



Morphological description and DNA barcoding of *Hydrobaenus majus* sp. nov. (Diptera: Chironomidae: Orthoclaadiinae) from the Russian Far East

EUGENYI A. MAKARCHENKO^{1,3}, MARINA A. MAKARCHENKO¹ & ALEXANDER A. SEMENCHENKO²

¹Institute of Biology and Soil Science, Far East Branch of the Russian Academy of Sciences, 100 let Vladivostoku 159, 690022 Vladivostok, Russia. E-mail: makarchenko@biosoil.ru

²Far Eastern Federal University, Suhanova St. 8, 690950 Vladivostok, Russia. semenchenko_alexander@mail.ru

³Corresponding author

Abstract. Illustrated descriptions of adult male, pupa and fourth instar larva, as well as DNA barcoding, of *Hydrobaenus majus* sp. nov. in comparison with the close related species *H. sikhotealinensis* Makarchenko et Makarchenko from the Russian Far East are provided. The species-specificity of *H. majus* sp. nov. COI sequences is analyzed and the sequences are presented as diagnostic characters—molecular markers of *H. majus* and *H. sikhotealinensis*.

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

Introduction

In the spring of 2013–2015 we collected adult males of *Hydrobaenus* Fries species in the Southern Primorye and the Amur River basin of the Russian Far East, which are close related to *H. sikhotealinensis* Makarchenko et Makarchenko and not readily separable from the latter. However, after analyzing the pupae and larvae of this species, as well as DNA barcoding, it was found that it is a new species, *Hydrobaenus majus* sp. nov. The morphological description and results of DNA barcoding of this species in comparison with the same of *H. sikhotealinensis* are presented below.

Material and methods

Larva, pupa and adults of *H. majus* were associated by rearing larvae individually to the mature pupae and from the pupae to adults. For morphological comparison, the analysed adult males of *H. majus* and *H. sikhotealinensis* were the same used for DNA barcoding.

The terminology follows Sæther (1980). 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).

Holotype and paratypes of the new species are deposited in the Institute of Biology and Soil Sciences, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia (IBSS FEBRAS).

Total genomic DNA was extracted from the samples using the Invitrogen (Invitrogen corp, Carlsbad, CA, 2007) protocol. The DNA precipitate was resuspended in TE buffer then stored in -20°C. The polymerase chain reaction (PCR) was used to amplify fragment of COI gene (mtDNA). The primers for amplification of the COI fragment were LCO1490 (5'-GGTCAACAAATCATAAAGAT ATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'), according to Folmer *et al.* 1994. 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 µl of each primer (100 ng/µl), 3 µl nuclease-free water and 1 µl of total 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 P.D.N. Hebert *et al.* 2003. Each fragment was purified using ethanol precipitated, air-dried and cycle sequenced on an ABI 3130x (Applied Biosystems) automated sequencer using BigDye terminator v3.1 cycle kit methods. Forward and reverse sequences were aligned and manually edited in MEGA 6. The taxon tree was generated using neighbour joining analysis and 1000 bootstrap replicates on Maximum Composite

Likelihood genetic distances in MEGA 6 (Tamura *et al.* 2013). Sequences of *H. majus* obtained in this study have been submitted to Gen-Bank KM081655–KM081657, KT285297–KT285300 and *H. sikhotealinensis*—KT285295–KT285296.

***Hydrobaenus majus* Makarchenko et Makarchenko, sp. nov.**

(Figs. 5–18).

Material. Holotype: adult male, Russian Far East, Primorye Territory, Ussuryisky District, Mountain-Taiga Station of the Far Eastern Branch Russian Academy of Sciences, Gornotaezhnoe Village, unnamed stream, N 43°41.675', E 132°09.293', 6.V. 2013, leg. E. Makarchenko & M. Makarchenko. 19 Paratypes: 5 adult males, 2 pupae with larval skins, 3 larvae, the same data as holotype, except 16.V. 2013, leg. E. Makarchenko; 1 adult male, the same data as holotype, except 27.V. 2013, leg. E. Makarchenko; 1 pupa, 4 larvae, the same data as holotype, except 10.V. 2014, leg. E. Makarchenko & M. Makarchenko; 3 adult males, Khabarovsk Territory, Bolshekhehtsyrsky Nature Reserve, Golovina Stream, Ussuri River basin, N 48°11.131', E 134°41.039', 13.V. 2015, leg. N. Yavorskaya.

