



## 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

ĽUBOŠ HRIVNIAK<sup>1,3,4</sup>, PAVEL SROKA<sup>1</sup>, ROMAN J. GODUNKO<sup>1,2</sup> & MARTINA ŽUROVCOVÁ<sup>1</sup>

<sup>1</sup>Institute of Entomology, Biology Centre CAS, Branišovská 31, CZ-37005 České Budějovice, Czech Republic.

E-mails: [lubos.hrivniak@gmail.com](mailto:lubos.hrivniak@gmail.com), [pavel.sroka@centrum.cz](mailto:pavel.sroka@centrum.cz), [martina@entu.cas.cz](mailto:martina@entu.cas.cz)

<sup>2</sup>State Museum of Natural History, National Academy of Sciences of Ukraine, Teatralna 18, UA-79008 Lviv, Ukraine.

E-mail: [godunko@seznam.cz](mailto:godunko@seznam.cz)

<sup>3</sup>Faculty of Sciences, University of South Bohemia, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic

<sup>4</sup>Corresponding author

### 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.) znojtkoi* (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).

Outside the Caucasus, *Caucasiron* also occurs in Greece (*E. (C.) znojkoii 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<sub>2</sub>O, 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](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://www.wabi.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

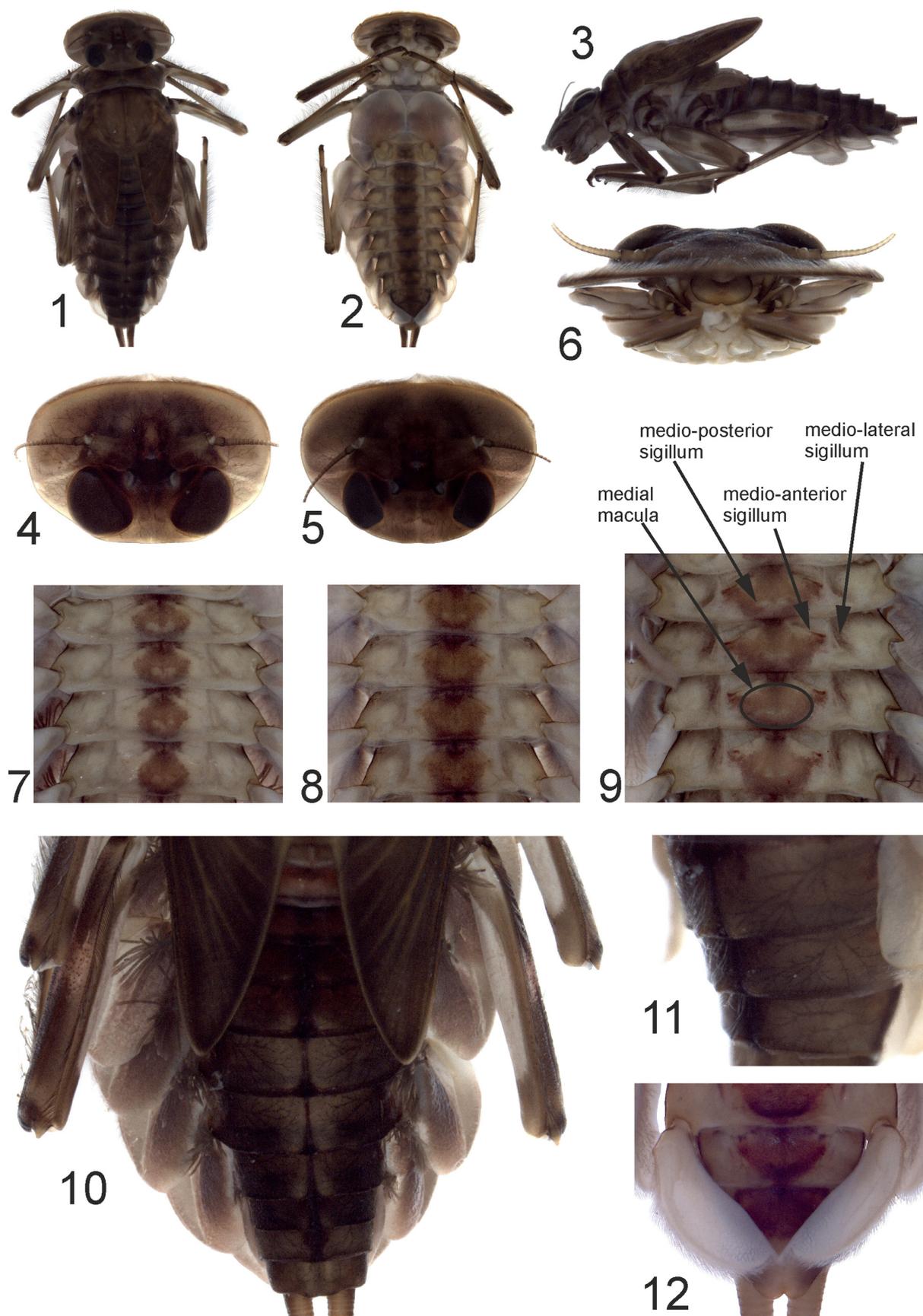
#### *Epeorus (Caucasiron) bicolliculatus* Hrivniak, sp. nov.

Figures 1–48

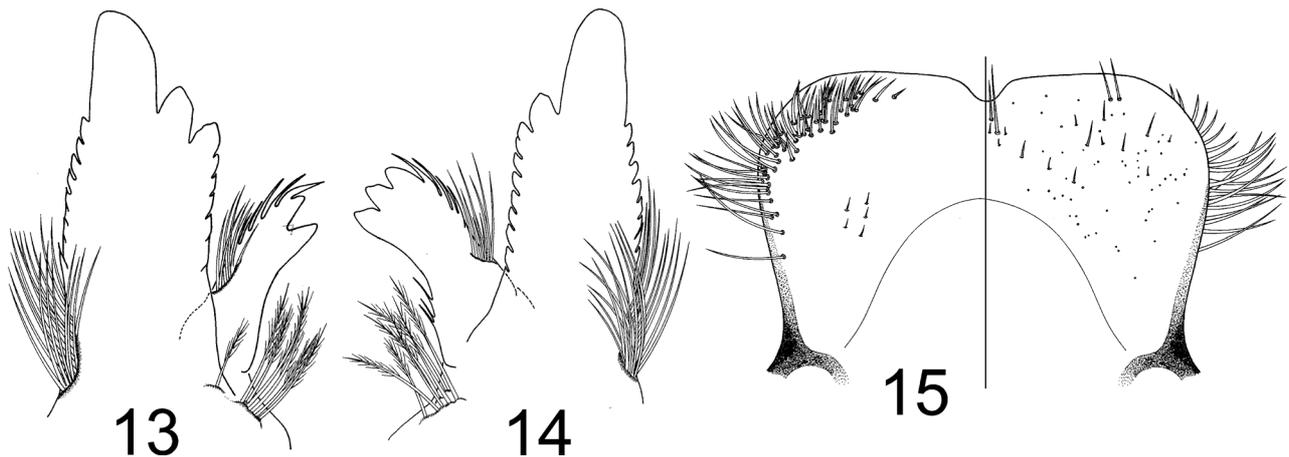
*Epeorus alpicola* (Eaton, 1871): Türkmen & Kazancı (2015: 44) [partim]

*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 Hamşıköy 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  $1.3 \times$  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.

