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Morphological description of a new species of *Capnia* (Plecoptera: Capniidae) with DNA barcoding of genus members from the Russian Far East

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Abstract

Capnia yavorskayae, a new species of the stonefly family Capniidae, is described from the Low Amur River Basin, Khabarovskiy Kray of the Russian Far East, on the basis of female morphological features. Confirmation of the uniqueness of the new species was also molecularly compared to other *Capnia*, including a few Far Eastern species, *C. aligera* Zapekina-Dulkeit, *C. bargusinica* Zapekina-Dulkeit, *C. khingana* Teslenko, *C. kurnakovi* Zhiltzova, *C. nearctica* Banks, *C. nigra* (Pictet), and *C. rara* Zapekina-Dulkeit for which DNA barcodes were obtained. We support the distinctiveness of the new species with mitochondrial DNA sequences, comparing it to *Capnia* from the eastern Palaearctic and Nearctic realms and one *Zwicknia* species. The new species forms a common clade with *C. khingana*, *C. kurnakovi* from the Russian Far East, and an undetermined *Capnia* species from Honshu, Japan. Each species from the Russian Far East has high interspecific distances from other *Capnia* species except *C. nearctica* which was close to *C. atra* Morton.

Key words: *Capnia yavorskayae*, **sp. nov.**, female, DNA barcoding, Russian Far East

Introduction

Capnia Pictet, 1841 represents the most species-rich genus in the stonefly family Capniidae and currently includes about 130 species in the world (DeWalt *et al.* 2022). Usually, identification is based solely on males, but in some females the structure of sternum eight and the shape of the subgenital plate may separate species. At least six valid *Capnia* species are described from females in Asia: *C. ansobiensis* Zhiltzova, 1974, *C. jankowskaja* Zhiltzova, 1974, *C. shugnanica* Zhiltzova, 1974, *C. tshukotica* Zhiltzova et Levanidovae, 1978, *C. montivaga* Kimmins, 1946, *C. femina* Kawai, 1968 (Zhiltzova 1974, Zhiltzova et Levanidovae 1978, Kimmins 1946, Kawai, 1968). All species were collected in hard-to-reach mountain lakes or streams, are known by the holotype or type series only, and are rare (Zhiltzova 2003, Murányi *et al.* 2015). The latter two species were recorded from Tibet and Hymalaya (Nepal). *Capnia ansobiensis*, *C. jankowskaja*, *C. shugnanica* are endemic to Central Asia (West Pamir, Alay-Pamir mountain system, and Tien-Shan). *Capnia tshukotica* is found in the Chukotka and the Koryak Highlands in the Asian North-East (Zhiltzova 2003, Levanidova 1982). In the present paper, we morphologically describe *Capnia yavorskayae* **sp. nov.** from the distinctive female collected from tributaries of the Gorin River (the Low Amur River Basin), Khabarovskiy Kray, in the Russian Far East. To confirm the uniqueness of a new species, DNA barcode sequences of *Capnia yavorskayae* **sp. nov.** as well as *C. aligera* Zapekina-Dulkeit, 1975, *C. bargusinica* Zapekina-Dulkeit, 1975, *C. khingana* Teslenko, 2019, *C. kurnakovi* Zhiltzova, 1978, *C. nearctica* Banks, 1918, *C. nigra* (Pictet, 1833), *C. rara* Zapekina-Dulkeit, 1970 (Zapekina-Dulkeit 1975a, 1975b, 1970, Teslenko 2019, Zhiltzova 1978, Banks 1918, Pictet 1833) are compared .

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Material and methods

Specimens were collected by a modified drift net with hole diameter 0.28 m and net length 0.8 m (Fig. 8), also by hand with entomological forceps from the surface film of water and foam that formed in calm areas of stream folds. Samples were fixed with 75% ethanol. Abdomens were removed and soaked in 10% NaOH overnight and rinsed with distilled water. Specimens were examined with the aid of a compound microscope in transmitted light. Illustrations were produced using digital cameras (Nikon Coolpix 995 and ToupView 3.7), stereomicroscope Olympus SZX1 6, the digital camera Olympus DP74, and stacked using Helicon Focus software. The final illustrations were post processed for contrast and brightness using Adobe® Photoshop® software. Photographs were taken in the Federal Scientific Center of the East Asia Terrestrial Biodiversity, FSC EATB FEB RAS.

