

ISOLATION AS A PHYLOGENY-SHAPING FACTOR: HISTORICAL GEOLOGY AND CAVE HABITATS IN THE MEDITERRANEAN TRUNCATELLOIDEA GRAY, 1840 (CAENOGASTROPODA)

ARTUR OSIKOWSKI

University of Agriculture in Krakow, Institute of Veterinary Science, Department of Animal Anatomy,
Al. Mickiewicza 24/28, 30-059 Cracow, Poland (e-mail: a.osikowski@ur.krakow.pl)

ABSTRACT: Geographical isolation is the main speciation-inducing factor. The current biogeographical and phylogenetic patterns of various organisms, among them gastropods, reflect past speciation events promoted by isolation resulting from geological processes. This is especially the case in the east of the Mediterranean Basin, where large-scale geological events, coupled with climatic changes, created a high environmental and topographic heterogeneity. This article reviews and summarises the studies on relationships between the phylogeography of the Truncatelloidea (Caenogastropoda) and the major geological events which have shaped the geomorphology of the Eastern Mediterranean and the Balkans. The evidence suggests that the geological events having the greatest impact on the biogeography of the truncatelloideans were: the initiation (~12 Mya) and end (~10 Mya) of the Mid-Aegean Trench; the beginning (5.96 Mya) and end (i.e. Zanclean Flood, 5.33 Mya) of the Messinian Salinity Crisis, and climatic oscillations during the Pleistocene (2.58–0.0117 Mya), especially the glaciations. Isolation shapes the pattern of inter-population diversity especially in the cave and spring fauna. The results of studies on the evolution of truncatelloideans in isolated cave habitats are also presented.

KEY WORDS: phylogeography, snails, Balkans, stygobionts, mtDNA

INTRODUCTION

All phylogenies can be described as sets of speciations (WILEY 1981, TEMPLETON 1989). Although cases of sympatric speciation, despite all the criticism, have been demonstrated (GITTENBERGER 1988, TAUBER & TAUBER 1989, ROLÁN-ALVAREZ 2007), allopatric speciation, following isolation, still seems to be the most common way of divergence. Thus it seems interesting to analyse the consequences of isolation: in the past – studying geological history – and present – studying the fauna of isolated habitats such as caves and springs.

Phylogenies of organisms are often closely correlated with historical geography of the areas they inhabit. Interpretative biogeographical studies concern historical biogeography which focuses on relationships between taxonomic groups and past geograph-

ic events. Historical biogeography is an integrative discipline which combines data from geology, geomorphology and biology. It deals with organisms' distributions in the context of evolution, focusing on possible relationships between phylogeography of the organisms and geological history of the areas of their occurrence.

Among the 25 originally proposed (MYERS *et al.* 2000), and 36 currently distinguished (CEPNET 2017) greatest biodiversity hotspots on Earth, the Mediterranean Basin is the only one located in Europe. The geological history of the area, including the Balkans, abounds in events which have influenced the species richness of various plant and animal groups. Dramatic changes of climate, sea level and topography of islands and mainland have caused



major changes in distribution ranges of organisms, resulting from extinction, isolation, colonisation and re-colonisation. The impact of such processes on the current biodiversity patterns has been subject to many studies, combining biological, geological and geographical data. In most cases, more than a single factor is responsible for evolutionary changes, but it is usually possible to discover some general patterns and correspondence between the biological events (e.g. estimated divergence times of the lineages) and the key geological processes.

The superfamily Truncatelloidea Gray, 1840 groups numerous minute and small-sized, dioecious, predominantly freshwater gastropods. Due to their miniaturisation, as well as to the inevitable adaptations to freshwater habitats (reproductive and

osmoregulatory organs) their morphology is simplified and uniform, and rich in parallelisms which often makes it difficult to identify the taxa, even to the family level (CRISCIONE & PONDER 2013). Thus, application of molecular techniques is necessary to infer relationships within the group and to study the phylogeny and phylogeography of its members. The Mediterranean Basin, which holds a rich truncatelloidean fauna, is an excellent area for studies on the evolution and distribution of this group of gastropods. The aim of this paper is to review phylogeographic studies on the Truncatelloidea inhabiting the NE. part of the Mediterranean Basin, including islands, Black Sea Basin, brackish water habitats, as well as springs and caves of the Balkans, to see how isolation has shaped the present diversity of those snails.

GEOLOGICAL HISTORY OF THE STUDIED AREA

The Aegean Sea harbours more than 7,000 islands and islets (TRIANANTIS & MYLONAS 2009), and connects three continents. In the Upper and Middle Miocene (23–12 Mya), however, the area formed a continuous landmass, *Ägäis*, which was situated on the northern coast of the Tethys Ocean (DERMITZAKIS & PAPANIKOLAOU 1981). Tectonic movements of 12–8 Mya caused a break-up of the southern part of *Ägäis*, starting formation of the Aegean region in its present form. The formation of the Mid-Aegean Trench, which started ~12 Mya and finished ~10 Mya (DERMITZAKIS & PAPANIKOLAOU 1981) (Fig. 1), separated the eastern and western Aegean islands, creating a barrier for the gene flow; its results are detectable in the current patterns of biodiversity of many organisms (e.g. LYMBERAKIS & POULAKAKIS 2010).

Closing of the Strait of Gibraltar in the Messinian (~6 Mya) led to an almost complete desiccation of the Mediterranean Sea. The Aegean Islands became mountain peaks, surrounded by steppe or salt desert. This drastic environmental change allowed some organisms (e.g. terrestrial mammals) to migrate freely, but dispersal of aquatic gastropods was negatively affected. The Messinian Salinity Crisis ended at 5.33 Mya, when the waters of the Atlantic Ocean refilled the Mediterranean after reopening of the Strait of Gibraltar in the so-called Zanclean Flood (GARCIA-CASTELLANOS *et al.* 2009). Later, during the Pleistocene, alternate glacial and interglacial pe-

riods caused fluctuations in the sea level, approaching 140 m. As a result, in the Aegean land and sea kept replacing each other. The present geography of the Aegean region is probably no more than 4–8 kya old (KOUGIOUMOUTZIS *et al.* 2014). Even Crete, far from the mainland, in the Middle Pleistocene (0.4–0.02 Mya), due to low sea levels was separated from the mainland only by a narrow strait. On the other hand, there were periods when Crete was divided in two or three smaller islands surrounded by the sea (CREUTZBURG 1963).

From the Late Jurassic, a large shallow sea, the Parathetys, covered the region from the Alps, through Central Europe, to the recent Aral Sea in central Asia. During the Pliocene, the Parathetys was divided into shallow inland seas. One of those was the Dacic Basin, a vast water body that separated the Carpathians from the recent central Bulgaria. The Dacic Basin was connected (through the present Iron Gate) with the Pannonian Basin in the west, with the Euxinian Basin in the east, and probably directly with the present-day Aegean Sea in the south (CLAUZON *et al.* 2005, SUC *et al.* 2011). With fluctuating water level, this water body existed from ~11 to 1.8 Mya (MATENCO & BEROTTI 2000). The connection between the Parathetys (Pannonian Basin) and the Aegean Sea, known as the Balkan Gateway, ran through the valley of the present Velika [Big] and Južna [South] Morava rivers (STOYANOV & GACHEV 2012).

MATERIAL AND METHODS

This review is entirely based on the materials published by SZAROWSKA *et al.* (2014a, b, c, d, 2016a, b), OSIKOWSKI *et al.* (2015, 2016, 2017) and FALNIOWSKI

et al. (2016a). To infer the phylogenetic relationships, various molecular markers were used: mitochondrial (cytochrome c oxidase subunit I (COI) and ribosomal

