



THE GENUS *DAPHNIOLA* RADOMAN, 1973 (CAENOGASTROPODA: HYDROBIIDAE) IN THE PELOPONNESE, GREECE

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ABSTRACT: A valvatiform hydrobiid gastropod, found in a spring at Dhiaselos, W of Sparta, N. Taigetos Mts., Peloponnese, Greece, was identified as *Horatia hadei* Gittenberger, 1982. Its protoconch sculpture, female reproductive organs and penis morphology are characteristic of *Daphniola* Radoman, 1973. Maximum likelihood phylogenetic analysis based on COI (cytochrome oxidase subunit I) fragments of mtDNA proved that the species is congeneric with *D. exigua* (A. Schmidt, 1856) and *D. louisi* Falniowski et Szarowska, 2000, and thus belongs to the genus *Daphniola*, and that *D. hadei*, *D. exigua* and *D. louisi* are species-level distinct taxa.

KEY WORDS: valvatiform, hydrobiid, protoconch, anatomy, mtDNA

INTRODUCTION

The genus *Daphniola* Radoman, 1973, type species *D. graeca* Radoman, 1973 from Daphne Spring in Témbi Valley, northern Greece, comprises freshwater stygobiont, valvatiform rissooids. In his review of the Greek Hydrobiidae, SCHÜTT (1980) assigned the valvatiform hydrobiids, which had been reported from that territory, to *Horatia* Bourguignat, 1887. Consequently, SCHÜTT (1980) synonymised *Daphniola graeca* with *Horatia exigua* (A. Schmidt, 1856). Hence, he expanded the range of this genus far to the south (RADOMAN, 1983). However, molecular data did not confirm any closer relationship between *Horatia* and *Daphniola* (SZAROWSKA 2006). GITTENBERGER (1982) described *Horatia hadei*, a new species of *Horatia* he found 5 km SW of Yíthion (Gythion), Peloponnese. FALNIOWSKI & SZAROWSKA (2000) described *Daphniola*

la louisi, a new species of *Daphniola* from Kessariani, Athens. The protoconch sculpture of *D. louisi* (FALNIOWSKI & SZAROWSKA 2000: Figs 5–7) resembled the one published by GITTENBERGER (1982: Fig. 2). Molecular data (FALNIOWSKI et al. 2007) confirmed that *D. exigua* and *D. graeca* were conspecific, and that the species was different from *D. louisi*.

In 2003, we found that the type locality of *D. hadei* was probably destroyed (SZAROWSKA & FALNIOWSKI 2004, SZAROWSKA 2006). In 2007, we found a valvatiform hydrobiid at one locality in Lakonia, Peloponnese, about 40 km from the type locality of *D. hadei* (GITTENBERGER 1982). The shell and protoconch sculpture of the gastropod resembled the shell and protoconch presented by GITTENBERGER (1982: Figs 1–2).

MATERIAL AND METHODS

The material was collected at two localities (Fig. 1): a spring at Dhiaselos, W of Sparta, N. Taigetos Mts, Peloponnese, 37°04'36.3"N, 22°21'56.4"E, 401 m a.s.l. (*D. hadei*, Fig. 1: H), and a large spring at Agia Para-

skevi, Témbi Valley, N of Larisa, northern Greece, 39°52'46.5"N, 22°35'06.9"E, 16 m a.s.l. (*D. exigua* Fig. 1: E). For phylogenetic analysis we used also sequences of *D. graeca* (Daphne Spring, Témbi Valley, N

