Two new Truncatelloidea species from Melissotrypa Cave in Greece (Caenogastropoda)

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Abstract
In the small lake located in the cave Melissotrypa in Thessalia, Greece, truncatelloidean gastropods representing two species were found, new to science. One of them, represented by two specimens only, has been described based on the shell characters only; with its cytochrome oxidase sequence it has been assigned to the genus Iglica, and to the family Moitessieriidae, Iglica hellenica sp. n. For the other species, represented by 30 collected specimens, the shell, protoconch, radula, head, penis and female reproductive organs have been described; all the morphological characters and cytochrome oxidase sequences have confirmed its assignment to the genus Daphniola (Hydrobiidae: Sadlerianinae), Daphniola magdalenae Falniowski, sp. n.

Keywords
Gastropoda, Hydrobiidae, Moitessieriidae, aquatic snails, morphology, cytochrome oxidase, taxonomy, troglobionts

Introduction
In June 2014, in Melissotrypa Cave in Greece (39°52′38″N and 22°02′58″E), several specimens of Truncatelloidea gastropods were collected. This was the third visit by the second author to this cave, but the snails were found for the first time.

The cave is located in Melissotrypa Kefalovriso Elassona, north of Larissa, and is the largest known underground karstic form of karst system Kranias Elassona, drilled
in marbles. The character of the cave is demonstrated by the remaining forms of dissolution and growth of the cave, the gypsum and detected hydrogen sulfide in the lakes of the cave. The cave covers an area 0.06 km² and has a total length of mapped passageways about 2103.6 m. The elevation in the region of the inlet orifice is 299 m while the interior reaches a depth up -47.3 m i.e. absolute altitude 251.7 m. The depth of the precipitous entry is 14.6 m (http://7gym-laris.lar.sch.gr/perivalon/spilaia.htm).

Many specimens of gastropods were concentrated in just one area in the sulfuric lake, close to the shore in a depth of approximately 10 cm. In the vicinity of the lake there are no terrestrial animals, although there are microbial biofilms and organic matter. The aquatic fauna is highly interesting: the most abundant form is an amphipod *Niphargus*, which swims upside down, seemingly an adaptation to such water chemistry. The snails do not live everywhere, but only in one place on a limestone wall, at 5–10 cm beneath the water surface. There were hundreds of individuals gathered in a compact group. Maybe there are more such groups, but the water is deep and one cannot reach the walls except by means of a small boat, the lake being very narrow. In this cave, there is also another lake, at several hundred meters away from the former, in which the water has no sulfur, and which is sometimes dry. No snails have been found in it.

Only two specimens with a turriform shell were collected, and approximately 30 specimens with a valvatiform shell. The aim of the paper is to describe these two snails collected in Melissotrypa Cave.

**Materials and methods**

The snails were collected by hand and placed directly in 95% ethanol. The ethanol was changed twice, and the material stored at -20 °C.

The shells were photographed with a CANON EOS 50D digital camera, attached to a NIKON SMZ18 stereoscope microscope with dark field. They were dissected using a NIKON SMZ18 stereoscope microscope with a NIKON drawing apparatus, and a NIKON DS-5 digital camera. Radulae and protoconchs were examined using a JEOL JSM-5410 scanning electron microscope, applying the techniques described by Falniowski (1990).

DNA was extracted from foot tissue of two specimens. The tissue was hydrated in TE buffer (3 × 10 min); total genomic DNA was then 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’-GGTCAACAAATCATAAGATATTGG-3’) (Folmer et al. 1994) and COR722b (5’-TAAACTTCAGGGTGACCAAAAAATYA-3’) (Wilke and Davis 2000) for the cytochrome oxidase subunit I (COI) mitochondrial gene.

The PCR conditions were as follows: 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. 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 ran on 1% agarose gel. The PCR products were purified using Clean-Up columns (A&A Biotechnology) and were then amplified in both directions 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 Ex Terminator Columns (A&A Biotechnology); DNA sequences then underwent electrophoresis on an ABI Prism sequencer.

