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## Morphological redescription and DNA barcoding of *Linevitshia prima* Makarchenko, 1987 (Diptera: Chironomidae: Diamesinae) from Amur River basin (Russian Far East), with notes on systematics of the genus

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### Abstract

Additions and corrections to the diagnosis of the genus *Linevitshia* for male adult, pupa and larva are given, and systematic position of the genus is discussed. Illustrated redescription of adult male and first description of 4<sup>th</sup> instar larva of *L. prima* Makarchenko from Amur River basin are provided. Comparison of data based on a new material with those of *L. yezoensis* Endo showed that the latter name is a junior synonym of *L. prima*. The species-specificity of *L. prima* COI sequences is analyzed and the sequences are presented as diagnostic characters—molecular markers of *L. prima*.

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

### Introduction

The genus *Linevitshia* Makarchenko was established for *L. prima* Makarchenko, the species described from the southern part of Russian Far East (Makarchenko 1987). First, the genus was placed in the subfamily Podonominae, partly due to the incompletely developed wing vein R<sub>2+3</sub> in the freshly emerged specimens originally described. Later, however, K. Endo collected very similar midges in Hokkaido with distinct R<sub>2+3</sub>. The male genitalia of these Japanese specimens differ slightly from *L. prima*, thus were described as *L. yezoensis* Endo, 2007, and consequently, the genus *Linevitshia* was provisionally transferred from the Podonominae to the Diamesinae (Endo *et al.* 2007). It was shown also that the genus *Linevitshia* and *Protanypus* Kieffer are closely related based on adult morphology, namely by complex of setae on pronotum (median and lateral Aps present) and mesonotum—Ac and Dc stripes connected in base, setae present on PAII and EII, and the presence of setae on alula of wing in adult males. This opinion was supported after examination of pupal morphology of *L. yezoensis* later (Makarchenko & Endo 2009).

In April 2014 we have got new materials consisted of adult males, pupae and larvae of *L. prima* from Amur River basin, including some males and larvae treated in DNA barcoding analysis. Characters of the *Linevitshia* larva, here described for the first time in the genus, confirm close relations to those of *Protanypus*, as was shown earlier for pupae and adults (Makarchenko & Endo 2009). Comparison of data based on a new material with those of *L. yezoensis* showed that the latter must be synonymized with *L. prima*. Additions and corrections to diagnoses for male adults, pupa and larva of the genus *Linevitshia* are also given, and systematics of the genus is discussed. The species-specificity of *L. prima* COI sequences is shown. These sequences could be used in future as diagnostic characters—molecular markers of *L. prima*.

## Material and methods

The larva, pupa and adult specimens of *L. prima* were associated by rearing larvae individually to the mature pupae and from the pupae to adults. 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). The material is deposited in the Institute of Biology and Soil Sciences, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia (IBSS FEB RAS).

For the sequence reaction one adult male and three larvae of *L. prima* were used. Reference DNA sequence database, based on partial sequenced from mitochondrial COI gene were obtained by preparing a 30 µl total DNA extract from body of insect using the Invitrogen (Invitrogen corp, Carlsbad, CA, 2007) protocol. The primers for amplification of the 686 bp fragment were LCO1490 (5'-GGTCAACAAATCATAAAGAT ATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3'), obtained from 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 5.1 (Tamura *et al.* 2011). All sequences obtained in this study have been submitted to Gen-Bank (KJ857066–KJ857069).

## Genus *Linevitshia* Makarchenko

*Linevitshia* Makarchenko, 1987: 205.

*Linevitshia* Makarchenko: Brundin 1989: 27, Fig. 4.3; Sæther *et al.* 2000: 134, Fig. 144; Ashe & O'Conner 2009: 292.

*Type species: Linevitshia prima* Makarchenko, 1987, by original designation and monotypy.

**Generic diagnosis** (emended). *Adult male*. Small to middle sized, up to 4.5 mm. Antenna with 13 flagellomeres. AR about 1. Eyes reniform, slightly pubescent. Clypeus without setae. Anteprenotum with U-shaped notch in frontal view. Anteprenotals in separate median and lateral clusters, the acrostichal and dorsocentral stripes connected posteriorly. Posterior anepisternum II and epimeron II with setae. Wing with setae on squama, alula and veins R, R<sub>1</sub>, R<sub>4+5</sub>; membrane without macrotrichia; R<sub>2+3</sub> indistinct; costa extends beyond R<sub>4+5</sub>. Tergite IX without anal point. Gonocoxite simple, inferior and superior volsellae reduced. Sternapodeme broadly arched; phallapodeme long; aedeagal lobe large, forked distally. Gonostylus in distal part with long and strong setae and 1–4 apical megasetae (Figs. 1–7, 24, 25).

