

SARAJANA RADOMAN, 1975 (CAENOGASTROPODA: TRUNCATELLOIDEA): PREMATURE INVALIDATION OF A GENUS

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Abstract *Sarajana apfelbecki* (Brancsik, 1888) was assigned by Radoman to the monotypic genus, closely related to *Belgrandiella* Wagner, 1927. Later the distinctness of the genus *Sarajana* was questioned, and *S. apfelbecki* classified within the genus *Belgrandiella*. Our study on *Sarajana* from five localities in Bosnia and Herzegovina including Vrelo Bosne (type locality of the *Sarajana apfelbecki*) confirms that the morphology of the characteristic penis, as well as the female reproductive organs, with an exception of the presence of small, vestigial proximal seminal receptacle, overlooked by Radoman contradict a close relationships with *Belgrandiella*. Two molecular markers: mitochondrial cytochrome c oxidase subunit I (COI) and nuclear histone 3 (H3) were used to infer phylogeny: *Sarajana* was placed within the Hydrobiidae, Sadlerianinae, but far from *Belgrandiella*, with *Graecoarganiella Falniowski et Szarowska*, 2011 as a sister taxon.

Key words Female reproductive organs, penis, cytochrome c oxidase subunit I (COI), histone H3, molecular phylogeny

INTRODUCTION

Radoman (1975) described the new genus *Sarajana* for *Frauenfeldia lacheineri* var. *apfelbecki* Brancsik, 1888, its type locality: a spring close to Vrelo Bosne, near Sarajevo, Bosnia and Herzegovina (Fig. 1). Previously these gastropods were classified as the representatives of *Belgrandiella* Wagner, 1927 (Schütt, 1959, Jaeckel, 1967), and Radoman (1975, 1983) still classified the species within the subfamily Belgrandiellinae, close to *Belgrandiella* and *Graziana* Radoman, 1975. In World Register of Marine Species – WoRMS (2018) (MolluscaBase, 2019) genus *Sarajana* is recognised as invalid, and *S. apfelbecki* assigned to the genus *Belgrandiella*, which follows Boeters, Glöer & Slavevska Stamenković (2017). Radoman (1975, 1983) listed four subspecies of *S. apfelbecki*, based on slight differences in shell characters coupled with geographic distribution. However, considering several reports on the

biodiversity overestimation in the Hydrobiidae (e.g. Wilke & Falniowski, 2001; Falniowski & Beran, 2015; Osikowski, Hofman, Rysiewska, Sket, Prevorčnik & Falniowski, 2018), with the present state of the arts it seems better to treat the genus *Sarajana* as monotypic. Recently we collected a few specimens of *S. apfelbecki*, which has made possible: (1) the assignment of the collected populations to *Sarajana* based on molecular and morphological characters; (2) to assess its distinctiveness with *Belgrandiella* following the same criteria; (3) to infer their phylogenetic position within the Hydrobiidae.

MATERIAL AND METHODS

40 specimens of *Sarajana* were collected from four springs situated in mountainous middle Dinaric region in the northwestern part of Bosnia and Herzegovina, and from Vrelo Bosne spring, the type locality of the *Sarajana apfelbecki* (Fig. 1):

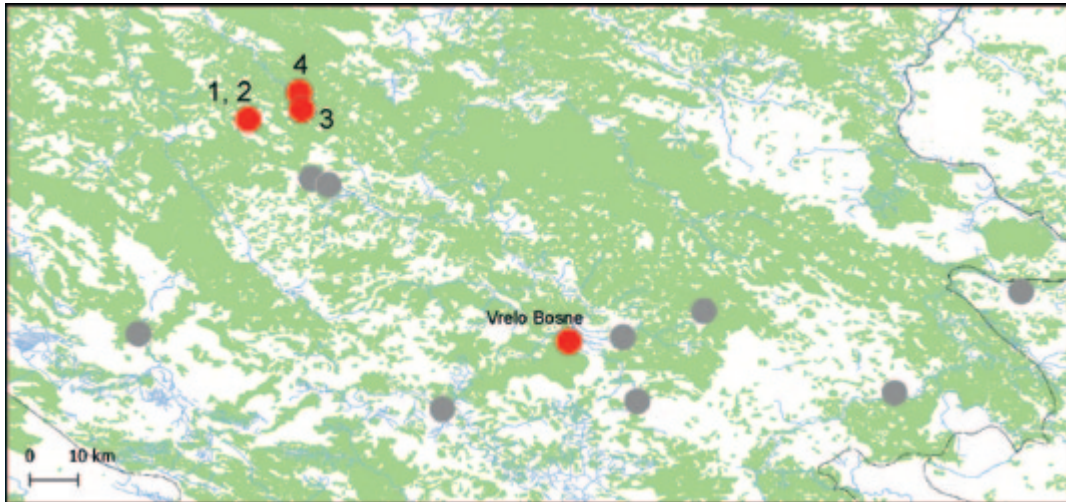


Figure 1 Map showing the localities of *Sarajana apfelbecki* listed by Radoman (1983), grey dots, and our localities, red dots



Figure 2 Photos of studied localities: a – Melina 1, b – Melina 2, c – Prisočka, d – Krusevo Brdo, e – Vrelo Bosne

