MOLECULAR PHYLOGENETICS OF THE NORTH AMERICAN STONEFLIES (INSECTA: PLECOPTERA), WITH DESCRIPTION OF A NEW SPECIES AND FAMILY

BY

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DISSERTATION

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ABSTRACT

Stoneflies (Insecta: Plecoptera) are vital to aquatic ecological systems worldwide. Their value as bioindicators and ideal subjects for biogeographic and phylogeographic studies is directly dependent on the integrity of their phylogeny. Yet, a fully-resolved and well-supported phylogeny of the order has eluded researchers for centuries. Previous phylogenetic hypotheses have shown incongruence and unresolved relationships, especially within the suborder Arctoperlaria. The primary objective of this dissertation was to examine relationships within the Arctoperlaria through construction of a robust molecular phylogeny of the North American Plecoptera. Live adult specimens, including 132 species across 92 of the 109 described North American genera, were collected from the United States and Canada. A total of 1400 orthologous genes selected from transcriptomes were used in maximum likelihood (ML) and multispecies coalescent (msc) analyses. High support was recovered for several family relationships including 1) Chloroperlidae + Perlodidae, 2) Peltoperlidae as sister to four infraorder Systellognatha families, and 3) Nemouridae + Capniidae instead of the traditionally accepted Leuctridae + Capniidae clade.

A fourth result of the North American analyses was recovery of a separate family level lineage for the genus *Kathroperla*. Therefore, the phylogenetic position of *Kathroperla* and its traditionally designated subfamily Paraperlinae were investigated further using analysis of 800 orthologues from 32 Systellognatha Plecoptera, including all ten species of Paraperlinae, seven of which were sequenced for this dissertation chapter. Results from ML and msc analyses supported a monophyletic *Kathroperla* as sister to the remaining superfamily Perloidea. Examination of specimens revealed postocular head length as a distinct character. Combined molecular and morphological evidence supported Kathroperlidae, fam. n., as the seventeenth family of extant Plecoptera.

Phylogenetic relationships were also examined at the species level within the Nearctic genus *Perlesta* Banks. A preliminary phylogenetic hypothesis for *Perlesta* was constructed for 17 congeners and outgroup taxa using mitochondrial cytochrome c oxidase subunit I (COI) barcoding fragment data for 66 specimens. Results of ML and Bayesian analyses were congruent with previous species groupings based on morphology of male genitalia. A significant outcome of this dissertation chapter was description of a new species, *Perlesta sublobata* South &

DeWalt, 2019. In addition to the molecular results, which included a monophyletic grouping of ten *P. sublobata* COI haplotypes, images and illustrations of a distinct prominent ventral caecum and a large basal dorsal spinulae patch of the aedeagus supported description of this new species.

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CHAPTER 1

PHYLOGENOMICS OF THE NORTH AMERICAN PLECOPTERA[1]

ABSTRACT

Stoneflies (Insecta: Plecoptera) provide essential ecosystem services and are vital components of aquatic ecological systems worldwide. Their value as bioindicators and integral subjects of biogeographic and phylogeographic studies is directly dependent on the integrity of their phylogeny. Despite this importance, a well-supported and fully-resolved phylogeny of the order has remained elusive for over a century. Using transcriptome data from 94 species, we performed maximum likelihood and multispecies coalescent analyses with 1400 orthologous genes. This taxon sample includes representatives of all families, subfamilies, and tribes of the North American fauna, providing the most complete molecular phylogenetic study of the North American Plecoptera to date. Analyses recovered high support for the resolution of previously unresolved or contested relationships and the elucidation of a few novel relationships among historically accepted clades. Results included recovering 1) Perlidae as the earliest diverging family of Perloidea, 2) the clade Nemouridae + Capniidae instead of the traditionally recognized Leuctridae + Capniidae, 3) Peltoperlidae as sister to four Systellognatha families, and 4) non-monophyly of Chloroperlidae due to placement of the genus *Kathroperla* Banks. The position of Taeniopterygidae and Leuctridae remain inconclusive, as the placement of these taxa was not consistent between analyses of different data types nor was strong support for their relationships to other stoneflies recovered in a four-cluster likelihood analysis. However, our results for the North American taxa establish a robust foundation for future phylogenetic studies of the Plecoptera world fauna.

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[1] This chapter appeared in its entirety in the journal Systematic Entomology and is referred to in the dissertation as "South et al. 2020." South, E.J., Skinner, R.K., DeWalt, R.E., Kondratieff, B.C., Johnson, K.P., Davis, M.A., Lee, J.J., & Durfee, R.S.(2020) Phylogenomics of the North American Plecoptera. *Systematic Entomology*. https://doi.org/ 10.1111/SYEN.12462.

INTRODUCTION

The order Plecoptera, commonly known as stoneflies, is a group of hemimetabolous aquatic insects containing approximately 3800 valid described extant species across 16 families, with a worldwide distribution on all continents except Antarctica (Zwick 2000, Fochetti & Tierno de Figueroa 2008, DeWalt & Ower 2019, DeWalt et al. 2020). Stoneflies have ancient origins, with 297 valid fossil species across 14 families (DeWalt et al. 2020). These insects are critical segments of global freshwater biodiversity, provide key ecosystem services, and are the most environmentally sensitive of the three orders (including Plecoptera, Ephemeroptera: mayflies, and Trichoptera: caddisflies) traditionally used for aquatic biomonitoring (Baumann 1979, Lenat & Resh 2001). They are an essential component of terrestrial and aquatic food webs, comprising a significant food resource for invertebrates and vertebrates (Bell et al. 1994, Wipfli 1997, Allan et al. 2003).

Plecoptera larvae inhabit cool to cold, well-oxygenated temperate lotic systems, though some taxa prefer warm tropical streams, cold lentic environments, or cool moist semi-terrestrial habitats (DeWalt et al. 2015). The terrestrial adults are typically macropterous or brachypterous with varying body lengths of 5–50 mm (Stark et al. 1998), but numerous species are apterous or micropterous (McCulloch et al. 2009) (Fig. 1.1). Adults remain close to sites of emergence, resting atop or beneath vegetative or rock substrates during a reproductive stage which lasts a few days or weeks. Life cycles are univoltine or semivoltine, with some taxa exhibiting diapause in egg or larval stages (DeWalt et al. 2015).

Stoneflies exhibit low vagility, making them excellent organisms for biogeographic and phylogeographic studies (Fochetti & Tierno de Figueroa 2008, Pessino et al. 2014, McCulloch et al. 2016, Stevens et al. 2018). However, the systematic study of stoneflies has been controversial and inundated with classification rearrangements and competing phylogenetic hypotheses for nearly three centuries. Early systematists explained stonefly evolutionary relationships based on preexisting classification schemes, even though many typological categories did not indicate phylogeny (Zwick 1973). Numerous previously proposed phylogenies have shown incongruence and/or unresolved relationships within the order and between other insect orders (Wipfler et al. 2019).

Plecoptera classification and phylogeny

Phylogenetic study of stoneflies was preceded and strongly influenced by the order's dynamic history of classification (Zwick 1973, 1980). Classification of the world fauna by Zwick (2000) is the current most widely accepted system. Two suborders were recognized: Arctoperlaria and Antarctoperlaria. The Arctoperlaria include 12 families, most with a Northern Hemisphere distribution, except Notonemouridae which is present only in the Southern Hemisphere, and three tribes within the Perlidae, Acroneuriini, Anacroneuriini, and Neoperlini, which have expanded Southern Hemisphere distributions. The suborder is further divided into the infraorder Euholognatha containing six families (Capniidae, Leuctridae, Nemouridae, Notonemouridae, Taeniopterygidae, and Scopuridae), and the infraorder Systellognatha containing six families (Chloroperlidae, Peltoperlidae, Perlidae, Perlodidae, Pteronarcyidae, and Styloperlidae). The Antarctoperlaria have a Southern Hemisphere distribution across four extant families: Austroperlidae, Diamphipnoidae, Eustheniidae, and Gripopoterygidae.

The monophyly of the Plecoptera is supported by a few apomorphies (Zwick 1973). These include the gonads forming loops, intersegmental musculature of the larvae allowing laterally undulating swimming, and the presence of a cercus heart. Additionally, the lack of an ovipositor is suggested as a part of the stonefly ground pattern (i.e., a plesiomorphy).

To date, Zwick's morphological analyses of the phylogenetic relationships within the Plecoptera (Zwick 1969, 1973, 1980, 2000) are considered the most complete (Stewart & Stark 2002) (Zwick 2000, our Fig. 1.2). At the subordinal level, the Arctoperlaria is recognized as monophyletic, and with the exception of the family Scopuridae, is defined primarily by the behavior and structures associated with vibrational communication (a.k.a. drumming) between adults of both sexes (Stewart & Sandberg 2006). The monophyly of Antarctoperlaria is supported by presence of a depressor muscle of the fore trochanter and floriform chloride cells. Within the Arctoperlaria, monophyly of the infraorder Systellognatha is supported by membranous adult mandibles and hard chorionic structure of the eggs. The infraorder Euholognatha is loosely defined by structure of the retrocerebral system and abandonment of the hard egg chorion. The highly heterogeneous Euholognathan Notonemouridae is suggested to be paraphyletic and consequently labeled as a gradotaxon (Zwick 2000, McCulloch et al. 2016). However, recent studies support the monophyly of Notonemouridae (Béthoux et al. 2015, McCulloch et al. 2016, Cui et al. 2019).

Relationships between the suborders and infraorders have withstood under detailed scrutiny. However, relationships between the lower level ranks are still contested. Zwick's morphological character data are recognized as inadequate for resolution of several problematic phylogenetic relationships (Nelson 1984). Zwick (2000) acknowledged the lack of morphological evidence from extant taxa and fossils to resolve some relationships, as well as the paramount need for molecular data to test his phylogeny.

Early molecular studies were limited to a single locus study by Thomas et al. (2000) and a multi-locus study by Terry (2003, PhD dissertation, unpublished). Thomas et al. used 18S sequence data, a noted source of alignment problems in insect datasets (Shull et al. 2001, Kjer 2004), to construct a phylogeny, but these results contradicted all prior family level Plecoptera phylogenetic hypotheses. Terry (2003) proposed a phylogeny using a parsimony-based analysis of three mitochondrial genes (12S, 16S, and COII), three nuclear genes (18S, 28S, and histone H3), and a morphological character matrix (Zwick 1973), incorporating taxa representing all global families and 159 of approximately 250 extant genera. Limitations of these data included use of rapidly evolving mitochondrial genes to discern deep level relationships (Ballard & Whitlock 2004) and missing nuclear sequence data for several taxa.

A few recent molecular studies including Arctoperlaria fauna have proposed phylogenies using mitochondrial genomic data and with limited taxon sampling (Chen et al. 2018, Wang et al. 2018, Ding et al. 2019, Wang et al. 2019). Incongruent topologies recovered in maximum likelihood (ML) and Bayesian analyses by Ding et al. (2019) led the authors to suggest that mitogenomes may be a poor data source for resolving Systellognatha family relationships. Other researchers have challenged the use of mitochondrial genetic data to infer deep level relationships (Ballard & Whitlock 2004, Cameron 2014, Barker et al. 2015).

Regarding relationships between families of the Arctoperlaria, the early contemporary phylogenies (Illies 1965, 1966, Brodskiy 1982, Nelson 1984, Uchida & Isobe 1989, Thomas et al. 2000, Zwick 2000, Terry 2003) all show incongruence and/or unresolved relationships (Illies 1965, our Fig. 1.3, Nelson 1984, our Fig. 1.4, Terry 2003, our Fig. 1.5). Leuctroidea, Leuctridae + Capniidae, is recognized by Illies, Brodskiy, Nelson, and Zwick, but dissolved by the placement of Nemouridae as sister to Capniidae by Terry. Nelson and Thomas et al. place Pteronarcyidae at the base of Systellognatha. In contrast, Peltoperlidae is placed at the base of Systellognatha by Terry, sister to Pteronarcyidae by Zwick, and uncertain placement by Illies.

Regarding the placement of Taeniopterygidae, Terry and Thomas et al. disagree with Illies, Nelson, and Zwick. The family relationships of Euholognatha as defined by Zwick, (Scopuridae, (Taeniopterygidae, ((Leuctridae, Capniidae), (Notonemouridae, Nemouridae)))) were completely rearranged by Terry (Leuctridae, (Notonemouridae, ((Nemouridae, Capniidae), (Scopuridae, Taeniopterygidae)))). Most dramatic was placement of the genus *Megaleuctra* Neave as sister to all of Plecoptera, though this relationship is not generally accepted by most stonefly researchers.

The most contentiously debated family level relationships of the Arctoperlaria are within the superfamily Perloidea: Perlidae, Perlodidae, and Chloroperlidae. All four possible topologies have been proposed: Perlidae as sister to Perlodidae + Chloroperlidae (Ricker 1952, Illies 1965, Chen et al. 2018, Wang et al. 2018, Wang et al. 2019), Perlodidae as sister to Perlidae + Chloroperlidae (Zwick 1973, 1980, Brodskiy 1982, Uchida & Isobe 1989), Chloroperlidae as sister to Perlidae + Perlodidae (Thomas et al. 2000, Terry 2003), and a trichotomy (Nelson 1984, Zwick 2000) (Fig. 1.6).

Relationships within and between subfamilies of the Perloidea have also been scrutinized. Sivec et al. (1988) proposed a world phylogeny of the Perlidae subfamily Perlinae using extensive morphological comparisons. Stark & Szczytko (1984, 1988) investigated the phylogeny of the Perlodidae subfamily Perlodinae using egg morphology. Similarly, Surdick (1985) used morphology-based study to present a phylogeny of the Chloroperlidae subfamily Chloroperlinae. Terry (2003) found paraphyly within the subfamilies Acroneuriinae of the Perlidae and Chloroperlinae of the Chloroperlidae, and two of the three tribes of Perlodinae within the Perlodidae.

Despite the substantial works of multiple stonefly researchers, a well-supported and fully-resolved phylogeny remains obscured. However, the advent of genomics/transcriptomics provides a new opportunity to reconstruct a robust stonefly phylogenetic hypothesis. Transcriptomes, complete sets of RNA molecules which reflect gene expression in an organism at a specific point in time, contain protein coding genes without introns and can provide hundreds of phylogenetically informative loci to yield robust phylogenetic hypotheses for key taxonomic groups (McCormack et al. 2013). Transcriptome data have been used for exploring the relationship of Plecoptera to other insect orders (Misof et al. 2014, Wipfler et al. 2019) and relationships between a small subset of taxa within the order (Davis 2013, Master's thesis, unpublished).

The objective of this study is to investigate evolutionary relationships within the Arctoperlaria through development of a well-supported and fully-resolved phylogeny of the North American Plecoptera using extensive taxon sampling and sequences of hundreds of single-copy orthologous genes selected from transcriptomes. We present the most complete molecular phylogenetic study of the North American fauna to date and provide a robust foundation for a future global phylogeny. We limited our efforts to the North American fauna due to focus on in-depth coverage of this taxonomically rich region. Sampling is also limited by the availability of live specimens from which to extract RNA, and we are hopeful that future sampling efforts will complement and add to the phylogeny of the North American taxa studied here.

METHODS

Study Area

Three geographic biota of North American Plecoptera are recognized (Hynes 1988): eastern boreal (states and provinces of the eastern United States and Canada), western boreal (states of northwestern Mexico and the states and provinces of the western United States and Canada), and austral (states of central and southern Mexico) (Stewart & Stark 2002, Nelson 2008). We focused our study on the eastern and western boreal areas, both comprising an ideal study system for stonefly phylogenetics, largely due to the dynamic phylogenetic history of this highly diverse endemic fauna. To date, there are approximately 724 described extant species across 109 genera, 14 subfamilies, and 12 tribes among nine families (Fig. 1.1) within the eastern and western boreal fauna (DeWalt et al. 2020). Furthermore, all genera of the austral fauna are represented in the other two regions.

Specimen collection

We collected 373 live adult specimens across 132 species from 92 genera from the United States and Canada, providing exemplars for all families, subfamilies, and tribes of the North American Plecoptera, including all North American genera for the families Taeniopterygidae, Peltoperlidae, Perlidae, and Pteronarcyidae. Adults were collected exclusively to optimize taxonomic resolution to species level, as larvae of many stonefly taxa can be identified only to genus. Specimens were collected from aquatic habitats using a beating sheet, by hand picking from rock or wood substrates, or by ultraviolet light trapping. Specimens were kept alive until laboratory processing to optimize RNA preservation.

RNA extraction

In the laboratory, live specimens were examined for species-level identification and mite infestation. Any observed mites were removed to minimize sample contamination. When available, males were selected for processing due to the highly specific diagnostic character of the genitalia. Voucher tissue, abdominal terminalia with genitalia and other taxonomically informative characters, was excised and subsequently stored in 2 mL vials of 95% EtOH and accessioned into the Illinois Natural History Survey (INHS) Insect Collection in Champaign, Illinois (USA). The head and prothorax of large specimens were retained as additional voucher tissue. Remaining tissue of the live specimens was immediately homogenized with a pestle in RNAlater® (Qiagen, Valencia, California, USA) and stored at -80^{o} C for optimal nucleic acid preservation. RNA was successfully extracted from 128 frozen samples using the Qiagen RNA extraction mini kit and following the manufacturer's protocol. Sample RNA concentration was determined using Qiagen Probit. For single species with multiple extractions, the sample with the highest RNA concentration was selected for sequencing.

Sequencing and transcript assembly

RNA samples for 94 stonefly species (Table 1.1) were processed at the W. M. Keck Center for Comparative and Functional Genomics (Keck Center) at the University of Illinois at Urbana-Champaign, Illinois (UIUC) via Advanced Analytical Technologies Inc. (Ames, Iowa, USA) fragment analysis, complementary deoxyribonucleic acid (cDNA) library construction, and sequencing of 150-bp paired-end reads. Illumina technology (Illumina, San Diego, California, USA) was used to sequence the initial sample set of 53 taxa via HiSeq 4000 and the subsequent sample set of 41 taxa via NovaSeq. Transcriptome assemblies for 20 outgroup taxa, including representatives from Paleoptera and Polyneoptera, were selected from data generated by Misof et al. (2014) (Table 1.1).

Raw reads received from the Keck Center were reviewed for quality using FASTQC (Andrews 2010) and trimmed using TRIMMOMATICPE (Bolger et al. 2014) with the following settings: headcrop of 12, lead and trail of 3, sliding window of 4 with minimum quality 15, minimum length 50, and an Illuminaclip of 2:30:10. Trimmed reads were assembled into contigs using TRINITY (Haas et al. 2013). Assembly quality was checked using BOWTIE v2 (Langmead & Salzberg 2012) and BUSCO (Simão et al. 2015). Decontaminated assemblies and raw reads were submitted to the Sequence Read Archive (SRA) and Transcriptome Shotgun Assembly (TSA) archives at the National Center for Biotechnology Information (NCBI).

Orthologues were identified following Johnson et al. (2018) using the software ORTHOGRAPH (Petersen et al. 2017) with a reference dataset of 2395 orthologues suggested by ORTHODB v.7 (Kriventseva et al. 2008) to be single-copy across the six arthropod taxa included in the reference set. Orthologous amino acid sequences were aligned individually by gene using PASTA (Mirarab et al. 2015) with default settings. Nucleotide sequences were aligned with reference to the amino acid sequence alignments using a custom Perl script. Amino acid and nucleotide sequences were trimmed using TRIMAL v.1.4 (Capella-Gutierrez et al. 2009) to remove poorly aligned positions. The dataset was then filtered to identify and retain genes present in ≥50% of ingroup taxa.

Guanine-Cytosine (GC) content analysis

We explored heterogeneity of guanine-cytosine (GC) content, a potential source of systematic bias in phylogenomic analyses of large datasets (Bossert et al. 2017). We used a custom Python script to measure the GC content of each codon across all orthologues of each species (for custom scripts, see File S1 in South et al. 2020). Results were visualized using the GGPLOT2 package in R (Wickham 2016). To further investigate GC content, a χ^2 test for homogeneity of nucleotide state frequencies across taxa was performed on the nucleotide and nt12 datasets using PAUP* v.4.0a (build 166) (Swofford 2002).

Maximum likelihood (ML) analyses

Amino acid and nucleotide alignments from the retained orthologues were concatenated using SEQUENCE MATRIX v. 1.8 (Vaidya et al. 2011). Subsequently, two datasets of concatenated nucleotide alignments were used for further downstream analyses: one with all three codon positions (hereafter complete nucleotide) and a second with the third codon position removed (hereafter nt12).

The concatenated amino acid, complete nucleotide, and nt12 alignments were used as input for ML analysis using select options and functions within IQ-TREE v. 2.0.5 (Nguyen et al. 2015). IQ-TREE analyses were performed using the -m TEST option, which performs model selection followed by ML tree inference using the best-fit model identified by the Bayesian Information Criterion (BIC). The best-fit models selected by BIC were JTT + F + I + G4 for the concatenated amino acid alignment and GTR + F + I + G4 for the concatenated complete nucleotide and nt12 alignments. To assess branch support, 3000 ultrafast bootstrap replicates (Minh et al. 2013, Hoang et al. 2018) were performed using the -bnni option to reduce the risk of branch support overestimation.

Four-cluster likelihood mapping

The phylogenetic content of the concatenated complete nucleotide, nt12, and amino acid alignments were evaluated using the four-cluster likelihood mapping (FCLM) (Strimmer & Von Haeseler 1997) option in IQ-TREE. FCLM is a cluster analysis of maximum likelihoods for the three possible resolved topologies of four alignments or alignment groups. The graphical output is an equilateral triangle subdivided into regions showing support values for the phylogenetic suitability of the alignment datasets for each of the three topologies. Support values were generated as percentages of 10 000 randomly chosen quartets for each analysis. We investigated all three of our concatenated alignments to more closely examine our recovered opposing topologies that show either Taeniopterygidae or Leuctridae as sister to Nemouridae + Capniidae. The four designated clusters were Taeniopterygidae containing six taxa, Leuctridae containing nine taxa, Nemouridae + Capniidae containing 21 taxa, and a group of all other 58 stonefly taxa.

Multispecies coalescent analysis

A set of individual gene trees and 100 bootstrap replicates for each orthologue were generated from trimmed nucleotide alignments using RAXML v.8.2.11 under a GTR + Γ model (Stamatakis 2014). A second set of gene trees with the third codon position removed was obtained similarly. After collapsing branches with less than 5% bootstrap support, the gene tree sets were input separately into ASTRAL v.5.15.1 (Mirarab & Warnow 2015) under default settings for multispecies coalescent analysis. ASTRAL was run using the -t 2 annotation option which provides several support values for each branch, including two in which we had primary interest: 1) local posterior probability (Sayyari & Mirarab 2016) and 2) effective number, the number of gene trees contributing information to a branch.

RESULTS

Transcriptome sequences from 94 stonefly individuals were included in our analyses (Table 1.1). Assessment of the complete transcriptomes found the average number of benchmarking universal single-copy orthologues (BUSCOs) per taxon was 942.2 with an average of 106.4 missing BUSCOs per taxon (Table S1, South et al. 2020). After orthologue identification and filtering, a total of 1400 orthologues were retained for all downstream analyses. Across concatenated alignments which included ingroup and outgroup taxa, the average number of taxa per locus was 106.3 and the average number of loci per taxon was 1303.1 (for gene occupancy details generated by SEQUENCE MATRIX, see Table S2 and Table S3 in South et al. 2020 for concatenated nucleotide and AA alignments, respectively). The final concatenated amino acid alignment contained 404 858 positions, while the complete nucleotide and nt12 alignments contained 1 214 574 and 809 716 nucleotide positions, respectively (see Table 1.2 for more details). The amount of missing data (ambiguity/gap) for the three alignments was 13.14%.

GC content

The third codon position was highly variable for GC content, ranging from 39% for *Anacroneuria wipukupa* Baumann & Olson to 68% for *Isocapnia grandis* (Banks) (Fig. 1.7). The GC content at the first codon position was less variable, ranging from 52% for M*egaleuctra williamsae* Hanson to 56% for *Soyedina vallicularia* (Wu). The second codon position was the least variable, ranging from 38% for *Doroneuria theodora* (Needham & Claassen) to 39% for *Nemoura arctica* Esben-Petersen. Results of the χ^2 test for homogeneity of nucleotide state frequencies across taxa were highly significant for the nucleotide alignment (χ^2 = 597 612.0, df = 339, $P < 1 \text{ x}10^{-8}$) and nt12 alignment (χ^2 = 27 853.7, df = 339, $P < 1 \text{ x } 10^{-8}$), suggesting that variation in %GC composition had the potential to influence phylogenetic results.

Maximum likelihood analyses

Maximum likelihood analyses of the concatenated amino acid, complete nucleotide, and nt12 alignments (Table 1.2) generated predominantly highly resolved trees with high bootstrap support. The complete nucleotide tree had 100% bootstrap support for all branches except two within the perlodid subfamily Perlodinae (Figs. 1.8A, 1.9). All but six branches in the nt12 tree (Figs. 1.8B, 1.10) and all but six branches in the amino acid tree (Fig. 1.11) also received 100% bootstrap support.

All four North American families of Euholognatha (hereafter Nemouroidea where used in reference to our study, in recognition that Euholognatha = Scopuridae + Nemouroidea, and further understood that Notonemouridae is not included in our dataset, see Fig. 1.2) were recovered as monophyletic in all three analyses. In addition, 100% bootstrap support existed for the sister relationship of Nemouridae and Capniidae in all concatenated analyses. The complete nucleotide analysis yielded 100% bootstrap support for Leuctridae as sister to the remaining Nemouroidea. In contrast, the nt12 and amino acid trees showed Taeniopterygidae as sister to the remaining Nemouroidea with less than maximum bootstrap support [bootstrap support (BS) = 79 for nt12, BS = 90 for amino acid], suggesting the variation in %GC composition at third codon positions may be driving some of the results for the complete nucleotide analysis. All analyses showed the Taeniopterygidae subfamilies Taeniopteryginae and Brachypterainae as monophyletic with 100% bootstrap support. Similarly, the Leuctridae subfamilies Megaleuctrinae and Leuctrinae received 100% bootstrap support for their monophyly in all three analyses. The Nemouridae subfamily Nemourinae was rendered paraphyletic in the three analyses by placement of *Amphinemura delosa* (Ricker) and *Malenka marionae* (Hitchcock), taxa comprising the subfamily Amphinemurinae. Amphinemurinae was recovered as monophyletic in the complete nucleotide and nt12 analyses (BS = 100 for complete nucleotide, BS = 72 for nt12), but paraphyletic in the amino acid analysis (BS = 98).

Among the five North American Systellognatha families (hereafter Systellognatha where used in reference to our study and understood that Styloperlidae is not included in our dataset, see Fig. 1.2), Peltoperlidae, Pteronarcyidae, Perlodidae, and Perlidae were highly supported as monophyletic in all the three analyses. Chloroperlidae and its subfamily Paraperlinae were rendered paraphyletic by the placement of *Kathroperla* Banks as sister to Chloroperlidae + Perlodidae in the amino acid and complete nucleotide analyses (BS = 100 for amino acid and complete nucleotide), or sister to all Perloidea in the nt12 analysis (BS = 91). All three analyses yielded 100% bootstrap support for placement of Perlidae as the earliest diverging family of Perloidea and Peltoperlidae as sister to the remaining Systellognatha.

Among the Perlidae subfamilies, paraphyly was recovered for Acroneuriinae in the three analyses by placement of a monophyletic Perlinae (BS = 100) containing *Claassenia sabulosa* (Banks), *Agnetina flavescens* (Walsh), *Neoperla osage* Stark & Lentz, and *Paragnetina media* (Walker). The Perlinae tribe Perlini containing *Agnetina flavescens* and *Paragnetina media* was rendered paraphyletic in the three analyses by the placement of *Neoperla osage*, the representative for the Perlinae tribe Neoperlini. The Acroneuriinae tribe Acroneuriini was rendered paraphyletic in all concatenated analyses by placement of Perlinae, *Perlesta teaysia* Kirchner & Kondratieff and *Anacroneuria wipukupa*.

Among the Perlodidae subfamilies, all three analyses gave maximum support for monophyly of the Isoperlinae containing *Cascadoperla trictura* (Hoppe), *Calliperla luctuosa* (Banks), *Clioperla clio* (Newman), and *Isoperla quinquepunctata* (Banks). Perlodinae was rendered paraphyletic in the amino acid and nt12 analyses by the placement of Isoperlinae. In addition, the complete nucleotide analysis recovered Perlodinae as paraphyletic by placement of *Setvena bradleyi* (Smith) outside the Perlodinae tribe Arcynopterygini which also includes *Arcynopteryx dichroa* (McLachlan), *Megarcys subtruncata* Hanson, *Skwala americana* (Klapálek), *Perlinodes aureus* (Smith), and *Salmoperla sylvanica* Baumann & Lauck. Furthermore, *Setvena bradleyi* was placed outside the Arcynopterygini in the amino acid and nt12 analyses. All three analyses recovered monophyly for the Perlodinae tribe Diploperlini containing *Diploperla robusta* Stark & Gaufin, *Pictetiella expansa* (Banks), *Kogotus modestus* (Banks), *Rickera sorpta* (Needham & Claassen), *Cultus tostonus* (Ricker), and *Remenus bilobatus* (Needham & Claassen). The nt12 and amino acid analyses recovered the Perlodinae tribe Perlodini as monophyletic. However, Perlodini was rendered paraphyletic in the complete nucleotide analysis due to placement of *Chernokrilus misnomus* (Claassen) (BS = 75).

Multispecies coalescent analyses

Multispecies coalescent analyses of alignments with all codon positions (hereafter msc) and without third codon position (hereafter msc12) recovered Nemouroidea and Systellognatha as monophyletic groups. Within the Nemouroidea, all but one branch in the msc (Figs. 1.12A, 1.13) and three branches in the msc12 (Figs. 1.12B, 1.14) received maximum (1.0) local posterior probability support. Both analyses showed maximum or near maximum local posterior probability support (local pP = 0.96 to 0.99) for all branches of Systellognatha except one within the Perlodinae of the msc12 tree (local pP = 0.80) and one in the Perlodinae of the msc tree (local pP = 0.75).

Similar to the ML analyses, multispecies coalescent analyses recovered all Nemouroidea families as monophyletic, a sister relationship between Nemouridae and Capniidae, and paraphyly for Nemourinae by placement of Amphinemurinae. Also similar to the ML analyses, multispecies coalescent analyses recovered two opposing positions for Taeniopterygidae and Leuctridae. Taeniopterygidae was placed sister to remaining Nemouroidea in the msc analysis (local pP = 0.69), whereas Leuctridae was placed sister to remaining Nemouroidea in the msc12 analysis (local pP = 0.55).

Similar relationships recovered for the Systellognatha between ML and multispecies coalescent analyses included 1) monophyly for Peltoperlidae, Pteronarcyidae, Perlodidae, and Perlidae, 2) paraphyly for the Chloroperlidae by placement of *Kathroperla* (local pP = 1.0), 3) Peltoperlidae as sister to the remaining Systellognatha (local pP = 1.0), and 4) paraphyly for some subfamily and tribal relationships within the Perloidea (Fig. 1.15). The Acroneuriinae tribe Acroneuriini was recovered as paraphyletic by the multispecies coalescent analyses due to the placement of Perlinae, *Perlesta teaysia*, and *Anacroneuria wipukupa*. The msc and msc12 analyses recovered paraphyly for Perlodinae and the tribe Arcynopterygini by placement of *Setvena bradleyi* (pP = 1.0 for msc, pP = 0.99 for msc12), and the tribe Perlodini by the placement of *Chernokrilus misnomus* (pP = 0.99 for msc and msc12).

Four-cluster likelihood mapping

The FCLM for the nt12 nucleotide alignment showed the largest percentage (52.9%) of the 10 000 randomly generated quartets between the four cluster groups supported Leuctridae as sister to Nemouridae + Capniidae (Fig. 1.16B), the same branching pattern supported by the corresponding ML analysis (Fig. 1.8B). In contrast, FCLM results for the amino acid alignment showed the largest support (43.5%) for Taeniopterygidae as sister to Nemouridae + Capniidae (Fig. 1.16C), a branching pattern incongruent with the corresponding ML analysis (Fig. 1.11). Moreover, the FCLM for the complete nucleotide alignment generated support (60.4%) for

Leuctridae as sister to Nemouridae + Capniidae (Fig. 1.16A), a branching pattern incongruent with the corresponding ML analysis (Fig. 1.8A).

DISCUSSION

We investigated many of the problematic relationships within the Arctoperlaria using the most extensive dataset for the North American Plecoptera (stoneflies) to date. Using transcriptome data, we performed multiple analyses using gene sequences from 1400 orthologues from 94 species representing all taxonomic levels of tribe and above for the North American stoneflies. We recovered evidence supporting the resolution of previously unresolved or contested relationships and the elucidation of new relationships among historically accepted clades.

Systellognatha

The clade Perloidea has been acknowledged by many stonefly systematists (Ricker 1952, Illies 1965, Zwick 1973, 1980, Brodskiy 1982, Uchida & Isobe 1989). Zwick (2000) stated "monophyly of Perloidea is beyond doubt," referencing the presence of carnivorous larval mouthparts that characterize the clade. However, the relationships between the Perloidea families, Perlidae, Perlodidae, and Chloroperlidae, have been highly contentious. The sister relationship between Chloroperlidae and Perlodidae was highly supported in all of our analyses and congruent with previous morphology-based research (Ricker 1950, 1952, Illies 1965). Molecular studies supported this sister relationship through analyses of mitochondrial genomic data (Chen et al. 2018, Wang et al. 2018, Wang et al. 2019). Stewart & Stark (2002) suggested a close relationship between these two families based on the presence of acanthae, cuticular mandibular spinules of chloroperlid and perlodid larvae. Although, these structures are lacking in the perlodid subfamily Isoperlinae. Zwick (2006) suggested that the acanthae argument is masked by two shared characters of Perlodidae and Perlidae: 1) absence of a functional articular membrane between cercal segments observed in larvae and exuviae, which is present in Chloroperlidae, and 2) the presence of anterior gut caeca, which is absent in Chloroperlidae. Therefore, Zwick (2006) suggested that Chloroperlidae is sister to Perlodidae + Perlidae.

Nelson (1984), using a parsimony analysis of Zwick (1980) character data, constructed the first computer generated phylogeny by comparing several competing hypotheses. The results showed a soft polytomy for four unresolved lineages within the Perloidea, including the Paraperlinae, the subfamily that contains the genus *Kathroperla*. In our analyses, Chloroperlidae and Paraperlinae were rendered non-monophyletic by the placement of *Kathroperla*, represented by the exemplars *K. takhoma* Stark & Surdick and *K. perdita* Banks, as either sister to Chloroperlidae + Perlodidae, or sister to the remaining Perloidea. Terry (2003) also detected the Chloroperlidae as non-monophyletic by placement of *Kathroperla* as sister to all of Perloidea, similar to our results from the analysis of the nt12 dataset. The Paraperlinae species *Paraperla frontalis* (Banks) was placed sister to a monophyletic Chloroperlinae with maximum support in all our analyses. Zwick (2006), using analysis of larval morphology, suggested that Paraperlinae may be sister to Chloroperlidae or a separate branch of Perloidea. Zwick (2000) proposed the monophyly of Paraperlinae partially based on the group's elongated postocular head region. However, species of the Paraperlinae genus *Utaperla* Ricker were noted to have only slightly elongated head shape and elongated heads have been found in some representatives of *Alloperla atlantica* Baumann (DeWalt, unpublished data). Zwick also recognized a second subfamily apomorphy, the presence of large flat sterna with slot-like longitudinally oriented furcal pits which anteriorly connect to the outward edge of the basisternum.

Within the Perlidae, all our analyses recovered a paraphyletic Acroneuriinae and a monophyletic Perlinae, compared to non-monophyly recovered for both of these subfamilies by Terry (2003). These molecular results contradict previous morphological studies which distinguished two groups based primarily on male terminalia (Sivec et al. 1988, Stark 2004). Male Perlinae were noted to possess hemitergal lobes of the 10th tergum, compared to Acroneuriinae males which lack them, but instead display sclerotized paraprocts. Within the Perlinae, the placement of *Claassenia* Wu as sister to the remaining Perlinae by all our analyses was congruent with the phylogenetic assessment by Sivec et al. (1988). Two characters were observed to separate *Claassenia* from the other Perlinae: 1) loss of abdominal pleural brushes typically present in most Systellognatha and 2) presence of long seta-like spines between ninth and tenth abdominal segments in females.

Stark & Szczytko (1984) proposed a new classification of the Perlodinae tribes, placing Arcynopterygini as sister to Perlodini + Diploperlini. Arcynopterygini taxa were observed to have eggs circular in cross section, whereas the Perlodini and Diploperlini were defined by eggs triangular or semicircular in cross section. In a subsequent study, Stark & Szczytko (1988) placed *Setvena* Ricker + *Pseudomegarcys* Kohno in the most basal position within Arcynopterygini, partially based on the secondary loss of the egg collar, a unique character of the tribe. Additionally, the peculiar morphology of *Setvena* has been recognized, notably the short submental gills and two pairs of thoracic gills (nymphs) or gill remnants (adults) (Baumann et al. 1977). In our study, Arcynopterygini was rendered non-monophyletic in all analyses by the placement of *S. bradleyi*, via a short branch, as sister to all Perlodidae.

Illies' (1965) phylogeny was based on Hennigian principles, assigning plesiomorphic and apomorphic polarity among three suborders (Fig. 1.3). However, the placement of Peltoperlidae was uncertain. All our analyses found high support for Peltoperlidae as sister to the remaining Systellognatha, a relationship congruent with the hypotheses of Ricker (1952) and Terry (2003). In contrast, Zwick (1973) placed Peltoperlidae as sister to Perloidea, largely based on synapomorphic reduction of gills and abdominal ganglia. Uchida & Isobe (1989) placed Peltoperlidae as sister group to Styloperlidae, elevated from Styloperlinae Illies of the peltoperlids, and proposed the superfamily Pteronarcyoidea, (Pteronarcyidae, (Peltoperlidae, Styloperlidae)). Pteronarcyoidea was differentiated from the Perloidea by the proximity of the paired corpora allata, noted as shared mesal contact in Pteronarcyoidea but widely separated in Perloidea. Peltoperlidae was suggested to be more closely related to Pteronarcyidae than Perloidea based on the synapomorphies of present tridentate lacinia and an apical spine-like process of the tenth tergite in the nymphs, and flattened egg shape (Stark & Stewart 1981).

GC content

Analyses of phylogenomic data can yield well-supported phylogenies, yet these large nucleotide datasets may harbor inherent challenges for phylogenetic analysis. One potential challenge is the introduction of phylogenetic bias due to heterogeneity of GC content (Weisberg et al. 1989, Hasegawa & Hashimoto 1993). Within a genomic dataset, variance of GC content can bias phylogenetic signal across taxa or between genes. Across taxa, GC heterogeneity can group taxa based on similarity of GC content instead of evolutionary relatedness (Collins et al. 2005, Simon et al. 2006). Among genes, phylogenetic signal can be obscured by GC-biased gene conversion (gBGC), a process that can preferentially increase alleles with higher GC content over lower GC content alleles through recombination during meiosis (Pessia et al. 2012). Recombination rates have been correlated with GC content in genomic regions exhibiting gBGC.

Furthermore, higher recombination rates at specific loci have been associated with lower species tree resolution accuracy due to systematic bias introduced from introgression (Martin et al. 2019), incomplete lineage sorting (Hobolth et al. 2011), and increased rate of multiple nucleotide substitutions at the same locus (Comeron et al. 2012). The GC content bias in insects has been addressed in genomic scale analyses of Hemiptera (Skinner et al. 2019), Hymenoptera (Singh et al. 2005, Kent et al. 2012, Bossert et al. 2017), and Diptera (Robinson et al. 2013).

We investigated GC content in our dataset as a potential source of systematic bias. We found significant variation at the third codon position. Our analyses of alignments with and without the third codon position recovered opposing topologies for the position of Taeniopterygidae and Leuctridae. These results prompted further investigation of possible bias from GC content on our dataset by using FCLM to test family relationships within Nemouroidea.

Nemouroidea

Multiple morphological studies supported Taeniopterygidae as an earlier diverging lineage than Leuctridae (Illies 1965, Brodskiy 1982, Nelson 1984, Zwick 2000), whereas molecular studies recovered Leuctridae as diverging earlier than Taeniopterygidae (Terry 2003, Ding et al. 2019). Within the Taeniopterygidae, we recovered the monotypic Taeniopteryginae as sister to a monophyletic Brachypterainae in all our analyses. This was in contrast to the paraphyly recovered for Brachypterainae by Terry. However, the phylogenetic placement of the two families was problematic in our study. Two opposing relationships were recovered: 1) Taeniopterygidae as sister to the remaining Nemouroidea in the ML analysis of the amino acid and nt12 datasets and msc coalescent analysis versus 2) Leuctridae as sister to the remaining Nemouroidea in the ML analysis of the concatenated complete nucleotide dataset and msc12 analysis. Branch lengths for these topological arrangements were very short in all our generated trees. Branch support was low to moderate in all analyses except in the concatenated complete nucleotide analysis.

Furthermore, recovered positions of Taeniopterygidae and Leuctridae were inconsistent in the FCLM analyses and incongruent with the corresponding concatenated complete nucleotide and amino acid ML analyses. Given the short branch lengths with less than maximum support and the contradictory results for this node in the phylogeny, we find placement of Taeniopterygidae and Leuctridae as inconclusive. We suggest that GC content variation at the third codon position may introduce systematic bias to elucidation of family level relationships within the base of the Nemouroidea phylogeny and that GC content variation at the third codon position may be responsible for the differences in the placements observed for Taeniopterygidae and Leuctridae between analyses.

