Jurnal Entomologi Indonesia
p-ISSN: 1829-7722 e-ISSN: 2089-0257
Terakreditasi Kemenristekdikti: 105/E/KPT/2022

Maret 2024, Vol. 21 No.1, 1–15
Online version: https://jurnal.pei-pusat.org
DOI: https://doi.org/10.5994/jei.21.1.1

The characterization of *Apis cerana* and *Apis mellifera mrjp2* gene in Indonesia and the phylogeny relationship with *mrjp* family genes

Karakterisasi gen *mrjp2 Apis cerana* dan *Apis mellifera* di Indonesia dan hubungan filogeni dengan gen *mrjp* famili

Nurul Insani Shullia[1,2], Tia Vina Febiriani[1], Rika Raffiudin[1]*, Chandra Widjaja[3], Tri Atmowidi[1]

[1]Departemen Biologi, Fakultas Matematika dan Ilmu Pengetahuan Alam, IPB University
Jalan Agatis, Kampus IPB Dramaga, Bogor 16680, Indonesia
[2]Program Studi Pendidikan Biologi, Fakultas Keguruan dan Ilmu Pendidikan, Universitas Jember
Jalan Kalimantan No. 37, Kampus Tegalboto, Jember 68121, Indonesia
[3]Asosiasi Perlebahan Indonesia
Jakarta 12930, Indonesia

(diterima Agustus 2023, disetujui Februari 2024)

ABSTRACT

The *major royal jelly protein 2* (*mrjp2*) gene is one of the molecular markers that can discriminate between *Apis cerana* Fabricius and *A. mellifera* Linnaeus. Due to the lack of *mrjp*2 gene sequences registered in GenBank for Indonesian *A. cerana* and *A. mellifera*, DNA characterization and bioinformatics analysis were needed. This research aimed to characterize the exon-intron organization of *mrjp2* genes for both Indonesian bee species and analyze the phylogenetic relationship with other *mrjp* family genes. DNA samples of *Apis cerana* and *A. mellifera,* collected from Bogor, were amplified using MF-MR primer at annealing temperatures of 47 °C and 50 °C, respectively. The length of *A. cerana* and *A. mellifera* DNA sequences were 579 and 597 bp, respectively. The DNA sequences of both species were comprised of partial exons 1, introns 1, exons 2, introns 2, and partial exon 3. The number of putative amino acids of *A. cerana* and *A. mellifera mrjp*2 genes were 111 and 123, respectively. We confirmed that the partial MRJP2 putative amino acids of both honey bees belong to the MRJP family and contained the peptide signal in the 14 first amino acid sites. Nucleotide variation of *the mrjp2* gene in *A. cerana* was higher than *A. mellifera*. Phylogenetic tree construction showed that *A. cerana* and *A. mellifera* form a monophyletic clade with the *A. mellifera mrjp7* gene and another *mrjp* family gene clustered as reported in a previous study.

Key words: evolution, exon-intron organization, honey bee, *mrjp* gene tree, royal jelly

ABSTRAK

Gen *major royal jelly protein 2* (*mrjp2*) merupakan salah satu marka molekular yang dapat membedakan lebah *Apis cerana* Fabricius dan *A. mellifera* Linnaeus. Karena keterbatasan sekuen gen *mrjp*2 yang terdaftar di GenBank untuk *A. cerana* dan *A. mellifera* asal Indonesia, sehingga karakterisasi DNA dan analisis bioinformatik diperlukan. Penelitian ini bertujuan untuk mengarakterisasi ekson-intron gen *mrjp2* untuk kedua spesies asal Indonesia tersebut dan dan menganalisis hubungan filogeni dengan gen *mrjp* famili lainnya. Sampel lebah *A. cerana* dan *A. mellifera* yang dikoleksi di Bogor diamplifikasi secara berturut-turut pada temperatur annealing (Ta) 47 °C dan 50 °C menggunakan primer

*Penulis korespondensi: Rika Raffiudin. Departemen Biologi, Fakultas Matematika dan Ilmu Penetahuan Alam, IPB University
Jalan Agatis, Kampus IPB Dramaga, Bogor 16680, Indonesia. Tel/Faks: +62-251-8622833, Email: rika.raffiudin@apps.ipb.ac.id

This is an open access article under the CC BY 4.0 License
(https://creativecommons.org/licenses/by/4.0/).

MF-MR. Panjang sekuen DNA *A. cerana* dan *A. mellifera* yang didapatkan secara berturut-turut 579 pb dan 597 pb. Sekuen DNA *A. cerana* dan *A. mellifera* terdiri dari sebagian ekson 1, intron 1, ekson 2, intron 2 dan sebagian ekson 3. Panjang asam amino putatif sampel *A. cerana* adalah 111 aa, sedangkan *A. mellifera* adalah 123 aa. Asam amino putatif parsial MRJP2 pada *A. cerana* dan *A. mellifera* yang dihasilkan masuk kedalam kelompok keluarga MRJP dan menunjukan signal pada 14 asam amino pertama. Variasi nukleotida dan asam amino putative gen *mrjp2* pada *A. cerana* lebih banyak daripada *A. mellifera.* Topologi pohon filogenetik menunjukkan gen *mrjp2 A. cerana* dan *A. mellifera* menempati satu cluster monofiletik bersama *A. mellifrea* gen *mrjp7*, sedangkan gen *mrjp* lainnya membentuk klaster sama seperti yang dilaporkan pada penelitian terdahulu.

Kata kunci: evolusi, lebah madu, organisasi exon intron, pohon gen *mrjp*, *royal jelly*

INTRODUCTION

The major royal jelly protein (MRJP) is a group of proteins for royal jelly (Wang et al. 2020); these proteins are produced only by honey bees (Corzo et al. 2023). MRJP proteins are encoded by nine related genes: *mrjp1-mrjp9* (Helbing et al. 2017). The *mrjp* family genes are grouped tandemly in a total of 60 kb base pairs and evolved from a single progenitor, i,e. the yellow protein gene family via gene duplication (Drapeau et al. 2006). This gene evolution conserves the exon/intron structure of the *mrjp* gene family (Drapeau et al. 2006). Although *mrjp* genes have similar exon/intron structures, each gene produces a protein with unique characteristics and functions (Botezan et al. 2023). The molecular weights of MRJP1, MRJP2, MRJP3, and MRJP5 proteins are 53 kDa, 46.5 kDa, 66.8 kDa, and 80.9 kDa, respectively (Koc Ucak et al. 2022).

Mrjp genes encode proteins that are responsible for honeybee nutrition, physiological function, and the development of social behaviors such as caste determination (Buttstedt et al. 2013), as well as the division of labor for nursing and foraging among honeybee workers (Fang et al. 2023). The proteins of MRJP2 (Feng et al. 2021), MRJP8, and MRJP9 (Lee et al. 2022) show antimicrobial activities that are important for honey bee larval immune systems. Besides antimicrobial activity, the recombinants of *Apis mellifera* Linnaeus MRJP's 1-7 provide antioxidant activity against H_2O_2 (Park et al. 2020). Additionally, MRJP proteins have several health benefits, e.g, anti-tumor (Abu-Serie & Habashy 2019), anti-aging (Jiang et al. 2018), anti-inflammatory (Hayashi et al. 2011), and antiapoptotic (Kim 2021); summarized by Mureşan et al. (2022).

The most common gene in the *mrjp* family is *mrjp2* which encodes *major royal jelly protein* 2 (MRJP2) (Schmitzova et al. 1998). *Mrjp2* gene sequences of *Apis cerana* Fabricius and *A. mellifera* show high polymorphism (Su et al. 2005). This gene was used to detect the entomological origin of *A. cerana* honey from China and *A. mellifera* honey from China, Brazil, Australia, and South Africa (Zhang et al. 2019) and also successfully distinguish the honey from those both bees in Java, Indonesia (Raffiudin et al. 2023). The first *mrjp2* gene DNA sequences of *A. cerana* and *A. mellifera* originated from Indonesia and have been published in the GenBank (NCBI) database (Raffiudin et al. 2023). Another study found that there are three specific peptide markers of *mrjp*1, *mrjp2*, and *mrjp3* genes for honey authentication, namely YNGVPSSLNVISK, TLQMIAGMK, and LTVAGESFTVK, respectively (Jiang et al. 2021).

