frontiers | Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 22 June 2023
DOI 10.3389/fevo.2023.1171242

OPEN ACCESS

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RECEIVED 21 February 2023
ACCEPTED 07 June 2023
PUBLISHED 22 June 2023

CITATION
Karuppaiah V, Gadge AS, Shirsat DV, Soumia PS, Mainkar P, Kumar S, Jaiswal DK and Mahajan V (2023) The complete mitochondrial genome of the Indian dammer bee, *Tetragonula iridipennis*, and the phylogenomics of Meliponini. *Front. Ecol. Evol.* 11:1171242. doi: 10.3389/fevo.2023.1171242

The complete mitochondrial genome of the Indian dammer bee, *Tetragonula iridipennis*, and the phylogenomics of Meliponini

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The Indian stingless bee *Tetragonula iridipennis* (Hymenoptera: Apidae), popularly recognized as the Indian dammer bee, is an economically important and widely distributed non-*Apis* bee species in India. The taxonomic gaps, systematics, evolutionary puzzles, and structural motifs within the mitogenomes of this species have rarely been examined and are not fully understood. Next-generation sequencing was employed to decipher the complete mitochondrial genome of *T. iridipennis* (15,045 bp). *De novo* genome assembly revealed that it encompasses 34 genes: protein-coding genes (13), transfer RNA (tRNAs) genes (19), and ribosomal RNA (rRNA) genes (2). Additionally, genome organization, including gene content, nucleotide composition, codon usage, and gene rearrangement, was investigated to better comprehend, utilize, and conserve this germplasm resource. The average gene length was 400 bp; maximum and minimum lengths were 1,530 bp (*cox1*) and 57 bp (tRNA-S1), respectively. Phylogenetic analysis has suggested that *T. iridipennis* is mostly closely related to *T. pagdeni* and *Lepidotrigona* species. All the stingless bee species (Meliponini) formed a distinct clade that shared a closer relationship with bumble bees (Bombini) than honey bees (Apini). The nucleotide composition of *T. iridipennis* was biased toward A+T, which accounted for 75.95% of the whole mitogenome. Length and compositional differences between *T. iridipennis* and other bees were detected, and gene order was compared. The mitogenome of *T. iridipennis* showed the highest gene rearrangement score (78), suggesting this species has a hyperactive evolutionary history. The variations of gene positions and gene rearrangement in the mitogenome could also aid in resolving the phylogenetic relationships and evolutionary history in Meliponini. Additionally, this is the first report of a complete mitochondrial genome sequence of *T. iridipennis*.

KEYWORDS

Indian stingless bee, Meliponini, mitogenome, next-generation sequencing, non-apis, phylogeny, systematics

1 Introduction

The stingless bees (Hymenoptera: Apidae: Meliponini) are an important group of non-*Apis* bees that visit many flowering plants in the tropics and subtropics (Roubik, 1993; Heard, 1999; Vossler, 2012; Layek and Karmakar, 2018). They are considered as a viable substitute for addressing the issue of pollination deficit in many agricultural, forest, and horticultural ecosystems (Garibaldi et al., 2013). Additionally, honey produced by stingless bees is very much in demand as it is rich in medicinal value (Al-Hatamleh et al., 2020). Furthermore, it is considered that uncovering the molecular features of this species is vital to understanding the molecular phylogeny and biodiversity of social insects (Wang et al., 2021). Wider adaptation to extreme environmental conditions and weedy types of reproduction in warmer conditions appears to be a superior trait of this species for their wide inhabitation. Among the corbiculate bees, enormous diversity is observed in stingless bees, which comprise over 600 distinct species (Gruter, 2020). The geospatial distribution of this species suggests that they are predominantly found in forests and agricultural ecosystems of tropical areas and urban realms of the Neotropical, Afrotropical, and Australasian regions (Gruter, 2020). The stingless bee species *Tetragonula iridipennis*, *T. bengalensis*, *T. praeterita*, *T. laeviceps*, *T. ruficornis*, and *Lepidotrigona arcifera* (Rahman et al., 2018) are widely distributed across India. Recently, two new species, *T. kyrdemkulaiensis* and *T.srikantanathi*, were identified in Northeast India (Viraktamath and Thangjam, 2021). Moreover, 17 diverse genera of stingless bees have reported in Indo-Malay/Australasia (Gruter, 2020; Rasmussen et al., 2017). Among *Tetragonula*, Moure is the most complex genus and alone comprises 34 species, predominantly inhabiting the Indian subcontinent. *Tetragonula iridipennis* (Smith) is an economically important stingless bee species natively known as the Indian stingless bee, Dammer bee, or Mosquito bee and is widespread in Asian countries, including Bangladesh, Bhutan, India, Indonesia, Myanmar, Nepal, Pakistan, and Sri Lanka (Rasmussen, 2013; Rahman et al., 2018; Engel et al., 2019; Bhatta et al., 2020; Nidup, 2021).

The smaller size of stingless bees means they are difficult to identify morphologically due to indistinctive differences and overlapping morphometric characteristics, leading to the misidentification of these species. Furthermore, the evolutionary histories of many taxa remain unexplored and are not obvious in the Meliponini tribe. As a result, there are many gaps in the taxonomic knowledge, which could have an impact on how efficiently stingless bee resources are protected and utilized (Wang et al., 2022). Again, morphology-based classification requires vast experience to effectively distinguish subtle differences between species. In this context, molecular features, such as complete mitochondrial DNA (mtDNA) sequences, are being efficiently utilized to distinguish the arthropod species and resolve contradictory evolutionary histories (Ohler and Delorme, 2006). In addition, the record of maternal inheritance obtained from mitochondrial genome is widely utilized in inferring phylogenetic relationships between diverse lineages. Furthermore, mtDNA poses several advantages as a biological marker, owing to its increased rate of mutation, nucleotide substitution rates, rare genetic recombination events, maternal inheritance, and easy availability (Howlader et al., 2015). Therefore, it has been extensively used in phylogenetic analysis, molecular evolution, genome structure analysis, and phylogeographic studies (Li et al., 2021). Additionally, mtDNA has been utilized to identify cryptic species, study population genetic structure, and evaluate micro-evolutionary processes (Zhang et al., 2018). Hence, the use of molecular datasets, especially mitogenomes, becomes essential to better understand stingless bee taxa and their molecular systematics, as well as conservation studies.