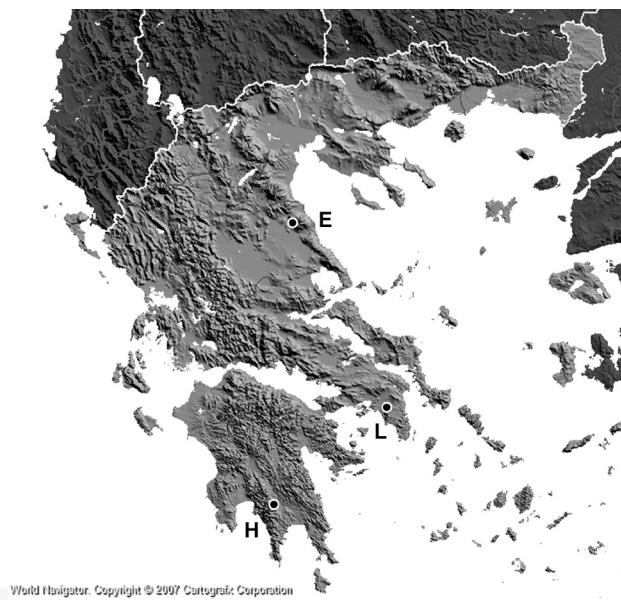


Fig. 1. Localities of the studied populations of *Daphniola*: E – *D. exigua*, Agia Paraskevi and Daphne Spring; H – *D. hadei*, L – *D. louisi*. Figure made using Cartografx Professional Software

of Larisa, 39°53'27.9"N, 22°36'26."E, 16 m a.s.l.; Fig. 1: E) and *D. louisi* (spring at Kesariani, Athens, 37°57'38.6"N, 23°47'54.8"E, 358 m a.s.l.; Fig. 1: L), from GenBank.

The snails were collected with a sieve, washed twice in 80% ethanol and left to stand in it for ca. 12 hours. Afterwards, the ethanol was changed twice in 24 hours and finally, after a few days, the 80% solution exchanged for a 96% one, stored at -20°C. For the morphological study we fixed additional material in 4% formalin and stored it in 80% ethanol.

The shells were cleaned in an ultrasonic cleaner and photographed with a CANON EOS 50D digital camera. Ten adults (five males, five females) were dissected using a NIKON SMZ-U stereomicroscope with a NIKON drawing apparatus, and a NIKON DS-5 digital camera. The protoconchs and radulae were examined using a JEOL JSM-5410 scanning electron microscope (SEM), applying the techniques described by FALNIOWSKI (1990).

DNA was extracted from the foot tissue of each snail. We hydrated the tissue in TE buffer (3×10 min.), extracted total genomic DNA with the SHERLOCK extracting kit (A&A Biotechnology), and dissolved the final product in 20 µm of TE buffer. The PCR reaction was performed with the following primers: LCOI490 (5'-GGTCAACAAATCATAAAGA TATTGG-3') and COR722b (5'-TAAACTTCAGGGTG ACCAAAAATYA-3') for the cytochrome oxidase subunit I (COI) mitochondrial gene (FOLMER et al. 1994). The PCR conditions were as follows: initial denaturation step of 4 min at 94°C, followed by 35 cycles at 94°C for 1 min., 55°C for 1 min., 72°C for 2 min.,

and a final extension of 4 min. at 72°C. The total volume of each PCR reaction mixture was 50 µl. To check the quality of the PCR products we ran 10 µl of the PCR product on 1% agarose gel. The PCR product was purified using Clean-Up columns (A&A Biotechnology) and afterwards 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. All the sequences are deposited in GenBank.

For phylogeny reconstruction we used 12 sequences of rissooid taxa, one sequence of *D. graeca*, and four of *D. louisi*, from GenBank (Table 1). The sequences were aligned by eye using BioEdit 5.0.0 (HALL 1999) and edited with MACCLADE 4.05 (MADDISON & MADDISON 2002). Mutational saturation for the COI dataset was examined by plotting the numbers of transitions and transversions for all the codon positions and separately for the 3rd position against the percentage sequence divergence with DAMBE 5.2.9 (XIA 2000). We used DAMBE 5.2.9 also to perform the saturation test (XIA et al. 2003). It revealed no saturation within *Daphniola*, but for all the studied sequences it revealed a significant degree of saturation in the third position of the sequences. However, to avoid substantial loss of information in the case of closely related species, belonging to *Daphniola* in this case, we did not exclude this position from the dataset and used it for the analysis.

