The tale of springs and streams. How different refugial ecosystems impacted the present molecular population structure of two riffle beetles in the Western Carpathians

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The Western Carpathians are particularly interesting part of the Carpathian Arc, which was, also according to recent molecular and fossil data, an important refugial area for the cold-stenotherm species during the Pleistocene glaciations. However, the Western Carpathians also include a rich system of karst springs inhabited by specific fauna, whose molecular diversity and phylogeographic patterns have not yet been explored. The relatively stable thermal and chemical conditions of these springs, which have persisted even throughout the Pleistocene and Holocene climate changes, make these highly specific lotic systems potentially ideal for the long-term survival of aquatic biota. This study aimed to compare the population genetic structure and molecular diversity of two related and commonly co-occurring riffle beetles - *Elmis aenea* (PWJ Müller, 1806) and *Limnius perrisi* (Dufour, 1843) - in the springs and streams using the mtDNA barcoding fragment I (COI). *E. aenea* significantly dominated in the springs, whereas *L. perrisi* preferred flows. The population of each species was relatively homogeneous genetically, with a single dominant haplotype. Nevertheless, the relative isolation of the springs and their stable conditions were reflected in the higher genetic variability of the *E. aenea* population in comparison to *L. perrisi*. The results of Bayesian Skyline Plot analyses also indicated the exceptional position of the springs regarding maintaining population size of the *E. aenea* throughout the Pleistocene climate fluctuations. On the other hand, streams provide more effective dispersal channels for the *L. perrisi*, whose population expanded during the period of postglacial global warming. Our findings suggest that the springs of the Western Carpathians may indeed have served as refugia for freshwater fauna, but not in the same way for different species, even belonging to one family.

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Abstract

The Western Carpathians are particularly interesting part of the Carpathian Arc, which was, also according to recent molecular and fossil data, an important refugial area for the cold-stenotherm species during the Pleistocene glaciations. However, the Western Carpathians also include a rich system of karst springs inhabited by specific fauna, whose molecular diversity and phylogeographic patterns have not yet been explored. The relatively stable thermal and chemical conditions of these springs, which have persisted even throughout the Pleistocene and Holocene climate changes, make these highly specific lotic systems potentially ideal for the long-term survival of aquatic biota.

This study aimed to compare the population genetic structure and molecular diversity of two related and commonly co-occurring riffle beetles - *Elmis aenea* (PWJ Müller, 1806) and *Limnius perrisi* (Dufour, 1843) - in the springs and streams using the mtDNA barcoding fragment I (COI). *E. aenea* significantly dominated in the springs, whereas *L. perrisi* preferred flows. The population of each species was relatively homogeneous genetically, with a single dominant haplotype. Nevertheless, the relative isolation of the springs and their stable conditions were reflected in the higher genetic variability of the *E. aenea* population in comparison to *L. perrisi*.

The results of Bayesian Skyline Plot analyses also indicated the exceptional position of the springs regarding maintaining population size of the *E. aenea* throughout the Pleistocene climate fluctuations. On the other hand, streams provide more effective dispersal channels for the *L. perrisi*, whose population expanded during the period of postglacial global warming. Our findings suggest that the springs of the Western Carpathians may indeed have served as refugia for freshwater fauna, but not in the same way for different species, even belonging to one family.

Introduction

Several studies pointed out that freshwater organisms reacted differently to Pleistocene climatic oscillations compared to terrestrial species (Taberlet et al., 1998; Previšić et al., 2009; Theissinger et al., 2012). In this period, the distribution of some aquatic species was reduced to various local refugia (including springs), but others completely disappeared from the glaciated regions and survived only in the southern outskirts of Europe (Pauls, Lumbsch & Haase, 2006; Macher et al., 2015; Sworobowicz et al., 2020). Springs function as ecotones between the surface and underground waters, which makes them an ecologically significant habitat (Gibert, 1991). They are characterized by chemical, physical, and trophic constancy over several geological periods (Minshall & Winger, 1968; Odum, 1971; Butler & Hobbs, 1982; Cushing & Wolf, 1984; Glazier & Gooch, 1987; Pringle et al., 1988; Gooch & Glazier, 1991; Orendt, 2000; Wood et al., 2005; Meyer et al., 2007), which in turn provided a stable environment for aquatic invertebrates throughout glaciation in Europe (Malicky, 2006; Ujvárosi et al., 2010). Moreover, springs support unique macroinvertebrate communities that are found nowhere else in a catchment and may also represent refugia for aquatic biota during adverse conditions (Lewin et al., 2015). In this context, it is unambiguous that studies upon the diversity and evolutionary history of aquatic biota studies should be more focused include not only streams but with no doubt also on springs, that are now heavily understudied.

Many mountain areas can be also considered as suitable refugia, where aquatic invertebrate species have often formed isolated populations within and/or between geomorphological units/subunits (Engelhardt, Haase & Pauls, 2011; Davis et al., 2013; Čiamporová-Zaťovičová & Čiampor Jr, 2017; Šípošová, Čiamporová-Zaťovičová & Čiampor Jr, 2017). Importantly, the historic isolation of individual species populations influences their genetic diversity and can be considered as the main force shaping the genetic structure of aquatic species in Central and Northern Europe (Bálint et al., 2011; Alp et al., 2012; Theissinger et al., 2012).

The Carpathians served as an important refugium for many plants (Magri et al., 2006) and animals (Schmitt, 2009; Schmitt & Varga, 2012) during Pleistocene glaciations, and could play the same role concerning biota inhabiting springs and small streams. The role of the Western Carpathians (W Carpathians) as a refugium is supported by the evidence that during the last glacial period (109 – 11.7 ka) the southern margin of the continental ice sheet was located some 150-200 km north from its area. During the Late Pleniglacial (29 - 14 ka) the ice sheet shifted south towards the W Carpathians, however, in the very next period (the Late Glacial, 14 – 11.7 ka) the continental glacier moved again north without a direct impact on the W Carpathians area

(Zasadni & Kłapyta, 2014; Marks et al., 2019). The local Carpathian glaciers disappeared completely from the higher altitudes of the Slovakian and Polish Tatra Mts at ca. 8500 years ago (Lindner et al., 2003). After the glacier retreat in the Holocene, many species recolonized formerly inhabited areas (Vila, Vidal-Romaní & Björklund, 2005; Kotlík et al., 2008; Rudolph et al., 2018).

The W Carpathians are considered an important refugium for a wide range of aquatic and terrestrial taxa (Neumann et al., 2005; Kotlík et al., 2006; Theissinger et al., 2012; Vörös et al., 2016; Copilaş-Ciocianu et al., 2017; Juřičková et al., 2017). However, the biodiversity of the W Carpathian springs and streams is still underexplored, especially in terms of the genetic diversity and population structure of aquatic species. Accordingly, the main objective of our study is to compare the genetic population structure and diversity patterns of two riffle beetles *Elmis aenea* (PWJ Müller, 1806) and *Limnius perrisi* (Dufour, 1843) in the W Carpathian area. The species are relatively closely related and commonly co-occur, yet represent a generally understudied family of freshwater beetles. Limited dispersal abilities, high habitat specificity, and more or less fragmented distribution make Elmidae an ideal taxon for studying genetic diversification through many geographic regions. Both the aforementioned elmid species are rheophilic, oligo-stenotherm and typical inhabitants of epirhithral streams at higher altitudes (Moog & Jäch, 1995; García-Criado, Fernández-Aláez & Fernández-Aláez, 1999) but potentially with different microhabitat preferences. They are relatively widespread, which guarantees detection of gene flow among geomorphological units/subunits (e.g. Mamos et al., 2016). In addition, riffle beetles are considered as a good indicator of water quality and perhaps also of climate change (Elliott, 2008), so our study can also provide valuable data for biodiversity conservation.

Our study aims at answering the following questions: (a) are the spring subpopulations genetically more variable if compared to subpopulations in the streams?; (b) do subpopulations of different geomorphological subunits of the W Carpathians maintain connectivity with each other?; (c) does genetic structuring of populations reflect population size change in the context of the Pleistocene climatic oscillations? And (d) are there interspecific differences in the population genetic structure among related, co-occurring beetle species?

