The genetic variations in the mitochondrial genomes of three Luciolineae fireflies

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Introduction

Lampyridae consists of nine subfamilies, 100 genera, and more than 2000 species. The taxonomy of Lampyridae has historically been based on morphological classification. As DNA barcode technology has matured, it has been used to identify insects and has shown good insect species identification capability in recent years in groups such as Coleoptera (Suzuki et al. 2002), thrips (Glover et al. 2010), and Trichoptera (Hogg et al. 2009). The mitochondrial genome and 18S rDNA are the most conserved genes. They are not only very efficient for classification but are also essential for understanding the evolution of firefly insects. Therefore, many scientists have studied the mitochondrial genome and the nuclear rDNA of fireflies. In recent years, 44 firefly mitochondrial genomes have been sequenced. Twenty six of these are complete mitochondrial genomes, and the rest are partial mitochondrial genomes (Wang et al. 2017; Chen et al. 2019; Wang and Fu 2019; Zhang and Fu 2019). In addition, 89 rDNA coding regions (18S regions) of fireflies have been sequenced thus far (Chen et al. 2019).

Emeia pseudosauteri is a special species of firefly that is only found in China. Fu et al. described Emeia gen. nov. for a single species pseudosauteri transferred from Curtos (Fu et al. 2012). P. qingyu was first found by Fu and Ballantyne. They described the morphology of its males, females (bursa structure) and larvae. In addition, P. qingyu is the first recorded synchronously flashing firefly from mainland China (Fu and Ballantyne 2008). Abscondita terminalis was found by Olivier in 1883. It was divided into Abscondita, a new genus of fireflies from Southeast Asia (Ballantyne et al. 2013). The mitochondrial genomes and the 18S rDNA of these three kinds of firefly have been studied (Chen et al. 2019).

We collected 5 firefly samples (P. qingyu: one, Abs. terminalis: one and E. pseudosauteri: three) from Sichuan and Hubei Province. Here, we assembled mitogenomes from P. qingyu, Abs. terminalis and E. pseudosauteri. And we assembled 18srDNA from the three E. pseudosauteri samples. Combining the three firefly species with those previously deposited in GenBank and then comparing different populations among the same species, we discussed the differences of the mitochondrial genome in different populations of each species.

Materials and methods

Firefly specimens

DNA extraction and sequencing

Whole-body genomic DNA was extracted from each individual using the Aidlab Genomic DNA Extraction Kit (Aidlab Co., Beijing, China) following the manufacturer’s protocols (Table 1).

Sequence processing and annotation

Primers were designed according to the mt genomic sequences of closely related species (and the 18S Primers were designed according to the genomic sequences of closely related species), and the long PCR (LA-PCR) amplification was performed using the LA Taq polymerase. The PCR conditions were as follows: initial denaturation 94°C for 2 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min/kb, followed by the final extension at 72°C for 10 min. The total volume for PCR
and LA-PCR was 50 μl, of which Takara LA Taq (5 U/μl) was 0.5 μl, 10 × LA Taq Buffer II (Mg2+) was 5 μl, dNTP mixture (2.5 mM) was 8 μl, template was 60 ng, and the total volume was then made up with distilled water. The final concentration of the forward and reverse primers was 0.2 μM, and that of MgCl2 was 2.0 mM. The PCR products were sequenced directly, or if needed first cloned into a pMD18-T vector (Takara, JAP) and then sequenced, by the dideoxynucleotide procedure, using an ABI 3730 automatic sequencer (Sanger sequencing) using the same set of primers.

After quality-proofing of the obtained fragments, the complete mt genome sequence was assembled manually using DNAstar v7.1 software (Burland 2000). Mt genome was annotated roughly following the procedure described before (Zou et al., 2017; Zhang et al., 2018b). First, raw mitogenomic sequences were imported into MITOS web servers to determine the approximate boundaries of genes. Exact positions of protein-coding genes (PCGs) were found by searching for ORFs (employing genetic code 5, the invertebrate mitochondrion). All tRNAs were identified using ARWEN, DOGMA and MITOS.

**Morphological identification**

We identified the morphological characteristics and dissected genital organs of male fireflies of each species by using a microscope. Then, the features were compared with previously published classification articles about the three kinds of fireflies.

