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PRIMARY RESEARCH PAPER

Isolation and endemism in the subterranean aquatic snails of the genus *Belgrandiella* A. J. Wagner, 1928 (Caenogastropoda: Truncatelloidea: Hydrobiidae)

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Abstract The Western Balkans hosts the richest subterranean aquatic gastropod fauna in the world. The main factors shaping intraspecies diversity are thought to be isolation and endemism. In the genus *Belgrandiella*, minute snails inhabiting subterranean waters and springs in Central Europe and Balkans, molecular studies have shown much fewer valid species than previously anticipated. The present study applies mitochondrial cytochrome c oxidase subunit I, histone 3, and RAPD analysis, to check the inter- and intraspecies genetic diversity in 36 *Belgrandiella* populations from caves, springs and interstitial aquifers. The level of gene flow is assessed to check if these snails form a widespread genetically uniform metapopulation or rather follow the highly endemic pattern. The studied populations have been assigned to six species. In the most widely distributed *B. kusceri* from 21 populations, 60 sequenced specimens represent 16 haplotypes. While the same haplotypes are present in distant populations, gene flow between the other populations is low. Nei distances for RAPD show no geographic pattern. The interspecies differences in COI evidently confirm the time of speciation in Pleistocene, before karstification, which rejects speciation within isolated caves. The pattern observed in *Belgrandiella* seems more similar to the one described in *Montenegrospeum* than in *Kerkia*.

Keywords Balkans · Gene flow · Phylogeny · Phylogeography · Pliocene speciation · Stygobite

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Introduction

Freshwater gastropods are a noteworthy element of the subterranean fauna. According to Illies (1978), from among 549 freshwater gastropod species of Europe as many as 106 inhabit subterranean waters, nearly all as stygobites. Stygobites are obligate, strictly subterranean aquatic animals which complete their entire life in the lightless environment. Only a few represent either stygophiles, i.e., they inhabit both surface and subterranean aquatic environments, but are not necessarily restricted to either, or stygoxenes, i.e., they are accidentally or occasionally present in subterranean waters. In the Balkans, the gastropod stygofauna is especially rich, comprising 169 species (Sket et al., 2004a). Even the representatives of two endemic families (Emmericiidae, Litthabitellidae) could be found. Now we can count more than 300 nominal species, although the species distinctness of many of them needs verification (Falniowski, 2018; Osikowski et al., 2018). In Slovenia, from 91 aquatic gastropod species, 46 are stygobionts (50%), and eight either stygoxenes or stygophiles (Sket, 1999). Nearly all of the gastropod stygofauna represents the minute, gill breathing Truncatelloidea ("Hydrobiidae *s. lato*" in Bernasconi & Riedel, 1994; Culver, 2012). The Truncatelloidea are one of the greatest examples of the Balkans biodiversity (Bănărescu, 2004). Surely, such species numbers are preliminary estimates; on the one hand, several cryptic species may still be recognized since the subterranean habitats are underexplored, but on the other hand, the number of already recognized species is presumably overestimated (Falniowski & Beran, 2015; Osikowski et al., 2018). Minute dimensions and simplified anatomy of these snails (Falniowski, 2018) coupled with low densities of their populations (e.g., Culver & Pipan, 2009, 2014), result in poor knowledge of their biology, speciation, and taxonomy. Descriptions based usually only on the shell morphology resulted in numerous nominal species representing no real biological entities (e.g., Wilke & Falniowski, 2001; Osikowski et al., 2018). Long lasting tendency to describe a new species at each locality, based on the assumption that it is isolated, has resulted in e.g., more than 60 nominal species (and many subspecies) of *Bythiospeum* Bourguignat, 1882 listed by Glöer (2002), many of them not representing distinct species (Richling et al., 2016).

Despite common reports on the hydrobioid stygobiotic species endemic to extremely restricted area—often just one cave (for review see Falniowski et al., 2021a), the hydrobioid snail *Fontigens tartarea* Hubricht, 1963 is known from dozens of caves in West Virginia (North America). Its geographic range reaches 200 km, and the species shows patchy distribution, sporadic occurrence, and high variability of the shell (Hershler & Holsinger, 1990; Culver, 2012). Similarly, in *Montenegrospeum bogici* (Pešić et Glöer, 2012) inhabiting caves and interstitial waters in the Western Balkans, and sporadically flooded out in springs, a wide geographic range coupled with high levels of gene flow between local populations was found (Falniowski et al., 2021a). Haase et al. (2021) suggest possible dispersal and radiation along the alluvial gravel interstitial, as well as the phreatic rhizosphere (i.e., roots of macrophytes, either aquatic or hygrophilous plants) for *Hauffenia* Pollonera, 1898, a stygobiont snail inhabiting only interstitial waters, not caves in Slovakia. Although stygobites (e.g., *Montenegrospeum* mentioned above) tend to have wider geographic ranges than subterranean terrestrial troglobites (Lamoreaux, 2004). that is not the universal pattern. Several species of *Kerkia* Radoman, 1978, that inhabit subterranean waters in the same region of the Balkans, are highly geographically restricted. Above the species-level they form three clades, also reflecting geography, thus the level of endemism in *Kerkia* is high (Hofman et al., 2022a, b).

The genus *Belgrandiella* A. J. Wagner, 1928, inhabits subterranean waters and springs in Slovenia, Austria and northern Italy. Some species are also known from Croatia, Bosnia and Herzegovina and Montenegro (Radoman, 1975, 1983; Giusti & Pezzoli, 1980; Kabat & Hershler, 1993; Haase, 1994, 1996). WORMS (WoRMS, 2021) lists 93 records for the genus. Although many of them are now classified as belonging to other genera, still more than 40 nominal *Belgrandiella* species exist: Nearly all of them were described based only on the characters of the shell. Thus, relatively rich reports on the distribution of the nominal species—even of more than one living in sympatry at some caves or springs (e.g., Radoman, 1975, 1983; Bole, 1979; Sket, 1986, 1993, 1999; Bole et al., 1993; Žagar, 2012), are not a reliable source of information about real endemism, intra- and interspecies diversity, gene flow or speciation. Some

molecular studies (Falniowski & Beran, 2015; Osikowski et al., 2018) have already reduced the number of valid species within this genus. The aim of the present paper is to check the inter- and intraspecies genetic diversity in (mostly Slovenian) *Belgrandiella* with mitochondrial cytochrome c oxidase subunit I—COI, nuclear histone H3, and RAPD analysis. The snails from caves, springs and interstitial aquifers are analysed to estimate the level of gene flow in order to determine whether these snails form a genetically uniform and widespread metapopulation (as *Montenegrospeum*), or they rather follow the highly endemic pattern found in *Kerkia*.

Material and methods

The snails were collected at 22 localities in Slovenia (Table 1, Figs. 1, 2). Seven of the localities (10–16) were in the cave Planinska jama ("jama" means cave in Slovenian). Planinska jama is the largest water cave in Slovenia, a karst cave that opens on the southern edge of Planinsko polje, near the village of Planina (Kogovšek et al., 2010). It consists of 6656 m of underground tunnels and is part of Postojna-Planinska jama System (hereafter referred to as PPCS). Into the Pivka channel of Planinska jama, flows the Pivka River that sinks in the cave Postojnska jama. Into the Rak channel of the Planinska jama, flows the Rak River that sinks into Tkalca jama. The Rak and Pivka Rivers both flow within their channels for about 2 km before they join together to form the Unica River about 500 m from the cave entrance. The side channels in both main channels are on upper level, they are older and mostly dry. They contain large amounts of cave sediments, especially alluvium brought in the cave by the underground Pivka River. The location of the debris indicates that parts of the cave have been completely filled at least once in the past. Most of the sediments are younger than 200,000 years, the cave developed over presumably more than two million years, with a more or less stable hydrological system between the Pivka Basin and Planinsko Polje (Kogovšek et al., 2010). Planinska jama is one of the underground habitats with high biodiversity, including the olm *Proteus anguinus* Laurenti, 1768, endemic species of amphipod *Niphargus* Schioedte, 1849, isopods *Monolistra* Gerstaecker, 1856, *Asellus aquaticus cavernicolus* Racovitza, 1925 and *Titanethes albus* (C. Koch, 1841), cave shrimp *Troglocaris planinensis* Birstein, 1948, cave hydrozoan *Velkovrhia enigmatica* Matjašič & Sket, 1971, and eight (Culver & Sket, 2000) mostly stygophile gastropod species (including terrestrial *Zospeum* Bourguignat, 1856 and pulmonate aquatic *Ancylus fluviatilis* O. F. Müller, 1774), whose empty, two to three millimeter-sized shells form dune-like thanatocenoses at some places. According to Sket (1999) in the Planinska jama PPCS 19, and according to Zagmajster et al. (2021) nine (in this seven Truncatelloid) aquatic gastropod species were found, and the number of troglobiotic and stygobiotic animal species approaches 120, similarly high number can be found only in Vjetrenica Cave in Bosnia and Herzegovina (Culver & Pipan, 2009).

Also the material from earlier studies, collected at 14 other localities in Slovenia, Croatia, and Bosnia and Herzegovina (Table 1, Fig. 1) was considered. Empty shells and live specimens were obtained from caves, springs and wells by different sampling techniques: manual collection, washing of vegetation and substratum, the use of either hand-net or metal sieve, sometimes fitted with an extended handle (3 m) to enable sweeping through the water and substrate at the bottom. The Bou–Rouch method (Bou & Rouch, 1967) was used to sample an interstitial fauna inhabiting the spring or river hyporheic zone, at the cca. 50 cm depth. The sampling procedure was repeated 5 times at each site, and 20 L of a mixture of water, sediments and particulate organic matter were pumped each time. The mixtures were sifted through a hand-net of 500 µm mesh size and fixed in 80% analytically pure ethanol, replaced two times. The samples were sorted later, and the snails were put in fresh 80% analytically pure ethanol and kept at -20 C in a freezer.

