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DNA BARCODING OF MAYFLIES (EPHEMEROPTERA) IN KAMCHATKA PENINSULA, RUSSIA

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Summary. DNA barcodes of 75 samples from 10 species were obtained, four of which were previously described from Kamchatka watercourses. Comparative analysis of the new sequences and data from the BOLD system made it possible to expand the ranges for *Acentrella diptera* Kluge et Novikova, 2011, *Baetis pseudothermicus* Kluge, 1983, *Cinygmula malaisei* Ulmer, 1927, *Ephemerella aurivillii* Bengtsson, 1908, and *E. mucronata* Bengtsson, 1908. Sequences XJDQD534-18 - XJDQD542-18 from China should be transferred from the genus *Baetiella* Ueno 1931 to the species *Baetis pseudothermicus*. Despite the high K2P distances for *Ephemerella mucronata* and *E. aurivillii*, we do not consider to divide them into several species and propose to increase the threshold of intraspecific differences by 6.47% or more for the genus *Ephemerella* Walsh of the family Ephemerellidae.

Key words: insects, Ephemeroptera, DNA barcodes, Kamchatka, Russian Far East.

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Резюме. Получены ДНК баркоды для 75 особей поденок относящимся к 10 видам, четыре из которых ранее были описаны из водотоков Камчатки. Сравнительный анализ полученных сиквенсов и данных BOLD позволил расширить ареалы для *Acentrella diptera* Kluge et Novikova, 2011, *Baetis pseudothermicus* Kluge, 1983, *Cinygmula malaisei*, Ulmer, 1927, *Ephemerella aurivillii* Bengtsson, 1908 и *E. mucronata* Bengtsson, 1908. Последовательности XJDQD534-18 - XJDQD542-18 из Китая следует перенести из рода *Baetiella* Ueno 1931 к виду *Baetis pseudothermicus*. Несмотря на высокие внутривидовые K2P дистанции для *Ephemerella mucronata* и *E. aurivillii*, мы не считаем оправданным разделение их на несколько самостоятельных видов и предлагаем увеличить порог внутривидовой дивергенции до 6.47% и более для рода *Ephemerella* Walsh семейства Ephemerellidae.

INTRODUCTION

The experience of research conducted on the mountain and foothill streams of Kamchatka has shown that when analyzing primary hydrobiological monitoring data, it is advisable to use a set of simple coefficients that provide an adequate assessment qualitative and quantitative change in benthic invertebrate communities in response to any type of anthropogenic impact (Chebanova, 2009). One of these indices is the *EPT* index, an indicator group of the invertebrate orders Ephemeroptera (mayfly), Plecoptera (stonefly), and Trichoptera (caddisfly), which are the least tolerant to various types of pollution (Rusanov *et al*., 1990; Lenat, 1994; Angradi, 1999; Mebane, 2001; Zweig & Rabeni, 2001; Tiunova *et al*., 2011; Teslenko *et al*., 2023). The reaction of the *EPT* indicator group to pollution is taken into account by changing their species diversity. Therefore, in order to achieve an accurate calculation of the *EPT* index, correct species diagnostics of mayflies at different stages of their development is required. However, reliable species characteristics are often absent in closely related species, and identification of larvae and imagos requires considerable time. In this case, DNA barcoding is the most acceptable and convenient method, which makes it possible to compare mayflies not only at the species level but also at different stages of development and sexes.

Currently, the fauna of the bottom streams of Kamchatka includes 36 species from 17 genera and 8 families (Tiunova, 2022). In terms of the number of species in Kamchatka's watercourses, the Heptageniidae family is the leader (12), followed by the Baetidae (9) and Ephemerellidae (6 species); the remaining families are represented by 1-3 species. The fauna consists mainly of widespread Circumboreal (3 species), Transpalearctic (14 species), and East Palearctic (14 species) species. Five species have a Paleoarchearctic distribution type. At the same time, for a number of species, such as *Rhodobaetis molecularis* Tiunova et Semenchenko, 2020; *Ameletus camtschaticus* Ulmer, 1927; *Cinygmula malaisei* Ulmer, 1927; and *Cinygmula cava* Ulmer, 1927, the Kamchatka watercourses are a typical habitat.

In this study, we provided the results of DNA barcoding using cytochrome oxidase subunit I (COI) of mayflies inhabiting the Kamchatka Peninsula. We also analyzed additional sequences of conspecific mayflies from other locations in Russia to obtain representative results.

MATERIAL AND METHODS

The material was collected in five regions of the Russian Far East: Primorsky and Khabarovsk Territory, Amur Region, Jewish Autonomous Region, Chukotka Autonomous Okrug, Kamchatka Territory, Magadan Region, Republic of Buryatiya, and Republic of Sakha-Yakutiya in 2014–2023. Material from the Kamchatka Peninsula was collected during expeditions in 2014, 2015, 2018, and 2022. Material for DNA analysis was preserved in 96% ethanol. The material was deposited in the collection of the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences, Vladivostok.

