Presence of *Meloidogyne enterolobii* on Jalapeño pepper (*Capsicum annuum* L.) in Sinaloa, Mexico

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Summary

Meloidogyne enterolobii (*Me*) is an emerging root-knot nematode (RKN) and the most important worldwide for its high aggressiveness and increasing geographical distribution. In Mexico only in watermelon and tomato has been reported. Here we report the presence of this nematode in Jalapeño pepper collected in the state of Sinaloa. The morphological and morphometric characteristics of the second-stage juveniles and adult females were similar to those described for *Me*. DNA sequencing and alignment analysis (BLAST/GenBank) showed that the SCAR and rDNA IGS2 fragments had a 99 % of identity, with *Me* DNA sequences present in the database. The results obtained indicate that the RKN detected corresponds to *M. enterolobii*. To our knowledge, this is the first report of this nematode in chilli pepper in this region of Mexico, considered of major economic importance for the variety of vegetables grown and the extension of the area sown.
Keywords: *Meloidogyne mayaguensis*; PCR; sequence-characterized amplified region; intergenic spacer region; root-knot nematodes

Introduction

Root knot nematodes (RKNs) are economically the most important by the great losses they cause in crop yields. Over 90 species (*Meloidogyne* spp.) attacking more than 3 000 species of plants have been described (Adams *et al.*, 2009). The most important and widespread species are *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949; *M. arenaria* (Neal, 1889) Chitwood, 1949; and *M. javanica* (Treub, 1885) Chitwood, 1949; however, currently *M. enterolobii* Yang and Eisenback, 1983 (=*M. mayaguensis* Rammah and Hirschmann, 1988) has become more important worldwide for its high aggressiveness, increasing geographical distribution and wide host range (Castagnone-Sereno, 2012). Aggressiveness is the ability of the nematode to invade and reproduce in a suitable host (Shaner *et al.*, 1992).

The host range of *M. enterolobii* includes several species of crop plants of great economic importance as pepper, tomato, soybean, guava, coffee, beans, watermelon and potato among others (Moens *et al.,* 2009). This species has the ability to successfully establish themselves in genotypes of plants carrying dominant genes conferring resistance to the three major species of RKNs on tomato (*Mi-1*), potato (*Mh*), soybeans (*Mir1*) and pepper (*N* and *Tabasco*) (Castagnone-Sereno, 2012). *Meloidogyne enterolobii* has been detected in Africa, Europe, USA, Central and South America (Brito *et al.,* 2007; Moens *et al*., 2009; Castagnone-Sereno, 2012); and recently was first reported in Mexico parasitizing watermelon (Veracruz state) and tomato plants (Sinaloa state) (Ramirez-Suarez *et al*., 2014; Martinez *et al*., 2015); however, its presence in other crops and in other regions is unknown. In the present research, we report the presence of this nematode in Jalapeño pepper collected in the state of Sinaloa.

Fig. 1. Jalapeño pepper roots severely galled by *Meloidogyne* spp.

Materials and Methods

Biological material

In the municipality of Ahome, Sinaloa, Mexico (25° 58' 33.86'' N and 109° 09' 21.04'' W), in December 2011, root samples of chilli pepper (cv. Jalapeño) heavily galled were collected (Fig.1). Eggs were extracted according to Vrain (1977) and incubated at 28±1 °C in Petri dishes containing sterile distilled water to obtain the second-stage juveniles (J_2). Mature females were obtained by dissecting root galls. The nematode population was increased on tomato plants (cv. Rio Grande) maintained in the greenhouse (Colegio de Postgraduados Campus Montecillo).

Morphological identification

The second-stage juveniles (J_2) and adult females were used. The process of fixation, dehydration and the mounting of J_2 was performed according to Seinhorst (1959) and to Cid del Prado Vera and Subbotin (2012); whereas the perineal patterns were prepared according to Riggs (1990). The observations and measurements of the preparations were carried out under a phase contrast microscope (Olympus BX41, Japan) equipped with a digital camera (Big Catch AM-423X, China). Morphological and morphometric identification was performed according to the original description of the species *M. enterolobii* (Yang & Eisenback, 1983; Rammah & Hirschmann, 1988) based on the distinctive characteristics of RKNs species, such as perineal pattern morphology and head of adult females; and body length, tail and hyaline region of J_2 (EPPO, 2011).

