BIOLOGY, MEDICINE, & NATURAL PRODUCT CHEMISTRY
Volume 15, Number 1, April 2026 | Pages: 39-44 | DOI: 10.14421/biomedich.2026.151.39-44

ISSN 2089-6514 (paper)
ISSN 2540-9328 (online)

Major Royal Jelly Protein 2 (*mrjp*2) Gene in *Apis cerana* from Central Sulawesi, Indonesia

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Manuscript received: 15 November, 2025. Revision accepted: 25 February 2026, Published: 01 April, 2026.

Abstract

Indonesia is recognized as one of the world's biodiversity hotspots, supporting a wide range of pollinating insects, including honey bee species of the genus *Apis*. Among these, *Apis cerana* is the most widely managed species and contributes significantly to national honey production, particularly in Sulawesi. However, the increasing economic value of honey has led to frequent cases of entomological origin fraud, in which honey is falsely labeled as being produced by a different bee species. This study aimed to determine the entomological origin of honey sold in Central Sulawesi using the Major Royal Jelly Protein 2 (*mrjp2*) gene as a molecular marker. Seven honey samples claimed as "forest honey" were analyzed through DNA extraction, PCR amplification using species-specific primers (CF-CR), and sequencing followed by BLAST-n analysis. All samples yielded high-quality DNA with purity ratios appropriate for PCR amplification. PCR successfully amplified the *mrjp2* gene, producing fragments of approximately 201-208 bp, consistent with the expected size for *A. cerana*. Sequence analysis showed percent identity values ranging from 99.20% to 100% when compared with reference sequences of *A. cerana*. Based on molecular verification, three samples (H3, H5, and H7) did not match the seller's claims and were confirmed to originate from *A. cerana* rather than *A. dorsata binghami*. These findings demonstrate that sequencing of the *mrjp2* gene is an effective and reliable approach to authenticate the entomological origin of honey and to detect fraudulent labeling in commercial honey products.

Keywords: *Apis* spp.; Central Sulawesi; entomological origin; honey; mrjp2.

INTRODUCTION

Indonesia is a tropical country with exceptionally high levels of floral and faunal diversity (Sun et al., 2024). Its geographical conditions, consisting of thousands of islands, diverse topography, and variations in microclimate across regions, make Indonesia an ideal habitat for numerous types of organisms, including pollinating insects (Ariyanto et al., 2021; Koneri & Nangoy, 2021; Winahyu et al., 2021). One of the insect groups exhibiting high species diversity and a wide geographical distribution is the honey bee (honey bees; Tribe: Apini). Honey bees are eusocial insects that live in colonies and are well known for producing honey as one of their major economically valuable products (Ji, 2021). To date, at least five native honey bee species belonging to the genus Apis have been identified in Indonesia and are distributed across various regions of the archipelago (Astuti & Laksmi, 2022; Al Awwally et al., 2023). The diversity of honey bee species in Indonesia is closely related to the country's vast geographical area, varied landscapes and environmental conditions, as well as its complex geological history, all of which have shaped the adaptation patterns and distribution of each species (Syarifudin & Prasetyo, 2021; Yusuf et al., 2024).

One of the regions that contributes significantly to Indonesia's total honey production is Sulawesi Island. According to production data, approximately 81.06% of the national honey yield originates from this region. The honey bee species that plays the most dominant role in this production is *Apis cerana*, which has long been recognized as the primary local honey-producing bee across various parts of Indonesia (Aisyah et al., 2022; Setyawan et al., 2023). This species is widely managed by local beekeepers due to its adaptability to diverse environmental conditions and its efficiency in collecting nectar from a wide range of flowering plants. In Sulawesi, honey produced by *A. cerana* is often marketed as "forest honey" because it is perceived as a more natural product with higher market value. The increasing production of A. cerana honey is also supported by the practice of migratory beekeeping in industrial forest areas, particularly in plantations of Acacia and Eucalyptus, which provide abundant sources of nectar and pollen throughout the year (Karnan et al., 2021).

