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Morphological description and DNA barcoding of *Hydrobaenus laticaudus* Sæther, 1976 (Diptera: Chironomidae: Orthocladiinae) from Amur River basin (Russian Far East)

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Abstract

Illustrated redescription of adult male and description for the first time of pupa and fourth instar larva, as well as DNA barcoding, of *Hydrobaenus laticaudus* Sæther in comparison with close related known species of *Hydrobaenus* Fries, and an updated part of a key to species of *Hydrobaenus* from the Russian Far East 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. For the estimation of interspecific distances and constructing detailed Bayesian tree within *Hydrobaenus*, we have obtained DNA barcoding for the two other species, *H. distinctus* and *Hydrobaenus* sp.1. The intraspecific sequence divergence of *H. laticaudus* based on the Kimura-2-parameter (K2P) distance ranged from 0.0–0.031, whereas the interspecific sequence divergence based on the K2P distance ranged from 0.106-0.197% between *H. laticaudus* and other species of genus *Hydrobaenus*. The well-supported monophyly as well as results of an ABGD analysis confirms the validity of *H. laticaudus*.

Key words: Diptera, Chironomidae, *Hydrobaenus,* taxonomy, DNA barcoding, Russian Far East

Introduction

This paper is a continuation of a series of articles based on the results of a revision of the genus *Hydrobaenus* Fries, 1830 of the Russian Far East using both morphological studies and DNA barcoding of adult males, pupae and larvae (Makarchenko *et al.* 2009, 2015, 2017b; Makarchenko & Makarchenko 2014).

DNA barcoding is a technique based on mass sequencing of 658bp from the 5' end of the mitochondrial cytochrome oxidase subunit I gene (COI, Hebert *et al.* 2003). This approach 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 orthoclads non-biting midges (Silva & Wiedenbrug 2014, Ekrem *et al.* 2010, Ekrem *et al.* 2018, Makarchenko *et al.* 2017a) as well as for genus *Hydrobaenus* (Makarchenko *et al.* 2015, 2017b, Wirta *et al.* 2016, Hebert *et al.* 2016).

To date, more than 44 species have been described in the genus *Hydrobaenus* (Ashe & O'Connor 2012). However, DNA barcoding was performed for only 7 described species, *H. conformis* (Holmgren), *H. fusistylus* (Goetghebuer), *H. golovinensis* Makarchenko *et* Makarchenko, *H. kondoi* (Sæther), *H. lapponicus* (Brundin), *H. majus* Makarchenko *et* Makarchenko and *H. sikhotealinensis* Makarchenko *et* Makarchenko, and 4 undescribed species (BOLD BIN numbers: AAI2245, AAI2246, AAI2247 and ACG3277).

The purpose of this paper is to perform redescription of adult male using additional material, description for the first time of pupa and fourth instar larva of *H. laticaudus* Sæther, to update part of a key to species of *Hydrobaenus* from the Russian Far East of Makarchenko & Makarchenko (2014), as well as to provide a DNA barcodes for *H. laticaudus*, *H. distinctus* and *Hydrobaenus* sp.1. Obtained DNA barcodes allowed to reconstruct phylogenetic relationships within the genus *Hydrobaenus* with using all possible for it species.

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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 specimens of *H. laticaudus* were associated by using of DNA barcoding as well as for comparing of this species with close related species. The terminology follows Sæther (1980).

All material 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).

We extracted genomic DNA 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. Polymerase chain reaction was performed to amplify the COI barcode fragment using the Go Taq Green Master Mix (Promega corp, Madison, WI, USA) and the primers LCO1490 (5'- GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'- TAAACTTCAGGGTGACCAAAAAATCA -3'), obtained from Folmer *et al.* (1994). PCR was carried out in a total volume of 10 µl and contained 5 µL of Master Mix, 0.5 µl of each primer (100 ng/µl), 3 µl nuclease-free water and 1 µl of total DNA. The PCR thermal conditions included an initial denaturation at 94 °C (2.5 min), followed by 35 cycles at 95 °C (denaturation, 30 s), 48 °C (annealing, 30 s), 72 °C (extension, 70 s), and a final extension step at 72 °C (10 min). PCR products were visualized on a 1.5% TBE agarose gel GelDoc XR+ imaging systems (BioRad). Each PCR fragment was purified using Exonuclease I (ExoI) and Thermosensitive Alkaline Phosphatase (FastAP) (Thermo Fisher Scientific Inc., USA). For additional purification AmpliClean Magnetic Bead-based PCR Cleanup (Nimagen, Netherlands) was used according to protocol. PCR products were cycle sequenced using BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.), and bidirectionally sequenced on an ABI 3130XL automated sequencer. MEGA7 (Kumar *et al.* 2016) and FinchTV were used to edit and assemble double stranded sequences. Also, MEGA7 was used for calculated inter- and intraspecific COI K2P distances. ABGD analysis (wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html, Puillandre *et al*., 2012) was used for species delamination and establish taxonomic status of sequenced specimens, using relative gap width (X = 1.0) and intraspecific divergence (P) values between 0.005 and 0.100 with the K2P model according to Song *et al.* (2017). The obtained sequences were checked aligned at the nucleotide level using MUSCLE (Edgar 2004). PartitionFinder 2.1.1 (Lanfear *et al.* 2012) was used to select the best-fit partitioning scheme and models separately for each codon position of COI using the greedy algorithm with linked branch lengths for the corrected Bayesian Information Criterion. The best models of nucleotide substitution for first COI codon positions were SYM+I (Zharkikh 1994), for second position - F81+I (Felsenstein 1981) and GTR+G for third position (Tavaré 1986).

