Journal of Tropical Biodiversity and Biotechnology
Volume 09, Issue 03 (2024): jtbb86256
DOI: 10.22146/jtbb.86256

Research Article

Detection of Entomological Origin of Honey Sold in Indonesia Based on *16S rRNA* Gene Analysis

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Keywords:
16S rRNA gene
Entomological origin of honey
Honey
Honey fraud
Submitted:
27 June 2023
Accepted:
08 December 2023
Published:
06 September 2024
Editor:
Furzani Binti Pa'ee

ABSTRACT
Honey is known for its various benefits for health, cosmetic ingredients, and other industrial materials. Especially, during the Covid-19 pandemic, many people consume honey to maintain body endurance. In Indonesia, the honey produced is dominated by *Apis mellifera* honey. With a cheaper price and a larger quantity, *A. mellifera* honey is often offered as forest honey or stingless bee honey to get more profit. Therefore, this study aims to determine the entomological origin of honey claimed as forest honey and stingless bee honey sold in the Indonesian market using the detection of *16S rRNA* gene amplicon. This study tested 30 samples of forest honey and 30 samples of stingless bee honey. DNA that has been isolated from honey samples was amplified by PCR using *16S rRNA* primers. The results from the sequence analysis showed that nine of honey samples were identified as honey fraud. Two samples were confirmed as falsification of the origin of honey-producing bees and four honey samples were confirmed as honey mislabelling. From this study it can be concluded that, it is possible to determine the entomological origin of honey molecularly by sequencing the *16S rRNA* gene. Therefore, this method can be used to identify honey fraud that may occur on the market.

INTRODUCTION
Honeybees (Apidae: Apini; *Apis*) and stingless bees (Apidae: Meliponini) belong to group of insects with a high diversity and wide distribution. As many as eight of the nine species belonging to the genus *Apis* in the world and about 46 species of stingless bees are widespread throughout Indonesia (Engel 2012; Kahono et al. 2018). The variety of honey bee species and stingless bees in Indonesia is also supported by Indonesia's geographical landscape, diverse landscape topography and environment, and the history of Indonesia's complex geological formation (Hall 2009). Honey bees and stingless bees are groups of bees that produce honey (Kahono et al. 2018; Gratzer et al. 2019; Buchori et al. 2022).

According to Codex Alimentarius (FAO 2019) honey is defined as a natural substance in the form of a sweet liquid produced by honey bees not only from plant nectar but also derived from plant secretions or plant -sucking insect excretions. Since a long time ago, Indonesian people have believed that honey, one of the traditional medicines, can cure various

diseases. Honey is beneficial both for health, cosmetics, and other industrial ingredients. In addition, honey can also act as an antioxidant, anti-inflammatory, anti-diabetic, and immune guard (Samarghandian et al. 2017). Therefore, during the Covid-19 pandemic, honey is sought after by the public to increase their body immunity to avoid the virus.

The highest honey production in Indonesia is produced in Java, 81.06% of the national production (Badan Pusat Statistik 2020). Mostly, it is produced by *Apis dorsata* and *Apis mellifera*. It has also been widely developed to increase honey production (Buchori et al. 2022). Honey production is also increasingly produced by grazing in Sumatra on industrial forest, *Accacia* and *Eucalyptus* (Pribadi 2016). Based on information from market players, some of *Apis mellifera* honey is forfeited as forest honey (honey from *Apis dorsata* or *Apis* spp) or stingless bee honey. Because the forest honey and stingless bees honey price is higher than *Apis mellifera* honey.

Honey fraud is a problem that often occurs. Based on APIMONDIA, honey fraud acts include dilution of honey with artificial syrup, premature harvesting of honey, use of ion-exchange resins, artificial feeding of honey-producing bees, and falsification or mislabeling of the origin of honey (geographical or botanical). This action can be considered fraud because, to gain more profit, honey is sold with a quality that does not meet global standards (APIMONDIA 2019). Therefore, it is necessary to have an effective method of determining the entomological origin of honey to avoid such fraud. Molecular identification of honey has been carried out using *16S rRNA*.

