Journal of Tropical Biodiversity and Biotechnology
Volume 09, Issue 02 (2024): jtbb85987
DOI: 10.22146/jtbb.85987

Research Article

Major Royal Jelly Protein 2 (*mrjp2*) Gene Detection in *Apis dorsata* Fabricius, 1793, *Apis dorsata binghami* Cockerell, 1906, *Apis florea* Fabricius, 1787, and *Apis nigrocincta* Smith, 1860

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Keywords:
Entomological origin
Honey
Honey bees
Indonesia
mrjp2 gene
Submitted:
20 June 2023
Accepted:
12 October 2023
Published:
05 April 2024
Editor:
Ardaning Nuriliani

ABSTRACT
Indonesian people's interest in honey, the product from honey bees, is quite high. It caused many cases of honey fraud such as mislabelling the entomological origin of honey. The Major Royal Jelly Protein 2 (*mrjp2*) gene, which encodes MRJP, can be used to determine the entomological origin of honey. The *mrjp2* gene, for example, can be detected in honey from *A. mellifera* and *A. cerana* using species-specific primers for *A. mellifera* (MF-MR) and *A. cerana* (CF-CR). This study aims to detect the *mrjp2* gene in several honey bee species native to Indonesia, namely *A. dorsata*, *A. dorsata binghami*, *A. florea*, *A. nigrocincta*, *A. mellifera*, and *A. cerana* as well as analyse the feasibility of MF-MR and CF-CR primers in determining the entomological origin of honey. The results showed that the MF-MR primers can amplify the DNA of A. *dorsata binghami*, *A. florea*, and *A. mellifera*, while CF-CR primers can amplify the DNA of both *A. nigrocincta* and *A. cerana*. The amplicons were subsequently sequenced. The phylogenetic tree and the genetic distance showed that there were differences and variation between each species of honey bee samples with the honey bee database. The data obtained from this research indicated that both primers could not determine the entomological origin of honey directly up to species level. The species level determination will only be possible using sequences information. However, in certain situations, the MF-MR and CF-CR primers were able to differentiate the honey bee species by including the information of the geographical origin of honey sample and the distribution area of each species of honey bees in Indonesia.

INTRODUCTION
Honey bees are insects that are members of the order Hymenoptera, the family Apidae, the subfamily Apinae, the tribe Apini, and the genus *Apis* (ITIS 2020). Honey bees are divided into two groups based on the type of hive in where they live: open nesting honey bees and cavity nesting honey bees (Hadisoesilo 2001). There are numerous species of honey bees found across Indonesia. In Indonesia, there are at least seven species of honey bees: *A. andreniformis*, *A. florea*, *A. dorsata*, *A. cerana*, *A. koschevni-*

kovi, *A. nigrocincta*, and *A. mellifera*. *A. andreniformis* is distributed throughout the western part of Indonesia as far as Makassar Strait in Sumbawa and westernmost Flores (Engel 2012). *A. dorsata* has a wide distribution and is commonly found in Indonesia, except Maluku and Papua (Hadisoesilo 2001). *A. dorsata* has three subspecies with two subspecies distributed in Indonesia, namely *A. dorsata dorsata* that is distributed in west part of Wallacea line and *A. dorsata binghami* that is distributed only in Sulawesi Island (Sakagami et al. 1980; Nagir et al. 2016). *A. cerana* is a honey bee that is widely distributed in Asia. One subspecies of *A. cerana*, *A. cerana nuluensis* can be found in Sabah, Kalimantan. *A. koschevnikovi* can be found on the Malay Peninsula, and throughout Kalimantan, Sumatra, and Java. *A. nigrocincta* is an endemic honey bee found on Sangihe Island, and Sulawesi. *A. mellifera* is a honey bee species introduced to Indonesia (Hadisoesilo 2001; Engel 2012).

Honey has been used by people from ancient times to serve nutritional needs and as a therapy. Honey is proven to be beneficial for health since it functions as an antioxidant, anti-inflammatory, antibacterial, antidiabetic, and overcomes many health problems. The macro and micronutrient of honey affected by various factors, such as bee type, flower source, and environmental and processing factors (Samarghandian et al. 2017; Ranneh et al. 2021).

Honey is commonly used by people all over the world as a natural product with numerous benefits. Because of the strong demand for honey, there have been examples of honey fraud involving modifying the composition and mislabelling the entomological origin of honey. Honey fraud is defined by Apimondia (International Federation of Beekeepers' Associations) as an illegal and purposeful act committed to gain an unfair economic advantage by manipulating and selling honey that do not match global honey standards. Honey derived from *A. mellifera* and *A. cerana* honey bees is often adulterated by mislabelling *A. mellifera* honey as *A. cerana* honey or modifying the composition of honey by mixing *A. mellifera* honey with *A. cerana* honey. Due to the limited production of *A. cerana* honey bees and consumer interest, honey generated from *A. cerana* honey bees sells for up to five times the price of *A. mellifera* honey on the market (Zhang et al. 2019).

