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Diversity and speciation of mayflies (Ephemeroptera) from the Caucasus and adjacent areas

Ph.D. Thesis

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Annotation

This dissertation thesis is focused on the diversity and evolutionary history of mayflies (Ephemeroptera) from the Caucasus, one of the most biologically diverse areas of the Palaearctic realm. The introduction details general information about the evolutionary mechanisms leading to the high diversity of mountain biota, the processes driving evolutionary diversification in aquatic insects, and the current state of knowledge on the diversity and distribution of mayflies in the Caucasus. The core of the thesis consists of five published articles and one manuscript, which are divided into four chapters. The first chapter deals with the diversity and distribution of mayflies from Armenia, the second and third are focused on diversity, integrative taxonomy, and identification of *Epeorus* (*Caucasiron*), one of the most diverse mayflies in the Caucasus. The last chapter is focused on the origin, historical biogeography, and evolutionary diversification of *Caucasiron*, the model group selected for the evolutionary study of mayflies from the Caucasus. The chapter describes when and under which circumstances Caucasian mayfly endemism and diversity developed. The thesis is concluded with a detailed summary of the results, and future perspectives in the research of Caucasian mayflies.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 20.07.2020

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Ľuboš Hrivniak

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. **Hrivniak Ľ.**, Sroka P., Godunko R. J., Žurovcová M. (2017) Mayflies of the genus *Epeorus* Eaton, 1881 s.l. (Ephemeroptera: Heptageniidae) from the Caucasus Mountains: a new species of Caucasiron Kluge, 1997 from Georgia and Turkey. Zootaxa 4341: 353-374 (copyrighted to www.mapress.com/j/zt) (IF = 0.990). *ĽH contributed to the study design and writing the manuscript, performed data analyses, morphological examinations, and created figures.*

II. **Hrivniak Ľ.**, Sroka P., Godunko R. J., Palatov D., Polášek M., Manko P., Oboňa J. (2018) Diversity of Armenian mayflies (Ephemeroptera) with the description of a new species of the genus *Ecdyonurus* (Heptageniidae). Zootaxa 4500: 195-221 (copyrighted to www.mapress.com/j/zt) (IF = 0.990). *ĽH contributed to the study design and writing the manuscript, participated on the field sampling, performed data analyses, morphological examinations, and created part of figures.*

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V. **Hrivniak Ľ.**, Sroka P., Bojková J., Godunko R. J., Soldán T., Staniczek A. H. (2020) The impact of Miocene orogeny for the diversification of Caucasian *Epeorus* (*Caucasiron*) mayflies (Ephemeroptera: Heptageniidae). Molecular Phylogenetics and Evolution 146: article number: 106735 (IF = 3.992). *ĽH contributed to the study design and writing the manuscript, participated on field sampling, performed data analyses, and created figures.*

VI. **Hrivniak Ľ.**, Sroka P., Bojková J., Godunko R. J. (2020) Identification guide to larvae of Caucasian *Epeorus* (*Caucasiron*) (Ephemeroptera: Heptageniidae) (manuscript). *ĽH contributed to the study design and writing the manuscript, performed mophological examinations, and created figures.*

Co-author agreement

Pavel Sroka, the supervisor of this thesis and co-author of all presented manuscripts, fully acknowledges the contribution of Ľuboš Hrivniak as the first author and his contributions as stated above.

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PART ONE: BACKGROUND

1. Introduction

Mayflies (Ephemeroptera) are an ancient order of merolimnic insects inhabiting freshwaters. With stem-groups more than 300 million years old, they represent one of the oldest lineages of extant insects (Bauernfeind and Soldán 2012). Their larvae are strictly aquatic and play an important role in freshwater ecosystems, significantly contributing to decomposition processes and representing a food source for diverse predators (Jacobus et al. 2019). Mayfly subimago, a winged stage unique in pterygote insects, and imago are terrestrial and have a lifespan of only a few hours or days (Brittain and Sartori 2009). Mayflies colonize all types of freshwaters, but are more diversified in lotic than in lentic habitats (Cardoni et al. 2015). Due to their sensitivity to water pollution and habitat degradation, mayflies are excellent bioindicators of the quality of aquatic ecosystems (Jacobus et al. 2019).

Mayflies are distributed worldwide, with the exception of Antarctica and some remote oceanic islands (Bauernfeind and Soldán 2012). The recent global fauna consists of approximately 3700 described species (Jacobus et al. 2019) and their taxonomic investigation is still in progress. The world diversity of mayflies follows a general geographical pattern of decreasing diversity from the tropics towards the poles (Brown 2013). The highest diversity occurs in the Neotropics with almost 900 described species, followed by the Palearctic (830), Oriental and Nearctic (620 and 610 respectively), Afrotropical (440), Australasian (250), and Pacific realm (48) (Jacobus et al. 2019).

The species composition of the Palearctic, and particularly European, mayfly fauna is relatively well-known. The level of endemism and species diversity in the western Palaearctic is high in the Mediterranean and European mountains (the Alps, the Pyrenees, the Carpathians), and in the Caucasus (Bauernfeind and Soldán 2012). The Caucasus is clearly the most neglected area in terms of the research of both mayflies and aquatic insects in general. Whereas regional diversity is relatively well-documented in vascular plants, vertebrates (e.g. Williams 2004), and some iconic insect orders, such as ground beetles (Tshikolovets and Nekrutenko 2012) or butterflies (Retezár 2011, 2015),

highly diverse aquatic insects remain comparatively less studied. Based on the existing biodiversity data, the Caucasus represents one of 25 world hotspots of biodiversity (Myers et al. 2000). The freshwaters of the Caucasus are tightly associated with high diversity and endemism (Kownacki 1987; Levin et al. 2019), but the evolutionary processes leading to this association are unknown, especially among insects. Due to their age, strong link to aquatic habitats with marked environmental and spatial gradients, and limited ability for overland dispersal in the adult stage, mayflies are excellent models for the research of evolutionary diversification (Dijkstra et al. 2014). Their extensive fossil records also allow the dating of evolutionary processes in time (Hubbard 1990).

The research of mayflies in the Caucasus can be highly beneficial for the region in several aspects. More information on their diversity and distribution, and an improved identification of species may enable the incorporation of mayflies as bioindicators into regional water quality assessments and conservation management. Moreover, research focused on their origin and the mechanisms driving their diversification will help us to understand their evolutionary history and the formation of their endemism, which may potentially help to guide prioritization in ecosystem conservation (Favre et al. 2015).

Therefore, the aim of this thesis is to provide complex information about the mayflies from the Caucasus mountains. It focuses on their diversity, distribution, identification, and evolutionary history leading to recent high regional and local endemism. It summarizes the current knowledge on the Caucasian mayfly fauna and describes several new species from this region based on an integrative taxonomic approach incorporating morphology and molecular data. Based on the knowledge on the Caucasian mayfly diversity, one model group (the genus *Epeorus*) was chosen for research into the origin of Caucasian mayflies and factors driving their evolutionary diversification. The aim was to clarify when, where, and how recent mayfly diversity has developed in the Caucasus, one of the most biologically diverse areas in the Palaearctic region.

2. Speciation and phylogeography of mountain aquatic insects

2.1. Mountain orogeny and speciation

Mountains play an important role in the determination of regional climate and spatial heterogeneity in acting as refugia, and barriers to and corridors for species dispersal, and are the source of most rivers (Perrigo et al. 2020). Despite covering a relatively small portion of the land surface, mountains host about one-third of terrestrial species and are considered hotspots of biodiversity and endemism (Körner 2004; Spehn et al. 2011). Mountains, as heterogeneous and complex geological systems, directly affect speciation processes, ecological interactions, adaptive and non-adaptive radiation, and stochastic processes co-acting in the diversification of lineages (Esquerré et al. 2019; Perrigo et al. 2020). As shown by several studies from the Andes (Esquerré et al. 2019), Borneo (Merckx et al. 2015), and Northern Hemisphere (Hagen et al. 2019), mountain-derived lineages can colonize other regions. Thus, mountains can be an important source of regional biodiversity for the surrounding areas.

The origin of montane clades is generally attributed to two basic models: **(1) dispersal with subsequent adaptation and diversification** (Fig. 1A), and to **(2) diversification during mountain building itself** (Heads 2019) (Fig. 1B).

In the first model (Fig. 1A), species colonize mountain ranges raised by an orogeny, which is similar to the colonization of newly formed islands, or any novel habitats or ecological niches that are opened up for colonization and diversification (e.g. Espeland and Johanson 2010). In this scenario, an assemblage of montane biota is explained by recent dispersal and colonization, rather than by events of the Earth´s tectonic history (Quintero et al. 2013).

In the second model (Fig. 1B), the montane lineage(s) are uplifted with the mountains during orogeny because their ancestors already occupied the region prior to it. Ancestral lowland lineages (which avoided extinction during the process of mountain uplift) gradually adapt to the montane conditions and, thus, separate from lowland populations. The

alternative process driving ancestral lineage diversification is isolation caused by increasing topographical heterogeneity during orogeny, i.e. the formation of geographical barriers, fragmentation of habitats, valley erosion, etc. (e.g. Favre et al. 2015; Xing and Ree 2017; Che et al. 2019; Heads 2019).

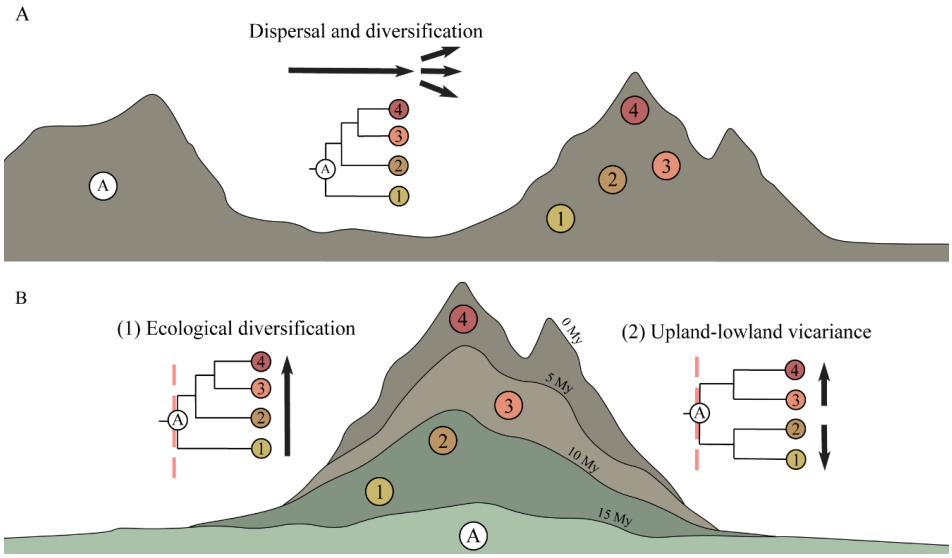


Figure 1. Hypothetical origin of montane biota with possible phylogenetic relationships. Explanations: white circles – ancestor; colored and numbered circles – hypothetical species; red dashed line – age of orogeny. A: dispersal, colonization and diversification after mountain orogeny. B: diversification from local lowland ancestor during mountain orogeny, (1) gradual ecological diversification along altitudinal gradient; (2) vertical vicariance of lowland and highland populations. Different layers in Fig. B correspond with mountain building process since 15 My.

The origin of montane clades is usually attributed to expansions from surrounding lowlands and long-distance dispersal from other mountains, but the effect of mountain orogeny has been largely overlooked (Heads 2019). Although the dispersal of lowland or pre-adapted montane lineages may explain the origin of local biota (e.g., the uplift of the North American Cordillera predates that of the Alpine belt and makes it a potential source of pre-adapted colonizing lineages (Mix et al. 2011; Favre et al. 2015), it does not explain how the lineages present in the mountains came to be there in the first place. The origin of montane biota via the dispersal model makes the assumption that recently mountainous

areas were empty of organisms prior to and during orogeny. However, plants and animals always occupy lowland areas before they are uplifted to an alpine elevation, and are uplifted together with the mountains themselves (Heads 2019). Recently, studies that have examined different taxa in the mountains of South America (e.g. Cadena 2007; Chaves et al. 2011; Ceccarelli et al. 2016; Testo et al. 2019), Europe (e.g. Ozenda 2009), Asia (e.g. cf. Favre et al. 2015), Africa (cf. Heads 2019), and New Guinea (e.g. Toussaint et al. 2014) considered the orogeny to be a significant factor in the diversification process, which can determine aspects of both ecology, such as the altitudinal distribution of clades, and evolution via vicariant events.

The extensive sampling of model groups and outgroups, the comparison of the age of montane clades with the age of uplift, and ancestral range reconstruction are currently used to infer diversification driven by mountain orogeny (Favre et al. 2015; Heads 2019). If the particular montane orogeny and age of clade coincide, and if the region was inhabited by the clade's ancestor prior to the uplift, diversification could well be driven by the orogeny (Heads 2019). The altitudinal diversification of lineages might also be coupled with orogenic processes if the lineages diversified during periods of orogeny (Cadena 2007). Passive uplift could also be indicated by altitudinal anomalies such as the occurrence of coastal elements at high altitudes (reviewed by Heads 2019).

As described here, two different mechanisms may be responsible for the origin of montane biota, adaptation and diversification during orogeny, or alternatively, immigration event(s) of lowland or pre-adapted montane lineages. If the former process dominates in the formation of local biodiversity it would also result in an increased diversification rate temporally coinciding with the process of orogeny (Favre et al. 2015). An increased diversification rate as a result of mountain uplift have been detected in several montane taxa e.g., the butterfly genus *Melitaea* (Nymphalidae) from the Qinghai-Tibetan Plateau (Leneveu et al. 2009), and the Neotropical *Phlegmariurus* (Lycopodiaceae) from the Andes (Testo et al. 2019).

Even if the initial evolution of a clade was promoted by mountain orogeny, its diversity may be younger than the mountains themselves, if the speciation process occurs after the uplift. Diversification may be driven by more recent events, such as the isolation of populations during the Pleistocene climatic cycles in glacial refugia, or any other recent speciation process (De-Silva et al. 2017). Quintero et al. (2013) suggested that the uplift of the different Cordilleras of the Andes (around 5 My) may have been responsible for the initial vicariant speciation of Andean parrots *Hapalopsittaca* (Psittacidae) into two clades. However, speciation within the clades took place within the last 0.5 My. This pattern was coupled with glacial and interglacial periods during the Pleistocene causing the fragmentation of Andean montane vegetation, which may have facilitated allopatry and subsequent differentiation. However, the combination of biogeographical, phylogenetic, and diversification rate analyses of a whole montane clade might clarify whether the initial diversification followed geological history or was a result of more recent dispersal.

Nevertheless, various processes may play a crucial role in various taxa in different regions (Antonelli 2015). It is very unlikely that all mountain biota originated via a single model. A general understanding of the complex evolution of montane biota will be achieved only by combining datasets derived from a variety of organisms (Favre et al. 2015). For instance, the phylogenetic and biogeographic study of multiple clades from the tropical mountain of Sabah in East Malaysia (Kinabalu Mountains) has shown that its local biota comprises a mix of both immigrant pre-adapted lineages, younger than the mountains itself, and descendants diversified from local lowland ancestors (Merckx et al. 2015).

Regarding the aquatic insects, the ecology of many groups is well-studied owing to their roles as bioindicators or disease vectors, but, despite their significant contribution to global biodiversity their evolutionary diversification has been largely overlooked (Dijkstra 2014). The process of mountain orogeny is closely associated with erosion and the formation of deep valleys and drainage systems, which significantly affect the evolutionary divergence of aquatic organisms (Favre et al.

2015). Drainage basins and dispersal barriers among them are important in the structuring of aquatic fauna with limited over-land dispersal, which disperse primarily along stream networks (Finn et al. 2007; Hughes et al. 2009). The formation of streams and river valleys during orogeny may cause vicariance as an indirect effect of the orogenic process (Heads 2019). Therefore, it can be assumed that the formation of stream networks and the separation of drainages can significantly affect the evolutionary history of aquatic organisms. The formation of mountain ranges is thus a potentially important driver of speciation in both terrestrial and aquatic biota (He and Chen 2006).

Only a few studies focused on historical biogeography and the speciation of aquatic insects have been published. Their results point to radiation driven by orogenic processes and diversification along the elevation gradient in Papua New Guinea (Toussaint et al. 2014: *Exocelina* diving beetles; Cozzarolo et al. 2019: *Thraulus* mayflies), and radiation driven by the epeirogeny of the Guyana plateau and vicariance after the formation of table mountains (De Marmels 2007: *Tepuibasis* dragonflies).

2.2. Effects of climatic changes

Pleistocene climatic cycles and the isolation of populations in allopatric refugia are important drivers of diversification and speciation (Hewit 1996; Stewart 2010). However, the role of Pleistocene glaciations in the speciation of montane aquatic insects in Europe has been questioned (Vuataz et al. 2016). Based on the traditional interpretation, terrestrial fauna survived glaciations in one or more (micro)refugia with favorable climatic conditions in Southern Europe and recolonized Central Europe during interstadials, interglacial, and postglacial periods (Hewitt 1999; Bunje 2005). The regression of populations in the Quaternary cold periods affected genetic divergences within isolated refugial populations (Taberlet et al. 1998) in species with discontinuous distribution or low dispersal ability (Pauls 2006). Despite some common patterns, many differences in the effects of climatic changes have been detected, because responses to Quaternary fluctuations were dependent on species´ life cycle, habitat preferences, dispersal ability (Lehrian et al. 2009), and

tolerance to climatic factors (Hänfling et. al. 2002; Pinceel et al. 2005; Stewart 2010).

The results of recent phylogeographic studies focused on montane aquatic fauna contradict the classical concept of refugia for terrestrial fauna (Hänfling et. al. 2002; Schmitt 2007). The life histories and responses of many aquatic species to Quaternary temperature fluctuation was fundamentally different to that of terrestrial species (Theissinger et al. 2012). This is particularly true for the cold-tolerant stream inhabiting aquatic insects (Pauls 2006; Danks 2007). Permanent running water provides relatively stable environmental conditions for the larvae of cold-tolerant aquatic insects. Such groups may be less exposed to temperature extremes over long time periods and can survive even in the vicinity of ice sheets (postulated in "Dinodal theory" by Malicky 1983) by way of only short-distance vertical migration, in contrast to the majority of terrestrial species with their large-scale distribution shifts (Miklos 2008). Therefore, such aquatic habitats become periglacial refugia during glaciation periods allowing in situ survival (Theissinger et al. 2012). Several studies on montane aquatic insects, Trichoptera (e.g. Pauls et al. 2006; Lehrian et al. 2009), Ephemeroptera (e.g. Theissinger et al. 2011), and Plecoptera (Theissinger et al. 2012), have documented high population divergence as a result of Pleistocene survival in multiple refugia over glacial cycles, which allowed the formation of many local endemic clades (Pauls 2006). Moreover, several studies have indicated a constant diversification rate of taxa and no impact of Quaternary climate change on the speciation or extinction of aquatic insects (e.g. Burbrink and Pyron 2010; Vuataz et al. 2016).

3. What makes the Caucasus so unique?

The Caucasus is a mountain region situated between the Black and Caspian Seas. The region covers a total area of 580 000 km^2 and includes the territory of Armenia, Azerbaijan, Georgia, the southwestern part of Russia, the northeastern part of Turkey, and a part of northwestern Iran (Williams 2004). The Caucasus Mountains consists of several mountain ranges with various connectivity. The Greater Caucasus, the most prominent mountain range in the region rising to 5, 642 m above sea level, is almost completely isolated by the Kuma-Manych Depression located in the north and the Transcaucasian Depression (Colchis plain, Kura-Aras Lowland) in the south. Other mountain ranges, namely the Lesser Caucasus, and the Pontic and Alborz Mountains are connected with the upland regions of western Asia. The Greater Caucasus connects to the Lesser Caucasus by the Likhi (Surami) Range located in central Georgia (Tarkhnishvili 2014); for the localization and topography of the region see Fig. 1 in Chapter III or IV.

The Caucasus is located in the central part of the Palearctic realm and represents a biogeographic crossroad between Europe (especially the Balkans and the Mediterranean) and Central Asia (Williams 2004). A high level of biodiversity and endemism in a relatively small area is what makes the Caucasus unique (Favre et al. 2015). The recent species richness of the Caucasus is comparable to that of the entire European continent and the level of regional endemism approaches or exceeds 50% (Tarkhnishvili 2014). Most significant factors responsible for such diversity and endemism are the geographic position of the region and its geological and climatic history, creating intricate environmental heterogeneity (Körner 2004; Levin et al. 2019).

3.1. History of the land development of the Caucasus

The Caucasus Mountains belong to the Alpine belt, an orogenic system stretching from the Pyrenees to the Himalayas and further to Southeast Asia (Gupta and Delany 2013). The system has been formed over the last 30 My (millions of years) with an uplift acceleration in the last 10 My (Popov et al. 2004).

The history of land development in the Caucasus region was very specific. The region was formed by the conjunction of several ancient land masses, originally scattered in the Paratethys Sea, which existed along the southern margin of Eurasia since the Eocene–Oligocene boundary (33 My). Since the Eocene, a land mass (coinciding with current Asia Minor) was located south of the current Black Sea (Fig. 2A, black arrow). Another land mass (coinciding with the present Alborz Mountains in northern Iran) was connected to Central Asia (Fig. 2A, red arrow). These two lands were originally separated by the Paratethys Sea and subsequently connected to each other by a land bridge in the early Oligocene (32 My) (Fig. 2B). Simultaneously, a small island started to emerge in the Paratethys Sea, forming the current Greater Caucasus range (Fig. 2B, arrow). In the late Oligocene, around 25 My, the Asia Minor Peninsula conjoined the Balkans. In the early to middle Miocene (20–15 My), the land masses of Asia Minor and the Balkans where again separated from Central Asia by a sea strait (Fig. 2C, D). In the middle Miocene (around 12–11 My) land masses corresponding to Asia Minor, the Lesser Caucasus, the Taurus Mountains, and the Iranian mountain ranges of the Alborz and Zagros were connected to each other (Fig. 2E). The Greater Caucasus was still an island in the Paratethys Sea (Fig. 2E, arrow). In the late Miocene (around 8 My), all ancient land masses (Asia Minor, Iran, the Arabian Peninsula, and the Greater Caucasus Island) began to connect to each other (Fig. 2F). This process is associated with the Messinian Salinity Crisis (around 6 My), a period of reduction of precipitation worldwide causing a decrease in sea levels (Manzi et al. 2013; Roveri et al. 2014). During this period, the Paratethys Sea was substantially reduced and fragmented into several separated basins such as the Black, Caspian, and Aral Seas (Abrantes et al. 2012). It was also a period of intensive orogenic activity in the region (Popov et al. 2004). The Greater Caucasus formed an island connected with the mainland forming an isthmus separating the Black and Caspian Seas. The uplift of the Caucasus continued throughout the Pliocene (Fig. 2G), and the region reached its current state by the beginning of Pleistocene (2.4 My) (Fig. 2H) (Popov et al. 2004; Tarkhnishvili 2014).

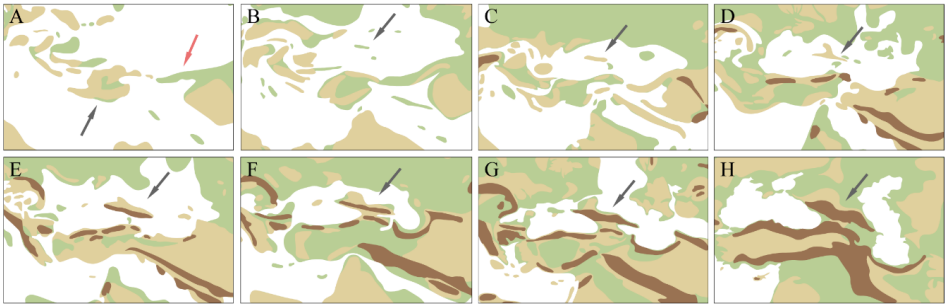


Figure 2. The history of the land development of the Caucasus region. Simplified according to Popov et al. (2004). Explanations: A: late Eocene (37–34 My); B: early Oligocene (34–32 My); C: early Miocene (20–19 My); D: early middle Miocene (16–15 My); E: late middle Miocene (12–11 My); F: middle late Miocene (8.5–7.0 My); G: late Pliocene (3.4 My); H: Holocene. Colors: white: sea; green: lowlands; pale brown: highlands; dark brown: mountain ranges. Black arrow in A points to a land massif coinciding with current Asia Minor. Red arrow in A points to a land massif coinciding with recent Alborz Mountains in northern Iran. Black arrow in B–H points on the Greater Caucasus.

3.2. Climatic history of the Caucasus

The Caucasus region is different from its surroundings in the presence of a humid climate and forest ecosystems in some areas, especially in the western part. It is connected to high precipitation at the eastern coast of the Black Sea. As a result, the western Caucasus and the coastal zone of the Black Sea have the most favorable moisture and temperature conditions in Northern Eurasia for the growth of broadleaved evergreen/mixed forests (Tarkhnishvili 2014). These areas were most likely a refuge for thermophilous biota during the coldest stage of the last glaciation (Tarasov et al. 2000). Although forests are currently absent from a large part of the East, North, and South Caucasus, this landscape is still typical for the region (Williams et al. 2006).

In the Miocene, Eurasia was largely covered with forests and a warm temperate climate of wet or humid character (Bruch and Zhilin 2007; Utescher et al. 2007). The global temperature and gradual humidity decline since the Middle Miocene Climate Transition (around 15 My), significantly affected the landscape and associated ecosystems (Zachos et al. 2001). This applies especially to the expansion of grasslands and fragmentation of forests throughout Eurasia (Ratellack 2001). The relatively humid mountainous landscape of the Caucasus, harboring forest biodiversity complexes, became increasingly isolated from the rest of

Eurasia. While the biota less dependent on high humidity levels and forest ecosystems could repeatedly expand to the region from Asia, Mediterranean, and Africa, the biota requiring humid conditions lost the connection to their geographically distant relatives and became isolated in the Caucasus (Tarkhnishvili 2014).

Aridification and deforestation intensified in the Miocene/Pliocene boundary (around 6 My) during the Messinian salinity crisis (Tarkhnishvili 2014; Favre et al. 2015). This process, together with intensive orogenic activity in the region, caused the further fragmentation of forests. Fossil records show that grasslands and scrublands started to expand into the Caucasus region during the Pliocene (around 4–3 My) and dominated in the region during the Pleistocene (Tarkhnishvili 2014). Glaciers covered only the Greater Caucasus at elevations above 1400–1900 m in the west and 2400–2900 m in the east (Museibov and Beruchashvili 1986).

Forest-associated retreating populations found refugia in habitats situated close to the seashore and isolated from each other by mountain ranges (Dubeya et al. 2006; Tarkhnishvili 2014). Phylogeographic studies suggest multiple forested refugia in the West Caucasus (the Pontic Mountains and Colchis Lowland), Alborz Mountains, and central Georgia based on data on reptiles and amphibians (Tarkhnishvili et al. 2000, 2001; Veith et al. 2003; Garcia-Porta et al. 2012), shrews (Dubeya et al. 2006), and land snails (Mumladze et al. 2013; Nieber and Hausdorf 2015). The paleoclimatic models of Tarkhnishvili et al. (2012) suggest that the West Caucasus and Alborz Mountains were important humid forest refugia during the last glacial maximum.

In Europe, many taxa survived the Quaternary cold periods at lower latitudes and altitudes in the three large peninsulas of South Europe: the Iberian and Apennine Peninsulas and the Balkans. These areas were isolated by the mountain ranges, which represented effective dispersal barriers. After the last glacial maximum, species requiring a mild humid climate started to expand from these refugia throughout Western Eurasia (Dapporto 2009). However, forest species from the Caucasus refugia, especially those with poor dispersal abilities, could expand only over relatively small areas due to the highly heterogeneous climate and

landscape mosaic in the region. Therefore, they could not create a large post-glacial distribution range and remained isolated as regional or local endemics (Tarkhnishvili 2014).

Several studies demonstrated the importance of dispersal barriers in the region. The Greater Caucasus mountain range was found to present an effective barrier for colonization after the last glacial maximum. Seddon et al. (2002) analyzed the mitochondrial sequences of the white-breasted hedgehog (*Erinaceus concolor*) from Eastern Europe, Turkey, and the Caucasus. The results show deep divergence among populations from the northern and southern slopes of the Greater Caucasus Mountains, and suggested two colonization routes, originating from separate refugial regions. The results of Babik et al. (2005) showed deep divergence between populations of Caucasian newt subspecies (*Triturus* v. *lantzi*) from the north and south of the Greater Caucasus suggesting that the mountains presented an effective barrier to dispersal. Dubeya et al. (2006) detected the Greater Caucasus as a dispersal barrier for populations of the Eurasian shrew (*Crodidura suaveolens* group).

3.3. Geological and climatic history of the Caucasus: a brief summary

The Caucasus region, as we know it from the current maps, consists of several ancient land masses that have split and merged in the course of geological history, and have probably exchanged their fauna and flora. The Greater Caucasus region was an isolated island in the ancient Paratethys Sea for a long time and conjoined with the mainland in the Miocene/Pliocene boundary, around 8 My. Climate changes since the Middle Miocene have caused the gradual fragmentation of humid forest ecosystems resulting in multiple forest refugia allowing the formation of the unique evolutionary lineages of present Caucasian biota. After the last glacial maximum, species from the Caucasus could not effectively expand because their habitats remained fragmented by grasslands or semidesert landscapes and evolved into endemic lineages. This is reflected by the general pattern of endemism in the Caucasus: more endemic taxa are distributed in the alpine grasslands and mountain forests, which are more fragmented and isolated, than in arid and semiarid landscape, scrublands,

and semideserts, which are widely distributed throughout the Mediterranean and Central Asia.

4. Mayflies (Ephemeroptera) of the Caucasus: the current state of knowledge

The mayflies of the Caucasus are extraordinarily diverse and significantly contribute to the overall biodiversity and endemism of aquatic ecosystems in the region. The Caucasus is characterized by all types of freshwater habitats: springs (often mineral), turbid glacial streams, various rhithral and epipotamal streams (of which the River Kura is the biggest in the region), and mountain lakes (including Lake Sevan, one of the largest mountain lakes in Eurasia) (Kownacki 1987).
Considering the lotic aquatic ecosystems, the most prevalent are springs, turbulent glacial streams and rivers, and large braided rivers (Fig. 3). These biotopes are located in different types of landscapes, ranging from forests (e.g. in the western Greater Caucasus; Fig. 3A, B, D) to open grasslands (e.g. in Armenia or Azerbaijan; Fig. 3C, E) and bare rocky landscapes of high altitudes. This large variety of aquatic habitats hosts a considerable number of invertebrates, of which mayflies are one of the most diversified.

The history of mayfly research in the Caucasus dates back to the 19th century when Georgi (1802) described a burrowing lowland species *Palingenia fuliginosa* from the Kura River. An intensive taxonomic investigation of the mayflies of this region was started by Tshernova (1931, 1938), who described several species of the families Leptophlebiidae, Heptageniidae, and Baetidae. After the study of Oligoneuriidae by Sowa and Zosidze (1973), mayfly research continued with numerous alpha-taxonomic studies regarding Baetidae and Heptageniidae, published by Sowa and Zimmermann (1976), Soldán (1977), Zimmermann (1977, 1981), Braasch (1978a,b, 1979a,b, 1980a,b, 1983a,b), Jacob and Zimmermann (1978), Zimmermann and Braasch (1979), and Sinitshenkova (1976, 1979a,b). Later on, Sowa (1984), Kluge (1987a,b, 1988, 1994), Kazancı and Thomas (1989), Kluge and Novikova (1992), and Sartori and Sowa (1992) significantly contributed to our knowledge of Caucasian mayfly diversity by the description of several additional species from the families Heptageniidae, Ephemeridae, and Leptophlebiidae.

The authors cited above described 51 species from the Caucasus region and closely adjacent areas. However, the more recent works published by Jacobus et al. (2009), Sroka and Godunko (2012), Kluge et al. (2013), Godunko et al. (2015a), Martynov et al. (2015), Martynov and Godunko (2017), and Bojková et al. (2018) describing 14 additional species indicated that mayfly research in the Caucasus is still far from being completed. A considerable part of the region is still poorly investigated. Whereas older studies focused on the Greater and Lesser Caucasus, representing the main mountain ranges of the region, the recent studies investigated the edges of the region, the Pontic and Alborz Mountains, as well as the surrounding Taurus and Zagros mountain ranges (e.g. Sroka et al. 2015, 2019a,b; Godunko et al. 2015b).

The traditional approach based solely on comparative morphology is now being replaced by an integrative taxonomy that defines species based on multiple and complementary perspectives (Dayrat 2005). This applies to the use of molecular data, representing a common component in the research of mayfly diversity taking place in European countries (e.g. Ståhls and Savolainen 2008; Vuataz et al. 2011; Moriniere et al. 2017; Tenchini et al. 2018; Weigand et al. 2019). As shown in Chapter II and IV, an incorporation of the molecular data to the research of the mayflies of the Caucasus may reveal new species and cryptic diversity. Moreover, this approach significantly improves the accuracy of the species identification that is fundamental to multiple basic and applied studies in taxonomy, evolution, ecology, and freshwater biodiversity assessment (Cardoni et al. 2015).

Several publications on Caucasian mayflies have focused on species inventories and summarized mayfly diversity in the north-eastern Turkey (e.g. Türkmen and Kazancı 2013, 2015), Georgia (Gabelashvili et al. 2018; Martynov et al. 2016;), Iran (Bojková et al. 2018), the Russian Federation (North Ossetia-Alania: Cherchesova 2004; Cherchesova et al. 2014; Prielbrusye National Park: Zalikhanov et al. 2016), Azerbaijan (Talysh Mountains: Palatov and Sokolova 2016), Armenia (Hrivniak et al. 2018; Chapter I), and various regions of Georgia, the Russian Federation, Armenia, Azerbaijan, and Turkey (Palatov et al. 2016).



Figure 3. An example of streams, rivers, and landscape variability in the Caucasus. A: glacial river in the western Greater Caucasus (Baksan River, Russia); B, C: brained rivers of the western (Teberda River, Russia) and eastern (Azerbaijan) Greater Caucasus, respectively; D: glacial river in the central Greater Caucasus (Belaya River, Adygea, Russia); E: steppe spring in the Lesser Caucasus (Armenian highlands, Armenia). Photos by J. Bojková and Ľ. Hrivniak.

Based on this recent knowledge, 130 mayfly species representing 15 families and 33 genera have been recorded in the Caucasus. All species are listed in Appendix. Nearly half of the species (61) are known only from the Caucasus region. Nevertheless, common Holarctic (6), Palearctic (28), Western Palearctic and European (18) species occur in the area, together with the species more or less widely distributed in the territories of Anatolia, Middle East (Turkey, Iran, Iraq, Syria, Israel, Jordan), and Central Asia (17).

No endemic families or genera occur in the Caucasus. Concerning Caucasian species, there are two types of endemism. A regional endemism encompassing endemic species widely distributed throughout the Caucasus region (21), and a local endemism referring to species restricted to the particular mountain ranges, the Greater Caucasus (19), the Lesser Caucasus and Pontic Mountains (13), and the Alborz (8).

In general, the greatest level of endemism was found in the family Heptageniidae with 42 described endemic species, followed by Baetidae (11), Leptophlebiidae (5), Ephemerelidae (2), and Oligoneuriidae (1). The families Heptageniidae and Baetidae are the most species-rich in the region and constitute nearly 70% of the entire mayfly fauna in the Caucasus. This diversity reflects the dominant representation of fast-flowing streams and rivers in the region. The majority of regional and local endemics in the Caucasus region are from the montane and submontane genera, namely *Rhithrogena* Eaton, 1882 (17), *Epeorus* Eaton, 1881 (10), *Electrogena* Zurwerra and Tomka, 1985 (11), *Ecdyonurus* Eaton,1868 (3) (Heptageniidae), and *Baetis* Leach, 1815 (8) (Baetidae).

5. *Epeorus* (*Caucasiron*): the best model group for the evolutionary study

The genus *Epeorus* Eaton, 1881 (Heptageniidae: Rhithrogeninae Lestage, 1917: Epeorini sensu Wang and McCafferty 2004) represents a group of "two tailed" heptageniid mayflies, i.e. with highly reduced paracercus in the larval stage. The larvae are scrapers inhabiting fast flowing mountain and submountain streams and rivers with cold water and stony bed substratum (Bauernfeind and Soldán 2012).

The larvae of *Epeorus* s.l. can be identified by (1) a reduced paracercus, (2) maxillae with a triad of greatly enlarged incisors, and (3) a dense row of setae along the anterior margin of the head. The imagines can be identified by a combination of the characters: (1) the absence of transverse suture on the mesonotum, (2) a first tarsal segment longer or equal in length to the second tarsal segment in the male, and (3) poorly developed basal cross veins in the forewings. Male genitalia are relatively variable within the genus. Titilators are present or absent and penis lobes are expanded or fused in their basal half (Webb and McCafferty 2008). The genus *Epeorus* currently includes more than 70 described species worldwide and, thus, ranks among the most diverse genera within mayflies (Barber-James et al. 2008).

Epeorus s.l. is one of the most diverse mayfly genera in the Caucasus. Except for a single representative of *Epeorus* s. str., *Epeorus* (*Epeorus*) *zaitzevi* Tshernova, 1981, all *Epeorus* species belong to the subgenus *Caucasiron* Kluge, 1997 (hereinafter *Caucasiron*). The global distribution of *Caucasiron* includes the eastern Mediterranean islands (Samos and Cyprus), Turkey, the Caucasus, Iraq, Iran, Central Asia (Kazakhstan, Tajikistan, Nepal, India), and south-western China (Guizhou province). The highest diversity is known in the Caucasus and adjacent areas, 15 species (including Samos Island) have been described to date (see Appendix). Central Asia and south-western China are each inhabited by a single species, making up altogether 17 confirmed *Caucasiron* species worldwide. A high species diversity in the Caucasus may be related to its specific geological history possibly affecting evolutionary processes, but may also be influenced by intensive research in this area. I

assume that the species diversity in Central Asia and China is underestimated at present. Moreover, based on the general habitus of larvae, at least three other species described in different genera from Central Asia (Braasch 1983c, 2006a) likely belong to *Caucasiron*. Their taxonomic revision is needed.

Caucasiron larvae are among the largest mayflies in the Caucasus (the larva of *E*. (*C*.) *magnus* grows up to 24 mm, without the length of cerci). Kluge (1997) defined the larvae of *Caucasiron* based on several characters: (1) the presence of a "suction disc" (Fig. 4B) (consisting of enlarged gill plate I, overlapping gill plates II–VI, and gill plate VII bent under the abdominal segments (Fig. 4C, D), (2) a projection on the costal margin of gill plates II–VI, representing unique apomorphy among Heptageniidae and mayflies in general (Fig. 4E, arrow), and (3) medio-dorsally directed hairs along the anterior margin of the head. Imagines are characterized by tubular penis lobes without dorso-lateral denticles (Fig. 4A,a) and well-developed median titilators (Fig. 4A,b).

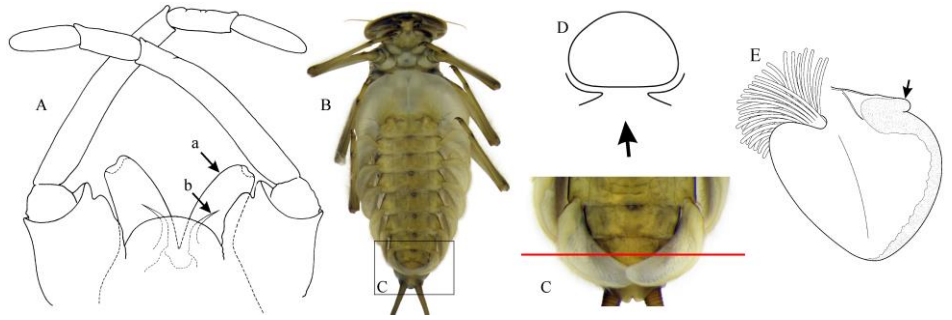


Figure 4. Main morphological diagnostic characters of *Caucasiron*, our model group for the evolutionary study in the Caucasus. A: male genitalia; a, points on tubular penis lobe; b, points on well-developed titilator; B: habitus of larvae from ventral view; C, D: cross section of abdomen and gill plate VII showing longitudinal fold (D); E: Gill plate III, arrow points on the projection on the costal margin.

For the study of the evolutionary history of mayflies from the Caucasus, I have chosen *Caucasiron* as the most appropriate model group, based on its biological features as well as for several practical reasons. Individual *Caucasiron* species possess various geographical and altitudinal distribution patterns. Some species are local endemics of the Greater Caucasus, Alborz Mountains, or Pontic Mountains inhabiting small high-

altitude streams and rivers, while other species are widespread throughout the Caucasus and adjacent areas and inhabit various lotic habitats, from small high-altitude streams to lowland braided rivers. The highest number of species and local endemics occur in the Greater Caucasus, the most prominent range in the region that was formed as an isolated island in the Paratethys Sea (Popov et al. 2004). I have assumed that different distributional patterns of species and a high level of endemism in the Greater Caucasus reflect various evolutionary histories.

I aimed to clarify: (1) where the closest relatives of *Caucasiron* are distributed, (2) when and how fast this group diversified, and (3) whether the high diversity and endemism in the Greater Caucasus reflect local speciation or have resulted from immigration from surrounding areas. Answers to these questions would significantly contribute to our knowledge of the evolution of the unique Caucasian mayfly fauna, which is currently unknown. From a general point of view, the results will clarify how, and to what extent, the specific geological history of the Caucasus affected evolution of its aquatic biota and endemism, and whether its diversification tracked the mountain orogeny, as was revealed in several taxa investigated so far from other mountain ranges.

The patterns of speciation of organisms within the Caucasus were generally coupled with the impact of climatic change during the Pliocene/Pleistocene. Regarding the speciation of cold-tolerant montane aquatic insects, it can be expected that Pleistocene climatic oscillations did not significantly affect their diversification, similarly as observed in montane mayflies of the genus *Rhithrogena* from the European Alps (Vuataz et al. 2016). An intensive Miocene orogeny and the isolation of mountain ranges might have a much greater influence on the diversification of Caucasian montane aquatic biota than climatic changes during the Pleistocene. However, these processes were not yet studied in the Caucasian aquatic insects and remain unknown. Therefore, I focus on this topic in Chapter IV based on *Caucasiron*. To analyze the evolutionary history of *Caucasiron* in time, the calibration of the molecular phylogenetic tree will be essential. Although fossil records of *Epeorus* are not available, several other fossils with an unambiguous systematic position within Heptageniidae (see Appendix A1 in Part

Chapter IV) allow us to perform a precise time calibration of diversification events.

Among more practical issues making *Caucasiron* a highly suitable model group is the possibility of comparing our newly collected specimens with the type material of most species obtained from the Stuttgart State Museum of Natural History. The accessibility of this material helped me to verify species identifications and prepare a precise taxonomic revision of all *Caucasiron* species (Chapter III).

Apart from using *Caucasiron* as a model group for studying the evolutionary history of mayflies in the Caucasus, I also aimed to clarify several systematic problems in the genus *Epeorus* s.l. In particular, the evolution of the larval suction disc, whose presence or absence are considered significant in the systematics of *Epeorus* s.l., and the relevance of which is extensively discussed in the literature. According to Braasch (2006b), the larval suction disc evolved only once within *Epeorus* s.l. and, thus, classified all species with this structure within a single (presumably) monophyletic genus *Iron* Eaton, 1885. This genus included subgenera *Iron* s.str. (distributed in Central Asia and the Nearctic Region), *Caucasiron*, *Ironopsis* Traver, 1935 (Nearctic region), and *Alpiron* Braasch, 2006 (Europe).

In contrast, Tshernova (1981) and Kluge (2004) considered a suction disc as a homoplasy of several unrelated lineages within *Epeorus* s.l. Furthermore, in contrast to Braasch (2006b), Kluge (2015) did not support the classification of European species with a suction disc in the subgenus *Alpiron*. According to him, these European species are rather related to Central Asian and Nearctic *Ironopsis*, and classified them into this subgenus. Whether the taxa with a suction disc represent a monophyletic group, and what are the actual relationships between European, Central Asian, and Nearctic species remain questionable and should be tested via the molecular approach. I therefore aimed to solve this problem in Chapter IV.

6. Scope and aims of the thesis

The overall goals of this thesis are to: (1) document mayfly diversity in the Caucasus Mountains by gathering all published data and extensive field research in the region, (2) revise the taxonomy and systematic position of *Caucasiron*, and (3) clarify the temporal and spatial origin of Caucasian mayflies based on a multi locus phylogenetic and biogeographical analyses of *Caucasiron*, the model group selected for the evolutionary study.

The thesis contains four chapters. The first chapter deals with the general diversity of Caucasian mayflies. The research is focused on Armenia, one of the least investigated countries within the Caucasus region, and aims to gather missing information on mayfly diversity and distribution in the Lesser Caucasus. The second and third chapters deal with the diversity and distribution of *Caucasiron*. Chapter two is focused on its taxonomy and distribution in the Caucasus and adjacent areas. As I care about the practical output of my research, chapter three is focused on the identification of *Caucasiron* species, which may be seen as a key component in research in the taxonomy and ecology of *Caucasiron* and stream ecology in general. In chapter four, I explore the effect of the geological history of the Caucasus on the species diversification of *Caucasiron*, and clarify the origin and age of *Caucasiron* lineage(s). In this chapter, I also analyze the phylogenetic relationships between *Epeorus* taxa with a suction disc and clarify their systematic positions within *Epeorus* s.l.

6.1. Specific aims and topics of the thesis

Chapter I

(1) Compilation of a comprehensive checklist of Armenian mayflies with a zoogeographical characteristic of their fauna.

(2) Description of the larva and male imago of a new species of *Ecdyonurus* (*Ecdyonurus*) based on morphology and molecular data.

(3) Provision of a differential diagnosis regarding other related *Ecdyonurus* species and the information on the habitat and distribution of the new species.

Chapter II

(1) Detailed taxonomic investigation of *Caucasiron* from the Caucasus and adjacent areas (Samos, Cyprus, Turkey, and Iran).

(2) Application of molecular data (*COI*) and various models of molecular species delimitation in an identification of individual species described based on comparative morphology.

(3) Provision of a morphological description for new lineages of *Caucasiron*, their morphological diagnostic characters, and comparison with related species.

(4) Give the information on the distribution and habitat preferences of all new species.

Chapter III

(1) Provision of the larval identification guide to all currently known *Caucasiron* species distributed in the Caucasus and adjacent areas.

(2) Summarization of the information on their distribution and habitat preferences.

Chapter IV

(1) Test the monophyly of three morphologically similar subgenera (with a suction disc) of *Epeorus* s.l. (*Caucasiron*, *Iron* and *Ironopsis*) and clarify their phylogenetic relationships and systematic position within *Epeorus* s.l.

(2) Estimate phylogenetic relationships and divergence times of *Caucasiron* based on the multi locus phylogeny and dataset covering the entire Caucasus and relevant adjacent mountains ranges.

(3) Explore the pattern of speciation and extinction rate over time and reconstruct ancestral distribution of individual *Caucasiron* lineages regarding the important geological events in the Caucasus region.

(4) Test whether orogeny forming the Caucasus mountain system promoted the diversification of *Caucasiron* species and whether the Miocene isolation of the Greater Caucasus caused the differentiation of mayfly lineages endemic to this mountain range.

(5) Explore possible effects of Pleistocene climatic oscillations on the speciation of *Caucasiron* in the Caucasus.

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PART TWO: RESULTS OF THE THESIS

Chapter I

Diversity of Armenian mayflies (Ephemeroptera) with the description of a new species of the genus *Ecdyonurus* (Heptageniidae)

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Diversity of Armenian mayflies (Ephemeroptera) with the description of a new species of the genus *Ecdyonurus* (Heptageniidae)

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Abstract

We provide the first commented checklist of Armenian mayflies, based on all relevant literature and recent extensive sampling of 72 localities throughout Armenia during 2011, 2014, and 2015. Altogether 46 species are listed, eight of them reported from Armenia for the first time. One new species, *Ecdyonurus* (*Ecdyonurus*) *eurycephalus* **sp. nov.** is described (larva and male imago) based on morphological and molecular (COI) data. The species is characterized by a unique head shape in male imago and by the presence of tracheal filaments on gill plate VII in the larval stage.

Key words: Caucasus, biogeography, taxonomy, faunistic research, molecular association

Introduction

Armenia is situated in the Lesser Caucasus mountain range, surrounded by Turkey and the Nakhichevan exclave to the west, Azerbaijan to the east, Georgia to the north, and Iran to the south. The territory of Armenia is located at the junction of the Circum-boreal, Irano-Turanian, and Southern Caucasic floristic regions, where mountainous landscape prevails (Sayadyan 2005). The Armenian Highlands are notable for their landscape and climatic diversity, the latter ranging from sub-tropical in the lowlands to alpine in the high mountains. The main landscapes are desert, semi-desert, steppe and meadow-steppe, forest, and sub-alpine and alpine meadows. A great variety of landscapes, a relatively mild climate, and the absence of recent complete glaciation in the geological past promote the existence of high biological diversity, in part relict.

Concerning mayfly fauna, Armenia has been studied only sporadically. Up to now, no extensive sampling has been done in the country with the aim of gathering complex information on mayfly biodiversity. The mayflies of Armenia have been studied either within the taxonomical research of individual genera (*Baetis*—Jacob & Zimmermann 1978, Zimmermann 1981; *Baetis* (*Acentrella*)—Zimmermann & Braasch 1979; *Electrogena*—Braasch 1983b; *Rhithrogena*—Kluge 1987a; Sinitshenkova 1979; *Epeorus*—Sinitshenkova 1976; *Heptagenia*—Kluge 1987b, 1989; *Ephemera*—Kluge 1988; *Oligoneuriella*—Soldán & Landa 1977), faunistic investigation (for references see in Table 1), or hydrobiological research as a component of macrozoobenthos (e.g. Kachvoryan *et al*.

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2008; Gabrielyan *et al*. 2010; Hakobyan *et al*. 2010; Shcherbina 2013; Hakobyan & Jenderedjian 2016; Asatryan *et al*. 2016; Hovhannisyan & Shahnazaryan 2016). If we take into account determination at the species level only, the results of the above-mentioned studies indicate the occurrence of altogether 38 mayfly species in the territory of Armenia.

In the present contribution we provide the first comprehensive list of taxa reported from Armenia up to now, based on a review of the relevant literature and data obtained from the field sampling of all Armenian provinces during 2011, 2014, and 2015.

Following the recently published checklists covering Georgia (Gabelashvili *et al*. 2018) and Iran (Bojková *et al*. 2018) this contribution represents a further step towards a more systematic approach to the study of mayflies in the Caucasus and surrounding regions, where the needs of hydrobiological research are constantly increasing. In this context, the lists of species enable easier orientation within the fauna and allow more accurate identification of individual species for both taxonomical and hydrobiological research.

Apart from the comprehensive Armenian checklist, we provide a description of a new species of *Ecdyonurus* (*Ecdyonurus*) which was discovered during our field trip to Armenia in 2015. We **(i)** describe the larva and the male imago (associated by DNA comparison), **(ii)** provide differential diagnosis with regard to related *Ecdyonurus* species, and **(iii)** give the basic information on its habitat and distribution.

Material and methods

Published records of mayfly species in Armenia were extracted from available literature and summarised in Table 1. The material was collected by Ľ. Hrivniak, P. Manko, J. Oboňa (2015) and D. Palatov (2011, 2014) from altogether 72 localities (Fig. 1). The samplings were performed using kick-sampling method and additional individual collections from different habitats using standard benthic net. Sampling of larvae was supplemented by sweeping of imagoes and subimagoes from riparian vegetation by a standard entomological sweeping net. All specimens were preserved in 96% EtOH in the field and stored in the laboratory at -20 °C. Some specimens were mounted on slides with HydroMatrix® (MicroTech Lab, Graz, Austria). The material was determined by P. Sroka, Ľ. Hrivniak, R.J. Godunko, D. Palatov and M. Polášek, and sums up to 3284 specimens. Determination was performed using a combination of the original taxonomic descriptions (list of geographically relevant species obtained from Ephemeroptera of the World online database; http://www.insecta.bio.spbu.ru/#Ephemeroptera) and publications specialized on particular genera (*Baetis*—Godunko *et al*. 2004a, Martynov & Godunko 2017; *Caenis*—Malzacher 1984, Buffagni 1997; *Habroleptoides*—Kluge 1994), supplemented by Bauernfeind and Humpesch (2001) and Bauernfeind & Soldán (2012).

All material is deposited in the collection of the Czech Academy of Sciences, Biology Centre, Institute of Entomology, České Budějovice, Czech Republic and Lomonosov Moscow State University.

Morphological analysis. Morphological characteristics for the description of a new *Ecdyonurus* species were used according to Bauernfeind and Humpesch (2001), Bauernfeind (1997) and Kluge *et al*. (2013).

Characters (including abbreviations) for the description of larval maxillae and labial palpi were adopted from Haybach (1999). To avoid distortions from microscopical slide, measuring of metafemora, labrum and pronotum (including recording of shapes) were performed *in situ* (see Bauernfeind 1997); gills, labial glossae and paraglossae were measured and drawn from slides. Expression "mature larva" refers to fully grown larvae (as on Figs 15–17) and also larvae of later instars, without fully developed wing pads. Abbreviation "n" refers to number of individuals investigated. Drawings were made using a stereomicroscope Olympus SZX7 and a microscope Olympus BX41, both equipped with a drawing tube. Photographs were made using a Canon EOS 1200D camera mounted on a Leica M205 C stereomicroscope. All photographs were subsequently enhanced with Adobe Photoshop™ CS5. For scanning electron microscopy, samples were gradually transferred to acetone, critical point dried and coated with gold by sputtering using a Baltec SCD050 Sputter Coater. Observations were taken on the scanning microscope Jeol JSM 7401F at 4 kV at the Institute of Parasitology, BC CAS, České Budějovice, Czech Republic.

Molecular analysis. To associate the larvae and imagoes of a new species, we used the mitochondrial Cytochrome oxidase I gene (COI). DNA was extracted from six specimens (four larvae of different instars and two male imagoes). DNA extraction, PCR amplification, sequencing and alignment construction were performed

according to Hrivniak *et al*. (2017). Obtained sequences are deposited in GenBank (see Appendix 2). Pairwise genetic distances were calculated in Mega version 7 (Kumar *et al*. 2016) using Kimura 2-Parameter model (K2P; Kimura 1980). The COI sequences were compared with the same fragment of COI gene of two morphologically related *Ecdyonurus insignis* (Eaton, 1870) (six individuals) and *Ecdyonurus asiaeminoris* Demoulin, 1973 (one individual) obtained from GenBank and Bold systems (http://www.boldsystems.org) (see Appendix 2). Taxonomic composition of the COI dataset was tested by an online version of the Poisson Tree Processes and its Bayesian implementation (bPTP; http://species.h-its.org/; Zhang *et al*. 2013) with 500000 MCMC generations and default settings. An input tree was a consensus tree generated by Bayesian analysis implemented in MrBayes 3.2.6. (Ronquist & Huelsenbeck 2003) with substitution model HKY + I (jModelTest 2.1.4; Darriba *et al*. 2012). Bayesian analysis was carried out by 2 independent runs of four MCMC chains for 1 million generations (sampling every 1000 generation); first 25% samples were discarded as a burnin. The species *Electrogena galileae* (Demoulin, 1973) (Ecdyonurinae) was selected as an outgroup; sequence was obtained from GenBank (accession no.: KX447085.1; Yanai *et al*. 2017).

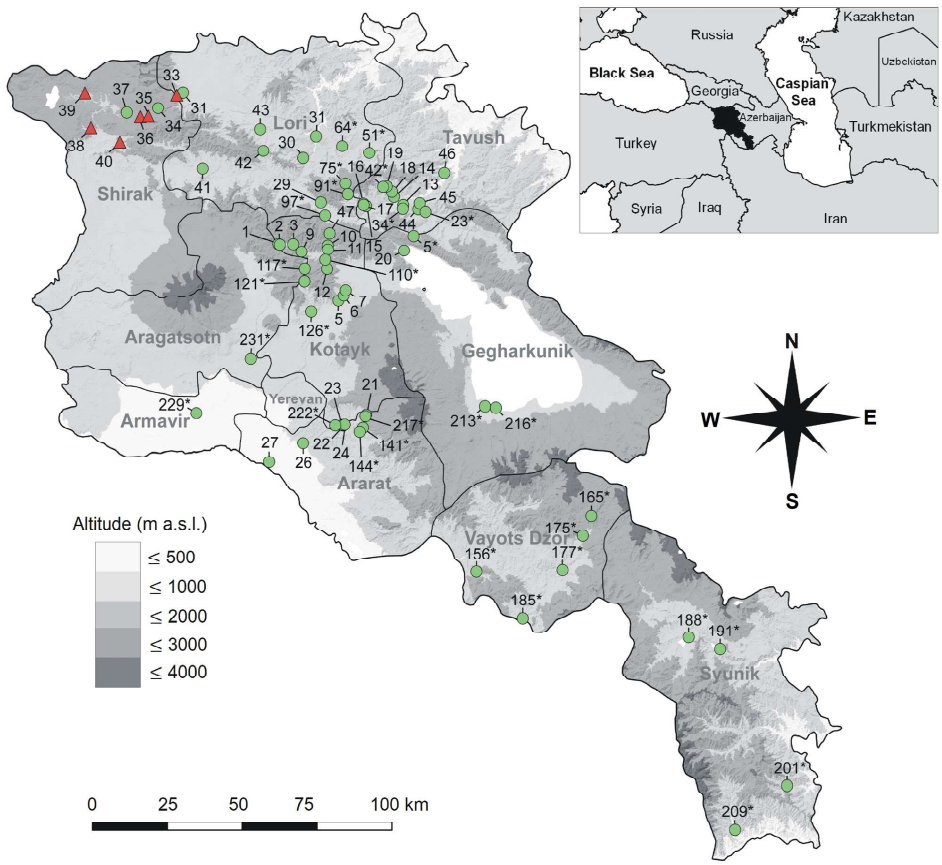


FIGURE 1. Distribution of sampling sites in Armenia. Occurrence of *E*. (*E*.) *eurycephalus* **sp. nov.** shown by red triangles. Sites sampled by D. Palatov marked with asterisk, other sites sampled by Ľ. Hrivniak, P. Manko & J. Oboňa. The numbers of sampling sites correspond to Appendix 1 and the codes on labels and field protocols of the respective collectors.

Results

I. Taxonomy

Morphological analysis

***Ecdyonurus* (*Ecdyonurus*) *eurycephalus* Hrivniak & Godunko, sp. nov.**
(Figs 2–45)

Type material (numbers in parentheses correspond to localities on Fig. 1 and Appendix 1).

Holotype: male imago: ARMENIA, Shirak province, NE of Musayelyan village; small steppe river, tributary of Akhurian River; 41°00'13.15" N, 43°57'24.87"E; 2195 m a.s.l.; 02/09/2015, **(36)**.

Paratypes: 2 male imagoes (genitalia and wings of 1 individual mounted on slide), 9 female (1 mounted on slide) and 6 male larvae (4 mounted on slide), same data as holotype **(36)**; 10 female (2 mounted on slide) and 8 male larvae (1 mounted on slide): ARMENIA, Shirak province, NE of Musayleyan village; steppe stream, tributary of Akhurian River; 41°00'20.12"N, 43°59'14.04"E; 2286 m a.s.l.; 02/09/2015, **(35)**; 3 female and 1 male larvae: ARMENIA, Shirak province, N of Amasia village, steppe stream, tributary of Akhurian River; 40°58'20.49"N, 43°46'06.88"E; 1987 m a.s.l.; 02/09/2015, **(38)**; 1 female larva: ARMENIA, Shirak province, E of Aghvorik village, steppe stream, tributary of Akhurian River; 41°04'21.19"N, 43°44'44.83"E; 2052 m a.s.l.; 03/09/2015, **(39)**; 3 female (1 mounted on slide) and 5 male larvae (1 mounted on slide): ARMENIA, Shirak province, E of Torosgyugh village, small steppe river, tributary of the Akhurian river; 40°55'54.99"N, 43°52'45.26"E; 1885 m a.s.l.; 03/09/2015, **(40)**.

Other material studied (not type): 3 female and 1 male larvae, **(36)**; 1 female larva: ARMENIA, Lori province, W of Katnarat, steppe stream, tributary of the Dzoraget River; 41°03'59.85"N, 44°05'44.24"E; 1949 m a.s.l.; 02/09/2015, **(33)**; 2 female and 1 male larvae, **(35)**; 1 female larva, **(40)**.

All types and other studied material were collected by P. Manko, J. Oboňa and Ľ. Hrivniak. Material is deposited in the collection of the Czech Academy of Sciences, Biology Centre, Institute of Entomology, České Budějovice, Czech Republic.

Description

Male imago (Figs 2–14).

Measurements (n=2): body length 9.2–10.3 mm; length of forewings 9.0–10.9 mm, hind wings 3.4–4.0 mm; cerci broken. General colour of body dark, dirty olive-brown to brownish-black. Marked reddish to red-orange spots and stripes on thorax and abdomen.

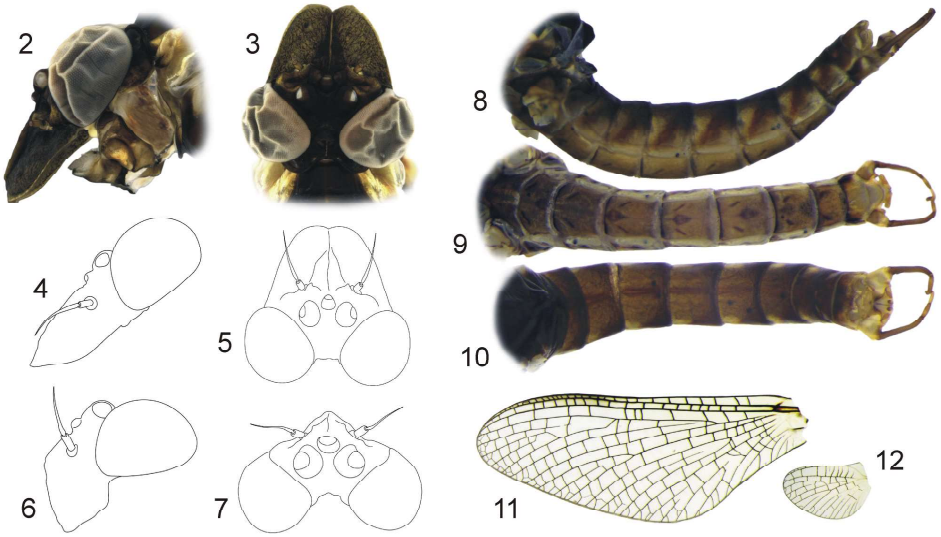
Head. General colour brown to blackish-brown and black. Facial plate with blackish maculations, oblong-shaped, directed anteriorly; anterior margin truncate, slightly concave medially (Figs 3, 5); in lateral view forming distinctly obtuse angle to body axis within 22–40° (Fig. 2, 4). Antennae light brown; flagellum slightly darker than scape and pedicel. Ocelli whitish-yellow apically, dark brown basally. Eyes widely separated; distance between eyes slightly less than 2× of central ocellus base width (Figs 3, 5). Eyes not large, moderately outwardly convex without distinct stripes or maculations laterally (Fig. 2). Indistinct basal reddish-brick to dirty violet stripe along anterior and inner margin of eyes; blurred dirty yellow band centrally; apical portion dirty violet; no distinct margin between differently coloured portions of eyes (colouration only faintly visible in fixed material).

Thorax. General colour dark, intensively brown to black. Ventral side of thorax brown with sparse blackish maculations. Pronotum brown to dark brown with two central elongated paler stripes. Mesothorax intensively blackish-brown; lateral sides yellowish-brown with several brick-reddish to brown maculations. Anepisternum and anterior paracoxal suture brownish-black. Ventral side of mesothorax same colour as dorsal side. Metathorax more or less unicolorous brownish-black.

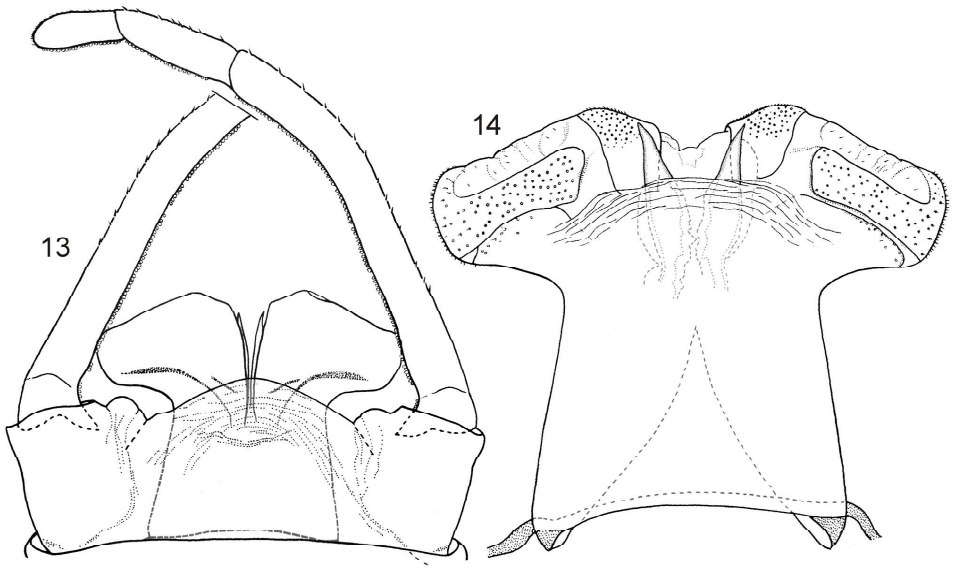
Wings. Forewings hyaline, translucent, with dirty yellow to dirty white shade (Fig. 11). Longitudinal venation dark brown proximally and centrally, brown to light brown distally; wing venation typical for Heptageniidae. Pterostigma slightly milky with 17–20 simple and forked cross veins. Hind wings same colour as forewings (Fig. 12).

Legs. Forelegs markedly darker than middle and hind legs, unicolorous, intensively brown to brownish-black.

Middle and hind legs of same colour: femora brown distally, with diffuse elongated bright maculation centrally; tibiae unicolorous yellowish-brown to light brown; tarsi brown, first segment slightly paler. Pretarsal claws of same colour as last segments of tarsi.



FIGURES 2–12. *Ecdyonurus* (*Ecdyonurus*) spp., male imagoes: 2–5, 8–12, *E.* (*E.*) *eurycephalus* **sp. nov.**: 2, 4, head lateral; 3, 5, head dorsal; 8, abdomen lateral; 9, abdomen ventral; 10, abdomen dorsal; 11, forewing; 12, hind wing; 6–7, *E.* (*E.*) *ornatipennis*: 6, head lateral; 7, head dorsal.



FIGURES 13–14. *Ecdyonurus* (*Ecdyonurus*) *eurycephalus* **sp. nov.**, genitalia of male imago: 13, styliger, forceps and penis ventral; 14, penis dorsal.

Abdomen. Terga with contrasting pattern (Fig. 10): tergum I darkest, unicolorous dark brown; terga II–VIII (IX) dirty brown to intensively brown with narrow brick-red longitudinal stripe centrally, lateral portion of terga with relatively large triangular brown spots posteriorly and brick-red to brown oblique stripes expanded posteriorly (oblique stripes surrounded with yellow, Fig. 8); terga IX and X unicolorous reddish-brown, slightly paler anteriorly. Abdominal sterna paler than terga, unicolorous light brown with pair of brown oblique strokes anteriorly on segments II–VIII; intensively brown spots anterolaterally on sternum IX. Dirty-violet nerve ganglia visible on sterna II–VIII (Fig. 9). Basal segments of cerci brown to intensively brown, yellow to whitish-yellow apically; joints of segments brownish or blackish.

Genitalia. Styliger light brown; two small slightly asymmetrical protuberances near forceps bases (Fig. 13). Forceps base yellowish-brown; forceps brown, two distal segments slightly paler; forceps segment II with indistinct hump at inner margin basally.

Penis lobes yellowish to light brown and brown; penial sclerites dirty-yellow to intensively brown. Penis lobes moderately expanded laterally and relatively elongated; outer outline nearly rectangular (Fig. 14). Basal sclerite poorly developed, with weakly expanded posterior margin; no prominent setae or spines along posterior margin of basal sclerite. Lateral sclerite relatively narrow, parallel sided centrally, slightly wider distally. Apical sclerite short, expanded apically; tip slightly projecting above lobes; surface of sclerite covered by small pointed setae. Titillators light brown, relatively narrow, with 1–2 small poorly visible subapical teeth.

Female imago, eggs and subimagoes: unknown

Larva (Figs 15–45).

Measurements: body length of mature larvae 11.6–12.9 mm (female; n=7), 9.1–10.7 mm (male; n=5). Cerci and paracercus broken. General colouration of body yellowish-brown with pale yellowish markings.

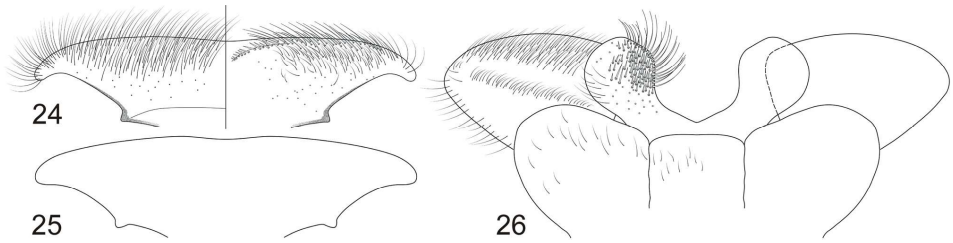


FIGURES 15–23. *Ecdyonurus* (*Ecdyonurus*) *eurycephalus* **sp. nov.**, larva: 15, habitus dorsal; 16, habitus ventral; 17, habitus lateral; 18, head dorsal; 19–21, semipronotum of mature larva; 22–23, semipronotum of early-instar larva.

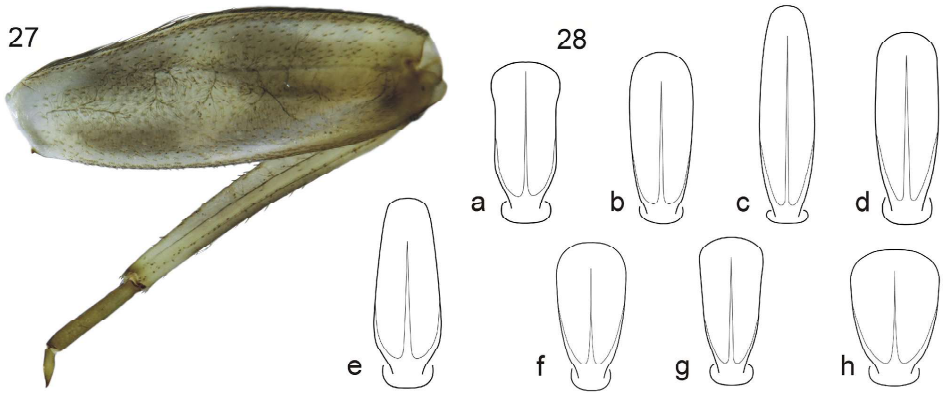
Head. Yellowish-brown, without distinct maculation. Anterior half slightly darker, with pale macula extending from medial ocellus; pair of pale spots situated above lateral ocelli (extent of macula and pale spots vary individually). Pair of dark brown maculae situated near inner edges of compound eyes. Pair of stripes extending from lateral ocelli to lateral margins of head (Fig. 18). Antennae unicolorous, of same color as head. Eyes blackish grey; ocelli black, whitish around. Shape of head ellipsoid; lateral margins widely rounded, posterior margin slightly concave medially.

Mouthparts (n=10). Labrum relatively slender and wide (Figs 24–25); lateral lobes more or less curved (subject to individual variation). Ratio of total labrum width to its width at the base 1.5–1.7. Number of bristles along sides of ventral groove 8–13, stout and arranged in one row. Number of bristles on left prostheca 9–13

(usually 10–13), on right prostheca 8–12 (usually 8–9). Longer hair-like setae on distal margins of hypopharyngeal superlingua more or less reaching its apex (subject to individual variation). Maxillae (abbreviations according to Haybach 1999): N_CBS = 20–24 (usually 20–21) + 1–3 setae; N_TCB$_5$ = 9–14 (usually 10–11); N_DOR = 11–17; N_OUT = 0–2; N_VEN = 5–16; N_PLP = >10; N_PLS = 37–52; N_PLH = 4–10 (without regular grouping; connected with N_PLS); N_PLBas = 6–16. Labial glossae rounded or slightly stretched laterally; paraglossae stretched laterally (Fig. 26); N_LPH = 24–46 (usually 31–37), arranged to 1–3 irregular rows.



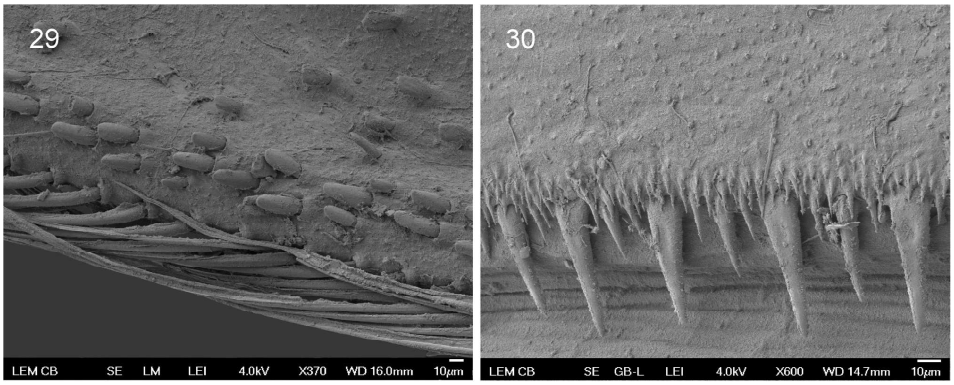
FIGURES 24–26. *Ecdyonurus* (*Ecdyonurus*) *eurycephalus* **sp. nov.**, mouthparts: 24, labrum dorsal (left) and ventral (right); 25, labrum contour; 26, labial glossae and paraglossae: left part ventral view, right part contour.



