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Chironomids are commensals of the larvae and pupae of Blephariceridae and Simuliidae from the North Caucasus (Diptera: Chironomidae: Orthocladiinae)

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Abstract

Illustrated morphological descriptions of chironomid larvae from subfamily Orthocladiinae *Cardiocladius* sp. 1, which as commensals live between ventral suckers of Blephariceridae larvae, as well as larvae and pupae of *Eukiefferiella claripennis* group inhabited of Simuliidae pupal cocoons, are given. DNA barcodes of these chironomid species and sequences of their hosts, three species of *Liponeura* Loew (Blephariceridae) and one species of *Simulium* aff. *variegatum* (Simuliidae), are provided.

Key words: Diptera, Chironomidae, *Cardiocladius*, *Eukiefferiella*, *commensals,* DNA barcoding, North Caucasus

Introduction

"In many different divisions of the animal kingdom there are to be found examples of symbiosis or commensalism, by which is meant the living together in close association of individuals of two (or more) quite unrelated species" (Edwards 1929). Representatives of the Chironomidae are no exception, among which commensalism is widespread, and Ephemeroptera, Plecoptera, Trichoptera, Diptera as well as other freshwater invertebrates can be their hosts (Tonnoir 1923; Kaiser 1947; Svensson 1986; Tokeshi 1993; Hayashi, Kobayashi 2000; Henriques-Oliveira & Nessimian 2011; Moubayed-Breil, J. & Ashe 2015 and others).

In the mountain rivers of North Ossetia and Dagestan (North Caucasus) in 2021 Dmitry Palatov collected chironomid larvae of *Cardiocladius* sp. 1 between ventral suckers of Blephariceridae larvae (Figs. 1–3) as well as larvae and pupae of *Eukiefferiella claripennis* group on cocoon of Simuliidae pupae (Figs. 4–6). There is not much information about such finds in the literature. So, for chironomids living on blephariceridae larvae only one species *Tonnoirocladius commensalis* (Tonnoir, 1923) from the subfamily Orthocladiinae is known, that inhabits the rivers of New Zealand (Cranston 2007). Cohabitation of chironomid larvae of *Cardiocladius* Kieffer and *Eukiefferiella* Thienemann with black fly pupae was noted by Edwards (1929), Grenier (1944, 1949) and Thienemann (1954). Therefore, we decided to give morphological descriptions of larvae and pupae of chironomids which were found on Blephariceridae and Simuliids. In addition we provided DNA barcodes of *Cardiocladius* sp. 1 and *Eukiefferiella claripennis* group as well as sequences of their hosts, three species of *Liponeura* Loew (Blephariceridae) and *Simulium* aff. *variegatum* (Simuliidae). DNA barcoding uses short standardized mitochondrial gene sequences to distinguish species. For invertebrate this marker is a 658-bp long fragment of the mitochondrial cytochrome c oxidase subunit 1 gene (COI) (Hebert *et al.* 2003). DNA barcoding has shown its reliability on chironomids of the subfamily Orthocladiinae (Vasquez *et al*. 2021, Stur & Ekrem 2020, Ekrem *et al.* 2018) as well as on Blephariceridae (Schröder *et al.* 2021) and Simuliidae (Thaijarern *et al.* 2019; Takaoka *et al.* 2020, Pramual *et al.* 2021).

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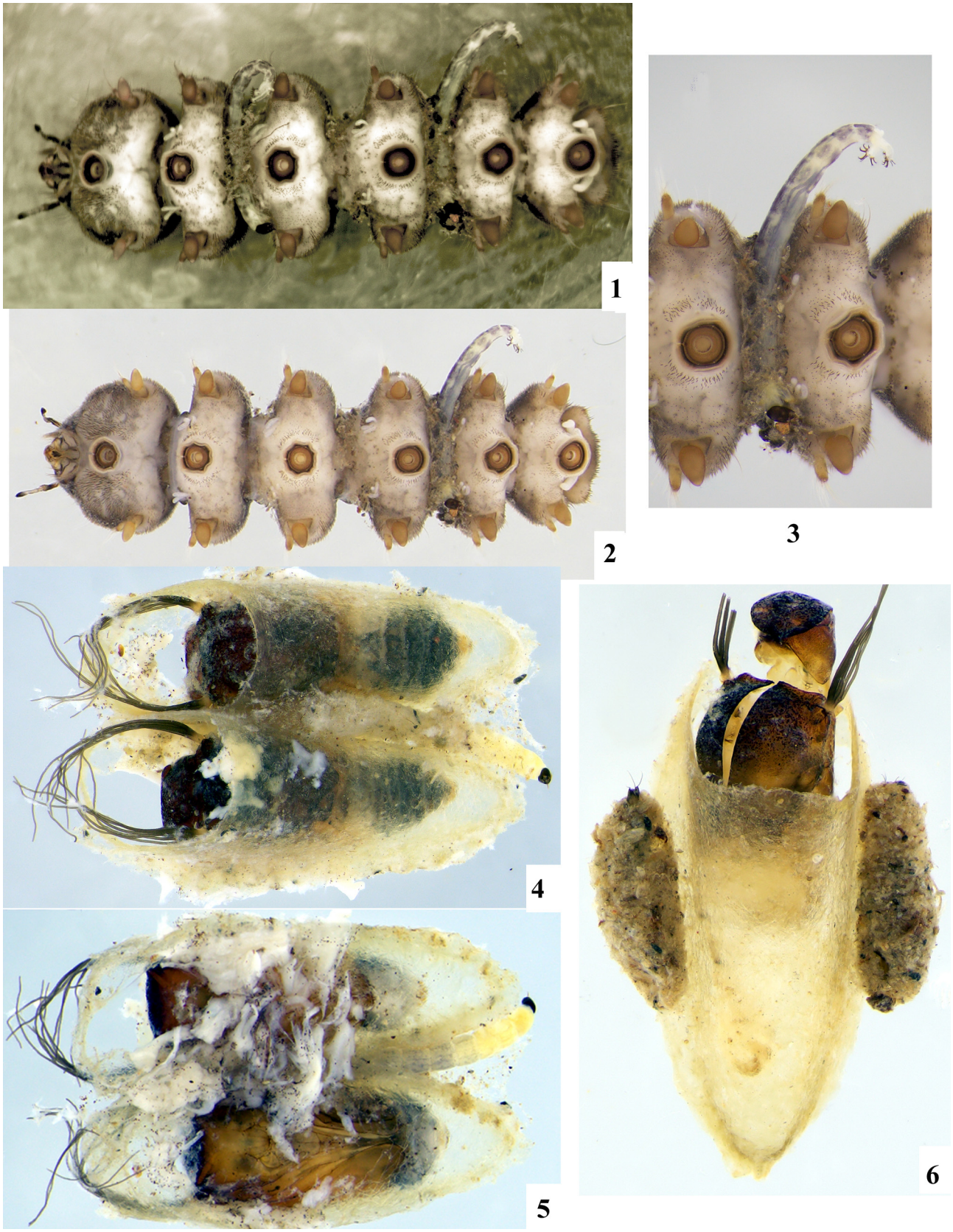


