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GENETIC DIVERSITY OF THE STONE CRAYFISH

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Abbreviations

16S rRNA – *16S* ribosomal RNA

ABGD – Automatic Barcode Gap Discovery

BA – Bayesian analysis

BEAST – Bayesian Evolutionary Analysis Sampling Trees

*BEAST – Star BEAST, Bayesian framework for species tree estimation

BIC – Bayesian information criterion

BIN – Barcode Index Number

bp – base pair

BPP – Bayesian posterior probability

bPTP – Bayesian implementation of the Poisson Tree Processes (PTP) model for species delimitation

COI – cytochrome c oxidase subunit I gene

DnaSP – DNA Sequence Polymorphism

ESS – Estimated Sample Size

F – empirical base frequencies

G – gamma distribution

GMYC – Generalized Mixed Yule Coalescent (GMYC) method

HKY – Hasegawa-Kishino-Yano evolution model

HPD - highest posterior density

I – invariant sites

ITS2 – Internal Transcribed Spacer 2

IUCN – International Union for Conservation of Nature

JC – Jukes and Cantor evolution model

Ka – thousand years ago

K2P – Kimura 2-parameter

K3Pu – three substitution types model and unequal base frequencies

Ma – million years ago

MAFFT – multiple sequence alignment based on fast Fourier transform

ML – maximum likelihood

MMCM – Metropolis-coupled Monte Carlo Markov chain

MP – maximum parsimony

NSV – node support value

OTU – operational taxonomic unit

PAUP – Phylogenetic Analysis Using Parsimony

PAUP* – Phylogenetic Analysis Using PAUP

PTP – Poisson Tree Processes

subs/s/my/l – substitution per site per million years per lineage

TBR – Tree-Bisection-Reconnection

TCS – Templeton, Crandall and Sing method

tcsBU – Templeton,Crandall and Sing (TCS) Beautifier

Contents

1. Introduction

The stone crayfish *Austropotamobius torrentium* (Schrank, 1803) (Figure 1) is the smallest of five European indigenous crayfish species and is considered a keystone species in freshwater ecosystems because of its role in preserving ecosystem stability and biodiversity (Füreder *et al.*, 2006; Reynolds, 2006; Reynolds *et al.*, 2013). It inhabits smaller streams and brooks at higher altitudes of central and south-eastern Europe (Holdich *et al.*, 2006; Kouba *et al*, 2014; Maguire *et al.*, 2018). The stone crayfish areal of distribution extends from Germany and the Czech Republic in the north, to Luxembourg in the west, to Greece in the south, and Turkey and Bulgaria in the east (Holdich *et al.*, 2006). Although their endemic areal of distribution is limited, they possess high genetic diversity preserved in eight distinct mtDNA lineages separated by prominent genetic gaps (Trontelj *et al.*, 2005; Klobučar *et al.*, 2013; Pârvulescu *et al.*, 2019).

Figure 1. Stone crayfish *Austropotamobius torrentium* (Schrank, 1803) (Photographed by Nataša Pršir)

In recent years many studies have shown that populations of *A. torrentium*, as well as other indigenous European crayfish species (*Astacus astacus, Astacus leptodactylus, Astacus pachypus, Austropotamobius pallipes*) are declining (Kouba *et al.*, 2014; Maguire *et al.*,

2018). They are threatened by both biotic and abiotic factors such as habitat deterioration (Holdich *et al.*, 2009; Weinländer *et al.*, 2014), water quality decline (Svobodová *et al.*, 2017), climate changes (manifested as prolonged/extreme droughts) (Maguire *et al.*, 2011; Kouba *et al.*, 2016), the presence/spread of non-indigenous invasive American crayfish species and their pathogens (eg. *Aphanomyces astaci*, the causative agent of the crayfish plague, disease that is lethal for indigenous crayfish species) (Souty-Grosset, 2016; Maguire *et al.*, 2016; Jussila *et al.*, 2017). Since *A. torrentium* is considered to be the most sensitive species of indigenous European crayfishes, and it is particularly sensitive to warm and polluted waters (Repina Potočki, 2016), it is protected by both national (NN 80/13, NN 144/2013) and international laws and listed in the Annexes II and V of the EU Habitats Directive (Council directive 92/43/EEC, 2000). The conservation status of *A. torrentium* is not yet resolved in all European countries since it is classified as data deficient on the IUCN Red List (Füreder *et al.*, 2010). In Croatia, as a result of the existence of historical and present data on the distribution and population size, the species is listed as vulnerable on the National Red List of Crustacea (VU A2ace) (Gottstein *et al.*, 2011; Maguire, 2014).

To develop adequate conservation plans and improve current protection of the species, it is necessary to determine the current state of genetic and morphological diversity within the species (Padial and De la Riva, 2006).

The first morphological studies aimed to distinguish different populations, presumably subspecies of stone crayfish (cf Crandall and De Grave, 2017). Three subspecies of *A. torrentium* were identified based on the morphological data: *A. t. torrentium* (von Paula Schrank, 1803), *A. t. macedonicus* (M. Karaman, 1929) and *A. t. danubicus* (S. Karaman, 1962; Starobogatov, 1996). Recently, analysing large number of morphometric and meristic characters per crayfish Maguire *et al.*, (2017) showed significant difference among distinct populations of *A. torrentium* in a small geographical region in Croatia.

Up till now, molecular phylogenetic studies of *A. torrentium* were based on the analyses of mtDNA (Trontelj *et al*., 2005; Schubart and Huber, 2006; Klobučar *et al.*, 2013; Pârvulescu *et al.*, 2019). In the first studies (Trontelj *et al.*, 2005) three divergent mtDNA phylogroups were discovered; one distributed in the south of the ákan Peninsula, another in a small area on the border between Slovenia and Croatia, and the third that encompasses the rest of Europe. This finding indicated that the stone crayfish should be considered a species complex. Later, the existence of four additional monophyletic phylogroups was discovered by Klobučar *et al.*,

(2013), with the highest genetic diversity found in the Dinaric region of Croatia. The phylogroups were named after geographical areas of distribution: central and south-eastern Europe (CSE), Gorski Kotar (GK), Lika and Dalmatia (LD), Žumberak, Plitvice and Bjelolasica (ŽPB), southern Balkans (SB), Banovina (BAN), Zeleni Vir (ZV). Phylogroups Gorski Kotar (GK), Lika and Dalmatia (LD), Žumberak, Plitvice and Bjelolasica (ŽPB), Banovina (BAN) and Zeleni Vir (ZV) are situated in the north and central Dinarides (NCD). Lately, Pârvulescu *et al.* (2019) discovered the existence of a new phylogroup, endemic to Romanian Apuseni Mountain region (APU). Combining the molecular mtDNA analyses with morphological data, the APU phylogroup was described as a new species of the genus *Austropotamobius* (*Austrobotamobius bihariensis*) (Pârvulescu, 2019).

Previous molecular phylogenetic studies have implied that *A. torrentium* phylogroups could be considered separate species (Klobučar *et al.*, 2013; Pârvulescu *et al.*, 2019; Pârvulescu, 2019). In order to successfully delimit evolutionary lineages, multiple characteristics should be considered. Morphological data (Maguire *et al.*, 2017; Pârvulescu, 2019) needs to be complemented with molecular approach for valid species identification (Puillandre *et al.*, 2012; Pante *et al.*, 2014). Integrative taxonomy, as an approach combines molecular, morphological, ecological, and geographical data to build species hypotheses. Integration of multiple approaches enables taxonomy to go beyond naming of a new species and brings to understanding of the processes that shape them (Schlick-Steiner *et al.*, 2010; Puillandre *et al.*, 2012).

Phylogenetic relationships between populations of *A. torrentium* can be reconstructed using the mitochondrial genes for cytochrome c oxidase subunit I (*COI*) and *16S* ribosomal RNA (Trontelj *et al.*, 2005; Schubart and Huber, 2006; Klobučar *et al.*, 2013). These mitochondrial genes are appropriate for resolving taxonomic relationships between genera and species (Harris and Crandall, 2000; Repina Potočki, 2016), but the need for a nuclear marker of *A.torrentium* genome to reconstruct the genetic relationships has been pointed out (Lefébure *et al.*, 2006). Unlike mitochondrial genes that are maternally inherited, nuclear genes are inherited biparentally. Therefore, they show deeper population structure related to both maternal and paternal lineages (Roberts *et al.*, 2004). Arthropod species delimitation studies showed that nuclear phylogenetic markers have lower failure rate at making species delimitation hypothesis than mtDNA markers (Schlick-Steiner *et al.*, 2010). When using multiple markers to delineate species it is recommended to include at least one nuclear marker, as two or more mitochondrial genes behave as a single locus marker because they are

linked on the same DNA molecule (Dellicour and Flot, 2018). As seen in previous studies, the second Internal Transcribed Spacer (*ITS2*) marker can be used as a complementary locus to mitochondrial *COI* and *16S* rRNA (Yao *et al.*, 2010). The *ITS2* is a spacer DNA located between structural ribosomal RNAs and has been used as a tool to define lower taxonomic categories such as genera, species and populations (Coleman, 2003) and elucidating relationships among closely related genera (Hao *et al.*, 2009). It is located between the 5.8S and 28S RNA genes and is easily amplified by PCR from small amount of DNA (Young and Coleman, 2004). Probably due to the large number of simple sequence repeats, the *ITS2* region of the ribosomal DNA repeat in crayfish is much longer (up to 1300 bp) than in other studied taxa sequences (200-600 bp) (Harris and Crandall, 2000; Young and Coleman, 2004). The *ITS2* sequence can also be used as a nuclear DNA marker to delineate species because of its concerted mode of evolution and fast mutation rates (Dellicour and Flot, 2018).

Cytochrome c oxidase subunit I (*COI*) gene has been used for DNA barcoding (Hebert *et al.*, 2003) and has found its applications as a marker for species delimitation, effective in revealing complex intraspecific relationships (Hebert *et al.*, 2003; Savolainen *et al.*, 2005; Hajibabaei *et al.*, 2007). Even more, databases cluster *COI* sequences into Barcode Index Numbers (BINs) which indicate species (Ratnasingham and Hebert, 2013). Single locus species discovery methods can be based either on genetic distances or allele sharing or tree-based approaches (Dellicour and Flot, 2018).

Methods based on genetic distances assign a threshold for identifying values that show intraspecific and interspecific divergence. The Automatic Barcode Gap Discovery (ABGD, Puillandre *et al.*, 2012) sorts the sequences into hypothetical species based on the barcode gap, which is observed when the intraspecific divergence is smaller than interspecific divergence. Tree based methods utilize phylogenetic analyses to identify species clusters. The Bayesian implementation of Poisson Tree Processes method (bPTP, Zhang *et al.*, 2013) deduces putative species based on a phylogenetic input tree. The General Mixed Yule Coalescent method (GMYC, Pons *et al.*, 2006) classifies branches in a phylogenetic tree as intraspecific, modelled with a neutral coalescent process or interspecific, modelled with a Yule model (Talavera *et al.*, 2013). The Templeton, Crandall and Sing method (TCS, Clement *et al.*, 2000) implements statistical parsimony to define the probability at which haplotypes connect with each other. It is used to construct haplotype networks but can also be used as a species delimitation tool (Hart and Sunday, 2007). Application of a wide range of different

species delimitation analyses reduces the impact of simplifying assumptions that each of the analyses makes which can affect the results. (Carstens *et al.*, 2013).

These species delimitation methods can be complemented with hypothesis-driven approaches like *BEAST that requires *a priori* assignment of samples to putative lineages (Bouckaert *et al.*, 2014). Bayes factor delimitation (BFD) approach can be used to compare candidate *BEAST species tree models based on Bayes factors (BF) (Grummer *et al*., 2013). Bayes factors are used as a model selection tool when models or phylogenetic hypothesis are compared. In order to calculate Bayes factor between two models, marginal likelihood estimation (MLE) is required (Kass and Raftery, 1995). A novel method for marginal likelihood estimation, nested sampling (NS), has been presented recently (Russel *et al.*, 2018). Unlike previous methods for marginal likelihood estimation (harmonic mean, path sampling and stepping-stone sampling and their different implementations), nested sampling provides the calculation of uncertainties in a single run (Russel *et al.*, 2018).

2. General and specific aims

With the increase of biotic and abiotic factors that threaten the indigenous stone crayfish *A. torrentium,* it is important to develop adequate conservation programs. The general aim of this research was to extend current knowledge about genetic diversity, an important segment in conservation programs development.

The specific aims of our study were: (a) to update previous phylogenetic findings through inclusion of previously unstudied populations of stone crayfish from Croatia, Slovenia and Macedonia, (b) to test *ITS2* as a marker for phylogenetic inference on *A. torrentium* and verify phylogenetic congruence between mitochondrial and nuclear DNA markers (c) to apply species delimitation methods on *A. torrentium* (d) to give new perspectives on *A. torrentium* conservation programs.

3. Methods

3.1. Sampling scheme

For this study 279 crayfish from 63 new locations were analysed. The localities from which the individuals were sampled are shown in Figure 2 and Table A1. One pereiopod from each individual was sampled and stored in 96% ethanol at 4 °C until DNA isolation.

This approach does not compromise crayfish integrity since the sampled appendage would regenerate upon next moulting. All sampling were conducted in accordance with ethical standards and all required permissions were obtained from Ministry of Environmental Protection and Energy of the Republic of Croatia (UP/I-612-07/18-48/148).

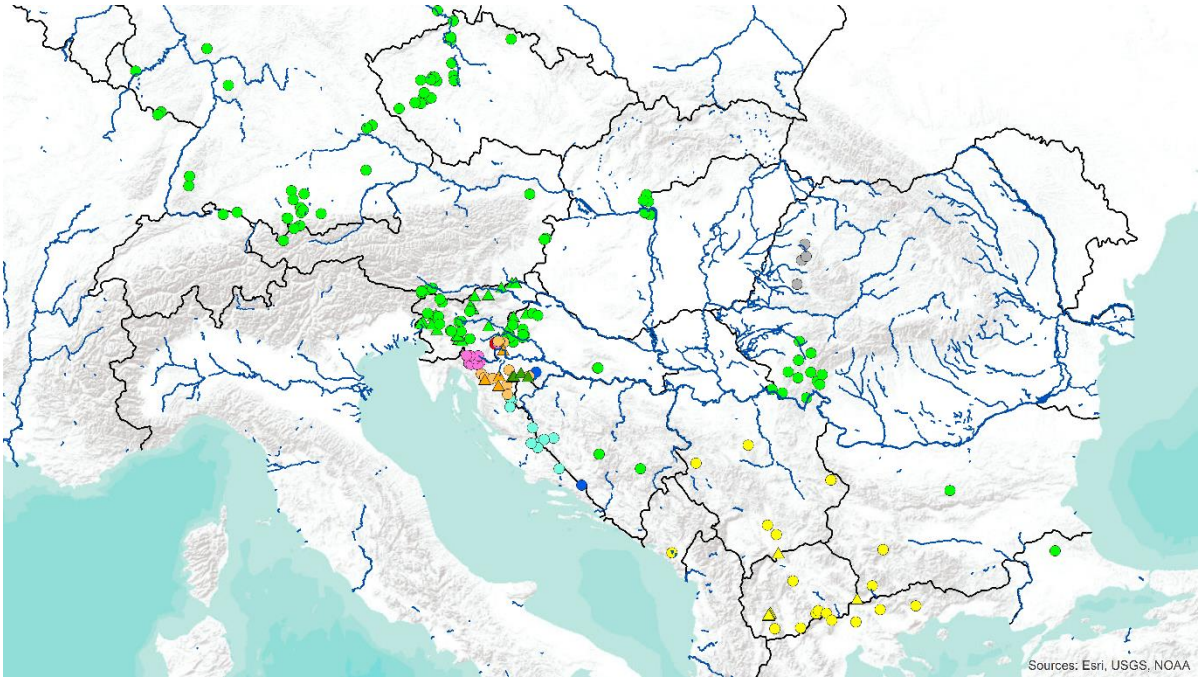

Figure 2. Geographical distribution of different *A. torrentium* phylogroups in Europe. Symbols used on the map: dots represent samples from previous research, and triangles samples from this study. Colours: lime green – central and south-eastern Europe (CSE), pink – Gorski Kotar (GK), aqua blue – Lika and Dalmatia (LD), orange – Žumberak, Plitvice and Bjelolasica (ŽPB), yellow – southern Balkans (SB), blue – Banovina (BAN), red – Zeleni Vir (ZV), gray – Apuseni Mountain (APU) and dark-green – Kordun (KOR), new phylogroup discovered in the present study.

3.2. DNA extraction, gene amplification and sequencing

Genomic DNA was extracted from muscle tissue using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol, and stored in a freezer until PCR amplification.

Mitochondrial *16S* rRNA and *COI*, and nuclear *ITS2* genes were amplified and sequenced. The *COI* primer sequences, LCO-1490 and HCO-2198, were adopted from Folmer *et al.* (1994) and the16S rRNA primer sequences, 16Sar and 16Sbr were used after Palumbi *et al*., 1991. The choice of these markers enabled the comparison of the newly obtained sequences with existing sequences from GenBank. The primer pair for the *ITS2* gene, ITSL2 and ITSH1b/m were adopted from Schubart *et al.*, 2010 and Jelić *et al.*, 2016 (Table 1). The *ITS2* gene was chosen as a nuclear marker because it shows sequence variability at the intraspecific level and it is used as an effective barcode locus complementing *COI* (Yao *et al.*, 2010).

Table 1. Sequences of primers used in PCR reactions and respective authors.

Primer	Sequence	Authors
LCO-1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	Folmer *et al.*, 1994
HCO-2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Folmer *et al.*, 1994
16S rRNAar	5'-CGCCTGTTTATCAAAAACAT-3'	Palumbi *et al.*, 1991
16S rRNAbr	5'-CCGGTCTGAACTCAGATCACGT-3'	Palumbi *et al.*, 1991
ITSL2	5'-AAGAATACCAGATACATCGACAA-3'	Schubart *et al.*, 2010
ITSH1b/m	5'-CCGGTTCAGTCGCCCTTACT-3'	Jelić *et al.*, 2016

Polymerase chain reaction (PCR) was carried out following the protocols. The final reaction mix in a total volume of 10 µL for *16S* rRNA gene contained 0.05 U/µL GoTaq G2 HotStart Polymerase, 1.5 mM GoTaq FlexiBuffer, 0.2 mM of each dNTP, 0.275 µmol/µL of each primer, and 10-50 ng/µL of DNA template. The PCR cycling protocol included: initial activation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 5 min. The reaction mix in a total volume of 25 µL for *COI* gene contained 0.04 U/µL HotStart Polymerase, 1.5 mM Promega Buffer, 0.15 mM of each dNTP, 0.400 µmol/µL of each primer, 0.7 mM $MgCl_2$, and 10-50 ng/µL of DNA template. The PCR cycling protocol included: initial activation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 48°C for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The reaction mix in a total volume of 20 µL for *ITS2* region contained 1.5 mM DreamTaq Mix, 0.400 µmol/µL of each primer, and 10-50 ng/µL of DNA template. The PCR

cycling protocol included: initial activation at 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 80 min and final extension at 72 °C for 10 min. The enzymatic purification of PCR products was performed using 2 µL of EXOAnP mix (containing 0.05 U/µL Antarctic Phosphatase and 0.5 U/µL Exonuclease I) for 20 µL of PCR product. The reaction was carried out on a PCR machine for 1 h at 37 °C followed by 20 min at 80°C. Sequencing of purified PCR products was performed by Macrogen Inc. (Amsterdam, Netherlands).

3.3. Sequence data

Sequences were edited using SEQUENCHER 5.4.6 (Gene Codes Corporation, Ann Arbor, MI USA) and aligned using MAFFT (Katoh and Standley, 2013). The final alignments were 582 and 476 bp long for *COI* and *16S* rRNA, respectively, while *ITS2* region was 1102 bp long. The *COI* and *16S* rRNA alignments did not contain any length variants or ambiguous sites, while the sequences of the *ITS2* region contained length variations and six ambiguous sites.

3.4. Phylogenetic reconstruction

Phylogenetic reconstruction analyses were performed on two data sets (Table 2). The first data set consisted of concatenated *COI* and *16S* rRNA sequences, and the second included only *ITS2* sequences. All phylogenetic trees were visualised in FigTree v1.4.4 (Rambaut, 2006) and edited in Inkscape 0.92.3 (Harrington *et al.*, 2005).

3.4.1. Mitochondrial DNA

The phylogenetic analyses included a total of 1114 *16S* rRNA and *COI* genes sequences of which 642 mtDNA sequences (211 *16S* rRNA and 431 *COI*) were downloaded from GenBank and 472 sequences (274 *16S* rRNA and 198 *COI*) were obtained in this study (Table A1). The sequences were collapsed to 55 unique *16S* rRNA haplotypes (16 new) and 153 *COI* haplotypes (57 new) using the software DnaSP 6.12.03 (Rozas *et al.*, 2017).

The final length of the alignment for concatenated mitochondrial sequences was 1058 bp. The data set included two *16S* rRNA and two *COI* sequences of *Austropotamobius pallipes* (KX370093, KX370094, KX369673, KX369674) as an outgroup. To assess congruence among the different partitions, the incongruence length difference test (Farris *et al.*, 1994) as implemented in PAUP* 4.0a164 (Swofford, 2002), was applied using 100 replicates. No significant heterogeneity amongst the partitions was detected (P = 0.78).

Phylogenetic relationships were reconstructed using three different optimality criteria: maximum parsimony (MP), maximum likelihood (ML) and Bayesian analysis (BA).

Maximum parsimony analysis was performed using a Tree-Bisection-Reconnection (TBR) algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates) in MEGA X (Kumar *et al.,* 2018). Support for individual clades in MP was evaluated using nonparametric bootstrapping (Felsenstein, 1985) obtained from 10000 bootstrap replicates. Values above 80 were considered well supported.

Maximum likelihood analysis was conducted on the IQ-TREE webserver (Trifinopoulos *et al.*, 2016). For this analysis we used the best-fit model for each partition (*16S* rRNA and *COI*) calculated using ModelFinder on the IQ-TREE webserver (Chernomor *et al.,* 2016; Kalyaanamoorthy *et al.,* 2017). For *16S* rRNA partition the three substitution types model, unequal base frequencies with empirical base frequencies and gamma distributed rate variation among invariable sites(K3Pu+F+I+G4) was used, while for *COI* partition the HKY model unequal base frequencies with empirical base frequencies and gamma distributed rate variation among invariable sites (HKY+F+I+G4) was used. We obtained branch supports with the 10000 bootstrap alignments using ultrafast bootstrap (Hoang *et al.*, 2018) implemented in the IQ-TREE software. The maximum likelihood tree was inferred using the edge linked partition model (Chernomor *et al.,* 2016). Bootstrap values above 90 were considered well supported.

For the Bayesian analysis the optimal model of nucleotide evolution for each partition of the concatenated *COI* and *16S* rRNA sequences was selected under the Bayesian information criterion (BIC) using the jModelTest 2.1.10 (Darriba *et al.,* 2012). The selected model for *COI* was HKY model with gamma-distributed rate variation among sites and unchanging sites (HKY + I + G), and for *16S* rRNA HKY model with gamma-distributed rate variation among sites (HKY + G).

