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The impact of Miocene orogeny for the diversification of Caucasian *Epeorus* (*Caucasiron*) mayflies (Ephemeroptera: Heptageniidae)

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ABSTRACT

A common hypothesis for the high biodiversity of mountains is the diversification driven by orogeny creating conditions for rapid in situ speciation of resident lineages. The Caucasus is a young mountain system considered as a biodiversity hotspot; however, the origin and evolution of its diversity remain poorly understood. This study focuses on mayflies of the subgenus *Caucasiron*, one of the most diversified stenotopic mayflies inhabiting various types of streams throughout the Caucasus. Using the time-calibrated phylogeny based on two mitochondrial (*COI*, 16S) and three nuclear (EF-1α, wg, 28S) gene fragments, we tested the role of Caucasian orogeny in biogeography, diversification patterns, and altitudinal diversification of *Caucasiron* mayflies. We found that orogeny promoted the lineage diversification of *Caucasiron* in the Miocene. The highest diversification rate corresponding with the uplift of mountains was followed by a significant slowdown towards the present suggesting minor influence of Pleistocene climatic oscillations on the speciation. The *Caucasiron* lineages cluster into three principal clades originating in the Upper Miocene. We found a strong support that one of the three clades diversified via allopatric speciation in the Greater Caucasus isolated in the Parathetys Sea. The other two clades originating most likely outside the Greater Caucasus diversified towards high and low altitude, respectively, indicating possible role of climatic factors and/or passive uplift on their differentiation. Current high *Caucasiron* diversity in the Greater Caucasus is a result of in situ speciation and later immigration from adjacent mountain ranges after the Parathetys Sea retreat. Our phylogeny supported the monophyly of Rhithrogeninae, *Epeorus* s.l., *Caucasiron*, and *Iron*. *Epeorus* subgenus *Ironopsis* was found paraphyletic, with its European representatives more closely related to *Epeorus* s.str. than to *Iron*. Therefore, we re-arranged taxa treated within *Ironopsis* to comply with the phylogeny recovered herein.

1. Introduction

Uplift and formation of mountain systems are crucial geological phenomena driving evolutionary diversification of organisms (Hoorn et al., 2013; Xing and Ree, 2017; Heads, 2019). Orogeny creates conditions favouring allopatric speciation and ecological adaptation of resident lineages via an increase of landscape heterogeneity, providing novel habitats free of strong competitors, and affecting dispersal of organisms by creation of either dispersal corridors or barriers (Dos Santos et al., 2018; Esquerré et al., 2019; Favre et al., 2015; Hoorn et al., 2018). Recent studies stress the importance of passive uplift of resident populations by raising of mountains and their vicariance resulting in rapid diversification of montane biota (Heads, 2019). Hence, it is not surprising that mountains harbour a large portion of biological diversity on Earth, being prominent global biodiversity hotspots (Myers et al., 2000; Jenkins et al., 2013; Hoorn et al., 2018). The origin and evolution of mountain biodiversity is recently intensively studied in two mountaineous biodiversity hotspots, the Andes (e.g., Chaves et al., 2011; Esquerré et al., 2019; Hughes and Eastwood, 2006; Lagomarsino et al., 2016) and the Qinghai-Tibetan Plateau (e.g., Wen et al., 2014;

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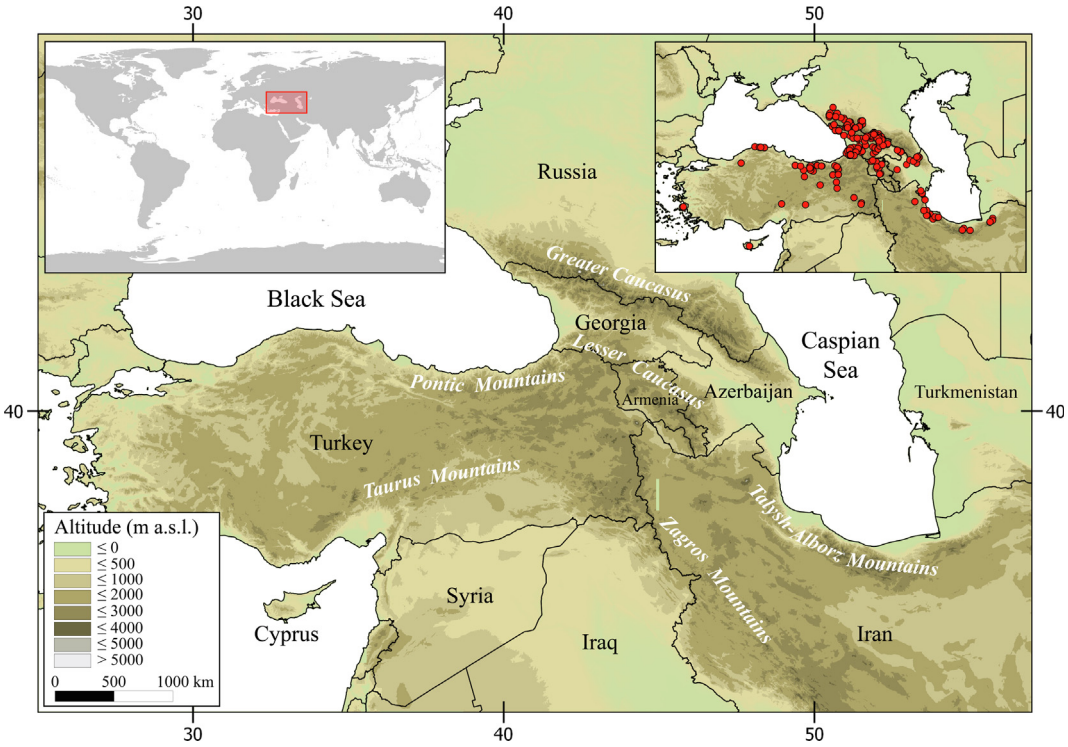

Fig. 1. Topographic map of the Caucasus and adjacent mountain ranges with the position of the study area (upper left part) and distribution of our sampling sites (upper right part).

Favre et al., 2015; Zhang et al., 2016; Xing and Ree, 2017). Another remarkable mountain hotspot with higher biodiversity than expected based on its area is the Caucasus (Myers et al., 2000; Williams, 2004; Kier et al., 2005). The origin of Caucasian biota and drivers of its evolution remain insufficiently understood despite large geographic extent and biogeographic significance of this mountain system (Tarkhnishvili, 2014).

The Caucasus, historically interpreted as the isthmus between the Black and Caspian Seas, covers a total area of 580,000 km^2, stretching over the south-western part of the Russian Federation, north-eastern Turkey, Georgia, Armenia, Azerbaijan, and part of north-western Iran (Williams, 2004). It consists of several mountain ranges with different connectivity, Greater and Lesser Caucasus, Pontic Mts., and Talysh-Alborz Mts. (Fig. 1). They are isolated by the large East European Plains in the north, but connected with Taurus and Zagros Mts. in the south (Fig. 1). Due to its location, the Caucasus represents a biogeographic crossroad between Europe, especially the Balkans and the Mediterranean, and central Asia (Williams, 2004; Tarkhnishvili, 2014). The Caucasus is relatively young, its current geology formed over the last 30 million years, with uplift acceleration in the last 10 million years (Mitchell and Westaway, 1999; Popov et al., 2004). Importantly, mountain building and interconnection of mountain ranges within the Caucasus developed progressively during gradual Paratethys Sea retreat and formation of terrestrial terrains (Popov et al., 2004; Tarkhnishvili, 2014). The Greater Caucasus, dominant and highest range in the region, formed a mountainous island in the Paratethys Sea during the Miocene (Popov et al., 2004). It was connected with the mainland (current Lesser Caucasus, Pontic and Talysh-Alborz Mts.) in the Upper Miocene. This was followed by the complete separation of the Black and Caspian Seas about 2.4 million years ago (Ma) (Popov et al., 2004). Strong orogenic activity in the Miocene-Pliocene boundary resulted in the formation of heterogeneous environments, with different climatic conditions between lowlands and highlands (Popov et al., 2004; Tarkhnishvili, 2014). It can be expected that uplift of the Caucasus and long isolation of the Greater Caucasus strongly influenced the evolution of Caucasian biota. However, this topic has not been addressed up to now. Available phylogenetic studies were focused on the phylogeography of different animal taxa, concerning particularly past climatic and landcover changes, and Pleistocene glacial refugia (Tarkhnishvili et al., 2000, 2001; Babik et al., 2005; Tarkhnishvili, 2014; Neiber and Hausdorf 2015; Levin et al., 2019).

