BIODIVERSITAS
Volume 24, Number 5, May 2023
Pages: 2612-2622

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240513

Molecular detection of the pathogen of *Apis mellifera* (Hymenoptera: Apidae) in honey in Indonesia

IGM RAKA ALPIN ADITYA[1,♥], HARI PURWANTO[2,♥♥]

[1]Undergraduate Program, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sleman 55281, Yogyakarta, Indonesia. Tel./fax.: +62-274-580839, ♥email: gm.raka.alpin@mail.ugm.ac.id

[2]Laboratory of Entomology, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sleman 55281, Yogyakarta, Indonesia. Tel./fax.: +62-274-580839, ♥♥email: hari.purwanto@ugm.ac.id

Manuscript received: 23 March 2023. Revision accepted: 11 May 2023.

Abstract. *Aditya IGMRA, Purwanto H. 2023. Molecular detection of the pathogen of* Apis mellifera *(Hymenoptera: Apidae) in honey in Indonesia. Biodiversitas 24: 2612-2622. Apis* honey bees play a major role in global crop pollination. The honey bee population faces various threats, one being entomopathogens, responsible for 13% of honey bees' declining population cases. Some major pathogens are *Nosema* sp., *Melissococcus plutonius,* and *Paenibacillus larvae*. Data on the prevalence of these pathogens is not available in Indonesia. Therefore, this study aimed to detect entomopathogen in Java, Bali, and Sumatra honey. We extracted 30 honey samples from Java, Bali, and Sumatra in this study. DNA from honey amplified by conventional Polymerase Chain Reaction (PCR) to detect *Nosema apis, Nosema ceranae, M. plutonius,* and *P. larvae*. Amplicons were sequenced to confirm the species of pathogens detected. Results showed that *N. ceranae* has a 20% prevalence, found in Java and Bali; *M. plutonius* was, for the first time, found in Indonesia and only in Java, with a 3.33% prevalence. In comparison, this study didn't find *N. apis* and *P. larvae*. Environmental factors were studied for their correlation with the incidence of pathogens. From several factors, only precipitation significantly correlates with the occurrence of *N. ceranae* and *M. plutonius*, while other factors do not. This research concludes that honey can be used as source of DNA for pathogens detection and monitoring in a certain area globally.

Keywords: European honey bees, foulbrood, honey, *Nosema*, PCR

Abbreviations: AFB: American Foulbrood; EFB: European Foulbrood

INTRODUCTION

Honey bees are eusocial insects in the genus of *Apis*, family Apidae, and tribe Apini. *Apis mellifera* Linnaeus 1758 is commonly farmed in Indonesia, especially in Java and Sumatra (Buchori et al. 2022). *Apis mellifera* belongs to medium-sized *Apis* with worldwide distribution because of its wide range tolerance to temperature and humidity (Li et al. 2019). Therefore, *A. mellifera* plays a major role in the global pollination of crop plants. Among 100 crop species that supplied 90% of our food, 71 were pollinated by honey bees (Paudel 2015). It makes honey bees become the key to our food security. Honey bees also impact other pollinators by increasing their diversity, eventually improving crop productivity (Paudel 2015).

The population of *A. mellifera* is facing several threats, including parasites, pesticides, loss of habitat, climate change, pests, weak queens, and entomopathogens (Goulson et al. 2015; Theisen-Jones and Bienefeld 2016). FAO's Global survey of honey bees and other pollinators in 2018 showed that pathogens threatened 13% of the honey bee population, becoming the third biggest threat causing the decline of the honey bee populations (FAO 2018). Pathogens commonly found infecting honey bees are *Paenibacillus larvae, Mellisococcus plutonius, Nosema apis,* and *Nosema ceranae* (Rivière et al. 2013). In addition, *P. larvae* cause American Foulbrood (AFB) disease, *M. plutonius* causes European Foulbrood (EFB) disease, and *N. apis* and *N. ceranae* cause nosemosis (Bailey and Ball 1991; Chantawannakul et al. 2015).

Nosemosis causes necrosis of the midgut's ventricular epithelial cell (Maiolino et al. 2014). Symptoms of nosemosis are abdominal dwelling, fecal stains at the hive's entrance, and a decrease in colony size, particularly in cold weather (Higes et al. 2010). *Nosema* can easily spread through saliva, oral apparatus, and potentially by honey or pollen (Higes et al. 2008). Nosema appearance in Indonesia was previously reported by Rice (2001) in Bogor and Botías et al. (2012) in Sulawesi and South Kalimantan. AFB and EFB attacked the broods; EFB killed the bee brood when they were 4-5 days old, while AFB infected older sealed larvae. Both diseases are widely distributed, especially where *A. mellifera* were kept. Both pathogens have not been reported in Indonesia due to a lack of data or research (Chantawannakul et al. 2015).

Detection of the pathogen plays an important role in preventing the spread of disease in a colony and provides prevalence information. In general, there are two methods for pathogen detection, microbiological and molecular methods (Higes et al. 2010; Fries et al. 2013). Nowadays, molecular detection is preferred because of its sensitivity, accuracy, and ability to detect pathogens as early as possible. The most frequently used method is Polymerase Chain Reaction (PCR) using larvae, gastrointestinal tract,

abdomen, or worker bees as biological samples. Alternatively, we could use environmental DNA (eDNA) to detect specific pathogens. eDNA is a collection of intracellular or extracellular DNA suspended in a matrix in an environment such as water, soil particles, or air. eDNA is often used to find the presence of a particular species in an environment (species-targeted) or to determine the richness of species in a community without the need to do a sampling of organisms (non-targeted) (Harper et al. 2018). eDNA has been applied to research on honey bees using honey samples as a source of DNA isolates. For example, Jain et al. (2013) and Hawkins et al. (2015) analyzed eDNA in honey to determine the composition of flora consumed by bees. Ribani et al. (2020) have used eDNA in honey to detect several honey bee pathogens due to honey's direct contact with larvae, worker bees, hives, and bee gastrointestinal tracts, the primary sources of microorganisms (Silva et al. 2016).

FAO's global survey, mentioned earlier, showed a lack of data on honey bee pathogens in Indonesia. Considering this, we aimed to detect four major pathogens of honey bees (*N. apis, N. ceranae, M. plutonius,* and *P. larvae*) using eDNA from *A. mellifera* honey. This study can fulfill the lack of data about pathogens by providing the prevalence of honey bees' major pathogens and validating the use of eDNA from honey for pathogens detection.

