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Research

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Characterization and Phylogenetic Analysis of the Mitochondrial Genome Sequence of *Nisia fuliginosa* (Hemiptera: Fulgoroidea: Meenoplidae)

Sha-Sha Lv, Yu-Jie Zhang, Nian Gong, and Xiang-Sheng Chen[1]

Institute of Entomology and Special Key Laboratory for Development and Utilization of Insect Resources of Guizhou, Guizhou University, Guiyang 550025, China and [1]Corresponding author, e-mail: chenxs3218@163.com

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Abstract

We explored characterization of the mitochondrial genome (mitogenome or mtGenome) and phylogenetic analysis between 32 Fulgoroid species by sequencing and analyzing the mitogenome of *Nisia fuliginosa* Yang and Hu, 1985 (Hemiptera: Fulgoroidea: Meenoplidae), thereby making it the first determined mitogenome from the family Meenoplidae. The mitogenome was found to be 15,754 bp in length and contained 13 protein-coding genes (PCGs), 22 tRNA genes, two ribosomal RNA genes (rRNAs), and a control region. All PCGs started with typical ATN codons, except for *nad1*, which used GTG as the start codon. Canonical TAA termination codons were found in 10 PCGs and the remaining three genes (*cox2*, *nad6*, and *nad1*) had incomplete stop codons T. All tRNAs could fold into typical cloverleaf secondary structures, with the exception of *trnC*, *trnV*, and *trnS1*. Additionally, we compared the AT and GC skews of 13 PCGs of 32 Fulgoroidea mitogenomes, on the L-strand, the AT and GC skews were negative and positive, respectively. However, on the H-strand, the AT skew could be positive or negative and the GC skew was always negative. Phylogenetic results showed that the eight families of Fulgoroidea were divided into two large groups. Delphacidae formed a monophyletic group sister to a clade comprising Meenoplidae and other six families (Fulgoridae, Ricaniidae, Flatidae, Issidae, Caliscelidae, and Achilidae). Meenoplidae was located near the clade of Delphacidae, and Fulgoridae was located near the clade of Meenoplidae. Furthermore, Caliscelidae, Issidae, Ricaniidae, and Flatidae are closely related and they collectively formed a sister group to Achilidae.

Key words: Fulgoroidea, Meenoplidae, mitochondrial genome, phylogeny

Meenoplidae is a relatively small family of planthoppers (Hemiptera: Fulgoroidea), with around 166 described species in 23 genera (Bourgoin 2021). It contains two subfamilies (Meenoplinae Fieber, 1872, and Kermesiinae Kirkaldy, 1906). Both lineages are mainly distributed in the Old World tropics and subtropics and are absent from the New World (Emeljanov 1984; Bourgoin 1997, 2021). It has been reported to harm a variety of economic crops, including Malvaceae (cotton), Gramineae (sugarcane, corn, sorghum, *Zizania latifolia*), Zygophyllaceae (*Tetraena mongolica*), Cyperaceae (*Cyperus rotundus*), and Caryophyllaceae (*Dianthus chinensis*) (Zhou et al. 1985, Hu and Yang 1993, Zhao and Liu 2010), and primarily feed on plants of the Gramineae family. During our investigation, we found that *N. fuliginosa* (Yang and Hu 1985) severely harmed the dominant aquatic plant, Cyperaceae, *Schoenoplectus tabernaemontani*, in the Caohai Wetland National Nature Reserve, Southwest China. Damage is caused to these plants when adults and nymphs suck the plant sap and rob plants of nutrients, causing plants to be malnourished and withered, with yellow or yellow-brown spots. Adult female planthoppers use the ovipositor to pierce plant tissues to lay eggs, causing plants to lose water. Adults and nymphs also secrete wax powder and honeydew, hindering the photosynthesis of plants and seriously affecting plant growth, sometimes to the point of plant death, and provide favorable conditions for the proliferation of fungi and bacteria.

In recent years, mitochondrial genome sequences have been widely used in the study of phylogenetic relationships, conservative genetics, and molecular evolution among insects (Crampton-Platt et al. 2015, Song et al. 2016a, Timmermans et al. 2016, Li et al. 2020, Liu et al. 2020), as well as in the study of phylogeography and population genetics (Bjork et al. 2011, Foote et al. 2011, James et al. 2016, Chang et al. 2017, Fields et al. 2018, Sun et al. 2019, Du et al. 2020). The mitogenome is a complete organelle genome sequence present in eukaryotic cells. Generally, the mitogenome is highly conserved and consists of a double-stranded circular molecule, encoding 37 genes, and ranges in length from approximately 14 to 20 kb, including 13 protein-coding genes (PCGs), 22 tRNA genes, two ribosomal RNA genes

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(rRNAs), and a control region (Boore 1999, Cameron 2014, Wang et al. 2015, Du et al. 2020). As a class of molecular markers, it has the characteristics of maternal inheritance, rapid evolution rate, relatively stable gene composition, and unambiguous orthologs (Avise 1986, Du et al. 2020). Compared with DNA fragments, the whole mitogenome can provide more detailed information, including the arrangement of gene sequences, RNA secondary structures, codon usage, and characteristics of the control region (Li et al. 2012a, Wang et al. 2019, Ma et al. 2020).

The study of mitogenomes started relatively late in the superfamily Fulgoroidea. Song and Liang (2009a) were the first to annotate the mitogenome of *Geisha distinctissima* in the family Flatidae and found that its gene composition and arrangement were similar to other Hemiptera. In the same year, the first annotated analysis of Delphacidae (*Laodelphax striatellus*) was performed and a phylogenetic tree was constructed for published Hemiptera insects (Song and Liang 2009b). Song et al. (2010) first studied the mitogenome of *Sivaloka damnosus* in Issidae and constructed a phylogenetic tree of Hemiptera. Song et al. (2012) annotated three new mitochondrial genomes, namely *Pyrops candelaria*, *Lycorma delicatula* (Fulgoridae), and *Ricania marginalis* (Ricaniidae), which further amplified the genome research of Hemiptera. Xu et al. (2019) were the first to annotate and analyze the mitogenomes of five species of Achilidae and constructed a phylogenetic tree of six families (Achilidae, Delphacidae, Fulgoridae, Issidae, Ricaniidae, and Flatidae). Gong et al. (2021) sequenced four new mitogenomes of Caliscelidae and constructed a phylogenetic tree of 28 Fulgoroidea species. Many ongoing studies continue to supplement the mitogenome data of Fulgoroidea. According to NCBI database statistics, the currently available mitogenome data of the superfamily Fulgoroidea has increased to 32 species in eight families (including this study).

At present, the research on Meenoplidae mainly consists of descriptions of new taxa and few reports on molecular studies have been reported. Some scholars have identified some gene fragments to study the phylogeny of the superfamily Fulgoroidea. However, based on the current research situation, the phylogenetic status and relationships of Meenoplidae based on the study of morphology and gene fragments remain unclear (Emeljanov 1991, Bourgoin 1997, Yeh et al. 2005, Urban and Cryan 2007, Song and Liang 2013) and research regarding the mitochondrial genome requires updating to further authenticate Meenoplidae's phylogenetic status and relationships.

In this study, we sequenced the mitochondrial genome of *N. fuliginosa* from southwestern China using next-generation sequencing technology, making it the first determined mitogenome from the family Meenoplidae. The gene organization, base composition, PCGs, codon usage, and the structure of the tRNAs and rRNAs of its mitochondrial genome were predicted and analyzed. We also compared the AT and GC skews of 13 PCGs of 32 Fulgoroidea mitogenomes. Besides, we constructed phylogenic trees of Fulgoroidea based on current mitogenomic information for Cercopidae (*Aeneolamia contigua*) and Cicadellidae (*Populicerus populi*) as outgroups under the Maximum Likelihood (ML) and Bayesian Inference (BI) criteria. This research can not only assist the understanding of the mitogenomic structure of the Meenoplidae, but also improve our understanding of the evolution of and relationships between different species of Fulgoroidea.

Materials and Methods

Sample Preparation and DNA Extraction

Samples of *N. fuliginosa* were collected in Caohai Wetland National Nature Reserve, Weining County, Guizhou Province, China. The collected samples were preserved in absolute ethanol, placed in a refrigerator at −40°C, and stored in the Institute of Entomology, Guizhou University. After morphological identification, total DNA was extracted from muscle tissue using the DNeasy DNA Extraction Kit (Qiagen, Hilden, Germany). Agarose (1%) electrophoresis was used to detect degradation and impurities in the extracted DNA, and the concentration and purity of samples were determined by Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA).

Sequencing and Assembling of Mitochondrial Genome

Next-generation sequencing technology was applied to obtain the mitogenome sequences. Qualified samples were constructed according to the standard procedure of the Illumina DNA library and to construct a paired-end (PE) sequencing library with an insert size of 350 bp. After the library was constructed, the quantitative polymerase chain reaction method and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) were used for quality control. The Illumina HiSeq 4000 (Illumina, San Diego, CA) high-throughput sequencing platform was used to sequence the qualified DNA library, the sequencing strategy was PE 150, and the quantity of sequencing data for each sample was at least 2 Gb. The high-quality reads were assembled using SPAdes version 3.5.0 (http://cab.spbu.ru/software/spades/) (Lapidus et al. 2014). According to the mitogenome sequence assembled, the DNA sequence alignment method was used to separate the mitogenome sequence from the total DNA sequencing data and then the captured mitochondrial genome sequence was subjected to data statistics and quality control.