Mayflies (Ephemeroptera), which are our model group to study species diversification in the Caucasus, are amphibiotic insects of ancient origin (about 300 Ma) with highly reduced terrestrial adult stage (Bauernfeind and Soldán, 2012). They are generally excellent models for research on diversification, due to their strong link to aquatic habitats with marked environmental and spatial gradients, and limited ability for overland dispersal in the adult stage (Bauernfeind and Soldán, 2012; Dijkstra et al., 2014). Caucasian mayflies are very diverse and include many endemic species (e.g., Türkmen and Kazancı, 2015; Bojková et al., 2018, Gabelashvili et al., 2018; Hrivniak et al., 2018). We chose mayflies of the genus *Epeorus*, subgenus *Caucasiron* Kluge, 1997 (Heptageniidae), as one of the most diverse mayfly groups in the Caucasus containing species with various distribution patterns that might reflect their different evolutionary history. *Caucasiron* mayflies include ten species and two subspecies, with distributional area ranging from species endemic to the Greater Caucasus to species widely distributed within the Caucasus and even in the adjacent mountains in Iran, Turkey and eastern Mediterranean islands (Hrivniak et al., 2019). The remaining five species are distributed in central Asia (Chen et al., 2010; Hrivniak et al., 2017). They are relatively stenotopic, restricted to lotic habitats of different altitudes. Their larvae are cold adapted and prefer streams with coarse, stony bed substrate, rapid current and turbulent flow. Kluge (1997) defined the subgenus *Caucasiron* based on a unique larval apomorphy, a projection on the costal margin of gill plates. However, the monophyly of *Caucasiron* as well as other subgenera of *Epeorus* s.l. has not yet been tested. To avoid possible paraphyletic classification, Webb & McCafferty (2008) synonymized all subgenera of the genus *Epeorus*.

This study aims to test the monophyly of three morphologically

similar subgenera of *Epeorus* s.l., namely *Caucasiron*, *Iron* and *Ironopsis* and clarify their systematic position. We then aim to estimate phylogenetic relationships and divergence times of *Caucasiron* based on the extensive dataset covering the entire Caucasus from northern Turkey to Iran and relevant adjacent mountains in Samos, Cyprus, and Turkey. We explore the pattern of speciation and extinction rate over time and reconstruct ancestral distribution and altitude of individual *Caucasiron* lineages. We focus on the comparison of patterns in evolutionary divergence in *Caucasiron* with the timing of important geological events in the Caucasus based on Popov et al. (2004). We hypothesize that orogeny forming the Caucasus mountain system promoted the diversification of *Caucasiron* species and Miocene isolation of the Greater Caucasus caused differentiation of mayfly lineages endemic to this mountain range. We intend to explore possible effects of Pleistocene climatic oscillations on the speciation of *Caucasiron*.

2. Material and methods

2.1. Sampling, identification, DNA isolation, and sequencing

Larvae of *Caucasiron* were collected from 523 streams in the Caucasus region and adjacent areas (Turkey, Georgia, Russia, Armenia, Azerbaijan, and Iran) and two Mediterranean islands (Samos and Cyprus) between 2008 and 2018 (Fig. 1). Sampling sites covered the geographical distributions of all known *Caucasiron* species occurring in the Caucasus. Larvae were collected by a hand net or a metal strainer. Subimagines and imagines were collected by sweeping net from riparian vegetation. All specimens were fixed in 96% ethanol and stored in the laboratory at −20 °C. Original descriptions of the species were used for species identification based on morphology (Sinitshenkova, 1976; Braasch, 1978, 1979, 1980; Braasch and Zimmermann, 1979; Braasch and Soldán, 1979; Hrivniak et al., 2017, 2019). Due to insufficient details given in some of these descriptions, some specimens were compared with the type material, holotypes and/or paratypes (namely, subspecies *E.* (*C.*) *caucasicus iranicus* and *E.* (*C.*) *znojkoi insularis*; and species *E.* (*C.*) *magnus*, *E.* (*C.*) *alpestris*, *E.* (*C.*) *soldani*, *E.* (*C.*) *longimaculatus*, and *E.* (*C.*) *sinitshenkovae*).

Representatives of all described species and morphologically distinct lineages not attributable to any described species were selected for DNA isolation. Details on DNA extraction were described in Hrivniak et al. (2017). Due to the incomplete taxonomic knowledge of *Caucasiron*, molecular delimitation of individual species was tested prior to the selection of specimens for phylogenetic analyses. For this initial species delimitation, mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) and single threshold General Mixed Yule Coalescent Model (GMYC) were used (Pons et al., 2006; Fujisawa and Barraclough, 2013). This method was found to be a useful tool for species delimitation within *Caucasiron* (Hrivniak et al., 2019) and mayflies in general (e.g., Monaghan et al., 2009; Vuataz et al., 2011; Rutschmann et al., 2017) when the single-locus data are available. The GMYC analysis was performed using the SPLITS package (Ezard et al., 2009) for R. Ultrametric *COI* gene input tree was reconstructed using BEAST 1.8.4 on CIPRES Science Gateway 3.3 (Miller et al., 2010), detailed settings of the analysis are described in Hrivniak et al. (2019). Molecular species delimitation was applied to 77 specimens (1–9 specimens per species or lineage depending on geographic distribution and material availability).

In all delimited GMYC-species, four additional loci were sequenced: mitochondrial large ribosomal subunit 16S rRNA (16S) and nuclear elongation factor 1-alpha (EF-1α), wingless (wg) and large ribosomal subunit 28S rRNA (28S). 16S was amplified according to Ogden and Whiting (2005), EF-1α according to Takemon et al. (2006), wg according to Vuataz et al. (2013), and 28S according to Pons et al. (2004). Annealing temperature for amplification of 16S and 28S was adopted from Yanai et al. (2017).

Sequencing using the forward primer was performed by the SEQme company (Dobříš, Czech Republic) with the Sanger method. Reverse primer sequencing was employed in a few samples in which the forward primer failed to produce high quality chromatograms. Unresolved double peaks in chromatograms were coded according to the IUPAC code. Additional sequences were obtained from GenBank. All sequences with GenBank accession numbers and references are listed in Table A1. Sequence data were edited and aligned in Jalview version 2.10.5 (Waterhouse et al., 2009) using Mafft (Kazutaka and Standley, 2013) with default settings. Coding alignments were checked for indels and stop codons in Mega 7 (Kumar et al., 2016). Characteristics of the final alignments were obtained in Mega 7 and FaBox 1.5 (Villesen, 2007).

2.2. Selection of outgroup taxa

Outgroups from all subfamilies of Heptageniidae (sensu Webb and McCafferty, 2008) were selected based on their systematic position and availability of fossil records in purpose of time tree calibration. The following taxa were used as outgroups: *Rhithrogena alpestris* Eaton, 1885, *Cinygmula* sp. (Rhithrogeninae), *Ecdyonurus eurycephalus* Hrivniak & Godunko, 2018, *Afronurus* sp. (Ecdyonurinae), *Stenonema meririvulanum* Carle & Lewis, 1978, and *Stenonema modestum* (Banks, 1910) (Heptageniinae). To test the monophyly of *Caucasiron* and its phylogenetic position among other subgenera of *Epeorus* s.l. (sensu Kluge, 1997, 2015), several species were included: Nearctic *Epeorus* (*Ironopsis*) *grandis* (Traver, 1935) and *E.* (*Iron*) *longimanus* (Eaton, 1883), central Asian *E.* (*Ironopsis*) *rheophilus* (Brodsky, 1930) and *E.* (*Iron*) *montanus* (Brodsky, 1930), European *E.* (*Ironopsis*) *alpicola* (Eaton, 1871), *E.* (*Ironopsis*) *yougoslavicus* (Šámal, 1935) and *E.* (*Epeorus*) *assimilis* Eaton, 1885. The final dataset used in the analyses comprised 19 ingroup and 14 outgroup taxa.

