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MORPHOLOGICAL REDESCRIPTION AND DNA BARCODING OF *TORLEYA PADUNICA* KAZLAUSKAS, 1963 (EPHEMEROPTERA, EPHEMERELLIDAE) FROM THE EAST PALAEARCTIC REGION

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Summary. The neotype of *Torleya padunica* is designated and description of all stages is given. The larvae *T. padunica* is most similar to *T. cohery*, *T. dibruensis, T. elissa* and *T. major*; the genitals of male imago – to *T. naga* and *T. mikhaili*; the eggs – to *T. lutosa*, *T. major, T. mikhaili, T. naga* and *T. nepalica*. *Torleya padunica* is genetically distant from other *Torleya* species showing uncorrected p-distances of >19 %, values well associated with interspecific variation. The well-supported monophyly as well as results of species delimitation analyses confirms the validity of *T. padunica*.

Key words: Ephemeroptera, mayfly, *Torleya*, morphology, taxonomy, neotype designation, barcoding, Russia.

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Резюме. Обозначен неотип *Torleya padunica* и приведено полное описание морфологии всех стадий развития этой поденки. Личинки *T. padunica* наиболее схожи с *T. cohery*, *T. dibruensis, T. elissa* и *T. major*; гениталии самцов имаго – с *T. naga* и *T. mikhaili*; яица – с яйцами *T. lutosa*, *T. major*, *T. mikhaili*, *T. naga* и *T. nepalica*. *Torleya padunica* генетически отличается от других видов рода *Torleya*, демонстрируя генетические расстояния более 19 %. Такие значения находятся на межвидовом уровне. Хорошо поддержанная монофилия *T. padunica*, как и результаты разделения видов, подтвердили его валидность.

INTRODUCTION

Mayfly *Torleya padunica* Kazlauskas, 1963 from a nameless brook that confluent to Angara River (Irkutsk Region, Eastern Siberia, Russia) was described as the first Siberian species of genus *Torleya* Lestage, 1917. A very short description and some figures of larvae made it possible to expand a range of this species to the Russian Far East (Levanidova, 1968) and to describe imaginal stages using the examples from this region (Tiunova, 1995). Published characters of *T. padunica* were make use in a key of larvae (Kluge, 1997), phylogenetic investigations (Jacobus & McCafferty, 2008; Zhou *et al*., 2000) and in a number of identification (Reznic, 2005; Tiunova & Potikha, 2005).

In the summer of 2020 as the result of rearing larvae (collected in the Amba River, Khasansky district, Primorsky Krai, Russia) were received imago that identified as *T. padunica*. The larvae also had characters of this species but more denticles on the claws then in the original description. The examinations of claws of larvae from Ussuri River, that was used for rearing imago and description imaginal stage by Tiunova (1995) also showed difference with original. Unfortunately, the holotype and paratypes of *T. padunica* from Angara River are lost (Kluge, 1995). But another larva from Eastern Siberia (from tributary of Vitim River, Buryatia) was examined. This specimen was identified as *T. padunica* by Kluge (1995) and the photograph of a claw courtesy by him. The number and form of denticles of the claw was coincident with the ones from the Russian Far East. Also, Jacobus *et al*. (2004) examined mouth parts of larva from Narva River, Khasansky district, Primorsky Krai and identified it as *T. padunica.* These evidences confirm the habitation of *T. padunica* in the currents of water in East Siberia and Primorsky Krai and suggest that original description of larva *T. padunica* consists inaccuracy. Modern methods of microscopy made it possible to achieve more detail images of anatomical structures and eggs of *T. padunica* from Amba River and made redescription. We also used this material for DNA barcoding which provides an efficient and reliable approach for species identification of mayflies of family Ephemerellidae (Selvakumar *et al*., 2016, Morinière *et al*., 2017, Tenchini *et al*., 2018, Martynov *et al*., 2023).

REMARK. In original description Kazlauskas (1963) stressed that the types of this species are kept in the Zoological Institute of USSR Academy of Sciences (ZIN). But Kluge (1995) carefully examined this collection and found that the types of *T. padunica* are lost. Here the neotype of this species is designated, described and illustrated for stability of nomenclature and according to Article 75 of the Code (ICZN, 1999).

