Three complete mitochondrial genomes of Hasora (Lepidoptera: Hesperiidae) and Phylogenetic analysis

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Abstract:

In the present study, the complete mitochondrial genomes of *Hasora chromus*, *Hasora badra* and *Hasora vitta* ALT525 have been sequenced. The complete mitogenome sequences of *H. chromus*, *H. badra* and *H. vitta* ALT525 are 15,413 bp, 15,324 bp and 15,290 bp in length, respectively. This three mitogenomes contain 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribo-somal RNA genes (rRNAs) and a non-coding control region (CR). Except for *COI*, all protein-coding genes of the three mitogenomes are initiated by the typical ATN codons. The CGA as the start codon of *COI* gene was found in the other Lepidoptera mitogenomes. All

tRNAs except $tRNA^{Ser(AGN)}$ were predicted with a typical clover-leaf secondary structure. There are diverse bases insertion between the conserved motif "ATAGAA" and poly T-stretch in the *Hasora* mitogenomes. The phylogenetic analyses of Hesperiidae showed that the relationship is Coeliadinae + (Euschemoninae+ (Pyrginae + (Heteropterinae +Hesperiinae))). *H. vitta* ALT525 was different from *H. vitta* ALT60 in some prime mitochondrial genes. *H. vitta* ALT525 was identified and classified as cryptic *Hasora vitta* ALT60 for the first time.

Keyword: Mitochondrial genome, Hesperiidae, Phylogenetic analyses, Cryptic *Hasora vitta*

1. Introduction

Insect mitochondrial genome is a double-stranded circular molecule with 14-20 kb in size and possesses 37 genes including 13 protein-coding genes (PCGs), 2 ribosomal RNA genes (lrRNA and srRNA), and 22 tRNA genes [1-3]. In addition, it contains a major non-coding region, known as the control region (or A+T-rich region), which harbors y the initiation sites of its replication and transcription [4-5]. Mitochondrial genome has been widely used to study on molecular evolutionary, phylogeny, systematics, population genetics, phylogeography and diagnostics for its maternal inheritance, sequence conservatism and rapid evolution [6-8].

The Hesperiidae is a large family of Lepidoptera and widely distributed around the world. The subfamilies and tribes have undergone many classificatory adjustments and various hypotheses of phylogenetic relationships [9-11]. Yuan et al considered four subfamilies of the Hesperiidae from China: Coeliadinae, Pyrginae, Heteropterinae, and Hesperiinae [12]. Vane-Wright et al inferred that the Hesperiidae was composed of Coeliadinae, Pyrrhopyginae, Pyrginae, Heteropterinae, Trapezitinae, Hesperiinae [13]. Warren et al inferred that Hesperiidae are divided into seven subfamilies: Coeliadinae, Euschemoninae, Eudaminae, Pyrginae, Heteropterinae,

Trapezitinae and Hesperiinae [14]. Despite Hesperiidae family contains more than 4,100 species, only more than 30 complete mitogenomes reported up to now. The number of mitogenome data is still quite limited compared to the diversity of the species, which severely confines our understanding of their evolution.

The genus *Hasora* Moore belongs to the subfamily Coeliadinae of Hesperiidae, which have seven species in China [12, 15]. One species of *Hasora* genus were found in the altitude of 525 meters in Hong Kong. Its morphological characters of the adults are strongly similar to *H. vitta* found in the lower altitude and at near sea level [16]. Its larval stages and host plant are distinctively different from *H. vitta*. Its larva has six instars and its host plant is one kind of *Millettia nitida* Benth with long silvery fibrous hair on its top. However, *H. vitta* has only five instars, the host plant is common *M. nitida* which have short fine hairs on its top. In the study, the species was found in the lower altitude and at near sea level was named *Hasora vitta* ALT60, another was found in the altitude of 525 meters was named *Hasora vitta* ALT525.