The holotype and paratypes are deposited in the collection of the Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia. Terminology follows Nelson & Baumann (1989) and Murányi *et al.* (2014).

TABLE 1. List of taxa, isolate numbers, sex, locations and GenBank accessions.

Species	Isolate	Sex	Country	Coordinates	Accession number
Capnia khingana	TVA62	Male	Russia: Amurskaya Oblast, Amur River Basin, Eracta River	49.09285 N 130.591083 E	OL343052
C. khingana	TVA95	Male	Russia: Amurskaya Oblast, Amur River Basin, Eracta River	49.09285 N 130.591083 E	OL343053
C. khingana	TVA160	Female	Russia: Khabarovskiy Kray, Amur River Basin, Khankuka stream	50.757554 N 137.413548 E	OL343054
C. yavorskayae **sp. nov.**	TVA163	Female	Russia: Khabarovskiy Kray, Amur River Basin, Khankuka Stream	50.756951 N 137.412730 E	OL343055
C. yavorskayae **sp. nov.**	TVA184	Female	Russia: Khabarovskiy Kray, Amur River Basin, Khankuka Stream	50.768183 N 137.421572 E	OL343062
C. nigra	TVA166	Male	Russia: Khabarovskiy Kray, Amur River Basin, Gorin River	50.890137 N 137.457746 E	OL343056
C. nearctica	TVA167	Male	Russia: Magadan Oblast, Ola River	59.576183 N 151.270017 E	OL343057
C. bargusinica	TVA168	Female	Russia: Magadan Oblast, Ola River Basin, Donyshko River	60.385667 N 151.475 E	OL343058
C. kurnakovi	TVA170	Female	Russia: Magadan Oblast, Ola River	59.576183 N 151.270017 E	OL343059
C. rara	TVA171	Female	Russia: Magadan Oblast, Ola River	59.576183 N 151.270017 E	OL343060
C. rara	TVA172	Male	Russia: Magadan Oblast, Ola River	59.576183 N 151.270017 E	OL343061
C. aligera	TVA186	Male	Russia: Khabarovskiy Kray, Amur River Basin, Gorin River	50.929194 N 137.754936 E	OL343063

Total genomic DNA was extracted from the thorax or legs of adult females using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) and the resultant DNA was eluted in 100 µl. The barcode fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene was amplified using the polymerase chain reaction (PCR) with the primers LCO1490 (5'–GGTCAACAAATCATAAAGATATTGG–3') and HCO2198 (5'–TAAACTTCAGGGTGACCAAAAAATCA–3') (Folmer *et al*. 1994). The PCR reactions comprised a heating step at 95°C for 30 s, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s and elongation at 72°C for 1 min, with a final extension phase of 72°C for 5 min. PCR was performed in a reaction volume of 10 µl using 5 µl Go Taq Green Master Mix (Promega corp, Madison, WI, USA), 0.5 µM of each primer, 3 µl nuclease-free water, and 1.5 µl of genomic DNA. The PCR products were confirmed by electrophoresis in 1.5% agarose gels and then sequenced bidirectionally. 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 PCR products were bidirectional sequenced on an ABI 3130x sequencer (Applied Biosystems) and were aligned in MEGA7 (Kumar *et al.* 2016).

The inter- and intraspecific genetic distances were calculated using p-distances in MEGA7. Automatic Barcode Gap Discovery (ABGD) analysis (Puillandre *et al*. 2012) is used for species delimitation and to establish taxonomic status of sequenced specimens, using relative gap width (X = 1.0) and intraspecific divergence (P) values between 0.001 and 0.100 with the p-distance model. PartitionFinder 2.1.1 (Lanfear *et al.* 2012) is used to select the best-fit partitioning scheme and models separately for each codon position of COI gene using the greedy algorithm with linked branch lengths for the corrected Bayesian Information Criterion as the optimality criterion for model selection. The best models for the first, second and third codon position of COI were SYM+G (Zharkikh 1994), F81+I (Felsenstein 1981) and GTR+G (Tavaré 1986) respectively. A Bayesian Inferences (BI) analysis was performed with MrBayes v.3.2.7 (Ronquist *et al.*, 2012) under the following conditions: 10 million generations with sampling every 500 generations, four chains and a burn-in of 25% trees. Trace files were visually inspected in Tracer v. 1.7 (Rambaut *et al.* 2018). Maximum likelihood (ML) analysis was conducted with RAxML v.8.2.7 with 1000 bootstrap replicates.

