

PSEUDORIENTALIA RADOMAN, 1973 (CAENOGASTROPODA: RISSOOIDEA) ON SAMOS ISLAND, AEGEAN SEA

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ABSTRACT: *Pseudorientalia* Radoman, 1973 is recorded from the Samos Island (East Aegean Islands). The genus was previously known from northern Turkey. The shells, protoconch, radula, penis, and female reproductive organs are described. All the morphological data confirm that the specimens belong to the genus *Pseudorientalia*, but for the moment its identity with *P. natolica* (Küster, 1852) from Turkey cannot be confirmed. Molecular data (mitochondrial cytochrome oxidase subunit I and nuclear 18S rRNA gene partial sequences) confirm the genus-level distinctness of *Pseudorientalia*, and its sister clade consists of *Grossuana* Radoman, 1973, *Trichonia* Radoman, 1973, and *Daphniola* Radoman, 1973.

KEY WORDS: Risssooidea, COI, 18S rRNA, radula, anatomy, phylogeny, Aegean Sea

INTRODUCTION

RADOMAN (1973) created a monotypic genus *Pseudorientalia* Radoman, 1973, for *Paludina natolica* Küster, 1852, described from “bei Bursa in Natolien” (KÜSTER 1852). *Pseudorientalia natolica* (Küster, 1852) is known from a few localities in northern Turkey (Asia Minor): great spring Pinar Basa in the Vedelek village, near Gemlik, and Inkaya, W of Bursa, and from two springs near Cav (SCHÜTT 1965, RADOMAN 1983). In general the species is distributed south-east of the Marmara Sea (SCHÜTT & BILGIN 1970, YILDIRIM 1999, YILDIRIM et al. 2006). Recently,

GLÖER & GEORGIEV (2012) described a new species of *Pseudorientalia*: *P. tzekovi*, from “thermal spring at fish tanks in the village Kefalari”, in North Greece. However, with no data on the anatomy of female organs, the genus assignment remains doubtful.

In 2012 we found representatives of *Pseudorientalia* on Samos, East Aegean Islands, Greece. The aim of the present paper is to describe the shell, protoconch, radula, penis and female reproductive organs of this *Pseudorientalia*, and to infer the relationships of the genus from molecular data.

MATERIAL AND METHODS

About fifty specimens of *Pseudorientalia* were collected, using a sieve (two meshes per mm), from Despoti Fountain between Koutsis and Pirogias, Samos Island: 37°42'59"N, 26°46'51"E, 490 m a. s. l., on the 20th of June 2012.

The snails were washed twice in 80% ethanol and left to stand in it for around 12 hours. Then the eth-

anol was changed twice more within 24 hours and finally, after a few days, the 80% solution was replaced with a 96% one, in which the samples were stored at -20°C. The shells were photographed with a CANON EOS 50D digital camera. Five adult males and five females were dissected, using a NIKON SMZ-U stereoscope microscope. The penis and female genitalia

(pallial oviduct) were examined using a MOTIC light microscope. The protoconch (after ultrasonic cleaning) and radulae were examined using a JEOL JSM-5410 scanning electron microscope, applying the techniques described by FALNIOWSKI (1990).

DNA was extracted from foot tissue of the specimen not presented in the photograph. 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'-GGTCAACAAATCATAAAGATATTGG-3') (FOLMER et al. 1994) and COR722b (5'-TAAACTTCAGGGTGACCAAAAAATYA-3') (WILKE & DAVIS 2000) for the cytochrome oxidase subunit I (COI) mitochondrial gene and SWAM18SF1

(5'-GAATGGCTCATTAATCAGTCGAGGTTCCCTTAGATGATCCAAATC-3'), and SWAM 18SR1 (5'-ATCCTCGTTAAAGGGTTTAAAGTGTACTCATTCCAATTACGGAGC-3') for the 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 were

