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Original Article

The mitochondrial genomes of three skippers: Insights into the evolution of the family Hesperiidae (Lepidoptera)

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ABSTRACT

We sequenced the mitogenomes of *Astictopterus jama*, *Isoteinon lamprospilus* and *Notocrypta curvifascia* to obtain further insight into the mitogenomic architecture evolution and performed phylogenetic reconstruction using 29 Hesperiidae mitogenome sequences. The complete mitogenome sequences of *A. jama*, *I. lamprospilus* and *N. curvifascia* are 15,430, 15,430 and 15,546 bp in size, respectively. All contain 13 protein-coding genes, 2 ribosomal RNA genes, 22 transfer RNA genes, and an A + T-rich region. Nucleotide composition is A + T biased, and the majority of the protein-coding genes exhibit a negative AT-skew, which is reflected in the nucleotide composition, codon, and amino acid usage. The A + T-rich region is comprised of nonrepetitive sequences, including the motif ATAGA followed by a poly-T stretch, a microsatellite-like element next to the ATTTA motif, and a poly-A adjacent to tRNAs. Although most genes evolve under a strong purifying selection, the entire *nad* gene family (especially *nad6*) exhibits somewhat relaxed purifying selection, and *atp8*, evolving under a highly relaxed selection, is an outlier in the family Hesperiidae. Several different approaches relatively consistently indicated that *nad6*, *atp8* and *nad4* are comparatively fast-evolving genes in this family, which may have implications for future phylogenetic, population genetics and species diagnostics studies. For phylogenetic analyses of Hesperiidae, we tested a few datasets, and found that the one comprising all 37 genes produced the highest node support, indicating that the inclusion of RNAs improves the phylogenetic signal. Results indicate that subfamilies Euschemoninae, Heteropterinae, and Coeliadinae are monophyletic with strong nodal support, but Pyrginae and Eudaminae are paraphyletic. Finally, we confirm that *A. jama* and *I. lamprospilus* are close relatives.

1. Introduction

The family Hesperiidae (Lepidoptera), or skippers, is a species-rich butterfly family that accounts for one fifth of the butterfly species in the world [1]. Traditionally, the group had been investigated within the morphology-based taxonomic framework established by Evans [2–4], where three geographic regions were treated separately and infra-subfamily groups were called genus-groups instead of tribes. Later, Warren et al. [5,6] put forward a phylogenetic and taxonomic framework based on a combination of molecular and morphological data. As some aspects of its taxonomy remained unresolved, the phylogeny of this family remained a popular study topic, with studies focusing on different taxonomic levels: genus [7,8], tribe [9,10], and the whole family [5,6,11–14].

Despite all these attempts, phylogeny and taxonomy of the Hesperiidae has not been resolved on the tribe level, particularly in the subfamily Hesperiinae, where many genera are left as *incertae sedis* [5]. Among these are the genera *Isoteinon* Felder, 1862 and *Astictopterus* Felder, 1860. The phylogenetic position of the genus *Astictopterus* also remains unresolved: Watson [3] originally assigned it to the Astictopterus subgroup of genera, but Chou [15] later replaced the subgroup with the tribe Astictopterini. Subsequent studies kept changing the taxonomic status of the genus, so some assigned it to the Isoteinon group [16] or the Ancistroides group [17]. Similarly, the genus *Isoteinon* was originally assigned to the Isoteinon group [3,15], to be later renamed into the tribe Isoteinonini. Most of subsequent attempts to resolve its phylogeny agreed that *Astictopterus* and *Isoteinon* are closely related to *Kedestes*, but they failed to resolve the phylogenetic position of the genus with confidence [6,12,18].

Most of these phylogenetic analyses relied on single-gene molecular

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markers, both mitochondrial (such as *cox1*, *cox2* and *16SrRNA*) and nuclear (*wingless* and *EF-1α*). Although mitogenomes have been widely used in evolutionary and phylogenetic studies of [19–21], relatively few studies have attempted to study the phylogeny of Hesperiidae using the mitochondrial phylogenomics approach [22]. The latest phylogenetic analysis using complete mitogenomes [13] used 27 skipper species, of which 12 were Hesperiinae. For this study, we sequenced three additional mitogenomes of species belonging to previously non-represented tribes of the subfamily Hesperiinae: Astictopterini (*Astictopterus jama*), Ancistroidini (*Notocrypta curvifascia*) and Isoteinonini (*Isoteinon lamprospilus*). Phylogenetic relationships of the family Hesperiidae were explored in combination with the 26 complete hesperiid mitogenomic sequences available in the GenBank.

As mitogenomes of Lepidoptera generally exhibit compositional heterogeneity, most previous studies attempted to reduce it by removing the third codon position of protein-coding genes, as well as excluding rRNAs and tRNAs [13,21]. However, indiscriminate exclusion of the third codon from PCGs may result in decreased phylogenetic resolution and reduced statistical support for topology [23,24], and RNAs can actually carry a considerable phylogenetic signal [25]. Thus, here we performed phylogenetic analysis of the family Hesperiidae using several different datasets to explore the impacts of inclusion or exclusion of RNA genes and the third codon position of PCGs on the phylogenetic resolution. Furthermore, based on comparative mitogenomics, we investigated whether mitochondrial gene fragments traditionally used as molecular markers are the most suitable mitogenomic tools for the task.

2. Materials and methods

2.1. Sample collection and DNA extraction

Specimens of *A. jama* and *I. lamprospilus* were collected in Matoushan, Fuzhou City, Jiangxi Province, China (27°84′ N; 117°13′ E), and *N. curvifascia* in Jiu Xian Mountain, Quanzhou City, Fujian Province, China (25°64′ N; 118°13′ E). Complete specimens were immediately preserved in 100% ethanol and stored at −20 °C in the Entomological Museum of the Northwest A&F University, Yangling, Shaanxi Province, China. The specimens were initially identified using morphological characteristics (particularly the genitalia) [26], and identity confirmed via *cox1* barcoding using the BOLD database [27]. The total DNA was extracted from the thoracic muscle using EasyPure^{R} Genomic DNA Kit following the manufacturer's instructions (TransGen, Beijing, China). As these three species are unprotected invertebrates, no permits were required for the study.

