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Spatial pattern of intraspecific mitochondrial diversity in the Northern Carpathian endemic spring snail, *Bythinella pannonica* (Frauenfeld, 1865) (Gastropoda: Hydrobiidae)

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Abstract *Bythinella* is a species-rich genus of spring-snails, having a wide range in Europe and Asia Minor. The genus contains several endemic species with narrow ranges, creating interest from a conservation perspective as well as their use as a model for research into biogeographical patterns and evolutionary development. Most of the species of the genus *Bythinella* are difficult to distinguish by traditional methods due to their similar shell morphology. In previous studies, molecular approaches often came to conclusions that contradicted those from a morphology-based approach, hence the classification of species of *Bythinella* has been in dispute. *Bythinella pannonica* has clearly distinct shell morphological features, and consequently is one of the few species of undisputed taxonomic status within this genus. As an important step towards clarifying the systematics of this genus, we have attempted to describe the structure and spatial pattern of diversity of the mitochondrial *COI* marker within this species, in the hope of applying these findings generally to the whole genus. Molecular results support the monophyly of *B. pannonica* within the genus. The largest pairwise intraspecific *COI* sequence difference was almost 5 %, much larger than the value of 1.5 % previously proposed as a barcoding threshold for delimitation of *Bythinella* species. This finding suggests that incautious application of barcoding might lead to incorrect taxonomic conclusions. Within *B. pannonica* there are two deeply divergent intraspecific clades, the spatial distribution of which has been shaped by fragmentation, as well as by short and long distance dispersal events. These two clades have not been found syntopically but, as a peculiar feature of this taxon, they are able to persist in nearby habitats. We have demonstrated that the patchy distribution of suitable habitats and the restricted, but non-zero gene flow amongst the populations might play a key role in maintenance of the observed genetic structure of this species.

Keywords Intraspecific diversity · COI · Barcoding · Phylogeography · Species delimitation · Cryptic lineage

Introduction

As species is the fundamental unit of taxonomy, biogeography, ecology and conservation biology, species delimitation is a crucial question in supra-individual biology (de Queiroz 2007; Flot et al. 2010; Petit and Excoffier 2009; Pons et al. 2006; Rissler and Apodaca 2007; Sites and Marshall 2003). Over ten thousand new taxa are described every year, most of them by traditional, morphology-based methods (Zhang 2008). Morphology, however, does not always reflect phylogeny, and those taxa for which morphology-based methods are of limited use are a challenge for taxonomists (Bickford et al. 2007; Havermans et al. 2010; Poisot et al. 2011; Weigand et al. 2011).

Among hidrobiid gastropods there are several problematic groups, including the most widespread and species-rich European spring-snail genus, *Bythinella* Moquin-Tandon, 1856. The majority of *Bythinella* species are quite similar in terms of their shell morphology, habitat preference, reproductive biological and behavioural characteristics (Wilke et al. 2010) and, due to the slightly differing and largely overlapping features of the genital organs, not even the genital morphology provides clear and unambiguous solution for taxonomic problems (Falniowski and Szarowska 2009; Giusti and Pezzoli 1977). In recent years, this genus

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has attracted increasing attention, as indicated by the number of new species descriptions (Boeters and Falkner 2008; Falniowski et al. 2009a; Georgiev 2009; Glöer and Georgiev 2009, 2011; Glöer and Pešić 2006, 2010; Prié and Bichain 2009). Due to its Europe-wide range (Bank 2012) and special habitat preference, this genus is a promising model taxon to study Pleistocene refugial patterns in Europe (Benke et al. 2009) and non-adaptive radiation in freshwater systems (Wilke et al. 2010). Due to their narrow ranges, several *Bythinella* species are of special conservation concern (Cuttelod et al. 2011). For the above reasons, clarification of the species boundaries and systematic relationships within *Bythinella* is more than a fundamental research objective in itself.

So far, the majority of phylogenetic studies related to European hidrobiids have focused on the most popular barcoding sequence, the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*). In related genera, the application of this marker gave somewhat inconsistent results. For instance, *COI* performed well for *Adriohidrobia* (Wilke and Falniowski 2001) but it was hardly convenient for *Hauffenia* (Šteffek et al. 2011). Studies on *Bythinella* (Bichain et al. 2007; Falniowski et al. 2009b, c, 2012; Falniowski and Szarowska 2011; Haase et al. 2007) have shown a certain degree of incongruence between *COI*-based phylogeny and morphology-based species delimitations. This phenomenon might be explained by the inadequacy of the marker used; however, a more concrete reason is difficult to define in a group like *Bythinella.* As the delimitation of *Bythinella* species is based largely on morphological characters of limited applicability, one might rather suppose that the incongruence is the result of incorrect taxonomy instead of an inappropriate marker.

In order to test the applicability of the molecular marker used, reliably delimited taxa are needed. As an important step towards the better understanding of the uses and limitations of the *COI* marker in the phylogenetic reconstruction of *Bythinella* and the clarification of the systematics of this genus, we aimed to investigate the intraspecific variability of such a species. *Bythinella pannonica* (Frauenfeld, 1865) seemed to be the most appropriate study system for this purpose. This is one of the few species within this genus that is distinguishable unambiguously by morphological features. By shell characters, *B. pannonica* is so strikingly different from other *Bythinella* species that, prior to the revision of Szarowska and Wilke (2004), it was classified in another genus, *Sadleriana* Clessin, 1890. Its distinct species status is, therefore, generally and undisputedly accepted (Falniowski and Szarowska 2009). At the same time, the number of known populations (>50) and the subdivided geographical range makes it feasible to get an insight into the structure and the spatial pattern of the intraspecific diversity.

We aimed (1) to test whether or not *COI*-based phylogeny is concordant with the morphology-based species delimitation (i.e. *B. pannonica* is monophyletic); (2) to measure the intraspecific variability of this marker and to test the applicability of the 1.5 % barcoding threshold value proposed by Bichain et al. (2007) for this genus; (3) to explore the spatial pattern of the intraspecific variability; and (4) to gain some insight into the biogeographic/demographic history of this species.

