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BYTHINELLA HANSBOETERSI GLÖER ET PEŠIĆ, 2006 (GASTROPODA: RISSOOIDEA) IN BULGARIA: ITS MORPHOLOGY, MOLECULAR DISTINCTNESS, AND PHYLOGEOGRAPHY

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ABSTRACT: For five populations of *Bythinella* from Bulgaria the shell, penis, and female reproductive organs are figured and briefly described. 27 sequences of COI (mtDNA), and 14 of ITS-1 (rRNA) are used to infer phylogenetic relationships among the studied populations and five Central European *Bythinella* species. All five studied populations belong to the same species: *B. hansboetersi* Glöer et Pešić, 2006, which is morphologically and molecularly distinct from *B. austriaca* earlier reported from Bulgaria. NCA analysis for the COI data revealed a pattern of restricted gene flow with isolation by distance.

KEY WORDS: freshwater snails, *Bythinella*, Bulgaria, morphology, DNA, phylogeography

INTRODUCTION

Data on the freshwater Rissooidea in Bulgaria are rather scarce. Among others, this concerns the genus *Bythinella* (WOHLBEREDT 1911, WAGNER 1927, JAECKEL et al. 1957, RADOMAN 1976, 1983, ANGELOV 2000, BANK 2004, GLÖER & PEŠIĆ 2006, HUBENOV 2007, SZAROWSKA et al. 2007, GEORGIEV & STOYCHEVA 2008). Much more is known on *Bythinella* in Central, South and West Europe (RADOMAN 1976, 1983, JUNGBLUTH & BOETERS 1977, GIUSTI & PEZZOLI 1977, 1980, FALNIOWSKI 1987, 1992, SZAROWSKA 1996, 2000, BOETERS 1998, FALNIOWSKI et al. 1998, 1999, BERNASCONI 2000, MAZAN 2000, MAZAN & SZAROWSKA 2000a, b, GLÖER 2002, SZAROWSKA & WILKE 2004). However, the systematic position of the genus and species discrimination within it have been somewhat less enigmatic since only recently (WILKE et al. 2001, SZAROWSKA & WILKE 2004, SZAROWSKA 2006, BICHAIN et al. 2007), the pattern of *Bythinella* speciation still remaining unclear (FALNIOWSKI & SZAROWSKA 2009).

This study includes five populations of *Bythinella* from Bulgaria. The morphological characters of the shell, penis and female reproductive organs, which are necessary to identify the studied taxon, are presented together with molecular data (mtCOI, rITS-1). Our aim was to answer the following questions: 1. how can the species be identified?; 2. is this only one or more than one species?; 3. is the species *B. austriaca* (Frauenfeld, 1857), usually reported from Bulgaria, or another one, and if so, what are its relationships with *B. austriaca* and some other Central European *Bythinella*?; 4. what is its pattern of interpopulation differentiation like?

MATERIAL AND METHODS

In 2005 *Bythinella* was collected at five localities in Bulgaria (Fig. 1): 1. Anton town, 1,036 m a.s.l., spring below Bolovan hill; 6.7.2005, 42°44'48.0"N, 24°16'50.7"E; 2. Anton town, 1,850 m a.s.l., spring below the top of Bolovan hill; 8.7.2005; 42°46'15.5"N, 24°16'11.4"E; 3. Smoljan town, 1,490 m a.s.l., small brook below Smoljanske Lake hotel; 1.7.2005; 41°37'01.0N", 24°40'31.3"E; 4. Smoljan town, small brook near Amzovo; 1.7.2005; 41°33'42"N, 24°41'41"E; 5. Mugla village, 1,730 m a.s.l.; base-rich spring fen; 1.7.2005; 41°37'43.0"N, 24°31'08"E. The snails were collected by hand and with a small sieve, and fixed with 80% ethanol.

MORPHOLOGICAL TECHNIQUES

Dissections were done using a NIKON SMZ-U stereo-microscope with a NIKON drawing apparatus, and a NIKON COOLPIX 4500 digital camera. Shells and reproductive organs were photographed with the same equipment.

