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Comparative analyses of mitogenomes in the social bees with insights into evolution of long inverted repeats in the Meliponini

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ABSTRACT

The insect mitogenome is typically a compact circular molecule with highly conserved gene contents. Nonetheless, mitogenome structural variations have been reported in specific taxa, and gene rearrangements, usually the tRNAs, occur in different lineages. Because synapomorphies of mitogenome organizations can provide information for phylogenetic inferences, comparative analyses of mitogenomes have been given increasing attention. However, most studies use a very few species to represent the whole genus, tribe, family, or even order, overlooking potential variations at lower taxonomic levels, which might lead to some incorrect inferences. To provide new insights into mitogenome organizations and their implications for phylogenetic inference, this study conducted comparative analyses for mitogenomes of three social bee tribes (Meliponini, Bombini, and Apini) based on the phylogenetic framework with denser taxonomic sampling at the species and population levels. Comparative analyses revealed that mitogenomes of Apini and Bombini are the typical type, while those of Meliponini show diverse variations in mitogenome sizes and organizations. Large inverted repeats (IRs) cause significant gene rearrangements of protein coding genes (PCGs) and rRNAs in Indo-Malay/Australian stingless bee species. Molecular evolution analyses showed that the lineage with IRs have lower d_N/d_S ratios for PCGs than lineages without IRs, indicating potential effects of IRs on the evolution of mitochondrial genes. The finding of IRs and different patterns of gene rearrangements suggested that Meliponini is a hotspot in mitogenome evolution. Unlike conserved PCGs and rRNAs whose rearrangements were found only in the mentioned lineages within Meliponini, tRNA rearrangements are common across all three tribes of social bees, and are significant even at the species level, indicating that comprehensive sampling is needed to fully understand the patterns of tRNA rearrangements, and their implications for phylogenetic inference.

Keywords: Social bees; Phylogeny; Mitogenome structure; Gene rearrangement; Inverted repeats

INTRODUCTION

The mitochondria are fundamental organelles in almost all eukaryotic cells with the function of energy conversion (Dowling & Wolff, 2023). As organelles of endosymbiotic origin, they contain their own genomic sequences, the mitogenomes, which are typically maternally inherited (Sato & Sato, 2012). Although mitochondria share the common ancestral origin as Alphaproteobacteria living within ancestral eukaryotes, mitogenome organizations show high diversity across eukaryotic lineages in both genome structures and gene rearrangements (Shtolz & Mishmar, 2023). Six major types of mitogenome structures have been recognized in all living organisms (Kolesnikov & Gerasimov, 2012), and insect mitogenomes correspond to type Ⅰ, with a compact circular molecule of 15–18 kb (Cameron, 2014). The gene content is

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highly conserved, with 37 unique genes, including 13 protein coding genes (PCGs), two ribosomal RNA genes (rRNAs), and 22 transfer RNA genes (tRNAs) (Boore, 1999). There is also a control region, which is assumed to be the largest non-coding segment within the mitogenome (Boore, 1999). This region is also called the D-loop region or the A+T rich region, and it has been shown to contain regulatory elements for the replication and expression of the mitogenome (Saito et al., 2005). Although containing five conserved elements, the control region is difficult to annotate, because their positions and structures are hypervariable across different lineages, and their lengths are variant as a result of variations of the repeat sequences (Dickey et al., 2015; Quinn & Mindell, 1996; Quinn & Wilson, 1993; Wei et al., 2010; Zhang et al., 1995; Zhang & Hewitt, 1997).

Since the first mitogenome of *Drosophila yakuba* was reported nearly 40 years ago (Clary & Wolstenholme, 1985), the number of insect mitogenomes has increased rapidly to more than 3 256 accessions by 2022 (https://www.ncbi.nlm.nih.gov/genome/browse#!/organelles/). Comparative studies of insect mitogenomes have revealed that structural variations and gene arrangements occur across lineages, although their organizations are generally conserved as the "normal" type. Major structural variations have been found in insect mitogenomes of some lineages including: (1) duplication of PCGs in the species *Microchorista philpotti* (Nannochoristidae) (Beckenbach, 2011), or inverted duplication of the nearly whole mitogenome or circular amphimeric mitogenome in two Australian stingless bees of *Tetragonula* (Hymenoptera) (Françoso et al., 2023); (2) multiple fragmented mitogenomes in order Thysanoptera (Dickey et al., 2015), Psocoptera (Shi et al., 2016), and Phthiraptera (Sweet et al., 2022); (3) gene or gene segment translocations or rearrangements in order Hemiptera (Li et al., 2012), Phthiraptera (Sweet et al., 2021), Psocoptera (Li et al., 2013), and Hymenoptera (de Paula Freitas et al., 2020; Oliveira et al., 2008; Xiao et al., 2011; Zheng et al., 2018), with massive rearrangements in *Tetragonula* spp. (Françoso et al., 2023; Wang et al. 2022), *Lepidotrigona* spp. (Wang et al., 2020, 2021). The circular amphimeric genome, two inverted repeats (IRs) separated by two single copy regions (SCs), is the typical feature of the chloroplast genome in land plants (Wicke et al., 2011), but is rather unusual in animal mitogenomes (Rayko, 1997; Kolesnikov & Gerasimov, 2012). Amphimeric genomes have structural isomers, which differ only in the orientation of the SC regions, and high frequency intramolecular recombination between two IRs could mediate structural heteroplasmy in the chloroplast genome of plants (Stein et al., 1986; Wang & Lanfear, 2019).

Hymenoptera is estimated to outnumber Coleoptera as the most species-rich insect order (Forbes et al., 2018), comprising 109 recognized extant families including species commonly known as sawflies, wasps, bees, and ants (Blaimer et al., 2023). Mitogenomes of investigated Hymenopteran lineages show high rates of gene rearrangements, which provide both opportunities and potential pitfalls for exploring homologous rearrangements and their implications in phylogenetic and evolutionary inferences (Dowton & Austin, 1999; Dowton et al., 2009). Most previous comparative analyses of mitogenomes were carried out with one or a few species to represent the whole genus, tribe, family, or even order (Dowton & Austin, 1999; Dowton et al., 2003, 2009; He et al., 2018; Silvestre & Arias, 2006; Wei et al., 2010; Zheng et al., 2018). This kind of sampling strategy could overlook mitogenome variations within family, tribe, genus, or species level, which may lead to some incorrect inferences. In this study, we focused on three social bee tribes, Apini (honeybees), Meliponini (stingless bees) and Bombini (bumblebees), to investigate mitogenome variations and their phylogenetic signature with extensive sampling at the species and population levels.

These three tribes and orchid bees (Euglossini) form a monophyletic group of (Euglossini+(Apini+(Bombini+Meliponini))) in the Apidae (Almeida et al., 2023). Species within this monophyletic group are known as "corbiculate bees" because, except for some cleptobiotic species in Meliponini (Melo, 2020) and Bombini (Lhomme & Hines, 2019), female workers of three social bee tribes and Euglossini females possess corbicula at the metatibia to carry plant resources. Taxa within these three tribes are iconic around the world for their roles in pollination and economic production, as well as their social behavior (Engel, 2023; Grüter, 2020; Michener, 2007). Stingless bees and honeybees are highly eusocial, living in perennial colonies and having two female castes, queens and workers (Roubik, 1989). Bumblebees are also eusocial, but their colonies are smaller and generally last less than a year, with only the newly mated young queen surviving to the next spring by hibernation and starting a new colony on its own (Michener, 2007). Among the three tribes, a large number of mitogenomes from Apini and Bombini have been decoded and show the typical type of mitogenome. By contrast, the mitogenomes of Meliponini have been less investigated (de Paula Freitas et al., 2020; Wang et al., 2020, 2021, 2022). Recent studies showed massive mitogenome variations in gene rearrangements and structural reorganizations in Meliponini. However, these studies only sampled five or fewer taxa of Meliponini for comparative analyses, so the evolutionary trends of mitogenome reorganizations within Meliponni have not been well understood. Bombini have shown pervasive tRNA rearrangements across lineages, which are synapomorphic among bumblebee subgenera, and are also regarded as an ideal group for mitogenome evolution (Gonçalves et al., 2023).

In this study, we *de novo* assembled 73 new mitogenomes and downloaded 81 previously reported mitogenomes (representing 9 honeybees, 40 bumblebees, and 23 stingless bee species) to reconstruct the phylogeny of three tribes of social bees and the orchid bees. Based on the phylogenetic framework, this study aimed to: (1) explore the occurrence of the IR regions in mitogenomic reorganizations within Meliponini, and to gain new insights into the evolutionary pattern; (2) compare the gene rearrangements across the three tribes of social bees to understand their evolutionary patterns at lower taxonomic levels, and their implications for phylogenetic inference.