*Pupa*. Middle sized, up to 4.7 mm. Exuviae yellow or brownish-yellow. Frontal short hair-like setae present on frontal apotome. Frontal apotome slightly granulated or smooth, without tubercles or warts (Fig. 8). Anteprenotum with 3 median and 2 lateral anteprenotals. Thoracic horn absent. Four precorneals present. Tergites and sternites I–VIII with slender shagreen better visible in lateral parts. Tergites III–VII and sternites III–VII with posterior transverse row of large and more dark thorn-like spines; size of these spines on tergites and sternites subequal (Figs. 9, 26); tergite VIII also with posterior row but small spines. Posterior margin of sternite VIII with long and triangular, apically rounded caudal lobe (male) (Fig. 10) or with 2 short and rounded caudal lobes (female) (Fig. 11). Abdominal setation: tergites I–VII with 5 D setae, some setae moved to lateral part; VIII with 2 D setae. Segments I with 2 L setae, II–VIII with 4 L setae. Anal lobe with 3 strong needle-shaped anal macrosetae and 2 very short and hair-like lateral setae (not well visible in all specimens); median setae and apical tubercle absent (Figs. 10–11). Male genital sac not extended beyond the anal lobe.

*Larva, 4<sup>th</sup> instar*. Size up to 7 mm. Head capsule with numerous pale, long, simple or divided into 2–3 branches setae; postoccipital margin without or with weak ventrolateral, posteriorly-directed projections, absent in some case (Figs. 13–14). Antenna with 4 segments; segment 3 non-annulate; style longer than segment 3 (Fig. 23). Labral setae S<sub>1</sub> long and divided into some branches; lamellae consisting of a semicircular row of broad, overlapping scales some of which apically serrate (Fig. 18). Pecten epipharyngis consists of 3 coarsely long and pointed scales. Premandible narrow, with 6 inner teeth; lateral spine absent (Fig. 17). Mandible with long apical

tooth and 4 subequal teeth; seta subdentalis reduced; seta interna with some simple branches (Fig. 19). Mentum with 3 pairs of lateral teeth and wide dome-shaped median tooth; ventromental plate small (Figs. 20–21, 27–28). Maxillary palp longer than wide. M appendage of the premento-hypopharyngeal complex deeply divided into lamellae (Figs. 22, 27). Abdominal setae short and thin, pale. Procercus longer than wide, bearing 5–6 anal setae. Anterior and posterior parapods separate, each bearing an apical group of irregularly placed claws.

**Comments.** After having analyzed all the data on adults, pupae and larvae the genus *Linevitshia* can now be assigned in the tribe Protanypodini, so far represented by the only genus *Protanypus* Kieffer. Main differences between these genera are given in the key below.

## Key to genera of the tribe Protanypodini Brundin

### Adult males

1. Thorax with setae on median and posterior anepisternum II, epimeron II and preepisternum. Gonocoxite strongly produced distal to gonostylus . . . . . *Protanypus* Kieffer
- Thorax with setae only on posterior anepisternum II and epimeron II. Gonocoxite not produced distal to gonostylus . . . . . *Linevitshia* Makarchenko

### Pupae

1. Thoracic horn present. Frontal setae present on prominent cephalic tubercles on frontal apotome. Tergites and sternites without posterior transverse row of thorn-like spines. Anal lobe with 7–13 anal macrosetae, some of them lamelliform . . . . . *Protanypus* Kieffer
- Thoracic horn absent. Frontal setae present on frontal apotome. Tergites and sternites III–VIII with posterior transverse row of dark thorn-like spines. Anal lobe with 3 strong needle-shaped anal macrosetae . . . . . *Linevitshia* Makarchenko

### Larvae

1. Head capsule with numerous closely-spaced short simple setae; postoccipital margin with long ventrolateral, posteriorly-directed projections on each side (Fig. 12). Labral setae  $S_1$  short and simple. Mandible with long apical tooth and 5 small inner teeth; seta interna absent. Anterior margin of middle 2/3 of mentum without teeth; 2 pairs of extreme lateral teeth present . . . . . *Protanypus* Kieffer
- Head capsule with numerous pale and long simple or divided into 2–3 branches setae; postoccipital margin without or with weak ventrolateral, posteriorly-directed projections (Figs. 13–14, 16, 28). Labral setae  $S_1$  long and divided into some branches (Fig. 18). Mandible with long apical tooth and 4 subequal inner teeth; seta interna with some simple branches (Fig. 19). Mentum with 3 pairs of lateral teeth and wide dome-shaped median tooth (Figs. 20–21) . . . . . *Linevitshia* Makarchenko

## Descriptions and taxonomic notes

### *Linevitshia prima* Makarchenko

(Figs. 1–28)

*Linevitshia prima* Makarchenko, 1987: 207, Fig. 1; Brundin 1989: 27, Fig. 4.3; Sæther *et al.* 2000: 134, Fig. 144; Ashe & Connor 2009: 292.

