The Complete Mitochondrial Genome of *Lepidotrigona flavibasis* (Hymenoptera: Meliponini) and High Gene Rearrangement in *Lepidotrigona* Mitogenomes

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**Abstract**

We reported the sequence and characteristics of the complete mitochondrial genome of an ecologically important stingless bee, *Lepidotrigona flavibasis* (Hymenoptera: Meliponini), that has suffered serious population declines in recent years. A phylogenetic analysis based on complete mitogenomes indicated that *L. flavibasis* was first clustered with another *Lepidotrigona* species (*L. terminata*) and then joined with the other two *Melipona* (Hymenoptera: Meliponini) stingless bees (*M. scutellaris* and *M. bicolor*), forming a single clade of stingless bees. The stingless bee clade has a closer relationship with bumblebees (*Bombus*) (Hymenoptera: Apidae) than with honeybees (*Apis*) (Hymenoptera: Apidae). Extremely high gene rearrangements involving tRNAs, rRNAs, D-loop regions, and protein-coding genes were observed in the *Lepidotrigona* mitogenomes, suggesting an overactive evolutionary status in *Lepidotrigona* species. These mitogenomic organization variations could provide a good system with which to understand the evolutionary history of Meliponini.

**Key words:** stingless bees, phylogeny, evolution, China

Stingless bees (Meliponini) are widely distributed in the tropical and subtropical regions of the world. These bees not only play important roles in tropical ecosystems and in the medical field but also constitute an important group for understanding the phylogeny and biodiversity of social insects. Due to their small size, indistinctive morphological differences and overlapping identification characteristics among species, some species have been misidentified, and the evolutionary histories of many taxa remain unexplored. These factors seriously affect integrated research and resource utilization regarding stingless bees. Previous studies (Rasmussen and Cameron 2007, 2010; Ramirez et al. 2010) have provided important references for the positions of high-level taxa, while internal taxonomic data are still scarce for some smaller genera, such as *Lepidotrigona*.

*Lepidotrigona flavibasis* (Cockerell, 1929) (Hymenoptera: Meliponini) is a species of stingless bee distributed in China (Wu 2000), Cambodia, Laos, Malaysia, and Thailand (Lee et al. 2016). In China, *L. flavibasis* is mainly distributed in Yunnan and Hainan Provinces (Wu 2000), and the bee plays numerous ecological roles in tropical and subtropical ecosystems. For example, *L. flavibasis* can pollinate tropical and subtropical plants (i.e., wampee, litchi, longan, chestnut, etc.) (personal observation), the honey produced by *L. flavibasis* has better quality than the honeys produced by *Apis cerana cerana* (Hymenoptera: Apidae) or *A. dorsata* (Wu et al. 2020). Unfortunately, the overexploitation of stingless bees for commercial purposes has caused a sharp decline in their population size and led *L. flavibasis* to a vulnerable conservation status in China, similar to other stingless bees elsewhere (Delgado et al. 2020, Lima and Marchioro 2021).

Morphology-based classification is important, but researchers engaging in morphological classification usually need a long time of experience to effectively distinguish the subtle differences between some species. For example, *L. flavibasis* males have asymmetric penis valves, which are important classification features (unpublished data). Male individuals of another species, *L. doipaensis*, also possess asymmetric penis valves, and the difference in the asymmetries between the two species is subtle and hard to distinguish (Attasopa et al. 2018). In contrast, molecular data are less dependent on experience and can straightforwardly
distinguish genetic differences. Therefore, molecular data, especially nonrecombinant and fast-evolving mitochondrial genome data, are particularly important for the classification of small stingless bee taxa.

Complete mitochondrial genome (mitogenome) sequences have been suggested to be particularly useful and effective for species conservation and biodiversity assessments (Galtier et al. 2009). The mitogenomes of insects are circular DNA molecules that code for 13 proteins, 22 tRNAs and two ribosomal RNAs (12S rRNA and 16S rRNA) (Boore 1999). Moreover, a mitogenome also contains one noncoding DNA region, the D-loop region (or A+T-rich region), which controls the replication and transcription of the whole mitogenome (Taanman 1999). Mitogenomes have also been widely used in phylogeny, population structure, and phylogeography analyses (Gissi et al. 2008).

