

**EVALUATING DNA BARCODING THRESHOLDS IN THE STONEFLY
GENUS *ALLOPERLA* BANKS, 1906 (PLECOPTERA: CHLOROPERLIDAE)
AND IMPLICATING FOR THE SYNONYMY OF *A. MEDIATA* (NAVÁS, 1925)**

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Summary. In this study, we successfully generated mitochondrial cytochrome *c* oxidase subunit I (*COI*) DNA barcodes for 10 stonefly specimens belonging to three Eastern Palearctic species of the genus *Alloperla* Banks, 1906: *A. mediata* (Navás, 1925), *A. rostellata* (Klapálek, 1923), and *A. teleckojensis* Šámal, 1939. Based on both morphological features and molecular evidence, *Alloperla ishikariana* Kohno, 1953 from Japan was found to be conspecific with the widespread *A. mediata* and is hereby formally downgraded to its junior synonym. A genus-wide phylogenetic reconstruction and species delimitation revealed three distinct types of incongruence between current public sequence repositories and morphological taxonomy: 1) several distinct morphospecies shared single mOTU, 2) a single morphospecies splitting into multiple distant non-monophyletic molecular operational taxonomic units (mOTUs), and 3) strictly monophyletic lineages of a single species being artificially fragmented into different mOTUs due to the over-sensitivity of the BOLD and ASAP frameworks. Pairwise divergence histograms combined with a blind kernel density estimation analysis via the spider library conclusively demonstrated that the macro-evolutionary barcoding gap occurs at exactly 6.1%. Implementing this empirically derived threshold successfully corrected algorithmic over-splitting and validated the conspecificity of deeply divergent allopatric populations within *Alloperla chloris* Frison, 1934, *A. petasata* Surdick, 2004, *A. teleckojensis*, and *A. mediata*.

Key words: Plecoptera, *Alloperla*, taxonomy, synonymy, DNA barcodes, barcoding gap, Eastern Palearctic.

А. А. Семенченко, В. А. Тесленко. Оценка пороговых значений ДНК-баркодинга в роде веснянок *Alloperla* Banks, 1906 (Plecoptera: Chloroperlidae) и обоснование синонимии *A. mediata* (Navás, 1925) // Дальневосточный энтомолог. 2026. N 560. С. 17-32.

Резюме. В настоящей работе впервые получены ДНК-баркоды по фрагменту митохондриального гена цитохромоксидазы I (*COI*) для 10 экземпляров веснянок, принадлежащих к трем восточнопалеарктическим видам рода *Alloperla* Banks, 1906: *A. mediata* (Navás, 1925), *A. rostellata* (Klapálek, 1923) и *A. teleckojensis* Šámal, 1939. На основании

комплекса морфологических и молекулярно-генетических данных установлено, что описанный из Японии вид *Alloperla ishikariana* Kohno, 1953 консpezifичен широко распространенному виду *A. mediata* и должен быть сведен в младшие синонимы. Филогенетический анализ и методы разграничения сиквенсов на молекулярные операционные таксономические единицы (МОТУ) для ДНК-баркодов рода выявил три основных типа таксономических несоответствий: 1) несколько видов объединились к одной МОТУ; 2) один вид разделился на немонофилетичные МОТУ; 3) произошло разделение монофилетических линий одного вида на разные МОТУ в силу избыточной чувствительности алгоритмов BOLD и ASAP. Построение гистограмм распределения попарных дистанций в сочетании со «слепым» анализом плотности Кернела в пакете spider показало, что порог разделения внутривидовой и межвидовой изменчивости находится на отметке 6.1%. Применение этого порога позволило скорректировать избыточную чувствительность автоматических программ и надежно подтвердить консpezifичность географически удаленных популяций с высокой внутривидовой дивергенцией для *Alloperla chloris* Frison, 1934, *A. petasata* Surdick, 2004, *A. teleckojensis* и *A. mediata*.

INTRODUCTION

The genus *Alloperla* Banks, 1906, belongs to the tribe Alloperlini Surdick, 1985 of the subfamily Chloroperlinae and family Chloroperlidae. *Alloperla* includes about 52 species distributed across the Nearctic and East Palearctic realms (DeWalt *et al.*, 2026). There are about 15 East Palearctic species in six countries: Japan (3), Korea (4), Mongolia (4), Kazakhstan (5), Russia (8), and China (9) (Shimizu *et al.*, 2005; Hwang & Murányi, 2020; Judson & Nelson, 2012; Devyatkov, 2022; Teslenko & Zhiltsova, 2009; Rehman *et al.*, 2025). Of them three species only, *Alloperla joosti* Zwick, 1972, *A. mediata* (Navás, 1925) and *A. rostellata* (Klapálek, 1923), are widespread and common to five countries. However, in Japan, *Alloperla* species widespread on the mainland have not yet been officially recorded, with the exception of the insular *A. kurilensis* Zhiltsova, 1978, known from the Southern Kuril Islands and Hokkaido. At the same time, despite the specific insular character of the Plecoptera fauna, about three dozen stonefly species from other genera and families shared Sakhalin Island, the Kuril Islands, and the Eurasian continent have been recorded in Japan (Teslenko & Zhiltsova, 2009). Recently, Yoshinari *et al.* (2024) discovered the Eastern Palearctic mainland species *Utaperla orientalis* Nelson & Hanson, 1969 (Plecoptera: Chloroperlidae) in the Satsunai River, Hokkaido, Japan, for the first time. Along with morphological characteristics, genetic information was presented, including for the species *Alloperla ishikariana* Kohno, 1953, collected from the same river. After comparison, we found that the DNA barcoding sequences of *A. ishikariana* and *A. mediata* are very similar. In this study, using our own collections, including those from the type locality, we conduct a thorough comparison of *A. mediata* and *A. ishikariana*, and taking into account the DNA barcoding data for both species, we propose considering *A. ishikariana* Kohno, 1953 as a junior synonym of *A. mediata* (Navás, 1925).