**Phylogenetic analyses**

To reconstruct the phylogenetic relationship among the species, the COI sequences of 22 firefly species, one Cantharidae and one Tenebrionidae were obtained from the GenBank database (Table 2). These sequences were divided into 5 subfamilies. The sequences of Tribolium castaneum (F20, NC003081) and Chaullognathus opacus (F21, FJ613418) were used as outgroups. The COI sequences from the 24 species were used in phylogenetic analysis, which was performed using maximum likelihood (ML) method with the MEGA version 7.0 program.

**Results**

**Species identification**

By analyzing these morphology features, we confirmed that samples F16-A, F16-B, F16-C were E. pseudosauteri, F10 was P. qingyu, and F12 was Abs. terminalis. To further confirm the identification of the three F16 samples, we analyzed the 18S rDNA sequences of the three samples by Clustal W, and the result showed that they are E. pseudosauteri.

**Basic description of the sequences**

The complete mitochondrial genome sequences of the three firefly species are similar to each other. As Metazoa, each of the sequences contains 13 protein-coding genes, 22 transfer RNA genes, 2 ribosomal RNA genes, and a non-coding AT-rich region, which together represent a typical insect mitochondrial genome (Wolstenholme 1992). The open frames of the 13 protein-coding genes were inferred from three other fireflies: Aquatica leii, Luciola substriata, and Pyrocoelia rufa (Lee et al. 2003; Jiao et al. 2015; Mu et al. 2015). Detailed
information on the complete mitochondrial genome of the three kinds of firefly is provided in Table 3.

### Table 3. Basic description of the complete mitochondrial genomes of the three species.

| Species                        | GenBank accession No. | Base length | Base composition | Initiation codon | Terminal codon |
|--------------------------------|-----------------------|-------------|------------------|------------------|----------------|
| Pygoluciola qingyu (F10)       | MN688374              | 17,332 bp   | A 45.59%         | ATT COI          | Single T       |
| Abscondita terminalis (F12)    | MN722653              | 16,398 bp   | C 11.66%         | ATA ND6          | COII, ND5, ND3, ND4L |
| Emeia pseudosauteri (F16)      | MN722654              | 16,956 bp   | G 7.96%          | ATG ND6, ND2, ND6 | ND6, ATP8, ATP6, ND4L |

**Results of phylogenetic analysis based on COI sequences**

Based on Figure 1, we can draw the following conclusions: first, the COI sequences of F11 and F12, F9 and F10 are exactly the same. Then, the sequences of F16-A, F16-B are the same. The COI sequence of F22 and F16-C is slightly far from those of F16-A and F16-B. Finally, the genetic distance between the COI sequence of F15 and the other four COI sequences of F16 and F22 is very far.

**BLAST results**

According to the BLAST results for the mitochondrial genome sequences, we can draw some conclusions as follows: first, the sequences of F11 and F12 are almost homologous, and their percent identity is 99.52%. Second, the sequences of F9 and F10 are very similar, and their percent identity is 98.43%. The differences are that the first 330 bases of F9 do not match those of F10, and the end of the F9 sequence lacks 919 bases.

**The results of multiple sequences alignment**

According to the multiple sequences alignment, the 18sDNA sequence of F22 is the same with F16, but the 18sDNA sequence of F15 has big difference with the F16. The MtDNA sequence of F16-A and F22 are almost the same, but they have big difference with the F15 (Tables 4 and 5).

**Discussion**

Abscondita terminalis is widely distributed in China, and it can survive in diverse habitat environments. To adapt to these diverse environments, the different geographical populations of Abs. terminalis may show some variations. We isolated two geographical populations that were about a thousand kilometers apart to compare the variations in their mitochondrial genomes. The results revealed that the genetic
distance between the two populations is very small. That means that the mitochondrial genome does not vary among different geographical populations and that the heredity of this species is very stable.

*Pygoluciola qingyu* is also widely distributed in China. In addition, the two samples (F9 and F10) were gathered from more than a kilometer apart. The variation analysis of the two populations suggests that the mitochondrial genome sequence of *P. qingyu* is very stable.

The F9 sequence is a partial mitochondrial genome sequence that was downloaded from Genbank, while F10 is a complete mitochondrial genome sequence that we had sequenced. Although the two sequences are very similar, they are still show important differences. The F9 lost the control region of a noncoding region that contains a repetitive sequence. Scientists have shown that any sequence of the mitochondrial genome is very important, especially the control region in noncoding sequences and the coding regions. In addition, any missing sequence will cause coding confusion and functional changes.

