Complete Mitochondrial Genomes of Three Skippers in the Tribe Aeromachini (Lepidoptera: Hesperiidae: Hesperiinae) and Their Phylogenetic Implications

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Abstract

The mitochondrial genome is now widely used in the study of the phylogenetics and molecular evolution due to its maternal inheritance, fast evolutionary rate and highly conserved gene content. To explore the phylogenetic relationships of the tribe Aeromachini within the subfamily Hesperiinae at the mitochondrial genomics level, we sequenced and annotated the complete mitogenomes of 3 skippers: *Amipittia virgata*, *Halpe nephele* and *Onryza maga*. All of these mitogenomes are double-stranded and have circular molecules with a total length of 15,333 bp, 15,291 bp and 15,381 bp, respectively. The mitogenomes all contain 13 protein-coding genes (PCGs), 22 transfer RNAs (tRNAs), 2 ribosomal RNAs (rRNAs) and a non-coding AT-rich region, and are consistent with other lepidopterans in gene order and type. In addition, we reconstruted the phylogenetic trees of Hesperiinae using maximum likelihood (ML) and Bayesian inference (BI) methods based on mitogenomic data. Results show that the 3 Aeromachini species in this study robustly constitute a monophyletic group in the subfamily Hesperiinae, with the relationships Coeliadinae + (Euschemoninae + ((Pyrginae + (Eudaminae + Tagiadinae)) + (Heteropterinae + (Barcinae + Hesperiinae)))). Moreover, our study supports the view that *Apostictopterus fuliginosus* and *Barca bicolor* should be placed out of the subfamily Hesperiinae.

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Abstract: The mitochondrial genome is now widely used in the study of the phylogenetics and molecular evolution due to its maternal inheritance, fast evolutionary rate and highly conserved gene content. To explore the phylogenetic relationships of the tribe Aeromachini within the subfamily Hesperiinae at the mitochondrial genomics level, we sequenced and annotated the complete mitogenomes of 3 skippers:*Amipittia virgata* , *Halpe nephele* and *Onryza maga.*All of these mitogenomes are double-stranded and have circular

molecules with a total length of 15,333 bp, 15,291 bp and 15,381 bp, respectively. The mitogenomes all contain 13 protein-coding genes (PCGs), 22 transfer RNAs (tRNAs), 2 ribosomal RNAs (rRNAs) and a non-coding AT-rich region, and are consistent with other lepidopterans in gene order and type. In addition, we reconstruted the phylogenetic trees of Hesperiinae using maximum likelihood (ML) and Bayesian inference (BI) methods based on mitogenomic data. Results show that the 3 Aeromachini species in this study robustly constitute a monophyletic group in the subfamily Hesperiinae, with the relationships Coeliadinae + (Euschemoninae + ((Pyrginae + (Eudaminae + Tagiadinae)) + (Heteropterinae + (Barcinae + Hesperiinae)))). Moreover, our study supports the view that *Apostictopterus fuliginosus* and*Barca bicolor* should be placed out of the subfamily Hesperiinae.

Keywords: Aeromachini; mitogenome; mitochondrial DNA; phylogeny

1. Introduction

The family Hesperiidae (skippers) is one of the speciose families in butterflies and consists of about 567 genera and more than 4000 species around the world (Warren, Ogawa, & Brower, 2008), accounting for one-fifth of the world's butterfly species, though the number is far underestimated. The higher classification of the family had mainly followed Evans (Evans, 1943, 1949, 1951) until Warren et al. inferred the phylogenetic relationship from molecular (three loci) and morphological data of 196 genera (Warren et al., 2008; Warren, Ogawa, & Brower, 2009). And the latest molecular study of 250 hesperiid species from all over the world (W. Li et al., 2019) and its supplementary study (Jing Zhang, Cong, Shen, Brockmann, & Grishin, 2019) showed that the family Hesperiidae should be classified into 12 subfamilies, with the relationship of (Coeliadinae + (Euschemoninae + ((Eudaminae + (Tagiadinae + (Pyrrhopyginae + Pyrginae))) + (Katreinae + (Chamundinae + (Heteropterinae + (Barcinae +Trapezitinae) + Hesperiinae)))))), but this higher classification as well as the phylogeny has not been approved generally. Hesperiinae, the largest subfamily, has been proved to be a distinctly monophyletic group by the previous studies (W. Li et al., 2019; Sahoo et al., 2016; Toussaint et al., 2018; Warren et al., 2009; Jing Zhang et al., 2019), we have provided more comprehensive data support for the phylogenetic research of the groups.

Aeromachini is a large and diversed tribe of the subfamily Hesperiinae and currently contains approximately 130 species in 12 genera, distributed in the Oriental Region, the Paearctic Region, and the Afrotropical Region (Cock & Congdon, 2012; Devyatkin, 1996; Evans, 1949; Huang et al., 2019; Warren et al., 2009; Yuan, Yuan, & Xue, 2015). Most of the genera, except for the genus *Halpe* , of Aeromachini are distributed in the Sino-Himalayan Subregion. In the previous phylogenetic studies, the tribe is always retrieved as a clade sister to the rest of the Hesperiinae. Two molecular studies within the tribe are known (Y. Li et al., 2019).