Research conducted by Kek et al. (2017) by detecting the *16S rRNA* and *CO1* genes, it was possible to determine the origin of honey from 14 samples obtained directly from the forest and one sample of commercial honey. In addition, research by Zhang et al. (2019) and Raffiudin et al. (2023) can also determine the origin of honey based on the presence of the *Major Royal Jelly Protein 2* (*mrjp2*) gene. However, previous research was still limited to honey samples from *A. mellifera* and *A. cerana*. Considering that the honey sold in the Indonesian market comes from various species of honey-producing bees, not only *A. mellifera* and *A. cerana* (Engel 2012; Kahono et al. 2018), therefore, this research is expanded on honey traded on the Indonesian market, marketed as forest honey and stingless bee honey.

MATERIALS AND METHODS

Materials

The tools used in the preparation of honey samples were a 50 mL falcon tube, 1.5 mL Eppendorf tube, semi-analytical balance (Ohaus), vortex, measuring cup, -4°C freezer, centrifuge (GyroZen), water bath, micropipette, pipette tips, and NanoDrop. For PCR analysis, the tools used were a PCR tool (Applied Biosystems™ 2720 thermal cycler), Spindown Mini Centrifuge, micropipette, 0.2 mL PCR tube, pipette tips, measuring cup, Erlenmeyer flask, spatula, semi-analytical balance, microwave, electrophoresis tool, UV transilluminator, and a smartphone camera.

The materials used in this study included 30 honey samples each claimed by the sellers or beekeepers to be forest honey (H1-H30) and stingless bees honey (K1-K30), absolute ethanol, sterile distilled water, DNA extraction kit FavorPrepTM Tissue Genomic DNA Extraction Mini Kit 100 Prep (Proteinase K) (animal tissue, Blood, Cell, fungus, bacteria), Gotaq® Green Master Mix Promega, primer (Table 1), agarose 1.5%, aquabidest, Nuclease Free Water (NFW), Fluorosafe, Geneaid 100 bp DNA Ladder and Tiangen 100bp DNA Ladder, and TBE buffer.

Table 1. Primers used in this study.

Primer	Sequence	Gene Marker	Product size (bp)	Reference
LR13107-F	TGG CTG CAG TAT AAC TGA CTG TAC AAA GG	16S rRNA *Tetragonula cf. pagdeni*	496	Thummajitsakul et al. 2013
LR12467-R	GAA ACC AAT CTG ACT TAC GTC GAT TTG A			

Methods

Sample collection

Samples were obtained from honey sold in online and offline shops directly from beekeepers in Klaten and Magelang that the seller claimed either as forest honey or stingless bee honey.

Sample preparation

The honey sample preparation method was carried out according to Thummajitsakul et al. (2013) with some modifications. A sample of 12.5 g of honey was put into a 50 ml falcon tube. Aquadest were added to the sample until it reached 50 ml. The honey solution is homogenized with a vortex. Samples were incubated at 40°C for 30 minutes in a water bath. The samples were centrifuged at 5000 rpm at 20°C for 20 minutes to precipitate the pellets. The supernatant was discarded, and the pellet was redissolved with distilled water. The honey sample was centrifuged at 5000 rpm at 20°C for 20 minutes to obtain pellets. 500 ul of aquabidest was added to the pellet and transferred to a 1.5 mL PCR tube. The sample can be extracted using Favorgen Tissue Genomic DNA Extraction Mini Kit 100 Prep (Proteinase K) (animal tissue, Blood, Cell, fungus, bacteria).

