

AQUATIC SNAILS *ECROBIA MARITIMA* (MILASCHEWITSCH, 1916) AND *E. VENTROSA* (MONTAGU, 1803) (CAENOGASTROPODA: HYDROBIIDAE) IN THE EAST MEDITERRANEAN AND BLACK SEA

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Abstract.— In the paper cytochrome oxidase subunit I (COI) in minute gastropods *Ecrobia* has been sequenced from five localities at the Black Sea (Pomorie Lake and Constanza) and eastern Mediterranean (Gulf of Corinth, Evvoia Island, Attica). Pomorie Lake, a hyperhaline lagoon in eastern Bulgaria, harbours a population of *Ecrobia maritima*, whose shell and penis morphology are presented. COI partial sequences of *E. maritima* from Pomorie Lake differ markedly from those in the two Aegean populations not studied so far (Evvoia Island, Attica), and from another four studied earlier, scattered across the Black Sea and Aegean Sea. Such a high level of divergence was unexpected, since Pomorie Lake is only about 20 km away from the closest known locality of *E. maritima* (Burgas, Bulgaria). The mean *p* distance within *E. maritima* was 0.0113. A similar value of *p* distance (0.0137) was found within the clade grouping all the haplotypes of *E. ventrosa*. Mean genetic distance between these two species is *p* = 0.048. *E. ventrosa* inhabits western Europe, Tunisia, the Peloponnesus and the Corinthian Gulf (Itea: present study), both in the Ionian Sea, and – surprisingly – the coast of the Black Sea in Romania (Constanza: present study). *E. maritima* was found in the Black Sea and the Aegean Sea. Estimated time of divergence between the species is 3.30 ± 0.23 Mya, and 1.00 ± 0.11 Mya between *E. maritima* from Pomorie Lake and the other studied localities. The observed pattern is discussed in the context of the geological history of the region, especially glaciation events. Speciation of *E. maritima* in the Ponto-Caspian waters isolated from the Mediterranean in the Late Piacenzian and divergence of the population presently occurring in Pomorie Lake during the local Calabrian salinity crisis are postulated.



Key words.— Black Sea, Late Pliocene, Mediterranean, molecular clock, phylogeography, Pleistocene, *Hydrobia*, *Ventrosia*, Pomorie Lake

INTRODUCTION

The Hydrobiinae, typical inhabitants of brackish waters, are a very popular subject of study for marine biologists (see Falniowski 1987 for a review of earlier studies), but exact data on their distribution are scarce, and there are numerous unreliable data. The numerous records that exist of *Peringia ulvae* (Pennant, 1777) from the eastern Mediterranean, despite the fact that the species does not occur in the Mediterranean Sea, may be a good example, as well as the monograph on the Greek Hydrobiidae (Schütt 1980), which does not mention any representatives of the Hydrobiinae. Although Muus (1963, 1967) already demonstrated that the shell characters are too variable to determine a species, thus penis morphology and head pigmentation pattern must be studied, many hydrobiologists and marine biologists still record those species determined on shell characters alone (e.g. Koutsoubas *et al.* 2000, Evagelopoulos *et al.* 2009). In fact, the differences shown by Muus (1963, 1967), and confirmed by Falniowski (1986, 1987, 1988), distinguish the genera: *Hydrobia* Hartmann, 1821, *Peringia* Paladilhe, 1874, and *Ventrosia* Radoman, 1977, but within those genera there are species, which cannot be distinguished with morphological data, because of morphostatic evolution (Davis 1992). Non-adaptive radiation marked by the rapid proliferation of species without ecological differentiation (Gittenberger 1991) results in a flock of species that need not differentiate morphologically or ecologically. Thus, at a species level in the Hydrobiinae, morphological characters cannot be used for species recognition alone and molecular characters are inevitably necessary to distinguish taxa (e.g. Wilke and Davis 2000, Wilke and Falniowski 2001, Wilke and Pfenniger 2002, Szarowska and Falniowski 2014). The shells of *Ventrosia maritima* and *V. ventrosa* cannot be distinguished from one another (Szarowska and Falniowski 2014), and the same concerns the penes.