Material and methods

The study species

B. pannonica is endemic to the southeastern part of the Northern Carpathian Mountains. As characteristic to the whole genus, it prefers clear, oligotrophic waters and inhabits springs and upper stream sections. Unlike other species of the genus, occurring in the Northern and Eastern Carpathians, *B. pannonica* exclusively inhabits limestone habitats. It is distributed in two well distinguishable subareas; a southern one in the Bükk Mts. (Hungary) and a northern one, which involves the Aggtelek Karst (Hungary) and the neighbouring Slovak Karst (Slovakia) (Fig. 1). Aggtelek and Slovak Karst form one continuous biogeographical subregion, also known as Gömör-Torna Karst (hereafter referred to as Karst). The two subareas are

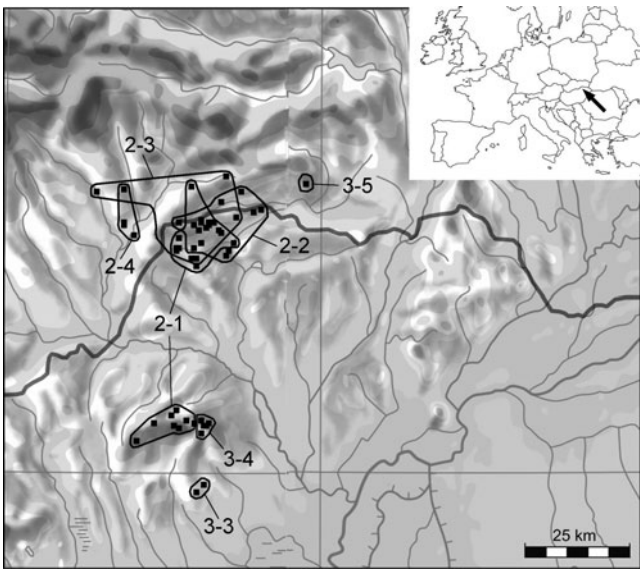

Fig. 1 Relief map of the northeastern part of the Carpathian Basin showing collection sites and the geographic distribution of intraspecific clades [at the level at which significant phylogenetic structure was found by nested clade phylogeographic analysis (NCPA)]. Clade names correspond to those in Figs. 2 and 3. More precise locality data are given in Table 1. *Thick solid line* Political border between Hungary and Slovakia

separated by a 25–30 km wide non-limestone zone. The number of known populations is>50 (Domokos 1992; Farkas 2005; Lisicky 1991; Lukács 1954; Pintér and Suara 2004; Szabó 1984; Wagner 1937). Its geographic range overlaps with that of *B. austriaca* (Frauenfeld 1857) but, except for some rare occasions, the two species are not syntopic (see also Wilke et al. 2010).

Samples were collected between 2006 and 2010 in 56 different Hungarian and Slovakian locations, which well represent the whole known range of the species. Sampling localities are listed in Table 1 and shown on the map in Fig. 1. The specimens, fixed and preserved in 96 % ethanol, have been deposited in the Mollusca Collection of the Hungarian Natural History Museum, Budapest (HNHM).

DNA extraction, amplification and sequencing

In total, 105 *B. pannonica* specimens were studied. Total DNA was extracted from the whole body after removing the shell using the CTAB protocol (Doyle and Doyle 1987; Bousquet et al. 1990) modified for pine needle. The detailed procedure can be found at: http://www.pierroton.inra.fr/genetics/labo/protocol. A 638-bp fragment of the mitochondrial cytochrome oxidase subunit I (*COI*) gene was amplified by polymerase chain reaction (PCR) using the primers LCO 1490 (Folmer et al. 1994) and COI-H (Machordom et al. 2003). The PCR reactions were carried out in 25 μl reaction volume using a PTC-200 (MJ Research, Waltham, MA) thermocycler. Each PCR reaction consisted of approximately 50 ng total DNA template, 0.35 μM of each primer, 0.25 mM dNTPs, 2 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany). The PCR amplification profile was 94 °C (1 min), then 39 cycles of 94 °C (1 min), 40 °C (90 s), and 72 °C (90 s), then a final extension at 72 °C for 6 min. PCR products were separated on 1.4 % agarose gel, and visualised with ethidium bromide under UV illumination. The amplified DNA was recovered using a Roche High Pure PCR Product Purification Kit (Roche, Mannheim, Germany). Purified PCR products were then sequenced on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Phylogenetic analyses

Sequences were aligned by Vector NTI Advance 9.0 to available *B. pannonica COI* gene fragments (GenBank AY222650–51, FJ029077–79) and were cut accordingly (638 bp). Identical sequences were deposited with GenBank (HQ149527–HQ149626) and those differing only in missing or unknown nucleotides were collapsed into haplotypes (Table 1). The appropriate model for nucleotide substitution (HKY+Γ) was selected by Modeltest version 3.7 (Posada and Crandall 1998) using Bayesian Information Criterion (BIC). Molecular clock likelihood ratio test (LRT) was performed by Tree-Puzzle version 5.2 (Schmidt et al. 2002) to test the clock hypothesis.

Using all *Bythinella COI* sequences available in GenBank, a preliminary NJ tree with 1,000 bootstrap replicates was constructed by MEGA version 5 (Tamura et al. 2011) to test the monophyly of *B. pannonica* and to locate its position within the tree of the genus.

After this, a constrained Bayesian tree was constructed from *B. pannonica* sequences by BEAST version 1.6.2 (Drummond and Rambaut 2007) with the following settings: HKY+Γ model of sequence evolution with five gamma rate categories, empirical base frequencies, strict clock model with a normal prior on clock rate (mean=2.2 % / My, SD= 0.1), and a Bayesian skyline plot coalescent tree prior. Following a burn-in of 100,000 cycles, every 1,000th tree was sampled from 10,000,000 Markov Chain Monte Carlo (MCMC) steps. Convergence of the chains to the stationary distribution was checked by visual inspection of plotted posterior estimates using the program Tracer version 1.5 (Rambaut and Drummond 2009). The effective sample size for each parameter sampled from the MCMC analysis was always found to exceed 100. Sampled trees were annotated to a maximum clade credibility tree.

Haplotype networks were constructed by statistical parsimony method at the 95 % confidence level using TCS version 1.21 (Clement et al. 2000).

Mantel tests with 1,000 permutations were performed by GenAlEx version 6.3 (Peakall and Smouse 2006). We intended to reveal the spatial dependence of genetic variability at different scales, therefore various datasets were tested; First, the whole dataset, then separately the two main clades that were unable to join in the same statistical parsimony network at the 95 % confidence level and, finally, the two geographic sub-areas of the Clade-A—i.e. the Bükk and the Karst—separately.

Arlequin version 3.11 (Excoffier et al. 2005) was used to describe intraspecific genetic variability [nucleotide diversity (π), number of polymorphic sites, mean pairwise genetic distances] and to perform Tajima's test of selective neutrality as well as the mismatch distribution analysis. The above analyses were done on the same datasets as the Mantel test. Test of goodness-of-fit (probability that simulated sum of squared deviations≥observed sum of squared deviations) was used to test whether observed data, derived from the mismatch distribution analysis, fitted a sudden expansion model.