For each maximum likelihood (ML) analysis, we used the best fit model of sequence evolution found by Modeltest v3.06 (POSADA & CRANDALL 1998, POSADA 2003). Following the recommendations of POSADA & BUCKLEY (2004) and SOBER (2002), we chose the best model for each dataset using the Akaike Information Criterion (AKAIKE 1974). We performed ML analyses in PAUP*4.0b10 (SWOFFORD 2002) 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). Nodal support was estimated using the bootstrap approach (FELSENSTEIN 1985). Bootstrap values for ML trees were calculated using 2000 bootstrap replicates, the "fast" heuristic search algorithm, and the same model parameters as for each ML analysis. We ran minimum evolution (ME) (for K2P distances) and maximum parsimony (MP) on PAUP*, and neighbor-joining (NJ) on MEGA4 (TAMURA et al. 2007); the bootstrap approach with full heuristic search, 2000 replicates, was applied to estimate nodal support. K2P distances were calculated with PAUP.



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

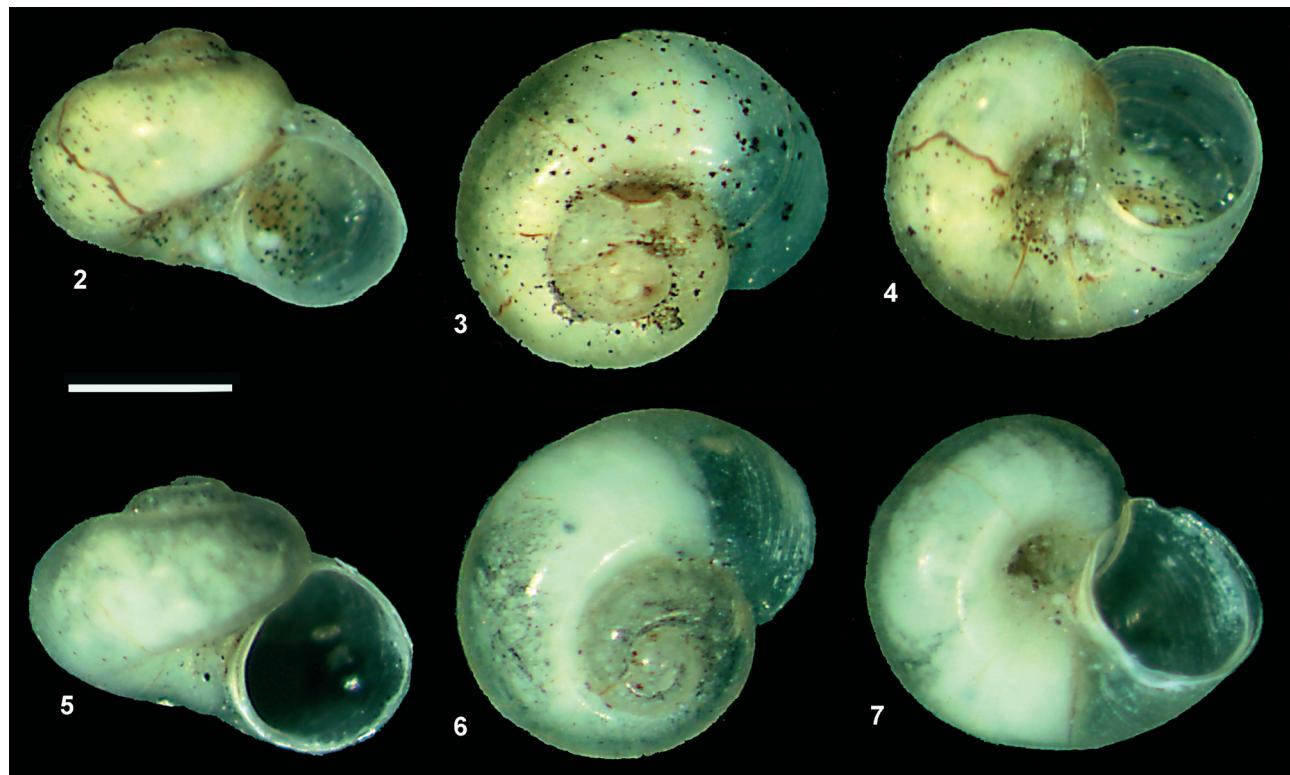
Species	GB#	References
<i>Alzoniella finalina</i> Giusti et Bodon, 1984	AF367650	WILKE et al. (2001)
<i>Anagastina zetavalis</i> (Radoman, 1973)	EF070616	SZAROWSKA (2006)
<i>Daphniola graeca</i> Radoman, 1973	EU047763	FALNIOWSKI et al. (2007)
<i>D. louisi</i> Falniowski et Szarowska, 2000	EF070618	SZAROWSKA (2006)
<i>D. louisi</i> Falniowski et Szarowska, 2000	EU047765-EU047767	FALNIOWSKI et al. (2007)
<i>D. exigua</i> (A. Schmidt, 1856)	JF916465-JF916470	present study
<i>D. hadei</i> (Gittenberger, 1982)	JF916471-JF916479	present study
<i>Dianella thiesseana</i> (Kobelt, 1878)	AY676127	SZAROWSKA et al. (2005)
<i>Graziana alpestris</i> (Frauenfeld, 1863)	AF367641	WILKE et al. (2001)
<i>Grossuana codreanui</i> (Grossu, 1946)	EF061919	SZAROWSKA et al. (2007)
<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF278808	WILKE & DAVIS (2000)
<i>Islamia piristoma</i> Bodon et Cianfanelli, 2001	AF367639	WILKE et al. (2001)
<i>Pseudamnicola lucensis</i> (Issel, 1866)	AF367651	WILKE et al. (2001)
<i>Radomaniola callosa</i> (Paulucci, 1881)	AF367649	WILKE et al. (2001)
<i>Rissoa labiosa</i> (Montagu, 1803)	AY676128	SZAROWSKA et al. (2005)
<i>Sadleriana fluminensis</i> (Küster, 1853)	AY273996	WILKE et al. (2001)
<i>Trichonia kephalovrissonia</i> Radoman, 1973	EF070619	SZAROWSKA (2006)

