

PHYLOGENETIC RELATIONSHIPS OF *BRACENICA* RADOMAN, 1973 (CAENOGASTROPODA: TRUNCATELLOIDEA)

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ABSTRACT: Shells of the representatives of the genus *Bracenicia* Radoman, 1973: *B. spiridoni*, *B. vitojaensis* and a new species, collected at the Kotor Bay (Montenegro) are presented. For the new species also the operculum (characteristic of the genus), penis and the female reproductive organs are described and figured. Considering the shell, operculum, and soft part morphology the new species belongs to the genus *Bracenicia*. Cytochrome oxidase subunit I (COI) of mitochondrial DNA sequence of the new species places it in the subfamily Sadlerianinae Szarowska, 2006, and *Bracenicia* is the sister genus of *Sadleriana* Clessin, 1890. COI sequences of *Hauffenia plana* Bole, 1961 clearly indicate its placement within the genus *Bracenicia*.

KEY WORDS: stygobiont, operculum, anatomy, mtDNA, COI, Hydrobiidae, Montenegro, Kotor Bay

INTRODUCTION

Bracenicia spiridoni Radoman, 1973 is a freshwater species of valvate-shelled truncatelloid, recorded from the subterranean habitats and springs. It is endemic to Montenegro, its known distribution is restricted to only two localities in the Lake Skadar area: Spirov Izvor (a small spring ca. 2 × 2 m) and to Karuč spring (a sublacustrine spring: RADOMAN 1983). GLÖER et al. (2015) described another species of *Bracenicia*: *B. vitojaensis* Glöer, Grego, Erőss et Fehér, 2015, from Vitoja Spring, Podgorica municipality, also close to Lake Skadar (Fig. 1). These descriptions were based on the shell morphology alone. However,

it was demonstrated that shell morphology was often not sufficient for species delimitation in Hydrobiidae (e.g. OSIKOWSKI et al. 2015, RYSIEWSKA et al. 2016). Moreover, members of the genus *Kerkia* Radoman, 1978 with very similar shell morphology are known from a closely situated area – southern Bosnia and probably northern Montenegro. We found members of the genus *Bracenicia* at a locality on the Kotor Bay, which extends its distribution range about 30 km northward. At another locality in the region we collected specimens of *Hauffenia plana* Bole, 1961 (BOLE 1961). The aim of this paper is to compare the