MATERIALS AND METHODS

Bee samplings, DNA extraction and sequencing

In total, 154 samples were included in this study, including 39 samples of nine Apini species, 50 samples of 42 Bombini species, and 65 samples of 23 Meliponini species (Tables 1, 2). Of those, 40 samples representing eight Meliponini species were newly sampled in Yunnan, Southwest China. Sampling sites are provided in Supplementary Table S1. Morphological and biological information for seven species were described in

Table 1 Information and features of mitogenomes assembled from SRA data or sequencing data by this study

Taxa	Voucher/SRA	Assessment of assembled mitogenomes		Genome size (bp)	IR (bp)	tRNA	GC content (%)
		Matched	Customized error rate				
Tribe Bombini							
Genus *Bombus*							
B. balteatus	SRR13788759	1474.15±229.72	0.0221±.1932	16871		+Met	13.0
B. balteatus	SRR13788762	1608.51±577.79	0.0162±.1437	17017		+Met	13.1
B. bohemicus	SRR12770612	1858.69±5291.58	0.0315±0.4228	20984		-Ser1 -Glu -Arg -Asn +Met	16.7
B. bifarius	SRR11404314	2250.03±934.00	0.0411±.3299	16714		22	14.2
B. bifarius	SRR12691508	1309.23±2389.85	0.0306±0.3136	16883		22	14.3
B. convexus	SRR12770609	3793.03±808.55	0.0315±.3398	16329		22	19.6
B. cullumanus	SRR12528003	285.93±15.82	0.0391±.4256	17418		22	15.7
B. impetuosus	SRR12770615	1356.22±349.93	0.0139±.1911	15767		22	13.8
B. laesus	SRR12770614	1270.73±93.00	0.0079±.0769	15732		22	12.6
B. lantschouensis	SRR12770617	952.42±18.92	0.0022±.0087	16431		+Met	14.0
B. melanopygus	SRR8700077	509.63±31.53	0.0144±.1477	17236		22	13.0
B. polaris	SRR12527998	1435.15±689.73	0.0172±0.1292	16374		-Met -Ile -Ala	12.8
B. sichelii	SRR12770616	1560.37±519.39	0.0017±0.0129	15675		-His	14.7
B. superbus	SRR12528014	2109.69±678.51	0.0060±0.0246	15738		22	15.8
B. sylvicola	SRR12691583	1358.14±06.26	0.0149±.0637	21701		+Met	11.1
B. terricola	SRR7696607	748.41±27.77	0.0123±.0930	16652		-Ala -Met	13.9
B. trifasciatus	SRR12770611	1043.36±28.48	0.0047±.0282	15871		-Met	14.6
B. turneri	SRR12528020	1410.97±63.59	0.0050±.0094	15689		-Ala	12.8
B. vancouverensis nearcticus	SRR11407481	1543.40±23.30	0.0146±.0497	19608		22	14.9
Tribe Meliponini							
Genus *Frieseomelitta*							
F. varia	SRR10065869	880.69±17.88	0.0107±.0167	15260		22	12.1
F. varia	SRR13310985	796.42±88.00	0.0167±.0275	15405		22	11.9
Genus *Nannotrigona*							
N. testaceicornis	SRR5651514	68.07±71.31	0.0443±0.131189202	15257		-Tyr -Lys -Ala -Gln -Met -Ile	13.7
Genus *Melipona*							
M. bicolor	SRR8735007	107.56±2.88	0.0068±.0114	15018		22	13.1
M. quadrifasciata	SRR1945415	742.44±77.06	0.0058±.0475	15246		22	12.0
M. variegatipes	ERR3255838	651.37±82.90	0.0044±.0066	15589		22	12.0
Genus *Heterotrigona*							
H. itama	ERR4276786	802.15±0.14	0.0043±.0041	26714	11275	22	23.7
Genus *Lepidotrigona*							
L. sp.	JHMW2	1199.01±29.44	0.0028±.0086	26566	10991	22	25.6
L. flavibasis	GLTMS1	1311.63±26.24	0.0023±.0050	26609	10821	22	22.3
L. flavibasis	HNBS1	413.78±69.90	0.0060±.0060	26599	10923	22	22.4
L. flavibasis	HNQZ1	620.67±34.07	0.0049±.0043	26588	10911	22	22.3
L. flavibasis	JHDDG4	1273.04±42.81	0.0025±.0036	26596	10920	22	22.3
L. flavibasis	LCCY9	1485.43±29.71	0.0050±.0030	26515	10881	22	22.2
L. flavibasis	MLBB1	884.29±64.70	0.0032±.0041	26600	10905	22	22.4
L. flavibasis	MLML6	2359.40±098.15	0.0018±.0019	26568	10904	22	22.4
L. flavibasis	MLMX13	1019.11±245.34	0.0035±0.0037	26595	10932	22	22.4
L. flavibasis	MLXTBG_R2	538.07±40.88	0.0035±.0048	26606	10938	22	22.4
L. flavibasis	MLYXC4	794.46±84.07	0.0040±.0068	26579	10898	22	22.4
L. flavibasis	MWDGP2	2373.33±93.75	0.0027±.0021	26589	10911	22	22.4
L. flavibasis	MWHTC19	2807.48±460.46	0.0037±.0095	26582	10912	22	22.3
L. terminata	GLTMS3	1346.40±94.36	0.0021±.0079	26626	11026	22	24.3
L. terminata	JHJH1	1134.30±74.75	0.0037±.0069	26628	11027	22	24.4
L. terminata	JHJN	1540.82±27.70	0.0020±.0071	29351	14310	22	24.0
L. terminata	MLMX15	1274.53±516.02	0.0018±0.0071	26630	11016	22	24.3
L. terminata	MLXTBG_L2	691.75±76.59	0.0040±.0079	26628	11020	22	24.3

Continued

Taxa	Voucher/SRA	Assessment of assembled mitogenomes		Genome size (bp)	IR (bp)	tRNA	GC content (%)
		Matched	Customized error rate				
L. terminata	MLYXC3	872.83±08.89	0.0020±.0082	26635	11028	22	24.4
Genus *Lisotrigona*							
L. carpenteri	YXYJ	580.58±76.25	0.0135±.0197	15543		22	11.6
Genus *Plebeina*							
P. hildedrandti	SRR5406061	27.19±4.16	0.0142±.0486	15165		22	13.3
Genus *Tetragonula*							
Subgenus *Tetragonilla*							
T. collina	MAMB2	2937.58±624.05	0.0040±.0198	22329	4681/ 1751	-Glu	20.4
T. collina	MHGDN13	2288.60±363.42	0.0035±.0191	22746	4746/ 1751	-Glu	20.5
T. collina	MHMB1_1	1157.15±73.49	0.0030±.0147	22337	4746/ 1751	-Glu	20.4
T. collina	MHMB1_3	1159.44±433.42	0.0026±0.0117	22337	4746/ 1751	-Glu	20.4
T. collina	MLYXC1_1	1460.65±015.20	0.0032±.0143	22321	4647/ 1750	-Glu	20.5
T. collina	MWHTC14	8323.51±970.48	0.0052±.0171	22335	4746/ 1751	-Gly	20.5
Subgenus *Tetragonula*							
T. carbonaria	SRR10389202	1936.93±620.20	0.0012±0.0357	30639	15309	-Glu	28.0
T. clypearis	SRR10411628	3232.83±340.39	0.0106±0.0163	24802	9373	-Gly	25.8
T. davenporti	SRR10405219	828.91±14.51	0.0030±.0049	30844	15268	-Glu	29.8
T. gressitti	JHMW12	2559.05±1319.9	0.0019±0.0083	24080	8297	-Glu	20.1
T. gressitti	LCCY6	2175.10±211.76	0.0040±.0067	24012	8247	-Gly	20.0
T. gressitti	LCY4	3582.91±177.19	0.0020±.0068	24012	18247	-Gly	20.0
T. gressitti	MWDGP1	822.28±63.19	0.0033±.0087	24077	8312	-Glu	20.1
T. gressitti	PEJC1	917.47±08.55	0.0055±.0069	24080	8093	-Glu	20.1
T. hockingsi	SRR10390715	1899.08±95.12	0.0011±.0018	30410	14992	-Glu	27.3
T. laeviceps	GLTMS2	998.11±01.15	0.0018±.0043	27607	12103	-Glu	25.9
T. laeviceps	GLTMS5	417.79±89.29	0.0018±.0047	29200	13659	-Gly	26.0
T. laeviceps	MLBB4	1044.40±73.04	0.0015±.0047	27699	12097	-Glu	25.9
T. laeviceps	MLML2	455.27±15.46	0.0023±.0047	27699	12097	-Glu	25.9
T. laeviceps	MLMX1	3298.87±230.10	0.0023±.0065	27693	12094	-Glu	25.9
T. laeviceps	MLXTBG1	1353.48±526.10	0.0049±0.0050	27719	12107	-Glu	25.9
T. mellipes	SRR10395358	550.55±7.60	0.0088±.0119	28477	12993	-Gly	27.6
T. mellipes	SRR10426324	1468.56±02.37	0.0063±.006	30828	15284	-Gly	28.0
T. pagdeni	LCCY4	1014.69±00.70	0.0045±.0061	23522	8342	-Glu	25.3
T. pagdeni	LCY2	2418.86±285.90	0.0025±.0077	23522	8342	-Gly	25.3
T. pagdeni	LCZK1	701.59±30.75	0.0051±.0075	23522	8342	-Gly	25.3
Tribe Euglossini							
Genus *Eufriesea*							
E. mexicana	SRR1945065	779.66.59±4523.87	0.0559±1.1037	15532			

There are typically 22 tRNAs which are identified by the one-letter for the corresponding amino acid according to the Rules (1968). 18 tRNAs each specify a single amino acid, while 2 tRNAs (Ser1 and Ser2) specify serine, and 2 tRNAs (Leu1 and Leu2) specify leucine. The "+" and "-" indicate the duplication or loss of tRNAs, and the number inside the brackets indicate the number of duplication or loss of tRNAs. The "IR" column shows the length of a single inverted repeat (IR) region.

a previous study (Li et al., 2021). To extend the sampling size and verify the accuracy of some published mitogenome sequences, we additionally downloaded the high throughput sequencing raw data of 19 Bombini samples (representing 18 species), and 14 Meliponini samples (representing 12 species) from the Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra). In addition, we also downloaded 39 Apini (representing nine species), 31 Bombini (representing 26 species), and 11 Meliponini (representing nine species) mitogenome sequences from the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/).