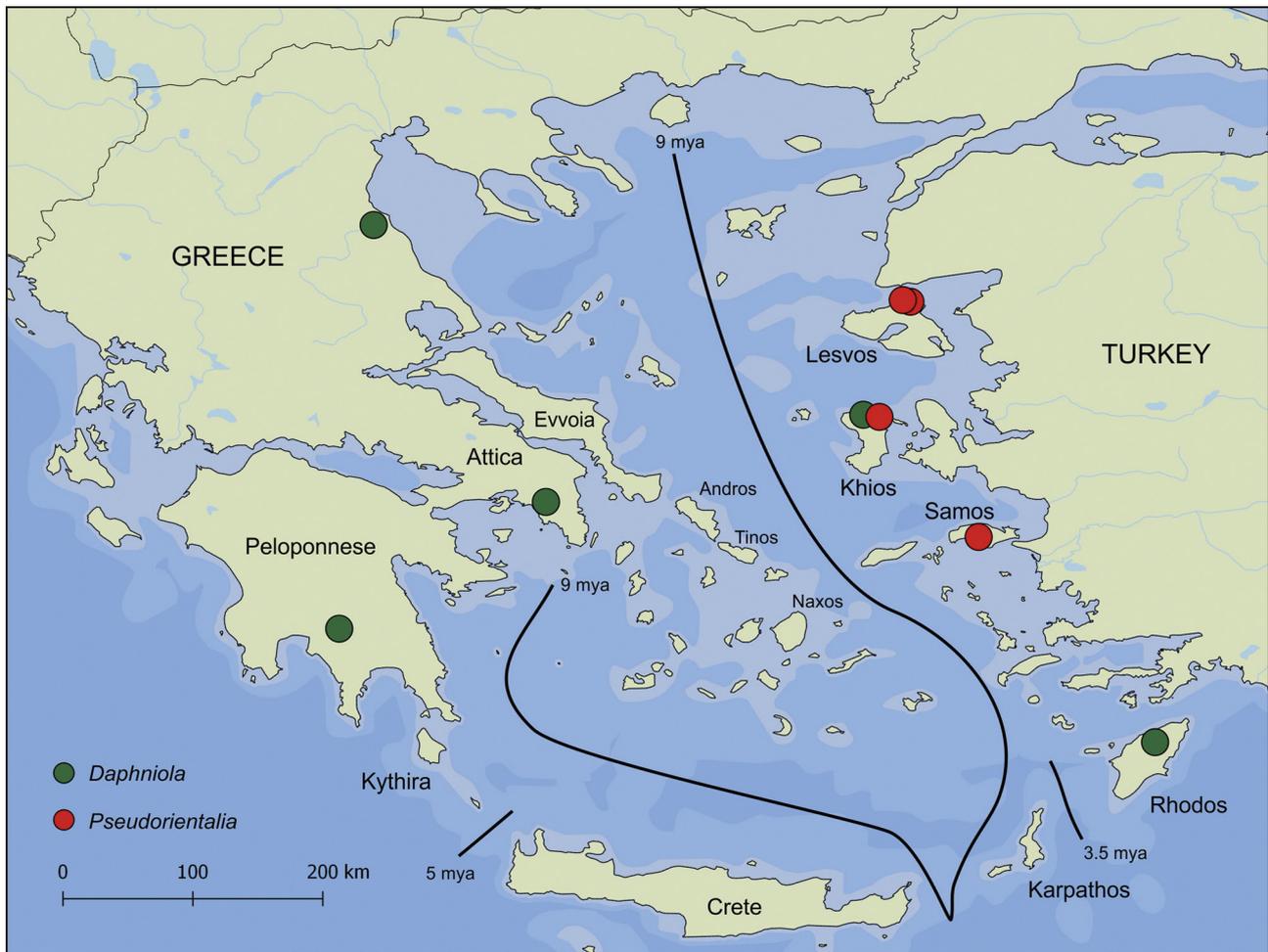


Fig. 1. Map of the Aegean Region. The important geographic locations appearing in the article, as well as the shape of the Mid-Aegean Trench, are shown. Sampling sites of two genera: *Pseudorientalia* and *Daphniola* are shown. For more information see: SZAROWSKA et al. 2014a, b (*Pseudorientalia*) and SZAROWSKA et al. 2014d (*Daphniola*)

16S RNA) and nuclear (internal transcribed spacer (ITS-1), ribosomal 18S and 28S RNA, and histone 3 (H3)). The phylogenetic trees were typically inferred with Maximum likelihood (ML) approach (RAxML, STAMATAKIS 2014). The trees with the highest likelihood score were regarded as the best representation of the phylogeny. In some of the studies, Bayesian inference (BI) was also used (HUELSENBECK & RANNALA 2004), and then the results obtained with ML and BI methods were compared and discussed. COI trees, calibrated with the data on *Salenthydrobia* (WILKE 2003), were used for time estimates with the correction according to FALNIOWSKI et al. (2008). To test the molecular clock, the likelihoods for trees with and without the molecular clock assumption for a Likelihood Ratio Test (LRT) (NEI & KUMAR 2000)

were calculated with PAUP (SWOFFORD 2002). The Relative Rate Test (RRT) (TAJIMA 1993) was performed in MEGA (TAMURA et al. 2013). As Tajima's RRTs and the LRT test rejected the equal evolutionary rate throughout the trees, time estimates were calculated using a non-parametric rate smoothing (NPRS) analysis with the recommended Powell algorithm, in r8s v.1.7 for Linux (SANDERSON 1997, 2003). Penalised likelihood analysis (SANDERSON 2002) which combines likelihood and nonparametric penalty function used in NPRS was also applied; the same analysis was done also on r8s with Powell and TN algorithms. The boundaries of periods, epochs and ages follow the chronostratigraphic chart (COHEN et al. 2013).

BIOGEOGRAPHY OF AQUATIC TRUNCATELLOIDEANS IN THE AEGEAN ISLANDS

The genus *Pseudorientalia* Radoman, 1983, inhabiting mostly Turkey (YILDIRIM et al. 2006), presents

a somewhat unexpected case. Despite the extensive field study in the Aegean islands, its representa-

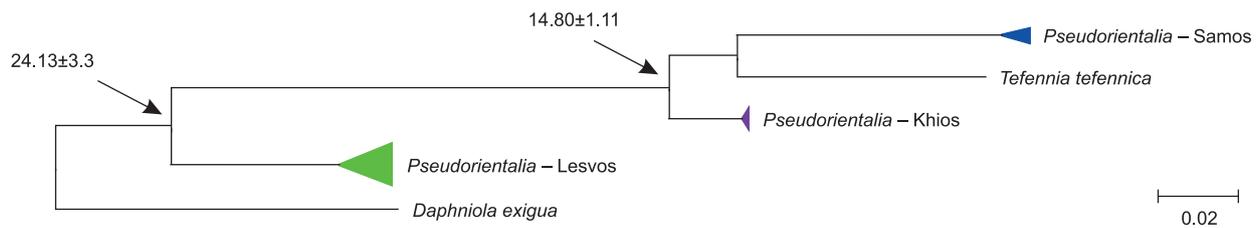


Fig. 2. COI based ML phylogeny of *Pseudorientalia* in the Aegean islands (SZAROWSKA et al. 2014b, modified)

tives were found only on three islands to the east of the Mid-Aegean Trench: Lesvos, Samos and Khios (SZAROWSKA et al. 2014a, b) (Fig. 1). The genetic analyses of the island populations of *Pseudorientalia* using two molecular markers (COI and 16S rRNA) gave consistent results, revealing a small or, in the case of 16S rRNA, no intra-population divergence and high inter-population divergence (SZAROWSKA et al. 2014b). For both markers, haplotypes were grouped in three clades, one on each island (Fig. 2). The genetic distances between the populations inhabiting each of the three islands were high enough to assume that they represented distinct species. The estimated divergence times of the three island populations, based on COI analysis, were 24.13 ± 3.30 Mya between Lesvos and Samos/Khios and 14.80 ± 1.11 Mya between Samos and Khios. These values, dating back to the period when the Aegean Islands formed a continuous landmass, were surprisingly high, considering the small geographical distances between the three islands (Lesvos – Samos: 175 km; Khios lying at about half of this distance). It suggests that the three populations have a relict character and that the lineages they represent diverged long before the islands' formation. The phenomenon of old species occurring on young islands is not unknown (HEADS 2011). The divergence of *Pseudorientalia* on those islands must reflect a high regional diversity in the past (most probably in the Miocene). If the populations from each island represent distinct species, which is strongly supported by molecular and morphological data, it is very likely that the genetic pools of the putative species may have been at least partly separated long before the land connection between the islands and the mainland disappeared in the Middle

Pleistocene (0.400–0.002 Mya) (POULAKAKIS et al. 2005).

The divergence of the genus *Daphniola* Radoman, 1973 in the Aegean Islands and continental Greece (Fig. 1) is intriguing. Until 2012, it was considered to be endemic to continental Greece (RADEA et al. 2013). Then, a single specimen was collected on the Khios Island and three juvenile snails on Rhodos (SZAROWSKA et al. 2014d). The shell morphology of the collected individuals was typical of *Daphniola*, the anatomy was unknown because of the lack of material. Two COI haplotypes were detected – one on Rhodos and another on Khios (Fig. 3). Phylogenetic study using these data, combined with reference sequences of *Daphniola* populations from continental Greece and the Peloponnesus, revealed an unusually close relationship between *Daphniola* from Khios and Rhodos: despite the relatively long geographical distance between the islands (more than 250 km), the p-distance was only 0.005, with the estimated separation time of 0.24 Mya, suggesting conspecificity. The most likely explanation of this small divergence is recent colonisation of one of the islands, either through the Anatolia peninsula or by a stepping stone migration through the neighbouring islands. In the COI ML tree, the Khios/Rhodos clade is grouped with *D. lousi* Falniowski et Szarowska, 2000 from Attica in the mainland Greece (FALNIOWSKI & SZAROWSKA 2000), with the separation time of 2.81 ± 0.86 Mya. Thus, the split of these two lineages occurred long after the Messinian Salinity Crisis which supports the hypothesis of recent colonisation of the eastern Mediterranean Islands by *Daphniola*.

