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Radiation and phylogeography in a spring snail *Bythinella* (Mollusca: Gastropoda: Rissooidea) in continental Greece

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We studied morphology (shell, penis and flagellum, female reproductive organs) and 395 partial sequences of mtDNA COI and 93 of ITS-1 in *Bythinella* from continental Greece. Molecular techniques inferred ten molecularly distinct species: two in the Peloponnese; one in the Parnassus and Attica; two sympatric/parapatric at Volos; one in the Lefkas; four in northern Greece. The differentiation was probably not older than 3.5 MYA, most species originated in the last 2 MYA (Pleistocene). Haplotype diversity, nucleotide diversity and mismatch distribution indicated common bottlenecks followed by fluctuations in population size. A nested-clade analysis indicated allopatric fragmentation with instances of long distance colonization, and restricted gene flow with isolation by distance. The decrease in *Bythinella* species richness from the north to the south was due to the geological history, colonization and recolonization, and short and long-distance dispersal, to survive in the unstable environment.

Introduction

The freshwater rissooideans in the Balkans appeared in the Palaeogene, if not earlier (Kabat & Hershler 1993). Therefore, they are suitable for evaluation of old (pre-Pleistocene) biogeographic relationships. The present-day representatives of the freshwater Rissooidea are considered relicts, the distributions of which trace the Neogene drainage patterns fragmented by subsequent changes in climate and landscape (Hershler & Liu 2004b). This assumption involves two conditions: (1) springs inhabited by those obligatorily aquatic animals are stable habitats; (2) gene flow among them, if at all present, is very low. In the rich literature covering the phylogeny and population genetic structure and gene flow of the spring fauna, the gastropods are well represented (e.g. Colgan & Ponder 1994, Ponder *et al.* 1995, Falniowski *et al.* 1998, 1999, 2009b, Bohonak 1999, Bilton *et al.* 2001, Myers *et al.* 2001, Finston & Johnson 2004, Hershler & Liu 2004a, 2004b, Brändle *et al.* 2005, Hershler *et al.* 2005). Most of the studies point to low levels of gene flow and high levels of endemism in spring snails (e.g. Colgan & Ponder 1994, Ponder *et al.* 1995, Finston & Johnson 2004), but on the other hand, some of the species the studies deal with are rather widespread, with much gene flow among their populations (Falniowski *et al.* 1998, 1999, Hershler *et al.* 2005).

The genus *Bythinella* is distributed from W Europe (Iberian Peninsula), to W Asia. The fossil shells of its representatives come from the

Palaeocene and Pleistocene (Kabat & Hershler 1993). These dioecious, oviparous snails with minute, dextral, ovoid shells inhabit freshwater springs (Giusti & Pezzoli 1980, Falniowski 1987), small brooks and subterranean waters. They may be very abundant on mosses and other aquatic plants, and appear among fallen leaves in spring-fed marshes. The rich literature on *Bythinella* (Radoman 1976, 1983, 1985, Jungbluth & Boeters 1977, Giusti & Pezzoli 1977, Falniowski 1987, 1992, Glöer 2002, Szarowska & Wilke 2004) pertains mainly to western, southern and central Europe. The earliest descriptions of the species employed shell morphology alone; later ones included some characters of soft-part morphology (especially the reproductive system) which did not, however, improve species delineation as the characters were too few and too variable (Falniowski 1987). Recently, Bichain *et al*. (2007), Haase *et al*. (2007), and Falniowski *et al*. (2009b) analysed molecular data and etablished the distinctness of several species of *Bythinella*. The ranges of some *Bythinella* species studied molecularly (e.g. Bichain *et al*. 2007, Falniowski *et al*. 2009b) are restricted to a single spring, spring complex, or local watershed, while those of other congeners stretch across one or more drainage divides. Our field observations and allozyme-based studies on the central European *Bythinella* (Falniowski 1987, Falniowski *et al*. 1998, 1999, Szarowska 2000), along the northern limit of the genus, indicated neither the complete isolation of those populations nor their stability and longevity. In central Europe, the recent fauna is moulded by glaciations (Falniowski *et al*. 1998, Benke *et al*. 2009, Falniowski *et al*. 2009b). It is probable that in some areas where *Bythinella* survived throughout the Pleistocene the populations are older and more relict in character.

There are dozens of *Bythinella* species described from western Europe and the former Yugoslavia (Radoman 1976, 1983, 1985, Giusti & Pezzoli 1977, Bichain *et al*. 2007), and also several species described and redescribed in central Europe (Jungbluth & Boeters 1977, Falniowski 1987, 1992, Glöer 2002, Szarowska & Wilke 2004, Haase *et al*. 2007). By contrast, information on the Greek *Bythinella* spp. is scarce. Schütt (1980) reported that *Bythinella charpentieri*, described from Attica, was the only *Bythinella* inhabiting continental Greece. Radoman (1976, 1983, 1985) who recorded *Bythinella* at two localities in Attica and Parnassos Mts., stated that the diversity of the genus in Greece was seemingly lower than in the more northern part of the Balkans. The opposite opinion was presented by Reischütz *et al*. (2008), who described two new species from the Peloponnese and supposed that there were many not yet described *Bythinella* species in that southernmost part of continental Greece.

The aims of the present study were: (i) to reveal, through molecular markers, the pattern of phylogeny of the *Bythinella* inhabiting the continental part of Greece; (ii) to find which of the two models is more applicable to the Greek *Bythinella*: either the one in which a relict fauna rich in endemics is differentiated in the way that reflects mostly the geological history of the area, or the model in which a relatively young fauna is composed of more or less widely distributed taxa, with relatively high levels of gene flow among the springs; (iii) to uncover the factors that shape interpopulation differentiation. To address the above problems, cytochrome oxidase subunit I (COI; mtDNA), and ribosomal internal transcribed spacer (ITS-1) were sequenced and analyzed in snails from 29 localities in continental Greece.

Material and methods

Material collection and fixation

We collected snails in 2003, 2007 and 2008 from 29 localities in Greece (Fig. 1 and Table 1), with a sieve, or by hand, washed them twice in 80% ethanol and left them to stand in it for ca. 12 hours. Afterwards, we changed the ethanol twice in 24 hours and finally, after a few days, replaced the 80% solution with a 96% one, and stored the samples at –20 °C. For the morphological study, we fixed additional material in 4% formalin and stored it in 80% ethanol.

Morphological data

We cleaned shells in an ultrasonic cleaner and

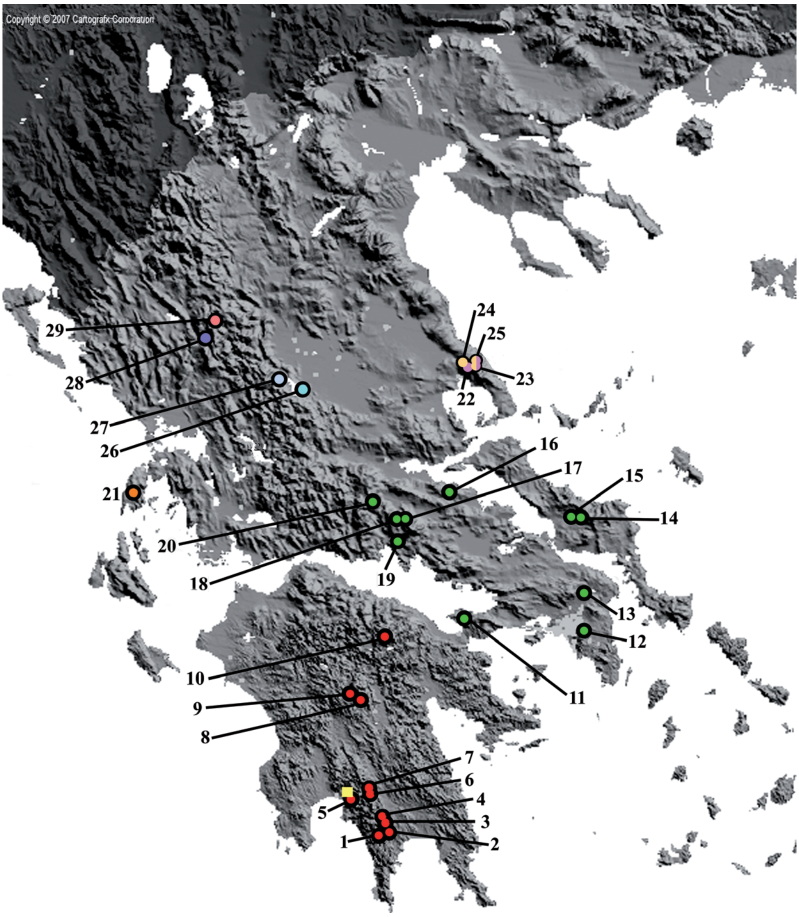


Fig. 1. Sampling localities of *Bythinella*. Figure produced using Cartografix Professional Software.

photographed them (all at the same magnification) with a NIKON DS-5 or CANON EOS 50D digital camera. We dissected ten adults (five males, five females) from each population, using a NIKON SMZ-U stereoscope microscope with a NIKON drawing apparatus, and a NIKON DS-5 digital camera. In each specimen, the penis with flagellum or the pallial section of the female reproductive organs were drawn.

Molecular data

We extracted DNA from foot tissue of each snail. We hydrated the tissue in TE buffer (3 × 10 min), extracted total genomic DNA using the SHERLOCK extracting kit (A&A Biotechnology), and dissolved the final product in 20 μl of TE buffer. We performed the PCR reaction (Palumbi 1996) with the following primers: LCOI490 (5´-GGTCAACAAATCATAAAGATATTGG-3´) and COR722b (5´-TAAACTTCAGGGTGACCAAAAAATYA-3´) for the COI gene (Folmer *et al*. 1994) and two *Bythinella*-specific primers ITS1D (5´-GTGGGACGGAGTGTTGTT-3´) and ITS1R (5´-CCACCGCCTAAAGTTGTTT-3´) for 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.