2.2. Sequence analysis

Three complete mitogenomes were extracted from whole genome sequence using Illumina HiSeq2000 system by Genesky Biotechnologies Inc. (Shanghai, China). First, the raw paired reads were retrieved and quality-trimmed using CLC Genomics Workbench v10.0 (CLC Bio, Aarhus, Denmark) with default parameters. Then the clean paired reads were used for mitogenome reconstruction using MITObim v1.7 software [28] with default parameters and the mitogenome of *Ampittia dioscorides* (Hesperiidae: Hesperiinae; KM102732) [29] as the reference. Annotation of the mitogenomes and comparative analyses were conducted following the methodology outlined before [30,31]. Briefly: mitogenomes were annotated using Geneious 8.1.3 (Biomatters, Auckland, New Zealand), also with *A. dioscorides* as the reference. Protein-coding genes (PCGs) were determined by finding the ORFs (employing codon table 5), and RNAs (rRNAs and tRNAs) were identified using MITOS Web Server [32]. 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/created using PhyloSuite [33]. To better understand the mitogenomic evolution of Hesperiidae, we selected a subset of eight mitogenomes, representing all six subfamilies (3 new mitogenomes representing the Hesperiinae and one mitogenome per each remaining subfamily). This dataset was used to calculate nucleotide diversity values for each gene and conduct a sliding window analysis (with a sliding window of 200 bp and a step size of 20 bp) to estimate the nucleotide diversity Pi between these eight mitogenomes (both conducted using DnaSP). Genetic distances among these eight mitogenomes were calculated based on Kimura-2-parameter using Mega 7.0 [34]. Non-synonymous (dN) / synonymous (dS) mutation rate ratios among the 13 PCGs of these eight mitogenomes were calculated with DnaSP v5 [35].

Table 1
Classification and origins of the mitogenomic sequences used in this study.

Taxonomy	Species	Accession number	Reference
Hesperiidae			
Coeliadinae	*Burara striata*	NC_034676	[42]
	Choaspes benjaminii	NC_024647	[22]
	Hasora anura	KF881049	[43]
	Hasora vitta	NC_027170	[44]
Euschemoninae	*Euschemon rafflesia*	NC_034231	[45]
Pyrginae	*Celaenorrhinus maculosus*	NC_022853	[46]
	Ctenoptilum vasava	JF713818	[47]
	Daimio tethys	KJ813807	[48]
	Erynnis montanus	NC_021427	[49]
	Pyrgus maculatus	NC_030192	Unpublished
	Tagiades vajuna	KX865091	[13]
Eudaminae	*Achalarus lyciades*	NC_030602	[50]
	Lobocla bifasciata	KJ629166	[22]
Heteropterinae	*Carterocephalus silvicola*	NC_024646	[22]
	Heteropterus morpheus	NC_028506	Unpublished
Hesperiinae	*Ampittia dioscorides*	KM102732	[29]
	Lerema accius	NC_029826	[51]
	Ochlodes venata	HM243593	Unpublished
	Parnara guttata	NC_029136	[52]
	Potanthus flavus	KJ629167	[22]
	Astictopterus jama	MH763663	This study
	Isoteinon lamprospilus	MH763664	This study
	Notocrypta curvifascia	MH763665	This study
	Agathymus mariae	KY630504	[53]
	Megathymus beulahae	KY630505	[54]
	Megathymus cofaqui	KY630503	[54]
	Megathymus streckeri	KY630501	[54]
	Megathymus ursus	KY630502	[54]
	Megathymus yuccae	KY630500	[54]
Outgroup			
Papilionidae	*Papilio machaon*	NC_018047	Unpublished
	Papilio helenus	NC_025757	[55]

2.3. Phylogenetic analysis

For the phylogenetic analysis, the three newly sequenced skippers were combined with 26 out of 28 Hesperiidae mitogenome sequences that are publicly available (Table 1). We excluded two species with very low homology to other species: *Polytremis jigongi* and *Polytremis nascens* [36]. Two Papilionidae species were selected as outgroups [37]. Statistics for the basic characteristics of the mitogenome and the extraction of PCGs and RNAs were produced by PhyloSuite [33]. PCGs were aligned in batches with MAFFT integrated into PhyloSuite [33], using codon-alignment mode. All RNAs were aligned with Q-INS-i algorithm (which takes secondary structure information into account) incorporated into MAFFT-with-extensions software [38].

Phylogenetic analyses were conducted using three different datasets: 13 protein-coding genes (PCG), removed third codon position of 13 PCGs + RNA genes (PCG12RT), and 13 PCGs + RNA genes (PCGRT). The GTR + I + G model of evolution was chosen for the three

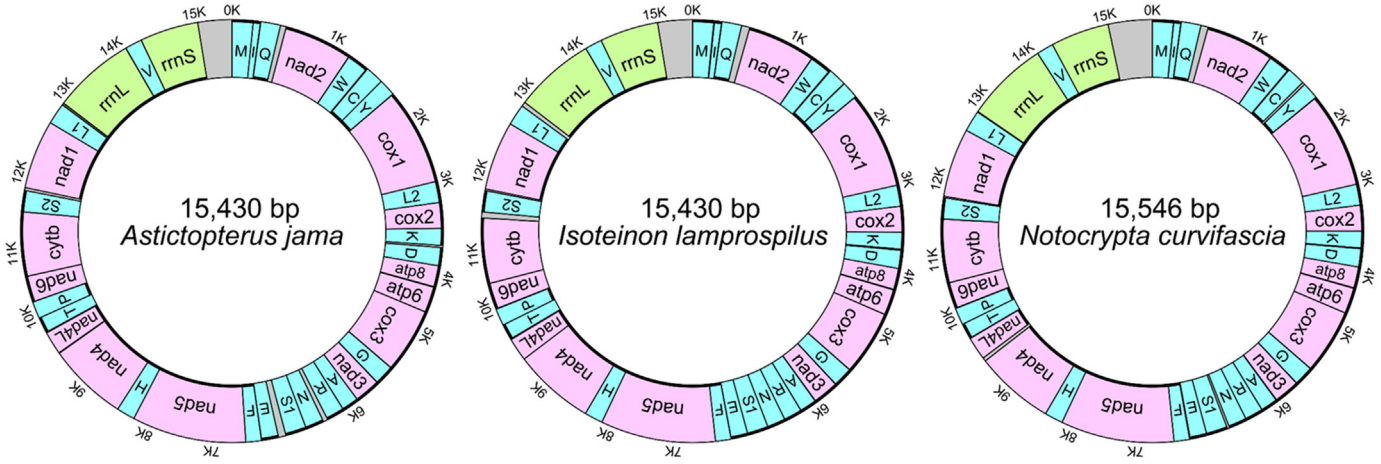

Fig. 1. Circular maps of the mitogenomes of *A. jama*, *I. lamprospilus* and *N. curvifascia*. Protein-coding and ribosomal genes are shown with standard abbreviations. The J-strand is visualized on the outer circle, and the N-strand on the inner circle.