Timing of main phylogenetic events (splits between clades) was estimated by the Bayesian method (Drummond et al. 2006) using BEAST. Alternatively, we also used population genetic methods to estimate the timing of the same events. From corrected mean pairwise difference we calculated this as $t=\max D_{corr} / 2\ \mu$, where $D_{corr}=(D_{xy} - (D_x+$

Table 1 *Bythinella pannonica* sampling locations and frequencies of sampled haplotypes. Locations are grouped by main subareas, countries are indicated in brackets; *HU* Hungary, *SK* Slovakia. Haplotype codes are the same as in the GenBank (HQ149527–HQ149626), except for the five sequences that were uploaded to the GenBank prior to this study (AY222650 and AY222651=bypa22, FJ029077 and FJ029079=bypa01, FJ029078=bypa00). Clade-B haplotypes are indicated in bold

Location	Geographic position	Haplotypes
Bükk Mountains		
Kács, Kácsi Stream near the spring (HU)	N47 57.658 E20 36.521	**bypa100 (5), bypa101 (2)**
Sály, Latorpuszta, Latori-vizfő (HU)	N47 58.919 E20 37.906	**bypa108 (1), bypa117 (3)**
Bükk, Szilvásvárad, Szalajka Stream (HU)	N48 04.065 E20 25.407	bypa00 (1), bypa01 (2)
Miskolc-Lillafüred, Szinva Stream, beneath the water plant (HU)	N48 05.315 E20 37.183	**bypa17 (8)**
Miskolc-Lillafüred, Szinva, S of the village (HU)	N48 05.467 E20 37.180	**bypa17 (2)**
Miskolc-Sebes, Felső-Sebes-víz, small stream above the reservoir (HU)	N48 05.983 E20 33.075	bypa01 (1), bypa18 (2)
Miskolc-Felsőhámor, Szinva Stream above the confluence with Garadna Stream (HU)	N48 06.374 E20 37.624	**bypa17 (1)**
Miskolc-Felsőhámor, Szinva Stream near Szikla restaurant (HU)	N48 06.393 E20 38.097	**bypa17 (1)**
Miskolc-Ómassa, Garadna spring (HU)	N48 06.496 E20 31.871	bypa01 (2)
Miskolc-Lillafüred, Eszperantó spring (HU)	N48 06.561 E20 36.869	bypa01 (2), bypa40 (1)
Miskolc-Alsóhámor, Szinva Stream (HU)	N48 06.671 E20 38.742	**bypa17 (1)**
Nagyvisnyó, Bán Stream (ca.6 km upstream of the village) (HU)	N48 06.750 E20 28.422	bypa01 (1)
Miskolc, Forrás-völgy, Közép spring (HU)	N48 06.984 E20 37.480	**bypa96 (1), bypa122 (2) bypa97 (1), bypa123 (1) bypa130 (1)**
Miskolc, Garadna Stream, at the Bánkút-junction (HU)	N48 07.004 E20 34.491	bypa01 (3)
Mályinka, Ágnes spring (HU)	N48 07.517 E20 31.461	bypa01 (3), bypa04 (1)
Mályinka, Mária spring (HU)	N48 07.565 E20 31.718	bypa01 (2)
Mályinka, Harica spring (HU)	N48 08.253 E20 32.600	bypa01 (1), bypa20 (1) bypa87 (1)
Gömör-Torna Karst (Aggtelek Karst, Slovakian Karst)		
Teresztenye, Kinizsi cave (HU)	N48 26.807 E20 36.143	bypa01 (1)
Tornakápolna, Vizetes-völgyi spring (HU)	N48 27.748 E20 36.554	bypa27 (1)
Tornakápolna, Kecske-kút (HU)	N48 27.920 E20 35.245	bypa01 (1)
Perkupa, Hideg spring (HU)	N48 28.192 E20 41.813	bypa22 (3)
Perkupa, Rahozna-kút (HU)	N48 28.747 E20 42.221	bypa77 (1), bypa77b (1)
Jósvafő, Komlós spring (HU)	N48 28.913 E20 32.684	bypa26 (1)
Szinpetri, Bolyamér Stream (HU)	N48 28.956 E20 35.742	bypa22 (1)
Jósvafő, Tengerszem (HU)	N48 28.983 E20 32.735	bypa27 (1)
Jósvafő, Kossuth cave, Nagy-Tohonya spring (HU)	N48 29.253 E20 33.026	bypa01 (1)
Szin, Nagy-Kopolya spring (HU)	N48 29.825 E20 37.414	bypa22 (1)
Dobódél, Szarukő-alji spring (HU)	N48 29.877 E20 43.156	bypa01 (1)
Jósvafő, Lófej spring (HU)	N48 30.544 E20 32.950	bypa01 (1)
Bohúňovo (SK)	N48 30.762 E20 24.449	bypa25 (1)
Szögliget, Szeszfőzdei spring (HU)	N48 31.209 E20 40.897	bypa01 (1), bypa22 (1)
Szögliget, Papkerti spring (HU)	N48 31.387 E20 40.700	bypa22 (2)
Szögliget, Patkós-völgy, Puska Pál spring (HU)	N48 31.554 E20 36.933	bypa01 (1)
Szögliget, Ménes-völgy, Fedor spring 1. (HU)	N48 31.767 E20 38.341	bypa01 (1)
Szögliget, Ménes-völgy, Fedor spring 2. (HU)	N48 31.767 E20 38.341	bypa01 (1)
Szögliget, Ménes-völgy, Hideg-kút (HU)	N48 31.978 E20 38.624	bypa22 (1)
Szögliget, Ménes-völgy, Medvekerti spring (HU)	N48 31.987 E20 35.638	bypa01 (1)
Gemerská Hôrka, a spring near the pub (SK)	N48 32.127 E20 22.691	bypa25 (1)
Szögliget, Ménes-völgy, Lizina spring (HU)	N48 32.136 E20 35.721	bypa01 (1)
Gemerská Hôrka, Päťročenka spring (SK)	N48 32.256 E20 22.723	bypa28 (2)
Szögliget, a spring beneath Éles-tető (HU)	N48 32.256 E20 37.285	bypa01 (1)
Aggtelek, Ménes-völgy, Mogyorós-kút (HU)	N48 32.350 E20 33.169	bypa72 (1)
Szögliget, Ménes-völgy, Tetves spring (HU)	N48 32.379 E20 39.802	bypa22 (1)

Table 1 (continued)