MATERIALS AND METHODS

Study area

This research was conducted from July to December 2022 on 20 apiaries in Indonesia. Four apiaries are in Sumatra, six in Bali, and ten in Java (Table 1 and Figure 1). Selected locations were the center of *A. mellifera* honey bee cultivation, who had agreed to be sampled and interviewed about their apiary. Honey samples and data collection of these apiaries was conducted from July to October 2022, while molecular analysis was performed from August to December 2022.

Procedures

Honey and worker bees collection

30 *A. mellifera* honey samples were collected for this research. Ten samples were collected from Java Island, eight from Bali Island, and 12 from Sumatra Island. Honey samples were collected when farmers harvest honey with an extractor, meaning the samples come from a mixture of several beehives. Honey samples from Manukaya Village (Apiary 1-3) came from one randomly selected beehive because the honey harvesting method in this apiary still uses conventional squeeze methods. Samples were transported to the Laboratory of Genetic Engineering, Biotechnology Study Center, Universitas Gadjah Mada, Yogyakarta, Indonesia. Honey samples were stored in 200 mL plastic bottles at room temperature until processed.

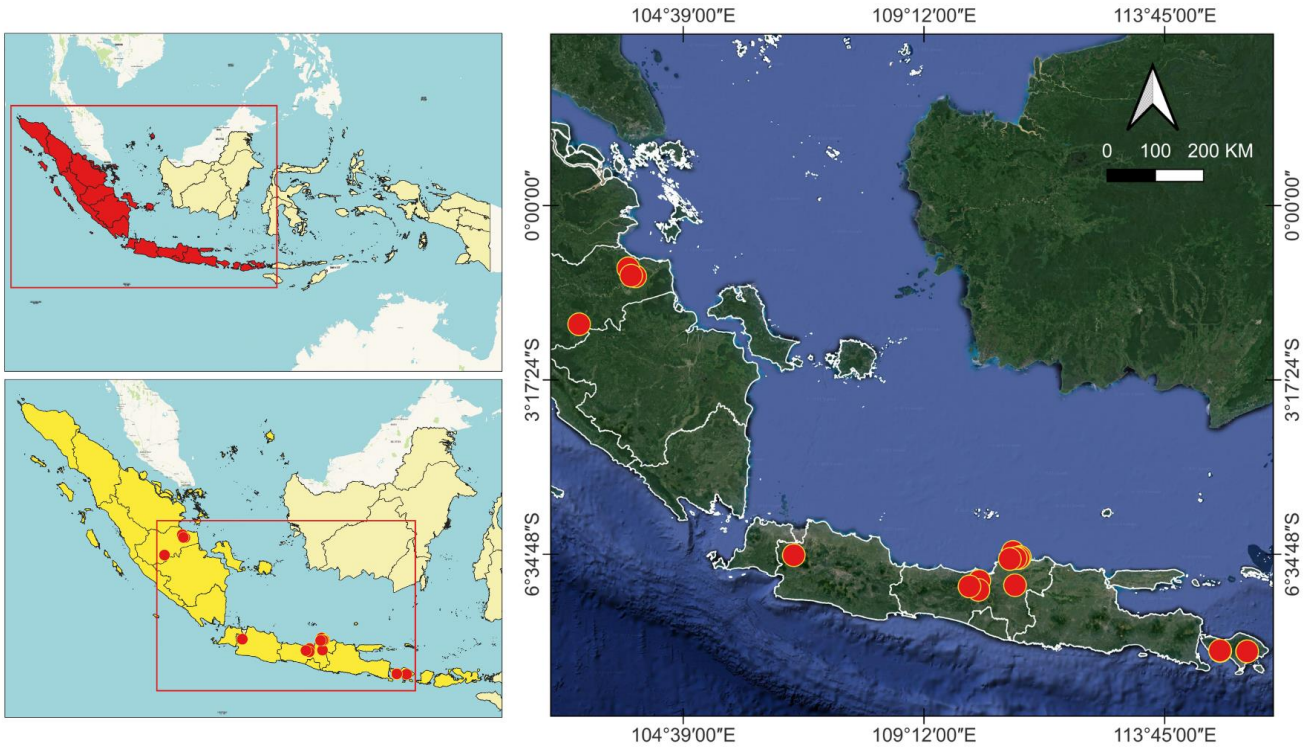

Figure 1. Map of 20 apiary locations across Java, Bali, and Sumatra (Indonesia)

Table 1. Coordinates of apiaries and sample codes from each apiary

Apiary number	Apiaries location	Longitudes	Latitudes	Sample code
Bali	Manukaya Village 1	115.3098	-8.41249	Gi1
	Manukaya Village 2	115.31009	-8.41188	Gi2
	Manukaya Village 3	115.31222	-8.41226	Gi3
	Yeh Sumbul Village 1	114.80068	-8.40447	Je1
	Yeh Sumbul Village 2	114.80129	-8.40445	Je2, Je3
	Yeh Sumbul Village 3	114.7977	-8.39501	Je4, Je5
Java	Pending Village	110.92342	-7.163485	Pu1
	Watuaji Village	110.883527	-6.535728	Jp1
	Suwatu Village	111.014632	-6.630794	Jp2
	Lahar Village	110.988728	-6.651618	Pa1
	Jollong Village	110.93465	-6.65182	Pa2
	Bategede Village	110.825061	-6.668817	Pa3
	Merbuh Village	110.247358	-7.083515	Ke1
	Tempuran Vilage	110.231464	-7.251326	Te1
	Candiroto Village	110.061867	-7.185617	Bo1
	Sukawening Village	106.740642	-6.596106	Te2
Sumatra	Suka Maju Village	103.743932	-1.333811	Ja1, Ja2, Ja3, Ja4, Ja5, Ja6
	Sinar Wajo Village	103.611539	-1.181458	Ja7, Ja8, Ja9, Ja10
	Bernai Village	102.682529	-2.237616	Su1
	Rantau Karya Village	103.667538	-1.31981	Su2

Data collection

Environmental, geographical, and threat data were collected from each apiary. These data include: the name of the owner, apiary location, longitude, latitude, altitude, mean annual temperature, annual precipitation, number of hives, number of frames per hive, the major source of nectar and pollen (monofloral or multifloral), *Varroa* infestation, and occurrence of other disturbance such as pesticide poisoning (Ansari et al. 2017; FAO 2018). Environmental data were collected from the climatecharts.net, and geographical data from My Elevation apps, while beekeepers and field surveys provided other information. For the apiary in Java and Bali, information was collected directly by observing several aspects of the apiary. While apiary in Sumatra, beekeepers were interviewed using an online meeting platform and were observed about their apiary condition and management.

