Identification of the exuviae of larva from *Teinopalpus aureus* Mell, 1923 using the complete mitochondrial genome [version 1; peer review: 2 approved with reservations]

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**Abstract**

*Teinopalpus aureus* Mell (Lepidoptera: Papilionidae) is distributed throughout China, Vietnam and Laos, and is listed as a Class I species in China. To identify whether the exuviae of larva is belonging to *Teinopalpus aureus* Mell, 1923 or not, and to compare the gene structure and genetic differences among the known populations, ten mitogenomes of *T. aureus* from the exuviae of larva collected in the *Michelia maudiae* were sequenced. This method of sequencing the mitogenomes of exuviae of larva can give us the chance to monitor the conservation of rare butterflies.

Ten mitogenomes of *T. aureus* showed typical gene arrangements and contained 13 protein-coding genes (PCGs), two ribosomal RNA genes (12S rRNA and 16S rRNA), 22 transfer RNA (tRNA) genes, and a non-coding control region (D-loop). The two haplotypes with one base different in *T. aureus* were found. We also conducted phylogenetic analyses including all different populations of *T. aureus* to assess the phylogenetic relationship of *T. aureus*. The lengths of the 12S rRNA and 16S rRNA genes from both haplotypes were 776 base pairs (bp) and 1,334 bp, respectively. The genetic distance of the ten samples was calculated as 0-0.000065 on the basis of the whole mitogenomes. *T. aureus* found in Taishun, Zhejiang province, China had a close phylogenetic relationship with the clade of *T. aureus* found in Pingshan, Jiangxi province, China, which was supported by neighbour-joining analysis.

**Keywords**

Golden Kaiserihind, Mitogenome, Phylogeny
Introduction

Because of its beautiful appearance and relative rarity in China, *Teinopalpus aureus* Mell, 1923 (Lepidoptera: Papilionidae) distributed in China, Vietnam and Laos is listed as a Class I species in China (Monta 1998; Masui 1999; Xing et al. 2019; Huang et al. 2015), which is one of three highest classifications for protection of endangered insect species in China. If we can identify the larva of *T. aureus* and obtain the mitochondrial genome using the exuviae of larva, we can determine its habitat sites and compare the genetic diversity of *T. aureus* using non-damage sampling to protect it in the larva stage.

No ethical approval was obtained for this study as we used wild exuviate gathered from the Wuyanling National Nature Reserve.

Methods

Ten exuviae of larva of *T. aureus* (No. ZJWYL20200601TP01- ZJWYL20200601TP10) were collected from wild host plants, *Michelia maudiae*, in Zhejiang Wuyanling National Nature Reserve, Taishun, Zhejiang province, China and stored in 100% absolute ethyl alcohol in the Natural Museum of Wuyanling National Nature Reserve, China.

A total of ten genomic DNAs from exuviae of larva (No. ZJWYL20200601TP01- ZJWYL20200601TP10) were extracted from partial exuviae using QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) according to the manufacturer instructions, and stored at -20 °C refrigerator in Key Lab of Wildlife Biotechnology, Conservation and Utilization of Zhejiang Province.

The mitochondrial genome was obtained by the Sanger method. Ten mt genomes were amplified by polymerase chain reaction (PCR) by using the normal primers (Zhang et al. 2018; Guan et al. 2021; Xu et al. 2021; Yu et al. 2021) and six specific primers according to the reported mitochondrial genomes of *T. aureus* (Huang et al. 2015). This study used both normal polymerase chain reaction (PCR) and long-and-accurate PCR (LA PCR) methods with Takara Taq or Takara LA Taq DNA polymerase (Takara, Dalian, China). Normal PCR (or Long PCR) was performed in a 50 μL reaction mixture consisting of 32.5 μL (or 26.5 μL) of sterilized distilled water; 5 μL MgCl₂, 25 mM; 5 μL 10 × PCR Buffer (or 5 μL 10 × LA PCR Buffer); 4 μL (or 8 μL) dNTP, 2.5 mM; 1 μL (or 2 μL) of each primer, 5 μmol; 1 μL DNA template; 0.5 μL Takara Taq enzyme (or Takara LA Taq enzyme) DNA polymerase (Takara Biomedical, Japan). Fragments were amplified using Takara Taq enzyme via normal PCR or Takara LA Taq enzyme: initial denaturation for 3 min at 95 °C, followed by 35 cycles of 40 s at 95 °C, 1 min or 3 min at 58 °C, and 50–90 s at 72 °C, and a subsequent 10 min final extension step at 72 °C. All PCR reactions were used in Applied Biosystems Veriti instrument (Singapore). PCR products were purified using the Axygen agarose-out kit (Axygen, Hangzhou, China), and sequenced using ABI 3730 system by primer walking with two-directions.

To further discuss the phylogenetic relationship of *T. aureus* found in Zhejiang Wuyanling National Nature Reserve, a total of 13 mitogenomes were analyzed, including 11 mt genomes of *T. aureus* downloaded from NCBI (Qin et al. 2012; Huang et al. 2015; Zou et al. 2021) and two mt genomes of *T. imperialis* (Huang et al. 2016) as outgroups (Figure 1). To align the all complete mt genomes, we used Clustal W in Mega 7.0 (Kumar et al. 2016). We constructed a Neighbour-Joining phylogenetic tree with the parameters as below: bootstrap replications (1000), substitution model (Kumira 2-parameter model), Rates among sites (Gamma distributed), other parameters used default parameters.