Such a case of honey fraud is also suspected to occur in Indonesia, which is adulteration of *A. mellifera* honey claimed to be forest honey. According to SNI 8664 (2018), forest honey is a natural liquid with a sweet taste produced by *A. dorsata* and/or *Apis* spp. from the juice of forest plant flowers (floral nectar) or other sections of forest plants (extra floral). Meanwhile, cultured honey is defined as a natural liquid with a sweet taste produced by cultivated bees *A. mellifera* or *A. cerana* from plant flower juice (floral nectar) or other plant components (extra floral).

Various methods have been used to determine the entomological origin of honey such as analysing the differences in molecular weight and surface structure of *mrjp1* (Won et al. 2008), analysis of honey bee protein profile using SDS-PAGE (Ramón-Sierra et al. 2015), and using *mrjp2* gene (Zhang et al. 2019). Major Royal Jelly Protein (MRJP) is the primary protein component in royal jelly, which represents approximately 90% of the total protein content (Drapeau et al. 2006). Major Royal Jelly Protein (MRJP) consists of nine types of proteins (MRJP1-MRJP9) with molecular weights of about 49 to 87 kDa and was encoded by 9 gene namely *mrjp1-mrjp9* gene (Drapeau et al. 2006; Buttstedt et al. 2013). Zhang et al. (2019) could determine the entomological origin of honey using species-specific primers of *A. mellifera* (MF-MR) and *A. cerana* (CF-CR) based on differences in *mrjp2* gene sequences in *A. mellifera* and *A. cerana*. Raffiudin

et al. (2023) have conducted research on the *mrjp2* gene in *A. mellifera* and *A. c. javana* from Indonesia and showed that CF and CR primers previously used in Zhang et al. (2019) research can amplify honey bee species *A. c. javana*.

In this study, following the previous study conducted by (Raffiudin et al. 2023; Zhang et al. 2019) this research will provide new information about the *mrjp2* gene present in honey bee species found in Indonesia such as *A. dorsata*, *A. dorsata binghami*, *A. florea*, and *A. nigrocincta*. New information provided by this research can be used to determine the entomological origin of honey sold in Indonesia as one way to avoid honey adulteration.

MATERIALS AND METHODS

Materials

All of the honey bee samples that used in this study were the collection of Laboratory of Entomology, Faculty of Biology, Universitas Gadjah Mada and were collected from Jambi, Central Java, East Java, and Central Sulawesi. All species samples that used in this research were identified molecularly based on 16S rRNA gene to ensure that it was identified correctly (unpublisihed data). In this study, *A. cerana* and *A. mellifera* were used as positive controls, and four species of honey bees namely, *A. dorsata*, *A. dorsata binghami*, *A. florea*, and *A. nigrocincta* were used as shown in Table 1. From each species, one honey bee samples were used for the analysis. In this study, species-specific primers of *mrjp2* gene for *A. mellifera* (MF-MR) and *A. cerana* (CF-CR) as shown in Table 2 were used.

Methods

The research method is designed based on Zhang et al. (2019) and Raffiudin et al. (2023) with some modification. This research was conducted from August 2022 to February 2023 in Laboratory of Genetic Engineering, Inter-University Centre, Universitas Gadjah Mada which included DNA extraction and isolation, DNA amplification and visualisation, and data analysis.

DNA extraction and isolation

DNA extraction and isolation was performed using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit according to the given protocol. One honey bee sample from each species were used to be extracted. The extraction procedure was carried out by cutting all the honey bee legs and grounding it in microcentrifuge tube using micropestle. The honey bee

Table 1. Honey bee samples' location.

Sample Code	Location	Species
AM	Kota Baru, Geragai, East Tanjung Jabung Regency, Jambi	*Apis mellifera*
AC	Petungkriyono, Pekalongan, Central Java	*Apis cerana*
AD	Petungkriyono, Pekalongan, Central Java	*Apis dorsata*
DB	Rogo Village, Central Sulawesi	*Apis dorsata binghami*
AF	Baluran National Park, East Java	*Apis florea*
AN	Bakubakulu Village, Central Sulawesi	*Apis nigrocincta*

Table 2. Species-specific primers of *mrjp2* gene for *A. mellifera* (MF-MR) and *A. cerana* (CF-CR) (Zhang et al. 2019)

