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Comparative analysis of mitochondrial genomes among the family Peltoperlidae (Plecoptera: Systellognatha) and phylogenetic implications

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Nowadays, the position of Peltoperlidae in Systellognatha has been resolved based on morphological analyses. However, there are different opinions based on molecular data. To date, only three peltoperlid mitogenomes are available, and more sampling is needed to obtain precise phylogenetic relationships. In this study, we obtained the complete mitogenomes of *Cryptoperla kawasawai* (15,832 bp) and *Peltoperlopsis sagittata* (15,756 bp). Our results show that gene content, gene order, DmTTF binding site, nucleotide composition, codon usage, ribonucleic acid (RNA) structure, and structural elements in the control region are highly conserved in peltoperlids. Heatmap analysis of codon usage shows that the AT-rich codons UUA, AUU, UUU, and AUA were commonly used codons in the Peltoperlidae. Evolutionary rate analyses of protein-coding genes reveal that different genes have been subject to different rates of molecular evolution correlated with the GC content. All tRNA genes in peltoperlid mitogenomes have a canonical cloverleaf secondary structure except for *trnS1*, whose dihydrouridine arm simply forms a loop. The control region of the family has several distinct structural characteristics and has the potential to serve as effective phylogenetic markers. Phylogenetic analyses support the monophyly of Perloidea, but the monophyly of Pteronarcyoidea is still not supported. The Peltoperlidae is placed as the earliest branch within the Systellognatha, and the estimated phylogenetic relationship is: Peltoperlidae + {(Styloperlidae + Pteronarcyidae) + [Perlidae + (Chloroperlidae + Perlodidae)]}. Our results provide new insight into the phylogeny of this group.

KEYWORDS

***Cryptoperla kawasawai*, *Peltoperlopsis sagittata*, mitochondrial genome, phylogeny, Plecoptera**

Introduction

The Plecoptera, also called stoneflies, are a small order of hemimetabolous insects of about 4,000 species worldwide (DeWalt et al., 2022). Because their nymphs dwell in aquatic habitats and are very sensitive to water quality, stoneflies can be used as monitors of healthy streams and rivers (Fochetti and Tierno de Figueroa, 2008). Stoneflies have a low level of vagility, making them ideal for biogeographic and phylogeographic research (Fochetti and Tierno de Figueroa, 2008; Pessino et al., 2014; McCulloch et al., 2016; Stevens et al., 2018). But the phylogenetic position of Plecoptera has long been under debate. Previously phylogenetic studies have indicated that Plecoptera is closely related to different insect taxa (Kukalová-Peck and Brauckmann, 1992). However, more conflicting hypotheses have been proposed by morphological evidence and the sister group of Plecoptera is still inconclusive (Terry and Whiting, 2005; Lin et al., 2010; Simon et al., 2012; Letsch and Simon, 2013; Misof et al., 2014; Song et al., 2016).

Several phylogenetic clades have been defined for Plecoptera, one of which is the infraorder Systellognatha. According to the morphological study of Zwick (2000), six extant families (Chloroperlidae, Perlidae, Perlodidae, Peltoperlidae, Pteronarcyidae, and Styloperlidae) in two superfamilies (Pteronarcyoidea and Perloidea) are included in Systellognatha. Recently, one new family (Kathroperlidae) was added to the superfamily Perloidea (South et al., 2021a). Finally, the infraorder includes seven families. Based on morphological evidence, Zwick (2000) reconstructed the broadly accepted phylogenetic relationship within Plecoptera. However, the phylogeny of Systellognatha remains debatable to date. As summarized in **Figure 1**, most studies placed Perloidea as a monophyletic group, but the monophyly of Pteronarcyoidea was not well supported. Meanwhile, the relationship among Perloidea was undetermined based on morphological data (Zwick, 2000), while it was recovered as Perlidae + (Chloroperlidae + Perlodidae) by most studies based on the mitochondrial genome (mitogenome) and transcriptome data (Chen et al., 2018; Shen and Du, 2019, 2020; Veale et al., 2019; Wang et al., 2019; South et al., 2021a,b; Mo et al., 2022). These results generated by mitogenome and transcriptome data are inconsistent with those by single or multiple genes and other transcriptome data (Thomas et al., 2000; Terry and Whiting, 2003; Davis, 2013). Therefore, the phylogeny of Systellognatha remains controversial, and more phylogenetic studies are needed.

Peltoperlidae is one of the smallest families of Plecoptera with approximately 50 species distributed in the Nearctic, Palearctic, and Oriental regions (Zwick, 2000; DeWalt et al., 2022). The nymphs are most common in very shallow running water, like seeps on rock faces, but they also occur in a variety of small streams and rivers (DeWalt et al., 2022). The nymphs are easily identified by their "roach-like" appearance, and the adults can be distinguished from other systellognathan stoneflies in the field by their small heads (Zwick, 2000). Peltoperlidae belongs to the superfamily Pteronarcyoidea, and the monophyly of Pteronarcyoidea was supported by Zwick's (2000) morphological phylogeny. However, phylogenetic relationships among three families in Pteronarcyoidea are very controversial all the time, as evidenced by molecular studies (**Figure 1**). Taxon sampling in these molecular studies has been limited, and not enough to obtain precise phylogenetic relationships within Systellognatha.

A well-constructed molecular phylogeny could substantially benefit the understanding of evolutionary relationships of major lineages and morphological character evolution, as in this case, resolving superfamily monophyly and the phylogenetic relationships within the Systellognatha. Complete mitogenomes contain more useful evolutionary information than single or multiple genes and have been widely used to investigate insect relationships at different taxonomic scales (Fenn et al., 2008; Dowton et al., 2009; Cameron, 2014), due to their small size, conserved gene components, maternal inheritance, rare recombination, and relatively high evolutionary rate (Boore, 1999; Curole and Kocher, 1999; Barr et al., 2005).

So far, there are approximately forty complete or near complete systellognathan mitogenomes available in GenBank, of which only nine species belong to Pteronarcyoidea. In this study, two complete mitogenomes from the family Peltoperlidae [*Cryptoperla kawasawai* Maruyama, 2002 and *Peltoperlopsis sagittata* (Cao et al., 2019)]. were sequenced. We conducted a comparative analysis of those newly sequenced mitogenomes and four published peltoperlid mitogenomes. Finally, we investigated the phylogenetic relationships within Systellognatha.

