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Two complete mitochondrial genomes of the subfamily Chloroperlinae (Plecoptera: Chloroperlidae) and their phylogenetic implications

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Received 18 November 2021
Accepted 4 March 2022
Published 20 May 2022

Academic Editors Christiane Weirauch, Klaus-Dieter Klass

Citation: Mo R-R, Wang Y, Cao J-J, Wang G-Q, Li W-H, Murányi D (2022) Two complete mitochondrial genomes of the subfamily Chloroperlinae (Plecoptera: Chloroperlidae) and their phylogenetic implications. Arthropod Systematics & Phylogeny 80: 155–168. https://doi.org/10.3897/asp.80.e78173

Abstract

Two new complete mitochondrial genomes (mitogenomes) of the subfamily Chloroperlinae, *Haploperla japonica* Kohno, 1946 and *Sweltsa* sp., were sequenced. The two species showed similar gene order, nucleotide composition, and codon usage. The *Sweltsa* sp. and *H. japonica* mitogenomes were typical circular DNA molecules, with lengths of 15,893 bp and 16,012 bp, respectively. Standard ATN start and TAN stop codons were present in most PCGs. All tRNA genes exhibited the cloverleaf secondary structure typical for metazoans except the $tRNA^{Ser(AGN)}$, which lacked the dihydrouridine arm. In both species, the secondary structure of *lrRNA* contained five structural domains, while the *srRNA* included three domains. The A+T-rich regions contained different repeat regions in each species. Phylogenetic analyses using Bayesian inference (BI) and maximum likelihood methods (ML) showed identical results. The family Chloroperlidae was sister to the Perlodidae. Our analyses inferred relationships between six of the seven Systellognatha families: (((Chloroperlidae + Perlodidae) + Perlidae) + (Styloperlidae + Pteronarcyidae)) + Peltoperlidae.

Keywords

Chloroperlinae, *Haploperla*, mitochondrial genomes, phylogenetics, Plecoptera, *Sweltsa*

1. Introduction

The mitochondrial genome (mitogenome) of metazoans is 14 to 20 kb in size, and usually a circular, double-stranded molecule. It contains 13 protein coding (PCGs), 2 ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) (altogether 37) genes, and an A+T-rich region which is known as the control (CR) or non-coding region (Wolstenholme 1992; Boore 1999; Gissi et al. 2008). The Plecoptera (stoneflies) constitutes an ancient order of hemimetabolous winged insects. Stoneflies include 308 existing genera in 17 families, including approximately 4,000 valid species worldwide (DeWalt et al. 2021). Chloroperlidae is one of the smaller families, with two subfamilies:

Chloroperlinae Okamoto, 1912 and Paraperlinae Ricker, 1943. In the Chloroperlinae, there are three tribes and 206 described species, while in the Paraperlinae, only two genera and six species are recorded (Alexander and Stewart 1999; Judson and Nelson 2012; Chen and Du 2015a; Li et al. 2015a, 2015b). In the present study, *Haploperla japonica* and *Sweltsa* sp. were analysed; both belong to Chloroperlinae. Zwick (2000) proposed a higher classification for the world fauna of Plecoptera, which is the current generally accepted system. However, the positions of four families (Chloroperlidae, Perlidae, Perlodidae, Notonemouridae) were still not clear (Zwick 2000). So far, the phylogenetic position of Plecoptera within the Neoptera, and phylogenetic relationships among some Plecoptera families have remained controversial, and more molecular data are needed to resolve them (Stewart and Stark 1988; Uchida and Isobe 1989; Terry and Whiting 2003; Chen et al. 2016, 2018; Wang et al. 2016, 2017a, 2019; Chen and Du 2017a, 2017b; Ding et al. 2019; Wipfler et al. 2019; South et al. 2020, 2021; Cao et al. 2021). In the superfamily Perloidea, the affinities of three families: Chloroperlidae, Perlidae and Perlodidae, have been proposed as a trichotomy (Nelson 1984; Zwick 2000), while Illies (1965) proposed Perlidae as sister to Chloroperlidae + Perlodidae. Most molecular data strongly supported the latter classification (Chen et al. 2016, 2018; Wang et al. 2016, 2017b, 2019; Chen and Du 2017b, 2017c; Ding et al. 2019; South et al. 2020, 2021; Cao et al. 2021).

At present, only three chloroperlid species, *Sweltsa longistyla* (Wu, 1938), *Suwallia bimaculata* (Okamoto, 1912) and *Suwallia errata* Li and Li, 2021, have been completely sequenced for mitogenomes (Chen and Du 2015b; Wang et al. 2018a; Cao et al. 2019a; Li et al. 2021). This limited mitogenomic data hinders the reconstruction of accurate phylogeny (Wu et al. 2014). In the present study, two complete mitogenomes from two genera, *Haploperla* and *Sweltsa*, were sequenced. Nucleotide composition, codon usage, RNA secondary structure, the evolutionary pattern among the PCGs, and structural elements within the control regions were analysed. Furthermore, a phylogenetic tree of the infraorder Systellognatha, excluding the family Kathroperlidae was also reconstructed based on the aligned PCGs using Bayesian inference (BI) and Maximum likelihood (ML) analyses.

2. Material and Methods

2.1. Specimen sample and DNA extraction

Adult specimens of *Haploperla japonica* were collected from Yoshino River, Minocho, Miyoshi City, Tokushima Prefecture, Japan, and adult samples of *Sweltsa* sp. were collected from a forested stream along the road No.328, Kasatori Mountain, Kumakogen, Ehime Prefecture, Japan. Species-level identification of *Sweltsa* species is unsure, due to taxonomic problems in Japanese representatives of the genus (Shimizu et al. 2005). All specimens were fixed in 100% ethanol and stored at –20°C. The examined specimens of *H. japonica* (No. VHem-0114) and *Sweltsa* sp. (No. VHem-0116) are deposited in the Entomological Museum of Henan Institute of Science and Technology (HIST), Henan Province, China. The full genomic DNA was isolated from thoracic muscle using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) and maintained at –20°C prior to PCR analysis.

2.2. Genome sequencing, assembly and annotation

The full mitogenomes of both Chloroperlidae species were amplified and sequenced as described in previous studies (Li et al. 2017; Wang et al. 2017a, 2017b; Liu et al. 2018) and uploaded in GenBank with the accession numbers: OL351265 (*Haploperla japonica*) and OL351266 (*Sweltsa* sp.) (Table 1). The program BioEdit version 7.0.5.3 was used to assemble raw sequences into contigs (Hall 1999). tRNA genes were identified with the software ARWEN using default setting (Laslett and Canbäck 2008). PCG and two rRNA genes were identified using BLAST searches in NCBI (http://www.ncbi.nlm.nih.gov), and confirmed by alignments of homologous genes from previously published stonefly mitogenomes. MEGA 5.0 was used to calculate nucleotide composition and codon usage for PCGs (Tamura et al. 2011). Strand asymmetry was measured by the following formulas: AT skew= [A−T]/ [A+T] and GC skew= [G−C]/ [G+C] (Perna and Kocher 1995). Tandem repeats in the A+T-rich region were identified using Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.advanced.submit.html).

