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Mitogenomes of Three Satyrid Butterfly Species (Nymphalidae: Lepidoptera) and Reconstructed Phylogeny of Satyrinae

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Abstract: Satyrinae is a 3000-species butterfly subfamily of Nymphalidae. The higher-level classification of this family is still controversial. In this research, we sequenced the complete mitogenomes of three satyrid butterfly species, *Hipparchia autonoe*, *Paroeneis palaearctica*, and *Oeneis buddha*, and studied the phylogeny of Satyrinae with all known complete mitogenomes. The results showed that the lengths of the three satyrid butterfly mitogenomes are 15,435 bp (*H. Autonoe*), 15,942 bp (*P. palaearctica*), and 15,259 bp (*O. buddha*). Gene content and arrangement of newly sequenced mitogenomes are highly conserved and are typical of Lepidoptera. These three mitogenomes were found to have a typical set of 37 genes and an A + T-rich region. The tRNA genes in these three mitogenomes showed a typical clover leaf structure, but the stem of tRNASer (AGN) was lacking dihydroacridine. In these three species, the lengths of the A + T-rich regions were different, which led to differences in mitochondrial genome sizes. The characterizations of the three mitogenomes enrich our knowledge on the Lepidopteran mitogenome and provide us genetic information to reconstruct the phylogenetic tree. Finally, the phylogenetic results confirmed the position of the genus *Davidina* in the subfamily Satyrini, had a closer phylogenetic relationship with *Oeneis*, and the phylogenetic analysis supported the formation of *Oeneis buddha* as an independent taxon in *Oeneis*.

Keywords: mitochondrial DNA; butterfly; Satyrinae

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Citation: Dan, Z.; Duan, L.; Chen, Z.; Guan, D.; Xu, S. Mitogenomes of Three Satyrid Butterfly Species (Nymphalidae: Lepidoptera) and Reconstructed Phylogeny of Satyrinae. *Diversity* **2021**, *13*, 468. https://doi.org/10.3390/d13100468

Academic Editor: Luc Legal

Received: 21 July 2021
Accepted: 23 September 2021
Published: 27 September 2021

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1. Introduction

Satyrinae is the most diversified butterfly group in Nymphalidae (Lepidoptera). It includes approximately 400 genera and 3000 species. They are distributed in all continents except Antarctica [1,2]. Satyrinae is traditionally divided into nine tribes [3,4]. However, the higher-level classification of this family is still controversial [2,5,6]. *Oeneis* is a type of butterfly that adapts to cold climates and is distributed at high altitudes. Due to their similarity in morphology, the boundaries at the species level have been very blurred. The early classification of this type of butterfly was mainly based on appearance and some gene fragments [7]. *Davidina* is a mysterious genus. The genus was mistaken as a Pieridae member early on because of the black veins and the absence of any eye spots on the wings [8]. Kusnezov [9] moved the genus into Satyrinae and later placed it in Satyrini (equivalent to the current Satyrinae) [10]. Similarly, *Paroeneis* had not yet been sampled in previous molecular phylogeny studies. This genus also lacks eye spots and has limited distribution. Due to the intricacy, it is essential to use mtDNA genome sequences of these species for classification and phylogenetic analysis.

The mtDNA of insects is approximately 16 kb in length. This circular DNA encodes 13 mitochondrial proteins, 22 mitochondrial tRNAs, and two mitochondrial-specific ribosomal RNAs, specifically 12S rRNA and 16S rRNA [11]. Additionally, it contains one non-coding DNA region, the A + T-rich region (or control region), which controls replication and transcription [12]. Due to rapid evolution, cellular abundance, and the absence of introns, mitochondrial sequences can be easily amplified. In addition, they have a

compact size, maternal inheritance, conservative features in the genetic organization, lack of extensive recombination, and a higher mutation rate than nuclear sequences. It is currently widely used in molecular evolution research, population genetic comparison, species identification, phylogenetic analysis, and evolutionary genomics [13–18]. In particular, phylogenetic analysis based on the mitochondrial whole genome demonstrated improved resolution of the inferred phylogenetic tree compared to phylogenetic trees based on partial gene fragments. In previous studies of mitochondrial genomes of Satyrinae, only partial genome sequences of limited taxon sampling have been performed. Therefore, it is necessary to study the phylogeny of Satyrinae according to denser taxon and mitochondrial sequence. To date, complete or near-complete mitogenomes have only been sequenced for 41 Satyrinae species, belonging to the subfamilies Satyrini, Melanitini, and Elymniini [19–36].

In the present study, three new sequences of Satyrinae were obtained, seven sequences of Satyrinae were extracted from the published butterfly genome data set, and we reannotated the published mitochondrial genomes of eight satyrid. To better understand the functions of related genes, we analyzed the relative synonymous codon usage (RSCU) and AT skew values of protein coding genes (PCGs) and compared them with those of other Lepidopteran sequences. Furthermore, the phylogeny of Satyrinae and related species was constructed, and the relationships between these taxa were discussed. The divergence time of three species in Satyrinae was evaluated.

2. Materials and Methods

2.1. Specimens and DNA Extraction

Specimens of *Hipparchia autonoe*, *Paroeneis palaearctica*, and *Oeneis buddha* were collected from the Qinghai Province of China in 2017. Genomic DNA was extracted from the 95–100% ethanol-preserved muscle tissue of three satyrid species using an EasyPure Genomic DNA Kit according to the manufacturer's instructions ((TransGen Biotech, Beijing, China).

2.2. Mitogenome Sequencing, Assembly, and Annotation

The entire mitochondrial genome was sequenced using an Illumina HiSeq2500platform (Biomarker Technologies, Beijing, China). We used Geneious 11.0.2 software to retrieve other Satyrinae species as reference sequences and used the default parameters to qualitatively prune the original sequence [37]. The 13 PCGs were predicted by comparison with the homologous sequence of reference mitogenomes and finding the open reading frames (ORFs) based on the invertebrate mitochondrial genetic code. The locations of 22 tRNAs were identified by using the MITOS WebServer (http://mitos.bioinf.uni-leipzig.de/index.py, accessed on 1 July 2021) [38]. The two ribosomal RNA genes (rrnS and rrnL) and the A + T-rich region were determined by the locations of adjacent genes (trnL1 and trnV) and alignment with the homologous sequences of reference mitogenomes. A Mitochondrial Genome ring map was constructed online utilizing Organellar genome-draw [39].

In this study, we found the genome data of seven Satyrinae species (*Coenonympha arcania*, *Lasiommata maera*, *Lasiommata megera*, *Maniola bathseba*, *Maniola Cecilia*, *Melanargia galathea*, *Melanargia ines*) from the published butterfly genome data set and selected the mitochondrial genome data we needed from them [40]. Mitochondrial genomes were spliced and annotated using Geneious11.0.2 and MITOS Web server (http://mitos.bioinf.uni-leipzig.de/index.py, accessed on 1 July 2021) [37]. The mitochondrial genome of *Lasiommata deidamia* was selected as the reference sequence, and mitochondrial reads were captured from the genome-wide data of seven satyrid. Mapping the whole genome data of seven satyrid to the mitochondrial genome of *L. deidamia*, high-coverage, and continuous mitochondrial reads formed sequence blocks (bins), the individual bins, or connected them to Contigs according to the overlap of bins to replace the original reference. The sequence was used as the target sequence for the next mapping (baiting sequencing), the whole genome data were sequentially and repeatedly mapped to the newly generated

target sequence to extend the sequence, and, finally, extended to the length of the complete mitochondrial genome [41].