For the phylogenetic analysis, we used our sequences, as well as sequences from GenBank and BOLD systems. We included one sample from each species of genus *Capnia* from GenBank and the BOLD system. For species that have formed more than one molecular taxonomic unit we use one sample from each BOLD BIN number. Also, we used *Zwicknia rupprechti* Murányi, Orci & Gamboa, 2014 as out-group. All sequences have been deposited in GenBank.

Results and discussion

Capnia yavorskayae Teslenko sp. nov.

(Figs. 1–7)

Material examined. Holotype: female, Russia, Far East, Khabarovskiy Kray, Komsomolskiy State Nature Reserve, Khankuka Stream Basin, Gorin River Basin, Amur River Basin, 50º76.818 N, 137º42.157 E, 14.05.2020, coll. N. Yavorskaya. Paratypes: 2 females the same place and data as holotype; 21 females, Khankuka Stream, low part, Gorin River Basin, Amur River Basin, 14.05.2020, coll. N. Yavorskaya; 1 female, quarry on the road 50º75.695 N, 137º41.273 E, Amur R. Basin, 14.05.2020; 3 females (2 in slides) unnamed stream near Kamennaya Pad cordon, Amur River Basin, 50°41’ N, 137°14’ E, 14.05.2020, coll. N. Yavorskaya; 2 females, Siutaru Stream, Gorin River Basin, Amur River Basin, 19.05.2020, coll. N. Yavorskaya.