*Linevitshia yezoensis* Endo in Endo, Makarchenko & Willassen, 2007: 93, Figs 1–4, 7–13; Ashe & Connor 2009: 292. **Syn. nov.**

**New material examined.** Far East of Russia. Jewish Autonomous Region, Obluchie District: 2 males, 1 pupa, 2 larvae, Fedotkin Spring of Bidzhan River basin (Amur River basin), N 48°38'409", E 131°37'217", 5.IV. 2014, leg. E. Makarchenko; 8 males, 6 pupal exuviae, 12 larvae of fourth instar, the same data except Lopatinskyi Spring, N 48°37'810", E 131°39'114", 7.IV. 2014, leg. E. Makarchenko. Kunashir Island (Kurile Islands), 3 larvae, unnamed stream of Kipiashee Lake, N 43°51'521", E 145°29'530", 28.VII. 2013, leg. D. Palatov.

**Description.** A detailed description of the adult male based on specimens from Japan is given by Endo *et al.* (2007) but due to slight differences found, we present a complemented redescription on the basis of the material from the Amur River basin.

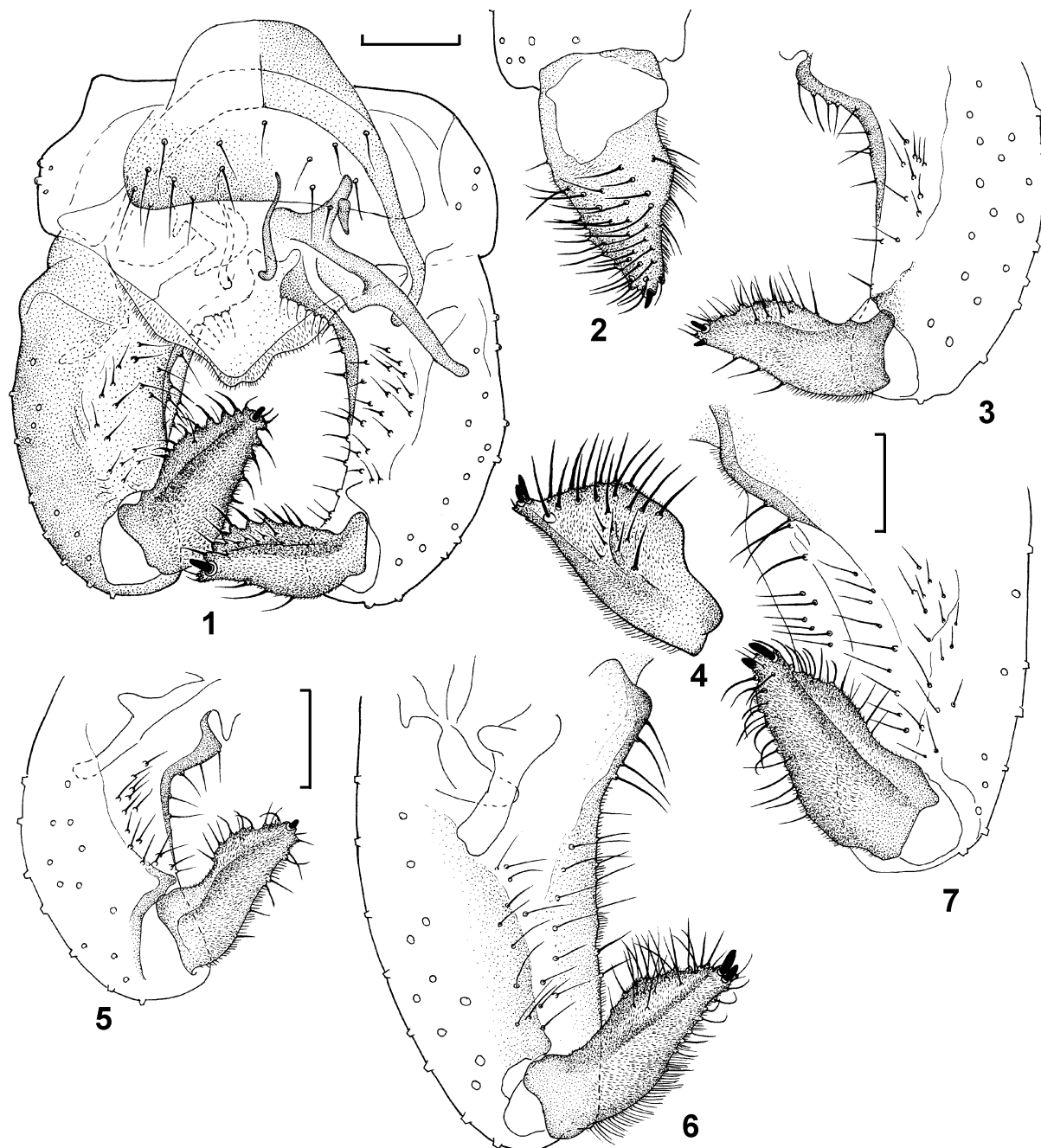
**Adult male** (n = 4, except when otherwise stated).

Total length: 3.8–4.3 mm. Wing length 3.2–3.3 mm. Total length/wing length 1.4–1.5. Coloration: body largely brown to dark brown; head and thorax grayish.