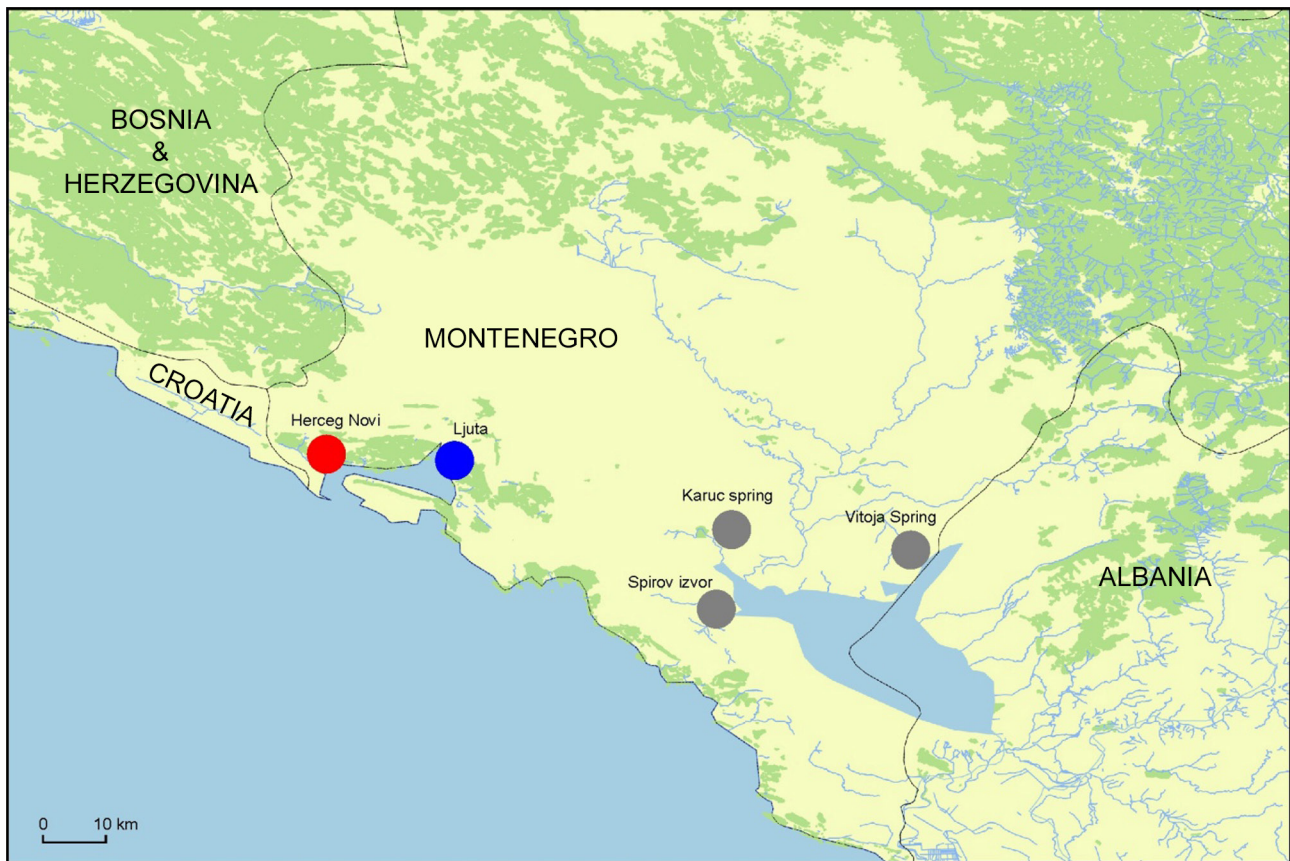


Fig. 1. Map with the type locality of *Bracenica gloeri* n. sp. (red dot), locality of *Bracenica plana* (Bole, 1961) (blue dot) and localities of other representatives of the genus (grey dots)

operculum and soft part morphology of our *Bracenica* with the other species of *Bracenica*, as well as to apply partial mitochondrial cytochrome oxidase subunit I

(COI) DNA sequence data to infer phylogenetic relationships of the newly found *Bracenica*, as well as of *Hauffenia plana*.

MATERIAL AND METHODS

Live individuals of *Bracenica* were collected at two localities on the Kotor Bay, Montenegro:

1. Spring behind the car wash Nickević at crossing of E65 and Bratsva Jedistva Street, Herceg Novi, Montenegro ($42^{\circ}27.72' N$, $18^{\circ}31.21' E$) (Fig. 1). A small spring, in the sand sediment and in dense web of roots hanging inside the spring water outlet (Figs 2–3);
2. Spring Ljuta between Orahovac and Dobrota, 7 km N of Kotor ($42^{\circ}29.13' N$, $18^{\circ}45.98' E$).

The shells were sorted under binocular microscope OLYMPUS SZ-11 and the live specimens fixed in 80% analytically pure ethanol. The shells were photographed with a CANON EOS 50D digital camera attached to NIKON SMZ-18 stereoscopic microscope with the dark field. The operculum was cleaned in ultrasonic cleaner and examined using a HITACHI S-4700 scanning electron microscope, applying the techniques described by FALNIOWSKI

(1990). The dissections of the soft parts were done with NIKON SMZ-18 stereoscopic microscope with the dark field. Morphological characters' terminology follows HERSHLER & PONDER (1998).

For DNA extraction, whole specimens with cracked shells were used. The tissue was hydrated in TE buffer (3×10 min.); then total genomic DNA was extracted with the SHERLOCK extracting kit (A&A Biotechnology), and the final product was dissolved in $20 \mu\text{l}$ of 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). A fragment of the mitochondrial cytochrome oxidase subunit I (COI) was 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) programme in MEGA 6 (TAMURA et al. 2013) and then checked

in BIOEDIT 7.1.3.0 (HALL 1999). Uncorrected p-distances were calculated in MEGA 6. The saturation test (XIA 2000, XIA et al. 2003) was performed using DAMBE (XIA 2013). In the phylogenetic analysis additional sequences from GenBank were used (Table 1). The data were analysed using approaches based on Bayesian inference (BI) and maximum

likelihood (ML). We applied the GTR + Γ model, as the main general whose certain parameters set either to 1 or 0, respectively, presented all the other stationary models of DNA evolution. The parameters of the model were estimated by the RaxML (STAMATAKIS 2014). The BI was run using MrBayes v. 3.2.3 (RONQUIST et al. 2012) with the default



