

# Do diversity patterns of the spring-inhabiting snail *Bythinella* (Gastropoda, Bythinellidae) on the Aegean Islands reflect geological history?

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**Abstract** To explain the origin of the differentiation of the spring-inhabiting fauna on an island system, this study focused on the Aegean Islands and the *Bythinella* snails as a model organism. We inferred the phylogeographic pattern of the Aegean *Bythinella* with two molecular markers, and compared the inferred pattern with the geological history of the region and the estimated levels of biodiversity of *Bythinella* in Greece. 95 sequences of COI and 60 of ITS-1 were obtained from samples collected from Andros, Crete, Naxos, Chios, Kithira, and western Turkey, and pooled with previously published sequences from continental Greece. Phylogenetic analysis based on the COI revealed seven new clades. On Crete, three distinct clades were identified. The estimated divergence time indicated that the

geological history of the island, which was divided into smaller islands until 2–3 Mya, was most probably responsible for such high diversity. *Bythinella* populations inhabiting Turkey were distinct from the Aegean populations, and the divergence began after the Zanclean flood (5.3 Mya). Shell morphology and anatomy of reproductive organs confirmed that molecularly distinct clades were real entities. Biodiversity analysis revealed that, in addition to five previously known *Bythinella* hotspots, there is a sixth in central Greece.

**Keywords** Origin and history of spring fauna · Springs as ephemeral habitats · mtDNA · COI · ITS-1 · Morphology · Phylogeography · Biodiversity

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## Introduction

Springs, as potentially isolated small habitats, are often considered the terrestrial analogues of islands at sea. The fauna of the “real” islands is known as interesting object of studies, focused mostly on the origin of differentiation within and between the islands. However, nearly all of the fauna that have been examined were terrestrial animals (e.g., Fattorini, 2002, 2007; Kasapidis et al., 2005; Klossa-Kilia et al., 2006). Thus, it could be expected that the spring-inhabiting fauna in “islands on islands” need not follow the same patterns that were inferred for terrestrial animals. To answer this question, we

studied the spring-inhabiting snail *Bythinella*, as a model organism, in the Aegean Islands, whose geological history is rather well known, and whose terrestrial fauna has been studied rather extensively.

The complex geographic history of the Aegean Islands, especially during the Pleistocene, led to the high diversification of the fauna and the flora. The current pattern of biodiversity in this area was shaped by substantial climatic changes as Messinian Salinity Crisis, Zanclean flood and repeated glaciations separated by warmer periods, causing fluctuations in the sea level that exceeded 200 m. The majority of studies on the Pleistocene phylogeography of European animals focus on the vertebrates and the terrestrial invertebrates (Dennis et al., 2000; Chatzimanolis et al., 2003; Benke et al., 2009, and the references therein), but to better understand the processes of diversification, species must be investigated that have different ecological restrictions. The freshwater snail species of cold springs are interesting examples of this kind, because these snails live in potentially isolated, unstable and ephemeral habitats.

In the eastern Mediterranean region, three basic patterns of relationships can be considered between the geological events and the phylogeography. The first pattern includes organisms that were present in this area before the opening of the Mid-Aegean Trench, which caused the separation of the Aegean Islands into western and eastern groups. The second concerns animals that arrived in the area after the formation of the Mid-Aegean Trench and which were affected by other geological or climatic events, such as the Messinian Salinity Crisis (Garcia-Castellanos et al., 2009) that transformed the Mediterranean Sea of today into a desert with lakes of low or high salinity. The third pattern is for the introductions through the transportation by animals or recently, by man. In most of the studies on the phylogeography of the eastern Mediterranean, one or more of these three patterns explained the distribution of an animal (e.g., Sfenthourakis et al., 1999; Parmakelis et al., 2006a, b; Simaiakis & Mylonas, 2008). Based on the geological and climatic events, some key dates can be highlighted as follows: the initiation of the split of the Mid-Aegean Trench (approximately 12 Mya), the end of the split (approximately 9 Mya) and the Messinian Crisis (approximately 5 Mya). Notably, the present geography of the Aegean is no more than 8–6 Kya old (Kougioumoutzis et al., 2014, and references therein).

Since the peak of the Wisconsin–Würm glaciation (24–10 Kya), the global sea level has risen by 120–130 m, and the Aegean Islands now lie in an area in which the land was once dry (Lykousis, 2009). In the Late Pleistocene, the eastern Aegean Islands were connected with Anatolia, and the strait between Europe and the Cyclade-mega-island (Cycladic plateau), which connected among others the present Naxos, Paros, Tinos and Andros islands, was rather narrow (Creutzburg, 1963; Douris et al., 2007; Kougioumoutzis et al., 2014).

The studies of the fauna and flora on islands often focus on the possible relationships of species richness with the area and the isolation of the islands (Fattorini, 2007). Of particular interest are the Aegean Islands, because they are located between two mainland areas (Turkey and continental Greece) and therefore may have a transitional character (Fattorini, 2002). The presence of land connections between the Aegean Islands and the Greek mainland from the Messinian to the Middle Pleistocene created opportunities for the colonisation of the islands from both directions.

There are several studies on land gastropods, but also on butterflies, beetles, terrestrial isopods, scorpions, frogs and lizards, and also plants (Beerli et al., 1996; Sfenthourakis, 1996; Douris et al., 1998, 2007; Chatzimanolis et al., 2003; Parmakelis et al., 2003, 2005, 2006a, b; Poulakakis et al., 2003, 2005a, b, c; Bittkau & Comes, 2005; Kasapidis et al., 2005; Klossa-Kilia et al., 2006; Lymberakis et al., 2007; Poulakakis & Sfenthourakis, 2008; Papadopoulou et al., 2009; Lymberakis & Poulakakis, 2010), which suggest that the present distributions of most terrestrial Aegean taxa were shaped primarily through vicariant phenomena that resulted from the complex geological history of the eastern Mediterranean and particularly that of the Aegean archipelago. Nevertheless, dispersal over sea or through occasional land bridges was also involved (see Douris et al., 1998; Dennis et al., 2000; Kasapidis et al., 2005).

The genus *Bythinella* is a group of freshwater, dioecious, oviparous snails that inhabit small springs and subterranean waters (Giusti & Pezzoli, 1977; Falniowski, 1987). The snails have a wide range that extends from the Iberian Peninsula to western Asia and from southern Poland to southern Greece. The divergence of *Bythinella* has primarily been studied in western, southern and central Europe (Boeters, 1973, 1998; Giusti & Pezzoli, 1977; Falniowski, 1987), but

these studies concentrated mostly on external morphology and anatomy, initially of the shell and later, of the soft body parts. However, morphology only cannot be used for the unequivocal delimitation of species because of the limited number of taxonomically useful characters and the high variability of those characters (Falniowski, 1987, 1992; Bichain et al., 2007; Falniowski et al., 2009a). In more recent studies with molecular data, several *Bythinella* species were identified and also several nominal species were synonymised (Bichain et al., 2007; Haase et al., 2007; Benke et al., 2009; Falniowski et al., 2009a, b, 2012b; Falniowski & Szarowska, 2011, 2012).

Several species of *Bythinella* were described in Europe, mainly in western and central parts of the continent (e.g., Radoman, 1976, 1983; Bichain et al., 2007). In contrast, relatively few *Bythinella* species have been identified in Greece and those species were initially identified based on morphological characters (Schütt, 1980; Radoman, 1976, 1983; Reischütz et al., 2008). In more recent research that combined morphology with the genetic markers (COI and ITS), ten putatively distinct species were identified in continental Greece: two in the Peloponnese, one each in the Attica and the Parnassos Mts. (central Greece), one on Lefkas Island and four in northern Greece (Falniowski & Szarowska, 2011, 2012). However, the diversity of *Bythinella* on the Aegean Islands remains to be uncovered, which would complete the phylogeographic picture of the genus.

