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A new hydrobiid species (Caenogastropoda, Truncatelloidea) from insular Greece

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Abstract

Daphniola dione **sp. nov.**, a valvatiform hydrobiid gastropod from Western Greece, is described based on conchological, anatomical and molecular data. *D. dione* is distinguished from the other species of the Greek endemic genus *Daphniola* by a unique combination of shell and soft body character states and by a 7–13% COI sequence divergence when compared to congeneric species. The only population of *D. dione* inhabits a cave spring on Lefkada Island, Ionian Sea.

Key Words

Freshwater diversity, Lefkada Island, taxonomy, valvatiform Hydrobiidae

Introduction

The Mediterranean Basin numbers among the first 25 Global Biodiversity Hotspots due to its biological and ecological biodiversity and the plethora of threatened biota (Myers et al. 2000). The region from the Iberian Peninsula to the Balkans is characterized by the high diversity and endemicity of freshwater flora and fauna (Cuttelod et al. 2008; De Figueroa et al. 2013; Smith et al. 2014) and the biota of Greek freshwater ecosystems are widely recognized as a major component of this diversity (Glöer and Maassen 2009; Glöer et al. 2010).

Among freshwater molluscs, the family Hydrobiidae (hydrobiids) is one of the largest and the most diverse gastropod family throughout the Mediterranean region (Cuttelod et al. 2008). The valvatiform hydrobiids is a group of minute gastropods with depressed trochiform shells resembling those of the genus *Valvata* O. F. Müller, 1773 (Heterobranchia, Valvatoidea). The species of this group are highly endemic and phylogenetically not related (Radea 2018). In Europe, 37 valvatiform genera have been described so far. More than 60% of these genera inhabit the freshwater systems of the Balkan Peninsula (Radea 2018; Boeters et al. 2019; Delicado et al. 2019).

The Mediterranean Basin, the Balkan, the Iberian and the Italian Peninsulas seem to be evolutionary centers of valvatiform hydrobiids throughout, especially of the stygophile and stygobiont taxa (e.g. Radoman 1983; Bodon et al. 2001; Arconada and Ramos 2001, 2002, 2006, 2007; Arconada et al. 2007; Callot-Girardi and Boeters 2012; Radea et al. 2016a; Quiñonero-Salgado and Rolán 2017; Boeters et al. 2019). To date, nine valvatiform hydrobiid genera live in lotic and lentic habitats of Greece, and five of them, namely *Daphniola* Radoman, 1973, *Graecoarganiella* Falniowski & Szarowska, 2011, *Isimerope* Radea & Parmakelis, 2013, *Myrtoessa* Radea, 2016 and *Pseudoislamia* Radoman, 1979, are endemic to Greece.

Daphniola (type species *Daphniola graeca* Radoman, 1973), which is the only non-narrow range Greek endemic valvatiform genus, was the first extant endemic valvatiform hydrobiid described from Greece. *Daphniola* is distributed in the eastern part of the central and southern main-

