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Phylogenetic Construction of Stingless Bees in Bandar Lampung based on 16S rRNA Gene

Priyambodo Priyambodo[1*], Elly Lestari Rustiati[2], Dian Neli Pratiwi[3], Alvin Wiwiet Susanto[4], Eko Agus Srihanto[5], Enny Saswiyanti[6]

[1, 2]Universitas Lampung, Indonesia
[3]Akar Lestari Indonesia Foundation, Indonesia
[4]Uppsala University, Sweden
[5, 6]Lampung Veterinary Investigation Center, Indonesia

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*Correspondence email:
priyambodo@fmipa.unila.ac.id

ABSTRACT

Stingless bees are one of the honey-producing groups that are currently in increasing demand. The significant rise in honey demand necessitates research into the variety of stingless bee species that have the potential to produce high-quality honey in large quantities. This study aims to determine the genetic relationship of stingless bees from two colonies in Rajabasa, Bandar Lampung. Samples were collected from two different colonies in Rajabasa, Bandar Lampung. Molecular analysis was conducted, including DNA extraction, amplification, DNA sequencing, and phylogenetic analysis using the 16S rRNA gene. The study assessed homology values, genetic distance, and the phylogenetic map. The results indicated that the individual samples, GB1 and GB2, found in Bandar Lampung, were closely related to the species Heterotrigona itama. This was confirmed by the query value of both samples being 100%, with similarity values of 96.99% and 97.20%, respectively. The genetic distance analysis, visualized in the phylogenetic map, also shows that the two samples are close relatives of Heterotrigona itama.

Konstruksi Filogenetik Lebah Tanpa Sengat di Bandar Lampung berdasarkan Gen 16S rRNA

ABSTRAK: *Lebah tanpa sengat menjadi salah satu kelompok penghasil madu yang saat ini makin banyak diminati masyarakat. Peningkatan kebutuhan madu yang signifikan, memerlukan penelitian tentang ragam spesies lebah tanpa sengat yang potensial dalam menghasilkan madu yang berkualitas prima dalam jumlah yang melimpah. Penelitian ini telah dilakukan dengan tujuan untuk mengetahui kedekatan genetik lebah tanpa sengat dari dua koloni di Rajabasa, Bandar Lampung. Sampel diambil dari dua koloni yang berbeda di Rajabasa, Bandar Lampung. Analisis molekuler telah dilakukan dengan tahapan ekstraksi DNA, amplifikasi DNA, sekuensing DNA, dan dilanjutkan dengan analisis filogenetik dengan menggunakan gen 16S rRNA. Analisis dilakukan untuk mengetahui nilai homologi, jarak genetik, dan peta filogenetik. Hasil menunjukkan bahwa individu sampel GB1 dan GB2 yang ditemukan di Bandar Lampung mempunyai kedekatan dengan spesies Heterotrigona itama. Hal ini ditunjukkan dengan nilai query keduanya sebesar 100% dan nilai similarity masing-masing adalah 96,99% dan 97,20%. Analisis jarak geneti yang divisualisasi dalam peta filogenetik juga menunjukkan bahwa kedua sampel yang diperoleh merupakan kerabat dekat dari Heterotrigona itama.*

INTRODUCTION

Honey has been used historically in various ancient civilizations for food and medicinal purposes (Qamar & Rehman, 2020). In terms of treatment, honey is known as a nutritious ingredient in healing various diseases, such as burns, ulcers, diabetes, and wounds (Zafar et al., 2020). Scientific studies have validated that honey can show effectiveness against digestive and cardiovascular diseases (Ahmed et al., 2023).

The value of honey trade, especially in Asia, has experienced rapid growth driven by increased global demand, thus opening up export opportunities for quality honey products (Pippinato et al., 2020). This opportunity encourages the spread of honey cultivation in Indonesia because it can improve the local economy (Harianja et al., 2023). This increase is also seen in the increasing practice of honey collection, especially from stingless bee types for rural communities (Fajari et al., 2023).