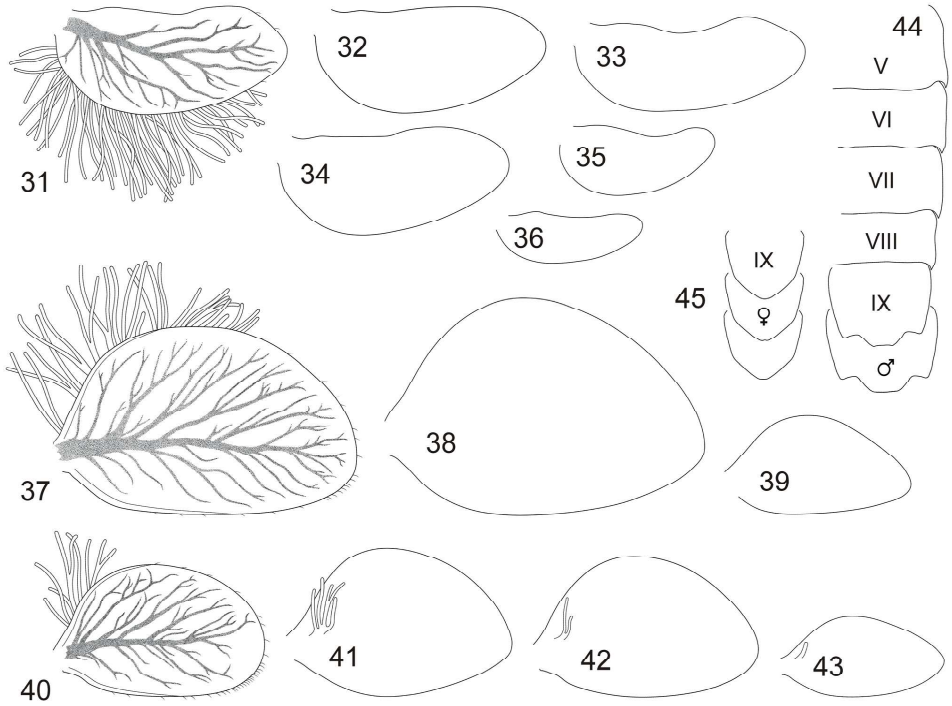
FIGURES 27–28. *Ecdyonurus* (*Ecdyonurus*) *eurycephalus* **sp. nov.**, leg: 27, hind leg dorsal; 28a–h, setae on dorsal surface of femora.

Thorax. Pronotum yellowish-brown with pale markings on dorsal margin; pronotal projections paler. Pronotal projections of mature larvae slightly curved inwards (Fig. 21) or more-less parallel to body axis (Figs 19–20); its apex blunt or bluntly pointed. Pronotal projections of early-instar larvae distinctly shorter, narrower and more acute apically (Figs 22–23). Width/length ratio of semipronotum to pronotal projection (a:b; see Bauernfeind and Humpesch, 2001: 69, fig. 196): mature larvae 2.4–2.9 (n=23), early-instar larvae 2.7–4.1 (n=10). Length of pronotal projection to total length of outer margin of semipronotum in mature larvae 2.3–2.9, in early-instar larvae 3.1–3.8. Mesonotum yellowish-brown with pale markings dorsally; metanotum yellowish.

Legs. Yellowish-brown to brown; colouration pattern on dorsal surface of femora as on Figs 17, 27 (colouration intensity of legs varies among individuals and instars). Trochanters with 2–3 irregular rows of blunt spatulate setae. Dorsal surface of femora with blunt spatulate setae on all legs; shape of setae varies among individuals and between distal and proximal portion from slender and elongated (Figs 28a–e) to widened apically or relatively wide (Figs 28f–h). Femoral setae arranged to 2–3 irregular rows near fore and hind margins (Fig. 29). Fore margin of femora with blunt spatulate setae (as on dorsal surface) and sparsely distributed blunt slender setae; hind margin of femora with row of blade-like setae and blunt stout setae (approx. in distal half of femora). Length/width ratio of metafemora in mature larvae 2.6–2.9 (n=14); widest part of metafemora in its midlength.



FIGURES 29–30. *Ecdyonurus* (*Ecdyonurus*) *eurycephalus* **sp. nov.**, larval abdomen and leg: 29, hind margin of middle femora; 30, posterior margin of tergum III.



FIGURES 31–45. *Ecdyonurus* (*Ecdyonurus*) *eurycephalus* **sp. nov.**, gills (flattened on slide) and larval abdomen: 31–36, gill plate I: 31–34 mature larva; 35–36, early-instar larva; 37–39, gill plate IV: 37–38, mature larva; 39, early instar larva; 40–43, gill plate VII: 40–42, mature larva; 43, early-instar larva; 44, abdominal sterna V–IX (male); 45, sternum IX (female).

Tibiae yellowish-brown, paler distally. Inner and ventral portion of tibiae with pointed or/and bluntly pointed setae (most numerous on hind legs); dorsal portion of tibiae with row of blunt spatulate setae and scattered

individual setae of same shape. Tarsi brownish with spatulate and bluntly pointed setae as on tibiae. Outer margin of tibiae and tarsi with row of setae. Claws brownish, usually with 3 denticles (individually up to 6).

Abdomen. Terga yellowish brown to brown with pale markings (Fig. 15). Tergum I yellowish, without distinct maculation (dark medial macula present in some specimens). Terga II–IX with colouration pattern consisting of: **(i)** variously contrasting dark medial macula on terga II–IX; **(ii)** yellowish macula around dark medial macula variously shaped—diffused on terga II and III (IV); U-shaped on terga (IV) V–VII or forming a pair of pale stripes (usually on tergum VI and VII); large U-shaped or diffused on terga VIII and IX; **(iii)** lateral oblique dark stripes on terga II–VIII (as in male imago, see Fig 8). Tergum X brown (with pair of pale medial maculae in some specimens). Early-instar larvae with pair of medio-lateral yellowish maculae near posterior margin of terga II–VII (on terga VIII and IX fused with larger yellowish macula). Posterior margin of abdominal terga with large pointed spines alternating with smaller spines; surface of terga rough and covered with sparse hair-like setae (Fig. 30).

Sterna yellowish-white, without distinct colouration pattern; dark nerve ganglion visible (Fig. 16) (visibility reduced in some individuals). Posterolateral projections poorly developed (Figs 16, 44). Sternum IX posteriorly narrowed, apically nearly straight, rounded or medially slightly concave in female (Fig. 45); trapezoid, medially slightly concave or straight in male (Fig. 44).

Gills greyish brown, dorsal portion distinctly darker (see Figs 15, 17). Gill plates relatively broad and asymmetric. Gill plate I dorsally more or less concave or nearly straight; ventrally rounded; distally narrowed and apically blunt (Figs 31–34). Gill plate IV dorsally rounded, ventrally nearly straight or slightly rounded; distally narrowed and apically rounded or bluntly pointed (Figs 37–38); length/width ratio 1.5–1.7; widest part in 1/2 of its length. Gill plate VII with tuft of tracheal filaments (Figs 40–43); its length and density vary among individuals and instars (sometimes reduced to short single filament, sporadically absent - observed in single specimen); shape similar to gill plate IV, widest part in 1/3–1/2 of its length. In early-instar larvae all gill plates distinctly elongated and narrowed (Figs 35–36, 39, 43).

Cerci and *paracercus* yellowish-brown; segments with pointed bristles alternating with hair-like setae.

Etymology. The name of the species refers to the shape of the head of male imago. The name is derived from the Latin „cephalus“ that refers to the head and prefix „eury-“ that express broad and elongated shape of its facial plate.

Diagnosis. The attribution of *E.* (*E.*) *eurycephalus* **sp. nov.** to the subgenus *Ecdyonurus* (*sensu* Bauernfeind & Soldán 2012) can be defined in *male imago* by **(i)** shape of apical sclerite of penis (i.e. apical sclerite of penis subparallel to the body axis, straight, relatively short and blunt), **(ii)** presence of oblique stripes on lateral portions of abdominal terga; in *larva* by presence of **(iii)** longer hair-like setae on the apical margins of hypopharyngeal superlinguae, **(iv)** one row of stout bristles on the ventral margin of labrum.

E. (*E*.) *eurycephalus* **sp. nov.** can be recognized from all other species within the subgenus by the combination of the following diagnostic characters:

Male imago: **(i)** general colour of body markedly dark, dirty olive-brown to brownish-black; **(ii)** head with facial plate directed anteriorly, forming distinctly obtuse angle to body axis in lateral view (Figs 2–5); **(iii)** wings slightly opaque, dirty yellow to dirty white (Figs 11–12); **(iv)** styliger plate with two small protuberances near forceps base; **(v)** forceps segment II with indistinct hump at inner margin basally (Fig. 13); **(vi)** penis lobes relatively elongated, moderately expanded laterally, with outer outline nearly rectangular; **(vii)** titillators relatively narrow with scarce denticulation (Fig. 14).

Larva: **(i)** shape of pronotal projections in mature larvae as on Figs 19–21 and in early instar larvae as on Figs 22–23; **(ii)** blunt spatulate bristles on the dorsal suface of femora of all legs (Figs 28–29); **(iii)** relatively broad shape of gill plates I in mature larvae (Figs 31–34); **(iv)** presence of a tuft of tracheal filaments on the gill plates VII (Figs 40–43); **(v)** absence of a colouration pattern on abdominal sterna (dark nerve ganglia often visible) (Fig. 16).

Distribution, habitat and biology. The data on the distribution of *E*. (*E*.) *eurycephalus* **sp. nov.** are rather limited. The species has been recorded from only six localities in northern Armenia (Provinces Shirak and Lori; see Fig. 1). The species was recorded only in small steppe brooks and rivers (Figs 46–48), which suggests its possible preference for smaller steppe watercourses with gravel substrate. The altitude of localities ranged from 1885 to 2286 m a.s.l. Since suitable habitats also occur in nearby countries (Georgia, Eastern Turkey), most probably the distribution of *E*. (*E*.) *eurycephalus* **sp. nov.** is not limited to the territory of Armenia.

The mature larvae and imagoes (together with early-instar larvae) were observed in August/September. The other mayfly species sporadically occurring in the same sites were *Habroleptoides confusa* Sartori & Jacob, 1986, *Ephemerella ignita* (Poda, 1761), *Baetis lutheri,* Müller-Liebenau, 1969, *B*. cf. *vadimi* Godunko, Palatov &

Martynov, 2015, *B. muticus* (Linnaeus, 1758), *B. ilex* Jacob & Zimmermann, 1978, *B. rhodani* (Pictet, 1843), *B. buceratus* Eaton, 1870, *B. samochai* Koch, 1981, *Epeorus caucasicus* Tshernova, 1981 and *Ecdyonurus* (*Ecdyonurus*) sp.



FIGURES 46–48. Sites of occurrence of *Ecdyonurus* (*Ecdyonurus*) *eurycephalus* **sp. nov.**: 46, site 36 (type locality); 47, site 38; 48, site 35.

Morphological affinities and discussion

Adult stage. The species *E.* (*E.*) *eurycephalus* **sp. nov.** occupies a relatively isolated position within all representatives of the genus *Ecdyonurus* (including both subgenera) by the structure of the facial plate and its position relatively to the body axis in male imago. Its facial plate is oblong, directed anteriorly, in lateral view forming a distinctly obtuse angle to the body axis, in contrast to the relatively narrow and truncate facial plate oriented more or less at right angle to the body axis in the overwhelming majority of Heptageniidae species (see Figs 6–7).

However, a similar shape and position of the facial plate is described for some extinct and extant taxa of Heptageniidae. Godunko (2007: 66, figs 1, 2) described and figured female imago of *Ecdyonurus* (*Nestormeus*) *groehnorum* Godunko, 2007 from Eocene Baltic amber, with a relatively enlarged facial plate directed anteriorly. The systematic position of this fossil taxon remains unclear. Nevertheless, the preliminary attribution of *Nestormeus* to Ecdyonurinae incertae sedis (*Ecdyonurus* s.l. in Godunko 2007) can be confirmed on the basis of the structure of parallel-sided furcasternal protuberances of the mesothorax.

The adults of two extant genera of Ecdyonurinae, namely *Notacanthurus* Tshernova, 1974 and *Thamnodontus* Kluge, 2004, known from the Southern and Eastern Palaearctic, are characterized by the presence of a more or less significantly expanded facial plate (see Kluge 1983: 28, fig. 1ж, е; Webb & McCafferty 2008: 39, fig. 131). Besides Ecdyonurinae, adult females of the Nearctic genus *Raptoheptagenia* Whiting & Lehmkuhl, 1987 (Heptageniinae) and adult males of Central Asian species *Cinygmula hutchinsoni* (Traver, 1939) (Rhithrogeninae)

possess a similar head structure (see Webb & McCafferty 2008: 39, fig. 132; Kluge 2015: 304, figs 2, 3). We can assume that the presence of the expanded facial plate as observed in *E.* (*E.*) *eurycephalus* **sp. nov.** represents a rare morphological trait occurring in several unrelated taxa within Heptageniidae.

In some details of the shape and structure of the genitalia, *E.* (*E.*) *eurycephalus* **sp. nov.** resembles some closely related species of the subgenus *Ecdyonurus*, namely *E.* (*E.*) *androsianus* (Braasch, 1983a), *E.* (*E.*) *asiaeminoris*, *E.* (*E.*) *insignis*, *E.* (*E.*) *starmachi* Sowa, 1971, and especially *E.* (*E.*) *solus* Godunko, Kłonowska-Olejnik & Prokopov, 2007. On the other hand, the new species clearly differs: **(i)** from *E.* (*E.*) *androsianus* in the colouration of the body (e.g. eyes, wings and especially the abdominal terga dorsally (see Braasch 1983a: 117–118, figs 1–4; Bauernfeind & Soldán 2012: 253, 584, fig. 113); **(ii)** from *E.* (*E.*) *asiaeminoris* in the shape of the shorter apical sclerite, less projecting and narrower, and especially in the colouration of the C and Sc fields of the forewings (see Demoulin 1973: 12–14, figs. 6a–b; Sartori 1992: 842, fig. 12); **(iii)** from *E.* (*E.*) *insignis* by weakly developed humps on forceps segment II, and especially in the colouration of abdominal sterna (see Bauernfeind & Soldán 2012: 261, 586, fig. 145); **(iv)** from *E.* (*E.*) *starmachi* in the colouration and shape of oblique strokes on the lateral sides of abdominal terga, the colour of the forewing, and in the absence of numerous distinct subapical teeth on the apical sclerite of the penis lobes (see Sowa 1971: 407–408, figs 1–3; Bauernfeind & Soldán 2012: 267, 268, 587, fig. 154); **(v)** from *E.* (*E.*) *solus* by the lighter colouration of the body, different colour and pattern combination on the lateral portion of the eyes, and by the arrangement of stripes and spots on the abdominal terga and sterna. Additionally *E.* (*E.*) *solus* differs from *E.* (*E.*) *eurycephalus* **sp. nov.** in the presence of robust titillators (see Kłonowska-Olejnik *et al*. 2007: 54–56, figs. 1–5; Bauernfeind & Soldán 2012: 266, 267, 587, fig. 153).

Only two species of the subgenus *Ecdyonurus* (until recently *Ecdyonurus venosus* species-group) have previously been described from the Caucasus. *E.* (*E.*) *ornatipennis* Tshernova, 1938 was described on the basis of two male imagoes from Disar village (Azerbaijan, Nakhichevan, Lesser Caucasus, Dernis-chai River), and reported later from the whole Caucasus (Zhadin 1940; Kasymov 1972; Cherchesova 2004; Puthz 1978; Palatov & Sokolova 2016). The species has also been collected in Iran (Braasch 1981; this record needs to be confirmed) and Turkey (for summary of references see Salur *et al*. 2016). The first description of larvae was based on non-associated material from Armenia (Garni village) and published by Braasch (1980a: 25–26, figs 1–12). The new species described here partially resembles to *E.* (*E.*) *ornatipennis* in the general shape of the penis lobes in male imago, slightly stretched laterally with the outer outline being nearly rectangular (see Tshernova 1938: 58, fig. 4). On the other hand, the apical sclerite in *E.* (*E.*) *ornatipennis*, distinctly projects above the penis lobes, in contrast to *E.* (*E.*) *eurycephalus* **sp. nov.** the apical sclerite of which projects only slightly. Although a relatively dark body colour makes these two species similar enough, *E.* (*E.*) *eurycephalus* **sp. nov.** clearly differs in the colour pattern of the lateral side of the eyes (in contrast to the uniformly blackish appearance in *E.* (*E.*) *ornatipennis*), and ornamentation of the abdominal sterna, in contrast to *E.* (*E.*) *ornatipennis* with more or less visible dark longitudinal stripe centrally on all sterna. This comparison is based on the observation of the fresh material of *E.* (*E.*) *ornatipennis* from Armenia: the Azat River near Garni village.

The second Caucasian species *E.* (*E.*) *autumnalis* Braasch, 1980 was described based on relatively limited material comprising male imagoes and larvae (Braasch 1980b) from Georgia (Autonomous Republic of Abkhazia; outflow of Amtkel Lake). Besides Georgia, where the species is known from the Imereti and Kakheti regions (Gabelashvili *et al*. 2018), *E. autumnalis* has also been reported from Turkey (Salur *et al*. 2016), and is reported by us for Armenia for the first time (see Table 1). The male imago of *E.* (*E.*) *eurycephalus* **sp. nov.** can be distinguished from *E.* (*E.*) *autumnalis* by the shape and structure of the penis lobes (relatively short, widely rounded distally, and deeply separated, with well projecting apical sclerite covered with numerous pointed setae in *E.* (*E.*) *autumnalis*). These species also differ in the shape of their titillators (being more robust, with obvious apical denticulation in *E.* (*E.*) *autumnalis,* see Braasch 1980a: 108, figs 1–2).

Larval stage. The presence of the tracheal filaments on gill plate VII clearly differentiates the larva of *E.* (*E.*) *eurycephalus* **sp. nov.** from the majority of the *Ecdyonurus* species described so far. Only two species, namely *E.* (*E.*) *insignis* and *E.* (*E.*) *asiaeminoris*, possess the same morphological feature. The larvae of *E.* (*E.*) *insignis* differs from *E.* (*E.*) *eurycephalus* **sp. nov.** by **i)** a characteristic hypodermal colouration pattern on the abdominal sterna (distinctly visible in mature larvae, see Haybach 1999: 130, fig. 5b; Hrivniak *et al*. 2015: 8, fig. 2B); **ii)** blunt and pointed setae on the dorsal surface of the hind femora; and **iii)** narrowed, tongue-shaped gill plate I in mature larvae (see Haybach 1999: 130, fig. 5d).

We did not find any distinct gap in the numerical characteristics of maxillae and labial palpi between *E.* (*E.*) *insignis* and *E.* (*E.*) *eurycephalus* **sp. nov.** We mostly observed overlapping ranges with slight differences in the

minimum and/or maximum values of particular diagnostics. The most pronounced differences were recorded in the number of comb-shaped bristles located apically on the maxilla (N_CBS): 16–20 in *E.* (*E.*) *insignis*, 20–24 (usually 20–21) in *E.* (*E.*) *eurycephalus* **sp. nov.**; the number of hair-like setae on the dorsal upper side of the maxilla (N_DOR): 7–16 in *E.* (*E.*) *insignis*, 11–17 in *E.* (*E.*) *eurycephalus* **sp. nov.** and the number of hair-like setae on the dorsal side of the first segment of the labial palp (N_LPH): 24–27 in *E.* (*E.*) *insignis*, 24–46 (usually 31–37) in *E.* (*E.*) *eurycephalus* **sp. nov.**

The larvae of *E.* (*E.*) *asiaeminoris* differ from *E.* (*E.*) *eurycephalus* **sp. nov.** in possessing: **i)** a distinctly narrowed gill plate I in mature larvae (see Demoulin 1973: 13, fig. 7g); **ii)** a characteristically narrow and apically pointed pronotal projections in mature larvae (see Demoulin 1973: 13, fig. 7n); and **iii)** an absence of denticulation in claws (see Demoulin 1973: 13, fig. 7l; corroborated by our study using a scanning electron microscope). An additional difference of *E.* (*E.*) *asiaeminoris* is a general colouration of the body with contrasting pale markings on the abdomen, legs, and head. We did not compare the numerical characteristics of maxillae and labial palpi, due to the absence of literary data and the unavailability of material.

Regarding the differentiation of the other two Caucasian *Ecdyonurus* species, *E.* (*E.*) *ornatipennis* possesses similar blunt spatulate bristles on the dorsal margin of all femora (see Braasch 1981: 24, fig.4) to those of *E.* (*E.*) *eurycephalus* **sp. nov**. The former species differs in possessing a characteristic hypodermal dark stripe along the midline of the abdominal sterna (Braasch 1980a: 23; 1981: 76), which is distinctly visible in the mature larvae, in contrast to *E.* (*E.*) *eurycephalus* **sp. nov**., where the dark stripe is always absent. *E.* (*E.*) *ornatipennis* also exhibits shorter pronotal projections in mature larvae (width/length ratio of semipronotum to pronotal projection at least 3.4; Kluge & Godunko unpubl.; see also Braasch 1980a: 24, fig. 1). The second Caucasian species, *E.* (*E.*) *autumnalis* is distinguishable from *E.* (*E.*) *eurycephalus* **sp. nov.** by the presence of pointed and bluntly pointed setae on the dorsal surface of femora (Kluge & Godunko unpubl.). Furthermore, the absence of a tuft of tracheal filaments on gill plate VII in both *E.* (*E.*) *ornatipennis* and *E.* (*E.*) *autumnalis* clearly differentiates these species from *E.* (*E.*) *eurycephalus* **sp. nov.**, where the tracheal filaments on gill plate VII are absent only sporadically.

Life stage association and molecular species delimitation. The molecular association of adults and immature stages represents a rapid and effective alternative to rearing immatures (Avelino-Capistrano *et al*. 2014) and is frequently used within the research of aquatic insects (e.g. Miller *et al*. 2005; Zhou *et al*. 2007; Gattolliat & Monaghan 2010; Massariol & Cruz 2015; Molina *et al*. 2017). We used COI sequences to associate the adults and larvae, and also the larvae of different instars, in order to describe morphological variability for more accurate species identification.

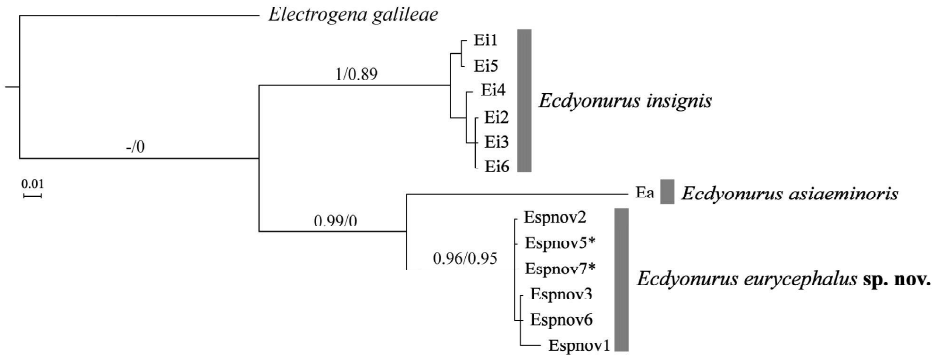


FIGURE 49. Bayesian majority-rule consensus *COI* gene tree of three *Ecdyonurus* species. Values above branches indicate posterior probabilities (MrBayes/bPTP).

The COI sequences of *E.* (*E.*) *eurycephalus* **sp. nov.** were successfully obtained from six specimens. Alignment consisted of 457 base pairs; 85 of 139 variable sites were parsimony informative. The K2P pairwise genetic distances between male imagoes and larvae, and the larvae of different instars of *E.* (*E.*) *eurycephalus* **sp. nov.** ranged from 0 to 1.3%. Such values for genetic distances fall into the generally observed variation of

intraspecific divergences, as reported in several studies focused on the molecular identification of mayflies (e.g. Ball *et al*. 2005; Webb *et al*. 2007; 2012; Massariol & Cruz 2015). The reconstruction of the phylogeny of *Ecdyonurus* is out of the scope of the present contribution; however we compared COI sequences of *E*. (*E.*) *eurycephalus* **sp. nov.** with two morphologically related species, namely *E*. (*E.*) *insignis* and *E*. (*E.*) *asiaeminoris*. The morphological similarity of these species was determined by the common presence of tracheal filaments on gill plate VII that represents a rare feature within the larvae of *Ecdyonurus*, shared only by the species analyzed. The analysis of COI data unambiguously recognized *E*. (*E.*) *eurycephalus* **sp. nov.** as a distinct lineage with Bayesian posterior probability (PP) = 0.96 and also as a distinct species according to bPTP model (PP = 0.95, Fig. 49). This result was also supported by high interspecific genetic distances (*E.* (*E.*) *asiaeminoris*: 15.9–17.9%, *E*. (*E.*) *insignis*: 17.9–19.2%). All inter- and intraspecific distances found in the study are listed in Appendix 2.

II. Check-list of Armenian mayflies

The literature published up to now contain records of 38 species occurring in Armenia, summarized in Table 1 (only taxa identified to the species-level are included). Our sampling provided records of nine new species for the Armenian fauna, including one species new to science (described above). A single record of *Ametropus fragilis* Albarda, 1878 by Eaton (1885) was considered as unclear, resulting altogether in 46 mayfly taxa present in the territory of Armenia. The systematic concept and general species distribution are based on Bauernfeind and Soldán (2012); other distribution information is supplemented by the Ephemeroptera of the World online database (see section Material and methods).

Zoogeographical characteristic of Armenian mayfly fauna. The mayfly fauna of the territory of Armenia is very diverse and comprises representatives of several distributional patterns. It includes the Caucasian species, the species distributed in adjacent areas, and the species more or less widespread throughout the Holarctic/Palaearctic biogeographical region. However, the majority of the species known from Armenia has an apparent pan-Caucasian core pattern. Based on the recent knowledge about the distribution ranges of individual species, the Armenian mayfly fauna may be divided into five distribution types:

i) Holarctic and Transpalaearctic species (9 species): *B. fuscatus*, *B. vernus*, *C. luteolum*, *P. pennulatum*, *C. dipterum*, *C. macrura*, *C. rivulorum*, *C*. cf. *pseudorivulorum*, *C. horaria*, *E. ignita*;

ii) common European and West Palaearctic species (10 species): *B. rhodani, B. muticus*, *B. buceratus*, *B. lutheri*, *B. pentaphlebodes*, *B. vardarensis*, *H. coerulans*, *H. confusa*, *C. beskidensis*, *R. iridina*. In four cases, Caucasian subspecies of the above-mentioned European species were reported from Armenia, namely: *B. vardarensis caucasicus*, *B. lutheri georgiensis* (both subspecies distributed throughout the Caucasus), *R. iridina kownackii* (distributed throughout the Caucasus and Turkey) and *H. coerulans micracantha* (distributed throughout the Caucasus);

iii) species widely distributed in the Caucasus region, known from both the Greater and Lesser Caucasus, sometimes with an area extension to the Middle East (Turkey, Iran, Iraq) or Central Asia (14 species): *B. braaschi*, *B. ilex*, *O. tskhomelidzei*, *E. pseudaffinis*, *E. squamata*, *R. caucasica*, *R. decolorata*, *E. zaitzevi, E. caucasicus*, *E. magnus*, *E. znojkoi*, *E. autumnalis*; *E. ornatipennis*, *H. samochai*;

iv) species distributed in the Lesser Caucasus, sometimes with an area extension to the Middle East (Turkey, Iran, Iraq) or Central Asia, but not reaching the Greater Caucasus (6 species): *R. binerve*, *E. romantzovi*, *B. petrovi*, *B. samochai*, *B. kars*, *E. kurarensis*;

v) species known only from Armenia (mostly only from the type series; 4 species): *B. joosti*, *E. eurycephalus* sp. nov., *E. armeniaca* and *R. zhiltzovae*.

Although our sampling sites cover a relatively wide area of Armenia, many parts of the country still remain poorly investigated. Therefore, we stress that the checklist provided above is certainly not complete and the discovery of more species may be expected. However, it summarizes the current state of knowledge and provides a basis for future research in the area. It will also be helpful for routine hydrobiological surveys and nature conservation purposes. We also wish to point out the need for further faunistic and taxonomic work, not only in the Caucasus region, but also in adjacent countries, the results of which would form the basis for a better understanding of the origin and uniqueness of the Caucasus fauna.

TABLE 1. Commented list of Ephemeroptera of Armenia. Species representing new records for Armenia—in bold; species whose distribution in Armenia is unclear and needs to be confirmed—without number. For the exact location of sampling sites see Appendix 1 and Fig. 1. Localities sampled by D. Palatov marked with asterisk, other localities sampled by Ľ. Hrivniak, P. Manko & J. Oboňa. Distribution "Caucasus" refers to species occurrence in at least in two countries within Caucasus region; "Armenia" refers to records only from Armenia.

	Species	Numbers of sampled sites	Distribution	References for Armenia
	Ametropodidae			
–	*Ametropus fragilis* Albarda, 1878[1]	–	Palaearctic	Eaton (1885)
	Baetidae			
1	*Baetis* (*Acentrella*) *joosti* Zimmermann & Braasch, 1979[2]	–	Armenia	Zimmermann & Braasch (1979); Zimmermann (1981); Kluge & Novikova (2011)
2	*Baetis* (*Baetis*) *buceratus* Eaton, 1870	20, 37, 38, 23*	West Palaearctic	Palatov (2013)
3	*Baetis* (*Baetis*) *pentaphlebodes* Ujhelyi, 1966	5, 7, 26, 126*, 231*	West Palaearctic	Palatov (2013)
4	*Baetis* (*Baetis*) *fuscatus* (Linnaeus, 1761)	1–3, 10, 11, 14, 15, 20, 24, 40, 23*	Palaearctic	Palatov (2013)
5	*Baetis* (*Baetis*) *lutheri* Müller-Liebenau, 1967[3]	1–3,10, 11, 14, 15, 17–23, 26, 29, 33, 36–41, 43, 47, 23*, 42*, 64*, 75*, 110*, 126*, 141*, 144*, 156*, 185*, 191*, 209*, 217*, 222*, 231*	Europe, Turkey, Caucasus, Iraq	Zimmermann (1981); Novikova (1987); Palatov (2013) – all sub nom. *Baetis* (*Baetis*) *lutheri georgiensis* Zimmermann, 1981
6	*Baetis* (*Baetis*) *petrovi* Tshernova, 1938[3]	–	Caucasus	Hakobyan *et al*. (2010)
7	*Baetis* (*Baetis*) *vardarensis* Ikonomov, 1960[4]	1, 2, 9, 14, 15, 20, 22, 24, 45, 126*, 191*	Europe, Caucasus	Zimmermann (1981); Palatov (2013) – both sub nom. *Baetis* (*Baetis*) *vardarensis caucasicus* Zimmermann, 1981
8	***Baetis* (*Baetis*) *samochai* Koch, 1981**	27, 37, 39	Turkey, Israel, Lebanon, Syria, Iran	**First record for Armenia**
9	*Baetis* (*Baetis*) *vernus* Curtis, 1834	–	Palearctic	Gabrielyan *et al*. (2010)
10	*Baetis* (*Nigrobaetis*) *kars* Kazanci & Thomas, 1989	24, 144*	Armenia, Turkey, Central Asia	Novikova & Kluge (1994); Palatov (2013); Martynov & Godunko (2017)

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TABLE 1. (Continued)

	Species	Numbers of sampled sites	Distribution	References for Armenia
11	*Baetis* (*Nigrobaetis*) *muticus* (Linnaeus, 1758)	1–3, 9–11, 18, 20, 21, 33, 34, 40, 41, 43–45, 5*, 23*, 42*, 64*, 75*, 110*, 177*, 217*	West Palaearctic	Zimmermann (1981); Novikova (1987); Novikova & Kluge (1994); Palatov (2013); Martynov & Godunko (2017) – sub nom. *Nigrobaetis* (*Takobia*) *muticus* (Linnaeus, 1758)
12	*Baetis* (*Rhodobaetis*) *braaschi* Zimmermann, 1980	45, 209*, 222*, 231*	Central Asia, Caucasus, Crimea, Turkey, Iran	Godunko *et al.* (2004b); Palatov (2013)
13	*Baetis* (*Rhodobaetis*) *ilex* Jacob & Zimmerman, 1978	2, 3, 8, 10, 24, 29, 31, 33, 36, 38–41, 43, 44, 46, 42*, 91*, 156*, 177*, 185*, 188*, 191*, 216*, 217*, 222*, 231*	Caucasus, Iran	Jacob & Zimmermann (1978); Zimmermann (1981); Jacob & Novikova (1987); Palatov (2013)
14	*Baetis* (*Rhodobaetis*) *rhodani* (Pictet, 1843)[5]	1–5, 10–12, 14–16, 18, 20–23, 26, 29, 37, 38, 40, 41, 43, 45, 47, 23*, 110*, 126*, 141*, 144*, 175*, 209*, 213*, 222*	West Palaearctic	Zimmermann (1981); Novikova (1987); Palatov (2013)
15	*Baetis* (*Rhodobaetis*) cf. *vadimi* Godunko, Palatov & Martynov, 2015	12, 15–18, 21, 23, 24, 29, 33, 35–37, 42, 43, 46, 47, 48*, 23*, 34*, 42*, 51*, 64*, 75*, 97*, 117*, 121*, 165*, 201*	unclear	Zimmermann (1981); Novikova (1987) – both sub nom. *Baetis gemellus* Eaton, 1885; Palatov (2013) – sub nom. *Baetis* (*Rhodobaetis*) *gemellus* Eaton, 1885[6]
16	*Centroptilum luteolum* (Müller, 1776)	7, 110*	Palaearctic	Palatov (2013)
17	*Cloeon* (*Cloeon*) *dipterum* (Linnaeus, 1761)[7]	5, 6, 13, 229*	Holarctic	Palatov (2013)
18	*Procloeon* (*Pseudocentroptilum*) *pennulatum* (Eaton, 1870)	191*, 222*	Holarctic	Palatov (2013)
	Oligoneuriidae			
19	*Oligoneuriella tskhomelidzei* Sowa & Zosidze, 1973	185*, 222*	Caucasus, Turkey, Iraq	Soldán & Landa (1977) – sub nom. *Oligoneuriella zanga* Soldán & Landa 1977 (synonymy proposed by Kluge 2004)
	Heptageniidae			
20	***Ecdyonurus* (*Ecdyonurus*) *autumnalis* Braasch, 1980**	5*, 23*, 42*, 64*, 75*, 91*, 97*, 110*, 121*, 141*, 144*	Caucasus, Turkey	**First record for Armenia**

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TABLE 1. (Continued)

	Species	Numbers of sampled sites	Distribution	References for Armenia
21	***Ecdyonurus* (*Ecdyonurus*) *eurycephalus* sp. nov.**	33, 35, 36, 38–40	Armenia	**First record for Armenia**
22	*Ecdyonurus* (*Ecdyonurus*) *ornatipennis* Tshernova, 1938	20, 24, 41, 45, 156*, 177*, 188*, 191*, 201*	Caucasus, Turkey, Iran	Zhadin (1940)
23	*Electrogena armeniaca* (Braasch, 1983)	–	Armenia	Braasch (1983b) – sub nom. *Ecdyonurus armeniacus* Braasch, 1983
24	*Electrogena kuraensis* (Braasch, 1978)	–	Caucasus	Hakobyan *et al.* (2010) – sub nom. *Ecdionurus* (sic!) *kuraensis*
25	***Electrogena pseudaffinis* (Braasch, 1980)**	45	Caucasus, Iran, Turkey, Central Asia	**First record for Armenia**
26	***Electrogena squamata* (Braasch, 1978)**	21, 5*, 23*, 34*, 42*, 51*, 75*, 91*, 97*, 117*, 121*, 141*, 144*, 165*, 177*, 191*, 201*, 209*, 213*, 217*, 222*	Caucasus, Iran	**First record for Armenia**
27	*Heptagenia* (*Dacnogenia*) *coerulans micracantha* Kluge, 1989	–	Caucasus	Kluge (1987b) – sub. nom. *Heptagenia* (*Dacnogenia*) *coerulans* Rostock, 1878; Kluge (1989)
28	*Heptagenia* (*Heptagenia*) *samochai* (Demoulin, 1973)	–	Crimea, Caucasus, Turkey, Iran, Israel	Zhadin (1940) – sub nom. *Heptagenia* (*Heptagenia*) *perflava* sensu Zhadin, 1940, non Brodsky, 1930[9]1; Kluge (1987b) – sub nom *Heptagenia* (*Heptagenia*) *lutea* Kluge, 1987 (synonymy proposed by Sartori 1992)
29	*Epeorus* (*Caucasiron*) *caucasicus* (Tshernova, 1938)	1, 12, 18, 19, 33, 36, 43, 42*, 51*, 64*, 75*, 91*, 97*, 117*, 121*, 177*, 201*	Caucasus, Turkey, Iran (ssp. *iranicus* Braasch & Soldán, 1979)	Sinitshenkova (1976) – sub nom. *Iron fuscus* Sinitshenkova, 1976 (synonymy proposed by Braasch 1979b)
30	*Epeorus* (*Caucasiron*) *magnus* (Braasch, 1978)	1, 12, 18, 22, 43	Caucasus, Turkey	Sinitshenkova (1976) – sub nom. *Iron znojkoi* Tshernova 1938 (probably misidentification, see Braasch, 1980c)
31	*Epeorus* (*Caucasiron*) *znojkoi* (Tshernova, 1938)	12, 18, 5*, 34*, 51*, 75*, 91*, 141*, 144*, 165*	Caucasus, Iran, Turkey	Sinitshenkova (1976) – sub nom. *Iron caucasicus* Tshernova 1938 (misidentification, see Braasch, 1980c)

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TABLE 1. (Continued)

	Species	Numbers of sampled sites	Distribution	References for Armenia
32	*Epeorus* (*Epeorus*) *zaitzevi* Tshernova, 1981	10, 11, 22, 24, 25, 177, 213, 222	Caucasus, Iran, Iraq, Israel, Syria, Turkey	Braasch (1978) – sub. nom. *Epeorus znojkoi* (misidentification, see Sartori 1992); Gabrielyan *et al.* (2010)
33	*Rhithrogena* (*Epeiron*) *binerve* Kluge, 1987	–	Caucasus, Himalayas	Kluge (1987a); Chun (1999)
34	***Rhithrogena* (*Rhithrogena*) *caucasica* Braasch, 1979**	34*, 91*, 97*	Caucasus, Turkey	**First record for Armenia**
35	***Rhithrogena* (*Rhithrogena*) *decolorata* Sinitshenkova, 1973**	43	Caucasus, Iran	**First record for Armenia**
36	*Rhithrogena* (*Rhithrogena*) *iridina kownackii* Sowa & Zimmermann, 1975	51*	Caucasus, Turkey	Braasch (1979a)
37	*Rhithrogena* (*Rhithrogena*) *zhiltzovae* Sinitshenkova, 1979[8]	185*	Armenia	Sinitshenkova (1979)
	Leptophlebiidae			
38	*Habroleptoides confusa* Sartori & Jacob, 1986	2, 12, 33, 42, 5*, 64*, 75*, 110*, 177*, 121*, 141*, 144*, 217*	Europe, Caucasus, Iran	Sartori & Jacob (1986); Kluge (1994) – sub nom. *Habroleptoides* (? *confusa*)
	Ephemeridae			
39	*Ephemera* (*Ephemera*) *romantzovi* Kluge, 1988	15, 16, 18, 19, 21, 22, 24, 43, 44, 5*, 42*, 191*, 144*, 201*, 209*, 222*	Caucasus, Turkey	Kluge (1988); Palatov *et al.* (2016)
	Ephemerellidae			
40	***Ephemerella ignita* (Poda, 1761)**	1–3, 9, 10, 12, 15–17, 24, 26, 33, 38, 41, 43, 45, 5*, 23*, 64*, 75*, 91*, 110*, 126*, 141, 177*, 185*, 188*, 191*, 209*, 213*, 222*, 231*	Palaearctic	**First record for Armenia**

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TABLE 1. (Continued)

	Species	Numbers of sampled sites	Distribution	References for Armenia
	Caenidae			
41	*Caenis beskidensis* Sowa, 1975	222*	Europe	Palatov *et al.* (2016)
42	*Caenis horaria* (Linnaeus, 1758)	–	Palaearctic	Gabrielyan *et al.* (2010); Shcherbina (2013); Hakobyan & Jenderedjian (2016); Bulysheva *et al.* (2016)
43	*Caenis luctuosa* (Burmeister, 1839)	–	Palearctic	Hakobyan & Jenderedjian (2016); Hakobyan *et al.* (2010) – sub nom. *Caenis moesta* Bengtsson 1917
44	*Caenis macrura* Stephens, 1835	10, 20, 24, 26, 5*, 23*, 42*, 110*, 141*, 144*, 156*, 177*, 185*, 217*, 222*, 231*	Palaearctic	Zhadin (1940); Palatov *et al.* (2016)
45	***Caenis* cf. *pseudorivulorum* Keffermüller, 1960**	45	Palaearctic	**First record for Armenia**
46	*Caenis rivulorum* Eaton, 1884	188	Palaearctic	Palatov *et al.* (2016)

[1] Distribution in Armenia can be assumed based on short unclear information published by Eaton (1885: 232). Other reports from the Caucasus (e.g. Yakobson & Bianki 1905: 877; Lestage 1917: 437) actually represent only citations of Eaton's record. Current distribution of *Ametropus* in Armenia needs to be confirmed. First confirmed Caucasian record of the genus *Ametropus* Albarda, 1878 was published by Sadovskii (1940) for Georgia (Kura River near Tbilisi). The closest record to Armenia was published by Braasch (1981) from Iran.

[2] Poorly known species with undescribed adult males (see Zimmermann & Braasch 1979; Zimmermann 1981; Kluge & Novikova 2011), known based on type series only (Armenia, Razdan).

[3] Novikova (1987: 84) proposed the synonymy of *Baetis petrovi* Tshernova, 1938 and *B. lutheri georgiensis* in her unpublished Ph.D. thesis; synonymy needs to be additionally testified. Delimitation between *B. lutheri lutheri* and *B. lutheri georgiensis* Zimmerman, 1981 also needs additional study.

[4] Delimitation between *B. vardarensis vardarensis* and *B. vardarensis caucasicus* Zimmerman, 1981 needs additional study.

[5] Actually constitute cryptic species complex (see Williams *et al.* 2006).

[6] For additional information concerning taxonomy of *B. gemellus* see Gabelashvili *et al.* (2018). Records from Armenia most probably belong to *B. braaschi, B. vadimi* or another undescribed closely related *Rhodobaetis* species.

[7] Actually constitute cryptic species complex (see Rutschmann *et al.* 2017).

[8] Chun (1999: 62) proposed the synonymy of *R. zhiltzovae* and *R. excisa* Sinitshenkova, 1979 in his unpublished Ph.D. thesis; synonymy needs to be additionally testified.

[9] All records from the Caucasus belong to *H. samochai* (for details see Kluge 1987b).

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APPENDIX 1. List of localities studied in Armenia in 2011, 2014 and 2015. Localities marked with asterisk sampled by D. Palatov. Other localities sampled by Ľ. Hrivniak, P. Manko & J. Oboňa. Abbrevations: N—north, S—south, W—west, E—east, T—tributary, RT– right tributary, LT—left tributary, prov.—province). The site numbers refer to the Fig. 1. and the codes on labels and field protocols of the respective collectors.

Site No.	Stream type	Stream name	Location	Latitude (N)	Longitude (E)	Altitude	Sampling date
1	River in middle mountains	Marmarik	Kotayk prov., Hankavan village	40°38'11.40"	44°28'58.29"	1975	26/08/2015
2	River in middle mountains	Marmarik	Kotayk prov., Hankavan village	40°38'04.74"	44°29'19.38"	1974	26/08/2015
3	Stream in middle mountains	RT of Marmarik	Kotayk prov., E of Hankavan village, above the reservoir	40°38'09.14"	44°32'23.19"	1913	26/08/2015
5	Stream with bedrocks and pools in middle mountains	Hrazdan	Kotayk prov., N of Solak village	40°28'19.72"	44°42'42.19"	1567	27/08/2015
6	Slowly flowing eutrophic river in upland	Hrazdan	Kotayk prov., SW of Hrazdan reservoir	40°29'12.75"	44°43'55.91"	1705	27/08/2015
7	Swampy stream in upland	LT of Hrazdan	Kotayk prov., S of Aghbuyrag village	40°30'10.17"	44°44'22.40"	1718	27/08/2015
9	River in middle mountains	Marmarik	Kotayk prov., NW of Artavaz village	40°36'49.92"	44°34'18.17"	1849	27/08/2015
10	Stream in middle-mountain valley	RT of Marmarik	Kotayk prov., NE of Mekhradzor village	40°37'53.02"	44°40'17.49"	1870	27/08/2015
11	Stream in upland	T of Marmarik	Kotayk prov., NE of Mekhradzor village	40°37'12.67"	44°40'18.30"	1825	27/08/2015
12	Stream in forested middle mountains	LT of Marmarik	Kotayk prov., S of Mamarik village	40°33'51.97"	44°40'09.13"	1872	27/08/2015
13	Eutrofic lake and forest stream	Parz Lake and its tributary	Tavush prov., SW of Haghartsin village	40°44'57.72"	44°45'33.30"	1376	28/08/2015
14	River in forested middle mountains	Aghstev	Tavush prov., SW of Teghut village	40°46'23.04"	44°55'25.71"	1102	28/08/2015
15	River in forested middle mountains	Bldan (RT of Aghstev)	Tavush prov., W of Diljan town	40°44'49.07"	44°49'03.47"	1354	28/08/2015
16	River in forested middle mountains	Bldan (RT of Aghstev)	Tavush prov., W of Diljan town	40°45'11.80"	44°48'25.69"	1411	29/08/2015
17	River in forested middle mountains	Bldan (RT of Aghstev)	Tavush prov., W of Diljan town	40°44'59.58"	44°48'29.21"	1392	29/08/2015
18	Stream in forested middle mountains	RT of Aghstev	Tavush prov., NW of Teghut village	40°47'15.24"	44°54'58.00"	1197	29/08/2015
19	Stream in forested middle mountains	RT of Aghstev	Tavush prov., NW of Teghut village	40°48'09.32"	44°53'43.68"	1382	29/08/2015
20	Stream in upland	Dzknaget	Gegharkunik prov., S of Tsovagyugh village	40°37'01.74"	44°57'44.23"	1930	29/08/2015
21	Stream in forested middle mountains	Azat	Ararat prov., E of Goght village, Geghard monastery	40°08'21.51"	44°49'07.08"	1770	30/08/2015

.....*continued on the next page*

APPENDIX 1. (Continued)

Site No.	Stream type	Stream name	Location	Latitude (N)	Longitude (E)	Altitude	Sampling date
22	Mountain river in deep canyon	Azat	Ararat prov., S of Garni town	40°06'33.22"	44°43'49.28"	1240	31/08/2015
23	Mountain stream (waterfall-like)	T of Azat	Ararat prov., S of Garni town	40°06'39.40"	44°43'45.28"	1249	31/08/2015
24	Mountain river in deep canyon	Azat and Geghard (confluence of both rivers)	Ararat prov., S of Garni town	40°06'42.72"	44°44'08.87"	1249	31/08/2015
26	River in Ararat valley	Azat	Ararat prov., Lanjazat village	40°03'26.95"	44°34'38.31"	976	31/08/2015
27	Canal parallel with Hrazdan River	Hrazdan chanel	Ararat prov., W of Araksavan town	40°00'13.68"	44°26'52.61"	842	31/08/2015
29	Small stream in upland	T of Aghstev	Lori prov., Lermontov village	40°45'24.63"	44°38'42.01"	1853	01/09/2015
30	River in forested middle mountains	Zamanlu	Lori prov., W of Vahagnadzor village	40°53'07.03"	44°34'38.99"	1092	01/09/2015
31	Small stream in forested middle mountains	T of Pambak	Lori prov., N of Dzoraget village	40°56'52.67"	44°37'37.22"	1030	01/09/2015
33	Small steppe stream in high mountains	T of Dzoraget	Lori prov., W of Katnarat village	41°03'59.85"	44°05'44.24"	1949	02/09/2015
34	Small steppe stream in mountain plateau	T of Dzoraget	Shirak prov., NE of Musayelyan village	41°01'43.99"	44°01'29.09"	2310	02/09/2015
35	Small steppe stream in mountain platcau	T of Dzoraget	Shirak prov., NE of Musayelyan village	41°00'20.12"	43°59'14.04"	2286	02/09/2015
36	Small steppe river in high mountains	T of Akhurian	Shirak prov., NE Musayelyan village	41°00'13.15"	43°57'24.87"	2195	02/09/2015
37	Steppe river in mountain plateau	T of Akhurian	Shirak prov., S of Zuygaghbyur village	41°00'56.12"	43°54'18.82"	2034	02/09/2015
38	Small stream in upland	T of Akhurian	Shirak prov., N of Amasia village	40°58'20.49"	43°46'06.88"	1987	02/09/2015
39	Small steppe stream in upland	T of Akhurian	Shirak prov., W of Aghvorik village	41°04'21.19"	43°44'44.83"	2052	03/09/2015
40	Steppe river in upland	T of Akhurian	Shirak prov., Torosgyugh village	40°55'54.99"	43°52'45.26"	1885	03/09/2015
41	River in upland	Pambak	Lori prov., NE of Geghasar village	40°51'17.42"	44°11'40.81"	1627	03/09/2015
42	Stream in middle mountains	T of Pambak	Lori prov., N of Aznvadzor village	40°54'22.87"	44°25'33.33"	1839	03/09/2015
43	Stream in middle mountains	T of Dzoraget	Lori prov., N of Pushkino village	40°58'04.80"	44°24'49.73"	1485	03/09/2015
44	Small stream in forested middle mountains	T of Gosh	Tavush prov., E of Gosh village	40°44'15.86"	45°01'01.21"	1039	04/09/2015

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APPENDIX 1. (Continued)

Site No.	Stream type	Stream name	Location	Latitude (N)	Longitude (E)	Altitude	Sampling date
45	River in low-mountain valley	T of Getik	Tavush prov., NE of Gosh village	40°45'16.48"	45°01'18.42"	940	04/09/2015
46	Stream in upland	T of Aghstev	Tavush prov., SW of Ijevan town	40°50'31.96"	45°06'56.82"	760	04/09/2015
47	Stream in high mountains	T of Marmarik	Kotayk prov., N of Meghradzor village	40°40'00.38"	44°40'41.28"	2380	05/09/2015
5*	River in forested middle mountains	Polad (T of Getik)	Gegharkunik prov., NE of Tsovagyugh village	40°39'32.13"	44°59'55.36"	1767	19/08/2011
23*	River in forested middle mountains	Getik	Tavush prov., NW of Khachardzan village	40°43'48.69"	45°2'38.82"	1008	21/08/2011
34*	River in forested middle mountains	Kebkhapijy (T of Aghstev)	Tavush prov., E of Dilijan town	40°44'20.66"	44°57'27.44"	1480	22/08/2011
42*	River in forested middle mountains	Haghartsin (T of Aghstev)	Tavush prov., NW of Teghut village, W of Haghartsin monastery	40°48'3.05"	44°52'30.05"	1574	23/08/2011
51*	River in forested middle mountains	Baboljan (T of Marts)	Lori prov., SE of Ahmidzor village	40°54'1.09"	44°49'46.77"	1942	26/08/2011
64*	River in forested middle mountains	Sorbulag (T of Marts)	Lori prov., SE of Marts village	40°55'10.25"	44°43'34.91"	1188	26/08/2011
75*	Stream in steppe high mountains	Chanakhchi	Lori prov., NW of Dilijan town	40°48'40.62"	44°44'19.22"	2150	28/08/2011
91*	Stream in steppe high mountains	Balan	Lori prov., NW of Dilijan town	40°46'49.87"	44°44'53.32"	2030	30/08/2011
97*	Stream in middle-mountain steppe	Unnamed stream	Lori prov.,SW of Margahovit village	40°43'12.02"	44°39'41.51"	1790	30/08/2011
110*	River in middle mountains	Marmarik	Kotayk prov., SE of Meghradzor town	40°35'24.47"	44°39'44.25"	1759	02/09/2011
117*	River in forested middle mountains	Korchlu	Aragatsotn prov., SW of Meghradzor town	40°33'51.53"	44°35'0.83"	2200	03/09/2011
121*	River in forested middle mountains	Arzakan (T of Hrazdan)	Kotayk prov., N of Arzakan village	40°31'43.90"	44°34'57.35"	1970	03/09/2011
126*	River in highland plain	Hrazdan	Kotayk prov., Arzakan village	40°26'25.51"	44°36'29.60"	1458	04/09/2011
141*	River in steppe middle mountains	Gilanlar (T of Karasu)	Ararat prov., NW of Mets Gilanlar village	40° 06'09.38"	44°48'19.29"	1606	06/09/2011
144	Mountain river in deep canyon	Karasu (T of Azat)	Ararat prov., SW of Mets Gilanlar village	40° 05'26.83"	44°47'35.86"	1400	07/09/2011
156*	Mountain river in deep canyon	Gnishik	Vayots Dzor prov., SE of Areni town	39°40'54.89"	45°14'17.37"	1478	07/07/2014

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APPENDIX 1. (Continued)

Site No.	Stream type	Stream name	Location	Latitude (N)	Longitude (E)	Altitude	Sampling date
165*	Mountain stream	T of Arpa	Vayots Dzor prov., NE of Jermuk town	39°50'46.33"	45°40'34.25"	2170	09/07/2014
175*	River at outlet of reservoir	Arpa	Vayots Dzor prov., SW of Kechut town	39°47'15.78"	45°38'36.86"	1911	11/07/2014
177*	River in middle mountains	Artavan (T od Darb, Arpa basin)	Vayots Dzor prov., NW of Artavan village	39°41'09.31"	45°33'58.74"	1386	11/07/2014
185*	River in middle mountains	Jahuk	Vayots Dzor prov., W of Nor Aznaberd village	39°32'40.36"	45°24'52.54"	1582	12/07/2014
188*	River in highland steppe	Sisian	Syunik prov., SE of Sisian town, below Tolors reservoir	39°29'18.30"	46° 02'49.37"	1605	15/07/2014
191*	Mountain river in deep canyon	Bazarçay	Syunik prov., NE of Ltsen village	39°27'14.47"	46°10'01.86"	1317	16/07/2014
201*	River in middle mountains	Tzav	Syunik prov., W of Tsav village	39°03'01.64"	46°25'19.43"	1200	20/07/2014
209*	River in middle mountains	Meghri	Syunik prov., NW of Meghri town	38°55'07.96"	46°13'26.55"	752	22/07/2014
213*	River in high plain	Argichi	Gegharkunik prov., N of Nerkin Getashen village	40° 09'51.26"	45°16'15.93"	1912	25/07/2014
216*	Swampy stream in high plain	Unnamed stream (T of Lake Sevan)	Gegharkunik prov., N of Martuni	40°09'36.36"	45°18'44.34"	1908	25/07/2014
217*	Mountain river in deep canyon	Azat	Ararat prov., SW of Geghard monastery	40°08'10.69"	44°48'54.33"	1680	26/07/2014
222*	Mountain river in deep canyon	Azat	Kotayk prov., SW of Garni village	40°06'34.76"	44°42'00.19"	1183	27/07/2014
229*	Canal from Akna lake in the Ararat plain	Nerkin Aygrlichskiy	Armavir prov., in Aknalich village	40°08'41.00"	44°10'15.29"	856	31/07/2014
231*	Mountain river in deep canyon	Kasakh	Aragatsotn prov., NE of Ashtarak town	40°18'13.86"	44°22'40.48"	1133	01/08/2014

APPENDIX 2. Sequences and K2P pairwise genetic distance values. Distances between larvae and imagoes of *E. (E.) eurycephalus* **sp. nov.** marked with asterisk

Species/abbrevation	**K2P pairwise genetic distance values (%)**												**GenBank accession and Bold sequence ID nos.**
Ecdyonurus asiaeminoris/Ea	Ea	Espnov2l	Espnov5i	Espnov7i	Espnov3l	Espnov1l	Espnov6l	Ei1	Ei5	Ei2	Ei3	Ei6	KX447091.1
Ecdyonurus eurycephalus **sp.nov.**/Espnov2l	15.9												MG972100
Ecdyonurus eurycephalus **sp.nov.**/Espnov5i	15.9	0.0*											MG972102
Ecdyonurus eurycephalus **sp.nov.**/Espnov7i	15.9	0.0*	0.0										MG972104
Ecdyonurus eurycephalus **sp.nov.**/Espnov3l	16.2	0.2	0.2*	0.2*									MG972101
Ecdyonurus eurycephalus **sp.nov.**/Espnov1l	17.9	1.3	1.3*	1.3*	1.1								MG972099
Ecdyonurus eurycephalus **sp.nov.**/Espnov6l	16.2	0.2	0.2*	0.2*	0.0	1.1							MG972103
Ecdyonurus insignis/Ei1	20.3	18.7	18.7	18.7	18.7	17.9	18.7						GBEPT1837-14
Ecdyonurus insignis/Ei5	20.6	19.0	19.0	19.0	19.0	18.2	19.0	0.2					FBAQU805-10
Ecdyonurus insignis/Ei2	20.4	19.3	19.3	19.3	19.3	18.5	19.3	1.3	1.1				GBEPT466-14
Ecdyonurus insignis/Ei3	20.3	19.2	19.2	19.2	19.2	18.5	19.2	1.6	1.3	0.0			GBEPT428-14
Ecdyonurus insignis/Ei6	20.3	19.2	19.2	19.2	19.2	18.5	19.2	1.6	1.3	0.0	0.0		FBAQU1254-12
Ecdyonurus insignis/Ei4	20.9	19.2	19.2	19.2	19.2	18.5	19.2	1.3	1.1.	0.4	0.7	0.7	GBEPT350-14

Chapter II

1. Mayflies of the genus *Epeorus* Eaton, 1881 s.l. (Ephemeroptera: Heptageniidae) from the Caucasus Mountains: a new species of *Caucasiron* Kluge, 1997 from Georgia and Turkey

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Mayflies of the genus *Epeorus* Eaton, 1881 s.l. (Ephemeroptera: Heptageniidae) from the Caucasus Mountains: a new species of *Caucasiron* Kluge, 1997 from Georgia and Turkey

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Abstract

The new species *Epeorus* (*Caucasiron*) *bicolliculatus* **sp. nov.** is described based on larvae and adults. Diagnostic characters are given with regard to the related species. The most pronounced difference is represented by protuberances on larval abdominal terga II–IX, present exclusively in *E.* (*C.*) *bicolliculatus* **sp. nov.** Primary data on the biology and distribution of the new species are also provided. Delimitation of the new species is verified by the analysis of COI (barcode) sequences. Barcode data for all Caucasian species of the subgenus *Caucasiron* are provided for the first time and compared with *E.* (*C.*) *bicolliculatus* **sp. nov.**

Key words: Ephemeroptera, taxonomy, barcoding, new species

Introduction

The mayflies of the genus *Epeorus* Eaton, 1881 (s.l.) are distributed in the Holarctic, Oriental, and northern Neotropic region (Webb & McCafferty 2008), and represent taxa occurring in the torrential sections of mountain and submountain streams and rivers (Nguyen *et al*. 2004; Bauernfeind & Soldán 2012). The Caucasus region, one of the global biodiversity hotspots (Myers *et al*. 2000) hosts a high number of *Epeorus* species within a relatively restricted area. The larvae of most species described from the Caucasus are characterised by the presence of gill plates which form a "suction disc" with the first pair of gill plates being highly enlarged under the thorax and the seventh pair possessing a longitudinal fold and being bent under the abdominal segments. Most of the species with such a shape of gill plates had originally been described in the genus *Iron* Eaton, 1883. However, the taxonomic rank and monophyly of *Iron* was subsequently discussed and still remains controversial. In summary, the species with the "suction disc" are treated either within subgenera of the genus *Iron* (Braasch 2006a), subgenera within the genus *Epeorus* (e.g. Kluge 1997; 2004) or just species within *Epeorus*, with no subgenera recognized (Webb & McCafferty 2008).

In this contribution we follow the concept of Kluge (1997; 2004), who recognized the "suction disc" as a diagnostic character for several subgenera within *Epeorus* (s.l.). From these subgenera, almost all representatives known from the Caucasus region bear characteristics of the subgenus *Caucasiron*, as defined by Kluge (1997). Until now, nine species have been described and recorded from the Caucasus and adjacent territories, namely *E.* (*C.*) *caucasicus* (Tshernova, 1938)—type species of the subgenus *Caucasiron*; *E.* (*C.*) *znojkoi* (Tshernova, 1938); *E.* (*C.*) *nigripilosus* (Sinitshenkova, 1976); *E.* (*C.*) *fuscus* (Sinitshenkova, 1976)—later synonymised with *E.* (*C.*) *caucasicus* in Braasch (1979); *E.* (*C.*) *magnus* (Braasch, 1978); *E.* (*C.*) *alpestris* (Braasch, 1979); *E.* (*C.*) *soldani* (Braasch, 1979); *E.* (*C.*) *sinitshenkovae* (Braasch & Zimmermann, 1979); *E.* (*C.*) *longimaculatus* (Braasch, 1980).

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Outside the Caucasus, *Caucasiron* also occurs in Greece (*E.* (*C.*) *znojkoi insularis* (Braasch, 1983a) from the island of Samos), Central Asia (*E.* (*C.*) *guttatus* (Braasch & Soldán, 1979) from Kazakhstan and Tajikistan) and South-western China (*E.* (*C.*) *extraordinarius* Chen *et al.*, 2010 from Guizhou province: Leigongshan Nature Reserve).

There are other species which likely belong to *Caucasiron*, but in these cases, only the description of the larval stage is available. Thus their attribution to *Caucasiron* will only be ascertained after the description of adults. This includes e.g. *Iron suspicatus* Braasch, 2006b from Nepal, *Iron kapurkipalanorum* Braasch, 1983b from the Himalayas, *Iron paraguttatus* Braasch, 1983b *sensu* Braasch (2006a) not Braasch (1981; 1983b) from the Himalayas; for details see Kluge (2015: 343) and *Iron caucasicus iranicus* Braasch & Soldán, 1979 from Iran. In the latter taxon, occurrence in the Caucasus is also possible.

Apart from *Caucasiron*, the genus *Epeorus* s.l. is represented by a single species of *Epeorus* s.str. in the Caucasian fauna, namely *E.* (*E.*) *zaitzevi* Tshernova, 1981.

Although the genus *Epeorus* s.l. is one of the well-known groups of mayflies in the temperate Holarctic region (Nguyen *et al.* 2004), the mountain ecosystems of the Caucasus region, Asia Minor, and Middle and East Asia are still insufficiently investigated and the occurrence of more species may be expected there.

The objectives of the present contribution are to: i) describe a new species from Georgia and north eastern Turkey, based on the morphological and molecular data; ii) provide differential diagnosis with regard to closely related taxa; iii) compare the morphology-based species identification of Caucasian *Epeorus* (*Caucasiron*) with the molecular results.

Material and methods

The material used for this study was collected by T. Soldán, J. Bojková, R.J. Godunko and P. Sroka (Georgia 2009, 2011, Turkey 2011) (larvae), V. Švihla (Armenia 1975) (larvae), G. Türkmen (Turkey 2009, 2016) (larvae), A.V. Martynov (Georgia 2013, 2016) (larvae, adults). Most of the material, including the holotype, is deposited in the collection of the Biology Centre, Czech Academy of Sciences, Institute of Entomology, České Budějovice, Czech Republic. Some paratypes are housed in the State Museum of Natural History, National Academy of Sciences of Ukraine (Lviv) (SMNH NASU) (8 larvae in EtOH), National Museum of Natural History, National Academy of Sciences of Ukraine (Kiev) (NMNH NASU) (18 larvae in EtOH and 3 larvae mounted on slides); and collection of N. Kazancı and G. Türkmen, Hacettepe University, Department of Biology, Biomonitoring Laboratory, Turkey (3 larvae in EtOH and 3 larvae mounted on slides).

Material of other *Epeorus* (*Caucasiron*) species for morphological and molecular comparisons used in the present study is deposited in the collection of the Institute of Entomology, České Budějovice, Czech Republic. Larvae and adults were associated by rearing in the field (male subimago, male imago) and COI sequence comparison (female imago). Abbreviation “n “ refers to the number of specimens examined.

Morphological study. Larval and subimaginal exuviae and specimens were preserved in 75–96% EtOH. Some specimens were mounted on slides with HydroMatrix® (MicroTech Lab, Graz, Austria). In order to remove the muscle tissues for an investigation of the cuticular structure, the specimens were left overnight in a 10% solution of NaOH prior to slide mounting. Drawings were made using a stereomicroscope Olympus SZX7 and a microscope Olympus BX41, both equipped with a drawing attachment. Photographs were made using a stereomicroscope Nikon SMZ 1500 with camera Infinity 2 and software QuickPhoto 3.0. All photographs were subsequently enhanced with CombineZP and Adobe Photoshop™ CS5. For scanning electron microscopy, samples were gradually transferred to acetone, critical point dried, and coated with gold by sputtering using a Baltec SCD050 Sputter Coater. Observations were made on the Jeol JSM 7401F scanning microscope at 4 kV at the Institute of Parasitology, BC CAS, České Budějovice, Czech Republic.

The eggs were dissected from female imago. Diagnostic characters for the description of larva were used according to Braasch & Soldán (1979). To name structures related to the cuticular texture of subimaginal tarsal segments and to the colouration, we mostly adopted the terminology previously used by Kluge & Novikova (2011) and Kluge (2004; 2015). The term medio-lateral sigilla is used for the first time in *Epeorus* s.l. and refers to the pair of coloured marks on the cuticle located on the abdominal sterna, corresponding to the muscle attachment of the gill plates (Fig. 9).

Molecular study. ***DNA extraction, amplification and sequencing.*** Total genomic DNA of 58 specimens was extracted from legs (in both larvae and adults) using the DEP-25 DNA Extraction Kit (TopBio s.r.o., Prague, Czech Republic) according to the manufacturer's protocol. To avoid contamination, all DNA laboratory work was conducted in sterile conditions with the use of barrier tips. PCR amplification was carried out using the Unis Taq system (TopBio s.r.o., Prague, Czech Republic) with forward primer F (5´-TTC AGC CAC TTT ACC GCG- 3´, Rutschmann *et al*. unpublished) and universal DNA barcode reverse primer HCO2198 (5´-TAA ACT TCA GGG TGA CCA AAA AAT CA- 3´, Folmer *et al*. 1994) for amplification of ca. 650 base pairs (bp) of the 5' end of mitochondrial cytochrome oxidase I gene (COI).

Reaction volumes (12.5 µl) consisted of 7.625 µl of ddH_2O, 1.25 µl of 10 × reaction buffer, 1 µl of dNTP mixture (2.5 mM each dNTP), 0.75 µl of each primer (5pmol/ µl), 0.125 µl UNIS Taq polymerase (0.05 U/µl) and 1 µl of template DNA (not quantified). The PCR reactions were carried out in a GenePro (Bioer Co., Ltd, China) thermocycler with the profile: 94 °C for 1 min followed by 40 cycles of 94°C for 30 s, 47–51°C for 40 s, 72°C for 1 min 30 sec, and final extension at 72°C for 1 min 30 s. In all sets of reactions, negative controls (reaction without DNA) were included to ensure that no cross-contamination occurred. After the verification on agarose electrophoresis, the PCR products were cleaned enzymatically with mixture of 0.5 µl Exonuclease I and 1 µl FastAP (Thermo Fisher Scientific Inc., USA) per sample (incubated at 37°C for 30 min followed by incubation at 80°C for 15 min) before direct sequencing. Sequencing using the forward primer was performed by the SEQme company (Dobříš, Czech Republic) with the Sanger method. Only in a few samples this primer failed to produce high quality chromatograms, in which case the reverse primer sequencing was employed.

Sequences were assembled in Geneious 7.0.6. (http://www.geneious.com; Kearse *et al*. 2012) and aligned in the same software using Mafft v7.017 (Katoh *et al*. 2002) plugin with default settings. An alignment was edited by online version of Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html; Castresana 2000). All sequences obtained in the present study are listed in Appendix 1 and deposited in GenBank.

Analyses of molecular data. Pairwise genetic distances for a COI dataset were calculated in Mega version 7 (Kumar *et al*. 2016) using the Tamura-Nei model (TN93) (Tamura & Nei 1993), which was the best substitution model according to a Model test conducted in Mega version 7. A pairwise deletion option was used for missing data. The distance matrix was analysed by Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al*. 2012) (online version: http://wwwabi.snv.jussieu.fr/public/abgd/) with default settings.

The hypothetical species composition generated by ABGD was compared with morphologically defined *Caucasiron* species, including the newly described species *E*. (*C*.) *bicolliculatus* **sp. nov.**

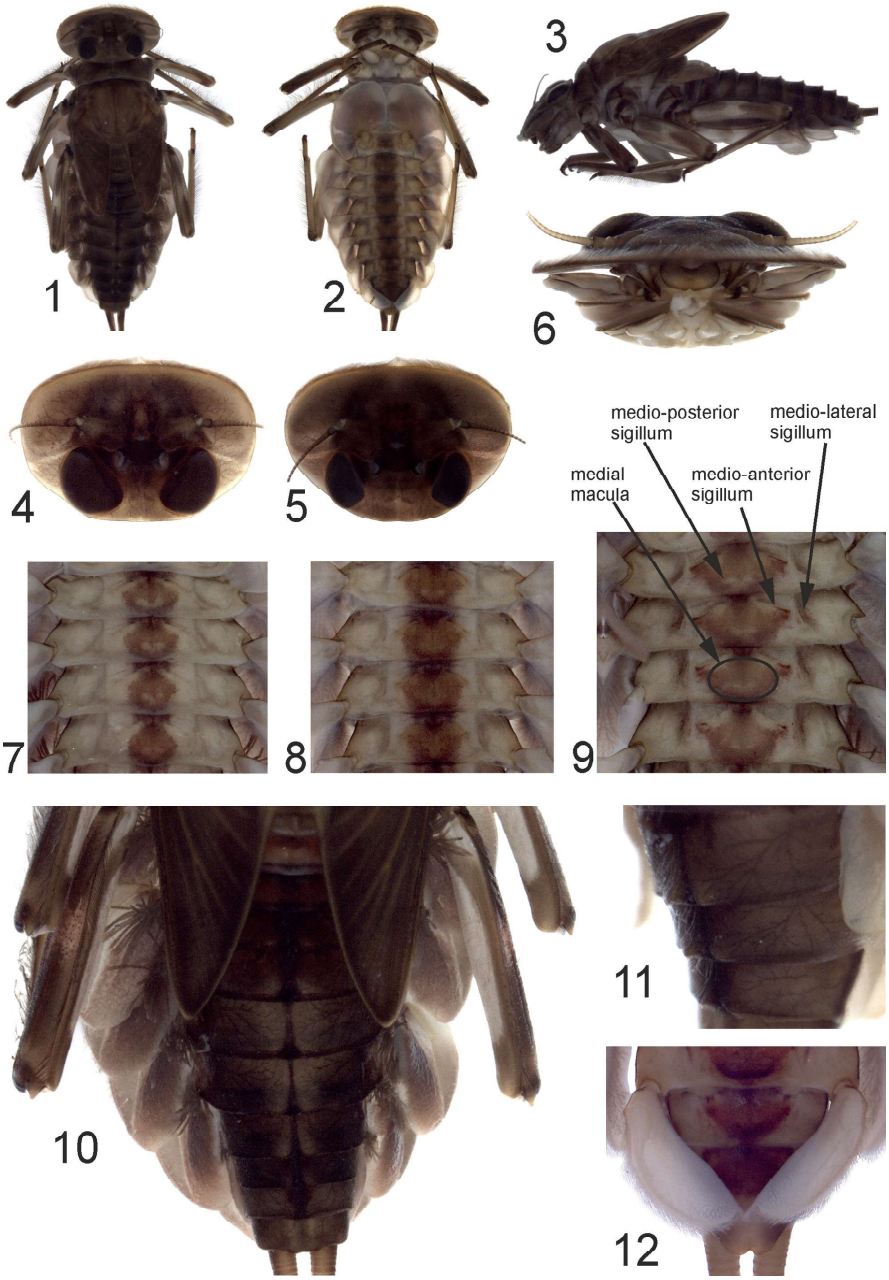
The histogram showing the distribution of the TN93 pairwise genetic distances was generated in PAST 3.14 (Hammer *et al*. 2001) (Fig. 50). The clustering was illustrated by a Neighbour joining tree (NJ) (Saitou & Nei 1987). In addition to NJ, the dataset was also analyzed using maximum likelihood analysis (ML) with the substitution model HKY+G+I generated by jModel test 2.14 (Darriba et. al. 2012; Guindon *et al.* 2003) following BIC criterion. Statistical support of individual clades for both NJ and ML was expressed by the bootstrap method (Felsenstein 1985) with 1000 replicates. Both analyses were run in Mega version 7 (Kumar *et al*. 2016). The representative of the related genus *Rhithrogena* (sequence obtained from GenBank, accession number: HM481145.1; Vuataz *et al.* 2011) was used as an outgroup. The NJ tree was edited in the Interactive Tree Of Life (iTol) version 3 (Letunic & Bork 2016) (online: http://itol.embl.de/).

Results

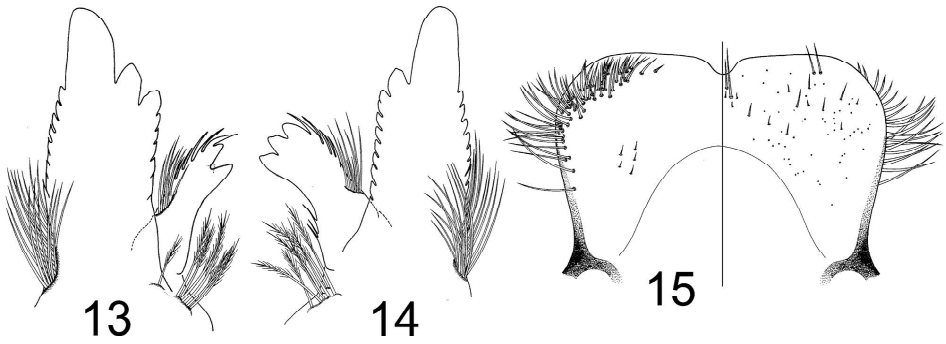
Taxonomy

***Epeorus* (*Caucasiron*) *bicolliculatus* Hrivniak, sp. nov.**
Figures 1–48

Epeorus alpicola (Eaton, 1871): Türkmen & Kazancı (2015: 44) [partim]
Epeorus sylvicola (Pictet, 1865): Türkmen & Kazancı (2015: 44, 45) [partim]
Epeorus (*Caucasiron*) sp.: Martynov *et al*. (2016: 170)



FIGURES 1–12. *Epeorus* (*Caucasiron*) *bicolliculatus* **sp. nov.**, larva: 1, female (dorsal); 2, female (ventral); 3, female (lateral); 4, male head (dorsal); 5, female head (dorsal); 6, male head (frontal view; labrum in natural position); 7–9, colouration of sterna II–V; 10, colouration of terga I–X; 11, tergal protuberances on terga VII–IX; 12, gill plates VII.



FIGURES 13–15. *Epeorus* (*Caucasiron*) *bicolliculatus* **sp. nov.**, mouthparts (flattened on slide): 13, right mandible incisors (ventral); 14, left mandible incisors (ventral); 15, labrum (left part ventral; right part dorsal).

Type material. Locality numbers (in square brackets) correspond to numbers on Fig. 51. Collectors of type material are listed in section Material and methods.

Holotype: female mature larva, **[1.]** GEORGIA: Autonomous Republic of Adjara (hereafter Adjara), vicinity of Chakhati village, Kintrishi Riv. (41°45'43"N 41°58'34"E), 325 m a.s.l., Martynov A.V. leg. 18–20.4.2016 (preserved in 96% EtOH).