Total DNA was extracted from the thorax or legs using the Qiagen DNeasy Blood and Tissue Kit or the Invitrogen PureLink Genomic DNA Mini Kit in accordance with the protocol in a final elution volume of 100 µL. The primers for amplification of the COI fragment were LCO1490 and HCO2198, according to Folmer *et al.* (1994). The PCR reaction for this fragment was run in a 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 template profile was as follows: 94.0°C for 5 min; 35 cycles at 94.0°C for 30 sec, 48.0°C for 30 sec, and 72.0°C for 60 sec; with a final extension at 72.0°C for 5 min. The PCR product was purified using Exonuclease I and Thermosensitive Alkaline Phosphatase

(ThermoFisher Scientific, Waltham, MA, USA) and sequenced for both directions. Sequencing reaction was performed using BigDye® Terminator v3.1 Cycle Sequencing Kits and run on an ABI 3130xl Genetic Analyzer Sequencer (Applied Biosystems, Foster City, CA, USA). Forward and reverse sequences were manually assembled and edited using Finch TV and MEGA 7 (Kumar *et al*., 2016). Based on the K2P, inter- and intraspecific genetic distances are calculated using MEGA7.

For reconstruction of phylogenetic relationships, we added sequences from the BOLD system (The Barcode of Life Data Systems). We selected each sequence longer than 500 base pairs and belonging to the same BIN (barcode index number) as the sequences obtained in this study. In the case of several samples from one localization belonging to the same haplotype, we selected only one DNA barcode. In some cases, molecularly conspecific species were representatives of other morphological species and even genera. For the remaining species of the genera *Acentrella* Bengtsson, 1912, *Ameletus* Eaton, 1885, *Rhodobaetis* Jacob, 2003, *Cinygmula* McDunnough, 1933, and *Ephemerella* Walsh, 1862, we selected one sequence from each BIN number defined to the species level. In the case of several BINs belonging to one morphological species, we selected samples collected closest to the type habitat. For species delimitation we use Assemble Species by Automatic Partitioning (ASAP) analysis that is implemented on the website (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html, Puillandre *et al*. 2021) with K2P distances.

Bayesian phylogenetic analyses were conducted with MrBayes v. 3.2.7 (Ronquist *et al*., 2012). 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 the *COI* gene. 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. The chains were run for 1 million generations and sampled every 500 generations. FigTree v. 1.4.4 was used to visualize the phylogenetic tree after analysis. Sequences have been deposited in GenBank under numbers PV259908–PV259944 and PV698190–PV698228.

RESULTS AND DISCUSSION

The final alignment of the *COI* gene yielded 597–658 bp. A total of 75 mayfly samples belonging to 10 species from 7 genera were sequenced in this study. The most samples (61) were larvae or mature larvae. Detailed descriptions of development stages, sampling locaions and dates, collectors, and geographical coordinates are given in the GenBank according to accession numbers.

The average intraspecific K2P distances of the samples sequenced in this study combined with molecularly conspecific samples after ASAP analyses (see below) were as follows: *Acentrella diptera* Kluge et Novikova, 2011 – 0.54%, *Ameletus camtschaticus* Ulmer, 1927 – 0.19%, *Rhodobaetis molecularis* Tiunova et Semenchenko, 2020 – 0.56%, *Baetis pseudothermicus* Kluge, 1983 – 1.68%, *Cinygmula cava* Ulmer, 1927 – 0.36%, *Cinygmula malaisei* Ulmer, 1927 – 0.56%, *Cinygmula putoranica* Kluge, 1980 – 0.68%, *Ephemerella aurivillii* Bengtsson, 1908 – 0.75%, *Ephemerella mucronata* Bengtsson, 1908 – 6.47% , and *Cinygma lyriformis* McDunnough, 1924. According to Moriniere *et al*. (2017), the maximum average values of intraspecific distances for Germany's mayflies that shared a single BIN (Ratnasingham &Hebert, 2013) were 0.1%, 1.55%, 0.50%, and 1.04% for Ameletidae, Baetidae, Ephemerellidae, and Heptageniidae, respectively.

Thus, the values of observed intraspecific variation fall within known mayfly's thresholds in accordance with families. High values (6.47%) were obtained for *Ephemerella mucronata*, while the minimum interspecific distances for Heptageniidae could fall to 2.49% (species pair

of comparison *Ecdyonurus helveticus* Eaton, 1886 and *E. zelleri* Eaton, 1885) or 4.77% (species pair of comparison *E. macani* Thomas et Sowa, 1970 and *E. torrentis* Kimmins, 1942) (Moriniere *et al*., 2017). Therefore, additional species delimitation analysis is required for this species, which is presented below. We also sequenced one sample of *Cinygma lyriformis* McDunnough. The sequence is new for the GenBank and BOLD systems. The latter database contains two additional sequences of this genus, not identified to the species level. Therefore, there are no data for calculating intraspecific distances and reconstructing a phylogenetic tree.

