PONTOBELGRANDIELLA RADOMAN, 1973 (CAENOGASTROPODA: HYDROBIIDAE): A RECENT INVADER OF SUBTERRANEAN WATERS?

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Abstract *The cytochrome oxidase c subunit I (COI) partial sequences of all the nominal species of* Belgrandiella *Wagner, 1927, and* Pontobelgrandiella *Radoman, 1973 described so far from Bulgaria, collected from 16 populations, have been studied. The results rejected the occurrence of any representative of the genus* Belgrandiella *in Bulgaria since all the populations belonged to* Pontobelgrandiella*. 60 sequences have been studied, among them eleven COI haplotypes (haplotype diversity Hd=0.870) have been identified. Divergence level has been small, with nucleotide diversity=0.0078 and 14 variable sites. With an exception of one population, no intrapopulation polymorphism has been found. The haplotypes formed four clades, with low p-distances between them (0.7–1.5%). The clade I, represented by eight populations, all found in the Balkan Mountains, has been characterised by the highest divergence (five haplotypes, p=0.6–1.2%, thus 3–6 point mutations). The clade II, with one haplotype, has been found at two localities distant one from another. The clade III with three haplotypes, and the clade IV with two haplotypes, have been found at small areas. The Principal Component Analysis of seven shell measurements showed some morphological distinctness of the representatives of the distinguished clades, although with some overlapping variability. The lack of polymorphism may reflect a founder effect, bottlenecks during subsequent local extinctions and/or drastic reductions of population size, and may also be a result of strong selection. The genetic distances between the clades: 0.7–1.5%, would indicate the time of divergence from 0.38–0.43 Mya to 0.81–0.92 Mya. Thus the divergence took place in the Pleistocene, more precisely in Calabrian and Middle Pleistocene. It suggests that the adaptation of Pontobelgrandiella to troglobiont and stygobiont habitats was quite recent, and still incomplete. The results seem to follow the "climatic relict" model. The Pleistocene unstable climatic conditions might have promoted the adaptation of this snail to underground waters.*

Key words *Balkans, troglobiont, stygobiont, Pleistocene, phylogeography*

Introduction

The genus *Belgrandiella* Wagner, 1927, with its type species *B. kusceri* (A. J. Wagner, 1914), was described from the Rakek Spring in Slovenia. Many minute truncatelloid gastropods with ovate-conic or conic shells have been assigned to *Belgrandiella*, without examining the anatomy. The range of the genus, given in the literature, spans from Slovenia (Ložek & Brtek, 1964) to Greece (Schütt, 1980). However, in Slovakia there is no *Belgrandiella* but *Alzoniella* (Szarowska, Falniowski & Šteffek, 2011), and no *Belgrandiella* in Greece (Radoman 1983). The genus *Pontobelgrandiella* Radoman, 1973, with its type species *Belgrandiella nitida* Angelov, 1972, was described from a spring at the cave entrance near village Polaten N of Teteven town (Radoman, 1973, 1983). From Bulgaria, many *Belgrandiella* species have been described (Wagner, 1927; Angelov, 1959, 1972, 1976; Pintér, 1968; Hubenov, 2005, 2007; Glöer & Georgiev, 2009; Georgiev, 2011 a, b, c, d; Georgiev, 2012; Georgiev, 2013a). Recently, a second *Pontobelgrandiella* species was described: *P. tanevii* Georgiev, 2013, from the pre-Balkan area of the northern Bulgaria (Georgiev, 2013b). All the Bulgarian *"Belgrandiella"/Pontobelgrandiella* nominal taxa are subterranean: either troglobiont or stygobiont, but there a few possible exceptions. At the surface in spring pools, especially after periods of high flow, numerous empty shells can be found along with the occassional live specimen. Although at a few localities living specimens can be more numerous. This makes the collection of these snails difficult, especially for any study of population genetic structure. This also means that the habitats of these snails, especially the troglobiont ones, have to be delineated. Current phylogeographic studies confirmed

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theoretical assumptions that cave animal taxa are often cryptic and possess highly restricted geographical distributions despite potential gene flow from surface populations (Juan, Guzik, Jaume & Cooper, 2010), which is not the case in the Bulgarian *"Belgrandiella"/Pontobelgrandiella*. Caves are relatively stable, long-lasting environments and individual ones often have an island character with no subterranean connections to any others. In some cases, particular caves can be characterized by endemic taxa with long, independent, evolutionary histories (e.g., Falniowski, Szarowska, Sirbu, Hillebrand & Baciu, 2008) that differ strongly from their sister taxa occurring outside caves. To our knowledge, there is no study on a group of widely (distances more than 200km) geographically distributed, phylogenetically close, subterranean populations, such as the snails presented in this paper.

The aim of the present study is: (i) to test the occurrence of *Belgrandiella* in Bulgaria; (ii) to describe a pattern of metapopulation genetic structure of this group of mostly subterranean gastropods; (iii) to infer the possible origin and history of *Pontobelgrandiella* in Bulgaria. To achieve this, the cytochrome oxidase c subunit I (COI) has been analysed.

Material and Methods

Sample Collection And Fixation

We collected "*Belgrandiella*" snails from 16 localities (including *B. angelovi*, *B. dobrostanica*, *B. pandurskii*, *B. stanimirae*, *B. zagoraensis* nominal species) and *Pontobelgrandiella tanevi*, from one locality from the Bulgaria (Fig. 1 and Table 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 hours. Afterwards, the ethanol was changed twice in 24 hours and, after few days, the 80% ethanol was replaced by 96%. Samples were then stored in -20°C prior to DNA extraction. Individuals for the morphological study were fixed in 4% formaldehyde and then stored in 80% ethanol.

