Falniowski A, Hofman S, Osikowski A (2019) New subterranean freshwater gastropod species from Montenegro (Mollusca, Gastropoda, Moitessieriidae, and Hydrobiidae). Ecologica Montenegrina 20: 71–90. https://doi.org/10.37828/em.2019.20.6

Haase M (1995) The stygobiont genus Bythiospaeum in Austria: a basic revision and anatomical description of B. cf. geyeri from Vienna (Caenogastropoda: Hydrobiidae). Bulletin of the American Malacological Union 11: 123–137.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Hofman S, Rysiewska A, Osikowski A, Grego J, Sket B, Prevorčnik M, Falniowski A (2018) Phylogenetic relationships of the Balkan Moitessieriidae (Caenogastropoda: Truncatelloidea). Zootaxa 4486: 311–339. https://doi.org/10.11646/zootaxa.4486.3.5

Hofman S, Osikowski A, Rysiewska, A, Grego J, Glöer P, Dmitrović D, Falniowski A (2019) Sarajana Radoman, 1975 (Caenogastropoda: Truncatelloidea): premature invalidation of a genus. Journal of Conchology 43: 407–418.

Hofman S, Rysiewska A, Osikowski A, Falniowski A (2020) A new species of Kerkia Radoman, 1978 (Caenogastropoda, Hydrobiidae) from Bosnia and Herzegovina. ZooKeys 973: 17–33. https://doi.org/10.3897/zookkeys.973.52788

Hurt C (2004) Genetic divergence, population structure and historical demography of rare springsnails (Pyrgulopsis) in the lower Colorado River basin. Molecular Ecology 13: 1173–1187. https://doi.org/10.1111/j.1365-294X.2004.02121.x

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33: 1870–1874. https://doi.org/10.1093/molbev/msw054

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov, New Orleans, 8 pp. https://doi.org/10.1109/GCE.2010.5676129

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403: 853–858. https://doi.org/10.1038/35002501

Nei M, Kumar S (2000) Molecular Evolution and Phylogenetics. Oxford University press, Oxford and New York, 333 pp.

Niero I, Pezzoli E (2016) Una nuova specie di Iglica Wagner, 1910 per la Lombardia (Gastropoda: Prosobranchia: Moitessieriidae). Bolletino Malacologico 42: 1–29.

Osikowski A, Hofman S, Georgiev D, Kalcheva S, Falniowski A (2016) Aquatic snails Ecrobia maritima (Milaschewitsch, 1916) and E. ventrosa (Montagu, 1803) (Caenogastropoda: Hydrobiidae) in the east Mediterranean and Black Sea. Annales Zoologici 66: 477–486. https://doi.org/10.3161/00034541ANZ2016.66.3.012
Two new species of Paladilhiopsis

Pavlović PS (1913) Pecinski puz Larretia serbica sp. nov. iž zapadne Srbije. Glas Srpska Kraljevske Akademije, Beograd, Prvi Razred 91: 71–75.

Pentinsaari M, Vos R, Mutanen M (2017) Algorithmic single-locus species delimitation: effects of sampling effort, variation and nonmonophyly in four methods and 1870 species of beetles. Molecular Ecology Resources 17: 393–404. https://doi.org/10.1111/1755-0998.12557

Pons J, Barraclough TG, Gómez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sumlin WD, Vogler AP (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Systematic Biology 55: 595–609. https://doi.org/10.1080/10635150600852011

Prevorčnik S, Hofman S, Delić T, Rysiewska A, Osikowski A, Falniowski A (2019) Lanzaiopsis Bole, 1989 (Caenogastropoda: Truncatelloidea): its phylogenetic and zoogeographic relationships. Folia Malacologica 27: 193–201. https://doi.org/10.12657/fomal.027.018

Puillandre N, Lambert A, Brouillet S, Acha G (2011) ABDG, Automatic Barcode Gap Discovery for primary species delimitation. Molecular Ecology 21: 1864–1877. https://doi.org/10.1111/j.1365-294X.2011.05239.x

Radoman P (1973) New classification of fresh and brackish water Prosobranchia from the Balkans and Asia Minor. Posebna Izdanja Prirodničesko muzeja, Beograd 32: 1–30.

Radoman P (1983) Hydrobioidea a superfamily of Prosobranchia (Gastropoda). I Systematics. Monographs 547, Serbian Academy of Sciences and Arts, Beograd.