However, the pattern of divergence in *Daphniola* was also shaped by the Messinian Salinity Crisis. All

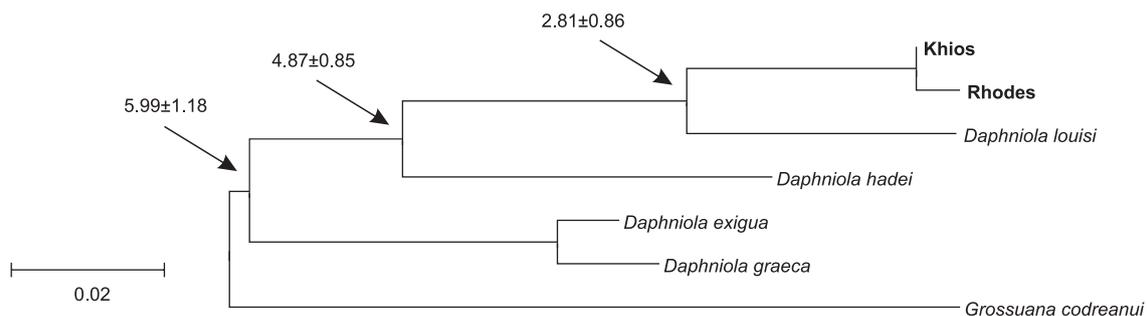


Fig. 3. COI based ML phylogeny of *Daphniola* in the Aegean islands and continental Greece (SZAROWSKA et al. 2014d, modified)

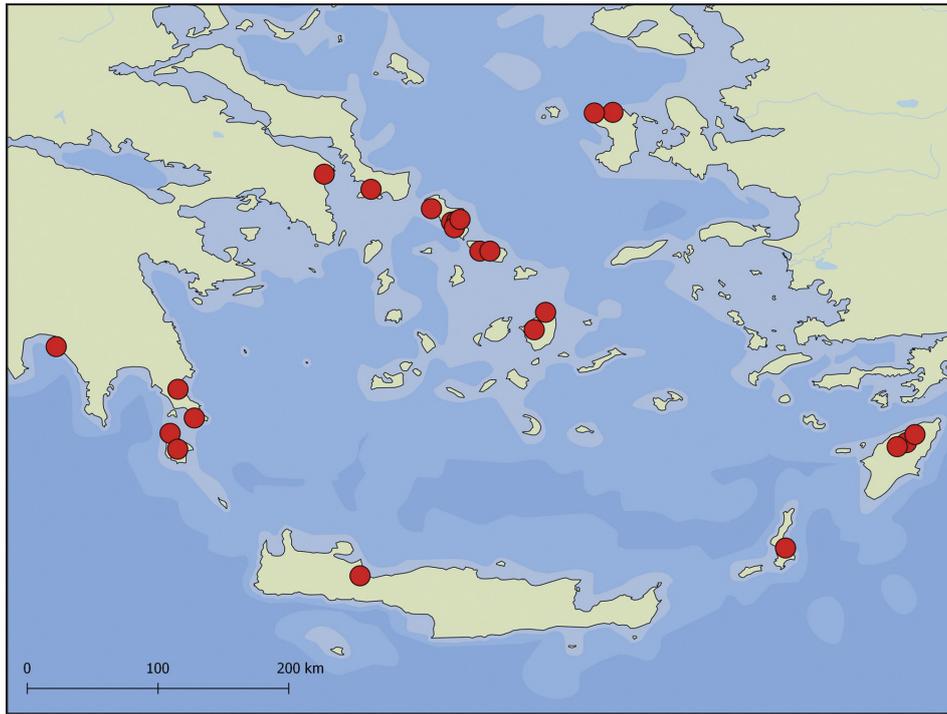


Fig. 4. Map of sampling sites of *Pseudamnicola*. See: SZAROWSKA et al. (2016a) for details

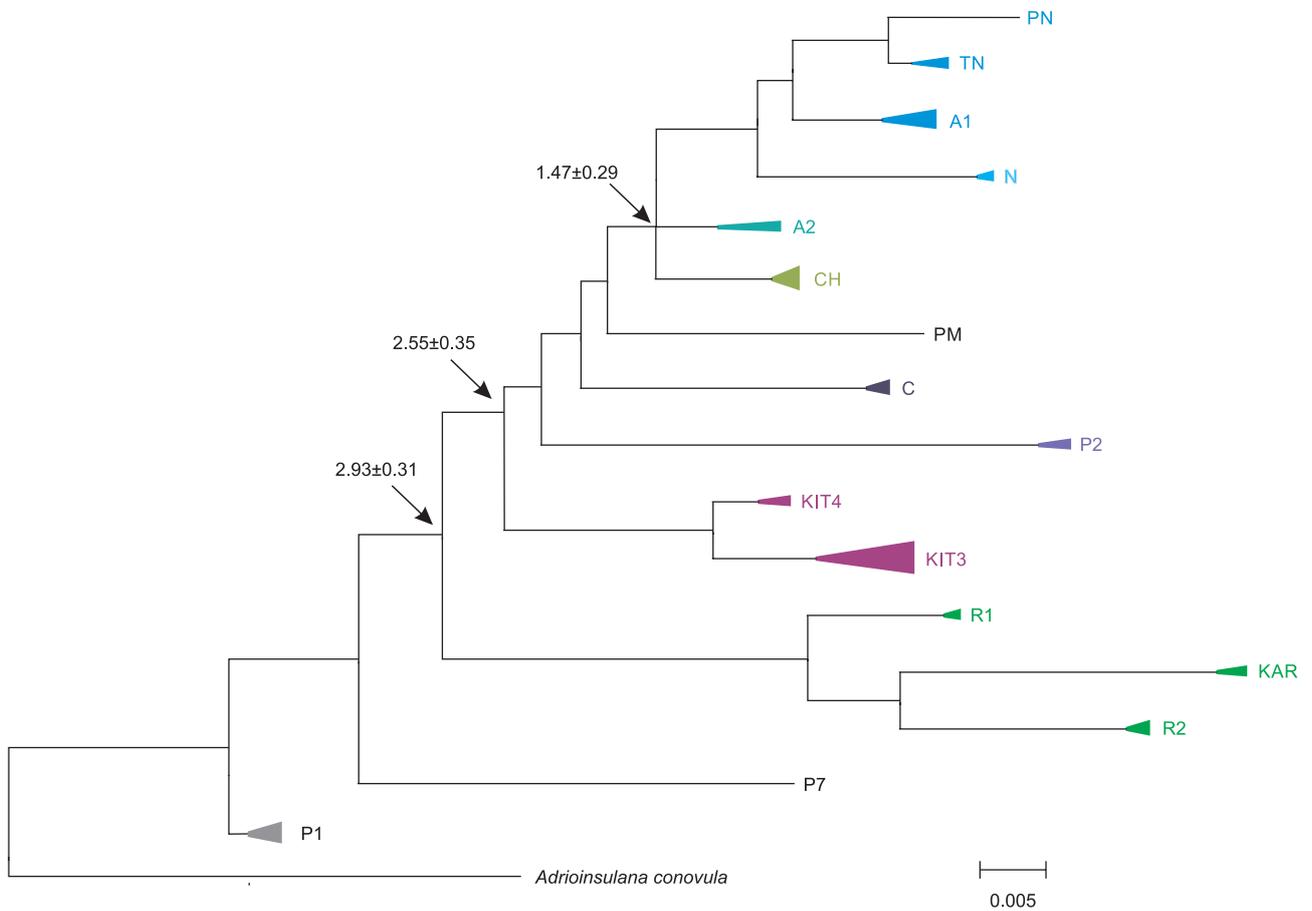


Fig. 5. COI based ML phylogeny of *Pseudamnicola* in the Aegean islands and continental Greece (SZAROWSKA et al. 2016a, modified)

