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The Complete Mitochondrial Genomes of *Aelia sibirica* and *A. fieberi* (Hemiptera, Pentatomidae), and Phylogenetic Implications

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Abstract: Species of genus *Aelia* are important pests of wheat crops in arid areas. In this study, the mitogenomes of *A. sibirica* and *A. fieberi* were sequenced using high-throughput sequencing technology. The mitochondrial genome characteristics of both *Aelia* species were compared and analyzed, and the phylogenetic relationships of Pentatomidae were constructed based on protein-coding genes. In addition, the taxonomic status of the genus *Aelia* was confirmed. The results showed that the total length of the mitogenome sequences of *A. sibirica* and *A. fieberi* were 15,372 bp and 15,450 bp, respectively, including 13 protein-coding genes, 22 tRNA genes, 2 rRNA genes, and a control region. By comparing the mitochondrial genome structure, base composition, codon usage, RNA secondary structure, and other characteristics, it was found that the mitochondrial genome characteristics of the two species were similar. Phylogenetic analysis showed that Phyllocephalinae and Asopinae both formed monophyletic groups, but the relationship between Podopinae and Pentatominae was not resolved. Within the subfamily Pentatominae, (Nezarini + Antestiini), (Aeliini + Carpocorini), and (Strachiini + *Pentatoma*) formed stable clades. *Aelia sibirica* and *A. fieberi* were found to be a stable sibling pair, and the clade was closely related to *Dolycoris baccarum*.

Keywords: *Aelia fieberi*; *Aelia sibirica*; mitochondrial genome; Pentatomidae; phylogeny

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1. Introduction

The mitochondrial genome is characterized by its compact size, genetic stability, rapid evolutionary rate, and maternal inheritance, and has emerged as one of the most commonly used molecular markers in evolutionary studies [1–6]. In hemipteran insects, the mitochondrial genome typically consists of a circular double-stranded DNA ranging from 14 to 20 kb and encompassing 13 protein-coding genes (PCGs), 22 tRNA genes, 2 rRNA genes, and a control region [7–9]. The known structure of hemipteran mitochondrial genomes exhibits a high degree of conservation, with a tight arrangement of genes and only sporadic occurrences of tRNA gene rearrangements observed among certain species [10]. The overall gene order remains largely consistent with that of ancestral insects [11,12]. The advent of high-throughput sequencing technologies has allowed the mitochondrial genome to be widely used in molecular evolutionary studies, phylogenetic analyses, and investigations pertaining to biogeography within hemipteran taxa [13–15].

The insect family Pentatomidae comprises a diverse range of species, and is the largest family in the superfamily Pentatomoidea. It is widely distributed across the world, with nearly 5000 species belonging to 896 genera and ten subfamilies [16]. The classification of higher taxonomic ranks within Pentatomidae has been the source of considerable disagreement among researchers, despite the increasing number of studies and abundant data. Based on morphological and anatomical characteristics, Pentatomidae has been successively divided into 11, 9, 8, and 10 subfamilies [17–20]. Originally, seven subfamilies were identified for Pentatomidae species in China; however, with the development of

molecular biology technology and the comprehensive analysis with multiple characters, the classification of Pentatomidae has been changed a lot [21–23]. Therefore, Chinese Pentatomidae currently includes four subfamilies: Phyllocephalinae, Asopinae, Pentatominae, and Podopinae. Previous phylogenetic studies of Pentatomidae subfamilies and tribes were constructed based on morphological characters. With the wide application of multiple molecular data, morphological characters were combined with molecular data for species classification [20,24]. Studies on the phylogenetic relationships within the subfamilies of Pentatomidae and the tribes within Pentatominae are insufficient, with most studies predominantly based on morphological characteristics or limited molecular data. Only a few taxa have been studied, and there is currently no widely recognized viewpoint [25–28]. Despite the vast number of Pentatomidae species, there is a lack of mitochondrial genomic research specifically targeting this important taxonomic group. To date, mitochondrial genome data are available for only 72 species (https://www.ncbi.nlm.nih.gov/, accessed on 2 January 2024); therefore, it is important to conduct mitochondrial genomic studies on Pentatomidae insects and gain comprehensive insights into the diversity and evolutionary characteristics of their mitochondrial genomes, with a view to enabling more accurate taxonomic assignments.

The genus *Aelia* belongs to the family Pentatomidae, and is widely distributed in the Palearctic region and North America [29,30]. It is an important agricultural pest in the arid areas of northern China and Central Asia, and primarily infests wheat crops, sucking on the ears of wheat and causing serious harm to wheat sprouts. It is essential to conduct comprehensive studies on this pest, which would be conducive to the long-term development of the agricultural economy. This study aimed to sequence the mitochondrial genomes of *A. sibirica* Reuter, 1884 and *A. fieberi* Scott, 1874 using Illumina MiSeq next-generation sequencing, and analyze the genomic composition and structural characteristics. By incorporating the mitochondrial sequence data from representative species of the Pentatomidae obtained from the GenBank database, Bayesian inference (BI) and maximum likelihood (ML) phylogenetic trees were constructed based on the PCG sequences.

2. Materials and Methods

2.1. Sampling, DNA Extraction, and Sequencing

The adult specimens of *A. sibirica* used in this experiment were collected on 14 August 2019, from the Sanjiangyuan National Nature Reserve in Qinghai Province. The adult specimens of *A. fieberi* were collected on 10 August 2019 from Shanfanao in Henan Province. Specimens were preserved in 100% ethanol and stored at −20 °C until used for DNA extraction. The leg muscle tissues of *A. sibirica* and *A. fieberi* were extracted, and genomic DNA extraction was performed following the instructions of the TIANamp Genomic DNA Kit (Tiangen biotechnology, Beijing, China). Voucher specimens were deposited at the Animal Systematics Laboratory, Department of Biology, Xinzhou Normal University, Xinzhou, China. The quality of the extracted DNA was assessed using a NanoDrop 2000 nucleic acid analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The mitogenome was sequenced using the whole-genome shotgun method on an Illumina Miseq platform (Personalbio, Shanghai, China).