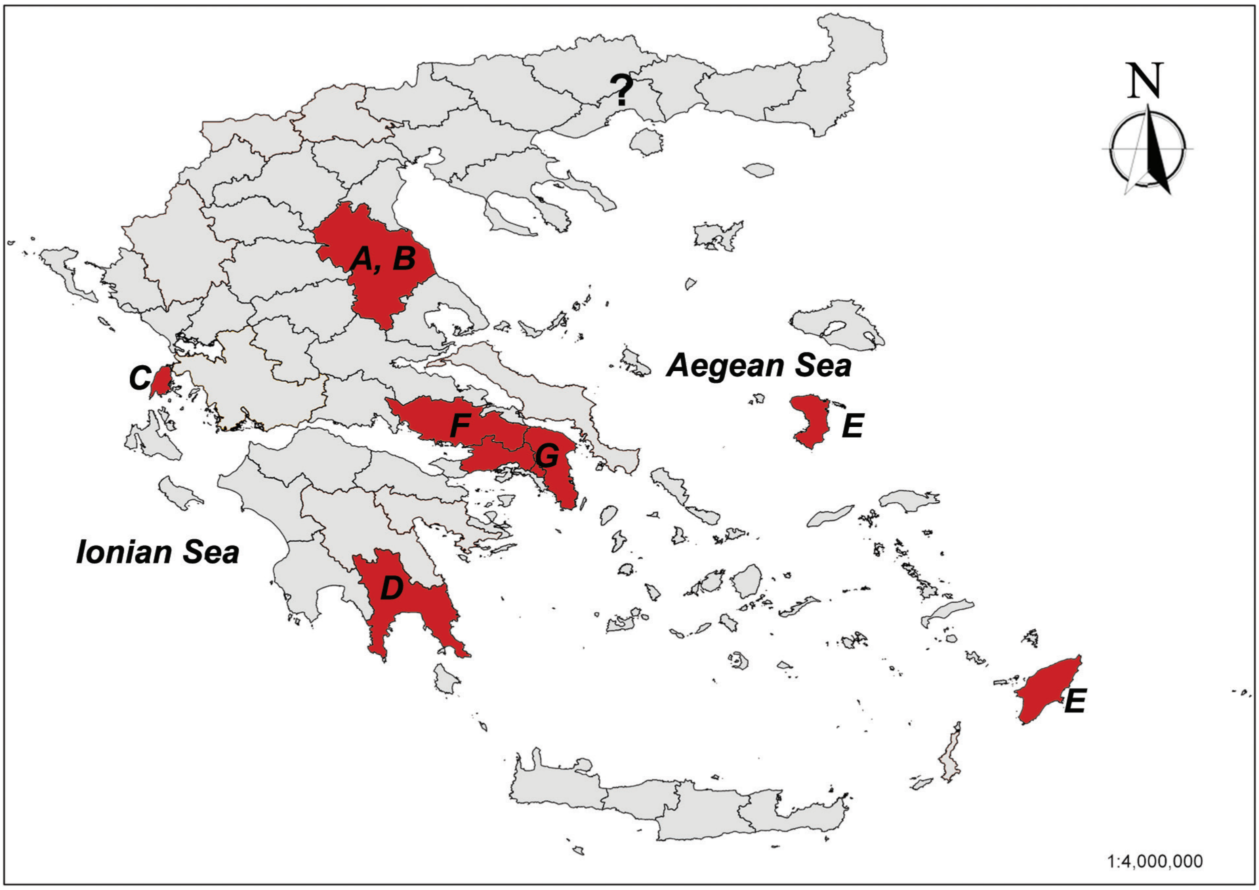

Figure 1. The distribution of the Greek endemic genus *Daphniola*. **A.** *D. exigua* and **B.** *D. magdalenae*, Larissa prefecture; **C.** *D. dione*, Lefkada Island; **D.** *D. hadei*, Lakonia prefecture; **E.** *Daphniola* sp., Rhodos and Chios Islands; **F.** *D. eptalophos* Parnassos Mt.; **G.** *D. louisi* Attiki prefecture; (?) the record of *D. exigua* (as *Horatia* (*Daphniola*) *exigua pangaea*) in Kavala prefecture (Reischütz 1984) should be re-examined because it is based on empty shells identification.

land and comprises five species: *D. exigua* (A. Schmidt, 1856) (as *Horatia* (*Daphniola*) *exigua* in Schütt 1980), *D. louisi* Falniowski & Szarowska, 2000, *D. eptalophos* Radea, 2011, *D. hadei* (Gittenberger, 1982) and *D. magdalenae* Falniowski, 2015. *D. graeca* Radoman, 1973 is accepted as synonym of *D. exigua* (MolluscaBase 2020). Another sequenced undescribed *Daphniola* species has been recorded in Chios and Rhodos Islands, Aegean Sea (Szarowska et al. 2014). Two *Daphniola* species, *D. exigua* and *D. louisi*, have been evaluated as Endangered and Critically Endangered in the IUCN Red List of Threatened Species, respectively (IUCN 2020.2).

Herein, we describe and analyze phylogenetically a new *Daphniola* species from Lefkada Island, Ionian Sea and delineate morphological and anatomical characters, which are evident and efficient to support the existence of the new species.

Materials and methods

Live specimens and empty shells of the new taxon were found on Lefkada Island, Ionian Sea (Fig. 1). All the material was collected by hand from a spring spouting inside a cave. Two specimens were stored at -20 °C and preserved in 100% ethanol to be used in subsequent molecular analyses, whereas the remaining specimens were preserved unrelaxed in 70% ethanol for further morphological and anatomical analysis.

DNA extraction, amplification and sequencing

Due to the small body size, the entire animals were used for genomic DNA isolation. DNA was extracted using the CTAB protocol as described in Parmakelis et al. (2003). Amplification of a fragment of approximately 700 bp long of the mitochondrial cytochrome C oxidase subunit I (COI), was carried out using the universal primers LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198 5′-TAAACTTCAGGGTGACCAAAAAAT-3′ (Folmer et al. 1994). Each PCR was performed in a 25 uL volume, where 2 uL of template DNA were mixed with 0.2 mM dNTPs, 3.5 mM MgCl2, 0.4 µM of each primer, and 0.5 unit of Taq Polymerase (Kappa). Thermocycling was performed in a BioRad MyCycler Thermal Cycler. The PCR program comprised an initial denaturation at 95 °C for 3 min, followed by 42 cycles of 15 sec at 94 °C, 1 min at 42 °C, and 1.5 min at 72 °C. The cycling was ended with 10 min sequence extension at 72 °C. PCR amplicons were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH and Co KG Neumann-Neander, Germany). Both strands of the PCR product were sequenced. The primers in the sequencing reactions were the same as in the amplification procedure. Out of the two specimens sequenced, only one generated a high-quality COI sequence.