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

Species	18S GB#	COI GB#	references
<i>Adriohydrobia gagatinella</i> (Küster, 1852)	AF367657	AF317881	WILKE & FALNIOWSKI (2001)
<i>Adrioinsulana conovula</i> (Frauenfeld, 1863)	AF367656	AF367628	WILKE et al. (2001)
<i>Agrafia wiktoria</i> Szarowska et Falniowski, 2011	JF906758	JF906762	SZAROWSKA & FALNIOWSKI (2011)
<i>Alzoniella finalina</i> Giusti et Bodon, 1984	AF367686	AF367650	WILKE et al. (2001)
<i>Anagastina zetavalis</i> (Radoman, 1973)	EF070622	EF070616	SZAROWSKA (2006)
<i>Bithynia tentaculata</i> (Linnaeus, 1758)	AF367675	AF367643	WILKE et al. (2001)
<i>Boleana umbilicata</i> (Kuščer, 1932)	JX982797	JX982795	FALNIOWSKI & SZAROWSKA (2012)
<i>Bythinella austriaca</i> (Frauenfeld, 1857)	AF212917	FJ545132	FALNIOWSKI et al. (2009)
<i>Bythiospeum</i> sp.	AF367664	AF367634	WILKE et al. (2001)
<i>Dalmatinella fluviatilis</i> Radoman, 1973	KC344539	KC344541	FALNIOWSKI & SZAROWSKA (2013)
<i>Daphniola graeca</i> Radoman, 1973	EF070624	EF070618	SZAROWSKA (2006)
<i>Dianella thiesseana</i> (Kobelt, 1878)	AY676125	AY676127	SZAROWSKA et al. (2005)
<i>Graecoarganiella parnassiana</i> Falniowski et Szarowska, 2011	JN202341	JN202348	FALNIOWSKI & SZAROWSKA (2011b)
<i>Graziana alpestris</i> (Frauenfeld, 1863)	AF367673	AF367641	WILKE et al. (2001)
<i>Grossuana codreanui</i> (Grossu, 1946)	EF061916	EF061919	SZAROWSKA et al. (2007)
<i>Hauffenia tellinii</i> (Pollonera, 1898)	AF367672	AF367640	WILKE et al. (2001)
<i>Heleobia dalmatica</i> (Radoman, 1974)	AF367661	AF367631	WILKE et al. (2001)
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF367680	AF278808	WILKE & DAVIS (2000)
<i>Islamia piristoma</i> Bodon et Cianfanelli, 2001	AF367671	AF367639	WILKE et al. (2001)
<i>Lithoglyphus naticoides</i> (C. Pfeiffer, 1828)	AF367674	AF367642	WILKE et al. (2001)
<i>Marstoniopsis insubrica</i> (Küster, 1853)	AF367676	AY027813	FALNIOWSKI & WILKE (2001)
<i>Pseudamnicola lucensis</i> (Issel, 1866)	AF367687	AF367651	WILKE et al. (2001)
<i>Pyrgula annulata</i> (Linnaeus, 1767)	AY676124	AY341258	SZAROWSKA et al. (2005)
<i>Radomaniola callosa</i> (Paulucci, 1881)	AF367685	AF367649	WILKE et al. (2001)
<i>Rissoa labiosa</i> (Montagu, 1803)	AY676126	AY676128	SZAROWSKA et al. (2005)
<i>Sadleriana fluminensis</i> (Küster, 1853)	AF367683	AY273996	WILKE et al. (2001)
<i>Trichonia kephalovrissonia</i> Radoman, 1973	EF070630	EF070619	SZAROWSKA (2006)
<i>Ventrosia ventrosa</i> (Montagu, 1803)	AF367681	AF118335	WILKE & DAVIS (2000)
<i>Vinodolia fumana</i> Radoman, 1973	KF359899	KF359900	SZAROWSKA et al. (2013)
<i>Pseudorientalia</i> sp.	KF982274	KF982276	present study
	KF982275	KF982277	present study

then amplified 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. The two sequences were deposited in GenBank (Table 1).

In the phylogeny reconstruction, we used 29 rissoid taxa sequences from GenBank (Table 1). Seven of them, used as an outgroup, represented the main non-hydrobiid lineages within the Rissooidea (WILKE *et al.* 2001); the other 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).

