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Morphological description and DNA barcoding of *Chaetocladius* (*Chaetocladius*) *elisabethae* sp. nov. (Diptera: Chironomidae: Orthocladiinae) from the Moscow Region

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Abstract

Illustrated descriptions of the adult male, pupa and fourth instar larva, as well as DNA barcoding results of *Chaetocladius* (*Chaetocladius*) *elisabethae* **sp. nov.** in comparison with closely related species of *Chaetocladius* s. str. from the Moscow Region are provided. A reference 658 bp barcode sequence from a fragment of the mitochondrial gene cytochrome oxidase I (COI) was used as a tool for species delimitation. Comparisons with corresponding regions of COI between *C.* (s. str.) *elisabethae* sp. nov. and other species of the subgenus produced K2P genetic distances of 0.11–0.16, values well associated with interspecific variation. The barcodes of the new species were identical to the *Chaetocladius* sp. 2ES in BOLD systems. Molecular data were also used for the reconstruction of the phylogenetic relationships within the subgenus *Chaetocladius* s. str.

Key words: Diptera, Chironomidae, *Chaetocladius* s. str., new species, taxonomy, DNA barcoding, Moscow Region

Introduction

The genus *Chaetocladius* Kieffer, 1911 is divided into two subgenera—*Amblycladius* Kieffer, 1923, including two species—*A. subplumosus* Kieffer, 1923 and *A. franzjosephi* Krasheninnikov, 2013 (Krasheninnikov & Gavrilo 2013) from the North of Russia, and *Chaetocladius* s. str., with 67 Palaearctic species (Stur & Spies 2011; Ashe & O'Connor 2012; Kobayashi 2012; Wang et al. 2012; Makarchenko & Makarchenko 2013a–c; Langton & Armitage 2015; Makarchenko *et al.* 2017a; Moubayed-Breil 2017; Moubayed-Breil & Dia 2017; Rossaro *et al.* 2017), 5 Nearctic, 7 Oriental and 2 Afrotropical species (Ashe & O'Connor 2012). Twenty five species of *Chaetocladius* s. str. are known from Russia (Zelentsov & Shilova 1996; Zelentsov 2007; Ashe & O'Connor 2012; Makarchenko *et al.* 2017a), of which 17 species were described as new from the Russian Far East and bordering territories (Makarchenko & Makarchenko 2001, 2003, 2004, 2006a–b, 2009, 2011a–b, 2012, 2017; Makarchenko *et al.* 2017a).

Despite a large number of published papers on the taxonomy of *Chaetocladius* s. str., there are still many unresolved problems in separating species on the basis of adult males and pupal morphology, and there is no information on the larvae of most species. We subscribe to the opinion of taxonomists studying Orthocladiinae that the subgenus needs revision using the material from adults and immature stages, as well as DNA analysis.

In the present paper, illustrated descriptions of the adult male, pupa and fourth instar larva, as well as DNA barcoding results of *Chaetocladius* (s. str.) *elisabethae* **sp. nov.** from the Moscow Region are provided in comparison with closely related species of *Chaetocladius* s. str. The DNA barcode corresponding to the 650-bp

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fragment of the mitochondrial gene cytochrome c oxidase I (COI) has been identified as the core of a global bio-identification system at the species level (Hebert *et al.* 2003) and has proved to be useful in the subfamily Orthocladiinae (Silva & Wiedenbrug 2014, Makarchenko *et al.* 2015, 2017a, 2017b).

After receiving the results of barcoding, analysis of the information from the papers and databases it turned out that a species with such sequences was already recorded from Norway as *Chaetocladius* sp. 2 (Ekrem *et al.* 2010) and in the Bold system registered as *Chaetocladius* sp. 2ES, but the morphological descriptions of the adult male or immature stages were not made, thus we fill this gap below.

Materials and methods

The material was preserved in 96% ethanol for DNA-analysis and in 70% ethanol for further study of morphology and slide-mounting, following the methods by Makarchenko (1985). The larva, pupa and adult male were associated by using of DNA barcoding as well as for comparing of a new species with closely related species. The terminology follows Sæther (1980).

Photographs were taken at the microscope Olympus BX53 + DeltaPix Invenio-8DII of the Interdepartmental laboratory "Biology of Marine Invertebrates", School of Natural Sciences, Far Eastern Federal University

Holotype and paratypes of the new species are 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).