Sequence alignment and genetic data analysis

Both strands of the sequenced fragment were combined in a single contig and edited with CodonCode Aligner v. 2.06. The generated sequence has been deposited in GenBank. To reconstruct the phylogenetic relationships of our specimen, to other published *Daphniola* species, *Daphniola* COI sequence data were obtained from GenBank. *Daphniola eptalophos*, which was recorded and described for the first time in 2011 from Mt. Parnassos, was not included in the phylogenetic analysis due to the lack of COI sequence data available for the species (Radea 2011, 2018). Phylogenetic relationships were inferred using Bayesian Inference (BI) framework. In order to select a proper outgroup taxon several preliminary analyses involving other hydrobiid genera, were performed. *Corbellaria celtiberica* Girardi & Boeters, 2012 was a valvatiform species leading to a well resolved phylogenetic tree, and thus was ultimately used as the outgroup species of our analyses. BI analysis was performed using MrBayes v.3.2.2 (Ronquist et al. 2012) following the selection of the best-fit model of molecular evolution using Partition Finder v2.1.1 (Lanfear et al. 2012).

The nucleotide substitution model suggested by Partition Finder was the HKY +G. No codon partition was implemented. For the BI analysis, the number of generations was set to 2×10^6. The first 25% of trees were discarded as burn-in and the analysis was summarized on a 50% majority-rule tree. Support for the nodes was assessed by posterior probabilities. Following the completion of the phylogenetic analysis, a rogue taxa analysis was performed using the RogueNaRok webserver (Aberer et al. 2013). Estimates of evolutionary divergence between species were estimated using both the p-distance and the HKY substitution model (Hasegawa et al. 1985) as implemented in SeaView v.4 (Gouy et al. 2010). The genetic distance estimates are presented in Table 2.

Shell morphology and soft body anatomy

Shell characters (shell height and width, aperture height and width) were taken from 10 specimens using the micrometer of a stereomicroscope Stemi 2000-C, Zeiss, Germany. Four ratios were generated from the raw data; these were SH/SW, AH/AW, SH/AH and SW/AW (see abbreviations at the end of this section). Before dissection, the shells were removed by soaking in Perenyi solution (Clayden 1971). Shell and soft body features were photographed using a Canon EOS 1000D camera attached to the stereomicroscope. The new taxon was compared with specimens of *D. exigua*, *D. eptalophos*, *D. graeca* and *D. louisi* which have been collected from

Table 1. Species, families, locality details, GenBank accession numbers and publication references for COI sequences used in the phylogenetic analysis of this study. The hydrobiid species *Corbellaria celtiberica* was used as outgroup.

Species	Family	Country	Region	Sampling locality	GenBank accession number	Reference
Daphniola exigua	Hydrobiidae	Greece	Thessaly	Large spring at Agia Paraskevi, Tembi Valley, N of Larisa	JF916467	Falniowski and Szarowska 2011
Daphniola graeca	Hydrobiidae	Greece	Thessaly	Daphne Spring at Tembi Valley	EU047763	Falniowski et al. 2007
Daphniola hadei	Hydrobiidae	Greece	Peloponnese	Spring at Dhiaselo, W of Sparta, N Taigetos Mts.	JF916479	Falniowski and Szarowska 2011
Daphniola louisi	Hydrobiidae	Greece	Attiki	Spring at Agia Paraskevi in Tembi Valley	EU047766	Falniowski et al. 2007
Daphniola magdalenae	Hydrobiidae	Greece	Thessaly	Melissotripa Cave	KT825580	Falniowski and Sarbu 2015
Daphniola dione	Hydrobiidae	Greece	Ionian Islands	Lefkada island	MW581160	Present study
Daphniola sp.	Hydrobiidae	Greece	Dodecanese Islands	Rhodes island	KM887915	Szarowska et al.2014
Corbellaria celtiberica	Hydrobiidae	Spain		Manubles River, Soria	MH350207	Delicado et al. 2019

Table 2. Pairwise genetic distances between *Daphniola* species and undescribed specimens estimated using both the p-distance and the HKY substitution model (p-distance/HKY).

Species	1	2	3	4	5	6
1. *D. exigua* JF916467						
2. *D. graeca* EU047763	0.02/0.02					
3. *D. louisi* EU047766	0.01/0.01	0.02/0.01				
4. *D. dione* sp.n. MW581160	0.07/0.07	0.08/0.09	0.07/0.08			
5. *Daphniola* sp. KM887915	0.09/0.09	0.10/0.11	0.10/0.10	0.09/0.08		
6. *D. magdalenae* KT825580	0.14/0.15	0.14/0.16	0.14/0.14	0.13/0.15	0.12/0.15	
7. *Corbellaria celtiberica* MH350207	0.18/0.21	0.18/0.21	0.18/0.20	0.17/0.20	0.17/0.21	0.21/0.25

