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Bee species authentication of commercial honey in Indonesia using the *mrjp2* gene

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Abstract. *Budiarsa IM, Trianto M, Kundera IN. 2026. Bee species authentication of commercial honey in Indonesia using the* mrjp2 *gene. Biodiversitas 27 (4): d270432. https://doi.org/10.13057/biodiv/d270432*. Authenticating the entomological origin of honey is essential to ensure product traceability, labeling accuracy, and the conservation of native honey bee species in Indonesia. This study aimed to authenticate the bee species origin of honey marketed in Indonesia using the major royal jelly protein 2 (*mrjp2*) gene as a molecular marker. A total of 21 honey samples, labeled as forest honey or specific bee-derived honey, were collected from Sulawesi, Sumatra, Kalimantan, Bali, Java, and Yogyakarta. Genomic DNA was extracted and amplified by PCR using two primer pairs targeting the *mrjp2* gene (MF-MR and CF-CR). PCR products were sequenced, and the resulting sequences were analyzed using BLAST-N against GenBank references and further evaluated through Maximum Likelihood phylogenetic analysis. The MF-MR primers generated amplicons of approximately 584-593 bp, while CF-CR primers produced fragments of 202-210 bp, consistent with the targeted *mrjp2* regions. Amplification patterns indicated that MF-MR primers amplified both *Apis mellifera* and *Apis dorsata* subsp. *binghami*, whereas CF-CR primers amplified *Apis cerana* and *Apis nigrocincta*. This cross-amplification among closely related *Apis* species indicates partial primer specificity rather than absolute species exclusivity. Sequence analysis confirmed high similarity to reference sequences, with BLAST-N identities of 99.58% (*A. mellifera*), 99.29% (*A. cerana*), 99.67% (*A. dorsata* subsp. *binghami*), and 91.25% (*A. nigrocincta*). Phylogenetic reconstruction revealed distinct clustering of samples with their respective reference taxa, supporting reliable species-level discrimination when PCR results are combined with sequencing and phylogenetic inference. These findings demonstrate that the *mrjp2* gene is a robust molecular marker for authenticating the entomological origin of honey in Indonesia. While primer-based PCR provides an initial indication of bee species origin, definitive authentication relies on sequence analysis and phylogenetic validation rather than PCR amplification alone.

Keywords: Apidae, entomological origin, honey, molecular marker, *mrjp2* gene

INTRODUCTION

Honey is a viscous and sweet solution produced by honeybees through the collection of floral nectar, biochemical transformation with the assistance of enzymes, and storage in honeycomb cells (Alaerjani et al. 2022; Rani et al. 2024). Chemically, honey is composed primarily of carbohydrates, particularly fructose (approximately 38-39%) and glucose (30-32%), along with maltose, sucrose, and other minor sugars (Hossain et al. 2023). Its water content ranges from 17-20%, with a pH of about 3.4-6.1 depending on the nectar source (Nicolson 2022). In addition, honey contains small amounts of protein (<0.5%), amino acids such as proline, vitamins (B1, B2, B3, B5, B6, and C), and essential minerals including potassium, calcium, magnesium, phosphorus, sodium, and iron (Da Silva et al. 2016; Cucu et al. 2021; Afroz et al. 2023). The organic acids present, such as gluconic, acetic, citric, malic, and succinic acids, contribute to the distinctive taste and aroma of honey (Rahman et al. 2023).

In addition to serving as an energy source, honey is known to exhibit significant antioxidant activity (Ahmed et al. 2018; Ilia et al. 2021). The antioxidant compounds in honey include flavonoids (e.g., flavones, flavonols, flavan-3-ols), phenolic acids, vitamin C, and antioxidant enzymes such as catalase and peroxidase (Zawawi et al. 2021). Specific phenolic compounds, including chrysin, pinobanksin, and pinocembrin, have been reported to play key roles in neutralizing free radicals and reducing oxidative stress (Qadir et al. 2020). The presence of these bioactive compounds renders honey not only a source of nutrients but also a potential therapeutic agent (Afrin et al. 2020).

The Indonesian Central Bureau of Statistics (BPS) recorded highly fluctuating honey production in Indonesia between 2016 and 2020. In 2016, national honey production reached 362.2 thousand liters, but this figure decreased by 85% to 54.3 thousand liters in 2017. In 2018, production rebounded by 171.3% to 147.3 thousand liters, followed by a significant increase of 238.1% to 498 thousand liters in 2019. However, in 2020, national honey production again dropped sharply by 89.7%, reaching only 51.34 thousand liters (Harianja et al. 2023). By island distribution, Java was the largest honey-producing region nationally in 2020, with a production of 41.6 thousand liters, accounting for 81.06% of total national output. Sumatra ranked second with 4.01 thousand liters (7.81% of the national total), while Kalimantan and Sulawesi produced 3 thousand liters and 500 liters, respectively (BPS 2020).

The high levels of honey production and consumption have contributed to an increase in honey adulteration in the

market. Such adulteration practices include mixing honey with artificial syrup, premature harvesting, the use of ion-exchange resins, feeding bees with artificial diets, and falsifying or misrepresenting the geographical or botanical origin of honey. These practices are considered fraudulent because honey is sold at a profit even though it does not meet global quality standards (APIMONDIA 2019). Although honey quality and purity standards have been established under SNI 8664 (2018), it may be difficult for the general public to assess authenticity and purity, especially for well-packaged honey. Therefore, effective methods are needed to determine the entomological origin of honey to prevent such adulteration.

