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First Record of the Complete Mitochondrial Genome for the Genus *Borbo* (Lepidoptera, Hesperiidae): Characterization and Comparative Genomic Analysis

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Abstract: Butterflies of the genus *Borbo* are mainly distributed in the Oriental and Australian regions and are considered pests of important crops. However, no mitochondrial genomes have been reported for this genus until now, leaving the evolutionary characteristics and differentiation patterns of their mitogenomes unclear. In this study, we present the first complete mitochondrial genome sequence of the rice swift, *Borbo cinnara*. The circular double-stranded mitogenome was 15,508 bp in length, comprising 13 protein-coding genes (PCGs), 2 ribosomal RNA genes (rRNAs), 22 transfer RNA genes (tRNAs), and 1 non-coding control region (CR). Among the mitogenomes of Hesperiinae, the *ND3* gene was found to be the most variable PCG, while *COX1* was the most conserved. Selection pressure analysis revealed that *ND3* was under relaxed purifying selection, whereas *COX1* was subjected to strong purifying selection. Phylogenetic trees reconstructed using both the Bayesian inference (BI) and maximum likelihood (ML) methods yielded robust and identical topologies, confirming the sister relationship between *B. cinnara* and *Pelopidas mathias* at the mitogenome level. Methodologically, this research enriches novel molecular markers for the species identification of butterflies and enhances our understanding of mitogenomic evolution in Lepidoptera.

Keywords: Lepidoptera; Hesperiinae; *Borbo cinnara*; mitochondrial genome; comparative analysis

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1. Introduction

Insect mitochondrial genomes are circular double-stranded DNA molecules, typically ranging in length from 14 to 20 kb in length and exhibiting remarkable conservation [1–3]. Mitogenomes generally consist of 37 genes: 13 protein-encoding genes (PCGs), 2 ribosomal RNA genes (rRNAs), 22 transfer RNA genes (tRNAs), and 1 non-coding A+T-rich region (or control region), which houses the initiation sites for transcription and replication [4,5]. Due to their cellular abundance, rapid evolution, and lack of introns, mitogenome sequences are easily amplified [6]. Compared to nuclear genes, mitogenomes have a compact size, maternal inheritance, conservative organization, lack of extensive recombination, and a higher mutation rate. Consequently, mitogenomes are widely used in studies of phylogenetic relationships, population genetics, and biogeography [3,7–9].

The butterfly genus *Borbo*, belonging to the tribe Baorini of the subfamily Hesperiinae, includes 17 known species worldwide, primarily distributed in the Oriental and Australian regions [10,11]. The larvae of many of these species feed on crops such as rice, corn, and sugarcane, causing significant agricultural damage during large outbreaks [11]. Despite their impact, current taxonomic studies on this genus rely mainly on morphological characteristics and a few partial gene fragments [10,12–14]. Previous research has shown that phylogenetic and evolutionary analyses based on whole mitogenome sequences are more reliable and stable than those based on single or partial gene fragments [6,15]. However, no mitogenomes from this group have been reported before, meaning that the evolutionary

characteristics and differentiation patterns of their mitochondrial genomes remain unclear and underexplored.

Borbo cinnara (Wallace, 1866), commonly known as the rice swift, is considered a minor pest of the economically important crop *Oryza sativa* L. (Asian rice) [16]. In this study, we sequenced, annotated, and analyzed the complete mitogenome of this species. We then conducted comparative analysis of mitogenome features among Hesperiinae species, focusing on genomic structure, nucleotide composition, and codon usage of the protein-coding genes (PCGs). Additionally, we reconstructed the phylogenetic relationships within the subfamily Hesperiinae using current mitogenome information, analyzed the taxonomic status of *B. cinnara*, and explored its phylogenetic relationships with related species at the mitogenome level.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

The *B. cinnara* samples utilized in this study were obtained from Luocheng (108°54′30″ E, 24°47′20″ N) on *Duranta erecta* in Guangxi Zhuang Autonomous Region, China. All fresh specimens were immediately soaked in 100% ethyl alcohol and then stored in a −20 °C refrigerator in our laboratory to facilitate DNA extraction. The identification of adult specimens was based on morphological characteristics as described by Yuan et al. (2015) and confirmed via *COX1* gene fragment using the NCBI database [17]. All specimens and vouchers were deposited at the School of Life Sciences, Qufu Normal University, Shandong, China.

Total genomic DNA was extracted from thorax and leg tissues using the Takara Genomic DNA Extraction Kit (Sangon Biotech, Shanghai, China) following the manufacturer's protocols. The quality of the extracted DNA was evaluated on a 1% agarose gel and further assessed using a Nanodrop 2000 spectrophotometer (Thermo, Wilmington, NC, USA). The extracted DNA was then preserved at −20 °C and used for future sequencing.

2.2. Primer Design, PCR Amplification and Sequencing

The complete mitogenome of *B. cinnara* was generated with overlapping fragments by means of the polymerase chain reaction (PCR) and Sanger sequencing [7]. Several pairs of universal primers for butterfly mitogenomes were used for PCR amplification [7,18]. Specific primers were designed by Primer Premier 5 to amplify overlapping segments of the whole mitogenome [19]. All fragments were amplified using Takara Ex-Taq polymerase (Takara, Dalian, China) under the conditions mentioned in a previous study [20]. The PCR products were electrophoresed on 1% agarose gels, and the target fragments were purified from excised pieces of gel using a SanPrep DNA Gel Extraction Kit (Sangon, Shanghai, China). Finally, the appropriate products were sequenced via the primer-walking strategy from both strands in Genscript Biotech Corp. (Nanjing, China) [20].