Species	Primer	Sequence	Product size (bp)
Apis cerana	C-F	TTTAACAATAAAAATAATCAGAAGA	212
	C-R	TTACATCCTAATTGATTTTAATGCG	
Apis mellifera	M-F	GCCATCCCTTGAAATTGTCACTCGT	560
	M-R	TCTGCAAACGACCAATCAGGATAT	

samples were then added 200 mL FATG1 Buffer and homogenised with vortex. Next, 20 µL Proteinase K (10 mg/mL) was added to the samples and then the samples were incubated at 60°C for 3 hours and inverted 10X every 30 minutes. Following the incubation period, 200 µL of FATG2 buffer was added, vortexed, and incubated at 70°C for 10 minutes. The sample was then added with 200 µL of cold 96% ethanol drop by drop, then inverted 10X and transferred to the FATG Mini Column. The sample was then centrifuged for 1 minute at 12,000 rpm at 4°C. After transferring the sample to a new Collection Tube, 400 µL of buffer W1 was added and centrifuged for 1 minute at 12,000 rpm at 4°C. The solution collected in the collection tube was discarded and 750 µL of Wash Buffer was added before centrifuging at 12,000 rpm for 3 minutes at 4°C. The preheated elution buffer was then added 100 µL to the FATG Mini Column membrane and left for 3 minutes for complete absorption. The sample was then centrifuged at 12,000 rpm at 4°C for 2 minutes. The DNA extraction results were then quantified using nanodrops and stored in a refrigerator at 4°C.

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 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C (for samples with *A. mellifera* primer) and 47°C (for samples with *A. cerana* primer) for 30 s, extension at 72°C for 30 seconds, and final extension at 72°C 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 visualised with a UV transilluminator.

Data analysis

The amplified DNA samples were sent to the Integrated Research and Testing Laboratory, Universitas Gadjah Mada for sequencing. The forward and reverse sequences from sequencing process were then edited using MEGA11 software. The consensus sequences were then analysed with BLAST-N on the NCBI website (http://blast.ncbi.nlm.nih.gov/) to find sequence similarities. The sequence results were also processed for phylogenetic tree analysis after being analysed with BLAST-N. The sequence results and the database sequence from GenBank were analysed using MEGA11 software and were aligned using ClustalW. The alignment result was then used in phylogenetic tree construction. The phylogenetic tree was constructed using the Maximum Likelihood (ML) algorithm with a bootstrap value of 1000.

RESULTS AND DISCUSSION

DNA amplification

DNA derived from four honey bee samples (*A. dorsata*, *A. dorsata binghami*, *A. florea*, and *A. nigrocincta*) and two control honey bees (*A. mellifera* and *A. cerana*) were successfully amplified using two primers, namely species-specific primers for *A. mellifera* (MF-MR) and species-specific primers for *A. cerana* (CF-CR) The concentration of extracted DNA used for amplification in this study ranged from 29.67-237.14 ng/µL with purity ranging from 1.914-2.195 (A260/230) and 1.703-2.079 (A260/280) as

shown in Table 3. The purity of the DNA that used in this research were good based on Abdel-Latif & Osman (2017) which is the range of good DNA purity 1.8 – 2 (A260/A280) and 2 – 2,2 (A260/230).

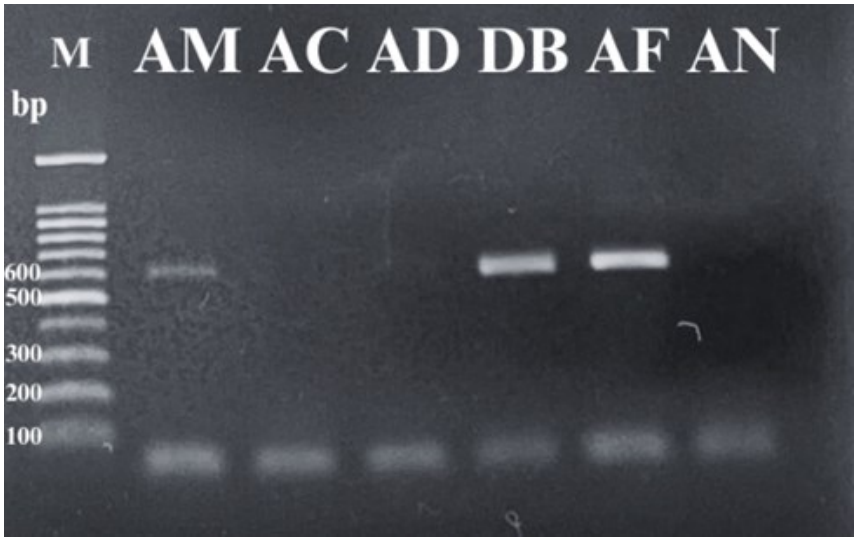

Figure 1. Results of DNA amplification in honey bees with MF-MR primer at annealing temperature 53°C. M: Marker, AM: *A. mellifera*, AC: *A. cerana*, AD: *A. dorsata*, DB: *A. dorsata binghami*, AF: *A. florea*, AN: *A. nigrocincta*