RESULTS

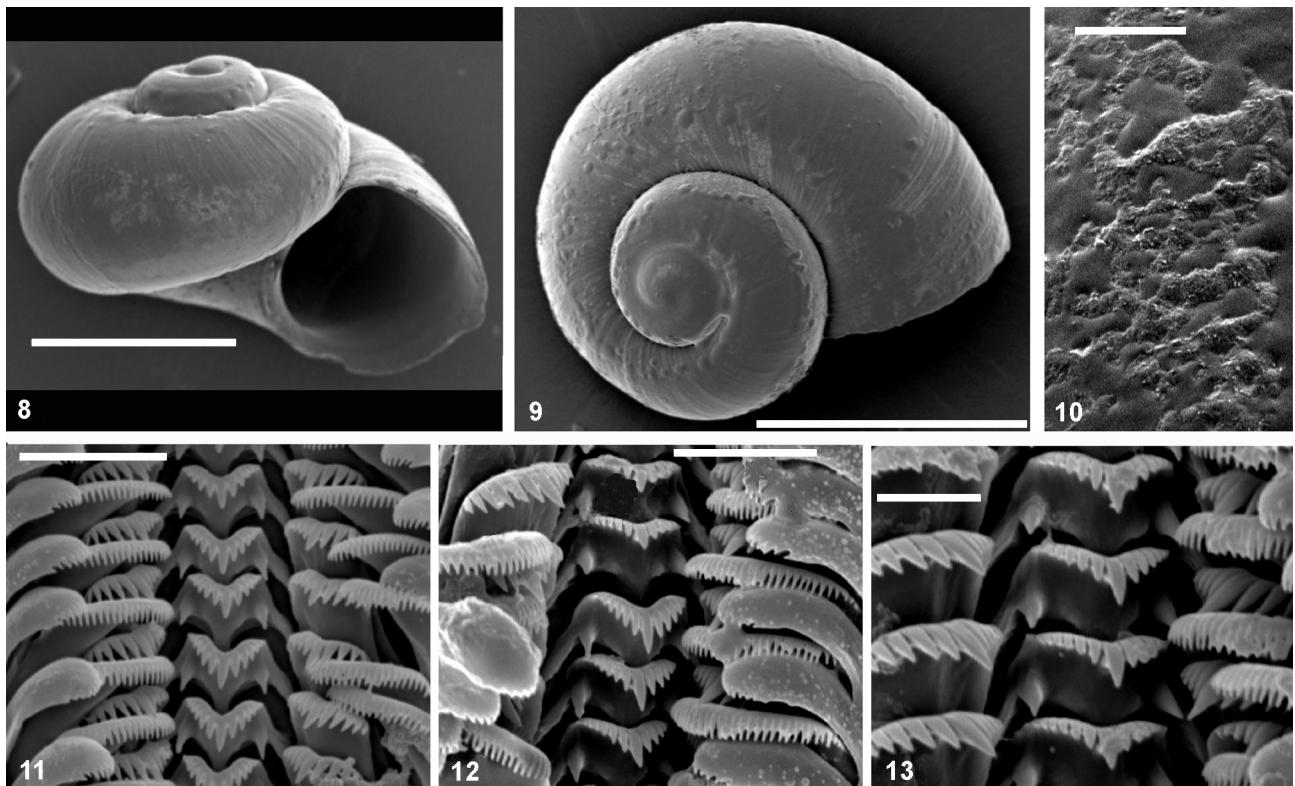
MORPHOLOGY OF *DAPHNIOLA HADEI*

Shell (Figs 2–7, 8–9) transparent, valvatiform, of $2\frac{1}{4}$ – $2\frac{1}{2}$ rapidly growing whorls; shell height:

0.84–0.85 mm; shell diameter: 1.14–1.15 mm; mouth height: 0.55–0.57 mm, mouth width: 0.52–0.54 mm; protoconch diameter: 0.52 mm. Protoconch sculpture (Fig. 10) reticulate.



Figs 2–7. Shells of *Daphniola hadei*. Scale bar 500 µm



Figs 8–13. SEM pictures of shell and radula of *Daphniola hadei*: 8–9 – shell, 10 – protoconch, 11 – 13 radula. Scale bars: 8–9 – 500 µm, 10–12– 10 µm, 13 – 5 µm

Body pigmentless, eyes present, rather small. Ctenidium present, number of ctenidial lamellae: 10–11. Osphradium elongate. Radula (Figs 11–13) taenioglossate, central tooth formula:

