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Phylogeny and evolutionary analysis of the whole mitochondrial genome of *Neohirasea stephanus* Redtenbacher, 1908 (Phasmatodea, Loncodidae)

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Abstract: *Neohirasea stephanus* belongs to the subfamily Necrosciinae in phasmida, which is the most abundant subfamily. In this study, the mitochondrial genome of *Neohirasea stephanus* was obtained, and its gene structure, protein-coding genes, nucleic acid composition and codon usage were analyzed, while the secondary structure of rRNAs was predicted, and the published complete mitochondrial genomes of 31 stick and leaf insects were compared and analyzed. In addition, the phylogenetic relationships were revealed based on 34 mitochondrial genome data sets by using the Bayesian inference method (BI). The results indicated that *N. stephanus* and *N. japonica* are the sister group, and the subfamily Clitumninae, Eurycanthinae and Lonchodinae are a polyphyletic group. According to the time calibrated phylogenetic estimation, the divergence time of Euphasmatodea is inferred to have started ~ 112.55 Mya. This study enriched the mitochondrial genome data of the subfamily Necrosciinae and provided a molecular reference for the future phylogenetic study of stick insects.

Keywords: divergence time; mitogenome; *Neohirasea stephanus*; phylogenetic analysis

1. Introduction

Insect mitochondria is a semi-autonomous organelle in eukaryotic cells, containings a set of mitochondrial genomes independent of nuclear genes (Nass MMK et al. 1963). The mitochondrial genomes of insects generally have a closed circular double-stranded structure, with a total of 37 genes, including 13 protein-coding genes (PCGs), 22 tRNA genes (tRNAs), 2 rRNA genes (*rrnS* and *rrnL*), and an A+T-rich region (Boore et al. 1999; Cameron et al. 2008, 2014). With the development of the Next-Generation Sequencing (NGS) technology, mitochondrial genome research has covered all orders of insects (Cameron et al. 201).

Phasmatodea is large stick- and leaf-like with chewing mouthparts, incomplete metamorphosis, and some species with wings, which is the longest existing insect species. At present, there are 3500 valid species in the world, divided into 14 families and mainly distributed in the tropics (Brock et al. 2023). With the development of molecular biology technology, more and more scholars used molecular classification to study the phylogenetic relationship of Phasmatodea. For example, Xu et al. (2021) successfully sequenced the complete mitogenome of *Eurycantha calcarata* Lucas, 1869 (Phasmatodea: Lonchodinae) and re-established the phylogenetic relationship of 27 species of Phasmatodea. The results showed that the monophyly of Lonchodinae and Necrosciinae was well supported, but the monophyly of Clitumninae was not recovered (Xu et al. 2021). Dong et al. (2021) sequenced and annotated two mitochondrial genomes for the first time, and found that Aschiphasmatidae and Neophasmatodea were a sister group, and the monophyly of Neophasmatodea and Phyllioidea was well supported (Dong et al. 2021).

Up to now, the NCBI database has recorded over 600 *COI* genes, over 300 *COII* genes and over 10 16S *rRNA* genes in Phasmatodea, as well as 20 complete mitochondrial genomes.

Bi and Li first reported the distribution of *Paracentema stephanus* in Guilin, Guangxi. The genus *Paracentema* is a junior synonym of the genus *Neohirasea* (Zompro 2001; Hennemann 2007), and *Neohirasea stephanus* Redtenbacher, 1908 was transferred to the genus *Neohirasea* (Ho 2012). The subfamily Necrosciinae is the most abundant subfamily with 110 genera (Brock et al. 2023). There has been a controversy about the monophyly of the subfamily Necrosciinae. Sellick thought that the subfamily Necrosciinae was multi-lineage (Sellick 1997), and the subsequent studies also supported the view (Bradler et al. 2014, 2009). Subsequently, Kômoto et al. (2011) failed to recover the monophyly of Necrosciinae based on 18 phasmid amino acid sequences (Kômoto et al. 2011). Up to now, although the species of Necrosciinae are abundant, only two complete mitochondrial genomes have been reported in NCBI.

The large scale analysis of the subfamily Necrosciinae is hindered by the lack of available systematic genome scale data sets to solve phylogenetic problems. At the same time, phylogenetic research using molecular data lacks substantial support and consistency for deep relationships, and the results are often inconsistent with morphological phylogenetic results (Buckley et al. 2010; Bradler et al. 2014, 2015). Therefore, it is necessary to estimate the time of system differentiation in order to have a deeper understanding of these groups and make accurate differentiation.

In this study, thirty-two mitochondrial genome data of Phasmatodea were selected to construct a molecular phylogenetic tree, and three fossil records of Euphasmatodea were used to correct the molecular clock and calculate its differentiation time. It is expected to provide data and method references for the classification and evolution of Phasmatodea.

2. Materials and methods

2.1. Taxon sampling and sequencing

In this study, the samples of *N. stephanus* were collected from Mao'er Mountain in Guangxi Zhuang Autonomous Region of China (25.887210°N, 110.483506°E) in July 2021 and stored in 100 % ethanol at −4°C. Total genomic DNA was extracted from the hind leg of an adult specimen of *N. stephanus* using TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). The genomic DNA was sequenced using 150 bp PE on the Illumina Novaseq platform (Berrygenomics, Beijing, China).

2.2. Genome annotation and sequence analysis

The raw paired-end reads were filtered to obtain high-quality clean reads by using CLC Genomics Workbench 12 (CLC Bio, Aarhus, Denmark) with default parameters. The filtered reads were aligned to the mitochondrial genome of *Micadina phluctainoides* (NC014673) as a reference for annotation from the basic local alignment search tool (BLAST) in the NCBI database. Then the mitochondrial genome was assembled by NOVOPLASTY (Dierckxsens et al. 2017), which was a Perl script that did not need to rely on other software. After changing the contents in a config .txt file, it will be operated directly, and the MITOS WebServer (http://mitos.bioinf.uni-leipzig.delindex.py) will be used for preliminary annotation (Bernt et al. 2013).

Secondary structures of all transfer RNA (tRNA) genes were predicted by MITOS WebServer (Bernt et al. 2013). The ring structure of the mitogenome was made by using the CGVIEW server (http://stothard.afns.ualberta.ca/cgview_server/) (Grant et al. 2008). In MEGA v.7.0 software, the invertebrate mitochondrial genetic code was used to translate the nucleotide sequences of PCGs, and the relative synonymous codon usage (RSCU) value was analyzed. The synonymous substitution rate (Ks) and non-synonymous substitution rate (Ka) were calculated by DNA SPV.5.10 (Kumar et al. 2016; Librado et al. 2009). The new mitogenome was deposited in GenBank under Accession Number OL405132.