Thus, the aim of this study was to explain the origin of the differentiation of the spring-inhabiting *Bythinella* on the Aegean Islands, the geological history of which is rather well known and whose terrestrial fauna is sufficiently studied for comparisons. Last but not least, the *Bythinella* in continental Greece, adjacent to the Aegean, was previously studied, and therefore, the differentiation could be compared between an archipelago and a landmass. To determine the origin of the differentiation of the *Bythinella* on the Aegean Islands, we (i) inferred the phylogeographic pattern of the Aegean *Bythinella* by examining two molecular markers, the Cytochrome c Oxidase subunit 1 (COI) mitochondrial gene and the Internal Transcribed Spacer (ITS-1) from the nuclear-encoded ribosomal gene region; (ii) compared the inferred pattern with the geological history of the region; and (iii) estimated the levels of biodiversity (as reflected by mean genetic distances) of *Bythinella* in Greece.

The morphology (shell and reproductive organs) of each clade inferred from the molecular analysis was also examined to assess whether the morphological differentiation reflected the genotypic one, and therefore, to determine whether the inferred clades represented not only haplotypes but also actual biological entities.

In general, our questions were as follows: (i) Are there differences in the patterns of diversity of this snail between the islands and the continent? (ii) How much of the differentiation in the spring-inhabiting snail could be explained by geological events? (iii) Is this differentiation similar to that of the terrestrial inhabitants of the archipelago?

## Materials and methods

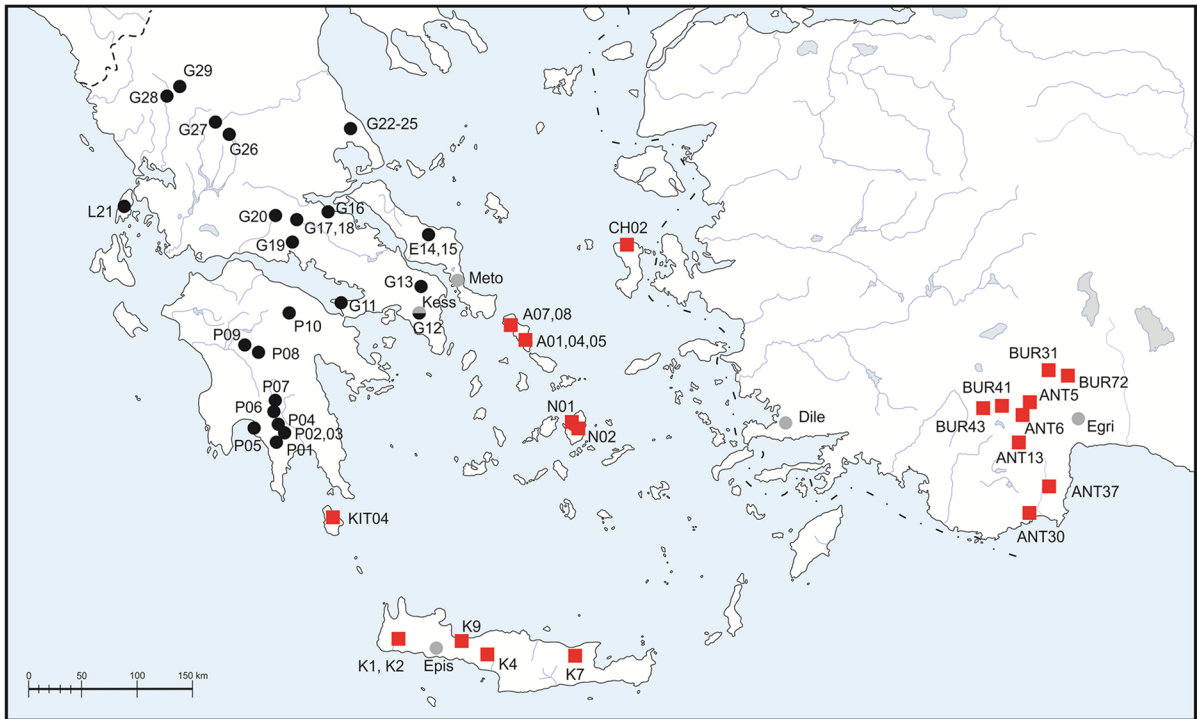
### Sample collection and fixation

During extended fieldwork in the Aegean Islands, we attempted to obtain snails from each part of the archipelago by searching on the islands of Rhodes, Samos, Chios, Lesbos, Crete, Karpathos, Milos, Naxos, Paros, Mykonos, Tinos, Andros, Evvoia and Kithira. However, the specimens of the genus *Bythinella* were found only on Andros, Chios, Crete, Kithira and Naxos. Additional samples were collected in the western region of Mediterranean Turkey. The geographic locations of the samples collected for this study are shown in Fig. 1 and are presented in Online Appendix 1.

Snails were collected by hand or with a sieve. Individuals to be used for molecular analyses were then washed in 80% ethanol and then left to stand in it for about 12 h. Afterwards, the ethanol was changed twice in 24 h and, after few days, the 80% ethanol was replaced by 96%. Samples were then stored in  $-20^{\circ}\text{C}$  prior to DNA extraction. Individuals for the morphological study were fixed in 4% formaldehyde and then stored in 80% ethanol.

### Morphological techniques

Snails were dissected using a NIKON SMZ18 stereomicroscope with dark field, drawings were done with a NIKON drawing apparatus, and shells and penes were photographed with a CANON EOS 50D digital camera.



**Fig. 1** Sites used in the phylogenetic analyses. *Red squares* sites sampled for the present study, *black dots* data taken from Falniowski & Szarowska (2011), *grey dots* data taken from Benke et al. (2011). Further details are given in Online Appendix 1

### Morphometric techniques

We used a NIKON DS-5 digital camera measurement system to measure seven shell morphometric parameters (Szarowska, 2006; Falniowski et al., 2012a) in ten specimens out of each of the nine molecularly distinguished clades (see below), represented by one population each. The linear measurements were then logarithmically transformed. For angular measurements, the arcsine transformation was applied. We calculated Euclidean distances and computed minimum spanning tree (MST) using NTSYSpc (Rohlf, 1998). The same programme was used to compute a principal component analysis (PCA), based on the matrix of correlation (Falniowski, 2003). The original observations were projected into PC space, with a superimposed MST (performed for clarity, not presented in the figures) to detect local distortions in the data. Such usage of the PCA in a descriptive approach makes it possible to detect morphologically distinct groups without any a priori classification.

### DNA extraction and sequencing

DNA was extracted from foot tissue using a SHER-LOCK extracting kit (A&A Biotechnology) and dissolved in 20  $\mu$ l of TE buffer. PCR was performed in the reaction mixture of total volume 50  $\mu$ l using the following primers: LCOI490 (5'-GGTCAACAAATCATAAAGATATTGG-3') (Folmer et al., 1994) and COR722b (5'-TAAACTTCAGGGTGACCAAAAAA TYA-3') (Wilke & Davis, 2000) for the COI gene and two *Bythinella*-specific primers ITS1D (5'-GTGGGACGGAGTGTGTT-3') and ITS1R (5'-CCACCGCCTAAAGTTGTTT-3') for the ITS-1 (Bichain et al., 2007). The PCR conditions were as follows: COI—initial denaturation step of 4 min at 94°C, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension of 4 min at 72°C; ITS-1—initial denaturation step of 4 min at 94°C, followed by 25 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final extension of 5 min at 72°C. Ten  $\mu$ l of the PCR product was run on 1% agarose gel to check the quality of the PCR product. The PCR product was

purified using Clean-Up columns (A&A Biotechnology). The purified PCR product was sequenced in both directions using BigDye Terminator v3.1 (Applied Biosystems), following the manufacturer's protocol and 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.

#### Data analyses

Sequences were initially aligned in the ClustalW programme (Thompson et al., 1997) in MEGA 6 (Tamura et al., 2013) and then checked manually in Bioedit 7.1.3.0 (Hall, 1999). Single ITS sequences were aligned using CodonCode Aligner 4.2.7 (CodonCode Corporation, Dedham, MA). Basic sequence statistics, including haplotype polymorphism and nucleotide divergence, were calculated in DnaSP 5.10 (Librado & Rozas, 2009). The saturation test was examined using DAMBE (Xia, 2013).

To infer the phylogenetic relationships, which reflected the history of the radiation, we inferred the phylogeny based on the molecular data. Sequences obtained from *Bythinella* specimens in the present work were used in a phylogenetic analysis with other sequences obtained from GenBank (Fig. 1, Online Appendix 1). The data were analysed using an approach based on Bayesian inference (BI) and maximum likelihood (ML). In the BI analysis, we applied the GTR + I +  $\Gamma$  model of nucleotide substitution because an over-parameterisation was apparently less critical for the BI analyses than an under-parameterisation (Huelsenbeck & Rannala, 2004). For the ML analyses, the GTR +  $\Gamma$  model was applied, as implemented in RaxML (Stamatakis, 2014).