2.3. Phylogenetic analysis

We used forty previously published and the two newly sequenced mitogenomes for phylogenetic analyses (Table 1). Two species from the family Nemouridae, *Amphinemura yao* Mo, Yang, Wang and Li, 2017 and *A. longispina* (Okamoto, 1922), were used as outgroup taxa (Table 1). Each PCG was aligned separately using the MAFFT algorithm implemented the TranslatorX online platform (Abascal et al. 2010). Poorly aligned regions were masked from the protein alignment before back-translation to nucleotides using GBlocks (implemented in the TranslatorX). Each rRNA gene was aligned separately by MAFFT v7.0 using G-INS-I strategy (Katoh and Standley 2013), and ambiguously aligned positions masked by GBlocks v0.91b (Castresana 2000).

We assembled the "PCG13 matrix" (11,049 bp in total) for the phylogenetic analyses, including 13 PCGs. GTR+I+G was the best-fit model for the nucleotide sequence alignments (jModelTest 0.1.1, Posada 2008). Bayesian analyses were conducted on the PCG13 dataset partitioned by gene was conducted Bayesian inference (BI), using MrBayes 3.2.6 and maximum likelihood (ML) using RAxML-HPC2 8.1.11 (Stamatakis 2006; Ronquist

Table 1. Classification of all Plecoptera species with sequenced mitogenomes, their genome lengths and accession numbers at Genbank.

Infraorder	Superfamily	Family	Subfamily	Species	Number (bp)	Accession Number
Systellognatha	Perloidea	Chloroperlidae	Chloroperlinae	*Haploperla japonica*	16,012	OL351265
				Sweltsa sp.	15,893	OL351266
				Sweltsa longistyla	16,151	KM216826
				Suwallia errata	16,146	MF198253
				Suwallia bimaculata	16,125	MN121757
		Perlidae	Acroneuriinae	*Acroneuria hainana*	15,804	NC_026104
				Acroneuria carolinensis	15,718	MN969989
				Caroperla siveci	15,353	MG677942
				Calineuria stigmatica	15,070	MG677941*
				Flavoperla hatakeyamae	15,730	MN821010
				Flavoperla sp. YW-2019	15,796	MN419916
				Flavoperla sp. YZD-2020	15,805	MK905206*
				Niponiella limbatella	15,924	MK686067
				Perlesta teaysia	16,023	MN627432
				Sinacroneuria dabieshana	15,752	MK492253
			Perlinae	*Claassenia sp.*	15,774	MN419914
				Dinocras cephalotes	15,666	NC_022843
				Etrocorema hochii	15,854	MK905888
				Kamimuria chungnanshana	15,943	NC_028076
				Kamimuria klapaleki	16,077	MN400755
				Kamimuria wangi	16,179	NC_024033
				Neoperlops gressitti	15,699	MN400756
				Paragnetina indentata	15,885	MN627431
				Neoperla sp.	15,667	KX091859*
				Neoperla ignacsiveci	15,777	KX091858
				Togoperla limbata	15,915	MN969990
				Togoperla sp.	15,723	KM409708
		Perlodidae	Isoperlinae	*Isoperla bilineata*	15,048	MF716959
				Isoperla eximia	16,034	MG910457
			Perlodinae	*Perlodes* sp.	16,039	MF197377
				Pseudomegarcys japonica	16,067	MG910458
	Pteronarcyoidea	Peltoperlidae	Peltoperlinae	*Cryptoperla stilifera*	15,633	KC952026*
				Peltoperlopsis cebuano	15,790	MK387068
				Soliperla sp.	15,877	MF716958
			Microperlinae	*Microperla geei*	15,216	MN096323
		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*
Euholognatha	Nemouroidea (Outgroup)	Nemouridae	Amphinemurinae	*Amphinemura yao*	15,876	MH085447
				Amphinemura longispina	15,709	MH085446
*Incomplete genome sequence						

et al. 2012). For the BI analyses, two simultaneous runs of 10 million generations were conducted, sampling every 1,000 generations and a burn-in rate of 25%. Stationarity was assessed using Tracer v.1.5 (Estimated sample size > 200). Bootstrap ML analyses of 1,000 replicates was performed with the fast ML method conducted in RAxML using the GTRGAMMA model for nucleotide sequences.

3. Results and Discussion

3.1. General characters of mitogenomes and the base composition

In this study, the full mitogenomes of two stonefly species in the subfamily Chloroperlinae: *Haploperla japonica* and *Sweltsa* sp., were studied for the first time (Table 2, Fig. 1). The new complete mitogenomes were 15,893 (*Swelt-*

Table 2. The size of nucleotide composition of the *Haploperla japonica* and *Sweltsa* sp. mitogenomes.

Family	Subfamily	Species	Genome	PCGs	tRNAs	lrRNA	srRNA	Control region
Chloroperlidae	Chloroperlinae	*Haploperla japonica*	16012	11247	1483	1324	776	1118
		Sweltsa sp.	15893	11244	1488	1325	799	1011

Table 3. Mitogenome structures of *Haploperla japonica* and *Sweltsa* sp.

Gene	*Haploperla japonica*				*Sweltsa* sp.		
	Direction	Location	Size	IGN	Location	Size	IGN
$tRNA^{Ile}$	F	1–67	67		1–67	67	
$tRNA^{Gln}$	R	65–133	69	–3	65–133	69	–3
$tRNA^{Met}$	F	139–207	69	5	136–204	69	2
ND2	F	208–1242	1035	0	205–1239	1035	0
$tRNA^{Trp}$	F	1241–1308	68	–2	1238–1305	68	–2
$tRNA^{Cys}$	R	1301–1367	67	–8	1298–1365	68	–8
$tRNA^{Tyr}$	R	1368–1433	66	0	1366–1433	68	0
COI	F	1426–2982	1557	–8	1426–2982	1557	–8
$tRNA^{Leu(UUR)}$	F	3003–3068	66	20	2995–3060	66	12
COII	F	3100–3783	688	31	3073–3760	688	12
$tRNA^{Lys}$	F	3788–3858	71	0	3761–3831	71	0
$tRNA^{Asp}$	F	3858–3926	69	–1	3831–3899	69	–1
ATP8	F	3927–4088	162	0	3900–4058	159	0
ATP6	F	4082–4759	678	–7	4052–4729	678	–7
COIII	F	4759–5547	789	–1	4729–5517	789	–1
$tRNA^{Gly}$	F	5550–5615	66	2	5517–5582	66	2
ND3	F	5616–5969	354	0	5583–5936	354	0
$tRNA^{Ala}$	F	5968–6033	66	–2	5935–6000	66	–2
$tRNA^{Arg}$	F	6034–6099	66	0	6001–6066	66	0
$tRNA^{Asn}$	F	6103–6168	66	3	6071–6136	66	4
$tRNA^{Ser(AGN)}$	F	6169–6235	67	0	6137–6203	67	0
$tRNA^{Glu}$	F	6236–6301	66	0	6204–6269	66	0
$tRNA^{Phe}$	R	6316–6380	65	14	6275–6339	65	5
ND5	R	6381–8115	1735	0	6340–8074	1735	0
$tRNA^{His}$	R	8116–8182	67	0	8075–8142	68	0
ND4	R	8187–9527	1341	4	8148–9488	1341	5
ND4L	R	9521–9817	297	–7	9482–9778	297	–7
$tRNA^{Thr}$	F	9820–9886	67	2	9781–9848	68	2
$tRNA^{Pro}$	R	9888–9955	68	1	9850–9917	68	1
ND6	F	9957–10481	525	1	9919–10443	525	1
CytB	F	10481–11617	1137	–1	10443–11579	1137	–1
$tRNA^{Ser(UCN)}$	F	11616–11685	70	–2	11579–11648	70	–1
ND1	R	11706–12656	951	20	11670–12620	951	21
$tRNA^{Leu(CUN)}$	R	12658–12723	66	1	12622–12687	66	1
lrRNA	R	12724–14047	1324	0	12688–14012	1325	0
$tRNA^{Val}$	R	14048–14118	71	0	14013–14083	71	0
srRNA	R	14119–14894	776	0	14084–14882	799	0
A+T-rich region		14895–16012	1118	0	14883–15893	1011	0

