A new species of *Kerkia* Radoman, 1978 (Caenogastropoda, Hydrobiidae) from Bosnia and Herzegovina

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Academic editor: Eike Neubert  |  Received 31 March 2020  |  Accepted 20 July 2020  |  Published 5 October 2020

http://zoobank.org/69C955E3-DC3A-43A9-A15D-7DA68EAF14EC

Citation: 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/zookeys.973.52788

Abstract
A new species of *Kerkia, K. briani* Rysiewska & Osikowski, sp. nov. is described from the spring Polički Studenac Vrelo (Crkvina), adjacent to the Trebišnjica River (Bosnia and Herzegovina) collected with Bou-Rouch technique, pumped from an interstitial habitat 50 cm below the bottom of the spring. The shell, female reproductive organs, and the penis are described and illustrated. Mitochondrial cytochrome oxidase subunit I (COI) and nuclear histone H3 partial sequences confirm the distinctness of the new species, and molecularly based phylogenetic relationships of *Kerkia* are briefly presented.

Keywords
Balkans, cytochrome oxidase, Gastropoda, histone, interstitial, molecular taxonomy, morphology, stygobiont

Introduction
Mud snails Hydrobiidae are very small or minute snails, whose shells are often approximately 1 mm high. They inhabit surface and subterranean freshwater habitats, although some can also be found in brackish and even marine environments. The
family comprises more than 400 extant genera (Kabat and Hershler 1993), many of which are stygobionts. The Balkans, especially their western region, harbours the world’s most diverse stygobiont malacofauna (e.g., Culver and Sket 2000; Culver 2012). The minute dimensions of those snails, coupled with low population densities (e.g., Culver and Pipan 2009, 2014), result in very poor knowledge of their biology, speciation, and taxonomy. A few specimens are sometimes flooded out of the substrate to the surface. Otherwise, extensive pumping of the interstitial habitats, applying the Bou and Rouch technique sometimes result in more numerous living specimens.

Radoman (1978) established the genus *Kerkia* Radoman, 1978, with the type species *Hauffenia kusceri* Bole, 1961, known only from the cave Krška jama in Slovenia. He described morphology and anatomy of those minute snails, clinging to the rocks in the underground section of the sinking river Krka (Radoman 1973, 1978, 1983). Later, another species of the genus, *K. brezicensis* Bodon & Cianfanelli, 1996, was described from a karstic spring at the entry to Dvorce village, southeast of Brežice in Slovenia. *Hauffenia jadertina* Kuščer, 1933 from the source of the river Jadro near Split in Croatia, as well as *H. jadertina sinjana* Kuščer, 1933 from a spring Zužino Vrelo in the Cetina valley also in Croatia, based on their anatomy, were synonymised and transferred to the genus *Kerkia* by Beran et al. (2014), who also described a new species *Kerkia kareli* Beran, Bodon & Cianfanelli, 2014, from an old well near Povljana on island Pag in Croatia. They provided descriptions and illustrations of the shells, protoconchs, radulae, and soft part morphology and anatomy as well for all the three Croatian taxa. Rysiewska et al. (2017) demonstrated molecular distinctness of those species of *Kerkia*.

In September of 2019, in the spring Polički Studenac Vrelo (Crkvina), adjacent to the Trebišnjica River, we found *Emmericia expansilabris* Bourguignat, 1880, *Sadleriana* sp., *Anagastina vidrovani* (Radoman, 1973), and *Ancylus recurvus* Martens, 1873. Pumping of the interstitial fauna from sediments below the spring resulted in the collection of a few most probably stygophilic *Radomaniola*, but also the typically stygobiont *Montenegrospenum* Pešić & Glöer, 2013 and *Kerkia*. The representatives of the latter genus did not belong to any species known so far, and in the present paper we describe this new species and discuss its relationships.

**Materials and methods**

The snails were collected at the spring Polički Studenac Vrelo (Crkvina), adjacent to the Trebišnjica River (42°42’46.4"N, 18°21’54.5"E), near Trebinje, Bosnia and Herzegovina (Fig. 1). The spring, situated at the right bank of the river (Fig. 2A, B) was in the form of a small shallow pool surrounded by a wall made of stones, with a gravel bottom (Fig. 2C). The Bou–Rouch method (Bou and Rouch 1967) was used to sample interstitial fauna below the spring bottom, at the depth of ca. 50 cm. The tube was inserted in the substrate five times, and 20 litres were pumped each time. Samples were sieved through 500 μm sieve and fixed in 80% analytically pure ethanol, replaced two
times, and later sorted. Next, the snails were put in fresh 80% analytically pure ethanol and kept in -20 °C temperature in a refrigerator.