Honey produced by stingless bees contains a wide range of bioactive compounds, including flavonoids and phenolic acids, which can aid in obesity management, have antimicrobial antioxidant properties, and show potential antihyperglycemic effects (Setiawan et al., 2024) (Melia et al., 2024);(Cabezas-Mera et al., 2024). Studies show that honey from stingless bees has potential benefits in regulating weight and gut microbiota (Mas'ud et al., 2023).

Remero et al. (2024) showed that variations in water content and antioxidant properties of stingless bees depend on their type and geographical origin. Therefore, research on species diversity and distribution of stingless bees is important. Given the need for a more comprehensive mapping of stingless bee farming practices to meet national standards and needs and global export potential (Rahmad et al., 2024).

Research on the diversity of species of stingless bees is important in cultivation and conservation strategies. Efforts to trace genetic diversity using molecular markers are crucial and accurate in characterizing germplasm, genetic variation, and optimization of breeding programs (Muthmainnah et al., 2023);(Bunjkar et al., 2024). High genetic diversity can support the adaptation process to population changes (Chika & Zahro, 2024);(Godiyal et al., 2024).

The molecular methods continue to grow in research on species diversity in biological studies. Genetic diversity research based on the 16s rRNA gene has been used in a wide variety of living things, including microbes associated with stingless bees (Trianto & Purwanto, 2020a);(Iskandar et al., 2021);(Tola et al., 2021);(Tarlinton et al., 2023). The genes used in the molecular detection of genetic diversity in stingless bees are also widely used, one is in research to analyze microbial diversity and relationships between species (Fatwa et al., 2021).

Indonesia is recorded to have 52 types of stingless bees, with a distribution in various regions in Indonesia, including Sumatra and Java (Trianto et al., 2023). Research in South Kalimantan documented ten species of stingless bees through their morphological and hive structure characterization methods, which are critical for species identification and conservation efforts (Purwanto et al., 2022). Molecularly, six types of stingless bees have been identified in Yogyakarta using the 16S rRNA gene (Trianto & Purwanto, 2020). Priyambodo et al. (2023) have amplified the 16S rRNA gene in several types of stingless bees in Pesawaran.

Bandar Lampung is a city that has a high potential for stingless bee cultivation (Lestari, 2022). Research on stingless honey's authenticity has been carried out using a portable LED-based fluorescence spectroscopy method (Suhandy et al., 2023).

In addition, Siti et al. (2023) have researched the characterization of beebread from stingless bees in Bandar Lampung. However, there has been no research on molecularly detecting stingless bee species in Bandar Lampung. Therefore, researchers are interested in conducting this research. This study aims to determine the genetic proximity of stingless bees from two colonies in the Rajabasa area, Bandar Lampung.

METHOD

Sampling

Samples of stingless bees were taken from two colonies in Rajabasa Jaya Village, Rajabasa District, Bandar Lampung City. The procedure is shown in Figure 1. The colonies were directly sampled using transparent plastic.