2.3. Sequence Analyses

The nucleotide composition and skew, codon usage of PCGs, and relative synonymous codon usage (RSCU) values of each PCG were calculated using PhyloSuite v1.2.1 [42], and tandem repeat units of the A + T-control region were analyzed with Tandem Repeats Finder online server (http://tandem.bu.edu/trf/trf.html, access on 1 July 2021) [43]. Strand asymmetry was calculated according to the following formulas: AT skew = [A − T]/[A + T] and GC skew = [G − C]/[G + C] [44].

2.4. Divergence Time Estimation

Divergence times were estimated by the LF method with a truncated Newton algorithm in R8S version 1.70, using as an input tree the consensus topology and branch lengths from MrBayes [45]. Four known divergence times were used as calibration points (*Lasiommata maera* with *Lasiommata megera* 14.18 MYA [46], *Melanargia ines* with *Melanargia galathea* 13.52 MYA [46], *Minois dryas* with *Oeneis urda* 15.86MYA [46], and *Melanitis leda* with *Elymnias hypermnestra* 47 MYA [5]).

2.5. Phylogenetic Analysis

A total of 54 mitogenomes of Nymphalidae insects were collected to analyze the phylogenetic relationships. Three newly sequenced specimens and 48 available mitogenomes were selected as ingroups (Tables S2 and S3). Three species, *Polyura arja, Polyura Schreiber*, and *Polyura nepenthe* (Nymphalidae) were employed as outgroup taxa. The nucleotide sequences of all 13 PCGs and two rRNA genes were used to elucidate the phylogenetic relationships of this tribe. All the available mitochondrial genomes were downloaded from GenBank for phylogenetic analyses.

Complete and partial mitogenome genes were extracted using PhyloSuitev1.2.1. The nucleotide sequences of all PCGs of the 54 species were aligned in batches with the MAFFT v7.313 (https://mafft.cbrc.jp/alignment/server/, access on 1 July 2021) algorithm integrated into PhyloSuitev1.2.1, using the codon alignment mode and G-INS-i (accurate) strategy. The alignment of all rRNAs was conducted in the MAFFT version 7 online service with the G-INS-i strategy (https://mafft.cbrc.jp/alignment/server/, access on 1 July 2021) [47].

The optimal partitioning scheme and nucleotide substitution model for Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyses with PartitionFinder2.1.1 incorporated into PhyloSuitev1.2.1 [48]. BI analyses was performed using MrBayesv3.2.2 [49]. The ML phylogenetic analysis was conducted by IQ-TREE v1.6.8 [50] using the ultrafast bootstrap (UFB) algorithm with 1000 replicates. Bootstrap support (BS) values were evaluated with 1000 replicates. BI analysis was conducted using four independent Markov chains set to run for 10 million generations with sampling every 1000 generations. The initial 25% of samples were discarded as burn-in and the remaining samples were used to generate a consensus tree and estimate the posterior probabilities (PP).

3. Results and Discussion

3.1. General Mitogenomic Features

H. autonoe, *P. palaearctica*, and *O. buddha* complete mitogenomes were found to be 15,435, 15,942, and 15,259 bp, respectively (Table S1). *P. palaearctica* Satyrinae mitogenomes were the largest of the 51 currently published Satyrinae genomes, and the other two mitogenome sizes were in the range of the 51 Satyrinae mitogenomes (from 14,675 bp of *Maniola bathseba* to 15,942 bp of *P. palaearctica*, Table S2). The genetic composition of the three species was found to be similar to that of other insect mitochondrial DNA, including 13 PCGs, two rRNA genes (16S *rrnL* and 12S *rrnS*), 22 tRNA genes, and an AT-rich region (A + T-rich region) (Figure 1). Among the 37 genes encoded by the mitochondrial genome, 14 were determined to be encoded by the N-strand (minority-strand), including

four PCGs, eight tRNA genes, and two rRNA genes; the other 23 genes are encoded by the J-strand. The A + T-rich region was found between the *rrnS* gene and $tRNA^{Met}$ gene. The arrangement of *trn M-trn I-trn Q* was different from that of *trn I-trn Q-rn M* [51], but this arrangement is typical in Lepidopterans.