Mitochondrial Genome Annotation and Analysis

Preliminary annotation of the mitochondrial genome was conducted using MITOS (http://mitos.bioinf.uni-leipzig.de/index.py) and ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) (Bernt et al. 2013). For the preliminary results of the annotation, BLASTP and BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to compare encoded proteins and rRNAs of the mitochondrial genomes with those of previously reported related species and to verify the accuracy of the results and make corrections. The base composition and relative synonymous codon usage (RSCU) were analyzed using MEGA 6.0 (Tamura et al. 2013). The secondary structure of the rRNAs was inferred and predicted based on the models of *Ugyops* sp. (Yu and Liang 2018) and *Taharana fasciana* (Wang et al. 2017) and the helix names refer to Gillespie et al. (2006). The annotation of the tRNAs was performed using ARWEN version 1.2 (http://mbio-serv2.mbioekol.lu.se/ARWEN/) (Laslett and Canbäck 2008). If there was an abnormality in the tRNA, we employed tRNAscan-SE 2.0 (http://lowelab.ucsc.edu/tRNAscan-SE/) to verify the prediction status. The tRNAs with unreasonable lengths and incomplete structures were discarded, and a tRNA secondary structure map was generated (Lowe and Chan 2016). AT Skew = (A − T)/(A + T), GC Skew = (G − C)/(G + C) (Perna and Kocher 1995).

Phylogenetic Analysis

Based on the nucleotide sequence of the 13 PCGs and 13 PCGs + 2 rRNAs of the mitogenome, we chose Cercopidae (*Aeneolamia contigua*) and Cicadellidae (*Populicerus populi*) as outgroups and used ML analysis and BI analysis methods to construct phylogenetic trees for eight families (Meenoplidae, Fulgoridae, Delphacidae, Achilidae, Issidae, Caliscelidae, Ricaniidae, and Flatidae) and 32 species of the superfamily Fulgoroidea (Table 1). The 13 PCGs

Table 1. List of taxa used for phylogenetic and skewness analysis

Superfamily	Family	Species	GenBank number GenBank accession
Outgroups	Cercopidae	*Aeneolamia contigua*	NC025495
	Cicadellidae	*Populicerus populi*	NC039427
Fulgoroidea	Meenoplidae	*Nisia fuliginosa*	MW192046
	Achilidae	*Peltatavertexalis horizontalis*	MH324929
		Betatropis formosana	MH324927
		Magadhaideus luodiana	MH324928
		Paracatonidia sp.	MH324931
		Plectoderini sp.	MH324930
	Fulgoridae	*Lycorma delicatula*	NC012835
		Lycorma meliae	MT079725
		Pyrops candelaria	FJ006724
		Aphaena discolor	MN025523
		Aphaena amabilis	NC045075
	Delphacidae	*Changeondelphax velitchkovskyi*	NC037181
		Laodelphax striatellus	NC013706
		Nilaparvata bakeri	NC033388
		Nilaparvata lugens	NC021748
		Nilaparvata muiri	NC024627
		Peregrinus maidis	NC037182
		Saccharosydne procerus	NC042179
		Sogatella furcifera	NC021417
		Sogatella vibix	NC042180
		Sogatella kolophon	MW009064
		Ugyops sp.	MH352481
	Issidae	*Hemisphaerius rufovarius*	MT210096
		Sivaloka damnosus	FJ360694
	Ricaniidae	*Ricania marginalis*	NC019597
		Ricania speculum	NC031369
		Ricania shantungensis	NC051496
	Flatidae	*Geisha distinctissima*	NC012617
	Caliscelidae	*Bambusicaliscelis flavus*	MW281858
		Bambusicaliscelis fanjingensis	MW281859
		Youtuus strigatus	MW281860
		Youtuus erythrus	MW281861

and two rRNAs were first aligned individually using MAFFT version 7.450 (Katoh and Standley 2013), then concatenated using SequenceMatrix 1.8 (Vaidya et al. 2011), which was also used for phylogenetic analyses. The best model (GTR + I + G4) for concatenate sequences under the corrected Akaike Information Criterion using jModeltest 2.1.10 (Darriba et al. 2012) was selected. The ML analysis was performed using IQ-TREE version 1.6.3 (Nguyen et al. 2015) and evaluated using the ultrafast bootstrap approximation approach for 10,000 replicates. The BI analysis was performed using MrBayes 3.2 (Ronquist et al. 2012), then four simultaneous Markov chains ran for 20 million generations and trees were sampled every 1,000 generations, with a burn-in of 25%.

Results

Genome Organization

The length of mitogenome of the *N. fuliginosa* was found to be 15,754 bp (GenBank MW192046), which consists of 37 genes, including 13 PCGs, 22 tRNA genes, two rRNA genes, and one control region (Fig. 1, Table 2). This is the first reported mitochondrial genome sequence from the family Meenoplidae. Unfortunately, we could not successfully sequence the complete control region, only 621 bp were sequenced. Notably, the gene arrangement of *N. fuliginosa* was consistent with the putative ancestral gene order.

The heavy chain (H-strand) encodes 23 genes, which comprise 9 PCGs (*nad2*, *cox1*, *cox2*, *atp8*, *atp6*, *cox3*, *nad3*, *nad6,* and *cytb*) and 14 tRNA genes (*trnI*, *trnM*, *trnW*, *trnL2*, *trnK*, *trnD*, *trnG*, *trnA*, *trnR*, *trnN*, *trnS1*, *trnE*, *trnT,* and *trnS2*). The remaining four PCGs (*nad5*, *nad4*, *nad4l,* and *nad1*), eight tRNA genes (*trnQ*, *trnC*, *trnY*, *trnF*, *trnH*, *trnP*, *trnL1,* and *trnV*), and two rRNA genes (*rrnL* and *rrnS*) are all encoded by the light chain (L-strand) (Fig. 1, Table 2). There are three gene overlapping regions in the mitochondrial genome, making a total of 11 bp. The longest overlapping sequence is between *atp8* and *atp6*, with a length of 7 bp. The mitogenome is relatively loose, with 25 gene spacer regions (854 bp), and the longest spacer sequence is between *nad4* and *nad4l* (148 bp). Eight regions have neither overlaps nor spacers.

Base Composition

Analysis of the base content of 13 PCGs, 22 tRNA genes, 2 rRNA genes, and a detected control region of the mitogenome sequence of the *N. fuliginosa* showed that (Table 3) the A, T, G, and C contents of the mitogenome (no control region) were 46.2%, 34.1%, 7.1%, and 12.6%, respectively. The AT content was found to be 80.3% and the GC content was found to be 19.7%, showing obvious AT preference. The content of AT and GC were found to be 85.5% and 14.5%, respectively, in the detected control region (621 bp), 79.2% and 20.8% in the 13 PCGs, 81.3% and 18.7%

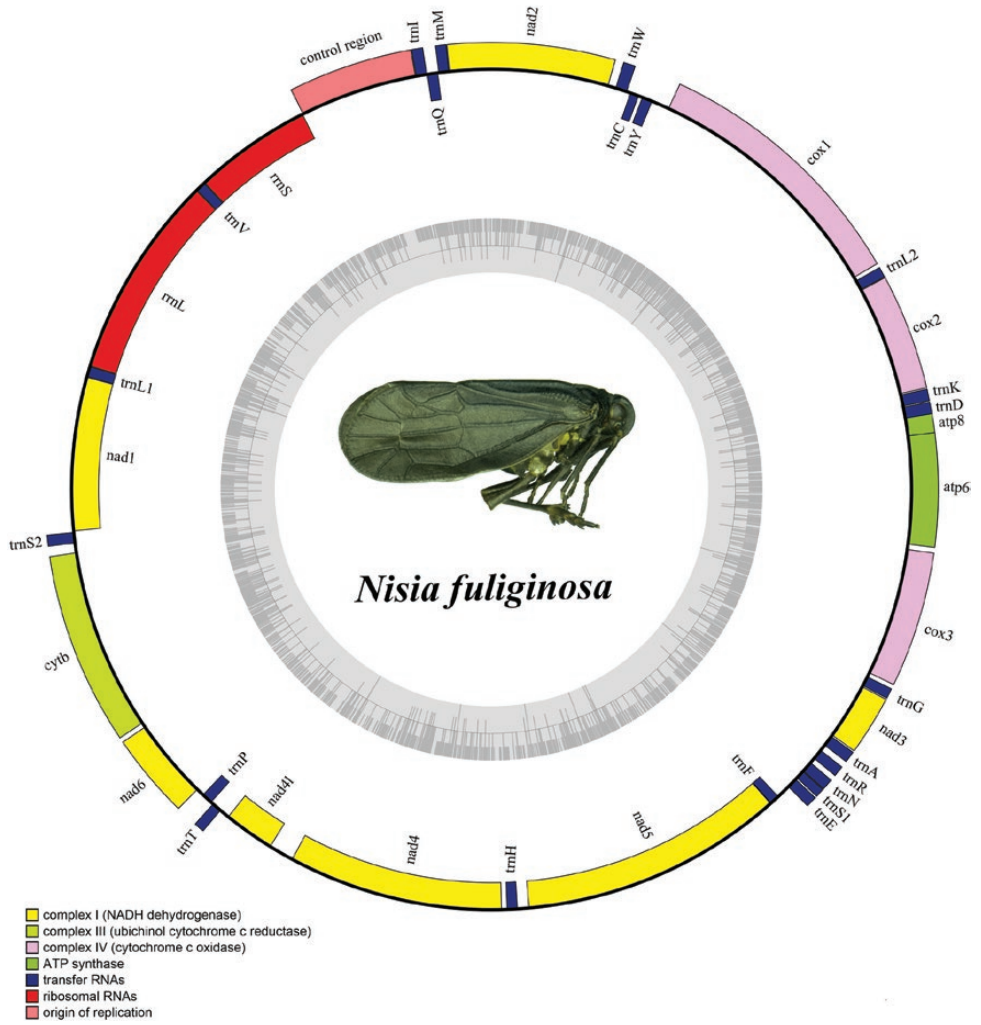

Fig. 1. Circular map of the mitogenome of *N. fuliginosa*. Protein coding and tRNA genes are shown with standard abbreviations. tRNA genes are exhibited as single-letter abbreviations, except for the S1 = AGN, S2 = UCN, L1 = CUN, and L2 = UUR. The thick lines outside the circle indicate the heavy strand, whereas those inside the circle indicate the light strand.

in the 22 tRNA genes, and 81.3% and 18.7% in the two rRNA genes, respectively.