2.2. Genome Annotations and Sequence Analyses

The sequencing data was assembled using A5-miseq v. 20150522 [31] and SPAdes v. 3.9 [32] software. The mitochondrial genome sequences were annotated by Geneious 10.1.3 [33]. The annotation of protein-coding genes was conducted by Open Reading Frame Finder on the NCBI website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html, accessed on 13 December 2021), as well as identifying start and stop codons. The tRNA genes were automatically annotated by MITOS (http://mitos.bioinf.uni-leipzig.de/index.py/, accessed on 13 December 2021) [34] with the invertebrate mitochondrial code. The annotation of rRNA genes was based on published reference sequence (*Eurydema dominulus* (Scopoli, 1763)) and combined with their putative secondary structures. The locations of the control

region were identified by the boundary of neighboring genes. The newly sequenced and annotated mitogenomes were submitted to GenBank with accession numbers NC_067883 (*A. sibirica*) and ON059969 (*A. fieberi*). The mitochondrial genome maps were generated by GCView Server [35]. The base composition and relative synonymous codon usage (RSCU) were performed using MEGA 7.0 [36]. Additionally, AT skew was calculated using the formula (A − T)/(A + T), while GC skew was calculated using (G − C)/(G + C) [37]. The number of tandem repeats of control region was investigated with Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.html, accessed on 20 March 2022) [38].

2.3. Phylogenetic Analysis

In order to investigate the phylogenetic relationships among different taxonomic levels within Pentatomidae, and the phylogenetic relationship between the genus *Aelia* and its closely related genera, this study selected 27 representative species from the family Pentatomidae to construct phylogenetic trees (Table 1). The selected 27 taxa covered 4 subfamilies of Pentatomidae and 13 tribes of Pentatominae which existed in the NCBI database. In addition, *Sastragala esakii* (Pentatomoidea: Acanthosomatidae) was selected as the outgroup. The maximum likelihood method (ML) and Bayesian inference (BI) were used to construct phylogenetic trees based on 13 protein-coding genes. Multiple alignments of protein-coding gene sequences were performed using MEGA 7.0, and the aligned gene sequence matrices were concatenated using the SequenceMatrix 1.8 [39]. The BI tree was generated using MrBayes v. 3.2 [40], with the GTR + I + G model selected and run for 10,000,000 generations. The program was stopped when the convergence diagnostic value was below 0.01. The ML tree was constructed using RAxML v. 7.0.3 [41], and branch support was assessed using 1000 bootstrap replicates.

Table 1. List of sequences used to reconstruct the phylogenetic tree.

Placement			Species	GenBank Accession Number
Acanthosomatidae			*Sastragala esakii*	NC_058975
Pentatomidae	Phyllocephalinae		*Gonopsis affinis*	NC_036745
			Dalsira scabrata	KX505855
	Asopinae		*Arma chinensis*	MW355500
			Cazira horvathi	MF497718
			Dinorhynchus dybowskyi	NC_037724
			Eocanthecona furcellata	MZ440302
			Stiretrus anchorago	BK059217
			Zicrona caerulea	MW847250
	Podopinae		*Deroploa parva*	MW679032
			Graphosoma rubrolineatum	KX267740
			Scotinophara lurida	MF497733
	Pentatominae	Aeliini	*Aelia fieberi*	ON059969
			Aelia sibirica	NC_067883
		Antestiini	*Plautia crossota*	MK757497
		Sephelini	*Brachymna tenuis*	MF497711
		Eysarcorini	*Carbula sinica*	NC_037741
		Caystrini	*Caystrus obscurus*	MF497717
		Carpocorini	*Dolycoris baccarum*	JQ743672
		Halyini	*Erthesina fullo*	MK374364
		Strachiini	*Eurydema dominulus*	MG584833
		Cappaeini	*Halyomorpha halys*	LC579925
		Hoplistoderini	*Hoplistodera incisa*	MF620037

Table 1. *Cont.*

Placement		Species	GenBank Accession Number
Pentatominae	Menidini	*Menida violacea*	MF497728
	Nezarini	*Nezara viridula*	NC_011755
		Chinavia impicticornis	MG253262
	Pentatomini	*Pentatoma semiannulata*	MT985377
		Placosternum urus	MF497730

3. Results

3.1. Genomic Features

The mitochondrial genomes of two *Aelia* species were obtained using high-throughput sequencing. The complete mitochondrial genome sequence of *A. sibirica* was 15,372 bp (Figure 1A), whereas that of *A. fieberi* was 15,450 bp (Figure 1B). Both genomes consisted of 13 PCGs, 22 tRNA, and two rRNA genes and a control region (Table 2). This gene arrangement was consistent with the typical order observed in Heteroptera insects without any specific rearrangements. The N-strand encoded fourteen genes, including four protein-coding, *nad1*, *nad4*, *nad4L*, and *nad5*; eight tRNA, *trnQ*, *trnC*, *trnY*, *trnF*, *trnH*, *trnP*, *trnL1*, and *trnV*; and the *12S rRNA* and *16S rRNA*. The remaining genes were encoded on the J-strand.

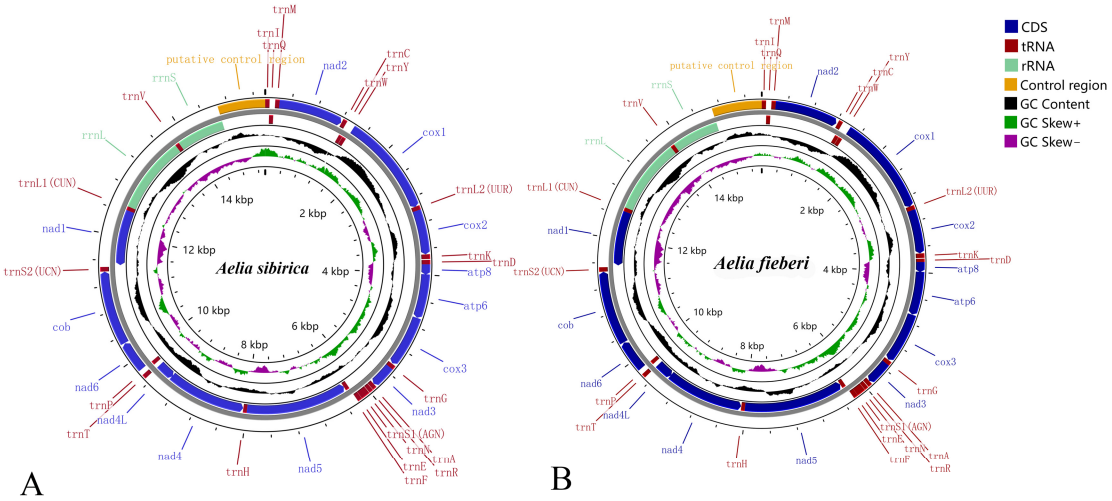

Figure 1. Mitochondrial genome structures of *A. sibirica* (**A**) and *A. fieberi* (**B**).