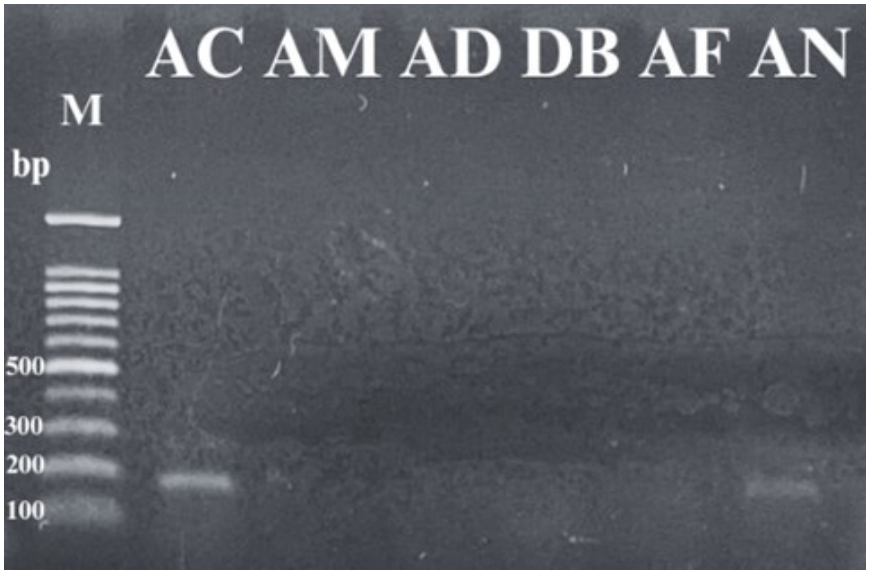

Figure 2. Results of DNA amplification in honey bees with CF-CR primer at annealing temperature 47°C. M: Marker, AC: *A. cerana*: AM: *A. mellifera*, AD: *A. dorsata*, DB: *A. dorsata binghami*, AF: *A. florea*, AN: *A. nigrocincta*

From four honey bee samples and two honey bee sample as control, this research succeeds in yielding DNA from three honey bee species, which are *A. dorsata binghami*, *A. florea*, and *A. mellifera* using species-specific primer of *mrjp2* gene for *A. mellifera* (MF-MR) at the annealing

Table 3. DNA extraction results of honey bee samples.

Species	Sample Code	A260/230	A260/280	Concentration (ng/µL)
Apis mellifera	AM	2,193	1,829	237,14
Apis cerana	AC	2,027	1,972	38,53
Apis dorsata	AD	2,049	2,079	105,60
Apis dorsata binghami	DB	2,195	1,982	187,88
Apis florea	AF	2,035	1,851	29,67
Apis nigrocincta	AN	1,914	1,703	53,25

temperature 53°C (Figure 1). This research also succeeded in yielding DNA from two honey bee species, which are *A. nigrocincta* and *A. cerana* using species-specific primers of *mrjp2* gene for *A. cerana* (CF-CR) at the annealing temperature 47°C (Figure 2). Raffiudin et al. (2023) was successfully amplified the DNA of *A. mellifera* using the MF-MR primer at the annealing temperature 50, 53, 55, 57, and 59°C, while the DNA of *A. cerana* was successfully amplified at the annealing temperature 47, 50, 53, and 55°C. The amplicon of the *mrjp2* gene in MF-MR primers was obtained at a size about 600 bp, while in CF-CR primers it was obtained at a size about 200 bp. The amplicon size of the results was as predicted as the product size of MF-MR primers and CF-CR primers which is 560 bp and 212 bp (Zhang et al. 2019). Visualisation of amplicon obtained in both MF-MR and CF-CR primers can be seen that the amplicon band is clearly visible and is a single band.

Based on these results, it can be concluded that the MF-MR primers can amplify not only *A. mellifera* DNA but also *A. dorsata binghami* and *A. florea* DNA, whereas CF-CR primers can amplify *A. cerana* and A. *nigrocincta* DNA from the cerana honey bee group. The results of this study agreed with Zhang et al. (2019) and Raffiudin et al. (2023) that the DNA from *A. cerana* could not be amplified using the MF-MR primers and the DNA from *A. mellifera* could not be amplified using the CF-CR primers. Consequently, the MF-MR primers are also species-specific for *A. dorsata binghami* and *A. florea* while the CF-CR primers are also species -specific for *A. nigrocincta*.