Although there have been numerous studies on arthropod mitochondrial genome sequencing, very few studies have been carried out with the Meliponini tribe. Six stingless bee species, *Melipona fasciculata* (MH680930.1), *Melipona bicolor* (AF466146.2), *Melipona scutellaris* (KP202303), *Lepidotrigona terminata* (MN737481.1), *Lepidotrigona flavibasis* (MN747147.1), and *Tetragonula pagdeni* (NC066054.1 and OK336459), have been characterized so far. Considering the distribution, diversity, and economic importance of *T. iridipennis* on the Indian subcontinent, molecular systematics of this species are vital for its characterization and conservation studies. Therefore, the complete mt genome of *T. iridipennis* was assembled using next-generation sequencing (NGS) for the first time. The sequence information and datasets generated will be helpful in uncovering the phylogenetics, and the evolutionary history of this species thereby aids conservation biology. Accordingly, the objectives of this study include: (i) characterization of the whole-mitochondrial genome of *T. iridipennis*; (ii) analyze the structural features of the mitochondrial genome, inherent nucleotide composition, codon usage bias, and the tRNA gene translocation; and (iii) establish the major phylogenetic relationship with the Apidae family and the gene order, as well as gene arrangement. The extensive genetic information generated from this study will aid in comparative genomics, molecular systematics, and population genetic studies of stingless bees.

2 Materials and methods

2.1 Sample collection

T. iridipennis-visited (workers bee) onion umbels were collected from onion seed production plots at the experimental farm of the Indian Council of Agricultural Research-Directorate of Onion and Garlic Research (ICAR-DOGR), located in Pune, Maharashtra, India (latitude of 18° 51' 59.99" N, longitude of 73° 53' 59.99" E, and 645 m above sea level). The insect sample was collected during the peak onion blooming period (February and March) and the voucher specimen was preserved (DOGR Voucher SHB01) following stipulated procedures. Species identity was confirmed by DNA barcoding at ICAR-DOGR, Pune, India, and the sequence information has been archived (NCBI GeneBank accession number: OQ103112).

2.2 Sample preparation and DNA extraction

Sample specimens collected from the onion field were initially preserved in 95% ethanol and stored at 4°C until experimentation.

Total genomic DNA was extracted from the legs of a single worker bee of *T. iridipennis* using a DNeasy Blood and Tissue Kit (QIAGEN, Germany). The quantitative and qualitative features of extracted DNA were confirmed through 1% agarose gel electrophoresis and spectrophotometric measurement (at 260 and 280 nm) using a SmartSpec 3000 UV/Visible Spectrophotometer (Bio-Rad, Hercules, CA, USA). Mitochondrial DNA was isolated from the genomic DNA using a REPLI-g Mitochondrial DNA Kit (QIAGEN, Germany).

2.3 Sequence assembly, annotation, and analysis

The sequence features from the mitochondrial genome library were obtained using an Illumina Next Seq 500 platform, and primary FASTQ data were generated. The FASTQ data were analyzed for total number of bases, the number of reads, GC% value, Q30, and the percentage of fuzzy bases. Then, the high-quality reads were generated by removing adapter and junk sequences and ambiguous reads, using the fastp tool (v0.12.4) (Chen et al., 2018). The resultant high-quality reads were further aligned with the reference sequences using BWA MEM (version 0.7.17) (Li and Durbin, 2009). Protein-coding and other RNA genes were identified from the consensus sequence extracted using SAM tools mpileup (Li et al., 2009). Genes from the invertebrate's mitochondrial genome were predicted using the MITOS web server (http://mitos.bioinf.uni-leipzig.de/index.py) (Bernt et al., 2013).

A *de novo* de-Brujin graph-based assembly was carried out to assemble the short reads into larger stretches of DNA contigs. These contigs were used for genome annotation, which assigns functions to various regions of the genome of the organism. The mitogenome was generated using the Megahit assembler (Li et al., 2015) as part of the MitoZ package (Meng et al., 2019). The assembly was further quality-checked using the Quast tool (Gurevich et al., 2013). Using MitoZ software (https://github.com/linzhi2013/MitoZ), the whole-mitochondrial genome was annotated (Meng et al., 2019) and subsequently verified with three mitogenome sequences of *Melipona bicolor* (AF466146.2), *Tetragonula pagdeni* (NC066054.1 and OK336459), and *Lepidotrigona flavibasis* (MN747147.1) available from the GeneBank database. The MITOS web server was used for assessing the assembled mitogenome and to predict the secondary structural aspects of tRNA genes (Bernt et al., 2013). A circular genome map, GC concentration, and GC skewness were generated using the molecular biology tool GCview server (http://stothard.afns.ualberta.ca/cgview_server/) (Grant and Stothard, 2008).

The base composition (A+T contents), codon usage, and relative synonymous codon usage (RSCU) of the protein-coding genes (PCGs) were analyzed using MEGA version XI (https://www.megasoftware.net/). The GC and AT skews were computed using the following formula: GC skew = [G-C]/[G+C] and AT skew = [A-T]/[A+T] (Perna and Kocher, 1995). The intergenic spacers and overlapping regions between the genes were manually computed. The complete mitogenome sequence of *T. iridipennis* following assembly and annotation was submitted to the NCBI sequence archive (GenBank accession no: OQ139639).

2.4 Phylogenetic analysis

The genetic relationships and taxonomy of *T. iridipennis* with other stingless bees (Meliponini) and hymenopteran bees (honey bees and bumble bees) were analyzed using 15 concatenated whole-mitogenome sequences of Hymenoptera species retrieved from the NCBI database.

Partition Finder 2.1.1 with the Bayesian information criterion (BIC) and greedy algorithm (www.phylo.org) was used to select the best partitioning schemes (Lanfear et al., 2017). The GTR+G model was preferred by the BIC for nucleotide alignment analysis. The nucleotide sequences from 15 different species were aligned using the MUSCLE tool implemented in MEGA 11 (https://www.megasoftware.net/) software. The phylogenetic tree was generated in MEGA 11 (Tamura et al., 2021) using the maximum likelihood (ML) method, general time reversible model with gamma distribution (GTR+G) default parameters, and bootstrap values from 500 iterations.

2.5 Gene rearrangement evaluation

The mitogenome of *T. iridipennis*, along with the mitogenomes of 15 chosen species, were examined for gene orders and potential gene rearrangement. This was carried out using the PhyloSuite software package (http://phylosuite.jushengwu.com/) (Zhang et al., 2020a). For quantitative analysis and comparison of the rearrangements among various mitogenomes, the rearrangement score (RS) of each individual gene was computed using qMGR software (http://qmgr.hnnu.edu.cn/) (Zhang et al., 2020b).