To date, 282 publicly available DNA barcodes of the genus *Alloperla* have been released, clustered into 25 BINs (BOLD system). Although these sequences represent 22 formally identified species, a significant portion of the records remains unidentified. Despite the substantial number of sequences, most studies incorporating these data focus on broad-scale invertebrate biodiversity and ecology across regions such as Canada (Hebert *et al.*, 2016; deWaard *et al.*, 2019; Erasmus & Huber, 2020; Cordero *et al.*, 2017) or local faunas the

USA, China, and Japan (Pilgrim *et al.*, 2011; Polato *et al.*, 2018; Yu *et al.*, 2019; Yoshinari *et al.*, 2023). In these studies, *Alloperla* stoneflies were treated as components of aquatic biocenoses without dedicated analysis of their specific molecular evolution or taxonomic boundaries.

In the present study, we provide 10 new DNA barcodes for three *Alloperla* species from Russia including the aforementioned *A. mediata*, and integrate them into a comprehensive analysis of phylogeny and species delimitation. Standard delimitation approaches, such as ASAP or the BOLD BIN system, often rely on a universal threshold of approximately 2% (Hebert *et al.*, 2003). However, for several genera within the family Chloroperlidae, the distance (p-distance or K2P) to the nearest neighbor (NN) sequence has been reported to be much higher, ranging from 7.94% to 17.04% (Zhou *et al.*, 2010; Morinière *et al.*, 2017; Hlebec *et al.*, 2022; Vuataz *et al.*, 2024). In this regard, we conducted a barcoding gap analysis to determine the precise threshold between intraspecific and interspecific K2P divergence. By comparing BOLD BIN assignments with morphologically determined species, we aim to clarify the COI evolution rate and resolve the taxonomic uncertainty currently surrounding the genus *Alloperla*.

MATERIAL AND METHODS

The specimens were collected through the sweeping of coastal vegetation using an entomological net. Samples were fixed with 75% ethanol.

The adults were studied in detail and documented by digital cameras (Nikon Coolpix 995 and Toup View 3.7) and with the stereomicroscope Olympus SZX16 and digital camera Olympus DP74, and stacked using Helicon Focus software. The final illustrations were post-processed for contrast and brightness using Adobe® Photoshop® software. The material is deposited in the collection of the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia.

Total genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) or ExtractDNA Blood & Cells (Evrogen, Moscow, Russia). We amplified the mitochondrial protein-coding gene cytochrome c oxidase I (COI) using polymerase chain reaction (PCR). For primers, reaction protocols, cycling programs, purification of PCR products, and sequencing details, see Teslenko & Semchenko (2022). The obtained sequences belonged to the ten specimens of *Alloperla* were deposited in GenBank under accession numbers PZ381789–PZ381798.

To assemble the comparative dataset, we retrieved all publicly available DNA barcodes for the genus *Alloperla* from the BOLD Systems database (as of May 2026), resulting in 282 records representing 25 BINs. Sequences were aligned, and genetic distances were calculated using the Kimura 2-parameter (K2P) model in MEGA7 (Kumar *et al.*, 2016). Molecular species delineation was achieved using Barcode Index Number (BIN) implemented in Barcode of Life Data System server (Ratnasingham & Hebert 2013) and Assemble Species by Automatic Partitioning (ASAP) (Puillandre *et al.*, 2021) using K2P distances as substitution model.

To visualize the barcoding gap, histograms of pairwise K2P distances were constructed in R v.4.6.0 (R Core Team, 2026) using the ggplot2 package. Pairwise K2P distances were categorized into intraspecific and interspecific groups according to two independent criteria: automated BOLD BIN assignments and morphological species identifications. For the latter, only specimens formally identified to the species level were included in the analysis. To perform a high-resolution barcoding gap analysis, separate detailed histograms restricted to a 10% divergence threshold were constructed for each approach. To provide a character-independent validation of these boundaries without prior taxonomic assumptions, a blind kernel density estimation (KDE) analysis was implemented using the spider v1.5.2 package

for *R.* The optimal macro-evolutionary divergence threshold for the genus *Alloperla* was mathematically determined via the localMinima function with a smoothing bandwidth of 1.0% (bw = 1.0). Based on the results of this combined barcoding gap visualization, an additional species delimitation analysis was performed using an empirically derived fixed distance threshold. This threshold was established to strictly separate intraspecific and interspecific K2P distances calculated between all individual sequences in the dataset.

To optimize dataset size and optimize space on the final phylogenetic reconstruction, a representative subset of sequences was selected. We included a single individual from each BIN. However, in cases where a single BIN contained multiple morphologically recognized species, one representative from each species within that BIN was selected to explicitly illustrate the lack of resolution between distinct morphological taxa. Specimens not identified to the species level (designated as *Alloperla* sp.) were excluded from the phylogenetic dataset, except for those that formed unique, distinct BINs.