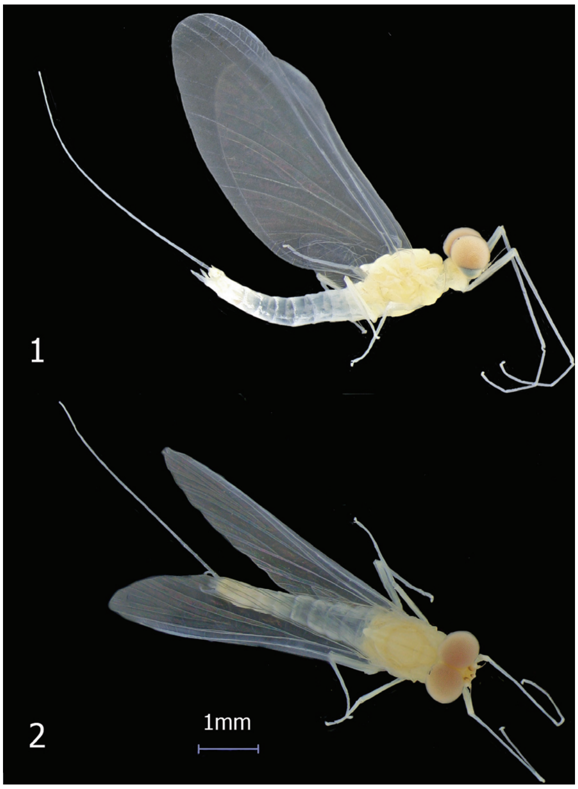
MATERIAL AND METHODS

Specimen collection and morphological identification. This paper is based on the material collected by E.A. Gorovaya (EG) in Khasansky district (Primorsky Krai, Russia). The rearing subimago from larvae took place in holding cages in the water flow of Amba River, rearing imago – in ambient room conditions. The material was preserved in 96% ethanol for DNA-analysis and in 70% ethanol for further study of morphology. Also, the materials collected by T. M. Tiunova (TT) in Far East of Russia were examined: specimens of *T. padunica* and *T. mikhaili* Tiunova, 1995 (the last one from typical habitat: Russia, Primorsky Krai, Ussuri River, 1.5 km below Utes mountain, 02.VIII 1992, TT) for detailed comparison of these two species.

Morphological characters were observed under Olympus CH light microscope. Photographs of specimens were taken with a stereomicroscope Olympus SZX16 and a digital camera Olympus DP74, and then stacked using Helicon Focus software. Microstructures were examined with a field-emission electron microscope MERLIN (CARL ZEISS, Germany) at the Center for Collective Use Biotechnology and Genetic Engineering of the Federal Scientific Center of the East Asia Terrestrial Biodiversity (FSC Biodiversity). The final illustrations were post-processed for contrast and brightness using Adobe® Photoshop® software. The morphological structures of the eggs are described using the terminology from the following publications: Koss & Edmunds, 1974; Ubero-Pascal & Puig, 2009. Material treated in this paper is deposited in the FSC Biodiversity, Vladivostok, Russia.

Molecular methods. DNA was extracted from thorax or legs using a Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The region of gene cytochrome c oxidase I (COI, length ca 650 bp) was amplified with the universal LC01490 and HC02198 (Folmer *et al*., 1994) primer pair. PCR products were performed with Go Taq Green Master Mix (Promega corp, Madison, WI, USA) and the thermal regime consisted of 1 cycle at 95°C for 3 min, 35 cycle at 95°C for 30 s, 48°C for 30 s, 72°C for 60s and 1 cycle at 72°C for 5 min. PCR products were confirmed by electrophoresis in 1.5% agarose gel. Amplified PCR bands were purified with 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). Based on the observed p-distances are calculated inter- and intraspecific genetic distances also using MEGA7. All sequences have been deposited in GenBank (PP175362-PP175369).

In the present study, we combined 51 previously published COI sequences of *Torleya* including *T.* (*Serratella*) *elissa* Jacobus, Zhou et McCafferty, 2009 from GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) and Barcode of Life Database (BOLD – www.boldsystems.org) with our 8 new COI sequences. Distance-based (ASAP, Puillandre *et al*., 2021) and tree-based methods (Multi-rate Poisson tree processes, mPTP, Kapli, 2017) were used for species delimitation. ASAP analyses were run using web service (https://bioinfo.mnhn.fr/abi/public/asap/) using p-distances substitution model. For the mPTP method we performed Bayesian ultrametric tree using BEAST v.1.10.4 (Suchard *et al*., 2018). Settings were as follows: strict clock, MCMC chain using 100 million generations, TN93 substitution model and Yule speciation model. The MCMC log-on posterior values were examined in Tracer v.1.7.1 (Rambaut *et al*., 2018) and a burn-in with 30% was set to obtain an optimal consensus tree. mPTP was run on the web servers https://mptp.h-its.org/ with default settings.



Figs 1–2. Male imago of *Torleya padunica*, habitus. 1 – lateral view; 2 – dorsal view.

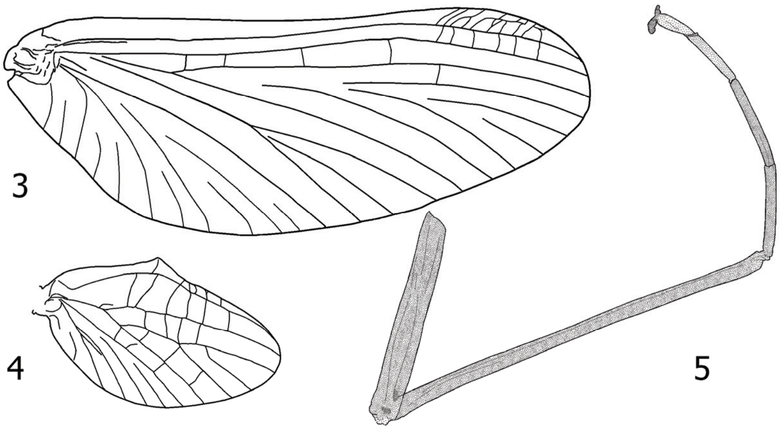
TAXONOMY

Torleya padunica **Kazlauskas, 1963**
Figs 1–35

Torleya padunica Kazlauskas, 1963: 584, figs 13–19 (holotype and paratype – mature larvae, Russia: Irkutskaya Oblast; lost); Kluge, 1995: 42; Tiunova, 1995: 56, figs 10–16; Jacobus *et al*., 2004: 170; Jacobus & McCafferty, 2008: 202.