Composition analysis (Table 3) revealed that the mitogenome of *N. fuliginosa* exhibited a positive AT skew (0.153) and a negative GC skew (–0.279) in the mitogenome. The 13 PCGs showed an AT skew of –0.139 and a GC skew of –0.067. The 22 tRNAs showed an AT skew of 0.058 and a GC skew of 0.134. The two rRNAs showed an AT skew of –0.171 and a GC skew of 0.339. Additionally, AT and GC skews (of 0.333 and –0.572, respectively) were detected in the detected control region. Positive and negative skews indicate the occurrence of more or less A(G) than T(C), which has also been observed in other examined Fulgoroidea mitogenomes.

We also compared the AT and GC skews of 13 PCGs of 32 Fulgoroidea mitogenomes (Figs 2 & 3, Tables 4 & 5). On the L-strand, the AT skew was negative and all skew values were high. Conversely, the GC skew was positive, only the skew value for the *nad4l* gene was high. On the H-strand, the AT skew could be positive or negative, but the GC skew was always negative. A and C bases were found to be more prevalent than T and G bases.

Protein-coding Genes

The 13 PCGs of the mitogenome of *N. fuliginosa* were found to be 10,839 bp in length, accounting for around 68.80% of the mitochondrial genome sequence (15,754 bp), encoding a total of 3,612 codons. Among them (Table 2), *nad5* (1,617 bp) was found to be the longest sequence and *atp8* (108 bp) was the shortest. Except for the start codon of the *nad1* gene (GTG), all other PCGs' start codons are ATN. The start codons of the *cox1*, *cox2*, *cox3*, *atp6*, *nad4*, *nad4l*, and *cytb* genes are ATG, whereas *nad2* and *nad5* use ATT as the start codon, *nad3* and *nad6* use ATA, and *atp8* use ATC. The termination codons of 10 PCGs are all TAA and the remaining genes (*cox2*, *nad6*, and *nad1*) use an incomplete stop codon T.

The amino acids usage of 13 PCGs and the RSCU frequency statistical analysis results are shown in Figures 4 & 5 and Table 6. The most frequently used amino acids were found to be Ile, Phe, and Leu2, the frequency of the codons AUU was found to be the highest (416 incidences). Cys, Arg, and Asp were found to be relatively scarce and the frequency of the codons CCG and CGC was zero. The most frequency of synonymous codons are UUA, UCA, and UCU, and UUA has the highest frequency of relative synonymous

Table 2. Summary of the mitogenome of *N. fuliginosa*

Gene	Strand	Location	Size (bp)	Anticodon	Start codon	Stop codon	Intergenic nucleotides
trnI	H	1–69	69	GAT			–3
trnQ	L	67–135	69	TTG			2
trnM	H	138–202	65	CAT			0
nad2	H	203–1,171	969		ATT	TAA	44
trnW	H	1,216–1,289	74	TCA			20
trnC	L	1,310–1,365	56	GCA			20
trnY	L	1,386–1,453	68	GTA			103
cox1	H	1,557–3,095	1,539		ATG	TAA	20
trnL2 (UUR)	H	3,116–3,180	65	TAA			0
cox2	H	3,181–3,853	673		ATG	T	3
trnK	H	3,857–3,929	73	CTT			2
trnD	H	3,932–4,003	72	GTC			0
atp8	H	4,004–4,111	108		ATC	TAA	–7
atp6	H	4,105–4,764	660		ATG	TAA	27
cox3	H	4,792–5,577	786		ATG	TAA	22
trnG	H	5,600–5,663	64	TCC			3
nad3	H	5,667–6,014	348		ATA	TAA	5
trnA	H	6,020–6,090	71	TGC			34
trnR	H	6,125–6,191	67	TCG			29
trnN	H	6,221–6,290	70	GTT			0
trnS1 (AGN)	H	6,291–6,348	58	GCT			6
trnE	H	6,355–6,422	68	TTC			81
trnF	L	6,504–6,566	63	GAA			–1
nad5	L	6,566–8,182	1,617		ATT	TAA	65
trnH	L	8,248–8,315	68	GTG			28
nad4	L	8,344–9,660	1,317		ATG	TAA	148
nad4l	L	9,809–10,081	273		ATG	TAA	56
trnT	H	10,138–10,201	64	TGT			11
trnP	L	10,213–10,282	70	TGG			43
nad6	H	10,326–10,821	496		ATA	T	24
Cytb	H	10,846–11,958	1,113		ATG	TAA	55
trnS2 (UCN)	H	12,014–12,082	69	TGA			3
nad1	L	12,086–13,025	940		GTG	T	0
trnL1 (CUN)	L	13,026–13,091	66	TAG			0
rrnL (16S)	L	13,092–14,343	1,252				0
trnV	L	14,344–14,407	64	TAC			0
rrnS (12S)	L	14,408–15,133	726				
Control region (CR)		15,134–15,754	621				

Strand of the genes is presented as H for majority and L for minority strand. In the column for intergenic length, a positive sign indicates the interval in base pairs between genes, while the negative sign indicates overlapping base pairs between genes.

Table 3. Composition and skewness of the *N. fuliginosa* mitogenome

N. fuliginosa	Size(bp)	A	T	G	C	A + T	G + C	AT-skew	GC-skew
Genome (no CR)	15,133	46.2%	34.1%	7.1%	12.6%	80.3%	19.7%	0.153	−0.279
Protein-coding genes	10,839	34.1%	45.1%	9.7%	11.1%	79.2%	20.8%	−0.139	−0.067
tRNA genes	1,473	43.0%	38.3%	10.6%	8.1%	81.3%	18.7%	0.058	0.134
rRNA genes	1,978	33.7%	47.6%	12.5%	6.2%	81.3%	18.7%	−0.171	0.339
Detected CR	621	57.0%	28.5%	3.1%	11.4%	85.5%	14.5%	0.333	−0.572

CR, control region.

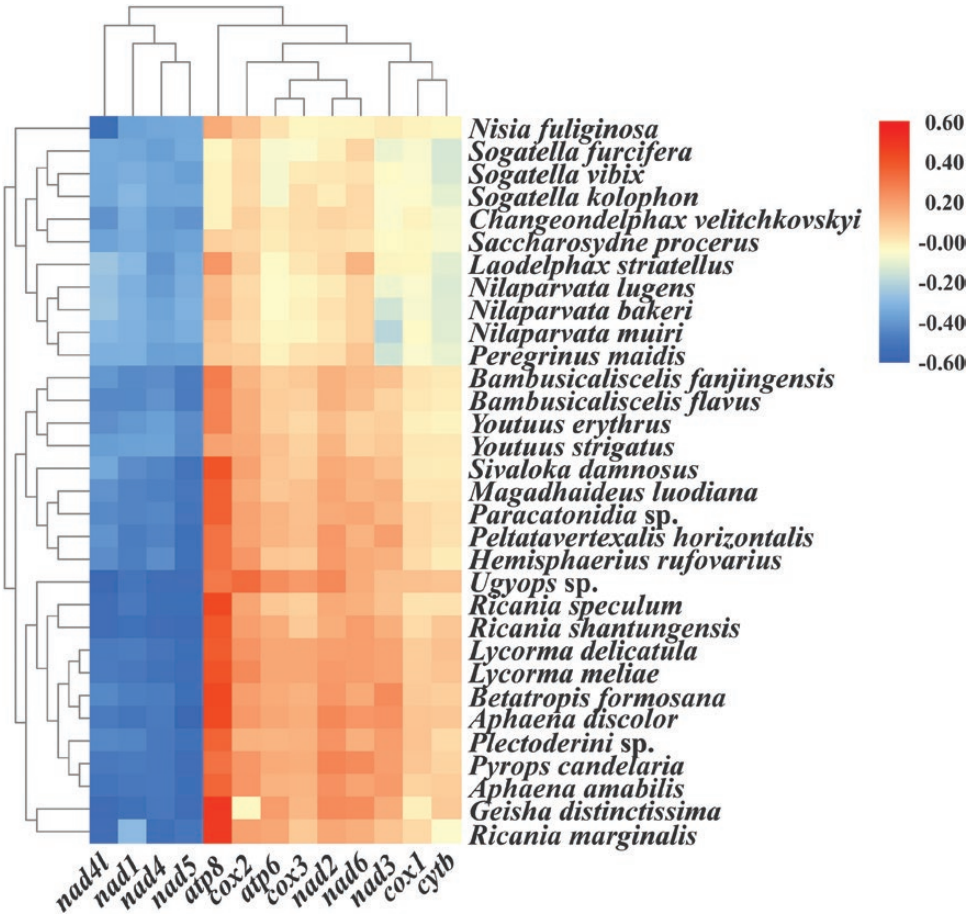

Fig. 2. The AT skew of 13 PCGs from fulgoroid mitogenomes.

codons, where the RSCU is 4.54. CGC and CCG, conversely, have a relatively low frequency of synonymous codons and the RSCU is 0.

tRNAs and rRNAs

The total length of the tRNA genes of the mitogenome of *N. fuliginosa* was found to be 1,473 bp, and the lengths of the tRNA genes are between 56 bp (*trnC*) and 74 bp (*trnW*) (Tables 2 & 3). Among these, 14 genes were located on the H-strand and 8 genes were located on the L-strand. The tRNAs that transport Leucine and Serine amino acids correspond to two tRNA genes, namely *trnL1* (UUR), *trnL2* (CUN) and *trnS1* (AGN), *trnS2* (UCN); the tRNAs transport other 20 amino acids and each correspond to a tRNA gene. Through the analysis of the secondary structure of the tRNAs (Fig. 6), we found that the three tRNA genes of *trnC*, *trnV*, and *trn S1* lacked the dihydrouridine (DHU) arm and the remaining 19 tRNA genes can form a typical cloverleaf structure.