Bayesian phylogenetic analyses were conducted with MrBayes v. 3.2.7 (Ronquist *&* Huelsenbeck 2003). In addition to our sequences, we used GenBank data for genus *Hydrobaenus* and only sequences longer than 650 bp were included in the final analyses respectively. Speciemens of *Parakiefferiella* used as an outgroup as the closest genus to the *Hydrobaenus* (Cranston *et al.* 2012). Bayesian Inference was performed with two independent runs of Metropolis-coupled Markov chain Monte Carlo analyses, with each run comprising one cold chain and three heated chains. Every chains were run for 5 million generations and sampled every 500 generations. A burn-in of 500000 generations (or 10% of the sampled trees) was used. Moreover, trace files were visually inspected in Tracer 1.7 (Rambaut *et al.* 2018). FigTree v. 1.4.4 was used to visualize phylogenetic trees after analysis.

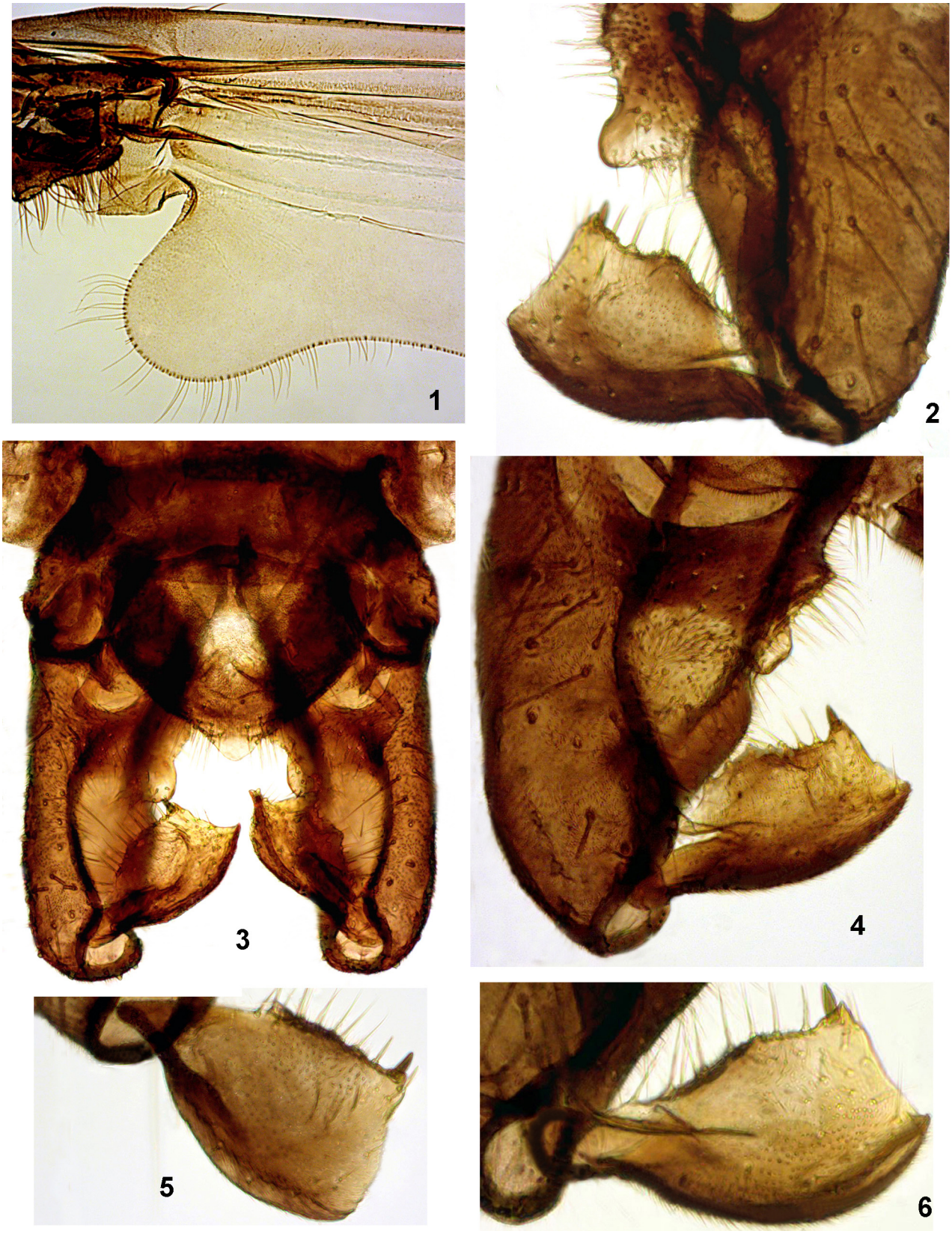
Sequences of *H. laticaudus* available in GenBank (NCBI) by numbers MN082695-MN082714. *H. distinctus* and *Hydrobaenus* sp.1, obtained in this study have been deposited by numbers MN082693 and MN082694 respectively.

