The first complete mitochondrial genome for the subfamily Limacodidae and implications for the higher phylogeny of Lepidoptera

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The mitochondrial genome (mitogenome) provides important information for understanding molecular evolution and phylogeny. To determine the systematic status of the family Limacodidae within Lepidoptera, we infer a phylogenetic hypothesis based on the complete mitogenome of *Monema flavescens* (Lepidoptera: Limacodidae). The mitogenome of *M. flavescens* is 15,396 base pairs (bp), and includes 13 protein-coding genes (PCGs), two ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes, and a control region (CR). The AT skew of this mitogenome is slightly negative and the nucleotide composition is also biased towards A + T nucleotides (80.5%). All PCGs are initiated by ATN codons, except for the cytochrome c oxidase subunit 1 (*cox1*) gene, which is initiated by CGA. All tRNAs display the typical clover-leaf structure characteristic of mitochondrial tRNAs, with the exception of trnS1 (AGN). The mitogenome CR is 401 bp and consists of several features common to Lepidoptera. Phylogenetic analysis using Bayesian Inference (BI) and Maximum Likelihood (ML) based on nucleotide and amino acid sequences of 13 mitochondrial PCGs indicates that *M. flavescens* belongs to Zygaenoidea. We obtain a well-supported phylogenetic tree consisting of Yponomeutoidea + (Tortricoidea + Zygaenoidea + (Papilionoidea + (Pyraloidea + (Noctuoidea + (Geometroidea + Bombycoidea)))))

The insect mitogenome is a circular molecule 14–19 kilobases in length. It contains 22 tRNAs, 13 PCGs, ATPase subunits 6 and 8 (*atp6* and *atp8*), *cox1*- *cox3*, cytochrome *B* (*cob*), NADH dehydrogenase subunits 1–6 and 4L (*nad1*-6 and *nad4L*), the small and large subunit rRNAs (*rrnL* and *rrnS*), and a non-coding element termed the A + T-rich region (CR), which contains initiation sites for transcription and replication. Because of their unique features, including coding content conservation, maternal inheritance, and rapid evolution, mitogenomes have been informative in diverse studies of molecular evolution, such as phylogenetics, population genetics, and comparative and evolutionary genomics.

Recent advances in sequencing technologies have led to the rapid increase in mitogenomic data in Genbank, including Lepidopteran mitogenomes. Lepidoptera is the second largest order of insects, accounting for more than 160,000 species. Zygaenidae is a species-rich superfamily of predominantly diurnal moths with a worldwide distribution. This family is particularly diverse in tropical and subtropical Asia and the Palaearctic region. Because of the broad geographical distribution of species, extensive variation in coloration patterns, and an intriguing chemical defence system, Zygaenidae is of great interest to lepidopterists and evolutionary biologists. To date, more than 200 complete or near-complete Lepidopteran mitogenomes are available. However, only one mitogenome of Zygaenoidea has been sequenced. *Monema flavescens* Walker, 1855 is a moth of the Limacodidae family found in Korea, Japan, China, and the Russian Far East. The mitogenome of *M. flavescens* has not been sequenced.

