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RESEARCH PAPER OPEN ACCESS

Filling the gaps in the genetic diversity of *Austropotamobius torrentium* (Astacidae, Decapoda) from one of the few unexplored hot spots

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Abstract – Recent molecular analyses of the stone crayfish have revealed a high degree of genetic diversity. The greatest diversity is found in the western Balkans (Dinarides), where more than half of the known phylogroups exist in a relatively small geographical area, some of them having smaller distribution range than the others. While the Croatian and Slovenian parts of the *Austropotamobius torrentium* areal are well described, data from Bosnia and Herzegovina (BA) are lacking. Here we provide data from 13 different localities in the northwestern parts of BA. We analysed two mtDNA markers and the results revealed high genetic diversity with a total of 12 *MT-COI* and nine *MT-16SrRNA* haplotypes, with the majority of novel haplotypes. Both genes confirmed the presence of two known phylogroups and the discovery of a new group named VOJ. The CSE phylogroup was the most widespread and restricted to the Vrbas basin. The first detection of the BAN phylogroup in BA indicates its wider distribution and connects previously isolated findings from Croatia. The discovery of 18 unique haplotypes as well as a new phylogroup is of particular interest, but further studies are needed to clarify their exact relationship to other lineages.

Keywords: native crayfish / mtDNA / Vrbas drainage / Una drainage / Bosnia and Herzegovina

1 Introduction

The family of freshwater crayfish (Astacidae) comprises four genera: *Austropotamobius*, *Astacus*, *Pontastacus*, and *Pacifastacus*. Three genera are native to Europe, while the genus *Pacifastacus*, native to North America, was introduced into European waters. The current distribution of freshwater crayfish in Europe is the result of natural processes that took place from the Miocene to the Pleistocene (Klobučar *et al.*, 2013; Pârvulescu *et al.*, 2019; Lovrenčić *et al.*, 2020a; Stanković *et al.*, 2024). As a result of taxonomic revisions over the last 60 years (summarised in Crandall and De Grave, 2017) and recent molecular analyses (Blaha *et al.*, 2021), seven species of freshwater crayfish have been identified as autochthonous to the European continent: *Astacus astacus* (Linnaeus, 1758), *Astacus colchicus* Kessler, 1876, *Pontastacus pachypus* (Rathke, 1837), *Pontastacus leptodactylus* (Eschscholtz, 1823), *Austropotamobius fulcisianus* (Ninni, 1886), *Austropotamobius torrentium* (Schrank, 1803), *Austropotamobius pallipes* (Lereboullet, 1858), and an additional endemic species *Austropotamobius bihariensis* Pârvulescu, 2019 with a narrow distribution range in Romania (Pârvulescu, 2019).

Austropotamobius torrentium is widespread species in central and southeastern Europe. The distribution area (Fig. 1) extends from Bulgaria in the east to Luxembourg and France in the west, from Germany and the Czech Republic in the north to Greece and Turkey in the south (Holdich, 2002; Kouba *et al.*, 2014; Ion *et al.*, 2024). Throughout its entire range, the species is classified as "Data Deficient" with a declining population status in the IUCN Red List of Threatened Species (Füreder *et al.*, 2017). In addition, stone crayfish is listed in Annex II of

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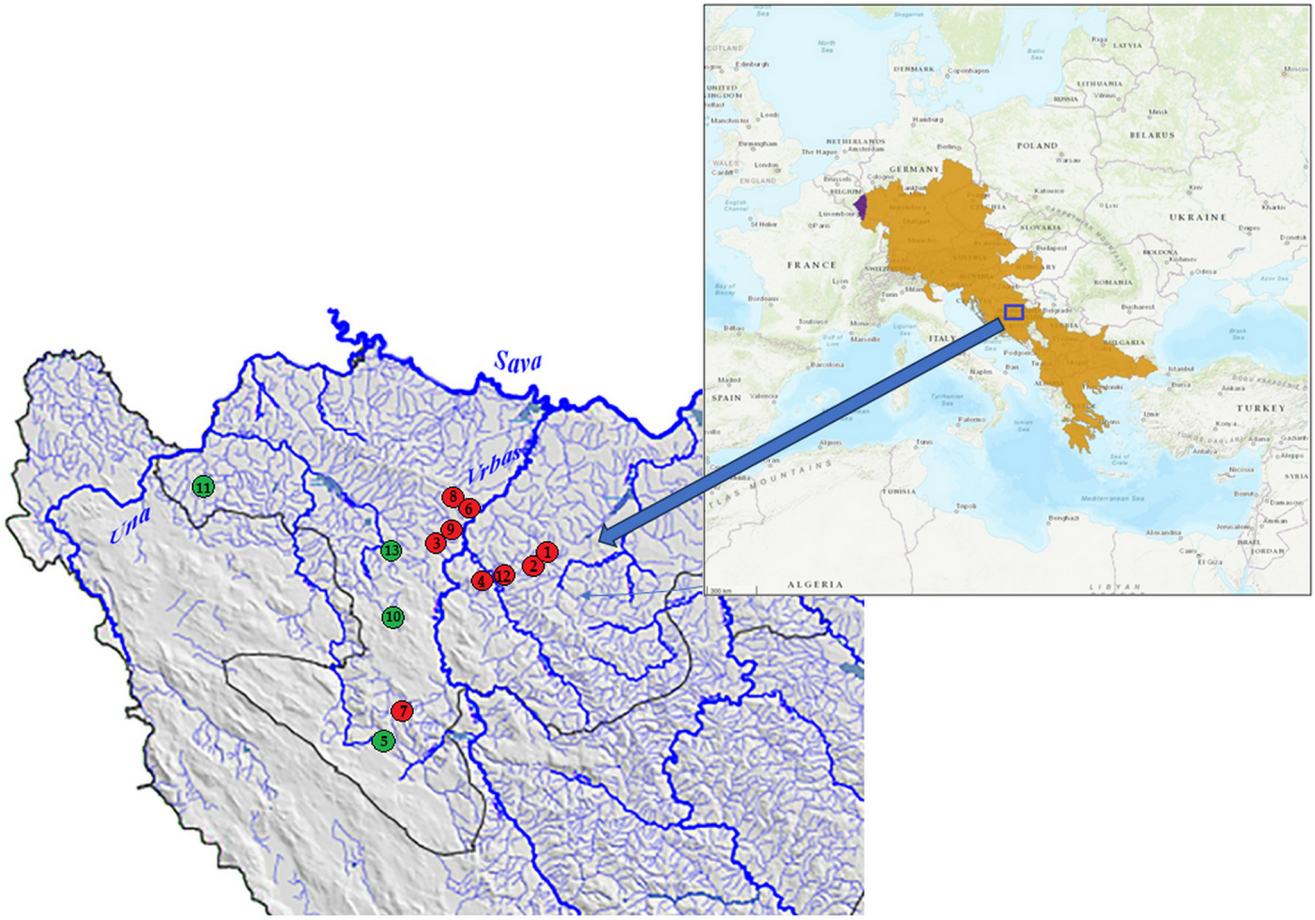