Descriptions

Hydrobaenus laticaudus Sæther

(Figs. 1–18)

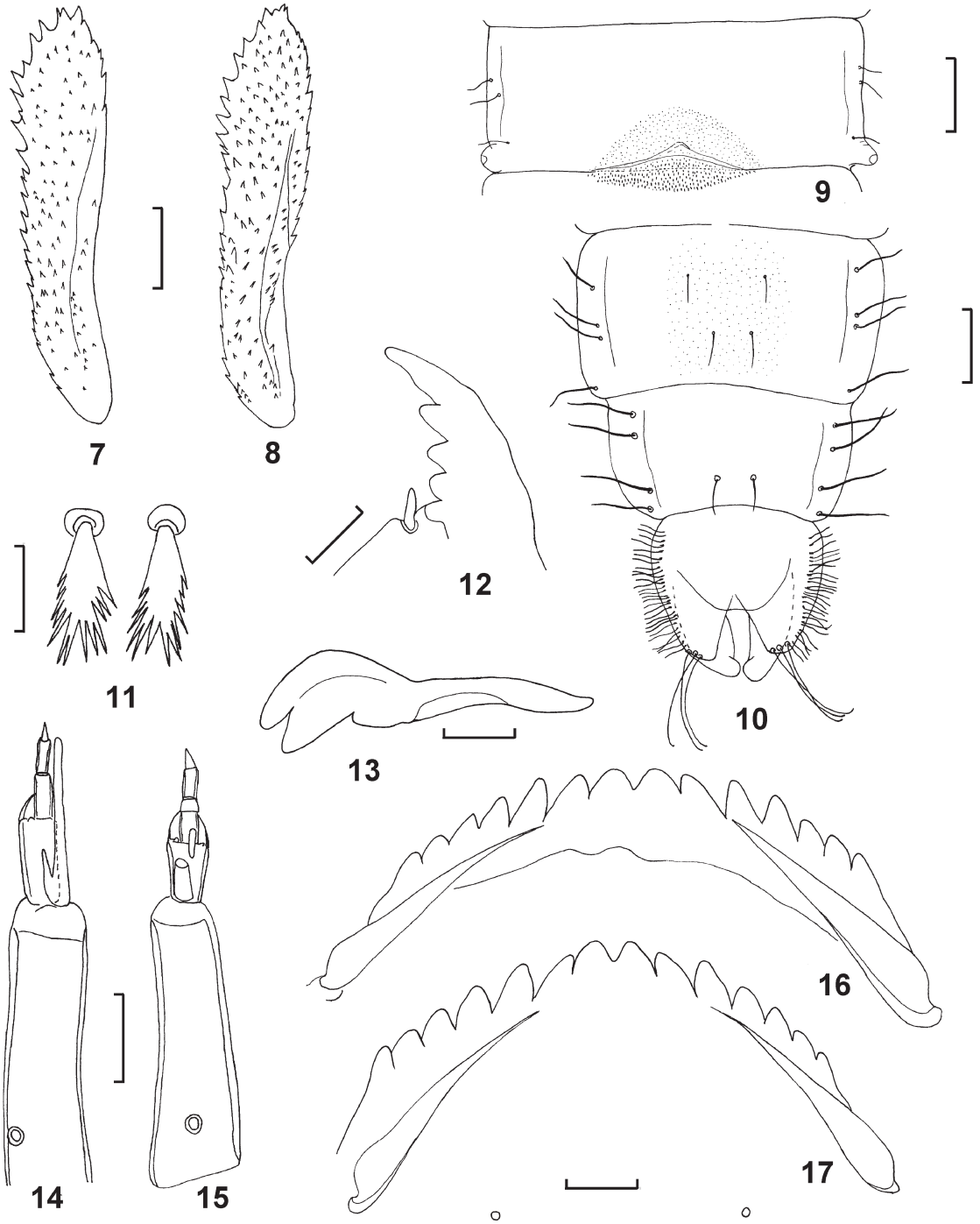
Hydrobaenus laticaudus Sæther, 1976: 80, Fig. 25; Makarchenko *et al.* 2009: 39, Figs. 19–24; Ashe & O'Connor 2012: 316.