2.3. Phylogenetic analyses

Phylogenetic analyses were performed using concatenated alignment approach. Alignments of individual gene fragments were concatenated using Sequence Matrix 1.7.8 (Vaidya et al., 2011). Analyses were conducted to three concatenated alignments: mitochondrial (mt; COI, 16S), nuclear (nu; 28S, EF-1α, wg), and all five gene fragments concatenated together (combined). Bayesian inference of phylogeny (BI) was carried out using MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003). The best substitution model for BI was selected using Partition Finder 2.1.1 (Lanfear et al., 2016) with models of evolution set for MrBayes and greedy algorithm (Lanfear et al., 2012). Codon positions in protein-coding gene fragments (*COI*, EF-1α, wg) were defined prior to model searching. The best substitution model was selected based on corrected Akaike information criterion (AICc). For BI we used two independent analyses with four MCMC chains. Both analyses were run for 10 million generations sampled every 1,000 generations. State frequencies, gamma shape, substitution rates, and the proportion of invariant sites were unlinked across partitions; ratepr parameter was set to variable. A 25% from each run was discarded as burn-in. Convergences and effective sample sizes (ESS > 200) of independent analyses were verified using Tracer 1.6 (Rambaut et al., 2018). Posterior probability above 0.9 was considered as a reliable node support.

Maximum likelihood tree reconstruction (ML) was conducted in IQ-Tree 1.6.8 (Nguyen et al., 2015) with 10,000 ultrafast bootstrap replicates (Hoang et al., 2018). The best substitution model for ML was selected using Model Finder (Kalyaanamoorthy et al., 2017) based on Bayesian information criterion (BIC). Codon positions in protein-coding gene fragments were defined prior to model searching. Bootstrap value above 70 was considered as a reliable node support.

2.4. Estimation of divergence times

We set priors for the divergence time estimations in *Caucasiron* based on the three fossil records (for details on the justification of fossils

phylogenetic placement see Appendix A1 in Supplementary material). The first fossil (No. 1 in Fig. 4c) used represents the undescribed (Staniczek and Godunko, in prep.) first Mesozoic record of the subfamily Ecdyonurinae in mid-Cretaceous Myanmar amber (dated at 98.79 ± 0.62 Ma; Shi et al., 2012). It was applied to an ancestral node of monophyletic subfamily Ecdyonurinae (Yanai et al., 2017), which comprised the genera *Ecdyonurus*, *Afronurus* and *Thalerosphyrus*. We used lognormal prior distribution, hard minimum age 98.17 Ma and 97.5% soft maximum age 99.4 Ma (mean = 0.62, standard deviation = 0.4, offset = 98.17). The second fossil (No. 2 in Fig. 4c) is a record of Heptageniinae from Miocene Mexican amber (ranging between 15 and 22.8 Ma; Huys et al., 2016; Solórzano-Kraemer, 2010) described as *Maccaffertium annae* Macadam & Ross, 2016. The fossil was used to constrain the age of the extant genus *Stenonema* and was placed at the ancestral node of *S. meririvulanum* and *S. modestum*. We used lognormal prior distribution, hard minimum age 15.0 and 97.5% soft maximum age 22.8 Ma (mean = 3.9, standard deviation = 0.39, offset = 15.0). As a third fossil (No. 3 in Fig. 4c), we used *Burshtynogena ferreci* Godunko & Sontag, 2004 embedded in Eocene Baltic amber (37.2–33.9 Ma; Aleksandrova and Zaporozhets, 2008a, 2008b). We used this fossil to calibrate the ancestral node of Rhithrogeninae with the lognormal prior distribution, hard minimum age 33.9 Ma and 97.5% soft maximum age 37.2 Ma (mean = 1.65, standard deviation = 0.39, offset = 33.9).

The time-calibrated phylogeny was generated in BEAST 2.4.8. (Bouckaert et al., 2014). The best substitution model for the analysis of combined dataset was selected by model averaging approach using bModelTest (Bouckaert and Drummond, 2017) with default settings. We ran two independent analyses under relaxed log normal molecular clock and Yule Model tree prior. Site and clock models were unlinked across partitions. Analyses were run for 500 million generations, sampled every 50,000 generations, resulting in 20,000 trees. Convergences of both runs and ESS were verified as in BI, first 10% were discarded as burn-in. Log and tree files were combined using LogCombiner 2.4.8. (Bouckaert et al., 2014). Maximum credibility (MCC) tree was constructed in TreeAnnotator 2.4.8. (Bouckaert et al., 2014). Codon positions in protein-coding gene fragments were initially defined, however we repeatedly failed to reach sufficiently high ESS of several parameters. Therefore, partition by gene fragment was employed, which resulted in adequate ESS values and convergences.

Aside from the fossil calibration, we conducted divergence time estimation using mitochondrial mutation rate of 0.0115 substitution/site/Ma, equal to 2.3% divergence/Ma (Brower, 1994). The time-calibrated phylogeny was generated in BEAST 2.4.8. We used mitochondrial concatenated alignment with relaxed lognormal molecular clock. The clock models and trees were linked and site models were unlinked across partitions. Two independent analyses were run for 300 million generations, sampled every 30,000 generations, resulting in 20,000 trees. The best substitution model search and the analysis of outputs were the same as in fossil calibrated phylogeny. Chronostratigraphic data for the interpretation of the results were based on Cohen et al. (2013).

2.5. Diversification rate analysis

To determine the diversification rates and rate shifts across phylogeny, we used BAMM 2.5.0 (Bayesian Analysis of Macro-evolutionary Mixtures; Rabosky, 2014). Priors for BAMM were set using SetBAMMpriors function implemented in R package BAMMtools (Rabosky et al., 2014). A prior "Expected number of shifts" was left to default value (1.0). Two independent analyses were run for 50 million generations, sampled every 5,000 generations. Convergences and ESS of both analyses were checked using R package Coda (Plummer et al., 2006), first 10% was then discarded as a burn-in. Output of BAMM was analysed with R package BAMMtools. The fit of the potential diversification rate shifts across lineages, relative to the null model with zero rate shifts among lineages, was tested by Bayes factors implemented in BAMMtools. Whether the diversification followed the constant rate was also tested using gamma (γ) statistic (Pybus and Harvey, 2000) with R package Phytools (Revell, 2012). Significantly negative γ indicates initially high speciation rate, significantly positive γ indicates an increased speciation. As an input tree for both analyses, BAMM and γ statistic, we used MCC tree with outgroups pruned using R package ape 5.0 (Paradis and Schliep, 2019). To account for phylogenetic uncertainty we calculated γ statistics on 1,000 randomly sampled trees from the posterior distribution of BEAST analysis and estimated mean γ and number of significant γ values. Both, BAMM analysis and γ statistics were assumed to be completely sampled.

2.6. Ancestral range estimation

The biogeographic history was analysed using RASP 4.0 (Yu et al., 2015). As input trees for analyses, 1,000 randomly sampled trees from the posterior distribution of BEAST analysis and MCC tree, as for the diversification rate analyses (see above), were used. The R script "BioGeoBEARS" (Matzke, 2013) was employed to select the best model for inferring ancestral ranges of internal nodes within phylogeny. Six models were evaluated within this likelihood framework (DEC, DEC + J, DIVALIKE, DIVALIKE + J, BAYAREALIKE, BAYAREALIKE + J). AICc was used to select the best model.

Six geographical areas (Figs. 1 and 5) were pre-defined based on current topography (Körner et al., 2017) and geological history of the region (Popov et al., 2004): Greater Caucasus (A), Lesser Caucasus (B), Pontic Mountains (C), Cyprus Island and Taurus-North Zagros Mountains (D), Talysh-Alborz Mountains (E), and Samos Island (F). Smaller topographic units forming geographic areas defined for the analyses are listed in Table A2.

Thanks to our collecting activity in the region, we have acquired reliable data on the presence/absence of individual species in all of these mountain ranges and coded the occurrences of individual species accordingly. To take into account possible wider distribution of individual species, the species recorded on the border of two areas was coded as present in both areas. The maximum possible number of ancestral areas at each internal node was set to six allowing every taxon to occur simultaneously in all areas defined for the analysis.