Bayesian analysis was performed in Mr.Bayes 3.2.6 (Ronquist *et al.,* 2012) with priors set according to the suggested model for each partition of concatenated data set. Two separate runs with four Metropolis-coupled Monte Carlo Markov chains (MMCM) were performed for 10000000 generations, and trees were sampled every 1000 generations. To check the convergence between the two runs we checked three diagnostic parameters: average standard deviation of split frequencies fell below 0.01 after 1866000 generations, Potential Scale

Reduction Factor (Gelman and Rubin, 1992) approached 1 and ESS (Estimated Sample Size) values were well above 200 for each parameter. We eliminated the first 25% of sampled trees as burn-in and a 50% majority-rule consensus tree was constructed, with nodal values representing the posterior probabilities.

3.4.2. Nuclear DNA

Phylogenetic analysis of the *ITS2* region was performed on 23 unique haplotype sequences, of which 22 were obtained in this study. They were selected as representatives of CSE, SB, BAN, ŽPB, ZV, GK and LD mitochondrial phylogroups. Four *A. pallipes* haplotypes were used as an outgroup. The optimal model of nucleotide evolution was selected under the BIC using jModelTest 2.1.10 (Darriba *et al.*, 2012). The proposed model was Jukes-Cantor (JC) with equal base frequencies, all substitutions equally likely assessed. Phylogenetic relationships were reconstructed using Bayesian analysis (BA). Before the Bayesian analysis *ITS2* dataset was analysed using FastGap (Borschenius, 2009) and the results were implemented in the Bayes block. Two separate runs with four MMCM chains were performed for 3000000 generations, and trees were sampled every 100 generations. All three diagnostic parameters of convergence were checked, average standard deviation of split frequencies fell below 0.01 after 190000 generations, Potential Scale Reduction Factor (Gelman and Rubin, 1992) approached 1 and ESS (Estimated Sample Size) values were well above 200 for each parameter. Bayesian posterior probabilities values above 0.9 were considered as supported.

3.5. Genetic diversity and haplotype networks

Pairwise comparison of uncorrected sequence divergences (p-distances) for *COI*, *16S* rRNA and *ITS2* sequences was performed in MEGA X (Kumar *et al.*, 2018). The rate variation among sites was modeled with a uniform distribution for all markers. The number of base substitutions per site from averaging over all sequence pairs between groups was calculated. Separate distance analyses were conducted using the Kimura 2-parameter model (Kimura, 1980) and the rate variation among sites was modeled with a uniform distribution for all markers.

The median joining network was built on concatenated *COI*/*16S* rRNA sequences using PopArt (Leigh *et al.*, 2014) to visualize the non-hierarchical haplotype relationships and their geographical distribution. This approach is recommended for analysis of intraspecific

evolutionary relationships (Posada and Crandall, 2001; Klobučar *et al.*, 2013; Jelić *et al.*, 2016).

Phylogenetic network using statistical parsimony was constructed for the *COI* gene using the TCS 1.21 software (Clement *et al.*, 2000). The network was visualized using tcsBU (Múrias dos Santos *et al.*, 2016).

3.6. Time of divergence

To estimate divergence times among phylogroups, concatenated data set (*COI* and *16S* rRNA) was used in the Bayesian statistical framework implemented in BEAST 2.5.2 (Drummond and Rambaut, 2007). The analyses were run on the Cipres Science Gateway (http://www.phylo.org/).

For this purpose, six different calibration approaches were employed (three molecular and three geological).

Molecular clock rate calibrations were based on the arthropod substitution rate of 2.3% pairwise sequence divergence (0.0115 subs/s/my/l) (Brower, 1994) and the decapod substitution rate of 1.4% for *COI* partition of mtDNA dataset (0.007 subs/s/my/l) (Schubart *et al.*, 1998). In both approaches we used an estimated molecular clock for the *16S* rRNA partition since no divergence rate is available for this gene. In the third approach we set the meanRate prior as a uniform distribution between 0.0083–0.01165 subs/s/my/l for *COI* and 0.00325–0.0044 subs/s/my/l for *16S* rRNA. Mid-points of these intervals (0.0099 for *COI* and 0.0038 for *16S* rRNA) were used as an ucld.mean prior (Klobučar *et al.*, 2013).

For the geological calibration of molecular clock, we used two previously described approaches. First, we used and event of intense uplifting of the Dinarids that took place around 12.5 Ma and caused the split between *A. pallipes* and *A. torrentium* (Pavelić and Belak, 2008; Klobučar *et al.*, 2013; Jelić *et al.*,2016). TreeModel prior distribution was set to normal, with a mean of 12.5 Ma and a standard deviation of 0.5. The second approach was based on the tectonic separation of the Apuseni Mountains from Dinarides (Tisza-Dacia microplate final tectonic rotation) that took place 16±1 Ma and it has been used as a calibration point for splitting between APU and other NCD phylogroups (Pârvulescu *et al.*, 2019), TreeModel prior distribution was set to normal, with a mean of 16 Ma and a standard deviation of 0.5. For the third geological calibration point rather than using orogenesis events, we used the formation of fluvial connection between the paleo-Danube River and paleo-Tisza

River systems that took place around 5.3 Ma (Magyar *et al.*, 2013). That event would enable colonization of nowadays north-eastern areal of *A. torrentium* distribution. TreeModel prior distribution was set to normal, with a mean of 5.3 Ma and a standard deviation of 0.5.

The molecular clock tests were performed using the maximum likelihood method in MEGA X by comparing the ML value for the given topology with and without the molecular clock constraints under HKY+G+I (for *COI* data set) and HKY+G (for *16S* rRNA data set) models. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level.

Prior was set to Yule model and clock model to relaxed molecular clock with an uncorrelated lognormal distribution for all tree calibrations. Independent substitution models were assigned to each gene partition (HKY + I + G for the *COI* partition and HKY + G for the *16S* rRNA partition). Molecular clock rate calibrations were run for 50000000 generations. Geological calibration using the uplift of Dinarides, tectonic separation of the Apuseni Mountains and the paleo-Danube/paleo-Tisza river systems connection were run for 50000000, 75000000 and 100000000 generations, respectively. The generated trace files were analysed using Tracer v1.6 (Rambaut *et al.*, 2018), and 25% of the sampled trees were subsequently discarded as burn-in and the consensus tree was produced in TreeAnnotator v2.5.2 (Drummond and Rambaut, 2007)

3.7. Species delimitation

Species delimitation analyses were performed only on *COI* sequences using four methods: ABGD, GMYC, bPTP and TCS.

For species delimitation The Automatic Barcode Gap Discovery (ABGD; Puillandre *et al.*, 2012) was performed on the web interface (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) using the *COI* sequence alignment file with no outgroups as input data. For all the parameters default values were employed, and analysis was conducted using Kimura 2 parameter (K2P) model. Values of prior intraspecific divergence P= 1.29×10^{-2} and P=4.64×10^{-3} were chosen as significant, because the value of recursive and initial partition were the same on those values.

GMYC (Generalized Mixed Yule Coalescent method) analyses were executed with the SPLITS package (Ezard *et al*., 2009) in R. The outgroups *A. astacus*, *A. leptodactylus* and *A. pallipes* were excluded from GMYC analysis using the function drop.tip( ) from the package

APE (Paradis *et al*., 2004). The ultrametric tree for GMYC was constructed using BEAST 2.5.2. as recommended in other studies (Monaghan *et al*. 2009; Ceccarelli *et al.,* 2012). BEAST analysis was run for 50000000 generations using Coalescence tree prior and a relaxed molecular clock with an uncorrelated log-normal distribution. The substitution model HKY + I + G was assigned to the *COI* datasets, with *Astacus leptodactylus, Astacus astacus* and *Austropotamobius pallipes* as outgroups. The substitution rate for *COI* was set to 0.007 subs/s/my/l and an estimated molecular clock was set for *16S* rRNA. We used the coalescence tree prior because it is considered to be a more adequate option and appears to fit better the majority of the data sets in model comparisons than the Yule prior which results in greater number of entities (Monaghan *et al*., 2009, Talavera *et al.,* 2013).

Bayesian implementation of the Poisson Tree Processes (bPTP) model for species delimitation (Zhang *et al.,* 2013) was run on the webserver at http://species.h-its.org using default parameters. Input tree for bPTP was the same as for GMYC analysis. The bPTP analysis was ran for 5000000 generations to reach convergence of log values. Again, outgroup sequences were excluded from this analysis.

3.8. Species validation

We estimated *A. torrentium* species tree using *BEAST v.2.5.2. The *COI* haplotypes were assigned into different species trees topologies according to the results of species delimitation analyses (ABGD (P=1.29e-02), TCS, GMYC and bPTP) as well as the consideration that all samples/crayfish belong to one/same species (details presented in Table 8). We performed multispecies coalescent Bayesian inference of tree topology, using an uncorrelated log-normal relaxed molecular clock with *COI* substitution rate of 0.007 subs/s/my/l. A Yule tree prior was used, and analysis was run for 50000000 generations. Sequences from *A. pallipes*, *A. astacus* and *A. leptodactylus* were used as an outgroup. We assessed ESS using Tracer v1.6 (Rambaut *et al.,* 2018). Marginal likelihood estimation of each species tree using nested sampling analysis was made. Chain length for nested sampling analysis was 200000, with sub chain length of 10000 and 20 particles, default value of epsilon (1^{-12}) was not changed. Bayesian factors (2*ln*Bfs) between two competing models were calculated as suggested by Kass and Raftery (1995). A positive BF (2*ln*Bfs) values reflect evidence in favor of the first model, to which the second model is compared, whereas negative BF values are considered as evidence favoring the second model. Values above 10 (2*ln*Bfs>10) are considered as decisive evidence in favor of the first model (Leaché *et al.,* 2014). Species tree was visualized in DensiTree v.2.2.6 (Bouckaert and Heled, 2014).

Table 2. Scheme depicting data set used in this study for different analyses. Different data sets were used according to the suitability for different analyses. Data set I was composed of concatenated *COI* and *16S* rRNA sequences and included *A. pallipes* sequences as outgroup. Data set II included a smaller number of *ITS2* sequences required to assess congruence between nuclear and mitochondrial genetic markers. Data set III consisted of concatenated *COI* and *16S* rRNA sequences, including 3 outgroups, and was used for divergence time analyses. Data set IV, for species delimitation analyses, was composed of *COI* sequences without outgroups, as it provided more accurate variation to study population processes and allowed comparison with previous studies and across different methodology. Data set V consisted of *COI* sequences for species validation with inclusion of *A. pallipes*, *A. astacus* and *A. leptodactylus* sequences as outgroups.

Data set	*16S* rRNA	*COI*	*ITS2*	Analyses	bp (n sequences)	Outgroup (n sequences)
I				Phylogenetic reconstruction and haplotype networks	1058 (144)	*A. pallipes* (2)
II				Nuclear genetic congruence	1102 (23)	*A. pallipes* (4)
III				Time of divergence	1059 (146)	*A. pallipes* (2), *A. astacus* (2), and *A. leptodactylus (2)*
IV				Species delimitation	582 (152)	no outgroup
V				Species validation	582 (158)	*A. pallipes* (2), *A. astacus* (2) and *A. leptodactylus (2)*

4. Results

4.1. Sequence data

We obtained a total of 54 *16S* rRNA and 163 *COI* unique haplotypes and 23 *ITS2* alleles. The concatenated *COI*/*16S* rRNA data set included 169 (80 new) haplotype combinations (Table 3). Analyses of *COI* gene revealed 166 (27.95%) variable sites, of which 141 (23.74%) were parsimony informative. For *16S* rRNA sequences 64 (13.42%) sites were revealed as variable with 49 (10.27%) containing parsimony informative sites. The combined data set contained 223 (21.07%) variable sites with 185 (17.48%) parsimony informative sites. Obtained *ITS2* alleles showed only 17 (1.54%) variable sites, and 11 (1.00%) were parsimony informative. Analysis of the *ITS2* sequences using FastGap revealed 24 (2.18%) gap informative sites.

Table 3. The number of haplotypes for *16S* rRNA and *COI* genes and for the concatenated dataset. The number of new haplotypes for each phylogroup is marked in blue.

	***16S* rRNA**		***COI***		**CON**	
Phylogroup	All	New	All	New	All	New
BAN	3	1	11	8	11	8
ZV	3	0	5	0	6	0
KOR	3	2	3	3	4	3
ŽPB	3	0	14	4	15	4
GK	2	0	13	3	14	3
LD	3	0	9	0	11	0
SB	11	2	24	3	27	6
CSE	25	11	72	35	90	52
APU	2	0	2	2	4	4

Phylogenetic reconstruction

4.1.1. Mitochondrial DNA

All implemented criteria of phylogenetic reconstruction (BA, MP and ML) yielded mostly congruent topologies for *COI*/*16S* rRNA concatenated data set (Figure 3). The majority of the newly obtained sequences nested within the existing phylogroups. The presence of eight previously reported monophyletic phylogroups (Klobučar *et al.*, 2013; Pârvulescu *et al.*, 2019) was confirmed. Moreover, a new phylogroup belonging to the Kordun region part of Dinarids, was discovered. The phylogroups belonging to the northern-central Dinaric (NCD) region ('Zeleni Vir' (ZV), 'Gorski Kotar' (GK), 'Žumberak, Plitvice and Bjelolasica' (ŽPB), 'Lika and Dalmatia' (LD), 'Banovina' (BAN), 'Kordun' (KOR)) and to the 'Apuseni Mountains' (APU) appear as monophyletic clades well supported by nonparametric bootstrap support values, ultrafast bootstrap support values and Bayesian posterior probabilities (Figure 3).'Southern Balkans' (SB) phylogroup was not supported as monophyletic, rather it represents a polytomy that consists of six sub-clades of which four are well supported and two represent separated lineages. Numerous sub-clades also exist within well supported monophyletic CSE phylogroup.

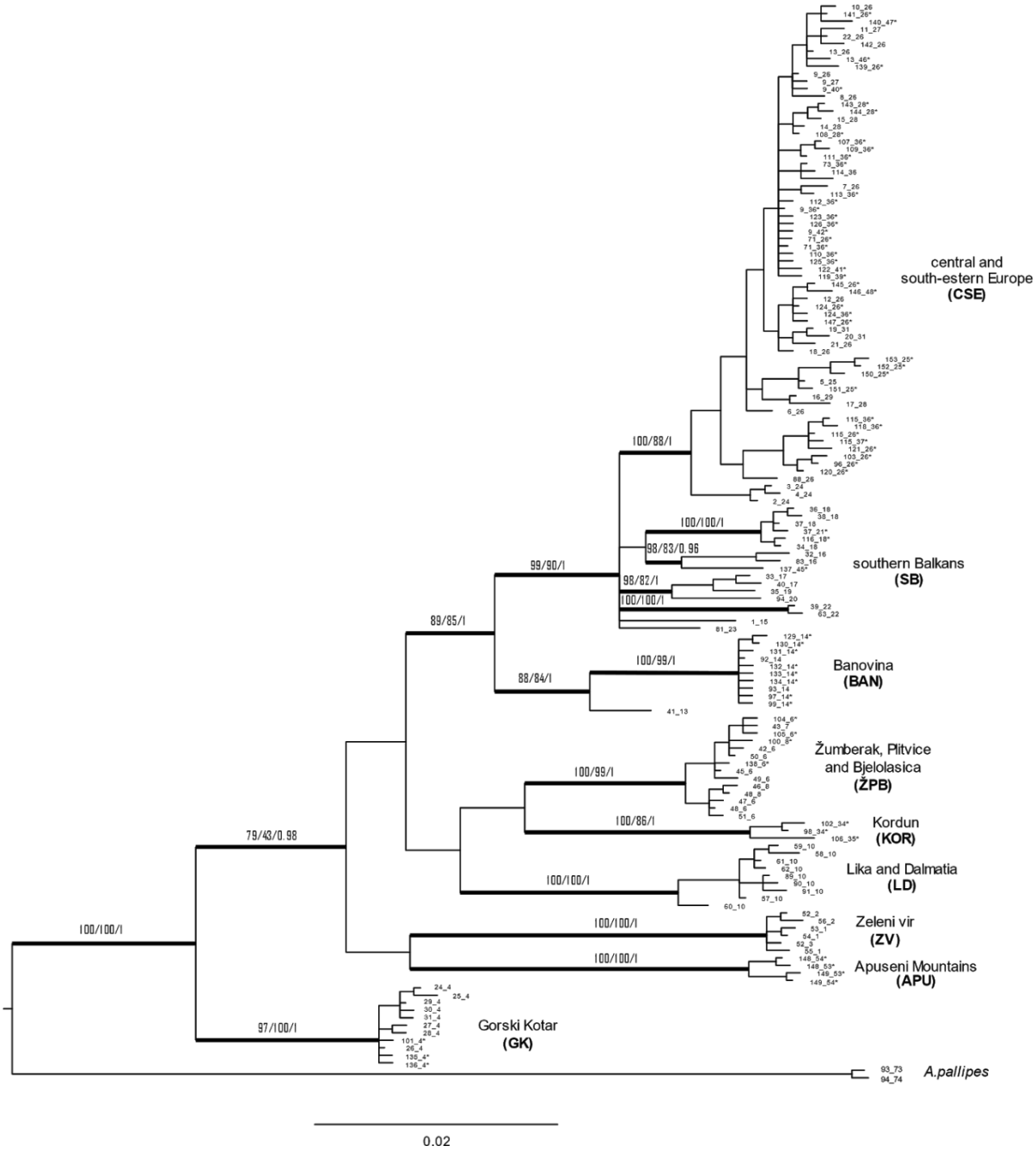

Figure 3. Bayesian phylograms of *A. torrentium* based on the concatenated *COI* and *16S* rRNA haplotypes. Numbers at the nodes represent maximum likelihood ultrafast bootstrap support values (%), maximum parsimony (MP) nonparametric bootstrap support values (%) and Bayesian posterior probabilities. Haplotypes obtained in this study are marked with an asterisk (*).

4.1.2. Nuclear DNA

The Bayesian phylogenetic analysis using *ITS2* data set yielded a tree topology with six monophyletic phylogroups. Sequences from five phylogroups belonging to the NCD region each form monophyletic clade, well supported by posterior probabilities. As in the analysis of *COI*/*16S* rRNA data set, 'southern Balkans' (SB) and 'central and south-eastern Europe' (CSE) haplotypes are comprised within one monophyletic phylogroup (Figure 4).

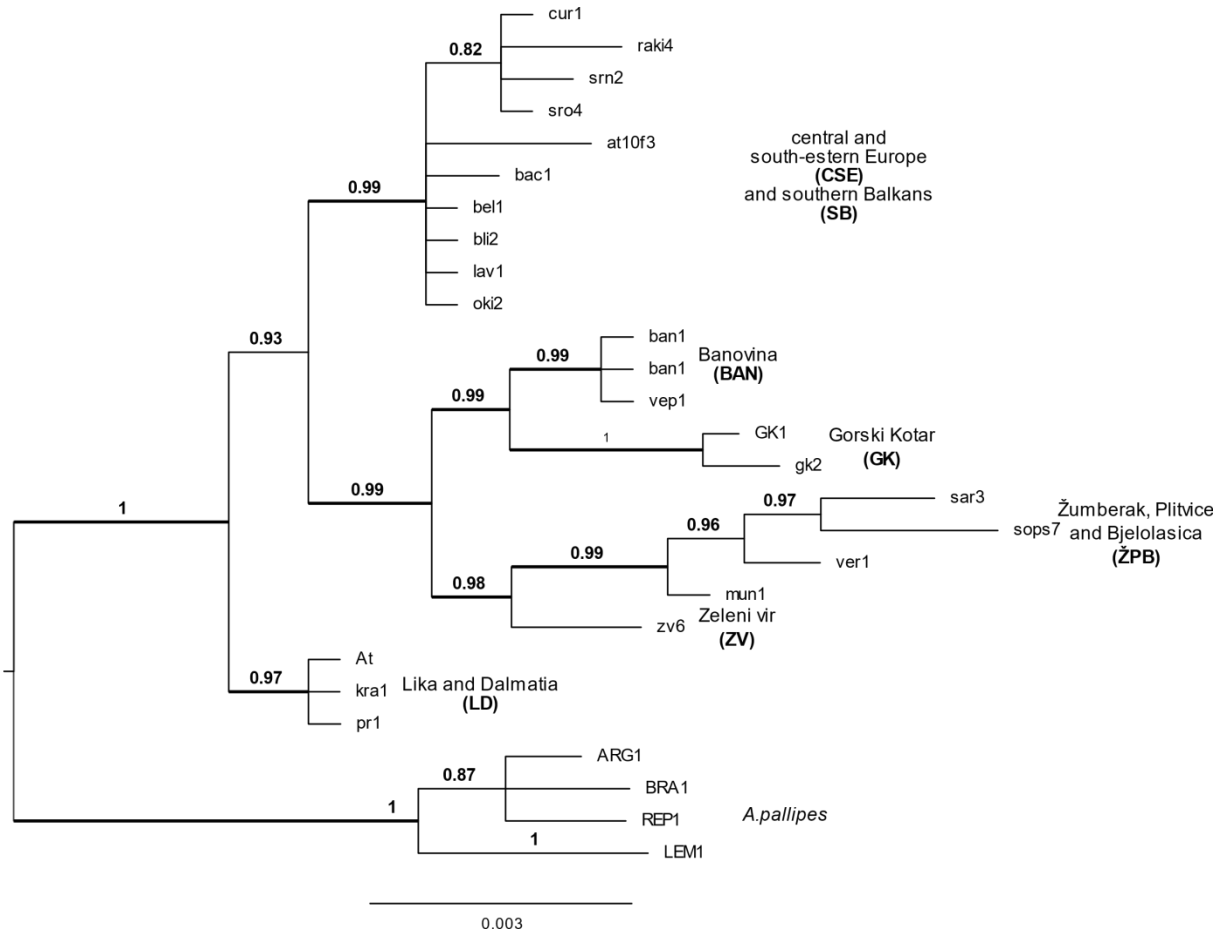

Figure 4. Bayesian phylogram of *A. torrentium ITS2* alleles. Numbers at nodes represent Bayesian posterior probabilities.

4.2. Genetic diversity

The genetic diversity analysis was made separately for *16S* rRNA, *COI* and *ITS2* data sets. The obtained uncorrected sequence divergences (p-distances) among phylogroups for *COI* and *16S* rRNA are shown in Table 4, while the K2P distances for the mitochondrial DNA markers are shown in Table 5.

The p and K2P distances for *ITS2* are shown in Table 7. The value of p-distances ranged from 4.21% to 8.83% for *COI* and from 0.72% to 4.79% for *16S* rRNA (Table 4). The smallest p-distance for *16S* rRNA was observed between KOR and ŽPB phylogroups (0.72%). Small genetic distance for both *16S* rRNA and *COI* markers was also observed between CSE and SB phylogroups (1.64% and 4.2%, respectively). ZV phylogroup showed the highest values of genetic distance from all the other phylogroups for both *16S* rRNA and *COI* marker (4.79% and 8.83%, respectively).