Materials & Methods

Study area

The Carpathian Arch stretches across Central and Eastern Europe and its main geomorphological units are the Western and Southeastern Carpathians (Kondracki, 1989). In general, the W Carpathians reach medium altitudes (ranging from 500 to 1300 m a.s.l.), only a few of their ranges exceed 1500 m a.s.l.; geologically the mountain system is characteristic by interactions of rock folding and horizontal shifts (Bielik, 1999).

The studied springs and streams are located mainly on the territory of the Slovak Republic, partially in Czech Republic and in Poland; in the geomorphological units/subunits of the Inner and Outer Western Carpathians. The exceptions are Vihorlat Mts (VM) being part of the Inner Eastern Carpathians and Poloniny Mts (PM) belonging to the Outer Eastern Carpathians (Fig. 1,

Table 1). Samples from these areas were included to explain the phylogenetic relationships in the broader context of the W Carpathians. Besides, some individuals from the Fagaraş Mts (the Southern Carpathians), the Apuşeni Mts (the Western Romanian Carpathians) and from the other major mountain chains in the Balkan region (Rila and Strandzha Mts) were also included in the haplotype maps.
More detailed characteristics of all sampling sites are available in Table 1 and BOLD datasets DS-SKLIMPER (DOI XXXX) and DS-SKELMAEN (DOI YYYY).

Sampling and morphological identification

Qualitative sampling of benthic invertebrates from the W Carpathian springs and streams took place in 2016 and 2017. The sampling was performed in the framework of a broader research, which was permitted on the basis of the permit issued by The District Office, Department of Environmental Care, No: OU-TN-OSZP1-2015/001937-12/Du (Supplemental file S1). Sampling of macrozoobenthos was carried out by the multi-habitat kick-sampling technique (Frost, 1971) using a hydrobiological hand-net with a mesh size of 0.5 mm. Organic material was fixed in 96 % ethanol directly in the field. In the laboratory, the invertebrates were picked off, sorted into higher taxonomic groups using stereomicroscope, prefixed with absolute ethanol and stored in a freezer at -25°C. Elmid beetles selected for molecular analysis were morphologically identified using the available determination keys (Więźlak, 1986; Jäch, 1992).

DNA extraction and PCR amplification

Total DNA was extracted from the legs or abdominal tissue of 560 individuals (297 sequences - *E. aenea*, 263 sequences - *L. perrisi*) using the Chelex protocol (Casquet, Thebaud & Gillespie, 2012), followed by PCR amplification of ca. 650 bp-long barcoding fragment of the mitochondrial cytochrome c oxidase subunit I (COI) using the primer pair LCO1490 and HCO2198 (Folmer et al., 1994). The PCR was performed in a total volume of 25 µl containing 5 µl of 5x DreamTaq™ Buffer, 1.5 µl of Mg+2(25 mM), 0.5 µl of each primer (concentration 5 lM), 0.5 µlof dNTP Mix (20 mM), 0.125 µl (0.625 U) DreamTaq™DNA Polymerase, 11.875 µl ultra-pure H2O and 5 µl of DNA template. The PCR cycling consisted of a 2-min initial denaturation at 94 °C, followed by 40 cycles of 94 °C (40 s) denaturation, 46 °C (40 s) annealing and 72 °C (1 min) extension and termination at 72 °C (10 min) for final extension. A 4 µl aliquot of the PCR products were visualized in GoldView (Solarbio) by electrophoresis on a 1 % agarose gel and GelLogic imaging equipment to check PCR product quality and length. The PCR products were purified with Exo-FastAP Thermo Scientific and were sent for sequencing to Macrogen Europe Inc., Amsterdam.

Data analyses

The obtained sequences were edited using SEQUENCHER v5.1 software and aligned using the MUSCLE algorithm (Edgar, 2004) in MEGA v7 (Kumar, Stecher & Tamura, 2016). The dataset

was complemented by BOLD sequences available for both species (18 of *E. aenea* from streams in Finland, Germany and France and 6 of *L. perrisi* from streams in Germany).
The haplotype data files were generated in DnaSP v5.10 (Librado & Rozas, 2009), the diversity indices were calculated in the same program. Haplotype networks were reconstructed using the median-joining method (MJN) in PopART v1.7 (Leigh & Bryant, 2015).
The population structure of both species was characterized by the analysis of molecular variance (AMOVA) and fixation indices (F_{ST}) using Arlequin 3.5 (Excoffier & Lischer, 2010). The AMOVA was used to estimate whether the observed genetic diversity may be attributed to the geographical partitioning of beetle populations in three levels: among geomorphological subunits, among subpopulations within subunits and within subpopulations. The subpopulation is defined as specimens from one locality within the geomorphological subunit (Table 1). F_{ST} is a measure of the genetic differentiation among subpopulations of individual localities by haplotype frequencies. To test the significance of covariance components and fixation indices, 1000 permutations were performed.
Further, the demographic and spatial dynamics of beetle populations was examined by the mismatch distribution analysis in Arlequin v3.5 (Excoffier & Lischer, 2010). The recent demographic expansion in both species was tested with Tajima's D (Tajima, 1989), Fu's Fs (Fu, 1997) and Fu and Li´s D (Fu & Li, 1993) tests of selective neutrality and population stability, performed in DnaSP. The significance of these tests was assessed with 10000 permutations.
The fluctuations of demography over time were identified with the extended Bayesian Skyline Plot (eBSP) in BEAST v2.6.2 software package (Bouckaert et al., 2019). The strict molecular clock was calibrated with the standard mitochondrial rate for arthropod COI equal to 0.0115 substitutions/site/Myr (Brower, 1994). The models of molecular evolution were set up through bModelTest (Bouckaert & Drummond, 2017). For comparison, two runs for each species of Monte Carlo Markov Chains (MCMC) were performed, each 40 million iterations long and sampled every 10000 iterations for eBSP log. The runs were examined in Tracer v1.7 (Rambaut et al., 2018) and all the parameters reached the effective sampling size (ESS) above 200. After removal of 10% burn-in, the eBSP plots were produced using R software (http://www.r-project.org). Both plots for each species were identical therefore only one is presented.
The phylogeny was reconstructed based on COI haplotypes using Bayesian approach in BEAST v2.6.2 (Bouckaert et al., 2019). The datasets were supplemented by outgroup consisting the European congeneric species: *Elmis perezi*, *E. rioloides*, *E. rietscheli*, *E. latreillei*, *E. obscura*, *E. maugeti*i, *Limnius opacus*, *L. muelleri* and *L. volckmari*. The model of substitution and molecular clock were set up identical as in the case of eBSP. The tree prior was set to Birth-Death following the Path Sampling selection. Two runs of Markov chain Monte Carlo (MCMC), each 20 million iterations long and sampled every 1000 iterations, were performed for both species. Runs were examined using Tracer v1.7 (Rambaut et al., 2018), and all the sampled parameters achieved a sufficient sample size (ESS > 200). Tree files were combined using Log-Combiner v1.8.1 (Drummond et al., 2012), with the removal of the non-stationary 25 % burn-in phase. The

maximum clade credibility chronogram was generated using TreeAnnotator v2.5.2 (Bouckaert et al., 2014).
All analysed sequences with GenBank accession numbers are available within two BOLD datasets: DS-SKLIMPER for *Limnius* samples (DOI XXXX) and DS-SKELMAEN for *Elmis* samples (DOI YYYY).

