A NEW SPECIES OF *DALMATINELLA* RADOMAN, 1973 (CAENOGASTROPODA: HYDROBIIDAE) FROM CROATIA

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Abstract *A new species of* Dalmatinella *Radoman, 1973 from Croatia, found at three localities: Donja Rošca and Trnbusi at Cetina River and Ploče, Baćina lakes (type locality), is described. The shell, female reproductive organs and penis are described and illustrated. New species is compared with the type species of* Dalmatinella*:* D. fluviatilis *Radoman, 1973, from its type locality: Jankovića Buk, waterfalls, the Zrmanja River, Croatia. The new species is characterised by somewhat different habitus of the penis, and by the shell with higher spire and aperture. The differences are confirmed by the principal component analysis (PCA). Mitochondrial cytochrome oxidase subunit I (COI) confirmed species distinctness of the new species (p-distance 0.039).*

Key words *Gastropod, taxonomy, reproductive organs, shell biometry, PCA, mtDNA, cytochrome oxidase, Balkans*

Introduction

Dalmatinella fluviatilis Radoman, 1973, the only representative of the genus *Dalmatinella* Radoman, 1973 described so far, can probably be considered one of the endemites of the Zrmanja River, in northern Croatia (Falniowski & Szarowska, 2013). Apart from this type locality (Fig. 1), it was reported from the lowest part of the Neretva River, between Kula and Opuzen (Radoman, 1983). Considering more than 200km distance between Zrmanja and Neretva, without any records along this distance, Falniowski & Szarowska (2013) raised doubt that the same species of *Dalmatinella* could be present at both localities *Dalmatinella* could be represented by the same species. Recently, one of us (LB) collected specimens of *Dalmatinella* at three new localities, unreported in Radoman (1983). The present paper deals with the systematic status of these gastropods.

Material and methods

Snails were collected at four localities (Fig. 1: localities' numbers as in the map):

1. Donja Rošca, 43°33'44,9''N, 16°43'03,8''E, the Cetina River in the Prančevići dam reservoir, stones on the bank of the dam reservoir (Fig. 2A), 275m a.s.l., 29.8.2018, about 40 specimens;

2. Trnbusi, 43°29'22,6''N, 16°48'59,8''E, the Cetina River to the east of hill Gradina (308m a.s.l.), stones on the bank of the river (Fig. 2B), 200m a.s.l., 22.8.2019, 1 specimen;

3. Ploče, 43°04'11,8''N, 17°25'22,2''E, the S part of lake Sladinac, one stone on the bank, Baćina lakes, 9m a.s.l., 22.8.2019, about 30 specimens; type locality;

4. Jankovića Buk waterfalls, 44°12'09.8''N, 15°43'16.9''E, Zaton Obrovački, the Zrmanja River (Figs 2C–D), 9m a.s.l. 4.6.2011, about 25 specimens, the locality and materials the same as in Falniowski & Szarowska (2013).

Snails were collected with a 0.5mm sieve and fixed in 80% analytically pure ethanol, replaced two times. Next, the snails were put in fresh 80% analytically pure ethanol and kept in -20ºC 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.

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Figure 1 Geographic distribution of the localities of *Dalmatinella*: *D. simonae* – red dots (3 – type locality); *D. fluviatilis* – black dot (type locality); localities' numbers – see the text.