Total DNA was extracted from specimens preserved in 96% ethanol using the Invitrogen PureLink Genomic DNA Mini Kit (Invitrogen corp, Carlsbad, CA 2007) according with the protocol, and the resultant DNA was eluted in 70 µl. The primers for amplification of the 658 bp fragment were COIF-ALT (5'-ACAAATCAYAARGAYATYGG-3') and COIR-ALT (5'-TTCAGGRTGNCCRAARAAYCA-3'), obtained from Mikkelsen *et al.* (2006). PCR reaction for this fragment was run in total volume of 10 µl with 5 µl Go Taq Green Master Mix (Promega corp, Madison, WI, USA), 0.5 µM of each primer, 3 µl nuclease-free water and 2-5 ng (1 µl) of genomic DNA. The PCR thermal regime consisted of one cycle of 1 min at 94° C; five cycles of 1 min at 94°C, 1.5 min at 45°C and 1.5 min at 72° C; 35 cycles of 1 min at 94°C, 1.5 min at 50°C and 1 min at 72°C and a final cycle of 5 min at 72° C, according to the PCR conditions in Hebert *et al.* 2003. Each PCR fragment was purified using Exonuclease I (ExoI) and Thermosensitive Alkaline Phosphatase (FastAP) (Thermo Fisher Scientific Inc., USA). Sequencing reactions had a total volume of 10 µl and included 10 pmol of each primer and reagents of BigDye terminator v3.1 cycle kit. The sequencing amplification protocol consisted of one cycle of 1 min at 98°C, followed by 30 cycles of 10 sec at 96°C, 5 sec at 50°C, and 4 min at 60°C. The PCR products were bidirectional sequenced on an ABI 3130x sequencer (Applied Biosystems) and were aligned in MEGA7 (Kumar *et al.* 2016). Sequence divergences among individuals were quantified by using the Kimura-2-Parameter distance model (Kimura 1980) and graphically displayed in a Maximum likelihood (ML) tree. All sequences of *C.* (s. str.) *elisabethae* sp. nov. have been deposited in GenBank (accession numbers KX270978-KX270993).

Descriptions

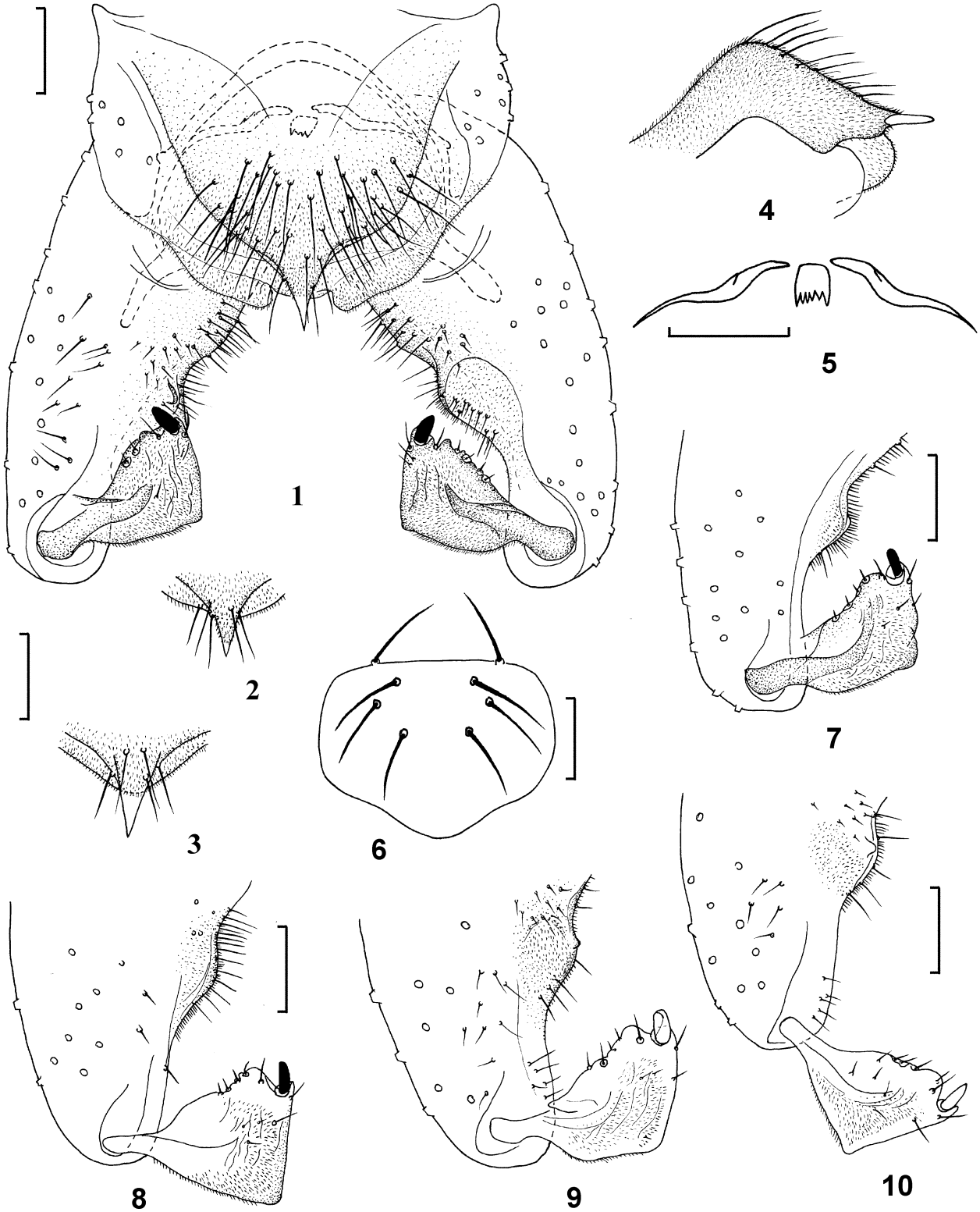
Chaetocladius (s. str.) *elisabethae* Makarchenko *et* Makarchenko, sp. nov.