The range of K2P distances for *COI* gene was between 4.42% and 9.8% and for the *16S* rRNA gene between 0.73% and 5.04%. The least divergent phylogroups were CSE and SB for the *COI* gene, and KOR and ŽPB for the *16S* rRNA gene. The highest value of genetic distance between phylogroups was observed between KOR and BAN, and between ZV and GK phylogroups for *COI* and *16S* rRNA genes, respectively (Table 5).

Table 4. Estimates of evolutionary divergence over sequence pairs. Average values of uncorrected (p) distances (in %) among nine *A. torrentium* mtDNA phylogroups for *COI* (below diagonal) and *16S* rRNA (above diagonal, marked in blue). The highest values are shown in red and the lowest in blue.

	CSE	**BAN**	**GK**	**LD**	**SB**	**ŽPB**	**KOR**	**ZV**	**APU**
CSE		2.03±0.56	3.81±0.86	2.22±0.57	1.64±0.35	2.34±0.62	2.82±0.68	3.96±0.80	3.54±0.78
BAN	6.47±0.94		3.64±0.79	1.94±0.57	1.89±0.47	1.87±0.57	2.45±0.67	3.74±0.81	2.56±0.68
GK	8.67±1.12	8.44±1,09		3.28±0.78	3.78±0.76	3.06±0.75	3.35±0.8	4.79±0.95	4.32±0.88
LD	7.26±0.99	7.58±0.95	7.59±1.03		2.26±0.53	1.75±0.58	2.23±0.66	3.05±0.71	3.06±0.75
SB	4.21±0.59	6.21±0.83	8.63±1.04	7.5±0.9		2.44±0.58	2.96±0.68	3.94±0.78	3.13±0.7
ŽPB	7.94±1.04	8.21±1.03	7.05±1.03	6.87±0.95	8.19±0.99		0.72±0.32	3.98±0.83	2.27±0.62
KOR	8.02±1.02	8.83±1.08	8.12±1.06	7.66±1.01	8.53±1.02	7.31±0.99		4.61±0.89	2.84±0.7
ZV	8.01±1.04	8.25±1.04	6.53±0.93	8.13±1.07	7.97±0.93	7.82±1.07	8.83±1.08		4.57±0.89
APU	8.61±1.08	8.22±1.06	8.51±1.14	8.09±1.03	8.42±0.97	8.22±1.06	8.36±1.08	7.59±1.08	

Table 5. Average values of Kimura two-parameter (K2-p) distances (in percentages) for *COI* gene (below diagonal) and *16S* rRNA (above diagonal, marked in blue). The highest values are shown in red and the lowest in blue.

	CSE	BAN	GK	LD	SB	ŽPB	KOR	ZV	APU
CSE		2.06±0.56	3.93±0.87	2.22±0.61	1.67±0.37	2.38±0.66	2.89±0.74	4.09±0.88	3.64±0.83
BAN	6.85±1.04		3.75±0.86	1.98±0.59	1.94±0.47	1.91±0.59	2.50±0.71	3.86±0.91	2.61±0.71
GK	9.4±1.30	9.15±1.26		3.37±0.82	3.91±0.83	3.14±0.81	3.45±0.85	4.98±1.06	4.48±0.97
LD	7.76±1.13	8.15±1.12	8.19±1.22		2.31±0.57	1.78±0.61	2.28±0.68	3.12±0.78	3.15±0.81
SB	4.37±0.64	6.59±0.92	9.37±1.21	8.07±1.06		2.50±0.63	3.05±0.74	4.08±0.85	3.22±0.74
ŽPB	8.53±1.18	8.87±1.22	7.56±1.17	7.35±1.1	8.86±1.14		0.73±0.32	4.13±0.93	2.31±0.65
KOR	8.61±1.17	9.59±1.23	8.78±1.25	8.25±1.18	9.24±1.17	7.83±1.14		4.80±1.01	2.92±0.73
ZV	8.58±1.21	8.87±1.19	6.93±1.08	8.77±1.28	8.55±1.09	8.42±1.24	9.57±1.29		4.77±0.97
APU	9.25±1.23	8.82±1.19	9.22±1.31	8.71±1.24	9.07±1.11	8.88±1.21	9.0±1.21	8.12±1.24	

Table 6. Average values of uncorrected (p) distances (in %) and average values of Kimura two-parameter (K2P) distances (in %) within nine *A. torrentium* phylogroups for *16S* rRNA and *COI* datasets. The highest values are shown in red and the lowest in blue

	***16S* rRNA**		***COI***	
	P	K2P	P	K2P
ZV	0.29±0.2	0.29±0.19	0.45±0.17	0.45±0.17
LD	0.29±0.19	0.29±0.19	0.89±0.21	0.91±0.24
BAN	0.43±0.24	0.43±0.24	0.96±0.19	0.97±0.18
SB	1.38±0.3	1.40±0.32	3.17±0.41	3.27±0.41
CSE	0.82±0.19	0.83±0.2	1.27±0.23	1.29±0.22
KOR	0.29±0.2	0.29±0.21	1.14±0.31	1.16±0.33
GK	0.22±0.2	0.22±0.2	0.61±0.12	0.61±0.17
ŽPB	0.29±0.19	0.29±0.19	0.74±0.17	0.74±0.21
APU	0.22±0.2	0.22±0.2	1.03±0.39	1.04±0.41

The range of p distances within phylogroups for *16S* rRNA gene was between 0.22% and 1.38% and for the *COI* gene between 0.45% and 3.17%. The values of K2P distances were similar to the values of p-distances. The lowest values for the *16S* rRNA gene were obtained for the NCD and APU phylogroups and the highest for the SB phylogroup (1.38% for p-distances and 1.40% for K2P distances). High values of genetic distances were also observed within the CSE phylogroup (0.82% for p-distances and 0.83% for K2P distances). The genetic distances within phylogroups for the *COI* gene were the lowest for ZV phylogroup (0.45 for both p- and K2P distances) and the highest for the SB phylogroup (3.17% for p-distances and 3.27% for K2P distances).

High values of genetic distances were also observed within the CSE (1.27% for p-distances and 1.29% for K2P distances) and KOR phylogroup (1.14% for p-distances and 1.16% for K2P distances).

Table 7. Average values of uncorrected (p) distances (in %) among seven *A. torrentium* phylogroups for *ITS2* region (below diagonal) and average values of Kimura two-parameter (K2P) distances (in percentages) for *ITS2* region (above diagonal, marked in blue). The highest values are shown in red and the lowest in blue

	LD	**SB**	**CSE**	**BAN**	**GK**	**ŽPB**	**ZV**
LD		0.493±0.22	0.295±0.15	0.098±0.09	0.394±0.19	0.345±0.16	0.295±0.16
SB	0.491±0.22		0.219±0.13	0.394±0.19	0.691±0.26	0.642±0.23	0.592±0.22
CSE	0.294±0.16	0.218±0.13		0.197±0.13	0.493±0.21	0.444±0.18	0.394±0.17
BAN	0.098±0.09	0.393±0.19	0.196±0.12		0.295±0.16	0.246±0.14	0.197±0.13
GK	0.393±0.19	0.688±0.26	0.491±0.21	0.295±0.17		0.542±0.22	0.493±0.21
ŽPB	0.344±0.16	0.638±0.23	0.442±0.18	0.246±0.14	0.540±0.23		0.246±0.14
ZV	0.295±0.16	0.589±0.22	0.393±0.18	0.196±0.13	0.491±0.21	0.246±0.13	

The values of p-distances and K2P distances for the *ITS2* gene, which shows a slower evolution rate then mitochondrial genes, showed congruent results to mitochondrial genes. Phylogroups SB and GK were most divergent, while CSE and SB phylogroups were closely related (Table 7). Genetic distances for all genes obtained using K2P and p distance methods were congruent.

4.3. Population structure

A median-joining (MJ) network for concatenated *COI*/*16S* rRNA mtDNA sequences was used to visualize haplotype relatedness and geographical haplotype distribution within *A. torrentium* (Figure 5). All nine phylogroups were highly divergent and separated by large number of mutational steps. The newly discovered KOR phylogroup was 42 mutational steps distant from the closely related ŽPB phylogroup. The CSE phylogroup showed a complex structure with a large number of closely related haplotypes, belonging to the widest range of localities. They were separated by small number of mutational steps (1-4) and did not form a star shaped topology. SB phylogroup consisted of six subclades separated by a large number of mutational steps (20-40). SB and CSE phylogroups showed the smallest (18) between-group number of mutational steps. ZV phylogroup showed the largest number (72) of mutational steps to its closest neighboring phylogroup BAN. Overall, net-like phylogenetic signals were dominant, and the star like signal was present only in the BAN phylogroup.

A TCS network based on *COI* dataset (used in species delimitation, Figure 9) indicated the existence of nine separated subclades within the SB phylogroup. Banovina phylogroup was split in to two clades. Haplotype 41 formed the first, and the second contained all other Banovina haplotypes. All other phylogroups (CSE, LD, KOR, APU, GK, ŽPB, ZV) formed a separate clade.

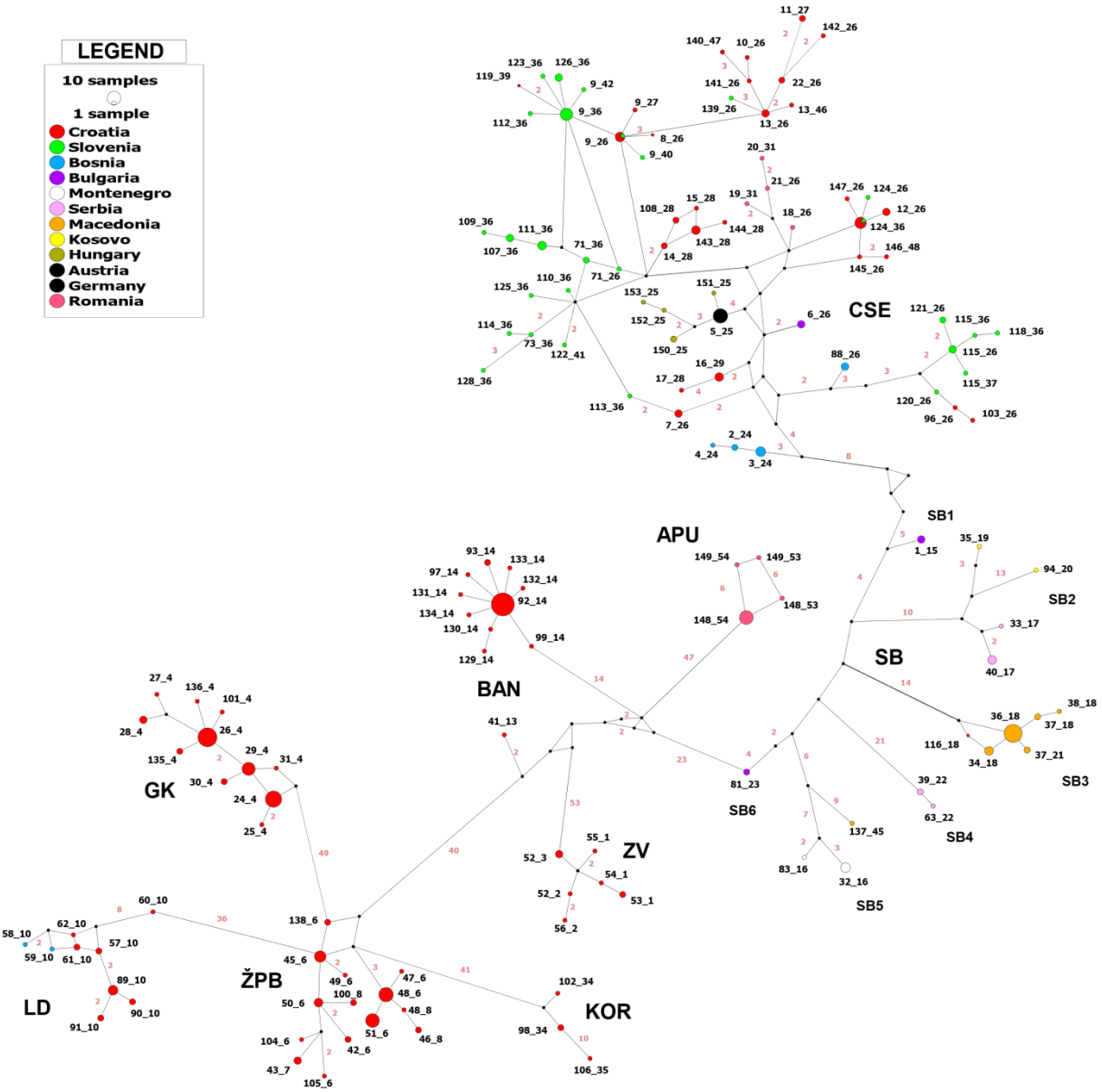

Figure 5. Median joinig (MJ) network for *COI/16S* rRNA concatenated dataset. The number of mutational steps is given in light red above branches. The size of the circle is proportional to the frequencies of the haplotype. Different coloured circles denote the share of distinct haplotypes within countries (legend is shown in the upper-left corner).

4.4. Time of divergence

Estimates of times of divergence based on mitochondrial dataset using three molecular clock calibrations and three geological calibrations are presented in Figure 6. The results of calibrations based on molecular clock calibrations overlaped. The mean values of all three molecular calibration methods for the key points in *A. torrentium* evolution were: (a) ~9.3 Ma for the split of *A. pallipes* and *A. torrentium* (b) ~6 Ma for the split between populations belonging to the NCD phylogroups, (c) ~4 Ma for the split of CSE/SB phylogroups from BAN phylogroup and (d) ~2.8 Ma for the split between CSE and SB phylogroups. Geological calibration points showed a wide range of different divergence times results. Geological calibration based on the tectonic displacement of the Tisza–Dacia microplate, that occurred ~16 Ma, gave the largest intervals of possible times of divergence and was not consistent with molecular clock calibrations. The 95% highest posterior density (HPD) range for divergence time between *A. pallipes* and *A. torrentium* was 23.1 – 49.4 Ma, a range of 26.3 Ma that is longer than the whole *A. torrentium* species complex evolutionary history. Other key points in *A. torrentium* evolution for tectonic displacement of the Tisza–Dacia microplate calibration were also inconsistent with molecular data, even if the results were considered at their youngest date estimates. The off-set of median values of key-events for this calibration, in comparison to the molecular clock calibrations, ranges from 24.8 to 7.5 Ma. On the other hand, geological calibration based on the uplift of the Dinaric Mountain range showed values closer to the molecular clock calibrations (especially arthropod evolutionary rate calibration). The off-set of median values of key-events for this calibration, in comparison to the molecular clock calibrations, ranged from 2.8 to 0.7 Ma. The new geological calibration point used in this research was based on the contact between the paleo-Tisza and paleo-Danube river systems accompanied by the process of desalination of the Lake Pannon (Figure 7). This event would enable the colonization of the Apuseni Mountains and the split of *A. bihariensis* from other NCD clades. This calibration point yielded the best results when compared to the molecular calibrations. The median values for the key points in *A. torrentium* evolution based on this geological calibration were: (a) 11.1 Ma (HPD 16.2 – 7.2 Ma) for the split of *A. pallipes* and *A. torrentium* is, (b) 6.9 Ma (HPD 9.8 – 5.2 Ma) for the split of NCD phylogroups, (c) 4.8 Ma (HPD 7.2 – 3.0) for the split of BAN and CSE/SB phylogroups and (d) 3.4 Ma (HPD 5.2 – 2.1 Ma) for the split of CSE and SB phylogroups. The 95% HPD intervals of this calibration overlapped with all molecular calibrations with an off-set of median values for key-events that ranged from 1.8 to 0.6 Ma. This calibration also overlapped

with the geological calibration based on uplift of the Dinaric Mountains with almost identical 95% HPD value ranges.

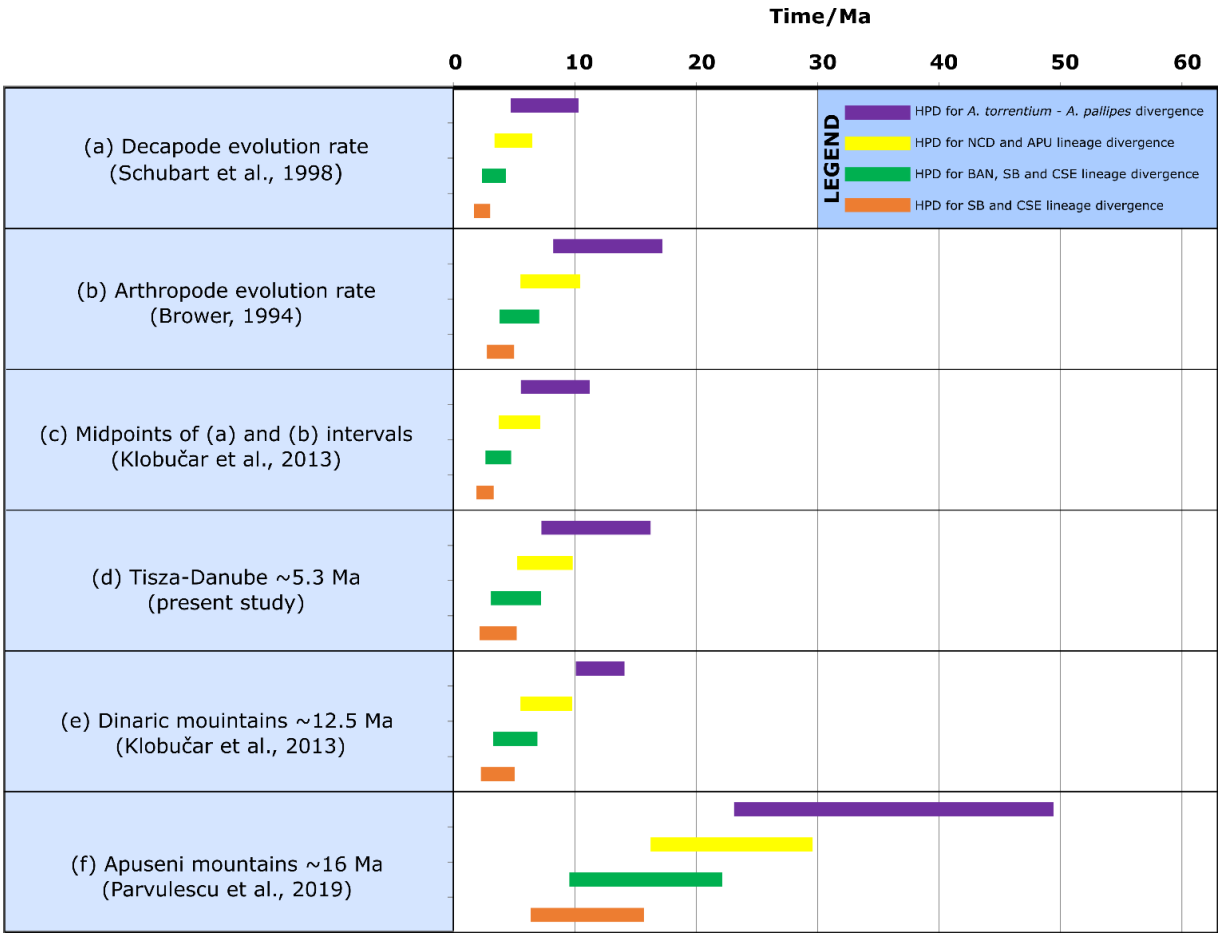

Figure 6. Chronogram of 95% highest posterior density intervals (HPD) of divergence time estimates obtained (a) using decapod evolutionary rate, (b) using arthropod evolutionary rate, (c) using mid-points of a uniform distribution, (d) in this study using geological calibration based on the contact of Tisza-Danube river systems (e) using geological calibration based on the uplift of the Dinaric Mountains (f) using geological event – separation of Tisza–Dacia microplate from Dinarides. Different colours denote the HPD of distinct lineages: purple – split of *A. pallipes* and *A. torrentium*; yellow - split of NCD phylogroups and APU phylogroup; green - split of BAN and CSE/SB phylogroups; dark red - split of CSE and SB phylogroups.

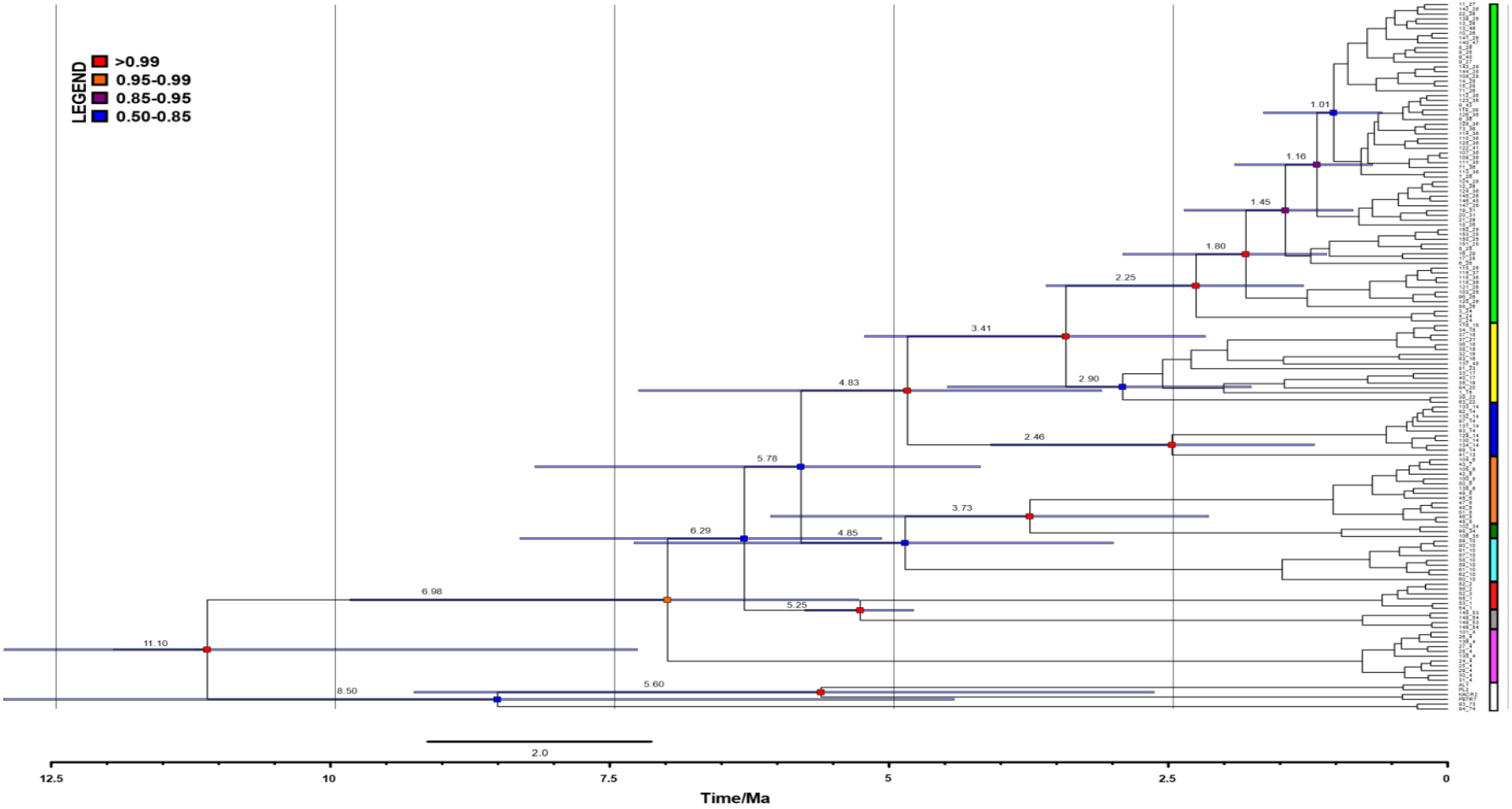

Figure 7. BEAST divergence times for *Austropotamobius torrentium* based on paleo-Danube-paleo-Tisza geological calibration. Nodes with support values over 0.5 are coloured as indicated in the legend. Node bars depict the 95% highest posterior density (HPD) interval with the median value of divergence time shown above each. Different phylogroups are indicated in different colours as in Figure 2, white indicates outgroups (AP, AA, AL).