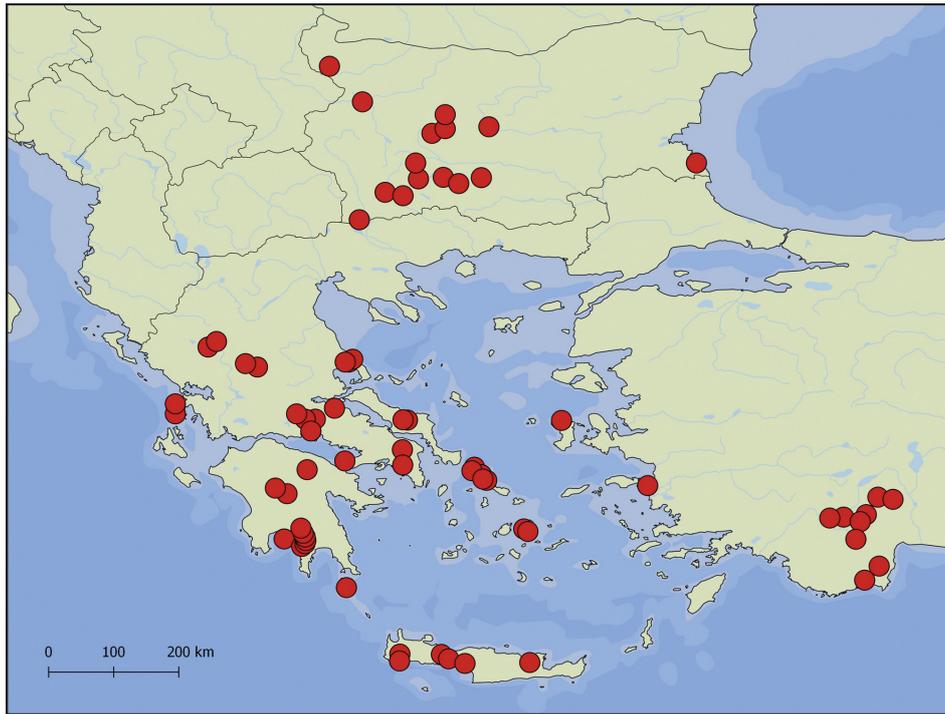


Fig. 6. Combined map of sampling sites of *Bythinella* studied by OSIKOWSKI et al. (2015) and SZAROWSKA et al. (2016b)

the known COI haplotypes from the region form two main clades (SZAROWSKA et al. 2014d) (Fig. 3). The first includes the putative species from Rhodos and Khios mentioned above, coupled with *D. louisi* from Attica and *D. hadei* (Gittenberger, 1982) from the Peloponnese. The second consists of *D. exigua* (A. Schmidt, 1856), including *D. graeca* Radoman, 1973 synonymised with *D. exigua*, both taxa inhabiting the Tembi Valley in Thessaly. The estimated divergence time of the two clades (5.99 ± 1.18 Mya) coincides with the beginning of the Messinian Salinity Crisis. In turn, the split of *D. hadei* from the Rhodos/Khios/*D. louisi* clade is estimated as 4.87 ± 0.85 Mya, suggesting that the separation of the Peloponnese lineage took place short after the Zanclean Flood.

The genus *Pseudamnicola* Paulucci, 1878 is widely distributed and includes species which inhabit springs of Spain, France, the Netherlands, Italy, the southern Balkans, Romania, Cyprus and Turkey. Until recently, its taxonomy was poorly understood and was based mainly on the shell morphology, which is, typically of the Truncatelloidea, highly variable, and on the anatomy of the reproductive organs, which is – as in the other truncatelloideans – simplified due to miniaturisation and shows similar adaptations to internal fertilisation and breeding in fresh water (SZAROWSKA & FALNIOWSKI 2011). Twenty four *Pseudamnicola* populations from the Aegean Islands (Andros, Khios, Crete, Karpathos, Kythira, Naxos, Rhodos, Evvoia and Tinos), and the mainland (Peloponnese and Attica) (Fig. 4) were extensively studied by SZAROWSKA et al. (2016a)

using mitochondrial and nuclear molecular markers, shell morphology and anatomy of male and female reproductive organs. The study led to some important conclusions regarding phylogeny of the genus in the studied area.

The haplotypes of cytochrome oxidase subunit I (COI) of *Pseudamnicola* form 16 clades (Fig. 5), which probably represent distinct species according to GMYC (PONS et al. 2006) analysis (SZAROWSKA et al. 2016a). However, they should be regarded only as putative species, since species distinctions based on a single molecular marker are always ambiguous. The COI dating analysis revealed that the majority of the clades split less than 3 Mya. The close relationships between the populations are confirmed by the differentiation which is very small or absent in the three studied nuclear loci. This strongly suggests that the present biodiversity pattern of the genus is a result of colonisations and re-colonisations due to the presence of land bridges between the islands in the Late Pliocene, and to the earlier major geological events such as the Messinian Salinity Crisis. The intra-population genetic diversity within *Pseudamnicola* is generally small. It shows a higher value only on the mainland and the islands situated close to it: Kythira (clades KIT3 and KIT4) and Rhodos (clades R1 and R2). This may suggest that the ecological conditions on the mainland are conducive to the longevity of populations. Such populations may act as a source of migrants, transferred to nearby islands by birds. It is noteworthy that two populations from Rhodos (R1 and R2), separated by only a few kilometres, are



genetically as distinct as to (presumably) represent distinct species. They differ more from each other than either differs from the population inhabiting the Karpathos Island (clade KAR).

The close relationship between the Andros/Naxos/Tinos group (clades TN, A1, A2, N) and Khios (CH), situated in the eastern part of the Aegean region, is also noteworthy. They form sister clades with the estimated divergence time of 1.47 ± 0.29 Mya. The pattern is most probably due to the lowering of sea levels in the Middle Pleistocene which resulted in formation of broad land bridges and thereby a drastic decrease in the barriers to gene flow between the eastern and western parts of the present Aegean Sea.

The biodiversity of island populations in the Aegean region was also studied for another truncatelloidean genus, *Bythinella* Moquin-Tandon, 1856 (SZAROWSKA et al. 2016b). Despite the extensive fieldwork on many Aegean Islands, the genus was found only on Evvoia, Andros, Khios, Crete, Kythira and Naxos. For the analysis, molecular data from these populations were combined with samples

from Turkey and continental Greece (FALNIOWSKI & SZAROWSKA 2011) (Fig. 6).

In general, the levels of diversity between the populations of *Bythinella* were similar within the mainland and within the island populations, which led to the conclusion that salt water and dry land formed comparable gene flow barriers for these spring-inhabiting gastropods (SZAROWSKA et al. 2016b). The genetic relationships between the studied populations, based on COI analysis, are mostly explainable by the geological history of the region, but in some cases are unexpected (Fig. 7). The haplotypes from Kythira are grouped into a single clade A with the Peloponnessus, which is not surprising considering the small geographical distance (115 km). Similarly, the haplotypes from the Andros Island (clade AND), which form a distinct clade, are closely related to the geographically closest ones from the Evvoia Island and Attica (clades B and B1).

The haplotypes from the Mediterranean part of Turkey (TU2) form the most divergent clade in the COI tree, with the estimated divergence time

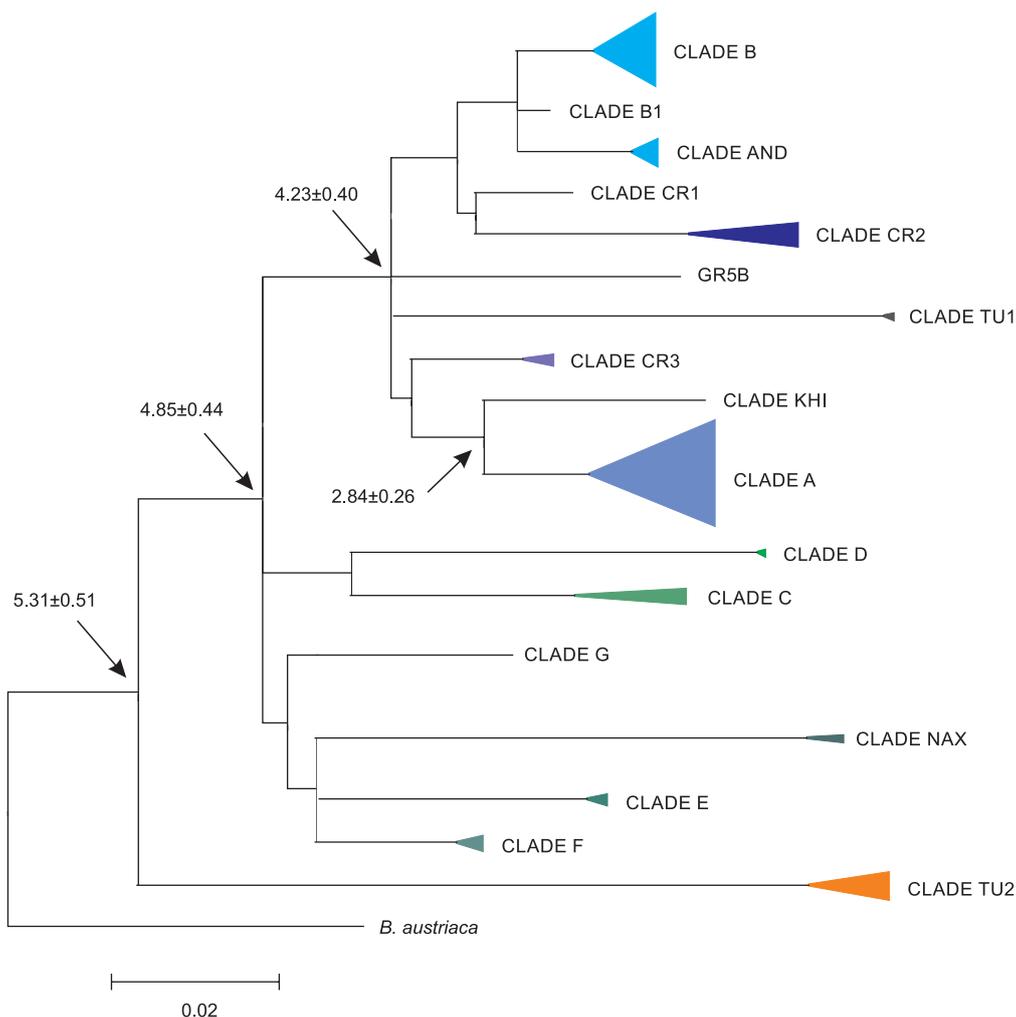


Fig. 7. COI based ML phylogeny of *Bythinella* in the Aegean islands, continental Greece and Turkey (SZAROWSKA et al. 2016B, modified)



