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Identification of the root-knot nematode species associated with *Carica papaya*

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Abstract. *Plena SNG, Indarti S, Widiastuti A, Putra NS. 2022. Identification of the root-knot nematode species associated with* Carica papaya. *Biodiversitas 23: 6212-6217.* Root-knot nematodes (*Meloidogyne* spp.) are important economic pests causing low production and yield losses in horticultural crops. *Meloidogyne* spp. are widespread and regarded as major pests since they are found all over the world in a variety of geographic locations, most frequently found in tropical and subtropical climate zones. They are obligate parasites that feed on a wide variety of plants, including papaya and other horticultural plants. They are root galling in host plants. Many species of root knot nematodes may be found in a suitable environment. Therefore, accurate identification of the root-knot nematode species in papaya plants is important for developing and applying control and management measures. Surveys and sampling of nematodes on papaya plants were performed at the Agrotechnology Innovation Center, Universitas Gadjah Mada, Yogyakarta, Indonesia, and its surroundings. Identification was performed both morphologically (based on the perineal pattern of female nematodes) and molecularly (using root-knot nematode species-specific primers). The results revealed that the species of root-knot nematode associated with papaya plants were *Meloidogyne javanica* based on the perineal pattern of female nematodes, specifically the lateral lines. This finding was also supported by the results of the DNA amplification at 670-bp product using specific primers for *M. javanica* (FJav/RJav). Consequently, plant protection efforts will need to focus on minimizing *M. javanica* damage in papaya cultivation.

Keywords: Identification, molecular, perineal pattern, root-knot nematode

INTRODUCTION

Papaya (*Carica papaya*) is a tropical fruit that is grown worldwide in tropical and subtropical climates (Koul et al. 2022). Papaya is widely cultivated since it has a high commercial value, due to the high market demand for fresh fruit and its processed products. Many traditional Asian dishes, including those from Thailand, Malaysia, Indonesia and India, contain green papaya fruit, young leaves or shoots, and can be eaten raw or cooked (Burns et al. 2022). Over the last 20 years, the global production of papaya has increased significantly on 2016 (Zhou et al. 2021), and reached 13,894,705 tons in 2020 (FAO 2020). The chief producers of papaya are India, Brazil, Mexico, Indonesia and Nigeria (FAO 2019). In Indonesia, the average production of papaya in the year of 1994-2020 was 690,765 tons (FAO 2020). Papaya could be found in all province in Indonesia but East Java, Central Java and West Java was the top 3 Province with the highest production of papaya. The production of papaya in 2021 from East Java, Central Java and West Java were 253,700 tons, 142,034 tons, 124,466 tons, respectively (BPS 2022).

The production of papaya plants is affected by various pathogens, including bacteria, viruses, fungi and nematodes, causing significant losses both during production and in postharvest contexts (Koul et al. 2022). Plant-parasitic nematodes cause damage plants and can cause substantial yield losses. Farming practices, surface water runoff and irrigation are responsible for the dissemination of nematodes (Bahadur 2021). The most prevalent nematodes in papaya crops across the world are root-knot nematodes, root lesion nematodes (*Pratylenchus* spp.), and reniform nematodes (*Rotylenchus* spp.). Root-knot nematodes are the most important cause of significant losses in agricultural crops, (Kesba et al. 2012; Lopes-Caitar et al 2019; Comejo-Condori et al. 2021), with losses caused from 15-20% (Padilla 2021). With root-knot nematode infestation, papaya roots atrophy, preventing the plant from absorbing sufficient water and this can lead to the death of the plant (Dagatti et al. 2014). This nematode has a stylet to pierces root cells and exude enzymatic secretions that lead to lesions, the induction of giant cells, and the development of root galls (Cornejo-Condori et al. 2021). Their infestation can lead to yellowing of plant leaves and stunted shoot development. Wilting can occur as a result of blocked xylem vessels (Ali et al. 2021). The second-stage juveniles penetrate roots and migrate between cells, and once feeding sites are established the roots respond with the formation of galls. They can infest all parts of the root system, including taproots and tubers, reducing the value of vegetable produce both economically and qualitatively. Root-knot nematodes could parasitize for about 3,000 record host plant species (Abad et al. 2003).

Infestation of *Meloidogyne* spp. in papaya plants have been reported worldwide with various species for example, in Brazil and Colombia, the most common root-knot nematode species identified are *Meloidogyne hapla, M. incognita, M. javanica*, and *M. arenaria* (Padilla 2021).