Currently, the *mrjp2* gene database at NCBI is limited to mRNA and Coding (CDS) sequences, with little information regarding exon/intron structure. Therefore, this study aims to characterize and analyze the exon/intron organization, nucleotide variation, variations in putative amino acids, and the phylogeny of *A. cerana* and *A. mellifera* bees in Indonesia based on their *mrjp2* genes. Relationships with other *mrjp* family genes is also examined.

MATERIAL AND METHOD

Research locations

A total of 30 individuals from each one colony of *A. cerana* and *A. mellifera* honeybees were collected from beekeeping in Bogor, West Java by direct hand sampling method. All samples were

paralyzed using 70% ethanol, and preserved in absolute ethanol at 4 °C until DNA extraction. This research was carried out at the Animal Molecular Laboratory, the Division of Animal Function and Behavior, Department of Biology, IPB University.

DNA extraction, amplification, and sequencing

Total DNA of each five individuals of *A. cerana* and *A. mellifera* honey bees were extracted from the thorax section based on the Phenol-Chloroform extraction method and ethanol precipitation (Sambrook et al. 1989) with modification (Raffiudin & Crozier 2007). The DNA was diluted in 0.5 mM TE buffer and stored at 4 ℃. The target *mrjp2* gene was amplified using MF-MR primer (Zhang et al. 2019). M-F primer '-GCCATCCCTTGAAATTGTCACTCGT-3') for forward and M-R primer 5'-GCCATCCCTTGAAATTGTCACTCGT-3) for reverse.

The MF-MR primer anneals at nucleotide positions 266 to 830 in the *A. mellifera* sequence (GeneID 406091), thus the target DNA comprised from exon 1 to exon 3 with an amplicon size target is ± 560 bp (Zhang et al. 2019). The annealing process was carried out at 47 ºC for *A. cerana* and 50 ºC for *A. mellifera*. DNA amplification was visualized using Diamond™ Nucleic Acid Staining in Gel Doc and was sequenced by 1stBASE, Singapore service company.

The DNA sequences of the chromatogram samples were edited using BioEdit Sequence and Alignment (Hall 1999) and Getenetyx-Win version 4.0.1 (https://www.genetyx.co.jp/). The contig results were analyzed for homology using BLAST-N at the NCBI website (http://blast.ncbi.nlm.nih.gov/). The results of the sample DNA sequences were aligned with the DNA sequences of the *mrjp2* gene of *A. mellifera* (406091) (Zhang et al. 2019) to determine the exon and intron organization using Clustal X software version 2.0 (Thompson et al. 1997). The intron positions were analyzed based on the GT/AG consensus patterns at the splice site (exons/introns) positions. Putative amino acid analysis was carried out using the Genetyx Win version 4.0.1 program. The exon positions were analyzed for nucleotide and putative amino acid variations using MEGA X software version 10.1.8 (Kumar et al. 2018). Protein signatures and families of MRJP2 putative amino acid sequences were explored using PROSITE (http://prosite.expasy.org/) and InterPro (https://www.ebi.ac.uk/interpro/), respectively. *N-glycosylation* in amino acids was predicted based on the study of Lin et al. (2019). The schematic of the exon and intron *mrjp* family genes was constructed using http://wormweb.org/exonintron.

Genetic distance and phylogenetic analysis

The phylogeny tree was constructed based on the nucleotide sequence of *mrjp2* exons 2 and 3 according to the primer design. The resulting sequences, along with corresponding GenBank nucleotide sequences from *A. cerana* and *A. mellifera*, were aligned to determine homology (Table 1). The maximum likelihood (ML) method for generating phylogenetic trees was based on MEGA X software (version 10.1.8) with 1000 bootstrap replications (Kumar et al. 2018).

RESULTS

Homology and exon-intron organization of *mrjp2* genes of *Apis cerana* and *A. mellifera* from Java

This study successfully characterized the 579 bp and 597 bp sequences of partial exon region 2 of *mrjp2* genes from *A. cerana* and *A. mellifera* in Bogor, Java (Figure 1 and 2, respectively). The homology analysis using online BLAST-N through NCBI (http://blast.ncbi.nlm.nih.gov/) showed that *the A. cerana* partial *mrjp2* gene sequence has 99% query cover and 100% identity with *A. cerana mrjp2* gene from Lebak Banten, East Java, Indonesia (LC596999.1) (Table 2). Comparing *A. mellifera* from Bogor with *A. mellifera* from Bondowoso, East Java, sequence identity was 100% and query cover was 99% (LC600169.1) (Table 3).

Both partial sequences of *A. cerana* and *A. mellifera mrjp2* genes showed high AT percentages: 67% and 66%, respectively. Determination of exon and intron organization was carried out using the *A. mellifera mrjp2* gene as a reference (Gen ID 406091). The resulting alignment showed that the DNA sequences of *A. mellifera* and *A. cerana* comprised partial exons 1, introns 1, exons 2, introns 2, and partial exon 3. The exon 2 region of *A. cerana* and *A.*

Table 1. *Apis mellifera* and *A. cerana* sequences were used for phylogenetic analysis using *mrjp* family genes

No.	Gene name	Species	Accession number	Location	Molecular type	References
Ingroup						
1.	*mrjp2*	*A. mellifera*	LC620983	Bogor, Indonesia	DNA	this study
2.	*mrjp2*	*A. cerana*	LC620984	Bogor, Indonesia	DNA	this study
3.	*mrjp2*	*A. mellifera*	NC_037648.1 (Gen ID 406091)	USA	*Gene Whole Genome*	Welberg et al. 2019
4.	*mrjp2*	*A. mellifera*	NM_001011580	Germany	mRNA	Dobritzsch et al. 2019
5.	*mrjp2*	*A. mellifera*	AF000632	Germany	mRNA	Schmitzova et al. 1998
6.	*mrjp2*	*A. mellifera*	GQ160519	South Korea	mRNA	Yoon & Nguyen et al. 2009 (unpublished)
7.	*mrjp2*	*A. mellifera carnica*	KX951418	Germany	CDS	Helbing et al. 2017
8.	*mrjp2*	*A. mellifera*	XM_026443530	USA	mRNA isoform X1	Wellberg et al. 2019
9.	*mrjp2*	*A. cerana*	NW_016019131 (Gen ID 107997173)	South Korea	mRNA	Park et al. 2019
10.	*mrjp2*	*A. cerana*	AF525777	Thailand	mRNA	Sittipraneed et al. 2004 (unpublished)
11.	*mrjp2*	*A. cerana*	AY392758	China	mRNA	Su et al. 2005
12.	*mrjp2*	*A. cerana*	AY515689	Thailand	CDS	Imjongjirak et al. 2005
13.	*mrjp2*	*A. cerana*	MH551225	South Korea	mRNA	Park et al. 2019
14.	*mrjp7*	*A. mellifera*	NM_001014429.1	*	DNA	Elsik et al. 2014
15.	*mrjp5*	*A. mellifera*	GU339164.1	*	mRNA	Yoon & Nguyen 2009 (unpublished)
16.	*mrjp5*	*A. mellifera*	NM_001011599.1	Germany	mRNA	Dobritzch et al. 2019
17.	*mrjp5*	*A. cerana*	AY392757.1	China	mRNA	Su et al. 2005
18.	*mrjp5*	*A. cerana*	NM_001328480.1	*	mRNA	Sittipraneed et al. 2004 (Unpublished)
19.	*mrjp3*	*A. mellifera*	NM_001011601.1	Germany	mRNA	Dobritzch et al. 2019
20.	*mrjp6*	*A. mellifera*	NM_001011622.1	*	DNA	Elsik et al. 2014
21.	*mrjp1*	*A. cerana*	AY279539.1	China	mRNA	Su et al. 2005
22.	*mrjp1*	*A. mellifera*	GQ160518.1	*	mRNA	Yoon dan Nguyen 2009 (unpublished)
23.	*mrjp1*	*A. mellifera*	NM_001011579.1	Germany	mRNA	Dobritzch et al. 2019
24.	*mrjp4*	*A. cerana*	MF402924.1	South Korea	mRNA	Kim et al. 2019
25.	*mrjp4*	*A. mellifera*	GU325612.1	South Korea	mRNA	Yoon & Nguyen 2010 (unpublished)
26.	*mrjp4*	*A. mellifera*	NM_001011610.1	*	DNA	Elsik et al. 2014
27.	*mrjp8*	*A. mellifera carnica*	EU703874.1	*	mRNA	Peiren et al. 2008
28.	*mrjp8*	*A. mellifera*	NM_001011564.2	Brazil	mRNA	Parpinelli et al. 2014
29.	*mrjp9*	*A. mellifera*	EU703875.1	*	mRNA	Peiren et al. 2008
30.	*mrjp9*	*A. mellifera*	NM_001024697.1	*	mRNA	Elsik et al. 2014
31.	*mrjp9*	*A. mellifera*	DQ000307.1	*	mRNA	Albert et al. 2007
31.	*mrjp9*	*A. mellifera*	DQ000307.1	*	mRNA	Albert et al. 2007
Outgroup						
32.	*mrjp2*	*Bombus impatiens*	NT_177730	USA	DNA *Whole Genome*	Robertson 2015 (unpublished)