Figs 2–3. Type locality of *Bracenicia gloeri* n. sp.: 2 – water outlet; 3 – rivulet from spring during the maximum water outlet during spring (Photo: Z. P. ERÖSS)

priors. Two simultaneous analyses were performed, each lasting 10,000,000 generations with one cold chain and three heated chains, starting from random trees and sampling trees every 1,000 generations. The first 25% of trees were discarded as burn-in. The analyses were summarized on a 50% majority-rule tree. Convergence was checked in Tracer v. 1.5 (RAMBAUT & DRUMMOND 2009). FigTree v. 1.4.4 (RAMBAUT 2010) was used to visualise the trees. The ML approach was applied with RAxML v. 8.0.24 (STAMATAKIS 2014). RAxML analyses were performed using the free computational resource CIPRES Science Gateway (MILLER et al. 2010).

Abbreviations: AMNH – American Museum of Natural History, New York, USA; HNHM – Hungarian Natural History Museum, Budapest, Hungary; NHMUK – Natural History Museum London, UK; NHMW – Naturhistorisches Museum Wien, Austria; NMBE – Naturhistorisches Museum, Bern, Switzerland; SMF – Senckenberg Museum, Frankfurt, Germany; AH – aperture height; AW – aperture width; BH – height of the body whorl; BW – width of the body whorl; H – shell height; W – shell width; LT – locus typicus.

Table 1. Taxa used for phylogenetic analyses with their GenBank accession numbers and references

Species	COI GB numbers	References
<i>Agrafia wiktoriae</i> Szarowska et Falniowski, 2011	JF906762	SZAROWSKA & FALNIOWSKI 2011
<i>Alzoniella finalina</i> Giusti et Bodon, 1984	AF367650	WILKE et al. 2001
<i>Anagastina zetavalis</i> (Radoman, 1973)	EF070616	SZAROWSKA 2006
<i>Avenionia brevis berenguieri</i> (Draparnaud, 1805)	AF367638	WILKE et al. 2001
<i>Belgrandiella kusceri</i> (Wagner, 1914)	KT218511	FALNIOWSKI & BERAN 2015
<i>Bithynia tentaculata</i> (Linnaeus, 1758)	AF367643	WILKE et al. 2001
<i>Bythinella austriaca</i> (von Frauenfeld, 1857)	JQ639858	FALNIOWSKI et al. 2012b
<i>Bythinella micherdzinskii</i> Falniowski, 1980	JQ639854	FALNIOWSKI et al. 2012b
<i>Bythiospeum acicula</i> (Hartmann, 1821)	KU341350	RICHLING et al. 2016
<i>Dalmanella fluviatilis</i> Radoman, 1973	KC344541	FALNIOWSKI & SZAROWSKA 2013
<i>Daphniola louisii</i> Falniowski et Szarowska, 2000	KM887915	SZAROWSKA et al. 2014
<i>Ecrobia maritima</i> (Milaschewitsch, 1916)	KJ406200	SZAROWSKA & FALNIOWSKI 2014b
<i>Emmericia expansilabris</i> Bourguignat, 1880	KC810060	SZAROWSKA & FALNIOWSKI 2013a
<i>Fissuria boui</i> Boeters, 1981	AF367654	WILKE et al. 2001
<i>Graecoarganiella parnassiana</i> Falniowski et Szarowska, 2011	JN202352	FALNIOWSKI & SZAROWSKA 2011
<i>Graziana alpestris</i> (Frauenfeld, 1863)	AF367641	WILKE et al. 2001
<i>Grossuana codreanui</i> (Grossu, 1946)	EF061919	SZAROWSKA et al. 2007
<i>Hauffenia tellinii</i> (Pollonera, 1898)	KY087861	RYSIEWSKA et al. 2017
<i>Hauffenia michleri</i> Kuščer, 1932	KY087865	RYSIEWSKA et al. 2017
<i>Heleobia dobrogica</i> (Grossu et Negrea, 1989)	EU938131	FALNIOWSKI et al. 2008
<i>Horatia klecakiana</i> Bourguignat 1887	KJ159128	SZAROWSKA & FALNIOWSKI 2014a
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF278808	WILKE et al. 2000
<i>Iglica</i> cf. <i>gracilis</i> (Clessin, 1882)	MH720985	HOFMAN et al. 2018
<i>Islamia zermanica</i> (Radoman, 1973)	KU662362	BERAN et al. 2016
<i>Kerkia jadertina</i> (Kuščer, 1933)	KY087874	RYSIEWSKA et al. 2017
<i>Lithoglyphus prasinus</i> (Küster, 1852)	JX073651	FALNIOWSKI & SZAROWSKA 2012
<i>Littorina littorea</i> (Linnaeus, 1758)	KF644330	LAYTON et al. 2014
<i>Marstoniopsis insubrica</i> (Küster, 1853)	AF322408	FALNIOWSKI & WILKE 2001
<i>Moitessieria</i> cf. <i>puteana</i> Coutagne, 1883	AF367635	WILKE et al. 2001
<i>Montenegrospium bogici</i> (Pešić et Glöer, 2012)	KM875510	FALNIOWSKI et al. 2014
<i>Paladilhopsia grobbeni</i> Kuščer, 1928	MH720991	HOFMAN et al. 2018
<i>Pontobelgrandiella</i> sp. Radoman, 1978	KU497024	RYSIEWSKA et al. 2016/GREGO et al. 2017
<i>Radomaniola curta</i> (Küster, 1853)	KC011814	FALNIOWSKI et al. 2012a
<i>Sadleriana fluminensis</i> (Küster, 1853)	KF193067	SZAROWSKA & FALNIOWSKI 2013b
<i>Tanousia zрманjae</i> (Brusina, 1866)	KU041812	BERAN et al. 2015