Fig. 1. Geographic distribution of investigated localities. Left: map of the investigated area that corresponds to blue square marked in the right map. Localities comprise two watercourses: Vrbas (red marks) – 1 – 4, 6 – 9, 12, and Una (green marks) – 5, 10, 11, 13. Locality numbers correspond to Table 1.

the EU Habitats Directive 92/43/EEC (Council of Europe, 1992) as a species requiring special conservation measures (Souty-Grosset *et al.*, 2006) and in Appendix III of the Bern Convention (Council of Europe, 1979). Although the assessment of the status of populations at European level was already carried out in 2010, no quantitative data on the rate of decline is available. The main threats identified were invasive species, habitat modification and degradation (habitat loss), pollution and eutrophication (Füreder *et al.*, 2006; Berger and Füreder, 2013). In the Republic of Srpska (Bosnia and Herzegovina), the species is listed in the Regulation on Strictly Protected and Protected Species (Official Gazette of the Republic of Srpska No. 65/20) (Anonymous, 2020).

In the last 20 years, great efforts have been made to determine the genetic profile of freshwater crayfish populations in the southeastern Europe (Trontelj *et al.*, 2005; Schubart and Huber, 2006; Klobučar *et al.*, 2013; Pârvulescu, 2019; Lovrenčić *et al.*, 2020a; Stanković *et al.*, 2024). Based on nucleotide sequence polymorphism analyses of two mitochondrial genes – cytochrome c oxidase I (*MT-COI*) and *MT-16SrRNA* – and a nuclear ITS2 (Lovrenčić *et al.*, 2020), a high genetic and haplotype diversity of the species has been reported, characterised by the existence of eight significantly divergent phylogroups. Six of them have a distribution range within the north-central Dinaric region (NCD), while the remaining two include populations from the southern Balkans (SB) and central and southeastern Europe (CSE) (Trontelj *et al.*, 2005; Klobučar *et al.*, 2013; Lovrenčić *et al.*, 2020a; Stanković *et al.*, 2024). Of particular interest for the present study are phylogroups belonging to the NCD lineages (situated in the northern and central Dinarides, in western and southern Croatia and the associated border areas in Slovenia and Bosnia and Herzegovina, Stanković *et al.*, 2024) as well as CSE lineage (SE Alps, Slovenia and upper Rhine Basin (Trontelj *et al.*, 2005) and also in central and eastern Bosnia and Herzegovina (Stanković *et al.*, 2024)).

Mitochondrial DNA (mtDNA) is a marker commonly used for genetic reconstruction of population history, demography, biogeography and speciation and is recommended for taxonomic studies (Hebert *et al.*, 2003). Here, we analyse the nucleotide sequence polymorphism of two mitochondrial DNA (mtDNA) genes, *MT-16SrRNA* and *MT-COI* of *A. torrentium* populations in the northwestern part of Bosnia and Herzegovina (BA), a central region of the Balkan Peninsula for which very few data are available. This approach has already proven successful in analysing the genetic polymorphism of *A. torrentium* (Trontelj *et al.*, 2005; Schubart and Huber, 2006; Klobučar *et al.*, 2013; Petrusek *et al.*, 2017;

Table 1. GenBank IDs with sampling localities of a total of 36 samples collected between 2018 and 2021 and analysed in this study.

MT-16S rRNA ID	*MT-COI* ID	Phylogroup	Sampling locality (No. from Fig. 1)	River basin	Sampling year
OP963758 h4	OQ048651 ba1	CSE	Stanikova rijeka (1)	Vrbas	2018
OP963759 h4	OQ048652 ba1	CSE	Stanikova rijeka (1)	Vrbas	2018
OP963760 ba1	OQ048653 ba1	CSE	Stanikova rijeka (1)	Vrbas	2018
OP963761 h4	OQ048654 ba1	CSE	Stanikova rijeka (1)	Vrbas	2018
OP963762 ba2	/	CSE	Rudnička rijeka (3)	Vrbas	2018
OP963763 h4	OQ048655 ba2	CSE	Rudnička rijeka (3)	Vrbas	2018
OP963764 ba3	/	CSE	Rudnička rijeka (3)	Vrbas	2018
OP963765 h4	OQ048656 ba3	CSE	Rudnička rijeka (3)	Vrbas	2018
OP963766 h4	/	CSE	Rudnička rijeka (3)	Vrbas	2018
OP963767 h4	OQ048657 ba1	CSE	Šargovački kanal (9)	Vrbas	2019
/	OQ048658 h41	BAN	Dževerov potok (7)	Vrbas	2020
/	OQ048659 ba4	BAN	Dževerov potok (7)	Vrbas	2020
OP963768 h27	/	BAN	Dževerov potok (7)	Vrbas	2019
OP963769 h27	/	BAN	Korana (5)	Una	2018
OP963770 h27	/	BAN	Korana (5)	Una	2018
OP963771 h27	/	BAN	Ledenac (10)	Una	2019
OP963772 ba4	OQ048661 ba1	CSE	Marjanovića potok (4)	Vrbas	2018
/	OQ048662 ba5	CSE	Marjanovića potok (4)	Vrbas	2018
OP963773 ba4	OQ048663 ba1	CSE	Marjanovića potok (4)	Vrbas	2018
OP963774 ba4	OQ048664 ba1	CSE	Marjanovića potok (4)	Vrbas	2020
OP963775 h4	OQ048665 ba6	CSE	Marjanovića potok (4)	Vrbas	2020
OP963776 h4	OQ048666 ba7	CSE	Mlinska rijeka (2)	Vrbas	2018
OP963777 h4	OQ048667 ba6	CSE	Mlinska rijeka (2)	Vrbas	2018
OP963778 h4	OQ048668 ba1	CSE	Mlinska rijeka (2)	Vrbas	2018
OP963779 h4	OQ048669 ba1	CSE	Mlinska rijeka (2)	Vrbas	2018
OP963780 ba5	OQ048670 ba1	CSE	Mlinska rijeka (2)	Vrbas	2018
OP963781 h4	OQ048671 ba8	CSE	Matića potok (6)	Vrbas	2018
OP963782 h4	OQ048672 ba9	CSE	Matića potok (6)	Vrbas	2018
OP963783 h4	OQ048673 ba8	CSE	Dobraš (8)	Vrbas	2019
OP963784 h4	OQ048674 ba8	CSE	Dobraš (8)	Vrbas	2019
OP963785 h4	OQ048675 ba8	CSE	Dobraš (8)	Vrbas	2019
OP963786 h4	OQ048676 ba8	CSE	Dobraš (8)	Vrbas	2019
OP963787 ba6	OQ048677 ba10	VOJ	Vojskova (11)	Una	2020
OP963788 ba6	/	VOJ	Vojskova (11)	Una	2020
OP963789 ba7	OQ048678 ba11	BAN	Subotica (13)	Una	2021
OP963790 h4	OQ048679 ba2	CSE	Opsečko (12)	Vrbas	2021