Diagnosis. Female subgenital plate short, transverse, truncated posteriorly, occupies anterior half of the sternum 8; unevenly pigmented, anterolateral edges pale, posterior margin straight or slightly rounded medially, and strongly sclerotized; inner sclerite of vaginal complex small, tooth-shaped, and black. Two or three thin transverse shallow membranous folds and a pair of lateral sclerites located below posterolateral margin the subgenital plate. Genital opening of the vaginal complex is broad.

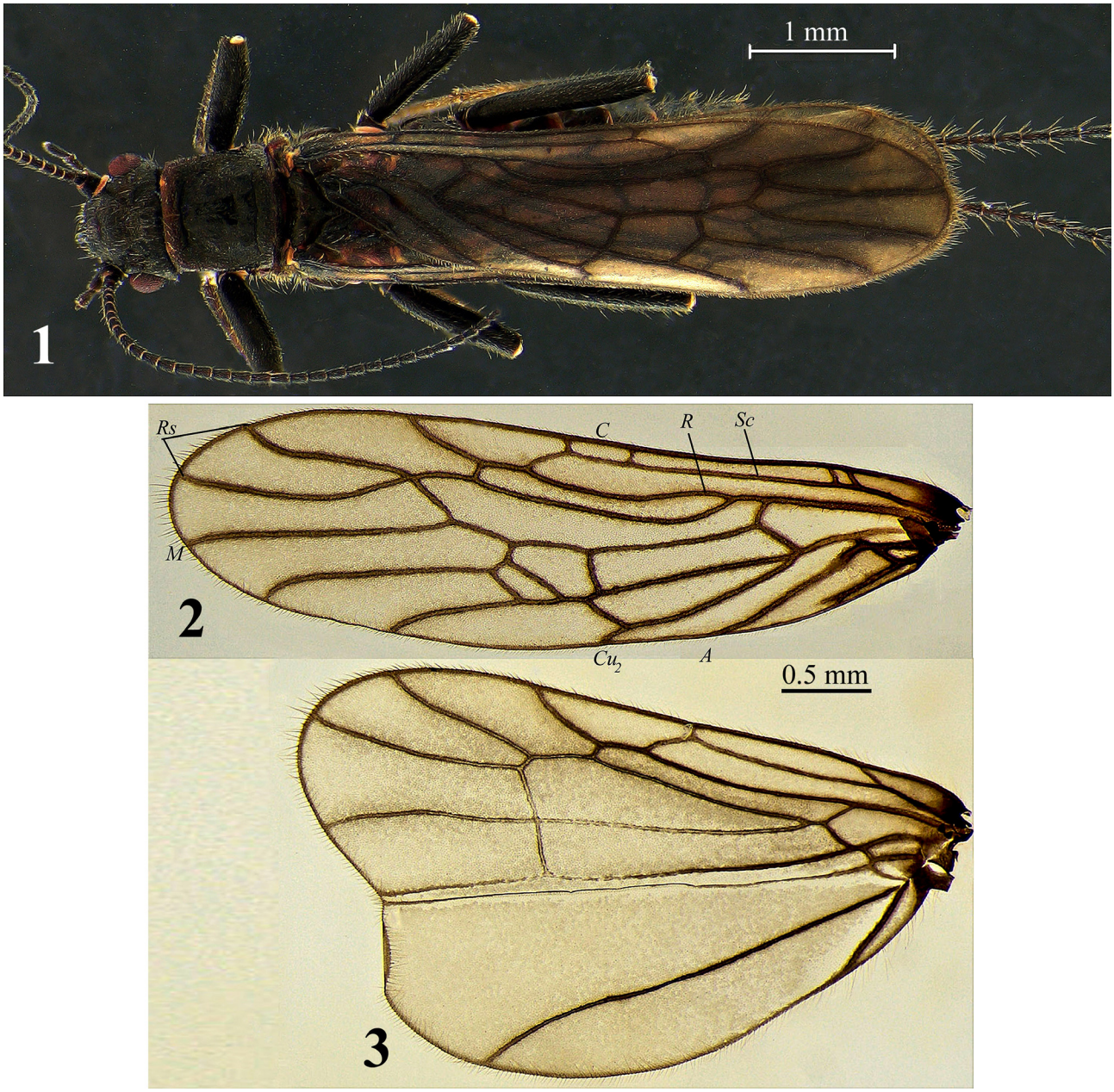
Description. Medium-sized species, body length 5.2–6.2 mm (n=7), darkly sclerotized, and overall brown color (Figs. 1, 4–6). Setation is dense and long, especially on abdomen and cerci (Figs. 1, 4–6). Antenna is moderately long, with 31 or more segments. Cercus slender and hairy, shorter than the abdomen length, with 11 or more club-shaped segments; third basal segment as long as wide, further ones are gradually elongated (Figs. 5, 6); apical cercal segments are the longest with their length about three times of their width, intercalary setae are dense and short (Fig. 1).