Various methods have been used to determine the entomological origin of honey, such as analyzing honeybee protein profiles using SDS-PAGE (Ramón-Sierra et al. 2015), examining differences in the molecular weight and surface structure of *mrjp1* (Won et al. 2008), and identification using the *COI* (Kek et al. 2017), *mrjp2* (Zhang et al. 2019; Raffiudin et al. 2023; Dewi and Purwanto 2024), and 16S rRNA (Indahsari and Purwanto 2024). Major royal jelly proteins (*mrjps*) are the primary protein components of royal jelly, accounting for approximately 90% of its total protein content (Drapeau et al. 2006). *mrjps* consist of nine proteins (*mrjp1-mrjp9*) with molecular weights ranging from 49 to 87 kDa, encoded by nine corresponding genes, *mrjp1* through *mrjp9* (Drapeau et al. 2006; Buttstedt et al. 2013). This study aimed to authenticate the bee species origin of honey marketed in Indonesia using the major royal jelly protein 2 (*mrjp2*) gene as a molecular marker.

MATERIALS AND METHODS

Sampling location

Honey samples were collected from several regions in Indonesia, including Sulawesi, Sumatra, Kalimantan, Bali, Java, and Yogyakarta (Table 1). These locations represent diverse ecological zones and beekeeping practices across the Indonesian archipelago. The samples were obtained from both online and offline markets, as well as directly from local beekeepers who claimed the products were forest honey bee products. Species information presented in Table 1 represents the declared bee species based on seller or beekeeper claims at the time of sampling.

Sample collection

Honey samples were collected using a purposive sampling approach to represent a wide range of honey products marketed across Indonesia. Sampling was conducted between August-September 2025, targeting both online marketplaces and offline retail outlets, as well as direct purchases from local beekeepers. A total of 21 honey samples were obtained from different sellers across six major regions: Sulawesi, Sumatra, Kalimantan, Bali, Java, and Yogyakarta. Sellers were selected based on the following criteria: (i) products labeled as forest honey or associated with specific bee species, (ii) availability of information regarding the claimed origin of honey, and (iii) accessibility for purchase either online or in local markets. To minimize sampling bias, efforts were made to include products from different vendors and geographical origins, avoiding multiple samples from the same seller whenever possible.

Information provided by sellers, including the declared bee species and origin of honey, was recorded at the time of purchase. However, these claims were not considered as verified evidence of authenticity, but rather as preliminary information to be tested through molecular analysis. No formal certification or laboratory authentication was requested from sellers, reflecting typical market conditions encountered by consumers. Although the sample size does not fully represent the entire honey-producing community in Indonesia, the selected samples encompass diverse geographic regions, marketing channels, and claimed bee species, thereby providing a reasonable cross-section of commercially available honey products. This approach allows for an initial evaluation of the reliability of seller-declared information and the applicability of molecular methods for honey authentication in real market conditions.

Table 1. Sampling locations and declared bee species based on seller claims

Sample code	Location/ Hive origin	Declared species (seller claim)
ACCS.1	Central Sulawesi	*Apis cerana* (Fabricius, 1793)
ACCS.2	Central Sulawesi	*Apis cerana* (Fabricius, 1793)
ACCS.3	Central Sulawesi	*Apis cerana* (Fabricius, 1793)
ADCS.4	Central Sulawesi	*Apis dorsata* subsp. *binghami* (Cockerell, 1906)
ADCS.5	Central Sulawesi	*Apis dorsata* subsp. *binghami* (Cockerell, 1906)
ANCS.6	Central Sulawesi	*Apis nigrocincta* (Smith, 1860)
AMCS.7	Central Sulawesi	*Apis mellifera* (Linnaeus, 1758)
AMWS.8	West Sulawesi	*Apis mellifera* (Linnaeus, 1758)
AMSS.9	South Sulawesi	*Apis mellifera* (Linnaeus, 1758)
ACSS.10	South Sulawesi	*Apis cerana* (Fabricius, 1793)
ACSS.11	West Sumatra	*Apis cerana* (Fabricius, 1793)
ACSS.12	South Sumatra	*Apis cerana* (Fabricius, 1793)
ADNS.13	North Sumatra	*Apis dorsata* subsp. *binghami* (Cockerell, 1906)
AMSK.14	South Kalimantan	*Apis mellifera* (Linnaeus, 1758)
ACSK.15	South Kalimantan	*Apis cerana* (Fabricius, 1793)
ACSK.16	South Kalimantan	*Apis cerana* (Fabricius, 1793)
ACCK.17	Central Kalimantan	*Apis cerana* (Fabricius, 1793)
ACB.18	Bali	*Apis cerana* (Fabricius, 1793)
AMY.19	Yogyakarta	*Apis mellifera* (Linnaeus, 1758)
ACWJ.20	West Java	*Apis cerana* (Fabricius, 1793)
ACCJ.21	Central Java	*Apis cerana* (Fabricius, 1793)