Paratypes: 1 female, 1 male imago, 1 male subimago (all reared from larvae and mounted on slides), 1 male, 6 female larvae (2 mounted on slide), same place and date as holotype **[1.]**; same place and date as holotype **[1.]**; 2 female larvae, GEORGIA, Adjara, vicinity of Chakhati village, Kintrishi Riv. (41°45'42"N 41°58'42"E; close to holotype locality), 325 m a.s.l., 6.6.2016; 12 female larvae (1 mounted on slide, 3 with only mouthparts mounted on slide), 1 male larva (mounted on slide), **[2.]** GEORGIA: Adjara, vicinity of Chakhati village, right tributary of Kintrishi Riv. (41°44'14"N 41°59'00"E), 380 m a.s.l., 19.4.2016; 1 male larva, **[4.]** GEORGIA: Imereti Region (hereafter Imereti), vicinity of Tskhomareti village, Jruchula Riv. (42°23'12.8"N 43°21'33.3"E), 505 m a.s.l., 23.6.2013; 4 male, 5 female larvae (1 mounted on slide), **[5.]** GEORGIA: Imereti, right tributary of Sairmegele Riv. (41°52'57.7"N 42°45'22.6"E), 1276 m a.s.l., 25.6.2013; 2 female larvae (1 mounted on slide), **[8.]** GEORGIA: Imereti, Sairmegele Riv. and its right tributary (41°52'45.2"N 42°45'32.2"E), 1285 m a.s.l., 25.6.2013; 1 female larva, **[9.]** GEORGIA: Imereti, app. 2 km S. Health Resort Sairme, Sairmegele Riv. (41°52'56"N 42°45'09"E), 1262 m a.s.l., 25.6.2013; 1 male, 2 female larvae (1 mounted on slide), GEORGIA: Adjara, vicinity of Chakhati village, right tributary of Kintrishi Riv. (41°45'23"N 41°58'41"E; close to holotype locality), 385 m a.s.l., 7.6.2013; 1 female larva, **[12.]** GEORGIA: Guria Region, Ozurgeti (41°54'29.64"N 42°03'52.44"E), 169 m a.s.l., 22.8.2009; 1 female larva, **[13.]** GEORGIA: Adjara, Zeda Erge village, near bridge on road Batumi–Akhaltsikhe, Jochostskali Riv. (right tributary of Tchorokhi Riv.) (41°34'02.04"N 41°41'16.20"E), 40 m a.s.l., 21.8.2009; 2 female larvae, **[14.]** GEORGIA: Adjara, Adzarisagmarti village, left tributary of Machakhlistskali Riv. (41°30'37.20"N 41°49'18.06"E), 207 m a.s.l., 20.8.2009; 1 female larva, **[15.]** TURKEY: Trabzon Province, vicinity of Hamşiköy village, right tributary of Maçka Riv. (40°40'45.36"N 39°26'55.74"E), 1460 m a.s.l., 29.6.2011; 1 female larva, **[16.]** GEORGIA: Adjara, Khala village, Kemerkobis Riv. (right tributary of Chekvistskali Riv.) (41°42'08.70"N 41°48'51.30"E), 100 m a.s.l., 18.8.2009; 5 female larvae (2 mounted on slides), 1 male larva (mounted on slide) **[19.]** TURKEY: Giresun Province, Kümbet Plateau, small tributary of Aksu Stream (40°32'19"N 38°21'35" E), 1570 m a.s.l., 02.6.2009; 2 female, 2 male larvae, **[20.]** TURKEY: Artvin Province, Artvin town, Gorgit Plateau Road, Didrelle Creek (41°25'13.65"N 41°55'29.57"E), 922 m a.s.l., 29.7.2016.

Other material examined (not type): 1 male larva, **[3.]** GEORGIA: Adjara, vicinity of Khino village, Kintrishi Riv. (41°43'01"N 42°02'41"E), 794 m a.s.l., 19.4.2016; 4 female, 3 male larvae, **[5.]**; 1 juv. larva, **[6.]** GEORGIA: Adjara, Kokoleti village, Machakhlistskali Riv. (41°29'54.7"N 41°51'26.1"E), 273 m a.s.l., 17.6.2013; 1 female larva, **[7.]** GEORGIA: Imereti, small stream, road Sairme-Abastumani (41°51'49.0"N 42°47'21.1"E), 1804 m a.s.l., 30.6.2013; 1 male larva, **[10.]** GEORGIA: Racha-Lechkhumi and Kvemo Svaneti Region, Legvanta village, Sakora Riv. (42°36'32.28"N 43°27'45.18"E), 837 m a.s.l., 25.8.2009; 4 juv. larvae, **[11.]** GEORGIA: Adjara, app. 5 km E. Goderdzi Pass, left tributary of Dzindzinstskali Riv. (41°38'00.18"N 42°33'24.6"E), 1801 m a.s.l., 28.8.2009; 1 female larva, **[14.]**; 1 female larva, **[15.]**; 1 female larva, ARMENIA: Kotayk Province, Geghard, Azat Riv., July 1975; 4 female larvae, **[17.]** TURKEY: Giresun Province, Dereli-Kümbet Road, Aksu

Stream (40°41'01.08"N 38°26'29.71"E), 557 m a.s.l., 1.6.2009; 1 female larva **[18.]** TURKEY: Giresun Province, Kümbet Plateau, Aksu Stream, (40°32'22"N 38°24'06"E), 1542 m a.s.l., 2.6.2009.

Description

Larva (females: n = 54; males: n = 15; juvenile larvae: n = 5)

Body length 10–13 mm (female), 9–12 mm (male). Length of cerci approximately 1.3× body length.

Head. Yellowish with brown to reddish-brown markings. Anterior margin slightly paler. Dorso-medial part with dark (brown or reddish) elongated macula, extending from lateral ocelli. Inner edges of eyes with dark triangular maculae. Pale stripes extending from lateral ocelli to lateral edges of head. Eyes dark grey to black, with (more or less visible) pale stripe around. Ocelli dark grey to black, paler basally. Antennae yellowish.

Shape of head trapezoidal, slightly different in male and female (Figs 4–5). Head width/length ratio 1.44–1.51 (female), 1.47–1.56 (male), head width of mature larvae 4–4.2 mm, length 2.7–2.8 mm (female), 3.1–3.7 mm and 2.0–2.5 mm (male). Anterior margin slightly convex (sometimes with shallow medial concavity), covered with dense hair-like setae extending to lateral margins and directed medio-dorsally. Lateral margins rounded, posterior margin convex (sometimes with shallow concavity medially) in female, slightly convex or nearly straight in male (Figs 4–5). Dorsal surface of head densely covered with basally widened setae (as on abdominal terga, see Figs 23, 30). Sparse pale fine setae located posteriorly to eyes.

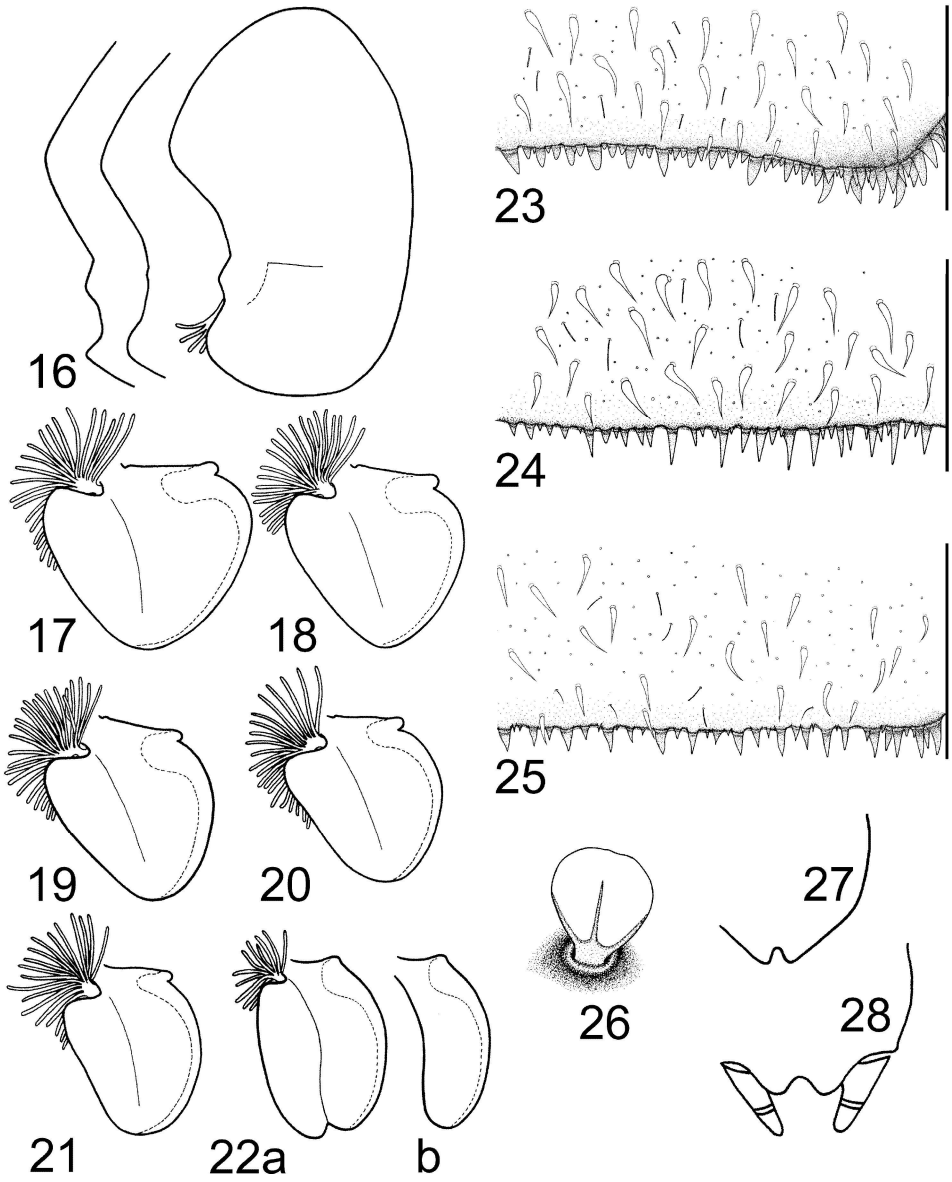
Mouthparts. Labrum (Figs 6, 15) widened distally, with anterior margin slightly concave or nearly straight; small medial emargination present. Antero-lateral margins rounded (shape of labrum may vary among individual specimens). Dorsal surface (Fig. 15, right) with sparse scattered setae and longer bristles antero-medially and antero-laterally. Ventral surface (Fig. 15, left) with longer shortly plumose bristles situated laterally and brush of fine setae medially (not figured). Posterior margin of labrum concave and rounded. Outer incisors of both mandibles (Figs 13–14) with three apical teeth, outer tooth of each mandible blunt. Inner incisors of left mandible with three apical teeth (Fig. 14), right inner incisors bifurcated (sometimes third short tooth is present in middle) (Fig. 13). Prostheca represented by tuft of plumose setae. Shape of maxillae and maxillary palpi as in congeners. Lingua of hypopharynx oblong-shaped, with two latero-apical lobes, covered with dense hair-like setae. Superlingua with apical lobes on inner margin, covered with hair-like setae that extend laterally; outer margin slightly extended, rounded and pigmented. Dorsal surface of glossae with longitudinal brush of fine setae; ventral surface with scattered short setae and longer setae on outer margins. Inner margins with pale bristles extending medially near apex. Paraglossae densely covered with fine setae on its anterior half. Shape of glossae, paraglossae, shape and setation of labial palpi as in congeners.

Thorax. Yellowish-brown to reddish-brown, with pale markings dorsally. Pronotum anteriorly narrowed, with lateral edges slightly rounded or straight. Posterior margin of metanotum slightly convex (sometimes bluntly pointed medially). Setation of thorax as on head and abdominal terga (see Figs 23, 30) (wing pads sparsely covered), pale hair-like setae present along mesonotal suture.

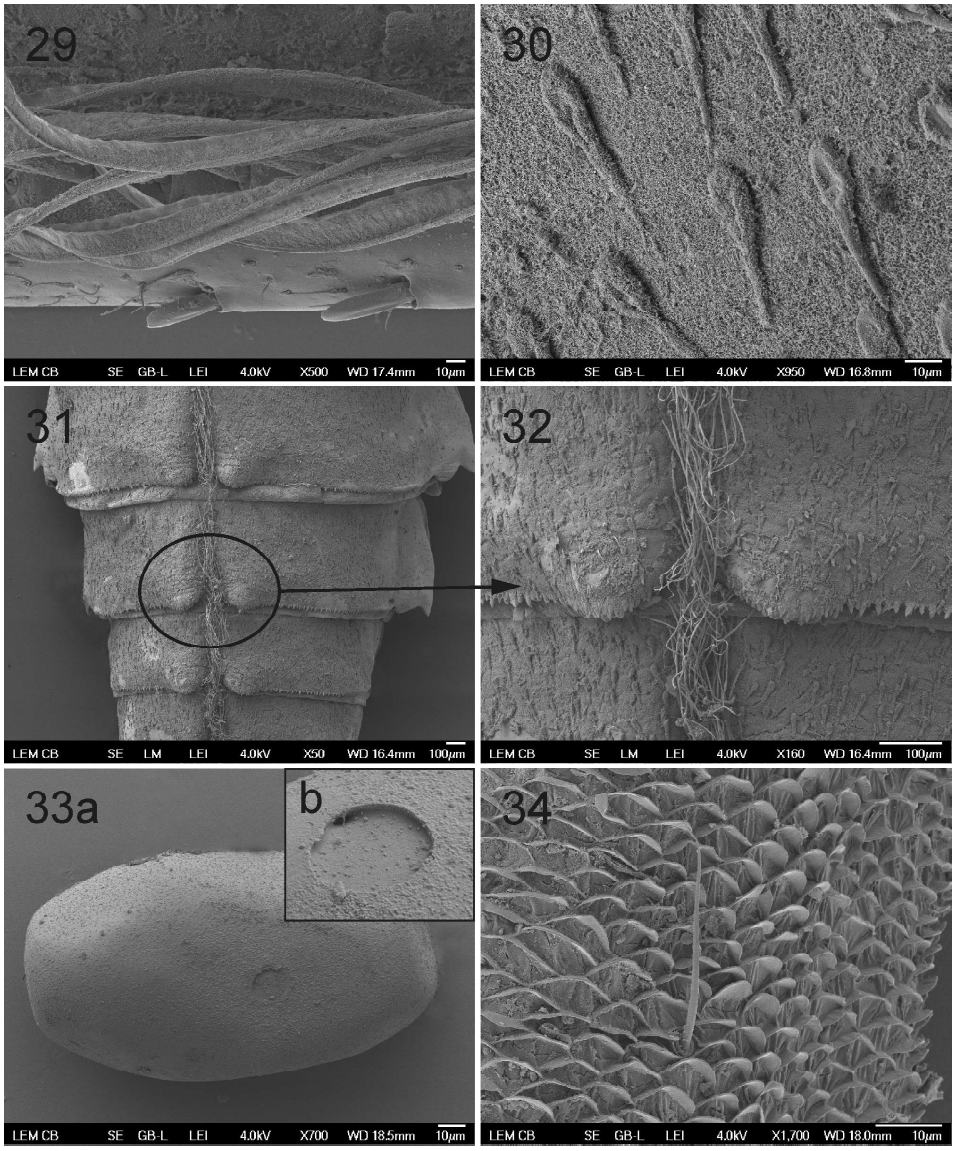
Legs. Yellowish-brown to reddish-brown. Femora with more or less contrasting markings dorsally (Fig. 3), without hypodermal medial spot (blurred macula may be present in darker specimens). Tibio-patellar suture darkened, tibia unicoloured, tarsi proximally and distally darkened. Coxal projections of forelegs pointed, of middle legs blunt and of hind legs pointed or bluntly pointed. Trochanters with spatulate setae (absent or sparsely present on forelegs). Tibiae of forelegs longer than femora (1.17–1.25× femur length), tibiae of middle legs nearly equal length as femora (1–1.08× femur length), tibiae of hind legs slightly shorter than femora (0.81–0.98× femur length). Tarsi in all legs 0.26–0.37× tibia length. Posterior margin of femora with a row of long blade-like setae and sparse row of short bluntly pointed spines (Fig. 29). Setation of dorsal surface of femora consists of scattered short spatulate setae (distally widened and apically rounded) (Fig. 26), basally widened hair-like setae and sparse stick-like setae. Anterior margin with short pointed spines. Tibiae and tarsi with row of long shortly plumose setae dorsally and irregularly situated spines of various sizes ventrally. Tarsal claws with 3–4 teeth.

Abdomen. Outer margins of individual segments with short and blunt (in lateral view) projections located anteriorly to gill bases.

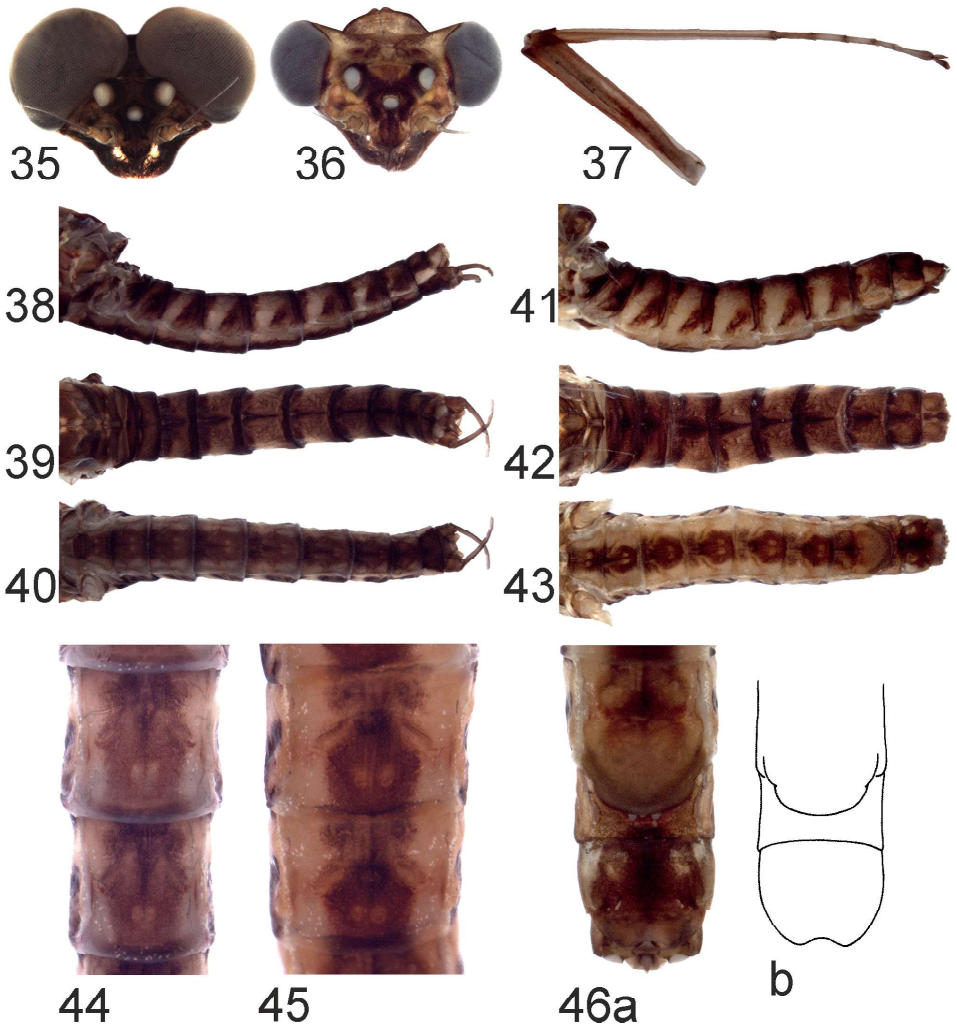
Abdominal terga yellowish-brown, with contrasting dark brown or reddish-brown pattern (Figs 1, 10), that consists of: a) narrow macula along anterior margin on terga I–IX; b) large medial macula on terga II–IV (shape of macula variable; usually in form of blurred stripe or triangle); c) anteriorly and posteriorly slightly widened medial longitudinal stripe stretching between anterior and posterior margin (posterior widening sometimes not clear) on terga V–VI (VII); d) elongated medial macula on terga (VII) VIII–IX; e) pair of sigilla (in form of short dark stripes or blanks) adjacent antero-laterally to medial maculae or stripes (in some individuals absent); f) pair of tiny blanks medially on tergum X; g) postero-lateral maculae on terga I–VII (VIII).



FIGURES 16–28. *Epeorus* (*Caucasiron*) spp.: 16–22a, *E.* (*C.*) *bicolliculatus* **sp. nov.**, gills I–VII (flattened on slide); 22b gill plate VII in natural position from ventral view; 23–25, tergum VII (flattened on slide; vertical line represents midline of tergum): 23, *E.* (*C.*) *bicolliculatus* **sp. nov.;** 24; *E.* (*C.*) *longimaculatus* (paratype); 25, *E.* (*C.*) *soldani*; 26, *E.* (C.) *bicolliculatus* **sp. nov.**, femoral spatulate seta (medial area of middle femur) (flattened on slide); 27, *E.* (*C.*) *bicolliculatus* **sp. nov.**, IX sternum of female; 28, *E.* (*C.*) *bicolliculatus* **sp. nov.**, IX sternum of male.

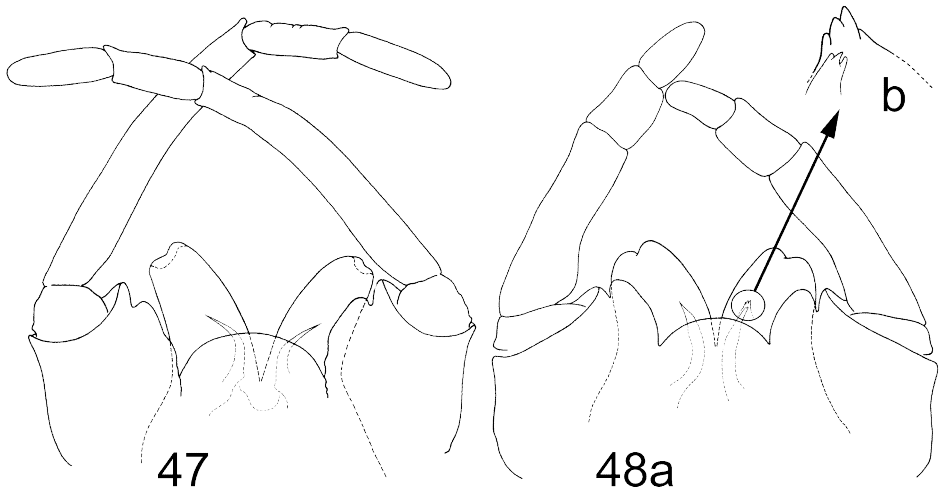


FIGURES 29–34. *Epeorus* (*Caucasiron*) *bicolliculatus* **sp. nov.**, larva, egg, subimago: 29, blade-like setae and bluntly pointed spine-like setae along posterior margin of femora (larva); 30, basally widened setae on tergum VII (larva); 31, tergal protuberances on terga VI–VIII (larva); 32, tergal protuberances in detail (larva); 33a, egg; 33b, detail of chorionic surface and micropyle; 34, detail of microlepides and microtrichia of 4th segment of foretarsus (male subimago).



FIGURES 35–46. *Epeorus* (*Caucasiron*) *bicolliculatus* **sp. nov.**, imago: 35, male head; 36, female head; 37, middle leg; 38, male abdomen (lateral); 39; male abdomen (dorsal); 40, male abdomen (ventral); 41, female abdomen (lateral); 42, female abdomen (dorsal); 43, female abdomen (ventral); 44–45, detail of colouration pattern (sterna V–VI); 46a, b, subgenital plate and sternum IX (female).

Terga II–IX with pair of postero-medial protuberances (Figs 1, 10, 11, 31, 32), which size varies between terga (gradually increasing in size, most developed on terga VI–VIII) and individuals; these protuberances usually paler in colour than adjacent area. Denticles on posterior margin of terga of various size, pointed or bluntly pointed, longer, denser, sometimes curved on protuberances (Fig. 23). Denticulation on posterior margin of terga interrupted medially. Each abdominal tergum with medial longitudinal stripe of relatively sparse hair-like setae (Figs 11, 31–32). Surface of terga covered (as head and thorax) with dense, basally widened setae (Figs 23, 30, 32) and sparsely with stick-like setae. Tergum X without pointed postero-lateral projections (slight convexity may be present).



FIGURES 47–48. *Epeorus* (*Caucasiron*) *bicolliculatus* **sp. nov.**, male genitalia: 47, imago; 48a, subimago; 48b, subimaginal titillator in detail (apex).

Abdominal sterna yellowish. Each sternum with more or less contrasting brown or reddish-brown pattern (Figs 2, 7–9). Sternum I with pair of blanks (medio-posterior sigilla) surrounded by dark medial macula. Pattern of sterna II–VIII consists of: a) pair of dark stripes located laterally from nerve ganglion (medio-anterior sigilla; in some individuals colourless); b) dark medial macula (shape may vary between specimens of both sexes, see Figs 7–9); c) pair of blanks (medio-posterior sigilla) inside medial macula (sometimes less contrasting due to paler medial macula or darker than medial macula); d) pair of dark medio-lateral stripes that represent medio-lateral sigilla (in some specimens colourless). Sternum IX usually with pair of blanks anteriorly (distinctly visible in female) and paler area posteriorly. Pattern of early-instar larvae sometimes colourless. Sternum IX with shallow medial notch (Figs 27–28) and covered by short setae and fine hair-like setae medially and medio-laterally.

Gills. Dorsally grey or brown-reddish (Figs 1, 10), ventrally whitish to pinkish (Fig. 2). Gill plates on segment I overlapping beneath thorax, shape of inner edge variable (Fig. 16). Projection on anterior margin of gill plates II–VII well developed (size of projection vary among specimens and segments) (Figs 17–22b). Gill plates VII relatively narrow (Figs 12, 22a, b). Each gill plate with filaments. Filaments of gills II–VII darkly pigmented and relatively dense. Filaments of gills II–VI reaching to 0.38–0.54× length of respective plate, filaments of gill VII to 0.2–0.33× (in late-instar larvae). Setation of gill plates as in congeners.

Cerci. Yellowish-brown, basally darkened. Dorsal margin with fine hair-like setae, segments with short, blunt and pointed teeth posteriorly.

Imago male (n = 1; reared from larva). Body length 14 mm. Length of forewings 15 mm, hind wings 5 mm. Cerci 2.35× body length. General colour of body yellowish-brown to brown with dark brown to reddish markings.

Head. Frontal fold dark brown. Medial area yellowish, with dark macula medially, extending from lateral ocelli. Antennae yellowish-brown. Ocelli basally brown, apically whitish. Eyes greyish, basally darkened, not touching each other. Distance between compound eyes approximately 1/2 of medial ocellus width (Fig. 35).

Thorax. Yellowish, with dark stripe along medial suture. Posterior margin of thorax, posterior scutal protuberances and area under lateroparapsidal suture darkened. Mesothoratic furcasternum dark brown. Wing membrane colourless. Veins dark brown, basally paler. C, Sc, RA slightly paler than remaining longitudinal and cross veins. Pterostigmatic area cloudy, with simple cross veins. Costal brace dark brown. Veins of hind wings brownish, basally paler.

Legs. Forelegs yellowish-brown, femora and tibiae darker, tarsi and tarsal claws paler. Middle and hind legs yellowish. All femora distally darkened, with brown elongated macula medially (distinctly visible on paler middle and hind legs; see Fig. 37).

Abdomen. Yellowish-brown. Colour pattern on abdominal terga and sterna as in mature larva (Figs 38–40, 44); longitudinal medial stripe of terga V–VI (VII) posteriorly not widened; tergum X with dark medial stripe. Medio-lateral sigilla absent. Styliger dark brown, convex medially, penis lobes apically slightly narrower, with shallow medio-apical emargination. Titillators curved outwards, distally narrower, apically sharply pointed, exceeding styliger to approximately 1/3 of penis lobes (Fig. 47). Forceps brownish dorsally, whitish ventrally. Cerci yellowish-brown, basally darkened.

Imago female (n = 1; molecular association). Body length 14 mm. Lenght of forewings 15 mm, hind wings and cerci lost. General colouration of body and markings as in male imago.

Head. Yellowish-brown, with dark macula extending from lateral ocelli. Eyes grey, ocelli as in male imago (Fig. 36).

Thorax. Colouration as in male imago. Veins of forewings dark brown (including C, Sc, RA), basally paler. Pterostigmatic area and costal brace as in male imago.

Legs. Forelegs yellowish-brown. Femora paler; tibiae, tarsi and tarsal claws darker. Colouration of middle and hind legs and markings on femora as in male imago (Fig. 37).

Abdomen. Yellowish-brown. Colouration pattern of terga and sterna (Figs 41–43, 45) as in mature larva and male imago. Posterior margin of subgenital plate slightly rounded, medio-lateral margins with notch. Sternum IX (Figs 46a, b) with shallow, U-shaped medial emargination.

Subimago male (n = 1; reared from larva). Body length 12 mm. Length of forewings 14 mm, hind wings 4 mm. Cerci broken. General colouration of body darker than in imago.

Head. As in male imago. Scapus and pedicellus yellowish, flagellum dark brown. Eyes and ocelli as in male imago.

Thorax. As in imago. Wing membrane greyish, veins dark brown, basally paler. Costal brace dark brown.

Legs. Forelegs brown to dark brown, femora brownish, tibiae, tarsi and tarsal claws dark brown. Middle and hind legs yellowish-brown. Markings on femora as in male and female imago. Tarsal segments of all legs covered by short blunt microlepides and sparse microtrichiae (Fig. 34).

Abdomen. Colouration pattern as in imago.

Styliger and genitalia as in imago (almost fully developed). Titillators dissimilar, left titillator apically serrated, right titillator apically pointed. Both titillators exceeding styliger to 1/3 of penis lobes (Figs 48 a, b). Forceps and cerci dark brown.

Subimago female. Unknown.

Eggs. Oval shaped, dimensions approximately 185 × 105 µm. Chorionic surface slightly granulate, without distinct structures. Micropyle shallow and rounded, located in equatorial position (Figs 33a, b).

Etymology. The name of the species refers to the presence of protuberances on the abdominal terga of larvae. The name is derived from the Latin expression “colliculus” for an elevated hump and the prefix “bi-“, referring to the number of these structures on each of the terga II–IX.

Morphological diagnosis and affinities. *E.* (*C.*) *bicolliculatus* **sp. nov.** is clearly attributable to the subgenus *Caucasiron* based on the presence of projections on the costal margin of gill plates II–VII, the medio-dorsal direction of hair-like setae on the anterior and antero-lateral margin of the head, tubular penis lobes and developed titillators.

Larva of *E*. (*C.*) *bicolliculatus* **sp. nov**. can be recognized from all other species within the subgenus *Caucasiron* sensu Kluge (1997) by the combination of the following diagnostic characters: i) the presence of paired postero-medial protuberances on abdominal terga II–IX (Figs 1, 10, 11, 31, 32); ii) head, thorax, and abdominal terga covered with flattened, basally widened setae (as on Figs 23, 30); iii) abdominal terga with a medial row of relatively sparse hair-like setae (Fig. 31); iv) gill plates VII relatively narrow (in natural position from ventral view; Figs 12, 22 b), v) the colouration pattern of the abdomen (Figs 1, 2, 7–10). The character i) represents a unique feature of *E*. (*C.*) *bicolliculatus* **sp. nov**., not shared by any other species within the subgenus.

Despite the relatively clear morphological characteristics of *E*. (*C.*) *bicolliculatus* **sp. nov.**, its identification may be confused with several superficially similar *Caucasiron* species. The colouration pattern of the abdominal sterna of *E*. (*C.*) *bicolliculatus* **sp. nov.** is similar to *E*. (*C.*) *alpestris* at first glance. However, the presence of characteristic flattened setae and protuberances on abdominal terga reliably distinguish *E.* (*C.*) *bicolliculatus* **sp. nov.** from *E*. (*C.*) *alpestris*.

Only two *Caucasiron* species, namely *E.* (*C.*) *soldani* and *E.* (*C.*) *longimaculatus,* possess similar flattened setae on the dorsal surface of the head, thorax, and abdominal terga. Apart from the most obvious lack of protuberances on the abdominal terga, several additional characters can be found to separate these two species from *E.* (*C.*) *bicolliculatus* **sp. nov.** *E.* (*C.*) *soldani* differs from *E.* (*C.*) *bicolliculatus* **sp. nov.** by the well-defined triangular medial macula adjacent to the anterior margin on abdominal terga V–VII, in contrast to the thin longitudinal stripe in *E.* (*C.*) *bicolliculatus* **sp. nov.** (Fig. 10) (the pattern of sterna in *E.* (*C.*) *soldani* may be similar to lighter specimens of *E.* (*C.*) *bicolliculatus* **sp. nov.**). Additionally, *E.* (*C.*) *soldani* also differs in possessing mostly pointed, triangular spines on the posterior margin of terga (Fig. 25), whereas these spines are more variable in shape, both pointed and blunt in *E.* (*C.*) *bicolliculatus* **sp. nov.** (Fig. 23). *E.* (*C.*) *longimaculatus* differs from *E.* (*C.*) *bicolliculatus* **sp. nov.** in the presence of a characteristic medially elongated femur spot (missing or unclear blurred macula in *E.* (*C.*) *bicolliculatus* **sp. nov.**) and the longitudinal medial macula on abdominal terga V–VII in the shape of a narrow triangle or thick stripe (instead of a thin longitudinal stripe in *E.* (*C.*) *bicolliculatus* **sp. nov.** see Fig. 10). Furthermore, the sterna are without a distinct pattern in *E.* (*C.*) *longimaculatus*, contrary to the clear pattern of *E.* (*C.*) *bicolliculatus* **sp. nov.** (Figs 2, 7–9). Further differences in *E.* (*C.*) *longimaculatus* include the less developed projection on the costal margin of gill plates II–VII, more slender and elongated teeth on the posterior margin of the abdominal terga (Fig. 24) in comparison with *E.* (*C.*) *bicolliculatus* **sp. nov**. (Fig. 23), and the absence of a row of hair-like setae along the abdominal terga, in comparison with *E.* (*C.*) *bicolliculatus* **sp. nov**. (see Figs 11, 31).

The main diagnostic characters for the identification of the winged stages of *E.* (*C.*) *bicolliculatus* **sp. nov.** are: i) colouration patterns of the abdominal terga and sterna (Figs 38–45); ii) the colouration of the legs (Fig. 37); iii) the shape of the male genitalia (penis lobes with shallow medio-apical emargination (Figs 47–48a). It should be noted that because of the limited material available, only three specimens of *E.* (*C.*) *bicolliculatus* **sp. nov.** were investigated (2 imagines, 1 subimago) in the present study. Therefore we did not recognize the possible variability in the colouration and shape of penis lobes. The combination of the colouration pattern of the abdominal terga and sterna clearly distinguishes *E.* (*C.*) *bicolliculatus* **sp. nov.** from *E.* (*C.*) *caucasicus* (sterna with dark coloured medio-anterior sigilla, that form a pair of stripes; area of nerve ganglion dark; see Braasch 1979: 284, fig. 3), *E.* (*C.*) *nigripilosus* (sterna with pair of dark medio-lateral spots or stripes; area of nerve ganglion dark; see Braasch 1979: 284, fig. 4), *E.* (*C.*) *sinitshenkovae* (terga with narrow triangular medial maculae adjacent to anterior margin and pair of spots adjacent by side to medial maculae; see Braasch & Zimmermann 1979: 105, fig. 1), *E.* (*C.*) *magnus* and *E.* (*C.*) *longimaculatus* (both species without distinct pattern on the abdominal sterna; for the colouration of terga see Braasch 1980: 172, figs 1, 2). The latter species possess similar dark elongated medial macula on the femora as *E.* (*C.*) *bicolliculatus* **sp. nov.** However, besides the different colouration of the abdominal sterna, *E.* (*C.*) *longimaculatus* additionally differs by a nearly straight medial margin of styliger as described by Braasch (1980) for male subimago (imago unknown). A similar colouration of terga to *E.* (*C.*) *bicolliculatus* **sp. nov.** occurs in *E.* (*C.*) *znojkoi*. This species can be separated from *E.* (*C.*) *bicolliculatus* **sp. nov**. by the following characteristics: i) the colouration of the sterna (blurred reddish macula, or macula in the form of a more or less well-defined large red triangle located behind medio-anterior sigilla, sigilla reddish or colourless; for the colouration of the sterna of *E.* (*C.*) *bicolliculatus* **sp. nov.** see Figs 40, 43, 44, 45; ii) the position of the eyes (dorsally touching each other or very closely separated in *E.* (*C.*) *znojkoi*, more widely separated in *E.* (*C.*) *bicolliculatus* **sp. nov.** (Fig. 35). Assuming from the variability of the colouration of the abdominal sterna in the mature larvae of *E.* (*C.*) *bicolliculatus* **sp. nov.**, the pattern of sterna in adults may in some specimens seem similar to *E.* (*C.*) *alpestris* and *E.* (*C.*) *soldani.* Both species differ from *E.* (*C.*) *bicolliculatus* **sp. nov.** by the absence of a medial stripe on the femora (Braasch 1979; Chen 1999). Additionally, *E.* (*C.*) *soldani* differs by the presence of a triangular medial macula (adjacent to anterior margin) on the abdominal terga. For the colouration of the abdominal terga of both species see Braasch (1979: 284, figs 1, 2).

Regarding the morphology of male genitalia, Braasch (1980) proposed two groups for the Caucasian species of *Caucasiron* (originally *Iron*): "*caucasicus* Gruppe" (penis lobes more or less apically bifurcated) with species *E.* (*C.*) *caucasicus* (syn. *fuscus*), *E.* (*C.*) *nigripilosus* and possibly *E.* (*C.*) *longimaculatus* (imago unknown), and "*znojkoi* Gruppe" (penis lobes only with shallow apical emargination) with species *E.* (*C.*) *znojkoi*, *E.* (*C.*) *alpestris*, *E.* (*C.*) *soldani*, *E.* (*C.*) *sinitshenkovae*, *E.* (*C.*) *magnus*. In this classification, *E.* (*C.*) *bicolliculatus* **sp. nov.** is congruent with the "*znojkoi* Gruppe". Based on the shape of the penis lobes, *E.* (*C.*) *bicolliculatus* **sp. nov.** can be distinguished from *E.* (*C.*) *alpestris* and *E. (C.) soldani.* The penis lobes are apically slightly narrowed in *E.* (*C.*) *bicolliculatus* **sp. nov.** (Fig. 47), whereas they are apically widened in *E.* (*C.*) *soldani*; see Chen (1999: 175,

fig. 201), and of mostly constant width in *E.* (*C.*) *alpestris;* see Chen (1999: 175, fig. 200).We refrain from generalizing the shape of titillators observed in *E.* (*C.*) *bicolliculatus* **sp. nov.** In the male imago, both titillators are apically pointed, whereas in the male subimago, one titillator bears several denticles on its apex (Figs 47, 48). Probably it represents a variable character within *E.* (*C.*) *bicolliculatus* **sp. nov.**; we found similar variations in other *Caucasiron* species we investigated.

The cuticular texture of subimaginal tarsal segments within Caucasian *Caucasiron* species are not described, therefore comparison is not possible. The Central Asian representative of the subgenus, namely *E.* (*C.*) *guttatus* has the first tarsal segments of all legs covered partly by microtrichiae, partly by blunt microlepides, whereas 2nd–5th segment are entirely covered by blunt microlepides (see Kluge 2015: 343). In the case of *E.* (*C.*) *bicolliculatus* **sp. nov.** no significant differences between subimaginal tarsal segments were observed; tarsal segments of all legs are covered by short blunt microlepides and sparse microtrichiae (as on Fig. 34).

A detailed description of the female genital apparatus and the structure of eggs are missing in the taxonomic literature; therefore, comparison with related species is not possible at present.

Analyses of molecular data

DNA sequencing. The „DNA Barcoding" fragment of COI was succesfully obtained from 47 specimens of *Caucasiron* spp. (see Appendix 1). Final alignment consisted of sequences with 621–659 base pairs. Of the 195 variable sites, 181 were parsimony informative positions. No insertions, deletions, or stop codons were identified. Nucleotide composition averaged overall showed an A+T bias (70.1%).

Data analyses. The ABGD analysis of the COI distance matrix generated eight stable groups (Fig. 49—A–H) within the initial partition. The seven groups corresponded with the morphologically defined *Caucasiron* species, namely *E.* (*C.*) *nigripilosus* (cluster A), *E.* (*C.*) *caucasicus* (cluster B), *E.* (*C.*) *znojkoi* (cluster C), *E.* (*C.*) *bicolliculatus* **sp. nov.** (cluster D), *E.* (*C.*) *soldani* (cluster F), *E.* (*C.*) *alpestris* (cluster G), and *E.* (*C.*) *magnus* (cluster H). Two species, namely *E.* (*C.*) *sinitshenkovae* and *E.* (*C.*) *longimaculatus,* were merged into one group (cluster E), where each species represented a separate lineage.

The genetic distances within groups generated by ABGD ranged between 0.00 and 7.14% and the distances between groups between 10.68 and 18.51% (Fig. 50). Thus, the barcoding gap detected by ABGD was 3.54% wide. The recursive (finer) partition delimited 10–21 groups. This partitioning reflected the intraspecific variability of COI both within and between geographically distinct populations of species, namely *E.* (*C.*) *caucasicus*, *E.* (*C.*) *znojkoi*, *E.* (*C.*) *nigripilosus*, *E.* (*C.*) *magnus* and *E.* (*C.*) *sinitshenkovae*.

The genetic differences between the newly proposed species *E.* (*C.*) *bicolliculatus* **sp. nov.** and all other *Caucasiron* species included in the present study ranged between 11.51 and 18.51%. Inter- and intraspecific genetic distances are shown in Table 1 and Table 2.

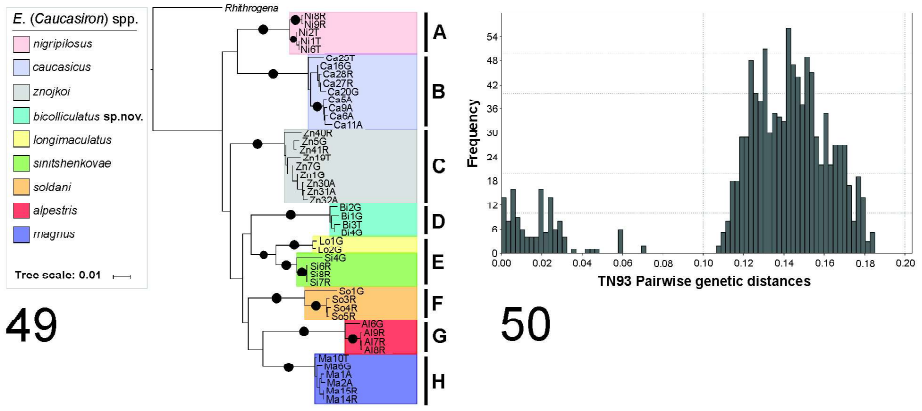


FIGURE 49–50. 49, Neighbour joining distance tree: (A–H) represents hypothetical species generated by ABGD, coloured clusters represents morphology-based species. Black circle correspond to bootstrap value ≥ 97%. 50, Distribution of the genetic variation (TN93 pairwise distances) within *Epeorus* (*Caucasiron*) spp.

TABLE 1. Mean/Standard deviation/minimum-maximum values of interspecific TN93 pairwise genetic distances.

Mean/SD **Min–Max** **Species**	*caucasicus*	*nigripilosus*	*longimaculatus*	*sinitshenkovae*	*znojkoi*	*soldani*	*bicolliculatus* **sp.nov.**	*magnus*
nigripilosus	12.40/0.56/ 11.42–13.53							
longimaculatus	12.74/0.43/ 12.05–13.35	15.83/0.46/ 15.10–16.37						
sinitshenkovae	13.81/0.85/ 12.76–15.87	14.46/0.43/ 13.93–15.54	6.17/0.55/ 5.79–7.14					
znojkoi	15.03/0.52/ 13.89–16.33	11.95/0.75/ 10.68–13.60	12.61/0.40/ 11.87–13.47	12.19/0.83/ 11.27–14.22				
soldani	16.20/0.47/ 15.45–17.56	15.42/0.38/ 14.85–16.05	12.47/0.61/ 11.80–13.53	12.12/0.47/ 11.50–13.05	13,86/0.50/ 13.16–15.25			
bicolliculatus **sp.nov.**	17.52/0.58/ 16.39–18.51	16.92/0.42/ 16.11–17.67	12.74/0.50/ 12.11–13.45	12.10/0.31/ 11.51–12.48	14.47/0.41/ 13.52–15.25	14.72/0.37/ 14.13–15.35		
magnus	14.54/0.50/ 13.15–15.42	14.23/0.28/ 13.76–14.62	13.56/0.08/ 12.97–13.97	14.07/0.32/ 13.51–14.45	12.65/0.37/ 11.81–13.34	13.36/0.37/ 12.44–13.82	15.18/0.40/ 14.29–15.91	
alpestris	17.04/0.38/ 16.48–17.97	17.66/0.27/ 17.13–18.05	16.60/0.45/ 15.80–17.13	15.71/0.52/ 15.34–16.83	16.26/0.40/ 15.40–17.27	15.74/ 0.44/ 15.74/ 0.44	16.15/0.73/ 15.22–17.83	12.89/0.44/ 11.86–13.45

The genetic distances between female imago associated with the larvae of *E.* (*C.*) *bicolliculatus* **sp. nov.** based on the COI sequence comparison exhibited range between 1.09 and 1.41%.

The monophyly of the groups recognized by ABGD and the morphology-based species including *E.* (*C.*) *bicolliculatus* **sp. nov.** were also recognized in the distance tree generated by NJ (Fig. 49) and gene tree generated by ML (not figured) analyses. All groups were supported by high bootstrap values (≥ 97%) in both approaches.

TABLE 2. Mean/Standard deviation/minimum-maximum values of intraspecific TN93 pairwise genetic distances and sample size

Species	Number of specimens	Mean/SD/Min–Max
caucasicus	9	1.69/0.85/0.15–3.14
nigripilosus	5	1.11/0.86/0.00–1.86
longimaculatus	2	0.62
sinitshenkovae	4	1.49/1.62/0.00–2.97
znojkoi	9	1.87/0.98/0.15–3.63
soldani	4	2.48/2.26/0.31–4.72
bicolliculatus **sp.nov.**	4	1.20/0.23/0.78–1.41
magnus	6	0.69/0.29/0.00–1.23
alpestris	4	1.29/1.30/0.00–2.59

Distribution, habitat and biology. *E.* (*C.*) *bicolliculatus* **sp. nov.** occurs in the streams and rivers of the Lesser Caucasus (NE Turkey/Eastern Black Sea Mountains and SW Georgia) and the southern slopes of the Central Greater Caucasus (N Georgia) (Fig. 51). Occurrence on the southern slopes of western Greater Caucasus is also possible; however the material from this area was not available for the present study. We found no specimens of *E.* (*C.*) *bicolliculatus* **sp. nov.** in the samples from the northern slopes of the Central (Russian Federation: Kabardino-Balkaria, North Ossetia-Alania) and Western (Russian Federation: Adygea, Krasnodar Province, Karachay-Cherkessia, see Fig. 51) Greater Caucasus during our investigation. These data indicate that the distribution of *E.* (*C.*) *bicolliculatus* **sp. nov.** in the Greater Caucasus is rather limited. The occurrence of *E.* (*C.*) *bicolliculatus* **sp. nov.** in Armenia is supported by only a single larva collected in 1975 (Kotayk Province: Geghard Monastery, Azat River). The species was not recorded in Armenia during our recent extensive sampling in 2015 (which also included the original locality from 1975) and its contemporary occurrence in this country remains doubtful.

The larvae of *E.* (*C.*) *bicolliculatus* **sp. nov.** inhabit the epirithral and metarithral zones of streams and rivers of various size in relatively broad altitudinal range (records from 40 to 1804 m a.s.l.). The species has been recorded in small and middle-sized streams (approx. 1–1.5 m wide, with current velocities of 0.3–1.1 m/s and maximum depths of 0.1–0.5 m), and larger rivers (approx. 10–20 m wide, with current velocities of 0.3–3 m/s and maximum depths of 0.4–1.5 m). As well as the other representatives of *Epeorus*, *E.* (*C.*) *bicolliculatus* **sp. nov.** occurs on the stony substrate of torrential sections. Water temperature in these localities ranged between 15 and 20°C in August. The environment around the sites was highly varied, from shores fully covered by riverbank vegetation to those completely exposed with 0% shade.

The mature larvae were observed in April and June. The emergence of subimagines was observed in the morning (7–10 am) and evening (4–7 pm). Other *Epeorus* species occurring in some localities were *E.* (*C.*) cf. *znojkoi* and *E.* (*C.*) *caucasicus*.

Discussion on species delimitation

Although the species delimitation based on the DNA barcoding alone is considered of rather limited value (DeSalle *et al*. 2005; Martinsson *et al.* 2013; Martinsson *et al.* 2015), barcoding can be regarded as supplementary evidence contributing to the delimitation of separately evolving lineages, if other independent characteristics are also analyzed (Will *et al*. 2005; Hajibabaei *et al.* 2007; Pires & Marinoni 2010; DeWalt 2011; Kučinić *et al.* 2013; White *et al*. 2014).

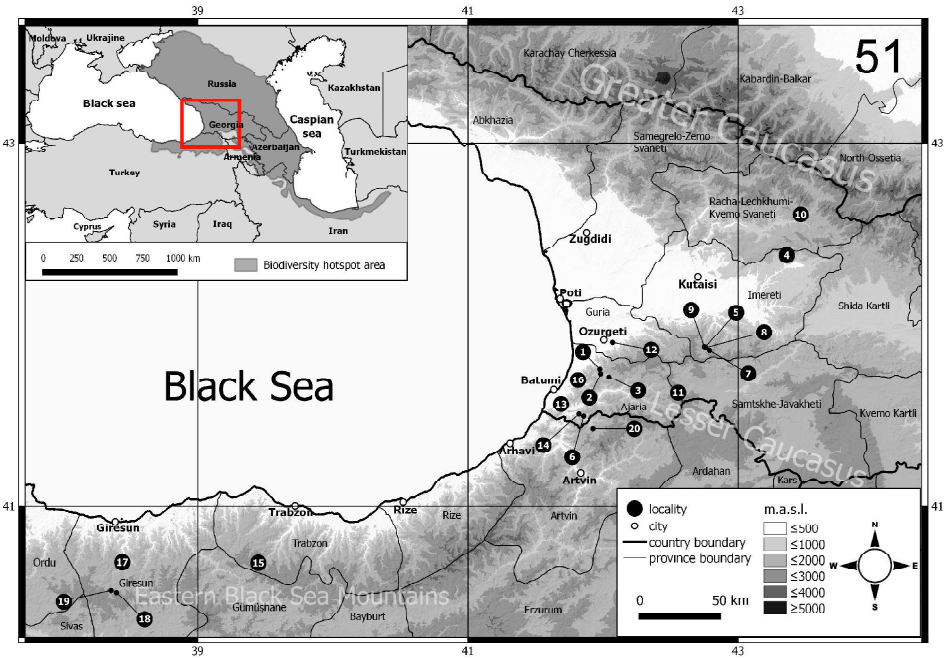


FIGURE 51. Map showing sampling sites with records of *Epeorus* (*Caucasiron*) *bicolliculatus* **sp. nov.** (locality numbers correspond to numbers given in square brackets in the list of material studied).

We used the morphological diagnostic characters integrated with the COI dataset for the purpose of the description of *E.* (*C.*) *bicolliculatus* **sp. nov.** and its delimitation within the subgenus *Caucasiron*. The analysis of phylogenetic relations between individual *Caucasiron* lineages would require a broader spectrum of genetic markers (DeSalle *et al*. 2005) and we refrain from making phylogenetic assumptions from our data.

Concerning morphology, *E.* (*C.*) *bicolliculatus* **sp. nov.** is well defined, representing the third species of *Caucasiron* with larvae bearing flat, basally widened setae on the head, thorax, and abdominal terga. The postero-medial protuberances on abdominal terga II–IX in larvae were found to be a unique morphological diagnostic character among all *Caucasiron* species known so far. All other morphological characters delimiting the new species are treated in detail in the chapter *Affinities*.

The analysis of the barcode data also unambiguously recognized *E.* (*C.*) *bicolliculatus* **sp. nov.** as a distinct monophyletic lineage within *Caucasiron* (NJ and ML bootstrap=100%). This result was also supported by relatively high genetic distances to other species (11.51–18.51%; see Table 1).

Several studies of the DNA barcoding highlighted the cases where this approach was found unsuitable for species identification (Meier *et al*. 2006, Whitworth *et al.* 2007). However, the results obtained in the studies on mayflies show a close correspondence between the morphologically defined species and the barcode clusters (Ball *et al.* 2005; Webb *et al*. 2007; Alexander *et al*. 2009; Zhou *et al*. 2009; Kjaerstad *et al*. 2012; Sroka 2012; Webb *et al*. 2012).

In our study, the ABGD analysis of the COI distance dataset successfully delimited almost all morphologically defined *Caucasiron* species from the Caucasus (see Fig. 49). The intraspecific divergences ranged between 0.00 and 7.14% and interspecific between 10.68 and 18.51%, meaning a width of barcode gap of 3.54% (see Fig. 50). Only two morphologically defined species were not successfully separated in the ABGD analysis, namely *E.* (*C.*) *sinitshenkovae* and *E.* (*C.*) *longimaculatus.* In terms of morphology, each of these two species is well distinguishable and both were also recognized as highly supported monophyletic groups in the analyses of

molecular data (see Fig. 49). However, they were not recognized by the ABGD due to the lower interspecific differences between them (5.79–7.14%; see Table 1) in contrast to the remaining species (10.68–18.51%) within our dataset. The relatively low interspecific divergences were also previously observed in other genera within mayflies e.g. *Baetisca* Walsh, 1862 and *Caenis* Stephens, 1835 (Ball *et al*. 2005), or *Siphlonurus* Eaton, 1868 (Webb *et al*. 2012), and may be explained by the young age of the lineages, or may reflect the lower substitution rate in these taxa (Ball *et al*. 2005; DeWalt 2011).

If we assume *E*. (*C*.) *sinitshenkovae* and *E*. (*C*.) *longimaculatus* to be separate species, intraspecific variation within our dataset ranged between 0.00 and 4.72% and interspecific between 5.79 and 18.51% (see Fig. 50). The barcode gap width formed 1.07%. The previous studies on mayflies show variable ranges of barcode gap, from much higher values (e.g. Kjaerstad *et al*. 2012; Webb *et al*. 2007) to no gap observed (e.g. Ball *et. al.* 2005; Alexander *et al*. 2009; Webb *et al*. 2012; Sroka 2012).

The mean interspecific divergence (14.33% with *E*. (*C*.) *sinitshenkovae* and *E*. (*C*.) *longimaculatus* constrained as separate species) obtained in our results showed similar value to other reported divergences for congeneric species within mayflies (Alexander *et al*. 2009; Webb et. al. 2007; Webb *et al*. 2012) or within Heptageniidae in particular (Ball *et al*. 2005). Therefore, our result confirmed the generally high level of COI sequence divergence within Ephemeroptera (Webb *et al*. 2012).

The range of barcode gap and the general identification success rate of barcoding depends (except from interspecific distances) on intraspecific genetic variation that should be accounted for in the analyses by sequencing a higher number of specimens from geographically distant populations (Meier *et al*. 2006; Meier 2008; DeWalt 2011). Our samples of *Epeorus* (*Caucasiron*) spp. included specimens from a relatively wide geographical range within the species' distribution area (Russia, Georgia, Armenia, Turkey; see Appendix 1) and intraspecific variations observed within the species showed geographically distinct patterns (Fig. 49). However, the degree of intraspecific variability (Table 2) did not hamper the delimitation of individual species.

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APPENDIX 1. Samples, localities and GenBank Accession Nos

Species	Specimen ID	Life Stage	Country	GPS coordinates	Leg.	Date	Gen Bank accesion number
E. (C.) caucasicus	Ca25T	Larva	Turkey	40°37'22"N 40°50'01"E	P. Sroka/ R.J. Godunko	28.06.2011	KY865682
E. (C.) caucasicus	Ca28R	Larva	Russia	43°14'31"N 42°33'49"E	V.V. Martynov	19.05.2013	KY865683
E. (C.) caucasicus	Ca16G	Larva	Georgia	42°36'28"N 44°38'05"E	P. Sroka/ R.J. Godunko	23.07.2010	KY865684
E. (C.) caucasicus	Ca20G	Larva	Georgia	42°33'30"N 44°30'35"E	P. Sroka/ R.J. Godunko	23.07.2010	KY865685
E. (C.) caucasicus	Ca27R	Larva	Russia	43°22'12"N 42°54'13"E	V.V. Martynov	10.05.2013	KY865686
E. (C.) caucasicus	Ca5A	Larva	Armenia	40°48'09"N 44°53'43"E	P. Manko/ J.Oboňa	29.08.2015	KY865687
E. (C.) caucasicus	Ca6A	Larva	Armenia	40°48'09"N 44°53'43"E	P. Manko/ J.Oboňa	29.08.2015	KY865688
E. (C.) caucasicus	Ca9A	Larva	Armenia	40°38'11"N 44°28'58"E	P. Manko/ J.Oboňa	26.08.2015	KY865689
E. (C.) caucasicus	Ca11A	Larva	Armenia	41°00'13"N 43°57'24"E	P. Manko/ J.Oboňa	02.09.2015	KY865690

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APPENDIX 1. (Continued)

Species	Specimen ID	Life Stage	Country	GPS coordinates	Leg.	Date	Gen Bank accesion number
E. (C.) nigripilosus	Ni8R	Larva	Russia	43°21'N 42°52'E	V.V. Martynov	09.05.2013	KY865691
E. (C.) nigripilosus	Ni9R	Larva	Russia	43°15'50"N 42°28'45"E	V.V. Martynov	12.05.2013	KY865692
E. (C.) nigripilosus	Ni2T	Larva	Turkey	40°26'29"N 40°54'24"E	P. Sroka/ R.J. Godunko	28.06.2011	KY865693
E. (C.) nigripilosus	Ni1T	Larva	Turkey	40°26'29"N 40°54'24"E	P. Sroka/ R.J. Godunko	28.06.2011	KY865694
E. (C.) nigripilosus	Ni6T	Larva	Turkey	40°26'35"N 40°57'53"E	P. Sroka/ R.J. Godunko	28.06.2011	KY865695
E. (C.) znojkoi	Zn1G	Larva	Georgia	42°21'51"N 44°40'50"E	P. Sroka/ R.J. Godunko	22.07.2010	KY865696
E. (C.) znojkoi	Zn7G	Larva	Georgia	42°10'58"N 44°40'49"E	P. Sroka/ R.J. Godunko	22.07.2010	KY865697
E. (C.) znojkoi	Zn5G	Larva	Georgia	42°56'32"N 42°11'04"E	P. Sroka/ R.J. Godunko	28.07.2010	KY865698
E. (C.) znojkoi	Zn30A	Larva	Armenia	40°38'37"N 44°32'34"E	A.V. Martynov	02.07.2015	KY865699
E. (C.) znojkoi	Zn31A	Larva	Armenia	40°38'37"N 44°32'34"E	A.V. Martynov	02.07.2015	KY865700
E. (C.) znojkoi	Zn32A	Larva	Armenia	40°36'47"N 44°34'14"E	A.V. Martynov	04.07.2015	KY865701
E. (C.) znojkoi	Zn40R	Larva	Russia	42°49'28"N 44°04'25"E	A.V. Martynov	25.07.2014	KY865702
E. (C.) znojkoi	Zn41R	Larva	Russia	42°51'17"N 44°09'07"E	A.V. Martynov	21.07.2014	KY865703
E. (C.) znojkoi	Zn19T	Larva	Turkey	40°42'19"N 40°39'00"E	P. Sroka/ R.J. Godunko	28.06.2011	KY865704
E. (C.) magnus	Ma1A	Larva	Armenia	40°08'24"N 44°49'04"E	P. Manko/ J.Oboňa	31.08.2015	KY865705
E. (C.) magnus	Ma2A	Larva	Armenia	40°58'04"N 44°24'49"E	P. Manko/ J.Oboňa	03.09.2015	KY865706
E. (C.) magnus	Ma6G	Larva	Georgia	41°50'34"N 44°35'29"E	P. Sroka/ R.J. Godunko	21.07.2010	KY865707
E. (C.) magnus	Ma15R	Larva	Russia	43°22'12"N 42°54'13"E	V.V. Martynov	10.05.2013	KY865708
E. (C.) magnus	Ma14R	Larva	Russia	43°14'31"N 42°33'49"E	V.V. Martynov	19.05.2013	KY865709
E. (C.) magnus	Ma10T	Larva	Turkey	42°00'04"N 33°21'06"E	P. Sroka/ R.J. Godunko	07.07.2011	KY865710
E. (C.) alpestris	Al7R	Larva	Russia	43°15'35"N 42°29'53"E	V.V. Martynov	12.05.2013	KY865711
E. (C.) alpestris	Al8R	Larva	Russia	43°16'26"N 42°29'32"E	V.V. Martynov	15.05.2013	KY865712
E. (C.) alpestris	Al9R	Larva	Russia	43°21'N 42°52'E	V.V. Martynov	09.05.2013	KY865713

.....continued on the next page

APPENDIX 1. (Continued)

Species	Specimen ID	Life Stage	Country	GPS coordinates	Leg.	Date	Gen Bank accesion number
E. (C.) alpestris	Al6G	Larva	Georgia	42°33'30"N 44°30'35"E	P. Sroka/ R.J. Godunko	23.07.2010	KY865714
E. (C.) soldani	So1G	Larva	Georgia	42°34'30"N 42°40'47"E	P. Sroka/ R.J. Godunko	29.07.2010	KY865715
E. (C.) soldani	So5R	Larva	Russia	43°16'30"N 41°48'31"E	R.J. Godunko	10.07.2010	KY865718
E. (C.) soldani	So3R	Larva	Russia	43°28'02"N 41°46'39"E	R.J. Godunko	10.07.2010	KY865716
E. (C.) soldani	So4R	Larva	Russia	43°27'38"N 41°44'52"E	R.J. Godunko	11.07.2010	KY865717
E. (C.) bicolliculatus **sp.nov.**	Bi1G	Imago female; paratype	Georgia	41°44'14"N 41°58'60"E	A.V. Martynov	19.04.2016	KY865722
E. (C.) bicolliculatus **sp.nov.**	Bi2G	Larva; paratype	Georgia	41°45'43"N 41°58'34"E	A.V. Martynov	20.04.2016	KY865719
E. (C.) bicolliculatus **sp.nov.**	Bi3T	Larva; paratype	Turkey	40°40'45"N 39°26'55"E	P. Sroka/ R.J. Godunko	29.06.2011	KY865720
E. (C.) bicolliculatus **sp.nov.**	Bi4G	Larva; paratype	Georgia	41°45'43"N 41°58'34"E	A.V. Martynov	20.04.2016	KY865721
E. (C.) longimaculatus	Lo1G	Larva	Georgia	42°04'58"N 45°02'19"E	P. Sroka/ R.J. Godunko	20.07.2010	KY865723
E. (C.) longimaculatus	Lo2G	Larva	Georgia	42°04'58"N 45°02'19"E	P. Sroka/ R.J. Godunko	20.07.2010	KY865724
E. (C.) sinitshenkovae	Si7R	Larva	Russia	43°14'31"N 42°33'49"E	V.V. Martynov	19.05.2013	KY865727
E. (C.) sinitshenkovae	Si8R	Larva	Russia	43°14'31"N 42°33'49"E	V.V. Martynov	19.05.2013	KY865728
E. (C.) sinitshenkovae	Si6R	Larva	Russia	43°14'39"N 42°31'32"E	V.V. Martynov	17.05.2013	KY865726
E. (C.) sinitshenkovae	Si4G	Larva	Georgia	42°32'42"N 44°29'29"E	P. Sroka/ R.J. Godunko	23.07.2010	KY865725

2. A new *Epeorus* (*Caucasiron*) (Ephemeroptera: Heptageniidae) species from Turkey based on molecular and morphological evidence

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A new *Epeorus* (*Caucasiron*) (Ephemeroptera: Heptageniidae) species from Turkey based on molecular and morphological evidence

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Abstract

Epeorus (*Caucasiron*) *turcicus* **sp. nov.** is described based on larvae from NE Turkey. The new species can be distinguished from other *Caucasiron* by a unique combination of several diagnostic characters: the presence of a rounded hypodermal medial femur spot, colouration of abdominal terga and sterna, narrow gill plate VII, fine hair-like setae on the surface of abdominal terga, and absence of postero-lateral projections on tergum X. In addition to morphological analysis, two single-locus analytical approaches are employed for delimiting the new species using *COI* sequences (Automatic Barcode Gap Discovery, ABGD; and General Mixed Yule Coalescent Model, GMYC). Both approaches unambiguously recognized *E.* (*C.*) *turcicus* **sp. nov.** as a distinct species. Our molecular dataset contains all *Caucasiron* species occurring in the Caucasus and the delimitation of individual species mostly follows the morphologically defined species. This study confirms the suitability of the GMYC approach for species delimitation within *Caucasiron.*

Key words: taxonomy, mayflies, new species, Caucasus, barcoding

Introduction

The heptageniid mayflies of the genus *Epeorus* Eaton, 1881, subgenus *Caucasiron* Kluge, 1997 (hereafter *Caucasiron*) are distributed in the Eastern Mediterranean, Asia Minor, the Caucasus, and Central and Western Asia. The global diversity of *Caucasiron* currently consists of eleven species and two subspecies, although more taxa from Central Asia originally described in the genus *Iron* Eaton, 1883 apparently belong to *Caucasiron* (see Chen *et al.* 2010; Hrivniak *et al.* 2017).

According to our knowledge at present, most of the *Caucasiron* diversity is concentrated in the Caucasus Mountains, where it represents the third most diverse mayfly taxon after the genera *Rhithrogena* Eaton, 1881 and *Electrogena* Zurwerra & Tomka, 1985. Until now, nine *Caucasiron* species and one subspecies have been recorded from the Caucasus; for the list of species see Hrivniak *et al.* (2017).

Individual *Caucasiron* species exhibit various distributional patterns. While the species *E.* (*C.*) *sinitshenkovae* Braasch & Zimmerman, 1979 and *E.* (*C.*) *soldani*, Braasch, 1979 are known only from the western and central section of the Greater Caucasus, other species (except *E.* (*C.*) *caucasicus iranicus* (Braasch & Soldán, 1979)) have been recorded from a broader area that includes both the Greater and Lesser Caucasus and also adjacent mountainous territories.

The diversity of *Caucasiron* mayflies have been relatively extensively studied in the Greater Caucasus (e.g. Braasch 1979; 1980; Braasch & Zimmermann 1979; Sinitshenkova 1976) and Lesser Caucasus (e.g. Tshernova

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1938; Braasch 1978, Martynov *et al.* 2016). In Turkey, the north-eastern region of which belongs to the Lesser Caucasus (Tarkhnishvili 2014), six *Caucasiron* species have been recorded so far: *E.* (*C.*) *caucasicus* Tshernova, 1938 (e.g. Kazancı 1986, 2009a; Türkmen & Kazancı 2015); *E.* (*C.*) *znojkoi* Tshernova, 1938 (e.g. Braasch 1981; Türkmen & Kazancı 2015); *E.* (*C.*) *nigripilosus* Sinitshenkova, 1976 (Kazancı 2001; Türkmen & Kazancı 2015); *E.* (*C.*) *magnus* Braasch, 1978 (Kazancı 2009a); *E.* (*C.*) *alpestris* Braasch, 1979 (Kazancı 1986; Aydınlı 2017); *E.* (*C.*) *longimaculatus* Braasch, 1980 (Kazancı & Braasch 1988) and *E.* (*C.*) *bicolliculatus* Hrivniak, 2017 (Hrivniak *et al.* 2017). However, it should be noted that the only record of *E.* (*C.*) *longimaculatus* from Uludağ Mountains was considered unclear (see Kazancı & Braasch 1988: 132). The other information on *E.* (*C.*) *longimaculatus* from Turkey (see e.g. Salur et al. 2016, with comments in Kazancı & Türkmen 2016) has been taken from the original record published by Kazancı & Braasch (1988).

Despite the existence of extensive published distributional records from Turkey, this region still needs further study, as do other mountain ranges in the proximity of the Caucasus (such as the Zagros and Alborz Mountains in Iran). During sampling in 2016 within NE Turkey, a new species of *Caucasiron* was discovered and is described in the present study as *E.* (*C.*) *turcicus* **sp. nov.** Following the integrative taxonomic approach using multiple lines of evidence for the species delimitation, we employ (in addition to the morphological study) an analysis of *COI* sequences using wide sampling of the species related to *E.* (*C.*) *turcicus* **sp. nov.**

Hrivniak *et al*. (2017) have already published sequences of the *COI* region of mtDNA for all *Caucasiron* species occurring in the Caucasus and analyzed the individual species delimitation using the Automatic Barcode Gap Discovery (ABGD) method (Puillandre *et al*. 2012). The analysis largely supported the existing morphology-defined species (for details see Hrivniak *et al*. 2017). In the present study, we use the same approach to test the delimitation of *E.* (*C.*) *turcicus* **sp. nov.** within *Caucasiron*. We also employ the General Mixed Yule Coalescent model (GMYC, see Pons *et al.* 2006; Fusijawa & Barraclough 2013), largely used for species delineation in mayflies when single-locus data are available (Monaghan *et al*. 2009; Vuataz *et al*. 2011, 2013; Pereira-da-Conceicoa *et al*. 2012, Rutschmann *et al.* 2014, 2017).

The main objectives of the present study can be summarized as follows: **i)** to describe the morphology of a new *Caucasiron* species; **ii)** provide differential diagnosis between new species and closely related taxa; **iii)** test the delimitation of the new species using analytical tools for the single-locus *COI* dataset; **iv)** provide the basic information on the distribution, habitat, and biology of the new species.

Material and methods

The material used for this study was collected by G. Türkmen and N. Kazancı in 2016. Specimens were preserved in 75–96% EtOH in the field. Material of other *Caucasiron* species used for the morphological and molecular comparisons was collected by T. Soldán, J. Bojková, R.J. Godunko and P. Sroka in 2008–2012 and is deposited in the collection of the Institute of Entomology, České Budějovice, Czech Republic.

Morphological examination. Slide mounting was performed using HydroMatrix® (MicroTech Lab, Graz, Austria) mounting medium. In order to remove the muscle tissues for an investigation of the cuticular structure, the specimens were left overnight in a 10% solution of NaOH prior to slide mounting. Drawings were made using a stereomicroscope Olympus SZX7 and a microscope Olympus BX41, both equipped with a drawing attachment. Photographs were obtained using a Canon EOS 1200D camera mounted on a Leica M205 C stereomicroscope. All photographs were subsequently enhanced with Adobe Photoshop™ CS5. Diagnostic characters for the description of larva were used according to Braasch & Soldán (1979) and Braasch (2006). The terminology was used mostly according to Kluge & Novikova (2011) and Kluge (2004, 2015). Abbreviation "n" refers to the number of specimens examined.

DNA extraction, PCR, sequencing and alignment. Total genomic DNA of seven specimens of a new putative species was extracted from legs using the DEP-25 DNA Extraction Kit (TopBio s.r.o., Prague, Czech Republic) according to the manufacturer's protocol. We sequenced mitochondrial cytochrome oxidase subunit I (*COI*) with forward primer F (5′-TTC AGC CAC TTT ACC GCG- 3′, Rutschmann *et al.* unpublished) and reverse primer HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA- 3′, Folmer *et al.* 1994). *COI* sequences of other *Caucasiron* species used for comparisons were obtained from Hrivniak *et al*. (2017) (GenBank accession nos KY865691–KY865725). The subspecies *E.* (*C.*) *znojkoi insularis* (Braasch, 1983) was additionally sequenced. The

subspecies *E.* (*C.*) *caucasicus iranicus* Braasch & Soldán, 1983 was not included in the analysis. PCR amplification of *COI* and reaction volumes was carried out as described in Hrivniak *et al*. (2017). Sequences were assembled in Geneious 7.0.6 (http://www.geneious.com; Kearse *et al*. 2012) and aligned in the same software using Mafft 7.017 (Katoh *et al.* 2002) plugin with default settings. Newly obtained sequences are deposited in GenBank with accession numbers MK140783-MK140789.

Molecular species delimitation. Species were delimited using single-locus (*COI*) coalescence based General Mixed Yule Coalescent model (GMYC, Pons *et al.* 2006; Fusijawa 2013). We used the single-threshold GMYC model as it has been found to outperform the multi-threshold (Fusijawa & Barraclough 2013).

The GMYC model identifies independent evolutionary clusters by detecting a threshold value at the transition from interspecific to intraspecific branching patterns (Bryson *et al*. 2013). A maximum likelihood approach is used to optimize the shift in branching patterns and likelihood ratio test assesses if the mixed model fits the data significantly better than a null model that assumes a single coalescent process for the entire tree (Pons *et al.* 2006; Monaghan *et al.* 2009). Analyses were performed using the SPLITS package for R (http:// r-forge.r-project.org/ projects/splits). Ultrametric *COI* gene tree was reconstructed under relaxed molecular clock (uncorrelated lognormal distribution) using BEAST 1.8.4 on CIPRES Science Gateway 3.3 (Miller *et al*. 2010). Input file was generated in BEAUti 1.8.4. The best substitution model, HKY+G+I was determinated using Jmodeltest 2.1.4 according to AICc. A coalescent constant size tree prior was prefered, because GMYC null model constitutes a single coalescent cluster (Monaghan *et al*. 2009; Zaldívar-Riverón *et al*. 2010; Vuataz *et al.* 2011, 2013). Other settings were default. Two analyses of MCMC chains were run for 50 milion generations sampled every 5000 generations. Convergence and effective sample size (ESS > 200) was verified using Tracer 1.6. The first 10% of trees (1000) from each run were discarted as burn-in. The files from both independent runs were combined using LogCombiner 1.8.4. The maximum clade credibility tree was constructed from 18,000 trees using TreeAnnotator 1.8.4 with default settings.

Inter- and intraspecific K2P pairwise genetic distances were calculated in MEGA 7 (Kumar *et al*. 2016). The distance matrix was analysed using Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al*. 2012) (online version: http://wwwabi.snv.jussieu.fr/public/abgd/) with default settings. The method identify so called barcode gap, that would correspond to threshold between intra- and interspecific genetic distances.

Results

Taxonomy

***Epeorus* (*Caucasiron*) *turcicus* Hrivniak, Türkmen & Kazancı, sp. nov.**
(Figs 1–18)

Type material (bold numbers in parentheses correspond to the localities in Fig. 20).

Holotype: female mature larva: TURKEY, Artvin Province, Camili Village, Merata Plateau, unnamed mountain stream, 2190 m a.s.l.; 41°26'30"N/42°04'41" E; code: CAM 10; 26.7. 2016, G. Türkmen & N. Kazancı leg. **(1)**.