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| Superfamily       | Family   | Subfamily | Species                  | Genbank No. |
|------------------|----------|-----------|--------------------------|-------------|
| Zygaenoidea      | Limacodidae |           | Momema flavescens        | KU946971    |
| Zygaenoidea      | Zygaenidae | Chalcisinae| Rhodoposoma rubiginosa    | KM244668    |
| Bombycoidea      | Bombycidae | Bombicinae| Bombyx mandarina         | AB070263    |
| Bombycoidea      | Bombycidae | Bombicinae| Bombyx mori              | AI49768     |
| Bombycoidea      | Bombycidae | Bombicinae| Bombyx huttoni           | KP216766    |
| Bombycoidea      | Saturniidae| Saturniinae| Samia cynthia ricini     | JN215366    |
| Bombycoidea      | Saturniidae| Saturniinae| Actias selene           | JX86589     |
| Bombycoidea      | Saturniidae| Saturniinae| Antherea pernyi         | AY242996    |
| Bombycoidea      | Saturniidae| Saturniinae| Eriogyna pyretorum       | FJ685653    |
| Bombycoidea      | Saturniidae| Saturniinae| Saturnia boishuvali      | EF622227    |
| Bombycoidea      | Sphingidae | Sphinginae| Mandra sexta             | EU286785    |
| Bombycoidea      | Sphingidae | Sphinginae| Sphinx morio             | KC470083    |
| Bombycoidea      | Saturniidae| Saturniinae| Antheraea fritilli       | KJ740437    |
| Bombycoidea      | Saturniidae| Saturniinae| Attacus atlas            | KF006336    |
| Bombycoidea      | Saturniidae| Saturniinae| Actias artemis aliena    | KJ720472    |
| Bombycoidea      | Saturniidae| Saturniinae| Samia camingi            | KJ159099    |
| Geometroidea     | Geometridae| Ennominae | Biston panterinaria      | JX401646    |
| Geometroidea     | Geometridae| Ennominae | Phthonandria atrilineata | EU569764    |
| Geometroidea     | Geometridae| Ennominae | Jankowskia athleta       | KR822683    |
| Geometroidea     | Geometridae| Ennominae | Apocheima cinerarium     | KF836545    |
| Geometroidea     | Geometridae| Larentiinae| Operophtera brumata      | KP027400    |
| Hepialoidea      | Hepialidae |           | Ahamus yunnanensis       | HM744695    |
| Hepialoidea      | Hepialidae |           | Thitarodes renchiensis   | HM744694    |
| Hepialoidea      | Hepialidae |           | Hepialus xiaojinensis    | KT836973    |
| Hepialoidea      | Hepialidae |           | Thitarodes pui           | KF908880    |
| Hepialoidea      | Hepialidae |           | Thitarodes gonggaoensis  | KP711817    |
| Hepialoidea      | Hepialidae |           | Napiusus humanensis      | KJ632665    |
| Noctuoidea       | Erebididae | Lymantriinae| Lymantri dispar          | FJ617240    |
| Noctuoidea       | Erebididae | Arctinae  | Hyphantria cunea         | GU592049    |
| Noctuoidea       | Erebididae | Aganainae | Asota plana lacteata     | KJ173908    |
| Noctuoidea       | Erebididae | Erebinae  | Catocala deuteronymphra  | KJ432280    |
| Noctuoidea       | Eutelidae  | Eutelinae | Eutelia adulatricoides   | KJ815311    |
| Noctuoidea       | Nolidae    | Chlorophorinae| Gabala argentata        | KJ410747    |
| Noctuoidea       | Nolidae    | Risobiniae| Risoba prominis          | KJ961997    |
| Noctuoidea       | Noctidae   | Amphipyrinae| Sesamia inferens         | JN039362    |
| Noctuoidea       | Noctidae   | Amphipyrinae| Spodoptera fragiperda    | KM362176    |
| Noctuoidea       | Noctidae   | Heliothinae| Helicoverpa armigera     | GU188273    |
| Noctuoidea       | Notodontidae| Phaleriniae| Phalerus flavescens      | JF403042    |
| Noctuoidea       | Notodontidae| Thaumetopoeiniae| Ochrogaster junifer    | AM946601    |
| Papilionoidea    | Hesperidae | Hesperinae| Ochlopes venata          | HM245593    |
| Papilionoidea    | Lycaenidae | Aphantinae| Spindasis takanomis      | HQ184266    |
| Papilionoidea    | Lycaenidae | Theclinae | Protantigius superans    | HQ184265    |
| Papilionoidea    | nymphalidae| Apatrinae | Apatura metis           | JF801742    |
| Papilionoidea    | nymphalidae| Apatrinae | Sasakia charonda        | AP011824    |
| Papilionoidea    | nymphalidae| Calpinae  | Calpnine davidis         | HQ658143    |
| Papilionoidea    | nymphalidae| Danaeinae | Euproca mulciber         | HQ378507    |
| Papilionoidea    | nymphalidae| Heliconinae| Fabriciana nigrrippe     | JF504707    |
| Papilionoidea    | nymphalidae| Libytheinae| Libythea celts          | HQ378508    |
| Papilionoidea    | nymphalidae| Satyrinae | Hipparchia autonoe       | GQ868707    |
| Papilionoidea    | Papilionidae| Papilioniinae| Graphium chronides      | KP159289    |
| Papilionoidea    | Papilionidae| Papilioniinae| Papilio marahu         | FJ810212    |
| Papilionoidea    | Papilionidae| Parnassiinae| Parnassius apollo        | KP746065    |
| Papilionoidea    | Papilionidae| Parnassiinae| Parnassius cephalus      | KP106555    |
| Papilionoidea    | Pieridae   | Pierinae  | Aporia crataegi         | JN796473    |

Continued
A better understanding of the Lepidopteran mitogenome requires an expansion of taxon and genome samplings. In this study, we sequence and describe the complete mitogenome of *M. flavescens*. We reconstruct a phylogenetic tree based on PCG sequences in order to analyse the evolutionary relationships among Lepidopteran insects. The assembly and annotation of the *M. flavescens* mitogenome will further the study of Zygaenidea mitochondrial genome architecture and phylogenetics. Furthermore, characterization of the *M. flavescens* mitogenome may provide novel insights into the mechanisms underlying mitogenome evolution.