Results

The two haplotypes with one base different in control region were found. The two obtained whole mt genome haplotypes were deposited in the National Center for Biotechnology Information with accession numbers OL449691 and OL449692. 

The two mt genome haplotypes of *T. aureus* in majority-strand was 15,243 base pairs (bp) in length with negative AT-skew and GC-skew, which were -0.0025 and -0.2376, respectively, which showed typical lepidopterous gene structure and contained 13 protein-coding genes (PCGs), two ribosomal RNA genes (12S rRNA and 16S rRNA), 22 transfer RNA (tRNA) genes, and one control region (D-loop). One base T replaced C in control region between two haplotypes was found. The start codons of PCGs were ATG (in COX2, COX3, ATP6, ATP8, ND1, ND4, ND4L, and Cytb), ATT (in ND2, ND3, ND5, and ND6), and CGA (in COX1). The stop codons of PCGs were TAA (in ND2, ATP6, ATP8, COX3, ND1, ND5, ND6, and Cytb), TAG (in ND4L), and the incomplete stop codon T- (in COX1, COX2, ND3, and ND4). The gene arrangement was identical to the reported mt genome of *T. aureus wuyiensis* from Lee (Zou et al. 2021). The overall nucleotide composition of A, T, C and G in majority-strand was 39.8%, 40.0%, 12.5%, 7.7%, respectively. The lengths of the 12S rRNA and 16S rRNA genes from two haplotypes were 776 bp and 1,334 bp, respectively. The genetic distance of between two haplotypes and other reported population of *T. aureus* based on Kimura 2-parameter model was calculated as 0.00007-0.01013 on the basis of the whole mitogenomes. According to genetic distance, we can identify that ten exuviae...
of larva is belonging to the exuviae from T. aureus. This method using the exuviae to identify species can help us to monitor and protect T. aureus.

In NJ tree, two haplotypes from Taishun, Zhejiang province and the sample from Wuyishan, Fujian province were formed one clade 1. Then this clade is closed to the clade 2 from Pingshan and Meihuashan. Clade 1 and clade 2 are sister clade to the clade 3 from Jiulianshan and Dayaoshan. The clade 4 is sister clade to other clade and formed the phylogenetic relationship of (Clade 4 + (Clade 3 + (Clade 1 + Clade 2).

In this study, the monophyly of T. aureus was well supported. The samples collected in Taishun, Zhejiang province can be identified as T. aureus wuyiensis because the genetic distance is below 0.00013 and the phylogenetic relationship of the samples collected in Taishun are clustered together with T. aureus wuyiensis. The new mitochondrial genomes obtained from the exuviae of larva of T. aureus can give us a further understanding of phylogenetic relationships of T. aureus to protect it.

Author contributions
All authors were involved in the conception and design, or analysis and interpretation of the data; LL Liu, LH Zhang, GH Weng, WQ Wang, and SS Zhang were involved in the drafting of the paper; all authors were involved in revising it critically for intellectual content; and all authors were involved in the final approval of the version to be published; and that all authors agree to be accountable for all aspects of the work.

Data availability
Source data
The mitochondrial genome data that support the findings of this study are openly available in GenBank of NCBI at [https://www.ncbi.nlm.nih.gov/nuccore/OL449691] and [https://www.ncbi.nlm.nih.gov/nuccore/OL449692] under the accession no. OL449691 and OL449692. The mt genome was obtained by the Sanger method, so no associated “BioProject”, “SRA” and “Bio-Sample” numbers should be shown.
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Version 1

Reviewer Report 27 March 2023

https://doi.org/10.5256/f1000research.133868.r163936

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This manuscript investigated a method to use exuviae of butterfly larva for species identification, which is useful especially for protected species. Nevertheless, the authors should describe more details of methods and explain some following points.

1. What does 'Class I species in China' mean?

2. The authors got ten mt genomes of *T. aureus* by the Sanger methods. Are all these sequences submitted to a public database? GenBank or other database?

3. Figure 1 include 11 mt genomes of *Teinopalpus aureus* and two mt genomes of *Teinopalpus imperialis*. For 11 mt genomes of *Teinopalpus aureus*, which one(s) are from this study? Which ones from published database? The authors said “including 11 mt genomes of *T. aureus* downloaded from NCBI”, please make the figure legend to be readable by itself.

Are the rationale for sequencing the genome and the species significance clearly described?
Partly

Are the protocols appropriate and is the work technically sound?
Yes

Are sufficient details of the sequencing and extraction, software used, and materials provided to allow replication by others?
Partly

Are the datasets clearly presented in a usable and accessible format, and the assembly and annotation available in an appropriate subject-specific repository?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Insect genome

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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Chang-Bae Kim
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The manuscript sequenced and analyzed the complete mitogenomes for identification of the exuviae of larva from *Teinopalpus aureus*. However, the authors did not explain why mitogenomes are necessary for this aim.

In the manuscript, the authors focus on describing mitogenome structure but this work was done 10 years ago by Qin *et al.* (2012): “Complete mitochondrial genome of the *Teinopalpus aureus guangxiensis* (Lepidoptera: Papilionidae) and related phylogenetic analyses”¹ and some following studies, compare your data to previous studies.

Moreover, all 10 samples for analyses in this study were collected in one region so there was almost no variation among mitogenome sequences. The authors describe these limitations and further studies.

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Are the rationale for sequencing the genome and the species significance clearly described?
No

Are the protocols appropriate and is the work technically sound?
Yes

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Yes

Are the datasets clearly presented in a usable and accessible format, and the assembly and annotation available in an appropriate subject-specific repository?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Genomics and bioinformatics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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