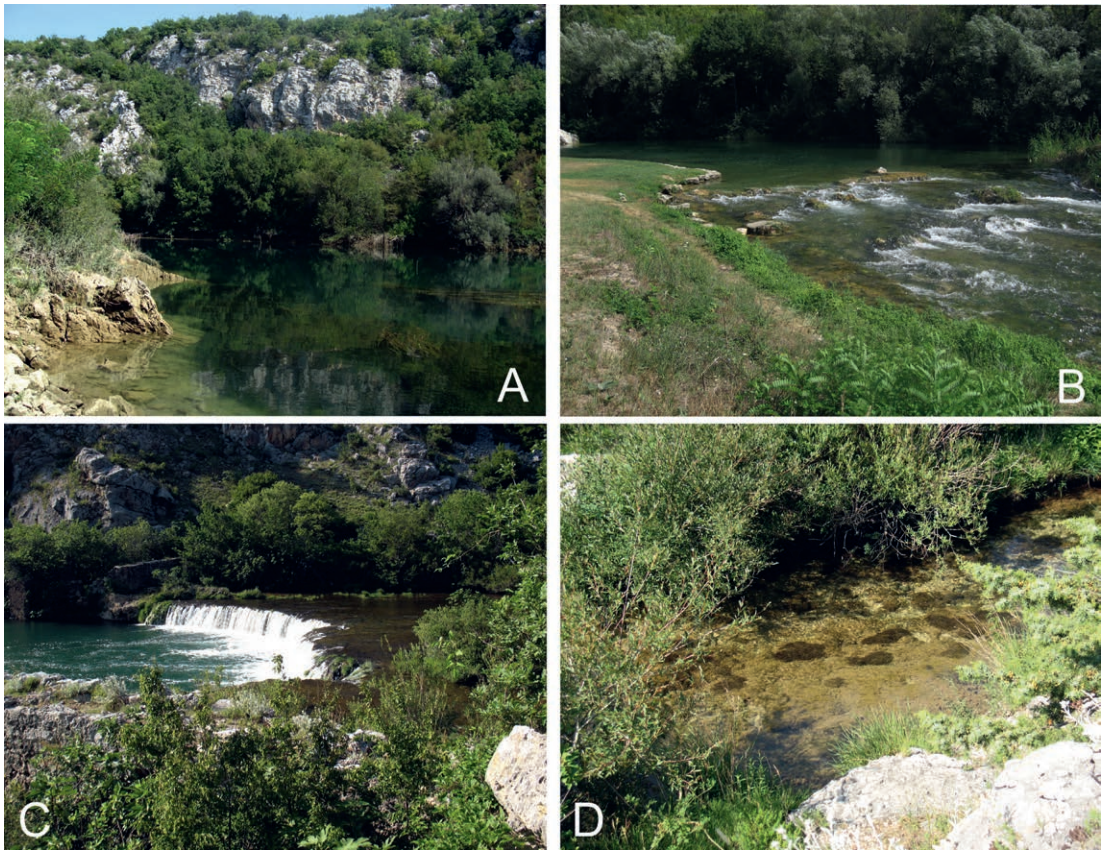

Figure 2 Localities of *Dalmatinella*: A–B – Cetina River: A – Prančevići dam reservoir, B – Trnbusi; C – Jankovića Buk waterfalls; D – Zrmanja River directly over the waterfalls, *Dalmatinella* was collected from this place.

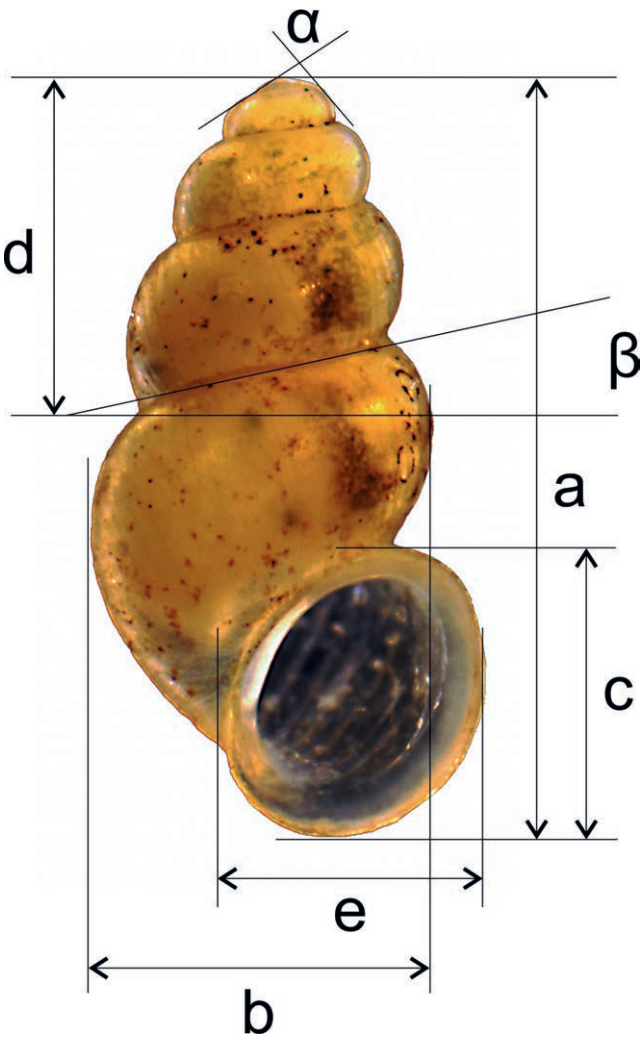

Figure 3 Morphometric measurements used in PCA analysis.