of 5.31 ± 0.51 Mya (SZAROWSKA et al. 2016b). This dating is consistent with the Zanclean flood, which supports the conclusion that the *Bythinella* populations from that region were isolated from the rest of their Aegean congeners by saltwater which filled the Aegean Sea after the Messinian Salinity Crisis. The other studied population from Turkey, from the Dilek Peninsula (clade TU1), is not closely related to those in the Mediterranean part of Turkey. The close relationship of the Dilek Peninsula population with the rest of the Aegean populations suggests that the western coast of Turkey was colonised (re-colonised?) by *Bythinella* long after the Zanclean Flood.

The sister clade relationship between the haplotypes of Khios (clade KHI) and Peloponnesus/Kythira (clade A), which are geographically remote and lie on the opposite sides of the Mid-Aegean Trench, is an unexpected result. The estimated divergence time of these two groups is 2.84 ± 0.26 Mya, thus the split took place after the Zanclean Flood. Another surprising finding is that the clade containing haplotypes from the Naxos Island (NAX) is closely related to the haplotypes from Thessaly and Epirus (continental Greece; clades E, F and G), and not to the haplotypes of the geographically close populations from Andros, Evvoia and continental Attica. The time of divergence of the Naxos clade from the rest of the Aegean lineages – 4.85 ± 0.44 Mya – suggests that this population has a relict character, especially when compared with the geographically close populations from Andros, Evvoia, and Attica. The distinctness of the Naxos populations of *Bythinella* was so high that it was described as a new species (FALNIOWSKI et al. 2016b). These findings indicate that the biogeographical pattern of *Bythinella* in the Aegean region is not a result of simple colonisation through a stepping stone scenario after a single geological event. Possibly, the biology of *Bythinella* makes them prone to long-distance, passive dispersal, for example by birds, due to their high tolerance to desiccation (e.g. FALNIOWSKI 1987, SZAROWSKA 2000). This ability may facilitate colonisation and/or re-colonisation.

The analysis of COI differentiation of *Bythinella* revealed an unexpectedly high diversification on Crete (SZAROWSKA et al. 2016b). Samples from five sites included haplotypes which belonged to three clades, not forming a monophyletic group. The hap-

lotypes from the central and eastern parts of the island formed two sister clades (CR1 and CR2 on Fig. 7) which were most similar to the clades from continental Greece and the Andros Island (clades B, B1 and AND). The haplotypes from the western part of Crete (CR3) formed a sister clade to the clades grouping the haplotypes from the Peloponnesus/Kythira (clade A) and, surprisingly, to the populations from Khios (KHI). The time of separation between the two lineages from Crete (central-eastern vs. western) was estimated as 4.23 ± 0.40 Mya. This dating indicates that the separation of the two lineages took place after the Messinian Salinity Crisis. Also later, in the Middle Pleistocene, the sea level was so low that the recent Crete was separated from the mainland only by a narrow strait; earlier, there were periods when Crete was divided in two or three smaller islands. Thus, the high level of genetic variation of the Cretan spring-inhabiting snails is not surprising, since there were probably many events of extinction and colonisation of the populations living there.

A comparison of the phylogeography of *Daphniola*, *Pseudamnicola* and *Bythinella* reveals an interesting, recurring pattern. The populations of the three genera inhabiting the eastern Aegean islands are closely related to those from the western part of the region, on the opposite side of the Mid Aegean Trench. In the case of *Daphniola*, the divergence time of the Khios/Rhodos clade and the Attica clade (i.e. *D. louisi*) is 2.81 ± 0.86 Mya. A very similar date was obtained for *Bythinella* – 2.84 ± 0.26 Mya between the Peloponnesus/Kythira and Khios. In *Pseudamnicola*, this time is even shorter: 1.47 Mya between the Khios and the Andros/Naxos/Tinos clades. These examples strongly suggest that long after the Messinian Salinity Crisis there were favourable conditions for long distance migrations of the Truncatelloidea, which had a strong impact on their current biogeography. The most probable explanation of this phenomenon is the lowered sea level during the Late Pliocene and, periodically, in the Pleistocene, when dry land occupied a larger area, and the distances between shores of the islands and the mainland were much shorter than today (POULAKAKIS et al. 2005), which increased the potential of freshwater gastropods to migrate between suitable habitats.

INFLUENCE OF THE PARATHETYS AND PLEISTOCENE GLACIATIONS ON THE BALKAN TRUNCATELLOIDEANS

The presence of the Dacic Basin was an important factor influencing the distribution of the truncatelloideans in the Balkans. The COI analysis shows that the populations of *Bythinella* from Romania

and Bulgaria form two distinct clades, with the estimated divergence time of ~ 8 Mya (OSIKOWSKI et al. 2015) (Figs 6, 8). The majority of the populations of *Bythinella* in Bulgaria inhabit western and

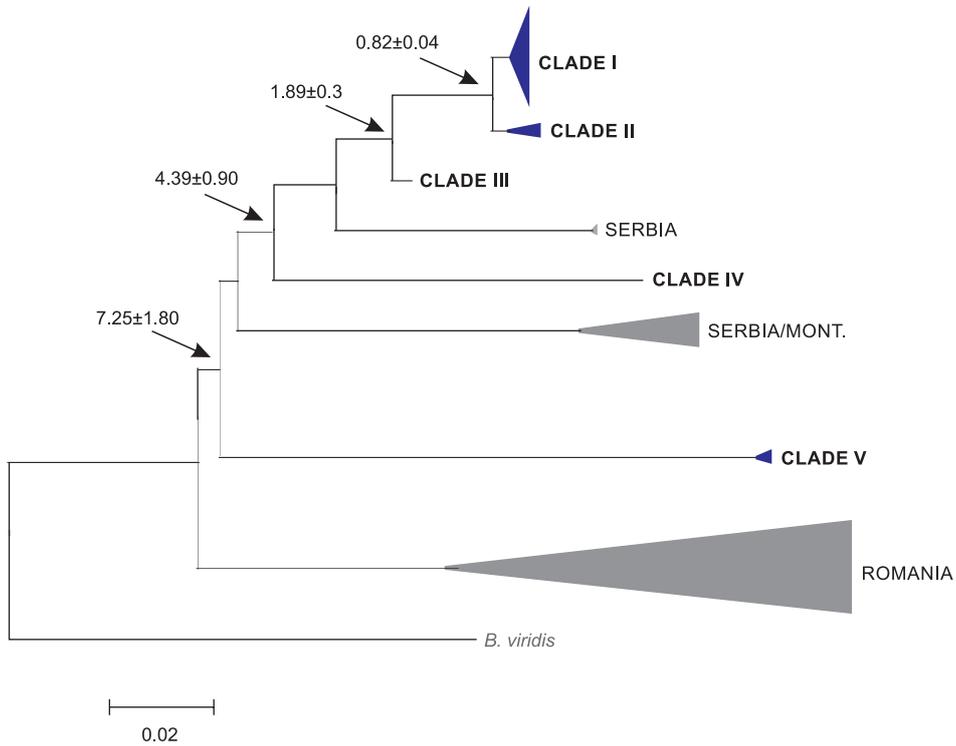


Fig. 8. COI based ML phylogeny of *Bythinella* in Bulgaria (OSIKOWSKI et al. 2015, modified)

central parts of the country (Fig. 6). Despite the extensive exploration, the members of the genus were not found in northern Bulgaria which had been covered by the Dacic Basin. Thus, it is very likely that this ancient water body divided the ancestors of the present-day *Bythinella* in two groups. Later, in the Pliocene, climatic circumstances, especially the presence of glaciers in South-Western Bulgaria

(and Northern Greece: ZAGORCHEV 2007), may have caused the extinction of *Bythinella* in this area. The low genetic diversity within the main *Bythinella* clade in Bulgaria, and its distinctness from the Romanian populations, indicates that the south-western and central Bulgarian populations are relatively young and colonised the area from the south, not earlier than during the late Pleistocene. This prediction is