Based on the BLAST-N result of honey bee samples, it was found that the sequence of the controlled honey bees which are *A. mellifera* and *A. cerana*, were confirmed to be the sequence of the *mrjp2* gene from *A. mellifera* and *A. cerana* available in database, as well as the sequence of honey bee samples *A. florea* which was confirmed to be the sequence of the *mrjp2* gene from *A. florea* available in database. The sequence of the *mrjp2* gene from *A. mellifera* sample was showing homology with the *A. mellifera mrjp2* sequence from Korea (GQ160519.1) with 96.58% percent identity. The sequence of the *mrjp2* gene from *A. dorsata binghami* sample was showing homology with the *A. dorsata mrjp5-like* sequence from Indonesia and Thailand (XM_031510778.1) with 98.67% percent identity. The sequence from *A. dorsata binghami* sample was showing homology with the *A. florea mrjp2* sequence from Korea (XM_003695113.3) with 95.45% percent identity. The sequence of the *mrjp2* gene from *A. florea* sample was showing homology with the *A. florea mrjp2* sequence from Korea (XM_003695113.3) with 98.34% percent identity. The sequence of the *mrjp2* gene from *A. cerana* sample was showing homology with the *A. cerana cerana mrjp2* sequence from China (AY392758.1) with 95.48% percent identity as well as *A. nigrocincta* sample showing 93.14% percent identity with the *A. cerana cerana* mrjp2 sequence from China (AY392758.1).

The only DNA that was unable to be amplified with two primers was *A. dorsata*. Meanwhile, the subspecies of *A. dorsata*, which is *A. dorsata binghami* was amplified by MF-MR primers. To ensure that the DNA of *A. dorsata* could not be amplified using MF-MR primer, we used the other DNA template of *A. dorsata* (AD1) with DNA concentration about 1.83 ng/µL and purity 2.031 (A260/230) and 1.970 (A260/280). The result showed that the DNA of *A. dorsata* (AD1) was also not amplified using MF-MR primers. In the results of the MF-MR Primer-BLAST, it was found that the sequence of MF-MR primers did not attach to any *A. dorsata* sequence and only attached to the *mrjp2* gene sequence of *A. mellifera* and *A. florea*. Meanwhile, the CF-CR Primer-BLAST only attached

to the *mrjp2* gene sequence of *A. cerana cerana*. The alignment results of MF-MR primers and CF-CR primers with the *mrjp2* gene sequence of *A. dorsata* (KY087957.1) showed that the primer sequences were not completely attached to *A. dorsata* sequence so that it can be confirmed that the MF-MR and CF-CR primers could not amplified the DNA of *A. dorsata*.

Sequences Differences Analysis Between Honey Bee Samples and Reference Sequences

Amplicons of *A. dorsata binghami*, *A. florea*, and *A. nigrocincta* produced final product size in 586, 600, and 187 bp respectively. Meanwhile, *A. mellifera* and *A. cerana* produced final product size in 619 and 186 bp respectively. The sequence results of this research were shown in Table 5. The samples and controls sequence obtained were aligned with the database sequence of *mrjp1-mrjp9* of *A. mellifera* and *mrjp2* of *A. cerana cerana* available in NCBI using ClustalW in MEGA11 software. The phylogenetic tree was then constructed using Maximum Likelihood (ML) algorithm with a bootstrap value of 1000 and a sequence from *Bombus ignitus* (EU391535.1) as the outgroup. The Maximum Likelihood (ML) algorithm is a phylogenetic construction method that looks for the model with the highest likelihood of producing the input sequence under a certain evolutionary model (Munjal et al. 2019).

Based on the phylogenetic tree between honey bee samples and honey bee database (Figure 3), the three honey bee samples amplified using MF-MR were incorporated into three different clades, which are clade 1, clade 2, and clade 4. The *mrjp2* gene of honey bee sample *A. florea* were incorporated in clade 1 with the *mrjp2* gene of *A. florea* (XM_003695113.3) with genetic distance 0.017 or 1.7% and showing high similiarities which value 99%. Also, the *mrjp2* gene of honey bee sample *A. florea* showing similiarities with *A. florea* (XM_031917464.1) with genetic distance 0.066 or 6.6%. The honey bee sample *A. mellifera* were incorporated in clade 2 with the *mrjp2* gene of *A. mellifera* (NM_001011580.1), showing high similarities which value 97% and genetic distance 0.039 or 3.9%. Meanwhile, the *mrjp2* gene of honey bee sample *A. dorsata binghami* were incorporated with the *mrjp5-like* gene of *A. dorsata* (XM_031510778.1) in clade 4. The *mrjp2* gene of *A. dorsata binghami* and the *mrjp5-like* gene of *A. dorsata* showing high similiarities with which value 99% and low genetic distance 0.013 or 1.3%.