Location	Geographic position	Haplotypes
Szögliget, Derenk, Kis-kút, (HU)	N48 32.517 E20 38.482	bypa01 (1)
Szögliget, Kecskés spring (HU)	N48 32.620 E20 37.437	bypa62 (1)
Bódvaszilas, Vecsem spring (HU)	N48 33.052 E20 43.909	bypa67 (1)
Tornanádaska, Kastély-kerti spring (HU)	N48 33.724 E20 47.081	bypa22 (1)
Tornanádaska, Rongyos-kút (HU)	N48 34.018 E20 48.345	bypa22 (1)
Jablonov nad Turňou, Jablanov spring (SK)	N48 35.021 E20 41.199	bypa37 (1)
Gemerské Teplice, Gemerskoteplická cave (SK)	N48 36.294 E20 17.687	bypa30 (1)
Hrhov, stream above the waterfall (SK)	N48 36.525 E20 44.933	bypa22 (3)
Kunova Teplica, Hučiaca spring (SK)	N48 36.617 E20 22.815	bypa31 (1)
Krásnohorská Dlhá Lúka, spring near Krásnahorská cave (SK)	N48 37.070 E20 35.387	bypa01 (1)
Krásnohorská Dlhá Lúka, Buzgó spring (SK)	N48 37.101 E20 35.168	bypa32 (1)
Drienovec, 2 km N of the village, Drienovecké kúpele (SK)	N48 37.482 E20 57.128	**bypa35 (4), bypa52 (1)**
Kováčová, spring in the village (SK)	N48 38.369 E20 41.792	bypa34 (1)

D_y)/2), D is mean pairwise difference (uncorrected p-distance) either between (D_{xy}) or within (D_x, D_y) populations and μ is the substitution rate. When statistical tests (Mantel, Tajima's) supported the recent exponential growth of a given clade, we calculated the time elapsed since these putative expansion events (t) using the parameter τ, which is an estimate of the mode of the mismatch distribution, using the formula $t=\tau/2\ \mu$. Both τ and D values were calculated by Arlequin. For all the three time estimation methods, substitution rate was fixed as 2.2 % / My multiplied by the length of sequences, which was also used by Benke et al. (2009) (see also Wilke et al. 2009).

A nested clade phylogeographic analysis (NCPA) was performed to infer general phylogeographic patterns (Templeton et al. 1995). Haplotypes of the statistical parsimony networks were nested into hierarchical clades following the nesting rules outlined in Templeton et al. (1987) and extended in Templeton and Sing (1993). The statistical test of geographical association among and between haplotypes and nested clades was performed by GeoDis version 2.0 (Posada et al. 2000) with 10,000 permutations. The inference key of 11/11/05, provided by GeoDis, was used to infer particular historical processes for clades where significant geographical association or dispersion was found.

In the ecological niche test, two variables were assigned to each haplotype: the flow rate (m^3/s) at the sampling site and the sampling site's distance from the source (m). The former was based on the data of National Park Directorates, and considering potential inaccuracies, it was converted to an ordinal variable (1–5), where 1 is the largest and 5 is the smallest flow rate. In case of haplotypes occurring more than one site, averages were taken. The null hypothesis was that the two main clades (Clade-A and Clade-B) prefer the same ecological conditions as regards the flow rate and the distance from the source. It was tested by multivariate nonparametric Cramér test as implemented in the "cramer" package (Franz 2012) in the R environment (R Development Core Team 2012).

Results

Among the *COI* sequences of our *B. pannonica* samples, a total of 56 segregating nucleotide positions defined 34 distinct haplotypes. The average pairwise difference was 11.5 bp (uncorrected p-distance=1.8 %), whereas the largest pairwise difference was 30 bp (4.7 %) (Table 2). *B. pannonica* forms a well supported (bootstrap value=100, not illustrated) monophyletic clade within the genus *Bythinella*. New sequences, obtained in the frame of the present study, did not influence the overall picture of the genus' phylogenetic tree (see Benke et al. 2009) in the sense that *B. austriaca* was found to be the sister group of *B. pannonica*. At the species level, two deeply diverged clades—hereafter referred to as Clade-A and Clade-B—can be distinguished (Fig. 2). These clades form two distinct statistical parsimony networks that were not connected at the 95 % confidence level. Within these main clades, Bayesian and statistical parsimony methods inferred very similar ingroup topologies; all 3-step clades and all but one 2-step clades of the network are monophyletic on the Bayesian tree (Figs. 2 and 3).

Compared to the other main clade, Clade-B branches deeper on the Bayesian tree and needed one more step to be grouped together during the nesting procedure of NCPA. This reflects the fact that the molecular diversity of Clade-B is larger than that of Clade-A (Table 2). The clock hypothesis could not be rejected under the HKY+Γ model of sequence evolution on a significance level of 5 % ($\log L_0=1{,}565.18$, $\log L_1=1{,}548.32$, $-2\log\Lambda=39.72$, df=33, $P=0.1958$).

Table 2 Genetic diversity of the studied *Bythinella pannonica* material based on *COI* gene sequences

Clade	Number of sampling sites	Number of individuals	Number of haplotypes	Number of polymorphic sites	Haplotype diversity (H)±SD	Nucleotide diversity (π)±SD	Mean pairwise differences (D)±SD
Total	56	105	34	56	0.8767 +/− 0.0230	0.017960 +/− 0.009091	11.458 +/− 5.237
Clade-B (= 4–2)	9	35	12	24	0.8336 +/− 0.0505	0.009325 +/− 0.005066	5.950 +/ –2.908
Clade-A (= 3–1)	47	70	22	24	0.7623 +/− 0.0453	0.002986 +/− 0.001909	1.905 +/− 1.099
Clade-A northern subarea	38	43	16	16	0.8106 +/− 0.0438	0.003215 +/− 0.002042	2.051 +/− 1.173
Clade-A southern subarea	9	24	7	8	0.5036 +/− 0.1226	0.001164 +/− 0.000995	0.743 +/− 0.569

Mismatch distribution analysis resulted in a bimodal distribution pattern, where the mode of the second wave at 22 bp was due to the mismatches between the two main clades (Table 3, Fig. 4). This finding, as well as Tajima's test of selective neutrality, rejects that the species as a whole went through a recent exponential population growth.

Both main clades are present in both geographic subareas, but the geographic distribution of the genetic variability is far from being panmictic (Fig. 1). Clade-A haplotypes were found in the majority of the species' range (in 9 of the 17 collecting sites in the Bükk Mts. and in 38 of the 39 collecting sites in the Karst, Tables 1 and 2). Clade-B haplotypes are restricted to three narrow and well distinguishable peripheral areas; 3–3 clade occurs in the southern Bükk Mts. (Kács and Sály), 3–4 clade occurs in the eastern Bükk Mts. (within the drainage of the Szinva Stream) and 3–5 clade occurs in the easternmost part of the Slovak Karst (Drienovec). It is worth noting that the haplotypes of the two main clades have not been found syntopically (Table 1).