The COI sequences were aligned by eye using BioEdit 5.0.0 (Hall 1999). The saturation test of Xia et al. (2003) was performed using DAMBE (Xia 2013). Sequences obtained from the snails from Melissotrypa Cave in the present work were used in a phylogenetic analysis with other sequences obtained from GenBank (Table 1). A maximum likelihood (ML) approach was conducted in RAxML v8.0.24 (Stamatakis 2014). One thousand searches were initiated with starting trees obtained through randomized stepwise addition maximum parsimony method. The tree with the highest likelihood score was considered as the best representation of the phylogeny. Bootstrap support was calculated with 1000 replicates and summarized onto the best ML tree. RAxML analyses were performed using free computational resource CIPRES Science Gateway (Miller et al. 2010). Genetic p-distances between the species of Daphniola were calculated using MEGA6 (Tamura et al. 2013), with standard errors estimated by 1,000 bootstrap replications with pairwise deletion of missing data. The maximum composite likelihood distance and Tajima relative rate tests of local clock-like behavior (Tajima 1993) were performed using MEGA6.

**Systematic part**

**Family Moitessieriidae Bourguignat, 1863**

**Genus Iglica Wagner, 1927**

**Iglica hellenica** sp. n.

http://zoobank.org/44EEDD4D-448D-4ABB-9128-E6AFC35F5B51

**Holotype.** Ethanol-fixed specimen, Melissotrypa Cave, Thessalia, Greece, 39°52’38”N, 22°02’58”E, sulphidic lake, near the shore, June 2014, S. Sarbu coll., ZMUJ-M.2107.

**Paratype.** One specimen destroyed for DNA extraction details as for holotype.

**Diagnosis.** One specimen destroyed for DNA extraction details as for holotype.

**Description.** Shell (Fig. 1) up to 4.04 mm tall, 5.5 whorls, spire height 281% width of shell. Holotype measurements: shell height 4.04 mm, spire height 1.85 mm, body whorl breadth 1.44 mm, aperture height 1.22 mm, aperture breadth 1.05 mm, whorls number 5½. Teleoconch whors highly convex, evenly rounded. Aperture nar-
Table 1. Taxa used for phylogenetic analyses, with their GenBank Accession Numbers and references.

