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# PHYLOGENETIC RELATIONSHIPS OF *DALMATINELLA FLUVIATILIS* RADOMAN, 1973 (CAENOGASTROPODA: RISSOOIDEA)

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ABSTRACT: Morphology of *Dalmatinella fluviatilis* Radoman, 1973, a putative endemite of the Zrmanja River (Croatia), was checked. Shell, radula, and soft part anatomy were considered. In both male and female genitalia *D. fluviatilis* resembled *Anagastina* and *Radomaniola*. Molecular phylogeny, inferred from the mitochondrial cytochrome oxidase subunit I (COI) and nuclear 18S rRNA gene partial sequences, confirmed morphological observations: the sister taxa of *Dalmatinella* being *Graecoarganiella*, *Radomaniola* and *Anagastina*, and *Dalmatinella* belonging to the Sadlerianinae.

KEY WORDS: Rissooidea, molecular phylogeny, DNA, COI, 18S rRNA, morphology

## INTRODUCTION

The river Zrmanja in Croatia, 69 km long, its catchment area covering 907 km$^2$, is one of the European diversity hot spots of freshwater gastropods. It is inhabited by 16 species, five of them endemic (STRONG et al. 2008; 22 species according to BERAN 2011). Rissooidea are represented by three species found in the brackishwater part below the Jankovica Buk waterfalls, and by eight species in the freshwater part above Jankovica Buk. Three of the freshwater species: *Belgrandiella zermanica* Radoman, 1973, *Islamia zermanica* Radoman, 1973, and *Lithoglyphulus tedanicus* Schlickum et Schütt, 1971 [=*Tanousia zrmanjae* (Brusina, 1866)] (RADOMAN 1983) are not found anywhere else. *Dalmatinella fluviatilis* Radoman, 1973, the only representative of *Dalmatinella* Radoman, 1973 described so far, can probably be considered another endemite of the Zrmanja. Apart from this river it was reported from the lowest part of the Neretva river, between Kula and Opuzen (RADOMAN 1983). We collected rissooids from that part of the Neretva twice (2001, 2004) and did not find any *Dalmatinella*. It seems hardly probable that the latter locality, isolated from the Zrmanja by a distance of more than 200 km with no record of *D. fluviatilis*, is inhabited by the same species. Passive transportation by birds (see FALNIOWSKI & SZAROWSKA 2011a for a review), not excluded, does not explain why *D. fluviatilis* should not be found in any other river of this region. Anyway, the possibility that there are two relic populations of the species cannot be excluded.

The aim of the present paper is to check the morphology of *D. fluviatilis* from its type locality, Jankovica Buk waterfalls, and to infer its phylogenetic relationships using molecular data.

## MATERIAL AND METHODS

The material was collected from Jankovica Buk waterfalls, the Zrmanja River, Croatia, 44°12'09.8"N, 15°43'16.9"E, 9 m a.s.l., with a sieve.

The snails were fixed with 80% ethanol. The shells were cleaned in an ultrasonic cleaner and photographed with a CANON EOS 50D digital camera.

Three adult males and three females were dissected under a NIKON SMZ-U stereomicroscope. Female genitalia (pallial oviduct) were examined using a MOTIC light microscope. Radulae were examined using a JEOL JSM-5410 scanning electron microscope, applying the techniques described by FALNIOWSKI (1990).

DNA was extracted separately from foot tissue of five specimens. 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 buffer. The PCR reaction was performed with the following primers: LCO1490 (5'-GGTCAACAAATCAT AAAGATATTGG-3') (FOLMER et al. 1994) and COR722b (5'-TAAACTTCAGGGTGACCAAAA AATYA-3') (WILKE & DAVIS 2000) for the cytochrome oxidase subunit I (COI) mitochondrial gene; SWAM18SF1 (5'-GAATGGCTCATTAAATCAGTCGA GGTTCCTTAGATGATCCAAATC-3') and SWA M18SR1 (5'-ATCCTCGTTAAAGGGTTTAAAGTGTA CTCATTCCAATTACGGAGC-3') for the nuclear 18S rRNA gene (PALUMBI 1996). The PCR conditions were as follows: COI – initial denaturation step of 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and a final extension of 4 min at 72°C; 18S – initial denaturation step of 4 min at 94°C, followed by 40 cycles of 45 s at 94°C, 45 s at 51°C, 2 min at 72°C and, after all cycles were completed, an additional elongation step of 4 min at 72°C was performed. The total volume of each PCR reaction mixture was 50 µl. To check the quality of the PCR products 10 µl of the PCR product was run on 1% agarose gel. The PCR products were purified using Clean-Up columns (A&A Biotechnology) and the purified PCR products were cycle-sequenced in both directions (HILLIS et al. 1996) using BigDye Terminator v3.1 (Applied Biosystems), following the manufacturer's protocol and with the primers described above. The sequencing reaction products were purified using ExTerminator Columns (A&A Biotechnology); DNA sequences then underwent electrophoresis on an ABI Prism sequencer. All the sequences were deposited in GenBank (Table 1).