Twenty-one wobble base pairs (Fig. 6) (G-U) were detected in 13 genes (*trnQ*, *trnC*, *trnY*, *trnF*, *trnH*, *trnL1*, *trnV*, *trnM*, *trnL2*, *trnD*, *trnG*, *trnA*, and *trnS1*) of the tRNA structure, in the amino acid-accepting arms, anticodon arms, TψC arms, and DHU arms all appeared, among which *trnQ* and *trnL1* had the highest rate (four pairs each). In addition, two pairs of U-U base mismatches (in *trnP* and *trnL2*) and four pairs of A-A base mismatches (in *trnW*, *trnA*, *trnE*, and *trnT*) were found in the amino acid-accepting arms and the TψC arms.

The *rrnL* (16S) and *rrnS* (12S) genes, which are adjacent to the *trnL1* and *trnV*, and *trnV* and control region, respectively, are

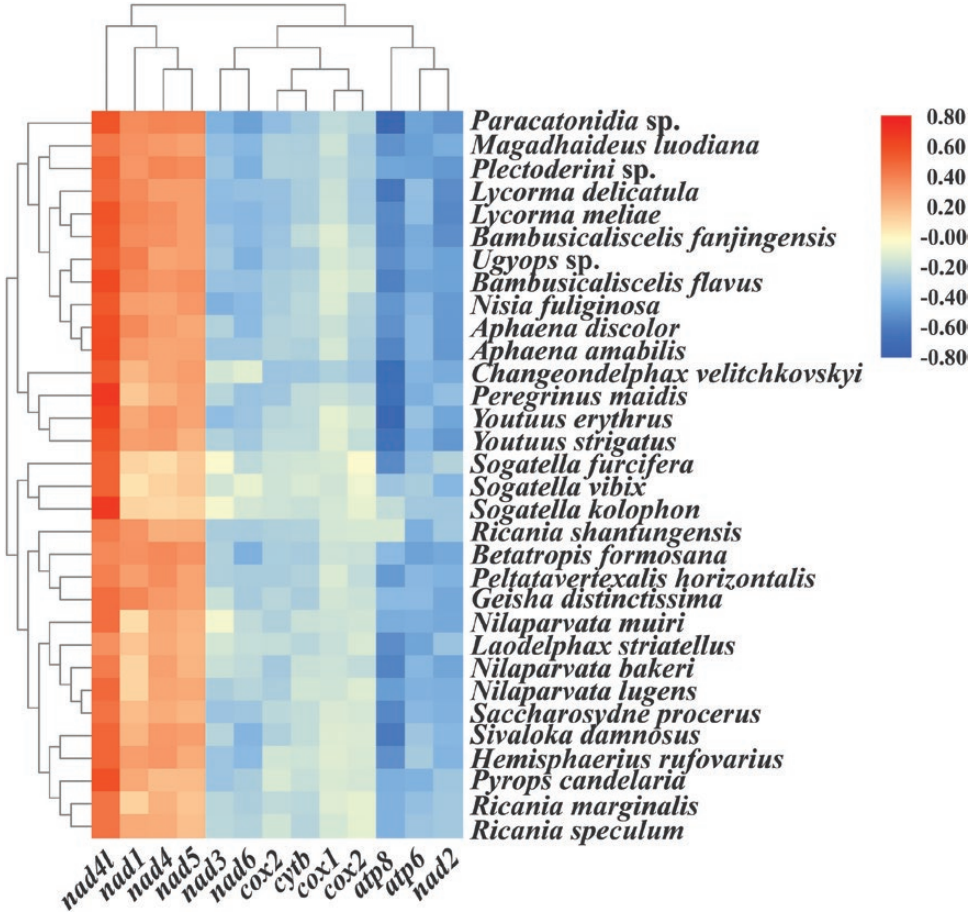

Fig. 3. The GC skew of 13 PCGs from fulgoroid mitogenomes.

located on the L-strand. The lengths of the two genes were 1,252 bp (*rrnL*) and 721 bp (*rrnS*), respectively (Table 2). The secondary structure of the *rrnL* gene (Fig. 7) was found to contain five structural domains (domain III is missing in arthropods) and 42 helices. The IV and V domains were found to be relatively conservative. The secondary structure of the *rrnS* gene (Fig. 8) was found to contain three domains and 27 helices; domain III was found to be more conservative than domains I and II.

Phylogenetic Analysis

Phylogenetic analyses were based on the nucleotide sequences of the 13 PCGs (Fig. 9, tree 1) and 13 PCGs + 2 rRNAs (Fig. 10, tree 2) used ML and BI methods to construct phylogenetic trees from the mitogenomes of 32 species of Fulgoroidea. In these four phylogenetic analyses, family-level topology was found to be the same and as follows: (Delphacidae + (Meenoplidae + (Fulgoridae + (Achilidae + (Caliscelidae + (Issidae + (Ricaniidae + Flatidae)))))). Phylogenetic results showed that the Fulgoroidea eight families were divided into two major groups, the Delphacidae formed a monophyletic group sister to a clade composed of Meenoplidae and six other families (Fulgoridae, Ricaniidae, Flatidae, Issidae, Caliscelidae, and Achilidae). Among them, Meenoplidae was located near the clade of Delphacidae, and Fulgoridae was located near the clade of Meenoplidae. Caliscelidae, Issidae, Ricaniidae, and Flatidae were closely related and formed the sister group to Achilidae. Besides, three families (Ricaniidae, Flatidae, and Issidae) formed the sister group to Caliscelidae.

Discussion

Among the Fulgoroidea, the mitogenome of the *N. fuliginosa* was found to be 15,754 bp in length, which was located between 14,367 bp in *Nilaparvata lugens* (Lv et al. 2015) and 17,619 bp in *Nilaparvata lugens* (Zhang et al. 2013). Unfortunately, we could not successfully sequence the complete control region, only 621 bp were sequenced, the longest known control region length is 2,429 bp (Zhang et al. 2013) in known Fulgoroidea insects. The gene arrangement of *N. fuliginosa* was found to be consistent with the putative ancestral gene order. Among the Fulgoroidea reported so far, gene rearrangement has been found in all Delphacidae examined except *Ugyops* sp. Compared with the original arrangement, the positions of three PCGs (*nad4*, *nad4l*, and *nad6*) and five tRNA genes (*trnC*, *trnW*, *trnH*, *trnP*, and *trnT*) were found to be translocated or inverted (Song and Liang 2009b, Zhang et al. 2013, Zhang et al. 2014, Lv et al. 2015). This gene rearrangement phenomenon is also found in other insects (Thao et al. 2004, Hua et al. 2008, Li et al. 2012b, Jiang et al. 2016, Song et al. 2016b, Du et al. 2017). Tandem duplications, nucleotide replacement and deletion, base mismatches, and gene spacers and overlaps are widely considered to be the cause of rearrangement phenomena (Mcknight and Shaffer 1997, Boore 2000, Song and Liang 2009b, Yu and Liang 2018, Xu et al. 2019).