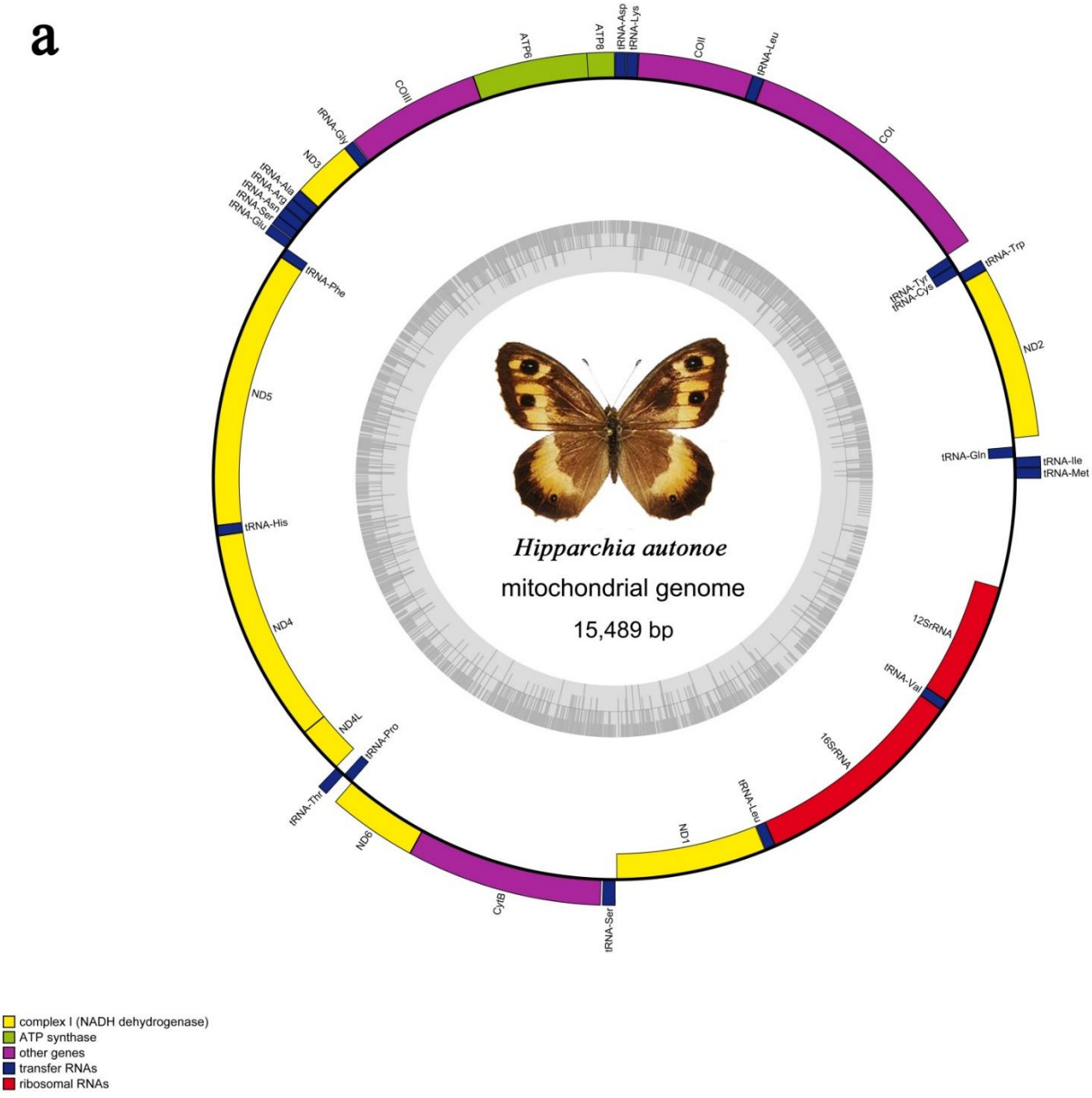
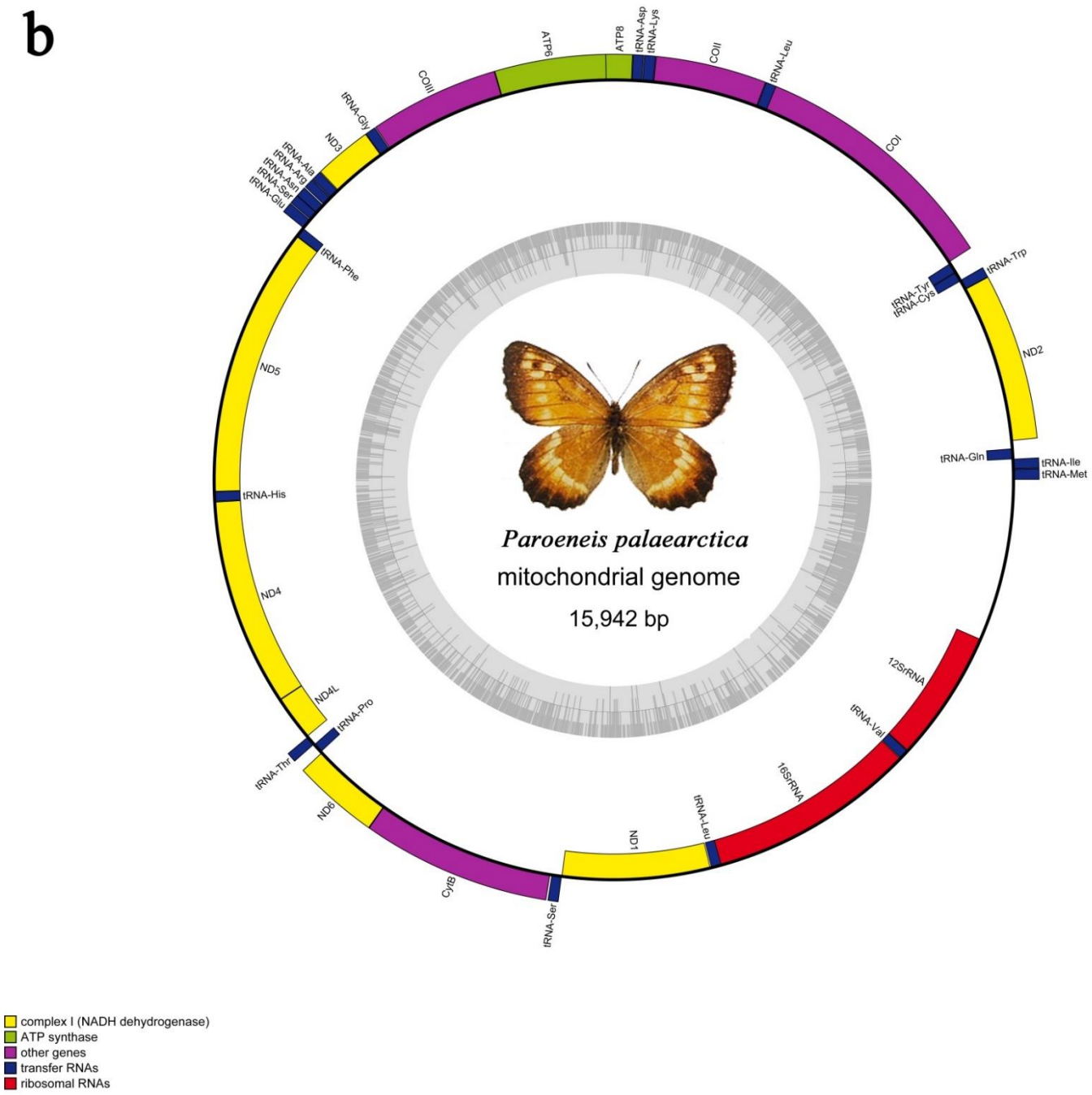


Figure 1. *Cont.*



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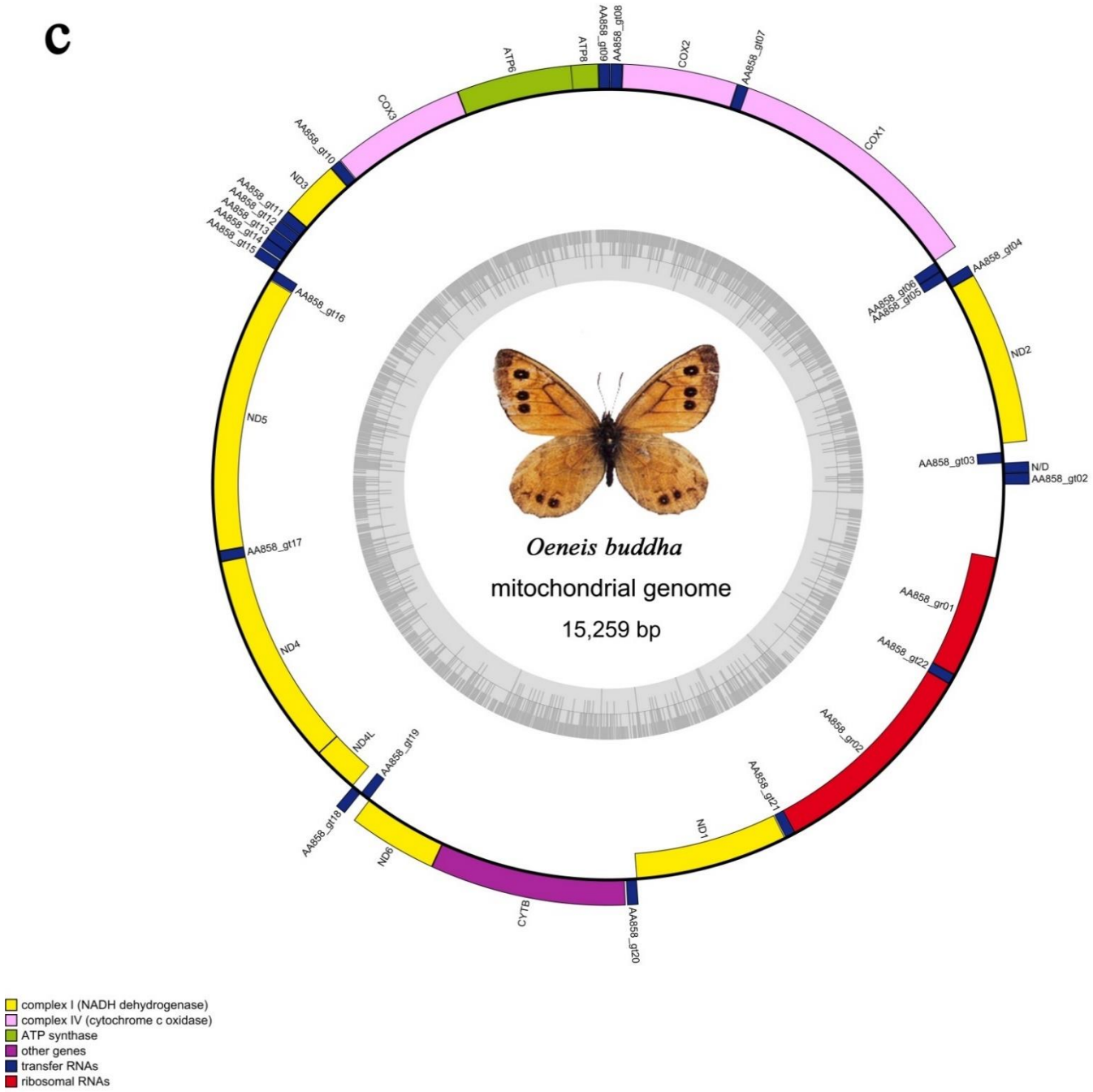