Seven morphometric parameters of the shell (Szarowska, 2006; Falniowski *et al*., 2007) were measured (Fig. 3) by one person using a Nikon DS-5 digital camera and ImageJ image analysis software (Rueden *et al*., 2017). The linear measurements were then logarithmically transformed; for angular measurements the arcsine transformation was applied. Principal component analysis (PCA), based on the matrix of correlation, was computed, applying a descriptive, non-stochastic approach. The original observations were projected into PC space, to show relationships between the specimens, without any classification given *a priori* (Falniowski, 2003; Rohlf, 1998). The transformations and PCA calculations were made by the ClustVis 2.0 (https://biit.cs.ut.ee/clustvis/) (Metsalu & Jaak, 2015). Shell character states after Hershler & Ponder (1998).

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).

A fragment of 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 estimation of the proportion of invariant sites and the saturation test for entire data sets (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). In the BI analysis, the GTR +I +Γ model of nucleotide substitution was applied. Model was selected using MrModelTest 2.3 (Nylander, 2004). The Bayesian analyses were run using MrBayes v. 3.2.3 (Ronquist *et al*., 2012) with default 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 & 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). We applied the GTR model which is the only nucleotide substitution model implemented in RaxML, whose parameters were estimated by RaxML (Stamatakis, 2014).

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

Species	COI GB numbers	References
Agrafia wiktori Szarowska et Falniowski, 2011	JF906762	Szarowska & Falniowski, 2011
Alzoniella finalina Giusti & Bodon, 1984	AF367650	Wilke *et al*., 2001
Anagastina zetavalis (Radoman, 1973)	EF070616	Szarowska, 2006
Avenionia brevis berenguieri (Draparnaud, 1805)	AF367638	Wilke *et al*., 2001
Belgrandiella kuesteri (Boeters, 1970)	MG551325	Osikowski *et al*., 2018
Ecrobia maritima (Milaschewitsch, 1916)	KX355835	Osikowski *et al*., 2016
Daphniola louisi Falniowski & Szarowska, 2000	KM887915	Szarowska *et al*., 2014a
Dalmatinella fluviatilis Radoman, 1973	KC344541-42	Falniowski & Szarowska, 2013
Fissuria boui Boeters, 1981	AF367654	Wilke *et al*., 2001
Graecoarganiella parnassiana Falniowski & Szarowska, 2011	JN202352	Falniowski & Szarowska, 2011b
Graziana alpestris (Frauenfeld, 1863)	AF367641	Wilke *et al*., 2001
Grossuana angeltsekovi Glöer & Georgiev, 2009	KU201090	Falniowski *et al*., 2016
Hauffenia tellinii (Pollonera, 1898)	KY087861	Rysiewska *et al*., 2017
Horatia klecakiana Bourguignat 1887	KJ159128	Szarowska & Falniowski, 2014
Islamia zermanica (Radoman, 1973)	KU662362	Beran *et al*., 2016
Montenegrospeum bogici (Pešić & Glöer, 2012)	KM875510	Falniowski *et al*. 2014
Pontobelgrandiella sp. Radoman, 1978	KU497024	Rysiewska *et al*., 2016
Pseudorientalia Radoman, 1973	KJ920477	Szarowska *et al*., 2014b
Radomaniola curta (Küster, 1853)	KC011814	Falniowski *et al*., 2012a
Sadleriana fluminensis (Küster, 1853)	KF193067	Szarowska & Falniowski 2013
Tanousia zrmanjae (Brusina, 1866)	KU041812	Beran *et al*., 2015

SYSTEMATICS

Family Hydrobiidae Stimpson, 1865

Subfamily Sadlerianinae Szarowska, 2006

Genus *Dalmatinella* Radoman, 1973

Dalmatinella simonae Beran et Rysiewska sp. n.

urn:lsid:zoobank.org:act:853CA97A-78CC-46E9-998C-48DB998E7AFB

Holotype Ethanol-fixed specimen (Fig. 3), collected at Ploče, 43°04'11,8''N, 17°25'22,2''E, the S part of lake Sladinac, one stone on the bank, Baćina lakes (Fig. 1 – locality 1), Museum of Natural History of the University of Wrocław, Poland, voucher number MNHW-1364.

Paratypes 20 ethanol-fixed specimens, collected at Ploče, 43°04'11.8''N, 17°25'22.2''E, the S part of Lake Sladinac, one stone on the bank, Baćina lakes, voucher number MNHW-1364.