The range of *Acentrella diptera* is in East Siberia and the Russian Far East (Kluge & Novikova, 2011). We obtained sequences for samples from Kamchatka, Primorsky, and Khabarovsk Territory and Chukotka Autonomous Okrug of Russia (Fig. 1). *Acentrella diptera* is not listed in the GenBank and BOLD system, but the results of the ASAP analysis indicate 5 samples from Alaska belonging to *A. turbida* McDunnough, 1924 (BOLD Process IDs: COLEC003-15, COLEC004-15, COLEC061-15, COLEC062-15, and UAMIC4410-21)

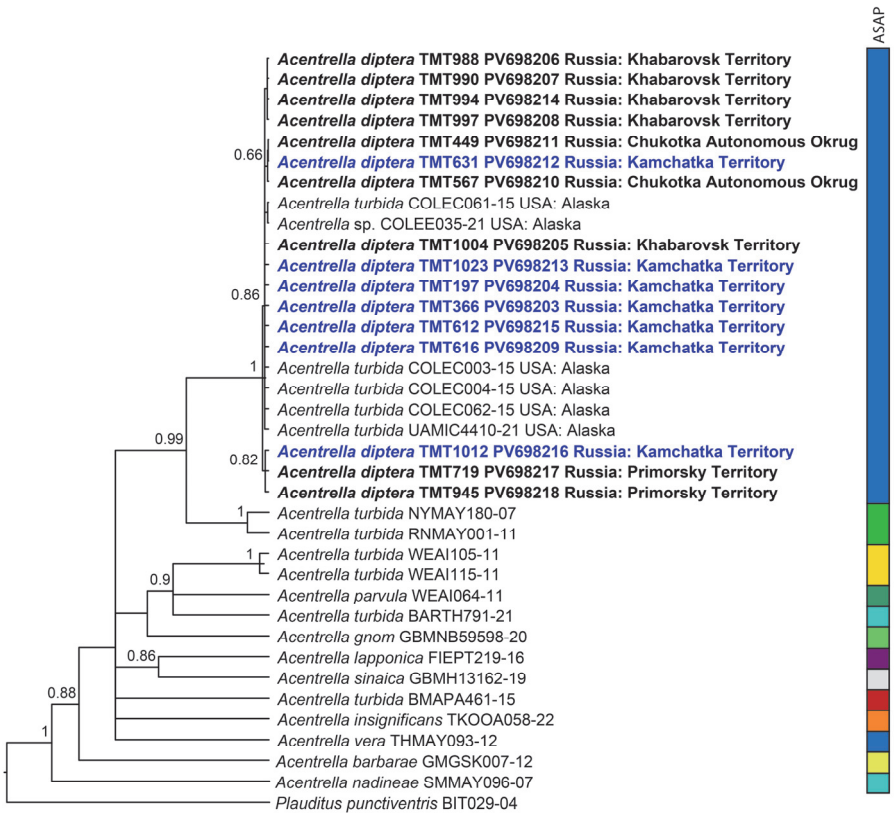

Fig. 1. Ultrametric Bayesian inference (BI) tree based on the cytochrome c oxidase I (COI) nucleotide sequence data of the genus *Acentrella* Bengtsson. Bayesian posterior probabilities (higher than 0.7) are given above tree nodes. Specimens obtained in this study are in bold. Samples from Kamchatka are highlighted in blue. *Plauditus punctiventris is* used as outgroup.

and *Acentrella* sp., also from Alaska (COLEC035-21), are conspecific with *A. diptera* (BIN AEH3172). Moreover, in addition to this BIN number, the Nearctic species *A. turbida* includes 8 additional BIN clusters. Thus, we assume that each sequence shared by BIN AEH3172 belongs to the species *A. diptera*, and its range should be extended to the northeastern Nearctic. The sister clade to *A. diptera* was *A. turbida,* belonging to two BIN clusters — AAC2912 from the eastern Nearctic and ABA1944 from the western Nearctic (Bayesian posterior probability, BPP = 0.99) (Fig. 1). The average K2P interspecific distances between these clusters were 18.18% and 17.03%, respectively. Most of the remaining species of the genus *Acentrella* Bengtsson, 1912 shared a polytomous clade without strong support (BPP < 0.7).