In this study, the mitogenomes of three *Hasora* species from Hong Kong were sequenced, which is *Hasora chromus*, *Hasora badra* and *Hasora vitta* ALT525. We had compared the genomic organization, nucleotide composition, codon usage and the control region of five *Hasora* mitogenomes and combined on 30 mitogenome data of Hesperiidae published in Genbank to reconstruct phylogenetic trees. In addition, *Hasora vitta* ALT60 and *Hasora vitta* ALT525 were compared and analyzed based on their mitogenomes.

2. Materials and methods

2.1. Sample collection and DNA extraction

The adults of *Hasora chromus*, *Hasora badra* and *Hasora vitta* ALT525 were collected from

Hong Kong, China in 2012. All fresh specimens were originally conserved in anhydrous ethanol, and then transferred to -20°C in the laboratory for cryopreservation. Total genomic DNA was extracted from thoracic muscle of a single adult using the OMEGA Insect DNA Kit (OMEGA, USA) following the manufacturer's instruction. The extracted DNA was checked by 1% agarose gel electrophoresis and was stored under -20°C preparing for PCR.

2.2. PCR amplification and sequencing

Twenty PCR universal primers were designed based on Simon et al. and known mitochondrial sequences of Hesperiidae insects [16-19], two primers were designed for two short fragments based on the conserved regions which we have determined and then amplified the remained mtDNA sequences. All PCR amplification were performed using TaKaRa LA Taq polymerase under the following conditions: initial denaturation for 5 min at 95℃, followed by 30 cycles of 50 s at 95℃, 50 s at 48℃, 2 min 30 s at 68℃, and a subsequent final extension at 68℃ for 10 min.

The PCR products were detected via electrophoresis in 1.0% agarose gel. All fragments were purified by OMEGA PCR purification kit (OMEGA, USA). DNA sequences were determined with an ABI 3730 semiautomated DNA sequencer (Applied BioSystems) at Invitrogen Inc in Shanghai, China. All fragments were sequenced by primer walking from double strands.

2.3. Sequence assembly, annotations and analysis

The overlapping fragments were assembled into a complete mitochondria DNA sequence using Sequencher 4.8 software (Gene Codes Corporation, 2008). The tRNA genes were verified through their proposed cloverleaf secondary structure and anticodon sequences using tRNAscan-SE 1.21) [20]. Some tRNA genes that could not be determined by tRNAscan-SE were identified

via aligning with the tRNA genes of other lepidopteran. Protein-coding and rRNA genes were identified via comparing the homologous regions of other Hesperiidae species. The tandem repeats in the A+T-rich region were predicted using the Tandem Repeats Finder available online (http://tandem.bu.edu/trf/trf.html) [21]. Nucleotide composition and codon usage were calculated using MEGA 7.0 [22].

2.4. Phylogenetic analysis

To construct the phylogenetic relationship of Hesperiidae, the complete mitogenomes of *H. chromus*, *H. badra*, *H. vitta* ALT525 and 30 Hesperiidae species were collected. The mitogenomes of *Adoxophyes honmai* (NC_008141) and *Grapholita molesta* (NC_014806) were selected as outgroups. 13 PCGs (excluding the termination codons) were aligned by Mega 7.0 software for Bayesian inference and Maximum Likelihood analysis [22].

The nucleotide alignments of protein-coding genes were concatenated for constructing the topology tree. The GTR+I+G model was optimal for the analysis of the nucleotide alignments according to the Akaike information criterion. Under the GTR+I+G model, MrBayes v.3.1.1 [23] and a PHYML online web server [24] were used to analyze this dataset. In Bayesian analysis, two simultaneous runs of 1,000,000 generations were conducted for the matrix and each set was sampled every 1000 generations, with the first 25% discarded as burn-in. In ML analysis, the parameters were estimated during analysis and the node support values were assessed by bootstrap resampling (BP) [25] calculated using 1000 replicates.