MOLECULAR TECHNIQUES

Snails were hydrated in TE buffer (3 × 10 min.) and their DNA was extracted with the SHERLOCK extracting kit (A&A Biotechnology), the final product was dissolved in 20 µl of TE buffer. The PCR reaction (PALUMBI 1996) was performed 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 the ITS-1 (BICHAIN et al. 2007). The PCR conditions were as follows: COI – initial denaturation step of 4 min at 94°C, followed by 35 cycles at 94°C for of 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. 10 µl of the PCR product was run on 1% agarose gel to check the quality of the PCR products.

Fig. 1. Localities of the studied populations of *Bythinella*; for the localities' numbers see the text

The PCR product was purified using Clean-Up columns (A&A Biotechnology). The purified PCR product was sequenced in both directions (HILLIS et al. 1996) using BigDye Terminator v3.1 (Applied Biosystems), following the manufacturer's protocol and with the primers described above. The sequencing reaction products were purified using ExTerminator Columns (A&A Biotechnology), and the sequences were read using the ABI Prism sequencer.

DATA ANALYSIS

The COI sequences were aligned by eye, using BioEdit 5.0.0 (HALL 1999) and edited with MACCLADE 4.05 (MADDISON & MADDISON 2002). For ITS-1 an initial alignment was performed using the CLUSTALX 1.82 (THOMPSON et al. 1997). Variable fragments that could not be aligned unambiguously were then removed with MACCLADE.

Despite all the criticism concerning the maximum likelihood techniques of phylogeny reconstruction (NEI 1987, 1996, GAUT & LEWIS 1995, YANG et al. 1995, SWOFFORD et al. 1996, YANG 1997, NEI et al. 1998, NEI & KUMAR 2000, TAKAHASHI & NEI 2000, FALNIOWSKI 2003), most of it applicable to the other techniques as well, we decided to use the ML approach, as implemented in PAUP*4.0b10 (SWOFFORD 2002), together with Modeltest (POSADA & CRANDALL 1998, POSADA 2003), to find the appropriate model of evolution, with the Akaike Information Criterion (POSADA & BUCKLEY 2004). The robustness of nodes was evaluated with bootstrap (10,000 replicates). In the phylogeny reconstruction for COI, five central European *Bythinella* species (Table 1) were used as outgroups.

Kimura's K2P distances (KIMURA 1980), as well as mean genetic diversities within and between populations were computed with the MEGA4 (TAMURA et al. 2007). Haplotype diversity Hd and nucleotide diversity π were computed with DNASP (ROZAS et al. 2003). To estimate gene flow between the populations, F_{st} of HUDSON et al. (1992), N_{st} of LYNCH & CREASE (1990), Δ_{st} and Γ_{st} and G_{st} of NEI (1982) were calculated with DNASP.

Haplotype network was inferred for COI with the TCS 1.21 (CLEMENT et al. 2000), with the connection limit excluding homoplastic changes set to 95%. There is no reliable technique of analysis of phylogeographic data (KNOWLES & MADDISON 2002). Nested clade analysis (NCA) was intended by TEMPLETON (1998) to separate population structure from population history. The technique lacks statistical inference (KNOWLES & MADDISON 2002), not always finds the correct, realistic interpretation (e.g. PANCHAL & BEAUMONT 2007, PETIT 2007, 2008, GARRICK et al. 2008) although some of the criticism is not necessarily justified (TEMPLETON 2008). Thus the NCA approach (TEMPLETON et al. 1987, 1992, TEMPLETON & SING 1993) was inferred with the ANeCA (automation of nested clade phylogeographic analysis, PANCHAL 2007). The programme implements the construction of haplotype networks (with the TCS), the nesting of clades, the calculation of summary statistics with a test of siginificance applying GeoDis 2.5 (POSADA et al. 2000), and also the interpretation of results with an inference key (TEMPLETON 2004).