Sample preparation

Honey samples were diluted by adding aquadest to reduce their viscosity. 15 g of honey was placed into a 50 mL falcon tube, and 35 mL of aquadest was added. The honey solution was vortexed and incubated at 40°C for 30 minutes in a water bath; these steps will completely dissolve the honey. After that, the honey solution was centrifuged for 25 minutes at 5,000 rpm at 20°C (Salkova et al. 2018; Ribani et al. 2020). The supernatant was discarded, and the pellets were dissolved in 2 mL aquadest. The pellet solutions were transported to the 2 mL PCR tube and centrifuged for 10 minutes at 12,000 rpm at 4°C. After the supernatant was discarded, pellets were flash-frozen using liquid nitrogen and crushed using a micropestle. These steps were repeated three times to break open the spore and cell wall of the entomopathogen (Fries et al. 2013). Pellets were re-suspended in 0.5 mL ddH_2O and extracted using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Proteinase K).

DNA extraction

DNA extraction was performed according to FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Proteinase K) user manual. First, 500 µL of pellets were placed into a 1.5 mL PCR tube, and 200 µL FATG1 was added. Next, the solution was vortexed, and 20 µL Proteinase K was added. Next, the solution was incubated at 60°C for 1 hour to activate the enzymes. After incubation, 200 µL FATG2 was added and incubated at 70°C for 10 minutes. Subsequently, absolute cold ethanol was added, and the solution was transferred into the FATG mini-column and collection tube. Next, the sample was centrifuged at 12,000 rpm at 4°C for 1 minute. After that, the sample was washed twice using 400 µL W1 buffer and 750 µL wash buffer. For the last step, the sample was eluted using 50 µL pre-heated elution buffer and centrifuged at 12,000 rpm at 4°C for 2 minutes. DNA concentration was evaluated using NanoDrop.

PCR analysis

DNA samples were analyzed using PCR for molecular identification using a set of primers summarized in Table 2. For positive control, extracted DNA sample was first amplified using *A. mellifera* 16S rRNA primer to confirm the presence of DNA in the sample. This study targeted *N. apis*, *N. ceranae*, *M. plutonius*, and *P. larvae*. For *N. apis* and *N. ceranae*, PCR analysis used two primers for a single reaction (multiplex PCR), while *P. larvae* and *M. plutonius* were analyzed using a single PCR analysis. Each PCR reaction consists of 25 µL GoTaq® Green Master Mix (Promega), 2 µL DNA template, 1 µL reverse primer, 1 µL forward primer, and 8.5 µL Nuclease Free Water.

Table 2. PCR primers used in this study to amplify extracted DNA

Species	Primer name	Sequence	Product size	Reference
Apis mellifera	LR13107-F	TGGCTGCAGTATAACTGACTGTACAAAGG	496	Thummajitsakul et al. (2013)
	LR12647-R	GAAACCAATCTGACTTACGTCGATTTGA		
Nosema apis	321APIS-FOR	GGGGGCATGTCTTTGACGTACTATGTA	321	Martín-Hernandez et al. (2007)
	321APIS-REV	GGGGGGCGTTTAAAATGTGAAACAACTATG		
Nosema ceranae	218MITOC-FOR	CGGCGACGATGTGATATGAAAATATTAA	218-219	Martín-Hernandez et al. (2007)
	218MITOC-REV	CCCGGTCATTCTCAAACAAAAAACCG		
Paenibacillus larvae	Han233PaeLarv16S_F	GTGTTTCCTTCGGGAGACG	233	Han et al. (2008)
	Han233PaeLarv16S_R	CTCTAGGTCGGCTACGCATC		
Melisococcus plutonius	MeliFORa	GTTAAAAGGCGCTTTCGGGT	281	Garrido-Bailon et al. (2013)
	MeliREVa	GAGGAAAACAGTTACTCTTTCCCCTA		

PCR profiles for *A. mellifera* 16SrRNA are as follows: predenaturation at 95°C for 2 min, 35 cycles (denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 30 s), final extension at 72°C for 5 min and final hold at 4°C (Thummajitsakul et al. 2013; Nuraini and Purwanto 2021; Rachmawati et al. 2022). PCR conditions for *N. apis and N. ceranae* 16S rRNA are as follows: predenaturation at 94°C for 5 min, 35 cycles (denaturation at 94°C for 30 s, annealing at 58°C (*N. ceranae*) or 66°C (*N. apis*) for 30 s, extension at 72°C for 1 min), and final extension at 72°C for 10 min (Ansari et al. 2017; Salkova et al. 2018). PCR profiles for *M. plutonius* are as follows: predenaturation at 95°C for 2 min, 35 cycles (denaturation at 95°C for 30 s, annealing for 30 s at 59°C, extension at 72°C for 45 s), final extension at 72°C for 7 min (Garrido-Bailon et al. 2013), and PCR profiles for *P. larvae* are as follows: 95°C for 2 min, 35 cycles (denaturation at 95°C for 30 s, annealing for 30 s at 61°C, extension at 72°C for 45 s), final extension at 72°C for 7 min (Garrido-Bailon et al. 2013; Ribani et al. 2020). First, the amplicon was separated using 1.2% agarose gel electrophoresis and visualized using a UV transilluminator. Next, 30-40 µL of PCR products that showed a positive result in electrophoresis were shipped to PT. Genetika Science for sequencing.

Data analysis

PCR results were used for disease prevalence calculation and were presented in a bar chart. Environmental conditions and beekeeping management data were analyzed for their relationship with the emergence of pathogens. The analysis was performed using SPSS 29. Fisher's exact test was carried out for nominal type data (nectar source, *Varroa* presence, and pesticide poisoning). This analysis resulted in the conclusion of a significant (S) or insignificant (NS) relationship. Quantitative data (altitude, precipitation, annual average temperature, number of hives, and number of frames) were analyzed using Spearman's correlation.