3. **Results and discussion**

3.1. Mitogenome organization

The complete mitogenome sequences of *H. chromus* (GenBank accession number

MN182753), *H. badra* (GenBank accession number NC_045249) and *H. vitta* ALT525 (GenBank accession number MK238675) are 15,413 bp, 15,324 bp and 15,290 bp in length, respectively (Fig. 1, Table 1). These three mitogenomes sizes are within the range of detected Hesperiidae mitogenomes from 15,267 bp of *Potanthus flavus* to 15,767 bp of *Heteropterus Morpheus* (Table 2). The three sequences contain 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribo-somal RNA genes (rRNAs) and a non-coding control region (CR). Among the 37 genes, 9 PCGs and 14 tRNAs are encoded on J-strand, 4 PCGs, 8 tRNAs and 2 rRNAs are encoded on N-strand (Fig. 1, Table 1). The control region is located between *rrnS* and $tRNA^{Met}$.

There are gene overlaps and intergenic spacers in five *Hasora* mitogenomes which are spread in whole mitogenomes. The longest intergenic spacer sequences are located between $tRNA^{Gln}$ and *ND2* in five *Hasora* mitogenomes, the spacer are 117 bp in *H. chromus*, 89 bp in *H. badra*, 83 bp in *H. vitta* ALT525 and *H. vitta* ALT60, 104 bp in *H. anura*. The gene overlaps are spread in 10 locations of *H. chromus*, 52 bp in 9 locations of *H. badra*, 40 bp in 9 locations of *H. vitta* ALT60, 32 bp in 8 locations of *H. vitta* ALT525 and *H. anura*, respectively. The total length of gene overlaps are from 32 bp in *H. vitta* ALT525 and *H. anura* to 52 bp at 9 locations in *H. badra*. Similar intergenic spacers and overlaps were also found in other lepidopteran insects [26-28].

3.2 Protein coding genes

The sizes and AT contents of PCGs in the five *Hasora* mitogenomes are 11,213 bp and 77.6% in *Hasora chromus*, 11,228 bp and 77.9% in *Hasora badra*, 11,224 bp and 78.2% in *H. vitta* ALT60, 11,213 bp and 78.2% in *H. vitta* ALT525, 11,207 bp and 77.8% in *H. anura*. The total codons numbers of PCGs, excluding the stop codons, are 3726 in *H. chromus*, 3731 in *H. badra*, 3729 in *H. vitta* ALT60, 3726 in *H. vitta* ALT525 and 3724 in *H. anura*, which are in the

range of the Hesperiidae species (from 3710 in *Ctenoptilum vasava* to 3732 in *Lerema accius*, Table 2).

Twelve protein-coding genes of the five *Hasora* mitogenomes are initiated by the typical ATN codons. The *COI* genes start with CGA. The CGA as the start codon of *COI* gene was found in most lepidopteran insects [29-30]. This codon is highly conserved in lepidopteran insects. Eleven PCGs use the complete stop codon TAN, *COII* and *ND4* are terminated with a single T (Table 1) [16-17].

The codon usage and the relative synonymous codon usage (RSCU) values are calculated in Fig. 2. Leu $^{(UUR)}$, Ile, Phe, Met and Asn are five frequently-used codons families in the five mitogenomes, the most frequently used codon is UUA(Leu), followed by AUU(Ile), UUU(Phe), AUA(Met) and AAU(Asn). The least used codon family is Cys (Fig. 2). These codons consist of A or T nucleotides, thus indicating the biased usage of A and T nucleotides in the PCGs of lepidopteran insects [31-32].

3.3 *Transfer RNAs and Ribosomal RNAs*

The five *Hasora* mitogenomes have the typical 22 tRNAs set, the 22 tRNAs were interspersed across the mitogenome and ranged from 61 to 71 bp. The order of 22 tRNAs is consistent with most Hesperiidae species [33-35]. The anticodons of 22 tRNAs are in accord with the most Lepidopteran insects [26, 28]. All tRNAs except $tRNA^{Ser(AGN)}$ were predicted with a typical clover-leaf secondary structure. The $tRNA^{Ser(AGN)}$ forms a simple loop and lacks the dihydrouridine (DHU) arm. The tRNA stem regions harbor unmatched base pairs, such as G·U, U·G, U·U, etc. The non-classical matched phenomena are present in the most other insects [29].