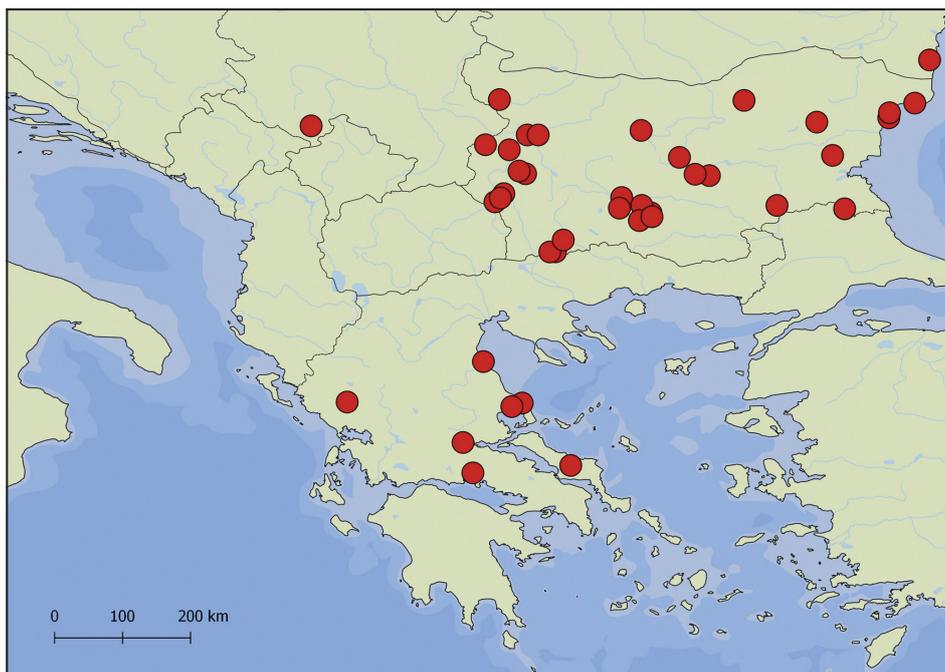


Fig. 9. Map of sampling sites of *Grossuana*. See: FALNIOWSKI et al. (2016a) for details

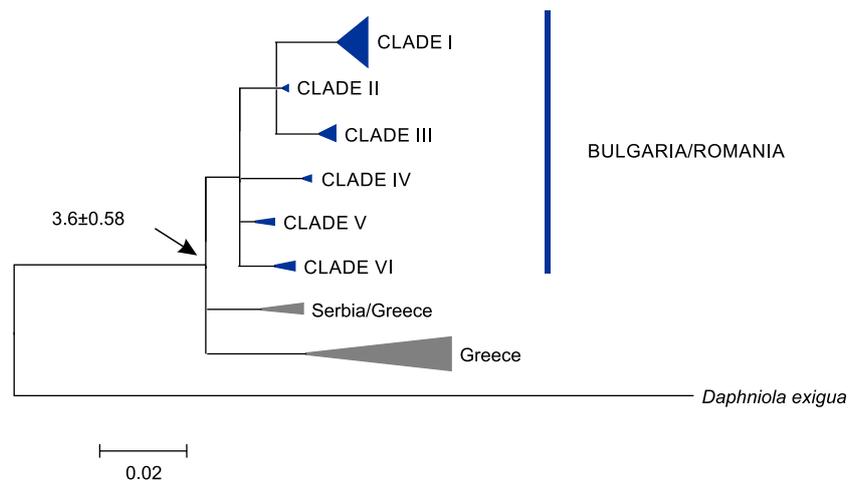


Fig. 10. COI based ML phylogeny of *Grossuana* (FALNIOWSKI et al. 2016a, modified)

also supported by the observation that the genetic divergence of *Bythinella* is much greater in Romania than in Bulgaria (FALNIOWSKI et al. 2009a, b, 2012b, OSIKOWSKI et al. 2015).

The traces of influence of the Dacic Basin on the biogeography are also visible in the case of minute, spring-inhabiting *Grossuana* Radoman, 1973. FALNIOWSKI et al. (2016a) studied the phylogeographic relationships of the genus in the Balkan Peninsula using new and previously published (SZAROWSKA et al. 2007, FALNIOWSKI et al. 2012a) molecular data (Fig. 9). Their COI analysis revealed a major clade grouping the haplotypes from Bulgaria and Romania (Fig. 10). The clade is clearly distinct from the remaining Balkan *Grossuana*, found in Serbia and Greece (continental part of the latter and on the Evvoia Island). The estimated divergence time of these two lineages was 3.6 ± 0.58 Mya which coincided with the existence of the connection between the Parathetycs and the Aegean Sea (Balkan Gateway). This saltwater connection formed a barrier between the *Grossuana* populations inhabiting the present-day Bulgaria and Romania (i.e., east of the Balkan Gateway) on the one hand, and those from the present-day Serbia and Greece on the other. The

present range of *Grossuana* seems to be limited, especially when compared to *Bythinella* or *Pseudamnicola*, which suggests that the dispersal potential of the genus is small. The isolation of the two *Grossuana* lineages was also maintained after the disappearance of the Balkan Gateway, when river valleys were cyclically filled with glaciers during the Pleistocene (STOYANOV & GACHEV 2012). Glaciations are also the most probable explanation of the lower genetic diversity within the Romania/Bulgaria clade of *Grossuana* in comparison with the higher diversity in the Greek populations (FALNIOWSKI et al. 2016a).

The majority of *Grossuana* haplotypes detected in Bulgaria are grouped in Clade I (Fig. 10), with the geographical range covering the Rhodopes, Rila and Pirin Mts in the SW. part of the country. The small genetic diversity within this clade suggests a short history of the genus in the area. Most probably, the Pleistocene glaciers in the Rila and Pirin Mts formed barriers for *Grossuana* and led to their extinction in SW. Bulgaria. The great haplotype diversity coupled with the small nucleotide diversity within Clade I strongly suggests a relatively recent colonisation of this area and rapid radiation from an ancestral population with a small evolutionarily effective size.

QUATERNARY HISTORY OF THE PONTO-CASPIAN BRACKISHWATER HABITATS

The Pleistocene glaciations seem to be the main factor shaping the divergence of the minute hydrobiid gastropod species: *Ecrobia maritima* (Milaschewitsch, 1916) and *E. ventrosa* (Montagu, 1803). The haplotypes of *Ecrobia* from Greece, Bulgaria, Romania, Tunisia, Ukraine (Black Sea), Italy, Germany, UK, Spain and USA (introduced there) confirmed the existence of two species (OSIKOWSKI et al. 2016) (Fig. 11). In the COI tree, the haplotypes formed two clades corresponding to *E. maritima* and *E. ventrosa*, with the estimated separation time of 3.30 ± 0.23 Mya

(Fig. 12) which coincides with the first Pliocene glaciation. The recent data of KEVREKIDIS et al. (2005), SZAROWSKA & FALNIOWSKI (2014) and OSIKOWSKI et al. (2016) on their distribution suggest that they are vicariant: *E. maritima* inhabits the Black Sea and the Aegean Sea, reaching the eastern shores of continental Greece, while *E. ventrosa* lives in the western part of the Mediterranean, and along the Atlantic coast reaching the Baltic and the White Sea. At the beginning of the Quaternary a wide area of the present-day Black Sea, the Caspian Sea and Lake Aral

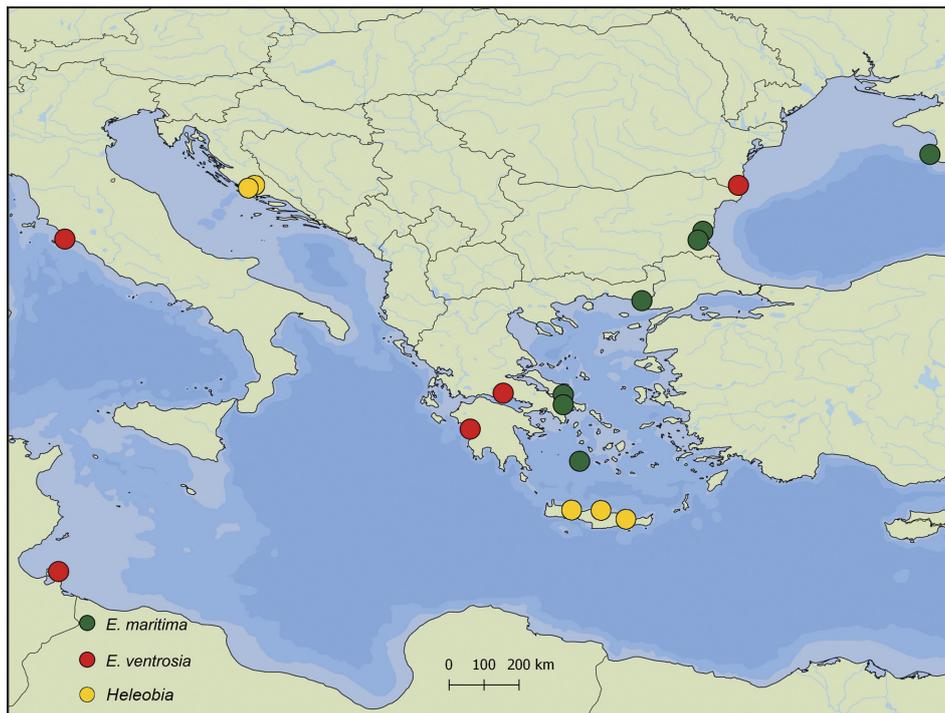


Fig. 11. Map of sampling sites of *Ecrobia maritima*, *E. ventrosa* and *Heleobia*. See: OSIKOWSKI et al. (2016) (*Ecrobia*) and SZAROWSKA et al (2014c) (*Heleobia*) for details

was covered by a shallow sea. After the Bosphorus sea link was closed, the connection between this basin and the Mediterranean Sea disappeared. Thus, *E. maritima* probably evolved there separated from the Atlantic/Mediterranean *E. ventrosa*.