For the newly sampled Meliponini, genomic DNA was extracted using the standard CTAB protocol (Hunt, 1997) from a single individual after removing the head and abdomen. The purified genomic DNA was used to prepare a sequencing library with an average insert size of 400 bp using Illumina TruSeq™ DNA Sample Prep Kit (USA), then sequenced by the Illumina NovaSeq 6000 platform (USA) to generate 4 Gb of 150 bp paired-end reads.

Mitogenome assembly and annotation

The cleaned raw reads were used to *de novo* assemble mitogenomes using the GetOrganelle toolkit (Jin et al., 2018).

Table 2 Information and features of mitogenomes downloaded from NCBI

Taxa	GenBank accession No.	Genome size (bp)	IR (bp)	tRNA	GC content (%)
Tribe Apini					
Genus *Apis*					
A. andreniformis	AP018490	16694		22	14.3
A. andreniformis	NC039709	16694		22	14.3
A. cerana	AP017314	15916		22	15.9
A. cerana	AP018450	15885		22	16.0
A. cerana japonica	AP017314	15917		22	15.9
A. dorsata	AP018369	15279		22	15.3
A. dorsata	NC037709	15892		22	15.0
A. florea	AP018491	17693		+Ser1(2)	13.7
A. florea	KC170303	15993		+Ser1(2)	14.8
A. florea	NC021401	17694		+Ser1(2)	13.9
A. koschevenikovi	KY348372	16049		+Met(1)	20.9
A. koschevenikovi	AP017643	15277		+Met(2)	16.0
A. laboriosa	NC036155	15510		22	15.0
A. laboriosa	KX908208	15621		22	15.1
A. mellifera adansonii	MN585109	16466		22	15.2
A. mellifera anatoliaca	MT188686	16256		22	15.4
A. mellifera capensis	MG552691	16515		22	17.7
A. mellifera carnica	MN250878	16358		22	17.1
A. mellifera carpatica	AP018403	16336		22	15.1
A. mellifera caucasica	AP018404	16341		22	14.5
A. mellifera caucasica	MN714160	16274		22	15.2
A. mellifera iberiensis	MN585110	16560		22	20.1
A. mellifera intermissa	KY926883	16343		22	14.9
A. mellifera jemenitica	MN714161	16427		22	15.4
A. mellifera lamarckii	KY464958	16589		22	15.1
A. mellifera ligustica	MT859135	16467		22	15.0
A. mellifera meda	KY464957	16248		22	15.5
A. mellifera monticola	MF678581	16343		22	15.2
A. mellifera ruttneri	MN714162	16577		22	15.6
A. mellifera sahariensis	MF351881	16569		22	15.2
A. mellifera scutellata	MG552698	16479		22	15.2
A. mellifera simensis	MN585108	16523		22	15.1
A. mellifera sinisxinyuan	MN733955	16886		22	14.8
A. mellifera syriaca	KY926882	16343		22	15.1
A. mellifera unicolor	MN119925	16373		22	15.4
A. nigrocincta	AP018398	15516		22	15.8
A. nigrocincta	NC038114	15855		22	15.4
A. nuluensis	AP018157	15921		22	15.5
A. nuluensis	MF565375	15843		22	16.1
Tribe Bombini					
Genus *Bombus*					
B. asiaticus	MH998259	15676		22	14.8
B. breviceps	MF478986	16014		22	16.4
B. campestris	HG995151	15573		22	12.8
B. canariensis	MW959771	17300		+Leu2	13.8
B. consobrinus	MF995069	16177		22	14.1
B. difficillimus	MZ352140	19072		+Met(2)	13.8
B. filchnerae	NC082116	18553		22	11.3
B. florilegus	AP018158	15763		+Met	14.4
B. haemorrhoidalis	MZ352141	15334		+Met(2)	14.5
B. hypocrita hypocrita	AP017662	15795		22	14.5
B. hypocrita sapporensis	AP017370	15826		22	14.5
B. hypocrita sapporensis	AP018339	16133		22	14.3
B. hypocrita sapporensis	AP018481	15835		22	14.5

Continued

Taxa	GenBank accession No.	Genome size (bp)	IR (bp)	tRNA	GC content (%)
B. ignitus	DQ870926	15937		22	13.5
B. kashmirensis	MH998261	15931		22	14.9
B. lantschouensis	MW839568	16292		+Met	14.1
B. lapidarius	KT164641	16543		22	14.8
B. longipennis	MW741884	18458		22	12.8
B. opulentus	MZ352142	17262		-Val -Gln	11.2
B. pascuorum	HG995285	15740		22	12.5
B. picipes	MZ352143	18057		+Met +Tyr -Ser2	13.5
B. pratotum	OV884001	21510		+Met -Ile	11.3
B. pyrosoma	MH998260	16262		22	17.3
B. schrencki	ENT01_LC702704	15793		22	12.7
B. schrencki	ENT01_LC702705	15793		22	12.7
B. sibiricus	MH998258	16287		22	15.0
B. soroeensis	MZ352146	17107		+Met(2)	17.0
B. superbus	MZ352147	15768		22	15.9
B. terrestris lusitanicus	MK570128	15888		+Leu2 +Met	14.6
B. terrestris terrestris	NC045179			+Leu2	
B. waltoni	NC045283	16012		22	16.7
Tribe Meliponini					
Genus *Frieseomelitta*					
F. varia	CM022150	15144		22	12.2
F. varia	WNWW01002174	15144		22	12.2
Genus Tetragonisca					
T. angustula	OR030859	15414		22	12.1
Genus *Melipona*					
M. bicolor	AF466146	15001		22	13.1
M. fasciculata	OQ225244	15206		+Trp	12.9
M. scutellaris	KP202303	14862		-Gln	13.2
M. scutellaris	NC026198	14862		-Gln	13.2
Genus *Lepidotrigona*					
L. flavibasis	MN747147	15408		22	21.7
L. terminata	MN737481	15431		22	23.5
Genus *Tetragonula*					
Subgenus *Tetragonula*					
T. carbonaria	OQ918628	30665	15215	-Glu/-Gly	27.9
T. hockingsi	OQ918629	30662	15224	-Glu/-Gly	27.8

Same with Table 1.

The main assembly process was conducted using the command "get_organelle_from_reads.py", which exploits Bowtie2 (Langmead & Salzberg, 2012) to recruit mitogenome-associated reads, then performs *de novo* assembling of all recruited reads using SPAdes (Bankevich et al., 2012). FASTA assembly Graph ("FASTAG") files from the outputs of SPAdes were trimmed by removing non-mitogenomic contigs, then were used to generate the complete mitogenome sequence. For samples whose complete mitogenome sequences could not be automatically exported, their FASTAG file was visualized by Bandage (Wick et al., 2015) and we manually filtered the path to export complete mitogenome sequences. Finally, we assessed all assembled mitogenome sequences using the command "evaluate_assembly_using_mapping.py".

Mitogenome annotation was performed using the MITOS web-server (http://mitos.bioinf.uni-leipzig.de/index.py) (Bernt et al., 2013). MITOS exploits MiTFi (Jühling et al., 2012) to annotate tRNAs. The open reading frames (ORFs) were annotated with the program Geneious Prime 2021.1.1 (Biomatters, New Zealand) to help adjusting start and stop codons manually. For mitogenome sequences downloaded from NCBI, their tRNAs were re-annotated using the same method. The gene maps of annotated mitogenomes were drawn on the online server OGDRAW (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html) (Greiner et al., 2019).

PCR verification of inverted repeats

The FASTAG file in Bandage (Wick et al., 2015) showed that some Meliponini species had amphimeric mitogenomes with one or two pairs of long IRs (Figure 1B). To verify the existence of IRs, we chose mitogenome sequences of six species (*Lepidotrigona flavibasis*, *Lepidotrigona terminata*, *Tetragonula collina*, *Tetragonula laeviceps*, *Tetragonula gressitti*, and *Tetragonula pagdeni*) with multiple samples as template sequences to design primers for PCR amplification and sequencing to verify the boundaries between the IR and SC regions. When these amplicon sequences were consistent with these template sequences, then the existence of the IRs were confirmed.