The gene orders of most invertebrate mitogenomes are highly conserved (Boore 1999). However, many gene rearrangement events have been discovered in invertebrate mitogenomes in recent years, including gene transpositions, gene losses, and gene duplications. It seems that a rearranged mitogenome has a limited effect on mitochondrial function rather than producing lethal effects, so these mitogenome rearrangements may not be erased by natural selection (Boore et al. 1995) and could reflect the evolutionary forces and rates in a given species or genus (Shao et al. 2003).

Prior to our research, only two complete stingless bee mitogenomes were available in GenBank (KP202303 and AF466146), greatly limiting phylogenetic and taxonomic analyses among stingless bees. In this study, we sequenced the complete mitogenome of *L. flavibasis*, and this sequence will be useful in studying conservation biology and population genetics in this species.

**Fig. 1.** The mitogenome map of *L. flavibasis*. All 37 genes were distributed on two different loops with different directions. The GC content and GC skew were calculated and are presented as sliding windows.
species. The gene order, nucleotide composition, and secondary structures of the tRNA genes in the *L. flavibasis* mitogenome were analyzed. In addition, the phylogenetic relationships between *L. flavibasis* and 11 other hymenopteran insects were reconstructed. The annotated genome was submitted to GenBank under accession number MN747147.

**Materials and Methods**

**Sample Collection and DNA Extraction**

The *L. flavibasis* specimens were obtained from Lincang, Yunnan, China (N 24°5′47″, E 99°46′24″) and deposited in the insect specimen room of the Research Institute of Resource Insects with accession number RIRI-w-20191013. The total genomic DNA was extracted from the chest muscles of the specimens with a Tissue DNA Kit (TIANGEN Biotech, Beijing) following the manufacturer’s instructions. The total DNA content was detected by using a Qubit dsDNA HS assay kit (Invitrogen).

**Sequencing and Assembly**

The library was constructed with the 1.0 µg genomic DNA by using KAPA Hyper Prep Kits (KAPA Biosystems). The average length of the inserted fragment was 350 bp. The sequencing work of the complete *L. flavibasis* mitogenome was performed by an Illumina Nextseq500 at Beijing Microread Genetics Co., Ltd., with a total data volume of 4G (150 bp reads). After removing the connector and the unmatched, short, and poor-quality reads, the remaining high-quality reads were assembled from scratch using IDBA-UD and SPAdes (Gurevich et al. 2013). After the mitogenome was generated by de novo assembly, the protein-coding genes (PCGs) of the *L. flavibasis* mitogenome were identified using a BLAST search in NCBI [https://www.ncbi.nlm.nih.gov], and the tRNA genes were identified using the tRNAscan-SE.