Ephemerella (*Torleya*) *padunica* Kluge, 1997: 213.

TYPE MATERIAL. Neotype – male imago (reared from larva), **Russia**: Primorsky Krai, Khasansky district, Amba River, 01.VII 2020, coll. E. Gorovaya, here designated; deposited in the collection FSC Biodiversity (Vladivostok).



Figs 3–5. Male imago of *Torleya padunica.* 3 – fore wing; 4 – hind wing; 5 – fore leg.

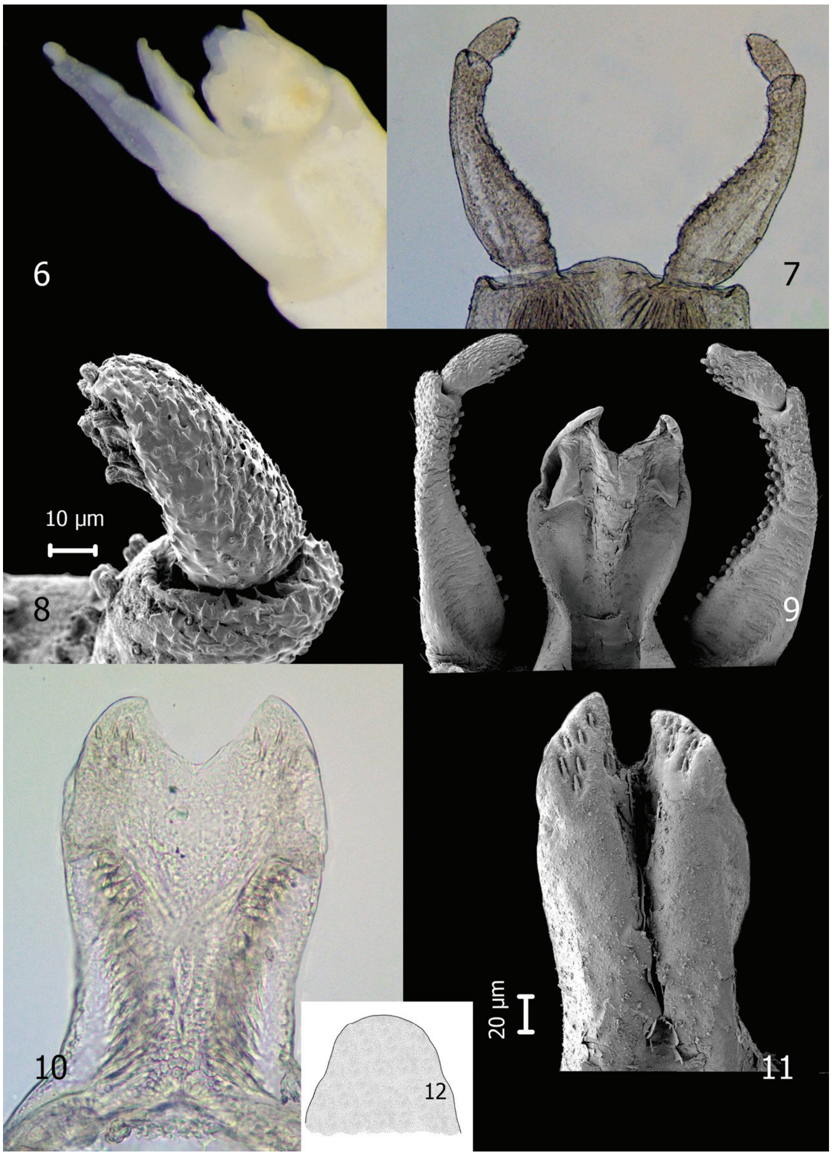
OTHER MATERIAL EXAMINATED. **Russia**: Primorsky Krai, Khasansky district, Amba River, 01.VII 2020, 13 larvae, 1 female subimago, 6 females imago,1 male subimago and 8 males imago (all reared) (EG); same locality, 06.VII 2020, 4 females subimago, 5 males subimago and 3 male imago (light) (EG); Republic of Sakha (Yakutiya), Aldan River basin, Ungra River, 28.VII 2006, 1 larva, 6 mails imago (TT); Zabaikalsky Krai, Chornaya River, 29–30.VII 2005, 1 mail imago (TT); Amurskaya Oblast, Bolshoi Oldoi River, 31.VII 2005, 6 mails imago (TT); Republic of Buryatiya, Vitim River, 57 km from the mouth of the Baisa River, 20VII 1960, 1 larva (I. Sukachova) (one claw of mid leg on slide) [collection of N. Kluge].