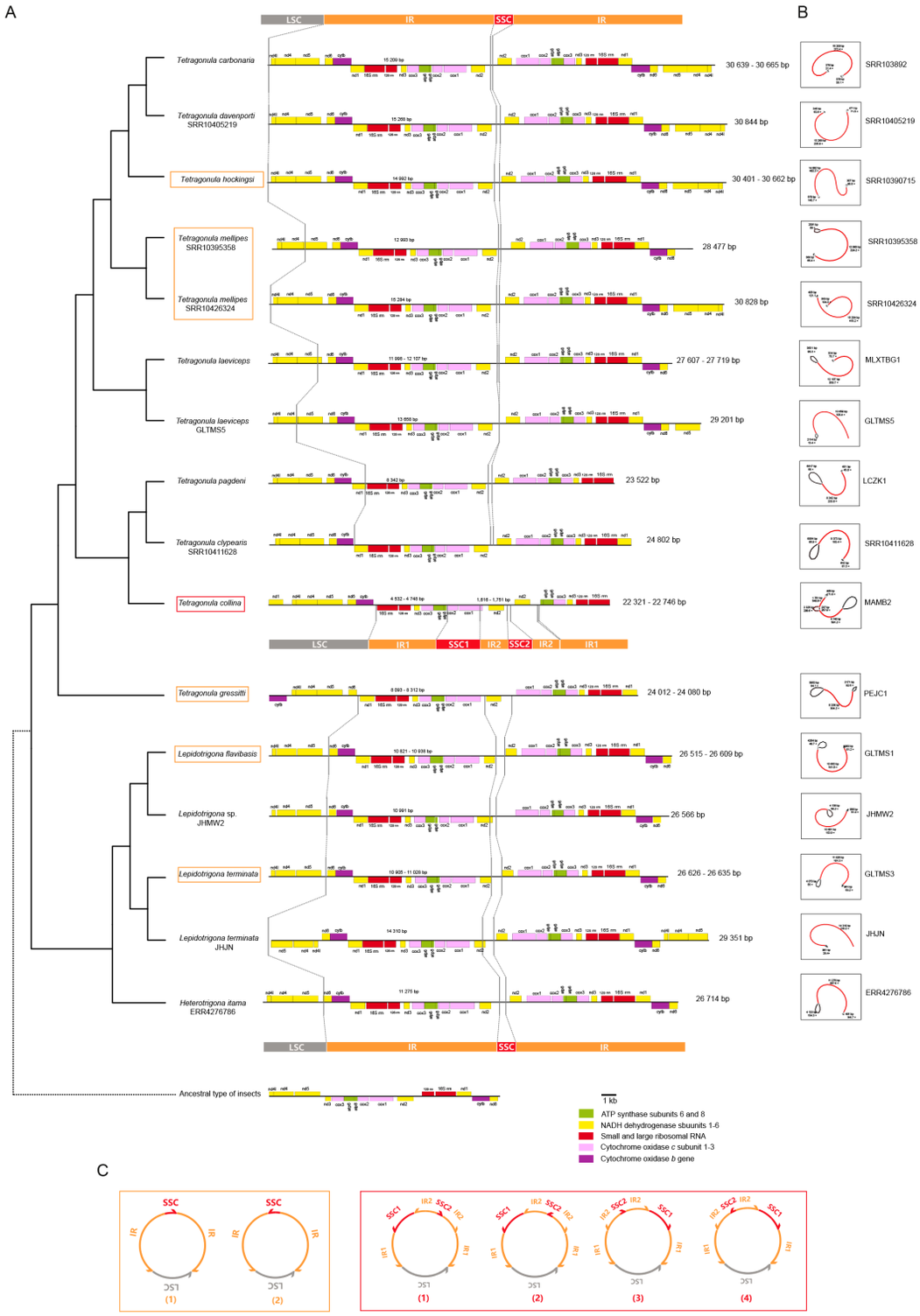

Figure 1 Mitogenome organizations for Meliponini taxa with inverted repeats

A: Linearized mitogenomic maps (in scale) of Meliponini taxa with inverted repeats and the typical type of insects. Gene directions are identified by whether they are above or below the black bar. Only rRNAs and PCGs are displayed in this map. Grey lines indicate inverted repeat boundaries in mitogenome sequences. The mitogenome lengths are given behind each map. For taxa with multiple samples, mitogenomic map of only one sample were drawn as a representative of the other samples which share the same IR boundaries, and the range of mitogenomes lengths are given. B: Target complete assembly graphs of mitogenomes of Meliponini taxa generated by GetOrganelle, with inverted repeated regions indicated by red lines and single copy regions indicated by black lines. The voucher numbers are given near the corresponding graph, and each graph corresponds to the mitogenomic map on the left in A. C: Two structural haplotypes (within the yellow box) detected in *Tetragonula hockingsi*, *Tetragonula mellipes*, *Tetragonula gressitti*, *Lepidotrigona terminata*, and *Lepidotrigona flavibasis* (framed by the yellow box in A), and four structural haplotypes (within the red box) detected in *Tetragonula collina* (framed by the red box in A). The gray regions are long single copy (LSC) regions, the yellow regions are inverted repeat (IR) regions, and the red regions are small single copy (SSC) regions.

When designing primers for the target regions, all mitogenome sequences of the same species were aligned, then we chose manually the highly conserved regions (usually within coding genes, nearly without nucleotide polymorphisms) to design primers with sizes of 20–25 bp. The GC contents (the content percent of guanine and cytosine in the nuclear sequence) of sense and anti-sense primers were designed to approximate to each other, so that they had similar annealing temperature. And adenine (A) was avoided at the 3' end of primers. The designed primers for each species are provided in Supplementary Table S2. For each species, three individuals from different sampling sites were used for PCR verifications. Genomic DNA of eighteen samples was extracted separately from a single individual by removing head and abdomen according to the protocol of the TIANamp Genomic DNA Kit. PCR amplification was performed with the designed primers using the reaction system (20 μL) as follows: ddH_2O (8 μL), 2×Taq PCR Mastermix (Tiangen, China) (10 μL), sense primers (10 P , 0.5 μL), anti-sense primers (10 P , 0.5 μL), and DNA templates (1 μL). After a series of PCR amplification tests, three PCR amplification programs could successfully amplify all pairs of primers with high quality amplicons. The detailed information for the three programs is as follows: (1) Program1: an initial denaturation step for 2 min at 95 °C, followed by 14 cycles of denaturation (95 °C,30 s), annealing (39 °C, 20 s), and elongation (72 °C, 20 s), followed by another 25 cycles of denaturation (94 °C, 1 min), annealing (46 °C, 20 s), and elongation (72 °C, 20 s), and a final extension for 10 min at 72 °C. (2) Program2: an initial denaturation step for 2 min at 95 °C, followed by 14 cycles of denaturation (95 °C,40 s), annealing (42 °C, 40 s), and elongation (72 °C, 2 min), followed by another 25 cycles of denaturation (95 °C,40 s), annealing (54 °C, 40 s), and elongation (72 °C, 2 min), and a final extension for 10 min at 72 °C. (3) Program3: an initial denaturation step for 2 min at 95 °C, followed by 32 cycles of denaturation (95 °C,30 s), annealing (temperature ranging from 42 to 54 °C, 30 s), and elongation (72 °C, 40 s), and a final extension for 10 min at 72 °C. All PCR products were preserved at 4 °C before further treatment. The specific PCR amplification program used for each pair of primers is listed in Supplementary Table S3.

All PCR products were evaluated using agarose gel electrophoresis to visualize the quality and length with the size marker. When the size of a high quality amplicon was consistent with the size of the temperate sequence, then the amplicon was selected to sequence. Agarose gel electrophoresis was performed on electrophoresis apparatus. The completely dissolved PCR products were mixed with the 6× Loading Buffer at a ratio of 5:1, and then were added into the gel point. The DL1000 Marker was added simultaneously. After connecting the positive and negative of the electrophoresis tank, the voltage was set at 160 V to perform electrophoresis for 40 min. After electrophoresis, the gel was removed and observed under the UV imaging system and photographed under the gel imager system. PCR products were purified using the Qingke PCR Purification Kit based on the paramagnetic particle method according to the manufacturer's protocol. DNA sequencing was carried out using the BigDye terminator kit on an ABI 3730 XL sequencer (USA).

Phylogenetic analyses

To infer phylogenetic relationships among the three tribes of social bees, 154 mitogenome sequences representing 72 species and one orchid bee *Eufriesea mexicana* were sampled, with the mitogenome of *Ceratina okinawana* (MW281319) from tribe Ceratinini as the outgroup. Thirteen PCGs were extracted from the annotated mitogenome separately and then each PCG aligned as codons using the invertebrate mitochondrial code for translation align. All PCG alignments were concatenated into a supermatrix dataset. The above operations were conducted in Geneious Prime 2021.1.1 (Biomatters, New Zealand). The supermatrix dataset was loaded into PartitionFinder2 (Lanfear et al., 2017) to select the best-fit partitioning schemes and nucleotide substitution models for downstream phylogenetic analyses. The partitioning schemes were based on 39 gene sets of sites, with one set defined separately for the 1st, 2nd and 3rd codon sites in each of 13 PCGs. The model selection and partitioning scheme comparison were performed using the Bayesian Information Criterion (BIC), and the scheme comparison were compared based on a heuristic search algorithm using the set "search=greedy" (Guindon et al., 2010; Lanfear et al., 2012).