The insect mitogenome is a double strand molecule about 15˜16Kb in size, typically containing 13 protein-coding genes (PCGs), 22 transfer RNAs (tRNAs), 2 ribosomal RNAs (rRNAs) and a non-coding AT-rich region. In the past few decades, due to its maternal inheritance, fast evolutionary rate and highly conserved gene content compared to nuclear genes, it has been widely utilized to investigate insect taxonomy, phylogenetic relationships, evolution and biogeography (Cameron, 2014; Galtier, Nabholz, Glémin, & Hurst, 2009). In this study we determine the complete mitochondrial genome sequences of 3 skipper species of the tribe Aeromachini and reconstruct the phylogenetic relationships of the family Hesperiidae, combined with other available sequence data in GenBank, and using maximum likelihood and Bayesian inference methods, aiming to provide new horizons and genomics data support for the phylogenetic research of the Aeromachini.

2. Materials and Methods

2.1 Sample Collection and DNA Extraction

Adult specimens of*Amipittia virgate* Leech, 1890 and*Halpe nephele* Leech, 1893 were sampled at Jiuxian Mountain and Wuyi Mountain in Fujian Province, China, respectively, in July, 2016. The adult *Onryza maga* Leech, 1890 were collected at Matou Mountain, Jiangxi Province, China in August, 2018. Specimen identification was based on morphological characteristics following Yuan et al. (Chou, 1994; Yuan et al.,

2015) and the identity was confirmed via *cox1* barcoding using the BOLD database (Ratnasingham & Hebert, 2013). All genetic materials were immediately preserved in 100% ethanol immediately after collecting and stored at -20@C at the Entomological Museum of the Northwest A&F University, Yangling, Shaanxi Province, China. The genomic DNA was isolated from the thoracic tissue using the EasyPure^{R} Genomic DNA Kit (TransGen Biotech, Beijing).

2.2 Sequencing, Assembly, Annotation and Bioinformatic Analyses

Three complete mitogenomes were sequenced using next-generation sequencing (NGS) on an Illumina HiSeq 2000 platform (Biomarker Technologies, Beijing). The raw paired reads were retrieved and quality-trimmed using CLC Genomics Workbench v10.0 (CLC Bio, Aarhus, Denmark) with default parameters. The clean paired reads were then used for mitogenome reconstruction using MITObim v1.7 software (Hahn, Bachmann, & Chevreux, 2013) with default parameters and the mitogenome of *Ampittia dioscorides* (KM102732) (Qin, Yang, Hou, & Li, 2017) as the reference. Annotation of the mitogenomes and comparative analyses were conducted following the methodology outlined above. The various genomic features were annotated using Geneious 8.1.3 (Biomatters, Auckland, New Zealand) and referenced to the complete mitogenome sequence of *A. dioscorides* . Protein-coding genes (PCGs) were determined by finding the ORFs based on the invertebrate codon table (codon Table 5) and RNAs (tRNAs and rRNAs) were identified using MITOS Web Server (Bernt et al., 2013). Transfer RNAs were manually plotted according to the secondary structure predicted by MITOS, using Adobe Illustrator CS5. Finally, all genes were visually inspected against the reference mitogenome in Geneious. Nucleotide composition, codon usage, comparative mitogenomic architecture tables for the three mitogenomes, and data used to plot RSCU (relative synonymous codon usage) figures were all calculated and created using PhyloSuite (D. Zhang et al., 2020). The AT-skew and GC-skew were computed according to the following formulas: AT-skew=[A-T]/[A+T] and GC-skew=[G-C]/[G+C] (Perna & Kocher, 1995). The three newly-sequenced mitogenome sequences of Aeromachini (*Amipittia virgata* , *Halpe nephele* and*Onryza maga*) have been uploaded onto GenBank with the accession number MW288057, MW288058 and MW288059, respectively.

2.3 Phylogenetic Analysis

A total of 35 species (3 newly determined in this study, 32 available from GenBank) representing seven subfamilies of Hesperiidae were used to construct the phylogenetic relationships. The ingroup contains 5 species of Coeliadinae, 1 species of Euschemoninae, 2 species of Pyrginae, 4 species of Tagiadinae, 2 species of Eudaminae, 3 species of Heteropterinae, 2 species of Barcinae and 16 species of Hesperiinae. The 4 Papilionidae species (*P. machaon* , *P. helenus* , *G. timur* and *P. apollo*) were selected as outgroups (Table 1).

The complete mitogenome genes were extracted using PhyloSuite v1.2.2 and the sequences of 13 PCGs of the 39 species were aligned in batches with MAFFT integrated into PhyloSuite. Nucleotide sequences were aligned using the G-INS-i (accurate) strategy and codon alignment mode. All rRNAs were aligned in the MAFFT with the Q-INS-i strategy (Katoh & Standley, 2013). Poorly matched sites in the alignments were removed using Gblocks v0.91b (Castresana, 2000). Individual genes were also concatenated using PhyloSuite v1.2.2.