Table 1. GenBank Accession Numbers and references of COI sequences of species used as outgroup

Species	GenBankAN	References
Bythinella austriaca (Frauenfeld, 1857)	FJ545132	FALNIOWSKI et al. (in press)
Bythinella compressa (Frauenfeld, 1857)	AF367653	SZAROWSKA and WILKE (2004)
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)

RESULTS

MORPHOLOGY

The shells of *B. hansboetersi* (Fig. 2) were similar to the ones figured by GLÖER & PEŠIĆ (2006), although their variability was wider. The penis and flagellum (Fig. 3) also resembled the one figured by GLÖER & PEŠIĆ (2006). The female reproductive organs (Fig. 4) were typical of *Bythinella*, with a J-shaped, moderately big and bulky bursa copulatrix with a long duct usually well demarcated from the bursa, and small to moderately big receptaculum seminis (Fig. 5).

MOLECULAR PHYLOGENY

Twenty seven sequences of COI, 546bp long (GenBank Aceesion numbers: GQ152518-GQ152 544), and 14 of ITS-1, 303bp long (GenBank Aceesion numbers: GQ152545-GQ152558), were used for phylogenetic inferrence.

For the COI the Akaike Information Criterion (AIC) with ModelTest found model TVM+I+G, with base frequencies: A = 0.2976, C = 0.2165, G = 0.1702, T = 0.3158; substitution rate matrix: [A-C] = 6983.3535, [A-G] = 60500.1562, [A-T] = 8945.5127, [C-G] = 2052.4856, [C-T] = 60500.1562, [G-T] =