Procedures

DNA extraction

Honey sample preparation followed the methods of Zhang et al. (2019) and Raffiudin et al. (2023), with minor modifications. Briefly, 12.5 g of honey was placed into a 50 mL Falcon tube and diluted with distilled water (aquadest) to a final volume of 50 mL. The solution was homogenized using a vortex mixer and incubated at 40°C for 30 min to reduce viscosity. Samples were centrifuged at 5,000 rpm at 20°C for 20 min to precipitate particulate material. The supernatant was carefully discarded, and the pellet was resuspended in distilled water. A second centrifugation step was performed under the same conditions to obtain a final pellet. The pellet was resuspended in 500 µL of aquadest and transferred to a 1.5 mL microcentrifuge tube. Genomic DNA was extracted using the Favorgen Tissue Genomic DNA Extraction Mini Kit (100 Prep, Proteinase K; applicable for animal tissue, blood, cells, fungi, and bacteria) following the manufacturer's instructions. No extraction blank (negative control) was included during the DNA extraction; however, PCR negative controls using nuclease-free water were included in all amplification assays to monitor for potential contamination. DNA concentration and purity were measured using a NanoDrop spectrophotometer (ND-2000, NanoDrop Technologies, Madison, WI, USA).

DNA amplification

DNA amplification was performed using two sets of primers, species specific primers for *Apis mellifera* (M-F: `GCC ATC CCT TGA AAT TGT CAC TCGT` and M-R: `TCT GCA AAC GAC CAA TCA GGA TAT`) and species specific primers for *Apis cerana* (C-F: `TTT AAC AAT AAA AAT AAT CAG AAGA` and C-R: `TTA CAT CCT AAT TGA TTT TAA TGCG`) (Zhang et al. 2019), to detect honey DNA using the *mrjp2* gene, following the methods of Zhang et al. (2019) and Raffiudin et al. (2023). PCR amplification of the *mrjp2* gene was performed in a total reaction volume of 25 µL, consisting of 12.5 µL of 2× PCR Master Mix (MyTaq™ HS Red Mix, Bioline), 1 µL of forward primer (10 pmol), 1 µL of reverse primer (10 pmol), 2 µL of template DNA, and 8.5 µL of nuclease-free water. Amplifications were carried out using a thermal cycler (Bio-Rad T100™) under the following conditions: an initial denaturation at 95°C for 3 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at a single temperature for 30 s, and extension at 72°C for 45 s; with a final extension at 72°C for 5 min. To determine the optimal annealing temperature, PCR reactions were conducted as separate gradient reactions, each using a single annealing temperature (47, 50, 53, 55, 58, or 59°C). Each temperature condition was run independently using identical reaction compositions.

Genomic DNA extracted from morphologically identified *Apis cerana* worker was used as a positive control, representing a known reference sample with confirmed species identity. This control served as an internal validation to ensure that the PCR system, primer specificity, and amplification conditions were functioning correctly. In addition, nuclease-free water was included as a negative control to monitor contamination. PCR products were visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and run alongside a 100 bp DNA ladder (GeneRuler™, Thermo Fisher Scientific) to estimate fragment size.

Data analysis

The amplified DNA samples were sent to PT Genetika Science in Jakarta for sequencing. The forward and reverse sequences obtained from the sequencing were edited using MEGA11. Consensus sequences were then analyzed using BLAST-N on the NCBI website (http://blast.ncbi.nlm.nih.gov/) to determine sequence similarities. The sequences were also used for phylogenetic tree analysis. Both the sequence results and the reference sequences from GenBank were aligned using ClustalW in MEGA11. The resulting alignments were used to construct phylogenetic trees. Phylogenetic trees were generated using the Maximum Likelihood (ML) algorithms with a bootstrap value of 1000.

RESULTS AND DISCUSSION

PCR amplification

DNA extracted from 21 honey samples was amplified using two species-specific primer pairs: MF-MR for *A. mellifera* and CF-CR for *A. cerana*. The concentration of extracted DNA ranged from 152.0 to 1050.2 ng/µL. Purity ratios ranged from 2.00-2.07 for A260/280 and 2.06-2.27 for A260/230, as summarized in Table 2. Based on Abdel-Latif and Osman (2017), A260/280 ratios of approximately 1.8-2.0 and A260/230 ratios of 2.0-2.2 are generally considered indicative of high-quality DNA. In this study, most samples fell within or slightly above these reference ranges. Elevated A260/230 values may indicate the presence of residual salts or organic compounds; however, all DNA extracts consistently yielded successful PCR amplification, indicating that DNA quality was sufficient for downstream molecular analyses.

PCR amplification of the *mrjp2* gene using MF-MR and CF-CR primers produced distinct single bands across annealing temperatures of 47, 50, 53, 55, 58, and 59°C. The observed amplicon sizes were approximately 584-593 bp for MF-MR and 202-210 bp for CF-CR primers (Figure 1), consistent with the predicted product sizes of 560 bp and 212 bp reported by Zhang et al. (2019). Clear amplification bands were observed in honey samples and positive controls, while no bands were detected in negative controls, confirming the absence of contamination and the specificity of amplification. Previous studies reported optimal amplification of *A. mellifera* DNA at 50-59°C and of *A. cerana* DNA at 47-55°C (Raffiudin et al. 2023; Dewi and Purwanto 2024), supporting the suitability of the temperature range used in this study for efficient primer binding.