Wings shortened, forewing length 4.3–4.6 mm, wingspan 9.6–10.2 mm; brownish along radial field, veins dark brown, margins with relatively long brown bristles (Figs. 2, 3). R_1 of forewing typical of *Capnia*, bent upward at its origin, four cross veins between *C* and *Sc*. *Rs* bears two apical branches, two veins between *M* and Cu_2; anal veins two (Fig. 2). Hindwing paler than forewing, veins brownish (Fig. 3).

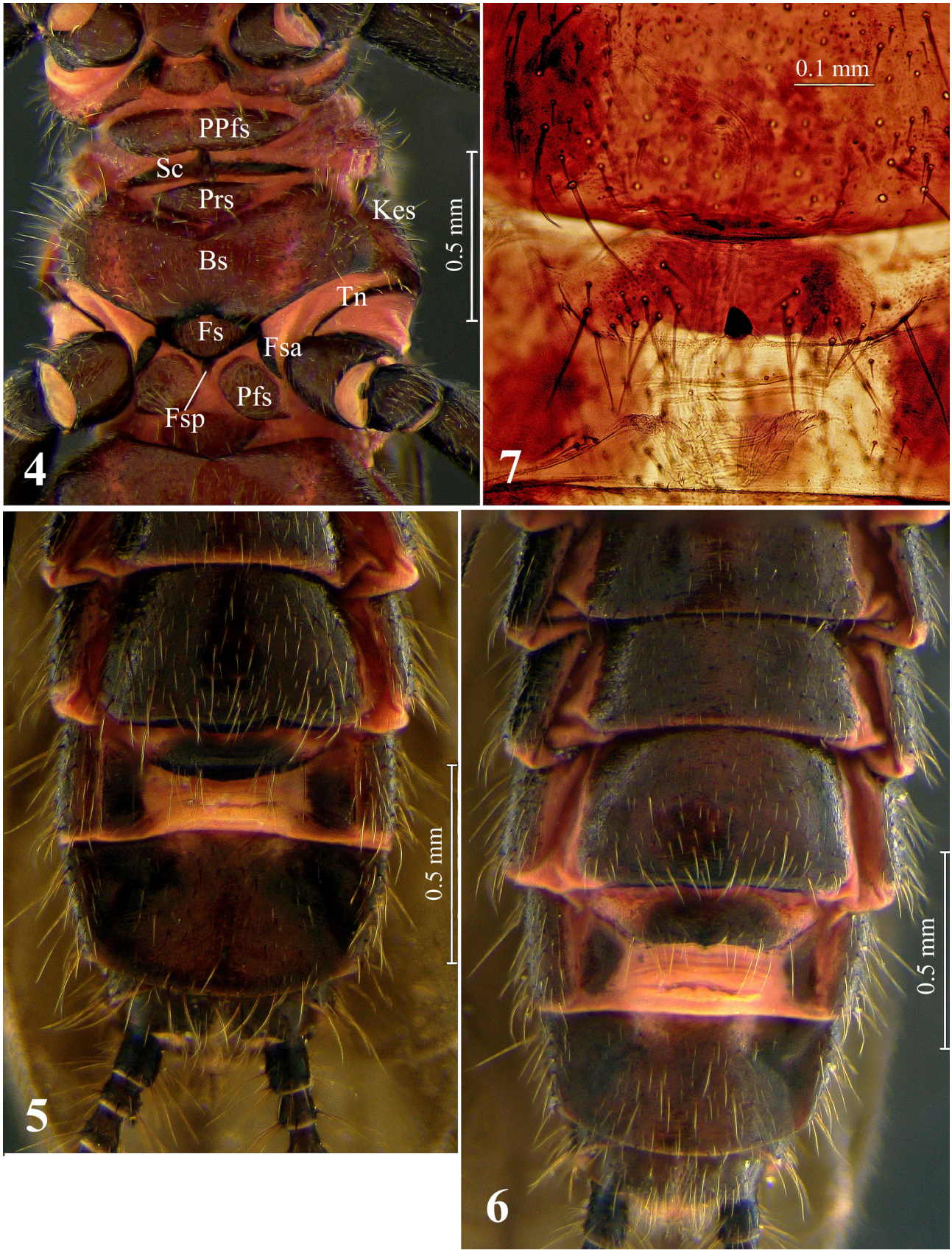
Mesothoracic sclerite (Fig. 4) from ventral view features narrow spinasternum (Sc), not fused with prothoracic postfurcasternum (PPfs) and large basisternum (Bs); median-sized subtriangular presternum (Prs) not fused with basisternum; subtriangular furcasternum (Fs) fused with basisternum, furcasternal arms (Fsa), and furcasternal pit

(Fsp); postfurcasternum (Pfs) divided into two lateral, suboval sclerites not fused with others; katepisternum (Kes) separated from basisternum and trochantin (Tn).

Abdominal terga 1−8 brown with broad longitudinal membranous areas along the midline, integument light, matte in appearance. Terga 9–10 are fully sclerotized. Abdominal sterna 1–7 with paired unsclerotized paralateral bands, sternum 7 slightly longer than others (Figs. 5, 6). Sternum 8 has a short, transverse subgenital plate, truncated posteriorly, length does not exceed ½ the length of the sternum 8 (Figs. 5, 6). An oval, unevenly pigmented spot occupies most of the subgenital plate, with pale anterolateral edges. Posterior margin strongly sclerotized, straight or slightly rounded medially, covered posterolaterally with long setae, and overlapping a small tooth-shaped black inner sclerite (Figs. 5, 6). Two or three thin transverse shallow membranous folds are below the subgenital plate. A pair of lateral sclerites located below posterolateral margins of subgenital plate at the membranous half of sternum 8 and do not exceed the length of sternum (Figs. 5, 6). Vaginal complex with broad genital opening, membranous genital cavity reaches the middle of the segment 7 where it branches into the oviducts (Fig. 7).



FIGURES 1–3. Female of *Capnia yavorskayae* **sp. nov.** Paratype. 1. Habitus, dorsal. 2. Right forewing. 3. Right hindwing.