(Figs. 1–28)

Chaetocladius sp. 2|SOE227|Male, Ekrem *et al.* 2010: 404, Fig.1.
Chaetocladius sp. 2|SOE131|Female, Ekrem *et al.* 2010: 404, Fig.1.
Chaetocladius sp. 2ES|Male, available from:
http://v3.boldsystems.org/index.php/Public_RecordView?processid=MIDGE631-08
Chaetocladius sp. 2ES|Female, available from:
http://v3.boldsystems.org/index.php/Public_RecordView?processid=MIDGE535-08
Chaetocladius sp. 2ES| available from: http://v3.boldsystems.org/index.php/Public_RecordView?processid=GMGMF495-14

Material. Holotype: adult male, Russia, vicinities of Moscow, Kaluga Region, Zhukovskyi District, unnamed

spring of the basin of Nara River (Raduzhnyi Falls) near Papino Village, N 55°12'42.05", E 36°57'15.39", 10.II. 2016, leg. D. Palatov. Paratypes: 52 adult males, 1 mature pupa (male), 6 larvae, the same data as holotype, 10.II. 2016, leg. D. Palatov; 12 adult males, Moscow Region, Ruzskyi District, unnamed spring near of Hrushevo Village, Moscow River basin, N 55°01'21.5", E 37°27'04.02", 13.I. 2016, leg. D. Palatov.



FIGURES 1–10. Adult male of *Chaetocladius* (s. str.) *elisabethae* **sp. nov. 1,** hypopygium in dorsal view; **2–3,** anal point in dorsal view; **4,** anal point in lateral view; **5,** virga and anterior part of fallapodemae; **6,** clypeus; **7–10,** gonocoxite and gonostylus. Scale bars 50 µm.

Derivatio nominis. The species is named in honour of Norwegian chironomidologist Dr. Elisabeth Stur, who was active in studying of Orthocladiinae together with Drs. T. Ekrem and P.D.N. Hebert who performed DNA barcoding, calling the species *Chaetocladius* sp. 2ES.

Adult male (n=4). Colouration dark brown, halters dark. Total length 3.2–3.4 mm. Wing length 2.72–2.84 mm. Total length/wing length 1.17–1.20.

Head. Eyes bare, with short dorsomedian prolongations. Temporal setae including 8–10 verticals and 6–7 postorbitals. Clypeus with 8–10 setae situated in basal half or one third of clypeus (Fig. 6). Antenna with 13 flagellomeres and well developed plume; apex of 13th flagellomere elongated and pointed, with 11–14 short white hairs; AR 1.88–2.21. Length of palpomeres 2–5 (in µm): 64, 164, 120–148, 256–260; palpomere 3 in subapical part with 12–14 sensitive hairs of sensilla capitata.

Thorax. Antepronotum with 5–8 lateral setae. Acrostichals 19–22 (beginning from border with pronotum), dorsocentrals 10–14, prealars 5–6, scutellum with 7–8 setae. Humeral pit like net spot.

Wing. Greyish, halters dark. R with 12–14 setae, R_1 with 2–3 setae, R_{4+5} without setae. R_{4+5} ending distal of apex M_{3+4}. Costa extension 60–84 µm. Cu_1 curved in apical part. Anal lobe well developed, rectangular-rounded. Squama with 10–13 setae.

Legs. BR_1 2.5–2.8, BR_2 1.8–2.5, BR_3 2.9–3.7 . Spur of fore tibia 68–84 µm long. Spurs of mid tibia 24–28 µm long. Spurs of hind tibia 64–70 µm and 20 µm long. Hind tibial comb with 18 setae. Fore leg on ta_1 with 0–1 pseudospur, mid leg on ta_1–ta_3 with 0–2 pseudospurs. Hind leg on ta_1–ta_2 with 2 pseudospurs, ta_3 with 0–2 pseudospurs; basal half of ta_1 with 2 or 5 sensilla chaetica. Small pulvilli present. Lengths and proportions of legs as in Table 1.