The COI sequences were aligned by eye using BioEdit 5.0.0 (HALL 1999) and edited with 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 third 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 rissoids, COI approaches saturation with about 18.6% or 120 nu-

cleotide 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. In fact, the analysis carried on the first and second positions only resulted in a similar deep phylogeny, but with several polytomies within more terminal nodes.

Initially, we performed phylogeny reconstruction for 18S rRNA and COI data separately, using the maximum likelihood (ML) technique. Next, the partition homogeneity test (FARRIS *et al.* 1995) was performed (1000 replicates) with PAUP*4.0b10 (SWOFFORD 2002), to check whether the two genes could be analysed together. Since $p > 0.769$, the maximum likelihood heuristic search was then run for the combined molecular data. Following the recommendations of POSADA & BUCKLEY (2004) and SOBER (2002), the best model for each dataset was chosen using the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC); both chose the same model. We performed ML analyses in PAUP* and used a heuristic search strategy with stepwise addition of taxa, 10 random-sequence addition replicates, and tree-bisection-reconnection (TBR) branch swapping (SWOFFORD *et al.* 1996), and with MEGA5.10 (TAMURA *et al.* 2011). Nodal support was estimated using the bootstrap (BS) approach (FELSENSTEIN 1985). Bootstrap values for ML trees were calculated using 10,000 bootstrap replicates, with MEGA5.10 and the same model parameters as for ML analysis.

RESULTS

MORPHOLOGY

Shell (Figs 1–9) ovate-conical, thin-walled and translucent, spire height variable. Operculum brownish. Mantle intensively pigmented black (Figs 1–9); black pigment present on the head as well. Ctenidium present.

Protoconch with regularly growing whorls (Figs 10–11), and characteristic, regular microsculpture (Figs 12–13).

Radula (Figs 14–16) taenioglossate, with many cusps on each tooth; central tooth formula:

$$\frac{5(6) - 1 - 5(6)}{1 - 1},$$

(sixth cusp rudimentary, if present), basal cusps moderately long, the other cusps on the rhachis rather sharp and tiny. Lateral tooth (Figs 14 and 16) with various numbers of cusps, its formula: 3 – 1 – 5, or 5 – 1 – 6, or 2 – 1 – 4, cusps sharp and longer than on the rhachis, biggest cusp much less than twice as