| Species                     | COI GB#       | References                        |
|-----------------------------|---------------|-----------------------------------|
| *Adrioinsulana conovula*    | AF367628      | Wilke et al. (2001)               |
| *Agrafia wiktori*           | JF906762      | Szarowska and Falniowski (2011)   |
| *Aloniella finalina*        | AF367650      | Wilke et al. (2001)               |
| *Anagastina setatalis*      | EF070616      | Szarowska (2006)                  |
| *Arenonius brevis*          | AF367638      | Wilke et al. (2001)               |
| *Belgrandiella kusceri*     | KT218520      | Falniowski and Beran (2015)       |
| *Bithynia tentaculata*      | AF367643      | Wilke et al. (2001)               |
| *Boleana umbilicata*        | KT218521      | Falniowski and Beran (2015)       |
| *Bythinella austriaca*      | FJ545132      | Falniowski et al. (2009)          |
| *Bythiopeum*                | AF367634      | Wilke et al. (2001)               |
| *Bythiopeum acutum*         | HM107120      | unpublished, from GenBank         |
| *Bythiopeum francomontanum* | HM107131      | unpublished, from GenBank         |
| *Bythiopeum hungaricum*     | KP296923      | unpublished, from GenBank         |
| *Bythiopeum humannii*       | HM107134      | unpublished, from GenBank         |
| *Bythiopeum pellucidum*     | HM107124      | unpublished, from GenBank         |
| *Bythiopeum suevicum*       | HM107118      | unpublished, from GenBank         |
| *Dalmatinella flaviatilis*  | KC344541      | Falniowski and Szarowska (2013)   |
| *Daphniola exigua*          | EU047767      | Falniowski et al. (2007)          |
| *Daphniola haderi*          | JF916477      | Falniowski and Szarowska (2011a)  |
| *Daphniola gneca*           | EF070618      | Szarowska (2006)                  |
| *Daphniola louisii*         | EU047769      | Falniowski et al. (2007)          |
| *Daphniola sp.*             | KM887915      | Szarowska et al. (2014)           |
| *Daphniola magdalaeae*      | KT825578-80   | present study                     |
| *Diatella thyesiana*        | AY676127      | Szarowska et al. (2005)           |
| *Fissuria boai*             | AF367654      | Wilke et al. (2001)               |
| *Graecoarganiella parnassiana* | JN202348 | Falniowski and Szarowska (2011b)  |
| *Grassianna alpestris*      | AF367641      | Wilke et al. (2001)               |
| *Grossuana codreanui*       | EF061919      | Szarowska et al. (2007)           |
| *Hauffenia tellini*         | AF367640      | Wilke et al. (2001)               |
| *Helobia dubitatica*        | AF367631      | Wilke et al. (2001)               |
| *Horatia kirczakia*         | KJ159128      | Szarowska and Falniowski (2014)   |
| *Hydrobia acuta*            | AF278808      | Wilke and Davis (2000)            |
| *Iglica hellenica*          | KT825581      | present study                     |
| *Islamia piristoma*         | AF367639      | Wilke et al. (2001)               |
| *Lithophagus naticeoides*   | AF367642      | Wilke et al. (2001)               |
| *Marstoniopsis insubrica*   | AY027813      | Falniowski and Wilke (2001)       |
| *Motteseria cf. puteana*    | AF367635      | Wilke et al. (2001)               |
| *Montenegropeus bogici*     | KM875510      | Falniowski et al. (2014)          |
| *Pseudannicola lucensis*    | AF367651      | Wilke et al. (2001)               |
| *Pyrgula annulata*          | AY341258      | Szarowska et al. (2005)           |
| *Radomaniola callosa*       | AF367649      | Wilke et al. (2001)               |
| *Rosa labiosa*              | AY676128      | Szarowska et al. (2005)           |
| *Sadleriana fluminensis*    | AY273996      | Wilke et al. (2001)               |
| *Tannusia zrmanjae*         | Xx            | Beran et al. (2015)               |
| *Trichonia kephalovrisiosa* | EF070619      | Szarowska (2006)                  |
| *Ventrosia ventrosa*        | AF118335      | Wilke and Davis (2000)            |
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Figure 1. Shells of Iglica hellenica sp. n.: A holotype B sequenced specimen. Scale bar 1 mm.

row, ovate, weakly angled adapically, separated from body whorl by a broad groove. Parietal lip complete, adnate, no umbilicus. Outer lip simple, orthocline. Shell glossy with no sculpture, periostracum yellowish. Soft parts pinkish, with no pigment. External morphology and anatomy unknown.

Etymology. The specific epithet (hellenica) is a Greek adjective meaning Greek.

Distribution and habitat. Known from two specimens from the type locality only.
Family Hydrobiidae Troschel, 1857
Subfamily Sadlerianinae Radoman, 1973
Genus Daphniola Radoman, 1973

*Daphniola magdalenae* Falniowski, sp. n.
http://zoobank.org/AF91ADE8-10B4-4737-8022-7EFDDC316EAD

**Types.** Ethanol-fixed specimens, Melissotrypa Cave, Thessalia, Greece, 39°52’38”N, 22°02’58”E, sulphidic lake, near the shore, June 2014, S. Sarbu coll., holotype: ZMUJ-M.2109; 20 paratypes: ZMUJ-M.2110–ZMUJ-M.2130.

**Diagnosis.** Shell relatively big, valvatiform-trochiform; soft parts with no pigment, no eyes, penis with long and slender filament and big outgrowth on the left side. Readily distinguished from geographically and closely related *D. exigua* (= *D. graeca*) by its bigger size (2.5 vs. 1.5 mm), reddish operculum, broader base and longer and thinner filament of the penis. Differentiated from *D. louisi* (from Kessariani at Athens) by its larger size, higher spire, longer and thinner filament and more prominent outgrowth on the left side of the penis. Differs from *D. hadei* (from Gythion at Peloponnese) by its double size, higher spire and much more prominent outgrowth on the left side of the penis.