TABLE 1. Lengths (in µm) and proportions of leg segments of male *Chaetocladius* (s. str.) *elisabethae* **sp. nov.**, (n=4).

	fe	ti	ta_1	ta_2	ta_3	ta_4	ta_5	LR	BV	SV
p_1	1040–1104	1152–1248	736–800	432–448	320–336	208–288	144–160	0.63–0.69	2.56–2.71	2.74–3.00
p_2	1056–1120	1120–1184	480–512	288–304	208–240	160–176	128–144	0.42–0.46	3.23–3.45	4.25–4.64
p_3	1200–1264	1296–1408	704–752	392–432	304–352	192–288	144–160	0.53–0.56	2.78–3.10	3.41–3.55

Hypopygium (Figs. 1–5, 7–13). Tergite IX in lateral view with a boss (Fig. 4), with *ca* 50 setae and bare or sometimes with some microtrichia, anal point 20–24 µm long, shape triangular in dorsal view and straight in lateral view (Figs. 1–4). Laterosternite IX with 8–10 setae on each side. Transverse sternapodeme 152–168 µm long, with poorly developed or reduced projections (Fig. 1). Virga 12–16 µm long and 12–16 µm wide, consists of 6–8 short and thin spines which appear as a dark spot (Fig. 5). Gonocoxite 280–292 µm long; inferior volsella in the form of a low tubercle covered by microtrichia and setae, in the anterior part with a small bare protuberance, sometime invisible (Figs. 1, 7–13). Gonostylus 104–120 µm long, in inner part with *ca* 4 short setae; megaseta 20 µm long, one strong seta in front of it and behind it; the shape of gonostylus is bound to its position, may be triangular, slightly rounded-triangular with a right or slightly rounded outer angle located in distal third; anterior edge with tubercle (Figs. 1, 7–13).

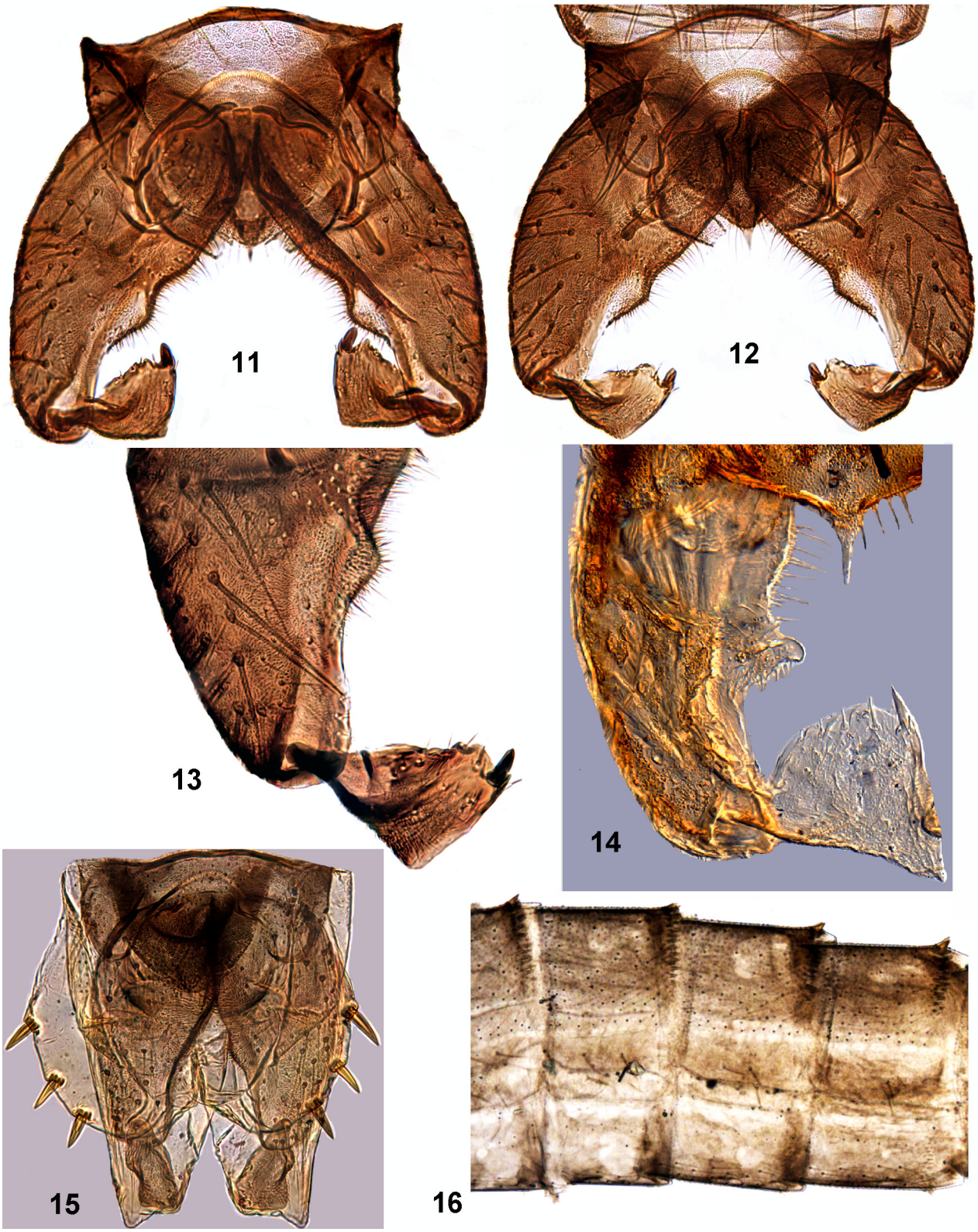
Pupa (n=1). Total length 3.1 mm. Colouration brownish. Exuviae yellowish.