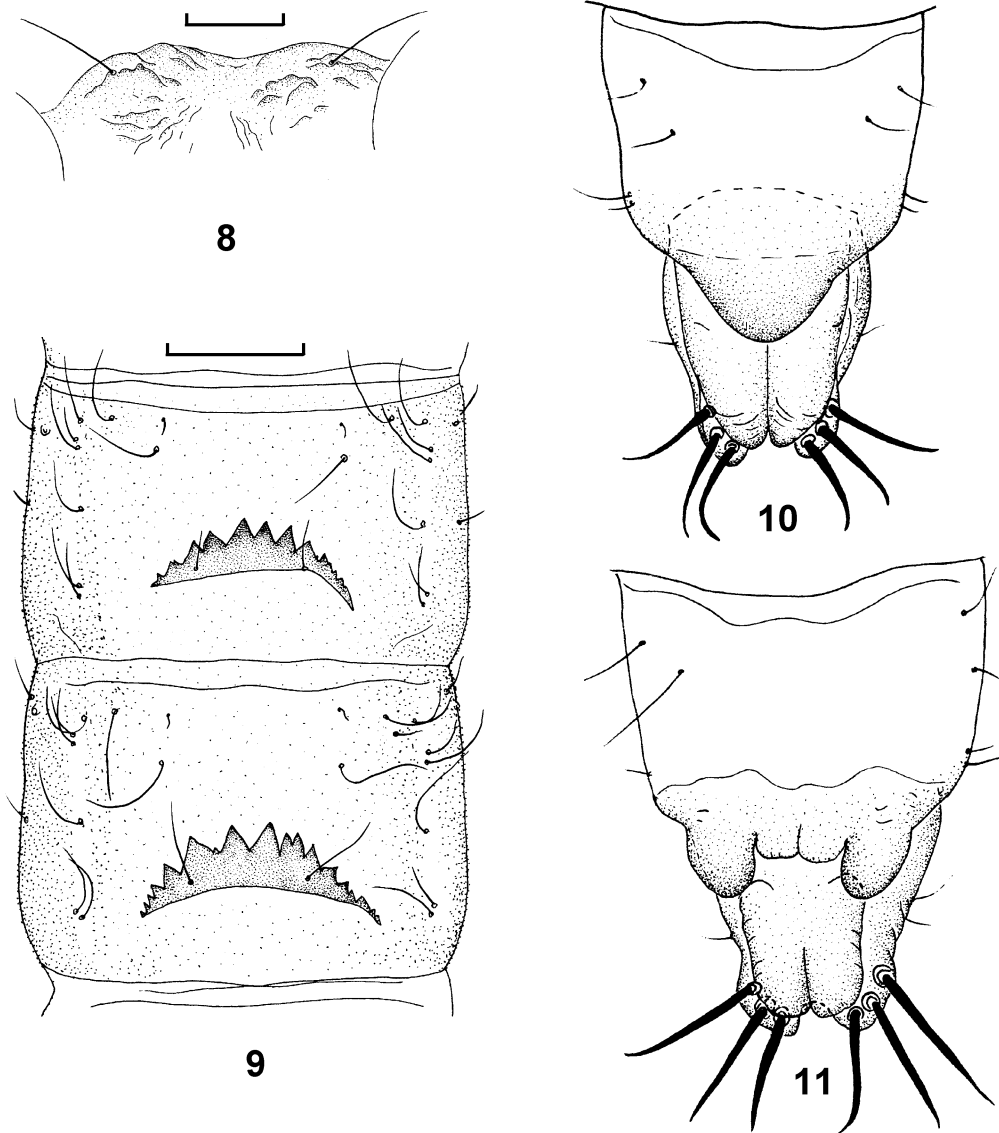
Head. Antenna with 13 flagellomeres and well-developed plume; ultimate flagellomere with 1–2 subapical setae 34–36  $\mu\text{m}$  long. AR 1.10–1.25. Temporal setae: 0–1 weak and short inner verticals and 3–5 stronger postorbitals. Length of palpomeres 1–5 ( $\mu\text{m}$ ): 40–48, 79–84, 146–152, 168–180, 240–272.

Thorax. Anteprepronotum with 2–3 dorsal and 8–14 lateral setae. Acrostichals 17–19, dorsocentrals 17–18, prealars 8–10, supralars 3, and scutellars 14–17. Posterior anepisternum II with 4–6 setae. Epimeron II with 8 setae.

Wing. Width 0.56–0.80 mm. Costa produced beyond  $R_{4+5}$  on 70–72  $\mu\text{m}$ . Anal lobe developed, round. Brachiolum with 3–5 setae. R with 24–29 setae,  $R_1$  with 9–15 setae,  $R_{4+5}$  with 2–5 setae subapically. Alula with 4–6 setae. Squama with 25–31 setae.



**FIGURES 1–7.** *Linevitshia prima* Makarchenko, male. **1**, hypopygium in dorsal view (Hokkaido, Japan); **2**, **4**, gonostylus in various aspects (holotype, Frolovka River, Primorye Territory, Russia); **3**, **5–7**, gonocoxite and gonostylus from Hokkaido, Japan (**3**, **5**) and from Amur River basin, Russia (**6–7**). Scale bars: 50  $\mu\text{m}$ .