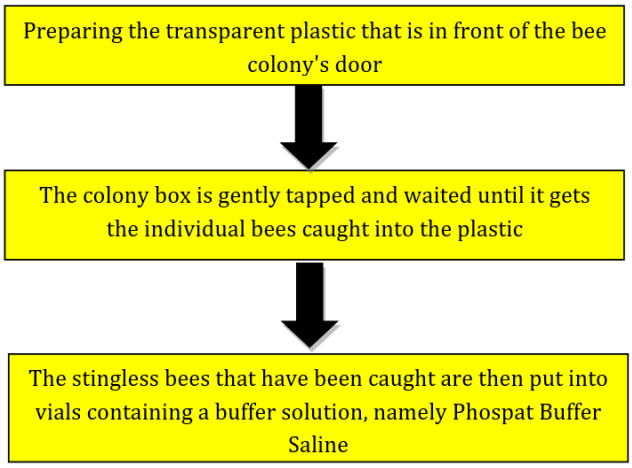

Figure 1. Sampling Procedure

DNA Extraction

DNA extraction was performed at the Biotechnology Laboratory, Lampung Veterinary Center, Bandar Lampung, using the GeneAid™ Genomic DNA Mini Kit (Tissue). The first stage in DNA extraction was tissue dissociation by grinding individual bees without stinging and adding 200µl of GT buffer and 20 µl of proteinase K. After that. It was incubated for five minutes. The second stage was lysis, in which 200 µl of GBT buffer was added, homogenized, and incubated at 60°C for 20 minutes. The sample was left at room temperature for five minutes. The third stage was DNA binding. At this stage, 200 µl of absolute ethanol was added and centrifuged at 15,000 × g for two minutes. The fourth stage was a cascade wash using 200 µl W1 buffer and 600 µl wash buffer. Each washing stage was centrifuged at 15,000 × g for 30 seconds. Next, DNA elution was carried out as the last stage by adding 100 µl of elution buffer. The success of the DNA extraction procedure was carried out qualitatively using the 1% agarose gel electrophoresis method.

DNA Amplification

Amplification of the 16S rRNA gene in the mitochondrial genes of individual bees without stings was performed using the LR13107-F primary pair as the primary forward with the sequence 5'-TGG CTG CAG TAT AAC TGA CTG TAC AAA GG - 3' and LR12647-R as a reverse primer with the sequence 5'-GAA ACC AAT CTG ACT TAC GTC GAT TTG A - 3' (Rachmawati et al., 2022). DNA amplification was carried out in a thermocycler machine for 35 cycles, with the pre-denaturation stage at 94°C for five minutes and denaturation at 94°C for one minute. Then, it was continued with annealing at 60°C for one minute, elongation at 72°C for two minutes, and finally, the post-elongation stage at 72°C for seven minutes. The results of the amplification process were qualitatively tested using 1% agarose gel electrophoresis.

Data Sequencing and Analysis

DNA sequencing of individual stingless bees was carried out through PT Genetics Science. Data analysis was carried out using gene bank data using the Basic Local Alignment Search Tool on the NCBI website. Molecular Evolutionary Genetics Analysis Software performed advanced analysis for genetic distance analysis and phylogenetic construction

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RESULTS AND DISCUSSION

Table 1 shows the results of DNA sequencing from two individuals of stingless bee samples in Bandar Lampung, based on the stages of DNA extraction, DNA amplification, DNA sequencing, and phylogenetic analysis using the 16S rRNA gene.

Table 1. Nucleotide Base Arrangement of Two Individuals of Stingless Bee DNA Samples in Bandar Lampung

No	Sample	Nucleotide Base Sequence
1	GB1 sample	TAGCATATCCAATTGGTTTTTAATTGGAATCTGGAATGAAAGGATTAATGAAATATGT ACTGTCTCAGTTGTACAATTATGAAATTAAAATTTTAATAAAAATGTTAAAATTAACT TATGGGACGATAAGACCCTATAGAATTTTATATTGAAATTACTCAGTAGTTAATTCAG AGATAGTTTCAATATTTGATTGGGAGGATTATTAAATTTAATTAACTTTAATTTAAAT TAACTTTGATTTAAGAGTAAATAATGATCTTCAATTTGAAATTGCTAGAATAAATTAC CTTAGGGATAACAGCGTAATACTTTTTTATAGGCCATATAGAAAAAAGTGGTTGCGAC CTCGATGTTGAATTAGGATAAATTTTAAATGCAGGAGTTTAATAATTAAGTCTGTTCG ACTTTTAACAATCCTACATGATTTGA
2	GB2 sample	CATAATCAATTGGGTTTTTAATGAAATCTGGAATGAAAGGATTAATGAAATATGTACT GTCTCAGTTGTACAATTATGAAATTAAAATTTTAATAAAAATGTTAAAATTAACTTAT GGGACGATAAGACCCTATAGAATTTTATATTGAAATTACTCAGTAGTTAATTCAGAGA TAGTTTCAATATTTGATTGGGAGGATTATTAAATTTAATTAACTTTAATTTAAATTAA CTTTGATTTAAGAGTAAATAATGATCTTCAATTTGAAATTGCTAGAATAAATTACCTT AGGGATAACAGCGTAATACTTTTTTATAGGCCATATAGAAAAAAGTGGTTGCGACCTC GATGTTGAATTAGGATAAATTTTAAATGCAGGAGTTTAATAATTAAGTCTGTTCGACT TTTAACAATCCTACCATGATTT