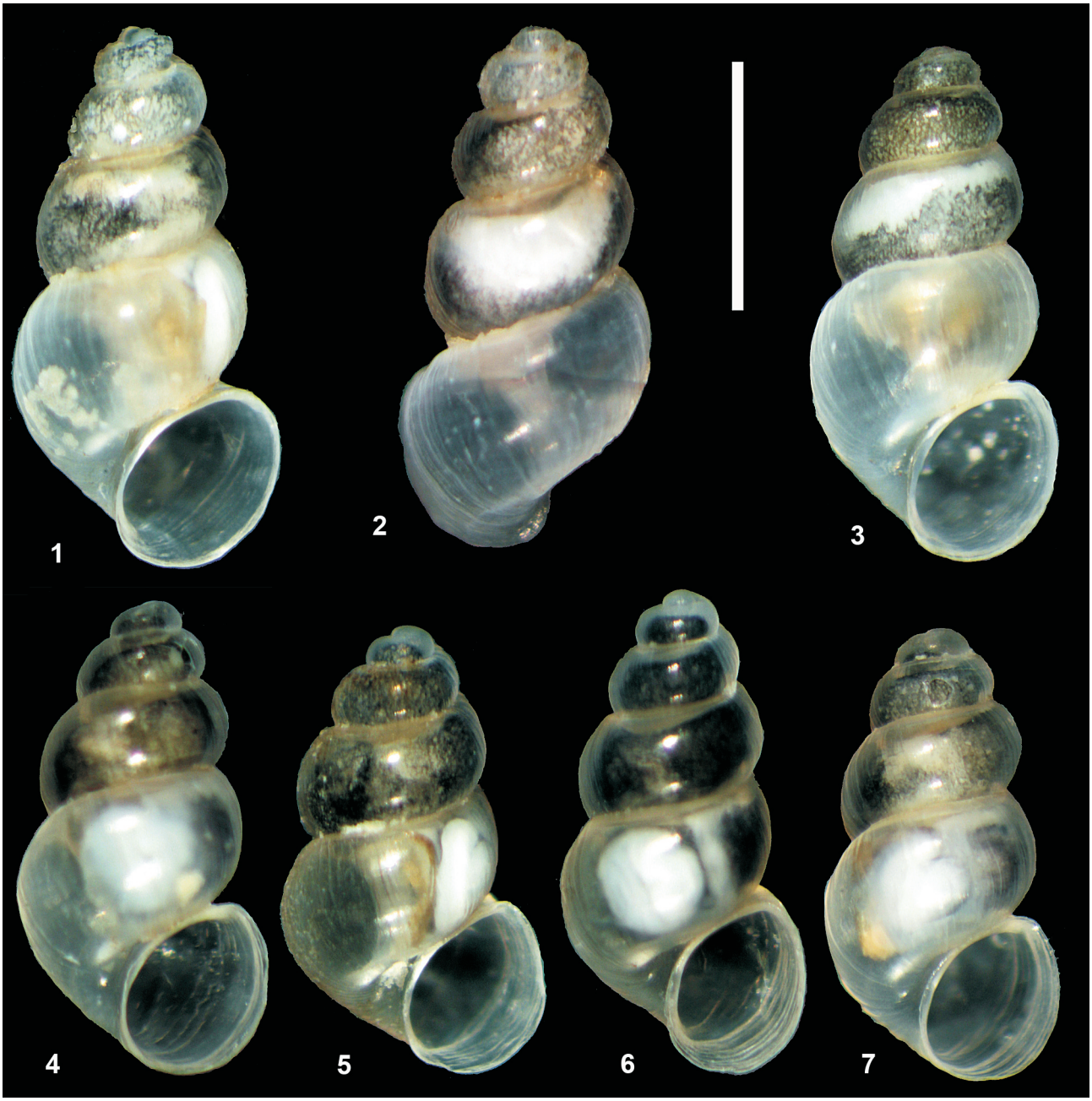
The COI sequences were aligned by eye using BioEdit 5.0.0 (HALL 1999) and edited with