2.3. Mitogenome Assembly and Bioinformatic Analysis

All DNA sequences were checked with the SeqMan program of DNASTAR (Madison, WI, USA) and assembled using Geneious R9 [21,22]. The assembled mitogenome was initially annotated using the MITOS web server (http://mitos2.bioinf.uni-leipzig.de/, with the invertebrate mitochondrial genetic code [23]. PCGs were further adjusted and corrected manually using reference mitogenomes of Hesperiidae to identify the non-normal start and stop codons. To verify the primary annotation results, tRNAs and rRNAs were identified by aligning them with homologous genes of previously sequenced hesperiid mitogenomes. Intergenic and overlapping regions of the genome were manually estimated. Secondary structures of 22 tRNAs were determined using tRNAscan-SE 1.21 and ARWEN 1.2 [24,25]. The mitogenome map was produced using the visualization module of MitoZ [26].

For the comparative analysis, sequences from 20 additional Hesperiinae species were downloaded from GenBank (Table S1). A total of 21 species were analyzed to assess nucleotide composition and skew, codon usage of PCGs, and relative synonymous codon

usage (RSCU) values. Codon usage statistics were calculated using MEGA X and PhyloSuite v1.2.1 [27,28], while strand asymmetry was determined using the following formulas: AT skew = [A − T]/[A + T] and GC skew = [G − C]/[G + C] [29]. Nucleotide diversity analysis, with a sliding window of 100 bp and a step size of 25 bp, was performed using DnaSP 5.0. Additionally, the number of synonymous substitutions (Ks) and non-synonymous substitutions (Ka) and the Ka/Ks ratios for each PCG were calculated using DnaSP 5.0 [30].

2.4. Phylogenetic Analysis

The mitogenome sequences of 24 Hesperiidae butterflies were used for phylogenetic analysis, and mitogenomes of *Heteropterus morpheus* (Heteropterinae), *Leptalina unicolor* (Heteropterinae) and *Rachelia extrusus* (Trapezitinae) served as outgroups. Detailed information and accession numbers of these mitogenomes are listed in Table S1. The concatenated nucleotide sequences of 13 PCGs and two rRNAs were used in our analyses. Sequences of each PCG were aligned individually with codon-based multiple alignments using MAFFT 7.205 by codon using the L-INS-i algorithm [31]. Sequences of each rRNA were aligned separately using the G-INS-i strategy. DAMBE 5 was then used to test the nucleotide saturation, and Gblocks 0.91b was used with default settings to identify the poorly aligned positions [32,33]. Individual alignment fragments were finally concatenated using PhyloSuite [28]. The phylogenetic trees were reconstructed using both the Bayesian inference (BI) and maximum likelihood (ML) methods. The optimal partition strategy and substitution models were selected using PartitionFinder 2 with the greedy algorithm [34]. ML analysis was performed using RAxML 8.2.0 with 1000 bootstrap replicates under the automatic model prediction [35]. BI analysis was carried out using MrBayes 3.2.215 under the following conditions: two independent Markov-chain Monte Carlo runs for 1,000,000 generations, sampling every 1000 generations, with a burn-in of 25% [36]. Finally, phylogenetic trees were checked and visualized in FigTree 1.4.2 [37].

3. Results and Discussion

3.1. General Mitogenomic Features

The complete mitogenome of *B. cinnara* was 15,508 bp in size, comprising 37 typical genes including 13 protein-coding genes (PCGs), 22 transfer RNAs (tRNAs), 2 ribosomal RNAs (rRNAs), and 1 long non-coding region known as the control region (CR) (Figure 1). Of these, 23 genes (9 PCGs and 14 tRNAs) were encoded on the majority strand (J-strand), while the remaining 14 genes (4 PCGs, 2 rRNAs, and 8 tRNAs) were located on the minority strand (N-strand) (Table 1). The organization and gene order were in keeping with those of other hesperiid butterflies previously reported (Table 1). All genes were arranged in the same order and direction as in the ancestral insect *Drosophila yakuba*, with the exception of the order among three (*trnI*, *trnQ,* and *trnM*) [38]. Comparative analysis showed that this rearrangement of tRNAs was extremely conservative within Lepidoptera, suggesting it might be regarded as a clear molecular synapomorphy for this order [39,40].

As is typical for Lepidoptera, the mitogenome of *B. cinnara* exhibited a significant bias towards A and T, with a nucleotide composition of 40.02% A, 40.90% T, 7.41% G, and 11.67% C. The highest A+T content was found in the control region (93.31%), while the lowest was in the PCGs (79.28%). The A+T content in each gene component showed a decreasing trend as follows: CR > rRNAs > tRNAs > PCGs. The AT skew and GC skew of the entire genome and PCGs were negative, while those of tRNAs and rRNAs were positive. This situation of nucleotide bias may be due to the asymmetric mutation during replication and transcription. Notably, the AT skew of CR was positive (0.104), and the GC skew was zero, indicating an equal number of Gs and Cs.

