Comparative Analysis of the *Ka/Ks* Ratio among Five Closely Related Species in the Subgenus *Bactrocera*

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Abstract [**Objectives**] To analyze the evolutionary rates of mitochondrial protein-coding genes across five closely related species of fruit flies, thereby providing a foundation for the molecular identification of these quarantine pests. [**Methods**] The newly identified species *Bactrocera latizona*, along with its closely related species within the same subgenus, namely *B. atrifemur*, *B. rubigina*, *B. thailandica*, and *B. tuberculata*, were selected as the subjects of this study. Utilizing the complete mitochondrial genome sequences of these five fruit fly species, the *Ka/Ks* ratios of 13 protein-coding genes were calculated to assess their selective pressures and degrees of conservation. [**Results**] The mitochondrial genome lengths of the five fruit fly species ranged from 15 603 to 15 972 bp. The *Ka/Ks* ratios of the *ND4L* and *ND4* genes for all species were generally elevated (values of the *ND4L* gene all exceeding 2), suggesting accelerated evolutionary rates. In contrast, the *COX1* gene exhibited the lowest *Ka/Ks* ratio, indicating it is the most conserved gene among those analyzed. The majority of genes displayed *Ka/Ks* ratios below 1, implying they are under purifying selection. [**Conclusions**] Among the mitochondrial genes of five fruit fly species, *COX1* is the most conserved, whereas *ND4L* exhibits the highest rate of evolution. These findings offer theoretical support for the development of molecular markers and the species identification of fruit flies.

Key words *Bactrocera*, Mitochondrial genome, *Ka/Ks*, Molecular identification

1 Introduction

Five species of fruit flies—*Bactrocera atrifemur*, *B. latizona*, *B. rubigina*, *B. thailandica*, and *B. tuberculata*—belonging to the genus *Bactrocera* Macquart within the subfamily Dacinae, family Tephritidae, order Diptera[1], are recognized as quarantine pests associated with imported plants. Among these, *B. atrifemur* was intercepted from radish mushrooms carried by inbound passengers from Malaysia, representing the first recorded occurrence in China[2]. Deng Yuliang *et al.*[3] documented the capture of 15 fruit fly species, including the quarantine species *B. rubigina* and *B. tuberculata*, at the Xishuangbanna border. Additionally, Xiao Shu *et al.*[4] captured and monitored five species of quarantine fruit flies, including *B. thailandica*, along the northeastern border of Myanmar. Huang Zhen *et al.*[5] identified a challenging species during border monitoring in Mengla, Yunnan Province, and subsequently described a new species, *B.* (*B.*) *latizona*. *Bactrocera* species primarily infest fruits and vegetables, causing substantial damage. Due to their significant economic impact, morphological similarities among species, the continual discovery of new and newly recorded species, and difficulties in accurate identification, these pests have become a focal point of research in many countries worldwide and are recognized as a group of economically important and hazardous pests[6].

This paper determined the complete mitochondrial genome sequences of a new species *B. latizona* and four other closely related species of the subgenus *Bactrocera*, *B. atrifemur*, *B. rubigina*, *B. thailandica*, and *B. tuberculata* discovered through monitoring in recent years. The complete mitochondrial gene sequences of five fruit fly species were analyzed to determine the ratios of their non-synonymous substitution rate (*Ka*) to synonymous substitution rate (*Ks*). Subsequently, the evolutionary rates of their protein-coding genes were evaluated and compared. This analysis aims to provide a scientific foundation for the development of species-specific molecular markers, DNA barcoding techniques, and phylogenetic studies through the application of *Ka/Ks* ratio analysis in molecular biology[7-8].

2 Materials and methods

2.1 Materials The fruit flies examined in this study comprised a newly identified species, *B. latizona*, along with its closely related species *B. atrifemur*, *B. rubigina*, *B. thailandica*, and *B. tuberculata*. Except for *B. atrifemur*, which was intercepted during quarantine inspections of imported fruits and vegetables at the port, all other specimens were obtained from the laboratory at Mengla Port, Yunnan Province, China. Professors Bai Yonghua and Yu Hui assisted in the identification and verification of fruit fly specimens.