The shells of the sequenced specimens were photographed with a CANON EOS 50D digital camera, under a NIKON SMZ18 microscope with dark field. Measurements of these shells, following the scheme given by Szarowska (2006; Fig. 44) were taken using ImageJ image analysis software (Rueden et al., 2017). The linear measurements were then logarithmically transformed; for angular measurements, the arcsine transformation was applied. Principal component analysis (PCA), based on the matrix of correlation, was computed, applying a descriptive, non-stochastic approach. The original observations were projected into PC space, to show the grouping of the

Table 1 Sampling localities with geographical coordinates

Localities of the new sampling		Coordinates		mOTU
Id	Locality			
1	Paka River – S36, Slovenia	46.3928	15.1671	F
2	Spring in Potoče – S27, Slovenia	46.3009	14.4595	B
3	Spring in Potoče – S26, Slovenia	46.3006	14.4614	B
4	Kokra River – S28, Slovenia	46.3003	14.4661	B
5	Močilnik spring – S32, Slovenia	45.9544	14.2923	A, F
6	Krška jama – S5, Slovenia	45.8898	14.7712	A, E
7	Krka River 1 – S2, Slovenia	45.8897	14.7714	A, E
8	Krka River 2 – S1, Slovenia	45.8866	14.7683	A
9	Fužina—S6, Slovenia	45.8595	14.8320	B
10	Planinska jama – next to entrance—Z, Slovenia	45.8209	14.2466	A
11	Planinska jama – 150 m S from entrance – PL1, Slovenia	45.8203	14.2465	A
12	Planinska jama – 300 m W from PL2 – PL4, Slovenia	45.8196	14.2457	A
13	Planinska jama – 300 m S from entrance – PL2, Slovenia	45.8196	14.2465	A
14	Planinska jama – 200 m E from PL2 –PL10, Slovenia	45.8194	14.2485	A
15	Planinska jama – 700 m S from PL4 – PL6, Slovenia	45.8169	14.2454	A
16	Planinska jama – 300 m S from PL6 – PL8, Slovenia	45.8148	14.2445	A
17	Rak River, Rakov Škocjan – S16, Slovenia	45.7915	14.3059	A
18	Žerovnica 1 – S9, Slovenia	45.7631	14.4356	A
19	Žerovnica 2 – S10, Slovenia	45.7619	14.4342	A
20	Žerovnica 3 – S11, Slovenia	45.7603	14.4260	A
21	Lipsenj 1 – S13, Slovenia	45.7471	14.4445	A
22	Lipsenj 2 – S12, Slovenia	45.7446	14.4454	A
	Localities of the reference sampling			
23	spring (2), Potoče, Preddvor, Kranj, Slovenia	46.3164	14.4606	B, C
24	spring (1), Potoče, Preddvor, Kranj, Slovenia	46.1864	14.2656	B
25	cave Babja luknja, Goričane, Medvode, Slovenia	46.1342	14.3917	A, C
26	cave Raja peč, Log, Sevnica, Slovenia	45.9947	15.2700	A, C
27	Dvorce, Čatež ob Savi, Slovenia	45.8858	15.6139	A
28	cave Rupa na Brodu, Brod, Novo mesto, Slovenia	45.7889	15.1444	A
29	Rakovski potok, Rakek, Slovenia	45.7775	14.1856	A
30	spring of river Žerovniščica, Žerovnica, Slovenia	45.7633	14.4363	A
31	spring of river Lipsenjščica, Cerknica, Slovenia	45.7450	14.4458	A
32	spring Veliki Obrh, Lož, Slovenia	45.6992	14.5108	A
33	Draga Bašćanska spring Žange, Krk Island, Croatia	45.0217	14.6825	A
34	spring by the Krupa River, Krupa, Croatia	44.1942	15.9094	A
35	Dramotići, a spring near Zrmanja River, Croatia	44.1919	15.7847	A
36	Izvor Plive 1A, Draganić, Bosina & Hercegovina	44.2406	17.0169	D

Haplotype and mOTU are also indicated. Grey font indicates localities where only H3 have been obtained. Localities 18 and 30, as well as 21 and 31 are very close

specimens, without any classification given a priori. For PCA analysis ClustVis 2.0 web tool (Metsalu & Vilo, 2015) was used.

DNA was extracted from whole specimens; tissues were hydrated in TE buffer (3×10 min); then total genomic DNA was extracted with the SHERLOCK extraction kit (A&A Biotechnology), and the final product was dissolved in 20 µl of tris–EDTA (TE) buffer. The extracted DNA was stored at − 80 °C at the Department of Malacology, Institute of Zoology

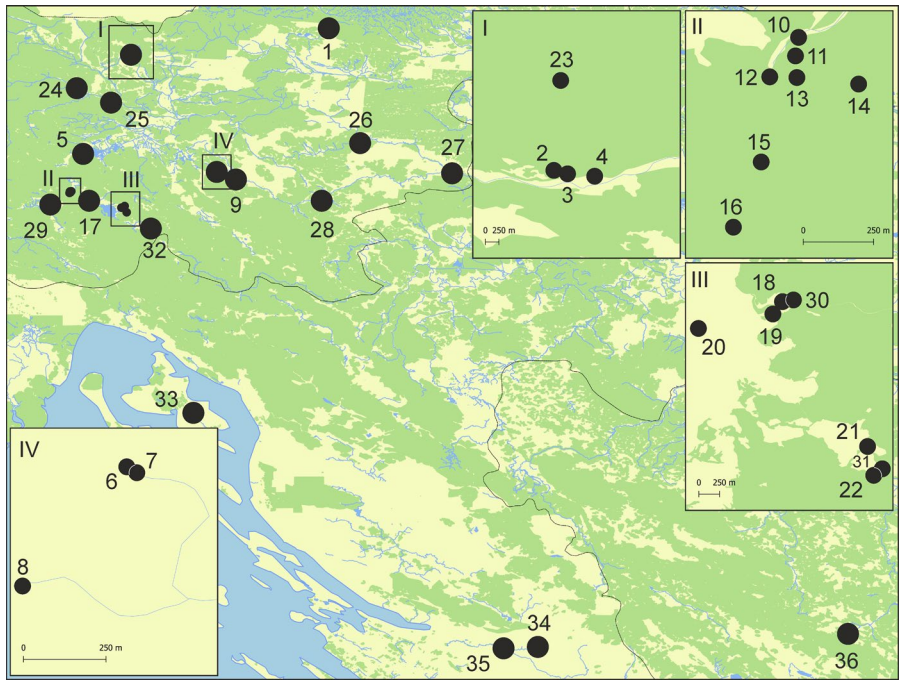

Fig. 1 Localities of the studied populations of *Belgrandiella*. Numbers correspond to localities list given in Table 1

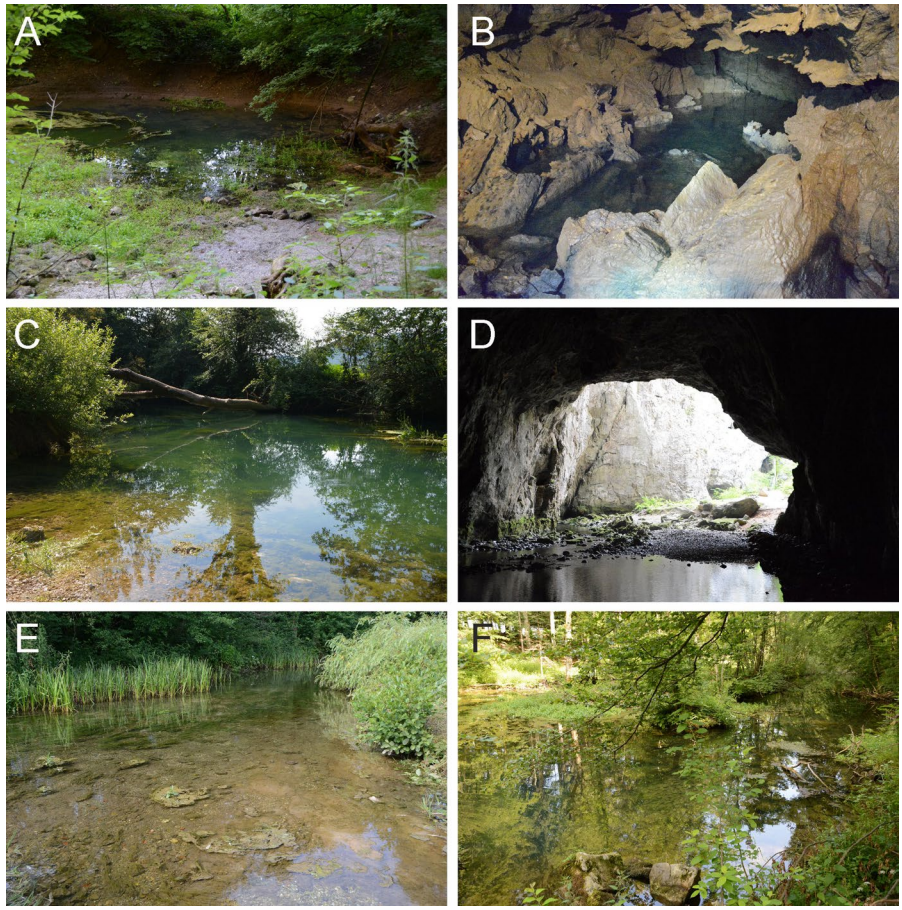

Fig. 2 Some of the studied localities: **A** locality 5, **B** locality 6, **C** locality 7, **D** locality 17, **E** locality 19, **F** locality 22

and Biomedical Research, Jagiellonian University in Kraków (Poland).

A fragment of mitochondrial cytochrome c oxidase subunit I (COI) and four nuclear fragments (18S ribosomal RNA – 18S, 28S ribosomal RNA – 28S, histone H3 – H3 and Internal Transcribed Spacer 2 – ITS2) were sequenced. Details of PCR conditions, primers used, and sequencing technique were given in Szarowska et al. (2016b), Hofman et al. (2022a, b). The Sanger sequencing was performed in Genomed Company in Warsaw, Poland. Sequences were initially aligned in the MUSCLE (Edgar, 2004) program accessed through MEGA 7 (Kumar et al., 2016). The correctness of the alignment was checked in BIOEDIT 7.2.5 (Hall, 1999), this program was also used to translate of sequences and check for reading frame and stop codons. Uncorrected p-distances were calculated in MEGA 7. The estimation of the proportion of invariant sites and the saturation test for the coding loci COI and H3 (Xia, 2000; Xia et al., 2003) were performed using DAMBE 7 (Xia, 2013). In the phylogenetic analysis additional sequences from GenBank were used (Table 2). The phylogenetic analysis was performed applying two approaches: Bayesian inference (BI) and maximum likelihood (ML). In the BI analysis, MRMODELTEST 2.3 (Nylander, 2004) was applied for each partition to find the best-fit model of nucleotide substitution.