Paratypes: 3 female (1 mounted on slide) and 1 male larvae, same locality as holotype.

1 male larva: TURKEY, Artvin Province, Camili Village, Merata Plateau, unnamed mountain stream, 2092 m a.s.l.; 41°26'08"N/42°04'00"E; code: CAM 8, 26.7. 2016, **(2)**.

4 female and 1 male larvae: TURKEY, Artvin Province, Camili Village, Lekoban Plateau, unnamed mountain stream, 2386 m a.s.l.; 41°24'05" N/42°02'18"; code: CAM 5, 25.7. 2016, **(3)**.

2 female larvae (1 mounted on slide): TURKEY, Artvin Province, Camili Village, Merata Plateau, unnamed mountain stream, 2050 m a.s.l.; 41°29'18" N/42°04'31"E; code: CAM 7, 26.7. 2016, **(4)**.

2 female larvae (both mounted on slides) and 3 male larvae (2 mounted on slides): TURKEY, Artvin Province, Camili Village, Lekoban Plateau, unnamed mountain stream, 2388 m a.s.l.; 41°24'04" N/42°24'04" E; code: CAM 6, 25.7. 2016, **(5)**.

All type material was collected by G. Türkmen and N. Kazancı. Holotype and 12 paratypes are deposited in the collection of the Biology Centre of the Czech Academy of Sciences, Institute of Entomology, České Budějovice,

Czech Republic. Four paratypes (2 larvae in EtOH and 2 larvae mounted on slides) are housed in the collection of N. Kazancı and G. Türkmen at Hacettepe University, Department of Biology, Biomonitoring Laboratory, Turkey.

Description. Larva (females: n=12; males: n=6).

Body length of mature larvae: 14 mm (female), 12–13 mm (male). Length of cerci approximately 1.2× body length.

Head. Shape trapezoidal; anterior and lateral margin rounded, posterior margin rounded in female, slightly rounded or nearly straight in male (Figs 4, 5). Anterior margin with shallow concavity medially (female). Head dimensions of mature larvae: length 2.9 mm, width 4.3–4.4 mm (female); length 2.6 mm, width 3.8 mm (male). Head width/length ratio: 1.41–1.51 (female); 1.42–1.47 (male). Colouration yellowish-brown with brown markings. Dorso-medial part with brown rectangular or oval pattern with edges often extending to antennae. Dark triangular maculae located near inner edges of compound eyes; pair of rounded maculae located anteriorly to triangular maculae. Pale stripes extending from lateral ocelli to lateral edges of head (Figs 4, 5). Pair of maculae between compound eyes. Compound eyes dark grey to black in female; grey to brown and basally darkened in male. Ocelli dark grey to black, basally pale. Antennae yellowish-brown. Anterior margin densely covered with hair-like setae extending to lateral margins and directed medio-dorsally. Dorsal surface of head covered with fine hair-like setae and sparsely distributed stick-like setae. Sparse longer fine hair-like setae located posteriorly to eyes.



FIGURES 1–5. *Epeorus* (*Caucasiron*) *turcicus* **sp. nov.**, mature larvae (holotype [female] and paratype [male]): 1, habitus, dorsal; 2, habitus, ventral; 3, habitus, lateral; 4, head of male, dorsal; 5, head of female, dorsal.

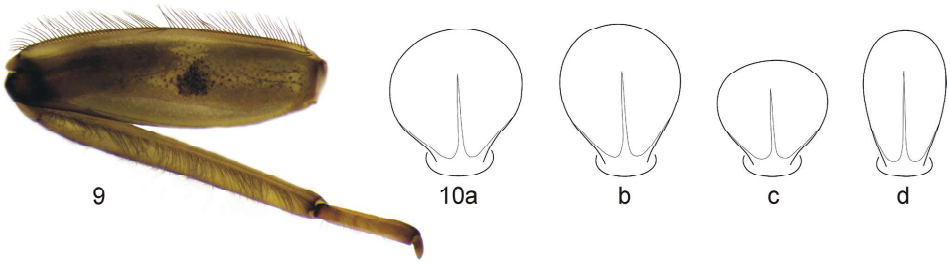
Mouthparts. Labrum (Fig. 6) widened anteriorly, with anterior margin slightly rounded or nearly straight (in dorsal view), medial emargination present. Lateral angles rounded (shape of labrum may vary among individual specimens). Dorsal surface (Fig. 6b, lower half) sparsely covered with setae of different size; 4–8 longer bristle-like setae located antero-medially and two antero-laterally (n=6). Ventral surface (Fig. 6a, upper half) with longer, shortly plumose bristles situated along lateral to anterior margin (only bases of setae figured), and brush of fine hair-like setae medially (not figured). Group of 6–12 setae located near brush of fine hair-like setae. Posterior margin of labrum irregularly concave. Outer incisors of both mandibles (Figs 7, 8) with three apical teeth; outer tooth blunt in both mandibles. Inner incisor of left mandible with three apical teeth, right inner incisor bifurcated; lateral margins of both inner incisors covered with variously situated setae and outgrowths. Prostheca represented by tuft of plumose setae. Maxillae and maxillary palpi without diagnostic characters. Lingua of hypopharynx oblong-shaped, with two latero-apical lobes, covered with dense hair-like setae. Each superlingua with inner apical lobe, covered with hair-like setae that extend laterally; outer margin of superlinguae slightly extended, rounded and pigmented. Dorsal surface of glossae with longitudinal brush of fine setae; ventral surface with scattered short setae; proximal half of outer margins covered by longer setae. Inner margins with long bristles extending medially near apex. Paraglossae densely covered with fine setae on its anterior half. Shape of glossae, paraglossae, shape and setation of labial palpi without diagnostic characters (for shape of glossae and paraglossae in Caucasiron see e.g Braasch 1979: 286, fig.1i).



FIGURES 6–8. *Epeorus* (*Caucasiron*) *turcicus* **sp. nov.,** mature larvae: 6, labrum (a) ventral, (b) dorsal); 7, incisors of right mandible, ventral; 8, incisors of left mandible, ventral.

Thorax. Yellowish-brown with pale markings dorsally. Pronotum anteriorly narrowed, lateral edges slightly rounded or nearly straight. Posterior margin of metanotum medially bluntly pointed. Dorsal surface covered with fine hair-like setae (as on abdominal terga and head); pro, meso- and metanotal suture with sparse longer hair-like setae.

Legs. Yellowish with dark brown markings; all legs with same colouration pattern. Femora with contrasting dark brown markings and hypodermal medial spot (Fig. 9). Shape of medial spot and colour intensity vary between specimens. Tibio-patellar suture and anterior margin of tibiae darkened; tarsi proximally and distally darkened. Coxal projections of forelegs pointed, of middle legs blunt and of hind legs pointed or bluntly pointed. Trochanters with spatulate setae. Tibiae of forelegs longer than femora (1.07–1.18× femur length), tibiae of middle legs nearly equal length as femora (0.97–1.01× femur length), tibiae of hind legs slightly shorter than femora (0.75–0.9× femur length). Tarsi of all legs 0.29–0.35× tibia length. Posterior margin of femora with row of long blade-like setae and sparse row of short, bluntly pointed spines. Dorsal surface of femora covered by flat, short (Fig. 10a–c), and sporadically elongated (Fig. 10d) spatulate setae, hair-like setae and sparsely distributed stick-like setae. Anterior margin of femora with short, pointed and/or bluntly pointed spines. Tibiae and tarsi with row of long, shortly plumose setae dorsally and irregularly situated spines of various size ventrally. Tarsal claws with 3–4 teeth.

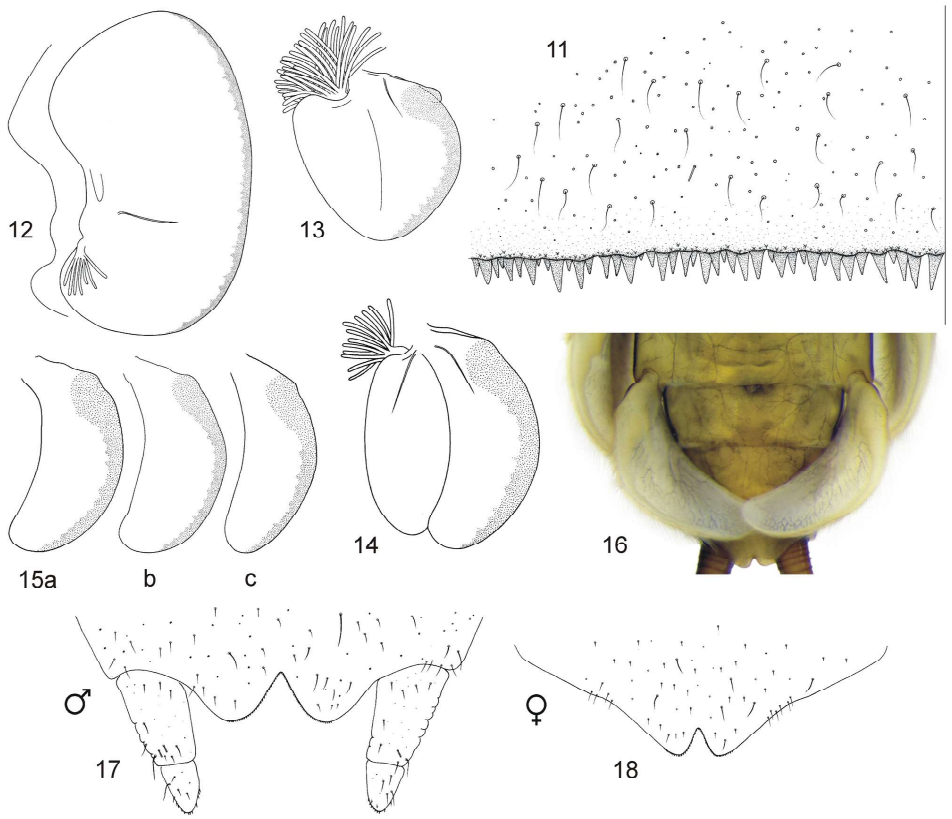


FIGURES 9–10. *Epeorus* (*Caucasiron*) *turcicus* **sp. nov.**, mature larvae: 9, middle leg, dorsal; 10, setae on dorsal surface of femora.

Abdomen. *Abdominal terga.* Yellowish-brown, with contrasting dark brown to blackish pattern, that consists of: **i)** narrow macula along anterior margin on terga II–IX (contiguous or medio-laterally interrupted by pair of blanks); **ii)** medial macula on terga II–IV (variable in shape, usually in form of blurred stripe, triangle or anteriorly and posteriorly widened macula); **iii)** medial longitudinal anteriorly widened stripe stretching between anterior and posterior margins on terga V–VII (VIII); **iv)** elongated medial macula on terga (VIII) IX (sometimes longitudinal stripe as on terga V–VII); **v)** lateral maculae on terga I–IX extending postero-medially, sometimes forming contiguous stripe along posterior margin (usually on terga II–V). Tergum X without distinct colouration. Pair of sigilla located antero-laterally to medial maculae usually not expressed (sometimes present in form of short dark stripes). Denticles on posterior margin on terga irregular and pointed; its density vary between terga and instars. Each abdominal tergum with medial longitudinal band of relatively sparse hair-like setae. Surface of terga covered with hair-like setae and sparsely with stick-like setae (as on head). Tergum X without postero-lateral projections**.** Supra-tergal projections short and blunt.

Abdominal sterna. Yellowish, without distinct colouration pattern. Nerve ganglia usually darkened; sigilla not expressed. Sternum IX with V-shaped medial emargination (shape may vary between individuals) and covered by scattered hair-like setae of different size (Figs 17–18).

Larval protopenis. Penis lobes with deep medio-apical emargination (n=2).



FIGURES 11–18. *Epeorus* (*Caucasiron*) *turcicus* **sp. nov.**, mature larvae: 11, abdominal tergum VII, surface and posterior margin; 12, gill I; 13, gill III; 14, gill VII (flattened on the slide); 15a–c, gill plates VII (in natural position from ventral view), variability in shape; 16, gill plates VII, ventral view; 17, sternum IX, male; 18, sternum IX, female.

Gills. Dorsally grey to brownish; ventrally yellowish. Gill plates I overlapping beneath thorax. Projection on costal margin of gill plates II–VII well developed (its size vary among specimens and segments) (Fig. 13). Gill plates VII relatively narrow (in natural position from ventral view) (Figs 14–16). Each gill plate with filaments, without distinct colouration. Filaments of gills II–VI reaching to 0.37–0.58× length of respective plate, filaments of gill VII to 0.23–0.26× (in late-instar larvae).

Cerci. Yellowish-brown, basally darkened. Dorsal surface with hair-like setae. Posterior margin of basal segments with pointed and bluntly pointed denticles.

Imago, subimago and egg. unknown.

Molecular analyses. Final alignment of *COI* gene fragment consisted of sequences with 631 base pairs. Of the 193 variable sites, 179 were parsimony informative positions. No insertions and deletions were identified.

GMYC analysis. The likelihood of GMYC model was significantly higher than the likelihood of null model expecting uniform coalescent braching rates across entire tree (Table 1). The GMYC estimated 11 species with a confidence interval ranging between 10–14. The newly described species *E.* (*C.*) *turcicus* **sp. nov.** was confirmed and the number of delimited GMYC species well correspond to morphologically defined species (Fig. 19), with monophyly of all species clusters highly supported (PP=1).

Pairwise genetic distances. The ABGD analysis of the *COI* distance matrix recognized 10 groups. Except for the two species, *E.* (*C.*) *sinitshenkovae* and *E.* (*C.*) *longimaculatus* that were merged into one group, all groups well correspond to morphologically defined species. The newly proposed species *E.* (*C.*) *turcicus* **sp. nov.** was recognised as a distinct group (Fig. 19).

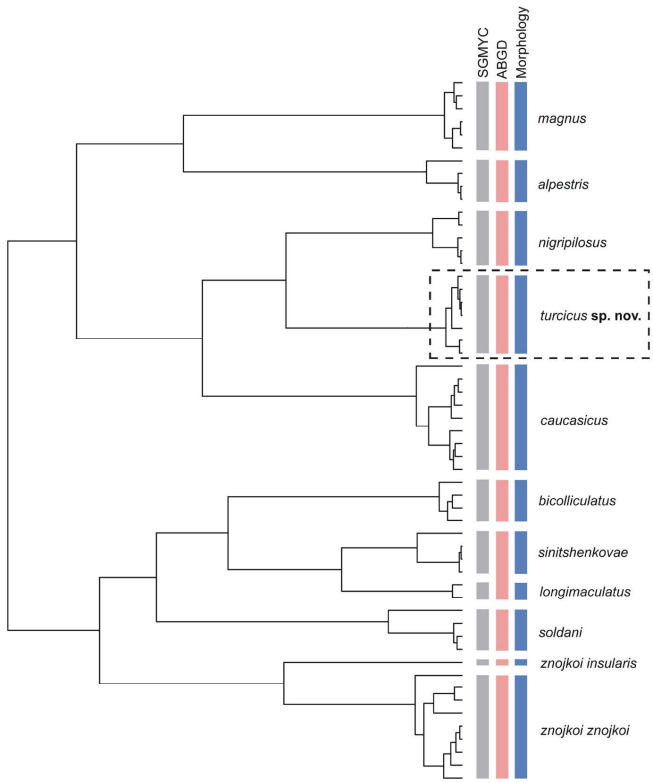


FIGURE 19. Bayesian maximum credibility *COI* gene tree of the subgenus *Caucasiron* with results of individual species

delimitation methods. Abbreviations: SGMYC–Single threshold General Mixed Yule Coalescent model, ABGD–Automatic Barcode Gap Discovery.

TABLE 1.

Threshold	T	N_{GMYC}	CI	L_0	L_{GMYC}	LR
single	0.02795126	11	10 – 14	335.4751	343.4486	0.0003444511***

T—threshold genetic distance from branch tips where transitions occured (shown only for single threshold analysis).
N_{GMYC}—number of delimited species (sum of clusters and singletons).
CI—confidence intervals of putative species.
L_0—likelihood for null model.
L_{GMYC}—likelihood for GMYC model.
LR—results of likelihood ratio test (*** indicates the test statistically significant).

The mean pairwise genetic distances between the newly proposed species *E.* (*C.*) *turcicus* **sp. nov.** and all other *Caucasiron* species included in the analysis ranged between 10.45% (species *nigripilosus*) and 16.95% (species *alpestris*).

Morphological diagnosis and affinities. *E.* (*C.*) *turcicus* **sp. nov.** belongs to the subgenus *Caucasiron* based on the presence of projections on the costal rib of gill plates II–VII and the medio-dorsal direction of hair-like setae located on the anterior margin of the head.

At the larval stage, the main diagnostic characters of *E.* (*C.*) *turcicus* **sp. nov.** are: **i)** presence of rounded hypodermal medial femur spot (Fig. 9, colouration intensity varies among specimens); **ii)** colouration of abdominal terga (Fig. 1); **iii)** absence of colouration pattern of abdominal sterna (Fig. 2); **vi)** shape of gill plate VII, relatively narrow in natural position from ventral view (Figs 14–16); **v)** fine hair-like setae on surface of abdominal terga (Fig. 11); **vi)** absence of postero-lateral projections on tergum X.

The presence of a rounded hypodermal medial femur spot, narrowed gill plates VII and the colouration of the abdominal terga and sterna clearly distinguish *E.* (*C.*) *turcicus* **sp. nov.** from the species *E.* (*C.*) *znojkoi* (see Braasch 1980: 172, fig. 4c) and *E.* (*C.*) *alpestris* (see Braasch 1979: 284, figs 1b, c).

However, *E.* (*C.*) *turcicus* **sp. nov.** is similar to several other *Caucasiron* species occurring in the Caucasus and adjacent areas. The rounded hypodermal medial femur spot is characteristic also for the species *E.* (*C.*) *nigripilosus* and *E.* (*C.*) *caucasicus* (this may be less visible in the latter species and not emphasized in the description of holotype, see Braasch 1979: 294). The larvae of both species differ from *E.* (*C.*) *turcicus* **sp. nov.** in the presence of a contrasting hypodermal colouration pattern on the abdominal sterna (see Braasch 1979: 284, figs 3b, 4b; Sinitshenkova 1976: 89, fig. 28). Additionally, *E.* (*C.*) *nigripilosus* differs by wider and apically rounded gill plate VII (in natural position from ventral view).

The gill plate VII in *E.* (*C.*) *turcicus* **sp. nov.** exhibits similar shape as in *E.* (*C.*) *longimaculatus*, *E.* (*C.*) *soldani*, *E.* (*C.*) *bicolliculatus* and *E.* (*C.*) *sinitshenkovae*. The first three species named above differ from *E.* (*C.*) *turcicus* **sp. nov.** by their basally, distinctly enlarged flattened setae on the surface of the abdominal terga (see Hrivniak *et al.* 2017: 359, figs 23–25) in contrast to the new species which bears only fine setae (Fig. 11). *E.* (*C.*) *longimaculatus* additionally differs in: **i)** displaying characteristic elongated medial femur spot (see Braasch 1980: 172, fig. 11); **ii)** absence of distinct projection on costal rib of gill plates (see Braasch 1980: 172, fig. 6b), and **iii)** absence of medial longitudinal stripe of hair-like setae along abdominal terga.

The species *E.* (*C.*) *sinitshenkovae* can be distinguished from *E.* (*C.*) *turcicus* **sp. nov.** by: **i)** absence of hypodermal medial femur spot and general colouration of femora (see Braasch & Zimmerman 1979: 106, fig. 10); **ii)** less developed projection on costal rib of gill plates (see Braasch & Zimmerman 1979: 106, fig. 8b); **iii)** only slightly enlarged setae on surface of abdominal terga.

Moreover, all four above mentioned species differ from *E.* (*C.*) *turcicus* **sp. nov.** in the colouration pattern of their abdominal terga (see Braasch 1979: 284, fig. 2b; Braasch & Zimmerman 1979: 105, fig. 1; Braasch 1980: 172, fig. 2B; Hrivniak *et al.* 2017: 356, fig. 10). The species *E.* (*C.*) *magnus*, which lacks the colouration pattern of abdominal sterna as well as *E.* (*C.*) *turcicus* **sp. nov.**, differs in the presence of a distinct posterolateral projection of abdominal segment X.

Distribution, habitat and biology. *E.* (*C.*) *turcicus* **sp. nov.** occurs in streams and brooks in the Eastern Black Sea Mountains of the Lesser Caucasus (NE Turkey—Eastern part of Black Sea Region of Turkey). Its presence on the northern slopes of the Lesser Caucasus (SW Georgia) and in other parts of the Eastern Black Sea Mountains (especially in Kaçkar Mountain Range) is possible (the materials from these areas were not available for the present study). However, the collection sites of *E.* (*C.*) *turcicus* **sp. nov.** are in a very restricted area in the Camili (Machakheli) District (near the border between Turkey and Georgia) (Fig. 20). We found no specimens of *E.* (*C.*) *turcicus* **sp. nov.** in the samples collected throughout the Caucasus region (Russian Federation, Georgia, Armenia, Azerbaijan, Iran) during our previous investigations. These data indicate that the distribution of *E.* (*C.*) *turcicus* **sp. nov.** might be restricted to the area of the Eastern Black Sea Mountains or distributed westwards, rather than in other parts of the Caucasian region.

The larvae of *E.* (*C.*) *turcicus* **sp. nov.** inhabit the hypocrenal and epirhithral zones of streams and brooks at relatively high altitudes (recorded in the alpine zone, between 2050 and 2388 m a.s.l.). The species was found in small-sized streams and brooks (approx. 1–2 m wide in the dry season and 1.5–3.5 m wide in the rainy season, at maximum depths of 0.1–0.5 m) (Figs 21, 22). Like many other *Epeorus* species, *E.* (*C.*) *turcicus* **sp. nov.** is a rheophilic species and prefers a moderate to fast current (current velocity recorded from 0.33 m/s to 2.3 m/s) with rocky and stony substrate. The environment around sampling sites varied, from riverbanks fully covered by riparian vegetation (small or large leaved-plants) with 100% shade, to exposed with 0% shade.

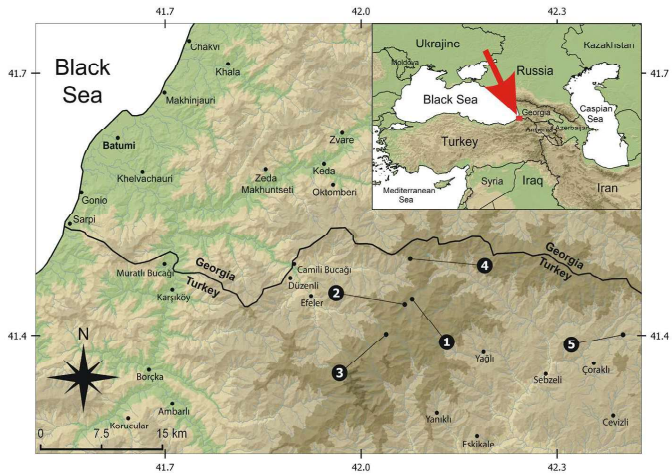


FIGURE 20. Sampling sites with records of *Epeorus* (*Caucasiron*) *turcicus* **sp. nov.** (locality numbers correspond to those given in parentheses in the list of material studied, arrow points to the area depicted on the larger map).

The larvae of *E.* (*C.*) *turcicus* **sp. nov.** were found in waters with a temperature range of 7–13°C. Therefore, the species was classified as a psychrophilic, preferring highly oxygenated water (concentrations of dissolved oxygen ranging from 9.8mg/l to 12.2mg/l, and the saturation of dissolved oxygen ranging from 87% to 102.2%). Electrical conductivity (spEC) values were 29–45µS/cm and TDS (total dissolved solid) values were 0.026–0.042 mg/l. All the values of physico-chemical variables mentioned above correspond to Class I water quality, according to the Legislation of Surface Water Quality Management of Turkey (Ministry of Forestry and Water Affairs, 2015). The pH values of the collecting sites ranged from 2.8 to 7.4. The lowest pH values were observed in localities CAM 8 and CAM 10 (sites 2 and 1, see section Type material) 2.8 and 3.7, respectively. These pH values correspond to Class IV water quality. We assume these values to be the result of the episodic acidification caused by melting snow mass. The melting period of the snow mass in the upper parts of the mountains was still in progress when the specimens were collected. Episodic acidification is a natural and a temporary situation observed in freshwater

ecosystems, resulting in low pH values and high PO_4-P concentrations during the melting period (Davies *et al.* 1993; Jacks *et al.* 1986). Episodic acidification is a common phenomenon for the Eastern Part of the Black Sea Region (Kazancı 2009b).

The other aquatic invertebrates frequently found with the larvae of *E.* (*C.*) *turcicus* **sp. nov.** were Heptageniidae, Baetidae, Leptophlebiidae (Ephemeroptera), Athericidae, Chironomidae, Tipulidae, Simuliidae, Blephariceridae (Diptera), Hydropsychidae, Rhyacophilidae, Brachycentridae, Sericostomatidae (Trichoptera), Perlidae, Perlodidae, Nemouridae, Chloroperlidae, Leuctridae (Plecoptera) and Oligochaeta. The taxa only occasionally found in the collecting sites included Empididae, Dixidae, Pediciidae, Limoniidae, Ceratopogonidae (Diptera), Elmidae, Curculionidae (Coleoptera), Dugesiidae (Plathelminthes), Asellidae (Isopoda), Gammaridae (Amphipoda), and Gordiidae (Nematomorpha).

The TR-BMWP, a national-based biotic index (Kazancı *et al.* 2016), was also applied to the collecting sites. The biotic index scores of the collecting sites ranged from 119 to160, which correspond to Class I water quality in all sites. This also reflects that all sites have high ecological status and hosts very diversified macroinvertebrate assemblages. Therefore, considering the results of physico-chemical variables and habitat quality evaluations, all collecting sites of *E.* (*C.*) *turcicus* **sp. nov.** are determined as reference sites.



FIGURE 21–22. *Epeorus* (*Caucasiron*) *turcicus* **sp. nov.,** sampling sites: 21, locality 1; 22, locality 3.

Discussion

In addition to morphological analysis, we used single locus *COI* data to test the delimitation of *E.* (*C.*) *turcicus* **sp. nov.** within the subgenus. Specifically in mayflies, single-locus mtDNA data has been repeatedly shown to be an appropriate means of delineating putative species, corroborated by nuclear markers and morphological characters (Monaghan *et al*. 2009; Vuataz *et al*. 2011, 2013; Pereira-da-Conceicoa *et al*. 2012; Rutschmann *et al.* 2017).

We used two species delimitation approaches based on the analysis of *COI*. The automatic barcode gap discovery (ABGD, see Puillandre *et al*. 2012) has already been used for *Caucasiron* in Hrivniak *et al*. (2017), where it recognized all previously known species, except *E.* (*C.*) *sinitshenkovae* and *E.* (*C.*) *longimaculatus*, that were merged into one group (for details and discussion on these two species see Hrivniak *et al.* 2017). In the present study, we newly included *E.* (*C.*) *turcicus* **sp. nov.** in the dataset and the new species was unambiguously recognized as a separate group (Fig. 19).

The second approach we used to delimit species was the Generalized Mixed Yule Coalescent model (GMYC, see Pons *et al.* 2006; Fujisawa & Barraclough 2013). Several studies on mayflies have provided strong evidence that the GMYC species correspond to biological species (Monaghan *et al*. 2009; Vuataz *et al*. 2011, 2013; Pereira-da-Conceicoa *et al*. 2012, Rutschmann *et al.* 2014, 2017). It is known that GMYC can delimit partially isolated

populations as separate species, especially if linking populations were not sampled (Fujisawa & Barraclough 2013). In our analysis, using the single threshold GMYC, all specimens sampled from geographically distinct populations were well-delimited and congruent with morphological identification. Therefore, we found the GMYC model to be highly suitable for species delimitation within *Caucasiron*.

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Diversity and distribution of *Epeorus* (*Caucasiron*) (Ephemeroptera, Heptageniidae) in Iran, with descriptions of three new species

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Abstract

Combining morphological and molecular data in an integrative approach, three new mayfly species of *Epeorus* (*Caucasiron*) are described. These include *Epeorus* (*Caucasiron*) *alborzicus* Hrivniak & Sroka, **sp. nov.** and *Epeorus* (*Caucasiron*) *shargi* Hrivniak & Sroka, **sp. nov.** from northern Iran, and *Epeorus* (*Caucasiron*) *zagrosicus* Hrivniak & Sroka, **sp. nov.** from central Iran. They are unambiguously delimited using both distance-based and likelihood-based approaches in the analyses of barcode COI sequences. Each new species is compared with other species of the subgenus and morphological diagnostic characters are provided. Based on extensive sampling of streams throughout the country, the distribution and habitat preferences of all *Caucasiron* species in Iran are assessed. Altogether, there are now six species recorded, among them also *E.* (*C.*) *nigripilosus* Sinitshenkova, 1976 is reported for the first time in Iran. Five species are distributed in the Alborz Mts. in northern Iran, one species was found in the Zagros Mts. in central Iran.

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Keywords
barcoding, Caucasus, diversity, mayflies, Middle East, taxonomy

Introduction

The genus *Epeorus* Eaton, 1881, subgenus *Caucasiron* Kluge, 1997 represents a group of mountainous mayflies distributed in Palaearctic region. Kluge (1997) defined *Caucasiron* based on a unique larval apomorphy, a projection on the costal margin of gill plates II–VII. Other larval diagnostic characters include the presence of medio-dorsally directed hair-like setae along anterior margin of head and gill plates forming a so-called "adhesive disc", consisting of enlarged gill plate I and overlapping gill plates II–VII. Gill plate VII has a longitudinal fold allowing to bend the plate ventrally under the abdominal segments. The systematic position of *Caucasiron* within *Epeorus*-related taxa was unclear for a long time (e.g., Braasch 2006, Kluge 2015). The recent study by Hrivniak et al. (2020) confirmed its monophyly and subgeneric position within *Epeorus* s.l. Moreover, the study pointed out its close phylogenetic relationship with the subgenus *Iron* Eaton, 1883 distributed in Central Asia and Nearctic realm.

Caucasiron occurs in the Eastern Mediterranean (Samos and Cyprus Island), Anatolia, Caucasus, and central and western Asia (Hrivniak et al. 2019, 2020). Their larvae inhabit riffle sections of montane and submontane streams with coarse bed substrate (Nguyen et al. 2004; Bauernfeind and Soldán 2012). At present there are 17 species described (Hrivniak et al. 2020), but apparently several Central Asian taxa described in the genus *Iron* rather belong to *Caucasiron* (Chen et al. 2010; Hrivniak et al. 2017). In any case, a taxonomic revision of these species is needed to clarify their systematic position.

The highest species richness of *Caucasiron* and a remarkable regional and local endemism was found in the Caucasus Mountains (Hrivniak et al. 2017; Hrivniak et al. 2020), which represent one of the world biodiversity hotspots (Myers et al. 2000). The 12 species known from the Caucasus and adjacent areas are as follows: *E.* (*C.*) *caucasicus* (Tshernova, 1938), *E.* (*C.*) *znojkoi* (Tshernova, 1938), *E.* (*C.*) *nigripilosus* (Sinitshenkova, 1976), *E.* (*C.*) *magnus* (Braasch, 1978), *E.* (*C.*) *alpestris* (Braasch, 1979), *E.* (*C.*) *soldani* (Braasch, 1979), *E.* (*C.*) *sinitshenkovae* (Braasch & Zimmermann, 1979), *E.* (*C.*) *longimaculatus* (Braasch, 1980), *E.* (*C.*) *bicolliculatus* Hrivniak, 2017, *E.* (*C.*) *turcicus* Hrivniak, Türkmen & Kazancı, 2019, *E.* (*C.*) *iranicus* (Braasch & Soldán, 1979), and *E.* (*C.*) *insularis* (Braasch, 1983). The latter two species for a long time were considered as subspecies of *E.* (*C.*) *caucasicus* and *E.* (*C.*) *znojkoi*, respectively. The recent molecular study of the Caucasian *Caucasiron* fauna, however, confirmed all morphologically defined species/subspecies as distinct evolutionary lineages and, consequently, both subspecies were raised to species level (Hrivniak et al. 2020). Moreover, the delimitation of several additional evolutionary lineages indicated that the diversity of *Caucasiron* in the Caucasus region could be even higher. However, these lineages have remained without formal description to date (Hrivniak et al. 2020).

Individual *Caucasiron* species exhibit different distribution patterns within the Caucasus region varying from an endemic distribution in the Greater Caucasus to a wide distribution covering distant regions in the Pontic Mountains, Lesser Caucasus, Zagros, and Alborz Mountains (Hrivniak et al. 2020). The highest species richness and endemism of *Caucasiron* is concentrated in the western and central part of the Greater Caucasus, the most prominent mountain range in the Caucasus region. However, the individual mountain ranges of the Caucasus have been studied to a different extent until now. Especially the Alborz Mountains, a southeast part of the Caucasus biodiversity hotspot, and the Zagros Mountains, a dominant part of the Irano-Anatolian biodiversity hotspot, have been left unattended without detailed investigation (Bojková et al. 2018). The only *Caucasiron* species described and known exclusively from Iran, *E.* (*C.*) *iranicus* (Braasch & Soldán, 1979), is distributed in the Alborz and most likely represents an endemic species of this mountain range. However, given the size and diversity of the Iranian territory and stream habitats, the diversity and endemism within *Caucasiron* can be expected to be much higher in Iran. Summarizing recent knowledge on the diversity and distribution of Iranian mayflies, Bojková et al. (2018) reported two species of *Caucasiron* from Iran, namely *E.* (*C.*) *iranicus* and *E.* (*C.*) *znojkoi*.

Based on morphology and molecular analyses, we describe in this integrative study, two new species of *Caucasiron* from the Alborz Mountains and one new species from the Zagros Mountains. We provide morphological diagnostic characters of the three new species and differential diagnoses between all species known from the Caucasus and adjacent areas, plus an analysis of respective COI sequences. Following recent studies on Iranian mayflies by Bojková et al. (2018), Sroka et al. (2019), and Staniczek et al. (2020), we also sum up all records of *Caucasiron* species from our recent Iranian field trips to further contribute to a systematic research of mayflies in Iran.

The main objectives of this study are to (i) describe the morphology of three new *Caucasiron* species and provide their differential diagnoses, (ii) apply the molecular species delimitation methods using analytical tools for the single-locus COI dataset, (iii) provide basic information about habitat requirements of the new species, and (iv) summarize the distribution of all *Caucasiron* species recently known from Iran.

Materials and methods

The material used for this study was collected by J. Bojková, T. Soldán, J. Imanpour Namin, and S. Bagheri in April and May 2016–2018, and A. Staniczek, M. Pallmann, R. J. Godunko, and F. Nejat in April and May 2017. All specimens were preserved in 75–96% EtOH and are deposited in the collections of the Biology Centre of the Czech Academy of Sciences, Institute of Entomology, České Budějovice, Czech Republic (**IECA**), State Museum of Natural History, Stuttgart, Germany (**SMNS**) and Natural History Museum and Genetic Resources, Department of Environment, Tehran, Iran (**MMTT_DOE**). Material of other *Caucasiron* species used for the morphological and molecular comparisons was obtained from the collection of IECA. This publica-

tion and the nomenclatural acts therein are registered with ZooBank under the LSID urn:lsid:zoobank.org:pub:3297FBE4-111C-4849-9533-225A53F7DB3C.

Morphological examination

Parts of specimens were mounted on microscopic slides using HydroMatrix (Micro-Tech Lab, Graz, Austria) mounting medium. In order to remove the muscle tissue for an investigation of the cuticular structures, specimens were left overnight in a 10% solution of NaOH prior to slide mounting. Drawings were made using a stereomicroscope Olympus SZX7 and a microscope Olympus BX41, both equipped with a drawing tube. Photographs were obtained using Leica DFC450 camera fitted with macroscope Leica Z16 APO and folded in Helicon Focus version 5.3 X64. All photographs were subsequently enhanced with Adobe Photoshop CS5. Diagnostic characters for the description of larva were chosen according to Braasch and Soldán (1979) and Braasch (2006). The terminology was used mostly according to Kluge and Novikova (2011) and Kluge (2004, 2015).

DNA extraction, PCR, sequencing and alignment

Total genomic DNA of the species (4–8 specimens/species) was extracted from legs using the DEP-25 DNA Extraction Kit (TopBio s.r.o., Prague, Czech Republic) according to the manufacturer's protocol. Mitochondrial cytochrome oxidase subunit I (COI) was sequenced according to Hrivniak et al. (2017). COI sequences of other *Caucasiron* species used for comparisons were obtained from Hrivniak et al. (2017) (GenBank accession nos KY865691–KY865725) and Hrivniak et al. (2019) (GenBank accession nos KY865691–KY865725). Three specimens of *E.* (*C.*) *iranicus* were additionally sequenced. The PCR amplification of COI and reaction volumes was carried out as described in Hrivniak et al. (2017). Sequences were assembled in Geneious 7.0.6 (http://www.geneious.com) and aligned in the same software using the Mafft 7.017 (Katoh et al. 2002) plugin with default settings. Newly obtained sequences are deposited in GenBank with accession numbers (GB) MN856180–MN856198.

Molecular species delimitation

Species were delimited using the single locus (COI) coalescence based General Mixed Yule Coalescent model (GMYC, Pons et al. 2006; Fusijawa and Barraclough 2013). We used the single-threshold GMYC model as it has been found to outperform the multi-threshold (Fusijawa and Barraclough 2013) and was found to be highly suitable for species delimitation within *Caucasiron* (Hrivniak et al. 2019). The GMYC model identifies independent evolutionary clusters by detecting a threshold value at the transition from interspecific to intraspecific branching patterns (Bryson et al. 2013). A maximum likelihood approach is used to optimize the shift in branching patterns. A likelihood ratio test assesses if the mixed model fits the data significantly better than a null model that

assumes a single coalescent process for the entire tree (Pons et al. 2006; Monaghan et al. 2009). Analyses were performed using the SPLITS package for R (http://r-forge.r-project.org/projects/splits). An ultrametric COI gene tree was reconstructed under relaxed molecular clock (uncorrelated lognormal distribution) using BEAST 2 (Bouckaert et al., 2014) on CIPRES Science Gateway 3.3 (Miller et al. 2010). An input file was generated in BEAUti 2. The substitution model was selected by bModelTest (Bouckaert and Drummond 2017) implemented in BEAUti 2 using a model averaging approach. A coalescent constant population tree prior was preferred, because the GMYC null model constitutes a single coalescent cluster (Monaghan et al. 2009; Zaldívar-Riverón et al. 2010; Vuataz et al. 2011). Other settings were default. Two analyses of MCMC chains were run for 50 million generations sampled every 5000 generations. Convergence and effective sample size (ESS > 200) were verified using Tracer 1.6. The first 10% of trees (1000) from each run were discarded as burn-in. The files from both independent runs were combined using LogCombiner 1.8.4. The maximum clade credibility tree was constructed from 18000 trees using TreeAnnotator 1.8.4 with default settings.

Inter- and intra-specific K2P pairwise genetic distances were calculated in MEGA 7 (Kumar et al. 2016). The distance matrix was analysed using Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012) (online version: http://wwwabi.snv.jussieu.fr/public/abgd/) with default settings. The method identifies so-called barcode gap that corresponds to threshold between intra- and inter-specific genetic distances and splits sequences to groups corresponding to putative species accordingly.

Results and discussion

Taxonomy

All of the species described below are attributed to the subgenus *Caucasiron* within the genus *Epeorus* based on the presence of projections on the costal rib of gill plates II–VII, and the presence of medio-dorsally directed hair-like setae located on the anterior margin of the head (see Kluge 2015 for a revision of the subgenus).

***Epeorus* (*Caucasiron*) *alborzicus* Hrivniak & Sroka, sp. nov.**
http://zoobank.org/F1721BB2-DC7C-4BBC-9AD2-8252A5D01EBF
Figures 1, 2

Type material. *Holotype*: female mature larva: IRAN, Mazandaran Province, Panjab village, unnamed brook (LT of Haraz River); 36°05'52.8"N, 052°15'16.0"E (locality no. 152); 955 m a.s.l.; J. Bojková, T. Soldán, J. Imanpour Namin, S. Bagheri leg., 9.5.2018, SMNS_EPH_010056.

Paratypes: 38 female larvae (3 mounted on slide), 10 male larvae (2 mounted on slide): same data as holotype, SMNS_EPH_010056. DNA extracted from 1 fe-

male (code: IR11, stored in EtOH) and 2 males (codes: IR12 and IR14, both stored in EtOH).

33 female larvae, 24 male larvae: IRAN, Tehran Province, Zayegan village, Lalan River; 35°58'39.2"N, 051°34'56.5"E (locality no. 55); 2290 m a.s.l.; A. Staniczek, M. Pallmann, R. J. Godunko, F. Nejat leg., 8.5.2017, SMNS_EPH_007617.

1 female larva: IRAN, Golestan Province, above Chah-e Ja village, unnamed brook (RT of river flowing to Fazelabad); 36°40'22.8"N, 054°46'37.9"E (locality no. 104); 1450 m a.s.l.; J. Bojková, T. Soldán, J. Imanpour Namin leg., 27.4.2018. DNA extracted specimen (code: IR13, stored in EtOH).

17 female larvae (3 mounted on slide), 6 male larvae: IRAN, Alborz Province, 2.5 km W of Asara village, Karaj River; 36°01'52.1"N, 051°13'10.0"E (locality no. 58); 1890 m a.s.l.; A. Staniczek, M. Pallmann, F. Nejat leg., 10.5.2017, SMNS_EPH_007627.

The holotype and 50 paratypes are deposited in SMNS, 50 paratypes (including DNA extracted specimens) are deposited in IECA and 29 paratypes in MMTT_DOE.

Other material examined. 8 larvae: same data as holotype, SMNS_EPH_010056; young instars or damaged specimens.

13 larvae: IRAN, Mazandaran Province, NE of Kahrud village, unnamed brook (LT of Haraz River); 36°03'42.7"N, 052°15'24.8"E (locality no. 153); 1020 m a.s.l.; J. Bojková, T. Soldán, J. Imanpour Namin, S. Bagheri leg., 9.5.2018.

2 larvae: IRAN, Mazandaran Province, 3.5 km E of Polour village, Lasem Rud (RT of Haraz River); 35°50'09.4"N, 052°04'38.4"E (locality no. 73); 2100 m a.s.l.; A. Staniczek, M. Pallmann, F. Nejat leg., 14.5.2017, SMNS_EPH_007680; 17 larvae: S. Bagheri leg., 16.4.2018.

1 larva: IRAN, Mazandaran Province, 1.5 km S of Part Kola village, Shirin Rud (LT of Sefidrud); 36°9'04.3"N, 053°20'54.7"E (locality no. 63); 750 m a.s.l.; A. Staniczek, M. Pallmann, F. Nejat leg., 11.5.2017, SMNS_EPH_007641; 10 larvae: S. Bagheri leg., 5.4.2018.

7 larvae: IRAN, Mazandaran Province, 3.5 km W of Razan village, Baladeh River; 36°11'39.6"N, 052°8'34.6"E (locality no. 73); 1360 m a.s.l.; A. Staniczek, M. Pallmann, F. Nejat leg., 14.5.2017, SMNS_EPH_007677.

1 larva: IRAN, Tehran Province, Lalan village, Lalan River; 35°59'50.3"N, 051°34'51.0"E (locality no. 53); 2438 m a.s.l.; A. Staniczek, M. Pallmann, R. J. Godunko, F. Nejat leg., 8.5.2017, SMNS_EPH_007613.

17 larvae: IRAN, Tehran Province, Igol village, Fasham River; 35°55'11.2"N, 051°28'51.3"E (locality no. 56); 2020 m a.s.l.; A. Staniczek, M. Pallmann, R. J. Godunko, F. Nejat leg., 8.5.2017, SMNS_EPH_007618.

10 larvae: IRAN, Alborz Province, 4 km NW of Shahrestanak village, Shahrestanak River; 35°59'01.2"N, 051°19'09.6"E (locality no. 57); 2100 m a.s.l.; A. Staniczek, M. Pallmann, F. Nejat leg., 10.5.2017, SMNS_EPH_007622.

Etymology. The species name refers to the type locality and distribution of the species in the Alborz mountain range.

Localities and habitat preferences of larvae. Larvae inhabit small streams (2–8 m width, 20–50 cm depth) at high altitudes (six of eleven localities at approx. 2000 m

a.s.l.) in the central Alborz (Fig. 9). One larva was found in the eastern Alborz (Fig. 9). Larvae were found only in cold and clear streams where they dwelled on large stones in riffles with very fast flow. All localities were situated in deep valleys with rivers draining high mountains. They were mostly treeless, only sometimes with sparse solitary shrubs and trees at the banks (Fig. 10A, B). Streams had a very coarse bed substrate with prevailing boulders and stones and a low share of fine sediments, and turbulent to strongly turbulent flow. They were characteristic of high fluctuation of discharge, with sudden peaks of discharge after spates on the mountains (Fig. 10A).

Description of larva. General colouration of larvae yellowish brown with dark brown maculation. Body length of mature larvae: 13.3–15.8 mm (female), 10.3–11.3 mm (male). Length of cerci approximately 1.3× body length.

Head. Shape trapezoidal; anterior and lateral margin rounded, posterior margin rounded in female, slightly rounded or nearly straight in male (Fig. 1D, E). Anterior margin with shallow concavity medially. Head dimensions of mature larvae: length 2.8–3.1 mm, width 4.0–4.6 mm (female); length 2.2–2.7 mm, width 3.2–3.7 mm (male). Head width/length ratio: 1.4–1.5 (both male and female). Dorso-medial part with pair of stripes. Pair of maculae located between ocelli (sometimes fused into single macula). Rounded maculae ventrolateral of lateral ocelli and blurred maculae near in-

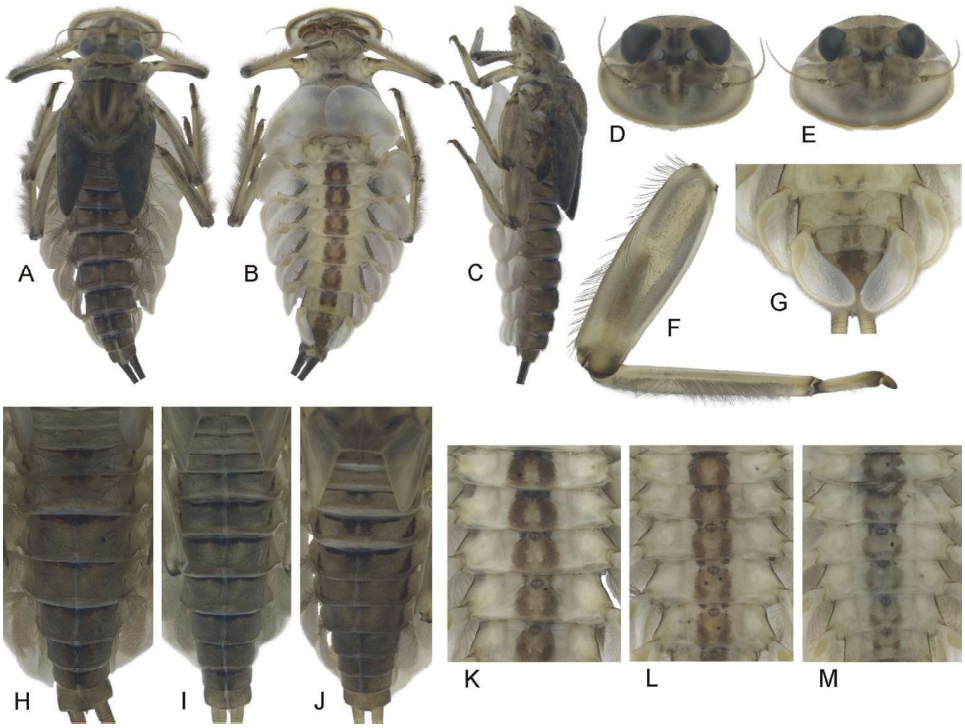


Figure 1. *Epeorus* (*Caucasiron*) *alborzicus* sp. nov., larva: **A** habitus in dorsal view **B** habitus in ventral view **C** habitus in lateral view **D** head of male in dorsal view **E** head of female in dorsal view **F** middle leg in dorsal view **G** distal part of abdomen in ventral view **H–J** colouration of abdominal terga **K–M** colouration of abdominal sterna.

ner edges of compound eyes. Pale stripes extending horizontally from lateral ocelli to lateral edges of head. Pair of elongated, curved maculae located along coronal suture. Compound eyes grey to black in female, brownish or greyish and basally black in male mature larva. Ocelli blackish, basally paler. Antennae yellowish brown, scapus and pedicellus darkened. Anterior margin of head densely covered with hair-like setae extending to lateral margins and directed medio-dorsally. Dorsal surface of head covered with fine hair-like setae and sparsely distributed stick-like setae. Sparse longer and fine hair-like setae located posteriorly to eyes.

Mouthparts. Labrum (Fig. 2A) widened anteriorly, with anterior margin slightly rounded or nearly straight (in dorsal view). Lateral angles rounded (shape of labrum may vary among individual specimens). Dorsal surface (Fig. 2A, right half) sparsely covered with setae of different size; 4–6 longer bristle-like setae located antero-medially and two bristles antero-laterally. Epipharynx with longer, slightly plumose bristles situated along lateral to anterior margin (Fig. 2A, left half, range of setation figured as large black dots), and cluster of fine, hair-like setae medially (not figured). Posterior margin of labrum irregularly concave; group of 6–17 setae of various size located on ventral surface close to posterior margin. Outer incisors of both mandibles (Fig. 2B, C) with three apical teeth; outer tooth blunt in both mandibles. Inner incisor of left mandible with three apical teeth, right inner incisor bifurcated.

Thorax. Pronotum anteriorly narrowed, lateral edges nearly straight. Metanotum with slight postero-medial projection. Dorsal surface covered with fine, hair-like setae (as on abdominal terga and head); sparse longer, hair-like setae along pro-, meso- and metanotal suture.

Legs. Colour pattern of femora as in Fig. 1F. Femora without medial hypodermal spot. Patella-tibial suture darkened; tarsi proximally and distally darkened. Coxal projections of fore- and hind legs pointed or bluntly pointed; in middle legs blunt. Trochanteres with spatulate setae as on dorsal surface of femora (Fig. 2D). Tibiae of forelegs 1.20–1.37× femur length, tibiae of middle legs 1.0–1.2× femur length, and tibiae of hind legs 0.92–1.08× femur length. Tarsi of all legs 0.26–0.34× tibia length. Dorsal surface of femora covered by short and sporadically elongated spatulate setae (Fig. 2D), hair-like setae, and sparsely distributed stick-like setae. Anterior margin of femora with short, pointed or bluntly pointed spine-like setae; posterior margin with row of long blade-like setae and sparse row of bluntly pointed, spine-like setae. Dorsal margin of tibiae and tarsi with row of long setae; ventral margin of both with irregular row of spine-like setae accumulated distally. Tarsal claws with 2–3 denticles.

Abdominal terga. Colour pattern of abdominal terga (Fig. 1A, H–J) consists of transversal stripe along anterior margin of terga I–IX (X), medially extending to single blurred macula or pair of rounded maculae on terga II–IV and short triangular or nearly rectangular macula on terga V–IX. Terga VIII and IX (X) medially darkened. Pattern of abdominal terga sometimes poorly expressed, only with medially thickened transversal stripe along anterior margin.

Lateral margins with oblique maculae on terga I–IX, sometimes dorso-posteriorly extended. Pair of sigilla sometimes coloured, in form of short stripes or spots located

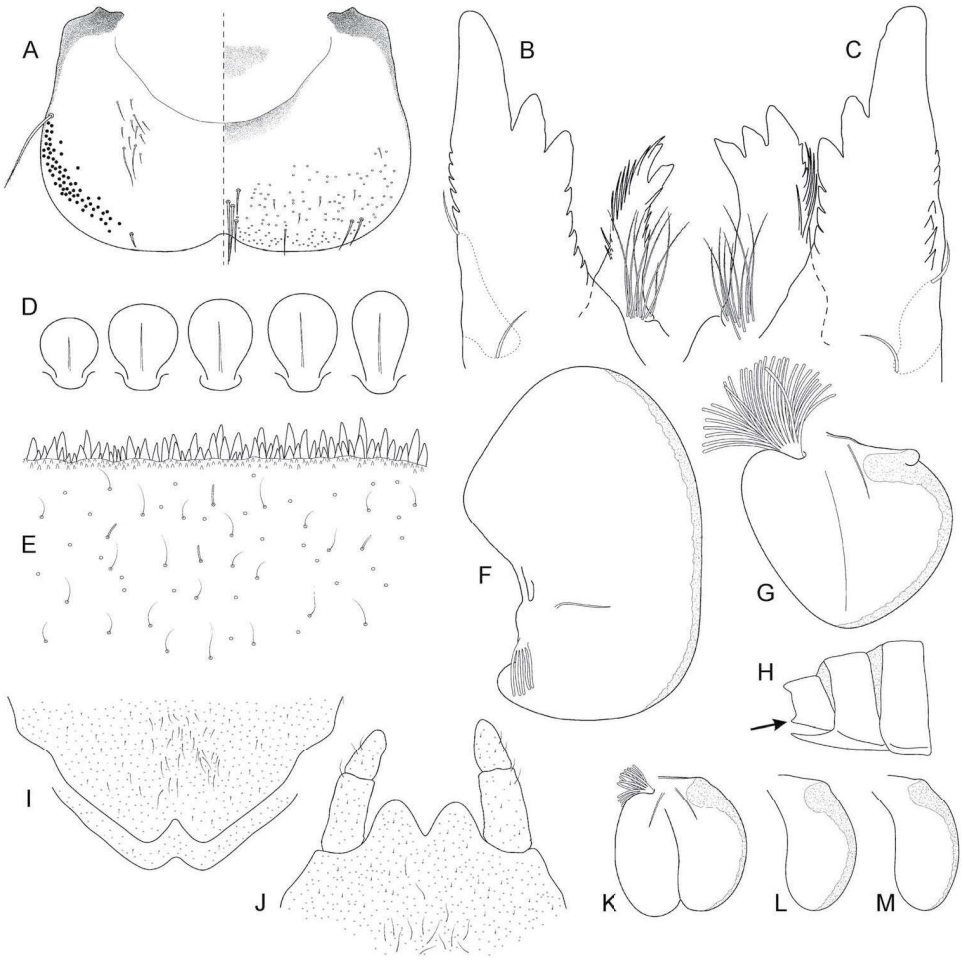


Figure 2. *Epeorus* (*Caucasiron*) *alborzicus* sp. nov., larva: **A** labrum (right half in dorsal view, left half in ventral view) **B** incisors of right mandible in ventral view **C** incisors of left mandible in ventral view (both flattened on slide) **D** setae on dorsal surface of femora **E** surface and posterior margin of abdominal tergum VII **F** gill I **G** gill III **H** abdominal segments VIII–X **I** sternum IX, female **J** sternum IX, male **K** gill VII (flattened on slide) **L–M** gill VII (in natural position from ventral view), variability in shape.

antero-laterally to medial macula. Denticles on posterior margin on terga of various size, irregular and pointed (Fig. 2E). Surface of terga covered with hair-like setae and sparsely with stick-like setae. Tergum X with distinct postero-lateral projections (Fig. 2H, arrow). Supra-tergalial projection (sensu Kluge 2004) short and blunt. Longitudinal row of hair-like setae along abdominal terga present medially.

Abdominal sterna. Yellowish, with distinct colour pattern in form of medial circular macula (Fig. 1B, G, K–M, best expressed on sterna II–VI). Medio-anterior sigilla partly pigmented, lateral sigilla not pigmented; medio-posterior sigilla in form of pale

spots in intensively pigmented specimens. Nerve ganglia occasionally darkened. Intensity of colouration varies among individuals (Fig. 1K–M). Sternum IX with V-shaped medial emargination; surface covered by irregularly distributed short hair-like setae, and medially accumulated longer hair-like setae (Fig. 2I, J).

Gills. Dorsal surface of gill plate I yellowish; of gill plates II–VII greyish on anterior half, brownish (sometimes reddish) on posterior half. Ventral margin of all gill plates yellowish. Projection of gill plate III well developed (Fig. 2G). Gill plate VII relatively wide (in natural position of ventral view, Figs 1G, 2L, M). Filaments of gills II–VI reaching 0.40–0.58× length of respective plate, filaments of gill VII reaching 0.18–0.24× (in late-instar larvae).

Cerci. Yellowish brown, basally darkened.

Subimago, imago and eggs. Unknown.

Morphological diagnostics of larvae. The main larval diagnostic characters of *E.* (*C.*) *alborzicus* sp. nov. are as follows: (i) colour pattern of abdominal terga (Fig. 1A, H–J) and sterna (Fig. 1B, K–M), (ii) presence of distinct postero-lateral projections on tergum X (Fig. 2H), (iii) absence of medial hypodermal femur spot (Fig. 1F), (iv) gill plate VII relatively wide (in natural position from ventral view; Figs 1G, 2L, M), and (v) fine hair-like setae on surface of abdominal terga (Fig. 2E).

Affinities. The combination of diagnostic characters mentioned above clearly distinguish larvae of *E.* (*C.*) *alborzicus* sp. nov. from all other *Caucasiron* species known so far. However, some of the diagnostic characters occur also in other *Caucasiron* species distributed in the Caucasus. The colour pattern of abdominal sterna in *E.* (*C.*) *alborzicus* sp. nov. is similar in *E.* (*C.*) *bicolliculatus* (Hrivniak et al. 2017: 356, fig. 8) and *E.* (*C.*) *alpestris* (Braasch 1979: 284, fig. 1d). Both species also lack a medial hypodermal femur spot. *Epeorus* (*C.*) *bicolliculatus* can be distinguished from *E.* (*C.*) *alborzicus* sp. nov. by (i) the presence of flattened setae on the surface of abdominal terga (Hrivniak et al. 2017: 359, fig. 23), (ii) the presence of paired postero-medial protuberances on terga II–IX (Hrivniak et al. 2017: 356, figs 10, 11; 360, figs 31, 32), and (iii) the absence of a postero-lateral projection on the tergum X.

Epeorus (*C.*) *alpestris* differs by the characteristic colour pattern of abdominal terga (Braasch 1979: 294, fig. 1c) and the absence of postero-lateral projections on the tergum X.

The presence of postero-lateral projections on the abdominal tergum X is characteristic for two species distributed in the Caucasus, *E.* (*C.*) *magnus*, *E.* (*C.*) *nigripilosus*, and sporadically also in *E.* (*C.*) *znojkoi*. *Epeorus* (*C.*) *magnus* differs from *E.* (*C.*) *alborzicus* sp. nov. in the absence of colouration of abdominal sterna and the characteristic setation on the dorsal margin of labrum (numerous thickened bristle-like setae, Hrivniak et al. in prep.). *Epeorus* (*C.*) *nigripilosus* can be separated from *E.* (*C.*) *alborzicus* sp. nov. by the presence of the distinct medial hypodermal femur spot and unique colour pattern of abdominal sterna (Sinitshenkova 1976: 89, fig. 28). *Epeorus* (*C.*) *znojkoi* can be clearly distinguished from *E.* (*C.*) *alborzicus* sp. nov. by the colour pattern of abdominal terga and conspicuous reddish colouration of abdominal sterna (Braasch 1980: 172, fig. 4b–c).

Two species, *E.* (*C.*) *soldani* and *E.* (*C.*) *sinitshenkovae*, are lacking a medial hypodermal femur spot just like *E.* (*C.*) *alborzicus* sp. nov. Both can be separated from the latter by the absence of postero-lateral projections on tergum X, narrower gill plates VII (in natural position from ventral view), and the absence of a distinct colour pattern of abdominal sterna. Additionally, *E.* (*C.*) *soldani* differs from *E.* (*C.*) *alborzicus* sp. nov. by the presence of flattened setae on the surface of abdominal terga (Hrivniak et al. 2017: 359, fig. 25).

Other *Caucasiron* species distributed in the Caucasus and adjacent areas do not share important diagnostic characters with *E.* (*C.*) *alborzicus* sp. nov. All of these species can be easily distinguished by the following combination of characters: (i) absence of the colour pattern of abdominal sterna and presence of the medial hypodermal femur spot in *E.* (*C.*) *turcicus*, *E.* (*C.*) *longimaculatus*, *E.* (*C.*) *shargi* sp. nov. and (ii) colour pattern of abdominal terga and sterna in *E.* (*C.*) *caucasicus* (Braasch 1979: fig. 3a), *E.* (*C.*) *iranicus* (Braasch and Soldán 1979: fig. 12), and *E.* (*C.*) *zagrosicus* sp. nov. (Fig. 5A–C, G, H–K). The larva of *E.* (*C.*) *insularis* is currently not described.

***Epeorus* (*Caucasiron*) *shargi* Hrivniak & Sroka, sp. nov.**
http://zoobank.org/6F5FE6F7-8710-416D-80DB-C202C71DE7FC
Figures 3, 4

Type material. ***Holotype***: female mature larva: IRAN, Golestan Province, Shirinabad village, unnamed river; 36°48'01.4"N, 055°01'05.8"E (locality no. 108); 740 m a.s.l.; J. Bojková, T. Soldán, J. Imanpour Namin leg., 27.4.2018, SMNS_EPH_010057.

Paratypes: 19 female, 11 male larvae: same data as holotype.

36 female (5 mounted on slide), 25 male (1 mounted on slide) larvae: IRAN, Golestan Province, above Chah-e Ja village, unnamed brook (RT of river flowing to Fazelabad); 36°40'22.8"N, 054°46'37.9"E (locality no. 104); 1450 m a.s.l.; J. Bojková, T. Soldán, J. Imanpour Namin leg., 27.4.2018. DNA extracted from 2 females (codes: IR23 and IR24, mounted on slides).

19 female (3 mounted on slide), 7 male (1 mounted on slide) larvae: IRAN, Golestan Province, below Chah-e Ja village (main valley), unnamed river flowing to Fazelabad, 36°41'46.3"N, 054°47'35.0"E (locality no. 105); 1240 m a.s.l.; J. Bojková, T. Soldán, J. Imanpour Namin leg., 27.4.2018. DNA extracted from 1 female (code: IR21, mounted on slide) and 1 male (code: IR22, stored in EtOH).

The holotype (SMNS_EPH_010057) and 50 paratypes (SMNS_EPH_010057) are deposited in SMNS, 50 paratypes (including DNA extracted specimens) are deposited in IECA, and 17 paratypes in MMTT_DOE.

Other material examined (not paratypes): 3 larvae: same data as holotype; young instars or damaged specimens.

Etymology. The species name derives from *shargi* (شرقی), which means eastern in Farsi. It refers to the distributional range of the species in the eastern part of the Alborz mountain range.

Localities and habitat preferences of larvae. Larvae were found in three clear streams at middle altitude (740–1450 m a.s.l.) in the eastern Alborz (Fig. 9). Habitat conditions of these streams differed from each other. Larvae were abundant in a cold, alkaline brook (water conductivity 1320 µS/cm) with patches of precipitated calcium crusts on the bed and in the non-alkaline water (with water conductivity reaching the values of clear montane streams in the region, 433 µS/cm) of the type locality. Both localities were characterised by stony bed sediment with leaf litter debris and fine gravel along the banks, and by fast, turbulent flow (Fig. 10C, D). Lower abundance of larvae was found in a river with uniform coarse substrate flowing in a wide gravel river channel. All streams were surrounded by deciduous forests (Fig. 10C, D). The species was not found in urban and agricultural areas in this region where many localities were investigated.

Description of larva. General colouration of larvae yellowish brown with dark brown maculation. Body length of mature larvae 13.7–15.6 mm (female), 11.7–13.0 mm (male). Length of cerci approximately 1.1× body length.

Head. Shape trapezoidal; anterior and lateral margin rounded, posterior margin rounded in female, slightly rounded in male (Fig. 3D, E). Anterior margin with shal-

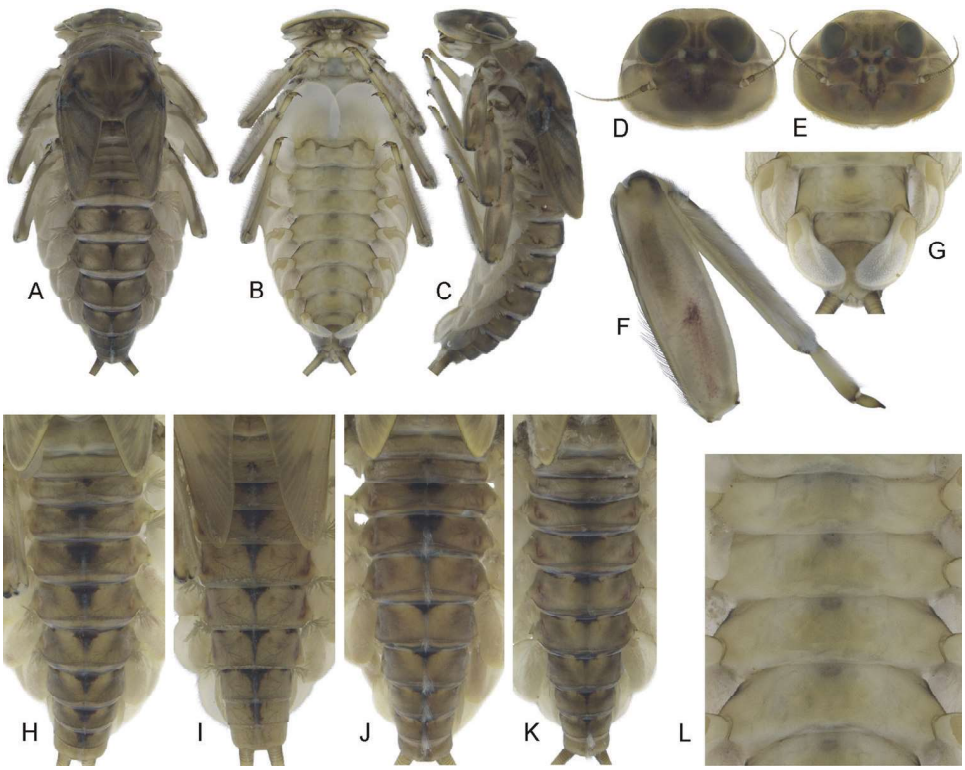


Figure 3. *Epeorus* (*Caucasiron*) *shargi* sp. nov., larva: **A** habitus in dorsal view **B** habitus in ventral view **C** habitus in lateral view **D** head of male in dorsal view **E** head of female in dorsal view **F** middle leg in dorsal view **G** distal part of abdomen in ventral view **H–K** colouration of abdominal terga **L** colouration of abdominal sterna.

low concavity medially. Head dimensions of mature larvae: length 3.0–3.2 mm, width 4.1–4.4 mm (female); length 2.70–2.95 mm, width 3.5–4.0 mm (male). Head width/length ratio: 1.33–1.40 (both male and female). Dorso-medial part with brown, rectangular or oval smudge, sometimes reduced to pair of stripes. Pair of maculae located between ocelli (sometimes fused into single macula). Rounded maculae lateroventral of lateral ocelli and blurred maculae near inner edges of compound eyes. Pair of pale stripes extending from lateral ocelli to lateral edges of head. Pair of maculae located along coronal suture. Compound eyes dark grey to black in female, brownish and basally blackish in male mature larva. Ocelli dark grey to black, basally paler. Antennae

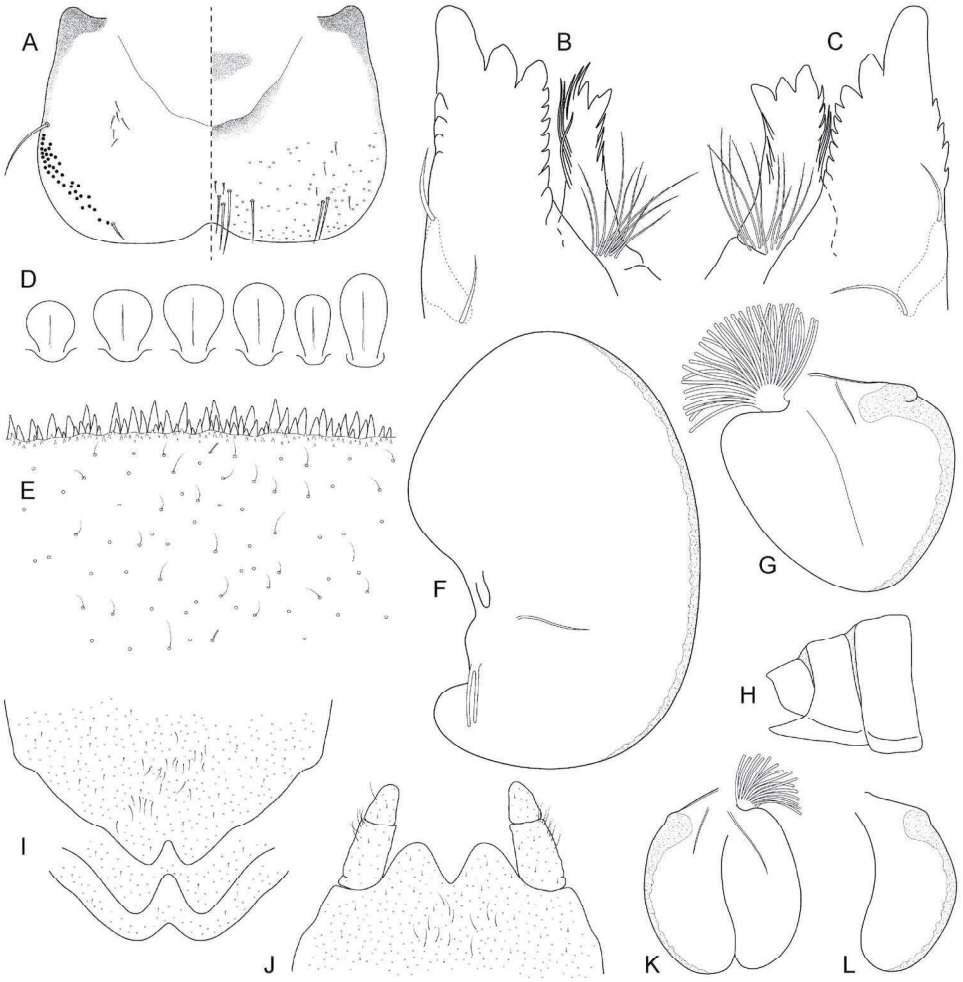


Figure 4. *Epeorus* (*Caucasiron*) *shargi* sp. nov., larva: **A** labrum (right half in dorsal view, left half in ventral view) **B** incisors of right mandible in ventral view **C** incisors of left mandible in ventral view (both flattened on slide) **D** setae on dorsal surface of femora **E** surface and posterior margin of abdominal tergum VII **F** gill I **G** gill III **H** abdominal segments VIII–X **I** sternum IX, female **J** sternum IX, male **K** gill VII (flattened on slide) **L** gill VII (in natural position from ventral view).

yellowish-brown, scapus and pedicellus darkened. Anterior margin of head densely covered with hair-like setae extending to lateral margins and directed medio-dorsally. Dorsal surface of head covered with fine hair-like setae and sparsely distributed stick-like setae. Sparse longer fine hair-like setae located posteriorly to eyes.

Mouthparts. Labrum (Fig. 4A) widened anteriorly, with anterior margin slightly rounded or nearly straight (in dorsal view). Lateral angles rounded (shape of labrum may vary among individual specimens). Dorsal surface (Fig. 4A, right half) sparsely covered with setae of different size; 4–6 longer bristle-like setae located antero-medially and two antero-laterally. Epipharynx with longer, shortly plumose bristles situated along lateral to anterior margin (Fig. 4A, left half), range of setation figured as large black dots), and brush of fine hair-like setae medially (not figured). Posterior margin of labrum irregularly concave; with group of 5–10 setae of various size located on ventral surface close to posterior margin. Outer incisors of both mandibles (Fig. 4B, C) with three apical teeth; outer tooth blunt in both mandibles. Inner incisor of left mandible with three apical teeth, right inner incisor bifurcated (inner side of right tooth usually with small denticle).

Thorax. Pronotum anteriorly narrowed, lateral edges nearly straight or slightly rounded. Metanotum with slight, blunt, postero-medial projection. Dorsal surface covered with fine hair-like setae (as on abdominal terga and head); sparse longer hair-like setae along pro-, meso- and metanotal suture.

Legs. Colour pattern of femora as in Fig. 3F. Femora with rounded or slightly elongated medial hypodermal femur spot. Patella-tibial suture darkened; tarsi proximally and distally darkened. Coxal projections of fore- and hind legs pointed or bluntly pointed; of middle legs blunt. Trochanteres with spatulate setae as on dorsal surface of femora (Fig. 4D). Tibiae of forelegs 1.23–1.28× femur length, tibiae of middle legs 1.03–1.50× femur length, and tibiae of hind legs 0.87–1.06× femur length. Tarsi of all legs 0.28–0.32× tibia length. Dorsal surface of femora covered by short, sporadically elongated spatulate setae (Fig. 4D), hair-like setae, and sparsely distributed stick-like setae. Anterior margin of femora with short, pointed and/or bluntly pointed spine-like setae; posterior margin with row of long blade-like setae and sparse row of bluntly pointed spine-like setae. Dorsal margin of tibiae and tarsi with row of long setae; ventral margin of both with irregular row of spine-like setae accumulated distally. Tarsal claws with 2–3 denticles.

Abdominal terga. Colour pattern of abdominal terga (Fig. 3A, H–K) consists of transversal stripe along anterior margin of terga I–IX (X) medially extending to i) triangular or blurred macula on terga (II) III–IV; ii) triangular or T-shaped macula on terga V–IX, reaching to half or stretching to posterior margin of corresponding tergum (medial macula of terga VIII and IX often widened). Transversal stripe along anterior margin of terga laterally extends to pair of short maculae. Medial maculae often surrounded by pale background. Tergum X without distinct maculation. Pair of sigilla sometimes coloured and forming pair of short stripes adjacent laterally to medial macula. Lateral margins of abdomen with oblique maculae on terga I–IX. Denticles along posterior margin on terga of various size, irregular and pointed (Fig. 4E). Surface

of terga covered with hair-like setae and sparsely with stick-like setae. Supra-tergalial projections short and blunt. Tergum X without distinct postero-lateral projections (Fig. 4H). Longitudinal row of hair-like setae along abdominal terga present medially.

Abdominal sterna. Yellowish, without distinct colour pattern. Nerve ganglia often dark brown pigmented (Fig. 3B, G, L). Sternum IX with V-shaped medial emargination; surface covered by irregularly distributed short hair-like setae and medially accumulated longer hair-like setae (Fig. 4I, J).

Gills. Dorsal surface of gill plate I yellowish, of gill plates II–VII greyish on anterior half and brownish to reddish on posterior half. Ventral margin of all gill plates yellowish. Projection of gill plate III well developed (Fig. 4G). Gill plate VII relatively wide (in natural position of ventral view, Figs 3G, 4L). Gill filaments reaching to 0.41–0.50× length of respective plate, filaments of gill VII to 0.24–0.28× (in late-instar larvae).

Cerci. Brownish, basally darkened.