Meloidogyne spp. can have multiple generations within single cropping seasons. Although papaya plants are widely cultivated in Indonesia since, studies on parasitic nematodes, especially the identification of root-knot nematodes in papaya plants has been limited. There was no information about species of *Meloidogyne* in papaya plants in Indonesia. Therefore, the purpose of this study was to determine the species of *Meloidogyne* spp. in papaya plants in Sleman, Yogyakarta, Indonesia.

MATERIALS AND METHODS

Root-Knot nematode sampling

Targeted sampling of symptomatic papaya plants by root-knot nematodes was undertaken at three locations (coordinates: 7°47'36.5"S 110°27'50.3" E; 7°47'46.9"S 110°27'48.2"E; and 7°47'21.6"S 110°28'09.6"E) at the Agrotechnology Innovation Center (Pusat Inovasi Agroteknologi) of Universitas Gadjah Mada (PIAT-UGM), Subdistrict of Berbah, District of Sleman, Special Region of Yogyakarta, Indonesia. The symptomatic papaya plants had common symptoms, such as chlorosis and swollen or galled roots.

Identification was performed using female nematodes dissected from the root galls. Female nematodes were chosen because they easily obtained and can be identified morphologically by perineal pattern.

Symptom observation and root staining

The symptoms of the infested papaya plants were observed and stained using a modified method by Bybd et al. (1983). The modification was on the root samples that not being cut into small pieces.

Species identification

Morphological identification

Morphological identification was performed by analyzing the female nematode perineal patterns. The preparation of perineal samples followed the method of Hasan and Abood (2018). The perineal pattern was observed under an Olympus CX-31 microscope (Tokyo, Japan) at a magnification of 40-400x. The perineal patterns were then compared with those described by Eisenback and Triantaphyllou (1991), Hunt and Handoo (2009) and Hasan and Abood (2018)

Molecular identification

The *Cetyl trimethylammonium bromide* (CTAB) method (Mondino et al. 2015) was used for DNA extraction from 10-15 female nematodes. This DNA was then processed by PCR using primers for *Meloidogyne* complex MIGF/MIGR (5'-ACACAGGGGGAAAGTTTGCCA/ GAGTAAGGCGAAGCATATCC-3') (Qiu et al. 2006) and species-specific primers for *M. javanica* FJav/RJav (5'-GGTGCGCGATTGAACTGAGC/CAGGCCCTTCAGTG GAACTATAC-3') (Zijlstra et al. 2000). The PCR program was run on a Bio-Rad T100 Thermal Cycler with a total volume of 25 µL (12.5 µL MyTaq HS RedMix BioLine, 2.5 µL of each primer at 10 pmol/µL, 5 µL DNA template and 2.5 µL nuclease-free water). Initial denaturation was performed at 94°C for 2 min followed by 35 cycles: denaturation for 30 s at 94°C, annealing for 30 s at 54°C (MIGF/MIGR) and 64°C (FJav/RJav), and the extension was at 72°C for 1 min. The DNA amplification products were then electrophoresed in 1% agar at 100 v for 25 min using a Mupid-EXu system. Visualization was achieved with a UV transilluminator.

Sequencing was performed at the Laboratory of Integrated Research and Testing (Laboratorium Penelitian dan Pengujian Terpadu, LPPT), Universitas Gadjah Mada. The sequence data were edited in BioEdit 7.2 before being blasted in the BLAST-N program at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov). Several specimens were randomly selected for comparison. A phylogenetic tree was then created with the neighbor-joining method using the Molecular Evolutionary Genetics Analysis 6.0 (Mega 6.0).

RESULTS AND DISCUSSION

Plant symptoms

Root-knot nematodes are parasitic nematodes that infest plant roots, causing yield losses in quality and quantity. Typical symptoms in papaya are root swelling and galling (Figure 1) and yellowing of plant leaves, stunted shoot growth, and wilting from blocked xylem vessels (Ali et al. 2021).

Secondary symptoms caused by root-knot nematodes are evident above ground but these are not specific, being similar to nutrient deficiency, including chlorosis, stunted or wilting. Infested plants are susceptible to moisture stress wilting more easily than healthy plants. Nematode infestation can inhibit water and nutrient uptake, which results in plant stunting. The fruit produced is also smaller and fruiting can be totally inhibited (Ralmi et al. 2016). Infection with other pathogens can also lead to pathogen-host complex that is difficult to manage or control, for example nematode infestation causes damage that allows the entry of *Phytophthora* (Padilla 2021).