Table 2. Organization of the mitochondrial genomes of *A. sibirica* and *A. fieberi*.

Gene	Size (bp)	Position		IGS (bp)	Anti-Codon	Start Codon	Stop Codon
		Start	Stop				
trnI	66/66	1/1	66/66	0/8	GAU	—	—
trnQ	69/69	64/75	132/143	−3/−3	UUG	—	—
trnM	66/66	152/150	217/215	19/6	CAU	—	—
nad2	987/987	218/216	1204/1202	0/0	—	ATC	TAA
trnW	66/66	1211/1221	1276/1286	6/18	UCA	—	—
trnC	62/66	1269/1279	1330/1344	−8/−8	GCA	—	—
trnY	65/65	1334/1348	1398/1412	3/3	GUA	—	—
cox1	1542/1542	1408/1422	2949/2963	9/9	—	TTG	TAA
$trnL2^{UUR}$	65/66	2945/2959	3009/3024	−5/−5	UAA	—	—

Table 2. *Cont.*

Gene	Size (bp)	Position		IGS (bp)	Anti-Codon	Start Codon	Stop Codon
		Start	Stop				
cox2	679/679	3010/3025	3688/3703	−5/0	—	ATA	T
trnK	71/70	3689/3704	3759/3773	0/0	CUU	—	—
trnD	66/66	3774/3770	3839/3835	14/−4	GUC	—	—
atp8	159/159	3840/3836	3998/3994	0/0	—	TTG/GTG	TAA
atp6	675/675	3992/3988	4666/4662	−7/3	—	ATG	TAA
cox3	789/789	4669/4665	5457/5453	2/2	—	ATG	TAA
trnG	63/63	5464/5458	5526/5520	6/4	UCC	—	—
nad3	352/377	5527/5521	5878/5897	0/0	—	ATA	TAG/T
trnA	64/66	5879/5898	5942/5963	0/0	UGC	—	—
trnR	64/61	5947/5973	6010/6033	4/9	UCG	—	—
trnN	68/67	6016/6037	6083/6103	5/3	GUU	—	—
$trnS1^{AGN}$	70/69	6083/6103	6152/6171	−1/−1	GCU	—	—
trnE	68/69	6155/6173	6222/6241	2/2	UUC	—	—
trnF	66/66	6221/6240	6286/6305	−2/−2	GAA	—	—
nad5	1707/1706	6286/6307	7992/8011	−1/1	—	ATT	TAA/T
trnH	64/64	7994/8013	8057/8076	1/1	GUG	—	—
nad4	1329/1329	8061/8080	9389/9408	3/3	—	ATT/ATG	TAA
nad4L	288/288	9383/9402	9670/9689	−7/−7	—	ATG/ATT	TAA
trnT	67/65	9673/9692	9739/9756	2/2	UGU	—	—
trnP	66/66	9740/9757	9805/9822	0/0	UGG	—	—
nad6	465/480	9816/9833	10,280/10,312	10/10	—	ATG/ATA	TAA
cytb	1137/1147	10,288/10,317	11,424/11,456	7/4	—	ATG	TAA
$trnS2^{UCN}$	69/69	11,426/11,465	11,494/11,533	1/8	UGA	—	—
nad1	924/924	11,522/11,558	12,445/12,481	27/24	—	TTG	TAG
$trnL1^{CUN}$	65/65	12,446/12,482	12,510/12,546	0/0	UAG	—	—
16S rRNA	1282/1272	12,510/12,546	13,791/13,817	−1/−1	—	—	—
trnV	68/68	13,792/13,818	13,859/13,885	0/0	UAC	—	—
12S rRNA	796/795	13,860/13,886	14,655/14,680	0/0	—	—	—
Control region	717/768	14,656/14,681	15,372/15,448	0	—	—	—

The mitochondrial genome of *A. sibirica* contained ten overlapping regions, ranging from 1 to 8 bp, and seventeen intergenic spacer regions, ranging from 1 to 27 bp. In contrast, the mitochondrial genome of *A. fieberi* had eight overlapping regions, ranging from 1 to 8 bp, and nineteen intergenic spacer regions, ranging from 1 to 24 bp.

3.2. Nucleotide Composition and Codon Usage

In the complete mitogenomes, nucleotide composition analysis revealed that both *A. sibirica* and *A. fieberi* had significantly higher AT than GC content. The nucleotide composition in the mitochondrial genomes of both species were similar, and the overall base composition was identical (Table 3). The AT content in the control region was slightly lower than that in the complete mitochondrial genome, PCGs, rRNAs, and tRNAs with values of 70.5% and 69.2%, respectively. Among the PCGs, nad2 had the highest AT content

in *A. sibirica* (79.8%), and nad6 had the highest AT content in *A. fieberi* (80.2%). The gene with the lowest AT content was cox1 (67.6% and 67.0%, respectively). The AT-skew values of the complete mitochondrial genome, 22 tRNAs, and the control region were positive in both *Aelia* species, whereas the AT-skew values for the two rRNAs and thirteen PCGs were negative. Among the PCGs, AT-skew values were negative for *nad1*, *nad4*, *nad4L*, *nad5*, and cytb in both *Aelia* species; *nad6* also had a negative AT-skew value in *A. fieberi*. The GC-skew values for the complete mitochondrial genome, thirteen PCGs, and the control region were negative, whereas the GC-skew values for the two rRNAs and twenty-two tRNAs were positive. Among the PCGs, the AT-skew values were negative, and the GC-skew values were positive for *nad1*, *nad4*, *nad4L*, *nad5*, and *cytb* in both *Aelia* species.