The secondary structure of rRNAs was first predicted by VIENNARNA online service website (Hofacker 2003), and the files in .ct format were saved. According to the SVG diagram (the secondary structure diagram of rRNA obtained from the MITOS online website) and the published secondary structure

diagram of related species, the structure was refined manually in RNAVIZ 2.0.3 software (Rijk et al. 2003). The control region was predicted by the online tool Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.advanced.submit.html), and ARWEN (http://mbio-serv2.mbioekol.lu.se/ARWEN/) (Laslett et al. 2008).

2.3. Phylogenetic analyses

Phylogenetic analysis was carried out on the newly obtained PCGs of the mitochondrial genome and 31 species of Phasmatodea downloaded from the GenBank database, *Aposthonia japonica* and *Aposthonia borneensis* were selected as the outgroup. The MEGA v. 7.0 was used to compare each protein-coding gene deduced from the amino acid comparison, and then the alignment results were connected. Bayesian inference (BI) analysis was used for phylogenetic reconstruction with MRBAYES 3.1.2 under the partition model selected by partition Finder 2 (Lanfear et al. 2012; Ronquist et al. 2012). In the BI analysis, using the GTR+G+I substitution model, four independent Markov chains were running for 10,000,000 generations and stopped when the average standard difference is less than 0.01. The first 25% of the trees were discarded, and the remaining trees were used to construct the 50% majority-rule consensus tree (Drummond et al. 2007).

2.4. Divergence time estimate

Divergence times among subfamilies were estimated using whole mitochondrial genome sequences with a relaxed clock log normal model in BEAST v.1.10.4 (Drummond et al. 2012). An uncorrelated lognormal relaxed clock model of rate variation across branches was implemented as a time prior and both Yule and Birth-Death speciation processes were used for the tree prior. Three fossil records were selected as calibration points: for the first, a fossil calibration was set applying a lognormal distribution (offset = 47) based on the leaf insect fossil *Eophyllium messelense* (Wedmann et al. 2007); for our second divergence time estimation and calibrated the root of the tree (Euphasmatodea) with a normal distribution (lower: 98.2 Mya; upper: 411.0 Mya) (Batelka et al. 2016); the last fossil calibration was set applying a lognormal distribution (offset = 44) based on the leaf insect fossil *Eophasma spp*.(Ghirotto et al. 2022). Four runs were performed for 700 million MCMC generations sampled every 5,000 generations with different calibrations to explore the divergence time of Phasmatodea. Results according to TRACER v.1.7.1 software test (Rambaut et al. 2018), ESS (effective sampling size) value should be greater than 200, which means it is reliable. Then, the software TREE ANNOTATOR 1.8.4 was used to generate the maximum confidence tree, and the software FIG TREE l.4.3 was used to open it and check the analysis results (Rambaut et al. 2014).

3. Results and discussion

3.1. Genome organization

The complete mitogenome of *N. stephanus* (GenBank No. OL405132) was a typical double-stranded circular DNA molecule with a length of 18, 201 bp and contained 13 protein-coding genes (PCGs), 22 tRNA genes (tRNAs), 2 rRNA genes (*rrnS* and *rrnL*), and an A＋T-rich region. Eight tRNA genes, four PCGs, and two rRNA genes were located in the N-strand and the remaining genes (fourteen tRNAs and nine PCGs) were in the J-strand (Table 1).

There were 13 gene overlaps in the mitochondrial genome of *N. stephanus*, with the length of 1–23 bp, of which the longest was the overlap between *ND1* and *trnL1* genes (23 bp). There were 9 gene spacer regions with a length of 1–29 bp, of which the largest spacer region was located between *trnL1* and *rrnL* (29 bp). The appearance of the genetic intervals may be beneficial to store more genetic information, and may also increase the mutation probability, which is beneficial to biological evolution.

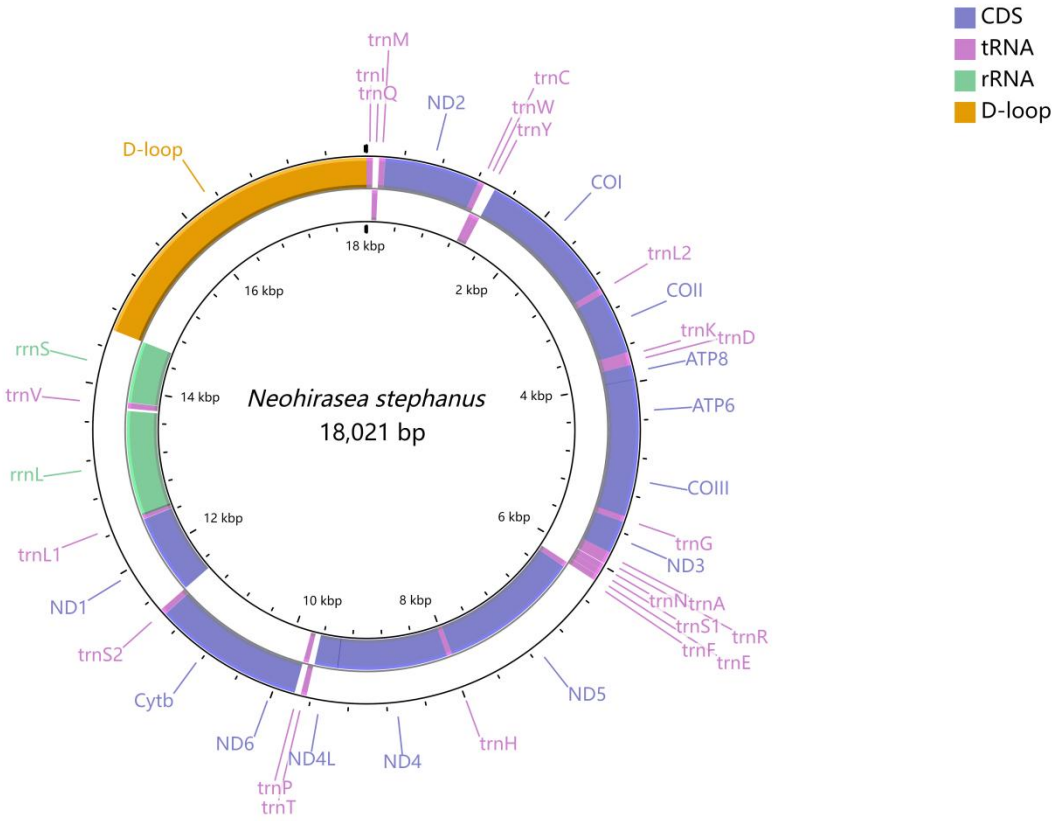

Figure 1. Circular visualization of the mitogenome of *Neohirasea stephanus*.