FIGURES 1–6. Adult male of *Hydrobaenus laticaudus* Sæther. **1,** anal lobe of wing; **2, 4,** gonocoxite and gonostylus; **3,** hypopygium in dorsal view; **5–6,** gonostylus.

Material. *Russian Far East, Amur River basin*: 8 adult males, Jewish Autonomous Region, Smidovichsky District, Nature Reserve "Bastak", Zabelovskoye Lake, N 48°25'57.3", E 134°12'50.0", 16.IV.2008, leg. V. Lubarets; 2

mature pupae (male), 1 mature pupa (female), 4 mature larvae, the same data, except 19.IV.2019, leg. E. Makarchenko; 28 adult males, the same data, except 20.IV.2019, leg. E. Makarchenko; 2 adult males, the same data, except Bolshoye Lake at the river channel Pemzemskaya, near Priamursky Village, 1.V. 2009, leg. N. Yavorskaya; 2 adult males, Khabarovsk Territory, Amursky District, Kharpi River (low stream), Bolon' Lake basin, 3.V.2008, leg. N. Yavorskaya; 7 adult males, Khabarovsk Territory, Khabarovsky District, Bolshekhekhtsirsky Nature Reserve, Chirka River, N 48°05'951", E 134°51'115", 29.IV.2019, leg. N. Yavorskaya; 2 adult males, the same data except unnamed stream near Korphovsky Village, N 48°12'939", E 135°02'619",11.V.2019, leg. N. Yavorskaya; 2 adult males, Khabarovsk Territory, Nanaisky District, Sindinskoye Lake, N 48°54′57.6″, E 136°11′09.6″, 12.V.2009, leg. N. Yavorskaya.



FIGURES 7–17. Pupa (7–10) and larva of fourth instar (11–17) of *Hydrobaenus laticaudus* Sæther. **7–8,** thoracic horn; **9,** tergite II; **10,** tergites VII–VIII and anal segment; **11,** S_1 of labrum; **12,** distal part of mandible; **13,** premandible; **14–15,** antenna; **16–17,** mentum. Scale bars: Figs. 7–8—100 µm; Figs. 9–10—200 µm; Figs. 11–17—20 µm.