Polymerase Chain Reaction (PCR)

DNA amplification was carried out by PCR using primers LR13107-F and LR12647-R to detect the presence of bee DNA based on the *16S rRNA* gene, according to Thummajitsakul et al. (2013). The results of DNA isolation were amplified using an Applied Biosystems™ 2720 Thermal Cycler PCR machine with a reaction volume of 25 µL. Each reaction volume contains 12.5 µL GoTaq® Green Master Mix, 5.5 µL NFW, 1.75 µL forward primer, 1.75 µL reverse primer, and 3.5 µL template DNA. PCR conditions for DNA amplification are adjusted to predenaturation at 95°C for 2 minutes, (denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 30 s with 35 cycles), and final extension at 72°C for 5 minutes.

Data visualization and analysis

PCR products with an amplicon of the *16S rRNA* gene of honey bees and stingless bees were electrophoresed on 1.2% agarose gel. PCR products showing positive electrophoresis results were sequenced at Integrated Laboratory for Research and Testing (LPPT) of Universitas Gadjah Mada. The sequencing results obtained from LPPT UGM were edited with MEGA X software. The sequences were then saved in FASTA format for analysis using NCBI Nucleotide BLAST (BLAST-n) page (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLST-n results show the sequence results that are most similar to the consensus sequences.

RESULTS AND DISCUSSION

The initial stage for molecular testing is the extraction of honey samples to obtain bee DNA contained in honey. The bee DNA in honey is an environmental DNA (eDNA). eDNA is a DNA fragment released by an organism into the environment. eDNA can be derived from nuclear or mi-

tochondrial DNA, which can be found in the cellular or extracellular matrix. Faecal secretions, mucus, gametes, loose hair and skin, and carrion organisms are some of the sources of DNA found in the environment (Barnes & Turner 2016). Many uses of bee eDNA in honey have been carried out, such as to detect the presence of parasites and pathogens and to determine the origin of nectar and pollen obtained by honey bees (Matsuzawa et al. 2020; Ribani et al. 2020). In addition, it has also been carried out to determine the entomological origin of honey (Kek et al. 2017; Zhang et al. 2019).

The success of DNA extraction is indicated by the good quality in terms of DNA concentration and the purity of the DNA obtained. Based on this study, the DNA concentration obtained ranged from 1.31 to 2181.71 ng/nL with A260/A280 ratio average is 2.06 and A260/A230 ratio average is 1.91. This indicates that most of the DNA has good purity with low level of protein and organic component contamination in DNA. DNA with good purity has an A260/A280 ratio ranging from 2.0-2.2 and an A260/A230 ratio ranging from 1.8-2.0. DNA. The purity and concentration of DNA affect PCR efficiency (Lucena-Aguilar et al. 2016).

DNA is amplified using the Polymerase Chain Reaction (PCR) method. PCR is a method used to replicate DNA in vitro, making it possible to obtain multiple copies of DNA fragments from samples. In general, PCR reagents consist of template DNA, Taq polymerase, primers, dNTPs, and buffer solutions (Kadri 2019). In this study PCR used primer to detect *16S rRNA* gene sequences.

The probability of the presence *16S rRNA* gene in a genome preparation is greater, it is because the ribosomal RNA have more DNA copies (Hori & Engel 2023). The characteristic of the *16S rRNA* gene is that it is easy to isolate, over time it may change its sequence, so that detection among distant species is possible with this gene. In addition, this gene is also composed of various parts that are conserved (Byrne et al. 2018). PCR using primers from mitochondrial DNA shows a relatively faster evolution than nuclear DNA. In addition, the number of DNA copies inside the cell is high, so it can be used to differentiate between animal species (Kim et al. 2017).