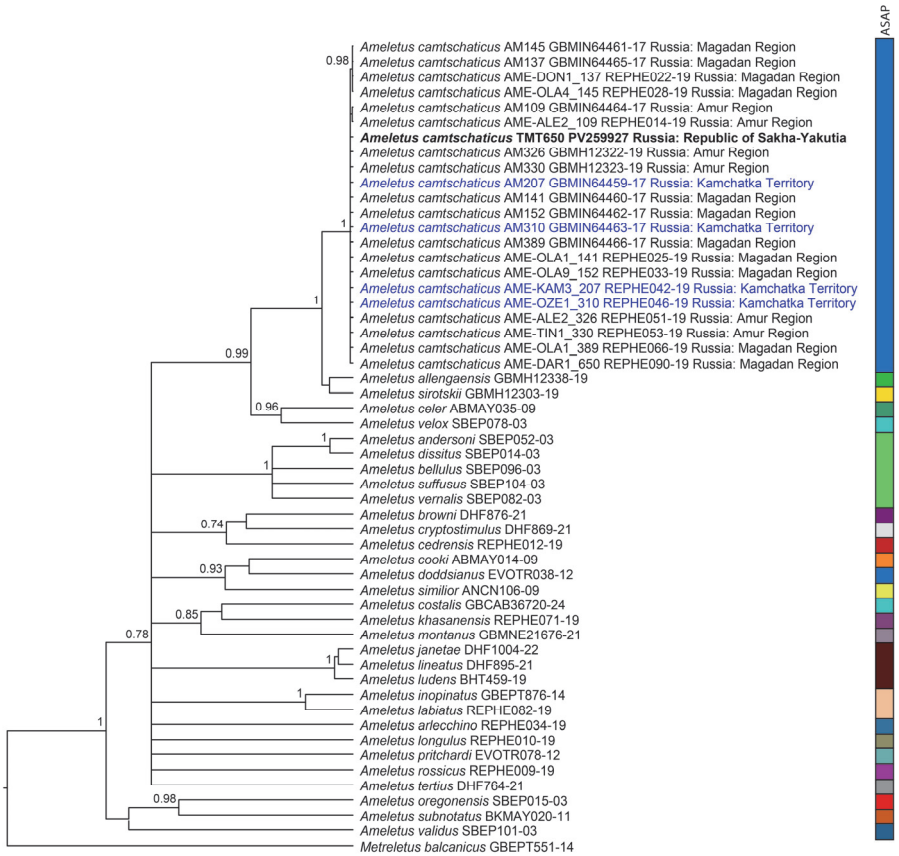

Fig. 2. Ultrametric Bayesian inference (BI) tree based on the cytochrome c oxidase I (COI) nucleotide sequence data of the genus *Ameletus*. Bayesian posterior probabilities (higher than 0.7) are given above tree nodes. Specimens obtained in this study are in bold. Samples from Kamchatka are highlighted in blue. *Metreletus balcanicus is* used as outgroup.

Ameletus camtschaticus was described from Kamchatka Territory by Ulmer in 1927. The species are distributed in the Far East of Russia: in the Khabarovsk Territory, Amur and Magadan Region, Kamchatka Territory, and the Republic of Sakha-Yakutiya. Tiunova *et al*. (2017) provide several descriptions of mayflies collected in the Magadan and Amur Region and Kamchatka Territory. In this study we obtained a single sequence from the Republic of Sakha-Yakutia related to the most common haplotype (Fig. 2). To date (July 2025), there are no available sequences from Khabarovsk Territory to cover all regions inhabited by *A. camtschaticus* with DNA barcodes.

The sister clade (BPP = 1) to *A. camtschaticus* was *A. sirotskii* Tiunova et Semenchenko, 2017 and *A. allengaensis* Tiunova et Semenchenko, 2017 also from the Russian Far East (Fig. 2). All three species are distinguished by the size and location of small denticles on the ventral plate of the penis in male imagoes and by the size of gills I and II in larvae. In *A. allengaensis* and *A. sirotskii* differ from *A. camtschaticus* by gill II, which does not have an anal rib on the anal margin (Tiunova *et al.*, 2017). Most of the remaining species of the genus *Ameletus* Eaton, 1885 shared a poorly supported polytomous clade (BPP = 0.78).

Rhodobaetis molecularis was recently described by Tiunova & Semenchenko (2020). This species is distributed in 6 regions of the Russian Far East: Chukotka Autonomous Okrug, Kamchatka and Khabarovsk Territory, Magadan Region, Jewish Autonomous Region, and Amur Region. Holotype specimens collected from Kamchatka Territory. In this study, we added 6 new sequences of this species from the same regions of the Russian Far East, as well as sample TMT737 from the Jewish Autonomous Region (Fig. 3).

The results of the ASAP analysis confirm conspecificity of all *R. molecularis* sequences (Fig. 3). Sister clade (BPP = 1) was *R. foemina* McDunnough, 1936 (BIN AAD4529); in turn, the sister of *R. molecularis* + *R. foemina* (BPP = 1) was one (BIN AAZ1614) of the seven currently known BINs, belonging to the *R. bicaudatus* Dodds, 1924. Most of the remaining species of the genus *Rhodobaetis* Jacob, 2003 shared a polytomous, highly supported clade (BPP = 1).