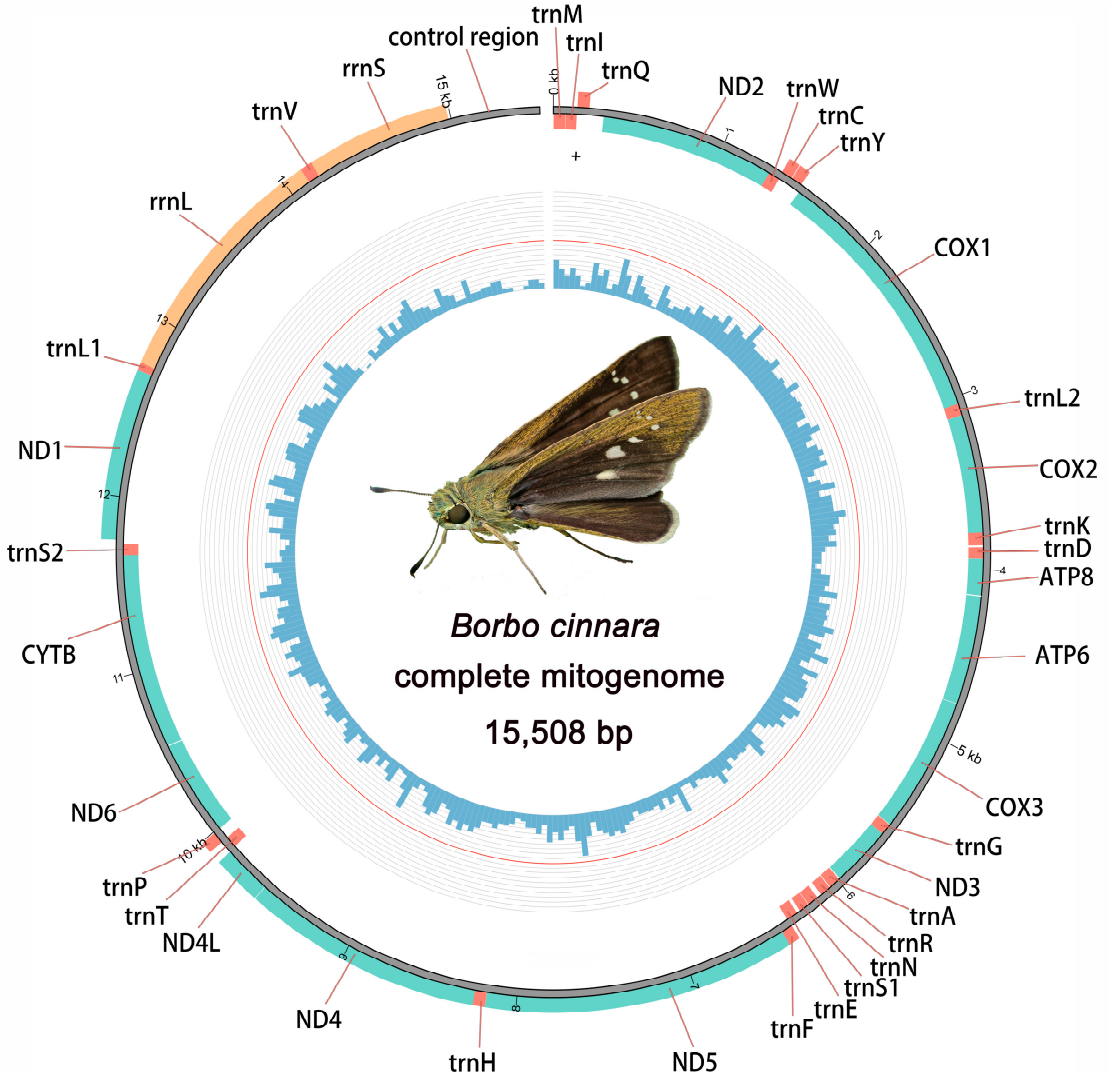

Figure 1. Complete mitochondrial genome map of *B. cinnara*. Genes transcribed anticlockwise outside and clockwise inside. The blue histogram on the inside indicates the GC content.

Table 1. Organization of the *B. cinnara* mitochondrial genome.

Gene	Strand	Location		Size (bp)	IN	Anticodon	Start Codon	Stop Codon
		From	To					
trnM	J	1	69	69	0	CAT		
trnI	J	71	135	65	1	GAT		
trnQ	N	137	205	69	1	TTG		
ND2	J	291	1304	1014	85		ATT	TAA
trnW	J	1303	1371	69	−2	TCA		
trnC	N	1364	1427	64	−8	GCA		
trnY	N	1431	1494	64	3	GTA		
COX1	J	1509	3039	1531	14		CGA	T--
trnL2	J	3040	3106	67	0	TAA		
COX2	J	3107	3766	660	0		ATG	TAA
trnK	J	3787	3857	71	20	CTT		
trnD	J	3870	3935	66	12	GTC		
ATP8	J	3936	4103	168	0		ATT	TAA
ATP6	J	4097	4774	678	−7		ATG	TAA
COX3	J	4774	5562	789	−1		ATA	TAA

Table 1. *Cont.*

Gene	Strand	Location		Size (bp)	IN	Anticodon	Start Codon	Stop Codon
		From	To					
trnG	J	5565	5631	67	2	TCC		
ND3	J	5632	5985	354	0		ATT	TAA
trnA	J	5991	6057	67	5	TGC		
trnR	J	6062	6124	63	4	TCG		
trnN	J	6152	6217	66	27	GTT		
trnS1	J	6220	6278	59	2	GCT		
trnE	J	6298	6365	68	19	TTC		
trnF	N	6364	6429	66	−2	GAA		
ND5	N	6430	8152	1723	0		ATT	T--
trnH	N	8171	8239	69	18	GTG		
ND4	N	8240	9578	1339	0		ATG	T--
ND4L	N	9581	9856	276	2		ATG	TAA
trnT	J	9862	9927	66	5	TGT		
trnP	N	9928	9993	66	0	TGG		
ND6	J	9996	10,520	525	2		ATT	TAA
CYTB	J	10,520	11,671	1152	−1		ATG	TAA
trnS2	J	11,670	11,736	67	−2	TGA		
ND1	N	11,758	12,696	939	21		ATG	TAA
trnL1	N	12,697	12,764	68	0	TAG		
rrnL	N	12,765	14,116	1352	0			
trnV	N	14,117	14,183	67	0	TAC		
rrnS	N	14,184	14,985	802	0			
A+T rich	J	14,986	15,508	523	0			

3.2. Protein-Coding Genes

The 13 protein-coding genes (PCGs) of *B. cinnara* span 11,148 bp, accounting for 71.87% of the total mitogenome sequence. Four of these genes (*ND1*, *ND4*, *ND4L* and *ND5*) were encoded on the N-strand, while the remaining nine were located on the J-strand, consistent with findings in other reported butterfly mitogenomes (Figure 1 and Table 1). The shortest PCG is *ATP8* (168 bp), while the longest is *ND5* (1723 bp). All PCGs, except for *COX1*, begin with typical ATN start codons (ATA/ATG/ATT). Notably, *COX1* uses an alternative start codon, CGA, a feature observed in many other insect mitogenomes [41,42]. Regarding stop codons, all PCGs terminated with the complete TAA codon, except for *COX2*, *ND4* and *ND5*, which end with a single T. These incomplete stop codons are likely to be completed through post-transcriptional polyadenylation [43].