We used 3 datasets to reconstruct the phylogenetic relationship: (1) PCG matrix, containing all codon positions of the 13 protein-coding genes; (2) PRT matrix, concatenating all codon positions of the 13 protein coding genes, 22 tRNAs and 2 rRNAs; and (3) 12PRT matrix, including the first and second codon positions of 13 protein-coding genes plus 22 tRNAs and 2 rRNAs. Based on 3 datasets, the maximum likelihood (ML) and Bayesian inference (BI) methods were used to reconstruct the phylogeny. The optimal partitioning scheme and nucleotide substitution model for ML and BI phylogenetic analyses were selected using PartitionFinder 2.1.1 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017) with the greedy algorithm and BIC (Bayesian information criterion) criteria (Tables S3 and S4). Maximum likelihood analysis was inferred using IQ-TREE (Nguyen, Schmidt, Von Haeseler, & Minh, 2015) with the ultrafast bootstrap (UFB) approximation approach (Minh, Nguyen, & von Haeseler, 2013), as well as the Shimodaira-Hasegawa-like approximate

likelihood-ratio test (Guindon et al., 2010), and the bootstrap value (BS) of each node of the ML tree was evaluated via the bootstrap test with 10,000 replicates. Bayesian inference was carried out using MrBayes 3.2.6 (Ronquist et al., 2012) with the following requirements: 2 independent runs of 1×10^7 generations were conducted with four independent Markov Chain Monte Carlo (MCMC) runs, including 3 heated chains and a cold chain, by sampling every 1,000 generations. A consensus tree was obtained from all the trees after the initial 25% of trees from each MCMC run was discarded as burn-in, with the chain convergence assumed after the average standard deviation of split frequencies fell below 0.01. The confidence value of each node of the BI tree was presented as the Bayesian posterior probability (BP).

3. Results and Discussion

3.1 Mitogenome Organization and Base Composition

The total lengths of the mitogenomes of *Amipittia virgata* ,*Halpe nephele* and *Onryza maga* are 15,333 bp, 15,291 bp and 15,381 bp, respectively (Figure 1). The gene order and organization are similar to those of other butterflies previously determined, containing 13 protein-coding genes (PCGs), 22 transfer RNAs (tRNAs), 2 ribosomal RNAs (rRNAs) and a non-coding AT-rich region. Among them, 14 genes (*trnQ* ,*trnC* , *trnY* , *trnF* , *nad5* , *trnH* ,*nad4* , *nad4L* , *trnP* , *nad1* , *trnL1* ,*rrnL* , *trnV* , *rrnS*) are encoded from the N-strand, and the remaining 23 genes (*trnM* , *trnI* , *nad2* ,*trnW* , *cox1* , *trnL2* , *cox2* , *trnK* ,*trnD* , *atp8* , *atp6* , *cox3* , *trnG* ,*nad3* , *trnA* , *trnR* , *trnN* , *trnS1* ,*trnE* , *trnT* , *nad6* , *cytb* , *trnS2*) are from the J-strand (Table 2).

Nucleotide composition of is *A. virgata* is A=39.7%, C=11.8%, G=7.5% and it is T=41.0%. The base composition of *H. nephele* is A=40.3%, C=12.3%, G=7.6% and T=39.7%. And A=39.8%, C=12.2%, G=7.7% and T=40.2% in *O. maga.* The A+T content are 80.7%, 80.0% and 80.0% respectively, showing a relatively strong AT bias (Table 3). Compared with the whole genome, the non-coding AT-rich region (NCR) has the highest AT content, up to 89.7%, 89.3%, and 91.9%, respectively. On the contrary, PCGs are the regions with the lowest AT content, which is 79.1%, 78.4%, 78.2% respectively. In addition, the T content of these mitogenomes is higher than that of A, with the exception of *H. nephele* (Table 3).

3.2 Protein-Coding Genes and Codon Usage

The total lengths of the 13 PCGs of *Amipittia virgata* ,*Halpe nephele* and *Onryza maga* are 11,190 bp, 11,202 bp and 11,187 bp, respectively (Table 3). In these 3 sequenced species, 9 of 13 PCGs (*nad2* , *cox1* , *cox2* , *atp8* , *atp6* ,*cox3* , *nad3* , *nad6* , *cytb*) are encoded in the J-strand, and the other 4 (*nad5* , *nad4* , *nad4L* ,*nad1*) are located on the N-strand. The size of the 13 PCGs with the smallest gene for the 13 PCGs is the *atp8* and the largest gene is the *nad5* ranging in size from 162 bp to 1,744 bp. The AT-skew and GC-skew indicate that the T content of PCGs is obviously higher than that of A among these 3 species, while the content of G and C is not much different. The AT bias of the bases is more significant in the third codon, and the AT content of the third codon (90.5%˜ 92.3%) is remarkably higher than that of the first codon (73.7%˜ 74.6%) and the second codon (70.1%˜ 70.5%), which is consistent with the higher mutation rate of the third codon site compared with the second and first codon sites (Table 3). All PCGs of these 3 mitogenomes start with typical ATN (ATG, ATT, ATA) codons except *cox1* using CGA, and all of them use TAA or TAG as the stop codons, with the exception for*cox1* , *cox2* , *nad4* and *nad5* , which use a single T as stop codons (Table 2). Statistics on the relative synonymous codon usage (RSCU) of the 3 skippers shows that the codon UUA (*Leu2*), UCU (*Ser2*) and CGA (*Arg*) are the 3 used most frequently, and the codons terminating with A and T also have a relatively higher frequency (Figure 2).

3.3 Transfer and Ribosomal RNA Genes

Each of the 3 skipper species harbor 22 tRNA genes, 14 of which (*trnM* ,*trnI* , *trnW* , *trnL2* , *trnK* , *trnD* ,*trnG* , *trnA* , *trnR* , *trnN* , *trnS1* ,*trnE* , *trnT* , *trnS2*) are encoded in the J-strand and 8 of them (*trnQ* ,*trnC* , *trnY* , *trnF* , *trnH* , *trnP* ,*trnL1* , *trnV*) are encoded in the N-strand, ranging from 63 bp to 69 bp in size (Table 2). The total lengths of the tRNA region of*A.virgata* , *H.nephele* and *O.maga* are 1,458bp, 1,460bp and 1,457bp, respectively. The AT content of tRNA is slightly higher than that of the PCGs (Table

3). Most tRNA genes of these 3 mitogenomes could be folded into a cloverleaf secondary structure, except for *trnS* (AGN), which lacks the DHU arm (Figure 3). The total number of unmatched base pairs found in the tRNAs of the 3 skippers was 28 in *O. maga* , 29 in *A. virgata* and 34 in*H. nephele* . Most of these unmatched base pairs occur on the amino acid acceptor arm, the DHU arm and the anti-codon arm, with only a few occurring on the TΨC arm. The majority of unmatched base pairs is U-G which is a semicompensatory substitution; the others being U-U A-C, U-C, A-A, and A-G mismatches (Figure 3).