Molecular identification

Genomic DNA extraction. Second-stage juveniles (J_2) were concentrated by centrifugation at 3 000 rpm for 5 min, and the supernatant was discarded. The precipitated pellet in Eppendorf tubes was frozen with liquid N_2 and macerated using a micropestle. The macerate was homogenized with 250 µL of extraction buffer [(500 mM KCl, 100 mM Tris HCl pH 8.0, 1 % Tritón X-100, 400 µg/mL Proteinase K (Sigma-Aldrich®)], and then this mixture was incubated at 60 °C for 4 h and the end at 90 °C for 10 min to inactivate the Proteinase K. Subsequently, the mixture was centrifuged at 5000 rpm for 5 min, the supernatant was placed in a new tube adding 600 µL of chloroform: isoamyl alcohol (24:1) and the new mixture was centrifuged at 10 000 rpm for 10 min. The DNA was then precipitated by adding 100 µL isopropanol and incubating for 30 min at -20 °C; then was centrifuged at 10 000 rpm for 10 min, the pellet was washed with 1 mL of 70 % ethanol (10 000 rpm for 10 min), dried at room temperature and resuspended in 30 µL of nuclease-free water.

Polymerase chain reaction.

PCR was performed using sequence-characterized amplified region (SCAR) specific primers: MK7-F (5'–GATCAGAGGCGGGCG-CATTGCGA-3') and MK7-R (5'-CGAACTCGCTCGAACTCGAC-3') (~520 pb) (Tigano *et al.*, 2010). rDNA-IGS2 (intergenic spacer region 2) was amplified using specific primers Me-F (5'-AACT-TTTGTGAAAGTGCCGCTG-3') and Me-R (5'-TCAGTTCAGG-CAGGATCAACC-3') (~236 pb) (Long *et al.*, 2006). The reaction mixture consisted of: buffer 10X; 1.5 mM $MgCl_2$; 0.2 mM dNTPs, amplicase (Biotecmol); 0.12 µM of IGS2 primers (Me-F/Me-R or SCAR MK7-F/MK7-R); 1 µL DNA and nuclease-free water to a final volume of 25 µL. PCR amplification conditions consisted of an initial denaturation at 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, annealing at 68 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min.

PCR products were separated by electrophoresis in a 1 % agarose gel stained with ethidium bromide; and were purified with QIAquick™ PCR purification Kit (Qiagen) according to the manufacturer's instructions and sequenced to confirm their identity. IGS2 PCR product was sequenced in both directions with IGS2 primers,

whereas the SCAR amplicon was in one direction (MK7-F). The IGS2 sequences were processed using the BioEdit software (version 7.2.5) to obtain a consensus sequence. Nucleotide sequences were aligned using BLAST (National Center for Biotechnology Information).

Results

Morphological identification
The morphological and morphometric characteristics of the sec-

ond-stage juveniles (J_2) and mature females of studied population of RKN were similar to those reported in the original description of *M. enterolobii* (*M. mayaguensis*) (Yang & Eisenback, 1983; Rammah & Hirschmann, 1988). The tail of J_2 was relatively thin, long and rounded end portion and with hyaline region, and sometimes with a lobed terminus (Fig. 2A and 2B).

Measures and ratios corresponded to those reported in the literature. Thus, the average body length was 414.5 ± 22.9 μm and an average body width of 14.05 ± 0.7 μm. The average length stylet 11.3 ± 0.8 μm and an average distance of DGO (dorsal esopha-

Table 1. Morphometry of second-stage juveniles and adult females from chilli pepper (cv. Jalapeño) compared with previous data of *M. enterolobii* described in the literature.