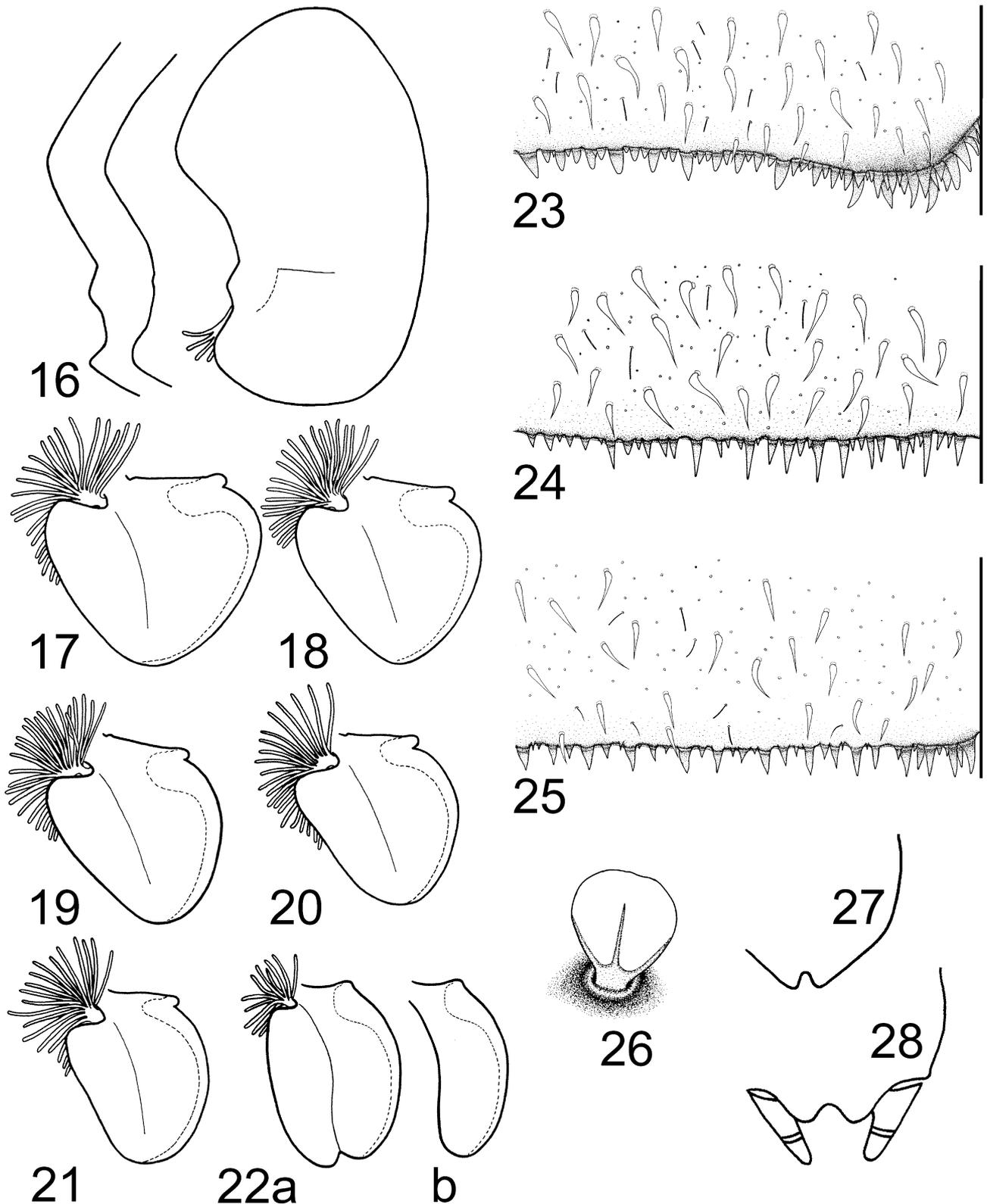
**Mouthparts.** Labrum (Figs 6, 15) widened distally, with anterior margin slightly concave or nearly straight; small medial emargination present. Antero-lateral margins rounded (shape of labrum may vary among individual specimens). Dorsal surface (Fig. 15, right) with sparse scattered setae and longer 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). Protheca 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.

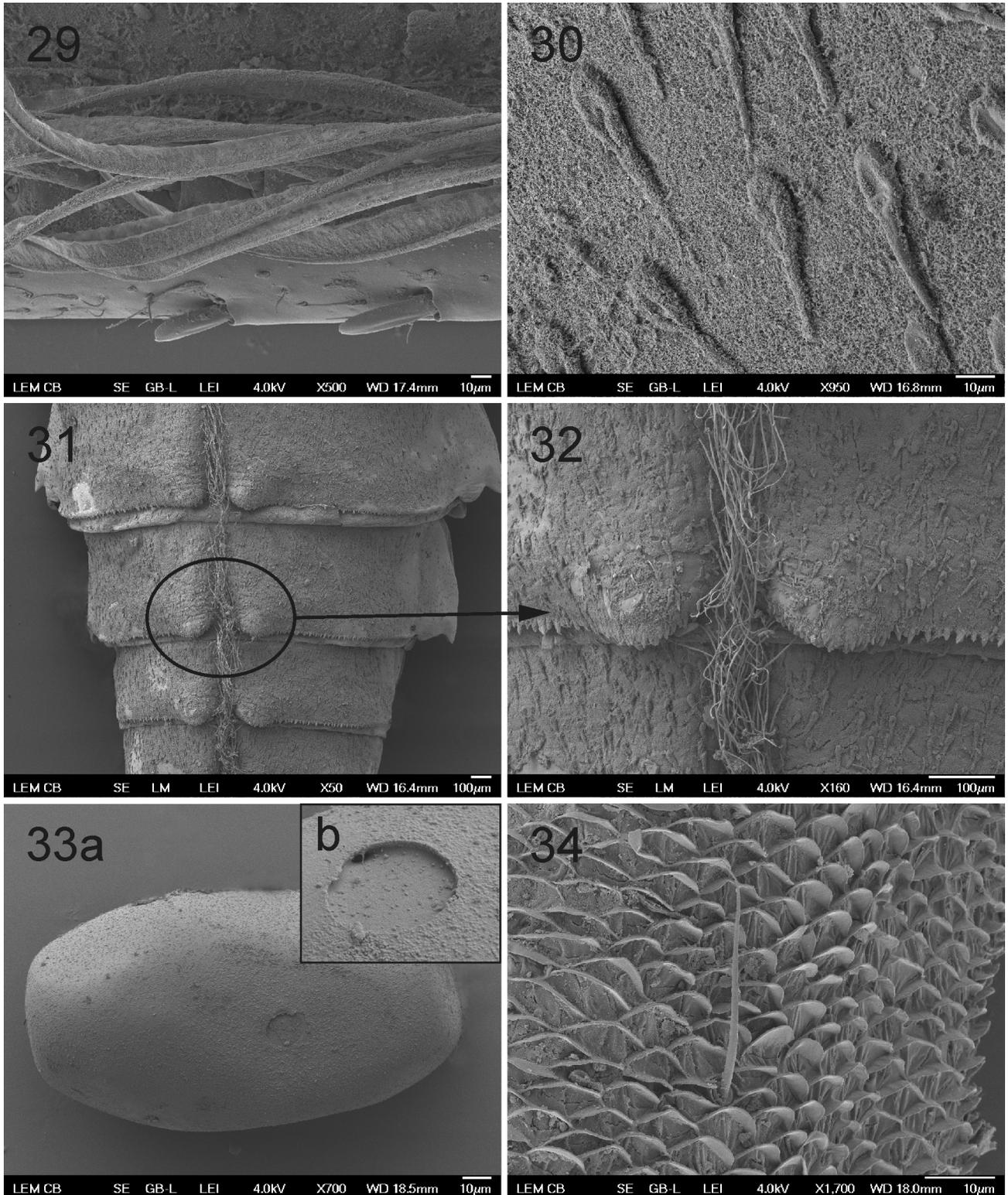
**Legs.** Yellowish-brown to reddish-brown. Femora with more or less contrasting markings dorsally (Fig. 3), without hypodermal medial spot (blurred macula may be present in darker specimens). Tibio-patellar suture darkened, tibia unicoloured, tarsi proximally and distally darkened. Coxal projections 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 ( $1.17$ – $1.25 \times$  femur length), tibiae of middle legs nearly equal length as femora ( $1$ – $1.08 \times$  femur length), tibiae of hind legs slightly shorter than femora ( $0.81$ – $0.98 \times$  femur length). Tarsi in all legs  $0.26$ – $0.37 \times$  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.