The 2 rRNA genes (*rrnL* , *rrnS*) encoded by the N-strand are located between *trnL* (CUN) and *trnV* , and between*trnV* and the AT-rich region respectively. The large subunit rRNA (*rrnL*) is 1,382/1,377/1,382 bp (*A.virgata/H.nephele/O.maga* , respectively) in length while the small subunit rRNA (*rrnS*) is 770/768/772 bp (Table 2). In addition, both tRNA and rRNA of the three mitogenomes show a strong AT bias, which is higher than that of the whole mitogenomes (Table 3).

3.4 Overlapping Sequences and Intergenic Spacers

There are 11, 8 and 5 gene overlapping regions in *A.virgata* ,*H.nephele* and *O.maga* mitogenomes, respectively, all ranging in size from 1 to 10 bp. The total lengths of the 3 mitogenomes ranges from 21 to 48 bp (Table 2). The longest of *A.virgata*mitogenomes is 10 bp located between *nad4* and *nad4L* , the longest of *H.nephele* is 25 bp located between*trnL1 -rrnL* , while the longest of *O.maga* is 8 bp located between *trnW-trnC* . Four identical overlapping regions, namely the *nad2-trnW* (2 bp), *trnW-trnC* (8 bp),*atp8-atp6* (7 bp) and *atp6-cox3* (1 bp) are all present in these 3 mitogenomes (Table 2). Nineteen, thirteen and sixteen intergenic spacers, ranging from 1 to 77 bp, from 2 bp to 53 bp and 1 bp to 78 bp, with their longest (77bp, 53bp, 78bp), are located between *trnQ*and *trnW* , are existed in *O.maga* , *A.virgata* and*H.nephele* mitogenomes, respectively (Table 2).

3.5 AT-rich Region

The AT-rich region is the longest non-coding region with a relatively high AT content, deemed to be related to the origin of replication and transcription (Boore, 1999; Cameron, 2014), and usually located between*rrnS* and *trnM* . In this study, this region ranges from 89.3% to 91.9%, with the longest (*A.virgata*) being 379 bp the second longest (*Halpe nephele*) being 374 bp, and the shortest (*O.maga*) being 369 bp in size (Table 3). In this study, a poly-T and poly-A stretches are all present with varying lengths in the AT-rich region. The poly-T length ranges from 16 bp to 22 bp, and the poly-A stretch ranges from 12 bp to 24 bp, often interrupted by the base T (Figure 4). These 2 types of T/A tandem repeats in the AT-rich region have been reported in other determined Hesperiidae mitogenomes (Han, Huang, Tang, Chiba, & Fan, 2018).

3.6 Phylogenetic Relationships

In this study, we conducted phylogenetic analysis on 39 butterfly species (including 4 outgroup species) with ML and BI methods based on three datasets (PCGs, PRT and 12PRT). Results show that the obtained phylogenetic trees harbored almost the same topological structures, with nodes of the tree being strongly supported (the bootstrap support values, BS, of ML trees and the posterior probability, PP, of the BI trees). Due to the limitation of the length of the paper, only one (PCGs-BI) of the six phylogenetic trees is shown here (Figure 5). The rest of the trees are in the supplementary materials (Figure S1—S5).

The phylogenetic tree consists of 8 clades corresponding to 8 major hesperiid subfamilies, and their relationships are (Coeliadinae + (Euschemoninae + ((Pyrginae + (Eudaminae + Tagiadinae)) + (Heteropterinae + (Barcinae + Hesperiinae))))) (Figure 5). The position of Eudaminae does not agree with that of previous studies where the subfamily is sister to the Pyrginae sensu lato, ie. Tagiadinae, Pyrrhopiginae, and Pyrginae sens Zhang et al (Jing Zhang et al., 2019). For the 3 Aeromachini species in this study, all results indicate that*H.nephele* and *O.maga* are sister groups (PP=1), and the*H.nephele* + *O.maga* calde is sister to *A.virgata*with strong support values (PP=1). The Aeromachini form an independent clade in the subfamily Hesperiinae, which is the subbasal lineage among them (PP=1). Although our analyses did not select sufficient samples of representative groups, for example the Eudaminae, a satisfactorily clustering and high node support values were present in all the obtained trees.

Apostictopterus fuliginosus and *Barca bicolor* had been placed in the subfamily Heteropterinae in previous research (Warren et al., 2008, 2009; Yuan et al., 2015) until Han et al. proposed that*A. fuliginosus* and *B. bicolor* should be placed in subfamily Hesperiinae through phylogenetic analysis of mitogenomes (Han et al., 2018). Subsequently Zhang et al, adding Trapezitinae in their analysis, raised them to a subfamily rank Barcinae (Jing Zhang et al., 2019). Since we could not include Trapezitinae in our analysis, we can only conclude that the hypothesis to place *A. fuliginosus* and *B. bicolor* out of the subfamily Heteropterinae is correct, and whether Barcinae is an independent subfamily or not needs further research.