Materials and methods

Specimens, deoxyribonucleic acid extraction, and sequencing

Adult male specimens of *C. kawasawai* were collected from Kumakogen town (Ehime Prefecture, Japan; May, 2016) and of *P. sagittata* from Gaoligong Mountain (Yunnan Provence, China; July, 2016). Before this study, all samples were stored in 100% alcohol and maintained at −20°C. Total genomic deoxyribonucleic acid (DNA) was extracted from the thoracic muscle using the DNeasy tissue kit (Qiagen, Hilden, Germany). NanoDrop One (Thermo Scientific, Waltham, MA, United States) was used to measure the DNA concentration for each sample. DNA samples with qualified concentration (>10 μg) were sent to Berry Genomics Co., Ltd. (Beijing, China) for further detecting. From the genomic DNA, an Illumina TruSeq library with an insert size of 480 bp was generated. The *de novo*

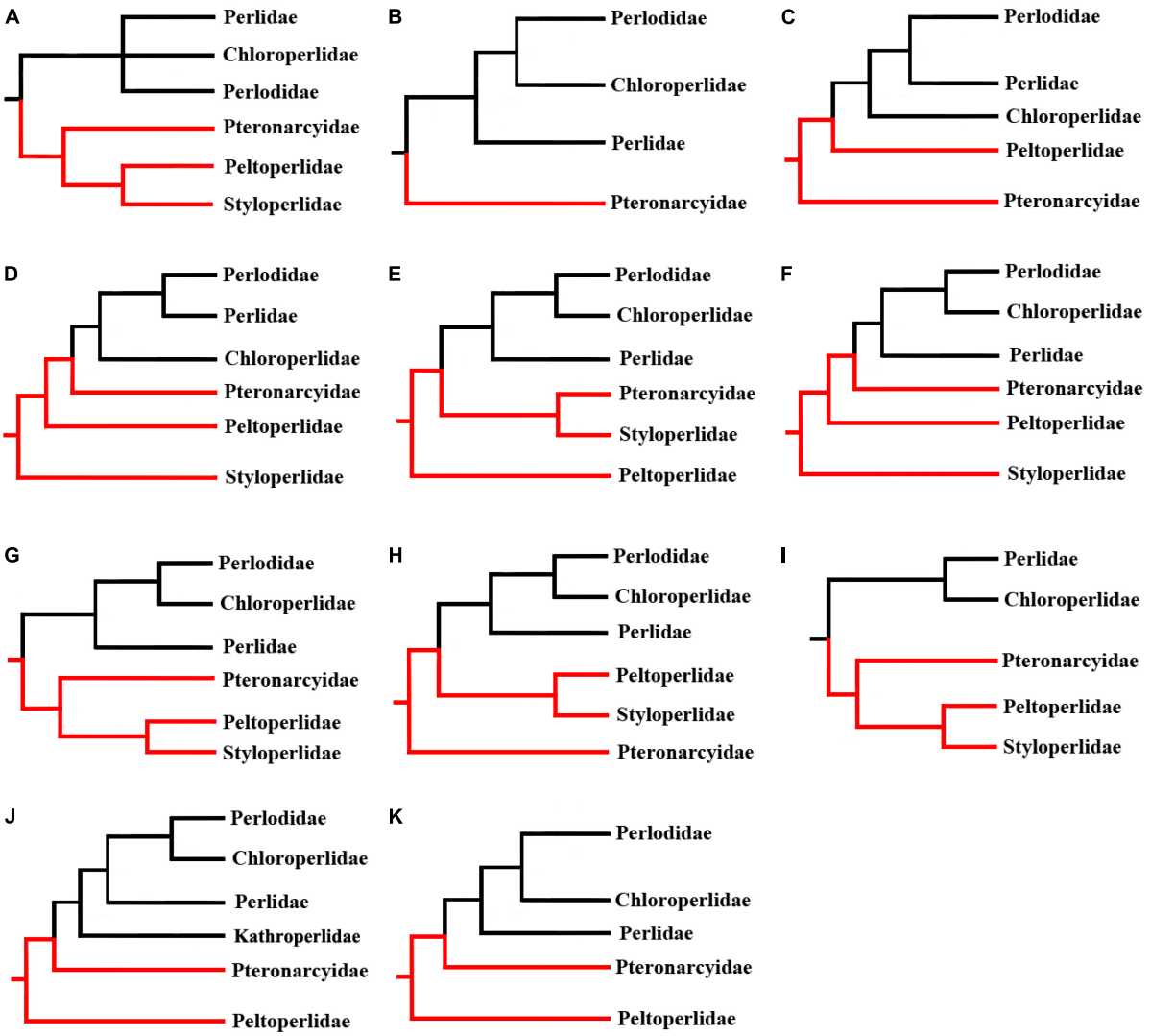

FIGURE 1
Representations of previous hypotheses for the family-level relationships within Systellognatha. **(A)** Zwick (2000), morphological data; **(B)** Davis (2013), transcriptome data; **(C)** Thomas et al. (2000), 18S gene; **(D)** Terry and Whiting (2003), six molecular markers; **(E)** Chen et al. (2018) and Mo et al. (2022), mitogenome data; **(F)** Shen and Du (2019) and Veale et al. (2019), mitogenome data; **(G)** Shen and Du (2020), mitogenome data; **(H)** Wang et al. (2019), mitogenome data; **(I)** Wang et al. (2018) and Ding et al. (2019), mitogenome data; **(J)** South et al. (2021a), transcriptome data; **(K)** South et al. (2021b), transcriptome data (concatenated complete nucleotide data set). Clades with black and red color belong to the superfamily Perloidea and Pteronarcyoidea, respectively.

genome sequencing was conducted on an Illumina Hiseq 2500 platform with 500 cycles of paired-end sequencing (250 bp reads).