**Methods**

**DNA Extraction.** The moths of *M. flavescens* were collected in Yancheng, Jiangsu Province. Total DNA was isolated using the Genomic DNA Extraction Kit (SangonBiotech, China) according to the manufacturer's instructions. Extracted DNA was used to amplify the complete mitogenome by PCR.
PCR Amplification and Sequencing. For amplification of the *M. flavescens* mitogenome, primer sets were designed based upon mitogenomic sequences obtained from other Lepidopteran insects\(^{10,11}\). PCR was performed under the following conditions: 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1–3 min at 48–60 °C, and 10 min at 72 °C. All amplifications were performed on an Eppendorf Mastercycler and Mastercycler gradient in 50 μL reaction volumes. The PCR products were separated by agarose gel electrophoresis (1% w/v) and purified using a DNA gel extraction kit (Transgene, China). The purified PCR products were ligated into the T-vector (SangonBiotech, China) and sequenced at least three times.

Sequence Assembly and Gene Annotation. Sequence annotation was performed using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast) and the DNAStar package (DNAStar Inc. Madison, WI, USA). The identity of tRNA genes was verified using the tRNAscan-SE program (http://lowelab.ucsc.edu/tRNAscan-SE/)\(^{12}\). The nucleotide sequences of PCGs were translated with the invertebrate mitogenome genetic code. Alignments of *M. flavescens* PCGs with various Lepidopteran mitogenomes were performed using Clustal X\(^{13}\). Composition skewness was calculated according to the following formulas: AT skew = \([A−T]/[A+T]\); GC skew = \([G−C]/[G+C]\). Codon usage was calculated using MEGA version 6.06. Tandem repeats in the A+T-rich region were predicted using the Tandem Repeats Finder program (http://tandem.bu.edu/trf/trf.html)\(^{14}\).

Phylogenetic Analysis. To reconstruct the phylogenetic relationships among Lepidopteran insects, the complete mitogenomes of Lepidopteran species were obtained from GenBank (Table 1). The amino acid

| Gene          | Direction | Location | Size | Anticodon | Start codon | Stop codon | Intergenic nucleotides |
|---------------|-----------|----------|------|-----------|-------------|------------|------------------------|
| trnM          | F         | 1–68     | 68   | CAT       | —           | —          | 2                      |
| trnI          | F         | 71–135   | 65   | GAT       | —           | —          | —3                    |
| trnQ          | R         | 133–201  | 69   | TTG       | —           | —          | 50                     |
| nad2          | F         | 252–1266 | 1015 | —         | ATT         | T          | —3                    |
| trnW          | F         | 1264–1332| 69   | TCA       | —           | —          | —9                    |
| trnC          | R         | 1324–1388| 65   | GCA       | —           | —          | 5                     |
| trnY          | R         | 1394–1462| 67   | GTA       | —           | —          | 3                     |
| cox1          | F         | 1466–2996| 1531 | —         | CGA         | T          | 0                     |
| trnL2(UUR)    | F         | 2997–3065| 69   | TAA       | —           | —          | 0                     |
| cox2          | F         | 3066–3747| 682  | —         | ATG         | T          | 0                     |
| trnK          | F         | 3748–3820| 73   | CTT       | —           | —          | 16                    |
| trnD          | F         | 3837–3899| 63   | GTC       | —           | —          | 0                     |
| atp8          | F         | 3900–4061| 162  | —         | ATT         | TAA        | —7                    |
| atp6          | F         | 4055–4728| 674  | —         | ATG         | TAA        | 3                     |
| cox3          | F         | 4732–5517| 786  | —         | ATG         | TAA        | 2                     |
| trnG          | F         | 5520–5588| 69   | TCC       | —           | —          | 0                     |
| nad3          | F         | 5589–5942| 354  | —         | ATT         | TAA        | 6                     |
| trnA          | F         | 5949–6018| 70   | TGC       | —           | —          | 36                    |
| trnR          | F         | 6055–6121| 67   | TCG       | —           | —          | 1                     |
| trnN          | F         | 6123–6196| 74   | GTC       | —           | —          | 1                     |
| trnM1(AGN)    | F         | 6198–6263| 66   | GCT       | —           | —          | 10                    |
| trnE          | F         | 6274–6343| 70   | TTC       | —           | —          | —2                    |
| trnF          | R         | 6342–6410| 69   | GAA       | —           | —          | 7                     |
| nad5          | R         | 6418–8136| 1719 | —         | ATC         | TAA        | 15                    |
| trnH          | R         | 8152–8219| 68   | GTG       | —           | —          | 0                     |
| nad4          | R         | 8220–9555| 1336 | —         | ATG         | T          | 0                     |
| nad4L         | R         | 9556–9839| 284  | —         | ATA         | A          | 24                    |
| trnT          | F         | 9864–9927| 64   | TGT       | —           | —          | 0                     |
| trnP          | R         | 9928–9993| 66   | TGG       | —           | —          | 8                     |
| nad6          | R         | 10,002–10,520| 519 | —     | ATG       | TAA       | 4                     |
| cob           | F         | 10,525–11,674| 1150 | —     | ATG       | T         | 0                     |
| trnM2(UCN)    | F         | 11,675–11,744| 70  | TGA       | —           | —          | −5                    |
| nad1          | R         | 11,770–12,708| 939 | —     | ATG       | TAA       | 0                     |
| trnL1(CUN)    | R         | 12,709–12,779| 71  | TAG       | —           | —          | 0                     |
| rnl           | R         | 12,780–14,138| 1359 | —     | —         | —          | 0                     |
| trnV          | R         | 14,139–14,203| 65  | TAC       | —           | —          | 0                     |
| rns5          | R         | 14,204–14,995| 792 | —     | —         | —          | 0                     |
| A+T-rich region|         | 14,996–15,396| 401 | —     | —         | —          | —                     |