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. Measurements of the shell (Fig. 3) were taken using ImageJ image analysis software (Rueden et al. 2017).

Snails for molecular analysis were fixed in 80% ethanol, changed twice, and later stored in 80% ethanol. DNA was extracted from whole specimens; tissues were hydrated in TE buffer (3 × 10 min), 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).

DNA coding for mitochondrial cytochrome oxidase subunit I (COI) and nuclear histone 3 (H3) were sequenced. Details of PCR conditions, primers used, and sequenc-
Figure 2. Type locality of Kerkia briani sp. nov.: A, B River Trebišnjica with the spring Polički Studenac Vrelo at its right bank C the spring from where interstitial snails were pumped.

Figure 3. Measurements of the shell.
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ing are given in Szarowska et al. (2016b). 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 estimation of the proportion of invariant sites and the saturation test (Xia 2000; Xia et al. 2003) were performed using DAMBE (Xia 2013). In the phylogenetic analysis additional sequences from GenBank were used as reference (Table 1). 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 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. 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). 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 (http://www.abi.snv.jussieu.fr/public/abgd/abgdweb.html) and the default parameters.

**Results**

**Systematic part**

**Family Hydrobiidae Stimpson, 1865**  
**Subfamily Sadlerianinae Szarowska, 2006**  
**Genus *Kerkia* Radoman, 1978**

*Kerkia briani* Rysiewska & Osikowski, sp. nov.  
http://zoobank.org/1F772BD0-3172-42E7-B559-E8A20773BCF1  
Figures 4, 5, 6A, B, 7

**Holotype.** Ethanol-fixed specimen (Fig. 4), spring Polički Studenac Vrelo (Crkvina), adjacent to the Trebišnjica River (42°42’46.4"N, 18°21’54.5"E), close to Trebinje (Bosnia and Herzegovina interstitially in the gravel 50 cm below the bottom of the spring. It is deposited in the Museum of Natural History of the University of Wrocław, Poland, signature: MNHW-1350.