Table 1. Taxa used for phylogenetic analyses, with their GenBank Accession Numbers and references

| Species | 18S GB# | COI GB# | References |
|---|---|---|---|
| *Adriohydrobia gagatinella* (Küster, 1852) | AF367657 | AF317881 | WILKE & FALNIOWSKI (2001) |
| *Adrioinsulana conovula* (Frauenfeld, 1863) | AF367656 | AF367628 | WILKE et al. (2001) |
| *Agrafia wiktori* Szarowska et Falniowski, 2011 | JF906758 | JF906762 | SZAROWSKA & FALNIOWSKI (2011) |
| *Alzoniella finalina* Giusti et Bodon, 1984 | AF367686 | AF367650 | WILKE et al. (2001) |
| *Anagastina zetavalis* (Radoman, 1973) | EF070622 | EF070616 | SZAROWSKA (2006) |
| *Bithynia tentaculata* (Linnaeus, 1758) | AF367675 | AF367643 | WILKE et al. (2001) |
| *Boleana umbilicata* (Kuščer, 1932) | JX982797 | JX982795 | FALNIOWSKI & SZAROWSKA (2012) |
| *Dalmatinella fluviatilis* Radoman, 1973 | KC344539 | KC344541 | present study |
| *Dalmatinella fluviatilis* Radoman, 1973 | KC344540 | KC344542 | present study |
| *Daphniola graeca* Radoman, 1973 | EF070624 | EF070618 | SZAROWSKA (2006) |
| *Dianella thiesseana* (Kobelt, 1878) | AY676125 | AY676127 | SZAROWSKA et al. (2005) |
| *Graecoarganiella parnassiana* Falniowski et Szarowska, 2011 | JN202341 | JN202348 | FALNIOWSKI & SZAROWSKA (2011b) |
| *Graziana alpestris* (Frauenfeld, 1863) | AF367673 | AF367641 | WILKE et al. (2001) |
| *Grossuana codreanui* (Grossu, 1946) | EF061916 | EF061919 | SZAROWSKA et al. (2007) |
| *Hauffenia tellinii* (Pollonera, 1898) | AF367672 | AF367640 | WILKE et al. (2001) |
| *Hydrobia acuta* (Draparnaud, 1805) | AF367680 | AF278808 | WILKE & DAVIS (2000) |
| *Islamia piristoma* Bodon et Cianfanelli, 2001 | AF367671 | AF367639 | WILKE et al. (2001) |
| *Pseudamnicola lucensis* (Issel, 1866) | AF367687 | AF367651 | WILKE et al. (2001) |
| *Pyrgula annulata* (Linnaeus, 1767) | AY676124 | AY341258 | SZAROWSKA et al. (2005) |
| *Radomaniola callosa* (Paulucci, 1881) | AF367685 | AF367649 | WILKE et al. (2001) |
| *Rissoa labiosa* (Montagu, 1803) | AY676126 | AY676128 | SZAROWSKA et al. (2005) |
| *Sadleriana fluminensis* (Küster, 1853) | AF367683 | AY273996 | WILKE et al. (2001) |
| *Trichonia kephalovrissonia* Radoman, 1973 | EF070630 | EF070619 | SZAROWSKA (2006) |
| *Ventrosia ventrosa* (Montagu, 1803) | AF367681 | AF118335 | WILKE & DAVIS (2000) |

MACCLADE 4.05 (MADDISON & MADDISON 2002). For 18S, an initial alignment was performed using CLUSTALX 1.82 (THOMPSON et al. 1997) and edited with MACCLADE. Mutational saturation for the COI dataset was examined by plotting the numbers of transitions and transversions for all the codon positions together, and for the 3rd position separately, against the percentage sequence divergence, using DAMBE 5.2.9 (XIA 2000). We also used DAMBE 5.2.9 to perform the saturation test (XIA et al. 2003). It revealed a significant degree of saturation in the third position of the sequences. In rissooids, COI approaches saturation with about 18.6% or 120 nucleotide differences (DAVIS et al. 1998), which seems to happen after approximately 10 million years. However, to avoid a substantial loss of information in the case of closely related species, this position was not excluded from the dataset and it was used for the analysis.