sa sp.) and 16,012 bp (*H. japonica*) in length (Fig. 1, Table 2). Genome sizes of these mitogenomes were in the middle of the range compared to mitogenomes of other stoneflies, that range from 15,048 bp (*Isoperla bilineata*) (Chen et al. 2018) to 16,602 bp (*Nemoura nankinensis*) (Chen and Du 2017a). Control region variation is the primary factor influencing size variation in Plecoptera mitogenomes (Table 2). The typical gene content found in most insects were found in these species: 37 mitochondrial genes (13PCGs, 22tRNAs and 2 rRNAs) and a large control region (Fig. 1). Twenty-three genes (9 PCGs and 14 tRNAs) were transcribed on the majority strand (J-strand), and the remaining fourteen genes (4 PCGs, 8 tRNAs, and 2 rRNAs) were encoded on the minority strand (N-strand) (Fig. 1).

Overlap between gene regions ranged from one to eight bp in length. Both species had seven bp overlaps

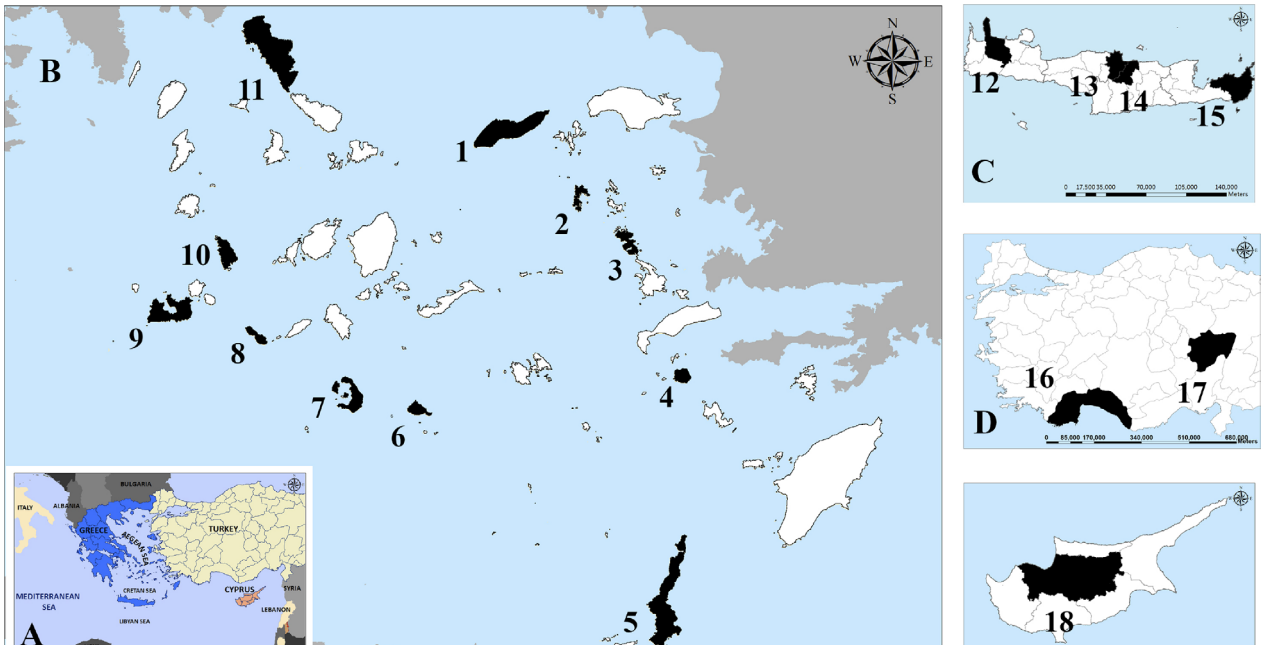

Figure 1. Map of the mitogenomes of *Haploperla japonica* and *Sweltsa* sp. Direction of gene transcription is indicated by the arrows. PCGs are shown as blue arrows, rRNA genes as purple arrows, tRNA genes as red arrows and CR as gray arrows. tRNA genes are labeled according to single-letter IUPAC-IUB abbreviations (L1: UUR, L2: CUN, S1: AGN, S2: UCN). The GC content is plotted using a black sliding window, as the deviation from the average GC content of the entire sequence. GC skew is plotted as the deviation from the average GC skew of the entire sequence.

in (*ATP6–ATP8*) and (*ND4–ND4L*), "ATGATAA" and "TTAACAT", respectively. This phenomenon was also found in the mitogenome of many insects (Yang et al. 2017; Cao et al. 2019a). An intergenic spacer sequence was found to be located between $tRNA^{Leu(UUR)}$ and *COII* in the two species, ranging from 12 bp in *Sweltsa* sp. to 31 bp in *Haploperla japonica* (Table 3).

3.2. Nucleotide composition

The A+T content in the *Haploperla japonica* and the *Sweltsa* sp. mitogenome is detailed in Table 4. The A+T content of *Sweltsa* sp. is similar to the base compositional biases reported for other chloroperlid species, that range from 66.7% (*Suwallia talalajensis* Zhiltzova, 1976) to 69.6% (*Sweltsa longistyla* (Wu, 1938)) (Chen and Du 2015b; Wang et al. 2018a), whereas A+T content in *H. japonica* is larger than other chloroperlids. The four major genome partitions (PCGs, tRNAs, rRNAs, and CR) and the whole mitogenome of both Chloroperlinae species were distinctly biased towards A and T. Among the four major partitions across Chloroperlinae species, PCGs consistently had the lowest A+T content, while the control region had the highest. A+T content of PCGs encoded on the minority strand (PCGs–N) was also higher than that of PCGs encoded on the majority strand (PCGs–J) (Table 4).