The sister relationship of Leuctridae + Capniidae, defined as Leuctroidea (Zwick 2000), has been proposed by several morphology-based hypotheses (Ricker 1952, Illies 1965, Brodskiy 1982, Nelson 1984, Zwick 1973, 1980, 2000). Characters used to define this group include modified paraproct lobes which facilitate sperm transfer, and reduced anal lobe of the hind wing to three anal veins (Zwick 2000). Prior molecular study did not support the clade Leuctroidea. Ding et al. (2019) recovered Capniidae and Taeniopterygidae as sister taxa and Leuctridae as sister to the remaining Nemouroidea, though the latter relationship received low branch support in their ML analysis. Similar to Terry (2003), our analyses provided strong support for the clade Nemouridae + Capniidae.

Megaleuctra has been regarded as the most primitive of the Leuctridae (Ricker & Ross 1969, Nelson & Hanson 1973) and it has been suggested to deserve designation as a subfamily, Megaleuctrinae (Zwick 2000), and even as a separate family (Terry 2003). Illies (1967) placed the *Megaleuctra* in the Notonemourinae. In contrast, Nelson & Hanson (1973) suggested placement of *Megaleuctra* within the Leuctridae. Thomas et al. (2000) referred to *Megaleuctra* as Megaleuctridae, and showed the taxon as a separate sister lineage to 13 other families. Terry (2003) placed *Megaleuctra* as sister to the remainder of Plecoptera. Béthoux (2005) refuted this placement by retaining *Megaleuctra* within the Leuctridae based on a detailed morphological comparative analysis of Plecoptera wing venation. Béthoux (2005) agreed with Zwick (2000), acknowledging the congruence of venation pattern between *Megaleuctra* and *Leuctra* Stephens and the absence of this defining character state in Antarctoperlaria and Systellognatha. Zwick (2000) also noted the bulbous structure and attached retractor musculature of the paraproct bases as consistent with the Leuctridae. Similarly, our analysis did not support family status for *Megaleuctra.* We found strong support for *M. williamsae* and *M. stigmata* (Banks) as the clade Megaleuctrinae within a monophyletic Leuctridae.

Study implications

Our extensive taxonomic coverage of the North American fauna facilitated phylogenetic examination of relationships between many Arctoperlaria families and most of the Nearctic genera. However, the geographical restriction of our study limits phylogenetic and phylogeographic implications for the diverse Plecoptera world fauna and may contribute to our inconclusive results for Nemouroidea and the paraphyly recovered for Acroneuriinae. The Nemouroidea include diverse groups throughout Europe and Asia, especially the speciose Leuctridae which have expanded distributions across northern Africa, Eurasia, and tropical southeast Asia. The genera *Leuctra* and taeniopterygid *Taeniopteryx* Pictet contain species groups with closely interrelated taxa between disjunct North American and European faunas (Zwick 2000). Within the Systellognatha, the perlid Acroneuriinae include taxa with expanded distributions into South America and Asia. The perlid genus *Neoperla* Needham of the Perlinae includes 16 American species and approximately 240 greatly different species in tropical and temperate Asia and Africa (DeWalt et al. 2020). Expansion of our taxonomic coverage to include European and Asian faunas in future phylogenetic analyses may provide more conclusive evidence to resolve the uncertain relationships that we recovered within nine families of the Arctoperlaria. Additionally, further expanded coverage to include the Antarctoperlaria fauna could provide substantial insight into the phylogeography of the order on a global scale.

CONCLUSIONS

Using data from transcriptome sequencing, we present a molecular phylogeny of the North American Plecoptera with extensive taxon sampling representing all families, subfamilies, and tribes of the North American stoneflies. Using multiple analyses of 1400 orthologues (more than 1.2 million aligned base pairs) from 94 species, we found strong support for the monophyly of previously accepted clades and elucidated novel relationships. Significant relationships recovered with high support include Perlidae as the earliest diverging family of Perloidea, the clade Nemouridae + Capniidae, Peltoperlidae as sister to remaining North American Systellognatha, and non-monophyly of the Chloroperlidae due to placement of the genus *Kathroperla*. The positions of Taeniopterygidae and Leuctridae remain inconclusive, as the placement of these taxa was not consistent between analyses of different data types nor was strong support for their relationships to other stoneflies recovered in the FCLM. Our results provide a robust framework for further in-depth exploration of Plecoptera phylogenetics and we encourage future global collaboration to expand the North American coverage into a phylogeny of the Plecoptera world fauna.

FIGURES AND TABLES

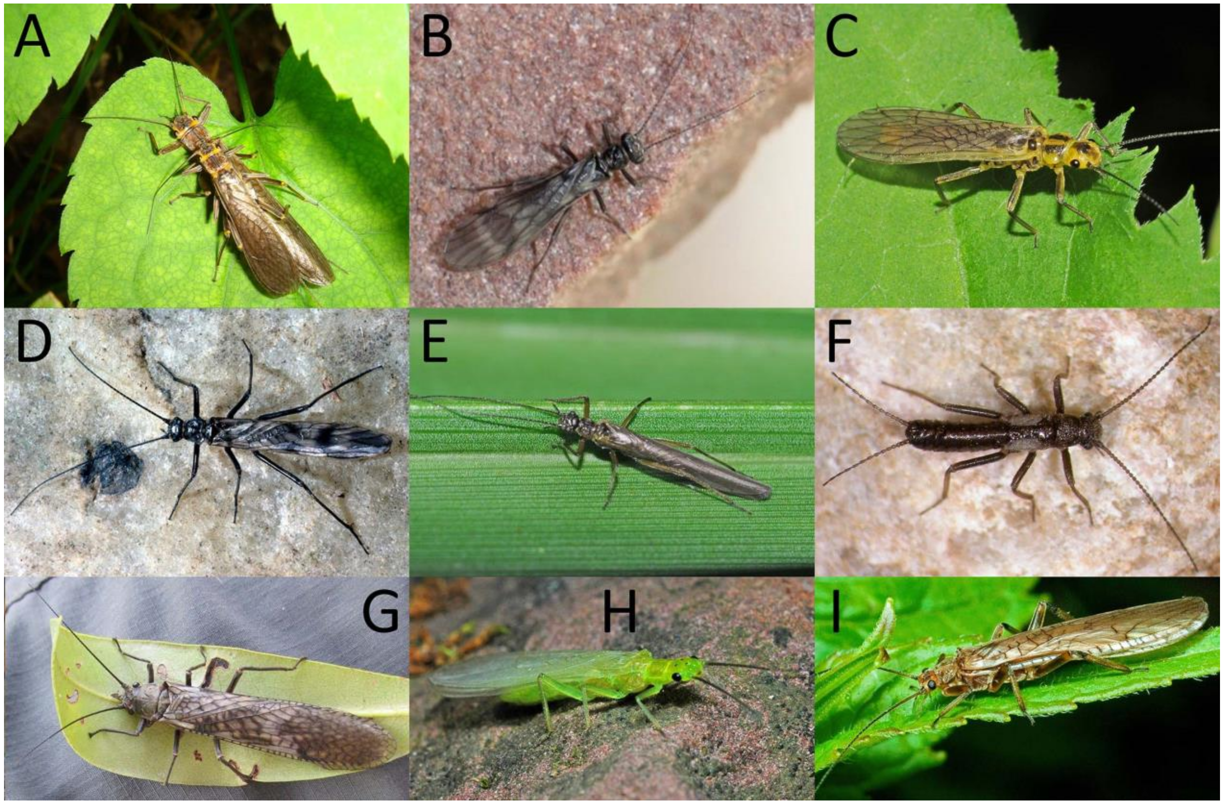

Figure 1.1. Adult stoneflies of North America representing nine families. (A) Perlidae: *Acroneuria carolinensis* (Banks). (B) Nemouridae: *Prostoia besametsa* (Ricker). (C) Perlodidae: *Isoperla stewarti* Szczytko & Kondratieff. (D) Taeniopterygidae: *Taeniopteryx* sp. Pictet. (E) Leuctridae: *Leuctra ferruginea* (Walker). (F) Capniidae: *Capnura wanica* (Frison). (G) Pteronarcyidae: *Pteronarcys scotti* Ricker. (H) Chloroperlidae: *Alloperla caudata* Frison. (I) Peltoperlidae: *Tallaperla cornelia* (Needham & Smith). Photos (A, C, D, E, H, I) by Bill Stark, (B) Dave Leatherman, (F) David Rees, and (G) Eric South.

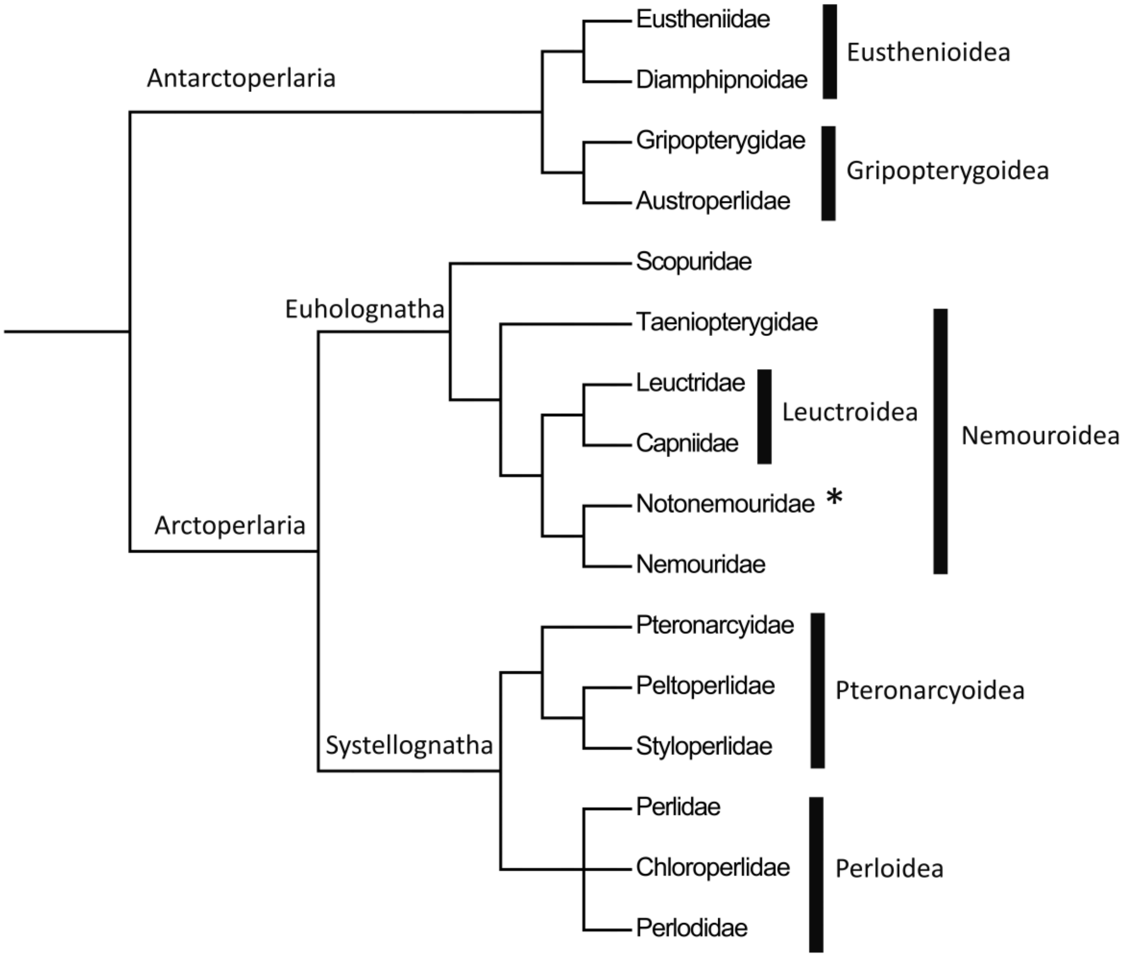

Figure 1.2. Phylogenetic hypothesis for Plecoptera family level taxa based on morphology (after Zwick, 2000). Uncertain monophyly of Notonemouridae is indicated by *.

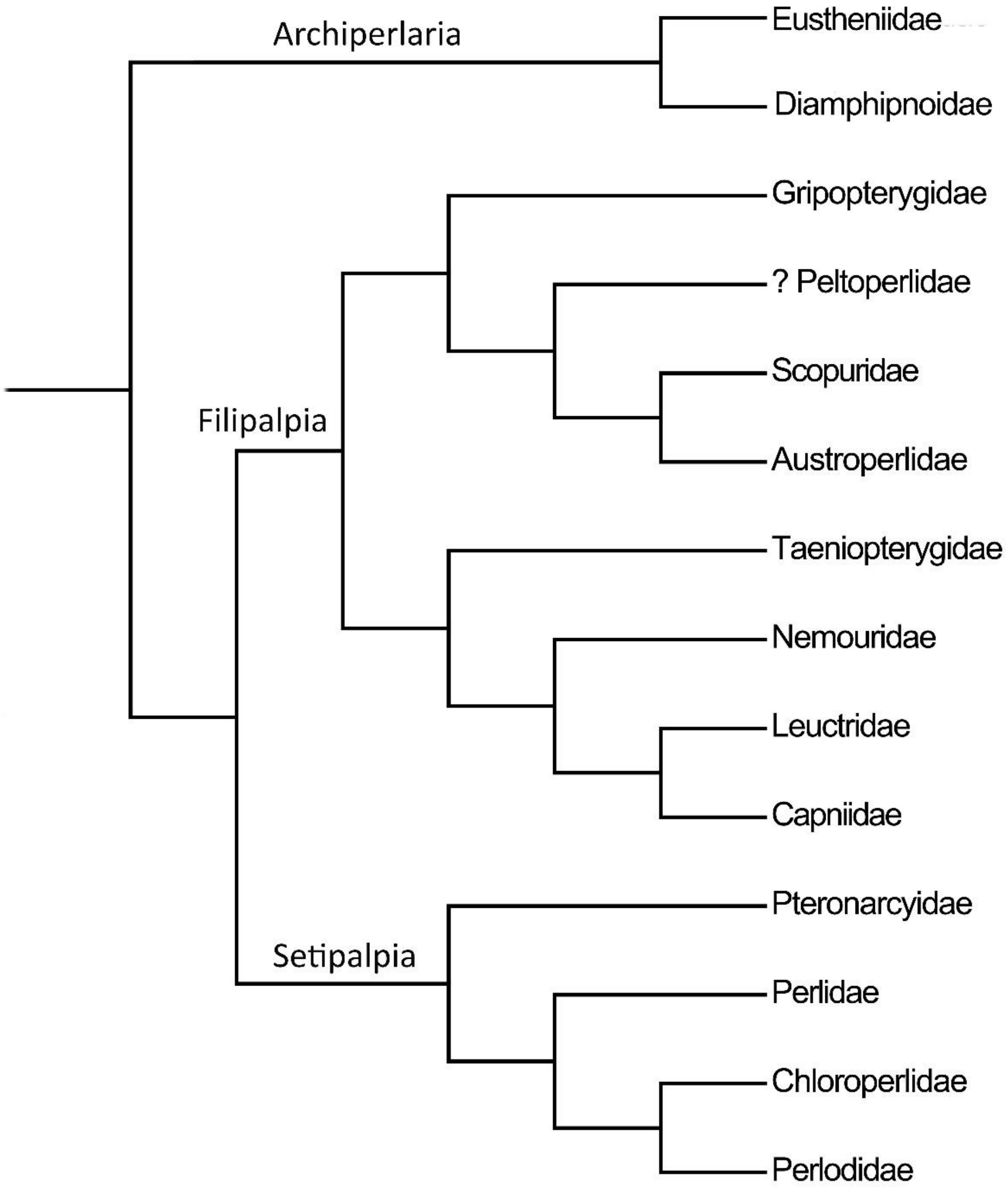

Figure 1.3. Phylogenetic hypothesis for Plecoptera family level taxa based on morphology (after Illies, 1965). Uncertain placement of Peltoperlidae is indicated with ?.

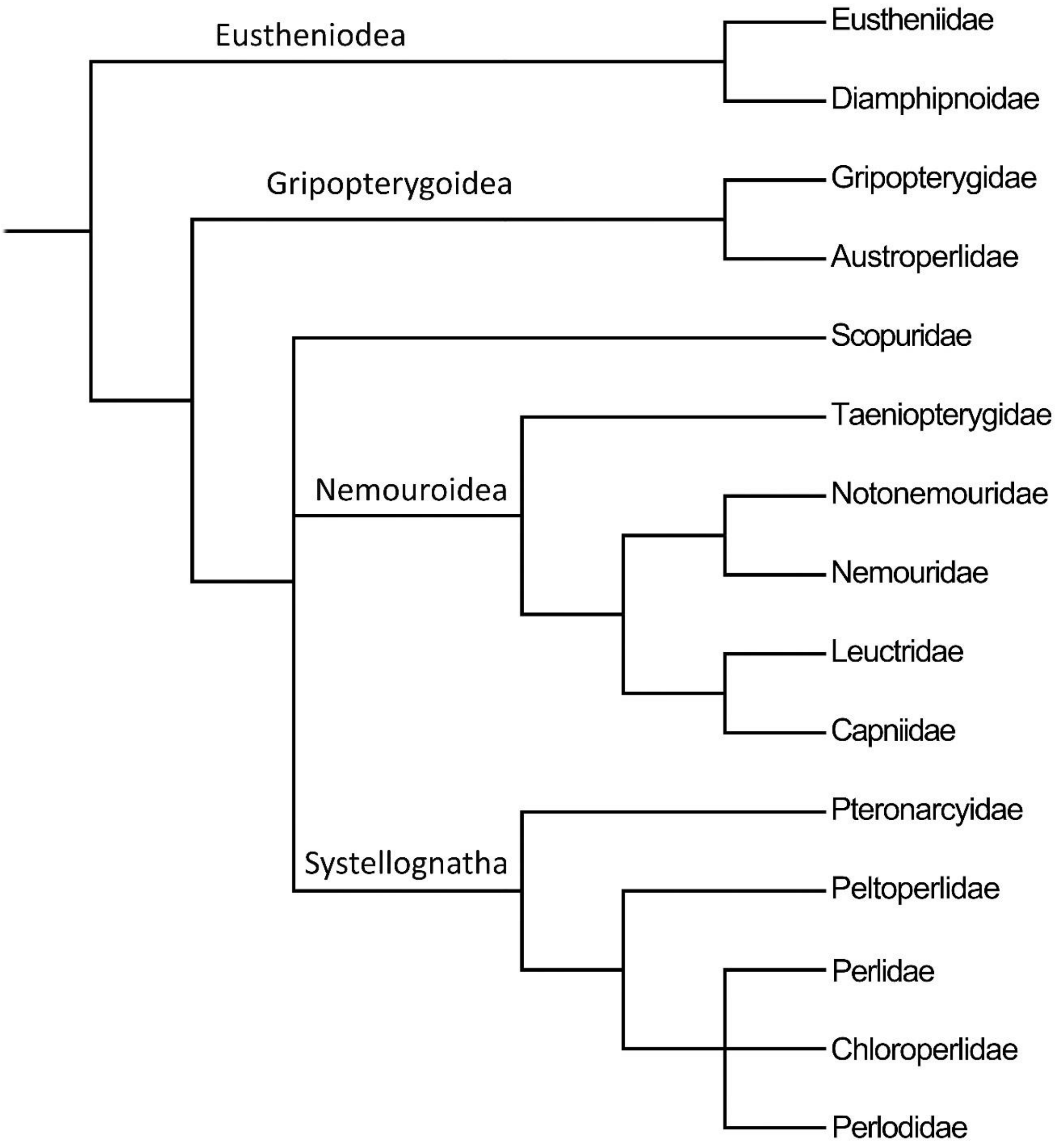

Figure 1.4. Phylogenetic hypothesis for Plecoptera family level taxa based on morphology (after Nelson, 1984).

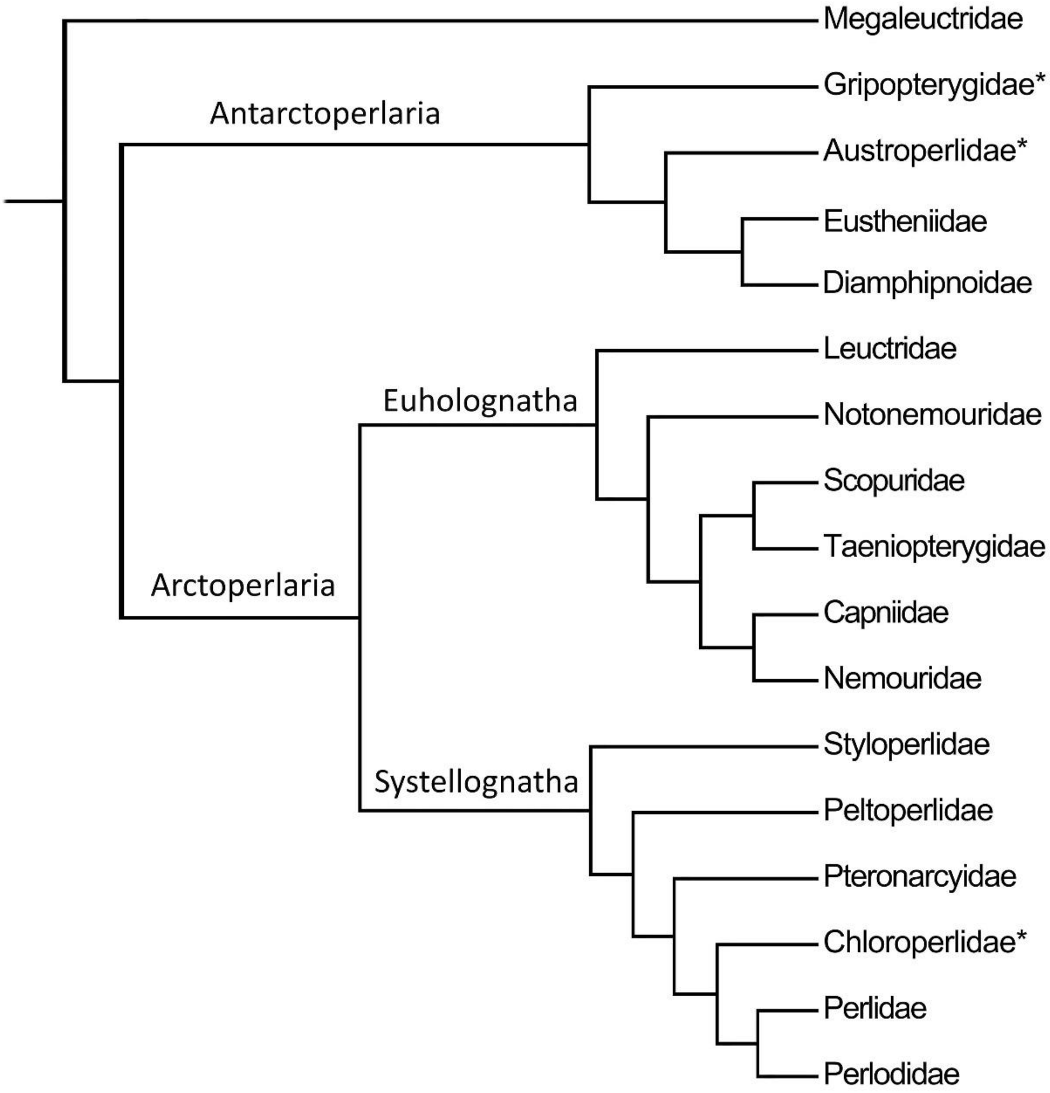

Figure 1.5. Phylogenetic hypothesis for Plecoptera family level taxa (Terry, 2003) based on three nuclear loci, three mitochondrial loci, and a morphological character matrix from Zwick (1973). Non-monophyletic groups are indicated by *.

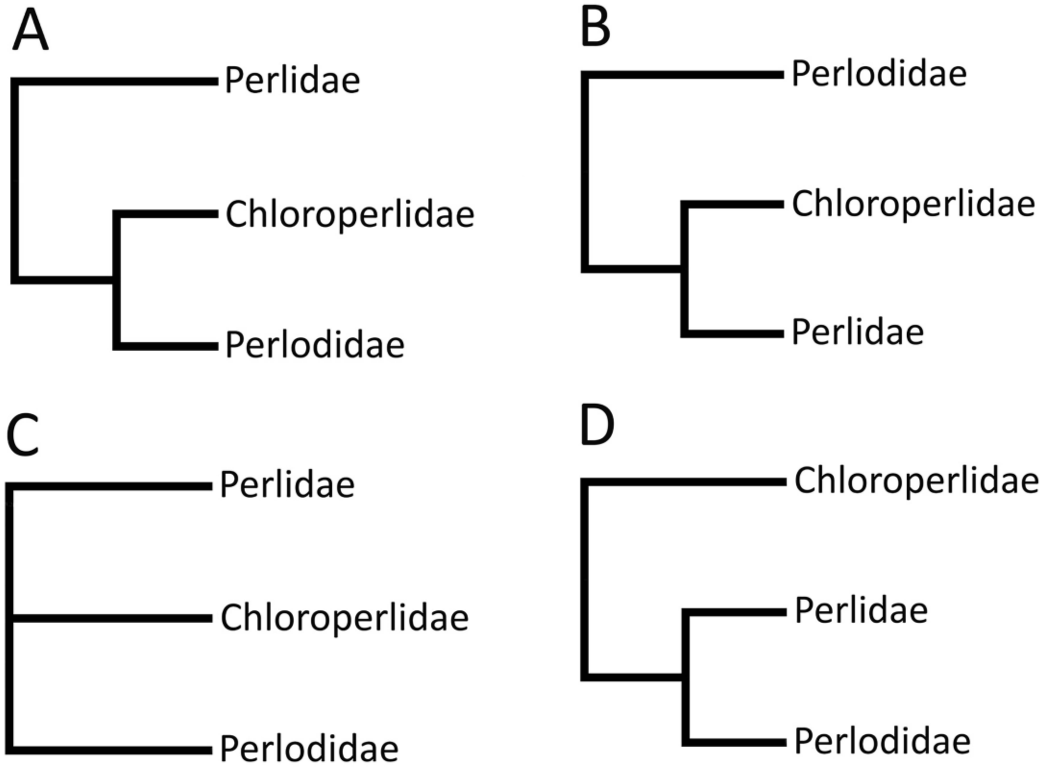

Figure 1.6. Phylogenetic hypotheses for family relationships of Perloidea. Topology consistent with (A) Ricker (1952), Illies (1965), Chen et al. (2018), Wang et al. (2018), Wang et al. (2019); (B) Zwick (1973, 1980), Brodskiy (1982), Uchida & Isobe (1989); (C) Nelson (1984), Zwick (2000); (D) Thomas et al. (2000), Terry (2003).

Table 1.1. National Center for Biotechnology Information (NCBI) accession numbers, collection localities, and percentage gap/ambiguity sites for all 94 ingroup (Plecoptera) and 20 outgroup (from Misof et al., 2014) specimens used in this study. Alignments: AA = amino acid, complete nt (nucleotide) = includes all three codon positions, nt12 = excludes the third codon position.

Family	**Species**	**BioSample no.**	**Run no.**	**TSA project no.**	**Locality**	**Gap/ambiguity data in AA, complete nt, and nt12 alignments (%)**
Plecoptera						
Capniidae	*Allocapnia granulata*	SAMN13118621	SRR10357449	GIDW00000000	United States: Illinois	5.1
Capniidae	*Arsapnia decepta*	SAMN13118625	SRR10357445	GIFA00000000	United States: Colorado	23.4
Capniidae	*Capnia confusa*	SAMN13118624	SRR10357446	GIDV00000000	United States: Colorado	4.5
Capniidae	*Capnura wanica*	SAMN13118619	SRR10357451	GIDY00000000	United States: Colorado	4.8
Capniidae	*Eucapnopsis brevicauda*	SAMN13118615	SRR10357456	GIEC00000000	United States: Nevada	6.9
Capniidae	*Isocapnia grandis*	SAMN11441126	SRR8924878	GHRB00000000	United States: Montana	12.3
Capniidae	*Mesocapnia frisoni*	SAMN13118620	SRR10357450	GIDK00000000	United States: Colorado	2.6
Capniidae	*Nemocapnia carolina*	SAMN13118622	SRR10357448	GIDX00000000	United States: Georgia	3.4
Capniidae	*Paracapnia angulata*	SAMN11441083	SRR8924842	GHPO00000000	United States: New York	15.5
Capniidae	*Sasquacapnia missiona*	SAMN11441097	SRR8924862	GHQA00000000	United States: Montana	4.8
Capniidae	*Utacapnia logana*	SAMN13118626	SRR10357443	GIDU00000000	United States: Colorado	18.4
Chloroperlidae	*Alloperla usa*	SAMN11441085	SRR8924844	GHPQ00000000	United States: Virginia	13.3
Chloroperlidae	*Haploperla brevis*	SAMN13118597	SRR10357475	GIEQ00000000	United States: Indiana	4.9
Chloroperlidae	*Kathroperla perdita*	SAMN11441113	SRR8924876	GHQO00000000	United States: Montana	26.7
Chloroperlidae	*Kathroperla takhoma*	SAMN11441118	SRR8924873	GHQT00000000	United States: California	19.3
Chloroperlidae	*Paraperla frontalis*	SAMN11441086	SRR8924843	GHPR00000000	United States: California	16.8
Chloroperlidae	*Plumiperla diversa*	SAMN13118609	SRR10357462	GIEG00000000	United States: Colorado	3.0
Chloroperlidae	*Sasquaperla hoopa*	SAMN11441122	SRR8924880	GHQX00000000	United States: California	8.4
Chloroperlidae	*Suwallia pallidula*	SAMN11441096	SRR8924869	GHPZ00000000	United States: Colorado	8.5
Chloroperlidae	*Sweltsa lamba*	SAMN13118588	SRR10357466	GIDO00000000	United States: Wyoming	10.1
Chloroperlidae	*Triznaka signata*	SAMN11441079	SRR8924834	GHPK00000000	United States: Colorado	4.2
Leuctridae	*Despaxia augusta*	SAMN11441117	SRR8924872	GHQS00000000	United States: Montana	6.6
Leuctridae	*Leuctra sibleyi*	SAMN11441093	SRR8924866	GHPX00000000	United States: Virginia	7.6
Leuctridae	*Megaleuctra stigmata*	SAMN11441074	SRR8924885	GHPF00000000	United States: Montana	5.4
Leuctridae	*Megaleuctra williamsae*	SAMN11441078	SRR8924835	GHPJ00000000	United States: North Carolina	4.6

Table 1.1 (cont.).

Leuctridae	*Moselia infuscata*	SAMN13118617	SRR10357453	GIEA00000000	United States: California	3.5
Leuctridae	*Paraleuctra sara*	SAMN13118610	SRR10357461	GIDQ00000000	United States: Georgia	3.9
Leuctridae	*Perlomyia collaris*	SAMN13118590	SRR10357444	GIEU00000000	United States: California	8.0
Leuctridae	*Pomoleuctra purcellana*	SAMN13118608	SRR10357463	GIEH00000000	United States: Montana	3.7
Leuctridae	*Zealeuctra claasseni*	SAMN13118595	SRR10357438	GIES00000000	United States: Indiana	5.7
Nemouridae	*Amphinemura delosa*	SAMN13118612	SRR10357459	GIEE00000000	United States: Missouri	4.0
Nemouridae	*Lednia tumana*	SAMN11441114	SRR8924877	GHQP00000000	United States: Montana	7.1
Nemouridae	*Malenka marionae*	SAMN11441089	SRR8924848	GHRF00000000	United States: California	9.0
Nemouridae	*Nemoura arctica*	SAMN13118606	SRR10357465	GIEX00000000	United States: Michigan	4.8
Nemouridae	*Ostrocerca foersteri*	SAMN13118592	SRR10357441	GIDM00000000	United States: California	4.7
Nemouridae	*Podmosta delicatula*	SAMN13118618	SRR10357452	GIDZ00000000	United States: California	3.8
Nemouridae	*Prostoia besametsa*	SAMN11441099	SRR8924864	GHRH00000000	United States: Colorado	11.9
Nemouridae	*Soyedina vallicularia*	SAMN13118623	SRR10357447	GIEZ00000000	United States: Indiana	8.9
Nemouridae	*Visoka cataractae*	SAMN13118586	SRR10357478	GIEV00000000	United States: Montana	18.3
Nemouridae	*Zapada oregonensis*	SAMN13118616	SRR10357454	GIEB00000000	United States: California	8.3
Peltoperlidae	*Peltoperla tarteri*	SAMN11441092	SRR8924838	GHPW00000000	United States: Virginia	7.8
Peltoperlidae	*Sierraperla cora*	SAMN13118603	SRR10357469	GIEW00000000	United States: California	4.2
Peltoperlidae	*Soliperla* sp.	SAMN13118593	SRR10357440	GIET00000000	United States: California	9.3
Peltoperlidae	*Tallaperla maria*	SAMN11441119	SRR8924870	GHQU00000000	United States: Virginia	13.2
Peltoperlidae	*Viehoperla ada*	SAMN13118587	SRR10357477	GIDP00000000	United States: North Carolina	5.0
Peltoperlidae	*Yoraperla nigrisoma*	SAMN11441088	SRR8924845	GHPT00000000	United States: California	8.8
Perlidae	*Acroneuria carolinensis*	SAMN11441100	SRR8924865	GHQC00000000	United States: Ohio	18.1
Perlidae	*Agnetina flavescens*	SAMN11441108	SRR8924855	GHQK00000000	United States: Virginia	30.2
Perlidae	*Anacroneuria wipukupa*	SAMN11441080	SRR8924887	GHPL00000000	United States: Arizona	9.5
Perlidae	*Attaneuria ruralis*	SAMN11441101	SRR8924860	GHQD00000000	United States: Missouri	13.7
Perlidae	*Beloneuria georgiana*	SAMN11441102	SRR8924861	GHQE00000000	United States: North Carolina	10.8
Perlidae	*Calineuria californica*	SAMN11441103	SRR8924852	GHQF00000000	United States: California	37.9
Perlidae	*Claassenia sabulosa*	SAMN11441081	SRR8924882	GHPM00000000	United States: Colorado	11.3
Perlidae	*Doroneuria theodora*	SAMN11441104	SRR8924851	GHQG00000000	United States: Montana	24.0
Perlidae	*Eccoptura xanthenes*	SAMN11441105	SRR8924850	GHQH00000000	United States: Virginia	35.9

Table 1.1 (cont.).

Perlidae	*Hansonoperla hokolesqua*	SAMN11441077	SRR8924836	GHPI00000000	United States: Kentucky	12.2
Perlidae	*Hesperoperla pacifica*	SAMN11441106	SRR8924849	GHQI00000000	United States: Wyoming	24.9
Perlidae	*Neoperla osage*	SAMN11441087	SRR8924846	GHPS00000000	United States: Arkansas	12.6
Perlidae	*Paragnetina media*	SAMN11441094	SRR8924867	GHPY00000000	United States: Virginia	5.4
Perlidae	*Perlesta teaysia*	SAMN11441095	SRR8924868	GHRG00000000	United States: Virginia	13.0
Perlidae	*Perlinella drymo*	SAMN11441107	SRR8924856	GHQJ00000000	United States: Illinois	24.9
Perlodidae	*Arcynopteryx dichroa*	SAMN13118604	SRR10357468	GIEK00000000	United States: Michigan	2.4
Perlodidae	*Calliperla luctuosa*	SAMN11441115	SRR8924874	GHQQ00000000	United States: California	13.6
Perlodidae	*Cascadoperla trictura*	SAMN13118596	SRR10357476	GIER00000000	United States: California	12.7
Perlodidae	*Chernokrilus misnomus*	SAMN13118591	SRR10357442	GIDN00000000	United States: California	11.8
Perlodidae	*Clioperla clio*	SAMN11441073	SRR8924886	GHOZ00000000	United States: Illinois	8.2
Perlodidae	*Cultus tostonus*	SAMN13118602	SRR10357470	GIEL00000000	United States: California	10.8
Perlodidae	*Diploperla robusta*	SAMN13118598	SRR10357474	GIEP00000000	United States: Indiana	9.4
Perlodidae	*Diura knowltoni*	SAMN13118614	SRR10357457	GIEY00000000	United States: Utah	23.3
Perlodidae	*Helopicus subvarians*	SAMN13118589	SRR10357455	GIDT00000000	United States: Georgia	5.8
Perlodidae	*Hydroperla crosbyi*	SAMN13118611	SRR10357460	GIEF00000000	United States: Illinois	18.9
Perlodidae	*Isogenoides zionensis*	SAMN11441124	SRR8924837	GHQZ00000000	United States: Utah	14.8
Perlodidae	*Isoperla quinquepunctata*	SAMN11441125	SRR8924859	GHRA00000000	United States: Colorado	8.8
Perlodidae	*Kogotus modestus*	SAMN11441120	SRR8924871	GHQV00000000	United States: Colorado	13.4
Perlodidae	*Malirekus Iroquois*	SAMN13118613	SRR10357458	GIED00000000	United States: Ohio	6.6
Perlodidae	*Megarcys subtruncata*	SAMN13118599	SRR10357473	GIEO00000000	United States: California	8.4
Perlodidae	*Oconoperla innubila*	SAMN11441076	SRR8924883	GHPH00000000	United States: North Carolina	11.6
Perlodidae	*Perlinodes aureus*	SAMN11441075	SRR8924884	GHPG00000000	United States: Montana	2.6
Perlodidae	*Pictetiella expansa*	SAMN13118594	SRR10357439	GIDL00000000	United States: Colorado	3.6
Perlodidae	*Remenus bilobatus*	SAMN11441091	SRR8924839	GHPV00000000	United States: Virginia	10.0
Perlodidae	*Rickera sorpta*	SAMN13118600	SRR10357472	GIEN00000000	United States: California	10.1
Perlodidae	*Salmoperla sylvanica*	SAMN13118605	SRR10357467	GIEJ00000000	United States: California	4.5
Perlodidae	*Setvena bradleyi*	SAMN13118607	SRR10357464	GIEI00000000	United States: Montana	4.0
Perlodidae	*Skwala americana*	SAMN11441116	SRR8924875	GHQR00000000	United States: Utah	7.9
Perlodidae	*Susulus venustus*	SAMN13118601	SRR10357471	GIEM00000000	United States: California	13.8

Table 1.1 (cont.).

Perlodidae	*Yugus bulbosus*	SAMN11441121	SRR8924879	GHQW00000000	United States: North Carolina	10.7
Pteronarcyidae	*Pteronarcella badia*	SAMN11441084	SRR8924841	GHPP00000000	United States: Utah	15.4
Pteronarcyidae	*Pteronarcys scotti*	SAMN11441090	SRR8924847	GHPU00000000	United States: Georgia	6.5
Taeniopterygidae	*Bolotoperla rossi*	SAMN11441109	SRR8924854	GHQL00000000	United States: North Carolina	11.9
Taeniopterygidae	*Doddsia occidentalis*	SAMN11441098	SRR8924863	GHQB00000000	United States: Colorado	5.0
Taeniopterygidae	*Oemopteryx glacialis*	SAMN11441110	SRR8924853	GHQM00000000	United States: Quebec	8.1
Taeniopterygidae	*Strophopteryx fasciata*	SAMN11441111	SRR8924858	GHQN00000000	United States: Kentucky	7.7
Taeniopterygidae	*Taenionema pallidum*	SAMN11441112	SRR8924857	GHRI00000000	United States: Montana	7.8
Taeniopterygidae	*Taeniopteryx nivalis*	SAMN11441082	SRR8924881	GHPN00000000	Canada: Quebec	6.8
Blattodea						
Blaberidae	*Blaberus Atropos*	SAMN02047121	SRR921572	GAYD00000000	Germany: North Rhine-Westphalia	42.4
Cryptocercidae	*Cryptocercus wright*	SAMN02047199	SRR921587	GAZN00000000	United States: Oregon	13.9
Termopsidae	*Zootermopsis nevadensis*	N/A	N/A	N/A	United States, California	1.4
Dermaptera						
Apachyidae	*Apachyus charteceus*	SAMN02047175	SRR921565	GAUW00000000	Malaysia: Selangor	23.0
Forficulidae	*Forficula Auricularia*	SAMN02047143	SRR921598	GAYQ00000000	Germany: North Rhine-Westphalia	27.2
Embioptera						
Oligotomidae	*Aposthonia japonica*	SAMN02047170	SRR921566	GAUW00000000	Japan: Kagoshima	24.1
Ephemeroptera						
Baetidae	*Baetis* sp.	SAMN02047149	SRR921569	GATU00000000	Germany: North Rhine-Westphalia	19.1
Ephemerellidae	*Eurylophella* sp.	SAMN02047200	SRR921596	GAZG00000000	United States: Pennsylvania	13.9
Grylloblattodea						
Grylloblattidae	*Galloisiana yuasai*	SAMN02047172	SRR921600	GAWN00000000	Japan: Nagano	22.0
Mantodea						
Mantidae	*Mantis religiosa*	SAMN02047157	SRR921615	GASW00000000	Germany: Rhineland-Palatinate	25.1
Metallyticidae	*Metallyticus splendidus*	SAMN02047174	SRR921620	GATB00000000	Malaysia: Selangor	33.5
Mantophasmatodea						
Tanzaniophasmatidae	*Tanzaniophasma* sp.	SAMN02047176	SRR921644	GAXB00000000	Mali: Labstock	28.3
Odonata						
Calopterygidae	*Calopteryx splendens*	SAMN02047184	SRR921575	GAYM00000000	Sweden: Skane	46.5

Table 1.1 (cont.).