3.2. Protein-coding genes

The start codon of most insect protein-coding genes is typical ATN, and some genes are TGG and GTG. Except that some genes are terminated with incomplete T and TA, most of the stop codons are TAA and TAG (Lanfear et al. 2012). In this study, all 13 protein-coding genes used ATN as the start codon, in which *ND2*, *COX2*, *ATP6*, *ND3*, and *ND1* were ATA, *COX1*, *COX3*, *ND4*, and *Cytb* were ATG, *ATP8*, *ND5*, *ND4L*, and *ND6* were ATT. Seven of the 13 protein-coding genes used typical TAA as the termination codon, of which *ATP8*, *ATP6*, *COX3*, *ND4*, *ND4L*, *ND6*, and *Cytb* were terminated by TAA, while *ND2*, *COX1*, *COX2*, *ND3*, *ND5*, and *ND1* used incomplete termination codon T (Table 1). Some researchers think that incomplete T will be polyadenylated by adding A to the 3' ends of mRNA after transcription and then converted into the complete stop codon to complete transcription termination (Zhao et al. 2018; Drummond and Rambaut 2007).

The Ka/Ks ratio of *N. stephanus* and each PCGs in the mitochondrial genomes of 31 stick insects downloaded from the GenBank database was calculated to evaluate the evolution rate of PCGs (Figure 4). The results showed that *ATP8* (3.72) of *Eurycantha calcarata* has the highest evolutionary rate, followed by *ND5* (3.47) of *Phraortes sp Iriomote Island*, whereas *COX1* (0.13) of *Timema californicum* appears to be the lowest. The Ka/Ks value of *ND4* and *ND5* was greater than 1, indicating that the gene had made a positive selection; the Ka/Ks value is equal to 1, indicating that the gene had undergone neutral evolution; the Ka/Ks value of other genes is less than 1, indicating that these genes had been purified selection.

Table 1. Organization of the *Neohirasea stephanus* mitogenome.

Gene	Strand	Position	Size	Start codon	Stop codon	Spacer/Overlap(-)
trnI	J	1—68	68			-3

trnQ	N	66－134	69			-1
trnM	J	134－200	67			0
ND2	J	201－1215	1015	ATA	T	0
trnW	J	1216－1282	67			-8
trnC	N	1275－1337	63			0
trnY	N	1338－1405	68			1
COX1	J	1407－2940	1534	ATG	T	0
trnL2	J	2941－3005	65			0
COX2	J	3006－3675	670	ATA	T	0
trnK	J	3676－3745	70			-1
trnD	J	3745－3812	68			0
ATP8	J	3813－3971	159	ATT	TAA	-4
ATP6	J	3968－4642	675	ATA	TAA	-1
COX3	J	4642－5427	786	ATG	TAA	-2
trnG	J	5426－5490	65			0
ND3	J	5491－5842	352	ATA	T	0
trnA	J	5843－5908	66			-1
trnR	J	5908－5972	65			5
trnN	J	5978－6045	68			0
trnS1	J	6046－6112	67			1
trnE	J	6114－6178	65			-2
trnF	N	6177－6242	66			0
ND5	N	6243－7965	1723	ATT	T	0
trnH	N	7966－8030	65			-1
ND4	N	8030－9361	1332	ATG	TAA	-7
ND4L	N	9355－9639	285	ATT	TAA	8
trnT	J	9648－9711	64			0
trnP	N	9712－9776	65			1
ND6	J	9778－10257	480	ATT	TAA	-1
CYTB	J	10257－11390	1134	ATG	TAA	1
trnS2	J	11392－11458	67			0
ND1	N	11459－12422	964	ATA	T	3
trnL1	N	12426－12493	68			-23
16S rRNA	N	12471－13744	1274			29
trnV	N	13774－13843	70			3
12S rRNA	N	13847－14592	746			0
CR	J	14593－18021	3429			0

Figure 2. Use of start codons of protein coding genes in mitochondrial genomes of 32 stick insects.

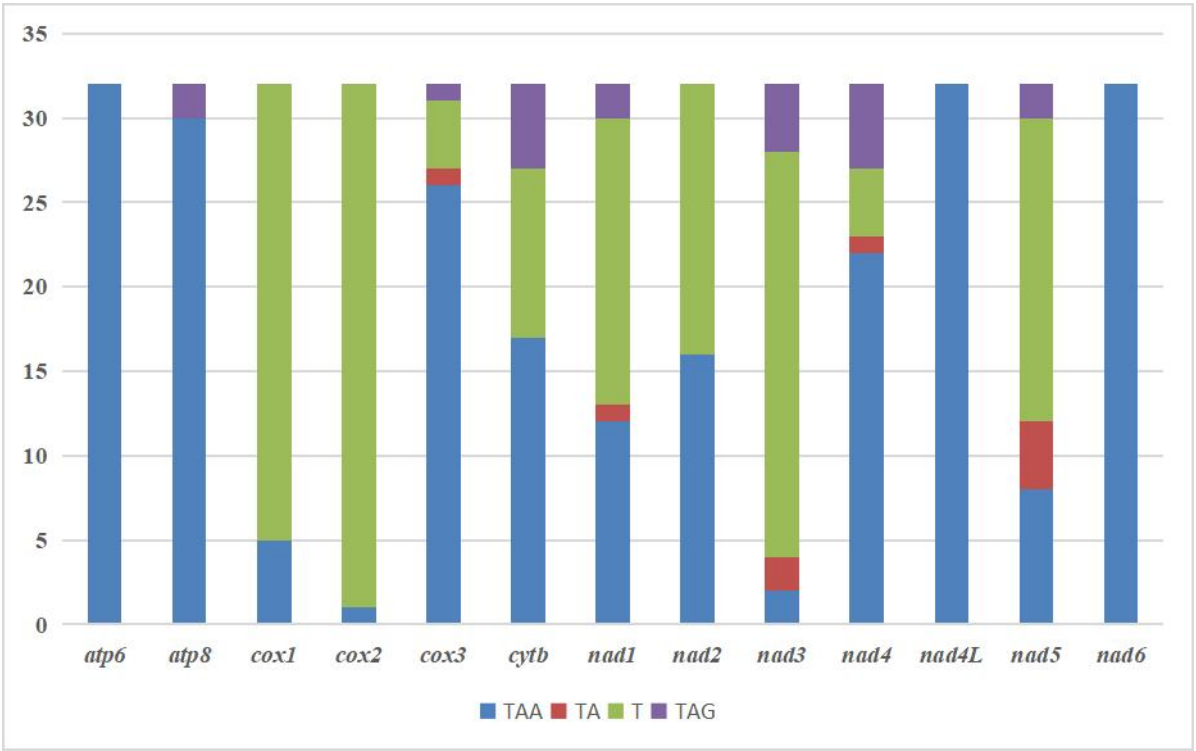

Figure 3. Use of stop codons of protein coding genes in mitochondrial genomes of 32 stick insects.