However, the growing demand and economic value of honey are often accompanied by the emergence of fraudulent practices in honey trading. One of the most common types of fraud is the falsification of the entomological origin of honey, also known as entomological origin fraud. This occurs when honey is falsely claimed to originate from a certain bee species, while in reality it does not (Purboyo et al., 2022). Other forms of honey adulteration include dilution with other substances, harvesting before maturity, and the feeding of artificial sweeteners to bees. Such practices are categorized as honey fraud because they are carried out to obtain greater financial profit at the expense of product quality and authenticity. Consequently, many honey products circulating in the market fail to meet global standards of purity and quality (Salsabilah & Hardiansyah, 2024).

To address these issues, several scientific approaches have been developed to accurately determine the entomological origin of honey. One of the most effective techniques is the molecular approach, which allows researchers to identify the bee species responsible for producing honey based on DNA traces that remain in the product. For example, Kek et al. (2017) successfully identified the entomological origin of 14 forest honey samples and one commercial honey sample using the 16S rRNA and CO1 genes. Similarly, studies by Zhang et al. (2019) and Raffiudin et al. (2023) demonstrated that the Major Royal Jelly Protein 2 (mrjp2) gene can serve as a reliable molecular marker for distinguishing honey bee species. The mrjp2 gene exhibits species-specific characteristics, making it a valuable tool for verifying the authenticity of honey based on its biological source. This study aims to detection of entomological origin of honey based on the Major Royal Jelly Protein 2 (mrjp2) gene.

MATERIALS AND METHODS

Tools and 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, -4ºC 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, electro phoresis tool, UV transilluminator, and a smartphone camera.

The materials used in this study included seven honey samples each claimed by the sellers or beekeepers to be forest honey (H1-H7), species specific primers for *A. cerana* CF-CR (C-F: TTTAACAATAAAAATAATCAGAAGA and C-R: TTACATCCTAATTGATTTTAATGCG) (Zhang et al., 2019), absolute ethanol, sterile distilled water, DNA extraction kit FavorPrepTM Tissue Genomic DNA Extraction Mini Kit 100 Prep (Proteinase K) (animal tissue, Blood, Cell, fungus, bac teria), 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.

Methods

Sample Collection

Samples were obtained from honey sold in online and offline shops di rectly from beekeepers in Central Sulawesi that the seller claimed either as forest 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 pre cipitate the pellets. The supernatant was discarded, and the pellet was re dissolved 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).

DNA Amplification and Visualisation

DNA samples and control of honey bees extracted were amplified using the Polymerase Chain Reaction (PCR) method. PCR is carried out by making a 25 μL PCR cocktail consisting of 12.5 μL PCR Promega GoTaq Green Master Mix, 9.5 μL Nuclease Free Water, 1.5 μL primer, and 1.5 μL DNA template. The mrjp2 gene was amplified with an initial pre-denaturation at 94ºC for 2 min, 35 cycles of denaturation at 94ºC for 30 s, annealing at 47°C for samples with *A. cerana* primer for 30 s, extension at 72ºC for 30 seconds, and final extension at 72ºC for 5 minutes (Zhang et al., 2019; Raffiudin et al., 2023). After going through the PCR process, 1.2% agarose gel electrophoresis was used to separate the amplicon, which was then visu alised with a UV transilluminator.

Data Analysis

The amplified DNA samples were sent to the PT Genetika Science for sequencing. The forward and reverse sequences from sequencing process were then edited using MEGA11 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 success of DNA extraction can be determined by the quality of the resulting DNA, particularly in terms of concentration and purity (Olson & Morrow, 2012). In this study, DNA extracted from seven honey samples showed optimal results and was successfully amplified using a species-specific primer pair. The extracted DNA concentration ranged from 28.55 to 225.02 ng/μL, with purity values between 2.02–2.15 (A260/230) and 2.00–2.04 (A260/280), as presented in Table 1. These values indicate that most of the extracted DNA possessed high purity, with low levels of protein, polysaccharide, and other organic compound contamination.

Table 1. DNA extraction results of honey bee samples.