DESCRIPTION. MALE IMAGO (in alcohol). General body color yellowish (Figs 1, 2). Length (mm): body 4.8–5.6; forewings 5.1–5.6, cerci 5.3–6.0.

Head: upper portion of the eyes brown (while alive ginger); lower one black.

Thorax: yellowish with light brown tint. Forewings hyaline, pterostigma and area between Sc and R dull white. Veins unpainted, poorly visualized (Fig. 3). Hind wings hyaline (Fig.4). Legs creamy white. Length (mm) of foreleg segments: femora 1.2; tibiae 2.4–2.6; tarsal segments 0.6–0.7, 0.5–0.6, 0.3–0.4, 0.2, 0.05 (Fig. 5). No maculation on femora surface.

Abdomen creamy white; terga I–VI hyaline. Styliger, gonostyli and penis creamy white, slightly hyaline (Fig. 6). Styliger with wide low rounded protrusion (Fig.7). Gonostyli tartareous, with papillae on inner surface. Gonostyli segments 1 and 2 poorly separated, segment 3 elongate, oval-shaped, slightly expanded at the base (Figs 7–9). Penis lobes wider in upper part, divided by less than a quarter, outer edges rounded, tops pointed (Figs 9–11). Each lobes with acute dorsal projection: low part triangular, slightly elongated (Fig. 9); on ventral side bear well developed spines in apical part (Figs.10, 11). Cerci creamy white, subequal to medial filament.



Figs 6–10. *Torleya padunica*: 6–11 male imago; 12 – female imago. 6 – genitalia; 7 – styliger and gonostyli; 8 – 3rd segment of gonostyli; 9 – genitalia; 10, 11 – penis; 12 – subanal plate. 6 – lateral view, 7, 10–12 – ventral view; 8, 9 – dorsal view.

MALE SUBIMAGO. Measurements and body color similar to those in male imago; abdomen and forewings creamy white, opaque.

FEMALE IMAGO (in alcohol). Length (mm): body 5.0–5.2; forewings 6.0–6.2; cerci 4.6. General color of body similar to that in male imago, but abdomen dark creamy due to transculent eggs. Eyes grey with ochroid tint. Subanal plate convex, roundish (Fig. 12).

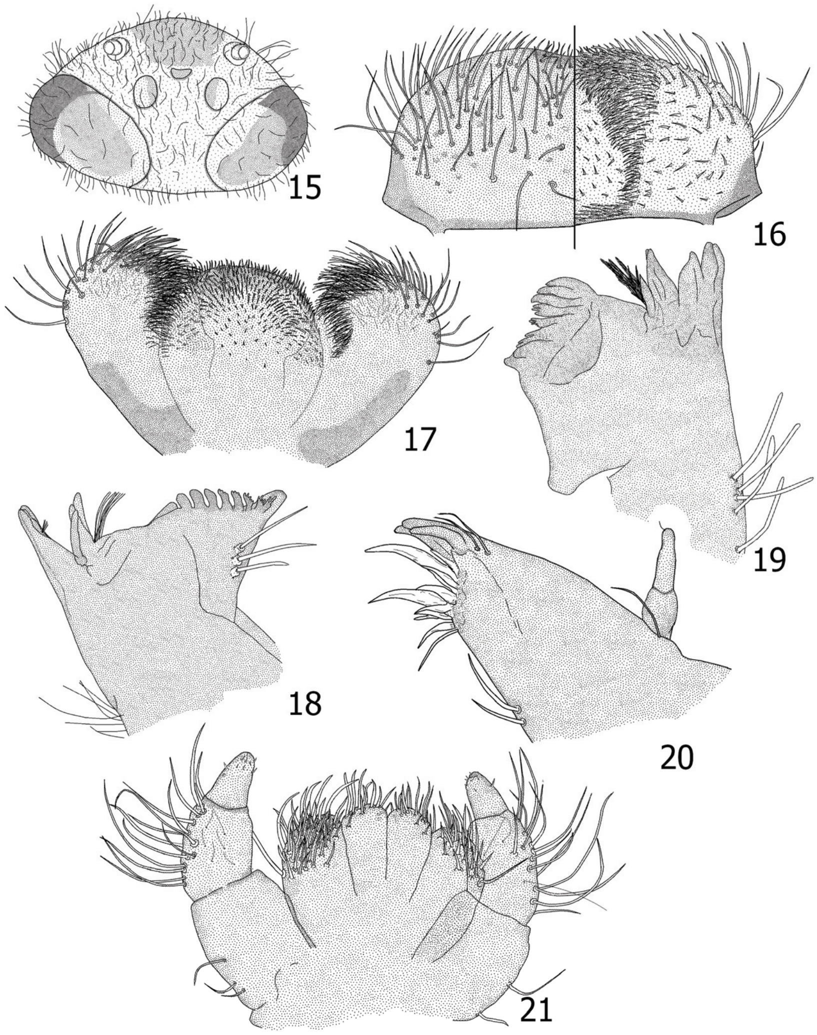
FEMALE SUBIMAGO. Measurements and body color similar to those in female imago; abdomen no hyaline. Forewings creamy white, opaque.

Figs 13–14. Larvae of *Torleya padunica*, habitus. 13 – dorsal view; 14 – ventral view.