**Paratypes.** Twelve paratypes, ethanol-fixed, in the collection of the Department of Malacology of Jagiellonian University.
| Species | COI/H3 GB numbers | References |
|---------|-------------------|------------|
| Agrafia wiktori Szarowska & Falniowski, 2011 | JF906762/MG543158 | Szarowska and Falniowski 2011/Grego et al. 2017 |
| Alzoniella finalina Giusti & Bodon, 1984 | AF367650 | Wilke et al. 2001 |
| Annicola limosa (Say, 1817) | AF213348 | Wilke et al. 2000b |
| Anagastina zetatalis (Radoman, 1973) | EF070616 | Szarowska 2006 |
| Avenionia brevis berenguieri (Draparnaud, 1805) | AF367638 | Wilke et al. 2001 |
| Belgrandia thermalis (Linnaeus, 1767) | AF367648 | Wilke et al. 2001 |
| Belgrandiella kuesteri (Boeters, 1970) | MG551325/MG551366 | Osikowski et al. 2018 |
| Bithynia tentaculata (Linnaeus, 1758) | AF367643 | Wilke et al. 2001 |
| Bythinella cretensis Schütt, 1980 | KT353689 | Szarowska et al. 2016a |
| Bythinella hansboetersi Gööer & Pešić, 2006 | KT381101 | Osikowski et al. 2015 |
| Bythispeum acicula (Hartmann, 1821) | KU341350/ MK609536 | Richling et al. 2016/Falniowski et al. 2019 |
| Bythispeum alzense Boeters, 2001 | KU341355 | Richling et al. 2016 |
| Ecrobia maritima (Milaschewitsch, 1916) | KX355835/MG551322 | Osikowski et al. 2016/Grego et al. 2017 |
| Daphniola louisi Falniowski & Szarowska, 2000 | KM887915 | Szarowska et al. 2014c |
| Dalmatinella fluviatilis Radoman, 1973 | KC344541 | Falniowski and Szarowska 2013 |
| Emmericia expansilabris Bourguignat, 1880 | KC810060 | Szarowska and Falniowski 2013a |
| Erhaia jianouensis (Y.-Y. Liu & W.-Z. Zhang, 1979) | AF367652 | Wilke et al. 2001 |
| Fissaria boui Boeters, 1981 | AF367654 | Wilke et al. 2001 |
| Graecanargiellina parrassiana Falniowski & Szarowska., 2011 | JN202352 | Falniowski and Szarowska 2011 |
| Graziana alpestris (Frauenfeld, 1863) | AF367641 | Wilke et al. 2001 |
| Grossuana angelsekovi Gööer & Georgiev, 2009 | KU201090 | Falniowski et al. 2016 |
| Hauffenia michleri (Kuščer, 1932) | KT236156/KY087878 | Falniowski and Szarowska 2015/Rysiewska et al. 2017 |
| Helobia maliziani (Westerlund, 1886) | KM213723/ MK609534 | Szarowska et al. 2014b/Falniowski et al. 2019 |
| Horatta kleckiana Bourguignat 1887 | KJ159128 | Szarowska and Falniowski 2014 |
| Hydrobia acuta (Draparnaud, 1805) | AF278808 | Wilke et al. 2000a |
| Iglica cf. gracilis (Clessin, 1882) | MH720985/MH721003 | Hofman et al. 2018 |
| Iglica bellenica Falniowski & Sarbu, 2015 | KT825581/MH721007 | Falniowski and Sarbu 2015/Hofman et al. 2018 |
| Islamia zermanica (Radoman, 1973) | KU662362/MG551320 | Beran et al. 2016/Grego et al. 2017 |
| Kerkia jadertina (Kuščer, 1933) | KY087868/KY087885 | Rysiewska et al. 2017 |
| Kerkia jadertina sinjana (Kuščer, 1933) | KY087873-74/KY087890-91 | Rysiewska et al. 2017 |
| Kerkia karsleri Beran, Bodon & Cianfanelli, 2014 | KY087875-77/KY087892-94 | Rysiewska et al. 2017 |
| Kerkia kusceri (Bole, 1961) | KY087867/KY087884 | Rysiewska et al. 2017 |
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| Species                        | COI/H3 GB numbers | References                                      |
|--------------------------------|-------------------|------------------------------------------------|
| Kerkia sp. Ljubač              | KY087872/KY087889 | Rysiewska et al. 2017                          |
| Littorina littorea (Linnaeus, 1758) | KF644330/KP113574 | Layton et al. 2014/Neretina 2014, unpublished |
| Littorina plena Gould, 1849     | KF643257          | Layton et al. 2014                             |
| Lithogyphus pratinus (Küster, 1852) | JX073651          | Falniowski and Szarowska 2012                  |
| Maestoniopsis insubrica (Küster, 1853) | AF322408          | Falniowski and Wilke 2001                      |
| Motesseria cf. puteana Courtagne, 1883 | AF367635/MH721012 | Wilke et al. 2001/Hofman et al. 2018           |
| Montenegropeum bogici (Pešić & Glöer, 2012) | KM875510/MG880218 | Falniowski et al. 2014/Grego et al. 2018       |
| Paladiliopsis grobbeni Kučer, 1928 | MH720991/MH721014 | Hofman et al. 2018                             |
| Peringia ulvae (Pennant, 1777)  | AF118302          | Wilke and Davis 2000                           |
| Pomatiopsis lapidaria (Say, 1817) | AF367636          | Wilke et al. 2001                              |
| Pontobelgrandiella sp. Radoman, 1978 | KU497024/MG551321 | Rysiewska et al. 2016/Grego et al. 2017        |
| Pseudamnicola chia (E. von Martens, 1889) | KT7710656        | Szarowska et al. 2016b                         |
| Pseudorientalia Radoman, 1973 – Lesvos | KJ920490        | Szarowska et al. 2014a                         |
| Radomaniola curta (Küster, 1853) | KC011814          | Falniowski et al. 2012                         |
| Sadleriana fluminensis (Küster, 1853) | KF193067          | Szarowska and Falniowski 2013b                 |
| Sadleriana robici (Clessin, 1890) | KF193071          | Szarowska and Falniowski 2013b                 |
| Salenhythrobia ferreri Wilke, 2003 | AF449213          | Wilke 2003                                     |
| Sarajana apfelbecki (Brancsik, 1888) | MN031432          | Hofman et al. 2019                             |
| Tanousia zrmanjae (Brusina, 1866) | KU041812          | Beran et al. 2015                              |
| Tricula sp. Benson, 1843        | AF253071          | Davis et al. 1998                             |

**Diagnosis.** Shell minute, nearly planispiral, distinguished from *K. kusceri* by its lower aperture of the shell and smaller non-glandular outgrowth on the left side of the penis, and from *K. jadertina* and *K. kareli* by its higher aperture of the shell and bigger the non-glandular outgrowth on the left side of the penis.