In addition to the stop codons, the mitogenome of *B. cinnara* encoded 3703 amino acids. Relative synonymous codon usage (RSCU) values were calculated and visualized in Figure 2. All possible synonymous codons of the 22 amino acids, except for AGG, were detected. Leucine (Leu2), phenylalanine (Phe), asparagine (Asn) and tyrosine (Tyr) were the most frequently encoded amino acids, with isoleucine (Ile) possessing the highest RSCU value (Table S2). Meanwhile, UUA (L), AUU (I), and UUU (F) were the most frequently used codons in this mitogenome. Comparative analysis suggested that codon frequencies might be one of the possible factors for the biased usage of A+T content.

3.3. Ribosomal and Transfer RNA Genes

Both the large rRNA (*rrnL*) and small rRNA (*rrnS*) genes were identified in the sequenced mitogenome, with rrnL located between trnL1 and trnV and rrnS positioned between trnV and the control region. Both genes were oriented on the N-strand (Figure 1 and Table 1). The lengths of *rrnL* and *rrnS* were 1352 bp and 802 bp, with A+T contents of 83.24% and 85.66%, respectively. Both rRNAs displayed positive AT skew and GC skew, consistent with observations in other lepidopteran mitogenomes [39,40]. All 22 typical tRNA genes were detected, ranging in size from 59 to 71 bp (Table 1). Fourteen genes

were encoded on the J-strand and eight on the N-strand, aligning with other Lepidoptera species. All tRNAs were found to be folded into typical cloverleaf secondary structures, except for *trnS1*, which lacked the dihydrouridine (DHU) arm and formed a simple ring instead (Figure 3). The missing DHU arm of *trnS1* is considered a common phenomenon in many other insect taxa [20,40]. Besides the normal base pairs (A-U and C-G), the secondary cloverleaf stems also included multiple non-Watson–Crick base pairs (15 G-U pairs and seven U-U pairs). These mismatches can be corrected through editing processes, ensuring that they do not affect the transport function in insect mitogenomes [44].

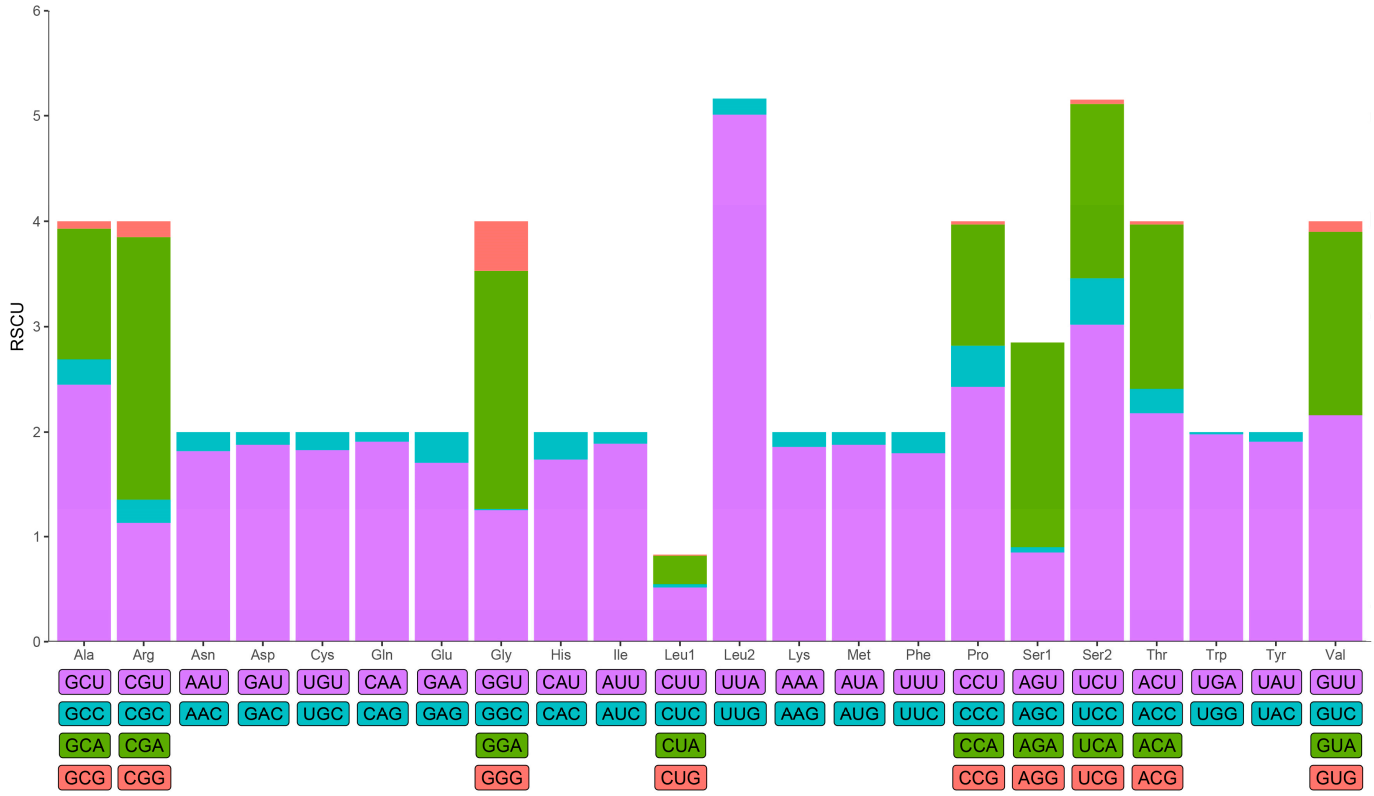

Figure 2. Relative synonymous codon usage (RSCU) of the mitogenome of *B. cinnara*.