4.5. Species delimitation

The results of the species delimitation analyses (ABGD, bPTP, GMYC, TCS) for mtDNA (*COI*) confirmed the existence of different *A. torrentium* operational taxonomic units (OTUs) (Table 8).

Two different approaches of ABGD method showed different results. We observed the existence of eight OTUs at the P value of $1.29x10^{-2}$. GK, APU, ZV, LD, ŽPB and KOR were each represented as one OTU. BAN phylogroup was split into two OTUs, one belonging to samples from BAN region, and one belonging to a sample haplotype 41. CSE and SB phylogroups were lumped into one OTU. At P value of $4.64x10^{-3}$ we observed the existence of 18 OTUs: the SB phylogroup was split in nine clades (potential species) and CSE formed a unique phylogroup.

The bPTP recognized between 27 and 46 molecular OTUs, 12 of which with Bayesian support values over 0.91. Only GK and ZV phylogroups were recognized as unique OTUs with support value of 0.733 and 0.697, respectively. BAN phylogroup had also high support value of 0.915 (haplotype 41 forms a unique OTU with support value of 1). SB phylogroup formed 13 distinct lineages, five with support value higher than 0.9, and eight with support value ranging from 0.73 to 0.845. CSE phylogroup presented six lineages, three with support value higher than 0.9 and three with support value ranging from 0.548 to 0.605. ŽPB phylogroup was split into two OTUs, one that was supported with value of 0.671 and the other with support value of 0.605. LD phylogroup was split into three OTUs of which one contained 7 haplotypes and had a support value of 0.497. The other two OTUs belonging to LD phylogroup contained a single haplotype with support values of 0.562 and 1. KOR and APU phylogroups each showed two highly supported OTUs, with support values of 0.872 and 0.993 for KOR phylogroup and 0.937 for both APU phylogroups.

The GMYC single threshold time approach identified 22 ML clusters (confidence interval: 19-36) and 29 entities (confidence interval: 25-53) (Figure 8). Threshold time was assessed at 0.93 Ma. Using this method, a total of 29 potential species was delimited (Table 8). SB phylogroups was split into 10 OTUs of which six showed support value higher than 0.8, two showed support values of 0.4-0.5, and two were unsupported. CSE was split into 8 OTUs of which two showed support value higher than 0.8, two showed support values of 0.4-0.55, two showed low support values of 0.1-0.25 and two were unsupported. ZV phylogroup was well supported as one OTU with support value of 0.88, as well as BAN phylogroup with support

value of 0.97 and unsupported haplotype 41. ŽPB phylogroup was split into two OTUs, one that is highly supported (0.87), and one with lower support value (0.52). GK phylogroup was delimited as one OTU with low support value (0.3). APU phylogroup showed a low support value of 0.38. LD phylogroup was also split into two OTUs of which one was supported with 0.44, and one was unsupported. KOR phylogroup showed two OTUs, one well supported (0.97) and one with support value of only 0.03.

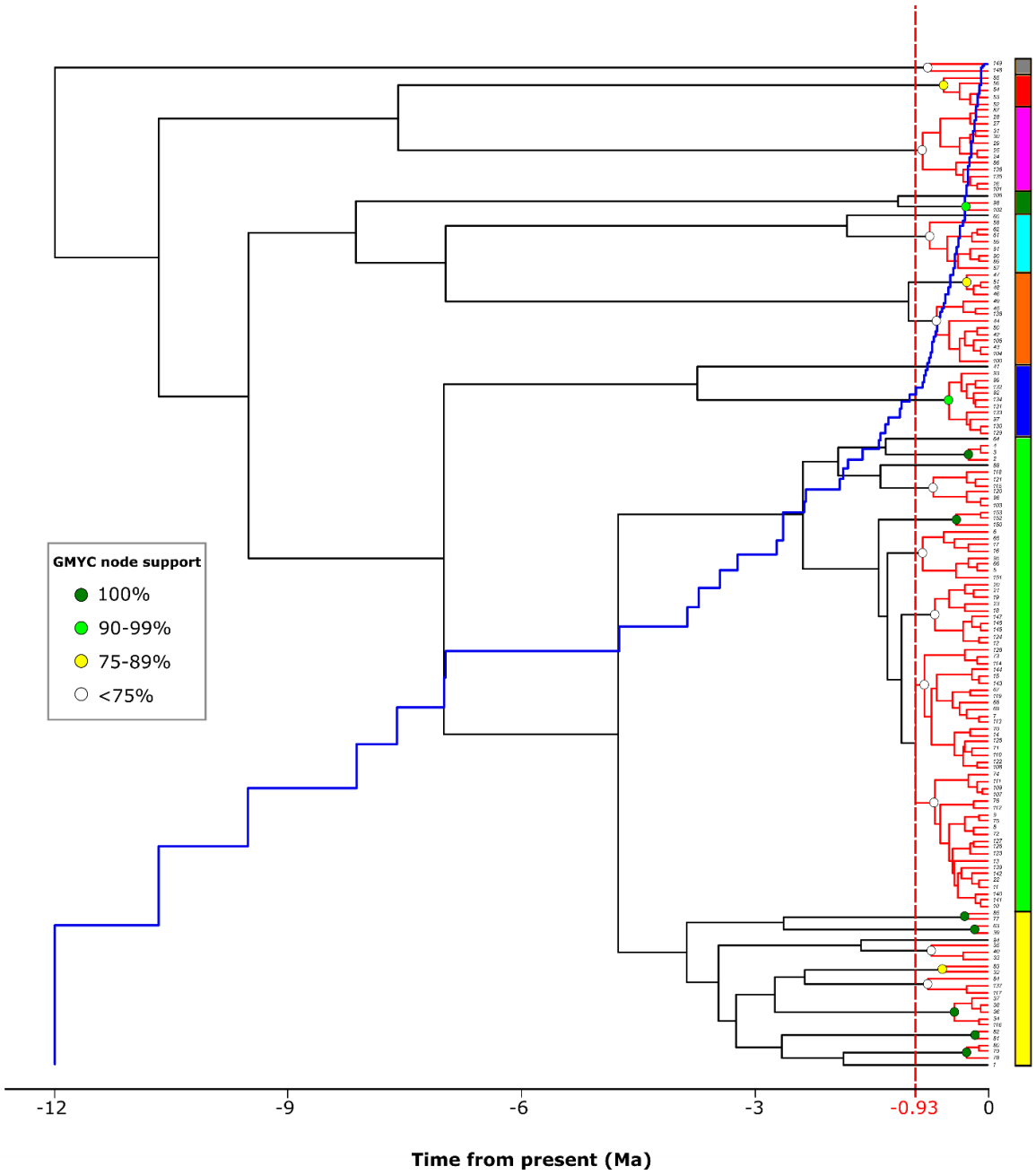

Figure 8. Species entities revealed by GMYC analysis and lineages-through-time plot based on *COI* sequences. The sharp increase in branching rate, shown with a red vertical dashed line, corresponds to the transition from interspecific to intraspecific events. Different phylogroups are indicated in different colour: lime green – central and south-eastern Europe (CSE), pink – Gorski Kotar (GK), aqua blue – Lika and Dalmatia (LD), orange – Žumberak, Plitvice and Bjelolasica (ŽPB), yellow – southern Balkans (SB), blue – Banovina (BAN), red – Zeleni Vir (ZV), grey – Apuseni Mountain (APU), dark-green – Kordun (KOR).

The results obtained using TCS network revealed the existence of 18 potential species/deeply divergent lineages with CSE, GK, ZV, LD, KOR, APU and ŽPB phylogroup each representing one OTU (Figure 9). BAN phylogroup was split into two OTUs (same as in ABGD) and SB phylogroup was split into nine OTUs.

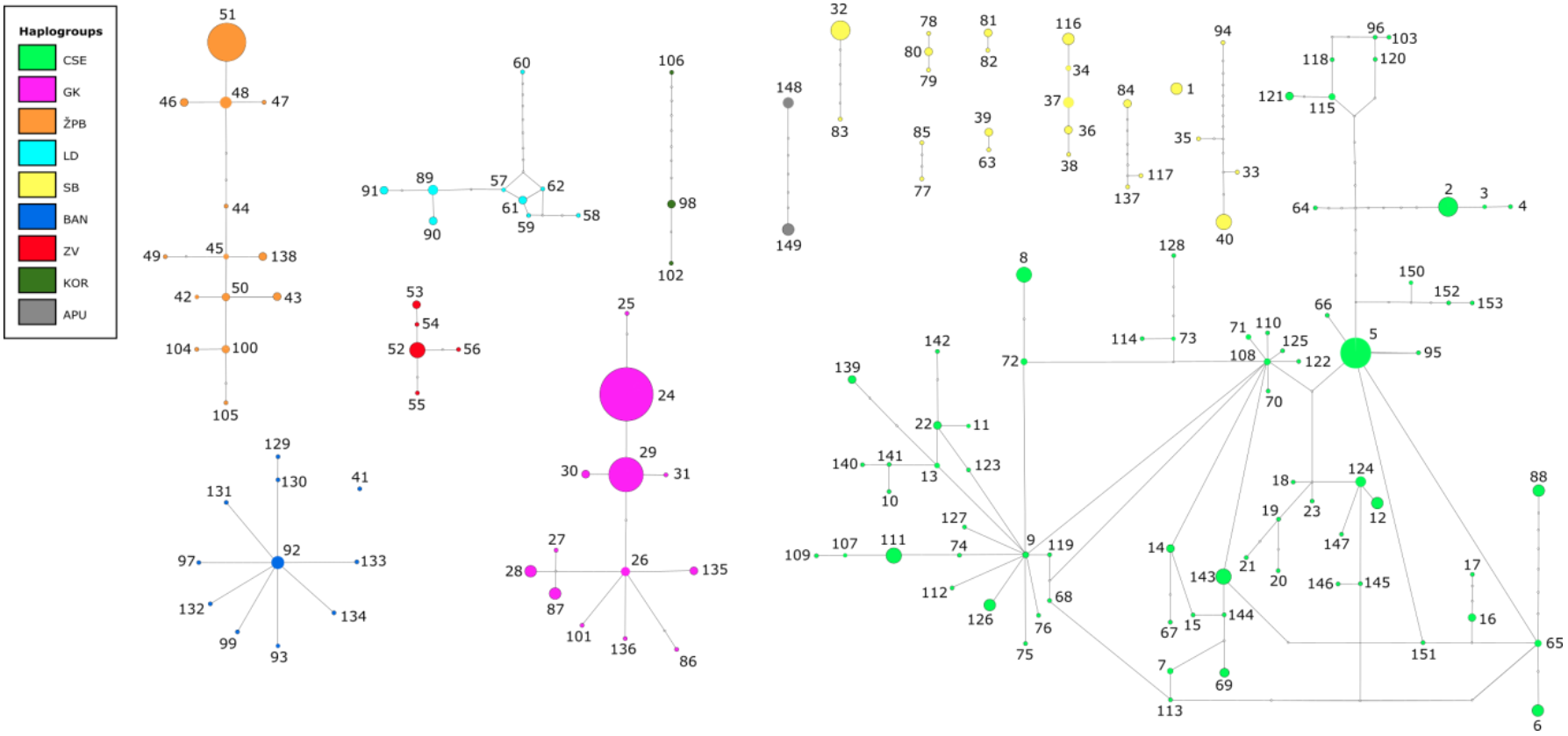

Figure 9. TCS phylogenetic network of *COI* sequences. The size of the circle is proportional to the frequency of the haplotype scaled with the number of localities in which it occurs. Different colours denote different phylogroups (legend is shown in the upper-left corner).

Table 8. Results of species delimitation analyses performed in ABGD, TCS, bPTP and GMYC. The columns marked in blue represent phylogroups as in Klobučar *et al*., (2013) and Pârvulescu *et al*., (2019). The columns represent groups detected by species delimitation analyses performed in this study: ABGD (P=1.29x10-2 and P=4.64x10-3), TCS, bPTP (BS - Bayesian support values) and GMYC (NSV – node support value shown in brackets). In the last column *COI* haplotypes, belonging to each phylogroup as arranged by GMYC (marked in olive green), are given.

Phylogroups as in Klobučar *et al*. (2013)	Phylogroups as in Pârvulescu *et al*. (2019)	ABGD P=1.29 x10^{-2}	ABGD P=4.64 x10^{-3}	TCS	bPTP (BS)	GMYC (NSV)	Haplotypes
CSE	CSE	G1	G1	G1	S32 (0.605)	S12 (1)	150, 152, 153
					S31 (0.548)	S11 (0.24)	95, 5, 66, 151, 16, 17, 65, 6
						S9 (0.12)	141, 140, 11, 22, 142, 139, 13, 123, 126, 127, 72, 8, 75, 9, 112, 76, 107, 109, 111, 74, 108, 122, 110, 71, 125, 14, 70, 113, 7, 69, 68, 119, 67, 143, 15, 144, 114, 73, 128
						S10 (0.49)	12, 124, 145, 146, 147, 18, 19, 20, 21, 23
					S18 (0.997)	S25 (n/a)	88
					S17 (0.555)	S13 (0.53)	103,96,120,115,118,121
					S14 (0.918)	S14 (1)	2, 3, 4
					S15 (0.999)	S26 (n/a)	64
SB	SB	G1	G2	G15	S5 (0.953)	S2 (1)	82,81
			G3	G13	S10 (0.916)	S1 (1)	78,80,79
			G4	G10	S9 (1)	S23 (n/a)	1
			G5	G12	S8 (0.845)	S3 (1)	37,36,34,116,38
			G6	G11	S16 (1)	S24 (n/a)	94
					S20 (0.772)	S6 (0.46)	35
					S19 (0.635)		40,33
			G7	G16	S30 (0.730)	S5 (0.85)	83
					S29 (0.730)		32
			G8	G17	S22 (0.835)	S4 (0.41)	84
					S21 (0.733)		117,137
			G9	G18	S6 (0.956)	S7 (1)	39,63
			G10	G14	S7 (0.832)	S8 (1)	85,77
BAN	BAN	G2	G11	G2	S3 (0.915)	S15 (0.97)	130, 129, 92, 131, 97, 132, 99, 93, 134, 133
		G9	G12	G3	S (1)	S27 (n/a)	41
ŽPB	ŽPB	G5	G15	G9	S28 (0.671)	S17 (0.87)	47, 51, 48, 46
					S27 (0.605)	S16 (0.52)	44, 49, 45, 138, 105, 43, 104, 42, 50, 100
LD	LD	G4	G14	G8	S11 (1)	S28 (n/a)	60
					S24 (0.562)	S18 (0.44)	58
					S23 (0.497)		62, 61, 59, 89, 91, 90, 57
n/a	n/a	G7	G17	G7	S12 (0.872)	S19 (0.97)	98, 102
					S13 (0.993)	S29 (0.03)	106
ZV	ZV	G6	G16	G6	S2 (0.697)	S21 (0.88)	52, 53, 54, 55, 56
n/a	APU	G8	G18	G5	S25 (0.937)	S22 (0.38)	148
					S26 (0.937)		149
GK	GK	G3	G13	G4	S1 (0.733)	S20 (0.3)	87, 28, 27, 136, 86, 26, 135, 101, 31, 29, 30, 25, 24

4.6. Species validation

Results of BFD* species delimitation are presented in Table 9. Nested sampling analysis yielded marginal likelihood estimations that ranged from -4607.94 to -5304.68 with associated SD ranged from 4.0 to 6.53.

Table 9. BFD* species delimitation results. Marginal likelihood estimates (MLE) and Bayes factors (2lnBf) are calculated for each species tree model. The model receiving the best marginal likelihood score is in bold (its BF is 0).

Model	MLE	BF	Rank
ABGD(P=1.23e-02)	-4720.05	224.21	3
TCS	-4908.0867	600.28	4
GMYC	**-4607.94**	**0**	**1**
bPTP	-4669.31	122.73	2
A. torrentium- one species	-5304.68	1393.47	5

The model receiving the highest marginal likelihood score was GMYC. Calculated Bayes factor values showed decisive support for species tree topology associated with GMYC species delimitation (Figure 10). *A. torrentium* as one species model obtained lowest marginal estimation value.

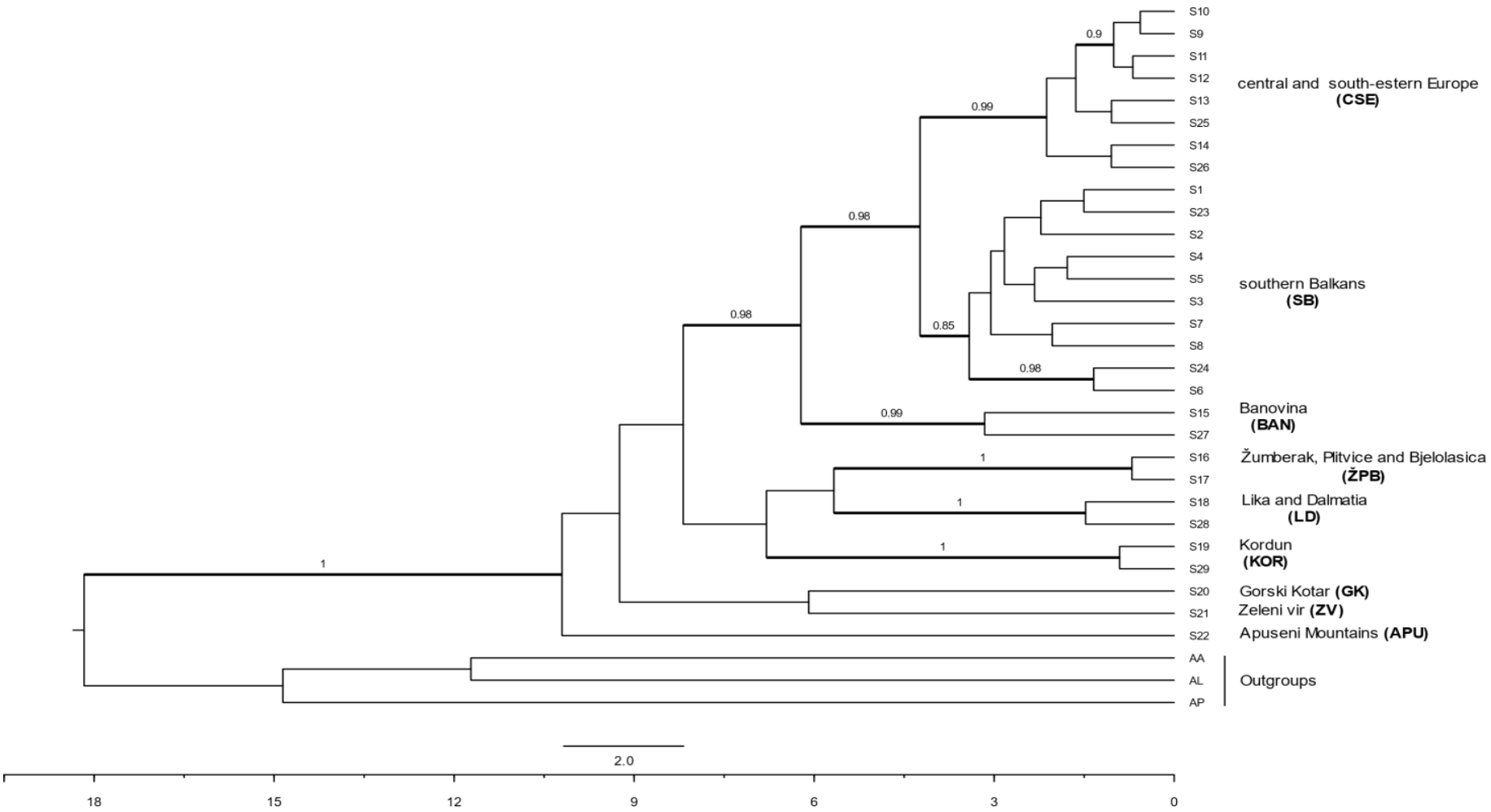

Figure 10. *BEAST species tree based on GMYC species delimitation model with tip labels corresponding to GMYC species numbers (see Table 8). Bayesian posterior probabilities above 0.9 were considered supported and are shown.

GMYC species tree (Figure 11) visualised in DensiTree v.2.2.6 showed that CSE phylogroup OTUs formed one clade, but relationships among them were not resolved. Similar was observed within SB clade and its OTUs, and within clades belonging to NCD phylogroups (KOR, ŽPB, LD, ZV and GK) and their OTUs. APU phylogroup OTU also interacts with ZV and GK OTUs. Interestingly, OTUs belonging to Banovina phylogroup did not show mixing with OTUs belonging to other phylogroups.