Figure 1. Mitochondrial gene map of three satyrid. (**a**) *Hipparchia autonoe*, (**b**) *Paroeneis palaearctica*, and (**c**) *Oeneis buddha*.

The A + T contents in the three satyrid mitogenomes were 79.1%, 78.2%, and 79.6%, which were significantly higher than those of G+C and had obvious AT-bias (Table S2). On the whole, the A + T content in each gene component showed a decreasing trend as follows: A + T-rich region > rRNA > tRNA > PCGs. The AT skews of the mitogenomes were −0.017, −0.022, and −0.029, respectively, whereas the AT skew of the PCGs and the first, second, and third codons were all negative, indicating that the T content is higher than that of A (Table 1). The AT skew values of all three mitogenomes were both within the corresponding values of other Satyrinae species, ranging from −0.055 in *Neope muirheadii* to −0.017 in *H. autonoe* (Table S2).

Table 1. Nucleotide composition of *Hipparchia autonoe* (Hip), *Paroeneis palaearctica* (Par), and *Oeneis buddha* (Oen).

Feature	Size			A + T%			AT-Skew			GC-Skew		
	Hip	Par	Oen	Hip	Par	Oen	Hip	Par	Oen	Hip	Par	Oen
Whole genome	15,435	15,942	15,259	79.1	78.2	79.6	−0.017	−0.022	−0.029	−0.238	−0.179	−0.225
Protein coding genes	11,208	11,212	11,205	76.8	78.8	781	−0.156	−0.157	−0.157	−0.007	0.026	−0.002
1st codon position	3735	3736	3734	72.4	73.2	73.1	−0.007	0	−0.001	0.186	0.195	0.194
2nd codon position	3735	3736	3734	70.1	70.3	70.3	−0.381	−0.371	−0.379	−0.089	−0.081	−0.089
3rd codon position	3735	3736	3734	87.8	92.8	90.7	−0.098	−0.121	−0.111	−0.245	−0.167	−0.293
tRNA genes	1442	1452	1450	80.6	80.6	80.3	0.015	0.017	0.013	0.164	0.163	0.172
rRNA genes	1358	1362	1362	83.5	81.3	84.0	0.095	0.064	0.078	0.339	0.265	0.355
A + T-rich region	896	1039	452	94.6	61.8	91.4	−0.016	−0.003	−0.026	−0.208	−0.003	0.076

3.2. Protein Coding Genes and Codon Usage

The lengths of the 13 PCGs of *H. autonoe*, *P. palaearctica*, and *O. buddha* were 11,205, 11,208, and 11,202 bp, respectively, accounting for 70.3%, 73.5%, and 73.4% of the total sequence. Except for *COX1*, all PCGs in the *H. autonoe* mitogenome were found to be initiated by typical ATN codons (*COX2*, *ATP6*, *COX3*, *ND4*, *ND4L*, and *Cyt b* with ATG; *ND2*, *ATP8*, and *ND6* with ATT; *ND3* and *ND5* with ATC; *ND1* with ATA). The start codons of 10 PCGs (*ND1*, *ND2*, *ND4*, *ND4L*, *COX1*, *COX2*, *COX3*, *ATP8*, *ATP6*, and *Cyt b*) in the three species were consistent with each other. Unlike that in *H. autonoe*, in *P. palaearctica* and *O. buddha*, *ND3* and *ND5* were determined to start with an ATT codon. Results showed that *COX1* in the three Satyrinae species starts with the alternative starting codon CGA, which is similar to that in all other known Lepidopteran mitogenomes. The termination codons of the three satyrids in this paper also had some similarities; except for the *COX2*, *ND4*, and *ND5* genes that terminate with a single T, the other PCGs terminate with a complete TAA codon.

The mitogenomes of *H. autonoe*, *P. palaearctica*, and *O. buddha* encode 3735, 3736, and 3734 amino acids (except stop codon), respectively. The number of amino acids is within the range of other Satyrinae species and is similar to that of other Satyrinae species (from 3621 in *Maniola bathseba* to 3744 in *Coenonympha arcania*, Table S2). Figure 2 summarizes the relative codon usage values for codon usage. Among them, AUU (I), UUU (F), UUA (L), and AAU (N) were determined to be the most frequently used codons in the three mitogenomes (Figure 2). Leucine (Leu) was highest in amino acid composition (15.4%, 15%, and 15.3%), followed by phenylalanine (Phe; 10%, 9.8%, and 10%), and serine (Ser; 8.6%, 8.9%, and 8.7%), whereas cysteine (Cys) was the lowest (1.1%, 1.1%, and 1%) (Figure 3).

3.3. The rRNAs and tRNAs

Like other Lepidopteran mitogenomes, the mitogenomes of these two species were found to have two ribosomal RNA genes (rrnL and rrnS) between $tRNA^{Leu}$ (CUN) and $tRNA^{Val}$ and between $tRNA^{Val}$ and the A + T rich region, respectively. The sizes of rrnL gene of *H. autonoe*, *P. palaearctica*, and *O. buddha* mitogenomes were 1358, 1362, and 1362 bp, respectively. The A + T contents of the rrnL genes in *H. autonoe*, *P. palaearctica*, and *O. buddha* were 83.4%, 83%, and 83.6%. The rrnS gene was found to be 498 bp long in *H. autonoe*, 852 bp in *P. palaearctica*, and 768 bp in *O. buddha*, and their A + T contents were 83.7%, 78%, and 86.2%, respectively (Table 1). These results were similar to those of other Satyrinae mitogenomes (Table S2).

H. autonoe, *p. palaearctica*, and *O. buddha* mitogenomes were all found to contain 22 tRNA genes. The length of the 22 tRNA genes ranged from 60 bp to 71 bp. All tRNA genes except $tRNA^{Ser}$(AGN) were determined to have a typical clover structure, whereas $tRNA^{Ser}$(AGN) lacked the dihydrouridine (DHU) stem arm and formed a simple ring instead.