Etymology. The species name is derived from the Latin word *majus*, which means May. All the examined material was collected in May.

Adult male (n=4). Total length 2.5–3.3 mm. Wing length 2.0–2.28 mm. Total length/wing length 1.25–1.45.

Colouration. Thorax, abdomen and legs brown to dark brown. Wing greyish.

Head. Eyes pubescence, with short dorsomedian prolongations Temporal setae 9–10, including 5–6 verticals and 4 postorbitals. Clypeus with 8–10 setae. Antenna with 13 flagellomeres and well developed plume; apex of 13th flagellomere pointed. AR 1.52–1.80. Length of 5 palpomeres (in μm): 20–32, 60–68, 116–120, 100–120, 136–160.

Thorax. Anteprepronotum with 3 lateral setae. Acrostichals 8–16, dorsocentrals 7–16, prealars 4–7, scutellum with 6–12 setae in one row.

Wing. R with 6–10 setae, R₁ and R₄₊₅ without setae. R₄₊₅ ending distal of apex M₃₊₄ and costa extension. Costa extension 48 μm . Anal lobe well developed, rounded. Squama with 11–16 setae.

Legs. Spur of fore tibia 56–60 μm long. Spurs of mid tibia 24–24 μm and 20 μm long. Spurs of hind tibia 56–60 μm and 20 μm long. Hind tibial comb with 10–11 setae. Front leg without pseudospurs on ta₁; mid leg with 0–1 pseudospur, hind leg with 2 apical pseudospurs on ta₁ and; sometimes hind legs with 1 apical spur on ta₁. Hind leg on ta₁ in basal part with 6–10 sensilla chaetica. Lengths and proportions of legs as in Table 1.

TABLE 1. Lengths (in μm) and proportions of leg segments of *Hydrobaenus majus* sp. nov., male (n = 2).

	fe	ti	ta ₁	ta ₂	ta ₃
P ₁	768–816	912–992	576–672	352–432	256–304
P ₂	800–864	848–928	400–480	224–272	176–192
P ₃	816–912	1008–1136	512–602	296–352	224–256

continued.