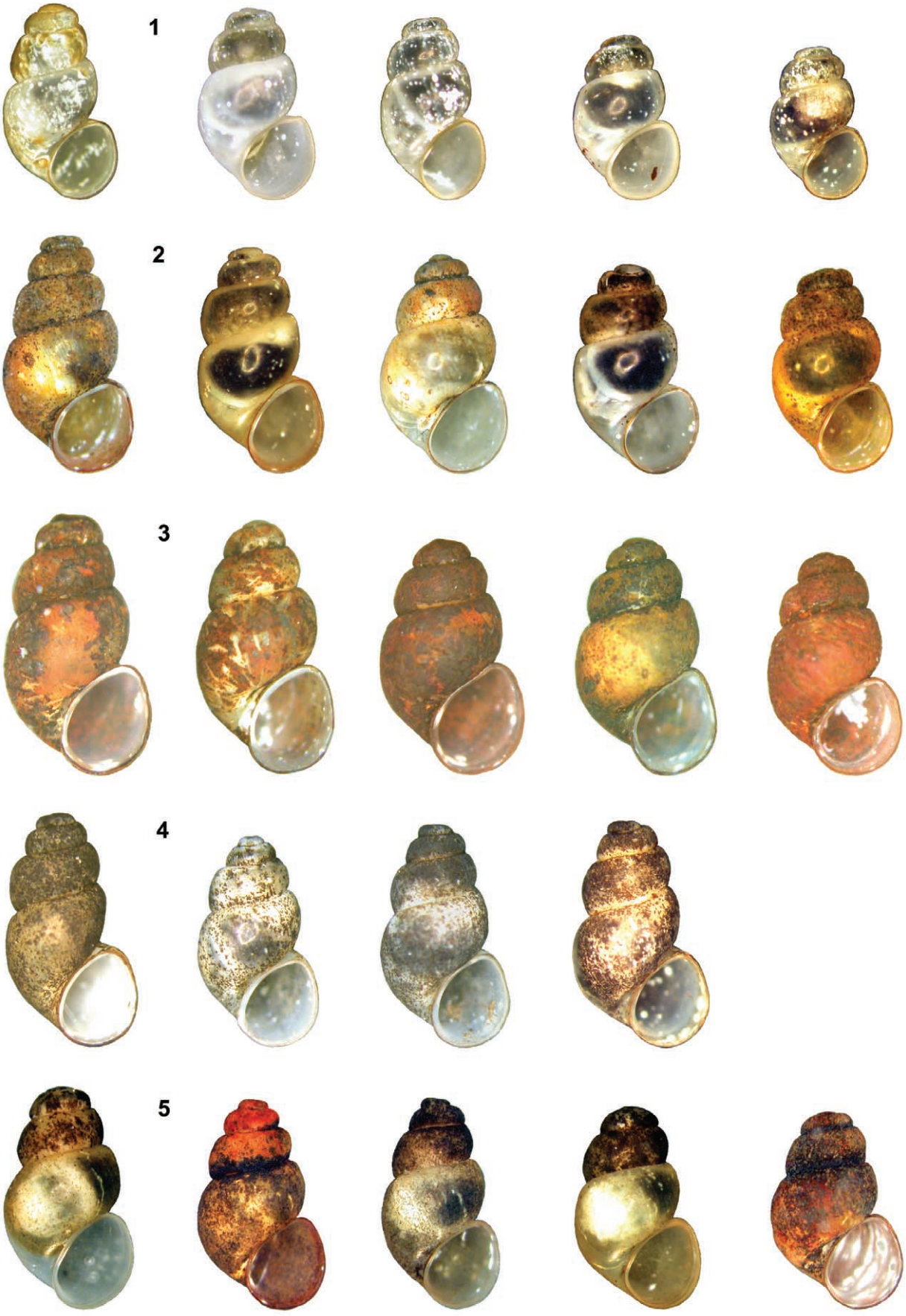

Fig. 2. Shells of *Bythinella hansboetersi*, numbers correspond to localities

1.0000, proportion of invariable sites: (I) = 0.5516, and Ă distribution with the shape parameter = 0.7776.

For the ITS-1 the Akaike Information Criterion (AIC) with ModelTest found model TrNef+I, with equal base frequencies; substitution rate matrix: [A-C] = 1.0000, [A-G] = 2.8961, [A-T] = 1.0000, [C-G] = 1.0000, [C-T] = 0.8079, [G-T] = 1.0000, proportion of invariable sites: (I) = 0.8697, and equal rates for all sites.

The inferred trees (Fig. 6) show similar topologies for the two sequence fragments. Population 1 was close to 2, and 3 to 4. In ITS-1, populations 3 with 4 were most distinct from the other populations. In COI all the populations of *B. hansboetersi* were close to each other, and formed a monophyletic group (bootstrap support 93), clearly different from e.g. *B. austriaca*.

The mean value of the K2P distance for all the COI haplotypes of *B. hansboetersi* equalled 0.008, the maximum reached 0.017. The mean value of the same distance between the other five species of *Bythinella* considered in the present analysis was 0.100, the values of the distance ranging from 0.036 (between *B. robiciana* and *B. schmidti*) to 0.126 (between *B. compressa* and *B. schmidti*).