Since the Piacenzian, Late Pliocene (~3 Mya), the development of the Black Sea has been shaped by tectonics and climatic changes. During the main glacial periods of the Pliocene and Quaternary, the sea level decreased to 140 m below the present level. As a consequence, the Black Sea was isolated from the Mediterranean Sea (IZDAR & ERGUN 1991) which resulted in a decrease in salinity of the Euxinian basin. During the interglacials the sea level rose and reached almost its present state, thus the connection with the Mediterranean Sea was periodically restored.

In these phases, the environmental conditions, flora and fauna of the Black Sea were under strong influence of the Mediterranean Sea.

The connection of the Black Sea with the Aegean Sea by the Bosphorus Strait, the Sea of Marmara and the Dardanelles, although sometimes thought to be a substantial barrier between the faunas of the two seas (e.g. DOBROVOLOV 2000), seems to have allowed some gene flow between the populations of *Ecrobia*. As demonstrated by OSIKOWSKI et al. (2016), the populations of *E. maritima* from continental Greece and from the Milos Island (Aegean Sea), as well as from Sevastopol and Burgas (Black Sea), are grouped in the same clade. On the other hand, one population of *E. ventrosa* was found in the Black Sea near Constanza (Romania), which probably was

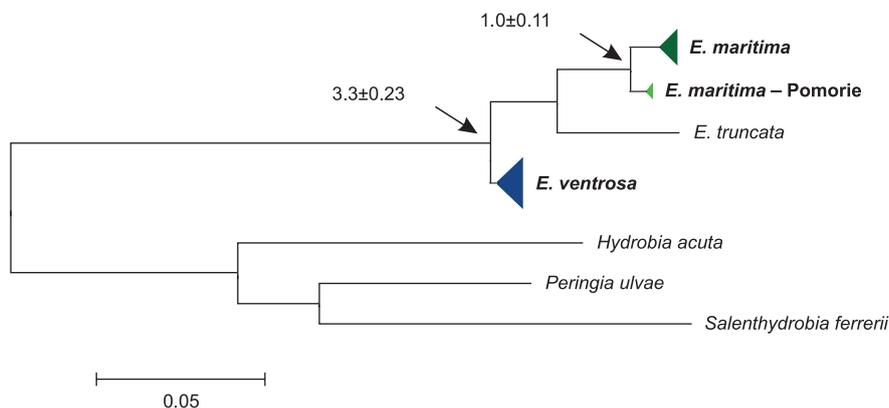


Fig. 12. COI based ML phylogeny of *Ecrobia* in the Black Sea and E. Mediterranean Sea (OSIKOWSKI et al. 2016, modified)



a result of passive transport by birds. It should be remembered, however, that *Ecrobia* are not truly marine, but brackish-water gastropods, inhabiting estuaries, lagoons, and other isolated brackish habitats. The presence of *E. maritima* in the Aegean Sea may be also explained by the peculiarity of water exchange between the Black and Marmara Seas through the Bosphorus. A surface current, cold and less saline, is directed from the Black Sea towards the Sea of Marmara, creating a possibility for passive transport of brackish-water organisms, such as *E. maritima*. A deeper current, running in the opposite direction, is warmer and more saline, thus much less suitable as a migration route for *Ecrobia*, especially since this current of heavy water tends to dive down into the un-oxygenated zone of the Black Sea, providing a slight chance for survival of an immigrant. Among the three studied populations of *E. maritima* from the Black Sea, one shows considerable distinctness. This is the population inhabiting Lake Pomorie, a natural lagoon, hypersaline in part, and in part brackish, separated from the sea by a narrow strip of sand and an artificial dyke. GEORGIEV & NIKOLOV (2010) reported there, based on morphology, the presence of *Ecrobia ventrosa* (as *Hydrobia ventrosa*), but OSIKOWSKI et al. (2016), applying molecular markers, assigned it to *E. maritima*, which confirms once again that the species delimitation based on morphological data in the Truncatelloidea may be confusing.

In the COI tree, the haplotypes from Lake Pomorie form a distinct subclade within *E. maritima* (Fig. 12). There is a substantial genetic distance between this population and the closest con-specific one, just 20 km away (Burgas, Bulgaria). This striking divergence is probably a consequence of combination of the complex geological history of the region and the specific environmental conditions in Lake Pomorie. The uncommon chemistry of the lagoon waters, especially their high salinity gradient varying from 1 to 140‰ (HIEBAUM 2010), may have led to emergence of specific adaptations in the local population. As a consequence, individuals of *E. maritima* from Lake Pomorie are unable to live outside this lagoon, and, analogously, migrants from outside populations cannot survive there. In this way, due to the limited gene

flow or its lack between the populations, *E. maritima* from Lake Pomorie remains clearly distinct.

The brackish-water Truncatelloidea, scattered in estuaries and brackish ponds and springs, do not necessarily show a similar pattern of a rather high geographic diversity. In case of the genus *Heleobia* Stimpson, 1865, only one species – brackish-water *H. maltzani* (Westerlund, 1886) – was found on Crete (Fig. 11). The COI analysis of populations from three distant localities of this species revealed a low level of genetic variation (SZAROWSKA et al. 2014c). Only two haplotypes, differing in one amino-acid substitution, were detected. In the phylogenetic analysis using the haplotypes from Crete pooled with the reference sequences of COI for the other studied *Heleobia* species from continental Europe and Turkey, the Cretan haplotypes formed a clade with one of the two sequences of *H. dalmatica* (Radoman, 1974) from Croatia. This clade was a sister one to another clade, grouping the second sequence of *H. dalmatica* and those of *H. scamandri* (Boeters, Monod et Vala, 1977) (France), *H. foxianensis* (De Stefani, 1884) (Italy) and *H. stagnorum* (Gmelin, 1791) (The Netherlands). The p-distance between these two clades was very small (0.008), indicating that the five nominal species were in fact closely related. The small level of genotypic differentiation of *Heleobia* in Europe is unexpected and may have emerged as a consequence of the bottleneck effect in a refugium, most probably in the Pleistocene. It is noteworthy that the small intra-population differentiation of *H. maltzani* on Crete, and its close relation to the populations from Croatia, Italy, France and the Netherlands, is not a typical pattern for the Cretan fauna. Due to the long isolation by the sea, there are many endemic species on this island, for example land snails (PARMAKELIS et al. 2005), or frogs and lizards (LYMBERAKIS & POULAKAKIS 2010). *Heleobia* is a brackish-water organism, however, thus sea water is not necessarily as strong a barrier for its dispersal as in the case of freshwater *Bythinella*, which are highly differentiated on Crete (SZAROWSKA et al. 2016b). On the other hand, in *Ecrobia*, inhabiting similar brackish-water habitats, the diversity is much higher than in *Heleobia*, which is not easy to explain.

ISOLATION IN LONG-LASTING STABLE HABITATS: TRUNCATELLOIDEAN DIVERSIFICATION IN CAVES

Cave-inhabiting organisms present an interesting case of influence of spatial isolation on genetic structure within and between populations. Stygobionts (also called stygobites) are obligatory subterranean aquatic animals, whose entire life cycle takes place in this environment. Among them, over 350 species

of gastropods have been described, 97% of them belonging to Hydrobiidae (CULVER 2012). Due to the common occurrence of karst topography, the stygobiont gastropod fauna of the Balkans is rich, with 169 species reported by SKET et al. (2004). Despite this richness, the knowledge of the Balkan cave truncatel-



loidean fauna is still very limited. The situation mainly results from the fact that cave-dwelling species are mostly known only as empty shells, washed out onto the surface. In the case of the Truncatelloidea, as already stated, the shell morphology is often not sufficient for reliable species delimitation (e.g. FALNIOWSKI et al. 2009a), and phylogenetic studies require the soft parts of the animals for anatomical studies and DNA extraction.