4. Conclusions

Three mitogenomes of species in the tribe Aeromachini (*Amipittia virgata* , *Halpe nephele* and *Onryza maga*) were sequenced to provide more comprehensive molecular data for phylogenetic status in this study. The size and structure of mitochondria, gene order, and AT content of these three species are highly consistent with other Lepidoptera species. We conducted the phylogenetic analyses using ML and BI methods, and the results show that Aeromachini is a monophyletic group and sister to the rest of Hesperiinae and that the relationships among hesperiid subfamilies is Coeliadinae + (Euschemoninae + ((Pyrginae + (Eudaminae + Tagiadinae)) + (Heteropterinae + (Barcinae + Hesperiinae)))). Moreover, we support the previous study placing *A. fuliginosus* and *B. bicolor* out of the subfamily Hesperiinae. Our research provides data and a framework for the phylogeny of the tribe Aeromachini as well as even the family Hesperiidae.

Data Availability Statement

The following information was supplied regarding the availability of DNA sequences: The complete mitogenome of *Amipittia virgata* ,*Halpe nephele* and *Onryza maga* is deposited in GenBank of NCBI under accession number MW288057, MW288058 and MW288059, respectively.

Author Contributions

Conceptualization, X.H., H.C. and X.Y.; methodology, J.L., J.X. and H.C.; software, X.H., J.X. and J.L.; validation, X.H. and X.Y.; resources, X.Y.; writing—original draft preparation, X.H.; writing—review and editing, X.Y. and H.C.; supervision, X.Y.; project administration, X.Y.; funding acquisition, X.Y. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

All authors report no conflicts of interest.

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Table 1. The mitochondrial genome sequences of the 35 Hesperiidae species and 4 Papilionidae outgroup species used in this study

Taxon	Species	Accession number	References
Hesperiidae			
Coeliadinae	*Burara striata*	NC_034676	(J. Zhang, Cong, Shen, Wang, & Grishin, 2017)
	Choaspes benjaminii	NC_024647	(Kim, Wang, Park, & Kim, 2014)
	Hasora anura	KF881049	(J. Wang, James John, Xuan, Cao, & Yuan, 2016)
	Hasora vitta	NC_027170	(Cao et al., 2016)
	Hasora badra	NC_045249	Unpublished
Euschemoninae	*Euschemon rafflesia*	NC_034231	(J. Zhang, Q. Cong, J. Shen, X. L. Fan, et al., 2017)
Tagiadinae	*Celaenorrhinus maculosus*	NC_022853	(K. Wang, Hao, & Zhao, 2015)
	Ctenoptilum vasava	JF713818	(Hao et al., 2012)
	Daimio tethys	KJ813807	(Zuo, Gan, Chen, & Hao, 2016)
	Tagiades vajuna	KX865091	(Liu, Li, Jakovlic, & Yuan, 2017)
Pyrginae	*Pyrgus maculatus*	NC_030192	Unpublished
	Erynnis montanus	NC_021427	(A. R. Wang, Jeong, Han, & Kim, 2014)
Eudaminae	*Achalarus lyciades*	NC_030602	(Shen, Cong, & Grishin, 2016)
	Lobocla bifasciata	KJ629166	(Kim et al., 2014)
Heteropterinae	*Carterocephalus silvicola*	NC_024646	(Kim et al., 2014)
	Heteropterus morpheus	NC_028506	Unpublished
	Leptalina unicolour	MK265705	(Jeong, Kim, Jeong, & Kim, 2019)
Barcinae	*Apostictopterus fuliginosus*	NC_039946	(Han et al., 2018)
	Barca bicolor	NC_039947	(Han et al., 2018)
Hesperiinae	*Amipittia virgata*	MW288057	This study
	Halpe nephele	MW288058	This study
	Onryza maga	MW288059	This study
	Lerema accius	NC_029826	(Cong & Grishin, 2016)
	Ochlodes venata	HM243593	Unpublished
	Parnara guttata	NC_029136	(Shao, Sun, & Hao, 2015)
	Potanthus flavus	KJ629167	(Kim et al., 2014)
	Astictopterus jama	MH763663	(Ma, Liu, Chiba, & Yuan, 2020)
	Isoteinon lamprospilus	MH763664	(Ma et al., 2020)
	Notocrypta curvifascia	MH763665	(Ma et al., 2020)
	Agathymus mariae	KY630504	(Shen, Cong, Borek, Otwinowski, & Grishin, 2017)
	Megathymus beulahae	KY630505	(Jing Zhang et al., 2017)
	Megathymus cofaqui	KY630503	(Jing Zhang et al., 2017)
	Megathymus streckeri	KY630501	(Jing Zhang et al., 2017)
	Megathymus ursus	KY630502	(Jing Zhang et al., 2017)
	Megathymus yuccae	KY630500	(Jing Zhang et al., 2017)
Outgroup			
Papilionidae	*Papilio machaon*	NC_018047	Unpublished
	Papilio helenus	NC_025757	(Tang et al., 2014)
	Graphium timur	NC_024098	(Y. Chen, Gan, Shao, Cheng, & Hao, 2016)
	Parnassius apollo	NC_024727	(Y. H. Chen, Huang, Wang, Zhu, & Hao, 2014)