Table 2. DNA extraction results of honey bee samples

Sample code	Species	A260/230	A260/280	Concentration (ng/µL)
ACCS.1	*Apis cerana* (Fabricius, 1793)	2.19	2.05	256.8
ACCS.2	*Apis cerana* (Fabricius, 1793)	2.13	2.06	1050.2
ACCS.3	*Apis cerana* (Fabricius, 1793)	2.12	2.00	626.1
ADCS.4	*Apis dorsata* subsp. *binghami* (Cockerell, 1906)	2.20	2.07	1021.8
ADCS.5	*Apis dorsata* subsp. *binghami* (Cockerell, 1906)	2.12	2.01	152.0
ANCS.6	*Apis nigrocincta* (Smith, 1860)	2.16	2.05	368.0
AMCS.7	*Apis mellifera* (Linnaeus, 1758)	2.21	2.00	437.7
AMWS.8	*Apis mellifera* (Linnaeus, 1758)	2.06	2.00	1001.9
AMSS.9	*Apis mellifera* (Linnaeus, 1758)	2.07	2.07	1054.4
ACSS.10	*Apis cerana* (Fabricius, 1793)	2.25	2.07	1016.2
ACSS.11	*Apis cerana* (Fabricius, 1793)	2.27	2.07	1001.7
ACSS.12	*Apis cerana* (Fabricius, 1793)	2.10	2.01	464.5
ADNS.13	*Apis dorsata* subsp. *binghami* (Cockerell, 1906)	2.23	2.01	1031.1
AMSK.14	*Apis mellifera* (Linnaeus, 1758)	2.13	2.06	138.1
ACSK.15	*Apis cerana* (Fabricius, 1793)	2.16	2.01	571.9
ACSK.16	*Apis cerana* (Fabricius, 1793)	2.17	2.03	416.4
ACCK.17	*Apis cerana* (Fabricius, 1793)	2.15	2.01	254.2
ACB.18	*Apis cerana* (Fabricius, 1793)	2.18	2.02	294.2
AMY.19	*Apis mellifera* (Linnaeus, 1758)	2.13	2.04	626.7
ACWJ.20	*Apis cerana* (Fabricius, 1793)	2.18	2.01	548.1
ACCJ.21	*Apis cerana* (Fabricius, 1793)	2.14	2.03	388.1

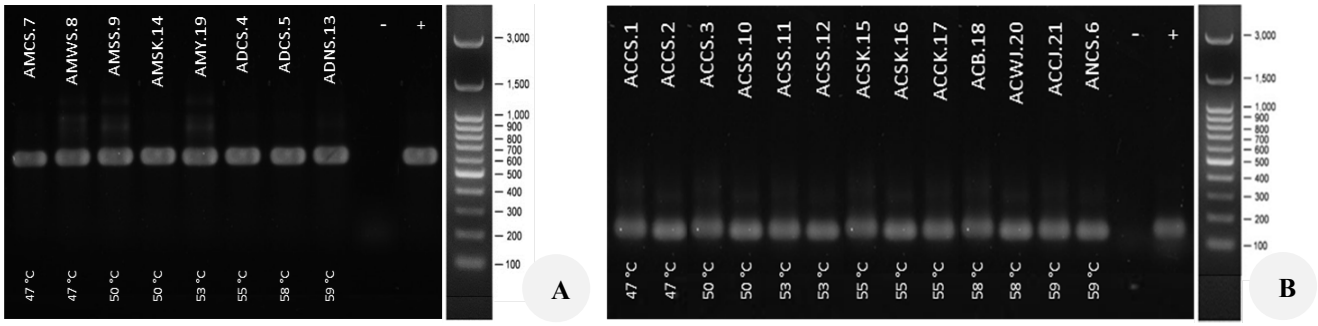

Figure 1. Amplified *mrjp2* gene of honey bee DNA using two sets of primers. A. M-F and M-R primers, B. C-F and C-R primers. M: Marker: 100 bp, AM: *Apis mellifera*, AD: *Apis dorsata* subsp. *binghami*, AC: *Apis cerana*, AN: *Apis nigrocincta*, -: Negative control, and +: Positive control, Ta (°C): 47, 50, 53, 55, 58, and 59

The amplification patterns revealed that MF-MR primers amplified both *A. mellifera* and *A. dorsata* subsp. *binghami*, whereas CF-CR primers amplified *A. cerana* and *A. nigrocincta*. This pattern indicates distinct affinities of each primer pair for specific *Apis* species, reflecting the compatibility between primer binding sites and target genomic regions. The observed cross-amplification among these species suggests that high nucleotide similarity within closely related taxa can promote primer annealing beyond the intended target species. These findings align with previous studies that *A. cerana* DNA could not be amplified with MF-MR primers, and *A. mellifera* DNA could not be amplified with CF-CR primers (Zhang et al. 2019; Raffiudin et al. 2023; Dewi and Purwanto 2024). The present study further extends these findings by demonstrating that MF-MR primers can also amplify *A. dorsata* DNA. In contrast, CF-CR primers can amplify *A. nigrocincta* DNA, providing new insights into interspecies primer compatibility within the *Apis* genus.