The holotype of *Baetis pseudothermicus* was collected in Primorsky Territory, Khasansky District, Kedrovaya River (Kluge ,1983). In this study, we obtained 13 sequences, including TMT544 from the Nezhinka River (Fig. 3), which is located 43 km north of the type locality. The new sequences were conspecific with our previously deposited data, including several samples from the Narva River (BP34, BP35), which is located 16 km south of the type locality. In addition to Primorsky Kray, conspecific samples by ASAP analyses were collected in the Irkutskaya Region, Republic of Buryatia, Kamchatka Kray, and Khabarovsk Territory. Thus, there is no doubt that the obtained sequences belong to the originally described *B. pseudothermicus* (BIN ADL2208). In turn, to date the BOLD system contains five additional BINs for *B. pseudothermicus*: BIN AEG6652 (Russia: Kamchatka, samples BP190 and BP672), BIN AEG1238 (Russia: Primorsky Territory, sample BP179), BIN AAA2041 (from Canada, USA, China, and Iraq, including species *B. phoebus* McDunnough, 1923, *B. flavistriga* McDunnough, 1921 and *B. notos* Allen and Murvosh 1984, used process ID GBMNF32766-22 on a Fig. 3 as an example), BIN AEB6454 (from South Korea, used process ID GBMNF32764-22 on a Fig. 3 as an example), and ADJ8883 (from Japan and South Korea, including species *B. sahoensis* Gose, 1980 used process ID GBMNF32767-22 on a Fig. 3 as an example). In our opinion the formation of the first and second BINs is associated with high sensitivity of the BIN-forming algorithm (Ratnasingham & Hebert, 2013). ASAP analysis does not confirm the results of BOLD analysis, and BIN ADL2208 differs from BIN AEG6652 and BIN AEG1238 by 4.27% and 4.78%, respectively. In turn, BIN ADL2208 differs from the third, fourth, and fifth BINs by 24.41%, 23.22%, and 20.19%. High values of K2P distances, not monophyletic with the type *B. pseudothermicus* cluster, and the remoteness of the sampling locations from the type habitat confirm that BINs AAA2041, AEB6454, and ADJ8883 do not belong to *B. pseudothermicus*.