**Table 1. Annotation of the *L. flavibasis* mitogenome**

| Locus     | Full name and function | Genome position | Codon |
|-----------|------------------------|-----------------|-------|
|           |                        | From | To   | Strand | Start | Stop | Anti-codon |
| tRNA-Met  | Transfer RNA for Methionine | 1    | 67   | -      | CAT   |       |           |
| tRNA-Lys  | Transfer RNA for Lysine  | 106  | 174  | +      | TTT   |       |           |
| tRNA-Ala  | Transfer RNA for Alanine | 202  | 267  | -      | TGC   |       |           |
| tRNA-Ile  | Transfer RNA for Isoleucine | 282  | 348  | -      | GAT   |       |           |
| ND2       | NADH dehydrogenase subunit 2 | 388  | 1093 | +      | GTG   | T(AA)# |           |
| tRNA-Cys  | Transfer RNA for Cysteine | 1094 | 1159 | +      | GCA   |       |           |
| tRNA-Trp  | Transfer RNA for Tryptophan | 1204 | 1269 | +      | TCA   |       |           |
| tRNA-Tyr  | Transfer RNA for Tyrosine | 1287 | 1354 | -      | GTA   |       |           |
| tRNA-Cox1 | Cytochrome c oxidase subunit I | 1421 | 2980 | +      | ATA   | TAA   |           |
| tRNA-Leu  | Transfer RNA for Leucine | 2992 | 3057 | +      | TAA   |       |           |
| tRNA-Cox2 | Cytochrome c oxidase subunit II | 3079 | 3732 | +      | ATA   | TAA   |           |
| tRNA-Asp  | Transfer RNA for Aspartic acid | 3742 | 3807 | +      | GTC   |       |           |
| ATP8      | ATP synthase F0 subunit 8 | 3808 | 3975 | +      | ATT   | TAG   |           |
| ATP6      | ATP synthase F0 subunit 6 | 4053 | 4652 | +      | ATT   | TAA   |           |
| COX3      | Cytochrome c oxidase subunit III | 4680 | 5435 | +      | ATA   | TAG   |           |
| tRNA-Gly  | Transfer RNA for Glycine | 5473 | 5541 | +      | TCC   |       |           |
| tRNA-Glu  | Transfer RNA for Glutamic acid | 5617 | 5686 | +      | TTC   |       |           |
| ND3       | NADH dehydrogenase subunit 3 | 5687 | 6040 | +      | ATT   | TAA   |           |
| tRNA-Arg  | Transfer RNA for Arginine | 6046 | 6111 | +      | TCG   |       |           |
| tRNA-Glu1 | Transfer RNA for Glutamine | 6215 | 6281 | +      | TTG   |       |           |
| 12S rRNA  | 12S ribosomal RNA | 6315 | 7082 | +      |       |       |           |
| 16S rRNA  | 16S ribosomal RNA | 7176 | 8545 | +      |       |       |           |
| tRNA-Val  | Transfer RNA for Valine | 7082 | 7147 | +      | TAC   |       |           |
| tRNA-Leu  | Transfer RNA for Leucine | 8523 | 8591 | +      | TAG   |       |           |
| ND1       | NADH dehydrogenase subunit 1 | 8604 | 9518 | +      | ATG   | TAA   |           |
| tRNA-Ser  | Transfer RNA for Serine | 9517 | 9583 | -      | TGA   |       |           |
| CYTB      | Cytochrome b | 9592 | 10689 | - | ATA | TAA |       |
| ND6       | NADH dehydrogenase subunit 6 | 10740 | 11210 | - | ATC | TAA |       |
| tRNA-Pro  | Transfer RNA for Proline | 11263 | 11330 | + | TGG |       |           |
| tRNA-Thr  | Transfer RNA for Threonine | 11336 | 11421 | + | TGT |       |           |
| tRNA-Asn  | Transfer RNA for Asparagine | 11484 | 11550 | - | GTT |       |           |
| tRNA-Ser  | Transfer RNA for Serine | 11598 | 11634 | + | TCT |       |           |
| tRNA-Phe  | Transfer RNA for Phenylalanine | 11672 | 11736 | - | GAA |       |           |
| ND5       | NADH dehydrogenase subunit 5 | 11739 | 13364 | - | ATA | TAA |       |
| tRNA-His  | Transfer RNA for Histidine | 13392 | 13455 | - | GTG |       |           |
| ND4       | NADH dehydrogenase subunit 4 | 13464 | 14684 | - | ATT | TAA |       |
| ND4L      | NADH dehydrogenase subunit 4L | 14806 | 15063 | - | ATT | TAG |       |
| D-loop    | A+T-rich region, which controls the replication and transcription of the whole mitogenome | 15064 | 15408 | - |       |       |           |

tRNA abbreviations followed the IUPAC-IUB three letter code.

# TAA stop codon was completed by the addition of 3′ A residues in polycistronic transcription cleavage and polyadenylation processes.
search server (Schattner et al. 2005). The final assembled mitogenome was verified, and the secondary structures of the tRNAs were predicted on the MITOS web server (Bernt et al. 2013). The mitogenome map was generated by the molecular biology tool CGView (Grant and Stothard 2008).

**Phylogeny Analysis**
To compare the *L. flavibasis* mitogenome with 11 other hymenopteran insects, concatenated sequences from 37 genes were aligned through ClustalW in the Mega 7.0 software package (Kumar et al. 2016). The maximum likelihood method was used to construct the phylogenetic relationships with default settings, and the bootstrap values were estimated using 500 replicates. The base composition and relative synonymous codon usage (RSCU) were also analyzed using the Mega 7.0 software package (Kumar et al. 2016) and PhyloSuite software package (Zhang et al. 2020a). The GC skew was computed according to the following formula: GC skew = \( \frac{G - C}{G + C} \) (Perna and Kocher 1995).

**Gene Rearrangement Analysis in the Mitogenome**
The related mitogenomes were downloaded from GenBank, and the gene orders were extracted by the PhyloSuite software package (Zhang et al. 2020a) and visualized on the iTOL website server (Letunic and Bork 2016). For a quantitative comparison of the rearrangement in each mitogenome, we calculated the rearrangement score (RS) and rearrangement frequency (RF) of each individual gene in all collected mitogenomes using a qMGR approach (Zhang et al. 2020b). By accumulating the RSs of all genes in one genome, the RS of each mitogenome could be calculated; this value can be used as a quantitative feature of mitogenome rearrangement.