Table 2. Summary of the mitogenome of *M. flavescens*.  

## PCR Amplification and Sequencing

For amplification of the *M. flavescens* mitogenome, primer sets were designed based upon mitogenomic sequences obtained from other Lepidopteran insects\(^{10,11}\). PCR was performed under the following conditions: 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1–3 min at 48–60 °C, and 10 min at 72 °C. All amplifications were performed on an Eppendorf Mastercycler and Mastercycler gradient in 50 μL reaction volumes. The PCR products were separated by agarose gel electrophoresis (1% w/v) and purified using a DNA gel extraction kit (Transgene, China). The purified PCR products were ligated into the T-vector (SangonBiotech, China) and sequenced at least three times.

## Sequence Assembly and Gene Annotation

Sequence annotation was performed using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast) and the DNAStar package (DNAStar Inc. Madison, WI, USA). The identity of tRNA genes was verified using the tRNAscan-SE program (http://lowelab.ucsc.edu/tRNAscan-SE/)\(^{12}\). The nucleotide sequences of PCGs were translated with the invertebrate mitogenome genetic code. Alignments of *M. flavescens* PCGs with various Lepidopteran mitogenomes were performed using Clustal X\(^{13}\). Composition skewness was calculated according to the following formulas: AT skew = \([A−T]/[A+T]\); GC skew = \([G−C]/[G+C]\). Codon usage was calculated using MEGA version 6.06. Tandem repeats in the A+T-rich region were predicted using the Tandem Repeats Finder program (http://tandem.bu.edu/trf/trf.html)\(^{14}\).

## Phylogenetic Analysis

To reconstruct the phylogenetic relationships among Lepidopteran insects, the complete mitogenomes of Lepidopteran species were obtained from GenBank (Table 1). The amino acid
sequences for each of the 13 mitochondrial PCGs were aligned using default settings and concatenated. This concatenated set of amino acid and nucleotide sequences was used for phylogenetic analysis, which was performed with the Bayesian inference (BI) and Maximum Likelihood (ML) methods using MrBayes v 3.2.2 and raxml-GUI, respectively. Alignments of individual genes were performed using MAFFT. Gblocks was used to identify conserved regions and remove unreliably aligned sequences within the datasets. For the BI and ML analyses, GTR + I + G was the appropriate model for the nucleotide sequences using MrModeltest 2.3 based on Akaike's information criterion (AIC).
information criterion (AIC)\textsuperscript{18}. MtArt + I + G + F was the appropriate model for the amino acid sequence dataset according to ProtTest 3.4 based on AIC\textsuperscript{19}. Four independent runs were conducted for 10,000,000 generations, and each was sampled every 1,000 generations. All analyses converged within 10,000,000 generations. We assessed the credibility of the results in two ways. First, the average standard deviation of split frequencies was less than 0.05 in the process of Bayesian. Second, we observed sufficient parameter sampling using software Tracer v1.6. The value of ESS was more than 200. This cumulatively suggested that our data was convergent. Posterior probabilities over 0.95 were interpreted as strongly supported. The mitogenomes of Hepialoidea insects were used as outgroups. The resulting phylogenetic trees were visualized in FigTree v1.4.2.