In the early stage of infestation, the visible symptoms in the roots can be difficult to observe. A gall usually forms 1-2 days after juvenile penetration, with gall size correlated with the number of nematodes present in the tissue (Eisenback and Triantaphyllou 1991). The presence of nematodes in plant root tissues is difficult to see directly, specifically for female nematodes, as they are in root tissue and have a color similar to that of plant root tissue (Mutalaliah et al. 2019). Therefore, the presence of root-knot nematodes in plant tissues can be observed through staining by the Bybd method. The staining of the root tissue aims to provide a contrasting color between the nematodes and the root tissues. The root tissues are cleared with NaOCl before staining with fuchsin acid, which reveals presence of the nematodes (Figure 2.A).

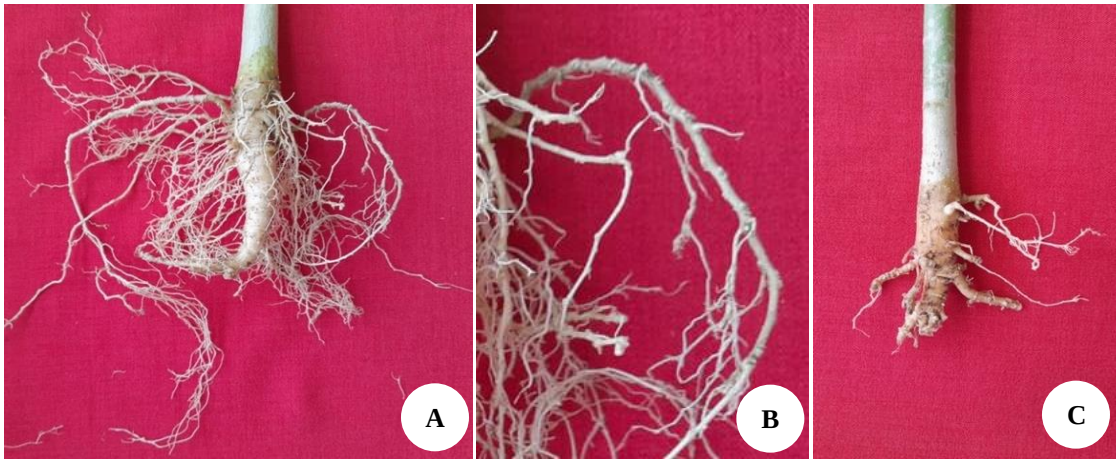

Figure 1. Symptoms of papaya roots infected by root-knot nematodes (*Meloidogyne* spp.). A-B. Gall; C. Root growth is disturbed and damaged

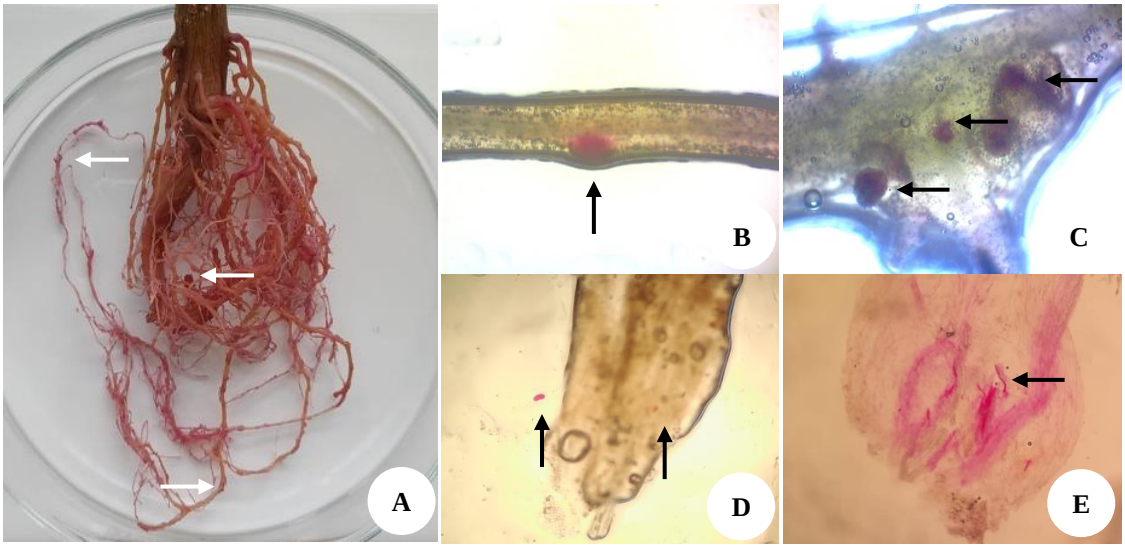

Figure 2. Staining of root-knot nematodes (*Meloidogyne* spp.) in papaya root tissue. A. Galled root system; B-C. Female nematodes in root tissue; D. Eggs; E. Second-stage juvenile in root tissue