3.4. Non-Coding Regions

Previous studies have shown that the number and size of overlapping regions and intergenic spacer regions vary significantly between species, contributing to differences in the overall length of the mitogenome [20]. In the mitogenome of *B. cinnara*, seven overlapping regions were identified, ranging in size from one to eight base pairs. Notably, the overlap at the *ATP6/ATP8* boundary is conserved across butterfly mitogenomes, with the 7 bp nucleotide fragment (5′-ATGATAA-3′) being identical to that found in other available mitogenomes [45]. Additionally, 19 intergenic regions of varying sizes (1–523 bp) are present in this mitogenome. The longest intergenic spacer (IGS) region, aside from the control region, is 85 bp and is located between *trnQ* and *ND2*. Among the non-coding regions, the largest is the control region, which spans 523 bp and is situated between the *rrnS* and *trnI* genes. This region, also known as the A+T-rich region due to its nucleotide composition, contains the highest percentage of A+T nucleotides and plays a crucial role in the regulation and initiation of mitochondrial DNA transcription and replication.

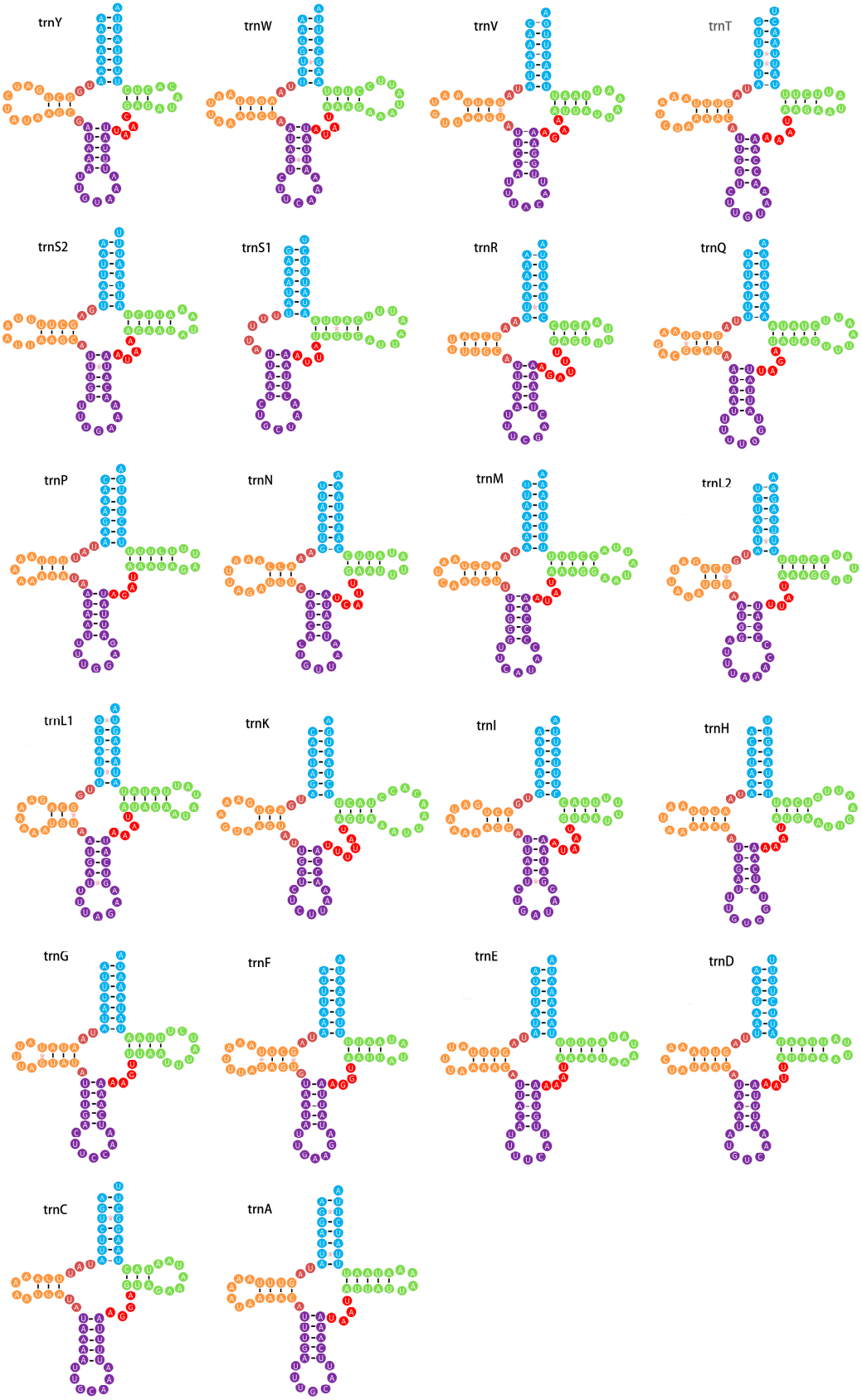

Figure 3. Secondary structures of 22 tRNAs in the mitogenome of *B. cinnara*. Blue: amino acid accepter arm; Green: TψC arm; Red: variable loop; Purple: anticodon arm; Orange: dihydorouidine arm.