Diagnosis Shell small, ovate-conic or conic, distinguished from similarly high and broad *D. fluviatilis* by proportionally higher spire, aperture higher and slightly broader, body whorl lower, and by the penis with smaller outgrowths.

Description *Shell* (Figs 3–4) ovate-conic or conic, up to 2.15mm tall, 4–5 whorls growing regularly, spire height approximately 86% width of shell. Holotype measurements: Table 2. Teleoconch whorls highly convex, evenly rounded. Aperture broad, ovate, very weakly angled ad apically, peristome continuous, in its upper part adjacent to body whorl. Parietal lip complete, adnate, umbilicus small and partly covered by lip. Outer lip fluted, orthocline. Shell glossy with very slightly marked growth lines, thin and translucent, periostracum whitish or pinkish.

Measurements of holotype and shells of the sequenced specimens: Table 2. Shell variability slight. Principal component analysis (PCA) of the measurements given in Table 2 (Fig. 5), of the shells presented in Fig. 4, illustrates variability and morphological distinctness of the two species of *Dalmatinella*, at all the four localities.

Soft parts morphology and anatomy Body yellowish with rather strong black pigmentation. Female reproductive organs (Fig. 6) with long and weakly swollen loop of oviduct, moderately large, spherical bursa copulatrix and two receptacula seminis, proximal (rs_2) big and distal (rs_1)