Both Chloroperlinae species showed positive AT-skew and negative GC-skew for the whole mitogenome. Total mitogenome of *Haploperla japonica*, the AT-skew and GC-skew were 0.065 and –0.223, respectively, whereas in *Sweltsa* sp., the AT-skew and GC-skew were 0.087 and –0.254, respectively. Slight T-skews and moderate C-skews were found in the majority strand PCGs while the reverse phenomenon was found for minority strand PCGs: both T-skews and G-skews were marked. For tRNAs and rRNAs, G-skews varied from slight to marked, while T-skews were slight to moderate. Modest A-skew and marked C-skew were found in the CR (Table 4).

For the majority strand PCGs, most known metazoan mitogenomes have positive AT-skew and negative GC-skew (Wei et al. 2010a). However, both Chloroperlinae species had negative AT-skews and negative GC-skews for the majority strand PCGs (Table 4). This difference has been noted for other published stonefly mitogenomes (Wu et al. 2014; Chen and Du 2017a, 2017c; Wang et al. 2017a, 2017b), as well as in other insects such as Philopteridae (bird lice), Aleyrodidae (whiteflies) and Braconidae (wasps); this phenomenon may be a result from replication direction charges (Wei et al. 2010a). For both Chloroperlinae species, the four major partitions (PCGs, tRNAs, rRNAs and CR) all had the same trends in the A+T content, AT and GC-skews which displayed similar patterns and were consistent with the common base composition biases of insect mitogenomes.

3.3. Protein-coding genes and codon usage

The total length of PCGs in the studied mitogenomes was 11,247 bp (*Haploperla japonica*) and 11,244 bp (*Sweltsa* sp.) (Table 2). In both mitogenomes from Chloroperlinae, typical start codons ATN (Met/Ile) were the most commonly used start codons. Additionally, TTG was inferred

Table 4. The nucleotide composition of the *Haploperla japonica* and *Sweltsa* sp. mitogenomes.

Region		*H. japonica*	*Sweltsa* sp.
Whole mitogenome	A+T%	69.7	68.2
	AT-skew	0.065	0.087
	GC-skew	–0.223	–0.254
PCGs	A+T%	68.2	66.5
	AT-skew	–0.173	–0.164
	GC-skew	–0.009	–0.047
PCGs–J	A+T%	66.4	64.5
	AT-skew	–0.096	–0.063
	GC-skew	–0.179	–0.235
PCGs–N	A+T%	71.0	69.6
	AT-skew	–0.289	–0.312
	GC-skew	0.307	0.305
tRNAs	A+T%	69.3	68.0
	AT-skew	–0.007	–0.022
	GC-skew	0.083	0.109
rRNAs	A+T%	72.4	72.1
	AT-skew	–0.100	–0.119
	GC-skew	0.283	0.318
CR	A+T%	80.6	80.0
	AT-skew	0.083	0.068
	GC-skew	–0.235	–0.257

Table 5. The start and stop codons of the *Haploperla japonica* and *Sweltsa* sp. mitogenomes.

Species	*ND2*		*COI*		*COII*		*ATP8*		*ATP6*		*COIII*		*ND3*	
	Start	**Stop**	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**
H. japonica	ATG	TAA	ATT	TAA	ATG	T-	ATG	TAA	ATG	TAA	ATG	TAA	ATT	TAG
Sweltsa sp.	ATG	TAA	ATT	TAA	ATG	T-	ATG	TAA	ATG	TAA	ATG	TAA	ATC	TAG
Species	***ND5***		***ND4***		***ND4L***		***ND6***		***CYTB***		***ND1***			
	Start	Stop	Start	Stop	Start	Stop	Start	Start	Start	Stop	Start	Stop		
H. japonica	GTG	T-	ATG	TAA	ATG	TAA	ATT	TAA	ATG	TAG	TTG	TAG		
Sweltsa sp.	GTG	T-	ATG	TAA	ATG	TAA	ATC	TAA	ATG	TAA	TTG	TAG		

as a start codon for *ND1*, and GTG as the start codon for *ND5* in both species. This phenomenon has been found in other stoneflies, and TTG is used in other insect species (Bae et al. 2004; James and Andrew 2006; Sheffield et al. 2008; Wei et al. 2010b; Elbrecht and Leese 2015; Elbrecht et al. 2015; Huang et al. 2015; Sproul et al. 2015; Chen et al. 2016; Chen and Du 2017c; Wang et al. 2017a, 2017b; Cao et al. 2019b). In both species, *ATP6*, *ATP8*, *COII*, *COIII*, *CYTB*, *ND2*, *ND4* and *ND4L* started with ATG (Met), while *COI*, *ND3* and *ND6* started with ATT (Ile) (Table 5).

The most commonly used stop codon in both Chloroperlinae species was TAA, found in *ATP6*, *ATP8*, *COI*, *COIII*, *ND2*, *ND4*, *ND4L* and *ND6*. In both species, the stop codon TAG was used by *CYTB*, *ND1* and *ND3* (except *CYTB* of *Sweltsa* sp., which used TAA). *COII* and *ND5* used a partial stop codon T in both species (Table 5). This phenomenon has also been recorded from other published stoneflies (James and Andrew 2006; Qian et al. 2014; Elbrecht and Leese 2015; Elbrecht et al. 2015; Huang et al. 2015; Wu et al. 2014; Chen et al. 2016; Chen and Du 2017c; Wang et al. 2017a, 2017b; Cao et al. 2019b). Truncated stop codons occur in many arthropod mitogenomes, and it is corrected by post-transcriptional polyadenylation (Ojala et al. 1981; Wolstenholme 1992). The two species had the same start and stop codons in ten genes (*COI*, *COII*, *COIII*, *ATP8*, *ATP6*, *ND1*, *ND2*, *ND4*, *ND4L* and *ND5*), while the remaining three genes (*ND3*, *ND6* and *CYTB*) only shared either the same start or stop codon (Table 5).

The genome-wide AT bias is reflected in codon usage. RSCU (relative synonymous codon usage) was calculated to identify the predominant synonymous codon (Grantham et al. 1980; Meganathan et al. 2012) (Fig. 2). The high AT base compositional biases was shown in the RSCU statistics for both Chloroperlinae mitogenomes. Codons ending with an A or U were favoured both four-fold and two-fold degenerate codons. RSCU demonstrates that codon usage patterns of these two stonefly species was consistent with other published Plecoptera species. All most commonly used codons: AAU (Asn), AUU (Ile), UUA (Leu1), AAA (Lys), AUA (Met), UUU (Phe) and UAU (Tyr), were always biased towards A and T at third codon positions (Fig. 2, Table 5).