$$\frac{5-1-5}{1-1},$$

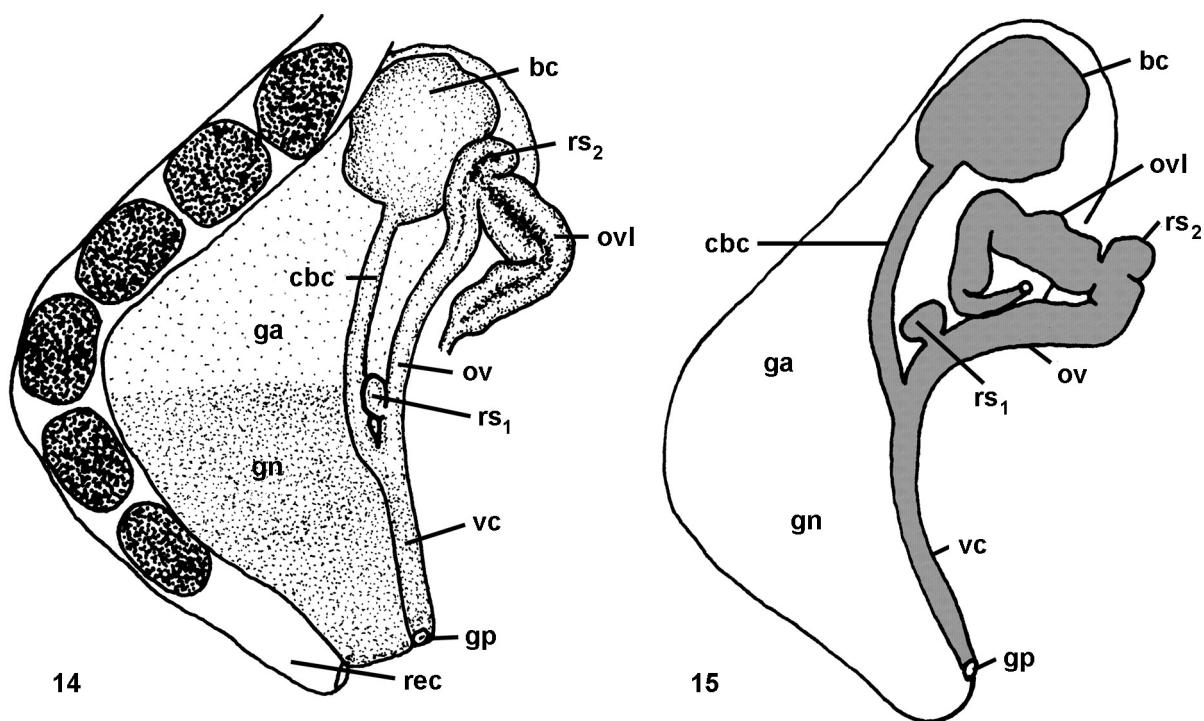
central cusp conspicuously longer than lateral cusps, some basal cusps look like merged of two cusps of uneven length (Figs 12–13). Lateral tooth formula: 4–1–5 or 4–1–6, one cusp conspicuously longer than others. Intestine course broadly u-shaped (Fig. 14). Female reproductive organs (Figs 