Radoman (1983) reported *V. ventrosa* (Montagu, 1803) from the Adriatic Sea. Radoman (1973) described a new species *V. pontieuxini* Radoman, 1973, postulating species-level distinctness of the Black Sea *Ventrosia*, formerly classified as *V. ventrosa*. Later, *V. pontieuxini* was considered a synonym of *V. maritima* (Milaschewitsch, 1916). Recently, Kevrekidis *et al.* (2005) reported this presumably Pontic species *V. maritima* from the Evros Delta lagoons at the border of Greece and Turkey, East Aegean Sea, and Szarowska and Falniowski (2014) recorded *V. maritima* from Milos Island in the Cyclades (Aegean Sea), and *V. ventrosa* from the west coast of the Peloponnese (Ionian Sea). Recently, *Ventrosia* Radoman, 1977 has been considered a junior synonym of *Ecrobia* Stimpson, 1865, since Davis *et al.* (1989) suggested that

North American *Ecrobia truncata* (Vanatta, 1924) was introduced from Europe and would then be a synonym of *Ventrosia ventrosa*. The cytochrome oxidase subunit I (COI) sequence of the North American *E. truncata* places this species within the clade of the European *Ventrosia*, thus confirming that *Ventrosia* is a junior synonym of *Ecrobia*.

The aim of this study is to expand knowledge on the distribution and genotypic diversification of the genus *Ecrobia* in the East Mediterranean and Black Sea, considering its possible geological background. We present the molecularly distinct local population of *E. maritima* from Pomorie Lake in Bulgaria and four hitherto unstudied local populations of *Ecrobia* (one from Romania and three from Greece), and compare with another 12 *Ecrobia* from GenBank.

MATERIAL AND METHODS

Snail sampling and fixation

The specimens of *Ecrobia* spp. were collected from five sites: three localities across Greece, one in Bulgaria and one in Romania (Fig. 1, Table 1). Snails were collected by hand or with a sieve. Specimens for the morphological study were fixed in 4% formaldehyde and stored in 80% ethanol, while specimens for molecular analyses were washed in 80% ethanol and left to stand in it for about 12 hours. The ethanol was then changed twice during 24 hours and, after a few days, the samples were transferred to 96% ethanol and stored at -20°C prior to DNA extraction. The shells were photographed with a CANON EOS 50D digital camera. Dissections were done, and penes photographed under a NIKON SMZ18 stereoscopic microscope with dark field and phase contrast, and CANON EOS 50D digital camera.

DNA extraction and sequencing

DNA was extracted from foot tissue using the Sherlock extracting kit (A&A Biotechnology) and dissolved in 20 µl TE buffer. PCR was performed in a reaction mixture of 50 µl total volume using the following primers: LCOI490 (Folmer *et al.* 1994) and COR722b (Wilke and Davis 2000). The PCR conditions were as follows. COI: initial denaturation step of 4 mins at 94°C, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 mins, and a final extension of 4 mins at 72°C. Ten µl of the PCR product was run on 1% agarose gel to check for quality. PCR products were purified using Clean-Up columns (A&A Biotechnology). The purified PCR products were sequenced in both directions using BigDye Terminator v3.1 (Applied Biosystems) following the manufacturer's protocol and



Figure 1. Map of all localities of *Ecrobia ventrosa* (red squares) and *Ecrobia maritima* (black dots) sampled in phylogenetic analyses. Localities 1–5 are represented by new COI sequences; localities 6–15 are represented by published sequences (Wilke and Davis 2000, Wilke 2003, Szarowska and Falniowski 2014, Wilke, unpublished). See Table 1 for locality details.

using the primers described above. The sequencing reaction products were purified using ExTerminator Columns (A&A Biotechnology), and the sequences were read using an ABI Prism sequencer.

Molecular data analysis

Sequences were aligned in Bioedit 7.1.3.0 (Hall 1999). Basic sequence statistics were calculated in DnaSP 5.10 (Librado and Rozas 2009) and MEGA 6.06 (Tamura *et al.* 2013). The saturation test was conducted using DAMBE (Xia 2013).

In a phylogenetic analysis we also used 17 sequences from the GenBank (Table 1) of *Ecrobia* from western Europe (Wilke and Davis 2000), Bulgaria (Wilke 2003), Greece (Szarowska and Falniowski 2014, Wilke, unpublished), Tunisia (Wilke, unpublished), Ukraine (Wilke, unpublished) and the USA (Wilke 2003). We also used three outgroup sequences (Table 1): *Hydrobia acuta* (Draparnaud, 1805) (Wilke *et al.* 2000), *Peringia ulvae* (Pennant, 1777) (Wilke and Davis 2000) and *Salenthydrobia ferrerii* Wilke, 2003 (Wilke 2003).