Table 4. The AT-skew of 13 PCGs in the 32 Fulgoroidea mitogenomes

Family	Species	*atp6*	*atp8*	*cox1*	*cox2*	*cox3*	*cytb*	*nad1*	*nad2*	*nad3*	*nad4*	*nad4l*	*nad5*	*nad6*
Meenoplidae	*Nisia fuliginosa*	0.02	0.24	−0.01	0.10	−0.02	−0.02	−0.37	−0.02	0.00	−0.35	−0.43	−0.35	−0.02
Achilidae	*Peltatavertexalis horizontalis*	0.14	0.32	0.05	0.19	0.12	0.02	−0.47	0.22	0.20	−0.46	−0.41	−0.53	0.14
	Betatropis formosana	0.16	0.44	0.09	0.20	0.15	0.08	−0.47	0.22	0.25	−0.48	−0.44	−0.54	0.17
	Magadhaideus luodiana	0.10	0.36	0.01	0.16	0.11	0.02	−0.45	0.18	0.15	−0.46	−0.41	−0.50	0.14
	Paracatonidia sp.	0.14	0.36	0.05	0.19	0.12	0.03	−0.46	0.17	0.14	−0.44	−0.44	−0.51	0.18
	Plectoderini sp.	0.14	0.36	0.11	0.15	0.15	0.04	−0.45	0.23	0.20	−0.49	−0.44	−0.53	0.15
Fulgoridae	*Lycorma delicatula*	0.18	0.38	0.08	0.23	0.18	0.11	−0.47	0.20	0.18	−0.51	−0.49	−0.54	0.20
	Lycorma meliae	0.18	0.40	0.08	0.25	0.17	0.10	−0.50	0.21	0.18	−0.52	−0.48	−0.55	0.20
	Pyrops candelaria	0.17	0.32	0.10	0.22	0.16	0.06	−0.48	0.27	0.20	−0.49	−0.49	−0.55	0.25
	Aphaena discolor	0.17	0.44	0.11	0.22	0.16	0.10	−0.50	0.26	0.23	−0.49	−0.48	−0.59	0.22
	Aphaena amabilis	0.15	0.35	0.07	0.22	0.14	0.08	−0.50	0.24	0.21	−0.49	−0.51	−0.54	0.19
Delphacidae	*Changeondelphax velitchkovskyi*	0.01	−0.01	−0.02	0.07	0.04	−0.07	−0.33	0.06	−0.05	−0.39	−0.41	−0.41	0.04
	Laodelphax striatellus	−0.04	0.22	−0.03	0.07	0.01	−0.11	−0.30	0.05	−0.02	−0.40	−0.25	−0.34	0.14
	Nilaparvata bakeri	−0.04	0.14	−0.06	0.05	−0.01	−0.12	−0.32	0.02	−0.14	−0.36	−0.26	−0.32	0.06
	Nilaparvata lugens	−0.04	0.13	−0.06	0.04	−0.02	−0.12	−0.32	0.00	−0.08	−0.38	−0.27	−0.33	0.06
	Nilaparvata muiri	−0.02	0.10	−0.03	0.04	−0.02	−0.11	−0.32	0.01	−0.20	−0.35	−0.30	−0.33	0.06
	Peregrinus maidis	−0.01	0.09	−0.06	0.07	0.03	−0.09	−0.32	0.03	−0.15	−0.38	−0.33	−0.37	0.10
	Saccharosydne procerus	0.00	0.07	−0.03	0.05	0.03	−0.06	−0.34	0.03	−0.03	−0.38	−0.36	−0.36	0.02
	Sogatella furcifera	−0.06	−0.03	−0.06	0.04	−0.05	−0.14	−0.35	−0.01	−0.09	−0.38	−0.34	−0.34	0.06
	Sogatella vibix	−0.07	−0.01	−0.05	0.05	0.01	−0.13	−0.33	0.01	−0.04	−0.35	−0.35	−0.38	0.03
	Sogatella kolophon	−0.07	−0.01	−0.04	0.04	0.04	−0.10	−0.30	0.00	−0.05	−0.35	−0.35	−0.35	0.05
	Ugyops sp.	0.24	0.30	0.11	0.33	0.21	0.10	−0.51	0.26	0.12	−0.55	−0.58	−0.55	0.16
Issidae	*Hemisphaerius rufovarius*	0.10	0.32	0.04	0.22	0.09	0.00	−0.47	0.21	0.15	−0.43	−0.43	−0.52	0.17
	Sivaloka damnosus	0.10	0.40	0.00	0.19	0.08	0.00	−0.43	0.17	0.11	−0.45	−0.35	−0.51	0.14
	Ricania marginalis	0.18	0.48	0.04	0.19	0.09	−0.05	−0.29	0.18	0.11	−0.53	−0.55	−0.50	0.15
Ricaniidae	*Ricania speculum*	0.09	0.44	0.02	0.18	0.07	0.03	−0.49	0.17	0.11	−0.54	−0.55	−0.54	0.15
	Ricania shantungensis	0.16	0.39	0.04	0.20	0.09	0.10	−0.53	0.16	0.15	−0.55	−0.55	−0.56	0.20
Flatidae	*Geisha distinctissima*	0.20	0.49	−0.01	−0.03	0.13	0.09	−0.52	0.25	0.16	−0.49	−0.56	−0.55	0.24
Caliscelidae	*Bambusicaliscelis flavus*	0.08	0.27	0.01	0.16	0.07	0.00	−0.44	0.15	0.06	−0.41	−0.44	−0.48	0.11
	Bambusicaliscelis fanjingensis	0.07	0.29	0.02	0.14	0.09	0.00	−0.44	0.15	0.11	−0.43	−0.40	−0.48	0.12
	Youtuus strigatus	0.11	0.19	0.01	0.17	0.07	0.01	−0.37	0.14	0.09	−0.36	−0.38	−0.43	0.07
	Youtuus erythrus	0.08	0.27	−0.01	0.16	0.05	−0.02	−0.40	0.12	0.08	−0.37	−0.42	−0.43	0.06

Table 5. The GC-skew of 13 PCGs in the 32 Fulgoroidea mitogenomes

Family	Species	*atp6*	*atp8*	*cox1*	*cox2*	*cox3*	*cytb*	*nad1*	*nad2*	*nad3*	*nad4*	*nad4l*	*nad5*	*nad6*
Meenoplidae	*Nisia fuliginosa*	−0.37	−0.44	−0.13	−0.27	−0.24	−0.22	0.30	−0.49	−0.39	0.28	0.56	0.32	−0.36
Achilidae	*Peltatavertexalis horizontalis*	−0.36	−0.49	−0.14	−0.27	−0.21	−0.25	0.30	−0.39	−0.26	0.36	0.40	0.28	−0.27
	Betatropis formosana	−0.45	−0.35	−0.18	−0.26	−0.18	−0.28	0.35	−0.42	−0.25	0.37	0.37	0.34	−0.40
	Magadhaideus luodiana	−0.46	−0.52	−0.18	−0.26	−0.27	−0.26	0.34	−0.41	−0.33	0.31	0.43	0.28	−0.37
	Paracatonidia sp.	−0.45	−0.75	−0.21	−0.34	−0.24	−0.28	0.36	−0.51	−0.39	0.39	0.56	0.38	−0.46
	Plectoderini sp.	−0.45	−0.44	−0.21	−0.25	−0.27	−0.26	0.33	−0.49	−0.31	0.38	0.51	0.36	−0.40
Fulgoridae	*Lycorma delicatula*	−0.33	−0.63	−0.18	−0.32	−0.29	−0.27	0.37	−0.54	−0.34	0.30	0.48	0.30	−0.33
	Lycorma meliae	−0.34	−0.55	−0.18	−0.33	−0.28	−0.27	0.39	−0.55	−0.35	0.35	0.57	0.30	−0.36
	Pyrops candelaria	−0.39	−0.40	−0.14	−0.14	−0.15	−0.19	0.26	−0.30	−0.31	0.19	0.58	0.19	−0.27
	Aphaena discolor	−0.35	−0.52	−0.18	−0.25	−0.25	−0.23	0.37	−0.49	−0.25	0.30	0.59	0.26	−0.35
	Aphaena amabilis	−0.35	−0.57	−0.15	−0.24	−0.27	−0.26	0.31	−0.49	−0.30	0.27	0.61	0.28	−0.30
Delphacidae	*Changeondelphax velitchkovskyi*	−0.38	−0.68	−0.26	−0.31	−0.30	−0.30	0.21	−0.42	−0.16	0.27	0.54	0.30	−0.12
	Laodelphax striatellus	−0.44	−0.54	−0.19	−0.20	−0.14	−0.23	0.17	−0.31	−0.15	0.25	0.35	0.21	−0.20
	Nilaparvata bakeri	−0.37	−0.57	−0.18	−0.28	−0.20	−0.19	0.11	−0.44	−0.19	0.29	0.42	0.22	−0.21
	Nilaparvata lugens	−0.39	−0.47	−0.17	−0.27	−0.13	−0.16	0.11	−0.40	−0.26	0.27	0.49	0.26	−0.23
	Nilaparvata muiri	−0.40	−0.41	−0.18	−0.25	−0.16	−0.19	0.08	−0.42	−0.06	0.26	0.47	0.22	−0.21
	Peregrinus maidis	−0.40	−0.67	−0.19	−0.30	−0.22	−0.21	0.15	−0.33	−0.23	0.23	0.68	0.28	−0.31
	Saccharosydne procerus	−0.36	−0.56	−0.14	−0.22	−0.15	−0.20	0.19	−0.39	−0.31	0.25	0.45	0.22	−0.30
	Sogatella furcifera	−0.31	−0.55	−0.15	−0.18	−0.04	−0.16	0.11	−0.24	−0.04	0.08	0.51	0.15	−0.22
	Sogatella vibix	−0.27	−0.30	−0.16	−0.17	−0.08	−0.14	0.06	−0.37	−0.16	0.12	0.51	0.16	−0.09
	Sogatella kolophon	−0.29	−0.20	−0.15	−0.17	−0.10	−0.19	0.11	−0.29	−0.06	0.10	0.67	0.12	−0.15
	Ugyops sp.	−0.40	−0.53	−0.15	−0.30	−0.20	−0.28	0.41	−0.45	−0.29	0.28	0.52	0.29	−0.39
Issidae	*Hemisphaerius rufovarius*	−0.28	−0.52	−0.12	−0.16	−0.14	−0.17	0.30	−0.38	−0.31	0.32	0.50	0.25	−0.36
	Sivaloka damnosus	−0.30	−0.62	−0.13	−0.25	−0.13	−0.20	0.22	−0.37	−0.23	0.32	0.50	0.30	−0.38
	Ricania marginalis	−0.33	−0.39	−0.12	−0.21	−0.10	−0.22	0.12	−0.29	−0.23	0.23	0.45	0.17	−0.26
Ricaniidae	*Ricania speculum*	−0.31	−0.39	−0.15	−0.16	−0.10	−0.24	0.25	−0.29	−0.22	0.24	0.45	0.18	−0.24
	Ricania shantungensis	−0.40	−0.14	−0.15	−0.25	−0.14	−0.25	0.34	−0.29	−0.27	0.24	0.41	0.24	−0.27
Flatidae	*Geisha distinctissima*	−0.35	−0.36	−0.16	−0.23	−0.18	−0.27	0.38	−0.43	−0.18	0.31	0.47	0.28	−0.28
Caliscelidae	*Bambusicaliscelis flavus*	−0.43	−0.58	−0.12	−0.27	−0.16	−0.24	0.37	−0.45	−0.32	0.32	0.61	0.35	−0.33
	Bambusicaliscelis fanjingensis	−0.41	−0.56	−0.12	−0.31	−0.23	−0.21	0.36	−0.53	−0.31	0.34	0.55	0.30	−0.36
	Youtuus strigatus	−0.37	−0.65	−0.12	−0.21	−0.20	−0.21	0.29	−0.49	−0.25	0.31	0.56	0.22	−0.29
	Youtuus erythrus	−0.32	−0.75	−0.10	−0.23	−0.16	−0.22	0.26	−0.44	−0.34	0.33	0.63	0.28	−0.31

