Daphniola Radoman, 1973 (Gastropoda: Hydrobiidae): shell biometry, mtDNA, and the Pliocene flooding

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Abstract
Shell biometry and cytochrome c oxidase subunit 1 (CO1) mtDNA were studied in Daphniola exigua (Schmidt, 1856), D. graeca Radoman, 1973, and D. louisi Falniowski and Szarowska, 2000 from Greece. Principal component analysis of shell morphometry confirmed the distinctness of D. louisi along the PC3 axis. Kimura 2-parameter (K2P) genetic distances within D. graeca and D. exigua were 0.016 and 0.003–0.008, respectively, all D. louisi sequences were identical. The distance between D. exigua and D. graeca was 0.013–0.027. The distances between D. louisi and D. graeca, and D. louisi and D. exigua, were 0.098–0.110 and 0.091–0.096, respectively. The mean distance between D. louisi and the other Daphniola species was 0.098 ± 0.007, while between the eight Daphniola specimens and the so far closest species Grossuana codreanui (Grossu, 1946) was 0.102–0.123. A maximum likelihood tree was constructed for all Daphniola, with Grossuana codreanui and Bythinella austriaca (Frauenfeld, 1856) as an outgroup. This confirmed that D. louisi is a distinct species, and must have diverged after the Pliocene marine transgression.

Keywords: CO1, cytochrome c oxidase subunit 1, Daphniola, Greece, phylogeny, rissooidean gastropods, shell biometry

Introduction
Within the “hydrobioid” gastropods there are numerous low-spired, valvatiform-shelled taxa, most of which belong to the smallest rissooideans. Many of the snails inhabit the Balkans. Formerly assigned mostly to the Valvatidae, presently they are classified among various clades of the Rissooidea. Most of them share a shell pattern without unique characters and (a result of miniaturization) a simplified anatomy. Hence, where one of these taxa is concerned, it is impossible, considering its morphological characters only, to establish either its phylogenetic position or species boundaries. One of these cases, in which molecular data may be of much use, is the genus Daphniola Radoman, 1973 inhabiting Greece.
Radoman (1973) described *Daphniola graeca* as the type species of a new genus *Daphniola*, found in Daphne spring north of Larissa. Schütt (1980) considered *D. graeca* a junior synonym of *Valvata exigua* Schmidt, 1856. In the original description of *V. exigua* the type locality given was “Greece”, but Schütt (1980) designated its neotype from a group of small springs at Agia Paraskevi railway station, Greece. In fact, the two localities, lying not far from each other, are situated in the valley of Tembe, Thessalia, Greece (but certainly not close to Thessaloniki as Kabat and Hershler 1993 state). Radoman (1983, 1985) criticized the synonymy introduced by Schütt (1980). Falniowski and Szarowska (2000) described *Daphniola louisi* from a small spring at the monastery at Kessariani, Athens, Attica, Greece. The description was not considered by Bodon et al. (2001), who followed either Schütt (1980) in synonymizing *D. graeca* with *D. exigua*, or Reischütz and Sattmann (1993) in including *Valvata (Cincinna) hellenica* Westerlund, 1898 in *Daphniola exigua*, thus rendering the genus *Daphniola* monotypic. They did not, however, examine the soft parts of any *Daphniola*. Employing molecular data (partial sequences of the mitochondrial CO1 and ribosomal 18S genes), Szarowska (2006) found *Grossuana* Radoman, 1973 to be the closest relative of *Daphniola*. The morphology and anatomy of *Daphniola* have been described by Radoman (1973, 1983), Schütt (1980), Falniowski and Szarowska (2000), Bodon et al. (2001), and Szarowska (2006). The radula and protoconch of *D. louisi* are described and illustrated by Falniowski and Szarowska (2000), and of *D. exigua* and *D. graeca*, by Szarowska (2006). Falniowski and Szarowska (2000) listed the anatomical differences between *D. louisi* and the other two *Daphniola*. The differences are so minor that the distinctness of the three nominal taxa of *Daphniola* cannot be proved based on morphology.

Despite numerous cases of incongruence, there is no intrinsic conflict concerning the phylogenies based on molecular versus morphological data (e.g. Hillis and Wiens 2000). However, where minute organisms with a simplified morphology are concerned, morphological data may be somewhat misleading, as they comprise too few characters, the states of which are prone to homoplasies. Such is the case of the soft part morphology of *Daphniola*. In this study, using partial mtDNA sequences of cytochrome c oxidase subunit 1 (CO1) and shell biometry, we try to answer the following questions:

1. Are the three nominal taxa of *Daphniola* distinct species?
2. How long ago may the fine morphological distinctness of these taxa have originated?
3. With which, if any, of the numerous geological events that affected the Mediterranean during the Caenozoic can that origin be correlated?