Table 2
Mitogenomic organization of *A. jama*, *I. lamprospilus* and *N. curvifascia*.

	Position		Size (bp)	Intergenic nucleotides	Codon		Strand
	From	To			Start	Stop	
A. jama/I. lamprospilus/N. curvifascia							
trnM	1/1/1	67/69/67	67/69/67				+/+/+
trnI	68/77/66	132/141/132	65/65/67	−/7/−2			+/+/+
trnQ	130/139/130	198/207/198	69/69/69	−3/−3/−3			−/−/−
nad2	268/305/286	1281/1327/1299	1014/1023/1014	69/97/87	ATT/ATA/ATA	TAA/TAA/TAA	+/+/+
trnW	1280/1326/1298	1347/1396/1365	68/71/68	−2/−2/−2			+/+/+
trnC	1340/1389/1358	1407/1452/1422	68/64/65	−8/−8/−8			−/−/−
trnY	1408/1456/1454	1472/1520/1522	65/65/69	−/3/31			−/−/−
cox1	1475/1523/1526	3005/3053/3056	1531/1531/1531	2/2/3	CGA/CGA/CGA	T/T/T	+/+/+
trnL2	3006/3054/3057	3073/3120/3123	68/67/67				+/+/+
cox2	3074/3121/3124	3752/3799/3802	679/679/679		ATG/ATG/ATA	T/T/T	+/+/+
trnK	3753/3800/3803	3823/3870/3873	71/71/71				+/+/+
trnD	3849/3885/3883	3915/3954/3948	67/70/66	25/14/9			+/+/+
atp8	3916/3955/3949	4083/4113/4116	168/159/168		ATT/ATC/ATT	TAA/TAA/TAA	+/+/+
atp6	4077/4107/4110	4754/4784/4787	678/678/678	−7/−7/−7	ATG/ATG/ATG	TAA/TAA/TAA	+/+/+
cox3	4754/4784/4787	5548/5569/5572	795/786/786	−1/−1/−1	ATA/ATG/ATG	TAA/TAA/TAA	+/+/+
trnG	5551/5572/5575	5615/5637/5640	65/66/66	2/2/2			+/+/+
nad3	5613/5635/5638	5969/5991/5994	357/357/357	−3/−3/−3	ATA/ATA/ATA	TAA/TAA/TAA	+/+/+
trnA	5969/5996/6001	6032/6063/6065	64/68/65	−1/4/6			+/+/+
trnR	6032/6063/6065	6095/6128/6132	64/66/68	−1/−1/−1			+/+/+
trnN	6150/6129/6133	6215/6195/6197	66/67/65	54/−/−			+/+/+
trnS1	6216/6196/6225	6274/6253/6283	59/58/59	−/−/27			+/+/+
trnE	6363/6254/6284	6429/6320/6351	67/67/68	88/−/−			+/+/+
trnF	6432/6319/6360	6498/6384/6424	67/66/65	2/−2/8			−/−/−
nad5	6499/6385/6425	8233/8122/8168	1735/1738/1744		ATT/ATT/ATT	T/T/T	−/−/−
trnH	8234/8123/8169	8298/8187/8236	65/65/68				−/−/−
nad4	8299/8188/8237	9637/9526/9575	1339/1339/1339		ATG/ATG/ATG	T/T/T	−/−/−
nad4L	9631/9527/9619	9909/9802/9894	279/276/276	−7/−/43	ATG/ATG/ATG	TAA/TAA/TAG	−/−/−
trnT	9919/9813/9900	9982/9876/9963	64/64/64	9/10/5			+/+/+
trnP	9983/9877/9964	10,048/9942/10,029	66/66/66				−/−/−
nad6	10,051/9948/10,035	10,578/10,478/10,562	528/531/528	2/5/5	ATA/ATT/ATT	TAA/TAA/TAA	+/+/+
cytb	10,578/10,478/10,562	11,726/11,632/11,716	1149/1155/1155	−1/−1/−1	ATG/ATG/ATG	TAA/TAA/TAA	+/+/+
trnS2	11,726/11,703/11,715	11,790/11,771/11,781	65/69/67	−1/70/−2			+/+/+
nad1	11,824/11,792/11,799	12,765/12,733/12,737	942/942/939	33/20/17	ATT/ATG/ATA	TAA/TAA/TAA	−/−/−
trnL1	12,766/12,734/12,738	12,834/12,802/12,810	69/69/73				−/−/−
rrnL	12,810/12,855/12,803	14,190/14,186/14,187	1381/1332/1385	−25/52/−8			−/−/−
trnV	14,193/14,188/14189	14,257/14,251/14,255	65/64/67	2/1/1			−/−/−
rrnS	14,257/14,252/14,256	15,029/15,020/15,023	773/769/768	−1/−/−			−/−/−
A + T-rich region	15,030/15,021/15,024	15,430/15,430/15,546	401/410/523				+/+/+

datasets using the Bayesian information criterion (BIC) implemented in jModelTest 2.1.7 [39]. Phylogenetic analyses were performed employing the best-fit model, using maximum likelihood (ML) and Bayesian inference (BI). The ML analyses were performed using RaxML GUI [40], with an ML + rapid bootstrap (BS) algorithm with 1000 replicates. The BI analyses were performed using MrBayes 3.2.6 [41] with default settings and 6×10^6 MCMC generations (average standard deviation of split frequencies < 0.01, estimated sample size > 200, and potential scale reduction factor ≈ 1).

Table 3
Nucleotide composition and skewness of mitogenomes of *A. jama*, *I. lamprospilus* and *N. curvifascia*.