Maximum Likelihood (ML) and Bayesian Inference (BI) phylogenies were inferred separately by RAxML-HPC2 v8.2.12 (Stamatakis, 2014) and MrBayes v3.2.7a (Ronquist & Huelsenbeck, 2003) with the online server CIPRES (http://www.phylo.org/). For ML analysis, the supermatrix dataset was divided into 12 partitioning schemes found by Partition Finder2. GTR+CAT was chosen as the model for the bootstrapping estimation, and the bootstraps replicates was set at 1 000 to estimate the Bootstrap support values (BS). For BI analysis, the dataset was divided into 12 subsets found by Partition Finder2, with either GTR+Γ+I or GTR+Γ as the nucleotide substitution model. Markov Chain Monte Carlo (MCMC) analyses were run for 4 000 000 generations, and the posterior probabilities (PP) were estimated. The BI analysis started with a random tree and sampled every 100 generations. The BI analysis found that the standard deviation of split frequencies was 0.0041 (<0.01 as requested), the potential scale reduction factor (PSRF) was equal to 1.00, and the estimated sample size (ESS) above 100, which indicated that the two independent runs were convergent. The first 10 000 trees were discarded as burn-in 25%, and the remaining 30 000 trees were used to calculate a majority-rule consensus tree. The phylogenetic trees were viewed and edited with the FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Molecular dating

We used the MCMCTree program of the PAML package (Yang, 1997, 2007) to estimate the species divergence times. We pruned the ML tree to include only one representative for each of the 72 species. We included five time calibrations in the tree based on molecular dating results in previous studies: (1) the most recent common ancestor (MRCA) of the three tribes was set at 80–90 Ma (Cardinal et al., 2018); (2) the MRCA of Bombini and Meliponini was set at 70–80 Ma (Cardinal et al., 2018); (3) the MRCA of the Meliponini clade was set at 70–80 Ma (Rasmussen & Cameron, 2010); (4) the MRCA of the Old World lineage of Meliponini was set at 50–60 Ma (Rasmussen & Cameron, 2010); (5) the MRCA of the Neotropical lineage of Meliponini was set at 30–40 Ma (Rasmussen & Cameron, 2010). The input sequence data was the alignment of 13 PCGs, which was divided into three partitions corresponding to the 1st, 2nd and 3rd codon sites

using the online tools Split Codons (https://www.bioinformatics.org/sms2/split_codons.html) within the Sequence Manipulation Suite (Stothard, 2000). We estimated the divergence times using the approximate likelihood method (Reis & Yang, 2011). We used the independent rates clock model and the GTR for the substitution model. For our analyses, MCMC ran for 400 000 +10×100 000=1 400 000 iterations, which means the program discarded the first 400 000 iterations as burn-in, and then sampled every 10 iterations until it had gathered 100 000 samples.

Reconstruction of ancestral mitogenome organization

We used the *ace* function in the R package *phytools* (Revell, 2012) to estimate the ancestral state of mitogenome organization in the three tribes. We pruned the BI tree to include only one representative for each of the 72 species. We fitted the Equal-Rates (ER) model and estimated ancestral states for three discrete characters of mitochondrial organization: (1) IR and gene rearrangement; (2) normal type; (3) gene rearrangement.

Molecular evolution estimations for the lineage with long inverted repeats

To test whether the nonsynonymous/synonymous rate ratios (d_N/d_S) of mitochondrial PCGs differ between lineages with or without IRs, we conducted codeml analysis (Yang, 1997) using EasyCodeML (Gao et al., 2019). Two phylogenetic trees were used for estimations: a big phylogeny of the three tribes of social bees and a small phylogeny of Meliponini. The BI tree topology was adopted for this analysis, but with one representative for each species. Two models were used for the likelihood analyses: (1) one-ratio model which assumes the same d_N/d_S ratio (ω) for all branches in the phylogeny; (2) two-ratio model which assumes that the branches with long IRs have a different d_N/d_S ratio (ω_1) from the background ratio (ω_0) (Yang, 1998). The lineage with IRs was specified as foreground branches in both big and small phylogenies. Each mitochondrial PCG was extracted from all taxa appearing in the tree to conduct translation alignment in Geneious Prime 2021.1.1 (Biomatters, New Zealand). Gaps and stop codons were removed from PCG alignments manually. The gene alignment file and labelled tree file were imported into EasyCodeML software and run using the preset branch model. The program conducted the likelihood ratio test to examine whether the two-ratio model fits the data better than the one ratio model.

RESULTS

Mitogenome organization

Mitogenome organizations of Apini and Bombini taxa are the typical type of insects, with only duplications or losses of tRNAs in some taxa (Tables 1, 2). For the Meliponini, mitogenomes are amphimeric circles with a pair of long IRs in *Tetragonula*, *Lepidotrigona*, and *Heterotrigona* species, and two pairs of long IRs in *Tetragonula collina* (Figure 1A, B). Duplications of large gene blocks enlarged mitogenome sizes ranging from 22 kb to 30 kb. The structure of mitogenomes with a single pair of IRs resemble the quadripartite structure of chloroplast genomes in land plants, with a large single copy (LSC) region and a small single copy (SSC) region separated by IRs. Among samples of *T. gressitti*, *T. hockingsi*, *T. mellipes*, *L. terminata*, and *Lepidotrigona flavibasis*, we found two structural isomers, which differ by the orientation of a single SSC region. For *T. collina*, we found four structural isomers formed by the permutation and combination of two opposite directed SSC regions (Figure 1C).

For lineages with IRs, the IR boundaries are variable among species. For example, *Tetragonula* species have varied IR boundaries: IRs of *T. carbonaria*, *T. davenporti*, *T. hockingsi*, and *T. mellipes* were almost completely expanded with nearly all genes duplicated. While, in contrast, IRs were shorter and only a part of all genes were duplicated in the remaining species (Figure 1A). IR boundaries also varied among samples from the same species as shown in the following examples: the enlarged IRs in sample GLTMS5 of *Tetragonula laeviceps* and sample JN of *L. terminata* compared with the other samples of the same species, and the different IR boundaries between samples SRR10426324 and SRR10395358 of *T. mellipes* (Figure 1A). The complete assembly graphs of the mitogenomes produced by the GetOrganelle toolkit showed that read depths of IRs are generally more than twice the read depth of SCs (Figure 1B), and the gel electrophoresis image of PCR products further confirmed the existence of IRs (Figure 2). The amplified sequences were sequenced and can be mapped to the expected regions in the mitogenomes. Thus, the existence of IRs in mitogenomes of Meliponini taxa was confirmed.

Phylogenetic analyses

Topologies of ML and BI trees are almost the same, only with some incongruences in weekly supported nodes within Bombini (Supplementary Figure S1). Both phylogenetic analyses strongly support the Meliponini as sister to Bombini, then the two tribes as sister to Apini (BS=100, PP=1.00), and *Eufriesea mexicana* sister to the three tribes of social bees. The Meliponini is clearly divided into three clades corresponding to their geographical distributions, with the Indo-Malay/Australian and Afrotropical clades forming the sister group to the Neotropical clade (BS=100, PP=1.00). The only exception was *Lisotrigona carpenteri* from Yunnan, China, which falls into the Afrotropical clade (BS=84, PP=0.95) (Supplementary Figure S1). Nine *Tetragonula* species (belonging to two subgenera *Tetragonula* and *Tetragonilla*) are clustered as a monophyletic clade, however two species belonging to subgenus *Tetragonula*, *T. pagdeni* and *T. clypearis*, form a clade which is sister to *T. (Tetragonilla) collina* (BS=53, PP=0.65), instead of clustering with the other species from subgenus *Tetragonula*. Both ML and BI topologies support *T. gressitti* as the sister group to the group consisting of the other species from genus *Tetragonula* (BS=90, PP=0.68) (Supplementary Figure S1). For Bombini, except for subgenera *Mendacibombus* and *Kallobombus*, which diverged early, the other taxa can be classified into the short-faced clade and long-faced clade (Supplementary Figure S1).

Molecular evolution analyses for the lineage with long inverted repeats

The branch model test showed that for the phylogenies of both the three social bee tribes and the Meliponini, the lineage with IRs has lower estimated d_N/d_S ratios for mitochondrial PCGs than lineages without IRs. The differences between the one-ratio model and two-ratio model were significant (likelihood ratio test P-value <0.05) in both phylogenies, indicating that the d_N/d_S ratios differ between lineages with and without IRs (Table 3).