*Sample locations are unknown.

mellifera yields 111 (Figure 1) and 123 putative amino acids, respectively (Figure 2), with introns 1 and 2 following the GT-AG consensus (Figure 1–2). Based on protein family analysis using InterPro, the exon 2 and partial exon 3 putative amino acids of *A. cerana* and *A. mellifera* MRJP2 belong to the protein family of major royal jelly protein/protein yellow (IPR017996). The protein structure analysis using PROSITE (https://prosite.expasy.org/) showed that the putative amino acids of *A. cerana* and *A. mellifera* MRJP2 from Bogor have signal peptides in the first 14 amino acid sites. The signal peptides of *A. cerana* partial MRJP2 are MTLWLFMVVCLGIA, while *A. mellifera* partial MRJP2 showed mutations in signal peptide at putative amino acid sites number 3 and 9. For amino acid number 3, lysine (K) in *A. cerana* (Figure 1) is replaced by arginine (R) in *A. mellifera* (Figure 2). Similarly, amino acid number 9 of *A. cerana* is valine (V) which is replaced by alanine (A) in *A. mellifera.* Another protein signature reported by PROSITE is a lipid that is placed in the 15th amino acid [Cysteine (C)] for both *A. cerana* and *A. mellifera*. Based on the previous study of N-glycosylation sites analysis (Lin et al. 2019), both *A. cerana* and *A. mellifera* showed the N-glycosylation site in the N_{92} putative amino acid (Figure 1–2).

Nucleotide and putative amino acid variation of *A. cerana* and *A. mellifera* partial *mrjp2* genes

Nucleotide variation analysis was carried out based on the 223 bp of exon 2 and 111 bp of exon 3. The results showed that in *A. cerana* there were 14 and 16 nucleotide variations in exons 2 and 3, respectively (Table 4). However, the *A. mellifera mrjp2* gene is more conserved, with only three nucleotide variations in exon 3. Nucleotide variations between *A. cerana* and *A. mellifera* occurred in 43 sites with more variation exon 2 than exon 3. Higher nucleotide variation in *A. cerana* resulted in higher variation in putative amino acids. *A. cerana* has 15 while *A. mellifera* has but one. The one mutation in *A. mellifera* occurs at site 96 in exon 3 of the *mrjp2* gene changing the amino acid glycine (G) to aspartic acid (D) (Table 5).

Genetic distance and phylogeny of *mrjp* genes

The genetic distance of the *mrjp* genes of *A. cerana* and *A. mellifera* was analyzed based on exon regions 2 and 3 using the Tamura 3-parameter analysis model (Nei & Kumar 2000). The genetic distance within *A. cerana* ranges from 0.0030 to 0.1132, while lower ranges of 0.000 to 0.0091 were observed in *A. mellifera* (Supplementary 1). Thus, the genetic distance between both honey bee *mrjp2* genes ranged from 0.0799 to 0.1015. The highest genetic distance within all *A. cerana* and *A. mellifera mrjp* genes (*mrjp1-9*) was 0.5479, which occurred from *A. mellifera mrjp1* and *mrjp9* gene (Supplementary 1).

Although the schematic structure of *the mrjp* family genes show that *mrjp1* to *mrjp9* have varied lengths and exon-intron structures, almost all coding regions (black boxes) of these gene have the similar patterns (Figure 3). Construction of an ML phylogenetic tree for *A. cerana* and *A. mellifera mrjp* family genes (*mrjp1-mrjp9*) revealed the presumptive evolution of *mrjp* genes. Sequences were clustered based on the gene with bootstrap values greater than 75%. All *A. cerana* and *A. mellifera mrjp2* genes are clustered in a single

Table 2. BLAST-N results of *Apis cerana* partial *mrjp*2 gene sequences from Bogor

Description	*Query cover*	*E value*	*Identity value*	Accession number
A. cerana mrjp2 gene Lebak Banten, partial cds	99%	0	100.00%	LC596999.1
A. cerana mrjp2 gene Bantul, partial cds	99%	0	99.83%	LC600197.1
A. mellifera carnica mrjp2 gene, complete cds	99%	0	90.89%	KX951418.1

Table 3. BLAST-N results of *Apis mellifera* partial *mrjp*2 gene sequences from Bogor

Description	*Query cover*	*E value*	*Identity value*	Accession number
A. mellifera mrjp2 gene Bondowoso*, partial cds*	99%	0	100.00%	LC600169.1
A. cerana carnica, complete cds	100%	0	98.99%	KX951418.1
A. cerana mrjp2 (*mrjp1*) *gene, complete cds*	96%	0	90.35%	AY515689.1

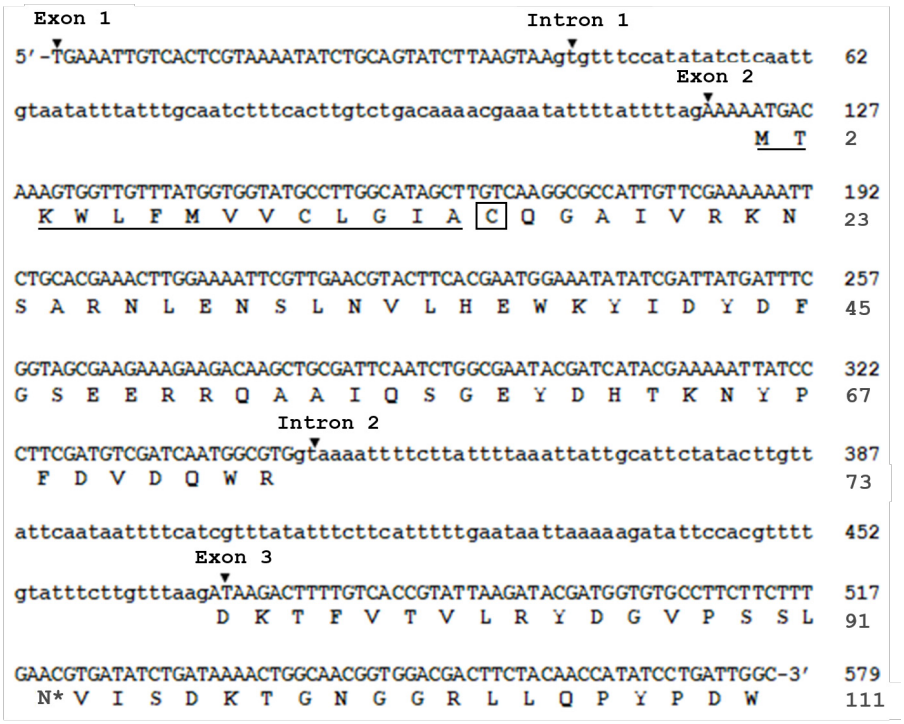

Figure 1. Structure of nucleotide sequences (up) and deduced amino acid (down) of partial *mrjp*2 gene in *Apis cerana* Bogor (LC620984). The numbering on the right indicates the position of the last nucleotide (up) or amino acid (down) in each line. Deduced amino acids with underline and box are signal and lipid-based on PROSITE, respectively. Deduced N-glycosylation in amino acids based on Lin et al. (2019) is indicated by an asterisk.