MATURE LARVA (in alcohol). Length (mm): body 3.7–4.4; cerci and paracercus 1.7–2.0. General body color creamy white (alive larvae with pale yellow tint) with light brown maculation (Figs 13, 14). All sclerotized structures abundantly covered with micro-thin transparent hairs.

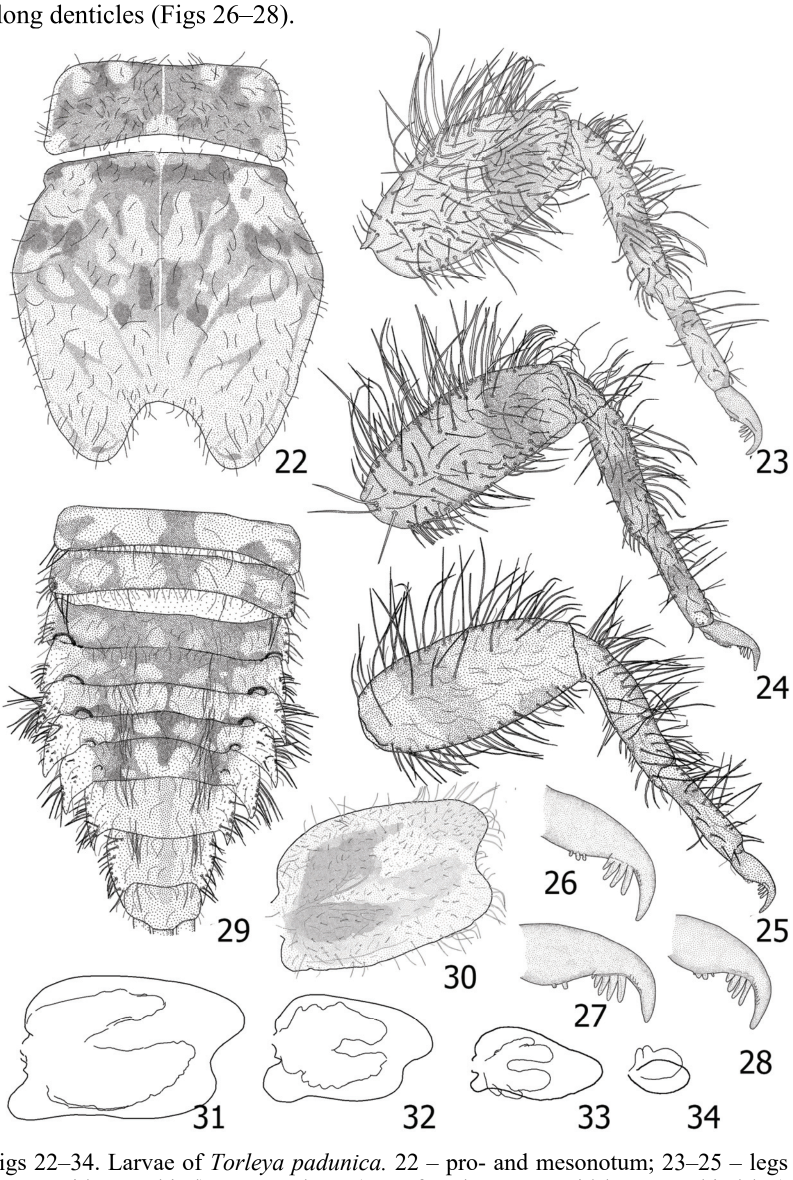
Head: without tubercles or projections, creamy white, with big brownish spot above labrum and sometimes, with small brownish mosaic spots between eyes (Fig. 15). Upper part of eyes of male brown (while alive ginger), lower one black. Antennae long, creamy white.

Mouthparts: Labrum pale, brownish; dorsal surface densely covered with long hairlike setae, ventral – small spikes; length to width ratio 1:2 anteromedian emargination well-marked (Fig.16). Hypopharynx wide, rounded, apical part densely covered with small spikes; superlinguae uniformly wide, with long thick setae along anterior and inner margins (Fig. 17). Mandible broad; right incisor with three (Fig. 18) rounded teeth; left incisor with three tapering teeth (Fig. 19). Maxilla wide, apex pointed with three teeth and some long thin setae near its base on external margin of maxilla (Fig. 20). Massing of multitudinal long thin setae is absent. Inner margin of maxilla with rows of strong dentiform setae. Maxillary palp 2-segmented with single thin setae on apex. Glossae closely approximate, some narrower and longer than paraglossae; both with long strong setae on distal parts. Labial palpus 3-segmented (Fig. 21); 1st and 2nd segments of labial palpus wide, rectangle; 3rd segment conical; transition from 2nd to 3rd segment smooth.



Figs 15–21. Larvae of *Torleya padunica.* 15 – head; 16 – labrum; 17 –hypopharynx and superlinguas; 18, 19 – mandibles, ventral view (18 – right, 19 – left); 20 – maxilla; 21 – labium.