The Bayesian analyses were run using MrBayes v. 3.2.3 (Ronquist et al., 2012) with defaults of most priors. Two simultaneous analyses were performed, each with 10,000,000 generations, with one cold chain and three heated chains, starting from random trees and sampling the trees every 1000 generations. The first 25% of the trees were discarded as burn-in. The analyses were summarised as a 50% majority-rule consensus tree. Convergence was checked in Tracer v. 1.5 (Rambaut & Drummond, 2009). FigTree v.1.4.4 (Rambaut, 2010) was used to visualise the trees. The maximum likelihood analysis was conducted in RAxML v. 8.2.12 (Stamatakis, 2014) using the 'RAxML-HPC v.8 on XSEDE (8.2.12) tool via the CIPRES Science Gateway (Miller et al., 2010). Rapid bootstrap and autoMRE bootstopping criterion were used. We applied the GTR + G model, whose parameters were estimated for each partition by the RAxML (Stamatakis, 2014). It has been suggested that using the most parameter-rich model GTR + I + G instead of conducting thorough model selection leads to phylogenies with topologies (although not necessarily branch lengths) the same as those obtained when model selection is performed, or better, since the programs like ModelTest are sometimes definitely

Table 2 Reference sequences used in phylogeny analysis

Species	COI GB numbers	COI references	H3 GB numbers	H3 References
Agrafia wiktori Falniowski and Szarowska (2011)	JF906762	Szarowska & Falniowski, (2011)	MG543158	Grego et al., 2017
Daphniola louisi Falniowski & Szarowska (2000)	KM887915	Szarowska et al., (2014a)	MZ265364	Hofman et al., (2021)
Dalmatinella fluviatilis Radoman (1975)	KC344541	Falniowski & Szarowska, (2013)	unpublished	Hofman et al., (2021)
Ecrobia maritima (Milaschewitsch, 1916)	KX355835	Osikowski et al., (2016)	MG551322	Grego et al., 2017
Graziana alpestris (Frauenfeld, 1863)	AF367641	Wilke et al., (2001)	-	
Graecoarganiella parnassiana (Falniowski & Szarowska, 2011)	JN202352	Falniowski & Szarowska, (2011b)	MZ265362	Hofman et al., (2021)
Hauffenia michleri (Kuščer, 1932)	KT236156	Falniowski & Szarowska, (2015)	KY087878	Rysiewska et al., (2017)
Horatia klecakiana Bourguignat, 1887	KJ159128	Szarowska & Falniowski, (2014b)	unpublished	Hofman et al., (2021)
Iglica cf. *gracilis* (Clessin, 1882)	MH720985	Hofman et al., (2021)	MH721003	Hofman et al., (2018)
Islamia zermanica (Radoman, 1973)	KU662362	Beran et al., (2016)	MG551320	Grego et al., (2017)
Montenegrospeum bogici (Pešić & Glöer, 2012)	KM875510	Falniowski et al., (2014)	MG880218	Grego et al., (2018)

misleading (our observations). This implies that the standard practice of model selection before phylogeny inference is unnecessary when a complex and parameter-rich model is applied to nucleotide data (Abadi et al., 2019; our results), as long as the parameters are estimated separately for each partition. However, combining a gamma distribution (G) with a proportion of invariant sites (I) has been criticized (e.g., Sullivan et al., 1999; Mayrose et al., 2005; Yang, 2006; Jia et al., 2014; Stamatakis, 2014) mainly since some of the parameters of the + G and + I models cannot be optimized independently of each other, thus rather GTR + G model should be applied. Two methods of species delimitation, analysing COI sequences, were performed: Poisson Tree Processes (bPTP) (Zhang et al., 2013) and Automatic Barcode Gap Discovery (ABGD). The bPTP approach (bPTP) was run using the web server https://species.h-its.org/ptp/, with 100,000 MCMC generations, 100 thinning and 0.1 burn-in. We used RAxML output phylogenetic tree. The ABGD approach using the web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) and the default parameters. To infer haplotype network of the COI marker, we used a median-joining calculation implemented in NETWORK4 (Bandelt & Röhl, 1999). The parameters describing a genetic variation and other statistics were calculated with DnaSP 6.12.03 software (Librado & Rozas, 2009) and Arlequin v. 3.5 (Excoffier & Lischer, 2010). The latter program was used to analyze molecular variance (AMOVA). The geographical distances between the localities were calculated with Geographic Distance Matrix Generator v. 1.2.3 (Ersts, 2020). Mantel tests for association between the matrices and UPGMA clustering were performed with NTSYSpc 2.02 g (Rohlf, 1998). To apply the molecular clock, the data from COI were used. Two hydrobiids, *Peringia ulvae* (Pennant, 1777) and *Salenthydrobia ferreri* Wilke, 2003 (AF118302, Wilke & Davis 2000; AF449213, Wilke 2003), were used as outgroups. The divergence time of the lineage of *Peringia* (Atlantic) with the linage comprising *Salenthydrobya* + *Achaiohydrobia* (Mediterranean) was used to calibrate the molecular clock, with correction according to Falniowski et al. (2008). The likelihood ratio test (LRT) (Nei & Kumar 2000) were calculated with PAUP. The relative rate test (RRT) (Tajima 1993) was performed in MEGA. As Tajima's RRTs and the LRT test rejected the equality of evolutionary rates throughout the tree for *Belgrandiella*, time estimates were calculated using a non-parametric rate smoothing (NPRS) analysis with the recommended Powell algorithm, in r8s v.1.7 for Linux (Sanderson 1997, 2003).

Details of randomly amplified polymorphic DNA (RAPD) analysis, primers used, and amplification technique were given in Falniowski et al. (2021a). The amplification products were separated in 2% agarose gel with ethidium bromide in TBE buffer. The banding patterns were visualised under UV light. The bands were scored visually, and those of similar molecular size (for the same primer) were assumed to be homologous. Each band was treated as an independent locus with two alleles: presence or absence of the band. The PopGen 32 software package (Yeh & Yang, 2000) was used to estimate the genetic variation. The following parameters were calculated: mean number of alleles per locus, effective number of alleles per locus, percentage of polymorphic loci and Nei's (1973) gene diversity, the Shannon index. This same software was used to calculate the Nei's genetic distances between populations.

Results

The shells of the studied *Belgrandiella* (Fig. 3) were either broadly conic or barrel-shaped, and highly variable in size. Some specimens were pigment- and eyeless (Figs. 3A, D, G-L, N-Q, and S-T), while the other showed no troglomorphism—their mantle was black pigmented, sometimes intensively, the eyes present. The principal component analysis (PCA) on seven shell biometric parameters (Fig. 4) demonstrated that the shell biometry of the six mOTUs (inferred from the mitochondrial COI, A-F, see below) slightly reflects their distinctness, since their variability ranges broadly overlap. The biggest ellipsoid contained specimens of *B. kusceri* (mOTU A), found in 23 from 36 (64%) populations. The PC1, reflecting mostly the size and describing about 65% of the total variance, ranged above three times more than each of the other five species. The PC2 and PC3, reflecting mostly differences in shell shape, showed different pattern. In the PC2 the range of the mOTU F was only slightly less wide than that of the mOTU A, and in PC3 the ranges were the same. For both PC1 and PC2 the highest character loadings were for the angles α (apex angle measured between the lines

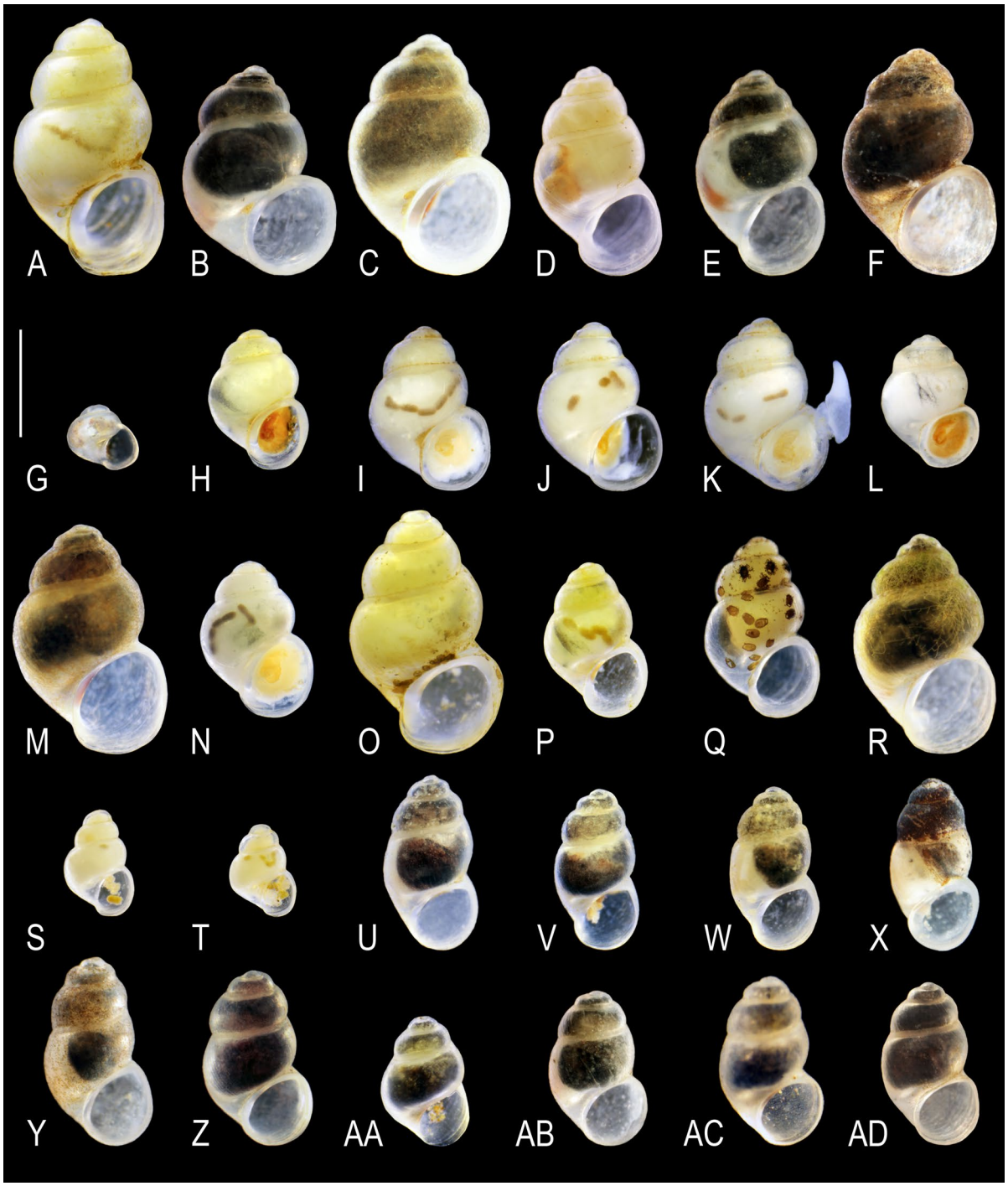

Fig. 3 Shells of the sequenced *Belgrandiella*: A-R – mOTU A, S-T – mOTU E, U-Y – mOTU F, Z-AD – mOTU B; A – 1T1, B – 2O36, C – 1U40, D – 1P28, E – 2O34, F – 2O40, G – 1S54, H – 1S46, I – 2O2, J – 203, K – 2O5, L – 2D72, M – 1U58, N – 1S17, O – 1T2, P – 1S51, Q – 1P12, R – 1U55, S – 1S18, T – 1S43, U – 1W22, V – 1W23, W – 1W24, X – 1W12, Y – 1W13, Z – 1U84, AA – 1S35, AB – 2O15, AC – 1U80, AD – 2O72. The extraction numbers correspond to Figs. 5–7. Bar equals: 1 mm