Phylogenetic analyses were performed using MrBayes v.3.2.7 (Ronquist *et al.*, 2012) which was preceded by the selection of the best-fit partitioning scheme and models separately for each codon position of COI using PartitionFinder 2.1.1 (Lanfear *et al.*, 2012). 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. For the BI, two separate runs with four Markov chain Monte Carlo (MCMC) simulations were performed for 1 million generations, sampling every 500 generations and discarding the first 25% trees as burn-in. Trace files were visually inspected in Tracer v.1.7 (Rambaut *et al.*, 2018). Phylogenetic trees were visualized using FigTree v.1.4.4. *Suwallia errata* Li et Li, 2021 was selected as out-group. Finally, the molecular operational taxonomic units (mOTUs) resolved by the BOLD BIN system, the ASAP algorithm, and the newly established empirical within a single consensus tree. threshold was mapped alongside the resulting Bayesian topology to evaluate and visualize species delimitation consistency.

TAXONOMY

Alloperla mediata (Navás, 1925)

Figs 1–12

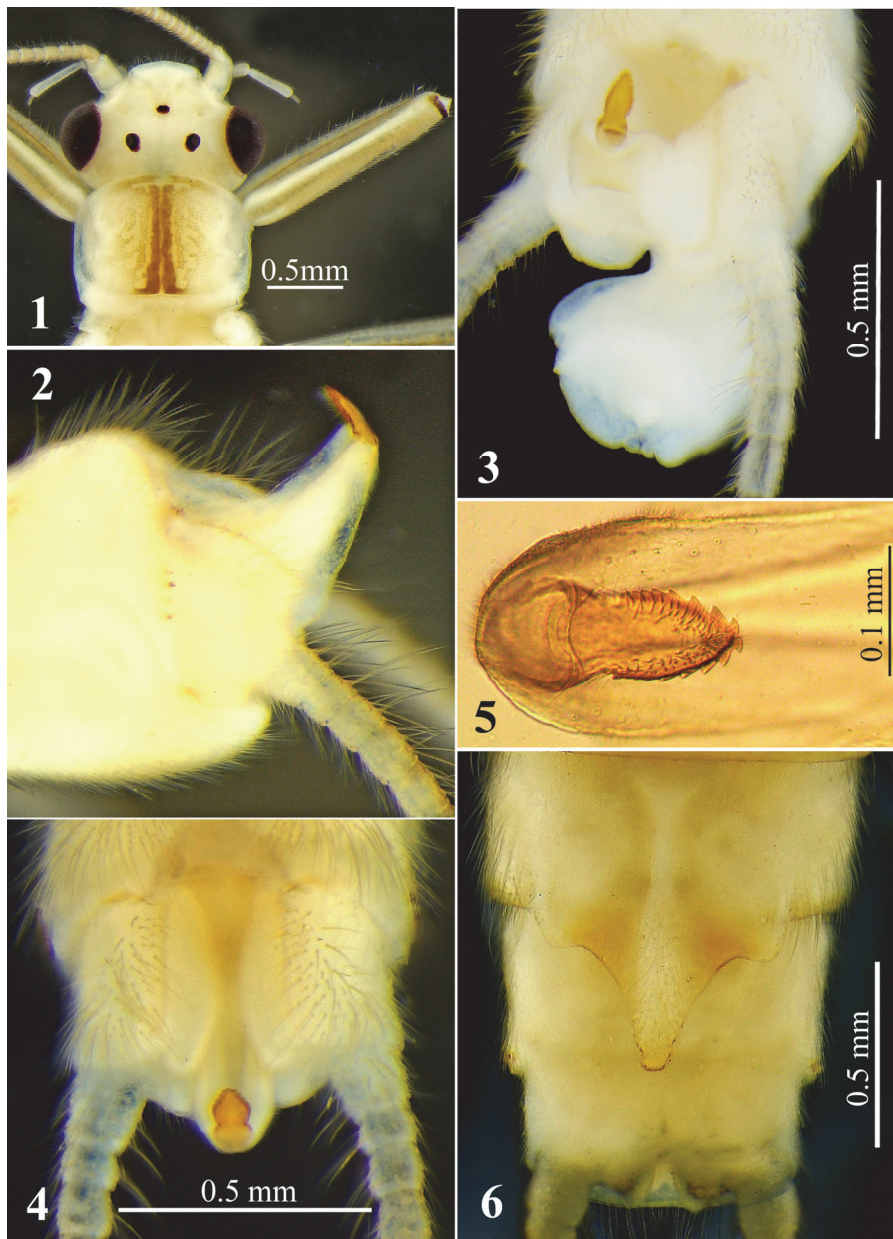
Chloroperla mediata Navás, 1925: 210, fig. 4a–b (original description of female from Russia); Zapekina-Dulkeit, 1961: 50 (distribution in Siberia).

Alloperla mediata: Zwick 1973: 285; Ham, 2008: 185; Teslenko, 2009: 699 (distribution); Teslenko & Zhiltzova 2009: 88, fig. 554–556 (key); Surenchorloo, 2009: 714 (first report for Mongolia); Judson & Nelson, 2012: 25, fig. 100–103 (male, female, distribution); Huo *et al.*, 2022: 6, 9, fig. 8 C–B (female, distribution); Rehman *et al.*, 2025: 368, fig. 4 A–D.