3 Results

3.1 Mitogenome organization and nucleotide composition

The circular mitogenome of the Indian stingless bee *T. iridipennis* was 15,045 bp in size (Figure 1). There were 34 sequence elements in total, comprising 13 protein-coding genes, 19 tRNA genes, and two rRNA genes. Analysis of the strand localization of 34 coding genes revealed that H-strand (+) harbors 28 genes whereas the L-strand (−) has six genes (Table 1). The *T. iridipennis* mitochondrion had an average gene length of 400 bp, while the maximum and minimum lengths were 1,530 bp (*cox1*) and 57 bp (tRNA-S1 genes), respectively. The base composition of the entire mitogenome of *T. iridipennis* was 40.66%, 35.29%, 12.65%, and 11.40% for A, T, C, and G nucleotides, respectively (Table 2). The nucleotide composition ranged from 64.94% to 85.47% for the 13 PCGs and two rRNAs, and it was 75.95% for

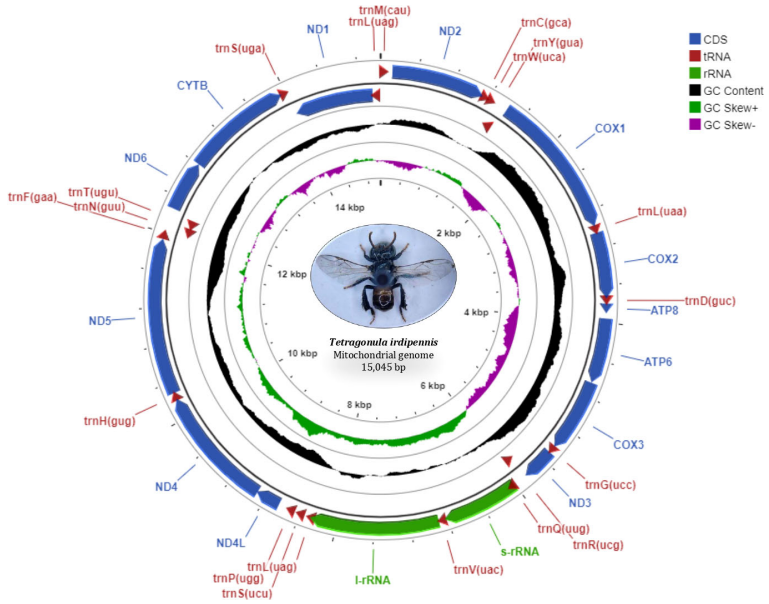

FIGURE 1
Mitochondrial genome map of *T. iridipennis*. From outer to inner, the first circle shows the gene map (PCGs, rRNA, tRNAs, and CR); tRNA genes are abbreviated by one letter symbols according to the IUPAC-IUB single-letter amino acid codes. The second circle shows the GC content and the third circle shows GC skew calculated as (G-C)/(G+C). GC content and GC skew are plotted as the deviation from the average value of the entire sequence.

the overall mitogenome. The AT skewness ranged between −0.050 to −0.212 for the 13 PCGs, which was relatively stronger than for the two rRNA genes, *rrnS* (0.047) and *rrnL* (0.032) (Table 2).

3.2 Protein-coding genes and codon usage bias

The total length of 13 PCGs of the mitogenome of *T. iridipennis* was 10,242 bp, which is nearly 68.08% of the genome. The length range of coding sequences varied from 168 bp (*atp8*) to 1,530 bp (*cox1*). Genes such as *cox1*, *cox2*, *cox3*, *nad2*, *nad3*, *nad4*, *nad4l*, *nad5*, *nad6I*, *CytB*, *atp6*, and *atp8* were located on the H-strand (+), while *nad1* alone was located on the L-strand (−). The four start codons, ATA, ATC, ATG, and ATT, were found in the *T. iridipennis* mitochondrial genome. Six genes (*cox1*, *cox2*, *atp8*, *nad4*, *nad4l*, and *nad5*) adopted ATT as their start codon, four genes (*atp6*, *cox3*, *nad6*, and *CytB*) used ATG, two genes (*nad3* and *nad1*) used ATA, and ATC was only used by *nad2*. Seven PCGs (*cox2*, *nad1*, *nad3*, *nad4l*, *nad5*, *nad6*, *and CytB*) are characterized with TAA as their termination codon, while four mitochondrial genes (*cox1*, *cox3*, *nad2*, and *atp6*) use TAG, and the incomplete termination codon 'T' is used by two genes (*atp8* and *nad4*).

The relative synonymous codon use (RSCU) and amino acid usage in the protein-coding genes of the *T. iridipennis* mitochondrion are summarized in Table 3. Leucine (L, Leu), isoleucine (I, Ile), phenylalanine (F, Phe), serine (S, Ser), tyrosine (Y, Tyr), and asparagine (N, Asn) were the most frequent amino acids, whereas tryptophan (W, Trp) and alanine (A, Ala) were the rarest (Figure 2). In general, UUU, AUU, UUA, UAU, and AAU were the most frequently used codons.

3.3 Transfer RNAs and ribosomal RNAs

The 19 tRNA genes, which vary in size from 57 to 69 bp, were scattered over the circular mitochondrial genome of *T. iridipennis*. The H-strand (+) of the mitochondria contained 14 tRNA genes, whereas the remaining five tRNA genes were found on the L-strand (−) (Figure 3; Table 1). The 19 tRNAs collectively comprised 1,255 bp covering 8.34% of the whole mitogenome. The characteristic clover-leaf secondary structures of tRNA genes were found except for $trnS1^{ser}$ (UCU), which lacked a dihydrouridine (DHU) arm. Four genes encoding tRNAs for Ala, Glu, Ile, and Lys were absent in the mitochondrial genome of *T. iridipennis*, whereas two copies of *trnL1* were found. A total of five mismatched base pairs ($tRNA^{Cys}$, $tRNA^{Gly}$, $tRNA^{Gln}$, $tRNA^{Thr}$, $tRNA^{Val}$) were present in the tRNAs of *T. iridipennis*.

The two rRNA genes (*rrnS*-and *rrnL*) located on the H-strand (+) constitute 14.16% of the total mitochondrial genome. The *rrnS* gene (763 bp long and an A+T content of 75.15%) was located between $tRNA^{Gln}$ and $tRNA^{Val}$. The *rrnL* gene (1,368 bp in size and an A+T content of 76.25%) was found between $tRNA^{Val}$ and $tRNA^{Leu.}$

3.4 The overlapping and intergenic spacer region

Two overlapping regions (26 bp in length) were identified in the mitogenome of *T. iridipennis*. These regions varied in length from 3 bp between *rrnS* and *trnV* and 23 bp between *rrnL* and *trnL1*. The intergenic spacers varied in length from 1 to 265 bp in the *T. iridipennis* mitogenome, constituting 1,412 bp, and were found interspersed in 27 regions of the genome. The longest intergenic

TABLE 1 Summary table of the characteristics of the complete mitogenome of *T. iridipennis*.