Table 3. Nucleotide composition and skewness of mitochondrial genome of *A. sibirica* and *A. fieberi*.

Gene	A/%	T/%	G/%	C/%	(A + T)/%	AT-Skew	GC-Skew
Whole genome	41.8/41.8	31.7/31.7	11.0/11.0	15.5/15.5	73.5/73.5	0.138/0.138	−0.167/−0.167
13PCGs	33.1/32.6	40.4/40.3	13.1/13.3	13.5/13.8	73.5/72.9	−0.100/−0.105	−0.015/−0.017
22tRNAs	38.4/37.6	37.1/37.2	13.4/13.9	11.1/11.3	75.5/74.8	0.017/0.005	0.094/0.106
2rRNAs	33.1/33.4	44.1/43.6	14.1/14.7	8.6/8.2	77.2/77.1	−0.142/−0.132	0.242/0.283
Control region	35.8/34.0	34.7/35.2	12.3/12.3	17.2/18.5	70.5/69.2	0.016/−0.017	−0.166/−0.203
nad1	24.5/24.7	50.0/50.2	16.8/16.6	8.8/8.5	74.5/74.9	−0.343/−0.341	0.313/0.319
nad2	44.2/44.3	35.6/34.5	8.9/9.2	11.3/12.0	79.8/78.8	0.108/0.123	−0.120/−0.129
nad3	38.4/39.6	35.8/34.2	10.8/11.2	15.1/15.0	74.2/73.8	0.034/0.074	−0.165/−0.146
nad4	26.4/25.8	49.3/50.6	14.6/13.9	9.6/10.3	75.7/75.8	−0.318/−0.335	0.137/0.149
nad4L	26.0/24.7	51.4/49.7	12.8/14.2	9.7/11.5	77.4/74.3	−0.327/−0.336	0.323/0.108
nad5	26.5/24.6	49.3/49.1	14.6/15.4	9.6/11.0	75.8/73.6	−0.301/−0.333	0.205/0.169
nad6	39.8/39.4	38.9/40.8	8.2/7.9	13.1/11.9	78.7/80.2	0.011/−0.0188	−0.232/−0.200
cox1	34.8/34.1	32.9/32.9	14.7/15.2	17.5/17.8	67.7/67.0	0.027/0.018	−0.091/−0.018
cox2	39.8/39.8	29.6/30.2	13.0/13.3	17.7/16.8	69.4/70.0	0.146/0.137	−0.154/−0.118
cox3	34.1/34.3	34.0/33.8	15.0/14.6	17.0/17.2	68.1/68.2	0.002/0.007	−0.063/−0.084
cytb	34.5/34.8	35.1/35.4	12.8/12.1	17.7/17.7	69.6/70.2	−0.009/−0.009	−0.162/−0.187
atp6	38.1/37.2	35.1/35.3	10.7/10.7	16.1/16.9	73.2/72.4	0.041/0.027	−0.204/−0.226
atp8	40.3/42.8	32.1/37.1	9.4/8.2	18.2/11.9	72.4/79.9	0.113/0.071	−0.318/−0.188

3.3. PCGs

The lengths of the 13 PCGs in *A. sibirica* and *A. fieberi* were 11,033 and 11,082 bp, respectively, accounting for 71.77% and 71.73% of the complete mitochondrial genome sequence, respectively, with the exception of *cox1*, *nad1*, and *atp8*, which started with the TTG, TTG/GTG, and TTG codons, respectively; the genes in both species started with the ATN codon. In *A. sibirica*, the numbers of genes starting with the ATC, ATG, ATT, and ATA codons were one, five, two, and two, respectively; whereas in *A. fieberi*, the numbers of genes starting with the ATC, ATG, ATT, and ATA codons were one, four, two, and three, respectively. With the exception of *nad3* and *nad5*, the PCGs in both species had identical stop codons. The preferred stop codon was TAA; however, some genes ended with TAG or an incomplete T.

The relative synonymous codon usage (RSCU) of PCGs for *A. sibirica* and *A. fieberi* were calculated (Figure 2). Excluding stop codons, *A. sibirica* contained 3456 codons, whereas *A. fieberi* contained 3478 codons. The most frequently used codons in *A. sibirica* were AUU (I), UAU (Y), and UUU (F), with 262, 251, and 243 occurrences, respectively. In contrast, the most frequently used codons in *A. fieberi* were UUU (F), AUU (I), and UUA (L), with 284,

248, and 224 occurrences, respectively. Codons with A/U nucleotides were predominant among those with higher usage frequencies.