Subimago, imago and eggs. Unknown

Morphological diagnostics of larvae. The main larval diagnostic characters of *E.* (*C.*) *shargi* sp. nov. are as follows: (i) colour pattern of abdominal terga (Fig. 3A, H–K) and no colouration of abdominal sterna (Fig. 3B, G, L), (ii) lack of distinct postero-lateral projections on tergum X (Fig. 4H), (iii) presence of medial hypodermal femur spot (Fig. 3F), (iv) relatively wide shape of gill plate VII (in natural position from ventral view; Figs 3G, 4L), and (v) fine hair-like setae on surface of abdominal terga (Fig. 4E).

Affinities. Based on the colour pattern of abdominal terga and sterna, *E.* (*C.*) *shargi* sp. nov. resembles several species distributed in the Caucasus and adjacent areas. At first glance, *E.* (*C.*) *soldani* and *E.* (*C.*) *turcicus* are most similar. Larvae of *E.* (*C.*) *soldani* possess triangular maculae on abdominal terga (Braasch 1979: 284, fig. 2b) and an indistinct, sometimes not expressed, colour pattern of abdominal sterna. It can be distinguished from *E.* (*C.*) *shargi* sp. nov. by a comparatively narrower gill plate VII (in natural position from ventral view), the presence of flattened setae on the surface of abdominal terga (Hrivniak et al. 2017: 359, fig. 25), and the absence of a medial hypodermal femur spot.

Epeorus (*C.*) *turcicus* shares with *E.* (*C.*) *shargi* sp. nov. the lack of colouration on abdominal sterna (Hrivniak et al. 2019: 61, fig. 2), the presence of a medial hypodermal femur spot (Hrivniak et al. 2019: 62, fig. 9), and fine hair-like setae on the dorsal surface of abdominal terga (Hrivniak et al. 2019: 63, fig. 11). Nevertheless, *E.* (*C.*) *turcicus* differs from *E.* (*C.*) *shargi* sp. nov. by the different colour pattern of abdominal terga, with anteriorly widened stripe stretching between anterior and posterior margins (Hrivniak et al. 2019: 61, fig. 1), in contrast to *E.* (*C.*) *shargi* sp. nov. with more or less triangular maculae on abdominal terga (Fig. 3A, H–K), and a distinctly narrower gill plate VII (in natural position from ventral view) (Hrivniak et al. 2019: 63, figs 15, 16).

Similar to *E.* (*C.*) *shargi* sp. nov., there is no colour pattern of abdominal sterna in several other species, namely *E.* (*C.*) *longimaculatus*, *E.* (*C.*) *sinitshenkovae*, and *E.* (*C.*) *magnus*. *Epeorus* (*C.*) *longimaculatus* can be clearly separated from *E.* (*C.*) *shargi* sp. nov. by (i) a distinctly narrower gill plate VII (in natural position of ventral view),

(ii) flattened setae on the surface of abdominal terga (Hrivniak et al. 2017: 359, fig. 25), (iii) poorly developed projection on the costal margin of gill plate III (Braasch 1980: 172, fig. 6b), and (iv) elongated medial hypodermal femur spot (Braasch 1980: 172, fig. 11).

Epeorus (*C.*) *sinitshenkovae* can be distinguished from *E.* (*C.*) *shargi* sp. nov. by the absence of a medial hypodermal femur spot, the characteristic colour pattern of femora (Braasch and Zimmerman 1979: 106, fig. 10), and the colour pattern of abdominal terga (Braasch 1979: 105, fig. 2).

Epeorus (*C.*) *magnus* can be reliably distinguished by the presence of distinct postero-lateral projections on abdominal tergum X and characteristic setation of labrum (numerous thickened bristle-like setae, Hrivniak et al. in prep.).

All other species distributed in the Caucasus and adjacent areas differ from *E.* (*C.*) *shargi* sp. nov. by the distinct colour pattern of abdominal sterna, namely *E.* (*C.*) *bicolliculatus* (Hrivniak et al. 2017: 356, figs 7–9), *E.* (*C.*) *alpestris* (Braasch, 1979: 284, fig. 1d), *E.* (*C.*) *alborzicus* sp. nov., (Fig. 1B, K–M), *E.* (*C.*) *caucasicus*, *E.* (*C.*) *iranicus* (Braasch 1979: 284, fig. 3b), *E.* (*C.*) *nigripilosus* (Sinitshenkova 1976: 89, fig. 28), *E.* (*C.*) *znojkoi* (Braasch, 1980: 172, 4b), and *E.* (*C.*) *zagrosicus* sp. nov. (Fig. 5B, K).

***Epeorus* (*Caucasiron*) *zagrosicus* Hrivniak & Sroka, sp. nov.**

http://zoobank.org/A49F6070-C918-4FA2-9287-D0B3D9BDBC01

Figures 5, 6

Type material. ***Holotype*:** female larva: IRAN, Lorestan Province, 4.5 km SW of Varayeneh village, Sarab-e Gamasiab River, 34°2'46.2"N, 048°22'32.6"E (locality no. 9); 1842 m a.s.l.; A. Staniczek, M. Pallmann, A. Abdoli, F. Nejat leg., 25.4.2017, SMNS_EPH_007520.

***Paratypes*:** 79 female larvae, 68 male larvae: same data as holotype, SMNS_EPH_007520. 6 female (2 mounted on slide), 5 male (2 mounted on slide) larvae: IRAN, Chaharmahal and Bakhtiari Province, Dimeh village, Chehme-Dimeh River, 32°30'11.6"N, 050°13'04.5"E (locality no. 45) ; 2220 m a.s.l.; A. Staniczek, M. Pallmann, R. J. Godunko, F. Nejat leg., 5.5.2017, SMNS_EPH_007707. DNA extracted from 3 females (code: IR32, stored in EtOH; codes: IR34 and IR35, mounted on slides) and 2 males (codes: IR33b and IR36, mounted on slides).

15 female (3 mounted on slide), 5 male larvae: IRAN, Kohgiluyeh and Boyer-Ahmad Province, 4 km E of Yasuj, Yasuj fall, 30°40'34.7"N, 051°37'35.6"E (locality no. 37); 2060 m a.s.l.; A. Staniczek, M. Pallmann, R. J. Godunko, F. Nejat leg., 4.5.2017, SMNS_EPH_007568. DNA extracted from 2 females (code: SP38, mounted on slide; code: IR33a, stored in EtOH) and 1 male (code: SP37, stored in EtOH).

2 female, 2 male larvae: IRAN, Chaharmahal and Bakhtiari Province, 5 km W of Chelgerd, Kouhrang River, 32°28'9.3"N, 050°5'26.2"E (locality no. 46); 2402 m a.s.l.; A. Staniczek, M. Pallmann, R. J. Godunko, F. Nejat leg., 5.5.2017, SMNS_EPH_007689.

The holotype and 100 paratypes are deposited in SMNS, 50 paratypes (including DNA extracted specimens) are deposited in IECA and 32 paratypes in MMTT_DOE.

Other material examined: 42 larvae: same data as holotype; young instars or damaged specimens.

1 male larva: IRAN, Chaharmahal and Bakhtiari Province, 4 km E. of Bajgiran, Dehno River, 31°54'26.2"N, 050°42'20.6"E (locality no. 50); 1721 m a.s.l.; A. Staniczek, M. Pallmann, R. J. Godunko, F. Nejat leg., 6.5.2017, SMNS_EPH_007606.

Etymology. The species name refers to its known records in the Zagros mountain range.

Localities and habitat preferences of larvae. Larvae were found in five streams of different size at high altitude, above 1700 m a.s.l. Three streams were strongly turbulent rivers with very coarse bed substrate flowing in high-mountain valleys (Fig. 10E). Larvae were found also in a shallow, slow-flowing brook with finer, gravel substrate flowing in the forest (locality near Yasuj fall, Fig. 10F), and in a small stream with moderate, slightly turbulent flow and stony bed substrate with fine gravel, silt, and macrophytes (Chehme-Dimeh River). The species was not found in streams that were polluted or seasonally drying out.

Description of larva. General colouration of larvae yellowish brown with dark brown maculation. Body length of mature larvae 13.5–14.5 mm (female), 10.0–11.0 mm (male). Length of cerci approximately 1.3× body length.

Head. Shape trapezoidal; anterior and lateral margin rounded, posterior margin slightly rounded or nearly straight (Fig. 5D, E). Anterior margin with shallow concavity medially.

Head dimensions of mature larvae: length 2.6–2.7 mm, width 3.6–4.0 mm (female); length 2.3–2.4 mm, width 3.3 mm (male). Head width/length ratio: 1.36–1.49 (both male and female).

Dorso-medial part with indistinct brown rectangular or oval macula, sometimes reduced to pair of stripes. Rounded maculae under lateral ocelli and blurred or triangular maculae near inner edges of compound eyes. Pair of pale stripes extending from lateral ocelli to lateral edges of head. Pair of maculae located along coronal suture. Compound eyes dark grey to black in female, brownish and basally blackish in male mature larva. Ocelli dark grey to black, basally paler. Antennae yellowish-brown, scapus and pedicellus darkened. Anterior margin densely covered with hair-like setae extending to lateral margins and directed medio-dorsally. Dorsal surface covered with fine hair-like setae and sparsely distributed stick-like setae. Sparse longer, fine, hair-like setae located posteriorly to eyes.

Mouthparts. Labrum (Fig. 6A) widened anteriorly, with anterior margin slightly rounded or nearly straight (in dorsal view). Lateral angles rounded (shape of labrum may vary among individual specimens). Dorsal surface (Fig. 6A, right half) sparsely covered with setae of different size; four longer, bristle-like setae located antero-medially and two antero-laterally. Epipharynx with longer, shortly plumose bristles situated along lateral to anterior margin (Fig. 6A, left half; range of setation figured as large black dots), and brush of fine hair-like setae medially (not figured). Posterior margin of

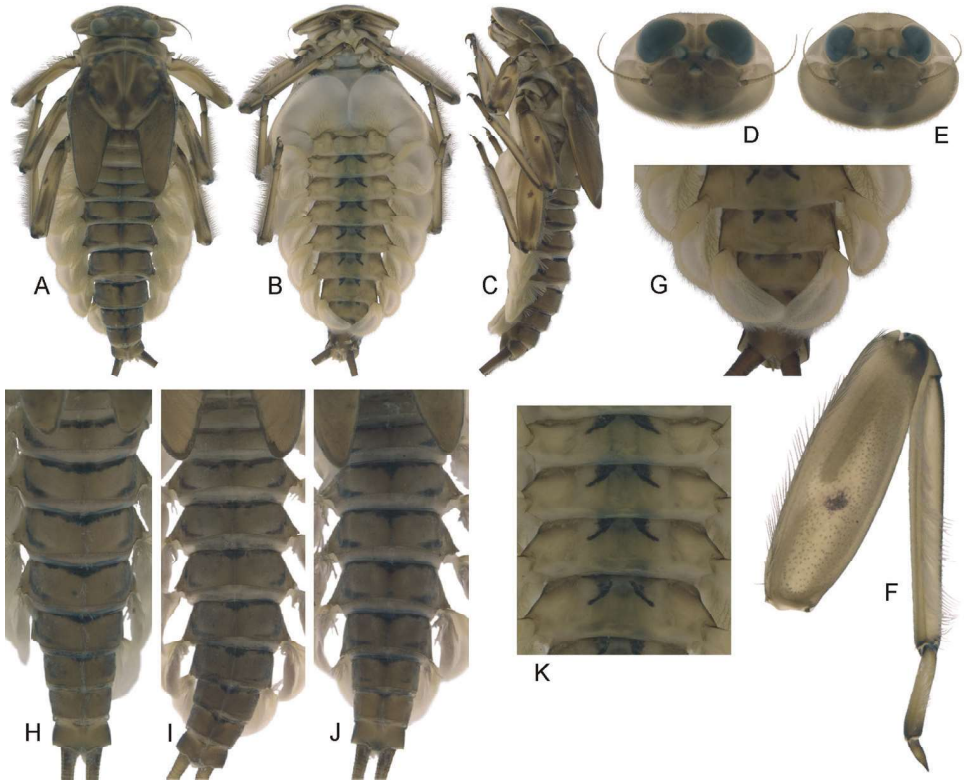


Figure 5. *Epeorus* (*Caucasiron*) *zagrosicus* sp. nov., larva: **A** habitus in dorsal view **B** habitus in ventral view **C** habitus in lateral view **D** head of male in dorsal view **E** head of female in dorsal view **F** middle leg in dorsal view **G** distal part of abdomen in ventral view **H–J** colouration of abdominal terga **K** colouration of abdominal sterna.

labrum irregularly concave; with group of 6–10 setae of various size located on ventral surface close to posterior margin. Outer incisors of both mandibles (Fig. 6B, C) with three apical teeth; outer tooth blunt in both mandibles. Inner incisor of left mandible with three apical teeth, right inner incisor bifurcated.

Thorax. Pronotum anteriorly narrowed, lateral edges nearly straight. Metanotum with slight postero-medial projection. Dorsal surface covered with fine hair-like setae (as on abdominal terga and head); sparse longer hair-like setae along pro, meso- and metanotal suture.

Legs. Colour pattern of femora as in Fig. 5F. Femora with rounded medial hypodermal femur spot. Patella-tibial suture darkened; tarsi proximally and distally darkened. Coxal projections of fore- and hind legs pointed or bluntly pointed; of middle legs blunt. Trochanteres with spatulate setae as on dorsal surface of femora (Fig. 6D). Tibiae of forelegs 1.20–1.31× femur length, tibiae of middle legs 1.06–1.14× femur length, and tibiae of hind legs 0.90–1.04× femur length. Tarsi of all legs 0.25–0.34× tibia length. Dorsal surface of femora covered by elongated and sporadically short

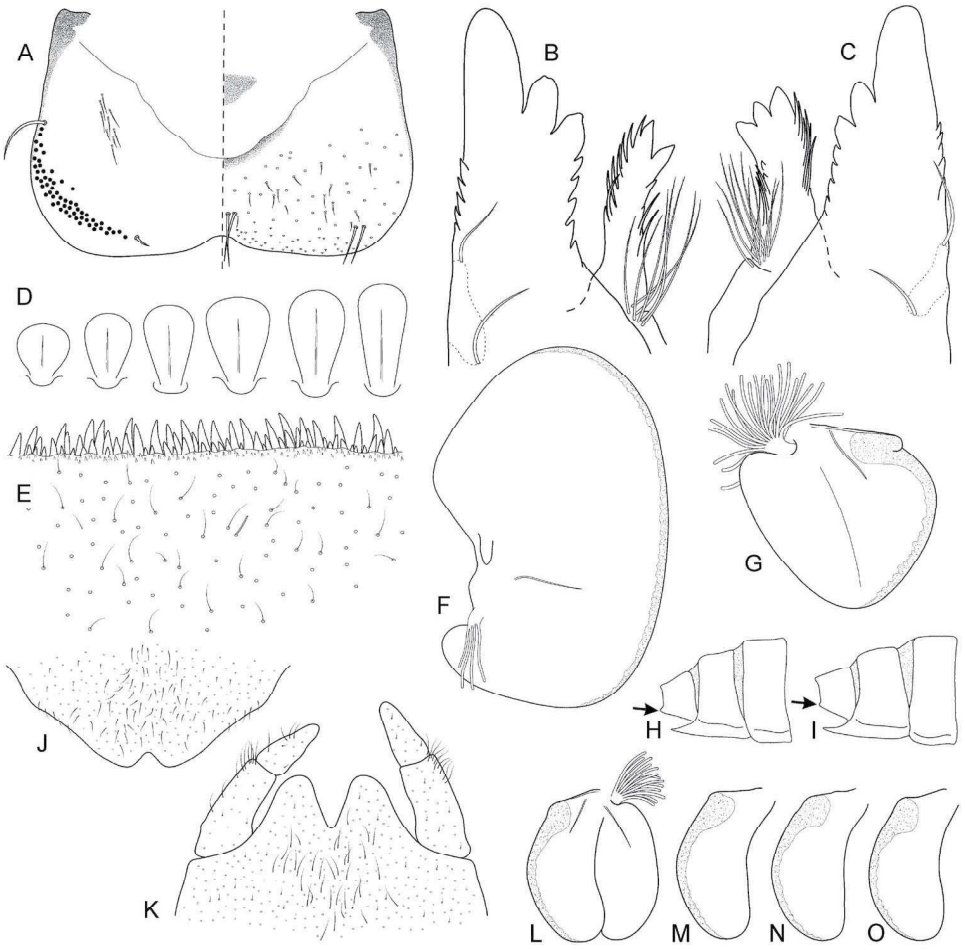


Figure 6. *Epeorus* (*Caucasiron*) *zagrosicus* sp. nov., larva: **A** labrum (right half in dorsal view, left half in ventral view) **B** incisors of right mandible in ventral view **C** incisors of left mandible in ventral view (both flattened on slide) **D** setae on dorsal surface of femora **E** surface and posterior margin of abdominal tergum VII **F** gill I **G** gill III **H–I** abdominal segments VIII–X **J** sternum IX, female **K** sternum IX, male **L** gill VII (flattened on slide) **M–O** gill VII (in natural position from ventral view), variability in shape.

rounded spatulate setae (Fig. 6D); hair-like setae and sparsely distributed stick-like setae. Anterior margin of femora with short, pointed and/or bluntly pointed spine-like setae; posterior margin with row of long blade-like setae and sparse row of bluntly pointed spine-like setae. Dorsal margin of tibiae and tarsi with row of long setae; ventral margin of both with irregular row of spine-like setae accumulated distally. Tarsal claws with two or three denticles.

Abdominal terga. Colour pattern of abdominal terga includes transversal stripe along anterior margin of terga I–IX (X) medially extending to triangular, short rectan-

gular or stripe-like medial macula on terga (III) IV–IX (transversal stripe sometimes not distinctly extended, for variability see Fig. 5A, H–J). Pair of sigilla sometimes coloured, in form of short stripes or spots located antero-laterally to medial macula. Tergum X without distinct maculation. Lateral margins of abdomen with oblique maculae on terga I–IX extending to dorso-posterior margin. Denticles along posterior margin on terga of various size, irregular and pointed, sometimes curved (Fig. 6E). Surface of terga covered with hair-like setae and sparsely with stick-like setae. Supra-tergalial projections short and blunt. Tergum X with more or less developed postero-lateral projections (Fig. 6H, I, arrows). Longitudinal row of hair-like setae medially along abdominal terga present.

Abdominal sterna. Yellowish, with distinct colouration pattern consisting of anteriorly widened pair of stripes (medio-anterior sigilla) on terga II–VIII (Fig. 5B, G, K). Sometimes only oblique stripes are present, without anterior widening (especially on sterna VI–VIII). Nerve ganglia occasionally darkened. Intensity of colouration varies among individuals. Sternum IX with V-shaped medial emargination; surface covered by irregularly distributed short hair-like setae, and medially accumulated longer hair-like setae (Fig. 6J, K).

Gills. Dorsal surface of gill plate I yellowish; of gill plates II–VII greyish on anterior half and brownish to reddish on posterior half. Ventral margin of all gill plates yellowish. Projection of gill plate III well developed (Fig. 6G). Shape of gill plate VII (in natural position from ventral view) varies from narrow to relatively wide (Figs 5G, 6M–O). Gill filaments reaching to 0.4–0.5× length of respective plate, filaments of gill VII to 0.24–0.30× (in late-instar larvae).

Cerci. Brownish, basally darkened.

Subimago, imago and eggs. Unknown

Morphological diagnostics of larvae. The main larval diagnostic characters of *E.* (*C.*) *zagrosicus* sp. nov. are as follows: (i) colour pattern of abdominal sterna (Fig. 5B, G, K) and abdominal terga (Fig. 5A, H–J), (ii) presence of postero-lateral projections on tergum X (Fig. 6H, I), (iii) presence of hypodermal medial femur spot (Fig. 5F), and (iv) fine hair-like setae on surface of abdominal terga (Fig. 6E).

Affinities. Based on the colour pattern of abdominal sterna, *E.* (*C.*) *zagrosicus* sp. nov. is most similar to *E.* (*C.*) *caucasicus* and *E.* (*C.*) *iranicus*. Both latter species possess pigmented medio-anterior sigilla forming a pair of oblique stripes on abdominal sterna II–VIII (e.g., Braasch 1979: 284, fig. 3b), and a medial hypodermal femur spot. However, *E.* (*C.*) *zagrosicus* sp. nov. differs by the distinct widening at the anterior margin of medio-anterior sigilla of abdominal sterna. If the sternal colour pattern is not fully developed (sporadically only stripes are present on all or several sterna), *E.* (*C.*) *zagrosicus* sp. nov. is distinguishable by the colour pattern of abdominal terga (Fig. 5A, H–J), which is different in *E.* (*C.*) *caucasicus* (Braasch 1979: 284, fig. 3a) and *E.* (*C.*) *iranicus* (Braasch and Soldán 1979: 264, fig. 12). In *E.* (*C.*) *zagrosicus* sp. nov., the postero-lateral projections on the tergum X are well-developed, whereas they are not significantly pronounced in either of the two species mentioned above (only small projections may be sporadically present).

Distinct postero-lateral projections on the tergum X are characteristic for *E.* (*C.*) *magnus*, *E.* (*C.*) *nigripilosus*, and *E.* (*C.*) *alborzicus* sp. nov. Small projections are also sporadically present in *E.* (*C.*) *znojkoi*. *E.* (*C.*) *magnus* can be easily distinguished from *E.* (*C.*) *zagrosicus* sp. nov. by the absence of colour pattern of abdominal sterna, the absence of a medial hypodermal femur spot, and setation on dorsal margin of labrum (numerous thickened bristle-like setae, Hrivniak et al., in prep.). *E.* (*C.*) *nigripilosus* and *E.* (*C.*) *alborzicus* sp. nov. differ by a typical colouration pattern of abdominal sterna (Sinitshenkova 1976: 89, fig. 28 for *E.* (*C.*) *nigripilosus* and Fig. 1B, G, K–M for *E.* (*C.*) *alborzicus* sp. nov.). *E.* (*C.*) *znojkoi* can be distinguished from *E.* (*C.*) *zagrosicus* sp. nov. by the colour pattern of abdominal terga and characteristic reddish colouration of abdominal sterna (Braasch 1980: 172, fig. 4b, c).

The presence of a medial hypodermal femur spot makes *E.* (*C.*) *zagrosicus* sp. nov. slightly similar to *E.* (*C.*) *turcicus* and *E.* (*C.*) *alborzicus* sp. nov. However, the presence of the characteristic pattern of abdominal sterna in *E.* (*C.*) *alborzicus* sp. nov. (Fig. 1B, G, K–M), and the absence of colouration pattern of abdominal sterna in *E.* (*C.*) *turcicus* reliably differentiate both species from *E.* (*C.*) *zagrosicus* sp. nov. Additionally, *E.* (*C.*) *turcicus* differs by the characteristic colour pattern of abdominal terga (Hrivniak et al. 2019: 61, fig. 1).

The other five species distributed in the Caucasus, namely *E.* (*C.*) *sinitshenkovae*, *E.* (*C.*) *alpestris*, *E.* (*C.*) *bicolliculatus*, *E.* (*C.*) *longimaculatus*, and *E.* (*C.*) *soldani*, do not share any important diagnostic characters with *E.* (*C.*) *zagrosicus* sp. nov. Nevertheless, *E.* (*C.*) *sinitshenkovae* and *E.* (*C.*) *alpestris* can be separated from *E.* (*C.*) *zagrosicus* sp. nov. by the absence of a medial hypodermal femur spot, overall colouration of the dorsal surface of femora (*E.* (*C.*) *sinitshenkovae*, Braasch and Zimmerman 1979: 106, fig. 10), and the different colouration of abdominal sterna (*E.* (*C.*) *alpestris*, Braasch 1979: 284, fig. 1d). Fine hair-like setae on the dorsal surface of abdominal terga clearly distinguish *E.* (*C.*) *zagrosicus* sp. nov. from *E.* (*C.*) *bicolliculatus*, *E.* (*C.*) *longimaculatus*, and *E.* (*C.*) *soldani*. All three species are characteristic by the presence of flattened setae on abdominal terga (Hrivniak et al. 2017: 359, figs 23–25).

Results from molecular species delimitation

The GMYC model provided significantly better fit to COI gene tree than the null model expecting uniform coalescent branching rates across entire tree (likelihood ratio test = 3.671927e-06***). The GMYC estimated 15 species (CI=13–19) consisting of 14 ML clusters and one singleton (CI = 12–16). All three newly described species were confirmed, and the overall number of delimited GMYC species corresponded well to morphologically defined species within *Caucasiron* (Fig. 7C). Monophyly of all species clusters were highly supported (PP = 1).

The ABGD analysis of the COI distance matrix recognized 15 stable groups within initial partition. All groups corresponded well to morphologically defined species and were congruent with GMYC analysis. All three newly proposed species were recognized as distinct groups (Fig. 7C). The mean pairwise genetic K2P distances between

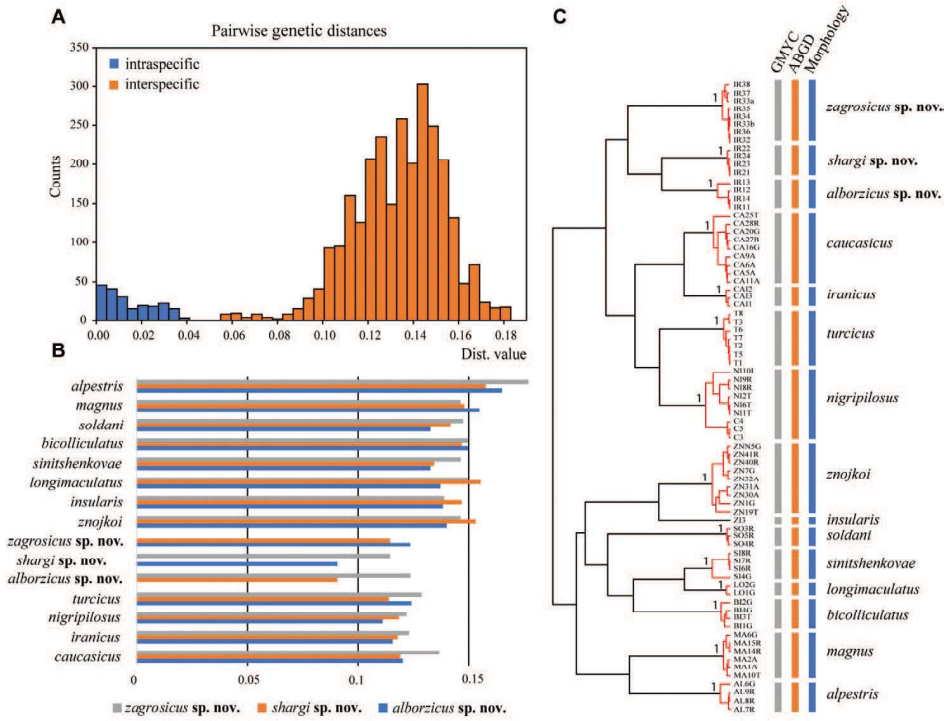


Figure 7. Results of the molecular species delimitation: **A** distribution of K2P pairwise genetic distances **B** mean pairwise genetic distances between new species and all Caucasian *Caucasiron* species known **C** COI gene tree with the results of molecular species delimitation analyses and morphology (node supports for species are indicated).

all *Caucasiron* species, including newly described, ranged between 6.71% (*E.* (*C.*) *caucasicus* / *E.* (*C.*) *iranicus*) and 17.68% (*E.* (*C.*) *alpestris* / *E.* (*C.*) *zagrosicus* sp. nov.). Maximum intraspecific and minimum interspecific distances were observed in *E.* (*C.*) *nigripilosus* (4.12%; Iran/Cyprus) and *E.* (*C.*) *caucasicus* / *E.* (*C.*) *iranicus* (5.48%), respectively. Overall distribution of K2P pairwise genetic distances is figured on Fig. 7A. Mean intraspecific genetic distances for all new species relative to individual *Caucasiron* species are shown in Fig. 7B.

Distribution of *Caucasiron* in Iran

Specimens of the genus *Epeorus* were found in 68 localities of all 254 localities investigated by us in 2016–2018 (Fig. 8) and in seven additional localities investigated by others (Braasch and Soldán 1979; Mousavi and Hakobyan 2017) (Table 1). Their occurrence was limited to streams with good water quality at altitudes between -4 and 2440 m a.s.l. (Table 1). They were neither found in polluted streams of agricultural and urban areas, nor in seasonally drying streams. Most of the species and



Figure 8. The map showing the occurrence of *Epeorus* (*Caucasiron*) spp. at all localities investigated in Iran. Colour of symbols shows the occurrence of species: green – *Epeorus* (*Caucasiron*) *zagrosicus* sp. nov., dark blue – *Epeorus* (*Epeorus*) *zaitzevi*, and violet – all other *Epeorus* (*Caucasiron*) species. Black symbols show collection points where no species of *Epeorus* was found. The letter H shows the locality of the respective holotype.

records were found in the Alborz in northern Iran (Fig. 9). These mountains host five species of the subgenus *Caucasiron* and one species of the subgenus *Epeorus* (*E. zaitzevi*). Except for the newly described species, **E.** (*C.*) *nigripilosus* found in five localities in the Alborz is new for Iran (its genetic data from the Alborz were used in phylogeographical analyses in Hrivniak et al. 2020). It is a widely distributed species ranging from Cyprus and Turkey to Georgia, Russia and Iraq (Sinitshenkova, 1976; Braasch 1979; Al-Zubaidi et al. 1987; Salur et al. 2016; Gabelashvili et al. 2018; Hrivniak et al. 2020). The identification of *E.* (*C.*) *nigripilosus* was confirmed by both morphological characters and molecular delimitation. The specimen from the

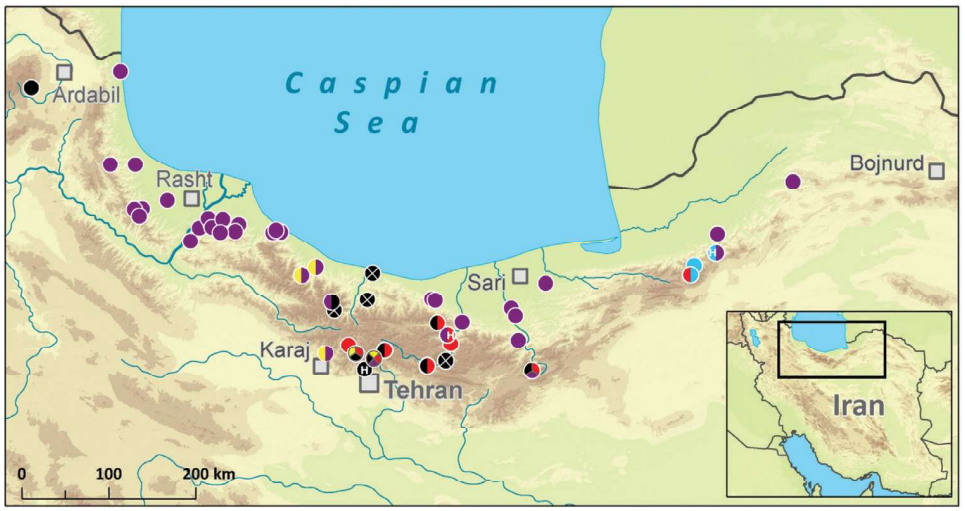


Figure 9. Distribution of *Epeorus* (*Caucasiron*) species in northern Iran. Colour of symbols shows the occurrence of species: red – *E.* (*C.*) *alborzicus* sp. nov., light blue – *E.* (*C.*) *shargi* sp. nov., violet – *E.* (*C.*) cf. *znojkoi*, black – *E.* (*C.*) *iranicus*, yellow – *E.* (*C.*) *nigripilosus*. The letter H shows the localities of holotypes. Black symbols with white cross show unrevised records of *E.* (*C.*) *iranicus*.

Alborz (coded as NI10I in Fig. 7C) clustered within the clade containing conspecific individuals from Russia and Turkey in the analysis of COI. It differed from these conspecifics in 2.8–3.6 % of K2P distance.

The most common *Caucasiron* species in the Alborz is *E.* (*C.*) cf. *znojkoi* distributed from the Talysh Mts. in the west to the Golestan NP in the east (Fig. 9). However, our study dealing with the molecular diversity of *Caucasiron* species in the Caucasus and adjacent regions (Hrivniak et al. 2020) indicated that *E.* (*C.*) *znojkoi* might represent a complex of cryptic species (only a subset of sequences included in the present study). The lineage *Caucasiron* sp. 4 (see Hrivniak et al. 2020) occurring in Iran (here called *E.* (*C.*) cf. *znojkoi*) differed from the Central Caucasian lineage. The delimitation of species within *E.* (*C.*) *znojkoi* s. l. requires further study. Nevertheless, the morphotype of *E.* (*C.*) cf. *znojkoi* has a wide geographical and ecological range, occurring at altitudes from -4 to 2290 m a.s.l. in northern Iran (Table 1). It was often found in shallow warm streams with good water quality flowing in humid broadleaved forests in the Caspian Sea lowland; approximately half of its localities was below 350 m a.s.l. At higher altitude, it may co-occur with *E.* (*C.*) *alborzicus* sp. nov., *E.* (*C.*) *shargi* sp. nov., *E.* (*C.*) *nigripilosus*, and *E.* (*C.*) *iranicus* (Fig. 9).

Three *Caucasiron* species, *E.* (*C.*) *iranicus*, *E.* (*C.*) *alborzicus* sp. nov., and *E.* (*C.*) *shargi* sp. nov., were described from the Alborz and are so far only known from there. *E.* (*C.*) *iranicus* is reliably reported from 12 localities, eight of them above 2000 m a.s.l. These include the Sabalan Mt. slopes in the western Alborz and the central Alborz, where it can co-occur with *E.* (*C.*) *alborzicus* sp. nov. (Fig. 9). It was found only in very cold streams fed by glaciers and melting snow from the highest mountains, with

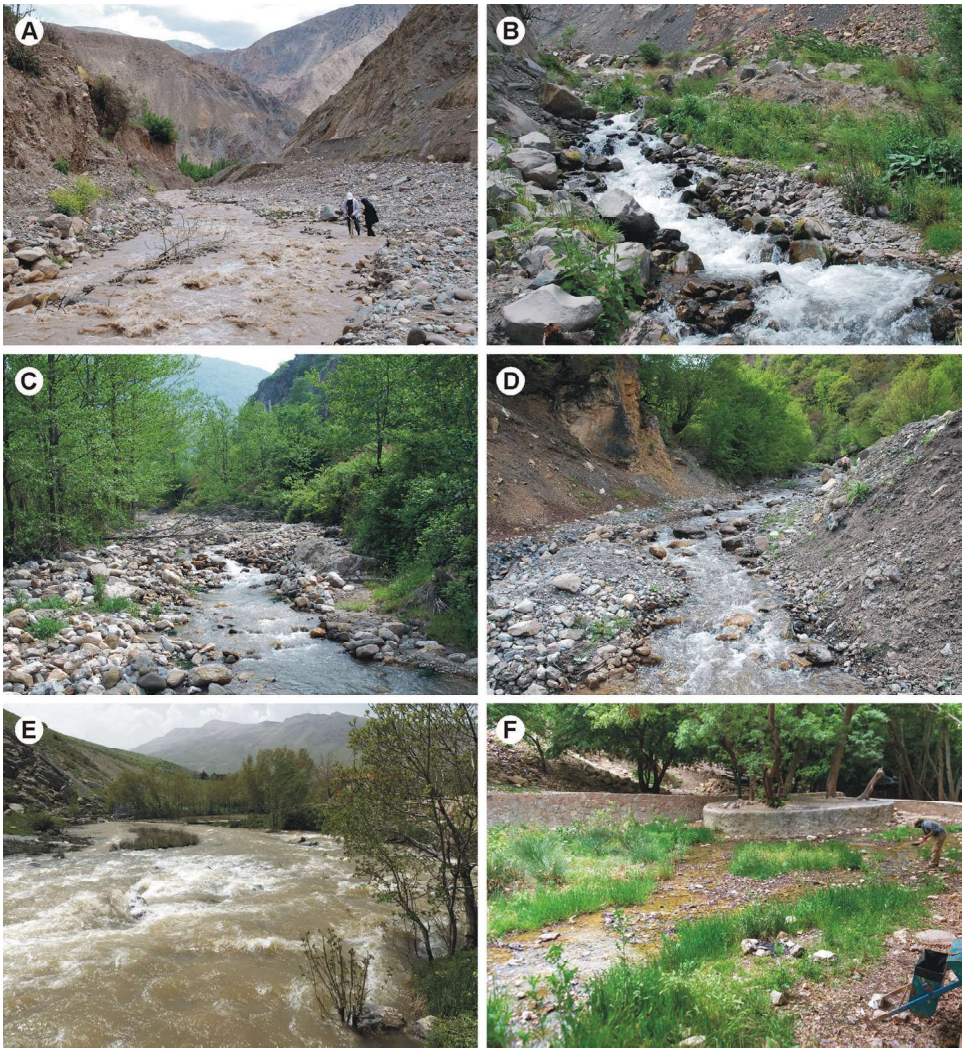


Figure 10. Photos of the localities of the new *Epeorus* (*Caucasiron*) species described herein: **A** unnamed brook near Panjab village – type locality of *E.* (*C.*) *alborzicus* sp. nov. **B** unnamed brook near Kahrud-e Bala village – locality of *E.* (*C.*) *alborzicus* sp. nov. **C** unnamed river near Shirinabad village – type locality of *E.* (*C.*) *shargi* sp. nov. **D** unnamed brook near Chah-e Ja village – locality of *E.* (*C.*) *shargi* sp. nov. **E** Gamasiab River near Varayeneh village – type locality of *E.* (*C.*) *zagrosicus* sp. nov. **F** Yasuj fall near Yasuj village – locality of *E.* (*C.*) *zagrosicus* sp. nov.

Alborz (coded as NI10I in Fig. 7C) clustered within the clade containing conspecific individuals from Russia and Turkey in the analysis of COI. It differed from these conspecifics in 2.8–3.6 % of K2P distance.

The most common *Caucasiron* species in the Alborz is *E.* (*C.*) cf. *znojkoi* distributed from the Talysh Mts. in the west to the Golestan NP in the east (Fig. 9). However,

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The most common *Caucasiron* species in the Alborz is *E.* (*C.*) cf. *znojkoi* distributed from the Talysh Mts. in the west to the Golestan NP in the east (Fig. 9). However, our study dealing with the molecular diversity of *Caucasiron* species in the Caucasus and adjacent regions (Hrivniak et al. 2020) indicated that *E.* (*C.*) *znojkoi* might represent a complex of cryptic species (only a subset of sequences included in the present study). The lineage *Caucasiron* sp. 4 (see Hrivniak et al. 2020) occurring in Iran (here called *E.* (*C.*) cf. *znojkoi*) differed from the Central Caucasian lineage. The delimitation of species within *E.* (*C.*) *znojkoi* s. l. requires further study. Nevertheless, the morphotype of *E.* (*C.*) cf. *znojkoi* has a wide geographical and ecological range, occurring at altitudes from -4 to 2290 m a.s.l. in northern Iran (Table 1). It was often found in shallow warm streams with good water quality flowing in humid broadleaved forests in the Caspian Sea lowland; approximately half of its localities was below 350 m a.s.l. At higher altitude, it can co-occur with *E.* (*C.*) *alborzicus* sp. nov., *E.* (*C.*) *shargi* sp. nov., *E.* (*C.*) *nigripilosus*, and *E.* (*C.*) *iranicus* (Fig. 9).

Three *Caucasiron* species, *E.* (*C.*) *iranicus*, *E.* (*C.*) *alborzicus* sp. nov., and *E.* (*C.*) *shargi* sp. nov., were described from the Alborz and are so far only known from there. *E.* (*C.*) *iranicus* is reliably reported from 12 localities, eight of them above 2000 m a.s.l. These include the Sabalan Mt. slopes in the western Alborz and the central Alborz, where it can co-occur with *E.* (*C.*) *alborzicus* sp. nov. (Fig. 9). It was found only in very cold streams fed by glaciers and melting snow from the highest mountains, with very rapid flow and strongly turbulent riffle sections. Four records of *E.* (*C.*) *iranicus* published by Mousavi and Hakobyan (2017) should be revised, because they included a wide range of altitude (20–2120 m a.s.l.) and were very close to our records of *E.* (*C.*) *alborzicus* sp. nov. and *E.* (*C.*) cf. *znojkoi*. The two new species from the Alborz seem to differ in habitat requirements. *Epeorus* (*C.*) *alborzicus* sp. nov. was only found in higher altitudes. These were all treeless localities in montane valleys with harsh climatic conditions, whereas *E.* (*C.*) *shargi* sp. nov. was found well below in submontane streams that were flowing in forests. The latter species was recorded only in the eastern Alborz near Gorgan (Fig. 9).

Other streams investigated in Iranian mountain ranges were dominated by Baetidae, and Heptageniidae were generally only scattered there. Larvae of *E.* (*C.*) *zagrosicus* sp. nov. and *E.* (*E.*) *zaitzevi* were found only in five and seven localities respectively, relatively distant to each other in the Zagros (Fig. 8). However, most of the streams explored in the Zagros were polluted or seasonally drying out due to the water storage in dams and water abstraction for irrigation of surrounding fields. Moreover, streams at higher altitude with presumably better water quality were almost inaccessible for us in April and May during our field trips. As *E.* (*C.*) *zagrosicus* sp. nov. was mostly found in natural streams in high-mountain valleys only with sparse villages, we expect that its distribution is limited to clear and cold mountain streams. However, a more detailed investigation of mayflies in high-mountain streams in Iran is needed.

Table 1. List of records of the *Caucasiron* species found in Iran (three new species are not included). Abbreviations: RT – right tributary; LT – left tributary; JB – Jindřiška Bojková; TS – Tomáš Soldán; IN – Javid Imanpour Namin; SB – Samereh Bagheri; AHS – Arnold H. Staniczek; MP – Milan Pallmann; RJG – Roman J. Godunko; FN – Farshad Nejat; AA – Ashgar Abdoli; HV – H. Valikhani; PT – P. Taban. Number of specimens includes larvae.

Species	Province	Stream	Locality	Nearest settlement	Altitude	Latitude (N) / Longitude (E)	Sampling date	Collector/reference	Number of specimens
E. (*C.*) *iranicus*	Alborz	Karaj R., Shahrestanak branch	SE of Shahrestanak	Chavar Chalun	2220	35°57'45.8"N, 051°21'59.7"E	1.9.2016	AA, HV, PT	4
	Ardabil	unnamed brook	in Alvaresi (below Alvares ski area)	Sarein	2235	38°09'38.0"N, 047°56'21.0"E	17.5.2016	JB, TS, IN; Bojková et al. 2018	2
	Mazandaran	Koshk Sara R.	in Kosh Sara	Chalus	18	36°37'57.7"N, 051°28'04.4"E	25.9.2013	Mousavi and Hakobyan 2017	2
	Mazandaran	Firuz Abad R.	near Dasht Nazir	Marzan Abad	929	36°24'33.8"N, 051°24'42.5"E	11.9.2014	Mousavi and Hakobyan 2017	80
	Mazandaran	Haraz R.	in Gazanak	Gazanak	1590	35°54'08.3"N, 052°13'30.0"E	10.7.2013	Mousavi and Hakobyan 2017	8
	Mazandaran	Dalir R.	above Dalir	Marzan Abad	2126	36°19'23.2"N, 051°04'27.5"E	26.7.2014	Mousavi and Hakobyan 2017	174
	Mazandaran	Lasem R.	E of Polour	Polour	2180	35°50'04.1"N, 052°05'07.6"E	14.5.2017	AHS, MP, FN	1
	Mazandaran	RT of Sardab Rud	SW of Kelardasht	Kelardasht	2020	36°26'06.5"N, 051°03'52.6"E	8.3.2018	SB	85
	Mazandaran	LT of Sardab Rud	NW of Vandarbon	Kelardasht	2250	36°25'53.7"N, 051°01'59.1"E	9.3.2018	SB	63
	Mazandaran	Sardab Rud	S of Vandarbon	Kelardasht	2290	36°25'23.0"N, 051°02'12.4"E	10.3.2018	SB	5
	Tehran	Darban valley (type locality)	N of Tehran	Tehran	2100	35°50'24.0"N, 051°25'19.9"E	18.7.1970	Braasch and Soldán 1979	14
	Tehran	Lalan R.	above Zayegan	Fasham	2290	35°58'39.2"N, 051°34'56.5"E	8.5.2017	AHS, MP, RJG, FN	158
	Tehran	Lalan R.	in Lalan	Lalan	2440	35°59'50.3"N, 051°34'51.0"E	8.5.2017	AHS, MP, RJG, FN	87
	Tehran	Ahar R.	near Igol	Fasham	2020	35°55'11.2"N, 051°28'51.3"E	8.5.2017	AHS, MP, RJG, FN	1
	Tehran	Shahrestanak R.	NW of Shahrestanak	Asara	2100	35°59'01.2"N, 051°19'09.6"E	10.5.2017	AHS, MP, FN	1
E. (*C.*) *nigripilosus*	Alborz	Kordan R.	N of Kordan	Kordan	1430	35°57'15.6"N, 050°50'25.3"E	10.5.2017	AHS, MP, FN	4
	Mazandaran	RT of Dohezar R.	N of Holu Kaleh	Tonkaboon	880	36°37'37.5"N, 050°44'30.2"E	16.6.2018	SB	1
	Mazandaran	Dohezar R.	SW of Parde Sar	Tonkaboon	450	36°40'07.0"N, 050°49'20.0"E	16.6.2018	SB	1
	Tehran	Ahar R.	near Igol	Fasham	2020	35°55'11.2"N, 051°28'51.3"E	8.5.2017	AHS, MP, RJG, FN	5
	Tehran	Shahrestanak R.	NW of Shahrestanak	Asara	2100	35°59'01.2"N, 051°19'09.6"E	10.5.2017	AHS, MP, FN	1

Species	Province	Stream	Locality	Nearest settlement	Altitude	Latitude (N) / Longitude (E)	Sampling date	Collector/reference	Number of specimens
E. (*C.*) cf. *znojkoi*	Alborz	Kordan R.	N of Kordan	Kordan	1430	35°57'15.6"N, 050°50'25.3"E	10.5.2017	AHS, MP, FN	4
	Gilan	RT of Khara Rud	S of Paein Khara Rud (S of Pashaki)	Sangar	210	37°02'29.0"N, 049°47'52.0"E	12.5.2016	JB, TS, IN	71
	Gilan	left fork of Khara Rud	in Madarsara (S of Pashaki)	Sangar	105	37°04'12.0"N, 049°46'36.0"E	12.5.2016	JB, TS, IN	27
	Gilan	right fork of Khara Rud	in Golestansara (S of Pashaki)	Sangar	201	37°02'20.0"N, 049°47'27.0"E	12.5.2016	JB, TS, IN	2
	Gilan	Zilaki River (RT of Sefid Rud)	in Mush Bijar (E of Shahr-e Bijar)	Shahr-e Bijar	120	37°00'28.0"N, 049°40'24.0"E	13.5.2016	JB, TS, IN	1
	Gilan	Sefidab (RT of Siah Rud)	in Divarsh (NE of Shirkuh)	Tutkabon	280	36°53'59.0"N, 049°35'06.0"E	13.5.2016	JB, TS, IN	149
	Gilan	Sangdeh (LT of Shafa Rud)	W of Punel	Punel	240	37°31'47.0"N, 049°00'52.0"E	15.5.2016	JB, TS, IN	10
	Gilan	Shafa Rud	W of Punel	Punel	240	37°31'47.0"N, 049°00'52.0"E	15.5.2016	JB, TS, IN	6
	Gilan	LT of Shafa Rud	NW of Sangdeh	Sangdeh	1345	37°31'46.0"N, 048°45'19.0"E	15.5.2016	JB, TS, IN	50
	Gilan	Shakhzar R.	NE of Fuman	Fuman	6	37°14'13.0"N, 049°20'43.0"E	15.5.2016	JB, TS, IN	1
	Gilan	LT of Bala Rud	S of Siahkal	Siahkal	490	37°00'31.0"N, 049°51'51.0"E	16.5.2016	JB, TS, IN	16
	Gilan	Lunak waterfalls	S of Siahkal	Siahkal	510	37°00'31.0"N, 049°51'49.0"E	16.5.2016	JB, TS, IN	5
	Gilan	Shamrud (RT of Sefid Rud)	S of Tushi (S of Siahkal)	Siahkal	315	37°03'00.0"N, 049°53'54.0"E	16.5.2016	JB, TS, IN	37
	Gilan	Chelavand R.	W of Chelvand	Lavandvil	-4	38°17'20.0"N, 048°51'35.0"E	19.5.2016	JB, TS, IN	2
	Gilan	unnamed brook	N of Chaldasht	Amlash	1255	36°59'33.0"N, 050°05'19.0"E	21.5.2016	JB, TS, IN	3
	Gilan	RT of Shalman Rud	in Bolurdekan	Amlash	345	37°01'09.0"N, 050°03'51.0"E	21.5.2016	JB, TS, IN	2
	Gilan	LT of Ghale Rudkhan	NE of Masuleh	Fuman	885	37°09'47.0"N, 049°00'17.0"E	22.5.2016	JB, TS, IN	66
	Gilan	RT of Ghale Rudkhan	NE of Masuleh	Fuman	705	37°09'42.0"N, 049°01'17.0"E	22.5.2016	JB, TS, IN	6
	Gilan	Ghale Rudkhan R.	E of Masuleh	Fuman	370	37°10'02.0"N, 049°05'03.0"E	22.5.2016	JB, TS, IN	4
	Golestan	unnamed river	in Shirinabad	Aliabad-e Katul	740	36°48'01.0"N, 055°01'05.0"E	27.4.2018	JB, TS, IN	50
	Golestan	Shirabad waterfalls	above Shirabad	Shirabad	140	36°57'33.0"N, 055°01'57.0"E	28.4.2018	JB, TS, IN	48
	Golestan	RT of Madarsu R.	E of Tangrah	Tangrah	495	37°23'27.0"N, 055°48'51.0"E	30.4.2018	JB, TS, IN	1

Species	Province	Stream	Locality	Nearest settlement	Altitude	Latitude (N) / Longitude (E)	Sampling date	Collector/reference	Number of specimens
E. (*C.*) cf. *znojkoi*	Mazandaran	Shirinrud	S of Part Kola	Farim	770	36°09'02.5"N, 053°20'58.1"E	11.5.2017	AHS, MP, FN	203
	Mazandaran	trib. Kashpel R.	SW of Chamestan	Chamestan	400	36°25'31.4"N, 052°03'38.4"E	13.5.2017	AHS, MP, FN	17
	Mazandaran	Chelav R.	N of Pasha Kola	Pasha Kola	820	36°12'24.7"N, 052°25'51.9"E	14.5.2017	AHS, MP, FN	63
	Mazandaran	Chelav R.	NW of Pasha Kola	Pasha Kola	570	36°13'28.9"N, 052°23'35.4"E	14.5.2017	AHS, MP, FN	2
	Mazandaran	Baladeh R.	W of Razan	Razan	1360	36°11'39.6"N, 052°08'34.6"E	14.5.2017	AHS, MP, FN	9
	Mazandaran	Chai Bagh R.	E of Andar Koli	Ghaem Shahr	200	36°20'30.0"N, 052°54'03.0"E	8.5.2018	JB, TS, IN, SB	27
	Mazandaran	RT of Haraz R.	NW of Pasha Kola	Amol	570	36°13'27.0"N, 052°23'36.0"E	9.5.2018	JB, TS, IN, SB	5
	Mazandaran	LT of Haraz R.	in Panjab	Amol	955	36°05'52.0"N, 052°15'15.0"E	9.5.2018	JB, TS, IN, SB	1
	Mazandaran	unnamed brook	above Darab Kola	Neka	135	36°33'10.0"N, 053°15'32.0"E	10.5.2018	JB, TS, SB	2
	Mazandaran	unnamed brook	in Momey Khal	Ghaem Shahr	760	36°04'24.0"N, 052°58'19.0"E	11.5.2018	JB, TS, SB	1
	Mazandaran	Palang Darreh R.	SE of Shirgah	Shirgah	320	36°16'31.0"N, 052°56'54.0"E	11.5.2018	JB, TS, SB	2
	Mazandaran	LT of Palang Darreh R.	SE of Shirgah	Shirgah	345	36°16'30.0"N, 052°56'51.0"E	11.5.2018	JB, TS, SB	1
	Mazandaran	RT of Sardab Rud	SW of Kelardasht	Kelardasht	2020	36°26'06.5"N, 051°03'52.6"E	9.3.2018	SB	9
	Mazandaran	Sardab Rud	S of Vandarbon	Kelardasht	2290	36°25'23.0"N, 051°02'12.4"E	16.6.2018	SB	57
	Mazandaran	RT of Dohezar R.	N Holu Kaleh	Tonkabon	880	36°37'37.5"N, 050°44'30.2"E	16.6.2018	SB	83
	Mazandaran	Dohezar R.	SW Parde Sar	Tonkabon	450	36°40'07.0"N, 050°49'20.0"E	16.6.2018	SB	46
	Mazandaran	RT of Sehezar R.	S Parde Sar	Tonkabon	570	36°38'41.5"N, 050°50'11.1"E	16.6.2018	SB	20
	Mazandaran	Sehezar R.	S Parde Sar	Tonkabon	540	36°39'01.0"N, 050°50'00.0"E	24.8.2018	SB	21
	Mazandaran	Lavij Rud	SE of Kiakola	Noor	820	36°21'33.1"N, 052°03'11.0"E	24.8.2018	SB	8
	Mazandaran	Vaz Rud	E of Vaz Oliya	Noor	1140	36°19'08.0"N, 052°08'24.1"E	15.6.2018	SB	33
	Mazandaran	Safarud	SW of Ramsar	Ramsar	490	36°52'55.8"N, 050°33'56.1"E	15.6.2018	SB	1
	Mazandaran	LT of Safarud	SW of Ramsar	Ramsar	610	36°53'24.4"N, 050°33'56.1"E	15.6.2018	SB	2
	Mazandaran	LT of Safarud	SW of Ramsar	Ramsar	330	36°54'06.1"N, 050°35'12.1"E	15.6.2018	SB	11
	Mazandaran	Chalak Rud	SW of Galeshmahalleh	Ramsar	100	36°49'13.0"N, 050°43'23.8"E	15.6.2018	SB	3
	Mazandaran	LT of Chalak Rud	NW of Talesh Sara	Ramsar	180	36°50'46.9"N, 050°40'25.9"E	8.3.2018	SB	8
	Tehran	Ahar R.	near Igol	Fasham	2020	35°55'11.2"N, 051°28'51.3"E	8.5.2017	AHS, MP, RJG, FN	4

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Chapter III

Identification guide to larvae of Caucasian *Epeorus* (*Caucasiron*) (Ephemeroptera: Heptageniidae)

Identification guide to larvae of Caucasian *Epeorus* (*Caucasiron*) (Ephemeroptera: Heptageniidae)

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Abstract
The Caucasus and adjacent areas are inhabited by fifteen species of mayflies of the genus *Epeorus*, subgenus *Caucasiron* Kluge, 1997 (Heptageniidae). This identification guide aims to facilitate an accurate species identification of their larvae and sum up all available information on their taxonomy and distribution. We provide an identification key and describe and illustrate the important diagnostic characters of all species. The larva of *E.* (*C.*) *insularis* (Braasch, 1983) is described for the first time. This study enables the routine identification of *Caucasiron* larvae necessary for biomonitoring and hydrobiological research in the Caucasus region.

Key words
aquatic insects, mayflies, morphology, identification key

Introduction
The knowledge facilitating the identification of mayflies inhabiting the Caucasus biodiversity hotspot (Myers et al. 2000) is limited to checklists (e.g., Bojková et al. 2018: Iran; Gabelashvili et al. 2018: Georgia; Hrivniak et al. 2018: Armenia) and alpha taxonomic papers focused

mostly on the delimitation of newly described species/taxa. The available identification keys deal with selected genera only (Sinitshenkova 1976, 1979: *Epeorus* Eaton, 1881 and *Rhithrogena* Eaton, 1882, respectively; Jacob and Zimmerman 1978: *Baetis* Leach, 1815) or mayfly fauna of the wider region without sufficient information on Caucasian species specifically (Kluge 1997a). These keys are largely outdated, because the number of species newly described from the Caucasus has been steadily increasing in recent years (e.g., Hrivniak et al. 2017; 2018; 2019; 2020a; Martynov and Godunko 2017; Bojková et al. 2018). Therefore, the identification of larvae to the species level is complicated due to the necessity of compiling information from original descriptions and requires advanced experience with the taxonomy of mayflies and comparative collections. Modern identification keys are needed especially for researchers implementing biomonitoring programmes and routine hydrobiological surveys in the region. They often use species data on the generic or family level only (e.g., Hakobyan et al. 2010; Asatryan et al. 2016; Hovhannisyan and Shahnazaryan 2016; Sharifinia et al. 2016) and often include numerous misidentifications (cf. Bojková et al. 2018). This study aims to partly fill this gap by providing a complex identification guide for the larvae of the genus *Epeorus*, subgenus *Caucasiron* Kluge, 1997 (Heptageniidae) (herineafter *Caucasiron*) occurring in the Caucasus and adjacent areas. The *Epeorus* s.l. larvae are known to be sensitive to pollution, are relatively stenotopic, restricted to lotic habitats, and form an ecologically important component in macroinvertebrate assemblages (Morisi et al. 2003). *Caucasiron* species, together with the remaining representatives of Heptageniidae, can, therefore, be used as indicators in water quality assessments and hydrobiological surveys in the Caucasus region.

Caucasiron ranks among the most diverse mayfly groups in the Caucasus region, together with the genera *Rhithrogena*, *Electrogena* Zurwerra & Tomka, 1985, and *Ecdyonurus* Eaton, 1868. It is a monophyletic subgenus, sister to the subgenus *Iron* distributed in Central Asia and the Nearctic Region (Hrivniak et al. 2020a). Kluge (1997b) defined *Caucasiron* based on the unique apomorphy among Heptageniidae (and mayflies in general), a projection on the costal margin

of the gill plates II–VII (see Fig. 4E, in *"Part one: Background, section 5"*). Other morphological characters of *Caucasiron* include: gill plates forming a "suction disc" (i.e., a structure consisting of enlarged gill plate I and overlapping gill plates II–VII, and gill plate VII with a longitudinal fold allowing it to be bent ventrally under the abdominal segments (see Fig. 4B–C, D, arrow in *"Part one: Background, section 5"*) and medio-dorsally directed hair-like setae along the anterior margin of the head (Kluge 2015: 346, fig. 178). Imagines are characterized by tubular penis lobes without dorso-lateral denticles and well developed median titilators (see Fig. 4A in *"Part one: Background, section 5"*).

The global diversity of *Caucasiron* comprises 17 species (Hrivniak et al. 2020b). This identification guide deals with 14 species inhabiting the Caucasus and neighbouring mountain ranges, such as the Zagros and Taurus Mountains, and one species inhabiting Samos Island. The extralimital species *E.* (*C.*) *guttatus* (Braasch, 1979) from Central Asia, *E.* (*C.*) *extraordinarius* Chen et al., 2010 from south-western China, and other Central Asian species presumably belonging to the genus *Caucasiron* (Hrivniak et al. 2017) are not included. All 15 species included in the guide are easily distinguishable based on both morphology and molecular data (Hrivniak et al. 2017, 2019, 2020a, b). Additionally, Hrivniak et al. (2020b) identified seven other distinct lineages based on molecular data only. Most of these lineages likely represent cryptic species or as yet have no formal morphological description. The distribution of possible cryptic lineages are to be found in the guide remarks of the respective morphotypes.

Individual species of *Caucasiron* have different distribution patterns in the Caucasus. Some species are local endemics to the Greater Caucasus, Pontic, Zagros, or Alborz Mountains. Others are widely distributed throughout the Caucasus and the adjacent areas of Anatolia, Cyprus, Iran, and Iraq (Hrivniak et al. 2017, 2019, 2020a, b). Their distribution and diversity patterns can be explained by geological history and land development in the region that have significantly affected the diversification of *Caucasiron* in the Caucasus (Hrivniak et al. 2020b).

We aim to provide information necessary for the accurate species identification of *Caucasiron* to the professional public in order to allow

the integration of *Caucasiron* into the hydrobiological surveys and biodiversity monitoring in the Caucasus. The main objectives of this study are to (i) form an identification key based on the reliable morphological characters of larvae, (ii) make an inventory of records of all species, and (iii) describe their geographic and altitudinal distribution based on our extensive data and available literature data. *Caucasiron* imagines are not described because of the lack of unambiguously associated specimens. Only information about whether the subimagines or imagines of a given species are described, how they were associated, and who described them, is provided.

Material and methods

Sampling

Larvae of *Caucasiron* were collected at 290 localities in Turkey, Georgia, Russia, Armenia, Azerbaijan, Iran, and Samos and Cyprus in 2008–2019 (Fig. 2). They were sampled by a hand net or a metal strainer and fixed in 96% ethanol in the field. Sampling sites fully covered Caucasus region and the geographical distribution of all known Caucasian *Caucasiron* species.

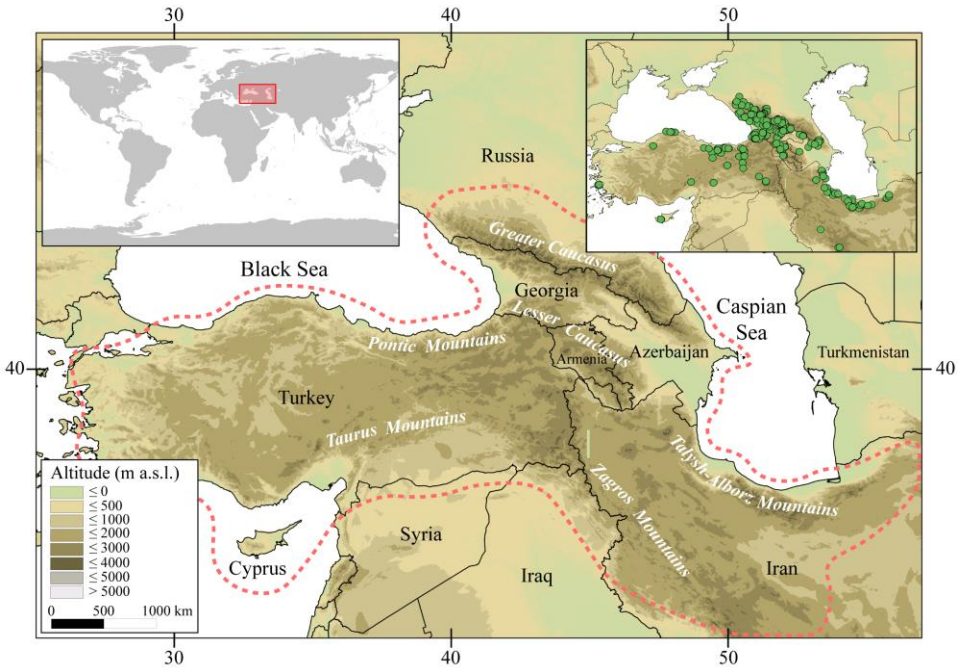


Figure 1. Topographic map of the Caucasus and adjacent mountain ranges with the position of the study area (upper left part) and distribution of our sampling sites (upper right part). Geographical coverage of identification guide of *Epeorus* (*Caucasiron*) larvae is defined by red dashed line.

Morphological examination

Original descriptions of individual species were used for the initial species identification based on morphology (Sinitshenkova 1976; Braasch 1978, 1979, 1980; Braasch and Zimmerman 1979; Braasch and Soldán 1979; Hrivniak et al. 2017, 2019, 2020a). Due to insufficient details given in several of these descriptions, newly collected specimens (both larvae

mounted on slides and larvae stored in ethanol) were compared with the type material (holotypes and/or paratypes) to accurately identify the species. Type series were studied in species recently described by us: *E*. (*C*.) *bicolliculatus* Hrivniak, 2017, *E*. (*C*.) *turcicus* Hrivniak, Türkmen & Kazancı, 2019, *E*. (*C*.) *alborzicus* Hrivniak & Sroka, 2020 , *E*. (*C*.) *shargi* Hrivniak & Sroka, 2020, and *E*. (*C*.) *zagrosicus* Hrivniak & Sroka, 2020. Type specimens were also studied in following species: *E*. (*C*.) *iranicus* (Braasch & Soldán, 1979), *E*. (*C*.) *insularis* (Braasch, 1983), *E*. (*C*.) *magnus* (Braasch, 1978), *E*. (*C*.) *alpestris* (Braasch, 1979), *E*. (*C*.) *soldani* (Braasch, 1979), *E*. (*C*.) *longimaculatus* (Braasch, 1980), and *E*. (*C*.) *sinitshenkovae* (Braasch & Zimmerman, 1979). Topotypes were collected and studied in several species: *E*. (*C*.) *insularis*, *E*. (*C*.) *alpestris*, *E*. (C.) *soldani*, *E*. (*C*.) *longimaculatus*, *E*. (*C*.) *iranicus*. The extent of morphological variability in each species was mostly determined based on specimens, which species identity was proved by molecular species delimitation (Hrivniak et al. 2017, 2019, 2020a, b).

Body parts with morphological structures requiring microscopical examination (i.e. mouthparts, femora, abdominal terga) were mounted on slide using HydroMatrix® (MicroTech Lab, Graz, Austria) mounting medium. In order to remove the muscle tissue for an investigation of the cuticular structure, the specimens were left overnight in a 10% solution of NaOH prior to slide mounting. Drawings were made using a stereomicroscope Olympus SZX7 and a microscope Olympus BX41, both equipped with a drawing attachment. Photographs were obtained using Leica DFC450 camera fitted with macroscope Leica Z16 APO and folded in Helicon Focus version 5.3 X64. All photographs were subsequently enhanced with Adobe Photoshop™ CS5. The terminology was used mostly according to Kluge and Novikova (2011) and Kluge (2004, 2015).

Results and discussion

Larval morphological diagnostic characters

A set of larval diagnostic characters used in the identification guide (listed below) was derived from Braasch and Soldán (1979), who proposed them for the distinguishing of larvae of the genus *Iron* Eaton, 1885. In the concept of Braasch and Soldán (1979), *Iron* included currently recognized taxa *Iron*, *Ironopsis* Traver, 1935, *Caucasiron*, and *Alpiron* Braasch, 2006 (see Hrivniak et al. 2020a for the revised concept and phylogeny of these taxa). Individual diagnostic characters are briefly described and figured for all species.

Morphological characters for the larval identification of *Caucasiron*:

i) coloration of abdominal terga: shape of medial macula (Fig. 3I, arrow) and length of lateral stripes (extended dorso-posteriorly or not; Fig. 12H–I and Fig. 15G respectively).

Sometimes the medial macula is visible only partly being concealed by a preceding abdominal segment due to the telescoping contraction of the abdomen. The abdomen must be sufficiently extended manually to expose all length of individual segments to recognize the shape of the medial macula.

ii) coloration of abdominal sterna: presence/absence and shape of pattern.

The coloration pattern of abdominal terga and/or sterna is often species-specific and valuable in the species identification of *Caucasiron* larvae. It is easily visible and, thus, valuable for the routine identification. However, it often fades in older material or in inadequately fixed larvae, and the intensity of coloration, especially of abdominal sterna, varies among specimens and instars, and may be poorly expressed in some specimens. Therefore, the combination of all characters provided in the guide should be considered for an accurate species identification. The coloration pattern is usually present on terga II–IX (X) and sterna II–VIII (IX). However, patterns vary among segments, therefore, for the purpose of the key we compare terga V–VII and sterna II–VI, which are most species-specific.

iii) surface of abdominal terga: presence/absence of outgrowths (protuberances, spines, etc.), shape of sensory setae (hair-like/wide; Fig. 4E and Fig.19E respectively), density, shape, and sclerotization of denticles along posterior margin of tergum VII (mounting on microscopic slide required).

Except *E*. (*C*.) *bicolliculatus* with a pair of postero-medial protuberances on abdominal terga II–IX (Hrivniak et al. 2017: figs 11, 31, 32; Fig. 33H, arrows), dorsal margin of abdominal terga of Caucasian *Caucasiron* species do not bear any outgrowths or spines.

Denticles along posterior margin of abdominal terga are pointed and irregular in size in all Caucasian *Caucasiron* species. However, the denticles of some species are denser and more sclerotized, e.g. in *E*. (*C*.) *znojkoi* Tshernova, 1938 (Fig. 7E) and *E*. (*C*.) *nigripilosus* (Sinitshenkova 1976) (Fig. 13E), strongly sclerotized, elongated and curved, e.g., in *E*. (*C*.) *magnus* (Fig. 10E) or less sclerotized and narrowed, e.g., in *E*. (*C*.) *longimaculatus* (Fig. 28E). The pattern of denticles slightly varies among terga and depends on a lateral distance from the midline of a given tergum. Thus, the reference part for the description of denticulation along the posterior margin of terga is used in the key. It is represented by tergum VII, the section from its midline to approximately half distance to the lateral margin.

iv) medial hypodermal femur spots: presence/absence and shape (rounded/elongated). The character is relatively stable and usually present on dorsal surface of femora of all leg pairs. Variability was observed in *E*. (*C*.) *caucasicus* (Fig. 3F–H) and includes absence on all or at least some of the legs.

v) mouthparts: setation on dorsal surface of labrum (sparse hair-like setae/dense bristle-like setae; Fig. 4A and Fig. 10A respectively) and shape of mandibular incisors (blunt/pointed) (mounting on microscopic slide required).

Mouthparts of Caucasian *Caucasiron* species are generally without distinct diagnostic characters in most of the species. The only exception is labrum, mandibles and maxillae of *E*. (*C*.) *magnus*. This species differs from all others by setation of dorsal surface of labrum (dense bristle-like setae; Fig. 10A), pointed mandibular incisors (Fig. 10B–C), and thickened

maxillary dentisetae (not figured). The shape of labrum is generally variable in most of the Caucasian *Caucasiron*. Exceptions are *E*. (*C*.) *magnus*, *E*. (*C*.) *alpestris*, and *E*. (*C*.) *sinitshenkovae*, the shape of labrum of which can be considered as one of the diagnostic characters. However, it should be noted that the shape of labrum is often distorted during the slide preparation and should be observed in natural position (not flattened), as well as suggested for other Heptageniidae (e.g. *Ecdyonurus*) (Bauernfeind 1997). Therefore, the shape of labra figured in the guide are not flattened on slide but drawn from dorsal view in natural position.

Drawings of the shape of mandibular incisors were based on flattened incisors on slides. Despite mandibular incisors are not considered as distinct character in the species identification, they are figured in the guide for comparison with *E*. (*C*.) *magnus*, and for purposes of further taxonomy, in case some new species with different incisors will be found in the future.

vi) gill plates: size of a projection on costal margin of gill plates III (with distinct/without distinct projection; Fig. 4G, arrow and Fig. 28G respectively) and shape of gill plates VII in natural position from ventral view (narrow/wide).

The shape of gill plates I–VI is more or less identical between individual species. However, the gill plate VII is specific for some species; e.g. narrow, banana-shaped plate in *E*. (*C*.) *soldani* (Figs 18L; 19H–K), *E*. (*C*.) *longimaculatus* (Figs 27L, M; 28H–L), or *E*. (*C*.) *sinitshenkovae* (Figs 24J; 25H–K); wider and rounded shape in *E*. (*C*.) *nigripilosus* (Figs 12K; 13H–J) or *E*. (*C*.) *alborzicus* (Figs 39J, K; 40H–J). Importantly, the shape of the gill plate VII must be observed in natural position from ventral view, without flattening on a slide (as shown e.g., in Fig. 6L–P). As a part of the gill plate VII is longitudinally bent under the abdomen (Fig. 1C–D), its shape is often deformed during the slide preparation by straightening of its inner margin.

vii) tarsal claw denticulation: number of denticles.

Denticulation of tarsal claws was omitted in the guide, due to its high overlap among species and frequent abrasion. Tarsal claws of all species usually possess 2–4 denticles.

viii) shape of head in fully grown larvae: ellipsoid/oval trapezoidal/sharply trapezoidal.

The shape of head (in dorsal view) may be used as one of the diagnostic characters in some species; e.g., *E.* (*C.*) *znojkoi* is characteristic by sharply trapezoidal head (Fig. 6D), *E.* (*C.*) *magnus* and *E.* (*C.*) *shargi* by oval trapezoidal head (Fig. 9D–E and Fig. 42D respectively), and *E.* (*C.*) *longimaculatus* by more or less ellipsoid shape of head (Fig. 27D). Interspecific differences in the shape of head are most distinct in fully grown or late instars of males (and females in *E.* (*C.*) *magnus*).

ix) postero-lateral projection on tergum X: presence/absence (Fig. 10K–M, arrows, and 16L respectively) and its size.

We also figure a shape of medial emargination of female sternum IX and spatulate setae on dorsal surface of femora (figures in the guide include the variability from proximal to distal margin of femora of all leg pairs). Despite a relatively wide range of variability in these characters, it may be helpful in identification of some species.

Identification guide to larvae of Caucasian species of Caucasiron

How to use the guide?

The dichotomous key divides *Caucasiron* species into two morphological groups, further divided into subgroups. They do not correspond with the phylogeny and merely represent groupings defined for the practical purpose of species identification.

Some characters within the key are subject to variation in some species. For instance, *E.* (*C.*) *caucasicus* usually has a median hypodermal femur spot, but in rare instances it is absent. We deal with this ambiguity by placing such species both in Group A (femur spot present) and B (femur spot absent). Thus, there are sometimes multiple paths leading to the same species in the key.

Most *Caucasiron* species are defined on the basis of a particular combination of several morphological characters. Following species identification using the dichotomous key, it is recommended to compare all the remaining diagnostic characters for a given species, provided in detail in the “Main morphological diagnostic characters of larvae” for

each species. Variability of morphological diagnostic characters is described in the remarks section.

The "main morphological diagnostic characters of larvae" were described based on late-instar larvae (fully-grown larvae). The order of characters is not concise in relation to all species; it always starts with the most prominent character for a given species after which the value of subsequent characters for species identification diminishes. For each species included in the guide, geographical and altitudinal distribution is provided. The construction of distribution maps was based on published records (Table 1) and our unpublished data. Brief information on distribution is also given directly in the key. Abbreviations correspond with points of the compass; central Greater Caucasus refers to area from Mount Elbrus to Mount Kazbek. Altitudinal distribution is divided into three categories: low (up to 500 m a.s.l.), middle (500–1500 m), and high (above 1500 m). This serves only for the purpose of rough orientation, since actual environmental conditions on a given altitude may vary significantly because of high climatic heterogeneity within the region. The list of synonyms given for each species includes all generic/subgeneric combinations under which the species is mentioned in the literature, always with the reference to the first study using a given combination.