Species	Sample code	A260/230	A260/280	Concentration (ng/μL)
Apis cerana	H1	2.15	2.03	767.6
Apis cerana	H2	2.11	2.04	962.5
Apis cerana	H3	2.14	2.01	644.8
Apis cerana	H4	2.13	2.05	1105.1
Apis cerana	H5	2.15	2.06	911.4
Apis cerana	H6	2.14	2.03	760.2
Apis cerana	H7	2.11	2.00	693.6

Generally, high-quality DNA exhibits an A260/A280 ratio between 1.8 and 2.0, which reflects minimal protein contamination, and an A260/A230 ratio between 2.0 and 2.2, indicating low levels of organic contaminants such as phenol or carbohydrates (Lucena-Aguilar et al., 2016). Therefore, the extracted DNA obtained in this study can be categorized as high quality and suitable for subsequent PCR amplification. High DNA quality, both in concentration and purity, is a crucial factor influencing the efficiency of PCR amplification. Poor-quality DNA may inhibit amplification, produce inconsistent results, or even cause amplification failure (Percze et al., 2024). Thus, the successful amplification using the CF–CR primer pair in this study demonstrates that the extraction method applied was effective in producing DNA of sufficient quality for further molecular analysis (Lucena-Aguilar et al., 2016).

DNA is amplified using the Polymerase Chain Reaction (PCR) method. PCR is a widely applied molecular technique that enables the replication of DNA in vitro, allowing researchers to generate millions of copies of a specific DNA fragment from even a very small amount of starting material (Singh et al., 2014; Zhu et al., 2020). This method is particularly valuable when the target DNA is present in low concentrations or when high quantities of DNA are required for downstream analyses (Amiteye, 2021). In general, PCR reagents consist of several essential components, including template DNA, which serves as the source of the target sequence; Taq polymerase, a heat-stable enzyme responsible for synthesizing new DNA strands; forward and reverse primers that provide starting points for DNA synthesis; deoxynucleotide triphosphates (dNTPs) as the building blocks for new DNA strands; and a buffer solution that maintains optimal conditions for enzyme activity (Kadri, 2019). These reagents work together through a series of thermal cycles denaturation, annealing, and extension to achieve exponential amplification of the target DNA region (Wheeler et al., 2018; Vidal et al., 2023). In this study, PCR was performed using specifically designed primers to detect the mrjp2 gene sequences. The use of these primers allows precise amplification of the gene of interest, thereby facilitating accurate identification and analysis of the mrjp2 gene in the examined samples.

The probability of detecting the mrjp2 gene in a genomic preparation is influenced by the fact that this gene plays an essential role in the biology of honeybee species, making it highly conserved within the genus Apis (Buttstedt et al., 2014). The mrjp2 gene exhibits distinctive characteristics, including its moderate evolutionary rate, which allows sufficient variation to differentiate closely related species while maintaining conserved regions suitable for primer binding (Han et al., 2014). These properties enable reliable detection and comparison of mrjp2 sequences among different honeybee species. In addition, the mrjp gene family contains multiple members with functional relevance, and mrjp2 specifically encodes proteins associated with royal jelly production and caste differentiation (Dewi & Purwanto, 2024). Its sequence contains both conserved motifs and variable regions, providing informative genetic markers for species identification and phylogenetic analysis. PCR using primers targeting the mrjp2 gene is advantageous because the gene exhibits species-specific sequence patterns, which improves the accuracy of distinguishing honey samples of different entomological origins (Helbing et al., 2017; Zhang et al., 2024).

The mrjp2 gene was detected in honey samples using the species specific primers for *A. cerana* CF-CR. PCR amplification of the mrjp2 gene using these primers produced distinct single bands at annealing temperatures of 47 °C. The amplicon sizes were approximately 201–208 bp (Figure 1), which are consistent with the predicted product sizes 212 bp reported by Zhang et al. (2019). These results confirm that the temperature range used in this study was suitable for achieving both specific and efficient primer binding, thereby ensuring accurate amplification of the mrjp2 gene without generating non-specific products. The consistent appearance of well-defined amplification bands further indicates that the samples indeed contained the mrjp2 gene, which is characteristic of honey bees (Mello et al., 2014). This provides strong molecular evidence supporting the presence of bee-derived genetic material within the analyzed honey samples.