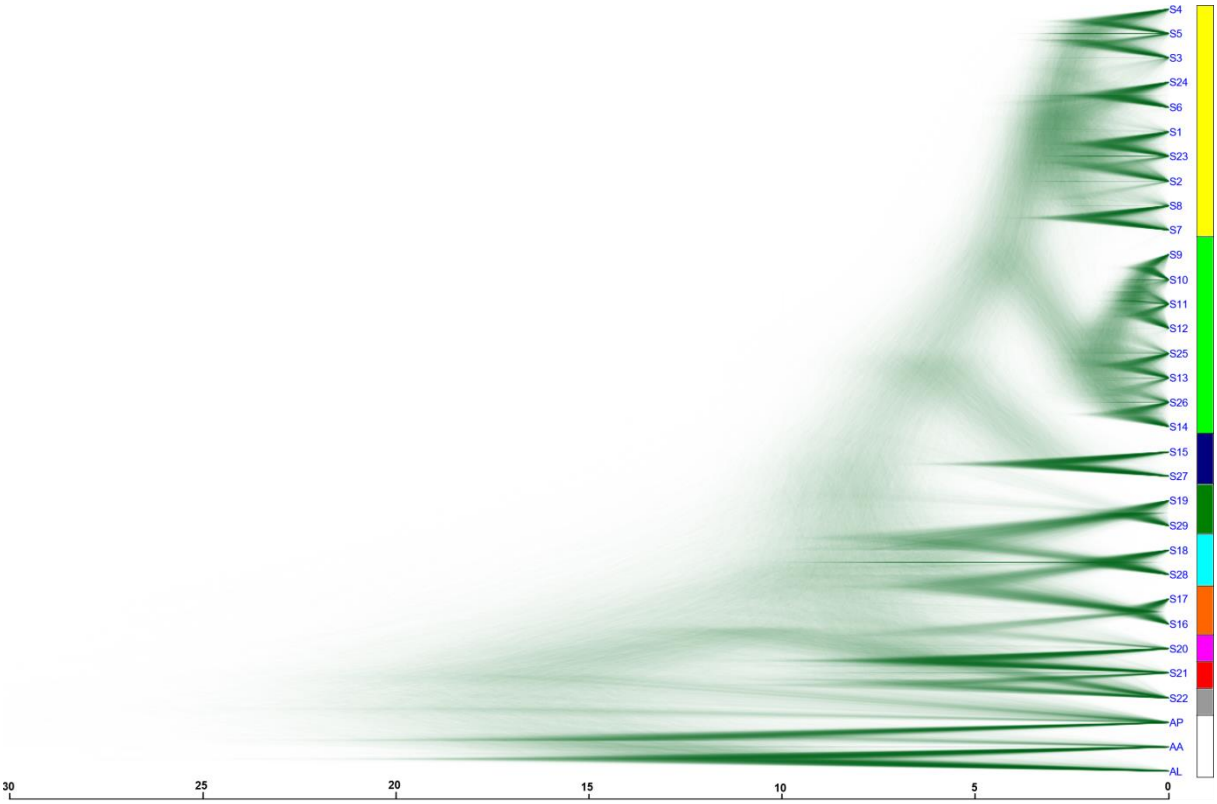

Figure 11. Species tree inferred with *BEAST visualized using DensiTree, based on GMYC species delimitation model. Tip labels correspond to GMYC species numbers (see Table 8). Different phylogroups are indicated in different colour: lime green – central and south-eastern Europe (CSE), pink – Gorski Kotar (GK), aqua blue – Lika and Dalmatia (LD), orange – Žumberak, Plitvice and Bjelolasica (ŽPB), yellow – southern Balkans (SB), blue – Banovina (BAN), red – Zeleni Vir (ZV), grey – Apuseni Mountain (APU), dark-green – Kordun (KOR) and white for outgroups (AP, AA, AL).

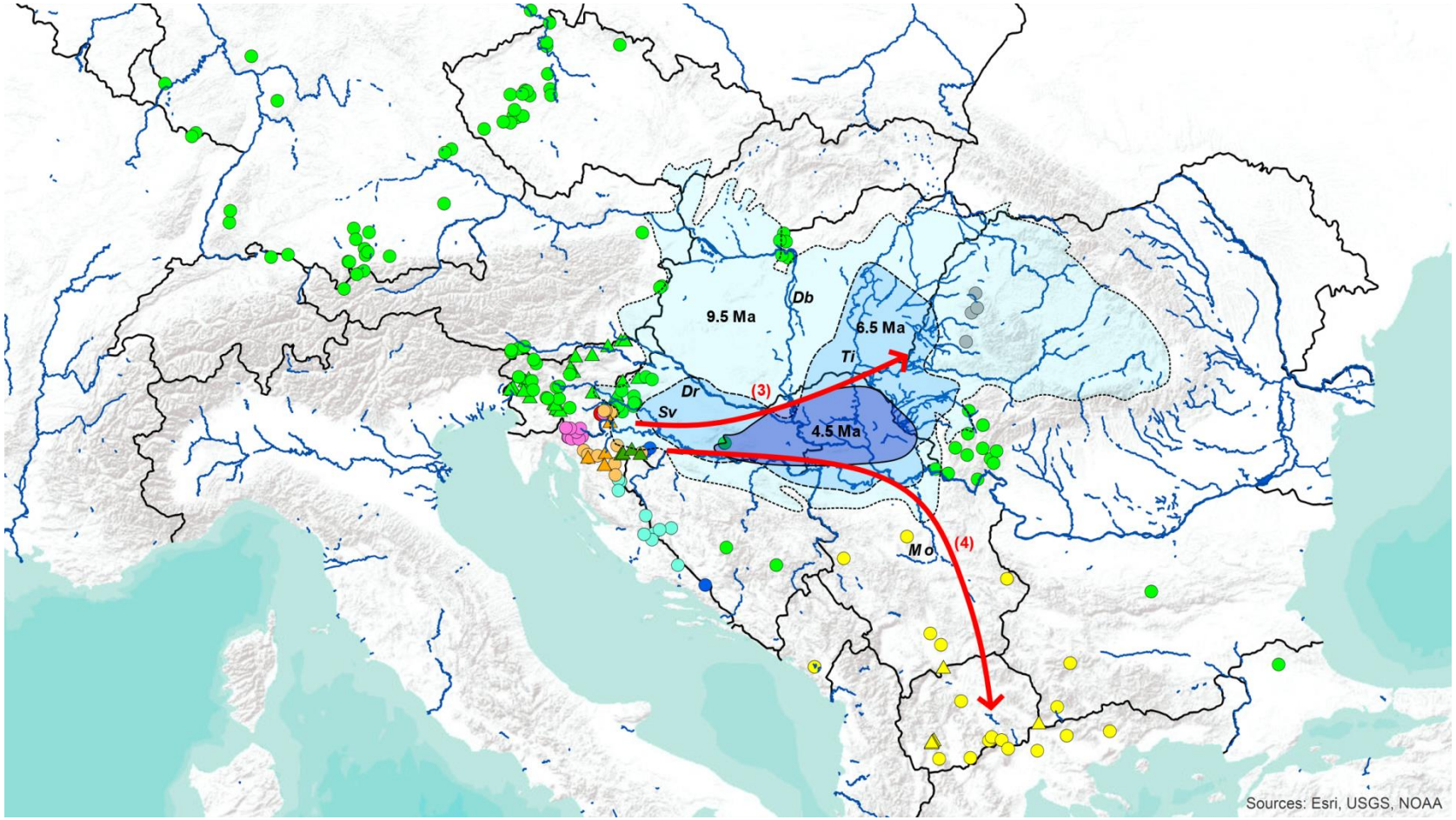

Figure 12. *Austropotamobius torrentium* possible pre-glacial dispersal route. The map shows present day stone crayfish distribution, and river systems. Major rivers are named in italic (*Db*-Danube River, *Dr*- Drava River, *Sv*- Sava River, *Ti*- Tisza River, *Mo*- Morava River). Extent of Lake Pannon at 9.5 Ma, 6.5 Ma and 4.5 Ma (in bold) is adapted from Magyar *et al.* (1999) and shaded in blue. Red arrows indicate: (3) colonization of the Apuseni Mountains through delta systems of paleo-Danube and paleo-Tisza on the northern shelf margin of the Lake Pannon that was possible after the end of MSC (~5.33 Ma) and (4) colonization of southern Balkan after formation of the freshwater Danube drainage system starting ~4.0 Ma. Different phylogroups are marked in different colours as in Figure 1.

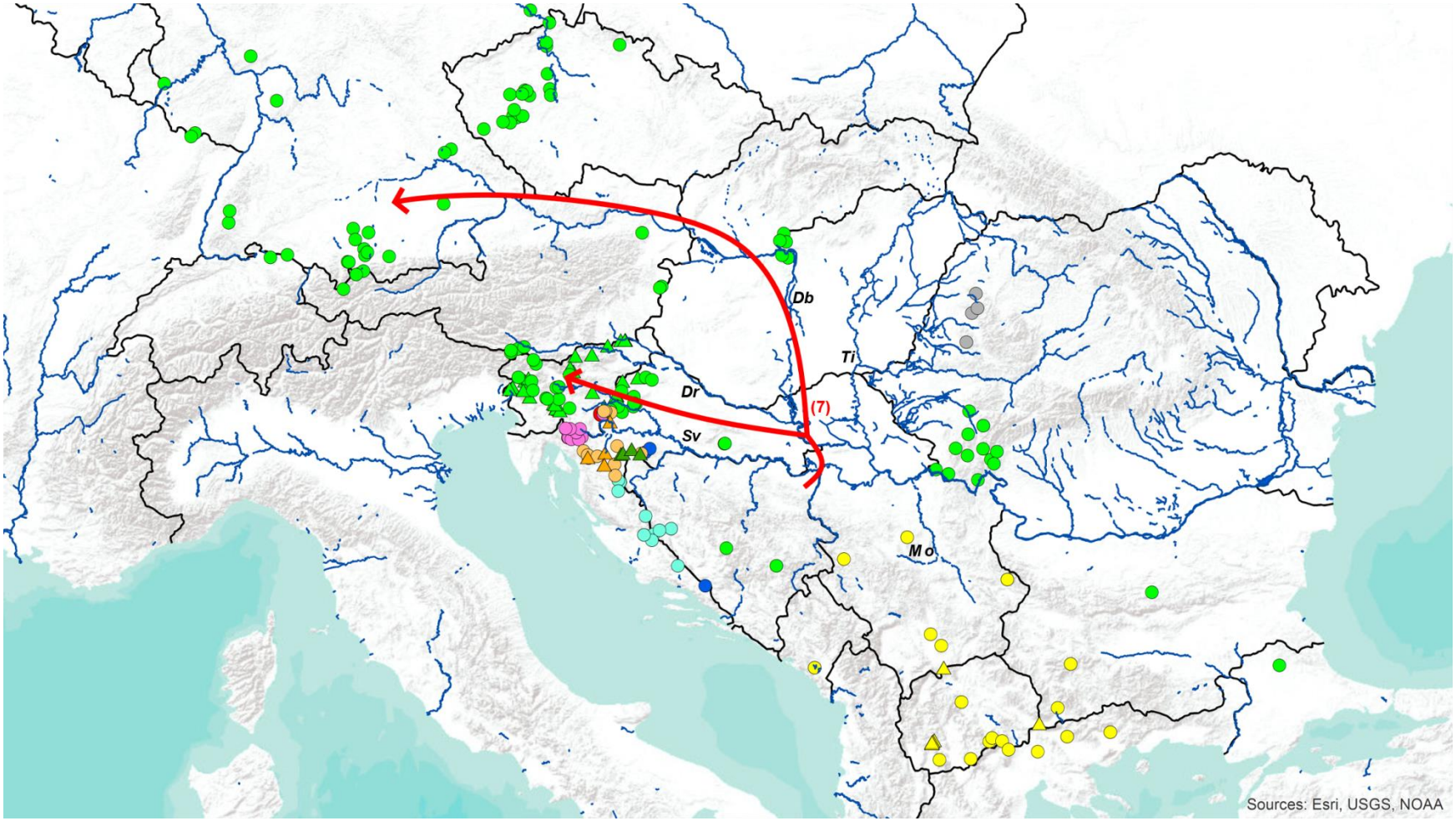

Figure 13. *Austropotamobius torrentium* possible post-glacial dispersal route. The map shows present day stone crayfish distribution, and river systems. Major rivers are named in italic (*Db*-Danube River, *Dr*- Drava River, *Sv*- Sava River, *Ti*- Tisza River, *Mo*- Morava River). Extent of Lake Pannon at 9.5 Ma, 6.5 Ma and 4.5 Ma (in bold) is adapted from Magyar *et al.* (1999) and shaded in blue. Red arrow indicates: (7) post-glacial recolonization of northern part of *A. torrentium* areal through leading edge expansion of CSE phylogroup (adopted from Klobučar *et al.*, 2013). Different phylogroups are marked in different colours as in Figure 1.

5. Discussion

5.1. Evolutionary history

It has been shown that south-eastern Europe and Balkan Peninsula are regions possessing high genetic diversity of both plant and animal organisms. As such, this region presents a 'hotspot' of European biodiversity (Hewitt, 2011). Previous studies of *Austropotamobius torrentium* (Trontelj *et al.,* 2005; Klobučar *et al.,* 2013; Pârvulescu *et al.,* 2019) revealed this species complex evolutionary history, as today's species range was formed through complex geological processes. Klobučar *et al.,* (2013.) suggested that the crucial events in the evolution of *A. torrentium* were: (a) Alpine and Dinaride orogeny (~12.5 Ma) (Rögl, 1998) which caused the separation of genus *Austropotamobius* into two species 'western' *A. pallipes* and 'eastern' *A. torrentium* (Trontelj *et al,* 2005); (b) intensification of Dinaric karstification (Trontelj *et al,* 2005; Pavelić and Belak, 2008) that resulted in heavily fragmented paleohydrography (Verovnik *et al.,* 2004) that enhanced allopatric speciation of the aquatic fauna, including stone crayfish (Klobučar *et al,* 2013); (c) creation of Danube drainage system (Gabris, 1994) that allowed the migration of stone crayfish towards SB and CSE (Klobučar *et al.,* 2013); (d) glacial contraction and interglacial recolonization of today's northern species range (Klobučar *et al.,* 2013).

Pârvulescu *et al.,* (2019) showed a new perspective on evolutionary history of *A. torrentium* through the inclusion of a new calibration point/approach for species divergence times. Their approach was focused on the separation of the Dinarides from the Tisza– Dacia mega-unit (including the Apuseni Mountains) from the Dinarides, which begun at ~16 Ma (Roşu *et al.,* 2004; Ustaszewski *et al.,* 2008; Balázs *et al.,* 2016). They believe that the Apuseni Mountains formed a large island inhibited by ancestors of today's APU phylogroup that was isolated from other phylogroups and that reconnect with other freshwater systems in the area ~5 Ma. This approach yielded much younger dates of separation for *A. torrentium* and its sister species *A. palllipes*, at ~42Ma (HPD 54-32 Ma) compared to previous estimations (Trontelj *et al.,* 2005; Klobučar *et al.,* 2013). Although, Pârvulescu *et al.,* (2019) brought a whole new perspective on geological history of *A. torrentium* species complex, they lacked congruence with previous research and molecular clock calibrations. In our study we tried to reconcile both geological calibration approaches and bring yet another perspective on *A. torrentium* evolutionary history.

Austropotamobius. torrentium is a freshwater cold-adapted species active at water temperatures > 5°C (Bohl 1987) with the mean annual water temperature that does not exceed 10 °C species (Maguire and Gottstein-Matočec, 2004). Species inhabits small and medium-sized rivers, brooks, and streams with pristine waters and moderate current speeds (Pöckl and Streissl 2005, Vlach *et al.,* 2009, Pârvulescu *et al.*, 2011). Due to its evolution in cold freshwater conditions, previous research also showed that its distribution is related to karstic formations, present in both the Dinarides and Apuseni mountains (Klobučar *et al*., 2013, Pârvulescu *et al.*, 2019). The Dinaric Karst possesses a high level of biodiversity (Gaston and David, 1994), with many endemic species of freshwater surface and subterranean fauna (Banarescu, 2004; Sket *et al.,* 2004; Previšić *et al*., 2009; Jelić *et al*., 2016). High level of endemism was also observed in the Apuseni Mountains, which represented refugium that preserved some endemic and relic species of Gastropoda, Isopoda and Diplopoda (Pullaiah, 2019). Both the Dinarides and Apuseni Mountains are rich in karst features as karren, dolines, ponors, springs and caves often with underground water systems (Bonacci, 2014; Ponta and Onac, 2019). Although *A. torrentium* is a species that usually inhabits small streams and brooks above-ground, reports of stone crayfish living deep inside caves (~7000 m from entrance) have been made in past (Koutrakis, 2005). On the other side some *A. torrentium* populations could also be found in rivers that flow through agricultural land and that lack typical stone crayfish habitats (Demers *et al*., 2006). Therefore, when assessing stone crayfish phylogeography all the above should be considered.

Both Klobučar *et al*., (2013) and Pârvulescu *et al.,* (2019) showed that evolutionary history of *A.torrentium* was shaped by the creation of the Lake Pannon. The Pannonian basin was isolated after the uplift of the Carpathian and Dinaric Mountains from the rest of the Paratethys (Rögl, 1996) and a large brackish to fresh water lake was formed (Magyar *et al*., 1999). The complete isolation of the Lake Pannon from influx of the saline water was estimated to around 11.7 Ma (Magyar et al., 2007; ter Borgh, 2013; ter Borgh *et al*., 2013). After the uplift of Dinarides, as mentioned above, the genus *Austropotamobius* split into ‘western’ *A. palllipes* and ‘eastern’ *A. torrentium.* (Klobučar *et al*., 2013). The paleo-Danube emerged at the beginning of the Late Miocene (10–11 Ma ago), and it discharged directly into the Lake Pannon through its delta (Magyar *et al*., 1999, Magyar *et al*., 2013.). This caused a change in water salinity turning the Lake Pannon into a brackish environment (Harzhauser *et al*., 2007). Together with its northern tributaries, such as the paleo-Tisza and other minor tributaries), it formed a shelf margin that prograde from the northwest towards the southeast

(Magyar *et al*., 2013). Despite that, during the lake's lifetime changes in southern shoreline of the lake were small (Magyar *et al*. 1999). Klobučar *et al*., (2013.) assumed that during this period (probably until ~6.5 Ma) the populations of *A. torrentium* in the NCD region were isolated between the Lake Pannon on the east and Dinarides/Alpes on the north and west. Magyar *et al*. (1999) observed that due to strong fluvial influence from surrounding rivers, freshwater mollusc genera were widespread in the shallow parts of the lake cca. 4.5 Ma ago. The same authors also observed that the paleo- Danube delta lobes in the central part of the Pannonian Basin approached lower flow of paleo- Tisza river. This was later confirmed as paleo-Danube shelf margin and the paleo-Tisza shelf margin were observed as coalesced and their original, almost perpendicular strike can be recognized until 5.3 Ma (Magyar *et al*., 2013). We argue that this connection between the paleo-Danube and Paleo-Tisza rivers could have allowed the ancestor of *A. torrentium* (now APU phylogroup) to colonize the Apuseni Mountains around 5.3 Ma. We choose this event as it represents the end of the Messinian Salinity Crisis (MSC) that lasted from ~5.96 Ma until ~5.33 Ma (Krijgsman *et al*., 1999). Messinian Salinity Crisis, apart a strong influence onto hydrology caused climate change in the Northern Hemisphere with an increased temperature, aridity and evaporation (Murphy *et al*., 2009). It is also speculated that the Messinian Salinity Crisis caused the Lake Pannon water level to drop, at least in its northern part (ter Borgh *et al.*, 2015). Thus, *A. torrentium* colonization of the Apuseni Mountains would be possible at the end of MSC (~5.3 Ma), thought the northern margin (Figure 12a) of Pannonian Lake, which is indicated by low genetic distances between ZV phylogroup and APU phylogroup, previously observed by Pârvulescu *et al.*, (2019), and confirmed in this study. Also, During MSC the sea-level dropped for 50–200 m in the Dacian Basin, that was situated to the east from Pannonian Lake and was connected to the Black Sea (Leever *et al*., 2010). It is believed that during the MSC paleo- Danube River run across the south Carpathians and overflew from the Pannonian into saline Dacian Basin (Clauzon *et al*., 2005). Therefore, we believe that the north dispersal route is more likely.

Most stone crayfish populations of the Carpathians are distributed in the mountain range of Romania (Holdich, 2002; Pârvulescu, 2010; Pârvulescu *et al.*, 2011). Pârvulescu *et al.*, (2019) pointed out that scenarios based on traditional molecular clock and geological (mainly Dinaride orogeny) calibrations failed to explain the endemism of the APU phylogroup. Two phylogroups of *A. torrentium* exist in Romania: populations of *A. biharensis* endemic to the Apuseni Mountains (APU phylogroup), distributed in the Tisza River drainage and

populations in the Anina Mountains (southwestern part) of Romania (CSE phylogroup), distributed in the Danube River drainage. Both, the Apuseni and Anina Mountains are karstic regions, therefore are suitable for *A. torrentium* colonization (Artugyan, 2016; Ilies and Josan, 2007). Although, Romania is entirely within the Danube watershed (Pârvulescu *et al.*, 2011) and conditions for crayfish dispersal should be homogeneous throughout history (Pârvulescu *et al*., 2013), colonization of the Anina Mountains would not be possible at the end of MSC, because Dacian Basin, unlike the Pannonian Lake, was still connected to the saline environment, and refiled with saltwater after the Strait of Gibraltar opened ~5.33 Ma ago. Ending of the MSC was followed by beginning of Pliocene Epoch that lasted from 5.33 Ma until 2.58 Ma. During that period conditions on the Earth changed; transition from relatively warm climate to a climate cooling with the numerous glacial-interglacial oscillations (Salzmann *et al*., 2011.).

Around 4 Ma brackish/saline environment in the Lake Pannon was replaced by the freshwaters and their biota (Magyar *et al*, 2013). This was accompanied with the connection of the paleo- Danube, paleo-Tisza, and minor river systems to the south (paleo- Velika Morava) and creation of the Danube River drainage system, as we know it today (Magyar *et al*., 2013; ter Borgh *et al*., 2014). We believe that this favourable climatic change together with the retraction and desalination of the Pannon Lake allowed the south-eastern colonization of *A. torrentium.* Our results indicate that the origin of SB and CSE phylogroup was mainly from the region of BAN phylogroup which, in phylogenetic analyses based on mtDNA, appears as genetically most closely related (Table 4 and Table 5) sister lineage to SB+CSE clade (Figure 3) and represents the most eastern NCD population (Figure 1). Therefore, CSE+SB emergence from the common ancestor with BAN was the most probable (Figure 12). This pattern of colonization was previously suggested by Klobučar *et al*. (2013). SB and CSE phylogroup colonization were probably occurring concurrently as indicated by unresolved polytomies of one CSE sub-clade and six divergent SB sub-clades comprised within monophyletic SB+CSE clade (Figure 3) Also, this substructure is visible on the results of MJ network where the BAN phylogroup is closely related to SB clades and CSE phylogroup (Figure 5). Phylogenetic reconstruction on *ITS2* nuclear DNA marker also indicates the polytomy of SB and CSE clade (Figure 4), although the inclusion of more sequences belonging to SB phylogroup is needed.

Glaciation in the Northern Hemisphere started during the Early Pleistocene (2.6-0.8 Ma) and were characterised by climatic fluctuations in 41 Ka precession cycle. Including only a few

glacials sufficiently cold and long enough to allow the development of substantial ice sheets (Ehlers *et al.*, 2011). Ice-sheet development on a continental scale, outside the polar regions, emerged in the "middle Pleistocene transition" when the glacial periods were regularly cold and long enough (Ehlers *et al*., 2011). Middle Pleistocene transition (1.25 – 0.7 Ma) could have affected the evolution of *A. torrentium*, as the Generalized Mixed Yule Coalescent method of species delimitation showed transition from interspecies to intraspecies level events at 0.93 Ma (Figure 8). This result could be probably a consequence of glaciation-related restriction of *A. torrentium* areal.

As Klobučar *et al*., (2013.) previously suggested, after the glaciations, the CSE clade of *A. torrentium* started spreading northwards through the Danube drainage, showing post-glacial leading-edge effect (Figure 13). CSE phylogroup does not show a star- shaped topology in MJ network, presumably because of glaciation range fragmentation that followed Pliocene expansion and post-glacial recolonization from multiple microrefugia of closely related haplotypes. Geographical distribution of CSE phylogroup follows present-day Danube river (Figure 2). We believe that NCD populations survived the adverse climatic conditions of the Pleistocene in allopatry, with limited or non-post-glacial expansion and contact indicating the existence of multiple 'refugia within refugia' (Gomez and Lunt, 2007), as previously suggested by Klobučar *et al*., (2013).