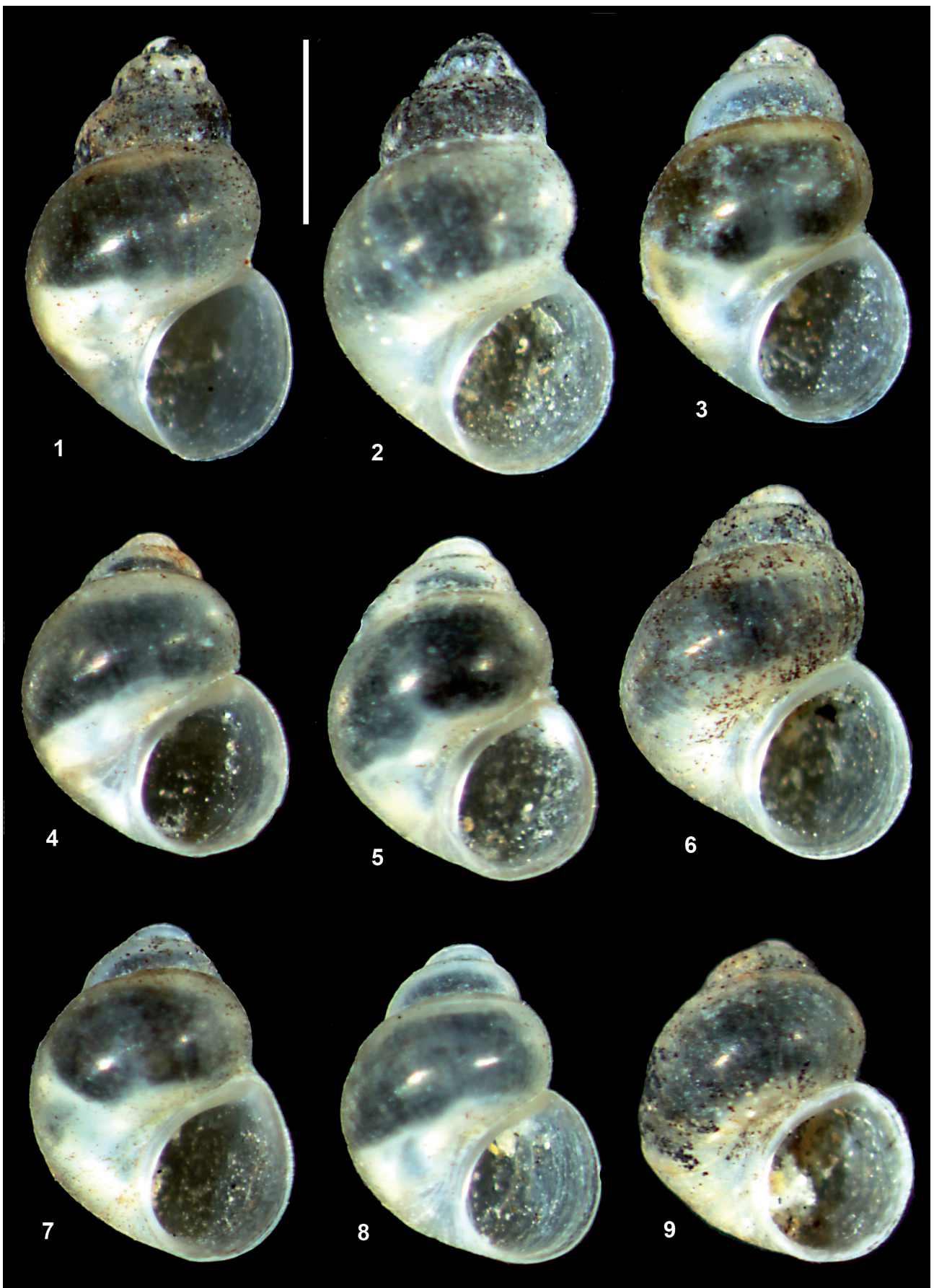
long as adjacent cusps. Inner marginal tooth (Figs 14–15) with 22 – 29 delicate and slender cusps, outer marginal tooth with about 10 small cusps.

Penis (Figs 17–18) simple, without any outgrowths, elongated, awl-like, with intensive black pigmentation in its middle section, vas deferens not visible inside.