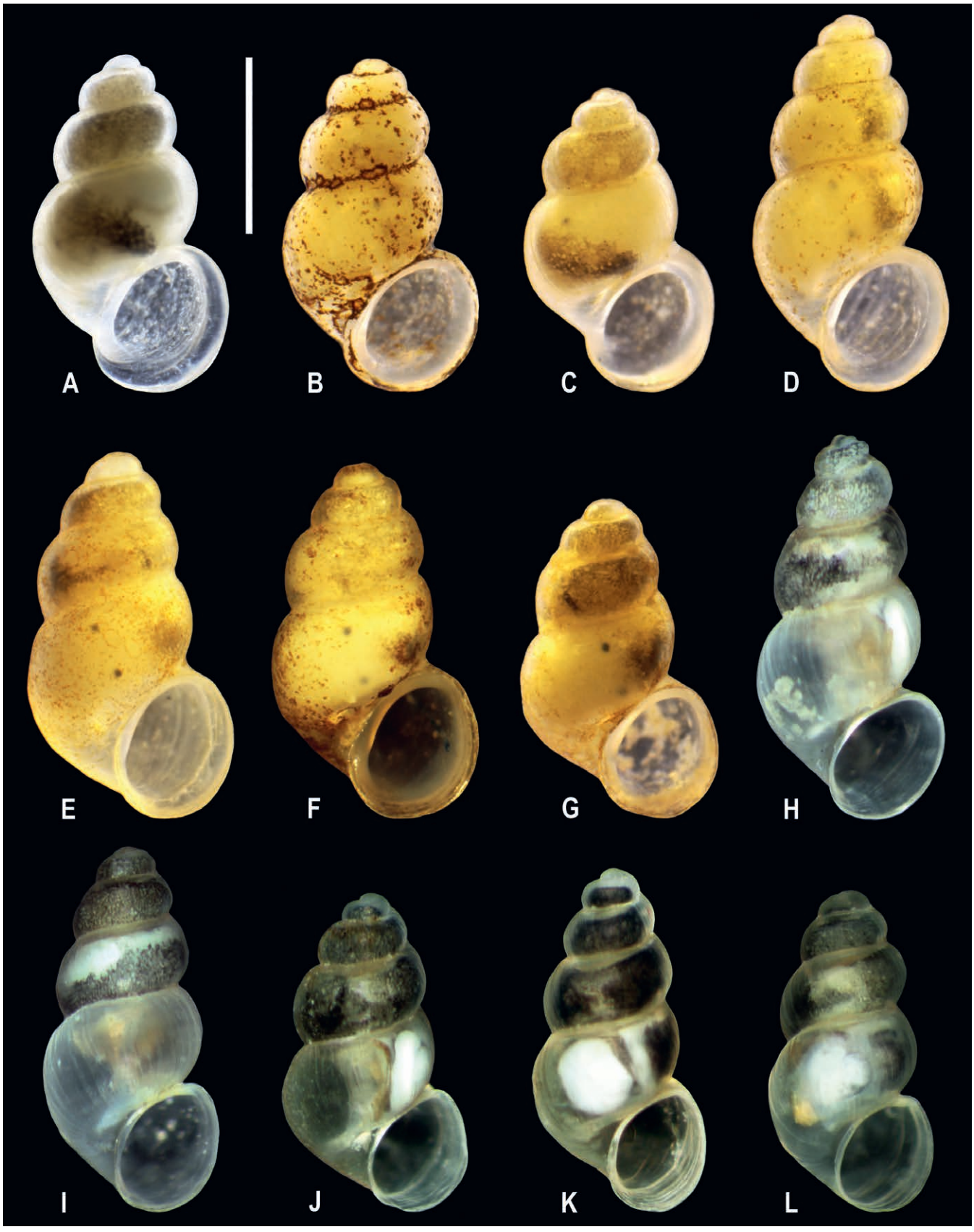

Figure 4 Shell variability of *Dalmatinella*: A–G – *D. simonae* n. sp. (A – holotype; B–G – specimens whose haplotypes are the same as in Fig. 8: site 1: B – 2E23, C – 2E24, D – 2E22; site 2: E – 1Z25, F – 1Z26; site 3: G – 2E19); H–L – *Dalmatinella fluviatilis* Radoman, 1973 (after Falniowski & Szarowska, 2013). Bar equals 1mm.

Table 2 Measurements of the shell. Measured parametres as shown in Fig. 3. Holotype and 2E19 from site 3, 2E22-24 – site 1; 1Z25-26 – site 2; M – mean, SD – standard deviation

	a	b	c	d	e	α	β
Dalmatinella simonae; n=7							
Holotype	1.94	0.96	0.84	0.72	0.79	97	16
2E23	1.90	0.91	0.76	0.74	0.72	97	17
2E24	1.74	0.87	0.74	0.60	0.72	97	17
2E22	2.15	0.97	0.82	0.94	0.72	89	16
1Z25	2.10	0.95	0.81	0.91	0.68	95	15
1Z26	1.99	0.89	0.85	0.86	0.72	95	16
2E19	1.83	0.88	0.74	0.77	0.67	93	19
M	1.95	0.92	0.79	0.79	0.72	94.71	16.57
SD	0.144	0.041	0.047	0.120	0.039	2.928	1.272
Min	1.74	0.87	0.74	0.60	0.67	89	15
Max	2.15	0.97	0.85	0.94	0.79	97	19
Dalmatinella fluviatilis; n=6							
D. f. 1	2.21	0.95	0.74	1.05	0.66	96	20
D. f. 2	2.09	0.93	0.73	0.97	0.60	96	14
D. f. 3	1.93	0.90	0.70	0.87	0.59	96	16
D. f. 4	1.83	0.88	0.71	0.87	0.58	96	18
D. f. 5	1.98	0.86	0.71	0.93	0.57	97	16
D. f. 6	1.84	0.86	0.67	0.87	0.57	99	19
M	1.98	0.90	0.71	0.93	0.60	96.67	17.17
SD	0.148	0.037	0.024	0.073	0.034	1.211	2.229
Min	1.83	0.86	0.67	0.87	0.57	96	14
Max	2.21	0.95	0.74	1.05	0.66	99	20