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 1,000 generations. The first 25% of trees were discarded as burn-in. The analyses were summarised 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 randomised 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 1,000 replicates and summarised onto the best ML tree. RAXML analyses were performed using free computational resource CIPRES Science Gateway (Miller et al., 2010).

Phylogenetic trees, as hierarchical and dichotomous, cannot reflect all the relationships between the haplotypes. Thus, we infer haplotype networks of the markers with a median-joining calculation that was implemented in NETWORK 4.6.1.1 (Bandelt et al., 1999). To find the estimated time frames for the main events in the inferred phylogeny, we were required to compute a time tree. To test the molecular clock, the data from COI were used. Two hydrobiids, *Peringia ulvae* (Pennant, 1777) and *Salenthydrobia ferreri* Wilke, 2003 (AF478401 and AF478410, respectively) were used as the outgroups. The divergence time between these two species was used to calibrate the molecular clock, with the correction according to Falniowski et al. (2008). The split between these two species occurred during the Messinian Salinity Crisis, which ended 5.33 Mya. In fact, the isolation of the Atlantic *Peringia* and the Mediterranean *Salenthydrobia* must have begun earlier, in time when the Mediterranean Basin started to separate from the Atlantic, and therefore, we extended this calibration point to 5.96 Mya.

The likelihoods for the trees with and without the molecular clock assumption for a likelihood ratio test (LRT) (Nei & Kumar, 2000) were calculated with PAUP. The relative rate test (RRT) (Tajima, 1993) was performed in MEGA. Because Tajima's RRTs and the LRT test rejected the equal evolutionary rate throughout the tree for *Bythinella*, the time estimates were calculated using a nonparametric rate smoothing (NPRS) analysis with the recommended Powell algorithm in r8s v.1.7 for Linux (Sanderson, 1997, 2003). We also used a penalised likelihood analysis (Sanderson, 2002) that combined the likelihood and the nonparametric penalty function used in NPRS. We also conducted this analysis in r8s with the Powell and TN algorithms. Because these analyses all produced consistent results, we choose to present the results based on NPRS for the comparisons with previous studies (see, e.g., Szarowska et al., 2014a, b). The generalised mixed Yule coalescent (GMYC) procedure that was introduced by Pons et al. (2006) was used in the attempt to delimit species-level entities.



To identify possible hot spots in the study area, we conducted an analysis following that of Benke et al. (2011) and used both our current data and previously published data (Falniowski & Szarowska, 2011). The populations were grouped according to  $1^\circ \times 1^\circ$  geographic grid cells. The mean pairwise genetic distances ( $K2P$ ) were calculated within each square of the grid. For the identification of the hot spots, we used the criteria proposed by Benke et al. (2011) as follows: (i) the highest level of genetic diversity ( $\geq 0.0348$ ) in at least one grid square in the studied area; (ii) the genetic diversity in a putative hot spot must be at least 10% of the total genetic diversity; and (iii) the absence of grid squares with no genetic diversity. With this approach, without the identification of species, the biological diversity was analysed for the genus *Bythinella* as an example of a case in which the species-level taxonomy was not clear.

## Results

### Morphology

The photographs present shells from all island populations that were not presented in earlier studies. For clarity, PCA and descriptive statistics (Table 1) were restricted to a single population from each of the molecularly distinguished clades (see below). PC1 explained 50.86% of the variance, and PC2 explained 19.98%; cumulatively, the two components explained 70.83% of the total variance. The shell height, spire height and aperture height had the highest loadings in PC1, and the angle between the body whorl suture and the horizontal surface had the highest loading in PC2. This approach was used to assess whether the molecularly distinct clades represented some real biological units that were identifiable based on the morphology and that could potentially corresponded to species-level entities.

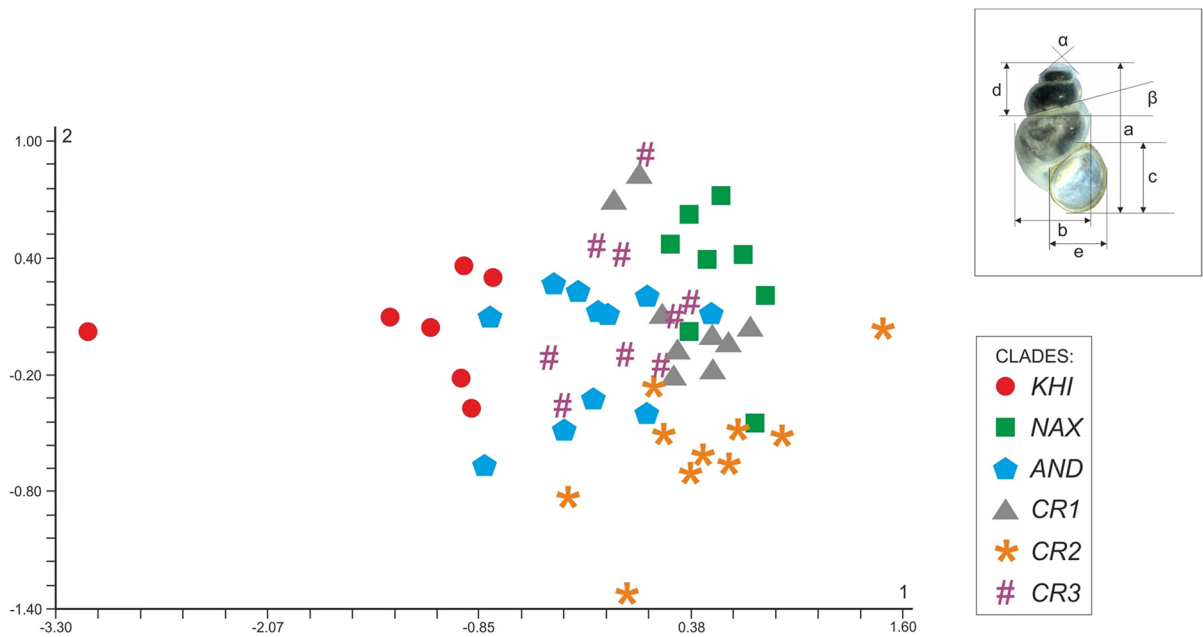
The shells from Andros Island (molecular clade *AND*), collected at five localities (Online Appendix 2A–ZZ), were slightly variable within a population, but no size differences were observed between populations. In the PCA (Fig. 2), they formed the group closest to the centroid and were mixed with the other molecular clades. Five populations of *Bythinella* from Crete (Online Appendixes. 3A–Z5 and 4A–E) represented three molecular clades (see below): *CR1*, *CR2*,

**Table 1** Parameters used in shell biometry (PCA) of *Bythinella*

Clades	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	$\alpha$	$\beta$
<i>AND</i> —Andros							
<i>M</i>	2.63	1.44	1.24	0.72	1.13	130.2	16.9
<i>SD</i>	0.11	0.06	0.08	0.06	0.47	6.58	2.08
<i>Max</i>	2.79	1.51	1.45	0.81	1.19	143	20
<i>Min</i>	2.49	1.35	1.16	0.63	1.05	122	13
<i>KHI</i> —Khios							
<i>M</i>	2.37	1.27	1.16	0.63	0.94	128.3	15
<i>SD</i>	0.19	0.09	0.10	0.07	0.15	4.75	1.53
<i>Max</i>	2.57	1.42	1.26	0.71	1.02	133	16
<i>Min</i>	2.00	1.19	0.96	0.50	0.60	121	12
<i>CR1</i> —Crete							
<i>M</i>	2.90	1.44	1.30	0.91	1.09	136	18.5
<i>SD</i>	0.09	0.08	0.05	0.10	0.05	10.8	1.27
<i>Max</i>	3.01	1.56	1.40	1.06	1.14	147	20
<i>Min</i>	2.77	1.31	1.24	0.76	0.97	114	17
<i>CR2</i> —Crete							
<i>M</i>	2.97	1.60	1.32	0.87	1.12	145.3	15.2
<i>SD</i>	0.20	0.06	0.06	0.15	0.05	11.5	1.62
<i>Max</i>	3.44	1.71	1.45	1.20	1.19	164	18
<i>Min</i>	2.77	1.51	1.20	0.70	1.06	125	12
<i>CR3</i> —Crete							
<i>M</i>	2.89	1.46	1.21	0.99	0.97	140.7	17.6
<i>SD</i>	0.16	0.08	0.04	0.12	0.05	14.7	1.78
<i>Max</i>	3.13	1.60	1.28	1.20	1.03	164	21
<i>Min</i>	2.65	1.37	1.13	0.84	0.90	114	15
<i>NAX</i> —Naxos							
<i>M</i>	2.94	1.49	1.26	0.88	1.10	129.1	18.12
<i>SD</i>	0.21	0.11	0.08	0.13	0.05	11.41	1.73
<i>Max</i>	3.16	1.64	1.37	1.05	1.17	145	21
<i>Min</i>	2.51	1.32	1.10	0.65	1.01	117	16