A. jama/I. lamprospilus/N. curvifascia								
Regions	Size (bp)	T (U)	C	A	G	AT (%)	AT skew	GC skew
PCGs	11,190/11,190/11,190	45.4/43.5/45.2	10.3/12.0/10.3	33.8/33.1/33.7	10.5/11.4/10.8	79.2/76.6/78.9	−0.147/−0.136/−0.145	0.007/−0.026/0.022
1st codon position	3730/3730/3730	36.7/36.2/37.3	10.5/10.9/9.8	37.7/37.1/37.3	15.1/15.8/15.5	74.4/73.3/74.6	0.013/0.012/0.000	0.183/0.186/0.225
2nd codon position	3730/3730/3730	48.3/48.1/48.2	16.2/16.6/16.2	22.3/21.7/22.2	13.2/13.5/13.4	70.6/69.8/70.4	−0.369/−0.378/−0.369	−0.103/−0.105/−0.094
3rd codon position	3730/3730/3730	51.1/46.1/50.0	4.4/8.5/4.9	41.4/40.5/41.7	3.2/4.9/3.4	92.5/86.6/91.7	−0.105/−0.065/−0.090	−0.160/−0.269/−0.187
A + T-rich region	401/410/523	45.6/48.8/45.3	4.2/5.1/4.0	47.4/44.6/46.8	2.7/1.5/3.8	93.0/93.4/92.1	0.019/−0.044/0.017	−0.214/−0.556/−0.024
tRNAs	1454/1466/1470	40.2/39.3/40.6	7.8/7.9/7.7	41.1/42.1/41.6	10.9/10.7/10.1	81.3/81.4/82.2	0.011/0.034/0.012	0.166/0.150/0.137
rRNAs	2154/2101/2153	41.8/40.6/40.6	4.9/5.0/5.0	41.7/41.6/44.2	11.6/12.7/10.2	83.5/82.2/84.8	−0.001/0.012/0.043	0.403/0.430/0.346
Full genome	15,430/15,430/15,546	40.8/39.3/41.3	12.0/13.7/11.8	39.8/39.2/39.4	7.4/7.7/7.4	80.6/78.5/80.7	−0.012/−0.001/−0.024	−0.238/−0.283/−0.228

3. Results and discussion

3.1. Genome structure and organization

The mitogenome lengths of *A. jama* and *I. lamprospilus* are the same (15,430 bp), while the length of *N. curvifascia* is 15,546 bp (Fig. 1). They are medium-sized when compared with the mitogenomes of other Hesperiidae, which range from 15,267 bp (*Potanthus flavus*) to 15,769 bp (*Heteropterus morpheus*) to date (2018.9.13). Each genome is composed of the typical 13 PCGs, 22 tRNA genes, two rRNA genes, and one major non-coding A + T-rich region which is considered to be the replication origin site [56]. Previously, the presence of a duplicated *tRNASer* (AGN) was reported from species of *Tagiades vajuna* [13] and *Ctenoptilum vasava* [47] (Hesperiidae: Tagiadini), but none of the three newly sequenced skipper mitogenomes had any duplicated genes. All three skippers display an identical gene arrangement, shared with a majority of lepidopteran mitogenomes (Table 2). Similar to many insect mitogenomes, the majority strand (J-strand) encodes 23 genes (9 PCGs and 14 tRNAs), whereas the minority strand (N-strand) encodes 14 genes (4 PCGs, 8 tRNAs and 2 rRNA genes) in these three mitogenomes. The A/T nucleotide composition is 78.5% in *I. lamprospilus*, 80.6% in *A. jama*, and 80.7% in *N. curvifascia*, indicating a strong A/T bias (Table 3).

3.2. Protein-coding genes (PCGs)

The 13 PCGs of three skipper mitogenomes all include 7 NADH dehydrogenase subunits, 3 cytochrome *c* oxidase subunits, 2 ATPase subunits, and one cytochrome *b* gene. Concatenated lengths of the 13 PCGs of *A. jama*, *I. lamprospilus* and *N. curvifascia* are 11,196 bp, 11,196 bp and 11,217 bp, encoding 3732, 3732 and 3739 amino acid residues respectively. Most PCGs start with ATN and stop with TAA or TAG codons (Table 2). An exception was *cox1*, which used the CGA start codon. The use of CGA in *cox1* has been reported before for Hesperiidae [57], but it is not conserved across the entire family, and it is not common in other insect groups, where other start codons were reported for *cox1*, such as TTA [58], TCG [59], TTG [60], and ACG [61]. With the exception of *cox1*, *cox2*, *nad5* and *nad4*, which use an incomplete stop codon T–, the remaining PCGs of three mitogenomes all use complete stop codons. In most lepidopteran species, *cox2* gene uses the abbreviated stop codon, believed to be completed by the addition of 3′ A residues to the mRNA [62,63]. This may have a function of minimizing the intergenic spacers and gene overlaps, thereby economizing the design of the mitogenome [64].

The amino acid composition and the codon usage of mitogenomes of eight species representing six subfamilies of Hesperiidae (3 new Hesperiinae and one mitogenome per each remaining subfamily) are shown in Fig. 2. The results show that the codon usage of all genes has a strong bias, and the RSCU (relative synonymous codon usage) values indicate a preference for NNU and NNA codons in skipper mitogenomes, which has been observed before [22,47]. Correspondingly, when the 13 PCGs of *A. jama*, *I. lamprospilus* and *N. curvifascia* were broken down by the codon position, the AT content of the third codon (92.5%, 86.6%, 91.7%) was much higher than the first (74.4%, 73.3%, 74.6%) and second positions (70.6%, 69.8%, 70.4%) (Table 2). In addition, Fig. 2 also shows that UUU (*Phe*), UUA (*Leu*), AUU (*Ile*), AUA (*Met*), and AAU (*Asn*) are the most frequently used codons. All these observations indicate a strong AT bias of the protein-coding genes in the skipper mitogenomes.