RESULTS

MOLECULAR PART

We obtained 3 new sequences of COI (382 bp, GenBank Accession numbers MT396209-MT396211). The tests of XIA *et al.* (2003) revealed little saturation. Results from the substitution saturation analysis showed an ISS = 0.75, significantly smaller than the critical ISS value (0.95), indicating that all the sequences are useful in phylogenetic reconstruction. The topologies of the resulting phylograms were identical in both the maximum likelihood (ML) and Bayesian inference (BI). The three new sequences (two of the new species of *Bracenic*

and one of *Hauffenia plana*) formed a distinct clade, closely related to *Sadleriana fluminensis* (Fig. 4).

The inferred tree reflects a common problem – saturation results in low resolution of deep nodes, showing one big polytomy, if only COI is applied. Unfortunately, our snails were not well fixed, thus it was possible to get only short sequences of COI. This was still enough to confirm that *Bracenic gloeri* and *B. plana* are congeners, and they are closely related with *Sadleriana* and *Horatia*, as well as to reject closer relationships of *Bracenic* with the other genera included in the tree.

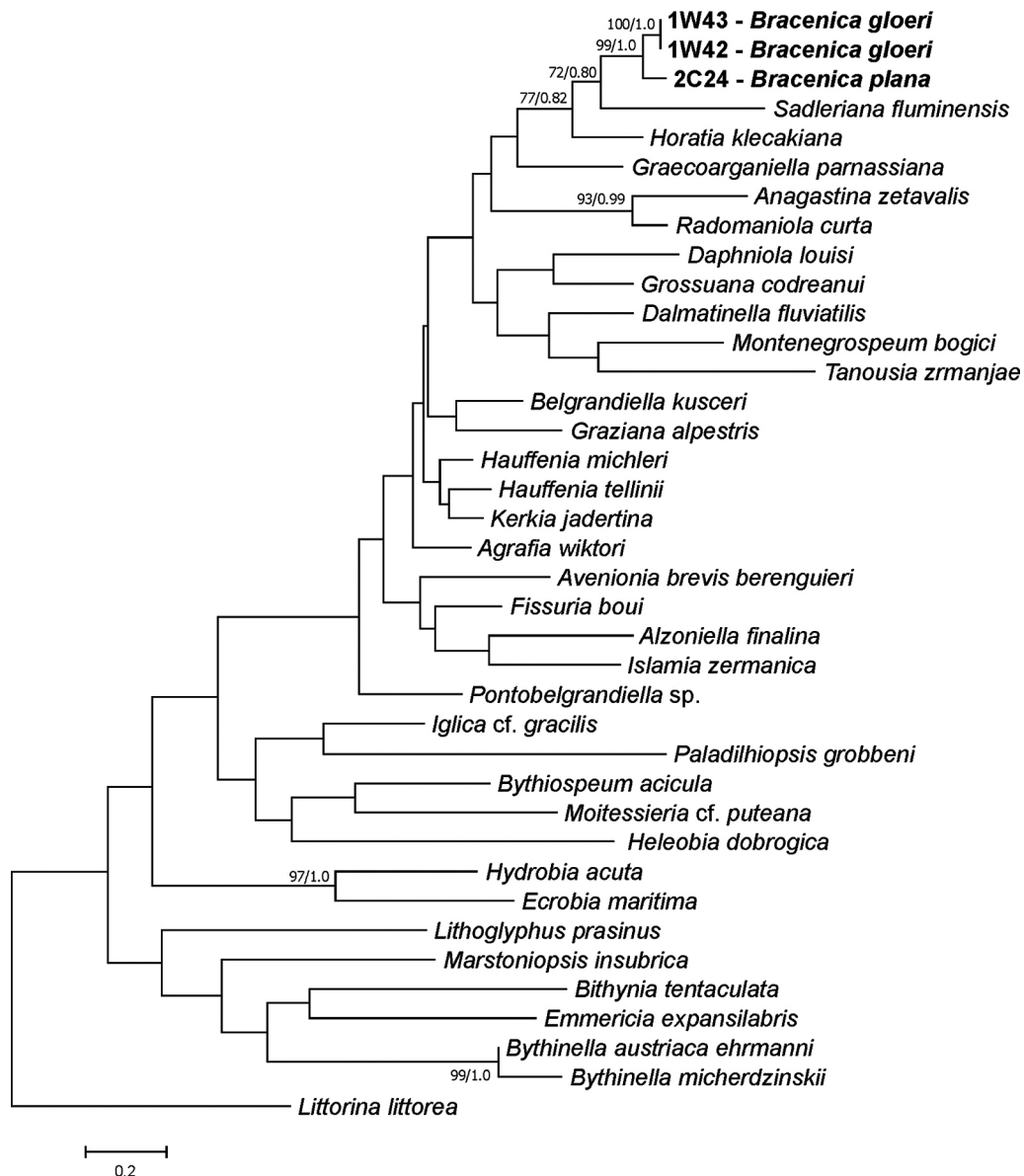


Fig. 4. Maximum likelihood tree based on mitochondrial COI. Bootstrap supports and Bayesian probabilities are given

TAXONOMIC PART

Superfamily Truncatelloidea Gray, 1840

Family Hydrobiidae Stimpson, 1865

Subfamily Sadlerianinae Szarowska, 2006

Genus *Bracenicia* Radoman, 1973

Type species: *Bracenicia spiridoni* Radoman, 1973

Bracenicia gloeri n. sp. Grego, Fehér et Eröss (Figs 5–12, 20–24)