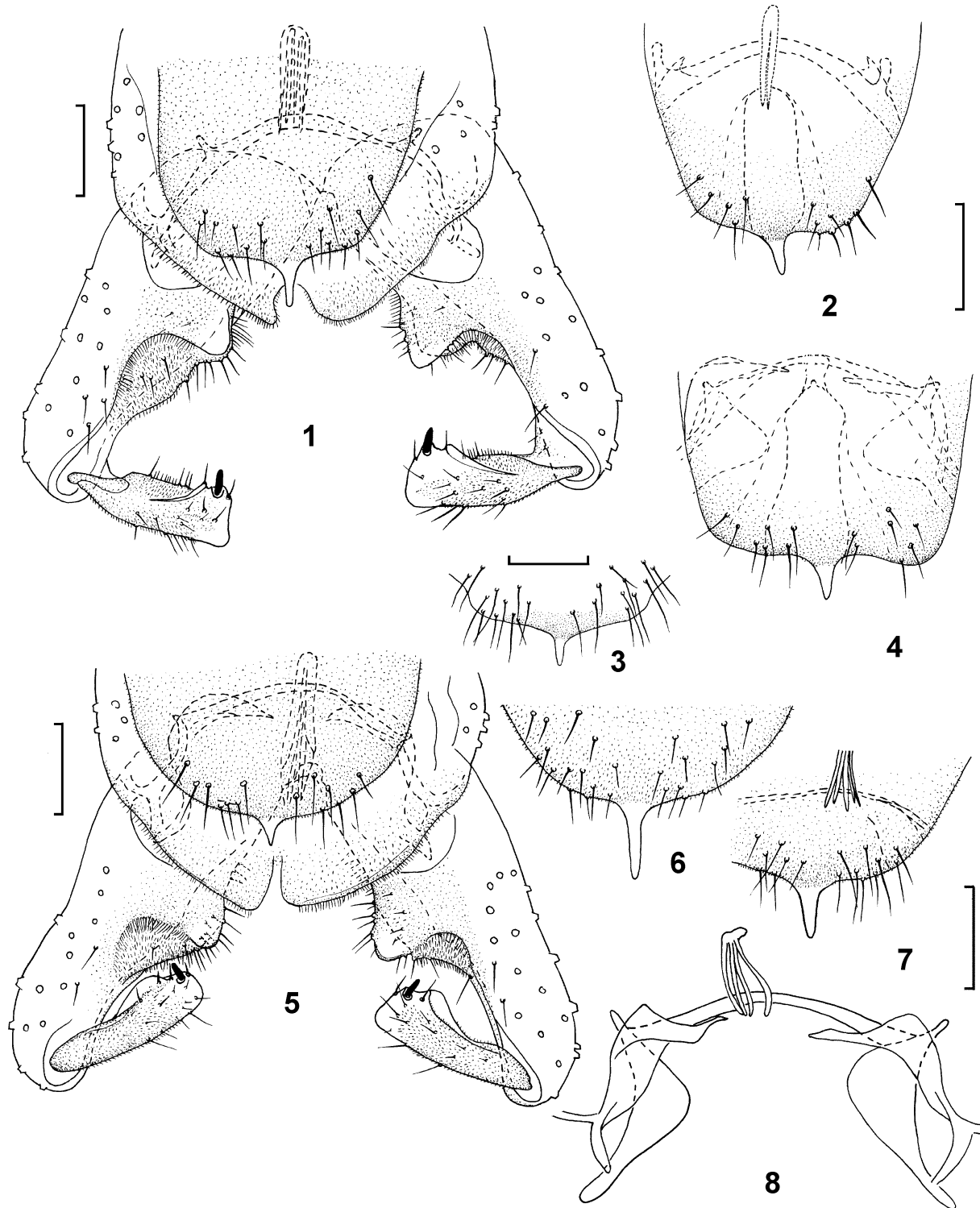
	ta ₄	ta ₅	LR	BV	SV
P ₁	160–176	120–128	0.63–0.68	2.38–2.54	2.69–2.92
P ₂	112–128	112	0.47–0.52	3.23–3.28	3.73–4.12
P ₃	128–144	120–128	0.51–0.53	3.02–3.04	3.37–3.56

Hypopygium (Figs. 5–8). Tergite IX roundish, with 13–28 setae. Anal point 16–36 μm long, with rounded apex. Laterosternite IX with 8–11 setae on each side. Transverse sternapodeme 128 μm long, with rod-like oral projections (Fig. 8). Virga 40–60 μm long, consists of 3–5 setae. Gonocoxite 216–268 μm long; inferior volsella like in Fig. 5. Gonostylus 88–116 μm long, with preapical crista dorsalis (Fig. 5); megaseta 12–16 μm long. One male has 2 megasetae. HV 2.41–3.37; HR 2.08–2.20.