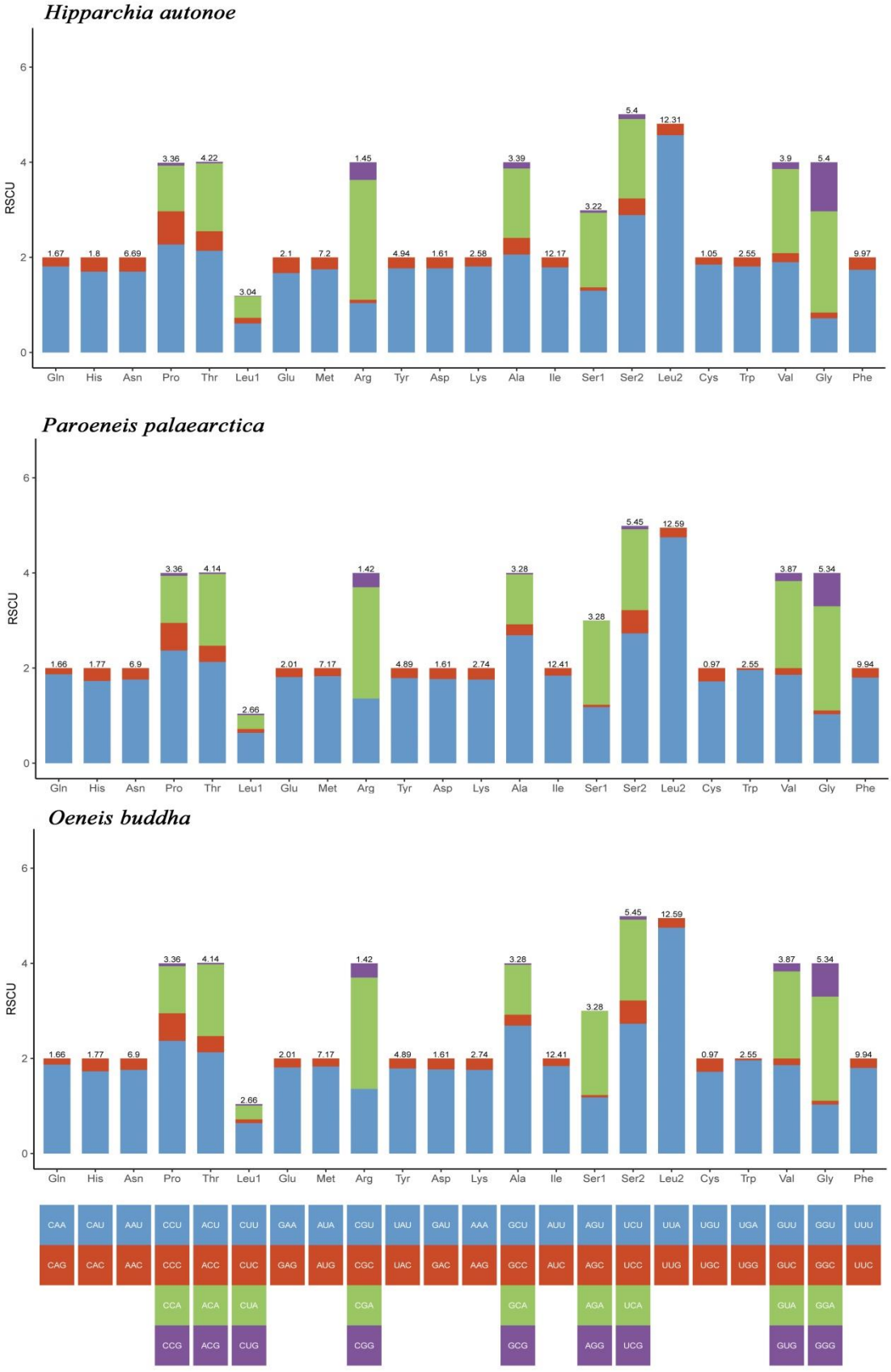

Figure 2. Relative synonymous codon usage (RSCU).

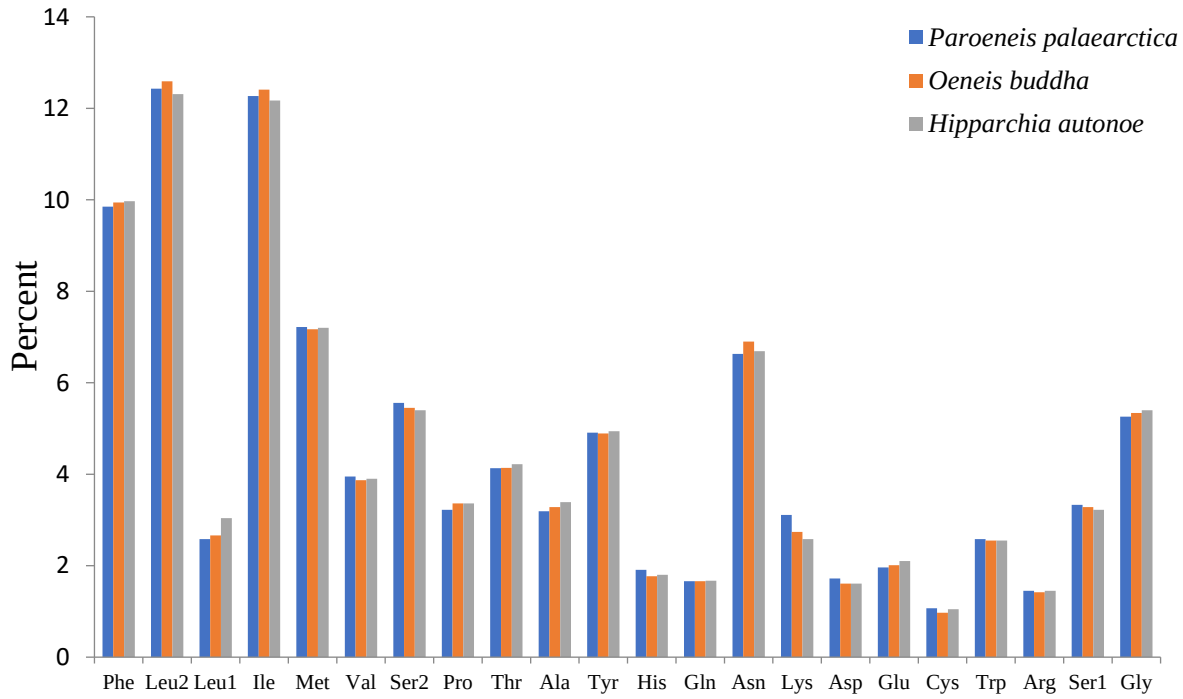

Figure 3. Composition of protein coding gene amino acids in the mitochondrial genomes.

3.4. Intergenic and Overlapping Spacer Regions

Although the mitogenomes of animals comprise a tightly arranged structure without introns, there are still multiple intergenic spacer sequences except for in the rich region. *H. autonoe* has 11 intergenic regions with a total length of 89 bp, and the interval length varies from 1 to 49 bp. The mitogenome of *P. palaearctica* has 12 intergenic regions with a total length of 86 bp, and the interval length varies from 1 to 49 bp. *O. buddha* has 12 gene spacer regions totaling 84 bp, and the spacer length varies from 1 to 51 bp. The largest gene interval of the three species was between $tRNA^{Gln}$ and *ND2* (49 bp in *H. autonoe*, 48 bp in *P. palaearctica*, and 51 bp in *O. buddha*), similar to the known spacing lengths of other Satyrinae species (Figure 4D), ranging in size from 40 bp in *Elymnias hypermnestra* to 53 bp in *Lethe albolineata*.