Results

The distribution of *Elmis aenea* and *Limnius perrisi* samples suggests different habitat preferences between the target species in the W Carpathians. *E. aenea* has a rather wide distribution in karst springs (31 sites), while it is less widespread in streams (15 sites). On the contrary, *L. perrisi* was located only in eight springs, but in 30 streams. *L. perrisi* was also found in four streams of VM (Inner Eastern Carpathians) and in one stream of PM (Outer Eastern Carpathians), while *E. aenea* was not recorded in these geomorphological subunits. Ultimately, both species co-occurred only in three springs and in 13 streams from the total of 73 sites sampled in W Carpathians (Table 1).
The W Carpathian population of *E. aenea* shares haplotypes with locations in the Fagaraş Mts, Apuseni Mts, as well as with localities outside the Carpathians (Rila and Strandzha Mts in the Balkan region, as well as Finland, Germany, France). 13 COI haplotypes of *E. aenea* were identified within 276 individuals collected from 46 localities in the W Carpathians (Fig. 2). The haplotype diversity was 0.34. Adding 39 sequences from the non-W Carpathian sites increased the number of haplotypes to 20 and the haplotype diversity to 0.36. Considerable genetic homogeneity of the *E. aenea* population in the W Carpathians resulted from the wide distribution of the dominant haplotype Ea1. The haplotype map (Fig. 2B) revealed that most haplotypes present in the southern part of the Carpathians Arc (Apuseni Mts) and the haplotypes of the Balkan region (Rila Mts, Strandzha Mts) were not reported from the W Carpathians. The exception was Ea14 shared between one stream in the Strandzha Mts and one spring (V050) in Slovakia (SOM). Individuals from Germany shared the haplotype Ea3 with a single locality in the geomorphological unit FTA (V070). In addition to dominant haplotype Ea1, another five haplotypes were found in FTA and seven in SOM. Haplotypes Ea5 and Ea11 were private and each occurred in one spring of FTA (V009, V086). The private haplotypes of SOM included Ea7, Ea12 and Ea13, while all of them were located in the springs of SOM1 subunit (V038, V048). Besides that, the one spring of SOM1 (V043) shared haplotype Ea8 with the spring of the mentioned geomorphological unit FTA (V020). In geomorphological unit WB, four haplotypes were found, while Ea9 was located exclusively at two localities (CZ03, CZ05). On the contrary, the haplotype Ea4 was also common in the SOM, FTA, CB and in the Apuseni Mts (Figs. 2A, 2B).
Compared to *E. aenea*, the population of *L. perrisi* in the W Carpathians was genetically more homogeneous. Just eight haplotypes with a haplotype diversity of 0.007 were found at 43 localities (245 sequences, Fig. 3). A group of five haplotypes (Lp5, Lp6, Lp7, Lp8, Lp9) recorded in the Apușeni, Fagaraș, Rila and the Strandzha Mts (Balkan region) was highly

divergent from the group found in the W Carpathians. Together with the non-W Carpathian haplotypes, the total haplotype diversity of *L. perrisi* was 0.2 (269 sequences). Haplotype Lp1 dominated in all geomorphological units of the W Carpathians; all German sequences also belonged to this haplotype. The presence of private haplotypes was lower compared to *E. aenea*: Lp14 from the one spring of SOM (V088), Lp10 in stream of WB (CZ06) and Lp11 from one spring of geomorphological unit FTA (V070). Besides that, two more haplotypes (Lp3, Lp13) were present in the FTA. Lp13 was shared with the locality of the different geomorphological unit SMC (SEL1). Lp3 was also present in unit WB, in addition to FTA (BEL3). The haplotype Lp2 was detected in a stream (CZ01) of WB and occurred also in one stream (KRV1) located in VM (Figs. 3A, 3B).

Overall, the comparison of haplotype maps of both elmid species (Figs. 2B, 3B) showed the same haplotype pattern - star-like topology with one dominant haplotype and low haplotype diversity values. The Bayesian time calibrated reconstruction of phylogeny suggested that the divergence of *E. aenea* 1 and 0.5 Mya while L. perrisi started between 1.6 and 0.6 Mya (Figs. 2C, 3C, S2, S3). Additionally, the sample GBCL24512-15IE_perezi from the GenBank is grouped with samples of *E. aenea*. Most likely this is due to misidentification/mislabelling of the deposited data, but we cannot resolve this without examination of the individual that the deposited sequence was produced from. The resulting phylogenetic trees are enclosed in the electronic supplementary material (Figs. S2, S3.).

The population-genetic analyses focused on the W Carpathian populations of both species (Table 2). The AMOVA showed that most of the observed molecular variance is generated within subpopulations (single localities). However, in *E. aenea*, the molecular variation among subpopulations within geomorphological subunits is more than twice (34.99 %) compared to *L. perrisi* (12.86 %).

Genetic differentiation between *E. aenea* and *L. perrisi* also consisted of different fixation index values (F_{ST}). The maximum value of fixation index for *E. aenea* was 0.8, for *L. perrisi* 0.4 (Fig. 4). The highest F_{ST} values of both species were found for pairs of subpopulations in the geomorphological subunits Slovak Karst (within the SOM1 (V038, V048) - *E. aenea*; (V088) - *L. perrisi*) and the Little Carpathians (within the FTA1 (V009) - *E. aenea*; (V070) - *L. perrisi*). Both species were characterized by the statistically significant, negative Fu´s Fs, Tajima´s D and Fu and Li´s D neutrality tests values (Table 3). This indicates a recent change in population size of both species. The Mismatch distribution analysis also confirmed recent demographic and spatial expansion for both species (Fig. 5). The eBSP of the mtDNA showed a signal of population growth in both species, although the pattern differed. The *E. aenea* population size was stable through the Ice Age and beginning of the Holocene, it is growth started roughly ca. 3000 years ago, whereas the population expansion of *L. perrisi* increased sharply around 8000 years ago (Fig. 6).

Discussion

Our study is focused on the two oligo-stenotherm riffle beetles, *Elmis aenea* and *Limnius perrisi*

(Elmidae), with a similar biotope preference and common occurrence (Moog & Jäch, 1995; García-Criado, Fernández-Aláez & Fernández-Aláez, 1999). However, we observed that their distribution patterns were quite different. While *E. aenea* occurred in karst springs and was less widespread in streams of the W Carpathians, the distributional pattern of *L. perrisi* was opposite. Such differences in distribution can be explained by different ecological demands, microhabitat preferences or altitude and flow type (Illies & Botosaneanu, 1963). Moreover, according to several studies, *E. aenea* is more sensitive to harsher conditions resulting from changes of the aquatic environment, manifested, for example, by the loss of macrophytes and moss (Maitland, 1967; Bradley & Ormerod, 2001; Hoffsten, 2003). These findings may explain much greater affinity of *E. aenea* to springs that generally, with respect to chemical, physical and trophic conditions, are more stable ecosystems compared to other lotic habitats (Minshall & Winger, 1968; Odum, 1971; Butler & Hobbs, 1982; Cushing & Wolf, 1984; Glazier & Gooch, 1987; Gooch & Glazier, 1991). This suggests that karst springs ensured a suitable environment for survival of some aquatic species even during the ice age (Thorup & Lindegaard, 1977). It supports the dinodal hypothesis (Malicky, 1983; Malicky, 2000) predicting that suitable habitats, such as headwaters, persisted throughout the Pleistocene within the periglacial area (dinodal biome), giving suitable conditions for the survival of specialized oligo-stenotherm communities in Central Europe.

In our study, the different results of the Bayesian Skyline Plot analyses between *E. aenea* and *L. perrisi* also confirmed the exceptional position of the springs. These findings indicate that springs could have a special status in terms of providing stable environmental conditions irrespective of the climatic changes during the glacial and interglacial periods which did not provoke a dramatic decline or increase of the *E. aenea* population size in the Western Carpathians during the Last Glacial Period (LGP) and beginning of Holocene. In contrast, the populations of *L. perrisi*, occurring in streams, began to expand rapidly after the LGP. Our findings are consistent with the results obtained by Haubrock et al. (2017), that suggested different evolutionary histories for several species of European trickle midges (Diptera: Thaumaleidae) of similar ecology. For example, *Thaumalea testacea* has survived in multiple Alpine refugia throughout the glacial maxima while *T. bezzi* has dispersed into Central Europe from the East Mediterranean area after the LGP. Moreover, the postglacial expansion may have a major impact on the genetic diversity of the affected species (Vila, Vidal-Romaní & Björklund, 2005; Schmitt, 2007; Kotlík et al., 2008). At the beginning of the Holocene (about 11.5 – 7.5 ka), a thermal maximum was recorded, which probably enhanced the expansion of species from its glacial refugia (Dabkowski et al., 2019). This corresponds to the sudden expansion of *L. perrisi*. Moreover, at that time the local Carpathian glaciers disappeared completely from the higher altitudes of the Tatra Mt (Lindner et al., 2003) which led to opening of, until then, inaccessible migration routes. Early-Holocene general warming is thought to be a major driving force for population divergence in temperate species (Hewitt, 1999). On one hand, with long glacials and shorter interglacials, temperate species spent much longer time in refugia than cold-adapted