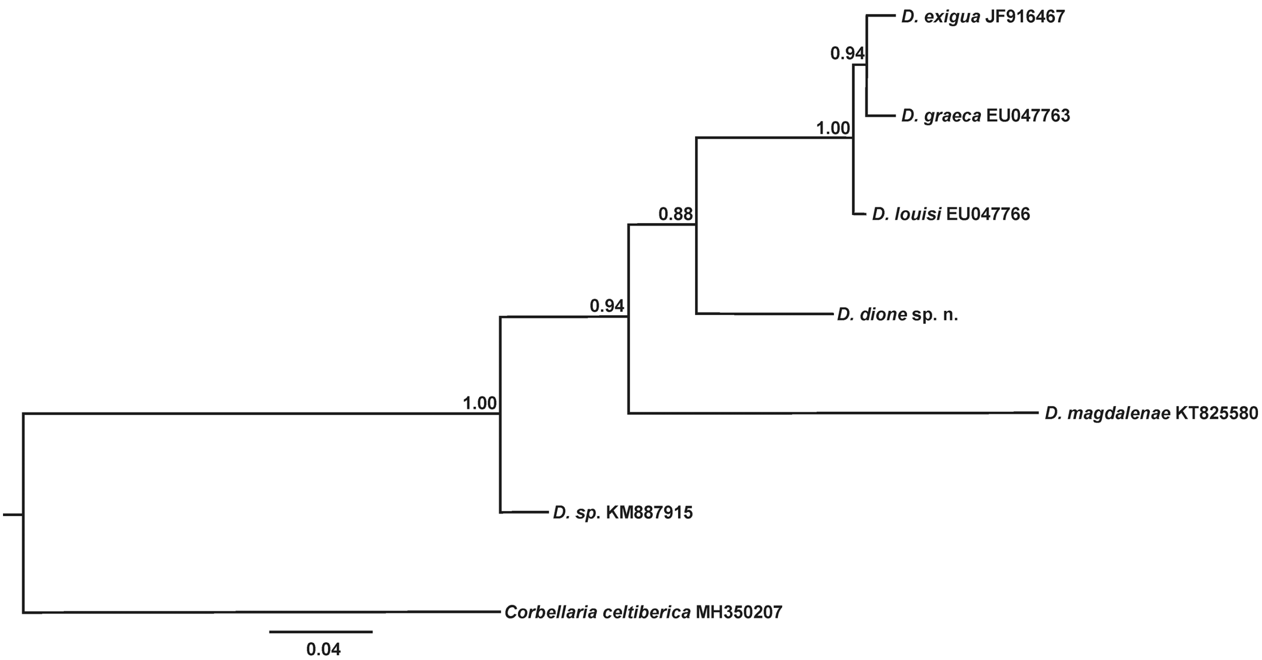

Figure 2. 50% majority-rule consensus tree of the BI analysis. Number on nodes denote the posterior probability values. Scale bar indicates number of substitutions per site. The valvatiform species *Corbellaria celtiberica* was used as outgroup.

their respective loci typici. These specimens are included in the personal collection of C. Radea deposited in the Section of Ecology and Systematics, Department of Biology, National and Kapodistrian University of Athens. Morphological terminology follows that of Hershler and Ponder (1998) and thus the character states may be different from those given by the authors which have described *Daphniola* species.

Abbreviations used in the text, Tables and Figures are: Ag = albumen gland, AH = aperture height, AW = aperture width, Bc = bursa copulatrix, Bd = bursal duct, Cg = capsule gland, CV* $(1+1/4n)*SD/\bar{X}$ = coefficient of variation corrected for sample size (Sokal and Rohlf 1995), GNHM = Goulandris Natural History Museum, Max = maximum, Min = minimum, n = number of specimens, NW = Number of whorls, NKUA = National & Kapodistrian University of Athens, Ov = Coiled oviduct, Pd = penial duct, Pl = penial lobe, rs1 = distal seminal receptacle, rs2 = proximal seminal receptacle, SD = standard deviation, SH = shell height, SW = shell width, $\bar{X}$ = mean.

Results

Phylogenetic tree and evolutionary divergence

The phylogenetic relationships of *Daphniola* species described so far are reflected in the tree shown in Fig. 2.

In a preliminary phylogenetic analysis, the species *Daphniola hadei* was identified as a rogue taxon. Thus, it was removed from the analysis since it was significantly distorting the phylogenetic information included in the dataset. To date, *D. hadei* is considered a valid *Daphniola* species, and for reasons of comparison with the new species described herein, it was maintained in the morphological assessment of the species. The tree obtained after excluding *D. hadei* from the analysis is quite well resolved with the majority of nodal support being above 0.90. *D. exigua* and *D. graeca* appear to be very closely related, whereas *D. louisi* is firmly associated with the former group of species. *D. dione* sp.n. seems to be a separate lineage that is well separated from the exigua-graeca-louisi group as well as from *D. magdalenae*. The latter is directly, albeit distantly, related to all the former. The most phylogenetically distant species of all *Daphniola* seems to be *Daphniola* sp. from Rhodes. The relationships reflected in the phylogenetic tree, are corroborated by the pairwise genetic distances of the sequences (Table 2) as well. *D. dione* is separated from all the other *Daphniola* species by distances ranging from 7% (*D. exigua*) to 13% (*D. magdalenae*).