**Material and methods**

In September 2003, the following material was collected from *Daphniola* type localities in Greece:

1. *Daphniola louisi* Falniowski and Szarowska, 2000, a small spring at the monastery of Kessariani, Athens, Attika, 37°57’39”N, 23°47’55”E, 358 m a.s.l.
2. *Daphniola graeca* Radoman, 1973, Daphne spring, a spring at the bottom of a natural pool rich in vegetation, about 30 km north of Larissa, 39°53’28”N, 22°36’26”E, 16 m a.s.l.
3. *Daphniola exigua* (Schmidt, 1856), two springs close to the railway station Agia Paraskevi, 39°52’47”N, 22°35’07”E, 16 m a.s.l.
For molecular study snails were fixed with 80% ethanol, for morphological study they were fixed with 4% formalin and after 24 h transferred to 80% ethanol for storage. Shells were photographed with a Nikon SMZ-U stereoscopic microscope with a Nikon Coolpix 4500 digital camera. To compare the shells, six morphometric parameters (Figure 1) were measured on 10 specimens of each of the three nominal species, with a COHU 3715 camera, connected to a frame-grabber and a PC equipped with the MultiScanBase v. 11.06 software. The linear measurements were logarithmically transformed; arcsine transformation was applied. Euclidean distances were calculated, and UPGMA clustering and minimum spanning tree was computed with NTSYSpc (Rohlf 1998). Principal component analysis (PCA) was computed based on the matrix of correlation, and the original observations were projected into PC space.

Ethanol-fixed snails were washed twice with ice-cold water, and then DNA was isolated according to the method described by Spolsky et al. (1996) and Davis et al. (1998), with modifications. Isolated DNA was used as a template in PCR reaction with primers: LCO1490 (5′-ggcaacacatgagaacctaatgg-3′) and COR722b (5′-taaatgtaggaacccattta-3′) for CO1 gene (Folmer et al. 1994; Davis et al. 1998). The PCR conditions were as described in Szarowska et al. (2007). Amplification was carried out in a 50 μl volume reaction. The PCR product was purified using the Clean-Up columns (A&A Biotechnology) according to the manuals. The purified PCR product was sequenced using the BigDye Terminator v3.1 (Applied Biosystems) according to the manufacturer’s protocols and the above-described primers. The reaction product was purified using the ExTerminator Columns (A&A Biotechnology) according to the manufacturer’s protocols, and sequences were read using an ABI Prism sequencer.

Figure 1. Shell morphometry measurements: a, height of shell; b, width of body whorl; c, height of mouth; d, height of spire; e, width of mouth; α, angle of spire.

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The sequences were aligned by eye using BioEdit 5.0.0 (Hall 1999) and edited with MacClade 4.05 (Maddison and Maddison 2002). MEGA3 (Kumar et al. 2004) was applied to calculate genetic distances (p and Kimura 2-parameter (K2P)) among the studied sequences. The maximum likelihood approach, not sensitive to violation of some of its assumptions (Swofford et al. 1996), may often find wrong reconstructions as in the case where one deals with many taxa and short sequences (Nei et al. 1998; Nei and Kumar 2000). There is no parameter connected with a tree topology in all the maximum likelihood theory: one must just believe that the tree with the most “true” branch lengths is, at the same time, the one with the best topology (Nei 1987, 1996; Yang et al. 1995). There is also strong evidence that the more complicated the model of evolution, the higher the variance of the resulting reconstructions. Our understanding of DNA evolution is not yet sufficient, thus all the models are far from realistic. Thus, it may happen that the simplest models will result in phylogeny reconstructions, which are closest to the real historical processes (Gaut and Lewis 1995; Yang 1997; Takahashi and Nei 2000; Falniowski 2003). Despite the above criticism, we decided to apply the maximum likelihood approach as implemented in PAUP*4.0b10 (Swofford 2002). PAUP together with Modeltest (Posada and Crandall 1998) were used to find the appropriate model of evolution.