316 species (Stewart et al., 2010). On the other hand, differences in species richness across biota may
317 also be due to variation in diversification rates (Ricklefs, 2007; Stadler, 2011).
318 We assume that the different historical dynamics of the two closely related elmid species was
319 reflected in their haplotype diversity. It is likely that *E. aenea* has utilized springs as glacial
320 refugia at a significantly higher rate, corresponding to its higher haplotype diversity when
321 compared with *L. perrisi* that prefers streams and their populations are much more uniform. In
322 line with our results, populations of two cofamilial caddisfly species in south-eastern UK showed
323 contrasting genetic patterns. *Polycentropus flavomaculatus* showed much more pronounced
324 genetic structure in the south-east of England than *Plectrocnemia conspersa* in the same region
325 (Wilcock et al., 2007). In another study on caddisflies of the Central European highlands, *Drusus*
326 *discolor* contained three times more haplotypes, which means much higher genetic diversity,
327 than *Hydropsyche tenuis*. Such findings suggest that the isolation among *D. discolor* populations
328 in Central Europe is stronger and persists for a longer time than in *H. tenuis* (Lehrian, Pauls &
329 Hasse, 2009). In both cases, the different phylogeographic histories of the species together with
330 their distinct ecological traits could be related to the present distinct patterns of haplotype
331 diversity (Wilcock et al., 2007; Lehrian, Pauls & Haase, 2009).
332 It is important to note that the temperate-adapted taxa were confined to refugia during glaciations
333 while the cold-adapted taxa retreated to refugia during interglacials (Stewart et al., 2010). During
334 the last glaciation and possibly for even longer time, the spring populations were unable to
335 spread extensively and, likely, persisted at the foothills of mountains (Schmitt, 2007). As a
336 consequence, many of the geomorphological units of the European high mountain systems have
337 their own genetic lineages or, at least, private haplotypes. What is confirmed by exceptionally
338 high diversity and local endemism of cold-adapted gammarids present in W Carpathians from
339 *Gammarus balcanicus* (Mamos et al 2014; Mamos et al., 2016) and *Gammarus fossarum* species
340 complexes (Copilaș-Ciocianu et al., 2017).
341 Another convincing example is caddisfly species *Drusus discolor* in the Tatra Mts persisted in
342 numerous refugia over multiple glacial cycles, allowing many local endemic clades to form
343 (Pauls, Lumbsch & Haase, 2006). In case of *E. aenea* and *L. perrisi*, the three localities in the
344 Slovak Ore Mts (SOM: V038, V048, V088) and two localities in the Fatra-Tatra area (FTA:
345 V009, V070) are remarkable with their strong fixation index (F_{ST}) in relation to other localities
346 (Fig. 4). Potentially, these areas could represent glacial or interglacial refugia, although
347 additional samples are needed to verify this hypothesis. However, the role of the W Carpathians
348 as a glacial refugium (Jamřichová, Potůčková & Horsák, 2014; Mráz & Ronikier, 2016;
349 Jamřichová, Petr & Jiménez-Alfaro, 2017) for various species or genetic lineages is, according to
350 several studies, undoubted (Pinceel et al., 2005; Magri et al., 2006; Wielstra, Babik & Arntzen,
351 2015; Mamos et al., 2016; Copilaș-Ciocianu et al., 2017).
352 *E. aenea* had a twice higher value of the fixation index compared to *L. perrisi*. It correlated with
353 the results of AMOVA when the genetic differentiation among *E. aenea* populations within
354 geomorphological subunits was relatively high (34.99 %). This indicates that there are some well
355 pronounced differences in the genetic composition among most of the spring subpopulations of

E. aenea within each geographical unit. Similar results emerged from the study on the black fly *Prosimulium neomacropyga* in the US Southern Rockies ecoregion with alpine tundra streams, where the differences among streams within the region were 24.58 % (Finn et al., 2006). However, the genetic differences between populations from different geomorphological subunits of the W Carpathians were very low in both elmid species, reflecting their overall low genetic variation within this region. In both species, only a single haplotype was abundant and widespread along the whole W Carpathians, surrounded by several rare peripheral haplotypes in a star-shaped topology (Figs. 2, 3). Similarly, lack of genetic population structure was also found in the W Carpathian populations of the blackfly *Simulium degrangei* (Jedlička et al., 2012). The maintenance of intraspecific genetic diversity is generally very important for the adaptation potential and long-term survival of species (Spielman, Brook & Frankham, 2004; Frankham, 2005). However, prolonged persistence is possible even despite low levels of genetic diversity (Johnson et al., 2009). The relatively homogeneous population patterns of both studied riffle beetles may reflect their short history in the W Carpathians, which was most probably recolonized no earlier than in the late Pleistocene. The comparatively low genetic differentiation among populations of trickle midges (Diptera: Thaumaleidae) in Northern Europe was also explained by relatively recent, possibly post-glacial dispersal (Haubrock et al., 2017).

Conclusions

In conclusion, despite the similarly low haplotype diversity, and absence of the more pronounced geographical pattern within populations, the studied species show different population dynamics through time (eBSP). We assume that distinct patterns may be related to the fact that *E. aenea* occurred mainly in the springs, while *L. perrisi* was found mostly in streams. These findings support the attribution of the W Carpathian springs to natural laboratories with a suitable environment for biota even during the ice age (Thorup & Lindegaard, 1977; Round, 1981). However, further studies should include more samples from Southern and Eastern Europe in order to understand the holistic biogeographic pattern of the target species and the spring fauna in general. The study of the local biota together with the history of climate change would be essential to unravel both regional and local diversity patterns (Calatayud et al., 2019).
Last but not least, the DNA barcoding proved to be a very useful tool in monitoring genetic diversity of species as well as improving and accelerating the process of taxonomic identification. From the past, we know examples where the solely morphology-based determination led to a significant error (Deichmann et al., 2017); this study revealed a questionably identified sample in the GenBank database. Such pitfalls, in turn, can bias markedly the results of the research. Therefore the importance of complementing traditional methods of characterizing biodiversity by approaches based on DNA sequencing should be emphasized. In addition, DNA barcoding is a key tool for assessing the health of animal populations in association with the ongoing biodiversity loss as well as with ecosystem degradation. However, the successful application of the DNA determination methods requires high quality reference data (Weigand et al., 2019). Therefore, it is necessary to publish sequences in the global

databases with the responsibility of assigning the correct taxonomic affiliation (Leray et al., 2019). This study added new and useful information about under-studied riffle beetle fauna of one of the world's biodiversity hotspots. The biota of the Carpathians is, with no doubt, invaluable and a high share of it belongs to freshwater fauna, but we can only preserve and protect it if we know it well.

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Table 1(on next page)

The list of all sampling sites and their geomorphological affiliation.