Staining revealed the presence of female and juvenile nematodes in the root tissue, as well as nematode eggs (Figure 2b-e.). However, no male nematodes were observed. According to Eisenback and Triantaphyllou (1991), infective-stage juveniles will group in the apical meristem, elongated cells near the lateral root meristems and will migrate between cells. Male nematodes will migrate from the roots whereas females remain sedentary and continue to feed on giant cells (Eisenback and Triantaphyllou, 1991). The egg sacs are typically attached to the posterior of the females and are slightly darker brown color than the gall tissue (Mutalaliah et al. 2019).

Species identification

Morphological identification

The root-knot nematode species identification is an important for determining effective nematode management, e.g., crop rotation and the use of resistant crops (Zijlstra et al. 2000). Morphological identification of *Meloidogyne* spp. is based on the perineal pattern, a fingerprint-like pattern on the perineal cuticle of female nematodes (Eisenback and Triantaphyllou, 1991). The perineal pattern is a characteristic feature of various female nematodes and is used to identify the nematode species. The perineal pattern of the nematodes from PIAT-UGM tended to be circular, with a dorsal part that is not particularly raised and slightly rough, with lateral line that separates the dorsal and ventral parts (Figure 3a). Based on these characteristics, the PIAT-UGM samples were identified as *M. javanica*. This finding is similar to the study by Eisenback and Triantaphyllou (1991), where *M. javanica* can be easily identified and distinguished from *M. arenaria* and *M. incognita* which have similar shapes through the presence of clear lateral lines.

Molecular identification

Two primer pairs were used in the identification of root-knot nematode species from papaya plants at PIAT-UGM. Amplification of the PCR product of *Meloidogyne* spp. from three locations in PIAT-UGM using the MIGF/MIGR primer pair produced a single band at 500 bp (Figure 4). The MIGF/MIGR primer set is a primer pair that can be used to amplify the *Meloidogyne* complex species. PCR products from *M. incognita*, *M. javanica* and *M. arenaria* can be amplified in the range of 500 bp band length (Qiu et al. 2006). Also, amplification using species-specific primers was performed to determine the *Meloidogyne* species. PCR products from samples from three locations were amplified forming as single bands at 670 bp using the species-specific primer pair, Fjav/Rjav. Fjav/Rjav are species-specific primers used to identify *M. javanica* as reported by El-Sagheer (2021).

DNA sequencing was performed to determine the genetic relationship with other nematodes within species or other species. Sequenced samples were from a location along Jalan Opak V because all the samples came from Berbah. Based on the results of phylogenetic analysis, samples of *M. javanica* from Berbah with a sequenced result of 500 bp were closely related to specimens from various countries but were in different groups or separate cladograms (Figure 5). In Figure 5, the branch length between the specimens of *M. javanica* from Berbah (Clade III) and the specimens in Clade II is longer than the branch length between Clade III and Clade IV. This indicates that the specimens of *M. javanica* from Berbah are more closely related with Clade II. The species with the longest branching in the cladogram has undergone the longest evolutionary process, resulting in more changes to the sequence of nucleotide bases. Conversely, short branching indicates that the evolutionary process has been relatively short, meaning that there is only a slight change in the sequence of nucleotide bases.

The process of evolution might occur due to genetic variation in a particular population. Evolutionary change usually caused by biotic interaction as major driver such as interactions of nematodes with competitors, parasites, predators, vectors, associated microorganisms and interaction between species (Schulenburg and Felix 2017). Accordingly, *M. javanica* from Berbah might be the evolution result of the specimens in Clade II, i.e., *M. javanica* from Brazil, China, Ethiopia, India, Israel, Malta, South Africa, USA or Vietnam. Also, specimens of *M. javanica* from Berbah are also considered to have undergone a long evolution to produce other specimens from Greece and Turkey. As shown in Figure 5, the specimens of *M. javanica* from the subdistrict of Berbah were separated from the specimens of *M. javanica* from various countries. Thus, genetically, *M. javanica* specimens from Berbah are quite different from those countries. Therefore, further research is needed on the genetic differences between *M. javanica* populations from Berbah and those from other countries.