Cephalothorax. Frontal apotome, slightly wrinkled, with 2 setae 60 µm long. Antepronotum with 2 median and 1 lateral antepronotals 92–128 µm long. Thoracic horn 225–240 µm long, slightly curved, expanding in the distal half, with pointed apex and covered small spinules in apical half, the largest on top (Figs. 17–18). Precorneal setae 80–88 µm long, hair-like, on a hump. Dorsocentrals hair-like, 40–60 µm long. Distance between Dc_1 and Dc_2 128–140 µm; between Dc_2 and Dc_3 16–28 µm; between Dc_3 and Dc_4 8 µm.

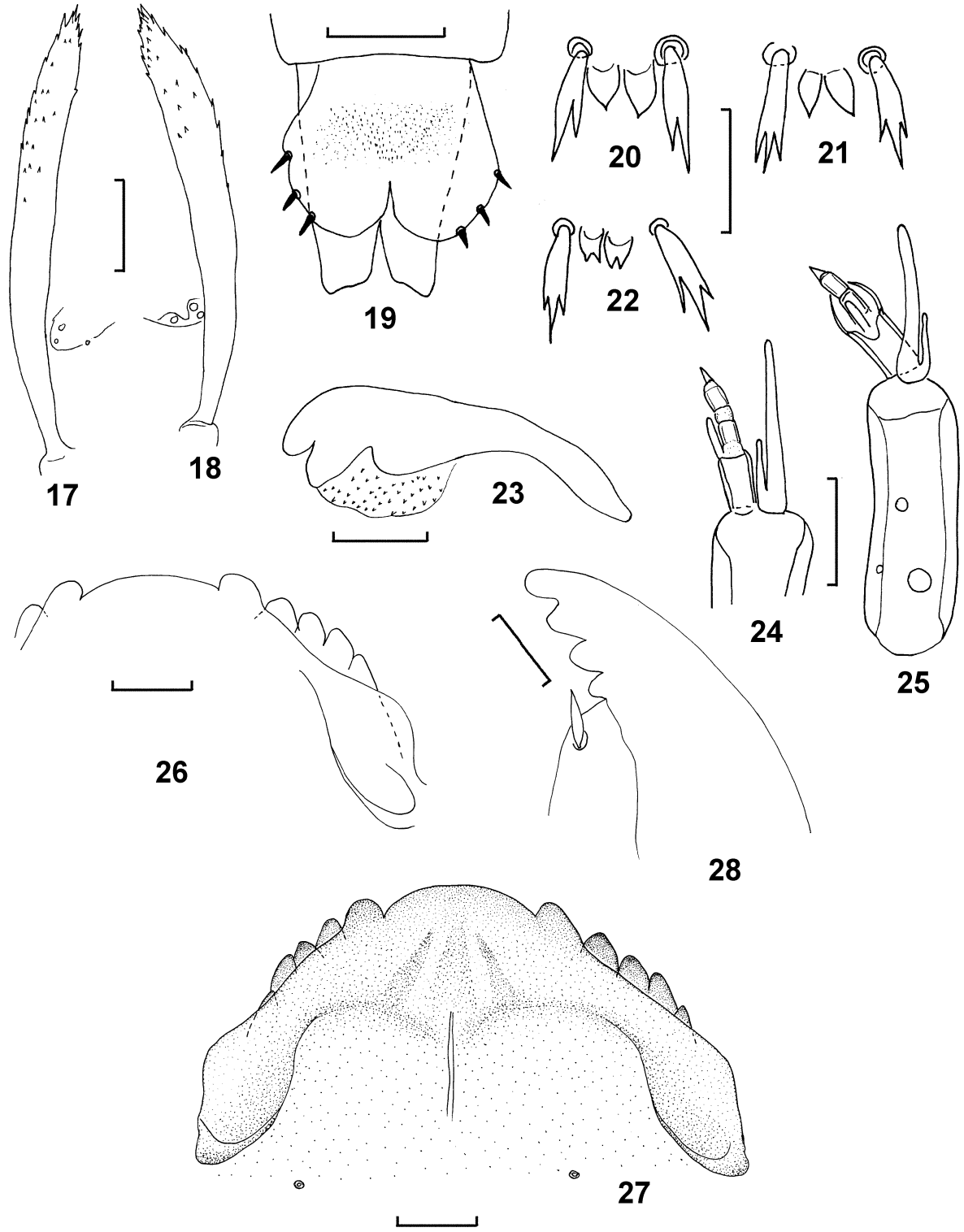
Abdomen. Tergite I with fine shagreen in anterior lateral corners and along posterior edge. Tergites II–VIII uniformly covered with shagreen, with spinules all of the same size and with 1 row of larger yellow spines near the posterior edge, small spinules are visible behind them (Fig. 16). Tergite IX with fine shagreen only in anterior half (Figs. 15, 19). Sternites I–III without shagreen; sternite IV with shagreen, with small spinules restricted to posterior edge; sternites V–VIII covered by shagreen as on tergites II–VIII, but spines along posterior edge shorter and paler. Segment I with 2 pairs of hair-like lateral setae. Segments II–VII with 4 pairs of lateral setae: 2 seta-like (LS_1 and LS_4) and 2 spine-like (LS_2 and LS_3) on small tubercles. Segment VIII with 2 pairs of hair-like lateral setae. Anal