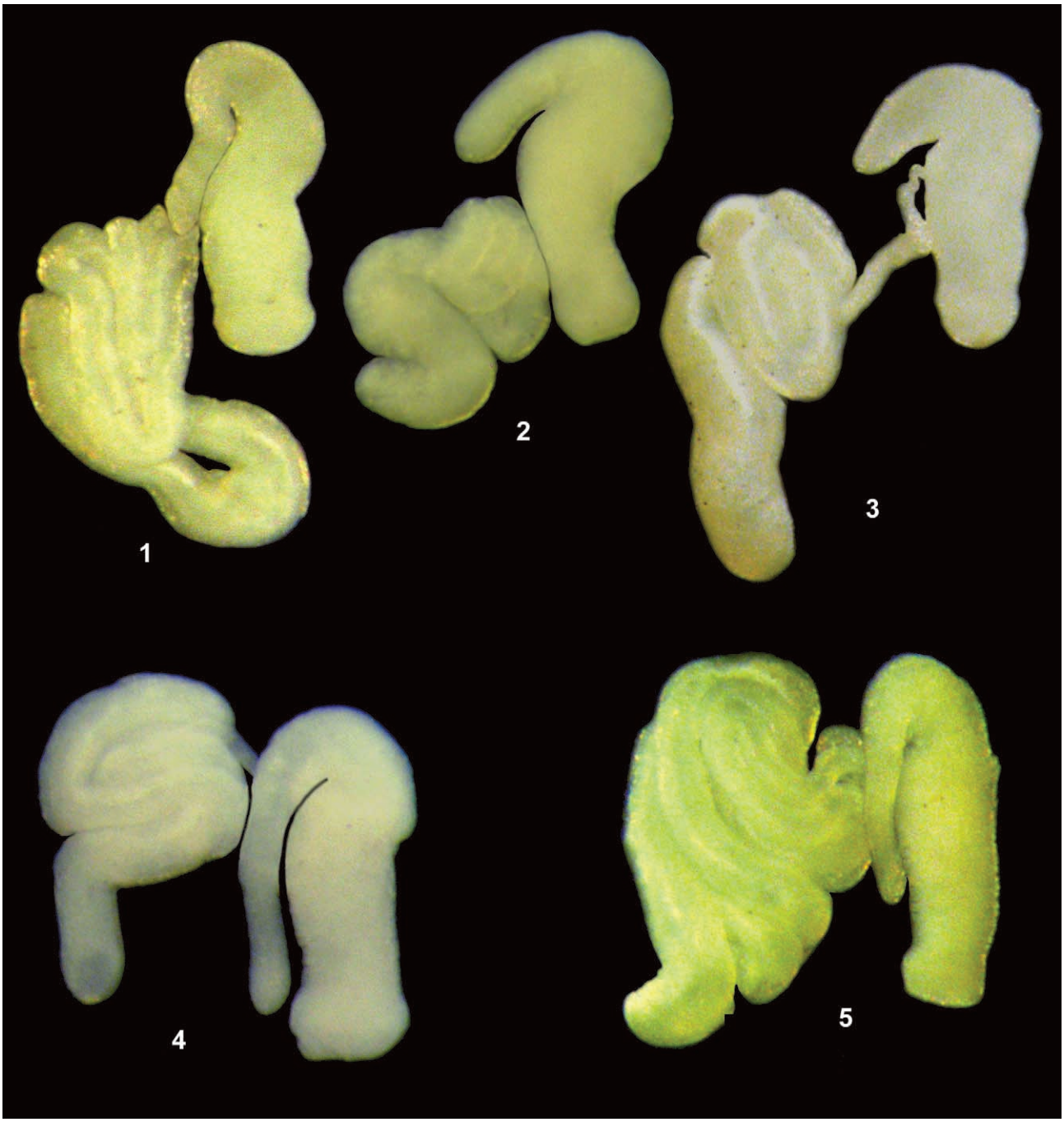

Fig. 3. Penes of *Bythinella hansboetersi*, numbers correspond to localities

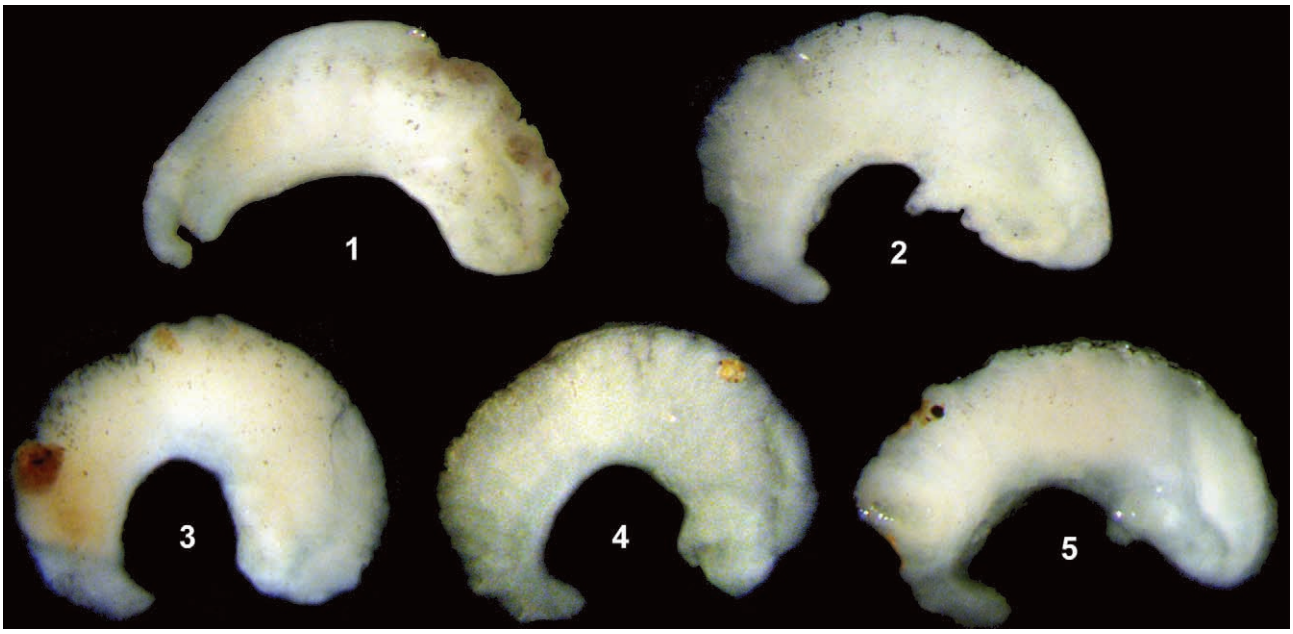

Fig. 4. Pallial section of the female reproductive organs of *Bythinella