**Description.** Shell (Fig. 4) up to 0.77 mm high and 1.39 mm broad, nearly planispiral, whitish, translucent, thin-walled, consisted of approximately five whorls, growing rapidly and separated by moderately deep suture. Spire low and flat, body

![Figure 4. Holotype of Kerkia briani. Scale bar: 0.5 mm.](image-url)
whorl large. Aperture prosccline, nearly circular in shape, peristome complete and thin, somewhat swollen, in contact with the wall of the body whorl; umbilicus wide, with the earlier whorls visible inside. Shell surface smooth, growth lines hardly visible.

**Measurements** of holotype, sequenced, and illustrated shells: see Table 2. Shell variability slight (Fig. 5).

**Soft parts morphology and anatomy.** Body white, without pigment, with no eyes. The ctenidium with twelve short lamellae, osphradium short and broad. Rectum forming characteristic broad loop (Fig. 6A). The female reproductive organs (Fig. 6A, B) with
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### Table 2.
Shell measurements (in mm) of *Kerkia briani*. For explanation of the symbols A–H, see Fig. 3.

|      | A    | B    | C    | D    | E    | F    | G    | H    |
|------|------|------|------|------|------|------|------|------|
| holotype | 0.77 | 1.39 | 0.87 | 0.62 | 0.60 | 1.09 | 0.41 | 0.97 |
| 2D44  | 0.72 | 1.12 | 0.73 | 0.54 | 0.55 | 0.93 | 0.37 | 0.75 |
| 2F59  | 0.73 | 1.26 | 0.82 | 0.54 | 0.55 | 0.95 | 0.41 | 0.80 |
| 2F62  | 0.72 | 1.35 | 0.86 | 0.57 | 0.57 | 1.03 | 0.36 | 0.73 |
| 2F70  | 0.67 | 1.12 | 0.72 | 0.52 | 0.48 | 0.97 | 0.40 | 0.72 |
| 2F71  | 0.75 | 1.37 | 0.85 | 0.46 | 0.60 | 1.02 | 0.41 | 0.84 |
| M     | 0.73 | 1.27 | 0.81 | 0.54 | 0.56 | 1.00 | 0.39 | 0.80 |
| SD    | 0.034 | 0.123 | 0.067 | 0.053 | 0.044 | 0.059 | 0.023 | 0.094 |
| Min   | 0.67 | 1.12 | 0.72 | 0.46 | 0.48 | 0.93 | 0.36 | 0.72 |
| Max   | 0.77 | 1.39 | 0.87 | 0.62 | 0.60 | 1.09 | 0.41 | 0.97 |

**Figure 6.** Renal and pallial section of the female reproductive organs of *Kerkia briani*: **A** the loop of oviduct in its normal position and the loop of the rectum **B** the loop of oviduct moved to show the receptaculum seminis and duct of bursa. Abbreviations: bc – bursa copulatrix, cbc – duct of bursa, ga – albuminoid gland, gn – nidamental gland, gp – gonoporus, ov – oviduct, ovl – loop of renal oviduct, rec – rectum, rs – receptaculum seminis. Scale bars: 1 mm.

*a long, moderately broad loop of renal oviduct and relatively big spherical bursa copulatrix (Fig. 6A) with a long bent duct (Fig. 6B), and one distal receptaculum seminis, long and worm-shaped. The penis (Fig. 7) elongated triangular, with a rather sharp tip and small non-glandular outgrowth on its left side, the vas deferens inside running in zigzags.*

**Derivatio nominis.** The specific epithet *briani* refers to our friend Brian Lewarne, Honorary Science Officer of The Devon Karst Research Society, and the Director for “Proteus Project in the Trebišnjica River Basin”, deeply devoted to the protection of *Proteus* as well as the study and protection of the subterranean habitats in Bosnia and Herzegovina.
Figure 7. Penis of *Kerkia briani*. Abbreviation: vd – vas deferens.Scale bar: 1 mm.