Type locality. Montenegro, Kotor Bay, Herceg Novi, spring behind the car wash Nickević at crossing of E65 and Bratsva Jedistva Street (42°27.72'N, 18°31.21'E).

Type material. Holotype: Leg. ERÖSS Z. P. and FEHÉR Z. 21.07.2018, HNHM-MOLL 104161; Paratypes: same data, HNHM-MOLL 104162/120 wet; NHMUK 20190541/3 dry; SMF 356764/3 dry; NMBE 561366/3 dry; SBMNH 633040/3 dry; AMNH_IZC 00331522/2 dry; coll. Eröss/20 wet and 3 dry; coll. Grego/88 dry).

Etymology. Named after our friend, German malacologist Peter Glöer from Hetlingen, who extensively contributed to the study of the Balkan freshwater gastropod fauna.

Description. Shell (Figs 5–12) translucent with smooth and shiny surface and reddish horny periostracum, with three convex whorls, separated by a deep suture forming elevated spire. Shell valvatoid, slightly conical with a sub-circular, slightly tear-shaped aperture and sharp peristome. Umbilicus widely open. In the holotype the aperture height to shell height ratio: 0.6, height of body whorl to height of spire ratio: 4.6, shell height 1.22 mm, shell width 1.65 mm. Operculum reddish-horny, circular, spiral, paucispiral; nucleus central in the form of characteristic, knob-like outgrowth (apophysis) on its inner side, spirally bent (Figs 20–22). The knobby structure spiral-wise elevated at its highest point. Body pigmentless, eyes absent. Female reproductive organs (Fig. 23) with big spherical bursa copulatrix and two seminal receptacles, the proximal one nearly vestigial, and the distal one big, sac-shaped. Penis (Fig. 24) with a characteristic outgrowth.