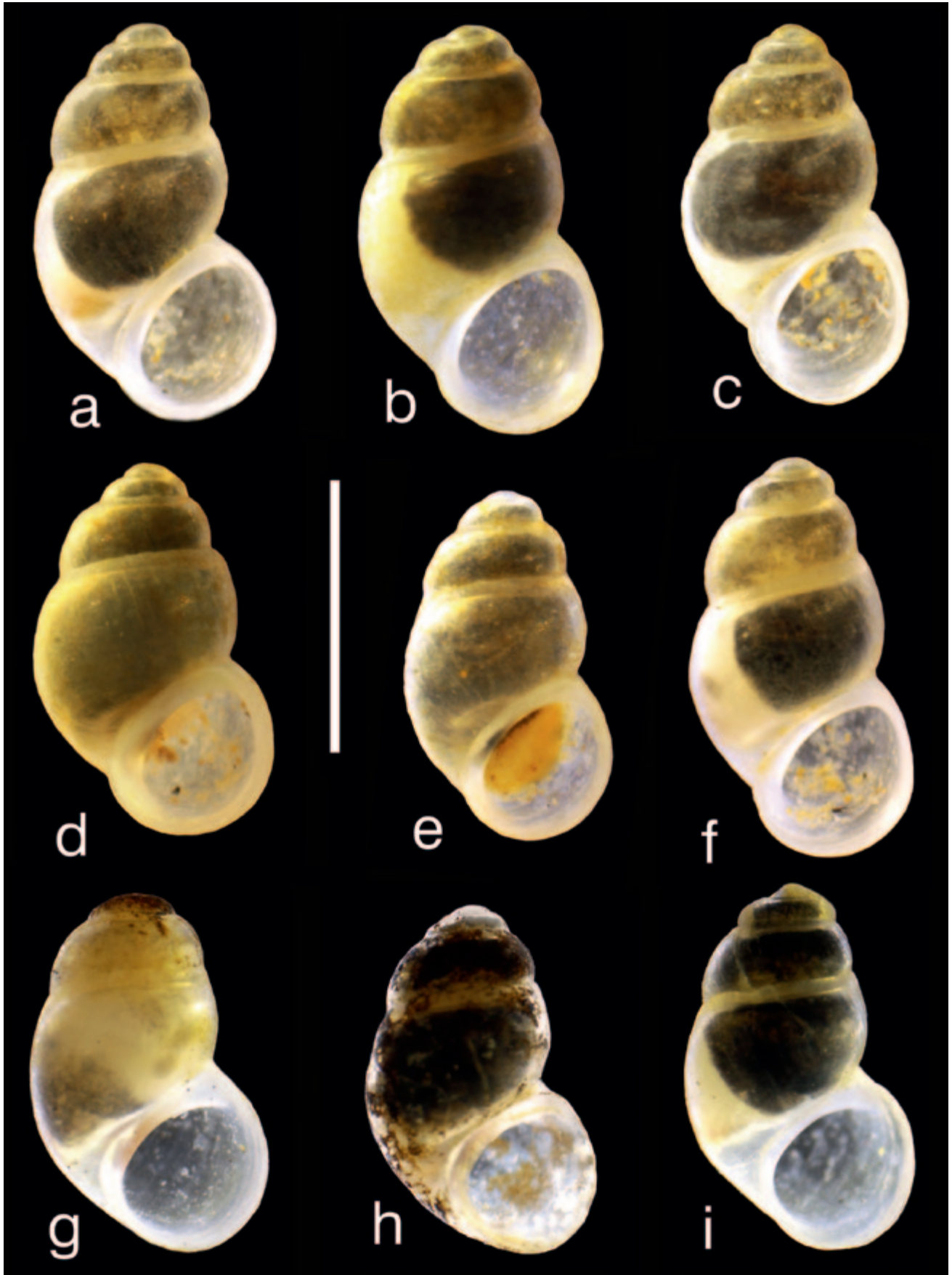


Figure 3 Shells of *Sarajana apfelbecki*: a-f – Vrelo Bosne, type locality; g-i – locality Melina 1; bar equals 500 μm

1 – Melina 1 (Fig. 2a); 44°N 23'3" 17°E 27'50"; 597m; 18.03.2017; ten specimens; extraction number – 1I7;

2 – Melina 2 (Fig. 2b); 44°N 23'10" 17°E 27'39"; 591m; 18.03.2017; 15 specimens; extraction number – 1I8;

3 – Prisocka (Fig. 2c); 44°N 27'19" 17°E 35'13"; 540m; 01.07.2017; one specimen; extraction number – 1I13;

4 – Krusevo Brdo (Fig. 2d); 44°N 24'38" 17°E 35'28"; 688m; 01.07.2017; eight specimens; extraction number – 1I14;

5 – Vrelo Bosne (Fig. 2e); 43°N 52'21" 18°E 25'26"; six specimens, extraction numbers – 2B16, 2B17.

Springs 1 and 2 belong to Ugar river basin, while springs 3 and 4 are from upper part of Vrbanja river basin. With the exception of spring 4, which is rheocrene, all the other springs are in varying degrees adjusted for water supply, with pipes.

The shells were photographed with a CANON EOS 50D digital camera, under a NIKON SMZ18 microscope with dark field. Dissections were done under NIKON SMZ18 microscope, and photographed with dark field.