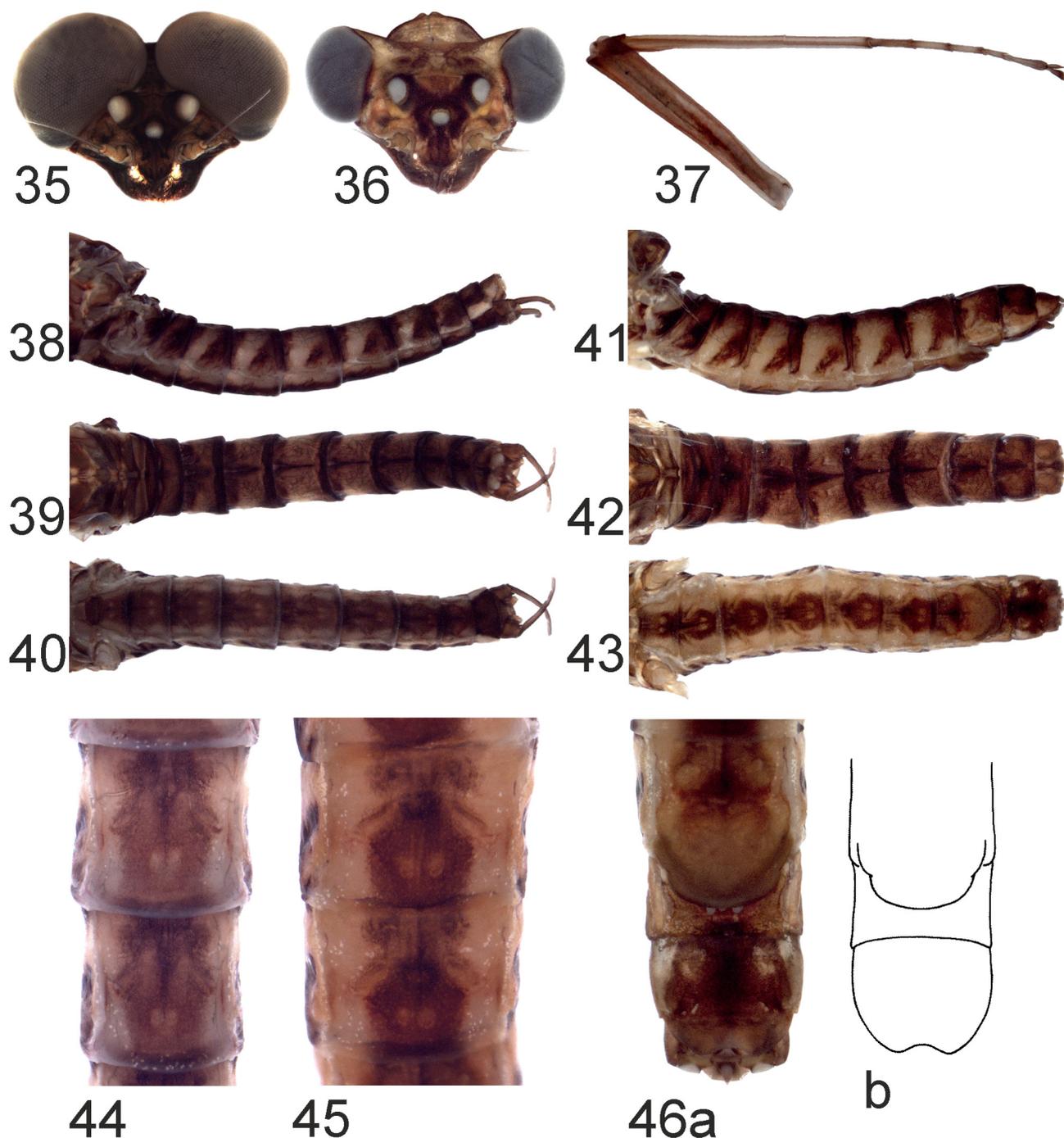
**Abdominal terga** yellowish-brown, with contrasting dark brown or reddish-brown pattern (Figs 1, 10), that consists of: a) narrow macula along anterior margin on terga I–IX; b) large medial macula on terga II–IV (shape of macula variable; usually in form of blurred stripe or triangle); c) anteriorly 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.