[h] – haplotype.

Pârvulescu *et al.*, 2019). In this study, we compare new BA samples with those imported from GenBank originating from other regions within the distribution range of *A. torrentium* with the aim of complementing the biodiversity data and filling a gap in the records of the genetic structure of this strictly protected species in the territory of BA (Anonymous, 2020) in the context of known mtDNA haplotypes and phylogroups from the surrounding regions.

2 Materials and methods

2.1 Sample collection, DNA extraction, and sequencing

In the period 2018–2021, a total of 36 crayfish samples from 13 different localities (Fig. 1, Tab. 1) were collected from the tributaries of the two rivers Vrbas and Una. The investigated habitats are located in a hilly region with beech forests at an altitude of 180–846 m above sea level, with megalithal and macrolithal dominate in the riverbeds with the lower proportion of psammal. The crayfish were caught by hand or with baited LiNi traps (Westmann *et al.*, 1978).

Total DNA was isolated from a small part of muscle tissue of a pereiopod segment preserved in 96% ethanol using the AccuPrep Genomic DNA Extraction Kit (Bioneer Corporation, Daejeon, Korea). The quantity and quality of all DNA extracts were measured using the NanoPhotometer N60/N50 (Impplen, GmbH) and visualised by 1% agarose gel electrophoresis. Polymerase chain reactions (PCRs) for the amplification of mtDNA gene fragments were performed with universal primers: *MT-16SrRNA* (16Sar, 16Sbr) and *MT-COI* (LCO-1490, HCO-2198) from Veith *et al.* (2003) and Folmer *et al.* (1994), respectively. The thermal PCR profiles were as follows: for *MT-16SrRNA*: initial denaturation (3 min at 94 °C),

followed by 40 cycles (30s at 94 °C, 30s at 52 °C, 40s at 72 °C) and a final elongation of 5 min at 72 °C; for *MT-COI*: initial denaturation (3 min at 94 °C), followed by 40 cycles (30s at 94 °C, 45s at 48 °C, 1 min at 72 °C) and a final elongation of 5 min at 72 °C. PCRs were performed using 1U Perpetual TaqDNA Polymerase (EurX, Poland) and approximately 100ng of genomic DNA in a final volume of 25 μL. Sanger sequencing was performed in one direction by a third party (Macrogen Europe). As universal primers for *MT*-16SrRNA gene were used, special attention was paid to sterile conditions to avoid contamination.

2.2 Genetic diversity and phylogenetic analysis

All sequences were visually examined with the FinchTV 1.4.0 chromatogram viewer (Geospiza Inc.), compared and analysed with BioEdit Ver. 7.2.5 (Hall, 1999). The presence of stop codons and chimeric sequences was examined and compared with the sequences currently available in Gen-Bank using Basic Local Alignment Search Tool (BLAST) analysis. They were then aligned using ClustalW, which is implemented in the MEGA Ver. XI software (Molecular Evolutionary Genetics Analysis across computing platforms) (Tamura *et al.*, 2021).

For the phylogenetic analysis of *MT-16SrRNA*, the final dataset contained a total of 88 sequences, *i.e.*, 33 from the sampled individuals (Tab. 1) and 55 imported from GenBank, NCBI (Tab. S1). For the phylogenetic *MT-COI* analysis, the final dataset comprised 179 sequences, *i.e.* 28 from the sampled individuals (Tab. 1) and 151 from GenBank (Tab. S1). Three species were used as outgroups for both datasets: *Austropotamobius pallipes*, *Astacus astacus* and *Pacifastacus leniusculus*.

Genetic diversity parameters (h- the number of haplotypes, Hd- haplotype (gene) diversity, Pi- nucleotide diversity, Eta-total number of mutations) were estimated using DnaSP Ver. 6 (Rozas *et al.*, 2017).

The estimation of evolutionary divergence between groups of sequences was calculated using the programme MEGA XI. The evolutionary divergence over sequence pairs between all detected phylogroups (p-distance) was calculated and the number of base differences per site from averaging over all sequence pairs between the groups is shown. The codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair ("pairwise deletion" option).

Prior to the phylogenetic analysis, a best-fit substitution model for the aligned sequences including the outgroups was performed using JModelTest v.2.1.4. (Darriba *et al.*, 2012). The best-fit substitution model in the aligned sequences tested was HKY+I+G (with gamma distribution and invariant sites). Phylogenetic analysis was performed using two different approaches to test the strength of the tree topology: Bayesian inference (BI) analysis in MrBayes (Ronquist and Huelsenbeck, 2003) and the Maximum Likelihood (ML) in PhyML (Guindon *et al.*, 2010). Trees were created using FigTree Ver. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) and MEGA XI. The BI analyses originated with random starting trees and were run for 20×10^6 generations, sampling every 1000th generation with the burn-in value set to 500. Combined trees of the various runs, a consensus tree was created using the 50% majority rule with the Bayesian posterior probability values of the relevant branches. The haplotype network was calculated and graphically presented using NETWORK Software (Bandelt *et al.*, 1999). It consists of nodes and links (nucleotide differences) connecting the nodes. The nodes are either sequences from the data set or median vectors (mv) – a hypothetical, often ancestral sequence required to connect existing sequences within the network with maximum parsimony.