The total volume of each PCR reaction mixture was 50 μl. To check the quality of the PCR products we ran 10 μl of the PCR product on 1%

Table 1. Localities sampled, sample sizes, and genetic parametres within studied populations of the Greek *Bythinella*; hn = number of haplotypes of COI, Mm = mismatch interpretation: 2 = two taxa of presumably species level, E = equilibrium, R = reduction in population size, B = bottleneck followed by population growth

	Locality (coordinates)	Altitude (m a.s.l.)	COI number	hn	*h* ± SD	π ± SD	θ_0	τ	Mm	ITS
1	Near Kastania, Taigetos Mts., Peloponnese (36°50´22´´N, 22°24´05´´E)	718	10	3	0.511 ± 0.164	0.00472 ± 0.00193	2.928	0.000	B	3
2	Near Arna, by Arna–Vasiliki road, Taigetos Mts. Peloponnese (36°52´44´´N, 22°25´47´´E	730	12	3	0.530 ± 0.136	0.00114 ± 0.00035	0.000	0.652	R	3
3	Ghorani, Taigetos Mts., Peloponnese (36°55´02´´N, 22°26´05´´E)	651	12	3	0.439 ± 0.158	0.00154 ± 0.00055	0.349	0.530	B	2
4	NNW of Ghorani. Taigetos Mts., Peloponnese (36°56´45´´N, 22°24´34´´E)	571	8	3	0.464 ± 0.200	0.00269 ± 0.00111	1.269	0.266	B	2
5	Agio Theodori, near Belitseika and Koutiveika Taigetos Mts., Peloponnese (36°56´56´´N, 22°10´01´´E)	238	9	2	0.286 ± 0.196	0.01303 ± 0.00896	11.723	0.000	2	4
6	S of Anavriti, Taigetos Mts., Peloponnese (37°01´49´´N, 22°22´10´´E)	863	20	5	0.726 ± 0.062	0.00582 ± 0.00079	1.746	1.569	B	5
7	Near road Dhiaselo – Pikoulianika, N. Taigetos Mts., Peloponnese (37°04´39´´N, 22°21´37´´E)	526	12	3	0.667 ± 0.091	0.00585 ± 0.00108	1.848	1.485	B	2
8	WSW of Piana, WNW of Tripolis, Peloponnese (37°34´15´´N, 22°11´19´´E)	1094	11	1	0	0	–	–	–	3
9	NE of Palaiokhorion, WNW of Tripolis, Peloponnese (37°37´21´´N, 22°02´59´´E)	894	8	4	0.750 ± 0.139	0.00338 ± 0.00061	0.374	1.554	B	4
10	Between Bouzion and Kalianci, N. Peloponnese (37°53´39´´N, 22°28´27´´E)	841	8	1	0	0	–	–	–	3
11	Platania Spring, Pisia, N of Loutrakion, Attica (38°01´02´´N, 22°59´20´´E)	506	9	2	0.500 ± 0.128	0.00088 ± 0.00023	0.000	0.500	R	3
12	Kessariani Monastery, Athens, Attica (37°57´39´´N, 23°47´55´´E)	358	10	2	0.500 ± 0.265	0.00088 ± 0.00047	0.000	0.500	R	4
13	Gerania Mt., N of Athens, Attica (38°09´49´´N, 23°47´22´´E)	473	14	4	0.714 ± 0.181	0.00301 ± 0.00113	0.837	0.878	B	3
14	E of Steni, Evvoia Island (38°35´12´´N, 23°50´44´´E)	417	14	2	0.264 ± 0.136	0.00046 ± 0.00024	0.000	0.264	E	3
15	Adimis, W of Steni, Evvoia Island (38°35´16´´N, 23°48´57´´E)	359	12	2	0.485 ± 0.106	0.00085 ± 0.00019	0.000	0.485	R	3
16	Spartias Mt., S of Agios Konstandinos (38°45´05´´N, 22°51´12´´E)	81	8	1	0	0	–	–	–	2
17	S of Ano Polidhrosos, Parnassos Mts. (38°34´50´´N, 22°33´57´´E)	1266	9	2	0.222 ± 0.166	0.00039 ± 0.00029	0.000	0.222	E	2
18	Agtrini, S of Eptalofos, Parnassos Mts. (38°34´58´´N, 22°29´25´´E)	1017	10	4	0.778 ± 0.091	0.00713 ± 0.00124	2.637	1.430	B	5
19	Kastalia Spring, Dhelfoi, Parnassos Mts. (38°29´01´´N, 22°30´14´´E)	531	11	2	0.436 ± 0.133	0.00077 ± 0.00023	0.000	0.436	R	2
20	S of Kaloskopi, Pindos Mts. (38°40´29´´N, 22°19´13´´E)	1090	14	1	0	0	–	–	–	5
21	Kerasias Spring, Sivros, Lefkas Island (38°40´15´´N, 20°39´01´´E)	260	10	1	0	0	–	–	–	3
22	Pilion Mt., E of Dhrakia, E of Volos (39°23´04´´N, 23°00´57´´E)	706	8	1	0	0	–	–	–	3
23	Pilion Mt., N of Dhrakia, E of Volos (39°23´36´´N, 23°02´33´´E)	1082	7	2	0.533 ± 0.172	0.02620 ± 0.00846	13.933	1.000	2	2
24	Makrinitsa–Koukourava, E of Volos (39°23´35´´N, 22°59´55´´E)	616	6	2	0.667 ± 0.314	0.00117 ± 0.00055	0.000	0.667	2	2
25	N slope of Pilion Mt, E of Volos (39°24´35´´N, 23°04´54´´E)	860	7	2	0.286 ± 0.196	0.01654 ± 0.01137	14.964	0.000	2	3
26	Between Kalivia Filaktis and Portitsa, SW of Kardhitsa, Thessalia (39°19´35´´N, 21°45´51´´E)	681	12	3	0.318 ± 0.164	0.00292 ± 0.00190	2.741	0.000	B	5
27	Palaiokhorion, W of Kardhitsa, Thessalia (39°22´07´´N, 21°37´53´´E)	774	12	1	0	0	–	–	–	4
28	Sirakon, Pindos Mts., Epirus (39°35´41´´N, 21°06´16´´E)	1032	8	4	0.786 ± 0.151	0.00990 ± 0.00526	6.960	0.000	B	3
29	Anthousa, Pindos Mts., Epirus (39°39´29´´N, 21°12´30´´E)	967	11	2	0.182 ± 0.144	0.00032 ± 0.00025	0.000	0.182	E	5
All populations (mean)			302	61	0.975 ± 0.002	0.03945 ± 0.00115				93

agarose gel. We purified the PCR product using Clean-Up columns (A&A Biotechnology) and amplified the purified PCR product in both directions (Hillis *et al*. 1996) using BigDye Terminator ver. 3.1 (Applied Biosystems), following the manufacturer's protocol and with the primers described above. We purified the sequencing reaction products using ExTerminator Columns (A&A Biotechnology); DNA sequences then underwent electrophoresis on an ABI Prism sequencer. All the sequences are deposited in GenBank.

In the phylogeny reconstruction for COI, we used five central European and one Bulgarian *Bythinella* species as outgroups, and *Salenthydrobia ferrerii* and *Peringia ulvae* to calibrate the molecular clock (Table 2).

Data analysis

We aligned the COI sequences by eye, using BIOEDIT 5.0.0 (Hall 1999) and edited them with MACCLADE 4.05 (Maddison & Maddison 2002). For ITS-1, we performed an alignment using CLUSTALX 1.82 (Thompson *et al*. 1997). We examined mutational saturation for the COI dataset with saturation test of Xia *et al*. (2003), performed with DAMBE 5.2.9 (Xia 2000).

We inferred the phylogenies of the COI and ITS-1 haplotypes separately using maximum-likelihood (ML), minimum evolution (ME), maximum parsimony (MP), and a Bayesian analysis (BA) and neighbor-joining (NJ) for COI.

For each maximum likelihood (ML) analysis, we used the best-fit model of sequence evolution found by MODELTEST ver. 3.06 (Posada & Crandall 1998, Posada 2003). Following the recommendations of Posada and Buckley (2004) and Sober (2002), we chose the best model for each dataset using the Akaike Information Criterion (Akaike 1974). We performed ML analyses in PAUP*4.0b10 (Swofford 2002) and used an heuristic search strategy with stepwise addition of taxa, 10 random-sequence addition replicates, and tree-bisection-reconnection (TBR) branch swapping (Swofford *et al*. 1996). We estimated nodal support using the bootstrap approach (Felsenstein 1985). We calculated bootstrap values for ML trees using 1000 bootstrap replicates, the "fast" heuristic search algorithm, and the same model parameters as for each ML analysis.

We ran minimum evolution (for maximum likelihood distances) and maximum parsimony in PAUP*, and neighbor-joining (for COI) in MEGA4 (Tamura *et al*. 2007); the bootstrap approach with full heuristic search, 1000 replicates was applied to estimate nodal support.

We also used MODELTEST with PAUP to select the best model of COI sequence evolution for all the studied *Bythinella*, with *Peringia* and *Salenthydrobia* as an outgroup, and we found the best ML trees, with and without an enforced molecular clock, to perform the Likelihood Ratio Test (LRT) (Nei & Kumar 2000, Posada 2003), to test the molecular clock hypothesis for COI. To infer the time of divergence, the pairwise ML distances for the selected model were calculated with PAUP, and Maximum Composite Likelihood (Γ) distances with standard errors (10 000 bootstrap replicates) were calculated with MEGA4. To calibrate the clock we applied the divergence time between *Peringia* and *Hydrobia* (Wilke 2003)

Table 2. GenBank Accession Numbers and references of COI sequences of species used as outgroup

Species	GenBankAN	References
Bythinella austriaca (Frauenfeld, 1857)	FJ545132	Falniowski *et al.* (2009a)
Bythinella compressa (Frauenfeld, 1857)	AF367653	Szarowska and Wilke (2004)
Bythinella hansboetersi Glöer et Pešić, 2006	GQ152518	Falniowski *et al* (2009b)
Bythinella pannonica (Frauenfeld, 1865)	AY222660	Szarowska and Wilke (2004)
Bythinella robiciana (Clessin, 1890)	AY273998	Szarowska and Wilke (2004)
Bythinella schmidti (Küster, 1852)	AY222649	Szarowska and Wilke (2004)
Salenthydrobia ferrerii Wilke, 2003	AF449200	Wilke 2003
Peringia ulvae (Pennant, 1777)	AF118288	Wilke 2003

with correction of Falniowski *et al*. (2008): 5.96 MYA instead of 5.33 MYA. We also applied the Γ distances to compute minimum evolution (ME) linearized tree (Nei and Kumar 2000, Tamura *et al*. 2007), on which divergence time of several clades was estimated with MEGA4.

We performed a Bayesian analysis (BA), commonly applied in phylogeny reconstruction (Huelsenbeck *et al*. 2001, 2002, Holder & Lewis 2003, Ronquist 2004) for each of the two datasets. We produced posterior probabilities of branches using MRBAYES ver. 3.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). We performed this with the same model of evolution as assumed for ML, a 4 chain (1 cold, three heated; $T = 0.15$) metropolis-coupled Monte Carlo analysis run twice in parallel for 30 000 000 generations, trees sampled every 1000 generations starting after a burn-in of 30 000 generations (the value chosen according to the log-likelihood values).

The sequences of ITS-1 in the GenBank are not numerous and their alignment may be ambiguous, thus we sequenced many fewer specimens for ITS-1 than for COI. ITS-1 was used to check distinctness of the groups inferred with mtCOI, analyzing a nuclear fragment. This difference in numbers made it impossible to perform a combined analysis or compute a consensus tree.

We used PAUP*4.0b10 (Swofford 2002) to calculate K2P (Kimura 1980) distances for the COI data. This distance is widely used in the literature for COI data, thus it is useful for comparisons of levels of differentiation.

For COI we computed haplotype diversity h and nucleotide diversity π with DNASP (Rozas *et al*. 2003). To estimate gene flow between the populations, we calculated AMOVA (Excoffier *et al*. 1992, Weir 1996) with estimates of gene flow with ARLEQUIN 3.1 (Excoffier *et al*. 2005); significance tests were run with 20 000 permutations.

We conducted mismatch distribution for populations (Harpending 1994, Schneider & Excoffier 1999, Harpending & Rogers 2000, Rogers & Harpending 1992, Rogers 1995, Rogers & Jordey 1995, Excoffier 2004) analyses using DNASP 4.0 (Rozas *et al*. 2003) and ARLEQUIN (Excoffier *et al*. 2005), with 20 000 permutations.

