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Molecular Identification of Root-Knot Nematodes Associated with Yellowing Symptoms in Black Pepper (*Piper nigrum* L.) on Bangka Island, Indonesia

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ABSTRACT

Root-knot nematodes (*Meloidogyne* spp.) are important pathogens of black pepper (*Piper nigrum*). They are often associated with yellowing disease, yet accurate species identification in Bangka Belitung Province, Indonesia, remains limited. This study aimed to identify *Meloidogyne* species from yellowing pepper plants in Petaling and Sungkap three plantations per location (sampled purposively without replication) using species-specific polymerase chain reaction (PCR) followed by sequencing of the SCAR marker (*M. incognita*) and the internal transcribed spacer (ITS) rDNA (*M. arenaria*). DNA was extracted from female nematodes dissected from root galls and amplified with species-specific primers Finc/Rinc (*M. incognita*) and FrDNA2/RrDNA1 (*M. arenaria*). *Meloidogyne incognita* and *M. arenaria* were identified in both locations. Primers Finc/Rinc amplified a fragment of approximately 900 bp from *M. incognita*, while primers FrDNA2/RrDNA1 amplified approximately 450 bp from *M. arenaria*. The *M. Incognita* SCAR sequences shared 99.4–100% similarity with isolates from other countries deposited in GenBank, whereas the *M. arenaria* ITS sequences were identical between locations (100%) and shared 99,7–100% similarity with isolates from Indonesia and China. These results show that *M. incognita* and *M. arenaria* occur in symptomatic pepper plants with root galls in Bangka and provide a molecular basis for nematode detection.

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INTRODUCTION

Black pepper (*Piper nigrum* L.) is an important spice crop and a major source of income for smallholders on Bangka Island, Indonesia. The province contributes about 39% of national pepper production (Kementan, 2025). However, the pepper area has declined sharply. BPS reported that the provincial area fell from 38,684 ha in 2023 to 28,097 ha in 2024 (BPS 2025). Yellowing disease is one of the most damaging constraints on its production in the region. The disease results from interactions among several factors, including the nematodes *Radopholus similis* and *Meloidogyne incognita*, the fungi *Fusarium solani* and *F. oxysporum*, and soil fertility and moisture. According to Mustika (1990), nematodes are the

main cause of yellow disease, while other factors exacerbate the condition of the plant.

Root knot nematodes (*Meloidogyne* spp.) are important pathogens of pepper and are consistently associated with yellowing disease. Species-level identification matters because *Meloidogyne* species differ in host range, pathogenicity, and response to control measures. Knowing which species occur in plantations is therefore needed to confirm the nematode component of the disease complex, to monitor how species spread between plantations, and to design management such as resistant cultivars, clean planting material, and crop rotation. Without species-level data, control recommendations for pepper in Bangka rest on assumptions rather than confirmed identifications.

Conventional identification techniques often include morphology and morphometry, host range, and isoenzyme phenotype. Morphological identification is relatively time-consuming and requires specialized expertise and skills. Identification techniques using molecular characters have helped nematode taxonomists identify nematode species more quickly and accurately.

Molecular methods allow faster and more accurate identification. These include PCR, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), sequence characterized amplified region (SCAR) markers, multiplex PCR, and amplified fragment length polymorphism (AFLP), and they have been used successfully for many *Meloidogyne* species. DNA-based identification has used mitochondrial DNA (Harris et al., 1990; Powers & Harris, 1993), ribosomal DNA (rDNA) (Zijlstra et al., 1995), and RAPD markers (Cenis, 1993). Williamson et al. (1997) identified *M. hapla* and *M. chitwoodi* using SCAR-specific primers, amplifying DNA from larvae prepared by the proteinase K method. Zijlstra (2000) used nested PCR with SCAR primers to identify *M. hapla*, *M. chitwoodi*, and *M. fallax*. In the same year, Zijlstra et al. (2000) used three pairs of specific primers to identify *M. incognita*, *M. arenaria*, and *M. javanica*, and these primers are useful for routine identification in nematology laboratories. Meng et al. (2004) designed SCAR-specific primers to identify *Meloidogyne* species from larval DNA extracts. Karajeh et al. (2010) identified *M. incognita*, *M. javanica*, and *M. arenaria* using SCAR-PCR and RAPD-PCR on DNA extracted from eggs and females.

