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Morphological, morphometrical and molecular identification of root-knot nematode (*Meloidogyne javanica*) infecting banana in Assiut governorate, Egypt

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Abstract

Plant-parasitic nematodes are extremely dangerous pests in a variety of economically important crops. Root-knot nematodes (RKN) *Meloidogyne* spp. are between the major important pests causing serious crops havoc worldwide because of their wide geographical distribution and variety of hosts. Therefore, both of identification that is true and trustworthy of these pests is required for evaluating various suitable management strategies. This study, aimed to characterize morphological, morphometric and molecularly isolate of *Meloidogyne* related to banana plants. Second-stage juveniles (body length, tail length, stylet length, hyaline terminus length, and DEGO) were used in morphometric and morphological studies and female in perineal patterns. The results revealed that the identified nematode species, *Meloidogyne javanica*, is the most common root knot nematode species in all three localities. To prove the identification, a polymerase chain reaction (PCR)-based experiment using a species-specific sequence described amplified regions (SCAR) primer series was used. The Fjav/Rjav primer effectively enhanced SCAR markers of 670 bp previously identified in *M. javanica*. This study confirms the use of an effective and reliable diagnosis of RKN using the three approaches.

Keywords: *Meloidogyne javanica*, root-knot nematodes, banana, morphometric, SCAR markers.

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1. Introduction

Banana (*Musa* sp.) is one of the world's most economic tropical fruit crops. It's an important source of carbohydrates, fibers, proteins, vitamins, and minerals. Bananas thrive in a wide range of soil conditions. It's cultivation area in Egypt reached approximately 447299 ha, with an average yield of 1359297 tons/ha. (FAO, 2019). Nematodes are one of the world's most significant limitations on banana production, with 146 species recorded in 43 genera. A most dangerous nematode species are that, devastate the primary roots, causing the anchorage system to fail and the plant to fall over (Gowen et al., 2005). When soil and root samples were analyzed, it was discovered that trees were severely infested with root knot nematodes. Various procedures were used to characterize the root-knot nematode species like, morphometric, morphological characters and molecular techniques. The perineal pattern is predominantly uncertain when used alone to make diagnostic deduction, but when used as an integral device in conjunction with morphometric characterization or molecular techniques, it is substantial for screening the morphological match-making of the identification (Carneiro et al., 2004). So that, molecular diagnostics of *Meloidogyne* sp. have been research as a surrogate. PCR-based disclosure methods, such as species specific or sequence characterized amplification region (SCAR) primers, have been advanced and vastly used for nematode identification (Daramola, et al., 2015). SCAR markers are preferred over RAPD markers because they detect only a single locus and are more specific. Furthermore, their PCR amplification is low sensible to reaction constraints and thus more reproducible. The objective of the present study was undertaken to characterize morphological (perineal patterns), morphometric and molecularly (PCR with species-specific primers) techniques to identify the root-knot nematode isolated from three localities (Assiut center, Alfath and Sahel sleim) cultivated with banana in Assiut governorate, Egypt.

2. Materials and methods

2.1 Isolated nematode identification

The soil and root samples of root-knot nematodes infecting banana were obtained from three localities (Assiut, Alfath and Sahel sleim) of Assiut governorate, Egypt cultivated with banana orchards. The morphological, morphometrical, and molecular characterization was carried out on second-stage juveniles (J2) gained from soil extraction and mature females obtained from infective roots.

2.2 Morphological characterization

Sections of infected roots should be immersed in 0.9% NaCl. Using a dissecting microscope, separate females from roots by needle and a scalpel and transfer the females to a petri dish with a small drop of 45% lactic acid. Push a female body out of a drop in a small isthmus of lactic acid solution, so that surface tension holds it in place. Insert the razor blade fragment into the slide and use a paper cutter to cut off the nematode's posterior. Using a dissecting needle, gently remove body tissue from the posterior section. In a small drop of glycerin, place the perineal pattern on a microscope slide. The internal surface of the cuticle should place against the glass then, cover slip placed on the glycerin

drop. (Taylor & Netscher, 1974).

2.3 Morphometrical characterization

Morphometric dimensions of *Meloidogyne* were specified on ten individual J2 from three localities. J2 was tentatively mounted in water on glass slides before being spotted and measured at 100 magnifications with a compound light microscope (OMAX 40X-2000X digital binocular biological compound microscope) linked to a computer working Scope- Image- 9.0 Professional Imaging software. The optical microscope was used to measure five morphometric variables (stylet length, tail length, body length, hyaline terminus length, and the distance between the stylet base and the dorsal esophageal gland orifice (DEGO)).