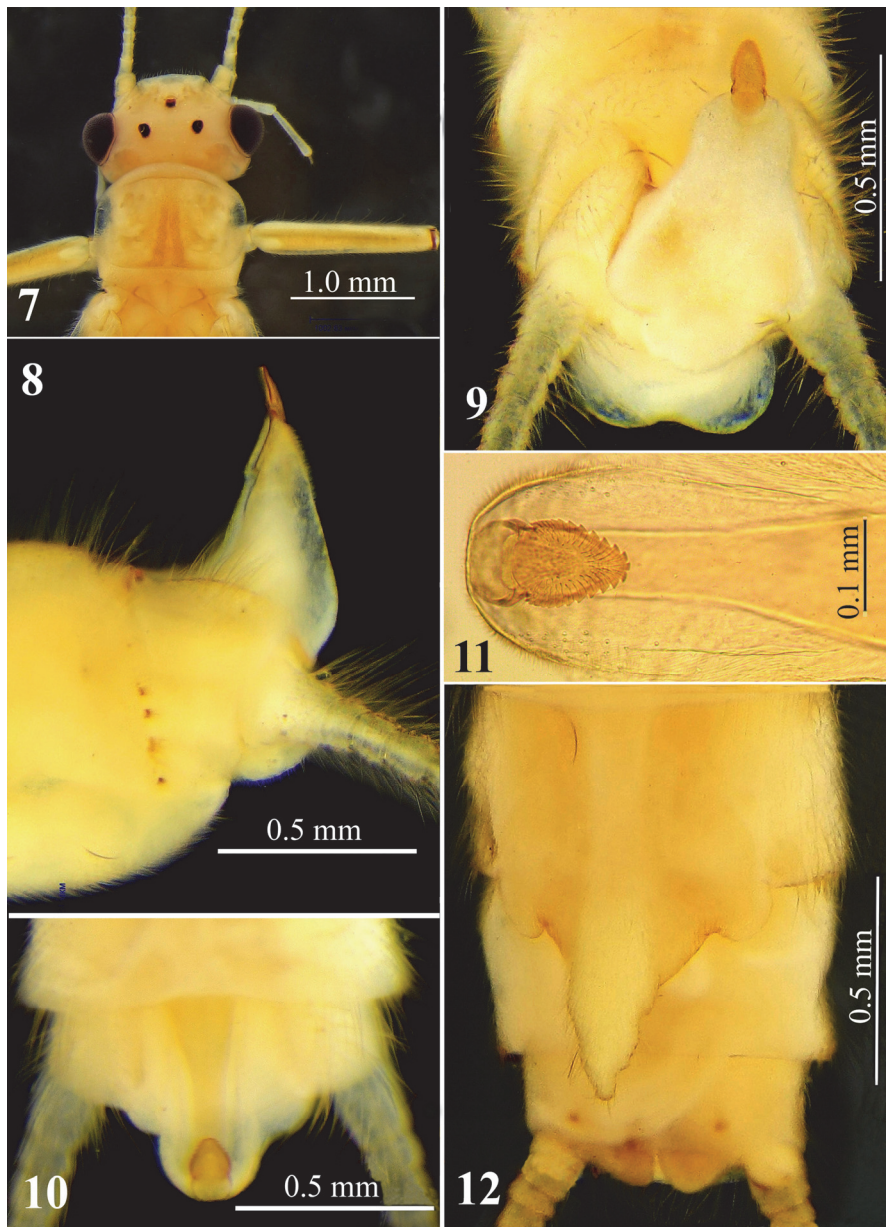
Alloperla (*Sweltsa*) *alexanderi* Nelson & Hanson, 1968: 425–428, fig. 4–6 (original description of male from China). Synonymized by Zwick *et al.* (1971: 855).

Alloperla ishikarianus Kohno, 1953: 67, Pl. 7 (original description of male); Illies, 1966: 432 (catalog); Shimizu *et al.*, 2005: 272, fig. 19–2–5 (male and female); Rahman *et al.*, 2021: 125886 (flight); Negishi *et al.*, 2022: 4203 (life cycle); Yoshinari *et al.*, 2024: 237, fig. 3, 5 (abundance, emergence, barcoding). **Syn. n.**

DIAGNOSIS. The general body color is pale yellow or yellow-green. Head pale brown, with a barely noticeable brownish M-line in front of the median ocellus and a pair of barely noticeable brownish round spots in the occiput closer to the eyes (Figs 1, 7). The pronotum has a characteristic dark medial stripe widened posteriorly (Figs 1, 7). Abdomen dorsally with



Figs 1–6. *Alloverla mediata* from type locality (Vladivostok): 1 – male head and pronotum, dorsal view; 2 – male epiproct with apical sclerite, lateral view; 3 – male epiproct with apical sclerite and everted penis, dorso-lateral view; 4 – male epiproct with apical sclerite, dorsal view; 5 – the apical sclerite, dorsal view; 6 – female subgenital plate, ventral view.