The accuracy of the molecular approach was supported by the successful amplification and correct identification of the positive control sample (*A. cerana*), which consistently produced amplicons of expected size and showed high sequence identity with reference sequences in GenBank. This confirms that the primers and PCR conditions accurately detect the target *mrjp2* gene in samples with known species identity. Furthermore, the absence of amplification in negative controls confirmed that the results were free of contamination, strengthening the method's reliability.

Sequence identification

The BLAST-N analysis of the honey bee DNA sequences revealed a strong correspondence between the amplified products and the *mrjp2* gene sequences available in the GenBank database for each respective *Apis* species (Table 3). The sequences obtained from the controlled honey bee samples, namely *A. mellifera* and *A. cerana*, were confirmed to match their reference *mrjp2* gene sequences with high accuracy.

Specifically, the *mrjp2* sequence from the *A. mellifera* sample exhibited 99.58% nucleotide identity with the *A. mellifera mrjp2* gene from Indonesia (accession number LC600176.1), confirming that the amplified fragment was highly conserved within the species. Similarly, the *A. dorsata* sample showed 99.67% sequence identity with the *A. dorsata* subsp. *binghami mrjp* gene reported from Indonesia and Malaysia (accession number XM_031510778.1), indicating that the MF-MR primers were capable of binding to a homologous region within the *mrjp* gene family of this species.

In addition, the *A. cerana* sample demonstrated 99.29% identity with the *A. cerana mrjp2* sequence from Indonesia (accession number LC600196.1), supporting the species-specific amplification of the CF-CR primer pair. Meanwhile, the *A. nigrocincta* sample exhibited 91.25% similarity with the *A. cerana mrjp2* sequence from Indonesia (accession number LC600187.1), suggesting partial homology between the two species. This moderate level of sequence similarity is consistent with the close phylogenetic relationship between *A. cerana* and *A. nigrocincta*, which may facilitate cross-amplification due to conserved regions within the *mrjp2* gene. Overall, these molecular findings validate the primer specificity and further demonstrate the evolutionary proximity among *A. mellifera*, *A. dorsata* subsp. *binghami*, *A. cerana*, and *A. nigrocincta*, as reflected by the high degree of sequence homology across the examined taxa.

The results of the BLAST-N analysis not only confirmed the accuracy of the amplified sequences but also provided insights into the evolutionary relationships among the four *Apis* species examined in this study. The high percentage of sequence identity observed between the *mrjp2* gene of *A. mellifera*, *A. cerana*, *A. dorsata* subsp. *binghami* and their corresponding reference sequences indicate that this gene is highly conserved across different populations of the same species, even those originating from different geographic regions. Such conservation is consistent with the essential role of *mrjp2* in royal jelly production and the regulation of caste differentiation within honey bee colonies, processes that are fundamental to the social organization of all *Apis* spp. (Dobritzsch et al. 2019).

The detection of a 91.25% sequence identity between *A. nigrocincta* and *A. cerana* further supports the close genetic and phylogenetic relationship between these two species. Previous studies have also suggested that *A. nigrocincta* is a sister taxon of *A. cerana*, sharing many morphological and behavioral traits as well as partial mitochondrial gene similarity (Lombogia et al. 2020). The observed cross-amplification of *A. nigrocincta* DNA using CF-CR primers designed for *A. cerana* likely reflects this evolutionary proximity, where conserved nucleotide motifs within the *mrjp2* gene allow partial primer binding (Shullia et al. 2024). This finding highlights the potential of *mrjp2*-based molecular markers to detect not only species-level differences but also interspecific genetic affinities within the genus (Alam et al. 2023).

Phylogenetic analysis

The amplicons of *A. mellifera* and *A. cerana* produced final products of 593 and 210 bp, respectively. Meanwhile, *A. dorsata* subsp. *binghami* and *A. nigrocincta* produced final product sizes of 584 and 202 bp, respectively. The samples and controls sequence obtained were aligned with the database sequence of *mrjp1-mrjp9* of *A. mellifera* and *mrjp2* of *A. cerana* available in NCBI using ClustalW in MEGA11 software. The phylogenetic tree was then constructed using the Maximum Likelihood (ML) algorithm with a bootstrap value of 1000 and a sequence from *Bombus ignitus* (Smith, 1869) (EU391535.1) as the outgroup.

Table 3. BLAST results and species verification

Sample code	BLAST			Species verification
	% Identity	% Query cover	Accession number Genbank	
ACCS.1	99.29	100	LC600196.1	*Apis cerana*
ACCS.2	99.29	100	LC600196.1	*Apis cerana*
ACCS.3	99.29	100	LC600196.1	*Apis cerana*
ADCS.4	99.67	100	XM_031510778.1	*Apis dorsata* subsp. *binghami*
ADCS.5	99.67	100	XM_031510778.1	*Apis dorsata* subsp. *binghami*
ANCS.6	91.25	97	LC600187.1	*Apis cerana*
AMCS.7	99.58	100	LC600176.1	*Apis mellifera*
AMWS.8	99.58	100	LC600176.1	*Apis mellifera*
AMSS.9	99.58	100	LC600176.1	*Apis mellifera*
ACSS.10	99.29	100	LC600196.1	*Apis cerana*
ACSS.11	99.29	100	LC600196.1	*Apis cerana*
ACSS.12	99.29	100	LC600196.1	*Apis cerana*
ADNS.13	99.67	100	XM_031510778.1	*Apis dorsata* subsp. *binghami*
AMSK.14	99.58	100	LC600176.1	*Apis mellifera*
ACSK.15	99.29	100	LC600196.1	*Apis cerana*
ACSK.16	99.29	100	LC600196.1	*Apis cerana*
ACCK.17	99.67	100	LC600196.1	*Apis cerana*
ACB.18	99.29	100	LC600196.1	*Apis cerana*
AMY.19	99.58	100	LC600176.1	*Apis mellifera*
ACWJ.20	99.29	100	LC600196.1	*Apis cerana*
ACCJ.21	99.29	100	LC600196.1	*Apis cerana*