3.3. Ribosomal and transfer RNA genes

The two rRNA genes, *rrnL* and *rrnS*, are located between *trnL* (CUN) and *trnV*, and *trnV* and the A + T-rich region respectively. Their precise boundaries were determined via a comparison with homologs. In the three newly sequenced mitogenomes, their lengths ranged from

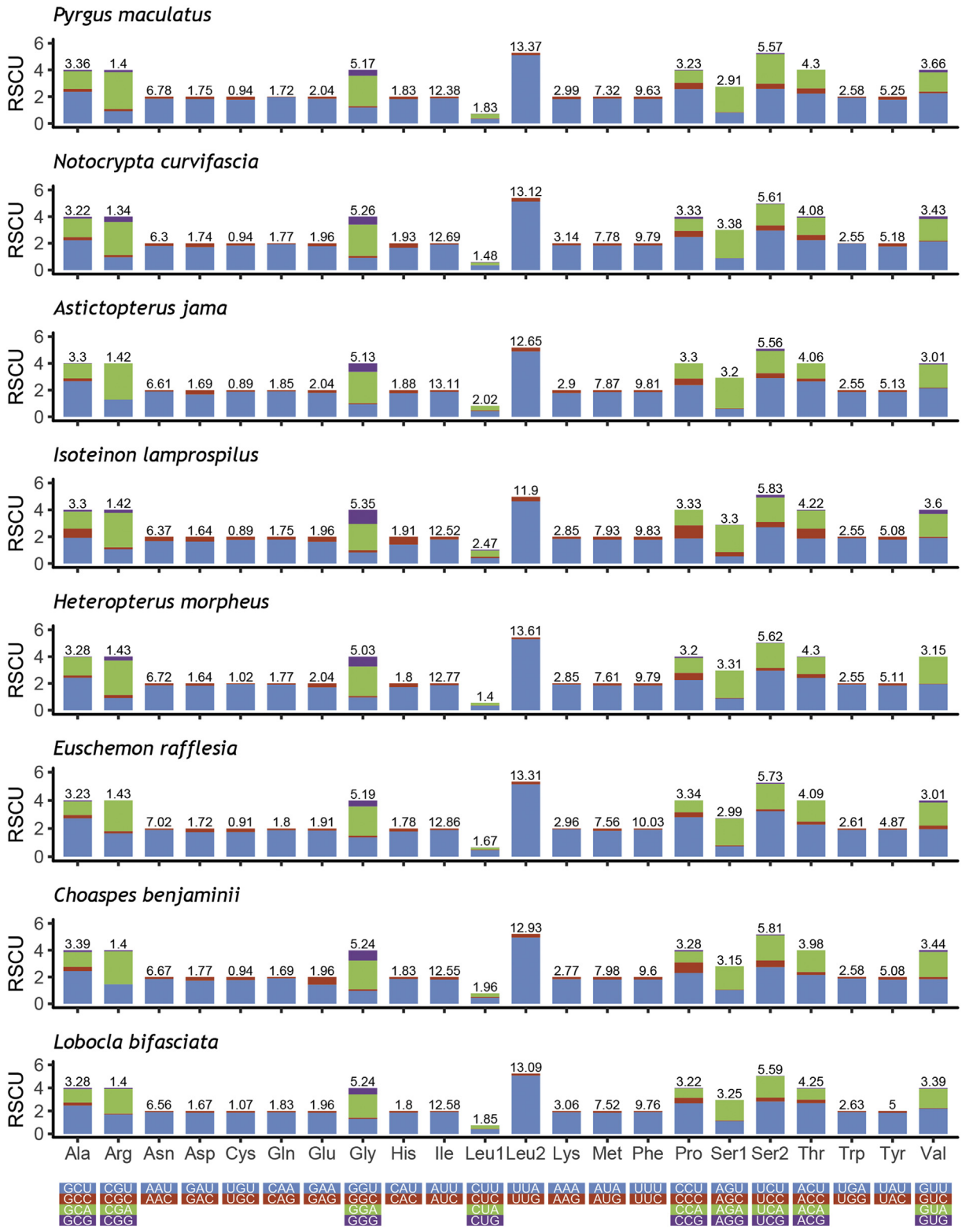

Fig. 2. Relative synonymous codon usage (RSCU) in the mitogenomes of eight species of Hesperiidae.

1332 bp (*I. lamprospilus*) to 1385 bp (*N. curvifascia*) for the *rrnL*, and from 768 bp (*N. curvifascia*) to 773 bp (*A. jama*) for the *rrnS* (Table 2).

The entire set of 22 tRNA genes was found, interspersed throughout the whole genome. Their length in the three newly sequenced skippers ranged from 58 to 71 bp in *I. lamprospilus*, from 59 to 71 in *A. jama*, and from 59 to 73 bp in *N. curvifascia* (Table 2). Most (21 of 22) tRNAs could be folded into a cloverleaf secondary structure; the only exception was *rrnS* (AGN), which lacked the DHU stem in all three mitogenomes (Fig. 3). The missing DHU stem of this tRNA gene evolved very early in the Metazoa [65], and it is the ancestral state in butterflies. The size of the amino acid acceptor stems and anticodon loops was highly conserved (all 7 bp), while the size of DHU and TΨC arms was variable. The total number of unmatched base pairs found in the three skippers was 30 in *A. jama*, 34 in *I. lamprospilus* and 26 in *N. curvifascia*. The