3 Results

3.1 MT-16SrRNA gene nucleotide sequence comparison

The 33 sequences obtained were deposited in the GenBank database under the accession numbers OP963758–OP963790 (Tab. 1). The final *MT-16SrRNA* dataset analysed here comprised 88 sequences together with 55 imported ones (Tab. S1). The data file with excluded outgroups contains 85 sequences, 493 sites and a total number of 464 nucleotide positions (excluding sites with gaps/missing data). There are 67 polymorphic and 397 invariable sites. The total number of mutations is 75 and a number of parsimony informative sites is 48. The overall number of haplotypes is $h = 60$; haplotype (gene) diversity Hd = 0.9442 ± 0.02; nucleotide diversity (per site) Pi = 0.01669 ± 0.00158. Among 33 newly sampled BA individuals nine haplotypes were detected, of which seven were new/original haplotypes labelled ba1-ba7 and only two were detected earlier: h4 and h27 (Klobučar *et al.*, 2013). The results are presented as the relatedness of all 60 haplotypes detected in this analysis with previously described phylogroups (Lovrenčić *et al.*, 2020): CSE – Central and Southeastern Europe; SB – South Balkans; ZV – Zeleni Vir; LD – Lika and Dalmatia; KOR – Kordun; BAN – Banovina; GK – Gorski Kotar and ŽPB – Žumberak, Plitvice and Bjelolasica.

The haplotypes analysed here, belonging to the CSE phylogroup, are: haplotype h4, which is the most common in the Vrbas samples from this study and also in several imported sequences; and a group of five original haplotypes ba1 – ba5, four of which are similar to haplotype h4 (with only one nucleotide change). Haplotypes h27 and ba7 belong to the BAN phylogroup and were found in the Una and Vrbas river basins. However, we also discovered a specific haplotype ba6–two samples from Vojskova in the Una river basin, which do not belong to any formerly determined phylogroups. Here they are regarded as a separate phylogroup VOJ.

The relationships between the haplotypes represented by a series of nucleotide changes between them, as well as the median vectors, are shown graphically in a Network Median Joining Haplotype Tree (Fig. 2a). All haplotypes from our sample are associated with previously defined phylogroups from the Balkans, CSE and BAN, with the exception of a new haplotype ba6 from Vojskova, Una (marked as VOJ), which does not belong to any known phylogroup. It is positioned between phylogroups BAN, SB and ŽPB, with five/six nucleotide substitutions and two/3 median vectors (symbolised by black *mv* dots in the tree). Between VOJ and ZV there is only one median vector, but with 11 nucleotide changes.

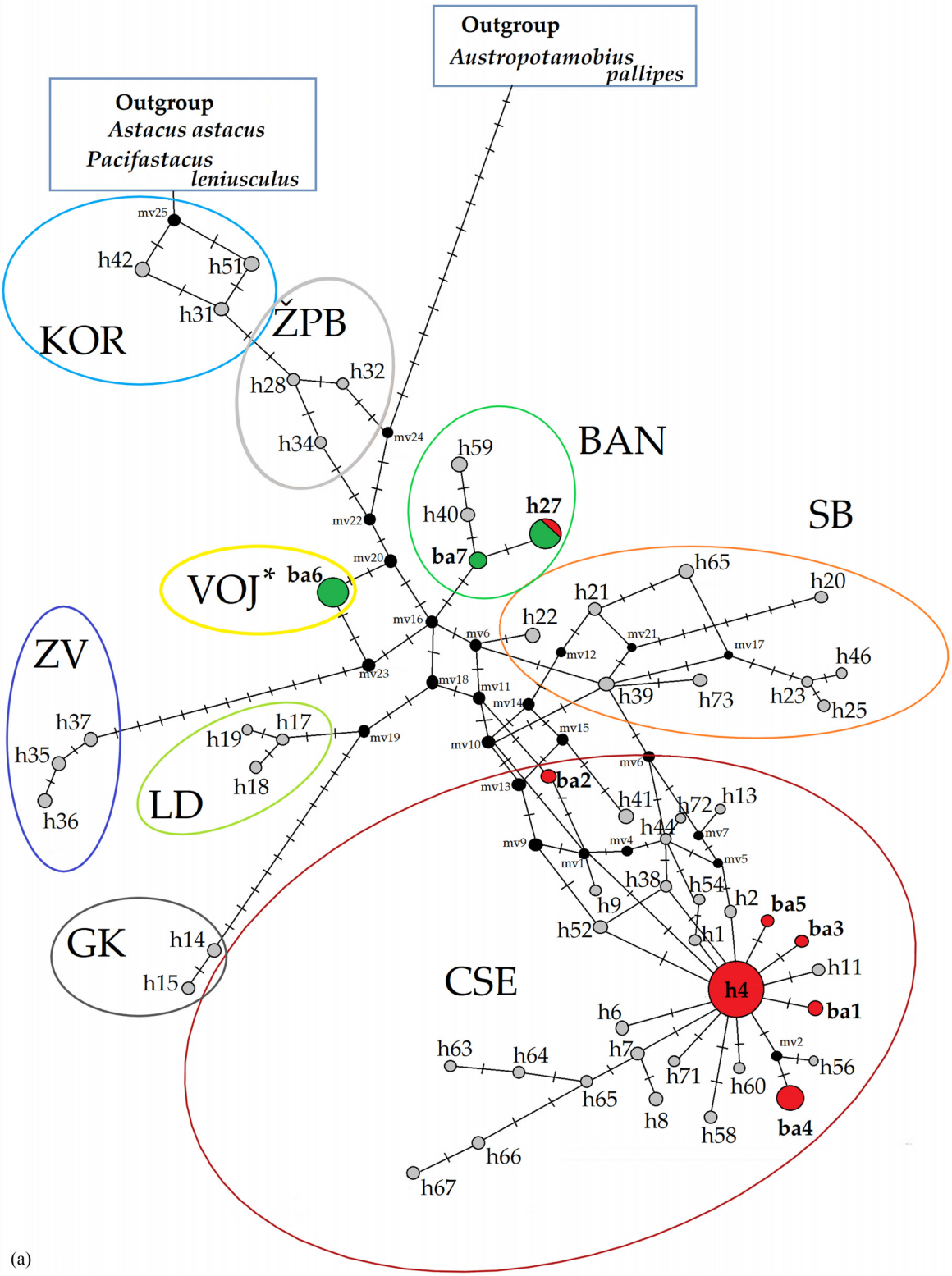


Fig. 2. Phylogenetic and haplotype network trees constructed using *MT-16SrRNA* dataset. a: Network Median Joining haplotype tree; red – Vrbas and green – Una. Haplotype node size is related to a sample size. Black dots (mv)-median vector. Haplotype ratio is distributed by phylogroups: CSE-central and south-eastern Europe; SB- south Balkan; ZV – Zeleni Vir; LD – Lika and Dalmatia; KOR- Kordun; BAN – Banovina; GK – Gorski Kotar and ŽPB – Žumberak, Plitvice and Bjelolasica; VOJ* – Vojskova. b: ML phylogenetic tree with bootstrap values of both methods, ML and BI, indicated in the nodes respectively. Dash indicates values <50. New samples from this study are marked with circles that correspond to phylogroup colors.