**FIGURES 8–11.** *Linevitshia prima* Makarchenko, pupa. **8**, frontal setae on frontal apotome; **9**, tergites III–IV; **10**, sternite VIII and anal segment in dorsal view, male; **11**, sternite VIII and anal segment in dorsal view, female. Scale bars: Fig. 8: 50 µm; Figs. 9–11: 200 µm.

Legs. Spurs of fore tibia 88–96 µm, of middle tibia 72–96 and 72–80 µm, of hind tibia 90–100 and 64–84 µm long. Hind tibial comb composed of 12–14 setae. Fore leg with 1 apical pseudospur on  $ta_1$ ; mid and hind legs with 2 apical pseudospurs on  $ta_1$  and  $ta_2$ . Sensilla chaetica absent.  $ta_4$  cylindrical,  $ta_5$  slightly curved. Pulvilli small. Tip of claws serrate, with about 5 teeth. Lengths and proportions of legs as in Table 1.

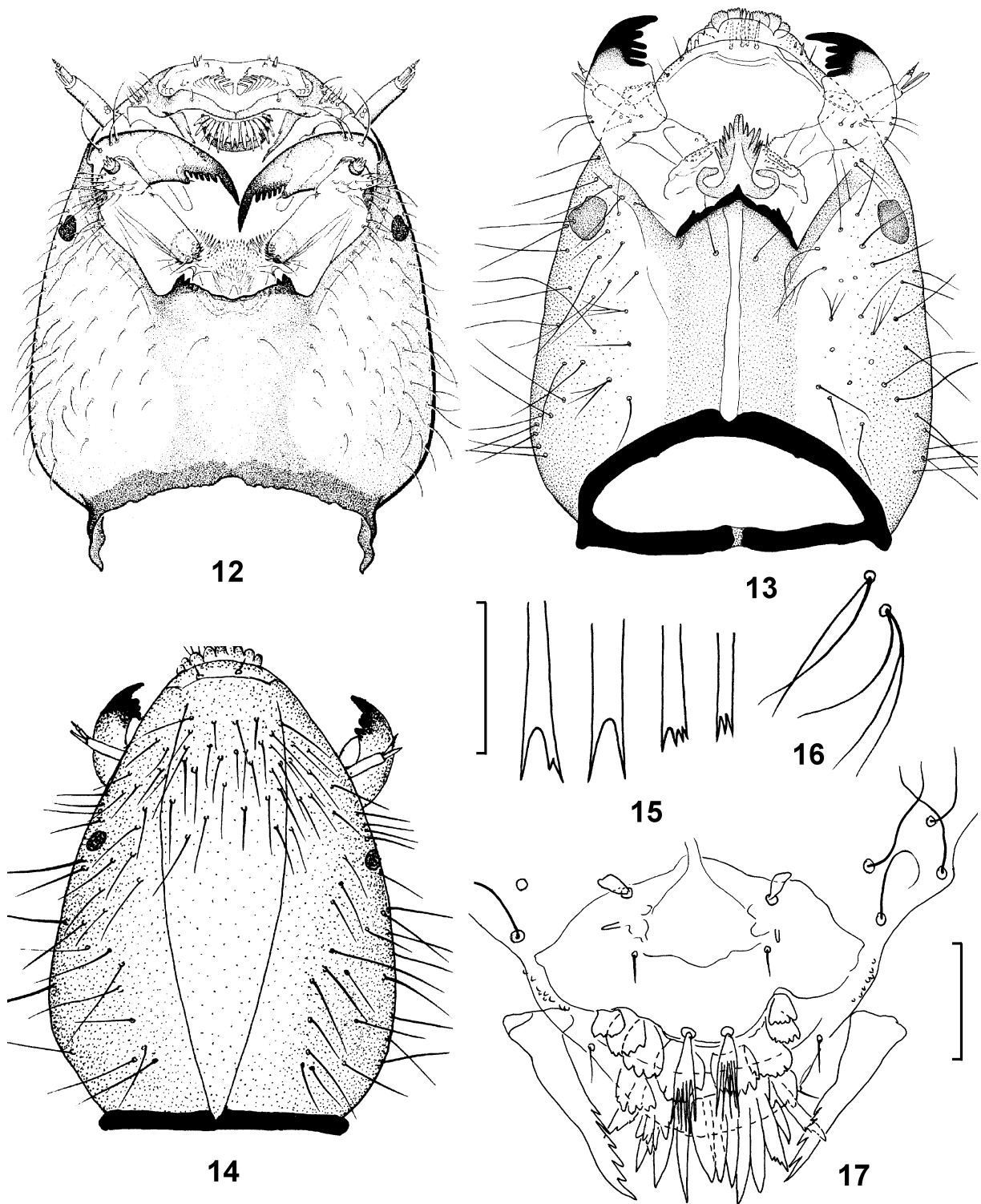
Hypopygium (Figs. 1–7, 24–25). Tergite IX with 11–18 setae. Anal point absent. Laterosternite IX with 8–13 setae. Gonocoxite 240–256 µm long. Sternapodeme broadly arched, 180–216 µm long and 36 µm wide. Phallapodeme 100–132 µm long; aedeagal lobe large, forked distally. Gonostylus 112–124 µm long; in distal part with long, strong setae and 1–2 apical megasetae 8–16 µm long. HR 1.94–2.29.