Cordulegastridae	*Cordulegaster boltoni*	SAMN02047156	SRR921583	GAYO00000000	Germany: Rhineland-Palatinate	21.9
Epiophlebiidae	*Epiophlebia superstes*	SAMN02047171	SRR921592	GAVW00000000	Japan: Kyoto	19.0
Orthoptera						
Gryllotalpidae	*Gryllotalpa* sp.	SAMN02047167	SRR921602	GAWZ00000000	Italy: Umbria	22.0
Tetrigidae	*Tetrix subulata*	SAMN02047150	SRR921646	GASQ00000000	Germany: North Rhine-Westphalia	14.9
Phasmatodea						
Pseudophasmatidae	*Peruphasma schultei*	SAMN02047114	SRR921632	GAWJ00000000	Germany: North Rhine-Westphalia	32.3
Timematidae	*Timema cristinae*	SAMN02047191	SRR921650	GAVX00000000	United States: California	44.0
Zoraptera						
Zorotypidae	*Zorotypus caudelli*	SAMN02047173	SRR921660	GAYA00000000	Malaysia: Selangor	28.2

Table 1.2. Summary counts and likelihood scores of the best trees obtained from maximum likelihood analysis using IQ-TREE.

Dataset	**Site total**	**Parsimony-informative sites**	**Singleton sites**	**Constant sites**	**Likelihood score of best tree**
Concatenated amino acid	404 858	214 665	54 646	135 547	-13 400 177.1
Concatenated complete nucleotide	1 214 574	768 043	94 917	351 614	-46 156 815.2
Concatenated nt12	809 716	373 088	92 314	344 314	-15 271 702.4

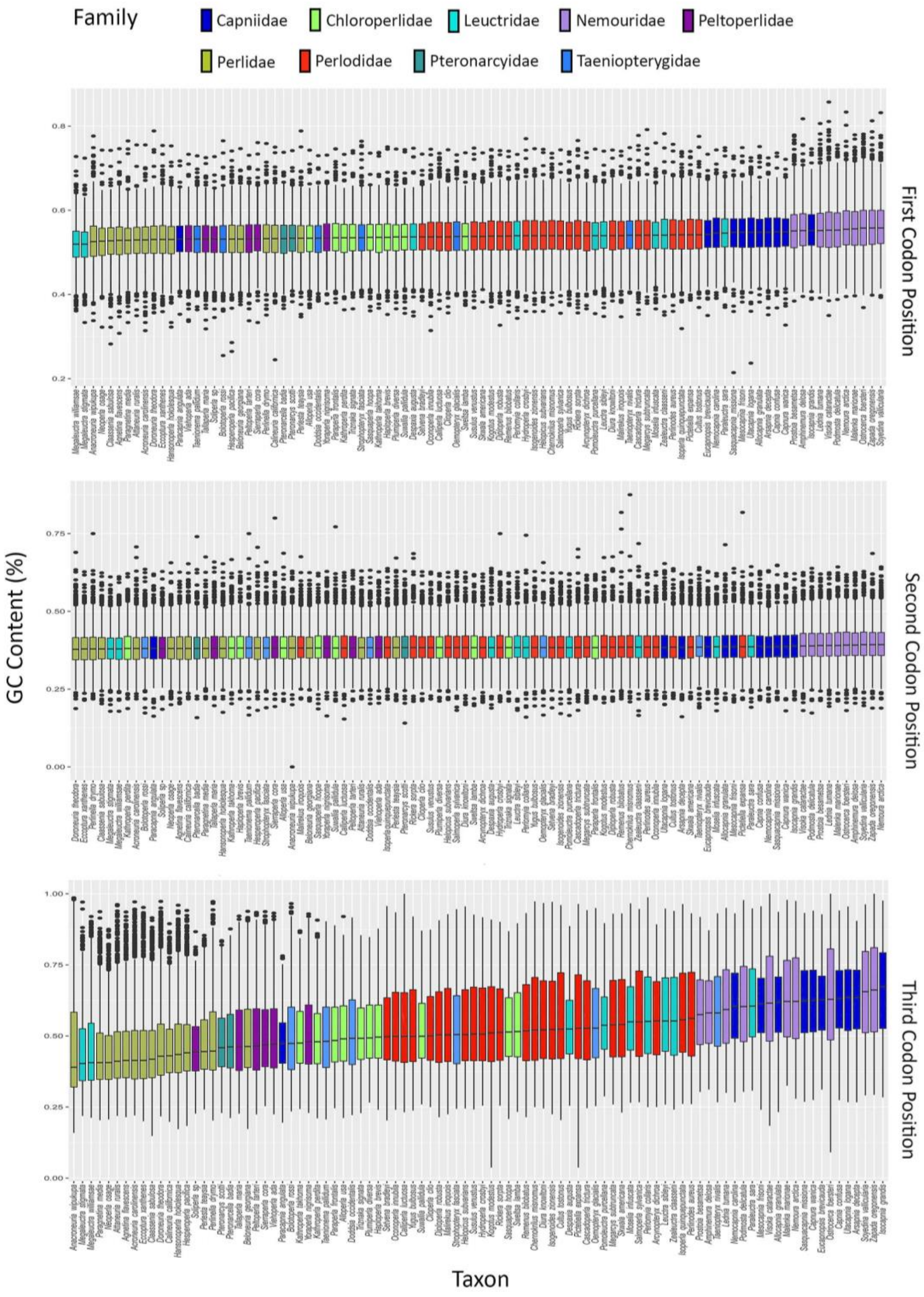

Figure 1.7. Variation of guanine-cytosine (GC) content across 94 North American Plecoptera species for first, second, and third codon positions of individual orthologues. Lines within boxes indicate the median. Boxes show range of data between first and third quartiles, while whiskers indicate 1.5 multiplied by the interquartile range (distance between first and third quartiles).

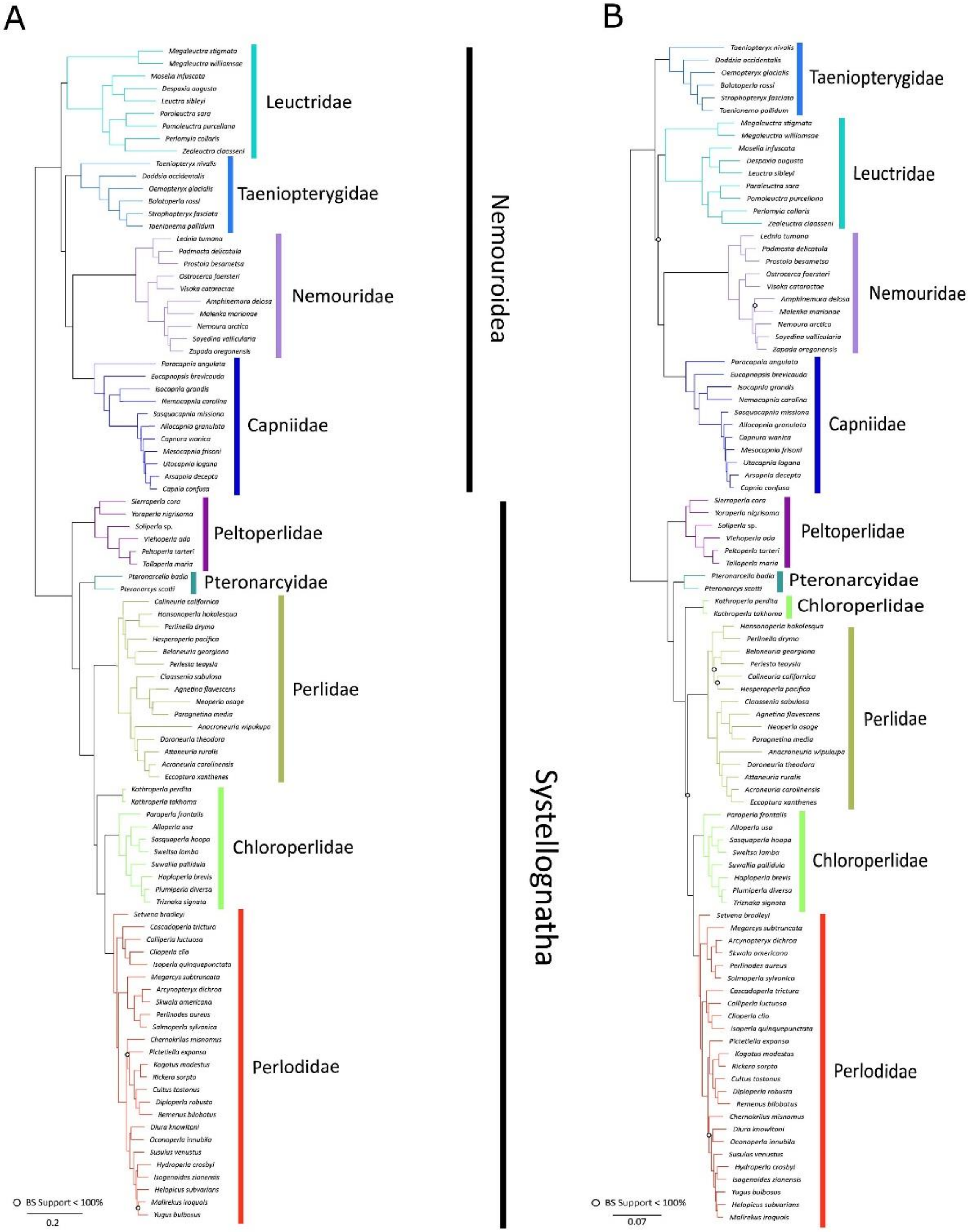

Figure 1.8. Maximum likelihood trees for North American Plecoptera generated from 1400 orthologues. (A) Best-scoring tree recovered from analysis of concatenated complete nucleotide dataset. (B) Best-scoring tree recovered from analysis of concatenated nucleotide dataset with the third codon position removed.

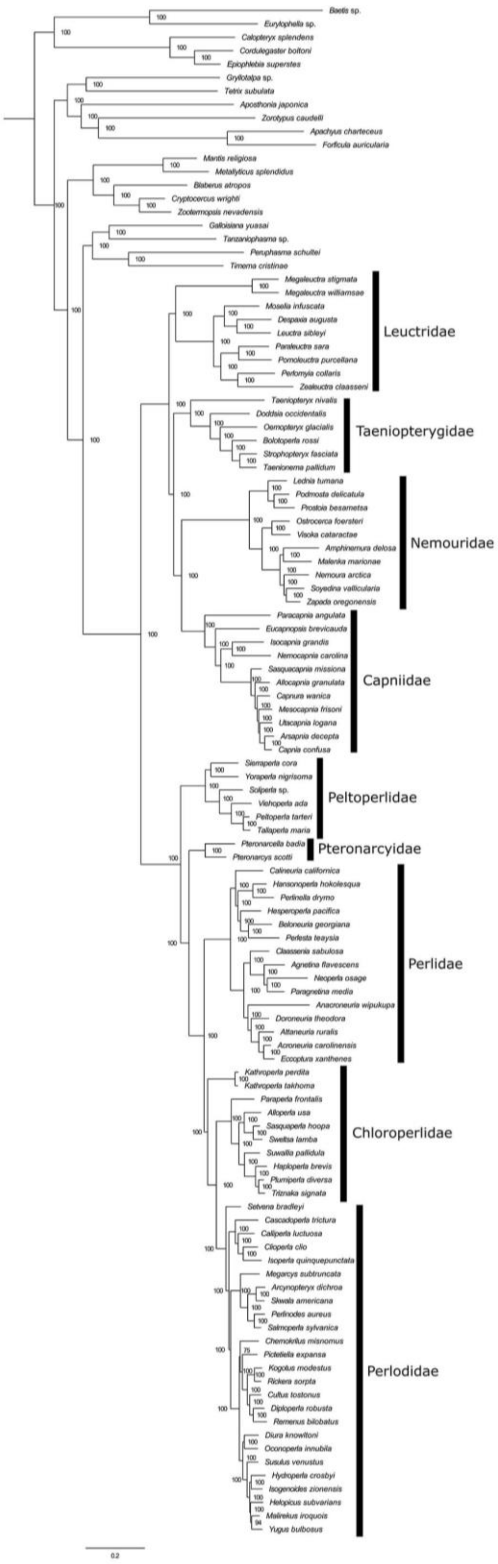

Figure 1.9. Best-scoring maximum likelihood tree including outgroup taxa recovered from analysis of concatenated complete nucleotide dataset.

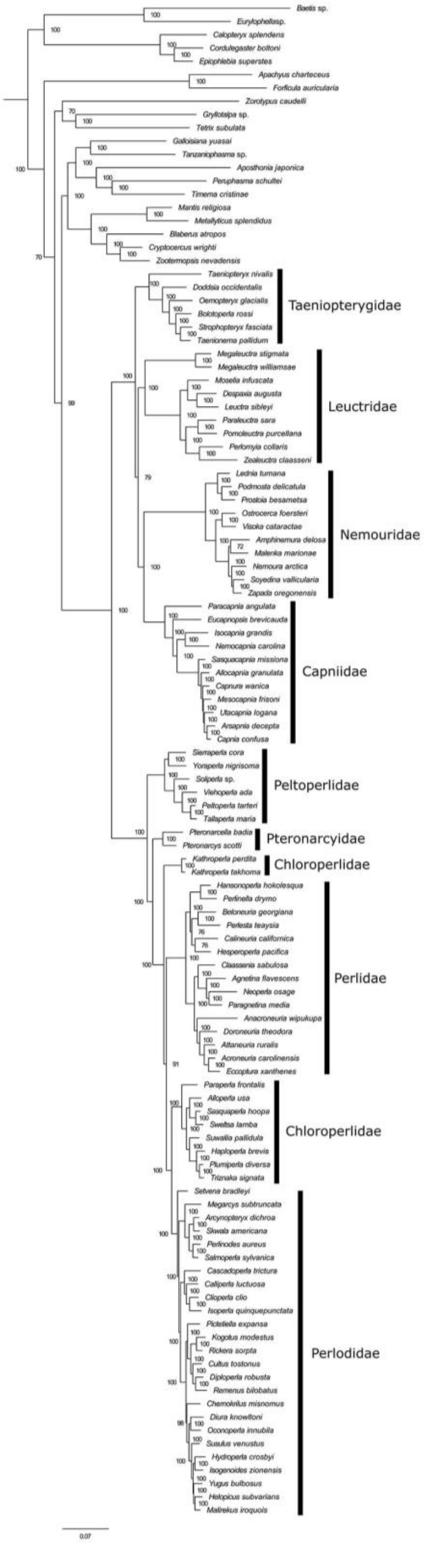

Figure 1.10. Best-scoring maximum likelihood tree including outgroup taxa recovered from analysis of concatenated nucleotide dataset with the third codon position removed.

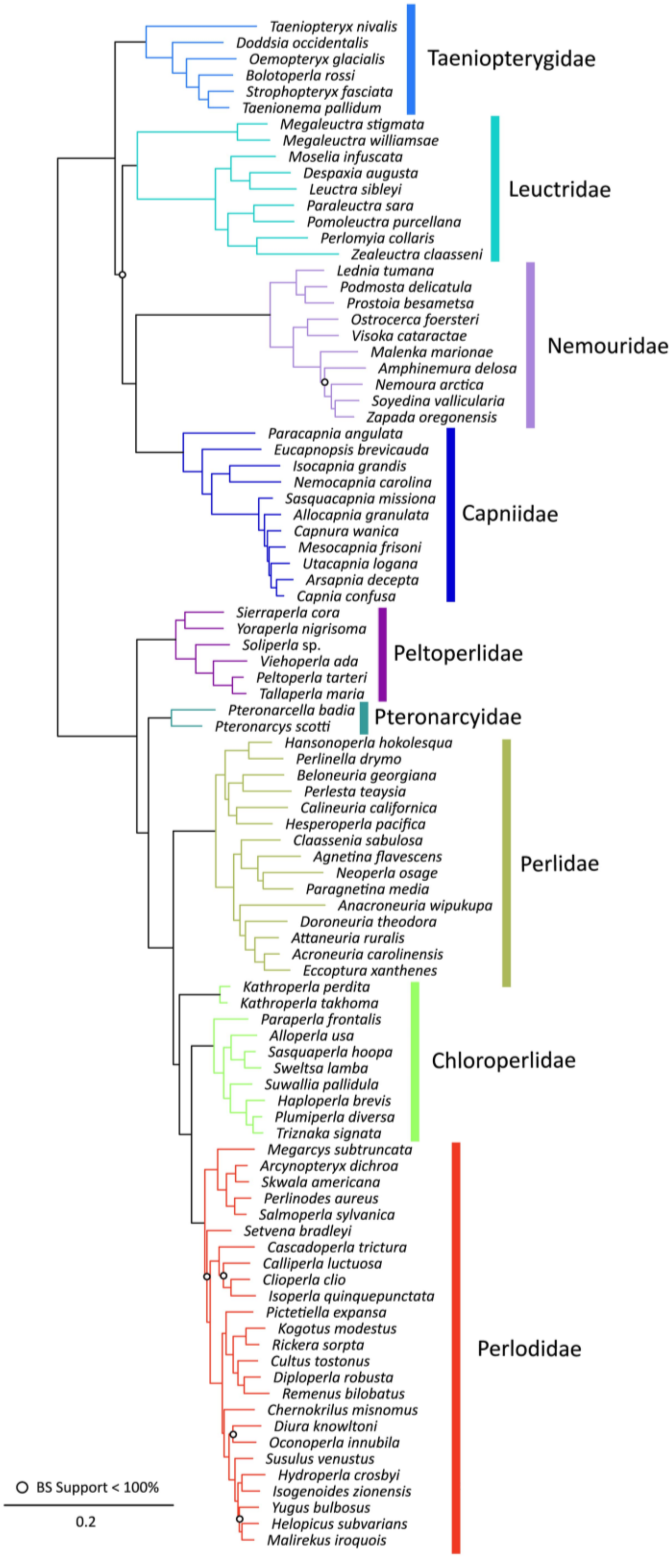

Figure 1.11. Best-scoring maximum likelihood tree recovered from analysis of the concatenated amino acid alignment of North American Plecoptera orthologues.

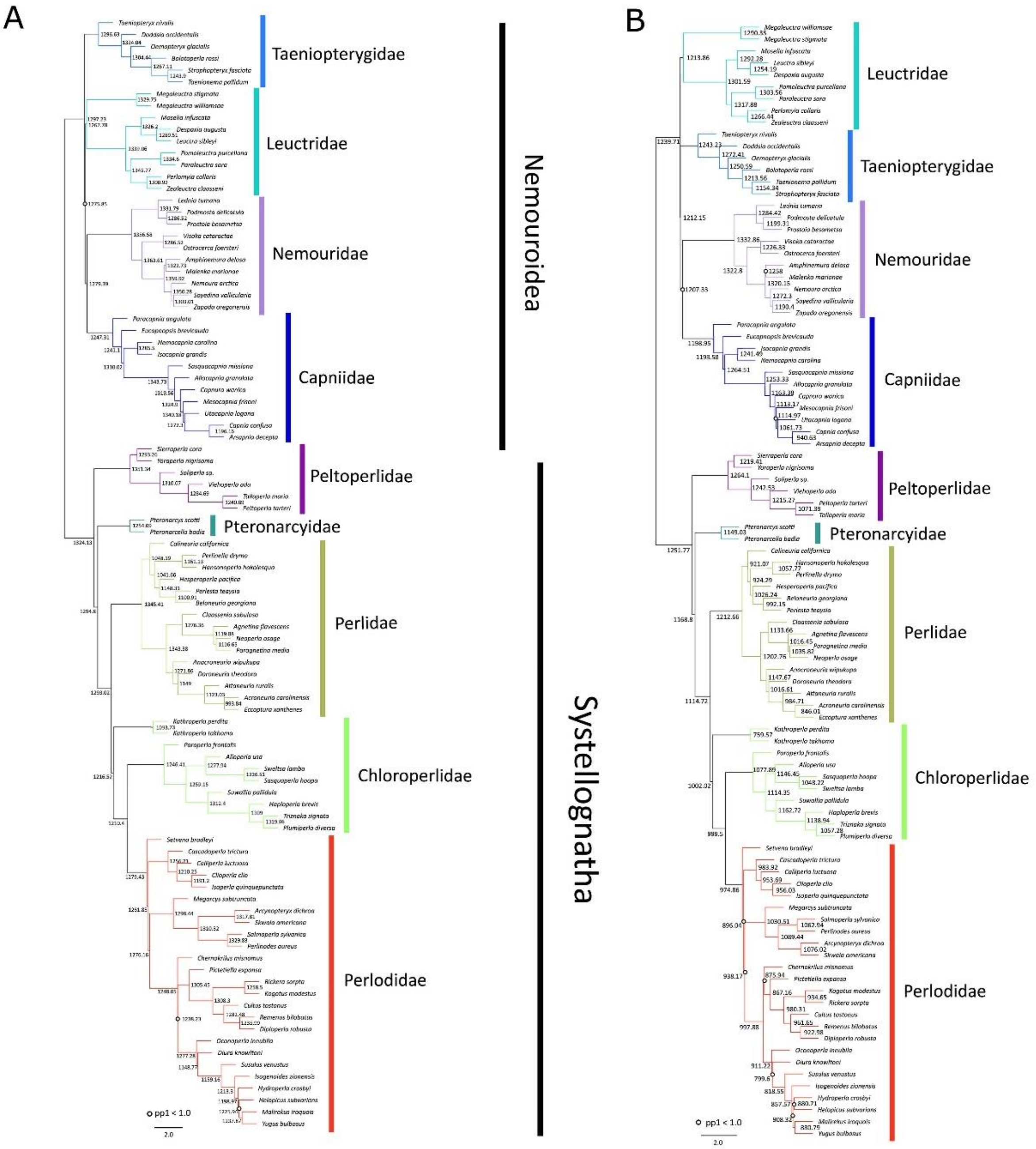

Figure 1.12. Multispecies coalescent tree for North American Plecoptera using ASTRAL. (A) Species tree obtained using gene trees from individual gene alignments including the third codon position. (B) Species tree obtained using gene trees from individual gene alignments excluding the third codon position. Branch values indicate the ASTRAL-calculated effective number, or how many gene trees contributed information to a quartet.

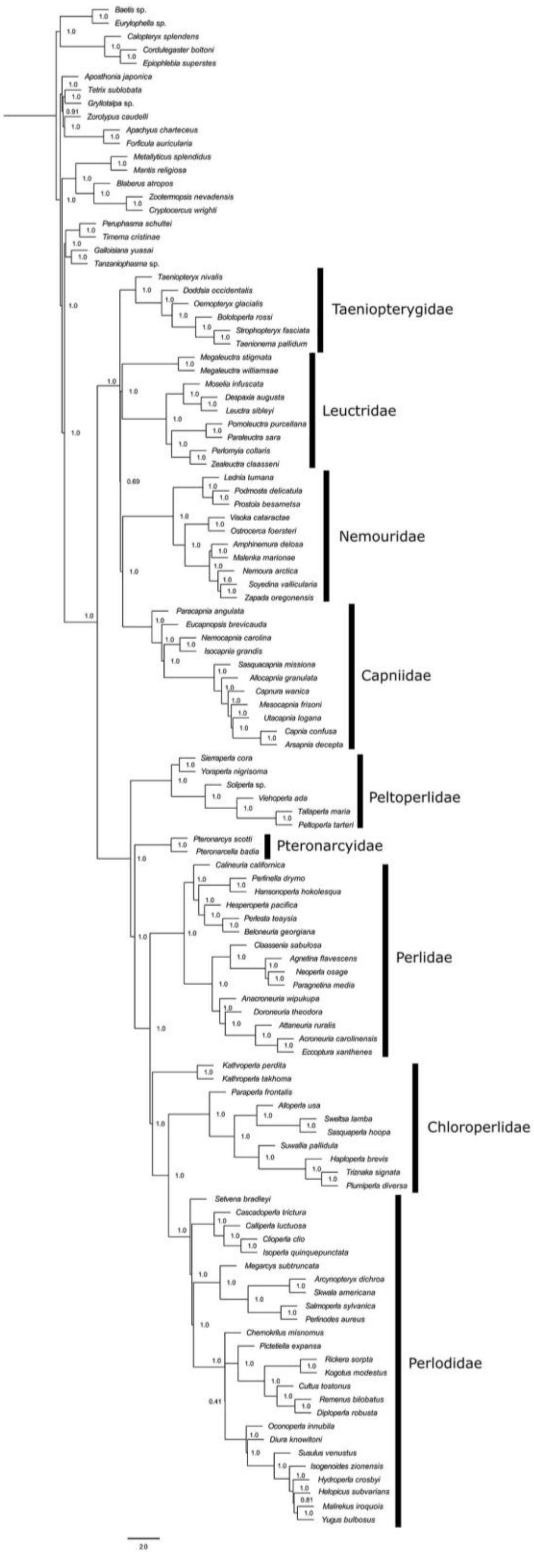

Figure 1.13. Multispecies coalescent tree including outgroup taxa using ASTRAL. Species tree obtained using gene trees from individual gene alignments including the third codon position.

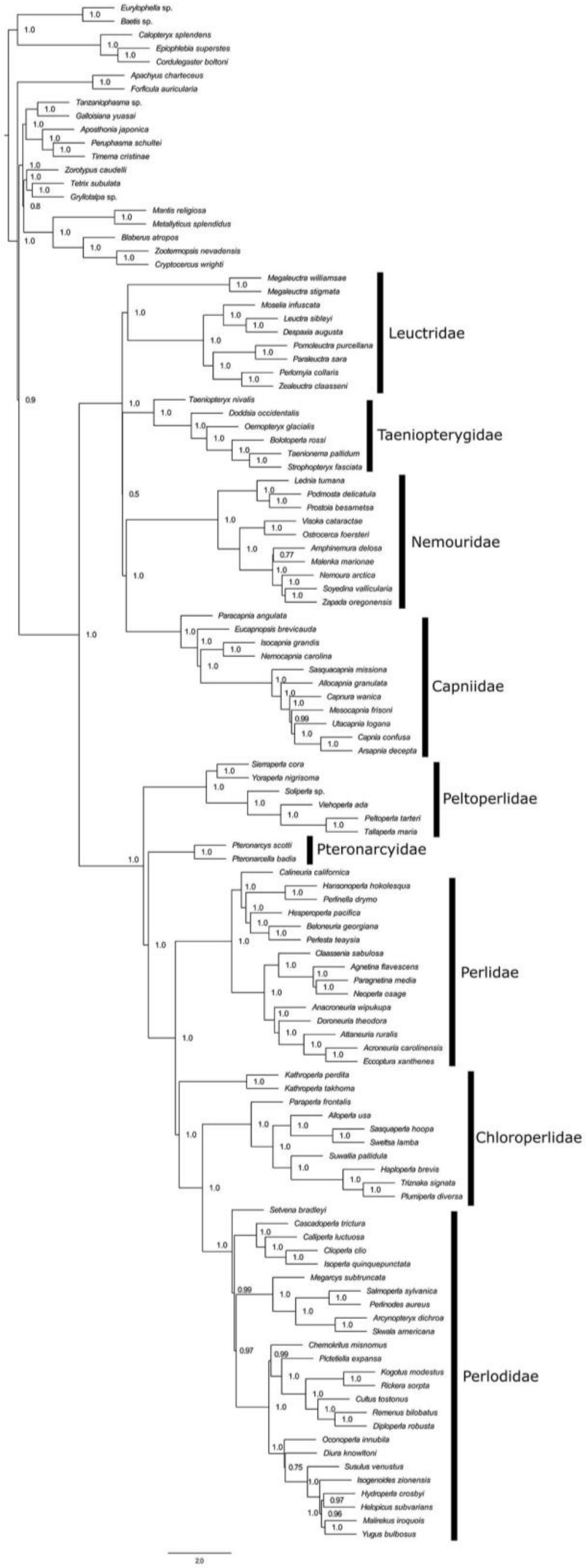

Figure 1.14. Multispecies coalescent tree including outgroup taxa using ASTRAL. Species tree obtained using gene trees from individual gene alignments excluding the third codon position.

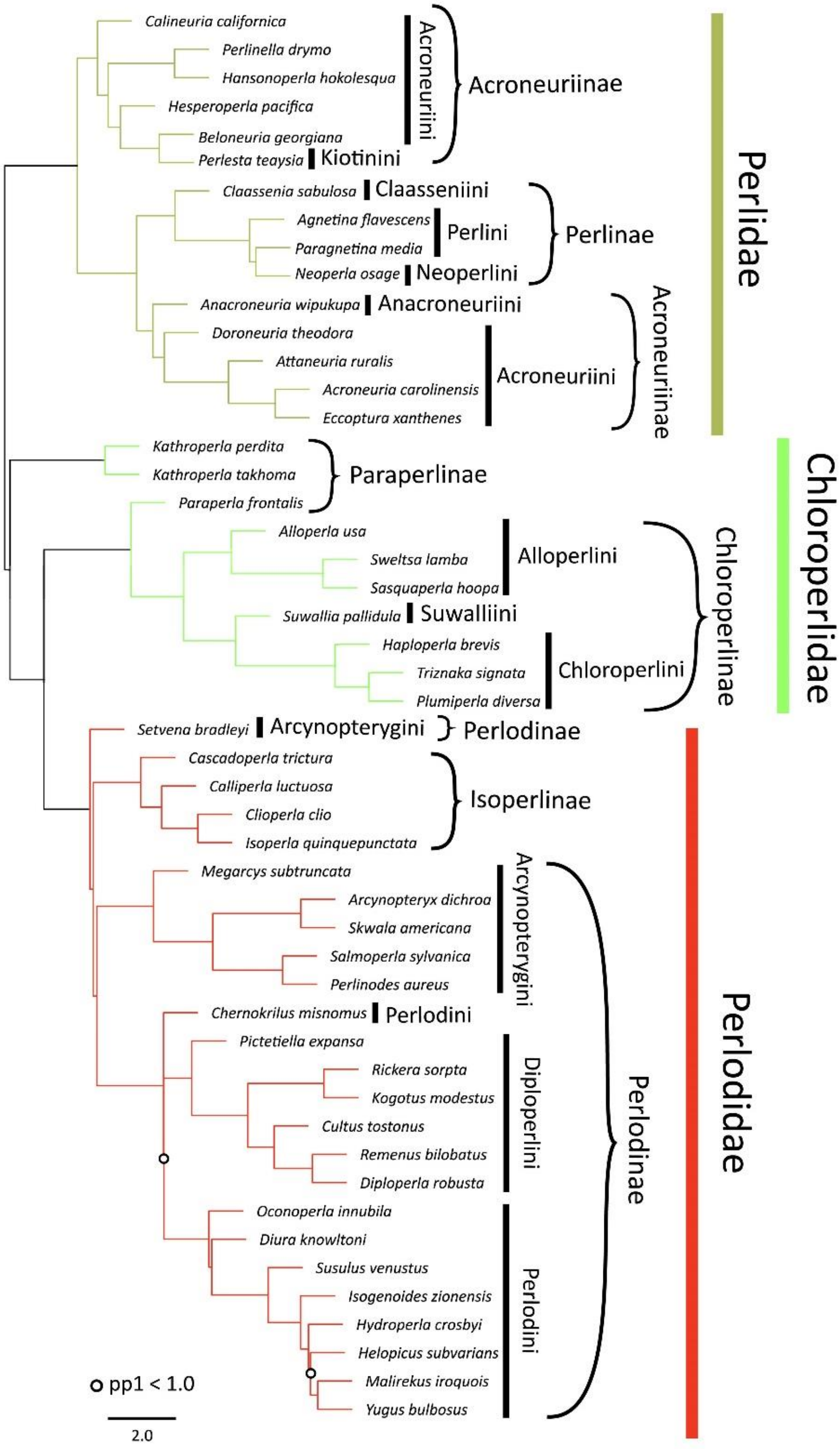

Figure 1.15. Multispecies coalescent tree for superfamily Perloidea of the North American Plecoptera extracted from an ASTRAL-generated species tree obtained using gene trees from individual gene alignments which include the third codon position (Fig. 1.12A). Color bars indicate families, brackets indicate subfamilies, and black bars indicate tribes.

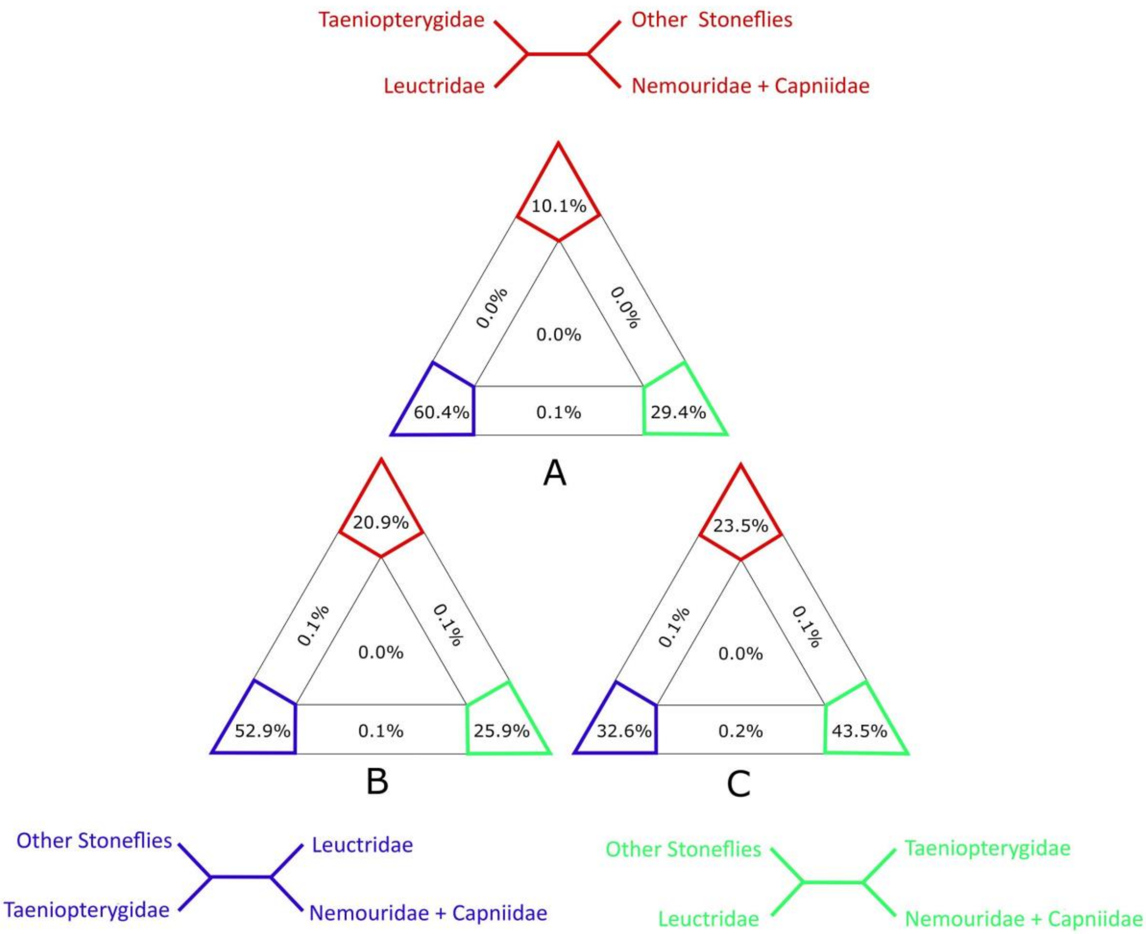

Figure 1.16. Four-cluster likelihood mapping of orthologue data for North American Plecoptera. Shown quartet topologies were tested using one of three alignments: (A) concatenated complete nucleotide, (B) concatenated nucleotide alignment without the third codon position, or (C) amino acid. Each triangle shows percentages of quartets that support unresolved topologies (triangle centers), partially resolved typologies (triangle lateral sections), and fully resolved topologies (triangle corners). Number of taxa comprising taxon groups is 6 for Taeniopterygidae, 9 for Leuctridae, 21 for Nemouridae + Capniidae, and 58 for the Systellognatha “other stoneflies.”

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CHAPTER 2

A NEW FAMILY OF STONEFLIES (INSECTA: PLECOPTERA), KATHROPERLIDAE, FAM. N., WITH A MOLECULAR PHYLOGENETIC ANALYSIS OF THE PARAPERLINAE (PLECOPTERA: CHLOROPERLIDAE)

ABSTRACT

Recent molecular analyses of transcriptome data from 94 species of North American Plecoptera recovered the genus *Kathroperla* Banks, 1920 as sister group to Chloroperlidae + Perlodidae. This discovery prompted further investigation of the genus and the subfamily Paraperlinae. To test phylogenetic placement of these taxa, multiple analyses were performed using 800 orthologous genes from 32 Systellognatha Plecoptera, including all species of the Paraperlinae. Morphological comparisons were made among all Paraperlinae using light microscopy. Molecular results support a monophyletic *Kathroperla* as sister to the remaining Perloidea. Postocular head length is determined to be a distinct morphological character. Combined molecular and morphological evidence support Kathroperlidae, fam. n., as the seventeenth family of extant Plecoptera.

INTRODUCTION

The Chloroperlidae Okamoto, 1912 are typically small, delicate stoneflies with adult coloration ranging from pale yellow to bright green, though some species are of moderate size and/or with dark coloration. Adults are highly susceptible to desiccation and seldom travel far from the protective riparian vegetation of their emergence sites. Nymphs of most species utilize the interstices of coarse-bottomed streams and rivers, or the hyporheic habitat, often many meters inland from the shoreline (Stanford & Ward 1993). Chloroperlid morphology is characterized by oval pronota, slender elongate bodies, and cerci shorter than abdominal length.

The Chloroperlidae have a Holarctic distribution, with 204 described extant species across 20 genera (DeWalt et al. 2020). Two chloroperlid subfamilies are recognized: Chloroperlinae

Okamoto, 1912 and Paraperlinae Ricker, 1943, containing 194 and 10 described extant species, respectively (DeWalt et al. 2020). Three tribes comprise the Chloroperlinae: Alloperlini Surdick, 1985, Chloroperlini Okamoto, 1912, and Suwalliini Surdick, 1985.

Chloroperlidae is one of three families that comprise the superfamily Perloidea Latreille, 1802 (including Chloroperlidae, Perlidae Latreille, 1802, and Perlodidae Klapálek, 1909) within the infraorder Systellognatha. Multiple maximum likelihood (ML) and multispecies coalescent (msc) analyses performed for Chapter 1 of this dissertation recovered a novel family-level phylogenetic relationship among the Perloidea: the genus *Kathroperla* Banks (1920) was removed from the Chloroperlidae and placed as sister to Perlodidae + the remaining Chloroperlidae. One of the ML analyses recovered *Kathroperla* as sister to all Perloidea, similar to the relationship recovered by Terry (2003, unpublished doctoral dissertation).

Kathroperla was proposed with the type species *K. perdita* Banks,1920, described from a single female specimen from British Columbia, Canada. The genus remained monotypic until Stark and Surdick (1987) described *K. takhoma* from Washington, USA. The genus was considered a Nearctic endemic until the description of *K. doma* Stark, 2010 from the Republic of Korea. Stark & Kondratieff (2015) described a fourth species, *K. siskiyou*, from southern Oregon, USA.

Kathroperla (Fig. 2.1A), along with the genera *Utaperla* Ricker, 1952 (Fig. 2.1B), and *Paraperla* Banks, 1906 (Fig. 2.1C), is currently classified (Stark et al. 2020) within the Paraperlinae. Adults of the Paraperlinae are morphologically differentiated from the Chloroperlinae by an elongated head (Zwick 1973). Nymphs are distinguished by lacinia shape: quadrate in *Kathroperla*; triangulate in *Utaperla, Paraperla,* and Chloroperlinae (Stewart & Stark 2002). *Kathroperla* is distinguished among the genera of Paraperlinae by the presence of a vesicle on the ninth male sternite, broad midlateral pigment bands of the pronotum, and postocular elongation of the adult head (Fig. 2.2A). The tuberculate chorion of the ova has also been noted as distinct among the Chloroperlidae (Stark et al. 2015).

Morphological adult characters distinguish *Kathroperla* among the stonefly families. The eyes are situated at the midpoint of an elongated head. Some taxa of other families, such as many perlodids and a few chloroperlids, have elongated heads, but the eyes are located behind the midpoint. The anterodorsal margin of the epiproct of the male genitalia has a transverse orientation (Fig. 2.2B). This is contrasted with other taxa which have a button-like or longitudinal dorsal epiproct shape.

In this study I propose a new stonefly family, Kathroperlidae, fam. n. The morphology of *Kathroperla* and all Paraperlinae species are compared and new light microscopy images of key morphological features and color patterns provided. Phylogenetic trees from multiple molecular analyses of transcriptome and whole genomic data are presented for all species of the Paraperlinae and select outgroup taxa comprising the Systellognatha of North America.

METHODS

Specimen collection and morphological analyses

Adult specimens for all ten described species of the Paraperlinae were obtained, including North American taxa collected for Chapter 1 and newly acquired specimens from the western United States, Republic of Korea, and Russia (Table 2.1). Specimens were collected using a beating sheet, by hand picking, or by Malaise trapping. Additional material was available from the INHS Insect Collection, the Colorado State University Collection (CSUC), and the Eric J. South Collection (EJSC).

Specimens were examined using a Nikon SMZ800 microscope and imaged with an Excelsis MPX-6C camera. Images were stacked and processed using Photoshop 2020. Complete intact single specimens were unavailable for *Kathroperla* and *Utaperla*. Therefore, detached structures from the most complete specimens were imaged separately and digitally stitched to their respective articulations. These modifications were performed to enhance the following two habitus images: Figure 2.1A, *K. takhoma* (left antenna stitched to scape; note: terminal segments of both cerci are missing); Figure 2.1C, *U. gaspesiana* Harper & Roy, 1975 (right antenna stitched to scape; right metathoracic leg stitched to coxa; distal left cercus stitched to tenth cercal segment).