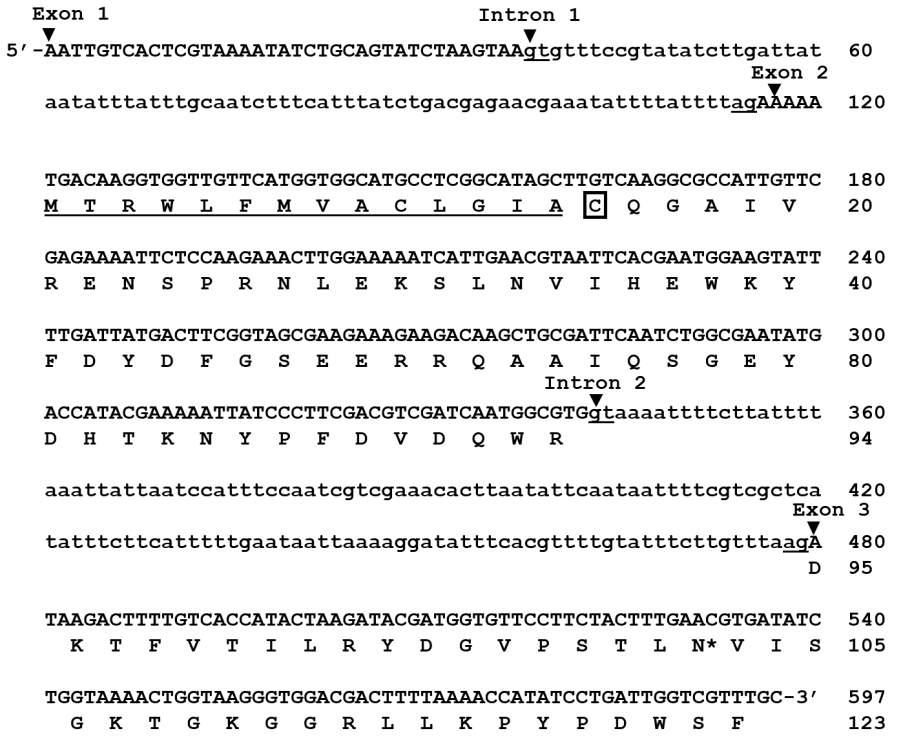

Figure 2. Structure of nucleotide sequences (up) and deduced amino acid (down) of partial *mrjp*2 gene in *Apis mellifera* Bogor (LC620983). The numbering on the right indicates the position of the last nucleotide (up) or amino acid (down) in each line. Deduced amino acids with underline and box are signal and lipid-based on PROSITE, respectively. Deduced N-glycosylation in amino acids based on Lin et al. (2019) is indicated by an asterisk.

Table 4. *Apis mellifera* and *A. cerana*'s nucleotide variations of *mrjp*2 gene in exon 2 and 3 region

No.	Sequences	Nucleotide site-																							
		Exon 2																							
			1	2	3	3	6	7	8	8	8	9	9	1	1	1	1	1	1	1	1	1	2	2	2	2
			8	6	3	6	0	4	0	7	9	5	6	0	1	2	3	4	4	5	5	9	0	2	4	6
		8	8	6	3	6	0	4	0	7	9	5	6	9	2	2	6	0	2	1	4	9	2	6	0	0
1.	*A. mellifera mrjp2 NM001011580*	G	C	C	C	C	C	G	G	C	C	A	G	A	A	A	G	T	T	C	C	T	C	C	G	A
2.	*A. mellifera Bogor mrjp2 exon 2 3 LC620983**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3.	*A. mellifera mrjp2 GQ160519.1*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4.	*A. mellifera mrjp2 AF000632.1*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5.	*A. mellifera mrjp2 KX951418.1*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
6.	*A. mellifera mrjp2 XM026443530.1*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7.	*A. mellifera mrjp2 406091*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8.	*A. cerana mrjp2 AY392758*	A	T	.	T	.	T	A	C	.	G	.	A	T	G	.	A	A	C	T	.	C	T	T	A	.
9.	*A. cerana mrjp2 AY515689*	A	T	.	T	.	.	A	C	.	G	.	A	T	G	.	A	A	C	T	.	C	T	T	A	.
10.	*A. cerana mrjp2 AF525777.2*	.	.	.	T	.	.	A	C	.	G	.	A	T	G	.	A	A	C	T	.	C	T	T	A	.
11.	*A. cerana mrjp2 MH551225.1*	.	.	.	T	G	T	A	C	.	G	.	A	T	.	.	A	A	C	T	.	C	T	T	A	.
12.	*A. cerana mrjp2 GenID 107997173*	A	T	T	T	.	.	.	A	T	G	C	.	T	G	C	A	A	C	T	T	C	T	T	.	G
13.	*A. cerana Bogor mrjp2 exon 2 3 LC620984**	A	T	T	T	.	.	.	A	.	G	C	.	T	G	C	A	A	C	T	.	C	T	T	.	G

Table 4. *Apis mellifera* and *A. cerana*'s nucleotide variations of *mrjp*2 gene in exon 2 and 3 regions (Continue...)

No.	Sequences	Nucleotide site- Exon 3																	
		2 6 3	**2 6 7**	**2 6 8**	**2 8 0**	**2 8 7**	**2 9 6**	**3 0 5**	**3 0 6**	**3 0 7**	**3 1 2**	**3 1 3**	**3 1 6**	**3 1 9**	**3 2 8**	**3 3 2**	**3 3 5**	**3 4 0**	**3 5 3**
1.	*A. mellifera mrjp2 NM001011580*	**C**	**G**	**A**	**T**	**A**	**G**	**G**	**G**	**T**	**C**	**T**	**T**	**G**	**A**	**T**	**A**	**A**	**T**
2.	*A. mellifera Bogor mrjp2 exon 2 3* LC620983*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3.	*A. mellifera mrjp2 GQ160519.1*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4.	*A. mellifera mrjp2 AF000632.1*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5.	*A. mellifera mrjp2 KX951418.1*	.	.	.	**G**	.	.	.	**A**	.	.	.	.	.	.	**C**	.	.	.
6.	*A. mellifera mrjp2 XM026443530.1*	.	.	.	**G**	.	.	.	**A**	.	.	.	.	.	.	**C**	.	.	.
7.	*A. mellifera mrjp2 406091*	.	.	.	**G**	.	.	.	**A**	.	.	.	.	.	.	**C**	.	.	.
8.	*A. cerana mrjp2 AY392758*	.	**A**	**G**	**G**	.	**A**	**A**	**A**	**C**	**T**	**C**	.	.	**C**	**C**	**C**	.	.
9.	*A. cerana mrjp2 AY515689*	.	**A**	**G**	**G**	.	**A**	**A**	**A**	**C**	**T**	**C**	.	.	**C**	**C**	**C**	.	.
10.	*A. cerana mrjp2 AF525777.2*	.	**A**	**G**	**G**	.	**A**	**A**	**A**	**C**	**T**	**C**	.	.	**C**	**C**	**C**	.	.
11.	*A. cerana mrjp2 MH551225.1*	.	**A**	**G**	**G**	.	**A**	**A**	**A**	**C**	**T**	**C**	.	.	**C**	**C**	**C**	.	.
12.	*A. cerana mrjp2 GenID 107997173*	**T**	.	.	**G**	**T**	.	.	**A**	.	.	.	**C**	**C**	.	**C**	**C**	**G**	.
13.	*A. cerana Bogor mrjp2 exon 2 3* LC620984*	**T**	.	.	**G**	**T**	.	.	**A**	.	.	.	**C**	**C**	.	**C**	**C**	.	**C**

clade along with the *mrjp7* gene (bootstrap results of 77%, Figure 4). As shown in Figure 4, despite lower bootstrap measures, the *mrjp2+7* gene clade was clustered with *mrjp1* (40% bootstraps). The *mrjp4* gene is the sister clade of *mrjp2+7* and *mrjp1* (34% bootstraps). The *mrjp6+mrjp3* clade was related to *mrjp5* (18% bootstraps). Finally, the *mrjp8+mrjp9* gene clade was placed at the base of the tree (41% bootstraps).