FIGURES 1–6. Arrangement of *Cardiocladius* sp. 1 larvae on the ventral side of Blephariceridae larvae **(1–3)** and of *Eukiefferiella claripennis* group larvae **(4–5)** and pupae **(6)** on cocoon of Simuliidae pupae.

Materials and methods

The larvae and pupae of Chironomidae, Blephariceridae and Simuliidae were preserved in 96% ethanol for DNA-analysis and in 70% ethanol for further study of morphology. All material was collected by Dmitry Palatov.

The material was slide-mounted in polyvinyl lactophenol. The terminology follows Sæther (1980). The photographs were taken using an Axio Lab.A1 (Karl Zeiss) microscope with an AxioCam ERc5s digital camera and an Olympus SZX16 stereomicroscope with an Olympus DP74 digital camera, and then stacked using Helicon Focus software. The final illustrations were post-processed for contrast and brightness using Adobe® Photoshop® software.

All material is deposited in the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia (FSCEATB FEB RAS).

TABLE 1. List of taxa, life stage, collection point and GenBank accessions of investigated material

Family	Species	Life stage	Country, collection point	Geographic coordinate	Accession number
Chironomidae (Orthocladiinae)	*Eukiefferiella claripennis* group	Larva	Russia: Dagestan. Avar Koysu River basin, left tributary of the Joahor River	41.948975 N 46.501026 E	ON149783
	Eukiefferiella claripennis group	Pupa	Russia: Dagestan. Avar Koysu River basin, left tributary of the Joahor River	41.948975 N 46.501026 E	ON149784
	Cardiocladius sp.1	Larva	Russia: Dagestan. Avar Koysu River basin, stream on the slope of the Dzhurmut River valley	41.978150 N 46.512333 E	ON149785
	Cardiocladius sp.1	Larva	Russia: Dagestan. Avar Koysu River basin, the left tributary of the Dzhurmut River	41.977567 N 46.49775 E	ON149786
	Cardiocladius sp.1	Larva	Russia: Dagestan. Avar Koysu River basin, the left tributary of the Dzhurmut River	41.977567 N 46.49775 E	ON149787
	Cardiocladius sp.1	Larva	Russia: Dagestan. Avar Koysu River basin, the left tributary of the Dzhurmut River	41.977567 N 46.49775 E	ON149788
	Cardiocladius sp.1	Larva	Russia: North Ossetia–Alania. Terek River basin, Shalatsikomdon River	42.792660 N 43.920051 E	ON149789
Simuliidae	*Simulium* aff. *variegatum*	Pupa	Russia: Dagestan. Avar Koysu River basin, left tributary of the Joahor River	41.948975 N 46.501026 E	ON149790
Blephariceridae	*Liponeura* sp.1	Larva	Russia: Dagestan. Avar Koysu River basin, the left tributary of the Dzhurmut River	41.978150 N 46.512333 E	ON149791
	Liponeura sp.1	Larva	Russia: North Ossetia–Alania. Skazdon River, Skazdon River	42.781545 N 43.901025 E	ON149792
	Liponeura sp.2	Larva	Russia: North Ossetia–Alania. Terek River basin, Shalatsikomdon River	42.792660 N 43.920051 E	ON149793
	Liponeura sp.3	Larva	Russia: North Ossetia–Alania. Terek River basin, Shalatsikomdon River	42.792660 N 43.920051 E	ON149794

Genomic DNA was extracted from 12 samples 10 of which were larvae and 2 pupae belongs to 3 families of Diptera (Table 1). DNA were extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) from whole bodies of larvae and pupae and the resultant DNA was eluted in 100 µl. The COI barcode fragment (658 bp) was amplified via PCR at the standard conditions for the reaction using the primers LCO1490 (5' GGTCAACAAATCATAAAGATATTGG 3') and HCO2198 (5' TAAACTTCAGGGTGACCAAAAAATCA 3') for the COI gene (Folmer *et al.* 1994). PCR amplifications used the following thermoprofile: an initial denaturation step at 95°C (1 min.), then 35 cycles of 95°C (30 sec.), 48°C (40 sec.), 72°C (1 min.) and a final extension at 72°C (5 min.). Successful amplifications (checked by TBE Gel Electrophoresis on a 1,5% agarose gel) were purified using Exonuclease I (ExoI) and Thermosensitive Alkaline Phosphatase (FastAP) (Thermo Fisher Scientific Inc., USA). Cycle sequencing reactions used 6.75 µl ddH2O, 1 µl ABI Big Dye Terminator ver. 3.1, 0.5 µl of primer at 10 µM, 1.25 µl BigDye