The two honey bee samples that amplified using CF-CR primers were incorporated in clade 6 with the *mrjp3* gene of *A. cerana* (AY663105.1) and the *mrjp3-like* gene of *A. cerana cerana* (AY394726.1). The *mrjp2* gene of honey bee sample *A. cerana* showing genetic distance 1.625 to the *mrjp3* gene of *A. cerana* (AY663105.1) and 2.056 to the *mrjp3 -like* gene of *A. cerana cerana* (AY394726.1). Meanwhile, the *mrjp2* gene of honey bee sample *A. nigrocincta* showing genetic distance 1.503 to the *mrjp3* gene of *A. cerana* (AY663105.1) and 2.230 to the *mrjp3-like* gene of *A. cerana cerana* (AY394726.1). The honey bee samples *A. cerana* and *A. nigrocincta* showing high similiarities which value 98% with genetic distance 0.195 or 19.5%.

Feasibility Analysis of *Apis mellifera* Species-Specific Primers (MF-MR) and *Apis cerana* Species-Specific Primers (CF-CR) in Determining the Entomological Origin of Honey

In this study, the MF-MR primers can amplify the DNA of other honey bee species besides *A. mellifera*, which are *A. dorsata binghami* and *A. florea*. In this case it can be said that the MF-MR primers cannot differenti-

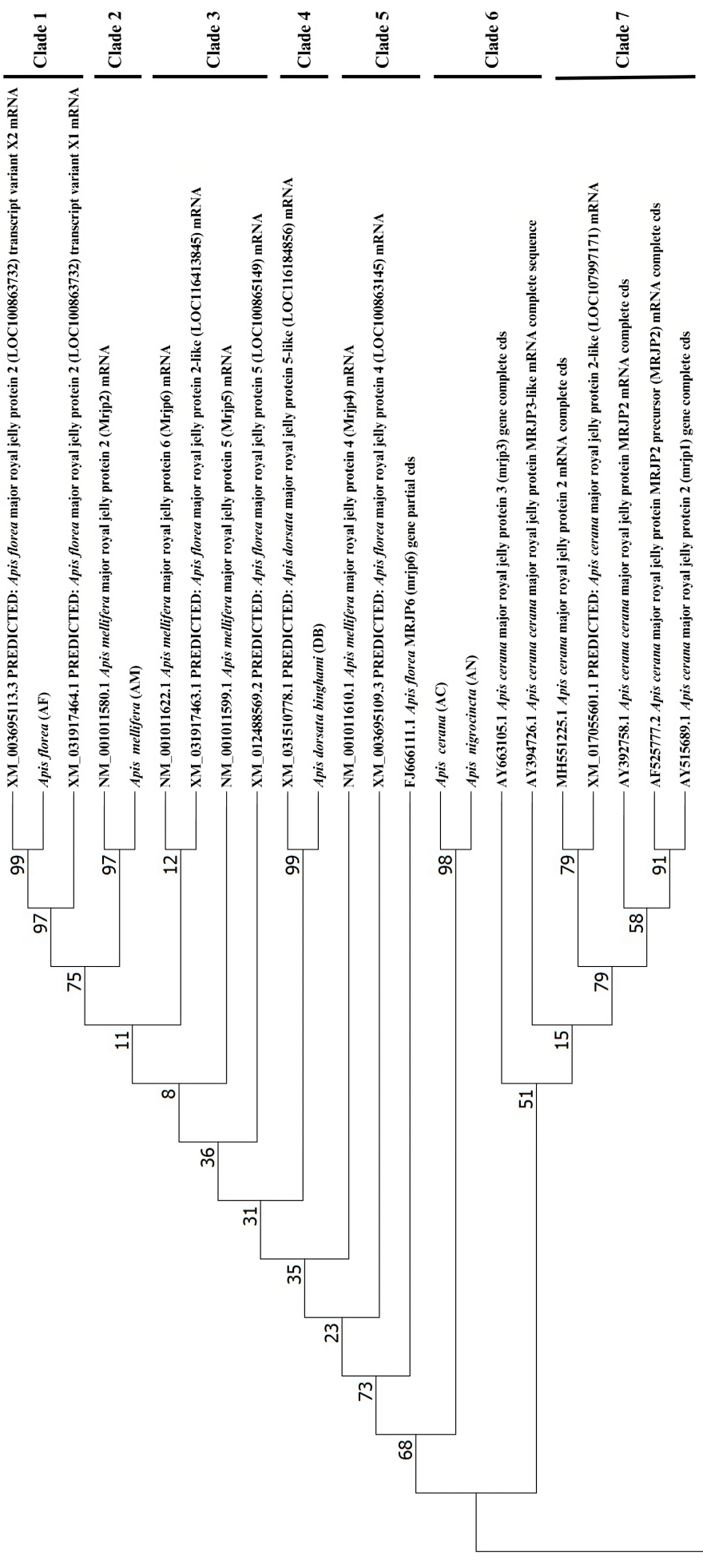

Figure 3. Phylogenetic tree of *mrjp2* gene of honey bee samples and *mrjp* gene database based on Maximum Likelihood (ML) algorithm with 1000 bootstrap value.