The *16S rRNA* gene was detected in honey samples using the primer LR13107-F/LR12647-R (Thummajitsakul et al. 2013). The PCR was divided into two batches and repeated twice. The results of the first batch of PCR (Figure 1) showed that there were 13 forest honey samples (H1, H2, H3, H5, H6, H7, H8, H9, H10, H12, H13, H14, H15, H16) and five samples stingless bees honey (K17, K18, K19, K21, K25) is positive. Meanwhile, in the second repetition (Figure 2), it showed that 12 forest honey samples (H1, H2, H3, H5, H6, H7, H10, H12, H13, H14, H15, H16) were positive, and five samples of stingless bee honey (K17, K18, K19, K21, K25) were positive. The bands appearance indicates that the sample contains the *16S rRNA* gene from honey bees or stingless bees. Based on these results, the appeared band was around 500bp.

Batch 2 PCR results (Figure 3) showed that nine samples of forest honey were positive (H17, H19, H20, H22, H25, H27, H28, H29, H30) and eight samples of positive stingless bee honey (K1, K2, K5), K6, K7, K9, K13, K14). Meanwhile, the second repetition in Figure 4 shows that ten samples of forest honey were positive (H17, H18, H19, H21, H22, H23, H24, H26, H28, H30) and eight samples of positive stingless bee honey (K1, K2, K3, K5, K9, K15). The band's appearance indicates that the sample contains the *16S rRNA* gene from honey bee or stingless bee. It showed the band that appears is around 500 bp in size.

Based on the result from PCR batch 1 and PCR batch 2, the num-

ber of positive samples are different in the first repetition and second repetition. In PCR batch 1, the first repetition of samples H8 and H9 showed positive results with the appearance of a DNA band, but in the second repetition the DNA band did not appear. The same thing also happened to the results of PCR batch 2. The absence of DNA bands in the PCR results may be due to the small number of DNA copies (copy number) in the sample. This is because the source of bee DNA in honey is eDNA, which does not only contain bee DNA in honey. Then, if it is related to the concentration of the DNA extraction results, even though it has quite good purity, there is a DNA concentration that is too low or too high. When the DNA concentration gets higher, the inhibitor concentration also increases. It can inhibit replication process in PCR. Meanwhile, when the DNA concentration is too low, too few DNA copy numbers are formed to be visualized.

Possible causes of DNA bands that initially appeared then did not reappear were DNA samples that had been stored for too long after extraction which affects DNA stability but depends on buffer composition used (Röder et al. 2010), repeated freeze-thawing, DNA samples kept at room temperature for too long, and inefficient purification of DNA samples, resulting in residues nuclease is still present in the sample. The more freeze-thawing cycles, the greater the degradation rate of genomic DNA. The concentration of DNA stored at 100 mg/mL was slightly more stable during freeze-thawing than DNA stored at 10 mg/mL. Thus, it can be said that the stability of DNA not to be degraded during the freeze-thawing phase is better when the DNA has a concentration of more than 100 mg/mL (Shao et al. 2012).

The research data showed that it was possible to determine the entomological origin of honey molecularly by detecting the *16S rRNA* gene. However, it is necessary to sequence the *16S rRNA* gene to know which species are identified because the size of the DNA amplicon is the same in all honey bee and stingless bee samples around 500bp.