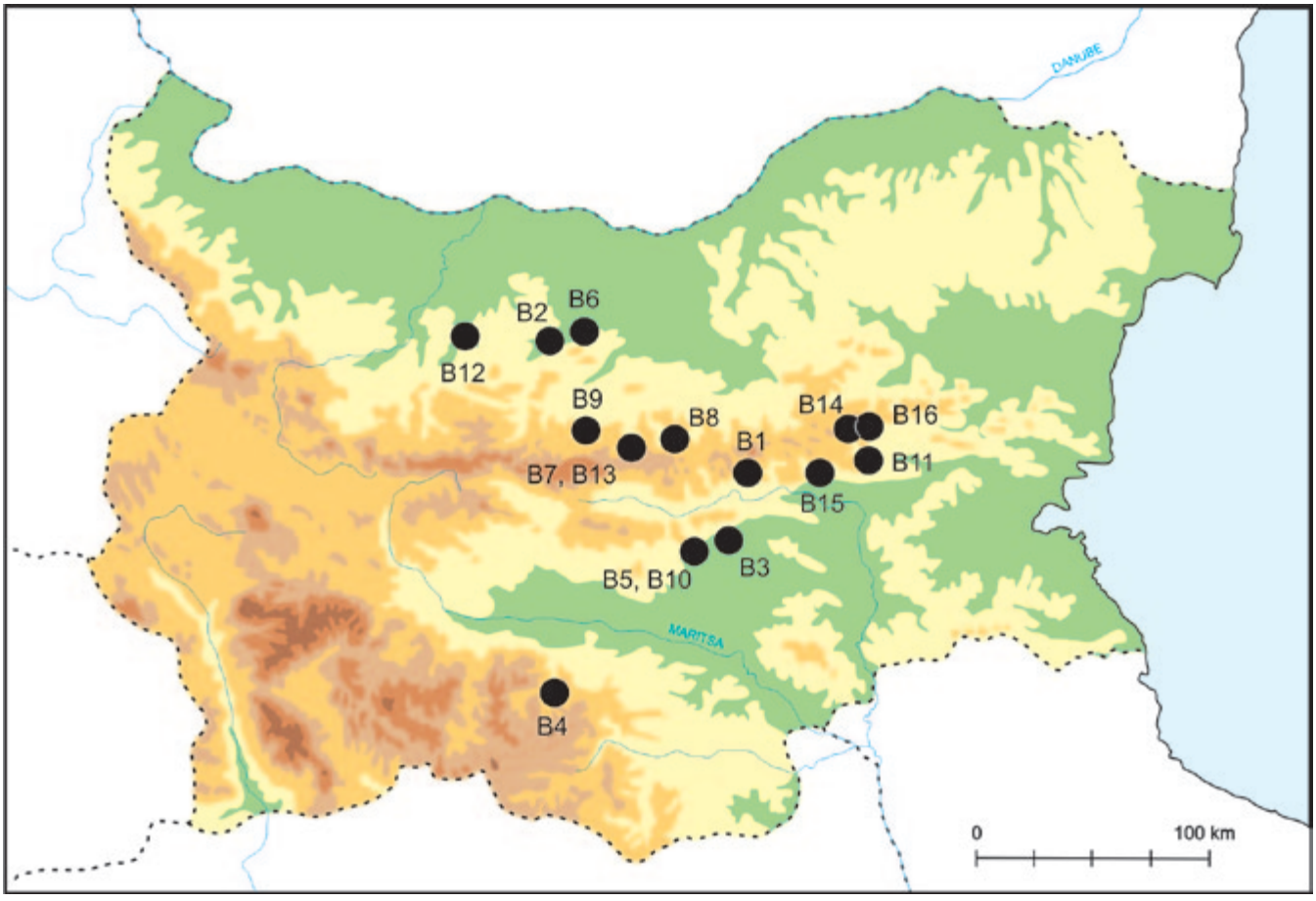

Figure 1 Sampling sites used in the present study. See also Table 1.

Table. 1 The sampling localities with their geographical coordinates, and the haplotypes for COI gene detected in each locality. Compare with Fig. 1.

ID	morphological taxon	Site	Coordinates		COI haplotypes
B1	*B. angelovi*	Bulgaria – Tvarditsa, spring	42°42'20''N	25°53'52''E	H1×6
B2	*B. pandurskii*	Bulgaria – Devetashka cave, entrance of the cave	43°14'03''N	24°53'04''E	H2A, H2B×3
B3	*Belgrandiella sp.*	Bulgaria – Dalboki, water source near the hut of village	42°28'50''N	25°46'14''E	H3×2
B4	*B. dobrostanica*	Bulgaria – Chardaka, water source	41°53'13''N	24°53'07''E	H4×2
B5	*B. zagoraensis*	Bulgaria – Stara Zagora, spring near the Park "Bedechka"	42°26'22''N	25°38'25''E	H3
B6	*B. pandurskii*	Bulgaria – Krushuna, spring near the entrance of Vodopada cave	43°15'05''N	25°02'09''E	H2B×7
B7	*B. angelovi*	Bulgaria – Zeleno Darvo, two springs in a beech forest	42°48'50''N	25°17'16''E	H7×4
B8	*B. stanimirae*	Bulgaria – Tryavna, Zmeyova Dupka cave	42°52'35''N	25°28'35''E	H8×1
B9	*B. angelovi*	Bulgaria – Stokite, stream in a mixed deciduous forest	42°53'32''N	25°04'10''E	H9×5
B10	*B. zagoraensis*	Bulgaria – Stara Zagora, spring near Bedechka River	42°25'53''N	25°39'14''E	H10×1
B11	*B. angelovi*	Bulgaria – Gradets, water source	42°47'53''N	26°33'15''E	H1×2
B12	*Pontobelgrandiella tanevi*	Bulgaria – Bezhanovo, stream at the entrance of Parnitsite cave	43°13'44''N	24°23'19''E	H4×11
B13	*B. angelovi*	Bulgaria – Shipka Pass, water source at the north mountain slope	42°49'02''N	25°19'28''E	H13×2
B14	*Belgrandiella sp.*	Bulgaria – Kotel, spring at river bank	42°53'20''N	26°26'20''E	H14×4
B15	*B. angelovi*	Bulgaria – Stara Planina, Sinite Kamani Nat. Park spring	42°43'43''N	26°18'09''E	H3×5
B16	*Belgrandiella sp.*	Bulgaria – Lednika Cave, Stara Planina Mts.	42°56'04''N	26°30'36''E	H1×3

Morphological Techniques

Snails were photographed with a CANON EOS 50D digital camera, attached to a NIKON SMZ18 stereo-microscope with dark field, measurements were taken with a NIKON DIGITAL SIGHT DS-2 camera measurement system.

Morphometric Techniques

We measured seven shell morphometric parameters (Szarowska, 2006; Falniowski, Szarowska, Glöer & Pešić, 2012) in ten specimens out of each of the nine clades, 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 program 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 minimum spanning tree (performed for clarity, not presented in the figures) to detect local distortions in the data.

DNA Extraction And Sequencing

DNA was extracted from foot tissue using a SHERLOCK extracting kit (A&A Biotechnology) and dissolved in 20 µl of TE buffer. PCR was performed in the reaction mixture of total volume 50 µl using the following primers: LCOI490 (5'- GGTCAACAAATCATAAAGATATTGG-3') (Folmer, Black, Hoeh, Lutz & Vrijenhoek, 1994) and COR722b (5'-TAAACTTCAGGGTGACCAAAAAATYA-3') (Wilke & Davis, 2000) for the COI gene. The PCR conditions were as follows: 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. Ten µl 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.