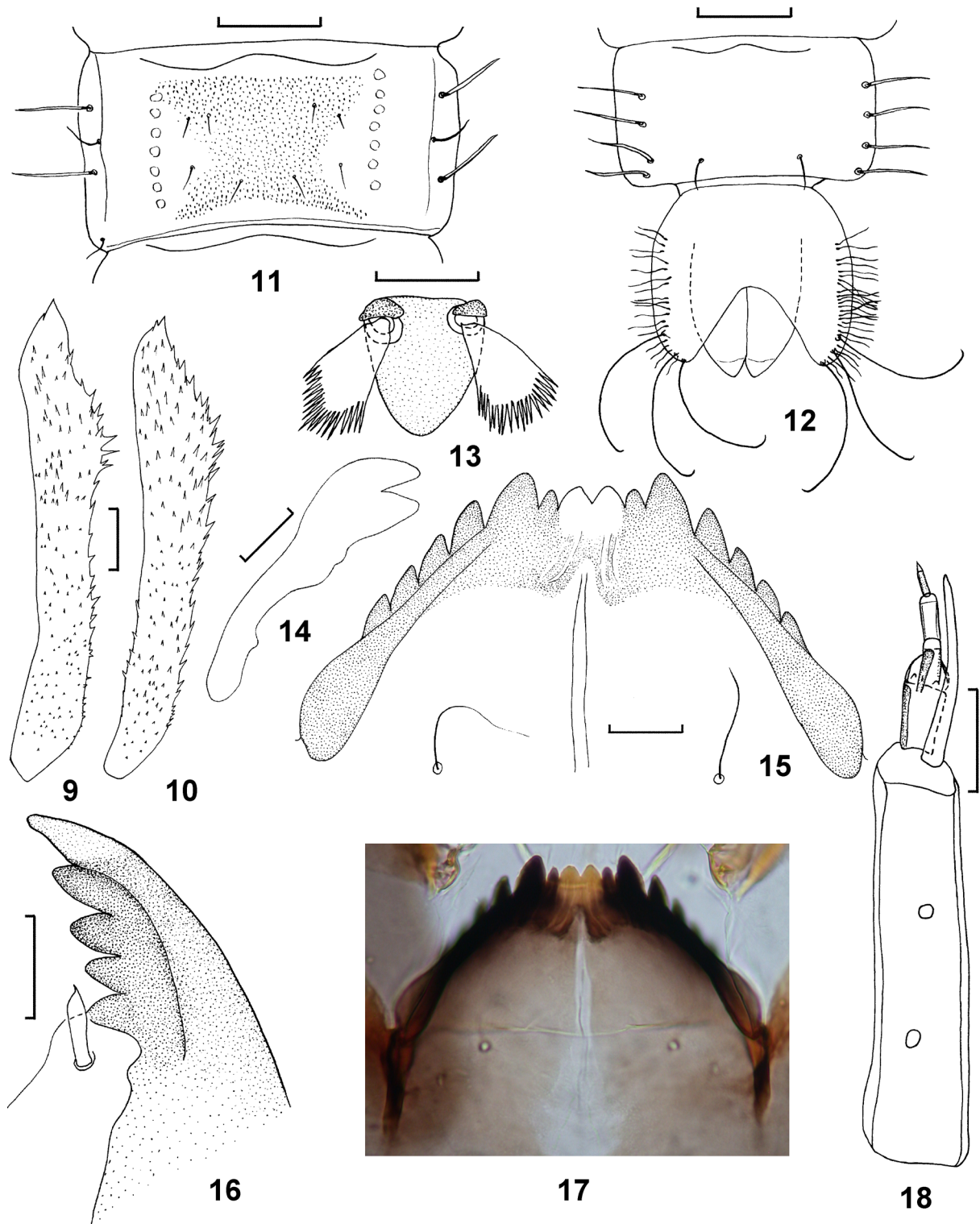
Pupa (n=3). Total length 4.0–4.3 mm. Colouration brownish, abdominal tergites light yellow to brown. Exuviae transparent. Tergites II–VIII and sternites II–VIII with brown or dark brown apophyses.

