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Mitochondrial 16S rRNA Characterization of *Heterotrigona itama* Colonies from Central Lampung, Indonesia

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Abstract

Stingless bees play important ecological and economic roles in tropical ecosystems, yet molecular baseline data for many regions in Sumatra remain limited. This study aimed to characterize mitochondrial genetic variation of *Heterotrigona itama* colonies from Punggur Subdistrict, Central Lampung, Indonesia, using partial 16S rRNA sequences. Three managed colonies were analyzed through PCR amplification and Sanger sequencing, followed by sequence alignment, BLAST comparison, genetic distance estimation using the Kimura-2-Parameter model, and phylogenetic reconstruction. All samples produced consistent amplicons of approximately 500 bp and showed complete nucleotide identity, indicating a single mitochondrial haplotype among the analyzed colonies. BLAST results revealed high similarity (96.91–98.38%) with reference sequences of *H. itama*. Genetic distance analysis showed zero divergence among the sampled colonies and low divergence (0.0069–0.0182) relative to other *H. itama* sequences, while higher divergence was observed with related taxa. Phylogenetic analysis placed all samples within a well-supported *H. itama* clade. These findings indicate mitochondrial uniformity among the sampled colonies and provide a preliminary genetic baseline for *H. itama* in Central Lampung. However, broader conclusions on population structure require further studies with larger sample sizes and additional molecular markers.

Keywords: genetic variation; haplotype; mitochondrial inharitance; stingless bees.

INTRODUCTION

Stingless bees (Hymenoptera: Apidae: Meliponini) represent one of the most diverse and ecologically significant groups of eusocial insects in tropical ecosystems (Straub et al., 2024). They function as key pollinators in both natural and agricultural landscapes, contribute to forest regeneration, and provide economic benefits through the production of honey and other bee products (Caballero Méndez et al., 2024). Indonesia is recognized as an important center of stingless bee diversity in Asia, with approximately 46–52 species distributed across major islands such as Sumatra, Java, Kalimantan, Sulawesi, and Papua (Trianto et al., 2023). Despite this richness, molecular data for many regions remain limited, constraining efforts to accurately document species identity and genetic variation.

Morphological identification is commonly used in stingless bee studies; however, it can be insufficient for distinguishing closely related taxa or detecting cryptic diversity (Anaktototy et al., 2021; Imtitsal et al., 2025). Molecular approaches, particularly mitochondrial DNA markers, have become essential tools for species confirmation and preliminary assessment of genetic variation. The mitochondrial 16S rRNA gene is widely applied due to its combination of conserved and variable regions, allowing reliable species-level identification in Meliponini (Urueña et al., 2022). Previous studies in Indonesia have reported molecular data for stingless bees from several regions, including Maluku, Yogyakarta, and East Lampung (Anaktototy et al., 2021; Imtitsal et al., 2025). However, comparable data from Central Lampung are still unavailable, limiting regional comparisons and baseline documentation.

In addition to species identification, mitochondrial markers can provide initial insights into genetic variation and maternal lineage patterns. In managed systems, such as meliponiculture, colony propagation practices, including brood division and colony exchange, may influence the distribution of mitochondrial haplotypes at local scales (Neumann and Blacquière, 2017; Santiago et al., 2016). Environmental conditions, including agricultural landscapes and habitat heterogeneity, may also contribute to shaping genetic patterns in stingless bee populations (Kay et al., 2020). Nevertheless, without baseline molecular data, it remains difficult to evaluate the extent of genetic variation within local populations.

Recent studies have emphasized the importance of region-specific molecular data to support biodiversity documentation and improve the accuracy of species identification in Southeast Asia (Henderson et al., 2025). Such baseline information is particularly relevant in areas where meliponiculture is developing, as it can provide a reference for future studies on genetic variation and colony management. Punggur Subdistrict in Central Lampung is one such area, where stingless bee cultivation is increasingly practiced, yet molecular data for local colonies are still lacking.