Systematic description

Family Hydrobiidae Stimpson, 1865

Daphniola dione sp. nov.

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Figs 3, 4

Etymology. The specific name (in apposition) derives from Greek mythology: Dione, (Διώνη in Greek), was the mother of the goddess Aphrodite according to the Greek poet Homer, author of Iliad and Odyssey.

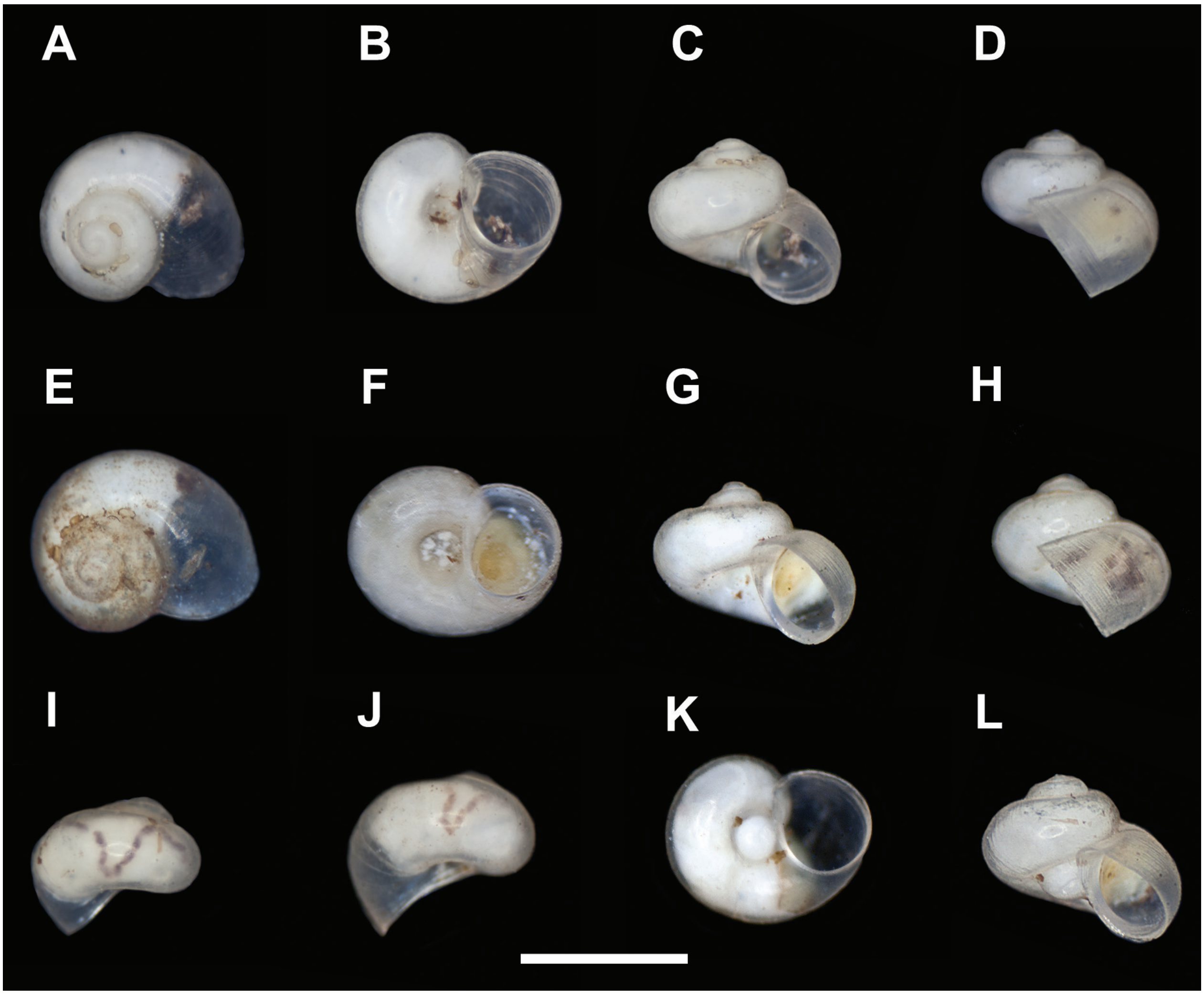

Figure 3. Shells of *Daphniola dione* sp. nov. **A.**, **E.** Dorsal view; **B.**, **F.**, **K.** Ventral view; **C.**, **D.**, **G.**, **H.**, **L.** Lateral view; **I.**, **J.** Shape of rectum in females and males respectively. Scale bar 1 mm.