When we consider all of the above, key-events for the *A. torrentium* species evolution based on our calibration (paleo-Tisza-paleo-Danube) and previous research (mainly Klobučar *et al.*, 2013.) are: (1) uplift of the Dinaride Mountains that caused split between *A. pallipes* and *A. torrentium*, estimated to 12.5 Ma (according to geological data), and ~11.1 Ma (HPD 16.2-7.2 Ma) (according to our calibration), (2) divergence of NCD phylogroups restricted to the area between the Lake Pannon and the Dinaride Mountains that lasted at least until ~6.5 Ma based on retrograding shoreline (Magyar *et al.*, 1999) and started ~7 Ma (HPD 9.8-5.2 Ma) according to our calibration (Figure 12) (3) colonization of the Apuseni Mountains through delta systems of paleo-Danube and paleo-Tisza on the northern shelf margin of the Lake Pannon (Figure 12) that was possible after the end of MSC (~5.33 Ma), and according to our estimation it took place ~5.3 Ma (HPD 4.7-5.7), (4) extinction of brackish biota in the Lake Pannon and formation of the freshwater Danube drainage system starting ~4.5 Ma and finished ~4.0 Ma (Figure 12), this event would allow CSE + SB to diverge from a common ancestor with BAN phylogroup, that according to our results took place ~4.8 Ma (HPD 7.2-3.1), (5) divergence between southern Balkan and central-southeastern Europe phylogroup, followed by widespread colonization and made possible by gradual lowering of

environmental temperature from 4 Ma until 2.7 Ma, according to our data took place ~3.4 Ma (HPD 5.2-2.2), (6) *A. torrentium* areal restriction caused by Pleistocene climatic oscillations, especially after Middle Pleistocene transition that was estimated to 1.3–0.7 Ma, also indicated in our research by threshold in GMYC analysis placed at 0.93 Ma, (7) post- glacial recolonization of northern part of *A. torrentium* areal through leading edge expansion of CSE phylogroup (Figure 13).

This timescale *of A. torrentium* species divergence based on the calibration with geological events is in accordance with molecular clock calibration based on all three clock rates (Figure 6). All molecular clock calibrations overlap in 95% HPD ranges for all key events with paleo-Danube-paleo-Tisza geological calibration, especially with the molecular clock calibration based on Arthropoda evolution rate (Brower, 1994).

Geological calibration based on the intense uplift of the Dinaride Mountains, applied by Klobučar *et al.* (2013), coincides with the results of the paleo-Tisza – paleo-Danube geological calibration. Placing of all key events for *A. torrentium* species evolution based on intense uplift of Dinaride Mountains geological calibration and paleo-Tisza – paleo-Danube geological calibration is highly similar with almost identical 95% HPD value ranges and the highest difference of 1.8 Ma for the median values of *A. pallipes* and *A. torrentium* time of divergence estimation. Similar results were obtained in the study conducted by Jelić *et al.* (2016). They placed the divergence of *A. pallipes* and *A. torrentium* at 95% HPD 11.1-19.1 Ma which corresponds to the 95% HPD (7.3-16.3 Ma) paleo-Danube-paleo-Tisza geological calibration. On the contrary, results obtained through calibration based on the geological event proposed by Pârvulescu *et al.*, (2019) does not overlap neither with the results of paleo-Tisza – paleo-Danube geological calibration nor with the other four calibrations (three molecular clock calibrations and intense uplift of the Dinaride Mountain geological calibration) at all. Even more, our results based on this calibration show a considerable offset to results presented by Pârvulescu *et al.*, (2019). They placed the diversification of the *Austropotamobius* genus at ~42 Ma (HPD 54–32 Ma) while our result suggests a later date placed at ~34 Ma (HPD 49.42-23.10). We also estimated significantly later divergence dates for all other *A. torrentium* phylogroups including NCD phylogroups, SB and CSE phylogroups, except APU phylogroup. It has been shown that, adding more informative data produces more accurate phylogenetic estimates and reduces the impact of stochasticity on parameter estimation (Sarver *et al.*, 2018). Therefore, we could attribute the difference between our results for this calibration and results obtained by Pârvulescu *et al.*, (2019) to the larger number of haplotypes and longer sequences of mtDNA used in our research, which

enabled better resolution of *A. torrentium* evolutionary history. Also, this could be a result of different tree evolution models in BEAST framework, Yule model, used in this study after Klobučar *et al*. 2013, opposed to Birth-death model applied in Pârvulescu *et al.*, (2019). Sarver *et al.* (2018) showed that the choice of tree prior has relatively small impact on the estimation of diversification rates on simulated data. The Yule model uses simple branching with only speciation as a considered parameter (Steel and McKenzie, 2001), while the Birth-death model enhances on the Yule model by addition of an extinction parameter (Rannala and Yang, 1996). In a study conducted by Condamine *et al*. (2015), younger divergence time estimation were obtained when using the Birth-death tree prior over the Yule tree prior. The observed phenomenon is described as "pull of the present" in which younger clades are not affected by the extinction parameter, while its effect is stronger on the older clades that are more susceptible to the extinction process (Etienne and Rosindell, 2012) Since the results of the key events in *A. torrentium* species evolution by Pârvulescu *et al*. (2019), compared to our results, are not affected by the "pull of the present" phenomenon, we conclude that the choice of a Yule prior did not affect the divergence time estimation in this study. This conclusion is supported by the observation that even though the Birth-death model may seem more biologically correct, it will not always fit the data better than the Yule model, especially for younger (pre-Triassic period) divergence times (Condamine *et al*., 2015).

5.2. Phylogenetic structure

In this study, as shown before, the phylogenetic structure of *A. torrentium* revealed its complexity. Trontelj *et al*. (2005), analysing the mtDNA, showed the existence of three phylogroups: Upper Kolpa Basin, Southern Balkans and Southeastern Alps + Slovenia (later named by Klobučar *et al.*, (2013) GK, SB and CSE). These phylogroups were later confirmed by Klobučar *et al.* (2013) with the addition of four new phylogroups: ZV, ŽPB, LD and BAN. The latest studies performed by Pârvulescu *et al.* (2019) discovered the existence of another phylogroup (APU) which was described as a new species *Austropotamobius bihariensis* (Pârvulescu, 2019). Almost all new sequences that were obtained in this study formed new haplotypes (a total of 72 new haplotypes) that nested within the existing phylogroups. Most of the new haplotypes belong to the CSE phylogroup, followed by BAN, ŽPB and GK. Our research revealed the existence of a previously unknown phylogroup belonging to the Kordun region (KOR) that was well supported by BA, ML and MP analysis (Figure 2). The sampled crayfish were found in streams of the Glina river in the Kordun region after which the phylogroup was named. These localities are part of the North-Central Dinaric region as ŽPB,

LD, BAN, GK and ZV. It has been shown that increased sampling of taxa is one of the most important ways to increase overall phylogenetic accuracy (Zwickl and Hillis, 2002). Our discovery was the result of broader sampling of newly discovered and previously unstudied populations. It is important to mention that in future studies the increase of the number of phylogroups could be expected. A possible example is the *COI* haplotype 41 that according to the current knowledge belongs to the BAN phylogroup. This haplotype, however, belongs to a sample found in a geographically distant location (Imotski region) and it is believed to be introduced from Bosnia and Hercegovina. Future research should focus on localities in Bosnia and Herzegovina, mainly north-western Krajina region, rich in freshwater karstic streams that are still understudied. This could potentially lead to discovery of new haplotypes (and possibly phylogroups) that could contribute to our current knowledge of species richness and genetic diversity.

As on the phylogenetic tree, the new KOR phylogroups forms a distinct phylogroup in the median joining network. All of the nine phylogroups are separated by a large number of mutational steps, with the smallest distance between CSE and SB (13 mutational steps), and highest between GK and ŽPB phylogroups. The CSE phylogroup shows the most diverse and complex structure having the highest number of haplotypes separated by small number of mutational steps, spread across a large number of countries. This phylogroup is closely related to the SB and BAN phylogroup, the former revealing a structure of six sub-clades. The BAN phylogroups shows a star shaped topology which implies that the central haplotype has been present in the populations for a longer period and the branching haplotypes formed and expanded recently (Slatkin and Hudson, 1991; Ferreri *et al.*, 2011). This expansion was probably the result of post-glacial recolonization at the beginning of Holocene (Klobučar *et al.*, 2013). This could indicate ancestral BAN-CSE+SB population capability for the large-scale range expansion that occurred during Pliocene and resulted in formation of SB and CSE phylogroups. The genetic vicinity of the CSE and SB phylogroups to the BAN phylogroup is a consequence of their evolutionary history which has been discussed above.

The genetic distances between the phylogroups are also shown by the p and K2P distances (Table 4 and Table 5). The new phylogroup KOR shows the smallest value of p and K2P distance to ŽPB phylogroup on the both mitochondrial markers. This close relationship between the KOR and ŽPB is also visible on the MJ network (Figure 5) and Bayesian and BEAST phylograms (Figure 3 and Figure 7) and it could be explained with their geographical vicinity. This relationship however, remains to be confirmed on the basis of nuclear markers

since KOR-ŽPB clade appear to be unsupported in phylogenetic analyses (Figure 3). The KOR phylogroup probably evolved together with the other NCD phylogroups.

For all phylogroups the values of p and K2P distances are similar. For the *COI* gene the smallest distance is between CSE and SB phylogroups (4.21% and 4.37% for p and K2P distances, respectively). For the *16S* rRNA gene, the greatest distance between phylogroups is present between ZV and GK (4.79%, 4.98% for p and K2P distances, respectively). That was already observed by Klobučar *et al.* (2013). All phylogroups, except SB, are well supported as a monophyletic clade by ML, MP and BA analyses (Figure 3). SB phylogroup shows a paraphyletic relationship towards CSE phylogroup on both nuclear and mitochondrial phylogenetic reconstruction (Figure 3, Figure 4). and it is closely related to *A. pallipes.* All others phylogroups (NCD and APU) show unresolved polyphyletic relationships. This lack of resolution could have emerged from a rapid divergence of the phylogroups (Whitfield and Lockhart, 2007; Klobučar *et al.*, 2013).

Genetic distances within phylogroups (Table 6) are lowest for NCD and APU phylogroups and highest for SB and CSE phylogroup. High values of genetic distances within CSE and SB phylogroup show that those phylogroups are more diverse. This could be the consequence of their evolutionary history. CSE phylogroup went through large areal expansion during post-glacial recolonization and shows several sub-clades, with the oldest clade located in Bosnia and Hercegovina (Figure 7). SB phylogroup also shows several sub-clades (Figure 5, Figure 7). In its evolutionary history it went through a southern areal expansion, presumably through paleo-Morava, as oldest SB clades are in Serbia on today's Morava tributaries. Populations of SB sub-clades may have been isolated in refugia during glaciations and did not came back in contact post-glacially which would result in high genetic distances among them. Genetic distances within SB phylogroup for *COI* (Table 6) are comparable with genetic distances between SB and CSE phylogroups (Table 5) and for *16S* rRNA are higher than genetic distances between KOR and ŽPB phylogroups. Higher within phylogroup genetic distances were also observed for BAN phylogroup, presumably because *COI* haplotype 41 is detached from other BAN haplotypes. The lowest within phylogroup genetic distances for ZV phylogroup (Table 6) could be a result of an isolated population belonging to one stream (Zeleni vir).

We have shown that *ITS2* nuclear marker is suitable for inferring *A. torrentium* phylogenetic tree reconstruction, as is shown on Figure 4. Although, *ITS2* region had a lower value of variable (1.54 %) and parsimony informative sites (1.00 %) than *COI* and *16S* rRNA mtDNA

markers, this was improved using gap analysis (2.18 % gap informative sites). *ITS2* alignment (1126 bp long) showed seven gap informative site segments for *A. torrentium* at: 245-259 bp, 521-524 bp, 637-640 bp, 670-677 bp, 803-845 bp, 932 bp and 946 bp. Long gaps indicate that *ITS2* segment of *A. torrentium* passed through rearrangements. In the *ITS2* analysis, we were able to obtain tree topology containing all NCD clades described in Klobučar *et al*. (2013). The phylogenetic tree based on the *ITS2* gene indicates the BAN, GK, ŽPB, ZV and LD phylogroups as monophyletic and well supported, while the SB and CSE groups are shown as one unique phylogroup. Clustering of SB and CSE phylogroup was expected as they share close evolutionary history as it was shown in previous studies (Trontelj *et al*., 2005; Klobučar *et al*., 2013). To improve the resolution of phylogenetic reconstruction, future studies should include APU and KOR phylogroups and higher number of sequences from other phylogroups. This should improve the assessment of structure and relationships between phylogroups and populations based on the *ITS2* gene.

We have observed that the values of p-distances and average values of Kimura two-parameter (K2P) distances for *ITS2* marker show are much lower than for mtDNA markers. Also, analyses of parsimony informative (PI) sites revealed the highest PI values for *COI* (23.74%), followed by *16S* rRNA (10.27%) and lastly *ITS2* (1.00%). These values indicate that the *COI* gene is two and 20 times more parsimony informative than the *16S* rRNA and *ITS2*, respectively. Slower evolution rate of non-coding nuDNA was previously observed across many different species (Guo *et al.*, 2015; Allio *et al.*, 2017). Nuclear DNA shows a slower evolution rate then mtDNA because among other it is not exposed to oxidation processes present in mitochondria, and the mutations are repaired through an effective repair mechanism (Larsen *et al.*, 2005). There is no broadly applicable mutation rate for *ITS2* gene, because of its overall length variation, which makes the calculation of evolutionary divergence among organisms harder (Coleman, 2003).

What is important to note is that the genetic divergences between ZV plus GK and other stone crayfish phylogroups calculated for *16S* rRNA data set was higher than in previous study (3.0–4.2%) by Klobučar *et al*. (2013.), ranging between 3.28% and 4.79% which put them closer to the range of genetic distances between *A. pallipes* and *A. italicus* (4.6–4.7%) observed by Grandjean *et al.* (2000) and Zaccara *et al.* (2004). Also, genetic distance for *COI* gene between most of the phylogroups (except between ZV + GK and SB, CSE and BAN pairs) is higher (Table 4 and Table 5) than those observed between *A. pallipes* and *A. italicus* (7.0 ± 1%) (Trontelj *et al.*, 2005). The K2P distances between most phylogroups exceeded the

intraspecific divergence (4.61%) observed in the study of *COI* diversity in Decapoda conducted by Matzen da Silva *et al*. (2011) and are similar to the intragenic K2P distances observed in that study.

The number of mutational steps in the MJ network dividing the phylogroups is comparable with those obtained by Pârvulescu *et al.*, 2019. The number between all phylogroups (except CSE, SB and BAN) is between 36 and 53, above the distance that divides the newly described species *A. bihariensis* (Pârvulescu, 2019; Pârvulescu *et al.*, 2019) from the other phylogroups.

Therefore, the large intraspecific phylogenetic gaps challenge whether the phylogroups should be considered as separate species.

5.3. Species delimitation

Following the hypothesis that the phylogroups could represent new species, various species delimitation methods were applied. These methods identify minimal phylogenetic units as operational taxonomic units (OTUs) (Goldstein *et al*., 2000; Luo *et al*., 2018) which generate a good estimate of species diversity for subsequent taxonomic revisions (Kekkonen *et al*., 2015).

The number of OTUs that the ABGD method yielded, agrees with the number of phylogroups known from previous studies (Klobučar *et al.*, 2013; Pârvulescu *et al.*, 2019). All the phylogroups are represented as a unique OTU, except SB and BAN. The former is split in nine sub-clades (observed also by Klobučar *et al.*, 2013) and the latter is split into two OTUs, one of which is the haplotype 41 already discussed above. These results are congruent with the TCS network analysis which can also indicate the presence of separate OTUs. Sequences from a single OTU form a single network (Hart and Sunday, 2007).

Unlike the distance-based species delimitation methods mentioned above, the tree-based methods (bPTP and GMYC) yielded a higher number of OTUs. The number of OTUs obtained by the bPTP and GMYC analyses is similar and shows the split of every phylogroup into several OTUs except ZV and GK. This observation could be explained from the fact that these two phylogroups are limited to a narrow geographic area (Figure 2) and they diverged from the common *A.torrentium* ancestor first (Figure 7). Bayesian support values higher than 0.91 for bPTP analysis were obtained for 14 OTUs and it has been suggested that support values are strongly correlated with the accuracy of the delimitation (Zhang *et al*, 2013). For GMYC analysis, 9 OTUs have been delimited as well supported separate OTUs, with the

probability of the clades being delimited as separate GMYC-species (within a 95% confidence set) higher than 0.9 (Fleck Fossen *et al.*, 2016). The higher number of OTUs obtained by the tree-based analyses could be a consequence of the fact that those methods tend to overestimate the number of species and they reflect the genetic structure of the data showing the population structure within the species (Sukumaran and Knowles, 2017). Their performance is also affected by the ratio of population sizes to species divergence times (Luo *et al.*, 2018).

For the validation of the species, we tested which of the applied method was best suitable for our data set. The result of BFD* species delimitation showed GMYC as the appropriate model (Table 9). Within the species tree yielded by BFD* high Bayesian posterior probabilities were obtained for monophyletic CSE, BAN, ŽPB, LD, KOR branches. For the monophyletic SB clade low Bayesian posterior probabilities were obtained, probably due to its high within population genetic distances (Table 6). Species tree on Figure 11, shows that deeper nodes in species tree topology among NCD+APU clades are unresolved, which is also indicated by phylogenetic reconstruction on concatenated dataset (Figure 3). A more resolved topology is obtained for BAN, SB and CSE clades, probably to more recant speciation events.

As mentioned before, the high number of OTUs obtained by GMYC is a result of a deep substructure between and within the phylogroups. According to the GMYC analysis the transition from interspecific to intraspecific events occurred around 0.93 Ma. This date should be taken with caution because it is based on the calibration of the molecular clock (decapod substitution rate) used for the tree as the input for BEAST. The choice of the value of 0.007 subs/s/my/l was arbitrary as all of the substitution rates (decapod, arthropod and mid-point of the two) showed similar results. The sharp increase in branching rate could explain the rapid change from species level evolution to population level evolution after the Mid Pleistocene transition and the beginning of the 100 Ka glacial cycles.

The genetic structure of *A. torrentium* and its evolutionary history were shown to be complex, intricate processes with an everlasting need for further studies. We hope that inclusion of microsatellite loci in the future will help with resolving the population structure of the stone crayfish and revel recent evolutionary changes and possible population-level hybridization events through secondary contacts. Moreover, the OTUs delimited by analysis of mtDNA represent hypothesis and should be taken with caution even if well-supported (Schlick-Steiner *et al.*, 2010). Molecular species delimitation should be employed as a part of an integrative

taxonomic approach (Luo *et al.*, 2018). These hypothesized species entities should be examined through morphological, biogeographical, distributional ranges data (Vogler and Monaghan, 2007) for a more accurate taxonomic definition. Additional studies on morphologic, meristic and cytogenetic data are needed. It was shown before that there is a morphological difference between the ZV, GK and ŽPB phylogroups (Maguire *et al.*, 2017); while Pârvuelscu (2019) described APU phylogroup as a distinct species (*A. bihariensis*). Considering the morphological and molecular diversity that has been shown in this and previous studies, the description of new species is possible. The description of new species must be a thoughtful process, that considers the whole genus *Austropotamobius* and not only the species within *A.torrentium* species-complex, so the number of species would not be over- or underestimated.

5.4. Conservation

The stone crayfish is listed as a vulnerable species, and it is protected by national laws in Croatia. However, it is listed as data deficient on IUCN Red List (Füreder *et al*., 2010). With the present research we expand the knowledge of the genetic diversity of the species to improve conservation planning. The results obtained using molecular phylogenetics can be used for identifying phylogroups some of which are of high conservation priority (Souty-Grosset and Reynolds, 2009). Moreover, an accurate estimate of species is a key factor in improving biodiversity assessment (Pante *et al*., 2014). This must be a basis for the species management and conservation actions (Berger *et al*., 2017, Puorto *et al*., 2001).

In order to develop adequate conservation plans, and find the best approach for species conservation, a better understanding of the threats and the conditions of the endangered species is needed (IUCN-SSC, 2008). Effective plans should consider current impacts on the threatened species habitat and geographic and spatio-temporal influences. Freshwater ecosystems are among the most endangered in the world, as these ecosystems are facing high levels of degradation and biodiversity loss, and are strongly affected by climate changes (Nel *et al.*, 2009). The vulnerability of these ecosystems is augmented by the poor level of protection of the habitat and the species present in them (Januchowski-Hartley *et al.*, 2011). Studies on freshwater biodiversity showed the need to monitor various parameters: habitat condition, biotic and abiotic factors, catchment disturbance, biotic indicators of water condition (Linke and Turak, 2011). In the conservation of the endangered species it is important to consider the habitat in which the species is distributed (Nel *et al.*, 2009). The habitat of the species should be considered as a protected area and also undergo a

reassessment within conservation programs because it is a key tool in preventing biodiversity loss (Januchowski-Hartley *et al.,* 2011). The freshwater crayfish and other freshwater taxa are at greater risk of extinction than terrestrial species (Richman *et al.,* 2015). The stone crayfish inhabits river springs and streams on higher altitudes and it is mostly threatened by human impacts, modification of water flow, polluted waters, invasive species and pathogens (e.g. *Aphanomyces astaci*) (Maguire, 2014). Also, the rate of diversification is crucial for assessing the risk of extinction of a species (Richman *et al.,* 2015). It is important to notice that several populations of the stone crayfish are distributed in the protected areas (e.g. in Croatia: Nature Park Žumberak Samoborsko gorje, National Park Risnjak, National Park Plitvička jezera, Nature Park Medvednica; in Romania: Apuseni Natural Park, Semenic - Cheile Carasului National Park, Nerei - Beușnița Ravine National Park, Domogled Valea Cernei National Park; in Slovenia: Triglav National Park, and many other) and areas included in the monitoring program. Many of those areas are part of part of Natura 2000 network (Maguire, 2014.). Still, areas like Banovina region and Kordun region, where the existence of the new phylogroup was observed in this research, should be considered in future monitoring and conservation programs as they represent distinct molecular-phylogenetic phylogroups. That need to be preserved.

6. Conclusion

Studying the mitochondrial and nuclear DNA of the stone crayfish by analysing three different markers *COI*, *16S* rRNA and *ITS2*, enabled us to obtain new information about the species genetic structure. Examining the *COI* and *16S* rRNA sequences we discovered the existence of a new phylogroup belonging to the Kordun region. In this study we applied a new calibration approach for estimation of the divergence time among stone crayfish phylogroups, and we concluded that the complex evolution of this vulnerable species is linked to the hydrogeographic history of the Pannon Basin area. The analysis of the *ITS2* nuclear marker, according to our knowledge, among the first ever done on *A. torrentium*, showed its suitability for assessing phylogenetic relationships. Combined with mitochondrial DNA marker, the *ITS2* marker, can be of a great help for the determination of potential species within the stone crayfish and studies of their evolutionary history. The species delimitation methods showed the existence of a great number (29) of operational taxonomic units. Integrating these findings with studies of morphologic and meristic characters, the description of new species is possible. To protect the genetic diversity of the species, it is important to include all

phylogroups in conservation programs, as they represent distinct evolutionary lineages and potentially new species.