Molecular Data Analysis

Sequences were aligned in Bioedit 7.1.3.0 (Hall, 1999). 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).

In the phylogenetic analysis three other sequences from GenBank were used as outgroups: *Alzoniella slovenica* (JF742656, Szarowska *et al*., 2011), *Avenionia brevis* (AF367638, Wilke, Davis, Falniowski, Giusti, Bodon & Szarowska, 2001) and *Belgrandiella kusceri* (KT218520, Falniowski & Beran, 2015). The data were analysed using an approach based on Bayesian inference (BI) and maximum likelihood (ML). We applied the GTR +I +Γ model because over-parameterization seems to be less dangerous for BI analyses than under-parameterization (Huelsenbeck & Rannala, 2004). For ML analyses, GTR +I +Γ is the only nucleotide substitution model implemented in RAxML.

The Bayesian analyses were run with MrBayes ver. 3.2.3 (Ronquist, Teslenko, van der Mark, Ayres, Darling, Hohna, Larget, Liu, Suchard & Huelsenbeck, 2012) with default priors. Two simultaneous analyses were performed, each lasting 10,000,000 generations with one cold chain and three heated chains, starting from random trees and sampling trees every 1000 generations. The first 25% of trees were discarded as burn-in. The analyses were 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 randomized stepwise addition maximum parsimony method. The tree with the highest likelihood score was considered as the best representation of the phylogeny. Bootstrap support was calculated with 1000 replicates and summarized onto the best ML tree. RAxML analyses were performed using free computational resource CIPRES Science Gateway (Miller, Pfeiffer & Schwartz, 2010).

To infer haplotype networks of the markers used, a median-joining calculation was implemented in NETWORK 4.6.1.1 (Bandelt, Forster & Röhl, 1999).

Results

In total we obtained 60 sequences of COI gene (552 bp, GenBank Accession numbers KU496965–KU497024). The saturation test by Xia, Xie, Salemi, Chen & Wang (2003) revealed no saturation. Among them, eleven COI haplotypes (haplotype diversity Hd=0.870) were identified (Table 1). All new sequences belonged to one mitochondrial lineage – *Pontobelgrandiella*, which is clearly distinct from *Belgrandiella* haplotypes (p=0.124 for *B. kusceri* from Rakek in Slovenia: Falniowski & Beran, 2015). The divergence level among *Pontobelgrandiella* lineage is small, with nucleotide diversity=0.0078 and 14 variable sites. With the exception of the population at locality B3, with two haplotypes, all other populations have been found to have only one haplotype, thus no infrapopulation polymorphism has been found. The *Pontobelgrandiella* haplotypes formed four clades (Fig. 2, 3), but differences between them are small (0.7–1.5%, Table 2). The geographic distribution of the clades is shown in Fig. 4.

Clade I (Figs 2–3) shows the highest divergence of the sequences (p=0.6%). All populations representing this clade inhabit central-eastern Bulgaria (Fig. 4). Population B8 represents the nominal species *Belgrandiella stanimirae*, B5 and B10 represent *Belgrandiella zagoraensis*, three

Table. 2 *p*-distances between main COI clades of *Pontobelgrandiella*. For details see Fig. 2. Mean distances within clades are also shown (italics).

	clade I	clade II	clade III	clade IV
clade I	*0.006*			
clade II	0.008	*0.003*		
clade III	0.014	0.007	–	
clade IV	0.015	0.007	0.008	*0.002*

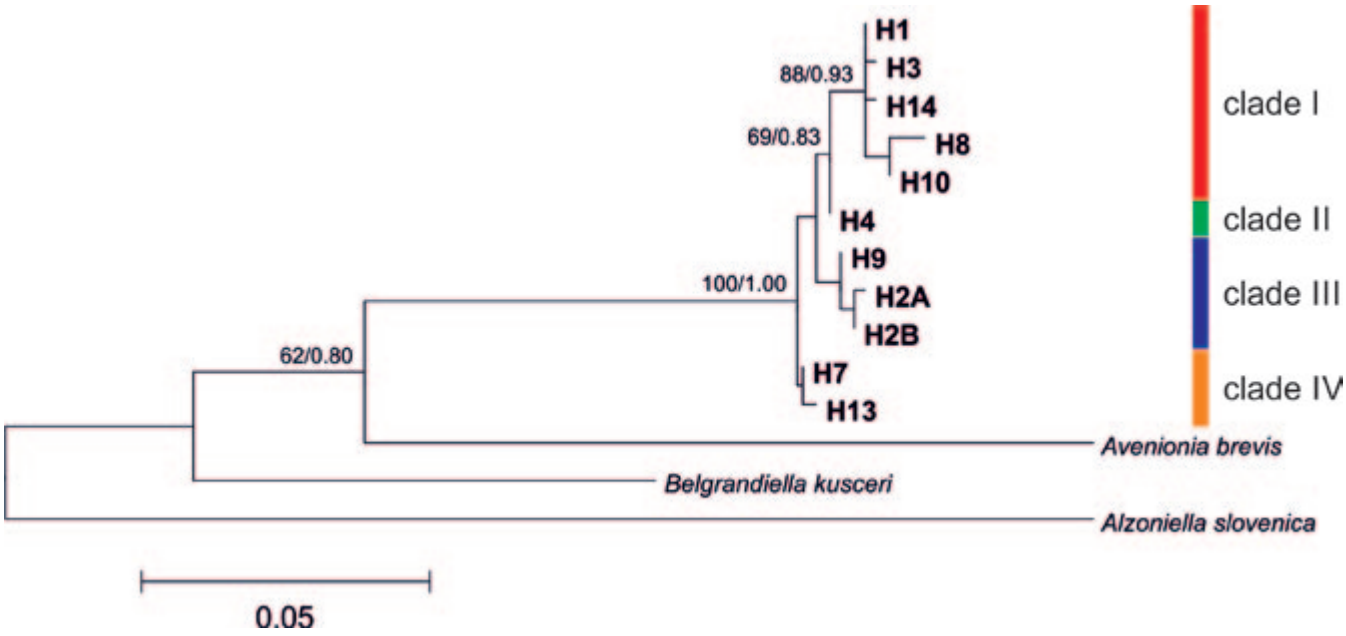

Figure 2 The maximum-likelihood phylogram for COI gene.