5x Sequencing Buffer and 0.5 µl purified PCR products. The products of sequence reactions were purified using D-Pure DyeTerminator Cleanup kit (Nimagen, Nijmegen, Netherlands) and sequenced on an ABI PRISM 3130xl (Applied Biosystems, Carlsbad, CA). Uncorrected *p*-distances between COI sequences for all taxa were calculated in MEGA ver. 7 (Kumar *et al.* 2016) with pairwise deletion of missing data, uniform rates among sites. ABGD analysis (https://bioinfo.mnhn.fr/abi/public/abgd/; Puillandre *et al.* 2012) was used for establish taxonomic status of chironomids, using relative gap width (X = 1.0) and intraspecific divergence (P) values between 0.005 and 0.100 with the p-distance model. These settings were successfully used for chironomids by Song *et al*. (2018). To achieve this task we used all available sequences in GenBank and BOLD systems of *Cardiocladius* and *Eukiefferiella* (accessed on May 09, 2022) and added the corresponding sequences to these datasets.

PartitionFinder 2.1.1 (Lanfear *et al.* 2012) is used to select the best-fit partitioning scheme and models separately for each codon position of COI gene using the greedy algorithm with linked branch lengths for the corrected Bayesian Information Criterion as the optimality criterion for model selection. A Bayesian Inferences (BI) analysis was performed with MrBayes v.3.2.7 (Ronquist *et al.* 2012) under the following conditions: 5 million generations with sampling every 500 generations, four chains and a burn-in of 25% trees. All sequences have been deposited in GenBank (Table 1).

Descriptions

Cardiocladius sp. 1

(Figs. 7–17)

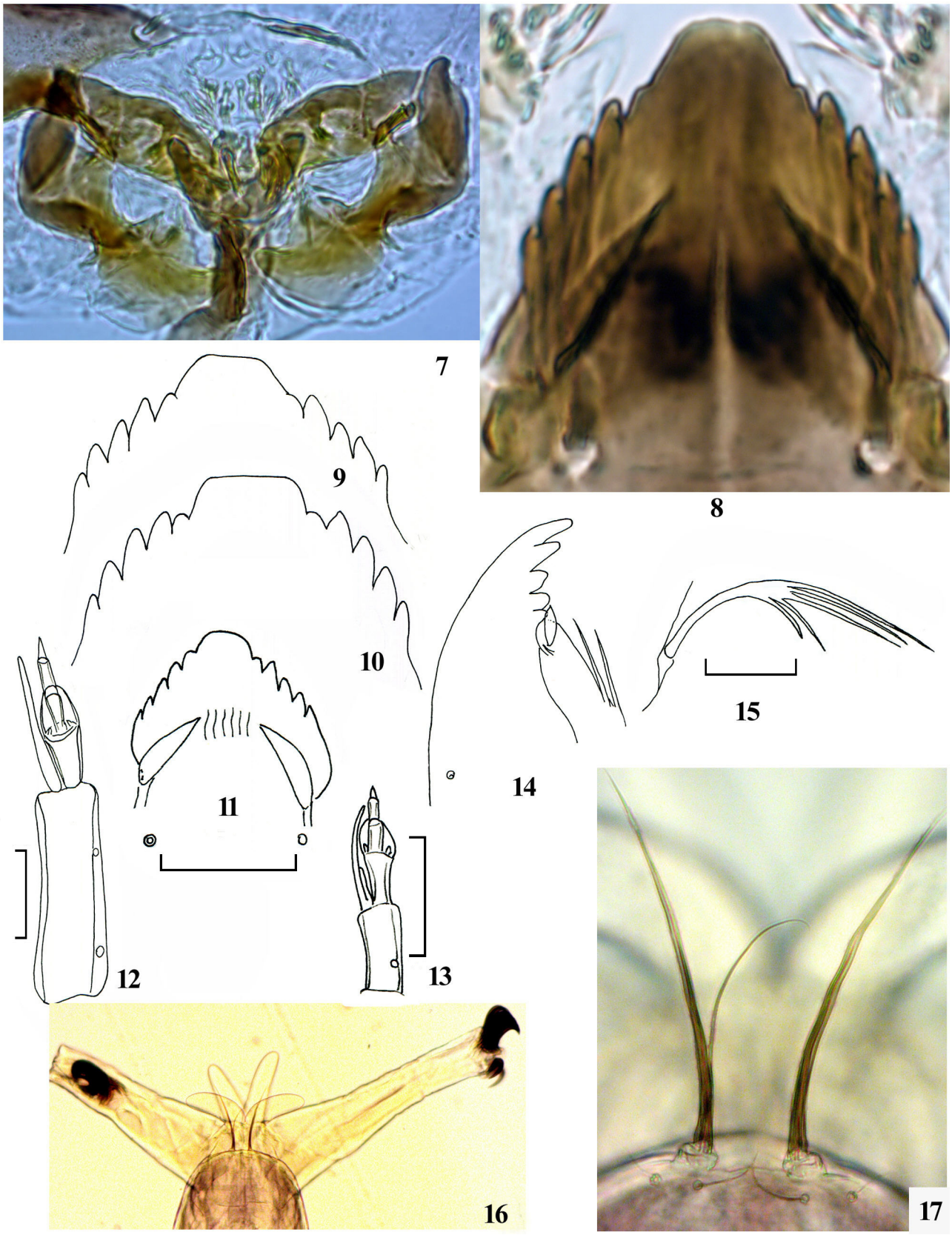
Material examined. 4 larvae of IV instar removed from larval Blephariceridae, RUSSIA: Republic of Dagestan, Tlyaratinsky District, Greater Caucasus, Avar Koysu River basin, the left tributary of the Dzhurmut River opposite of Choroda Village, altitude 1696 m above sea level, 41º58.654'N, 46º29.865'E, 08.V.2021; 3 larvae of III instar and 1 larva of IV instar removed from larval Blephariceridae, the same locality, except stream on the slope of the Dzhurmut River valley near of the Salda Village, altitude 1763 m above sea level, 41º58.046'N, 46º31.029'E, 09.V.2021. 3 larvae of IV instar removed from larval Blephariceridae, RUSSIA: Republic of North Ossetia–Alania, Alagirsky District, Greater Caucasus, Tseyskoye Gorge, Terek River basin, waterfall on the Shalatsikomdon River, altitude 1755 m above sea level, 42.79266 N, 43.920051 E, 29.VII.2021; 2 larvae of II instar removed from larval Blephariceridae, the same locality, except Skazdon River near the Tsey (Tseyskoe gorge) ski resort, altitude 2025 m above sea level, 42.781545 N, 43.901025 E, 29.VII.2021.