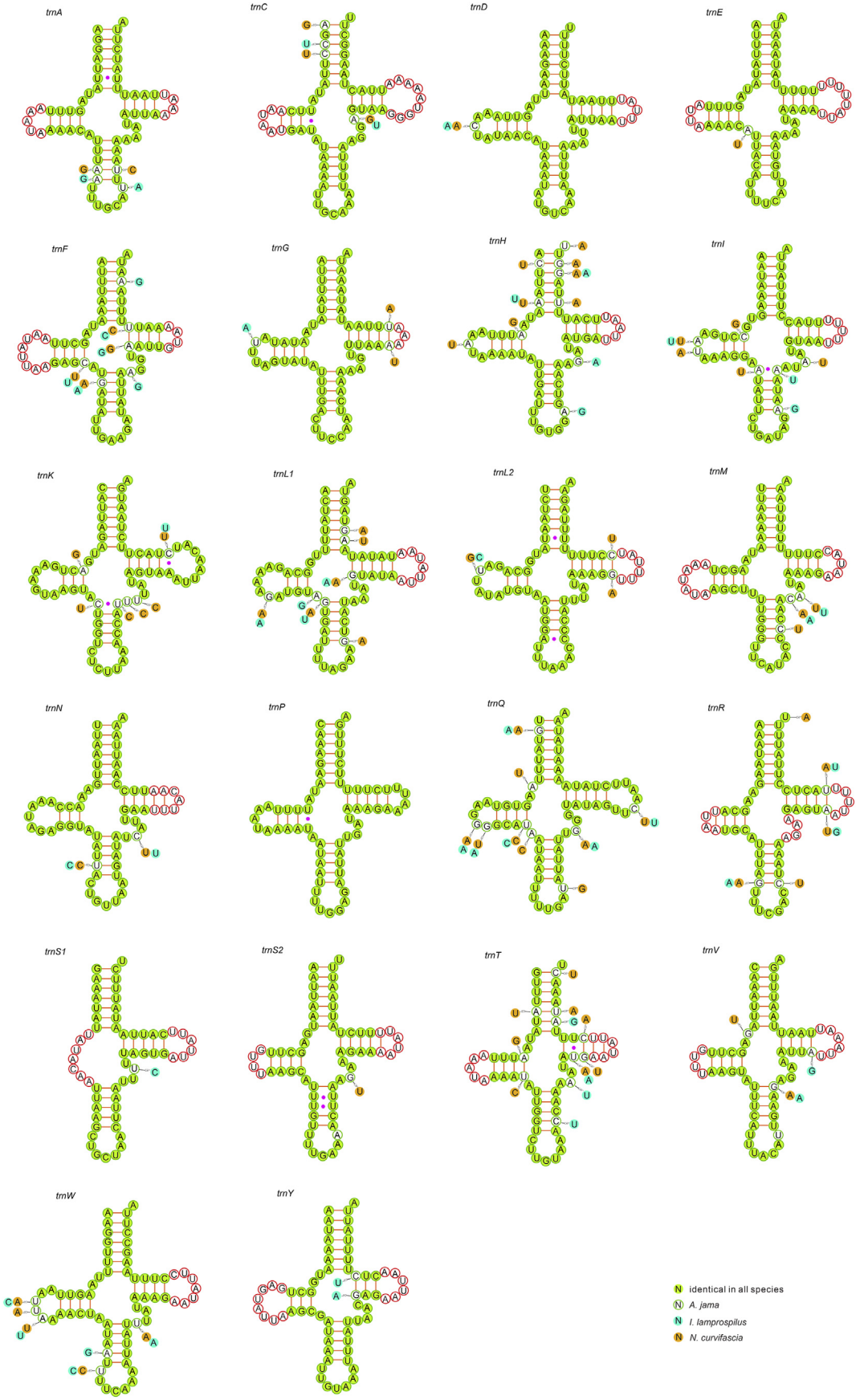

Fig. 3. Predicted secondary cloverleaf structure for the tRNAs of *A. jama*, *I. lamprospilus* and *N. curvifascia*.

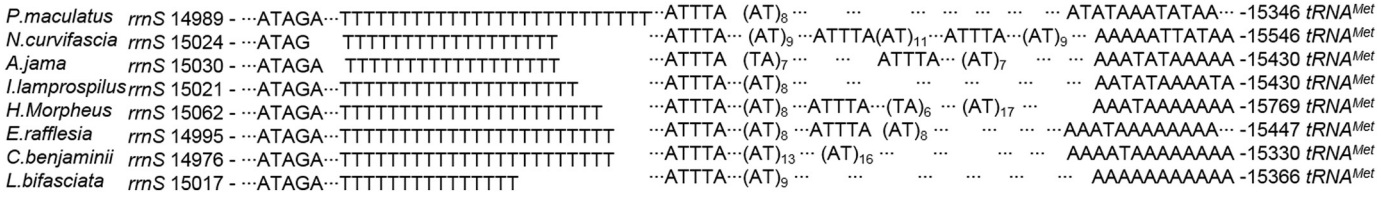

Fig. 4. Structural elements found in the A + T-rich region of eight skippers.