**Pupa.** Specimens from the Amur River basin do not differ from those described from Japan (Endo *et al.* 2007).

**Fourth instar larva** (n=6).

Total length 6.4–7.1 mm.

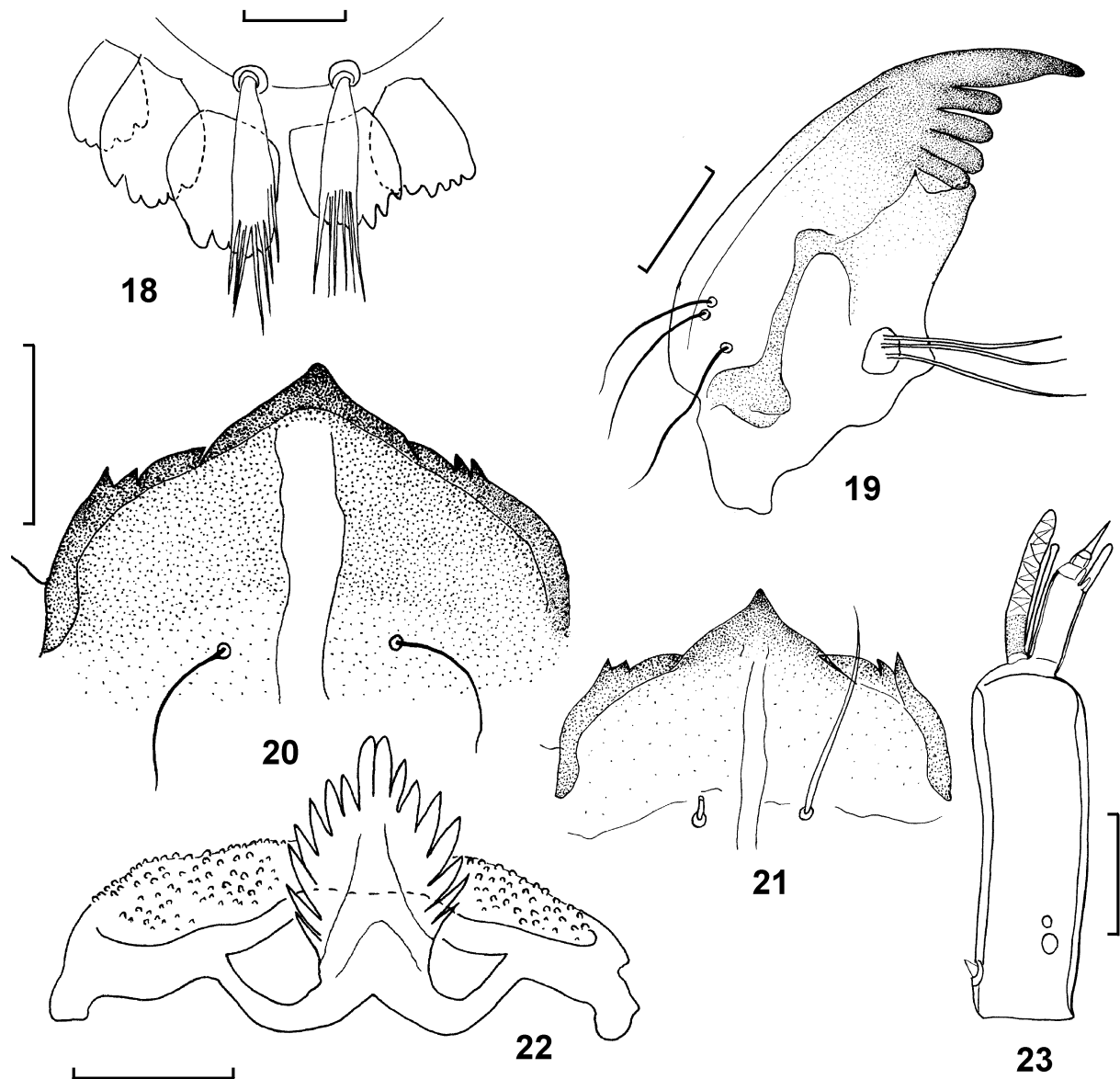
Coloration brownish; head capsule light yellow with postoccipital margin black and mandible dark brown or black in apical part; procercus light-yellow.