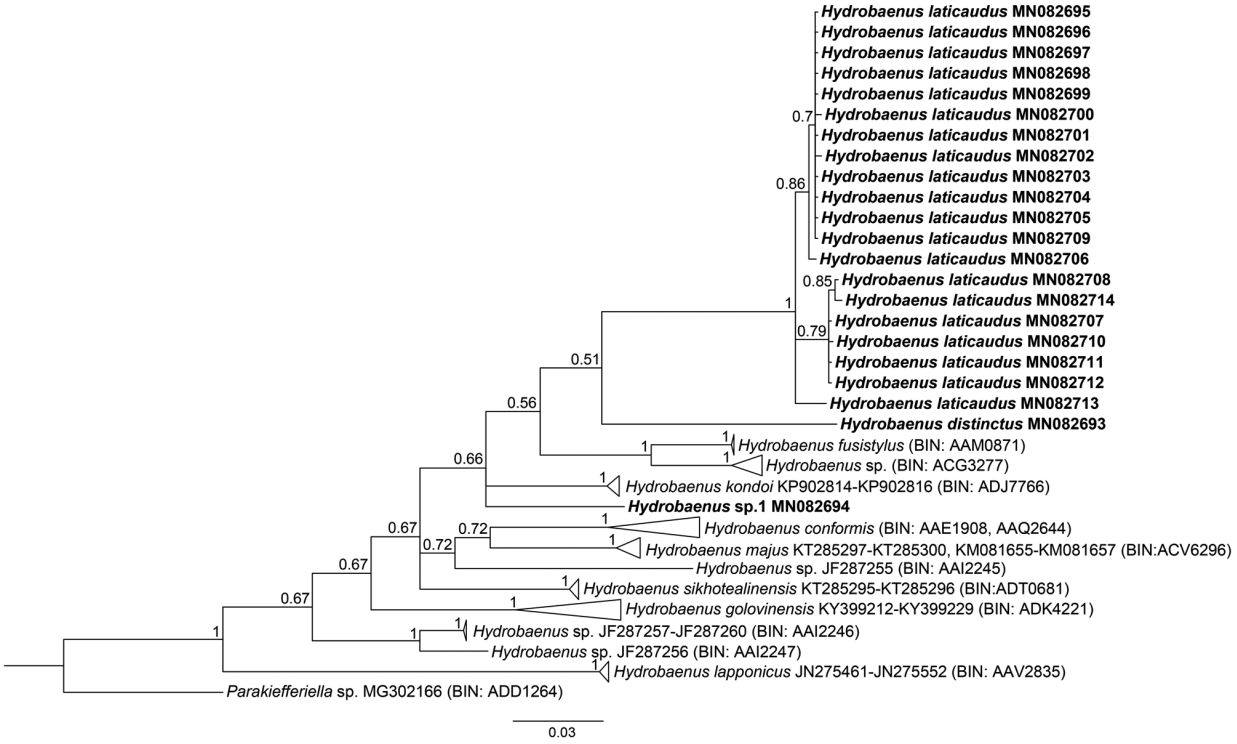


FIGURE 18. Bayesian phylogenetic tree of the genus *Hydrobaenus* and *Parakifferiella* sp. as outgroup based on the mitochondrial cytochrome c oxidase I (COI) barcode gene sequences (657-658 bp). The phylogenetic reconstruction was performed with using SYM+I, F81+I and GTR+G for each codon position. Bayesian posterior probabilities (PP) given in tree nodes. Specimens obtained in this study are in bold.

Adult male (n=7). Total length 3.5–4.7 mm. Total length/wing length 1.39–1.57.

Head. Eyes naked, with short dorso-median prolongations. Temporal setae: 1–3 outer verticals, 4–5 inner verticals and 5–8 postorbitals. Clypeus with 10–17 setae. Antenna with 13 flagellomeres and well developed plume; 13th flagellomere 860–880 µm long; AR 2.45–2.56. Length of palpomeres (in µm): 30–34, 70–96, 120–152, 92–140, 144–176; palpomere 3 with 1–3 sensitive hairs in apical part; length of 1–5 palpomeres/head width 0.87.

Thorax. Dark brown. Antepronotum with 7–10 lateral setae. Ac 2–8 (very short and situated in middle of mesonotum), Dc 9–17, Pa 7–13, Scts 9–18 in one row.

Wing. Greyish. Length 2.5–3.1 mm. R with 5–9 setae, R_1 without setae, R_{4+5} with 0–1 seta apically. R_{4+5} ending distally of apex M_{3+4}. Costa extension 40–100 µm long. Cu_1 curved in apical part. Anal lobe well developed and strongly protrude (Fig. 1). Squama with 23–44 setae.

Legs. Spur of fore tibia 68–88 µm long. Spurs of mid tibia 25–28 µm and 30–36 µm long. Spurs of hind tibia 30–100 µm and 16–25 µm long. Hind tibial comb with 12–14 setae. Basitarsus of hind leg with 4 sensilla chaetica in basal part. Lengths and proportions of legs as in Table 1.

TABLE 1. Lengths (in µm) and proportions of leg segments of *Hydrobaenus laticaudus* Sæther, male (n=5)

	fe	ti	ta_1	ta_2	ta_3	ta_4	ta_5	LR	BV	SV	BR
P_1	896-1088	1040-1312	704-896	400-528	304-370	208-224	168-170	0.68-0.74	2.39-2.59	2.49-2.75	2.9-4.3
P_2	928-1120	1008-1200	480-592	288-336	208-260	144-176	144-168	0.48-0.52	2.98-3.11	3.71-4.03	2.0-3.8
P_3	1008-1248	1184-1472	608-800	336-448	288-330	160-190	144-180	0.51-0.54	2.93-3.14	3.40-3.61	3.1-5.2

Hypopygium (Figs. 2–6). Tergite IX roundish, with 35–54 setae and short, poorly developed, rounded triangular and bare in distal part anal point. Laterosternite IX with 12–15 setae. Transverse sternapodeme 156–160 µm long, with rounded triangular projections. Virga 18–32 µm long, consisted of 4 setae. Gonocoxite 300–372 µm long; inferior volsella as shown in Figs. 2–4. Gonostylus 128–160 µm long, wide, distally angular, with small protrusion on outer edge (Figs. 2–6); megaseta 8–13 µm long.