Molecular study

Genomic DNA was extracted from seven Paraperlinae species (*K. doma, K. siskiyou, Paraperla wilsoni* Ricker, 1965*, Utaperla gaspesiana, U. lepnevae* (Zhiltzova, 1970)*, U. orientalis* Nelson & Hanson, 1969*, U. sopladora,* Ricker, 1952) using the Qiagen Blood and

Tissue extraction kit (Qiagen, Valencia, California, USA) and following the manufacturer's protocol. DNA was extracted from excised thoracic tissue of the larger *Kathroperla* and *Paraperla* specimens and from the entire thorax of the smaller *Utaperla* specimens. Remaining unexcised tissue, including abdominal terminalia, was stored in 2 mL vials of 95% EtOH and accessioned as specimen voucher material into the Illinois Natural History Survey (INHS) Insect Collection in Champaign, Illinois (USA) or the Colorado State University Collection (CSUC) in Fort Collins, Colorado (USA). DNA samples were measured for concentration with Qiagen Probit and checked for quality with gel electrophoresis.

The seven genomic DNA samples were processed at the W. M. Keck Center for Comparative and Functional Genomics (Keck Center) at the University of Illinois at Urbana-Champaign, Illinois (UIUC). Shotgun genomic libraries were constructed via the Hyper Library construction kit from Kapa Biosystems (Wilmington, Massachusetts, USA) and 150-bp paired-end reads were sequenced via an Illumina NovaSeq 6000 sequencer (Illumina, San Diego, California, USA). Raw reads received from the Keck Center were reviewed for quality using FASTQC (Andrews 2010). Trimming of raw reads was performed in two steps. First, bases with quality scores below 35 were removed using the fastq_quality_trimmer tool from the FASTX-Toolkit 0.0.14 (Gordon & Hannon 2010). Following this step, the FASTX-Toolkit tool fastx_trimmer was used to remove the first ten bases of each read to reduce noise.

Assembly of target orthologues

A targeted assembly method was used to avoid the computational requirements of full genome assembly and annotation. The software aTRAM (Automated Target Restricted Assembly Method) v2.3.3 (Allen et al. 2018) was used for this purpose, and requires a set of reference sequences for targeted assembly. To build this reference sequence set, a "best orthologue" set for the chloroperlid taxa was generated from ten chloroperlid taxa (7 Chloroperlinae; 3 Paraperlinae) that were included in the dataset generated in Chapter 1. For each orthologue included in the original dataset, the longest sequence with the fewest ambiguities (undetermined X residues in the sequence) was selected from these ten species to serve as the reference sequence for that orthologue.

The aTRAM libraries for each taxon were constructed from the trimmed Illumina reads using the atram_preprocessor.py script. After library construction, assemblies were performed using the atram.py script with the abyss assembler option and 10 iterations. The atram_stitcher.py script was then used to obtain the final assembled sequences for downstream analyses. Amino acid sequences for the assembled nucleotide sequences were obtained using TransDecoder v5.5.0 (Haas & Papanicolaou 2018). A total of 1445 orthologues were obtained following these procedures and were included in the alignment and trimming steps.

Alignment, trimming, and taxon-presence filtering

Amino acid sequences for each orthologue were aligned individually using MAFFT v7.471 (Katoh et al. 2002, Katoh & Standley 2013) and trimmed using trimal v1.4.rev15 with the --gappyout option (Capella-Gutierrez et al. 2009). Nucleotide sequences were aligned with respect to the amino acid sequences using PAL2NAL (Suyama et al. 2006) and trimmed using the same settings. In order to reduce the potential effect of missing data while still retaining sufficient orthologues for gene tree analysis, both amino acid and nucleotide orthologues were filtered by species presence following trimming so that only orthologues containing at least 70% of species in Paraperlinae, at least one member of the genus *Kathroperla*, and at least half of all total species were retained. This filtering step resulted in a total of 800 orthologues being retained for maximum likelihood and gene tree analyses.

Maximum likelihood (ML) analyses

Amino acid and nucleotide alignments were concatenated using SEQUENCE MATRIX v. 1.8 (Vaidya et al. 2011). Because high variability in the third codon position was recovered in stonefly orthologues (see Chapter 1, Fig. 1.7) and has been considered a potential source of systematic bias in phylogenomic analyses (Weisberg et al. 1989, Hasegawa & Hashimoto 1993, Collins et al. 2005, Simon et al. 2006), two datasets of concatenated nucleotide alignments were used for further downstream analyses: one with all three codon positions (hereafter complete nucleotide) and a second excluding the third codon position (hereafter nt12).

Maximum likelihood (ML) analyses were conducted on the concatenated amino acid, complete nucleotide, and nt12 alignments using IQ-TREE v. 2.1.1 (Nguyen et al. 2015) via the CIPRES Science Gateway v3.3 (Miller et al. 2012). Analyses were performed under the -m TEST option, which executes model selection followed by ML tree inference using the best-fit model identified by the Bayesian Information Criterion (BIC). The best-fit models selected by

BIC were JTT + F + I + G4 for the concatenated amino acid alignment and GTR + F + I + G4 for the concatenated complete nucleotide and nt12 alignments. Branch support was assessed with 3000 ultrafast bootstrap replicates (Minh et al. 2013, Hoang et al. 2018) under the -bnni option to reduce the risk of branch support overestimation.

The nucleotide partitioning schemes were obtained from the concatenated complete nucleotide and nt12 nexus files output from SequenceMatrix. ML analyses were performed on the partitioned complete nucleotide and nt12 datasets via the CIPRES Science Gateway. Within IQ-TREE, a model search using the -TESTMERGE option was performed using MODELFINDER (Chernomor et al. 2016). Due to computational limitations, the relaxed hierarchical clustering algorithm (Lanfear et al. 2014) was used to limit partition merge examinations to the top 10% of partition merging schemes under the -rcluster option. The -Q partition option was used to allow each partition to have its own branch lengths to account for within-site evolutionary rate variation. Branch support was assessed by generation of 3000 ultrafast bootstrap replicates within partitions, reducing the risk of overestimating branch support by using the -bnni option. Trees were rooted with outgroup taxa from Peltoperlidae and Pteronarcyidae.

Multispecies coalescent analysis

Individual model tests and maximum likelihood analyses with 1000 ultrafast bootstraps for all 800 orthologues were performed in IQTree v1.6.9. After collapsing branches with less than 5% bootstrap support, the concatenated gene tree sets were used as input for ASTRAL II v.5.15.1 (Mirarab & Warnow 2015) under default settings for multispecies coalescent analysis (hereafter msc). Support values were generated as local posterior probabilities (Sayyari & Mirarab 2016).

RESULTS

Taxonomy

Kathroperlidae South et al., fam. n.

Included genera: *Kathroperla* Banks, 1920

Examination of *Kathroperla* specimens revealed intraspecific variation and morphological detail not provided in the original descriptions. Slight differences were observed in shape of the head, epiproct, and subgenital plate. Additionally, first-time images of key structures generated for this chapter displayed greater detail of coloration patterning than that shown in previous illustrations.

Kathroperla doma **Stark**

Figs. 2.3–2.4

Kathroperla doma Stark, 2010. Holotype ♂ (National Museum of Natural History, Smithsonian) Dungeon-li near Doma Pass, Sangchon-Myeon, Chungbuk, Republic of Korea

Distribution:

Republic of Korea (Stark 2010).

Remarks:

Male. The head is broad and subquadrate with slightly rounded lateral occipital margins (Fig. 2.3A). The postocular region is elongate, and slightly less than two eye lengths from the posterior eye margin to the posterior head capsule margin, a proportional length less than that previously illustrated (Stark 2010, his Fig. 1, unspecified sex). Head coloration shows diffuse dark pigmentation over the occiput and a dark quadrate patch covering the interocellar area which extends anteriorly to the frons, connecting with a darker transverse oblong patch and two anterior dark ovoid patches. This is contrasted with the more uniform head coloration pattern illustrated by Stark (2010, his Fig. 1). Anterior and posterior pronotal margins are darker than illustrated by Stark (2010, his Fig. 1). An image of the thoracic sterna is provided for the first time (Fig. 2.3B). Stark (2010) described the epiproct as butterfly-shaped with lateral margins curved mesially (his Figs. 2, 4). In contrast, Fig. 2.3C depicts a lateral margin that is not curled mesially, but is straight, the lateral margins expanding laterally and widening apically to form a wide V-shape (Fig. 2.3C). The imaged vesicle on the ninth sternum appears stalked (Fig. 2.3D), a feature not illustrated by Stark (2010, his Fig. 3).

Female. The head is similar to that of the male, but postocular length is approximately two eye lengths from posterior eye margin to the posterior head capsule margin (Fig. 2.4A). The pronotal margins (Fig. 2.4A) exhibit paler lateral pronotal margins than the male. Mesosternal suture anterior parallel furcal pits is curved posteriorly (Fig. 2.4B). Both subgenital plates (Figs. 2.4C, 2.4D) show dark sclerotization of the lobes which is not illustrated by Stark (2010, his Fig. 10). The specimen from Gyeongsangnam-do is a new province record (Figs. 2.4A, 2.4B, 2.4C).

Material examined:

Republic of Korea: **Gangwon-do:** Jeongseon-gun, Jeongseon-eup, unnamed stream, Gariwangsan Recreational Forest, 37.41001, 128.53417, 12.v–21vi.2019, D.S. Ham, S.H. Park, ♂, ♀ (INHS Insect Collection 658552, 658553). **Gyeongsangnam-do:** (new province record) Hamyang, Macheon, unnamed stream, Samjeong-li, 35.35917, 127.64722, 8.v.–5.vi.2004, P. Tripotin, ♀ (INHS Insect Collection 658551).

Kathroperla perdita Banks

Figs. 2.5–2.6

Kathroperla perdita Banks 1920: 315. Holotype ♀ (MCZ Museum of Comparative Zoology), Kaslo, British Columbia, Canada
Kathroperla perdita: Needham & Claassen, 1925: 132. Description of male
Kathroperla perdita: Neave, 1934: 2. Description of nymph
Kathroperla perdita: Ricker, 1943: 141. Illustration of nymph
Kathroperla perdita: Stark & Surdick, 1987: 530. SEM image of egg
Kathroperla perdita: Stewart & Stark, 2002: 263–265. Description and illustration of nymph
Kathroperla perdita: Stark et al., 2015: 96. SEM image of epiproct

Distribution:

Canada: Alberta, British Columbia, Yukon. United States: Alaska, California, Idaho, Montana, Nevada, Oregon, Washington (DeWalt et al. 2020).

Remarks:

Male. The head (Fig. 2.5A) shows “great length of the head behind the eyes,” as described by Banks (1920). However, the lateral margins of the occiput are slightly rounded and not straight as illustrated by Stark & Surdick (1987, their Fig. 9, unspecified sex). Present images provide detailed information not previously given for segmentation of the antennal flagellum (Fig. 2.5B), lateral pronotal profile (Fig. 2.5C), sclerotization of the thoracic dorsum (Fig. 2.5D), and sternal sclerite configuration (Fig. 2.5E). Stark et al. (2015) provided SEM images for the epiproct (their Figs. 7–8), but light microscopy images demonstrate terminalia coloration (Figs. 2.5F, 2.5G, 2.5H).

Female. The head (Fig. 2.6A) exhibits a more anteriorly truncated interocellar pigment patch than the male. Additionally, the female mesosternal plate (Fig. 2.6B) is more quadrate than the male. The posterior margin of the subgenital plate (Fig. 2.6C) shows a more pronounced median notch than previously illustrated (Needham & Claassen 1925, their Plate 23, Fig. 8).

Material examined:

United States: **California:** Butte Co., Butte Creek, 39.6 km NNE Paradise at Cherry Hill C. G., 40.10273, -121.49932, 26.v.2019, E.J. South, B.C. Kondratieff, J.B. Sandberg, ♀ (EJSC Ka_pe_01). **Montana:** Flathead Co., Middle Fork Flathead River aquifer, 12.3 km ESE West Glacier at well HA18, 400 m from river, 48.46684, -113.81898, 1.vi.2018, W. Sigl, 2♂ (INHS Insect Collection 658497, 658498). **Oregon:** Benton Co., Rock Creek, 30.iii.1938, E. Yeaman, ♂ (INHS Plecoptera 13546).

***Kathroperla siskiyou* Stark & Kondratieff**

Figs. 2.7–2.8

Kathroperla siskiyou Stark et al., 2015: 96–99. Holotype ♀ (National Museum of Natural History, Smithsonian) Split Rock Creek, Jackson County, Oregon, United States

Distribution:

United States: Oregon (Stark & Kondratieff 2015).

Remarks:

Male. The head (Fig. 2.7A) shows two pigment spots lateral to the interocellar patch and one on the anterior frons, features not described or illustrated by Stark et al. (2015, their Fig. 15). The mesosternum (Fig. 2.7B) shows the connection between the furcal pits and the spina, a character not mentioned or illustrated by Stark et al. (2015). Lateral margins of the epiproct (Fig. 2.7C) are directed slightly less caudad than shown in Stark et al. (2015, their Fig. 16). First images of ventral and dorsal views of the extruded aedeagus are provided (Figs. 2.7C, 2.7D).

Female. The first images of *Kathroperla* wings are provided (Figs. 2.8A, 2.8B). Similar to the male, the female head shows two pigment spots lateral to the interocellar pigment patch (Fig. 2.8C). The notched apex of the subgenital plate is bordered by rounded lobes (Fig. 2.8D) instead of pointed projections as illustrated by Stark et al. (2015, their Fig. 19).

Material examined:

United States: Oregon: Jackson Co., MacDonald Creek, 18.6 km S Talent at Wagner Gap Rd., 42.07864, -122.78843, 22.v.2014, B.C. Kondratieff, C.J. Verdone, ♂ (CSUC); Split Rock Creek, 19.3 km S Talent at Wagner Gap Rd., 42.0948, -122.77397, 22.v.2014, B.C. Kondratieff, B.P. Stark, J.B. Sandberg, C.J. Verdone, 2♀ (CSUC).

***Kathroperla takhoma* Stark & Surdick**

Figs. 2.9–2.10

Kathroperla takhoma Stark & Surdick, 1987: 527. Holotype ♀ (National Museum of Natural History, Smithsonian) Falls Creek, Mount Rainier National Park, Washington, United States
Kathroperla takhoma Stark et al., 2015: 100–101. SEM and description of egg

Distribution:

United States: California, Washington (DeWalt et al. 2020).

Remarks:

Male. Head (Fig. 2.9A) is more quadrate than illustrated by Stark & Surdick (1987, their Fig. 8, unspecified sex). First images of dorsal, lateral, and ventral views of male terminalia are provided (Figs. 2.9B, 2.9C, 2.9D).

Female. Variation in the interocellar pigment patch (Figs. 2.10A, 2.10B, 2.10C) and the mesosternum (Fig. 2.10D) are provided for the first time. The subgenital plate notch (Fig. 2.10E) is deeper than that illustrated by Stark & Surdick (1987, their Fig. 4).

Material examined:

United States: California: Humboldt Co., Upper Willow Creek, 12.3 km WSW Willow Creek at Hwy 299, 40.90095, -123.76742, 1.v.2018, J.J. Lee, 3♂ (INHS Insect Collection 658451, 658478, EJSC Ka_ta_01), same but Boise Creek, 26.iv.2007, J.J. Lee, ♀ (INHS Insect Collection 658554), same but Red Mountain Creek, J.J. Lee, ♀ (INHS Insect Collection 658555). **Oregon**: Hood River Co., Casey Creek, Columbia Gorge, 27.v.2007, ♀ (INHS Insect Collection 658556).

Molecular analyses

A total of 800 orthologous genes from 32 stonefly individuals were included in all analyses. The final concatenated amino acid alignment contained 353 872 positions, while the complete nucleotide and nt12 alignments contained 1 061 616 and 707 743 nucleotide positions, respectively (see Table 2.2 for more details). The amount of missing data (ambiguity/gap) was 26.68%. for the nt and amino acid alignments and 26.73% for the nt12 alignment.

Maximum likelihood analyses of the concatenated complete nucleotide, nt12, partitioned nucleotide, and partitioned nt12 datasets generated trees showing *Kathroperla* as the monophyletic sister group to the remaining Perloidea with 100% bootstrap support (Figs. 2.11A, 2.11B, 2.12A, 2.12B, respectively). The ML analysis of the amino acid alignment showed 82% bootstrap support for a monophyletic *Kathroperla* as sister to Chloroperlidae + Perlodidae (Fig. 2.13). Within *Kathroperla*, two topologies were recovered: 1) *K. doma* + *K. siskiyou* as sister to *K. perdita* + *K. takhoma*, by the AA, nt12, and nt12 partitioned analyses and 2) *K. doma* as sister to *K. siskiyou* + (*K. perdita* + *K. takhoma*) by the nt and nt partitioned analyses. The ML analyses of all alignment datasets generated maximum support for a monophyletic Paraperlinae as sister to Chloroperlinae. Within the Paraperlinae, the *Utaperla* were supported as monophyletic in all ML analyses. Relationships between the *Utaperla* species varied among the datasets. However, a sister relationship between *U. gaspesiana* + *U. orientalis* and *U. lepnevae* + *U. sopladora* was supported by the AA, nt12, and nt12 partitioned analyses. A second topology, *U. lepnevae* as sister to *U. orientalis* + (*U. sopladora* + *U. gaspesiana*), was supported by the nt and nt partitioned analyses. *Paraperla frontalis* was recovered as the earliest diverging lineage within the Paraperlinae clade by all five analyses.

Similar to the ML analyses, the msc analysis recovered a monophyletic *Kathroperla* as sister to the remaining Perloidea and a monophyletic Paraperlinae as sister to Chloroperlinae (Fig. 2.14). Within the *Kathroperla* clade and dissimilar to the ML analyses, the msc analysis recovered *K. doma* as sister to *K. takhoma* + (*K. perdita* + *K. siskiyou*). Within the Paraperlinae, the topology recovered for *Utaperla* was the same as the ML analyses of the nt and nt partitioned datasets. However, unlike the paraphyly recovered for *Paraperla* in the ML analyses, *P. frontalis* and *P. wilsoni* were recovered as a monophyletic sister group to Chloroperlinae.

DISCUSSION

Morphology

Several adult morphological characters separate *Kathroperla* from the Paraperlinae including head configuration, male terminalia, wing venation, female subgenital plate shape, and the connection of the mesosternal furcal pits to the spina (Zwick 2006, Stark et al. 2015). In *Kathroperla*, postocular head elongation is approximately two eye lengths from posterior margin of the eye to posterior head capsule margin, whereas this measurement is approximately one eye length or less in *Paraperla* (Figs. 2.15A, 2.15B) and *Utaperla* (Figs. 2.16A, 2.16B, 2.16C, 2.16D). For males, a transverse epiproct characterizes *Kathroperla*, compared to an upturned supra-anal process in *Paraperla* (Figs. 2.17A, 2.17B) or upturned bifurcated supra-anal process in *Utaperla* (Figs. 2.18A, 2.18B, 2.18C, 2.18D, 2.18E, 2.18F). The tenth tergite is prominently cleft in *Paraperla* and *Utaperla*, but only partially cleft in *Kathroperla.* Additionally, a vesicle on the ninth sternite is present in *Kathroperla* (Figs. 2.3D, 2.5F, 2.5H, 2.7D, 2.9D) but absent in *Paraperla* and *Utaperla*. Wing venation differentiates *Kathroperla* (Figs. 2.8A, 2.8B) from *Paraperla* and *Utaperla*, the latter two genera having fewer than four veins in the anal lobe of the hindwing (*P. wilsoni*, Fig. 2.19B; *U. gaspesiana*, Fig. 2.19D) and fewer cross veins in the forewing (*P. wilsoni*, Fig. 2.19A; *U. gaspesiana*, Fig. 2.19C). Posterior emargination of the subgenital plate of *Kathroperla* forms a V-shape bordered by pointed lobes (*K. perdita*, Fig. 2.6C; *K siskiyou*, Fig. 2.8D) or pronounced projections (*K. doma*, Figs. 2.4C, 2.4D; *K. takhoma*, Fig. 2.10E). The Paraperlinae subgenital plates are either emarginate with broadly rounded lobes *(P. frontalis*, Fig. 2.20A), slightly emarginate (*P. wilsoni*, Fig. 2.20B; *U. sopladora*, Fig. 2.21D), or entire (*U. gaspesiana*, Fig. 2.21A; *U. lepnevae*, Fig. 2.21B; *U. orientalis*, Fig. 2.21C). The furcal pits of the mesosternum are connected posteriorly to the spina (Zwick 2006, his Fig. 10), whereas this connection is absent in *Paraperla* and *Utaperla* (Zwick 2006) (*Paraperla*, Figs. 2.22A, 2.22B; *Utaperla*, Figs. 2.23A, 2.23B, 2.23C, 2.23D).

Head configuration and epiproct shape distinguish *Kathroperla* from the Chloroperlidae. Elongated heads of *Kathroperla* adults and larvae have been recognized to be distinct from all other Chloroperlidae (Baumann et al. 1977). Although Zwick (2006) stated that head length is a poor diagnostic character mainly due to allometry, meaning early instar nymphs can be indistinguishable. Head length was measured from the anterior ocellus to the rear of the head capsule. In this study, postocular head length of adults was determined using compound eye length. This measurement accounts for eye size (relatively small in *Kathroperla*) and the position of the eyes relative to the posterior head capsule margin. Examination of the taxonomic literature and all available specimens suggest that postocular elongation of approximately two eye lengths is distinct in *Kathroperla*. Other taxa, including a few perlodids and chloroperlids, have long heads. But posterior elongation is usually less than one, or rarely one and half eye lengths. The dorsal epiproct margin of *Kathroperla* is elongated and transverse, contrasted with the longitudinally elongated or button shaped dorsal epiproct aspect of the Chloroperlinae, or the upturned supra-anal process of *Paraperla* and *Utaperla*.

Phylogeny

Molecular phylogenetic analyses performed in this study support evidence from previous morphology-based studies (Zwick 2000, 2006). Zwick (2006) identified apomorphic characters for adult and larval Paraperlinae (see his Table 1) and transferred *Paraperla lepnevae* to *Utaperla*. This transfer is congruent with the monophyly recovered for *Utaperla* by all molecular analyses in this study. Zwick also noted the lack of unique apomorphies for *Paraperla*, suggesting possible paraphyly for the genus. This was supported in the present study by the non-monophyly recovered for *Paraperla* in four of the five analyses. Unique apomorphies among the Paraperlinae for *Kathroperla* adults included presence of the ninth sternite vesicle, epiproct transformed into a short open bowl, forward median lobe of the basisternum, and forward curvature of the suture in front of the furcal pits (Zwick 2006). However, the later character no longer applies since the description of *K. doma* which displays a suture with a posterior curvature. Additionally, semi-quadrate lacinia with no subterminal tooth was identified as a larval autapomorphy (Zwick 2006).

Kathroperla doma is distinct among the *Kathroperla*. Morphological distinctions include a broad subquadrate head, cerci with only four segments, a female subgenital plate with extended triangulate lobes, and a four-sided egg. Moreover, *K. doma* has an Asian distribution, contrasted to the Nearctic distribution of the other species. Additionally, three of the six molecular analyses in this study placed *K. doma* at the base of the *Kathroperla* clade. Further investigation is needed to determine the phylogenetic position of *K. doma*.

CONCLUSIONS

Molecular and morphological evidence from this study support the designation of a seventeenth extant stonefly family, Kathroperlidae, fam. n**.** All molecular analyses show a monophyletic group, five of which recovered *Kathroperla* as sister group to the remaining Perloidea. Observed morphology shows postocular head length of *Kathroperla* as distinct among the Perloidea.

Material examined of the Paraperlinae:

Paraperla frontalis (Banks, 1902)

United States: California: Plumas Co., Sulphur Creek, 47.6 km W Cold Springs at CA-89, 39.70722, -120.53188, 25.v.2019, E.J. South, B.C. Kondratieff, 2♂, ♀ (INHS Insect Collection 658513, EJSC Pa_fr_01, Pa_fr_02); Sierra Co., North Yuba River, 3.0 km SW Bassetts at CA-49, 39.59619, -120.61041, 21.v.2016, E.J. South, R.E. DeWalt, ♀ (793097).

Paraperla wilsoni Ricker, 1965

United States: Alaska: East Fork Six Mile Creek, 5 km N Hope turnoff at mile marker 59 AK 1 south, 16.v.2006, D.W. Webb, ♂ (INHS Insect Collection 487620). **Montana**: Lincoln Co., Ross Creek, Ross Creek Cedars Scenic Area, 48.20782, -115.91459, 27.iv.2008, R.S. Durfee, ♂ (CSUC). **Oregon**: Hood River Co., Herman Creek, near Oxbow Fish Hatchery, 14.v.2003, B.C. Kondratieff, R.W. Baumann, ♀ (CSUC).

Utaperla gaspesiana Harper & Roy, 1975

United States: New York: Delaware Co., East Branch Delaware River, Rt. 28, SW Margaretville, 42.1242, -74.6726, 27.v.2009, L.W. Myers, B.C. Kondratieff, ♂, ♀ (INHS 658543), same but 27.v.2010, L.W. Myers, B.C. Kondratieff, ♂, ♀ (INHS Insect Collection 658549, 658550); Ulster Co., Lucas Creek, E Manorville at Ralph Vedder Rd., 42.1408, -74.0331, 21.v.2008, L.W. Myers, B.C. Kondratieff, R.W. Baumann, ♀ (INHS Insect Collection 658544).

Utaperla lepnevae (Zhiltzova, 1970)

Russia: Khabarovsky Region, Anyui River, Anyui National Park, 6.iv.2019, N.M. Yavorskaya, ♂, ♀ (INHS Insect Collection 658548, 658547).

Utaperla orientalis Nelson & Hanson, 1969

Russia: Khabarovsky Region, Anyui River, Anyui National Park, 6.iv.2019, N.M. Yavorskaya, ♂, ♀ (INHS Insect Collection 658545, 658546).

Utaperla sopladora Ricker, 1952

United States: Montana: Mineral Co., St. Regis River, Haugan, exit 16, 47.38418, -115.39988, 16.vi.2006, R.S. Durfee, ♀ (CSUC); Ravalli Co., Bitterroot River, River Park, Hamilton, 46.24185, -114.16839, 9.vi.2009, R.S. Durfee, ♂ (CSUC), same but Little Sleeping Child Creek, Sleeping Child Rd., 46.13021, -114.04882, 13.vi.2020, B.C. Kondratieff, A. Mousa, 2♂ (CSUC).

Modified key to Stark et al. 2015 for species of *Kathroperla*

0) Adult cerci four-segmented (Figs. 2.3C, 2.4C, 2.4D); dark lateral pronotal bands cover more than 2/3 dorsal surface (Figs. 2.3A, 2.4A); head broad and subquadrate (Figs. 2.3A, 2.4A); egg four-sided (Stark 2010, his Fig. 8) .... *K. doma*

0′) Adult cerci with more than four segments (> 12); dark lateral pronotal bands cover less than 2/3 dorsal surface; head less broad; egg circular in cross section (Stark et al. 2015, his Figs. 1–6, 9–14, 20–25) 1

1) Lateral pronotal margin of adults usually entirely yellow: female subgenital plate usually reaching posterior margin of sternum 9 (Fig. 4 in Stark & Surdick 1987); egg collar absent and chorionic striations absent (Figs. 20–25, Stark et al. 2015); dorsal margin of epiproct usually bicolored with a black anterior band adjacent to a dark brown band (Fig. 1 in Stark & Surdick 1987); basal male cercal segments about 3 times long as wide .... *K. takhoma*

1′) Lateral pronotal margins of adults with black pigment extending at least a third of the margin length, but often completely dark (Fig. 15, Stark et al. 2015); egg collar present, chorionic striations present although sometimes indistinct (Figs. 1–6, 9–14, Stark et al. 2015); dorsal margin of epiproct narrow and black; basal male cercal segments about 2–2.5 times long as wide 2

2) Dorsolateral margin of male epiproct slanted conspicuously caudad (Fig. 16, Stark et al. 2015); egg chorion covered with fine bead-like tubercles poorly organized into striations (Figs. 9–14, Stark et al. 2015) .... *K. siskiyou*

2′) Dorsal margin of male epiproct almost straight; egg chorion covered with thick tubercles organized into well-developed longitudinal striations (Figs. 1–6, Stark et al. 2015) ...... *K. perdita*

Key to families of Perloidea adults

1) Thoracic remnant gill tufts present. Costal cross veins numerous .... Perlidae

1′) Thoracic remnant gill tufts absent, though finger-like projections may be present. Costal cross veins less numerous, usually <10, present in basal half of wing 2

2) Postocular head length from posterior margin of eye to posterior margin of head capsule approximately two eye lengths .... Kathroperlidae, fam. n.

2′) Postocular head length from posterior margin of eye to posterior margin of head capsule less than one and a half eye lengths, usually less than one eye length 3

3) Hindwing with less than four anal veins. Last segment of maxillary palps peg-like and small, connected asymmetrically atop the penultimate segment...Chloroperlidae

3′) Hindwing with more than four anal veins. Last segment of maxillary palp attached symmetrically to penultimate segment .... Perlodidae

FIGURES AND TABLES

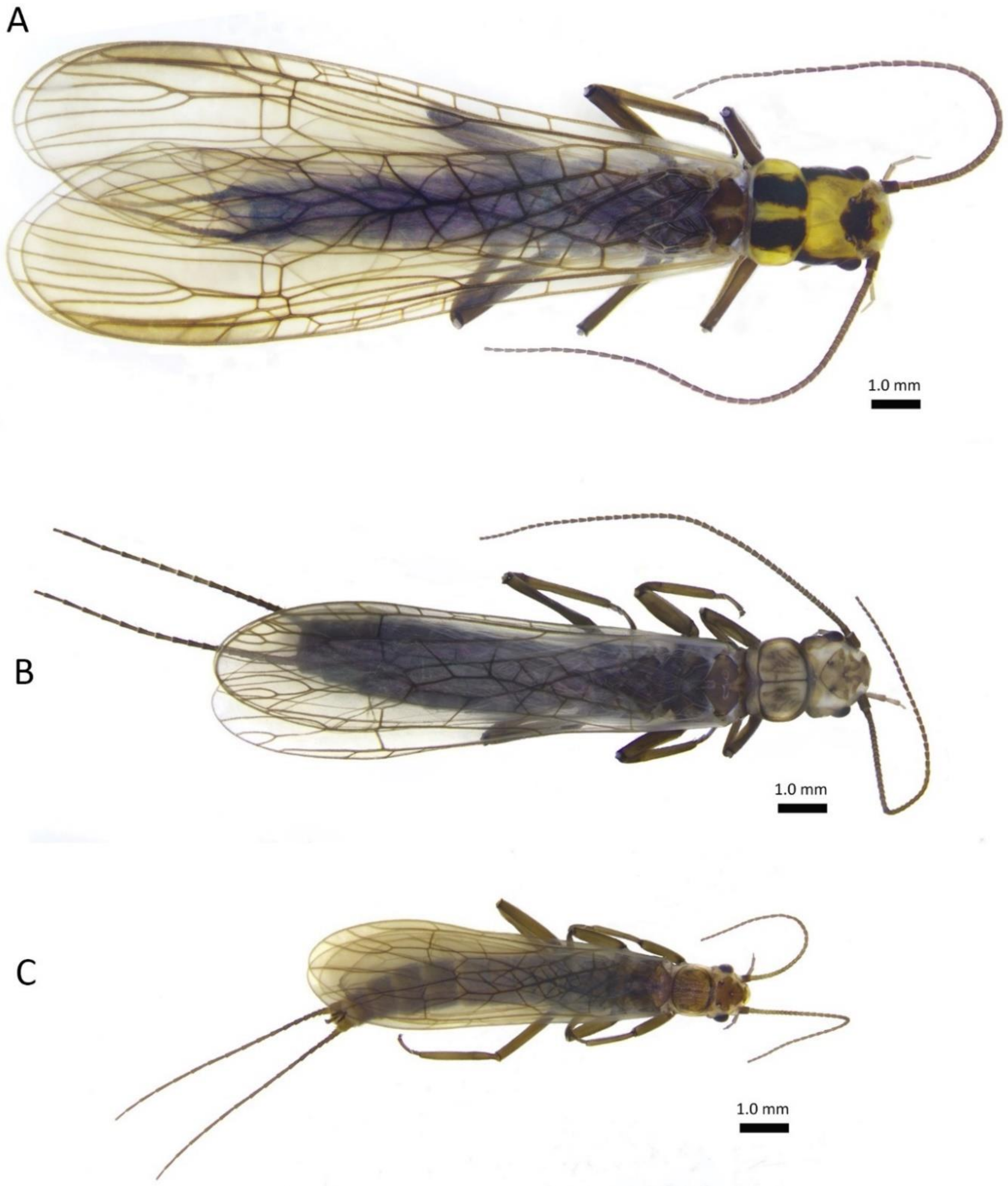

Figure 2.1. Habitus of select Paraperlinae. (A) *Kathroperla takhoma*, male, Upper Willow Creek, California, USA (EJSC Ka_ta_01). (B) *Paraperla frontalis*, female, Sulphur Creek, California, USA (EJSC Pa_fr_02). (C) *Utaperla gaspesiana*, male, East Branch Delaware River, New York, USA (INHS Insect Collection 658549).

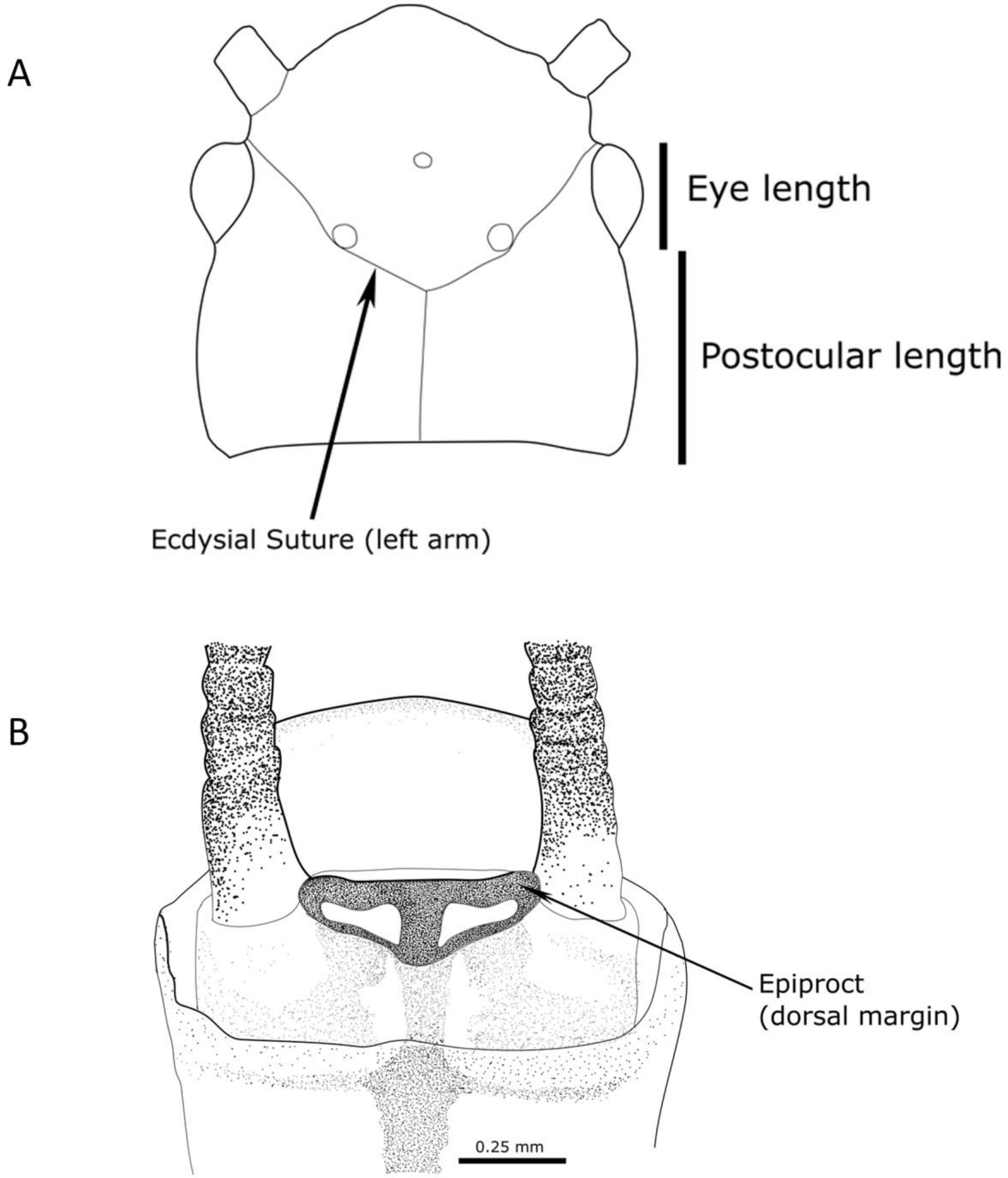

Figure 2.2. *Kathroperla*, adult structures, dorsal. (A) *K. takhoma*, female, head, Boise Creek, California, USA (INHS Insect Collection 658554). (B) *K. perdita*, male, terminalia, Middle Fork Flathead River aquifer, Montana, USA (INHS Insect Collection 658497).

Table 2.1. Stonefly specimens used in phylogenetic analyses. INHS = Illinois Natural History Survey Insect Collection, CSUC = Colorado State University Collection, Ortho. = number of orthologues used in analysis.

Species	**INHS/CSUC**	**Sex**	**Locality**	**Lat./Lon.**	**Ortho.**
Chloroperlinae					
Alloperla usa Ricker, 1952	793088	♂	USA: VA	36.96919, -80.16414	738
Haploperla brevis (Banks, 1895)	658505	♀	USA: IN	39.30893, -86.72016	770
Plumiperla diversa (Frison, 1935)	658536	♂	USA: CO	40.57301, -105.86258	788
Sasquaperla hoopa Stark & Baumann, 2001	658483	♀	USA: CA	40.94097, -123.66079	755
Suwallia pallidula (Banks, 1904)	793178	♂	USA: CO	39.98273, -105.45035	763
Sweltsa lamba (Needham & Claassen, 1925)	658442	♂	USA: WY	41.37398, -106.25403	739
Triznaka signata (Banks, 1895)	658433	♂	USA: CO	40.59895, -105.08371	780
Paraperlinae					
Kathroperla doma Stark, 2010	658552	♂	KOR: Gangwon-do	37.41001, 128.53417	789
Kathroperla perdita Banks, 1920	658497	♂	USA: MT	48.46684, -113.81898	633
Kathroperla siskiyou Stark & Kondratieff, 2015	CSUC	♂	USA: OR	42.07864, -122.78843	791
Kathroperla takhoma Stark & Surdick, 1987	658478	♂	USA: CA	40.90095, -123.76742	682
Paraperla frontalis (Banks, 1902)	793097	♀	USA: CA	39.59619, -120.61041	706
Paraperla wilsoni Ricker, 1965	CSUC	♂	USA: MT	48.20782, -115.91459	798
Utaperla gaspesiana Harper & Roy, 1975	658543	♂	USA: NY	42.1242, -74.6726	799
Utaperla lepnevae (Zhiltzova, 1970)	658548	♂	RUS: Khabarovsky Region		800
Utaperla orientalis Nelson & Hanson, 1969	658545	♂	RUS: Khabarovsky Region		792
Utaperla sopladora Ricker, 1952	CSUC	♂	USA: MT	46.1302, -114.04882	777
Peltoperlidae					
Peltoperla tarteri Stark & Kondratieff, 1987	793136	♂	USA: VA	37.17601, -80.06274	762
Sierraperla cora (Needham & Smith, 1916)	658522	♂	USA: CA	41.32703, -122.32647	773
Viehoperla ada (Needham & Smith, 1916)	658422	♂	USA: NC	35.07265, -83.60309	774

Table 2.1 (cont.).