Two time slices (12.1–9.75 Ma and 9.75 Ma–present) were defined based on changes in the connectivity of individual pre-defined areas in time, as inferred from Popov et al. (2004). A matrix with dispersal probabilities between individual areas was assembled (Table A2). The Greater Caucasus had at first formed an island, and the land connection appeared approx. 9.75 Ma (Popov et al., 2004). Therefore, we set the probability of the colonisation of Greater Caucasus lower prior to this age. The topography of other mountain ranges did not substantially differ between time slices. However, the probability of a direct dispersal between more distant ranges was set lower than between neighbouring ranges. We also analysed an unconstrained model with equal probabilities of dispersal between all pre-defined mountain ranges through time. Besides models evaluated by BioGeoBEARS, we also computed Statistical Dispersal-Vicariance Analysis (S-DIVA) as implemented in RASP, not included in the BioGeoBEARS script. We compared results of both approaches.

2.7. Ancestral altitude estimation

Ancestral altitude estimation was performed to reconstruct the origin of the *Caucasiron* lineages and pattern of their diversification in altitude. Minimum, maximum and mean altitude of the occurrence of each lineage were obtained from our field data (Table A3). Maximum likelihood ancestral state reconstruction for continuous characters was computed using R package ape (Paradis and Schliep, 2019), with the ace function and Restricted maximum likelihood (REML) method under Bownian motion model. An input tree was MCC *Caucasiron* tree. Colour

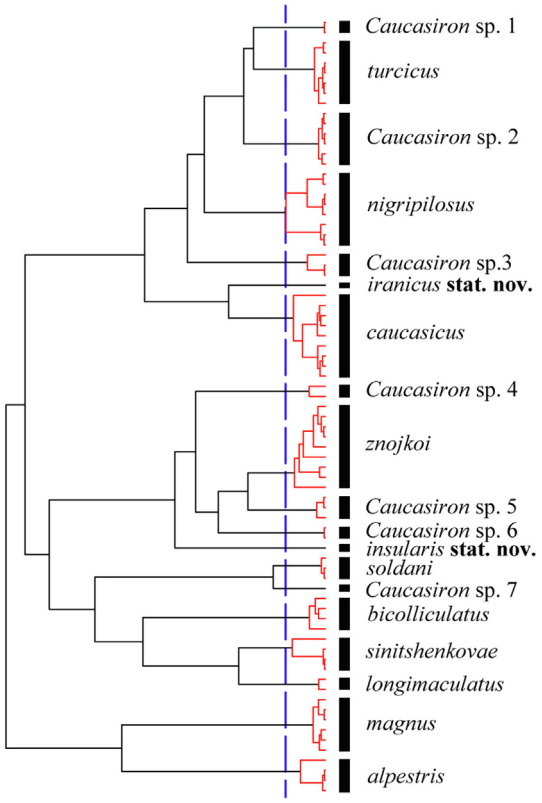

Fig. 2. Results of GMYC species delimitation analysis applied to MCC tree of the complete *COI* dataset of *Caucasiron*. Blue line indicates the point of transition from inter- to intraspecies branching events. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gradient of mean ancestral altitudes was plotted using ContMap function in R package Phytools (Revell, 2012).

3. Results

3.1. Sequencing and species delimitation

COI was successfully amplified from 77 specimens and altogether 19 GMYC-species were delimited within this dataset (Fig. 2). They included all ten described *Caucasiron* species and two subspecies (from here treated as distinct species, for justification see *4.5. Systematic implications*). In addition, seven lineages were delimited as distinct species that remain without formal description at present. They are labelled as *Caucasiron* sp. 1–7 for the purposes of the analyses.

Sequencing of additional four gene fragments (16S, EF-1α, wg, 28S) from each delimited species and all the outgroup taxa was almost completely successful. We only failed to obtain the wg sequences from two species (*E. grandis*, *Caucasiron* sp. 2) and 28S sequences from two species (*Rhithrogena alpestris* and *Cinygmula* sp.). No stop codons in protein-coding gene fragments were detected. Missing data in *COI* and 28S mostly included lacking 3′and/or 5′ ends in several sequences; gaps were detected in wg and 16S. Complete characteristics of individual gene fragments and concatenated datasets are summarised in Table A4. Substitution models and partitioning schemes for phylogenetic analyses are summarised in Table A5.

3.2. Phylogenetic analyses

The BI and ML phylogenetic analyses of individual datasets (mt, nu, combined) resulted in the same or highly similar topologies, differing only in poorly supported nodes (Fig. 3, A1 – A2). The monophyly of subfamily Rhithrogeninae was well supported based on all analyses, except BInu (Figs. 3, A1). All phylogenetic analyses clearly supported the genus *Epeorus* s.l. as a monophyletic group, and the monophyly of subgenus *Caucasiron* was supported by analyses of combined and mt dataset (Fig. 3). Phylogenetic reconstruction of nu dataset placed subgenera *Caucasiron* and *Iron* with a central Asian species *E.* (*Ironopsis*) *rheophilus* into one well supported clade (BS = 88, PP = 0.99) (Figs. A1, A2).

Three main clades were recognized within the subgenus *Caucasiron*. Clade A included seven species: *E.* (*C.*) *alpestris*, *E.* (*C.*) *bicolliculatus*, *E.* (*C.*) *longimaculatus*, *E.* (*C.*) *magnus*, *E.* (*C.*) *sinitshenkovae*, *E.* (*C.*) *soldani*, and *Caucasiron* sp. 7. Clade B included seven species: *E.* (*C.*) *caucasicus*, *E.* (*C.*) *iranicus*, *E.* (*C.*) *nigripilosus*, *E.* (*C.*) *turcicus,* and *Caucasiron* sp. 1–3. Clade C included five species: *E.* (*C.*) *znojkoi* and *E.* (*C.*) *insularis*, and *Caucasiron* sp. 4–6 (Fig. 3). Clades B and C were supported by all analyses, Clade A was well supported by ML analysis (combined and mt). Phylogenetic relationships among clades A, B and C, and species within the respective clades were not fully resolved. The clades A, B and C have no equivalents in the current taxonomy of *Caucasiron*. Two morphological species groups, *caucasicus* group and *znojkoi* group, proposed by Braasch (1980) were found polyphyletic and neither of them corresponded with any of the clades recognized herein (Fig. 3).

Concerning other subgenera of *Epeorus* s.l. included into our phylogenetic analyses, subgenus *Iron,* here represented by the Nearctic *E.* (*I.*) *longimanus* and central Asian *E.* (*I.*) *montanus*, was well supported as monophyletic by all analyses, being a sister lineage to the subgenus *Caucasiron* (BIcombined, MLcombined and MLmt). Subgenus *Ironopsis*, here represented by four of five known species (European *E.* (*I.*) *alpicola* and *E.* (*I.*) *yougoslavicus*, central Asian *E.* (*I.*) *rheophilus* and Nearctic *E.* (*I.*) *grandis*) was found to be paraphyletic. The European species *E.* (*I.*) *alpicola* and *E.* (*I.*) *yougoslavicus* formed a monophyletic group sister to the subgenus *Epeorus*, represented by *E.* (*E.*) *assimilis*, based on all analyses. Nearctic species *E.* (*I.*) *grandis* was sister to all other *Epeorus* s.l. species included into the analyses. The phylogenetic position of central Asian *E.* (*I.*) *rheophilus* was not reliably resolved due to poor node support.

3.3. Estimation of divergence times

Fossil-calibrated phylogenetic analysis in BEAST revealed topology and node supports highly congruent with BI and ML phylogenetic analyses; all three principal clades of *Caucasiron* were well supported (Fig. 4; for full calibrated tree see Figs. A3). The root of *Caucasiron* was dated in the Miocene, 12.4 Ma with a 95% high posterior density interval (95% HPD) ranging between 15.9 and 9.1 Ma (Fig. 4a). Three principal clades of *Caucasiron*, denoted as A, B, and C, were dated in the Miocene, specifically 9.9 Ma (95% HPD, 13.4–6.6 Ma) in Clade A, 7.3 Ma (95% HPD, 9.7–5.0 Ma) in Clade B and 6.6 Ma (95% HPD, 9.2–4.2 Ma) in Clade C. Divergence time estimates in subgenus *Caucasiron* based on mt dataset and standard mitochondrial rate (0.0115 substitution/site per million years) yielded highly similar results, with only small deviations (Figs. A4). The root of *Caucasiron* was dated to 9.8 Ma (95% HPD, 12.1–7.6 Ma), Clade A to 8.1 Ma (95% HPD, 10.2–6.3 Ma), Clade B to 6.1 Ma (95% HPD, 7.8–4.6 Ma) and Clade C to 5.3 Ma (95% HPD, 6.9–3.9 Ma). The results of both analyses indicate that the diversification within the clades B and C were probably younger than the diversification within the Clade A. However, the confidence intervals were partly overlapping (Fig. 4a). Most of the divergence events within the clades A, B and C took place in the Upper Miocene, Pliocene and Lower Pleistocene (Fig. 4a).