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9. Appendix

Table A1. List of sampling locations and *COI*, *16S* rRNA and *ITS2* haplotypes used in this study.

Locality	Phylogroup	*COI* haplotype	*16S* rRNA haplotype	*COI_16S* rRNA	*ITS2*	Number of individuals (samples)	Authors
Macedonia: River Mrenoshtica	SB	n/a	18	_18	n/a	2	present study
Macedonia: River Klucka	SB	n/a	38	_38	at10f3	1	present study
Slovenia: Sromljica River, Curnovec	CSE	n/a	26	_26	n/a	2	present study
Slovenia: Koričanski Stream, Koritno	CSE	n/a	26	_26	n/a	3	present study
Croatia: Okićnica	CSE	n/a	28	_28	n/a	4	present study
Slovenia: Srna Grapa River, Gornja Trebuša	CSE	n/a	33	_33	n/a	3	present study
Slovenia: Tributary of Bloščica, Mlaka	CSE	n/a	36	_36	n/a	2	present study
Slovenia: Tributary of Bloščica, Povirje	CSE	n/a	36	_36	n/a	2	present study
Slovenia: Barbarski Stream, Slovenj Gradec	CSE	n/a	36	_36	n/a	3	present study
Slovenia: Domaček Stream, Morsko	CSE	n/a	36	_36	n/a	2	present study
Slovenia: Bača River, Koritnica	CSE	n/a	36	_36	n/a	4	present study
Croatia: Bresni Stream, Gerovo	GK	n/a	4	_4	n/a	1	present study
Croatia: Blate, Malinci	GK	n/a	4	_4	n/a	7	present study
Croatia: Bela vodica Stream, Vela Voda	GK	n/a	4	_4	n/a	2	present study
Croatia: Mrzlica Stream, Mrzla Vodica	GK	n/a	4	_4	n/a	1	present study
Croatia: Bela vodica Stream, Vela Voda	GK	101	4	101_4	GK1_8	1	present study
Croatia: Delnice Stream	GK	135	4	135_4	GK2_1	2	present study
Croatia: Vele vode	GK	136	4	136_4	n/a	1	present study
Croatia: Blate, Malinci	GK	53	1	53_1	n/a	9	Klobučar *et al.* 2013
Croatia: Ruševica Stream, Maljevačko selište/Čulibrci	KOR	n/a	34	_34	n/a	1	present study
Croatia: Glinica River, Gojkovac	KOR	n/a	34	_34	n/a	1	present study
Croatia: Sopotski slap	ŽPB	n/a	6	_6	n/a	2	present study
Croatia: Sopotski slap, Sošice	ŽPB	n/a	6	_6	n/a	4	present study
Croatia: Bilić Stream	ŽPB	n/a	6	_6	n/a	1	present study
Croatia: Dodigovac, village Jagetići	ŽPB	n/a	8	_8	n/a	2	present study
Croatia: Tounjčica River	ŽPB	100	8	100_8	MUN1	1	present study
Croatia: Tounjčica River	ŽPB	100	8	100_8	MUN1	1	present study
Croatia: Glinica River, Gojkovac	KOR	102	34	102_34	n/a	1	present study
Croatia: Draga Stream, tributary of Vera, Vera	ŽPB	104	6	104_6	sar3	1	present study
Croatia: Bilić Stream	ŽPB	105	6	105_6	sops7	1	present study
Croatia: Pecka Stream, Topusko	KOR	106	35	106_35	n/a	1	present study
Croatia: Dodigovac, Jagetići	ŽPB	138	6	138_6	VER1	2	present study
Croatia: Blate, Malinci	GK	54	1	54_1	n/a	2	Klobučar *et al.* 2013

Croatia: Munjava, Cerovnik	ŽPB	42	6	42_6	n/a	1	present study
Croatia: Blate, Malinci	GK	24	4	24_4	n/a	3	present study
Croatia: Unnamed Stream, Vrelo	ŽPB	55	1	55_1	n/a	1	Klobučar *et al.* 2013
Croatia: Blate, Malinci	GK	57	10	57_10	n/a	1	Klobučar *et al.* 2013
Croatia: Unnamed Stream, Vele Vode	GK	57	10	57_10	n/a	1	Klobučar *et al.* 2013
Slovenia: Kolpa River, Kočevje	GK	58	10	58_10	n/a	1	Klobučar *et al.* 2013
Croatia: Vele vode	GK	26	n/a	26_	n/a	1	present study
Slovenia: Belca River, Idrijska Bela	CSE	n/a	36	_36	n/a	1	present study
Croatia: Vele vode	GK	26	n/a	26_	n/a	1	present study
Croatia: Unnamed Stream, Plaška Glava	ŽPB	59	10	59_10	n/a	1	Klobučar *et al.* 2013
Croatia: Bresni Stream, Gerovo	GK	60	10	60_10	n/a	1	Klobučar *et al.* 2013
Croatia: Unnamed Stream, Crni Lug	GK	61	10	61_10	n/a	1	Klobučar *et al.* 2013
Bulgaria: Sedrac, Razlog	SB	62	10	62_10	n/a	3	Klobučar *et al.* 2013
Macedonia: River Mrenoshtica	SB	116	18	116_18	n/a	3	present study
Macedonia: River Strumica	SB	117	n/a	117_	n/a	1	present study
Macedonia: River Plavaja	SB	137	45	137_45	n/a	1	present study
Montenegro: River Crnojevića, Cetinje	SB	89	10	89_10	n/a	5	Klobučar *et al.* 2013
Serbia: Grošnička reka, Kragujevac	SB	89	10	89_10	n/a	1	Klobučar *et al.* 2013
Macedonia: Unnamed Stream, Jakupica Mountain	SB	90	10	90_10	n/a	1	Klobučar *et al.* 2013
Slovenia: Belca River, Idrijska Bela	CSE	n/a	36	_36	n/a	1	present study
Macedonia: Unnamed Stream, Jakupica Mountain	SB	91	10	91_10	n/a	1	Klobučar *et al.* 2013
Macedonia: Kadina River, pritoka Vardara	SB	34	18	34_18	n/a	2	present study
Kosovo: Unnamed Stream, Rashan	SB	n/a	10	n/a_10	n/a	1	Klobučar *et al.* 2013
Croatia: Javnica Stream	BAN	n/a	14	_14	n/a	1	present study
Croatia: Vera Stream, Vera	ŽPB	43	7	43_7	n/a	1	present study
Croatia: Unnamed Stream, Plaška Glava	ŽPB	n/a	10	n/a_10	n/a	2	Klobučar *et al.* 2013
Croatia: Unnamed Stream, Drežnica	ŽPB	n/a	10	n/a_10	n/a	1	Klobučar *et al.* 2013
Croatia: Jaruga River, Lug polje	ŽPB	45	6	45_6	n/a	1	present study
Croatia: Unnamed Stream, Drežnica	ŽPB	n/a	11	n/a_11	n/a	2	Klobučar *et al.* 2013
Croatia: Brebornica, Krnjak	ŽPB	n/a	12	n/a_12	n/a	1	Klobučar *et al.* 2013
Croatia: Unnamed Stream, Krakar	ŽPB	41	13	41_13	n/a	2	Klobučar *et al.* 2013
Croatia: Ratković, Donji Puškarići	ŽPB	92	14	92_14	n/a	1	Klobučar *et al.* 2013
Croatia: Jarak, Jelenići	ŽPB	92	14	92_14	n/a	2	Klobučar *et al.* 2013
Croatia: Jarak, Sošice	ŽPB	92	14	92_14	n/a	6	Klobučar *et al.* 2013
Croatia: Sopotski slap, Sošice	ŽPB	92	14	92_14	n/a	3	Klobučar *et al.* 2013
Croatia: Suvaja, Sošice	ŽPB	93	14	93_14	n/a	1	Klobučar *et al.* 2013
Croatia: Sopotski slap	ŽPB	48	6	48_6	n/a	1	present study

Croatia: Sopotski slap, Sošice	ŽPB	48	6	48_6	n/a	1	present study
Slovenia: Belca River, Idrijska Bela	CSE	n/a	36	_36	n/a	3	present study
Slovenia: Left tributary of Črmenica River, Verdinek	CSE	n/a	36	_36	n/a	2	present study
Slovenia: Helenski Stream, Podpeci	CSE	n/a	36	_36	n/a	3	present study
Slovenia: Rakiški graben, Rakitna	CSE	n/a	36	_36	n/a	3	present study
Slovenia: Rakiški graben	CSE	n/a	36	_36	n/a	1	present study
Slovenia: Zamedvejski Stream, Zamedveje	CSE	n/a	36	_36	n/a	4	present study
Slovenia: Srna Grapa River, Gornja Trebuša	CSE	n/a	43	_43	n/a	1	present study
Croatia: Gračanski Stream, Zagreb	CSE	1	15	1_15	SRO4	1	Klobučar *et al.* 2013
Croatia: Sopotnica Stream, Sopot, Višnjevac	CSE	103	26	103_26	n/a	1	present study
Slovenia: Tributary of Bloščica, Mlaka	CSE	107	36	107_36	n/a	3	present study
Slovenia: Tributary of Bloščica, povirje	CSE	107	36	107_36	n/a	1	present study
Croatia: Okićnica	CSE	108	28	108_28	n/a	3	present study
Slovenia: Dreta River, Gornji Grad	CSE	108	36	108_36	n/a	1	present study
Slovenia: Tributary of Bloščica, Mlaka	CSE	109	36	109_36	n/a	1	present study
Croatia: Ivanečka Željeznica, Ivanec	CSE	32	16	32_16	BAC1	1	Klobučar *et al.* 2013
Croatia: Lonja, Paka	CSE	83	16	83_16	BAC1	1	Trontelj *et al.* 2005
Slovenia: Tributary of Bloščica, povirje	CSE	110	36	110_36	n/a	1	present study
Slovenia: Tributary of Bloščica, Runavščica Stream, Runarsko	CSE	111	36	111_36	n/a	5	present study
Slovenia: Domaček Stream, Morsko	CSE	113	36	113_36	n/a	1	present study
Slovenia: Domaček Stream, Morsko	CSE	114	36	114_36	n/a	1	present study
Slovenia: Sromljica River, Curnovec	CSE	115	26	115_26	n/a	2	present study
Slovenia: Curnovščica River, Curnovec	CSE	115	26	115_26	n/a	1	present study
Slovenia: Dreta River, Gornji Grad	CSE	115	36	115_36	n/a	1	present study
Slovenia: Sromljica River, Curnovec	CSE	115	37	115_37	n/a	1	present study
Slovenia: Bača River, Koritnica	CSE	118	36	118_36	n/a	1	present study
Slovenia: Left tributary of Črmenica River, Verdinek	CSE	119	39	119_39	n/a	1	present study
Croatia: Jarak, Stojdraga	CSE	n/a	16	n/a_16	BEL1	3	Klobučar *et al.* 2013
Slovenia: Curnovščica River, Curnovec	CSE	120	26	120_26	n/a	1	present study
Slovenia: Curnovščica River, Curnovec	CSE	121	26	121_26	n/a	2	present study
Slovenia: Dreta River, Gornji Grad	CSE	122	41	122_41	n/a	1	present study
Slovenia: Helenski Stream, Podpeci	CSE	123	36	123_36	n/a	1	present study
Slovenia: Koričanski Stream, Koritno	CSE	124	26	124_26	n/a	1	present study
Croatia: Jarak, pritoka Bregane, Stojdraga	CSE	124	26	124_26	n/a	6	present study
Slovenia: Bača River, Kuk	CSE	124	36	124_36	n/a	1	present study
Slovenia: Bača River, Kuk	CSE	125	36	125_36	n/a	1	present study

Slovenia: Lučnica River, Luče	CSE	126	36	126_36	n/a	2	present study
Slovenia: Tributary of Mura River, Robičevi gozd	CSE	126	36	126_36	n/a	1	present study
Slovenia: Rakiški graben, Rakitna	CSE	127	n/a	127_	n/a	1	present study
Slovenia: Zamedvejski Stream, Zamedveje	CSE	128	36	128_36	n/a	1	present study
Croatia: Kraljevec, Zagreb	CSE	33	17	33_17	CUR1	1	Klobučar *et al.* 2013
Croatia: Bliznec, Medvednica	CSE	13	26	13_26	CUR1	3	present study
Croatia: Bliznec, Medvednica	CSE	139	26	139_26	n/a	2	present study
Croatia: Okićnica, Donji Gorički	CSE	40	17	40_17	LAV1	2	Klobučar *et al.* 2013
Croatia: Bliznec, Medvednica	CSE	140	47	140_47	n/a	1	present study
Croatia: Bliznec, Medvednica	CSE	141	26	141_26	n/a	1	present study
Croatia: Bliznec, Medvednica (lov ruke)	CSE	142	26	142_26	n/a	1	present study
Croatia: Okićnica	CSE	143	28	143_28	n/a	3	present study
Croatia: Okićnica	CSE	144	28	144_28	n/a	1	present study
Croatia: Jarak, pritoka Bregane, Stojdraga	CSE	145	26	145_26	n/a	1	present study
Croatia: Jarak, pritoka Bregane, Stojdraga	CSE	146	48	146_48	n/a	1	present study
Croatia: Jarak, pritoka Bregane, Stojdraga	CSE	147	26	147_26	n/a	1	present study
Croatia: Okićnica, Donji Gorički	CSE	n/a	17	n/a_17	RAKI4	1	Klobučar *et al.* 2013
Croatia: Unnamed Stream, Crkveni Vrhovci	CSE	34	18	34_18	SRN2	2	Klobučar *et al.* 2013
Croatia: Vučjak, Jagodnjak	CSE	34	18	34_18	SRN2	2	Klobučar *et al.* 2013
Croatia: Bukovica, Novo Selo	CSE	36	18	36_18	BLI2	1	Klobučar *et al.* 2013
Romania: Cerna, Caras Severin	CSE	36	18	36_18	oki2	1	Klobučar *et al.* 2013
Romania: Cerna, Caras Severin	CSE	38	18	38_18	n/a	1	Klobučar *et al.* 2013
Bosnia&Herzegovina: Crna River, Trnovo	CSE	n/a	18	n/a_18	n/a	5	Klobučar *et al.* 2013
Romania: Cerna, Caras Severin	CSE	35	19	35_19	n/a	1	Klobučar *et al.* 2013
Romania: Cerna, Caras Severin	CSE	52	2	52_2	n/a	1	Klobučar *et al.* 2013
Croatia: Sarni, Kraljev Vrh	CSE	56	2	56_2	n/a	2	Klobučar *et al.* 2013
Croatia: Veliki Stream, Zagreb	CSE	94	20	94_20	n/a	1	Klobučar *et al.* 2013
Bosnia&Herzegovina: Crna River, Trnovo	CSE	37	21	37_21	n/a	1	Klobučar *et al.* 2013
Bosnia&Herzegovina: Sutjeska, Tjentište	CSE	3	24	3_24	n/a	1	present study
Bosnia&Herzegovina: Crna River, Trnovo	CSE	39	22	39_22	n/a	1	Klobučar *et al.* 2013
Germany & Switzerland: Rhine & Danube systems	CSE	63	22	63_22	n/a	19	Klobučar *et al.* 2013
Austria & Germany: Haldensee + Algäu	CSE	n/a	22	n/a_22	n/a	11	Klobučar *et al.* 2013
France: Schlierbach, Bliesbruck, Sarreguemines	CSE	81	23	81_23	n/a	1	Schubart & Huber 2006
France: Gailbach, Obergailbach, Sarreguemines	CSE	2	24	2_24	n/a	1	Klobučar *et al.* 2013
Germany: Freiburg im Breisgau	CSE	3	24	3_24	n/a	1	Klobučar *et al.* 2013
Czech Republic: Bertinský brook	CSE	4	24	4_24	n/a	10	Klobučar *et al.* 2013

Czech Republic: Bojovka	CSE	5	25	5_25	n/a	14	Klobučar *et al.* 2013
Czech Republic: Huníkovský brook	CSE	5	25	5_25	n/a	10	Schubart & Huber 2006
Czech Republic: Chocenický brook	CSE	n/a	25	_25	n/a	10	COI present study 16S rRNA Parvulescu *et al.* 2019
Czech Republic: Luční brook, Bohemian Uplands	CSE	n/a	25	_25	n/a	2	COI present study 16S rRNA Parvulescu *et al.* 2019
Czech Republic: Míza	CSE	151	25	151_25	n/a	2	COI present study 16S rRNA Parvulescu *et al.* 2019
Czech Republic: Oupoř	CSE	n/a	25	_25	n/a	15	COI present study 16S rRNA Parvulescu *et al.* 2019
Czech Republic: Padrťský brook	CSE	n/a	25	_25	n/a	10	COI present study 16S rRNA Parvulescu *et al.* 2019
Czech Republic: Příchovický brook	CSE	n/a	25	_25	n/a	5	COI present study 16S rRNA Parvulescu *et al.* 2019
Croatia: Jarak, Sošice	ŽPB	n/a	25	_25	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Croatia: Unnamed Stream, Krakar	ŽPB	n/a	25	_25	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Croatia: Sartuk, Sertić Poljana	ŽPB	150	25	150_25	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Croatia: Sartuk, NP Plitvice	ŽPB	50	6	50_6	n/a	3	present study
Croatia: Zeleni Vir, Radatovići	ZV	n/a	25	_25	ZV6	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Croatia: Zeleni Vir, Radatovići	ZV	153	25	153_25	n/a	3	COI present study 16S rRNA Parvulescu *et al.* 2019
Croatia: Zeleni Vir, Radatovići	ZV	n/a	25	_25	n/a	2	COI present study 16S rRNA Parvulescu *et al.* 2019
Croatia: Zeleni Vir, Radatovići	ZV	n/a	25	_25	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Croatia: Zeleni Vir, Radatovići	ZV	150	25	150_25	n/a	1	COI present study 16S rRNA Parvulescu *et*

							al. 2019
Croatia: Zeleni Vir, Radatovići	ZV	n/a	25	_25	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Croatia: Unnamed Stream, Čujića Krčevina	LD	152	25	152_25	kra1	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Croatia: Source lake, Sinj	LD	n/a	25	_25	kra1	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Bosnia&Herzegovina: Unnamed Stream, Preodac	LD	10	26	10_26	pr1	1	Klobučar *et al.* 2013
Bosnia&Herzegovina: Unnamed Stream, Preodac	LD	12	26	12_26	AT_234	1	Klobučar *et al.* 2013
Czech Republic: Příchovický brook	CSE	13	26	13_26	n/a	3	Klobučar *et al.* 2013
Croatia: Unnamed Stream, Čujića Krčevina	LD	18	26	18_26	n/a	1	Klobučar *et al.* 2013
Croatia: Kobilica, Palanka	LD	21	26	21_26	n/a	2	Klobučar *et al.* 2013
Croatia: Kobilica, Palanka	LD	22	26	22_26	n/a	1	Klobučar *et al.* 2013
Macedonia: Bošava, Kožuf Mountain	SB	6	26	6_26	n/a	1	Klobučar *et al.* 2013
Czech Republic: Příchovický brook	CSE	7	26	7_26	n/a	4	Klobučar *et al.* 2013
Czech Republic: Rakovský brook	CSE	8	26	8_26	n/a	9	Klobučar *et al.* 2013
Czech Republic: Radotínksý brook	CSE	88	26	88_26	n/a	8	Klobučar *et al.* 2013
Czech Republic: Stroupínský brook	CSE	88	26	88_26	n/a	3	Klobučar *et al.* 2013
Macedonia: Bošava, Kožuf Mountain	SB	9	26	9_26	n/a	1	Klobučar *et al.* 2013
Macedonia: Crna Reka , v. Zeleznec	SB	37	18	37_18	n/a	2	present study
Czech Republic: Stroupínský brook	CSE	n/a	26	n/a_26	n/a	1	Klobučar *et al.* 2013
Macedonia: Crna Reka , v. Zeleznec	SB	37	18	37_18	n/a	2	present study
Macedonia: Bela Reka, v. Sloeshtica	SB	37	18	37_18	n/a	3	present study
Macedonia: Bela Reka, v. Sloeshtica, Germanski vir	SB	37	18	37_18	n/a	1	present study
Macedonia: Bela Reka, v. Sloeshtica, near hydropower station	SB	37	18	37_18	n/a	1	present study
Macedonia: River Mrenoshtica, near the road toward v. Mrenoga	SB	37	18	37_18	n/a	2	present study
Croatia: Unnamed Stream, Crni Lug	GK	n/a	26	n/a_26	n/a	1	Klobučar *et al.* 2013
Croatia: Gerovčica, Gerovo	GK	n/a	26	n/a_26	n/a	1	Klobučar *et al.* 2013
Croatia: Unnamed Stream, Bela Vodica	GK	n/a	26	n/a_26	n/a	1	Klobučar *et al.* 2013
Croatia: Unnamed Stream, Vele Vode	GK	n/a	26	_26	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Czech Republic: Stroupínský brook	CSE	n/a	26	_26	n/a	7	COI present study 16S rRNA Parvulescu *et al.* 2019

Croatia: Kobilica, Palanka	LD	11	27	11_27	n/a	1	Klobučar *et al.* 2013
Croatia: Krasulja, Korenica	LD	11	27	11_27	n/a	2	Klobučar *et al.* 2013
Czech Republic: Trebušín	CSE	9	27	9_27	n/a	3	Klobučar *et al.* 2013
Czech Republic: Úpořský brook	CSE	14	28	14_28	n/a	10	Klobučar *et al.* 2013
Croatia: Orašnica, Knin	LD	15	28	15_28	n/a	2	Klobučar *et al.* 2013
Croatia: Srebrenica, Donji Srb	LD	17	28	17_28	n/a	2	Klobučar *et al.* 2013
Croatia: (Maja Stream)	BAN	n/a	14	_14	n/a	2	present study
Croatia: Brućina, Mali Gradac	BAN	n/a	14	_14	n/a	1	present study
Croatia: Oblakovića vrelo, Gornja Bačuga	BAN	n/a	14	_14	n/a	1	present study
Croatia: Maja (kod ušća pritoke Slatine)	BAN	129	14	129_14	vep1	1	present study
Macedonia: River Mrenoshtica, near the road toward v. Mrenoga	SB	37	18	37_18	n/a	1	present study
Croatia: Unnamed Stream, Čujića Krčevina	LD	16	29	16_29	n/a	1	Klobučar *et al.* 2013
Croatia: Kobilica, Palanka	LD	16	29	16_29	n/a	1	Klobučar *et al.* 2013
Croatia: Rječica, Jezerce	LD	52	3	52_3	n/a	2	Klobučar *et al.* 2013
Croatia: Rakova voda, Strmica	LD	n/a	30	_30	n/a	5	COI present study 16S rRNA Parvulescu *et al.* 2019
Bosnia&Herzegovina: Korana, Bosansko Grahovo	LD	n/a	30	_30	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Macedonia: in the confluence of rive Bela Reka and River Mrenoshtica	SB	37	18	37_18	n/a	5	present study
Croatia: Orašnica, Knin	LD	n/a	10	_10	n/a	3	present study
Croatia: Bela vodica Stream, Vela Voda	GK	26	4	26_4	n/a	1	present study
Croatia: Unnamed Stream, Čujića Krčevina	LD	n/a	10	_10	n/a	3	present study
Croatia: Krasulja, Korenica	LD	89	10	89_10	n/a	2	present study
Croatia: Krasulja, Korenica	LD	n/a	10	_10	n/a	1	present study
Macedonia: in the confluence of rive Bela Reka and River Mrenoshtica	SB	37	18	37_18	n/a	1	present study
Czech Republic: Všenorský	CSE	19	31	19_31	n/a	7	Klobučar *et al.* 2013
Croatia: Sopotski slap, Sošice	ŽPB	20	31	20_31	n/a	2	Klobučar *et al.* 2013
Czech Republic: Všenorský	CSE	n/a	32	n/a_32	n/a	2	Klobučar *et al.* 2013
Croatia: Bela vodica Stream, Vela Voda	GK	26	4	26_4	n/a	1	present study
Croatia: Delnički Stream	GK	26	4	26_4	n/a	6	present study
Croatia: Vele vode	GK	26	4	26_4	n/a	3	present study
Croatia: Maja (kod ušća pritoke Slatine)	BAN	130	14	130_14	BAN1_1	1	present study
Croatia: Maja (kod ušća pritoke Slatine)	BAN	131	14	131_14	BAN1_2	1	present study
Croatia: Brućina, Mali Gradac	BAN	132	14	132_14	n/a	1	present study