2.2 Methods

2.2.1 Sequencing. DNA extraction was performed using the Rapid Animal Genomic DNA Isolation Kit in accordance with the manufacturer's instructions. Following quality assessment of the extracted DNA, high-throughput sequencing was conducted using the Illumina NovaSeq 6000 platform, with sequencing services provided by Sangon (Shanghai) Biotech Co., Ltd. The resulting sequencing data were assembled, spliced, and subjected to gene annotation. The complete mitochondrial genome sequence obtained was submitted to the NCBI GenBank database to acquire the corresponding accession number.

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2.2.2 Calculation method of *Ka/Ks* ratio. The ratio of the non-synonymous substitution rate (*Ka*) to the synonymous substitution rate (*Ks*) for protein-coding sequences (CDS) was calculated based on the mitochondrial genome sequences of the examined fruit fly species using *KaKs*_Calculator 3.0 software. Concurrently, the nucleotide diversity index was determined employing the dnaml software. The evolutionary rate of protein-coding genes in fruit fly species was assessed through the calculation and analysis of the *Ka/Ks* ratio[9].

3 Results and analysis

3.1 Sequence and accession number This study determined the complete mitochondrial genome sequences of five closely related species within the subgenus *Bactrocera*, namely *B. atrifemur*, *B. latizona*, *B. rubigina*, *B. thailandica*, and *B. tuberculata*. The lengths of the complete mitochondrial genomes were 15 603, 15 936, 15 972, 15 903, and 15 943 bp, respectively. All sequences were submitted to the NCBI GenBank database, with the corresponding accession numbers MZ202395, MZ221972, MW892729, MW929333, and MW892726.

3.2 Analysis of the *Ka/Ks* ratios reflecting evolution rates of protein-coding genes in five fruit fly species

3.2.1 Protein-coding genes of *B. latizona*. Among the 13 protein-coding genes in the mitochondrial genome of *B. latizona*, *ND4L* exhibited the highest evolutionary rate, with a ratio of 2.75. *ND4* ranked second, with a ratio of 1.143. The genes *ATP8*, *ND2*, and *ND3* demonstrated the lowest evolutionary rates, each with a ratio of 0, indicating highly conserved evolutionary rate. The evolutionary rates of the remaining genes were ordered as follows: *ND5* > *COX2* > *ND6* > *ND1* > *COX1* > *COX3* > *CYTB* > *ATP6* (Fig. 1). Among the 13 protein-coding genes analyzed, only the *Ka/Ks* ratios of the *ND4L* and *ND4* genes exceeded 1, whereas the *Ka/Ks* ratios of the remaining 11 genes were all below 1, indicating that these genes may have been subject to purifying selection. These findings suggest that the selective pressures acting on the protein-coding genes within the mitochondrial genome of *B. latizona* vary, as does their degree of conservation. Notably, *ATP8*, *ND2*, and *ND3* were identified as the most conserved genes, while *ND4L* and *ND4* exhibited relatively accelerated evolutionary rates.

3.2.2 Protein-coding genes of *B. atrifemur*. The evolutionary rates (*Ka/Ks*) of 13 protein-coding genes within the mitochondrial genome of *B. atrifemur* were analyzed, revealing variability in evolutionary rates among these genes. Notably, the *ND4L* gene exhibited the highest evolutionary rate (*Ka/Ks* = 2.167), followed by the *ND4* gene (*Ka/Ks* = 0.778). The *ATP6* gene demonstrated a *Ka/Ks* ratio of 0, indicating it is the most evolutionarily conserved. The evolutionary rates of the remaining genes ranked as follows: *ND3* > *ATP8* > *ND2* > *COX2* > *CYTB* > *COX3* > *COX1* > *ND1* (Fig. 1). Among the 13 protein-coding genes analyzed, only the *Ka/Ks* ratios of the *ND4L* and *ND4* genes exceeded 1, whereas the *Ka/Ks* ratios of the remaining 11 genes were all below 1, indicating that these genes may have been subject to purifying selection. These findings suggest differential selective pressures acting on the protein-coding genes within the *B. atrifemur* mitochondrial genome, reflecting varying degrees of evolutionary conservation. *ATP6* was identified as the most conserved gene, while *ND4L* and *ND4* exhibited relatively accelerated evolutionary rates.