3.4. Diversification rate analysis

The BAMM analysis did not detect any significant diversification rate shifts among lineages within MCC tree of the subgenus *Caucasiron*. Bayes factors also did not support any of the BAMM rate shift model

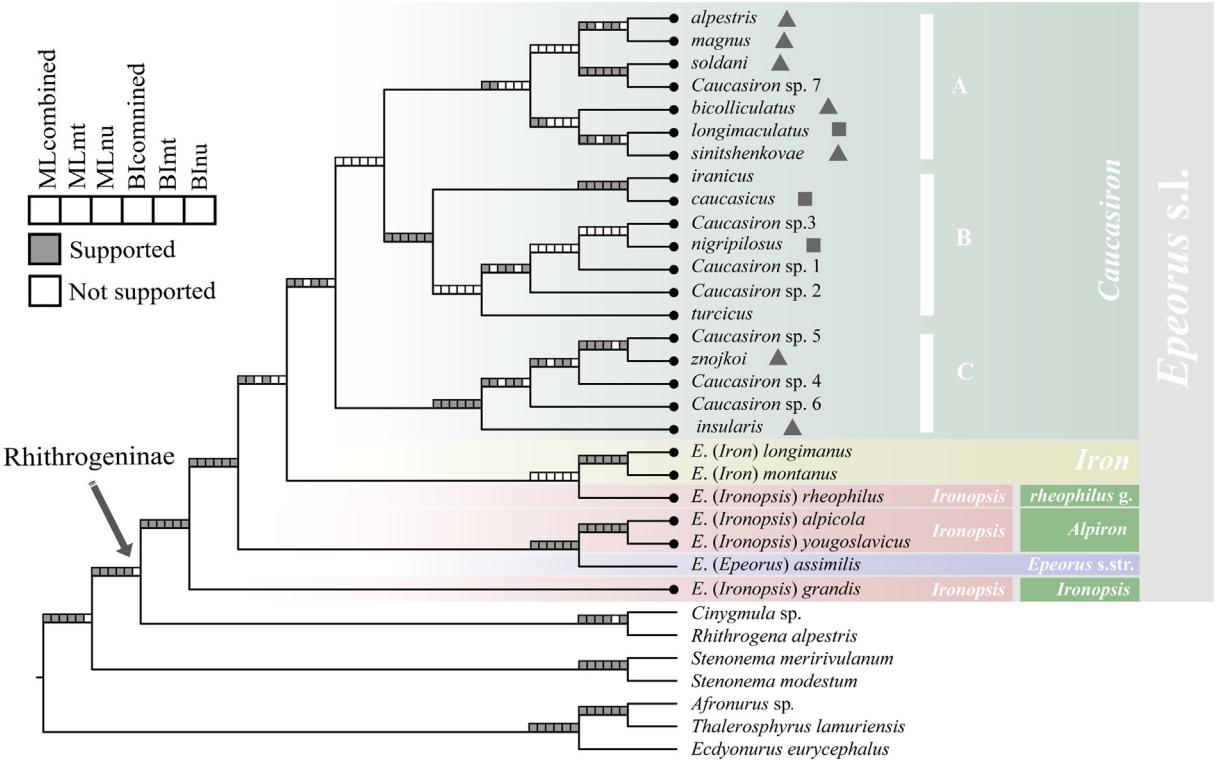

Fig. 3. Maximum likelihood (IQ-tree) cladogram reconstructed based on two mitochondrial (*COI*, 16S) and three nuclear (28S, EF-1α, wg) gene fragments. For each node, bootstrap value (> 70) and posterior probability (> 0.9) from all analyses are figured. Black circles at the end of terminal nodes in *Epeorus* s.l. indicate presence of larval "suction disc". Species groups proposed in the subgenus *Caucasiron* are indicated by a square (*caucasicus* group) and a triangle (*znojkoi* group). The proposed systematic concept based on our results is highlighted by green colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

over the null model with zero rate shifts among lineages. The BAMM analysis suggested a slow decrease in diversification rate through time with the highest rate at the root of *Caucasiron* (Fig. 4b); extinction rate was nearly constant through time, not exceeding speciation rate (Figs. A6). The results of γ statistics applied on a single MCC tree showed significantly negative values (γ = −2.8311; p-value = 0.0046), thus constant speciation rate through time was rejected. The γ statistics applied to 1,000 trees randomly sampled from the posterior distribution of phylogenetic analysis in BEAST also consistently rejected null hypothesis of constant rate and by resulting of 95% significant negative γ values with the mean value −2.726679. These results also supported that speciation rate in Caucasian species of subgenus *Caucasiron* has slowed down towards the present.

3.5. Ancestral range estimation

The BioGeoBEARS selected the best model for the ancestral range estimation as DIVALIKE + J according to AICc (Table A6). The likelihood ratio test did not reject the null hypothesis that without J and + J confer equal likelihoods on the data (p-value 0.06). Whether dispersal constraints within pre-defined time slices were applied or not, had no effect on the model selection. The DIVALIKE + J model suggested the same pattern as S-DIVA, only with higher level of uncertainty, particularly in the clades B and C (Figs. A6). According to the most probable biogeographic scenario of S-DIVA analysis, the common ancestor of *Caucasiron* occupied most of mountain ranges in the Caucasus, including Greater Caucasus ancient island. Clade A speciated in the Greater Caucasus, with some lineages secondarily dispersing outside this mountain range (Fig. 5). The origin of Clade B is ambiguous, being placed to a relatively wide area, whereas the most likely area of origin of clade C was situated in the area encompassing Pontic Mts. and Samos Island. Our analysis does not reveal major diversification within clades B and C in Greater Caucasus (Fig. 5); some lineages of these clades managed to colonize Greater Caucasus only secondarily as reflected in their current distributional pattern.

3.6. Ancestral altitude estimation

The common ancestor of Caucasian *Caucasiron* most likely originated from mid altitude (mean 1,120 m; 95% CI 1,020–1,220 m). Clade A diversified from mid altitude (mean 1,160 m, 95% CI 1,070–1,250 m) and diversification to higher altitude was indicated in three lineages (*E. alpestris*, *E. soldani,* and *E. sinitshenkovae*; Fig. 6). Clade B diversified at high altitude (mean 1,493 m, 95% CI 1,410–1,576 m) with both downward and upward trend of diversification. The ancestor of clade C diversified at lower altitude (mean 577 m, 95% CI 492–661 m) with upward trend of diversification in one lineage (*E. znojkoi*).

4. Discussion

4.1. The origin of Caucasiron

The origin and phylogeography of the Caucasian aquatic fauna is virtually unknown, available knowledge is limited to freshwater fishes and amphibians (cf. Tarkhnishvili, 2014; Levin et al., 2019). In barbels of the genus *Barbus*, the Ponto-Caspian clade is sister to the Balkan clade, with closer relations of Balkan species inhabiting the Aegean Sea basin to Caucasian barbels (Levin et al., 2019). Similar close relations of Caucasian and Balkan (or east Mediterranean) lineages were found in various terrestrial groups (cf. Tarkhnishvili, 2014). The *Caucasiron* mayflies are sister group to central Asian and Nearctic *Iron*, being less related to European and Balkan *Epeorus* lineages. It shows that they originated from Asia, even though the precise phylogenetic relationships within central Asian *Caucasiron* species (listed in Chen et al.,