The *rrnL* and *rrnS* are lay between $tRNA^{Leu(CUN)}$ and $tRNA^{Val}$, and between $tRNA^{Val}$ and

control region, respectively (Fig. 1 and Table 1). This arrangement is consistent with other lepidopteran insects [31-32]. The lengths and the A+T contents of *rrnL* gene are 1402 bp and 83.9% in *H. chromus*, 1432 bp and 84.3% in *H. badra*, 1397 bp and 84.4% in *H. vitta* ALT60, 1400 bp and 85.0% in *H. vitta* ALT525, and 1387 bp and 83.5% in *H. anura* (Table 2); The sizes of rrnS gene are 794 bp in *H. chromus*, 798 bp in *H. badra*, 797 bp in *H. vitta* ALT60 and *H. vitta* ALT525, and 799 bp in *H. anura*, and their A+T contents are 85.4% in *H. chromus*, 85.3% in *H. badra*, 85.2% in *H. vitta* ALT60 and *H. vitta* ALT525, and 85.4% in *H. anura* (Table 2). The A+T contents in *rrnS* genes of five *Hasora* mitogenomes are higher than *rrnL* genes which is consistent with published Hesperiidae species (Table 2).

3.4 Control region

The control regions of five *Hasora* mitogenomes are located between the *rrnS* gene and $tRNA^{Met}$ (Table 1; Fig. 1). Their A+T contents are 96.1% in *H. chromus*, 96.0% in *H. badra*, 94.1% in *H. vitta* ALT60, 93.3% in *H. vitta* ALT525 and 95.8% in *H. anura*, respectively (Table 2). The A+T contents is the highest in the whole mitogenome. All control regions of Hesperiidae mitogenomes indicated a forceful A+T bias (Table 2) [36-37].

The control region of five *Hasora* mitogenomes contain several features which are common to the Lepidoptera control region. The poly-T stretch and several tandemly-repeated elements were found in the control region which had been mentioned frequently in other insects. The conserved motif “ATAGAA” + poly-T as the origin of the N-strand DNA replication was found downstream from the *rrnS* gene. There is base insertion between the "ATAGAA" and poly T-stretch in five *Hasora* mitogenomes, “TTAA” was inserted in *H. anura*, *H. chromus* and *H. vitta* ALT525, “TTATTATTATTA” was inserted in *H. badra*, “TTAATTA” was inserted in *H. vitta*

ALT60 (Fig. 3) .Some microsatellite-like $(AT)_n$ elements are present in the control region. *H. anura* contains two microsatellite-like repeats of $(TA)_9$ and $(TA)_{12}$, *H. chromus* contains two microsatellite-like elements of $(TA)_7$ and $(TA)_{12}$, *H. badra* harbors two microsatellite-like elements of $(TA)_9$ and $(TA)_{12}$, *H. vitta* ALT60 contains two same microsatellite-like elements of $(TA)_9$ and one $(TA)_7$, *H. vitta* ALT525 harbors two same microsatellite-like elements of $(TA)_9$. Finally, the poly-A element was present in control region of *H. vitta* ALT60 and *H. vitta* ALT525 and present in upstream $tRNA^{Met}$ (Fig 3).

3.5. Phylogenetic analyses

Up to now, some studies on the phylogenetic analyses of Hesperiidae have been reported using morphological data, partial mitochondrial genes or the combination of morphological data, partial mitochondrial genes and a few nuclear genes [11, 14]. However, the phylogenetic relationship of Hesperiidae has yet a controversial issue. In Vane-Wright et al. reconstructed the phylogenetic tree of Hesperiidae based on 103 morphological characters and the result is: (Coeliadinae+ (Pyrrhopyginae+ Pyrginae+ (Heteropterinae+ (Trapezitinae+ Hesperiinae)))) [13]. Warren et al. proposed the phylogenetic relationship of Hesperiidae in the world base on three gene regions and 49 morphological characters and the results showed: (Coeliadinae + (Euschemoninae+ (Eudaminae+ (Pyrginae + (Heteropterinae + (Trapezitinae + Hesperiinae)))))) [14]. Yuan et al. inferred the phylogenetic relationships of the subfamilies of Chinese Hesperiidae based on *Cytb*, *ND1* and *COI* and the results showed: (Coeliadinae + (Eudaminae + (Pyrginae + (Heteropterinae + Hesperiinae)))) [38].