Therefore, this study aimed to characterize mitochondrial genetic variation of *Heterotrigona itama* colonies from Punggur Subdistrict, Central Lampung, Indonesia, using 16S rRNA sequences. Specifically, this study sought to (i) confirm species identity, (ii) examine mitochondrial sequence variation among sampled colonies, and (iii) provide a preliminary genetic baseline for future studies on stingless bee diversity in the region.

MATERIALS AND METHODS

Study area and sample collection

This study was conducted in Punggur Subdistrict, Central Lampung Regency, Lampung Province, Indonesia. The area is characterized by an agricultural landscape where meliponiculture practices are increasingly developed. Samples of stingless bees were obtained from three managed colonies maintained by local beekeepers.

A single worker bee was collected from each colony (HB1, HB2, and HB3) at the hive entrance using sterile forceps and preserved in 96% ethanol. Sampling was carried out in April to June 2023. The samples were transported to the Biotechnology Laboratory, Lampung Veterinary Investigation Center, Bandar Lampung and stored at -20 °C until further analysis.

Because mitochondrial DNA is maternally inherited and generally homogeneous within colonies, one worker per colony was used to represent colony-level mitochondrial haplotypes.

DNA extraction and PCR amplification

Genomic DNA was extracted from the whole body of individual worker bees using the QIAGEN DNeasy Blood & Tissue Kit, following the manufacturer's protocol. DNA quality and quantity were assessed using agarose gel electrophoresis.

A fragment of the mitochondrial 16S rRNA gene was amplified using the primer pair described by Wang et al. (2020), consisting of forward primer 5'-TGG CTG CAG TAT AAC TGA CTG TAC AAA GG-3' and reverse primer 5'-GAA ACC AAT CTG ACT TAC GTC GAT TTG A-3'.

PCR amplification was performed in a total reaction volume of 25 µL containing PCR master mix, 10 pmol of each primer, genomic DNA template, and nuclease-free water. The amplification conditions included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 5 min.

PCR products were visualized on 1.5% agarose gel to confirm successful amplification. Successful amplicons were purified and sequenced in both directions using a commercial sequencing service.

Sequence processing and genetic analysis

Raw sequence chromatograms were inspected and edited to remove ambiguous bases and low-quality regions. Forward and reverse sequences were assembled into consensus sequences. Multiple sequence alignment was performed using the ClustalW algorithm implemented in MEGA XI.

The final aligned fragment length used for analysis was approximately 500 bp after trimming. Sequence similarity was assessed using BLAST searches against the NCBI database to confirm species identity.

Genetic distances were calculated using the Kimura-2-Parameter (K2P) model in MEGA XI. Pairwise distances were computed using pairwise deletion for gaps and missing data. Reference sequences of *Heterotrigona itama*, *H. erythrogaster*, *Tetragonula minangkabau*, and *Apis cerana* were retrieved from GenBank to support comparative analysis.

Phylogenetic analysis

Phylogenetic relationships were reconstructed using both Neighbour-Joining (NJ) and Maximum Likelihood (ML) methods implemented in MEGA XI. The best-fit nucleotide substitution model was selected based on the lowest Bayesian Information Criterion (BIC).

Bootstrap analysis with 1000 replicates was performed to assess node support, and values ≥70% were considered indicative of strong support. *Apis cerana* was used as an outgroup to root the phylogenetic tree.

The resulting tree topology was used to evaluate the placement of sampled colonies relative to reference sequences.

RESULTS AND DISCUSSION

The results are organized into four sections, including PCR amplification and sequence characteristics, sequence similarity and species identification, genetic distance analysis, and phylogenetic relationships, to describe the molecular features of the analyzed *Heterotrigona itama* colonies.

PCR amplification and sequence characteristics

PCR amplification of the mitochondrial 16S rRNA gene was successfully performed for all sampled colonies (HB1, HB2, and HB3). The amplification results were visualized using agarose gel electrophoresis to assess the presence and size of the PCR products (Figure 1).