Results and Discussion

Genome Organization and Base Composition. The mitogenome of \textit{M. flavescens} is a closed circular molecule 15,396 bp in size. The gene content is typical of other Lepidopteran insect mitogenomes, including 22 tRNA genes (one for each amino acid and two each for leucine and serine), 13 PCGs (\textit{cox1-3}, \textit{nad1-6}, \textit{nad4L},

Figure 3. Predicted secondary structures for the tRNA genes in the \textit{M. flavescens} mitogenome.
cob, atp6, and atp8), two mitochondrial rRNA genes (rrnS and rrnL), and a major non-coding region known as the CR. The majority strand (J strand) encodes 23 genes, while the opposite (N) strand encodes 14 genes (Fig. 1, Table 2). The arrangement of the genes within Lepidopteran mitogenomes is usually highly conserved. While the order and orientation of genes in the M. flavescens mitogenome are identical to the only other Zygaenoidea insect sequenced to date, this gene order differs from ancestral insects. Specifically, the placement of the trnM gene between the CR and trnI in the M. flavescens mitogenome (CR, trnM, trnI, trnQ, nad2) differs from ancestral insects in which trnM is located between trnQ and nad2 (CR, trnI, trnQ, trnM, nad2)20. However, the ancestral arrangement of the trnM gene cluster was also found in ghost moths21. This result in M. flavescens supports the hypothesis that the ancestral arrangement of the trnM gene cluster underwent rearrangement after Hepialoidea diverged from other Lepidopteran lineages. The tRNA gene rearrangements are commonly considered to be a consequence of tandem duplication in a portion of the mitogenome, followed by random or non-random loss of the duplicated copies22.

Skewness, Overlapping, and Intergenic Spacer Regions. The mitogenome of M. flavescens has a 29 bp overlap between genes in six locations, with the longest 9 bp overlap located in between trnW and trnC. The mitogenome of M. flavescens contains 167 bp of intergenic spacer sequence spread over 17 regions, ranging in size from 1 to 50 bp (Table 2). The longest spacer sequence is 50 bp located between the trnQ and nad2 genes, and it is extremely A + T rich. The nucleotide composition of the M. flavescens mitogenome is as follows: A = 6,275 (40.8%), T = 6,115 (39.7%), G = 1,164 (7.5%), and C = 1,842 (12.0%). As observed in other Lepidopterans, the nucleotide composition of the M. flavescens mitogenome is A + T rich (80.5%). This enrichment is lower than in other species, such as D. punctiferalis (80.6%), M. vitrata (80.7%), M. testulalis (80.8%), L. haraldusalis (81.5%), and T. hypsalis and N. noctuella (both 81.4%). In contrast, this enrichment is slightly higher compared to S. incertulas (77.1%), C. suppressalis (79.7%), and D. saccharalis (80.0%). Additionally, the AT skew for the M. flavescens mitogenome is slightly positive, indicating a higher occurrence of A compared to T nucleotides. The GC skew values

Figure 4. Features present in the A + T-rich region of the M. flavescens mitogenome. The reverse strand sequence is shown. Coloured nucleotides indicate the ATATG motif (red), the poly-T stretch (blue), two microsatellite T/A repeat sequences (green), and the poly-A stretch (pink). Two tandem repeats 51 bp in length are indicated in red and black single underline.
for the *M. flavescens* mitogenome are negative, indicating a higher content of C compared to G nucleotides. This is similar to GC skew values observed in all sequenced Lepidopteran mitogenomes to date.