For Bayesian inference (BA) we used MRBAYES 3.1.2 (HUELSENBECK & RONQUIST 2001, RONQUIST & HUELSENBECK 2003). We selected the best model of sequence evolution for each data set using MRMODELTEST 2.2 (NYLANDER 2004), applying the Akaike Information Criterion (POSADA & BUCKLEY 2004). The Bayesian inference was performed with the following parameters: 4 chains in two parallel analyses (1 cold, three heated; heating temperature = 0.15) Metropolis-Coupled Monte Carlo analysis run twice in parallel for 80,000,000 generations, trees sampled every 1,000 generations starting after a




Figs 1–7. Shells of *Dalmatinella fluviatilis* from Jankovica Buk, bar represents 1 mm

burn-in of 30,000 generations (the value chosen according to the log-likelihood values). The Bayesian inference was run unless the parallel runs achieved convergence (split frequency standard deviations <0.001). The partition was set, with COI treated as coding and 18S as uncoding. We inferred majority-rule consensus tree with Bayesian (posterior) probabilities.

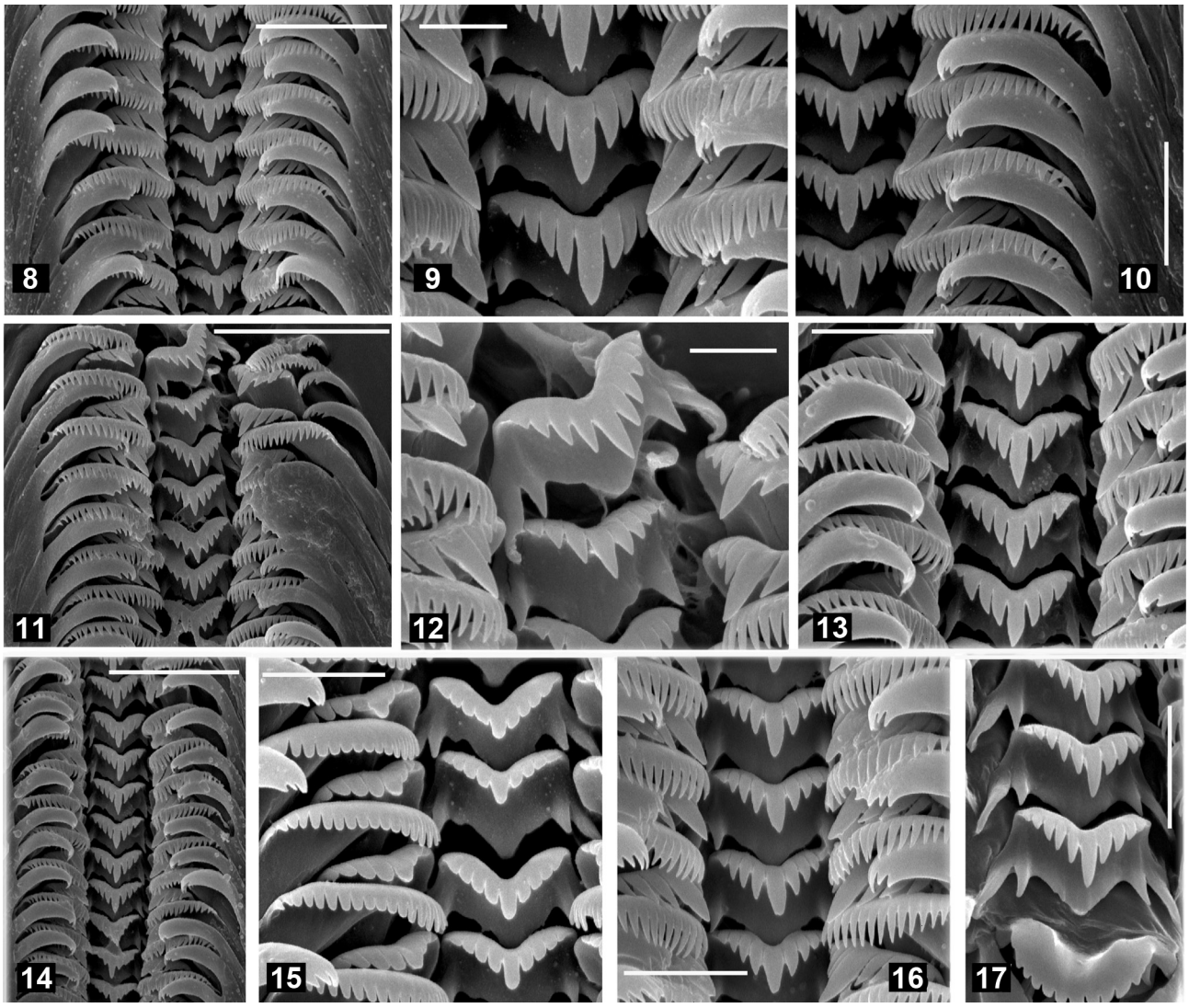
In the phylogeny reconstruction, we used GenBank sequences from 22 rissooid taxa (Table 1). Two of these, used as an outgroup, represented the main non-hydrobiid lineages within the Rissooidea (WILKE et al. 2001); another seven taxa represented the Hydrobiinae (including "Pyrgulinae"; SZAROWSKA et al. 2005). The remaining taxa were chosen to represent all the main lineages within the European Sadlerianinae (SZAROWSKA 2006).