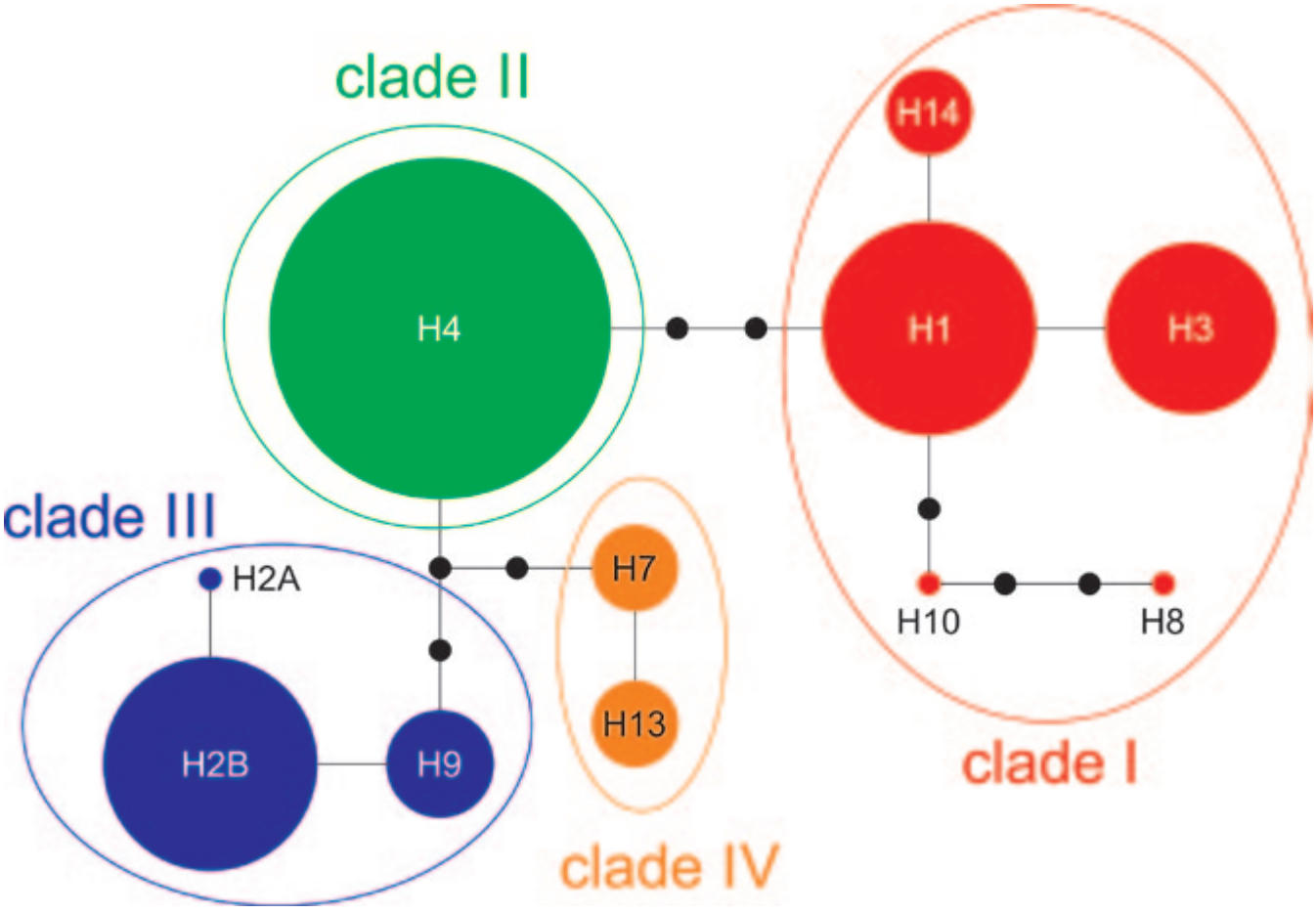

Figure 3 The median-joining haplotype network of COI haplotypes. The shades/colours indicate the specific clades, see Fig. 2.