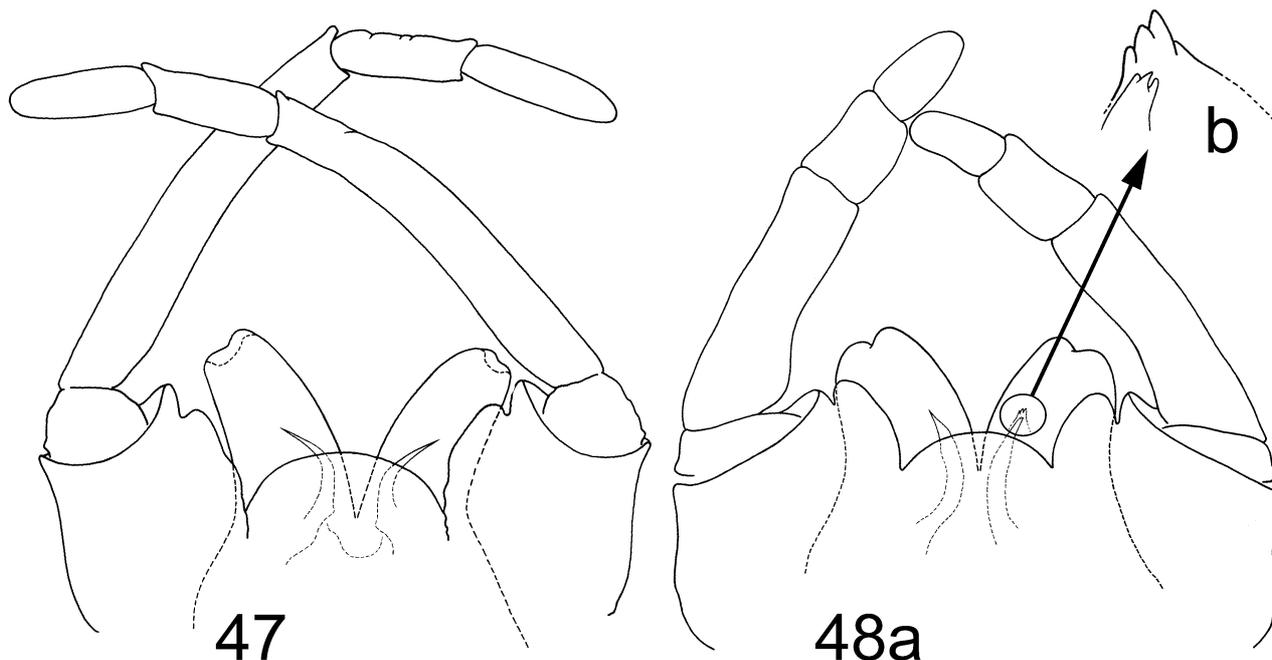


**FIGURES 29–34.** *Epeorus (Caucasiron) bicolliculatus* sp. nov., larva, egg, subimago: 29, blade-like setae and bluntly pointed spine-like setae along posterior margin of femora (larva); 30, basally widened setae on tergum VII (larva); 31, tergal protuberances on terga VI–VIII (larva); 32, tergal protuberances in detail (larva); 33a, egg; 33b, detail of chorionic surface and micropyle; 34, detail of microlepid 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  $0.38\text{--}0.54\times$  length of respective plate, filaments of gill VII to  $0.2\text{--}0.33\times$  (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  $2.35\times$  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. Mesothoracic 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. Length 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.) znojkoii*. 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.) znojkoii*, 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 “*znojkoii* Gruppe“ (penis lobes only with shallow apical emargination) with species *E. (C.) znojkoii*, *E. (C.) alpestris*, *E. (C.) soldani*, *E. (C.) sinitshenkovae*, *E. (C.) magnus*. In this classification, *E. (C.) bicolliculatus* **sp. nov.** is congruent with the “*znojkoii* 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 microlepidides, whereas 2nd–5th segment are entirely covered by blunt microlepidides (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 microlepidides 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.

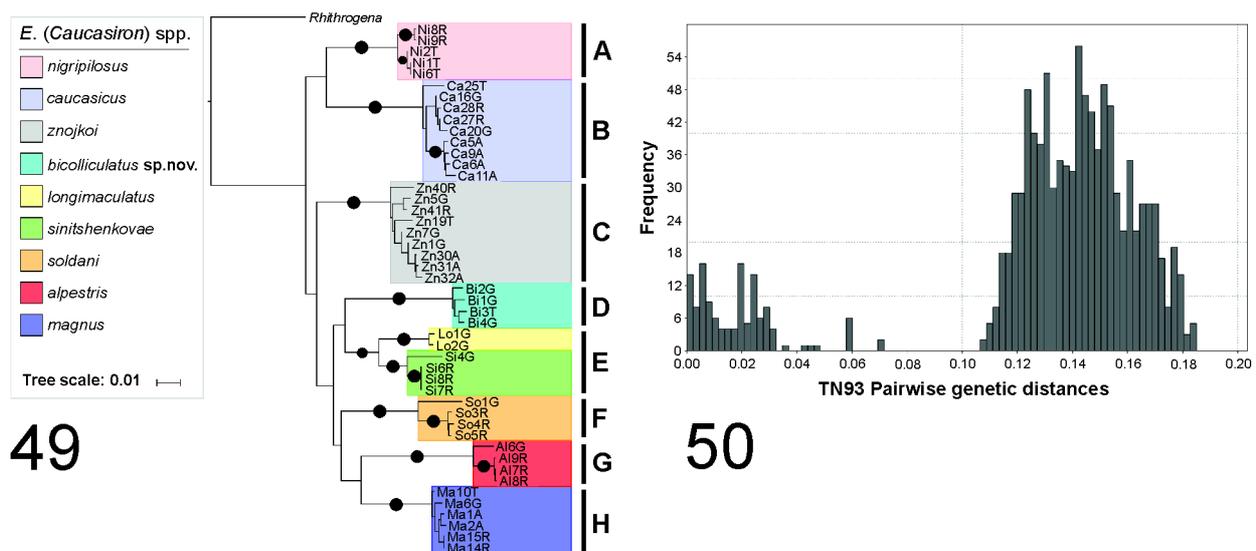
## Analyses of molecular data

**DNA sequencing.** The „DNA Barcoding“ fragment of COI was successfully 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.) znojkoii* (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.) znojkoii*, *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  $\geq 97\%$ . 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	<i>caucasicus</i>	<i>nigripilosus</i>	<i>longimaculatus</i>	<i>sinitshenkovae</i>	<i>znojkoii</i>	<i>soldani</i>	<i>bicolliculatus</i> <b>sp.nov.</b>	<i>magnus</i>
<i>nigripilosus</i>	12.40/0.56/ 11.42–13.53							
<i>longimaculatus</i>	12.74/0.43/ 12.05–13.35	15.83/0.46/ 15.10–16.37						
<i>sinitshenkovae</i>	13.81/0.85/ 12.76–15.87	14.46/0.43/ 13.93–15.54	6.17/0.55/ 5.79–7.14					
<i>znojkoii</i>	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				
<i>soldani</i>	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			
<i>bicolliculatus</i> <b>sp.nov.</b>	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		
<i>magnus</i>	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	
<i>alpestris</i>	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 ( $\geq 97\%$ ) 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
<i>caucasicus</i>	9	1.69/0.85/0.15–3.14
<i>nigripilosus</i>	5	1.11/0.86/0.00–1.86
<i>longimaculatus</i>	2	0.62
<i>sinitshenkovae</i>	4	1.49/1.62/0.00–2.97
<i>znojko</i>	9	1.87/0.98/0.15–3.63
<i>soldani</i>	4	2.48/2.26/0.31–4.72
<i>bicolliculatus</i> <b>sp.nov.</b>	4	1.20/0.23/0.78–1.41
<i>magnus</i>	6	0.69/0.29/0.00–1.23
<i>alpestris</i>	4	1.29/1.30/0.00–2.59