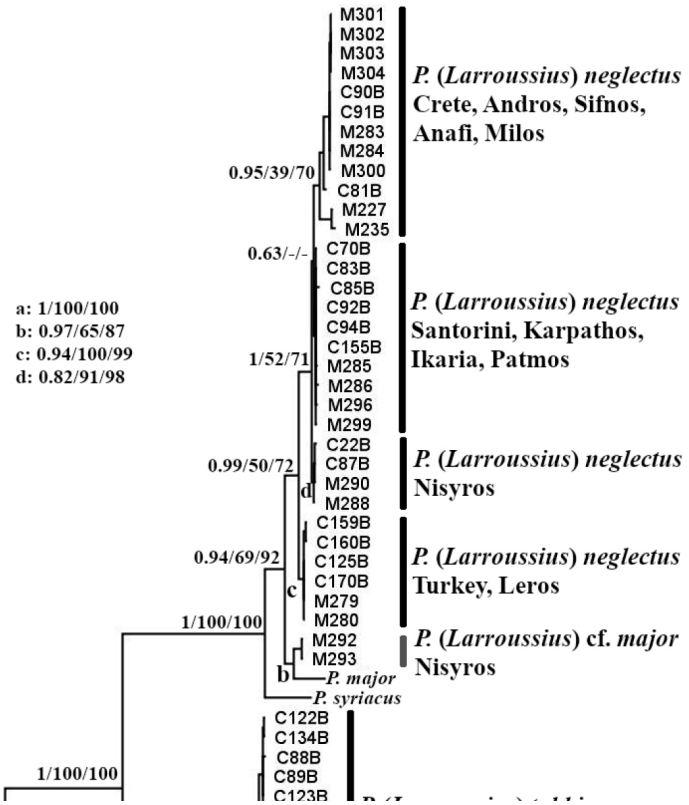

Figure 2. Relative synonymous codon usage (RSCU) in *Haploperla japonica* and *Sweltsa* sp. mitogenomes. Codon families are provided on the X-axis.

3.4. Transfer and ribosomal RNAs

In the present study, the tRNA genes in both species ranged from 65 to 71 bp, and comprised 9.26% (1,483 bp in *H. japonica*) and 9.36% (1,488 bp in *Sweltsa* sp.) of the complete mitogenomes (Tables 2 and 3). $tRNA^{Ser(AGN)}$, which could not be detected by ARWEN software, was identified by alignment with homologous genes from other published stoneflies (James and Andrew 2006; Chen et al. 2016; Wang et al. 2017a, 2018a, 2018b; Cao et al. 2019b; Guo et al. 2021). Most tRNAs had the typical cloverleaf structure, except $tRNA^{Ser(AGN)}$ that lacks the dihydrouridine (DHU) arm that formed a simple loop. In most insect mitogenomes, it is common for $tRNA^{Ser(AGN)}$ to lack the DHU stem (Cameron and Whiting 2007; Wan et al. 2013). The length of the different nucleotides of tRNAs between *H. japonica* and *Sweltsa* sp. ranged from 1 bp ($tRNA^{Ser(AGN)}$, $tRNA^{Ser(UCN)}$, $tRNA^{Val}$) to 13 bp ($tRNA^{His}$). By comparison, the $tRNA^{Cys}$ and $tRNA^{His}$ had the most variation with more than ten indels or substitutions; the nucleotide insertion-deletion appears in $tRNA^{Cys}$, $tRNA^{His}$, $tRNA^{Phe}$, $tRNA^{Thr}$, $tRNA^{Tyr}$ (Fig. 3).

Based on the secondary structures of tRNA, eight mismatched base pairs are found in *H. japonica*, which are U-U (2 bp), A-A (3 bp) and A-C (3 bp) (Fig. 3). And they have 24 G-U pairs, 7 located in the AA, 7 in the DHU, 8 in the AC and 2 in the TΨC stem. In *Sweltsa* sp., there are nine mismatches in tRNAs: U-U (1 bp), A-A (5 bp), C-C (1 bp) and A-C (2 bp) (Fig. 3); the 29 G-U pairs are located in the AA (5 bp), the DHU (8 bp), the AC (12 bp) and the TΨC stem (4 bp).

Similar to the mitogenomes of most other insects, the large and small rRNA subunits (*lrRNA* and *srRNA*) in both species were located between $tRNA^{Leu(CUN)}$ – $tRNA^{Val}$ (*lrRNA*) and $tRNA^{Val}$ – the control region (*srRNA*) (Fig. 1, Table 3). The lengths of *lrRNA* and *srRNA* of *Haploperla japonica* were 1324 bp and 776 bp, and the lengths of *lrRNA* and *srRNA* of *Sweltsa* sp. were 1325 bp and 799 bp (Table 2). Secondary structures of both *lrRNA* and *srRNA* were highly similar between these species (Pairwise sequence identity was 87.82% (*lrRNA*) and 87.98% (*srRNA*)).

Haploperla japonica was used as model for comparison of rRNA secondary structures. *lrRNA* consists of 5 structural domains (I–II, IV–VI), while domain III was absent, as usual in arthropods (Fig. 4) (Cannone et al. 2002). Domains I, II and IV were variable between these species, while eight helices (H2064, H2347, H2395, H2455, H2507, H2520, H2547 and H2588) within domain V had high similarity (Wang et al. 2017a).

srRNA consists of three domains (Fig. 5). Domains I and II were more variable than domain III in both species, while Helix 1399 region was the most conserved (Wang et al. 2017a). Furthermore, *Sweltsa* sp. possessed 21 more bp nucleotides in Helix 577 than *Haploperla japonica*.

3.5. The control region

In a previous study, the A+T-rich region (CR) was found to contain essential elements for the initiation of tran-

Node	Ancestral aeras	Probability
1	A (100%)	1
2	A (100%)	1
3	A (100%)	1
4	A (100%)	~1
5	AE (50%), AD (50%)	0.5
6	A (99.95%), AE (0.05%)	0.5
7	A (100%)	1
8	AB (100%)	1
9	A (100%)	1
10	A (100%)	1
11	A (100%)	1
12	A (100%)	1
13	A (100%)	1
14	A (100%)	1
15	A (100%)	1
16	AC (20%), AB (20%), AE (20%), AD (20%), A (20%)	0.2