DISCUSSION

Both *mrjp2* gene sequences of *A. cerana* and *A. mellifera* bees have higher AT nucleotide content than GT nucleotide content, consistent with Raffiudin et al. (2022). The whole genome *A. mellifera* also reported that honey bees contain high amounts of adenine (A) and thymine (T) compared to other insect genome sequences (Honeybee Genome Sequencing Consortium 2006). The results of the BLAST-N DNA sequences of *A. cerana* and *A. mellifera* showed that both were homologous to the samples of *A. cerana* (LC596999.1) and *A. mellifera* (LC600169.1) from Java, Indonesia with 100% homology values and an E-value of 0. A nucleotide sequence can be said to be homologous if the results of homology analysis using BLAST-N show an E-value close to zero (Pearson 2013) or an identity value greater than 70% for nucleotide sequence data and more than 25% for the analyzed amino acid sequence data (Claviere & Notredame 2007). The high homology of *A. cerana* is consistent with the previous results that found there is no variation of *the mrjp2* gene in *A. cerana* from several provinces in Indonesia (Raffiudin et al. 2022)

Alignment results showed that *A. cerana* and *A. mellifera* sequences comprised the exon 1 to partial exon 3, this result confirmed the targeted *mrjp2* region of MF-MR primer (Zhang et al. 2019). The intron region of *A. cerana* and *A. mellifera mrjp2* gene sequences have fulfilled the GT-AG consensus (Kitamura-Abe et al. 2004), that flank the exon region (Breathnach et al. 1978). The resulting of 112 and 123 putative amino acids in exon 2 and 3, respectively of both honey bee were confirmed by using InterPro as the MRJP family member. The MRJP protein contains around 400–578 putative amino acids (Buttstedt et al. 2013) with the *mrjp2* gene having six exons and five introns (Drapeau et al. 2006). This study found that the first 14 putative amino acids were detected as the N-terminal signal peptide both in *A. cerana* and *A. mellifera*. This signal is supported by the study that revealed the MRJP family consists of 16–20 amino acids N-terminal signal peptide (Schmitzova et al. 1998; Buttstedt et al. 2014). A recent study found that there were three N-glycosylation sites in MRJP2 isolated from fresh royal jelly of *A. mellifera*, which are N_{145}, N_{178}, and N_{92} (Lin et al. 2019). Based on Lin et al. (2019), our result confirmed that the MRJP2 putative amino acid of *A. cerana* and *A. mellifera* showed the expected N-glycosylation site in the asparagine (N) amino acid number 92. The N-glycosylation has been conserved and is important since these asparagine residues are the attachment sites for complex sugars or glycosylation (Park & Zhang 2011).

Variations between *A. cerana* and *A. mellifera* in their nucleotide sequences and putative amino acids may reflect differences in the quantity and composition of the MRJP proteins that make up royal jelly (Yu et al. 2009). Moreover, the results show that *A. cerana* has more within-species nucleotide and amino acid variation than *A. mellifera*. This high genetic variation in *A. cerana* nucleotide sequences might be due to a wider geographical distribution and the presence of different subspecies. Variation within subspecies of *A. cerana* in Indonesia is low (Raffiudin et al. 2022).

The genetic distance results show that *A. cerana* bee samples have a genetic distance that was closer to the DNA sequences of the *A. cerana mrjp2* gene than to the DNA sequences of the *A. mellifera mrjp2* gene (Supplementary 1). This result follows Su et al. (2005) that the *mrjp2* gene of *A. cerana* and *A. mellifera* has a large polymorphism.

Although the *mrjp2* gene of *A. cerana* and *A. mellifera* have a high polymorphism, however, they formed the monophyletic clade. Uniquely, the phylogenetic topology of *A. cerana* based on exons 2 and 3 of the *mrjp2* gene showed that *A. cerana* from Bogor clustered with *A. cerana* (Gen ID 107997173) with a bootstrap value of 77%. This

Table 5. *Apis cerana* and *A. mellifera* putative amino acid variations of exon 2 and 3 regions of *mrjp*2 gene

No.	Species	Putative amino acid site-																	
		Exon 2										Exon 3							
		3	9	20	22	24	25	26	30	35	41	74	81	83	90	93	96	98	100	106
1.	*A. mellifera mrjp2 NM001011580*	R	A	V	E	S	P	R	K	I	F	R	I	R	T	V	G	T	K	K
2.	*A. mellifera Bogor mrjp2 exon 2 3* LC620983*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3.	*A. mellifera mrjp2 GQ160519.1*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4.	*A. mellifera mrjp2 AF000632.1*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5.	*A. mellifera mrjp2 KX951418.1*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.
6.	*A. mellifera mrjp2 XM026443530.1*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.
7.	*A. mellifera mrjp2 406091*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.
8.	*A. cerana mrjp2 AY392758*	K	.	I	Q	.	A	K	N	.	I	H	.	K	.	M	N	I	.	Q
9.	*A. cerana mrjp2 AY515689*	K	.	I	Q	.	A	K	N	.	I	H	.	K	.	M	N	I	.	Q
10.	*A. cerana mrjp2 AF525777.2*	.	.	I	Q	.	A	K	N	.	I	H	.	K	.	M	N	I	.	Q
11.	*A. cerana mrjp2 MH551225.1*	.	.	I	Q	.	A	K	N	.	I	H	.	K	.	M	N	I	.	Q
12.	*A. cerana Bogor mrjp2 exon 2 3* LC620984*	K	V	.	K	.	A	.	N	L	I	.	V	.	S	.	D	.	N	Q
13.	*A. cerana mrjp2 GenID 107997173*	K	V	.	K	F	A	.	N	L	I	.	V	.	S	.	D	.	N	Q