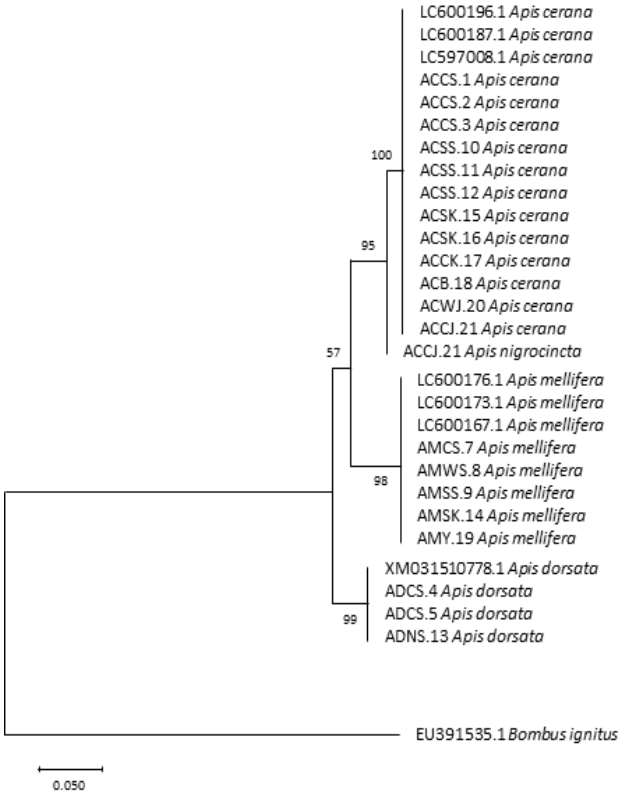

Figure 2. Phylogenetic tree of *mrjp2* gene of honey bee samples and *mrjp* gene database based on the Maximum Likelihood (ML) algorithm with 1000 bootstrap values

The Maximum Likelihood (ML) method is a statistically robust approach widely used in molecular phylogenetic analysis to reconstruct evolutionary relationships among species based on DNA or protein sequence data (Soares et al. 2019). It operates by identifying the tree topology that maximizes the probability (likelihood) of producing the observed data under a given model of nucleotide or amino acid substitution. Compared to distance-based methods, ML provides higher accuracy because it incorporates complex models of molecular evolution, accounts for variable substitution rates among sites, and allows hypothesis testing through likelihood ratio tests (Jacobson et al. 2022). This method also yields reliable bootstrap support values indicating the confidence level for each branching point in the phylogenetic tree. Overall, the ML method is considered one of the most powerful and reliable tools for phylogenetic reconstruction, offering statistically sound and biologically meaningful insights into evolutionary history (Munjal et al. 2024).

Based on the phylogenetic tree between honey bee samples and the honey bee database (Figure 2), the two honey bee samples amplified using CR-CR were incorporated into two different clades, which are clade 1 and clade 2. The *mrjp2* gene of honey bee sample *A. cerana* was incorporated into clade 1 with the *mrjp2* gene of *A. cerana* (LC600196.1, LC600187.1, and LC597008.1), with a genetic distance of 0% and showing high similarity (100%). Meanwhile, the *mrjp2* gene of honey bee sample *A. nigrocincta* shows a genetic distance 3.5% to the *mrjp3* gene of *A. cerana* (LC600196.1).

The two honey bee samples amplified using MF-MR were assigned to clades 3 and 4. The honey bee sample *A. mellifera* was incorporated into clade 3 with the *mrjp2* gene of *A. mellifera* (LC600176.1, LC600173.1, and LC600167.1), showing high similarity (97%) and a genetic distance of 2.3%. Meanwhile, the *mrjp2* gene of the honey bee sample *A. dorsata* subsp. *binghami* were incorporated with the *mrjp5*-like gene of *A. dorsata* (XM031510778.1) in clade 4. The *mrjp2* gene of *A. dorsata* subsp. *binghami* and the *mrjp5*-like gene of *A. dorsata* show high similarity (99%) and a low genetic distance (1.7%).