Rambaut A, Drummond AJ (2009) Tracer v1.5. http://beast.bio.ed.ac.uk/Tracer

Richling I, Malikowsky Y, Kuhn Y, Niederhöfer H-J, Boeters HD (2016) A vanishing hotspot – impact of molecular insights on the diversity of Central European Bythiospeum Bourguignat, 1882 (Mollusca: Gastropoda: Truncatelloidea). Organisms Diversity and Evolution 17: 67–85. https://doi.org/10.1007/s13127-016-0298-y

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539–542. https://doi.org/10.1093/sysbio/sys029

Rueden DT, Schindelin J, Hiner MC, Dezonia BE, Walter AE, Arena ET, Eliceiri KW (2017) ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics 18: 529. https://doi.org/10.1186/s12859-017-1934-z

Rysiewska A, Osikowski A, Pesic V, Grego J, Falniowski A, Hofman S (2021) Plagigeyeria montenegrina Bole, 1961 (Caenogastropoda: Truncatelloidea: Moitessieriidae): morphology and molecules in the species and genus taxonomy. Journal of Conchology 44: 37–51.

Skel B, Paragamian K, Trontelj P (2004) A Census of the obligate subterranean fauna of the Balkan Peninsula. In: Griffiths HI, Kryštufek B, Reed JM (Eds) Balkan Biodiversity: Pattern and Process in the European Hotspot. Kluwer Academic Publishers, 309–322. https://doi.org/10.1007/978-1-4020-2854-0_18

Skel B, Velkovrh F (1981) Podzemeljske živali v termalnih vodah. Biološki Vestnik 29: 91–120.

Slatnik R (1995) Razširjenost podrobu Bythiospeum (Paladilhiopsis) Pavlović 1913 (Gastropoda, Prosobranchia, Hydrobiidae) v osamelem krasu vzhodne Slovenije = Distribution of the subgenus Bythiospeum (Paladilhiopsis) Pavlović 1913 (Gastropoda, Prosobranchia, Hy-
drobiidae) in isolated karst of eastern Slovenia. Razprave Sazu [Academia Scientiarum et Artium Slovenica], Razred za Naravoslovne Vede [Classis 4] 36: 59–89.

Stamatakis A (2014) RaxML Version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30: 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Szarowska M (2006) Molecular phylogeny, systematics and morphological character evolution in the Balkan Rissooidea (Caenogastropoda). Folia Malacologica 14: 99–168. https://doi.org/10.12657/folmal.014.014

Szarowska M, Falniowski A (2011) Destroyed and threatened localities of rissooid snails (Gastropoda: Rissooidea) in Greece. Folia Malacologica 19: 35–39. https://doi.org/10.2478/v10125-011-0010-y

Szarowska M, Osikowski A, Hofman S Falniowski A (2016) Pseudamnicola Paulucci, 1878 (Caenogastropoda: Truncatelloidea) from the Aegean Islands: a long or short story? Organisms Diversity and Evolution 16: 121–139. https://doi.org/10.1007/s13127-015-0235-5

Wilke T, Davis GM, Falniowski A, Giusti F, Bodon M, Szarowska M (2001) Molecular systematics of Hydrobiidae (Mollusca: Gastropoda: Rissoidea): testing monophyly and phylogenetic relationships. Proceedings of the Academy of Natural Sciences of Philadelphia 151: 1–21. https://doi.org/10.1635/0097-3157(2001)151[0001:MSOHMG]2.0.CO;2

Xia X (2000) Data analysis in molecular biology and evolution. Kluwer Academic Publishers, Boston, Dordrecht & London, 280 pp.

Xia X (2013) DAMBE: A comprehensive software package for data analysis in molecular biology and evolution. Molecular Biology and Evolution 30: 1720–1728. https://doi.org/10.1093/molbev/mst064

Xia X, Xie Z, Salemi M, Chen L, Wang Y (2003) An index of substitution saturation and its application. Molecular Phylogenetics and Evolution 26: 1–7. https://doi.org/10.1016/S1055-7903(02)00326-3

Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A General Species Delimitation Method with Applications to Phylogenetic Placements. Bioinformatics 29: 2869–2876. https://doi.org/10.1093/bioinformatics/btt499