The data were analysed using an approach based on Bayesian inference (BI) and maximum likelihood

(ML). The Bayesian analyses were run with MrBayes ver. 3.2.3 (Ronquist *et al.* 2012) with default priors. Two simultaneous analyses were performed, each lasting 10,000,000 generations with one cold chain and three heated chains, starting from random trees, and sampling trees every 1000 generations. The first 25% of trees were discarded as burn-in. The analyses were summarized on a 50% majority-rule tree.

A maximum likelihood (ML) approach was applied in RAxML v8.0.24 (Stamatakis 2014). One thousand searches were initiated with starting trees obtained through the 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 the free computational resource CIPRES Science Gateway (Miller *et al.* 2010).

To test the molecular clock, *Peringia ulvae* and *Salenthydrobia ferrerii* (Wilke 2003) were used as outgroups. The divergence time between these two species was used to calibrate the molecular clock, with correction according to Falniowski *et al.* (2008).

Table 1. Sampling localities of *Ecrobia* with their geographical coordinates. Sequences of *Ecrobia* from GenBank and outgroups are also included.

Locality	Site	Coordinates/GB numbers	
new <i>Ecrobia</i>			
1	Pomorie Lake, Bulgaria	42°34'40''N	27°36'52''E
2	Constanza, Romania	43°47'46''N	28°31'02''E
3	Itea, Gulf of Corinth, Greece	38°25'37''N	22°27'22''E
4	Aliveri, Evvoia, Greece	38°23'31''N	24°00'57''E
5	Kato Souli, Attica, Greece	38°09'07''N	24°01'48''E
references <i>Ecrobia</i>			
6	Bulgaria, Burgas	AF449216	
7	Germany, Boiensdorfer Werder	AF118369	
8	Greece, Evros Delta	AY616140	
9	Greece, Milos	KJ406197- KJ406200	
10	Greece, Peloponnisos, Kato Xirokhorion	KJ406201- KJ406204	
11	Italy, Orbetello Lagoon	AF118327	
12	Spain, Bay of Cadiz	AF118331	
13	Tunisia, Medenine, Djerba, Sebkh de Sidi	AY616138	
14	Ukraine, Sevastopol	AY616139	
15	United Kindom, Holme Broadwater	AF118337	
16	USA, Stone Harbor, New Jersey	AF449217	
outgroups			
	<i>Hydrobia acuta</i> (Draparnaud, 1805)	AF278812	
	<i>Peringia ulvae</i> (Pennant, 1777)	AF118302	
	<i>Salenthydrobia ferrerii</i> Wilke 2003	AF449200	

The likelihoods for trees with and without the molecular clock assumption in a likelihood ratio test (LRT) (Nei and Kumar 2000) were calculated with PAUP. The relative rate test (RRT) (Tajima 1993) was performed in MEGA. As Tajima's RRTs and the LRT test rejected an equal evolutionary rate throughout the tree for *Ecrobia*, time estimates were calculated using a penalized likelihood method (Sanderson 2002) in r8s v.1.7 for Linux (Sanderson 2003).

RESULTS

The shells of *E. maritima* from Pomorie Lake (Fig. 2A–I) were typical of *Ecrobia*, and the same concerns the penes (Fig. 3A–B). Thus, the morphological characters cannot be used to distinguish a species within the genus.

In the present study we obtained COI (552 bp) sequences from 14 specimens of *Ecrobia* (GenBank Accession nos. KX355830–43). *E. ventrosa* was found at the Corinthian Gulf (Itea; locality 3) in the Ionian Sea, and – surprisingly – the western coast of the Black Sea in Romania (Constanza, locality 2). *E. maritima*

was discovered in the Black Sea region (Pomorie Lake, locality 1) and in the Aegean Sea region, Greece (Attica, locality 5 and Evvoia Island, locality 4).