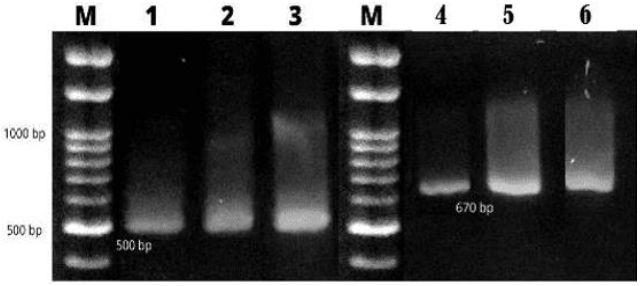

Figure 4. Visualization of PCR for papaya root-knot nematode DNA from three locations using primers MIGF/MIGR (500 bp) for samples 1 to 3 and Fjav/Rjav (670 bp) for samples 4 to 6. M, DNA ladder 100 bp; lanes 1-6, samples (1 and 4 from Jl. Opak V, 2 and 5 from block 2 Agrotechnology Innovation Center (Pusat Inovasi Agroteknologi) of Universitas Gadjah Mada, 3 and 6 from block 3 PIAT UGM

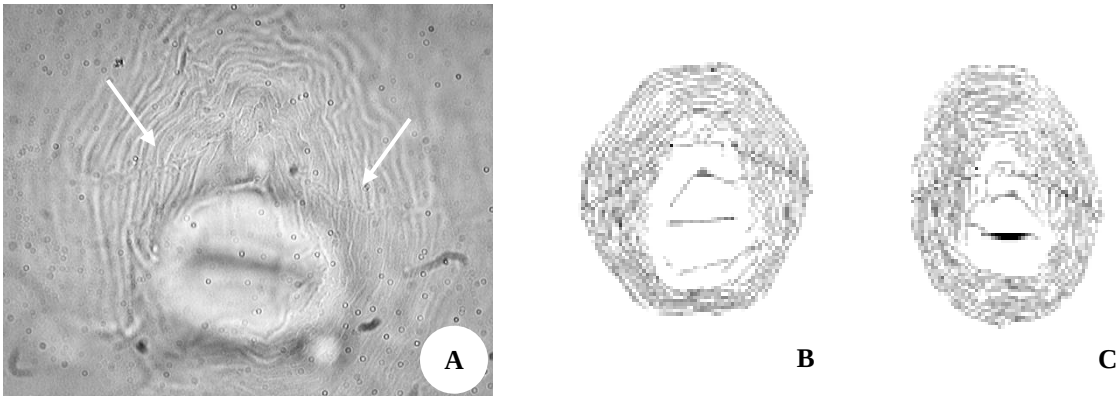

Figure 3. *Meloidogyne javanica* perineal pattern. A. Lateral line at *Meloidogyne* sampled from (Pusat Inovasi Agroteknologi) of Universitas Gadjah Mada, Yogyakarta, Indonesia; B-C. Illustration of *M. javanica* by Hunt and Handoo (2009)