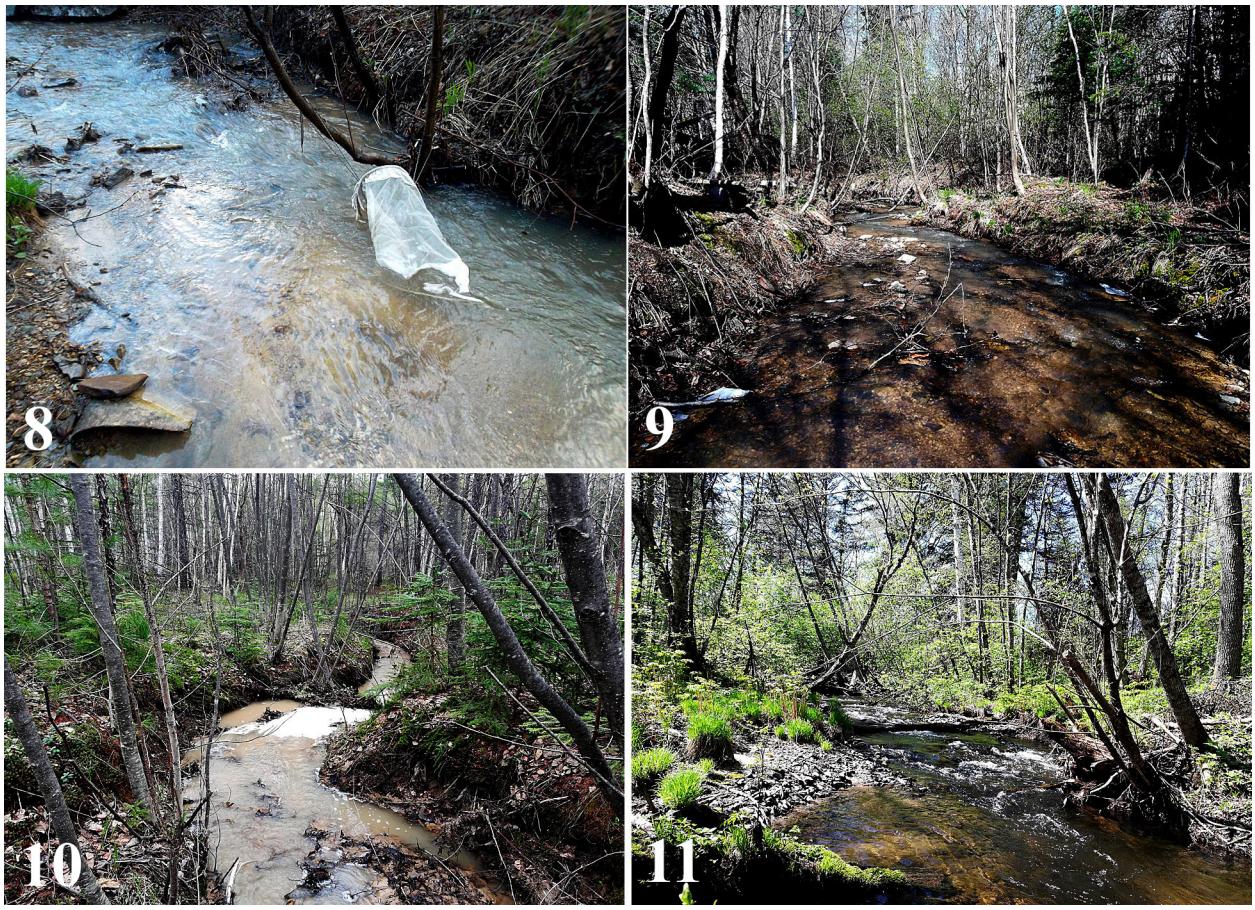
FIGURES 4–7. Female of *Capnia yavorskayae* **sp. nov.** Paratype. 4. Mesothoracic sclerite, ventral: Bs—basisternum; Fs—furcasternum; Fsa—furcasternal arm; Fsp—furcasternal pit; Kes—katepisternum; Pfs—postfurcasternum; Prs—presternum; PPfs—prothoracic postfurcasternum; Sc—spinasternum; Tn—trochantin. 5–6. Abdominal tip, variation of the subgenital plate. 7. Vaginal complex (cleared) with genital opening, membranous genital cavity, inner tooth-shaped black sclerite.

Affinities. Based on external female morphology, no closely related species are indicated. The noticeably short and truncated subgenital plate is not similar to other *Capnia* species, and only remotely resembles that of *C. kurnakovi*, from the Kolyma River Basin, North-East Russia (Zhiltzova 1978). *Capnia kurnakovi* has a shortened subgenital plate but posterior obtuse-angled margin, its base and medial parts are dark, thin and transverse; and membranous folds below the subgenital plate are absent.