Mountain System[1]	Geomorphological Unit	Geomorphological Subunit	Code	Locality	Habitat[2]	GPS Coordinates	Altitude	Species[3]
IWC	**FTA** Fatra-Tatra Area (Fatránsko-tatranská oblasť)	**FTA1** Little Carpathians (Malé Karpaty)	V006	Stužková	SP	48.595833, 17.453567	250 m	EA
			V009	Chrenkech jarok	SP	48.654283, 17.619650	328 m	EA
			V070	Orešanská	SP	48.451800, 17.346483	324 m	EA, LP
			KCH3	Kuchyňa	ST	48.403491, 17.154763	241 m	EA
			LIB1	Libuša 1	ST	48.500500, 17.324700	374 m	LP
			LIM1	Limbašský 1	ST	48.294700, 17.175000	342 m	LP
			LOZ4	Lozorno 4	ST	48.324600, 17.090000	265 m	LP
			SPV1	Stupavský 1	ST	48.252500, 17.112217	292 m	EA, LP
		FTA2 High Tatras (Vysoké Tatry)	BEL3	Belá	ST	49.113900, 19.834700	809 m	LP
		FTA3 Low Tatras (Nízke Tatry)	V020	Bukovinka I	SP	49.003350, 19.282567	653 m	EA
			V022	Bukovinka III	SP	49.003300, 19.285083	631 m	EA
			BOC2	Boca 2	ST	48.996700, 19.763200	697 m	LP
			BYS4	Nižné Bystré	ST	48.944000, 19.632800	1638 m	LP
			HOD1	Hodruša 1	ST	48.960300, 19.826000	877 m	LP
			SVA1	Svarinka 1	ST	48.959900, 19.891900	983 m	LP
		FTA4 Western Tatras (Západné Tatry)	V027	Prosiek 2	SP	49.157917, 19.497483	642 m	EA
			V028	Prosiek 3	SP	49.157383, 19.498019	639 m	EA
			V086	Liptovská Anna	SP	49.159783, 19.462033	845 m	EA, LP
		FTA5 Great Fatra (Veľká Fatra)	V033	Jazierce	SP	49.018200, 19.281900	589 m	EA
		FTA6 Belianske Tatras (Belianske Tatry)	V036	Dolina 7 prameňov	SP	49.222800, 20.277600	1208 m	LP
		FTA7 Strážov Mts (Strážovské vrchy)	NIT1	Nitra 1	ST	48.657484, 18.637691	671 m	EA, LP
			SVI1	Svinianka 1	ST	49.149437, 18.650388	456 m	EA
			TUR1	Turiec 1	ST	48.964780, 18.727228	575 m	EA, LP
	SCM Slovak Central Mts (Slovenské stredohorie)	**SCM1** Podpoľanie	V087	Oravická	SP	48.700667, 19.272938	394 m	EA
		SCM2 Poľana Mts (Poľana)	KAM1	Kamenistý	ST	48.662400, 19.628500	884 m	LP
	SOM Slovak Ore Mts (Slovenské rudohorie)	**SOM1** Slovak Karst (Slovenský kras)	V037	Prameň sv. Jána	SP	48.653850, 20.974667	264 m	EA
			V038	Drieňovská	SP	48.607583, 20.964650	187 m	EA
			V042	Fej	SP	48.609367, 20.749017	222 m	EA
			V043	Tapolča I	SP	48.583600, 20.686883	198 m	EA
			V044	Tapolča II	SP	48.584050, 20.688838	204 m	EA
			V045	Eveteš	SP	48.598633, 20.643438	255 m	EA
			V046	Čierna	SP	48.562683, 20.465317	248 m	EA
			V047	Biela	SP	48.567583, 20.468050	237 m	EA
			V048	Kečovská	SP	48.500100, 20.485817	331 m	EA
			V049	Krásnohorská	SP	48.617600, 20.587233	336 m	EA
			V050	Brzotínska	SP	48.608783, 20.470933	247 m	EA
			V051	Vidová	SP	48.564317, 20.440167	238 m	EA
			V052	pod Vápenkou	SP	48.554933, 20.419170	212 m	EA
			V073	Studený	SP	48.571583, 20.400850	224 m	EA
			V075	Hučiaca B	SP	48.625167, 20.389900	269 m	EA
			V088	Drieňov kúpele	SP	48.624500, 20.952000	257 m	EA, LP
		SOM2 Muráň Plateau (Muránska planina)	V057	Brusík	SP	48.831400, 20.010100	574 m	LP
			V058	pod Javorníčkovou	SP	48.724100, 20.013800	413 m	LP
			V060	Havraník lúka	SP	48.813400, 20.071400	768 m	LP
			V061	Havraník les	SP	48.813300, 20.071700	766 m	LP
			V062	Jelšavská teplica	SP	48.605017, 20.295092	255 m	EA
			V065	Tisovec	SP	48.692317, 19.967417	576 m	EA
			V066	Rejkovský	SP	48.668283, 19.925367	400 m	EA
			V067	Teplice - Furmanec	SP	48.688833, 19.898817	476 m	EA
			V074	Kunova teplica	SP	48.607333, 20.390933	248 m	EA
			HAV1	Havraník 1	ST	48.824000, 20.071600	761 m	LP
			HDZ1	Hrdzavý 1	ST	48.768200, 19.986800	868 m	LP
		SOM3 Volovec Mts (Volovské vrchy)	SMO1	Smolník 1	ST	48.709000, 20.700700	635 m	LP
OWC	**SMC** Slovak-Moravian Carpathians (Slovensko-moravské Karpaty)	**SMC1** Maple Mts (Javorníky)	VAH1	Váh 1	ST	49.325346, 18.511067	582 m	EA, LP
			VYD1	Vydrňanka	ST	49.217800, 18.252780	523 m	EA, LP
		SMC2 White Carpathians (Biele Karpaty)	SEL1	Selecký 1	ST	48.777800, 17.998700	374 m	LP
	WB Western Beskids (Západné Beskydy)	**WB1** Moravian-Silesian Beskids (Moravsko-sliezske Beskydy)	CZ05	Kněhyně	ST	49.462546, 18.278190	570 m	EA, LP
			KYS1	Kysuca 1	ST	49.431857, 18.626540	570 m	EA, LP
			CZ01	Lomná	ST	49.547710, 18.650423	538 m	EA, LP
			CZ02	Příslopský	ST	49.624213, 18.575444	497 m	LP
			CZ03	Satina	ST	49.565317, 18.422775	772 m	EA, LP
			CZ04	Černa Ostravice	ST	49.456600, 18.470900	816 m	LP
			CZ06	Malá Bystřička	ST	49.394775, 18.053709	456 m	EA, LP
			CZ07	Bystřička	ST	49.371673, 17.750556	563 m	EA, LP
		WB2 Orava Magura (Oravská Magura)	BRE1	Brezovica 1	ST	49.343800, 19.662100	687 m	LP
		WB3 Silesian Beskids (Sliezske Beskydy)	PL04	Żylica	ST	49.693800, 18.984000	609 m	LP
			PL05	Labajów	ST	49.622821, 18.869191	523 m	EA, LP
	CB Central Beskids (Stredné Beskydy)	**CB1** Kysucké Beskydy	OSC1	Oščadnica	ST	49.421200, 18.910810	822 m	EA, LP
IEC	**VM** Vihorlat Mts (Vihorlatské vrchy)	**VM1** Vihorlat Mts (Vihorlatské vrchy)	BAR1	Barnov 1	ST	48.938400, 22.160300	434 m	LP
			HRA1	Hrabový 1	ST	48.878000, 22.297500	412 m	LP
			KRV1	Krivec 1	ST	48.907300, 22.203700	569 m	LP

			ROV1	Rovný 1	ST	48.887200, 22.324200	311 m	LP
OEC	**PM** Poloniny Mts (Poloniny)	**PM1** Poloniny Mts (Poloniny)	ZBJ2	Zbojský	ST	49.050500, 22.513700	773 m	LP

Mountain System[1]: IWC Inner Western Carpathians, **OWC** Outer Western Carpathians, **IEC** Inner Eastern Carpathians, **OEC** Outer Eastern Carpathians**; Habitat[2]: SP** spring, **ST** stream**; Species[3]: EA** *Elmis aenea***, LP** *Limnius perrisi*

Table 2(on next page)

Analysis of molecular variation (AMOVA) calculated from COI mtDNA sequences for *Elmis aenea* and *Limnius perrisi* Western Carpathian populations.

Groups - populations of *E. aenea* and *L. perrisi* of different geomorphological subunits. The subpopulation is defined as specimens from one locality within the geomorphological subunit in Table 1.

E. aenea

Source of variation	df[1]	SS[2]	Variance components	% of variation	F value	P - value
Among subunits	10	5.704	0.00206	1.02	F_{CT} = 0.010	> 0.352
Among subpopulations within subunits	36	19.768	0.07080	34.99	F_{SC} = 0.353	> 0.000
Within subpopulations	230	29.784	0.12950	64	F_{ST} = 0.360	< 0.000

L. perrisi

Source of variation	df[1]	SS[2]	Variance components	% of variation	F value	P - value
Among subunits	12	0.657	-0.00148	-3.16	F_{CT} = 0.010	> 0.335
Among subpopulations within subunits	29	2.145	0.00602	12.86	F_{SC} = 0.125	> 0.097
Within subpopulations	192	8.117	0.04227	90.3	F_{ST} = 0.097	< 0.074

df[1] Degree of freedom, **SS[2]** Sum of squares

Table 3(on next page)

Values of neutrality tests (Fu´s *Fs*, Tajima´s *D*, Fu and Li´s *D* test) for *Elmis aenea* and *Limnius perrisi* mtDNA COI sequences.