The test of Xia *et al.* (2003) revealed no saturation. Topology of the trees obtained from BI and ML analyses were identical. New and reference sequences (Table 1) of the European *Ecrobia* are grouped in two distinct clades, corresponding to *E. ventrosa* and *E. maritima*, with an estimated divergence time of 3.30 ± 0.23 Mya. In the tree, the North American *E. truncata* is placed within the European *Ecrobia*, closest to *E. maritima* (although this placement is not well supported) (Fig. 4). Mean genetic distance between these two clades is $p = 0.048$. The mean p distance within these groups was similar: 0.0113 and 0.0137 for *E. maritima* and *E. ventrosa*, respectively. The most divergent sequence within *E. ventrosa* came from Tunisia ($p = 0.0222$). The phylogeny of the rest of the *E. ventrosa* subclades was not fully resolved. The close relationship between sequences from Itea (Corinthian Gulf) and the sequence from Romania is noteworthy.

Within the *E. maritima* clade, two well supported subclades were distinguished with a p distance of

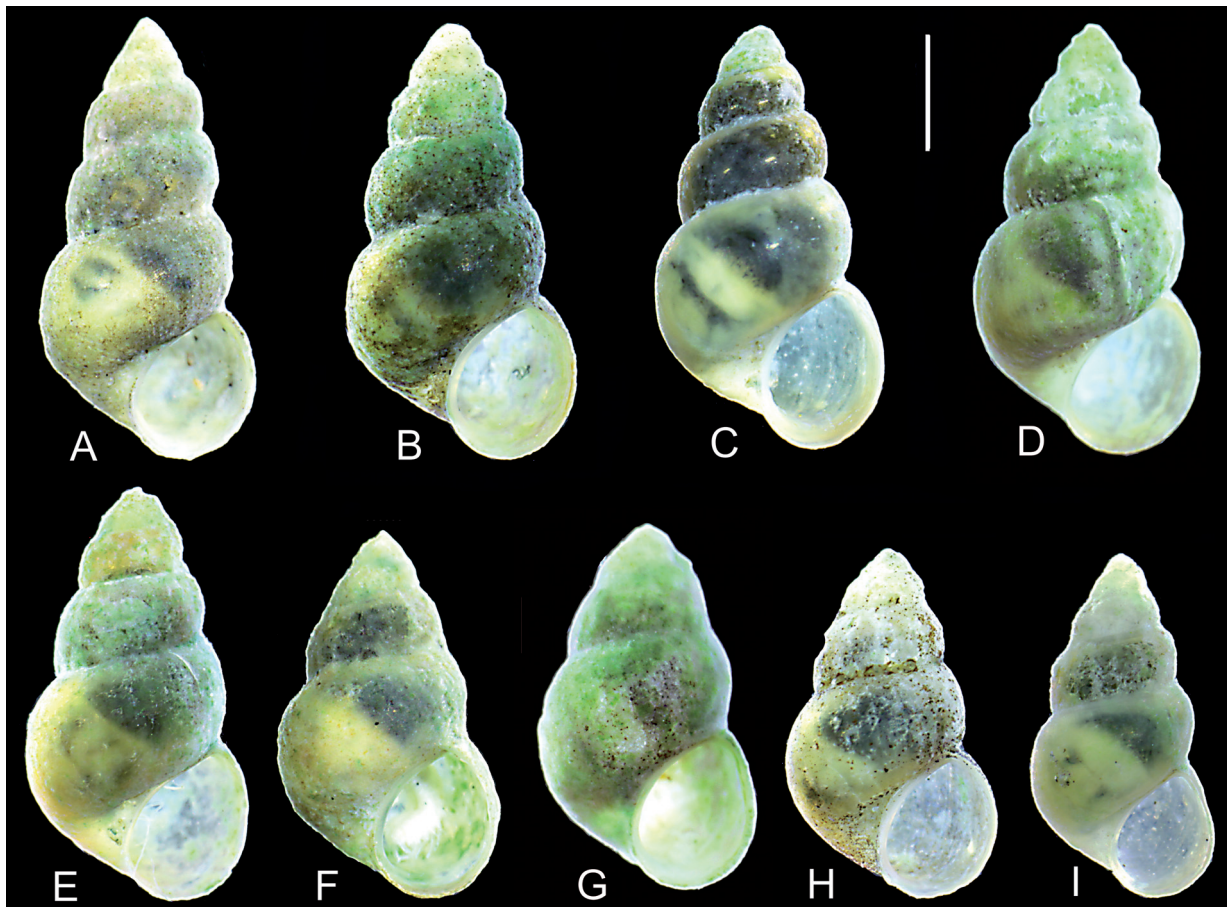


Figure 2. Shells of *Ecrobia maritima* from Pomorie Lake, scale bar equals 1 mm.