Key to species

Medial hypodermal femur spots present (e.g. Fig. 12F–G): **group A**

– Coloration pattern on abdominal sterna present (Figs 3; 12; 45B): **subgroup A1, p. 156.**

– Coloration pattern on abdominal sterna absent (Figs 27; 36; 42B): **subgroup A2, p. 156.**

Medial hypodermal femur spots absent (e.g. Fig. 15F): **group B**

– Coloration pattern on abdominal sterna present (Figs 3; 6; 15; 30; 33; 39B; 18J–K): **subgroup B1, p. 157.**

– Coloration pattern on abdominal sterna absent (e.g. Figs 9; 25B): **subgroup B2, p. 158.**

subgroup A1

1 abdominal sterna II–VI with a pair of oblique stripes (Figs 3J; 21I, J; 45I): **2**

– abdominal sterna II–V (VI) with a pair of triangular spots (Fig. 12J) and abdominal terga with lateral stripes extended dorso-posteriorly (Fig. 12H–I, arrows): ***E*. (*C*.) *nigripilosus* (widespread in the Caucasus; Cyprus), p. 170.**

2 stripes on abdominal sterna II–VI widened anteriorly (Fig. 45I, arrows) and abdominal terga with lateral stripes extended dorso-posteriorly (Fig. 45H, arrows): ***E*. (*C*.) *zagrosicus* (S and SW Iran), p. 203.**

– stripes on abdominal sterna II–VI not widened anteriorly (Figs 3J; 21I–J): **3**

3 abdominal terga V–VII with crown-like medial macula (Fig. 3I): ***E*. (*C*.) *caucasicus* (widespread in the Caucasus), p. 159.**

– abdominal terga V–VII with stripe-like medial macula and a pair of distinct antero-lateral stripes (Fig. 21G, arrows): ***E*. (*C*.) *iranicus* (N Iran), p. 179.**

subgroup A2

1 medial hypodermal femur spots distinctly elongated (Fig. 27F–H); setae on abdominal terga wide and denticles along posterior margin of tergum VII narrow (Fig. 28E); gill plates III without distinct projections (Fig. 28G); gill plates VII narrow (Figs 27L–M; 28H–L): ***E*. (*C*.) *longimaculatus* (central Greater Caucasus), p. 185.**

– medial hypodermal femur spots rounded, not distinctly elongated, gill plates III with well-developed projections; setae on abdominal terga hair-like (e.g. Fig. 37E): **2**

2 abdominal terga V–VII with stripe-like medial macula and lateral stripes extended dorso-posteriorly (Fig. 36G, arrows); gill plates VII narrow (Figs 36I; 37H–K): ***E*. (*C*.) *turcicus* (NE Turkey), p. 194.**

– abdominal terga V–VII with more or less triangular or T-shaped medial macula, lateral stripes not extended dorso-posteriorly (Fig. 42I–K); gill plates VII wide (Figs 42M; 43H–J): ***E*. (*C*.) *shargi* (N Iran), p. 200.**

subgroup B1

1 setae on abdominal terga wide: **2**

– setae on abdominal terga hair-like: **3**

2 abdominal terga II–IX with a pair of postero-medial protuberances (Fig. 33H, arrows; protuberances are most developed on terga VI–VIII and best visible from dorso-lateral view); abdominal terga V–VII with stripe-like medial macula, which is often anteriorly and posteriorly widened (Fig. 33G, H); abdominal sterna II–VI as on Figs 33J–L: ***E*. (*C*.) *bicolliculatus* (NE Turkey, Georgia, Armenia), p. 191.**

– abdominal terga without postero-medial protuberances; terga V–VII with well-defined triangular maculae (Fig. 18H, I); sterna not intensively pigmented, pattern of sterna II–VI as on Fig. 18J, K: ***E*. (*C*.) *soldani* (W and central Greater Caucasus), p. 176.**

3 postero-lateral projections on tergum X distinct (Fig. 40K, arrow); abdominal sterna II–VI with circular medial macula (Fig. 39L–N); gill plates VII wide (Figs 39J–K; 40H–J): ***E*. (*C*.) *alborzicus* (N Iran), p. 197.**

– postero-lateral projections on tergum X absent or indistinct, coloration pattern of abdominal sterna different: **4**

4 abdominal sterna II–VI yellowish, with a pair of black oblique stripes or brownish rounded medial macula: **5**

– all or at least abdominal sterna VIII–IX intensively red (Fig. 6L), with reddish to brownish maculation (Fig. 6M) including a longitudinal stripe (Figs 6N–P; 30J) and a pair of reddish oblique (Fig. 6K, a) and/or medio-lateral stripes (Fig. 6K, b): **6**

5 abdominal sterna II–VI with a pair of black oblique stripes (Fig. 3J); abdominal terga V–VII with crown-like medial macula (Fig. 3I): ***E*. (*C*.) *caucasicus* (widespread in the Caucasus), p. 159.**

– abdominal sterna II–VI with brownish rounded medial macula (Fig. 15I); abdominal terga V–VII with narrow stripe-like medial macula (widened on terga VIII–IX, Fig. 15G–H, arrows): ***E*. (*C*.) *alpestris* (W and central Greater Caucasus), p. 173.**

6 gill plate VII wide (Figs 6J, L–P; 7H–L); denticles along posterior margin of tergum VII relatively long, strongly sclerotized and dense (Fig. 7E); postero-lateral projections on tergum X present or absent (Fig. 7M, N): ***E*. (*C*.) *znojkoi* s.l. (widespread in the Caucasus), p. 162.**

– gill plates VII narrow (Figs 30K; 31H, I); denticles along posterior margin of tergum VII relatively short and poorly sclerotized (Fig. 31E); postero-lateral projections on tergum X absent (Fig. 31J): ***E*. (*C*.) *insularis* (Samos Island, Greece), p. 188.**

subgroup B2

1 postero-lateral projections on tergum X present (Fig. 10K–M); dorsal surface of labrum with dense bristle-like setae (Fig. 10A); gill plates VII wide or slightly narrowed (Figs 9K; 10H–J): ***E*. (*C*.) *magnus* (widespread in Caucasus, Anatolia), p. 167.**

– postero-lateral projections on tergum X absent (Figs 19, 25L); gill plates VII distinctly narrowed (Figs 18L; 19H–K; 24J; 25H–K); dorsal surface of labrum with sparse hair-like setae (Figs 19A; 25A): **2**

2 abdominal terga V–VII with narrowed triangular medial macula and a pair of anterolateral spots (Fig. 24H; arrows); gill plates III without distinct projections (Fig. 25G); setae on terga not widened, often elongated (Fig. 25E): ***E*. (*C*.) *sinitshenkovae* (W and central Greater Caucasus), p. 182.**

– abdominal terga V–VII with well-defined triangular medial maculae, without a pair of anterolateral spots (Fig. 18H–I); setae on terga wide (Fig. 19E); gill plates III with well-developed projections (Fig. 19G): ***E*. (*C*.) *soldani* (W and central Greater Caucasus), p. 176.**

Morphological diagnostics, distribution, and habitat of individual species

***Epeorus* (*Caucasiron*) *caucasicus* (Tshernova, 1938)**

Cynigma caucasica Tshernova, 1938
Epeorus (*Iron*) (Tshernova, 1938); in Tshernova (1974)
Iron fuscus Sinitshenkova, 1976; jun. syn.; in Braasch (1979)
Epeorus (*Caucasiron*) *caucasicus* (Tshernova, 1938); in Kluge (1997b)
Figs 2–4

Type locality: Azerbaijan, a stream in the vicinity of Göygöl Lake.
Distribution: Georgia, south-western Russia, Azerbaijan, Armenia, eastern Turkey (Fig. 2). One of the most widespread species in the Caucasus.
Habitat: Larvae inhabit small streams and rivers at middle and high altitude, most frequently found above 1000 m a.s.l. Altitudinal range of sampling sites 496–2474 m a.s.l. (Fig. 2).

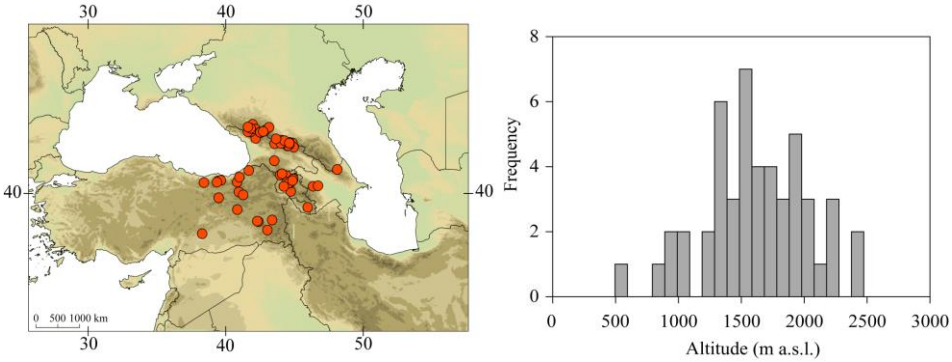


Figure 2. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *caucasicus.*

Main morphological diagnostics of larvae: (i) abdominal sterna II–VI with a pair of oblique stripes; nerve ganglia often with stripes or spots (Fig. 3B, J); (**ii)** abdominal terga V–VII with crown-like medial macula (Fig. 3A, I, arrow); (**iii)** femora with medial hypodermal spot (Fig. 3G–H), sporadically absent or poorly visible (Fig. 3F); **(iv)** setae on abdominal terga hair-like (Fig. 4E); **(v)** gill plates III with well-developed

projections (Fig. 4G); **(vi)** tergum X with poorly developed postero-lateral projections (Fig. 4M, arrow) or without postero-lateral projections (Fig. 4L).



Figure 3. *Epeorus* (*Caucasiron*) *caucasicus*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F–H, middle leg in dorsal view; I, abdominal terga (arrow points on medial macula); J, abdominal sterna II–VI; K–L, gills VII (in natural position from ventral view).

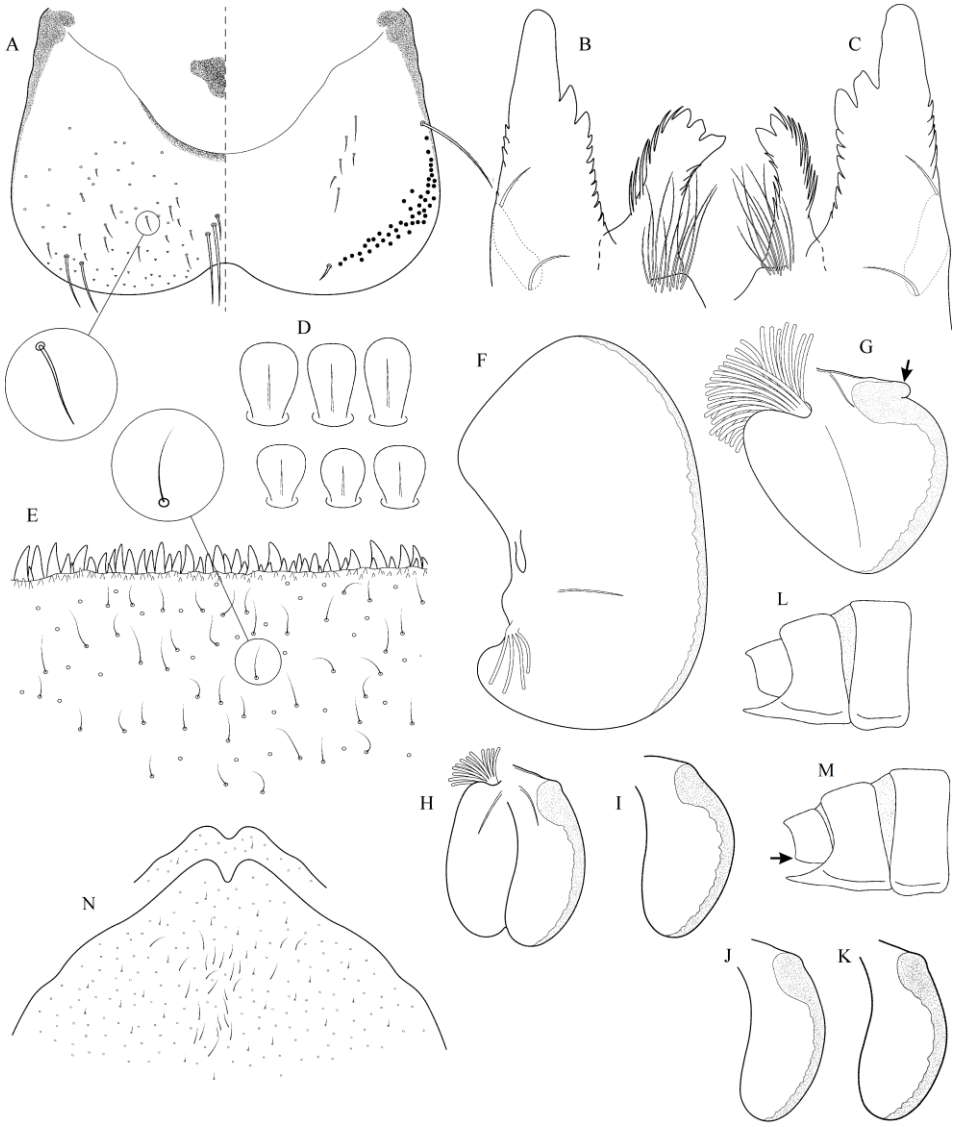


Figure 4. *Epeorus* (*Caucasiron*) *caucasicus*, larva: A, labrum (left half in dorsal view, right half in ventral view) with detail of hair-like seta; B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII with detail of hair-like seta; F, gill I; G, gill III (arrow points on distinct projection on costal margin); H, gill VII (flattened on slide); I–K, gill VII (in natural position from ventral view), variability in shape; L–M, abdominal segments VIII–X in lateral view (arrow points on postero-lateral projection); N, sternum IX of female with observed variability.

Remarks: ***Morphology*****.** Coloration pattern of abdominal sterna as in *E*. (*C*.) *iranicus* (p. 179), similar pattern in *E*. (*C*.) *zagrosicus* (p. 203). Lateral stripes on abdominal terga sporadically dorso-posteriorly extended as in *E*. (*C*.) *nigripilosus* (Fig. 12H–I, arrows). A projection on gill plates III usually well-developed, a slight reduction observed in specimens collected from central Armenia. ***Taxonomy*****.** Species was described based on male imagines from Azebarbaijan (the vicinity of Göygöl Lake) and Nakhchivan (Sakarsu River) (Tshernova 1938). The type series is deposited in the Institute of Zoology, Russian Academy of Sciences, Sankt Petersburg (IZ) (Kluge 1995). Female imago not described; the larva described by Sinitshenkova (1976) from several localities in Russia, Armenia, and Azerbaijan. Larvae and imagines were associated based on the same sampling sites (a part of the material originated from the vicinity of the type locality) and a similarity in the coloration of abdomen of the larva and imagines. The description and validity of larval diagnostic characters were discussed by Braasch (1979, 1980). According to him, Sinitshenkova (1976) described the larva of *E*. (*C*.) *znojkoi* under the name *E*. (*C*.) *caucasicus* by mistake. This opinion was supported by the investigation of imagines reared from larvae corresponding to *E*. (*C*.) *caucasicus* described by Sinitshenkova (1976). Imagines corresponded to *E*. (*C*.) *znojkoi* as were described by Tshernova (1938). The larva belonging to *E*. (*C*.) *caucasicus* was also described in Sinitshenkova (1976), but under erroneous attribution to newly proposed species *E*. (*C*.) *fuscus*. Later, *E*. (*C*.) *fuscus* was considered as a synonym of *E*. (*C*). *caucasicus* (Braasch 1979; Braasch and Soldán 1979).

***Epeorus* (*Caucasiron*) *znojkoi* (Tshernova, 1938) s.l.**

Iron znojkoi Tshernova, 1938
Epeorus (*Iron*) *znojkoj* (Tshernova, 1938); in Tshernova (1974)
Iron caucasicus (Tshernova, 1938); in Sinitshenkova (1976) partim
Iron znojkoi Tshernova, 1938; in Sinitshenkova (1976) partim
Epeorus (*Caucasiron*) *znojkoi* (Tshernova, 1938); in Kluge (1997b)
Figs 5–7

Type locality: Azerbaijan, Nakchivan Autonomous Republic, Giljan-tshaj (Gilljak).

Distribution: Georgia, south-western Russia, Azerbaijan, Armenia, Turkey, northern Iran (Fig. 5). The most widespread species in the Caucasus.

Habitat: Larvae inhabit streams and rivers of various sizes, from larger braided low altitude rivers to small streams at high altitude. Altitudinal range of sampling sites -6–2453 m a.s.l. (Fig. 5). Most frequently found in low and middle altitudes. Often syntopic with *E*. (*C*.) *magnus*.

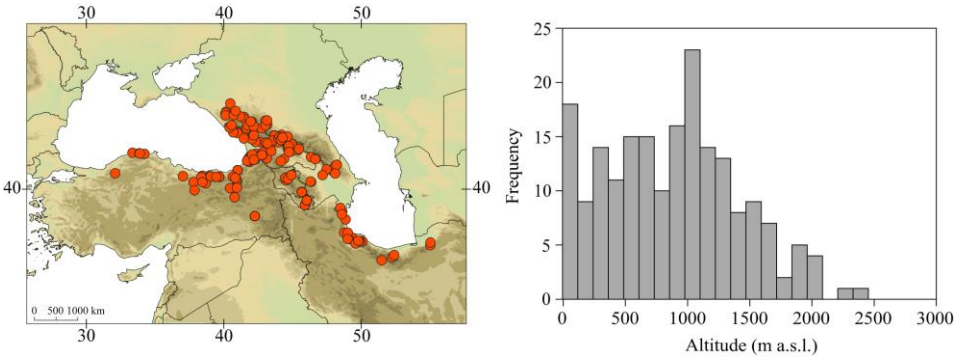


Figure 5. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *znojkoi*.

Main morphological diagnostics of larvae: (i) abdominal terga II–IV with triangular medial macula and terga V–VII with T shaped medial macula (Figs 6A; G–I); **(ii)** abdominal sterna intensively red or reddish (Fig. 6B, L, M), with a pair of reddish oblique stripes (Fig. 6K, a) and/or reddish medio-lateral stripes (Fig. 6K, b), or with reddish to brownish longitudinal stripe on all sterna or at least on sterna VIII and IX (Fig. 6N–P); **(iii)**; tergum X with short postero-lateral projections (Fig. 7M, arrow) or without postero-lateral projections (Fig. 7N) ; **(iv)** femora without medial hypodermal spot (Fig. 6F); **(v)** gill plates VII (in natural position from ventral view) wide (Figs 6J, L–P; 7H–L); **(vi)** denticles along posterior margin of tergum VII strongly sclerotized and dense (Fig. 7E); **(vii)** gill plates III with well-developed projection (Fig. 7G); **(viii)** shape of head sharply trapezoidal in males (Fig. 6D).



Figure 6. *Epeorus* (*Caucasiron*) *znojkoi*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F, middle leg in dorsal view; G–I, abdominal terga; J, gills VII (in natural position from ventral view); K, abdominal sterna II–VI (a, position of oblique stripes; b, position of medio-lateral stripes); L–P, abdominal sterna, variability in coloration pattern (L, Georgia; M, O, P, Iran; N, Turkey).

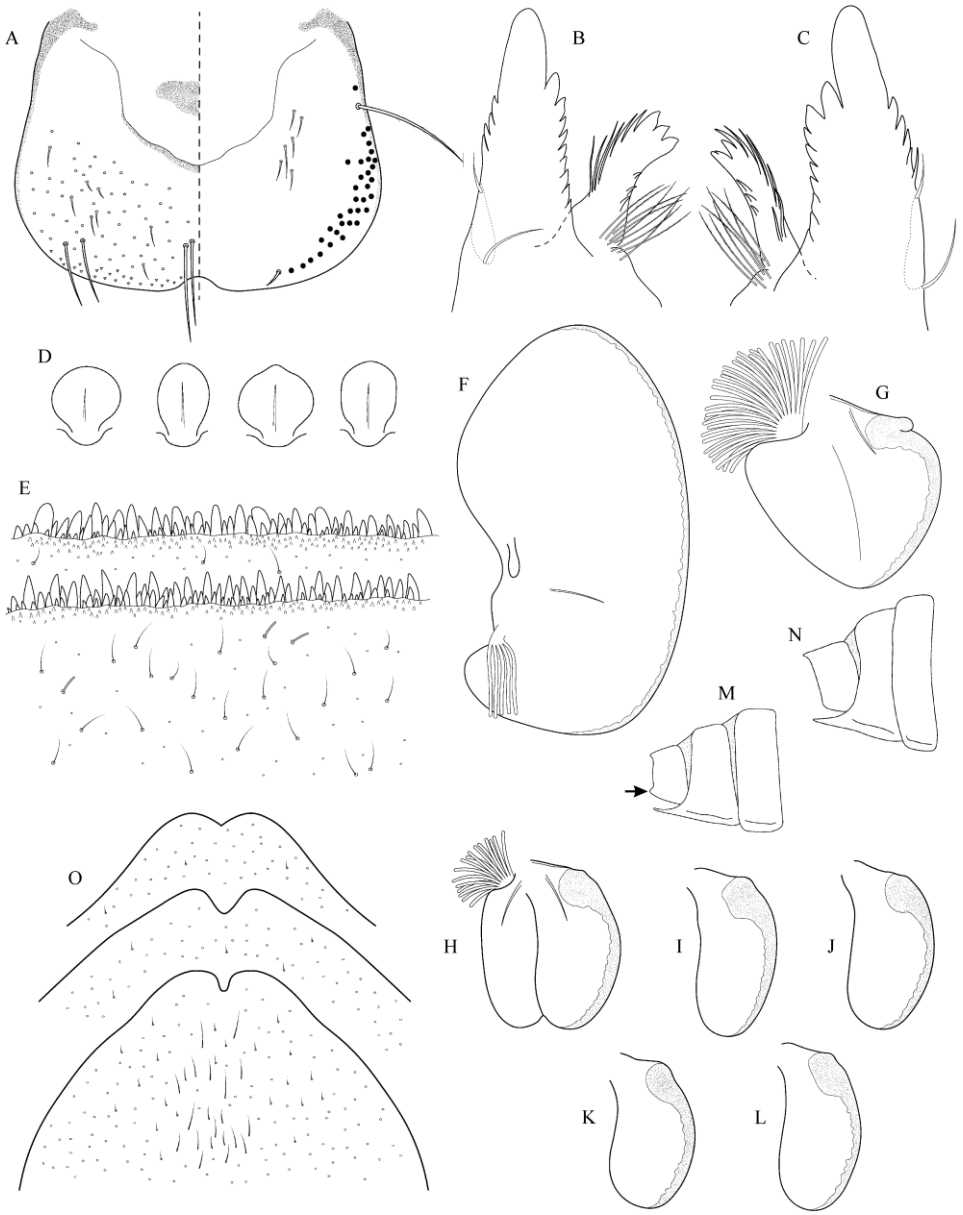


Figure 7. *Epeorus* (*Caucasiron*) *znojkoi*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII; F, gill I; G, gill III; H, gill VII (flattened on slide); I–L, gill VII (in natural position from ventral view), variability in shape; M–N, abdominal segments VIII–X in lateral view (arrow points on postero-lateral projection); O, sternum IX of female with observed variability.

Remarks*: Morphology.* The reduction of reddish coloration of abdominal sterna observed particularly in specimens collected from northern Iran (Fig. 6P). Similar coloration pattern of sterna as present in *E*. (*C*.) *insularis* (p. 188).

***Taxonomy*.** Species was described based on male and female subimagines and imagines from Nakhchivan and Azerbaijan (Tshernova 1938). The type series is deposited in IZ (Kluge 1995). The larva was described by Sinishenkova (1976) based on material collected in Georgia, Russia (the central Greater Caucasus), Armenia and the type locality. Larvae were identified as species *znojkoi*, based on the proximity of its type locality and the similarity of markings on abdominal terga. However, the description of larva is confusing, because the larva of *E*. (*C*.) *znojkoi* was erroneously described under the name *E*. (*C*.) *caucasicus* by Sinishenkova (1976) (Braasch, 1980). Therefore, the larva described by Sinishenkova (1976) as *E*. (*C*.) *znojkoi* should belong to a different species. Its diagnostic characters correspond to those of *E*. (*C*.) *magnus* that was later described by Braasch (1978). These characters include: **(i)** body length: Tshernova (1938) noted 9.5–12 mm for imagines of species (*E*. (*C*.) *znojkoi*; contrary to Sinitshenkova (1976) who noted 14–19 mm for the larvae. Larvae of species *magnus* exhibit 20–24 mm as described by Braasch (1978); **(ii)** shape of head: trapezoidal head with rounded edges as figured by Sinitshenkova (1976) is typical for *E*. (*C*.) *magnus* (Fig. 9D–E), not to *E*. (*C*.) *znojkoi* with more angular edges of head (Fig. 6D); **(iii)** setation of labrum: the shape of labrum and dense setae on its dorsal surface as figured by Sinitshenkova (1976) is characteristic for *E*. (*C*.) *magnus* (Fig. 10A); **(iv)** coloration of abdominal sterna: an absence of coloration on abdominal sterna as described by Sinitshenkova (1976) is typical for *E*. (*C*.) *magnus* (Fig. 9B); *E*. (*C*.) *znojkoi* possess reddish sterna and gills.

Distribution. *E*. (*C*.) *znojkoi* is considered as a species complex containing several lineages (Hrivniak et al. 2020a). They are distributed in the Pontic Mts. in Turkey (*Caucasiron* sp. 5 in Hrivniak et al. 2020a), the Alborz Mts. in Iran (*Caucasiron* sp. 4 in Hrivniak et al. 2020a), and the Lesser Caucasus in Georgian Adjara (*Caucasiron* sp. 6 in Hrivniak et

al. 2020a). The lineages are not formally described now and fall into the group *E*. (*C*.) *znojkoi* s.l. in this identification guide.

***Epeorus* (*Caucasiron*) *magnus* (Braasch, 1978)**

Iron znojkoi Tshernova, 1938; in Sinitshenkova (1976), partim
Iron magnus Braasch, 1978
Epeorus (*Iron*) *magnus* (Braasch, 1978); in Kluge (1988)
Epeorus (*Caucasiron*) *magnus* (Braasch, 1978); in Kluge (1997b)
Figs 8–10

Type locality: Russia, Western Caucasus, Sochi River (20 km above Sochi; 800 m a.s.l.).
Distribution: Georgia, Russia, Azerbaijan, Armenia, Turkey (Fig. 8). One of the most widespread species in the Caucasus.
Habitat: Larvae inhabit streams and rivers of various sizes, from larger braided low-altitude rivers to small streams at high altitude. Altitudinal range of sampling sites 6–2474 m a.s.l. (Fig. 8). Most frequently found at low and middle altitude. Often syntopic with *E*. (*C*.) *znojkoi*.

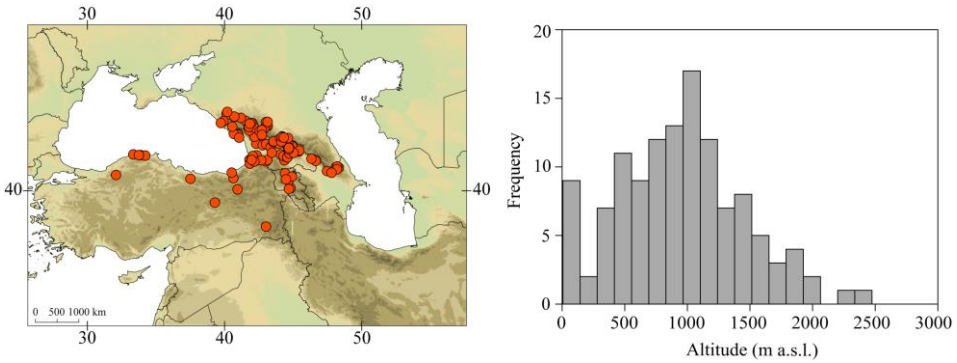


Figure 8. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *magnus*.

Main morphological diagnostics of larvae: (i) shape of head in male and female oval, trapezoidal (Fig. 9D–E); **(ii)** tergum X with well-developed postero-lateral projections (Fig. 10K–M, arrows), sporadically poorly developed; **(iii)** abdominal sterna without coloration pattern (Fig. 9B, J); **(iv)** abdominal terga V–VII with triangular medial macula (Fig.

9H), sporadically poorly visible (Fig. 9I); **(v)** femora without medial hypodermal spot (Fig. 9F–G); **(vi)** dorsal surface of labrum densely covered by bristle-like setae (Fig. 10A); **(v)** setae on abdominal terga hair-like (Fig. 10E); **(vi)** gill plates III with well-developed projections (Fig. 10G); **(vii)** denticles along posterior margin of tergum VII strongly sclerotized, dense and curved (Fig. 10E).

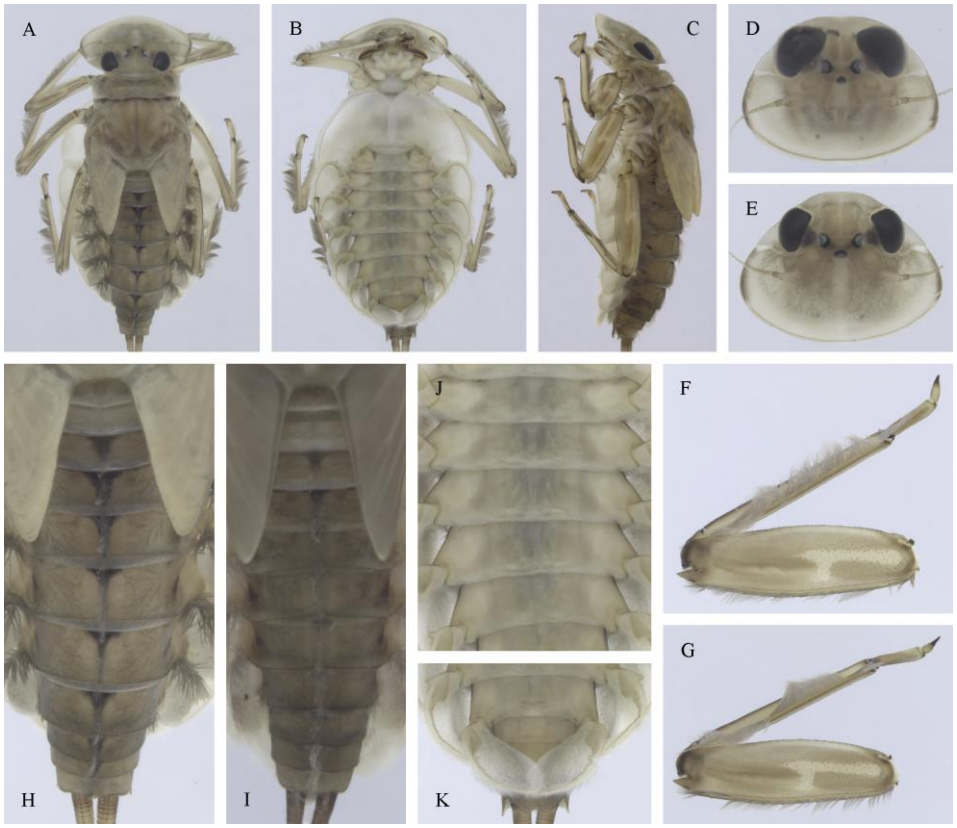


Figure 9. *Epeorus* (*Caucasiron*) *magnus*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F–G, middle leg in dorsal view; H–I, abdominal terga; J, abdominal sterna II–VI; K, gills VII (in natural position from ventral view).

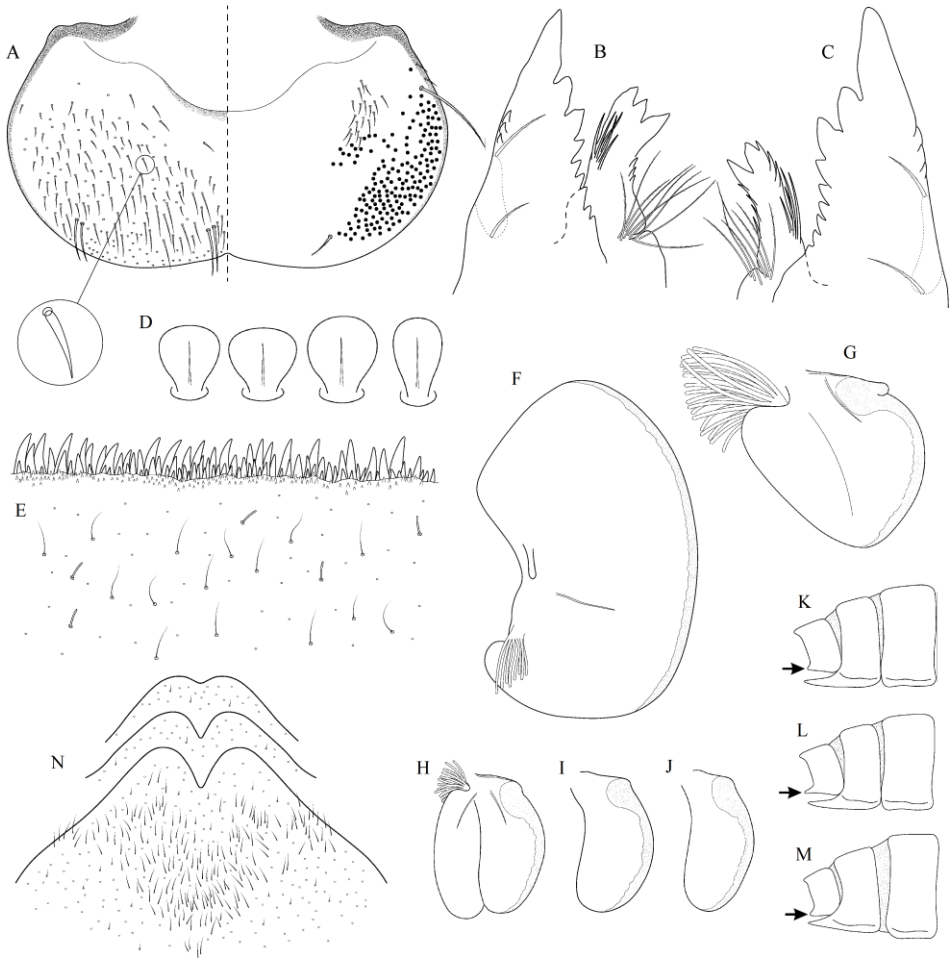


Figure 10. *Epeorus* (*Caucasiron*) *magnus*, larva: A, labrum (left half in dorsal view, right half in ventral view) with detail of bristle-like seta; B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII; F, gill I; G, gill III; H, gill VII (flattened on slide); I–J, gill VII (in natural position from ventral view), variability in shape; K–M, abdominal segments VIII–X in lateral view (arrow points on postero-lateral projection); N, sternum IX of female with observed variability.

Remarks: ***Morphology*****.** The largest species occurring in the Caucasus. The body size of larvae 20–24 mm, cerci 20–22 mm (Braasch 1978).

Taxonomy**.** Original description based on the larvae from Russia (western Caucasus) (Braasch 1978). The type series is currently deposited in the collection of Stuttgart State Museum of Natural History, Stuttgart, Germany (SMNS). Imagines (male and female) and female subimago described by Braasch (1980) based on material from Russia, Armenia and

Georgia. We assume the larva of *E*. (*C*.) *magnus* was erroneously described under the name *znojkoi* by Sinitshenkova (1976) (see remarks to *E*. (*C*.) *znojkoi* s.l. for details).

***Epeorus* (*Caucasiron*) *nigripilosus* (Sinitshenkova, 1976)**

Iron nigripilosus Sinitshenkova, 1976
Epeorus (*Iron*) *nigripilosus* (Sinitshenkova, 1976); in Kluge (1995)
Epeorus (*Caucasiron*) *nigripilosus* (Sinitshenkova, 1976); in Kluge (2004)
Figs 11–13

Type locality: Georgia, Kistinka River (along the military road).
Distribution: Georgia, Russia, Turkey, Cyprus Island, northern Iraq, northern Iran (Fig. 11).
Habitat: Larvae inhabit small streams and rivers at middle and high altitude. Altitudinal range of sampling sites 450–2100 m a.s.l. (Fig. 11). Most frequently found above 1000 m a.s.l.

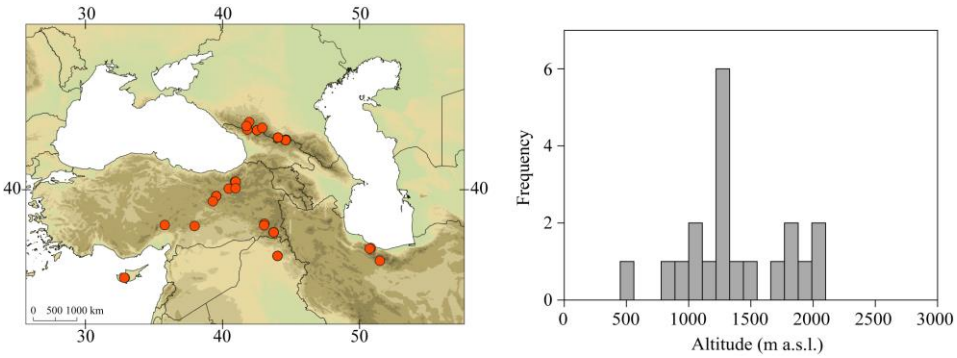


Figure 11. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *nigripilosus.*

Main morphological diagnostics of larvae: **(i)** abdominal sterna II–VI with a pair of triangular spots; nerve ganglia often with spots (Fig. 12B, J); **(ii)** abdominal terga V–VII as on Fig. 12H–I, with lateral stripes extended dorso-posteriorly (Fig. 12H–I, arrows); **(iii)** tergum X with postero-lateral projections (Fig. 13K–L, arrows); **(iv)** femora with rounded medial hypodermal spot (Fig. 12F–G); **(v)** setae on abdominal

terga hair-like (Fig. 13E); **(vi)** denticles along posterior margin of tergum VII strongly sclerotized and dense (Fig. 13E); **(vii)** gill plates VII (in natural position from ventral view) wide (Figs 12K; 13H–J); **(viii)** gill plates III with developed projections (Fig. 13G).

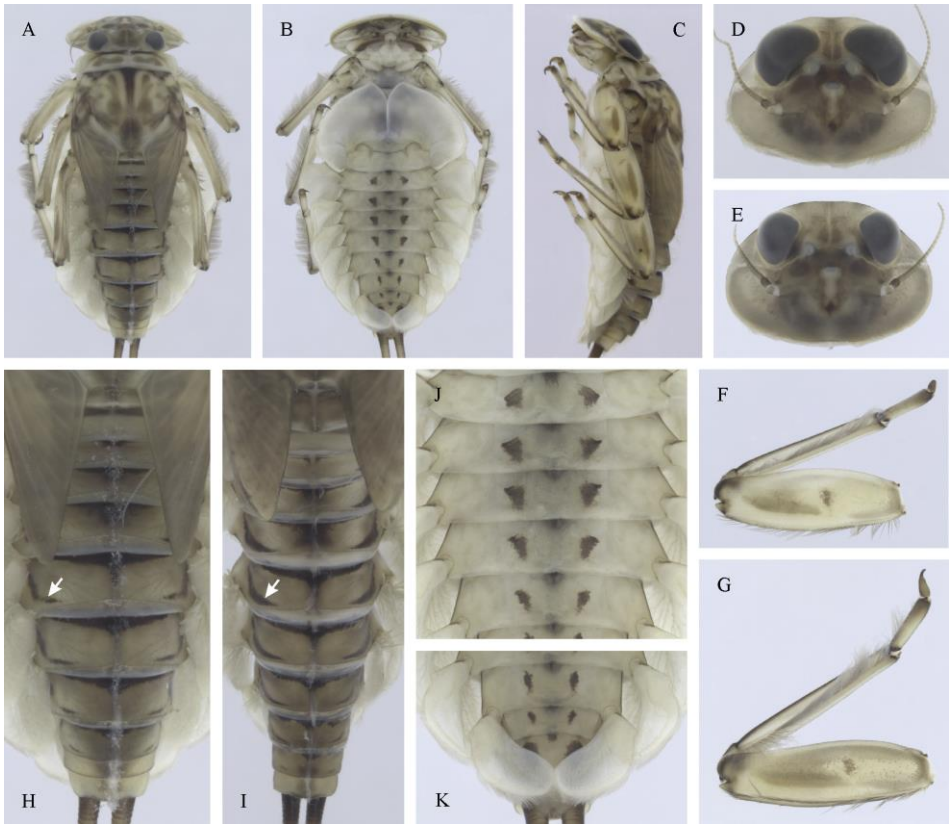


Figure 12. *Epeorus* (*Caucasiron*) *nigripilosus*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F–G, middle leg in dorsal view; H–I, abdominal terga (arrows point on dorso-posteriorly extended lateral stripes); J, abdominal sterna II–VI; K, gills VII (in natural position from ventral view).

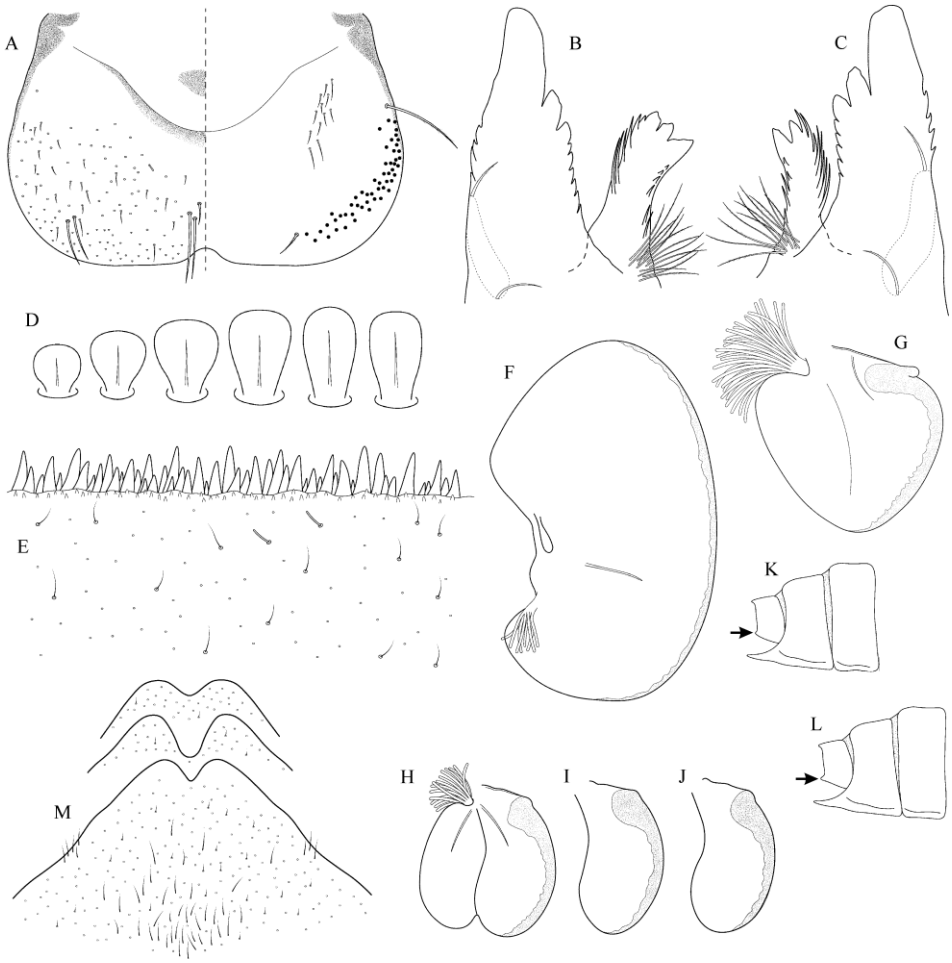


Figure 13. *Epeorus* (*Caucasiron*) *nigripilosus*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII; F, gill I; G, gill III; H, gill VII (flattened on slide); I–J, gill VII (in natural position from ventral view), variability in shape; K–L, abdominal segments VIII–X in lateral view (arrows point on postero-lateral projection); M, sternum IX of female with observed variability.

Remarks: ***Taxonomy*.** Species was described based on larvae from Georgia (Kistinka River). Type series is deposited in IZ (Kluge 1995). Male imago was described by Braasch (1979) based on the material from the western Caucasus (Teberda River) associated with larvae according to similar coloration of abdominal terga and sterna. Female imago not described. Male genitalia similar to *E*. (*C*.) *caucasicus* (Braasch (1979).

***Epeorus* (*Caucasiron*) *alpestris* (Braasch, 1979)**

Iron alpestris Braasch, 1979

Epeorus (*Iron*) *alpestris* (Braasch, 1979); in Kluge (1988)

Epeorus (*Caucasiron*) *alpestris* (Braasch, 1979); in Kluge (1997b)

Figs 14–16

Type locality: Russia, western Greater Caucasus, Teberda (Glacier Alibek – stream).

Distribution: Georgia, Russia. Species endemic to the Greater Caucasus (Fig. 14).

Habitat: Larvae inhabit small streams and rivers at middle and high altitude in the western and central Greater Caucasus. Altitudinal range of sampling sites 570–2580 m a.s.l (Fig. 14). Most frequently found at altitudes above 1200 m a.s.l. Often syntopic with *E*. (*C*.) *soldani* and at higher altitude with *E*. (*C*.) *sinitshenkovae*.

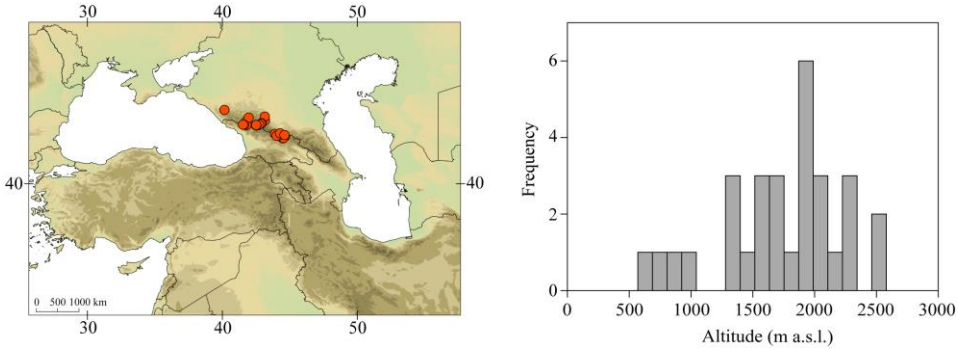


Figure 14. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *alpestris*.

Main morphological diagnostics of larvae: (i) abdominal terga V–VII with narrow stripe-like medial macula; widened on terga VIII–IX (Fig. 15G–H, arrows); **(ii)** abdominal sterna II–VI with rounded medial macula (Fig. 15B, I); **(iii)** femora without medial hypodermal spot (Fig. 15F); **(iv)** tergum X without postero-lateral projections (Fig. 16L); **(v)** gill plates III with well-developed projections (Fig. 16G); **(vi)** setae on abdominal terga hair-like (Fig. 16E); **(vii)** dorsal surface of labrum with sparse hair-like setae (Fig. 10A); **(viii)** gill plates VII (in natural position of ventral view) wide (Figs 15J–K; 16H–K).



Figure 15. *Epeorus* (*Caucasiron*) *alpestris*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F, middle leg in dorsal view; G–H, abdominal terga (arrows point on widened medial maculae); I, abdominal sterna II–VI; J–K, gills VII (in natural position from ventral view).

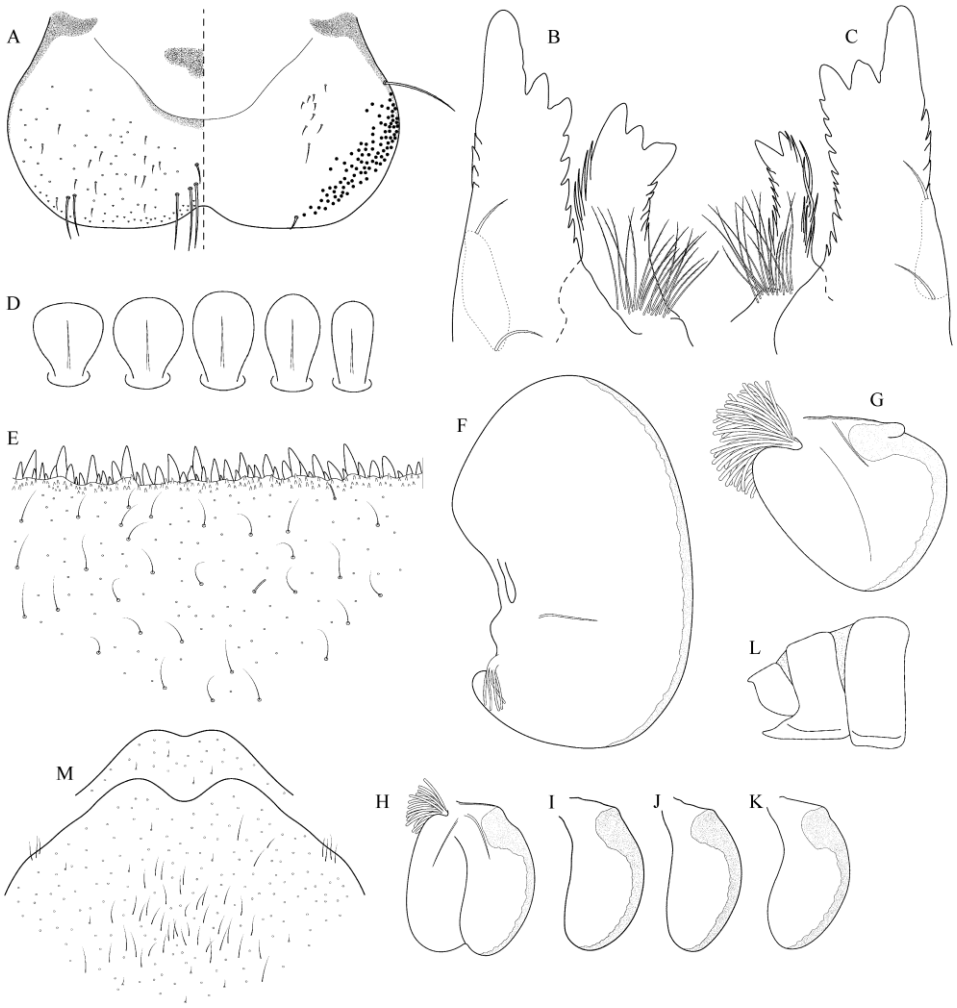


Figure 16. *Epeorus* (*Caucasiron*) *alpestris*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII; F, gill I; G, gill III; H, gill VII (flattened on slide); I–K, gill VII (in natural position from ventral view), variability in shape; L, abdominal segments VIII–X in lateral view; M, sternum IX of female with observed variability.

Remarks: ***Taxonomy.*** Species described based on the male imago and larva from western Greater Caucasus (Braasch 1979). The type series is currently deposited in SMNS. Imagines and larvae were associated based on the coloration of abdomen. Female imago not described.

***Epeorus* (*Caucasiron*) *soldani* (Braasch, 1979)**

Iron soldani Braasch, 1979

Epeorus (*Iron*) *soldani* (Braasch, 1979); in Kluge (1988)

Epeorus (*Caucasiron*) *soldani* (Braasch, 1979); in Kluge (1997b)

Figs 17–19

Type locality: Russia, western Greater Caucasus, Teberda (Glacier Alibek - stream).

Distribution: Georgia, Russia. Species endemic to the Greater Caucasus (Fig. 17).

Habitat: Larvae inhabit small streams and rivers at middle and high altitudes in the western and central Greater Caucasus. Frequently found above 1000 m a.s.l. Altitudinal range of sampling sites 426–1900 m a.s.l. (Fig. 17). Often syntopic with *E*. (*C*.) *alpestris* and *E*. (*C*.) *sinitshenkovae*.

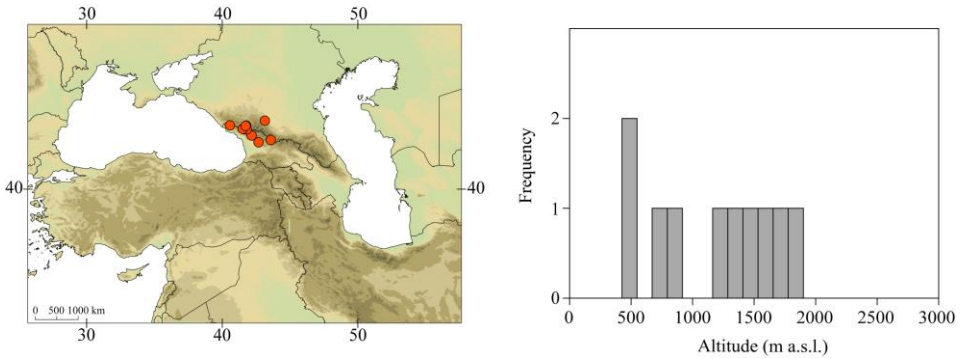


Figure 17. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *soldani*.

Main morphological diagnostics of larvae: (i) abdominal terga V–VII with well-defined triangular medial maculae (Fig.18H–I); **(ii)** abdominal sterna II–VI either without pattern or with indistinct pattern as on Fig. 18J–K; **(iii)** setae on abdominal terga wide (Fig. 19E); **(iv)** femora without medial hypodermal spot (Fig. 18F–G); **(v)** tergum X without postero-lateral projections (Fig. 19L); **(vi)** gill plates III with well-developed projections (Fig. 19G); **(vii)** gill plates VII (in natural position of ventral view) narrow (Figs 18L; 19H–K); **(viii)** denticles along posterior margin of tergum VII relatively sparse and triangular (Fig. 19E).

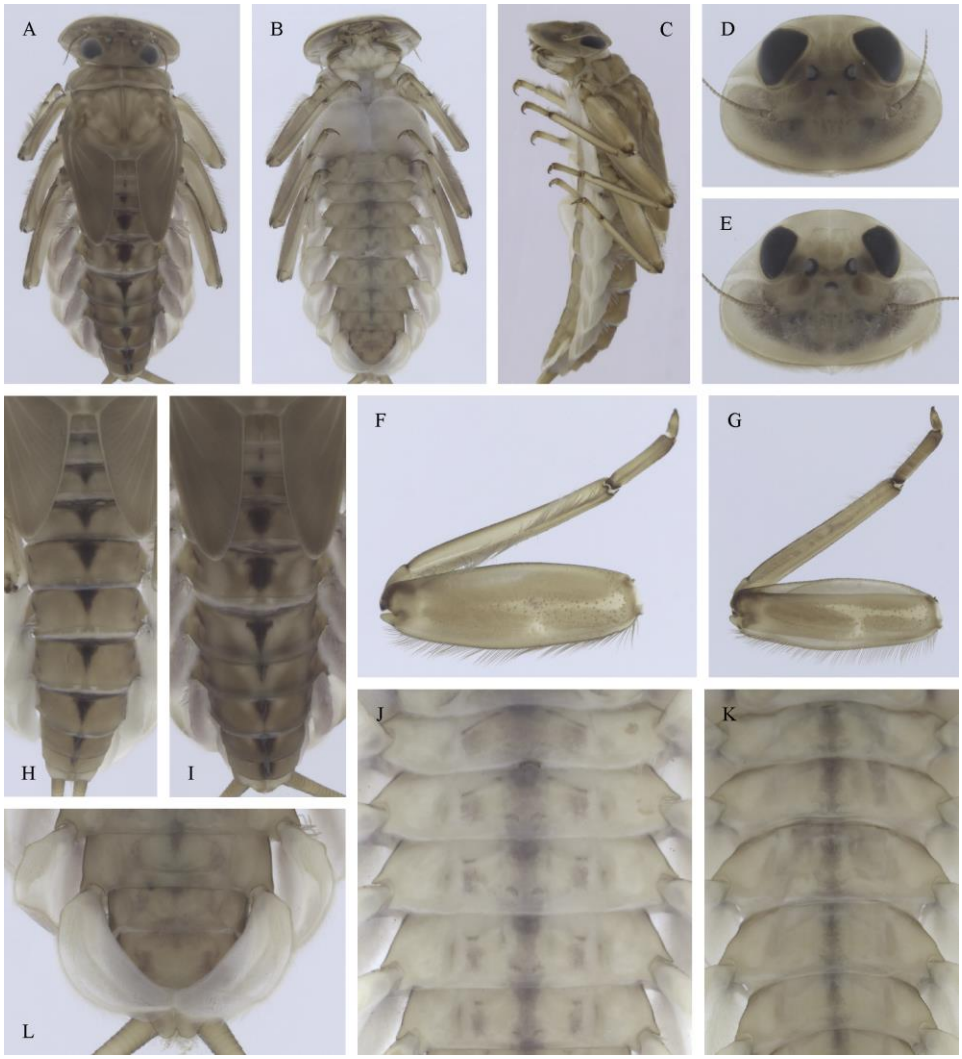


Figure 18. *Epeorus* (*Caucasiron*) *soldani*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F–G, middle leg in dorsal view; H–I, abdominal terga; J–K, abdominal sterna II–VI; L, gills VII (in natural position from ventral view).

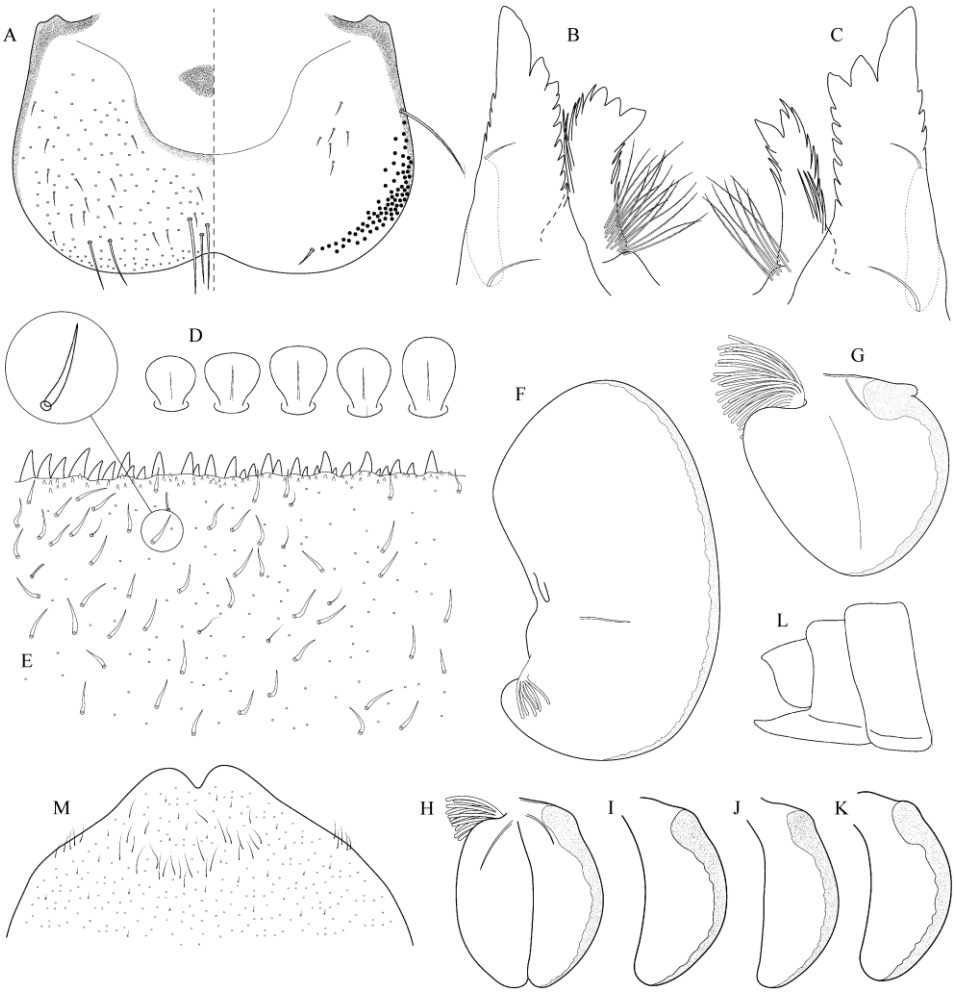


Figure 19. *Epeorus* (*Caucasiron*) *soldani*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII with detail of wide seta; F, gill I; G, gill III; H, gill VII (flattened on slide); I–K, gill VII (in natural position from ventral view), variability in shape; L, abdominal segments VIII–X in lateral view; M, sternum IX of female.

Remarks: ***Taxonomy.*** Species described based on male imago and larva from the western Greater Caucasus (Braasch 1979). The type series is currently deposited in SMNS. Larva associated with imago based on the coloration of abdomen. Female imago not described.

Distribution. The lineage *Caucasiron* sp. 7 detected by Hrivniak et al. (2020b) is distributed in Georgia and morphologically correspond to *E.* (*C.*) *soldani*. Therefore, *E.* (*C.*) *soldani* may represent a species complex.

***Epeorus* (*Caucasiron*) *iranicus* (Braasch & Soldán, 1979)**

Iron caucasicus iranicus Braasch and Soldán (1979)
Epeorus (*Caucasiron*) *caucasicus iranicus*; in Bojková et al. (2018)
Epeorus (*Caucasiron*) *iranicus*; in Hrivniak et al. (2020b)
Figs 20–22

Type locality: Iran, Tehran, river in the Darban-Tal, 2100 m a.s.l.
Distribution: northern Iran. Species endemic to the Alborz Mountains (Fig. 20).
Habitat: Larvae inhabit streams at altitudes above 2000 m a.s.l. in the western and central Alborz. Altitudinal range of sampling sites 2020–2440 m a.s.l. (Fig. 20). Often syntopic with *E.* (*C.*) *alborzicus.*

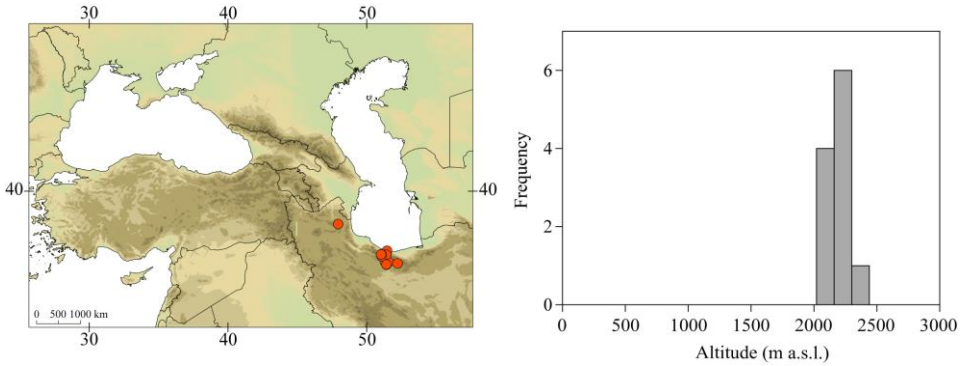


Figure 20. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *iranicus.*

Main morphological diagnostics of larvae: (i) abdominal sterna II–VI with a pair of oblique stripes; nerve ganglia often with stripes or spots (Fig. 21B, I–J); **(ii)** abdominal terga V–VII with stripe-like medial macula and a pair of distinct antero-lateral stripes (Fig. 21G, arrows); **(iii)** femora with rounded medial hypodermal spot (Fig. 21F); **(iv)** gill III with well-developed projections (Fig. 22G); **(v)** setae on abdominal terga hair-like (Fig. 22E); **(vi)** tergum X with poorly developed postero-lateral projections (Fig. 22K, arrow) or without postero-lateral projections (Fig. 22L).

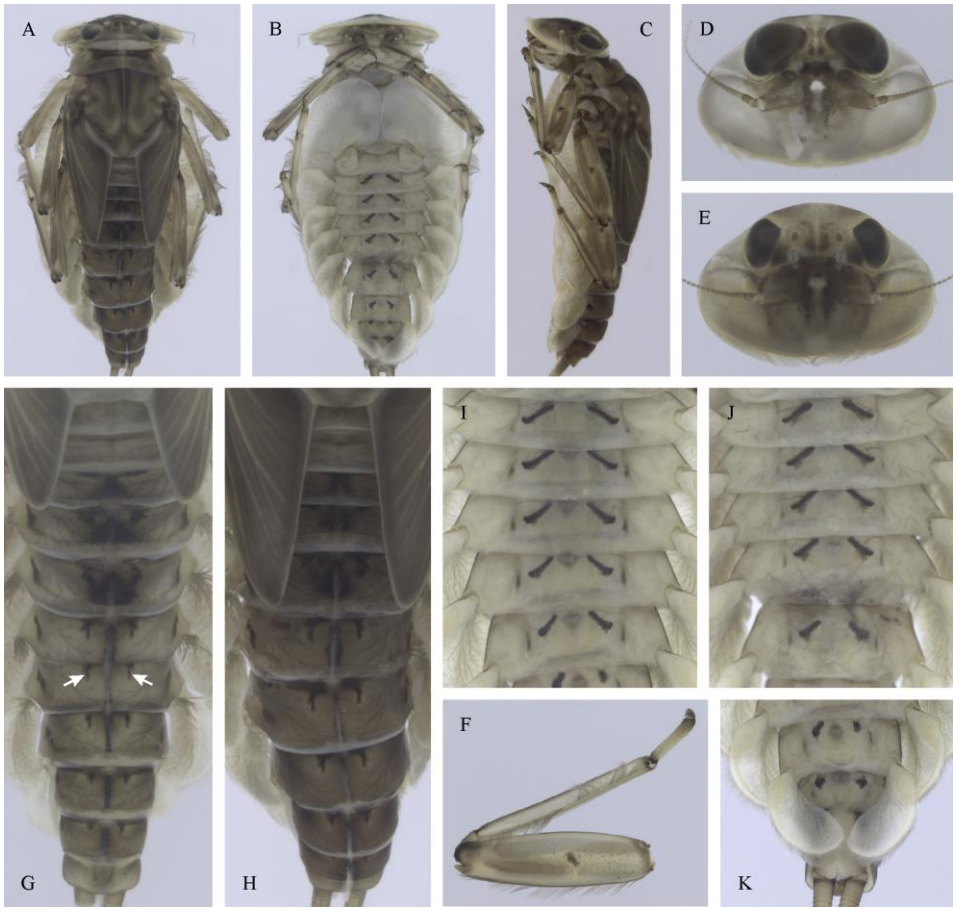


Figure 21. *Epeorus* (*Caucasiron*) *iranicus*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F, middle leg in dorsal view; G–H, abdominal terga (arrows point antero-lateral stripes of medial macula); I–J, abdominal sterna II–VI; K, gills VII (in natural position from ventral view).

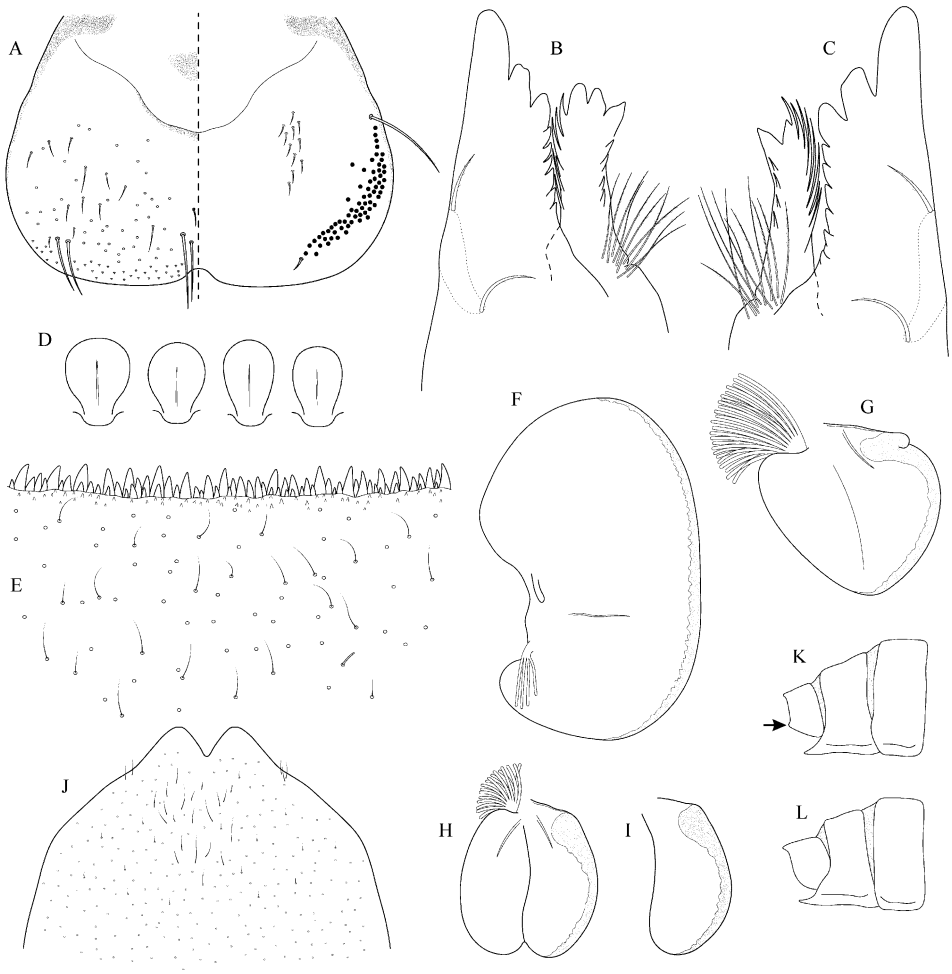


Figure 22. *Epeorus* (*Caucasiron*) *iranicus*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII; F, gill I; G, gill III; H, gill VII (flattened on slide); I, gill VII (in natural position from ventral view); J, sternum IX of female; K–L, abdominal segments VIII–X in lateral view (arrow points on postero-lateral projection).

Remarks: ***Morphology.*** Coloration pattern on abdominal sterna as in *E.* (*C.*) *caucasicus* (p. 159) similar pattern in *E.* (*C.*) *zagrosicus* (p. 203).

***Taxonomy*.** Species described as a subspecies of *E.* (*C.*) *caucasicus* based on larvae collected in the Alborz Mts. Elevated to species level by Hrivniak et al. (2020a) based on a phylogenetic analysis of all Caucasian *Epeorus* (*Caucasiron*) species. The holotype probably lost. Paratypes are currently deposited in SMNS and Biology Centre of the Czech Academy

of Sciences, Institute of Entomology, České Budějovice, Czech Republic (IECA). Imagines and subimagines not described.

***Epeorus* (*Caucasiron*) *sinitshenkovae* (Braasch & Zimmerman 1979)**

Iron sinitshenkovae Braasch & Zimmermann, 1979
Epeorus (*Iron*) *sinitshenkovae* (Braasch & Zimmermann, 1979); in Kluge (1995)
Epeorus (*Caucasiron*) *sinitshenkovae* (Braasch & Zimmermann, 1979); in Kluge (2004)
Figs 23–25

Type locality: Russia, central Greater Caucasus, right tributary of Dongoserun River.
Distribution: Georgia, Russia. Species endemic to the Greater Caucasus (Fig. 23).
Habitat: Larvae inhabit small streams and rivers at middle and high altitude in the western and central Greater Caucasus. Altitudinal range of sampling sites 760–2580 m a.s.l. (Fig. 23). Most frequently found above 1800 m a.s.l. Often syntopic with *E.* (*C.*) *alpestris* and at lower altitude with *E.* (*C.*) *soldani*.

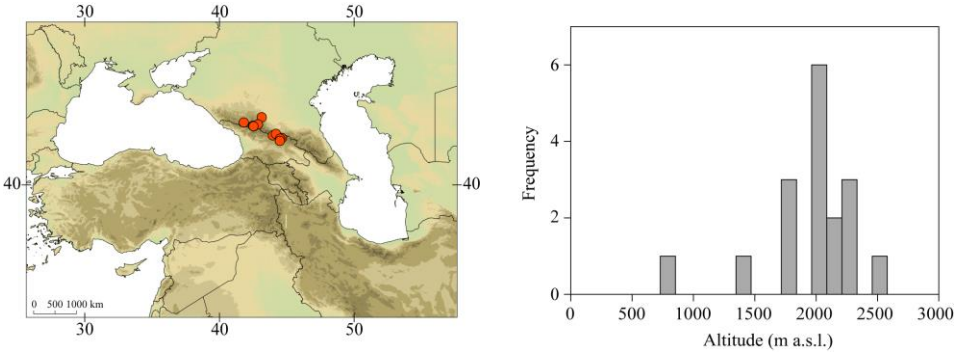


Figure 23. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *sinitshenkovae*.

Main morphological diagnostics of larvae: (i) abdominal terga V–VII with narrowed triangular medial macula and a pair of anterolateral spots (Fig. 24H; arrows); **(ii)** abdominal sterna without coloration pattern (Fig. 24B, I); **(iii)** femora without medial hypodermal spot (Fig. 24F); **(iv)** gill

plates VII (in natural position from ventral view) narrow (Figs 24J; 25H – K); **(v)** gill plates III with poorly developed projections (Fig. 25G); **(vi)** setae on abdominal terga slightly widened, often elongated (Fig. 25E); **(vii)** tergum X without postero-lateral projections Fig. 25L).

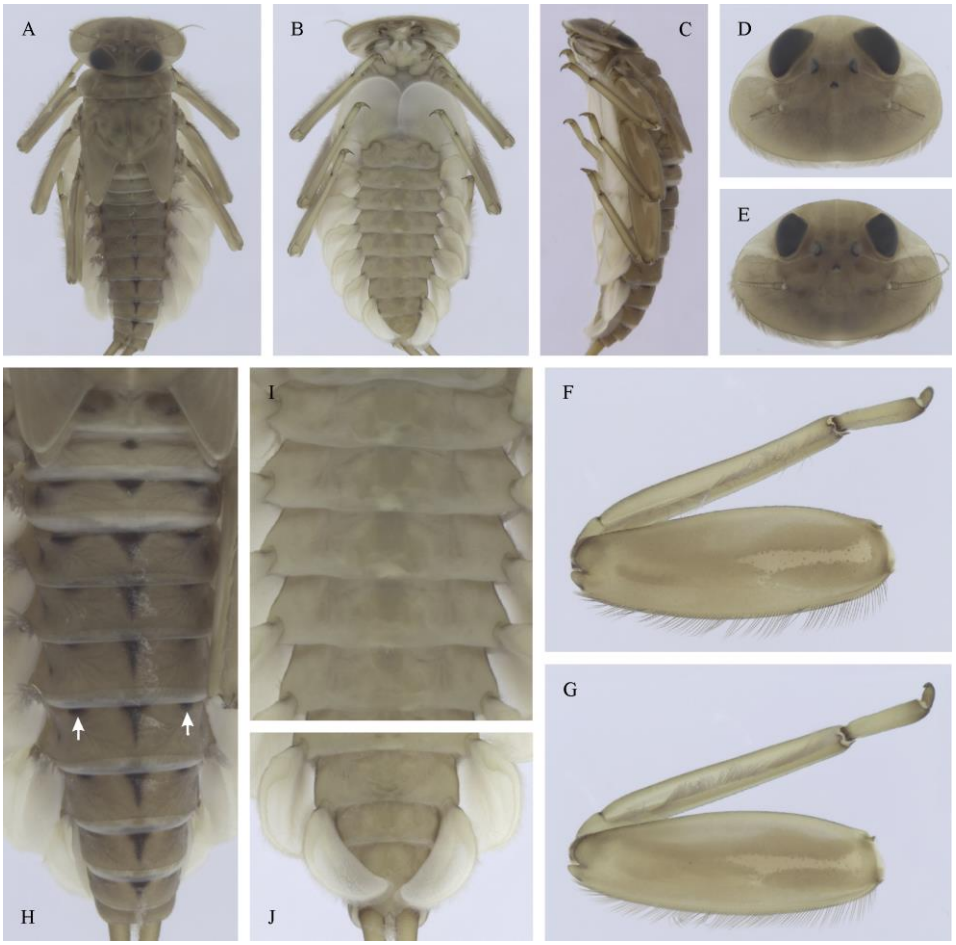


Figure 24. *Epeorus* (*Caucasiron*) *sinitshenkovae*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F–G, middle leg in dorsal view; H, abdominal terga (arrows point on anterolateral spots); I abdominal sterna II–VI; J, gills VII (in natural position from ventral view).