Sequence assembly, annotation, and analyses

Trimmomatic v0.30 (Lohse et al., 2012) was used for *de novo* assembly of high-quality data. A total of 6 Gb clean data were obtained and used in the *de novo* assembly using IDBA-UD (Peng et al., 2012) with minimum and maximum *k* values of 45 and 145 bp. *COI* and *srRNA* fragments were amplified as bait sequences using PCR (Li et al., 2012). Then, the mitogenome sequence was searched with the bait sequences using BLAST with at least 98% similarity. Two newly sequenced mitogenomes have been deposited in GenBank (**Table 1**). MITOS web server was used to identify transfer ribonucleic acid genes (tRNAs) (Bernt et al., 2013). Protein-coding genes (PCGs) and ribosomal RNA genes (rRNA) were identified by alignment with homologous genes of previously sequenced stonefly mitogenomes. The graphical maps of two mitogenomes were depicted with CGView Server (Grant and Stothard, 2008). Nucleotide composition and codon usage

TABLE 1 List of taxa used in this research.

Superfamily	Family	Species	Number (bp)	Accession number
Perloidea	Perlidae	*Acroneuria hainana*	15,804	NC_026104
		Acroneuria carolinensis	15,718	MN969989
		Caroperla siveci	15,353	MG677942
		Calineuria stigamata	15,070	MG677941*
		Flavoperla hatakeyamae	15,730	MN821010
		Flavoperla sp.	15,796	MN419916
		Flavoperla biocellata	15,805	MK905206*
		Niponiella limbatella	15,924	MK686067
		Sinacroneuria dabieshana	15,752	MK492253
		Claassenia sp.	15,774	MN419914
		Dinocras cephalotes	15,666	NC_022843
		Kamimuria chungnanshana	15,943	NC_028076
		Kamimuria klapaleki	16,077	MN400755
		Kamimuria wangi	16,179	NC_024033
		Paragnetina indentata	15,885	MN627431
		Neoperla sp.	15,667	KX091859*
		Togoperla limbata	15,915	MN969990
		Togoperla sp.	15,723	KM409708
	Perlodidae	*Isoperla bilineata*	15,048	MF716959
		Isoperla eximia	16,034	MG910457
		Perlodes sp.	16,039	MF197377
		Pseudomegarcys japonica	16,067	MG910458
	Chloroperlidae	*Haploperla japonica*	16,012	OL351265
		Sweltsa sp.	15,893	OL351266
		Suwallia errata	16,146	MF198253
		Suwallia bimaculata	16,125	MN121757
Pteronarcyoidea	Peltoperlidae	*Cryptoperla stilifera*	15,633	KC952026*
		Peltoperlopsis cebuano	15,790	MK387068
		Soliperla sp.	15,877	MF716958
		Microperla geei	15,216	MN096323
		Cryptoperla kawasawai	15,832	ON854136
		Peltoperlopsis sagittata	15,756	ON854137
	Pteronarcyidae	*Pteronarcys princeps*	16,004	NC_006133
		Pteronarcella badia	15,585	NC_029248
	Styloperlidae	*Styloperla* sp.	15,416	KR088971*
		Styloperla spinicercia	16,129	KX845569
		Cerconychia flectospina	15,188	MF100783*
Nemouroidea (Outgroup)	Leuctridae	*Paraleuctra cercia*	15,625	MK492251
		Perlomyia isobeae	15,795	MK492252

*Incomplete mitochondrial genome sequence.

was obtained using MEGA 6.0 (Tamura et al., 2013). The codon usage was visualized by a heatmap using the online tool CIMminer.[1] AT and GC skew were calculated *via* the following formula: AT-skew = (A − T)/(A + T), GC-skew = (G − C)/(G + C) (Perna and Kocher, 1995). The rates of Ka (the non-synonymous substitution rate) and *Ks* (the synonymous substitution rate) for each PCG were determined with DnaSP 5.0 (Librado and Rozas, 2009). The tandem repeats in the control region were predicted using the Tandem Repeat Finder server (Benson, 1999).

Phylogenetic analyses

To analyze the phylogenetic relationships among Systellognatha, thirty-seven systellognathan mitogenomes were involved in our phylogenetic analysis. Two leuctrid

1 http://discover.nci.nih.gov/cimminer/home.do

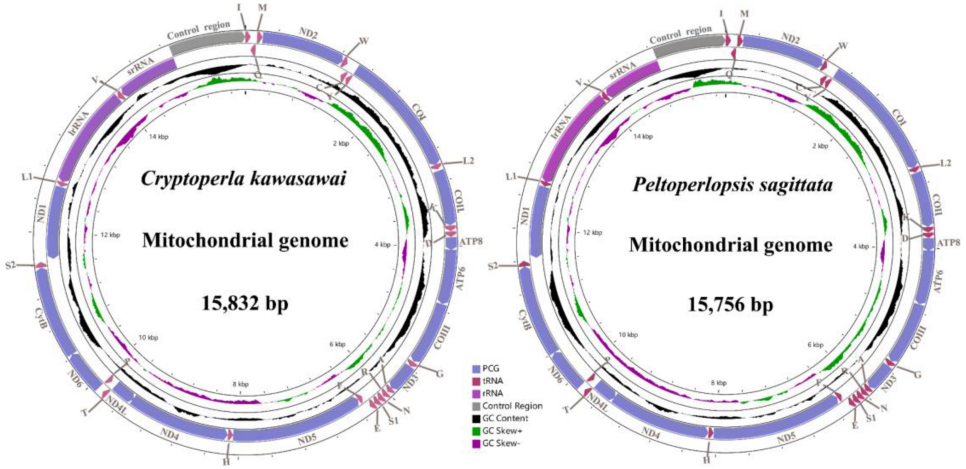

FIGURE 2
Mitochondrial maps of *Cryptoperla kawasawai* and *Peltoperlopsis sagittata*. The direction of gene transcription is indicated by the arrows. tRNA genes are labeled according to single-letter International Union of Pure and Applied Chemistry-International Union of Biochemistry (IUPAC-IUB) abbreviations (L1, UUR; L2, CUN; S1, AGN; S2, UCN).

mitogenomes (*Paraleuctra cercia* and *Perlomyia isobeae*) from infraorder Euholognatha were used as outgroups (**Table 1**). Two datasets were assembled for phylogenetic analyses: (1) the "PCG matrix" (10,971 bp), including 13 PCGs; (2) the "13 PCGs and two rRNAs (PCGRNA) matrix" (12,750 bp), including 13 PCGs and two rRNAs. PCGs were aligned independently using the MAFFT algorithm within the TranslatorX online platform (Abascal et al., 2010). Before the protein alignment was back-translated to nucleotides, GBlocks (in TranslatorX) with default settings were used to remove ambiguously aligned areas. The G–INS–I alignment strategy in MAFFT 7.0 online was used for rRNA alignment (Katoh and Standley, 2013), and ambiguously aligned sites masked with Gblocks (Castresana, 2000).

Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference (BI). The best-fit model for each dataset was determined using ModelFinder applying the Akaike Information Criterion (AIC) (Nguyen et al., 2015). ML analyses were inferred using IQ–TREE (Nguyen et al., 2015), and an ultrafast bootstrap approximation with 1,000 replicates. Bayesian analyses were carried out using MrBayes v3.2.6 (Ronquist et al., 2012) with the selected model (GTR + I + G). For MrBayes, runs were as follows: 10 million generations with four chains, sampling every 100 generations, and the first 25% discarded as burn-in.

Results and discussion

General features of mitogenomes

We successfully obtained two complete mitogenomes of the family Peltoperlidae and submitted the sequences to GenBank (accession numbers: ON854136-ON854137). The genome sizes were 15,832 bp (*C. kawasawai*) and 15,756 bp (*P. sagittata*), respectively (**Supplementary Tables 1, 2** and **Figure 2**). The gene order and content of two mitogenomes were typical of Plecoptera (Chen et al., 2018; Cao et al., 2019; Wang et al., 2019) and highly conserved, including 37 genes (22 tRNAs, 13 PCGs, and two rRNAs) and a non-coding control region (CR) (**Figure 2**). Compared with other systellognathan species, the sizes of two newly sequenced peltoperlid mitogenomes were close to average (15,832 bp, only counting the complete mitogenomes) (**Table 1**). In the peltoperlid mitogenomes, variation in the length of PCGs, tRNAs, *lrRNA* (large subunit ribosome gene), and *srRNA* (small subunit ribosome gene) was inconspicuous. But the length variation was very different in the control region (**Table 2**), which differed in both the replicates and length of various short repeat sequences within it (Dotson and Beard, 2001).

Gene overlaps and spacers were presented in several conserved positions in the peltoperlid mitogenomes, such as *trnI-trnQ* (3 bp), *trnW-trnC* (−8 bp), *COI-trnL2* (−5 bp), *ATP8-ATP6* (−7 bp), *ND4-ND4L* (−7 bp), *ND4L-trnT* (2 bp), etc (**Supplementary Table 3**). In *Drosophila melanogaster*, two conserved non-coding intergenic regions (*trnE-trnF* and *trnS2-ND1*) have been considered to be bidirectional transcription termination factor (DmTTF) binding sites (Beckenbach, 2012). In this study, we aligned the sequences of these two regions in six peltoperlid species and *D. melanogaster* (**Supplementary Figure 1**). Like other stonefly and insect mitogenomes (Beckenbach and Stewart, 2009; Beckenbach, 2012; Wang et al., 2018; Cao et al., 2019), the DmTTF binding site of *trnE-trnF* was absent in six peltoperlid mitogenomes (**Supplementary Figure 1**). However, the DmTTF binding site of *trnS2-ND1* (the longest spacer sequence except for the control region) was found in the six species and is highly conserved across

TABLE 2 Structural features of the mitochondrial genomes across six species of family Peltoperlidae.

Region	Feature	CK	CS	MG	PC	PS	SS
Whole mitogenomes	Size (bp)	15,832	15,633	15,216	15,790	15,756	15,877
	A + T%	68.5	69.3	68.3	69.4	68.3	69.8
	AT-skew	0.082	0.079	0.082	0.049	0.094	0.064
	GC-skew	−0.282	−0.264	−0.230	−0.286	−0.327	−0.255
PCGs	Size (bp)	11,229	11,208	11,226	11,229	11,229	11,232
	A + T%	66.8	67.6	68.1	67.8	66.3	68.0
	AT-skew	−0.161	−0.160	−0.171	−0.177	−0.164	−0.161
	GC-skew	−0.048	−0.018	−0.021	−0.026	−0.060	−0.033
PCGs-J	Size (bp)	6,906	6,897	6,906	6,903	6,906	6,909
	A + T%	64.7	65.9	66.5	65.8	64.7	66.2
	AT-skew	−0.068	−0.068	−0.077	−0.109	−0.068	−0.091
	GC-skew	−0.258	−0.219	−0.196	−0.240	−0.258	−0.223
PCGs-N	Size (bp)	4,323	4,311	4,320	4,326	4,323	4,323
	A + T%	70.1	70.4	70.6	70.9	70.1	70.7
	AT-skew	−0.299	−0.298	−0.311	−0.275	−0.299	−0.267
	GC-skew	0.348	0.351	0.298	0.375	0.348	0.315
tRNAs	Size (bp)	1,480	1,479	1,467	1,484	1,482	1,477
	A + T%	70.0	70.9	69.7	70.5	71.9	70.5
	AT-skew	−0.012	−0.034	0.003	0.000	0.002	0.004
	GC-skew	0.185	0.167	0.131	0.142	0.154	0.125
tRNAs-J	Size (bp)	941	945	935	939	941	937
	A + T%	71.3	72.4	70.7	70.9	71.3	71.4
	AT-skew	0.028	0.033	0.041	0.032	0.028	0.022
	GC-skew	0.081	0.025	0.000	0.010	0.081	0.022
tRNAs-N	Size (bp)	539	537	532	545	539	540
	A + T%	67.7	69.5	68.0	69.7	67.7	69.1
	AT-skew	−0.085	−0.099	−0.066	−0.059	−0.085	−0.029
	GC-skew	0.345	0.366	0.341	0.360	0.345	0.293
rRNAs	Size (bp)	2,143	2,117	2,118	2,143	2,143	2,140
	A + T%	71.9	72.8	72.6	72.0	71.9	72.6
	AT-skew	−0.121	−0.135	−0.142	−0.089	−0.121	−0.127
	GC-skew	0.372	0.360	0.321	0.393	0.372	0.328
CR	Size (bp)	938	777	>393	914	901	1,013
	A + T%	78.5	80.2	–	81.1	77.9	81.6
	AT-skew	0.084	0.025	–	0.018	0.103	0.100
	GC-skew	−0.188	−0.172	–	−0.237	−0.327	−0.311

PCGs-J, PCGs encoded by the majority strand; PCGs-N, PCGs encoded by the minority strand; CR, control region; CK, *Cryptoperla kawasawai*; CS, *C. stilifera*; MG, *Microperla geei*; PC, *Peltoperlopsis cebuano*; PS, *P. sagittata*; SS, *Soliperla* sp.

insects (Cameron and Whiting, 2008; Beckenbach, 2012; Wang et al., 2018; Cao et al., 2019). In addition, the 7 bp gene pairs (*ATP8*/*ATP6* and *ND4*/*ND4L*) are often been found across the Metazoa (Stewart and Beckenbach, 2005; Carapelli et al., 2006), and thought to be translated as a bicstron (Stewart and Beckenbach, 2005).