Gene name	Full name and function	Position		Length (bp)	Strand*	Intergenic spacers	Codon		Anticodon
		Start	End				Start	Stop	
trnM	Transfer RNA for Methionine	3	69	67	+	83			cat
nad2	NADH dehydrogenase subunit 2	153	1055	903	+	62	atc	tag	
trnC	Transfer RNA for Cysteine	1118	1182	65	+	8			gca
trnW	Transfer RNA for Tryptophan	1191	1258	68	+	21			tca
trnY	Transfer RNA for Tyrosine	1280	1345	66	–	69			gta
cox1	cytochrome c oxidase subunit I	1415	2944	1530	+	36	att	tag	
trnL2	Transfer RNA for Leucine	2981	3045	65	+	15			taa
cox2	cytochrome c oxidase subunit II	3061	3708	648	+	22	att	taa	
trnD	Transfer RNA for Aspartic acid	3731	3797	67	+	0			gtc
atp8	ATP synthase F0 subunit 8	3798	3965	168	+	11	att	t	
atp6	ATP synthase F0 subunit 6	3977	4639	663	+	40	atg	tag	
cox3	cytochrome c oxidase subunit III	4680	5414	735	+	20	atg	tag	
trnG	Transfer RNA for Glycine	5435	5502	68	+	0			tcc
nad3	NADH dehydrogenase subunit 3	5503	5847	345	+	6	ata	taa	
trnR	Transfer RNA for Arginine	5854	5918	65	–	87			tcg
trnQ	Transfer RNA for Glutamine	6006	6073	68	+	1			ttg
rrnS	12S ribosomal RNA	6075	6837	763	+	-3			
trnV	Transfer RNA for Valine	6835	6900	66	+	0			tac
rrnL	16S ribosomal RNA	6901	8268	1368	+	-23			
trnL1	Transfer RNA for Leucine	8246	8313	68	+	55			tag
trnS1	Transfer RNA for Serine	8369	8425	57	+	50			tct
trnP	Transfer RNA for Proline	8476	8539	64	+	109			tgg
nad4l	NADH dehydrogenase subunit 4L	8649	8906	258	+	83	att	taa	
nad4	NADH dehydrogenase subunit 4	8990	10207	1218	+	10	att	t	
trnH	Transfer RNA for Histidine	10218	10283	66	+	27			gtg
nad5	NADH dehydrogenase subunit 5	10311	11687	1377	+	265	att	taa	
trnF	Transfer RNA for Phenylalanine	11953	12017	65	+	20			gaa
trnN	Transfer RNA for Asparagine	12038	12106	69	–	30			gtt
trnT	Transfer RNA for Threonine	12137	12202	66	–	117			tgt
nad6	NADH dehydrogenase subunit 6	12320	12793	474	+	74	atg	taa	
cytB	cytochrome b	12868	13905	1038	+	50	atg	taa	
trnS2	Transfer RNA for Serine	13956	14022	67	+	41			tga
nad1	NADH dehydrogenase subunit 1	14064	14948	885	–	0	ata	taa	
trnL1	Transfer RNA for Leucine	14949	15016	68	–	0			tag

*"+" and "–" indicates the heavy and light strands of the circular mitochondrial genome, respectively.

spacer was 265 bp and located between *nad5* and *trnF*. Similarly sized intergenic spacer regions were also located between *trnT* and *nad6*, and *trnP* and *nad4l*, with lengths of 117 bp and 109 bp, respectively.

3.5 Phylogenetic analysis

Molecular phylogenomics were evaluated from the phylogenetic tree constructed using the concatenated complete mitogenome

TABLE 2 AT/CG skews in the 13 PCGs and two rRNAs of the *T. iridipennis* mitogenome.

Gene	Length	T%	C%	A%	G%	AT%	GC%	GC Skew	AT Skew
nad2	903	40.66	15.96	30.82	12.55	71.49	28.51	−0.120	−0.138
cox1	1560	37.56	19.62	27.37	15.45	64.94	35.06	−0.119	−0.157
cox2	699	36.48	18.60	30.33	14.59	66.81	33.19	−0.121	−0.092
atp8	103	46.60	14.56	33.98	4.85	80.58	19.42	−0.500	−0.157
atp6	687	39.59	20.96	26.06	13.39	65.65	34.35	−0.220	−0.206
cox3	780	36.28	20.26	30.00	13.46	66.28	33.72	−0.202	−0.095
nad3	354	39.55	21.19	25.71	13.56	65.25	34.75	−0.220	−0.212
nad4l	273	49.82	4.40	35.53	10.26	85.35	14.65	0.400	−0.167
nad4	1315	46.92	7.91	36.20	8.97	83.12	16.88	0.063	−0.129
nad5	1653	44.10	7.80	39.93	8.17	84.03	15.97	0.023	−0.050
nad6	537	46.55	8.01	38.92	6.52	85.47	14.53	−0.103	−0.089
cytB	1149	45.34	11.58	33.59	9.49	78.94	21.06	−0.099	−0.149
nad1	930	45.70	8.17	35.91	10.22	81.61	18.39	0.111	−0.120
rrnS	829	35.83	9.29	39.32	11.22	75.15	20.50	0.094	0.047
rrnL	1385	36.90	10.40	39.35	12.22	76.25	22.61	0.080	0.032
Overall Mitogenome	15045	40.66	12.65	35.29	11.40	75.95	24.05	−0.052	−0.071

sequences of 15 Hymenopteran bees, including seven stingless bees (Figure 4). Phylogenetic analysis revealed three major clades based on the tribes (Meliponini, Bombini, and Apini) along with an outgroup comprising *Megachile sculpturalis* (KT223644) and *Colletes gigas* (KM978210). The Meliponini tribe was grouped together, forming a major monophyletic clade of stingless bees. *T. iridipennis* from the current study (OQ139639) was closely related to *T. pagdeni* (OK336459) and clustered together with two *Lepidotrigona* species (MN747147.1 and MN737481.1) under sub-clade Ia. Meanwhile, three *Melipona* species (MH680930, AF466146, and KP202303) were grouped under sub-clade Ib. At the genus level, *Tetragonula* exhibited greater molecular proximity to *Lepidotrigona* than to the *Melipona* taxa. The phylogenetic analysis also showed that stingless bees (Meliponini) have a closer relationship with the clade corresponding to bumble bees (*Bombus*) of the Bombini tribe than with the clade belonging to honey bees (*Apis*) of the Apini tribe in the Apidae family.

3.6 Gene rearrangement evaluation

The order of genes of the mitogenome of *T. iridipennis* was quite distinct from the six previously reported stingless bee mitogenomes (OK336459, MN747147, MN737481, AF466146, MH680930, AF466146, and KP202303), and dramatic gene rearrangement was observed in *T. iridipennis* (Figure 5). Genetic rearrangement events were remarkably consistent with the output of the phylogenetic tree (Figures 5, 6), supporting the idea that the order of genes in the mitogenome may contain crucial evolutionary data for phylogenetic research. The RS value, which describes the extent of gene rearrangement of each mitogenome, was determined. *T. iridipennis* had the highest RS value (78) among all Apidae insects considered in the current study (Figure 6), indicating an active evolutionary past of *T. iridipennis*. The mitochondrial gene rearrangement score of *T. iridipennis* was relatively higher among the Hymenopteran insect species. Among the stingless bee (Meliponini) clade, the locations of tRNA-K differed. Gene rearrangement showed M-K-A-I block in *Lepidotrigona*, whereas the reversed order I-A-K-M block was present in *Melipona*, and K-A-I-M was present in *T. pagdeni*. By contrast, *T. iridipennis* had I-M block instead on the usual gene rearrangement in Hymenopteran orders due to missing tRNA-A and tRNA-K (Figure 5).