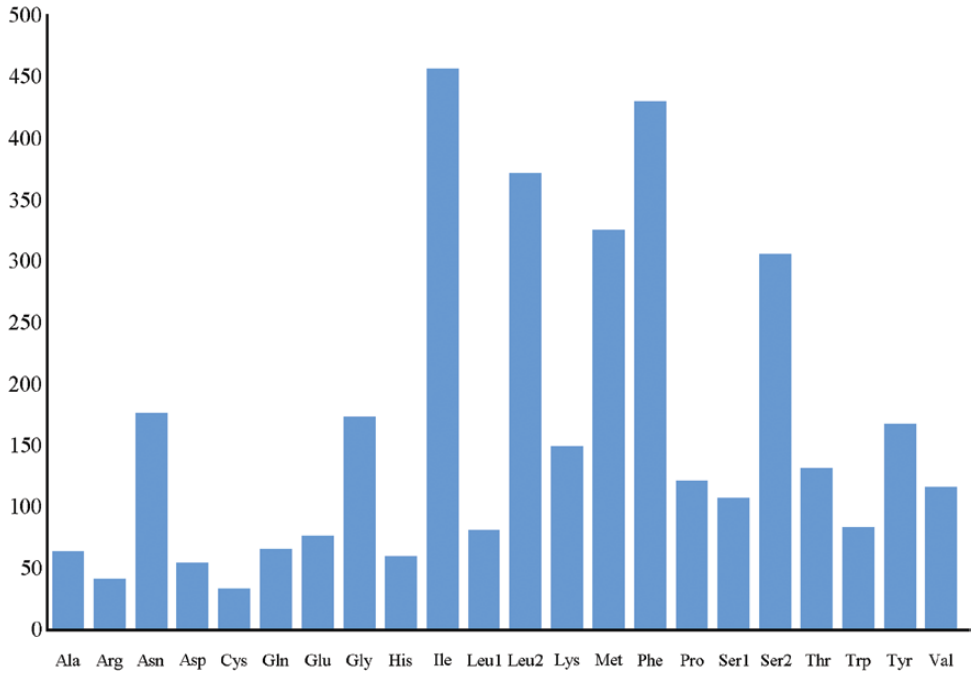

Fig. 4. Usage of amino acids of 13 PCGs in the *N. fuliginosa* mitogenome. Numbers to the left refer to the total number of the codon. Codon families are indicated below the x-axis.

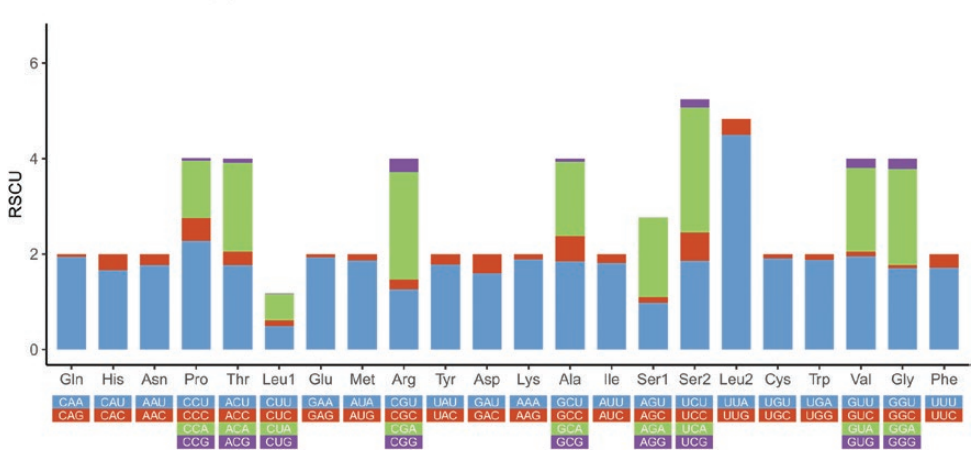

Fig. 5. Relative synonymous codon usage of amino acids of 13 PCGs in the mitochondrial genome of *N. fuliginosa*. Codon families are indicated below the x-axis.

Whether other, as yet undiscovered, factors affect gene rearrangement requires more in-depth research (Song and Liang 2009b). Currently, data of eight known families of Fulgoroidea show that only Delphacidae have rearrangements. Although base mismatches and gene spacers/overlaps have also appeared in other families, no rearrangements have been detected. Therefore, extensive research is still required to accurately study the rearrangement mechanisms of Fulgoroidea genomes.

There are three gene overlapping regions in the mitochondrial genome of *N. fuliginosa*, the longest overlapping sequence is located between *atp8* and *atp6* and has a length of 7 bp. Among the Fulgoroidea insects, *atp8* and *atp6* overlap by 7 bp in most species, whereas they overlap by 4 bp in some species and by 1 bp in *Nilaparvata lugens*. The AT content of the mitogenome (no control region) of *N. fuliginosa* was found to be 80.3% and the GC content was found to be 19.7%, showing obvious AT preference. This percentage is between the AT content of *Magadhaideus luodiana*, *Pyrops candelaria* (both 74.3%), and *Saccharosydne procerus* (80.5%) (Song et al. 2012, Huang and Qin 2018, Xu et al. 2019). We also compared the AT and GC skews of 13 PCGs of 32 Fulgoroidea mitogenomes. We observed on the L-strand, the AT skew was negative and the GC skew was positive. On the H-strand, the AT skew could be positive or negative, but the GC skew was always negative. The gene set on the H-strand was C-skewed and that on the L-strand was G-skewed (Yu and Liang 2018).

Table 6. Codon number and RSCU in *N. fuliginosa* mitochondrial PCGs

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	400	1.86	UCU(S)	118	2.27	UAU(Y)	152	1.81	UGA(W)	77	1.83
UUC(F)	30	0.14	UCC(S)	16	0.31	UAC(Y)	16	0.19	UGG(W)	7	0.17
UUA(L)	342	4.54	UCA(S)	168	3.24	CAU(H)	44	1.47	CGU(R)	18	1.71
UUG(L)	29	0.38	UCG(S)	4	0.08	CAC(H)	16	0.53	CGC(R)	0	0.00
CUU(L)	35	0.46	CCU(P)	48	1.57	CAA(Q)	59	1.79	CGA(R)	23	2.19
CUC(L)	2	0.03	CCC(P)	14	0.46	CAG(Q)	7	0.21	CGG(R)	1	0.10
CUA(L)	41	0.54	CCA(P)	60	1.97	AAU(N)	151	1.72	AGU(S)	27	0.52
CUG(L)	3	0.04	CCG(P)	0	0.00	AAC(N)	25	0.28	AGC(S)	1	0.02
AUU(I)	416	1.82	ACU(T)	54	1.64	AAA(K)	140	1.87	AGA(S)	74	1.43
AUC(I)	42	0.18	ACC(T)	14	0.42	AAG(K)	10	0.13	AGG(S)	7	0.13
AUA(M)	296	1.82	ACA(T)	63	1.91	GAU(D)	43	1.56	GGU(G)	74	1.70
AUG(M)	30	0.18	ACG(T)	1	0.03	GAC(D)	12	0.44	GGC(G)	6	0.14
GUU(V)	59	2.02	GCU(A)	30	1.88	GAA(E)	68	1.77	GGA(G)	83	1.91
GUC(V)	1	0.03	GCC(A)	7	0.44	GAG(E)	9	0.23	GGG(G)	11	0.25
GUA(V)	53	1.81	GCA(A)	26	1.62	UGU(C)	30	1.76	UAA(*)	10	0.34
GUG(V)	4	0.14	GCG(A)	1	0.06	UGC(C)	4	0.24	UAG(*)	0	0.00

*Termination codons.

The 13 PCGs of the mitogenome of *N. fuliginosa* were found to be 10,839 bp in length, except for the start codon of the *nad1* gene (GTG), all other PCG start codons are ATN. In Fulgoroids, it has been found that the *nad1* gene of four species also use GTG as the start codon (Song et al. 2010, Xu et al. 2019) and the *nad5* gene of the seven species use GTG as the start codon (Zhang et al. 2014, Huang and Qin 2018, Yu and Liang 2018, Gong et al. 2021); in addition, the *nad5* gene starts with TTG (Xu et al. 2019, Yang et al. 2020). The termination codons of 10 PCGs are all TAA and the remaining genes (*cox2*, *nad6*, and *nad1*) use an incomplete stop codon T. In Fulgoroids, most PCGs use TAA as the stop codon, some use incomplete T, and a few use TAG.

Through the analysis of the secondary structure of the tRNAs, we found that the three tRNA genes of *trnC*, *trnV*, and *trnS1* lacked the DHU arm and the remaining 19 tRNA genes can form a typical cloverleaf structure. In Fulgoroidea, barring the fact that the *trnS1* genes in most insects lack DHU arms, the *trnV* genes in *Aphaena discolor*, *A. amabilis, Bambusicaliscelis flavus*, *B. fanjingensis*, *Youtuus Strigatus*, and *Y. erythrus*; the *trnG* and *trnS2* genes in *Sogatella furcifera*; the *trnH* in *Laodelphax striatellus*; and the *trnC* in *Youtuus Strigatus* and *Y. erythrus* also cannot form a typical cloverleaf structure (Song and Liang 2009a, 2009b; Zhang et al. 2014; Zhang et al. 2016; Yu and Liang 2018; Wang et al. 2019; Xu et al. 2019; Gong et al. 2021). Twenty-one wobble base pairs (G-U) were detected in 13 genes of the tRNA structure, among which *trnQ* and *trnL1* had the highest rate (four pairs each). In addition, two pairs of U-U base mismatches (in *trnP* and *trnL2*) and four pairs of A-A base mismatches (in *trnW*, *trnA*, *trnE,* and *trnT*) were found. Wobble and mismatched pairs, which commonly occur in insect tRNAs, are usually corrected during the editing processes (Lavrov et al. 2000).