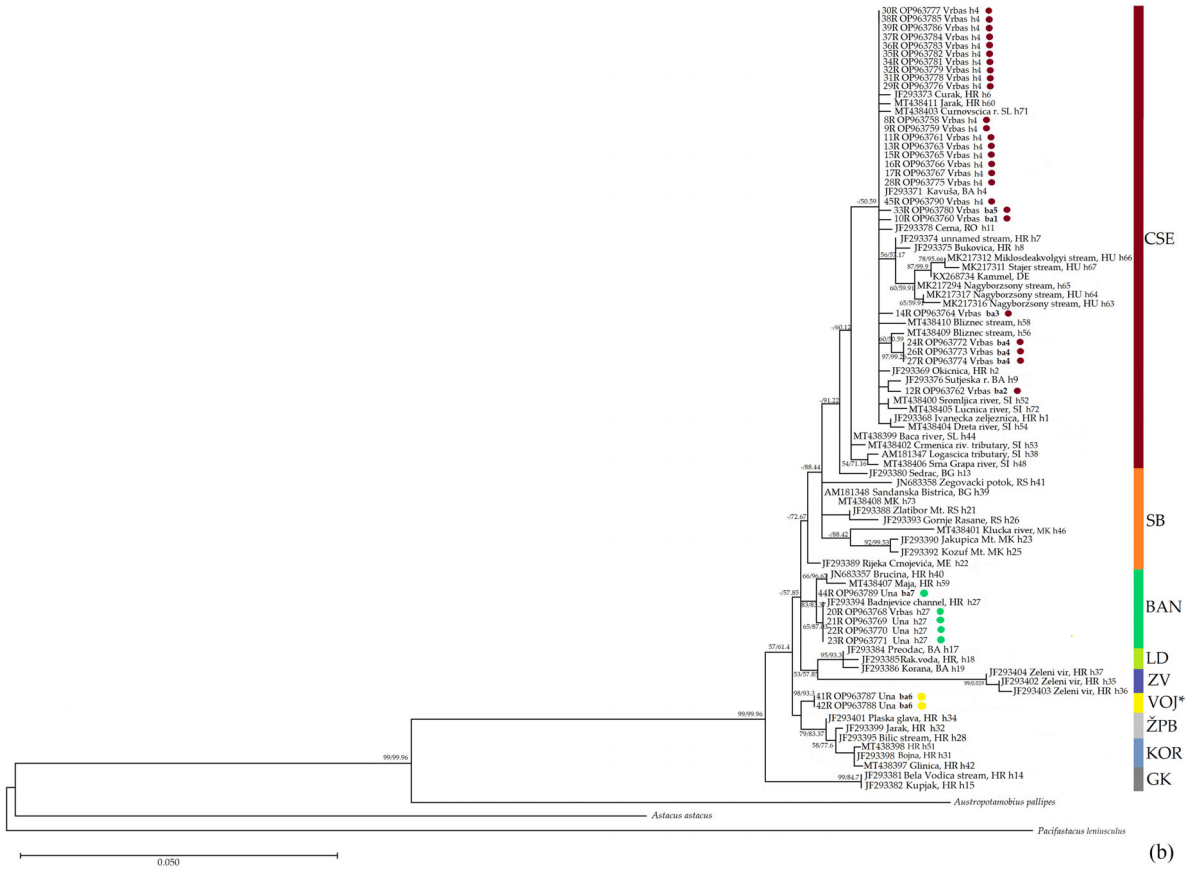


Fig. 2. (Continued).

Maximum likelihood (ML) and Bayesian (BI) inference yielded similar phylogenetic tree topologies; therefore, only the ML tree is presented in Figure 2b. The phylogenetic tree inferred from the *MT-16SrRNA* dataset illustrates that phylogroups CSE and SB are evolutionarily youngest, with the dominant haplotype h4 together with ba1 – ba5 in CSE. Next to them is phylogroup BAN, comprising haplotypes h27 and ba7 and then phylogroups LD and ZV. The newly discovered phylogroup VOJ is positioned between ZV and ŽPB. The evolutionarily oldest is the phylogroup GK, placed in the base of the tree.

The estimates of evolutionary divergence over sequence pairs (p-distances) between all detected phylogroups are shown in Table 2. This analysis included 85 nucleotide sequences and a total of 497 positions in the final dataset. Among the lowest observed p-distances values, besides ŽPB and KOR are those between the new phylogroup VOJ (haplotype ba6) and ŽPB/ BAN phylogroups.

3.2 MT-COI gene nucleotide sequence comparison

The final *MT-COI* gene dataset comprises 28 obtained sequences deposited in the GenBank database under accession numbers OQ048651 – OQ048659 and OQ04861–OQ04879 (Tab. 1) and 151 imported sequences listed in Table S1. The final dataset without outgroups contains 642 sites with 322 nucleotide positions (excluding sites with gaps/missing data) with 171 polymorphic sites and a total number of mutations, Eta = 119. The total number of haplotypes is $h = 134$; haplotype diversity, Hd = 0.9932 ± 0.0023; and nucleotide diversity (per site) Pi = 0.05591 ± 0.00267. Among the 28 newly sampled individuals from BA, 12 haplotypes were identified, of which 11 are new/original and only one h41 (Klobučar *et al.*, 2013) was previously discovered.

The estimates of evolutionary divergence over sequence pairs (p-distances) between all detected phylogroups are shown in Table 3. This analysis included 176 nucleotide sequences and a total of 636 positions in the final dataset. These values are higher than those calculated from *MT-16SrRNA* dataset, with the lowest distance provided among CSE and SB.

Most haplotypes are associated with CSE phylogroup: the most abundant is ba1 and the others are ba2, ba3, ba5–ba9, all of which were sampled in the Vrbas river basin (Fig. 3a). These are all new haplotypes. The newly discovered haplotypes ba4 (Dževerov potok, Vrbas basin) and ba11 (Subotica, Una basin) as well as h41 from the Vrbas basin, shared with an imported sample JF293445 from Badnjevice, Croatia, are all linked to BAN phylogroup. *MT-COI* gene analysis confirms the specific position of haplotype ba10 (Vojskova, Una river basin), *i.e.* it

Table 2. The estimates of Evolutionary Divergence over Sequence Pairs between Groups (*p*-distance) calculated from the *MT-16SrRNA* dataset. The number of base differences per site from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown above the diagonal.