4 Discussion

In general, the insect mitogenome is characterized as a circular double-stranded polymer 14–19 kb in length and encodes 37 genes, including 22 tRNA genes, two rRNA genes, and 13 PCGs (Boore, 1999; Cameron, 2014; De Mandal et al., 2014). The size of the whole mitogenome of *T. iridipennis* from the present study (15,045 bp) is in accordance with the mitogenome of other stingless bees, such as *T. pagdeni* (16,061 bp), *L. flavibasis* (15,408 bp), *L. terminata* (15,431 bp), *M. bicolor* (15,001 bp), *M. fasciculata* (14,753 bp), and *M. scutellaris* (14,862 bp), belonging to the same tribe, Meliponini, in the Apidae family.

The nucleotide base composition of *T. iridipennis* revealed an obvious bias toward A and T. Among the four nucleotides, T and G were the most and least favored, respectively. According to previous

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TABLE 3 Codon usage and relative synonymous codon usage (RSCU) pattern of the 13 mitochondrial protein-coding genes from *T. iridipennis*.

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	21	1.5	UCU(S)	6	1.64	UAU(Y)	12.5	1.49	UGU(C)	3.5	1.5
UUC(F)	6.9	0.5	UCC(S)	2.1	0.57	UAC(Y)	4.3	0.51	UGC(C)	1.2	0.5
UUA(L)	18.1	2.54	UCA(S)	7.9	2.17	UAA(*)	13.4	1.76	UGA(*)	5.7	0.75
UUG(L)	6.5	0.92	UCG(S)	1.5	0.4	UAG(*)	3.8	0.49	UGG(W)	1.8	1
CUU(L)	6.2	0.87	CCU(P)	2.6	1.86	CAU(H)	3.7	1.63	CGU(R)	1	0.82
CUC(L)	3.6	0.51	CCC(P)	1	0.71	CAC(H)	0.8	0.37	CGC(R)	0.1	0.06
CUA(L)	4.8	0.68	CCA(P)	1.5	1.1	CAA(Q)	4.9	1.33	CGA(R)	1	0.82
CUG(L)	3.5	0.49	CCG(P)	0.5	0.33	CAG(Q)	2.5	0.67	CGG(R)	0.4	0.32
AUU(I)	18.8	1.44	ACU(T)	5.5	1.91	AAU(N)	10.2	1.53	AGU(S)	3.6	0.99
AUC(I)	4	0.31	ACC(T)	1.8	0.64	AAC(N)	3.1	0.47	AGC(S)	0.8	0.23
AUA(I)	16.5	1.26	ACA(T)	3.8	1.34	AAA(K)	6.8	1.33	AGA(R)	3.5	2.84
AUG(M)	4.8	1	ACG(T)	0.3	0.11	AAG(K)	3.5	0.67	AGG(R)	1.4	1.14
GUU(V)	2.7	1.4	GCU(A)	1.7	2.05	GAU(D)	4.5	1.49	GGU(G)	1.2	1.03
GUC(V)	1.2	0.6	GCC(A)	0.4	0.47	GAC(D)	1.5	0.51	GGC(G)	0.4	0.32
GUA(V)	2.7	1.4	GCA(A)	1.2	1.4	GAA(E)	4.3	1.3	GGA(G)	2.3	1.94
GUG(V)	1.2	0.6	GCG(A)	0.1	0.09	GAG(E)	2.3	0.7	GGG(G)	0.8	0.71

Average Codons = 271.
Asterisks * indicate termination codon.

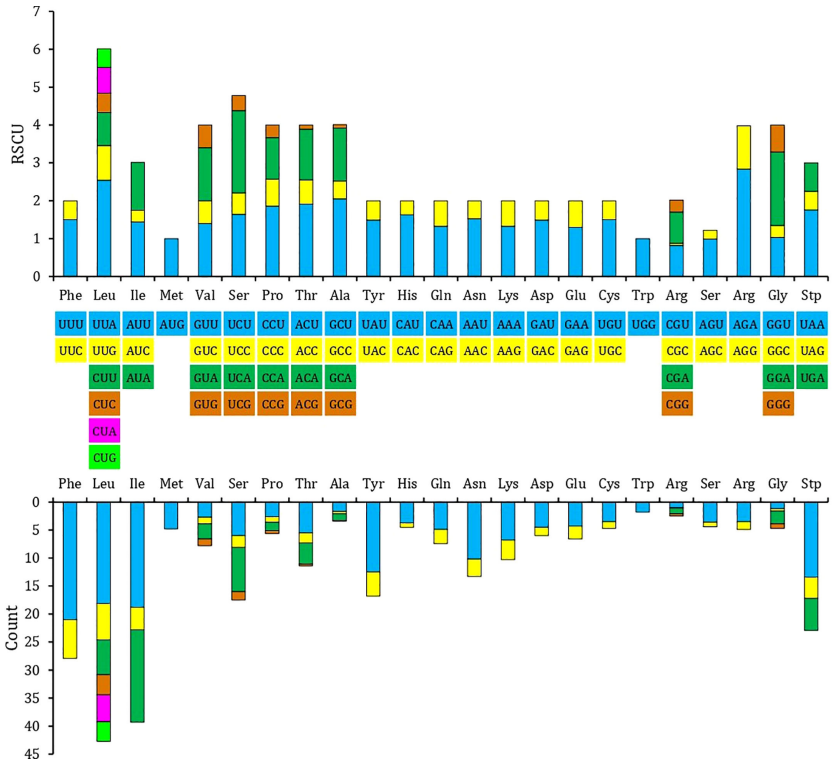

FIGURE 2
Relative synonymous codon usage (RSCU) in the mitogenome of *T. iridipennis*. The different colors in the column chart represent the codon families corresponding to the amino acids below and consistent colors are used to represent the same codon families.