3.2.3 Protein-coding genes of *B. rubigina*. The evolutionary rates (*Ka/Ks*) of 13 protein-coding genes within the mitochondrial genome of *B. rubigina* were examined, revealing significant variation in evolutionary rates among the genes. Notably, the *ND4L* gene exhibited the highest evolutionary rate (*Ka/Ks* = 2.665), followed by the *ND4* gene (*Ka/Ks* = 1.368). The *ATP6*, *ATP8*, and *ND2* genes displayed *Ka/Ks* ratios of 0, indicating strong evolutionary conservation. The evolutionary rates of the remaining genes were ranked as follows: *COX2* > *ND3* > *ND5* > *ND6* > *COX3* > *ND1* > *COX1* > *CYTB* (Fig. 1). Among the 13 protein-coding genes analyzed, only the *Ka/Ks* ratios of the *ND4L* and *ND4* genes exceeded 1, whereas the *Ka/Ks* ratios of the remaining 11 genes were all below 1, indicating that these genes have likely undergone purifying selection. These findings suggest differential selective pressures acting on the protein-coding genes within the mitochondrial genome of *B. rubigina*, resulting in varying degrees of conservation. *ATP6*, *ATP8*, and *ND2* were identified as the most conserved genes, while *ND4L* and *ND4* exhibited relatively accelerated evolutionary rates.

3.2.4 Protein-coding genes of *B. thailandica*. The evolutionary rates (*Ka/Ks*) of 13 protein-coding genes within the mitochondrial genome of *B. thailandica* were examined, revealing variability in evolutionary rates among the genes. The *ND4L* gene exhibited the highest evolutionary rate (*Ka/Ks* = 2.563), followed by the *ND4* gene (*Ka/Ks* = 1.078). The *ATP8* and *ND3* genes demonstrated *Ka/Ks* ratios of 0, indicating strong evolutionary conservation. The evolutionary rates of the remaining genes were ranked as follows: *COX2* > *ND5* > *ND2* > *ND6* > *CYTB* > *ND1* > *COX3* > *COX1* > *ATP6* (Fig. 1). Among the 13 protein-coding genes analyzed, only the *Ka/Ks* ratios of the *ND4L* and *ND4* genes exceeded 1, whereas the *Ka/Ks* ratios of the remaining 11 genes were all below 1. This pattern suggests that the majority of these genes have been subject to purifying selection. These findings indicate variability in the selective pressures acting on the protein-coding genes within the mitochondrial genome of *B. thailandica*, as well as differences in their degrees of conservation. *ATP8* and *ND3* were identified as the most conserved genes, while *ND4L* and *ND4* exhibited relatively higher evolutionary rates.

3.2.5 Protein-coding genes of *B. tuberculata*. The evolutionary rates (*Ka/Ks*) of 13 protein-coding genes within the mitochondrial genome of *B. tuberculata* were examined, revealing variability in evolutionary rates among the genes. The *ND4L* gene exhibited the highest evolutionary rate (*Ka/Ks* = 2.667), followed by the *ND4* gene (*Ka/Ks* = 1.875). The *ATP6*, *ATP8*, and *ND2* genes dis-

played Ka/Ks ratios of 0, indicating strong evolutionary conservation. The evolutionary rates of the remaining genes ranked as follows: *COX2* > *ND5* > *ND6* > *ND3* > *COX1* > *COX3* > *ND1* > *CYTB* (Fig. 1). Among the 13 protein-coding genes analyzed, only the *ND4L* and *ND4* genes exhibited *Ka/Ks* ratios greater than 1, whereas the *Ka/Ks* ratios for the remaining 11 genes were all below 1, indicating that these genes have likely undergone purifying selection. These findings suggest differential selective pressures acting on the protein-coding genes within the mitochondrial genome of *B. tuberculata*, reflecting varying degrees of evolutionary conservation. Specifically, *ATP6*, *ATP8*, and *ND2* were identified as the most conserved genes, while *ND4L* and *ND4* demonstrated relatively accelerated evolutionary rates. The *Ka/Ks* ratios for the 13 protein-coding genes across five fruit fly species, including *B. atrifemur*, *B. latizona*, *B. rubigina*, *B. thailandica*, and *B. tuberculata*, are presented in Fig. 1.