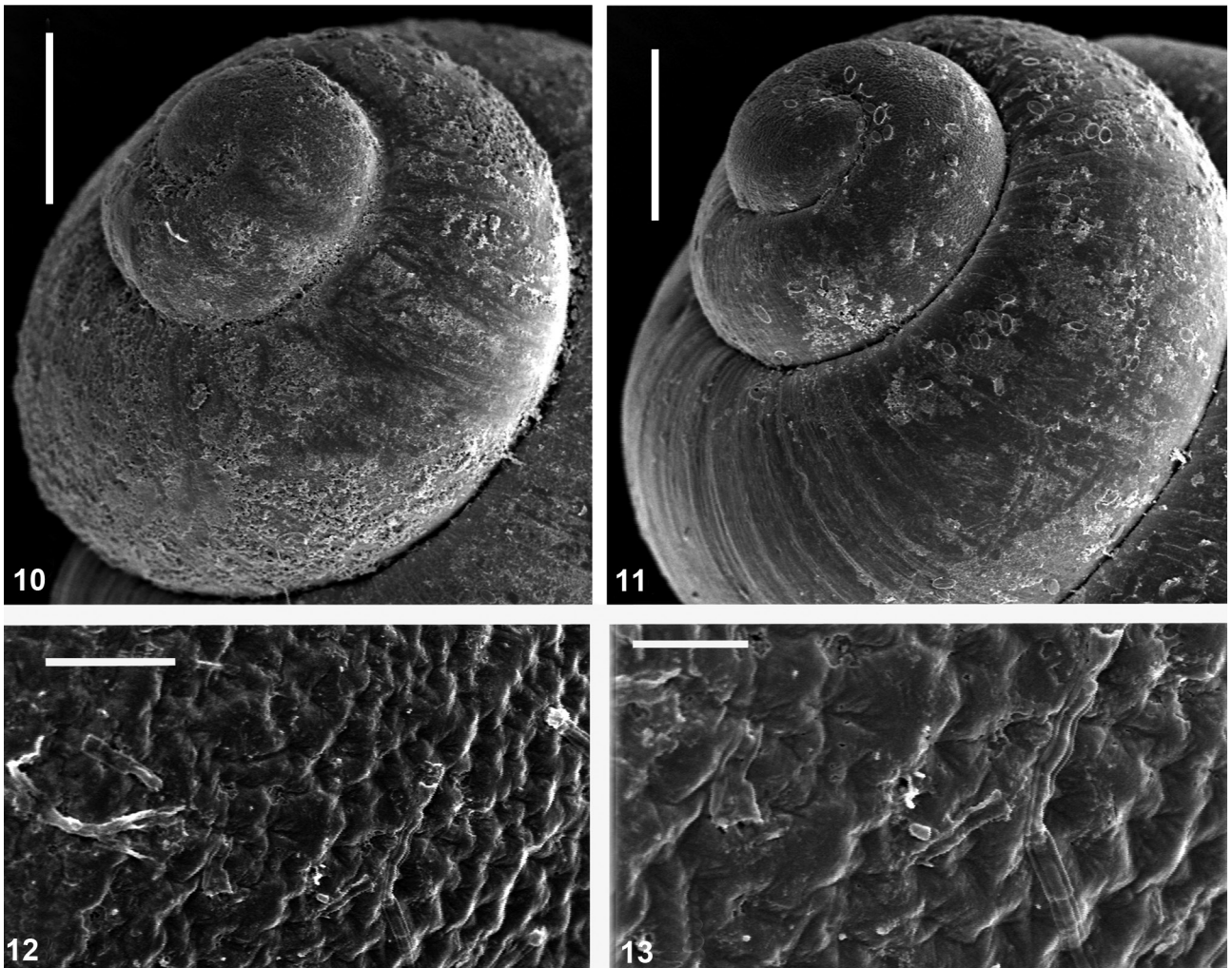
Female reproductive organs (Fig. 19), with two seminal receptacles, rs_2 size similar as the size of rs_1 , both moderately big, coil of oviduct massive. The most striking character state of the female reproductive organs is the huge bursa copulatrix, whose size approaches the size of the accessory gland complex (Fig. 19); the bursa is flattened spherical in shape.