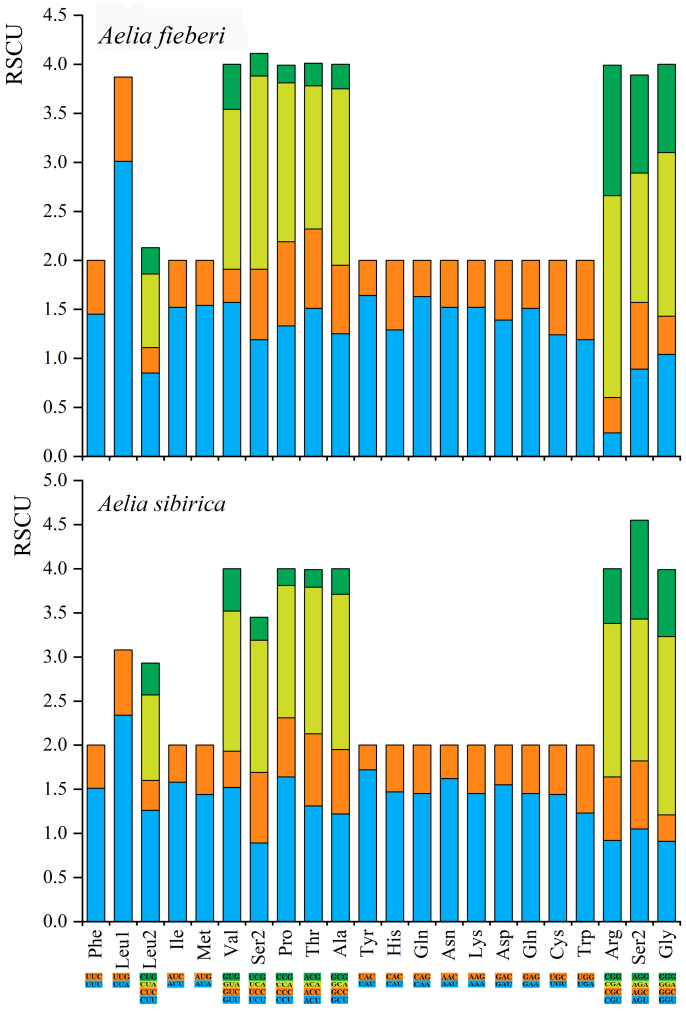

Figure 2. The relative synonymous codon usage (RSCU) in the mitogenomes of *A. sibirica* and *A. fieberi*.

3.4. Ribosomal and Transfer RNA Genes

The mitogenomes of *A. sibirica* and *A. fieberi* contained 22 tRNA genes with total lengths of 1458 and 1453 bp, respectively. The length ranges of the tRNA genes in *A. sibirica* were from 62 bp (*trnC*) to 71 bp (*trnK*), whereas in *A. fieberi*, they ranged from 61 bp (*trnR*) to 70 bp (*trnK*). A comparison of the tRNA secondary structures found minimal differences between the two *Aelia* species. Apart from *trnS1* and *trnV*, which lacked a dihydrouridine arm, the remaining 20 tRNA genes exhibited a typical cloverleaf structure (Figure 3). The number of nonclassical pairings of *A. sibirica* and *A. fieberi* was 18 and 26, respectively, with multiple occurrences of G-U non-canonical pairing.

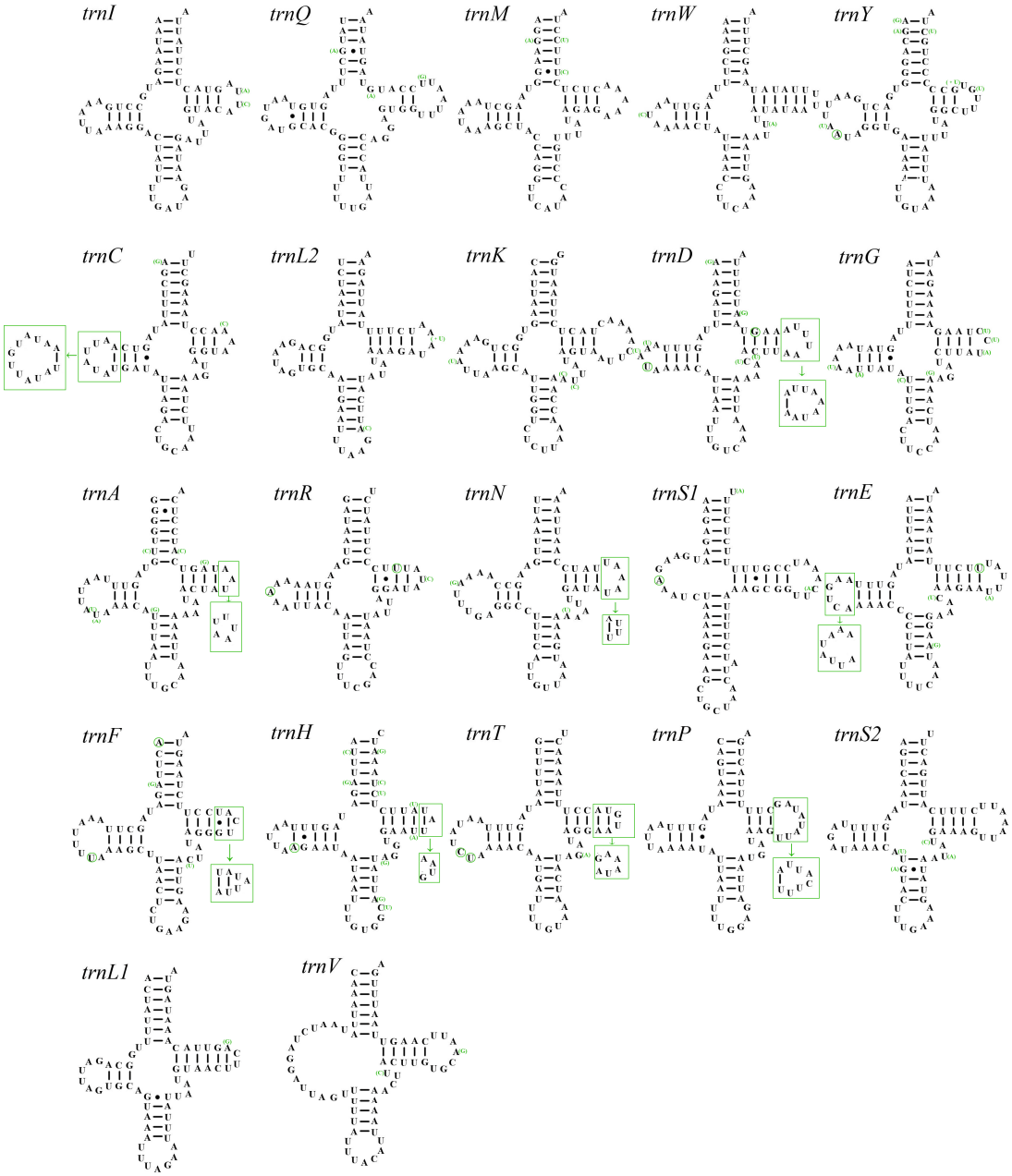

Figure 3. Secondary structures of tRNAs of *A. sibirica* and *A. fieberi* (*A. sibirica* as the template).