lobe 304 µm long, rounded in apical part, with 3 spine-like macrosetae (AM) 40–48 µm long and *ca* 8–10 µm wide. Distance between AM_1 and AM_2 48–52 µm, between AM_2 and AM_3 36 µm, see Figs. 15, 19. Male genital sac extending 84 µm beyond anal lobe.

Forth instar larva (n=3). Total length 4.8–6.4 mm.



FIGURES 11–16. Adult male (11–14) and pupa (15–16) of *Chaetocladius* (s. str.) *elisabethae* **sp. nov.** (11–13, 15–16) and *Chaetocladius* (s. str.) *laminatus* Brundin (holotype) (14). **11–12, 14,** hypopygium in dorsal view; **13,** gonocoxite and gonostylus; **15,** anal segment; **16,** segments II–V in lateral view.



FIGURES 17–28. Pupa (17–19) and larva of fourth instar (20–27) of *Chaetocladius* (s. str.) *elisabethae* **sp. nov**. **17–18,** thoracic horn; **19,** anal segment; **20–22,** S_I and labral lamellae; **23,** premandible; **24,** distal part of antenna; **25,** antenna; **26–27,** mentum; **28,** distal part of mandible. Scale bars: Figs. 17–19—50 μm; Figs. 20–28—20 μm.

Head. Head capsule yellowish; 352–384 μm long and 304–320 μm wide; cephalic index (IC) 0.792–0.869. Labrum: S_I divided into 2–3 pointed, not equal branches (Figs. 20–22), S_{II} simple and strong, S_{III} simple and thin, S_{IV} simple and short. Labral lamellae paired, located between base of S_I, tapering to apex, sometimes apically bifurcated (Figs. 20–22). Pecten epipharyngis consists of 3 scales, middle of which little longer. Premandible with two apical teeth and one inner tooth, with well developed brush consisting of small spines (Fig. 23). Antenna with

5 segments; one large and one small ring organs in proximal half of basal segment, third ring organ in middle of segment; segment 2 on apex with large Lauterborn organ, its tip reaches the base of segment 4, with style whose length is equal to the length of the 3rd segment; longest branch of blade slightly over the apex of the 5th segment (Figs. 24–25); AR 1.71–2.0. Mandible with 4 teeth, apical tooth shorter than the combined width of the 3 inner teeth; seta subdentalis tapering to pointed apex reaching the 3rd inner tooth (Fig. 28); seta interna with 5–6 simple branches. Pecten galearis developed. Mentum with 1 median tooth and 5 pairs of lateral teeth; middle tooth 4–5 times wider than the 1st lateral tooth; 5th lateral teeth small and sometimes are well visible. Ventromental plate large and elongate, extends beyond the lateral teeth (Figs. 26–27). Anal tubules shorter than posterior parapods, these 160–180 µm long. Procercus 28–32 µm long and 20–24 µm wide, bearing 7 apical anal setae of different length, the longest setae 250–360 µm long, the shortest setae160–192 µm long; 2 lateral setae short and thin, supraanal seta 92–140 µm.