Species	Fu´s *Fs* test (p-value)	Tajima´s *D* test (p-value)	Fu and Li´s *D* test (p-value)
Elmis aenea	-17.331 (0.000)	-2.047 (0.001)	-3.323 (< 0.02)
Limnius perrisi	-14.064 (0.000)	-2.004 (0.002)	-3.320 (< 0.02)

1

Figure 1

Map of the studied area and 73 sampling sites (36 springs and 37 streams) divided into eight geomorphological units represented by different colours (white line represents state borders).

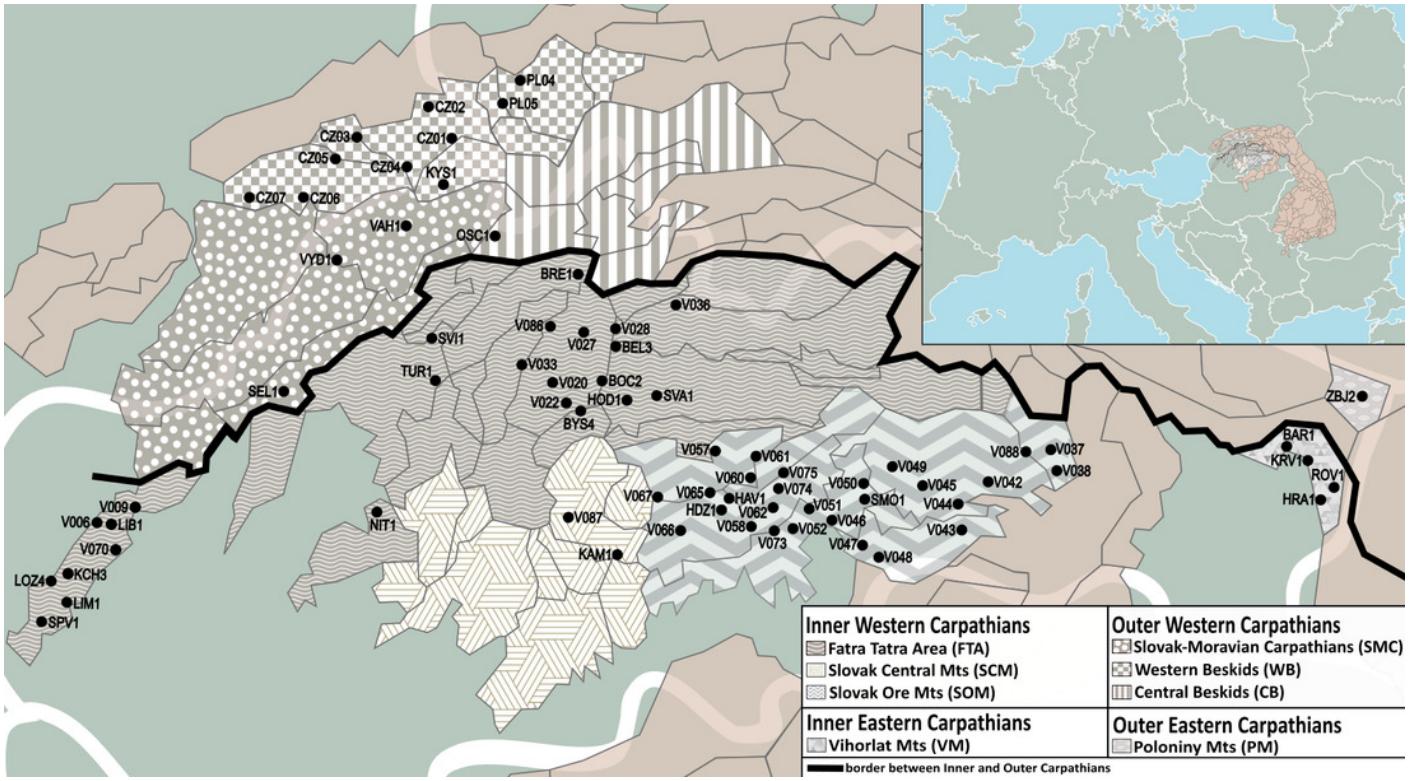

Figure 2

Elmis aenea

(A) map with mtDNA haplotypes distribution in the Western Carpathians; (B) median-joining network (circle size is proportional to sample size for each haplotype, colors indicate geomorphological units, mutational steps are indicated with bars, black dots represent undetected haplotypes); (C) time-calibrated phylogenetic tree of *E. aenea* haplotypes and outgroup species *E. perezi*, *E. rioloides*. Bayesian maximum clade credibility tree obtained from the BEAST analysis based on the COI marker. 95% HPD of estimated divergence ages are illustrated as violet interval bars at selected calibrated nodes, posterior probabilities (PP) >0.5 are given above each branch.

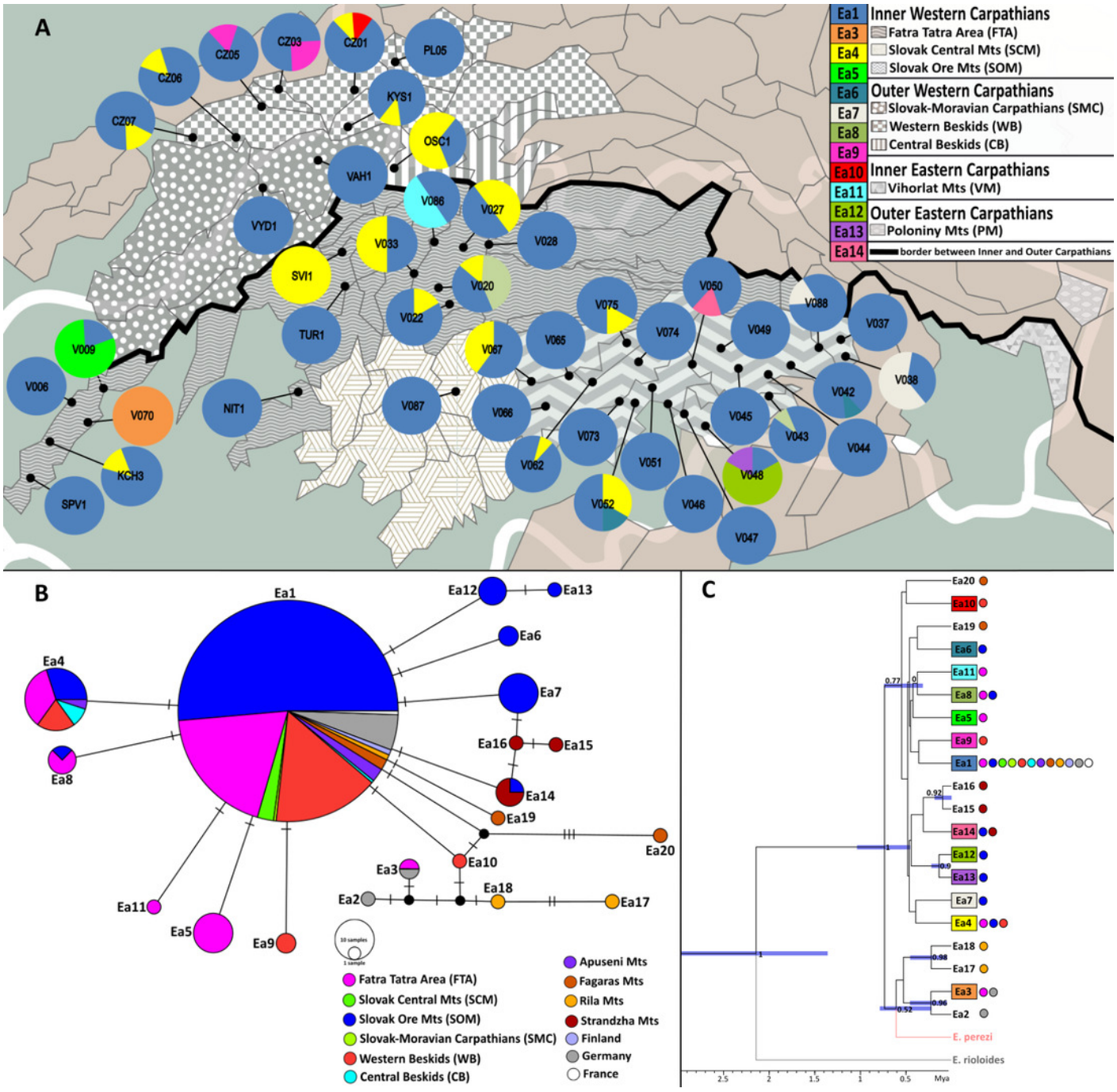
A
Ea1 Inner Western Carpathians
Ea3 Fatra Tatra Area (FTA)
Ea4 Slovak Central Mts (SCM)
Ea5 Slovak Ore Mts (SOM)
Ea6 Outer Western Carpathians
Ea7 Slovak-Moravian Carpathians (SMC)
Ea8 Western Beskids (WB)
Ea9 Central Beskids (CB)
Ea10 Inner Eastern Carpathians
Ea11 Vihorlat Mts (VM)
Ea12 Outer Eastern Carpathians
Ea13 Poloniny Mts (PM)
Ea14 border between Inner and Outer Carpathians
CZ03
CZ01
CZ05
PL05
CZ06
KYS1
CZ07
OSC1
VAH1
V086
V027
VYD1
V033
V028
SVI1
V020
V022
V050
V075
V088
V037
TUR1
V074
V049
V009
V065
V087
V038
V006
V042
NIT1
V087
V066
V045
V070
V073
V043
V044
V062
V051
KCH3
V048
V052
V046
SPV1
V047
B
Ea1
Ea12
Ea13
Ea6
Ea4
Ea7
Ea16
Ea15
Ea8
Ea14
Ea19
Ea20
Ea3
Ea10
Ea18
Ea2
Ea17
Ea11
Ea5
Ea9
10 samples
1 sample
Fatra Tatra Area (FTA)
Slovak Central Mts (SCM)
Slovak Ore Mts (SOM)
Slovak-Moravian Carpathians (SMC)
Western Beskids (WB)
Central Beskids (CB)
Apuseni Mts
Fagaras Mts
Rila Mts
Strandzha Mts
Finland
Germany
France
C
Ea20
Ea10
Ea19
Ea6
Ea11
Ea8
Ea5
Ea9
Ea1
Ea16
Ea15
Ea14
Ea12
Ea13
Ea7
Ea4
Ea18
Ea17
Ea3
Ea2
E. perezi
E. rioloides
0.77
0.92
0.9
0.98
0.96
0.52
2.5
2
1.5
1
0.5
Mya

