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RESEARCH PAPER

Molecular identification and characterization of *Maconellicoccus multipori* (Takahashi) (Hemiptera: Pseudococcidae) on *Piper nigrum* L.

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ABSTRACT

The mealybug *Maconellicoccus multipori* (Takahashi) was identified on black pepper (*Piper nigrum* L.) seedlings in a greenhouse in Bogor, West Java, using both molecular and morphological characterization. Two mealybug isolates were successfully amplified with primer pairs targeting the LCO region of the mitochondrial cytochrome oxidase I (COI) gene, yielding a 491 bp PCR product. The nucleotide sequences of both isolates (GenBank accession numbers LC666906 and LC666907) were identical. Phylogenetic analysis revealed that the Bogor isolates clustered closely with *M. multipori* populations from China and Thailand, with high sequence homology of 99.30% and 99.10%, respectively. Morphological observations of the adult female specimens further confirmed their identity as *M. multipori*, based on key diagnostic features including body size, antennal segmentation, cerarii pattern, and distribution of pores and ducts, which correspond to descriptions in established taxonomic keys. This study provides the first molecular characterization of *M. multipori* in Indonesia. The COI sequence data obtained enhances reference databases for DNA barcoding and strengthens early detection strategies for pest monitoring. These findings are crucial for supporting quarantine inspections, management, and control of *M. multipori* in black pepper nurseries and preventing its spread to other crops.

Key words: Black pepper, COI gene, female imago, mealybug, PCR

INTRODUCTION

Mealybugs (Hemiptera: Pseudococcidae), including species such as *Pseudococcus madeirensis* (Green), *Pseudococcus solani* (Cockerell) (Mazzeo et al., 1999), and *Pseudococcus comstocki* (Kuwana) (Pellizzari, 2005), are serious pests of crops and ornamental plants. Pest outbreaks involving mealybugs have also been reported in tropical regions (Halima-Kamel et al., 2015). In addition to their role as pests, certain mealybug species, such as *Ferrisia virgata* (Cockerell) and *Planococcus minor* (Maskell), serve as vectors for the piper yellow mottle virus (PYMoV), the primary cause of mottle disease in black pepper (*Piper nigrum* L.) plants (Miftakhurohmah et al., 2022a). *Maconellicoccus multipori* (Takahashi) is another notable quarantine pest (Williams, 1996).

Mealybugs are among the pests that attack black pepper plants. Nine species have been identified on black pepper in Indonesia: *Pl. minor*, *F. virgata*, *Pseudococcus jackbeardsleyi* (Gimpel & Miller), *Paracoccus marginatus* (Williams & Granara de Willink), *Pseudococcus longispinus* (Targioni Tozzetti), *Dysmicoccus brevipes* (Cockerell), *M. multipori*, *Paracoccus interceptus* (Lit), and *Phenacoccus parvus* (Morrison) (Balfas et al., 2002; Sartiami et al., 2008; Miftakhurohmah et al., 2022b; Morales et al., 2016). *Planococcus citri* (Risso) and *F. virgata* are found in Malaysia, while *Planococcus* sp., *Pl. citri*, *Pl. minor, Pseudococcus* sp., *Ps. longispinus,* and *Pseudococcus orchidicola* (Takahashi) are present in India (Devasahayam, 2000). Globally, *Formicoccus polysperes* (Williams), *Pseudococcus elisae* (Borchsenius), and *Pseudococcus orchidicola* (Takahashi) are known to attack black pepper plants (Morales et al., 2016).

In Bogor, West Java, Indonesia, mealybugs were recently found infesting black pepper seedlings plant. The mealybug were concentrated at the base of the stem internodes of seedlings in the greenhouse (Figure

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1A). This infestation has the potential to damage the seedlings. Accurate identification of the species is necessary to assess its pest status. Mealybug species identification can be challenging, particularly for males and nymphs, requiring significant experience and time (Beltrà et al., 2012). Additionally, their wax-covered bodies and subtle morphology make them difficult to identify based solely on visual observation. Molecular markers, such as DNA barcoding, offer a more rapid and accurate method of identification. Recent studies have employed Cytochrome Oxidase I (COI) gene barcoding to distinguish between closely related mealybug species, important for both economic and quarantine concerns (Ren et al., 2018). This study presents both morphological and molecular characteristics of the mealybug species found on black pepper in Indonesia, using primer pairs targeting the LCO region of the insect COI gene.