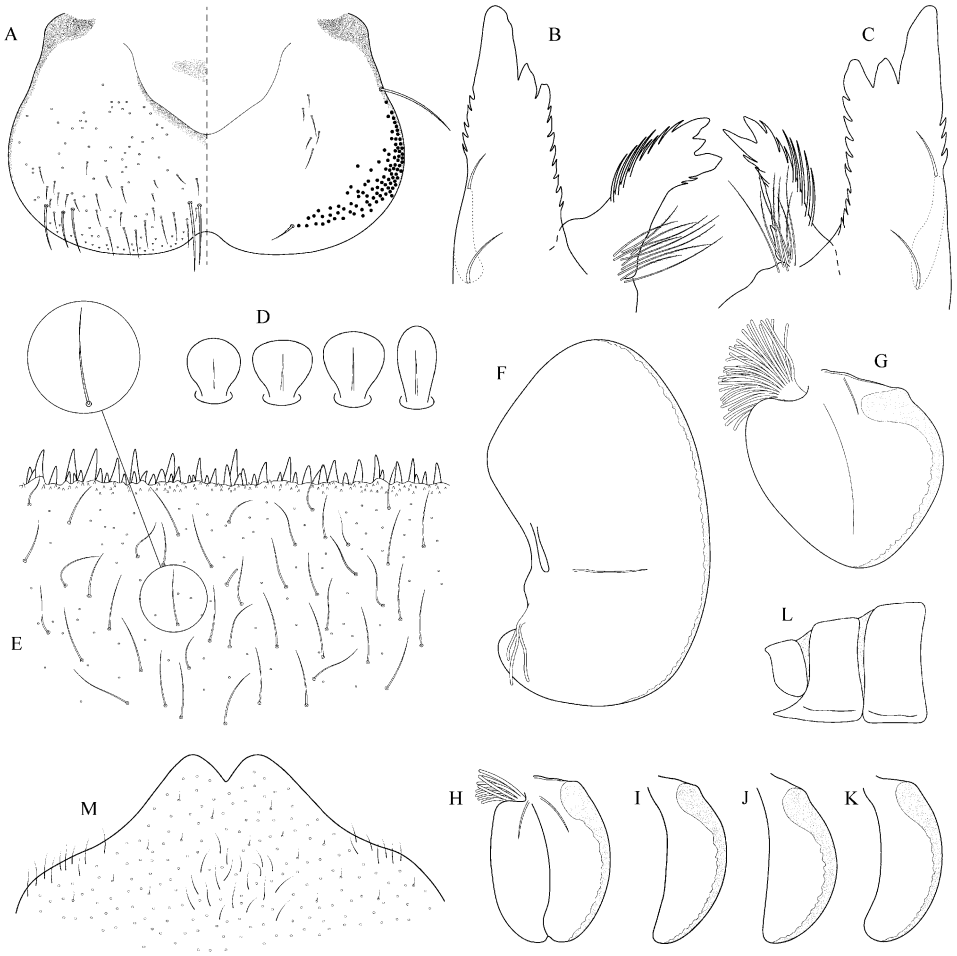


Figure 25. *Epeorus* (*Caucasiron*) *sinitshenkovae*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII with detail of slightly widened elongated seta; F, gill I; G, gill III; H, gill VII (flattened on slide); I–K, gill VII (in natural position from ventral view), variability in shape; L, abdominal segments VIII–X in lateral view; M, sternum IX of female.

Remarks: ***Taxonomy.*** Original description based on male imago and larva from the Greater Caucasus (Braasch and Zimmermann 1979). The type series is currently deposited in SMNS. Female imago not described in detail. The association of imagines and larvae based on the colour pattern of abdominal terga and sterna in material from the same locality.

***Epeorus* (*Caucasiron*) *longimaculatus* (Braasch, 1980)**

Iron longimaculatus Braasch, 1980

Epeorus (*Caucasiron*) *longimaculatus* (Braasch, 1980); in Kluge (2004)

Figs 26–28

Type locality: Georgia, central Greater Caucasus, tributary of Aragvi river, 3 km above Pasanauri (1400–1500 m a.s.l.).

Distribution: Georgia. Species endemic to the Greater Caucasus (Fig. 26).

Habitat: Larvae inhabit small streams and rivers at middle altitude in the central Greater Caucasus. Altitudinal range of sampling sites 903–1193 m a.s.l. (Fig. 26).

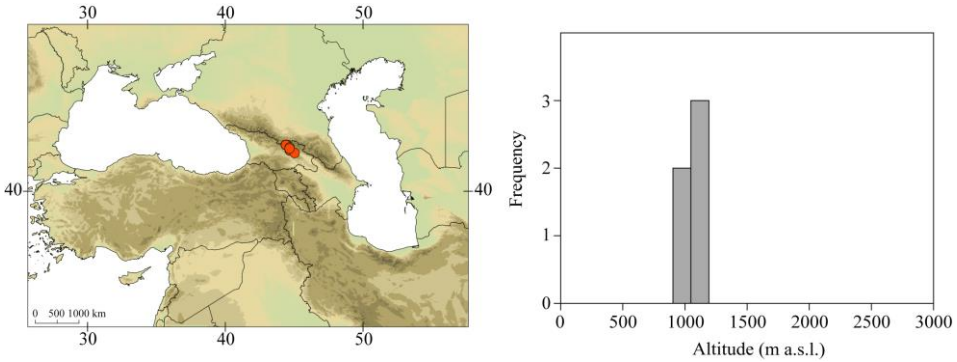


Figure 26. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *longimaculatus*.

Main morphological diagnostics of larvae: (i) femora with elongated medial hypodermal spot (Fig. 27F–H); **(ii)** setae on abdominal terga wide (Fig. 28E); **(iii)** gill plates III without distinct projections (Fig. 28G); **(iv)** gill plates VII (in natural position from ventral view) narrow (Figs 27L–M; 28H–L); **(v)** denticles along posterior margin of tergum VII narrowed (Fig. 28E); **(vi)** abdominal terga V–VII with narrowed triangular medial macula (Fig. 27I–K); **(vii)** abdominal sterna without coloration pattern (Fig. 27B, N–P); **(viii)** tergum X without postero-lateral projections (Fig. 28N); **(ix)** shape of head of male ellipsoid (Fig. 27D).

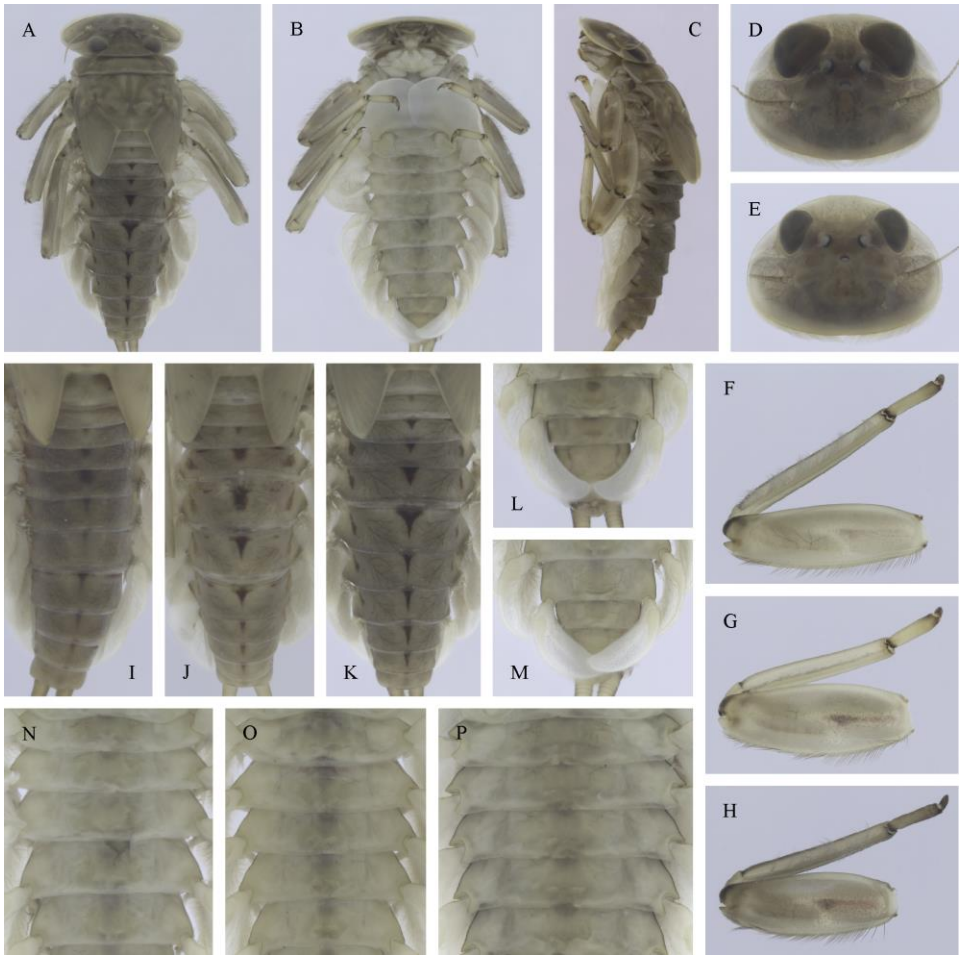


Figure 27. *Epeorus* (*Caucasiron*) *longimaculatus*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F–H, middle leg in dorsal view; I–K, abdominal terga; L–M, gills VII (in natural position from ventral view); N–P, abdominal sterna II–VI.

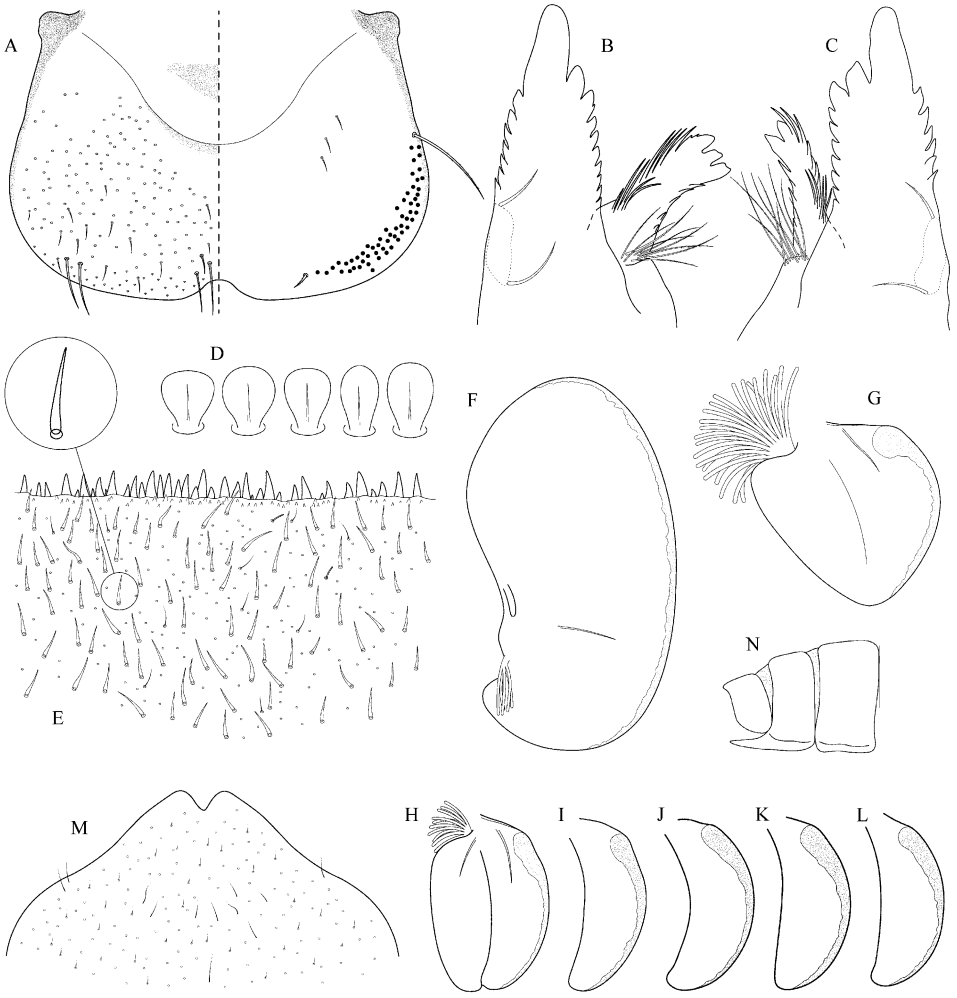


Figure 28. *Epeorus* (*Caucasiron*) *longimaculatus*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII with detail of wide seta; F, gill I; G, gill III; H, gill VII (flattened on slide); I–L, gill VII (in natural position from ventral view), variability in shape; M, sternum IX of female; N, abdominal segments VIII–X in lateral view.

Remarks: ***Taxonomy.*** Species described based on male subimago and larva collected in central Greater Caucasus (Braasch 1980). The type series is currently deposited in SMNS. Larva associated with the subimago according to the coloration of abdomen. Male and female imagines not described.

***Epeorus* (*Caucasiron*) *insularis* (Braasch, 1983)**

Iron znojkoi insularis Braasch (1983)

Epeorus (*Caucasiron*) *insularis* in Hrivniak et al. (2020b)

Figs 29–31

Type locality: Greece**,** Samos Island, stream east of Pirgos, 37°3'N/26°49'E; 300 m a.s.l.

Distribution: Recently known only from few sites in Samos Island (Fig. 29).

Habitat: Larvae inhabit small forested streams at 128–440 m a.s.l. (Fig. 29).

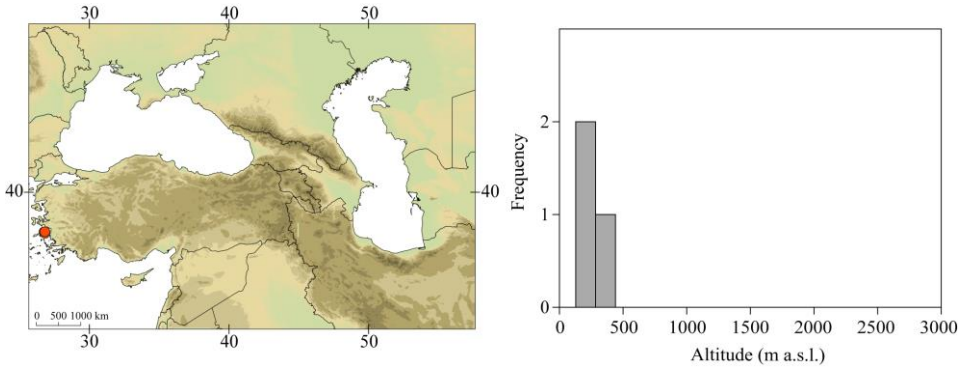


Figure 29. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *insularis*.

Main morphological diagnostics of larvae: (i) abdominal terga V–VII with T-shaped medial macula (Fig. 30I); **(ii)** abdominal sterna V–VII with reddish to brownish longitudinal stripe (Fig. 30B, J); **(iii)** tergum X without postero-lateral projections (Fig. 31J); **(iv)** gill plates VII (in natural position from ventral view) narrow (Figs 30K; 31H–I); **(v)** gill plates III with well-developed projections (Fig. 31G); **(vi)** setae on abdominal terga hair-like (Fig. 31E); **(vii)** denticles along posterior margin of tergum VII relatively short and poorly sclerotized (Fig. 31 E).

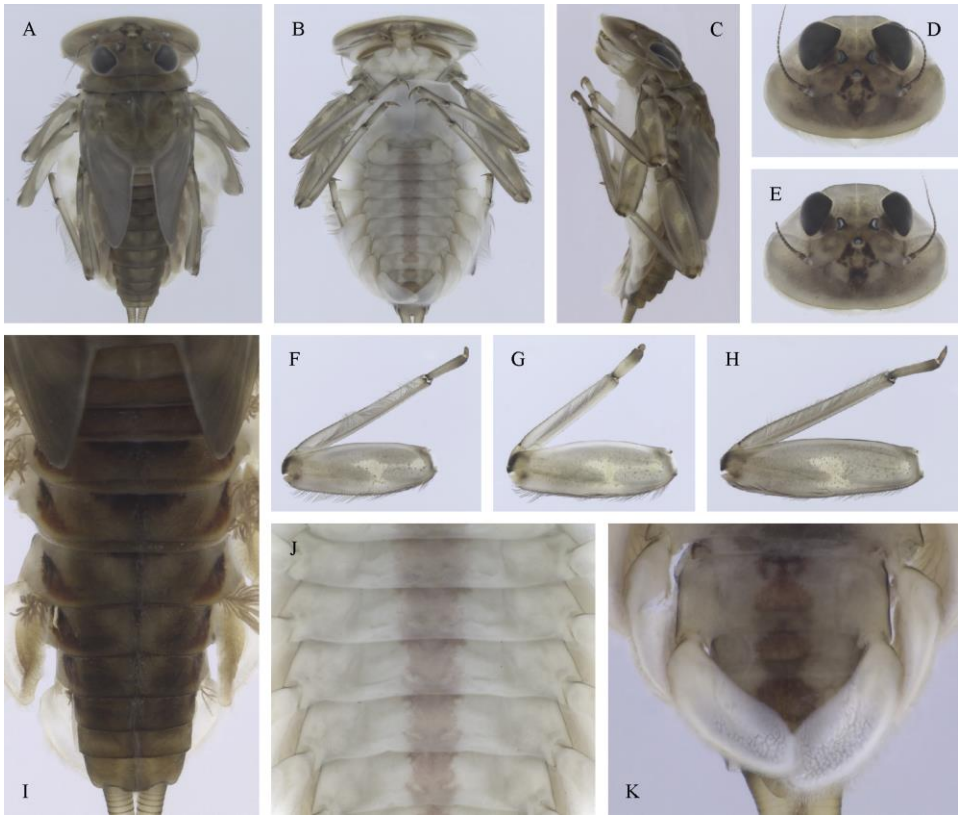


Figure 30. *Epeorus* (*Caucasiron*) *insularis*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F–H, middle leg in dorsal view; I, abdominal terga; J, abdominal sterna II–VI; K, gills VII (in natural position from ventral view).

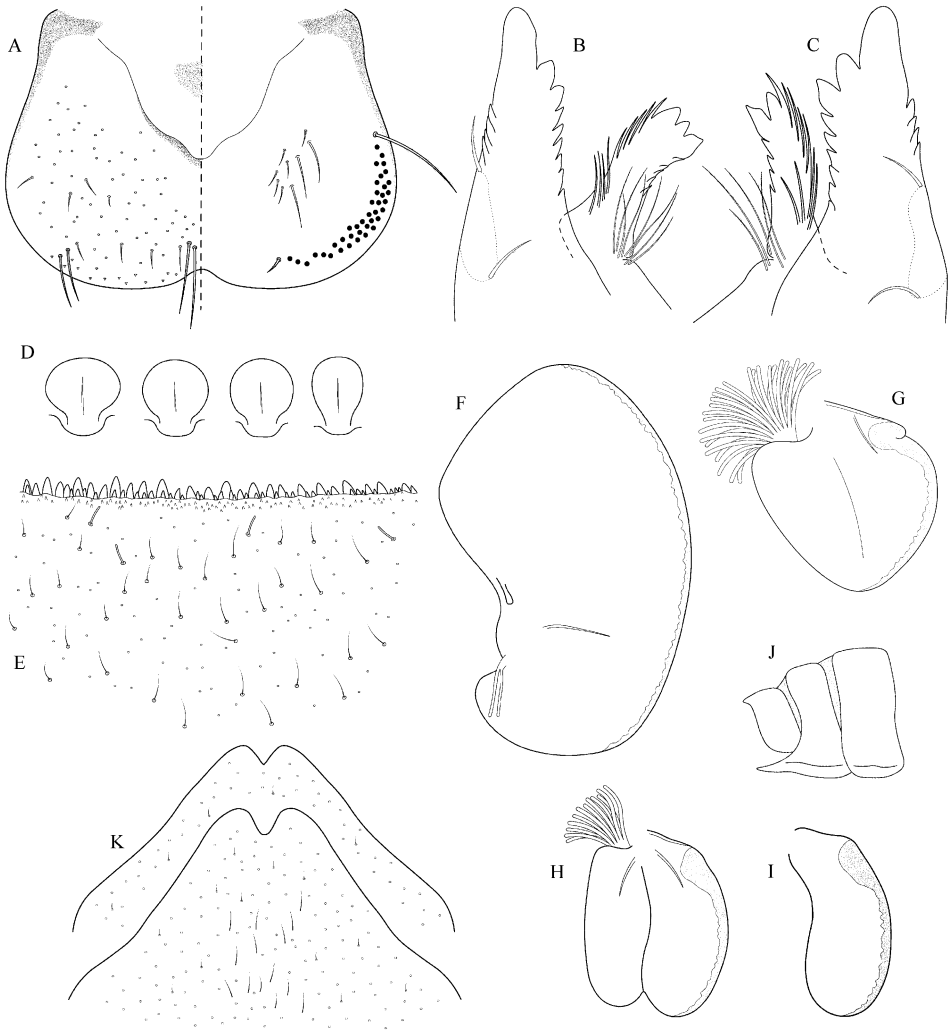


Figure 31. *Epeorus* (*Caucasiron*) *insularis*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII; F, gill I; G, gill III; H, gill VII (flattened on slide); I, gill VII (in natural position from ventral view); J, abdominal segments VIII–X in lateral view; K, sternum IX of female with observed variability.

Remarks: *Morphology*: Coloration of abdominal terga and sterna as in *E.* (*C.*) *znojkoi* s.l. (p. 162).

Taxonomy. Species described based on imagines as a subspecies of *E.* (*C.*) *znojkoi* (Braasch 1983). Elevated to species level in Hrivniak et al. (2020b) based on a phylogenetic analysis of all Caucasian *Epeorus* (*Caucasiron*) species. The type series is currently deposited in SMNS.

***Epeorus* (*Caucasiron*) *bicolliculatus* Hrivniak, 2017**

Epeorus alpicola (Eaton, 1871); in Türkmen and Kazancı (2015), partim
Epeorus sylvicola (Pictet, 1865); in Türkmen and Kazancı (2015), partim
Epeorus (*Caucasiron*) sp.; in Martynov et al. (2016)
Figs 32–34

Type locality: Georgia, Autonomous Republic of Adjara, vicinity of Chakhati village, Kintrishi River; 41°45'43"N/41°58'34"E; 325 m a.s.l.
Distribution: Georgia, north-eastern Turkey, Armenia (Fig. 32).
Habitat: Larvae inhabit streams and rivers of different sizes, from to middle-sized rivers at low altitude to small streams at high altitudes. Altitudinal range of sampling sites 40–1804 m a.s.l. (Fig. 32).

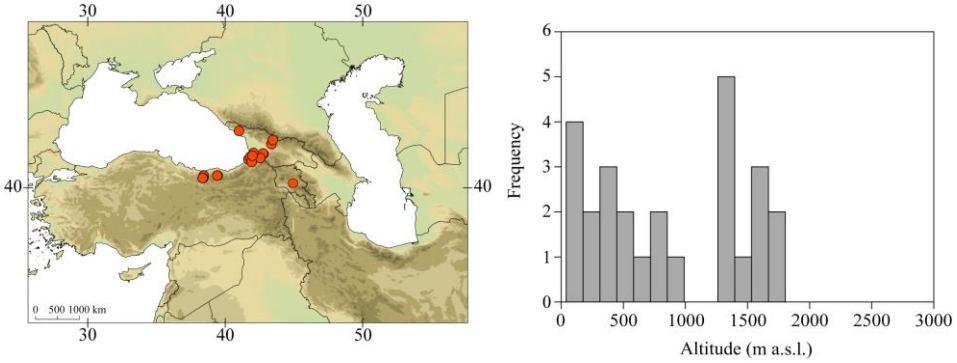


Figure 32. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *bicolliculatus*.

Main morphological diagnostics of larvae: (i) abdominal terga II–IX with paired postero-medial protuberances (Fig. 33H, arrows); **(ii)** abdominal terga V–VII with stripe-like medial macula, often anteriorly and posteriorly widened and with antero-lateral stripes (Fig. 33G–H); **(iii)** abdominal sterna as on Fig. 33B, J–L; **(iv)** setae on abdominal terga wide (Fig. 34E); **(v)** gill plates VII (in natural position from ventral view) narrow (Figs 33I; 34I–J); **(vi)** femora without medial hypodermal spot (Fig. 33F); **(vii)** tergum X without postero-lateral projections (Fig. 34H); **(viii)** gill plates III with well-developed projections (Fig. 34G).



Figure 33. *Epeorus* (*Caucasiron*) *bicolliculatus*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F, middle leg in dorsal view; G, abdominal terga; H, abdominal terga VI–X (arrows point on postero-medial protuberances); I gills VII (in natural position from ventral view); J–L; abdominal sterna II–VI.

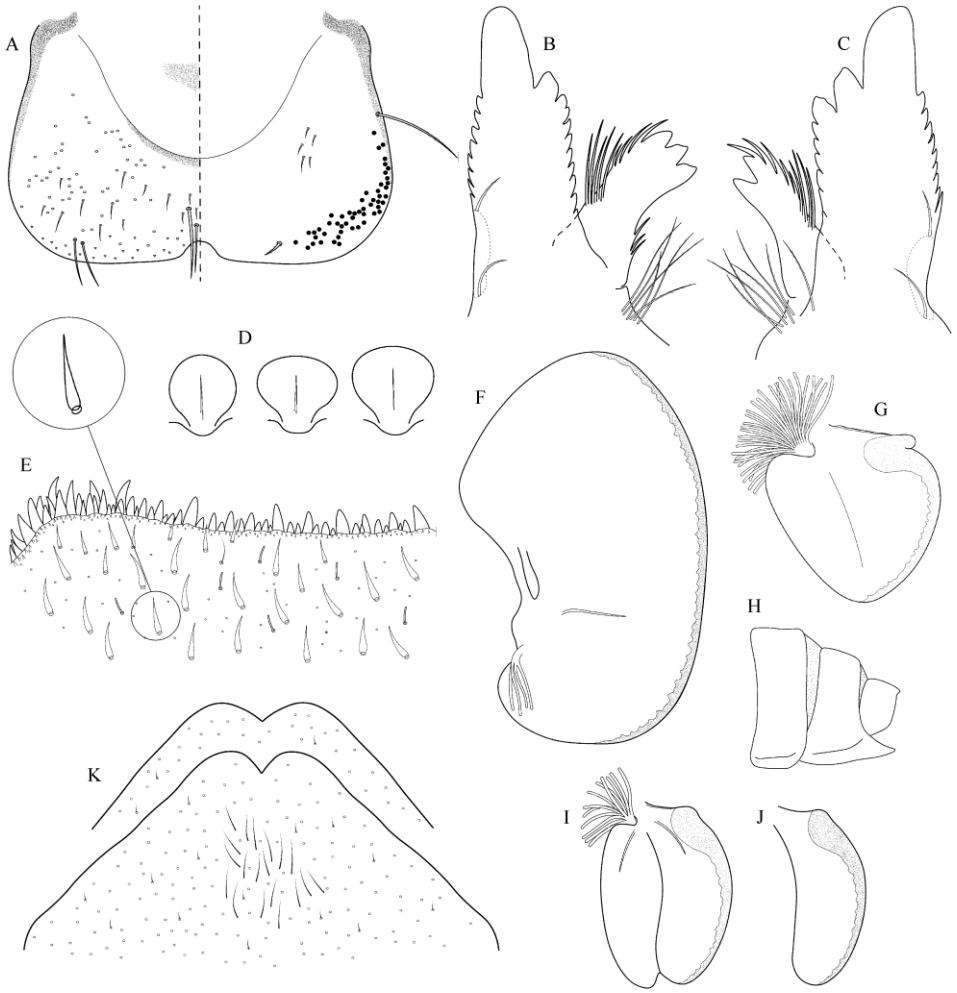


Figure 34. *Epeorus* (*Caucasiron*) *bicolliculatus*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII with detail of wide setae; F, gill I; G, gill III; H, abdominal segments VIII–X in lateral view; I, gill VII (flattened on slide); J, gill VII (in natural position from ventral view); K, sternum IX of female with observed variability.

Remarks: ***Taxonomy.*** Species described based on the larva, male subimago and imago (associated by rearing), female imago (associated by DNA analysis) and eggs. The type series is currently deposited in IECA.

***Epeorus* (*Caucasiron*) *turcicus* Hrivniak, Türkmen & Kazancı, 2019**
Figs 35–37

Type locality: Turkey, Artvin Province, Camili Village, Merata Plateau, unnamed mountain stream; 41°26'30"N/42°04'41"E; 2190 m a.s.l.
Distribution: North-eastern Turkey (Fig. 35). Known only from few sites in the Camili (Machakheli) District in Turkey.
Habitat: Larvae inhabit small streams at high altitude, 2080–2388 m a.s.l. (Fig. 35).

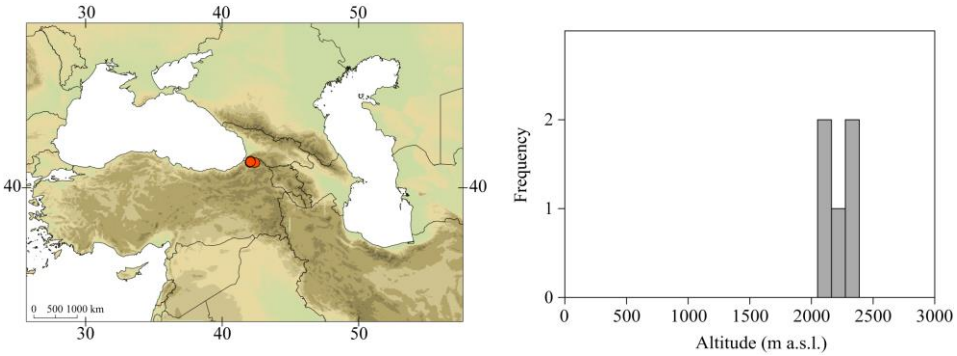


Figure 35. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *turcicus*.

Main morphological diagnostics of larvae: **(i)** femora with medial hypodermal spot (Fig. 36F); **(ii)** abdominal terga V–VII with stripe-like medial macula with lateral stripes extended dorso-posteriorly (Fig. 36G, arrows); **(iii)** abdominal sterna without coloration pattern (Fig. 36B, H); **(iv)** gill plates VII (in natural position from ventral view) narrow (Figs 36I; 37H–K); **(v)** setae on abdominal terga hair-like like (Fig. 37E); **(vi)** tergum X without postero-lateral projections (Fig. 37L); **(vii)** gill plates III with well-developed projections (Fig. 37G).



Figure 36. *Epeorus* (*Caucasiron*) *turcicus*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F, middle leg in dorsal view; G, abdominal terga (arrows point on dorso-posteriorly extended lateral stripes); H, abdominal sterna II–VI; I, gills VII (in natural position from ventral view).

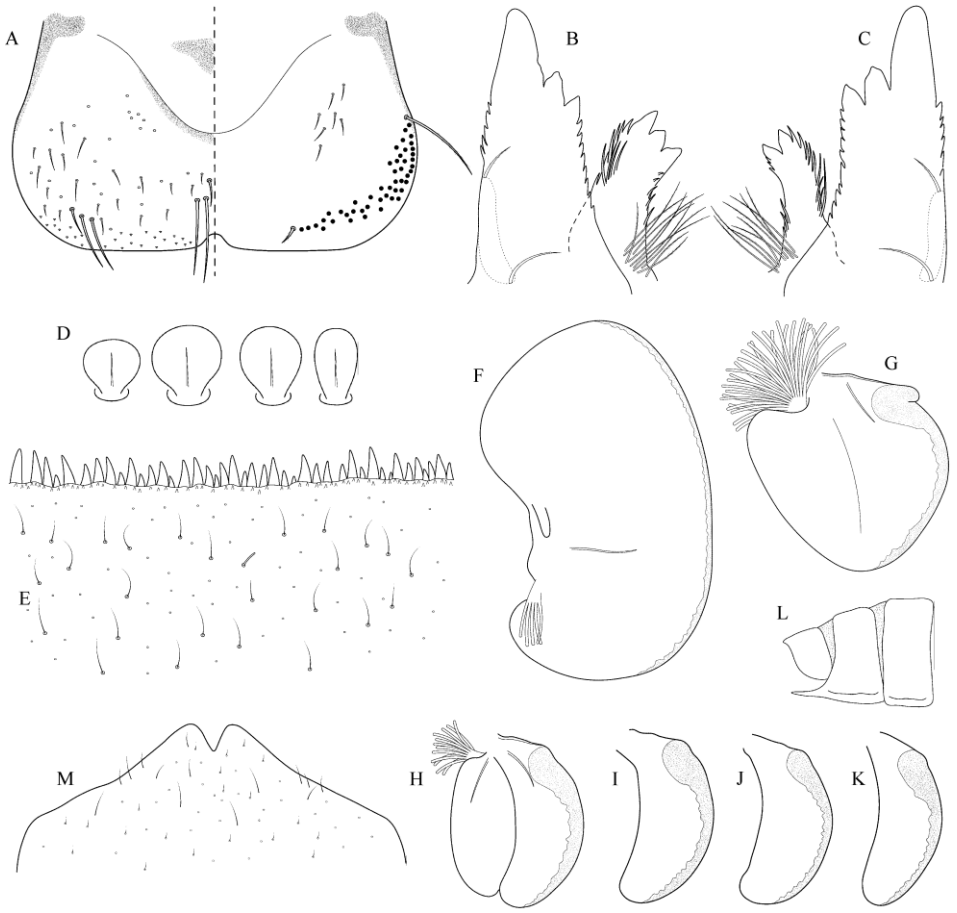


Figure 37. *Epeorus* (*Caucasiron*) *turcicus*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII; F, gill I; G, gill III; H, gill VII (flattened on slide); I–K, gill VII (in natural position from ventral view), variability in shape; L, abdominal segments VIII–X in lateral view; M, sternum IX of female.

Remarks: ***Taxonomy.*** Species described based on larvae. Imagines not described. The type series is currently deposited in IECA.

***Epeorus* (*Caucasiron*) *alborzicus* Hrivniak & Sroka, 2020**

Figs 38–40

Type locality: Iran, Mazandaran Province, Panjab village, unnamed brook (left tributary of Haraz River); 36°05'52.818"N/52°15'15.987"E (locality no. 152); 955 m a.s.l.

Distribution: Northern Iran. Species endemic to the Alborz Mountains (Fig. 38).

Habitat: Larvae inhabit small rivers at middle and high altitude in the central Alborz. Altitudinal range of sampling sites 750–2438 m a.s.l. (Fig. 38). Most frequently found at altitudes above 1000 m a.s.l. At high altitudes often syntopic with *E*. (*C*.) *iranicus.*

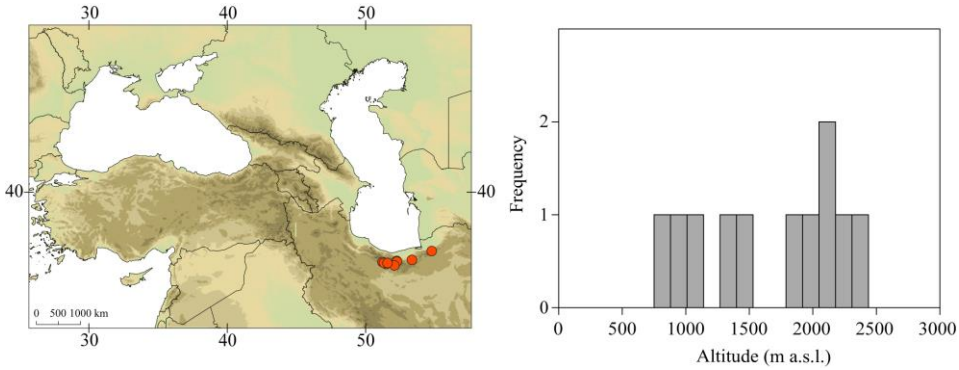


Figure 38. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *alborzicus*.

Main morphological diagnostics of larvae: (i) abdominal terga as on Fig. 39H–I; **(ii)** abdominal sterna II–VI with circular central medial macula of various intensity (Fig. 39B, L–N); **(iii)** tergum X with postero-lateral projections (Fig. 40K, arrow), **(iv)** femora without medial hypodermal spot (Fig. 39F–G); **(v)** gill plates VII (in natural position from ventral view) wide (Figs 39J–K; 40H–J); **(vi)** setae on abdominal terga hair-like (Fig. 40E); **(vii)** gill plates III with well-developed projections (Fig. 40G).



Figure 39. *Epeorus* (*Caucasiron*) *alborzicus*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F–G, middle leg in dorsal view; H–I, abdominal terga, J–K, gills VII (in natural position from ventral view); L–N, abdominal sterna II–VI.

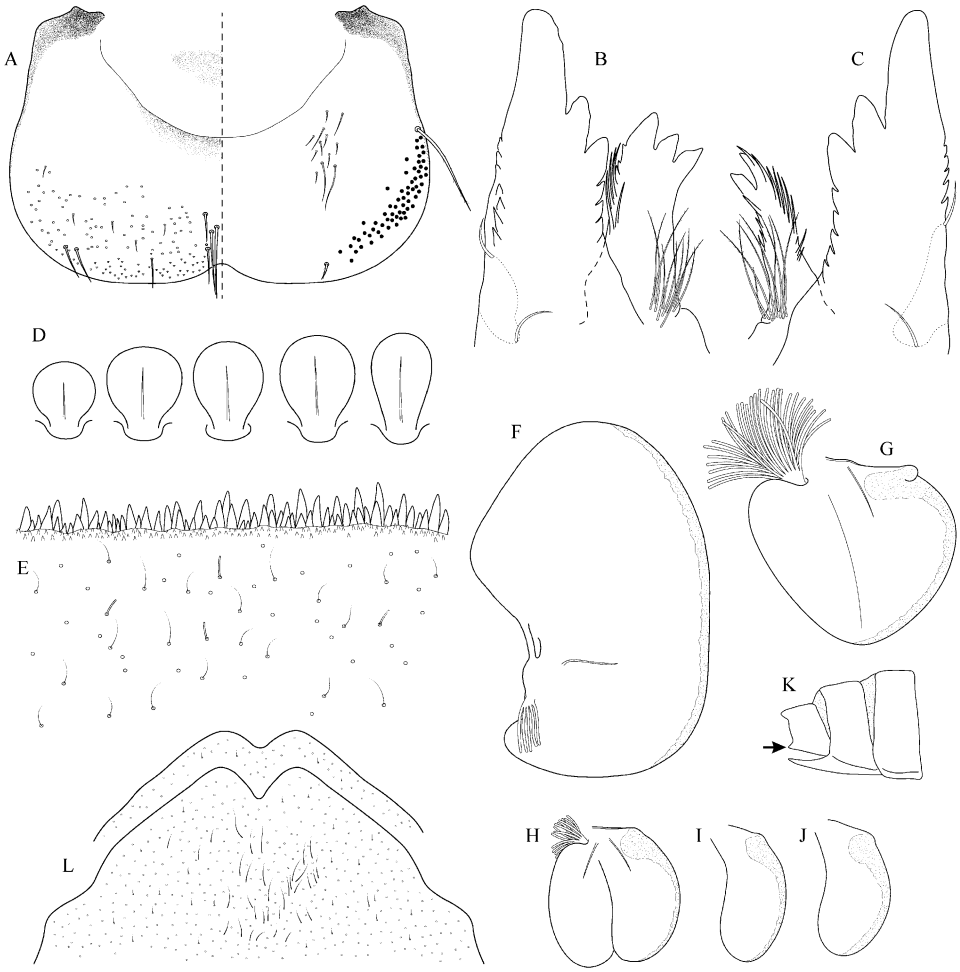


Figure 40. *Epeorus* (*Caucasiron*) *alborzicus*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII; F, gill I; G, gill III; H, gill VII (flattened on slide); I–J, gill VII (in natural position from ventral view), variability in shape; K, abdominal segments VIII–X in lateral view (arrow points on postero-lateral projection); L, sternum IX of female with observed variability.

Remarks: ***Taxonomy*.** Species described based on larvae. Imagines not described.

The type series is currently deposited in SMNS, IECA, and Natural History Museum and Genetic Resources, Department of Environment, Tehran, Iran (MMTT_DOE).

***Epeorus* (*Caucasiron*) *shargi* Hrivniak & Sroka, 2020**

Figs 41–43

Type locality: Iran, Golestan Province, Shirinabad village, unnamed river; 36°48'01.44"/ 55°01'05.78"E (locality no. 108); 740 m a.s.l.

Distribution: Northern Iran. Known only from three sites in the eastern Alborz (Fig. 41).

Habitat: Larvae inhabit streams at middle altitude, 740–1450 m a.s.l. (Fig. 41).

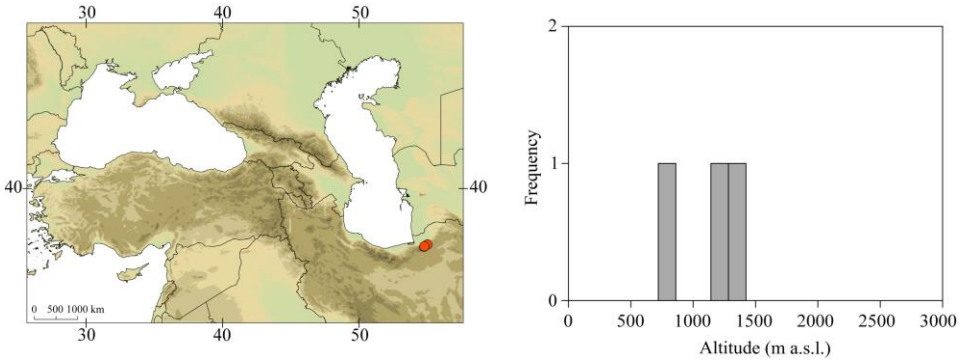


Figure 41. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *shargi*.

Main morphological diagnostics of larvae: (i) abdominal terga V–VII with triangular or T-shaped medial macula (Fig. 42I–K); **(ii)** abdominal sterna without coloration pattern (Fig. 42B, L); **(iii)** tergum X without postero-lateral projections (Fig. 43J); **(iv)** femora with medial hypodermal spot (Fig. 42E–H); **(v)** gill plates VII (in natural position from ventral view) wide (Figs 42M; 43H–I); **(vi)** setae on abdominal terga hair-like (Fig. 43E); **(vii)** gill plates III with well-developed projections (Fig. 43G); **(viii)** shape of head of male oval trapezoidal (Fig.42D).



Figure 42. *Epeorus* (*Caucasiron*) *shargi*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F–H, middle leg in dorsal view; I–K, abdominal terga; L, abdominal sterna II–VI; M, gills VII (in natural position from ventral view).

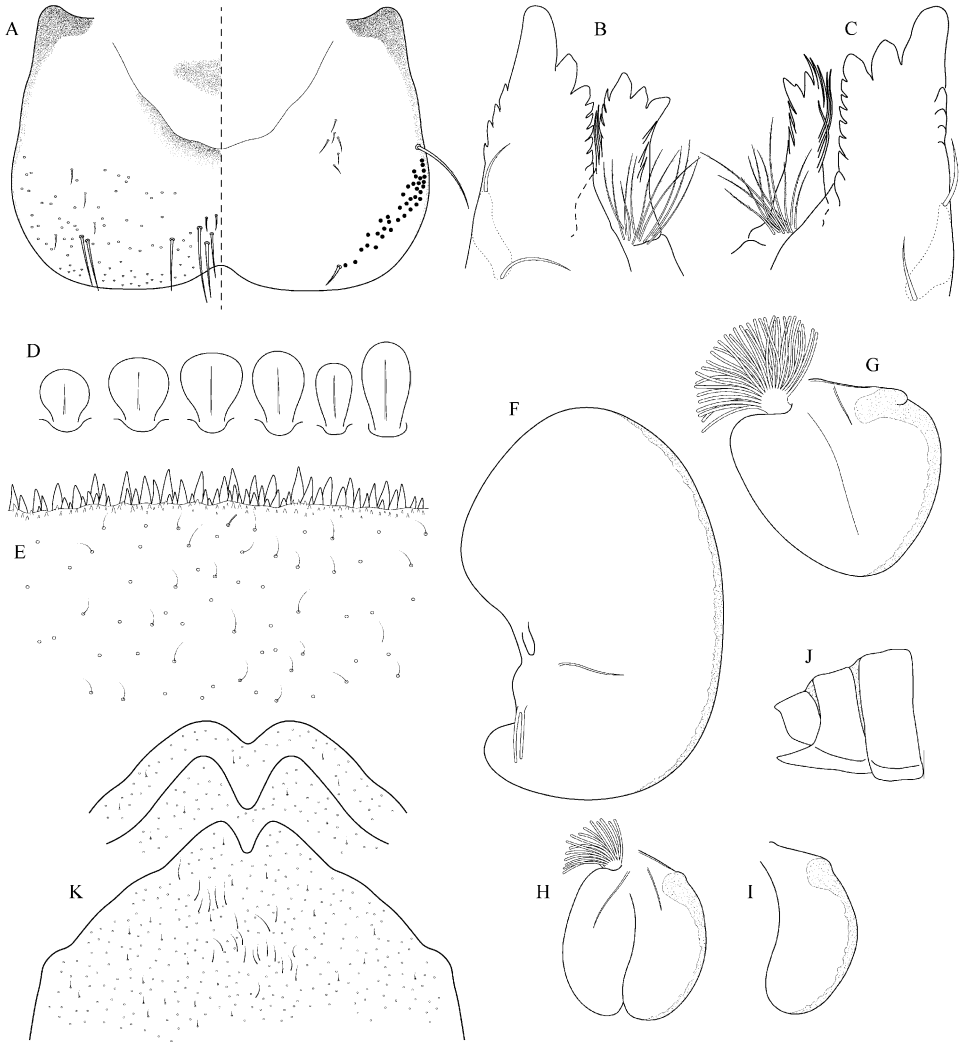


Figure 43. *Epeorus* (*Caucasiron*) *shargi*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII; F, gill I; G, gill III; H, gill VII (flattened on slide); I gill VII (in natural position from ventral view); J, abdominal segments VIII–X in lateral view; K, sternum IX of female with observed variability.

Remarks: ***Taxonomy.*** Species described based on larvae. Imagines not described. The type series is currently deposited in SMNS, IECA, and MMTT_DOE.

***Epeorus* (*Caucasiron*) *zagrosicus* Hrivniak & Sroka, 2020**

Figs 44–46

Type locality: Iran, Chaharmahal and Bakhtiari Province, Dimeh village, Chehme-Dimeh River, N32°30'11.62", E50°13'04.45"; 2220 m a.s.l.

Distribution: South-western Iran. Known only from few sites in the central Zagros (Fig. 44).

Habitat: Larvae inhabit streams and rivers at high altitude, 1721–2402 m a.s.l. (Fig. 44).

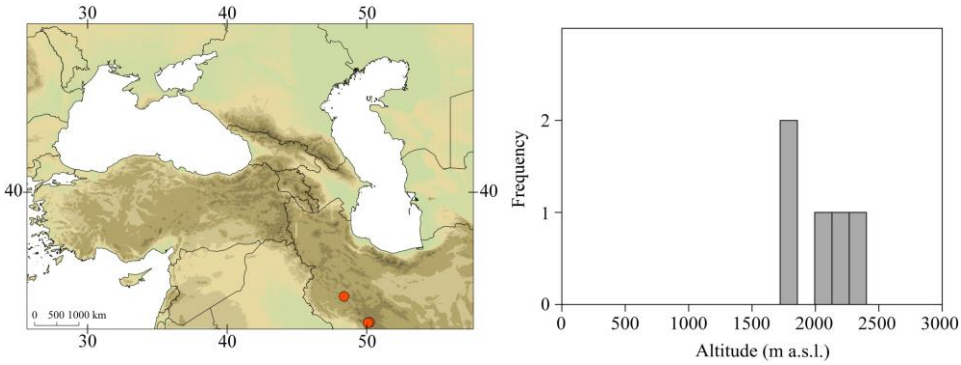


Figure 44. Geographical (left) and vertical (right) distribution of *Epeorus* (*Caucasiron*) *zagrosicus*.

Main morphological diagnostics of larvae: (i) abdominal sterna II–VI with a pair of anteriorly widened oblique stripes (Fig. 45B, I, arrows); **(ii)** abdominal terga V–VII with triangular, stripe-like or crown-like medial macula (Fig. 45G–H), often with lateral stripes extended dorso-posteriorly (Fig. 45H, arrows); **(iii)** tergum X with postero-lateral projections (Fig. 46L–M, arrows); **(iv)** femora with medial hypodermal spot (Fig. 45F); **(v)** setae on abdominal terga hair-like (Fig. 46E); **(vi)** gill plates III with well-developed projections (Fig. 46G); **(vii)** gill plates VII (in natural position from ventral view) relatively wide (Figs 45J–K; 46H–K).

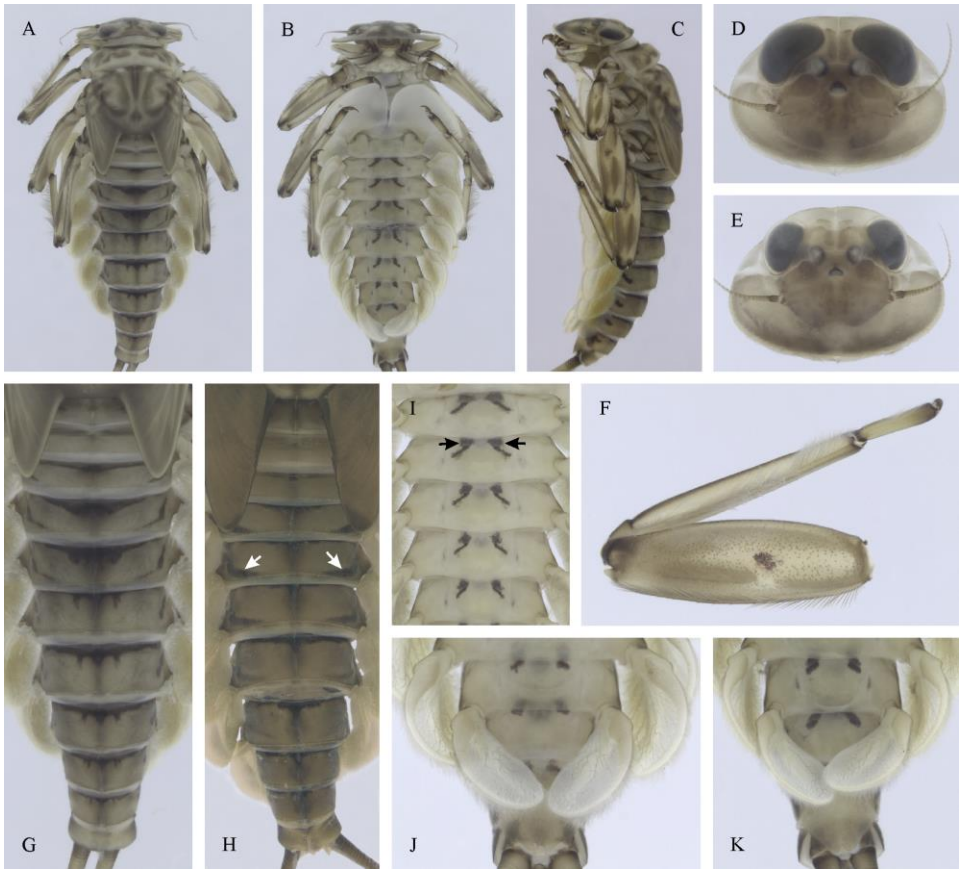


Figure 45. *Epeorus* (*Caucasiron*) *zagrosicus*, larva: A, habitus in dorsal view; B, habitus in ventral view; C, habitus in lateral view; D, head of male in dorsal view; E, head of female in dorsal view; F, middle leg in dorsal view; G–H, abdominal terga (arrows point on dorso-posteriorly extended lateral stripes); I, abdominal sterna II–VI; J–K, gills VII (in natural position from ventral view).

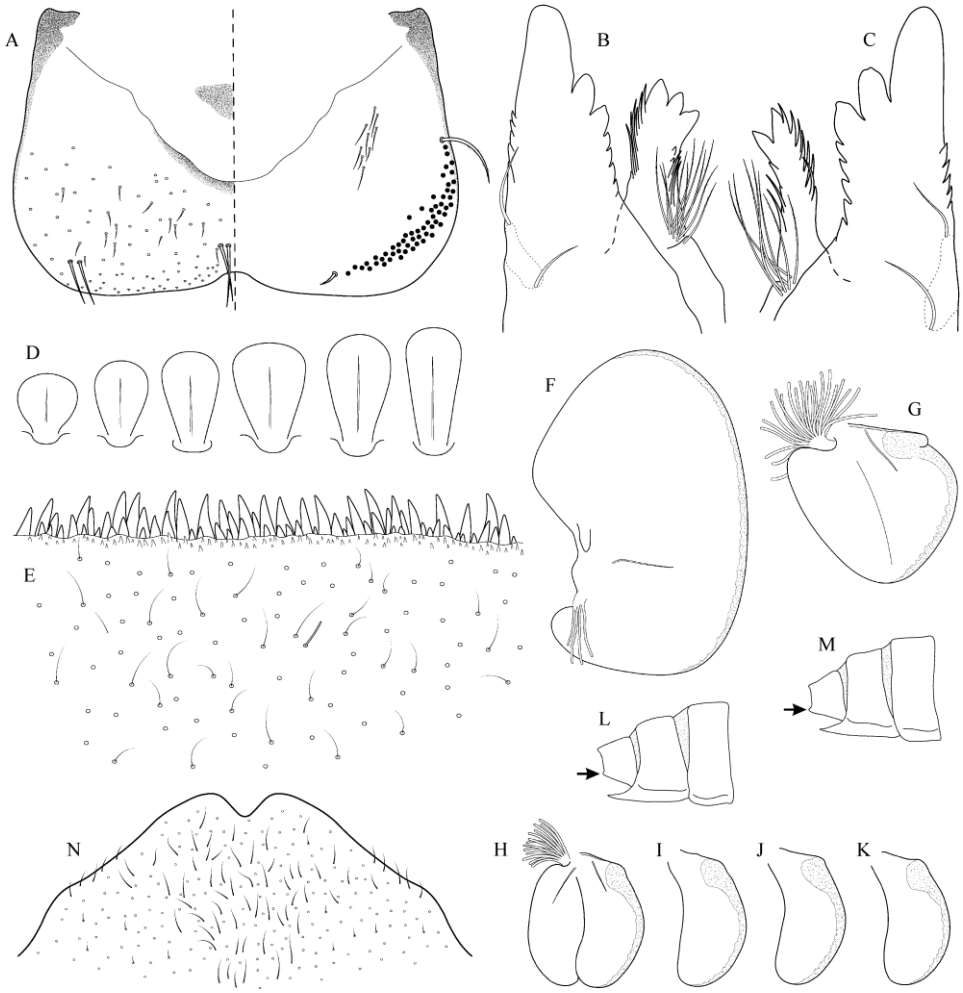


Figure 46. *Epeorus* (*Caucasiron*) *zagrosicus*, larva: A, labrum (left half in dorsal view, right half in ventral view); B, incisors if left mandible; C, incisors of right mandible; D, setae on dorsal surface of femora; E, surface and posterior margin of abdominal tergum VII; F, gill I; G, gill III; H, gill VII (flattened on slide); I–K, gill VII (in natural position from ventral view), variability in shape; L–M, abdominal segments VIII–X in lateral view (arrow point on postero-lateral projections); N, sternum IX of female.

Remarks: ***Taxonomy.*** Species described based on larvae. Imagines not described. The type series is currently deposited in SMNS, IECA, and MMTT_DOE.

Distribution. The lineage *Caucasiron* sp. 2 detected by Hrivniak et al. (2020a) is distributed in Turkey (Taurus Mts.) and morphologically corresponds to *E.* (*C.*) *zagrosicus*. Therefore, *E.* (*C.*) *zagrosicus* may represent a species complex.

Conclusion

This contribution represents the first complex source of information for the routine identification of the larvae of all fifteen *Caucasiron* species occurring in the Caucasus and adjacent areas. It is possible that additional new *Caucasiron* species will be described from the region and some morphologically and genetically variable taxa, such as *E*. (*C*.) *znojkoi*, will be split into several species. This identification guide describes the state of the art at the time of publication.

All species of *Caucasiron* mayflies are charismatic animals, unique to the region. Some of them are endemic in a relatively limited area (especially for the Greater Caucasus and the Alborz Mts.) and may have considerable conservation value. We hope that this work will contribute to an increase in the knowledge of *Caucasiron* mayflies among hydrobiologists and ecologists. We would also like to encourage regional researchers to incorporate *Caucasiron* species as indicators in their biomonitoring surveys and water quality assessments.

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Table 1. Records of *Caucasiron* species from the Caucasus and adjacent areas. Abbreviations used: **A**-Armenia; **N**-Nakhchivan; **Te**-eastern Turkey; **T**-Turkey*; **G**-Georgia; **AZ**-Azerbadijan; **I**-Iran; **Iq**-Iraq; **Is**-Israel; **S**-Syria; **Rw**-Russia (western Caucasus); **Rc**-Russia (central Caucasus); **Sa**-Samos Island; **C**-Cyprus Island.
* without exact localization, not included in distribution maps.
** doubtful record not included in distribution maps.
*** unpublished record included in the distribution map.

Species	Records and references
E* (*C.*) *caucasicus (Tshernova, 1938)	**N**-Tshernova (1938); **Rw**,**A**,**G**,**N**-Sinitshenkova (1976); **AZ**-Sinitshenkova (1976); **Rc**-Cherchesova (2004); **Te**-Braasch (1981), Koch (1988), Kazancı (2001); **T**-Kazancı and Türkmen (2012)*, Türkmen and Kazancı 2015
E* (*C.*) *znojkoi (Tshernova, 1938)	**N**,**AZ**-Tshernova (1938); **G,Rc**-Sinitshenkova (1976), Braasch (1980), Cherchesova (2004), Khazeeva (2010); **A**-Sinitshenkova (1976); **Te**-Braasch (1981), Türkmen and Kazancı (2015), Aydinli (2017); **T**-Kazancı and Türkmen (2012)*; **I**-Bojková et al. (2018)
E* (*C.*) *nigripilosus (Sinitshenkova, 1976**)**	**G**-Sinishenkova (1976); **Rc**-Sinishenkova (1976), Braasch (1979), Khazeeva (2010); **Rw**-(Braasch 1979); **Iq**-Al-Zubaidi et al. (1987); **Te**-Kazanci (2001); **T**-Kazancı and Türkmen (2012)*; **I**-Hrivniak et al. (2020a, b); **C**-Hrivniak et al. (2020a, b)
E* (*C.*) *magnus (Braasch, 1978)	**Rw**-Braasch (1978,1980); **G**-Braasch (1980); **A**-Braasch (1980); **T**-Kazancı and Türkmen (2012)*, **Rc**-Cherchesova (2004)
E* (*C.*) *alpestris (Braasch, 1979)	**Rw**; **Rc**-Braasch (1979); **Te**-Kazanci (1986, 2001)**; **T**-Kazanci and Turkmen (2012)*, Aydinli (2017)**
E* (*C.*) *soldani (Braasch, 1979)	**Rw**; **Rc**-Braasch (1979)
E* (*C.*) *sinitshenkovae (Braasch & Zimmerman, 1979)	**Rc**; **Rw**; **G**-Braasch and Zimmermann (1979)
E* (*C.*) *longimaculatus (Braasch, 1980)	**G**-Braasch (1980), Martynov et al. (2016)**; **Te**-Kazanci and Braasch (1988)**, Kazanci (2001)**; **T**-Kazanci and Turkmen (2012)**
E*. (*C.*) *iranicus (Braasch & Soldán, 1979)	**I**-Braasch and Soldán (1979), Mousavi and Hakobyan (2017), Bojková et al. (2018), (Hrivniak et al. 2020a,b)
E*. (*C.*) *insularis (Braasch, 1983)	**Sa**-Braasch (1983), Hrivniak et al. (2020a,b)
E*. (*C.*) *bicolliculatus Hrivniak 2017	**G**-Martynov et al. (2016), Hrivniak et al. (2017); **Te**-Türkmen and Kazancı (2015), Hrivniak et al. (2017); **A**-Švihla (1975)***
E*. (*C.*) *turcicus Hrivniak, Türkmen & Kazancı, 2019	**Te**-Hrivniak et al. (2019)
E*. (*C.*) *alborzicus Hrivniak & Sroka, 2020	**I**-Hrivniak et al. (2020a)
E*. (*C.*) *shargi Hrivniak & Sroka, 2020	**I**-Hrivniak et al. (2020a)
E*. (*C.*) *zagrosicus Hrivniak & Sroka, 2020	**I**-Hrivniak et al. (2020a)

Chapter IV

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

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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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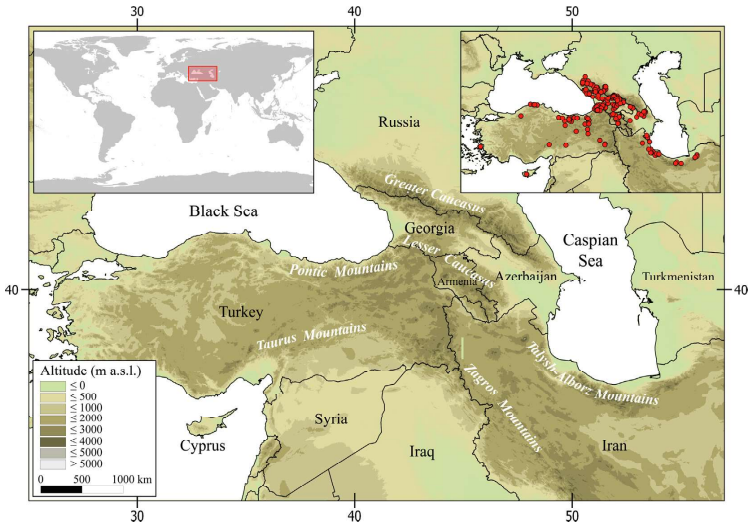


Fig. 1. Topographic map of the Caucasus and adjacent mountain ranges with the position of the study area (upper left part) and distribution of our sampling sites (upper right part).

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

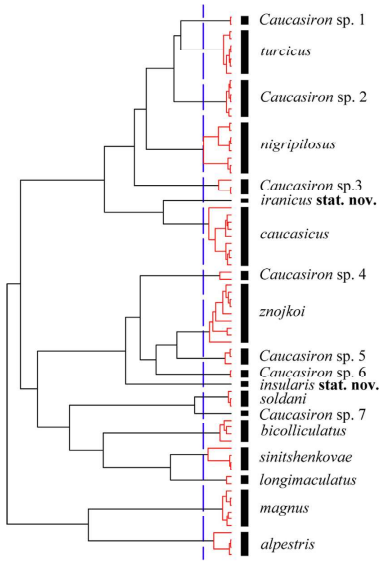


Fig. 2. Results of GMYC species delimitation analysis applied to MCC tree of the complete *COI* dataset of *Caucasiron*. Blue line indicates the point of transition from inter- to intraspecies branching events. (For interpretation of the references to colour in this figure legend, the reader is referred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

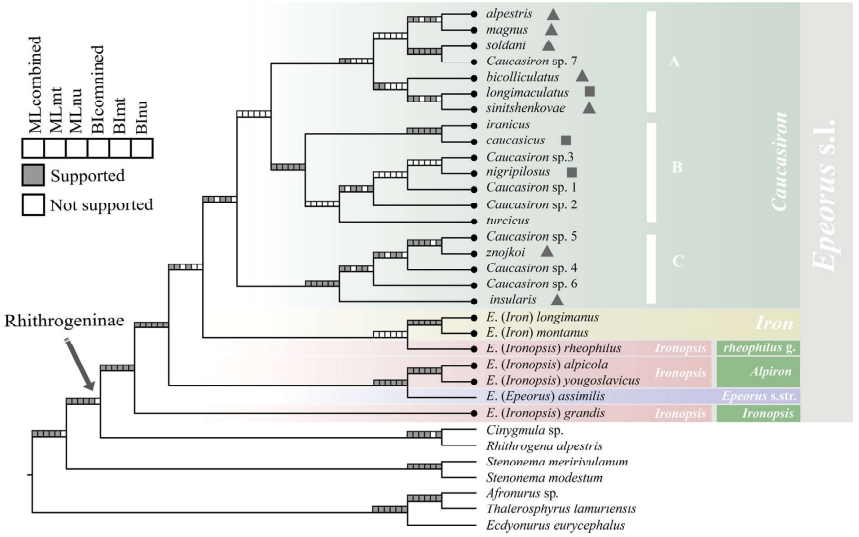


Fig. 3. Maximum likelihood (IQ-tree) cladogram reconstructed based on two mitochondrial (*COI*, 16S) and three nuclear (28S, EF-1α, wg) gene fragments. For each node, bootstrap value (> 70) and posterior probability (> 0.9) from all analyses are figured. Black circles at the end of terminal nodes in *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.,

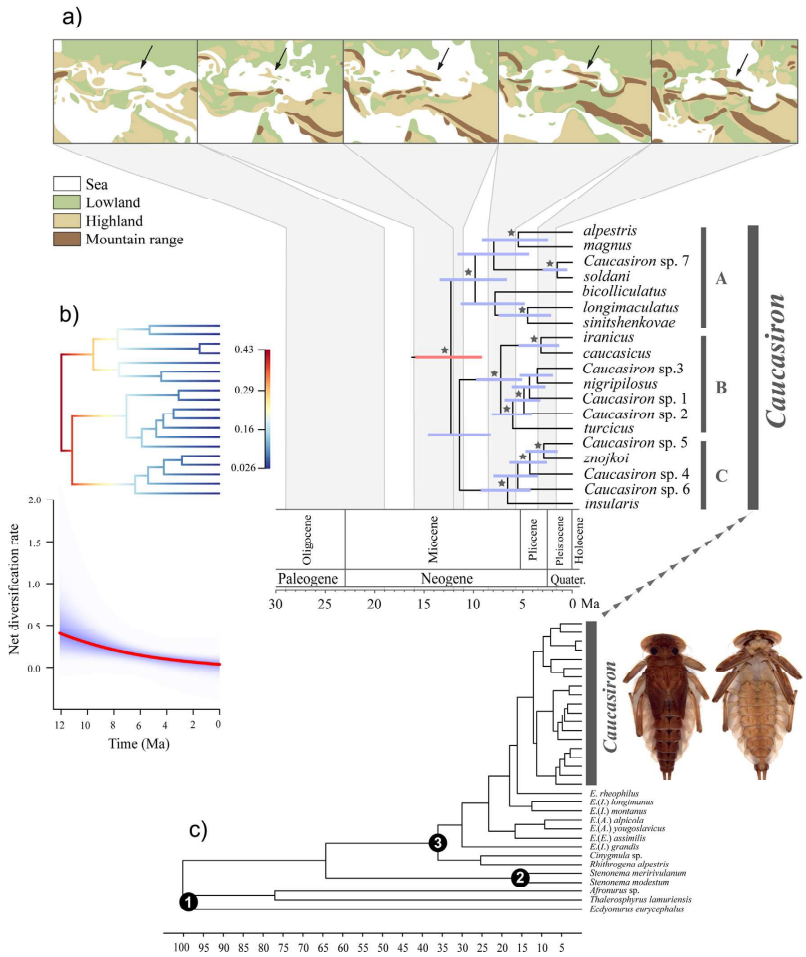


Fig. 4. A. Time-calibrated MCC tree of *Caucasiron* generated by BEAST from combined dataset. Stars above nodes indicate posterior probability (> 0.9). Basic geological scenarios in the Caucasus based on Popov et al. (2004, simplified) are figured at the top. Black arrow shows the position of Greater 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,

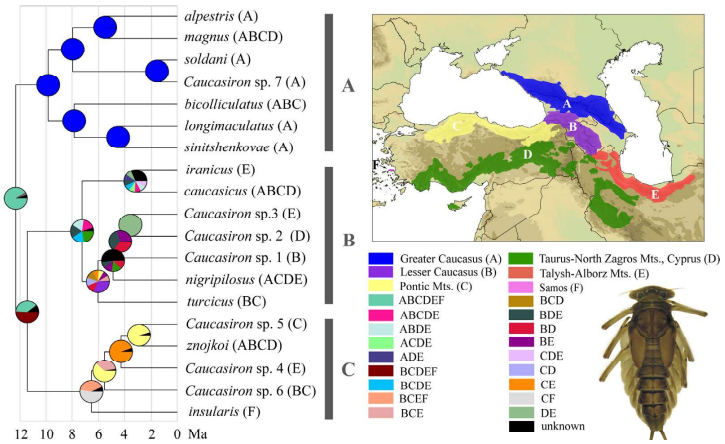


Fig. 5. Ancestral range estimation of *Caucasiron* based on RASP using S-DIVA analysis. Location of mountain ranges used in the analysis are shown in the map. Colours and letters codes under map correspond to colours on tree nodes. The recent distribution of species is given in parentheses next to species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

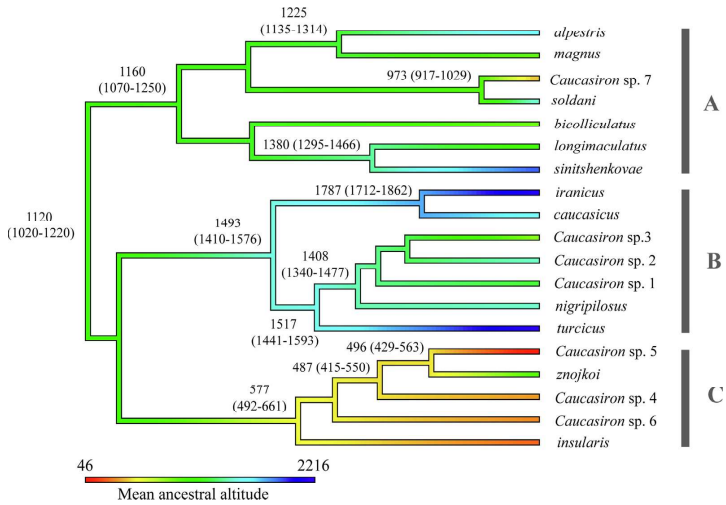


Fig. 6. MCC tree of *Caucasiron* constructed from combined dataset showing ancestral altitude estimates. Colour gradient corresponds to mean ancestral altitude. For each well-supported node mean ancestral altitude and 95% confidence interval are shown.

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

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Appendix A. Supplementary material

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

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Appendix A1. Phylogenetic relationships of the fossils used in the calibration.

Here we provide justification for the phylogenetic placement of the fossils used as calibration points. One of the fossils we use is not yet described; we therefore summarize its key morphological characters and provide photographic documentation. A comprehensive description is in preparation (Staniczek and Godunko in prep.). The remaining two fossils are already described; however, we provide some additional morphological characters based on our study of these fossils.

As the first calibration fossil, we used an undescribed fossil male imago in mid-Cretaceous Myanmar amber (98.79±0.62 Ma; Shi et al., 2012). This specimen is deposited in the collection of State Museum of Natural History Stuttgart, Germany under inventory number BU-163 and represents the oldest reliable representative of the subfamily Ecdyonurinae known so far (Staniczek and Godunko in prep.). Its placement within Heptageniidae can be confirmed by the presence of two pairs of intercalaries in the cubital field of the forewing (the basal pair being longer than the apical pair) and the presence of an distinctly articulated first tarsomere on all legs, which is not fused with the respective tibia (Fig. A.a–b). Moreover, penis lobes are deeply separated and exhibit a pattern of sclerites typical for Heptageniidae, with presence of ventral and outer sclerites; ventral side of genitalia bears developed prominent titilators medially.

The placement of this specimen within Ecdyonurinae is based on the shape of the median impression of furcasternum (MiFS), parallel-sided almost for its entire length, and slightly widened in the anterior part (Fig. A.e, see also Kluge, 2004: fig. 57A, B), in contrast to Rhithrogeninae and Heptageniinae, with a median impression of furcasternum narrowed

anteriorly. Additionally, in contrast to Heptageniinae with a distinct transverse ridge on the imaginal prosternum, the fossil specimen discussed herein possesses a flat prosternum without any ridges or projections (see Fig. A.c,e; Kluge, 2004, fig. 56A, B). The mesonotum of this Cretaceous Ecdyonurinae specimen possesses a clearly visible lateroparapsidal suture (LPs in the Fig. A.d), with its posterior end curved laterally from the medioparapsidal suture (MPs), reaching the area of the dorsal attachment of the posterior scuto-coxal muscle (S.CmP) as defined by Kluge (2004: fig. 63A–C). This character state is listed by Kluge (2004) as an apomorphy for Rhithrogenini s. Kluge (1988). Nevertheless, a relatively short, deep lateroparapsidal suture, slightly curved and not contiguous with medioparapsidal suture is described for the Eocene taxon *Nestormeus* Godunko, 2004, which is attributed to Ecdyonurinae (see Godunko, 2004, 2007). Therefore, we do not consider this character as an apomorphy of Rhithrogenini.

As a second calibration fossil, we used the species described as *Maccaffertium annae* Macadam & Ross, 2016. The relatively well-preserved male (holotype) and female (paratype) adult specimens are embedded in Mexican amber (Mexico, Chiapas, Early Miocene) (Macadam and Ross, 2016). The age of Mexican amber was indicated by Solórzano-Kraemer (2010) as ranging between 15 and 20 Ma, although Huys et al. (2016) stated that amber from the Finca Carmitto Member (Chiapas) was dated as Aquitanian (Early Miocene) in age (22.8 Ma), based on the analysis of strontium isotope ratios in some fossil remnants.

It should be noted that Zembrzuski and Anderson (2018) recently redefined the genus *Stenonema* Traver, 1933 to include also *Maccaffertium* Bednarik, 1979 as a subgenus. Their study did not deal with fossil taxa, thus we keep the original combination *Maccaffertium annae* for the purpose of this text. The attribution to *M. annae* to Heptageniidae can be confirmed by the presence of two pairs of intercalary veins in the cubital field of forewing (the shorter pair adjacent to Cu1) and five-segmented tarsi in all legs, the first tarsomere not being fused with the tibia. The placement of this fossil species within the genus *Maccaffertium* was justified by Macadam and Ross (2016) based on details of the forewing venation (e.g. cross veins of forewings are

crowded in the first three bulla interspaces, as indicated for *Maccaffertium* by Bednarik and McCafferty, 1979), and the genitalia closely resemble those in *S. mexicanum* Ulmer. Despite the detailed description of both fossil specimens of *M. annae*, the structure of thorax, which could provide additional evidence of their attribution to Heptageniinae was not discussed in the original description. According to our study of the high-resolution photographs, the ventral side of mesothorax in the holotype of *M. annae* exhibits furcasternal protuberances strongly convergent anteriorly (posterior parts of protuberances are poorly preserved and deformed, but clearly separated by a narrow impression, see Fig. A.f). The prosternum of the male holotype is characterized by a distinct transverse ridge and concave surface posterior to it (Fig. A.f). Both of these newly observed thoracic characters support the attribution of *M. annae* to Heptageniinae.