Despite these advances, the *Meloidogyne* species associated with yellowing pepper on Bangka Island have not been characterized at the molecular level. Without molecular confirmation, it remains uncertain which species are present in pepper plantations, whether more than one species occurs, and how local populations relate to those reported elsewhere. PCR with specific primers directly detects target genes, and variation in the size or sequence of the amplified products can be used to characterize populations and infer relationships among isolates.

This study, therefore, aimed to identify the *Meloidogyne* species associated with yellowing pepper in Petaling and Sungkap villages, Bangka Island, using perineal pattern morphology, species-specific PCR (SCAR for *M. incognita*, ITS for *M. arenaria*), and nucleotide sequencing with phylogenetic analysis against GenBank reference.

MATERIALS AND METHODS

Sampling was conducted in two pepper (*Piper nigrum*) production centers on Bangka Island, Bangka Belitung Islands Province, Indonesia: Petaling Village, Mendo Barat District, Bangka Regency (2°06’46”S 105°59’37”E), and Sungkap Village, Simpang Katis District, Central Bangka Regency (2°20’32.24”S 106°09’30.78”E). Bangka Island is one of the main pepper-growing areas in Indonesia and has long been reported to have yellowing disease of pepper (Mustika, 1990). In both locations, pepper is grown in smallholder plantations. Samples were collected using

purposive sampling, in which roots were taken from more than one-year-old pepper plants of the Lampung Daun Lebar (LDL) and Nyelungkup varieties that showed leaf yellowing and root galls.

Nematode extraction. Sampling was carried out in January 2025. Three plantations were sampled at each location (six in total). In each plantation, 20 pepper plants of the Lampung Daun Lebar (LDL) or Nyelungkup cultivar were selected by purposive sampling. Selected plants were more than one year old and showed leaf yellowing and root galls. This gave 60 plants per location and 120 plants overall. The sampling unit was one plant, from which a composite sample of galled roots was collected. No replication was used: the three plantations per location were purposively sampled and are not treated as statistical replicates; no technical replicates were used. The plantations were the independent biological replicates (n = 3 per location); no technical replicates were used. Root samples were wrapped in newspaper and transported in a cool box to avoid direct sunlight, pressure, and high temperature. Root galls were soaked in water for 24 h and dissected with a surgical needle to obtain female nematodes, following a modified method of Hikmia et al. (2012).

Polymerase Chain Reaction. Before DNA extraction, each female was identified by perineal pattern. The posterior end was cut and mounted for morphological examination, and the anterior end of the same female was used for DNA extraction. Twenty females from each location were placed in microtubes containing 150 µL of extraction buffer (200 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS) and ground with a sterile pestle. An equal volume (150 µL) of chloroform: isoamyl alcohol (24:1) was added, vortexed for 3 min, and centrifuged at 11,000 rpm for 10 min. A 100 µL aliquot of the supernatant was transferred to a new tube, mixed with 50 µL of 3 M sodium acetate (pH 5.2) and 67 µL of isopropanol, and then stored at −20 °C for 18 h. After centrifugation at 12,000 rpm for 20 min, the supernatant was discarded, and the pellet was washed with 80% ethanol (equal volume to the pellet) and centrifuged at 12,000 rpm for 15 min. The pellet was air-dried, dissolved in 20–50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), depending on pellet size, and stored at −20 °C.

The *M. incognita*-specific SCAR primers Finc and Rinc (Zijlstra et al., 2000) (5’-CTCTGCCCAATGAGCCGTTCC-3’) and Rinc (5’-CTCTGCCCTCACATTAGG-3’). Amplification consisted of initial denaturation at 94 °C for 2 min, followed by [45] cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. The ITS-rDNA region was amplified separately with primers FrDNA2 (5’-TTGATTACGTCCCTGCCCTTT-3’) and RrDNA1 (5’-ACGAGCCGAGTGATCCACCG-3’) for *M. arenaria,* using 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min (Vrain et al., 1992).