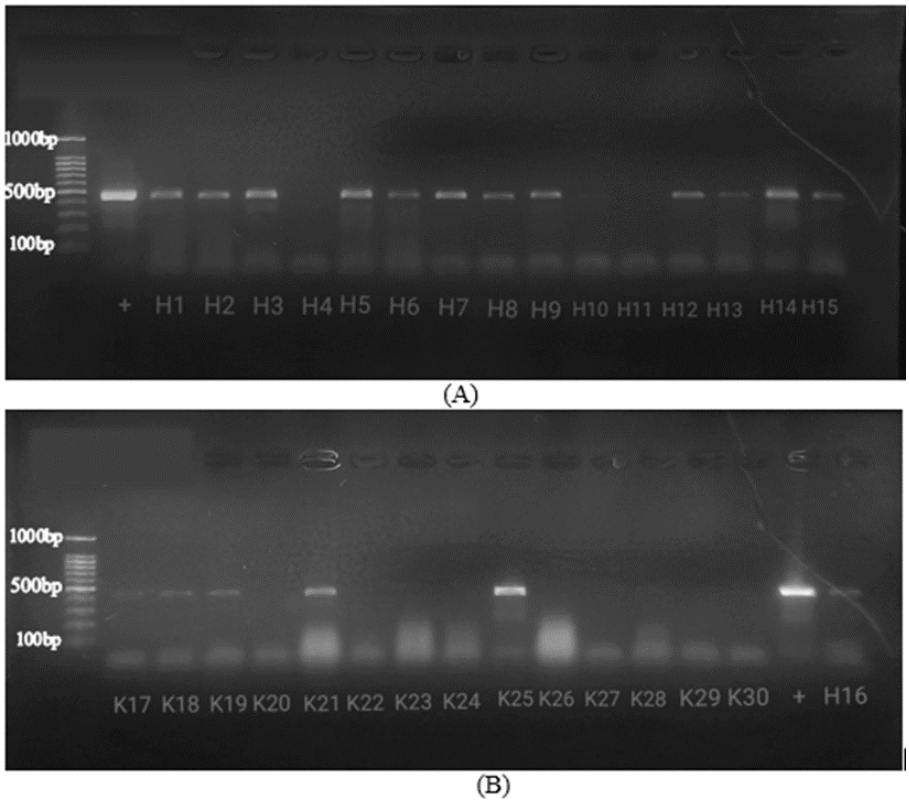

Figure 1. Results of Batch 1 DNA amplification of forest honey (A) and stingless bees honey (B) with primer LR13107-F/LR12647-R for the first repetition.