Figs 7–12. *Alloverla mediata* from Masunogawa River, Takino, Sapporo, Hokkaido, Japan: 7 – male head and pronotum, dorsal view; 8 – male epiproct with apical sclerite, lateral view; 9 – male epiproct with apical sclerite, dorso-lateral view; 10 – male epiproct with apical sclerite, dorsal view; 11 – apical sclerite, dorsal view; 12 – female subgenital plate, ventral view.

a median longitudinal black stripe. The erected epiproct is large, triangular, and directed upward with an extremely sclerotized small sclerite at the apex (Figs 2, 8). Sclerite is divided into two portions, nearly equal in width, separated by noticeable constriction (Figs 3–5, 9–11). The posterior portion of the sclerite is short and rounded; the anterior portion is oval with a pointed tip, bearing numerous symmetrical serrations along lateral margins, easily visible from the dorsal view (Figs 5, 11), and tiny setae ventrally (Figs 5, 11). The female subgenital plate (Figs 6, 12) is a long, narrow triangle extending almost the entire length of the sternum 9; the base of the subgenital plate is wide with a pair of rounded lobes laterally; the triangular apex of the subgenital plate is sometimes dark brown, sometimes with a rib in the middle, and covered with pale hairs; the lateral edges are uneven, especially in island populations of this species (Figs 6, 12).

MATERIAL EXAMINED. Russia: Primorsky Krai, vicinity of Vladivostok, the Chernaya Rechka River, 1.V 1987, 5♀, coll. TI Arefina; the same place, 12.VII 1994, 1♂, 2♀, coll. TI Arefina; a stream at the Chaika railway station near the FSC EATB FEB RAS, 5.VI 1995, 3♀, coll. TI Arefina; the Lazurnaya River, Shamora Bay, the Sea of Japan basin, 27.V 2007, 1♂, coll. VA Teslenko; an unnamed stream above the Energetik holiday home, the Sea of Japan basin, 3–16.VI 2014, 1♂, 2♀, coll. VA Teslenko; Mertvaya Pad' stream, above the Rybachy settlement, the Sea of Japan basin, 2.VI 2016, 1♀, coll. EA Gorovaya; Partizansk District, Shkotovka River, Shkotovo settlement, the Sea of Japan basin, 25–29.V 2015, 4♂, 6♀, coll. VA Teslenko; the same place, 1.VI 2025, 14♂ 16♀, coll. VA Teslenko. **Japan:** Hokkaido, Masunogawa River, Takino, Sapporo, 28.V 1991, 13♂, 26♀, coll. VA Teslenko; Eniva, Izari River, Okuizarigawa Bridge, 29.V 1991, 3♂, 1♀, coll. VA Teslenko.

DISTRIBUTION. East Palearctic species distributed widely: Republic of Altai, Irkutsk Oblast, Krasnoyarsk Krai, Republic of Buryatia, Republic of Tuva, and Russian Far East, including Chukotka Autonomous Okrug, Magadan Oblast, Kamchatka Krai, Khabarovsk Krai, Sakhalin Island, Kuril Islands, and Primorsky Krai. Occurs in the Eastern Kazakhstan (Devyatkov, 2022), Mongolia, China, South Korea, and Japan.

REMARKS. *Chloroperla mediata* was described by Navás (1925) from a single female collected in Vladivostok. The original description included two illustrations of a head with a pronotum and forewing tip, but the characteristic female subgenital plate was not illustrated. Zwick studied the holotype of *Chloroperla mediata*, and a drawing of the distinctive long subgenital plate extending from a broad base was first presented in a paper on the stonefly fauna of the Soviet Far East (Zwick *et al.*, 1971, fig. 19). In the same paper, a female was associated with a male (Zwick *et al.*, 1971, fig. 20) from numerous fresh specimens from Kamchatka. Additionally, a comparison of the *A. mediata* holotype with the topotype of a male *Alloperla alexandrii* Nelson et Hanson, 1968, described from the Greater Khingan, China, established the identity of both species and that *A. alexandrii* is a junior synonym of *A. mediata*. It was later established that *A. mediata* adults of the Kuril and Sakhalin populations exhibit considerable variability in a number of characteristics, including body color, shape and color of the female genital plate, color of the medial part of the pronotum and distal parts of the forewing veins (Levanidova & Zhiltzova, 1979). Shimizu *et al.* (2005) provided excellent illustrations of *Alloperla ishikariana* Kohno, 1953, described from Hokkaido, which were in agreement with illustrations of *A. mediata*, indicating they may refer to the same species. Although the similarity of the two species was not in doubt, no nomenclatural changes were made for them. The Japanese researchers noted that the relationship of insular *A. ishikariana* to mainland *A. mediata* requires further study (Shimizu *et al.*, 2005).

Recently, Yoshinari *et al.* (2024) discovered the Eastern Palearctic mainland species *Utaperla orientalis* in the Satsumai River, Hokkaido, Japan, for the first time. Along with morphological characteristics, genetic information was presented, including for the species

Alloperla ishikariana, collected from the same river. After comparison, we found that the DNA barcoding sequences of insular *A. ishikariana* and mainland *A. mediata* are very similar. In this study, using our own collections, including those from the type locality, we conduct a thorough comparison of *A. mediata* and *A. ishikariana*, and taking into account the DNA barcoding data for both species, we propose considering *A. ishikariana* as a junior synonym of *A. mediata*.