Cephalothorax. Frontal apotome almost smooth, with 2 tubercles and 2 setae 144 μm long. Anteprepronotum with two

median and two lateral anteprenotal setae. One median anteprenotal seta thin and hair-like, another seta strong, *ca* 4 μm wide and sometimes branched near apex (one female). Both lateral anteprenotals thin and hair-like. Thoracic horn 368–400 μm long (males) or 384 μm long (female), with strongly pointed apex and with extension in distal third, covered by teeth (Figs. 9–10). Precorneal setae lengths (in μm): Pc_1 —72–100, Pc_2 —136–140, Pc_3 —20–36. Dorsocentrals thin and hair-like; $\text{Dc}_{1,3,4}$ 56–50 μm long (males) and 48–68 μm long (females), Dc_2 40 μm long. Distance between Dc_1 and Dc_2 44–52 μm ; between Dc_2 and Dc_3 36–62 μm ; between Dc_3 and Dc_4 32–36 μm .



FIGURES 1–8. Adult males of *Hydrobaenus sikhotealinensis* Makarchenko *et* Makarchenko [KT000584] (1–4) and *H. majus* sp. nov. [KM081655–KM081657] (5–8). **1, 5**, hypopygium in dorsal view; **2–4, 6–7**, tergite IX with anal point; **8**, TSA, phallapodemes, aedeagal lobes and virga. Scale bars 50 μm .



FIGURES 9–18. *Hydrobaenus majus* sp. nov., pupa (9–12) and larva of fourth instar (13–18). 9–10, thoracic horn of male (9) and female (10); 11, tergite VI; 12, tergite VIII and anal segment; 13, S, and labral lamella; 14, premandible; 15, 17, mentum; 16, distal part of mandible; 18, antenna; Scale bars: Figs. 9–10—50 μ m; Figs. 11–12—200 μ m; Figs. 13–16, 18—20 μ m.

Abdomen. Tergite I without shagreen. Tergite II with shagreen of spinules which in middle part small, at anterior edge spinules larger; at posterior edge shagreen with relatively large spines situated in 3 rows and their apex pointed orally. Shagreenation of tergites III–IV is same as tergite II, but the spinules at posterior edge of the same size as in the middle part. Tergites V–VI with shagreen of spinules as tergites III–IV but at posterior edge without spinules pointed orally (Fig. 11). Tergites VII–IX with weak shagreen of spinules in the middle part. Sternites without shagreen but

sometimes some spinules situated on sternites I–V. Segment II with weak PSB. Sternites IV–VII with PSA. Segment I with 0–1 pairs of lateral setae. Segments II–V with 3 pairs of hair-like lateral setae. Segment VI with 1–2 pairs of short hair-like lateral seta and 2 pairs long taeniate lateral setae (Fig. 11). Segments VII–VIII with 4 pairs of long taeniate lateral setae (Fig. 12). Anal lobe 320–380 μm long, with fringe of 28–30 setae which 68–100 μm long, occupy two-thirds of the anal lobe and extend to anal macrosetae, often these setae 12–64 μm long situated between anal macrosetae (Fig. 12). Male genital sac overreaching anal lobe 24 μm . Anal macrosetae 272–304 μm long.

Fourth instar larva (n=4). Colouration of head brown, body segments yellowish or yellowish-brown. Total length 4.7–6.0 mm.

Head. Brownish. Labral setae S_I plumose (Fig. 13), S_{II} strong, S_{III} weak and hair-like, S_{IV} short; labral lamella abutting sockets of S_I roundish-triangular (Fig. 13). Pecten epipharyngis consisting of 3 equal scales. Premandible brown, distally with 2 teeth (Fig. 14). Antenna 112–128 μm long, with 6 segments. Length of antennal segments (in μm): 76–80, 13–20, 8–12, 8–10, 4–5, 1.5–1.7. AR 1.58–2.22. Apex of segment 2 with stylus 7.5–8.4 μm long and lauterborn organs which ending at apex of 3rd segment; antennal blade ending near mid or apex of 5th segment; one large and small ring organs in proximal 1/3 of basal segment and sometimes one ring organ in distal half of basal segment (Fig. 18). Mandible dark brown, with yellowish apical tooth, which shorter of combined width of inner teeth; seta interna with 6 plumose branches, seta subdentalis long, with beak-shaped apex (Fig. 16). Mentum with 2 yellow or yellowish median teeth and 6 pairs of dark brown lateral teeth; median teeth lower than the second laterals; first lateral teeth length equal or slightly lower of median teeth and slightly narrower of median teeth. Ventromental plates extend beyond last lateral tooth (Figs. 15, 17). Maxilla with pecten galearis.