0.0188 and a divergence time of 1.00 ± 0.11 Mya. The first subclade is formed by specimens from Pomorie in Bulgaria, the second by the remainder of the *E. maritima* samples. The high level of divergence between these two subclades was unexpected, since the Pomorie Lake is only about 20 km away from the bay between Strandzha and Kraimorie, home to one of the populations belonging to the second subclade (Burgas, Bulgaria; see Table 1), but the p distance between these two populations is 0.0207.

DISCUSSION

Complex geological history of the Aegean Sea and Black Sea region affected biogeographical patterns of several organisms, including gastropods (e.g. Kalkan *et al.* 2011, Szarowska *et al.* 2014). The evolution of the Black Sea is still a controversial subject, with many contradictory models of its time of origin, ranging from Precambrian to early Quaternary (Izdar and Ergun 1991, Dimitrov and Dimitrov 2004, Panin 2008). A part

of the Thethys Sea later became an Euxinian part of the eastern Paratethys, a complex of numerous water bodies forming a passage between the present Caspian and Mediterranean Seas. This contained freshwater and brackish water phases, connected with the Caspian, Mediterranean, or both. Later, as a consequence of subduction, the sea gradually became deeper, over 2000 m, with no oxygen below about 140 m. There is a broad shelf in the NW part of the Black Sea, which dried up several times during the glacial regressions and the sea level was lower than the contemporary one, reaching depths of 90, 120 and 170 m (Dimitrov and Dimitrov 2004). Transgressions and regressions determined the facies pattern of the Black Sea sediments during the Quaternary (Izdar and Ergun 1991). The Quaternary period witnessed a series of glacial and interglacial events accompanied by widespread tectonism. It should be noted, however, that the series of glaciations had already started in the Pliocene, about 3 Mya. Therefore, the paleogeographic development of the Black Sea region is mainly a function of tectonism and climate. The main glacial periods