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 µl TE

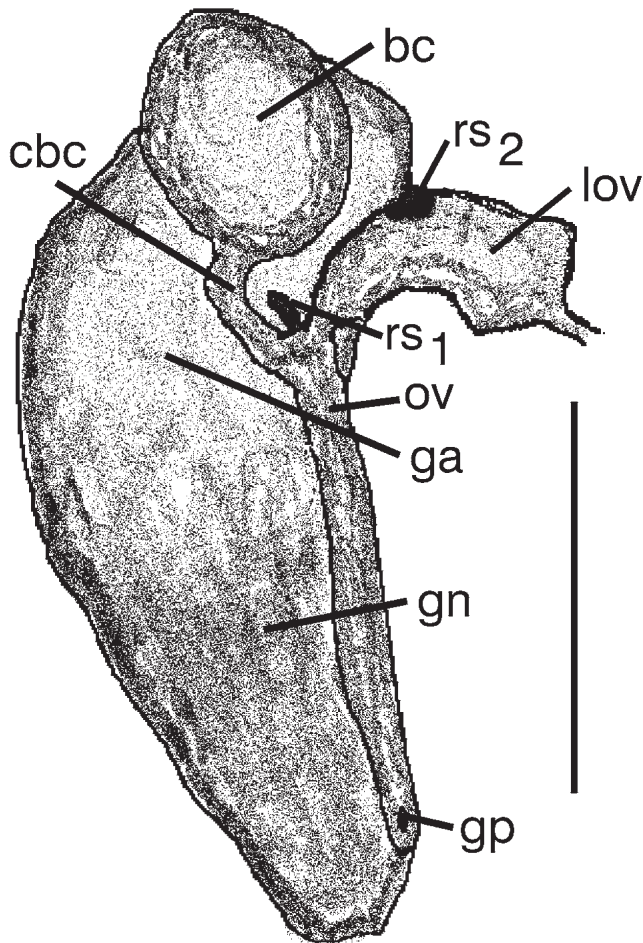


Figure 4 Renal and pallial section of the female reproductive organs of *Sarajana apfelbecki* from the locality Melina 2 (bc – bursa copulatrix, cbc – duct of bursa, ga – albuminoid gland, gn – nidamental gland, gp – gonoporus, ov – oviduct, ovl – loop of (renal) oviduct, rs₁ – distal seminal receptacle, rs₂ – proximal seminal receptacle); bar equals 500 µm

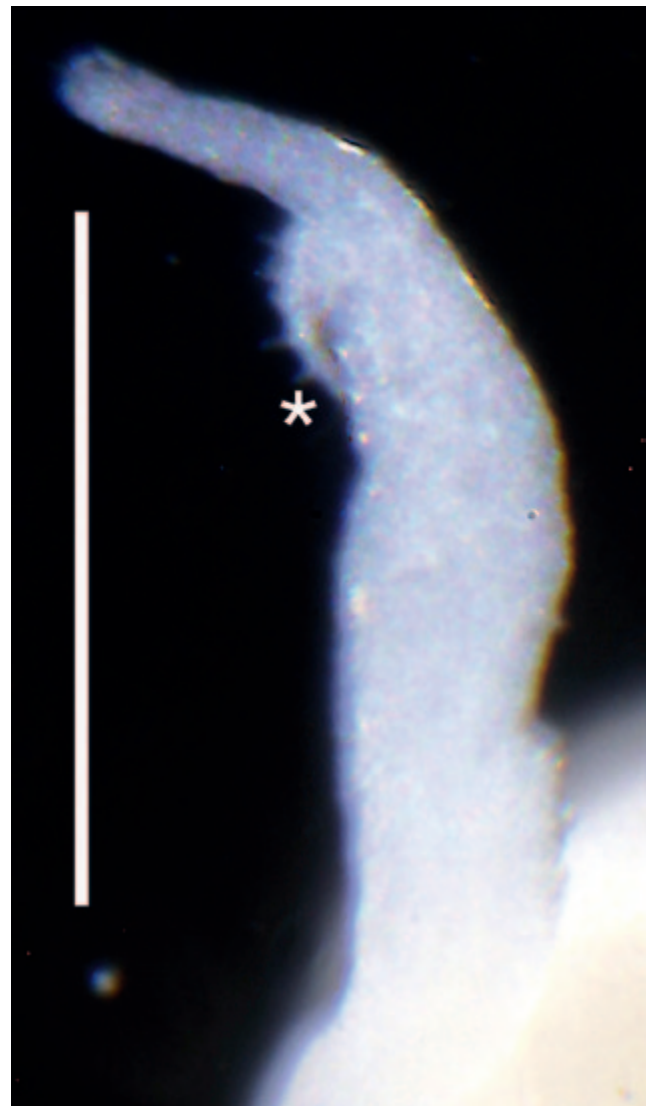


Figure 5 Penis of *Sarajana apfelbecki* from the locality Melina 2, asterisk indicates filamentous outgrowth, bar equals 200 µm