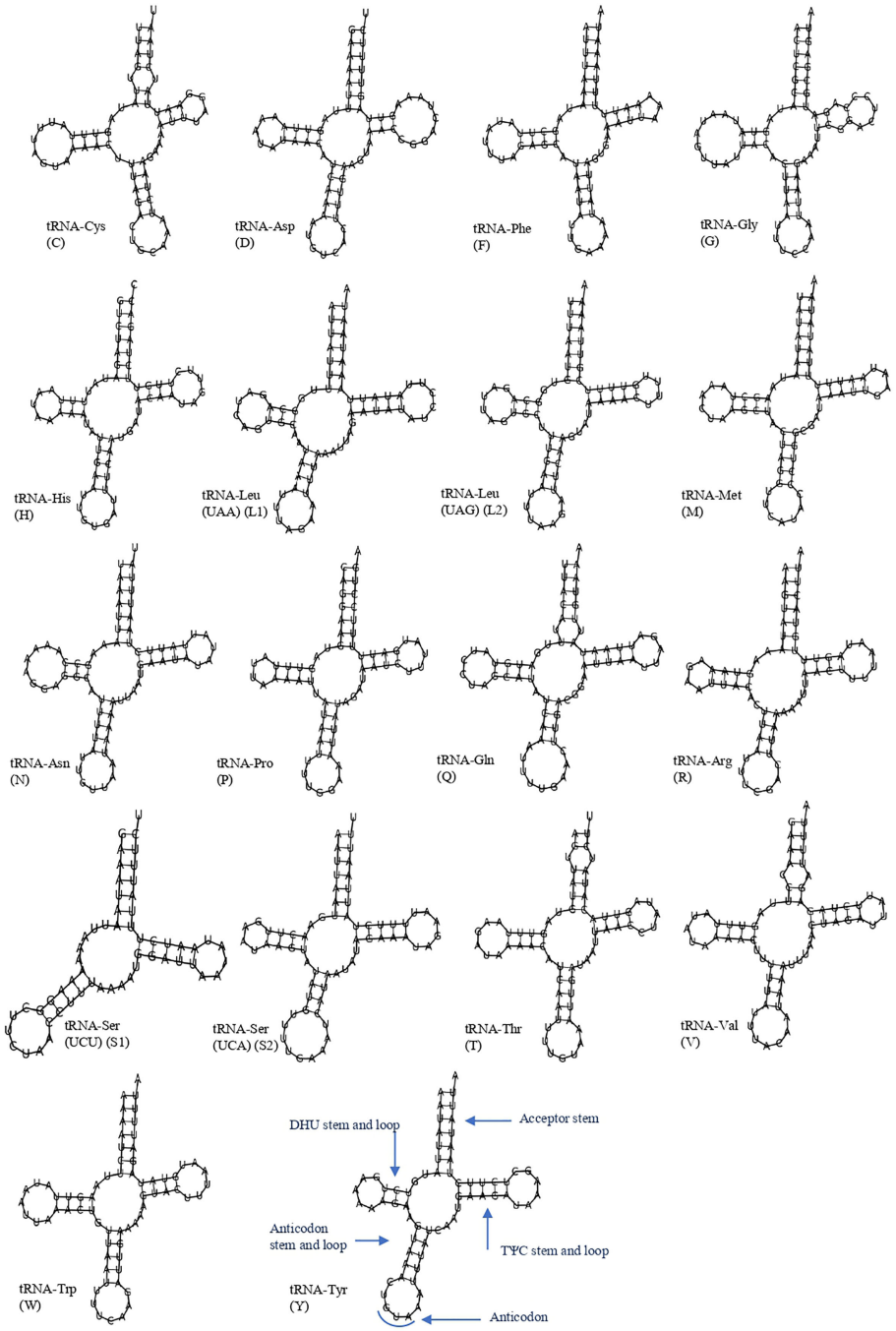

FIGURE 3
Putative secondary structures for 19 tRNA genes of *T. iridipennis*. The uppercase alphabet letters are the single letter abbreviation of the amino acids.

reports, the nucleotide composition of stingless bees belonging to different species was A+T rich (Wang et al., 2021; Wang et al., 2022). A similar trend was observed in the *T. iridipennis* mitogenome. The bias of the base composition of an individual strand can be described by skewness. The AT (−0.071) and GC (−0.052) skewness values were both negative, indicating a bias towards Ts and Cs rather than As and Gs, respectively, in the mitogenome of *T. iridipennis*. According to the hypothesis, biasness might be due to the active use of these bases (Ts and Cs) by DNA polymerase during mtDNA replication (Clary and Wolstenholme, 1985). Furthermore, less energy is required to break the A-T linkages, and the subsequent transcription will generate an AT bias in species that depend on mitochondrial efficiency to maintain a high metabolic rate (Xia, 1996).

The PCGs [three *Cytochrome oxidases* (*cox1*, *cox2*, and *cox3*), seven *NADH dehydrogenases* (*nad1*, *nad2*, *nad3*, *nad4*, *nad4l*, *nad5*, and *nad6*), one *Cytochrome B* gene (*CytB*), and two *ATPases* (*atp6* and *atp8*)] of the mitochondrial genome of *T. iridipennis* are distributed throughout. Most species belonging to the Hymenoptera, such as *T. pagdeni* (Wang et al., 2022), *L. flavibasis* (Wang et al., 2021), *L. terminata* (Wang et al., 2020), *M. bicolor*

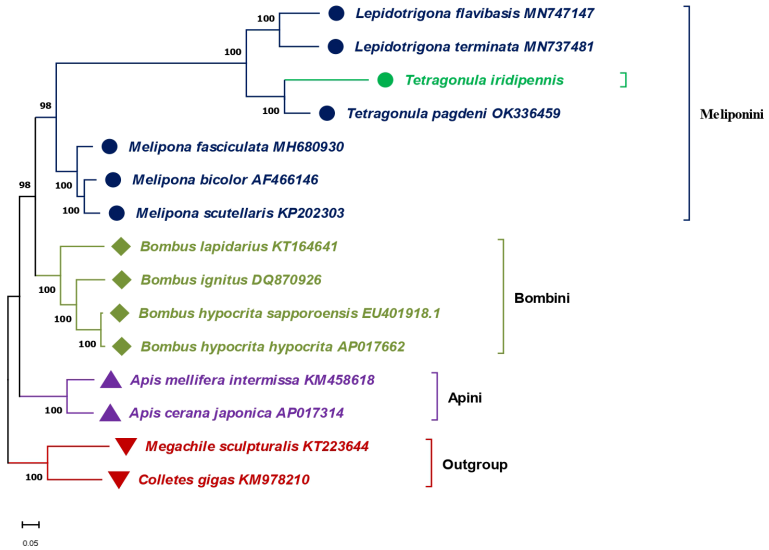

FIGURE 4
Phylogenetic tree showing the relationship between *T. iridipennis* and 14 other Hymenopteran species based on the concatenated nucleotide sequences of the whole mitogenome using maximum likelihood (ML) and Bayesian inference (BI) analysis. Megachilidae (*Megachile sculpturalis*) and Colletidae (*Colletes gigas*) were used as an outgroup. GenBank accession numbers of each sequence are displayed in the tree beside their corresponding species names.