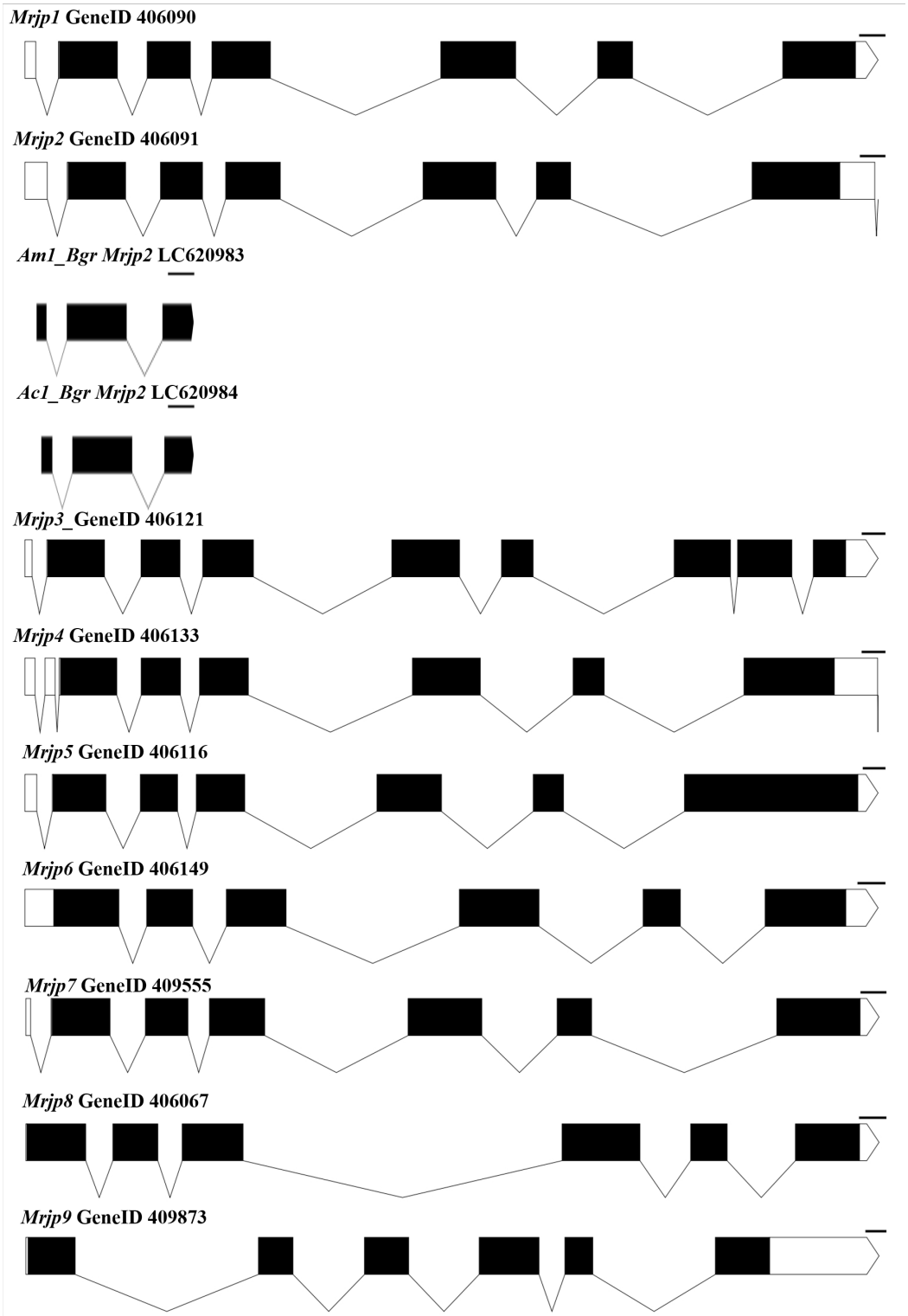

Figure 3. Schematic position of *Apis mellifera* (LC620983) and *A. cerana* (LC620984) partial *mrjp*2 gene in Bogor compared to *A. mellifera mrjp* gene family. The box indicates the exon region and the line indicates the intron. Scale 100 bp.

clade was separate from the other *mrjp2* genes of *A. cerana* and *A. mellifera* (Figure 4). Furthermore, the topology of the *mrjp* family gen tree showed that the *A. cerana* and *A. mellifera mrjp*2 clustered with the *A. mellifera mrjp7* gene with a low bootstrap value of 41% (Figure 4). The entire *mrjp2* and *mrjp7* family genes in the phylogenetic tree can still be classified as one cluster because the resulting bootstrap value is relatively low (Figure 4). Clusters in a phylogenetic tree are said to be real and reliable if they have a bootstrap proportion value of more than 70% (Hillis & Bull

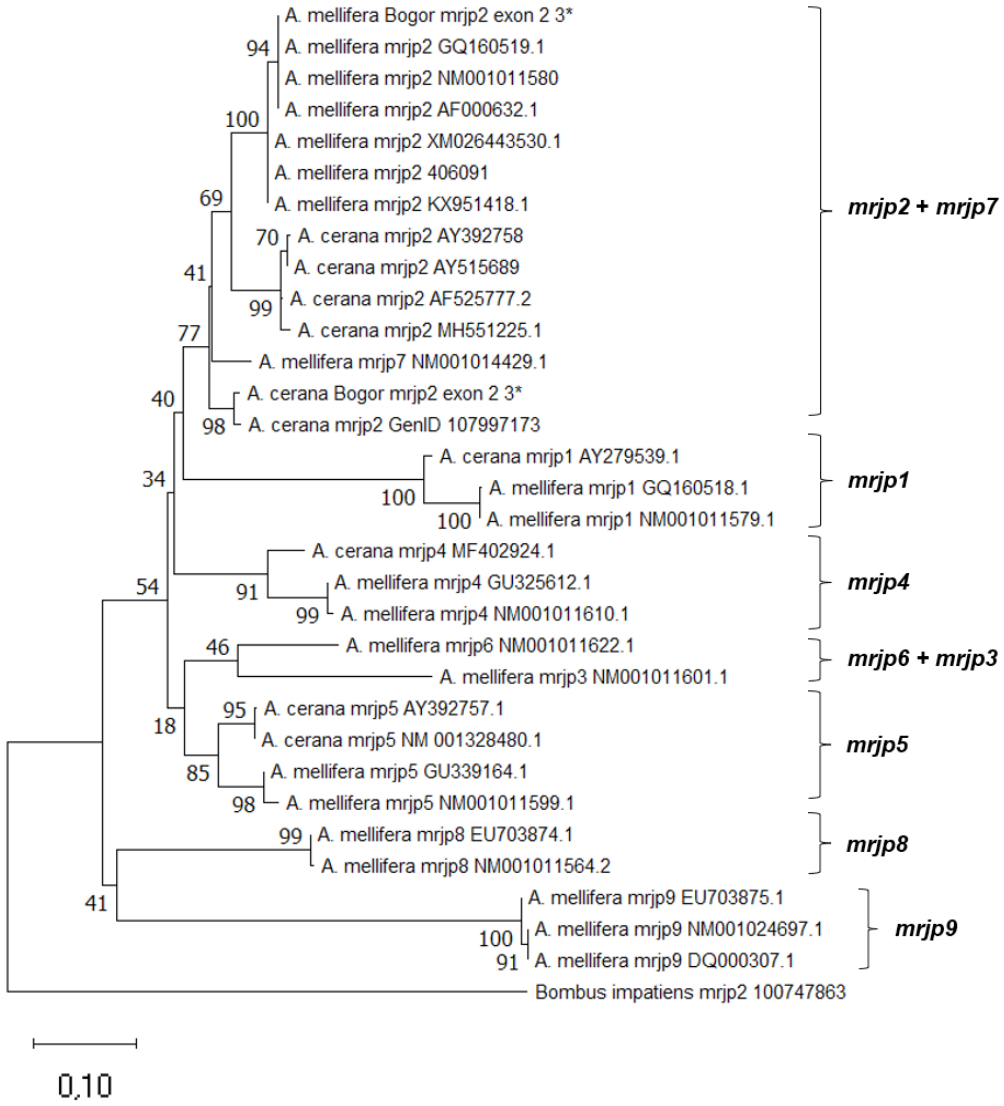

Figure 4. The nucleotide sequence-based phylogenetic tree of *mrjp* family genes in *Apis cerana* and *A. mellifera* using the asterisks code (*) indicates current samples.

1993). The clustering of *mrjp2* and *mrjp7* genes are congruent with the phylogenetic tree of *Apis* spp. using the *mrjp* family that showed *mrjp*2 is grouped in the same cluster as *mrjp*7 (Drapeau et al. 2006; Buttstedt et al. 2014).