Each 25 µL reaction contained 12.5 µL of 2× DreamTaq Green Master Mix (Thermo Scientific, USA), 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 2 µL of DNA template, and 8.5 µL of nuclease-free water. Reference isolates of *M. incognita* and *M. arenaria* from the Plant Nematology Laboratory collection, Department of Plant Protection, IPB University, obtained from *Impatiens balsamina* in 2025 and confirmed by perineal pattern, served as the positive control, and nuclease-free water replaced the template in the negative control. PCR products were separated on 1% (w/v) agarose gels in [1×] TBE buffer at 100 V for 30 min (Advanced Mupid-Exu electrophoresis system), and visualized on a Bio-Rad UView Mini transilluminator, with amplicon sizes estimated against a 100 bp DNA marker (Thermo Scientific, USA).

Nucleotide Sequencing. Nucleotide sequencing was performed using an ABI-Prism 3100-Avant Genetic Analyzer sequencer at the Research and

Development Center of PT. Genetika Science Indonesia. The obtained electrophoresis sequences were viewed using the Sequence Scanner v1.0 program (Applied Biosystems, USA). The nucleotide sequencing results were then confirmed in GenBank *using* the *Basic Local Alignment Search Tool* (BLAST) program available at *the National Center for Biotechnology Information* (NCBI, 2014). The confirmed nucleotide sequences were then aligned using the ClustalW multiple alignment algorithm in the *BioEdit Sequence Alignment Editor* v7.0.5.3 (Hall, 1999). Multiple sequence alignments were generated using ClustalW implemented in MEGA version 12 (Kumar et al., 2024), with default parameters. Phylogenetic analyses were conducted using the Maximum Likelihood (ML) method in MEGA 12. The fittest nucleotide substitution model for each dataset was selected based on the lowest Bayesian Information Criterion (BIC) score (Nei & Kumar, 2000). The Tamura-Nei (TN93) model was applied for *M. arenaria* and *M. incognita,* with 1000 bootstrap replicates (Kimura, 1980; Tamura & Nei, 1993; Kumar et al., 2024). The sequence of *M. javanica* (PZ805061.1) was used as an outgroup taxon for *Helicotylenchus crenacauda. H. crenacauda* (PP177318.1) served as an outgroup taxon for *M. arenaria,* and (OP972600.1) was used as an outgroup taxon for *M. incognita*.

RESULTS AND DISCUSSION

The perineal pattern is located at the posterior end of the female nematode and serves as a morphological characteristic for species of the genus *Meloidogyne*. *M. incognita*, the dorsal arch is high and accompanied by striae. *M. arenaria* is characterized by a low-rounded to concave dorsal arch near the lateral with irregular branches in the lateral fields, with smooth striae (Figure 1).

Species-specific PCR identified two *Meloidogyne* species in both Petaling and Sungkap. Primers Finc and Rinc amplified the SCAR region of *M. incognita* as a band of approximately 900 bp, and the ITS rRNA region of *M. arenaria* as a band of approximately 450 bp (Figure 2).

The SCAR marker sequences of the Petaling and Sungkap isolates were 100% identical to each other and to *M. incognita* from Indonesia (KP253749.1) (Table 1). Similarity with other *M. incognita* references ranged from 99.4% to 99.8%, including isolates from China (OQ919497.1, JN005841.1, MK410954.1, MZ596342.1), the USA (PV151353.1), India (KP411873.1), and Indonesia (LC894941.1, LC894942.1). In the phylogenetic tree (Figure 3), all isolates formed a single *M. incognita* clade. The Petaling and Sungkap isolates grouped with KP253749 in a subclade distinct from the remaining references.