The lengths of *12S rRNA* and *16S rRNA* genes in *A. sibirica* were 796 and 1282 bp, respectively, whereas those in *A. fieberi* were 795 and 1272 bp, respectively. The 16S rRNA gene was located between trnL1 and trnV, whereas the *12S rRNA* gene was located between *trnV* and the control region. The secondary structures of the rRNA in *A. sibirica* were compared with those in *A. fieberi* and identified five domains in the *16S rRNA*, whereas the *12S rRNA* contained three domains (Figures 4 and 5). The *12S rRNA* secondary structure in both *Aelia* species showed minimal changes in the structural domains and nucleotide positions, particularly in the loop region. In contrast, domains IV and VI in the secondary structure of *16S rRNA* were more conserved than domains I, II, and V, with variable sites concentrated in various loop regions.

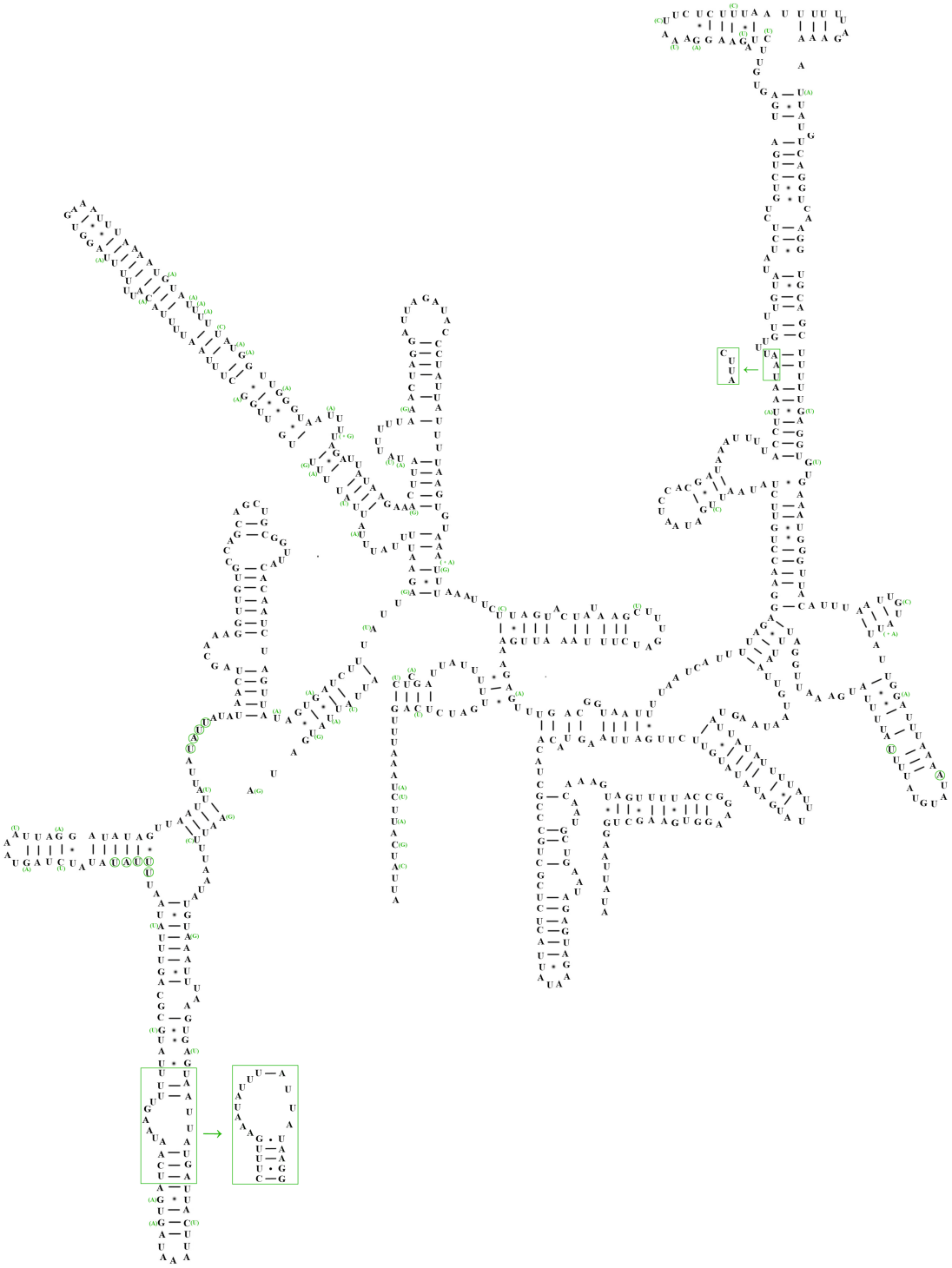

Figure 4. Secondary structure of *12S rRNA* gene of *A. sibirica* and *A. fieberi* (*A. sibirica* as the template).