Perlidae					
Acroneuria carolinensis (Banks, 1905)	793036	♂	USA: OH	40.61294, -82.31696	687
Anacroneuria wipukupa Baumann & Olson, 1984	658439	♀	USA: AZ	34.28371, -111.06792	758
Perlesta teaysia Kirchner & Kondratieff, 1997	793170	♂	USA: VA	37.14702, -81.26314	726
Agnetina flavescens (Walsh, 1862)	793083	♂	USA: VA	37.31615, -80.64498	621
Claassenia sabulosa (Banks, 1900)	658446	♂	USA: CO	40.62089, -105.13942	725
Neoperla osage Stark & Lentz, 1988	793104	♂	USA: AR	35.77562, -94.37487	727
Perlodidae					
Isoperla quinquepunctata (Banks, 1902)	658431	♂	USA: CO	40.59895, -105.08371	753
Arcynopteryx dichroa (McLachlan, 1872)	658526	♂	USA: MI	47.45269, -88.19545	788
Diploperla robusta Stark & Gaufin, 1974	658507	♂	USA: IN	39.30893, -86.72016	749
Isogenoides zionensis Hanson, 1949	793073	♂	USA: UT	40.08458, -111.35519	721
Pteronarcyidae					
Pteronarcella badia (Hagen, 1874)	793074	♂	USA: UT	40.08458, -111.35519	728
Pteronarcys scotti Ricker, 1952	793132	♂	USA: GA	34.68791, -84.20182	755

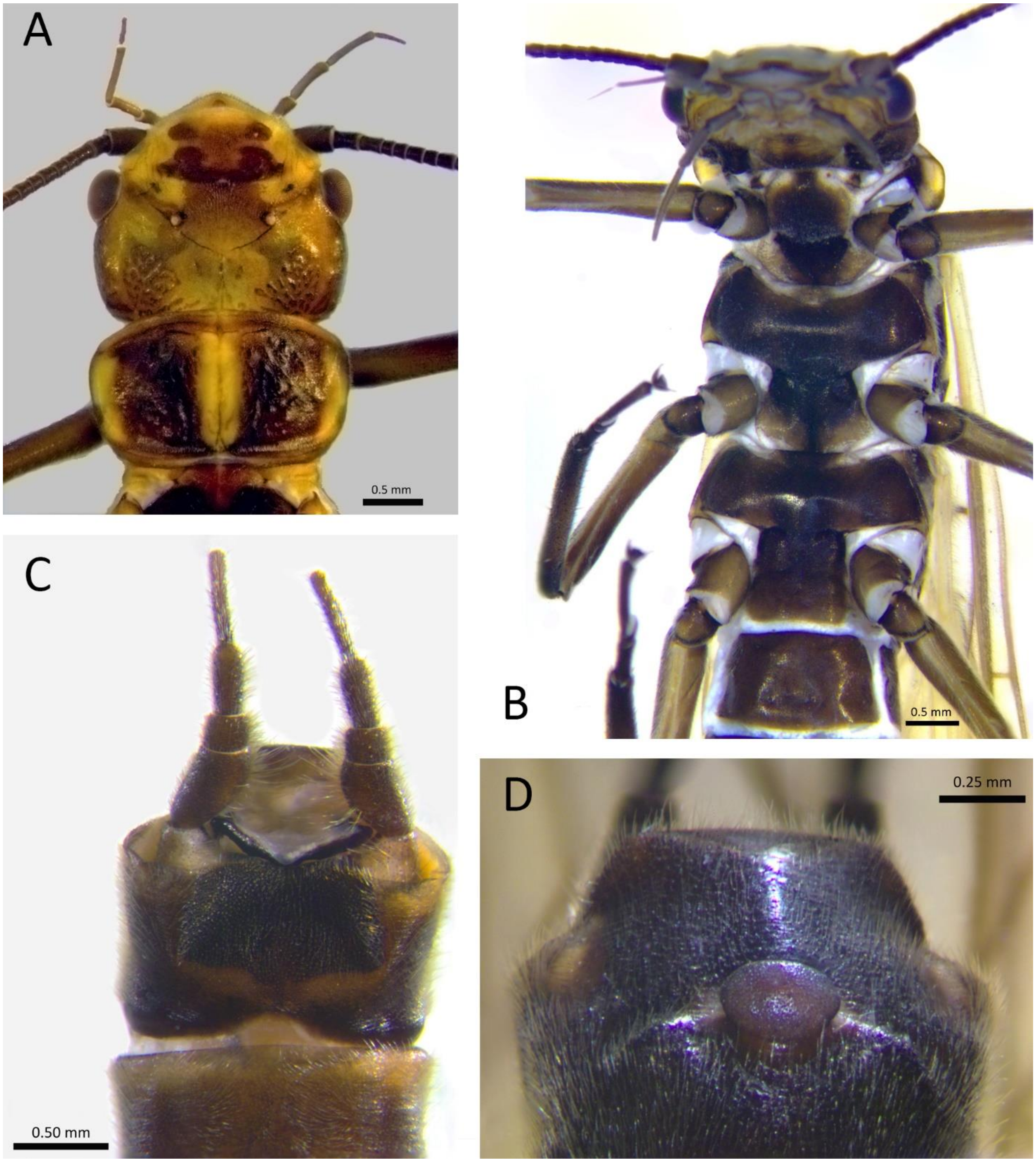

Figure 2.3. *Kathroperla doma*, male, unnamed stream, Gangwon-do, Republic of Korea (INHS 658552). (A) Head and pronotum. (B) Venter. (C) Terminalia, dorsal. (D) Vesicle.

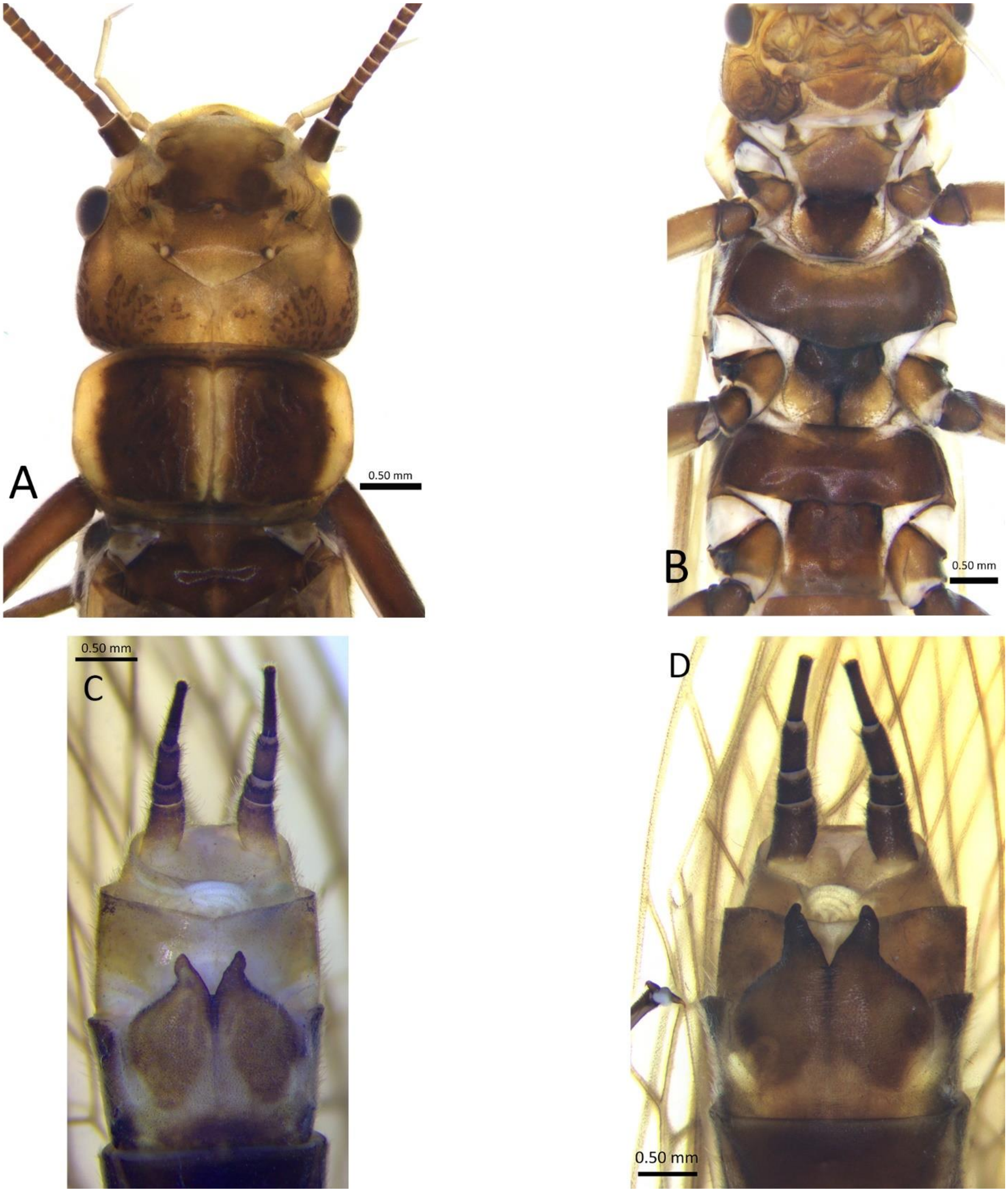

Figure 2.4. *Kathroperla doma*, females. (A) Head and pronotum, unnamed stream, Gyeongsangnam-do, Republic of Korea (INHS Insect Collection 658551). (B) Venter. (C) Terminalia, ventral. (D) Terminalia, ventral, unnamed stream, Gangwon-do, Republic of Korea (INHS Insect Collection 658553).

Figure 2.5. *Kathroperla perdita*, male, Rock Creek, Oregon, USA (INHS Plecoptera 13546). (A) Head and pronotum. (B) Left antenna. (C) Head and pronotum, lateral. (D) Dorsum.

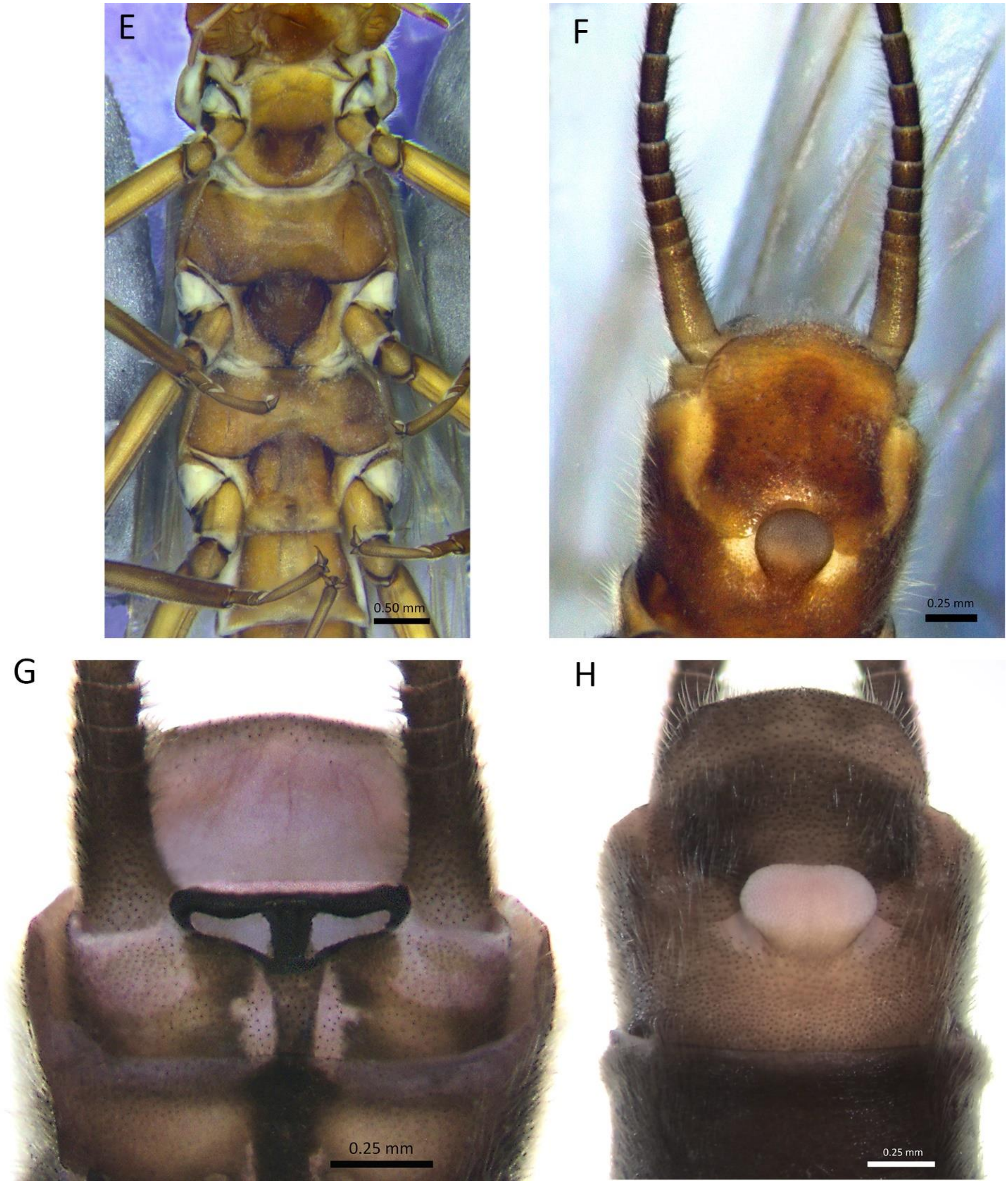

Figure 2.5 (cont.). *Kathroperla perdita*, males. (E) Venter, Rock Creek, Oregon, USA (INHS Plecoptera 13546). (F) Terminalia, ventral. (G) Terminalia, dorsal, Middle Fork Flathead River aquifer, Montana, USA (INHS Insect Collection 658497). (H) Vesicle.

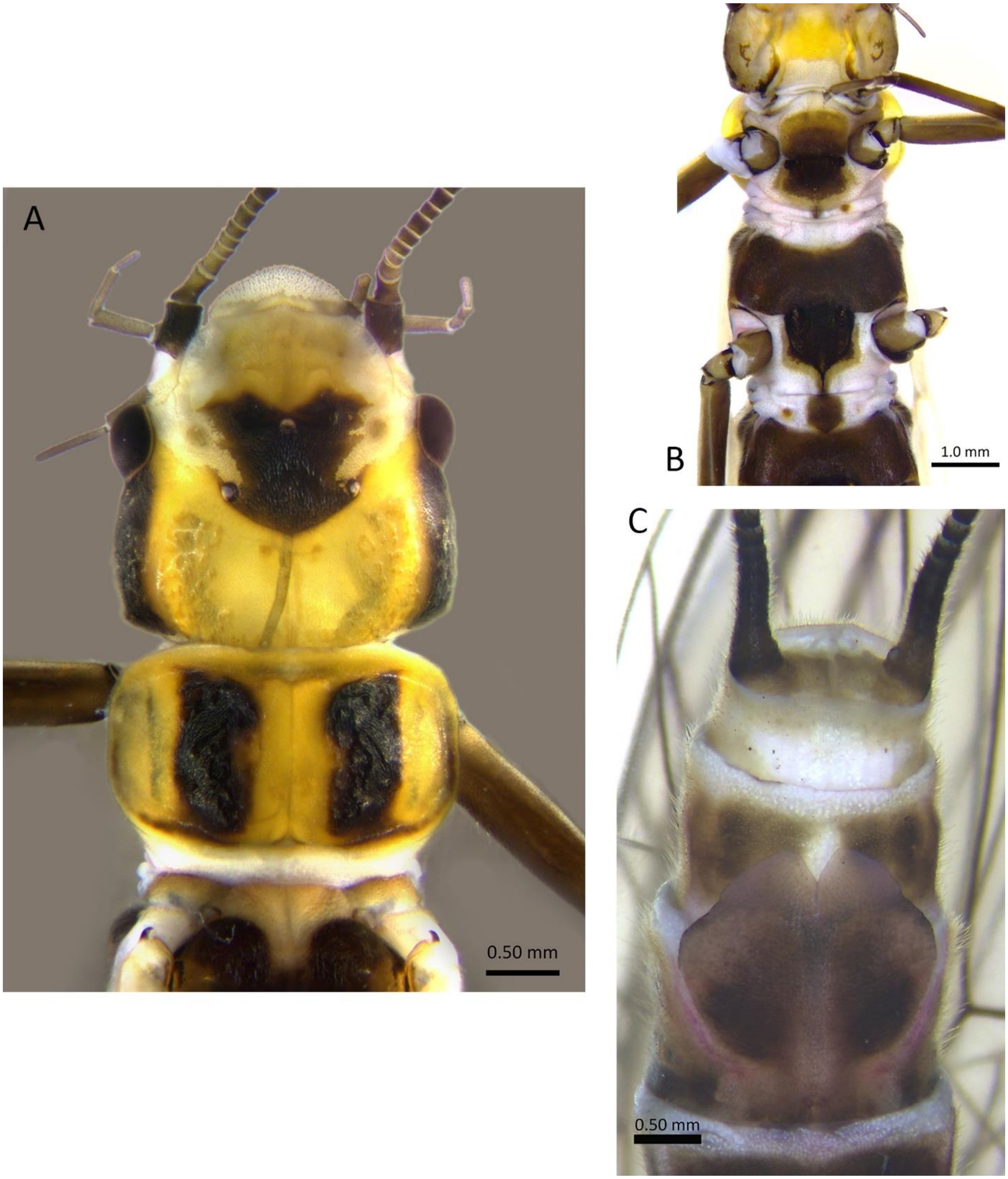

Figure 2.6. *Kathroperla perdita*, female, Butte Creek, California, USA (EJSC Ka_pe_01). (A) Head and pronotum. (B) Venter. (C) Terminalia, ventral.

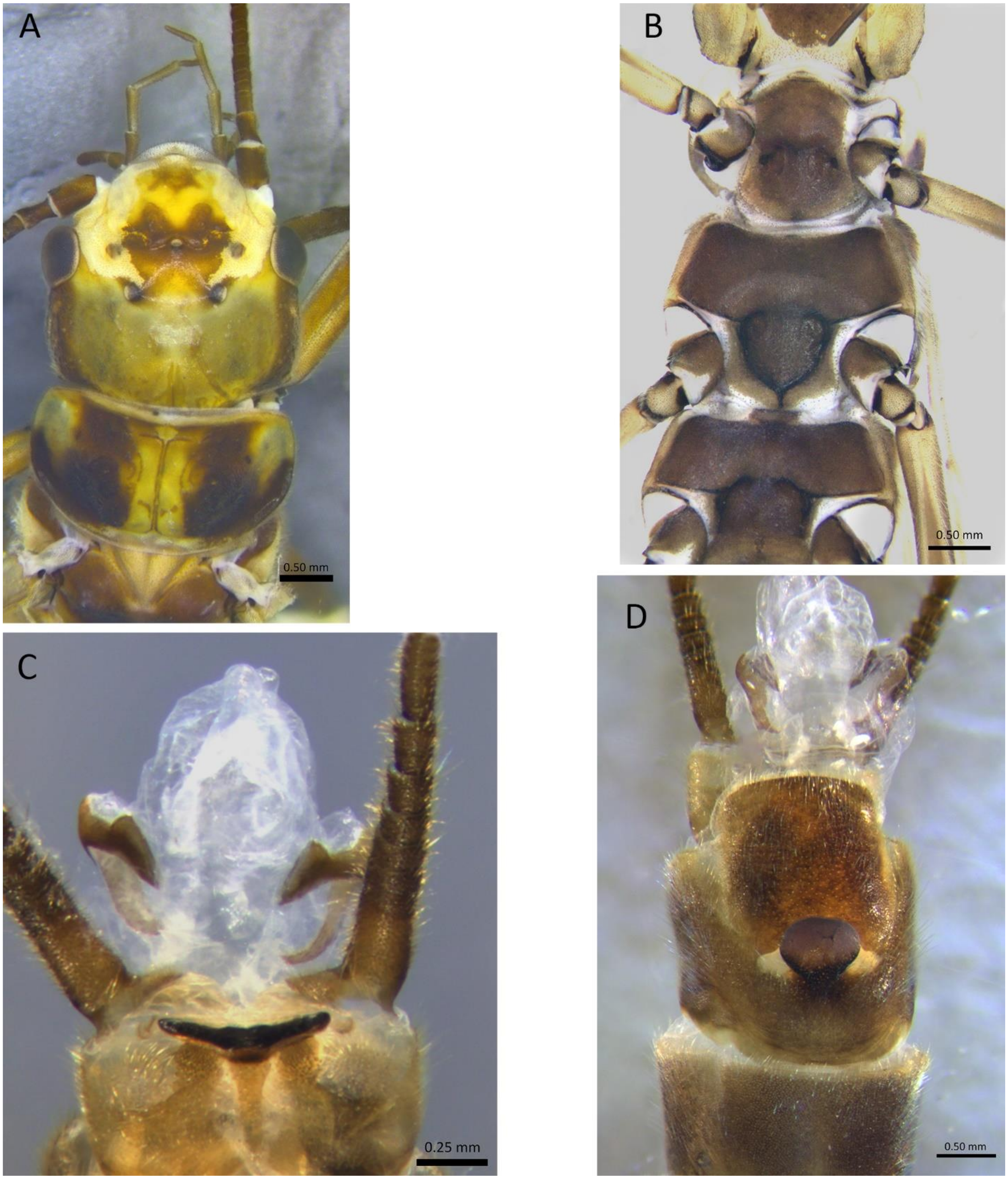

Figure 2.7. *Kathroperla siskiyou*, male, McDonald Creek, Oregon, USA (CSUC). (A) Head and pronotum. (B) Venter. (C) Terminalia with extruded aedeagus, dorsal. (D) Terminalia with extruded aedeagus, ventral.

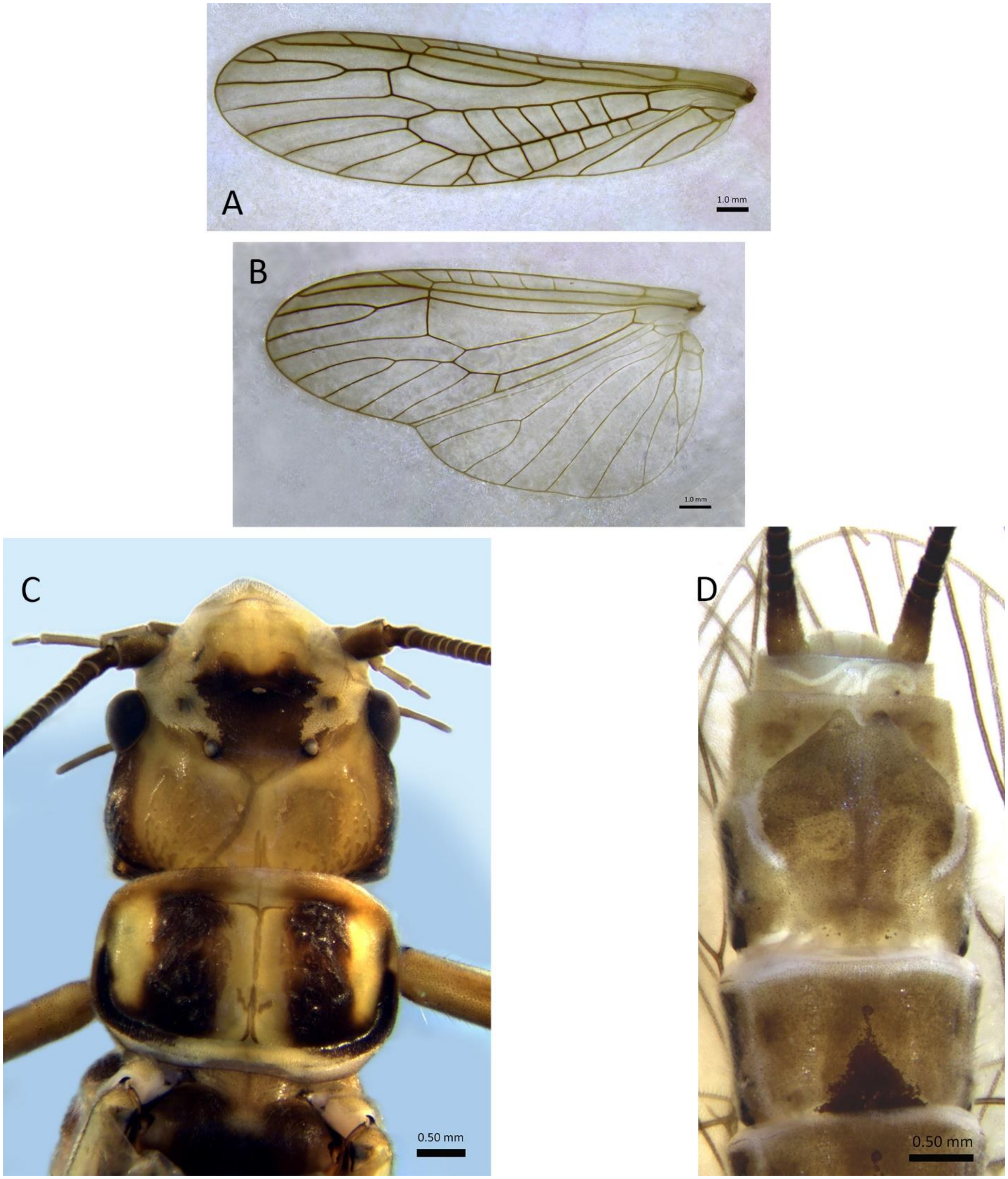

Figure 2.8. *Kathroperla siskiyou*, female, Split Rock Creek, Oregon, USA (CSUC). (A) Left forewing. (B) Left hindwing. (C) Head and pronotum. (D) Terminalia, ventral.

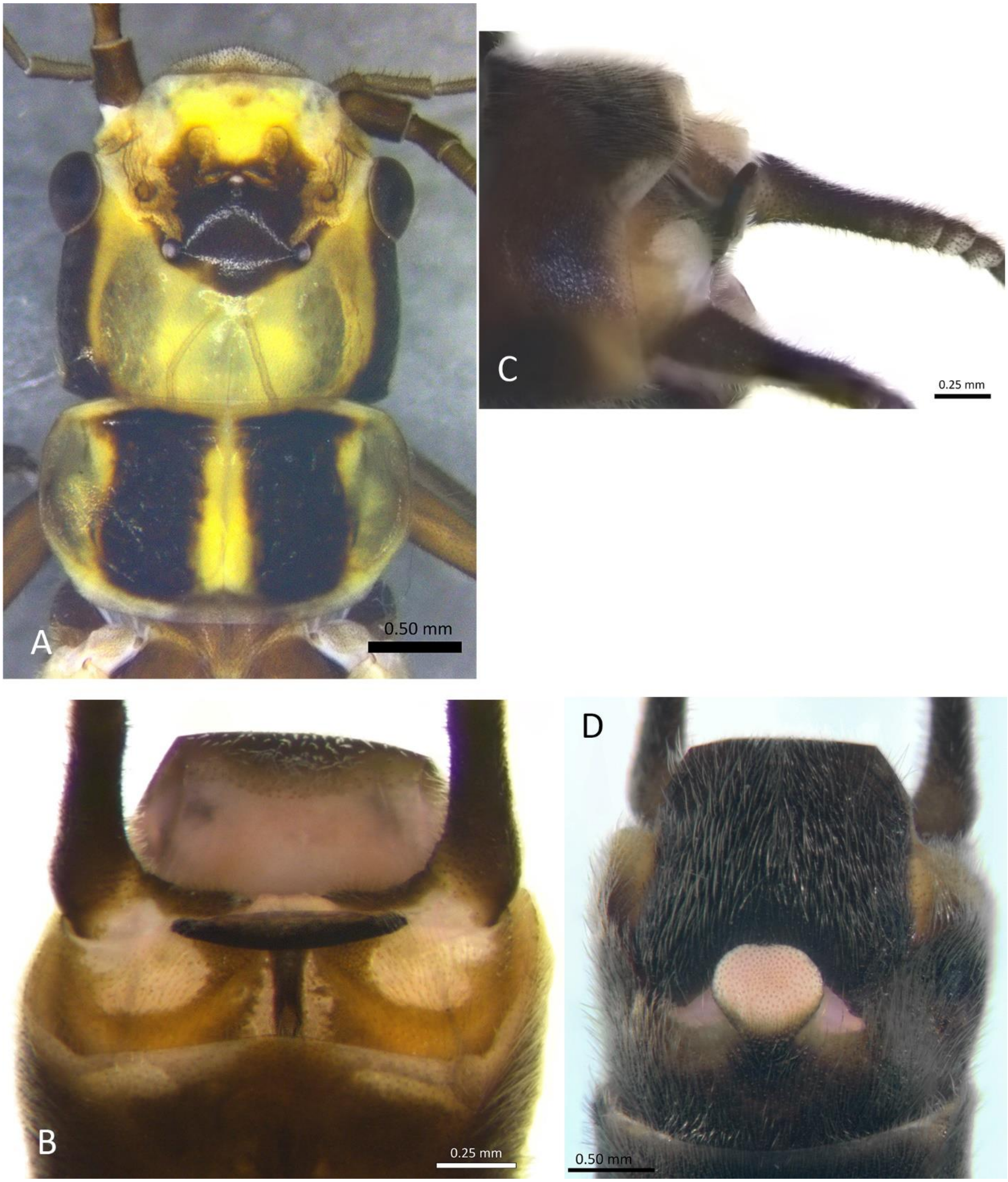

Figure 2.9. *Kathroperla takhoma*, male, Upper Willow Creek, California, USA (INHS Insect Collection 658451). (A) Head and pronotum. (B) Terminalia, dorsal. (C) Terminalia, lateral. (D) Vesicle.

Figure 2.10. *Kathroperla takhoma*, females, head and pronotum. (A) Boise Creek, California, USA (INHS Insect Collection 658554). (B) Red Mountain Creek, California, USA (INHS Insect Collection 658555). (C) Casey Creek, Oregon, USA (INHS Insect Collection 658556).

Figure 2.10 (cont.). *Kathroperla takhoma*, female, Red Mountain Creek, California, USA (INHS Insect Collection 658555). (D) Venter. (E) Terminalia, ventral.

Table 2.2. Summary counts and likelihood scores of the best trees obtained from maximum likelihood analysis using IQ-TREE.

Dataset	Site total	Parsimony-informative sites	Singleton sites	Constant sites	Likelihood score of best tree
Concatenated amino acid	353 872	96 369	69 581	187 922	-3 793 979.5
Concatenated complete nucleotide	1 061 616	446 397	143 575	471 644	-10 460 540.5
Complete Partitioned nucleotide					-10 100 860.6
Concatenated nt12	707 743	151 584	109 411	446 748	-4 089 834.7
Partitioned nt12					-3 828 928.6

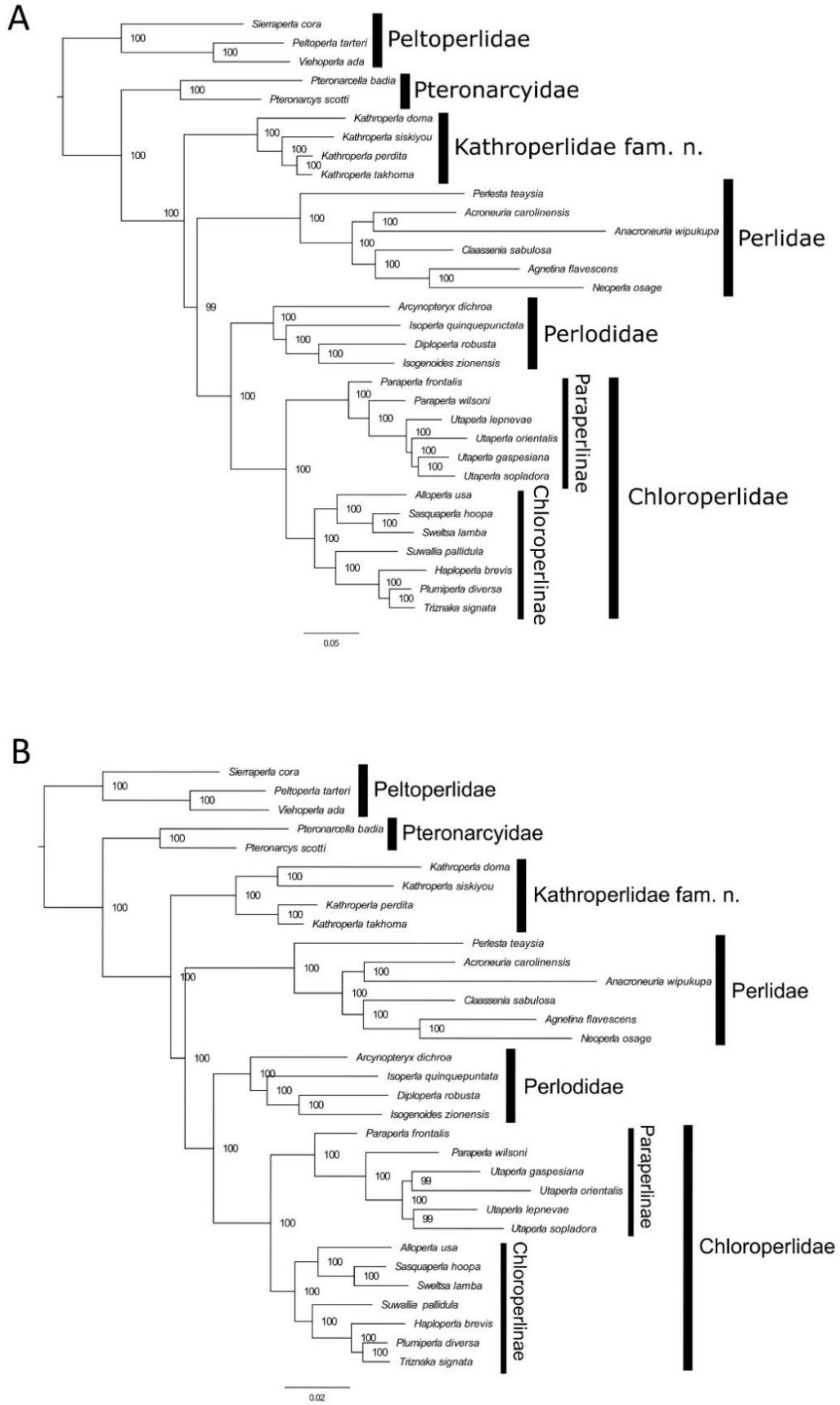

Figure 2.11. Best tree for maximum likelihood analysis of (A) complete concatenated nucleotide dataset and (B) nucleotide dataset with third codon position removed.

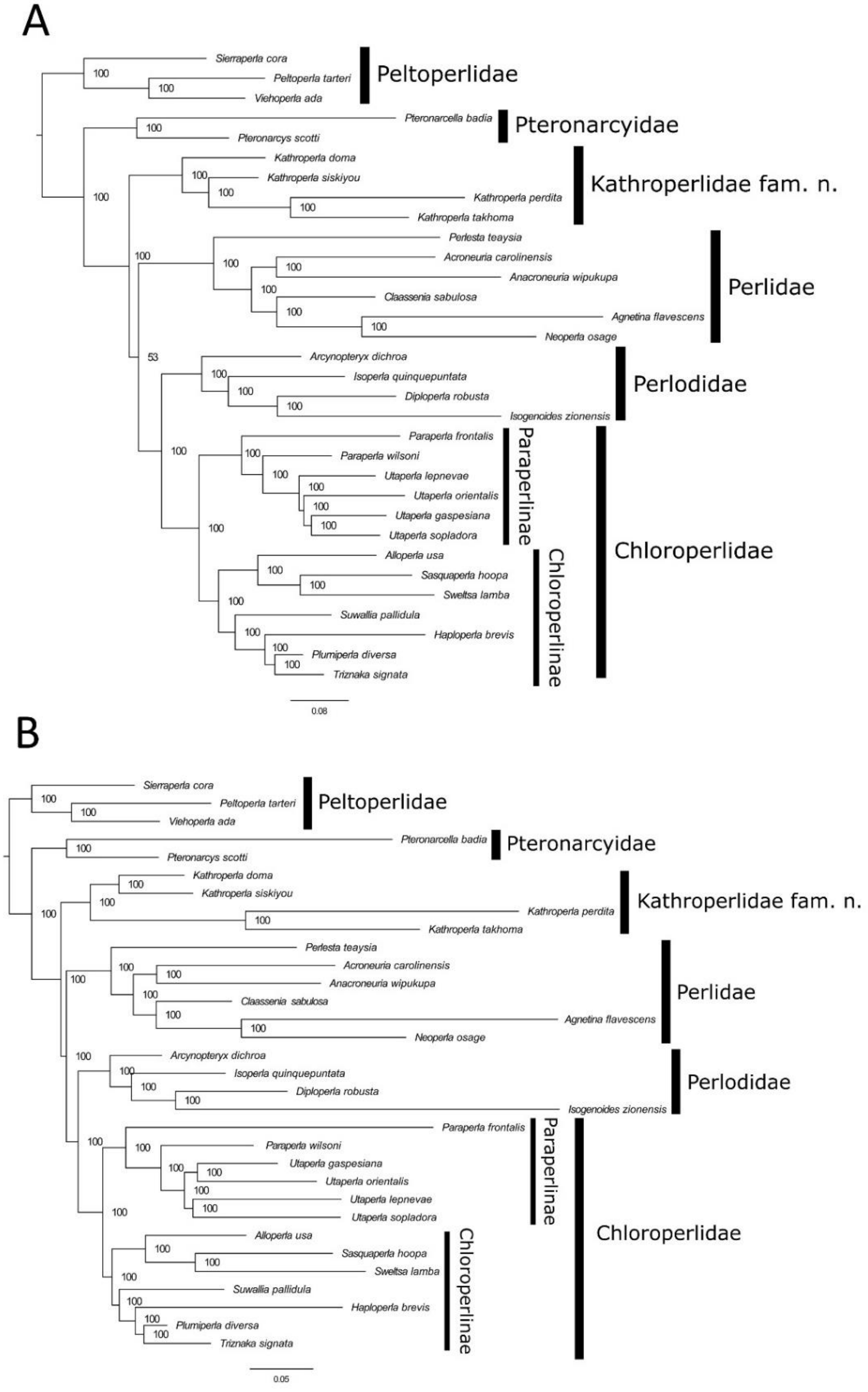

Figure 2.12. Best tree for maximum likelihood analysis of partitioned datasets for (A) complete nucleotide and (B) third codon position removed.

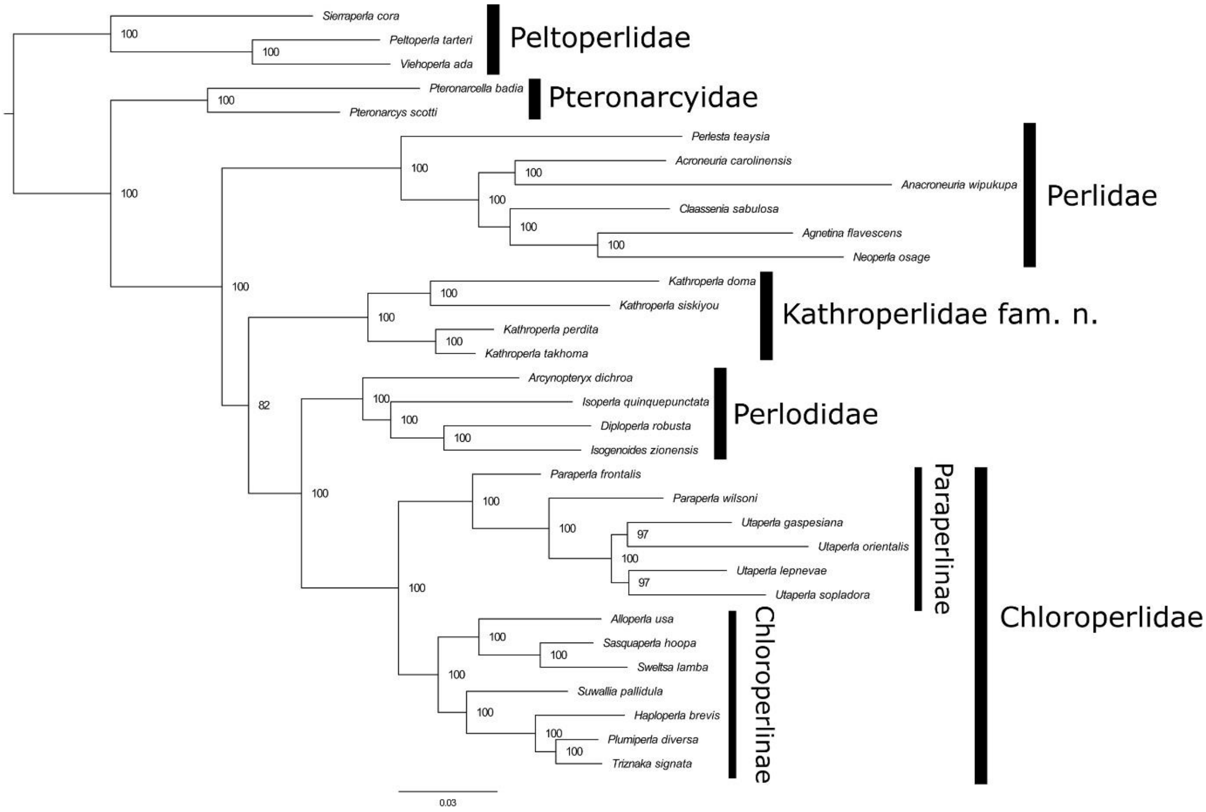

Figure 2.13. Best tree for maximum likelihood analysis of amino acid alignment.

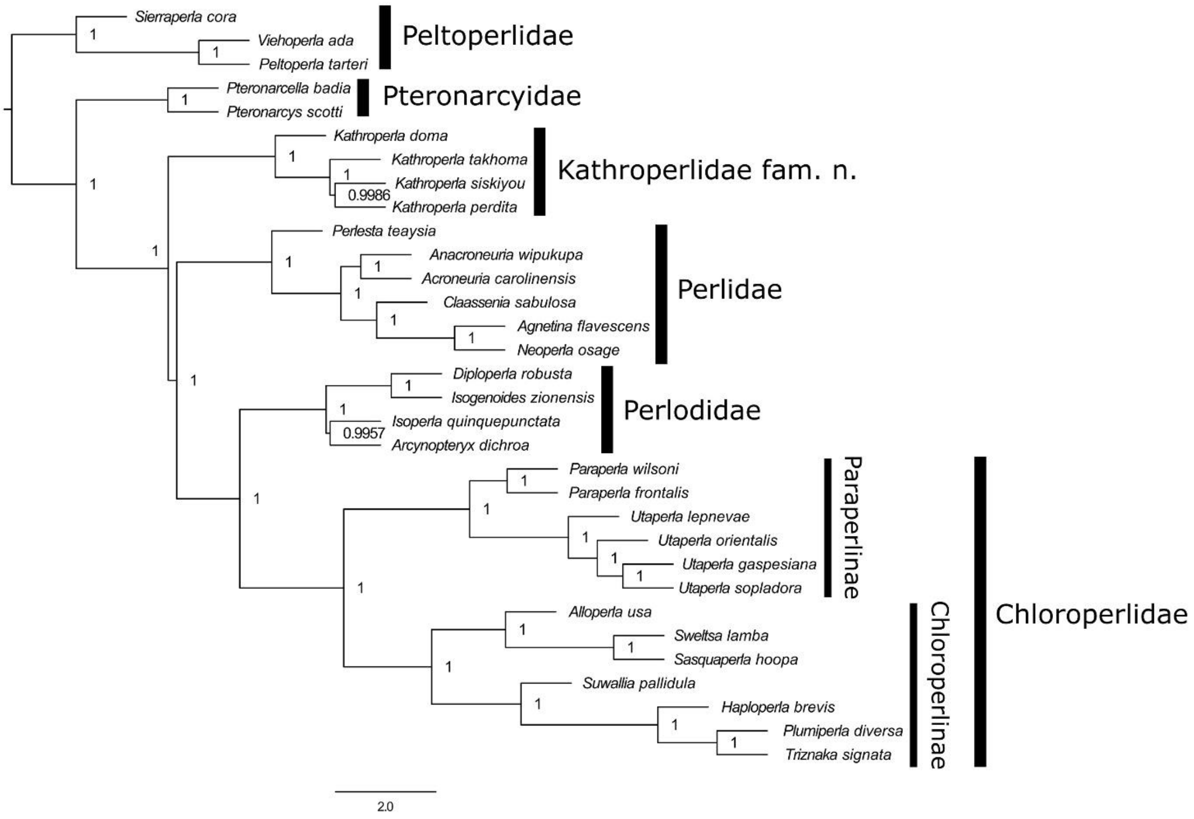

Figure 2.14. Multispecies coalescent tree for 32 Systellognatha taxa using ASTRAL.