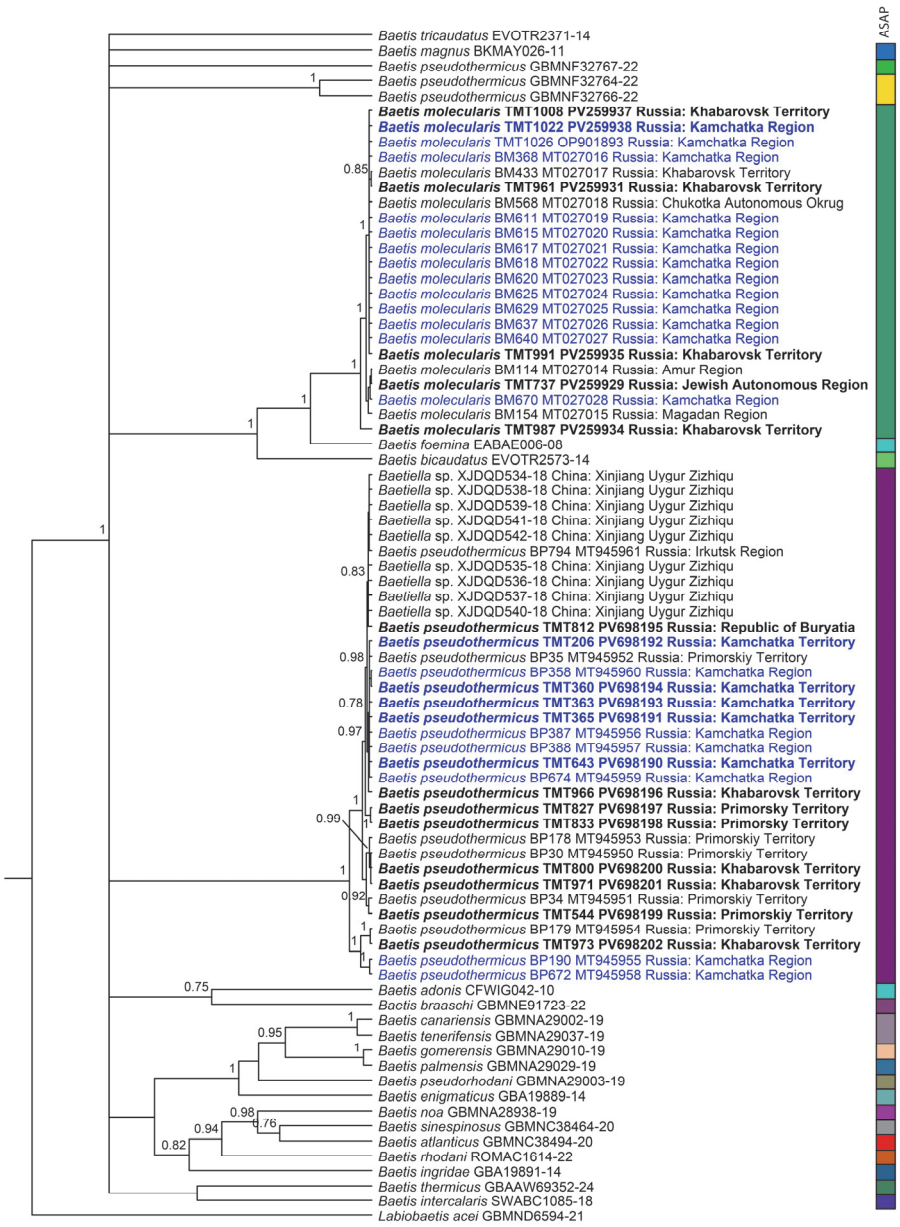

Fig. 3. Ultrametric Bayesian inference (BI) tree based on the cytochrome c oxidase I (COI) nucleotide sequence data of the genus *Baetis*. Bayesian posterior probabilities (higher than 0.7) are given above tree nodes. Specimens obtained in this study are in bold. Samples from Kamchatka are highlighted in blue. *Labiobaetis acei was* used as outgroup.

There are 9 sequences deposited in the BOLD system, identified as *Baetiella* sp. from China, Xinjiang Uygur Autonomous Region (Fig. 3). These sequences share a single BIN with the type *Baetis pseudothermicus* (BIN ADL2208). Based on this, we assume that sequences XJDQD534-18 - XJDQD542-18 are erroneously assigned to the genus *Baetiella* sp. and belong to *B. pseudothermicus*, expanding the species range to northwestern China.

In 2022 we deposited many sequences of *Cinygmula putoranica* and *C. cava* in GenBank, which have not been mined into the BOLD systems database to date. In turn, DNA barcodes of *C. malaisei* were deposited in GenBank for the first time in this study. *C. putoranica* was described from the Taimyr Peninsula of Krasnoyarsky Territory. Morphology and molecular data are expanding the species range in the Kamchatka Territory, Chukotka Autonomous Okrug, Amur Region, and Khabarovsk Territory. The closest collection point to the typical habitat in our collections is a sample from the watercourses of the Chukotka Autonomous Okrug. The results of the ASAP analysis confirm the conspecificity of all the samples of *C. putoranica* (Fig. 4).

Cinygmula cava was sister to *C. putoranica* (BPP = 0.99) (Fig. 4). *C. cava* was described from Kamchatka but is widespread in East Siberia and the Russian Far East: Primorsky and Khabarovsk Territory, Jewish Autonomous and Sakhalinskaya Regions, Republic of Sakha (Yakutiya), Kamchatka Territory and Altai Republic, Republic of Buryatiya, and Irkutsk Territory. According to the results of the ASAP analysis, all samples of *C. cava* belong to a single species. *C. malaisei* was sister to the clade containing *C. cava* + *C. putoranica*. This species is also described from Kamchatka, but conspecific DNA barcodes were also collected in the Khabarovsk Territory, Magadan and Amur Region according to the results of ASAP analysis. *Cinygmula* sp. collected in Yukon, Canada (GenBank KJ675257, BOLD Process ID: GBMH12106-19), also belongs to the species *C. malaisei* according to the species delimitation method, which significantly expands the range of this species. Apparently, *C. malaisei* is also distributed in the Chukotka Autonomous Okrug and Alaska.

Ephemerella mucronata was described from Sweden by Bengtsson in 1909. Subsequently, the range of this species has significantly expanded to the Western and Eastern Palaearctic up to the Russian Far East. Fragments of the *COI* gene of *E. mucronata* from Finland (Kjaerstad *et al*., 1999), Germany (Moriniere *et al*., 2017), and Slovakia (Macko *et al*., 2024) were sequenced and deposited in GenBank. We have obtained DNA barcodes for four samples of *E. mucronata* from Kamchatka and Khabarovsk Territory (Fig. 5). The samples used in the phylogenetic tree belonged to the same mOTU (molecular operational taxonomic unit) according to the results of the ASAP analysis. However, this species was divided into 4 different BINs by BOLD systems. The first number, BIN AAU0444 (process ID EPHFI065-11 used in Fig. 5 as an example), is a sister to the obtained DNA barcodes. Specimens were distributed in Norway, Finland, China, and Russia. The second BIN AAL6162 is known from Bulgaria: Sofiya (used process ID BGMAY003-10 used in Fig. 5 as an example). The third BIN AAL0654 was collected in Germany, Switzerland, Slovakia, Poland, and Czechia (process ID GBEPT079-13 used in Fig. 5 as an example). Finally, the fourth BIN AAY4985 was collected in Bulgaria, Slovakia, Romania, Ukraine, and Serbia (process ID BGMAY288-11 used in Fig. 5 as an example). Despite significant intraspecific distances (reaching 6.47%, see above) and dividing into four different BINs, we consider it appropriate to consider all samples belonging to a single species, *E. mucronata*. The reasons for this are 1) the uniting of each sequence into a single mOTU according to ASAP analysis, 2) the monophyly of the *E. mucronata* clade by Bayesian inference, and 3) the absence of morphological differences between the species described in Swedish and Russian samples (Tshernova, 1952; Tshernova *et al*., 1986; Kluge, 1997).