Nucleotide composition

The nucleotide compositional behavior of mitogenomes can be analyzed by A + T content, AT skew, and GC skew (Hassanin et al., 2005; Wei et al., 2010). The nucleotide composition of the *C. kawasawai* mitogenome (A = 37.1%, T = 31.4%, G = 11.3%, C = 20.2%) was similar to that of *P. sagittata* (A = 37.4%, T = 30.9%, G = 10.6%, C = 21.0%). Similar to other published stoneflies, the nucleotide compositions of the six peltoperlid species revealed a strong A and T base bias in all six mitogenomes (Chen et al., 2018; Wang et al., 2018, 2019; Ding et al., 2019; Shen and Du, 2019, 2020; Mo et al., 2022). By comparison, the control region of all six peltoperlid mitogenomes showed a higher A + T content than other major partitions (e.g., PCGs, tRNAs, rRNAs, etc.) (**Table 2**).

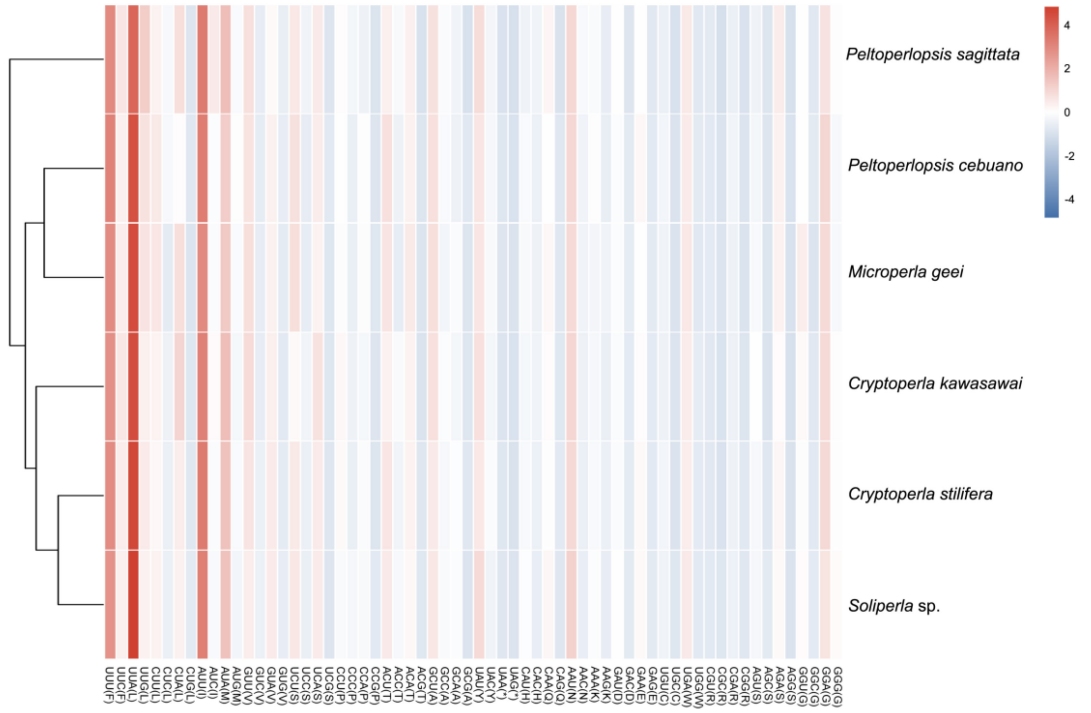

FIGURE 3
Heatmap of codon usage for protein-coding genes in Peltoperlidae. Red and blue in the heatmap indicate high and low absolute correlation, respectively. The species in the rows of the heat map are sorted by the corresponding cluster tree based on codon usage. The columns indicate 64 codons of the invertebrate mitochondrion.

The nucleotide compositions were all strongly skewed away from T in favor of A (the AT-skews were from 0.049 to 0.094) and from G in favor of C (the GC-skews were from −0.230 to −0.327) (**Table 2**). In most metazoan mitogenomes, the strand skew biases are found to be weakly positive AT-skew and strongly negative GC-skew for the J-strand (Hassanin et al., 2005). Our results show that all the peltoperlid PCGs have a negative AT-skew and negative GC-skew for the J-strand, while all the peltoperlid tRNAs have a positive AT-skew and positive GC-skew for the J-strand (**Table 2**). This pattern is inconsistent with most insect mitogenomes, but it is also found in many stonefly mitogenomes (Chen et al., 2018; Wang et al., 2018, 2019; Cao et al., 2019). This phenomenon may result from the gene direction, replication, and codon positions (Lindahl, 1993; Wei et al., 2010).

Protein-coding genes

We detected 13 protein-coding genes in two newly sequenced mitogenomes. Similar to other peltoperlid mitogenomes, nine PCGs were encoded on the majority strand (J-strand), and the remaining four PCGs were encoded on the minority strand (N-strand) (**Figure 2**). Six out of thirteen PCGs (*COI*, *ND1*, *ND2*, *ND4*, *ND5*, and *ND6*) differed in size among the six peltoperlid species (**Supplementary Table 4**). But in general, the length variation in those genes was limited.