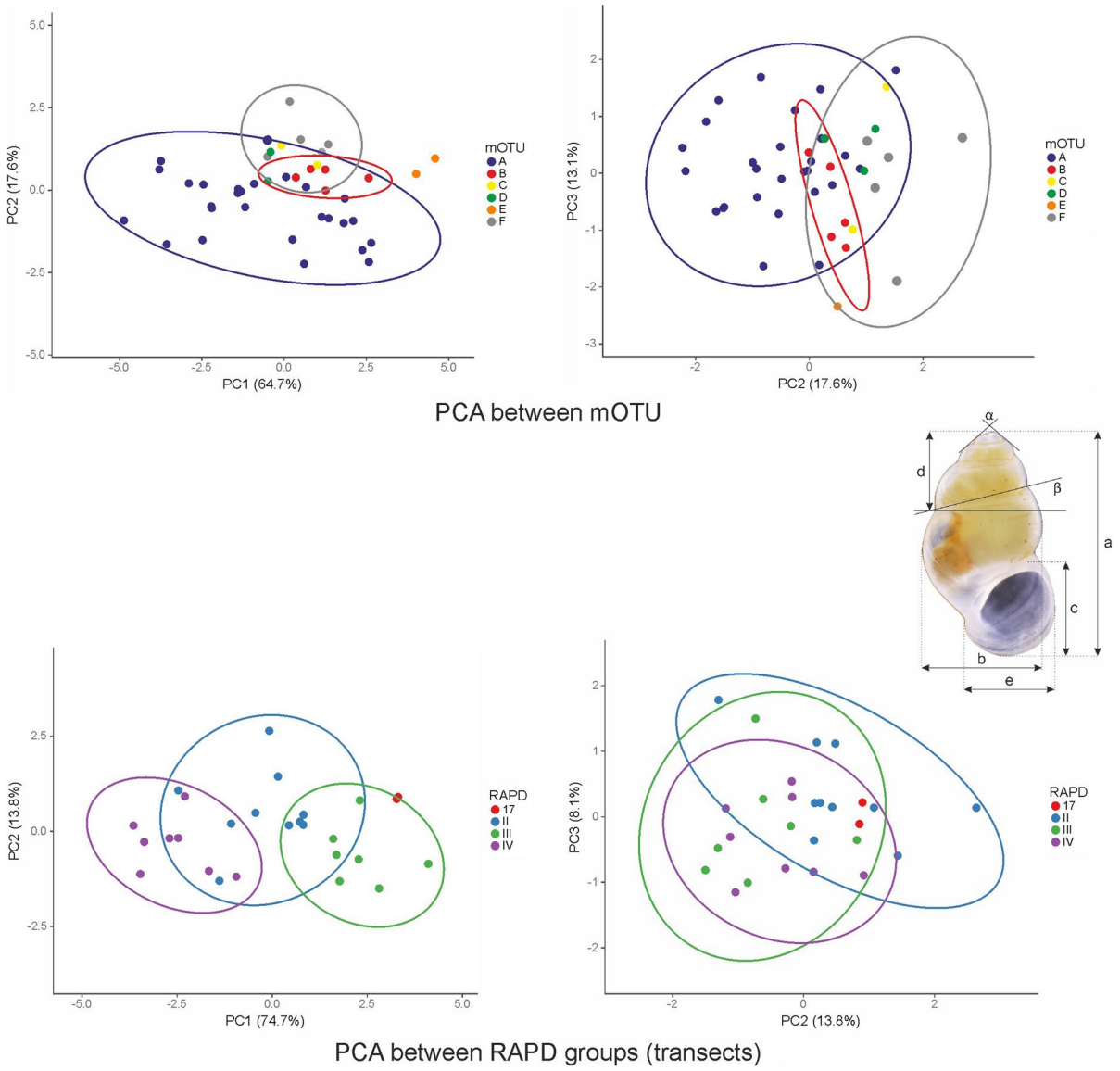

Fig. 4 Principal component analysis (PCA) on the shell of *Belgrandiella*, for different mOTUs (above) as well as for different groups within *B. kusceri*, distinguished with RAPD analysis for transects II, III, IV and locality 17 (below). Measured variables: a – shell height, b – body whorl breadth, c – aperture height, d – spire height, e – aperture breadth, α – apex angle measured between the lines tangential to the spire, β – angle between the body whorl suture and the line perpendicular to the columella

tangential to the spire) and β (angle between the body whorl suture and the line perpendicular to the columella). The loading of all the other characters on the PC3 were similarly low. For the PC2 higher values were computed for the body whorl breadth and aperture height, and stronger but negatively correlated—for the spire height. The three transects found for the RAPD in *B. kusceri* (Fig. 1) differ only in PC1 (Fig. 4). The transects III and IV did not overlap, but the transect II overlapped with both III and IV.

We obtained 56 new sequences of COI (457 bp, GenBank accession numbers OP804353-OP804408), 28 of H3 (310 bp, GenBank accession numbers OP824660-OP824687), 28 of the ITS2

(130 bp, GenBank accession numbers OP829059-OP829086), 28 of 28S (497 bp, GenBank accession numbers OP829115-OP829142), and 28 of 18S (401 bp, GenBank accession numbers OP829087-OP829114). The tests by Xia et al. (2003) revealed no saturation for COI and H3. Results from the substitution saturation analysis showed an ISS (0.65 for COI; 0.47 for H3) significantly smaller than the critical ISS value (ISSC: 0.94 for COI; 0.62 for H3). In all analyses, the topologies of the resulting phylograms were identical in both the ML and BI, for both COI and H3 sequences. For 18S, ITS2 and 28S all sequences were identical.

For all the sequences of cytochrome c oxidase subunit I (COI) for *Belgrandiella* (new and reference ones) we obtained 21 haplotypes with haplotype diversity (Hd) 0.895 ± 0.024. In total 61 sites were polymorphic (segregating) site and nucleotide diversity per site (Pi) was 0.0334 ± 0.0045. For H3 we obtained seven haplotypes with Hd 0.701 ± 0.051. In total 16 sites were polymorphic (segregating) site and Pi was 0.0117 ± 0.0013.

All the sequences of *Belgrandiella* belonged to one monophyletic clade in the tree based on histone H3 (Fig. 5). However, in the tree computed for COI (Figs. 6, 7), not all sequences of *Belgrandiella* formed a monophyletic clade, sister to *Graziana alpestris* (Frauenfeld, 1863), p-distance 0.11. There were four deviating sequences, identified as *Belgrandiella* cf. *fontinalis* (Schmidt, 1847) (mOTU C) and *B.* cf. *koprivnensis* Radoman, 1975 (mOTU D), which did not confirm their assignment to the genus *Belgrandiella*. In the clade of *Belgrandiella* inferred from COI, four mOTUs could be distinguished: *B. kusceri* (A. J. Wagner, 1914) (mOTU A, in 28 populations, although the COI sequences only for 23 of them) and *B. cf. kuesteri* (Boeters, 1970) (mOTU B). mOTU E was identified as *B. crucis* (Kuščer, 1928), and mOTU F as *Belgrandiella.* sp. Within the clade of *Belgrandiella* inferred from COI, the p-distances between mOTUs varied from 0.052 to 0.099 (Table 3). *B.* cf. *koprivnensis* and *B.* cf. *fontinalis* were more distinct from the other mOTUs (p-distance 0.082–0.121, Table 3). The species distinctness was confirmed by both used methods of species delimitation (for bPTP posterior probabilities were higher than 0.95). The geographic distribution of the mOTUs is shown in Fig. 8. While the mOTU A is distributed throughout the studied area, the mOTU B is restricted to the central Slovenia. The mOTU C is found at two localities in northern Slovenia and the mOTU D at a single, southernmost locality. Both the mOTUs E and F are also restricted to two Slovenian localities; the former to locations less than 20 m apart and the latter to localities 80 km apart. The estimated

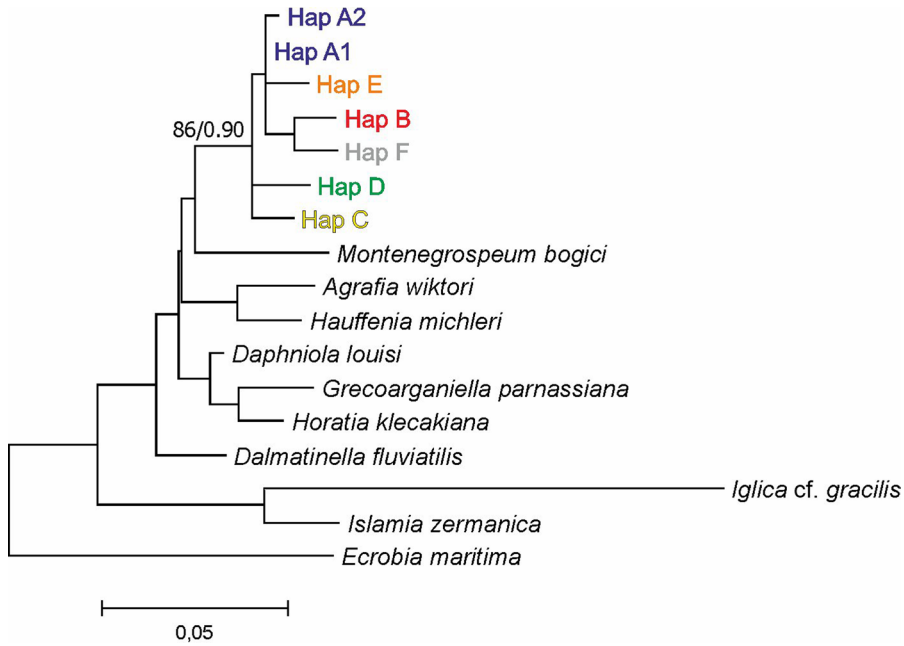

Fig. 5 Maximum likelihood H3 tree showing phylogenetic relationships between the studied snails. Bootstrap support (> 70%) and Bayesian posterior probabilities (0.95) are shown