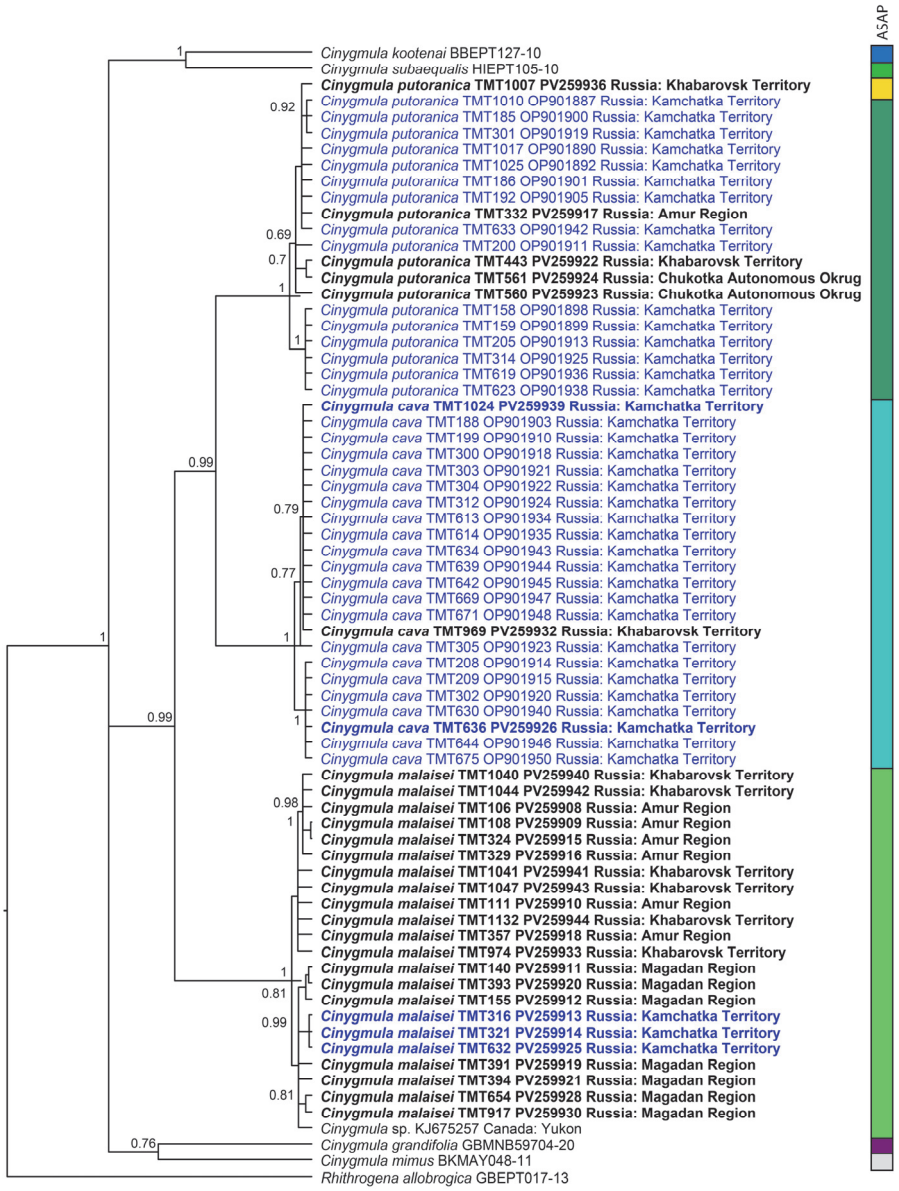

Fig. 4. Ultrametric Bayesian inference (BI) tree based on the cytochrome c oxidase I (COI) nucleotide sequence data of the genus *Cinygmula*. Bayesian posterior probabilities (higher than 0.7) are given above tree nodes. Specimens obtained in this study are in bold. Samples from Kamchatka are highlighted in blue. *Rhithrogena allobrogica is* used as outgroup.