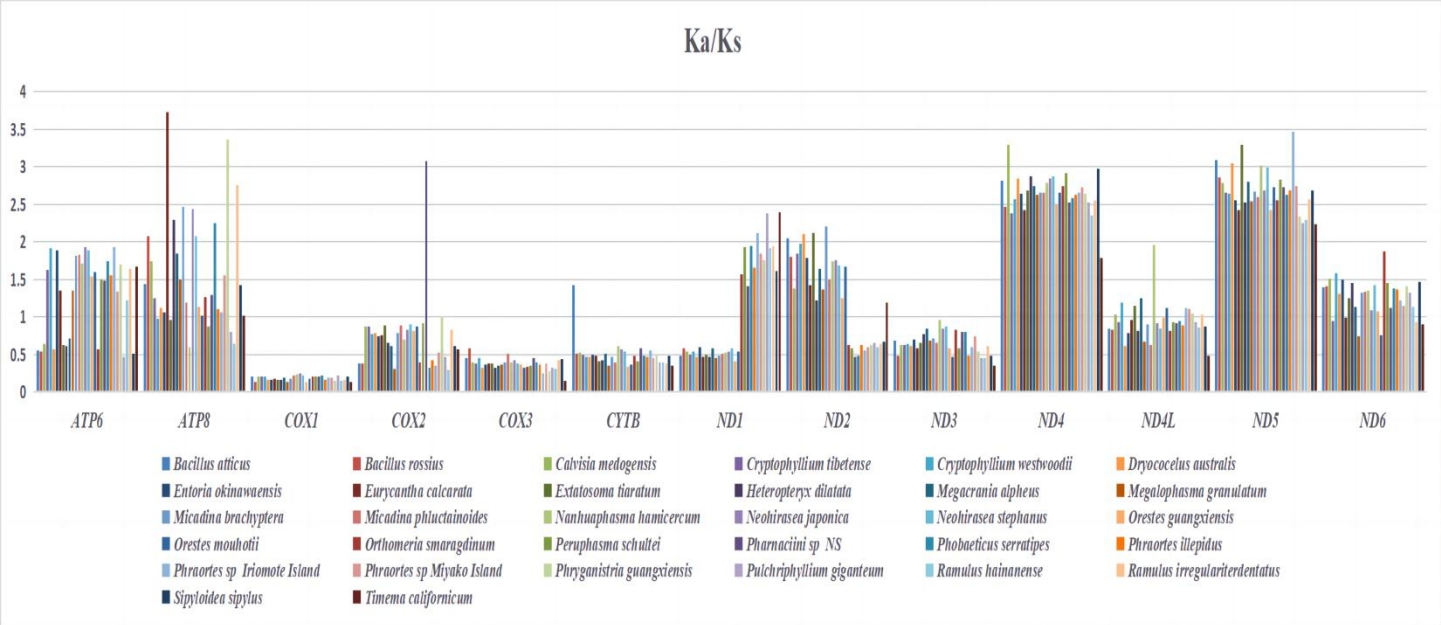

Figure 4. Evolutionary rates of PCGs in 32 species of stick insects. The bar indicates each gene's Ka/Ks value.

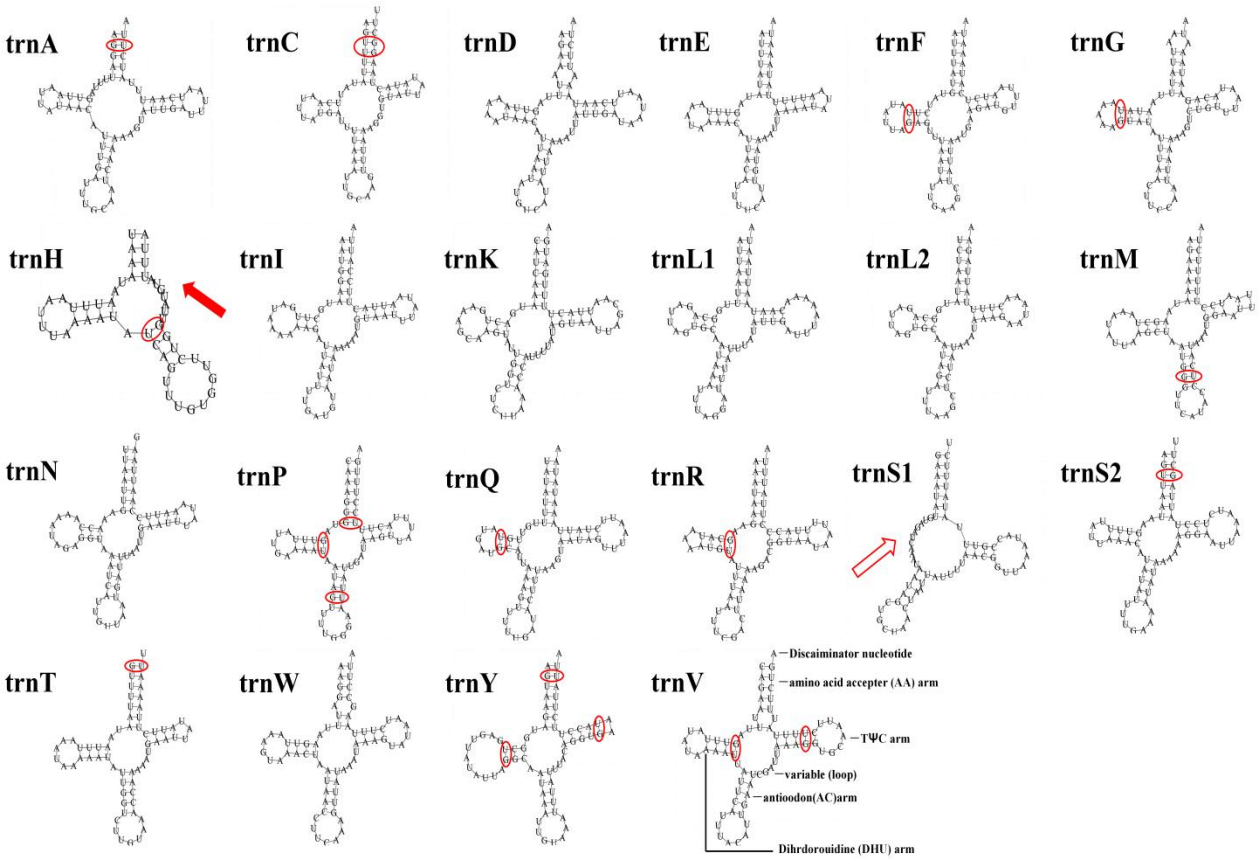

Figure 5. Secondary structures of tRNA genes in the mitogenome of *Neohirasea stephanus*. Mismatched base pairs are indicated by red circles; reduced arms are indicated by red arrowheads.

3.3. Nucleotide composition and codon usage

The base content and skewness of the genes in the *N. stephanus* mitogenome were shown in Table 2. The base composition of the complete mitogenome was 45.7% of A, 32.7% of T, 11.8% of C, and 9.7% of G,

with a bias toward A + T. The relative synonymous codon usage (RSCU) values for the *N. stephanus* mitogenome were summarized in Figure 3 and Table 2, which showed that the six most frequently used codons were AUA(I), UUA(L), AUUA(I), UUU(F), AAU(N), and UAU(Y), while CGC(R),UGC(C),GCG(A), ACG(T), CCG(P) and CUG(L) were rarely used. The RSCU analysis showed that codons including A or T at the third position were always used more frequently than other synonymous codons in *N. stephanus*. Meanwhile, the overall analysis of RSCU showed that codons ending in T or A were more frequently used than codons ending in C or G (Figure 5).