We inferred haplotype networks for COI with TCS 1.21 (Clement *et al*. 2000), with the connection limit excluding homoplastic changes set to 95% (99% for the networks presented graphically). There is no reliable technique for analysing phylogeographical data (Knowles & Maddison 2002). Templeton (1989) introduced a nested clade analysis (NCA). The technique (Templeton *et al*. 1987, 1992, Templeton & Sing 1993, Crandall & Templeton 1993) lacks statistical inference (Knowles & Maddison 2002), simulations proved that it often does not find a realistic interpretation of the data, although those simulations are also criticised as not necessarily realistic (Panchal & Beaumont 2007, Petit 2007, 2008, Garrick *et al*. 2008, Templeton 2008). Thus we inferred the NCA approach for populations with the ANeCA (automation of nested clade phylogeographical analysis: Panchal 2007), implementing the construction of haplotype networks (with TCS), nesting of clades, calculation of summary statistics with tests of siginificance applying GEODIS 2.5 (Posada *et al*. 2000), and the interpretation of results with an inference key (Templeton 2008). Neither Crandall and Templeton's criteria (Crandall & Templeton 1993), nor those of Pfenninger and Posada (Pfenninger & Posada 2002) made it possible to resolve loops, thus we enumerated and nested all the possible trees. To carry out tests of significance, we ran 10 000 random permutations of geographical locations of individuals.

Results

Morphology

Bythinella shells in populations 1–10 from the Peloponnese (Fig. 2) varied in size: in some populations (especially 1, 6 and 9) dwarf shells were found. In population 5 there were two shell morphotypes (Fig. 2: 5A, 5B *vs*. 5C, 5D). Shell habitus was more diverse within the group of populations from Attica, Evvoia and Parnassus (Fig. 3: populations 11–20), and among the other nine populations from Lefkas island (21) and northern Greece (Fig. 4).

The penis and flagellum showed no constant differences between populations (although some interpopulation differences were found in all but

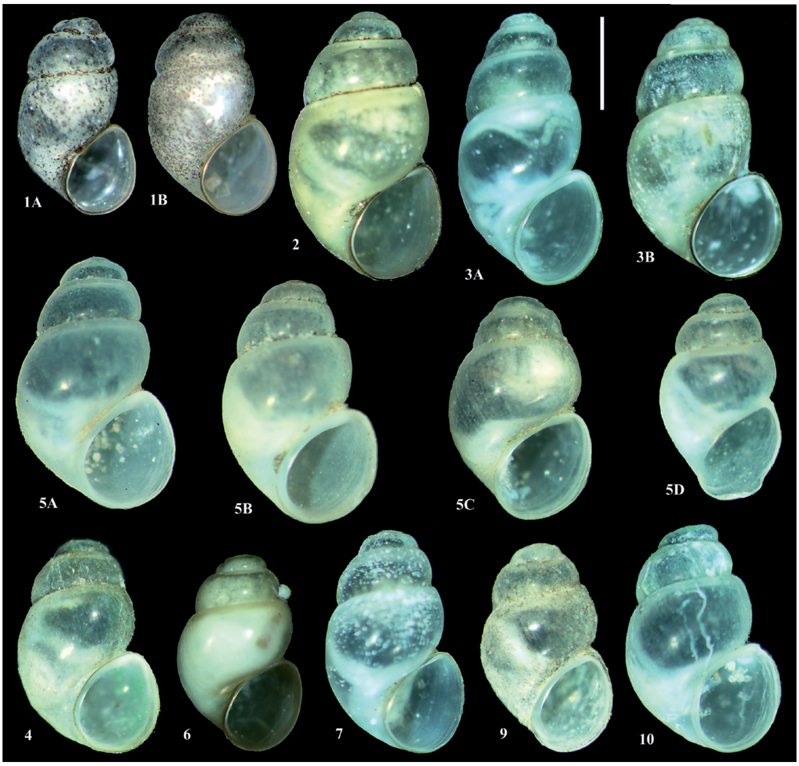


Fig. 2. Shells of *Bythinella* from localities 1–10; locality number is given below each shell; bar equals 1 mm.

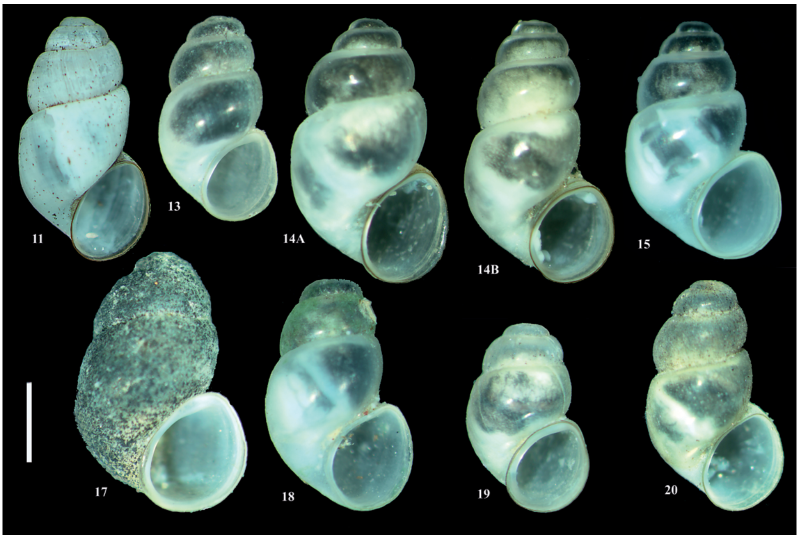


Fig. 3. Shells of *Bythinella* from localities 11–20; locality number is given below each shell; bar equals 1 mm.

some specimens), and the same was true of the proximal part of the pallial section of the female reproductive organs (Figs. 5–7). The two morphs from locality 5 (Fig. 2: 5A, 5B *vs*. 5C, 5D) differed in their female organs (Fig. 6: 5B *vs*. Fig. 6: 5A and Fig. 7: 5).

Molecular phylogeny and phylogeography

In total, we analyzed 302 sequences of COI, 570 bp long (GenBank Accession Numbers: JF314040–JF314315), and 93 sequences of

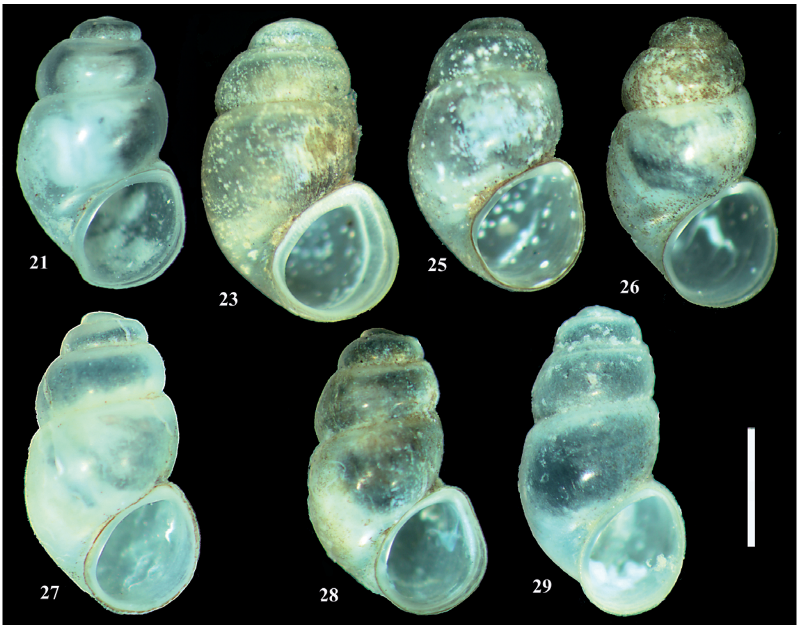


Fig. 4. Shells of *Bythinella* from localities 21–29; locality number is given below each shell; bar equals 1 mm.

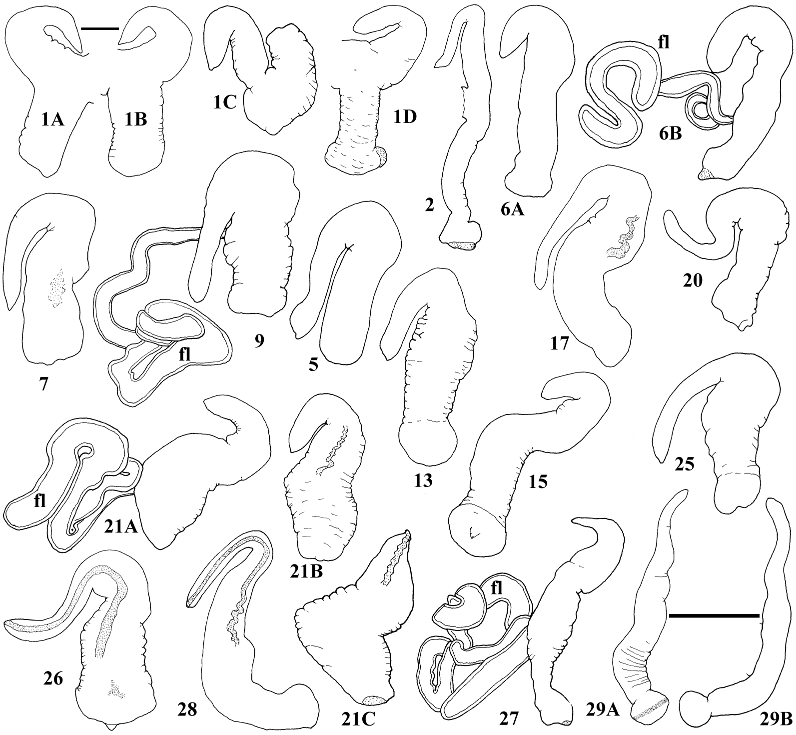


Fig. 5. Penes of *Bythinella*; locality number is given below each penis, 1A, 29A — ventrally, the other — dorsally, fl = flagellum (penial gland).

ITS-1 (from the same specimens as for COI), 322 bp long (GenBank Accession Numbers: JF313943–JF314039).

Saturation test of Xia *et al*. (2003) showed little saturation in our COI data: $I_{ss} = 0.067$, $I_{ss.c} = 0.710$ for symmetrical topology, df = 484, $p < 0.001$; and $I_{ss.c} = 0.389$ for asymmetrical topology, df = 484, $p < 0.001$.