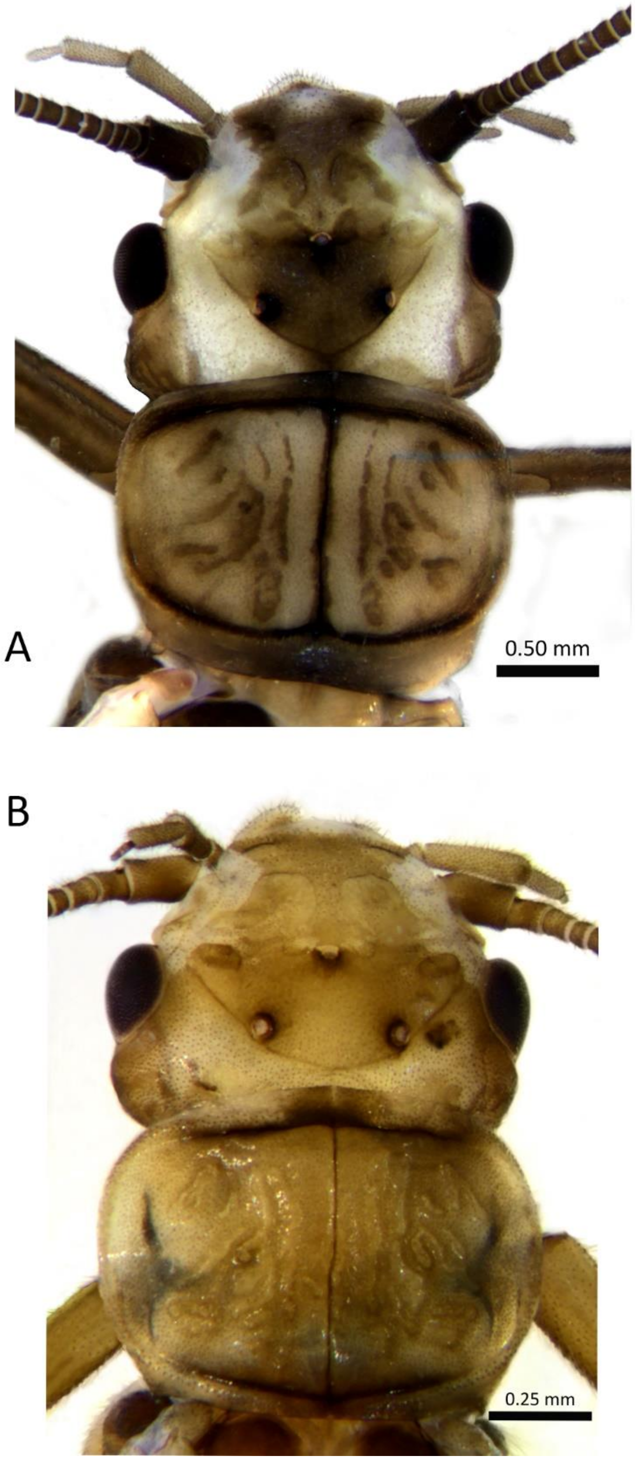

Figure 2.15. *Paraperla*, head and pronotum. (A) *P. frontalis*, female, Sulphur Creek, California, USA (EJSC Pa_fr_02). (B) *P*. wilso*ni*, male, Ross Creek, Montana, USA (CSUC).

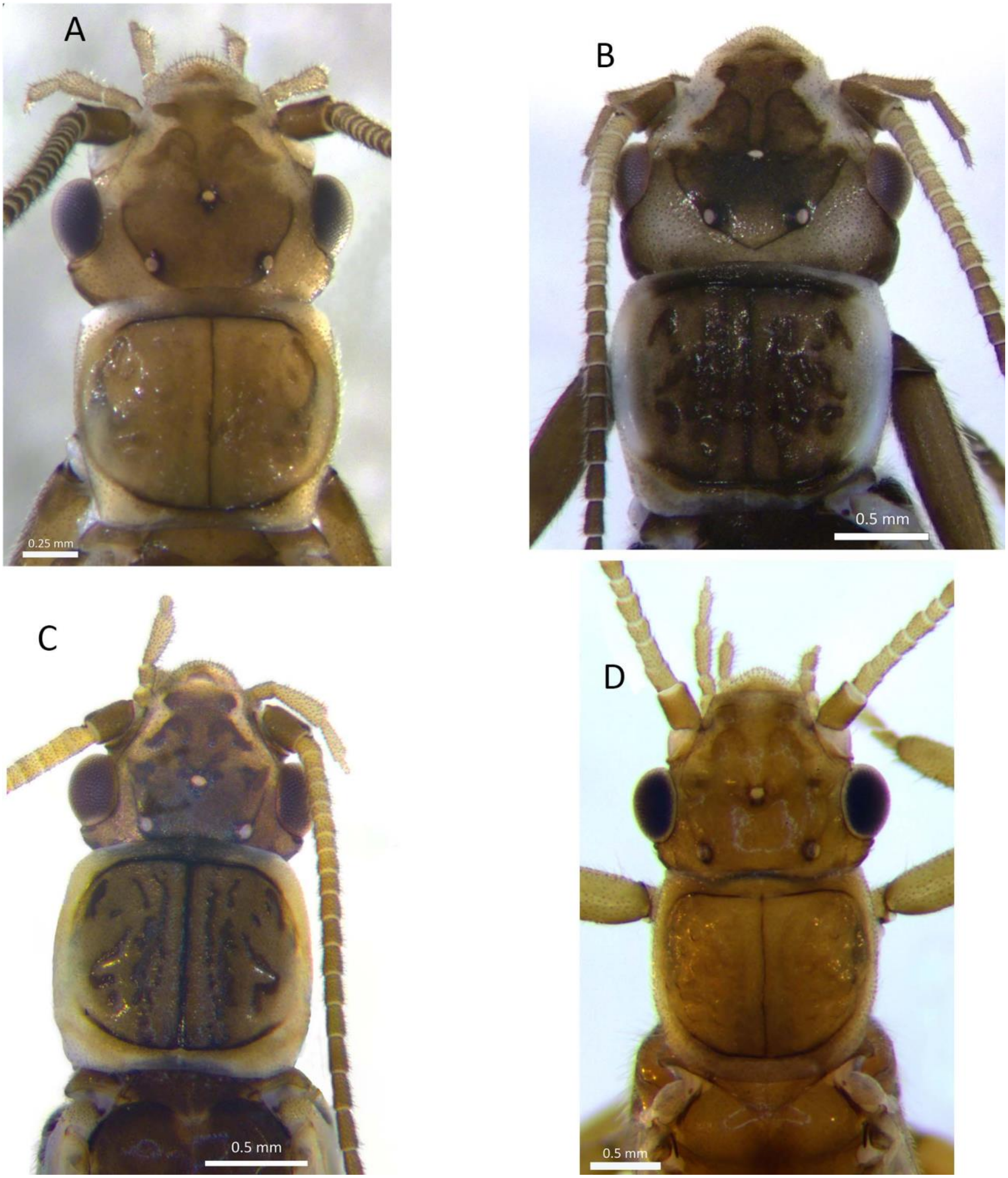

Figure 2.16. *Utaperla*, head and pronotum. (A) *U. gaspesiana*, female, Lucas Creek, New York, USA (INHS Insect Collection 658544). (B) *U. lepnevae*, male, Anyui River, Khabarovsky Region, Russia (INHS Insect Collection 658548). (C) *U. orientalis*, female, Anyui River, Khabarovsky Region, Russia (INHS Insect Collection 658546). (D) *U. sopladora*, male, Bitterroot River, Montana, USA (CSUC).

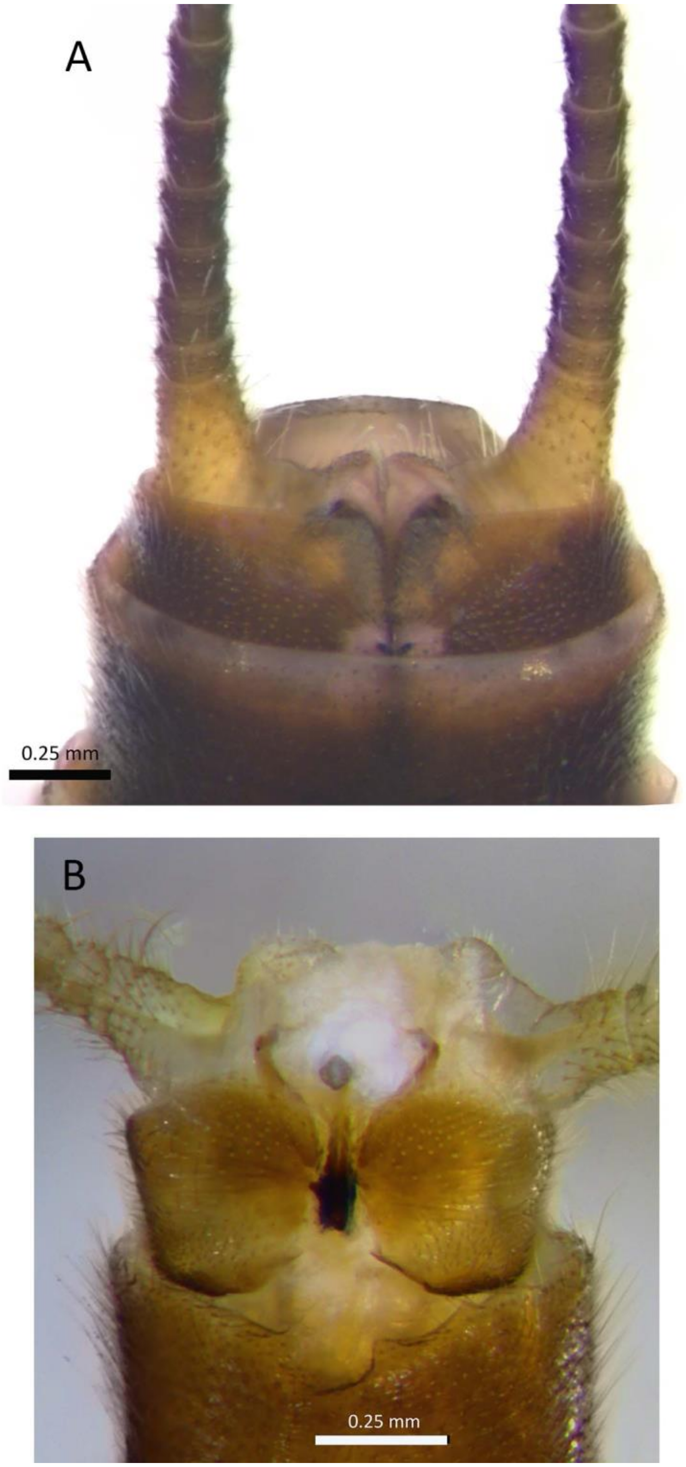

Figure 2.17. *Paraperla*, male terminalia, dorsal. (A) *P. frontalis*, Sulphur Creek, California, USA (EJSC Pa_fr_01). (B) *P. wilsoni*, Ross Creek, Montana, USA (CSUC).

Figure 2.18. *Utaperla gaspesiana*, male, terminalia, East Branch Delaware River, New York, USA (INHS Insect Collection 658543). (A) Dorsal. (B) Caudal. (C) Lateral.

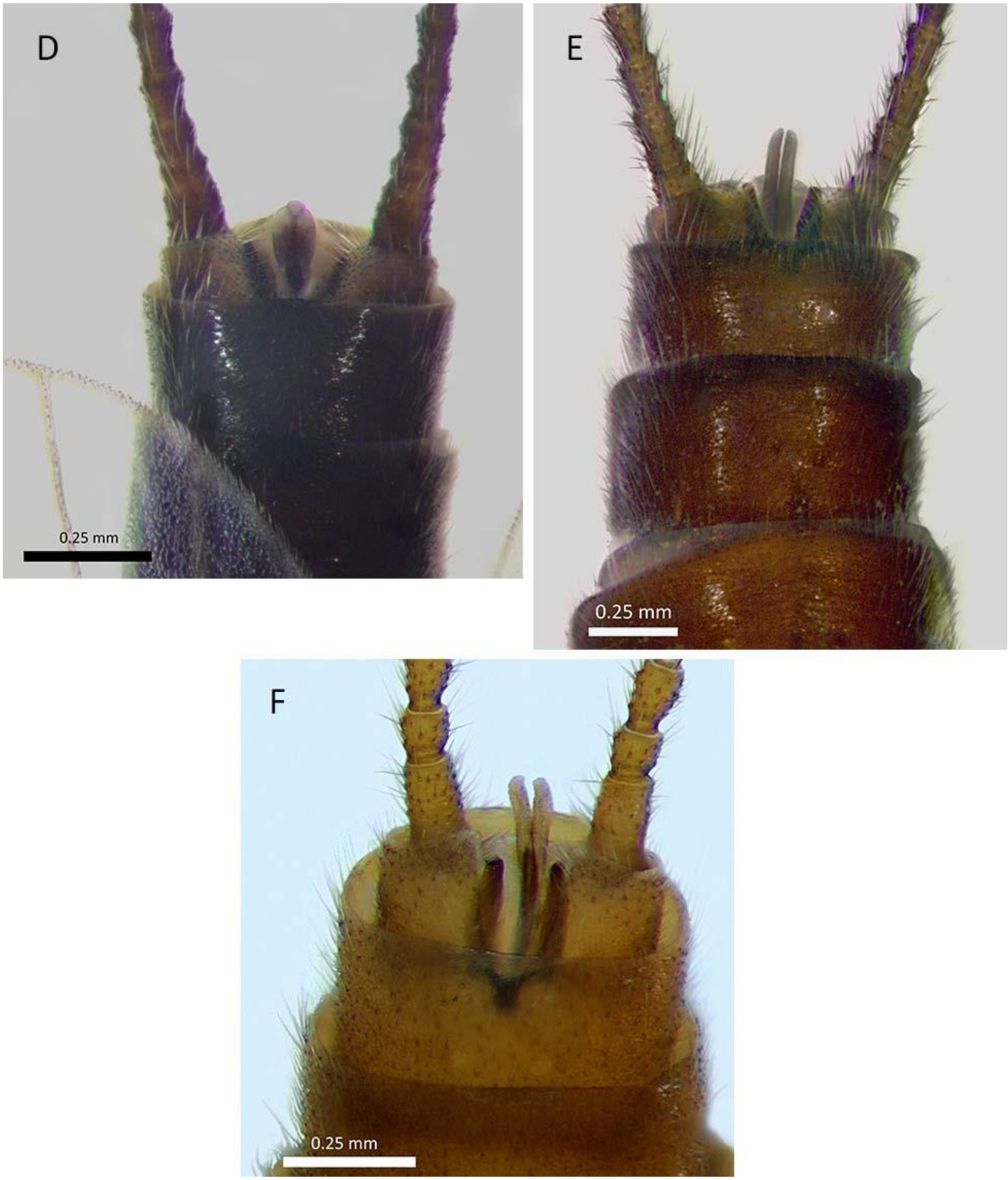

Figure 2.18 (cont.). *Utaperla*, male terminalia, dorsal. (D) *U. lepnevae*, Anyui River, Khabarovsky Region, Russia (INHS Insect Collection 658548). (E) *U. orientalis*, Anyui River, Khabarovsky Region, Russia (INHS Insect Collection 658545). (F) *U. sopladora*, Bitterroot River, Montana, USA (CSUC).

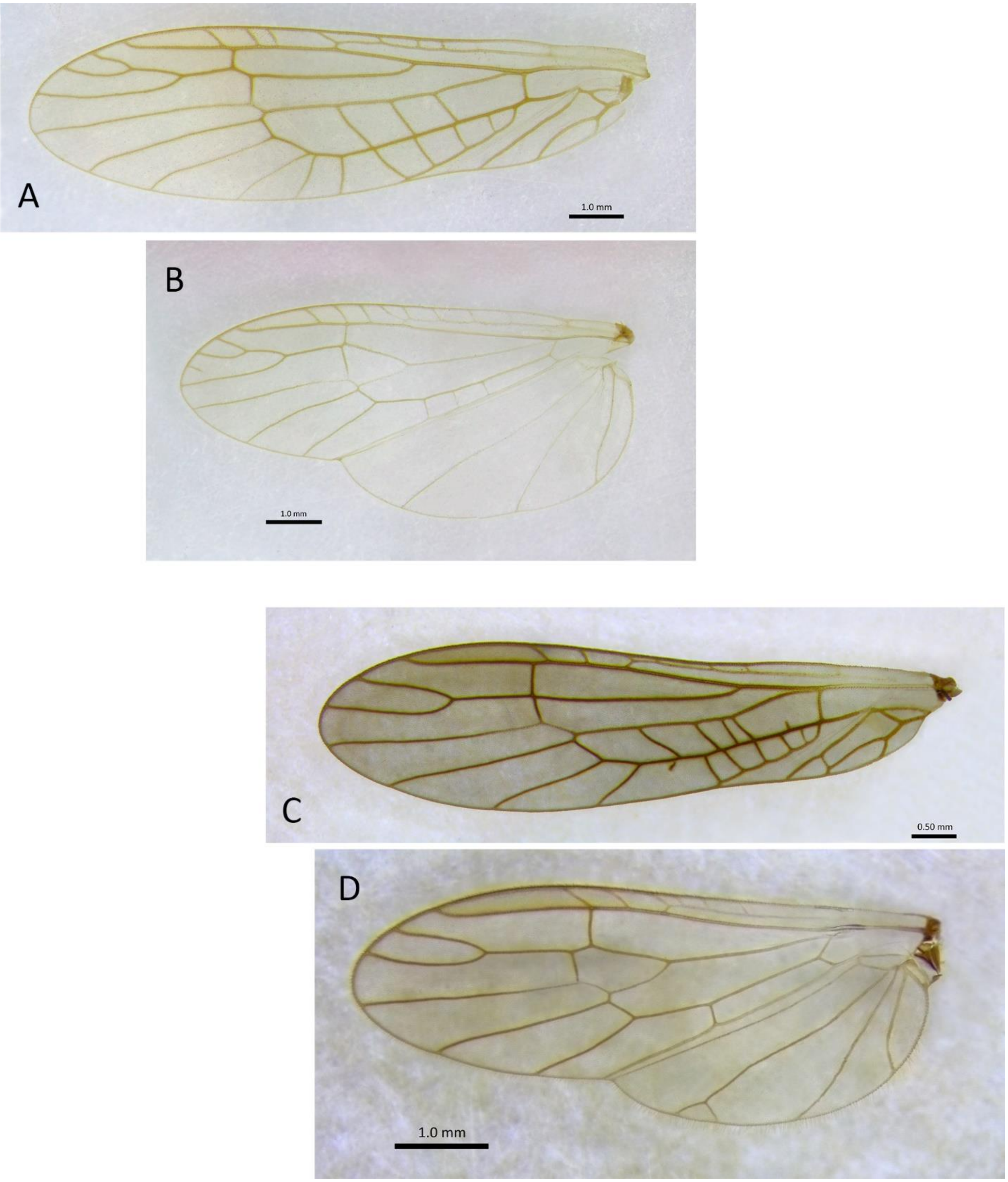

Figure 2.19. *Paraperla* and *Utaperla*, wings. (A) *P. wilsoni*, male, left forewing, East Fork Six Mile Creek, Alaska, USA (INHS Insect Collection 487620). (B) Left hindwing. (C) *U. gaspesiana*, female, left forewing, Delaware River, New York, USA (INHS Insect Collection 658542). (D) Left hindwing.

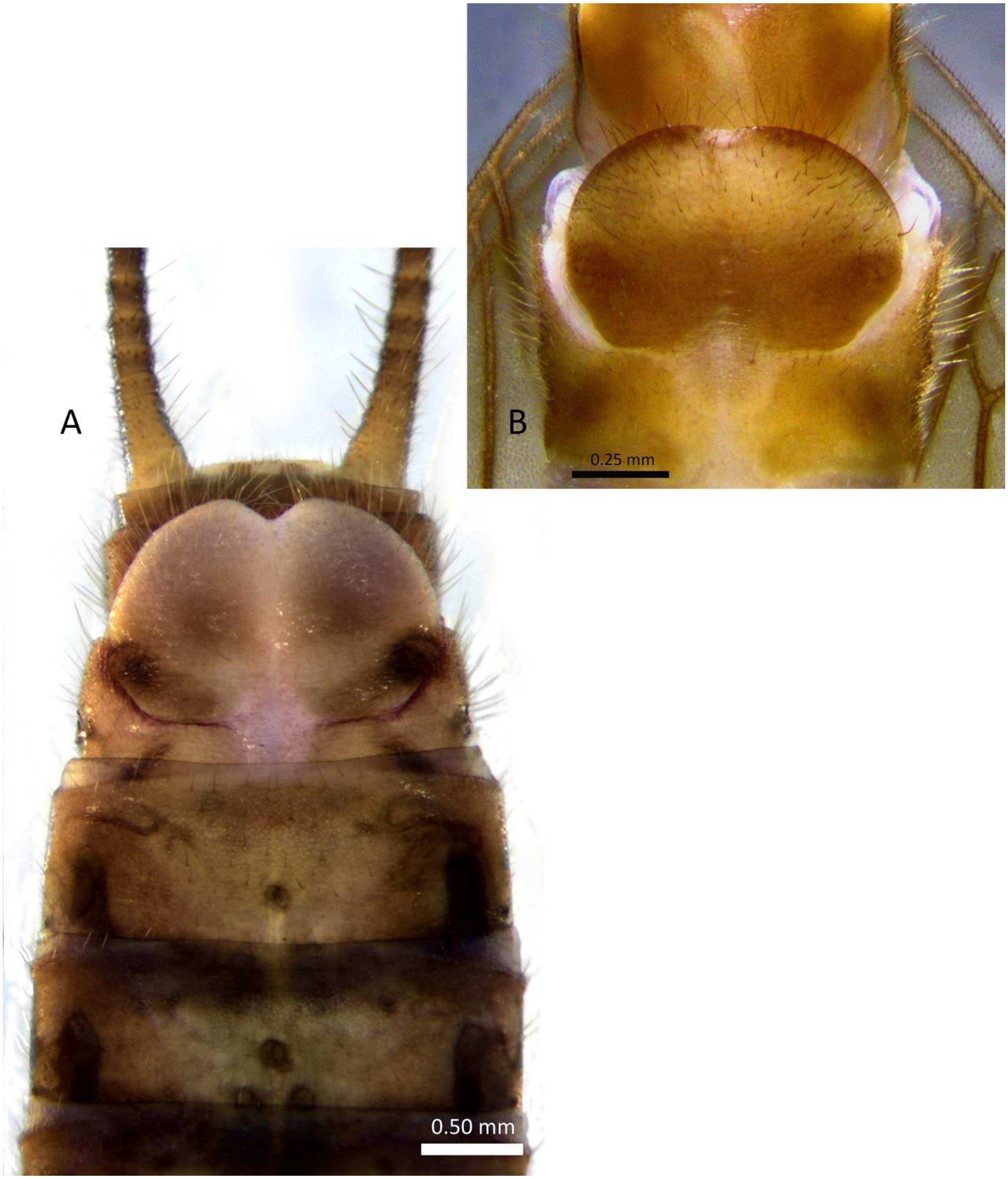

Figure 2.20. *Paraperla*, female terminalia, ventral. (A) *P. frontalis*. (B) *P. wilsoni*.

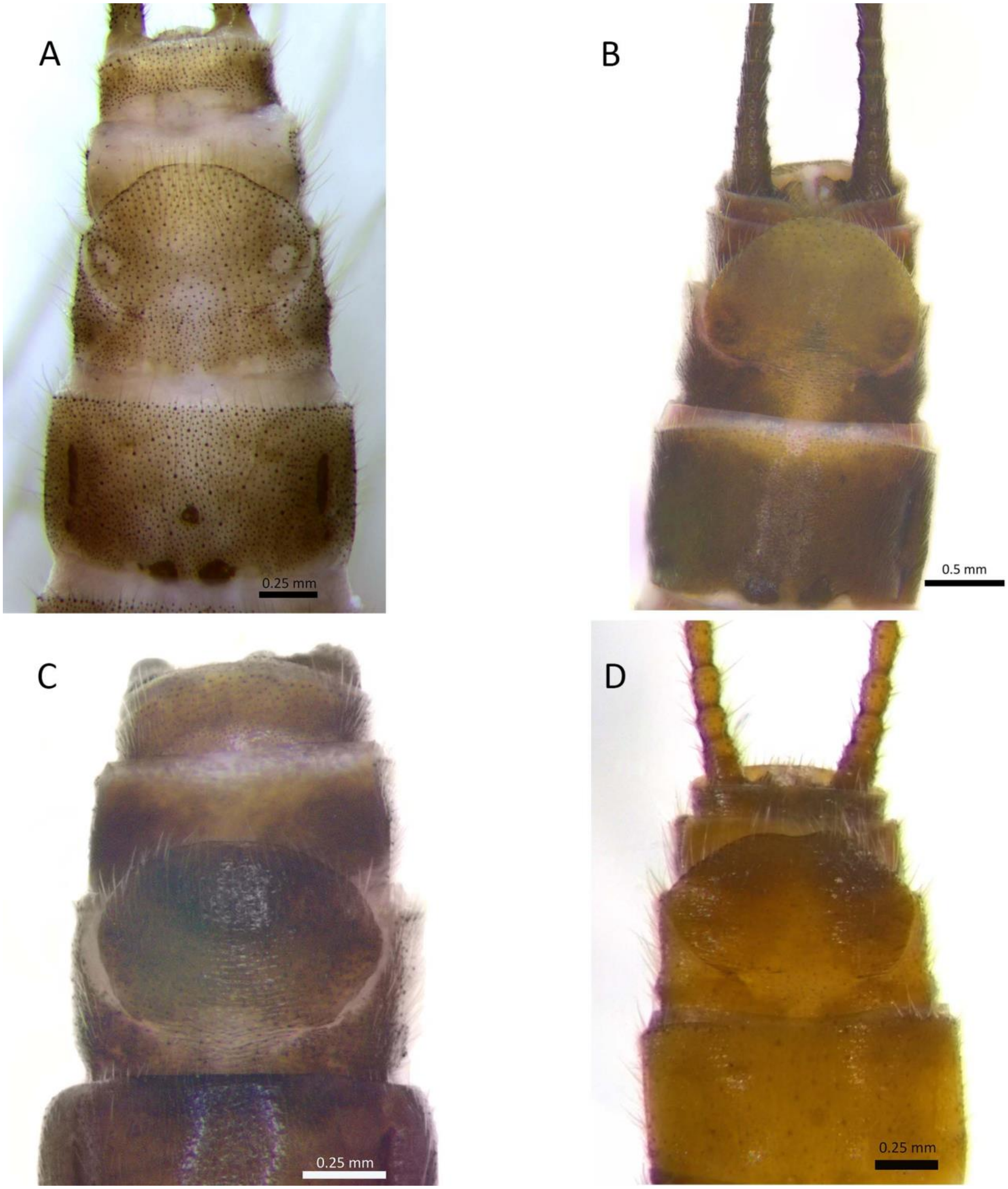

Figure 2.21. *Utaperla*, female terminalia, ventral. (A) *U. gaspesiana*, Lucas Creek, New York, USA (INHS Insect Collection 658544). (B) *U. lepnevae*, Anyui River, Khabarovsky Region, Russia (INHS Insect Collection 658547). (C) *U. orientalis*, Anyui River, Khabarovsky Region, Russia (INHS Insect Collection 658546). (D) *U. sopladora*, St. Regis River, Montana, USA (CSUC).

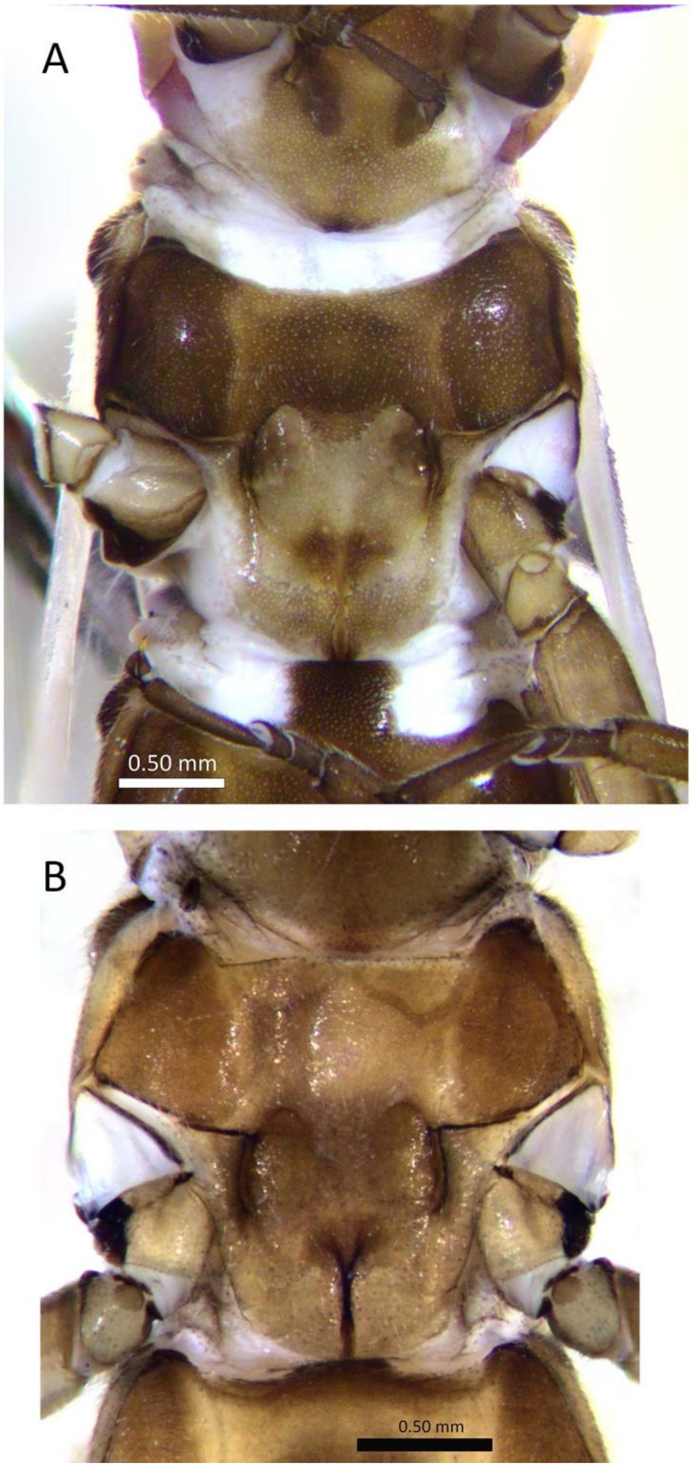

Figure 2.22. *Paraperla*, venter. (A) *P. frontalis*, female, Sulphur Creek, California, USA (EJSC Pa_fr_02) (B) *P. wilsoni*, male, Ross Creek, Montana, USA (CSUC).

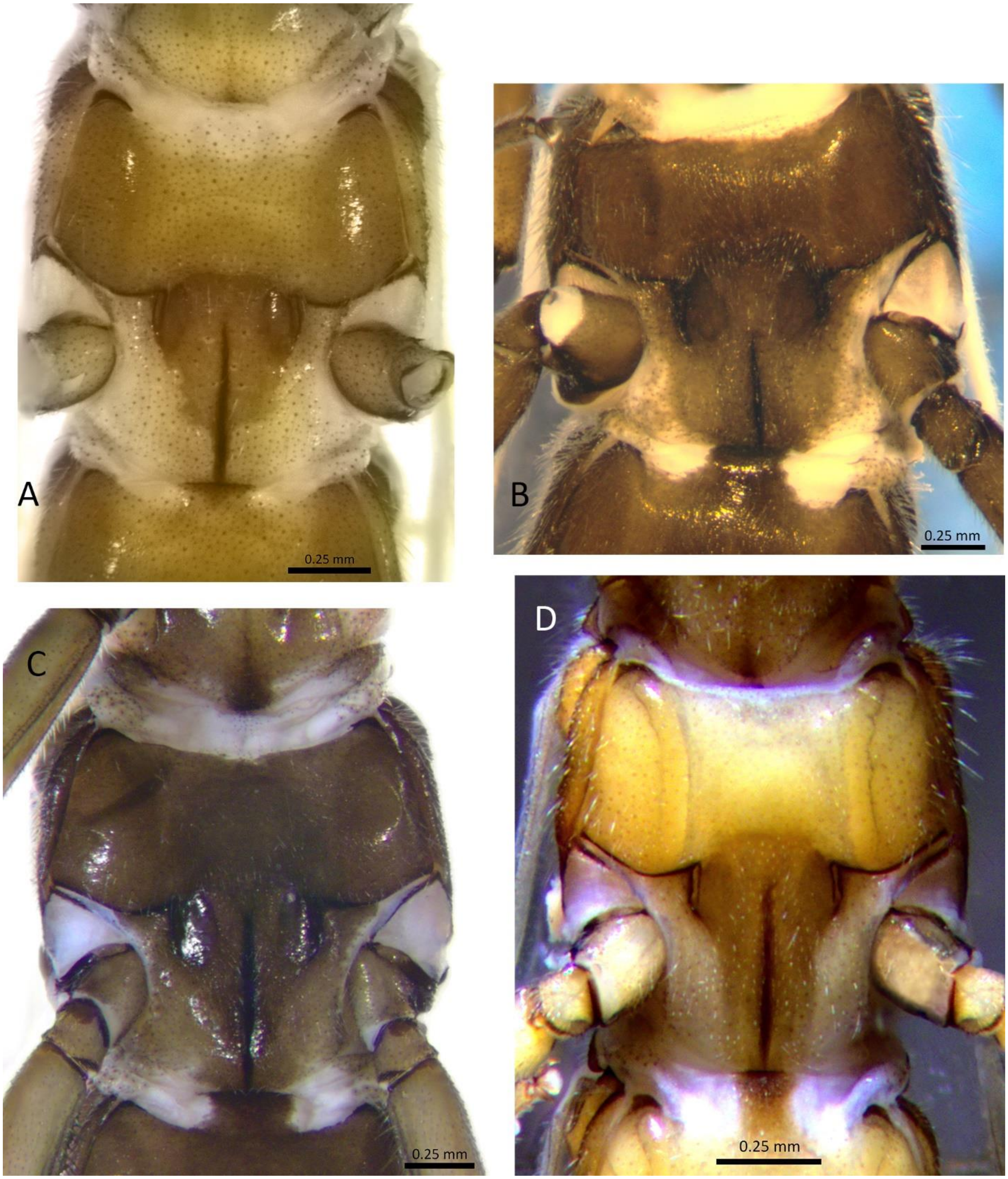

Figure 2.23. *Utaperla*, venter. (A) *U. gaspesiana*, male, East Branch Delaware River, New York, USA (INHS Insect Collection 658549). (B) *U. lepnevae*, male, Anyui River, Khabarovsky Region, Russia (INHS Insect Collection 658548). (C) *U. orientalis*, female, Anyui River, Khabarovsky Region, Russia (INHS Insect Collection 658546). (D) *U. sopladora*, male, Bitterroot River, Montana, USA (CSUC).

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CHAPTER 3

A NEW STONEFLY SPECIES (PLECOPTERA: PERLIDAE) FROM THE INTERIOR HIGHLANDS USA, WITH MORPHOLOGICAL AND MOLECULAR COMPARISON TO OTHER CONGENERIC SPECIES[2]

ABSTRACT

Thirty-one species of Nearctic *Perlesta* Banks, 1906 (Plecoptera: Perlidae) are recognized. A new species is described from western Arkansas and eastern Oklahoma, USA, *Perlesta sublobata* South & DeWalt, sp. nov., from the adult male, adult female, and egg. *Perlesta sublobata* males are differentiated from other congeners by a combination of a prominent ventral caecum and a distinct dorsal extension of the lateral sclerites of the aedeagus. A preliminary molecular phylogenetic hypothesis is proposed for *Perlesta* based on 17 congeners and three outgroup taxa using partial mitochondrial cytochrome c oxidase subunit I sequence data. Illustrations, stereomicroscope images, and scanning electron micrographs support the description and comparison to other *Perlesta*.

INTRODUCTION

Needham and Claassen (1925) described *Perlesta* Banks, 1906 as a Nearctic genus of small, brown, triocellate stoneflies with yellow costal wing margins, long cerci, and highly variable coloration of the head and wing membrane. For over a century, the name of the type species of the genus, *Perlesta placida* (Hagen, 1861), has been used for innumerable specimens that once critically reviewed, were revealed to encompass many cryptic species (Stark 1989, DeWalt et al. 2001). Stark (1989) revised the genus, removing several species from synonymy, describing

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[2] This chapter appeared in its entirety in: South, E.J., DeWalt, R.E., Davis, M.A., & Thomas, M.J. (2019) A new stonefly species (Plecoptera: Perlidae) from the Interior Highlands USA, with morphological and molecular comparison to other congeneric species. *Zookeys*, **858**, 45–70.

seven new species, recognizing a total of 12 species, and providing the first useful key to the *P. placida* complex. Stark's revision prompted additional work, with eight new species described over the next 14 years (Poulton & Stewart 1991, Kirchner & Kondratieff 1997, Stark & Rhodes 1997, DeWalt et al. 1998, Kondratieff & Baumann 1999, Kondratieff & Kirchner 2002, 2003). Subsequently, a revised taxonomic key was necessary, in which was included 21 Nearctic species (Stark 2004). The genus has since expanded to 31 Nearctic species through the works of Kondratieff et al. (2006, 2008, 2011), Kondratieff & Myers (2011), and Grubbs & DeWalt (2011, 2012, 2018).

Two species are recognized from China (Murányi & Li 2016) and undetermined nymphs have been reported from Costa Rica (Gutiérrez-Fonseca & Springer 2011). Described species could easily surpass 40, given the amount of presumed new, undescribed *Perlesta* material currently present in North American collections (Grubbs & DeWalt 2018).

At least eight species of *Perlesta* co-occur in the United States Interior Highlands, a mountainous region defined by the United States Geological Survey as encompassing southern Missouri, western Arkansas, eastern Oklahoma, and extreme southeastern Kansas (Omernik 1987). This area of the central United States was extensively examined for stoneflies by Poulton & Stewart (1991). Surprisingly, a remarkably distinct and undescribed *Perlesta* species from the Interior Highlands was revealed through recent examination of undetermined Arkansas material donated to the Illinois Natural History Survey (INHS) Insect Collection by the late Kenneth W. Stewart (DeWalt et al. 2018) and from eastern Oklahoma material borrowed from the K. C. Emerson Entomological Museum, Oklahoma State University (OKSU) at Stillwater. Using freshly collected and properly prepared specimens, we describe this new species, *Perlesta sub-lobata* sp. nov., and compare it to similar regional congeners. Moreover, we provide the first comparative molecular study of the genus by exploring partial mitochondrial cytochrome c oxidase subunit I (COI) DNA sequence data to examine monophyly of the new species, delimit congeners, and construct a preliminary phylogeny.

The holotype male and all paratypes are deposited in the Illinois Natural History Survey (INHS) Insect Collection. Other material is deposited in the INHS Insect Collection with the exception of nine vials borrowed and returned to the OKSU Insect Collection.

METHODS

Collection and morphological analyses

Terminology of all stages follows Stark (1989). Fresh specimens of the new species (108 males and 40 females) were collected from six Arkansas stream systems, 13–19 June 2016 (Fig. 3.1). Methods included sweep netting during the day and ultraviolet light trapping at night. Live male specimens were anesthetized in a dry ice CO_2 chamber and subsequently squeezed with forceps to evert the aedeagus, the source of the most informative morphological characteristics distinguishing species. All specimens were preserved in 95% EtOH. Select individuals of the fresh material and several related species were stack photographed and processed with a Zeiss AxioCam HRc Rev. 3 digital camera and Helicon Focus 6 software in the Sam W. Heads laboratory, INHS. The aedeagus and paraproct were sketched from the stereomicroscope images using Adobe Illustrator CC 2018. Scanning electron micrographs (SEM) of eggs and female terminalia were prepared at the Beckman Institute Microscopy Suite, University of Illinois by critical point drying, placing on an aluminum carbon disk, sputter coating with gold-palladium alloy, and imaged with a Thermo-Fisher FEI Quanta FEG 450 ESEM.

Molecular studies

Genomic DNA from 20 *P. sublobata* specimens, 17 congeners, and three outgroup taxa was extracted using the Qiagen DNeasy Kit, amplified for a fragment of the mitochondrial gene encoding for the COI subunit via polymerase chain reaction using either primers LCO1490 and HCO2198 (Folmer et al. 1994) or jgLCO1490 and jgHCO2198 (Geller et al. 2013), and sequenced with Sanger technology at the University of Illinois W. M. Keck Core Sequencing Facility. Thermocycling conditions consisted of one 94 °C for 5 min denaturation cycle, 40 cycles at 94 °C for 45 s, 50 or 53 °C for 1 min, 72 °C for 1.5 min, and one 72 °C for 5 min extension cycle. Amplification success was verified with gel electrophoresis. Forward and reverse sequences were aligned to create contigs, and all 63 aligned contigs were truncated to a uniform length of 606 nucleotides, visually edited with Sequencher 5.4, aligned in MUSCLE 3.8, and the sequences and supporting data deposited in GenBank (Table 3.1). Sequences were tested to determine the model of evolution in jModelTest2 (Darriba et al. 2012), and a gamma distribution with a proportion of invariable sites was used to model rate variation across sites (invgamma).