Table 2. Codon number and RSCU in the *Neohirasea stephanus* mitochondrial PCGs

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	291.3	1.69	UCU(S)	93.3	2.12	UAU(Y)	157.4	1.75	UGU(C)	41.8	1.81
UUC(F)	52.6	0.31	UCC(S)	10.5	0.24	UAC(Y)	22.7	0.25	UGC(C)	4.4	0.19
UUA(L)	339.6	4.12	UCA(S)	117.3	2.66	UAA(*)	7.3	0.24	UGA(*)	83.2	2.74
UUG(L)	51	0.62	UCG(S)	3.9	0.09	UAG(*)	0.7	0.02	UGG(W)	11.6	1
CUU(L)	37.1	0.45	CCU(P)	56.9	1.71	CAU(H)	48.4	1.45	CGU(R)	17.7	0.83
CUC(L)	5.7	0.07	CCC(P)	11.9	0.36	CAC(H)	18.3	0.55	CGC(R)	1.1	0.05
CUA(L)	56.5	0.69	CCA(P)	61.4	1.84	CAA(Q)	56.1	1.75	CGA(R)	29.7	1.4
CUG(L)	4.3	0.05	CCG(P)	3.3	0.1	CAG(Q)	7.9	0.25	CGG(R)	3.2	0.15
AUU(I)	320.2	1.35	ACU(T)	63.1	1.24	AAU(N)	159.9	1.64	AGU(S)	35.5	0.81
AUC(I)	47.8	0.2	ACC(T)	18	0.35	AAC(N)	35.4	0.36	AGC(S)	3.7	0.08
AUA(I)	341.7	1.44	ACA(T)	119.2	2.34	AAA(K)	91.3	1.69	AGA(R)	70.7	3.33
AUG(M)	29.9	1	ACG(T)	3.4	0.07	AAG(K)	16.8	0.31	AGG(R)	4.9	0.23
GUU(V)	77	1.85	GCU(A)	49.7	1.71	GAU(D)	61.1	1.69	GGU(G)	81.5	1.62
GUC(V)	4.5	0.11	GCC(A)	10.4	0.36	GAC(D)	11.4	0.31	GGC(G)	6.8	0.14
GUA(V)	78.1	1.87	GCA(A)	54	1.85	GAA(E)	63.9	1.71	GGA(G)	86.7	1.73
GUG(V)	7	0.17	GCG(A)	2.5	0.09	GAG(E)	10.9	0.29	GGG(G)	25.8	0.51

Note: *indicates the stop codons; RSCU indicates the Relative Synonymous Codon Usage.

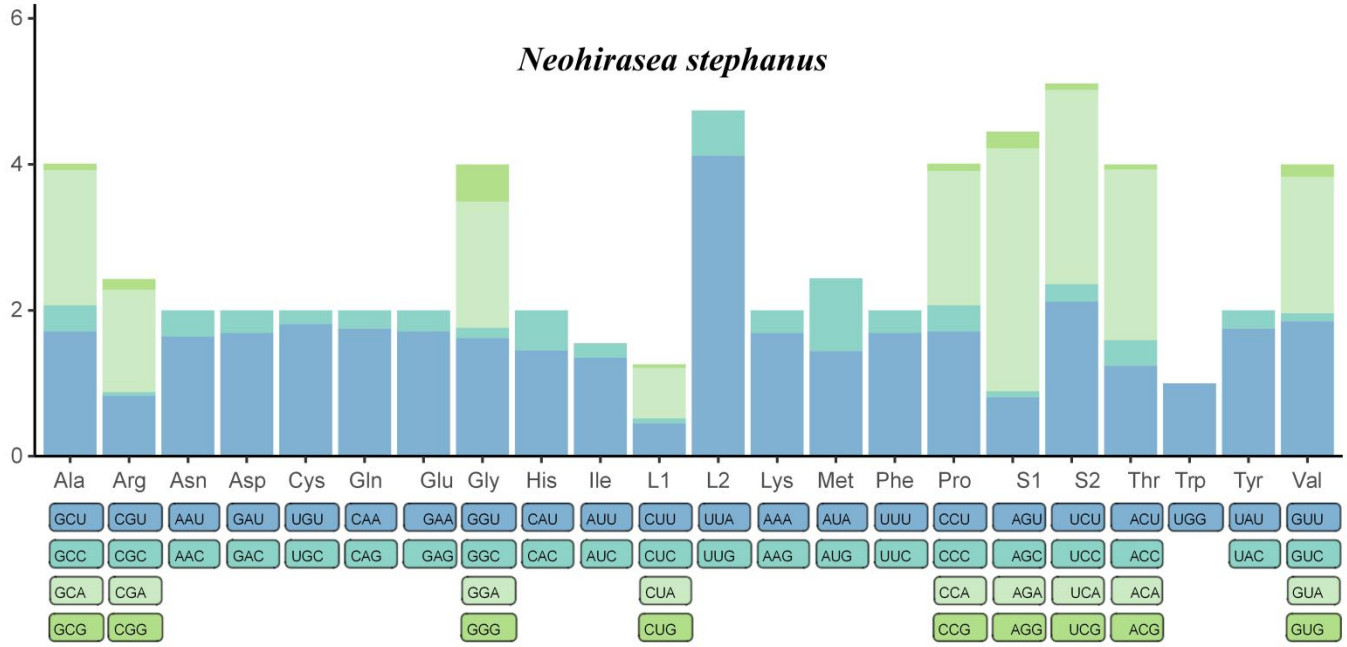

Figure 6. Relative synonymous codon usage of *Neohirasea stephanus* mitochondrial protein-coding genes. Condon families are provided on the x-axis.

3.4. tRNA genes

There are 22 potential tRNA genes in *N. stephanus* respectively (Table 1), ranging from 63 to 70 bp in size. A schematic drawing of their respective secondary structures was shown in Figure 5. *trnS1* lacked a DHU arm and *trnH* lacked a TΨC arm, so it can't form a secondary structure, which was a common phenomenon in metazoa (He et al. 2011). Other tRNA genes had the classic clover-leaf secondary structure. Besides the typical base pairs (A-U and G-C), G-U pairing was found. In insects, in addition to G-U pairing, there are also U-C and U-U pairs. Some scholars had suggested that those tRNAs with non-Watson-Crick matching can be transformed into fully functional proteins through a post-transcriptional mechanism (Yokobori and Pääbo 1997; Ojala et al. 1981).