From the analyses of the mitochondrial genome sequences and 18S rDNA sequences of the three firefly samples (F15, F16-A, and F22) as well as the results for the two species of fireflies (*Emeia pseudosauteri* and *Vesta saturnalis*) in the paper published by Xing Chen et al. (Chen et al. 2019), we conclude that the mitochondrial genome sequences and 18S rDNA sequences of the two fireflies should be interchanged.

**Table 4.** Estimates of evolutionary divergence between 18sDNA sequences.

| Species | 1 | 2 | 3 | 4 | 5 |
|---------|---|---|---|---|---|
| *Emeia pseudosauteri* in this study F16-A | | | | | |
| *Emeia pseudosauteri* in this study F16-B | 0.000 | | | | |
| *Emeia pseudosauteri* in this study F16-C | 0.000 | 0.000 | | | |
| *Vesta saturnalis* MK292083 F22 | 0.000 | 0.000 | 0.000 | | |
| *Emeia pseudosauteri* MK292085 F15 | 0.582 | 0.582 | 0.582 | 0.582 | |

**Table 5.** Estimates of evolutionary divergence between MitDNA sequences.

| Species | 1 | 2 | 3 |
|---------|---|---|---|
| *Emeia pseudosauteri* in this study F16-A | | | |
| *Vesta saturnalis* MK292083 F22 | 0.022 | | |
| *Emeia pseudosauteri* MK292085 F15 | 0.234 | 0.234 | |
Eumeia pseudosauteri is only distributed in Sichuan Province and Hubei Province, where there are many mountains, causing habitat isolation. The results above show that the COI sequence of F16-C is very different from the COI sequences of the other two populations, F16-A and F16-B. This means that the mitochondrial genome sequence of E. pseudosauteri shows great variation among different geographical populations.

The above discussion suggests that classification based on the mitochondrial genome is not very accurate and that human factors causing upload errors in Genbank may lead to incorrect identification. Scientists should combine morphological classification and DNA barcoding to classify firefly species in order to make the classification more accurate.

The above discussions reveal that the COI sequences in different populations have great variation. It means that the mtDNA may have variation among different geographical populations. In this paper we only sequenced the differences of COI sequences of E. pseudosauteri. We did not discuss the differences of complete MtDNA sequences of E. pseudosauteri in details, especially the differences of D-loop and AT-reach region of MtDNA sequences of E. pseudosauteri in different geographical populations.