## RESULTS AND DISCUSSION

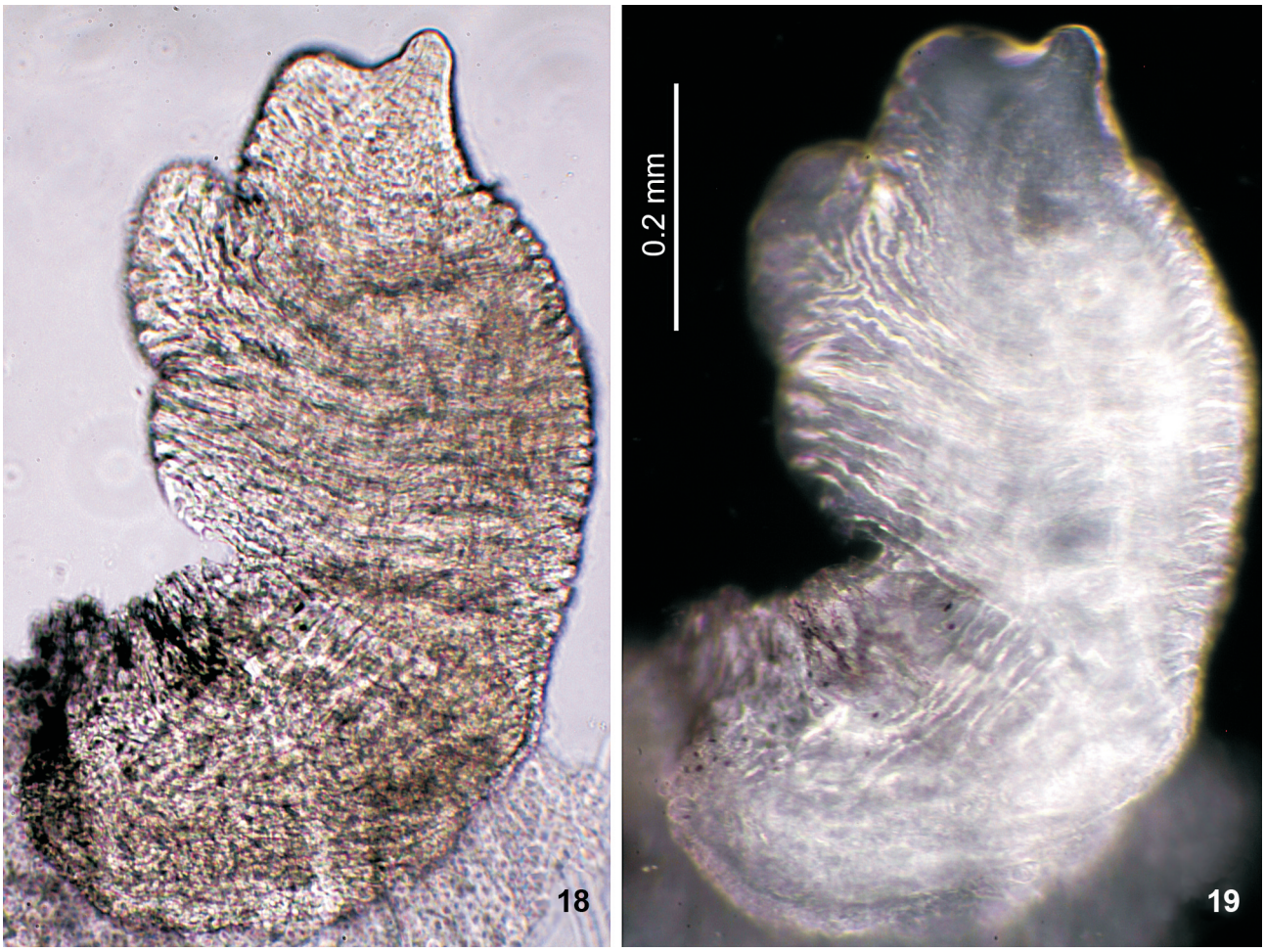
Shell of *Dalmatinella fluviatilis* (Figs 1–7) little variable, turriform; mouth rounded, ovoid, as described by RADOMAN (1973, 1974, 1983). Mantle black-pigmented (Figs 1–7); head whitish, almost lacking pigment (Figs 5–7). Ctenidium absent.