Thorax: Pronotum light brown with small creamy white roundish spots. Mesonotum and rudiments of fore wings maculation (Fig. 22). All legs creamy white with light brown belts: more clearly expressed on femurs and tarsi, less – on tibiae (especially hind leg); densely covered by long setae and hairs (Figs 23–25). Length (mm) of segments of legs (femur; tibia; tarsus): fore: 0.5–0.7; 0.4–0.5; 0.25–0.35; mid: 0.6–0.8; 0.4–0.5; 0.3–0.35; hind: 0.7–0.8; 0.4–0.5; 0.25–0.35. Femurs of fore leg wide, oval; femurs of mid and hind legs more elongate. All claws with two rows of denticles: three to four short basal denticles and preapical palisade with four to five long denticles (Figs 26–28).



Figs 22–34. Larvae of *Torleya padunica.* 22 – pro- and mesonotum; 23–25 – legs (23 – fore, 24 – mid, 25 – hind); 26–28 claws (26 – fore leg, 27 – mid leg, 28 – hind leg); 29 – abdomen, dorsal view; 30– gill I pair; 31–34 gills, sketchily (31 – II pair, 32 – III pair, 33 – IV pair, 34 – V pair).

Abdomen: Tergites I–IV, VI light brown with small creamy white roundish spots along middle line, terga V lighter, tergites VII–X all over creamy white. Tergites III–IIX with well-developed acuminate creamy white posterolateral projections. Lateral margins of tergites II–IX with strong, long and middle setae and hairs. Abdominal tergal paired spines absent; on its position long, thin, hard setae. Posterior margins of abdominal tergites V–IIX with paired tufts of long, thin, hard setae (Fig. 29). Sternites creamy white without maculation. Abdominal gills on tergites IV–VII imbricated. Length of gills of larvae 5 mm (mm): I, II–0.7; III – 0.5; IV – 0.35; V – 0.1. Gill V consists of two large and three small but quite wide plate (Figs 30–34). Caudal filaments creamy white; every fourth segment brownish (Figs 13, 14).

Egg (Taken from imago). Measurements: length 157−166 µm; width 100−109 µm. Shape oviform with one polar cap. Form of polar cap depends from the quality of cleaning and can be wide and flat or a bit fastigiate (Fig. 35). Surface of the chorion with geometric relief: wide reticulation with mesh about 11.4–12.3 µm formed by raised porous ridges with thickness 4.5–4.9 µm. Bottom of each mesh unit hillocky. Papillae of inner margin of ridges are not explicitly expressed. Lateral attachment structures represented by multitreads-folded with terminal fibre cluster (MFT) which widely-spaced all over the surface. Micropyle (two to three in the field of vision) pear shaped with narrower part near micropylar opening in shape. All micropyles with porous rim and situated in the equatorial part of egg.

DISTRIBUTION. **Russia**: Irkutskaya Oblast, Republic of Buryatiya, Zabaikalsky Krai, Republic of Sakha (Yakutiya), Amurskaya Oblast, Jewish Autonomous Oblast, Khabarovsky Krai, Primorsky Krai.

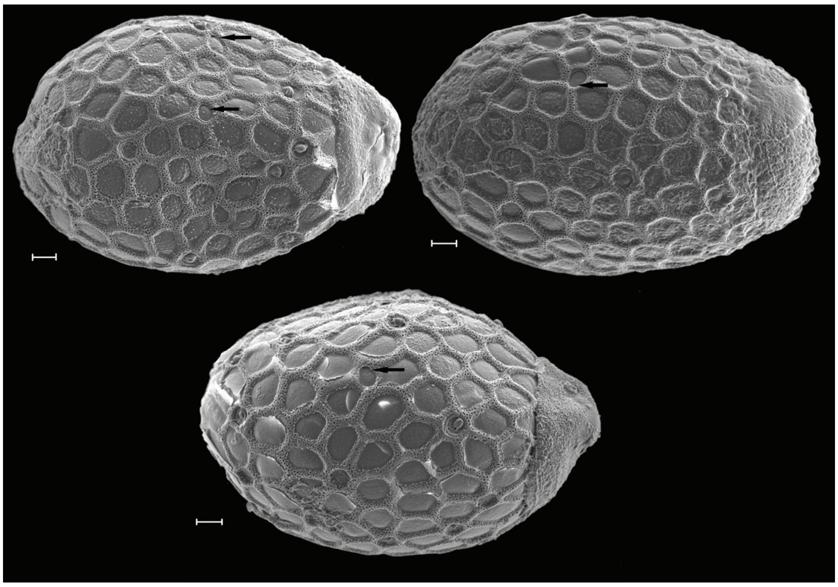
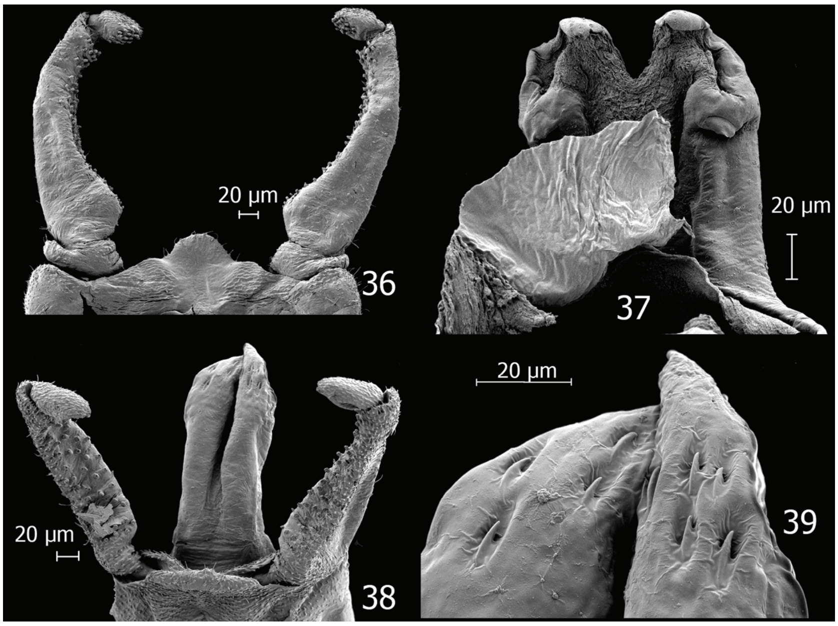