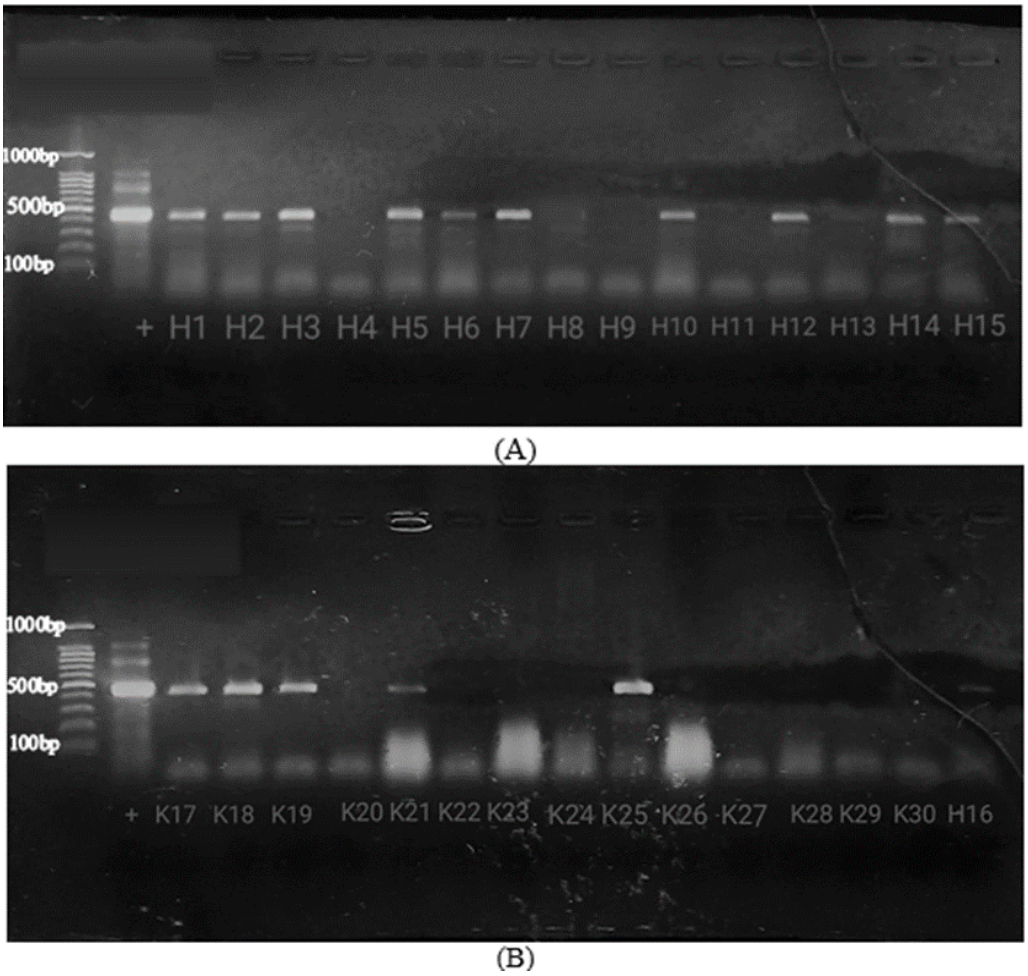

Figure 2. Results of Batch 1 DNA amplification of forest honey (A) and stingless bees honey (B) with primer LR13107-F/LR12647-R for the second repetition.