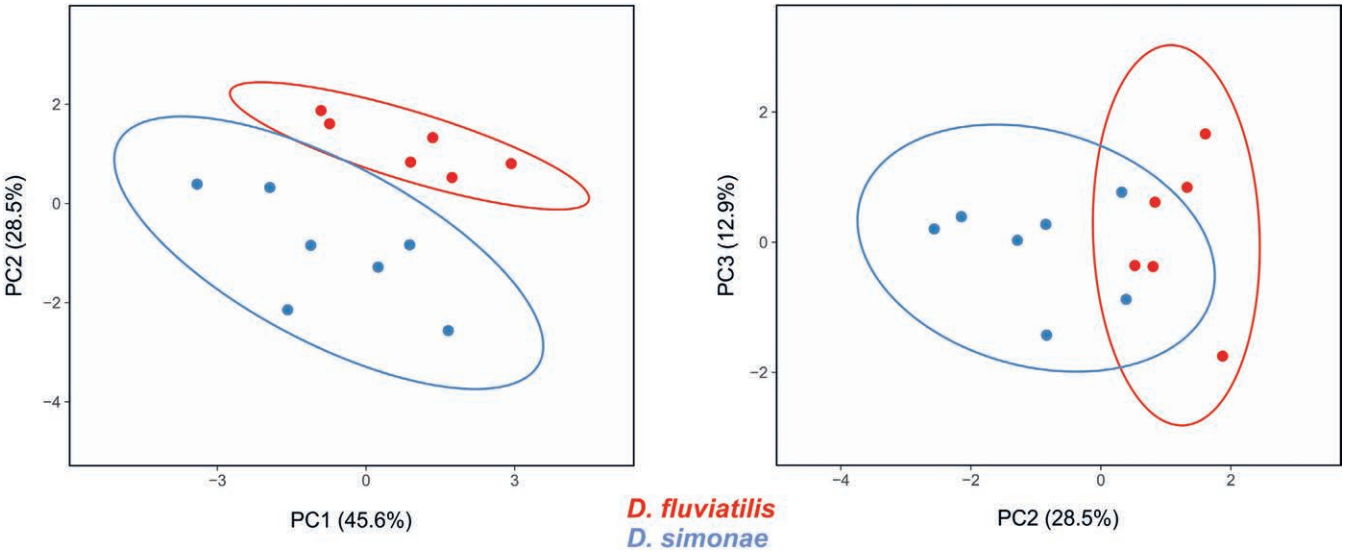

Figure 5 Principal component analysis (PCA) on the shells of *Dalmatinella* based on the measurements given in Table 2.

nearly vestigial, female organs not different from the ones of *D. fluviatilis* (Radoman 1973, 1974, 1983–Falniowski & Szarowska 2013). Penis (Fig. 7) with two small, non-glandular lobes, lobes less prominent than in *D. fluviatilis* (Radoman 1973, 1983–Falniowski & Szarowska 2013).

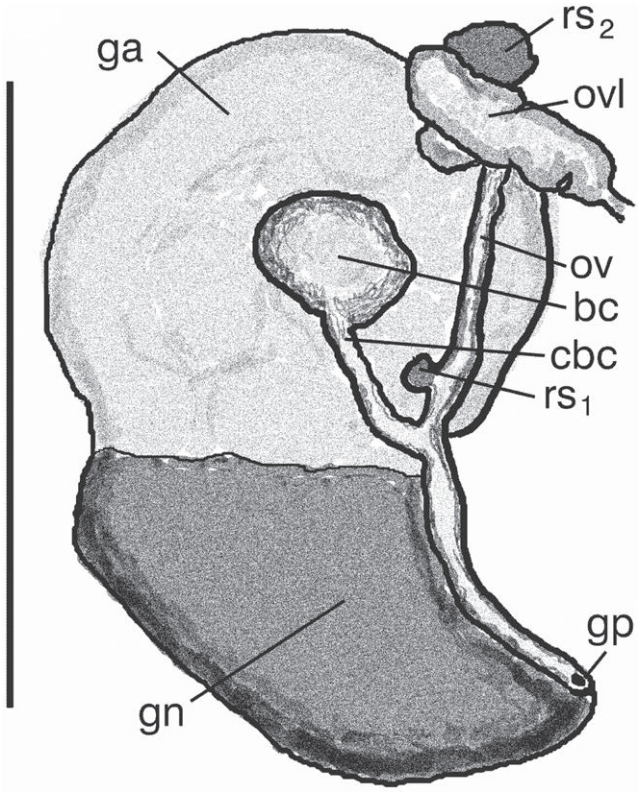

Figure 6 Renal and pallial section of the female reproductive organs of *Dalmatinella simonae*, a paratype (bc – bursa copulatrix, cbc – duct of bursa copulatrix, ga – albuminoid gland, gn – nidamental gland, gp – gonoporus, ov – oviduct, ovl – loop of (renal) oviduct, rs_1 rs_2 – receptaculum seminis, terminology after Radoman (1973): rs_1 – distal, rs_2 – proximal); bar equals 0.5mm.

Derivation of name The specific epithet *simonae* refers to Simona Prevorčnik from the University of Ljubljana, our friend and contributor to our study of stygobiont Truncatelloidea of the Balkans.

Distribution and habitat Found at type locality (Ploče, S part of lake Sladinac, Baćina lakes, Fig. 1 – site 3), but also in Cetina River (Donja Rošca and Trnbusi, Fig. 1 – sites 1 and 2), on stones of the banks of the river, dam reservoirs or lakes.