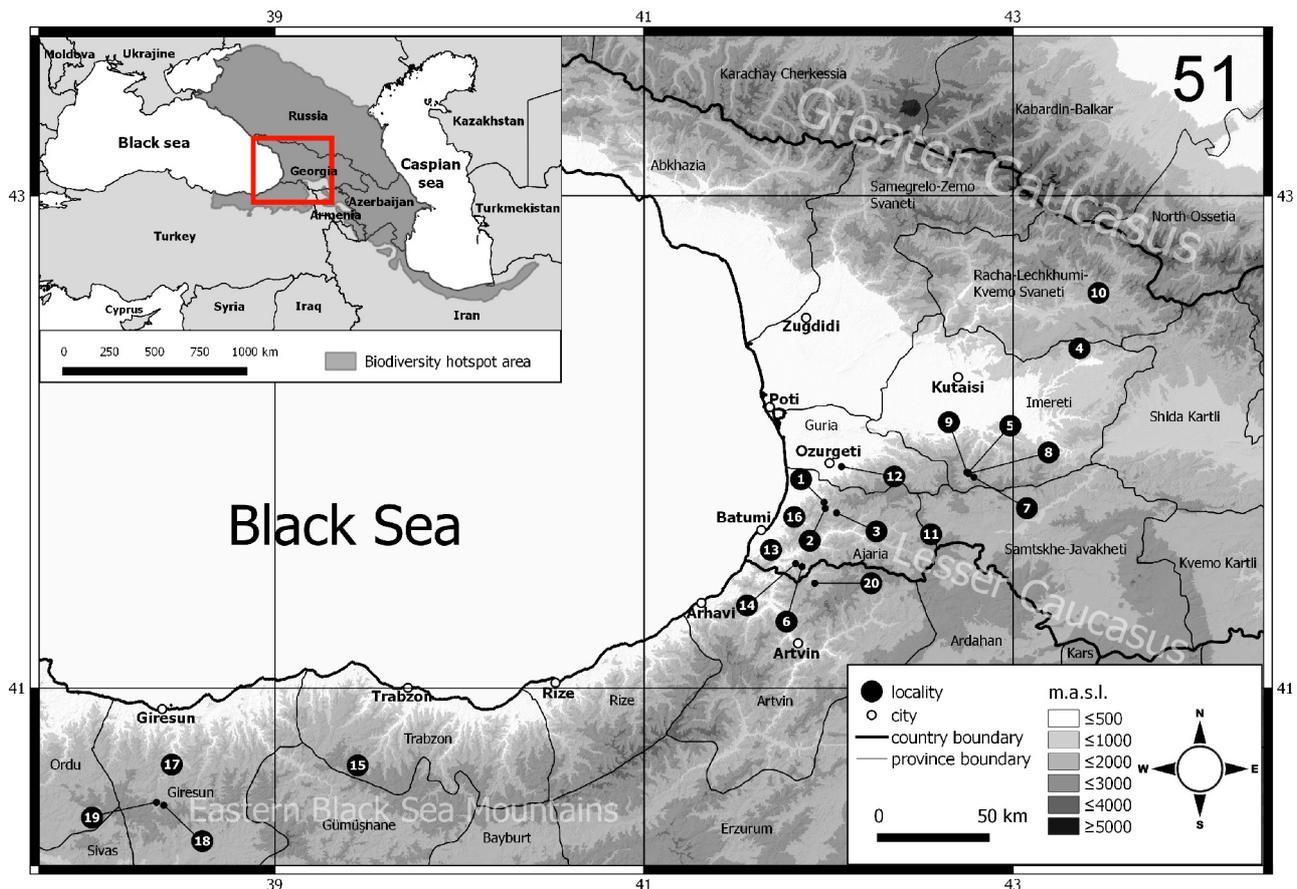
**Distribution, habitat and biology.** *E. (C.) bicolliculatus* **sp. nov.** occurs in the streams and rivers of the Lesser Caucasus (NE Turkey/Eastern Black Sea Mountains and SW Georgia) and the southern slopes of the Central Greater Caucasus (N Georgia) (Fig. 51). Occurrence on the southern slopes 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. *znojko* 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.) sinitschenkova* 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 *Siphonurus* 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.

## Acknowledgements

We thank our colleagues T. Soldán, J. Bojková, A.V. Martynov, V.V. Martynov and G. Türkmen for kindly providing material at our disposal, and to O. Nedvěd for help with photographic documentation. We are also grateful to J. Oboňa and P. Manko for the opportunity to participate in the sampling of comparative material in Armenia, to L. Mocek and P. Černuška for consultation regarding Latin, to P. Kokořová for help with laboratory procedures and to S. Rutschmann for providing unpublished primer sequences. S. Gabelashvili is thanked for checking the spelling of localities in Georgia. We thank reviewers J. Webb and C. Belfiore for constructive comments. This research was conducted with institutional support RVO: 60077344 (IE, BC CAS), grant GAJU 152/2016/P provided by the University of South Bohemia for LH and President's of Ukraine grant for competitive projects (18426) of the State Fund for Fundamental Research for RJG.

## References

- Alexander, L.C., Delion, M., Hawthorne, D.J., Lamp, W.O. & Funk, D.H. (2009) Mitochondrial lineages and DNA barcoding of closely related species in the mayfly genus *Ephemerella* (Ephemeroptera: Ephemerellidae). *Journal of the North American Benthological Society*, 28 (3), 584–595.  
<https://doi.org/10.1899/08-150.1>
- Ball, S.L., Hebert, P.D.N., Burian, K. & Webb, J.M. (2005) Biological identifications of mayflies (Ephemeroptera) using DNA barcodes. *The North American Benthological Society*, 24 (3), 508–524.  
<https://doi.org/10.1899/04-142.1>
- Bauernfeind, E. & Soldán, T. (2012) *The mayflies of Europe (Ephemeroptera)*. Apollo Books, Ollerup, 781 pp.  
<https://doi.org/10.1163/9789004260887>
- Braasch, D. (1978) *Epeorus znojko* Tshernova und *Iron magnus* n. sp. (Heptageniidae, Ephemeroptera) aus dem Kaukasus. *Entomologische Nachrichten*, 22 (5), 65–70.
- Braasch, D. (1979) Beitrag zur Kenntnis der Gattung *Iron* Eaton im Kaukasus (UdSSR) (III) (Ephemeroptera, Heptageniidae). *Reichenbachia*, 17 (33), 283–294.
- Braasch, D. (1980) Beitrag zur Kenntnis der Gattung *Iron* Eaton (Heptageniidae, Ephemeroptera) im Kaukasus (UdSSR), 2.