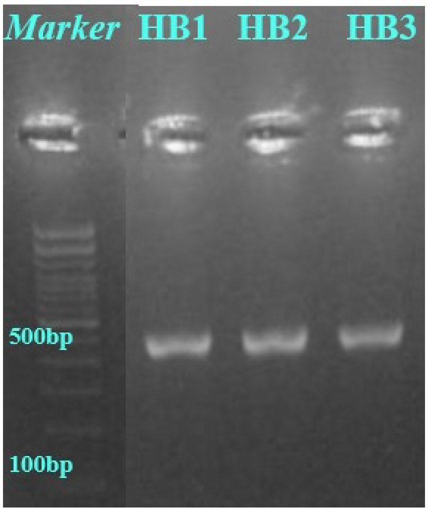

Figure 1 Agarose gel electrophoresis of 16S rRNA PCR amplicons from stingless bee colonies HB1, HB2, and HB3.

The electrophoresis results showed that all samples produced clear and distinct DNA bands at approximately 500 bp. The bands corresponding to HB1, HB2, and HB3 were consistently observed at the same position on the gel, indicating similar fragment lengths among all samples. No additional bands or visible smearing were detected in any of the lanes.

The DNA bands appeared sharp and well-defined, with comparable intensity across the three colonies. The absence of non-specific amplification products and primer-dimer formations indicates that the PCR amplification yielded specific target fragments for all analyzed samples.

Sequence similarity and species identification

Sequence similarity analysis was conducted by comparing the obtained 16S rRNA sequences with reference sequences available in the NCBI database. The results of this comparison, including accession numbers, sequence lengths, and percentage identity values, are presented in Table 1.

Table 1 Sequence similarity analysis.

No	Species	Acc. Number	Acc. Length	Percent Identity (%)
1.	*H. itama*	KU571761	528	98.38
2.	*H. itama*	KU571733	507	96.91
3.	*H. itama*	KX113624	506	96.91
4.	*H. itama*	DQ790396	528	96.91
5.	*H. erythogaster*	DQ790395	528	93.16
6.	*Apis cerana*	L22892	470	-
7.	*Tetragonula minangkabau*	MH453962	446	-

The analyzed sequences showed high similarity to *Heterotrigona itama* reference sequences. The highest similarity value was observed with accession KU571761 (98.38%), followed by KU571733, KX113624, and DQ790396, each showing 96.91% identity. The corresponding sequence lengths of these references ranged from 506 to 528 base pairs.

In comparison, the sequence similarity with *H. erythrogaster* (DQ790395) was lower at 93.16%. The reference sequences of *Apis cerana* (L22892) and *Tetragonula minangkabau* (MH453962) were included in the analysis, with sequence lengths of 470 bp and 446 bp, respectively, although percentage identity values were not reported for these taxa in the table.

Genetic distance analysis

Genetic distance among the analyzed sequences was calculated using the Kimura-2-Parameter (K2P) model. Pairwise distance values between samples and reference sequences are presented in Table 2.

Table 2 Genetic distance analysis.

Species	HB1	HB2	HB3	KU571761	KU571733	KX113624	DQ790396	DQ790395	L22892	MH453962
HB1		0.0000	0.0000	0.0069	0.0069	0.0069	0.0069	0.0123	0.0215	0.0183
HB2	0.0000		0.0000	0.0069	0.0069	0.0069	0.0069	0.0123	0.0215	0.0183
HB3	0.0000	0.0000		0.0069	0.0069	0.0069	0.0069	0.0123	0.0215	0.0183
KU571761 *H. itama*	0.0182	0.0182	0.0182		0.0000	0.0061	0.0061	0.0134	0.0216	0.0181
KU571733 *H. itama*	0.0182	0.0182	0.0182	0.0000		0.0061	0.0061	0.0134	0.0216	0.0181
KX113624 *H. itama*	0.0182	0.0182	0.0182	0.0156	0.0156		0.0000	0.0132	0.0215	0.0181
DQ790396 *H. itama*	0.0182	0.0182	0.0182	0.0156	0.0156	0.0000		0.0132	0.0215	0.0181
DQ790395 *H.*	0.0625	0.0625	0.0625	0.0729	0.0729	0.0729	0.0729		0.0226	0.0171