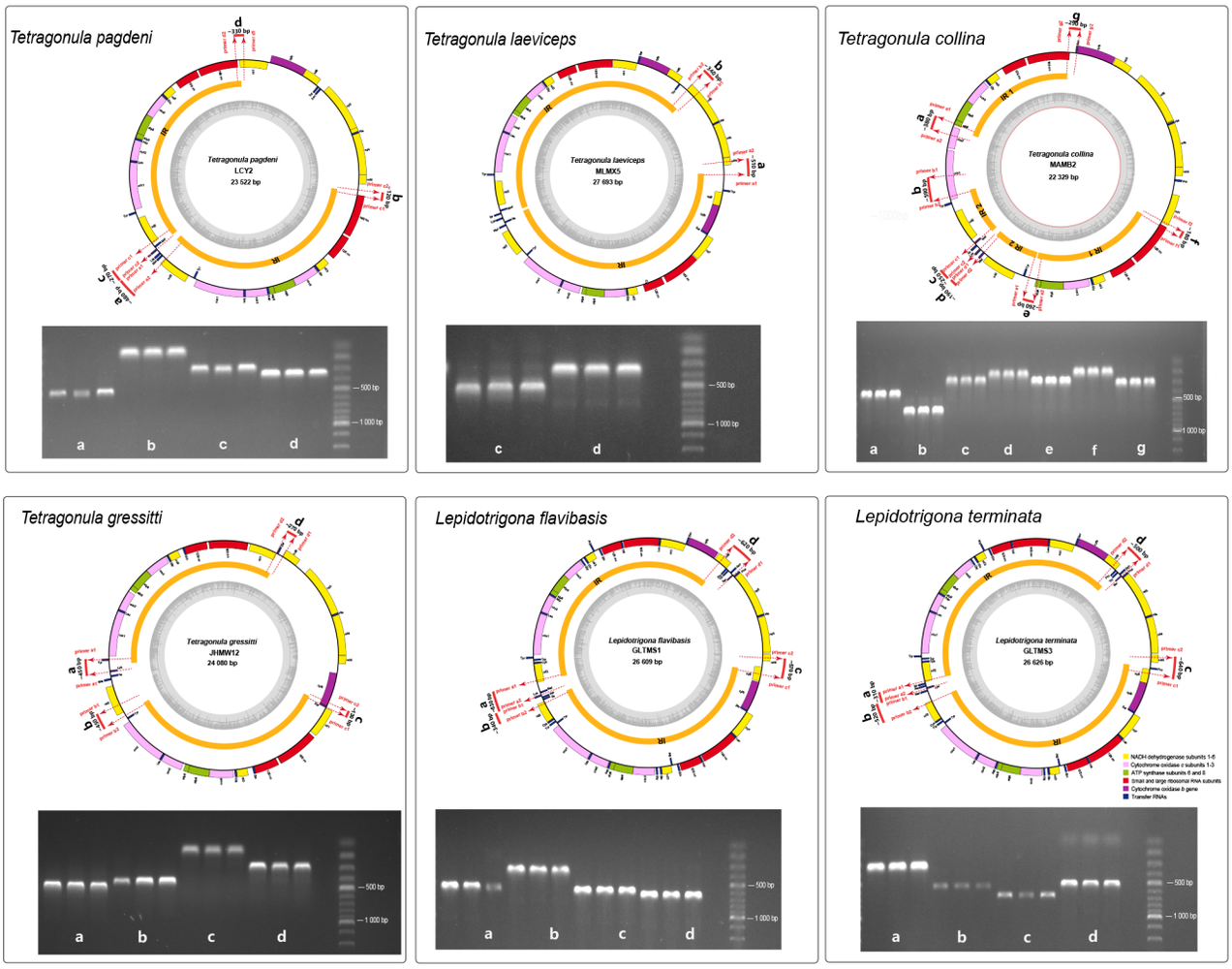

Figure 2 Gel electrophoresis image of PCR products amplified by designed primers and the distribution of primers in the mitogenomes of six Meliponini taxa with inverted repeats.

Gene rearrangements in the social bees

In comparison with the typical insect mitogenome, two monophyletic lineages within Meliponini showed extensively reorganized mitogenomes. One is the lineage consisting of species with long IRs in the Indo-Malay/Australian clade. For this lineage, gene block *nd6-cytb-nd1-srRNA-lrRNA was* inverted, and swapped position with gene block *nd2-cox1-cox2-atp8-atp6-cox3-nd3.* Another is the lineage consisting of *Frieseomelitta varia*, *Tetragonisca angustula* and *Nannotrigona testaceicornis* within the Neotropical clade, for which the gene block *nd2-cox1-cox2-atp8-atp6-cox3-nd3* was inverted, and the gene block *nd6-cytb* was reversely transposed, leading to the same direction of all PCGs (Figures 3, 4).

The tRNAs show rearrangements in all three tribes and some hotspots were found. One hotspot is the cluster *trnE-trnS1*. In Apini, *trnE-trnS1* moved from cluster *trnF-trnE-trnS1-trnN* to upstream of *srRNA*. Additionally, *trnE* and *trnS1* swapped positions in *A. mellifera*, and *trnS1* was tandemly duplicated in *A. florea.* In Bombini, *trnE* and *trnS1* swapped positions in most taxa, and *trnE* also moved to downstream of *nd3* in the subgenus *Psithyrus*. In *Bombus polaris,* the *trnS1-trnE-trnN-trnR* block inversed and moved to the downstream of *nd6*. Cluster *trnE-trnS1* has not rearranged in taxa within Meliponini, except for taxa belonging to the two lineages with extensively reorganized mitogenomes (Figure 3). Another hotspot was cluster *trnP-trnT*. In the short-faced clade of Bombini, *trnP* and *trnT* swapped positions, while *trnP* was also tandemly duplicated in *Bombus vancouverensis*, and *trnT* and *trnF* inversed and swapped positions with each other in *B. polaris*. In Meliponini, *trnT* inversed in *L. carpenteri*, *Plebeina hildedrandti*, *Melipona bicolor*, *Melipona scutellaris*, *F. varia*, and *T. angustula*; *trnT* inversed and swapped positions with *trnP* in *Melipona quadrifasciata, Melipona fasciculata* and *Melipona variegatipes* (Figure 3). The last one is cluster *trnD-trnK*. Swapped positions of *trnK* and *trnD* were found across the whole tribe of Apini and Bombini, while transposition of *trnK* was also found in *L. carpenteri*, *P. hildedrandti*, and *Melipona* species in Meliponini (Figure 3). There were also some shared rearrangements among the three tribes. For example, *trnA* moved to the cluster *trnM-trnQ-trnI*, though the gene positions within this cluster varied a lot across lineages (Figure 3).

For taxa in the Indo-Malay/Australian clade of Meliponini, tRNA rearrangements occur with large blocks of genes. These rearrangements were so dramatic that it was nearly impossible to compare them with the ancestral type. One interesting finding is an ambiguous tRNA in genus *Tetragonula* species with multiple samples, including *T. laeviceps*, *T. pagdeni*, *T. gressitti*, and *T. collina*. Some samples within the species presented *trnG* (glycine) between *cox3* and *nd3* genes, while other samples presented *trnE*

Table 3 Estimated d_N/d_S ratios of branches under different models and *P*-value of likelihood ratio test (LRT) of the branch model using EasyCodeml

	Estimated d_N/d_S ratio (omega) for two ratio Model 2		Estimated d_N/d_S ratio for one ratio Model 0	LRT *P*-value (Model 0 vs Model 2)
	Omega of foreground branch (ω1)	Omega of background (ω0)	Omega of all branches (ω)	
Phylogeny of Meliponini				
atp6	0.00614	0.05818	0.05534	0.000670464**
cox1	0.00082	0.05814	0.05534	0.000690563**
cox 2	0.00300	0.04307	0.03931	0.000000118**
cox 3	0.00498	0.07305	0.03492	0.000000000**
cytb	0.00907	0.03685	0.03492	0.000232998**
nd1	0.00163	0.03708	0.03229	0.000002739**
nd2	0.00209	0.13369	0.11868	0.00000000**0
nd3	0.01195	0.08911	0.08421	0.010207148*
nd4	0.00435	0.03319	0.03212	0.000580657**
nd4L	0.00075	0.03218	0.02988	0.026699323*
nd5	0.00368	0.02748	0.02642	0.000315853**
nd6	0.00498	0.07305	0.06688	0.000000000**
Phylogeny of three tribes				
atp6	0.03803	0.00091	0.03690	0.000004161**
cox1	0.00027	0.00963	0.00916	0.000000000**
cox 2	0.00177	0.02611	0.02503	0.000000004**
cox 3	0.00181	0.05632	0.05456	0.001325546**
cytb	0.00503	0.02007	0.00916	0.000000000**
nd1	0.00177	0.02611	0.02503	0.000000004**
nd2	0.00058	0.08153	0.07673	0.000000000**
nd3	0.00181	0.05632	0.05456	0.001325544**
nd4	0.00081	0.02502	0.02461	0.000001683**
nd4L	0.00101	0.01809	0.04750	0.000000000**
nd5	0.00067	0.02124	0.02069	0.000000405**
nd6	0.00181	0.04130	0.03936	0.001551061**

atp6: ATP synthase subunits 6; *cox1-3*: Cytochrome oxidase subunit Ⅰ, Ⅱ, Ⅲ; *cytb*: Cytochrome *b* apoenzyme; *nd1-6,4L*: NADH dehydrogenase subunits 1-6, 4L. *: $P<0.05$; **: $P<0.01$

(glutamic acid) between *cox3* and *nd3* genes (Figure 3). The defining difference between the two tRNA is whether the second position of the anticodon is a cytosine (for *trnG*) or a thymine (for *trnE*) (Supplementary Figure S2).