*a* shell height, *b* body whorl breadth, *c* aperture height, *d* spire height, *e* aperture breadth,  $\alpha$  apex angle,  $\beta$  angle between body whorl suture and horizontal surface, *M* mean, *SD* standard deviation, *max* maximum, *min* minimum. The *a–e* in mm,  $\alpha–\beta$  in degree angle

and *CR3*. The shells of the specimens belonging to *CR3* (Online Appendix 3A–M) mostly had narrower apertures than those belonging to clade *CR2* (Online Appendixes 3N–W and 4A–E, Table 1), although this was not systematic. The clade *CR1* (Online Appendix 3X–Z5) was characterised by a wider variability of the shell than that found in the other two clades from Crete (Table 1). The PCA (Fig. 2) showed that the specimens of the three clades were somewhat mixed, but



**Fig. 2** Shell biometry (PCA) of *Bythinella* for the six main clades (see COI phylogenetic tree, Fig. 3). Shell measurements are as shown in the inset: *a* shell height, *b* body whorl breadth, *c* aperture height, *d* spire height, *e* aperture breadth,  $\alpha$  apex

angle,  $\beta$  angle between body whorl suture and horizontal surface. PC1 explains 50.86%, and PC2 19.98%, cumulatively they explain 70.83% of the total variance

their centroids did not overlap; *CR2* was not mixed with the other two clades.

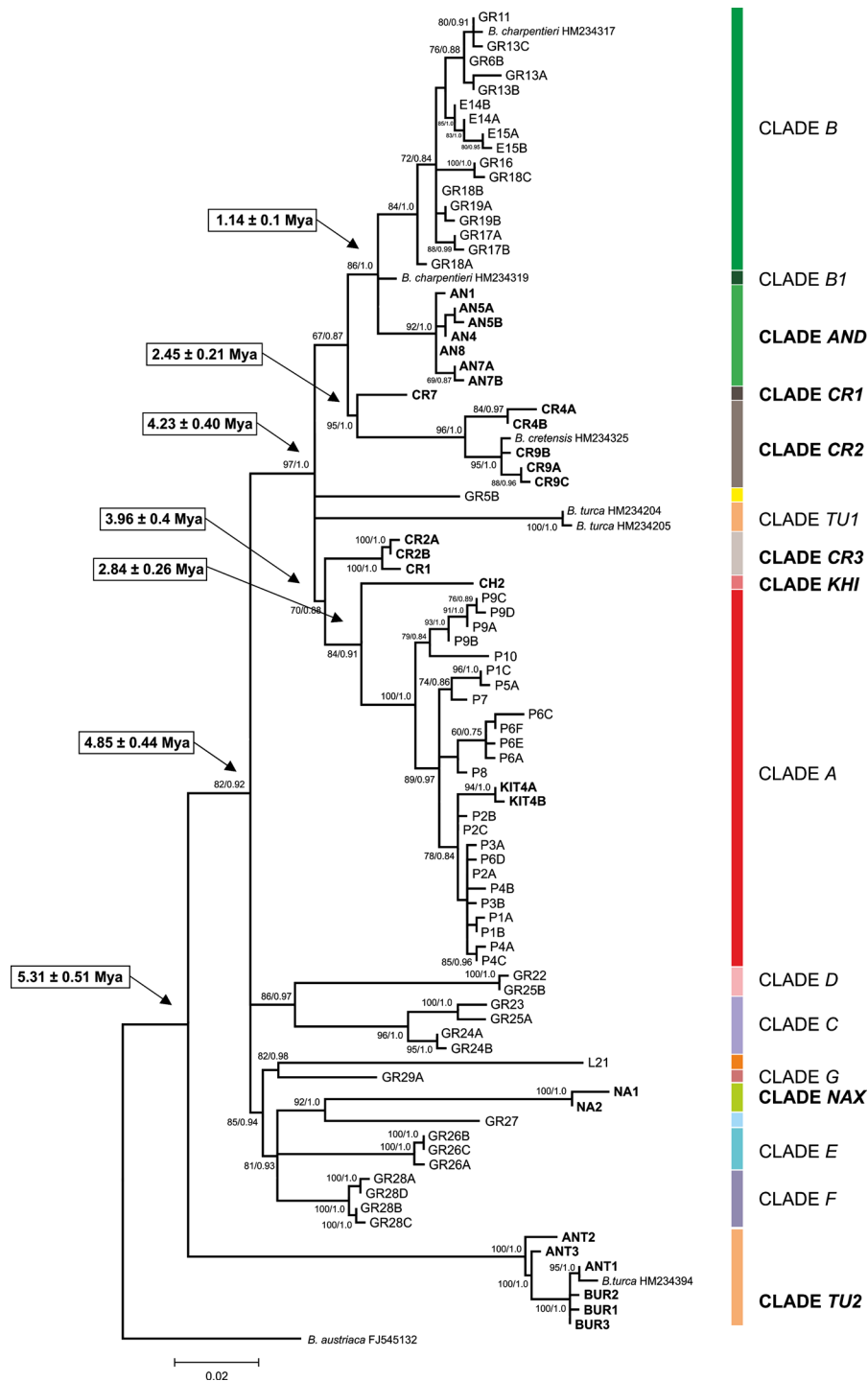
The shells from Khios Island (Online Appendix 4F–H) were characteristic, with relatively conical, narrow apertures (Table 1), which was later confirmed by PCA (Fig. 2). A dwarf specimen of unknown origin was an outlier of this population (Fig. 2), which perhaps represented another generation. Another dwarf specimen was used for PCR and represented the same haplotypes as the other gastropods from the population. The shells from Naxos Island (Online Appendix 4I–T) were similar to those from clades *CR1* and *CR3* on Crete, which was confirmed by PCA (Fig. 2).

The female reproductive organs, the bursa copulatrix, the receptaculum seminis and the loop of the oviduct (Online Appendix 5A–G), did not show differences between the clades. In general, the penes and penial glands (Online Appendixes 6–7) did not show consistent or well-marked differences among the clades. The penes of the snails from Andros Island (Online Appendix 6A–E) had a relatively short left arm, containing the vas deferens, and in the snails from Khios Island (Online Appendix 6I), the penes were

exceptionally slender. The morphology of the penis and the penial gland was not different among the Cretan clades *CR1* (Online Appendix 7H–I), *CR2* (Online Appendix 7F–G) and *CR3* (Online Appendix 7A–E).