Species	HB1	HB2	HB3	KU571761	KU571733	KX113624	DQ790396	DQ790395	L22892	MH453962
erythrogaster										
L22892 *A. cerana*	0.2336	0.2336	0.2336	0.2388	0.2388	0.2388	0.2388	0.2336		0.0225
MH453962 *T. minangkabau*	0.1545	0.1545	0.1545	0.1518	0.1518	0.1518	0.1518	0.1283	0.2500	

The genetic distance among the sampled colonies (HB1, HB2, and HB3) was 0.0000, indicating identical sequences across all three samples. Distances between the sampled colonies and *H. itama* reference sequences (KU571761, KU571733, KX113624, and DQ790396) ranged from 0.0069 to 0.0182. The distances among the reference *H. itama* sequences themselves varied between 0.0000 and 0.0061.

Higher genetic distances were observed between the sampled colonies and other species. The distance between the sampled colonies and *H. erythrogaster* (DQ790395) was 0.0625. Greater distances were recorded with *Tetragonula minangkabau* (MH453962), ranging from 0.1545, and the highest values were observed with *Apis cerana* (L22892), reaching 0.2336. Distances among non-*H. itama* reference sequences also showed variation, including values such as 0.0226 between *H. erythrogaster* and *A. cerana*, and 0.0171 between *H. erythrogaster* and *T. minangkabau*.

Phylogenetic relationships

Phylogenetic relationships among the analyzed samples and reference sequences were reconstructed based on mitochondrial 16S rRNA sequences. The resulting tree topology, including bootstrap support values, is presented in Figure 2.

The phylogenetic tree shows that the three sampled colonies (HB1, HB2, and HB3) are grouped together in a single cluster labeled as *H. itama* from Punggur, with a bootstrap support value of 95. This cluster is positioned within a larger clade that includes multiple *H. itama* reference sequences, such as KU571761, KU571733, KX113624, and DQ790396, which are supported by bootstrap values of 98.

The broader tree structure indicates that the *H. itama* group is separated from *H. erythrogaster* (DQ790395), which forms a distinct branch with bootstrap support values of 89 and 98 in different parts of the tree. More distant taxa, including *T. minangkabau* (MH453962) and *Apis cerana* (L22892), are located in separate branches, with *A. cerana* positioned as the outgroup. The scale bar shown in the figure represents a genetic distance of 0.02 substitutions per site.