Most PCGs in six peltoperlid species initiated with a typical ATN codon, while *ND2* in *Peltoperlopsis cebuano* initiated with GTG, *ND5* in five peltoperlids (one exception: *Soliperla* sp.) started with GTG, and *ND1* in all peltoperlids initiated with TTG (**Supplementary Table 4**). Most PCGs terminated with the canonical TAA/TAG stop codon in six peltoperlid species. The incomplete stop codon T was found

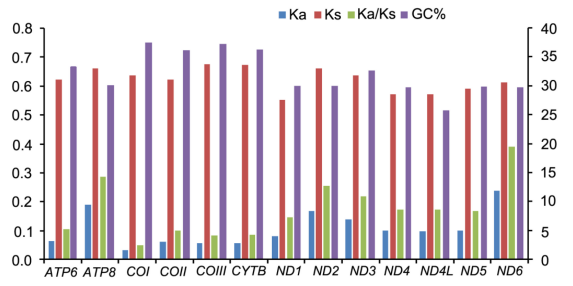

FIGURE 4
Evolutionary rates of 13 protein-coding genes in the mitochondrial genomes of six peltoperlid species. The left Y-axis provides the substitution rate of the mitochondrial gene, while the right Y-axis provides the G + C content.

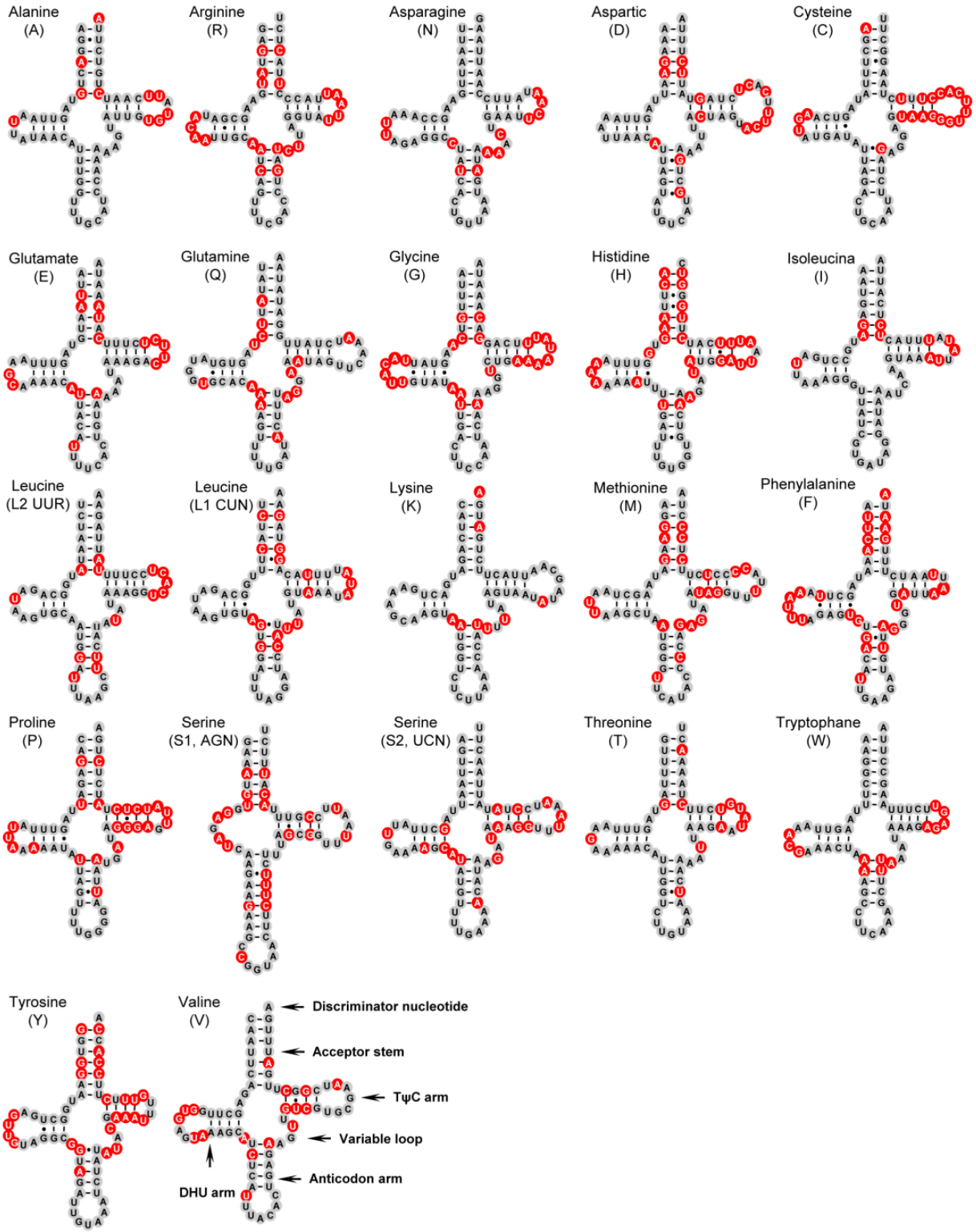

FIGURE 5
Putative secondary structures of tRNAs found in the *Cryptoperla kawasawai* mitogenome. Completely conserved sites within the six peltoperlid species were marked by black nucleotides within gray spheres. Inferred Watson–Crick bonds are illustrated by lines, whereas guanine–uracil (GU) bonds are illustrated by dots.

in *COII* and *ND5* genes in most of the six peltoperlid mitogenomes (**Supplementary Table 4**). These incomplete codons may be the product of the selective pressure to economize the mitogenome size and are presumed to be completed *via* post-transcriptional polyadenylation (Ojala et al., 1981). In addition, each of the five genes (*ATP6*, *COII*, *COIII*, *ND4L*, and *ND6*) used the same start and stop codons, indicating these genes were highly conserved among all species (**Supplementary Table 4**).

In our study, a heatmap was used to visualize codon usage for the 13 PCGs available in the Peltoperlidae, with the color representing the frequency of codon usage (**Figure 3**). Within peltoperlids, similar but slightly different patterns were observed. Heatmap analysis showed that the AT-rich codons UUA, AUU, UUU, and AUA were commonly used codons in the Peltoperlidae. Similarly, the biased use of A + T nucleotides was reflected in the codon frequencies. The dendrogram based on codon usage showed a close relationship between *C. kawasawai* and the clade of *Soliperla* sp. plus *Cryptoperla stilifera*, and *P. sagittata* is the earliest branch within Peltoperlidae. The monophyly of *Cryptoperla* and *Peltoperlopsis* was not supported.