**Results and Discussion**

**Genome Structure and Nucleotide Composition**
The mitogenome was 15,408 bp in size, including 13 typical invertebrate PCGs, 22 transfer RNA genes, two ribosomal RNA genes and...
a noncoding control region (D-loop). Among the 37 coding genes, 23 genes were located on the H-strand, and the remaining 14 genes were coded on the L-strand (Fig. 1). The base composition of the whole \textit{L. flavibasis} mitogenome was 38.93\% for A, 10.96\% for G, 39.41\% for T, and 10.70\% for C. The A+T content of the whole \textit{L. flavibasis} mitogenome was 78.34\%, showing an obvious AT mutation bias (Nguyen et al. 2020). The D-loop region exhibited the highest A+T content (85.80\%) in the \textit{L. flavibasis} mitogenome.

Protein-coding Genes and Codon Usage

The total length of the 13 PCGs in the \textit{L. flavibasis} mitogenome was 10,387 bp. Among the 13 PCGs, 12 PCGs used the standard ATN as their start codon, while \textit{ND2} used GTG as a start codon. For the stop codon, nine PCGs had the common mitochondrial stop codon TAA, while \textit{ATP8}, \textit{COX3}, and \textit{ND4L} were terminated with the stop codon TAG, and \textit{ND2} ended with a single T (Table 1). Truncated stop codons also exist in the mitochondrial genomes of vertebrates, such as in human mitochondria (Andrews et al. 1999). It has been demonstrated that incomplete stop codons can produce functional stop codons in polycistronic transcription cleavage and polyadenylation processes (Ojala et al. 1981).

The total number of codons in the 13 PCGs was 3,451. The codon usage and RSCU values are summarized in Supp Table S1 (online only). In general, AUU, AUA, UUU, AAU, and UUA were the most frequently used codons. AUU (Ile) was the most abundant codon. The RSCU analysis indicated that A and T were used more frequently than G and C in degenerate codons (Fig. 2). GC-rich codons were used less commonly than AT-rich codons in the \textit{L. flavibasis} mitogenome; for example, no GCG or GGC codons were observed, and only two codons each were counted for CGC, GCC, and CGG. Considering the high AT content in the whole \textit{L. flavibasis} mitogenome, this codon usage bias should mainly result from the obvious AT mutation bias (Nguyen et al. 2020).

Ribosomal and Transfer RNAs

The two rRNA genes found on the H-strand had the second-highest A+T contents (79.61\%). The \textit{rrnS} gene was 768 bp long and was located between \textit{tRNA^{Glu}} and \textit{tRNA^{Val}}; the A+T content of this gene was 79.43\%. The \textit{rrnL} gene was 1370 bp in size, found between \textit{tRNA^{Val}} and \textit{tRNA^{Leu}}; and the A+T content of the \textit{rrnL} gene was 79.78\%.

The 22 tRNA genes were distributed throughout the \textit{L. flavibasis} mitogenome, ranging from 57 to 70 bp in size. Thirteen tRNA genes were located on the H-strand, and the remaining nine tRNA genes were located on the L-strand (Table 1). All the tRNAs except \textit{tRNA^{Ser}(UCU)} can be folded into typical cloverleaf secondary structures (Supp Fig. S1 [online only]). The unusual \textit{tRNA^{Ser}(UCU)} lacks the dihydrouridine (DHU) arm and cannot be folded into the typical clover-leaf structure. This feature of \textit{tRNA^{Ser}} has been witnessed in many insect and metazoan mitogenomes (Wolstenholme 1992, Cameron 2014).

Phylogenetic Analysis

The whole mitogenome sequence is a better choice for a phylogenetic analysis than partial mitochondrial sequences or a combination of partial mitochondrial sequences and a few nuclear genes. To date, the phylogenetic relationships between stingless bees and other hymenopteran insects have not been analyzed based on complete mitochondrial genomes. It has been reported that the concatenated
nucleotide sequences of 37 mitochondrial genes yield topologies with high support values (Wang et al. 2019). Therefore, we performed phylogenetic analyses on the concatenated nucleotide sequences of 37 genes from L. flavibasis and 11 representative hymenopteran insects whose complete mitogenomes have been reported.