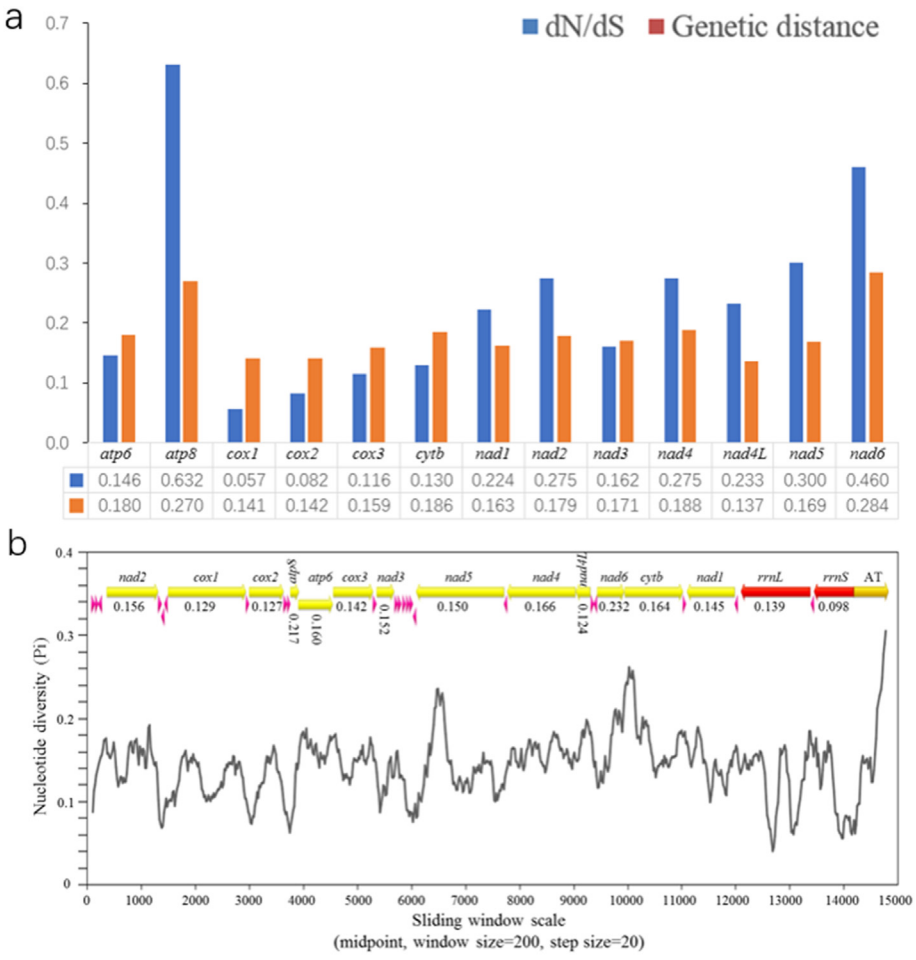

Fig. 5. a Genetic distances and the ratio of non-synonymous (dN) to synonymous (dS) substitution rates of protein-coding genes among eight Hesperiidae species. The average value for each PCG is shown under the gene name. b Sliding window analysis of the alignment of complete mtDNAs of the eight Hesperiidae species. The black line shows the value of nucleotide diversity Pi (p) in a sliding window analysis, with a window size 200 bp, step size 20, and the value inserted at the midpoint. Gene names, boundaries/direction (colored arrows) and nucleotide diversity values are indicated above the graph.

types of unmatched pairs include U—U, U—C, A-A, A-C, A-G and G-U pairs, all of which form a stable hydrogen-bonded pair (Fig. 3).

3.4. Intergenic spacers and overlapping sequences

We identified 11 to 13 intergenic spacers in the three mitogenomes, with sizes ranging from 1 to 97 bp. The longest (97 bp) intergenic spacer was identified in *I. lamprospilus*, between *trnQ* and *nad2* genes (Table 2). This intergenic spacer, found in most lepidopteran mitogenomes [56], seems to be fundamental to the transcription termination site recognition by the transcriptional machinery [56,66]. The spacer between *trnS* (UCN) and *nad1* is also present in most insect mitogenomes, and although its size is relatively variable, the existence of a highly conserved ATACTAA motif is an indication of its functional role [56]. The three skippers had 9 to 13 overlapping genes, with overlaps ranging from 1 bp to 25 bp (Table 2). Gene overlaps occurred more frequently between tRNA genes, which may be related to the lesser evolutionary constraints of tRNA genes [64].

3.5. A + T-rich region

The A + T-rich region, believed to be involved in the control of transcription in insects [67], is located between *rrnS* and *trnM* in these three skippers. Its length and A + T content range from 401 bp (*A. jama*) to 523 bp (*N. curvifascia*), and 92.1% (*N. curvifascia*) to 93.4% (*I. lamprospilus*) respectively (Table 2). Sequence analysis of the A + T rich regions in the eight skippers from six subfamilies of Hesperiidae revealed the presence of conserved structures, including a poly-T stretches of varying length (15 to 26 bp), several microsatellite-like A/T sequences following the motif ATTTA, and either a complete or an interrupted poly-A stretch immediately upstream of *trnM*. In addition, the motif ATAGA near the 5′-end of the *rrnS* is the origin of the minority

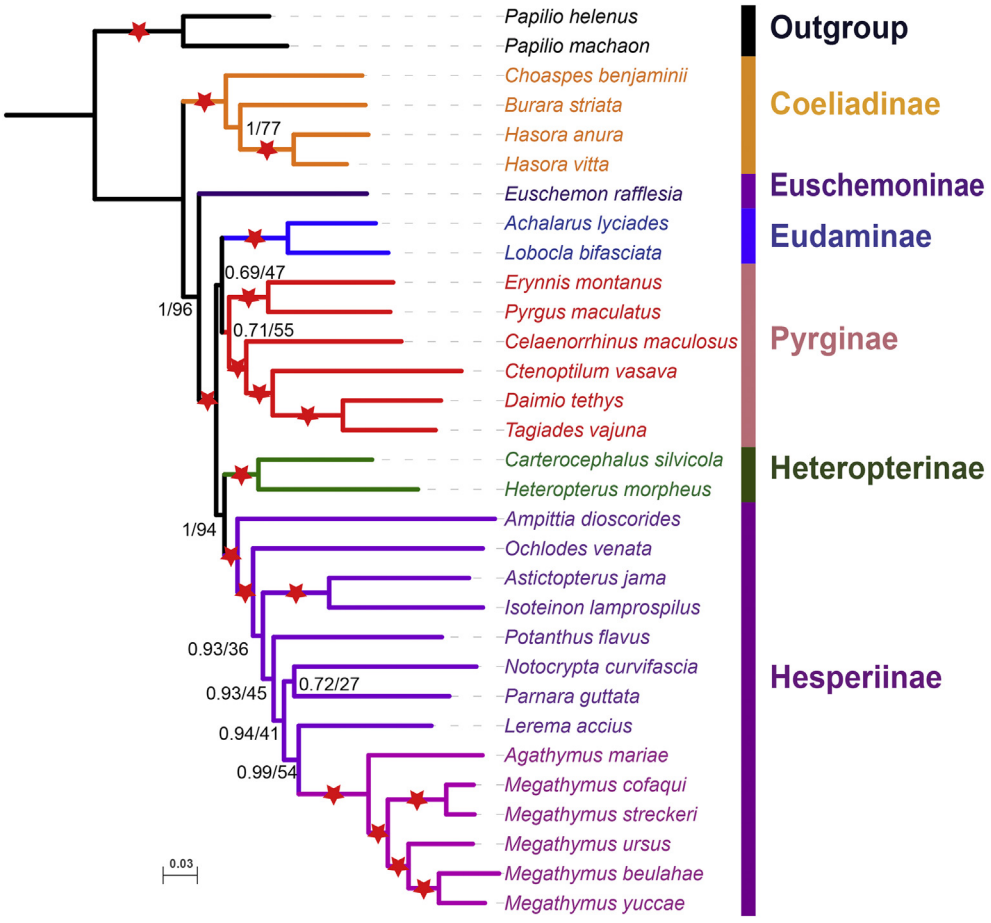

Fig. 6. Phylogenetic tree produced by maximum likelihood and Bayesian inference analyses of the PCGRT dataset. Bootstrap (BS) and posterior probability (BPP) support values lower than the maximum support (BS = 100, BPP = 1.0) are shown above the branches. Star symbol indicates that both methods produced a maximum support value.

strand replication in lepidopteran mitogenomes [56,68]. However, in *N. curvifascia* we found ATAG motif instead of the ATAGA (Fig. 4).