- Entomologische Nachrichten*, 24 (10–11), 166–173.
- Braasch, D. (1981) Eintagsfliegen (Gattungen *Epeorus* und *Iron*) aus Nepal (II) (Ephemeroptera, Heptageniidae). *Reichenbachia*, 19 (18), 105–110.
- Braasch, D. (1983a) Zwei neue Heptageniidae von den griechischen Inseln (Ephemeroptera). *Reichenbachia*, 21 (11), 69–74.
- Braasch, D. (1983b) Eintagsfliegen (Gattungen *Epeorus* und *Iron*) aus Nepal und Indien (Ephemeroptera, Heptageniidae). *Reichenbachia*, 21 (34), 195–196.
- Braasch, D. (2006a) Neue Eintagsfliegen der Gattungen *Epeorus* und *Iron* aus dem Himalaja (Ephemeroptera, Heptageniidae). *Entomologische Nachrichten und Berichte*, 50 (1–2), 79–88.
- Braasch, D. (2006b) *Iron suspicatus* n. sp. (Ephemeroptera, Heptageniidae) aus Nepal und aus dem Kulu-Valley des Himalaja in Indien. *Entomologische Nachrichten und Berichte*, 50 (3), 125–128.
- Braasch, D. & Soldán, T. (1979) Neue Heptageniidae aus Asien (Ephemeroptera). *Reichenbachia*, 17 (31), 261–272.
- Braasch, D. & Zimmermann, W. (1979) *Iron sinitshenkovae* sp.n. – eine neue Heptageniide (Ephemeroptera) aus dem Kaukasus. *Entomologische Nachrichten*, 23 (7), 103–107.
- Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution*, 17 (4), 540–552.  
<https://doi.org/10.1093/oxfordjournals.molbev.a026334>
- Chen, D. Ch. (1999) *Mayflies of the tribe Rhithrogenini (Ephemeroptera, Heptageniidae) of Russia and adjacent countries* (Поденки трибы Rhithrogenini (Ephemeroptera, Heptageniidae) территории России и сопредельных стран). Institute of Zoology, Russian Academy of Sciences, St. Petersburg, 209 pp. [dissertation thesis in Russian; unpublished manuscript]
- Chen, P., Wang, Y., Zhou, C-F. (2010) A New Mayfly Species of *Epeorus* (*Caucasiron*) from Southwestern China (Ephemeroptera: Heptageniidae). *Zootaxa*, 2527, 61–68.  
<https://doi.org/10.5281/ZENODO.196454>
- Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9 (8), 772.  
<https://doi.org/10.1038/nmeth.2109>
- DeSalle, R., Egan, M.G. & Siddall, M. (2005) The unholy trinity: taxonomy, species delimitation and DNA barcoding. *Philosophical transactions of the Royal Society of London, Series B, Biological sciences*, 360 (1462), 1905–1916.  
<https://doi.org/10.1098/rstb.2005.1722>
- DeWalt, R.E. (2011) DNA barcoding: a taxonomic point of view. *Journal of the North American Benthological Society*, 30 (1), 174–181.  
<https://doi.org/10.1899/10-021.1>
- Eaton, A.E. (1868) An outline of a re-arrangement of the genera of Ephemeridae. *Entomologist's Monthly Magazine*, 5, 82–91.
- Eaton, A.E. (1871) *A monograph on the Ephemeridae*. Transactions of the Entomological Society of London for the year 1871, London, 164 pp.
- Eaton, A.E. (1881) An announcement of new genera of the Ephemeridae. *Entomologist's Monthly Magazine*, 17, 21–27.
- Eaton, A.E. (1883–1888) A revisional monograph of recent Ephemeridae or mayflies. *Transactions of the Linnean Society of London*, 3 (2), 1–352.
- Felsenstein, J. (1985) Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution*, 39 (4), 783–791.  
<https://doi.org/10.2307/2408678>
- Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (2004) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3 (5), 294–299.
- Guindon, S. & Gascuel, O. (2003) A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Systematic Biology*, 52 (5), 696–704.  
<https://doi.org/10.1080/10635150390235520>
- Hajibabaei, M., Singer, G.A.C., Hebert, P.D.N. & Hickey, D.A. (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics*, 23 (4), 167–172.  
<https://doi.org/10.1016/j.tig.2007.02.001>
- Hammer, Ø., Harper, D.A.T. & Ryan, P. D. (2001) PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica*, 4 (1), 1–9.
- Katoh, K., Misawa, K., Kuma, K. & Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, 30 (14), 3059–3066.  
<https://doi.org/10.1093/nar/gkf436>
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P. & Drummond, A. (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28 (12), 1647–1649.  
<https://doi.org/10.1093/bioinformatics/bts199>
- Kjaerstad, G., Webb, J.M. & Ekrem, T. (2012) A review of Ephemeroptera of Finnmark—DNA barcodes identify Holarctic relations. *Norwegian Journal of Entomology*, 59 (2), 182–195.
- Kluge, N.J. (1997) New subgenera of Holarctic mayflies (Ephemeroptera: Heptageniidae, Leptophlebiidae, Ephemerellidae). *Zoosystematica Rossica*, 5 (2), 233–235.