Phylogenetic analyses were based on the nucleotide sequences of the 13 PCGs (tree1) and 13 PCGs + 2 rRNAs (tree 2) from the mitogenomes of 32 species of Fulgoroidea. In this study, both tree 1 and tree 2 showed that the families Delphacidae formed monophyletic group and was located at the base of the tree, which is consistent with the results of many previous phylogenetic studies based on the external morphological characteristics, single-nuclear genes and mitochondrial genes (Muir 1923, Emeljanov 1991, Bourgoin 1993, Yeh et al. 2005, Urban and Cryan 2007, Yu and Liang 2018, Xu et al. 2019, Gong et al. 2021). Meenoplidae and six other families formed another group that was located near the clade of Delphacidae, consistent with findings of previous studies (Muir 1923, Emeljanov 1991, Bourgoin 1993, Yeh et al. 2005, Urban and Cryan 2007). Three families (Ricaniidae, Issidae, and Flatidae) formed a consistent clade, in concordance with previous studies (Asche 1987, Huang and Qin 2018, Wang et al. 2019, Xu et al. 2019, Gong et al. 2021). Caliscelidae, Issidae, Ricaniidae, and Flatidae are closely related and they gathered to form a sister group to Achilidae, this was consistent with the research of Gong et al. (2021). In some studies, Achilidae was located near the clade of Fulgoridae, whereas, in our study, it was far from the clade of Fulgoridae; however, our findings are consistent with those of some previous studies (Song et al. 2012, Xu et al. 2019, Yang et al. 2020), which may be caused by different taxa sampling or tree construction methods. Although the known mitogenomes from Fulgoroidea are still limited, our study will be helpful to provide a molecular basis for the classification and phylogeny of Fulgoroidea. Besides, further mitochondrial genome research is needed to better understand the evolutionary status and phylogenetic relationships of Fulgoroidea in the future.

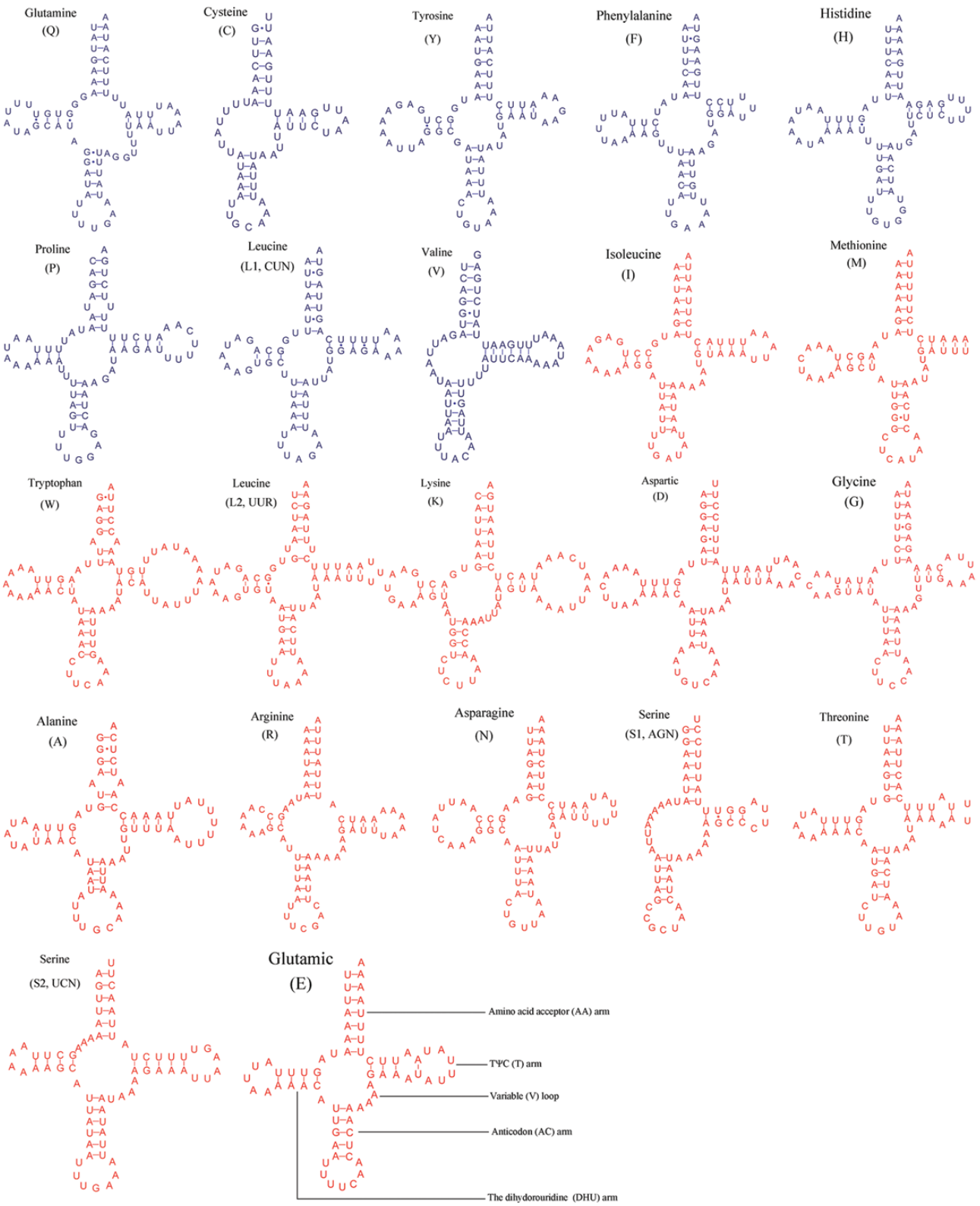

Fig. 6. Secondary structure of 22 tRNA genes from *N. fuliginosa* mitogenome. (The standard base-pairing bonds of A-U and G-C are represented by straight lines, while G-U pairings are indicated by points. The tRNAs located on the L-strand are shown in blue, while those located on the H-strand are shown in red.)

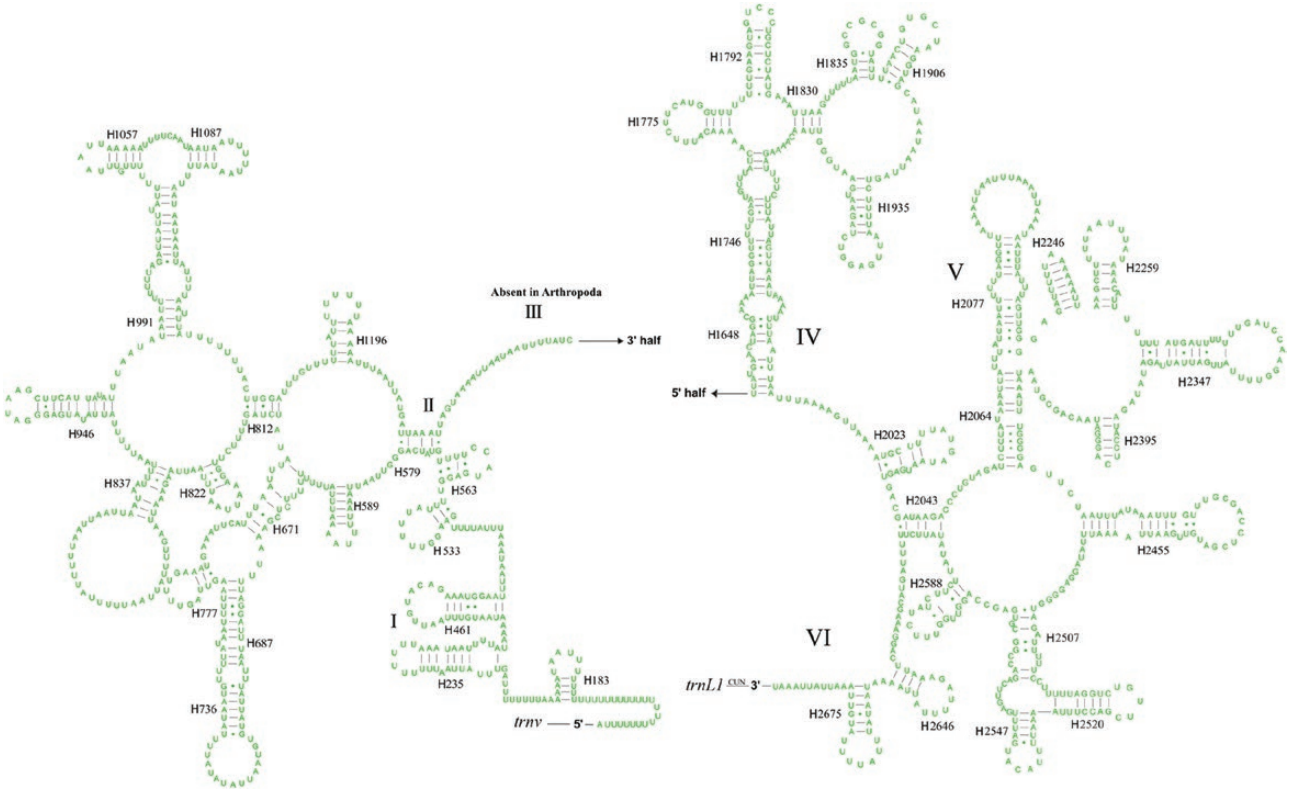

Fig. 7. Predicted secondary structure of *rrnL* in the *N. fuliginosa* mitogenome. (The standard base-pairing bonds of A-U and G-C are represented by straight lines, while G-U pairings are indicated by points.)