Pupa (n=3). Total length 4.0–4.9 mm. Cephalothorax brownish, abdominal tergites light brownish or yellowish. Exuviae transparent. Tergites II–VIII and sternites II–VIII with brown or dark brown apophyses.

Cephalothorax. Frontal apotome with 2 setae 100–140 μm long and with poorly developed or reduced warts. Antepronotum with two median antepronotals setae *ca* 100 μm long and two lateral antepronotal setae *ca* 70 μm long; both median and lateral antepronotals thin and hair-like. Thoracic horn 448–464 μm long, covered by spinules, expands to the rounded apex (Figs. 7–8). Precorneal setae lengths (in μm): Pc_1 80–92, Pc_2 72–120, Pc_3 40–60. Bases of setae arranged in form of triangle. Dorsocentrals hair-like; Dc_{1-4} 48–80 μm long. Distance between Dc_1 and Dc_2 32–64 μm; between Dc_2 and Dc_3 48–66 μm; between Dc_3 and Dc_4 36–92 μm.

Abdomen. Tergite I without shagreen. Tergite II with shagreen of spinules in middle of posterior half; shagreen at posterior edge with relatively large spinules in 4 rows, their apex pointed orally (Fig. 9). Tergites III–IV with shagreen of spinules at least covered of ½ tergite surface, sometimes whole surface of tergite; size of spinules slightly increases toward the posterior margin; 2–3 rows of larger spinules along posterior margin with apex pointed orally. Shagreenation of tergites V–VI similar to the same of tergites III–IV but spinules of posterior margin are much smaller and tergite VI without rows of spinules along posterior margin. Tergite VII with fine shagreen in middle part from anterior to posterior margins. Tergites VIII–IX without shagreen but sometimes tergite VIII may be with very tender shagreen. Sternites I–IX without shagreen. Segment II with PSB. Sternites IV–VII with PSA. Segment I with 0–1 pair of lateral setae. Segments II–III with 2–3 pairs of hair-like lateral setae. Segments IV–VI with 3 pairs of hair-like lateral seta. Segments VII–VIII mostly with 4 pairs of taeniate lateral setae, and one specimen with 3 pairs of taeniate lateral setae on segment VIII from one side only (Fig. 10). Anal lobe 368–416 μm long and 192–216 μm width, with fringe of 37–40 setae from one side, which 72–104 μm long, occupy all lateral part of anal lobe edge to base of megasetae (Fig.10); anal lobe length/anal lobe width 1.7–2.0. Male genital sac overreaching anal lobe on 60 μm, without apical papilla. Anal macrosetae 236–260 μm long.

Fourth instar larva (n = 4). Total length 5.4–7.2 mm.

Head. Light-yellowish, with postoccipital margin, teeth of mentum, mandible and premandible dark brown or almost black; head length 320–368 μm. Labral setae S_I plumose, with 12–14 unequal branches (Fig. 11); labral lamella simple; $S_{II} – S_{IV}$ simple and hair-like. Pecten epipharyngis consisted of 3 equal pointed scales. Premandible distally with 2 apical teeth (Fig. 13). Antenna with 5 segments; length 1–5 segments (in μm): 59–62 : 12–16 : 8–10 : 6–8 : 3–5; AR 1.59–1.91; apex of segment 2 with stylus and lauterborn organs ending at apex of 3rd segment; antennal blade 32–38 μm long, ending near base of 5th segment; one large ring organ in proximal 1/3 of basal segment and one small ring organ in apical half (Figs.14–15). Mandible with apical tooth, which equal or shorter of combined width of three inner teeth; seta interna with 5–7 of 5 poorly serrated branches, seta subdentalis long and wide, with rounded apex (Fig. 12). Mentum with 2 median and 6 pairs of dark brown or black lateral teeth; median teeth well divided, one of them in 1.5 time wider than first lateral tooth; first lateral tooth is strongly inclined to median tooth. Setae submenti at level of posterior edge of ventromental plates. Ventromental plates extending beyond last lateral tooth (Figs. 16–17); ventromental plate maximum width/one median tooth width 0.67–1.0. Postmentum 144–168 μm long. Maxilla with pecten galearis.

Abdomen. Procercus 32–44 μm long (length/width 2.0), with 7 anal setae 400–560 μm long and 2 thin lateral setae. Supraanal setae 304–352 μm long; Sa/An 0.54–0.88. Anal tubules elongated, egg-shaped, shorter than posterior parapods. Posterior parapods with simple hooks in apex.