(Silvestre et al., 2008), *M. scutellaris* (Pereira et al., 2016), and *Apis dorsata* (Chhakchhuak et al., 2016), have the same number of PCGs, with little variation in their size and location. Like most Hymenopterans, the initiation codon (ATN) and the incomplete stop codon (T) were observed in *T. iridipennis*. However, no anomalous codon was observed in *T. iridipennis* PCGs, as found in *L. flavibasis* (GTG start codon for *nad2*) and *A. cerana* (GTG start codon for *nad2*) (Wang et al., 2021). In *T. iridipennis*, the degenerate codons frequently used A and T rather than G and C. Similar results were reported by Wang et al. (2022) in *T. pagdeni*. In addition, the incomplete stop codon (T) can give rise to functional stop codons due to processes such as polyadenylation and polycistronic transcription cleavage (Ojala et al., 1981). RSCU analysis indicated a bias toward AT-rich codons, which mainly results from an AT mutation bias (Wang et al., 2022).

The predicted secondary structures of the tRNAs of *T. iridipennis* were very similar to *T. pagdeni* and *L. flavibasis* (Wang et al., 2021; Wang et al., 2022). Nonetheless, a few differences were present in the TΨC and D arms (Silvestre et al., 2008). The tRNA-Ser1 (UCU) lacks the dihydrouridine (DHU) arm and thereby a simple loop is formed rather than the typical clover-leaf shape. The mitogenomes of many stingless bees showed similar results (Wang et al., 2020; Wang et al., 2021; Wang et al., 2022). The anticodons were the same for all the stingless bee species studied by various researchers (Silvestre et al., 2008; Wang et al., 2021; Wang et al., 2022). In confirmation with Silvestre et al. (2008), the anticodon loop of 14 tRNAs of *T. iridipennis* had seven nucleotides and its arm had five base pairs, except for 5 tRNAs (tRNA-Leu1, tRNA-Gln, tRNA-Arg, tRNA-Ser2 and tRNA-Val) that only had four base pairs on the anticodon loop, similar to *A.*

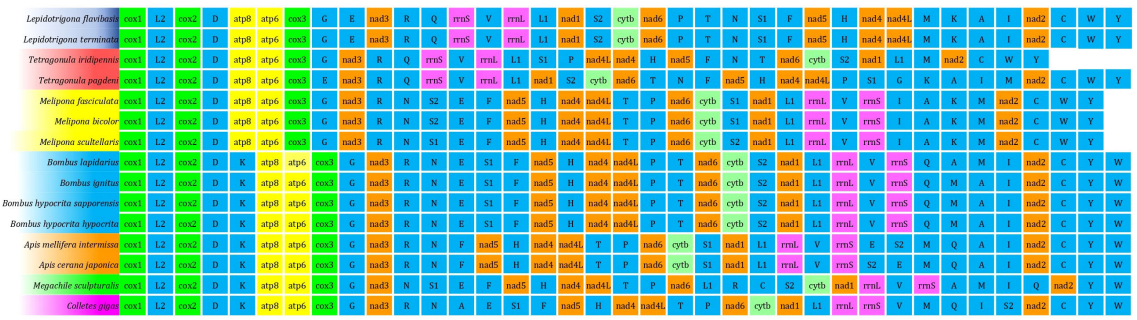

FIGURE 5
The gene order of each mitogenome for comparison of the mitochondrial gene orders between *Tetragonula* stingless bees and other Hymenopteran insects. The same colour indicates the sequence of the similar gene. The uppercase alphabet letters are the single letter abbreviation of the amino acids.

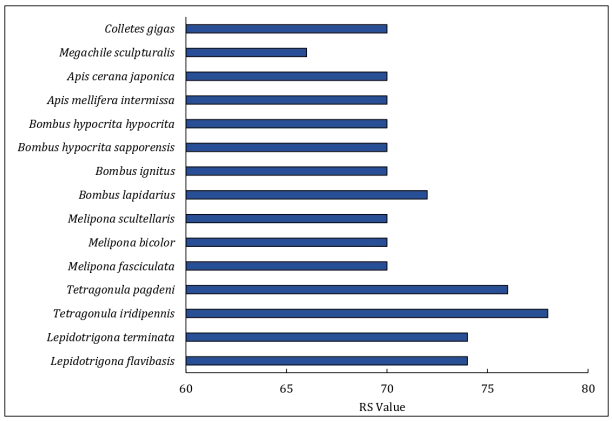

FIGURE 6
The Rearrangement Score (RS value) of each mitogenome for comparing *Tetragonula* stingless bees and other Hymenopteran insects.

mellifera tRNA-Val (4 bp). Additionally, the acceptor arm had a conserved size (7 bp) in most Meliponini stingless bees, except in *M. bicolor* tRNA-Arg (6 bp) (Silvestre et al., 2008). The two copies of *trnL1* reported in the mitochondrial genome of *T. iridipennis* might have occurred due to the partial duplication that commonly occurs in insect mtDNA (Zhang and Hewitt, 1997). Unlike the 22–23 tRNA genes commonly found in animal mitochondrial genomes, 19 tRNAs were identified in *T. iridipennis* mtDNA in the present study. The four missing tRNA genes coded Ala, Glu, Ile, and Lys amino acids. Similar results were observed by Silvestre et al., 2008 in *M. bicolor* with the absence of tRNA-Cys, tRNA-Gly, tRNA-Ile, and tRNA-Ser1. The mitochondrial tRNAs inferred for Hymenopteran insects *T. pagdeni*, *L. flavibasis*, *M. bicolor*, *A. mellifera*, *A. cerana*, and *Bombus ignites* were found to have mismatched base pairs (Silvestre et al., 2008; Tan et al., 2011; Wang et al., 2021; Wang et al., 2022). Mitochondrial tRNAs of Hymenopteran species are characterized by stem mismatches and sequences that are rectified later by a posttranscriptional editing mechanism (Tan et al., 2011). The relative locations of two genes encoding ribosomal RNAs in the *T. iridipennis* mitogenome were identical to those in *T. pagdeni* and *L. flavibasis* but different from *Melipona* species, thereby clustering them in different clades of the phylogenetic tree (Silvestre et al., 2008; Wang et al., 2021; Wang et al., 2022).

Two overlapping regions of 26 bp in length have been reported in the mitogenome of *T. iridipennis*. Similar overlapping regions have also been recorded in the mitogenomes of *T. pagdeni* and *L. flavibasis*, with lengths of 25 and 24 bp, respectively (Wang et al., 2021; Wang et al., 2022). These regions of *T. iridipennis*, *T. pagdeni*, and *L. flavibasis* ranged in length from 1–3 bp between *rrnS* and *trnV* and 23 bp between *rrnL* and *trnL1*, constituting a single clade in evolutionary history. Owing to the apparent lack of strong purifying selection and accumulation of multiple base changes and insertion/deletions, this region is referred to as a second origin of mtDNA replication and transcription (Cornuet et al., 1991).