Reconstruction of ancestral mitogenome organizations

We estimated the evolution of mitochondrial organizations among three tribes of social bees. The results showed that long IRs may have occurred once in the common ancestor of the Indo-Malay/Australian clade of Meliponini at 48.36 Ma, along with duplications and rearrangements of genes (Figure 4). The gene rearrangements found in the monophyletic lineage consisting of *F. varia*, *T. angustula* and *N. testaceicornis* may have occurred once in their common ancestor at 22.70 Ma (Figure 4).

DISCUSSION

Significance of mitogenome phylogeny

Meliponini and Apini are the only two highly eusocial groups within Apidae, but all studies, including this one, support a topology of (Meliponini+Bombini)+Apini (Almeida et al., 2023; Cardinal et al., 2018, 2010; Danforth et al., 2013; He et al., 2018; Kapheim et al., 2015; Kawakita et al., 2008). The phylogenetic relationships within Bombini and Apini based on mitochondrial genes reported here are largely consistent with previous studies based on nuclear and/or mitochondrial genes (Arias & Sheppard, 2005; Cameron et al., 2007; Lo et al., 2010; Ramírez et al., 2010), showing that mitochondrial genes can recover a robust phylogeny and evolutionary history for the three tribes of social bees.

Among the three tribes, Meliponini is the largest with 605 described species in 45 extant genera throughout the tropical and subtropical regions (Engel et al., 2023). Phylogenies of Meliponini have been reported in several previous studies (Christy & Roesma Dahelmi, 2019; Costa et al., 2003; Ramírez et al., 2010; Rasmussen & Camargo, 2008; Rasmussen & Cameron, 2007), but the global phylogeny is still not well resolved. The phylogenetic relationships between genera based on mitochondrial genes reported here (Supplementary Figure S1) are largely consistent with the global phylogeny based on five mitochondrial and nuclear gene sequences (Rasmussen & Cameron, 2010). An interesting finding is the phylogenetic placement of *T. gressitti*, which was not included in previous phylogenies. According to the most updated classification, *T. gressitti* belongs to the genus *Tetragonula*, which consists of two subgenera, *Tetragonula* and *Tetragonilla* (Engel et al., 2023). However, our phylogeny strongly supports that *T. gressitti* is sister to the group consisting of the other sampled genus *Tetragonula* species (Supplementary Figure S1). Moreover, workers of *T. gressitti* differ from the other subgenus *Tetragonula* species in several characters: 1) the whole body of *T. gressitti* is black vs

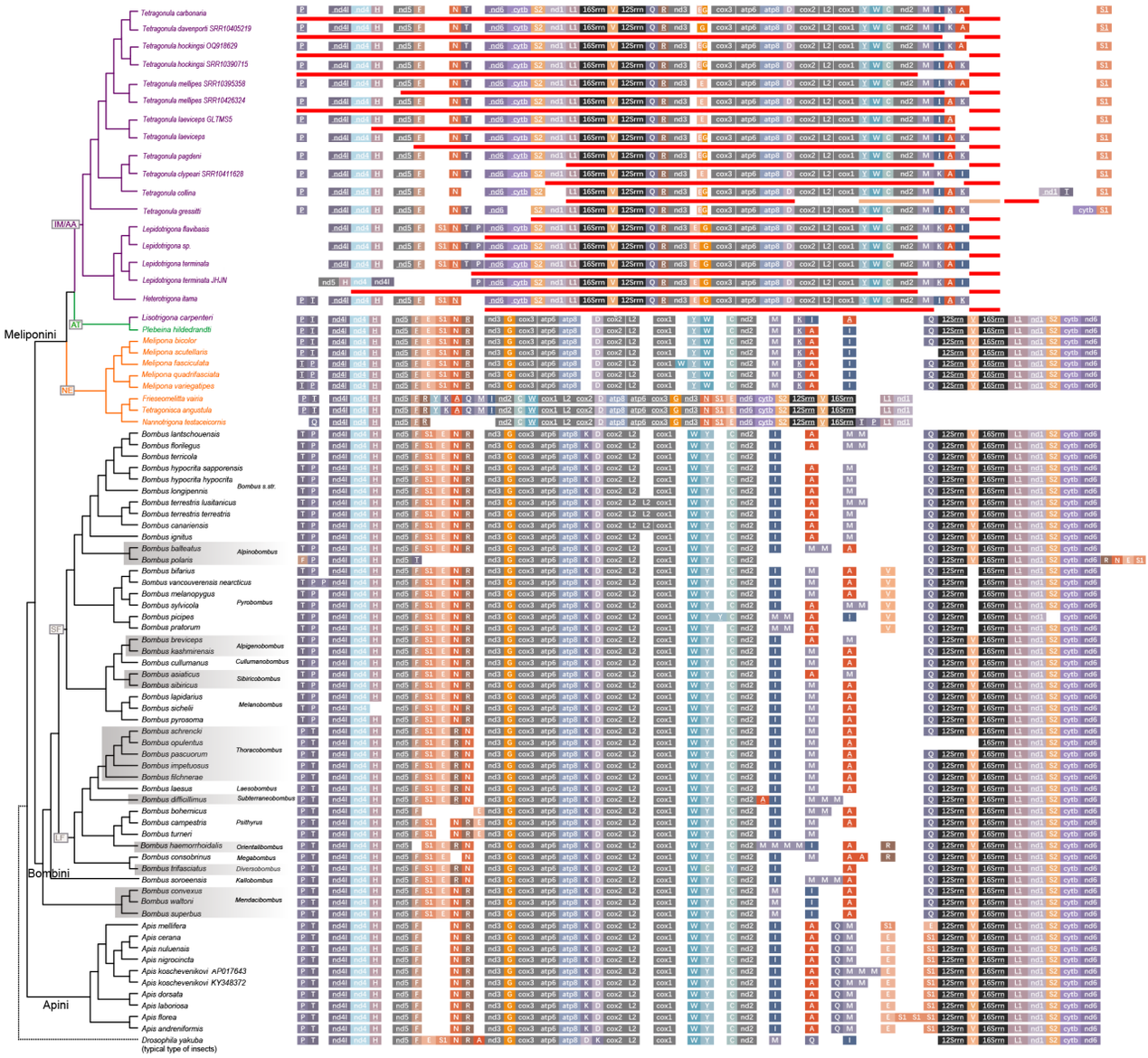

Figure 3 Phylogenetic patterns of mitochondrial gene rearrangements of the three tribes of the social bees

The topology was modified from the Bayesian tree. The colored rectangles represent mitochondrial genes. PCGs and rRNAs are abbreviated as in the text, while tRNAs are identified by the one-letter for the corresponding amino acid according to the Rules (1968). Lines under gene names indicate that the reading directions of the genes are right-to-left. The red lines under the gene block regions indicate the IR regions, with only one set of genes in IRs shown. Abbreviations of geographical distribution for lineages of Meliponini: IM/AA, Indo-Malay/Australasia clade; AT, Afrotropical clade, NE; Neotropical clade. Abbreviations of morphological lineages of Bombini: SF, short-faced clade; LF, long-faced clade.

more or less pale in other species; 2) the head of *T. gressitti* is relatively round vs more or less transverse in other species (Sakagami, 1978). Both morphology and mitogenomics therefore suggest that *T. gressitti* may represent a new subgenus within genus *Tetragonula* (Engel et al., 2023), but more detailed studies are needed to verify this.

Evolution of long inverted repeats in the mitogenome of Meliponini

In this study, long IRs were found exclusively in the Indo-Malay/Australian clade of Meliponini and are estimated to have originated once in their common ancestor at 48.36 Ma (Figure 4). Assembly graphs displayed the amphimeric mitogenomes with one or two pairs of long IRs, which have been verified by PCR amplification and sequencing. Recently, Françoso et al. (2023) also found long IRs in the mitogenomes of Australian species *T. hockingsi* and *T. carbonaria*, which have been confirmed from different samples of the two same species in this study. Although mitogenomes of *L. terminata* and *T. pagdeni* have long IRs in our assemblies, previous studies failed to identify IRs in *L. terminata* (Wang et al., 2020), *L. flavibasis* (Wang et al. 2021), and *T. pagdeni* (Wang et al., 2022). Firstly, it is only possible to detect IRs using *de novo* assembly. Assembly methods that depend on a reference mitogenome may not produce the real structure of assembled mitogenomes. Secondly, as IRs have not been reported before, mitogenome assembly programs which only take known mitogenome structures into consideration cannot produce complete mitogenome sequences with IRs directly. It is therefore necessary for mitogenome assembly programs to re-recognize the variation of mitogenome organizations, and to bring the uncommon structure reported here into their reference database of animal mitogenomes.