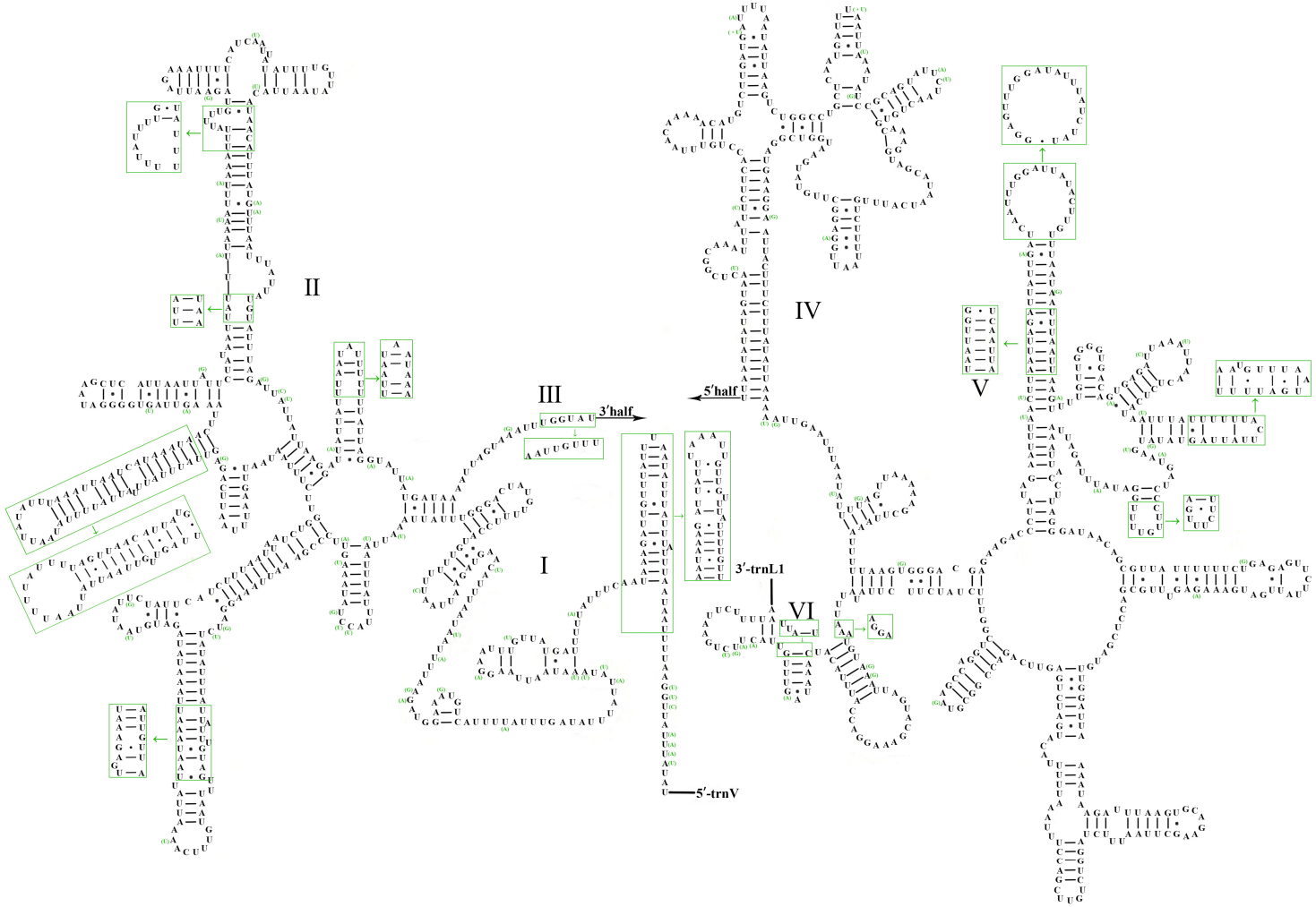

Figure 5. Secondary structure of *16S rRNA* gene of *A. sibirica* and *A. fieberi* (*A. sibirica* as the template).

3.5. Control Region

Complete sequences of the control region in *A. sibirica* and *A. fieberi* were obtained, resulting in lengths of 717 bp and 768 bp, respectively. The control region was located between the *12S rRNA* gene and trnI. The control region of *A. sibirica* had an AT content of 70.5%, whereas the control region of *A. fieberi* had an AT content of 69.2%, both of which were lower than the overall AT content of the complete mitogenomes. The repeat unit of *A. fieberi* was 54 bp, with five copies located between 462 and 731 bp; the repeat unit of *A. sibirica* was 55 bp, with four copies located between 487 and 706 bp.

3.6. Phylogenetic Analyses

Phylogenetic trees of the Pentatomidae were constructed based on 13 PCGs using both the ML and BI methods (Figure 6). The topological structures of the phylogenetic trees were consistent, and most internal nodes had high posterior values. The results showed that the subfamily Phyllocephalinae was the earliest diverging lineage and formed the basal clade of the Pentatomidae. Phyllocephalinae were a sister group to *Placosternum*, currently classified in Pentatominae. Both the subfamilies Phyllocephalinae and Asopinae were found to be monophyletic and showed clear relationships within the subfamilies, supported by high branch support values. In the subfamily Podopinae, *Scotinophara lurida*, *Graphosoma rubrolineata*, and *Deroploa parva* did not cluster into a monophyletic group, but formed sister-group relationships with Pentatominae species. Within the subfamily Pentatominae, some tribes formed stable sibling clades, such as (Aeliini + Carpocorini), (Nezarini + Antestiini), and (Strachiini + *Pentatoma*).

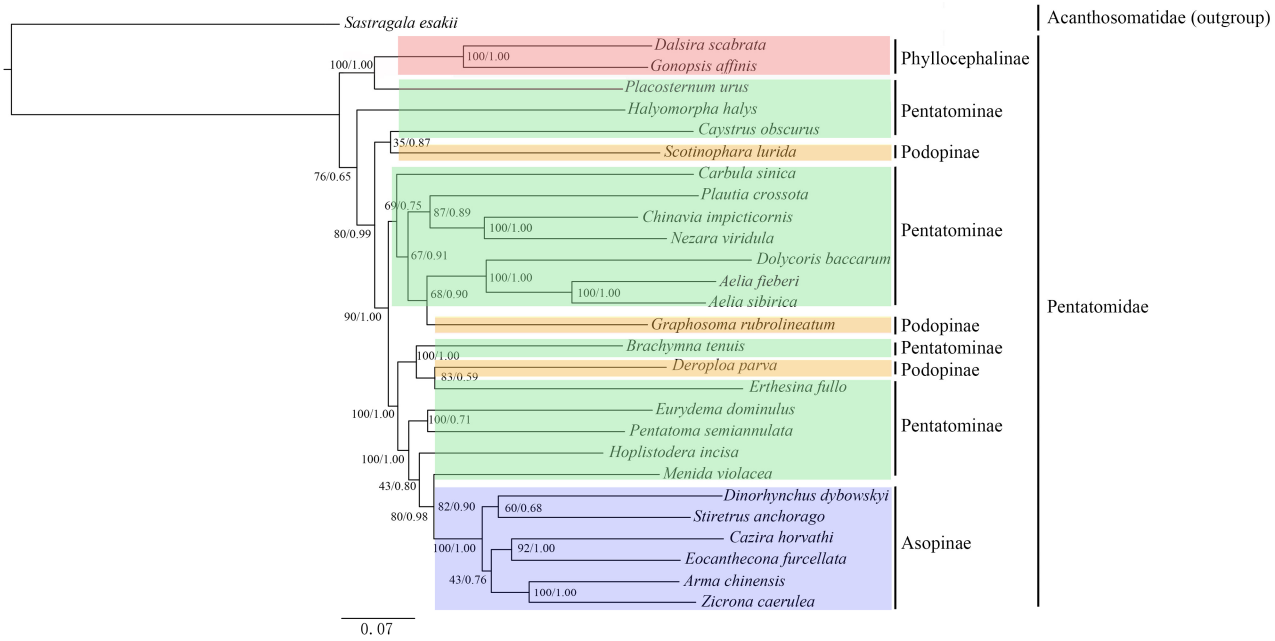

Figure 6. ML and BI phylogenetic trees of Pentatomidae based on the protein-coding genes. Numbers above each node indicate Bayesian posterior probabilities values and ML bootstrap values.