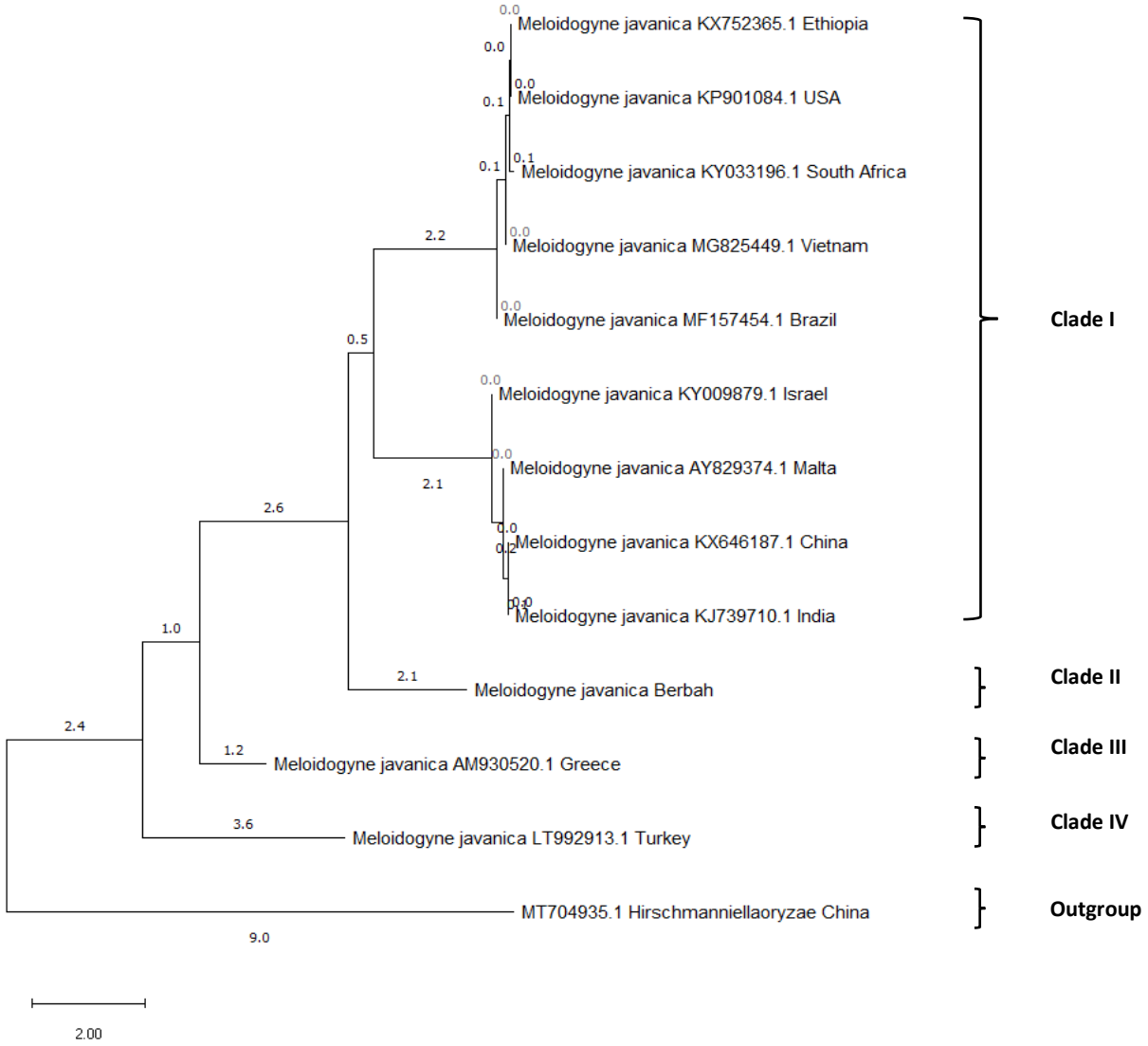

Figure 5. Neighbor-joining phylogenetic tree of *Meloidogyne javanica* from Berbah. The best fit Kimura 2 parameter model with 1000 bootstraps was used to construct the tree with other available *M. javanica* and *Hirschmaniella oryzae* as outgroup taxa from the GenBank NCBI database. Phylogenetic tree was conducted by MEGA6 (Molecular Evolutionary Genetics Analysis Version 6.0)

The genetic relationship of nematode populations from various countries might occur due to the migration process or the spread of a nematode species worldwide. However, the origin and mechanism of this spread is unknown. However, this might have happened due to the illegal distribution of seeds. In addition, *M. javanica* has a wide range of hosts because it reproduces quickly, spreads, and adapts easily to new habitats, which allows it to have many hosts. It is an extremely polyphagous, apomictic species, widely distributed with a wide host range and is responsible most of crop losses caused by root-knot nematodes (Karajeh 2015) Hence, there is a high possibility that *M. javanica* can reproduce in various places. In addition to papaya, other host plants of *M. javanica* are carrots (Hikmia et al. 2012), potatoes (Mutalaliah et al. 2019), soybeans (de Sá et al. 2012). Several weed hosts also reported to be hosts of *M. javanica* with different susceptibility levels. High nematode population density can be maintained by highly susceptible weeds such *as Chenopodium album, Anagallis arvensis* and *Portulaca oleracea* (Öztürk et al. 2020). Further, climatic conditions also affect the distribution of root-knot nematodes. The distribution of root-knot nematodes is commonly in the tropics and temperate regions. Additionally, the nematodes also spread through infested planting materials, water drains from infested areas, soils, tires of motor vehicles and through human activities (Bahadur 2021).

The findings from this study demonstrated that the root-knot nematode specie from papaya roots in Berbah, Yogyakarta, Indonesia were *M. javanica* based on the perineal pattern of female nematodes and molecular methods. The identification of root-knot nematode species associated with papaya is important because *M. javanica* is a damaging species and causes losses to horticultural crops

in Indonesia. Further studies need to be conducted to determine appropriate control for this species in papaya.

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