Lepidotrigona flavibasis was first clustered with another Lepidotrigona species (L. terminata) and then joined with the other two stingless bees (Melipona scutellaris and M. bicolor) (Hymenoptera: Meliponini), forming a single clade of stingless bees (Fig. 3a). This relationship is consistent with the phylogeny reconstructed with a multigene approach based on nuclear, mitochondrial, and ribosomal loci (Ramírez et al. 2010). The phylogenetic tree also indicated that stingless bees have a closer relationship withbumblebees (Bombus) (Hymenoptera: Apidae) than with honeybees (Apis). Zhao et al. (2017) also discovered a closer relationship between stingless bees and bumblebees than between bumblebees and honeybees.

Gene Order and Rearrangement of the L. flavibasis Mitogenome

The gene order of the L. flavibasis mitogenome was identical to that of another Lepidotrigona stingless bee (GenBank accession number MN737481) but was different from the gene orders of the other two previously reported Melipona stingless bee mitogenomes (GenBank accession numbers KP202303 and AF466146), with several rearranged genes. The rearrangement events also supported the phylogenetic relationships that were reconstructed based on the mitogenome sequences (Fig. 3a and b). The conserved tRNA D-K block in other hymenopteran insects was changed in the stingless bee clade, indicating that the translocation of tRNA K occurred after the stingless bee ancestor diverged from the other Apidae insects. Inside the stingless bee clade, the locations of tRNA K also differed. In the stingless bee ancestor diverged from the other Apidae insects. In addition to tRNA genes, protein-coding genes (PCGs) and rRNA genes, K, A, and W showed the highest rearrangement frequencies. Especially for the two analyzed Lepidotrigona species of Lepidotrigona flavibasis, an M-K-A-I block was formed, while in Melipona, the block was reversed as I-A-K-M (Fig. 3a and b).

Relative to the ancestral insect mitogenome (Boore 1999), many genes have been rearranged in the stingless bee mitogenomes. For example, for the two analyzed Lepidotrigona species, the positions of two ribosomal RNAs and several tRNAs changed dramatically, suggesting an overactivated status in Lepidotrigona mitogenomes. We calculated the RS and RF of each individual gene in all the collected mitogenomes. The RS of each mitogenome was also calculated by accumulating the RSs of all the genes in that mitogenome. The results indicated that Lepidotrigona was a very active group, with the highest RS among Apidae insects (Fig. 3c).

For individual genes, tRNA genes had obviously higher rearrangement frequencies than protein-coding genes (Fig. 3d). Among tRNA genes, K, A, and W showed the highest rearrangement frequencies. In addition to tRNA genes, protein-coding genes (PCGs) and tRNA genes were also rearranged dramatically in Lepidotrigona. The conserved Nad1-L1-rnlL-vrrnS block in Apidea was transposed and inverted in Lepidotrigona. Moreover, the T-P-nad6-cytb block was also transposed and inverted in Lepidotrigona. A tRNA-E gene was inserted into the conserved cox3-G-nad3 block to form a new cox3-G-E-nad3 block (Fig. 3d). These events indicated that Lepidotrigona species have had very active mitogenomes.

Consistent with the high observed gene rearrangement rates, the Lepidotrigona mitogenomes also had high nucleotide substitution rates, which manifested as longer branches in the phylogenetic tree (Fig. 3a and c). The correlation between the substitution rate and RF has also been observed in other insect mitogenomes (Shao et al. 2003), indicating that they were influenced by the same evolutionary force in different species. In the future, we will further investigate whether a high nucleotide substitution rate and gene rearrangement are related to the sharp population decline in stingless bees.

Supplementary Data

Supplementary data are available at Journal of Insect Science online.

Figure S1. Predicted secondary cloverleaf structures for the 22 transfer RNA genes of the L. flavibasis mitogenome.

Table S1. The codon usage and the relative synonymous codon usage (RSCU) values of the L. flavibasis mitogenome.

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Author Contributions

Conceptualization, C.-Y.W., M.Z., S.-J.W., and H.-L.X.; Methodology, C.-Y.W. and L.-N.L.; Validation, C.-Y.W.; Formal Analysis, C.-Y.W.; Investigation, C.-Y.W., M.Z., H.-L.X., and L.-N.L.; Resources, S.-J.W. and Y.-M.Y.; Data Curation, C.-Y.W.; Writing—Original Draft Preparation, C.-Y.W. and M.Z.; Writing—Review and Editing, C.-Y.W., M.Z., H.-L.X., S.-J.W., and Y.F.

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