The nucleotide sequences of the two sample individuals were entered into the National Center for Biotechnology Information (NCBI) website to determine the homology. Based on the results from BLAST, it was found that the bee sample from Bandar Lampung, which lacked GB1 stingers, had the closest homology to *Heterotrigona itama* with accession number KX113624.1 (Table 2).

Table 2. Homological Values of GB1 Stingless Bee DNA Samples in Bandar Lampung with Data from NCBI

Comparator Species	Homology Values	
	Query (%)	***Similarity (%)***
Heterotrigona itama (KX113624.1)	100	96.99
Heterotrigona itama (DQ788141.1)	100	96.76
Heterotrigina bakeri (DQ790397.1)	100	94.68
Heterotrigona erythrogaster (DQ790395.1)	100	93.75
Sundatrigona sp. (KU571795.1)	100	91.69
Sundatrigona moorei (DQ790402.1)	100	91.45
Platytrigona hobbyi (DQ790401.1)	100	89.86
Lepidotrigona ventralis (DQ790400.1)	100	89.68
Lepidotrigona terminate (MG543810.1)	100	88.97
Geniotrigona thoracica (KU571732.1)	100	88.53
Geniotrigona incasa (DQ790392.1)	97	88.97

Based on the results of BLAST analysis, individual samples of bees without GB1 stingers had the highest similarity with the comparison species H. *Itama*, with a query value of 100% and a similarity value of 96.99. These results are consistent with the analysis of individual bee samples without GB2 stingers (Table 3).

Table 3. Homological Values of GB1 Stingless Bee DNA Samples in Bandar Lampung with Data from NCBI

Comparator Species	Homology Values	
	Query (%)	***Similarity (%)***
Heterotrigona itama (KX113624.1)	100	97.20
Heterotrigona itama (DQ788141.1)	100	96.96
Heterotrigina bakeri (DQ790397.1)	100	94.86
Heterotrigona erythrogaster (DQ790395.1)	100	93.93
Sundatrigona sp. (KU571795.1)	100	91.84
Sundatrigona moorei (DQ790402.1)	100	91.61
Platytrigona hobbyi (DQ790401.1)	100	90.00
Lepidotrigona ventralis (DQ790400.1)	100	89.81
Lepidotrigona terminate (MG543810.1)	100	89.10
Geniotrigona thoracica (KU571732.1)	100	88.66

The results from the GB1 sample individuals show that, like the GB2 sample, the highest homological value was found with the comparison species Heterotrigona itama. Based on the data obtained from the analysis, both samples had homological values 100% similar to those of Heterotrigona itama. A 100% similarity indicates that each DNA sequence from both sample individuals fully matched the Heterotrigona itama reference sequence in the NCBI database. Additionally, the high similarity values suggest that most of the nucleotides in the DNA sequences of both sample individuals were identical to those in the Heterotrigona itama reference. The data also revealed that the GB1 sample had a slightly lower similarity to Heterotrigona itama than the GB2 sample. This difference in similarity values may be due to factors affecting evolutionary patterns, such as intraspecific variations, minor mutations, or different environmental adaptations in each population (Kamarudin et al., 2016). The analysis of genetic distance is presented in Table 4.