hansboetersi*, numbers correspond to localities

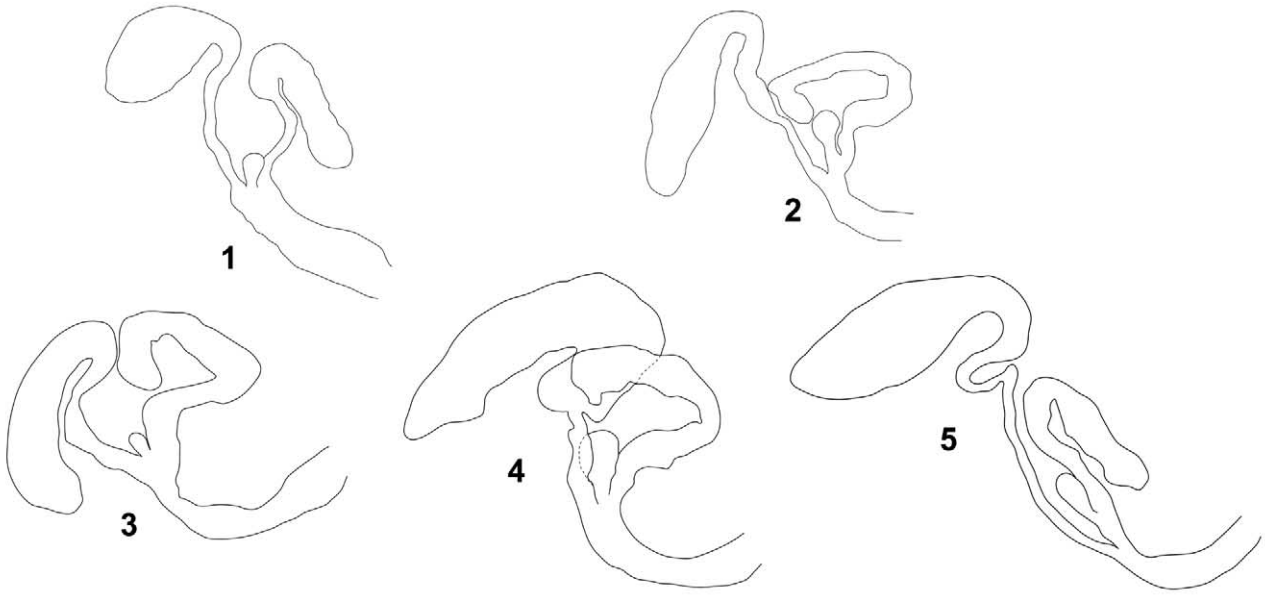

Fig. 5. Bursa copulatrix with duct, receptaculum seminis and loop of (renal) oviduct of *Bythinella hansboetersi*, numbers correspond to localities