Diagnosis. Shell minute (maximum height 0.98 mm, maximum width 1.31 mm), valvatiform; soft body without any pigmentation; eyes small; penis long, narrow, tapered, with wider wrinkled proximal portion, filamentous distal portion and an obtuse outgrowth on the left side forming an acute angle with the penis distal portion; female genitalia with large pyriform bursa copulatrix, renal oviduct coiled in a equilateral triangle. Differentiated from *D. exigua* by its smaller size, wider and more open umbilicus, paler operculum, lack of pigmentation in soft body, pyriform-shaped bursa copulatrix, wider bursal duct, triangle-shaped oviduct and obtuse rather than pointed outgrowth at the distal penis portion. Differentiated from *D. louisi* by its smaller size, lack of pigmentation in soft body, large, pyriform and protruding bursa copulatrix, triangle-shaped oviduct, nearly centered penial duct, and more prominent penial outgrowth. Differentiated from *D. eptalophos* by its smaller size, paler operculum, lack of pigmentation in soft body, protruding bursa copulatrix, wider bursal duct, triangle-shaped oviduct, more pointed penial apex, outgrowth on distal rather than proximal penial portion and nearly centered penial duct. Differentiated from *D. hadei* by its larger size, wider and more open umbilicus, pyriform and protruding bursa copulatrix, more pointed penial apex and more prominent penial outgrowth. Differentiated from *D. magdalenae* by its smaller size, wider and more open umbilicus, paler operculum, protruding bursa copulatrix, more pointed penial apex and presence of eyes.

Type-locality. Cave spring on Lefkada Island, Ionian Sea, Greece, 38.722532°N, 20.682713°E, 240 m a.s.l., 15.V.2017, K. Bakolitsas leg.

Type material. ***Holotype***. Ethanol-fixed specimen, GNHM 39587. ***Paratypes*** (from the same lot). Two ethanol-fixed specimens, GNHM 39588. Seven ethanol-fixed mature specimens dissected for anatomical study. Three mature and seven immature specimens are in the personal collection of C. Radea deposited in the Section of Ecology and Systematics, Department of Biology, NKUA.

Table 3. Shell morphometry of *Daphniola dione* sp. nov. Measurements are in mm. For abbreviations see the section of Materials and methods.

n=10	SH	SW	AH	AW	SH/SW	AH/AW	SH/AH	SW/AW
Min	0.83	1.14	0.47	0.52	0.68	0.79	1.62	2.05
Max	0.98	1.31	0.53	0.61	0.80	1.02	2.08	2.21
$\bar{X}$	0.91	1.21	0.51	0.57	0.75	0.90	1.78	2.13
SD	0.04	0.05	0.03	0.03	0.03	0.07	0.12	0.06
CV	0.05	0.05	0.06	0.06	0.05	0.08	0.07	0.03

Description. *Shell* (Fig. 3A–L). Valvatiform with up to 3.25 whorls, thin, colorless, transparent and crystalline when fresh, finely striated; spire more or less depressed; whorls rounded, regularly growing with shallow sutures. Measurements are given in Table 3. Periostracum light cream-colored; aperture adhering to the last whorl, prosocline, roundish to ovate; peristome continuous, slightly thickened at columellar margin, the outer margin simple; umbilicus open, deep, wide so that the first whorls can be seen through it (Fig. 3B, F).

Operculum (Fig. 3F). Ovate, thin, pliable, corneous, paucispiral, yellowish darker at the nucleus, with weakly convex inner face without any peg, nucleus sub-central.

Soft body pigmentation (Fig. 3A–L). Soft body totally unpigmented and visible under the transparent shell; snout longer than wide, parallel-sided with medium distal lobation; eye spots very small.

Nervous system. Cerebral ganglia of the same size, white-colored; supraoesophageal and suboesophageal ganglia of the same size, smaller than cerebral ganglia, white-colored; supraoesophageal connective longer than suboesophageal connective.

Ctenidium-Osphradium. Ctenidium with ca 8–12 lamellae. Osphradium of intermediate width, opposite approximate middle of ctenidium.

Digestive system. Radula very fragile, not investigated; stomach unpigmented. Style sac smaller than stomach, not protruding to the intestinal loop; rectum (Fig. 3I, J) with V-shaped bend, in some specimens with irregular V-shaped bend. The V-shaped bend is narrower in males (Fig. 3J) than in females (Fig. 3I). Faecal pellets are longitudinally packed.

Male reproductive system (Fig. 4A, B) Penis whitish, medium-sized, narrow, long, gradually tapering bearing an obtuse outgrowth on the left side forming an acute angle with the penis distal portion; distal portion filamentous and usually bent; proximal portion bent upon itself and wrinkled; base of penis of intermediate width, its attachment area behind the right eye; penial duct strongly undulating especially in the proximal penis portion, near outer edge positioned, penial opening terminal; prostate like an elongate bean.