Fig. 35. Eggs of *Torleya padunica*. The arrows indicate the micropyles.

TAXONOMIC NOTES. Larvae only five species of the genus *Torleya* have relatively well-developed maxillary palp: *T. padunica*, *T. cohery* (Allen et Edmunds, 1963) (Oriental region), *T. dibruensis* Selvakumar, Martynov et Jacobus, 2021 (Oriental region), *T. elissa* (Jacobus, Zhou, et McCafferty, 2009) (East Palaearctic region) and *T. major* Klapálek, 1905 (West Palaearctic region). All these species differs well from *T. padunica* in a number of characters. So, *T. cohery* has special form of mandibles and labium; tarsal claws with a significant number of long denticles in palisade (more than five); abdominal segment 8 with a narrow acute posterior projection, posterolateral projection on segment 9 extends posterolaterally beyond margin of segment 10 (Allen & Edmunds, 1963: figs 15, 17–19; Martynov *et al*., 2021: fig. 10). *T. dibruensis* has terga IV–VII and IX with paired posterior protuberances and dorsal lamella of gill III which extending to middle of tergum VIII (Tiunova, 2024: fig. 12B). *T. elissa* has maxillary palp with three segments, specific form and location of the denticles on claws (Martynov *et al*., 2023: figs 7, 9d, 9e). Micro-thin transparent hairs on all sclerotized structures are absent (Jacobus *et al*., 2009). Claws of *T. major* without palisade of the denticles (Jacobus &McCafferty, 2008: fig. 41), gill V consists of two large and six small plates (Mikulskij, 1936: fig. 114b).

Form of styliger and genitals of male's imago *T. padunica* close to the ones of *T. naga* Jacobus et McCafferty, 2004 (Oriental region). But apical parts of penis lobes of *T. naga* very narrow, widely spaced; the outer edges of the penis straight;



Figs 36–39. *Torleya mikhaili*, genitalia. 36 – styliger and gonostyli; 37 – penis; 38 – penis and gonostyli; 39 – penis lobes. 36, 38–39 – ventral view; 37 – dorsal view.

the third segment of gonostyles is curved, and its base strongly narrowed (Jacobus *et al.,* 2004: fig. 6). The greatest similarity male imago *T. padunica* has with *T. mikhaili* Tiunova, 1995 from East Palaearctic (Figs 36–39). But *T. mikhaili* has styliger with high conical rounded protrusion (Fig. 36), third segment of gonostyles oval-shaped with narrow base, the outer edge of the penis lobes with a hollow (Tiunova, 2024: figs 3–5) and spines on ventral side of penis lobes smaller ones (Fig. 39).

All known eggs of the genus *Torleya* have close to oval shape and one polar cap. But eggs of *T. lutosa* Kang et Yang, 1995, *T. nepalica* (Allen et Edmunds, 1963) (= *T. glareosa* Kang et Yang, 1995, synonymized by Jacobus & McCafferty, 2008) (both from East Palaearctic and Oriental regions) have teardrop-shaped micropyle and high ridges (Kang & Yang, 1995: figs 20–24), that are wider than those of *T. padunica*. The eggs of *T. major* also have wide, but only slightly raised ridges (Ubero-Pascal & Puig 2009: fig. 4c). The ridges of reticulation of *T. naga* very narrow, micropyle oval or teardrop-shaped (Jacobus & McCafferty, 2004: fig. 5). The eggs of *T. mikhaili* are smaller and ridges are more subtle with numerous papillae on inner margin (Tiunova, 2024: figs 23–26).

RESULTS OF DNA BARCODING

Overall, we have sequenced fragments of the cytochrome oxidase subunit I (658 bp in length) of 8 samples of *T. padunica* that generated 7 unique haplotypes. Intra-specific genetic p-distances within samples ranged between 0.00 and 0.61%, with average value 0.3%. Eight observed mutations were parsimony- uninformative (singleton) synonymous transitions that are homogeneously distributed among samples despite significant geographic distance between Primorsky Krai and Amurskaya Oblast (more than 700 km).