For COI the Akaike Information Criterion (AIC) with ModelTest selected the model GTR

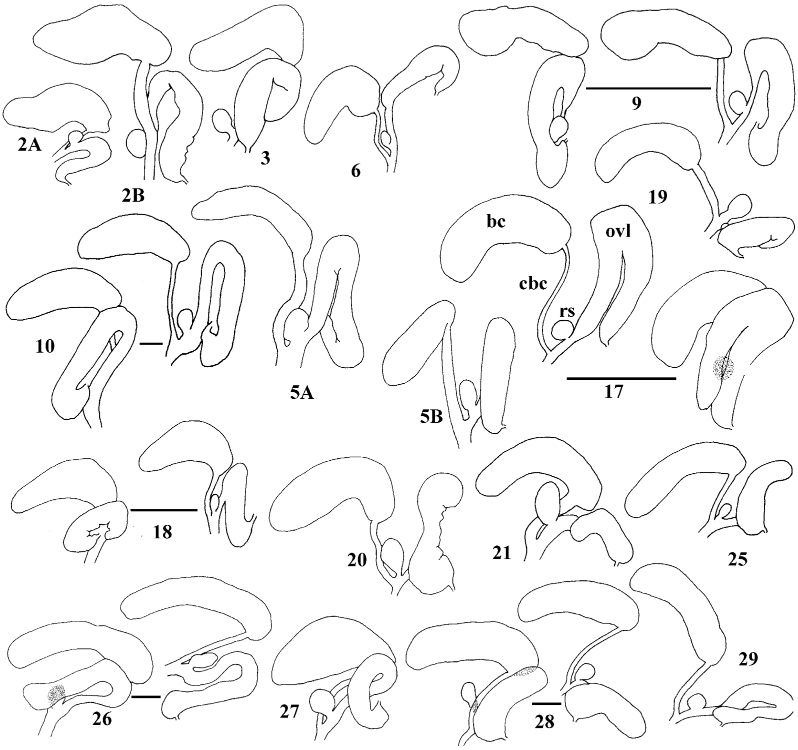


Fig. 6. Female reproductive organs (proximal section of pallial part) of *Bythinella*; locality number is given below each drawing; 5A, 5B — organs of two species cooccurring at locality 5: 5A represents specimens with shells 5C, 5D in Fig. 2; 5B represents specimens with shells 5A, 5B in Fig. 2; abbreviations as in Fig. 7 (below).

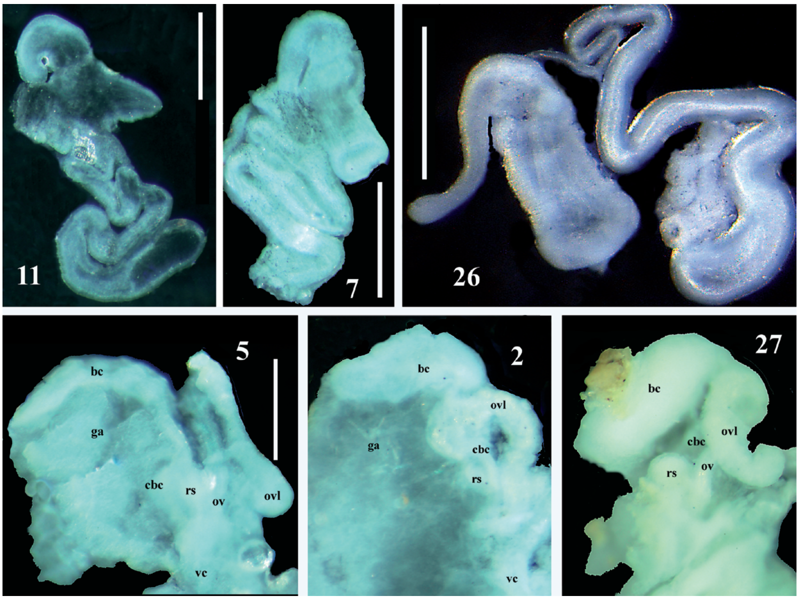
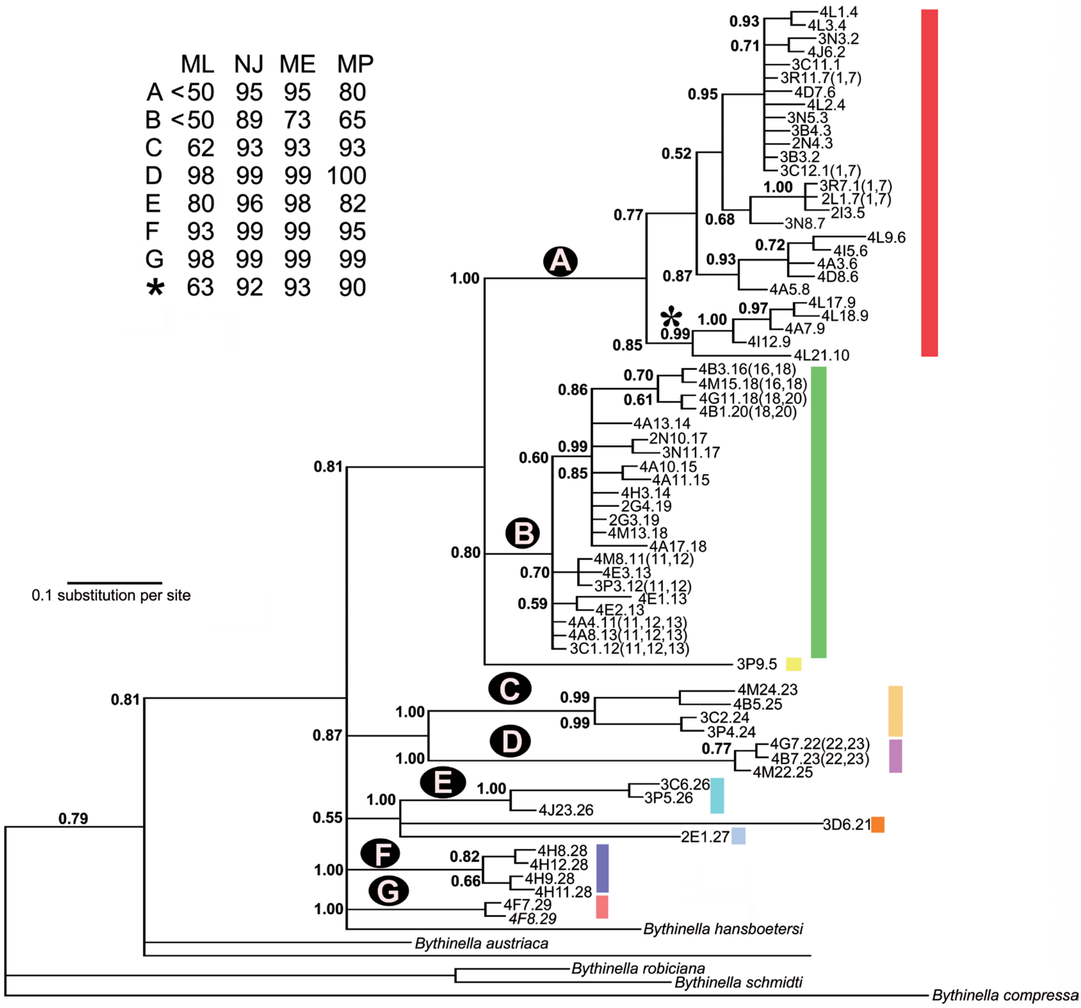


Fig. 7. Penes with flagella (above) and female reproductive organs (proximal section of pallial part: below); locality number is given in each photograph (5: shell morph as in Fig. 2: 5C, 5D); bar equals 0.5 mm for penes and 0.25 mm for female organs; abbreviations: bc = bursa copulatrix, cbc = duct of bursa copulatrix, ga = albumen gland, ov = pallial oviduct, ovl = coil of (“renal”) oviduct, rs = seminal receptacle, vc = ventral channel.

+ I + Γ, the same model was found for the tree with *Peringia* and *Salenthydrobia* as outgroup, with base frequencies: A = 0.3164, C = 0.1920, G = 0.1349, T = 0.3566; substitution rate matrix: [A–C] = 35.2722, [A–G] = 641.8149, [A–T] = 25.0159, [C–G] = 33.1934, [C–T] = 333.2993, [G–T] = 1.0000, proportion of invariable sites: (I) = 0.6007, and Γ distribution with the shape parameter = 0.9321.

For ITS-1, the Akaike Information Crite-



	ML	NJ	ME	MP
A	<50	95	95	80
B	<50	89	73	65
C	62	93	93	93
D	98	99	99	100
E	80	96	98	82
F	93	99	99	95
G	98	99	99	99
*	63	92	93	90

Fig. 8. Bayesian 50% majority rule consensus tree for COI haplotypes. Locality number is given at the end of each haplotype label; Bayesian probabilities are given for each clade; in parentheses localities' numbers if haplotype found at more than one locality; A–G and asterisk — major clades in the tree; in the table bootstrap supports for each major clade (ML = maximum likelihood, NJ = neighbor-joining, ME = minimum evolution, MP = maximum parsimony).

rion (AIC) with ModelTest selected the model SYM + Γ, with equal base frequencies; substitution rate matrix: [A–C] = 0.8103, [A–G] = 1.0609, [A–T] = 1.0389, [C–G] = 0.5660, [C–T] = 1.6248, [G–T] = 1.0000, proportion of invariable sites: (I) = 0.0000, and Γ distribution with the shape parameter = 0.5313.

For COI, we found three ML trees, their topology almost the same as that of the Bayesian tree (Fig. 8), differing only in some branch lengths. In the Bayesian tree, there is one large clade (A) that represents all the Peloponnesian populations (1–10) except one of the two haplotypes (3P9_5R) from locality 5. We found haplotype 3P9_5R in specimens whose shell (Fig. 2: 5C, 5D), and female reproductive organs (Figs. 6: 5A, 7: 5) differed from the shell and organs of the specimens with the other haplotype recorded at that locality (Figs. 2: 5A, 5B and 6: 5B). Within the clade representing the Peloponnese in the COI tree, there is a subclade grouping the haplotypes from localities 9 and 10 (marked with an asterisk in Figs. 8–9). The ITS-1 tree clade A is paraphyletic (Fig. 9). Its subclade (*),

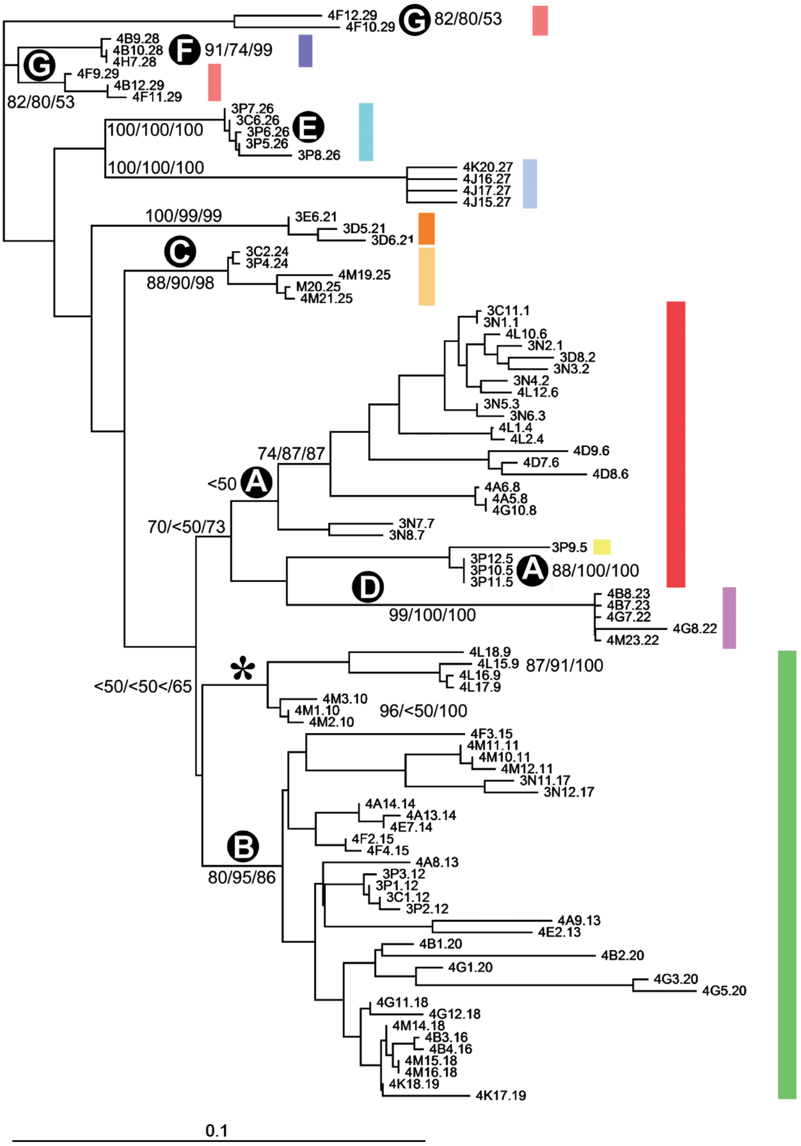


Fig. 9. Maximum likelihood tree for ITS-1 haplotypes; locality number is given at the end of each label; major clades (A–G and asterisk) as in Fig. 8, for each of them bootstrap supports (ML/ME/MP) given.