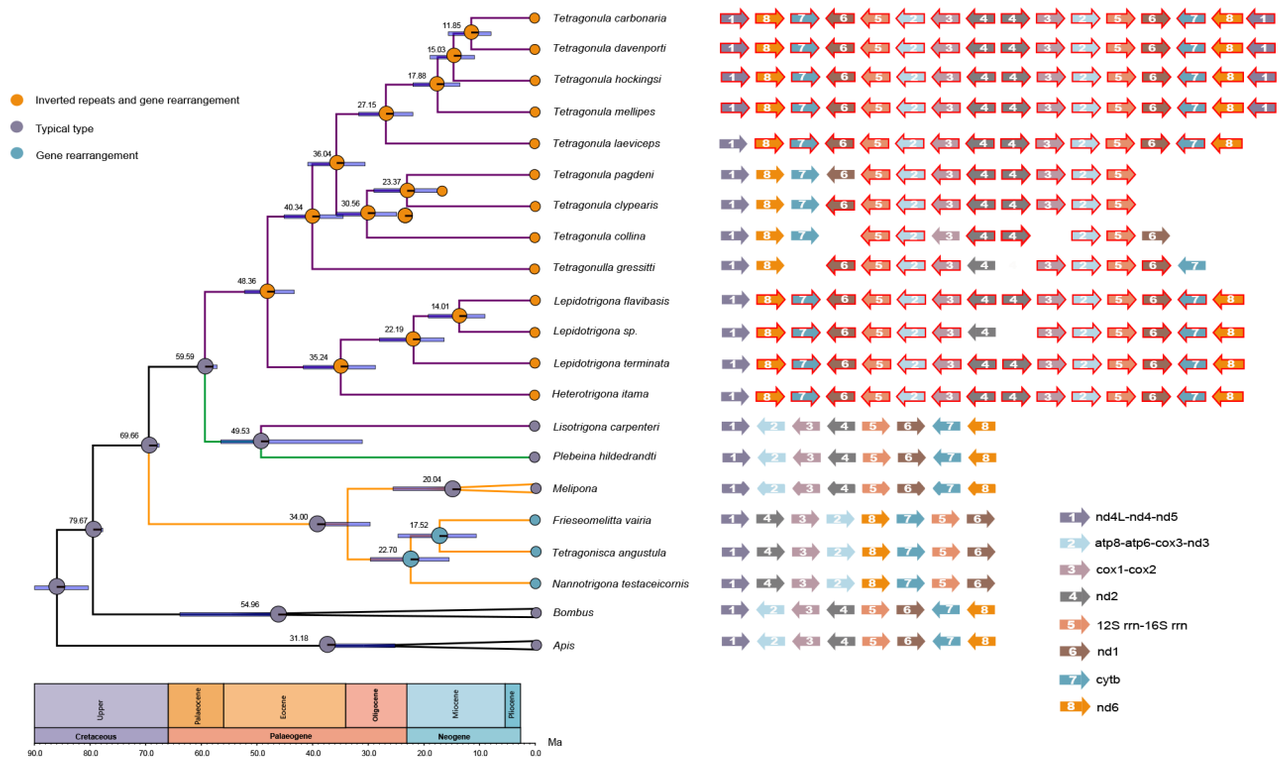

Figure 4 Ancestral reconstruction of mitogenome structures and gene rearrangement of three tribes of social bees

Gene blocks are represented by arrows with numbers, and the directions of arrows indicate the reading direction. Arrows with red boundaries represent gene blocks that are inversely repeated. PCGs and rRNAs are abbreviated as in the text. Abbreviations of geographical distribution for lineages of Meliponini: IM/AA, Indo-Malay/Australasia; AT, Afrotropical; NE, Neotropical.

The amphimeric mitogenome is a rare organization in insects, as well as animals (Cameron, 2014; Kolesnikov & Gerasimov, 2012; Rayko, 1997), therefore the evolutionary significance of the long IRs in mitogenomes remains unclear. Françoso et al. (2023) found long IRs in *T. hockingsi* and *T. carbonaria* and compared their mitogenomes with published mitogenomes of another two stingless bee species, *L. flavibasis* (MN747147) and *M. bicolor* (AF466146). This showed that the mitogenome of *L. flavibasis* shares the same specific rearrangements with *T. hockingsi* and *T. carbonaria*, but has no IRs in the sequence. In this study, we assembled the mitogenome of *L. flavibasis* from 12 samples having IRs, and phylogenetic analyses supported *L. flavibasis* as monophyletic including the published mitogenome (Supplementary Figure S1). Based on large-scale taxa samplings and accurate assemblies, ancestral state inference suggested the IRs of amphimeric mitogenomes are more likely to have occurred once in the common ancestor of the Indo-Malay/Australian clade, not only restricted to the Carbonaria species complex (including at least *T. carbonaria*, *T. hockingsi*, *T. davenporti*, and *T. mellipes*) as proposed by Françoso et al. (2023). The lengths of IRs show variations within the genus or species because of IR elongation or constriction, which should cause gene translocations or gene losses.

The results of the branch model test showed that for the phylogenies of both the three tribes and of the Meliponini, the lineages with IRs have lower estimated d_N/d_S ratios for mitochondrial PCGs than the lineages without IRs (Table 3), indicating the potential effect of IRs on gene evolution. There are 98 extant species from 11 extant genera in the Indomalayan, Papuasian, and Australian regions (Engel et al., 2023), while two genera, *Lisotrigona* and *Austroplebeia*, appear in the Afrotropical clade instead of the Indo-Malay/Australian clade (Rasmussen & Cameron, 2010). This study only sampled 13 species from 3 genera in this clade, so more comprehensive sampling is needed to understand the comprehensive pattern of mitogenome structures within the Meliponini and how this structural variation evolved across lineages.

Evolutionary significance of gene rearrangements

There are three types of gene rearrangements: transpositions (changes of gene positions), inversions (changes of gene directions), and inverse transpositions (changes of both directions and positions) (Cameron, 2014). Mechanisms proposed to explain these rearrangements include tandem duplication and random loss model (TDRL) (Moritz et al., 1987), tandem duplication and non-random loss model (TDNR) (Lavrov et al., 2002), and inter/intramolecular recombination (Yokobori et al., 2004). TDRL and TDNR models can explain the transpositions, while recombination can explain the inversions and inverse transpositions (Cameron, 2014).

PCGs and rRNAs are usually conserved within mitogenomes, but in the Meliponini, significant gene rearrangements of PCGs and rRNAs occur alongside IRs for taxa in the Indo-Malay/Australian clade (Figure 4). For this lineage, unknown mechanisms aside from the above-mentioned models might contribute to the formation of IRs and significant gene rearrangement. Another pattern of significant gene rearrangements of PCGs and rRNAs was found in a monophyletic lineage within the Neotropical clade (Figure 4) and a process of inter/intramolecular recombination might contribute to this. de Paula Freitas et al. (2020) speculated

that the Meliponini are a hotspot in mitogenome evolution based on the mitogenome in *F. varia*. In this study, our results strongly support that extensive gene rearrangements occurred once in the common ancestor of the monophyletic lineage within the Neotropical clade, and that the Indo-Malay/ Australian clade also had amphimeric mitogenomes and dramatic gene rearrangements.

Previous studies (Dowton & Austin, 1999; Dowton et al., 2003, 2009; Peters et al., 2017; Silvestre & Arias, 2006; Wang et al., 2020, 2021, 2022; Wei et al., 2010; Zheng et al., 2018) suggest that tRNA rearrangements are prevalent across different taxonomy levels within Hymenoptera, and can provide phylogenetic information. However, all these studies only sampled one or very few species to represent the whole genus or tribe, ignoring the influence of gene rearrangements at lower taxonomic level. Through denser taxonomic sampling across three tribes, this study shows that tRNA rearrangements are significant even at the species level (Figure 3). Some tRNA rearrangements are consistent across all samples as previous studies have reported, such as the inversion of *trnR* reported in Apoidea (Dowton et al., 2009). However, most tRNA rearrangements reported in specific lineages by previous studies are not consistent across all taxa within the lineage. For example, the inverse transposition of *trnK* reported in Meliponini (Cameron, 2014) is invalid for taxa with IRs and *F. varia*. The rearrangement of *trnI-trnQ-trnM* to *trnM-t trnqQ-trnA-trnI* reported in *Apis* is consistent in most species, but for *A. koschevenikovi*, *trnM* is also tandemly duplicated (Cameron, 2014). Additionally, we found an ambiguous tRNA in the *Tetragonula* lineage within Meliponini: either *trnG* or *trnE* presented between *cox3* and *nd3* in samples from the same species. Françoso et al. (2023) have found this in *T. carbonaria* and *T. hockingsi*, and proved the polymorphism even exists within individuals by PCR amplification and sequencing. Thus, without a relatively comprehensive sampling of investigated lineages, the result can be misleading when exploring the phylogenetic implications of tRNA rearrangements.

DATA AVAILABILITY

The newly sequenced and assembled mitogenomes of Meliponini can be downloaded from NCBI (accession numbers in Supplementary Table S4). All the newly assembled mitogenomes can be downloaded from China National Center for Bioinformation (Supplementary Table S5) (PRJCA021965) and Science Data Bank databases (DOI: 10.57760/sciencedb.j00139.00088).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

W.B.Y., R.T.C., and Y.R.L. conceived and designed the research; Z.W.W. and Y.R.L. collected and identified samples; Y.R.L. and W.B.Y. analyzed the data and interpreted results; Y.R.L. and W.B.Y. wrote draft of the manuscript; all authors revised and approved the final version of the manuscript.

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