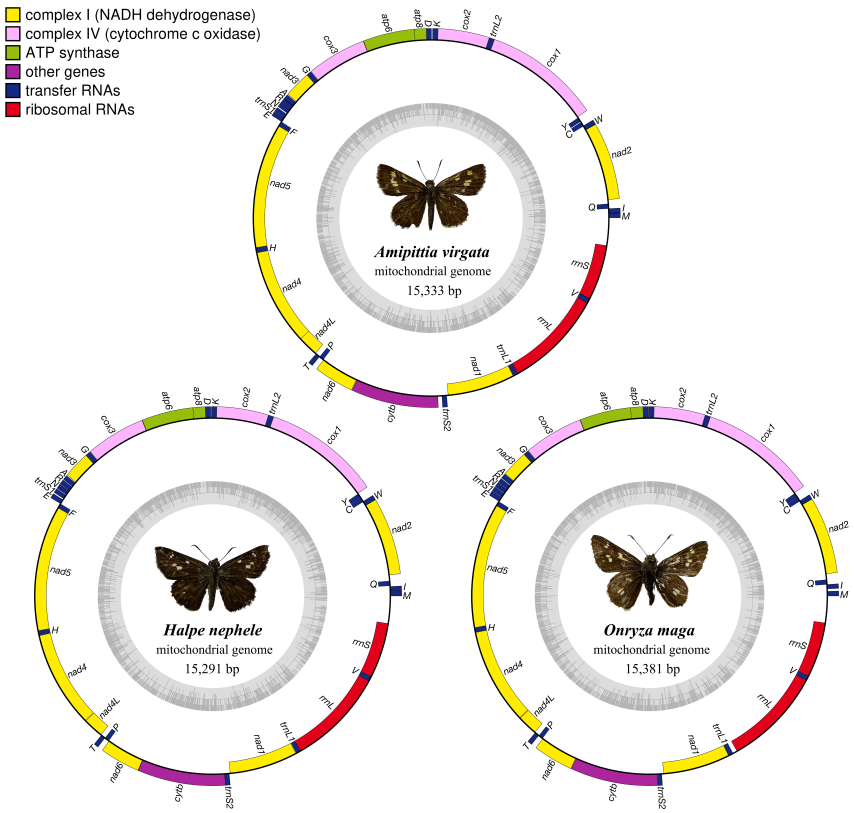
Table 2. Mitogenomic organization of *A.virgata, H.nephele and O.maga.*