Larva of III–IV instar *(n* = 9). Total length 2.6–3.2 mm. Head dark brown to black, thoracic segments greenish, abdominal segments bluish-violet.

Head. Length 295–3328 µm, width 197–213 µm. Labral setae S_1–S_2 simple (Fig. 7). Premandible distally with 1 wide apical tooth (Fig. 7). Antenna with 5 segments; length 1–5 segments (in µm) (n=5): 39–44 : 11–15 : 8–9 : 7 : 5; AR 1.22–1.39; apex of segment 2 with large Lauterborn organs ending at base of 4th segment; antennal blade 31 µm long, its apex reaches the base of the 5th segment, its internal branch reaches the middle of the 3rd segment; one large ring organs in proximal 1/3 of basal segment and one small in distal 1/3 (Fig. 12). Mandible with apical tooth and 3 inner teeth (Fig. 14); seta interna with long basal part (18–20 µm) and distal with 5 branches (Fig. 15); seta subdentalis leaf-shaped, with pointed apex; inner margin of mandible with 2 long serrations. Mentum with 5 pairs of lateral teeth; median tooth single, truncated or trapezoidal, in 3.3–4 times wider than 2nd lateral tooth; 1st lateral tooth reduced and appressed to median tooth; ventromental plates large, pointed basally (Figs. 8–10). Maxilla without pecten galearis.

Abdomen. Anal tubules subequal, 120–124 µm long. Posterior parapods long, 400–408 µm long (Fig. 16), length/width ratio 3.5–5,7; posterior parapods length/anal tubules length 3.2–3.3. Procerci greatly reduced, hardly protruding, in the form of tubercles or incompletely sclerotized ring (as in some *Diamesa*), each bearing 4 anal setae, 3 of which are 96–124 µm long and 1 is 60–84 µm long; lateral seta 20–32 µm long sits directly on body (Fig. 17).

Larva of II instar (n=2). Total length 1.6–2.0 mm. Head dark brown to black, thoracic segments greenish, abdominal segments bluish-violet.



FIGURES 7–17. Larva of *Cardiocladius* sp. 1. **7,** labrum with S-setae; **8–11,** mentum; **12–13,** antenna; **14,** mandible; **15,** inner setae of mandible; 16, anal segment with procercus, posterior parapods and anal tubules; **17,** procercus with anal setae.

Head. Premandible distally with 1 wide apical tooth. Antenna with 5 segments; length 1–5 segments (in μm) : 15–23 : 9–10 : 4–7 : 3–4 : 2–4; AR 0.83–0.92; apex of segment 2 with large Lauterborn organs ending at base of 4th segment; apex of antennal blade reaches middle of 4th segment, its internal branch reaches 2/3 of the 3rd segment; one large ring organs in proximal 1/3 of basal segment (Fig. 13). Mentum with 5 pairs of lateral teeth; median tooth single, truncated or trapezoidal, in 4.2–4.3 times wider than 2nd lateral tooth; 1st lateral tooth reduced and appressed to median tooth (Fig. 11).

Abdomen. Anal tubules subequal, 56–84 μm long. Posterior parapods long, 200–280 μm long; posterior parapods length/anal tubules length 2.9–3.3. Procerci greatly reduced, hardly protruding, in the form of tubercles or incompletely sclerotized ring, each bearing 4 anal setae, 2 of which are 56–68 μm long and 2 are 28–32 μm long; lateral seta 12–14 μm long sits directly on body.

Remarks. The larvae of *Cardiocladius* sp. 1, which found on blepharicerids, according to the identification key of Cranston (1982) and Schmid (1993) are closely related to *C. capucinus* (Zetterstedt) but without a pupa and an adult male we cannot confirm it. Also, this is not supported by the results of DNA barcoding (Fig. 34). In the process of identifying the larvae of *Cardiocladius* sp. 1 we also used the work of Bode (1983), according to which our species could be attributed to the *Eukiefferiella cyanea* group. In our opinion, this group of species should be transferred from the genus *Eukiefferiella* to *Cardiocladius*.

Ecology. Chironomid larvae on blepharicerids were collected on large stones and rocks of mountain rivers and in a waterfall at a current speed of 0.6–1 m/s, at an altitude of 1696 m a.s.l. in Dagestan and 2025 m a.s.l. in North Ossetia (Figs. 30–32).

Eukiefferiella claripennis group

(Figs. 18–29)

Material examined. 2 larvae of IV instar and 2 pupae of female removed from pupal Simuliidae, RUSSIA: Republic of Dagestan, Tlyaratinsky District, Greater Caucasus, Avar Koysu River basin, left tributary of the Joahor River, 3 km upstream of its mouth on the Jurmut River, altitude 2218 m above sea level, 41.948975 N, 46.501026 E, 14.VIII.2021.

Pupa (n=2). Total length 1.8–1.9 mm. Cephalothorax greyish brown, abdominal segments greenish brown. Exuviae greyish yellow.