MATERIALS AND METHODS

Research Site. Mealybugs were collected from black pepper seedlings in the greenhouse of the Indonesian Spice and Medicinal Crops Research Institute (ISMECRI), Bogor, West Java, Indonesia (6°34′36″S, 106°47′06″E). Both, nymph and adult insects were collected using a wet brush and preserved either in absolute ethanol (for molecular identification) (Beltra et al., 2012) or in 70% ethanol (for morphological identification) (Malausa et al., 2011).

Molecular Characterization. Total DNA was extracted from individual nymphs or female adults using CTAB buffer (Rohimatun et al., 2024). Each insect was placed in an Eppendorf tube containing 100 µL of CTAB extraction buffer [1% (w/v) CTAB; 1 M NaCl; 100 mM Tris-HCl (pH 8.0); 20 mM EDTA (pH 8.0); 1% polyvinylpyrrolidone (PVP)] and crushed with a micro pestle. The mixture was incubated at 65 ºC for 45 min, cooled to room temperature, and extracted with 100 µL of chloroform:isoamyl alcohol (24:1). After vortexing for 10 sec, the mixture was centrifuged at 12,000 rpm for 5 min at room temperature. The aqueous (upper) phase was carefully transferred to a new tube, and DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and an equal volume of cold isopropanol. The tube was inverted gently until the solution was mixed and a DNA pellet became visible, then centrifuged at 12,000 rpm for 15 min. The supernatant was discarded, and the DNA pellet was washed twice with 70% ethanol, air-dried, and dissolved in 20–30 µL of nuclease-free water. DNA samples were stored at –20 ºC until use. DNA quantity and quality were assessed using an Implen NanoPhotometer (Germany).

PCR amplification targeted the LCO region of the mitochondrial cytochrome oxidase I (COI) gene using primer pairs LCO-M-2d-F (ATAACTATACCTATYATTATTGGAAG) and LCO-M-2d-R (AATAAATGTGATATAAAATTGG), yielding an expected fragment size of approximately 491 bp (Malausa et al., 2011). Each 20 µL PCR reaction contained 10 µL of 2× MyTaq HS Red mix (Bioline, Germany), 0.4 µM of each primers, 1 µL of DNA template, and nuclease-free water. Amplification was conducted in a Labcycler Gradient PCR machine (SensoQuest, Germany) under the following conditions: initial denaturation at 95 ºC for 5 min, 30 cycles of denaturation at 95 ºC for 1 min, annealing at 48 ºC for 15 sec, and extension at 72 ºC for 1 min, followed by a final extension at 72 ºC for 10 min. Nuclease-free water served as a negative control. PCR products were visualized by electrophoresis on a 1.5% agarose gel

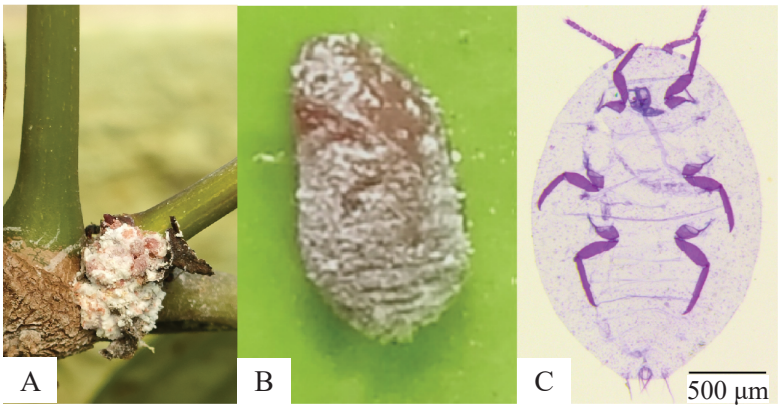

Figure 1. *Maconellicoccus multipori* (*Takahashi*). A. Colonies at the base of black pepper (*Piper nigrum* L.) stem segment. B. Adult female of *M. multipori.* C. Slide-mounted specimen of the adult female of *M. multipori*.

in 0.5× TAE buffer, stained with RedSafe™ (Intron Biotechnology, South Korea), and documented using a GelDoc Fire Reader V4 (Uvitec Cambridge).