Croatia: Brućina, Mali Gradac	BAN	133	14	133_14	n/a	1	present study
Croatia: Brućina, Mali Gradac	BAN	134	14	134_14	n/a	1	present study
Croatia: Badnjevice channel, Donji Proložac	BAN	n/a	33	n/a_33	n/a	1	Schubart & Huber 2006
Croatia: Maja, Glina	BAN	24	4	24_4	n/a	1	Klobučar *et al.* 2013
Czech Republic: Všenorský	CSE	24	4	24_4	n/a	1	Klobučar *et al.* 2013
Croatia: Sopotski slap, Sošice	ŽPB	25	4	25_4	n/a	2	Klobučar *et al.* 2013
Croatia: Maja, Glina	BAN	26	4	26_4	n/a	1	Klobučar *et al.* 2013
Croatia: Slatina, Dvor	BAN	26	4	26_4	n/a	1	Klobučar *et al.* 2013
Croatia: Sopotski slap, Sošice	ŽPB	26	4	26_4	n/a	1	Klobučar *et al.* 2013
Croatia: Vele vode	GK	26	4	26_4	n/a	2	present study
Croatia: Gramaljska Dobra, Gramalj	GK	26	4	26_4	n/a	1	Klobučar *et al.* 2013
Croatia: Unnamed Stream, Leskova Draga	GK	26	4	26_4	n/a	3	Klobučar *et al.* 2013
Croatia: Unnamed source, Malinci	GK	26	4	26_4	n/a	3	Klobučar *et al.* 2013
Czech Republic: Zbirožský brook	CSE	27	4	27_4	n/a	2	Klobučar *et al.* 2013
Croatia: Unnamed source, Malinci	GK	28	4	28_4	n/a	2	Klobučar *et al.* 2013
Croatia: Sopotski slap, Sošice	ŽPB	51	6	51_6	n/a	5	present study
Czech Republic: Zbirožský brook	CSE	29	4	29_4	n/a	3	Klobučar *et al.* 2013
Czech Republic: Zákolanský brook	CSE	29	4	29_4	n/a	11	Klobučar *et al.* 2013
Czech Republic: Zubřina	CSE	29	4	29_4	n/a	1	Klobučar *et al.* 2013
Germany: Dresden	CSE	n/a	4	n/a_4	n/a	12	Klobučar *et al.* 2013
Macedonia: Unnamed Stream, Kožuf Mountain	SB	n/a	4	n/a_4	n/a	1	Klobučar *et al.* 2013
Macedonia: Bošava, Kožuf Mountain	SB	n/a	49	_49	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Serbia: Toplodolska reka, Temska	SB	n/a	5	n/a_5	n/a	2	Klobučar *et al.* 2013
Serbia: Unnamed Stream, Zlatibor Mountain	SB	n/a	50	_50	n/a	4	COI present study 16S rRNA Parvulescu *et al.* 2019
Serbia: Toplodolska reka, Temska	SB	n/a	51	_51	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Greece: Maras, Pige, Drama	SB	n/a	51	_51	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Greece: Mílli, Angístro, Sidirókastro	SB	n/a	51	_51	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Greece: Mílli, Angístro, Sidirókastro	SB	n/a	52	_52	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019

Greece: Ramna, Akritohóri, Sidirókastro	SB	149	53	149_53	n/a	2	COI present study 16S rRNA Parvulescu *et al.* 2019
Bulgaria: Sandanska Bistrica, Sandanski	SB	148	53	148_53	n/a	2	COI present study 16S rRNA Parvulescu *et al.* 2019
Greece: Koursovit, Karidohóri, Sidirókastro	SB	148	54	148_54	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Montenegro: River Crnojevića, Cetinje	SB	149	54	149_54	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Greece: Batania, Koúpa, Políkastro	SB	148	54	148_54	n/a	2	COI present study 16S rRNA Parvulescu *et al.* 2019
Greece: Ano Kefalari, Drama	SB	n/a	54	_54	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Kosovo: Lunni, Zhegovc	SB	148	54	148_54	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Montenegro: River Crnojevića, Cetinje	SB	42	6	42_6	n/a	2	Klobučar *et al.* 2013
Germany: Dresden	CSE	43	6	43_6	n/a	9	Klobučar *et al.* 2013
Austria: Archbach, Reutte (greška sa COI seq)	CSE	45	6	45_6	n/a	1	Klobučar *et al.* 2013
Germany & Switzerland: Rhine & Danube systems	CSE	45	6	45_6	n/a	10	Klobučar *et al.* 2013
Bulgaria: Rositsa, Rositsa	CSE	45	6	45_6	n/a	3	Klobučar *et al.* 2013
Turkey: Velika, Demirköy, Kirklareli	CSE	45	6	45_6	n/a	1	Klobučar *et al.* 2013
Germany: Dachs See, Algäu	CSE	47	6	47_6	n/a	3	Klobučar *et al.* 2013
Germany: Auerberg, Algäu	CSE	48	6	48_6	n/a	1	Klobučar *et al.* 2013
Luxembourg: Rouderbaach, Grevenmacher	CSE	48	6	48_6	n/a	1	Klobučar *et al.* 2013
Czech Republic: Radotínksý brook	CSE	49	6	49_6	n/a	2	Klobučar *et al.* 2013
Austria: Wienerwald	CSE	50	6	50_6	n/a	1	Klobučar *et al.* 2013
Slovenia: Zala Creek, Godovič, Idrija	CSE	51	6	51_6	n/a	1	Klobučar *et al.* 2013
Slovenia: Dovje, Jesenice	CSE	51	6	51_6	n/a	1	Klobučar *et al.* 2013
Slovenia: Bohinjska Bela, Bled	CSE	51	6	51_6	n/a	2	Klobučar *et al.* 2013
Croatia: Dolje, Podsused	CSE	n/a	6	n/a_6	n/a	1	Klobučar *et al.* 2013
Croatia: Dolje, Medvednica	CSE	7	26	7_26	n/a	2	present study
Slovenia: Zaplana, Logatec	CSE	43	7	43_7	n/a	1	Klobučar *et al.* 2013
Slovenia: Baškagrapa, Tolmin	CSE	46	8	46_8	n/a	1	Klobučar *et al.* 2013
Slovenia: Lavegraben,	CSE	71	26	71_26	n/a	1	present study

Trate							
Slovenia: Bača River, Kuk	CSE	71	36	71_36	n/a	2	present study
Austria: Zainer Bach, Arnoldstein	CSE	48	8	48_8	n/a	3	Klobučar *et al.* 2013
Italy: Tributary of the Slizza, Tarvisio	CSE	n/a	9	n/a_9	n/a	1	Klobučar *et al.* 2013
Austria: Schinzengraben, Pressegger See, Hermangor	CSE	26	n/a	26_	n/a	1	Klobučar *et al.* 2013
Slovenia: River Cerknica, Cerkno	CSE	26	n/a	26_	n/a	1	Trontelj *et al.* 2005
Slovenia: Domaček Stream, Morsko	CSE	73	36	73_36	n/a	1	present study
Slovenia: Left tributary of Črmenica River, Verdinek	CSE	73	36	73_36	n/a	1	present study
Slovenia: Rakitna, Ljubljana	CSE	44	n/a	44_	n/a	1	Klobučar *et al.* 2013
Slovenia: Gorenji Lazi, Ribnica	CSE	23	n/a	23_	n/a	1	Klobučar *et al.* 2013
Slovenia: Glinščica Creek, Ljubljana	CSE	5	n/a	5_	n/a	1	Schubart & Huber 2006
Croatia: Dolje, Podsused	CSE	5	n/a	5_	n/a	1	Schubart & Huber 2006
Croatia: Dolje, Medvednica	CSE	8	26	8_26	n/a	6	present study
Croatia: Dolje, Medvednica	CSE	8		8_	n/a	1	present study
Bosnia&Herzegovina: Kavuša, Gornji Vakuf	CSE	5	n/a	5_	n/a	2	Trontelj *et al.* 2005
Serbia: Grošnička reka, Kragujevac	SB	5	n/a	5_	n/a	2	Trontelj *et al.* 2005
Bosnia&Herzegovina: Kavuša, Gornji Vakuf	CSE	5	n/a	5_	n/a	1	Trontelj *et al.* 2005
Slovenia: Logatec	CSE	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Croatia: Bručina, Glina	BAN	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Croatia: Javnica Stream	BAN	92	14	92_14	n/a	2	present study
Croatia: Maja Stream	BAN	92	14	92_14	n/a	1	present study
Croatia: Stupnica Stream	BAN	92	14	92_14	n/a	1	present study
Croatia: Maja	BAN	92	14	92_14	n/a	9	present study
Croatia: Brućina, Mali Gradac	BAN	92	14	92_14	n/a	5	present study
Croatia: Oblakovića vrelo, Gornja Bačuga	BAN	92	14	92_14	n/a	6	present study
Croatia: Maja	BAN	92	44	92_44	n/a	2	present study
Croatia: Bručina, Glina	BAN	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Croatia: Brućina, Mali Gradac	BAN	93	14	93_14	n/a	1	present study
Croatia: Radošnica Stream, pritoka Žirovnice	BAN	97	14	97_14	n/a	1	present study
Croatia: Velika Petrinjčica Stream, Piramida	BAN	99	14	99_14	n/a	1	present study
Croatia: Unnamed source, Malinci	GK	5	n/a	5_	n/a	7	Petrušek *et al.* 2017
Slovenia: Grivački Stream, Grivac, Kočevje	GK	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Slovenia: Belica Creek, Kočevje	GK	5	n/a	5_	n/a	3	Petrušek *et al.* 2017
Croatia: Unnamed Stream, Crni Lug	GK	5	n/a	5_	n/a	2	Petrušek *et al.* 2017
Croatia: Gramaljska Dobra, Gramalj	GK	5	n/a	5_	n/a	2	Petrušek *et al.* 2017
Croatia: Unnamed Stream, Kupjak	GK	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Macedonia: Unnamed	SB	5	n/a	5_	n/a	1	Petrušek *et*

Stream, Pelister Mountain							*al.* 2017
Serbia: Toplodolska reka, Temska	SB	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Croatia: Ruševica Stream, Maljevačko selište/Čulibrci	KOR	98	34	98_34	n/a	2	present study
Croatia: Unnamed Stream, Plaška Glava	ŽPB	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Croatia: Bojna, Bojna	KOR	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Slovenia: Rakitna, Ljubljana	CSE	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Slovenia: River Iška, Ljubljana	CSE	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Slovenia: Hotenjka Creek, Logatec	CSE	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Slovenia: Jazbinški Stream, Žerjav	CSE	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Italy: Piano di Fusine,Tarvisio	CSE	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Czech Republic: Luční brook, Giant Mts.	CSE	5	n/a	5_	n/a	5	Petrušek *et al.* 2017
Croatia: Dubravica, Pušća	CSE	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Slovenia: Koričanski Stream, Koritno	CSE	9	26	9_26	n/a	1	present study
Croatia: Veliki Stream, Zagreb	CSE	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Slovenia: Barbarski Stream, Slovenj Gradec	CSE	9	36	9_36	n/a	2	present study
Slovenia: Dreta River, Gornji Grad	CSE	9	36	9_36	n/a	2	present study
Slovenia: Helenski Stream, Podpeci	CSE	9	36	9_36	n/a	1	present study
Slovenia: Lučnica River, Luče	CSE	9	36	9_36	n/a	2	present study
Slovenia: Curnovščica River, Curnovec	CSE	9	40	9_40	n/a	1	present study
Slovenia: Lučnica River, Luče	CSE	9	42	9_42	n/a	1	present study
Czech Republic: Zbirožský brook	CSE	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Croatia: Sopotnica Stream, Sopot, Višnjevac (Zagorje)	CSE	96	26	96_26	n/a	1	present study
Slovenia: Bača River, Kuk	CSE	n/a	42	kratki4_42	n/a	1	present study
Slovenia: Srna Grapa River, Gornja Trebuša	CSE	n/a	43	kratki5_43	n/a	1	present study
Croatia: Bregana, Grdanjci	CSE	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Croatia: Curak, Glušinja	CSE	5	n/a	5_	n/a	2	Petrušek *et al.* 2017
Croatia: Mrzlak, Zagreb	CSE	5	n/a	5_	n/a	2	Petrušek *et al.* 2017
Croatia: Izvir, Mrzlo Polje Žumberačko	CSE	5	n/a	5_	n/a	1	Petrušek *et al.* 2017
Croatia: Curak, Glušinja	CSE	77	n/a	77_	n/a	1	Trontelj *et al.* 2005
Slovenia: Reka, Logaščica tributary, Logatec	CSE	78	n/a	78_	n/a	3	Trontelj *et al.* 2005
Romania: Rănușa, Crișul Alb	APU	79	n/a	79_	n/a	1	Trontelj *et al.* 2005
Romania: Rănușa, Crișul Alb	APU	80	n/a	80_	n/a	1	Trontelj *et al.* 2005
Romania: Rănușa, Crișul Alb	APU	82	n/a	82_	n/a	1	Trontelj *et al.* 2005
Romania: Rănușa, Crișul Alb	APU	84	n/a	84_	n/a	1	Trontelj *et al.* 2005
Romania: Șoimușurilor, Crisul Negru,	APU	85	n/a	85_	n/a	1	Trontelj *et al.* 2005

Romania: Damiș, Crișul Negru	APU	5	n/a	5_	n/a	4	Petrušek *et al.* 2017
Romania: Damiș, Crișul Negru	APU	64	n/a	64_	n/a	1	Trontelj *et al.* 2005
Romania: Răchiteasca, Crisul Repede	APU	65	n/a	65_	n/a	5	Schubart & Huber 2006
Hungary: Bernece-Stream	CSE	65	n/a	65_	n/a	2	Schubart & Huber 2006
Hungary: Miklósdeákvölgyi-Stream	CSE	65	n/a	65_	n/a	1	Trontelj *et al.* 2005
Hungary: Stájer-Stream	CSE	65	n/a	65_	n/a	1	Petrušek *et al.* 2017
Hungary: Malomvölgyi-Stream	CSE	66	n/a	66_	n/a	1	Schubart & Huber 2006
Hungary: Nagybörzsöny-Stream	CSE	67	n/a	67_	n/a	1	Trontelj *et al.* 2005
Hungary: Miklósdeákvölgyi-Stream	CSE	68	n/a	68_	n/a	2	Trontelj *et al.* 2005
Hungary: Miklósdeákvölgyi-Stream	CSE	69	n/a	69_	n/a	1	Trontelj *et al.* 2005
Hungary: Bükkös-Stream	CSE	70	n/a	70_	n/a	2	Trontelj *et al.* 2005
Hungary: Miklósdeákvölgyi-Stream	CSE	71	n/a	71_	n/a	2	Trontelj *et al.* 2005
Hungary: Malomvölgyi-Stream	CSE	72	n/a	72_	n/a	2	Trontelj *et al.* 2005
Hungary: Bagolybükki-Stream	CSE	72	n/a	72_	n/a	1	Trontelj *et al.* 2005
Hungary: Nagybörzsöny-Stream	CSE	72	n/a	72_	n/a	1	Trontelj *et al.* 2005
Hungary: Bükkös-Stream	CSE	73	n/a	73_	n/a	1	Trontelj *et al.* 2005
Hungary: Bagolybükki-Stream	CSE	74	n/a	74_	n/a	1	Trontelj *et al.* 2005
Hungary: Bagolybükki-Stream	CSE	75	n/a	75_	n/a	1	Trontelj *et al.* 2005
Hungary: Miklósdeákvölgyi-Stream	CSE	76	n/a	76_	n/a	1	Trontelj *et al.* 2005
Hungary: Miklósdeákvölgyi-Stream	CSE	9	n/a	9_	n/a	1	Schubart & Huber 2006
Hungary: Bükkös-Stream	CSE	86	n/a	86_	n/a	2	Trontelj *et al.* 2005
Hungary: Bagolybükki-Stream	CSE	87	n/a	87_	n/a	1	Trontelj *et al.* 2005
Hungary: Nagybörzsöny-Stream	CSE	9	n/a	9_	n/a	2	Trontelj *et al.* 2005
Hungary: Nagybörzsöny-Stream	CSE	9	n/a	9_	n/a	1	Trontelj *et al.* 2005
Hungary: Nagybörzsöny-Stream	CSE	9	n/a	9_	n/a	1	Trontelj *et al.* 2005
Hungary: Nagybörzsöny-Stream	CSE	9	n/a	9_	n/a	1	Trontelj *et al.* 2005
Hungary: Miklósdeákvölgyi-Stream	CSE	9	n/a	9_	n/a	1	Trontelj *et al.* 2005
Hungary: Malomvölgyi-Stream	CSE	9	n/a	9_	n/a	1	Petrušek *et al.* 2017
Hungary: Miklósdeákvölgyi-Stream	CSE	95	n/a	95_	n/a	1	Petrušek *et al.* 2017
Hungary: Nagybörzsöny-Stream	CSE	149	n/a	149_	n/a	1	COI present study 16S rRNA Parvulescu *et al.* 2019
Hungary: Bernece-Stream	CSE	5	n/a	5_	n/a	1	COI present study 16S rRNA

							Parvulescu *et al.* 2019

10.Summary

Genetic diversity of the stone crayfish

Lena Bonassin and Ljudevit Luka Boštjančić

Austropotamobius torrentium (Schrank, 1803) is a freshwater species distributed across central and south-eastern Europe that inhabits smaller waterbodies at higher altitudes. This research represents a most comprehensive study of its genetic diversity. In this study, we analysed 279 crayfish from 69 new locations from Croatia, Slovenia and Macedonia. Our datasets consisted of 1114 mitochondrial DNA (mtDNA) sequences and 23 nuclear DNA (nuDNA) sequences. Previous studies of *A. torrentium* phylogeography revealed the existence of eight geographically localised phylogroups, based on mtDNA. Although most of the newly sampled localities nested within previously described phylogroups, we discovered a new phylogroup located in Kordun region, Croatia. We report the first successful application of nuDNA marker (Internal transcribed spacer 2 (*ITS2*)) for phylogenetic inference of *A. torrentium* and discuss its application in future research. A range of molecular clock and geological calibrations were applied to asses times of divergence and unravel *A. torrentium* evolutionary history. For the first time, a geological calibration based on hydrogeological event was applied. Contact between paleo-Danube and paleo-Tisza ~5.3 Ma that was accompanied by favourable climatic and environmental factors for colonisation. This approach enabled us to reconcile previous conflicting views on *A. torrentium* evolutionary history. A wide range of species delimitation methods (ABGD, TCS, GMYC, bPTP, BFD*) were applied on mtDNA dataset, with the highest support for 29 operational taxonomic units (OTUs) within *A. torrentium* species complex. We discuss the prospect of this discovery and its application in for future species description within the genus *Austropotamobius*. Over last decades, a decline in freshwater crayfish populations has been observed. Therefore, the application of acquired results in the future *A. torrentium* conservation programs is discussed. Their focus should be on preserving current species range and habitats with emphasis on phylogroups in currently unprotected areas.

Keywords: *Austropotamobius torrentium*, conservation, biodiversity, *ITS2*, species delimitation

11. Sažetak

Genska raznolikost potočnog raka

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Austropotamobius torrentium (Schrank, 1803) je slatkovodna vrsta rasprostranjena u centralnoj i jugoistočnoj Europi, koja nastanjuje manje vodotoke na većim nadmorskim visinama. Ovo istraživanje predstavlja najopsežniju studiju genske raznolikosti ove vrste. Analizirano je 279 rakova s 69 novih lokaliteta iz Hrvatske, Slovenije i Makedonije. Naš skup podataka čine 1114 sekvenci mitohondrijske DNA (mtDNA) i 23 sekvence nuklearne DNA (nuDNA). Prijašnja istraživanja filogeografije potočnog raka na temelju mtDNA otkrila su postojanje osam geografski lokaliziranih filogrupa. Iako većina uzoraka s novih lokacija pripada već otkrivenim filogrupama, uočili smo postojanje nove filogrupe u Hrvatskoj na području Korduna. U našem istraživanju prvi puta smo uspješno upotrijebili nuDNA marker *ITS2* (engl. Internal transcribed spacer 2) za određivanje filogenetskih odnosa kod potočnog raka i predložili smjernice za njegovu uporabu u budućim istraživanjima. Upotrijebljen je niz geoloških kalibracija i kalibracija molekularnog sata kako bi se odredila evolucijska povijest potočnog raka. Po prvi puta smo primijenili geološku kalibraciju utemeljenu na hidrogeološkom događaju, povezivanju rijeka, paleo-Dunava i paleo-Tise, prije ~5.3 Ma. Događaj koji je popraćen povoljnim klimatskim i okolišnim uvjetima za kolonizaciju. Ovim pristupom pomirili smo prijašnje nesuglasne poglede na evolucijsku povijest potočnog raka. Širok raspon metoda razgraničenja vrsta (ABGD, TCS, GMYC, bPTP, BFD*) primijenjen je na mitohondrijskom setu podataka, s najvećom podržanošću za postojanje 29 operativnih taksonomskih jedinica (engl. operational taxonomic units) unutar kompleksa vrsta potočnog raka. Istaknuta je njihova važnost i primjena u budućim istraživanjima s ciljem utvrđivanja novih vrsta unutar roda *Austropotamobius*. Tijekom prošlog desetljeća zamijećen je značajan pad broja populacija slatkovodnih rakova. Iz tog razloga, potreban je okvir za primjenu rezultata dobivenih u ovom istraživanju, unutar budućih konzervacijskih programa. Njihov fokus treba biti na očuvanju sadašnjeg areala vrste i staništa s posebnim naglaskom na populacije koje čine jedinstvene filogrupe, a nalaze se na nezaštićenim područjima.

Keywords: *potočni rak*, konzervacija, bioraznolikost, *ITS2*, razgraničenje vrsta

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