Table 4. The *mrjp2* gene sequence results of honey bee samples.

Apis mellifera (AM)
TGCCATCCCTTGAAATTGTCACTCGTAAAATATTCGCAGTATA- TAAGTAAGTGTTTCTAATTGTGTCGGTTGCAGCATTTATTTGCAATCTTTCATTTATCTGCATAC AAAAAAGATATTTTATTTAAGAAAAATGACAAGGTGGTTGTTCATGGTGGCATGCCTCGGCATAG CTTGTCAAGGCGCCATTGTTCTAGAAAATTCTCCAATAAACTTGGAAAAATCCTTGAACGTAATTC ACGAATGGATTTATTTTGATTATGACTTCGGTAGCGAAGAAAGAAGACAAGCTGCGATTCAATCT GGCGAATATGACCATACGAAAAATTATCCCTTCGACGTCGATCAATGGCGTGGTAAAATTTTCTTA TTTTAAATTATTAATCCATTTCCAATCGTCGAAACACTTAATATTCAATAATTTTCGTCGCTCATA TTTCTTCATTTTTGAATAACTAAAAGGATATTTCACGTTTTGTATGTCTTGTTTAAAATAAGACT TTTGTCACCATACTAAGATACGATGGTGTTCCTTCTACTTTGAACGTGATATCTGGTAAAACTGG TAATGCTGTACGTCAGACGATTGCATATCCTGATTGGACGTTTTGCAGAATCT
Apis cerana (AC)
CTTTTACATCCTAATTGATTTTAATGCGATTTTGAAGAACGACGAACTTGAT- TATCATTCTGATTGTTAGGATTCTGATTGTTATTTTTCTGATTGTTATTCTTCTGATTGTTATTC TTCTGATTGTTATTGTTCTGATTGTTATTCTTCTGATTGTTATTCTTCTGATTATTTTTATTGTT AAAA
Apis dorsata binghami (DB)
TGCCATCCCTTGAAATTGTCACTCGTAAAATATTTGCAGCATCCTACGTAAGTGTTTCTA- TATATCTTAATTGTATTTATTTGCAATCTTTCATTTGTCTAACACCCATTTAATATTTTATAGAAA AATGACAAACTGGTTACTATTGATAGTGTGTCTTGGCATAGCTTGTCAAGGTATCACAGGCGCTG TTGTTCGAGAAAATTCCTCGAGAAAAAACTTGACAAATTCGAAGAACGTGATTTACGAATGGAAG TATCTTGAATATGACTTCAGTAACGCGATTCAATCTGACGAATATGACCACACAAAAAATTATCCT TTCGATATCGATCAATGGCATGGTAAAATATTCGCATTTCATTTAATATTACATTTTATTTATCAA ATCAATTTAATATTCAATTATTTTTGTCATTCAAATTTTTTTATTTTTGTATCGTTATAAAATATC AAACATTTTGCATTTCTTCAAGATAAGATTTTTGTCGCCGTACCAATAAATTAAGGTGTTACCTTC CTCTTTAAATATGATATCTGACAAAACTGGGTAAGGTAGACGCTCTTCAGGAAATCCAGGGTGAA G
Apis florea (AF)
GCCATCCCTTGAAATTGTCACTCGTAAAATATCTGCCGCACACCCCCCTAACGTCCTTAA- TATATTTCAATTGTAATATTTATTTTCATTCTTTTAATCTATCAAAACGAAATATTTTATAGAAAA ATGATAAAGTGGGTGTTTATGGTGGCATGCCTTGGCATAACTTGTCAAGGCGCAACTGTTCGAGA AAATTCTGCAAAAAACTTGGAAAATTCTTTGAACATAATTCACGAATGGAAATATATTGATTATGA TTTCGGTAGCGACGAAAGAAGACAAGCTGCGATTCAATCTGGCGAATATGACTATAGAAGAAATT ATCCCTTCGATGTCGATCAATGGCGTGGTAAAATTTTCGTATTTTAAATTATTATTGCATTCTGA TCGTCGAAACATTTAATATTCAATAATTTTCATCATTCATATTCCTTCATTTTTGAATCATTAAAA ATGATATTCCACTTCTTGTTTAAGATAAGACTTTTGTCACCGTATTAAGATACGATGGTGTGCCT TCTTCTTTGAACCGTGATATCTGAGAAAAATGGTAAGAGAGCGAGAAAGCCACAACCATATCCTGA TTGGTCGTTTGCATAA
Apis nigrocincta (AN)
CATTTTACATCGACTTGATTATCATTCTGATTGATTAGTATTCTGATTGTTAG- TATTCTGAACTTTGTTATTCTTCTGATTGTTATTCTTCTGATTGTTATTCTTCTGATTGTTATTT TTCTGATTGTTATTTTTCTGATTGTTATTCTTCTGATTGTTATTTTTCTGATTGTTTTTATTGTT AAAA