**FIGURES 12–17.** Larva of *Protanypus* sp. (12) and *Linevitshia prima* Makarchenko (13–17). 12, 13—head, in ventral view; 14, head, in dorsal view; 15, apical part of head setae; 16, branched head setae; 17, labrum. Scale bars: Figs. 15: 20  $\mu$ m; Figs. 16–17: 50  $\mu$ m. Fig. 12 after Oliver & Roussel (1983).

Head capsule 560–576  $\mu$ m long and 360–440  $\mu$ m wide; postoccipital margin narrow. Some setae of head with divided apex (Fig. 15). Labral setae  $S_1$  long, divided into 6–8 unequal sized branches. Lamellae consisted of a semi-circular row of broad, overlapping scales apically serrate (Fig. 18). Premandible narrow, with 6 inner teeth (Fig. 17). Length of antennal segments ( $\mu$ m): 62–64, 17, 3–4, 8. Longest branch of antennal blade 267  $\mu$ m long, shorter branch 217  $\mu$ m long. AR 2.17–2.38. Mandible with apical tooth in 1.5–1.6 times longer of combined width of inner

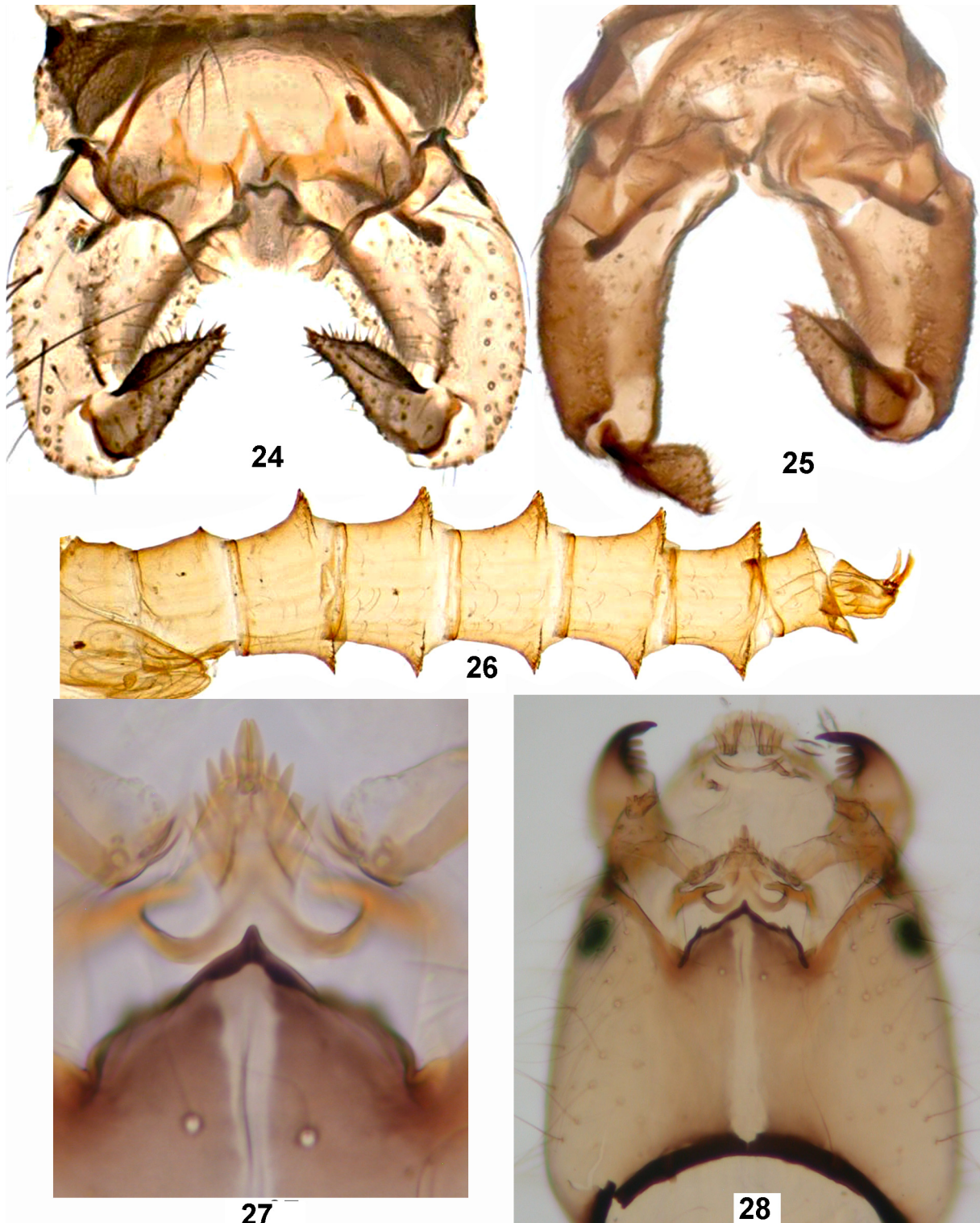
teeth; seta interna with 3 simple branches (Fig. 19). Mentum with 3 pairs of lateral teeth and wide dome-shaped median tooth; ventromental plate small (Figs. 20–21). Maxillary palp 2–2.5 times longer than wide, with 5 short setae in apical part and 4–5 short setae laterally, basally with ring organ. M appendage of premento-hypopharyngeal complex with 2 median lamellae and 6–8 pairs of lateral lamellae (Figs. 22, 27). Abdominal setae short and thin, pale. Procercus 2–2.5 times longer than wide, bearing 5–6 apical setae.