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

**Molecular distinctness and relationships of *Kerkia briani***

We obtained six new sequences of COI (479 bp, GenBank Accession Numbers MT780191–MT780196), and six new sequences of H3 (309 bp, GenBank Accession Numbers MT786730–MT786735). The tests by Xia et al. (2003) for COI and H3 revealed no saturation. Phylograms were constructed for COI, H3 and for combined COI-H3 dataset. In all analyses, the topologies of the resulting phylograms were identical in both the ML and BI. The ABGD and PTP approaches gave the same results (Fig. 8).

The sequences of the *Kerkia briani* formed distinct clade on COI, H3 as well as combined phylograms (Fig. 8). At the same time all *Kerkia* sequences formed distinct lineage with five different species. The p-distance of *Kerkia briani* with other *Kerkia* species varied from 0.123 to 0.146 for COI and from 0.007 to 0.023 for H3 (Table 2). The sister clade of *Kerkia* (bootstrap support 98%) were *Islamia* Radoman, 1973, *Ponto belgrandiella* Radoman, 1978, *Belgrandiella* Wagner, 1927, *Montenegrospeum* Pešić & Glöer, 2013, *Hauffenia* Pollonera, 1898, and *Agrafia* Szarowska & Falniowski, 2011 (Fig. 8, the tree for concatenated COI and H3 sequences).
Figure 8. Maximum Likelihood tree inferred from mitochondrial COI. Bootstrap supports above 60% with corresponding Bayesian probabilities are given.
Table 3. p-distances between *Kerkia* mOTUs for the COI (below diagonal) and H3 genes.

|         | mOTU – A | mOTU – B | mOTU – C | mOTU – D | mOTU – E |
|---------|----------|----------|----------|----------|----------|
| mOTU – A | –        | 0.010    | 0.007    | 0.010    | 0.023    |
| mOTU – B | 0.135    | –        | 0.017    | 0.020    | 0.033    |
| mOTU – C | 0.126    | 0.124    | –        | 0.010    | 0.023    |
| mOTU – D | 0.146    | 0.138    | 0.124    | –        | 0.013    |
| mOTU – E | 0.123    | 0.110    | 0.095    | 0.093    | –        |

**Discussion**

Following the terminology of Hershler and Ponder (1998), the habitus of the shell of *Kerkia* is depressed valvatiform (trochiform) or just planispiral. However, the latter term should not be used, since there is no planispiral shell in any recent gastropod (e.g., Falniowski 1993). The ctenidium, osphradium, and loop of oviduct are as in the other species of *Kerkia* (Bodon et al. 2001; Beran et al. 2014). The female reproductive organs are also typical of *Kerkia* (Radoman 1978, 1983; Bodon et al. 2001; Beran et al. 2014). The single receptaculum seminis is situated distally, in the position of rs1 after Radoman (1973, 1983). The penis is similar to that described and drawn by Radoman (1978, 1983), Bodon et al. (2001), and Beran et al. (2014), but the outgrowth of its left side in *K. briani* is smaller than in *K. kusceri*, but larger than that in *K. jadertina* and *K. kareli* (in the latter the outgrowth is nearly vestigial).

Falniowski (1987) demonstrated high variability of the shell, but also of the morphology and anatomy of the soft parts in the Truncatelloidea. In the latter, miniaturisation is one more a source of slight morphological diversity, decreasing the number of possible taxonomically useful characters (Falniowski 2018); in this regard, Szarowska and Falniowski (2008) stressed the narrow limits of morphology-based taxonomy within the Truncatelloidea. On the other hand, Szarowska (2006) demonstrated that such simple structures like the outgrowths on the penis and bursae/receptacula in the female reproductive organs are surprisingly evolutionary stable in position, although not in size and shape, whose variability – physiologically, ontogenetically, and artifactually (as a result of fixation of the snails) based – is striking. Moreover, problems can increase with taxa living in habitats of limited accessibility (such as caves and/or interstitial habitats) for which molecular studies often reveal numerous species but only a few or single living specimens of each species could be found. Thus, the anatomy is basic in distinction of the families and even genera, but the stable and reliable differences between congeneric species are hardly observable. However, the molecular distinctness of *Kerkia briani* is clear.

Finally, it has to be pointed out that *K. briani* inhabits the southernmost locality of *Kerkia*, expanding the range of the genus ca. 190 km ESE.

**Acknowledgments**

The study was supported by a grant from the National Science Centre 2017/25/B/ NZ8/01372 to AF.
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