- Kluge, N.J. (2004) *The phylogenetic system of Ephemeroptera*. Springer, Dordrecht, 456 pp.  
<https://doi.org/10.1007/978-94-007-0872-3>
- Kluge, N.J. (2015) Central Asian mountain Rhithrogenini (Ephemeroptera: Heptageniidae) with pointed and ephemeropteroid claws in the winged stages. *Zootaxa*, 3994 (3), 301–353.  
<https://doi.org/10.11646/zootaxa.3994.3.1>
- Kluge, N.J. & Novikova, E.A. (2011) Systematics of the mayfly taxon *Acentrella* (Ephemeroptera, Baetidae), with description of new Asian and African species. *Russian Entomological Journal*, 20 (1), 1–56.
- Kučinić, M., Szivák, I., Pauls, S.U., Bálint, M., Delić, A. & Vučković, I. (2013) *Chaetopteryx buhari* sp. n., a new species from the *Chaetopteryx rugulosa* group from Croatia (Insecta, Trichoptera, Limnephilidae) with molecular, taxonomic and ecological notes on the group. *ZooKeys*, 320, 1–28.  
<http://doi.org/10.3897/zookeys.320.4565>
- Kumar, S., Stecher, G. & Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, 33 (7), 1870–1874.  
<https://doi.org/10.1093/molbev/msw054>
- Letunic, I. & Bork, P. (2016) Interactive Tree Of Life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research*, 44 (1), 242–245.  
<https://doi.org/10.1093/nar/gkw290>
- Martinsson, S., Achurra, A., Svensson, M. & Christer, E. (2013) Integrative taxonomy of the freshwater worm *Rhyacodrilus falciformis* s.l. (Clitellata: Naididae), with the description of a new species. *The Norwegian Academy of Science and Letters*, 42 (6), 612–622.  
<https://doi.org/10.1111/zsc.12032>
- Martinsson, S., Rhodén, C. & Erséus Ch. (2015) Barcoding gap, but no support for cryptic speciation in the earthworm *Aporrectodea longa* (Clitellata: Lumbricidae). *Mitochondrial DNA*, 28, 1–9.  
<https://doi.org/10.3109/19401736.2015.1115487>
- Martynov, A.V., Godunko, R.J. & Palatov D.M. (2016) Kintrishi State Nature Reserve—a hotspot for mayfly (Insecta: Ephemeroptera) diversity in Adjara (Georgia). *Zoosymposia*, 11, 168–173.  
<https://doi.org/10.11646/zoosymposia.11.1.18>
- Meier, R. (2008) DNA Sequences in Taxonomy Opportunities and Challenges. In: Wheeler, Q.D. (Ed.), *The new taxonomy. Systematic Association. Special Volume 76*. CRC Press, Boca Raton, pp. 95–127.
- Meier, R., Kwong, S., Vaidya, G. & Ng, P.K.L. (2006) DNA barcoding and taxonomy in Diptera: A tale of high intraspecific variability and low identification success. *Systematic Biology*, 55 (5), 715–728.  
<https://doi.org/10.1080/10635150600969864>
- Myers, N., Mittermeier, R.A., Mittermeier, C.G., Da Fonseca, G.A.B. & Kent, J. (2000) Biodiversity hotspots for conservation priorities. *Nature*, 403 (6772), 853–858.  
<https://doi.org/10.1038/35002501>
- Nguyen, V.V. & Bae, Y.J. (2004) Larvae of the heptageniid mayfly genus *Epeorus* (Ephemeroptera: Heptageniidae) from Vietnam. *Journal of Asia-Pacific Entomology*, 7 (1), 19–28.  
[https://doi.org/10.1016/S1226-8615\(08\)60197-1](https://doi.org/10.1016/S1226-8615(08)60197-1)
- Pictet, E. (1865) *Synopsis des Névroptères d'Espagne*. Georg, Genève, Suisse, 123 pp.
- Pires, A.C. & Marinoni, L. (2010) DNA barcoding and traditional taxonomy unified through Integrative Taxonomy: a view that challenges the debate questioning both methodologies. *Biota Neotropica*, 10 (2), 339–346.  
<https://doi.org/10.1590/s1676-06032010000200035>
- Puillandre, N., Lambert, A., Brouillet, S. & Achaz, G. (2012) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology*, 21 (8), 1864–77.  
<https://doi.org/10.1111/j.1365-294x.2011.05239.x>
- Saitou, N. & Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4 (4), 406–425.
- Sinitshenkova, N.D. (1976) Mayflies of the Genus *Iron* Eaton (Ephemeroptera, Heptageniidae) in the Fauna of the Caucasus. *Entomologicheskoye obozreniye*, 55 (4), 853–862.
- Sroka, P. (2012) Systematics and phylogeny of the West Palaearctic representatives of subfamily Baetinae (Insecta: Ephemeroptera): combined analysis of mitochondrial DNA sequences and morphology. *Aquatic Insects*, 34, 23–53.  
<https://doi.org/10.1080/01650424.2012.718081>
- Stephens, J.F. (1836) Family III.—Ephemeridae, Leach. In: *Illustrations of British Entomology, Mandibulata 6*, pp. 53–70.
- Tamura, K. & Nei, M. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, 10 (3), 512–526.  
<https://doi.org/10.1093/oxfordjournals.molbev.a040023>
- Türkmen, G. & Kazancı, N. (2015) Additional records of Ephemeroptera (Insecta) species from the Eastern Part of Black Sea Region (Turkey). *Review of Hydrobiology*, 8, 33–50.
- Tshernova, O.A. (1938) [Zur Kenntnis der Ephemeropteren Ost-Transkaukasien]. *Trudy Azerbajdshanskogo Filiala AN SSSR, Baku*, 7 (42), 55–64.
- Tshernova, O. A. (1981) On the systematics of adult mayflies of the genus *Epeorus* Eaton 1881 (Ephemeroptera,

- Heptageniidae). *Entomologicheskoe Obozrenie*, 60 (2), 323–336.
- Vuataz, L., Sartori, M., Wagner, A. & Monaghan, M.T. (2011) Toward a DNA Taxonomy of Alpine *Rhithrogena* (Ephemeroptera: Heptageniidae) Using a Mixed Yule-Coalescent Analysis of Mitochondrial and Nuclear DNA. *PLoS One*, 6 (5), e19728.  
<https://doi.org/10.1371/journal.pone.0019728>
- Walsh B.D. (1862) List of the Pseudoneuroptera of Illinois contained in the cabinet of the writer, with descriptions of over forty new species, and notes on their structural affinities. In: *Proceedings of the Academy of Natural Sciences of Philadelphia*, 1862 (printed for the academy 1863), Philadelphia, pp. 361–402.
- Webb, J.M., Sun, L., McCafferty, W.P. & Ferris, V.R. (2007) A new species and new synonym in *Heptagenia* Walsh (Ephemeroptera: Heptageniidae: Heptageniinae) based on molecular and morphological evidence. *Journal of Insect Science*, 7 (63), 1–16.  
<https://doi.org/10.1673/031.007.6301>
- Webb, J.M. & McCafferty, W.P. (2008) Heptageniidae of the world. Part II. Key to the genera. *Canadian Journal of Arthropod Identification*, 7, 1–55.
- Webb, J.M., Jacobus, L.M., Funk, D.H., Zhou, X., Kondratieff, B., Geraci, C.J., DeWalt, R.E., Baird, D.J., Richard, B., Phillips, I. & Hebert, P.D. (2012) A DNA barcode library for North American Ephemeroptera: progress and prospects. *PLoS One*, 7 (5), e38063  
<https://doi.org/10.1371/journal.pone.0038063>
- White, B., Pilgrim, E., Boykin, L., Stein, E. & Mazor, R. (2014) Comparison of four species-delimitation methods applied to a DNA barcode data set of insect larvae for use in routine bioassessment. *Freshwater Science*, 33 (1), 338–348.  
<https://doi.org/10.1086/674982>
- Whitworth, T.L., Dawson, R.D., Magalon, H. & Baudry, E. (2007) DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). *Proceedings of the Royal Society B-Biological Sciences*, 274 (1619), 1731–1739.  
<https://doi.org/10.1098/rspb.2007.0062>
- Will, K.W., Mishler, B.D. & Wheeler, Q.D. (2005) The perils of DNA barcoding and the need for integrative taxonomy. *Systematic Biology*, 54 (5), 844–851.  
<https://doi.org/10.1080/10635150500354878>
- Zhou, X., Adamowicz, S., Jacobus, L., DeWalt, R.E. & Hebert, P.D.N. (2009) Towards a comprehensive barcode library for arctic life—Ephemeroptera, Plecoptera, and Trichoptera of Churchill, Manitoba, Canada. *Frontiers in Zoology*, 6 (1), 30.  
<https://doi.org/10.1186/1742-9994-6-30>