As a third calibration fossil, we used *Burshtynogena ferreci* Godunko & Sontag, 2004, whose description is based on an adult female specimen embedded in Eocene Baltic amber (Priabonian stratigraphic level, 37.2–33.9 Ma; Aleksandrova and Zaporozhets, 2008a, 2008b). This fossil was placed within the family Heptageniidae based on the presence of two pairs of intercalary veins in the cubital field of the forewing (the basal pair being longer than the apical pair), five-segmented tarsi with articulated first segment, and the absence of a terminal filament. The presence of a deep mesonotal suture, median impression of furcasternum narrowed anteriorly, and the lack of the transverse and longitudinal ridge of the prosternum (Godunko and Sontag, 2004) points to the assignment of *B. ferreci* within the subfamily Rhithrogeninae (sensu Webb and McCafferty, 2008).

Several other Heptageniidae are known from the fossil record and were excluded from analysis due to specific reasons. The oldest Heptageniidae fossil was described as *Amerogenia macrops* Sinitshenkova, 2000 from the Upper Cretaceous New Jersey amber (Turonian stage, Raritan Formation, USA, 94.3–89.3 Ma; Sinitshenkova, 2000; Nicholson et al., 2015). Kluge (2004) listed this taxon within Pentamerotarsata incertae sedis sensu Kluge (2000), corresponding to the subfamily Heptageniinae sensu Needham 1901 (in Needham and Betten,

1901). The placement of *Amerogenia* within Heptageniidae can be confirmed based on the structure of tarsomere 1, which distinctly articulates with tibia (not fused), and 5-segmented tarsi. Nevertheless, based on available characters described and figured by Sinitshenkova (2000), it is impossible to attribute *Amerogenia* to any subfamily of Heptageniidae.

We exclude from our analysis two compressed larval fossils attributed to Heptageniidae by Bell et al. (2013), discovered from the Late Cretaceous Campanian-Maastrichtian Wapiti Formation (Alberta, Canada). Many characters support their placement within Heptageniidae (e.g. shape of the head, abdomen, and remnants of abdominal appendages, which probably represent gills). However, the attribution to any of the discussed subfamilies is not possible due to the poor preservation of these fossils.

We also exclude Miocene fossil larvae from the analysis, which were attributed to Heptageniidae. Most of these were described based on poorly preserved material and placed within extant genera (e.g. *Heptagenia* or *Ecdyonurus*), however with insufficient justification (Hong, 1983; Zhang, 1989; Godunko, 2004). Additionally, two extinct mayfly genera were described based on larval material from the Middle Miocene of the Russian Federation (*Miocoenogenia* Tshernova, 1962) and the Upper Miocene of Murat, France (*Pseudokageronia* Masselot & Nel, 1999). The genus *Miocoenogenia* is placed within the group Radulapalpata incertae sedis sensu Kluge (2000), corresponding to the subfamilies Heptageniinae + Rhithrogeninae, see Tshernova, 1962; Kluge, 2004), and *Pseudokageronia* is listed as a member of the subfamily Ecdyonurinae incertae sedis (Masselot and Nel, 1999; Kluge, 2004).

Several fossil taxa were described from the Eocene based on both larval and winged stages (winged stages include imaginal as well as subimaginal specimens). Demoulin (1965) described *Succinogenia larssoni* Demoulin, 1965 on a relatively complete larval specimen and suggested its attribution to subfamily Heptageniinae. Nevertheless, Kluge (2004) placed this taxon within Radulapalpata incertae sedis. Other Eocene Heptageniidae taxa are known as adults and were described by

Demoulin (1968), Kluge (1986), and Godunko (2004, 2007). Their original placements formally comprise the extant genera *Heptagenia*, *Kageronia*, *Cinygma*, *Rhithrogena*, and *Ecdyonurus* (*Nestormeus*). However, only for two of them, i.e. *Kageronia* and *Ecdyonurus* s.l., the presence in the Eocene is reliable, based on an analysis of a set of morphological characters of the thorax summarized by Kluge (2004). The remaining Eocene taxa described by Demoulin (1968) should be reinvestigated, as their systematic position is unclear.

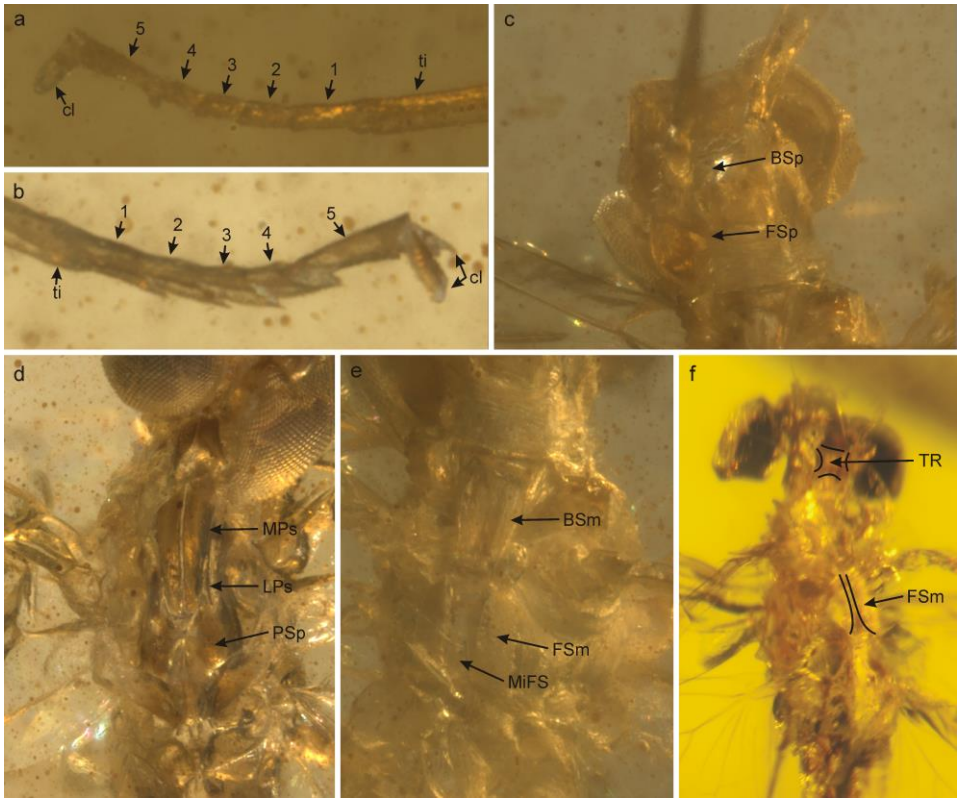


Fig. A. Morphology of specimens used as calibration fossils. a–e. Ecdyonurinae sp., male imago, mid-Cretaceous Myanmar amber. a, tarsus of left middle leg; b, tarsus of right hind leg; c, head and prothorax in ventral view; d, thorax in dorsal view; e, thorax in ventral view; f. *Maccaffertium annae* Macadam & Ross, 2016, holotype (NMS G.2008.34.3.1), male imago, early Miocene Mexican amber, head and thorax in ventral view (lines added to highlight important structures). Abbreviations: BSm, basisternum of mesothorax; BSp, basisternum of prothorax; cl, claw; FSm, furcasternum of mesothorax; FSp, furcasternum of prothorax; LPs, lateroparapsidal suture; MiFS, median impression of furcasternum; MPs, medioparapsidal suture; PSp, posterior scutal protuberance; ti, tibia; TR, transversal ridge. Numbers indicate individual tarsomeres.

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Table A1. GenBank accession numbers for each gene fragment for all analysed specimens. Already published data are provided with primary reference.

Species/genus	**COI**	**16S**	**28S**	**EF-1α**	**wg**
Ecdyonurus eurycephalus	MG972100 (Hrivniak et al., 2018)	MN298791	MN298847	MN271453	MN271426
Afronurus sp.	HE651387 (Vuataz et al., 2013)	HE651424 (Vuataz et al.,2013)	HE651449 (Vuataz et al., 2013)	HE651531 (Vuataz et al., 2013)	HE651479 (Vuataz et al., 2013)
Thalerosphyrus lamuriensis	HE651388 (Vuataz et al., 2013)	HE651425 (Vuataz et al.,2013)	HE651450 (Vuataz et al., 2013)	HE651532 (Vuataz et al., 2013)	HE651480 (Vuataz et al., 2013)
Stenonema meririvulanum	JN200389 (Pilgrim et al., 2011)	MN298818	MN298845	MN271454	MN271427
Stenonema modestum	MN271424	MN298819	MN298846	MN271455	MN271428
Rhithrogena alpestris	LN868543 (Vuataz et al., 2016)	LN868657 (Vuataz et al., 2016)	missing data	LN868579 (Vuataz et al., 2016)	LN868618 (Vuataz et al., 2016)
Cinygmula sp.	JF423908 (Vuataz et al., 2011)	LN868675 Vuataz et al., 2016)	missing data	LN868597 (Vuataz et al., 2016)	LN868636 (Vuataz et al., 2016)
Epeorus (*Ironopsis*) *grandis*	MN271425	MN298792	MN298843	MN271456	missing data
Epeorus (*Iron*) *longimanus*	JN287282 (Webb et al., 2012)	MN298793	AY749917 (Ogden & Whiting, 2005)	MN271481	MN271452
Epeorus (*Ironopsis*) *rheophilus*	MN271396	MN298794	MN298828	MN271472	MN271447
Epeorus (*Iron*) *montanus*	MN271397	MN298795	MN298839	MN271459	MN271448
Epeorus (*Ironopsis*) *alpicola*	MN271421	MN298815	MN298841	MN271458	MN271451
Epeorus (*I.*) *yougoslavicus*	MN271422	MN298816	MN298842	MN271457	MN271450
Epeorus (*Epeorus*) *assimilis*	MN271423	MN298817	MN298830	MN271471	MN271449
Caucasiron sp. 1	MN271398	MN298796	MN298840	MN271480	MN271432
Caucasiron sp. 1	MN271399				
E. (*Caucasiron*) *turcicus*	MK140789 (Hrivniak et al., 2019)				
E. (*C.*) *turcicus*	MK140785 (Hrivniak et al., 2019)				
E. (*C.*) *turcicus*	MK140783 (Hrivniak et al., 2019)				
E. (*C.*) *turcicus*	MK140784 (Hrivniak et				

	al., 2019)				
E. (*C.*) *turcicus*	MK140786 (Hrivniak et al., 2019)				
E. (*C.*) *turcicus*	MK140788 (Hrivniak et al., 2019)	MN298797	MN298834	MN271474	MN271441
E. (*C.*) *turcicus*	MK140787 (Hrivniak et al., 2019)				
Caucasiron sp. 2	MN271400				
Caucasiron sp. 2	MN271401				
Caucasiron sp. 2	MN271402				
Caucasiron sp. 2	MN271403				
Caucasiron sp. 2	MN271404				
Caucasiron sp. 2	MN271405	MN298799	MN298838	MN271476	missing data
E. (*C.*) *nigripilosus*	KY865691 (Hrivniak et al., 2017)				
E. (*C.*) *nigripilosus*	KY865692 (Hrivniak et al., 2017)				
E. (*C.*) *nigripilosus*	KY865694 (Hrivniak et al., 2017)				
E. (*C.*) *nigripilosus*	KY865695 (Hrivniak et al., 2017)	MN298802	MN298833	MN271479	MN271431
E. (*C.*) *nigripilosus*	KY865693 (Hrivniak et al., 2017)				
E. (*C.*) *nigripilosus*	MN271406				
E. (*C.*) *nigripilosus*	MN271407				
E. (*C.*) *nigripilosus*	MN271408				
Caucasiron sp. 3	MN271409				
Caucasiron sp. 3	MN271410	MN298798	MN298837	MN271475	MN271433
Caucasiron sp. 3	MN271411				
E. (*C.*) *iranicus*	MN271412	MN298800	MN298836	MN271478	MN271430
E. (*C.*) *caucasicus*	KY865682 (Hrivniak et al., 2017)				
E. (*C.*) *caucasicus*	KY865690 (Hrivniak et al., 2017)				
E. (*C.*) *caucasicus*	KY865688 (Hrivniak et al., 2017)	MN298801	MN298835	MN271477	MN271429
E. (*C.*) *caucasicus*	KY865687 (Hrivniak et al., 2017)				
E. (*C.*) *caucasicus*	KY865689 (Hrivniak et al., 2017)				
E. (*C.*) *caucasicus*	KY865683 (Hrivniak et al., 2017)				
E. (*C.*) *caucasicus*	KY865686 (Hrivniak et al., 2017)				

E. (*C.*) *caucasicus*	KY865684 (Hrivniak et al., 2017)				
E. (*C.*) *caucasicus*	KY865685 (Hrivniak et al., 2017)				
Caucasiron sp. 4	MN271413				
Caucasiron sp. 4	MN271414	MN298810	MN298825	MN271470	MN271442
E. (*C.*) *znojkoi*	KY865696 (Hrivniak et al., 2017)				
E. (*C.*) *znojkoi*	KY865701 (Hrivniak et al., 2017)	MN298811	MN298826	MN271468	MN271443
E. (*C.*) *znojkoi*	KY865699 (Hrivniak et al., 2017)				
E. (*C.*) *znojkoi*	KY865700 (Hrivniak et al., 2017)				
E. (*C.*) *znojkoi*	KY865697 (Hrivniak et al., 2017)				
E. (*C.*) *znojkoi*	KY865704 (Hrivniak et al., 2017)				
E. (*C.*) *znojkoi*	KY865698 (Hrivniak et al., 2017)				
E. (*C.*) *znojkoi*	KY865703 (Hrivniak et al., 2017)				
E. (*C.*) *znojkoi*	KY865702 (Hrivniak et al., 2017)				
Caucasiron sp. 5	MN271415				
Caucasiron sp. 5	MN271416				
Caucasiron sp. 5	MN271417	MN298812	MN298821	MN271469	MN271444
Caucasiron sp. 6	MN271418				
Caucasiron sp. 6	MN271419	MN298813	MN298831	MN271467	MN271446
E. (*C.*) *insularis*	MN271420	MN298814	MN298824	MN271466	MN271445
E. (*C.*) *soldani*	KY865717 (Hrivniak et al., 2017)				
E. (*C.*) *soldani*	KY865716 (Hrivniak et al., 2017)	MN298804	MN298829	MN271465	MN271436
E. (*C.*) *soldani*	KY865718 (Hrivniak et al., 2017)				
Caucasiron sp. 7	KY865715 (Hrivniak et al., 2017)	MN298803	MN298827	MN271460	MN271435
E. (*C.*) *bicolliculatus*	KY865722 (Hrivniak et al., 2017)				
E. (*C.*) *bicolliculatus*	KY865720 (Hrivniak et al., 2017)				

E. (*C.*) *bicolliculatus*	KY865721 (Hrivniak et al., 2017)	MN298809	MN298822	MN271461	MN271439
E. (*C.*) *bicolliculatus*	KY865719 (Hrivniak et al., 2017)				
E. (*C.*) *shinitshenkovae*	KY865725 (Hrivniak et al., 2017)	MN298807	MN298832	MN271464	MN271440
E. (*C.*) *shinitshenkovae*	KY865726 (Hrivniak et al., 2017)				
E. (*C.*) *shinitshenkovae*	KY865727 (Hrivniak et al., 2017)				
E. (*C.*) *shinitshenkovae*	KY865728 (Hrivniak et al., 2017)				
E. (*C.*) *longimaculatus*	KY865724 (Hrivniak et al., 2017)				
E. (*C.*) *longimaculatus*	KY865723 (Hrivniak et al., 2017)	MN298808	MN298844	MN271462	MN271434
E. (*C.*) *alpestris*	KY865714 (Hrivniak et al., 2017)				
E. (*C.*) *alpestris*	KY865712 (Hrivniak et al., 2017)				
E. (*C.*) *alpestris*	KY865711 (Hrivniak et al., 2017)	MN298806	MN298820	MN271473	MN271437
E. (*C.*) *alpestris*	KY865713 (Hrivniak et al., 2017)				
E. (*C.*) *magnus*	KY865709 (Hrivniak et al., 2017)				
E. (*C.*) *magnus*	KY865708 (Hrivniak et al., 2017)				
E. (*C.*) *magnus*	KY865707 (Hrivniak et al., 2017)				
E. (*C.*) *magnus*	KY865706 (Hrivniak et al., 2017)	MN298805	MN298823	MN271463	MN271438
E. (*C.*) *magnus*	KY865710 (Hrivniak et al., 2017)				
E. (*C.*) *magnus*	KY865705 (Hrivniak et al., 2017)				

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Table A2. Dispersal rate scaling matrices used for DIVALIKE+J model. Two time slices are shown, providing the connectivity between the biogeographical areas along the time. Biogeoraphical areas defined for the analyses are **A** – Greater Caucasus, **B** – Lesser Caucasus, **C** – Pontic Mts. (includes: Turkey: Pontic, Allahu Ekber, Yalnizcam, Kargapazari, Ilgaz, Mescit, Balaban, Koroglu, Botu, Gavur, Sündiken, Kesis, Cosan Mts.), **D** – Cyprus Island and Taurus-North Zagros Mountains (includes: Iran: Zagros, Iri, Küh-e Mishab, Küh-e Mishab, Qaflankuh Mts., **Turkey**: Taurus, Hama Dag, Ispiriz, Agri, Hakkari, Doganli, Ala, Muratbasi, Mardin Platosu, Süphan, Tendürük, Malatya, Malendiz, Erciyas, Bolkar Mts., **Cyprus**: Troödos Mts.), **E** – Talysh-Alborz Mountains (includes: **Iran**: Alborz, Küh-e Bozqush, Talysh, Qareh Dagh, Küh-e haye Sabalan Mts.), **F** – Samos Island (see Fig. 5).

9.75-0 Ma						
	A	B	C	D	E	F
A0-1	1	0.9	0.7	0.7	0.7	0.6
B0-1	0.9	1	1	1	1	0.7
C0-1	0.7	1	1	1	0.8	0.9
D0-1	0.7	1	1	1	1	0.9
E0-1	0.7	1	0.8	1	1	0.7
F0-1	0.6	0.7	0.9	0.9	0.7	1
12.5-9.75 Ma						
	A	B	C	D	E	F
A1-2	1	0.45	0.35	0.35	0.35	0.3
B1-2	0.45	1	1	1	1	0.7
C1-2	0.35	1	1	1	0.8	0.9
D1-2	0.35	1	1	1	1	0.9
E1-2	0.35	1	0.8	1	1	0.7
F1-2	0.3	0.7	0.9	0.9	0.7	1

Table A3. Mean (mean), minimum (min) and maximum (max) altitude of all records and number of sampling sites (n) of *Caucasiron* species/GMYC putative species (species) used in ancestral state reconstruction.

species	**mean**	**min**	**max**	**n**
alpestris	1664	570	2580	25
magnus	936	35	2210	104
soldani	1477	730	1900	7
Caucasiron sp. 7	426	426	426	1
bicolliculatus	895	40	1804	25
longimaculatus	1085	1025	1145	2
sinitshenkovae	1930	730	2580	13
iranicus	2183	2100	2292	3
caucasicus	1622	496	2474	60
Caucasiron sp. 3	839	252	1345	4
Caucasiron sp. 2	1445	1090	1800	2
Caucasiron sp. 1	1237	1231	1243	2
nigripilosus	1463	970	2024	14
turcicus	2216	2050	2388	6

Caucasiron sp. 5	46	39	53	2
znojkoi	965	7	2453	115
Caucasiron sp. 4	359	-6	1345	18
Caucasiron sp. 6	325	325	325	1
insularis	250	128	440	3

Table A4. Datasets and alignments characteristics; number of sequences (nuclear and combined include missing data) (n), size of aligned matrices (bp), number of haplotypes (K), number of variable sites (V), parsimony-informative sites (number and %) (Pi), number of positions with gaps/missing data (I).

Dataset	**n**	**bp**	**K**	**V**	**Pi**	**Pi%**	**I**
COI	33	631	33	248	236	37	34
16S	33	470	33	149	111	23	8
wg	31	465	28	138	99	21	21
28S	31	608	27	78	65	11	152
EF-1α	33	115	17	24	14	12	0
mt	33	1101	33	397	346	31	42
nu	33	1188	32	240	178	15	173
combined	33	2289	33	637	524	23	215

Table A5. Partitioning schemes and substitution models used in Bayesian (BI) and maximum likelihood (ML) phylogenetic analyses.

Combined dataset (BI):

Subset	**Best Model**	**# sites**	**Partition names**
1	GTR+I+G	470	16S
2	GTR+I	608	28S
3	SYM+I+G	250	COI_1stpos, EF_1stpos
4	F81+I	210	COI_2ndpos
5	GTR+G	210	COI_3rdpos
6	GTR+I	193	WG_2ndpos, EF_2ndpos
7	SYM+G	38	EF_3rdpos
8	GTR+G	155	WG_1stpos
9	HKY+I	155	WG_3rdpos

mt datased (BI):

Subset	**Best Model**	**# sites**	**Partition names**
1	GTR+I+G	470	16S
2	SYM+G	211	COI_1stpos
3	F81+I	211	COI_2ndpos
4	GTR+G	210	COI_3rdpos

nu dataset (BI):

Subset	Best Model	# sites	Partition names
1	HKY+I	608	28S
2	GTR+G	194	WG_1stpos, EF_1stpos
3	GTR+I	193	EF_2ndpos, WG_2ndpos
4	SYM+G	38	EF_3rdpos
5	HKY+G	155	WG_3rdpos

Combined dataset (ML):

ID	Model	Partition name
1	TPM2u+F+I+G4	16S
2	K2P+I	28S
3	TIM3e+I+G4	COI_1stpos
4	F81+F+I	COI_2ndpos
5	TIM2+F+G4	COI_3rdpos
6	TNe	EF_1stpos
7	JC+I	EF_2ndpos
8	TNe+G4	EF_3rdpos
9	TIM2+F+G4	WG_1stpos
10	JC+I	WG_2ndpos
11	HKY+F+I	WG_3rdpos

mt dataset (ML):

ID	Model	Partition name
1	HKY+F+I+G4	16S
2	TIM3e+I+G4	COI_1stpos
3	F81+F+I	COI_2ndpos
4	TIM2+F+G4	COI_3rdpos

nu dataset (ML):

ID	Model	Partition name
1	K2P+I	28S
2	TNe	EF_1stpos
3	K3P+I	EF_2ndpos
4	TNe+G4	EF_3rdpos
5	TIM2+F+I	WG_1stpos
6	JC+I	WG_2ndpos
7	HKY+F+I	WG_3rdpos

Table A6. Results from model test with parameters of all the models evaluated by BioGeoBEARS for ancestral range estimation.

model	LnL	numparams	d	e	j	AICc	AICc_wt
DEC	-58.4	2	0.040	1.0e-12	0	121.6	0.060

DEC+J	-55.33	3	0.034	1.0e-12	0.11	118.3	0.31
DIVALIKE	-56.97	2	0.045	1.0e-12	0	118.7	0.25
DIVALIKE+J	-55.2	3	0.038	1.0e-12	0.071	118	0.36
BAYAREALIKE	-66.46	2	0.041	0.14	0	137.7	1.9e-05
BAYAREALIKE	-58.08	3	0.026	1.0e-07	0.26	123.8	0.020

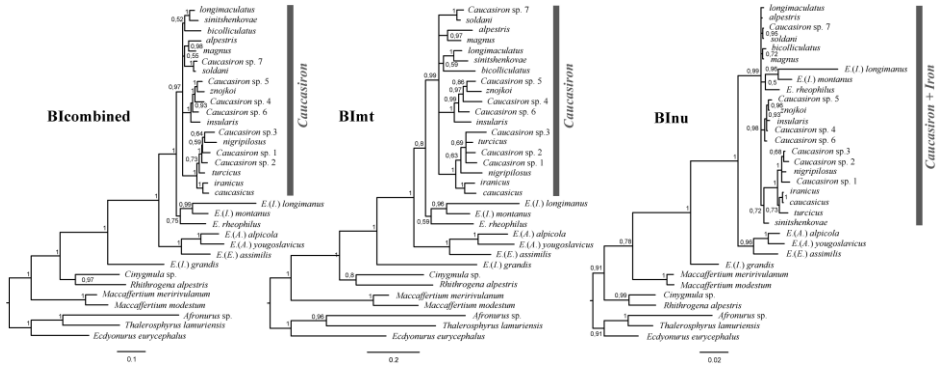


Fig. A1. Bayesian inference of phylogeny of individual datasets with all taxa included. For each node posterior probability is figured.

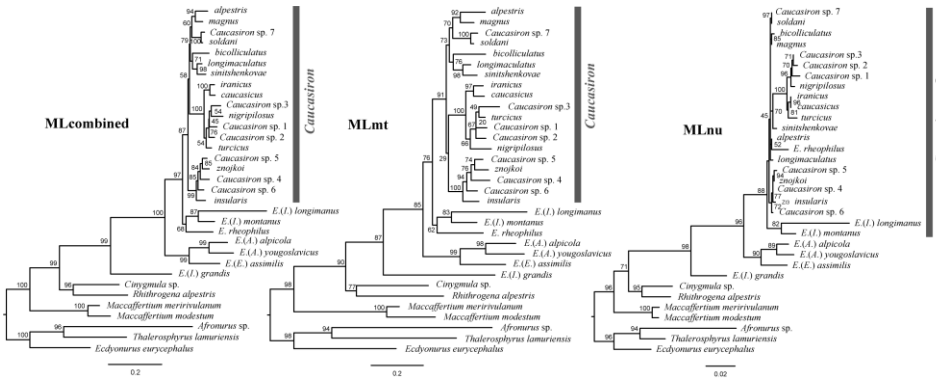


Fig. A2. Maximum likelihood tree reconstruction of individual datasets with all taxa included. For each node bootstrap value is figured.

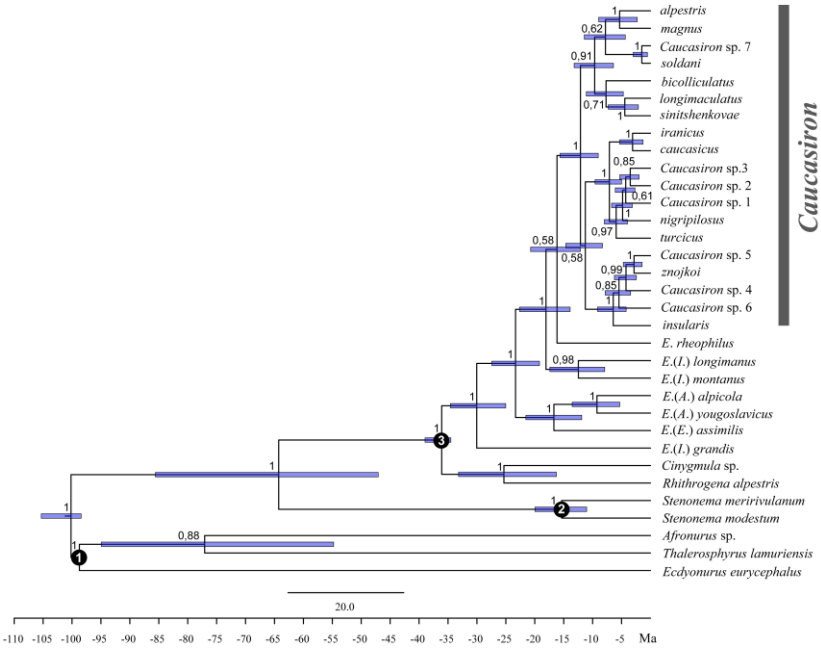


Fig. A3. Fossil calibrated maximum clade credibility tree of combined dataset with all taxa included. Numbered black circles correspond to fossil calibration points. For each node posterior probability is figured.

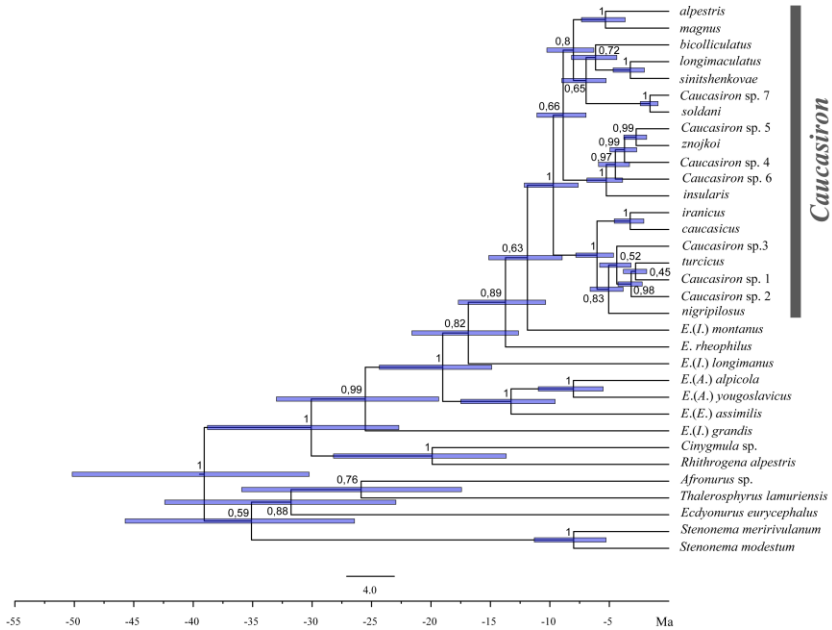


Fig. A4. Maximum clade credibility tree of mitochondrial dataset calibrated by standard mitochondrial mutation rate with all taxa included. For each node posterior probability is figured.

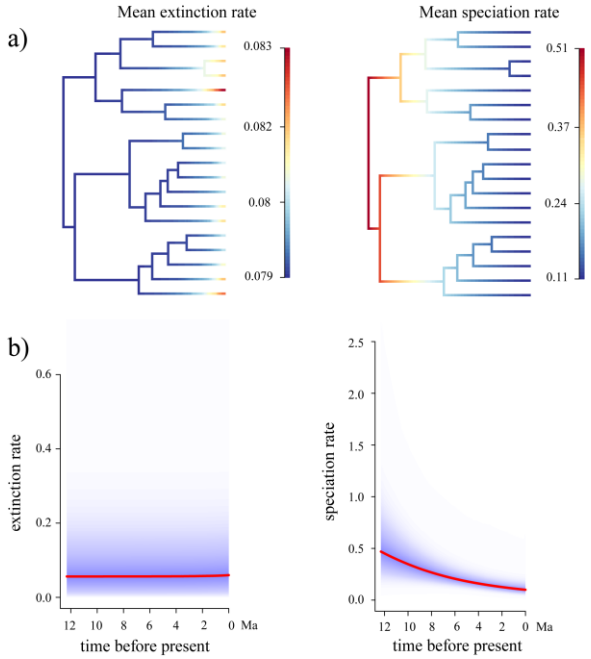


Fig. A5. Mean extinction and speciation rate (a) and extinction and speciation through time (b) of *Caucasiron* generated by BAMM analysis.

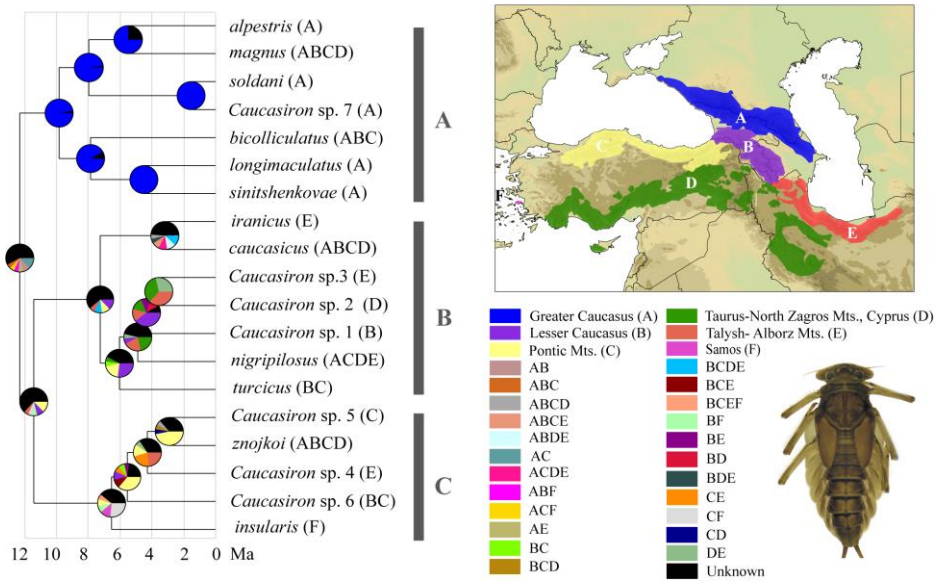


Fig. A6. Ancestral range estimation of *Caucasiron* based on DIVALIKE+J model. Location of mountain ranges used in the analysis are showed in the map. Colours and letters codes under map correspond to colours on tree nodes. The recent distribution of species is given in the parentheses next to species name.

PART THREE: SUMMARY AND FUTURE PERSPECTIVES

1. Summary

This dissertation thesis is focused on mayflies (Ephemeroptera) of the Caucasus, an ancient merolimnic insect order inhabiting freshwater habitats. The Caucasus is one of the most biologically diversified mountainous areas in the Palearctic region, but is still poorly investigated in many aspects. The results of the thesis significantly contribute to our knowledge of the diversity, distribution, and evolutionary history of mayflies, and shed more light on the evolution of the unique mountain biota of the Caucasus in general.

The thesis comprises four chapters. The first chapter is focused on the diversity of mayflies of Armenia. The country is situated in the central part of the Lesser Caucasus mountain range and was among the least explored countries within the Caucasus. An attached publication entitled "*Diversity of Armenian mayflies (Ephemeroptera) with the description of a new species of the genus Ecdyonurus (Heptageniidae)* " is a part of a set of works focused on mapping mayfly species diversity in the Caucasus (e.g. Bojková et al. 2018; Gabelashvili et al. 2018), and constitutes the first complex study of the diversity and biogeographic composition of the mayflies of Armenia, based on our extensive sampling throughout the entire territory in 2011, 2014, and 2015. Altogether 46 species were confirmed, and eight of them were reported from the country for the first time. The dominant part of the fauna were regional endemics of the Caucasus, distributed in both the Greater and Lesser Caucasus, sometimes with an area extension to the Middle East (Turkey, Iran, Iraq) or Central Asia, and common European and West Palearctic species. Moreover, one species, *Ecdyonurus* (*Ecdyonurus*) *eurycephalus*, was described as a new species for science. Description was carried out based on the combination of morphology and molecular data, which were also used for the association between larval instars and life stages. The species occurs in the high-altitude steppe streams and rivers of the Armenian highlands, and is most likely endemic to this area.

The goal of the second chapter was a detailed investigation of the diversity of Caucasian *Epeorus* (*Caucasiron*), the model group selected for the research of mayfly evolutionary history in the Caucasus. Based on the sequencing of specimens collected from a wide geographical range within the Caucasus region, we aimed to clarify whether the species described based on comparative morphology correspond to species clusters delimited by molecular data, to describe the morphological variability of individual species for the purposes of species identification in Chapter III, and to capture as many independent

Caucasiron lineages as possible for the analyses of evolutionary diversification events.

The results showed high congruence between morphological and molecular species delimitation, which points to the effective use of molecular data in species identification. Moreover, several independent lineages were discovered and, after detailed morphological investigation, described as new species for science. This included the new species *Epeorus* (*Caucasiron*) *bicolliculatus* and *E.* (*C.*) *turcicus* distributed in the western Caucasus and Pontic Mountains, and three new species from Iran, *E.* (*C.*) *alborzicus* and *E.* (*C.*) *shargi* distributed in the Alborz, and *E.* (*C.*) *zagrosicus* occurring in the Zagros Mountains. These results are summarized in three published papers attached to Chapter II.

Chapter III has a more practical function. The attached manuscript is focused on the identification, habitat preferences, and distribution of all currently known species from the Caucasus and adjacent areas. I have summarized all knowledge on *Caucasiron* morphology, the variability of individual species and their identification, and prepared an identification guide. The guide is designed for the wider professional public and non-taxonomic research in the fields of hydrobiology, ecology, and faunistics. An accurate species identification is of crucial importance in these fields and a lack of suitable literature hampers research in the Caucasus. I believe that this contribution will help to move the research of Caucasian mayflies beyond alpha taxonomy and enable us to use the potential of mayflies in the monitoring of water quality and conservation of habitats.

The goal of the last chapter was to reveal the geographical and temporal origin of Caucasian mayflies and examine the course of their evolutionary diversification over time. Using multi locus time-calibrated phylogeny and biogeographical analyses, I tested the role of geological history of the Caucasus in the biogeography, speciation patterns, and altitudinal diversification of *Caucasiron* mayflies, as one of the most diversified mayfly taxa in the Caucasus and adjacent areas. By incorporating other *Caucasiron*-related taxa sampled over the Holarctic region (Central Europe, the Balkans, Central Asia, and North America) I also clarified their geographical origin.

I found that *Caucasiron* mayflies are sister to Central Asian and Nearctic *Iron*, being less related to Central European and Balkan lineages, indicating that they originated from Asia. Biogeographic analyses indicated that the ancestor of all Caucasian *Caucasiron* lineages was widely distributed in the pre-Caucasian region, including the ancient Greater Caucasus island, and rapidly diversified in the Middle Miocene, during the mountain building of the Caucasus. This

suggested that the uplift and formation of the Caucasus mountain system promoted the evolutionary diversification of a widespread *Caucasiron* ancestor. The highest diversification rate, corresponding to the uplift of mountains, was followed by a significant slowdown towards the present, suggesting the minor influence of Pleistocene climatic oscillations on their speciation.

The Caucasian *Caucasiron* lineages clustered into three principal clades originating in the Upper Miocene. Biogeographical analyses found strong support for the notion that one of the three clades diversified in the Greater Caucasus mountain range, isolated in the ancient Paratethys Sea. It indicated that environmental changes caused by mountain building and the long isolation of the Greater Caucasus led to the vicariance of the ancestral lineage with subsequent diversification of the clade. All species of this clade originated in the Greater Caucasus, and four of them, namely *E.* (*C.*) *alpestris*, *E.* (*C.*) *soldani*, *E.* (*C.*) *sinitshenkovae*, and *E.* (*C.*) *longimaculatus*, are endemic to this mountain range, especially to its western and central part. Two species, *E.* (*C.*) *magnus* and *E.* (*C.*) *bicolliculatus*, dispersed towards the south-west, to the Lesser Caucasus and Pontic Mts. (and Taurus Mts. in the former species). *E.* (*C.*) *magnus* is recently widely distributed throughout Anatolia, far beyond the boundary of the Caucasus region. This indicated that the Greater Caucasus acted as a diversification centre and source of diversity for surrounding areas.

The radiation of the other two clades seemed to be relatively younger. Both clades originated most likely outside the Greater Caucasus and diversified towards high and low altitude, respectively. This could be related to the passive uplift of a mid-altitude *Caucasiron* ancestor and its subsequent diversification or to ecological speciation through climatic niche divergence. Three species from these clades, *E.* (*C.*) *znojkoi*, *E.* (*C.*) *caucasicus*, and *E.* (*C.*) *nigripilosus*, are currently widely distributed in the Caucasus region, including the Greater Caucasus. Current high *Caucasiron* diversity in the Greater Caucasus was therefore the result of two events; in situ speciation and later immigration from adjacent mountain ranges.

To conclude, this chapter shows that the Miocene/Pliocene orogeny of the Caucasus most likely triggered the diversification of freshwater montane biota by the isolation of mountain ranges leading to allopatric speciation and the creation of environmental gradients vacant for speciation. Our results indicate that the speciation of Caucasian cold-adapted aquatic insects was influenced by the history of the land mass development of the Caucasus and late orogeny, rather than later climatic oscillations in the Quaternary.

Regarding the systematic problems concerning the evolution of the suction disc within *Epeorus* s.l., our study did not support Braasch´s hypothesis incorporating all species with a suction disc into the monophyletic genus *Iron*. The European species *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 a cluster within European species with a suction disc. This indicates the possible reduction of a suction disc in at least one lineage within *Epeorus* s.l.

Our study supports the monophyly of *Caucasiron*. The European species with a suction disc, belonging to *Ironopsis* according to Kluge´s classification were found to be sister to *Epeorus* s. str., not to Nearctic and Central Asian *Ironopsis*, or any other *Epeorus* taxa with a suction disc. Thus, the subgenus *Ironopsis*, as proposed by Kluge, is paraphyletic. We finally accepted the Braasch´s concept of the subgenus *Alpiron* (containing European species with a suction disc) and proposed nomenclatural changes according to the phylogeny set out in our study.

2. The future direction of the Caucasian mayfly research

This thesis constitutes the first comprehensive study of the diversity, distribution, and evolution of mayflies from the Caucasus. It is mostly focused on *Caucasiron*, and the same research approach should be applied to the other groups in the future.

The diversity of mayflies in the Caucasus region is still underestimated and the final number of species is far from being complete. Our results point to the fact that a detailed taxonomic investigation of individual genera may reveal new species for science from the region, especially when the integrative approach is included in the species delimitation.

The research of mayfly diversity, with the use of both morphology and molecular data should be gradually applied to other genera. These should include especially *Rhithrogena*, *Electrogena*, and *Ecdyonurus*, which represent diverse, frequent, and abundant, but still poorly investigated mayflies in the region. After processing the revision of these genera, specialists should focus on species identification, because the absence of identification keys significantly hampers further research beyond the alpha taxonomy. We are currently working on the revision and species identification of the genus *Ecdyonurus*, and the revision of the other diverse genera named above will be prioritized in future research.

Other significant hurdles regarding mayfly diversity research are the inadequate existing investigation of low altitude biotopes in the Caucasus and the absence of species inventories from some Caucasian countries. Most studies focus on dominant mountain habitats and lowlands are largely omitted. I assume though that such biotopes likely host unknown diversity from the mayfly families of Baetidae, Siphlonuridae, and Ephemeridae.

The investigation of the whole area of the Caucasus and all of the aquatic biotopes there is essential for a complex analysis of the species distribution and ecological preferences needed to provide the relevant data for species and habitat conservation. Part of this thesis was focused on the species inventory of Armenia. Currently, the species inventory of mayflies from Azerbaijan based on our extensive sampling in 2017–2019 is being worked upon. To complete the overall picture of Caucasian mayflies, some parts of the Russian Federation remain to be studied.

Information on the origin and diversification of *Caucasiron* provides an initial insight into the formation of the Caucasian mayfly fauna. However, biological evolution is complex and consists of various evolutionary histories. The evolution might vary among model groups depending on its specific features

possibly affecting their evolutionary history such as habitat preferences, ecology, and dispersal ability. The analyzes of environmental data from sampling sites and a detailed investigation of the habitat preferences of individual species is therefore among our future goals. After the systematic revisions of other genera, we plan to explore their origin and diversification mechanisms and compare them with those found in *Caucasiron*. As we included only limited material from Central Asia in our analyses, our future goal is to incorporate *Caucasiron* from the Pamir and Himalayas in our evolutionary study and clarify their biogeography and phylogenetic relationships to the Caucasian lineage. Our recent molecular dataset did not allow us to fully reconstruct phylogenetic relationships among principal clades and some species. We assume that the inclusion of a modern sequencing method, such as new generation sequencing technology, would solve this problem and complete our phylogenetic analyses. Improving the research by the updating of methodology is therefore among the future priorities.

Although the results from our diversification rate analyses did not indicate a significant impact for the Pleistocene climatic cycles on the speciation of *Caucasiron*, we hypothesize that climatic oscillations have affected the distribution and genetic structure on the level of populations. We therefore aim to supplement our species-level study of *Caucasiron* with the phylogeography and clarify whether its populations followed a similar pattern as observed in other groups of biota in the Caucasus. It may be expected that the landscape fragmentation during Pliocene/Pleistocene has resulted in fragmented distribution of some species, such as *E.* (*C.*) *nigripilosus.* We also aim to analyze populations of other species widespread throughout the Caucasus, such as *E.* (*C.*) *znojkoj*, *E.* (*C.*) *caucasicus*, or *E.* (*C.*) *magnus*, and investigate their genetic variability and possible dispersal routes throughout the region.

All of the above mentioned research topics indicate that the Caucasus region is still a highly attractive area for a wide variety of research activities ranging from taxonomy to evolutionary biology. Our intensive research on Caucasian mayflies will, therefore, continue in the future and I believe that a combination of studies on the species and population level focused on multiple model groups will finally provide a complex picture of the formation of the highly diverse Caucasian aquatic fauna.

APPENDIX

Checklist of mayflies (Ephemeroptera) of the Caucasus and adjacent areas

Species distribution is adopted from Bauernfeind & Soldán (2012) and the Ephemeroptera of the World online database (www.insecta.bio.spbu.ru/#Ephemeroptera). Caucasian endemics (i.e. species recorded only from the Caucasus region up to now) are in bold. Systematic classification is adopted from Bauernfeind & Soldán (2012), Gabelashvili et al. (2018), or follows original species description. Explanations for distribution: **HOL**: Holarctic; **PAL**: Palearctic; **WPAL**: western Palearctic; **EU**: Europe; **CAU**: Caucasus region; **GEO**: Georgia; **RUS**: Russian Federation; **ARM**: Armenia; **AZE**: Azerbaijan; **TUR**: Turkey; **IR**: Iran; **IS**: Israel; **SY**: Syria; **IQ**: Iraq; **GR**: Greece; **UA**: Ukrajine; **CP**: Crimean Peninsula; **CY**: Cyprus; **LEB**: Lebanon; **JOR**: Jordan; **IN**: India; **KAZ**: Kazakhstan; **TURK**: Turkmenistan; **TAJ**: Tajikistan; **AFG**: Afghanistan; **AM**: Asia Minor.

Family: SIPHLONURIDAE Ulmer, 1920
Genus: *Siphlonurus* Eaton, 1868
1. *Siphlonurus* (*Siphlurella*) *alternatus* (Say, 1824)
Distribution: HOL; CAU: GEO (Adjara, Guria), AZE, RUS (Krasnodar Krai, Stavropol Krai)
2. *Siphlonurus* (*Siphlonurus*) *lacustris* Eaton, 1870
Distribution: PAL; CAU: GEO (Mtskheta-Mtianeti), AZE (Nagorno-Karabakh), RUS (North Ossetia)

Family: AMETROPODIDAE Bengtsson, 1913
Genus: *Ametropus* Albarda, 1978
3. *Ametropus fragilis* Albarda, 1878
Distribution: PAL; CAU: RUS (North Ossetia, Krasnodar Krai)

Family: AMELETIDAE McCafferty, 1991
Genus: *Ameletus* Eaton, 1885
4. *Ameletus inopinatus* Eaton, 1887
Distribution: HOL; CAU: AZE (Nakhchivan, Nagorno-Karabakh)

Family: BAETIDAE Leach, 1815
Genus: *Baetis* Leach, 1815
5. *Baetis* (*Acentrella*) *joosti* Zimmermann & Braasch, 1979
Distribution: CAU: ARM
6. *Baetis* (*Acentrella*) *inexpectatus* (Tshernova, 1928)
Distribution: EU; CAU: RUS (Krasnodar Krai)
7. *Baetis* (*Baetis*) *baksan* Soldán, 1977
Distribution: CAU: GEO (Mtskheta-Mtianeti, Abkhazia, Guria), RUS (Kabardino-Balkaria, North Ossetia, Krasnodar Krai)
8. *Baetis* (*Baetis*) *baroukianus* Thomas & Dia, 1984
Distribution: LEB; CAU: IR
9. *Baetis* (*Baetis*) *buceratus* Eaton, 1870
Distribution: PAL; CAU: ARM, GEO (Imereti), AZE (Talysh Mts.), RUS (North Ossetia), IR

10. *Baetis* (*Baetis*) *fuscatus* (Linnaeus, 1761)
Distribution: PAL; ARM, GEO (Adjara, Guria, Samtskhe-Javakheti, Imereti), RUS (Krasnodar Krai), TUR, IR

11. *Baetis* (*Baetis*) *ilex* Jacob & Zimmermann, 1978
Distribution: CAU: RUS (Kabardino-Balkaria, Krasnodar Krai, North-Ossetia), ARM, GEO (Samtskhe-Javakheti, Racha-Lechkhumi, Samegrelo-Svaneti, Shida Kartli, Kvemo Kartli, Mtskheta-Mtianeti)

12. *Baetis* (*Baetis*) *lutheri georgensis* Zimmermann, 1981
Distribution: CAU: ARM, AZE (Talysh Mts.), RUS (Krasnodar Krai, Karachay Cherkess), GEO (Adjara, Abkhazia, Samegrelo-Svaneti, Racha-Lechkhumi, Shida Kartli, Mtskheta-Mtianeti, Kakheti, Imereti, Guria)

13. *Baetis* (*Baetis*) *monnerati* Gattolliat & Sartori, 2012
Distribution: JOR; IS; CAU: IR

14. *Baetis* (*Baetis*) *pentaphlebodes* Ujhelyi, 1966
Distribution: WPAL; CAU: ARM, AZE (Talysh Mts.), GEO (Samtskhe-Javakheti), IR, TUR

15. *Baetis* (*Baetis*) *petrovi* Tshernova, 1938
Distribution: CAU: ARM, AZE (Nagorno-Karabakh)

16. *Baetis* (*Baetis*) *samochai* Koch, 1981
Distribution: TUR; IS; LEB; SY; CAU: IR, ARM

17. *Baetis* (*Baetis*) *vardarensis caucasicus* Zimmermann, 1981
Distribution: CAU: ARM, AZE (Talysh Mts., Kura-Aras lowland), RUS (Krasnodar Krai), GEO (Shida Kartli, Kvemo Kartli, Kakheti), TUR

18. *Baetis* (*Baetis*) *vernus* Curtis, 1834
Distribution: PAL; GEO, RUS (Krasnodar Krai)

19. *Baetis* (*Rhodobaetis*) *braaschi* Zimmermann, 1980
Distribution: UA (Donesk, Kharkov); CP; UZ; KAZ; TURK; TAJ; CAU: GEO (Mtskheta-Mtianeti, Imereti, Kakheti, Shida Kartli, Kvemo Kartli), ARM, RUS (Kabardino-Balkaria, Krasnodar Krai), TUR, IR

20. *Baetis* (*Rhodobaetis*) cf. *qadeai* Thomas, 1999
Distribution: CAU: GEO (Imereti, Samtskhe-Javakheti, Abkhazia, Adjara)

21. *Baetis* (*Rhodobaetis*) *gemellus* Eaton, 1885
Distribution: WPAL; CAU: GEO (Racha-Lechkumi, Samegrelo-Svaneti, Samtskhe-Javakheti, Shida Kartli, Kvemo Kartli, Mtskheta-Mtianeti, Kakheti, Abkhazia), ARM, AZE (Talysh Mts.) RUS (Kabardino-Balkaria, Karachay-Cherkess, Krasnodar Krai, North Ossetia), TUR

22. *Baetis* (*Rhodobaetis*) *rhodani* (Pictet, 1843)
Distribution: WPAL; CAU: AZE (Nakhchivan, Nagorno-Karabakh, Greater Caucasus), ARM, GEO (Kvemo Kartli, Abkhazia, Racha-Lechkhumi, Samegrelo-Svaneti, Shida Kartli, Kakheti, Mtskheta-Mtianeti, Adjara, Guria, Samtskhe-Javakheti), RUS (Kabardino-Balkaria, Krasnodar Krai, North Ossetia), TUR, IR

23. *Baetis* (*Rhodobaetis*) *vadimi* Godunko, Palatov & Martynov, 2015
Distribution: CAU: GEO (Adjara, Imereti), TUR

24. *Baetis* (*Labiobaetis*) *tricolor* Tshernova, 1928
Distribution: PAL; CAU: RUS (Krasnodar Krai)

Genus: *Nigrobaetis*

25. *Nigrobaetis* (*Nigrobaetis*) *digitatus* (Bengtsson, 1912)
Distribution: PAL; CAU: GEO (Guria, Adjara), RUS (Krasnodar Krai)

26. *Nigrobaetis* (*Nigrobaetis*) *gracilis* Bogoescu & Tabacaru, 1957
Distribution: PAL; CAU: AZE (Talysh Mts.), RUS (North Ossetia, Stavropol

Krai), GEO (Kvemo Kartli, Kakheti, Guria, Adjara), IR
27. *Nigrobaetis* (*Takobia*) *katerynae* Martynov & Godunko, 2017
Distribution: CAU: GEO (Adjara)
28. *Nigrobaetis* (*Takobia*) *muticus* (Linnaeus, 1758)
Distribution: WPAL; CAU: ARM, AZE (Talysh Mts.), GEO (Racha-Lechkhumi, Samegrelo-Svaneti, Kakheti, Mtskheta-Mtianeti, Samtskhe-Javakheti, Imereti, Adjara), RUS (Kabardino-Balkaria, Karachay-Cherkess, Krasnodar Krai, North Ossetia), TUR, IR
29. *Nigrobaetis* (*Nigrobaetis*) *niger* (Linnaeus, 1761)
Distribution: PAL; CAU: RUS (Kabardino-Balkaria, North Ossetia), GEO (Kvemo Kartli, Adjara, Guria)
30. *Nigrobaetis* (*Nigrobaetis*) *kars* (Kazancı & Thomas, 1989)
Distribution: TUR; CAU: TUR, ARM

Genus: *Centroptilum* Eaton, 1869
31. *Centroptilum luteolum* (Müller, 1776)
Distribution: HOL; CAU: AZE (Nagorno-Karabakh, Kura-Aras Lowland, Talysh Mts), GEO (Adjara, Guria, Abkhazia), ARM, RUS (Krasnodar Krai, North Ossetia, Stavropol Krai)

Genus: *Cloeon* Leach, 1815
32. *Cloeon* (*Cloeon*) *cognatum* Stephens, 1836
Distribution: HOL; CAU: RUS (North Ossetia, Kabardino-Balkaria, Krasnodar Krai), IR
33. *Cloeon* (*Cloeon*) *dipterum* (Linnaeus, 1761)
Distribution: HOL; CAU: AZE (Greater Caucasus, Nagorno-Karabakh, Talysh Mts., Kura-Aras Lowland), GEO (Samtskhe-Javakheti, Kakheti, Adjara, Guria), RUS (Adygea, Chechen Republic, Dagestan, Kabardino-Balkaria, North Ossetia, Stavropol Krai), IR, TUR
34. *Cloeon* (*Cloeon*) *inscriptum* (Bengtsson, 1914)
Distribution: PAL; CAU
35. *Cloeon* (*Similicloeon*) *simile* Eaton, 1870
Distribution: PAL; CAU: GEO (Adjara, Guria, Abkhazia, Kvemo Kartli), AZE (Kura-Aras Lowland, Nagorno-Karabakh), RUS (Stavropol Krai, Ingushetia), IR

Genus: *Procloeon* Bengtsson, 1915
36. *Procloeon* (*Procloeon*) *bifidum* (Bengtsson, 1912)
Distribution: PAL; CAU: RUS (Krasnodar Krai), GEO (Samtskhe-Javakheti)
37. *Procloeon* (*Pseudocentroptilum*) *caspicum* Sroka, 2018
Distribution: CAU: IR (Gilan province)
38. *Procloeon* (*Procloeon*) *heterophyllum* Kluge & Novikova, 1992
Distribution: CAU: RUS (Krasnodar Krai)
39. *Procloeon* (*Pseudocentroptilum*) *pulchrum* (Eaton, 1885)
Distribution: WPAL; CAU: GEO (Adjara, Imereti), RUS (Krasnodar Krai)
40. *Procloeon* (*Pseudocentroptilum*) *pennulatum* (Eaton, 1870)
Distribution: HOL; CAU: AZE (Talysh Mts.), ARM
41. *Procloeon* (*Pseudocentroptilum*) *unguiculatum* (Tshernova, 1941)
Distribution: PAL; GEO (Imereti), RUS (Krasnodar Krai)

Family: ISONYCHIIDAE Ulmer, 1914
Genus: *Isonychia* Eaton, 1871
42. *Isonychia ignota* (Walker, 1853)
Distribution: PAL; CAU: RUS (WGC, CGC), GEO (Imereti, Kakheti), AZE (Nakhchivan, Greater Caucasus), RUS (Chechen Republic)

Family: OLIGONEURIIDAE Ulmer, 1914
Genus: *Oligoneuriella* Ulmer, 1924
43. *Oligoneuriella rhenana* (Imhoff, 1852)
Distribution: EU; CAU: AZE (Kura-Aras Lowland, Talysh Mts.), RUS (Krasnodar Krai), GEO (Kvemo Kartli, Adjara, Guria)
44. *Oligoneuriella tskhomelidzei* Sowa & Zosidze, 1973
Distribution: CAU: GEO (Adjara, Guria), ARM, RUS (North Ossetia, Dagestan)

Family: HEPTAGENIIDAE Needham & Betten, 1901
Genus: *Ecdyonurus* Eaton, 1868
45. *Ecdyonurus* (*Helvetoraeticus*) *adjaricus* Kluge, Godunko & Apanaskevich, 2013
Distribution: CAU: GEO (Adjara), TUR
46. *Ecdyonurus* (*Ecdyonurus*) *autumnalis* Braasch, 1980
Distribution: CAU: GEO (Imereti and Kakheti), TUR, ARM
47. *Ecdyonurus* (*Ecdyonurus*) *eurycephalus* Hrivniak & Godunko 2018
Distribution: CAU: ARM
48. *Ecdyonurus* (*Ecdyonurus*) *ornatipennis* Tshernova, 1938
Distribution: TUR; CAU: GEO (Shida Kartli, Mtskheta-Mtianeti, Kvemo Kartli), ARM, AZE (Nagorno-Karabakh, Talysh Mts.), RUS (North Ossetia), IR

Genus: *Electrogena* Zurwerra & Tomka, 1985
49. *Electrogena armeniaca* (Braasch, 1983)
Distribution: CAU: ARM
50. *Electrogena azerbajdzanica* (Braasch, 1978)
Distribution: CAU: AZE (Greater Caucasus), GEO (Kakheti)
51. *Electrogena bothmeri* (Braasch, 1983)
Distribution: CAU: IR
52. *Electrogena qibedede* Sroka & Godunko, 2012
Distribution: CAU: RUS (Adygea)
53. *Electrogena kurarensis* (Braasch, 1978)
Distribution: CAU: GEO (Shida Kartli)
54. *Electrogena meyi* (Braasch, 1980)
Distribution: CAU: RUS (Karachay Cherkess)
55. *Electrogena monticola* (Braasch, 1980)
Distribution: CAU: RUS (Karachay Cherkess, North Ossetia)
56. *Electrogena pseudaffinis* (Braasch, 1980)
Distribution: CAU: GEO (Mtskheta-Mtianeti, Kvemo Kartli, Adjara), RUS (Krasnodar Krai), IR, TUR, ARM
57. *Electrogena ressli* (Braasch, 1981)
Distribution: CAU: IR, TUR
58. *Electrogena squamata* (Braasch, 1978)
Distribution: CAU: GEO (Mtskheta-Mtianeti, Abkhazia, Imereti, Kvemo Kartli, Adjara), RUS (Kabardino-Balkaria, North Ossetia), IR, ARM, AZE (Talysh Mts.)
59. *Electrogena zimmermanni* (Sowa, 1984)
Distribution: CAU: RUS (Karachay Cherkess), GEO (Abkhazia), AZE (Talysh Mts.)

Genus: *Rhithrogena* Eaton, 1882
60. *Rhithrogena (Rhithrogena) caucasica* Braasch, 1979
Distribution: CAU: AZE (Talysh Mts.), GEO (Mtskheta-Mtianeti, Abkhazia), RUS (Krasnodar Krai, North Ossetia), ARM, TUR
61. *Rhithrogena* (*Rhithrogena*) *dagestanica* Braasch, 1979
Distribution: CAU: AZE (Greater Caucasus)
62. *Rhithrogena* (*Rhithrogena*) *decolorata* Sinitshenkova, 1973

Distribution: CAU: ARM, AZE (Talysh Mts.), RUS (North Ossetia, Kabardino-Balkaria), IR, GEO (Adjara)

63. *Rhithrogena* (*Rhithrogena*) *expectata* Braasch, 1979

Distribution: CAU: RUS (Krasnodar Krai, Karachay Cherkess), GEO (Shida Kartli, Kvemo Kartli, Mtskheta-Mtianeti, Abkhazia), TUR

64. *Rhithrogena* (*Rhithrogena*) *excisa* Sinitshenkova, 1979

Distribution: CAU: AZE (Nagorno-Karabakh), RUS (North Ossetia, Kabardino-Balkaria)

65. *Rhithrogena* (*Rhithrogena*) *iranica* Braasch, 1983

Distribution: CAU: TUR, IR

66. *Rhithrogena* (*Rhithrogena*) *iridina kownackii* Sowa & Zimmermann, 1975

Distribution: CAU: GEO (Mtskheta-Mtianeti, Adjara), ARM

67. *Rhithrogena* (*Rhithrogena*) *joostiana* Sowa & Zimmermann, 1976

Distribution: CAU: RUS (Kabardino-Balkaria)

68. *Rhithrogena* (*Rhithrogena*) *klausniczeriana* Braasch, 1979

Distribution: CAU: RUS (Karachay Cherkess)

69. *Rhithrogena* (*Rhithrogena*) *laciniosa* Sinitshenkova, 1979

Distribution: CAU: AZE (Nagorno-Karabakh, Talysh Mts.), RUS (North Ossetia)

70. *Rhithrogena* (*Rhithrogena*) *lucida* Braasch, 1979

Distribution: CAU: GEO (Mtskheta-Mtianeti)

71. *Rhithrogena* (*Rhithrogena*) *potamalis* Braasch, 1979

Distribution: CAU: GEO (Shida Kartli, Kvemo Kartli), TUR

72. *Rhithrogena* (*Rhithrogena*) *teberdensis* Zimmermann, 1977

Distribution: CAU: RUS (Kabardino-Balkaria, North Ossetia, Karachay Cherkess)

73. *Rhithrogena* (*Rhithrogena*) *umbrosa* Braasch, 1979

Distribution: CAU: RUS (Karachay Cherkess)

74. *Rhithrogena* (*Rhithrogena*) *zhiltzovae* Sinitshenkova, 1979

Distribution: CAU: ARM

75. *Rhithrogena* (*Epeiron*) *binerve* Kluge, 1987

Distribution: CAU: GEO (Kvemo Kartli), ARM

76. *Rhithrogena* (*Epeiron*) *paulinae* Sartori & Sowa, 1992

Distribution: CAU: IR

77. *Rhithrogena* (*Epeiron*) *znojkoi* (Tshernova, 1938)

Distribution: LBN; IS; CAU: AZE, GEO (Kvemo Kartli), TUR

Genus: *Epeorus* Eaton, 1881

78. *Epeorus* (*Caucasiron*) *alborzicus* Hrivniak & Sroka, 2020

Distribution: CAU: IR

79. *Epeorus* (*Caucasiron*) *alpestris* (Braasch, 1979)

Distribution: CAU: GEO (Mtskheta-Mtianeti), RUS (Karachay Cherkess)

80. *Epeorus* (*Caucasiron*) *bicolliculatus* Hrivniak, 2017

Distribution: CAU: TUR, GEO (Abkhazia, Guria, Adjara, Imereti, Samtskhe-Javakheti, Racha-Lechkhumi-Kvemo Svaneti), ARM

81. *Epeorus* (*Caucasiron*) *caucasicus* (Tshernova, 1938)

Distribution: CAU: ARM, AZE (Nagorno-Karabakh), GEO (Adjara, Samtskhe-Javakheti, Mtskheta Mtianeti, Racha-Lechkhumi-Kvemo Svaneti), RUS (Kabardino-Balkaria, North Ossetia, Karachay Cherkess), TUR

82. *Epeorus* (*Caucasiron*) *iranicus* (Braasch & Soldán, 1979)

Distribution: CAU: IR

83. *Epeorus* (*Caucasiron*) *longimaculatus* (Braasch, 1980)

Distribution: CAU: GEO (Mtskheta-Mtianeti)

84. *Epeorus* (*Caucasiron*) *magnus* (Braasch, 1978)
Distribution: AM; CAU: AZE (Greater Caucasus), ARM, RUS (Krasnodar Krai, Kabardino-Balkaria, North Ossetia), GEO (Mtskheta-Mtianeti, Abkhazia, Adjara, Guria, Samtskhe-Javakheti, Imereti, Racha-Lechkhumi-Kvemo Svaneti, Shida Kartli, Kvemo Kartli, Kakheti), TUR
85. *Epeorus* (*Caucasiron*) *nigripilosus* (Sinitshenkova, 1976)
Distribution: TUR; CY; CAU: GEO (Mtskheta-Mtianeti), RUS (Karachay Cherkess), TUR, IR
86. *Epeorus* (*Caucasiron*) *shargi* Hrivniak & Sroka, 2020
Distribution: CAU: IR
87. *Epeorus* (*Caucasiron*) *sinitshenkovae* (Braasch & Zimmerman, 1979)
Distribution: CAU: RUS (Kabardino-Balkaria, Karachay Cherkess), GEO (Mtskheta-Mtianeti)
88. *Epeorus* (*Caucasiron*) *soldani* (Braasch, 1979)
Distribution: CAU: GEO (Samegrelo-Zemo Svaneti, Racha Lechkhumi Kvemo Svaneti), RUS (Karachay Cherkess)
89. *Epeorus* (*Caucasiron*) *turcicus* Hrivniak, Türkmen & Kazancı, 2019
Distribution: CAU: TUR
90. *Epeorus* (*Caucasiron*) *zagrosicus* Hrivniak & Sroka, 2020
Distribution: IR (Zagros Mts.)
91. *Epeorus* (*Caucasiron*) *znojkoi* (Tshernova, 1938)
Distribution: AM; CAU: GEO (Mtskheta-Mtianeti, Abkhazia, Adjara, Guria, Semegrelo -Zemo Svaneti, Imereti, Racha-Lechkhumi Kvemo Svaneti, Kvemo Kartli, Kakheti), AZE (Greater Caucasus, Nagorno-Karabakh, Talysh Mts.), ARM, IR, RUS (Karachay Cherkess, Kabardino-Balkaria, North Ossetia), TUR
92. *Epeorus* (*Epeorus*) *zaitzevi* Tshernova, 1981
Distribution: AM; IS; IQ; SY; CAU: GEO (Adjara, Imereti, Shida Kartli, Mtskheta-Mtianeti), ARM, IR, TUR

Genus: *Heptagenia* Walsh, 1863
93. *Heptagenia* (*(Dacnogenia*) *coerulans micracantha* Kluge 1987
Distribution: CAU: GEO (Kvemo Kartli), ARM
94. *Heptagenia* (*Heptagenia*) *flava* Rostock, 1878
Distribution: PAL; CAU: RUS (North Ossetia)
95. *Heptagenia* (*Kageronia*) *fuscogrisea* (Retzius, 1783)
Distribution: PAL; CAU: RUS (North Ossetia)
96. *Heptagenia* (*Heptagenia*) *samochai* Demoulin, 1973
Distribution: AM; CP; IS; TUR; CAU: RUS (North Ossetia), GEO (Imereti, Kakheti, Adjara, Mtskheta-Mtianeti, Kvemo Kartli), IR, ARM, AZE (Talysh Mts.)
97. *Heptagenia* (*Heptagenia*) *sulphurea* (Müller, 1776)
Distribution: PAL; CAU: GEO (Kakheti, Adjara, Guria), AZE (Greater Caucasus, RUS (Kabardino-Balkaria, North Ossetia)

Family: LEPTOPHLEBIIDAE Banks, 1900
Genus: *Habroleptoides* Schoenemund, 1929
98. *Habroleptoides caucasica* Tschernova, 1931
Distribution: CAU: GEO (Abkhazia, Guria), RUS (Krasnodar Krai, North Ossetia), TUR
99. *Habroleptoides confusa* Sartori & Jacob, 1986
Distribution: EU; CAU: ARM, AZE (Talysh Mts.), GEO (Adjara), RUS (North Ossetia), IR, TUR
100. *Habroleptoides kavron* Kazanci & Turkmen 2011
Distribution: CAU: TUR

101. *Habroleptoides pontica* Kluge, 1934
Distribution: CAU: RUS (Krasnodar Krai)

Genus: *Leptophlebia* Westwood, 1840
102. *Leptophlebia vespertina* (Linnaeus, 1758)
Distribution: WPAL; CAU: GEO (Adjara and Guria)

Genus: *Habrophlebia* Eaton 1881
103. *Habrophlebia lauta* Eaton, 1884
Distribution: WPAL; CAU: IR, TUR, AZE (Talysh Mts)
104. *Habrophlebia fusca* (Curtis, 1834)
Distribution: WPAL; CAU: TUR, RUS (Kabardino-Balkaria, North Ossetia)

Genus: *Paraleptophlebia* Lestage, 1917
105. *Paraleptophlebia submarginata* (Stephens, 1835)
Distribution: WPAL; CAU: RUS (North Ossetia), GEO (Adjara, Guria), IR, TUR
106. *Paraleptophlebia werneri* Ulmer, 1920
Distribution: WPAL; CAU: GEO (Abkhazia), AZE (Talysh Mts.)

Genus: *Choroterpes* Eaton, 1881
107. *Choroterpes* (*Euthraulus*) *caucasicus* Kluge, 2012
Distribution: CAU: RUS (Krasnodar Krai)
108. *Choroterpes* (*Choroterpes*) *picteti subcostalis* Kluge, 2012
Distribution: CAU: RUS (Krasnodar Krai)

Family: EPHEMERIDAE Latreille, 1810
Genus: *Ephemera* Linnaeus, 1758
109. *Ephemera danica* Müller, 1764
Distribution: WPAL; CAU: GEO (Kvemo Kartli, Samtskhe-Javakheti), IR, TUR
110. *Ephemera romantzovi* Kluge, 1988
Distribution: TUR; CAU: ARM, AZE (Talysh Mts.), RUS (Krasnodar Krai)
111. *Ephemera vulgata* Linnaeus, 1758
Distribution: PAL; CAU: RUS (Adygea, Krasnodar Krai), AZE (Kura-Aras Lowland), GEO (Adjara, Guria), TUR

Family: POLYMITARCYIDAE Ulmer, 1920
Genus: *Ephoron* Williamson, 1802
112. *Ephoron virgo* (Olivier, 1791)
Distribution: PAL; CAU: RUS (Adygea)

Family: PALINGENIDAE Albarda, 1888
Genus: *Palingenia* Burmeister, 1839
113. *Palingenia fuliginosa* Georgi 1802
Distribution: UA; SK; IR; CAU: AZE (Nagorno-Karabakh), RUS (Krasnodar Krai, North Ossetia)
114. *Palingenia longicauda* (Olivier, 1791)
Distribution: EU; TUR; CAU: AZE (Kura-Aras Lowland), RUS (North Ossetia), GEO (Adjara, Guria), IR

Family: POTAMANTHIDAE Albarda, 1888
Genus: *Potamanthus* Pictet, 1843
115. *Potamanthus luteus* (Linnaeus, 1767)
Distribution: PAL; OR; CAU: GEO (Kvemo Kartli), AZE (Kura-Aras Lowland), TUR

Family: EPHEMERELIIDAE Klapálek, 1909
Genus: *Ephemerella* Walsh, 1863
116. *Ephemerella ignita* (Poda, 1761)
Distribution: PAL; CAU: ARM, AZE (Greater Caucasus, Kura-Aras Lowland, Nagorno-Karabakh), GEO (Kakheti, Adjara, Guria), RUS (Krasnodar Krai, North Ossetia, Kabardino-Balkaria), IR, TUR
117. *Ephemerella maculocaudata* Ikonomov, 1961

Distribution: EU; IR; CAU

Genus: *Eurylophella* Tiensuu, 1935
118. *Eurylophella korneyevi* Martynov, Palatov & Godunko, 2015
Distribution: CAU: GEO (Adjara)

Genus: *Serratella* Edmunds, 1959
119. *Serratella elissa* Jacobus, Zhou & McCafferty, 2009
Distribution: CAU: IR

Genus: *Torleya* Lestage, 1917
120. *Torleya major* Klapálek, 1905
Distribution: WPAL; CAU: AZE (Talysh Mts.)

Genus: *Teloganopsis* Ulmer, 1939
121. *Teloganopsis subsolana* (Allen, 1973)
Distribution: AFG; CAU: IR

Family: CAENIDAE Newman, 1853
Genus: *Caenis* Stephens, 1836
122. *Caenis beskidensis* Sowa, 1975
Distribution: EU; CAU: ARM, GEO
123. *Caenis horaria* (Linnaeus, 1758)
Distribution: PAL; CAU: ARM
124. *Caenis luctuosa* (Burmeister, 1839)
Distribution: PAL; CAU: GEO (Kvemo Kartli), ARM
125. *Caenis macrura* Stephens, 1836
Distribution: PAL; CAU: ARM, AZE (Kura-Aras Lowland, Nagorno-Karabakh, Nakhchivan), GEO (Imereti, Shida Kartli, Kvemo Kartli, Kakheti, Adjara, Guria, Samtskhe-Javakheti), RUS (Adygea, Krasnodar Krai, North Ossetia, Kabardino-Balkaria), IR, TUR
126. *Caenis pseudorivulorum* Keffermüller, 1960
Distribution: PAL; CAU: GEO (Adjara), ARM
127. *Caenis robusta* Eaton, 1884
Distribution: PAL; CAU: GEO (Guria)
128. *Caenis rivulorum* Eaton, 1884
Distribution: PAL; CAU: ARM

Genus: *Clypeocaenis* Soldán, 1978
129. *Clypeocaenis bisetosa* Soldán, 1978
Distribution: IN; CAU: IR

Family: PROSOPISTOMATIDAE Laméere, 1917
Genus: *Prosopistoma* Latreille, 1833
130. *Prosopistoma pennigerum* (Fourcroy, 1785)
Distribution: PAL; CAU: GEO (Kakheti, Kvemo Kartli, Samtskhe-Javakheti)
Distribution: BU; GR; TUR; MAC; CAU: GEO (Adjara)

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The list of the species is compiled from references cited in section "*Part one: Background, 4. Mayflies (Ephemeroptera) of the Caucasus: the current state of knowledge*".

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Hrivniak Ľ., Sroka P., Godunko R. J., Žurovcová M., Bojková J., Soldán T., Staniczek A. (2018). Diversity, distribution and molecular phylogeny of Epeorus (Caucasiron) Kluge, 1997 (Ephemeroptera: Heptageniidae) in the Caucasus Mountains and adjacent areas.
Talk at XV International Conference on Ephemeroptera & XIX International Symposium on Plecoptera, 3–8 June 2018, Aracruz, Brazil.

Hrivniak Ľ., Sroka P., Godunko R.J. Mayflies of the Caucasus: diversity and perspectives.

Poster presentation at Fresh Blood for Freshwater, 9.4.–13.4. 2017, České Budějovice, Czechia.

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Armenia, Ukrajine (2015), Turkey, Greece (2016), Azerbaijan, Georgia (2017, 2018, 2019), Argentina (2019, 2020).

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