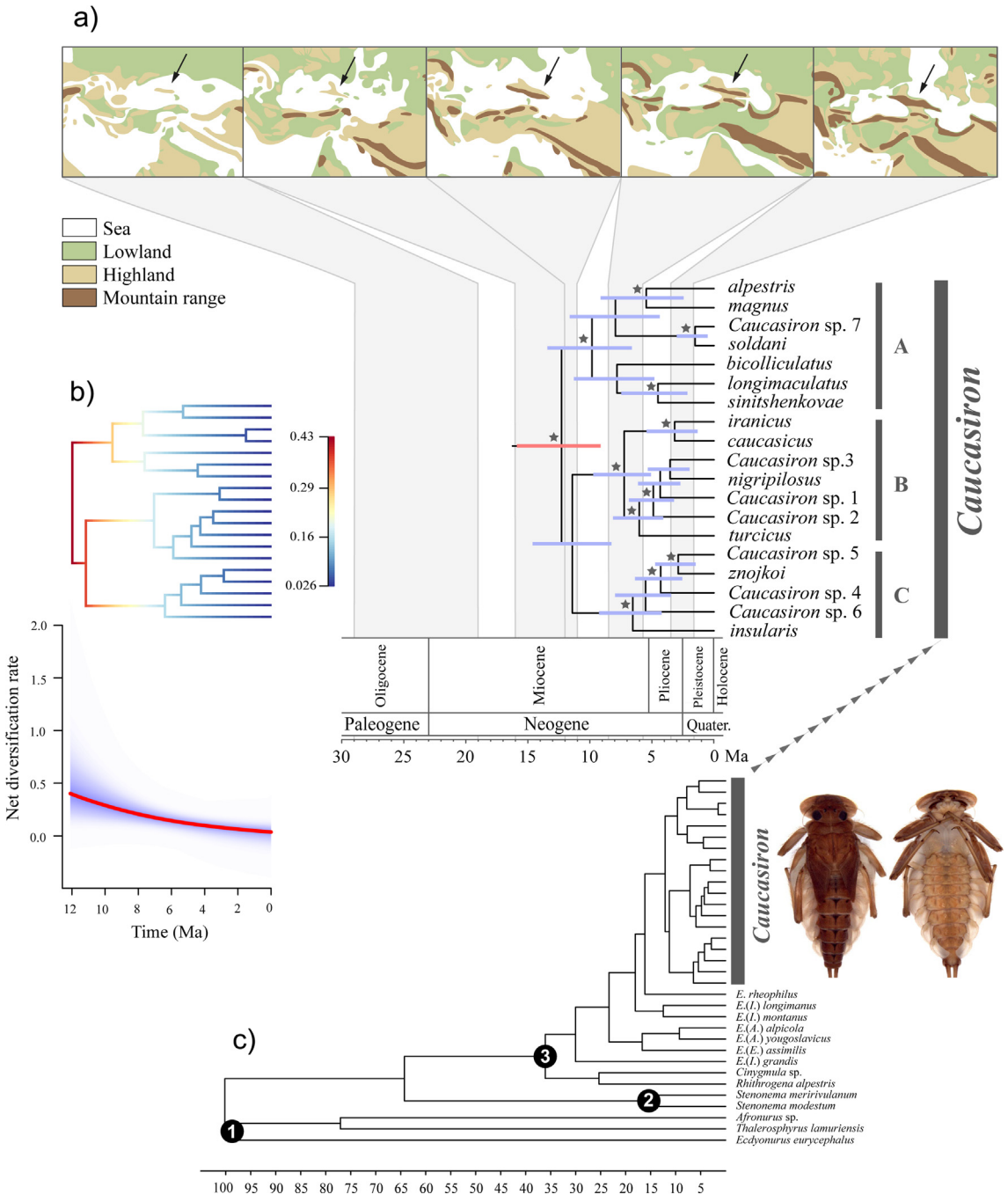

Fig. 4. A. Time-calibrated MCC tree of *Caucasiron* generated by BEAST from combined dataset. Stars above nodes indicate posterior probability (> 0.9). Basic geological scenarios in the Caucasus based on Popov et al. (2004, simplified) are figured at the top. Black arrow shows the position of Greater Caucasus. B. Net diversification rate of *Caucasiron* generated by BAMM analysis. C. MCC tree of all taxa generated by BEAST. Black circles correspond to fossil calibration points. Their numbers are described in the text (chapter Estimation of divergence times in Methods).

2010; Hrivniak et al. 2017) not included in our study remain unclear. Concerning the ancestor of all Caucasian *Caucasiron* lineages, our results suggest that their common ancestor was widely distributed in the pre-Caucasian region, including the ancient Greater Caucasus Island, and likely occupied mid altitudes, around 1,020–1,220 m a. s. l. Its origin dated at ~12.1 Ma fell within the period of Miocene climate and landscape changes opening up new habitats and niche space for more temperate organisms (e.g., Flower and Kennett, 1994; Frigola et al., 2018).

4.2. Diversification of Caucasiron and orogeny of the Caucasus

Our results brought clear evidence that the uplift and formation of the Caucasus mountain system promoted diversification in *Caucasiron*. The net diversification rate was highest in the period of rapid changes in topographic and environmental conditions in the area in the Upper Miocene (Popov et al., 2004; Tarkhnishvili, 2014) and then significantly decreased towards the present. This slowdown was not related to an increase in extinction rate, because extinction rate was nearly constant over time. We assume that the Caucasus orogeny triggered the diversification of *Caucasiron* by isolation of mountain ranges or valleys leading to allopatric speciation (Antonelli and Sanmartín,

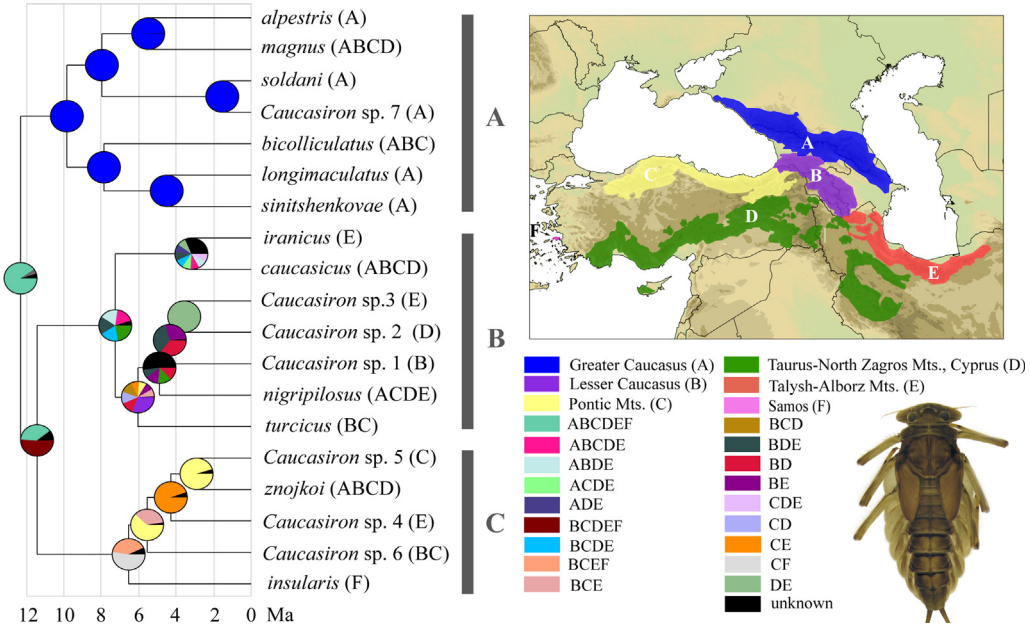

Fig. 5. Ancestral range estimation of *Caucasiron* based on RASP using S-DIVA analysis. Location of mountain ranges used in the analysis are shown in the map. Colours and letters codes under map correspond to colours on tree nodes. The recent distribution of species is given in parentheses next to species name. For the results from analysis using DIVALIKE + J model see Figs. A6.

2011; Favre et al., 2015; Heads, 2019) and creation of environmental gradients, especially climatic zonation, along which speciation occurred (Hua and Wiens, 2013; Moen and Morlon, 2014).

The origin of the Caucasian *Caucasiron* overlaps with an intensive land formation in the Parathetys Sea region in the Middle Miocene (~15–10 Ma). Large terrain comprising the area of current southern Black Sea, Lesser Caucasus, Taurus, Alborz and Zagros Mts. was still separated from the Balkans and Arabian Peninsula, and Greater Caucasus was isolated, forming an island in the sea (Popov et al., 2004; Fig. 4a). The diversification of the presumably older *Caucasiron* lineage (clade A) was dated at 9.9 Ma (13.4–6.6 Ma), which coincided with the uplift of the isolated Greater Caucasus mountain range. It indicates that environmental changes caused by mountain building and long isolation from other parts of the Caucasus led to vicariance of ancestral lineage of *Caucasiron* and diversification of the clade A. Hence, all species of the clade originated in the Greater Caucasus. Four of them (*Epeorus alpestris*, *E. soldani*, *E. sinitshenkovae*, and *E. longimaculatus*) are endemic to the Greater Caucasus, especially to its western and central part. Two species, *E. magnus* and *E. bicolliculatus*, dispersed towards south-west, to the Lesser Caucasus and Pontic Mts. (and Taurus Mts. in the former species). The ancestor of clade A likely came from mid altitude, and most lineages were diversified there (Fig. 6). Upward diversification trend was found in three lineages, *E. alpestris*, *E. sinitshenkovae,* and *E. soldani,* which inhabit cold high-altitude streams.