Results

The shells in the studied populations of *Daphniola* (Figures 2–13) showed a wide range of variability within the valvatiform habitus. The widest variation was found in *D. exigua* from Agia Paraskevi (Figures 2–6). It was only in this population that either high- or low-spired specimens were observed (Figures 2–4 and 5, 6, respectively). In *D. louisi* (Figures 7–10) the shells were less variable and had low spires. In *D. graeca* from Daphne spring (Figures 11–13) variation was very restricted and all the shells low-spired. The variability ranges, mean values and standard deviations of all the six measured parameters for each taxon are given in Table I. The shells of the three nominal taxa are closely similar (Figures 2–13) and can easily be assigned to one species.

UPGMA clustering yielded several clusters, some of which included mixed nominal taxa, thus not confirming the species distinctness of any of them. In PCA the first three principal components (PC) explained cumulatively as much as 91.59% of total variation (as compared with 80.83% predicted by the broken-stick model). Along the first PC axis all the three nominal taxa are mixed up, which can be interpreted as showing that none of the differences among them reflect size, polymorphism, or sexual dimorphism. On the other hand, in the space of PC2 and PC3 (Figure 14) *D. louisi* is clearly distinct (with just one outlier of *D. louisi* and one of *D. exigua*) from the other two taxa along the PC3 axis (although PC3 explains only 9.90% of the total variation, which is even less than the 15.83% predicted by the broken-stick model). Along the PC2 axis *D. exigua* somewhat overlaps *D. graeca* and *D. louisi*, the latter mixed with *D. graeca* along this axis.

Two CO1 sequences of *D. graeca*, three of *D. exigua*, and three (all of them identical) of *D. louisi* (Table I) were used, together with sequences of *Grossuana codreanui* (Grossu, 1946) and *Bythinella austriaca* (Frauenfeld, 1856) as an outgroup. The pattern reflected by p-distances was similar to the one reflected by K2P distances (Table II), the latter more congruent with the model of evolution found (see below), thus we discuss only the K2P distances. There simply was no differentiation, the same haplotype being represented by the three sequenced specimens of *D. louisi*. Within *D. graeca* the distance was 0.016, and within *D. exigua* 0.003–0.008 (Table II). The distances between *D. exigua* and *D. graeca*
were within the range 0.013–0.027, overlapping the range for *D. graeca*. On the other hand, the distance between *D. louisi* and *D. graeca* was 0.098–0.110, and between *D. louisi* and *D. exigua*, 0.091–0.096. The mean distance between *D. louisi* and the other *Daphniola* was 0.098 ± 0.007. The distances between *Grossuana* and the eight specimens of *Daphniola*...
Hierarchical Likelihood Ratio Tests calculated with PAUP* and Modeltest found TVM+G model of evolution, with the base frequencies: A=0.2824, C=0.1757, G=0.1825, and T=0.3594, rate matrix: [A-C]=0.7517, [A-G]=5.6616, [A-T]=2.4539, [C-G]=0.1928, [C-T]=5.6616, and [G-T]=1.0000, proportion of invariable sites=0, and \( \gamma \) distribution with the shape parameter \( \alpha = 0.3683 \). The model was applied to compute the maximum likelihood tree (Figure 15). The phylogram, the branch lengths of which reflect the amount of evolution along a branch, clearly shows close relationships of all the five specimens of D. graeca and D. exigua, a distinct position of D. louisi and the monophyly (in the sense of no support for non-monophyly) of the genus Daphniola (bootstrap support 94).

Table I. Shell biometry of Daphniola.

|         | a    | b    | c    | d    | e    | z      |
|---------|------|------|------|------|------|--------|
| Daphniola exigua | 1.17–1.58 | 0.99–1.28 | 0.64–0.81 | 0.158–0.294 | 0.63–0.79 | 91.61–124.21° |
|         | 1.36±0.123 | 1.14±0.071 | 0.72±0.045 | 0.210±0.035 | 0.73±0.041 | 109.43±12.452° |
| Daphniola graeca  | 0.99–1.48 | 0.96–1.29 | 0.63–0.87 | 0.099–0.200 | 0.68–0.87 | 123.82–160.70° |
|         | 1.26±0.139 | 1.13±0.097 | 0.74±0.060 | 0.144±0.032 | 0.76±0.051 | 130.66±32.763° |
| Daphniola louisi  | 1.10–1.45 | 0.91–1.19 | 0.74–0.96 | 0.077–0.196 | 0.66–0.85 | 118.03–144.33° |
|         | 1.25±0.098 | 1.06±0.075 | 0.82±0.057 | 0.145±0.037 | 0.75±0.051 | 129.52±8.125° |

a, height of shell; b, width of body whorl; c, height of mouth; d, height of spire; e, width of mouth; z, angle of spire. Values (in mm except for z) are ranges and means ± SD.