4. Discussion

The sequence lengths of insect mitochondrial genomes are relatively stable and primarily influenced by the length of the control region [42,43]. In this study, the complete mitochondrial genome sequences of two species belonging to the genus *Aelia* were obtained, with lengths of 15,372 and 15,450 bp. The lengths of the control regions were 717 and 768 bp, showing only minor differences. The 37 genes were tightly arranged, and their genomic structure was consistent with that of most Pentatomidae insects, without gene rearrangements or losses [14,44–46]. The longest overlapping region was located between *trnC* and *trnY*, and the longest intergenic region was located between *nad1* and *trnL1*, which

is a common feature of the mitochondrial genomes of Pentatomidae [47]. The A + T content of both species of *Aelia* was significantly higher than the G + C content, indicating a clear AT bias. For most genes, the usage of T exceeded that of A bases, whereas the usage of G exceeded that of C bases. The compositional bias of bases has important implications for studying the mechanisms of mitochondrial genome replication, transcription, and phylogenetic relationships [48].

In addition to TTG and GTG, other start codons are preferred by Pentatomidae [49,50], and ATN is the most commonly used start codon in *Aelia* species. In the present study, *cox1*, *nad1*, and *atp8* used TTG, TTG/GTG, and TTG start codons, respectively. In total, 11 of the 13 PCGs had identical stop codons, and the most frequently used stop codon was TAA, followed by TAG and incomplete T. PCGs ending with a T codon are completed with a TAA stop codon during transcription through polyadenylation [51]. The AT bias in the mitochondrial genome was also reflected in the frequency of synonymous codon usage by PCGs. Codons with higher frequencies often ended with an A or U, and their relative synonymous codon usage values were greater than one.

The lengths and nucleotide compositions of RNA genes showed high similarity between *A. sibirica* and *A. fieberi*. The secondary structures of rRNA genes play a crucial role in the functionality of PCGs. Most nucleotide position variations existed in the ring region, whereas the stem regions tended to be more conserved. In addition, the *12S rRNA* gene was more conserved than the *16S rRNA* gene. G-U pairing was observed multiple times in the secondary structure of tRNA, and such mismatches have been shown to play an important role in maintaining secondary structure stability [52].

In the phylogenetic analysis, there were relatively few species from Asopinae and Phyllocephalinae, and they exhibited distinct differences from other subfamilies within the family Pentatomidae, making their classification less controversial and more stable. The phylogenetic relationships obtained by different methods supported the monophyly of Asopinae and Phyllocephalinae, which was consistent with the results based on mitochondrial RNA genes and aligned with current mainstream views [20,53].

Species in the subfamily Pentatominae are abundant, and their morphological variations are great. Owing to the lack of stable and reliable identification characters for phylogenetic reconstructions, different researchers have had different opinions on the division of tribes and the monophyly of Pentatominae, and the relationships between the various groups within Pentatominae remain uncertain [24,54]. Based on morphological studies, Yang divided the Chinese Pentatominae species into seven tribes [55]. Whereas Rider recorded 42 tribes about the worldwide Pentatominae species [18]. In the 7-tribe classification system, the genus *Dolycoris* was classified under the tribe Dolycorini, whereas in the 42-tribe classification system, it was placed in the tribe Carpocorini, together with *Carpocoris*, *Palomena*, and *Rubiconia*. In this study, Carpocorini and Aeliini were found to be sister groups, this is congruent with Roca-Cusachs et al. [20]. However, Lian et al. support the finding that Eysarcorini and Carpocorini are sister groups based on mitochondrial genomes [56]. The external morphological features of *Placosternum urus* are similar to those of the tribe Pentatomini; however, shorter or ear-shaped scent gland openings are observed. The results of this study indicated that *Placosternum urus* forms a sister group relationship with the subfamily Phyllocephalinae, whereas *Pentatoma semiannulata* forms a sister group relationship with *Eurydema dominulus* (belonging to Strachiini). *Graphosoma rubrolineata* and *Scotinophara lurida*, both of which belong to the subfamily Podopinae, have a complex relationship with Pentatominae. Some Podopinae species were previously assigned to Scutelleridae or Pentatominae, and were even separately promoted to family level; however, there is no widely accepted view of Podopopinae based on a comprehensive analytical approach. Yang previously classified *Graphosoma* and *Eysarcoris* in the tribe Graphosomini of the subfamily Pentatominae [55]. In addition, Zhao et al. and Li et al. also support a close relationship between the genus *Graphosoma* and species of Pentatominae, which is corroborated by the results of this study [57,58]. In this study, the representative species of Podopinae mixed in the Pentatominae species, the result combined with pre-

vious studies indicate that the classified position of *Graphosoma rubrolineata* could not be determined [26,56]. Owing to the limitations of the sample size in this study, further investigation of the monophyly of the subfamily Pentatominae and the validity of taxonomic units within the subfamily Podopinae require additional mitochondrial sequence information.

5. Conclusions

Currently, there are few phylogenetic studies within Pentatomidae, and the use of molecular data is limited. This study determined the mitochondrial genome sequences of *A. sibirica* and *A. fieberi*. The sequence features, base composition, codon usage, and RNA secondary structures were found to be highly similar between the two species, with no significant differences observed within the genus. The comprehensive analysis of the *Aelia* mitochondrial genomes performed in this study contributes new information which can be used in molecular evolutionary studies and phylogenetic analyses. More mitogenomes or other molecular data are needed to conduct further exploration of the taxonomic status and phylogenetic history of Pentatomidae species.

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