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References

Amano H, Matsui M, Igarashi M, Tanaka M, Safrizal E. 2013. Complete mitochondrial genome sequence of Luciola cruciata. Res Rep Fukushima Natl Technol. 54:149–152.
Bae JS, Kim I, Sohn HD, JIN BR. 2004. The mitochondrial genome of the firefly, Pyrocoelia rufa: complete DNA sequence, genome organization, and phylogenetic analysis with other insects. Mol Phylgenet Evol. 32(3):978–985.
Ballantyne L, Fu X, Lambkin CL, Faust LYNN, Wijekoon WMCD, Li D, Zhu T. 2013. Studies on South-east Asian fireflies: Abscondita, a new genus with details of life history, flashing patterns and behaviour of Abs. chinensis (L.) and Abs. terminalis (Olivier) (Coleoptera: Lampyridae: Luciolinae). Zootaxa. 3721(1):1–048.
Burland TG. 2000. DNASTAR’s Lasergene sequence analysis software. Bioinformatics methods and protocols. Totowa: Humana Press; p. 71–91.
Chen X, Dong Z, Liu G, He J, Zhao R, Wang W, Peng Y, Li X. 2019. Phylogenetic analysis provides insights into the evolution of Asian fireflies and adult bioluminescence. Mol Phylgenet Evol. 140:106600.
Fan Y, Fu X. 2017. The complete mitochondrial genome of the firefly, Pteropteryx maipo (Coleoptera: Lampyridae). Mitochondr DNA. 2(2):795–796.
Friedrich M, Muqim N. 2003. Sequence and phylogenetic analysis of the complete mitochondrial genome of the flour beetle Tribolium castaneum. Mol Phylgenet Evol. 26(3):502–512.
Fu XH, Ballantyne LA. 2008. Taxonomy and behaviour of Lucioline fireflies (Coleoptera: Lampyridae: Luciolinae) with redefinition and new species of Pygoluciola Wittmer from mainland China and review of Luciola LaPorte. Zootaxa. 1733(1):1–44.
Fu XH, Ballantyne LA, Lambkin CL. 2012. Eumeia gen. nov., a new genus of Luciolinae fireflies from China (Coleoptera: Lampyridae) with an unusual trilobite-like larva, and a redescription of the genus Curtos Motsch. Zootaxa. 3403(1):1–53.
Glover RH, Collins DW, Walsh K, Boonham N. 2010. Assessment of loci for DNA barcoding in the genus Thrips (Thysanoptera:Thripidae). Mol Ecol Resour. 10(1):51–59.
Hogg ID, Smith BJ, Banks JC, Dewaard JR, Hebert PDN. 2009. Testing use of mitochondrial COI sequences for the identification and phylogenetic analysis of New Zealand caddisflies (Trichoptera). N Z J Mar Freshwater Res. 43(5):1137–1146.
Hu J, Fu XH. 2018. The complete mitochondrial genome of the firefly, Abscondita anceyi (Olivier) (Coleoptera: Lampyridae). Mitochondr DNA Part B. 3(1):442–443.
Jiao H, Ding M, Zhao H. 2015. Sequence and organization of complete mitochondrial genome of the firefly, Aquatica leii (Coleoptera: Lampyridae). Mitochondr DNA. 26(5):775–776.
Lee S-C, Bae J-S, Kim I, Suzuki H, Kim S-R, Kim J-G, Kim K-Y, Yang W-J, Lee S-M, Sohn H-D, et al. 2003. Mitochondrial DNA sequence-based population genetic structure of the firefly, Pyrocoelia rufa (Coleoptera: Lampyridae). Biochem Genet. 41(11/12):427–452.
Luan X, Fu X. 2016. The complete mitochondrial genome of the firefly, Asymmetricatina circundata (Motschulskey) (Coleoptera: Lampyridae). Mitochondr DNA. 1(1):533–555.
Maeda J, Kato DI, Arima K, Ito Y, Toyoda A, Noguchi H. 2017. The complete mitochondrial genome sequence and phylogenetic analysis of Luciola lateralis, one of the most famous firefly in Japan (Coleoptera: Lampyridae). Mitochondr DNA. 2(2):546–547.
Mu FJ, Ao L, Zhao HB, Wang K. 2015. Characterization of the complete mitochondrial genome of the firefly, Luciola substriata (Coleoptera: Lampyridae). Mitochondr DNA. 27(5):1–3.
Sheffield NC, Song H, Cameron SL, Whiting MF. 2009. Nonstationary evolution and compositional heterogeneity in beetle mitochondrial phylogenomics. Syst Biol. 58(4):381–394.
Suzuki H, Sato Y, Ohba N. 2002. Gene diversity and geographic differentiation in mitochondrial DNA of the Genji firefly, Luciola cruciata (Coleoptera: Lampyridae). Mitochondr DNA. 3(3):71–79.
Wang J, Fu X. 2019. The complete mitochondrial genome of the firefly, Abscondita chinensis (Coleoptera: Lampyridae). Mitochondr DNA Part B. 4(1):1599–1600.
Wang K, Hong W, Jiao H, Zhao H. 2017. Transcriptome sequencing and phylogenetic analysis of four species of luminescent beetles. Sci Rep. 7(1):1814.
Wolstenholme DR. 1992. Animal mitochondrial DNA: structure and evolution. Int Rev Cytol. 141:173–216.
Yang Z, Fu XH. 2019. The complete mitochondrial genome of the firefly, Diaphanes citrinus (Olivier), (Coleoptera: Lampyridae). Mitochondr DNA Part B. 4(2):2986–2987.
Zhang D, Li W X, Zou H, Wu S G, Li M, Jaković I, Zhang J, Chen R, Wang G T. 2018b. Mitochondrial genomes of two dipelcandids (Platyhelminthes: Monogononta) expose paraphyly of the order Dactylogyridae and extensive rRNA gene rearrangements. Parasit Vectors. 11(1):601
Zhang J, Fu X. 2019. The complete mitochondrial genome of the firefly, Curtos costipennis, (Coleoptera: Lampyridae). Mitochondr DNA Part B. 4(1):1578–1579.
Zhou H, Jaković I, Chen R, Zhang D, Zhang J, Li W-X, Wang G-T. 2017. The complete mitochondrial genome of parasitic nematode Camallanus cotti: extreme discontinuity in the rate of mitogenomic architecture evolution within the Chromadorea class. BMC Genomics. 18(1):840.