3.5. rRNA genes

There are two rRNA genes in the mitochondrial genome of *N. stephanus*, namely the *rrnL* gene (*16S rRNA*) and the *rrnS* gene (*12S rRNA*) in the N-chain. The *rrnL* gene was between *trnL1* and *trnV* with a length of 1247 bp. The *rrnS* gene is located between *trnV* and the control region with a length of 746 bp. The AT content of *rrnL* and *rrnS* was 81% and 78.6%, respectively.

In addition to the typical Watson-Crick pairing (G-C and A-U), the secondary structure of the rRNA gene of *N. stephanus* also found some wobble G-U pairs, which can form stable chemical bonds between G and U. The secondary structure of *rrnS* consists of three structural domains, among which 6 pairs of G-U pairing were found. While, the secondary structure of the *rrnL* gene includes 6 domains, and 15 G-U pairs were found. In addition, no other types of mismatched base pairs were found.

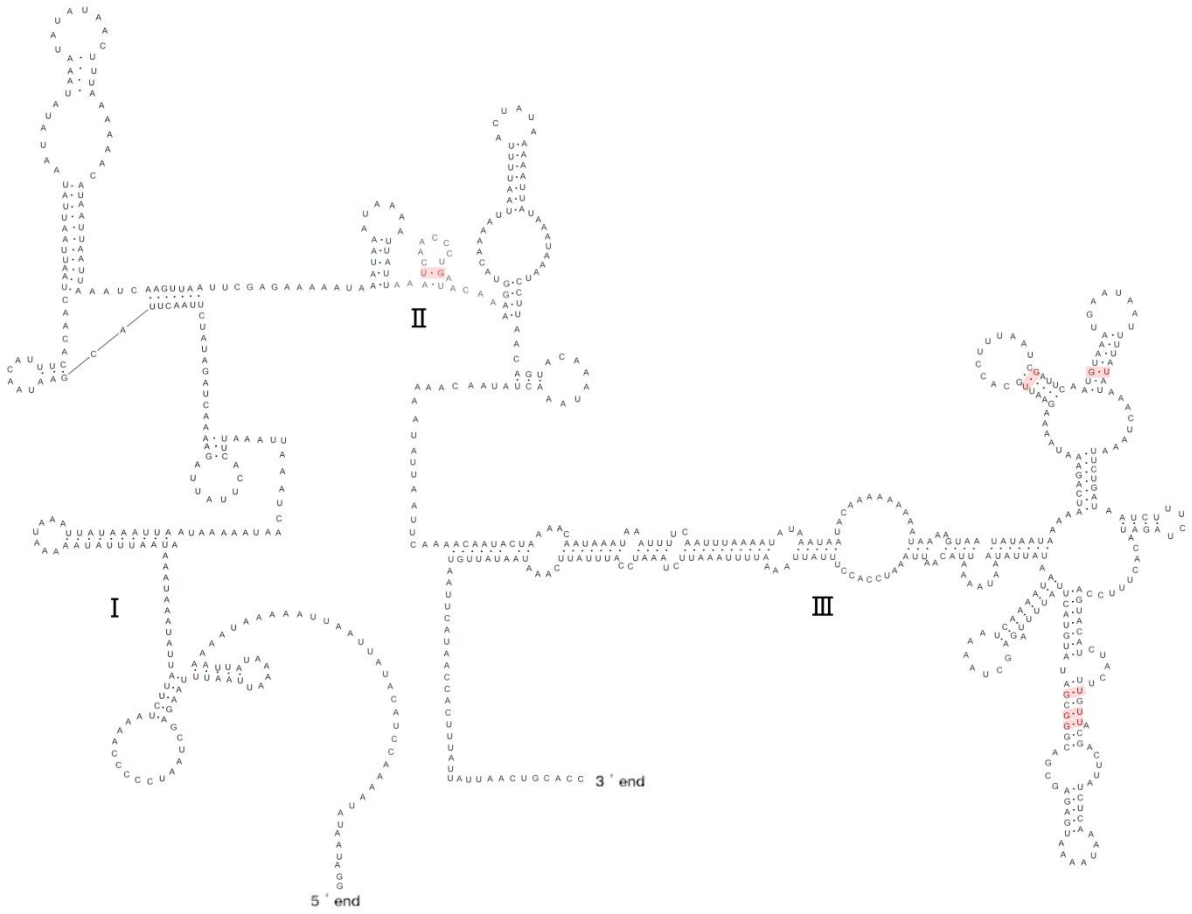

Figure 7. Predicted secondary structure of the *rrnS* gene in the *N. stephanus* mitogenome. Roman numerals denote the conserved domain structure. The red area indicates G-U pairing.