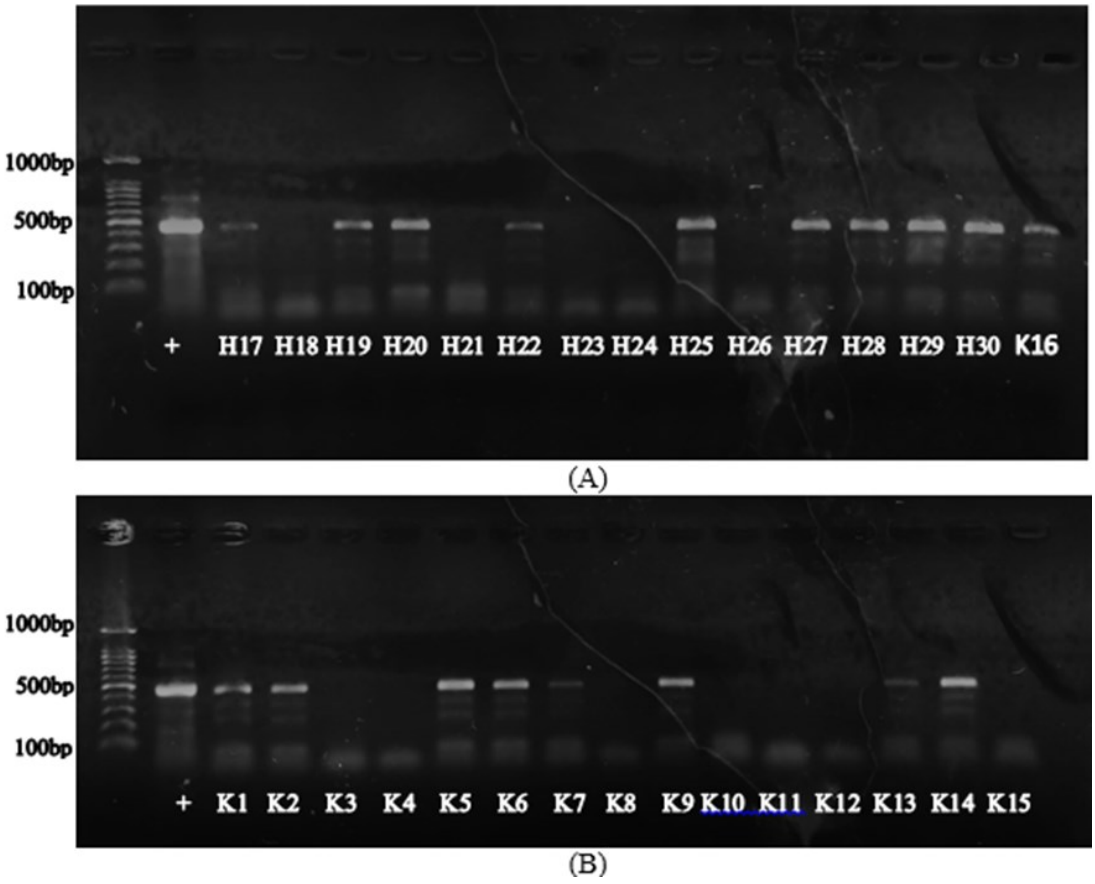

Figure 3. Results of Batch 2 DNA amplification of forest honey (A) and stingless bees honey (B) with primer LR13107-F/LR12647-R for the first repetition.

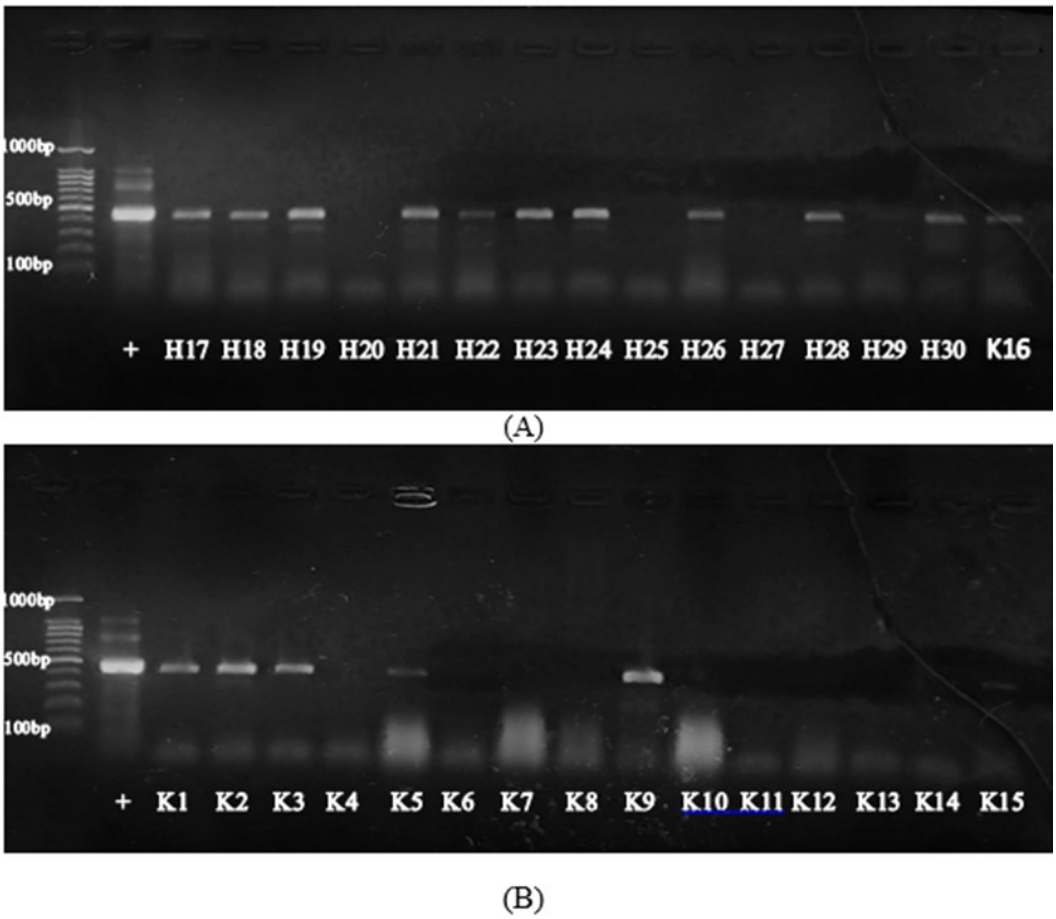

Figure 4. Results of Batch 2 DNA amplification of forest honey (A) and stingless bees honey (B) with primer LR13107-F/LR12647-R for the second repetition.