2.4 Molecular characterization

2.4.1 DNA extraction

The CTAB (cetyltrimethylammonium bromide) method was used to extract DNA from nematode isolates (Mondino et al., 2015) with some modifications. Many adult females gained from each isolate were frozen in liquid nitrogen then, crushed using a suitable pestle and mortar. 600µl of CTAB extraction buffer was added to each sample and the mixture was then transferred to 1.5 ml Eppendorf tube. A volume of 50µl β-mercaptoethanol was added and all tubes were well vortexed for 15 sec and then incubated for about 40 min at 65° C in a water bath. After incubation, the tubes were kept at room temperature for 5-10 min, and 600µl chloroform: isoamyl alcohol solution (24:1 v/v) was then added to each tube, and the solution was gently mixed. The tubes were then subjected to a centrifugation (8,000 rpm at 4° C for 15 min). After the centrifugation, approximately 500µl of the upper aqueous phase (without any solid material) was transferred to a new 1.5 tube and an equal volume (500 µl) of cold isopropanol was added to each tube. The tubes were then slowly inverted several times and stored in the refrigerator overnight. A centrifugation (13,000 rpm at 4° C for 10min) was performed for the tubes. After the centrifugation, the supernatant was discarded and the DNA pellet was then washed by adding 1 ml of 70% cold ethanol, and a centrifugation (13,000 rpm at 4° C for 5 min) was performed. The tubes were kept at room temperature to allow the DNA pellet to air-dry (approximately 15 min). The dried DNA pellet was then resuspended in 100 µl TE buffer. DNA concentration (µg/ml) was determined for each sample by using spectrophotometer, and required dilutions were then performed to be used later for PCR.

2.4.2 Species-specific PCR assay

A species-specific SCAR primer collection (Table 1), selected from previous studies (Zijlstra et al., 2000) as a specific marker for *Meloidogyne javanica*, namely Fjav/Rjav, was used to confirm morphological identification of nematode isolates. Amplifications were carried out in 25µl reaction mixtures containing 5-10 ng of genomic DNA, 1X PCR buffer, 1.5 mM MgCl2, 200 µM of each dNTP, 0.8 µM of each primer, and 1 U Taq DNA-polymerase. Amplifications were carried out using the following PCR software in a Senso Quest

Lab Cycler (SensoQuest GmbH, Göttingen, Germany): 5 minutes at 95° C, then 35 intervals of 1 minute at 94° C, 1 minute at 58° C, and 1 minute at 72° C, followed by one final extension period at 72° C for 10 minutes. PCR products were separated on a 1.5 percent agarose gel stained with ethidium bromide in 0.5 X TBE buffer using a horizontal gel electrophoresis unit. The size of each amplified DNA fragment was determined using a DNA ladder. The gel was run for about an hour at a constant voltage of almost 80 V, and then photographed using a gel documentation device under UV light. For each SCAR marker, the same band with the predicted size was then detected separately.

Table 1: SCAR primers were used to identify *Meloidogyne javanica* at the molecular level.

Name of Primer	Fragment size (bp)	Sequence (5'-3')	Reference
Fjav/Rjav	670	GGTGCGCGATTGAACTGAGC CAGGCCCTTCAGTGGAACTATAC	Zijlstra et al. (20000

3. Results

3.1 Morphometric characters

Second-stage juveniles were vermiform and slender ranged from 400-550 µm in length and a head that was not offset from the body. Stylet knobs transversely elongate and are offset from the stylet shaft, stylet length ranged from 9.6-12.4 µm. The distance between the dorsal esophageal gland and the base of the stylet was 3-4 m. Tail length was 50-62.2 µm with rounded tip. The hyaline tail length ranged from 10.2 to 18.4 µm, with a long slender tapering tail and a delicately curved tail tip, which corresponded to the characterization set for *M. javanica* by (Karssen & Moens 2006; Eisenback, 1985). Morphometris performed on J2 are reported in Table (2).

Table 2: Morphometric comparison of second-stage juveniles of the Assiut isolate and *Meloidogyne javanica* reported values.

Characteristic	Assiut isolate	*M. javanica* reported values
Body length	400 – 550 (475) µm	400 – 560 µm
Stylet length	15 – 17 (16) µm	14 –18 (16) µm
Tail length	50.0 - 62.2 (56.1) µm	51.0 – 63.0 (57) µm
Hyaline terminus	10.2 – 18.4 (14.3) µm	10.0 – 19.0 (14.5) µm
Dorsal esophageal Gland Orifice (DEGO)	3 – 4 (3.5) µm	3 – 4 (3.5) µm

All measurements in micrometers with range (mean).

3.2 Perineal pattern morphology

When comparing to previous reports, assessment of the perineal pattern's morphology of adult females from three localities, selected by hand from infected banana roots, revealed brow model of *M. javanica* (Eisenback, 1985). *Meloidogyne javanica* was dominant in the three localities. The perineal patterns of *M. javanica* are unrivaled because they consist of side ridges that part the dorsal and ventral lines. In generally, the ridges run the entire width of the pattern, but progressively die out near the tail end. The dorsal arch is faint and rounded to

high and squarish and often contains a whorl in the tail terminal area.