14–15) with coil of oviduct, two seminal receptacles (rs_1 , rs_2), and bursa copulatrix. Penis (Figs 16–18) pigmentless with long and narrow filament and small and blunt lobe.

MOLECULAR PHYLOGENY

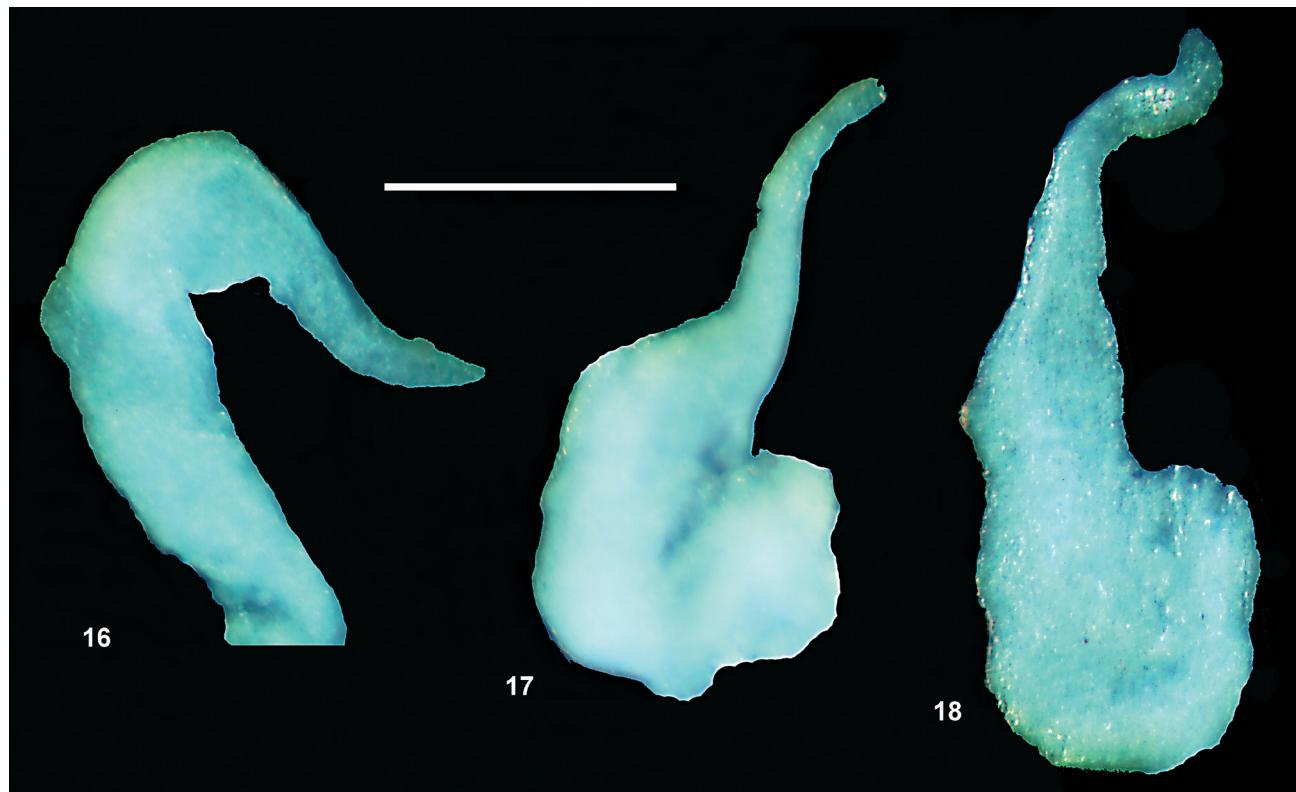
The Akaike Information Criterion (AIC), obtained with ModelTest, selected the model K81uf+I+Γ, with base frequencies: A=0.2948,