**Description.** Shell (Fig. 2A–D) valvatiform-trochiform, up to 2.68 mm tall, having 3.5–3.75 whorls, spire height 16% height of shell, and 13–16% width of shell. Teleoconch whorls moderately convex, evenly rounded, growing rapidly in diameter. Aperture circular, parietal lip complete, umbilicus very broad, outer lip simple, orthoeline. Teleoconch with delicate growth lines, periostracum pinkish. Shell parameters for a series of paratypes are given in Table 2. On the surface there are numerous pellets of sediment, most probably of sulfuric bacteria.

Inner and outer sides of operculum smooth. Operculum pink (Fig. 2A–D). Protoconch of 1.25–1.40 whorls growing slowly (Fig. 3), with a net-like pattern of dense depressions, their shape irregular (Fig. 4), covering all the protoconch and initial part of the teleoconch.

Radula (Figs 5–7): taenioglossate, typically hydrobiid; the cusps on the central, lateral and inner marginal teeth prominent, long and sharp; the central tooth trapezoid (Figs 5–6), with one pair of big basal cusps arising from the tooth face (Fig. 5) and numerous long cusps along the cutting edge, the basal tongue broadly V-shaped and about equal in length to the lateral margins, lateral cusps five–six. Lateral teeth (Figs 6–7) having four cusps on inner, and five cusps on outer side, central cusp broad and blunt. Inner marginal tooth (Fig. 7) with 35–36 cusps, outer marginal teeth (Figs 6–7) with 21–23 cusps.

Animal brownish, with no pigment, and no eyes (Fig. 8). Penis (Figs 9–11) having broad base bent U-shaped in natural position (Fig. 8), long and narrow filament and prominent outgrowth on its left edge. Female reproductive organs (Fig. 12) with big bursa copulatrix with long duct and two small receptacula seminis.

**Etymology.** Named in memory of Dr Magdalena Szarowska, a malacologist, wife and best friend of the first author.

**Distribution and habitat.** Known from the type locality only.
Molecular relationships of the new taxa

The saturation test of Xia et al. (2003) revealed a significant degree of saturation in the third position of the sequences. In rissooids, COI approaches saturation with approximately 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. The maximum likelihood tree (Fig. 13) was characterized by low bootstrap values at deep nodes, which is typical of cytochrome oxidase-based phylogenies, but clearly showed that *Daphniola magdalenae* sp. n. belonged to the genus *Daphniola* (bootstrap value 63%), although it was clearly a distinct species. Its closest relatives were *Daphniola* sp. from Khios and Rhodes islands, and *D. exigua/D. graeca* from Tembi valley (bootstrap support 79%). The bootstrap support of the clade of *Daphniola*, *Trichonia* Radoman, 1973, and *Grossuana* Radoman, 1973 was 89%. The p-distance between *Daphniola magdalenae* sp. n. and *D. exigua* was p = 0.1325. The relative rates test for all the *Daphniola* species confirmed the ultrametricity of the data. The tree also confirmed close relationships of *Iglica hellenica* sp. n. with “*Bythiospeum*” *hungaricum* (bootstrap value/support 64%), and that both *Iglica hellenica* and “*Bythiospeum*” *hungaricum* do not belong to the genus *Bythiospeum* Bourguignat, 1882.

**Discussion**

With one (since the other had to be destroyed for DNA extraction) available specimen of *Iglica hellenica* sp. n. it has not been possible to study its soft parts. However, nearly all the representatives of *Bythiospeum*, *Paladilhoiopsis*, *Iglica*, etc. are known as empty...
shells only. The distinction between these genera remains unclear. The molecular tree, as well as the phylogeny presented by Wilke et al. (2013), does not confirm even the close relationships between *Bythiospeum*, *Iglica hellenica* sp. n., and *Moitessieria*. It also does not confirm that “*Bythiospeum* hungaricum” belongs to the genus *Bythiospeum*, but confirms its close relationships with *Iglica hellenica*. From Greece there are four known species of *Iglica*: *I. sidarensis* Schütt, 1980 from Corfu, *I. maasseni* Schütt, 1980 from Rhodes, and two species from the Peloponnese: *I. wolfischeri* A. & P. Reischutz, 2004 and *I. alpheus* A. & P. Reischutz, 2004. With the exception of *I. alpheus*, the shells of all are similar to the one of *I. hellenica*, but much smaller with shell heights of 1.5–2.3 mm, compared with 4.04 mm in *I. hellenica*. The representatives of another cave-inhabiting genus *Paladilhiopsis* Pavlovic, 1913 should also be considered. From Greece there are three species in this genus: *P. blanci* (Westerlund, 1886) from the is-