For COI, within *B. hansboetersi* the mean genetic diversity for all the metapopulation was 0.008, mean within populations: 0.002, and mean between the (sub)populations was 0.006. 16 haplotypes were found, differing in 13 sites. Haplotype diversity Hd = 0.954±0.021, nucleotide diversity π = 0.00783±0.00071. Within the populations, haplotype diversity Hd was 0.50000±0.265 for population 1, 0.83333±0.222 for 2, 0.90000±0.161 for 3, 0.80952±0.130 for 4, and 0.66667±0.160 for population 5. Nucleotide diversity π was 0.00092±0.00049 in population 1, 0.00214±0.00067 in 2, 0.00366±0.00108 in 3, 0.00227±0.00060 in 4, and 0.00209±0.00080 in population 5. Gene flow estimates calculated for COI with DNASP were low (0.09-1.39, according to the technique applied). For all the tests of genetic differentiation between the studied populations the permutation test with 5,000 replicates indicated high significance ($P<0.001$).

The haplotype network (Fig. 7) generally mirrored the pattern shown by the ML tree for COI (Fig. 6). Restricted gene flow with isolation by distance was found for the total cladogram by the inference key.

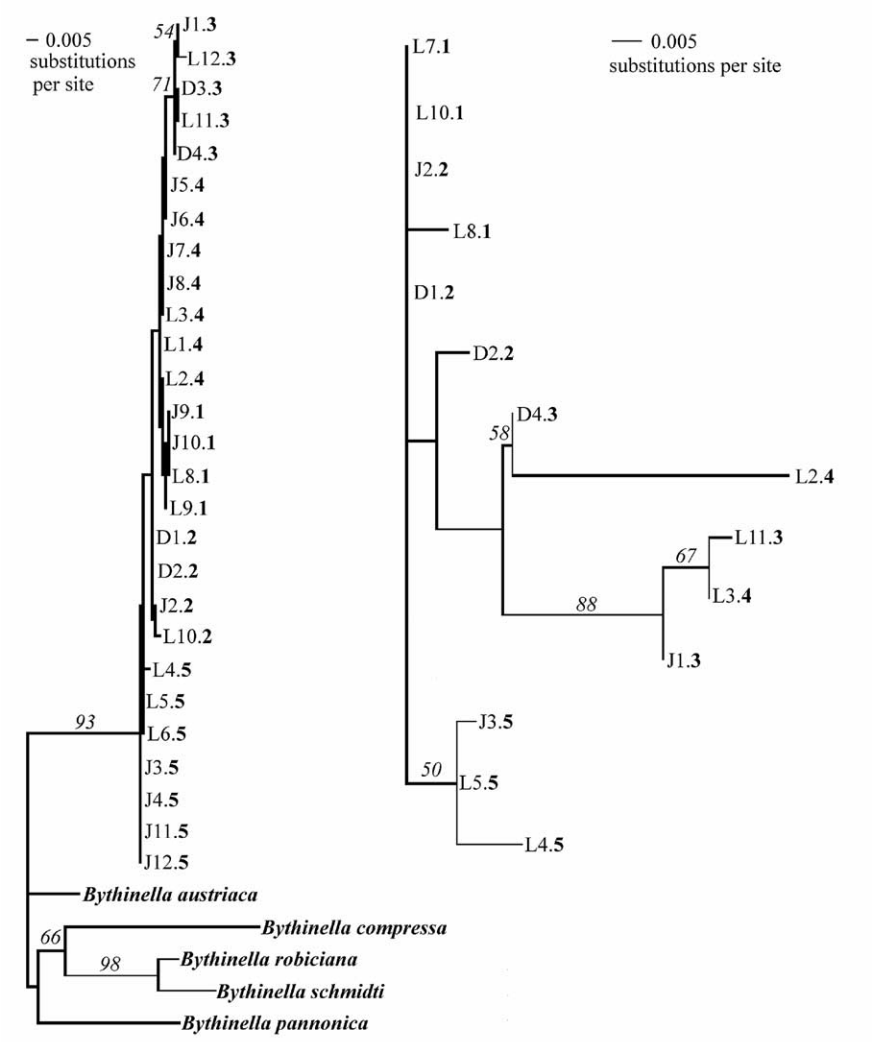

Fig. 6. Maximum-likelihood trees for the COI (left) and ITS-1 (right). Localities' numbers in bold. Bootstrap support indicated (10,000 replicates) when > 50%. See text for details

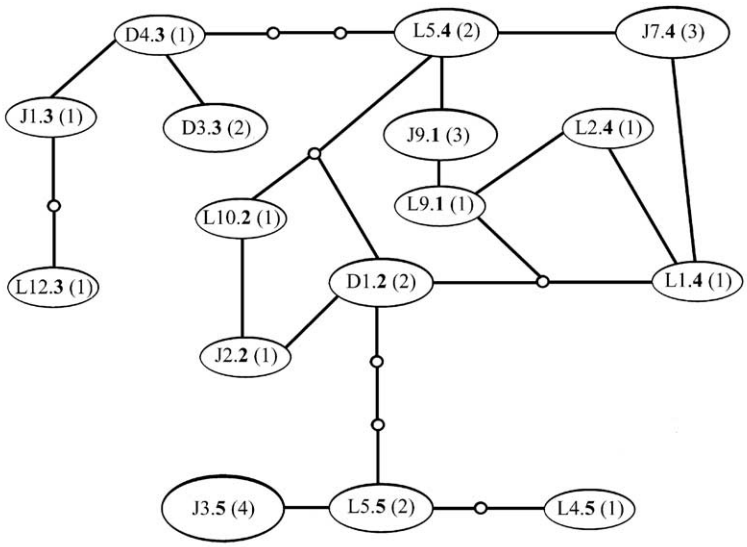

Fig. 7. Haplotype network for the COI, localities' numbers in bold, ellipse size proportional to the number of specimens representing a haplotype (given in brackets)

DISCUSSION

The morphology, as well as geographic distribution of the studied populations of *Bythinella* confirm their identity with *Bythinella hansboetersi*, described recently by GLÖER & PEŠIĆ (2006) from this part of Bulgaria.