Figure 14. Shell morphometry of Daphniola: projection of objects into space of PC2 and PC3. (○) D. graeca; (●) D. exigua; (X) D. louisi.
Table II. Pairwise distances between studied taxa.

|        | 1     | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|--------|-------|---|---|---|---|---|---|---|---|----|
| 1. *Daphniola graeca* 1 | – | 0.016 | 0.013 | 0.016 | 0.017 | 0.091 | 0.091 | 0.091 | 0.103 | 0.219 |
| 2. *Daphniola graeca* 2 | 0.016 | – | 0.022 | 0.025 | 0.027 | 0.102 | 0.102 | 0.102 | 0.113 | 0.229 |
| 3. *Daphniola exigua* 1 | 0.013 | 0.022 | – | 0.003 | 0.008 | 0.085 | 0.085 | 0.085 | 0.094 | 0.212 |
| 4. *Daphniola exigua* 2 | 0.016 | 0.026 | 0.003 | – | 0.005 | 0.088 | 0.088 | 0.088 | 0.097 | 0.215 |
| 5. *Daphniola exigua* 3 | 0.017 | 0.027 | 0.008 | 0.005 | – | 0.089 | 0.089 | 0.089 | 0.099 | 0.213 |
| 6. *Daphniola louisi* 1 | 0.098 | 0.110 | 0.091 | 0.095 | 0.096 | – | 0.000 | 0.000 | 0.108 | 0.224 |
| 7. *Daphniola louisi* 2 | 0.098 | 0.110 | 0.091 | 0.095 | 0.096 | 0.000 | – | 0.000 | 0.108 | 0.224 |
| 8. *Daphniola louisi* 3 | 0.098 | 0.110 | 0.091 | 0.095 | 0.096 | 0.000 | 0.000 | – | 0.108 | 0.224 |
| 9. *Grossuana codreanui* | 0.112 | 0.123 | 0.102 | 0.105 | 0.107 | 0.118 | 0.118 | 0.118 | – | 0.205 |
| 10. *Bythinella austriaca* | 0.261 | 0.274 | 0.250 | 0.255 | 0.252 | 0.269 | 0.269 | 0.269 | 0.242 | – |

Above diagonal: p distances; below diagonal: K2P distances. GenBank accession numbers: *Daphniola graeca* 1, EU047763; 2, EU047764; *D. exigua* 1, EU047765; 2, EU047766; 3, EU047767; *D. louisi* 1, EU047768; 2, EU047769; 3, EF070618; *Grossuana codreanui*, EFO61919; *Bythinella austriaca*, EFO70617.

Figure 15. Phylogram computed with maximum likelihood, *Grossuana codreanui* and *Bythinella austriaca* as outgroup; bootstrap supports (10,000 replicates) given for all branches whose support exceeded 50%.
Discussion

Falniowski and Szarowska (2000) stated that the shells of *D. louisi* have lower spires than those of *D. exigua* (including *D. graeca*). This is generally confirmed in the present study: the majority of the shells of *D. exigua* from Agia Paraskevi had higher spires, but the variation within the species was so wide that it also covered the typical shell habitus of *D. louisi*; the shells of *D. graeca* from Daphne spring were all characterized by a low spire. On the other hand, PCA showed the evident distinctness of *D. louisi* in shell shape characters (Rohlf 1998; Falniowski 2003), especially along the PC3 axis. However, PC3 explained less variability than predicted by the broken-stick model, assuming that all relationships between the variables are less significant than expected by chance.

Genetic distances have been invented to reflect the amount of evolutionary processes going on after the divergence of the considered clades, but neither reflect those amounts precisely, nor is there the same evolutionary rate in various lineages. On the other hand, we can always scale those distances for a given case. Certainly the distances between *D. graeca* and *D. exigua* are of the same magnitude as those within the two taxa, thus *D. graeca* and *D. exigua* are conspecific, both belonging to *D. exigua*. This confirms the opinions of Schütz (1980) and Bodon et al. (2001) and contradicts Radoman (1983). On the other hand, the distances between *D. louisi* and the other *Daphniola* are of the same magnitude as the distances between *Daphniola* and *Grossuana*. The distances between *Bythinella* and all the other taxa given in Table I are about twice the distances between *D. louisi* and the other *Daphniola*, but *Bythinella* is phylogenetically far from the Hydrobiidae (Wilke et al. 2001; Szarowska 2006). All the above considered, *Daphniola louisi* is a distinct species and can be assigned to the genus *Daphniola*. The maximum likelihood phylogram presents the same pattern.