although has very low support, forms a sister clade with clade B representing the populations from Attica, Evvoia and Parnassus. All the other populations from the Peloponnese in the ITS-1 tree form a clade together with some of the haplotypes from the vicinity of Volos (D). Haplotype 3P9_5T for ITS-1 is the sister clade of the other haplotypes found at locality 5 (Fig. 9).

All the Attic, Evvoia and Parnassian populations (11–20) form another large clade (B) in the COI tree (Fig. 8). Both its Bayesian probability and bootstrap supports are low. In the ITS-1 tree (Fig. 9) clade B is better supported.

For four neighbouring populations from the vicinity of Volos (populations 22–25), the COI tree (Fig. 8) shows two distinct sympatric/parapatric haplotype groups (C and D). The groups are equally distant from each other as the Peloponnesian populations are from the Attic, Evvoia and Parnassian ones. In the ITS-1 tree (Fig. 9), the distinctness of C and D is even better supported.

In populations 21 and 27, we found only one distinct COI haplotype in each (Fig. 8). The ITS-1 tree (Fig. 9) confirms the distinctness of

Table 3. K2P distances between (below diagonal) and within (at diagonal) presumed species, A–G = clades as in Figs 8 and 9.

	A	B	C	D	E	F	G	3P9_5	3D6_21
A	0.00176–0.02699								
B	0.03256–0.05144	0.01760–0.02167							
C	0.04574–0.07508	0.05161–0.06928	0.01064–0.02330						
D	0.06528–0.08133	0.05728–0.06916	0.05161–0.06340	0.00035–0.00176					
E	0.04010–0.06318	0.02829–0.05703	0.05356–0.06496	0.04968–0.06705	0.00352–0.01777				
F	0.04186–0.06318	0.04180–0.05330	0.05144–0.05747	0.05533–0.06131	0.02691–0.04161	0.00176–0.00706			
G	0.04752–0.06093	004944–0.06102	0.04567–0.05347	0.05533–0.05924	0.03615–0.04924	0.02872–0.03620	0.00176		
3P9_5	0.03997–0.05330	0.03615–0.04364	0.06517–0.07508	0.07496–0.07699	0.04944–0.05501	0.05122–0.05516	0.05898–0.06093		
3D6_21	0.05719–0.07902	0.05516–0.06299	0.07105–0.07699	0.07485–0.07687	0.04745–0.06077	0.06290–0.06676	0.06299–0.06496	0.07474	
3E1_27	0.05758–0.07318	0.06142–0.07331	0.04783–0.05944	0.06528–0.06727	0.04968–0.05524	0.04951–0.05347	0.04759–0.04951	0.07318	0.06905

these populations. In the COI tree, the haplotypes from populations 26, 28 and 29 form distinct, well supported clades (Fig. 8). The ITS-1 tree (Fig. 9) confirms the distinctness of clades E and F. However, the haplotypes from locality 29 (clade G in the COI tree) that are mixed-up with clade F in the ITS-1 tree, are not monophyletic (Fig. 9).

The phylogenies inferred with ME, MP and NJ were, in general, similar to the ones described above, thus they were used only to estimate bootstrap supports of the distinguished clades (Figs. 8 and 9).

The K2P distances for COI between and within the recognised clades are listed in Table 3. The distance between the haplotype 3P9R_5 from locality 5 and co-occurring haplotype 2I3R_5, equalled 0.04759. The distance between *Bythinella robiciana* and *B. schmidti* was 0.03428, and between the other outgroup species 0.07645–0.12452. The distances among the haplotypes from northern Greece (outside the Peloponnese and Attica with Evvoia and Parnassus) were between 0.00176 and 0.06705. Finally, the distances between the two groups of populations, one from the Peloponnese plus one from Attica, Evvoia and Parnassos, *versus* all the other (northern) ones ranged from 0.01418 to 0.08133 (in almost all cases exceeding 0.04).

Ln-likelihood for the best found tree (for COI) not assuming molecular clock equalled –3221.2118, and with the clock assumption it equalled –3268.3617, Δ = 94.2998, df = 76, the Likelihood Ratio Test (LRT) resulted in p = 0.0759. The value of p was just above the formal significance level, so we applied the molecular clock with caution. The observed differences reflected no more than about 4 MYA (Table 4), most of them within or slightly above the last 2 MYA that correspond to the Pleistocene.

The values of haplotype diversity h and nucleotide diversity π in the studied populations are listed in Table 1. The total fixation index F_{ST} equalled 0.91832, within population variation accounted for 8.17% of the total variance, and between the populations 91.83% ($p < 0.001$). Pairwise F_{ST} values (Table 5) were high, and the estimates of gene flow Nm (Table 5) were low, although there were some cases of high gene flow between quite distant populations.

Mismatch distribution curves (not shown) reflected equilibrium only in populations 14, 17 and 29 (Table 1 and Appendix). We inferred population reduction in populations 2, 11, 12, 15 and 19, and a bottleneck followed by population growth in populations 1, 3, 4, 6, 7, 9, 13, 18, 26 and 28.

From among the 61 haplotypes found in COI, 54 occurred at one locality each (Fig. 10). Haplotype networks constructed with the connection limit excluding homoplastic changes conventionally set to 95% showed a very complicated pattern, with several loops. Some of them remained with the limit set to 99% (Fig. 10). The group of haplotypes from the Taigetos Mts. (populations 1–7), with the exception of one of the two haplotypes present at locality 5 (3P9_5), formed three networks (99% connection limit): one including localities 1, 5 and 7, one comprising only locality 6, and one including all the other haplotypes (Fig. 10). The haplotypes from Attica, Evvoia and the Parnassus (localities 11–20) formed a single network with several loops (Fig. 10 B1, one of the alternative trees: B2), with several distant connections (across a geographical distance approaching 150 km). The haplotypes of one of the two species from Volos (clade C), and those of population 26 (clade E), did not form single networks with the same 99% connection limit. The other of the two Volos species (clade D) and population 29 (clade G) were represented by a one-step network each (Fig. 10). The four haplotypes of population 28 (clade G) were interconnected (Fig. 10).

For the Peloponnesian haplotypes (except 3P9_5), a nested clade analysis (NCA) together with an inference key indicated allopatric fragmentation, with two cases of long-distance colonization possibly coupled with subsequent fragmentation, or past fragmentation followed by range expansion, between localities 8, 9 and 10, across a geographical distance of 48 km. Within Attica, Evvoia and Parnassos (populations 11–20) the inference key indicated allopatric fragmentation for the total cladogram. At the lower nesting levels, we found some cases of long-distance dispersal and some of restricted gene flow with isolation by distance. For the other nine northern populations, we inferred allopatric fragmentation.

Discussion

Taxonomic implications

From among the concepts of species, we prefer the cohesive one proposed by Templeton (1989). Unfortunately, we know too little about the ecology and biology of the *Bythinella* taxa under consideration to apply this concept. In practice, in the case of the Greek *Bythinella* we could use three criteria. One of them is the relative range of genotypic differentiation. There are many data on the genetic distances in COI. Most studies apply K2P distances (e.g. Bichain *et al*. 2007). It must be stressed that the values of the distances are applicable only within a group of rather closely related species. The differences among the seven distinguished clades (A–G), haplotype 3P9_5R, and populations 21, 26, 27, 28 and 29, are within the range found among the six *Bythinella* outgroup species, as

Table 4. Estimates of divergence time, in MYA: Lin. tree = estimated in the linearized tree, Comp. lik. = calculated from composite likelihood distances, with standard error for higher value, ML = estimated from maximum likelihood distances; dating assumes divergence time between *Salenthydrobia* and *Peringia* as 5.96 MYA, not 5.33 MYA (following Falniowski *et al.* 2008)

	Lin. tree	Comp. lik. ± SE	ML
All populations max.	3.340	4.341 ± 0.753	3.757
G1–7 *vs.* G8–10	0.909	0.438–1.511 ± 0.382	0.376–0.989
G1–10 *vs.* G11–20	2.142	1.913–2.190 ± 0.130	1.194–1.995
G21 *vs.* mainland	2.808	2.573–4.341 ± 0.747	2.046–3.757
Clade C *vs.* clade D	3.257	2.742–3.382 ± 0.634	2.029–2.580
26 *vs.* 27	2.430	2.624–2.980 ± 0.583	1.909–2.275
28 *vs.* 29	1.730	1.536–1.933 ± 0.454	1.085–1.239

well as between species within other genera in the Rissooidea (e.g. Wilke 2003, Falniowski *et al*. 2007, 2009b, Szarowska *et al*. 2007). Bichain *et al*. (2007) reported that the species threshold value of K2P distance in the west-European *Bythinella* was 0.015. The second criterion, of morphological differences between molecularly distinct taxa, may not be useful in the case of