	CSE	SB	BAN	LD	ŽPB	KOR	GK	ZV	VOJ
CSE		0.36%	0.54%	0.56%	0.60%	0.68%	0.81%	0.85%	0.58%
SB	1.51%		0.48%	0.54%	0.59%	0.67%	0.78%	0.81%	0.51%
BAN	1.83%	1.80%		0.58%	0.54%	0.65%	0.83%	0.85%	0.52%
LD	2.00%	2.20%	1.83%		0.55%	0.63%	0.78%	0.79%	0.63%
ŽPB	2.27%	2.49%	1.74%	1.79%		0.28%	0.76%	0.89%	0.52%
KOR	2.71%	2.94%	2.28%	2.26%	0.63%		0.80%	0.94%	0.63%
GK	3.61%	3.69%	3.48%	3.19%	3.05%	3.28%		0.95%	0.76%
ZV	4.08%	4.05%	3.80%	3.18%	4.21%	4.79%	4.88%		0.78%
VOJ	2.01%	1.99%	1.46%	2.04%	1.37%	1.91%	2.85%	3.16%	

Table 3. The estimates of Evolutionary Divergence between Groups of Sequences (p-distance) calculated from *MT-COI* dataset. The number of base differences per site from estimation of net average between groups of sequences are shown. Standard error estimate(s) are shown above the diagonal.

	CSE	ZPB	SB	GK	BAN	KOR	LD	ZV	VOJ
CSE		0.97%	0.43%	1.03%	0.78%	0.98%	0.93%	1.00%	0.99%
ZPB	6.87%		0.89%	0.94%	0.97%	0.95%	0.93%	1.00%	1.04%
SB	1.80%	6.07%		0.92%	0.62%	0.91%	0.84%	0.89%	0.91%
GK	7.62%	6.36%	6.59%		1.03%	1.04%	1.02%	0.90%	1.15%
BAN	4.77%	7.04%	3.66%	7.31%		0.98%	0.94%	0.98%	1.01%
KOR	6.66%	6.38%	6.22%	7.24%	7.38%		0.98%	1.13%	1.10%
LD	6.05%	6.06%	5.34%	6.83%	6.41%	6.63%		1.01%	1.09%
ZV	7.00%	7.23%	6.01%	6.02%	7.26%	8.04%	7.46%		1.13%
VOJ	6.53%	7.24%	5.99%	8.69%	7.09%	8.36%	7.59%	8.02%	

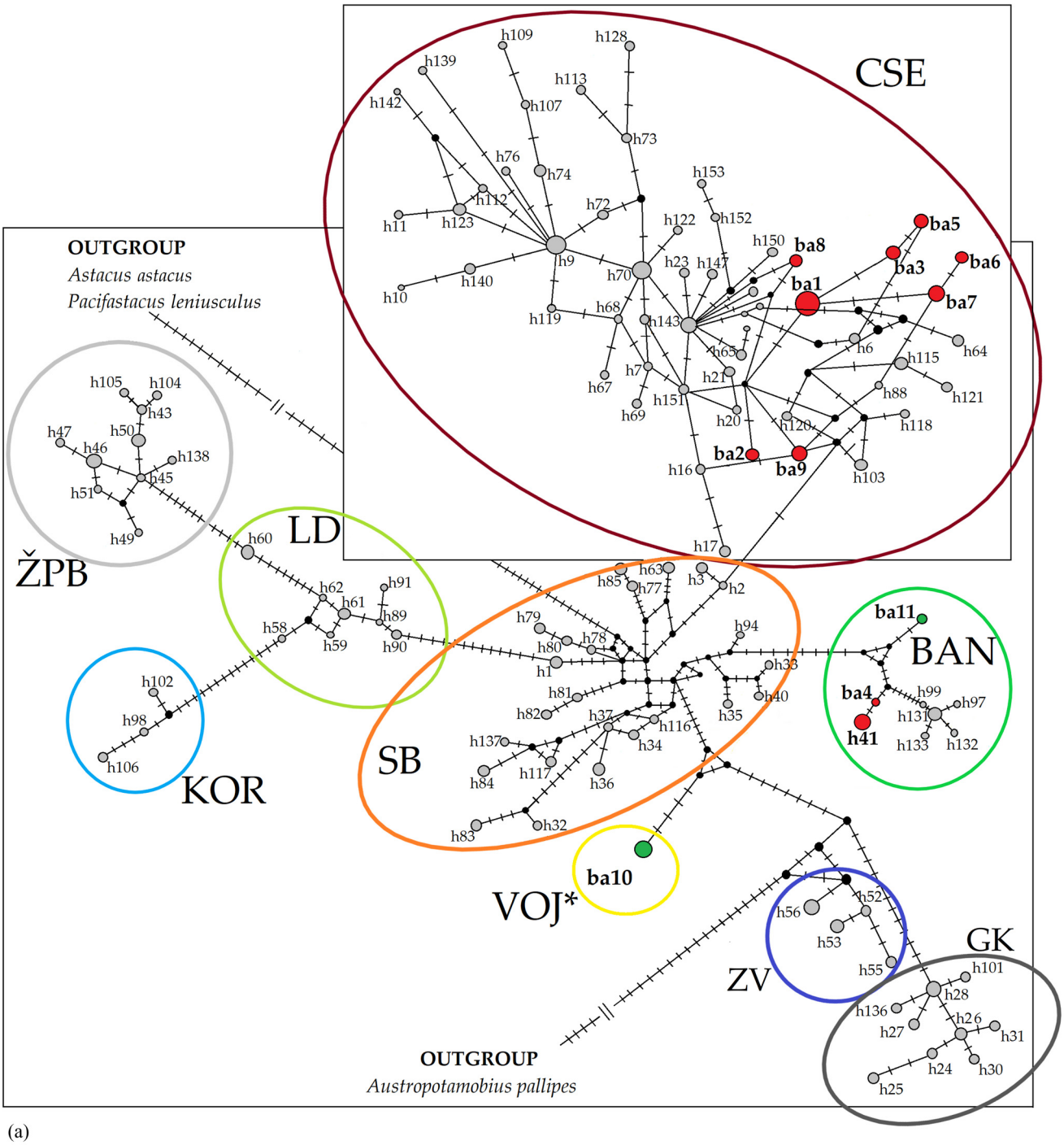
does not belong to any earlier described phylogroup. It is located between the SB and ZV groups, but is more distantly related to them compared to the *MT-16SrRNA* gene analysis.