Diagnostic characters. The new species could be assigned to the *dentiforceps* group (Cranston *et al.* 1983, 1989; Coffman *et al.* 1986). However, we support the opinion of Stur (pers. comm.) and Moubayed-Breil (2017) that the species grouped around the *C. laminatus* Brundin be better regarded as a "*laminatus* group s. str." Indeed the new species is most closely related to *C. laminatus* Brundin, *C. lopatinskiy* Makarchenko et Makarchenko, *C. purbeckensis* Langton et Armitage and *C. guisseti* Moubayed-Breil. The adult male of *C. elisabethae* **sp. nov.** is separated from all these species by features of hypopygium, namely the shape of inferior volsella, gonostylus and anal point and by structure of virga. Pupa is distinguished by the length of thoracic horn and anal macrosetae. Unfortunately, there is no reliable data for larvae of *C. laminatus* and other related species, so a comparison cannot be made. A more detailed comparative characteristic of closely related species is given in Table 2, as well as in the results of DNA barcoding (Fig. 29).

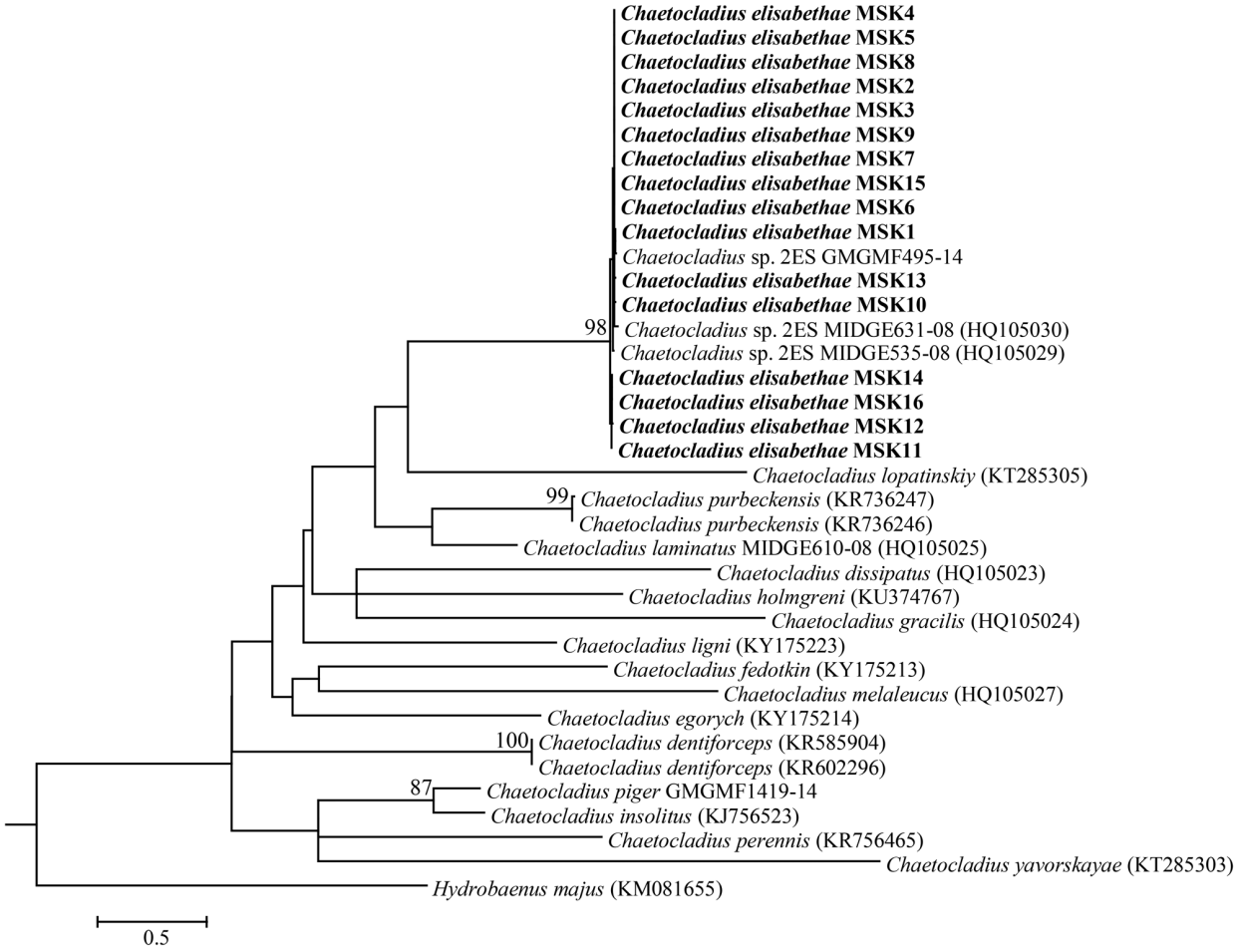


FIGURE 29. Maximum likelihood (ML) tree (-Ln likelihood = 3624.3) of the genus *Chaetocladius* s. str. and one outgroup, *Hydrobaenus majus* (Orthocladiinae) inferred from the cytochrome c oxidase I (COI) nucleotide sequence data (658 bp). Numbers are bootstrap support of 1000 replicates, bootstrap values only on branches that were supported in more than 80 % of the bootstrap replicates. Specimens obtained in this study are in bold.