3.6. Nucleotide diversity and evolutionary rate analysis

All analyses were conducted using eight skipper mitogenomes representing the six subfamilies. The plot of sequence variation ratio (sliding-window analysis) exhibited highly variable nucleotide diversity (Pi values), not only among different genes, but also among different parts of genes (Fig. 5b). Nucleotide diversity values calculated for individual genes ranged from 0.098 (*rrnS*) to 0.232 (*nad6*), where other genes with comparatively low values included *cox2* (0.121), *nad4L* (0.124), *cox1* (0.129) and *rrnL* (0.139), whereas *atp8* (0.217), *nad4* (0.166), *cytb* (0.164) and *atp6* (0.160) exhibited comparatively high values. Pairwise genetic distances among these eight mitogenomes produced congruent results: average values indicate that *cox1* (0.141), *cox2* (0.142) and *nad4L* (0.137) are evolving comparatively slowly, while *atp8* (0.270), *nad6* (0.284) and *nad4* (0.188) are evolving comparatively fast (Fig. 5a, Table S1). Non-synonymous/synonymous (dN/dS) substitution ratio can be used to estimate whether a sequence is undergoing purifying, neutral, or positive selection. We conducted pairwise dN/dS analyses for these eight mitogenomes, and found that all genes are evolving under a purifying selection: *cox1* exhibited the strongest purifying selection, whereas the *nad* family genes (especially *nad6*) exhibited a slightly relaxed purifying selection; *atp8* was an outlier, with pairwise comparison values ranging from strong purifying (0.246) to positive selection (1.415), and the average value indicating a highly relaxed purifying selection (close to neutral selection; Fig. 5a, Table S2).

Nucleotide diversity analyses are useful for designing species-specific markers, especially in taxa where morphological identification is difficult and ambiguous [69,70] and for functional studies [69–71]. The *cox1* gene is often used as a universal barcode for species identification in animals [72], including insects [73–75], so its low variability indicates that its utility as a barcode for the family Hesperiidae, or even the entire Lepidoptera, needs to be carefully tested and revised. If its resolution power indeed proves to be too low, we propose that genes exhibiting an optimal combination of fast evolution and sufficiently large size (*atp8* is too small for example), notably *nad6* and *nad4*, should be evaluated as potential DNA markers for species and/or population identification.

3.7. Phylogenetic analyses

All six topologies produced by the phylogenetic analyses (three datasets × two methods) were largely congruent, regardless of the analytical method and datasets used. PCGRT dataset produced the most consistent topologies and higher node support values than PCG and PCG12RT datasets, indicating that the RNAs increased the phylogenetic resolution [25]. Therefore, only the phylogenetic tree constructed using the PCGRT dataset is shown (Fig. 6), whereas the remaining dendrograms are shown in supplementary data: PCG dataset in Figs. S1 and S2, and PCG12RT dataset in Figs. S3 and S4.

Topologies of all six phylograms are almost identical to the one obtained in the most recent mitochondrial phylogenomic study [13], with Coeliadinae as the basal branch, followed by Euschemoninae, Eudaminae + Pyrginae, Heteropterinae, and finally Hesperiinae. Discordant is only the position of Eudaminae, and phylogenetic relationships among/within the Pyrginae and Eudaminae varied among the six phylograms. The Pyrginae subfamily was monophyletic only in ML and BI analyses of the PCGRT dataset, but with weak statistical support (BS = 55, BPP = 0.71), and polyphyletic in PCG12RT and PCG

datasets, with a sister group relationship between (Erynnini + Pyrgini), and (Eudaminae + (Celaenorrhinini + Tagiadini)) in the PCG12RT dataset (BS = 50, BPP = 0.57). The following topology was obtained using the PCG dataset: ((Erynnini + Pyrgini) + ((Eudaminae + (Celaenorrhinini + Tagiadini)) + (Heteropterinae + Hesperiinae))) (BS = 97, BPP = 1). Currently, studies based on mitochondrial genomic data and combined mitochondrial/nuclear datasets have not yet produced results supporting the monophyly of Pyrginae [11,22,64]. In order to resolve the status of Pyrginae, larger datasets, such transcriptome or genomics data, possibly in combination with morphological features, will be required.

Within the Hesperiinae, *A. jama* and *I. lamprospilus* formed a well-supported clade (BS = 100, BPP = 1), which is consistent with previous studies [5,11,18]. *Isoteinon* is a monotypic genus, and two genera mainly distributed in Asia Minor, *Eogenes* and *Actinor*, are considered to be close relatives. In addition to *A. jama*, *Astictopterus* has eight African species. Touissaint et al. [18] showed that *A. punctulata* forms a clade with *A. jama*, *I. lampropilus*, and *Kedestes callicle*. Therefore, we propose placing *Astictopterus* in the Isoteinonini clade. The placement of *N. curvifascia* remains unresolved. Though statistical support is low, *Notocrypta* is closely related to *Lerema* in the results of the PCG dataset (Figs. S1 and S2). Interestingly, *N. curvifascia* and L. *accius* have some similar morphological features: larval head capsule, pupal head projection, and peculiar violet gray scaling on the ventral hindwing of adults. As many lineages of the family Hesperiidae remain underrepresented or not represented at all in terms of the availability of complete mitochondrial genomes, sequencing of additional mitogenomes is needed to resolve the complex taxonomic sub-division of the Hesperiidae.

4. Conclusions

In this study, the mitogenomes of *A. jama*, *I. lamprospilus* and *N. curvifascia* were sequenced, and the phylogenetic relationships of the family Hesperiidae were analyzed using different methods and datasets. Comparative analyses of the 37 genes demonstrate that evolutionary rates differ among individual genes. Combined results of the sliding window analysis, dN/dS ratios and genetic distances indicate that *atp8*, *nad4* and *nad6* are the most variable PCGs, whereas *cox1*, *cox2* and *nad4L* are the most conserved PCGs in Hesperiidae. Intriguingly, as opposed to other genes, which evolve under purifying selection, *atp8* appears to evolve under a highly relaxed, almost neutral, selection in Hesperiidae.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2019.03.006.

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