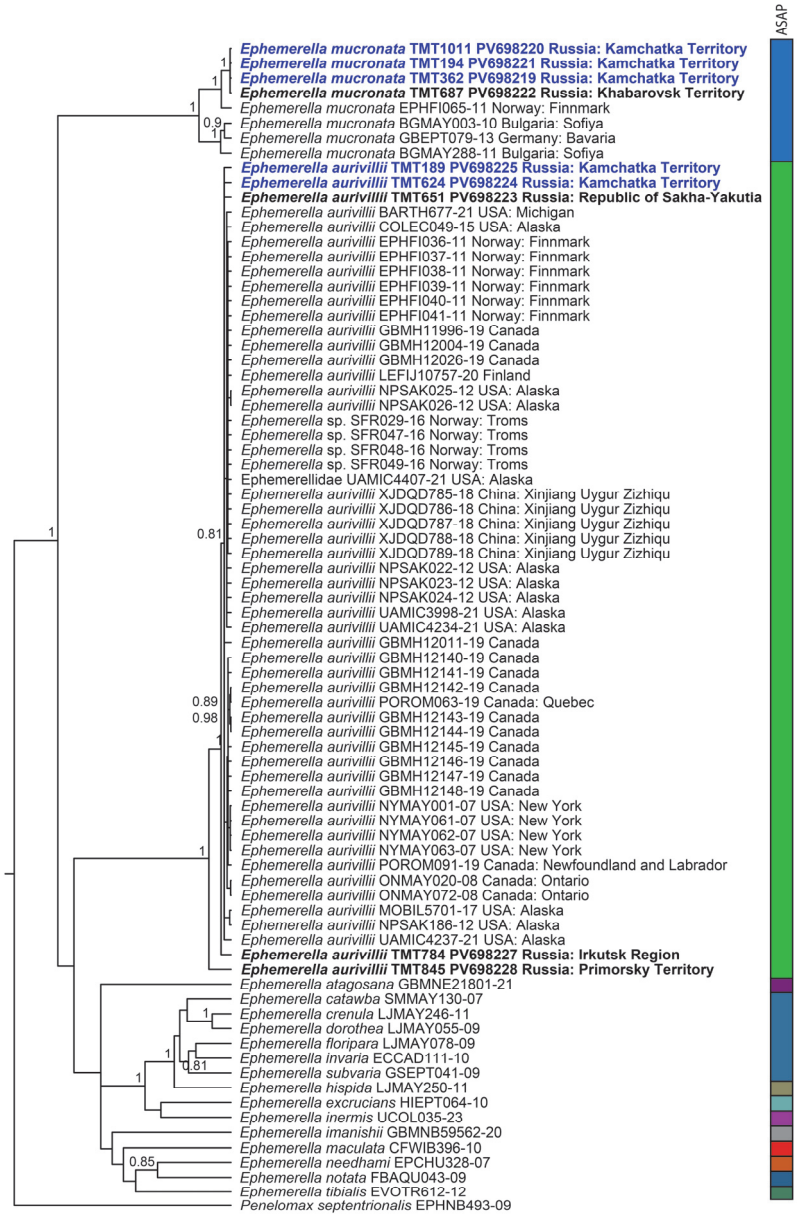

Fig. 5. Ultrametric Bayesian inference (BI) tree based on the cytochrome c oxidase I (COI) nucleotide sequence data of the genus *Ephemerella*. Bayesian posterior probabilities (higher than 0.7) are given above tree nodes. Specimens obtained in this study are in bold. Samples from Kamchatka are highlighted in blue. *Penelomax septentrionalis was* used as outgroup.

Ephemerella aurivillii was also described from Sweden; the species is distributed in the Western Palaearctic and Northern Nearctic, which is confirmed by taxonomic studies and DNA barcodes (Webb *et al*., 2012, Kjaerstad *et al*., 1999, Fig. 5). Obtained DNA barcodes extend the range of this species to the Russian Far East, including the Kamchatka Territory, Republic of Sakha-Yakutiya, and Irkutsk and Primorsky Territory. The sample TMT845 from Primorsky Territory significantly differed from the other samples; the average K2P distances were 4.51%. Despite this, the amino acid sequence coincided with most of the DNA barcodes of *E. aurivillii* except for three samples that had non-synonymous substitutions. In our opinion, each DNA barcode of *E. aurivillii* used in analysis, as in the case of *E. mucronata*, belongs to one species, and the level of intraspecific distances in the Ephemerellidae family is above 6.47%.

One sample of *Cinygma lyriformis* McDunnough from Kamchatka Territory was sequenced. DNA barcode was new for BOLD and GenBank. To date 18 DNA barcodes of genus *Cinygma* Eaton from Nearctic available in BOLD, but none of them has been identified to species level.

In the course of this study, we obtained DNA barcodes from 75 samples of mayflies belonging to 10 species, which is less than a third of the known mayfly species in the Kamchatka Territory. Comparative analysis of new sequences and BOLD system data allowed us to expand the range of *Acentrella diptera*, *Baetis pseudothermicus*, *Cinygmula malaisei*, *Ephemerella aurivillii,* and *E. mucronata*. Sequences XJDQD534-18 - XJDQD542-18 from China should be transferred from the genus *Baetiella* Ueno 1931 to the species *Baetis pseudothermicus*. Despite the high K2P distances for *Ephemerella mucronata* and *E. aurivillii*, we do not consider to divide them into several species and propose to increase the threshold of intraspecific differences by 6.47% or more for the genus *Ephemerella* Walsh of the family Ephemerellidae.

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