Sequence retrieved from sequencing is processed with BioEdit v.7.2.5 software. First, the forward and reverse sequences are edited by trimming both ends. The two sequences are then aligned to create a consensus sequence. This sequence is then analyzed using Nucleotide BLAST (BLASTn) to confirm the species. In addition, consensus sequences are also processed for phylogenetic tree analysis. Finally, the sequence is analyzed using the MEGA X software and aligned with sequences available in the GenBank database using CLUSTAL W. The alignment results are then used for the construction of phylogeny trees. Phylogeny trees are created by using the Maximum-Likelihood (ML) algorithm. The confidence level of the phylogeny tree was tested with a bootstrap value of 1000.

RESULTS AND DISCUSSION

A previous study reported *Nosema apis* in Bogor, West Java, Indonesia (Rice 2001), *N. ceranae* in South Kalimantan, and Sulawesi, Indonesia (Botías et al. 2012), while both *P. larvae* and *M. plutonius* never been reported found in Indonesia. The current study confirmed the presence of *N. ceranae* in Bali and Java, while *M. plutonius* was positively found in Bogor, West Java.

Apiary condition

The surveys of 20 apiaries were conducted through field survey for apiaries located in Bali and Java, Indonesia, while data collection for apiaries located in Sumatra were conducted through an online interview. From this survey, we collected several information including hives number, number of frames, source of nectar, pesticide poisoning, and *Varroa* infestation. While, physicochemical parameters such as altitudes, mean temperatures, and precipitation were collected from climatecharts.net and "My Elevation" apps (Table 3).

DNA extraction

DNA extracted from the honey was analyzed using NanoDrop to evaluate the quality of DNA. Three parameters are used: concentration (ng/nL), A260/A230, and A260/280. We were more focused on the A260/230 and A260/280 ratio because these ratios explained the purity of our extraction results. A260/280 shows the ratio of DNA and protein in the sample, while A260/230 shows the DNA to organic compounds ratio in the samples. A lower A260/230 or A260/280 ratio indicates a high amount of protein or organic compounds. Otherwise, a higher ratio indicates a high amount of nucleic acid, usually in the form of RNA. The normal range for A260/230 is around 2.0-2.2,

while A260/280 is 1.8-2.0 (Abdel-Latif and Osman 2017). Our extracted DNA concentration averages 82.63 ng/nL, while its purity ranges from 1.88-2.22 (A260/230) and 1.749-2.501 (A260/280). Therefore, from 30 honey samples, only one sample from Tempuran Village has high organic matter and RNA content contamination, and 29 other samples have good purity.

Molecular detection of entomopathogens

Molecular detection was performed using conventional PCR methods; all the primers used in this study were described in Table 2 earlier. First, the amplicon was visualized using agarose gel electrophoresis, as shown in Figure 2**.** Positive results in PCR are then further analyzed by sequencing to confirm the species.

Table 3. Environmental description of each apiary that supplies honey samples

Apiaries location	Hive number	Number of frames	Source of nectar	Varroa infestation	Pesticide poisoning	Altitudes (MASL)a	Annual mean temp. (°C)b	Annual precipitation (mm)b
Bali								
Manukaya Village 1	3	7	Multifloral	No	Yes	543	25.1	2413.8
Manukaya Village 2	5	7	Multifloral	No	No	546	25.1	2413.8
Manukaya Village 3	5	7	Multifloral	No	No	547	25.1	2413.8
Yeh Sumbul Village 1	20	8	Multifloral	Yes	No	29	26.4	1839.5
Yeh Sumbul Village 2	100	8	Monofloral	Yes	Yes	30	26.4	1839.5
Yeh Sumbul Village 3	40	8	Multiflora	Yes	No	61	26.4	1839.5
Java								
Pending Village	200	8	Monofloral	Yes	Yes	82	27.3	2296.8
Watuaji Village	200	8	Monofloral	Yes	No	380	27.5	2702
Suwatu Village	200	8	Monofloral	Yes	No	121	27.5	2702
Lahar Village	200	8	Monofloral	Yes	No	210	27.6	1742.5
Jollong Village	200	8	Monofloral	Yes	No	641	27.5	2702
Bategede Village	200	8	Monofloral	Yes	No	446	27.5	2702
Merbuh Village	140	9	Multifloral	Yes	No	287	25.5	2522.9
Tempuran Vilage	140	9	Monofloral	Yes	No	676	25.5	2522.9
Candiroto Village	150	6	Multifloral	Yes	Yes	747	25.8	3423.6
Sukawening Village	150	6	Multifloral	Yes	Yes	226	25.5	2522.9
Sumatra								
Suka Maju Village	50	7	Multifloral	Yes	Yes	32	28.1	2576.5
Sinar Wajo Village	200	9	Monofloral	Yes	Yes	28	28.1	2576.5
Bernai Village	200	9	Monofloral	Yes	Yes	53	28.1	2576.5
Rantau Karya Village	400	8	Multifloral	Yes	Yes	8	28.1	2871.6

Note: [a] = Altitude of each location was measured using "My Elevation" apps; [b] = Monthly temperature and precipitation data were collected from climatecharts.net by Zepner et al. (2020), using CRU Time Series v 4.05 dataset (Harris et al. 2021) as their data reference. The average value was calculated using data from 2009 to 2019