MOLECULAR PHYLOGENY

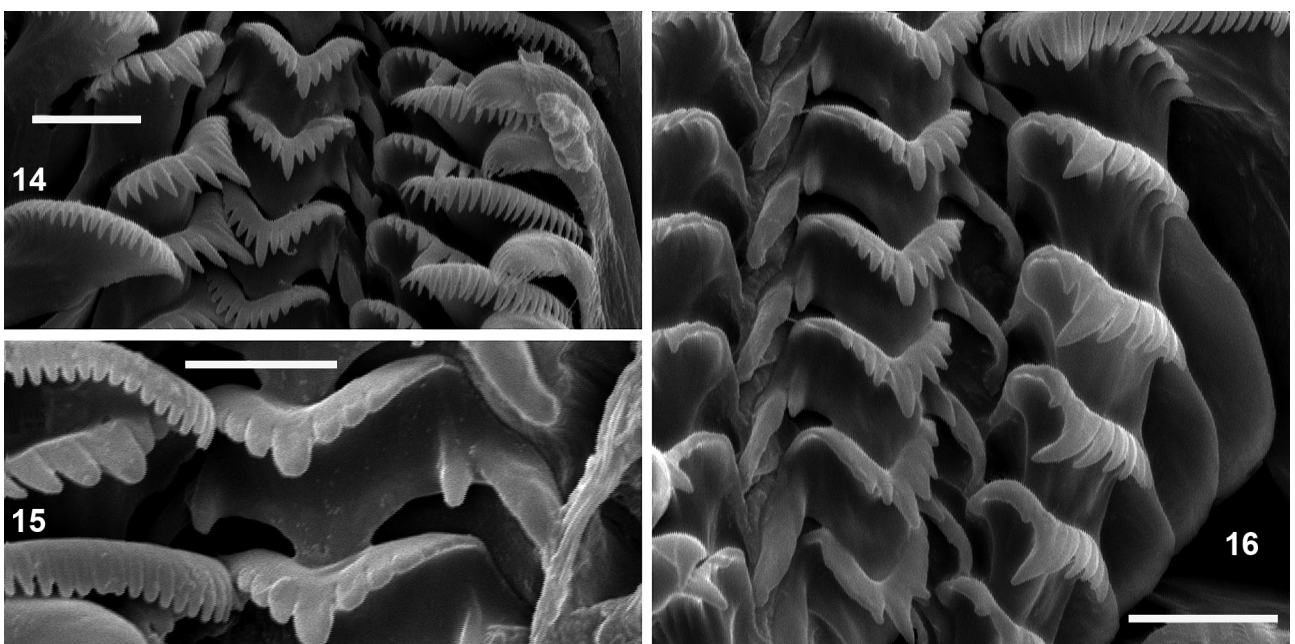
For the combined data set the Bayesian Information Criterion (BIC) and corrected Akaike Information Criterion (AICc) with MEGA5 found model T92 (Tamura 3-parameter: NEI & KUMAR 2000) + I + Γ ,



Figs 1–9. Shells of *Pseudorientalia* from Samos, scale bar equals 1 mm



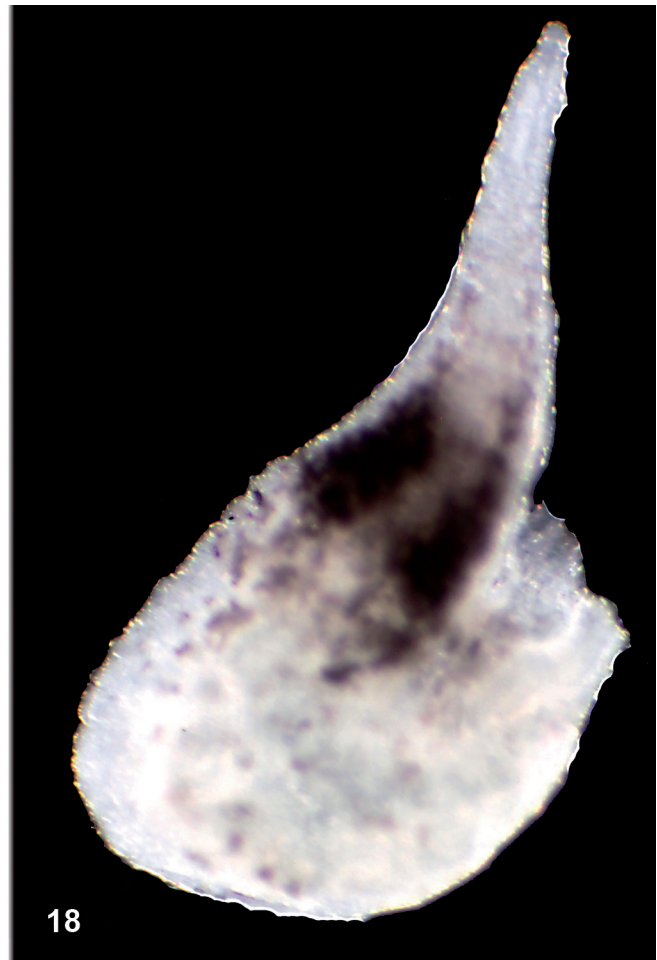
Figs 10–13. Protoconchs of *Pseudorientalia* from Samos, scale bars equal 200 μm , 200 μm , 10 μm , and 5 μm , respectively