The *mrjp* family gene tree also revealed that *mrjp3+mrjp6* and *mrjp5* genes were closely related. The *mrjp3+mrjp6* group is similar to Buttstedt et al. (2014) study, while the position of *mrjp5* is inconsistent (Drapeau et al. 2006). On the other hand, *mrjp8* and *mrjp9* were clustered forming a group that is separated from other *mrjp* family members (Figure 4). This is consistent with previous studies (Drapeau et al. 2006; Buttstedt et al. 2014). Perhaps the functions of *mrjp8* and *mrjp 9* differ from *mrjp1-7*. Unlike other *mrjp* family genes, the expression of *mrjp8* and *mrjp9* were low both in the brain and hypopharyngeal gland, while expressed in another organ, leading to the hypothesis that the function of those genes differs from other *mrjp* genes (Dobritzsch et al. 2019). The gene sequence of the *mrjp* families *A. cerana* and *A. mellifera* can group in the same cluster because the *mrjp* family genes evolved from one gene of the same family. The *mrjp* gene family of *Apis* spp. evolved from a common background, thus they have a close relationship (Drapeau et al. 2006). The *mrjp* gene family forms a large cluster with the *mrjpl/yellow* gene family in the Hymenopteran species, which includes *Apis* honey bees, non-*Apis* bees, and ants (Buttstedts et al. 2014). Knowledge of *the mrjp* gene family's structure and evolution is important for our understanding of their roles in honey bee development and nutrition.

CONCLUSION

This study successfully isolated 579 bp and 597 bp of *mrjp*2 genes from *A. cerana* and *A. mellifera*, respectively. Those sequences are homologs with the *mrjp2* gene of *A. cerana* and *A. mellifera* from Indonesia. They are comprised of partial exons 1 up to partial exon 3, and produce 111 and 123 putative amino acids of *A. cerana* and *A. mellifera*, respectively. Nucleotide and putative amino acid variation in *A. cerana* is higher than in *A. mellifera*. Phylogenetic tree construction showed that *the A. cerana* and *A. mellifera mrjp2* genes have a close phylogenic relationship with *the A. mellifera mrjp7* gene. The structure and phylogenetic relationships confirm that this family of genes has conserved exon/intron structure through gene duplication.

ACKNOWLEDGMENTS

We thank The Ministry of Research and Technology/National Research and Innovation Agency (Indonesia) (Kementerian Riset Teknologi dan Pendidikan Tinggi Republik Indonesia) for the research grant entitled "Entomological detection of *Apis cerana* and *Apis mellifera* honey based on *Major Royal Jelly Protein 2* gene" No: 4049/IT3. L1/PN/2020.

REFERENCES

Abu-Serie MM, Habashy NH. 2019. Two purified proteins from royal jelly with in vitro dual anti-hepatic damage potency: Major royal jelly protein 2 & its novel isoform X1. *International Journal of Biological Macromolecules.* 128:782–795. DOI: https://doi.org/10.1016/j.ijbiomac.2019.01.210.

Albert S, Klaudiny J. 2007. MRJP9, an ancient protein of the honeybee MRJP family with non-nutritional function. *Journal of Apicultural Research.* 46:99–104. DOI: https://doi.org/10.3896/IBRA.1.46.2.06.

Botezan S, Baci GM, Bagameri L, Pașca C, Dezmirean DS. 2023. Current status of the bioactive properties of royal jelly: A comprehensive review with a focus on its anticancer, anti-inflammatory, and antioxidant effects. *Molecules.* 28:1510. DOI: https://doi.org/10.3390/molecules28031510.

Breathnach R, Benoist C, O'Hare K, Gannon F, Chambon P. 1978. Ovalbumin gene: Evidence for leader sequence in mRNA and DNA sequences at exon- intron boundaries. *Proceedings of the National Academy of Sciences.* 75:4853-4857. DOI: https://doi.org/10.1073/pnas.75.10.4853.

Buttstedt A, Moritz RF. Erler S. 2013. More than royal food-Major royal jelly protein genes in sexuals and workers of the honeybee *Apis mellifera*. *Frontiers in Zoology*. 10:72. DOI: https://doi.org/10.1186/1742-9994-10-72.

Buttstedt A, Moritz RFA, Erler S. 2014. Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the yellow gen family. *Biological Reviews.* 89:255–269. DOI: https://doi.org/10.1111/brv.12052.

Claviere JM, Notredame C. 2007. *Bioinformatics for Dummies.* 2nd Ed. Indiana: Willey Publishing Inc.

Corzo E, Clement H, Corzo G, Peña G, Cid-Uribe JI. 2023. Transcriptomic comparison of the royal jelly proteins coded in the hypopharyngeal glands of *Apis mellifera* and *Geotrigona acapulconis*. *Research Square Preprint.* DOI: https://doi.org/10.21203/rs.3.rs-2948844/v1.

Dobritzsch D, Aumer D, Fuszard M, Erler S, Buttstedt. 2019. The rise and fall of major royal jelly proteins during a honeybee (*Apis mellifera*) workers' life. *Ecology and Evolution.* 9:8771–8782. DOI: https://doi.org/10.1002/ece3.5429.

Drapeau MD, Albert S, Kucharski R, Prusko C, Maleszka R. 2006. Evolution of the yellow/major royal jelly protein family and the emergence of social behavior in honey bees. *Genome Research.* 16:1385–1394. DOI: https://doi.org/10.1101/gr.5012006.

Elsik CG, Worley KC, Bennett AK, Beye M, Camara F, Childers CP, de Graaf DC, Debyser G, Deng J, Devreese B et al. 2014. Finding the missing honey bee genes: Lessons learned from a genome upgrade. *BMC Genomics.* 15:86. DOI: https://doi.org/10.1186/1471-2164-15-86.

Fang Y, Feng M, Ma C, Rueppell O, Li J. 2023. Major royal jelly proteins influence the neurobiological regulation of the division of labor among honey bee workers. *International Journal of Biological Macromolecules.* 15:848–860. DOI: https://doi.org/10.1016/j.ijbiomac.2022.11.150.

Feng M, Fang Y, Ma C, Duan X, Zhang Y, Han B, Hu H, Meng L, Wang F, Li J. 2021. Mechanistic insight into royal protein inhibiting the gram-

positive bacteria. *Biomolecules*. 11:64. DOI: https://doi.org/10.3390/biom11010064.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*. 41:95–98.

Hayashi T, Takamatsu N, Nakashima T, Arita T. 2011. Immunological characterization of honey proteins and identification of MRJP 1 as an IgE-binding protein. *Bioscience, Biotechnology, and Biochemistry.* 75:556–560. DOI: https://doi.org/10.1271/bbb.100778.

Helbing S, Lattorff HMG, Mortz RF, Buttstedt A. 2017. Comparative analyses of the major royal jelly protein gene cluster in three Apis species with long amplicon sequencing. *DNA Research.* 24:279–287. DOI: https://doi.org/10.1093/dnares/dsw064.

Hillis DM, Bull JJ. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology.* 42:182–192. DOI: https://doi.org/10.1093/sysbio/42.2.182.

Imjongjirak C, Klinbunga S, Sittipraneed S. 2005. Cloning, expression and genomic organization of genes encoding major royal jelly protein 1 and 2 of the honey bee (*Apis cerana*). *BMB Report.* 38:49–57. DOI: https://doi.org/10.5483/BMBRep.2005.38.1.049.

Jiang CM, Liu X, Li CX, Qian HC, Chen D, Lai CQ, Shen LR. 2018. Anti-senescence effect & molecular mechanism of the major royal jelly proteins on human embryonic lung fibroblast (HFL-I) cell line. *Journal of Zhejiang University SCIENCE B.* 19:960–972. DOI: https://doi.org/10.1631/jzus.B1800257.

Jiang W, Ying M, Zhang J, Cui Z, Chen Q, Chen Y, Wang J, Fang F, Shen L. 2021. Quantification of major royal jelly proteins using ultra performance liquid chromatography tandem triple quadrupole mass spectrometry and application in honey authenticity. *Journal of Food Composition and Analysis*. 97:103801. DOI: https://doi.org/10.1016/j.jfca.2021.103801.

Kim BY. 2021. Antiapoptotic role of major royal jelly protein 8 of honeybee (*Apis mellifera*) venom. *Journal of Asia-Pacific Entomology.* 24:666–670. DOI: https://doi.org/10.1016/j.aspen.2021.05.014.