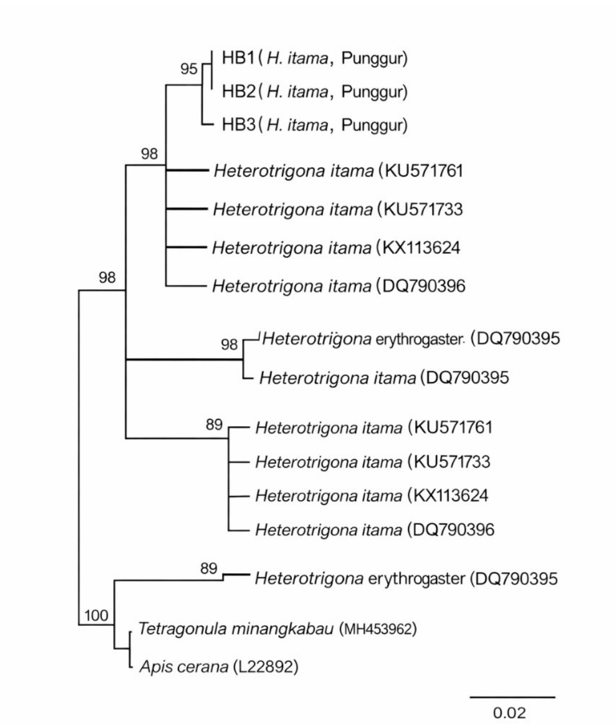

Figure 2 Maximum Likelihood phylogenetic tree based on mitochondrial 16S rRNA sequences showing the evolutionary placement of *Heterotrigona itama* colonies from Punggur, Central Lampung (HB1–HB3) relative to reference sequences retrieved from GenBank. Bootstrap values (1000 replicates) ≥70% are indicated at nodes. *Apis cerana* was used as the outgroup. Scale bar represents nucleotide substitutions per site.

Discussion

The results of this study show that all sampled colonies (HB1–HB3) share identical mitochondrial 16S rRNA sequences, resulting in a single detected haplotype. This pattern indicates mitochondrial uniformity among the analyzed colonies and suggests the absence of detectable variation within the sampled dataset (Marquis et al., 2017). Similar observations have been reported in other stingless bee populations, particularly in managed systems, where colony propagation practices may influence haplotype distribution and reduce local genetic variability (Neumann and Blacquière, 2017; Santiago et al., 2016). In meliponiculture, colony multiplication through brood division, artificial splitting, and exchange among beekeepers may lead to repeated use of the same maternal lineage, thereby contributing to reduced variation in mitochondrial lineages at local scales (Quezada-Euán & Alves, 2021).

In addition, the relatively homogeneous genetic pattern observed in this study may also reflect the combined effects of limited dispersal, localized colony management, and potential founder effects. In managed environments, colonies are often derived from a restricted number of initial stocks, which may result in reduced haplotype diversity over time (Szűcs et al., 2017). However, it is important to emphasize that the present findings are based on a limited number of colonies from a single locality. Therefore, the observed uniformity should be interpreted as a preliminary pattern within the sampled colonies rather than a comprehensive representation of mitochondrial variation across the broader population. A more extensive sampling design, including multiple colonies from different sites and management systems, would be necessary to determine whether similar patterns are consistently observed at larger spatial scales.

The sequence similarity analysis further supports the identification of the sampled colonies as *H. itama*, with BLAST identity values ranging from 96.91% to 98.38% compared to reference sequences. These values fall within the range commonly reported for mitochondrial markers in Meliponini, where the 16S rRNA gene is frequently used for species-level identification and comparative analyses (Urueña et al., 2022; Marconi et al., 2022). The phylogenetic clustering of the samples within the *H. itama* clade provides additional support for this identification, reinforcing the consistency between similarity-based and tree-based approaches.

Nevertheless, the variation in similarity values among reference sequences suggests the presence of some level of sequence divergence within the species. This divergence may reflect regional variation, differences in sequence length or coverage, or methodological differences in alignment and trimming Portik & Wiens, 2021). Comparable patterns have been documented in other studies of stingless bees, where mitochondrial markers reveal moderate intraspecific variation across geographic regions and environmental gradients (Maia et al., 2022; Wongsa et al., 2024). Such variation is not uncommon in widely distributed species and may indicate the existence of geographically structured lineages, although confirming this would require broader sampling and additional genetic markers.

The genetic distance analysis reveals a clear and consistent pattern in which divergence values are lowest among the sampled colonies and increase progressively when compared with other species. The zero genetic distance among HB1–HB3 confirms the absence of detectable variation in the analyzed fragment and is consistent with the presence of a single haplotype. At the same time, low divergence values relative to *H. itama* reference sequences indicate close genetic relationships within the species. This pattern is in line with expectations for intraspecific comparisons, where genetic distances are generally low but not necessarily identical due to geographic and evolutionary factors (Lawrence & Fraser, 2020).