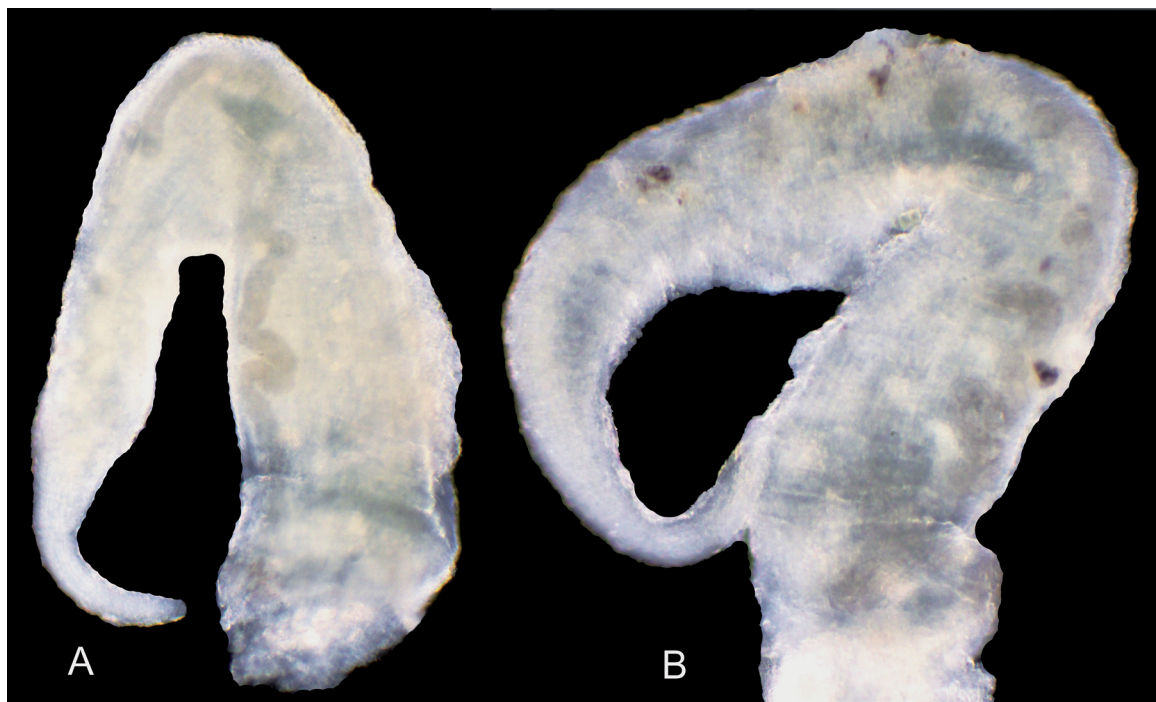


Figure 3. Penes of *Ecrobia maritima* from Pomorie Lake.

of the Quaternary (and late Pliocene) in Europe (Danube, Günz, Mindel, Riss and Würm) corresponded to the regressive phases of the Black Sea, with low-stands of the water level down to -120 m. The regressions represent phases of isolation of the Black Sea from the Mediterranean Sea and the World Ocean. Only the connection with the Caspian Sea was sometimes continued through Manytch Valley. Correspondingly, during regressions, under fresh water conditions, the faunal assemblages had a pronounced Caspian character. On the contrary, during the interglacials, the water level rose to close to the present level; the Black Sea was reconnected to the Mediterranean Sea, and the environmental conditions as well as the fauna came under marine Mediterranean influences.

The divergence time estimate of *Ecrobia ventrosa* and *E. maritima* in our tree is 3.30 ± 0.23 Mya, in the Pliocene epoch, Piacenzian stage. This coincides with the first Pliocene glaciation. A number of studies in other organisms inhabiting European seas, have also indicated younger, middle Pleistocene divergences between Atlantic, Mediterranean, and Black Sea populations, thus population divergences need not necessarily be erased by subsequent glacial cycles (Papadopoulou *et al.* 2005 and references herein).

In this region, the Quaternary started with the marine Akyaglayan transgression (2.6 Mya), which transformed wide areas of Russia into a shallow sea by

uniting the Black Sea, the Caspian Sea and Lake Aral. A gradual deepening of the Black Sea during this period led to the formation of a basin structure which, after closure of the Bosphorus sea link, developed into a non-marine but salinar environment. The tectonic elements affecting Quaternary palaeogeography were principally due to subsidence in the south and uplift in the north of Europe. This caused opening and closing of communication paths between the Odessa – Azov depression and the Black Sea and the Caspian Sea, respectively. *E. maritima* most probably speciated during this period of isolation from the Atlantic/Mediterranean *E. ventrosa*. The data of Kevrekidis *et al.* (2005), Szarowska and Falniowski (2014) and the present study suggest that the current range of *E. maritima* covers the Black Sea and Aegean Sea (Milos, Evvoia, Attica), with the western border at eastern continental Greece. *E. ventrosa* is a vicariant species, inhabiting the West Mediterranean, with the Ionian Sea (including the Gulf of Corinth) as the eastern border of the range.

The Bosphorus Strait and the Sea of Marmara can be considered as the gene flow barrier between Aegean and Black Sea maritime fauna (e.g. Dobrovolov 2000, Viaud-Martinez *et al.* 2008, Turan *et al.* 2009). However, our data do not confirm this hypothesis, since sequences from Attica, Milos and Evvoia formed one subclade with sequences from Burgas, Evros Delta and Sevastopol. On the other hand, *Ecrobia* is not truly

marine, but a brackish-water species, inhabiting habitats with similar salinity in the Black Sea and Mediterranean. The Bosphorus Strait has also been seen not to restrict gene flow in some other animals (e.g. Shemesh *et al.* 2008, Kalkan *et al.* 2011).

The exchange of water along the shallow and narrow Bosphorus Strait is limited and two-directional. The upper current of colder and less saline water runs

from the Black Sea into the Marmara Sea, and transports twice as much water as the lower, bottom current of warmer, more saline water from the Marmara Sea. The water from the Marmara Sea flows down into anoxic zone of the Black Sea, thus restricting passive transportation into the Black Sea. This may explain the expansion of *E. maritima* into the Aegean Sea.