The gene overlapping sequences of the three mitogenomes had a total length of 33 bp at 10 locations in *H. autonoe*, 36 bp at 11 locations in *P. palaearctica*, and 36 bp at 11 locations in *O. buddha*. One was found to be composed of "AAGCCTTA" at the $tRNA^{Trp}$ and $tRNA^{Cys}$ junction (Figure 4A); the second was a shorter sequence of "TCTAA" located at the *COX1* and $tRNA^{Leu}$ (CUN) junction (Figure 4C). Like previously described Lepidopteran mitogenomes, the overlapping sequences (a 7-bp overlap) in the three species were found to be present between *ATP8* and *ATP6* genes (Figure 4B).

3.5. A + t-Rich Region

The lengths of the A + t-rich region of the three Satyrinae mitochondrial genomes were as follows: *H. autonoe* = 896 bp, *P. palaearctica* = 1039 bp, and *O. buddha* = 452 bp, located between srRNA and tRNAIle (Figure 1 and Table 1). The A + t content was 94.6% in *H. autonoe*, 61.8% in *P. palaearctica*, and 91.4% in *O. buddha*. The A + t content of *P. palaearctica* was the smallest of the 49 Satyrinae A + T contents published to date.

Figure 4. Gene overlapping and intergenic regions among three sequenced Satyrinae mitogenomes. Nucleotides colored red indicate the sequences of the overlapping or intergenic regions except where further explained. (**A**) The overlapping region between tRNATrp and tRNACys. (**B**) The overlapping region between *ATP8* and *ATP6*. (**C**) The overlapping region between *COX1* and tRNALeu (CUN). (**D**) The intergenic region between tRNAGlu and *ND2*.

3.6. Phylogenetic Analyses

This study was based on the aforementioned three newly acquired species of Satyrinae, combined with the known complete mitochondrial genome sequences of 48 other Satyrinae species, whereas the mitogenomes of Nymphalidae (*P. arja*, *P. schreiber* and *P. nepenthe*) were used as outgroups (Figures 5 and 6). The connected data set was analyzed by Bayesian inference (BI) and maximum likelihood (ML). Satyrinae is one of the most abundant taxa of butterfly insects and is an important model material for many research fields such as ecology, functional genomics, and bionics. However, there are many problems that need to be resolved in research on the system classification of Satyrinae low-level and high-level

elements [2–4,52]. To further understand their evolutionary relationship, we conducted a preliminary investigation based on mitochondrial data.

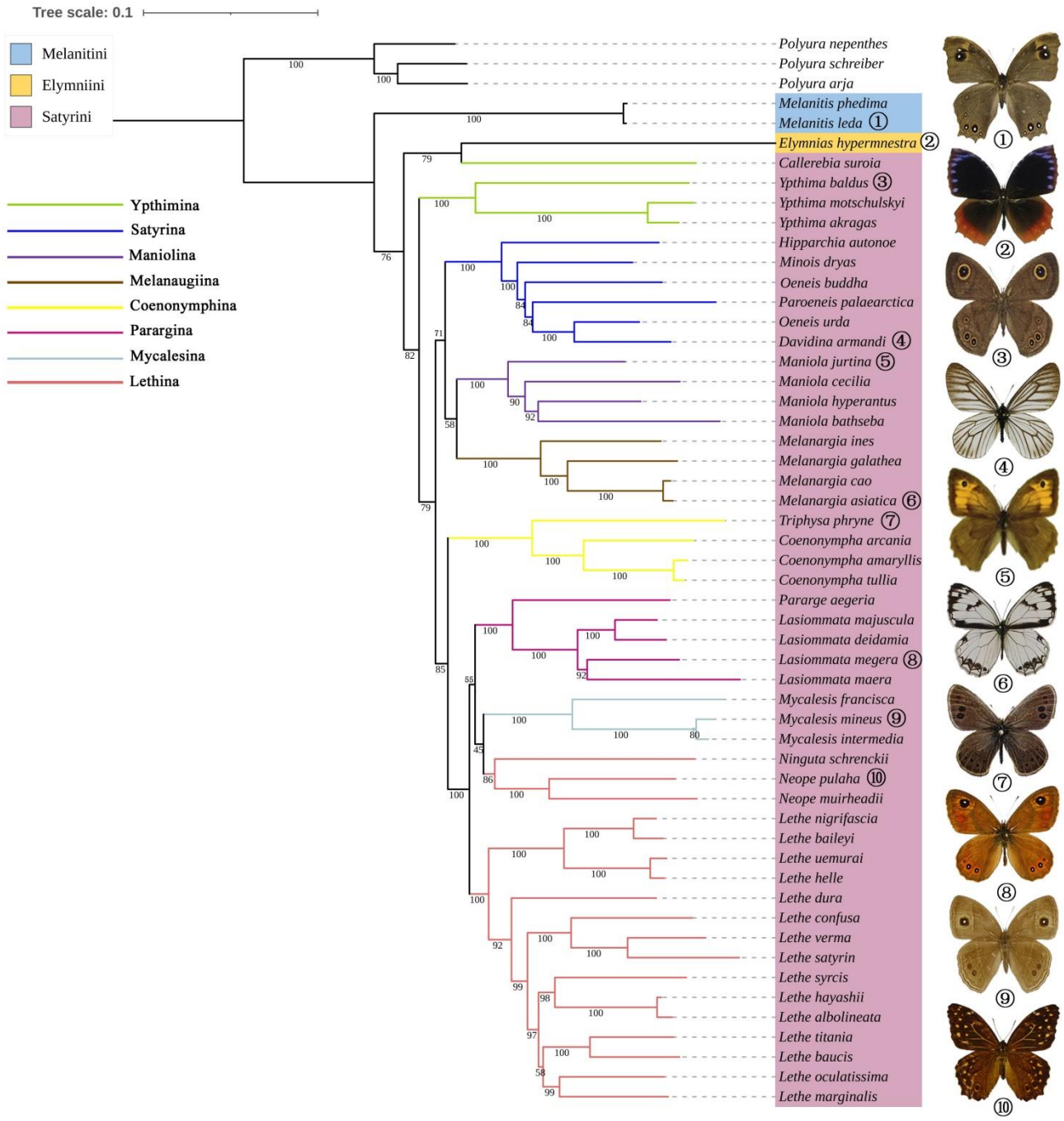

Figure 5. Phylogenetic tree inferred from nucleotide sequences of 13 PCGs and two rRNA genes using the ML. Bootstrap support values are indicated on branches.