PCR products were sequenced by First Base Laboratory (Selangor, Malaysia). Sequence data were initially analyzed using the BLAST tool (Altschul et al., 1990). The sequences were edited, aligned, and compared with related sequences from GenBank using Bioedit. Sequence alignments was visualized with GeneDoc. Phylogenetic analysis was conducted using the neighbor-joining method with 1000 bootstrap replicates in MEGA X software (Kumar et al., 2018).

Morphological Characterization. Morphological identification was performed to support the molecular characterization results, following the keys and methods described by Williams & Watson (1988) and Williams (2004). Eleven adult female mealybugs were selected for slide mounting. The specimens were placed in a Ciracus cup containing 6–8 mL of Essig's fluid and one drop of chloroform. Each mealybugs was pricked dorsally (submarginal area) and stained with 1–2 drops of acid fuchsin. The samples were heated at 60–70 ºC for 15–30 min and then cooled. Internal contents were gently removed with a fine brush. The mealybugs were then transferred to a fresh Ciracus cup containing Essig's fluid and chloroform and cleaned again until transparent. Finally, the specimens were mounted on microscope slides using Heinz mounting medium, covered with a cover glass, and dried at 60 ºC for 5 min.

RESULTS AND DISCUSSION

Molecular Characterization. DNA amplification of the Bogor mealybugs samples using the LCO-M-2d primer pair successfully yielded the expected target band of approximately 491 bp (data not shown), consistent with the predicted amplicon size (Malausa et al., 2011). Previously, this primer set has been used successfully to identify *F. virgata, Pl. minor*, and *Pa. marginatus* (Miftakhurohmah et al., 2022b). Preliminary BLAST analysis of the sequencing results identified the Bogor samples as *M. multipori*. Consequently, phylogenetic analysis was conducted. In the resulting phylogenetic tree, the two Bogor isolates clustered with *M. multipori* isolates from Thailand and China, clearly separated from other Pseudococcidae species such as *Ferrisia malvastra*, *Pl. citri, Planococcus ficus*, *Pseudococcus cryptus*, and *Nipaecoccus bromelicola*. Two isolates of *Nilaparvata lugens* (Hemiptera: Delphacidae) served as outgroups and formed a distinct clade (Figure 2).