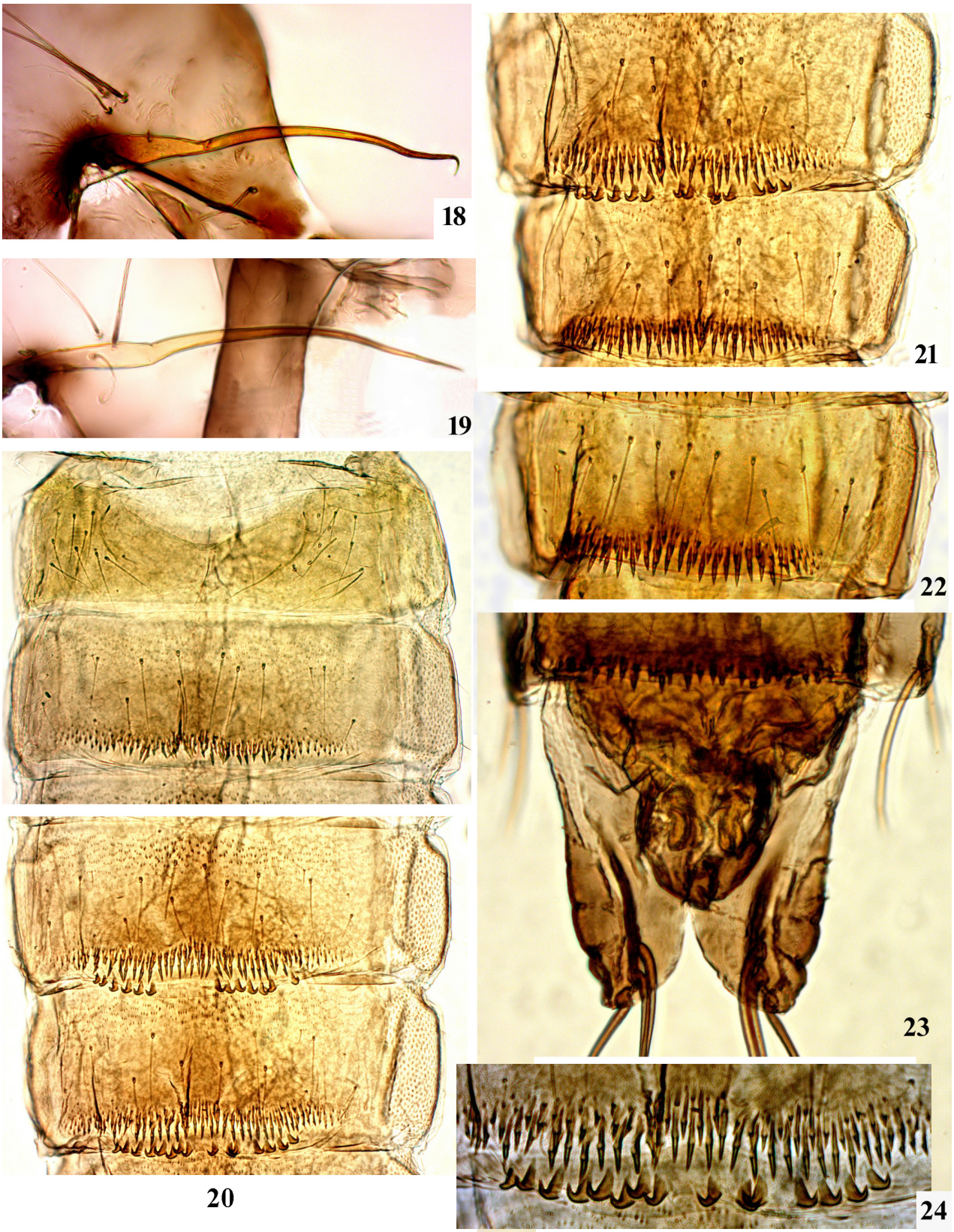
Cephalothorax. Frontal apotome without setae, tubercles and warts. Antepronotum with 2 thin and hair-like median antepronotal setae 85–92 μm long and with 3 lateral antepronotal setae 112–120 μm long. Surface of thorax dorsally smooth. Thoracic horn light brown to brown, 224–232 μm long, broadest at base (30–32 μm), tapering to apex; length horn base 72 μm; horn smooth (Figs. 18–19). Length horn base/length horn 0.31–0.32. Precorneal setae lengths (in μm): Pc_1 40, Pc_2 112–120, Pc_3 88–100. Bases of setae arranged in form of triangle. Dorsocentrals hair-like, Dc_1 48 μm long, Dc_2 16 μm long, Dc_3 32 μm long, Dc_4 44–48 μm long. Distance between Dc_1 and Dc_2 12–16 μm; between Dc_2 and Dc_3 184 μm; between Dc_3 and Dc_4 16 μm.

Abdomen. Tergite I without shagreen. Tergites II–VIII with weak shagreen of small spinules in anterior third and with 1–2 transverse rows of long needle-like spines, longest of which are 20–28 μm (Figs. 20–22). In addition, tergites III–V behind straight spines with row of hooked spines intermittent in middle, apex of which directed anteriorly; their number on these tergites accordingly 13 : 15 : 14 (Figs. 21, 24). Shagreen of small spinules in posterior part of sternites V–VII are presented. Segments without PSB and PSA. Segments I–VII with 4 pairs of hair-like lateral setae; length of L_1 of these segments (in μm)—24–32, L_2—16–24, L_3—16–24, L_4—18–20. Segments VIII with 4 pairs of lateral setae; length of $L_{1–4}$ of this segment (in μm) accordingly—16–32 : 16–28 : 80–86 : 96–112. Anal segment 180–190 μm long, with dorsal and lateral shagreen. Anal lobe with 3 macrosetae 180–200 μm long. Anal segment length/anal macroseta length 0.95–1.11. Female genital sac rounded and short (Fig. 23).

Larva of IV instar *(n* = 2). Total length 3.1–3.2 mm. Head brown to dark brown, antenna light brown or yellowish brown, mandible and mentum brown to dark brown; thoracic segments brownish yellow, abdominal segments brownish brown.