**Protein-Coding Genes.** The start and stop codons of the 13 PCGs in the *M. flavescens* mitogenome are shown in Table 2. Like invertebrate mitogenomes, 12 of these PCGs begin with the standard ATN start codon, except for *cox1*. Sequence alignment revealed that the open reading frame of *cox1* starts with a CGA codon, which encodes arginine. The putative start codon CGA is common in insects\(^{10,23,24}\). An unusual start codon for the *cox1* gene has also been described in various arthropods\(^{25–27}\). In the *M. flavescens* mitogenome, the canonical termination codon, TAA, occurs in seven PCGs. However, the *nad4L* gene utilizes A and the *cox1, cox2, nad2, nad4*, and *cob* genes utilize T as a truncated stop codon instead. Similar results have also been found in other animal mitochondrial genes\(^{28–31}\). Relative synonymous codon usage values for the *M. flavescens* mitogenome are summarized in Table 3 and Fig. 2. The total number of codons in PCGs is 3,716, and the codons CUC, GUC, CCG, UGG, CGG, and AGG are not represented. The most common amino acids in mitochondrial proteins are leucine 2 (Leu 2, 484), isoleucine (Ile, 455), and phenylalanine (Phe, 393), which are likewise highly abundant in mitochondrial proteins in other animals\(^{32–34}\). The average AT content of the 13 PCGs is 78.7%. Furthermore, the AT skew of these PCGs is slightly positive, while the GC skew is slightly negative (Table 4).

**Transfer RNA Genes and Ribosomal RNA Genes.** The tRNAscan-SE Search Server was used to predict the structure of the 22 tRNAs present in the *M. flavescens* mitogenome. Eight tRNAs are encoded by the L-strand and the remaining 14 are encoded by the H-strand. This tRNA genomic architecture is identical to that found in all Lepidopteran species examined to date. Furthermore, all *M. flavescens* tRNAs display the typical clover-leaf secondary structure observed in most mitochondrial tRNAs with the exception of the *trnS1 (AGN)* gene. Interestingly, *trnS1 (AGN)* lacking a stable dihydrouridine arm has been observed in several insects, including Lepidopteran species and metazoan mitogenomes\(^{35–38}\). A 7 bp amino acid acceptor stem, in addition to the anticodon stem and loop (7 bp), are both conserved in all tRNAs. While a total of 25 unmatched base pairs were detected in these tRNAs (Fig. 3), 18 of them are G-U pairs, which form a weak bond and are well-known non-canonical pairs in tRNA secondary structures. The remaining seven mismatches include one C-U and six U-U pairs. 22 tRNAs in the *M. flavescens* mitogenome are 1,513 bp long, each of which range in size from 63 to 73 bp. The A + T content is 82.4%. The AT skew for both tRNAs and rRNAs is slightly positive, indicating a higher occurrence of A compared to T nucleotides. The GC skew for both tRNAs and rRNAs is slightly negative, indicating a higher occurrence of C compared to G nucleotides. The two rRNA genes (*rrnS* and *rrnL*) present in *M. flavescens* mitogenome are located between *trnL1 (CUN)* and *trnV* or between *trnV* and the A + T-rich region, respectively. The sizes of *rrnL* and *rrnS* are 1,359 bp and 792 bp, respectively. The A + T content of the two rRNAs is 84.5% (Table 4).
Control Region. The CR possesses essential elements involved in the initiation of replication and transcription of the mitogenome. The CR of the *M. flavescens* mitogenome extends over 401 bp and is located between *rrnS* and *trnM*. The CR contains the highest A + T content (93.3%) in the entire mitogenome. Both the AT skew and GC skew for the CR are slightly negative, indicating that T and C are more abundant than A and G, respectively. Several conserved structures found in other Lepidopteran mitogenomes are also observed in the A + T-rich region of *M. flavescens*. This includes the motif ‘ATAGA’ and a poly-T stretch downstream of *rrnS*, which is widely conserved in Lepidopteran mitogenomes and may represent the origin of minority or light strand replication. A poly-A commonly observed in other Lepidopteran mitogenomes is also found immediately upstream of the *trnM* gene. We identified microsatellite (AT)$_{10}$ elements in the A + T-rich region. Multiple tandem repeat elements are typically present in the A + T-rich region of most insects. However, only three tandem repeats are found in the CR of the *M. flavescens* mitogenome (Fig. 4).

Phylogenetic Analyses. Phylogenetic relationships within the Zygaenoidea superfamily are highly debated. In the present study, concatenated amino acid and nucleotide sequences of the 13 PCGs from mitogenomes obtained from nine Lepidopteran superfamilies are used to reconstruct phylogenetic relationships by the BI and ML methods (Figs 5 and 6). The monophyly of each superfamily is generally well supported. The best-supported phylogenetic relationship found in this study is as follows: Yponomeutoidea + (Tortricoidea + Zygaenoidea + (Papilionoidea + (Pyraloidea + (Noctuoidea + (Geometroidea + Bombycoidea))))). The analyses show that *M. flavescens* belongs in the Zygaenoidea superfamily. Both Papilionoidea and Tortricoidea superfamilies are most closely related to Zygaenoidea. More mitogenomes from Zygaenoidea insects were required to resolve the position of Zygaenoidea and the relationships among these superfamilies. Our phylogeny clearly separates and demonstrates a similar topology as that derived from traditional classifications and other molecular data.