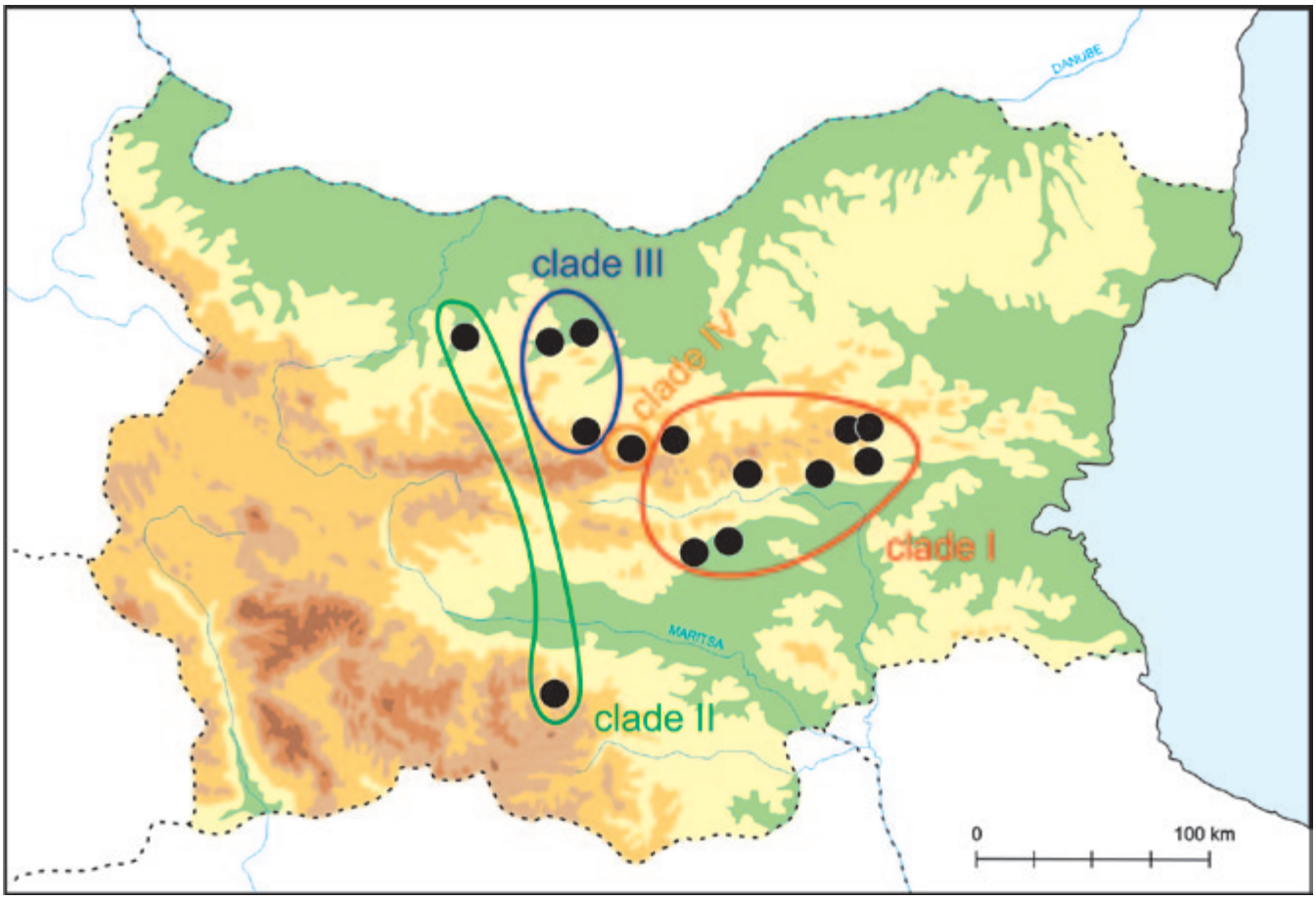

Figure 4 Geographical distribution of COI clades. Compare with Fig. 1.

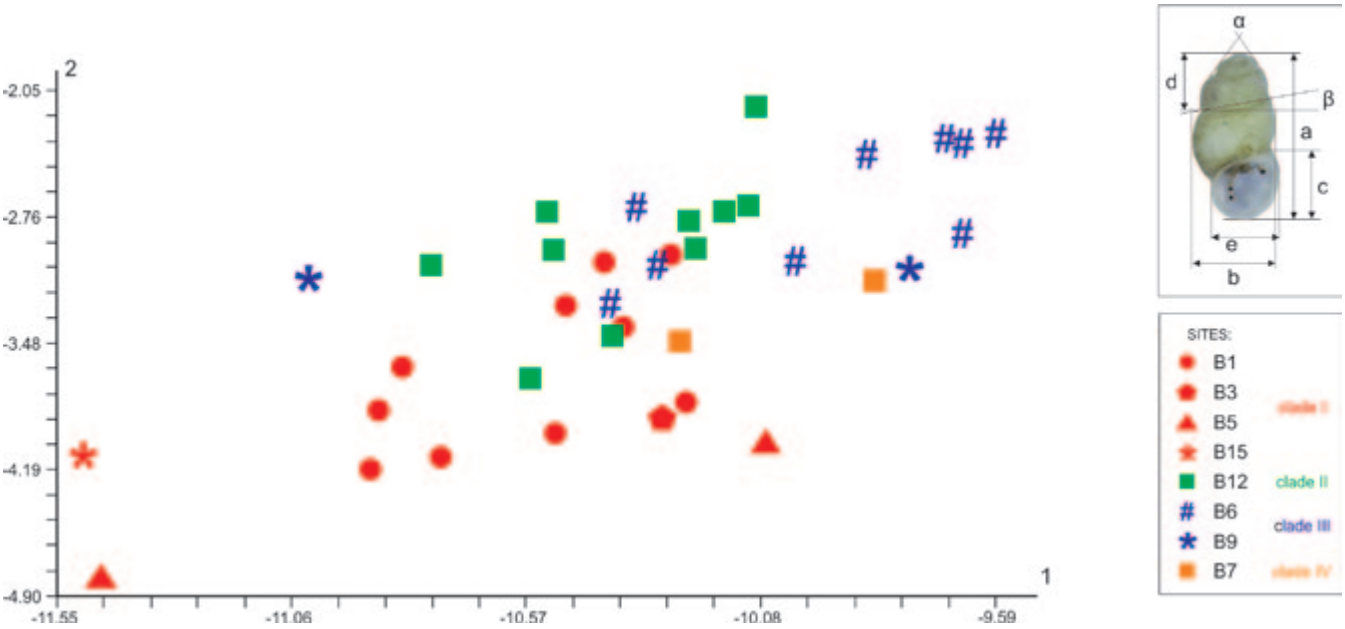

Figure 5 Shell biometry (PCA) of *Pontobelgrandiella* for the four main clades (see COI phylogenetic tree, Fig. 2). Shell measurements were as shown in the inset: a – shell height, b – body whorl breadth, c – aperture height, d – spire height, e – aperture breadth, α – apex angle, β – angle between body whorl suture and horizontal surface.