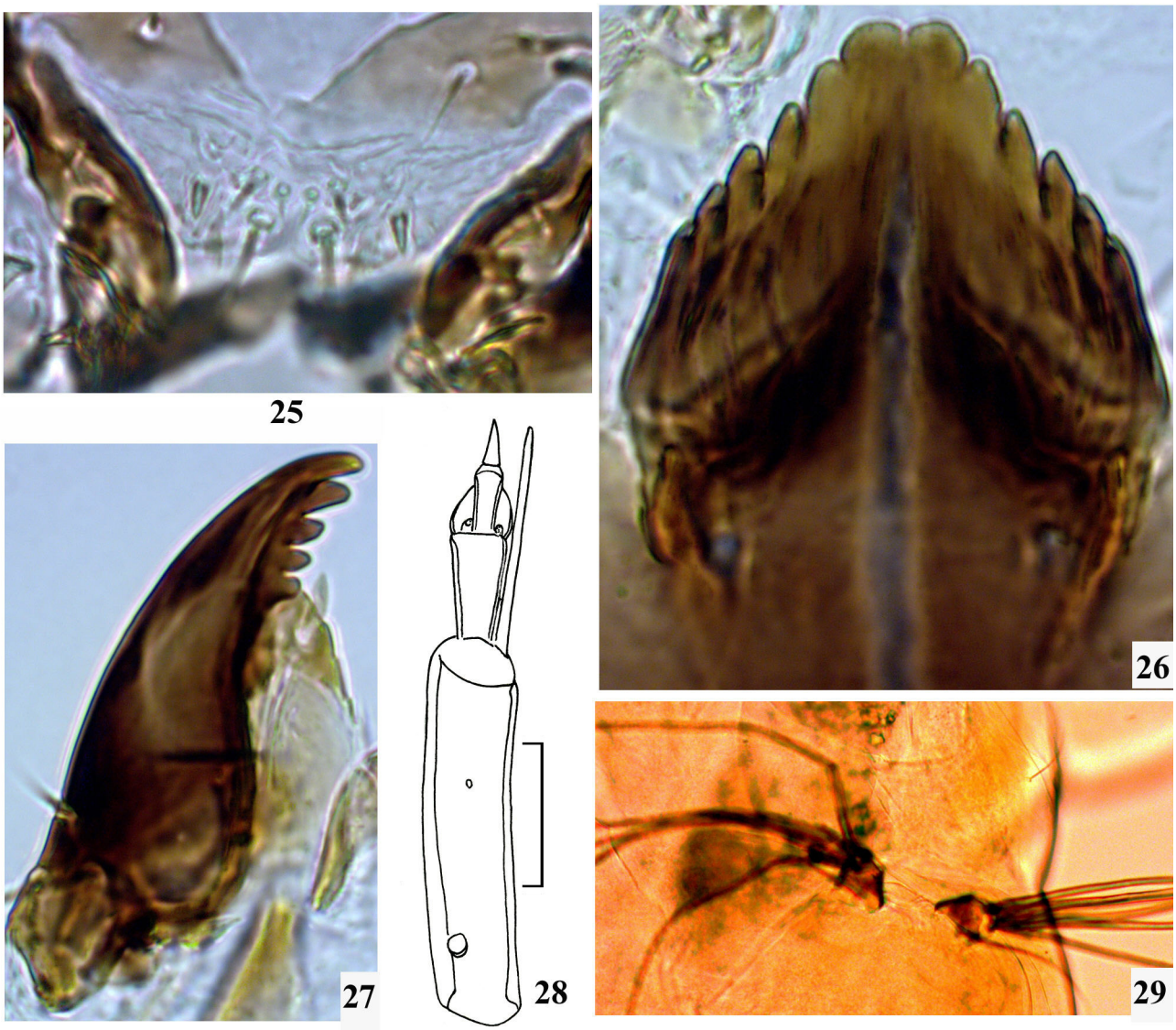
Head. Labral S-setae simple (Fig. 25). Premandible distally with 1 wide apex apical tooth. Antenna with 4 segments; length 1–4 segments (in μm): 49–51 : 13–14 : 7 : 5–6; AR 1.88–1.96; apex of segment 2 with Lauterborn organs ending at 2/3 or base of 3rd segment; antennal blade 25 μm long, its apex slightly not reach apex of 4th segment; one large ring organs in proximal 1/4 of basal segment and one small in distal 1/3 (Fig. 28). Mandible with

apical tooth and 3 true inner teeth and one false tooth; seta interna with 3–4 branches; seta subdentalis leaf-shaped, with pointed; inner margin of mandible (mola) with 2–3 short serrations (Fig. 27). Mentum with 2 median teeth and 5 pairs of lateral teeth; median tooth in 2.0–2.6 times wider than 1st lateral tooth; ventromental plates large, pointed basally (Fig. 26). Maxilla without pecten galearis.



FIGURES 18–24. Pupa of *Eukiefferiella claripennis* group. **18–19,** thoracic horn; **20,** tergites I–IV; **21,** tergites V–VI; **22,** tergite VIII; **23,** anal segment; **24,** spines and spinules of posterior part of tergites IV.

Abdomen. Anal tubules 72–88 µm long. Posterior parapods long, 208–216 µm long; posterior parapods length/anal tubules length 2.3–3.0. Procerci are typical for genus, each bearing 7 long anal setae; lower lateral seta 60–68 µm long (Fig. 29).



FIGURES 25–29. Larva of *Eukiefferiella claripennis* group. **25,** S-setae of labrum; **26,** mentum; **27,** mandible; **28,** antenna; **29,** procercus with anal setae.