MOLECULAR DISTINCTNESS AND RELATIONSHIPS OF *DALMATINELLA SIMONAE*

We obtained six new sequences of COI (457 bp, GenBank Accession Numbers MT773271–MT773276). The tests by Xia *et al.* (2003) revealed no saturation. In all analyses, the topologies of the resulting phylograms were identical in both the ML and BI. The new sequences clearly belonged to the genus *Dalmatinella* (Fig. 8). The sequences of cytochrome oxidase COI differ by the p-distance 0.039 between *Dalmatinella simonae* and *D. fluviatilis*, confirming species distinctness of the two taxa. Within the species there was nearly no haplotype diversity, the p-distance between the haplotype from the locality 1 and the haplotype from the localities 2 and 3 (the same haplotype at the latter two localities) was only 0.003 (Fig. 8). *Dalmatinella* clearly belongs to the Sadlerianinae, but exact phylogenetic relationship with other genera is uncertain due to low bootstrap values.

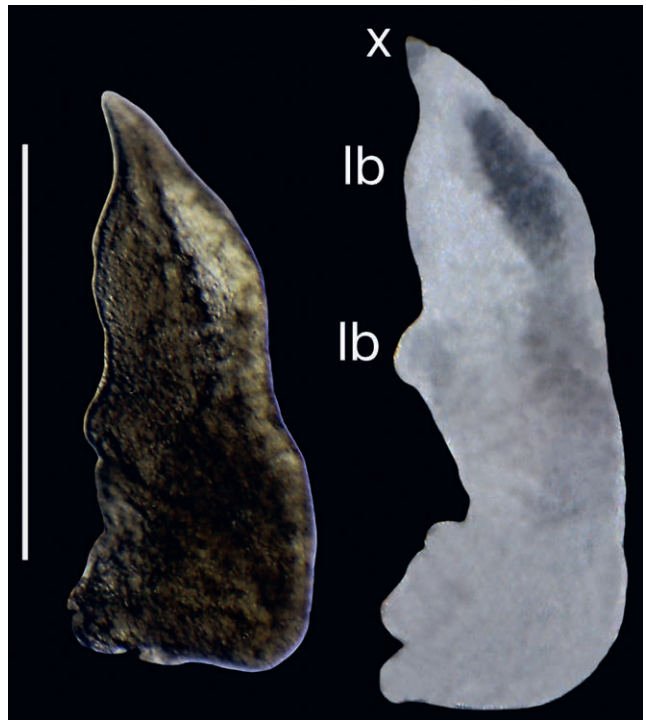

Figure 7 Penis of *Dalmatinella simonae*, two paratypes (x – tip, lb – non-glandular lobe); bar equals 0.5mm.

DISCUSSION

Falniowski (1987) in his monograph of the Polish Hydrobioidea illustrated and described the variability of the characters used in the truncatelloid taxonomy (male and female reproductive organs, ctenidium, osphradium, soft part pigmentation, etc.) and considered such characters, like the proportions of the penis, receptacula, bursa, etc., much more variable that it had been usually noticed in the literature. Szarowska (2006) reconstructed character evolution in the Balkan Truncatelloidea, and found long-lasting