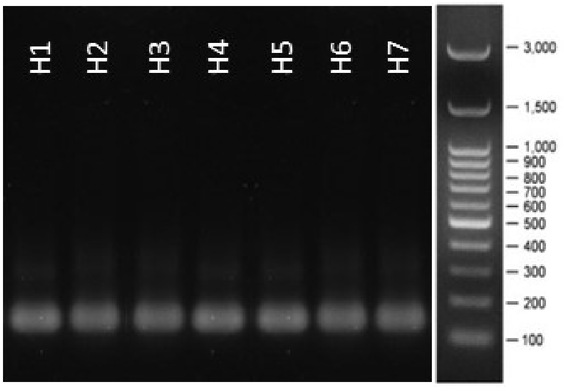

Figure 1. Amplified mrjp2 gene of honey bee DNA using species specific primers for *A. cerana* CF-CR. M (Marker = 100 bp)

The research data demonstrated that determining the entomological origin of honey at the molecular level is feasible through the detection of the mrjp2 gene. The successful amplification of this gene indicates the presence of genetic material derived from honey-producing bee species, thereby providing a reliable molecular basis for species verification (Buttstedt et al., 2014; Han et al., 2014). However, PCR amplification alone cannot fully resolve species identity, as closely related bee species may share similar amplicon sizes or conserved gene regions. Therefore, sequencing of the mrjp2 gene is essential to accurately determine which specific species are present in the honey samples. Sequence analysis would allow for precise comparison with reference databases, enabling definitive species identification and improving the reliability of entomological authentication.

Using the mrjp2 gene as a molecular marker, taxonomic identification can be based on sequence similarity. The similarity of around 97% indicates the same genus, while a similarity of about 99% is required to confirm the same species. These thresholds help distinguish taxonomic levels and improve accuracy in identifying the entomological origin of honey (Petti, 2007). The highest percent identity is for samples H3, H5, and H7 at 100%, so it can be said that 100% probability that the honey samples come from *A. cerana*. The H1, H2, and H4 sample shows 99.55% percent identity, so it can be said that 99.55% probability that the honey sample comes from *A. cerana*. Meanwhile, the lowest percent identity is shown by the H6 sample of 99.20%. From these results, the sample has a similarity with *A. cerana* (Table 2).

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

Sample code	**BLAST**			**Identification**
	% Identity	**% Query Cover**	**Accession Number GenBank**	
H1	99.55	100	LC600196.1	*Apis cerana*
H2	99.55	100	LC600196.1	*Apis cerana*
H3	100	100	LC600196.1	*Apis cerana*
H4	99.55	100	LC600196.1	*Apis cerana*
H5	100	100	LC600196.1	*Apis cerana*
H6	99.20	100	LC600196.1	*Apis cerana*
H7	100	100	LC600196.1	*Apis cerana*

From seven samples that were sequenced, it was shown that three samples of honey did not match with the seller's claims. Based on Table 3, samples of forest honey (H3, H5, and H7) resulted in contradiction between the seller's claims and the identification results. Samples H3, H5, and H7 labelled as forest honey samples claimed as honey from *A. dorsata binghami*, while the identification results showed that it came from *A. cerana*. Table 2 shows the percent identity of the three samples is 100%. Thus, it can be said with certainty that three samples were honey derived from *A. cerana*. 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. cerana* bees on the market has a much lower selling value than forest honey (Zhang et al., 2019).

Table 3. Verification of claimed entomological origin of honey samples.

Sample code	**Claim**	**Species verification**
H1	*Apis cerana*	*Apis cerana*
H2	*Apis cerana*	*Apis cerana*
H3	*Apis dorsata binghami*	*Apis cerana*
H4	*Apis cerana*	*Apis cerana*
H5	*Apis dorsata binghami*	*Apis cerana*
H6	*Apis cerana*	*Apis cerana*
H7	*Apis dorsata binghami*	*Apis cerana*

CONCLUSIONS

Based on this research, it can be concluded that, it is possible to determine the entomological origin of honey molecularly by sequencing the mrjp2 gene. Three honey samples, namely H3, H5, and H7, committed fraud by claiming honey from *A. cerana* honey bees as forest honey specifically *A. dorsata binghami* honey.

Acknowledgements: The authors would like to express their gratitude to Tadulako University for providing financial support for the implementation of the 2025 Research Program.

Authors' Contributions: Conceptualization, I Made Budiarsa and Manap Trianto; methodology, I Made Budiarsa, Manap Trianto, and Akram; analysis, Yulia Windarsih and Abdul Ashari; writing original draft preparation, Akram, Yulia Windarsih, and Abdul Ashari; writing, review and editing, I Made Budiarsa, Manap Trianto, and Akram.

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

Funding: This research was funded by Tadulako University, grant number 4621/UN28/HK.02/2025.

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