Table 4. Genetic Distance of Two Individuals of a Stingless Bee DNA Sample in Bandar Lampung with a Database on NCBI

	1	2	3	4	5	6	7	8	9	10	11	12
Sampel GB1		0.007	0.011	0.040	0.037	0.038	0.036	0.035	0.039	0.033	0.030	0.030
Sampel GB2	0.017		0.010	0.040	0.036	0.036	0.035	0.033	0.038	0.031	0.029	0.028
KX113624.1 *Heterotrigona itama*	0.037	0.031		0.038	0.034	0.033	0.033	0.031	0.036	0.031	0.029	0.028
MF661805.1 *Tetragonula carbonaria*	0.221	0.222	0.209		0.021	0.019	0.026	0.026	0.020	0.030	0.035	0.035
MN659662.1 *Tetragonula davenporti*	0.204	0.197	0.182	0.098		0.021	0.029	0.029	0.024	0.030	0.032	0.033
MN659624.1 *Tetragonula hockingsi*	0.208	0.201	0.181	0.089	0.099		0.023	0.023	0.024	0.028	0.031	0.031
MG543811.1 *Tetragonula laeviceps*	0.205	0.197	0.185	0.139	0.164	0.124		0.005	0.028	0.029	0.029	0.031
NC 066054.1 *Tetragonula pagdeni*	0.192	0.185	0.173	0.142	0.160	0.121	0.008		0.028	0.029	0.029	0.030
DQ790431.1 *Tetragonula sirindhornae*	0.217	0.209	0.201	0.101	0.124	0.121	0.156	0.156		0.024	0.036	0.036
MG543813.1 *Tetragona apicalis*	0.184	0.177	0.176	0.166	0.162	0.148	0.159	0.158	0.125		0.027	0.027
MN747147.1	0.159	0.152	0.152	0.195	0.177	0.166	0.159	0.158	0.201	0.140		0.004

	1	2	3	4	5	6	7	8	9	10	11	12
Lepidotrigona flavibasis MG529489.1 *Lepidotrigona ventralis*	0.152	0.145	0.145	0.195	0.184	0.166	0.166	0.165	0.201	0.141	0.006	

The genetic distance values confirm the homology between the two sample individuals, GB1 and GB2, and *Heterotrigona itama* (KX113624.1). The genetic distances for the two samples are the lowest compared to other species, with values of 0.037 for GB1 and 0.031 for GB2, respectively. This indicates that both sample individuals have a high genetic affinity with *Heterotrigona itama*. Such small genetic distances support the identification of the samples as *Heterotrigona itama*, as values below 0.05 generally suggest intraspecific genetic variation, which is within the natural genetic limits of a single species.

In contrast, a significant difference is observed between species from other genera, such as *Tetragonula carbonara* (MF661805.1), which show genetic distance values of 0.221 for GB1 and 0.222 for GB2. This suggests that the two stingless bee individuals from Bandar Lampung are genetically distinct from species in the *Tetragonula* genus, further confirming the identity of the samples as *Heterotrigona*.

The genetic distance values also show high divergence with other *Tetragonula* species, such as *Tetragonula davenporti* and *Tetragonula sirindhornae*, further underscoring the cladistic differences between *Heterotrigona* and *Tetragonula*.

The genetic distance data was then visualized in a phylogenetic map to represent the closeness of kinship between the two stingless bee samples from Bandar Lampung and the other stingless bee species recorded in the NCBI (Figure 2).