#### APPENDIX 1. Samples, localities and GenBank Accession Nos

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

.....continued on the next page

**APPENDIX 1. (Continued)**

Species	Specimen ID	Life Stage	Country	GPS coordinates	Leg.	Date	Gen Bank accession number
<i>E. (C.) nigripilosus</i>	Ni8R	Larva	Russia	43°21'N 42°52'E	V.V. Martynov	09.05.2013	KY865691
<i>E. (C.) nigripilosus</i>	Ni9R	Larva	Russia	43°15'50"N 42°28'45"E	V.V. Martynov	12.05.2013	KY865692
<i>E. (C.) nigripilosus</i>	Ni2T	Larva	Turkey	40°26'29"N 40°54'24"E	P. Sroka/ R.J. Godunko	28.06.2011	KY865693
<i>E. (C.) nigripilosus</i>	Ni1T	Larva	Turkey	40°26'29"N 40°54'24"E	P. Sroka/ R.J. Godunko	28.06.2011	KY865694
<i>E. (C.) nigripilosus</i>	Ni6T	Larva	Turkey	40°26'35"N 40°57'53"E	P. Sroka/ R.J. Godunko	28.06.2011	KY865695
<i>E. (C.) znojkoii</i>	Zn1G	Larva	Georgia	42°21'51"N 44°40'50"E	P. Sroka/ R.J. Godunko	22.07.2010	KY865696
<i>E. (C.) znojkoii</i>	Zn7G	Larva	Georgia	42°10'58"N 44°40'49"E	P. Sroka/ R.J. Godunko	22.07.2010	KY865697
<i>E. (C.) znojkoii</i>	Zn5G	Larva	Georgia	42°56'32"N 42°11'04"E	P. Sroka/ R.J. Godunko	28.07.2010	KY865698
<i>E. (C.) znojkoii</i>	Zn30A	Larva	Armenia	40°38'37"N 44°32'34"E	A.V. Martynov	02.07.2015	KY865699
<i>E. (C.) znojkoii</i>	Zn31A	Larva	Armenia	40°38'37"N 44°32'34"E	A.V. Martynov	02.07.2015	KY865700
<i>E. (C.) znojkoii</i>	Zn32A	Larva	Armenia	40°36'47"N 44°34'14"E	A.V. Martynov	04.07.2015	KY865701
<i>E. (C.) znojkoii</i>	Zn40R	Larva	Russia	42°49'28"N 44°04'25"E	A.V. Martynov	25.07.2014	KY865702
<i>E. (C.) znojkoii</i>	Zn41R	Larva	Russia	42°51'17"N 44°09'07"E	A.V. Martynov	21.07.2014	KY865703
<i>E. (C.) znojkoii</i>	Zn19T	Larva	Turkey	40°42'19"N 40°39'00"E	P. Sroka/ R.J. Godunko	28.06.2011	KY865704
<i>E. (C.) magnus</i>	Ma1A	Larva	Armenia	40°08'24"N 44°49'04"E	P. Manko/ J.Oboňa	31.08.2015	KY865705
<i>E. (C.) magnus</i>	Ma2A	Larva	Armenia	40°58'04"N 44°24'49"E	P. Manko/ J.Oboňa	03.09.2015	KY865706
<i>E. (C.) magnus</i>	Ma6G	Larva	Georgia	41°50'34"N 44°35'29"E	P. Sroka/ R.J. Godunko	21.07.2010	KY865707
<i>E. (C.) magnus</i>	Ma15R	Larva	Russia	43°22'12"N 42°54'13"E	V.V. Martynov	10.05.2013	KY865708
<i>E. (C.) magnus</i>	Ma14R	Larva	Russia	43°14'31"N 42°33'49"E	V.V. Martynov	19.05.2013	KY865709
<i>E. (C.) magnus</i>	Ma10T	Larva	Turkey	42°00'04"N 33°21'06"E	P. Sroka/ R.J. Godunko	07.07.2011	KY865710
<i>E. (C.) alpestris</i>	A17R	Larva	Russia	43°15'35"N 42°29'53"E	V.V. Martynov	12.05.2013	KY865711
<i>E. (C.) alpestris</i>	A18R	Larva	Russia	43°16'26"N 42°29'32"E	V.V. Martynov	15.05.2013	KY865712
<i>E. (C.) alpestris</i>	A19R	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 accession number
<i>E. (C.) alpestris</i>	A16G	Larva	Georgia	42°33'30"N 44°30'35"E	P. Sroka/ R.J. Godunko	23.07.2010	KY865714
<i>E. (C.) soldani</i>	So1G	Larva	Georgia	42°34'30"N 42°40'47"E	P. Sroka/ R.J. Godunko	29.07.2010	KY865715
<i>E. (C.) soldani</i>	So5R	Larva	Russia	43°16'30"N 41°48'31"E	R.J. Godunko	10.07.2010	KY865718
<i>E. (C.) soldani</i>	So3R	Larva	Russia	43°28'02"N 41°46'39"E	R.J. Godunko	10.07.2010	KY865716
<i>E. (C.) soldani</i>	So4R	Larva	Russia	43°27'38"N 41°44'52"E	R.J. Godunko	11.07.2010	KY865717
<i>E. (C.) bicolliculatus</i> <b>sp.nov.</b>	Bi1G	Imago female; paratype	Georgia	41°44'14"N 41°58'60"E	A.V. Martynov	19.04.2016	KY865722
<i>E. (C.) bicolliculatus</i> <b>sp.nov.</b>	Bi2G	Larva; paratype	Georgia	41°45'43"N 41°58'34"E	A.V. Martynov	20.04.2016	KY865719
<i>E. (C.) bicolliculatus</i> <b>sp.nov.</b>	Bi3T	Larva; paratype	Turkey	40°40'45"N 39°26'55"E	P. Sroka/ R.J. Godunko	29.06.2011	KY865720
<i>E. (C.) bicolliculatus</i> <b>sp.nov.</b>	Bi4G	Larva; paratype	Georgia	41°45'43"N 41°58'34"E	A.V. Martynov	20.04.2016	KY865721
<i>E. (C.) longimaculatus</i>	Lo1G	Larva	Georgia	42°04'58"N 45°02'19"E	P. Sroka/ R.J. Godunko	20.07.2010	KY865723
<i>E. (C.) longimaculatus</i>	Lo2G	Larva	Georgia	42°04'58"N 45°02'19"E	P. Sroka/ R.J. Godunko	20.07.2010	KY865724
<i>E. (C.) sinitschenkova</i>	Si7R	Larva	Russia	43°14'31"N 42°33'49"E	V.V. Martynov	19.05.2013	KY865727
<i>E. (C.) sinitschenkova</i>	Si8R	Larva	Russia	43°14'31"N 42°33'49"E	V.V. Martynov	19.05.2013	KY865728
<i>E. (C.) sinitschenkova</i>	Si6R	Larva	Russia	43°14'39"N 42°31'32"E	V.V. Martynov	17.05.2013	KY865726
<i>E. (C.) sinitschenkova</i>	Si4G	Larva	Georgia	42°32'42"N 44°29'29"E	P. Sroka/ R.J. Godunko	23.07.2010	KY865725