The radiation of the clades B and C dated at 7.3 and 6.6 Ma, respectively, seems to be relatively younger, belonging to the period

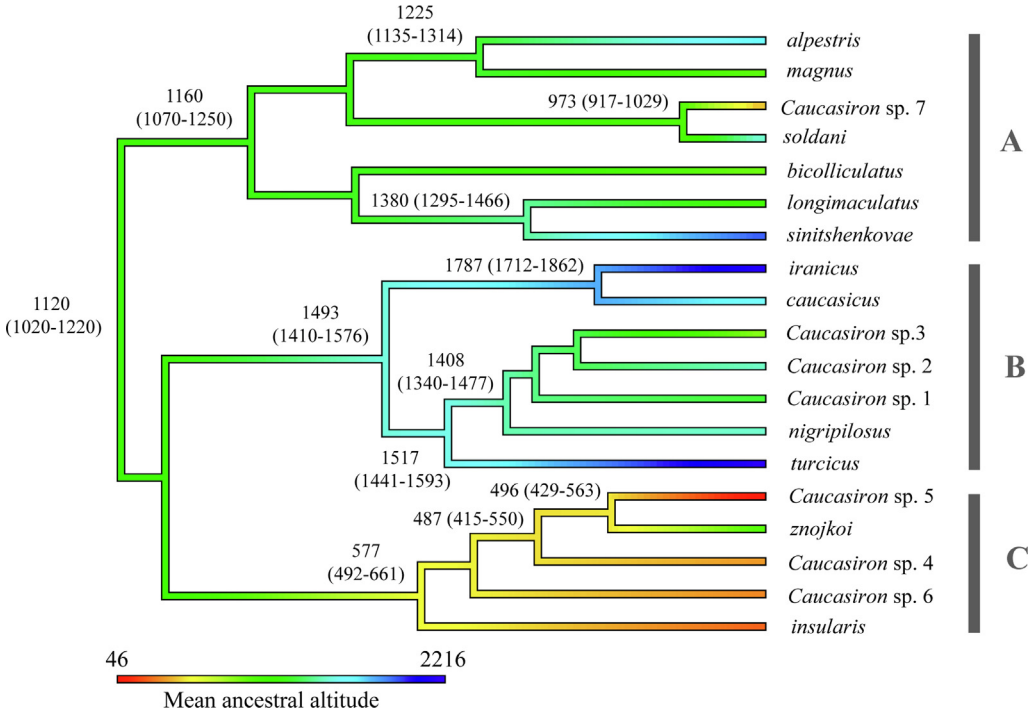

Fig. 6. MCC tree of *Caucasiron* constructed from combined dataset showing ancestral altitude estimates. Colour gradient corresponds to mean ancestral altitude. For each well-supported node mean ancestral altitude and 95% confidence interval are shown.

when most mountain ranges currently known from the region were already formed and areas of Asia Minor, Iran, and Greater Caucasus were connected (Popov et al., 2004; Fig. 4a). Clades B and C differ in the ancestral altitude suggesting that their ancestors inhabited different type of streams, presumably warmer streams at lower altitudes (clade C) and colder streams of higher altitudes (clade B), and further diversified towards low and high altitudes, respectively (Fig. 6). We assume that their diversification was triggered by the continuing uplift of mountains providing new habitats for the montane lineage. It may be related either to passive uplift of mid-altitude *Caucasiron* ancestor to higher altitude in southern part of the Caucasus and its subsequent diversification (Heads, 2019) or to ecological speciation of low-altitude and montane ancestors of both lineages through climatic niche divergence (Hua and Wiens, 2013; Castro-Insua et al., 2018). However, insufficiently resolved phylogenetic relationship and ancestral origin of the clades prevent us from discerning between these possibilities.

The most probable ancestral origin and current distribution of species suggest that both clades, B and C, originated outside of the Greater Caucasus (Fig. 5). The low-altitude clade C most likely originated in western Anatolia and the Pontic Mts. It subsequently dispersed eastward to the neighbouring Lesser Caucasus, Talysh-Alborz Mts. and, in the case of *E. znojkoi*, also to the Greater Caucasus. One species of the clade, *E. insularis*, dispersed westward to Samos Island most likely from the western Anatolian mainland. The montane clade B showed no consistent pattern in the biogeographic analysis. Two well-defined species from this clade, *E. caucasicus* and *E. nigripilosus*, are currently widely distributed, including the Greater Caucasus. Mountain ranges outside the Greater Caucasus were not separated by distinct barriers during their development around the Miocene-Pliocene boundary, only the eastern part (Talysh-Alborz Mts.) was possibly partly separated by a narrow sea strait (Popov et al., 2004). Thus, the estimation of the origin of clade B can be confounded by dispersal among mountain ranges. Alternatively, the results obtained using DIVALIKE + J model can be influenced by coarse resolution of our coding procedure, if the areas of ancestral origin are not defined by topographic barriers separating mountain ranges, but by other boundaries, such as those separating drainage basins. Drainage basins and dispersal barriers among them are important in structuring of aquatic fauna with limited over-land dispersal, which disperse primarily along stream network (Finn et al., 2007; Hughes et al., 2009; Dijkstra et al., 2014). For example, the Caucasian barbels form two lineages of Black Sea and Caspian Sea basins that split in the Upper Pliocene about 4.9 Ma, i.e. around the time when the Parathethys Sea was divided in several inland seas, including modern Black and Caspian Sea, and separated the basins (Levin et al., 2019). Further investigation into the spatial structuring of aquatic fauna and dispersal would be an interesting line of research for future studies.

4.3. The influence of climatic changes

Tarkhnishvili (2014) thoroughly examined the role of climate in the diversification of the Caucasian biota in his book summarising the historical biogeography of the Caucasus and emphasizing the joint effect of climate and landscape changes. Gradual decline of temperature and precipitation since the Middle Miocene Climate Transition (~15–13 Ma) accelerating after the Messinian Salinity Crisis (~6.0–5.3 Ma) caused the expansion of arid and semiarid landscapes and fragmentation of humid habitats in the Caucasus (Frenzel et al., 1992; Tarkhnishvili, 2014). Consequent range fragmentation in terrestrial species dependent on humid habitats caused their diversification and split of Caucasian lineages from their relatives in Europe or central Asia even before the first glacial waves, mainly in ~7–3 Ma (Tarkhnishvili, 2014).

In *Caucasiron*, most speciation events outside the Greater Caucasus fell within this period and potentially were driven by aridisation and hydrological changes in some areas. For example, the divergence of *E. insularis* in the low-altitude clade C was dated at approximately 6.6 Ma (Fig. 4a), which is close to the period of the Messinian Salinity Crisis (Hsü et al., 1977). The dramatic retreat of the Parathetys Sea promoted dispersal of freshwater invertebrates to the Mediterranean islands and the following refilling of the sea led to their isolation and vicariance (e.g., Sola et al., 2013). A similar mechanism was proposed to drive the speciation of Corso-Sardinian mayflies (Gattolliat et al., 2015; Vuataz et al., 2016), and it is also a plausible explanation for the split of *E. insularis*, a species currently known only from Samos Island. The remaining lineages of the low-altitude clade diversified from the Pontic or Talysh-Alborz Mts. (Fig. 5), humid mountains serving as Pleistocene and possible even pre-Pleistocene humid temperate climate refugia (Tarkhnishvili et al., 2000, 2012; Tarkhnishvili, 2014). This might indicate the role of climatic fragmentation in their diversification.