Mantel test indicated significant spatial dependence of both main clades, but the r-value was much higher for Clade-B. Mismatch analyses, performed separately on the main clades, resulted in a multimodal distribution and significant test of goodness-of-fit for Clade-B. This indicates a

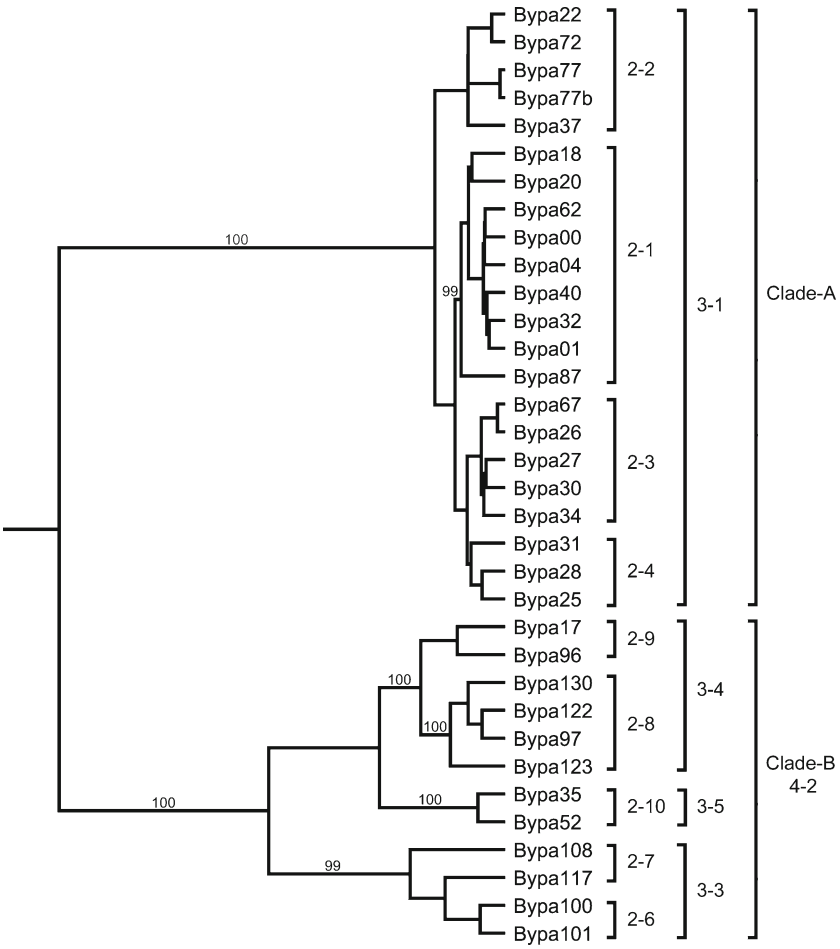

Fig. 2 Constrained Bayesian tree of *Bythinella pannonica* mitochondrial cytochrome *c* oxidase subunit I gene (*COI*) haplotypes. The tree was rooted by *Bythinella austriaca* (FJ028978) (not illustrated). Numbers at branches are Bayesian posterior probabilities. For reasons of comparability, 2nd and higher step clades, defined by nested clade analysis, are indicated. Posterior probabilities above 95 % are given only for 2nd and higher step clades (see Fig. 3 for nested clade structure). Note that clade 2–7 is inferred to be paraphyletic by this analysis. This tree has been deposited in the TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S10789)

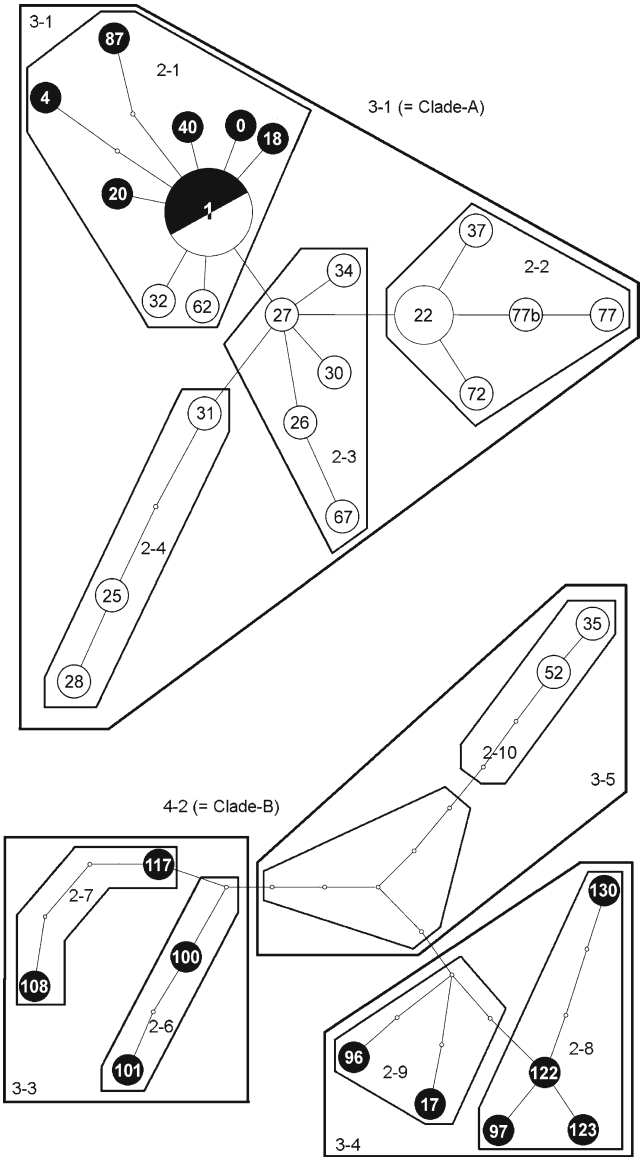

Fig. 3 *Bythinella pannonica COI* haplotype network. *Circles* Haplotypes occurring in the Karst (*white*) and in the Bükk Mts. (*black*). Circle sizes are proportional to the frequencies of the haplotypes. Haplotypes were nested into hierarchical clades following the rules of Templeton et al. (1987) and Templeton and Sing (1993). The two main clades, which could not be connected at the 95 % confidence level, are considered as distinct 4-step clades. For reasons of simplicity, only 2nd and higher step clades are indicated here. See Table 4 for phylogeographic inferences

stable population structure for this subset, which is also supported by the non-significant Tajima's test. By contrast, Clade-A exhibits unimodal mismatch distribution, which closely approximates the ideal Poisson-like distribution, and the significant negative values of the Tajima's test also infer a recent population expansion for this subset (Fig. 4, Table 3).