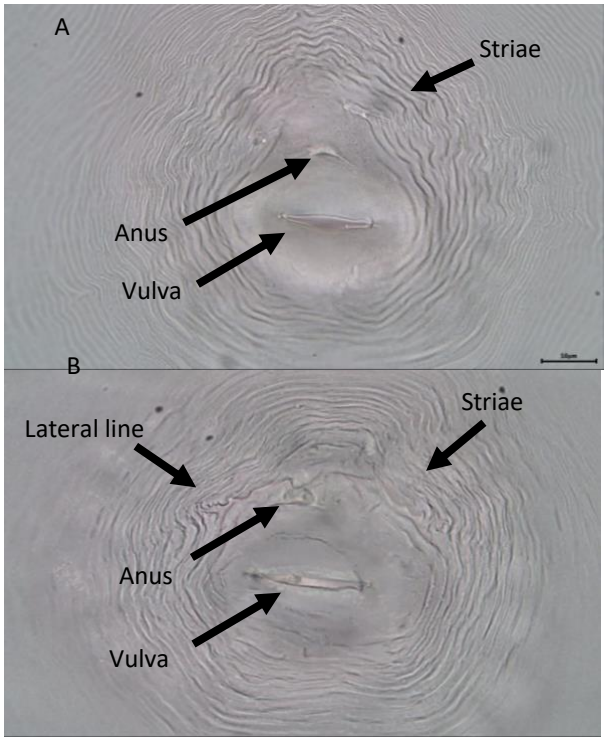


Figure 1. The perineal pattern *Meloidogyne* from Sungkap and Petaling: A, *M. incognita*; B, *M. arenaria.*

Alignment analysis was conducted using isolates from other countries for comparison. BLAST analysis revealed that the *Meloidogyne arenaria* sample from Petaling and Sungkep exhibited the highest sequence similarity (100%) with an *M. arenaria* isolate from Indonesia (KP253749.1). Phylogenetic analysis of the ITS

rRNA region placed both root-knot nematode isolates from black pepper, Petaling and Sungkap, in a single clade with *Meloidogyne arenaria* reference sequences from GenBank (KP253748.1 and KP234264.1 from Indonesia; MZ522722.1, OQ408134.1, and OQ408135.1 from China) (Figure 4). This clade was clearly separated from *M. javanica* (OP972600.1, Kenya), which served as the outgroup. Branch lengths within the *M. arenaria* clade were negligible, so the two isolates are identical or nearly identical to each other and to the reference sequences in this region. The ITS sequences of the Petaling and Sungkap isolates were 100% identical to each other (Table 2). Both isolates showed 100% similarity with *M. arenaria* from Indonesia (KP253748.1) and China (OQ408134.1 and OQ408135.1), and 99.7% similarity with *M. arenaria* from China (MZ522722.1) and Indonesia (KP234264.1).

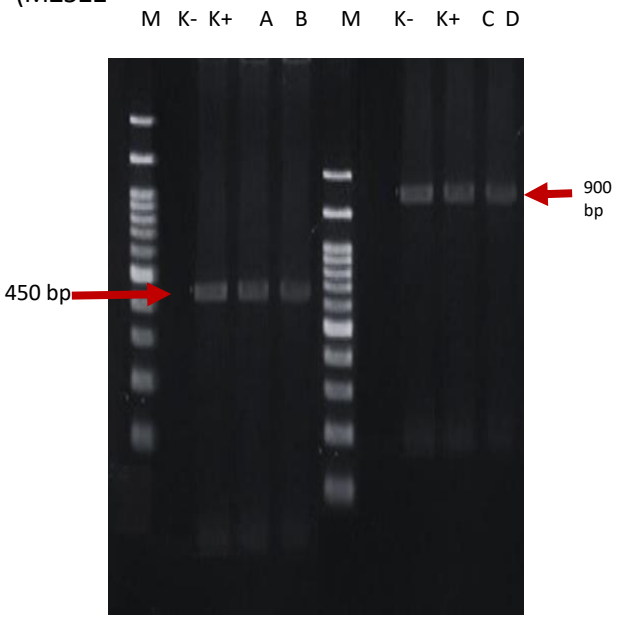


Figure 2. DNA amplification results of root knot nematodes from Petaling Village and Sungkap Village, Bangka Belitung Province, using specific primers—*t*he perineal pattern *Meloidogyne* from Sungkap and Petaling: A, *M. incognita*; B, *M. arenaria*.