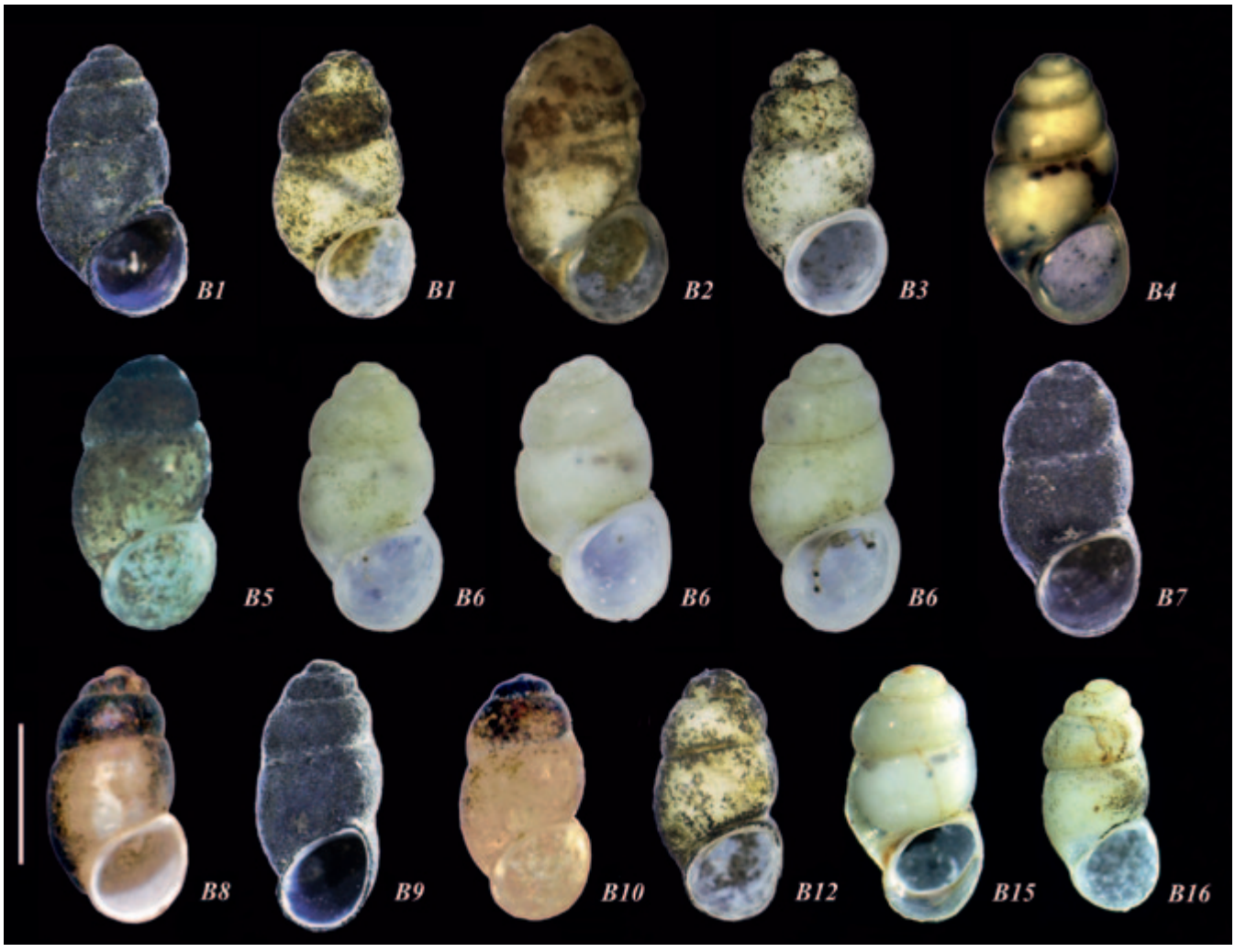

Figure 6 Shells of *Pontobelgrandiella*; scale bar equals 1mm.

populations of *B. angelovi* and two *Belgrandiella* sp. also belong to this clade. The locality B8 and the other populations belonging to clade I, are characterized by relatively high genetic distance (*p*-distance: 0.6–1.2%, respectively, thus 3–6 point mutations). PCA on the seven shell characters (Fig. 5) also shows that morphological variability is the highest in clade I.

Clade II is represented by single haplotype (Figs 2–3), found in two populations: B4 and B12 (Fig. 4), separated by a long distance, meridionally. The shells within this clade are characterized by wide variability, partly overlapping clade I (Fig. 4). The representatives of the other two clades have been found close to each other, in the northern part of the central Bulgaria (Fig. 4).

Clade III, represented by three haplotypes (Figs 2–4 ; p=0.3%) has been found at three localities (B2, B6, B9), and clade IV, with two haplotypes (p=0.2%), at two localities (B7 and B13). Shell variability within clade III is also wide, but nearly not overlapping with clade I (Fig. 6).

Discussion

The results undoubtedly reject the occurrence of representatives of the genus *Belgrandiella* in Bulgaria. This confirms the opinion of Radoman (1985), but contradicts the allegations of Schütt (1980) who expanded the range of *Belgrandiella* as far as to Greece, and including in it several tiny representatives of various families such as *Litthabitella* or *Grossuana*. All the Bulgarian populations studied here belong to the genus *Pontobelgrandiella*, and, which should be stressed, they cover all the known range of the genus.

In general, the nominal morphospecies of "*Belgrandiella*" molecularly do not form distinct clades, since sequences of "*B. angelovi*" belonged to three clades (I, III and the most distinct clade

IV). It is noteworthy that "*B". pandurskii* has been found in caves, springs and streams at the surface, thus its genetic variability may be accompanied by the suite of inhabited habitats. The "*B. pandurskii*" formed a subclade with one of "*B. angelovi*" within clade III. The "*B. dobrostanica"* was identical to *Pontobengrandiella* sp. and both formed clade II. The "*Belgrandiella stanimirae"* and *"B. zagoraensis"* belonged to clade I, with "*B. angelovi*", and two "*Belgrandiella"* sp. populations. Interestingly, "*B. stanimirae"* (from the cave) is distant from the rest of the clade I with *p*-distance 0.006–0.012.

As already pointed out in the introduction, *Pontobelgrandiella* is an inhabitant of subterranean waters, either stygobiont or troglobiont, thus at the surface (usually in springs) available populations are formed by single or a few living specimens, only empty shell may be numerous. At a few localities some more living specimens were found at the surface, although it seems dubious that they survive or could reproduce there. This makes it difficult to study the genetic structure of those populations, as is the case of other inhabitants of caves and subterranean waters as reflected in the sparse literature (Falniowski *et al*., 2008, Juan *et al*., 2010: references therein). For cavernicolous gastropods the following studies are representative; Kano & Kase (2004) (neritopsid *Neritilia cavernicola* from Philippines), Schilthuizen, Cabanban & Haase (2006) (caenogastropod representative of Hydrocenidae *Georissa* from Borneo), and Bichain *et al*., 2007 a, b (*Bythinella* from France).

With the exception of one population, there was no intrapopulation polymorphism. This, in part, could be explained by low numbers of sequenced specimens per population, as a consequence of low numbers of collected animals. On the other hand, in some cases there were six to eleven sequences in single population, but there was also no polymorphism. Low polymorphism or no polymorphism at all is characteristic of troglobiont populations (Juan *et al*., 2010). The lack of polymorphism may reflect a "founder effect" resulting from the initial colonization by a few specimens. Alternatively a "bottleneck" during subsequent local extinctions and/or drastic reductions of population size may result in low polymorphism. Strong selection pressures may also result in low polymorphism.

The genetic *p*-distances between the clades: 0.7–1.5%, applying the calibration of the molecular clock given by Wilke (2003) and Hershler & Liu (2008), would indicate the time of divergence from 0.38–0.43 Mya, respectively, to 0.81–0.92 Mya. Thus the divergence took place in the Pleistocene, more precisely in the Calabrian and Middle Pleistocene. Subsequent interglacials and glacials, the latter forming local glaciers in Bulgaria (Zagorchev, 2007) obviously resulted in formation of numerous barriers, promoting allopatric differentiation. Anyway, the history of the studied populations may not be long. The observed diversity within clade I, with relatively high haplotype diversity coupled with low nucleotide diversity, is in accordance with the model of rapid population growth from the ancestral population characterized by low evolutionary effective size (Avise, 2000). There was enough time to bring back haplotype diversity through mutations, but too less to accumulate the mutations to restore higher distances between the haplotypes. Such interpretation seems justified in case of those subterranean gastropods, whose populations probably are established by a few immigrants. Similar remarks could concern clade III.

The representatives of clade II have been found at two localities only, nearly 200km distant one from another, between this localities, *Pontobelgrandiella* have not been found. Perhaps the sampling has not been dense enough. Despite such high distance, between the two localities the haplotype is the same.