RESULTS OF COI DNA BARCODING

We sequenced fragments of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene (622–658 bp in length) from 10 specimens of the genus *Alloperla*, belonging to three species: *A. mediata*, *A. rostellata*, and *A. teleckojensis*. The average intraspecific K2P sequence divergence within these three species was 2.20%, 0.18%, and 3.12%, while the maximum intraspecific divergence reached 4.11%, 0.30%, and 3.12%, respectively. For the three newly sequenced species, *A. rostellata*, *A. mediata*, and *A. teleckojensis*, the minimum interspecific K2P distances to their nearest neighbors were *Alloperla delicata* (BOLD:AAJ2684) (11.08%) for the former, while both latter species shared the same closest match toward *Alloperla serrata* (BOLD:AAQ2344) at 12.61% and 6.79%, respectively.

Moreover, specimen of *Alloperla ishikariana* from Japan (BOLD process ID: GBAAX68627-24, BIN BOLD:AGN9030, GenBank accession number LC818982) was found to be conspecific with *Alloperla mediata*. The average K2P genetic distance between *A. ishikariana* and *A. mediata* was 1.16%, whereas the *A. mediata* specimen TVA402 from Kamchatka shared an identical haplotype with the Japanese *A. ishikariana* sequence.

Bayesian phylogenetic inference of the genus *Alloperla*, using *Suwallia errata* Li, Li as an outgroup, suggests that the *A. mediata* + *A. ishikariana* cluster and *A. rostellata* represent early-branching clades (Bayesian posterior probability, BPP = 0.94 and 0.86 respectively) (Fig. 13). *A. teleckojensis*, in turn, nested within a well-supported clade (BPP = 0.91) that comprises ten distinct Barcode Index Numbers (BINs) belonged to seven species.

Molecular species delimitation approaches implemented via the BOLD BIN system and the ASAP algorithm demonstrated near-complete congruence. However, in several instances, multiple morphologically recognized species were assigned to a single MOTU. For example, the top-clade on the consensus tree encompasses four distinct morphological species: *A. hamata*, *A. caudata*, *A. atlantica*, and *A. banksi*.

Conversely, the phylogenetic tree also revealed incongruencies where identical morphological species designations appeared across different MOTUs, as observed for *A. caudata*, *A. pilosa* and *A. severa*. Finally, the tree topology resolved four cases where monophyletic lineages belonging to the same morphospecies were partitioned into different MOTUs due to high genetic distances; these cases include *A. chloris*, *A. petasata*, *A. teleckojensis*, and *A. mediata*.

To establish an empirical barcoding gap for the genus, we constructed three pairwise K2P distance histograms utilizing the complete *Alloperla* dataset, incorporating also a blind kernel density analysis via the SPIDER library (Figs 14 A, B, C). The first histogram (Fig. 14 A) illustrates the frequency distribution of all pairwise comparisons across the entire genus. To evaluate the sensitivity of the BOLD algorithm, comparisons within a single BIN were color-coded in blue, while comparisons between different BINs were color-coded in red. The BIN algorithm implemented in BOLD strictly partitioned intra- and inter-species distances at approximately the 2% threshold; however, the overall distribution revealed that the barcoding gap occurs at the 6.1% K2P divergence level. To clarify these boundaries, a high-resolution histogram was restricted to K2P distance values below 10% (Fig. 14 B). This high-detail analysis corroborated the primary frequency drop at 6.1%, while resolving two secondary declines in pairwise comparison frequencies around 1.8% and 3.0%.

To contrast the automated algorithms with expert taxonomy, we constructed an additional pairwise K2P distance histogram (Fig. 14 C) utilizing exclusively the specimens formally identified to the species level. In these plots, the frequency distributions were color-coded to differentiate between comparisons within the same morphological species (green) and those between distinct morphological species (orange). The primary barcoding gap remained discernable at the 6.1% K2P divergence level. However, a strict boundary between the two categories was obscured due to a pronounced overlap at low divergence levels. In numerous instances, specimens assigned to different morphological species exhibited genetic distances well below the barcoding threshold, extending down to zero. This significant conflict between morphological designations and COI divergence further underscores the necessity for a comprehensive taxonomic revision of the genus *Alloperla*.

DISCUSSION

DNA barcoding using the mitochondrial *COI* gene remains a primary tool for the precise species-level identification of various invertebrate groups (Hebert et al., 2003). For stoneflies, which serve as crucial bioindicators within the standardized EPT (Ephemeroptera, Plecoptera, Trichoptera) index (Lenat, 1988; Plafkin et al., 1989), robust molecular identification is of paramount importance to ensure accurate biodiversity and water quality assessments. For the practical and reliable application of DNA barcoding in environmental monitoring, the creation of a high-quality, curated reference database is an absolute prerequisite. Such baseline libraries must be rigorously verified by expert taxonomists rather than relying on automated or unverified submissions.