Distribution and ecology. The Gorin River is a left tributary (facing downstream) of the Amur River (the Low Amur Basin) about 390 km long. It flows in the Komsomolskiy State Nature Reserve in the Khabarovskiy Kray, of the Russian Far East. The mountain ranges belong to the Sikhote-Alin folded region. *Capnia yavorskayae* **sp. nov.** was collected in tributaries of the Gorin River downstream: in the Khankuka Stream (13 km long, altitude 360 m above sea level), Siutaru Stream (13 km long, 480 m above sea level), and small unnamed stream in tract Kamennaya Pad, which flows down in Amur River independently (Figs. 9–11). Stream water temperature did not exceed 5–7°C.

Capnia yavorskayae **sp. nov.** seems to be a cold stenothermal species occurring in small mountain streams at altitudes of 300–500 m above sea level. The flight period is difficult to understand from the collection method and narrow window of time it was in operation; all females were collected between 14–19 May, and at that time appeared to be nearing completion. The new species was collected along with females of *C. khingana*, *Capniella nodosa* Klapálek, 1920, *Paracapnia leisteri* Zhiltzova and Potikha, 2005, and males and females of *Paraleuctra cercia* (Okamoto, 1922) (Klapálek 1920, Potikha and Zhiltzova 2005, Okamoto 1922).

Etymology. The new species is named for Nadezhda Yavorskaya, a Russian chironomidologist, who actively collects stoneflies, including this interesting species.



FIGURES 8–11. 8. Drift net for collecting stoneflies. 9–11. Holotype and paratype localities of *Capnia yavorskayae* **sp. nov.**, the Komsomolskiy State Nature Reserve, Gorin River Basin, the Low Amur River Basin, Khabarovskiy Kray, Far East of Russia. 9. Paratype. The unnamed stream near Kamennaya Pad cordon. 10. Holotype. The unnamed stream, Khankuka Stream Basin. 11. Paratype. The Siutaru Stream.

Results of DNA barcoding

Russian Far Eastern Species. The 12 new barcode sequences (Table 1), representing seven existing species and one proposed new species, ranged in size from 617-658 bp. After assembly and alignment, nucleotide frequencies across these sequences were as follows: A=26.5%, T=34.5%, G=18.4% and C= 20.6%.

Interspecific pairwise distances between the eight Far East *Capnia* species ranged from 2.8 to 14.2 (mean 11.4%). For most species, interspecific distances ranged from 10.2 to 15.2 (mean 12.0%) while two pairs of species - *C. bargusinica* - *C. rara* and *C. nigra* - *C. aligera* differed by 3.2 and 2.8%, respectively. According to Murányi et al. (2014) these values are sufficiently different to support species level distinction.

The high differences between eight investigated *Capnia* species and 43 GenBank & BOLD sequences (Fig. 12) were confirmed by ABGD analysis, which yielded 47 operational taxonomic units (OTU) using a 0.001–0.0028 intraspecific divergence. All eight species were included as separate OTUs.