The perineal pattern is located at the posterior end of female nematodes and serves as a morphological characteristic of species of the genus *Meloidogyne* (Taylor & Netscher, 1974; Eisenback et al., 1981; Hartman & Sasser, 1985). In *M. incognita*, the dorsal arch is high and squarish with striae. In *M. arenaria*, the dorsal arch is low and rounded, with irregular branching striae in the lateral fields and smooth striae, consistent with the descriptions of Eisenback et al. (1981) and Jepson (1987). However, perineal patterns vary within and among species, particularly in *M. arenaria* (Karssen, 2002; Hunt & Handoo, 2009), so morphology alone is insufficient for reliable identification and was complemented by molecular analysis.

Two root-knot nematode species, *M. incognita* and *M. arenaria*, were detected in pepper plants from Petaling and Sungkap. *Meloidogyne incognita* has long been implicated in yellowing disease of pepper in Bangka, together with *Radopholus similis* and *Fusarium* spp. (Mustika 1990), Moreover, our results extend this by showing that *M. arenaria* also occurs in the same pepper-growing areas. *Meloidogyne arenaria* has been reported on black pepper in other countries, for example, in southern Bahia, Brazil, where it was identified from galled roots using biochemical and morphological characters (De Souza et al., 2021), and in Vietnam, where it was recorded on black pepper and coffee and characterized with morphology and several gene regions, including ITS (Le et al., 2022). Both species were found at the two locations, suggesting they may be present in other pepper plantations in the area. However, sampling was purposive and limited to three plantations per location.

Table 1. Homology of the nucleotide sequence of *M. incognita,* the cause of yellowing disease in pepper plants in Bangka Belitung province (*M. incognita* Petaling and Sungkap

No	Isolate	Number accession	Homology (%)											
			1	2	3	4	5	6	7	8	9	10	11	12
1	*Meloidogyne incognita* Petaling		ID											
2	*M. incognita* Sungkap		100	ID										
3	*M. incognita* Indonesia	KP253749.1	100	100	ID									
4	*M. incognita* China	OQ919497.1	99,8	99,8	99,6	ID								
5	*M. incognita* China	JN005841.1	99,5	99,5	99,5	99,4	ID							
6	*M. incognita* China	MK410954.1	99,5	99,5	99,5	99,4	100	ID						
7	*M. incognita* China	MZ596342.1	99,5	99,5	99,5	99,4	100	100	ID					
8	*M. incognita* USA	PV151353.1	99,8	99,8	99,8	100	100	100	100	ID				
9	*M. incognita* Indonesia	LC894942.1	99,8	99,8	99,8	100	100	100	100	100	ID			
10	*M. incognita* Indonesia	LC894942.1	99,8	99,8	99,8	100	100	100	100	100	100	ID		
11	*M. incognita* India	KP411873.1	99,4	99,4	99,4	99,7	99,8	99,9	99,9	99,9	99,9	99,9	ID	
12	*M. javanica Kenya*	OP972600.1	37,3	37,3	37,3	41,9	41,9	41,9	41,9	41,9	41,9	41,9	41,9	ID

The *M. incognita* SCAR sequences showed 99.4–100% similarity to isolates from China, the USA, India, and Indonesia, indicating that the Bangka isolates belong to the same species and that this region is highly conserved across countries. The *M. arenaria* ITS sequences showed 99.7–100% similarity with references from Indonesia and China. Small differences between isolates are not unusual because the ITS of *Meloidogyne* can show high rRNA variation, including intragenomic polymorphism that may be widespread in the genus (Gu et al., 2020). The small differences observed may therefore reflect intraspecific or intragenomic variation, and cloning of the PCR products would be needed to confirm this.