3.5. Comparative Analysis of Hesperiinae Mitogenomes

The mitogenome sequences of 21 Hesperiinae butterflies displayed a significant AT nucleotide bias, ranging from 71.99% to 78.57%, with the AT content in the protein-coding genes (PCGs) being slightly lower than in other regions (Figure 4). Compositional asymmetry, measured by AT and GC skews, showed that the AT skew for tRNAs and rRNAs was predominantly positive, while other regions exhibited negative values, indicating a higher number of T nucleotides compared to As (Figure 5). The GC skew for the entire sequences of the 21 genomes was consistently negative (−0.18 to −0.25), while it was positive for tRNAs and rRNAs. Interestingly, the GC skew across the three matrices of PCGs varied among species, even among closely related ones, suggesting that GC content is not conserved within Hesperiinae. Additionally, the AT content of PCG3 was significantly higher than that of PCG123 and PCG12. Codon usage patterns and the most frequently used codons across the 21 mitogenomes were highly conservative. The most frequently used amino acids were isoleucine (Ile), leucine (Leu), and phenylalanine (Phe), which were encoded by codons with high A or T contents (Figure 6). This AT content and codon usage bias might result from synonymous codon usage in mitogenomes favoring codons ending in A or T, which could facilitate transcription [46,47].

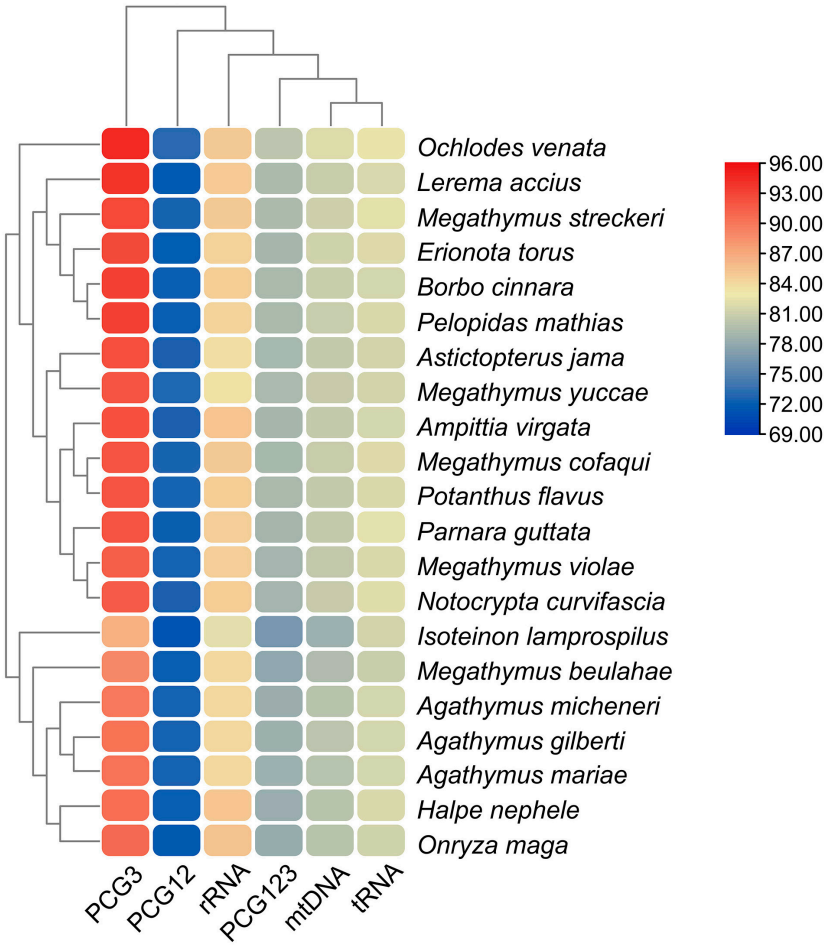

Figure 4. Nucleotide composition across different datasets of 21 Hesperiinae mitogenomes.

Figure 7A illustrates the nucleotide diversity of Hesperiinae mitogenomes (PCGs) using a sliding window of 100 bp. The most variable region was identified in *ND3*, while *COX1* exhibited the lowest nucleotide diversity (Pi), consistent with observations in other insects [48]. The Ka (nonsynonymous substitutions) and Ks (synonymous substitutions) values are widely used as indicators of selective pressure [49]. To evaluate the evolutionary rates of Hesperiinae PCGs, we calculated the average Ka/Ks ratios, which ranged from 0.07

(*COX1*) to 0.79 (*ND3*), indicating that all PCGs are under purifying selection (Ka/Ks < 1). Our in-depth analysis showed that *ND3* is subject to relaxed purifying selection, whereas *COX1* and *COX2* are under the strongest purifying selection (Figure 7B), highlighting the strong functional constraints on Hesperiinae genes.