According to Wang et al. (2019); Wang et al. (2021), complete mitochondrial genome sequences are usually preferred for phylogenetic analysis over partial or incomplete combination sequences and a few nuclear genes as they yield topologies with strong support values. The complete mitogenomes of *T. iridipennis* and 14 common Hymenopteran species retrieved from the NCBI database were used for the phylogenetic analysis. In accordance with other studies, the current phylogenetic analysis of stingless bees (Meliponini) revealed the following relationships: (*Tetragonula* + *Lepidotrigona*) + *Melipona*) + *Bombus*) + *Apis*) + (*Megachile* + *Colletes*) (Wang et al., 2020; Wang et al., 2021; Wang et al., 2022). At the genus level within the Meliponini tribe, *Tetragonula* is more closely linked to *Lepidotrigona* than to *Melipona*, and these findings closely align with some earlier reports by Wang et al. (2022); Wang et al. (2021); Wang et al. (2020). Additionally, Zhao et al. (2017) and Wang et al. (2020); Wang et al. (2021); Wang et al. (2022) have provided evidence that stingless bees are phylogenetically more closely related to bumble bees than honey bees. The phylogenetic relationships were reconstructed based on the complete mitogenome, supporting the inferences obtained from the conventional morphology-based phylogenetics of the Apidae family.

The gene order of most insect mitogenomes is the same; nevertheless, gene rearrangements in the mitogenomes of some species are very common. In general, rearrangements of genes involve gene inversion, genetic transposition, inverse transposition, and tandem duplication, causing loss of gene function (Cameron, 2014; Chen et al., 2018). The absence of the super conserved tRNA D-K block in the stingless bee group implies that *tRNA-K* movement could have occurred early in the progenitor of the stingless bee, thereby phylogenetically separating the stingless bee from other Hymenopteran insects (Figure 5). Similar findings were found in *T. pagdeni* and *L. flavibasis* (Wang et al., 2021; Wang et al., 2022). Furthermore, *T. iridipennis* was found to exhibit unique translocation, which broke the relatively frequent intact block *Nad1-L1-rrnL-V-rrnS* box in the mitogenomes of Hymenopteran insects, suggesting that *T. iridipennis* had a very active mitogenome. However, in *Lepidotrigona* and *Tetragonula*, this box had moved and turned upside down (Wang et al., 2021; Wang et al., 2022). The translocated *Nad-1* moved to the position next to *S-2* (Figure 5). The super-conserved *atp8-atp6-cox3-G* block in Hymenopteran mitogenomes was retained in *T. iridipennis*. However, Wang et al. (2022) found an uncommon *tRNA-Gly* translocation by substituting E for G in *T. pagdeni*. The rRNA, tRNA, and (PCG) genes of *T. iridipennis* experienced considerable rearrangement. These occurrences imply that the *Tetragonula* species' mitogenomes have been very active. Most invertebrate mitogenomes have largely retained gene ordering. Natural selection may not completely eradicate mitogenome rearrangements, and they may reflect the dynamics and rates of evolution within a given species or genus (Shao et al., 2003).

Many cultivated crops are cross pollinated and mainly depend on insect pollinators, especially from the order Hymenoptera, for their ecosystem services. Among insect pollinators, honey bees are important pollinators belonging to the Apidae family. Nevertheless, non-*Apis* species of stingless bees are imperative in the pollination of agricultural, forest, and horticultural crops in the tropics and subtropics. Mitochondrial genomics has helped greatly in

elucidating the evolutionary relationships of Hymenopteran insects at multiple taxonomic levels, especially for Apoidea. Hence, the scientific literature is replete with mitochondrial genomics pertaining to the Apidae family; however, very few reports are available pertaining to the Meliponini tribe. This study provides novel molecular insights into the genetic architecture of the mitogenomic and phylogenetic framework of *T. iridipennis* in Hymenoptera and facilitates future studies on the evolution of invertebrates. In this study, the entire *T. iridipenni* mitogenome sequence was made available for the first time. The circular mitogenome of *T. iridipennis* is 15,045 bp in length and contains 34 genes, comprising 13 protein-coding genes, *NADH dehydrogenase* genes, *cytochrome c oxidase* genes, *ATPase* genes, a *Cytochrome B* gene, two ribosomal RNA genes, and 19 transfer RNA genes. These findings provide crucial information for upcoming mtDNA analysis of other Meliponini species, helping to resolve the intraspecific and interspecific study of biological, ecological, and evolutionary issues. Because many stingless bee species are endangered, the application of molecular tools in deciphering the population genomics of the species is crucial for biodiversity conservation. Furthermore, the greater degree of gene rearrangements found in tRNA genes of the *T. iridipennis* mitochondrial genome underscores the genetic fluidity of the species. Phylogenetic analysis conducted using complete mitogenomes of 15 Hymenopteran species confirmed the phylogenetic position of *T. iridipennis* as a sister species to *T. pagdeni.* Furthermore, phylogenetic analysis of gene order could help in resolving the molecular evolutionary genomics of bees and could shed light on the emergence of eusociality among corbiculate bees (Silvestre et al., 2008). It is anticipated that the incorporation of more molecular data and genetic dissection of tRNA gene clusters in the mitogenomes of several species will provide novel insights into the evolution and biogeography of the Meliponini tribe of the Apidae family.

5 Conclusions

In summary, the complete mitochondrial genome of *T. iridipennis* was sequenced using next-generation sequencing for the first time. The circular mitogenome of *T. iridipennis* is 15,045 bp in length and contains 34 sequence elements, comprising 13 protein-coding genes, 19 tRNA genes, and two rRNA genes. Phylogenetic analysis revealed that *T. iridipennis* is closely related to *T. page* and *Lepidotrigona* species. The genetic information generated from this study will aid in comparative genomics, molecular systematics, and population genetic studies of many species of the *Tetragonula* genus in particular and hymenopterans bees in general.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

Ethical review and approval was not required for the study on the insect pollinator, *Tetragonula iridipennis*, in accordance with the local legislation and institutional requirements. The insect specimens were collected from the onion field by net sweeping, and no specific permissions were required for these locations/activities. The species in our study are common pollinators and did not involve endangered or protected species in India. The ICAR-Directorate of Onion and Garlic Research, Pune, Maharashtra, India, approves the work.

Author contributions

VK and PS conceptualized the study and designed the experiment. VK, AG, and DS carried out the methodology. AG, DS, PM, and SK analyzed and interpreted the data with inputs. VK, AG, DS, PS, and DJ wrote and edited the manuscript and prepared display elements. VM provided resources. All authors contributed to the article and approved the submitted version.

Funding

The research was funded by the Department of Science and Technology (DST) – Science and Engineering Research Board (SERB), New Delhi (CRG/2021/004267).

Acknowledgments

We are grateful to the Department of Science and Technology (DST) – Science and Engineering Research Board (SERB), New Delhi, India for financial grant (CRG/2021/004267) support and the Indian Council of Agricultural Research and Directorate of Onion and Garlic Research (ICAR-DOGR), Pune, Maharashtra, India, for providing lab facilities.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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