Figs 14–16. Radulae of *Pseudorientalia* from Samos, scale bars equal 5 μm

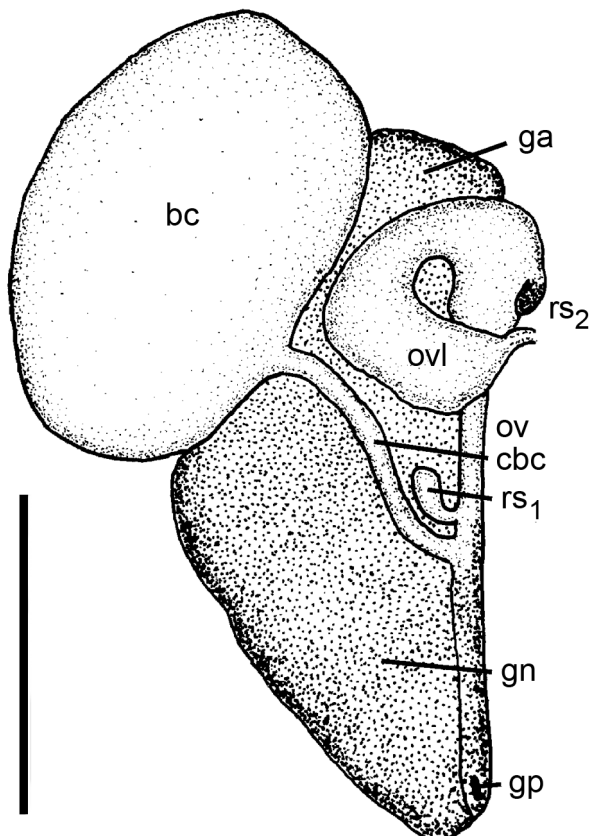


17



18

Figs 17–18. Penis of *Pseudorientalia* from Samos, photographed on bright and dark field, respectively; scale bar equals 0.5 mm



with base frequencies: A = 0.284, C = 0.216, G = 0.216, T = 0.284; substitution rate matrix: [A-C] = 0.027, [C-A] = 0.036, [A-G] = 0.162, [G-A] = 0.213, [A-T] = 0.036, [T-A] = 0.036, [C-G] = 0.027, [G-C] = 0.027, [C-T] = 0.213, [T-C] = 0.162, [G-T] = 0.036, [T-G] = 0.027; proportion of invariable sites: (I) = 0.62, Γ distribution with the shape parameter = 0.58, and transition/transversion bias R = 2.92.

In the inferred molecular tree (Fig. 20) our *Pseudorientalia* belongs to the family Hydrobiidae, and subfamily Sadlerianinae, as defined by SZAROWSKA (2006). Its sister clade consists of *Grossuana* Radoman, 1973, *Trichonia* Radoman, 1973, and *Daphniola* Radoman, 1973 (bootstrap support 56%). The molecular data seem to confirm the genus-level distinctness of *Pseudorientalia*.

Fig. 19. Female reproductive organs of *Pseudorientalia* from Samos (bc – bursa copulatrix, cbc – duct of bursa copulatrix, ga – albuminoid gland, gn – nidamental gland, gp – gonoporus, ov – oviduct, ovl – loop of oviduct, rs₁, rs₂ – receptaculum seminis 1 and 2, respectively), scale bar equals 0.5 mm