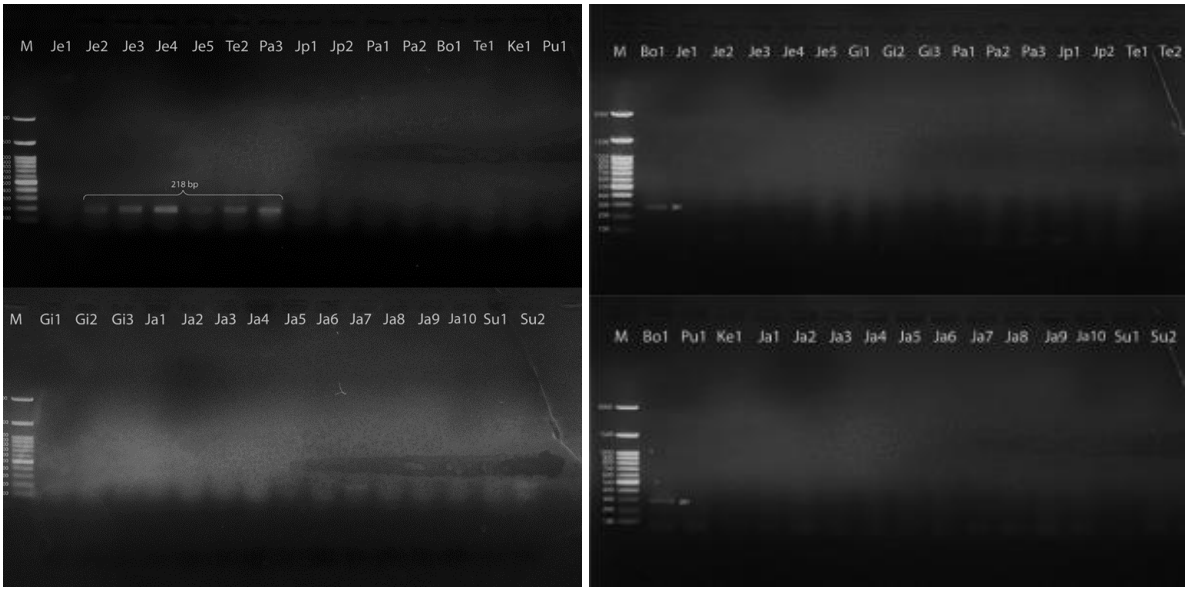

Figure 2. PCR results from each honey sample were amplified using 218MITOC primer (*left*) and MeliFOR/REVa (*right*). Note: M (Marker), sample codes Gi1 (Manukaya 1), Gi2 (Manukaya 2), Gi3 (Manukaya 3), Je1 (Yeh Sumbul 1), Je2-Je3 (Yeh Sumbul 2), Je4-Je5 (Yeh Sumbul 3), Pa1 (Lahar), Pa2 (Jollong), Pa3 (Bategede), Jp1 (Watuaji), Jp2 (Suwatu), Te1 (Tempuran), Te2 (Candiroto), Pu1 (Pending), Ke1 (Merbuh), Bo1 (Sukawening), Ja1-Ja6 (Suka Maju), Ja7-Ja10 (Sinar Wajo), Su1 (Bernai), and Su2 (Rantau Karya)

The results of molecular detection show that *Nosema ceranae* was found in 6 out of 30 samples, or 20% prevalence among the studied sample. Six positive samples came from Yeh Sumbul Village (Je2, Je3, Je4, and Je5), Candiroto Village (Te2), and Jollong Village (Pa3). That means *N. ceranae* is found in Bali and Java, while no such pathogen is found in Sumatra. *M. plutonius* is only found in Sukawening Village (Bo1) in Java and not in Bali or Sumatra. *P. larvae* and *N. apis* are not found anywhere in the studied area. The number of positive PCR results is divided by the total samples for each area to calculate the prevalence of each pathogen (Figure 3).

Sequence analysis

Honey samples from Yeh Sumbul Village, Jollong Village, and Candiroto Village showed a band measuring 218 bp while analyzed with 218MITOC primer; this sample was suspected of containing DNA from *N. ceranae*. In comparison, the honey sample from Sukawening Village was the only sample to show a band measuring 281 bp when amplified with MeliFOR/REVa primer. Therefore, the amplicon from these samples was sequenced to confirm the species and compared with other available sequences using BLASTn (Table 4).

Environmental

Statistical analyses were conducted for environmental factors, including altitudes, mean temperature, precipitation, parasites (*Varroa* sp.), source of nectar, and pesticide poisoning, and also for beekeepers' management factors, including the number of hives in one apiary and number of the frames in each hive (Table 5).

Phylogenetic analysis

Phylogenetic analyses were performed using the ML algorithm by comparing the sequence retrieved from this research with sequences from the same species in the NCBI database. We also included outgroup species for the phylogenetic tree (Figure 4).

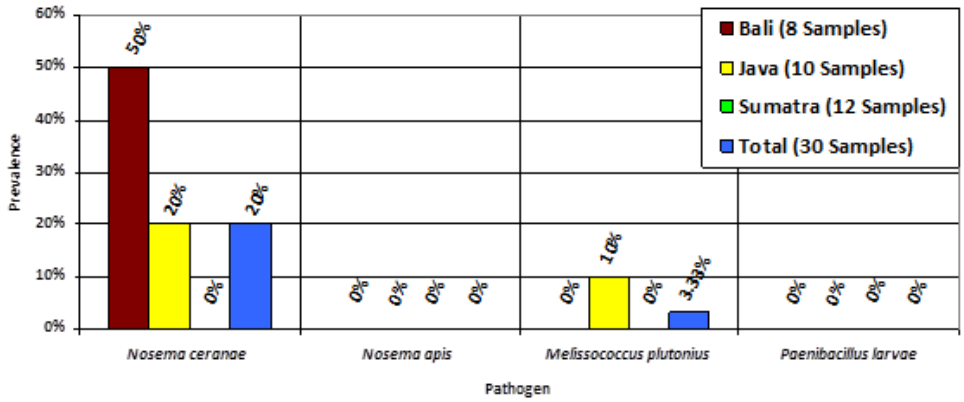

Figure 3. Prevalence of *Nosema apis, N. ceranae, Melisococcus plutonius*, and *Paenibacillus larvae*

Table 4. BLASTn result of Candiroto Village (Te2), Yeh Sumbuh Village (Je3), and Sukawening Village (Bo1) honey sample

Scientific Name	Accession Number	Max Score	Total Score	Query Cover	E-value	Percent Identity
Candiroto (Te2)						
Nosema ceranae	ON596641.1	409	409	100%	10^{-109}	100%
Nosema ceranae	MN715145.1	409	409	100%	10^{-109}	100%
Nosema ceranae	MN715144.1	409	409	100%	10^{-109}	100%
Nosema ceranae	MN715143.1	409	409	100%	10^{-109}	100%
Nosema ceranae	MN715142.1	409	409	100%	10^{-109}	100%
Yeh Sumbul (Je3)						
Nosema ceranae	OP081021.1	401	401	100%	$2x10^{-107}$	99.55%
Nosema ceranae	OP081020.1	401	401	100%	$2x10^{-107}$	99.55%
Nosema ceranae	OP081019.1	401	401	100%	$2x10^{-107}$	99.55%
Nosema ceranae	OP081018.1	401	401	100%	$2x10^{-107}$	99.55%
Nosema ceranae	OP081017.1	401	401	100%	$2x10^{-107}$	99.55%
Sukawening (Bo1)						
Melissococcus plutnoius	AP021885.1	475	1897	100%	10^{-129}	100%
Melissococcus plutnoius	AP019526.1	475	1897	100%	10^{-129}	100%
Melissococcus plutnoius	AP018524.1	475	1902	100%	10^{-129}	100%
Melissococcus plutnoius	AP018492.1	475	1902	100%	10^{-129}	100%
Melissococcus plutnoius	CP006683.1	475	1902	100%	10^{-129}	100%