Akaike Information Criterion (AIC) results indicated that the General Time Reversible nucleotide substitution model (GTR+I+G) was best for the Maximum Likelihood and Bayesian analyses. These models were applied in subsequent phylogenetic tree generation analyses.

We generated a maximum likelihood tree using MEGA 7.0 (Kumar et al. 2016) and calculated pairwise genetic distances for both sequences generated for this study, as well as additional sequences accessioned from GenBank, using the Kimura 2-parameter model (K2P) (Kimura 1980), the de facto standard for measuring mitochondrial pairwise distances (Collins et al. 2012). A Bayesian analysis was performed for all haplotypes using MrBayes 3.2.6 (Huelsenbeck & Ronquist 2001) with a burn-in length of 500,000, subsampling frequency of 500, and a chain length of 5,100,000.

RESULTS

Perlesta sublobata **South & DeWalt, sp. nov.**

http://zoobank.org/1FB6141B-3C6E-4B64-983C-406649DE6830
http://lsid.speciesfile.org/urn:lsid:Plecoptera.speciesfile.org:TaxonName:505368
Figs. 3.1–3.6

Diagnosis

Males are distinguished by a combination of a prominent ventral caecum with a broad ventral setal patch and a distinct dorsal extension of the lateral sclerites of the aedeagus. Females possess a subgenital plate with a deep V-shaped notch and truncate lobes. Eggs have a smooth chorion and a well-developed, distally flanged collar.

Male. Habitus moderately dark (Fig. 3.2A). ***Wings*****:** Membrane brown with dark brown venation and pale intercostal margin (Figs. 3.2B, 3.2C). ***Forewing*****:** Length 8–9 mm (mean = 8.3 ± 0.3 SD, n = 95); membrane with two lightly pigmented longitudinal bands: one posterior to the posterior cubital vein and a second anterior to the median vein (Fig. 3.2B). ***Head*****:** Pale with dark brown quadrangular patch covering interocellar region; brown subtriangular patches anterolateral and anteromedial to median ocellus (Figs. 3.2D, 3.2E); diffuse brown pigmentation posterior to ecdysial suture (Fig. 3.2D); ecdysial suture extends slightly to moderately beyond ocelli as a distinct dark line; antenna darkly pigmented on ca. distal 2/3 of flagellum and dorsomedian region of scape; proximal antennal segments pale with tan dorsal pigmentation. ***Thorax*****:** Pronotum brown with vermiculated rugosities and faint, pale median stripe (Figs. 3.2D, 3.2E); mesothoracic and metathoracic nota brown; mesothoracic and metathoracic basisterna pale; femur and tibia pale, brown dorsally. ***Abdomen*****:** Sterna pale; terga pale medially and light brown laterally, or uniformly brown. ***Terminalia*****:** Tergum 10 with dark subquadrate pigment patch (Fig. 3.3A) and 10–20 small, sensilla basiconica (visible at 80× magnification); cercus long (holotype = 15 segments), pale proximally and dark brown distally; paraprocts broad basally and narrowed distally in caudal aspect (Fig. 3.3B); anteapical paraproct spine and carina directed anteromedially—best visible in oblique lateral view of unextruded individuals (Figs. 3.3C, 3.3D). ***Aedeagus*****:** Dorsal caecum moderately produced, ca. as long as wide and broad apically (Figs. 3.4A, 3.3B); dorsal patch broad over sac, moderately expanded proximally, constricted subapically, and broadly expanded over caecum; prominent lateral sclerites merge dorsally to form a distinct V-shaped pattern extending more than 1/2 tube length (Figs. 3.4C, 3.4D); prominent ventral caecum, narrowed apically, with a broad patch of fine seta-like spines covering venter and apex, length ca. 2/5 sac width; sac with fine seta-like spines covering venter (Figs. 3.4E, 3.4F).

Female. Female habitus similar to male, but of larger size and wings of lighter pigmentation (Fig. 3.5A). Pronotum tan with brown vermiculated rugosities and pale median stripe (Fig. 3.5B). Wings with subhyaline membrane, tan venation, and pale intercostal margin (Figs. 3.5C, 3.5D). Forewing length 9–11 mm (mean = 9.8 ± 0.57 SD, n = 40); often with two unpigmented longitudinal bands: one posterior to the posterior cubital vein and a second anterior to the median vein (Fig. 3.5C). Subgenital plate lobes truncate medially and truncate to slightly rounded laterally, slightly to moderately pigmented, covered with long bristle-like hairs, and separated by a deep V-shaped notch (Figs. 3.5E, 3.5F).

Egg. Length ca. 360 μm, width ca. 280 μm. Chorion smooth with fine pitting (Fig. 3.6A). Collar well developed, ribbed, and flanged distally (Fig. 3.6B). Micropylar orifices distinct near anterior pole (opposite collar) (Fig. 3.6C).

Molecular analyses

Perlesta sublobata formed a monophyletic group with strong support (ML bootstrap support = 97%, Bayesian posterior probability = 92%). The nearest neighbor species to *P. sublobata* was

P. decipiens (Walsh, 1862) at 1.8% sequence divergence. Maximum intraspecific COI genetic distances were less than minimum interspecific distances within all tested *Perlesta* (Table 3.2). All intraspecific distances were less than the arbitrary threshold of 3.5%, suggesting that the new species was monophyletic without other cryptic species present within the new taxon (Hebert et al. 2003, Zhou et al. 2010). All haplotypes (total = 47) were confined to their respective genera and presumptive species in the ML and Bayesian analyses (Figs. 3.7, 3.8, respectively). The three tested species within the *P. frisoni* group, consisting of five Nearctic species that lack an aedeagal dorsal caecum, formed a monophyletic grouping. Four of the five "dark" species studied in Grubbs & DeWalt (2018) also formed a monophyletic grouping. The placement of *P. adena* Stark, 1989 outside this group may be spurious, indicating additional genes or populations are needed for further refinement. The relatively distant placement of *P. golconda* DeWalt & Stark, 1998 from *P. sublobata* is congruent with the species' distinctly different morphologies, apart from the male genitalic similarities.

DISCUSSION

Remarks

The shape and armature of the aedeagus are the most distinct morphological features of *P. sublobata.* Stark (1989) illustrated a lateral view of an undetermined species from Arkansas (*P. sublobata*), demonstrating spinule patterns and shape of the aedeagal telescoping sections: envelope, tube, and sac. He noted that lateral sclerites of the tube joined dorsally. This dorsal extension of the lateral sclerites was not illustrated or specified in the literature for any other *Perlesta*. Furthermore, a ventral caecum is present in *P. sublobata* and only one other described congener, *P. golconda.* However, the ventral caecum of *P. golconda* is less prominent and without a distinct ventral patch of fine seta-like spines. Additionally, the dorsal caecum of *P. sublobata* is moderately developed, compared to the poorly developed dorsal caecum of *P. golconda* (Fig. 3.9).

The known distribution of *P. golconda*, originally limited to Illinois (DeWalt & Stark 1998), has expanded to include Iowa, Indiana, Michigan, and Nebraska (DeWalt et al. 2019), as well as Missouri (Stark 2004) and Louisiana (INHS Insect Collection 564765). Arkansas is bordered by Missouri to the north and Louisiana to the south. A sympatric distribution with *P. sublobata* is expected due to this geographic adjacency and overlap of the Interior Highlands' habitat. Consequently, re-examination of some museum specimens may be required. The male and female habitus easily distinguish *P. golconda* from *P. sublobata*. The ocelli of *P. golconda* are usually connected by a moderately dark V-shaped pattern on a pale background (Figs. 3.10A, 3.10B), whereas *P. sublobata* has a dark subquadrate interocellar region. The pronotum of *P. golconda* is primarily pale with light tan rugosities on the lateral margins, whereas *P. sublobata* has a dark pronotum with a pale narrow median stripe. Additionally, *P. golconda* females are distinguished by a very short egg collar (Grubbs and DeWalt 2008, their Fig. 17) and rounded subgenital plate lobes (Figs. 3.11A, 3.11B).

The female habitus of *P. sublobata* resembles two Interior Highlands congeners, *P. decipiens* and *P. ephelida* Grubbs & DeWalt, 2012. However, *P. sublobata* differs from *P. decipiens* and *P. ephelida* by subgenital plate morphology. *Perlesta decipiens* has a deep U-shaped notch bordered by truncate lobes, typically with darker pigmentation on the posterior margins (Fig. 3.12). *Perlesta ephelida* has a shallow V-shaped notch enclosed by truncate lobes, usually pale to lightly pigmented with posteromedially upturned margins (Fig. 3.13; Grubbs & DeWalt 2012, their Fig. 7). These characters are contrasted to the deep V-shaped notch and moderately pigmented, truncate lobes of *P. sublobata*. Furthermore, *P. sublobata* has a shorter forewing length than *P. decipiens* (*P. sublobata* = 9–11 mm; *P. decipiens* = 12–13 mm, Stark 2004). Egg chorion and collar are similar to *P. decipiens* (Stark 2004, his Figs. 7.397–7.399) and *P. ephelida* (Grubbs & DeWalt 2012, their Figs. 14–21).

Habitat

With the exception of one locality (OK, Washington Co., Caney River), all collection sites for *P. sublobata* are within or closely adjacent to the Interior Highlands, a region containing four contiguous U. S. Environmental Protection Agency (EPA) Level III Ecoregions: Ozark Highlands, Boston Mountains, Arkansas Valley, and Ouachita Mountains. Collection sites for *P. sublobata* within the Interior Highlands are partially canopied, hardwood forested, wadeable, low gradient streams (ca. 15–20 m wide) with substrata composed mostly of sand, gravel, and cobble. The type locality is a low gradient run (ca. 25 m wide) of the Little Missouri River (Fig. 3.14), located 45 km downstream of Lake Greeson and 65 km upstream from its confluence with the Ouachita River in the extreme north EPA Level III Ecoregion 35 (South Central Plains). The substrate is primarily gravel and sand, with some large woody debris. Other stonefly species collected with the new species at the type locality included *Acroneuria frisoni* Stark & Brown, 1991, *Acroneuria* nr. *ozarkensis* Poulton & Stewart, 1991, *Agnetina flavescens* (Walsh, 1862), *Neoperla falayah* Stark & Lentz, 1988, *N. robisoni* Poulton & Stewart, 1986, *P. decipiens*, and *Perlinella ephyre* (Newman, 1839).

Etymology

The specific epithet is derived from *sub*, Latin for under, and *lobata*, the feminine adjectival form of *lobus*, Latin for a rounded projection or protuberance (Brown 1956). The name references the ventral caecum of the aedeagus, a character shared by only one other described congener, *P. golconda*, though it is most prominent in *P. sublobata*.

Material examined of *Perlesta sublobata*:

Holotype: ♂, in 95% ethanol, **USA: Arkansas:** Pike Co., Little Missouri River, 10.0 km SSE Delight at AR-19, 33.95608, -93.44362, 15.vi.2016, E. J. South (INHS Insect Collection 793224).

Paratypes. USA: Arkansas: Clark Co., Caddo River, 6.7 km NNW Arkadelphia at Super 8 Motel at US-67, 34.17985, -93.07021, 14.vi.2016, E. J. South, 6♂ (INHS Insect Collection 793213–793218); Franklin Co., Mulberry River, 2.3 km SSW Cass at AR-23, 35.66984, -93.82962, 13.vi.2016, E. J. South, 3♂ (INHS Insect Collection 793209– 793211); Madison Co., War Eagle Creek, 5.8 km NE Huntsville at AR-412, 36.12076, -93.69354, 13.vi.2016, E. J. South, 3♂ (INHS Insect Collection 793206–793208), same but 16.vi.2016, E. J. South, ♂ (INHS Insect Collection 793228), same but 17.vi.2016, E. J. South, 4♂ (INHS Insect Collection 793229–792232); Pike Co., Antoine River, Antoine at AR-26, 34.03899, -93.41803, 14.vi.2016, E. J. South, ♂ (INHS Insect Collection 793212). Same data as holotype, E. J. South 10♂ (INHS Insect Collection 793219–793227), same but 18.vi.2016, E. J. South, 71♂, 20♀ (INHS Insect Collection 793261–793343); Sevier Co., Cossatot River, 5.1 km W Lockesburg at AR-24, 33.97145, -94.22274, 18.vi.2016, E. J. South, 9♂, 19♀ (INHS Insect Collection 793233–793260).

Other material examined:

USA: Arkansas: Franklin Co., Mulberry River, Hwy 23 at Turner's Bend, 35.66984, -93.82962, 5.vii.1986, B. C. Poulton, 4♂ (INHS Insect Collection 795241); Howard Co., Cossatot River, 12.9 km W Umpire at Hwy 4, 34.29584, -94.17787, 26.vi.1981, H. W. Robison, 10♂ (INHS Insect Collection 794630), same but Saline River, 8 km S Umpire at Hwy 4, 34.21096, -94.05099, 9.vii.1982, H. W. Robison, D. Koym, 7♂, 5♀ (INHS Insect Collection 794640), same but 1.6 km W Athens at Hwy 84, 34.31498, -93.99048, 9.vii.1984, H. W. Robison, D. Koym, 3♂, 10♀ (INHS Insect Collection 794629, 794634); Johnson Co., Mulberry River, 4.8 km W Ozark at Wolf Pen, 35.67376, -93.63271, 16.vii.1983, H. W. Robison, D. Koym, 8♂ (INHS Insect Collection 794643); Madison Co., War Eagle Creek, 4.8 km NE Huntsville at Hwy 68, 36.12076, -93.69354, 27.v.1978, J. McGraw, 17♂ (INHS Insect Collection 794636); Nevada Co., Little Missouri River, 17.7 km N Prescott at AR-19, 33.95571, -93.44388, 3.vii.1982, D. Koym, 8♂, 11♀ (INHS Insect Collection 794641), Little Missouri River, Nubbin Hill Rd., 33.93804, -93.35393, 1.vi.1982, D. Koym, 8♂ (INHS Insect Collection 794637); Pike Co., Antoine River, Antoine at AR-26, 34.03899, -93.41803, 18.vi.1982, D. Koym, 10♂, 8♀ (INHS Insect Collection 794638); Saline Co., Middle Fork Saline River, 1.6 km NW Owensville, 34.63066, -92.82711, 10.vii.1981, H. W. Robison, S. Harris, 4♂ (INHS Insect Collection 794631); Scott Co., Shadley Creek, 0.4 km S Bates, 34.90626, -94.38661, 12.vi.1983, H. W. Robison, D. Koym, 10♂ (INHS Insect Collection 794642); Sevier Co., Cossatot River, AR-24, 33.97145, -94.22274, 26.vi.1982, H. W. Robison, 10♂ (INHS Insect Collection 794632); Van Buren Co., South Fork Little Red River, 4 km NE Scotland at AR-95, 35.54868, -92.58541, 22.vi.1985, H. W. Robison, 15♂ (INHS Insect Collection 793774); Washington Co., Cove Creek, 24.1 km S Prairie Grove, 35.79531, -94.36519, 6.vi.1962, O. Hite, M. Hite, 2♂, 2♀ (INHS Insect Collection 794639). **Oklahoma:** Atoka Co., motel, Atoka, 34.38538, -96.12788, 4.vi.1969, D. C. Arnold, 2♂ (OKSU Midwest Plecoptera 19534); Le Flore Co., Big Creek, Page, 34.71595, -94.55016, 23.vi.1937, Standish, Kaiser, 8♂ (OKSU Midwest Plecoptera 19529); McCurtain Co., Broken Bow, 34.02983, -94.73871, 29.vii.1937, Standish, Kaiser, 34♂, 30♀ (OKSU Midwest Plecoptera 19517, 19518), Sherwood, 34.33121, -94.77833, 27.vi.1937, Standish, Kaiser, 5♂, 24♀ (OKSU Midwest Plecoptera 19521, 19522), West Fork Glover River, Battiest, 34.39393, -94.94166, 14.vi.1972, D. C. Arnold, 2♂ (OKSU Midwest Plecoptera 19523), Mountain Fork, Beaver's Bend State Park, 34.13960, -94.70704, 11.vi.1985, D. C. Arnold, 3♂

(OKSU Midwest Plecoptera 19526, 195277), same but 10.vi.1985, D. C. Arnold, ♂, 4♀ (OKSU Midwest Plecoptera 19528); Pontotoc Co., Ada, 34.77447, -96.67892, 16.vii.1937, Standish, Kaiser, 2♂, 2♀ (OKSU Midwest Plecoptera 19519, 19520); Washington Co., Caney River, Bartlesville, 36.75401, -95.97137, 31.v.1978, D. C. Arnold, ♂ (OKSU Midwest Plecoptera 19525).

Material examined of *Perlesta golconda*:

USA: Illinois: Carroll Co., Mississippi River, Savanna, 42.09622, -90.16227, 19.vi.1999, R. E. DeWalt, ♂ (INHS Insect Collection 566462). **Indiana:** Ohio Co., Arnold Creek, 6.9 km WSW Rising Sun at IN-262 and White Rd., 38.93676, -84.93167, 14.v.2018, E. A. Newman, ♀ (INHS Insect Collection 660320). **Iowa:** Cedar Co., Cedar River, Cedar Bluff at Hwy F28, 41.78790, -91.31340, 2.viii.2000, D. Heimdal, ♂, 2♀ (INHS Insect Collection 36061). **Louisiana:** East Baton Rouge Co., Mississippi River, Baton Rouge at Centroplex Pier N I-10Br., 30.44532, -91.19184, 12.vi.1992, R. E. DeWalt, ♂, ♀ (INHS Insect Collection 564765). **Minnesota:** Winona Co., Mississippi River, 3.6 km N La Crescent, rest stop at I-90, 43.85981, -91.30351, 18.vi.2012, R. E. DeWalt, ♀ (INHS Insect Collection 577372). **Nebraska:** Nemaha Co., Missouri River, Brownville, 200 m downstream US-136, 40.39335, -95.64948, 17.vi.2018, R. E. DeWalt, 6♂, ♀ (INHS Insect Collection 660253, 660254), same but 24.vi.2018, R. E. DeWalt, 8♂, 10♀ (INHS Insect Collection 660209–660220).

Modified key to first couplet in Stark (2004) for identification of males of *Perlesta sublobata* and *P. golconda*

1 Fully everted aedeagus with dorsal caecum (fig. 7.273).. **1a**

– Fully everted aedeagus without dorsal caecum (fig. 7.361)..**17**

1a Aedeagus with ventral caecum...**1b**

– Aedeagus without ventral caecum...**2**

1b Ventral caecum prominent, length ca. 2/5 aedeagal sac width, fine ventral seta-like spines present; dorsal caecum moderately developed (Fig. 3.4A); distinct dorsal extension of the aedeagal lateral sclerites with proximal V-shaped pattern (Fig. 3.4C); ocellar area with dark subquadrate patch... ***P. sublobata***

– Ventral caecum less prominent, length ca. 1/3 aedeagal sac width, without distinct fine ventral seta-like spines; dorsal caecum poorly developed; dorsal aedeagal patch with lateral margins darker than mesal field, appearing as two tracks (fig.7.364); ocelli usually connected by V-shaped area with pale center..***P. Golconda***

FIGURES AND TABLES

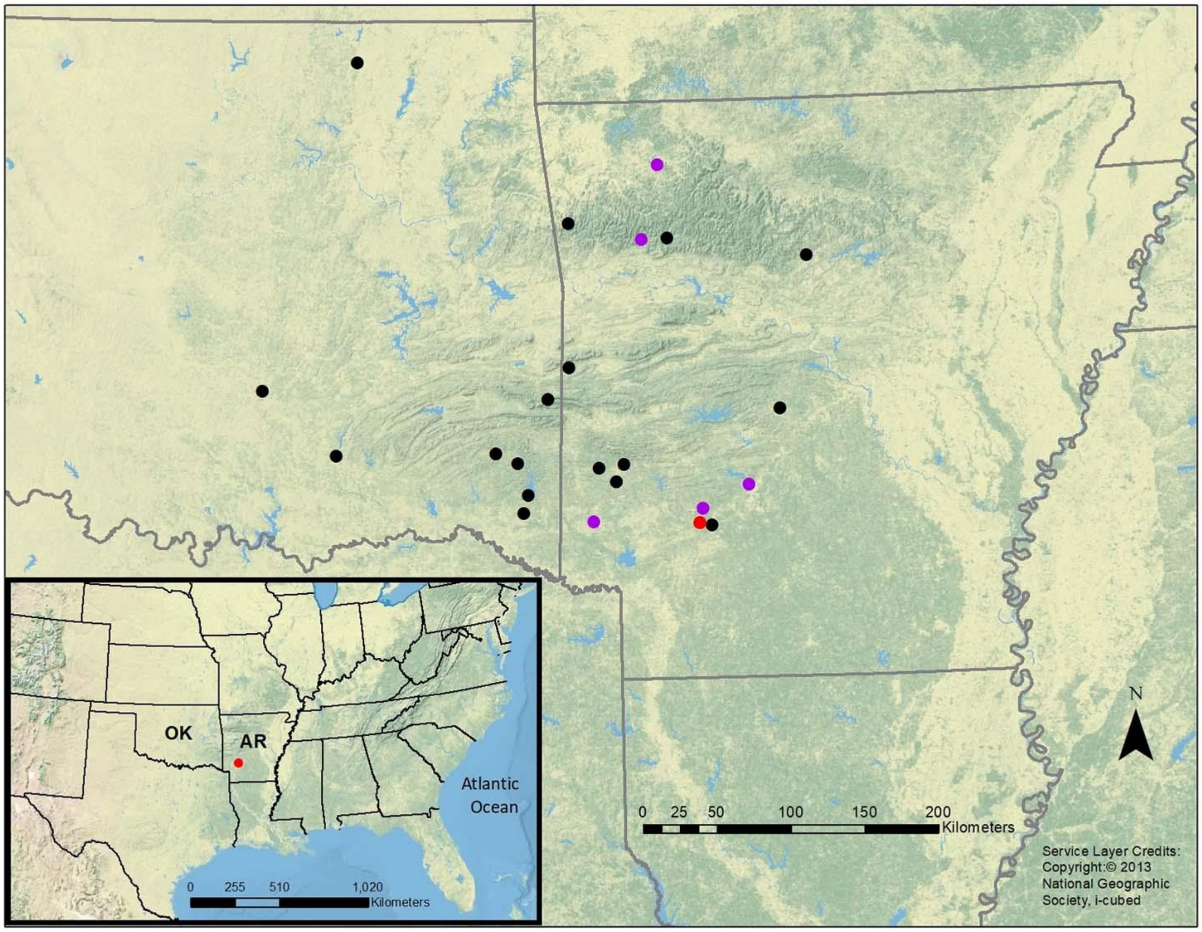

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Figure 3.1. Collection sites (1932–2016) for *Perlesta sublobata* sp. n. in Oklahoma (OK) and Arkansas (AR), USA. Red circle represents type locality, 2016. Purple circles represent paratype localities, 2016.

Table 3.1. Description of COI haplotypes for 18 *Perlesta* species and three outgroup taxa. Number of specimens for each haplotype (N) is listed beside corresponding GenBank accession number. Multiple specimens sharing the same haplotype are listed consecutively. All specimens collected from USA except *Perlesta nelsoni* (Canada). Sequences obtained from GenBank are denoted with *. INHS = Illinois Natural History Survey Insect Collection record number.

Species	**Sex**	**GenBank**	**N**	**INHS**	**Lat. / Lon.**	**Stream**	**State/ Prov.**	**Collector(s)**
Beloneuria georgiana (Banks, 1914)	♂	MH778486	1	909265	34.69804 -83.78149	Tributary of Dukes Creek	GA	E. J. South
Perlesta adena Stark, 1989	♂	MH778426	1	793345	36.39021 -86.25096	Rocky Creek	TN	S. A. Grubbs
Perlesta armitagei Grubbs and DeWalt, 2018	♂	MH778427	1	457510	39.0342 -86.16788	Little Salt Creek	IN	R. E. DeWalt
Perlesta bjostadi Kondratieff and Lenat, 2006	♂	MH778428	1	793346	36.84673 -77.56095	Nottoway River	VA	B. C. Kondratieff
Perlesta browni Stark, 1989	♀	MH778429	1	658464	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
Perlesta cinctipes (Banks, 1905)	♂	MH778430	1	658465	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♂	MH778431	1	658466	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♀	MH778432	1	658467	38.45202 -92.48643	South Moreau Creek	MO	E. J. South

Table 3.1 (cont.).

	♂	MH778433	1	658468	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
Perlesta decipiens (Walsh, 1862)	♂	MH778434	1	658778	41.3337 -88.18761	Kankakee River	IL	A. Yanahan
	♀	MH778435	1	658777	41.3337 -88.18761	Kankakee River	IL	A. Yanahan
Perlesta ephelida Grubbs and DeWalt, 2012	♂	MH778436	1	658780	44.72652 -86.14303	Platte River	MI	R. E. DeWalt, S. K. Ferguson
	♀	MH778437	1	658781	44.72652 -86.14303	Platte River	MI	R. E. DeWalt, S. K. Ferguson
	♂	MH778438	1	658469	37.49727 -92.63033	Osage Fork of Gasconade River	MO	E. J. South
	♂	MH778439	1	658470	37.49727 -92.63033	Osage Fork of Gasconade River	MO	E. J. South
	♀	MH778440	1	658477	37.49727 -92.63033	Osage Fork of Gasconade River	MO	E. J. South
Perlesta frisoni Banks, 1948	♂	*HQ568861	2	NA	35.62276 -83.44288	West Prong Little Pigeon River	TN	R. E. DeWalt
	♂	*JF884174	2	NA	35.4968 -83.8337	Twentymile Creek	NC	R. E. DeWalt
Perlesta golconda DeWalt and Stark, 1998	♂	MH778441	1	550392	41.67397 -91.56452	Iowa River	IA	M. Kippenhon
Perlesta lagoi Stark, 1989	♂	MH778442	9	658456	38.45202 -92.48643	South Moreau Creek	MO	E. J. South

Table 3.1 (cont.).

	♂	MH778443	9	658457	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♂	MH778444	9	658460	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♂	MH778445	9	658461	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♂	MH778446	9	658462	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♀	MH778447	9	658471	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♀	MH778448	9	658472	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♀	MH778449	9	658473	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♂	MH778450	9	658474	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♂	MH778451	1	658459	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♂	MH778452	1	658475	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♂	MH778453	1	658476	38.45202 -92.48643	South Moreau Creek	MO	E. J. South
	♂	MH778454	1	658776	41.66065 -81.11747	Bates Creek	OH	E. J. South, R. E. DeWalt

Table 3.1 (cont.).

Perlesta mihucorum Kondratieff and Myers, 2011	♂	MH778455	1	793347	42.4401 -73.8137	Hannacroix Creek	NY	L. Myers, J. Myers
Perlesta nelsoni Stark, 1989	♂	*KR144298	1	NA	45.976 -66.719	St. John River	NB	K. Heard et al.
Perlesta ouabache Grubbs and DeWalt, 2011	♂	MH778456	1	516699	42.45994 -89.23985	Sugar River	IL	R. E. DeWalt et al.
Perlesta roblei Kondratieff and Kirchner, 2003	♂	MH778457	1	793348	36.4684 -77.1443	Kirbys Creek	NC	B. C. Kondratieff et al.
Perlesta sublobata sp. n.	♂	MH778458	8	793207	36.12052 -93.69319	War Eagle Creek	AR	E. J. South
	♂	MH778459	8	793212	34.03869 -93.41752	Antoine River	AR	E. J. South
	♂	MH778460	8	793218	34.17985 -93.07021	Caddo River	AR	E. J. South
	♂	MH778461	8	793230	36.04161 -93.70482	War Eagle Creek	AR	E. J. South
	♂	MH778462	8	793266	33.95608 -93.44362	Little Missouri River	AR	E. J. South
	♂	MH778463	8	793271	33.95608 -93.44362	Little Missouri River	AR	E. J. South
	♂	MH778464	8	793273	33.95608 -93.44362	Little Missouri River	AR	E. J. South

Table 3.1 (cont.).

	♂	MH778465	8	793288	33.95608 -93.44362	Little Missouri River	AR	E. J. South
	♂	MH778466	1	793208	36.12052 -93.69319	War Eagle Creek	AR	E. J. South
	♂	MH778467	3	793209	35.66925 -93.83033	Mulberry River	AR	E. J. South
	♂	MH778468	3	793233	33.97121 -94.22292	Cossatot River	AR	E. J. South
	♀	MH778469	3	793324	33.95608 -93.44362	Little Missouri River	AR	E. J. South
	♂	MH778470	2	793211	35.66925 -93.83033	Mulberry River	AR	E. J. South
	♂	MH778471	2	793224	33.95608 -93.44362	Little Missouri River	AR	E. J. South
	♂	MH778472	1	793214	34.17985 -93.07021	Caddo River	AR	E. J. South
	♂	MH778473	1	793234	33.97121 -94.22292	Cossatot River	AR	E. J. South
	♂	MH778474	1	793299	33.95608 -93.44362	Little Missouri River	AR	E. J. South
	♂	MH778475	1	793312	33.95608 -93.44362	Little Missouri River	AR	E. J. South
	♀	MH778476	1	793328	33.95608 -93.44362	Little Missouri River	AR	E. J. South
	♀	MH778477	1	793331	33.95608 -93.44362	Little Missouri River	AR	E. J. South

Table 3.1 (cont.).

Perlesta teaysia Kirchner and Kondratieff, 1997	♂	MH778478	1	515560	39.1355 -86.1601	Tributary of Middle Fork Salt	IN	R. E. DeWalt
	♀	MH778479	1	457522	39.1355 -86.1601	Tributary of Middle Fork Salt	IN	R. E. DeWalt
Perlesta WI-1 (undescribed)	♂	MH778480	1	516410	46.07721 -92.24608	St. Croix River	WI	R. E. DeWalt et al.
	♀	MH778481	1	552631	46.07721 -92.24608	St. Croix River	WI	R. E. DeWalt, S. K. Ferguson
	♂	MH778482	1	576963	45.57953 -87.78796	Menominee River	WI	R. E. DeWalt et al.
	♂	MH778483	1	658779	45.77348 -92.78164	St. Croix River	MN	R. E. DeWalt
	♀	MH778484	1	583370	45.82306 -92.77001	Snake River	MN	R. E. DeWalt
Perlesta xube Stark and Rhodes, 1997	♂	MH778485	1	790543	39.40911 -88.89952	Mud Creek	IL	E. J. South, R. E. DeWalt
Perlinella drymo (Newman, 1839)	♂	MH778487	1	514716	40.2942 -87.2546	Wabash River	IN	R. E. DeWalt, M. Pessino
Perlinella ephyre (Newman, 1839)	♂	MH778488	1	548835	42.32815 -83.8595	Huron River	MI	R. E. DeWalt et al.

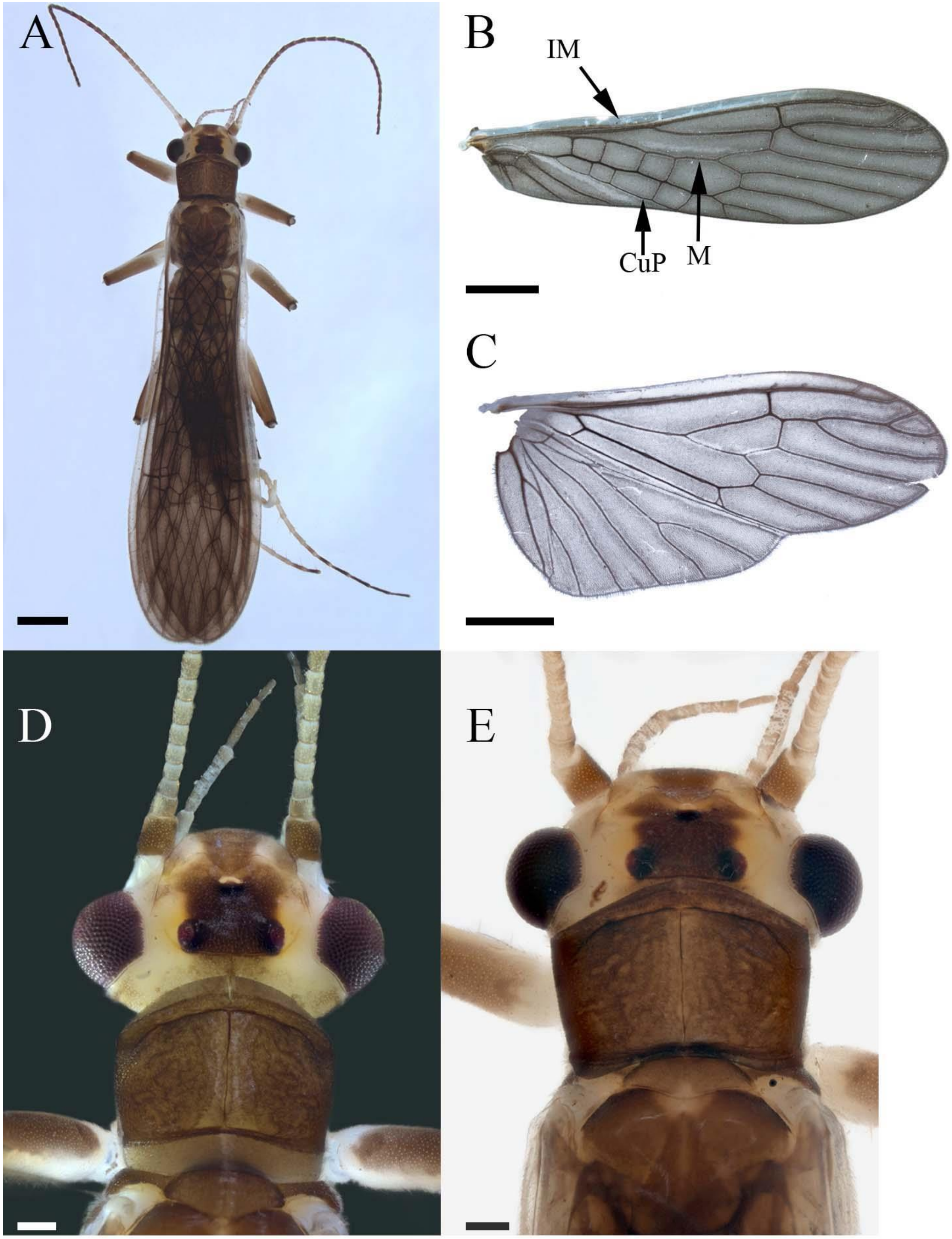

Figure 3.2. *Perlesta sublobata* sp. n**.,** paratype males from Little Missouri River, Arkansas. (A) Habitus, dorsal view (INHS Insect Collection 793329) (scale 1 mm). (B) Dorsal view of right forewing showing intercostal margin (IM), posterior cubital vein (CuP), and median vein (M) (scale 1.2 mm). (C) Right hind wing, dorsal view (INHS Insect Collection 793270) (scale 1.2 mm). (D) Head and pronotum (INHS Insect Collection 793226) (scale 200 µm). (E) Head and pronotum (INHS Insect Collection 793329) (scale 200 µm).

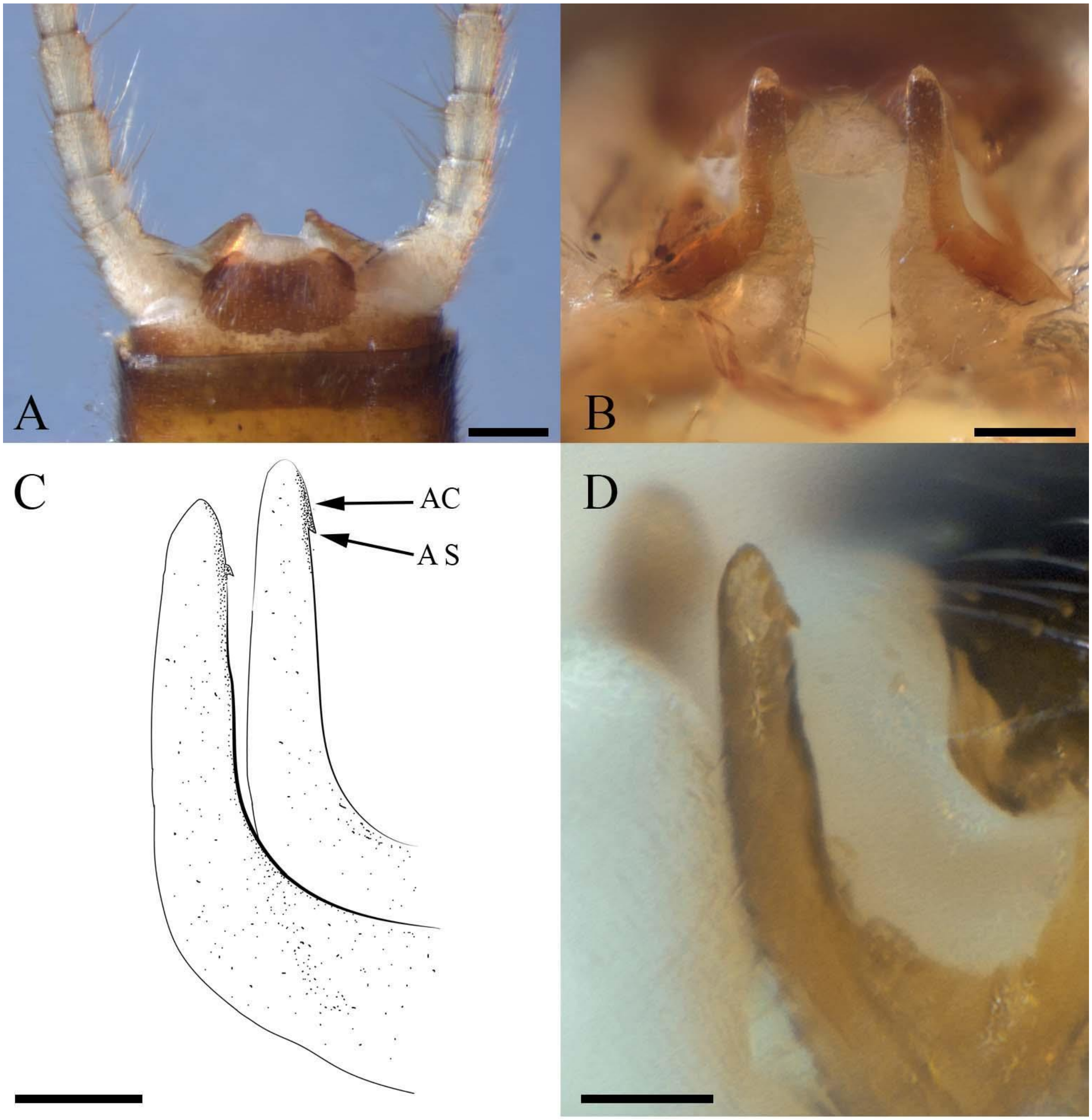

Figure 3.3. *Perlesta sublobata* sp. n., terminalia of paratype males from Little Missouri River, Arkansas. (A) Tenth tergite and paraprocts, dorsal view (INHS Insect Collection 793335) (scale 200 µm). (B) Paraprocts, caudal view (scale 100 µm). (C) Paraprocts, oblique lateral view showing anteromedially directed carina (AC) and spine (AS) (scale 50 µm). (D) Right paraproct of extruded male, oblique lateral view (INHS Insect Collection 793226) (scale 50 µm).