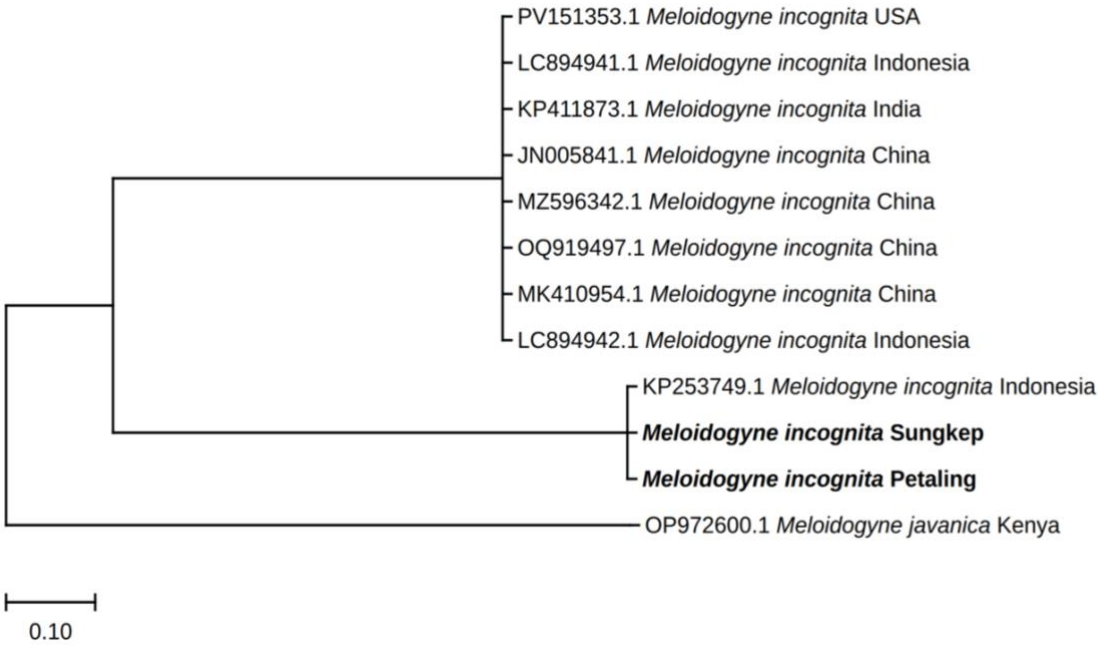


Figure 3. Phylogenetic tree *of M. incognita* isolates from Petaling and Sungkap, along with *M. incognita* isolates from other countries found in *GenBank,* based on nucleotide sequences using the MEGA v6.06 program with the Maximum Likelihood method. The scale below the image shows the genetic distance coefficient, which describes the average number of nucleotide changes between isolates.

The rDNA contains conserved genes (18S, 5.8S, and 26S/28S) separated by the ITS-1 and ITS-2 spacers (Gomes et al., 2002; O'Brien et al., 2005). The conserved genes are suitable for higher-level comparisons, whereas the ITS and IGS regions evolve more rapidly and exhibit variation among species and populations (Jamil, 2005). This explains why the sequenced markers (ITS for *M. arenaria*, SCAR for *M. incognita*) could separate *M. incognita* and *M. arenaria* from the outgroup *M. javanica* and also reveal small variation among isolates. The species-specific primers, based on SCAR markers (Zijlstra et al., 2000), produced single bands of the expected sizes, which show that this approach is fast and practical for routine identification of *Meloidogyne* species, compared with morphological identification, which is time-consuming and requires specialized expertise.