Character	This work	Yang and Eisenback, 1983	Rammah and Hirschmann, 1988
	Second-stage juveniles (J_2)		
	n= 28	n= 30	n= 35
Body length	414.5 ± 22.9[#] (391.5 – 486.8)	436.6 ± 16.61 (405 – 472.9)	453.6 ± 28.4 (390.4 – 528.0)
Greatest body width	14.05 ± 0.7 (13.2 – 15.6)	15.3 ± 0.89 (13.9 – 17.8)	14.7 ± 0.5 (13.8 – 15.8)
Stylet length	11.3 ± 0.8 (10.1 – 13.0)	11.7 ± 0.45 (10.8 – 13.0)	11.6 ± 0.3 (11.1 – 12.2)
DGO	3.4 ± 0.4 (2.6 – 4.2)	3.4 ± 0.33 (2.8 – 4.3)	3.9 ± 0.2 (3.3 – 4.3)
Tail length	54.4 ± 5.5 (40.7 – 61.6)	56.4 ± 4.48 (41.5 – 63.4)	54.4 ± 3.6 (49.2 – 62.9)
Hyaline region length	14.2 ± 0.8 (11.5 – 15.0)	---------	-------
a	29.5 ± 1.6 (26.3 – 33.5)	28.6 ± 1.88 (24.0 – 32.5)	30.9 ± 1.9 (26.4 – 34.7)
c	7.9 ± 0.7 (6.4 – 9.6)	7.8 ± 0.65 (6.8 – 10.1)	8.3 ± 0.4 (7.0 – 9.2)
	Females		
	n= 8	n= 20	n= 35
Stylet length	14.5 ± 1.7 (12.8 – 17.3)	15.1 ± 1.35 (13.2 – 18.0)	15.8 ± 0.8 (13.8 – 16.8)
DGO	4.7 ± 1.1 (3.2 – 6.4)	4.9 ± 0.78 (3.7 – 6.2)	4.8 ± 0.8 (3.5 – 6.7)
Vulva-anus distance	18.5 ± 1.0 (17.4 – 19.6)	22.2 ± 1.76 (19.7 – 26.6)	18.4 ± 1.5 (12.7 – 21.1)
Vulval slit length	23.5 ± 1.9 (20.2 – 25.3)	28.7 ± 1.96 (25.3–32.4)	26.1 ± 1.9 (20.9–30.4)

[#]Average measurements in μm ± standard deviation (range)
a: body length/greatest body width, *c*: body length/tail length
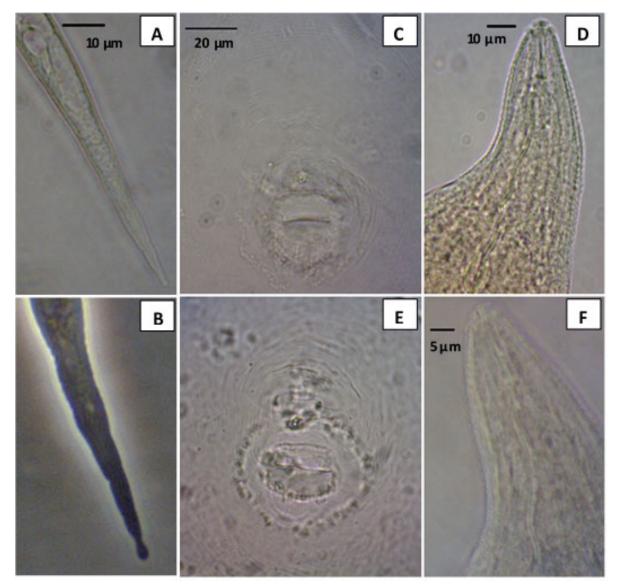DGO: dorsal esophageal gland orifice

Fig. 2. *M. enterolobii*. Second-stage juveniles (J$_2$): Tail region (A), tail terminus (B). Adult females: perineal patterns (C and E), necks (D and F)

geal gland orifice) to the stylet base of 3.4 ± 0.4 µm. The average tail length was 54.4 ± 5.5 µm and an average hyaline region 14.2 ± 0.8 µm. The average of body length/greatest body width (index *a*) was 29.5 ± 1.6; and the body length/tail length (index *c*) was 7.9 ± 0.7 (Table 1). Adult females showed a perineal pattern of oval shape, dorsal arch usually high and round, weak lateral lines sometimes present, large phasmids (Fig. 2C and 2E), typical characters of the species *M. enterolobii*. Also showed a lip region not annulated and an elongated neck (Fig. 2D and 2F). The average stylet length was 14.5 ± 1.7 µm, the average distance of DGO to the stylet base was 4.7 ± 1.1 µm, the average vulva-anus distance was 18.5 ± 1.0 µm, and average vulva slit length was 23.5 ± 1.9 µm (Table 1).