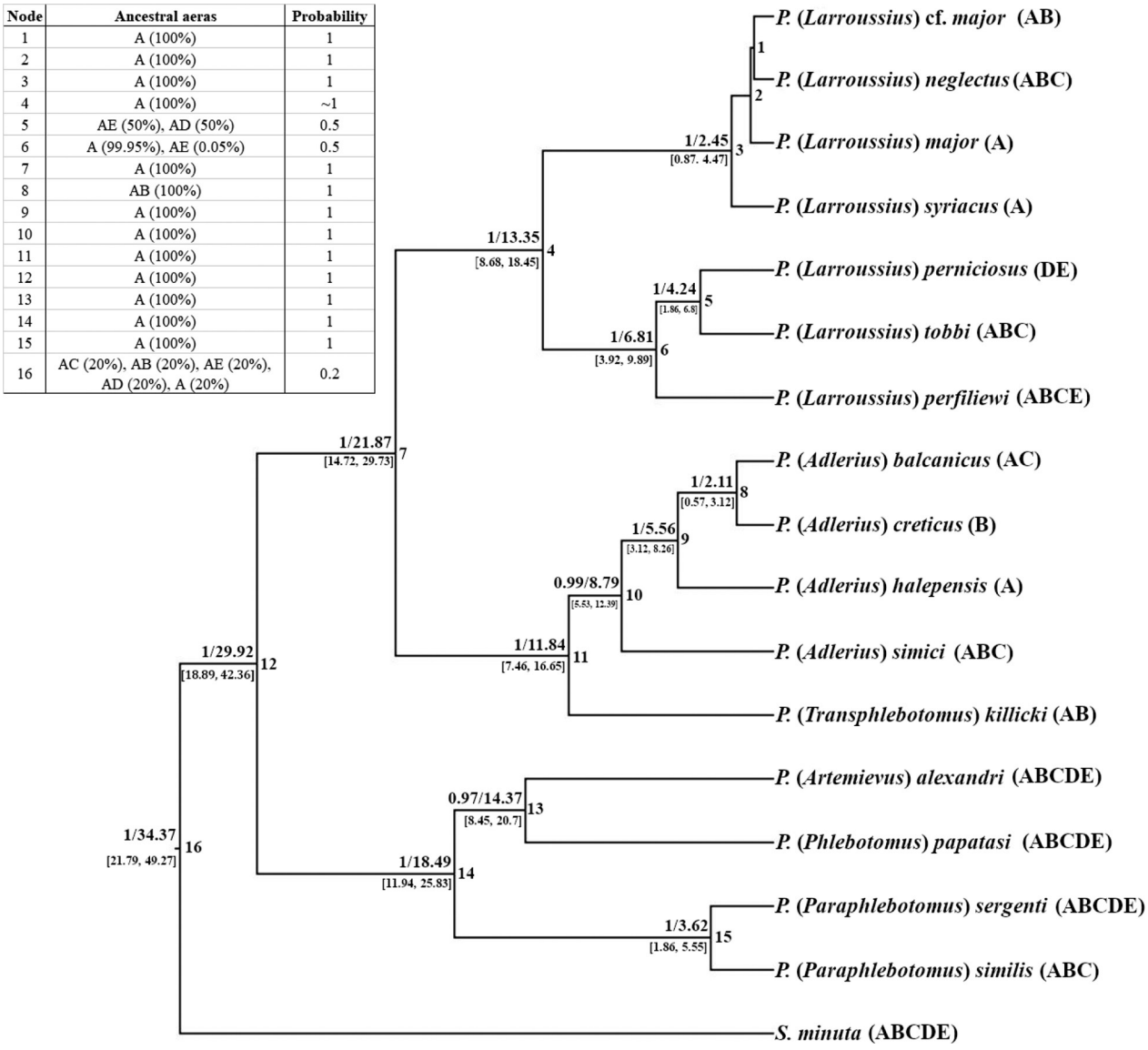

Figure 3. Secondary structures of 22 tRNAs of *Haploperla japonica* and *Sweltsa* sp. All tRNAs are labeled with the abbreviations of their corresponding amino acids. Dashes (–) indicate Watson-Crick base pairing and dots (•) indicate G-U base pairing. Both species are represented in gray; blue circle only on *H. japonica*, yellow only on *Sweltsa* sp.; green means not on *H. japonica*, red means not on *Sweltsa* sp.

scription and for replication (Zhang and Hewitt 1997). *Haploperla japonica* mitogenome had an A+T-rich region of 1,118 bp, with an A+T content of 80.6%; while *Sweltsa* sp. it was 1,011 bp long, with an A+T content of 80.0% (Tables 2 and 4). The A+T-rich region of both species is located between the *srRNA* and *tRNA^{Ile}* gene cluster (Fig. 1). The following structural elements were found in the control region of *H. japonica* and *Sweltsa* sp. (Fig. 6): (1) a leading sequence (588 bp (*H. japonica*), 496 bp (*Sweltsa* sp.)) adjacent to *srRNA*; (2) two large tandem

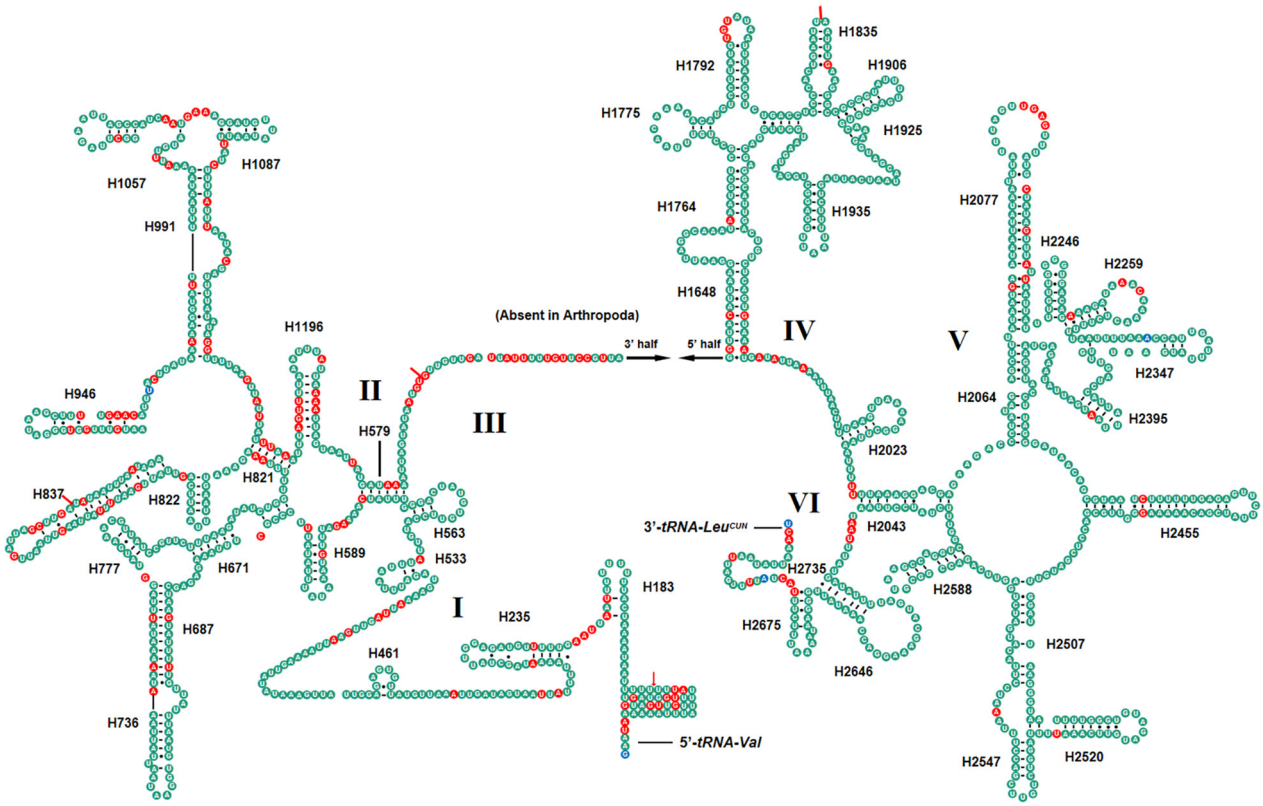

Figure 4. Predicted secondary structure of the *lrRNA* in *Haploperla japonica* and *Sweltsa* sp. Roman numerals represent the conserved domain structures. Dashes (–) indicate Watson-Crick base pairings and dots (•) indicate G-U base pairing. Different nucleotides of the two species are shown in red. Missing nucleotides in *H. japonica* are indicated by red arrows, missing nucleotides in *Sweltsa* sp. are shown in blue.

repeats (TRs), while (3) the remaining control region was 321 bp (*H. japonica*) and 372 bp (*Sweltsa* sp.).