According to Bodon et al. (2001) *Daphniola* inhabits almost the entire Peloponnissos, Attika without its easternmost part, the western half of Evvia Island, and southeast Thessalia. In the geologic history of the region (e.g. Rögl 1998, 1999) there were many events, such as transgressions of the sea, that must have created conditions that would promote speciation. Unfortunately, the studied material does not cover all the range of the genus but represents only its northeast part. The two type localities in the valley of Tembe are close to each other. On the other hand, the distance between the two localities and Kessariani, the latter situated in a different geological formation, is more than 300 km. The geographical and geological circumstances may have resulted in allopatric speciation between the Kessariani population and two Thessalian populations, the genetic data presented above strongly supporting this speciation event. The evident genotypic differences are weakly reflected in morphology, which is common within the Rissooidea. On the other hand, those differences are reflected by PCA on the shell morphometric characters.

The genetic distances between *D. louisi* and other *Daphniola* equalled 0.091–0.110, mean 0.098 ± 0.007. Corrected for the supposed ancestral polymorphism (thus subtracting the mean polymorphism within the studied populations, π = 0.005), this equals 0.093 ± 0.007. Despite all the precautions concerning the scaling of the molecular clock (Avise 2000; Nei and Kumar 2000), the latter has been calibrated for *Hydrobia* (Wilke 2003), a genus that is quite close to *Daphniola* (Szarowska 2006). The estimated rate of evolution for the CO1 mitochondrial gene is 1.83 ± 0.21% of population divergence per million years. Applying this value to our data we can estimate the time of divergence at 5.35 ± 0.38 million years ago (mya) (or 5.08 ± 0.38 mya after correction for the supposed ancestral polymorphism).
This coincides with 5.33 mya: the time of the Oligocene flooding that ended the Messinian salinity crisis.

The Messinian salinity crisis affected all the Recent basin of the Mediterranean (Krijgsman et al. 1999; McKenzie 1999). The uplift of the northern African and southern Iberian margins, which was probably due to the roll back of the Tethys oceanic lithosphere delaminating bands of lithospheric mantle from beneath the continental margin (Duggen et al. 2003), blocked the passage between the Atlantic and Mediterranean, about 5.96 mya. This resulted in the regression of the sea, whose water level decreased by more than 1000 m. In place of the Recent Mediterranean there was a desert, crossed by the vast canyons of big rivers, with some water bodies too rich and others too poor in salt, thus making it impossible for marine organisms to inhabit the area (Figure 16). There is a discontinuity in the marine sediments corresponding to that crisis. There were at least 10 sea transgressions in the Mediterranean during the Messinian (Hsu¨ 1983). The region of the Recent Sea of Marmara served as a gateway between the Paratethys and Mediterranean; in the north Aegean region frequent marine incursions occurred during the Messinian stage (Çağatay et al. 2006). They fed the big brackishwater Egemar basin (of “Lago-Mare” character: a big and deep, though brackish, habitat) in the north of the Recent Aegean (Sakinç and Yaltırak 2005).

Later on, 5.33 mya, the Pliocene transgression delimited the Miocene from the Pliocene. There was an abrupt catastrophic transgression of water from the Atlantic, probably caused by gravity-induced slumping from the western margin of the Gibraltar arch into the Atlantic abyssal plains (Duggen et al. 2003). The rapid, drastic changes in hydrographic conditions caused by the Pliocene flooding, which restored the sea in the Mediterranean, thus forming barriers for freshwater fauna and changing climatic conditions, promoted speciation processes in many fresh- and brackishwater animals (e.g. Wilke 2003; Huys“¢ et al. 2004). The area between the Recent Attica and the valley of Tembe (Figure 16) was covered by the sea in the Early Pliocene and later.

Certainly, the real rates of the molecular clock may be different, and not necessarily equal in all the lineages of Daphniola, thus we can only hypothesize that the Pliocene flooding was the direct cause of the isolation of the discussed phylogenetic lineages. Nevertheless, the isolation must have happened not long after that, so to resolve the origin and phylogeny of

Figure 16. The geography of the Mediterranean in the Late Miocene (Messinian salinity crisis; after Banarescu 1992) and the localities of the studied populations in the Recent.
the Recent Greek malacofauna one must consider not only its present distribution as proposed e.g. by Schütt (1980). The resolution is to be sought deep in the Tertiary, in the history of that malacofauna.

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