#### Molecular data

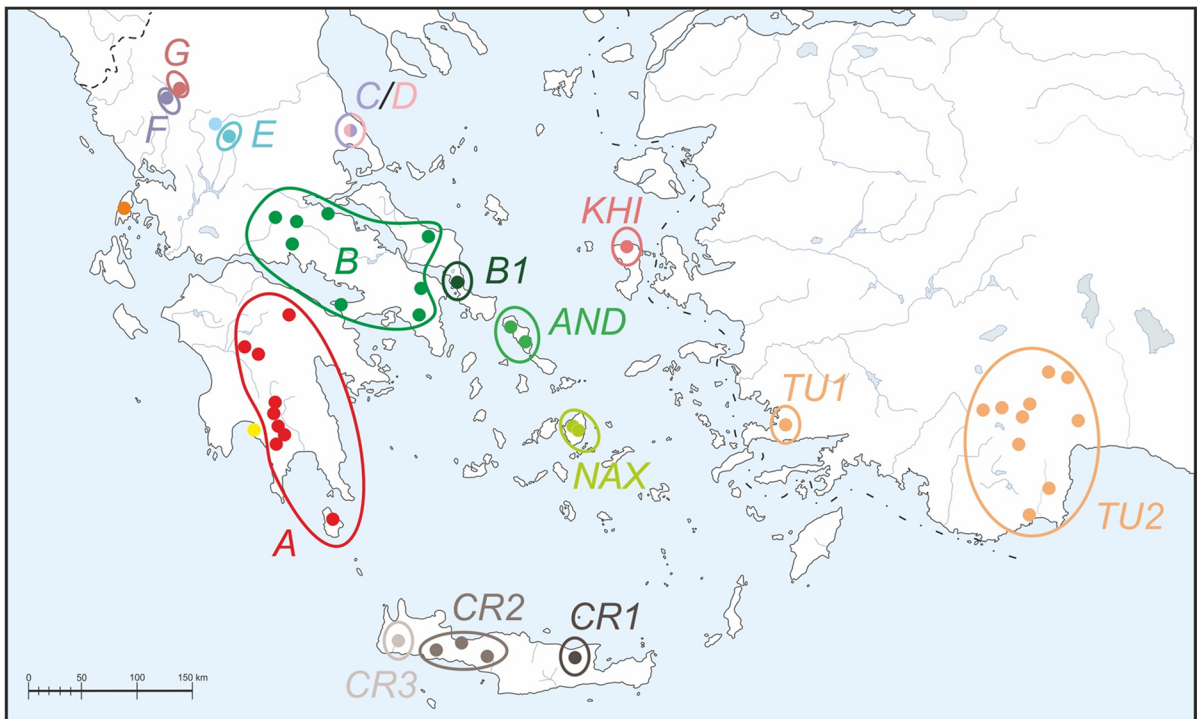
We obtained 95 sequences of COI (552 bp; GenBank Accession numbers KT353624–KT353718), which represented 27 haplotypes, and 60 sequences of ITS-1 (varying from 233 to 276 bp; GenBank Accession numbers KT353564–KT353623), which represented 21 haplotypes. The saturation test by Xia et al. (2003) revealed no saturation for COI. In all analyses, the topologies of the resulting phylograms were identical in both the ML and Bayesian inference. For the phylogenetic and phylogeographic analyses, we used reference sequences (Online Appendix 1). We added 288 COI and 97 ITS-1 sequences from continental Greece and the Peloponnese (Falniowski & Szarowska, 2011) and 6 COI sequences of *B. charpentieri*, *B. cretensis* and *B. turca* (Benke et al., 2011). *B. austriaca* and *B. dacica* were used as the outgroups.



**Fig. 3** The maximum-likelihood phylogram for COI haplotypes. Bootstrap support and Bayesian posterior probabilities were shown. Division into 16 clades and divergence times of

main clades are also indicated. *Normal font* indicates reference haplotypes, *bold font* haplotypes obtained in present study





**Fig. 4** Geographic distribution of COI clades. Compare with Fig. 3

Thus, we performed the phylogenetic analysis for 96 haplotypes of the COI and for 83 haplotypes of the ITS-1 genes.

For the COI, with sequences from more populations, and possibility of dating, the tree enabled the reconstruction of the history of the group. The best RAxML tree for COI comprised 16 main clades (Figs. 3, 4). The  $p$ -distances are given in Table 2. The most divergent clade was TU2 from southern Turkey, with a separation time of 5.31 Mya. It was the sister clade to the other COI haplotypes, which formed 15 clades, the majority of which were identical to those in Falniowski & Szarowska (2011). The clade A from the Peloponnese and the clade B from continental Greece aggregated the majority of the haplotypes. The estimated time of separation between them was 4.23 Mya. As expected, our two Kithira haplotypes were grouped with clade A. Our sequences from Andros (clade AND) and the reference sequence of *B. charpentieri* were closely related to clade B.

Unexpectedly, the haplotypes from Crete were not monophyletic, and the sequences from this island were the most diverse when compared with the sequences from the other island populations (Fig. 5). The

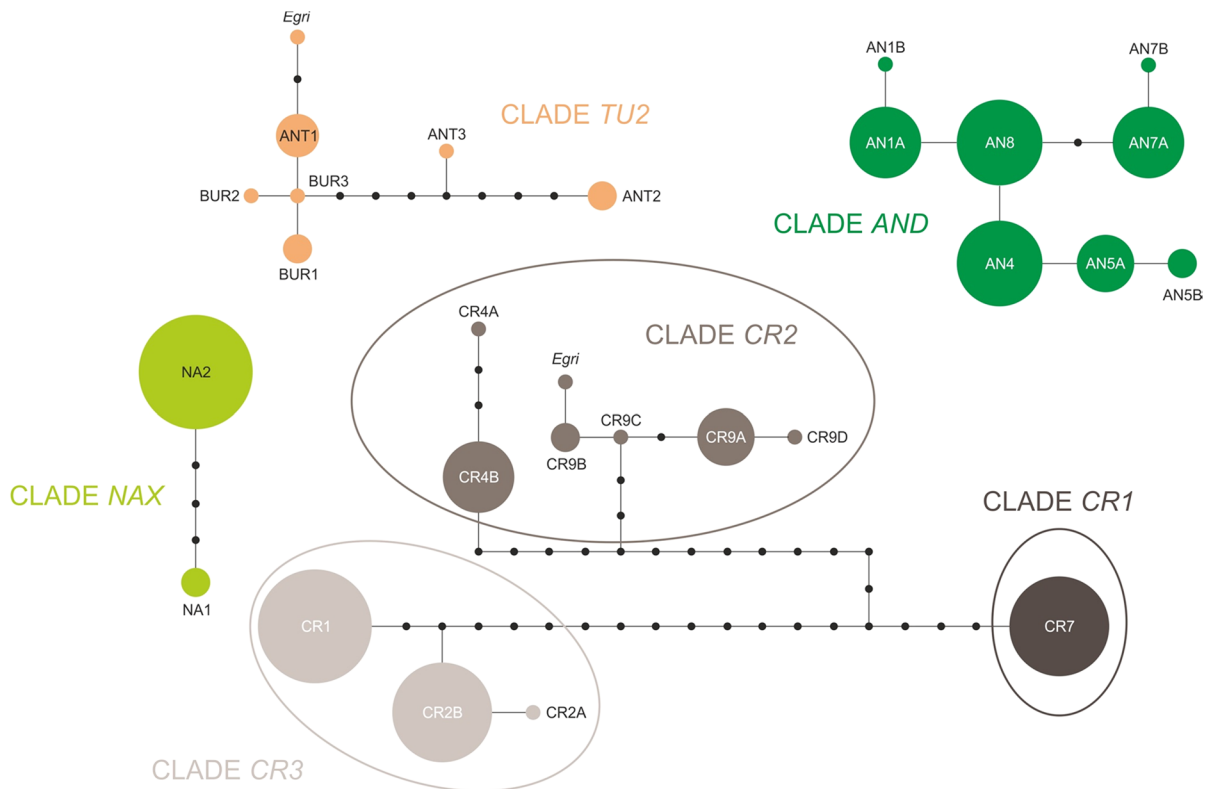
haplotypes from the central and eastern parts of Crete formed a separated group that consisted of two clades CR1 and CR2. The CR2 was the most similar to the reference sequence of *B. cretensis*. These two clades were most similar to the B/B1/AND group of clades. From the western part of the island (clade CR3), the haplotypes formed a sister clade to clade A and to clade KHI from Khios Island, with a divergence time of 3.96 Mya. The clades A and KHI diverged 2.84 Mya. The Naxos haplotypes formed a distinct clade (clade NAX), which was closely related to population G27 and the clades E and F from central Greece. The GMYC analysis resulted in 93 (confidence interval 29–93) entities for a single threshold analysis and 44 (33–46) for a multiple threshold analysis.