Table 5. The statistical value of each factor shows their significant correlation to the emergence of pathogens

Factors		*Nosema ceranae*		*Melissococcus plutonius*	
		Statistical value	**Annotation**	**Statistical value**	**Annotation**
Environmental	Varroa infestation	0.254*	NS	1*	NS
	Source of Nectar	0.641*	NS	1*	NS
	Pesticide poisoning	0.660*	NS	1*	NS
	Altitude	0.125**	NS (p = 0.507)	0.119**	NS (p = 0.532)
	Mean temperature	-0.325**	NS (p = 0.079)	-0.189**	NS (p = 0.316)
	Precipitation	-0.382**	S (p = 0.037)	0.320**	NS (p = 0.084)
Beekeeping management	Number of hives	-0.146**	NS (p = 0.439)	0.043**	NS (p = 0.819)
	Number of frames per hive	0.212**	NS (p = 0.260)	-0.177**	NS (p = 0.349)

Note: *= p-value; **= r value; NS= Not Significant; S= Significant

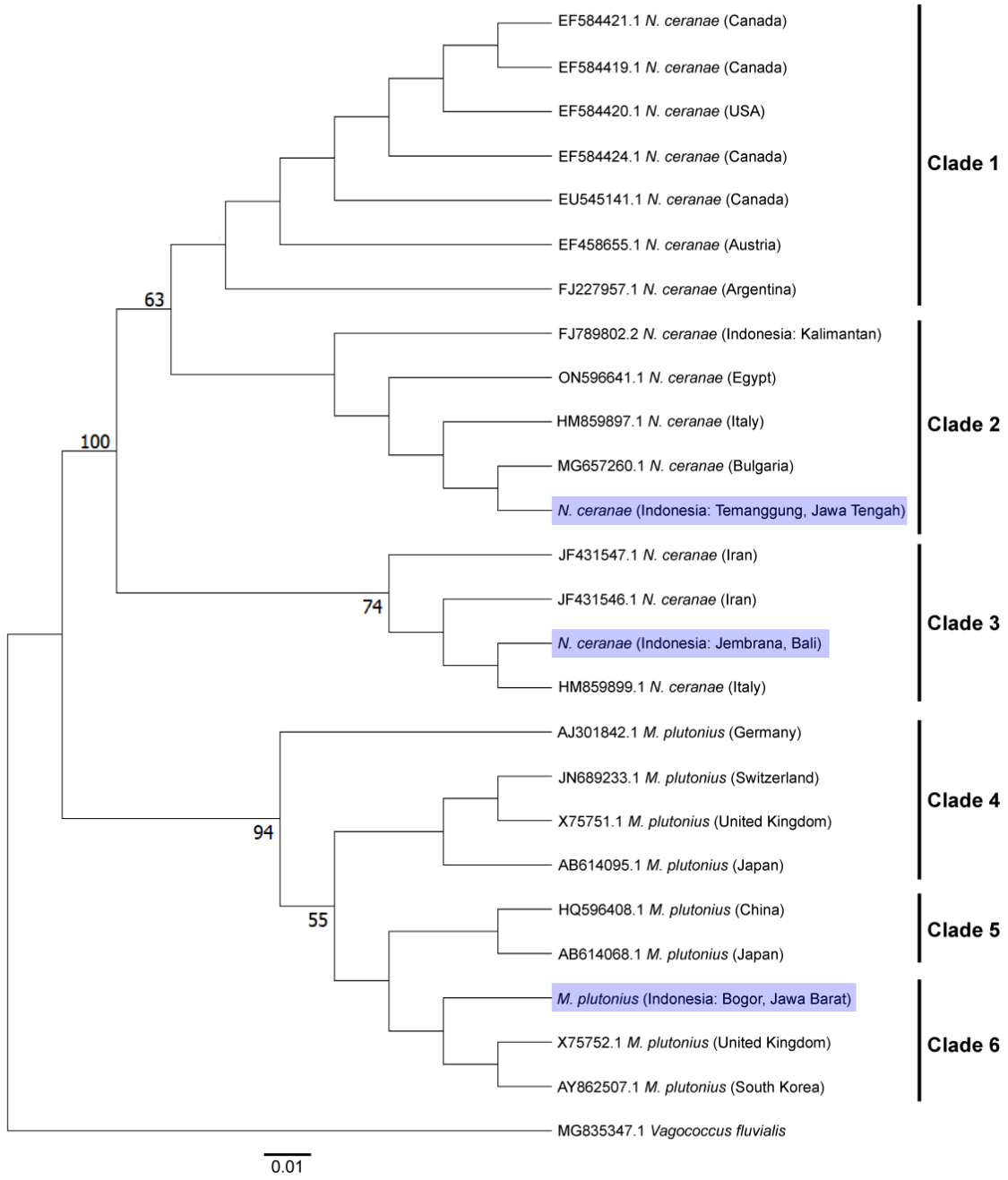

Figure 4. Phylogenetic tree of *N. ceranae* and *M. plutonius* based on Maximum-Likelihood (ML). The sequence inside the blue box indicates the sequence retrieved in this research, while other sequences were retrieved from the NCBI database.