Using the 16S rRNA gene method, a genus is declared similar if it has 97% similarity and is said to be a species if the similarity is 99% (Petti 2007). Table 2. shows that the highest percent identity is for samples H19 and H25 at 100%, so it can be said that 100% probability that the honey samples come from *A. mellifera*. The H28 sample shows 99% percent identity, so it can be said that 99% probability that the honey sample comes from *A. cerana*. Meanwhile, the lowest percent identity is shown by the K25 sample of 96.33%. From these results, the sample has a similarity of 96.33% with *T. laeviceps*.

From nine samples that were sequenced, it was shown that six samples of honey did not match with the seller's claims. Based on Table 2. samples of forest honey (H16, H19, H20, H25, H27, and H28) resulted in contradiction between the seller's claims and the identification results. Samples H16, H20, and H27 labelled as forest honey samples claimed as honey from *A. dorsata*, while the identification results showed that it came from stingless bees. Meanwhile, samples H19 and H25, which claimed to be honey from *A. dorsata*, were identified as honey from *A. mellifera*. Table 2 shows the percent identity of the two samples is 100%. Thus, it can be said with certainty that both samples were honey derived from *A. mellifera*. This finding implied that there was an act of falsification of the origin of honey. This is one of honey fraud because honey from *A. mellifera* bees on the market has a much lower selling value than forest honey (Zhang et al. 2019). The seller gains more profit by selling honey produced by *A. mellifera* bees as forest honey.

Based on the same Table 2. mislabelling honey was also detected. Therefore, the honey-producing bees from the honey sample may have a close relationship with the identified species.

Table 2. BLAST-n results of positive sample sequences.

Sample Code	Identification	Claim	*Accession Number*	*Query Cover*	*E Value*	*Percent Identity*
H16	*Tetragonula cf. pagdeni*	*Apis dorsata* honey	DQ790437.1	99%	0.0	96.78%
H19	*Apis mellifera*	*Apis dorsata* honey	AP018434.1	95%	0.0	100%
H20	*Heterotrigona itama*	*Apis dorsata* honey	KU571761.1	100%	0.0	96.88%
H25	*Apis mellifera*	*Apis dorsata* honey	MN714160.1	100%	0.0	100%
H27	*Heterotrigona itama*	*Apis dorsata* honey	KU571761.1	97%	0.0	97.91%
H28	*Apis cerana*	*Apis dorsata* honey	AP017984.2	99%	0.0	99%
K5	*Tetragonula cf. pagdeni*	Stingless bees honey	DQ790437.1	100%	0.0	97.22%
K9	*Tetragonula cf. pagdeni*	Stingless bees honey	DQ790437.1	99%	0.0	96.60%
K25	*Tetragonula laeviceps*	*Tetragonula laeviceps* honey	KU571748.1	100%	0.0	96.33%

CONCLUSIONS

Based on this research, it can be concluded that, it is possible to determine the entomological origin of honey molecularly by sequencing the *16S rRNA* gene. Two honey samples, namely H19 and H25, committed fraud by claiming honey from *A. mellifera* honey bees as forest honey specifically *A. dorsata* honey. Four samples claimed to be honey from *A. dorsata*, were mislabelled, namely forest honey H16, H20, and H27, that were identified as stingless bees honey, and the sample number H28, identified as honey from *A. cerana*.

AUTHOR CONTRIBUTION

A.N. collected and analysed the data and wrote the manuscript, H.P. designed the research, corrected the manuscript and supervised all the processes.

ACKNOWLEDGMENTS

This research was funded by Final Assignment Recognition Grant (RTA) 2022 batch 1 from Universitas Gadjah Mada and Student-Lecturer Collaboration Research Grant 2022.

CONFLICT OF INTEREST

The authors declared there are no conflicts of interest regarding the research.

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