The analyses of the ITS-1, a nuclear locus that might have a different history than the mitochondrial COI, indicated slightly different phylogenetic relationships for the *Bythinella* (Fig. 6); however, the topology of the tree was not fully resolved because of low support values. Unfortunately, for technical reasons, we lacked ITS-1 sequences from Turkey; thus, the most divergent clade was formed by the haplotypes from Naxos, which was similar to the COI

**Table 2** *p*-Distances between main COI clades of *Bythimella*

	<i>B</i>	<i>BI</i>	<i>AND</i>	<i>CRI</i>	<i>CR2</i>	<i>TUI</i>	<i>CR3</i>	<i>KHI</i>	<i>A</i>	<i>D</i>	<i>C</i>	<i>L21</i>	<i>G</i>	<i>NAX</i>	<i>GR27</i>	<i>E</i>	<i>F</i>
<i>BI</i>	0.014	<i>0.017</i>															
<i>AND</i>	0.023	0.021	<i>0.005</i>														
<i>CRI</i>	0.029	0.024	0.031	–													
<i>CR2</i>	0.042	0.041	0.046	0.034	<i>0.014</i>												
<i>TUI</i>	0.057	0.056	0.052	0.052	0.062	<i>0.002</i>											
<i>CR3</i>	0.038	0.034	0.041	0.034	0.047	0.055	<i>0.005</i>										
<i>KHI</i>	0.043	0.039	0.042	0.034	0.047	0.054	0.040	–									
<i>A</i>	0.041	0.040	0.044	0.039	0.054	0.057	0.034	0.039	<i>0.019</i>								
<i>D</i>	0.062	0.061	0.061	0.064	0.066	0.084	0.065	0.067	0.069	<i>0.002</i>							
<i>C</i>	0.062	0.059	0.066	0.064	0.069	0.077	0.053	0.051	0.056	0.059	<i>0.016</i>						
<i>L21</i>	0.061	0.061	0.072	0.065	0.074	0.079	0.063	0.068	0.068	0.073	0.073	–					
<i>G</i>	0.053	0.048	0.056	0.049	0.061	0.071	0.043	0.048	0.053	0.058	0.049	0.063	–				
<i>NAX</i>	0.076	0.077	0.080	0.074	0.079	0.090	0.071	0.068	0.075	0.085	0.070	0.086	0.067	<i>0.008</i>			
<i>GR27</i>	0.069	0.064	0.066	0.065	0.071	0.071	0.065	0.055	0.067	0.064	0.052	0.070	0.048	0.065	–		
<i>E</i>	0.055	0.055	0.063	0.048	0.064	0.066	0.053	0.057	0.056	0.064	0.061	0.063	0.048	0.068	0.057	<i>0.003</i>	
<i>F</i>	0.046	0.045	0.053	0.046	0.061	0.061	0.037	0.049	0.050	0.060	0.054	0.063	0.033	0.065	0.049	0.035	<i>0.004</i>
<i>TU2</i>	0.087	0.087	0.090	0.089	0.089	0.088	0.081	0.084	0.083	0.090	0.077	0.090	0.075	0.087	0.081	0.082	0.077

For details see Fig. 3. Mean distances within clades are also shown (italics)



**Fig. 5** The median-joining haplotype network of COI haplotypes for clades discovered in present study. The *colours* indicate the specific clades, see Fig. 3. *Italics* indicate samples from Benke et al. (2011)

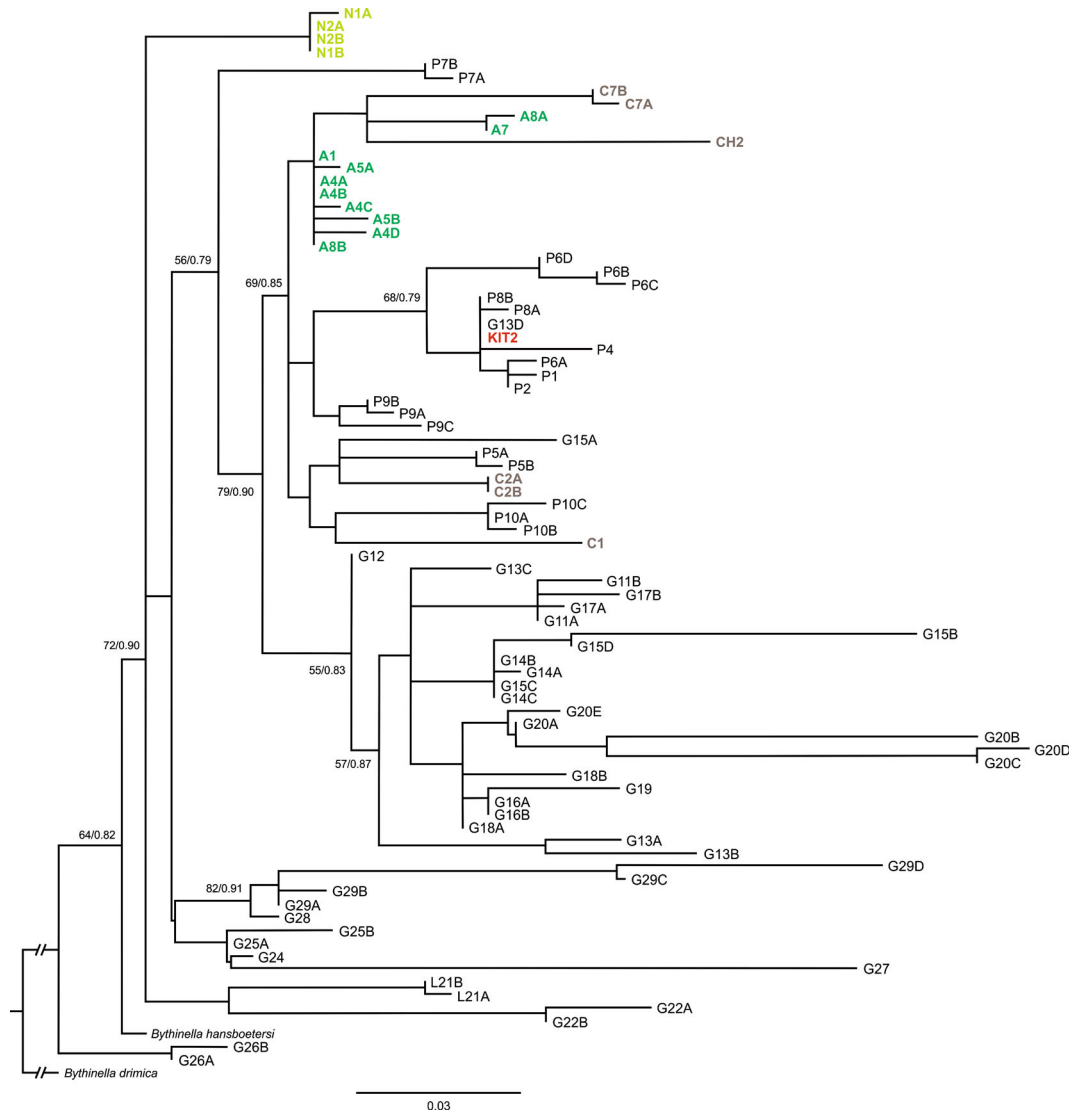
analysis. However, the haplotypes from Andros were grouped into a separate clade that also contained the only haplotype from Khios and two of the haplotypes from Crete. In a comparison with the COI analyses, the haplotypes from Crete were arranged into distinct lineages, but the lineages reflected different phylogenetic topology. The first group, listed above, from the easternmost Cretan population was close to the Andros and Khios haplotypes. The second, consisting of two groups, was placed with the Peloponnese haplotypes; the sequences from Kithira also grouped with the Peloponnese haplotypes.

Our analysis of this extended dataset revealed a new *Bythinella* hot spot in central Greece and the Peloponnese (Fig. 7), which met all criteria listed by Benke et al. (2011). The hot spot contained one grid cell with the highest level of genetic diversity (0.0401), the genetic diversity of all included grid cells totalled much more than the required 10% of the total sampled genetic diversity of all *Bythinella* sequences, and no grid cells were found that lacked genetic diversity.

## Discussion

The morphology of the shell and the morphology and the anatomy of the reproductive organs were highly variable but nevertheless also showed interclade differences in some cases. Similarly, the PCA showed rather distinct groups for some of the molecularly distinct clades. However, the differences were small and the within-clade variation was high, and therefore, the morphology cannot be the basis to delimit species in *Bythinella* (Falniowski & Szarowska, 2011; Falniowski et al., 2012a, b). Evidently, *Bythinella* is a further example of morphostatic radiation, as defined by Davis (1992), which is very common among truncatelloid snails (e.g., *Hydrobia*: Wilke & Davis, 2000; *Adriohydrobia*: Wilke & Falniowski, 2001; *Bythinella*: Falniowski & Szarowska, 2011; *Radomaniola* and *Grossuana*: Falniowski et al., 2012a).