**Figures 8–11.** Head and penes of *Daphniola magdalenae*: 8 head with penis, 9–11 penes. Scale bar: 250 µm.
lands Cephalonia and Lefkada, *P. janinensis* Schütt, 1962 from the springs at the shore
of Pamvotis Lake (now the springs are completely dry), and *P. thessalica* Schütt, 1970,
from Pyrgetos at Tembi Valley. This locality is only 46 km away from Melissotrypa
Cave. However, the shell but especially the aperture of *I. hellenica* is typical of *Iglica*,
not of *Paladilhiopsis* (e. g. Schütt 1980). Moreover, the 18S sequence of *I. hellenica* (unpublished data)
was very different from the one of *Paladilhiopsis carpatica* Soós, 1940 from Vadu Crisul Cave in Romania (Szarowska 2006). Thus the assignment of *I.
hellenica* to the genus *Iglica* remains justified based on the available data.

The shells of *Daphniola exigua* are highly variable (Falniowski et al. 2007), including
the similar shells of *D. magdalenae* sp. n., but are much smaller (maximum 1.58 mm *vs.*
2.68 in *D. magdalenae*). The shells of the other species of *Daphniola* have lower spires,
and are also maximum 1.5 mm tall (Falniowski et al. 2007, Falniowski and Szarowska
2011a). The penis of *Daphniola magdalenae* sp. n. differs in its long and narrow, sharply
pointed filament of the penes from those of *D. exigua* and *D. graeca* (Radoman 1983,
Szarowska 2006), and *D. louisi* (Falniowski & Szarowska, 2000). A similar filament,

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**Figure 12.** Renal and pallial section of female reproductive organs of *Daphniola magdalenae* (bc – bursa copulatrix, cbc – duct of bursa copulatrix, ga – albuminoid gland, gn – nidamental gland, gp – gonoporus, ov – oviduct, ovl – loop of (renal) oviduct, rs₁, rs₂ – receptacula seminis, nomenclature after Radoman (1973, 1983), vc – ventral canal). Scale bar 1 mm.
Figure 13. Maximum likelihood tree computed for cytochrome oxidase I sequences, bootstrap supports given if > 50%.
but less prominent outgrowth on the left side of the penis is characteristic of *D. hadei* (Falniowski and Szarowska 2011a). The female reproductive organs of *D. magdalenae* are characteristic of *Daphniola* (Radoman 1973, 1983, Szarowska 2006). Some differences in size proportions of the receptacula and bursa could be observed between the species, but the variability is high; even the genera of the Hydrobiidae with two receptacula could not always be recognized with this character (Falniowski et al. 2012). *Daphniola exigua* inhabits two springs in Tembi Valley, approximately 50 km from Melissotripa cave, but in the molecular tree it is not the sister species of *D. magdalenae* sp. n.. The genetic distance between *D. magdalenae* and *D. exigua* is $p = 0.1325$. Based on mtCOI clock calibrations of 1.83% per million years for European Hydrobiidae (Wilke 2003) and 1.62% per million years for *Pyrgulopsis* (Hershler and Liu 2008), the estimated divergence times of the two species ranged from 7.24 to 8.20 mya, thus the very beginning of the Messinian or even upper Tortonian in the Miocene.

The molecular tree confirms relationships of both new species *Iglesia hellenica* and *Daphniola magdalenae*. As it is based on one short fragment of mitochondrial DNA, it presents the phylogeny of this fragment, certainly not of the species/genera (e.g., Avise 2000), and its deep nodes are not supported. Thus the tree cannot be interpreted as phylogeny of the Truncatelloidea. However, it seems sufficient to detect the closest relatives of the new species described in this paper.

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