To better investigate the evolutionary patterns across the 13 PCGs in peltoperlid species, the values of *Ka* (rates of non-synonymous mutations), *Ks* (rates of synonymous mutations), and the ratio of *Ka*/*Ks* (ω) were calculated for each PCG, respectively (**Figure 4**). In all PCGs, *COIII* had the highest *Ks*, whereas *ND6* had the highest *Ka* and ω values. The ω values for 13 PCGs were far lower than 1 (<0.40), indicating the existence of purifying selection in these genes (Roques et al., 2006). Therefore, all mitochondrial PCGs could be used to analyze phylogenetic relationships within Peltoperlidae. In addition, we found a negative correlation between ω and the G + C content of each PCG in peltoperlid species ($y = -24.37x + 36.38$, $R^2 = 0.89$, $P < 0.05$). This result is similar to previous studies and indicates that G + C content may be one important element in determining the evolutionary patterns of PCGs (Li et al., 2012; Yuan et al., 2015; Zhang et al., 2016).

Transfer and ribosomal ribonucleic acids

All tRNA genes in six peltoperlid species showed classical cloverleaf structures except for *trnS1*, whose dihydrouridine (DHU) arm simply formed a loop (**Figure 5**). The lack of a DHU arm in *trnS1* was also found in sequenced stonefly mitogenomes, and this phenomenon has been considered a typical feature of metazoan mitochondrial DNA (Lavrov et al., 2004). Although it is not clear if the aberrant tRNAs lose function in this case, some studies proposed a post-transcriptional RNA editing mechanism to keep these tRNA genes functional (Tomita et al., 2001).

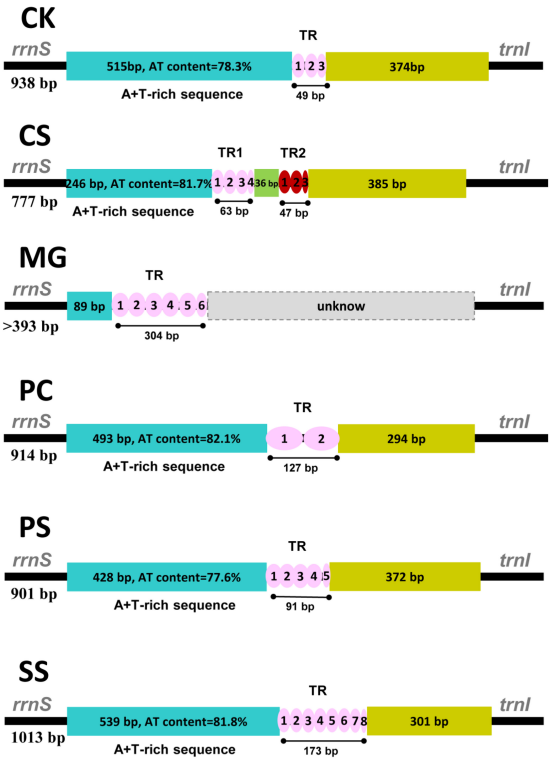

FIGURE 6
Control region structure of six peltoperlid species. TR, tandem repeat.

We calculated the percentage of identical nucleotides (%INUC) for each tRNA family of the six peltoperlid mitogenomes (**Supplementary Table 5**). The%INUC ranged from 54.2% in *trnH* to 88.7% in *trnK*, with an average of 72.8%. Eleven tRNAs displayed high levels of conservation (%INUC ≥ 75.0%). Nucleotides in the stems and loops of the tRNAs were relatively conserved (>70%). The most conserved site was the anticodon (AC) loop with an average of 96.1%, and the most variable region was the TψC loop (with an average of 33.2%). In addition, the conservation of each stem, with the exception of the AC loop, was always higher than that of its corresponding loop.

As in the inferred ancestral insect mitogenome pattern, the two rRNA genes were usually separated by a single *trnV* gene. The lengths of *lrRNA* in the two newly sequenced mitogenomes were 1,334 and 1,344 bp, and the lengths of *srRNA* were 809 and 796 bp, respectively (**Supplementary Tables 1, 2**). The multiple alignments of peltoperlid *lrRNAs* had 1382 positions and contained 837 conserved positions (60.6%), 518 nucleotide substitutions (37.5%), and 27 indels (1.9%), respectively. The multiple alignments of peltoperlid *srRNAs* possessed 822 positions and contained 491 conserved positions (59.7%), 319 nucleotide