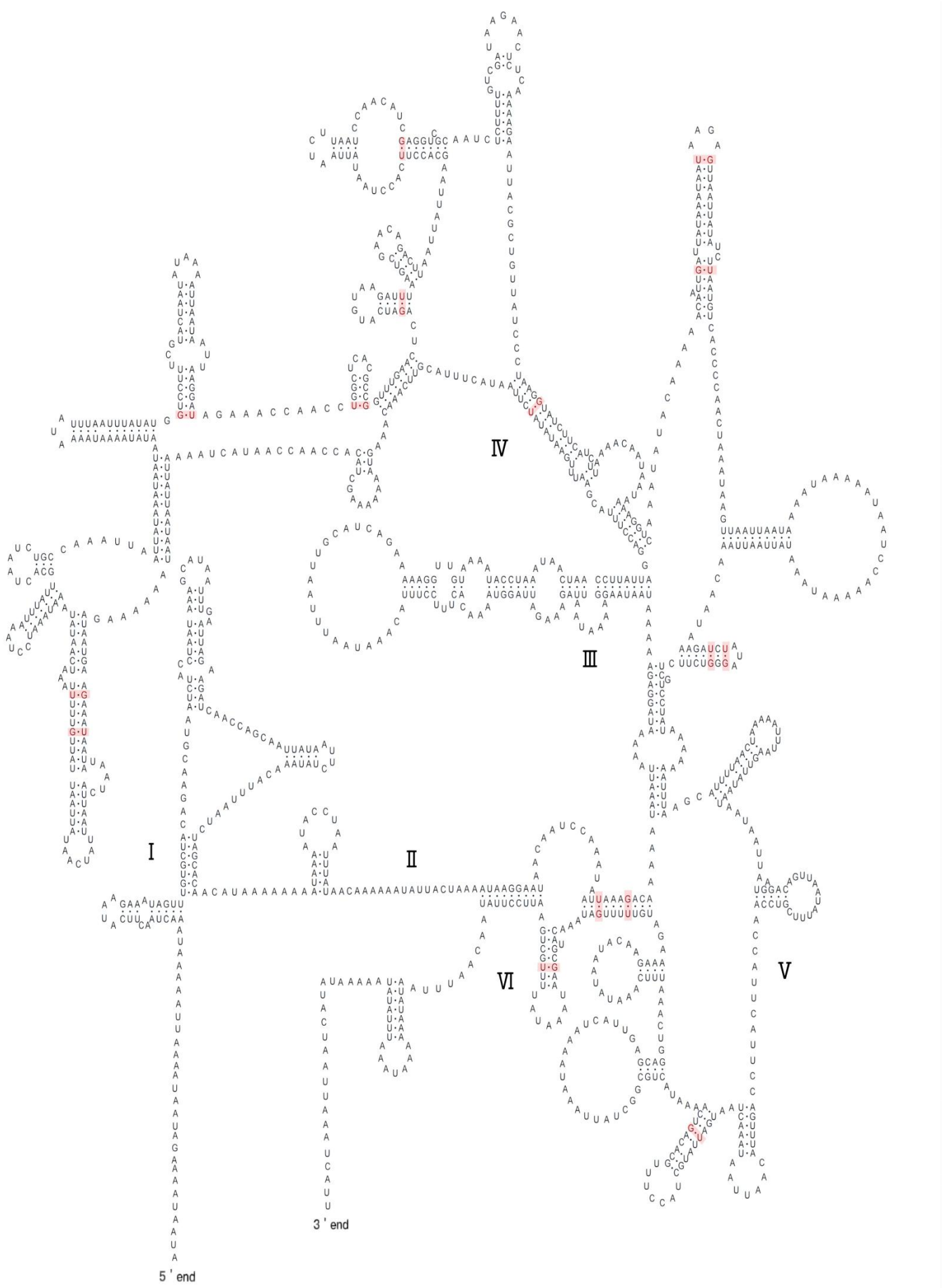

Figure 8. Predicted secondary structure of the *rrnL* gene in the *N. stephanus* mitogenome. Roman numerals denote the conserved domain structure. The red area indicates G-U pairing.

3.6. Non-coding region

The non-coding region of *N. stephanus* mitochondrial genome was located between *rrnS* and *trnI*. The non-coding region was 3429 bp in length, which was the longest region in the mitochondrial genome of *N. stephanus*, with an A+T content of 76% and a G+C content of 22%.

The non-coding region contains tandemly repeated sequences (8.3×204 bp), and the repeated unit is 'TGCAATTAACATTAGCTCAGAAATAATTTTAATTGCAAATAATTGTAAAAAATGGTAAAAAACTG ATTTGATTAAGCAAGAAAATATCAACTAGAAAGCCCAAATTGATCAAAAGTATAATCAATTTTGA AAAAAAGTATCCGTTTAGATCGATGGAAATAAATGAAATTTATAAATCAGATTTTGATAAATAACC AATTTAGG'. Tandem repeating units in the non-coding region had been shown to be useful in the inference of the genetic structure of closely related or identical species (Mancini et al. 2008).

4. Phylogenetic relationships and Divergence-time estimation

Bayesian inference was performed based on 13 PCGs. As shown in Figure 10. In addition to the outgroup, Phasmatodea are divided into two clades, one consisting of the subfamily Timematinae, and the other clade including twelve subfamilies (Aschiphasmatinae, Phylliinae, Lonchodinae, Eurycanthinae, Heteropteryginae, Dataminae, Pseudophasmatinae, Bacillinae, Necrosciinae, Clitumninae, Platycraninae, Tropidoderinae), this was consistent with the results by Bradler and Buckley (Bradler and Buckley 2018). The subfamily Aschiphasmatinae and the remaining subfamilies of the Euphasmatodea were sister group, which was consistent with previous classification results of Engel et al. based on morphological features (Engel et al. 2016). Dataminae + Heteropteryginae received high support (PP=1), consistent with the phylogenetic results by Büscher et al. and Robertson et al. (Büscher et al. 2018; Robertson et al. 2018). Prior to this, there were two topological inferences about the sister group of the subfamily Dataminae and the subfamily Heteropteryginae: Dataminae + (Heteropteryginae + Obriminae) (Bradler et al. 2015, 2009; Simon et al. 2019) and Heteropteryginae + (Obriminae + Dataminae) (Zompro 2004). Regarding the controversy between these two subfamilies, molecular data validation should be increased in the future.

The species of the subfamily Necrosciinae were well grouped together to form a monophyletic group, *N. stephanus* and *N. japonica* (AB477469) were a sister group with a high bootstrap value. Few species have been detected in the complete mitochondrial genome sequence of the insects of the subfamily Necrosciinae, and the analysis of the phylogenetic relationship of the biological system is mainly dominated by a single gene or multiple gene sequences. Mitochondrial genes frequently used in the phylogenetic analysis include the *COI* gene, *COII* gene, and so on. Therefore, attention should be paid to the study of the mitochondrial genome of insects belonging to Necrosinae in order to obtain a more phylogenetic relationship of Necrosinae.

Moreover, it is clear that the subfamily Clitumninae, Lonchodinae and Eurycanthinae were a polyphyletic group. Our data supported the monophyly of the subfamily Heteropteryginae, which was also reported by Xu et al. (Xu et al. 2021). At the same time, this study also found that the family Lonchodidae includes the subfamily Necrosciinae and the subfamily Lonchodinae, but the research results did not form a clade, which was also reported by Song et al. (Song et al. 2016).

Divergence time estimates indicated that the Euphasmatodea clade and the *Timema* lineage diverged 112.55 Mya (95% HPD = 89.5–142.52 Mya), Simon et al. showed that the *Timema* and the Euphasmatodea diverged 121.8 Mya (Simon et al. 2019), and Forni et al. deduced 273.8 Mya, speculated the Triassic-Jurassic mass extinction and the breakup of Pangea could have contributed to the process (Forni et al. 2021). The subfamilies of the Euphasmatodea diverged between the Middle Cretaceous and Late Neotertiary periods. In this study, the divergence time of the subfamily Bacillinae was about 12.13 Mya, while Mantovani et al. (2001) found that the divergence time of this subfamily through phylogenetic reconstruction of the *COII* gene was 22.79 +/- 2.65 Mya (Mantovani et al. 2001). Based on fossil evidence, the Tertiary period was believed to be a flourishing period for the differentiation of Phasmatodea. In the Tertiary period, the dominant ones began in the Late Cretaceous period Angiosperms flourished more and plant partitions were closer to modern times (Pohl et al. 2009). In recent years, more and more studies have begun to use molecular clock methods and fossil record tests to estimate the differentiation time of

Phasmatodea, and some people have reconstructed ancestral distribution areas and carried out the biogeographic analysis, which is another idea to explore the origin and evolution of Phasmatodea.