Abdomen. Procercus 28–52 μm long, 28–36 μm wide, with 7 anal setae 430–496 μm long and 2 thin lateral setae. Supraanal setae 160–224 μm long. Anal tubules shorter than posterior parapods. Posterior parapods with simple hooks in apex.

Diagnostic characters and taxonomic notes. The new species is closely related to Far Eastern species *H. sikhotealinensis* and *H. maiorovi* Makarchenko et Makarchenko but adult males of these species are not readily separable from the *H. majus* by morphological features. Male of *H. maiorovi* has narrower tergite IX and more massive rounded oral projections of transverse sternapodeme than other two species, subapical part of gonostylus with microtrichia (Khamenkova *et al.* 2014, Fig.2), as well as the lack of pseudospurs on ta_1 and ta_2 of middle and hind legs. Males of *H. sikhotealinensis* and *H. majus* are closely related because of bare subapical part of gonostylus and rod-like projections of transverse sternapodeme, ta_1 and ta_2 of middle and hind legs with pseudospurs (Makarchenko & Makarchenko 2006; Makarchenko *et al.* 2009). These two species differ with each other only by shape the posterolateral edge of tergite IX, that is angular in the male of *H. sikhotealinensis* (Figs. 1–4) and rounded in *H. majus* (Figs. 5–7). Data for all other features are overlapped. In such cases, a great help in the identification of species can provide a comparative study of the morphology of the pupae and larvae, as well as DNA barcoding. Preimaginal stages for *H. maiorovi* are unknown but pupae and larvae of *H. sikhotealinensis* and *H. majus* are well separated by many features.

H. sikhotealinensis has a pupa with thoracic horn which tapers towards the top (Makarchenko & Makarchenko 2009, Fig. 48), length 310–320 μm . Abdominal segment VI with 3 pairs of simple lateral setae, segment VIII with 5 pairs of taeniate lateral setae (Makarchenko & Makarchenko 2009, Fig. 49). Mentum of larva with one light-brown or brown median tooth and six pairs of lateral teeth the same colour.

H. majus has a pupa with thoracic horn with extension in distal third (Figs. 9–10), length 368–400 μm . Abdominal segment VI with 1–2 pairs of short hair-like and 2 pairs long taeniate lateral setae, segment VIII with 4 pairs of taeniate lateral setae (Fig. 12). Mentum of larva with 2 yellow or yellowish median teeth and 6 pairs of dark brown lateral teeth (Figs. 15, 17).

Results of DNA barcoding of *H. majus* and *H. sikhotealinensis* are given below.

Results of DNA barcoding

Sequences of a 686 bp segment at the 5'-end of the cytochrome c oxidase I were determined for 7 individuals (6 adult males and 1 pupa) of *H. majus* and 2 individuals (adult males) of *H. sikhotealinensis*. The nucleotide composition of the studied sequences of *H. majus* COI gene fragments deviated from an equilibrium one, comprising 25.6 % of A, 40.2 % of T, 16.9 % of C, and 17.2 % of G. For *H. sikhotealinensis* the values were: 25.7 % of A, 41.5 % of T, 16.1 % of C, and 16.7 % of G. Total pairwise sequence divergence within *H. majus* ranged from 0.001 to 0.013, which is based on nineteen nucleotide substitutions. Two individuals of *H. sikhotealinensis* differ in three nucleotides substitutions (0.004). Most substitutions within species were observed only in the third position of codons.