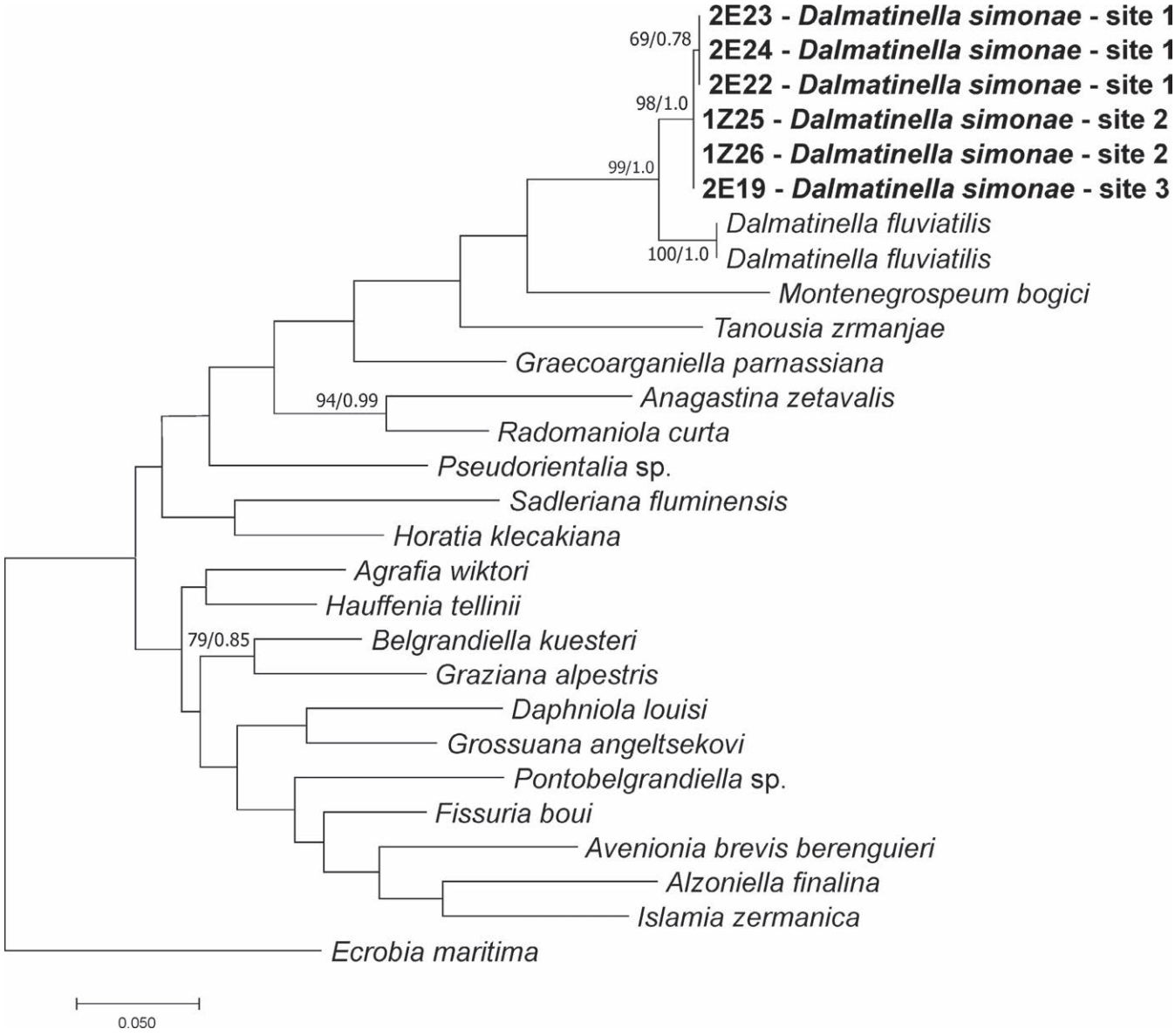

Figure 8 Phylogenetic relationships of *Dalmatinella*: maximum likelihood (ML) phylogram based on COI; *Ecrobia maritima* (Hydrobiinae) given as outgroup.

evolutionary stability of the presence and topological position of bursae, receptacula and penis outgrowths, but high variability of their shape, proportions and relative size. There are several factors shaping this variability, coupled with relative simplicity of these structures (Szarowska & Falniowski 2008–Falniowski 2018), but slight differences observed between *Dalmatinella fluviatilis* and *D. simonae* are not surprising.

Our results confirm the opinion of Falniowski & Szarowska that *Dalmatinella* inhabiting Neretva River may not belong to *D. fluviatilis*. Our findings at two localities in Cetina River suggest more continuous geographic distribution of the genus. Interestingly, the geographic distance between the most distant localities of *D. simonae* (1 and 3) is similar to the one between the westernmost locality of *D. simonae* (locality 1) and the locality inhabited by *D. fluviatilis*. At the same time, the genetic p-distance between the localities 1 and 2/3 approaches 0.003, while the p-distance between the species equals 0.039.

The above value of the genetic distance fall within the zone characteristic either of weakly marked interspecific values or high intraspecific variation, but rather characterise interspecific values (e.g. Perez *et al*., 2005; Falniowski *et al*., 2007; Szarowska *et al*., 2007; Falniowski & Szarowska,

2011a; Szarowska & Falniowski, 2013). Bichain *et al*. (2007) reported the threshold value 0.015 in the west-European *Bythinella* species. In case of our *Dalmatinella* the virtual lack of intraspecific genetic variation between three localities of *D. simonae* supports the interspecies range of the observed p-distance 0.039. Obviously, the evolution of a gene need not necessarily reflect the evolution of species (e.g. Avise, 2000), but in the case of *Dalmatinella* the genetic differences is coupled with the differences in the proportions of the shell, clearly illustrated by PCA, and by different habitus of the penis, which confirm species distinctness of the two taxa.

Acknowledgments

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