Similar to the *MT-16SrRNA* dataset, maximum likelihood (ML) and Bayesian (BI) inference from the *MT-COI* gene data yielded similar tree topologies, which is why only the ML tree is shown in Figure 3b. The phylogenetic tree shows phylogroups CSE and SB as evolutionarily youngest, with high significance values in the branch nodes. Phylogroup BAN is clustered with these groups, also with high significance. The new phylogroup VOJ with the haplotype ba10 (Vojskova, Una) is placed among Dinaric karst lineages, between BAN and ŽPB and at the base of the phylogenetic ML tree are the phylogroups ZV and GK as the evolutionarily oldest.

4 Discussion

The study area presented here is positioned in the middle of the distribution range of *A. torrentium* in central and southeastern Europe (Fig. 1). As the species is classified as "Data Deficient" with a declining population status in the IUCN Red List of Threatened Species (Füreder *et al.*, 2017) and requires special conservation measures (Souty-Grosset *et al.*, 2006) and is listed in Appendix III of the Bern Convention (Council of Europe, 1979), it was necessary to investigate the genetic status of samples from Bosnia and Herzegovina and their relationship with populations from the surrounding regions. The results of our study confirm the presence of high genetic diversity in stone crayfish from the Western Balkans. Eleven out of 12 *MT-COI* haplotypes and seven out of nine *MT-16SrRNA* haplotypes detected in this analysis were formerly unknown. The identified mtDNA phylogenetic tree topologies are generally consistent with previous data on the relationships between the known phylogroups (Trontelj *et al.*, 2005; Klobučar *et al.*, 2013; Petrusek *et al.*, 2017; Pârvulescu *et al.*, 2019; Lovrenčić *et al.*, 2020a; Groza *et al.*, 2021; Stanković *et al.*, 2024), with GK representing the evolutionarily oldest and CSE and SB the youngest groups.

Given the high genetic diversity and the importance of the region for the diversification and evolution of this crustacean species, even smaller, understudied areas have the potential for some important additions to species knowledge (new phylogroups), as Lovrenčić *et al.* (2020a) have shown. It is worth mentioning that so far only two phylogroups – CSE and LD, have been confirmed from Bosnia and Herzegovina (Klobučar *et al.*, 2013; Lovrenčić *et al.*, 2020a). Our study shows the presence of another previously known phylogroup



(a)

Fig. 3. Phylogenetic and haplotype network trees constructed using *MT-COI* dataset. a: Network Median Joining haplotype tree; red – Vrbas basin and green – Una. Haplotype node size is related to a sample size. Black dots (mv)-median vector. Haplotype ratio is distributed by phylogroups: CSE- central and south-eastern Europe; SB- south Balkan; ZV- Zeleni Vir; LD- Lika and Dalmatia; KOR- Kordun; BAN- Banovina; GK- Gorski Kotar; ŽPB- Žumberak, Plitvice and Bjelolasica; APU- Apuseni Mt.; Vojskova – VOJ*. b: ML phylogenetic tree with bootstrap values of both methods, ML and BI, indicated in the nodes. Dash indicates values <50. Some branches that contain only sequences imported from the GenBank are condensed. New samples from this study are marked with circles that correspond to phylogroup colors.

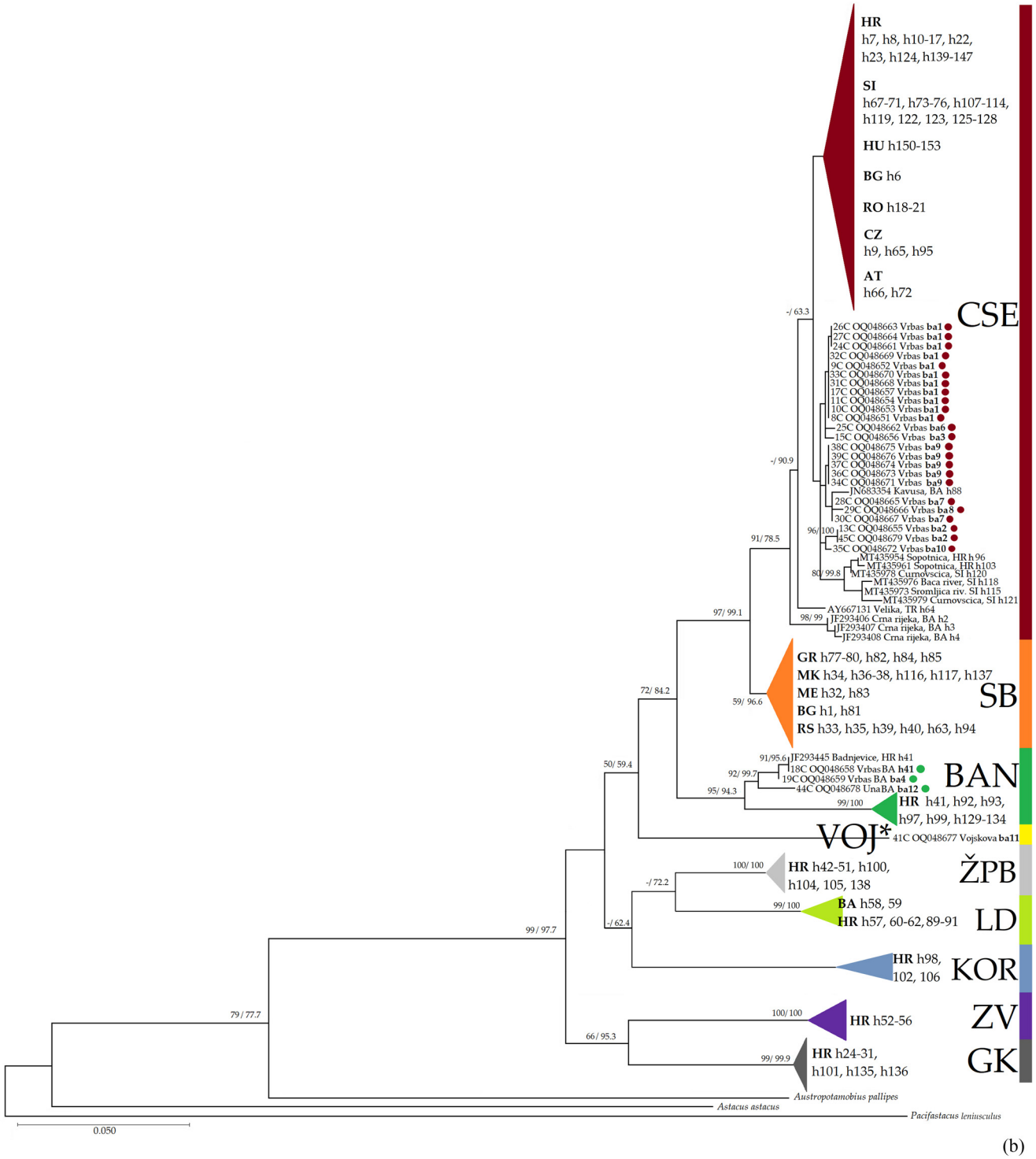


Fig. 3. (Continued).