Figure 3

Limnius perrisi

(A) map with mtDNA haplotype distribution in the Western Carpathians, (B) median-joining network (circle size reflects haplotype abundance - number of individuals that had the haplotype, colors indicate geomorphological units, mutational steps are indicated with bars) and (C) time-calibrated phylogenetic tree of *L. perrisi* haplotypes. Bayesian maximum clade credibility tree obtained from the BEAST analysis based on the COI marker. 95% HPD of estimated divergence ages are illustrated as violet bars at selected calibrated nodes. Posterior probabilities (PP) >0.5 are given above each branch.

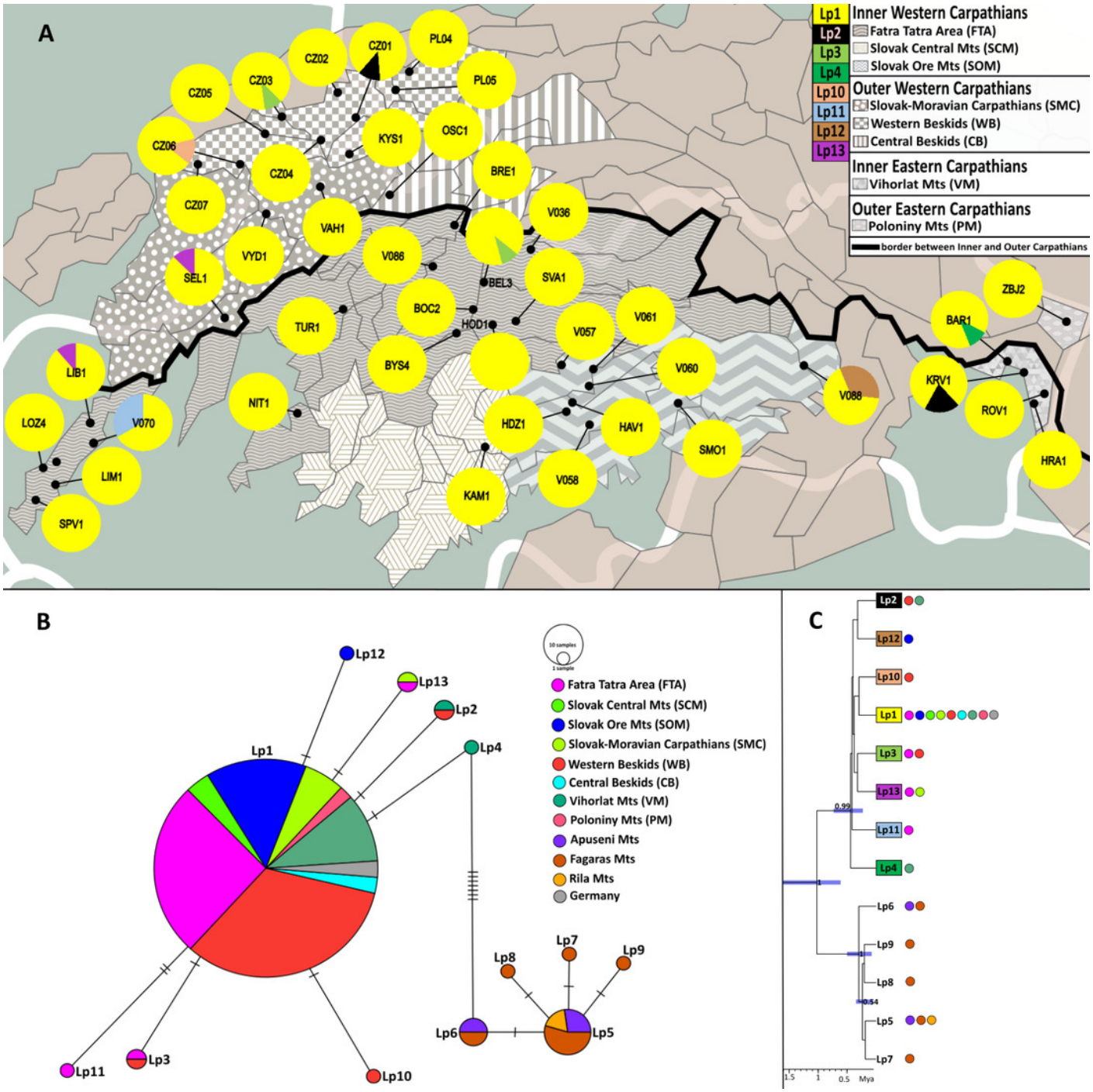
A
Lp1 Inner Western Carpathians
Lp2 Fatra Tatra Area (FTA)
Lp3 Slovak Central Mts (SCM)
Lp4 Slovak Ore Mts (SOM)
Lp10 Outer Western Carpathians
Lp11 Slovak-Moravian Carpathians (SMC)
Lp12 Western Beskids (WB)
Lp13 Central Beskids (CB)
Inner Eastern Carpathians
Vihorlat Mts (VM)
Outer Eastern Carpathians
Poloniny Mts (PM)
border between Inner and Outer Carpathians
CZ01
CZ02
CZ03
CZ04
CZ05
CZ06
CZ07
PL04
PL05
KYS1
OSC1
BRE1
V036
VAH1
V086
VYD1
SEL1
BEL3
SVA1
BOC2
HOD1
TUR1
V061
V057
V060
BYS4
LIB1
NIT1
V088
KRV1
ROV1
ZBJ2
BAR1
HRA1
LOZ4
V070
HDZ1
HAV1
SMO1
LIM1
KAM1
V058
SPV1
B
Lp12
Lp13
Lp2
Lp4
Lp1
Lp11
Lp3
Lp10
Lp6
Lp8
Lp7
Lp9
Lp5
10 samples
1 sample
Fatra Tatra Area (FTA)
Slovak Central Mts (SCM)
Slovak Ore Mts (SOM)
Slovak-Moravian Carpathians (SMC)
Western Beskids (WB)
Central Beskids (CB)
Vihorlat Mts (VM)
Poloniny Mts (PM)
Apuseni Mts
Fagaras Mts
Rila Mts
Germany
C
Lp2
Lp12
Lp10
Lp1
Lp3
Lp13
Lp11
Lp4
Lp6
Lp9
Lp8
Lp5
Lp7
0.99
1
0.54
1.5
1
0.5
Mya

Figure 4

Heat map of pairwise F_{ST} values among the localities of *Elmis aenea* and *Limnius perrisi*.