Table 5. Below diagonal: matrix of *M* values (*M* = Nm for haploid data); along diagonal: population specific F_{ST} indices, above diagonal: matrix of coancestry coefficients F_{ST}.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
01	0.9147	0.7587	0.8322	0.6129	1.5885	1.0234	0.4473	1.7100	1.5972	2.2418	2.5329	2.2886	2.2443	2.8711	2.7605	2.4145
02	0.4403	0.9239	0.6160	1.1718	1.9591	1.2536	1.0599	2.9865	2.2721	3.5462	3.5319	3.4768	3.0073	3.8900	3.7044	3.7551
03	0.3851	0.5872	0.9229	1.2133	1.9092	1.3093	1.1227	2.8575	2.2659	3.3350	3.3551	3.2502	2.9051	3.7007	3.5435	3.4804
04	0.5912	0.2244	0.2114	0.9201	1.6992	1.1892	0.9086	2.5082	1.9663	2.9638	3.0581	2.8421	2.6071	3.4498	3.2850	3.0556
05	0.1283	0.0821	0.0870	0.1119	0.8946	1.5203	1.5537	2.0196	1.6224	2.0557	1.7708	1.4314	1.5694	2.1289	2.0443	1.5330
06	0.2805	0.1998	0.1849	0.2189	0.1399	0.9110	1.0390	1.0066	1.5646	1.8320	2.0927	1.9340	1.9654	2.3335	2.2975	2.0325
07	0.8865	0.2651	0.2412	0.3376	0.1341	0.2738	0.9114	1.3497	1.3991	1.9250	2.3232	2.0945	2.1111	2.6258	2.5174	2.1722
08	0.1104	0.0266	0.0305	0.0443	0.0765	0.2880	0.1751	0.9270	2.5689	∞	4.4648	5.1122	3.4435	4.9417	4.4771	∞
09	0.1269	0.0575	0.0579	0.0814	0.1230	0.1323	0.1639	0.0415	0.9184	2.2877	2.9276	2.6827	2.5347	3.3089	3.1617	2.8676
10	0.0595	0.0149	0.0185	0.0272	0.0734	0.0953	0.0854	0.0000	0.0565	0.9270	4.5141	5.0828	3.4737	5.0174	4.5261	∞
11	0.0431	0.0151	0.0181	0.0247	0.1026	0.0704	0.0543	0.0058	0.0283	0.0055	0.9247	0.0000	0.5357	2.3190	2.5024	3.0143
12	0.0564	0.0160	0.0202	0.0310	0.1570	0.0845	0.0702	0.0030	0.0367	0.0031	inf	0.9250	0.3990	2.4660	2.5223	3.3673
13	0.0593	0.0260	0.0290	0.0398	0.1315	0.0815	0.0689	0.0165	0.0431	0.0160	0.7057	1.0199	0.9195	1.8350	2.0171	2.0682
14	0.0300	0.0104	0.0127	0.0164	0.0675	0.0537	0.0390	0.0036	0.0190	0.0033	0.0546	0.0464	0.0950	0.9257	1.9112	3.4741
15	0.0338	0.0126	0.0149	0.0195	0.0744	0.0559	0.0439	0.0058	0.0221	0.0055	0.0446	0.0436	0.0767	0.0868	0.9247	2.6568
16	0.0491	0.0120	0.0159	0.0247	0.1377	0.0754	0.0643	0.0000	0.0301	0.0000	0.0258	0.0179	0.0724	0.0160	0.0377	0.9270
17	0.0362	0.0106	0.0133	0.0184	0.0865	0.0624	0.0513	0.0023	0.0218	0.0024	0.0355	0.0297	0.0715	0.0262	0.0368	0.0135
18	0.0917	0.0572	0.0593	0.0759	0.1673	0.1045	0.0999	0.0499	0.0776	0.0484	0.2676	0.3801	0.3031	0.2418	0.3759	2.8438
19	0.0362	0.0128	0.0153	0.0203	0.0819	0.0601	0.0472	0.0052	0.0232	0.0049	0.0669	0.0634	0.1316	0.0819	0.1076	0.0335
20	0.0252	0.0067	0.0088	0.0117	0.0595	0.0485	0.0352	0.0000	0.0143	0.0000	0.0128	0.0062	0.0342	0.0110	0.0271	0.0000
21	0.0199	0.0052	0.0069	0.0096	0.0414	0.0362	0.0273	0.0000	0.0112	0.0000	0.0039	0.0021	0.0111	0.0024	0.0042	0.0000
22	0.0248	0.0066	0.0086	0.0137	0.0604	0.0392	0.0332	0.0000	0.0156	0.0000	0.0050	0.0033	0.0152	0.0029	0.0049	0.0000
23	0.1241	0.0927	0.0918	0.1305	0.1868	0.1072	0.1281	0.0895	0.1237	0.0956	0.1133	0.1865	0.1416	0.0775	0.0854	0.1739
24	0.0462	0.0115	0.0144	0.0232	0.0971	0.0595	0.0618	0.0021	0.0308	0.0024	0.0088	0.0093	0.0236	0.0053	0.0082	0.0041
25	0.1070	0.0750	0.0746	0.0959	0.1412	0.0983	0.1228	0.0716	0.0966	0.0734	0.0719	0.1100	0.0917	0.0523	0.0611	0.1052
26	0.0390	0.0205	0.0218	0.0279	0.0733	0.0570	0.0482	0.0162	0.0300	0.0154	0.0221	0.0268	0.0308	0.0160	0.0194	0.0246
27	0.0173	0.0045	0.0059	0.0080	0.0377	0.0326	0.0258	0.0000	0.0119	0.0000	0.0030	0.0015	0.0085	0.0020	0.0032	0.0000
28	0.0878	0.0597	0.0598	0.0739	0.1520	0.0961	0.0981	0.0499	0.0722	0.0475	0.0719	0.1041	0.0924	0.0489	0.0572	0.0891
29	0.0245	0.0073	0.0090	0.0123	0.0522	0.0436	0.0362	0.0017	0.0170	0.0017	0.0060	0.0047	0.0136	0.0040	0.0057	0.0023

	17	18	19	20	21	22	23	24	25	26	27	28	29
01	2.6960	1.8647	2.6955	3.0356	3.2622	3.0542	1.6154	2.4692	1.7358	2.6267	3.3981	1.9014	3.0645
02	3.8743	2.2760	3.6898	4.3322	4.5752	4.3458	1.8554	3.7949	2.0366	3.2368	4.7257	2.2380	4.2391
03	3.6539	2.2441	3.5182	4.0543	4.2905	4.0809	1.8638	3.5753	2.0416	3.1747	4.4600	2.2368	4.0350
04	3.3362	2.0263	3.2453	3.7752	3.9686	3.6277	1.5753	3.1182	1.8269	2.9417	4.1461	2.0498	3.7319
05	1.9141	1.3834	1.9610	2.2408	2.5708	2.2280	1.3019	1.8167	1.5129	2.0569	2.6585	1.4560	2.3588
06	2.1996	1.7556	2.2326	2.4260	2.6963	2.6213	1.7340	2.2413	1.8062	2.2795	2.7928	1.8250	2.5229
07	2.3751	1.7928	2.4510	2.7210	2.9626	2.7758	1.5898	2.2069	1.6238	2.4307	3.0155	1.8076	2.6958
08	5.4005	2.3990	4.5799	∞	∞	∞	1.8849	5.4629	2.0773	3.4602	∞	2.3997	5.6971
09	3.1740	2.0073	3.1146	3.5821	3.8181	3.5010	1.6178	2.8457	1.8205	2.8711	3.7636	2.0700	3.4172
10	5.3473	2.4268	4.6296	∞	∞	∞	1.8292	5.3292	2.0562	3.5099	∞	2.4440	5.6727
11	2.7132	1.0538	2.1368	3.6887	4.8588	4.6070	1.6891	4.0616	2.0738	3.1643	5.1221	2.0740	4.4403
12	2.8820	0.8397	2.1852	4.4007	5.4861	5.0439	1.3031	3.9990	1.7129	2.9802	5.7933	1.7587	4.6718
13	2.0785	0.9744	1.5687	2.7494	3.8344	3.5265	1.5111	3.0987	1.8642	2.8467	4.0963	1.8583	3.6294
14	2.9985	1.1211	1.9604	3.8412	5.3517	5.1538	2.0086	4.5629	2.3578	3.4729	5.5532	2.4174	4.8435
15	2.6805	0.8459	1.7309	2.9664	4.7985	4.6291	1.9247	4.1324	2.2175	3.2856	5.0634	2.2758	4.4926
16	3.6381	0.1620	2.7693	∞	∞	∞	1.3546	4.8071	1.7495	3.0594	∞	1.8888	5.4051
17	0.9259	0.9610	2.2972	4.4277	5.6626	5.3726	1.6720	4.5512	2.0145	3.2165	5.8917	2.0956	4.9420
18	0.3098	0.9084	0.6414	0.5174	2.7716	2.5446	1.4653	2.2771	1.7265	2.2674	2.9939	1.6330	2.6940
19	0.0559	0.5561	0.9249	3.1088	4.9142	4.7308	1.8678	4.2382	2.1966	3.2442	5.1893	2.1998	4.5539
20	0.0060	0.7378	0.0234	0.9270	∞	∞	2.0869	5.9324	2.4574	3.6201	∞	2.5654	6.0059
21	0.0017	0.0334	0.0037	0.0000	0.9270	∞	1.9998	5.7619	2.3210	3.5678	∞	2.6328	5.8758
22	0.0023	0.0426	0.0045	0.0000	0.0000	0.9270	0.1754	5.0188	1.6660	3.3722	∞	2.1885	5.5050
23	0.1157	0.1502	0.0913	0.0708	0.0783	2.6087	0.8638	0.9720	0.6317	1.7454	1.8998	1.1683	1.7399
24	0.0053	0.0572	0.0073	0.0013	0.0016	0.0033	0.3043	0.9247	0.8234	3.0712	5.5170	1.9127	4.5422
25	0.0770	0.1082	0.0626	0.0468	0.0544	0.1165	0.5676	0.3913	0.8859	1.9897	2.2471	1.3708	2.0481
26	0.0209	0.0578	0.0203	0.0138	0.0145	0.0178	0.1058	0.0243	0.0792	0.9192	3.5752	1.8101	3.2846
27	0.0014	0.0264	0.0028	0.0000	0.0000	0.0000	0.0880	0.0020	0.0591	0.0144	0.9270	2.5639	5.7081
28	0.0701	0.1214	0.0623	0.0417	0.0387	0.0631	0.2256	0.0866	0.1702	0.0978	0.0417	0.9019	2.0445
29	0.0036	0.0363	0.0053	0.0012	0.0014	0.0020	0.1065	0.0054	0.0740	0.0195	0.0017	0.0744	0.9261