The phylogenetic analysis based on the *mrjp2* gene sequences clearly demonstrates genetic differentiation among the analyzed *Apis* spp. The clustering of *A. cerana* with its respective database sequences and the low genetic distance indicate a high degree of genetic conservation within this species. This close relationship confirms that the *mrjp2* gene is a reliable molecular marker for identifying *A. cerana*, as it maintains sequence stability across populations (Raffiudin et al. 2023; Dewi and Purwanto 2024). In contrast, *A. nigrocincta* showed a higher genetic distance from *A. cerana*, supporting its recognition as a distinct species despite its close phylogenetic affinity (Figure 2). This finding aligns with previous reports that *A. nigrocincta* and *A. cerana* share a recent common ancestor but have diverged through allopatric speciation, likely influenced by the geographic isolation of Sulawesi Island. The moderate divergence observed in *A. nigrocincta* suggests evolutionary differentiation at the molecular level, possibly related to local adaptation and reproductive isolation mechanisms (Raffiudin and Shullia 2020; Smith et al. 2020; Su et al. 2023).

The separation of *A. dorsata* subsp. *binghami* and *A. mellifera* into distinct clades further reinforces the phylogenetic validity of the analysis. *A. mellifera* sample grouped closely with *A. mellifera* database sequences, consistent with known inter-subspecies variability within this cosmopolitan species (Raffiudin et al. 2023; Dewi and Purwanto 2024). Meanwhile, *A. dorsata* subsp. *binghami* exhibited high similarity and low genetic distance with the *mrjp5-like* gene of *A. dorsata*, indicating strong sequence conservation within the *A. dorsata* group. This close genetic relationship suggests that *mrjp* genes are evolutionarily conserved among giant honey bees, potentially reflecting similar ecological and behavioral traits. Overall, the phylogenetic pattern obtained supports the distinct taxonomic identity of each *Apis* spp. analyzed and highlights the *mrjp2* gene potential as a molecular marker for resolving species-level relationships and evolutionary divergence within the genus *Apis* (Dobritzsch et al. 2019; Stączek et al. 2023).

Feasibility analysis of specific primers MF-MR and CF-CR in determining the entomological origin of honey

The reliability of the *mrjp2*-based molecular method applied in this study is supported by both internal validation and consistency with previously published studies, which together serve as a practical "gold standard" for method verification. Internally, the use of a morphologically identified

A. cerana specimen as a positive control demonstrated that the primers and PCR system could accurately amplify and identify DNA from a known species. Externally, the high sequence identity values obtained in this study (≥99% for *A. mellifera*, *A. cerana*, and *A. dorsata* subsp. *binghami*) are consistent with previous studies that validated the *mrjp2* gene as a reliable marker for honey bee species identification (Zhang et al. 2019; Raffiudin et al. 2023; Dewi and Purwanto 2024). These studies successfully applied the same genetic marker and primer systems to distinguish *Apis* species, thereby providing independent validation of the method. Moreover, the combination of PCR amplification, DNA sequencing, and phylogenetic analysis constitutes a widely accepted molecular framework for species authentication, reducing the likelihood of misidentification when relying on a single analytical approach. Unlike conventional methods that rely solely on PCR band detection, integrating sequence-based confirmation increases accuracy and reproducibility. Therefore, although no universal gold standard exists for entomological honey authentication, the approach used in this study aligns with established molecular identification practices and provides robust evidence for reliable species determination.

Based on the observed amplification patterns and sequencing outcomes, species attribution using the *mrjp2* marker followed a stepwise diagnostic workflow. First, both MF-MR and CF-CR primer sets were applied to each honey DNA extract to verify amplification of the target region. Second, all PCR products were subjected to Sanger sequencing to confirm amplicon identity. Third, species assignment was determined based on BLAST sequence similarity thresholds, where identities ≥99% supported confident species-level attribution, identities between 95-98% indicated close relatedness within the same species group, and identities <95% suggested higher interspecific divergence or limited availability of reference sequences. This combined approach ensures reliable authentication of the entomological origin of honey while accounting for potential cross-amplification among closely related *Apis* species.

The feasibility of using species-specific primers MF-MR and CF-CR in determining the entomological origin of honey was assessed based on their amplification performance, specificity, and cross-reactivity among *Apis* species (Soares et al. 2019). The MF-MR primer pair, designed for *A. mellifera*, and the CF-CR primer pair, designed for *A. cerana*, target the major royal jelly protein 2 (*mrjp2*) gene, a highly conserved yet species-distinguishable molecular marker (Raffiudin et al. 2023; Dewi and Purwanto 2024). The amplification results, BLAST-N sequence validation, and phylogenetic clustering collectively demonstrated the effectiveness and potential limitations of these primers when applied to honey authentication (Indahsari and Purwanto 2024). The PCR amplification results confirmed that both primer sets successfully amplified DNA fragments of expected sizes from honey samples representing different *Apis* species. The MF-MR primers produced amplicons of approximately 584-593 bp, while the CF-CR primers generated products of 202-210 bp. These values are consistent with the findings of Zhang et al. (2019), who reported similar amplification sizes for *A. mellifera* and *A. cerana*. The successful amplification across a range of annealing temperatures (47-59°C) indicated that both primer pairs possess robust binding affinity and thermal stability, making them suitable for standard PCR assays under varying laboratory conditions (Li et al. 2020).