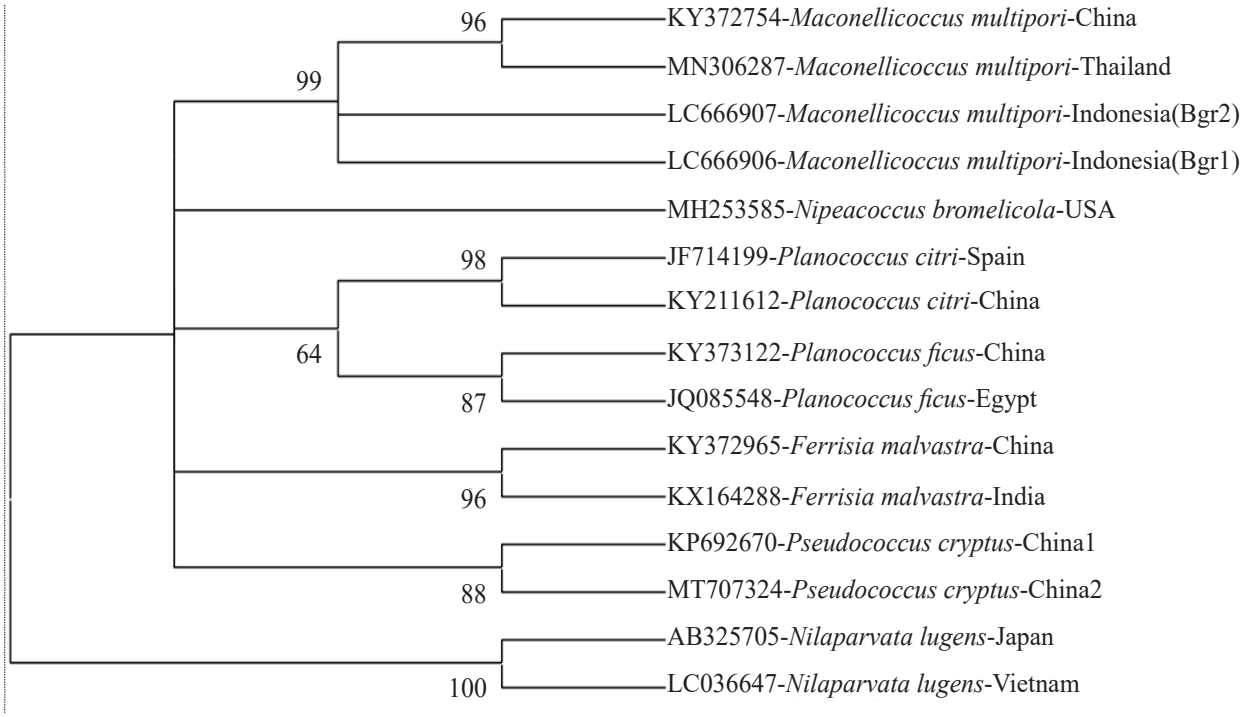

Figure 2. Phylogenetic tree of two *Maconellicoccus multipori* (*Takahashi*) isolates from Bogor, along with *M. multipori* isolates from China and Thailand, and several Pseudococcidae species [*Ferrisia malvastra* (McDaniel), *Planococcus citri* (Risso), *Planococcus ficus* (Signoret), *Pseudococcus cryptus* (Hempel), and *Nipaecoccus bromelicola* (Ellenreider, Watson & Kinnee)]. The tree was constructed with 1000 bootstrap replicates. Two isolates of *Nilaparvata lugens* (Stål, 1854) were used as outgroups.

The sequences of the Bogor *M. multipori* isolates have been deposited in GenBank under accession numbers LC666906 and LC666907.

The COI (HCO-LCO region) nucleotide sequences of the two Bogor isolates were identical and exhibited high sequence similarity with Chinese and Thai isolates, showing 99.30% and 99.10% identity, respectively. Nucleotide divergence between the Bogor and Chinese isolates occurred at three positions: 25, 469, and 472. Divergence with the Thai isolates occurred at four positions: 25, 253, 472, and 481. At positions 25 and 472, both Chinese and Thai isolates contained thymine (T), while the Bogor isolates had adenine (A). At positions 253, 469, and 481, the Bogor isolates had thymine (T), while the Chinese or Thai isolates had cytosine (C) (Figure 3).

Morphological Characterization. Observations of the adult specimens revealed that the live adult female had a pink body covered with white wax (Figure 1B). Slide-mounted specimens measured 1304-2119 µm in length and 959–1538 µm in width (Figure 1C). The antennae were 9-segmented and measured 290–366 µm in length. Four pairs of cerarii were present only on the posterior abdominal segments, each bearing two conical setae measuring 10–14 µm. The anal ring measured 60–79 µm in width, with six setae measuring 82–135 µm in length). On the ventral surface, multilocular disc pores measured 6–9 µm in diameter, and trilocular pores were evenly distributed. Several discoidal pores were also observed. Wide and conspicuous oral rim tubular ducts (9–14 µm) were present, noticeably larger than trilocular pores (3–4 µm) (Figure 4A-4L). The legs were well developed, with the hind trochanter and femur measuring 138–232 µm, the hind tibia and tarsus measuring 160–259 µm, the claw measuring 20–39 µm. The ratio of tibia+tarsus to hind trochanter+femur was 1.02–1.23, while the tibia-to-tarsus length ratio was 1.68–2.69. Translucent pores were present on both the hind femur and tibia (Figure 5). These characteristics are consistent with the taxonomic descriptions of *M. multipori* by Williams (1996) and Miller (1999).

Currently, two species of *Maconellicoccus* are known in Indonesia: *M. hirsutus* and *M. multipori* (García-Morales et al., 2016; Zarkani et al., 2021). *M. hirsutus* can be distinguished from *M. multipori* by the absence of a circulus (present in *M. multipori*) and by the variable presence of dorsal oral collar tubular ducts (Williams, 2004).

PCR-based molecular identification using the COI gene in this study confirmed the identity of the Bogor samples as *M. multipori*. This identification was strongly supported by the corresponding morphological characteristics. Recently, DNA barcoding and barcode index numbers (BNI) have been increasingly used to accelerate quarantine inspections and detect non-native mealybugs (Ren et al., 2018). However, the utility of this approach for mealybugs remains limited due to a lack of reference sequence data. Although, 154 species of mealybugs have been documented in China, many,—including *M. multipori*—lack sufficient genetic data (Wang et al., 2016). Previous studies (Ren et al., 2018) also noted that, unlike *M. hirsutus*, *M. multipori* has relatively sparse genetic data in reference databases.