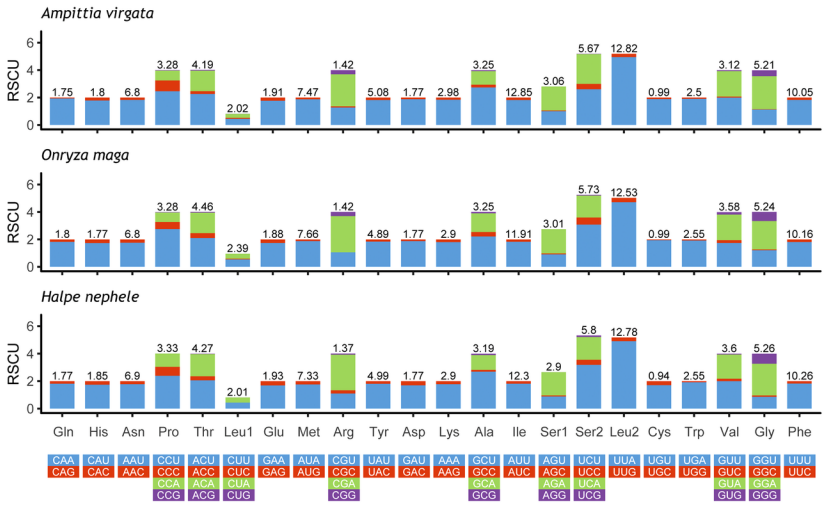
Gene	Position	Position	Size	Intergenic nucleotides	Intergenic nucleotides	Codon	Codon	Strand
	From	To				Start	Stop	
A.virgata/H.nephele/O.hugae	*A.virgata/H.nephele/O.hugae*	*A.virgata/H.nephele/O.hugae*	*A.virgata/H.nephele/O.hugae*	*A.virgata/H.nephele/O.hugae*	*A.virgata/H.nephele/O.hugae*	*A.virgata/H.nephele/O.hugae*	*A.virgata/H.nephele/O.hugae*	*A.virgata/H.nephele/O.hugae*
trnM	1/1/1	67/68/68	67/68/68	67/68/68				J/J/J
trnI	67/69/99	130/133/162	64/65/64	64/65/64	-1/-/30			J/J/J
trnQ	128/141/160	196/209/228	69/69/69	69/69/69	-3/7/-3			N/ N / N
nad2	250/288/306	1263/1301/1319	1014/1014/1014	1014/1014/1014	53/78/77	ATT/ATT/ATT	TAA/TAA/TAA	J/J/J
trnW	1262/1300/1318	1328/1366/1384	67/67/67	67/67/67	-2/-2/-2			J/J/J
trnC	1321/1359/1377	1385/1423/1441	65/65/65	65/65/65	-8/-8/-8			N/ N / N
trnY	1395/1425/1443	1460/1490/1507	66/66/65	66/66/65	9/1/1			N/ N / N
cox1	1471/1493/1514	3001/3023/3044	1531/1531/1531	1531/1531/1531	10/2/6	CGA/CGA/CGA	T/T/T	J/J/J
trnL2	3002/3024/3045	3068/3090/3111	67/67/67	67/67/67				J/J/J
cox2	3069/3091/3112	3744/3769/3787	676/679/676	676/679/676		ATG/ATG/ATG	T/T/T	J/J/J
trnK	3745/3770/3788	3815/3840/3858	71/71/71	71/71/71				J/J/J
trnD	3827/3845/3861	3893/3914/3929	67/70/69	67/70/69	11/4/2			J/J/J
atp8	3894/3915/3930	4055/4079/4094	162/165/165	162/165/165		ATA/ATT/ATT	TAA/TAA/TAA	J/J/J
atp6	4049/4073/4088	4726/4750/4765	678/678/678	678/678/678	-7/-7/-7	ATG/ATG/ATG	TAA/TAA/TAA	J/J/J
cox3	4726/4750/4765	5511/5535/5550	786/786/786	786/786/786	-1/-1/-1	ATG/ATG/ATG	TAA/TAA/TAA	J/J/J
trnG	5514/5538/5553	5577/5604/5618	64/67/66	64/67/66	2/2/2			J/J/J
nad3	5578/5605/5619	5931/5958/5972	354/354/354	354/354/354		ATT/ATT/ATT	TAA/TAA/TAA	J/J/J
trnA	5939/5967/5976	6005/6032/6043	67/66/68	67/66/68	7/8/3			J/J/J
trnR	6005/6038/6051	6067/6103/6115	63/66/65	63/66/65	-1/5/7			J/J/J
trnN	6068/6106/6118	6133/6172/6182	66/67/65	66/67/65	-/2/2			J/J/J
trnS1	6147/6178/6186	6207/6238/6246	61/61/61	61/61/61	13/5/3			J/J/J
trnE	6209/6253/6249	6273/6319/6317	65/67/69	65/67/69	1/14/2			J/J/J
trnF	6274/6318/6319	6341/6382/6383	68/65/65	68/65/65	-/-2/1			N/ N / N
nad5	6342/6383/6384	8076/8123/8127	1735/1741/1744	1735/1741/1744		ATT/ATT/ATT	T/T/T	N/ N / N
trnH	8077/8124/8128	8145/8188/8192	69/65/65	69/65/65				N/ N / N
nad4	8146/8189/8193	9493/9527/9531	1348/1339/1339	1348/1339/1339		ATT/ATG/ATG	T/T/T	N/ N / N
nad4L	9484/9534/9536	9771/9818/9820	288/285/285	288/285/285	-10/6/4	ATG/ATG/ATG	TAA/TAA/TAA	N/ N / N
trnT	9781/9824/9826	9845/9887/9890	65/64/65	65/64/65	9/5/5			J/J/J
trnP	9846/9888/9891	9910/9952/9955	65/65/65	65/65/65				N/ N / N
nad6	9913/9955/9958	10443/10485/10488	531/531/531	531/531/531	2/2/2	ATT/ATT/ATT	TAA/TAA/TAA	J/J/J
cytb	10446/10485/10491	11594/11636/11639	1149/1152/1149	1149/1152/1149	2/-1/2	ATA/ATG/ATA	TAA/TAA/TAA	J/J/J
trnS2	11647/11635/11641	11714/11698/11705	68/64/65	68/64/65	52/-2/1			J/J/J
nad1	11733/11709/11728	12674/12659/12666	942/951/939	942/951/939	18/10/22	ATT/ATA/ATG	TAA/TAG/TAA	N/ N / N
trnL1	12675/12663/12667	12743/12730/12734	69/68/68	69/68/68	-/3/-			N/ N / N