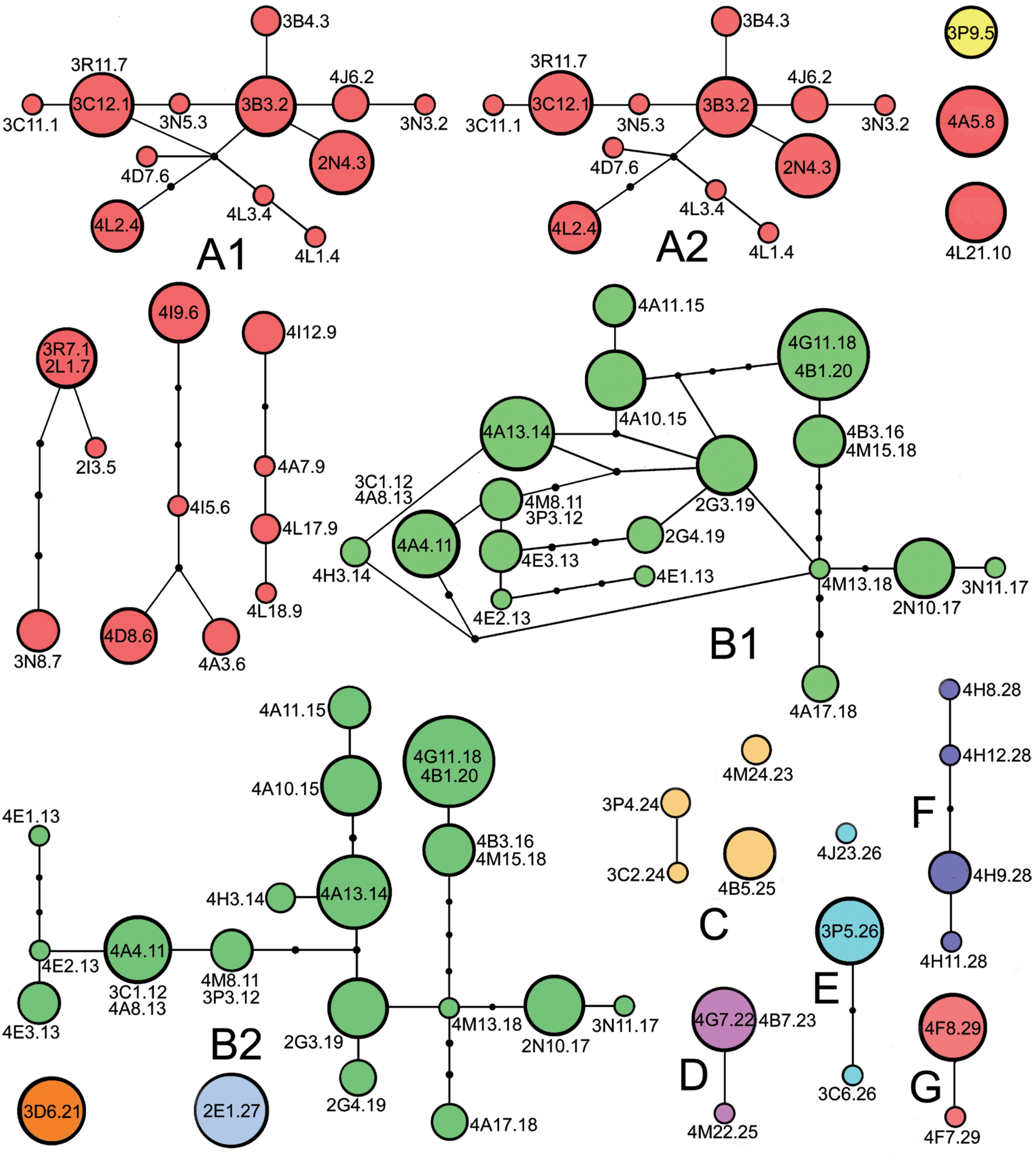


Fig. 10. Haplotype networks for COI, connection limit excluding homoplastic changes set to 99%; haplotype labels as in the cladogram; circles representing haplotypes proportional in size to haplotype frequencies; A2, B2: networks A1, B1, respectively, with resolved loops.

morphostatic evolution. In practice, it is not easy to assess distinctness of species in more or less different yet allopatric taxa. Where there is sympatry combined with well-marked differences (morphology included), species distinctness is well supported. Fortunately, we could apply this third criterion (of sympatric occurrence) to the studied *Bythinella*. In population 5, there were two distinct morphotypes (Fig. 2: 5A and 5B *vs*. 5C and 5D), their shell variability not overlapping between, and the female reproductive organs different from each other (Fig. 6: 5A *vs*.

5B). Their genetic distinctness, marked well in COI and much less in ITS-1, reflected their morphological distinctness. These two taxa inhabited the same small spring, which proved the species distinctness. Our material was, unfortunately, too scarce to examine the morphological differences between clades C and D that occurred in sympatry/parapatry in the vicinity of Volos (populations 22–25). In *Bythinella*, however, it is common that molecular differences are not well reflected in morphology, which confirms the morphostatic model of evolution, with numerous cryptic species within the genus (Falniowski *et al*. 2009b). The photographs and drawings in the present paper show that the Greek *Bythinella* is not an exception.

All the above considered, the COI data suggest the species distinctness of all the ten clades (A–G, haplotype 3P9_5, populations 21, and 27). In the ITS-1 tree, group A does not form a clade. The story inferred from ITS-1 is, in general, similar to the one inferred from COI, but there are some differences. In the ITS-1 tree, clades A and G are not monophyletic, the distinctness of the "asterisk" group is more evident than in the COI tree, and the distinctness of haplotype 3P9_5T less marked than for 3P9_5R one.

There are several explanations of the differences between the COI and ITS-1 trees. Firstly, a gene tree need not reflect another gene tree or a species tree (e.g. Avise 2000). Secondly, the mitochondrial DNA presents one locus as a whole and tells us nothing about the history as represented by the males (Freeland 2005). More than in other cases the problem arises when migration rates for the males and the females are different. Obviously, *Bythinella* cannot actively migrate, but one can speculate about, say, sex-dependent survival during passive transport (presumably by birds: *see* below). Obviously, more data are necessary to get a complete picture of *Bythinella* taxonomy in Greece, but in the present state of understanding we assume that our populations represent ten distinct species. Clade B, representing Attica, Evvoia and the Parnassus Mts., can be identified as *Bythinella charpentieri*; the other nine species are probably new ones.

Interestingly, most of the species occur in northern Greece, with only two (or possibly three) species in the Peloponnese. The Taigetos Mts. harbour a very rich land snail fauna (Sfenthourakis & Legakis 2001), but in spite of our extensive sampling (seven *Bythinella* localities) we found only one widespread and one narrowly endemic species. There is also only one species in Attica, Evvoia and the Parnassus Mts.

We noted two cases of sympatry (locality 5 and localities 23 and 25). This sympatry was confirmed by distinct sequences of both COI and ITS-1. The sympatric occurrence of two *Bythinella* species at three localities is noteworthy. For long the one-species-at-one-locality view was widely accepted concerning *Bythinella* (for references *see* Falniowski 1987, Falniowski *et al*. 2009b). This argument assumed that: (i) closely phylogenetically related snails have most probably the same or almost the same ecological niche; (ii) there is little or no spatial differentiation inside small and simple habitats like springs. Neither argument need be true. Firstly, the ecological niches of closely related species do not necessarily overlap. Secondly, even the smallest spring may contain more than one microhabitat. On the other hand, one can assume that two species whose ecological niches overlap will coexist if their densities are limited not by their competition, but some other factors (e.g. parasites). Falniowski *et al*. (2009b, 2009c) found a case of sympatric occurrence of two *Bythinella* species in Romania. Carlos *et al*. (2000) found similarly unexpected co-occurrence of two or more closely related, congeneric species in a very small habitat in a *Symbiodinium* species inhabiting the *Tridacna* clam.

Geological history of continental Greece

In the late Tortonian (8 MYA), the modern post-Alpine European topography in the Mediterranean emerged (Kostopulos 2009). In the upper Miocene (about 6 MYA) the Peloponnese, Evvoia and Lefkas were part of a continuous land mass in the continent (Popov *et al*. 2004). In the middle-upper Pliocene (3.5–1.8 MYA), freshwater lakes and marshes stretched along the present shore of the Aegean Sea; at the same time another such strip of lakes and marshes stretched along the eastern border of the Hellenids ridge (Popov *et al*. 2004). Evvoia was still a part of the continent, but the Peloponnese was separated from the continent by a broad strait (Creutzburg 1963, Dermitzakis & Papanikolaou 1981, Dermitzakis 1990, Popov *et al*. 2004). The present Gulf of Corinth (105 km long and 30 km wide, the maximum depth of about 900 m) is separated from the open sea by the Rion Strait, with a sill depth of 62 m marked by an extensive submarine terrace. During eustatic sea-level lowstands, the Gulf of Corinth was a lake, the last lacustrine condition in the Gulf occurred about 12 000 years ago. In periods when the sea level was a few metres above the Rion Strait sill, the Gulf of Corinth was a marine area with high fresh water content (Perissoratis *et al*. 2000). During the glacial maxima, the sea level was as much as 200 m lower than today (Beerli *et al*. 1996). In the valley surrounding Megalopolis in the Peloponnese (thus between our localities G1–7 and G8–10) in the upper Pliocene and Pleistocene there was a big, deep freshwater lake; later, in the middle Pleistocene, there were two sea transgression on the Peloponnese (Siavalas *et al*. 2009).

During the glacial maxima in the Pleistocene, in northern Europe the temperature was as much as 21 °C lower than today (Hofreiter & Stewart 2009). Obviously, in Greece the climatic conditions were less severe. On the Greek island Andikithira 16 000 yr BP (thus during the last glacial maximum: LGM) temperatures were some 5–8 °C below the present, and the climatic conditions were slightly drier (confirmed by Digerfeldt *et al*. 2000). Thus that large temperature depression, well documented in north and central Europe, affected also the Mediterranean climate (Gittenberger & Goodfriend 2006). Tzedakis (1993) demonstrated the long-term occurrence of forests in Greece during the glacial period, but Willis *et al*. (2000) demonstrated that at least isolated refugia of forest trees existed as far to the north as Hungary.

Near Ioannina Lake, trees persisted throughout the LGM and probably much before. The glacial vegetation history of north-west Greece differs substantially from that of most of Europe (Lawson *et al*. 2004). The diversity of microclimates present within the mountainous Ioannina catchment, characteristic of the Pindus Mts., makes them a patchwork of varied habitats able to support a number of distinct vegetation communities (Lawson *et al*. 2004), from maquis, through mixed deciduous woodland and boreal coniferous forest, to alpine shrub and herb communities. This diversity promoted species survival during the glacial periods, but also promoted speciation. On the other hand, unstable fluvio-lacustrine system in SW Bulgaria and northern Greece, with glaciers in the Pirin and Rila Mts. (Zagorchev 2007) probably formed effective, temporary barriers for *Bythinella*.

To our knowledge, there are no data on the fossil occurrence of *Bythinella* in Greece. In Poland, the oldest record is 7750 ± 130 years old (Falniowski *et al*. 1998). On the other hand, in Italy *Bythinella* was found in the Pliocene/Pleistocene boundary (southern margin of the Alps, north of Bergamo: Esu & Gianolla 2009). The estimated times of divergence (Table 4) suggest, unexpectedly, a rather short history of *Bythinella* in the studied area. Schilthuizen *et al*. (2004) estimated a similar short time for the Cretean *Albinaria*. Obviously the time frame cannot exceed about 4 MYA (Table 4). In fact, 3.5 MYA, in the Pliocene, the connection between the mainland and Lefkas Island disappeared due to a rise in sea level (Dermitzakis & Papanikolaou 1981, Dermitzakis 1990), and this coincides with the estimates for the Lefkadian population 21 *vs*. the ones from the mainland. However, 3.5 MYA the present Peloponnese was likewise isolated from the mainland, but the divergence time between the Peloponnesian and Attic, Evvoia and Parnassian populations (Table 4) oscillates around 1 MYA. This may be due to several episodes of land bridges at both (E and W) borders of the present Gulf of Corinth. The distances between the two (C and D) clades found near Volos may reflect a secondary contact of the lineages separated 2.5–3 MYA by the strip of lakes/marshes along the present coast of the Aegean Sea. Interestingly, in the same region there occur three lineages of *Grossuana*/*Radomaniola* (unpublished data), which in other areas are vicariant. The other strip of lakes, running along the eastern edge of the Hellenids, may be responsible for the observed distance between populations 26 and 27 (2–3 MYA). The split between the southern (1–7) and northern (8–10) Peloponnese populations, dated to about 1 MYA (Table 4), thus may reflect isolation by the lake in the region of Megalopolis. The divergence time between the two Epirot populations (28 and 29), corresponding to about 1–1.5 MYA, may reflect the unique conditions that existed in this region during the Pleistocene (*see* above). In general, the distances among the northern Greek populations are larger. This suggests a longer period of isolation. The distinct groups of haplotypes found in the restricted areas in northern Greece point to northern Greece as a source of both Attic (plus Evvoia and Parnassian) and Peloponnesian populations.