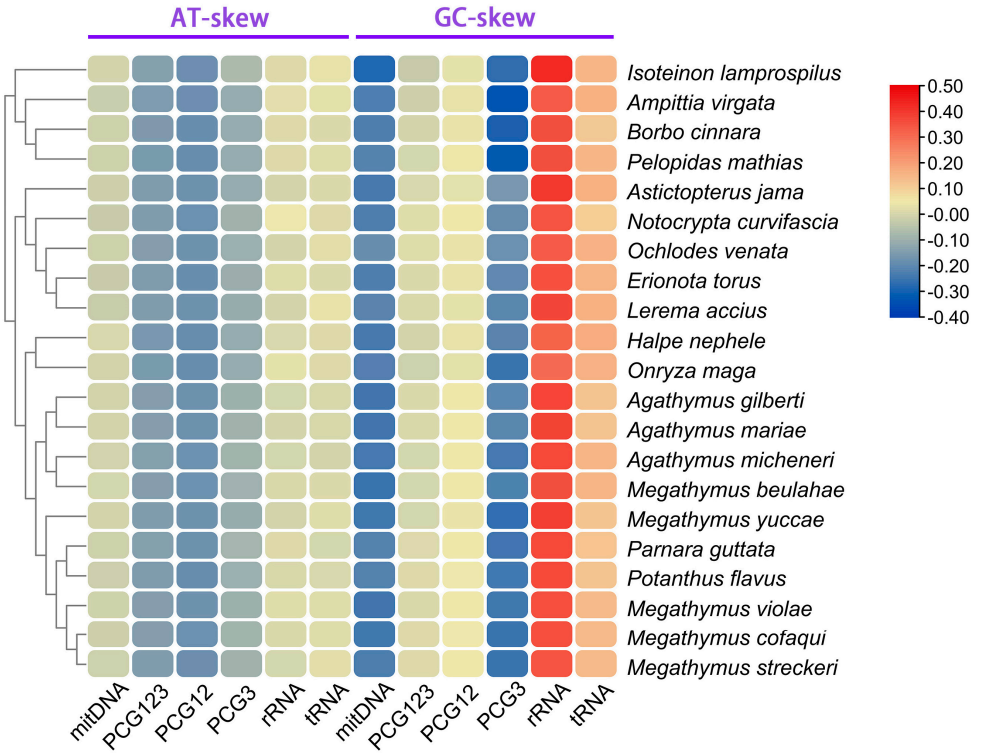

Figure 5. Nucleotide composition of various datasets of 21 Hesperiinae mitogenomes.

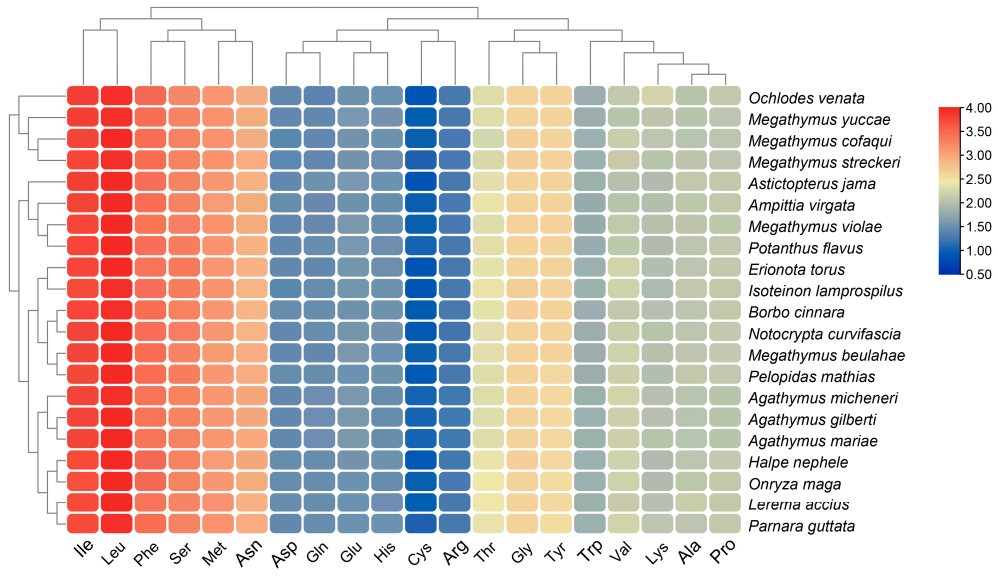

Figure 6. Amino acid composition of all protein-coding genes (PCGs) across the 21 Hesperiinae mitogenomes.

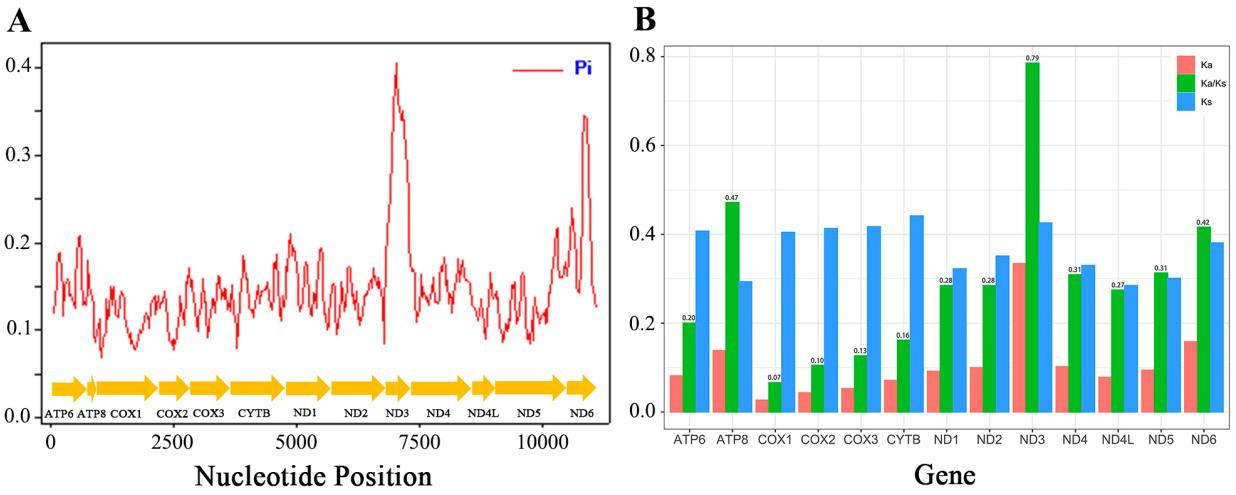

Figure 7. (**A**) Sliding window analysis conducted on 13 aligned protein-coding genes (PCGs), with the blue line representing nucleotide diversity (Pi). (**B**) The ratio of non-synonymous (Ka) to synonymous (Ks) substitution rates was calculated across the 13 PCGs for 21 species within the Hesperiinae subfamily.