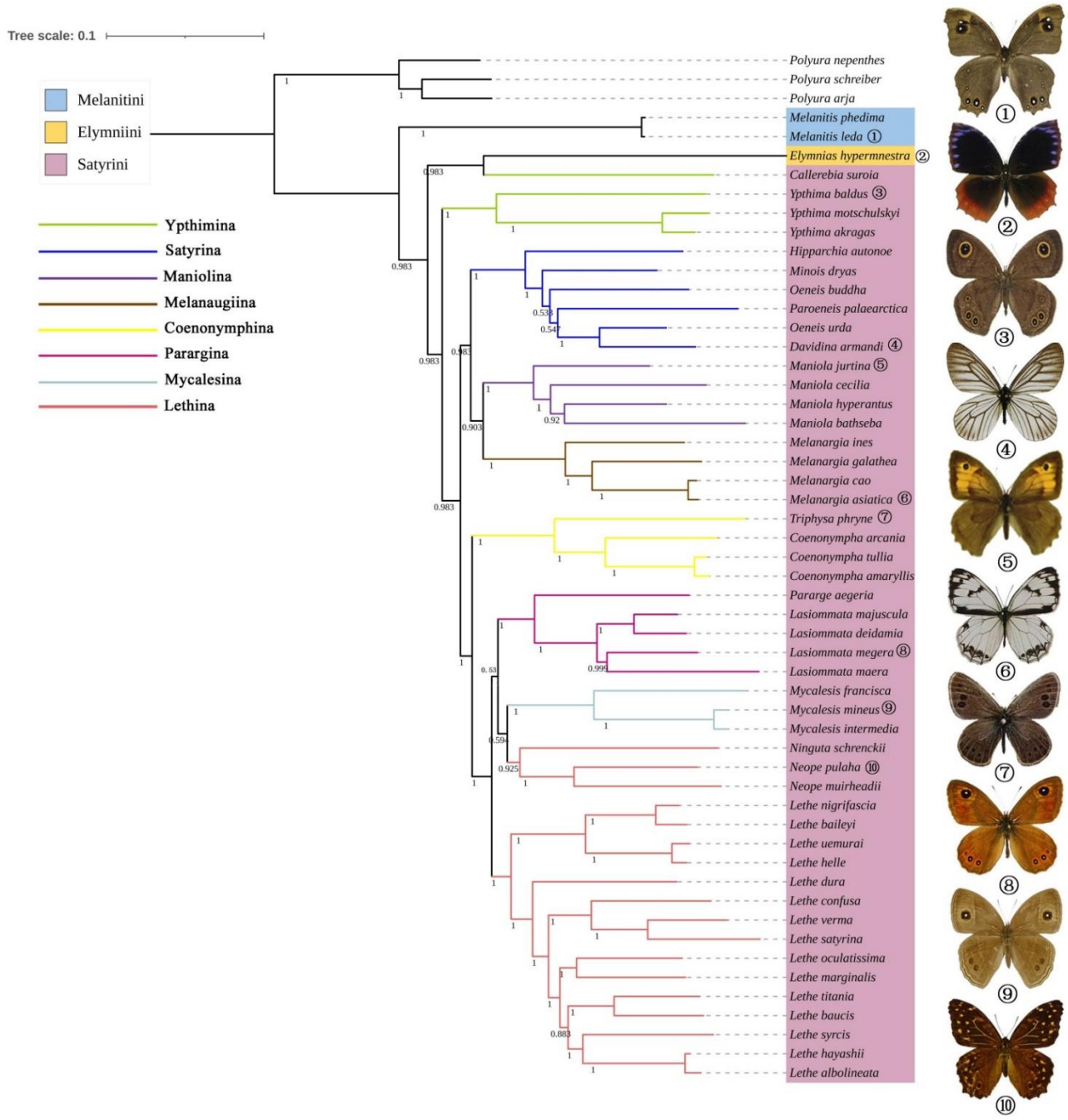

Figure 6. Phylogenetic tree inferred from nucleotide sequences of 13 PCGs and two rRNA genes using the BI. Posterior probabilities are indicated on branches.

In our phylogenetic relationship, Lethina is closer to Parargina and Mycalesina, and Satyrina is closer to Melanargia and Maniolina. This kind of relationship is similar to Marín's research results [6]. In this study, we selected 18 species of Lethina. In the obtained phylogenetic relationship, *Neope* and *Ninguta* are sister groups of *Lethe*, and similar relationships have been established in previous studies [3,28].

Davidina is a mysterious genus. The genus was mistaken as a Pieridae member early on because of the black veins and the absence of any eye spots on the wings [8]. Kusnezov [9] moved the genus into Satyrinae and later placed it in Satyrini (equivalent to the current Satyrinae) [10]. Additionally, the latest research proved *Davidina* is not a local monotypic Chinese endemic genus, as has been previously supposed, but is composed of nine species and has a broad distribution area in the Holarctic region including Europe

and America [53]. Similarly, *Paroeneis* had not yet been sampled in previous molecular phylogeny studies. This genus also lacks eye spots and has limited distribution. In this study, we found that it is very close to *Davidina*.

Due to the wide-ranging variations, the classification of the genus Oeneis is difficult. There are still many unresolved problems: (1) Wing patterns are quite similar among different species, whereas (2) great variations in wing patterns are noted even within the same species, for example, in *Oeneis urda*. In addition, in the morphological features, *Davidina* and *Oeneis* share common morphological characteristics. Their first thoracic limbs are significantly degenerated and traced, male and female genitalia are similar to those of the genus *Oeneis*, and the wing venation is also similar to that of the genus *Oeneis* [9]. In our phylogenetic relationships, *O. urda* and *Davidina* have a closer phylogenetic relationship. A similar relationship was established in previous research [53,54]. In the genus *Oeneis*, the morphological characteristics of male genitalia in *O. buddha* were classified as an independent group due to the presence of features not found in other *Oeneis* groups (valve without serrations) [55]. The present phylogeny supports the morphological classification of *O. buddha* forming an independent group.

Satyrini represents the most diverse tribe in Satyrinae (approximately 2200 species) and is divided into 13 subtribes [6]. In this study, three new mitochondrial genomes of Satyrini were determined, increasing the number of published Satyrini mitogenomes to 51. In previous studies, the close relationships among Parargina, Lethina, Mycalesina, Melanargiina, Satyrina, Coenymphina, Maniolina, and Ypthimina groups have been verified and were supported in our analyses [2,4]. However, it is important to note that only eight of the 13 subtribes have been analyzed. To better deal with the phylogenetic relationships within Satyrinae, more mitochondrial genome data need to be added for analysis.

3.7. Divergence Time Estimation of Satyrinae Species

Times of divergence within Satyrinae were estimated using a Bayesian approach in r8s (Figure 7). The divergence time of Satyrinae was approximately 47 MYA. Among them, Melanitini divergence time was approximately 0.5057MYA, whereas Satyrini divergence time was approximately 43.2742 MYA.

The divergence time of Satyrinae was in the Paleogene Period. This is related to the rapid evolution of angiosperms in terrestrial ecosystems during this period. According to the fossil record, the tertiary period was the prime of species evolution and angiosperms began to dominate [56–59]. At the same time, one of the fastest and most intense global warming events occurred during this period. During this time, high temperatures and a warm ocean created a moist and mild earth environment; except for the deserts, the surface was completely covered by forests and there was a great abundance of plants to provide food for a wide range of plant-feeding insects.

In this study, the divergence time of *H. autonoe*, *P. palaearctica*, and *O. buddha* was approximately 27.8749 MYA (in the Paleogene Oligocene). The three Satyrinae samples were from Qinghai Province in the northeast of the Qinghai-Tibet Plateau, and *P. palaearctica* and *O. buddha* are alpine species with special living environments. According to the history of the evolution of the global environment, during the Oligocene (33.7 MYA-23.5 MYA), the temperature was generally low and the Qinghai-Tibet Plateau began to lift up. The Paleozoic era had prosperous angiosperms, providing prerequisites for the evolution of alpine species of butterflies towards high-altitude environments adapted to low temperature, hypoxia, and climate change, as well as rapid adaptation to radiation events that occurred in the Quaternary period. In this study, the divergence time of three satyrids was predicted to coincide with the geological and environmental events mentioned previously herein.