Caves are long-lasting, stable and isolated habitats. Thus, it is not surprising that the current studies confirm the theoretical predictions that stygobiont fauna would be rich in cryptic species (e.g. JUAN et al. 2010). Two alternative scenarios were proposed to explain the origins of the cave fauna (JUAN et al. 2010). In the “climatic-relict” hypothesis, some pre-adapted organisms passively colonise cave habitats. In the next step, their surface-living (epigeal) ancestors become extinct, due to drastic climatic changes, which cuts off the gene flow between the

cave populations and leads to their independent evolution. The “adaptive-shift” scenario assumes that pre-adapted organisms actively colonise cave habitats in search of resources and, despite the gene flow, diverge from the surface ancestors due to adaptations to cave environments.

Distinguishing between these two hypotheses typically is not easy and requires knowledge of the relationships between the taxa involved. Coexistence in time of highly divergent epigeal and stygobiont taxa in allopatry may indicate the “adaptive-shift” mechanism. Alternatively, existence of stygobiont species without any zone of contact with closely related epigeal ones may be a product of the “climatic-relict” mechanism.

Seven truncatelloidean species from 13 caves in Bulgaria were analysed using molecular (OSIKOWSKI et al. 2017) and morphological (GEORGIEV et al. 2017) techniques to reveal their phylogeny. According to COI analysis, all the stygobiont taxa formed one,

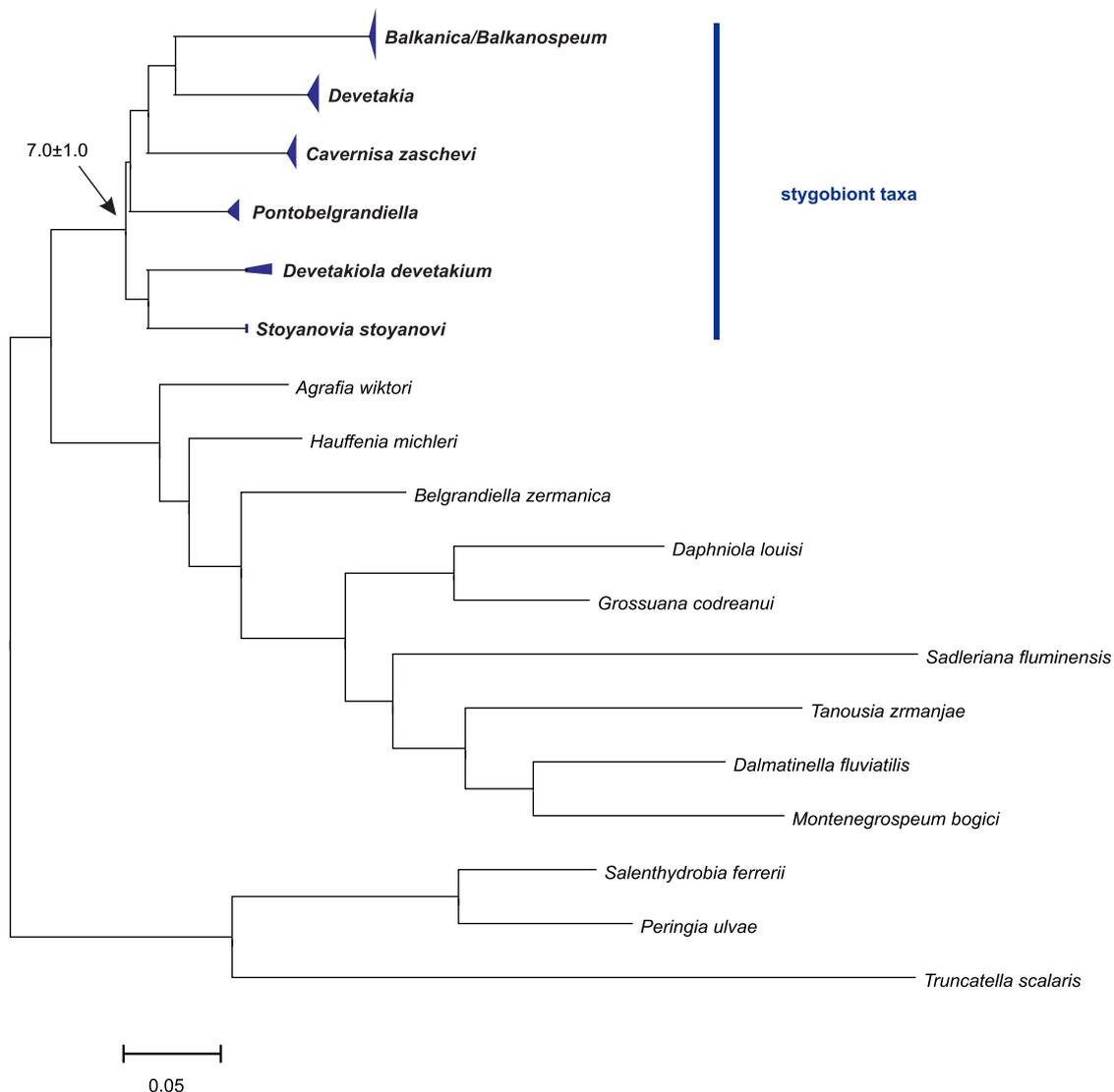


Fig. 13. COI based ML phylogeny of the Bulgarian cave Hydrobiidae (OSIKOWSKI et al. 2017, modified)



highly supported, relatively old monophyletic clade, divided into six subclades (Fig. 13: *Balkanica* Georgiev, 2011/*Balkanospeum* Georgiev, 2012; *Pontobelgrandiella* Radoman, 1978; *Cavernisa* Radoman, 1978; *Devetakia* Georgiev & Glöer, 2011; *Devetakiola* Georgiev, 2017; *Stoyanovia* Georgiev, 2017) (OSIKOWSKI et al. 2017). In general, there are no epigeal, closely related species, parapatric to the studied cave forms, which strongly suggests that the “climatic-relict” scenario may have been at work. Interestingly, the “cave clade” does not include any surface-living taxa, with one possible exception – *Pontobelgrandiella* Radoman, 1978. In contrast to the other studied cave-dwelling truncatelloideans, live individuals of the genus are sometimes found in surface waters; it is doubtful, however, whether they can live and reproduce there (RYSIEWSKA et al. 2016). Sixteen populations of *Pontobelgrandiella* were studied in Bulgaria (RYSIEWSKA et al. 2016), and COI analysis revealed four clades, separated by small genetic distances, but their distinctness was confirmed by PCA analysis of seven shell characters. The *Pontobelgrandiella* clade in the study of OSIKOWSKI et al. (2017) is as old as the other five cave clades, thus the most likely hypothesis explaining the presence of individuals of the genus in surface waters is a relatively new adaptation to epigeal habitats. The small genetic divergence of *Pontobelgrandiella* may also be a result of other factors: adaptation to interstitial habitats, i.e. underground connections between cave systems, allowing an extensive gene flow between the local populations. A somewhat different diversity pattern was observed in cave-dwelling *Bythinella* from Bulgaria, where OSIKOWSKI et al. (2015) studied gastropods from 15 sites, including four caves. Among the 17 COI haplotypes discovered in their study, seven came from cave populations. Three of them, from the Vodni Pech Cave in north-western Bulgaria, formed a separate clade (clade V, Fig. 8). Its substantial distinctness (estimated divergence time: 7.25 ± 1.8 Mya) and the coexistence of three haplotypes (unusual for cave populations, which are usually monomorphic; JUAN et al. 2010), suggest a long isolation and a strictly stygobiontic character of the population. The presence of three different haplotypes may also be a consequence of the occurrence of more than one

species in the cave, but the intra-population genetic differentiation within this clade is small (p -distance=0.4%), much below the proposed, estimated species threshold for *Bythinella* (K2P=1.5%; BICHAIN et al. 2007). It is noteworthy, however, that all the studied specimens were collected not from inside of the cave, but from the water flowing out of it. There are no morphological and ecological data to indicate if the *Bythinella* population from the Vodni Pech Cave is strictly stygobiontic or not. It is very likely that the existence of the cave population of *Bythinella* in the Vodni Pech Cave is a result of “climatic-relict” scenario. The single COI haplotype of *Bythinella gloeri* Georgiev, 2009 from the Lepenitza cave also formed a separate clade (clade III, Fig. 8), with the estimated time of separation of 1.89 ± 0.3 Mya. In this case, also the “climatic-relict” scenario is the most probable explanation. The remaining three COI haplotypes of cave populations of *Bythinella* from Bulgaria formed clades with other haplotypes from epigeal populations; thus, it can be concluded that they are in an early phase of the “climatic-relict” scenario, or alternatively, the situation is a result of an “adaptive-shift”. More data, especially concerning adaptations to cave environment, are necessary to resolve this problem.

It is striking that in both studies described above, the divergence time of the clades grouping stygobiont populations are similar (7–7.5 Mya). It strongly suggests that some drastic climatic changes at that time led to extinction of surface-dwelling populations, reducing the gene flow between the existing cave populations, leading to their independent evolution.

Considering all the facts and hypotheses listed above, it is evident that isolation, past and present, is the main factor shaping phylogenies of the Truncatelloidea.

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