Table 6. Genetic distance matrix between clades

	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6
Clade 1	-					
Clade 2	0.017 (0.000-0.029)	-				
Clade 3	0.037 (0.021-0.045)	0.024 (0.020 - 0.031)	-			
Clade 4	0.916 (0.897-0.945)	0.916 (0.897 - 0.945)	0.862 (0.838 - 0.881)	-		
Clade 5	0.930 (0.972-0.909)	0.929 (0.897 - 0.972)	0.873 (0.838 - 0.904)	0.005 (0.002 - 0.010)	-	
Clade 6	0.980 (0.953-1.048)	0.969 (0.927 - 1.048)	0.888 (0.802 - 0.928)	0.002 (0.000 - 0.008)	0.003 (0.000 - 0.004)	-

The phylogeny trees of *N. ceranae* and *M. plutonius* show a relationship between the sequences obtained in this study and other sequences available in the database. The tree is divided into three more branches, separating *N. ceranae*, *M. plutonius*, and the outgroup (*Vagococcus fluvialis*). Furthermore, it is known that the sample of *N. ceranae* from Temanggung is similar to the sample from Bulgaria, while *N. ceranae* from Jembrana is similar to the sequence from Italy. The *M. plutonius* obtained from Bogor is similar to sequences from the United Kingdom and South Korea. The Phylogeny tree formed 6 clades; clade 1-3 belongs to *N. ceranae*, while clade 4-6 belongs to *M. plutonius*. Genetic distance between clades was calculated between each clade (Table 6). Genetic distance shows the base difference and variation between clades.

Discussion

The population of *A. mellifera* (European honey bees) faces various threats from climate change, pesticide poisoning, parasites, and pathogens (FAO 2018). Pathogens contribute to 13% of honey bees' significant threats; the most common diseases are nosemosis and foulbrood (FAO 2018). Nosemosis was caused by *N. apis* or *N. ceranae*, while foulbrood was caused by *M. plutonius* (European Foulbrood) or *P. larvae* (American Foulbrood) (Bailey and Ball 1991).

Detection of this disease is usually carried out using bees for analysis. However, a recent study has shown honey has the potential to be utilized as a source of environmental DNA (Salkova et al. 2018). Honey could contain DNA from honey bees, pollen, or microorganisms, including the microorganisms that infect honey bees. This allows the detection of diseases in honey bees using honey samples. Several studies have successfully utilized honey for disease detection, such as Salkova et al. (2018) in Bulgaria and Ribani et al. (2020) in Europe, Asia, Oceania, Africa, and South America. However, no recent studies on disease detection in Indonesian honey bees exist. The latest study was conducted by Botías et al. (2012), which investigated the presence of *N. ceranae* in Asia and Oceania.

In this study, we successfully extract DNA from 30 honey samples from Bali (n=8), Java (n=10), and Sumatra (n=12). Honey samples from Sumatra were harvested by beekeepers and shipped to the Laboratory of Entomology, Universitas Gadjah Mada. These samples were collected from one apiary, meaning the samples come from several beehives. Java honey samples are pre-harvested as honey stocks, except for samples from Pending Village. Therefore, this sample could have been a mixture of several apiaries in the same area. Meanwhile, samples from Pending Village were harvested from one apiary in that region. Honey samples from Bali were collected under different conditions. Samples from Yeh Sumbul Village were collected directly when harvesting in one apiary. Meanwhile, samples from Manukaya Village came from one randomly selected beehive in each apiary. This collection state also follows the study conducted by Ribani et al. (2020). Therefore, honey samples are suitable for monitoring pathogens' presence and health conditions at the apiary level or surrounding area but not for single colony inspection. This is due to honey samples derived from a mixture of several beehives, as mentioned earlier.

Extracted DNA from honey samples was further analyzed for pathogen detection. We amplified DNA using a qualitative single PCR reaction using a specific primer targeting each pathogen. This preliminary study aimed to evaluate honey's use in pathogens detection and calculate the prevalence of each pathogen in Bali, Java, and Sumatra. This information will be useful for the global monitoring of specific pathogens in Indonesia. However, this study has limitations in the number of samples, so it cannot adequately represent epidemiological conditions.

This study used five sets of primers: four primer pairs to detect four different pathogens and one primer (LR13107-F/LR12467-R) to detect the 16S rRNA sequence from *A. mellifera*. This primer was designed to determine stingless bee species (Thummajitsakul et al. 2013), but it can also be used to detect *A. mellifera* sequences with the product of 496 bp. Detection of *A. mellifera* sequences aims to ensure that the extracted DNA from honey is good quality and feasible for the PCR reaction. That is because the DNA of honey bees will always be carried into the honey they produce (Ribani et al. 2020). This evaluation is necessary because honey has a low content of DNA and is mainly composed of sugars (>80%), minerals, vitamins, organic acids, amino acids, and protein (Samarghandian et al. 2017). This content and secondary metabolites from

pollen could inhibit the PCR reaction (Soares et al. 2015). Nevertheless, the positive result of the PCR reaction using this primer indicates that the PCR reaction is working well.

In this study, we successfully detected *N. ceranae* and *M. plutonius*, while *Paenibacillus larvae* and *N. apis* were not detected. As expected, *P. larvae* are undetected in Java, Bali, or Sumatra. Instead, *P. larvae* are distributed worldwide in temperate and sub-tropic regions (Bailey and Ball 1991). In Asia, these pathogens can be found in Japan, China, Taiwan, India, Turkey (Chantawannakul et al. 2015), and Jordan (Nizar et al. 2015). Meanwhile, this disease has never been reported in Southeast Asia, including Indonesia. That is in line with the findings in this study.

Melissococcus plutonius, the causative agent of European Foulbrood (EFB) disease, was expected not to be found in Indonesia. This pathogen was distributed globally wherever *A. mellifera* is kept (Forsgren et al. 2013). However, no reports have mentioned this pathogen's presence in Africa and several Middle Eastern countries (Beverley 2012). In Asia, *M. plutonius* was recently reported to be found in several Southeast Asian countries, such as Vietnam (Forsgren et al. 2014), Thailand, and Malaysia (Chantawannakul et al. 2015). Meanwhile, *M. plutonius* has never been reported to be found in Indonesia. In our study, we successfully amplified the PCR product of MeliFORa/MeliREVa primer pairs in 1 out of 30 honey samples. Samples contaminated with *M. plutonius* DNA came from Sukawening Village (Bogor, West Java, Indonesia). That suggests *M. plutonius* has spread in Indonesia despite its very low prevalence of 3.33%. This finding is somewhat expected because M. plutonius recently began to spread to Southeast Asia, including Malaysia, which is directly adjacent to Indonesia. The phylogenetic tree reveals that the *M. plutonius* sequence found in Sukawening Village (Bo1) is closely related to the sequence from the United Kingdom (X75752.1) and South Korea (AY862507.1). This may indicate that *M. plutonius* in Indonesia came from *A. mellifera* honey bees imported from Europe or South Korea. Even though it still needs more investigation to determine where the pathogen came from. This can also be an early warning for beekeepers and regulators to monitor honey bees' transport between regions. The phylogeny tree also shows that the genetic distance between the clades *of M. plutonius* is very low (0.002-0.005), which means there are only 2 to 5 different bases every 1000 bases. According to Vetrovsky and Baldrian (2013), the mean sequence similarity for intraspecies 16S rRNA variation is 0-1%. Therefore, all *M. plutonius* sequences studied in this research are still considered one species.