Female reproductive system (Fig. 4C). Albumen and capsule glands very small, total mean height 0.38 mm (three specimens); bursa copulatrix large-sized, pyriform, posteriorly positioned and fully protruding from the posterior end of the albumen gland with longitudinal orientation relative to albumen gland; bursal duct anterior, wide and longer than bursa length; coiled oviduct unpigmented, well-developed forming an equilateral triangle with a vertical, V-shaped primary loop; two seminal receptacles; distal seminal receptacle (rs_1) very small, globular without duct; proximal seminal receptacle (rs_2) larger than distal one and smaller than bursa copulatrix, pyriform, with very short duct and a pink pearly shine, lying over the renal oviduct and against bursa copulatrix; an egg

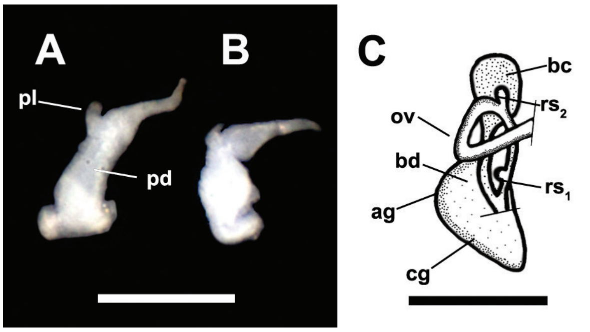

Figure 4. A., **B**. Penis (dorsal aspect); **C**. Female genitalia. Abbreviations are given in the section of Materials and Methods. Scale bar 0.5 mm.

Table 4. Morphometry of *Daphniola* species. Measurements are in mm. For abbreviations see Materials and methods.

Species	NW	SH	SW	AH	AW
D. dione sp. nov.	2.75–3.25	0.83–0.98	1.14–1.31	0.47–0.55	0.52–0.61
D. eptalophos	3.25–3.50	0.90–1.25	1.10–1.90	0.50–0.80	0.50–0.75
D. exigua	3.00–3.50	1.10–1.52	1.10–1.40	0.60–0.80	0.60–0.76
D. hadei	2.25–2.50	0.84–0.85	1.14–1.15	0.55–0.57	0.52–0.54
D. louisi	3.50	1.09–1.45	1.17–1.69	0.59–0.98	0.59–0.79
D. magdalenae	3.50–3.75	2.34	-	1.35	1.28

Table 5. Morphological and anatomical diagnostic characters of *D. dione* sp. nov. and its congeneric species.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
D. dione sp. nov.	0	0	0	2	1	1	2	3	0	0	1	0	1	1	1
D. eptalophos	0	1	2	1,2	0	0	1	1	1	2	1	2	0	1	0
D. exigua	1	2	2	0,1	1	1	0	0	1	0	0	1	0	1	0
D. hadei	1	0	0	1	0	0	–	–	–	1	2	0,1	–	1	0
D. louisi	0	0	1	1	0	0	2	2	2	0	2	0	0	1	0
D. magdalenae	1	3	0	2	0	0	–	–	–	1	1	0	–-	0	1

Umbilicus: wide, open (0); semi-open (1)
Operculum: light yellowish darker on the nucleus (0); orange; darker on the nucleus (1); yellow; orange on the nucleus (2); pink reddish (3)
Soft body pigmentation: no pigmentation (0); light pigmentation (1); pigmentation (2)
Bursa copulatrix shape: globular (0); ovoid (1); pyriform (2)
Bursa copulatrix size: medium (0); large (1)
Bursa copulatrix position relative to posterior end of albumen gland: non-protruding (0); protruding (1)
Bursal duct width: narrow (0); medium wide (1); wide (2)
Coiled oviduct shape: circular-ovoid (0); Ovoid (1); ellipsoid, transverse relative to albumen gland (2); triangular (3)
Primary oviduct loop shape: V (0); U (1); circular (2)
Penis pigmentation and shape: unpigmented with pointed apex (0); unpigmented with long, narrow filament (1); pigmented with pointed apex or short filament (2)
Penial outgrowth shape: prominent; forming a right angle with the distal part of penis (0); prominent; forming an acute angle with the distal part of penis (1); slightly prominent (2)
Penial outgrowth position: distal penis portion (0); medial (1); basal penis portion (2)
Penial duct: near center (0); near outer edge (1)
Eyes: absent (0); present (1)
Habitat: spring (0); cave spring (1)
–: No data

capsule with a single egg was found inside the umbilicus of one specimen (Fig. 3K, L).

Morphometric data along with conchological and anatomical characters of the nominal *Daphniola* species are provided in Tables 4 and 5, respectively.

Distribution and habitat. So far, the distribution of *Daphniola dione* sp. nov., is restricted to the type locality. The live specimens of the new species were found crawling on the roots of woody plants reaching the bottom of the spring.