3.6. Phylogenetic relationships

Phylogenetic relationships were reconstructed for the clade Systellognatha using 42 species: 36 complete (including the two species herein studied) and 6 nearly complete mitogenome sequences (Table 1). *Amphinemura yao* Mo, Yang, Wang and Li, 2017 and *A. longispina* (Okamoto, 1922) were used as outgroup taxa. Bayesian inference (BI) and maximum likelihood (ML) analyses, of the aligned 13 PCGs dataset, generated trees with identical topologies (Fig. 7).

Systellognatha includes seven families: superfamily Perloidea with Chloroperlidae, Kathroperlidae, Perlidae, Perlodidae; and superfamily Pteronarcyoidea with Peltoperlidae, Pteronarcyidae, Styloperlidae; all except Kathroperlidae (no mitogenomes yet sequenced) were represented in our study. We obtained all families as monophyletic (colors in Fig. 7). Our analyses strongly supported Peltoperlidae as the sister group of a clade containing the five other families. Similar relationships were recovered in previous phylogenetic analyses (Chen et al. 2018; South et al. 2020, 2021; Cao et al. 2021). A sister-group relationship between Perloidea (monophyletic herein: Fig. 7) and Pteronarcyoidea was not supported, as the latter was obtained as paraphyletic, which is inconsistent with morphological hypotheses (Zwick 2000). Phylogenetic analyses based on morphological characters proposed a clade Peltoperlidae + Styloperlidae, with Pteronarcyidae as their sister group (Zwick 2000). According to the present studies, Styloperlidae is sister to Pteronarcyidae rather than Peltoperlidae albeit with poor nodal support (as in Ding et al. 2019 Wang et al. 2019; Cao et al. 2019a; 2021). Perlidae was sister to Chloroperlidae + Perlodidae with high support (Fig. 7) (confirming Ricker 1952; Illies 1965; Chen et al. 2018; Wang et al. 2019, 2018c; South et al. 2020, 2021). Our analyses found relationships within Systellognatha to be (((Chloroperlidae + Perlodidae) + Perlidae) + (Styloperlidae + Pteronarcyidae)) + Peltoperlidae (Fig. 7). Recent phylogenetic analyses based on mitochondrial data have inferred the same topology (Chen et al. 2018; Cao et al. 2021), however, our results differ from the older morphology-based classification. The different results may be due to the limited mitogenomic data yet published about the Systellognatha, being especially scarce in Pteronarcyidae and Styloperlidae, and lacking in Kathroperlidae.

At the generic level, one pattern is the disjunct phylogenetic positions for Asian and North American members of the same genus (Fig. 7). For example, regarding *Isoperla* Banks, 1906, *Pseudomegarcys japonica* Kohno, 1946 was sister to the Asian *I. eximia* Zapekina-Dulkeit, 1975, rather than the North American *I. bilineata* (Say, 1823), the type species of genus *Isoperla*. Additionally, regarding *Acroneuria* Pictet, 1841, *Sinacroneuria dabieshana*