The MF-MR primers specifically amplified DNA from *A. mellifera* and *A. dorsata* subsp. *binghami*, whereas the CF-CR primers amplified *A. cerana* and *A. nigrocincta* (Raffiudin et al. 2023; Dewi and Purwanto 2024). This cross-species amplification pattern reflects partial homology in the *mrjp2* gene among closely related taxa within the *Apis* genus. The ability of MF-MR primers to amplify *A. dorsata* DNA and of CF-CR primers to amplify *A. nigrocincta* DNA suggests that while the primers are species-specific in design, conserved nucleotide regions may allow binding to homologous gene segments in genetically similar species. Such cross-reactivity is not necessarily a drawback but rather demonstrates evolutionary proximity among *Apis* species and highlights the conserved nature of the *mrjp* gene family. The BLAST-N analysis provided molecular validation of amplification accuracy (Bogaerts et al. 2021; Adelino et al. 2024). The sequences of *A. mellifera* samples amplified using MF-MR primers showed 99.58% identity with the *mrjp2* gene of *A. mellifera* (GenBank accession number LC600176.1). Similarly, *A. cerana* samples amplified with CF-CR primers exhibited 99.29% identity with *A. cerana mrjp2* sequences (LC600196.1). These high identity values confirm the reliability of both primer sets in accurately detecting their intended target species. Meanwhile, *A. dorsata* subsp. *binghami* amplified with MF-MR primers showed 99.67% similarity to the *mrjp5-like* gene (XM_031510778.1), indicating that these primers can anneal to closely related regions within the *mrjp* gene cluster. For *A. nigrocincta*, amplification using CF-CR primers produced 91.25% identity to the *A. cerana mrjp2* sequence, signifying strong genetic relatedness but also confirming species-level distinction.

These findings emphasize that primer feasibility is influenced not only by design specificity but also by interspecies genomic conservation (Antil et al. 2023). The *mrjp2* gene, central to royal jelly protein synthesis, is functionally conserved across *Apis* species. However, subtle variations within its coding and flanking regions enable discrimination among species when amplified and sequenced (Feng et al. 2015; Alam et al. 2023). Thus, the combined use of MF-MR and CF-CR primers provides a complementary molecular approach capable of distinguishing between *A. mellifera*, *A. cerana*, *A. dorsata* subsp. *binghami*, and *A. nigrocincta*, while also reflecting phylogenetic relationships among them. The phylogenetic analysis further corroborated the feasibility of using these primers for honey authentication. Samples amplified with CF-CR primers clustered into two distinct clades: *A. cerana* grouped closely with reference *mrjp2* sequences (LC600196.1, LC600187.1), while *A. nigrocincta* formed a separate but proximal clade, indicating evolutionary divergence. Similarly, MF-MR-amplified samples were separated into two groups: *A. mellifera* aligned with *mrjp2* reference

sequences, and *A. dorsata* subsp. *binghami* aligned with *mrjp5-like* sequences of *A. dorsata*. The low genetic distances (1.5-3.5%) within each clade demonstrate strong conservation, while the clear inter-clade separation validates the discriminatory capacity of both primers.

From an applied perspective, these results confirm that MF-MR and CF-CR primers are feasible molecular tools for authenticating the entomological origin of honey in Indonesia (Raffiudin et al. 2023; Dewi and Purwanto 2024). Their successful amplification across multiple *Apis* taxa and high sequence congruence with GenBank references support their utility in routine honey authentication and species verification (Moškrič et al. 2021; Zhang et al. 2024). Moreover, the observed interspecific amplification, particularly between *A. cerana-A. nigrocincta* and *A. mellifera-A. dorsata* subsp. *binghami* expands the potential applications of these primers for detecting hybridization or mislabeling in commercial honey. However, further refinement is recommended to enhance species discrimination. The use of additional *mrjp* gene regions or multilocus approaches (e.g., *mrjp1*, COI, or 16S rRNA) could complement current findings and reduce ambiguities arising from conserved regions (Helbing et al. 2017). Despite these limitations, the present analysis demonstrates that both primer sets MF-MR and CF-CR exhibit high specificity, reproducibility, and evolutionary relevance, confirming their feasibility for molecular identification and authentication of honey based on its entomological origin (Soares et al. 2019).

In conclusion, the MF-MR primers generated amplicons of approximately 584-593 bp, while CF-CR primers produced fragments of 202-210 bp, consistent with the targeted *mrjp2* regions. Amplification patterns indicated that MF-MR primers amplified both *A. mellifera* and *A. dorsata* subsp. *binghami*, whereas CF-CR primers amplified *A. cerana* and *A. nigrocincta*. This cross-amplification among closely related *Apis* species indicates partial primer specificity rather than absolute species exclusivity. Sequence analysis confirmed high similarity to reference sequences, with BLAST-N identities of 99.58% (*A. mellifera*), 99.29% (*A. cerana*), 99.67% (*A. dorsata* subsp. *binghami*), and 91.25% (*A. nigrocincta*). Phylogenetic reconstruction revealed distinct clustering of samples with their respective reference taxa, supporting reliable species-level discrimination when PCR results are combined with sequencing and phylogenetic inference. These findings demonstrate that the *mrjp2* gene is a robust molecular marker for authenticating the entomological origin of honey in Indonesia. While primer-based PCR provides an initial indication of bee species origin, definitive authentication relies on sequence analysis and phylogenetic validation rather than PCR amplification alone. Future work should expand reference datasets and adopt multilocus assays to improve discrimination and routine surveillance.

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