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

Species	COI/H3 GB numbers	References
<i>Agrafia wiktoria</i> Szarowska & Falniowski, 2011	JF906762/MG543158	Szarowska & Falniowski, 2011/ Grego <i>et al.</i> , 2017
<i>Alzoniella finalina</i> Giusti & Bodon, 1984	AF367650	Wilke <i>et al.</i> , 2001
<i>Anagastina zetavalis</i> (Radoman, 1973)	EF070616	Szarowska, 2006
<i>Avenionia brevis berenguieri</i> (Draparnaud, 1805)	AF367638	Wilke <i>et al.</i> , 2001
<i>Belgrandiella cf. kusceri</i> (Wagner, 1914)	KT218511/MG551366	Falniowski & Beran, 2015/ Osikowski <i>et al.</i> , 2018
<i>Belgrandiella cf. kuesteri</i> (Boeters, 1970)	MG551411	Osikowski <i>et al.</i> , 2018
<i>Bithynia tentaculata</i> (Linnaeus, 1758)	AF367643	Wilke <i>et al.</i> , 2001
<i>Bythinella austriaca</i> (von Frauenfeld, 1857)	JQ639858	Falniowski <i>et al.</i> , 2012b
<i>Bythinella micherdzinskii</i> Falniowski, 1980	JQ639854	Falniowski <i>et al.</i> , 2012b
<i>Bythiospeum acicula</i> (Hartmann, 1821)	KU341350/MK609536	Richling <i>et al.</i> , 2016/ Falniowski <i>et al.</i> , 2019
<i>Bythiospeum alzense</i> Boeters, 2001	KU341354	Richling <i>et al.</i> , 2016
<i>Dalmatinella fluviatilis</i> Radoman, 1973	KC344541	Falniowski & Szarowska, 2013
<i>Daphniola lousi</i> Falniowski & Szarowska, 2000	KM887915	Szarowska <i>et al.</i> , 2014a
<i>Emmericia expansilabris</i> Bourguignat, 1880	KC810060	Szarowska & Falniowski, 2013a
<i>Fissuria boui</i> Boeters, 1981	AF367654	Wilke <i>et al.</i> , 2001
<i>Graecoarganiella parnassiana</i> Falniowski & Szarowska, 2011	JN202352	Falniowski & Szarowska, 2011a
<i>Graecoarganiella</i> sp. Falniowski & Szarowska, 2011	JN202354	Falniowski & Szarowska, 2011a
<i>Graziana alpestris</i> (Frauenfeld, 1863)	AF367641	Wilke <i>et al.</i> , 2001
<i>Grossuana codreanui</i> (Grossu, 1946)	EF061919	Szarowska <i>et al.</i> , 2007
<i>Hauffenia tellinii</i> (Pollonera, 1898)	KY087861	Rysiewska <i>et al.</i> , 2017
<i>Hauffenia michleri</i> Kuščer, 1932	KY087865/KY087878	Rysiewska <i>et al.</i> , 2017
<i>Heleobia dobrogica</i> (Grossu & Negrea, 1989)	EU938131	Falniowski <i>et al.</i> , 2008
<i>Heleobia maltzani</i> (Westerlund, 1886)	KM213723/ MK609534-MK609535	Szarowska <i>et al.</i> , 2014b/ Falniowski <i>et al.</i> , 2019
<i>Horatia klecakiana</i> Bourguignat 1887	KJ159128	Szarowska & Falniowski, 2014a
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF278808	Wike <i>et al.</i> , 2000
<i>Iglica cf. gracilis</i> (Clessin, 1882)	MH720989/MH721004	Hofman <i>et al.</i> , 2018
<i>Iglica cf. hauffeni</i> (Brusina, 1886)	-/MH720995	Hofman <i>et al.</i> , 2018
<i>Iglica cf. forumjuliana</i> (Pollonera, 1887)	-/MH721006	Hofman <i>et al.</i> , 2018
<i>Iglica hellenica</i> Falniowski & Sarbu, 2015	KT825581/MH721007	Falniowski & Sarbu, 2015/ Hofman <i>et al.</i> , 2018
<i>Islamia zermanica</i> (Radoman, 1973)	KU662362/MG551320	Beran <i>et al.</i> , 2016/Grego <i>et al.</i> , 2017
<i>Kerkia jadertina</i> (Kuščer, 1933)	KY087868	Rysiewska <i>et al.</i> , 2017
<i>Lithoglyphus prasinus</i> (Küster, 1852)	JX073651	Falniowski & Szarowska, 2012
<i>Littorina littorea</i> (Linnaeus, 1758)	KF644330/KP113574	Layton <i>et al.</i> , 2014/ Neretina 2014, unpublished
<i>Marstoniopsis insubrica</i> (Küster, 1853)	AF322408	Falniowski & Wilke, 2001
<i>Moitessieria cf. puteana</i> Coutagne, 1883	AF367635/MH721012	Wilke <i>et al.</i> , 2001/Hofman <i>et al.</i> , 2018
<i>Montenegrospeum bogici</i> (Pešić & Glöer, 2012)	KM875510/MG880218	Falniowski <i>et al.</i> , 2014/Grego <i>et al.</i> , 2018
<i>Paladilhiopsis bosniaca</i> (Clessin, 1910)	-/MH721020	Hofman <i>et al.</i> , 2018
<i>Paladilhiopsis bosnica</i> Bole, 1970	-/MH721021	Hofman <i>et al.</i> , 2018
<i>Paladilhiopsis grobbeni</i> Kuscer, 1928	MH720991/-	Hofman <i>et al.</i> , 2018
<i>Paladilhiopsis turrita</i> (Kuščer, 1933)	MH720992/MH721015	Hofman <i>et al.</i> , 2018
<i>Paladilhiopsis gittenbergeri</i> (A. Reischutz & P. L. Reischutz, 2008)	MH720993/MH721025	Hofman <i>et al.</i> , 2018