Interspecific average distance between *H. majus* and *H. sikhotealinensis* was 8.9%. Between species one non-synonymous substitution at position 32 was observed, which in *H. majus* was Valine and in *H. sikhotealinensis* Isoleucine. Comparison of obtained species with other members of the genus *Hydrobaenus* showed the following results: *Hydrobaenus* sp. (JF287257- JF287260)—10.5–10.8%, *H. biwaquartus* (Sasa et Kawai) (AB704940)—9.2–10.8%. High differences between the groups can argue species independence of *H. majus* and *H. sikhotealinensis* (Ekrem *et al.* 2007). The neighbour joining tree of *H. majus* and *H. sikhotealinensis*, as well as individuals from the GenBank are shown in Figure 19.

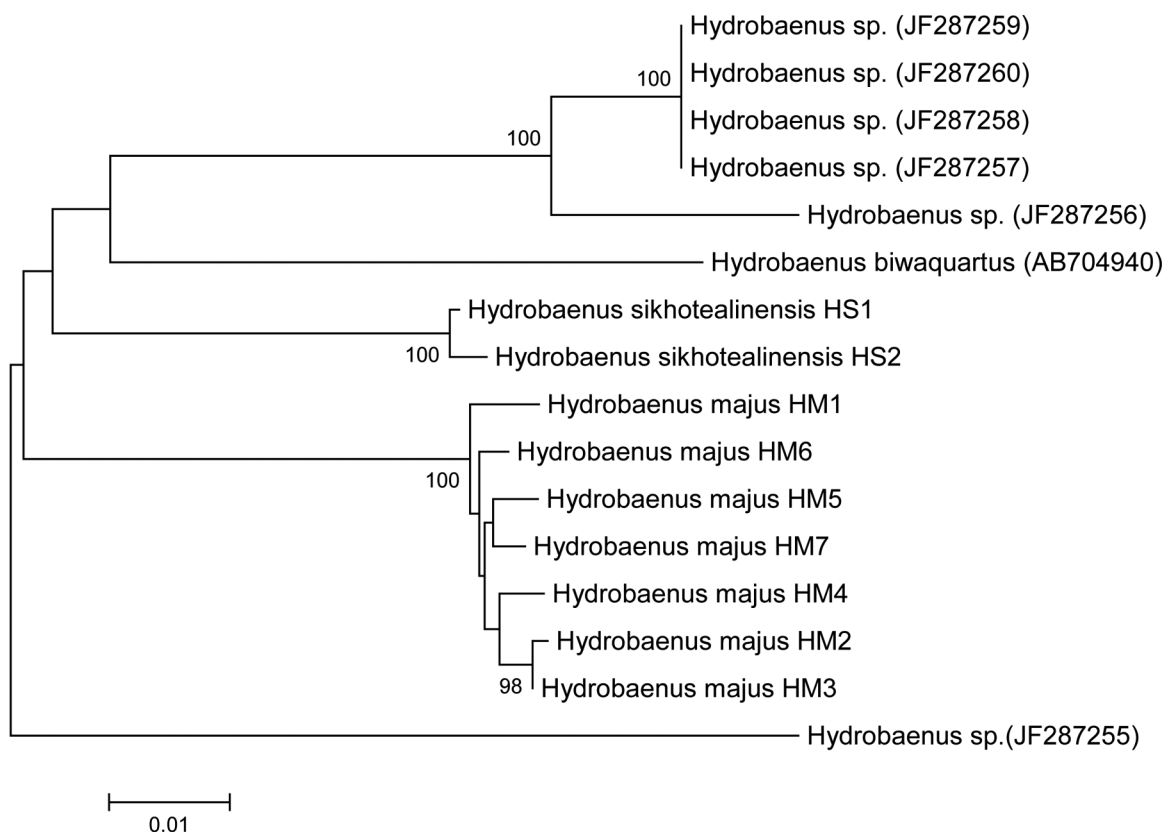


FIGURE 19. Taxon tree from Neighbour Joining analysis on Maximum Composite Likelihood model using 1000 bootstrap replicates. Bootstrap values >70 are given on branches.

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