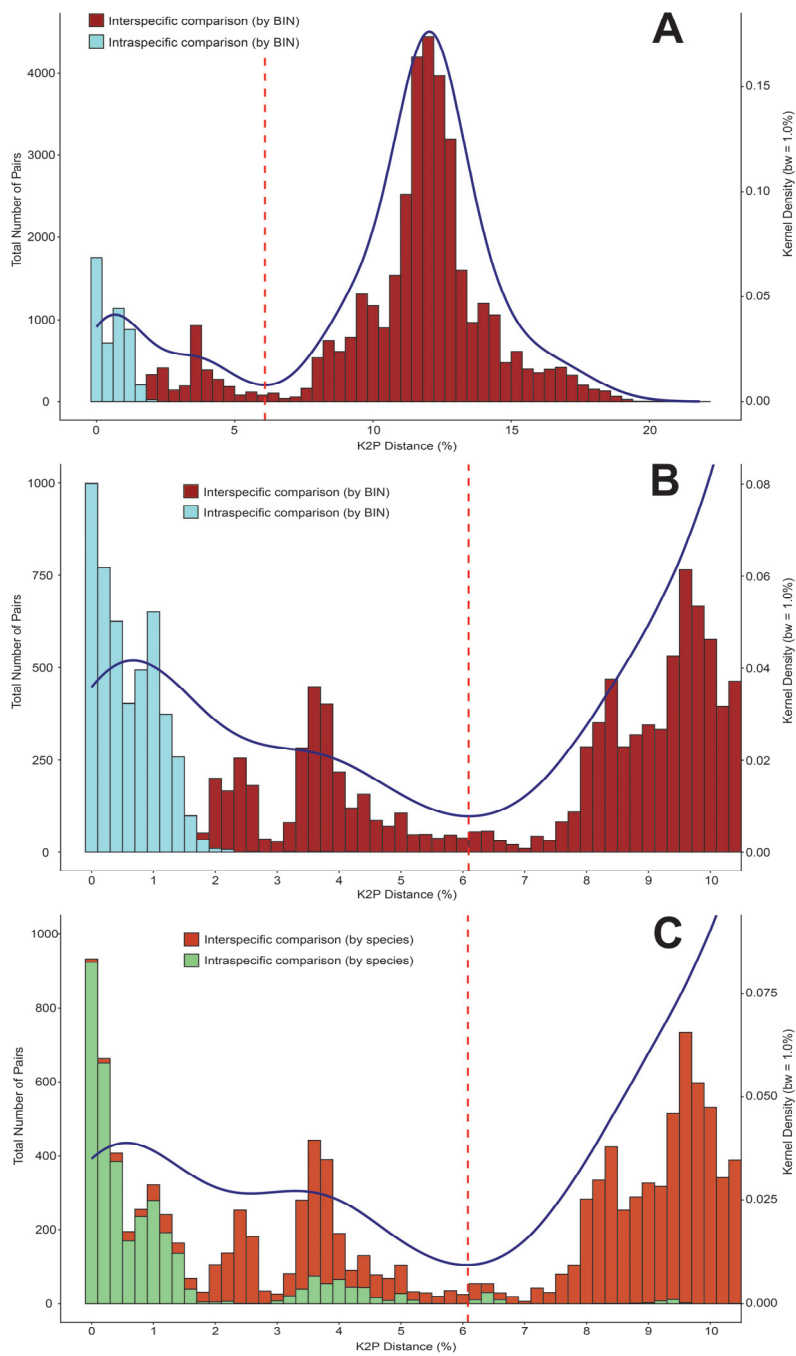
This study represents the first comprehensive generation of DNA barcodes for the genus *Alloperla* from the Eastern Palearctic realm. Prior to our work, the entire global dataset of public *Alloperla* barcodes was heavily biased toward the Nearctic realm, with 281 publicly available sequences and only a single Palearctic sequence of *A. ishikariana* from Japan (Yoshinari et al., 2024). We generated new DNA barcodes for three Eastern Palearctic species, *A. mediata*, *A. rostellata*, and *A. teleckojensis*, and implemented a comprehensive comparative analysis incorporating all available DNA barcodes for this genus.

DNA barcoding results conclusively demonstrated that the Japanese sequence of *A. ishikariana* is conspecific with the widespread *A. mediata*. Consequently, *A. ishikariana* is hereby formally designated as a junior synonym of *A. mediata*.

By constructing a phylogenetic tree and species delimitation algorithms using a single representative from each morphologically identified species per BIN, we revealed three types of incongruencies. In the first case, a single BIN shares multiple morphospecies, such as *A. hamata* Surdick, 1981, *A. caudata* Frison, 1934, *A. atlantica* Baumann, 1974, and *A. banksi* Frison, 1942, or *A. biserrata* Nelson et Kondratieff, 1980, and *A. imbecilla* Say, 1823 (Fig. 13). This may require synonymization of these species and/or detailed comparison with type specimens for accurate identification.

In the second case, conversely, a single morphological species shares multiple non-monophyletic BINs, as observed for *A. caudata* and *A. severa* (Hagen, 1861). This is usually associated with cryptic species or hidden morphological traits. Similarly, this requires meticulous comparison of morphological features with type specimens, using methods like scanning electron microscopy (SEM) of male epiprocts and female eggs.