C=0.1335, G=0.1473, T=0.4244; substitution rate matrix: [A–C]=1.0000, [A–G]=7.9929, [A–T]=0.3377, [C–G]=0.3377, [C–T]=7.9929, [G–T]=1.0000, proportion of invariable sites: (I)=0.5393, and Γ distribution with the shape parameter = 0.7889.

The phylogenetic ML analysis resulted in a tree (Fig. 19), the topology of which is very similar to the ones inferred with NJ, ME and MP (not presented). Like the above mentioned morphological characters, the tree (Fig. 19) indicates that *D. graeca* belongs to *D. exigua*, and that each of the taxa: *D. louisi*, *D. exigua* and *D. hadei*, represents a highly supported monophyletic group of a species level (except ML support of *D. hadei* being 67 only). Each technique grouped together the three species, this grouping, however, was not supported with ML (but other techniques resulted in supports 77–82). In the tree the sister clade of *Daphniola* is the clade of *Grossuana* Radoman, 1973/*Trichonia* Radoman, 1973 (Fig. 19). The K2P distances between the three *Daphniola* species were all about 0.09–0.11.



Figs 14–15. Renal and pallial section of female reproductive organs of *Daphniola hadei*: bc – bursa copulatrix, cbc – canal of bursa copulatrix, ga – albumen gland, gn – capsule gland, gp – gonopore, ov – oviduct, ovl – coil (loop) of oviduct, rec – rectum, rs₁ – proximal seminal receptacle, rs₂ – distal seminal receptacle