ate whether a honey bee specimen is an *A. mellifera*, *A. dorsata binghami*, or *A. florea*. However, MF-MR primers can differentiate honey bees that belong to the cerana group, *A. cerana* and *A. nigrocincta* because the DNA of the two honey bees are not amplified using MF-MR primers. Meanwhile, the CF-CR primers themselves could not differentiate honey bees from the Cerana Group, which are *A. cerana* and *A. nigrocincta*. The MF-MR primers can amplify the DNA of other honey bee species beside the *A. mellifera* species, same with the CF-CR primers can amplify the DNA of other honey bee species beside the *A. cerana* species. The two primers showed that the primers were not species-specificied to one species because of the amplification process producing amplicons in the same size and after sequencing process results all amplicon produces sequence are

different. Based on this study, it can be said that the two primers cannot be used to differentiate honey bee species directly, and a sequencing process is needed to confirm the species of the honey bee sample.

The used of the MF-MR primer and CF-CR primer as a method to determine the entomological origin of honey can be compared with other primer such as the LR13107-F and LR12647-R primer (Thummajitsakul et al. 2013). The LR13107-F and LR12647-R primer were primers that can be used to identify honey bee species based on the 16S rRNA gene. In terms of determining the entomological origin of honey, we suggest that it is better to use the LR13107-F and LR12647-R primers because of the MF-MR and CF-CR primer, based on this study, could not determining the entomological origin of honey directly. To be able to find out the entomological origin of honey using MF-MR primers and CF-CR primers, after going through the PCR, it must go further through the sequencing process. If the sequencing process is required to distinguish the honey bee species, the used of the LR13107-F and LR12647-R primer would be an accurate method to determine the entomological origin of honey. Furthermore, if we're sequencing the 16S rRNA gene of a honey bee sample, we can discover the precise sequence on the database because of the 16S rRNA gene data is already available, as opposed to the *mrjp2* gene which is rarely utilised and hence more difficult to find on the database.

Indonesia has numerous honey bee species that are found all over the islands. Indonesia has at least seven species of honey bees that are found throughout the country, namely *A. andreniformis*, *A. florea*, *A. dorsata*, *A. cerana*, *A. koschevnikovi*, *A. nigrocincta*, and *A. mellifera* (Engel 2012). The large number of honey bee species distributed in Indonesia makes the determination of the entomological origin of honey from Indonesia more difficult. However, since each species of honey bee in Indonesia has a distinctive distribution area, by observing the information of the distribution area and geographical origin of honey, it is still possible to determine the entomological origin of honey.

Based on the research that has been done, it can be concluded that the feasibility of using MF-MR primer and CF-CR primer in determining the entomological origin of honey is feasible provided that the geographic origin of the honey is known. Because of the MF-MR primers and CF-CR primers could amplified the DNA of other honey bee species, the use of the two primers in determining the entomological origin of honey need to be evaluated. In the research of Zhang et al. (2019), MF-MR primers and CF-CR primers were able to determine the entomological origin of honey derived from *A. mellifera* and *A. cerana* because they only concern on the two species of honey bees, namely *A. mellifera* and *A. cerana*. This is different from Indonesia which has a variety of honey bee species so that MF-MR primers and CF-CR primers did not show the same results as in the previous study.

CONCLUSIONS

Based on the research that has been carried out, it is concluded that the species-specificied primers of *mrjp2* gene for *A. mellifera* (MF-MR) can detect the *mrjp2* gene not only in *A. mellifera* but also in *A. dorsata binghami* and *A. florea*. Meanwhile, the species-specific primers of *mrjp2* gene for *A. cerana* (CF-CR) can detect the *mrjp2* gene not only in *A. cerana* but also in *A. nigrocincta*. The MF-MR primers and CF-CR primers are feasible in determining the entomological origin of honey sold in the Indonesian market as long as the information of the geographical origin of honey sample and the distribution area of each species of honey bees in Indonesia are provided.

AUTHOR CONTRIBUTION

Y.D. collected and analysed the data and wrote the manuscript, H.P. designed the research 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 from Faculty of Biology Universitas Gadjah Mada.

CONFLICT OF INTEREST

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

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