TABLE 2. Comparison of adult males and pupae of some species of *Chaetocladius* "*laminatus* s. str. group"

Characters	*C. elisabethae* **sp. nov.** (orig.)	*C. lopatinskiy* Makarchenko *et* Makarchenko, 2017 (orig.)	*C. laminatus* Brundin, 1947*	*C. purbeckensis* Langton *et* Armitage, 2015 (orig.)	*C. guisseti* Moubayed-Breil, 2017 (orig.)
Adult males					
Total length, mm	3.2–3.4	3.5–4.0	**4.0–4.1,** 3.9–4.2	3.2	4.1–4.4
Wing length, mm	2.72–2.84	2.28–2.76	**3.2,** 1.10–1.20	2.0	2.00–2.10
AR	1.88–2.21	2.14–2.33	**1.54–1.73**, 1.55–1.75	1.07	1.15–1.35
Subapical seta of 13th flagellomere	Absent	Absent	Absent	Absent	Present
Acrostichals	Present	Present	Present	Absent	Present
Anal point length, µm	20–24	28–40	30–35	20	30–35
Shape of anal point in lateral view	Straight	Straight	Straight	–	Curved
Inferior volsella	In the form of low tubercle, with small bare and poorly developed protuberance in inner anterior part	As a rounded projection, lacking protuberance in inner part	Subtriangular, with swollen posterior margin, bearing a beak-like to curved nose-like inner apex	Broad, triangularly produced in its distal end, with nose-like inner apex	Large, lobe-like, not contrasting and lacking protuberance in inner part
Virga	With 6–8 short and thin spines	With 2–3 small spines	With 2 large spines and 1 smaller spine placed medially	With 2 separate short spines	With 3 small unequal spines
Gonostylus	Triangular or slightly rounded-triangular with right or slightly rounded outer angle, located in distal third; anterior edge with tubercle	Triangular, sometimes may be projecting posteriorly; anterior edge straight; outer edge extends almost at right angle	Broadly, sub-triangular, slightly spherical medially, posteriorly tapering to pointed apex	Triangularly expended with broadly rounded outer corner	Triangular, spherical medially, projecting posteriorly; posterior tip rounded, hyaline and bare
Pupae					
Total length, mm	3.1	4.0–4.65	3.0–3.5	–	4.0–4.2
Thoracic horn length, µm	225–240	232–280	120–138	–	115–120
Anal macrosetae length, µm	40–48	104–164	25–30	–	100–110

*Adult male after Brundin (1947) (in bold), Moubayed-Breil (2017) and according to Fig. 14 of the present paper; pupa after Langton & Visser (2003).

Results of DNA barcoding

The final alignment of the COI gene yielded 658 bp for 16 specimens of *C.* (s. str.) *elisabethae* **sp. nov.** with 7 haplotype, one of which detected in 9 specimens. Total pairwise sequence divergence within the new species ranged from 0.0000 to 0.0106, which is based on nine variable sites. Only one substitution was non-synonymous and changed in an isolate MSK10 Alanine to Valine in protein sequences (position 23). All the substitutions were transitions except for the mutation A–C at position 421.

The sequences of *C.* (s. str.) *elisabethae* sp. nov. were identical to the *Chaetocladius* sp. 2ES in BOLD systems consisting of tree specimens: ID GMGMF495-14, MIDGE535-08 and MIDGE631-08 (Fig. 21). Average interspecies K2P distance between *C.* (s. str.) *elisabethae* **sp. nov.** and other species of *Chaetocladius* s. str. were 0.11–0.16 (mean 0.14). According to Montagna *et al.* (2016) and Ekrem *et al.* (2010) these values are sufficient to maintain the species level. The monophyly of the *C.* (s. str.) *elisabethae* **sp. nov.** strongly supported on ML tree (Fig. 29).

Distribution and biology. West Palaearctic species, known from Moscow Region of Russia, Norway (Ekrem et *al.* 2010) and Germany (Sequence ID in BOLD: GMGMF495-14).

The pupa and larvae from Moscow Region were collected in springs with water temperature 1–6°C, on gravel and stones covered with moss *Fontinalis* sp. Adult males were caught during the swarming and from snow in the February, at air temperature 0–1°C.

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We much grateful to Dr. Elisabeth Stur for useful discuss of some taxonomical problems in *Chaetocladius* s. str. and for sending photos of hypopygium *C. laminatus* (holotype), *C. purbeckensis* (paratype) and some other unnamed species.

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