The dichotomous key to species of the genus *Daphniola*

1	Soft body totally unpigmented	2
–	Soft body more or less pigmented	4
2	Bursa copulatrix fully protruding from the posterior end of the albumen gland	*D. dione*
–	Bursa copulatrix non-protruding from the posterior end of the albumen gland	3
3	Penial lobe slightly prominent	*D. hadei*
–	Penial lobe prominent forming an acute angle with the distal part of penis	*D. magdalenae*
4	Penis black with tapered distal portion and unwrinkled proximal portion	*D. eptalophos*
–	Penis unpigmented with tapered distal portion and wrinkled proximal portion	5
5	Penial lobe slightly prominent*Daphniola dione* sp. nov. by original designation	*D. louisi*
–	Penial lobe prominent forming a right angle with the distal part of penis	*D. exigua*

Discussion

Based on the phylogenetic relationships and the COI genetic distances, it can be claimed that *D. dione* sp. nov. is a well-established and separated genetic lineage and should be considered as different species. The genetic distances (p-distance: 7–13%, HKY: 7–14% Table 2) of *D. dione* from the other *Daphniola* species are much higher than those for conspecific populations of *Daphniola* (p-distance: 1.3–2.7%, Falniowski et al. 2007) and are either higher or fall within the range of intra-generic variation estimated for various hydrobiids: *Hauffenia* Pollonera 1898 (p-distance: 6.7% in Falniowski and Szarowska 2015), *Agrafia* Szarowska & Falniowski, 2011 (p-distance: 9.5% in Grego et al. 2017), *Islamia* Radoman, 1973 (p-distance: 11.9% in Beran et al. 2016) and *Pseudamnicola* Paulucci, 1878 (p- distance: 2.9–7.7% in Radea et al. 2016b).

D. dione exhibits a unique combination of shell morphometry and anatomical character stages, not allowing its inclusion in any other known *Daphniola* species. Thus, the morphology and anatomy corroborate the results of the molecular analysis supporting the distinctiveness of this taxon.

Some interesting remarks derive from the data presented in Table 5. A notable variability is observed both in penis and penial lobe shape of *Daphniola* species. Radoman (1983) mentioned that the penis is “rather narrow and elongated, with a prominent point and a rather long and pointed outgrowth on the left side”; in fig. 45, p. 84, he depicted a penis gradually tapering with its basal and distal portions not clearly differentiated. The penis of *D. louisi* (figs 13–15 and 18–25, p. 184, Falniowski and Szarowska 2000; specimens from locus typicus dissected by C. Radea), *D. hadei* (figs 16–18, p. 135, Falniowski and Szarowska 2011) and *D. magdalenae* (figs 8–11, p. 9, Falniowski and Sarbu 2015) is rather wide and robust with the distal portion well demarcated from the proximal portion in the latter two species. The penial lobe in all species of the genus is rather short and blunt; especially in *D. louisi* and *D. hadei* this lobe is very short and not clearly distinct (Falniowski and Szarowska 2000, Although the looping pattern and the orientation of primary loop of renal oviduct are suggested by Hershler and Ponder (1998) for distinguishing hydrobiid species, these characters are usually not reported. In *Daphniola*, the orientation of primary oviduct loop is vertical. However, the overall shape of oviduct differs between the species being triangular in *D. dione*, circular-ovoid in *D. exigua* (fig. 45, p. 84, Radoman 1983 as *D. graeca*; specimens from loci typici of *D. exigua* and *D. graeca* dissected by C. Radea), ovoid in *D. eptalophos* (fig. 11, p. 59, Radea 2011) and ellipsoid in *D. louisi* (specimens from locus typicus dissected by C. Radea) (Table 5). A similar differentiation has also been recorded between the Greek species of the genus *Pseudamnicola* (Radea et al. 2016b).

The molecular analysis conducted in the present study confirms the findings of Falniowski et al. (2007), which claimed that *D. graeca* and *D. exigua* are conspecific taxa both belonging to *D. exigua*. Although *D. louisi* is genetically closely-related to *D. exigua* (Fig. 2), the combination of diagnostic characters’ states (Table 5) easily distinguishes these species.

Lefkada is a densely populated Ionian island and a well-known summer tourist destination very close to the Greek mainland. However, the type locality of *D. dione* is not vulnerable to anthropogenic stressors because it is located on a hilly and woody area far away from villages, cultivations and tourism infrastructures. Currently, the single population of the new species seems not to face any obvious threat.

The discovery of the new *Daphniola* species expands the distribution of the genus westwards (Fig. 1). *Daphniola* was previously thought to be restricted to the eastern part of Greece along with two other hydrobiid genera of the eastern Balkan Peninsula *Graecoanatolica* Radoman, 1973 and the Greek endemic *Graecorientalia* Radoman, 1973 (Radoman 1985).

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