The general pattern of the genotypic divergence in *Pontobelgrandiella* indicates a short lifetime for all the populations, and their presence over a relatively big area suggests the ability of the snail to passively invade new localities. This ability seems rather unexpected for a representative of a subterranean fauna. The route of expansion to new localities, and forming new populations, remains enigmatic in *Pontobelgrandiella*. The few observed cases of rather numerous living specimens at the surface may reflect the ability of *Pontobelgrandiella* to survive some time at the surface. It has to be noted that in case of troglobionts inhabiting caves one could expect stabile, long lasting habitats, but in case of stygobionts inhabiting ground and interstitial waters rather drastic instability may be typical. The latter may substantially influence the pattern

of differentiation. We cannot specify the factors that preclude inhabiting surface waters by *Pontobelgrandiella*. We could only speculate that there might be: high temperature, solar radiation, competition with the other spring inhabitants, or predation. It could be supposed that, during glaciations, all the above factors had a lower effect on *Pontobelgrandiella* than in interglacials, which thus promoted expansion of *Pontobelgrandiella* during glaciations. The low genetic divergence may also indicate quite recent adaptation of *Pontobelgrandiella* to troglobiont and stygobiont habitats. The Calabrian and Middle Pleistocene may have been times of adaptation of this snail to underground waters, which may be promoted by the Pleistocene unstable climatic conditions. Perhaps the surface ancestral population, before its shift to the underground waters, during the more and more severe conditions on the surface, was drastically bottlenecked; the shift to the underground conditions was also, possibly, accompanied by selection. However, it seems that the subterranean populations were formed by one or only a few haplotypes, and that later mutations restored slight polymorphism.

Two alternative models of the speciation of the cave fauna have been proposed: "climatic relict" and "adaptive shift" (Howarth, 1973; Holsinger, 2000; Rivera, Howarth, Taiti & Roderick, 2002). The first of them, proposed for continental ecosystems of the temperate zone (Holsinger, 1988, 2000; Peck & Finston, 1993) assumes that invasion of subterranean habitats, with the gene flow from the surface populations still present after invasion, is followed by later speciation of the subterranean populations in strict allopatry, after extinction of the surface populations caused by the climatic shifts, like glaciations or aridity. This model most probably holds for *Pontobelgrandiella*, although the process seems unfinished.

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References

Angelov A 1959 Neue Gastropoden aus den unterirdischen Gewässern Bulgariens. *Archiv für Molluskenkunde* **88**: 51–54.

Angelov A 1972 Neue Hydrobiidae aus Höhlengewässern Bulgariens. *Archiv für Molluskenkunde*, **102**: 107–112.

Angelov A 1976 Ein neuer Vertreter der Gattung *Belgrandiella* A. Wagner, 1927 (Gastropoda, Hydrobiidae) von Grundwassern Bulgariens. *Acta Zoologica Bulgarica* **4**: 78–80.

Avise JC 2000 *Phylogeography. The history and formation of species* Harvard University Press, Cambridge, MA and London, 447 pp.

Bandelt Hj, Forster P & Röhl A 1999 Median–joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37–48.

Bichain JM, Gaubert P, Samadi S & Boisselier-Dubayle MC 2007a A gleam in the dark: Phylogenetic species delimitation in the confusing spring-snail genus *Bythinella* Moquin-Tandon, 1856 (Gastropoda: Rissooidea: Amnicolidae). *Molecular Phylogenetics and Evolution* **45**: 927–941.

Bichain JM, Boisselier-Dubayle MC, Bouchet P & Samadi S 2007b Species delimitation in the genus *Bythinella* (Mollusca: Caenogastropoda: Rissooidea): A first attempt combining molecular and morphoetrical data. *Malacologia* **49**: 291–311.

Falniowski A 2003 *Metody numeryczne w taksonomii [Numerical techniques in taxonomy]* Wydawnictwo Uniwersytetu Jagiellońskiego, Kraków.

Falniowski A & Beran L 2015 *Belgrandiella* A. J. Wagner, 1928 (Caenogastropoda: Truncatelloidea: Hydrobiidae): how many endemics? *Folia Malacologica* **23**: 187–191.

Falniowski A, Szarowska M, Glöer P & Pešić V 2012 Molecules vs morphology in the taxonomy of the *Radomaniola/Grossuana* group of Balkan Rissoiidea (Mollusca: Caenogastropoda). *Journal of Conchology* **41**: 19–36.

Falniowski A, Szarowska M, Sirbu I, Hillebrand A & Baciu M 2008 *Heleobia dobrogica* (Grossu & Negrea, 1989)(Gastropoda: Rissooidea: Cochliopidae) and the estimated time of its isolation in a continental analogue of hydrothermal vents. *Molluscan Research* **28**: 165–170.

Folmer O, Black M, Hoeh W, Lutz R & Vrijenhoek RC 1994 DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294–299.

Georgiev D 2011a Check list of the Bulgarian minor freshwater snails (Gastropoda: Risooidea) with some ecological and zoogeographical notes. *Zoonotes*, **24**: 1–4.

Georgiev D 2011b. A New Species of *Belgrandiella* (Wagner 1927) (Mollusca: Gastropoda) from Caves in Northern Bulgaria. *Acta Zoologica Bulgarica* **63**: 7–10.

Georgiev D 2011c New species of snails (Mollusca: Gastropoda: Risooidea) from cave waters of Bulgaria. *Buletin Shkenkor Ser Shkenkat Natyrore* **61**: 83–96.

Georgiev D 2011d New localities of four Bulgarian Hydrobiidae species (Gastropoda: Risooidea). *Zoonotes* **24**: 1–4.

GEORGIEV D 2012 New species of stygobiotic *Belgrandiella* from north Bulgaria (Gastropoda: Hydrobiidae). *Biologica Nyssana* **3**: 105–107.

GEORGIEV D 2013a The Bulgarian Endemic *Belgrandiella angelovi* Pintér 1968 (Gastropoda: Risooidea): Taxonomical Features, Ecology and Distribution. *Acta Zoologica Bulgarica*, **65** (2): 265–269.

GEORGIEV D 2013b Catalogue of the stygobiotic and troglophilous freshwater snails (Gastropoda: Rissooidea: Hydrobiidae) of Bulgaria with descriptions of five new species. *Ruthenica* **23**: 59–67.

GLÖER P & GEORGIEV D 2009 New Rissooidea from Bulgaria (Gastropoda: Rissooidea). *Mollusca* **27**: 123–136.

HALL TA 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NY. *Nucleic Acids Symposium Series* **41**: 95–98.