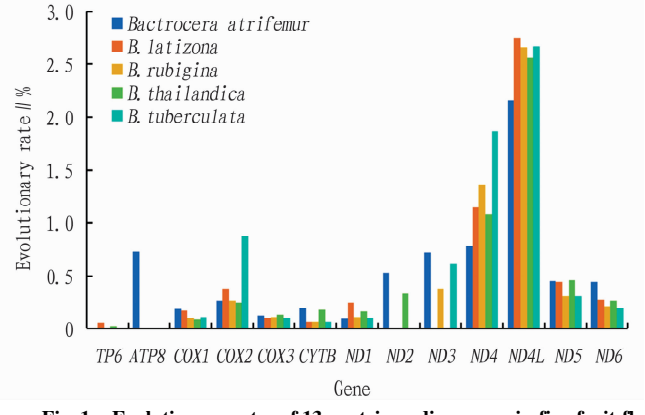

Fig. 1 Evolutionary rates of 13 protein-coding genes in five fruit fly species

4 Conclusions and discussion

The complete mitochondrial genomes of five fruit fly species exhibited sequence lengths of 15 603, 15 936, 15 972, 15 903, and 15 943 bp, respectively. Analysis of the *Ka/Ks* ratios for 13 protein-coding mitochondrial genes revealed that the highest *Ka/Ks* ratios were observed in the *ND4L* gene, followed by the *ND4* gene. Specifically, the *Ka/Ks* ratios for the *ND4L* gene ranged from 2.167 to 2.75, all exceeding 2. For the *ND4* gene, the *Ka/Ks* ratios in four of the species ranged from 1.078 to 1.875, all greater than 1, whereas *B. atrifemur* exhibited a ratio of 0.778, less than 1. The *Ka/Ks* ratios for the remaining protein-coding genes varied across different fruit fly species. For instance, in the *ATP8* gene, the *Ka/Ks* ratio for *B. atrifemur* was 0.727, whereas the ratios for *B. latizona*, *B. rubigina*, *B. thailandica*, and *B. tuberculata* were all 0. In the *ND2* gene, the *Ka/Ks* ratio was 0.531 for *B. atrifemur*, 0.329 for *B. thailandica*, and 0 for *B. latizona*, *B. rubigina*, and *B. tuberculata*. Regarding the *ND3* gene, the *Ka/Ks* ratios were 0.714, 0.615, and 0.369 for *B. atrifemur*, *B. tuberculata*, and *B. rubigina*, respectively, while the remaining two fruit fly species exhibited ratios of 0. These findings suggest that the selective pressures acting on the protein-coding genes within the mitochondrial genomes of these five fruit fly species vary, as does the degree of gene conservation. The *Ka/Ks* ratio serves as an effective indicator of selective pressure on protein-coding genes, thereby providing insight into their evolutionary conservation.

Analysis of the *Ka/Ks* ratios across five species of fruit flies revealed that the ratios for the three cytochrome oxidase subunit genes (*COX1*, *COX2*, and *COX3*) were relatively stable and generally low. Notably, the *COX1* gene exhibited the lowest *Ka/Ks* ratio, followed by the *COX3* gene, while the *COX2* gene displayed a comparatively higher ratio. These findings suggest that within the *COX* gene family, the *COX1* gene evolves at the slowest rate and is the most highly conserved. Consequently, numerous researchers predominantly utilize the *COX1* gene as a molecular marker in studies such as DNA barcoding and species identification. This preference is attributed to the relatively conserved nature of the mitochondrial DNA *COX1* gene, its stable and compact sequence arrangement, its compatibility with amplification using universal primers, and its adequate specificity for distinguishing among species. Thus, the mtDNA *COX1* gene serves as an informative classification standard and an effective tool for biological taxonomy. It has become one of the most widely employed genes in contemporary research on insect classification, identification, and molecular evolution, and has been applied extensively in phylogenetic analyses of closely related fruit fly species[10-12].

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Furthermore, longstanding issues such as inadequate road infrastructure and limited water sources in certain forested areas hinder the early detection and prevention of fires once they have started.

In the future, the management area can further optimize the intelligent sentry system by attempting to integrate it into the scenic area's big data center and exploring its compatibility with UAVs and other security systems. This approach aims to enhance the system's intelligence in forest fire prevention as well as its comprehensive prevention and control capabilities. Additionally, efforts will be made to overcome the limitations associated with monitoring a single type of disaster. Building upon the existing hardware infrastructure, system architecture, and early warning procedures, the system will be advanced toward a "Smart Forest and Grassland Resources Supervision Platform". This platform will integrate forest fire early warning, ecological environment monitoring, and pest and disease control, thereby establishing a robust safety defense mechanism to support the sustainable development of the Mount Tai Scenic Area.

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