Table 1 Continued

Species	COI/H3 GB numbers	References
<i>Peringia ulvae</i> (Pennant, 1777)	AF118302	Wilke & Davis, 2000
<i>Pontobelgrandiella</i> sp. Radoman, 1978	KU497024/MG551321	Rysiewska <i>et al.</i> , 2016/Grego <i>et al.</i> , 2017
<i>Pseudamnicola</i> sp. Paulucci, 1878	-/KT710579	Szarowska <i>et al.</i> , 2016
<i>Radomaniola curta</i> (Küster, 1853)	KC011814	Falniowski <i>et al.</i> , 2012a
<i>Sadleriana fluminensis</i> (Küster, 1853)	KF193067	Szarowska & Falniowski, 2013b
<i>Sadleriana sadleriana</i> (Frauenfeld, 1863)	MG922569	Delicado, 2018
<i>Sadleriana robici</i> (Clessin, 1890)	KF193076	Szarowska & Falniowski, 2013b
<i>Salenthydrobia ferrerii</i> Wilke, 2003	AF449213	Wilke, 2003
<i>Tanousia zrmanjiae</i> (Brusina, 1866)	KU041812	Beran <i>et al.</i> , 2015
<i>Ecrobia maritima</i> (Milaschewitsch, 1916)	KJ406200/MG551322	Szarowska & Falniowski, 2014b/Grego <i>et al.</i> , 2017

buffer. The extracted DNA was stored at -80°C at the Department of Malacology of Institute of Zoology and Biomedical Research of the Jagiellonian University in Kraków (Poland). Mitochondrial cytochrome *c* oxidase subunit I (COI) and nuclear histone H3 loci were sequenced. Details of PCR conditions, primers used and sequencing are given in Szarowska *et al.* (2016).

Sequences were initially aligned in the MUSCLE (Edgar, 2004) program in MEGA 6 (Tamura, Peterson, Peterson, Stechner, Nei & Kumar, 2013) and then checked by eye 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, Xie, Salemi, Chen & Wang, 2003) were performed using DAMBE (Xia, 2013). In a phylogenetic analysis additional sequences from GenBank were used (Table 1). The taxa were chosen to represent all the European families of the Truncatelloidea, as well as all the main lineages of the Hydrobiidae, especially the subfamily Sadlerianinae. The data were analysed using approaches based on Bayesian inference and maximum likelihood (ML). In the BI analysis, we applied the GTR model of nucleotide substitution because an over-parameterisation was apparently less critical for the BI analyses than an underparameterisation (Huelsenbeck & Rannala, 2004). For the ML analyses, the GTR model was also applied, as implemented in RaxML (Stamatakis, 2014). The Bayesian analyses (BI) were run using MrBayes v. 3.2.3 (Ronquist, Teslenko, van der Mark, Ayres, Darling, Höhna, Larget, Liu, Suchard & Huelsenbeck, 2012) with the default 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 1000 generations. The first 25% trees were discarded as burnin. The analyses were summarised on 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, Pfeiffer & Schwartz, 2010).

RESULTS AND DISCUSSION

Morphology

The shells of *Sarajana apfelbecki* (Fig. 3a–f) resembled the ones shown and illustrated by Radoman (1975, 1983). Also the shells of *Sarajana* from the locality Melina 1 (Fig. 3g–i) and whose DNA was sequenced, resembled the ones from type locality (Vrelo Bosne). They were minute, conic (shell form after Hershler & Ponder 1998), with moderately high spire, resembling the one of *Belgrandiella*. The operculum was faded red, the mantle intensively black pigmented, the eyes big. The female reproductive organs (Fig. 4) from all the five localities, resembled the ones described and drawn by Radoman (1983): with large, nearly spherical bursa copulatrix with short and broad duct, moderately thick and rather short loop of the (renal) oviduct, and small, almost vestigial distal receptaculum seminis (rs_1). However, we