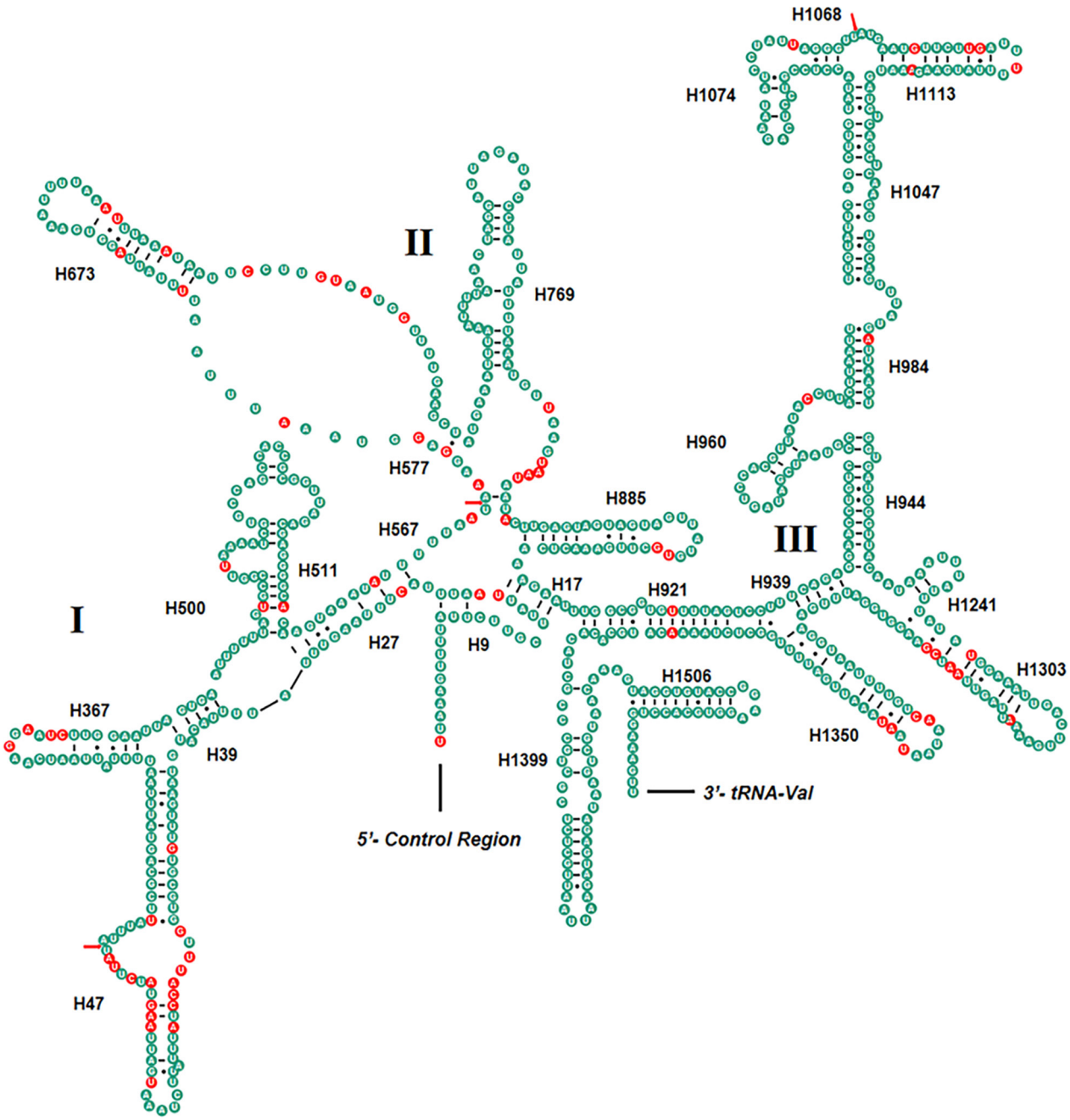

Figure 5. Predicted secondary structure of the *srRNA* in *Haploperla japonica* and *Sweltsa* sp. Roman numerals denote the conserved domain structure. Dashes (–) indicate Watson-Crick base pairing and dots (•) indicate G-U base pairing. Different nucleotides of the two species are shown in red. Missing nucleotides in *H. japonica* are indicated by red arrows, missing nucleotides in *Sweltsa* sp. are shown in blue.

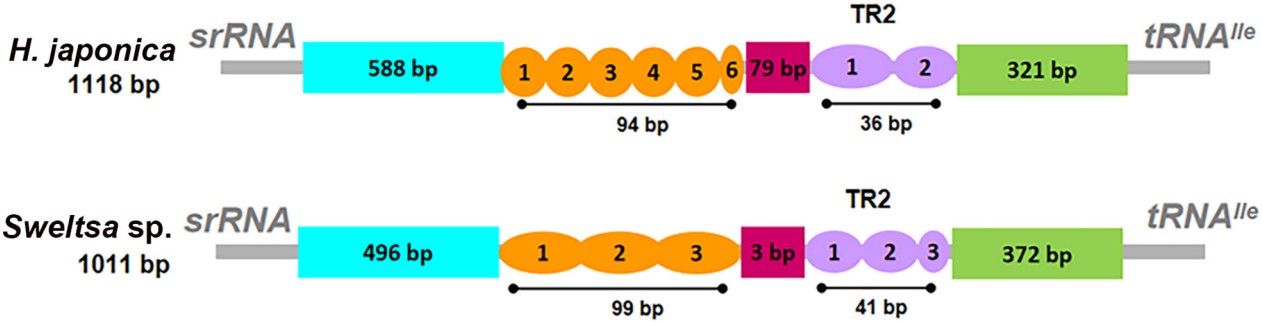

Figure 6. Structure elements found in the control region of *Haploperla japonica* and *Sweltsa* sp.

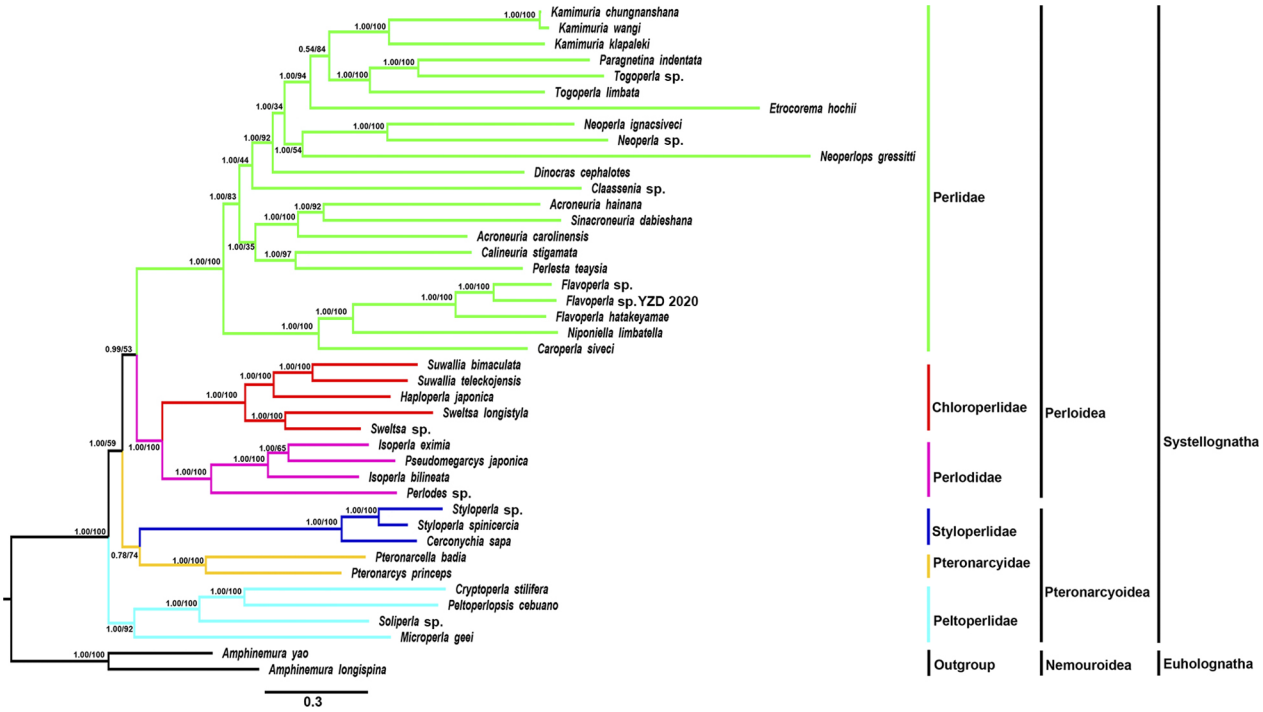

Figure 7. Phylogenetic tree inferred from the sequences of the mitogenomes of 40 Systellognatha species and 2 outgroup species. Numbers at the nodes are Bayesian posterior probabilities (left) and ML bootstrap values (right).

Li and Murányi, 2014 was sister to the Asian species *A. hainana* Wu, 1938, rather than the North American *A. carolinensis* (Banks, 1905). Regarding their morphology, both *Pseudomegarcys* and *Sinacroneuria* are rather distinctive genera, in addition, *Pseudomegarcys* belongs to the subfamily Perlodinae Klapálek, 1909, while *Isoperla* belongs to Isoperlinae Frison, 1942. Inclusion of these genera in *Isoperla* and *Acroneuria*, respectively, is not likely, and additional molecular studies are needed to clarify their phylogenetic position.

4. Competing Interests

The authors have declared that no conflict of interest exists.

5. Acknowledgments

The research was partially supported by the National Natural Science Foundation of China (No. 31970402), the Graduate Education Innovation Training Base Project of Henan Province in 2021 (107020221005) and the Program for Science & Technology Innovation Talents in Universities of Henan Province (No. 21HASTIT042).

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