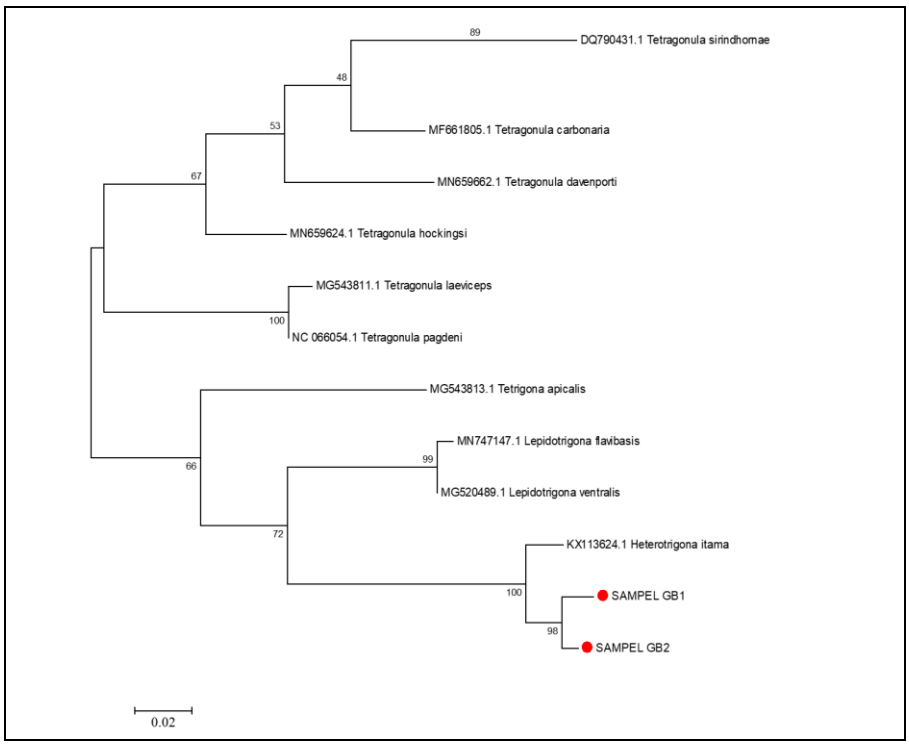

Figure 2. Phylogenetic Map of Stingless Bees in Bandar Lampung

This phylogenetic map places the GB1 and GB2 sample individuals in a clade close to Heterotrigona itama. Both samples show a strong kinship with Heterotrigona itama, further supported by the low genetic distance in the previous data. This suggests

that the origin of these samples is most likely Heterotrigona itama. The close phylogenetic relationship places GB1 and GB2 in the same clade as Heterotrigona itama, indicating a similar evolutionary and adaptive proximity to this species in the Bandar Lampung environment.

Heterotrigona itama is important in Indonesia's biodiversity and stingless beekeeping practices. This species is valuable for honey production and serves as an environmental bioindicator. According to Kadarsah et al. (2024), *Heterotrigona itama* can be found in rubber plantations with variations in body size. Morphologically, *Heterotrigona itama* has a nest entrance dimension ranging from 2.50 to 18.00 cm (Joaty et al., 2022). In stingless bee cultivation studies, this species can effectively return to its hive from distances of up to 1000 m, with the optimal placement recommended to be within 500 m of the food source (Rusdimansyah et al., 2024). In behavioral studies, *Heterotrigona itama* shows peak daily foraging activity between 09:00 to 10:00 and 13:30 to 14:30 (Sriwahyuni et al., 2023).

The phylogenetic map also reveals the presence of several other species within the genus *Heterotrigona*, which appear in different clades. These differences in branching indicate a divergence among species within the genus. This divergence suggests the existence of adaptive or geographic variation among *Heterotrigona* species, which may have originated from different regions or ecosystems. Although these species share a common ancestor, they have undergone speciation, leading to distinct morphological and genetic adaptations in response to their respective environments.

In the phylogenetic map, the genus *Tetragonula* is positioned in a different clade from *Heterotrigona*, indicating a greater evolutionary distance between the two genera. The more distant position of *Tetragonula* suggests that these genera had different ancestors and diverged much earlier in their evolutionary history. This phylogenetic distance supports the idea that, although both belong to the *Meliponini* tribe, they exhibit adaptive differences that allow them to occupy distinct ecological niches, including differences in foraging patterns and environmental preferences.

The close phylogenetic relationship of the GB1 and GB2 sample individuals with *Heterotrigona itama* suggests that this species may be highly adapted to the local environmental conditions in Bandar Lampung. These adaptations could include preferences for local vegetation, foraging capabilities, or interactions with local flora species for pollination. This evolutionary proximity may also indicate low gene flow or migration from stingless bee populations in other regions, suggesting that the local species focus more on specific environmental adaptations within the Bandar Lampung area.

CONCLUSIONS AND SUGGESTIONS

Based on the analysis of nucleotide base sequences in the 16S rRNA gene of individual stingless bee samples GB1 and GB2 that have been analyzed for homology, genetic distance, and phylogenetic maps, the two samples are most closely related to the species *Heterotrigona itama.*

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