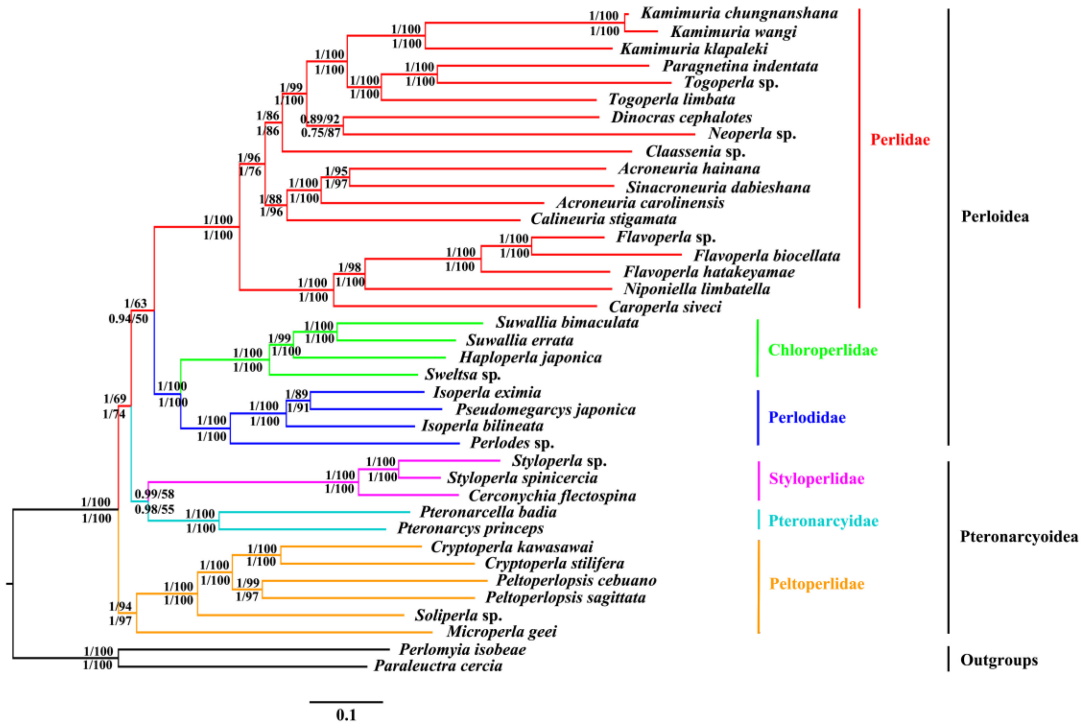

FIGURE 7
Phylogenetic tree of the thirty-nine sequenced stoneflies. Bayesian inference and Maximum Likelihood analysis inferred from Protein-coding gene (PCG) and 13 PCGs and two rRNAs (PCGRNA) datasets supported the same topological structure. Values at nodes are ML bootstrap values and Bayesian posterior probabilities using the PCG (up) and PCGRNA (down) datasets. The tree was rooted with two outgroups (*Paraleuctra cercia* and *Perlomyia isobeae*).

substitutions (38.8%), and 12 indels (1.5%), respectively (**Supplementary Figure 2**).

The control region

The control region is located between *srRNA* and *trnI*, including the origin of replication and promoters for transcription initiation (Zhang et al., 1995). A comparison of the control region sequences of six peltoperlid species revealed a few structural elements: (1) a leading sequence adjacent to *srRNA* with high AT content; (2) one or two tandem repeated sequence blocks consisting of repeat units; (3) the remainder of the control region (**Figure 6**).

Large tandem repeats with two or more copies were detected in all control region sequences examined here. The size and copy number of the repeat unit are different in six peltoperlids, and the size variation of the control region is largely caused by this discrepancy. Overall, the control region of the family exhibited a number of distinctive structural and evolutionary characteristics, such as variable size, conserved structural elements, and abundant tandem repetitions. These properties made this region an effective phylogenetic marker for evolutionary and population genetic studies.

Molecular phylogeny

Two datasets (PCG and PCGRNA) were used in the present analyses. The phylogenetic trees generated from BI and ML inferences had identical topologies based on different datasets (**Figure 7**). Our results showed support values were higher in the BI tree than in the ML tree using the same dataset. All phylogenetic analyses supported the monophyly of each family, although some nodes have lower bootstrap values (BP).

The monophyly of two superfamilies, namely, Perloidea and Pteronarcyoidea are widely accepted and supported by morphological data (Zwick, 2000). However, this has never been well–supported by molecular evidence, especially the monophyly of Pteronarcyoidea (Thomas et al., 2000; Terry and Whiting, 2003; Davis, 2013; Chen et al., 2018; Ding et al., 2019; Wang et al., 2019; Cao et al., 2021; South et al., 2021a,b; Mo et al., 2022). In the current study, the monophyly of the superfamily Perloidea was recovered. The relationships of three families in Perloidea were recovered as: [Perlidae + (Chloroperlidae + Perlodidae)]. Our results are consistent with previous morphological studies (Illies, 1965), transcriptome (Davis, 2013; South et al., 2021a), and mitogenome analyses (Wang et al., 2019; Cao et al., 2021; Mo et al., 2022). In Zwick's (2000) study, the monophyly of Perloidea

was supported based on morphological characteristics, but the relationships within Perloidea were not fully resolved. Our results confirm Chloroperlidae as a sister to Perlodidae [supported in all analyses, BP = 100, Bayesian posterior probability values (PP) = 1.0] and also provide an increasingly clearer view of relationships within Perloidea.

The phylogenetic position of Peltoperlidae has long been under debate. Based on synapomorphic reduction of gills and abdominal ganglia, Zwick (1973) placed Peltoperlidae as sister to Perloidea. Stark and Stewart (1981) suggested Peltoperlidae to be more closely related to Pteronarcyidae than Perloidea based on some characters, such as the synapomorphies of present tridentate lacinia and an apical spine-like process of the tenth tergite in the nymphs and flattened egg shape. Uchida and Isobe (1989) elevated the subfamily Styloperlinae of the Peltoperlidae to familial rank as Styloperlidae and placed Peltoperlidae as sister group to Styloperlidae. In this study, a sister group relationship between Peltoperlidae and remaining Systellognatha was highly supported by all analyses (**Figure 7**), which was consistent with the hypotheses of Ricker (1952), Terry and Whiting (2003), and South et al. (2021a,b).

Unfortunately, the monophyly of Pteronarcyoidea was still not supported in this study. The Peltoperlidae was placed as the earliest branch within the Systellognatha, and all analyses generated the same relationships within Pteronarcyoidea [the relationship is Peltoperlidae + ((Styloperlidae + Pteronarcyidae) + Perloidea)]. These relationships are well-supported by BI analyses (PP $\geq$ 0.98). However, the posterior probabilities on some nodes are very low (PP = 0.37–0.52), and relationships within Pteronarcyoidea are still not exactly solved. Although this result differs from the generally accepted hypothesis that the Pteronarcyoidea are monophyletic (Zwick, 2000), no molecular study has proposed this relationship until now. Our results provide new insight into the phylogeny of this group, and analyses with more systellognathan taxa in future studies are needed to test the conclusion from the present study.

Conclusion

In this study, two complete mitogenomes from the family Peltoperlidae (*C. kawasawai* and *P. sagittata*) were sequenced. We present the comparative analysis of six peltoperlid mitogenomes and our results show that gene content, gene order, DmTTF binding site, nucleotide composition, codon usage, RNA structure, and structural elements in the control region are highly conserved in peltoperlids. Phylogenetic relationships within Systellognatha support the monophyly of Perloidea, but the monophyly of Pteronarcyoidea is still not supported. Sequencing more mitogenomes representing various taxonomic levels will greatly improve our understanding of phylogenetic relationships in Systellognatha.

Data availability statement

The datasets presented in this study can be found in online repositories and the study is deposited in the NCBI repository with accession numbers: ON854136 and ON854137.

Author contributions

YW, JC, and WL conceived and designed the study and critically revised the manuscript. YW, JC, XG, and CG performed the experiments. YW and JC analyzed the data. YW and WL drafted the manuscript. WL and DM helped in the study design. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.979847/full#supplementary-material

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