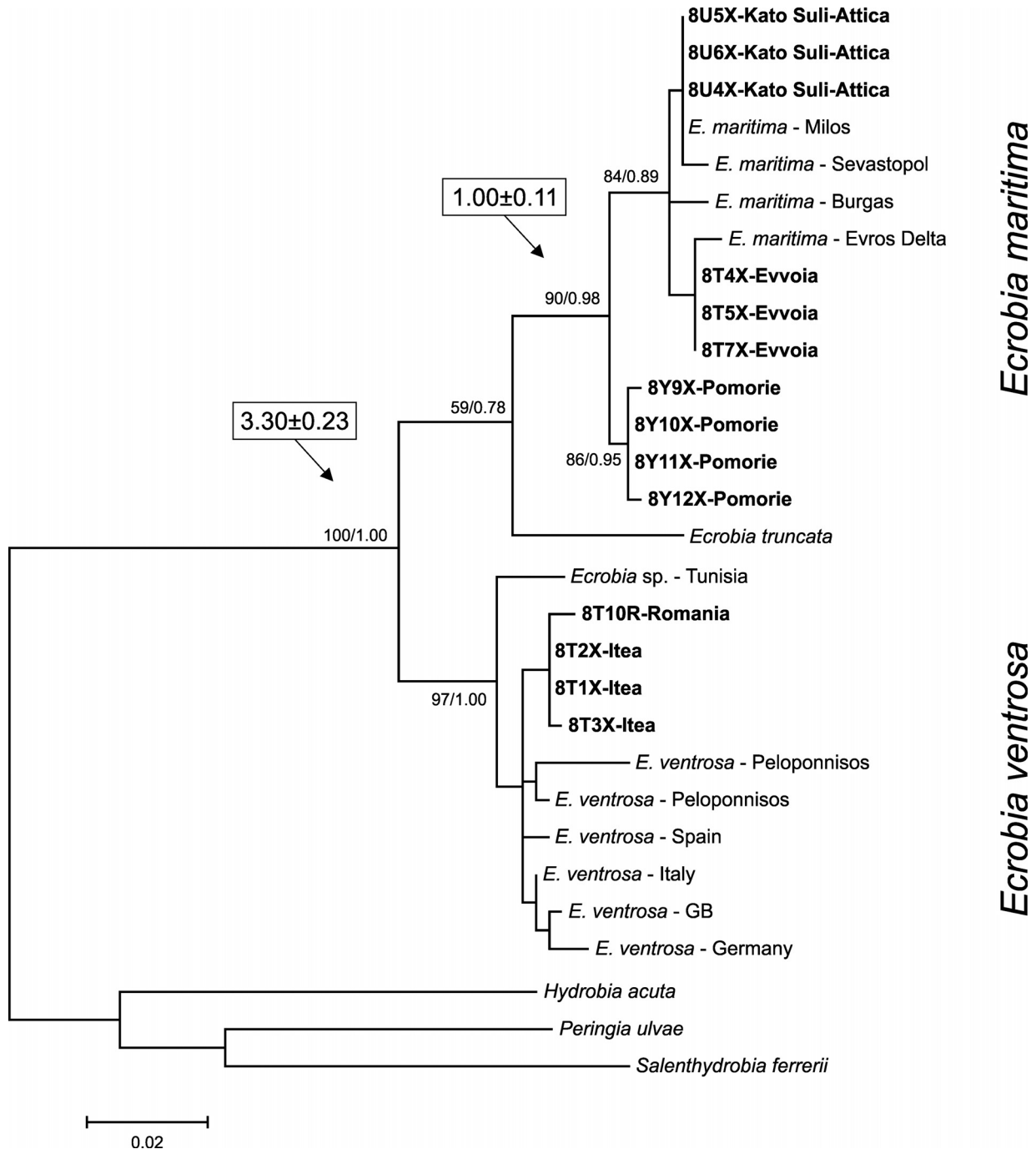


Figure 4. Maximum-likelihood phylogram for the COI gene. New COI sequences in bold.

The maintenance of the genetic divergence observed between the Aegean-Black Sea *E. maritima* and the Mediterranean *E. ventrosa* can also be attributed to the existence of separate marine water masses. The possibility of gene flow between these two basins could be reduced by the limited water exchange between the Aegean-Black Sea and the eastern Mediterranean. Moreover, the circulation between the Ionian and Levantine basins of the Mediterranean and the Aegean basin is limited by the arc of the Cycladic archipelago. In addition, water from the Black Sea is moved toward the Mediterranean by the surface circulation in the Aegean basin, influenced by cold northern winds and by upwelling of cold water along the Anatolian coast (Shemesh *et al.* 2008).