All the studied populations are conspecific. This species is certainly molecularly not identical with *B. austriaca* usually reported from Bulgaria (ANGELOV 2000, BANK 2004, HUBENOV 2007). It is quite distinct from all the Central European *Bythinella* species considered, showing a level of distinctness typical of interspecific level within the Rissooidea (e.g. FALNIOWSKI et al. 2007, SZAROWSKA et al. 2007).

Any existing technique of estimating gene flow between (sub)populations is reliable in any conditions (for a review see AVISE 2000, FALNIOWSKI et al. 1999, 2004). However, the so-called direct techniques (based on direct observations of migration of individuals) cannot be used instead, since they do not provide information about the fate of the genes of immigrants (e.g. FALNIOWSKI et al. 1999, 2004). In the Bulgarian *Bythinella*, the level of gene flow was low, which was also confirmed by the inference key (TEMPLETON 2004).

The number of studied populations (5) is certainly too low for any more sound conclusion, but no more material was available. In fact, two of them were situated very close to each other, and the other three were very close to each other also. On the other hand, the geographic distance between the two groups of populations approached 135 km. The inference key (TEMPLETON 2004) found the observed interpopulation differentiation to be due to isolation by distance with restricted gene flow. In the case of such isolated habitats like springs one should rather predict the infinite island pattern, not the isolation by distance one (or, considering the low level of gene flow, the stepping stone model), but the pattern discovered in the present study is the same as that found in the Polish *Bythinella* in our earlier study based on allozymes (FALNIOWSKI et al. 1998).

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