In the Pleistocene, the Caucasus was not strongly glaciated – the ice shield covered only the Greater Caucasus between 1,400 and 1,900 m a. s. l. in its western part and between 2,400 and 2,900 m in its eastern part (Museibov and Beruchashvili, 1986; cf. Tarkhnishvili, 2014). Grasslands and scrublands dominated in the landscape and aridisation could influence aquatic biota by drying out or freezing out of streams. However, we did not find signs of the influence of Pleistocene climatic oscillations for the diversification and extinction rates of *Caucasiron*. Likewise, a constant speciation rate over time found in *Rhithrogena*, a close relative of *Caucasiron*, suggested limited influence of Pleistocene glacial cycles on the pace of the diversification in the Alps (Vuataz et al., 2016). However, substantial number of lineages (more than 50% of the sampled species) were diversified in 3.0–1.5 Ma (Vuataz et al., 2016), which was not the case in the Caucasian *Caucasiron* with prevailing older lineages. Nevertheless, climatic changes could fragment the distribution of some species. For example, *E. nigripilosus* is widely distributed in central and western Greater Caucasus, Pontic Mts, Taurus (including Cyprus island), and Alborz Mts., but avoids arid areas of the Lesser Caucasus in Armenia, Azerbaijan, and in north-west Iran. Exploring of the range fragmentation, origin of species outside the Greater Caucasus, and climatic refugia requires more and detailed genetic data. Future work should concentrate on these topics to improve the knowledge on the phylogeography of the Caucasian fauna, not only in mayflies, but also in the aquatic fauna in general.

4.4. Fossil calibration

Due to the lack of fossil records in *Epeorus* and *Caucasiron*, fossils representing outgroup lineages were used. In order to achieve accurate divergence time estimates, we included three amber fossils, one of each subfamily within Heptageniidae. These fossils represent the best-preserved specimens within Heptageniidae in general, allowing unambiguous phylogenetic assignment of these taxa. Although several other Heptageniidae are known from the fossil record, we excluded them from the calibration due to their unclear phylogenetic position (see Appendix A1 in Supplementary material). We found a high congruence between fossil-calibrated results and timing calculated by using the standard mitochondrial mutation rate (Brower, 1994), which represents a commonly applied method of dating diversification events, when no fossils or geological events are available (Papadopoulou et al., 2010). Our data on *Caucasiron* verified by independent fossil evidence can serve as an example that mt mutation rate may provide accurate results for divergence time estimates of closely related taxa. Our study thus provides additional support for the previous analyses of other Rhithrogeninae taxa, where timing of branching events was estimated using mt mutation rate only, as in Vuataz et al. (2016).

4.5. Systematic implications

Previous authors considered the taxa *Epeorus* s.str., *Iron*, *Ironopsis,* and *Caucasiron* as either separate genera, subgenera or synonyms (Traver, 1935; Edmunds and Allen, 1964; Tshernova, 1981; Kluge,

1997, 2004, 2015; Braasch, 2006; Webb and McCafferty, 2008). The most discussed morphological structure, often considered significant for systematics of all *Epeorus*-related taxa, is the presence or absence of an abdominal "suction disc" in larvae. The disc is formed by gill plates: considerably enlarged first pair, overlapping second to sixth pair, seventh pair with a longitudinal fold and bent under abdominal segments (cf. Fig. 4 and Hrivniak et al., 2019). Suction disc represents a friction pad, adaptation related to benthic exposure to swift current (Wang and McCafferty, 2004). Braasch (2006) hypothesized that this structure evolved only once and proposed a monophyletic group containing all *Epeorus* s.l. taxa with a suction disc. An alternative hypothesis considered a suction disc as a homoplasy of several unrelated lineages within *Epeorus* s.l. (Tshernova, 1981; Kluge, 2004). Although suction disc in all species of *Epeorus* s.l. included in our analysis is morphologically identical, our results do not support the concept of Braasch (2006). *E. assimilis* (belonging to *Epeorus* s.str., i.e. without a suction disc) does not form a separate sister clade to all remaining *Epeorus* s.l., but clusters within European species with a suction disc (Fig. 3). Therefore, the absence of a suction disc in *Epeorus* s.str. is most likely a secondary reduction in one lineage within *Epeorus* s.l. Similar gill structure occurs also in other, unrelated genera such as *Rhithrogena* (Heptageniidae) or *Lepeorus* and *Deleatidium* (Leptophlebiidae), however its arrangement is slightly different from *Epeorus* s.l. (Kluge, 2004).

Our study supports the monophyly of *Caucasiron* as defined by Kluge (1997). The taxonomic recognition of subgenera *Caucasiron* and *Iron* as sister groups within the monophyletic *Epeorus* s.l. is well supported. The delimitation of *Ironopsis* is rather ambiguous due to unresolved position of *E.* (*I.*) *rheophilus*. However, the position of *E.* (*I.*) *grandis* as a sister lineage to all remaining *Epeorus* s.l. is well supported. As *E.* (*I.*) *grandis* is the type species of *Ironopsis* Traver, 1935, we limit the subgenus *Ironopsis* to two Nearctic species, *E.* (*I.*) *grandis* and *E.* (*I.*) *permagnus*, as was proposed by Braasch (2006). The European species with a suction disc, previously belonging to *Ironopsis* (e.g., Kluge, 2004), form a well-supported monophyletic sister group to *Epeorus* s.str. without a disc, not to any other *Epeorus* taxa with a suction disc. The same results have been found by Zurwerra et al. (1986, 1987) based on enzyme electrophoresis. In order to avoid a paraphyletic *Ironopsis*, we accept the concept of subgenus *Alpiron* proposed by Braasch (2006), which includes species with a suction disc, as a sister group to *Epeorus* s.str. Proposed nomenclatural changes are as follows: *Epeorus* (*Alpiron*) *alpicola* (Eaton, 1871) comb. nov. and *Epeorus* (*Alpiron*) *yougoslavicus* (Šámal, 1935) comb. nov. As the position of *E.* (*I.*) *rheophilus* was not resolved, we keep the separate position of this species in the "*rheophilus* group", as proposed by Braasch (2006).

The two subspecies, *E.* (*C.*) *caucasicus iranicus* and *E.* (*C.*) *znojkoi insularis*, included in the dataset along with the respective nominotypical species were recovered as distinct species in the GMYC analysis (Fig. 2). They were also morphologically defined by Braasch (1983) and Braasch and Soldán (1979). Therefore, we raise them to species level as *Epeorus* (*Caucasiron*) *iranicus* Braasch & Soldán 1979 stat. nov. and *Epeorus* (*Caucasiron*) *insularis* Braasch 1983 stat. nov. The existence of several undescribed species within *Caucasiron* was indicated by the GMYC analysis. They are left without morphological definition and formal description for the time being.

5. Conclusions

Our time-calibrated phylogeny does not pose a major conflict with the current taxonomy of *Epeorus* s.l. and supports a close relationship between the subgenera *Caucasiron* and *Iron*. The subgenus *Ironopsis* is found paraphyletic, with its European representatives more closely related to *Epeorus* s.str. than to the remaining *Ironopsis*. We thus assign European *Ironopsis* species to the separate subgenus *Alpiron*.

The *Caucasiron* lineages cluster into three principal clades. One of them diversified in the Greater Caucasus isolated in the Parathetys Sea and some species then dispersed outside this mountain range after its connection with eastern Anatolia in the Upper Miocene. The remaining two *Caucasiron* clades diversified most likely outside the Greater Caucasus and dispersed there later. Hence, current high *Caucasiron* diversity in the Greater Caucasus is a result of both in situ speciation and later immigration.

Our fossil-calibrated timing of diversification events corresponds with time estimates obtained using universal mt mutation rate calculation. Net diversification rate of the *Caucasiron* mayflies significantly decreased after initial radiation in the Miocene. The period of the highest *Caucasiron* diversification overlaps with the formation of the Caucasus mountain system. Miocene and Pliocene orogeny of the Caucasus most likely triggered the diversification of freshwater montane biota by isolation of mountain ranges leading to allopatric speciation and creation of environmental gradients, especially climatic zonation, along which speciation occurred. Our results indicate that the speciation of Caucasian cold adapted aquatic insects was influenced more by late Tertiary orogeny than later climatic oscillations in the Quaternary.

CRediT authorship contribution statement

Ľuboš Hrivniak: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Project administration. **Pavel Sroka:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization, Project administration, Supervision. **Jindřiška Bojková:** Investigation, Resources, Writing - original draft. **Roman J. Godunko:** Investigation, Resources, Writing - review & editing. **Tomáš Soldán:** Investigation, Resources. **Arnold H. Staniczek:** Writing - review & editing, Funding acquisition.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106735.

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