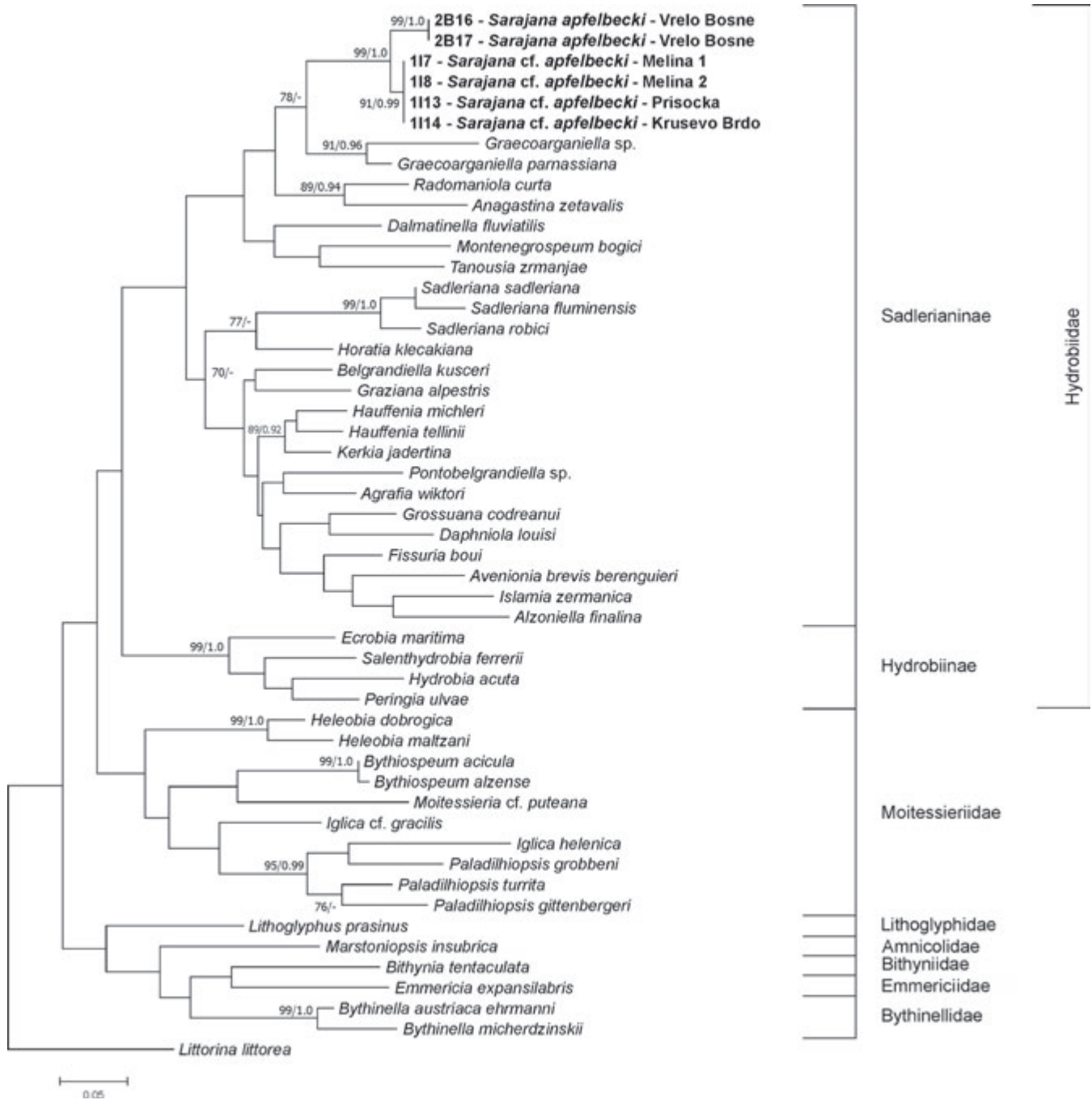


Figure 6 The maximum-likelihood tree of the COI gene. Bootstrap support and Bayesian posterior probabilities are shown

have also found, similarly vestigial, the proximal receptaculum (rs_2), overlooked by Radoman (1975, 1983) and Boeters *et al.* (2017). This structure, whose position is unexpectedly evolutionarily stable, parallelly lost in some lineages like *Belgrandiella* (Szarowska, 2006), places *Sarajana* far from *Belgrandiella*, among the genera possessing two seminal receptacles. The penis (Fig. 5), identical at all the five localities, was similar

to the one drawn by Radoman (1983), but half the size. It was characteristically long and narrow, with a filamentous outgrowth on its left side (marked with asterisk in Fig. 5). Such a penis is not characteristic for *Belgrandiella*.

Molecular relationships

In both coding loci the tests of Xia *et al.* (2003) revealed little saturation. Results from the