Measurements. Holotype: H 1.22 mm; W 1.65 mm; BH 1.08 mm; BW 1.10 mm; AH 0.73 mm; AW 0.62 mm.

Differentiating features. The shell of *Bracenicia gloeri* n. sp. (Figs 5–9) differs in proportions from that of *B. spiridoni* (Fig. 19), also that figured by RADOMAN (1983: plate IV, fig. 58), as well as from the one of *B. vitojaensis* (Fig. 18), also that figured by GLÖER et al. (2015: figs 12–14): the body whorl is much higher, and the peristome is ovate, not circular. The bursa copulatrix is spherical, proportionally bigger than the ellipsoid one in *B. spiridoni*. The COI sequences-based phylogeny (Fig. 4) indicate *Sadleriana fluminensis* (Küster, 1853) as the sister clade of our *Bracenicia* clade, close to *Horatia klecakiana* Bourguignat, 1887, within the subfamily Sadlerianinae Szarowska, 2006. *B. gloeri* and *B. plana* are sister taxa, with p-distance = 4.7%.

Habitat. The live animals were found at the point of spring water outlet covered by a dense web of tree and bush roots. Live animals were found directly among the fine roots, or in the fine sandy sediment immediately below the root zone.

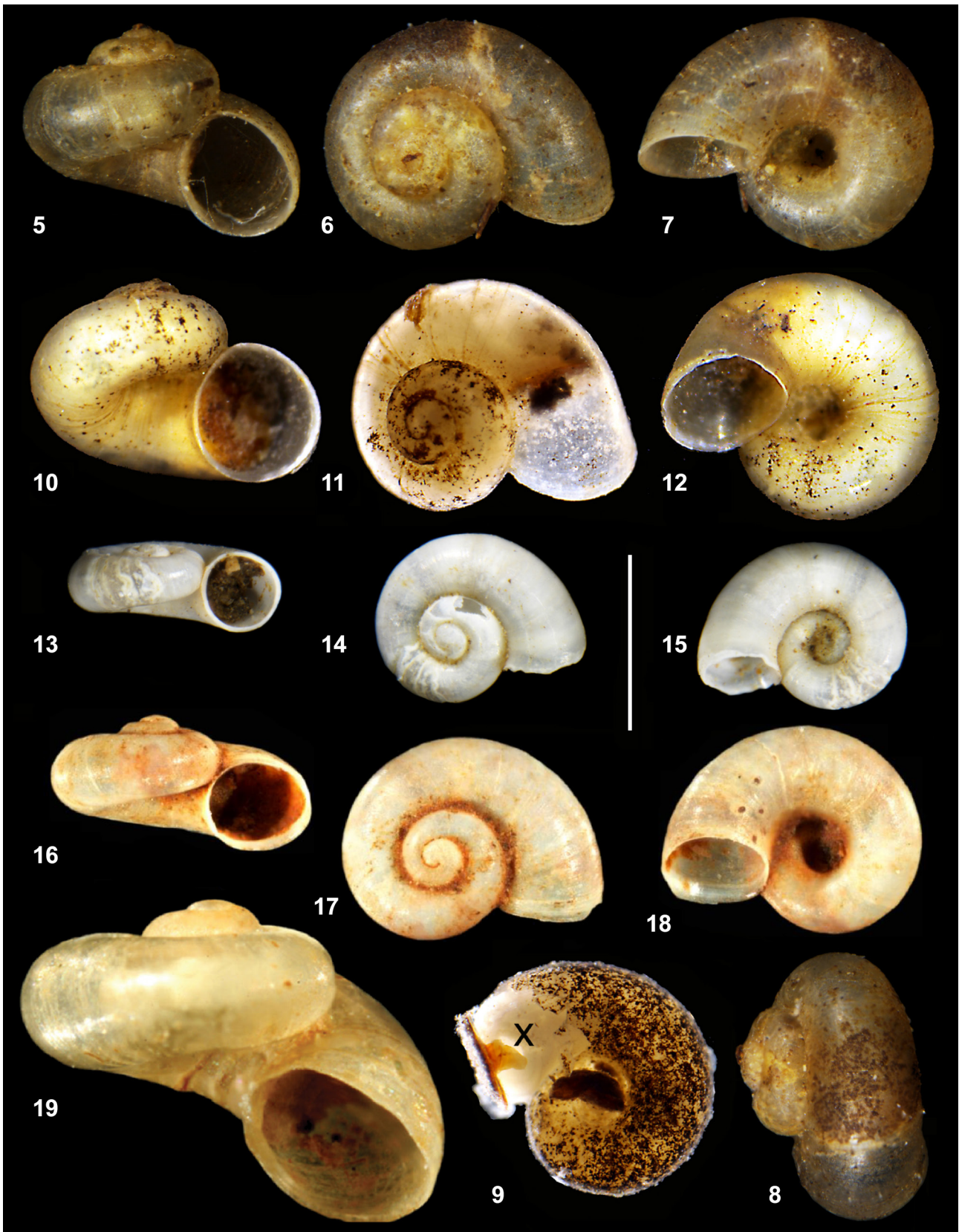
Distribution. Only known from the type locality.