HERSHLER R & LIU H–P 2008 Ancient vicariance and recent dispersal of springsnails (Hydrobiidae: *Pyrgulopsis*) in the Death Valley system, California-Nevada. *The Geological Society of America Special Paper* **439**: 91–101.

HOLSINGER JR 1988 Troglobites: the evolution of cave-dwelling organisms. *American Scientist* **76**: 146–153.

HOLSINGER JR 2000 Ecological derivation, colonisation and speciation. *In* H. Wilkens, D. Culver & W. Humphreys (eds) *Subterranean Ecosystems*: 399–415 Elsevier, Amsterdam.

HOWARTH FG 1973 The cavernicolous fauna of Hawaiian lava tubes. I. Introduction. *Pacific Insects* **15**: 139–151.

HUBENOV Z 2005 Malacofaunistic diversity of Bulgaria. In: Current state of Bulgarian biodiversity – problems and perspectives *In* A. Petrova (ed.) *Bulgarian Bioplatform*, Sofia, 199–246. (in Bulgarian).

HUBENOV Z 2007 Fauna and zoogeography of marine, freshwater, and terrestrial mollusks (Mollusca) in Bulgaria. *In* V. Fet, A. Popov (eds.) *Biogeography and ecology of Bulgaria*. Springer, Dodrecht, 141–198.

HUELSENBECK JP & RANNALA B 2004 Frequent properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. *Systematic Biology* **53**: 904–913.

JUAN C, GUZIK MT, JAUME D & COOPER SJB 2010 Evolution in caves: Darwin's 'wrecks of ancient life' in the molecular era. *Molecular Ecology* **19**: 3865–3880.

KANO Y & KASE T 2004 Genetic exchange between anchialine cave populations by means of larval dispersal: the case of a new gastropod species *Neritilia cavernicola. Zoologica Scripta* **33**: 423–437.

LIBRADO P & ROZAS J 2009 DnaSP v5 a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451–1452.

LOŽEK V & BRTEK J 1964 Neue *Belgrandiella* aus den Westkarpaten. *Archiv für Molluskenkunde* **93**: 201–207.

MILLER MA, PFEIFFER W & SCHWARTZ T 2010 Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov., New Orleans, LA pp 1–8.

PECK SB & FINSTON TL 1993 Galapagos islands troglobites: the questions of tropical troglobites, parapatric distributions with eyed-sister-species, and their origin by parapatric speciation. *Memoires de Biospieliologie* **20**: 19–37.

PINTÉR L 1968 Eine neue Wasserschnecke aus Bulgarien. *Archiv für Molluskenkunde* **98**: 61–63.

RADOMAN P 1973 New classification of fresh and brackish water Prosobranchia from the Balkans and Asia Minor. *Posebna Izdanja Prirodnjacki muzej u Beogradu* **32**: 1–30.

RADOMAN P 1983 *Hydrobioidea a superfamily of prosobranchia (Gastropoda). I. Systematics.* Monographs Serbian Academy of Sciences and Arts 547, Department Sciences **57**: 1–256.

RADOMAN P 1985 *Hydrobioidea, a superfamily of prosobranchia (Gastropoda). II. Origin, zoogeography, evolution in the Balkans and Asia Minor.* Faculty of Science, Department of Biology Monographs, Institute of Zoology Beograd **1**: 1–173.

RIVERA MAJ, HOWARTH FG, TAITI S & RODERICK GK 2002 Evolution in Hawaiian cave isopods. (Oniscidea: Philosciidae): vicariant speciation or adaptive shifts? *Molecular Phylogenetics and Evolution* **25**: 1–9.

ROHLF FJ 1998 *NTSYSpc, Numerical Taxonomy and Multivariate Analysis System. Version 2.0.* [Computer software and manual] Exeter Software, Seatuket, NY.

RONQUIST F, TESLENKO M, VAN DER MARK P, AYRES D, DARLING A, HOHNA S, LARGET B, LIU L, SUCHARD MA & HUELSENBECK JP 2012 MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**: 539–542.

SCHILTHUIZEN M, CABANBAN AS & HAASE M 2005 Possible speciation with gene flow in tropical. cave snails. *Journal of Zoological and Evolutionary Research* **43**: 133–138.

SCHÜTT H 1980 Zur Kenntnis griechischer Hydrobiiden. *Archüv für Molluskenkunde* **110**: 115–149.

STAMATAKIS A 2014 RAxML Version **8**: A tool for Phylogenetic Analysis and Post- Analysis of Large Phylogenies. *Bioinformatics* **30**: 1312–1313.

SZAROWSKA M 2006 Molecular phylogeny, systematics and morphological character evolution in the Balkan Rissooidea (Caenogastropoda). *Folia Malacologica* **14**: 99–168.

SZAROWSKA M, FALNIOWSKI A & ŠTEFFEK J 2011 Phylogenetic relationships of *Alzoniella slovenica* (Ložek & Brtek, 1964) (Caenogastropoda: Hydrobiidae). *Folia Malacologica* **19**: 87–95.

WAGNER A 1927 Studien zur Molluskenfauna der Balkanhalbinsel mit besonderer Berücksichtigung Bulgariens und Thraziens, nebst monographischer Bearbeitung einzelner Gruppen. *Annales Zoologici Muzei Polonici Historiae Naturalis* **6**: 263–399.

WILKE T 2003 *Salenthydrobia* gen. nov. (Rissooidea: Hydrobiidae): a potential relict of the Messinian

Salinity Crisis. *Zoological Journal of the Linnean Society* **137**: 319–336.

Wilke T & Davis GM 2000 Infraspecific mitochondrial sequence diversity in *Hydrobia ulvae* and *Hydrobia ventrosa* (Hydrobiidae: Rissoacea: Gastropoda): Do their different life histories affect biogeographic patterns and gene flow? *Biological Journal of the Linnean Society* **70**: 89–105.

Wilke T, Davis GM, Falniowski A, Giusti F, Bodon M & Szarowska M 2001 Molecular systematics of Hydrobiidae (Mollusca: Gastropoda: Rissooidea): testing monophyly and phylogenetic relationships *Proceedings of the Academy of Natural Sciences of Philadelphia* **151**: 1–21.

Xia X 2013 DAMBE: A comprehensive software package for data analysis in molecular biology and evolution. *Molecular Biology and Evolution* **30**: 1720–1728.

Xia X, Xie Z, Salemi M, Chen L & Wang Y 2003 An index of substitution saturation and its application. *Molecular Phylogenetics and Evolution* **26**: 1–7.

Zagorchev I 2007 Late Cenozoic development of the Strouma and Mesta fluviolacustrine systems, SW Bulgaria and northern Greece. *Quaternary Science Reviews* **26**: 2783–2800.