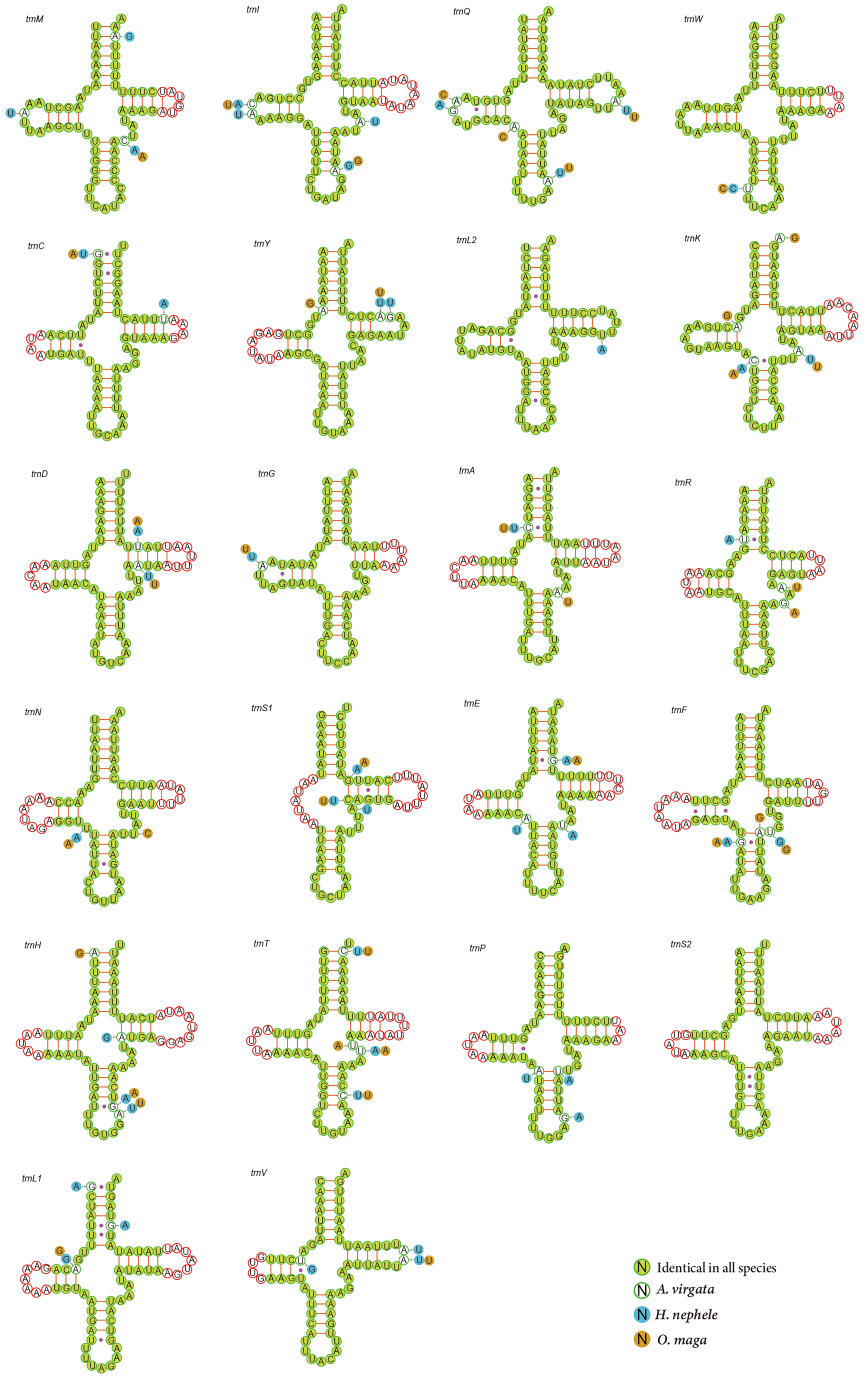
Gene	Position	Position	Size	Intergenic nucleotides	Intergenic nucleotides	Codon	Codon	Strand
rrnL	12739/12706/12792	14120/14082/14187	1382/1377/1382	2382/1377/1382	-25/-25/59			N/ N / N
trnV	14121/14083/14178	14185/14149/14246	65/67/65	65/67/65				N/ N / N
rrnS	14185/14150/14254	14917/14917/15012	768/768/772	770/768/772	-1/-/-			N/ N / N
NCR	14955/14918/15013	15291/15291/15388	337/374/369	379/374/369				J/J/J

Table 3. Nucleotide composition and skewness of different elements of mitogenomes of *A.virgata, H.nephele and O.maga.*

Regions	Size (bp)	T(U)%	C%	A%	G%	A+T%	AT skew	GC skew
A.virgata/ H.nephele/ O.maga								
PCGs	11190/11202/11457	45.3/45.1/45.1	10.6/10.9/11.0	33.6/33.1/33.1	10.3/10.8/10.8	79.1/78.4/78.2	-0.150/-0.155/-0.153	-0.012/-0.002/-0.011
1st codon position	3730/3734/3729	37.3/37.5/37.0	10.2/10.3/10.6	37.3/36.5/36.7	15.2/15.7/15.7	74.6/74.0/73.7	0.000/-0.013/-0.005	0.196/0
2nd codon position	3730/3734/3729	48.2/48.2/48.1	16.4/16.6/16.7	22.3/22.3/22.0	13.2/13.0/13.2	70.5/70.5/70.1	-0.368/-0.367/-0.372	-0.108/-0.121/-0.117
3rd codon position	3730/3734/3729	51.0/50.1/50.2	5.1/5.7/5.7	41.3/40.4/40.7	2.6/3.7/3.	92.3/90.5/90.9	-0.105/-0.107/-0.105	-0.329/-0.207/-0.253
NCR	379/374/369	48.3/45.7/46.1	6.6/7.2/5.7	41.4/43.6/45.8	3.7/3.5/2.4	89.7/89.3/91.9	-0.076/-0.024/-0.003	-0.282/-0.350/-0.400
tRNAs	1458/1460/1453	39.6/40.3/40.1	7.7/7.5/7.8	42.0/41.4/41.2	10.7/10.7/10.9	81.6/81.7/81.3	0.029/0.014/0.011	0.164/0
rRNAs	2152/2145/2154	41.5/42.1/41.4	4.9/5.1/5.1	43.7/42.8/44.1	9.9/9.9/9.5	85.2/84.9/85.5	0.026/0.008/0.032	0.333/0
Full genome	15333/15291/15481	43.8/39.7/40.2	11.8/12.3/12.2	39.7/40.3/39.8	7.5/7.6/7.7	80.7/80.0/80.0	-0.017/0.007/-0.005	-0.220/-0.237/-0.222







Amipittia virgata	*rrnS*-14955	···ATAGA···	TTTTTTTTTTTTTTTTTTTTT	···ATTTA···(TA)$_7$···	AATAAATATAAA	15333-*tRNA*^{Met}
Halpe nephele	*rrnS*-14918	···ATAGA···	TTTTTTTTTTTTTTTTTT	···ATTTA···(TA)$_7$··· ··· ATTTA···(TA)$_8$···(TA)$_2$···	ATAATAAATAAAAAAAAATAATTT	15291-*tRNA*^{Met}
Onryza maga	*rrnS*-15013	···ATAGA···	TTTTTTTTTTTTTTT	···ATTTA...(AT)$_6$···	···AATAAATATAAATTATAAA	15381-*tRNA*^{Met}