Our molecular data from the five Aegean islands and from Turkey complemented the data from continental Greece (Falniowski & Szarowska, 2011), which addressed one of our major questions (i). Overall, the



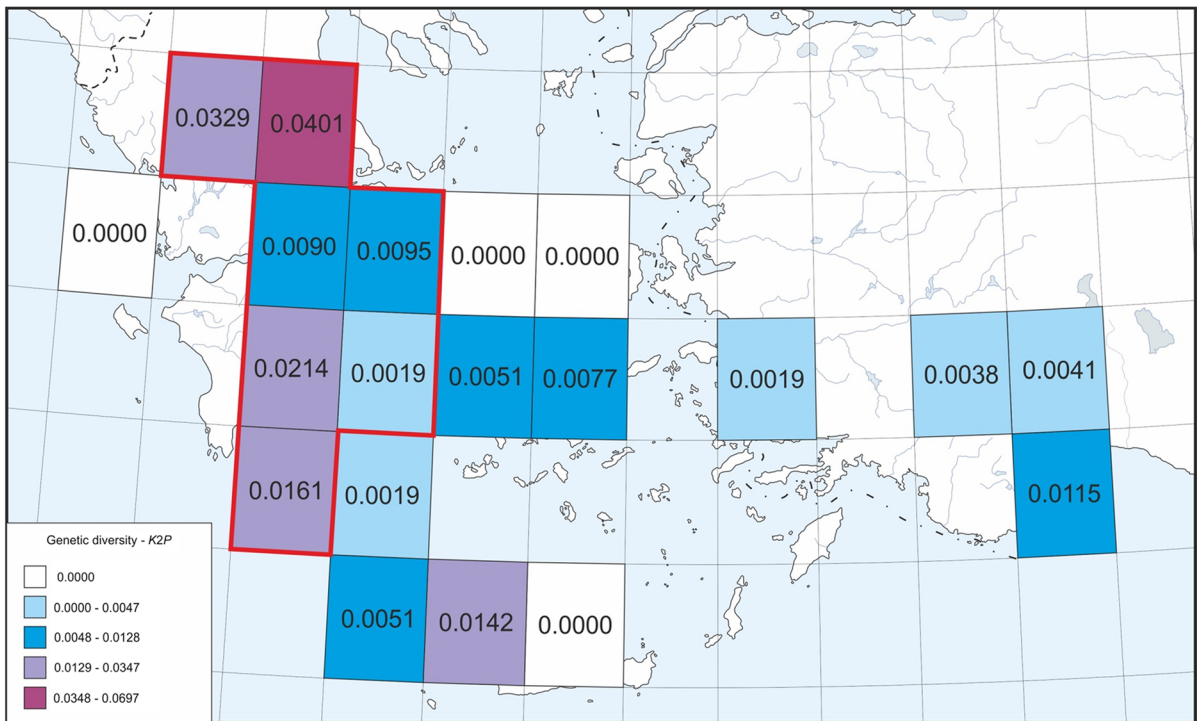
**Fig. 6** The maximum-likelihood phylogram for the ITS-1 haplotypes. Bootstrap support and Bayesian posterior probabilities were shown. *Normal font* indicates reference haplotypes, *bold font* haplotypes obtained in current study

topology of the COI tree was similar to the findings of the previous study (Falniowski & Szarowska, 2011), with the addition of seven new clades that represented the haplotypes from Andros, Khios, Naxos, Turkey, Kithira and the three distinct clades from Crete. In general, the levels of diversity in the island and land populations were similar, which suggested that the sea and the dry land formed similar barriers to gene flow. The haplotypes from Andros formed a distinct clade, which was most similar to the geographically closest clade of Attica and the regions of central Greece as well as to *B. charpentieri* specimens from Evvoia

Island. The divergence among these haplotypes varied from 2.0 to 2.3%.

The haplotypes from Kithira were closely related to the haplotypes from the Peloponnese and formed a subclade within clade A, as described by Falniowski & Szarowska (2011), with slight differentiation (1–2%). This result was not surprising because Kithira is situated about 115 km from the Peloponnese shores, and therefore, the Peloponnese could have been the source of the population from Kithira.

Our second major question (ii) was: does the observed diversity reflect the geological events? The



**Fig. 7** Distribution of *Bythinella* spp. diversity in studied area divided into  $1 \times 1$  geographic grid cells. The region identified as hotspot is outlined in red

answer to this question is complex, and the relationships among the other clades distinguished in this study were somewhat unexpected considering the geographic distributions of the populations but could be explained in part by some geological events. The first surprising result was the sister clade relationship between the haplotypes from Khios and those from Peloponnese/Kithira, despite the large geographic distance between Khios and the other two localities. The estimated time of divergence of these clades was 2.45 Mya, and  $p$ -distance = 0.39, which suggested that the divergence of the Khios and the Peloponnese/Kithira clades occurred after the Zanclean Flood. The distance was much higher than the one corresponding to the distance  $K2P = 0.015$ , given by Bichain et al. (2007) as the threshold value of the distance between species of western European *Bythinella*.

The COI analysis revealed an interesting pattern of genetic diversity of *Bythinella* on Crete. Our results indicated that there were representatives of two separate lineages, with a separation time of 4.23 Mya. The first of the lineages consisted of two Cretan clades (*CR1* and *CR2*), and the second lineage

included the clade *CR3*. For freshwater organisms, a high level of genotypic differentiation among the populations that inhabited particular islands, among the populations on different islands, and between the island and the mainland or the populations from other islands might be a typical pattern that resulted from the frequent events of colonisation and recolonisation from various source populations, in addition to the climatic/hydrologic fluctuations (Szarowska, 2000). Springs are ephemeral rather than long-lasting habitats, and therefore there must be some mechanisms for the colonisation/recolonisation. These mechanisms worked apparently not only between closely situated springs on an island but also for longer distances between islands. The passive transportation by birds, over the sea as well, was particularly likely (Falniowski & Szarowska, 2011, and the references therein). However, there is also some evidence for the opposite phenomenon. In a recent study (Szarowska et al., 2014a, b), we found very little differentiation within the Cretan populations of the brackish-water genus *Heleobia* Stimpson, 1865, which belongs to the same Truncatelloidea superfamily as *Bythinella*.

These results indicated that such scenarios were also possible and were most likely a result of a recent colonisation of Crete. The high level of differentiation of the Cretan *Bythinella* presented in this study confirmed the general predictions on the mechanisms of differentiation for freshwater organisms on islands. Thus, the freshwater snails, particularly those that inhabited springs and subterranean waters, were particularly prone to fast divergence caused by the isolation between habitats (populations).

The haplotypes from central and eastern Crete were more closely related to the populations from Andros, Attica and Evvoia, which was in contrast to the clade from western Crete that was more closely related to the Kithira, Peloponnese and Khios haplotypes [according to Schütt (1980), *B. kosensis* occurred at Khios]. Similarly, Szarowska et al. (2015) demonstrated the close relatedness of the Cretan *Pseudamnicola brachia* (Westerlund, 1886) and *P. chia* (Martens, 1889) from Khios. This suggests that the current pattern of *Bythinella* variation on Crete might be a result of a few possible scenarios. The differentiation might result from more than a single colonisation of Crete by the mainland snails. The estimated time of divergence (4.23 Mya) indicated that the separation of the two Cretan lineages was younger than the Messinian Salinity Crisis (5.96–5.33 Mya). Although the Messinian Salinity Crisis was the most important geological event in this area with great effects on the fauna and the flora of the Aegean region, our results indicated that other events were also important. The geological history of Crete is complex, with some periods of connection with the continent, the last of which occurred during the Late Serravallian (12 Mya). However, during the Messinian (5.5 Mya) and also in the Middle Pleistocene (0.4–0.02 Mya), only a narrow straight occurred between the Recent Crete and the mainland. Moreover, a few periods also occurred when Crete was divided into two or three smaller palaeo-islands that were surrounded by the sea (Creutzburg, 1963). From the lower Tortonian (11 Mya) until the late Pliocene (1.8 Mya), Crete was an archipelago that was composed of a small number of islands (Dermitzakis & Papanikolaou, 1981; Dermitzakis, 1987, 1990; Douris et al., 1998). Thus, the high diversity of *Bythinella* on Crete was not surprising, and the simplest explanation for this phenomenon might be a relict character of the populations that were isolated on mountain peaks surrounded by salt water in

the Neogene. This prediction was supported by the estimated time of divergence for the clades *CR1* and *CR2* (2.45 Mya), which closely coincided with the time when the sea isolated these two parts of Crete.