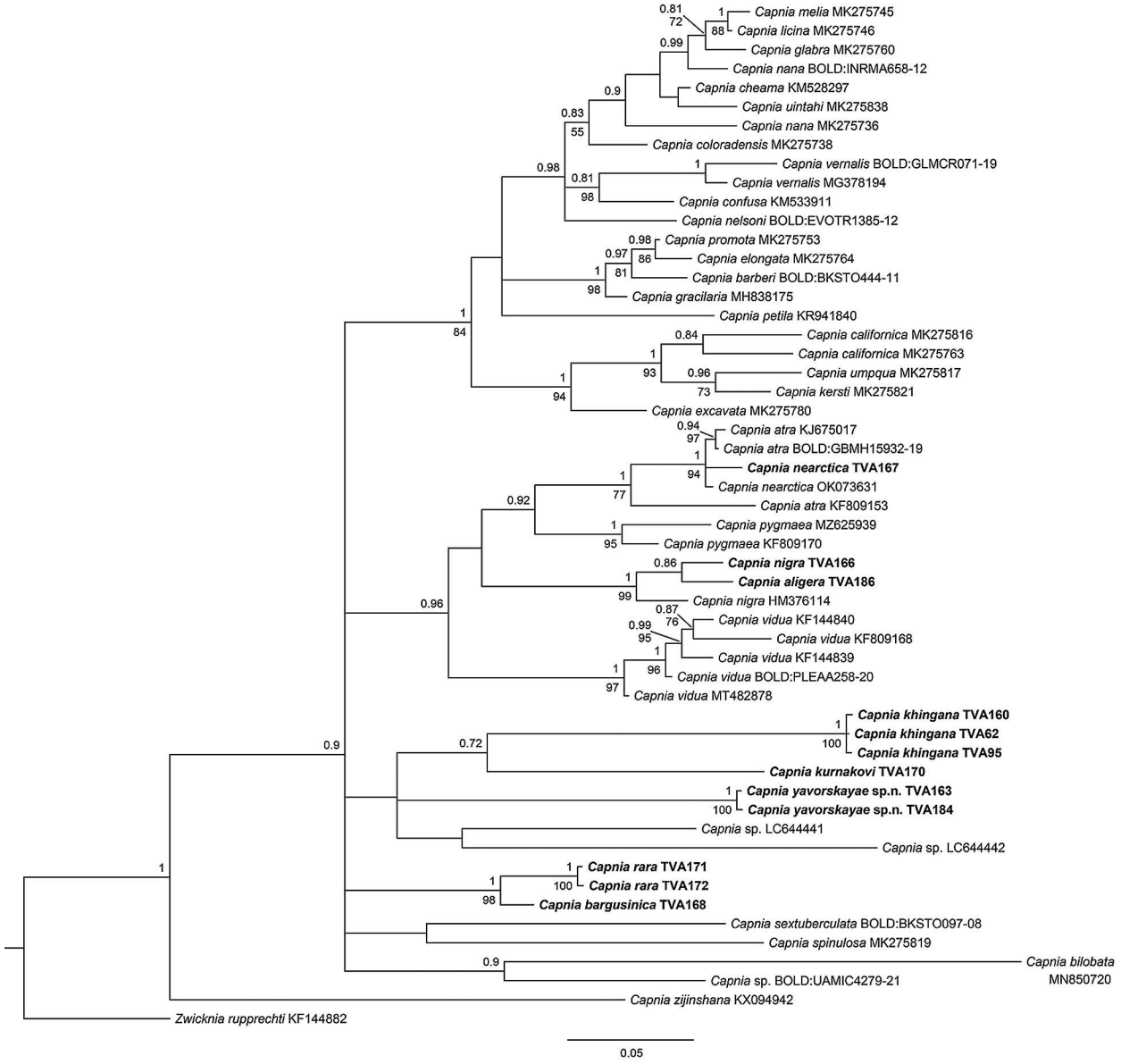


FIGURE 12. Bayesian inference (BI) tree based on the cytochrome c oxidase I (COI) nucleotide sequence data of the genus *Capnia* and outgroup *Zwicknia rupprechti* Bayesian posterior probabilities (≥0.7) are given above tree nodes and bootstrap support values found in the ML analysis (≥70 %) are shown below nodes. Specimens obtained in this study are in Bold.

We used COI to reconstruct the species relationships in the genus *Capnia*. Bayesian inference (BI) and Maximum Likelihood (ML) trees had a nearly similar topology, so we present only the Bayesian tree with posterior

probability (PP) above nodes, including bootstrap support for ML tree under nodes (Fig. 12). The BI phylogeny revealed a well-supported polytomic primary clade (PP = 0.9) uniting six clades of different *Capnia* species (Fig. 12). *Capnia zijinshana* Du & Chen was the earliest branching lineage and sister to the polytomic primary clade. The *C. bargusinica* and *C. rara* were placed in one of the six primary clades. *Capnia yavorskayae* **sp. nov.**, *C. khingana*, *C. kurnakovi* as well as *Capnia* sp. (LC644441) and *Capnia* sp. (LC644442) were placed in the independent clade (Fig. 12). *Capnia nearctica*, *C. nigra* and *C. aligera* were placed into a large clade that also includes many other *Capnia* species. It is noteworthy that the sample *C. nigra* (TVA166) were not conspecific to *C. nigra* (HM376114). These two samples did not form a monophyletic clade, and the p-distances were 4.4% which requires further research. In turn, *C. nearctica* (TVA167) were probably conspecific to *C. nearctica* (OK073631, p-distance = 1.8%) as well as to *C. atra* (ADO9186) but this is contradicted by the results of ABGD analysis. To solve this problem, additional sequences of *C. nearctica* and *C. atra* from different locations of the range are required.

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