Radula (Figs 8–17) taenioglossate; central tooth formula:

$$\frac{(4-5)-1-(4-5)}{1-1},$$




Figs 8–17. Radulae of *Dalmatinella fluviatilis*: 8, 11 and 14 – whole row; 9, 12 and 17 – central tooth; 10, 13 and 15–16 – central, lateral and marginal teeth (bar equals: 50 μm in 8, 11 and 14; 9 μm in 9 and 12; 10 μm in 10, 13 and 15–17)




Figs 18–19. Penis of *Dalmatinella fluviatilis*

basal cusps prominent, all cusps long and sharp; lateral tooth formula: 3 – 1 – (3–5), biggest cusp nearly twice as long as adjacent cusps; inner marginal tooth with 18–21 long and sharp cusps; outer marginal tooth with 6–9 relatively massive cusps.

Penis (Figs 18–19). In its general habitus it resembles the penis illustrated by RADOMAN (1973, 1983), but instead of "two similar symmetrical outgrowths on the left and right side of its distal end, between whom a three-angle point is located" (RADOMAN 1983: p. 33, fig. 22), there is a double outgrowth on the left side, and the penis tip situated on the right (Figs 18–19), with the vas deferens slightly visible inside, running in zigzag (Fig. 19). Thus, the penis resembles the one typical of *Anagastina* Radoman, 1978 or *Radomaniola* Szarowska, 2006, and is quite similar to that of *Graecoarganiella* Falniowski et Szarowska 2011, the

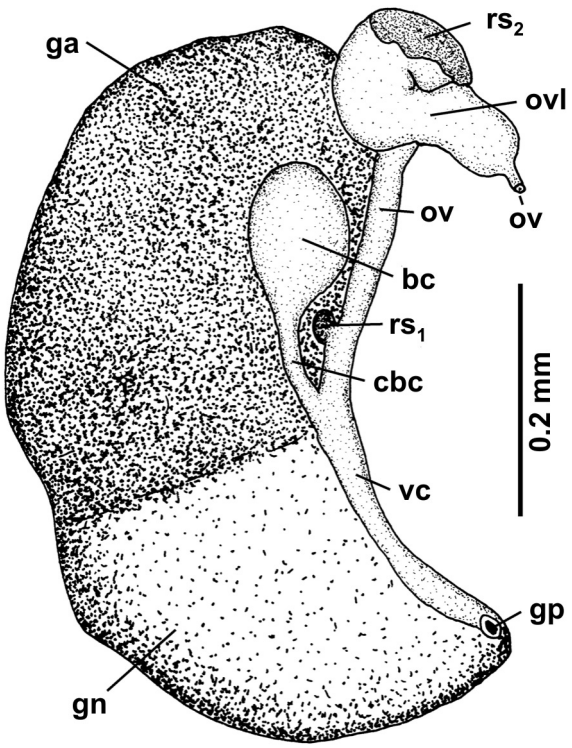



Fig. 20. Renal and pallial section of female reproductive organs of *Dalmatinella fluviatilis* (bc – bursa copulatrix, cbc – duct of bursa copulatrix, ga – albuminoid gland, gn – nidamental gland, gp – gonoporus, ov – oviduct, ovl – loop of (renal) oviduct, $rs_1$ and $rs_2$ – receptaculum seminis, distinction after RADOMAN 1983, vc – ventral channel)