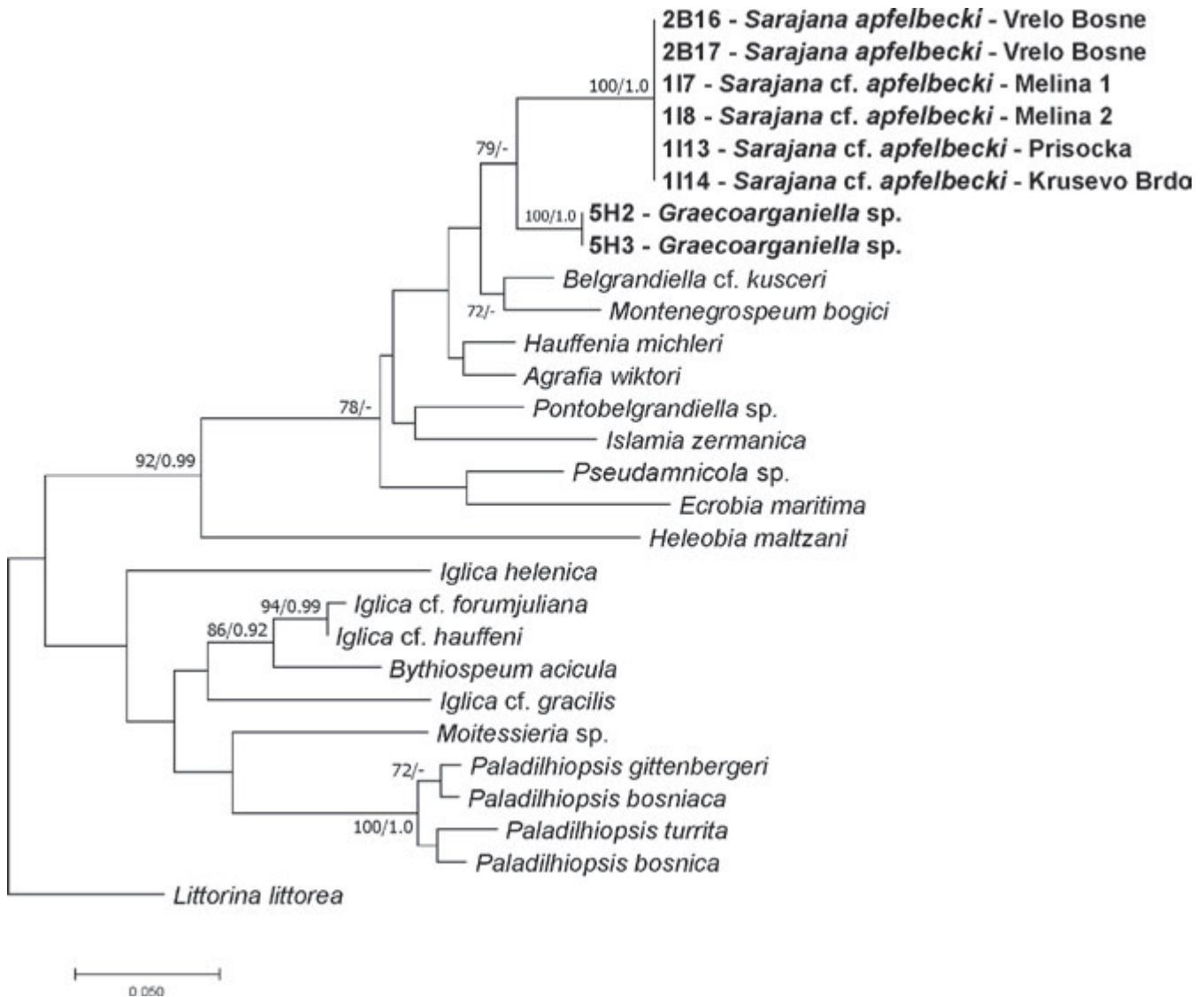


Figure 7 The maximum-likelihood tree of the H3 gene. Bootstrap support and Bayesian posterior probabilities are shown

substitution saturation analysis showed an ISS (0.72 for COI; 0.47 for H3) significantly smaller than the critical ISS value (ISSC: 0.97 for COI; 0.59 for H3), indicating that all sequences are useful in phylogenetic reconstruction

Topology of the trees obtained from BI and ML analyses were identical. In total we obtained six *Sarajana* sequences of COI (552 bp, GenBank Accession numbers MN031428–MN031433) and eight sequences of histone 3 (283 bp, GenBank Accession numbers MN031434–MN031441), six for the *Sarajana* and two for the *Graecoarganiella* sp. In the latter, for H3 we used the DNA extracts previously used by Falniowski & Szarowska, 2011a.

For both markers (Figs 6–7), sequences of *Sarajana* were closest to *Graecoarganiella*, with high bootstrap support (71 for the COI and 79 for the H3). These values were much higher for the combined tree from these two markers (96, Fig. 8). However, p-distance between these two genera was rather high: 0.137 for COI; p-distance between *Sarajana* and *Belgrandiella* was 0.176 for COI and 0.068 for H3 (compare with: Perez, Ponder, Colgan, Clark & Lydeard, 2005, Bichain, Gaubert, Samadi, Boisselier-Dubayle, 2007, Falniowski, Szarowska & Grzmil, 2007, Szarowska, Grzmil, Falniowski & Sirbu, 2007, Falniowski & Szarowska, 2011a) and 0.055 for H3. It should be noted that the