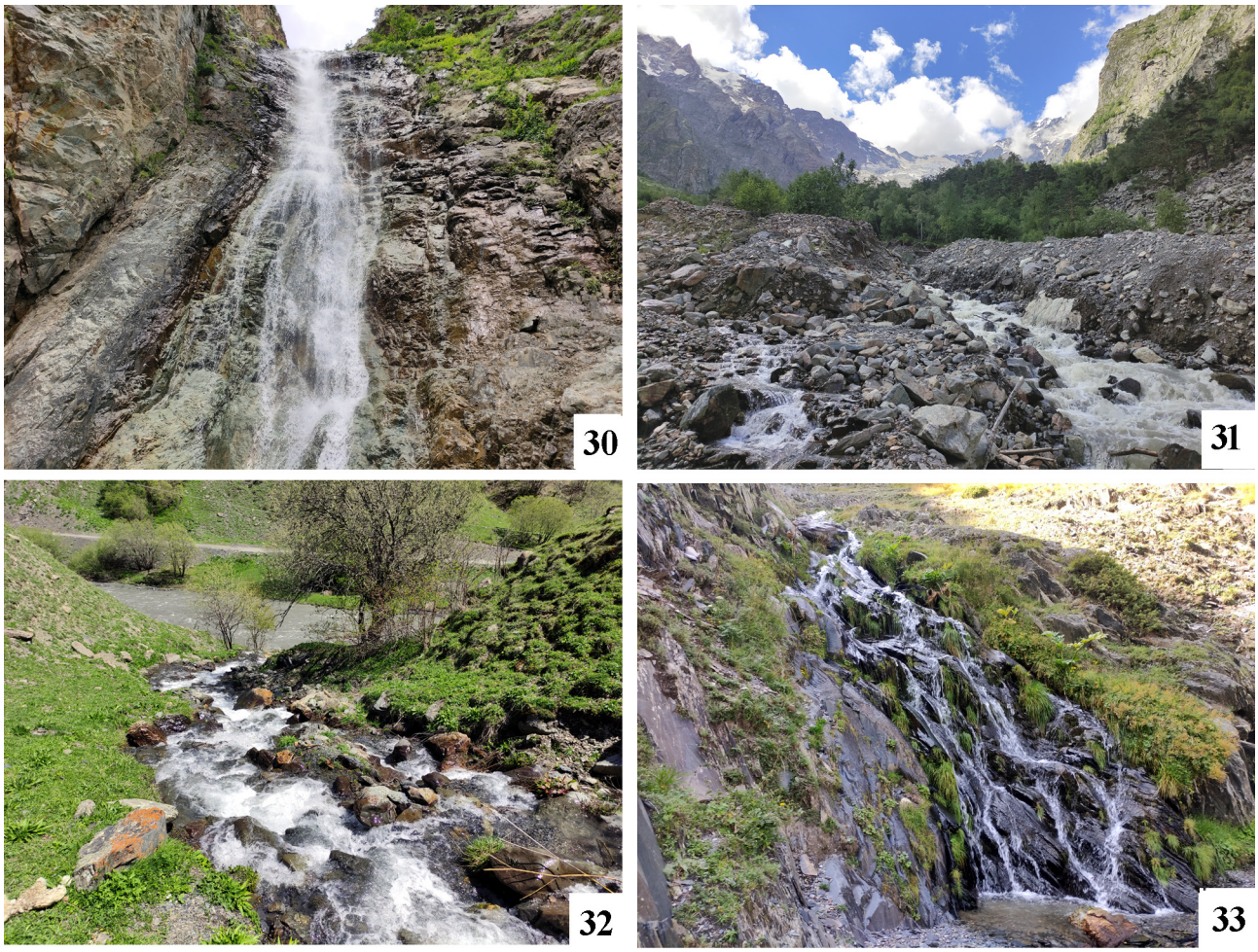
Remarks. Since the pupae of *Eukiefferiella claripennis* group were immature and of female, we could not accurately determine their species identity but the larva and pupa are typical representatives of the *claripennis* group and according to identification keys of Schmid (1993) and Lehmann (1972) similar to *E. lobifera* Goetghebuer. Unfortunately, there are no data for *E. lobifera* in the GenBank, and the closest sequence to our *E. claripennis* group is *E. claripennis* (Lundbeck). For more details see section "Results of DNA barcoding".

Ecology. The larvae and pupae of *Eukiefferiella claripennis* group in cocoon of black flies pupae were collected on stones with a small amount of algae fouling of a waterfall in a mountain stream at a current speed of 0.6–1 m/s at an altitude of 2218 m a.s.l. (Fig. 33).

Results of DNA barcoding

A total of 12 barcodes 560–628 bp in length were obtained belonging to 3 family and 6 species (see Table 1). We made a comparison of the obtained sequences with data of NCBI GenBank and BOLD systems. The closest se-

quence to our *Eukiefferiella claripennis* group were *Eukiefferiella claripennis* (Lundbeck) (GenBank №KJ439944, BOLD ID GBDP44017-19, BIN BOLD:ADT8287), the average p-distances between them were 7.42% which probably corresponding to species level (Imada, 2020). ABGD analysis yielded 35 operational taxonomic units (OTU) of the genus *Eukiefferiella* using a 0.0129–0.0215 intraspecific divergence. Sequences of *Eukiefferiella claripennis* group were placed as separate OTU. However, with an increase in intraspecific divergence to 0.0359—0.1 three sequences uniting into single OTU.



FIGURES 30–33. Localities of *Cardiocladius* sp. 1 and Blephariceridae larvae **(30–32)**, *Eukiefferiella claripennis* group and Simuliidae larvae with pupae **(33)**. **30,** Greater Caucasus, Tseyskoye Gorge, Terek River basin, Waterfall on the Shalatsikomdon River (Russia: Republic of North Ossetia–Alania); **31,** Greater Caucasus, Skazdon River near the Tseyskoe Gorge (Russia: Republic of North Ossetia–Alania); **32,** Greater Caucasus, Avar Koysu River basin, the left tributary of the Dzhurmut River opposite of Choroda Village (Russia: Republic of Dagestan); **33,** Greater Caucasus, Avar Koysu River basin, left tributary of the Joahor River, 3 km upstream of its mouth on the Jurmut River (Russia: Republic of Dagestan). (Photos by D.M. Palatov).