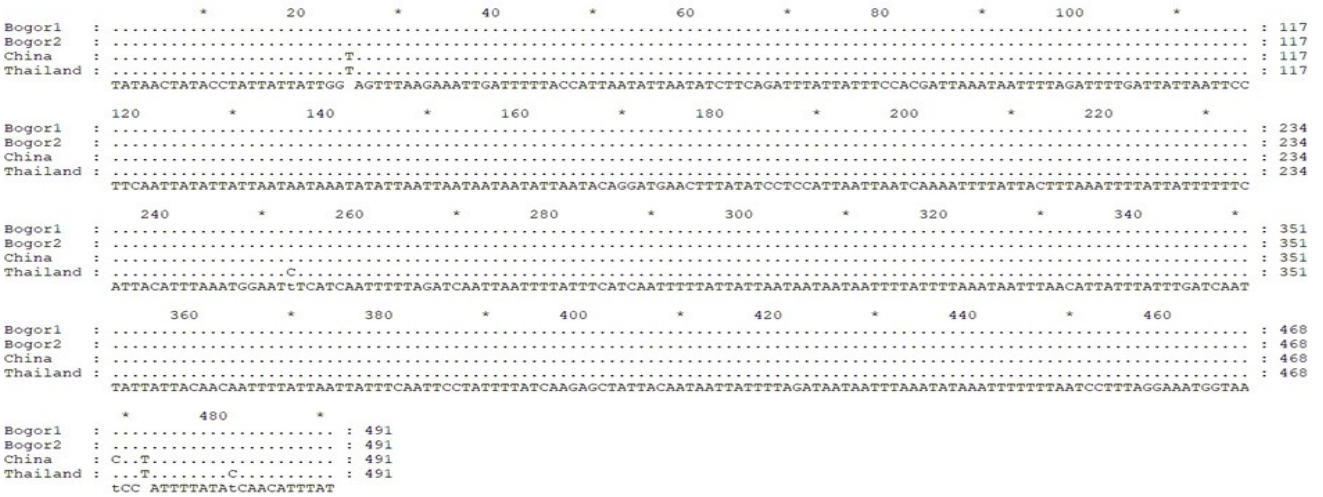

Figure 3. Alignment of the nucleotide sequences of the COI (HCO-LCO) regions from two *Maconellicoccus multipori* isolates from Bogor (GenBank accession numbers LC666906 and LC666907) with Chinese (KY372754) and Thai (MN306278) isolates, generated using the GeneDoc program. Dots indicate conserved nucleotides.

In this study, the COI sequences of the two Bogor isolates successfully confirmed the identity of *M. multipori* infesting black pepper seedlings in Bogor. This is the first report describing the molecular characteristics of *M. multipori* in Indonesia. These data will contribute valuable reference sequences to support COI-5P barcode databases and validate the BIN technique for identifying *Maconellicoccus* species, particularly *M. multipori*. Accurate molecular identification also supports early detection efforts, which are critical for control strategies and for assessing genetic diversity of the species.

In Indonesia, *M. multipori* has previously been reported on soursop (*Annona muricata*) (Sartiami et al., 1999), *P. nigrum*, *Crypteronia griffithii, Daemonorops* sp., and *Neonauclea* sp. (Williams, 2004). Outside of Indonesia, it has been reported on black pepper in Thailand and on *Piper betle* in India (Williams, 1996; Williams, 2004; García-Morales et al., 2016). However, no prior studies have documented the damage caused by *M. multipori* to these host plants. In this study, *M. multipori* appears to pose a potential threat to black pepper seedling production in greenhouses and shade nurseries. As black pepper seedlings are commonly propagated in shaded nurseries, management of this pest is necessary to minimize damage, prevent spread to other economically important crops, and safeguard export commodities.

CONCLUSION

The mealybug infesting black pepper seedlings in a greenhouse in Bogor was successfully identified as