Finally, the third type of incongruence is associated with the separation of monophyletic individuals into different MOTUs by the BOLD and ASAP algorithms. The tree revealed four cases where sister (monophyletic) individuals were placed in different MOTUs due to high pairwise distances: *A. chloris* Frison 1934, *A. petasata* Surdick, 2004, *A. teleckojensis*,



and *A. mediata* (Fig. 13). The latter two species were sequenced in the present study. For *A. teleckojensis*, two specimens collected from Khabarovsk and Primorsky territories (unfortunately, both were imago females) were assigned to different mOTUs despite having identical morphological characteristics. For *A. mediata*, a specimen from the Primorsky territory was placed in a separate mOTU relative to the Kamchatka and Khabarovsk Kray samples. Adult males were analyzed from both the Primorsky and Kamchatka populations, and their morphological traits unequivocally confirm their conspecificity. For the remaining stonefly species, specimens were also collected from distant localities across their ranges, while apparently sharing identical morphological characters. Obviously, these examples demonstrate that the BOLD and ASAP algorithms possess an unjustifiably high sensitivity, leading to the excessive fragmentation of sequences into potential species and an unwarranted search for non-existent morphological diagnostic characters.

Neglecting minor mathematical discrepancies between the p-distance and K2P models, it is evident that interspecific NN distances for chloroperlids never fall below 7.94% (between *Siphonoperla torrentium* (Pictet, 1841) and *S. italica* (Aubert, 1953) from Switzerland (Vuataz *et al.*, 2024) under the p-distance model, which scales up to 9.15% when recalculated using K2P distances. In turn, literature data indicate that maximum intraspecific distances can reach up to 2.99%, as documented for *S. torrentium* (Morinière *et al.*, 2017). Consequently, the literature confirms that the standard ~2% threshold implemented by BOLD (Hebert *et al.*, 2003; Ratnasingham & Hebert, 2013) is excessively sensitive, whereas the actual K2P interspecific boundary lies above 9%.

However, a dedicated divergence threshold had never been calculated specifically for the genus *Alloperla*, and the aforementioned literature values represent isolated pairwise comparisons rather than a comprehensive genus-wide statistical analysis supported by hundreds of reference sequences. To address this limitation, we constructed three pairwise K2P distance histograms utilizing all available *Alloperla* DNA barcodes to visualize the frequency of total comparison pairs and localize the true barcoding gap (according to Hebert *et al.*, 2003).

Our compiled histograms demonstrate that the true drop in pairwise transition frequencies occurs within a broad range between 6% and 7%. The localMinima algorithm from the R package spider allowed us to precisely calculate this baseline, which was localized at 6.1% (Figs 14 A, B, C).

We analyzed literature data reporting the minimum genetic distances of Chloroperlidae species to their nearest neighbors (NN) to evaluate the baseline for intraspecific and interspecific divergence within the family. Canadian stoneflies of genera *Hastaperla* Ricker, 1935 and *Haploperla* Navás, 1934 exhibited K2P NN values of 10.89% (Zhou *et al.*, 2010). In the Croatian fauna, chloroperlids of the genus *Siphonoperla* Zwick, 1967 displayed p-distances to their NN of 11.46%, while *Xanthoperla apicalis* (Newman, 1836) differed

Fig. 14. Combined frequency distribution histograms and kernel density estimation (KDE) overlay of pairwise K2P genetic distances for the genus *Alloperla* based on the mitochondrial *COI* gene. (A) Full-scale interspecific (red) and intraspecific (blue) comparisons according to BOLD BIN. (B) High-resolution subset restricted to 0–10% divergence with inter- and intraspecific comparisons according to BOLD BIN. (C) Morphological validation analysis restricted to 0–10% divergence, color-coded by interspecific (orange) and intraspecific (green) comparisons. The smooth dark blue curve tracks the kernel density estimation (bw = 1.0%). The vertical red dashed line indicates the mathematically resolved global minimum of the macro-evolutionary barcoding gap at 6.1%, computed via the localMinima function.

from *Siphonoperla* by 17.04% (Hlebec *et al.*, 2022). German chloroperlids spanning two species of *Chloroperla* Newman, 1836 and one species of *Siphonoperla* yielded K2P NN values of 10.18–12.96% (Morinière *et al.*, 2017). For Swiss chloroperlids of the same genera, but with broader species coverage, these NN values ranged between 7.94% and 10.33% using p-distances (Vuataz *et al.*, 2024).

Although the automated ASAP framework utilizes the same underlying distance dataset, it favors a much more sensitive partition (1.53%). This discrepancy occurs because the ASAP algorithm is fundamentally designed to maximize the partition of sequences into the tightest possible subgroups based on immediate genetic similarities, heavily penalizing any group mergers. Based on our empirically derived 6.1% threshold, we mapped an additional species delimitation partition onto our consensus tree (Fig. 13). This custom molecular partition demonstrated that strictly monophyletic individuals belonging to a single morphological species successfully collapsed into unified, stable molecular operational taxonomic units (mOTUs), which is taxonomically more conservative. Clear examples of this algorithmic correction on the tree topology include *A. chloris*, *A. petasata*, *A. teleckojensis*, and *A. mediata*.

Finally, when mapping intraspecific and interspecific distances based on morphologically defined species onto the histograms (Fig. 14 C), we observe numerous incongruencies that align with the first two types discussed above. Most of these incongruencies are driven by the splitting of individuals with very low or zero pairwise distances into separate morphological species, a phenomenon prominently visible within the highly compressed terminal clades at the top of the phylogenetic tree. Conversely, instances of unusually high intraspecific divergence exceeding the 6.1% threshold are less common. Such cases are restricted to *Alloperla pilosa*, *A. chloris*, *A. serrata*, and *A. caudata*, all of which clearly require comprehensive taxonomic revision.

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