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


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
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 (Exol) 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:
Chaetocladius sp. 2ES/Female, available from:
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

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, gonoxite 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, halteres 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.


Legs. BR₁, 2.5–2.8, BR₂ 1.8–2.5, BR₃ 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 t₃ with 0–1 pseudospur, mid leg on t₃–t₄ with 0–2 pseudospurs. Hind leg on t₃–t₄ with 2 pseudospurs, t₄ with 0–2 pseudospurs; basal half of t₃ 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 | t₁ | t₂ | t₃ | t₄ | t₅ | t₆ | t₇ | t₈ | t₉ | t₁₀ | t₁₁ | t₁₂ | t₁₃ | t₁₄ | t₁₅ | t₁₆ | t₁₇ |
| p₁             | 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 |

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 poorly developed or reduced projections (Fig. 1). Virga 12–16 µm long and 12–16 µm wide, consists of 6–8 short and bare or with 2–3 setae, R₄ without setae. R₉ ending distal of apex M₃₄. Costa extension 60–84 µm. Cu₁ curved in apical part. Anal lobe well developed, rectangular-rounded. Squama with 10–13 setae.

**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 antenontalos 92–128 µm long. Thoracic horn 225–240 µm long, slightly curved, expanding in the distal half, with pointed apex and covered small spnines in apical half, the largest on top (Figs. 17–18). Precoerenal setae 80–88 µm long, hair-like, on a hump. Dorsoventrals hair-like, 40–60 µm long. Distance between Dc₁ and Dc₂ 128–140 µm; between Dc₁ and Dc₃ 16–28 µm; between Dc₃ and Dc₄ 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 (LS₁ and LS₂) and 2 spine-like (LS₃ and LS₄) 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 AM₁ and AM₂, 48–52 µm, between AM₂ and AM₃, 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.
DESCRIPTION OF *CHAETOCLADIUS ELISABETHAE* SP. NOV.

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, S₁ 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: S₁ divided into 2–3 pointed, not equal branches (Figs. 20–22), S₂ simple and strong, S₃ simple and thin, S₄ simple and short. Labral lamellae paired, located between base of S₁, 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
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 setae 160–192 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.](image-url)
**TABLE 2.** Comparison of adult males and pupae of some species of *Chaetocladius “laminatus s. str. group”*

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

*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 GMGFM495-14, 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: GMGFM495-14).

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.

**Acknowledgments**

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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