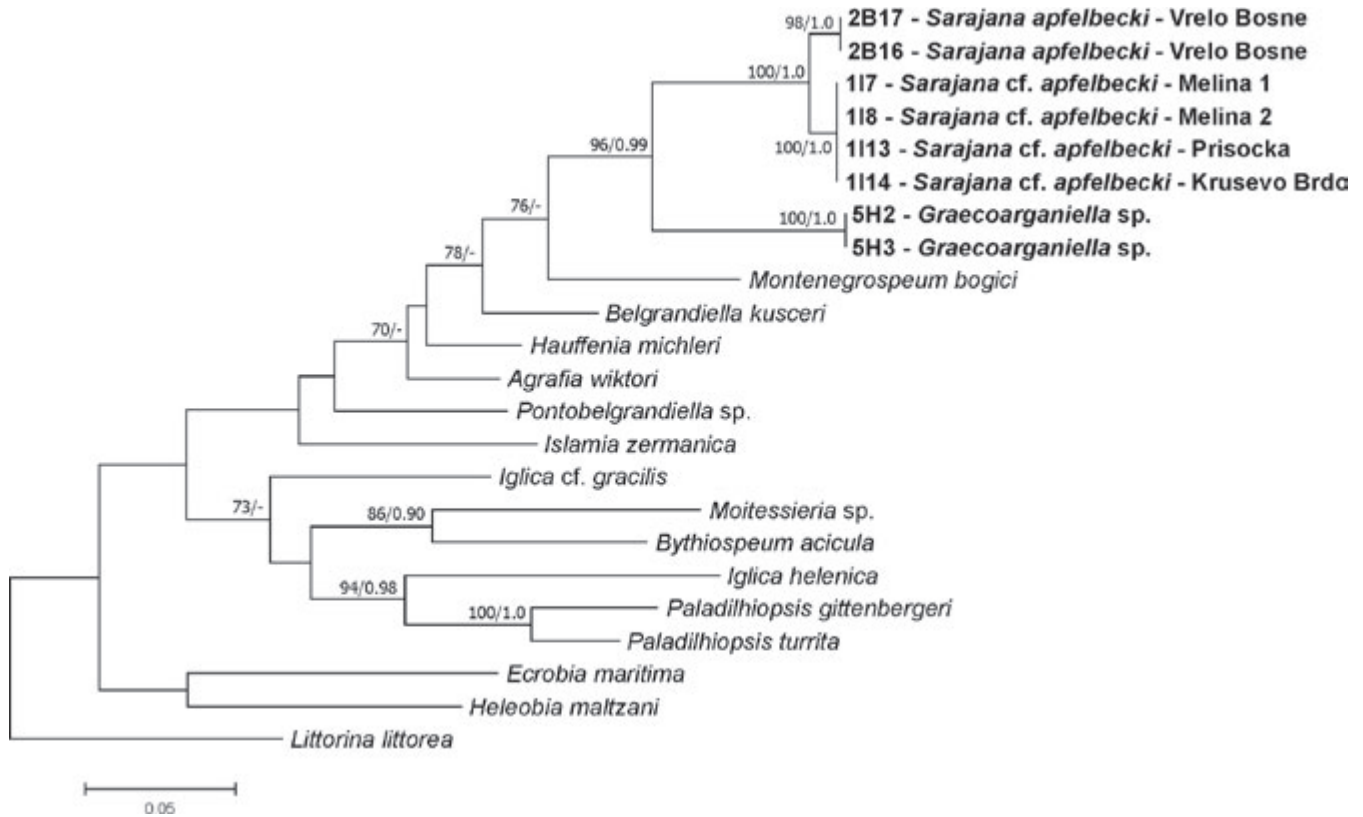


Figure 8 The maximum-likelihood tree of the COI and H3, concatenated sequences gene. Bootstrap support and Bayesian posterior probabilities are shown

morphology – including the valvatiform shell of *Graecoarganiella* Falniowski et Szarowska, 2011, lack of the ctenidium in the latter, etc. – was markedly different in the two genera. This is one more example of limited usefulness of the morphology in the phylogenetic inference in the Truncatelloidea (Szarowska & Falniowski, 2008), although the inferred sister-clade relationship is coupled with a rather high genetic distance.

In the COI tree (Fig. 6) all the deeper nodes were not supported, which is typical of this locus. In the H3 tree (Fig. 7) all the Hydrobiidae formed a well-supported (bootstrap support 78%) clade, with *Sarajana* within it. In the tree computed for the two loci (Fig. 8) there was well-supported sister-clade relationship of *Sarajana*/*Graecoarganiella* clade with *Montenegrospeum* Pešić & Glöer, 2013, and for these three taxa with *Belgrandiella*, thus the latter was not closely related with *Sarajana*.

As already stated above, the morphology of the gastropods studied in the present paper corresponded with the one of the genus *Sarajana*, as defined by Radoman (1975, 1983), with the exception of the proximal receptaculum which

was overlooked in Radoman's description. The latter clearly distinguishes *Sarajana* from *Belgrandiella*. *Sarajana* inhabits a relatively wide range in central Bosnia, from the border with Serbia in the east, to Šuica in the west (Radoman, 1985). Our samples came from the sites close to the ones listed by Radoman (1975, 1983, 1985), around 20–25km away from the type locality of *S. apfelbecki travnicensis* Radoman, 1975 (Travnik: Fig. 1). For COI, the sequences were identical in the populations at the four localities, but not identical between them and the ones from the type locality: the genetic distance $p=0.035$. In the Truncatelloidea this value is within the zone of ambiguity: such values are considered as still infraspecies in some cases, but as already higher than the threshold values estimated for some genera, like *Bythinella* Moquin-Tandon, 1855 (e.g. Bichain, Boisselier-Dubayle, Bouchet & Samadi, 2007; Szarowska, Grzmił, Falniowski & Sirbu, 2007; Falniowski, Szarowska & Grzmił, 2007; Falniowski & Szarowska, 2011b). However, considering the complete lack of the polymorphism, with the same haplotype of COI in the four

northern populations (from Melina 1, Melina 2, Prisocka and Krusevo Brdo), and with another single haplotype at the type locality of *S. apfelbecki* (Vrelo Bosne), suggests rather a species-level distinctness of the two *Sarajana*. The molecular phylogeny also clearly contradicts the status of this genus in the WoRMS as invalid (“unaccepted”) and the assignment of *S. apfelbecki* to the genus *Belgrandiella*. *Sarajana* is clearly a distinct genus, not closely related to *Belgrandiella*.

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