3.6. Phylogenetic Analysis

In the present study, the mitogenomes of 24 Hesperiinae species and three outgroup species (one species of Trapezitinae and two of Heteropterinae) were used to reconstruct the phylogenetic relationships of Hesperiinae. The matrix contained 12,333 sites, including all codon positions of 13 PCGs plus 2 rRNAs. DAMBE analysis confirmed the suitability of the nucleotide's unsaturation of the concatenated data for phylogenetic analysis (ISS < ISS.c). Using two analytical methods, Bayesian inference (BI) and maximum likelihood (ML), we generated phylogenetic trees that exhibited unique topologies. These trees were subsequently integrated (Figure 8). The results demonstrated high stability, as indicated by the robust nodal support values derived from mitogenome data.

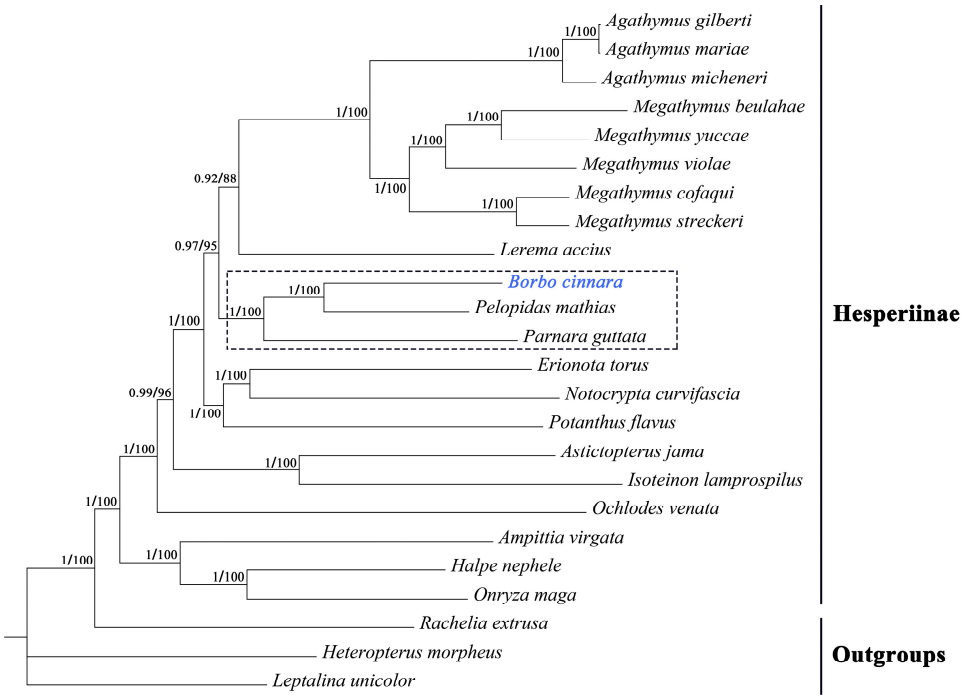

Figure 8. Phylogenetic trees obtained from ML and BI analysis. Numbers on nodes are the posterior probability (PP) and bootstrap value (BV).

The analysis revealed a large disparity in the number of available mitogenome sequences among different genera, with most genera having only one sequence available. Two genera with more than two species were found to be monophyletic in our trees. Three genera (*Borbo*, *Pelopidas* and *Parnara*) with only one species belong to the same tribe Baorini [50]. Among the three species in Baorini, the following relationships were reconstructed with the support of high nodal values (PP = 1 and BV = 100):((*B. cinnara* + *Pelopidas mathias*) + *Parnara guttata*), which indicated that *Borbo* was more closely related to *Pelopidas* than *Parnara*. Our results supported those from the previous study based on several genes [11,14,51,52].

4. Conclusions

In this study, we successfully determined and analyzed the complete mitogenome of *B. cinnara*. The gene characteristics and arrangements were consistent with those reported in other butterfly mitogenomes. The nucleotide composition of the mitogenome was relatively conserved, displaying a preference for A and T nucleotides. All PCGs started with the canonical ATN start codon, except for *COX1*, which utilized CGA. Three genes (*COX2*, *ND4* and *ND5*) were terminated with the incomplete codon T. The nucleotide diversity of Hesperiinae PCGs revealed that *ND3* was the most variable gene, while *COX1* was the most conserved. Furthermore, selection pressure analysis for each gene showed that *ND3* was under relaxed purifying selection, whereas *COX1* experienced the strongest purifying selection. These comparative analyses contribute to our understanding of butterfly mitochondrial genome evolution. Phylogenetic analyses based on PCGs and rRNAs from the mitogenomes of 24 species provided clarity on the phylogenetic relationships within Hesperiinae. The sister relationship between *B. cinnara* and *P. mathias* was corroborated at the mitogenome level. Our findings suggest that whole mitogenomes are effective molecular markers that can be used to study phylogenetic relationships within Hesperiinae. Due to extremely limited available data and uneven sequencing among different genera, larger samples across more taxa are necessary for future research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16090560/s1, Table S1. Complete mitogenomes used in this study. Table S2. Codon number and RSCU in the mitogenome of *B. cinnara*.

Author Contributions: Conceptualization, C.X., R.L. and X.Y.; methodology, R.L.; software, C.X. and D.L.; validation, D.Z. and D.L.; formal analysis, C.X. and D.Z.; investigation, C.X. and L.J.; resources, L.J. and R.L.; data curation, C.X. and R.L.; writing—original draft preparation, C.X. and D.Z.; writing—review and editing, R.L. and X.Y.; visualization, C.X. and R.L.; supervision, X.Y.; project administration, R.L. and X.Y. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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