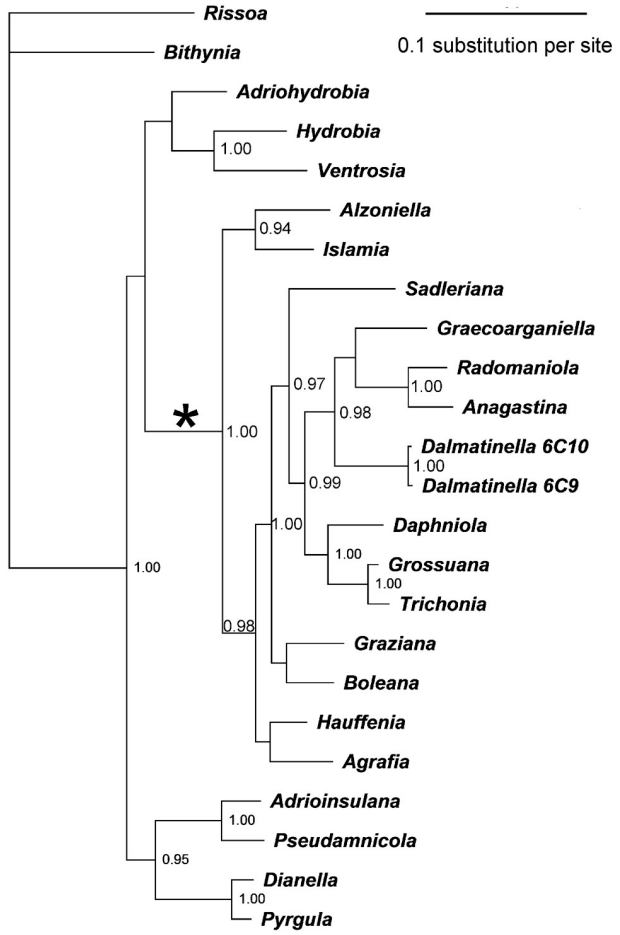



Fig. 21. Bayesian tree computed for the COI and 18S sequences, Bayesian probabilities given near the relevant node if >0.90; asterisk indicates the clade representing Sadlerianinae

genera to which *Dalmatinella* is closely related in our inferred molecular phylogeny (see below).

The female reproductive organs (Fig. 20) correspond with RADOMAN's (1973, 1983) description: two seminal receptacles, $rs_1$ vestigial, $rs_2$ prominent; bursa copulatrix moderately big, pear-shaped, bursal duct short. Contrary to RADOMAN's (1973, 1983) figure the outlet of $rs_2$ is far more distant from $rs_1$.

For the COI GTR + I + Γ model was selected (with base frequencies: A=0.3563, C=0.1300, G=0.1166, T=0.3971, and substitution rate matrix: [A – C]=0.4614, [A – G]=8.0529, [A – T]=0.0994, [C – G]=1.4309, [C – T]=21.7321, [G – T]=1.0000, proportion of invariable sites: (I)=0.5264, and Γ distribution with the shape parameter =0.4721. For 18S the SYM + I + Γ model was selected (with invariable sites and Γ distribution, assuming the equal base frequencies, and substitution rate matrix: [A – C]=1.0000, [A – G]=1.8443, [A – T]=1.0000, [C – G]=1.0000, [C – T]=4.9724, [G – T]=1.0000, proportion of invariable sites: (I)=0.7143, and Γ distribution with the shape parameter =0.5232.

In the Bayesian tree computed for the molecular data (Fig. 21), *Anagastina*, *Radomaniola* and *Graecoarganiella*, are the sister clade of *Dalmatinella* (Bayesian probability 0.98). This corresponds with the male and female genitalia described above. The tree (Fig. 21) shows *Dalmatinella* belonging to the Sadlerianinae Szarowska, 2006 (Bayesian posterior probability of the clade representing the subfamily equals 1.0 in the tree).

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