The high level of the biodiversity of land snails on Crete was proposed to be the result of such a geological history (Welter-Schultes & Williams, 1999; Schilthuizen et al., 2004), but some other research stressed that ecological factors might be responsible for the intensive radiation of these snails (Sauer & Hausdorf, 2010). However, comparisons between the land and the spring-inhabiting snails should be interpreted with caution because of the differences in habitat characteristics and dispersal potential. Our results for the diversity of *Bythinella* confirmed rather the opinion of Welter-Schultes & Williams (1999) and reflected the geological history of the island.

The high level of divergence among the three Cretan clades (3.4–4.7%) strongly suggested that more than one species inhabited this island. The name *B. cretensis* Schütt, 1980 should be limited to the clade closest to the *locus typicus*—Mesa Potami (Schütt, 1980), situated about 2–3 km from the population K07, which formed our clade *CR1*. Thus, the name *B. cretensis* should be used for the clade *CR1* only. The '*B. cf. cretensis*' that was studied by Benke et al. (2011) was in the clade *CR2*, which represented a distinct *Bythinella* lineage. However, a complete delimitation of the *Bythinella* species would require more detailed studies.

The position of the Naxos branch on the COI tree was also notable. Two haplotypes from this island formed a distinct clade that was closely related to the haplotypes from Epirus and Thessaly. This result strongly indicated that the pattern of diversification of *Bythinella* in the Aegean Islands was not a result of the simple stepping stone dispersal scenario after a large geological event. The estimated divergence of this clade from the rest of the Aegean lineages occurred 4.85 Mya, which suggested that the Naxos population might possess more relict characters than the *Bythinella* populations on the geographically close Attica, and Andros and Evvoia. No close relationships were found among the *Bythinella* from Naxos, Andros and Tinos, as was found for *Pseudamnicola* (Szarowska et al., 2015), despite the relatively recent connection of all these islands (Kougioumoutzis et al., 2014). Moreover, our *Bythinella* on Naxos was distinct



from all other clades and perhaps reflected the same factors that caused Naxos to be one of the centres of diversity for plants (Kougioumoutzis et al., 2014). In general, the pattern of interpopulation diversity in *Bythinella* apparently reflected geological history, particularly the earlier events, much more than that in *Pseudamnicola*. Perhaps, this result was a reflection of the differences in the biology between these two genera. *Bythinella* may survive some desiccation (e.g. Falniowski, 1987; Szarowska, 2000), which is not necessarily typical of spring-inhabiting fauna, but the snail is certainly not as amphibious as *Pseudamnicola*. We found *Pseudamnicola* at some localities creeping above the waterline. At locality A04, where the spring was in the form of a pond, the *Bythinella* inhabited the submerged fallen leaves of *Platanus* on the bottom and the submerged macrophytes, but *Pseudamnicola* was found only on leaves floating at the surface. Thus, the diversity pattern of *Bythinella*, despite the very likely transportation of this snail by birds, resembled that of the flightless tenebrionid beetles (Papadopoulou et al., 2009), in contrast to the pattern of the flying beetles and somewhat unexpectedly, *Pseudamnicola*.

The most divergent clade on the COI tree was formed by our new haplotypes from Turkey. The divergence time between this clade and the rest of the tree was calculated at 5.31 Mya, which was notable, because this estimation corresponded to the end of the Messinian Salinity Crisis. Thus, the indication was strong that the *Bythinella* populations that inhabited the Mediterranean region of Turkey have been separated from the Aegean populations since the Zanclean flood, which refilled the dried Mediterranean Sea and eliminated the land connection between the current mainland and the islands.

Two reference *B. turca* sequences (Benke et al., 2011) were used in the present work. One *B. turca* sequence was from the Dilek Peninsula and formed the clade *TU1*, which was clearly distinct from the clade *TU2* from the Mediterranean region of Turkey and was grouped with the rest of Aegean clades. However, the clades *TU1* and *TU2* could not be assigned to one species. A clear distinction from the *TU2* clade suggested that the western coast of Turkey was colonised with the western *Bythinella* long after the Messinian crisis. To confirm or reject this hypothesis, additional sampling in this area of Turkey would be required. The second *B. turca* haplotype from Eğirdir Lake was in the clade *TU2*. Radoman (1976) described

*B. turca* Radoman, 1976 from the Cire spring (Eğirdir, Province Isparta), which was within the range of our clade *TU2*; thus, our data indicated that *B. turca* was restricted to the Burdur and Antalya subregions of Turkey.

The topology of the ITS-1 tree revealed some differences in a comparison with the COI tree, as was also observed in another study on *Bythinella* (Falniowski & Szarowska, 2011). Several explanations can be proposed for the differences between these COI- and ITS-1-based results. Some of these differences might result from the limited amount of data (e.g., the lack of ITS-1 sequences from Turkey). Clearly, gene trees are not species trees (e.g., Avise, 2000). The mitochondrial DNA presents only one locus that is inherited through the female, and therefore, the differences in dispersal and selection rates, among others, between males and females may affect the differences between the mitochondrial and the nuclear phylogenies. However, the divergence of several of the clades that were distinguished in the COI analysis was confirmed; for example, the haplotypes from Andros and Naxos formed distinct clades, and the haplotypes from Crete formed three clades with a high level of divergence.

The genetic diversity in the genus *Bythinella* was not equally distributed, and quantifiable areas of increased diversity likely occur in Europe. Overall, five hot spots were identified in European mountains: the Pyrenees, the western and eastern Alps, and the western and eastern Carpathians (Benke et al., 2011). However, in a hot spot analysis of the Mediterranean region (e.g., the Balkans), a relatively low level of diversity for *Bythinella* species was found (Benke et al., 2011). Our analysis followed the approach of Benke et al. (2011), which was based on the haplotypes and without the delimitation of species. Considering all the data on the morphological and the genotypic diversity of *Bythinella*, in addition to our results for the GMYC, which showed wide confidence intervals, we share the opinion that the delimitation of species in this genus remains still an open question. Our analysis that used the extended data presented in this paper and that from Falniowski & Szarowska (2011) revealed a new *Bythinella* hot spot in central Greece and the Peloponnese. The new hot spot met all the criteria listed by Benke et al. (2011) and contained one grid cell with the highest level of genetic diversity and the genetic diversity of all included grid cells totalled more than the required 10% of the total

sampled genetic diversity of all *Bythinella* sequences (Wilke et al., 2000, 2001; Szarowska & Wilke, 2004; Bichain et al., 2007; Haase et al., 2007; Benke et al. 2009, 2011; Falniowski et al. 2009a, b; Falniowski & Szarowska, 2011 and present data). According to Benke et al. (2011), the relatively low degree of biodiversity in the analysis of the Balkans might be partly caused by limited sampling, resource partitioning and even competitive exclusion. Our results indicated that the first explanation was correct because the analysis of the extended dataset revealed another hot spot in this area. The analysis confirmed that there were regions in which the diversity of *Bythinella* was much higher than that in others. The nonrandom distribution of spring habitats across Europe was a natural explanation of this phenomenon. These small springs are more common in mountainous areas, and therefore, it was not surprising that all the *Bythinella* hot spots in Europe were identified in the mountains only. Almost all the hot spots that were identified by Benke et al. (2011) were previously described as glacial refugia of *Bythinella* (Benke et al., 2009; Falniowski et al., 2009b), which strongly suggested that hot spots might reflect glacial refugia. We have no direct evidence that central Greece was another glacial refugium for *Bythinella*, but this scenario is highly likely because in this region there were the refugia for many plants (Kougioumoutzis et al., 2014), vertebrates (e.g., Weiss & Ferrand, 2007) and invertebrates (Sfenthourakis & Legakis, 2001).

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