(BAN), but also an additional potentially new phylogroup (VOJ) belonging to Dinaric karst lineages, which could be of particular interest. Namely, in both genes *MT-16SrRNA* and *MT-COI*, the haplotypes ba6 and ba10 were found, respectively, which cannot be assigned to any known phylogroup. According to the phylogenetic tree topologies derived from both genes, the VOJ haplotypes belong to the evolutionarily older branches. These individuals were sampled from the Vojskova River (Una basin). The river harbours numerous streams and karst springs and exhibits a rather heterogeneous structure, while remaining relatively untouched by negative anthropogenic influences. The sampling site (the village of Donji Dubovik) is geographically the furthest from the other sites investigated. In addition, no stone crayfish (and no other

astacids) were found during several field surveys in the immediate vicinity (personal communication with fishermen).

The BAN phylogroup was first discovered by Klobučar *et al.* (2013) and described with only 3 haplotypes from two isolated populations near the border between Croatia and Bosnia and Herzegovina (Banija/Banovina and Imotski). The data presented here confirm the broader occurrence and present two new *MT-16SrRNA* haplotypes (h27, distributed in the Una and Vrbas basins and ba7 in the Una basin) and a new *MT-COI* haplotype (ba11 in the Una basin). The haplotype h41 from the BAN phylogroup found in the Vrbas basin is shared with the Badnjevice locality in Croatia. Based on the available data, we suspect that in Bosnia and Herzegovina (at least in the Neretva and Bosna basins) an even greater presence of the BAN phylogroup is to be expected.

All other BA haplotypes belong to the CSE phylogroup. Five unique *MT-16SrRNA* haplotypes and one of the most common h4 haplotype are identified in the Vrbas basin, which is in common with a sample JN683358 from Žegovački potok, Serbia. Although the CSE phylogroup was the most abundant and widespread, it was restricted to the Vrbas basin. The reason for this could be the lower number of populations sampled in the Una basin (four) compared to the Vrbas basin (nine). As in the previous case, with further sampling we could probably assume that this widespread haplogroup is more abundant in the Una basin and perhaps also in the Sava, Bosna and Drina drainages.

The results of genetic diversity and evolutionary divergence contribute to our assumption of a high genetic diversity of stone crayfish in this area. The total nucleotide diversity per site was high in our study, especially that of *MT-COI*. Both values are significantly higher than those reported in the literature (Klobučar *et al.*, 2013). The values of evolutionary *MT-16SrRNA* divergence (average values of uncorrected (*p*) distances (in percent) between all phylogroups from the literature data (Klobučar *et al.*, 2013) are highly consistent with the values from our study. The results from the *MT-COI* dataset yielded three to four times higher values for p-distances, genetic diversity parameters and haplotypes, which is to be expected considering that the *MT-16SrRNA* gene is more conserved. For this reason, some inconsistencies in the relationships between the phylogroups can be detected when the results from two genes are compared. The *MT-COI* evolutionary divergence found here between all analysed phylogroups is slightly lower than in the literature (Klobučar *et al.*, 2013), but basically their relationships are the same. CSE and SB are the closest phylogroups with the lowest evolutionary divergence, followed by the divergence of BAN-SB and BAN-CSE. However, it is important to note that the values for the evolutionary distance between VOJ and all other phylogroups provided by both genes correspond to the values among all recognised lineages, *i.e.* phylogroups. This fact and the clear separation of the VOJ haplotypes in the Network Median Joining haplotype trees and the BI/ML phylogenetic trees resulting from both genes, are evidence that it is a separate new phylogroup. Nevertheless, the exact position of the VOJ phylogroup in the phylogenetic trees and its connection to other phylogroups cannot be determined with only two samples. The evolutionary divergence of the *MT-16SrRNA* gene of the new VOJ phylogroup shows a closer connection to ŽPB and BAN and is furthest away from phylogroups ZV and GK. The *MT-COI* dataset shows a slightly closer relationship of VOJ to BAN and SB. This suggests that a broader sample is required for further studies in this geographic region.

The three phylogroups ŽPB, LD and KOR occur in the close proximity to Bosnia and Herzegovina, in the border regions (Lovrenčić *et al.*, 2020a), but were not detected in BA samples in this study. Given the geographical/hydrogeographical characteristics of the regions, we could expect that, if not all, at least some of these groups will be found in future studies in Bosnia and Herzegovina. Considering the limited dispersal potential of crustaceans in general (Kerby *et al.*, 2005; Bubb *et al.*, 2006) and the geographical/hydrogeographical aspects of the area (Karst Dinarides in Bosnia and Herzegovina) that favour speciation and diversification of species (Bănărescu, 2004; Marčić *et al.*, 2011), the discovery of additional new phylogroups might not be far away.

Given the limited study area, which is in close proximity to a well-studied region in Croatia, the high genetic diversity in stone crayfish from the Western Balkans, which was also found in this study, was to be expected. Of particular interest however, is the discovery of original evolutionarily older haplotypes possibly belonging to a new phylogroup and its origin. VOJ could be a remnant population, diverged from BAN or SB. Following the Paratethys's continued retraction and the formation of modern river networks, including the Danube around 4.36 million years ago (de Leeuw *et al.*, 2018), the SB lineage started to colonize the Danube basin, leading to the CSE lineage (Pârvulescu *et al.*, 2019). This period could have isolated the VOJ lineage within its current karstic habitats, suggesting it originated from initial colonization attempts by BAN or SB.

A larger study area encompassing other main catchments of Bosnia and Herzegovina (Neretva, Bosna, and Drina) should shed more light on the status of this species in the region and could eventually modify the preserved topologies and phylogroup relationships.

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Supplementary material

Table S1. List of MT-16S rRNA and MT-COI sequences imported from the GenBank.

The Supplementary Material is available at https://www.kmae-journal.org/10.1051/kmae/2024023/olm.

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