Darker shades of blue indicate higher values of F_{ST}. The maximum F_{ST} values were 0.8 for *E. aenea* and 0.4 for *L. perrisi.*

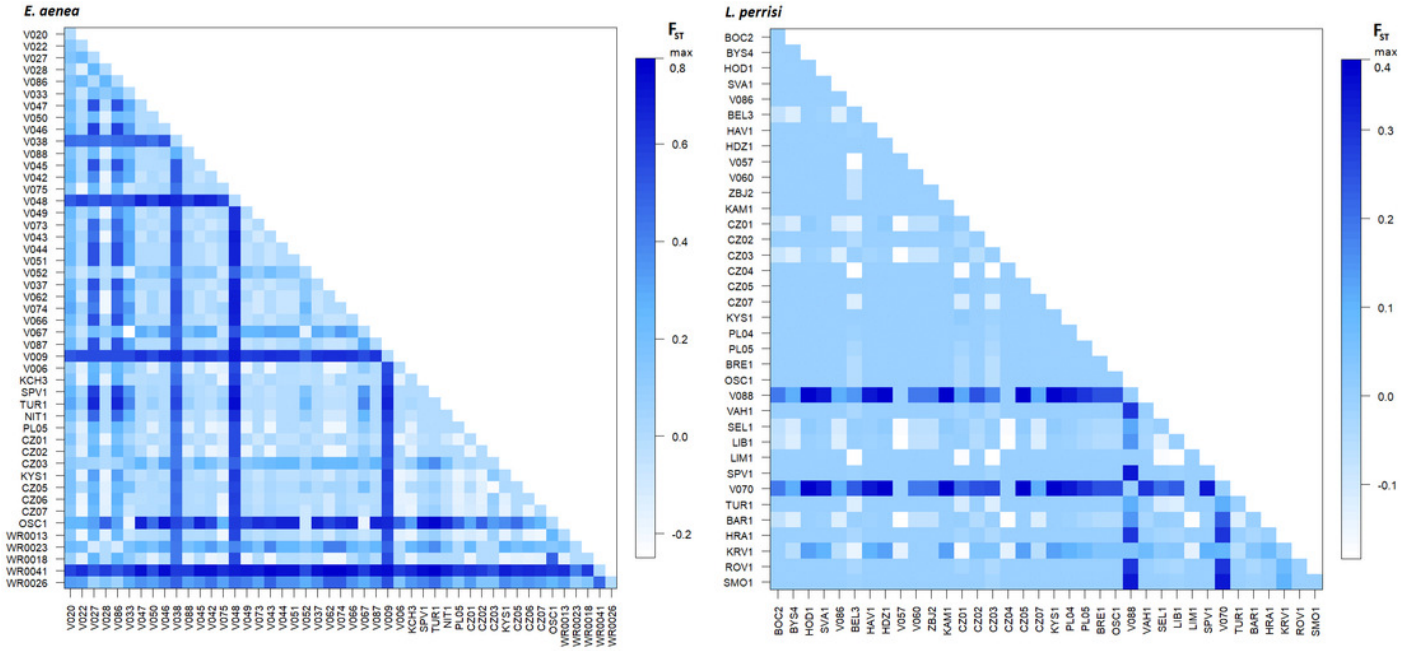

Figure 5

Mismatch distribution analysis (MDA) for *Elmis aenea* and *Limnius perrisi* from the Western Carpathian springs and streams.

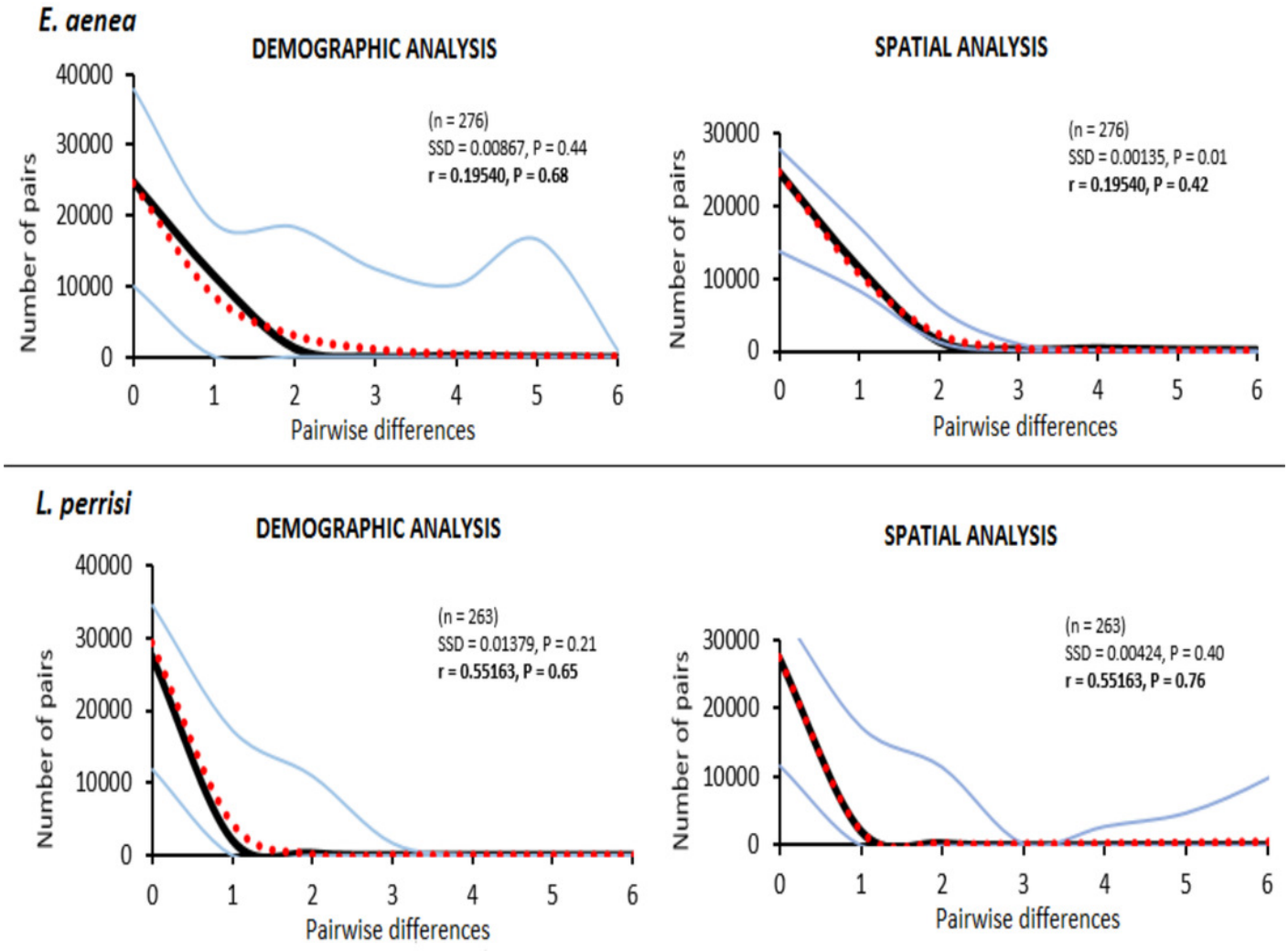

Figure 6

Bayesian skyline plot for *Elmis aenea* and *Limnius perrisi* from the Western Carpathian springs and streams, reconstructing the population size history using an evolutionary rate 0.0115 substitution/site/Myr (Brower 1994).