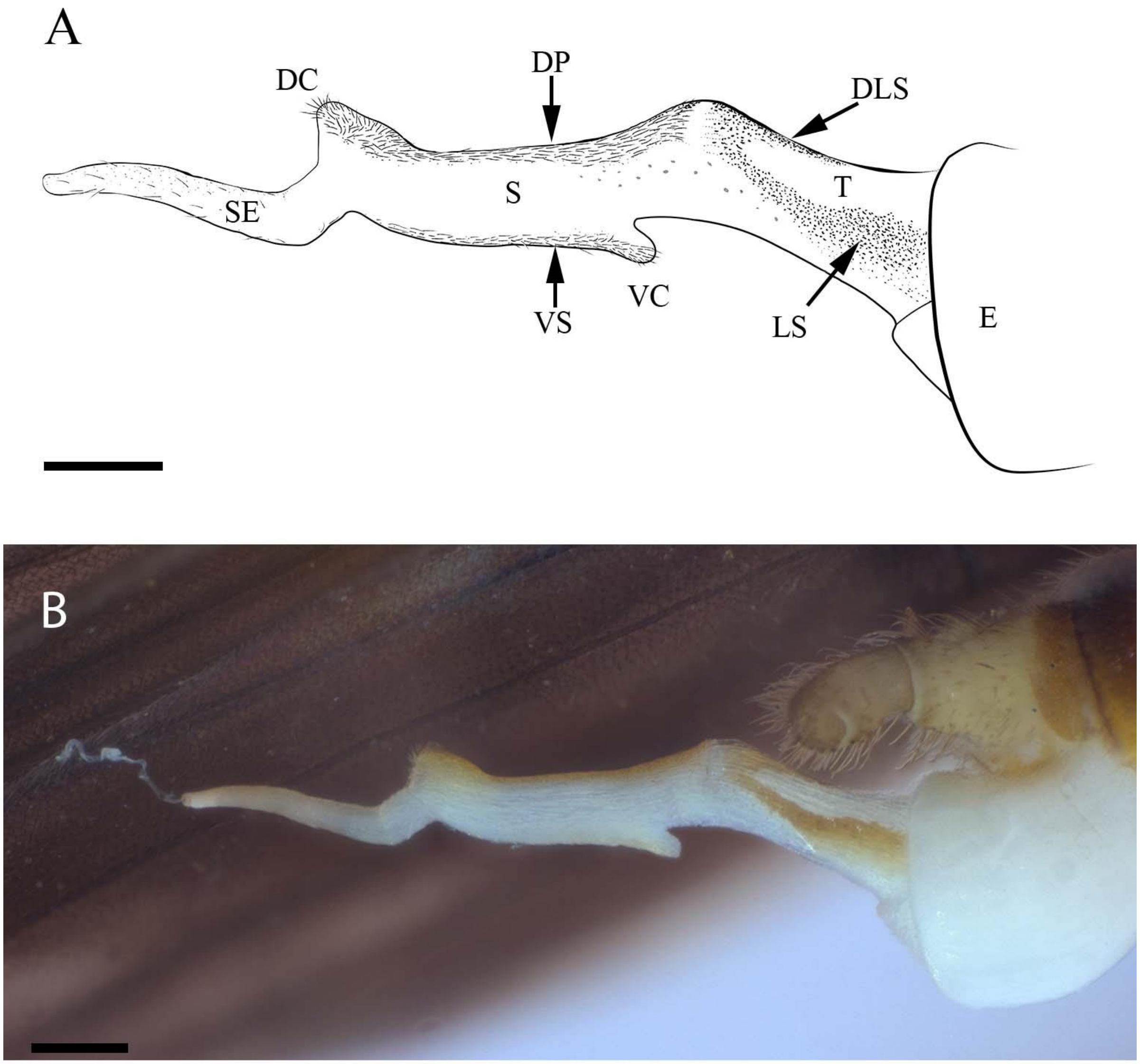

Figure 3.4. *Perlesta sublobata* sp. n.**,** aedeagus of paratype male from Little Missouri River, Arkansas (INHS Insect Collection 793295). (A) Lateral view showing dorsal caecum (DC), ventral caecum (VC), envelope (E), tube (T), sac (S), sac extension (SE), dorsal patch (DP), ventral seta-like spines (VS), lateral sclerite (LS), and dorsal extension of the lateral sclerites (DLS) (scale 200 µm). (B) Lateral view showing partially extruded dorsal caecum (scale 200 µm).

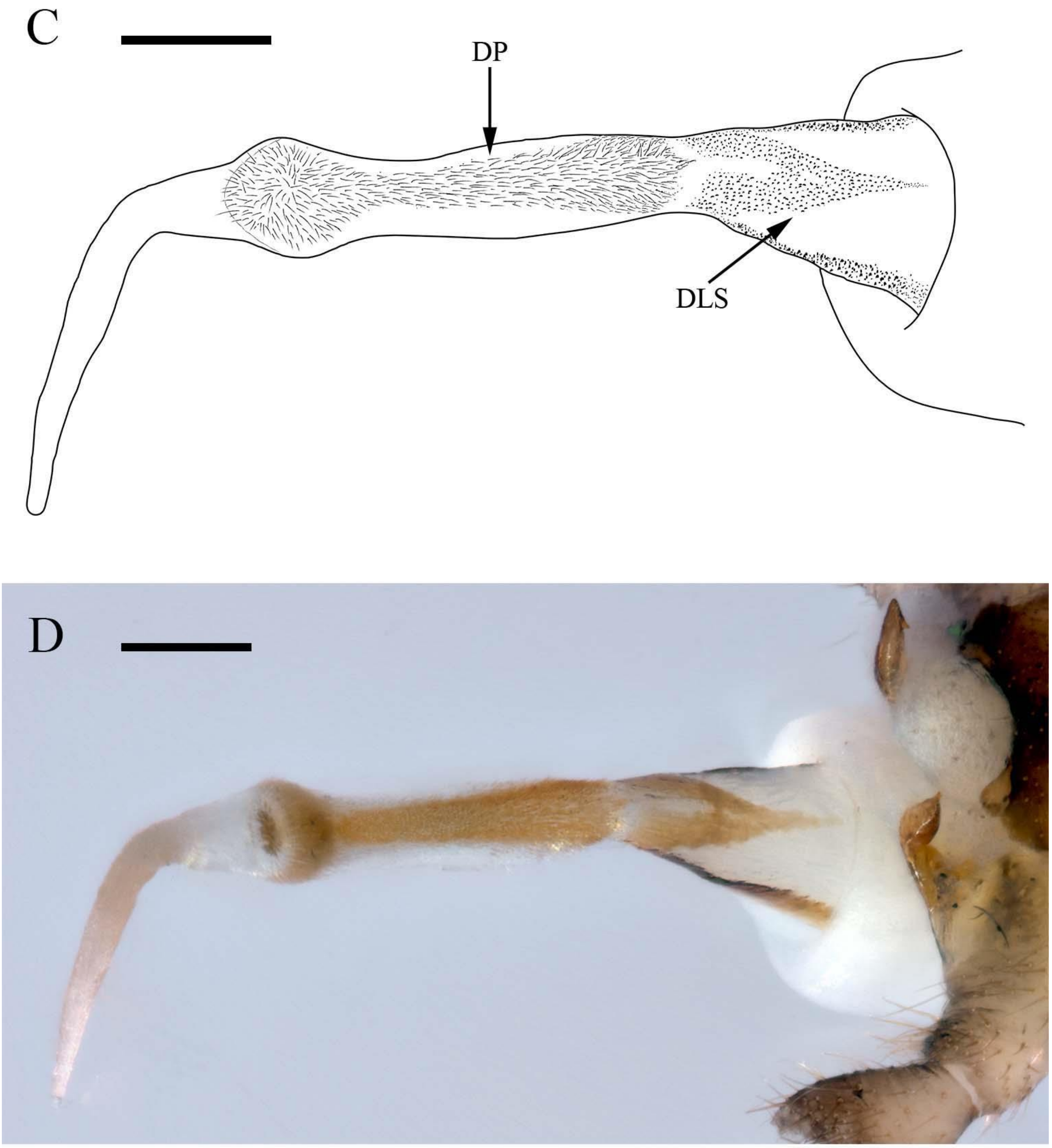

Figure 3.4 (cont.). (C) Dorsal view showing dorsal patch (DP) and dorsal extension of the lateral sclerites (DLS) (scale 200 µm). (D) Dorsal view showing partially extruded dorsal caecum (scale 200 µm).

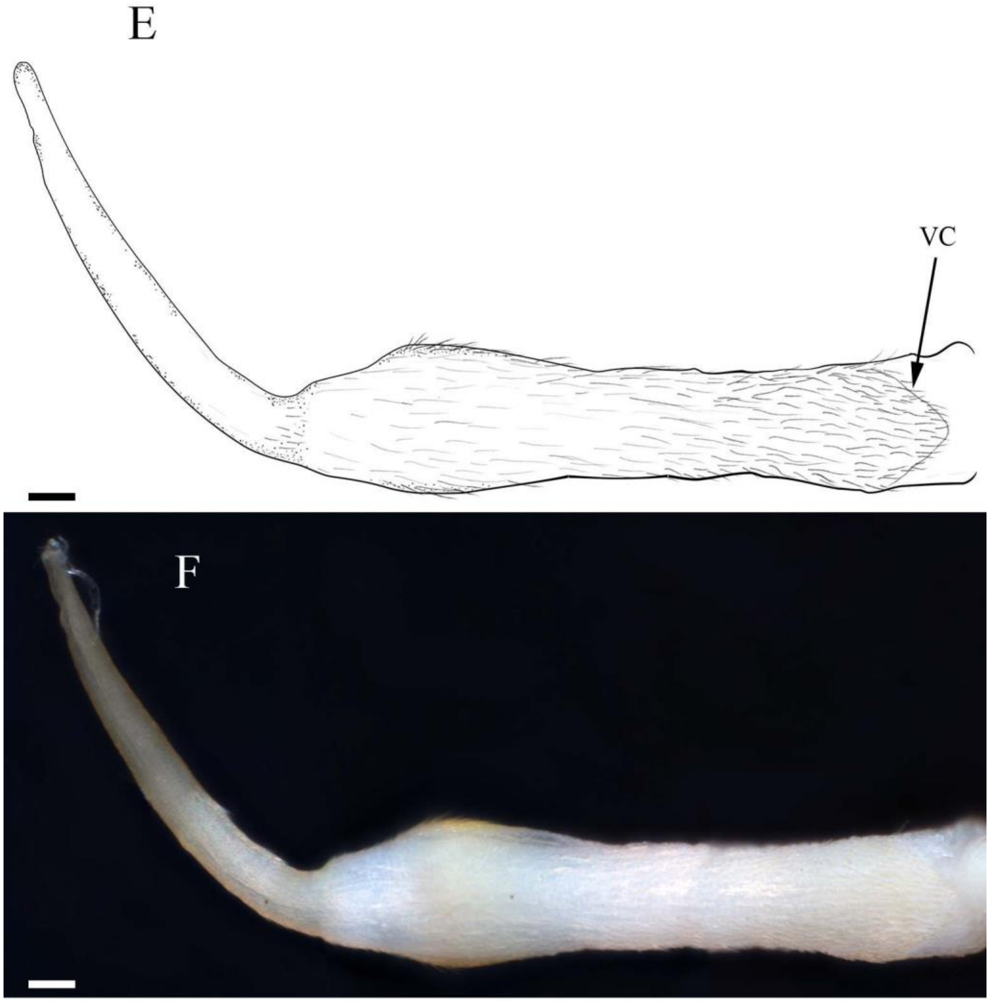

Figure 3.4 (cont.). (E) Ventral view showing fine seta-like spines covering sac venter and ventral caecum (VC) (scale 50 µm). (F) Ventral view (scale 50 µm).

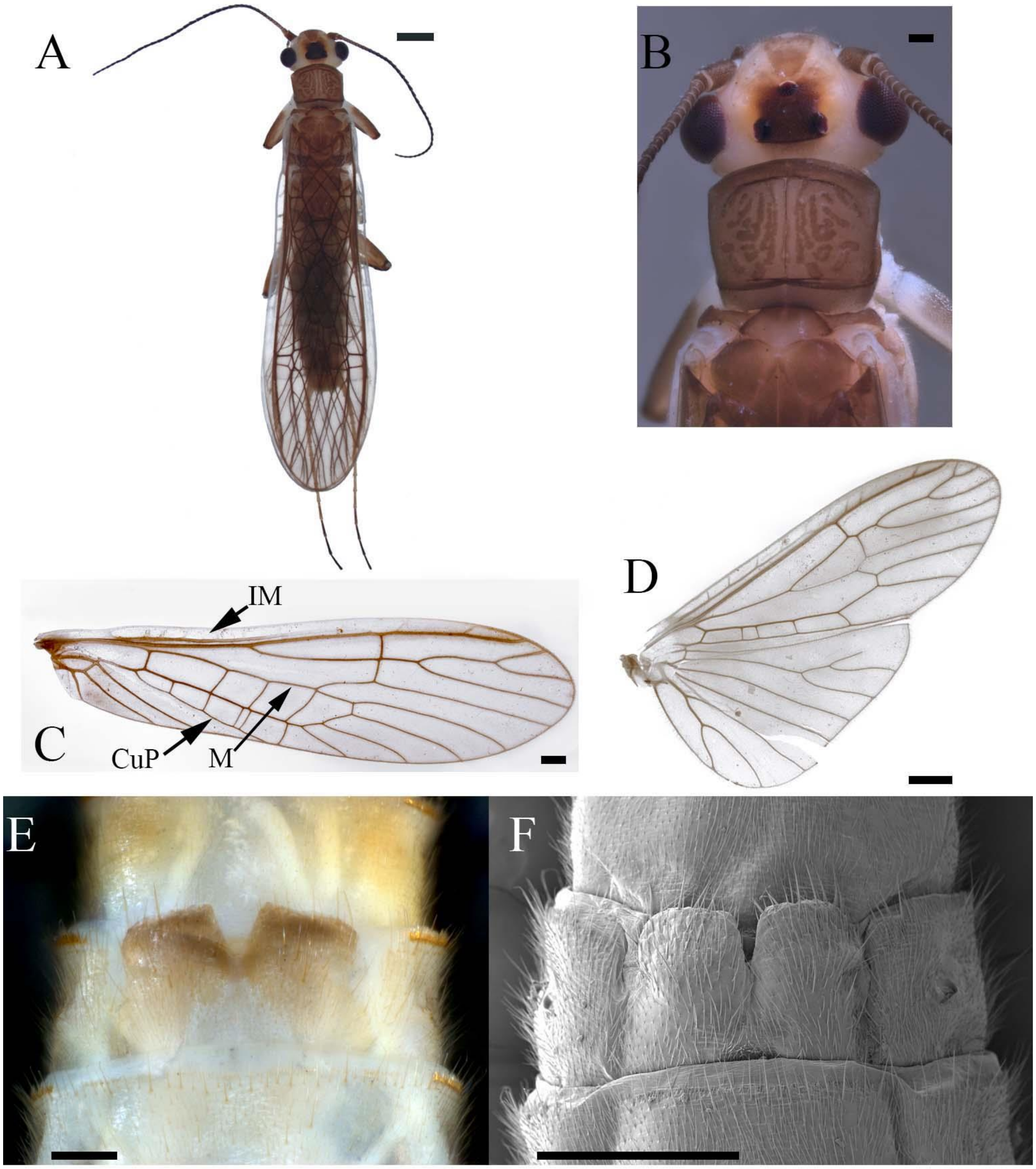

Figure 3.5. *Perlesta sublobata* sp. n., paratype females from Little Missouri River, Arkansas. (A) Habitus, dorsal view (scale 1 mm). (B) Head and pronotum (INHS Insect Collection 793329) (scale 200 µm). (C) Dorsal view of right forewing showing intercostal margin (IM), posterior cubital vein (CuP), and median vein (M) (scale 500 µm). (D) Right hind wing, dorsal view (INHS Insect Collection 793332) (scale 750 µm). (E) Subgenital plate (INHS Insect Collection 793329) (scale 200 µm). (F) subgenital plate, SEM (INHS Insect Collection 793328) (scale 500 µm).

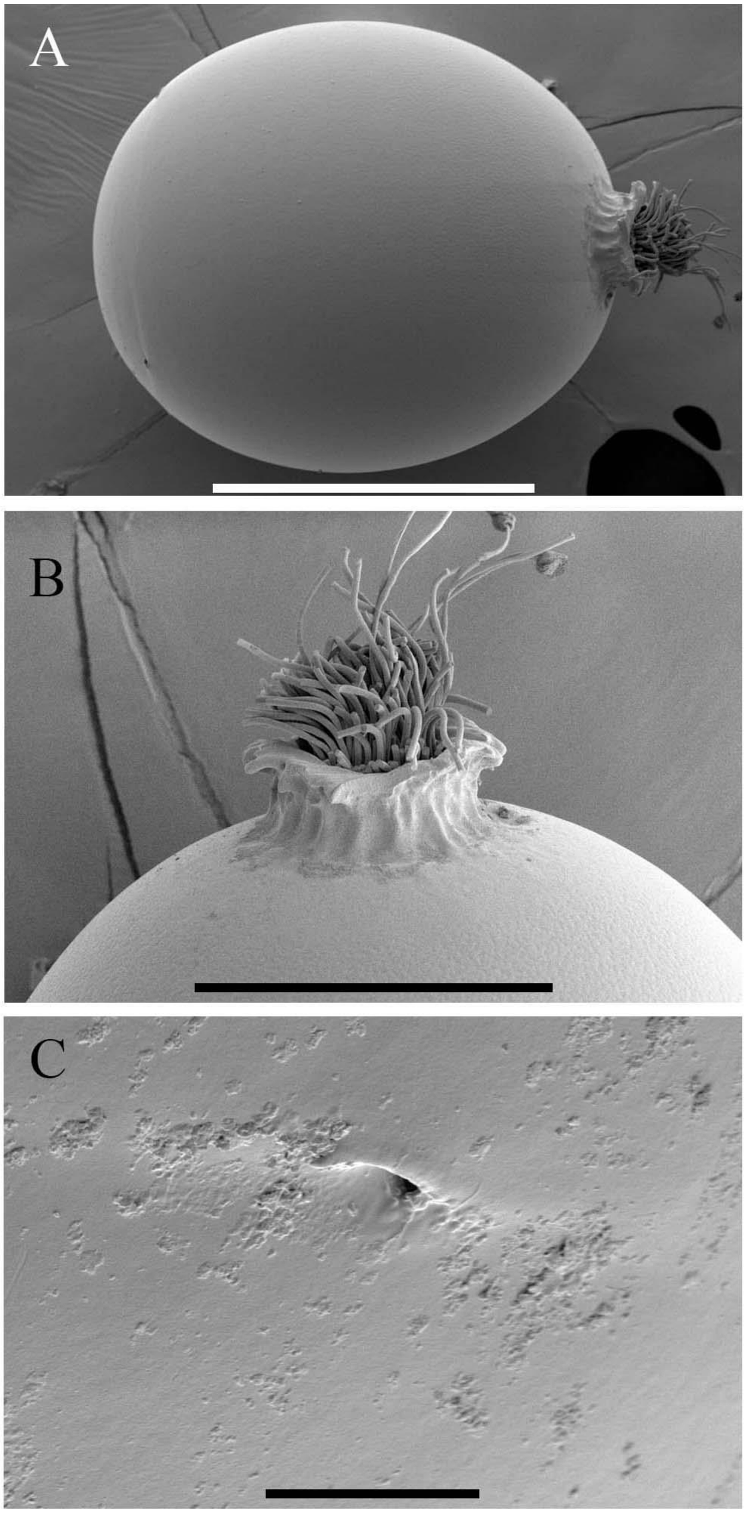

Figure 3.6. *Perlesta sublobata* sp. n., egg of paratype female from Little Missouri River, Arkansas (INHS Insect Collection 793316). (A) Entire egg (scale 200 µm). (B) Posterior pole and collar (scale 100 µm). (C) Micropyle (scale 10 µm).

Table 3.2. Intra and interspecific distance. Maximum intraspecific and minimum interspecific (nearest neighbor) Kimura 2-parameter values for COI within *Perlesta*. N = number of specimens. *P.*WI-1 is an undescribed species from Wisconsin.

Species	**N**	**Maximum intraspecific distance (%)**	**Nearest neighbor**	**Nearest neighbor distance (%)**
P. adena	1	0	*P. nelsoni*	19.3
P. armitagei	1	0	*P. xube*	7.2
P. bjostadi	1	0	*P. decipiens*	4.5
P. browni	1	0	*P. armitagei*	15.1
P. cinctipes	4	0.7	*P. armitagei*	10.4
P. decipiens	2	1.2	*P. lagoi*	1.5
P. ephelida	5	0.7	*P. ouabache*	4.8
P. frisoni	2	0	*P. nelsoni*	14.9
P. golconda	1	0	*P.* WI-1	17.8
P. lagoi	13	0.8	*P. decipiens*	1.5
P. mihucorum	1	0	*P. sublobata*	4.8
P. nelson	1	0	*P. teaysia*	9.4
P. ouabache	1	0	*P. sublobata*	2
P. roblei	1	0	*P. ephelida*	15.6
P. sublobata	20	0.8	*P. decipiens*	1.8
P. teaysia	2	0.2	*P. frisoni*	15.9
P. WI-1	5	2.4	*P. ouabache*	3.9
P. xube	1	0	*P. armitagei*	7.2

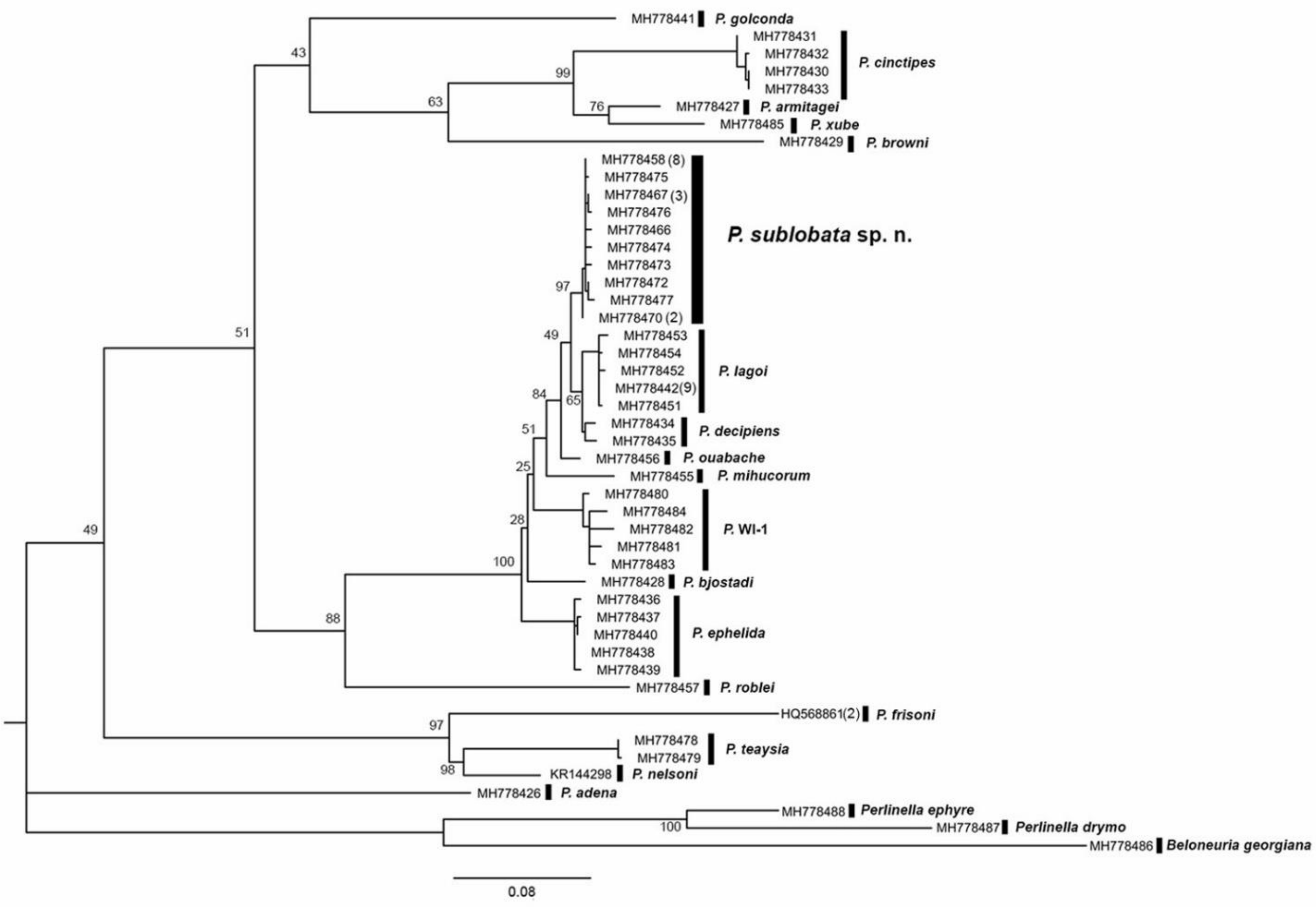

Figure 3.7. Maximum Likelihood phylogenetic reconstruction of 44 unique *Perlesta* CO1 haplotypes using the GTR+I+G nucleotide substitution model. Haplotypes represented by more than one specimen are indicated in parentheses beside corresponding GenBank accession numbers. Outgroup taxa: *Beloneuria georgiana*, *Perlinella drymo*, and *Perlinella ephyre*. Bootstrap scores from 1,000 replicates are displayed at nodes. Scale bar represents the estimated number of nucleotide substitutions per site.

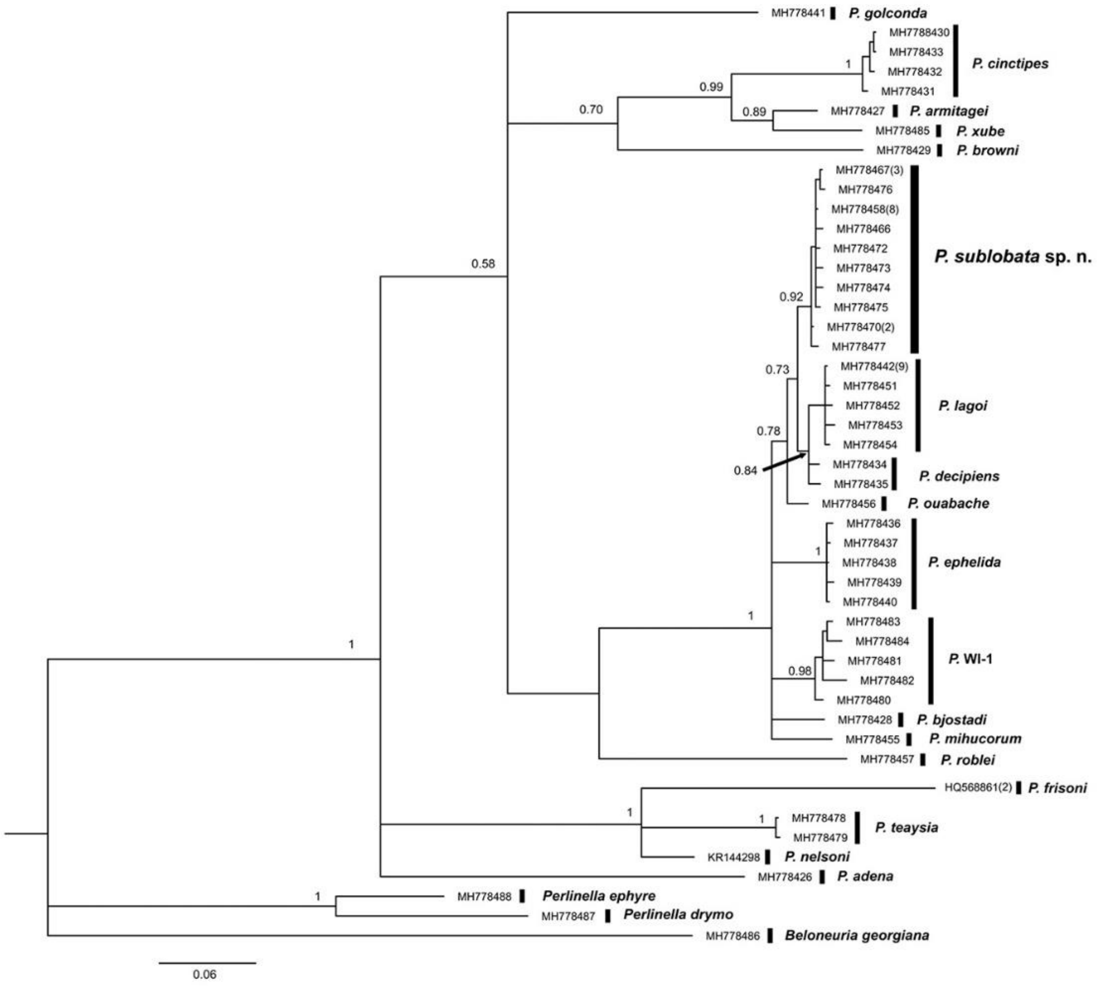

Figure 3.8. Bayesian phylogenetic reconstruction of 44 unique *Perlesta* CO1 haplotypes using the GTR+I+G nucleotide substitution model. Haplotypes represented by more than one specimen are indicated in parentheses beside corresponding GenBank accession numbers. Outgroup taxa: *Beloneuria georgiana*, *Perlinella drymo*, and *Perlinella ephyre*. Posterior probabilities are indicated at nodes. Scale bar represents the estimated number of nucleotide substitutions per site.

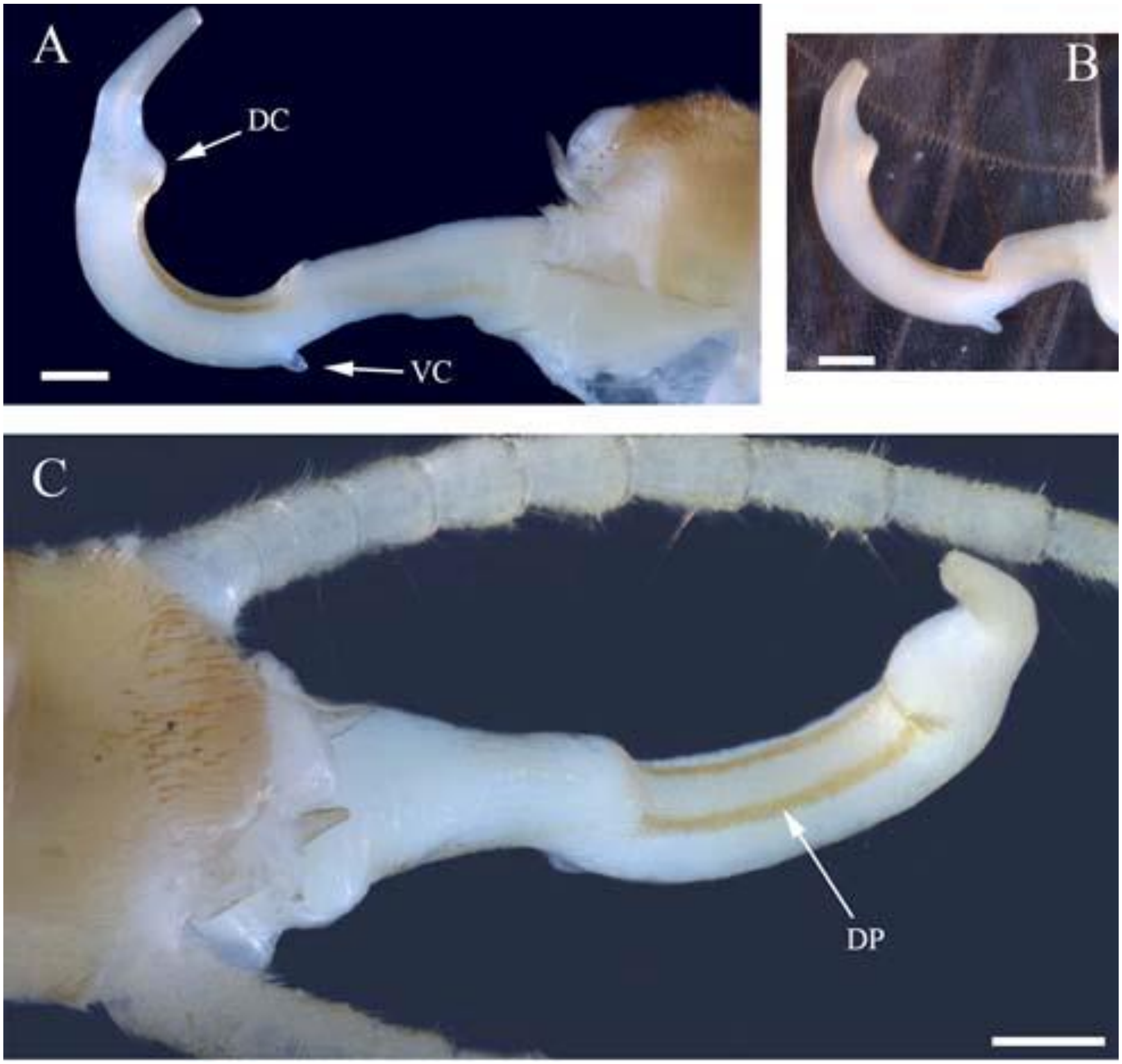

Figure 3.9. *Perlesta golconda*, aedeagi of males from Missouri River, Nebraska. (A) Lateral view showing dorsal caecum (DC) and ventral caecum (VC) (INHS Insect Collection 660209) (scale 200 µm). (B) Lateral view (INHS Insect Collection 660210) (scale 200 µm). (C) Dorsal view showing dorsal patch (DP) (INHS Insect Collection 660209) (scale 200 µm).

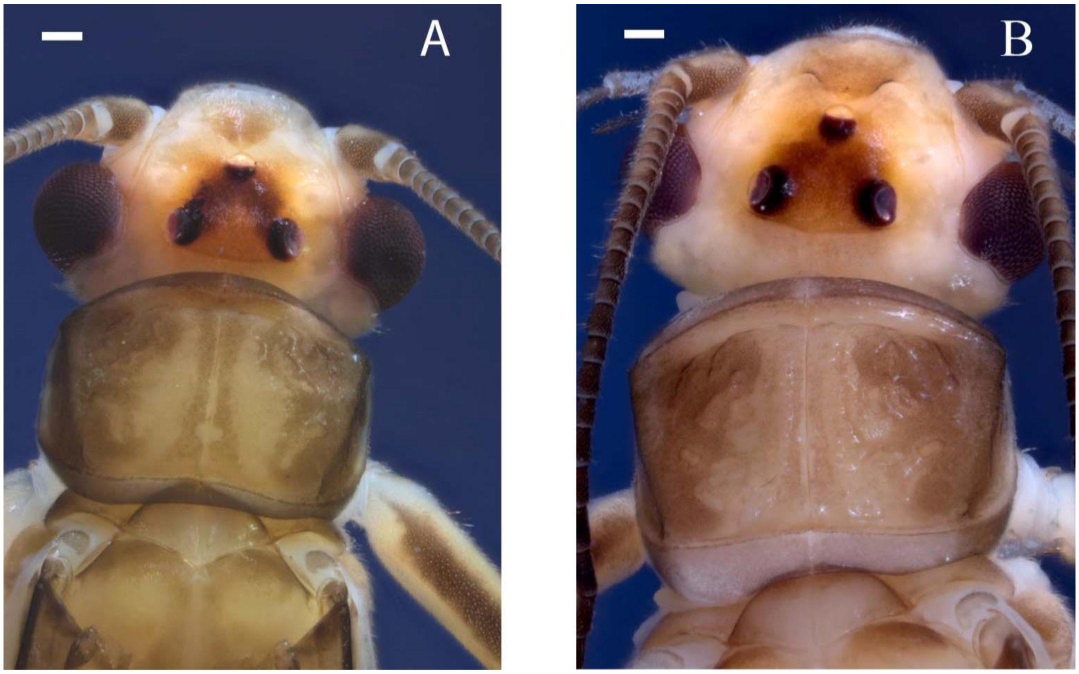

Figure 3.10. *Perlesta golconda*, head and pronotum, Missouri River, Nebraska. (A) Male (INHS Insect Collection 660210) (scale 200 µm). (B) Female (INHS Insect Collection 658790) (scale 200 µm).

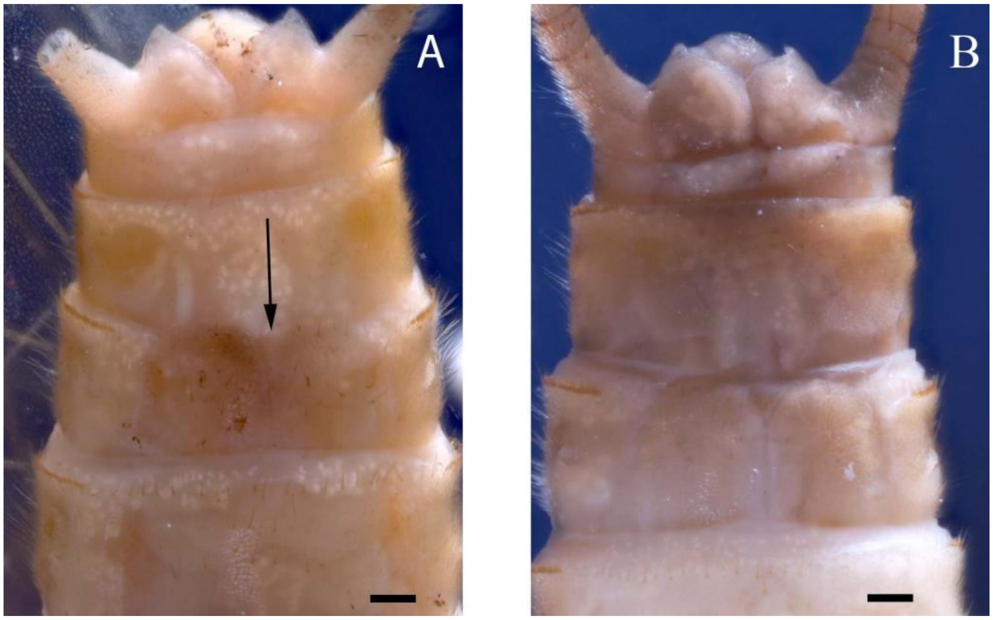

Figure 3.11. *Perlesta golconda*, subgenital plates of females from Missouri River, Nebraska. (A) Notch indicated by arrow (INHS Insect Collection 658788) (scale 200 µm). (B) (INHS Insect Collection 658789) (scale 200 µm).

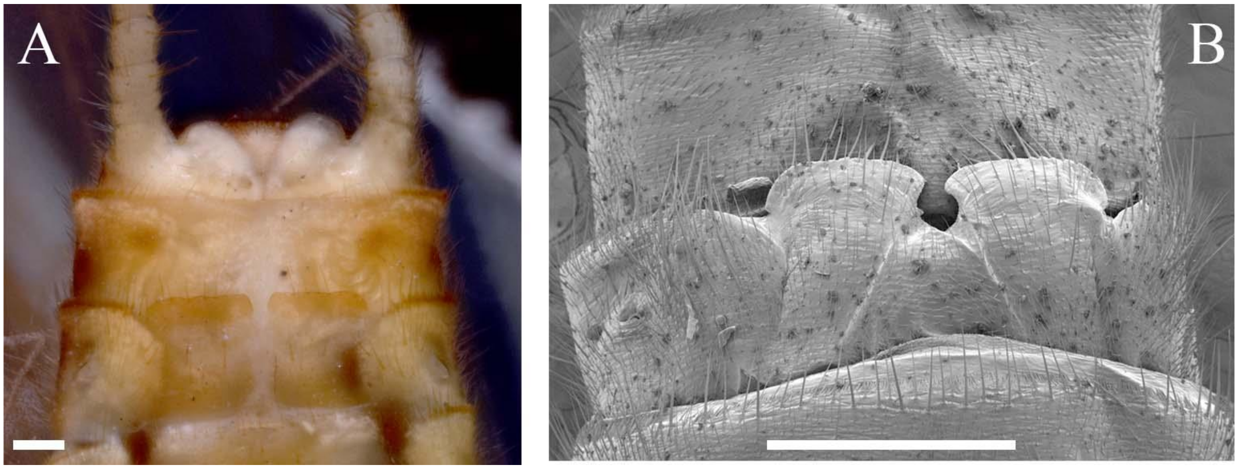

Figure 3.12. *Perlesta decipiens*, female subgenital plates. (A) Kankakee River, Illinois (INHS Insect Collection 577949) (scale 200 µm). (B) Caddo River, Arkansas, SEM (INHS Insect Collection 793908) (scale 500 µm).

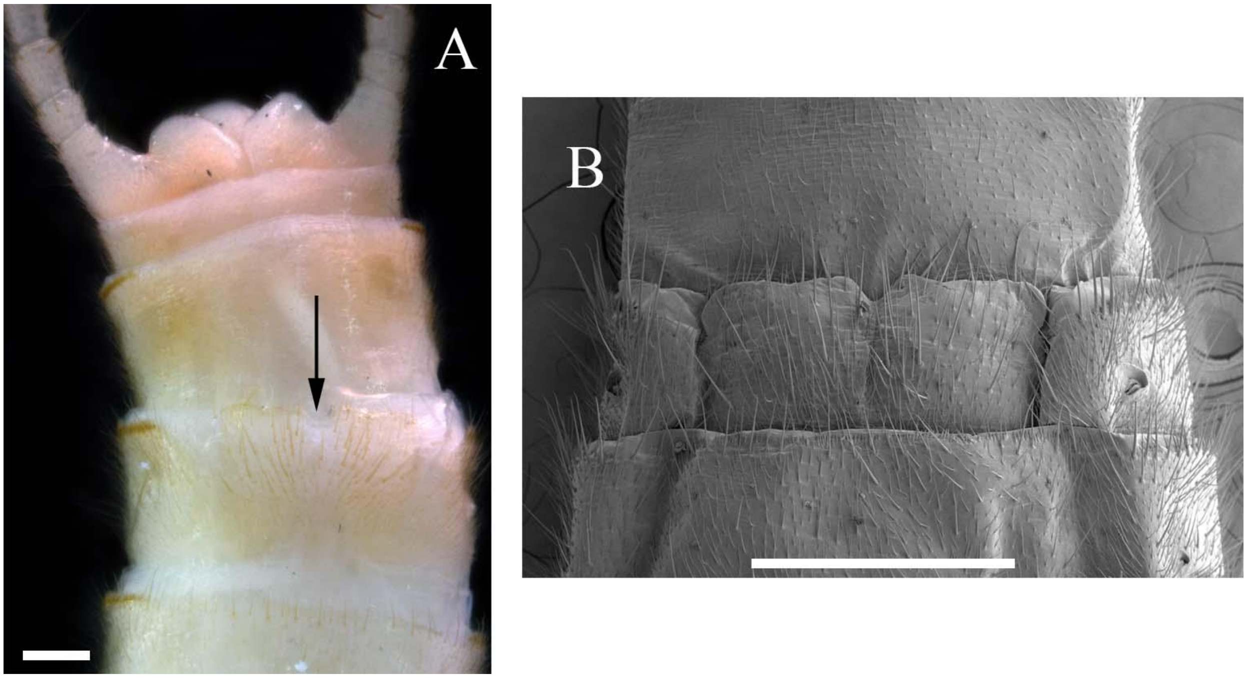

Figure 3.13. *Perlesta ephelida*, female subgenital plates. (A) Platte River, Michigan, notch indicated by arrow (INHS Insect Collection 658781) (scale 200 µm). (B) Sugar Creek, Indiana, SEM (INHS Insect Collection 658791) (scale 500 µm).

Figure 3.14. Little Missouri River, Pike County, Arkansas, USA. Type locality for *Perlesta sublobata* sp. n.

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