***Bracenicia plana* (Bole, 1961) comb. nova**

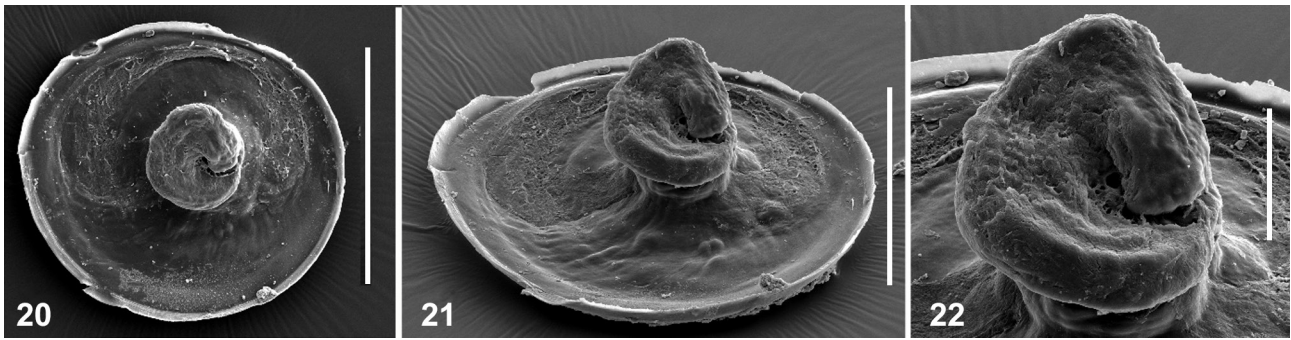
Hauffenia plana Bole, 1961 (BOLE 1961) (Figs 13–15)

Type locality. Ljuta pri Kotoru = Spring Ljuta between Orahovac and Dobrota, 7 km N of Kotor (42°29.14'N, 18°45.99'E). A seasonally very large spring emerging in a short gorge under limestone cliff of eastern Kotor Bay. In dry season the water is brackish; however, a few smaller freshwater outlets are permanently maintained throughout the year.

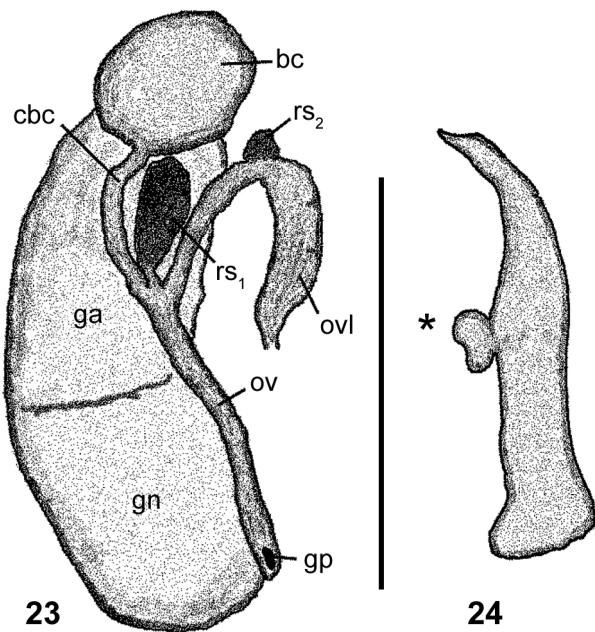
Remarks. The species was originally assigned to the genus *Hauffenia*; however, the molecular data of the topotype (whose morphology was in accordance with the description of BOLE 1961) refute its assignment to *Hauffenia* by SCHÜTT (2000) and HIRSCHFELDER (2017), and suggest its placement within the genus *Bracenicia*. Unfortunately, there was no material fixed well enough for morphological study. The genetic differentiation between *B. gloeri* and *B. plana* described above is 4.7% (as opposed to 14.7% from *Hauffenia*), so they probably belong to *Bracenicia*, not to *Hauffenia*. The valvatiform-shelled specimens from spring Ombla and from Popovo Polje were assigned to *Hauffenia plana* only based on the resemblance in shell morphology (SCHÜTT 2000, HIRSCHFELDER 2017). Considering the hydrological isolation of Ombla and Popovo Polje situated in Trebišnjica drainage basin from the Kotor Bay drainage area (STEVANOVIĆ et al. 2014) with the Ljuta spring and the type locality of our new species, it is highly unlikely that they would represent *Bracenicia plana*.