**FIGURES 18–23.** *Linevitshia prima* Makarchenko, larva. **18**, labral lamellae and  $S_1$ ; **19**, mandible; **20**, mentum of 4<sup>th</sup> instar larva; **21**, mentum of 3<sup>rd</sup> instar larva; **22**, M appendage of premento-hypopharyngeal complex; **23**, antenna. Scale bars: Figs. 18, 23: 20  $\mu\text{m}$ ; Figs. 19–22: 50  $\mu\text{m}$ .

**Taxonomic notes.** In the remarks to description of *Linevitshia yezoensis* we wrote that “males of *L. yezoensis* differ from *L. prima* in the shape of gonostylus and the sternapodeme. *L. yezoensis* has one apical megaseta whereas *L. prima* has three to four” (Endo *et al.* 2007). Additional study of *L. prima* holotype, Japanese material of *L. yezoensis* and the new material of *L. prima* from Amur River basin showed that shape of transverse sternapodeme of males from all populations are very similar or the same. In the holotype (freshly emerged male specimen), apical part of sternapodeme was not good visible. Shape of the hypopygial gonostylus from all populations are also very similar or the same and depends on its position on slides. Some males of *L. yezoensis* from Hokkaido have 2 megasetae on gonostylus (Fig. 3), whereas specimens of *L. prima* from Amur River basin

have 1–2 megasetae (Figs. 6–7). All other features of adult males of known populations of *Linevitshia* completely overlap, justifying the presently proposed synonymization of *Linevitshia yezoensis* Endo and *Linevitshia prima* Makarchenko.



**FIGURES 24–28.** *Linevitshia prima* Makarchenko, adult male (24–25), pupa (26) and larva (27–28). 24–25, hypopygium in dorsal view from Amur River basin (24) and Hokkaido (25); 26, abdomen of pupa in lateral view; 27, mentum and of premento-hypopharyngeal complex; 28, head in ventral view.

**TABLE 1.** Lengths (in  $\mu\text{m}$ ) and proportions of leg segments of *Linevitshia prima* Makarchenko, male (n = 4).

	fe	ti	ta <sub>1</sub>	ta <sub>2</sub>	ta <sub>3</sub>
P <sub>1</sub>	1168–1336	1376–1520	960–1069	451–518	301–351
P <sub>2</sub>	1152–1269	1269–1403	560–618	304–359	217–251
P <sub>3</sub>	1376–1503	1664–1787	832–935	464–534	272–301

continued.

	ta <sub>4</sub>	ta <sub>5</sub>	LR	BV	SV
P <sub>1</sub>	200–288	150–160	0.68–0.70	2.74–3.28	2.65–2.76
P <sub>2</sub>	142–150	134–150	0.44–0.46	3.67–4.12	4.20–4.34
P <sub>3</sub>	167–184	150–167	0.50–0.52	3.56–3.61	3.52–3.65

### Results of DNA barcoding

The final alignment of the COI gene yielded 686 bp for 4 individuals of *L. prima* that were 4 haplotypes. The nucleotide composition of the studied sequences of *L. prima* COI gene fragments deviated from an equilibrium one, comprising 25.8 % of A, 36.6 % of T, 20.0 % of C, and 17.6 % of G. Total pairwise sequence divergence within *L. prima* ranged from 0.0015 to 0.0029, which is based on three nucleotide substitutions. All the substitutions were synonymous transitions (A-G) and observed only in the third codon positions. The HKY (Hasegawa, Kishino, & Yano 1985) model was the best-fit in describing the pattern of nucleotide variation in the *L. prima* COI sequences.

Average intergenus P-distance between *L. prima* and other genera of the subfamily Diamesinae showed the following results: *Potthastia* Kieffer (JF287767)—15.9%, *Pseudodiamesa* Goetghebuer (JF764760, JF764764, JF764771)—16.7%, *Pagastia* Oliver (JF287653)—17.2%, *Diamesa* Meigen (AB704934)—17.4%, *Sympotthastia* Pagast (JF288065)—18.2%. High differences between the groups can argue genus independence of *Linevitshia* (Ekrem *et al.* 2007).

**Distribution and biology.** East Palaearctic species, known from Japan, China and the Russian Far East (Kunashir Island, Primorye Territory, Amur River basin) (Wang 2000, Endo *et al.* 2007, Ashe & Connor 2009). Pupae and larvae from Amur River basin were collected in springs with water temperature 5–7°C, from gravel and stones covered with moss *Brachythecium rivulare* Bruch *et al.* Larvae in Kunashir Island were collected from small stones covered with moss in spring with water temperature 2°C.

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