Our phylogenetic reconstruction (Fig. 40) based on the COI gene revealed two primary clades, first including *T. nepalica* (GenBank №KM262827) and *Torleya* sp. (BOLD ID THMAY132-12) and second polytomic clade includes the remaining taxa (Bayesian posterior probability, BPP=0.99). This clade consisted of six independent branches with various combinations of samples and species of the genus *Torleya*. The Bayesian inference confirmed close relation of *T. elissa* and *Torleya* sp. from Caucasian range and significant divergence between samples of type species *T. major* (Martynov *et al*., 2023). *T. padunica* formed one of the six clades (BPP=1).

Results of molecular species delimitation using ASAP and mPTP generated 21 and 18 mOTUs respectively. For the most species produced mOTUs did not correspond to the species. Species *Torleya major* was split into five different mOTUs while *T. nepalica* and *T. coheri* were split into three mOTUs for each species. Such a split may be caused by incorrect identification of mayflies (apparently *T. nepalica* and *T. coheri*), or by high intraspecific sequence divergence (apparently *T. major*). According to mPTP samples *T. grandiforceps* (MT274131) and *T. nepalica* (MT274132) were merged into single mOTU although ASAP didn't support this merge.

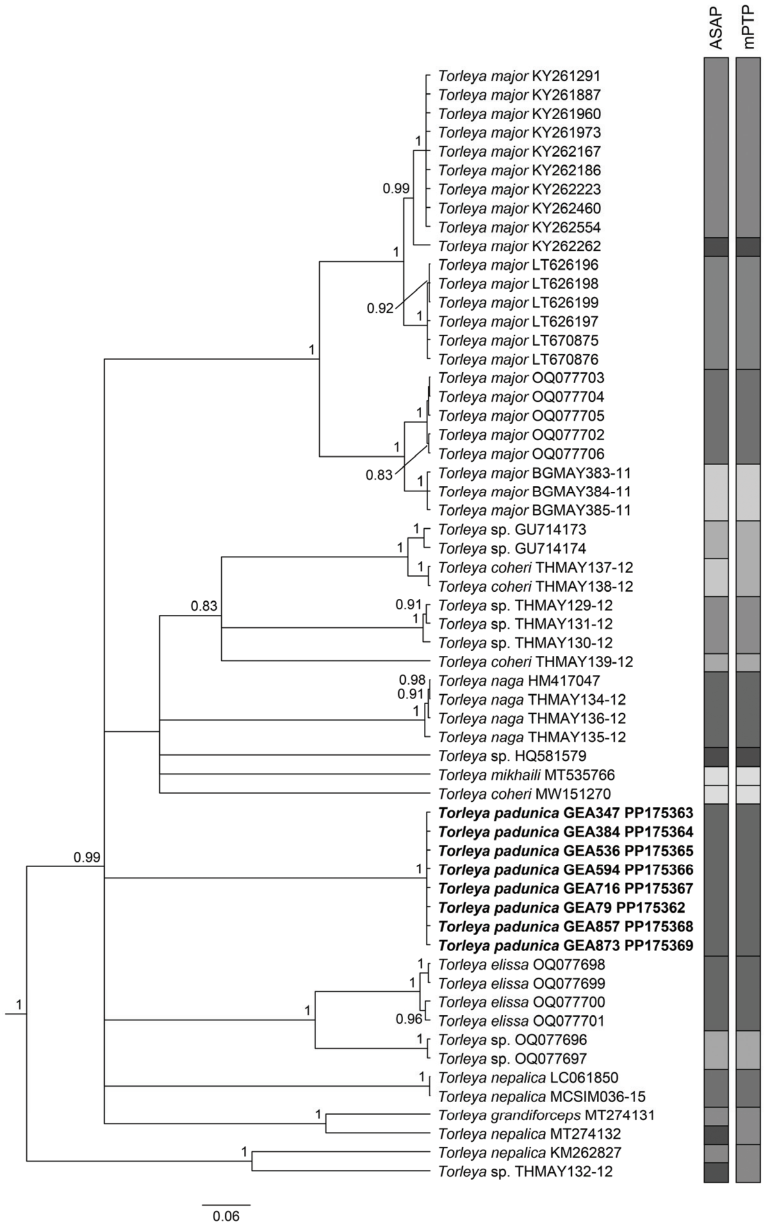


Fig. 40. Ultrametric Bayesian inference (BI) tree based on the cytochrome c oxidase I (COI) nucleotide sequence data of the genus *Torleya* Lestage. Bayesian posterior probabilities (higher than 0.7) are given above tree nodes. Specimens obtained in this study are in bold.

To calculate interspecific distances, we grouped samples according to the results of ASAP analysis instead of grouping them by species. The interspecific divergences between *T. padunica* and other mOUTs of *Torleya* mayflies ranged from 19.35 (*T. elissa*) to 29.45 (*Torleya* sp. THMAY132-12) that correspond to species level (Morinière *et al*., 2017, Tenchini *et al*., 2018, Martynov *et al*., 2023). The mean of all interspecific divergences was computed as 20.08%. The average sequence divergence between 5 mOTUs of *T. major* were 9.41%

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