A high number of samples included the *Cardiocladius* sp.1 made it possible to evaluate the intraspecific distances which were ranged from 0.71 to 2.62%, with an average of 1.45%. Most of the identified mutations were synonymous transitions. The sequences of this species did not match to any *Cardiocladius* from GenBank and BOLD systems. Two sequences of *Cardiocladius* sp. (GenBank № HQ738736 and EU669980, BOLD ID GBDP11445-12 and GBDP6813-09, BIN BOLD:AAW4309) from Australia were closest to *Cardiocladius* sp.1, the average p-distances were 15.55%. The high differences between *Cardiocladius* sp.1 and other *Cardiocladius* sequences were also confirmed by ABGD analysis, which yielded 9–11 operational taxonomic units (OTU) for initial and recursive partition respectively using a 0.001–0.100 intraspecific divergence.

We used Bayesian Inference to reconstruct relationships of the genus *Cardiocladius* (Fig. 34). To do this, we selected one sample from each BIN number from the BOLD systems and *Eukiefferiella claripennis* as outgroup.

Cardiocladius sp. 1 was placed as sister to *Cardiocladius* sp. (BIN BOLD:AAW4309) with high support (Bayesian posterior probability, PP=0.99). Clade formed by the remaining *Cardiocladius* species as sister groups (PP=1.00).

Sequence of *Simulium* aff. *variegatum* was close to BIN BOLD:ADK2119 which includes 15 sequences of *Simulium variegatum* and 7 sequences *of Simulium argyreatum*. The average p-distances were 2.98%. which support their conspecificity (Thaijarern *et al.* 2019).

The comparison of *Liponeura* sp.1, *Liponeura* sp. 2 and *Liponeura* sp. 3 revealed similarities with *Liponeura brevirostris* (BIN BOLD:ADM6413, p-distance 12.84%), *Liponeura cinerascens* (BIN BOLD:AEG4784, 13.70%) and *Liponeura decipiens* (BIN BOLD:ADM5805, 14.54%) respectively. Moreover, distances between *Liponeura* sp. 1—*Liponeura* sp. 2, *Liponeura* sp. 1—*Liponeura* sp. 3 and *Liponeura* sp. 2—*Liponeura* sp. 3 were 14.95%, 14.72% and 9.42% respectively which support the distinctiveness of each species (Zhang & Bu 2022).

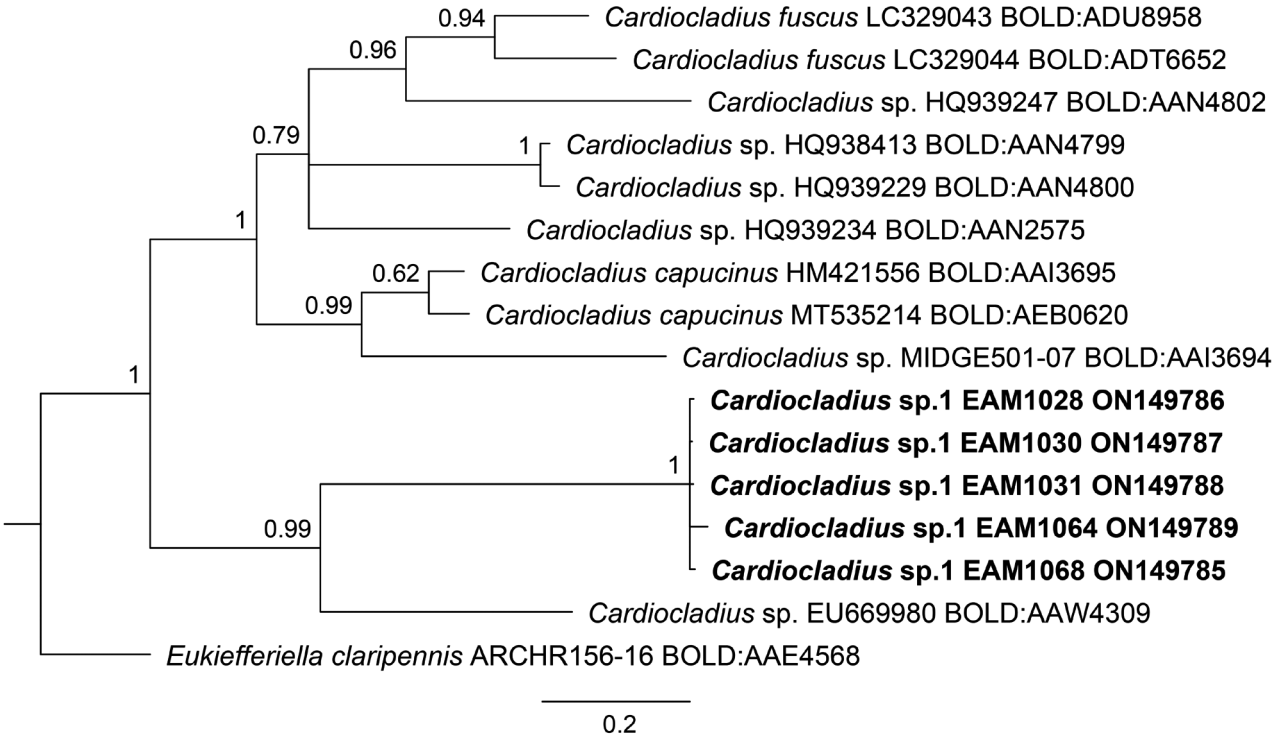


FIGURE 34. Bayesian tree based on mitochondrion COI gene for obtained sequences and each available BIN number of the genus *Cardiocladius* Kieffer. *Eukiefferiella claripennis* was used as outgroup to root the tree. Bayesian posterior probabilities (PP) are given above tree nodes. Specimens obtained in this study are in bold.

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