Nosema ceranae, a microsporidian that causes nosemosis, was expected to be found in Indonesia. Its host, *Apis cerana* Fabricius 1793, is the most commonly reared *Apis* species among Indonesian beekeepers (Buchori et al. 2022). Although its primary host is *A. cerana*, this pathogen *N. ceranae* also infects *A. mellifera* (Higes et al. 2006). The prevalence of *N. ceranae* in *A. mellifera* continues to increase because *N. ceranae* is considered to have a higher virulence and cause more severe damage to a colony than *N. apis* (Martín-Hernandez et al. 2007). *N. ceranae* is distributed widely across Asia, Australia, and Oceania. In Indonesia, *N. ceranae* was previously reported to be found in Sulawesi and South Kalimantan, Indonesia (Botías et al. 2012). This study successfully amplified the 16S rRNA locus from *N. ceranae* in 6 of 30 honey samples. All Sumatran honey appears to be uncontaminated with *N. ceranae*. Contaminated samples came from Yeh Sumbul Village (Jembrana, Bali, Indonesia), Jollong Village (Pati, Central Java, Indonesia), and Candiroto Village (Temanggung, Central Java, Indonesia). This result suggests that *N. ceranae* has a high prevalence on Bali Island (50%) and a lower prevalence on Java Island (20%). Phylogeny results show that *N. ceranae* found in Candiroto Village, Temanggung District (Te2), is incorporated in Clade 2 and is closely related to the sequence from Bulgaria. In contrast, the sequence from Yeh Sumbul Village, Jembrana District (Je3) is more closely related to *N. ceranae* from Italy in Clade 3. The genetic distance between the *N. ceranae* clade (Clade 1-3) is relatively high compared to *M. plutonius*. The average distance between clades 1 and 2 was 0.017 (1.7%), between clades 1 and 3 was 0.037 (3.7%), and between clades 2 and 3 was 0.024 (2.4%). High genetic variation may happen due to a host switch from *A. cerana* to *A. mellifera* (Higes et al. 2006), which might cause them to adapt to new hosts. Besides that, the distribution of *A. mellifera* worldwide causes *N. ceranae* to be exposed to various climatic conditions, which can enhance their genetic variation (Wei et al. 2022).

N. apis, the cause of nosemosis, was expected to be more prevalent than *N. ceranae* since the honey is extracted from the *A. mellifera* colony. *N. apis* was previously found in various areas in Indonesia, such as Semarang, Bogor, and Batang (Rice 2001; Maharani 2017). Research has shown that *N. apis* are less common than *N. ceranae* (Martín-Hernández et al. 2007; Emsen et al. 2016; Pacini et al. 2016). A host-switch event on *N. ceranae* precedes it initially only infected *A. cerana* but now also infects *A. mellifera* (Higes et al. 2006). *N. apis* is likely to be outmatched by *N. ceranae* due to virulence factors (Higes et al. 2010) accompanied by the ability of *N. ceranae* to grow throughout the year while *N. apis* only grow in certain seasons (Higes et al. 2010). In this research, we cannot amplify the 16S rRNA sequence of *N. apis* from 30 honey samples because the result indicates that *N. apis* have a 0% prevalence in Java, Bali, and Sumatra, Indonesia. However, this result is not absolute. Amplification failure is also influenced by several factors, such as primer specificity, annealing temperature, and failure to break down the cell/spore wall, so DNA is not extracted properly.

Environmental factors, such as annual temperature and climate, have been studied for their relationship to pathogenic infections in honey bees. Pacini et al. (2016) found lower temperature increases the prevalence of *N. ceranae* infection; a similar point was suggested by Ansari et al. (2017), who found that *N. ceranae* infection is more prevalent in cold climates. However, Ansari et al. (2017) state that these results are not evenly distributed and suggest that it's due to various unknown environmental

factors. In this study, we tried to analyze the relationship between ecological factors and beekeeper management practices to the incidence of pathogen infection. Environmental factors include annual mean temperature, annual precipitation, altitudes, parasites (*Varroa* sp.), sources of nectar, and pesticide poisoning. At the same time, beekeepers' management factors include the number of hives in one apiary and the number of frames in each hive. Statistical analysis shows that precipitation significantly correlates with the emergence of *N. ceranae*. In contrast, other factors are not significantly related to the pathogen emergence. Previous studies about precipitation and pathogen emergence were reported by Pacini et al. (2016) which showed no correlation between precipitation and the emergence of *N. ceranae* and by Rowland et al. (2021), which stated incidence of EFB was positively correlated with the rain volume. However, these results should be studied more intensively to ascertain the influence of a factor on the emergence of pathogens. We recommend conducting further intensive studies on several apiaries with more conditioned external factors in a certain area.

This is the first study using the molecular technique to detect major pathogens of *A. mellifera* in Indonesia. The results show that *P. larvae* and *N. apis* have not yet been found. *M. plutonius* was found in Indonesia for the first time and only in Java. In contrast, *N. ceranae* was found in the Java and Bali regions, with the highest prevalence in the Bali region. Further research is needed to provide a more comprehensive understanding of the prevalence of the diseases in Indonesia, especially outside Java, Bali, and Sumatra. More honey samples from different areas will better understand this pathogens' distribution. In addition, more intensive and detailed research can be carried out on the influence of environmental factors on the incidence of disease infection. For this purpose, we suggest conducting the study in fewer apiaries so that all environmental factors can be observed properly.

ACKNOWLEDGEMENTS

This research was funded by Final Assignment Recognition (RTA) 2022 batch 1 from Universitas Gadjah Mada, Yogyakarta, Indonesia. We also would like to thank beekeepers across Bali, Java, and Sumatra who provided honey samples for this research and the staff of the Laboratory of Entomology, Faculty of Biology, Universitas Gadjah Mada and Laboratory of Genetic Engineering, Biotechnology Research Center, Universitas Gadjah Mada who provided facilities during the study.

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