The majority of the rich literature on the phylogeography and history of the fauna of the region covers the islands (e.g. Parmakelis *et al*. 2005, 2006a, 2006b, Poulakakis *et al*. 2003, 2005), and only a few deal with continental Greece (e.g. Moorsel *et al*. 2000, De Weerd *et al*. 2004).

Phylogeography

Haplotype diversity (*h*) presents information on the numbers and frequencies of different alleles at a locus, regardless of their sequence relationships; nucleotide diversity π is a weighted sequence divergence between individuals in a population, regardless of the number of different haplotypes (Avise 2000). A population with a low *h* and π probably experienced a prolonged or severe bottleneck (or selective sweep) in recent times (populations 8, 10, 16, 20, 21, 22 and 27, and also 14, 17, 26, and 29). The high values of both *h* and π in populations 18 and 28 may reflect stable populations with large long-term evolutionary effective population size N_e (Avise 2000), but at the locality 23 can be rather assigned to an admixed sample of individuals of another species (Avise 2000); the same suggestion applies to the low values of *h* coupled with high values of π in populations 5 and 25 (Avise 2000). The high *h* and low π, found in the other populations, may suggest a rapid population growth from an ancestral population with a small N_e, if time was sufficient for haplotypes variation to recover through mutation, yet insufficient for large sequence differences to accumulate.

The analysis of mismatch distribution, indicating equilibrium only in three populations, also reflects drastic changes in population density and diversity in *Bythinella*. These are most probably due to the instability of the spring environment. Among the five populations in which mismatch distribution suggested a reduction of population size, in two cases, Kessariani Monastery (12) and Kastalia Spring at Dhelfoi (19) the springs were heavily affected due to human impact (Szarowska & Falniowski 2004). Severe bottlenecks followed by population growth were found in ten populations. τ values varied among populations, suggesting local bottleneck events, instead of any more global one. Almost all of the springs that *Bythinella* inhabits in Greece are very small, the water percolating from the ground only in winter and spring. The population in such a spring must have a low value of N_e, and thus prone to stochastic factors. Ponder *et al*. (1995) found higher differences between snail populations from neighbouring small springs than between populations of the same snails that inhabited bigger springs.

In an allozyme-based study of the central European *Bythinella* (Falniowski *et al*. 1998, 1999), we reported low levels of gene flow coupled with some long-distance dispersal. It is noteworthy, however, that the pattern observed in central Europe corresponds not to the infinite island model (random dispersal regardles of distance) but to the stepping-stone model (isolation by distance with restricted gene flow). The same concerns Bulgarian populations (Falniowski *et al*. 2009a) and the Greek *Bythinella* in the present study. Haplotypes 3C12_1 and 3R7_1 were common to localities 1 and 7 situated 26 km from each other. Haplotype 4A4_11 occurred at localities 11, 12 and 13 separated by distances of 23–72 km; haplotype 4M8_11 at localities 11 and 12 situated 71 km far from each other. Haplotype 4B3_16 was present at localities 16 and 18; 4G11_18 at 18 and 20; 4G7_22 at 22 and 23; the distances were 6, 17 and 2.5 km, respectively. The few cases of presumed passive transportation (the sea included) we found in this study confirm that passive dispersal, most probably by birds (Rees 1965, Wesselingh *et al*. 1999, Charalambidou & Santamaria 2002, Figuerola & Green 2002) is an important factor that moulds the geographical distribution of haplotypes. There are no observations on the bird transport of *Bythinella*, but the data on snails transported by birds are numerous. Lyell (1832) mentioned the possibility of transport by waterfowl to distant islands for a land snail *Succinea putris* through the attachment of eggs to feathers, and Darwin (1859) observed ducks emerging from a pond with duckweed adhering to their backs. For the Rissooidea, Bondesen and Kaiser (1949) mentioned a possibility for *Potamopyrgus antipodarum* and other prosobranchs of attaching themselves to feathers by trapping them between operculum and shell. Cadée (1988, 1994) observed living *Hydrobia ulvae* emerging from faeces of the shelduck, *Tadorna tadorna*, indicating that they survived the passage of the digestive tract. The numbers of *Hydrobia* observed alive in the shelduck's faeces were considerable: three droppings contained in total 140 living specimens and 960 empty and/or broken *Hydrobia* shells (Cadée 1988).

General pattern of differentiation

Hershler and Liu (2004a, 2004b) found a similar pattern of gene flow in another rissooid over a large distance. The levels of gene flow, as well as metapopulation structure in general, depend on several species-specific traits (Bohonak 1999, Bilton *et al*. 2001, Myers *et al*. 2001). Allopatric fragmentation as the main mechanism responsible for the observed pattern of interpopulation differences points to mostly vicariant, allopatric speciation in the Greek *Bythinella*. However, this model disagrees with the mtCOI sequence divergence data suggesting the relatively recent origin of the observed differentiation which only in some cases is attributable to geologic events (*see* above). In the same manner, vicariance cannot explain the whole pattern in the land snail *Albinaria* (Douris *et al*. 1998, 2007), and other invertebrates and vertebrates in the Aegean (Dennis *et al*. 2000; Kasapidis *et al*. 2005). Just as in the central-European *Bythinella* (Falniowski 1987, Falniowski *et al*. 1998, 1999, Szarowska 2000) the present data did not confirm either the complete isolation of the Greek populations or their stability and longevity. For inhabitants of such miniature and changeable habitats survival must depend on dynamic processes of colonization and recolonization events coupled with short- or (in some cases) long-distance dispersal.

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Appendix. Mismatch distribution. RI = Raggedness index; p_R = p value of raggedness; Mp = model (SSD).

Stat.	G_1	G_2	G_3	G_4	G_5	G_6	G_7	G_9	G_11	G_12	G_13	G_14
τ	0.0	0.8	2.9	0.0	3.0	5.9	6.1	3.4	0.8	0.8	4.0	0.3
$\tau_{2.5}$	0.0000	0.0000	0.0000	0.0000	0.3535	0.1250	0.1016	0.0000	0.0000	0.0000	0.0000	0.0000
τ_{5}	0.0000	0.1309	0.0000	0.0000	0.3535	0.9199	0.6309	0.3613	0.0000	0.0000	0.3301	0.0000
τ_{95}	0.2051	1.6816	4.1836	0.2832	3.0000	9.8965	9.1582	5.9863	1.6367	1.8066	7.2344	0.9219
$\tau_{97.5}$	0.6289	1.8848	24.9023	0.5703	3.0000	10.8613	10.9512	7.4102	1.7637	2.2090	53.6816	0.9805
θ_{0}	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
$\theta_{0\_2.5}$	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
θ_{0_5}	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
θ_{0_95}	0.0000	0.1441	0.6688	0.0000	0.0000	1.9336	2.6719	2.2377	0.0070	0.0035	1.6418	0.0106
$\theta_{0\_97.5}$	0.0035	0.1441	0.9000	0.0000	0.0018	2.6364	4.3119	3.9481	0.0070	0.0035	4.0500	0.3498
θ_{1}	99999.0000	99999.0000	1.1317	99999.0000	0.2254	5.7599	6.1877	4.8981	99999.0000	99999.0000	2.7480	99999.0000
$\theta_{1\_2.5}$	99869.0000	5.2500	0.0000	99869.0000	0.0000	2.5543	2.8383	1.1659	25.4453	349.0000	0.2327	7.4277
θ_{1_5}	99869.0000	5.2500	0.0000	99869.0000	0.0000	3.5773	3.9246	2.5641	25.4453	349.0000	0.4166	14.1172
θ_{1_95}	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000
$\theta_{1\_97.5}$	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000
SSD	0.3842	0.0031	0.1636	0.3087	0.1153	0.1619	0.1631	0.0210	0.0219	0.0219	0.0291	0.0010
Mp	0.0000	0.7760	0.1400	0.0010	0.0270	0.0340	0.0410	0.5860	0.2850	0.6750	0.6440	0.6340
RI	0.3052	0.1012	0.7004	0.3431	0.6735	0.4779	0.3829	0.0651	0.2500	0.2500	0.1179	0.2928
p_R	0.9660	0.7430	0.1350	0.9510	0.6360	0.0050	0.0320	0.9000	0.3550	0.9360	0.7900	0.3950

	G_15	G_17	G_18	G_19	G_23	G_24	G_25	G_26	G_28	G_29	Mean	SD
τ	0.7	2.9	8.7	0.6	0.0	1.1	3.0	3.0	1.8	3.0	1.81816	2.19289
$\tau_{2.5}$	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.3535	0.3789	0.0000	0.0000	0.0453	0.1114
τ_{5}	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.3535	0.3789	0.4902	0.0000	0.1362	0.2347
τ_{95}	1.5293	87.9297	53.6953	1.3672	0.4141	86.0527	3.0000	3.0000	6.7637	15.4648	10.5245	23.0601
$\tau_{97.7}$	1.8594	87.9297	97.6953	1.4531	0.7266	86.0527	3.0000	3.1914	8.0234	45.5898	15.6678	28.4951
θ_{0}	0.0000	0.9000	0.0033	0.0000	0.0000	0.0000	0.0000	0.0000	1.3254	0.4500	0.0924	0.2949
$\theta_{0\_2.5}$	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
θ_{0_5}	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
θ_{0_95}	0.1266	4.0500	5.5266	0.0932	0.0000	0.0000	0.0000	0.0018	0.9869	0.4500	0.7088	1.3471
$\theta_{0\_97.5}$	0.1266	7.2000	8.1000	0.0932	0.0088	0.0000	0.0018	0.0018	1.5504	1.0125	1.1880	2.1851
θ_{1}	99999.0000	3.6000	7.1277	99999.0000	99999.0000	99999.0000	0.2254	0.3434	3.7744	0.4500	34483.6714	47529.8149
$\theta_{1\_2.5}$	6.2363	1.4302	2.4947	14.6836	99869.0000	429.0000	0.0000	0.0000	0.9516	0.0000	10360.5418	30404.6698
θ_{1_5}	6.7783	2.3416	3.8394	14.6836	99869.0000	429.0000	0.0000	0.0000	2.2659	0.0000	10361.0415	30404.4996
θ_{1_95}	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	75861.3103	42791.5502
$\theta_{1\_97.5}$	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	99999.0000	75861.3103	42791.5502
SSD	0.0188	0.3067	0.0747	0.0111	0.5689	0.0898	0.1153	0.0741	0.0477	0.0176	0.0938	0.1360
Mp	0.2290	0.1000	0.2100	0.2790	0.0010	0.6190	0.0200	0.0650	0.5000	0.2950	0.2125	0.2575
RI	0.2360	0.3580	0.1551	0.2066	0.7867	0.5556	0.6735	0.5572	0.0689	0.4380	0.2757	0.2424
p_R	0.2200	0.2890	0.3210	0.3410	0.9350	0.8920	0.6250	0.4480	0.8500	0.4380	0.4208	0.3619