In this study, the phylogenetic analyses of Hesperiidae were conducted based on 13 protein-coding genes of 33 known Hesperiidae, including *H. chromus*, *H. badra* and *H. vitta* ALT525,

which five taxon (Nymphalidae, Pieridae, Lycaenidae, Papilionidae and Danaidae) were selected. The result showed that the family-level relationship is: ((((Nymphalidae + Danaidae) + (Pieridae + Lycaenidae)) + Hesperiidae) + Papilionidae). Hesperiidae was identified as a sister to Nymphalidae, Danaidae, Pieridae and Lycaenidae. In Hesperiidae, the relationship of the subfamilies is follow: Coeliadinae + (Euschemoninae+ (Pyrginae + (Heteropterinae +Hesperiinae))) (Fig. 4). This result is consistent with the previous reports based on morphological or partial molecular characters [14, 38].

The Coeliadinae subfamily was located in the basal position as the sister to the other subfamilies in Hesperiidae. In Coeliadinae, the phylogenetic relationship is *Choaspes benjaminii*+ (*Burara striata*+ (*H. badra*+ (*H. chromus*+ (*H. anura*+ (*H. vitta* ALT60+ *H. vitta* ALT525))))). Five sequenced *Hasora* species belong to the Coeliadinae clade. The clade was strong support at 100% by BI analyses and ML analyses. The conclusion is consistent with the morphological classification. The Coeliadinae subfamily is considered as the oldest subfamily of Hesperioidea. In Hasora clade, *H. vitta* ALT60 and *H. vitta* ALT525 were identified as a sister relationship with high support on BI analyses and ML analyses at 100%, *H. badra*, *H. chromus* and *H. anura* are closer to *H. vitta* ALT60 and *H. vitta* ALT525 with strong supports in both ML and BI trees, respectively (Fig. 4).

Conclusion

This study sequenced the complete mitochondrial genomes of three *Hasora* species. The phylogenetic relationships of the Hesperiidae based on the complete mitochondrial genomes provides a strong support on BI analyses and ML analyses. The analysis result supported some previous reports based on morphological or partial molecular characters [14, 38].

The study discovered hidden *Hasora* species and tested the presence of cryptic species. This results indicated *H. vitta* ALT525 is different from *H. vitta* ALT60 in molecular level. Some prime molecular characters of *H. vitta* ALT525 have been mutated, genetic differentiation among *H. vitta* ALT60 and *H. vitta* ALT525 was obvious. Meanwhile, the difference of the two species is mainly reflected at the larval stage in biology and morphology. The additional six instar larval stage of *H. vitta* ALT525 may be caused by the harsh climatic condition at the high altitude where it is very hot in summer but is cold and windy in winter. The larval duration of *H. vitta* ALT525 had obviously prolonged caused inconsistent eclosion and mating time with *H. vitta* ALT60. During the evolution of the species, reproductive isolation developed and a new species finally formed. *H. vitta* ALT525 which was collected at 525 metres elevation was identified as a cryptic species of *H. vitta* ALT60.

At present, the mtDNA was used for comparative and evolutionary genomics, molecular evolution, phylogenetics and population genetics. Although the phylogenetic analysis based on the complete genome sequence is ideal, the species of whole mitogenome sequences was limited. With the rapid development of sequencing technologies, the complete genomes of more and more species will be sequenced, the species will be identified and classified more correctly.

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Declaration of competing interest

The authors declare that they have no competing interests.

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