In contrast, higher divergence values observed with *H. erythrogaster*, *T. minangkabau*, and *A. cerana* reflect increasing taxonomic separation, a pattern commonly observed in mitochondrial-based analyses across insect taxa (Kim et al., 2019). The stepwise increase in genetic distance from intra-colony comparisons to interspecific and intergeneric comparisons provides a consistent framework for interpreting the genetic relationships among the analyzed sequences (Ellegaard & Engel, 2019). However, it is important to recognize that this analysis is based on a single mitochondrial marker, which reflects only maternal lineage information and does not capture the full extent of genomic variation. As such, the results should be considered as an initial approximation of genetic relationships rather than a comprehensive assessment of population structure or evolutionary history.

The phylogenetic reconstruction further illustrates the placement of the sampled colonies within a well-supported *H. itama* clade, in agreement with both sequence similarity and genetic distance results. The clustering pattern shows that the sampled colonies form a closely related group nested within a broader lineage of *H. itama*, while other species are positioned in clearly separated branches. This topology is consistent with previous phylogenetic studies of Meliponini, which demonstrate clear separation among species and genera based on mitochondrial data (Sann et al., 2018; Urueña et al., 2022).

At the same time, the phylogenetic tree also highlights the limitations of the current dataset. The resolution of phylogenetic relationships is influenced by the number of sequences, the length of the analyzed fragment, and the diversity of reference taxa included in the analysis. With a limited number of samples and a single gene region, the resulting tree provides a general framework for species placement but does not allow detailed inference of phylogeographic patterns or lineage diversification. Expanding the dataset to include additional sequences from different regions would likely improve the resolution and provide a more comprehensive understanding of evolutionary relationships within *H. itama*.

Taken together, these findings provide an initial molecular baseline for *Heterotrigona itama* colonies in Central Lampung, particularly within the context of managed meliponiculture systems. Such baseline data are important for supporting future studies on genetic variation, species identification, and biodiversity documentation in Indonesian stingless bees (Trianto et al., 2023; Henderson et al., 2025). In regions where molecular data are still limited, even small-scale studies can contribute valuable reference information for subsequent research and comparative analyses.

Nevertheless, several limitations should be acknowledged. The study is based on a small number of colonies from a single locality and relies on a single mitochondrial marker, which restricts the scope of inference. The absence of nuclear genetic data further limits the ability to assess overall genetic diversity, population connectivity, and potential gene flow among colonies. In addition, the lack of detailed information on colony origin, management history, and landscape context prevents a more comprehensive evaluation of the factors influencing genetic patterns.

Therefore, future research should incorporate larger sample sizes, include both managed and wild populations, and utilize additional molecular markers such as nuclear genes, microsatellites, or SNPs to provide a more comprehensive understanding of genetic structure and evolutionary patterns in stingless bee populations. Integrating molecular data with ecological, geographic, and management information will also be essential for developing more robust interpretations and for supporting sustainable meliponiculture practices in Indonesia and other tropical regions.

CONCLUSIONS

This study demonstrates that the analyzed *Heterotrigona itama* colonies from Central Lampung share identical mitochondrial 16S rRNA sequences, indicating a single haplotype among the sampled colonies. Sequence similarity, genetic distance, and phylogenetic analyses consistently support the identification of these colonies as *H. itama*. These findings provide a preliminary molecular baseline for stingless bee colonies in the region. However, due to the limited sample size and the use of a single mitochondrial marker, broader conclusions regarding population structure require further investigation using larger datasets and additional molecular markers.

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Authors' Contributions: Priyambodo contributed to the research concept, manuscript drafting, and sample collection. Nindy Permatasari participated in sample collection. Elly Lestari Rustiati provided critical review and manuscript refinement. Aditya Fahrezi, Enny Saswiyanti, and Aulia Imtitsal conducted laboratory analyses, while Eko Agus Srihanto and Dian Neli Pratiwi performed data analys.

Competing Interests: The authors declare that there are no competing interests.

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