References
1. Cameron, S. L. Insect mitochondrial genomics: implications for evolution and phylogeny. *Annu. Rev. Entomol.* 59, 95–117 (2014).
2. Wolstenholme, D. R. Animal mitochondrial DNA: structure and evolution. *Int. Rev. Cytol.* 141, 173–216 (1992).
3. Boore, J. L. Animal mitochondrial genomes. *Nucleic Acids Res.* 27, 1767–1780 (1999).
4. Lavrov, D. V. et al. Mitochondrial DNA of Clathrina clathrus (Calcarea, Calcinea): six linear chromosomes, fragmented rRNAs, tRNA editing, and a novel genetic code. *Mol. Biol. Evol.* 30, 865–880 (2013).
5. Kristensen, N. P., Scoble, M. J. & Karsholt, O. Lepidoptera phylogeny and systematics: the state of inventorying moth and butterfly diversity. *Zootaxa* 1668, 699–747 (2007).
6. Epstein, M. E., Geertsema, H., Naumann, C. M. & Tarmann, G. M. The Zygaenoidea in *Handbook of Zoology* (ed. Kristensen, N. P.) 59–180 (De Gruyter, 1999).
7. Naumann, C. M., Tarmann, G. M. & Tremewan, W. G. The Western Palaearctic Zygaenidae. *Apollo Books, Sterndrup* (1999).
8. Tang, M. et al. Multiplex sequencing of pooled mitochondrial genomes—a crucial step toward biodiversity analysis using mito
targetingomics. *Nucleic Acids Res.* 42, e166 (2014).
9. Pan, Z., Zhu, C. & Wu, C. A review of the genus Monema Walker in China (Lepidoptera, Limacodidae). *ZooKeys* 306, 23–36 (2013).
10. Liu, Q. N., Zhu, B. J., Dai, L. S., Wei, G. Q. & Liu, C. L. The complete mitochondrial genome of the wild silkworm moth, *Actias selene*. *Gene* 505, 291–299 (2012).
11. Liu, Q. N. et al. Characterization of the complete mitochondrial genome of the Oriental armyworm, *Mythimna separata* (Lepidoptera: Noctuidae). *Eur. J. Entomol.* 112, 399–408 (2015).
12. Lowe, T. M. & Eddy, S. R. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res.* 25, 955–964 (1997).
13. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876–4882 (1997).
14. Benson, G. Tandem repeats finder: A program to analyze DNA sequences. *Nucleic Acids Res.* 27, 573–580 (1999).
15. Ronquist, F. et al. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61, 539–542 (2012).
16. Katoh, K. et al. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30, 3859–3866 (2002).
17. Castresana, J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17, 540–552 (2000).
18. Nylander, J. MrModeltest v2. Program distributed by the author, vol. 2. Evolutionary Biology Centre, Uppsala University (2004).
19. Abascal, F., Zardoya, R. & Posada, D. ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* 21, 2104–2105 (2005).
20. Boone, J. L., Lavrov, D. V. & Brown, W. Gene translocation links insects and crustaceans. *Nature* 392, 667–668 (1998).
21. Cao, Y. Q., Ma, C., Chen, J. Y. & Yang, D. R. The complete mitochondrial genomes of two ghost moths, *Thitarodes renzhiensis* and *Thitarodes yunnanensis*: the ancestral gene arrangement in Lepidoptera. *BMC Genomics* 13, 276 (2012).
22. Juhling, F. et al. Improved systematic tRNA gene annotation allows new insights into the evolution of mitochondrial tRNA structures and into the mechanisms of mitochondrial genome rearrangements. *Nucleic Acids Res.* 40, 2833–2845 (2012).
23. Negrisolo, E., Minelli, A. & Valle, G. Extensive gene order rearrangement in the mitochondrial genome of the centipede *Scutigera coleoptrata*. *J. Mol. Evol.* 58, 413–423 (2004).
24. Zhu, B. J. et al. Characterization of the complete mitochondrial genome of *Diaphania pylooids* (Lepidoptera: Pyralidae). *Gene* 527, 283–291 (2013).
25. Dai, L. S. et al. Characterization of the complete mitochondrial genome of *Cerura menciana* and comparison with other Lepidopteran insects. *PLoS One* 10, e0132951 (2015).