Further subdivision based on the geographical subareas has shown that the Karst subsample has almost the same mismatch distribution pattern, and its mode is almost at the same value as that of the whole Clade-A, though the p-value of the Tajima's test was slightly above the 5 % significance level (Table 3). The Bükk subset of Clade-A, which comprised only clade 2–1 haplotypes, shows lower diversity (Table 2) and a more perfect fit to a Poisson-like mismatch distribution with a mode much closer to zero (Fig. 4), inferring a more recent expansion. Mantel test indicates very strong spatial dependences for Clade-B and for the Karst subset of Clade-A, whereas it indicates the complete absence of spatial dependence for the Bükk subset ($r=-0.001$ and $P=0.535$) (Table 3).

The nested clade phylogeographic analysis (NCPA) resulted in five nesting levels (Fig. 3). The fifth level connects the final two clades, 3–1 (= Clade-A) and 4–2 (= Clade-B), which could not be connected at the 95 % confidence level of the statistical parsimony procedure. Out of the 17 nested clades, only five could reject the null hypothesis of no phylogeographic structure, all of which were 3-step or higher level clades (Table 4). The NCPA result suggests that the species' split into the two main clades was due to fragmentation. Clade-B appears to be the result of fragmentation events followed by long distance colonisation of the eastern part of the Karst by clade 3–5. The phylogeographic structure of Clade-A appears to result from restricted gene flow (within sub-areas) and long distance dispersal (between subareas). Clade 3–3 seems to be the result of allopatric fragmentation on a small scale, because the two springs in the southern Bükk Mts., which are inhabited by the allopatric populations (clades 2–6 and 2–7), are separated by ca. 4 km.

There were slight differences between divergence times, estimated by different population genetic and phylogenetic methods. The Bayesian method generally calculated earlier values. The split between the two main clades is estimated to have happened around the turn of the Lower and Middle Pleistocene (ca. 1 to 0.65 My). Clade-B apparently diverged deeper, based on the population genetic estimates about three times earlier than Clade-A. According to the Bayesian method, Clade-B diverged only 1.5 times earlier than Clade-A. There was no difference found between the expansion times of the whole Clade-A and its Karst subset, whereas the expansion of the Bükk subset seems to be a much more recent event (Table 3).

Cramér test did not reject the null hypothesis ($P=0.119$), that is, in terms of the examined ecological factors, no difference was revealed between the ecological preferences of the two clades.

Discussion

Studying French *Bythinella*, Bichain et al. (2007) delimited species "as sets of organisms that have genealogic

Table 3 Tests of the spatial dependence of genetic variability and results of various divergence time estimations. Divergence time estimations are given in million years (My). For all the three estimation methods the substitution rate was fixed as 2.2 % / My. 95 % C.I. values are given in brackets

Clade	Mantel test	Tajima's D	Test of goodness-of-fit	Divergence time estimated from D	τ-value	Divergence time estimated from τ	Divergence time estimated by Bayesian analysis
Total (4–2 / 4–1)	r=0.272; *P*<0.001	0.221; *P*=0.655	*P*=0.05	D=18.29; t=0.651	–	–	1.020; (0.638–1.423)
Clade-B (3–3 / 3–4 / 3–5)	r=0.487; *P*<0.001	0.072; *P*=0.601	*P*=0.00	3–3 / 3–5; D=6.67; t=0.238	–	–	0.358; (0.207–0.534)
Clade-A (2–1 / 2–2 / 2–3 / 2–4)	r=0.183; *P*<0.001	−1.916; *P*=0.006	*P*=0.26	2–3 / 2–4; D=2.37; t=0.084	2.53; (0.6–4.4)	0.090; (0.022–0.157)	0.205; (0.104–0.320)
Clade-A northern subarea	r=0.425; *P*<0.001	−1.414; *P*=0.053	*P*=0.11	–	2.37; (0.7–3.7)	0.084; (0.025–0.132)	–
Clade-A southern subarea	r=−0.001; *P*=0.535	−2.096; *P*=0.002	*P*=0.92	–	1.00 (0–2.5)	0.035; (0–0.089)	–

relationships and form isolated, irreversible evolutionary lineages (diagnosable monophyletic groups)" (see also Samadi and Barberousse 2006). They considered those groups of *COI* haplotypes, which were not connected into a statistical parsimony network at the 95 % confidence level, as distinct species. This practically meant 14 mutational steps or more, equivalent to 1.5 % or higher p-distance.

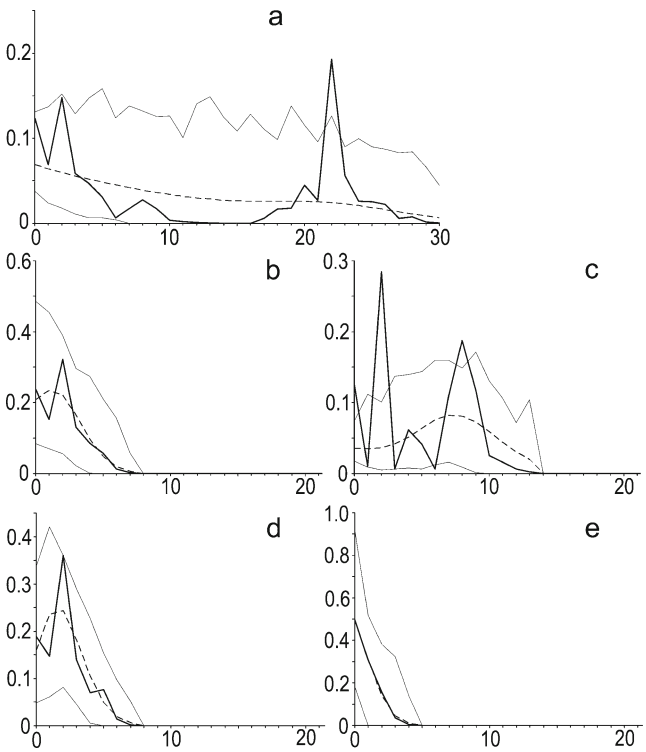

Fig. 4 Mismatch frequency distributions of pairwise mutational differences for different datasets of *Bythinella pannonica* samples. **a** Whole dataset, **b** clade-A (see Figs. 2 and 3) **c** Clade-B **d** Subset of Clade-A sampled in the Karst, **e** Subset of Clade-A sampled in the Bükk Mts. *Thick solid lines* Observed frequency of pairwise differences, *dashed lines* expected distribution under the sudden expansion model, *thin lines* lower and upper bounds of the 95 % confidence interval

In the beginning of this study, our premise was that *B. pannonica* is one species. We found two deeply diverged clades, between which the average pairwise p-distance was 2.9 % (largest pairwise p-distance was 4.7 %). According to Bichain et al. (2007), one might presume that one of these clades corresponds to a cryptic species, therefore, it was necessary to critically examine this hypothesis.