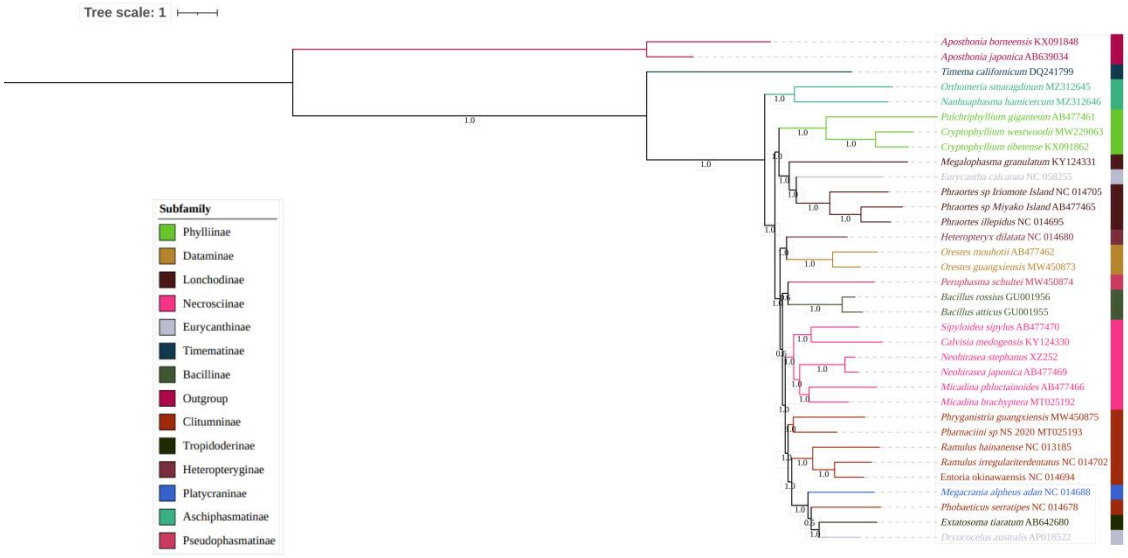

Figure 9. Phylogenetic tree obtained from BI analysis based on 13 concatenated mitochondrial PCGs. Values at nodes indicate BI posterior probabilities (PP).

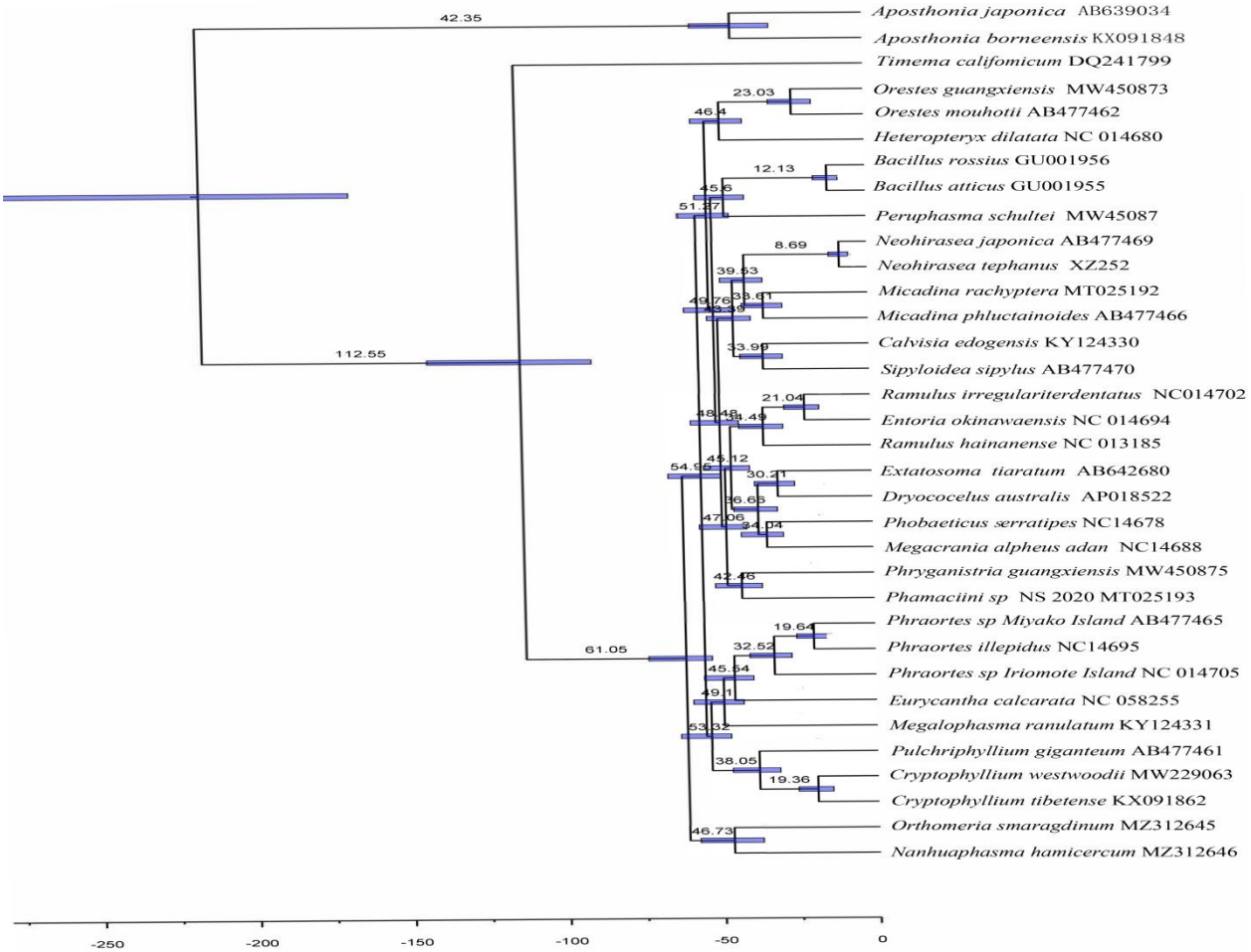

Figure 10. Chronogram with estimated divergence time based on fixed rate calibration among Phasmatodea using BEAST v.1.10.4. Horizontal bars represent 95% credibility intervals of time estimates. Numbers on

the nodes indicate the mean divergence times.

5. Conclusions

In this study, a total of 32 mitochondrial genomes were used in Phasmatodea, including 20 whole mitochondrial genomes and 12 linear mitochondrial genomes, involving 13 subfamilies. The newly sequenced mitogenome had similar structural features and nucleotide composition to previously published mitochondrial genomic data, and *N. stephanus* has not found the phenomenon of gene rearrangement, it may be that the measured data of the mitochondrial genome of insects belonging to Necrosciinae is too little to fully reflect the arrangement order of mitochondrial genes of this subfamily. The secondary structure of rRNAs was predicted for the first time, which laid the foundation for the subsequent disclosure of the mitochondrial genome structure characteristics of Phasmatodea. Our phylogenetic results showed the subfamily Clitumninae, Lonchodinae and Eurycanthinae were polyphyletic group, the newly sequenced species *N. stephanus* formed a sister group with *N. japonica.* Divergence time estimates indicated that the Euphasmatodea may have started ~ 112.55 Mya, and the divergence time of the subfamily Necrosciinae has started ~ 39.53 Mya. In the future, more mitochondrial genome data from Phasmatodea will be supplemented for more accurate phylogenetic analysis.

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