26. Wang, Y., Liu, X. & Yang, D. The first mitochondrial genome for caddisfly (insecta: Trichoptera) with phylogenetic implications. *Int. J. Biol. Sci.* 10, 53–63 (2013).
27. Liao, F. et al. The complete mitochondrial genome of the fall webworm, *Hyphantria cunea* (lepidoptera: Arctiidae). *Int. J. Biol. Sci.* 6, 172–186 (2010).
28. Liu, Q. N., Zhu, B. J., Dai, L. S. & Liu, C. L. The complete mitogenome of *Bombyx mori* strain Dazao (Lepidoptera: Bombycidae) and comparison with other lepidopteran insects. *Genomics* 101, 64–73 (2013).
29. Cameron, S. L. & Whiting, M. F. The complete mitochondrial genome of the tobacco hornworm, *Manduca sexta* (insecta: lepidoptera: Sphingidae), and an examination of mitochondrial gene variability within butterflies and moths. *Gene* 408, 112–113 (2008).
30. Pan, M. H. et al. Characterization of mitochondrial genome of Chinese wild mulberry silkworm, *Bomys mandarinia* (lepidoptera: Bombycidae). *Sci. China C. Life Sci.* 51, 693–701 (2008).
31. Dai, L. S., Zhu, B. J., Zhao, Y., Zhang, C. F. & Liu, C. L. Comparative mitochondrial genome Analysis of *Euglena narcissus* and other Lepidopteran insects reveals conserved mitochondrial genome organization and phylogenetic relationships. *Sci. Rep.* 6, 26387 (2016).
32. Song, S. N., Tang, P., Wei, S. J. & Chen, X. X. Comparative and phylogenetic analysis of the mitochondrial genomes in basal hymenopterans. *Sci. Rep.* 6, 20972 (2016).
33. Liu, Q. N. et al. The complete mitochondrial genome of *Eriocher japonica sinensis* (Decapoda: Varunidae) and its phylogenetic analysis. *Biochem. Syst. Ecol.* 62, 241–248 (2015).
34. Yong, H. S. et al. Complete mitochondrial genome of *Bactrocera areacea* (Insecta: Tephritidae) by next-generation sequencing and molecular phylogeny of Dacini tribe. *Sci. Rep.* 5, 15155 (2015).
35. Liu, Q. N., Chai, X. Y., Bian, D. D., Zhou, C. L. & Tang, B. P. The complete mitochondrial genome of *Plodia interpunctella* (Lepidoptera: Pyralidae) and comparison with other Pyraloidea insects. *Genome* 59, 37–49 (2016).
36. Ma, H. et al. First mitochondrial genome for the red crab (*Charybdis feriata*) with implication of phylogenomics and population genetics. *Sci. Rep.* 5, 11524 (2015).
37. Liu, Q. N., Chai, X. Y., Ge, B. M., Zhou, C. L. & Tang, B. P. The complete mitochondrial genome of fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Genes Genom.* 38, 205–216 (2016).
38. Li, H. H. et al. Higher-level phylogeny of paraneopteran insects inferred from mitochondrial genome sequences. *Sci. Rep.* 5, 8527 (2015).
39. Zhang, D. X. & Hewitt, G. M. Insect mitochondrial control region: A review of its structure, evolution and usefulness in evolutionary studies. *Biochem. Syst. Ecol.* 25, 99–120 (1997).
40. Taaman, J. W. The mitochondrial genome: structure, transcription, translation and replication. *Biochim. Biophys. Acta.* 1410, 103–123 (1999).
41. Kristensen, N. P. & Skalski, A. W. Phylogeny and palaeontology. In: Kristensen, N. P. (ed.), Lepidoptera: Moths and Butterflies, 1 Evolution, Systematics, and Biogeography, *Handbook of Zoology* Vol IV, Part 35. Berlin and New York, De Gruyter, 7–25 (1999).
42. Timmermans, M. J., Lees, D. C. & Simonsen, T. J. Towards a mitogenomic phylogeny of Lepidoptera. *Mol. Phylogen. Evol.* 79, 169–178 (2014).

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**Author Contributions**

Q.-N.L. and B.-P.T. conceived and designed the experiments. Q.-N.L., Z.-Z.X., D.-D.B. and X.-Y.C., performed the experiments. Q.-N.L. and Z.-Z.X. analyzed the data. C.-L.Z. and B.-P.T. contributed reagents and materials. Q.-N.L. and Z.-Z.X. wrote the paper. Z.-Z.X., Q.-N.L. and B.-P.T. revised the paper.

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