Figs 16–18. Penes of *Daphniola hadei*. Scale bar 0.25 mm

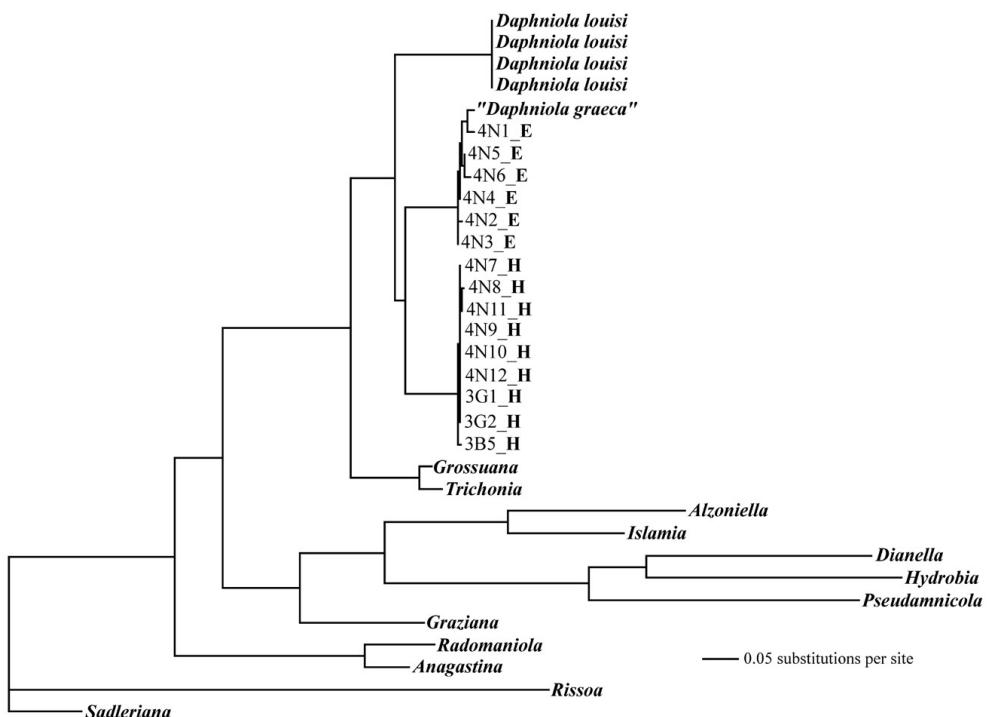


Fig. 19. Maximum likelihood phylogram for COI sequences, ML (in bold), NJ, ME and MP supports >50 given for main clades; E – *Daphniola exigua*, H – *Daphniola hadei*

DISCUSSION

The type locality of *D. hadei* (GITTENBERGER 1982), and our locality at Dhiaselo, W of Sparta, were situated close enough to each other (about 40 km) for one hydrobiid species to occur at both of them. The shell presented in our Fig. 2 looks much like the holotype of *Horatia hadei* (GITTENBERGER 1982: fig. 1). In the specimen in Fig. 10 the protoconch sculpture is worn and only small fragments are preserved, but what is left of it resembles the reticulate protoconch sculpture of both *H. hadei* (GITTENBERGER 1982: fig. 2) and *Daphniola* (FALNIOWSKI & SZAROWSKA 2000: figs 5–6, SZAROWSKA 2006: figs 70–71).

The radula (Figs 11–13) resembles in general the radulae of *D. louisi* (FALNIOWSKI & SZAROWSKA 2000: figs 9–12) and *D. exigua* (SZAROWSKA 2006: figs 133–134). A characteristic trait not found in the radulae of the two *Daphniola* species is the strange, “merged” appearance of some basal cusps (Figs 12–13).

The female reproductive organs and penis shown in Figs 14–18 do not differ much from the corresponding organs of *Daphniola*, portrayed in RADOMAN (1983: fig. 20), FALNIOWSKI & SZAROWSKA (2000: figs 15, 18–29) and SZAROWSKA (2006: fig. 225).

Neither morphology (RADOMAN 1973, 1983, BODON et al. 2001, SZAROWSKA 2006), nor molecular data (SZAROWSKA 2006) confirm closer relationships between *Horatia* Bourguignat, 1887, and *Daphniola*

Radoman, 1973. Thus the specimens from Dhiaselo, morphologically similar and molecularly close to *Daphniola exigua* and *D. louisi*, must belong to the same genus. There are four *Daphniola* localities studied molecularly so far, out of which Agia Paraskevi and Daphne Spring are situated very close (a few km) to each other, and harbour one species (*D. exigua*). The remaining two localities (Kessariani and Dhiaselo) harbour a distinct species each. The K2P distances between the populations are like typical distances between congeneric species within the Rissooidea (FALNIOWSKI et al. 2007, 2009, SZAROWSKA et al. 2007). FALNIOWSKI et al. (2007) found molecularly an estimate of the time of divergence between *D. exigua* and *D. louisi*, which suggested the divergence time coinciding with the Pliocene flooding that terminated the Messinian salinity crisis. In the area of the present Mediterranean Sea the latter affected directly the marine fauna, but also expanded the land area. The Pliocene flooding fragmented land habitats which consequently may have brought about several cases of vicariant speciation, an example of which may be the *Daphniola* species.

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