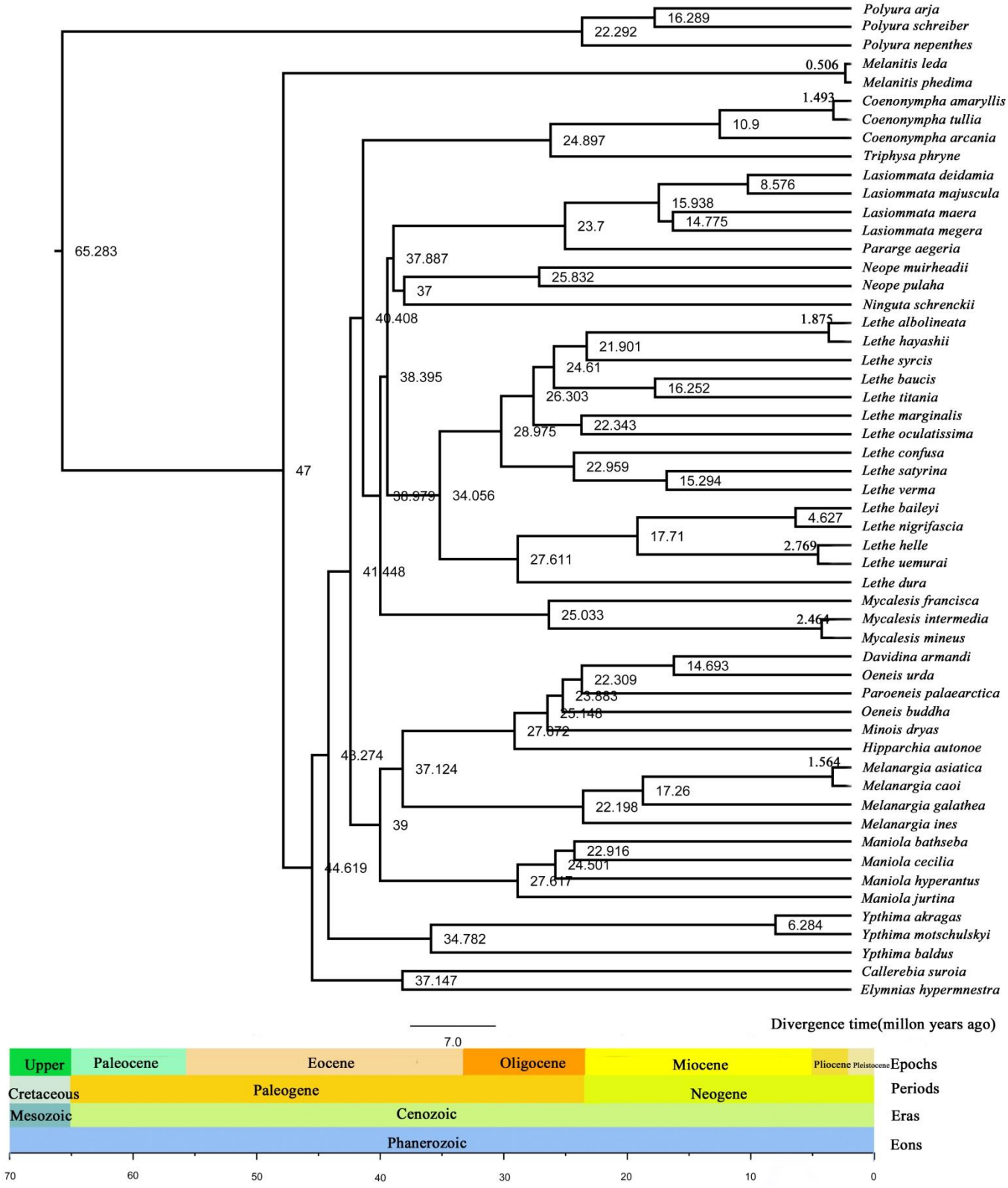

Figure 7. Divergence times of Satyrinae: maximum clade credibility tree with median age and 95% confidence interval estimated using a Bayesian uncorrelated relaxed clock implemented in r8s.

However, combined with previous research results, it is shown that the divergence time of Satyrinae predicted in this paper is similar to Espeland et al. [60] and Pena et al. [5] and slightly later than other research results. For example, Wahlberg et al. believed that

the divergence time of Satyrinae was about 60.7 MYA [4], while Marianne et al. believed that the divergence time of Satyrinae was about 80 MYA [4].

The reasons for the differences between the research results of different scholars and this study may be as follows.

(1) When the phylogenetic tree is reconstructed, the choice of sequence data will inevitably have a certain impact on the research results, because of the difference in the evolution rate of different sequences. This study used mitochondrial genome protein-coding genes for phylogenetic tree reconstruction and molecular clock analysis, while previous studies all used partial mitochondrial genome sequence fragments or mitochondrial partial genes combined with one or two nuclear genes to reconstruct the phylogenetic tree. The molecular clock analysis results are slightly different.
(2) The results of the study are affected by the different embedding locations of the fossil correction points and the different setting of the time during molecular clock calculation.
(3) The value given by the molecular clock calculation is the average value of a confidence interval, that is, the divergence time of each clade in the research results is not a given value but a time range, and the data results given only provide convenience for intuitive comparison.

4. Conclusions

In this study, we identified three new mitochondrial whole genomes, including *H. autonoe*, *P. palaearctica*, and *O. buddha*. At the same time, we also re-spliced and annotated the mitochondrial genomes of seven Satyrinae species, which are very similar to the sequence structures of other Satyrinae species. Their mitochondrial genomes are highly conserved in base content and composition, genome size and sequence, protein coding genes and codon usage, and tRNA secondary structure. Based on 13 PCGs and two rRNA phylogenetic analyses of BI and ML, a well-resolved topological structure was obtained, with high support for each branch. The divergence time of Satyrinae is the Paleogene Period. The phylogenetic results confirmed the position of the genus *Davidina* in the subfamily Satyrini and had a closer phylogenetic relationship with *Oeneis*. The phylogenetic analysis supported the formation of *Oeneis buddha* as an independent taxon in *Oeneis*.

In this study, the number of mitochondrial genomes in Satyrini was increased to 51. Satyrinae is a large subfamily of Nymphalidae with abundant species' resources, but there are few species for which completed mitochondrial genome sequencing has been performed. Therefore, it is urgent to obtain more mitochondrial genome sequences of Satyrinae insects to better solve the phylogenetic relationship of Satyrinae.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13100468/s1. Table S1: Organization of the three Satyrinae species (*Hipparchia autonoe* (Hip), *Paroeneis palaearctica* (Par), and *Oeneis buddha* (Oen)) mitochondrial genomes. Table S2: Summarized mitogenomic characteristics of the 51 Satyrinae species investigated in this study. Table S3: List of taxa used for the phylogenetic analyses in this study.

Author Contributions: Conceptualization, S.X. and D.G.; formal analysis, Z.D., L.D., and Z.C.; writing—original draft preparation, Z.D.; writing—review and editing, S.X. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported financially by the Excellent Doctor Innovation Project of Shaanxi Normal University (S2015YB03) and Fundamental Research Funds for the Central Universities (2018CSLZ004). In addition, this work was partly supported by the National Natural Science Foundation of China (NSFC 31872273).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All sequences files are available from the GenBank (Table S3).

Acknowledgments: We sincerely thank all fund providers.

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

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