Table 2. Homology of the nucleotide sequence of *M. arenaria,* the cause of yellowing disease in pepper plants in Bangka Belitung province (*M. arenaria* Petaling and Sungkap)

No	Isolate	Accession number	Homology (%)							
			1	2	3	4	5	6	7	8
1	*M. arenaria* Petaling		ID							
2	*M. arenaria* Sungkap		100	ID						
3	*M. arenaria* Indonesia	KP253748.1	100	100	ID					
4	*M. arenaria* China	MZ522722.1	99,7	99,7	99,7	ID				
5	*M. arenaria* China	OQ408134.1	100	100	100	99,7	ID			
6	*M. arenaria* China	OQ408135.1	100	100	100	99,7	100	ID		
7	*M. arenaria* Indonesia	KP234264.1	99,7	99,7	99,7	100	99,7	99,7	ID	
8	*M. javanica* Pakistan	PZ805061.1	49,6	49,6	49,6	49,6	49,6	49,6	49,6	ID

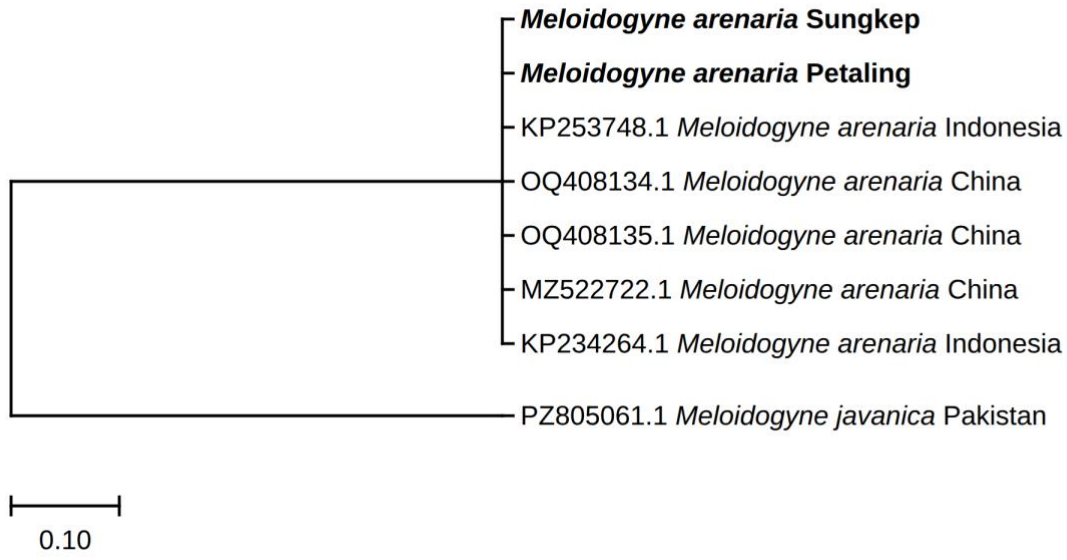


Figure 4. Phylogenetic tree of *M. arenaria* Petaling and Sungkap isolates with isolates from other countries found in *GenBank* based on nucleotide sequences using the MEGA v 6.06 program with the Maximum Likelihood methods. The scale below the image shows the genetic distance coefficient, which describes the average number of nucleotide changes between isolates.

Phylogenetic trees group sequences by their number of nucleotide differences, so isolates with high similarity appear on adjacent branches (Wasserman & Sandelin, 2004; Ubaidillah & Sutrisno, 2009). In both trees, the Bangka isolates clustered with foreign and Indonesian isolates of the same species without geographic structuring. In *M. incognita*, the Petaling and Sungkap isolates grouped with KP253749 in a subclade separated from the other references by only 0.2–0.6% divergence, which is more likely to reflect minor intraspecific variation than a distinct lineage.

Knowing which *Meloidogyne* species occur in pepper plantations is a useful basis for nematode management, since species differ in host range and virulence (Perry et al., 2009). Management options such as microbial agents have been reported to reduce nematode multiplication and root galling in black pepper (Saad et al., 2022). However, their efficacy should be tested against the species found here.

CONCLUSIONS

1. Species-specific PCR followed by sequencing identified *Meloidogyne incognita* and *M. arenaria* in galled roots of symptomatic pepper plants from Petaling and Sungkap, Bangka.
2. The *M. incognita* SCAR sequences were 99.4–100% similar to reference sequences in GenBank, and the *M. arenaria* ITS sequences were 99.7–100% similar.

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