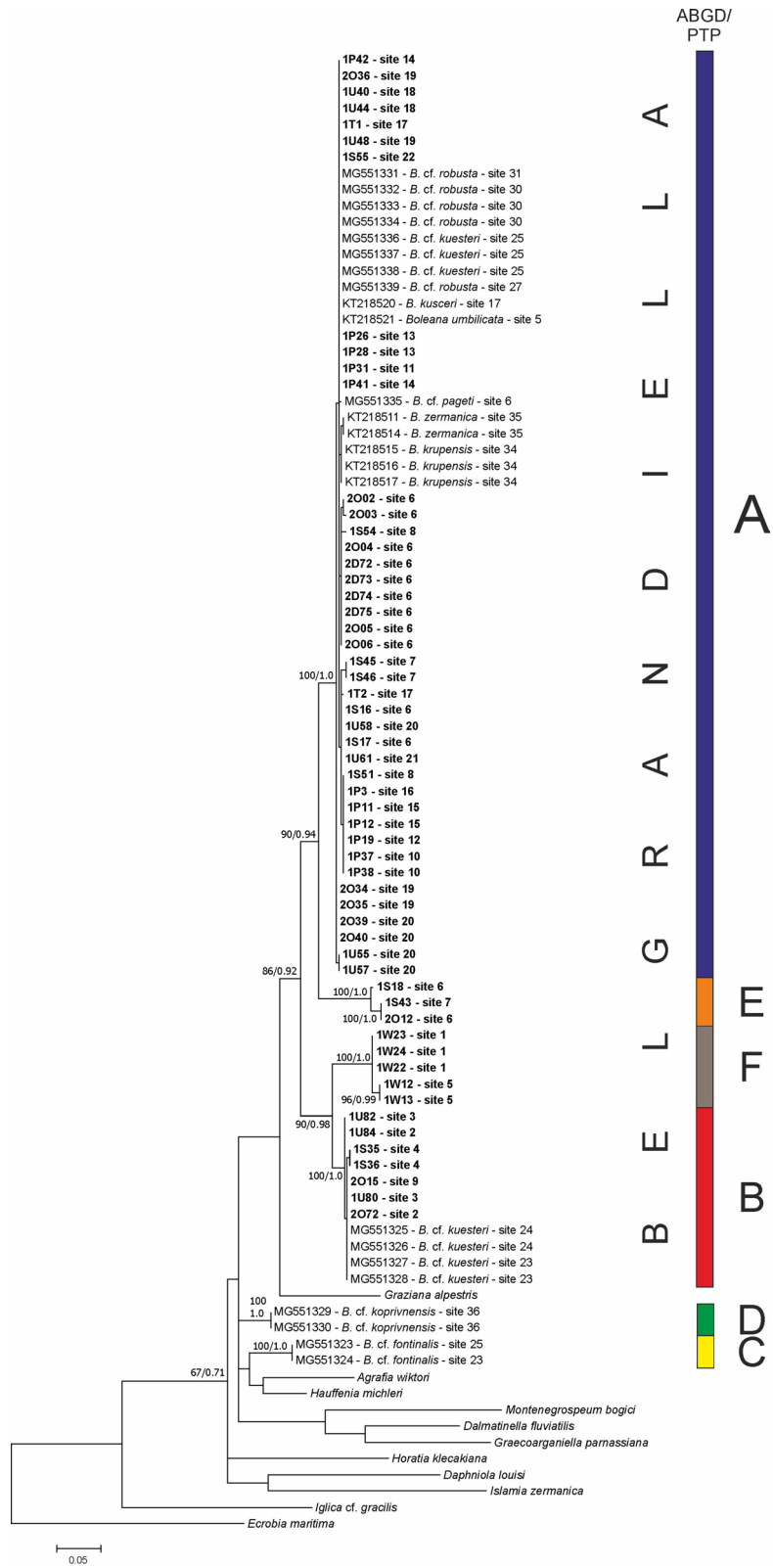

Fig. 6 Maximum likelihood COI tree showing phylogenetic relationships between the studied snails. Bootstrap support (> 65%) and Bayesian posterior probabilities (> 0.9) are shown. Results of the ABGD and bPTP analysis and division into the mOTUs are also shown

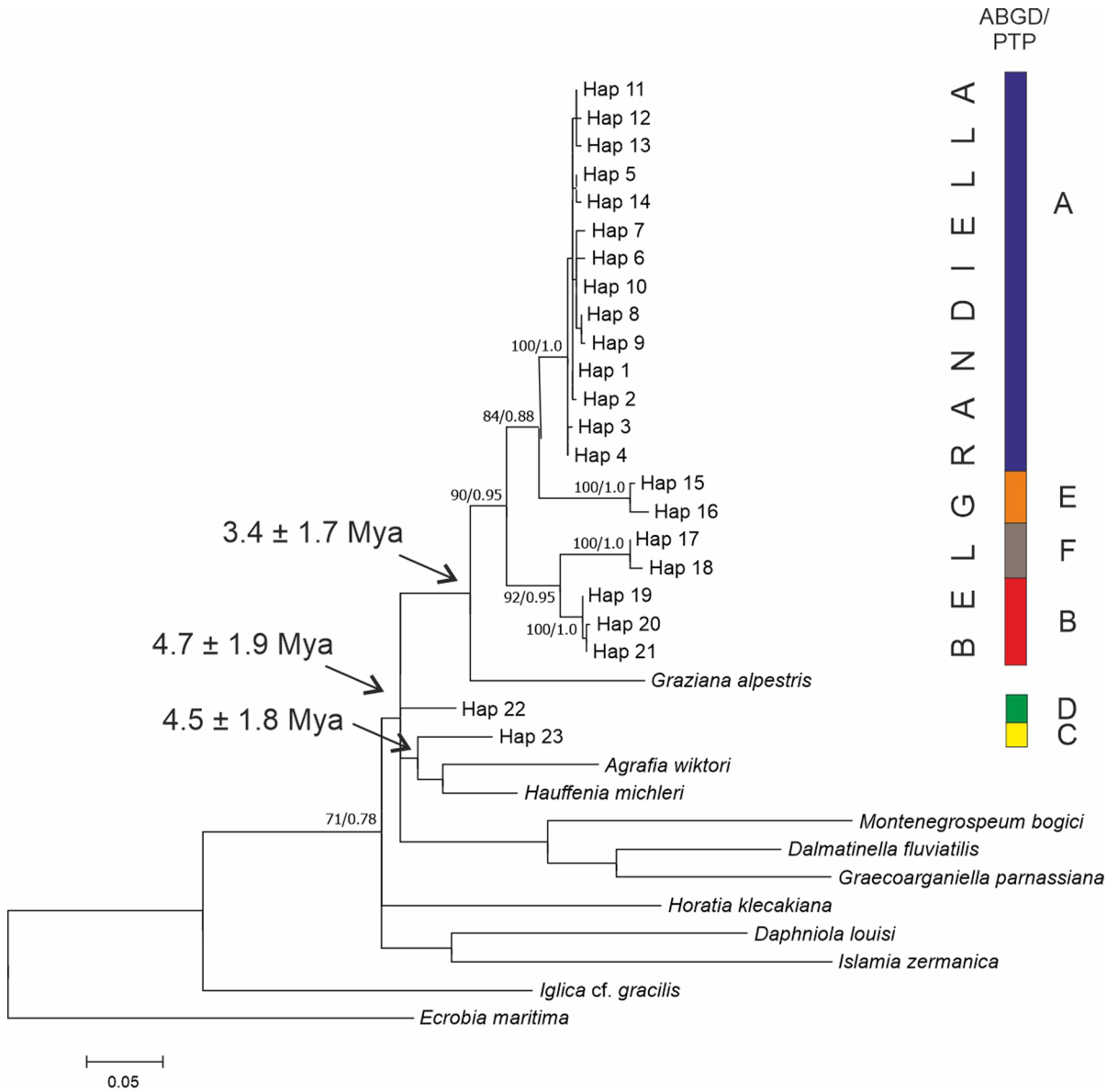

Fig. 7 Maximum likelihood COI tree for haplotypes. Bootstrap support (>70%) and Bayesian posterior probabilities (>0.75) are shown. Results of the ABGD and bPTP analysis and division into the mOTUs are also shown. The estimated divergence times between the three main lineages are also indicated

Table 3 Pairwise p-distances for the studied populations, below diagonal for COI, above diagonal for K3; intrapopulation p-distances for COI along diagonal (italic, bold)

	mOTU A	mOTU B	mOTU E	mOTU F	mOTU C	mOTU D
mOTU A	***0.005***	0.019	0.012	0.019	0.015	0.019
mOTU B	0.072	***0.002***	0.024	0.020	0.031	0.034
mOTU E	0.066	0.099	***0.009***	0.027	0.024	0.024
mOTU F	0.084	0.052	0.097	***0.005***	0.031	0.027
mOTU C	0.093	0.101	0.107	0.121	***0.000***	0.024
mOTU D	0.085	0.082	0.094	0.099	0.066	***0.000***