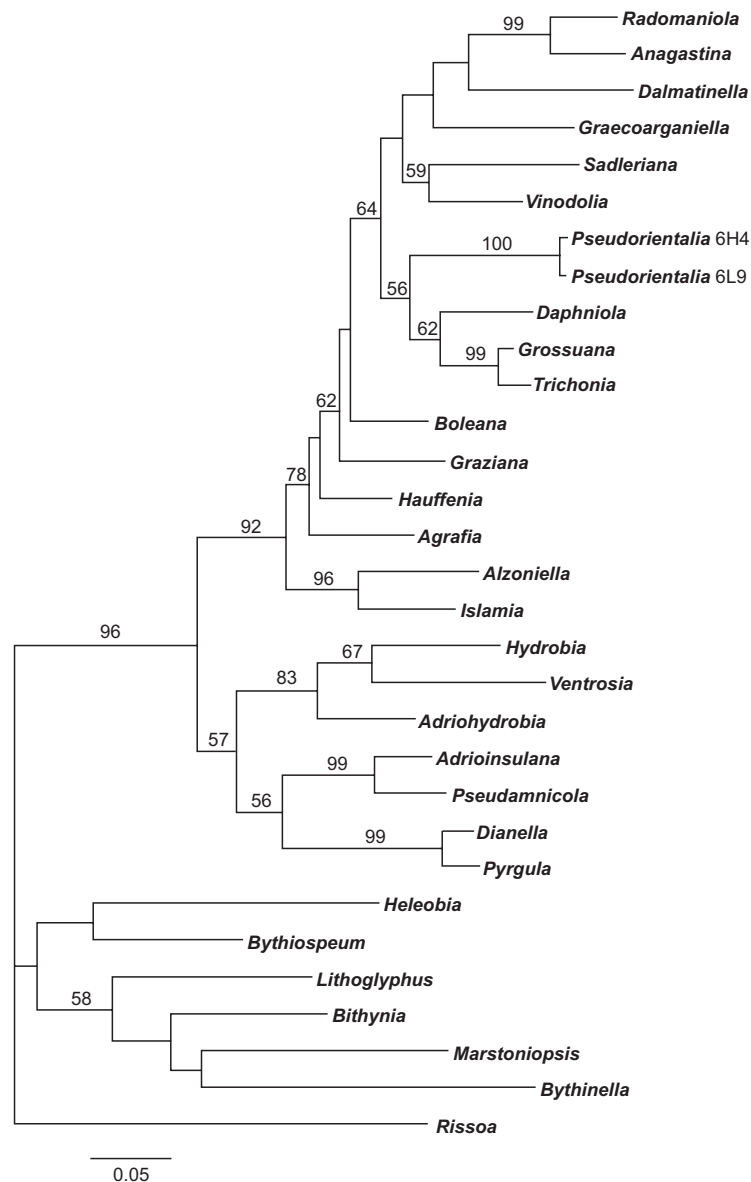


Fig. 20. Maximum likelihood tree of the two concatenated sequences (18S and COI), bootstrap supports (10,000 replicates) given if >50%

DISCUSSION

The shell resembles the one photographed by RADOMAN (1983) for *P. natolica*, but the spire height in the shells from Samos varies widely (compare Figs 1 and 9, both of adult specimens with complete peristome).

RADOMAN (1983) noted that all the cusps on the radula were tiny, as they could be seen only under immersion. In the radula of our *Pseudorientalia* the cusps are rather small, indeed, but perhaps not as tiny as in the radula studied by Radoman.

The penis is similar to the one drawn and described by RADOMAN (1983), and GLÖER & GEORGIEV (2012). Similarly, the female reproductive organs re-

semble the ones described and figured by RADOMAN (1973, 1983). It should be noted that the female reproductive organs of *Pseudorientalia* are quite characteristic, especially the enormously big bursa copulatrix, but the penis is very simple, and its morphology cannot be conclusive – especially in fixed material such simple morphology can be observed in some other hydrobiid taxa.

Evidently, all the morphological character states confirm the assignment of our specimens from the Samos Island to the genus *Pseudorientalia*. However, their possible identity with *P. natolica* remains open, as long as there are no molecular data on the lat-

ter species from its type locality. Our *Pseudorientalia* may represent a distinct species, and the representatives of the genus may occur on some other Aegean Islands. Theoretically, island populations should be isolated from the ones from the mainland, which may promote speciation. However, all the spring populations are similarly isolated, even at the same continuous land, and, on the other hand, such factors as bird passive transport, weaken this isolation (FALNIOWSKI & SZAROWSKA 2011a). There are several examples in the Greek *Bythinella* (FALNIOWSKI & SZAROWSKA 2011a, FALNIOWSKI et al. 2012) of rela-

tively high gene flow across a sea. Thus, for the moment, we prefer to determine it as *Pseudorientalia* sp.

The inferred molecular tree confirms the morphology-based systematics proposed by RADOMAN (1973, 1983). Thus, in this case, the systematics of Radoman, often criticised, remains justified.

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