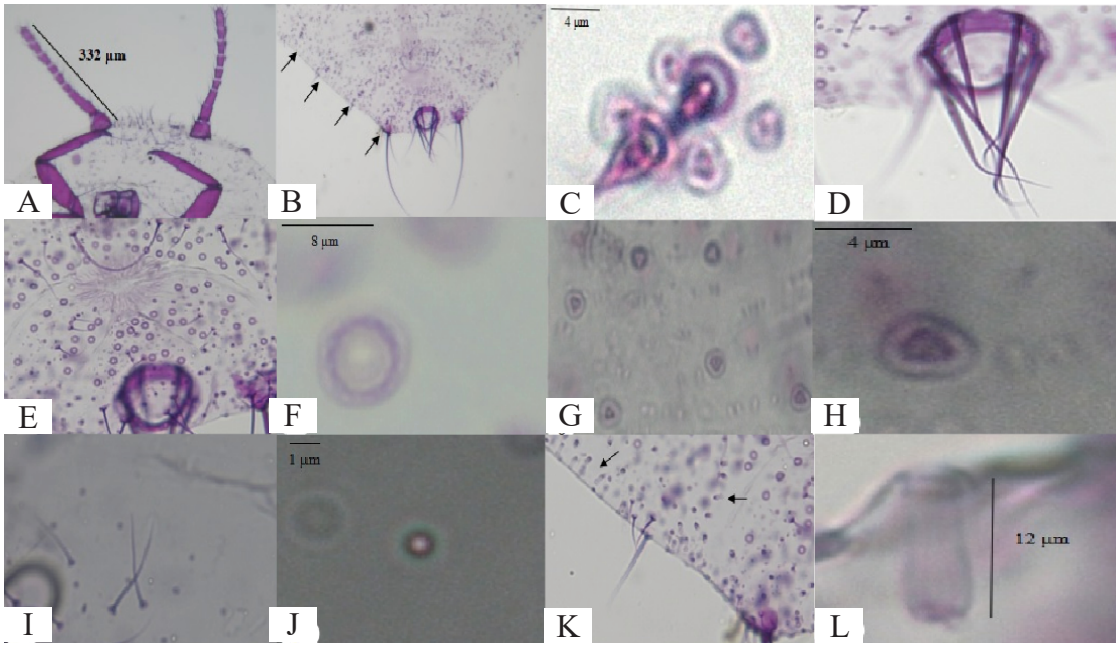

Figure 4. Slides -mounted morphological characters of *Maconellicoccus multipori* (*Takahashi*). A. Antenna with 9 segments; B. Cerarii with 4 pairs; C. Conical setae; D. Anal ring; E, F. Multilocular disc pores; G, H. Trilocular pores; I, J. Discoidal pores; K, L. Oral rim tubular ducts.

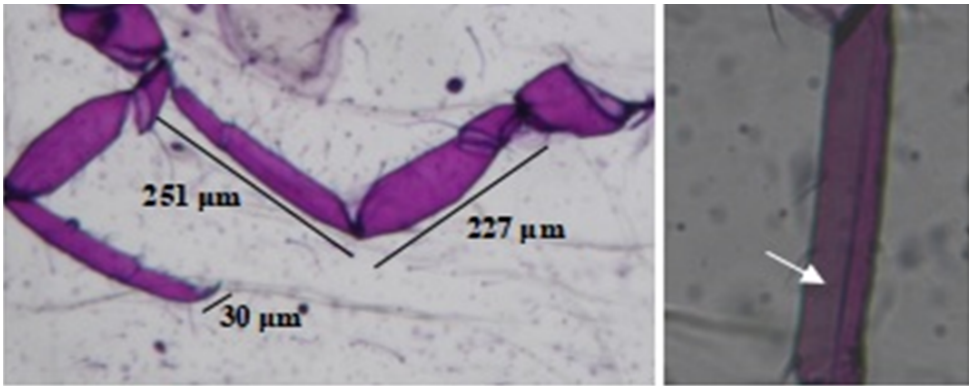

Figure 5. Hind leg (left) and translucent pores on hind femur and hind tibia (right) of *Maconellicoccus multipori* (Takahashi).

Maconellicoccus multipori through molecular analysis of the COI gene region. Morphological examination of adult female specimens corroborated the molecular identification. The COI sequences of the Bogor isolates were identical and showed high similarity to isolates from China and Thailand. These COI sequences are expected to enhance early detection methods for *M. multipori* and support efforts in monitoring and management of this species.

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AUTHORS' CONTRIBUTIONS

TLM and DS considered and planned the experiment and worked on morphological identification. TLM prepared the manuscript. M as the leader of molecular identification activities and analyzed molecular data. SRD carried out the data interpretation. R performed on collected mealybugs on the black pepper plant and morphological identification. SH collected mealybugs and worked on DNA isolation. RH, WA and S performed DNA isolation and PCR of insect samples. The final manuscript has been read and approved by all of the authors.

COMPETING INTEREST

The authors declare that they have no competing interests.

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