Kim CK, Lee DC, Choi SH. 2017. Detection of Korean native honey and European honey by using duplex polymerase chain reaction and immunochromatographic assay. *Korean Journal for Food Science of Animal Resources.* 37:599–605. DOI: https://doi.org/10.5851/kosfa.2017.37.4.599.

Kim BY, Lee KS, Jung B, Choi YS, Kim HY, Yoon HJ, Gui Z, Lee J, Jin BR. 2019. Honeybee (*Apis cerana*) major royal jelly protein 4 exhibits antimicrobial activity. *Journal of Asia Pacific Entomology.* 22:175-182. DOI: https://doi.org/10.1016/j.aspen.2018.12.020.

Kitamura-Abe S, Itoh H, Washio T, Tsutsumi A, Tomita M. 2004. Characterization of the splice sites in GT-AG and GC-AG introns in higher eukaryotes using full-length cDNAs. *Journal of Bioinformatics and Computational Biology.* 2:309–331. DOI: https://doi.org/10.1142/S0219720004000570.

Koc Ucak A, Karacaoglu M, Bakır ZB, Kızılkaya K. 2022. Determination of total protein, trans-10-Hydroxy-2-Decenoic Acid (10-HDA) and major royal jelly proteins in royal jelly produced at different harvest times in queenless and queenright colonies. *Harran Tarım ve Gıda Bilimleri Dergisi.* 26:109–116. DOI: https://doi.org/10.29050/harranziraat.1016909.

Kumar S, Stecher G, Li M, Knyaz C,Tamura K. 2018. Mega x: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution.* 35:1547–1549. DOI: https://doi.org/10.1093/molbev/msy096.

Lee S, Lee KS, Ok M, Kim BY, Jin BR. 2022. Antimicrobial activity of major royal jelly protein 8 and 9 of honeybee (*Apis mellifera*) venom. *Journal of Asia-Pacific Entomology.* 25:101964. DOI: https://doi.org/10.1016/j.aspen.2022.101964.

Lin N, Li J, Shao R, Zhang H. 2019. Site-Specific analysis of N-linked glycosylation heterogeneity from royal jelly glycoproteins. *Journal of Agricultural and Food Chemistry.* 67:9411–9422. DOI: https://doi.org/10.1021/acs.jafc.9b03080.

Mureşan CI, Dezmirean DS, Marc BD, Suharoschi R, Pop OL, Buttstedt A. 2022. Biological properties and activities of major royal jelly proteins and their derived peptides. *Journal of Functional Foods.* 98:105286. DOI: https://doi.org/10.1016/j.jff.2022.105286.

Nei M, Kumar S. 2000. *Molecular Evolution and Phylogenetics.* New York: Oxford University Press. DOI: https://doi.org/10.1093/oso/9780195135848.001.0001.

Park C, Zhang J. 2011. Genome-wide evolutionary conservation of N-glycosylation sites. *Molecular Biology and Evolution.* 28:2351–2357. DOI: https://doi.org/10.1093/molbev/msr055.

Park MJ, Kim BY, Deng Y, Park H, Choi YS, Lee KS, Jin BR. 2020. Antioxidant capacity of major royal jelly proteins of honeybee (*Apis mellifera*) royal jelly. *Journal of Asia-Pacific Entomology.* 23:445–448. DOI: https://doi.org/10.1016/j.aspen.2020.03.007.

Park MJ, Kim BY, Park HG, Deng y, Yoon HJ, Choi YS, Jin BR. 2019. Major royal jelly protein 2 acts as an antimicrobial agent and antioxidant in royal jelly. *Journal of Asia-Pacific Entomology.* 22:684–689. DOI: https://doi.org/10.1016/j.aspen.2019.05.003.

Parpinelli RS, Ruvolo-Takasusuki MC, Toledo VA. 2014. MRJP microsatellite markers in Africanized *Apis mellifera* colonies selected on the basis of royal jelly production. *Genetics and Molecular Research.* 13:6724–33. DOI: https://doi.org/10.4238/2014.August.28.16.

Pearson WR. 2013. An introduction to sequence similarity ('homology") searching. *Current Protocols in Bioinformatics*. 42:311–318. DOI: https://doi.org/10.1002/0471250953.bi0301s42.

Peiren N, de Graaf DC, Vanrobaeys F, Danneels EL, Devreese B, Van Beeumen J, Jacobs FJ. 2008. Proteomic analysis of the honey bee worker venom gland focusing on the mechanisms of protection against tissue damage. *Toxicon.* 52:72–83. DOI: https://doi.org/10.1016/j.toxicon.2008.05.003.

Raffiudin R, Crozier RH. 2007. Phylogenetic analysis of honey bee behavioral evolution. *Molecular Phylogenetics and Evolution.* 43:543–552. DOI: https://doi.org/10.1016/j.ympev.2006.10.013.

Raffiudin R, Shullia NI, Damayanti, A.U. Wahyudi DT. Febiriani TV, Atmowidi T, JSA, Widjaja MC. 2022. New haplotypes of *Apis cerana* in Indonesia: Identification from mitochondrial and major royal jelly protein 2 genes. *International Journal of Tropical Insect Science.* 42:389–401. DOI: https://doi.org/10.1007/s42690-021-00556-x.

Raffiudin R, Shullia NI, Febiriani TV, Nisa NR, Rahmadini J, Purwanto H, Atmowidi T. 2023. Entomological origin detection of honey from *Apis mellifera* and *Apis cerana javana* in Indonesia based on the *Major Royal Jelly Protein 2* (*mrjp*2) gene. *Journal of Apicultural Research.* 62:330–333. DOI: https://doi.org/10.1080/00218839.2021.1989795.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular Cloning: A Laboratorium Manual.* 2nd Ed. New York: Cold Spring Harbor Laboratory Press.

Schmitzova J, Klaudiny J, Albert S, Schroder W, Schreckengost W, Hanes J, Judova J, Simuth J. 1998. A family of major royal jelly proteins of the honeybee *Apis mellifera* L. *Cellular and Molecular Life Sciences.* 54:1020–1030. DOI: https://doi.org/10.1007/s000180050229.

Su S, Albert S, Chen S, Zhong B. 2005. Molecular cloning and analysis of four cDNAs from the heads of *Apis cerana* cerana nurse honeybees coding for major royal jelly proteins. *Apidologie.* 35:389–401. DOI: https://doi.org/10.1051/apido:2005026.

The Honeybee Genome Sequencing Consortium. 2006. Insight intosocial insects from the genome of the honeybee *Apis mellifera*. *Nature.* 443:931–949. DOI: https://doi.org/10.1038/nature05260.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research.* 25:4876–4882. DOI: https://doi.org/10.1093/nar/25.24.4876.

Wallberg A, Bunikis I, Pettersson OV, Mosbech MB, Childers AK, Evans JD, Mikheyev AS, Robetson HM, Robinson GE, Webster MT. 2019. A hybrid de novo genome assembly of the honeybee, *Apis mellifera*, with chromosome-length scaffolds. *BMC Genomics*. 20:275. DOI: https://doi.org/10.1186/s12864-019-5642-0.

Wang X, Dong J, Qiao J, Zhang G, Zhang H. 2020. Purification and characteristics of individual major royal jelly protein 1–3. *Journal of Apicultural Research.* 1–12. DOI: https://doi.org/10.1080/00218839.2020.1761071.

Yu F, Mao F, Jianke L. 2009. Royal jelly proteome comparison between *A. mellifera* and *A. cerana* cerana. *Journal of Proteome Research.* 9:2207–2215. DOI: https://doi.org/10.1021/pr900979h.

Zhang YZ, Wang S, Chen YF, Wu YQ, Tian J, Si JJ, Zhang CP, Zheng HQ, Hu FL. 2019. Authentication of *Apis cerana* and *Apis mellifera* honey based on major royal jelly protein 2 gene. *Molecules.* 24:1–10. DOI: https://doi.org/10.3390/molecules24020289.