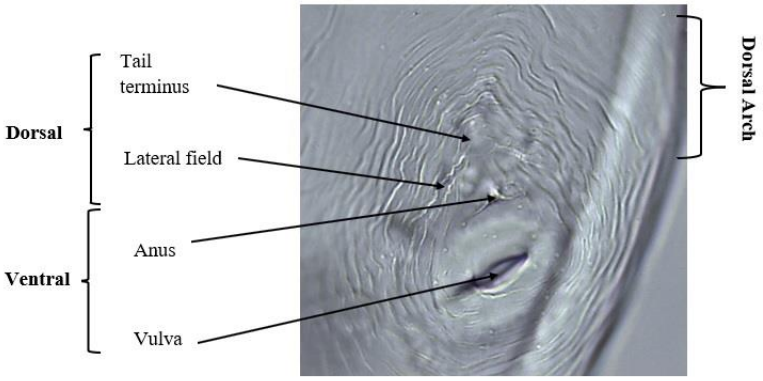

Figure 1: Perineal pattern of *Meloidogyne javanica*.

The striae are sleek to little adverse, and several striae may curvature across the vulval edges (Figure 1).

3.3 Molecular identification of *Meloidogyne javanica*

One species-specific SCAR primer pairs, namely Fjav/Rjav were used for molecular diagnosis of nematode isolates to further confirm species identification. The PCR assay was performed on one sample from each locality. The three nematode isolates clearly amplified the expected specific DNA fragment of 670 bp (Figure 2) which confirms the identification of *M. javanica* as recorded by (Zijlstra et al., 2000).

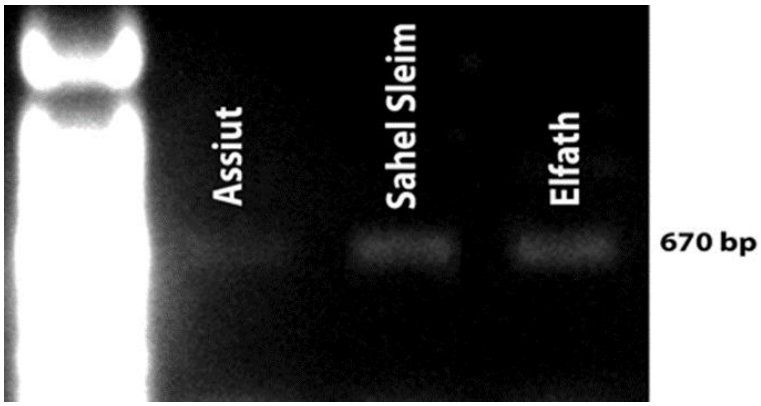

Figure 2: Amplification items (670 bp) for *Meloidogyne javanica* species – Fjav/Rjav SCAR primers.

4. Discussion

Morphological and morphometric studies necessitate a significant amount of endeavor and are not always simple, attributed to the prevalence of intra-specific variations. As the previously reported, the morphometric values overlap, and the morphology of the perineal pattern, while extra helpful but,

remains indecisive due to individual variability, the assorted practice of those describing the patterns, and the increased numbering of species. The combination of morphology and morphometrics may provide a small hint toward species identification. This result is agreement with (Phan et al., 2021; Katooli et al., 2020, Pehlivan et al., 2020; Rusinque et al., 2018; Eisenback & Triantaphyllou, 1991). *M. javanica* is the most frequent *Meloidogyne* sp. found in tropical and subtropical areas (Moens et al., 2009), such as Egypt, where annual temperatures range between 17-32˚C. It is important to note that, while PCR is quick, simple, and capable of determining species identity regardless of developmental phase and from tiny portions of tissue. Because of intraspecific variability and species closeness, its dependability is uncertain. Thus, morphology, morphometrics, and molecular analysis work in tandem to provide more accurate and reliable identification. The PCR examination for the nematode isolates with the specific SCAR primer Fjav/Rjav clearly produces a specific DNA piece of 670 bp (Figure 2) which confirms the identification of *M. javanica* such results were in harmony with those (Mwesige et al., 2016; Naz et al., 2012; Devran & Sȍgüt, 2009; Zijlstra et al., 2000). SCAR markers have been to a large degree used in molecular identity of root-knot nematodes, both to prove morphological identifications and to set apart unknown isolates in genomic analysis (Naz et al., 2012; Devran & Sȍgüt, 2009; Jones et al., 2009; Randig et al., 2002; Zijlstra et al., 2000). From the results we concluded that the morphometric, morphological, and molecular identification were harmonic with one another, implying that molecular analysis of root-knot nematodes using SCAR markers could be used as a supplement to morphometric and morphological identification.

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