Figs 5–19. *Bracenic* species: 5–8 – holotype of *Bracenic gloeri* n. sp. HNHM-MOLL 104161, Montenegro, Kotor Bay, Herceg Novi; 9–12 – *B. gloeri* n. sp. live preserved specimens used for molecular and anatomical investigations, X indicates operculum peg; 13–15 – *Bracenic plana* (Bole, 1961), Kotor Bay, Spring Ljuta, topotype; 16–18 – paratype of *B. vitojaensis* Gloer, Grego, Eröss and Fehér, 2015, Montenegro, Podgorica, Spring Vitoja near Skadar Lake, coll. Grego; 19 – *B. spiridoni* Radoman, 1973, Montenegro, Karuč Spring. Scale bar 1 mm (Photo: A. RYSIEWSKA, P. GLÖER and J. GREGO)



Figs 20–22. SEM photographs of the *Bracenicia gloeri* n. sp. operculum. Scale bars 500 μ m



Figs 23–24. Reproductive organs of *Bracenicia gloeri* n. sp.: 23 – renal and pallial section of female reproductive organs; 24 – penis (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; asterisk – outgrowth of the penis). Scale bar 500 μ m

DISCUSSION

The operculum (Figs 9 and 20–22) with a characteristic outgrowth on its inner side was mentioned by RADOMAN (1983). The female reproductive organs (Fig. 23) are similar to the ones described and figured by RADOMAN (1983), in *B. gloeri* with a bigger, spherical bursa copulatrix and two seminal receptacles: proximal (rs₂ after RADOMAN 1973) small, nearly vestigial, and distal (rs₁ after RADOMAN 1973) very big, sac-shaped (Fig. 23), like in *B. spiridoni*. The penis (Fig. 24), long and slender, with a medium-sized outgrowth on its left side, is similar to the one described and drawn by RADOMAN (1983).

Many live animals of *B. gloeri* n. sp. were found directly among the fine roots, or in the sandy sediment just below the root zone. The find at this habitat confirmed our previous observations on valvatiform-shelled gastropods living in similar habitats, associated with plant (tree and bush) roots. We observed identical associations also for the representatives of

the genus *Hauffenia* Pollonera, 1899 from Slovakia and Hungary (*H. kisdalmae* Eröss et Petró, 2008), of the genus *Kerkia* Radoman, 1978 in north-western Bosnia, *Daphniola* Radoman, 1973 in Greece and *Pontohoratia* Vinarski, Palatov et Glöer, 2014 in Georgia. In all cases abundant populations were found in association with submerged fine roots. It is very likely that all five subterranean valvatiform-shelled genera prefer the shallow stygobiont habitat in the spring zone, where the water-saturated debris and gravel are within the zone of the fine plant roots, which are, in fact, the main source of organic matter in this subterranean habitat (CULVER & PIPAN 2009, 2014). As all five genera share the similar valvatiform shell morphology, but, on the other hand, differ distinctly in both their anatomy and molecularly inferred phylogenetic position, it could be speculated that the root-associated habitat could support, as adaptation, such shell morphology. Further studies will be needed to



understand the geographical distribution of *Bracenic* in the Dinaride Karst. The two genera, *Bracenic* and *Hauffenia*, are very distinct (p-distance = 0.157), and probably belong to different genetic lineages (Fig. 4).

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