First of all, it should be noted that the majority of the observed variability occurred in the 3rd codon position (in 42 out of the 56 variable sites), which is particularly variable at this mitochondrial marker (Pons et al. 2010). Most of these mutations did not lead to amino acid changes (e.g. there is no amino acid difference between bypa27, the central haplotype of Clade-A, and bypa100, a representative haplotype of the other main clade) (Fig. 3).

In the opinion of Wilke et al. (2010) the diversification of *Bythinella* genus is driven primarily by non-adaptive mechanisms and there are no apparent differences between the ecological preferences of different species. In those rare occasions, however, when two *Bythinella* species syntopically occur in the Northern or in the Eastern Carpathians (e.g. *B. austriaca* and *B. pannonica* at Gemerské Teplice or *B. molcsanyi* H. Wagner 1941 and *B. grossui* Falniowski, Szarowska & Sirbu 2009 at Statiunea Izvoare, Igniş Mountains, Romania) the relative abundances of co-occurring species seem to be dependent on the distance from the source (Z.F., unpublished observations). These observations indicate that different *Bythinella* species might have somewhat different ecological requirements and prompted us to compare the two clades' habitats. With this object, we have studied two variables, the flow rate of the water at the collecting site and the distance from the source. Climatic data (precipitation, air temperature, solar radiation, etc.), which are used widely in ecological niche modelling (Kozak et al. 2008), were disregarded because they were expected to have no significant effects on animals living in constant temperature freshwater habitats. Data on water chemistry were not

Table 4 Results of the nested clade analysis of *Bythinella pannonica* *COI* haplotypes (Fig. 3). Only those clades that could reject the null hypothesis of no phylogeographic structure are shown. *Dc* Geographical spread within a clade, *Dn* geographical spread between nested clades, *I-T* average distance between interior and tip nodes in a given nested clade. Abbreviations for the phylogeographic mechanism inferred by the nested clade analysis are as follows: *AF* allopatric fragmentation, *PF/LDC* past fragmentation and/or long distance colonisation, *RGF/D* restricted gene flow/dispersal but with some long distance dispersal over intermediate areas not occupied by the species, *IGR* insufficient genetic resolution to discriminate between range expansion/colonisation and restricted dispersal

Clade	Interior haplotypes /clades	D_c (km)	D_n (km)	Inference chain
Total	4-1	24	24**	1–2–3–5–15–No:
	4-2	15*	22	PF/LDC
	I-T	–	–	
4-2 (= Clade-B)	3-3	1*	18	1–19–20–2–3–5–15–No:
	3-4	1*	4*	PF/LDC
	3-5	0*	58**	
	I-T	−1	49**	
3-1 (= Clade-A)	2-1	23**	21**	1–2–3–5–6–7–8–Yes:
	2-2	6*	12*	RGF/D
	2-3	12	13	
	2-4	3*	19	
	I-T	−4	−5	
3-3	2-6	0*	2**	1–19–No: AF
	2-7	0	2*	
	I-T	0*	0**	
3-4	2-8	0*	2**	1–2–3–5–6: IGR
	2-9	1	1	
	I-T	1**	−1*	

*Distances significantly ($P<0.05$) smaller than expected, ** distances significantly ($P<0.05$) larger than expected

available, but the springs of the geological sub-areas are fed by the same groundwaters and, therefore, we assumed no fundamental differences in water chemistry parameters within the sub-areas. Beside the morphological identity, amino acid sequence identity and overlapping ranges of the main *B. pannonica* clades, the fact that no difference was found between their niche preferences (at least in terms of the examined variables) is an additional argument against the cryptic species hypothesis.

Therefore, we assume that both clades belong to *B. pannonica* and in this species, nearly 5 % *COI* p-distance is still within the threshold of intraspecific variability. Considering that such intraspecific *COI* variability is not unusual among pulmonate snails [e.g. *Arianta arbustorum* (Gittenberger et al. 2004; Haase et al. 2003); *Cepaea nemoralis* (Davison 2000); *Lozekia deubeli* (Fehér et al. 2009); *Trochoidea geyeri* (Pfenninger et al. 2003)] and that there are also similar examples among dioecious gastropods [e.g. *Theodoxus fluviatilis* (Bunje 2005); *Oncomelania hupensis* (Hauswald et al. 2011)], this finding is not surprising at all. Thus, we can assume that some other *Bythinella* species might also have similar genetic structure and similarly a high intraspecific diversity. Consequently, applying a 1.5 % threshold value would result in an unrealistic oversplitting of the genus. At the same time, we do not believe that the 5 % value for species delimiting threshold should be applied generally since the example of *B. robiciana* and *B. opaca*—the former being a rapidly evolved young species derived from the latter—indicates that, in some other cases, interspecific *COI* divergence can be far below 5 % (Haase et al. 2007). Rather, we think that *Bythinella* is another example of a taxonomic group in which intra- and interspecific variations overlap, i.e. the intraspecific variation of some species exceeds the interspecific divergence between other species (Meyer and Paulay 2005; Wiemers and Fiedler 2007). It seems that, in this group, the *COI* marker in itself is a very limited tool to distinguish species, and does not provide an alternative to morphology-based taxonomy (see also Bauer et al. 2011; Galtier et al. 2009).

Despite the fact that both clades occur in both geographic sub-areas, Mantel test has shown that the distribution of genetic variability is not panmictic, but is spatially structured. The NCPA results are not easy to interpret, as 12 out of the 17 nested clades have not shown any significant phylogeographic structure. This might result from the fact that subsequent colonisation, extinction and dispersal events within the relatively narrow geographic range of the species obscured most of the phylogeographic structures. This indicates the limited applicability of this method, at least in the case of narrow range groups (see also Knowles and Maddison 2002; Knowles 2008 and Petit 2007 for further criticism, but see also Garrick et al. 2008 and Templeton 2008). We therefore drew tentative conclusions on the biogeographical history of *B. pannonica* interpreting NCPA outcomes in the context of the results of Tajima's D test, divergence time estimations and mismatch analyses.