Taxonomic notes. *H. laticaudus* was described by Sæther (1976) from Alaska on adult males and placed in *H. conformis* group. But since the pupa and larva were not known, the author had some doubts and wrote in the remarks that "At first glance the hypopygium of this species appears to resemble some members of *Zalutschia* more than other species of *Hydrobaenus"*. After studying the pupa and larva, we confirm the position of *H. laticaudus* in the genus *Hydrobaenus*.

In our earlier work (Makarchenko & Makarchenko 2014) we compared adult males from Alaska with those from the Amur River basin but did not note the characteristic feature of males from the Amur River basin, which have a more developed and protruding forward anal lobe of the wing (Fig. 1). In description of males from Alaska Sæther (1976) recorded that "anal lobe ... slightly protruding". The characteristics of pupae and larvae, comparing of *H. laticaudus* with closely related species, are given below in our updated keys based on that by Makarchenko & Makarchenko (2014: 431), as well as in the results of DNA barcoding.

Distribution. *H. laticaudus* is known only from Alaska (U.S.A.) and Amur River basin (Russian Far East).

Updated keys to species of *Hydrobaenus* Fries from the Russian Far East (part)

Pupae

4. Anal lobe fringe located around lateral edge to base of megasetae 5
– Anal lobe fringe located in anterior two-thirds of anal lobe and not reaching base of megasetae 9
5. Thoracic horn almost one width, with rounded apex. Anal lobe fringe with 15–23 setae . . . . . *H. maladistinctus* Makarchenko *et* Makarchenko
– Thoracic horn with pointed apex tapering from base to top or if thoracic horn with a rounded apex, then it expands to the apex. Anal lobe fringe with various number of setae 6
6. Tergite VI with 3 pairs of hair-like lateral setae 7
– Tergite VI with 4 pairs of hair-like and taeniate lateral setae 8
7. Thoracic horn 310–320 µm long, with pointed apex. Anal lobe fringe with 15–24 setae *ca* 150 µm long . . . . . *H. sikhotealinensis* Makarchenko *et* Makarchenko
– Thoracic horn 448–464 µm long, expands to apex. Anal lobe fringe with 37–40 setae 72–104 µm long . . . . . *H. laticaudus* Sæther
8. Thoracic horn 368–400 µm long, with extension in distal third (Makarchenko *et al.* 2015: Figs. 9–10) . . . . . *H. majus* Makarchenko *et* Makarchenko
– Thoracic horn 260–320 µm long, almost parallel-sided , slightly narrowing subapically (Makarchenko *et al.* 2009: Fig. 13) . . . . . *H. kisosecundus* Sasa *et* Kondo
9. Anal lobe fringe located in anterior one-third of anal lobe. Segment V with 2 pairs of hair-like lateral setae and 2 pairs of taeniate lateral setae . . . . . *H. conformis* (Holmgren)
– Anal lobe fringe located in anterior two-thirds of anal lobe. Segment V with 4 pairs of hair-like lateral setae, sometimes setae of one pair taeniate 10
10. Thoracic horn leaf-shaped. Anal lobe fringe with 4–12 setae 8–76 µm long … *H. golovinensis* Makarchenko *et* Makarchenko
– Thoracic horn never leaf-shaped. Anal lobe fringe with 16–25 setae 100–130 µm long 11
11. Segment VI with 2 pairs of hair-like and 2 pairs of taeniate lateral setae. Segment VIII with 5 pairs of taeniate lateral setae. Genital sheath of male with dark papilla. Tip of anal lobe smooth . . . . . *H. fusistylus* (Goetghebuer)
– Segment VI with 1 pair of hair-like and 3 pairs of taeniate lateral setae. Segment VIII with 4 pairs of taeniate lateral setae. Genital sheath of male without papilla. Tip of anal lobe rugulose . . . . . *H. biwaquartus* Sasa *et* Kawai