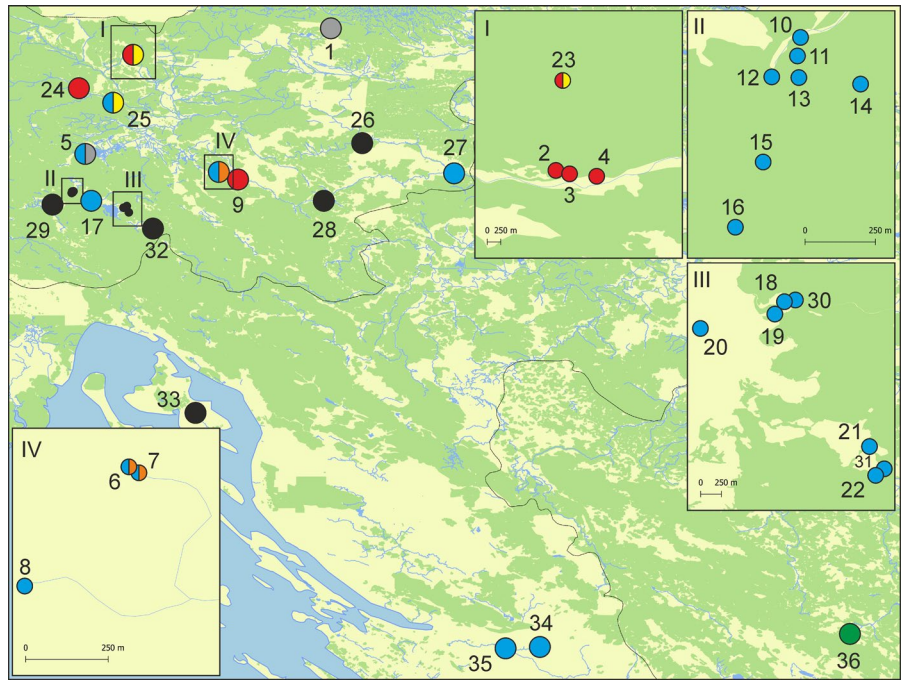

Fig. 8 Geographic distribution of the mOTUs of the *Belgrandiella*. Dot colors correspond with Figs. 6 and 7

divergence times between the three main lineages are shown in Fig. 7.

Two of H3 haplotypes were characteristic for mOTU A, the other were characteristic each for one of the mOTUs. Individuals from five populations, not sequenced for the COI fragment, will cluster within mOTU A or C on the basis of H3 sequences (Table 1). For this locus all sequences belonged to one, well supported lineage. The p-distances between mOTUs varied from 0.012 to 0.034 (Table 3). From among 36 localities studied, at six (5, 6, 7, 23, 25 and 26) two species occurred in sympatry. It has to be stressed that interspecies distances for histone H3 were unusually high (Table 3). For COI AMOVA (Table 4) clearly assigned 94.34% of the total variance to the interspecies (between the mOTUs) differences. Within the mOTUs only 2.90% of the total variance was estimated, and nearly the same (2.76%) within populations.

The mOTU A, identified as *B. kusceri*, was most rich in the sequenced specimens (60) and in the studied populations (23), as well as most widely distributed geographically. Thus, it was possible to analyse its genetic structure, especially the pattern of the gene flow between the local (sub)populations. In this mOTU, 60 sequences represented 16 haplotypes, with $Hd = 0.847 \pm 0.036$. Number of polymorphic (segregating) sites was 35 and nucleotide diversity (per site) 0.015 ± 0.003. Haplotypes network is shown in Fig. 9. Neighbouring haplotypes differed mainly by one substitution, two times by two substitutions. The geographic distribution of the haplotypes is shown in Fig. 10. The most numerous haplotype 1 was found at twelve localities across Slovenia. The most interesting

Table 4 Results of AMOVA for COI: percentage of variation in all data and in three separated species

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among groups (mOTUs)	5	521.865	13.80547 Va*	94.34
Among populations within groups (mOTUs)	30	40.210	0.4243 Vb*	2.90
Within populations	44	17.750	0.4034 Vc*	2.76
Total	79	579.825	14.6332	

Statistical significance
*$P < 0.001$

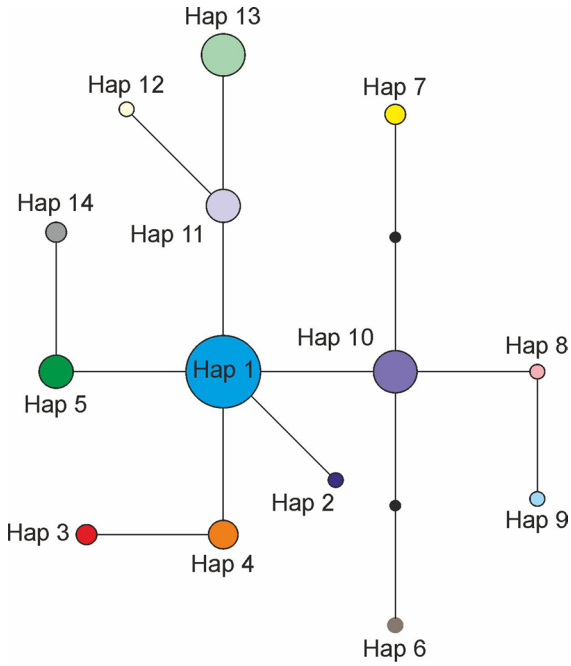

Fig. 9 Haplotype network (non-hierarchical, no dichotomous tree) of the studied populations of *Belgrandiella kusceri*. The dots colors correspond to Fig. 10

population inhabited the cave Krška jama (locality 6), where five different haplotypes were found, four of them belonging to *B. kusceri*. Moreover, the latter haplotypes were unique for this population. In Žerovnica (locality 20), *B. kusceri* was represented by three haplotypes, and at the locality 8 it was represented by two haplotypes (different by five substitutions), 17, 12 and 19. Interestingly, in seven subpopulations (10–16) inhabiting the biggest water cave Planinska jama, only two haplotypes were found.

Pairwise p-distances between the populations of *B. kusceri* in some cases approached 0.032. Mantel test, checking the association between the matrices of pairwise p-distance and geographic distance, thus testing the isolation-by-distance model, with 9999 permutations, resulted in p [random Z ≤ observed Z] = 0.6799, thus no significant association was found. The test between the matrices of geographic distance and pairwise F_{ST} (θ) (Table 5, Fig. 11), with 9999 permutations, resulted in p [random Z ≤ observed Z] = 0.0066, thus the association was highly significant. Pairwise theta (F_{ST}), Fisher's estimator of the amount of structuring of a population into subpopulations, in general was high, with exception of populations sharing the same haplotype, then F_{ST} equalled 0, thus Nm infinity. In all the other cases Nm were low. Thus, this

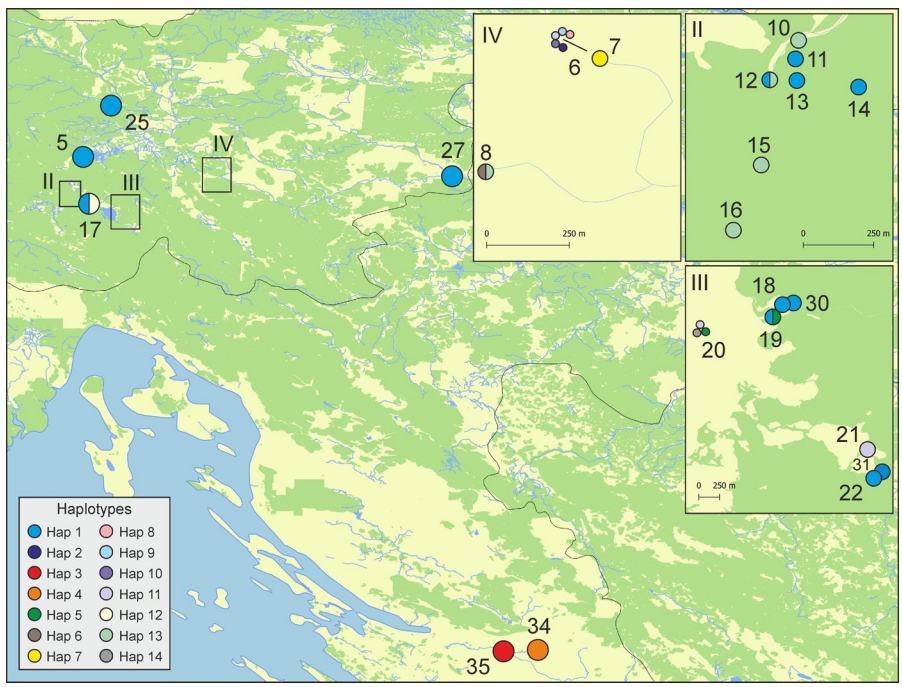

Fig. 10 Geographic distribution of the haplotypes of *Belgrandiella kusceri* (mOTU A)

Table 5 Pairwise F_{ST} (θ) between populations within mOTU A

	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	25	27	30	31	34
6	− 0.0788																					
7	1.0000	0.5378																				
8	− 1.0000	0.2262	0.2857																			
10	1.0000	0.6106	1.0000	0.0000																		
11	0.0000	− 0.0788	1.0000	− 1.0000	1.0000																	
12	1.0000	0.5377	1.0000	− 1.0000	0.0000	1.0000																
13	0.0000	0.1800	1.0000	0.0000	1.0000	0.0000	1.0000															
14	0.0000	0.1800	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000														
15	1.0000	0.6106	1.0000	0.0000	0.0000	1.0000	0.0000	1.0000	1.0000													
16	1.0000	0.5377	1.0000	− 1.0000	0.0000	1.0000	0.0000	1.0000	1.0000	0.0000												
17	− 1.0000	0.2558	0.7108	0.0282	0.5714	− 1.0000	0.3333	− 0.2000	− 0.2000	0.5714	0.3333											
18	0.0000	0.1800	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	0.0000	1.0000	1.0000	− 0.2000										
19	− 0.3333	0.3618	0.8597	0.3253	0.8049	− 0.3333	0.7333	0.1111	0.1111	0.8049	0.7333	0.1765	0.1111									
20	0.2857	0.4997	0.7590	0.3617	0.6552	0.2857	0.5455	0.4737	0.4737	0.6552	0.5455	0.3213	0.4737	0.2308								
21	1.0000	0.2964	1.0000	− 1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	− 0.3333	1.0000	0.5556	0.1667							
22	0.0000	− 0.0788	1.0000	− 1.0000	1.0000	0.0000	1.0000	0.0000	0.0000	1.0000	1.0000	− 1.0000	0.0000	− 0.3333	0.2857	1.0000						
25	0.0000	0.2568	1.0000	0.2500	1.0000	0.0000	1.0000	0.0000	0.0000	1.0000	1.0000	0.0000	0.0000	0.2500	0.5522	1.0000	0.0000					
27	0.0000	− 0.0788	1.0000	− 1.0000	1.0000	0.0000	1.0000	0.0000	0.0000	1.0000	1.0000	− 1.0000	0.0000	− 0.3333	0.2857	1.0000	0.0000	0.0000				
30	0.0000	0.2568	1.0000	0.2500	1.0000	0.0000	1.0000	0.0000	0.0000	1.0000	1.0000	0.0000	0.0000	0.2500	0.5522	1.0000	0.0000	0.0000	0.0000			
31	0.0000	− 0.0788	1.0000	− 1.0000	1.0000	0.0000	1.0000	0.0000	0.0000	1.0000	1.0000	− 1.0000	0.0000	− 0.3333	0.2857	1.0000	0.0000	0.0000	0.0000	0.0000		
34	1.0000	0.5499	1.0000	0.4828	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.6000	1.0000	0.7391	0.7321	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	
35	1.0000	0.6476	1.0000	0.4444	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.6757	1.0000	0.8049	0.7727	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000