The striking genetic distinctness of the *E. maritima* inhabiting Pomorie Lake, from all the other populations of this species inhabiting the Black and Aegean Seas, is noteworthy. At the Pliocene-Pleistocene boundary, regression took place in the Black Sea, simultaneously with the Late-Calabrian regression in the Mediterranean under glacial Gunz conditions approximately 1.7 Mya. The Black Sea level reached to 170 m below present sea level. The Late Calabrian includes 1.00 ± 0.11 Mya, the estimate of the divergence time between our *E. maritima* from Pomorie Lake and the other populations of *E. maritima*. More precisely, 1 Mya was reported as a local Salinity Crisis, marked by the evaporites in the sediment (Degens *et al.* 1981). The population from Pomorie Lake is markedly different from the one from Burgas, a few kilometres away. However, the lake is too young to have harboured this isolated population long enough.

Pomorie lake, which is part of the Burgas Wetlands situated along the Bulgarian Black Sea coast, is a natural, hypersaline lagoon, divided from the sea by a narrow strip of sand and an artificial dike. This waterbody is characterized by the salinity gradient varying from 1 to 140‰ (Hiebaum 2010), which maintains a high diversity of habitats (Raynova 2010). Georgiev and Nikolov (2010), describing invertebrate fauna of Pomorie Wetland, listed three species belonging to Hydrobiidae: *Hydrobia* sp., *H. ulvae* (*Peringia ulvae*) and *H. ventrosa* (*Ecrobia ventrosa*). Our molecular analysis clearly indicates that Pomorie Lake is inhabited by *E. maritima* rather than *E. ventrosa*, which confirms that previous studies based on morphological characters alone may be confusing.

The distinctness of the *E. maritima* population inhabiting Pomorie Lake from even the closely situated population of this species found in Burgas may result from the combination of complex geological history of the region and specific environmental conditions within the hypersaline water body. Complex hydrochemistry of the waters of Pomorie Lake (Hiebaum 2010) may prevent migration of snails from other

populations. High salinity gradient and other characters of the lagoon may lead to the emergence of various adaptations in the local population, limiting the gene flow and promoting selection. Unfortunately, our knowledge about the environmental requirements of *Ecrobia* snails is limited and cannot explain the possible ecological constraints capable of maintaining the distinctness of *E. maritima* in Pomorie Lake. With our present knowledge, we can say that this population is unique and deserves to be protected and studied further.

E. maritima was found in the Black Sea region (from Ukraine: Sevastopol and Bulgaria: Burgas, Pomorie and Evros Delta) and in the Aegean Sea region (Greece: Attica and two islands – Milos and Evvoia). *E. ventrosa* was discovered in western Europe (including the type locality in the United Kingdom), Tunisia, the Peloponnesus and the Corinthian Gulf (Itea), both in the Ionian Sea, and the western coast of the Black Sea in Romania. The somewhat unexpected occurrence of *E. ventrosa* at Constanza, on the Black Sea, is most probably a result of long-range passive transportation by birds (Rees 1965, Wesselingh *et al.* 1999, Charalambidou and Santamaria 2002, Figuerola and Green 2002). Lyell (1832) mentioned the possibility that waterfowl could transport land snail *Succinea putris* to distant islands through the attachment of snail eggs to feathers, and Darwin (1859) observed ducks emerging from a pond with duckweed adhering to their backs. For the Risssoidea, Bondesen and Kaiser (1949) mentioned the possibility of *Potamopyrgus antipodarum* (Gray, 1843) and other prosobranchs attaching themselves to feathers by trapping them between the operculum and shell. Cadée (1988, 1994) observed living *Hydrobia ulvae* emerging from faeces of the shelduck, *Tadorna tadorna* (Linnaeus, 1758), indicating that they had survived passage through the digestive tract. The numbers of *Hydrobia* observed alive in shelduck faeces were considerable: three droppings were found to contain a total of 140 living specimens and 960 empty and/or broken *Hydrobia* shells (Cadée 1988). It should be noted that only a few specimens of *E. ventrosa* were found at Constanza even after a long search, thus the population was either young or close to extinction.

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