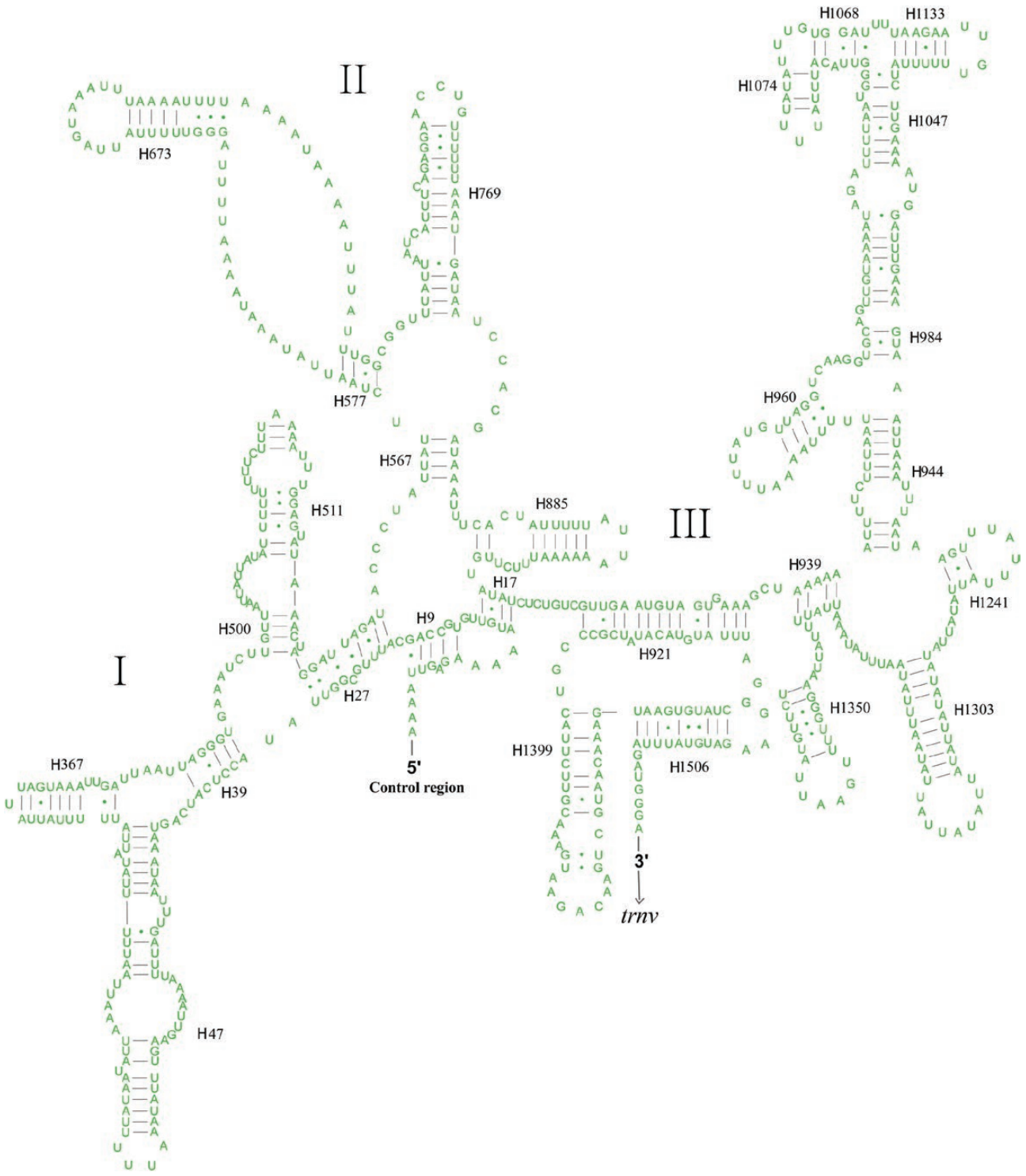

Fig. 8. Predicted secondary structure of *rrnS* in the *N. fuliginosa* mitogenome. (The standard base-pairing bonds of A-U and G-C are represented by straight lines, while G-U pairings are indicated by points.)

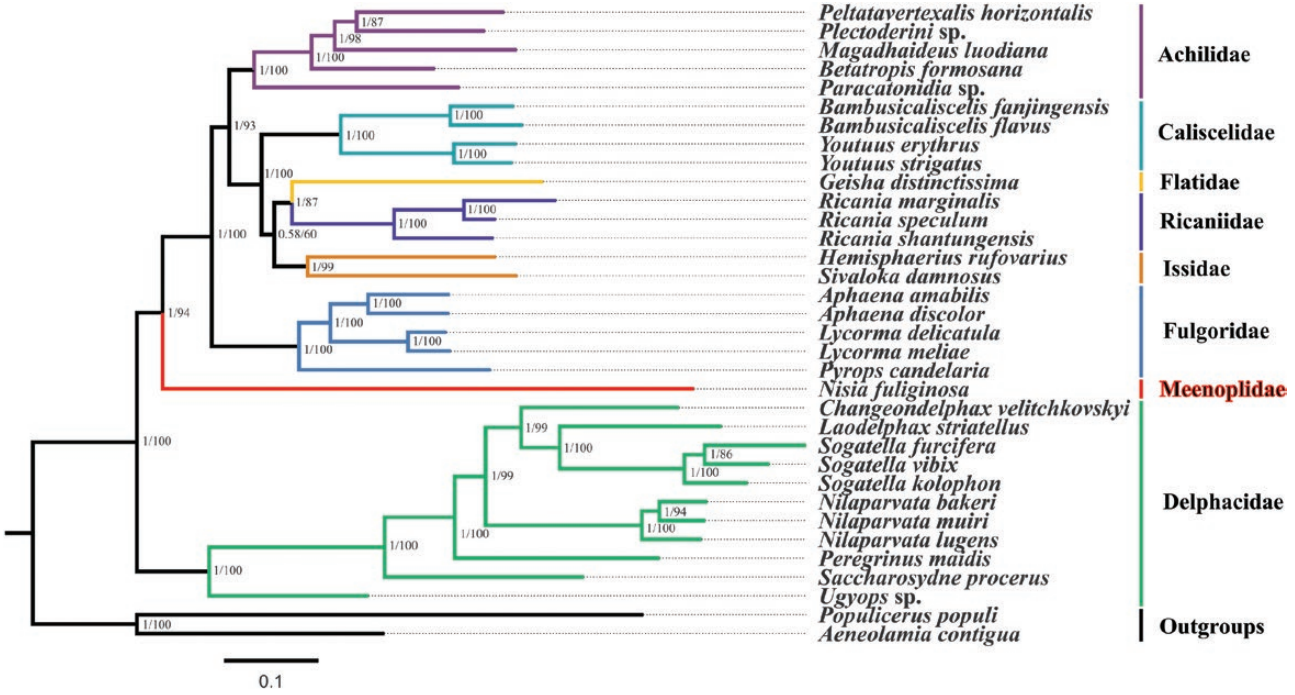

Fig. 9. Phylogenetic tree of 32 species of Fulgoroidea based on the nucleotide sequences of mtDNA 13 PCGs using ML. Numbers on the branches indicate Bayesian Inference posterior probability (PP, left) and ML bootstrap support (BS, right).

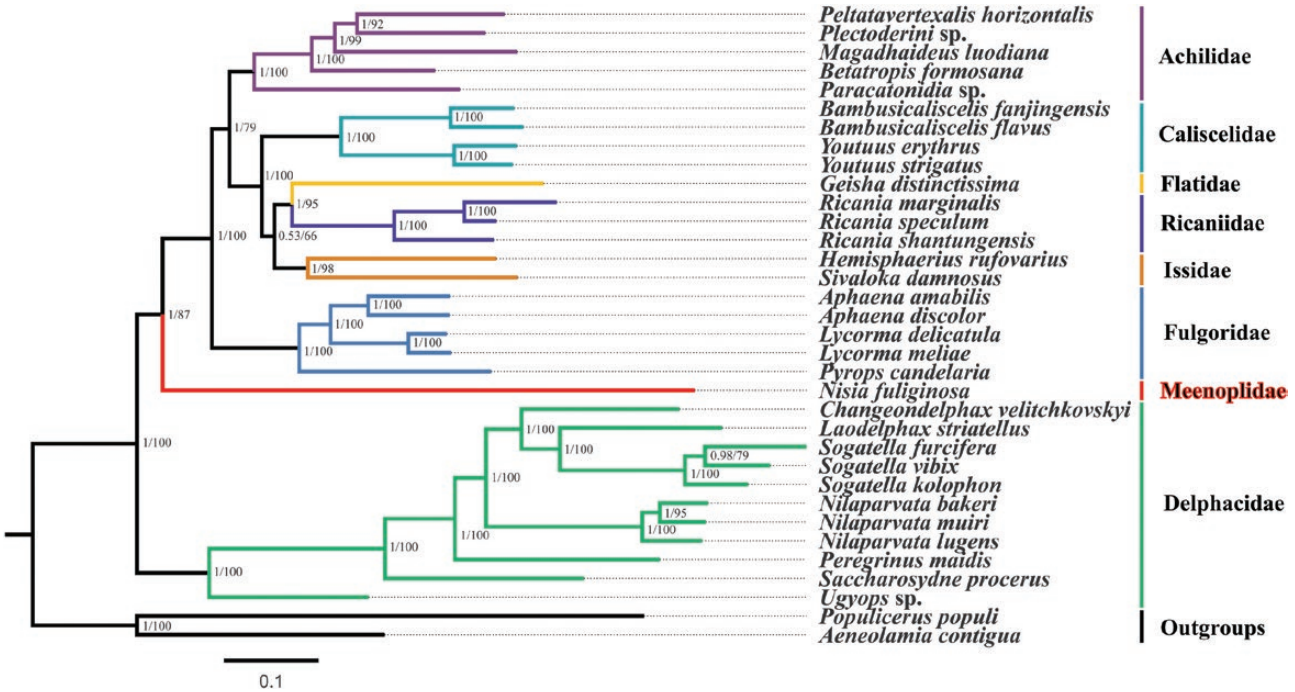

Fig. 10. Phylogenetic trees of 32 species of Fulgoroidea based on the nucleotide sequences of mtDNA 13 PCGs + 2 rRNAs using ML. Numbers on the branches indicate Bayesian Inference posterior probability (PP, left) and ML bootstrap support (BS, right).

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Author Contributions

S.-S.L. and X.-S.C. conceived the original idea. S.-S.L. carried out the experiment. S.-S.L. wrote the manuscript with support from Y.-J.Z. and X.-S.C. Y.J.Z. and N.G. offered great in data analysis.

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