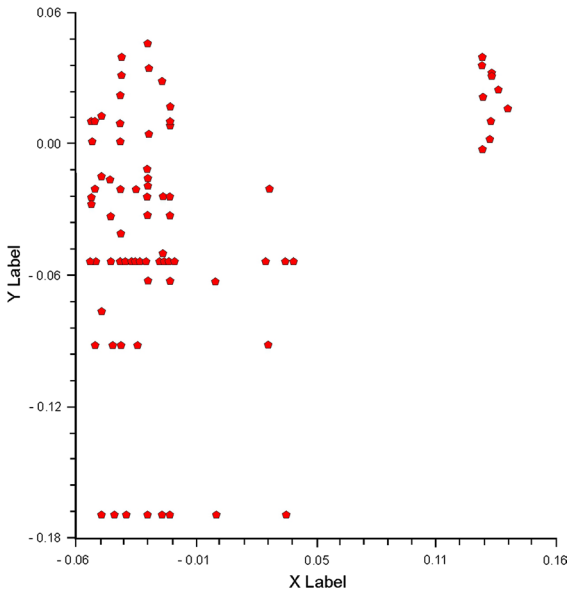

Fig. 11 Pairwise F_{ST} vs geographic distance within *Belgrandiella kusceri*

estimator derived from Fisher's F-statistics, reflected low migration in subdivided population.

In the RAPD analysis, each primer amplified from five to eight fragments, with an average of eight (overall 33 fragments), and among them 32 bands (96.97%) were polymorphic. Number of individuals and basic parameters of diversity are given in Table 6. Nei's (1973) gene diversity ranged from 0.014 to 0.137, and the Shannon index ranged from 0.020 to 0.265. Mantel test of association between the matrices of geographic distance and Nei's genetic distances (Table 7) with 9999 permutations, resulted in p [random Z ≤ observed Z] = 0.1531 thus not significant association was found. UPGMA clustering calculated for these Nei distances (Fig. 12) showed geographic pattern: (1) the cave Krška jama with two localities at the Krka River (populations 6–8, transect IV), (2) Žerovnica with Lipsenj (populations 18–22, transect III), (3) six populations in the Planinska jama (populations 10–16, transect II), and (4) one population from Rakov Škocjan (population 17). The geographical distribution of the RAPD bands for each of the primer are shown in Supp. Figure 1. It presents a similar differentiation, dependent on geographical distribution. In general, all three transects and population 17 differed in frequencies of particular bands and in populations there were often private bands. However, some bands were identified in all transects.

Discussion

Osikowski et al. (2018) partly revised the Balkan *Belgrandiella*, demonstrating its diversity overestimation, chaos in the species-level taxonomy and high variation in the shell morphology, with its ranges'

Table 6 Genetic variation parameters

Population	*N*	*Nm*	*Ne*	*P*	*He*	*Ho*
6	5	1.030	1.026	3.03	0.014	0.020
7	2	1.152	1.107	15.15	0.063	0.092
8	2	1.212	1.150	21.21	0.088	0.128
10	2	1.061	1.043	6.06	0.025	0.037
11	4	1.364	1.229	36.36	0.137	0.203
12	4	1.182	1.140	18.18	0.075	0.108
13	4	1.242	1.161	24.24	0.092	0.136
14	4	1.454	1.326	45.45	0.182	0.265
15	4	1.152	1.079	15.15	0.049	0.075
16	4	1.273	1.192	27.27	0.107	0.156
17	2	1.000	1.000	0.00	0.000	0.000
18	2	1.061	1.043	6.06	0.025	0.037
19	3	1.000	1.000	0.00	0.000	0.000
20	5	1.152	1.044	15.15	0.034	0.057
21	1	1.000	1.000	0.00	0.000	0.000
22	1	1.000	1.000	0.00	0.000	0.000

N number of individuals, *Nm* mean number of alleles per locus, N_e effective number of alleles per locus, *P* percentage of polymorphic loci, H_e Nei's (1973) gene diversity, H_o the Shannon index

Table 7 Nei's genetic distances between populations of *Belgrandiella kusceri* based on RAPDs analysis

	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21
7	0.0446														
8	0.2111	0.1694													
10	0.1428	0.1297	0.2141												
11	0.2627	0.2095	0.1927	0.1607											
12	0.2173	0.1459	0.1791	0.1438	0.0688										
13	0.2225	0.1779	0.1893	0.1381	0.0452	0.0352									
14	0.3137	0.2495	0.2102	0.1410	0.0802	0.1276	0.1097								
15	0.2177	0.1485	0.1698	0.1451	0.0486	0.0246	0.0296	0.1234							
16	0.2474	0.1745	0.1767	0.1604	0.0676	0.0183	0.0404	0.1082	0.0515						
17	0.4625	0.4057	0.3729	0.3483	0.3342	0.4260	0.3674	0.3185	0.3838	0.4244					
18	0.4498	0.3659	0.3794	0.3356	0.3681	0.3353	0.3425	0.3577	0.3064	0.3455	0.6167				
19	0.4625	0.4057	0.3098	0.3483	0.4383	0.3789	0.4016	0.3992	0.3771	0.4043	0.5521	0.1165			
20	0.4149	0.3063	0.2687	0.3071	0.3329	0.2591	0.3176	0.3119	0.2588	0.2906	0.6017	0.1617	0.0788		
21	0.5122	0.3865	0.3540	0.3928	0.4176	0.3339	0.4016	0.3992	0.3328	0.3697	0.6061	0.1023	0.0308	0.0528	
22	0.3700	0.3160	0.2268	0.3483	0.4383	0.3789	0.4016	0.3992	0.3771	0.4043	0.5521	0.2649	0.1292	0.0778	0.1643

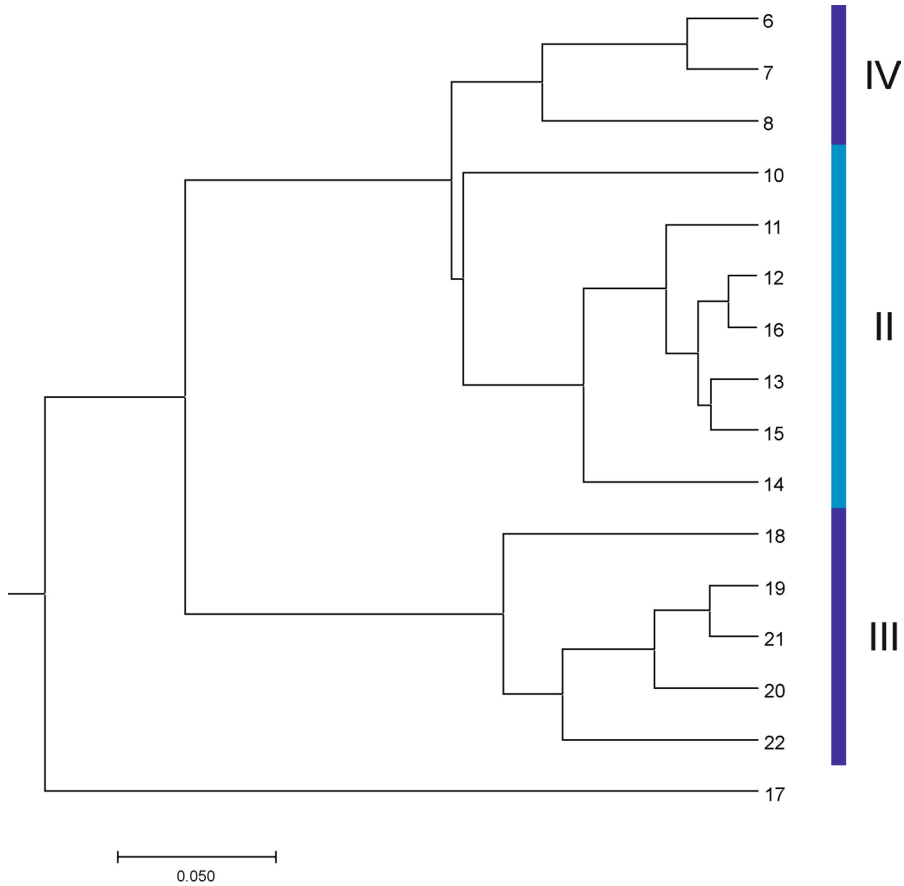

Fig. 12 UPGMA dendrogram for populations of *Belgrandiella kusceri* using RAPD data. The Arabic letters indicates population numbers, Roman letters numbers of the transects, see Fig. 1

overlap. The same can be seen in our present materials. We follow the nomenclature proposed in that study. *Belgrandiella* cf. *fontinalis* (Schmidt, 1847) is mOTU C, as in Osikowski et al. (2018), and *B.*cf. *koprivnensis* Radoman, 1975 is mOTU D as in that study. *B. kusceri* (Wagner, 1914) is mOTU A as there, and *B.* cf. *kuesteri* (Boeters, 1970) is their clade B. The representatives of two other mOTUs, E and F, have not been studied by Osikowski et al. (2018). Considering descriptions, drawings and photographs, as well as the data on geographic distribution given by Kuščer (1928, 1932), Radoman (1975, 1983), Bole (1979), De Mattia (2005), Žagar (2012) and Sket & Stoch (2014), we identified mOTU E as *B. crucis* (Kuščer, 1928). In the case of the mOTU F, we have not been able to perform identification. Its shell has no distinct characteristics and may resemble either to *B. fontinalis* – but this name was previously assigned to the mOTU C (Osikowski et al., 2018), or to *B. robusta* Radoman, 1975. However, the histone H3 sequences of the topotypes of *B. robusta*, presented in Osikowski et al. (2018), were different from the ones of the mOTU F. Considering all the ambiguities in the species-level taxonomy of *Belgrandiella* and the nomenclatorial chaos, it seemed safer to leave this species unidentified.