Larvae of fourth instar

1. Head capsule light-yellow. Labral setae S_I palmate, with 12–14 more or less equal branches. Mentum with 2 median and 5 pairs of lateral teeth (Figs. 22–23, 27–28) . . . . . *H. distinctus* (Makarchenko *et* Makarchenko)
– Head capsule from light-yellow to dark brown. S_I plumose or coarsely plumose, with various numbers of branches. Mentum with 1–2 median and 6 pairs of lateral teeth 2
2. Mentum with 2 median teeth 3
– Mentum with 1 median tooth 8
3. Head capsule dark brown. Median tooth of mentum lower than or equal to height of first lateral tooth . . . . . *H. jacuticus* Makarchenko *et* Makarchenko
– Head capsule brownish-yellow. Median tooth of mentum higher of first lateral tooth 4
4. Median teeth of mentum divided by small notch. S_I with 6–8 unequal branches . . . . . *H. conformis* (Holmgren, 1869)
– Median teeth of mentum well divided. S_I plumose, with equal or unequal branches 5
5. One median tooth of mentum 2.8–3 times wider than first lateral tooth ... *H. maladistinctus* (Makarchenko *et* Makarchenko)
– One median tooth of mentum 1.5 times wider than first lateral tooth 6
6. S_I with more or less equal branches. Sa/An 0.23–0.25. Procercus 40–44 µm long . . . . . *H. fusistylus* (Goetghebuer)
– S_I with unequal branches. Sa/An 0.54–0.88. Procercus 32–64 µm long 7
7. First lateral tooth of mentum is strongly inclined to median tooth. Procercus 32–44 µm long. Postmentum 144–168 µm long . . . . . *H. laticaudus* Sæther
– First lateral tooth of mentum is not inclined to median tooth. Procercus 56–64 µm long. Postmentum 195 µm long . . . . . *H. biwaquartus* Sasa *et* Kawai
8. Median tooth of mentum brown, as all lateral teeth, with rounded tip 9
– Median tooth of mentum lighter than lateral teeth, with convex or straight tip 10
9. Median tooth of mentum with rounded tip, 4–4.5 times wider than first lateral tooth. Head yellowish. Apical tooth of mandible lighter than other teeth . . . . . *H. sikhotealinensis* Makarchenko *et* Makarchenko
– Median tooth of mentum with slightly convex tip, 1.75–2.3 times wider than first lateral tooth. Head brownish. Apical tooth of mandible brown as other teeth . . . . . *H. golovinensis* Makarchenko *et* Makarchenko
10. Median tooth of mentum with straight tip, 4–4.5 times wider than first lateral tooth. AR 2.7–2.8. Body length 4.8–5.2 mm . . . . . *H. kisosecundus* Sasa *et* Kondo
– Median tooth of mentum with convex tip, 2–2.3 times wider than first lateral tooth. AR 1.1–1.3. Body length 3.1–3.3 mm . . . . . *H. monodentatus* Makarchenko *et* Makarchenko

Results of DNA barcoding

Overall, we have sequenced fragments of the cytochrome oxidase I (658 bp) of 20 specimens of *H. laticaudus* and one specimens for the two remaining species*, H. distinctus* and *Hydrobaenus* sp.1. The nucleotide composition of the studied sequences has following values: 39,8 % of T, 17,5 % of C, 25.7 % of A, 17,0% of G. Totally, we have determined 109 variable sites, most of which were interspecific. Pairwise sequence divergence (K2P) within *H. laticaudus* ranges from 0.0 to 0.031 (average 0.011). The intraspecific divergence values are based on 26 synonymous substitutions of which 25 transitions and one transversion.

The average interspecific K2P distance (COI) between *H. laticaudus* and other *Hydrobaenus* species was 0.131 (0.106-0.197). The interspecific distances within *Hydrobaenus* was 0.130 in average, which is acceptable for an intraspecific level (Montagna *et al*. 2016). Sequences of *H. distinctus* and *Hydrobaenus* sp.1 differ from other species on 0.133 and 0.120 respectively and these values are sufficient to maintain the species level.

Species independence was also confirmed by ABGD analysis. We created a dataset of all *Hydrobaenus* sequences from GenBank longer than 650 bp. The ABGD of the entire dataset separated *H. laticaudus*, *H. distinctus* and *Hydrobaenus* sp.1 in different groups (OTU) with a prior intraspecific divergence of P=0.0514.

The total length of the COI after alignment with the GenBank samples was 657-658 bp. Most of the external nodes were moderately-supported (Bayesian posterior probability, PP=0.51 - 0.72) whereas internal nodes were often well-supported (PP=0.7 - 1) (Fig. 18). The monophyly of each *Hydrobaenus* species was supported.

H. laticaudus was placed as sister to the *H. distinctus* and consist of three clades two of which uniting specimens from the Chirka River (MN082707, MN082708, MN082710-MN082714) and one clade includes specimens from Korphovsky Village and Zabelovskoe Lake (MN082695-MN082706, MN082709). The geographical distance between the last two localizations is more than 60 km while between Chirka River and Korphovsky Village less than 30 km. Thus, there is no pattern in the mutations from the geographical distance. In turn, *Hydrobaenus* sp.1 was placed to the polytomy node with *H. kondoi* and clade uniting *Hydrobaenus* sp. (BIN: ACG3277), *H. fusistylus* (BIN: AAM0871) and *H. distinctus–H. laticaudus* clade.

In general, the resulting tree did not allow to resolve the phylogeny of the genus *Hydrobaenus.* Future molecular studies of chironomids will need to incorporate multi-locus methods to resolve questions regarding relationships the genus *Hydrobaenus* and other orthoclads.

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