The main problem with the time estimations is the lack of reliable calibration, as there are no available fossils (Krolopp 1992), or any well-defined biogeographical events. In the absence of something better, we followed Wilke et al. (2009) and calculated with a fixed 2.2 % substitution rate (also used by Benke et al. 2009). We were well aware of the fact that, without solid reference points, these analyses provide meaningful information only about the relative, rather than the absolute, values of the divergence times. The split of the two main clades was estimated to a date ca. three times earlier than the radiation of Clade-B, and the latter was dated to take place much (i.e., 2–3 times) earlier than that of Clade-A. It is noteworthy that even the radiation of clade 2–1 in the Bükk Mts. was estimated to have taken place during the Pleistocene. These results, however, should be treated with care, as some authors report much higher rates for *COI* divergence

[e.g. Pons et al. (2010) calculated 8.5 % in insects], and there is accumulating evidence of the time dependency of the rate (Ho et al. 2005). For instance, Burridge et al. (2008) calculated a ca. ten times higher value for the evolution rate of fishes when calibrated to a Holocene, as opposed to a Pliocene event. As we used a rate that was calibrated to events of a few My, it is not inconceivable that time estimates of more recent events have an error factor of an order of magnitude. In the light of this, we have to take into account that not only the demographic expansion of Clade-A in the Bükk Mts., but also the whole Clade-A expansion might have been a Holocene event. The time dependency of the rate might affect the estimates of earlier events as well, but it seems probable that the radiation of Clade-B and the split of the main clades took place during the Pleistocene.

Based on the available data, the following biogeographical history can be hypothesised. The two currently existing main lineages have developed, probably due to fragmentation, some time in the Pleistocene (probably earlier than 1 My). As the original picture was obscured by more recent events (long and short range dispersals), we can only suppose, on the basis of higher haplotype diversity, that the early phase of Clade-B evolution took place in the Bükk Mts. Accordingly, we might think that Clade-A evolved originally in the Karst. Further history of Clade-B is difficult to infer, as currently there are only three remnant lineages surviving in three narrow refugia. Our data suggest at least one long distance colonisation event in the Pleistocene (the transfer of clade 3–5 to the Karst) and clade 3–3 shows, on a small scale, the sign of allopatric fragmentation in the southern Bükk Mts. Clade-A most probably went through a bottleneck effect sometimes in the Late Pleistocene or in the beginning of the Holocene. This population then expanded rapidly, as indicated by the significant Tajima's D test, the star-like genealogy (Fig. 3), and the unimodal mismatch distribution (Fig. 4, Table 3) (Rogers and Harpending 1992; Slatkin and Hudson 1991). The nested clade structure (Fig. 3) suggests that Clade-A expansion started originally in the Karst, and the Bükk Mts. were colonised later, because interior clades of a haplotype network are thought to be more ancient than the tip clades (Castelloe and Templeton 1994; Templeton 2004). The higher τ value of the Karst subset also supports this scenario. Based on the current spatial distribution of the haplotypes, it can be supposed that bypa01 haplotype has colonised the Bükk Mts., as this is the one that occurs in both subareas, whereas its descendants are found in only one of the two sub-areas. As the colonisation of the Bükk Mts. is inferred to have been a Holocene event, it is noteworthy that Clade-A occupied within a few thousand years almost the whole Bükk sub-area, indicating a capacity for rapid dispersal (in a phylogeographic sense).

As an empirical addition to the dispersal and colonisation ability of *B. pannonica*, there are cases observed in the past few decades in which a known population has disappeared from a given location without any apparent environmental change (e.g. from Sv. Ján Spring, Debrad' in the Slovak Karst, J. Grego personal communication; or Mária Spring in the Bükk Mts., S. Szabó, personal communication). Other cases show either the spontaneous colonisation of a previously unpopulated habitat (e.g. Ágnes Spring in the Bükk Mts.), or the disappearance and re-appearance of populations (Ámor Spring and Szilvia Spring in the Bükk Mts., S. Szabó, personal communication). These cases indicate that local populations might be subject to periodic extinctions and recolonisations.

The persistence of deeply diverged intraspecific lineages is probably not exceptional in the case of *B. pannonica*. Similar structures have been revealed in several other species, e.g. in a grasshopper (*Dichroplus*) species (Rosetti and Remis 2012), the Brazilian merganser (Vilaça et al. 2012), a landsnail (*Albinaria*) species (Páll-Gergely et al. 2012), or some butterfly (*Heliconius*) species (Muñoz et al. 2011), to mention some recent findings. The uniqueness of *B. pannonica* is that, although the different lineages are not found syntopically, they are able to persist in nearby locations. Although this feature is seemingly contradictory to the hypothesis of rapid dispersal, it can be explained by the population structure and the rate of gene flow.

It is well known that active dispersal of the spring-snails is limited, and it seems to be an improbable way to overcome barriers between adequate habitats (Kappes and Haase 2012). However, it is assumed that *B. pannonica*, similarly to many other gastropod species, is able to spread by random passive dispersal (Wilke et al. 2010). Though there are some assumptions about the role of birds or mammals as vectors (Gittenberger et al. 2006; Green and Figuerola 2005; Groh and Fuchs 1988; Maciorowski et al. 2012; Wada et al. 2012), the dispersal mechanisms are not exactly known. Apparently the number of migrant specimens, and therefore the rate of gene flow, is very limited (Ponder and Colgan 2002).

If we suppose that a population is stable, the number of generations (G) to the common ancestor can be estimated by the effective population size (N_F) (Avise 2000: 37–49). In the case of small populations, like those of *Bythinella*, this mechanism maintains a homogenous intra-population structure, as any variation (the result of mutation or migration) is soon sorted out. This explains why no syntopic occurrences of the two lineages were found among our samples. As a result of random passive dispersal, migrant individuals arrive at populated habitats from time to time. As long as the number of migrants remains low, it is much more probable that the original lineage persists and newcomer lineages and/or new mutations are sorted out. Therefore, the patchy distribution of potential habitats combined with the small population sizes could effectively preserve deeply diverged lineages even in nearby habitats, provided that the rate of

migration is low. At the same time, a lineage carried by a migrant specimen can easily become established in an unpopulated habitat. Thus, it can be assumed that local extinctions and recolonisations of unpopulated habitats are the main factors that make the dispersal of intraspecific lineages possible.

Since *B. pannonica* is listed by Annex II of the European Habitat and Species Directive, and also legally protected in Hungary, our findings might also have conservation implications. We found both a high genetic diversity in this species and the presence of deeply diverged intraspecific clades, some of which are of narrow distribution. Any conservation actions regarding this species should focus on the preservation of this intraspecific diversity, because it is believed to have an important role in the adaptability and the long-term survival of species (Frankham 2005; Pertoldi et al. 2007). As our study has inferred that the meta-population structure plays a key role in maintenance of intraspecific diversity, conservation action should focus on its preservation in general, rather than concentrating on the protection of some individual populations and habitats.

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Ethical standards The authors declare that the experiments comply with the current laws of the country in which they were performed.

Conflict of interest The authors declare that they have no conflict of interest.

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