The results of AMOVA strongly confirmed high interspecies differences parallelly to markedly low percent of variance explained by interpopulation and intrapopulation differences within the species. Somewhat unusually, the values of intra- and interpopulation variance proportion were nearly the same.

The genetic monomorphism in the stygobiotic organisms is often recorded (e.g., Culver & Pipan, 2009), but usually only a single or few specimens are available, allowing the possibility that the picture is biased at the expense of deficient material. However, sometimes the samples richer in number are available, and also then the monomorphism holds. Hofman et al. (2021) sequenced COI in as many as 13 specimens of *Paladilhiopsis stellatus* Hofman et al., 2021 from Zvezda Spring in Croatia, finding

no polymorphism. Thus, the sympatric occurrence of as many as four unique haplotypes of *B. kusceri* in the cave Krška jama, in sympatry with *B. crucis*, is unusual and noteworthy. Three sympatric haplotypes at Žerovnica, and two at other four localities confirm rather common genetic polymorphism in *Belgrandiella*. Only two found haplotypes within the vast and microhabitat rich Planinska jama in contrast with five haplotypes from the small Krška jama, are hard to explain.

Low values of p-distance and low levels of intrapopulation polymorphism in *B. kusceri* may suggest rather high levels of gene flow, although selection cannot be excluded, and Nm estimates derived from F_{ST} were not high. As was stressed by Endler (1977), adaptive differences among populations can be maintained by natural selection even under high levels of gene flow, as well as selection may mimic effects of gene flow. This similarity may be also due to numerous stochastic factors, area effects, etc. No significant association between the p-distances and geographic distances may suggest rather the infinite-island model of interpopulation differentiation (Wright, 1978), expected for isolated populations, but the statistically significant association between pairwise F_{ST} and geographic distances suggests rather the stepping-stone model. If two populations did not share the same haplotype, the estimates of gene flow (Nm) derived from F_{ST} were low. If $Nm > 1$, the allele frequencies in the subpopulations remain homogenized (Wright, 1931, 1969). If $Nm < 1$ but still positive, an equilibrium based on the rate of mutation, migration, and genetic drift will be established. The haplotype network confirms low genetic diversification, but rather of dumbbell, not star-phylogeny pattern (Avise, 2000), with two starbursts, but close to each other – differing only by one mutation. Evidently there is a signature for a species that has expanded its range rather recently, but rather from more numerous founders than in the case of star-phylogeny. Longer time may also suggest the existence of two haplotypes in slowly evolving histone H3.

The RAPD results show finer resolution than COI, and the picture does not follow the one gained on the oxidase. Such dissimilarity was also observed in Planinska jama for *Asellus aquaticus* (Linnaeus, 1758) (Verovnik et al., 2003; Verovnik, 2012). Association between Nei and geographic pairwise distances was not significant, but the clustering presented nearly strictly geographic pattern.

In stygobites the pigmentation and eyes are sometimes still present (e.g., Culver & Pipan, 2009; Culver, 2012), but the pigmentation characterizing many populations of *Belgrandiella* may also suggest their stygophily. There are a few stygophile Balkan truncatelloid species like *Sadleriana cavernosa* Radoman, 1975 (Delicado, 2018), *Litthabitella chilodia* (Westerlund, 1886) or *Radomaniola* spp. (Falniowski et al., 2021b). However, *Belgrandiella* can be found in springs as single/a few specimens, rather overflooded from the subterranean waters, which rather contradicts possible stygophily of *Belgrandiella*.

Belgrandiella can be found in caves and springs, sometimes also as an element of the meiofauna, but in epikarst, only its empty shells can be found (Velkovrh, 1974). High intraspecies variability of the shell coupled with interspecies overlap of its variation forces caution with interpretation of all the faunistic records based solely on the shell morphology. Anyway, there are interesting observations on the geographic distribution of the aquatic subterranean snails, showing the same pattern as for the crustaceans (Sket, 1986, 1993). Species determinations in the latter group, based on several characters of their hard parts must be more reliable than the ones of the gastropods, based mostly on their shells, often even empty ones. Contrary to expectations, the species ranges in both groups do not follow the recent hydrographical units, but the presumed Pliocene ones. Karstification began in the end of Pliocene and continued through the Pleistocene (Matvejev 1961; Sket 1999). This may suggest that speciation took place in epigean streams before karstification, later the taxa invaded subterranean habitats and parallelly gained troglomorphic character states (many of them losing eyes and pigment, elongating appendages, etc.), but after hydrographical changes they have not changed their distribution ranges along the newly established water connections (Sket, 1986, 1993). The Pliocene lasted 5.33–2.58 Mya (Cohen et al., 2013), thus, assuming 1.83% per Mya estimate for the Hydrobiinae given by Wilke (2003), the time of divergence between our mOTUs would be 3.6–5.4 Mya (3.4 ± 1.7 Mya for our estimates) for the monophyletic clade inferred for COI (mOTUs A, B, E and F), and 4.4–5.8 Mya (4.7 ± 1.9 Mya and 4.5 ± 1.8 Mya for our estimates) for the other two mOTUs. Considering the

non-ultrametricity of our molecular tree, as well as the rate of mutation not necessarily the same as in *Hydrobia*, these estimates are very rough, but still sufficient to confirm the hypothesis of Sket (1986, 1993) about the time and place of their speciation, preceding invasions of the subterranean habitats. Caves are often seen as analogues to the islands in the sea, long lasting in isolation. However, sometimes old species can be found on the islands much younger than these species (Szarowska et al., 2014). The case of *Belgrandiella* seems similar: already distinct, "good" species colonized caves. The pairwise p-distances within *B. kusceri* in several cases also clearly suggest the Pliocene as the time of divergence.

The competing "climate-relict" and "adaptive-shift" hypotheses have been proposed to explain the origins of cave organisms (Juan et al., 2010). According to the "climate-relict" model, preadapted ancestors took refuge (or simply colonized) in caves before the surface climate was altered by glaciation or aridification, and gradually adapted to the cave environment. Climatic oscillations caused local extinctions of the surface populations, leaving each relict population to evolve in allopatry, in a separate cave system. The "adaptive-shift" model supposes that preadapted ancestral species actively colonized caves to exploit novel resources and diverged under a gene flow scenario. Divergent selection between surface and subterranean habitats gradually overcame the homogenizing process of gene flow and eventually led to parapatric speciation (Zhang & Li, 2013). Molecular phylogenies are helpful in testing those hypotheses, since they allow reconstruction of sister relationships between cave and surface taxa (Rivera et al, 2002; Leys et al., 2003). The presence of either surface and cave populations in allopatry, or only relict subterranean species, or parapatric distributions of both surface and cave populations, may be criteria of distinction between the two hypotheses. In *Belgrandiella*, despite often pigmented populations, stygophily remains doubtful – the specimens in springs are few and they seem rather flooded from the subterranean waters. Nevertheless, *Belgrandiella* certainly does not reach the high abundance of e.g., *Bythinella* or *Radomaniola* that both inhabit also caves (Giusti & Pezzoli, 1977, 1980; Bichain et al., 2007; Falniowski et al., 2021b), thus should be classified as stygophiles. It has to stressed, however, that high densities of the latter are typical of springs, whereas *Belgrandiella* could be find in spring as single/a few specimens overflooded from the subterranean waters. Thus, a "climatic-relict "hypothesis seems more probable, but we should note again that the speciation preceded invasion of "isolated" caves (although earlier the snails may might inhabit interstitial habitats, subterranean but rather continuous) and later, in caves, only slight genetic changes took place.

Animals adapted to cave environments are traditionally thought to be highly geographically isolated because of their limited dispersal ability, resulting from limited physiological tolerances and, especially in the case of snails, physical limitations of their locomotion. The available data on the molecular genetics of the stygobiont gastropods did not confirm either the complete isolation of populations or their stability and longevity (Falniowski, 1992; Falniowski et al., 1998, 1999; Bilton et al., 2001; Brändle et al., 2005; Falniowski & Szarowska, 2011). For inhabitants of such miniature and changeable habitats (Szarowska, 2000) survival must depend on dynamic processes of colonization and recolonization events, coupled with short- or (in some cases) long-distance dispersal (Szarowska et al., 2016a, b). There are subterranean habitats that make migration between caves and springs possible – those are interstitial habitats, unconsolidated sediments bordering and underlying streams and rivers. They are neither rare nor discontinuous (Culver & Sket, 2000; Lamoreaux, 2004; Culver et al., 2009; Culver, 2012; Rysiewska et al., 2017; Haase et al., 2021). Falniowski et al. (2021a) reported high levels of gene flow, resulting in more than 200 km wide range of one haplotype of the truncatelloid *Montenegrospeum bogici* (Pešić et Glöer, 2012). On the other hand, in *Kerkia* inhabiting nearly the same territory as our *Belgrandiella* (and, in part, *Montenegrospeum*) the pattern was completely different (Hofman et al., 2022a, b). Like in *Paladilhiopsis* (Hofman et al., 2021), confirming the most widespread opinions on the stygobiont fauna, the intrapopulation genetic diversity does not (or nearly does not) exist, and the same concerns the intraspecies variation between populations. At the same time the differences between the species are high, the species have narrow geographic ranges restricted to the small geographic areas, and grouped also geographically, in three clades, thus the high level of endemism could be confirmed in *Kerkia*.

The pattern observed in our *Belgrandiella* species seems rather similar to the one described for *Montenegrospeum*, not for *Kerkia*, although in *Belgrandiella kusceri* (mOTU A) the geographic range is narrower – covering only about 42 km, and the levels of gene flow are lower. But as in *Montenegrospeum*, there are also some analogies in *Belgrandiella* distribution with the famous European Cave Salamander *Proteus anguinus* Laurenti, 1768 with wide and discontinuous range and cryptic species (Sket, 1997; Gorički & Trontelj, 2006), or the moitessieriid snail *Bythiospeum* (Richling et al., 2016).

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Data Availability Sequence data are also downloaded at GenBank; accession numbers OP829115-OP829142, OP829059-OP829086, OP829087-OP829114, OP804353-OP804408, OP824660-OP824687.

Code availability Software application—BioEdit 7.2.5

Declarations

Conflict of interest The authors declare that they have no competing interests.

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