Molecular identification

PCR amplification was positive with SCAR and IGS2 specific primers, where in both cases a single fragment was observed (Fig. 3A and 3B).

DNA sequencing analysis of the PCR products, and subsequent alignment using the BLAST program indicated that the fragment generated with Me-F/Me-R primers exhibited 99 % identity with rDNA IGS2 sequences of *M. enterolobii* (Accession numbers: KM008548, JN005846, GQ395554, GQ395547, GQ395531-GQ395533, and GQ395524). The fragment generated with the SCAR primers (MK7-F/MK7-R) showed 99 % identity with the MK7-SCAR sequence of *M. enterolobii* (supplied by Dr. Valdir Correa and Dr. Regina Carneiro. Embrapa Recursos Genéticos e Biotecnologia, Brazil).
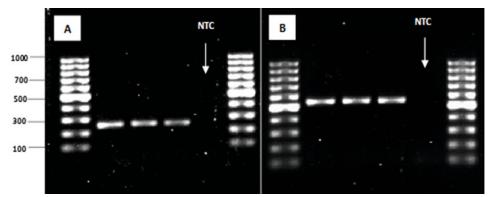
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Fig. 3. Fragments amplified from DNA of second-stage juveniles (J$_2$) using *M. enterolobii*-specific primers: IGS2 Me-F/Me-R (~236 pb) (A) and SCAR MK7-F/MK7-R (~520 pb) (B). NTC: negative control without DNA template. 100-pb ladder (Fermentas™)

Discussion

Meloidogyne enterolobii (=*M. mayaguensis*) is an emerging species, and due to its wide host range is considered to be polyphagous. It is further characterized by its remarkable pathogenicity to overcome resistance to *M. incognita*, *M. arenaria* and *M. javanica* in pepper and tomato genotypes (Brito *et al.,* 2007; Castagnone-Sereno, 2012). The increasing geographical distribution of *M. enterolobii* threatens the production of crops that are of economic importance worldwide. In Mexico, the state of Sinaloa ranks first in tomato and green pepper and second in potato production, these vegetable crops are potential hosts of *M. enterolobii* (SIAP, 2013). In the present study, the presence of *M. enterolobii* parasitizing chilli pepper plants (cv. Jalapeño) in Sinaloa, Mexico was confirmed. Morphometric characters of second-stage juveniles (J$_2$) and adult females usually were within the range reported in the original description of this species (Yang & Eisenback, 1983; Ramma & Hirschmann 1988). A peculiarity of J$_2$ of *M. enterolobii* is the body length (405 – 472.9 μm), which is larger than that of *M. incognita* (360 – 393 μm) (Yang & Eisenback, 1983). In addition to this, the J$_2$ have a greater length of the tail compared to the species *M. incognita* (46 μm), *M. javanica* (49 μm) and *M. hapla* (43 μm), but very close to the value of *M. arenaria* (55.8 μm) (EPPO, 2011). In our case, the average value of the tail was 54.4 μm very close to the populations of *M. enterolobii* from China (Yang & Eisenback, 1983) and *M. mayaguensis* from Costa Rica (Ramma & Hirschmann, 1988). Perineal patterns showed a dorsal arch rounded which is typical of *M. enterolobii*, and usually sets it apart from the common species *M. incognita*, *M. arenaria*, *M. javanica* and *M. hapla* (EPPO, 2011). It should be mentioned that it is not unusual that overlaps occur between the values of the different species of root-knot nematodes. Such situations do not allow a correct differentiation between these species, so the use of more than one identification technique is necessary, as was carried out for the population we analyzed.

Molecular analysis of the DNA amplified fragments of ~236 and ~520 bp obtained by using IGS2 and SCAR primers, respectively, revealed that they corresponded to those reported (Long *et al*., 2006; Tigano *et al*., 2010). The percentages of similarity of the IGS2 amplicon with the corresponding sequences found in Genbank (NCBI) were high (99 %), whereas for the SCAR amplicon (~520 bp) no corresponding sequences were found in the database. The reason for this is that in such database SCAR sequences of this species have not been registered yet; however, the amplicon obtained exhibited 99 % similarity to the sequence SCAR-MK7 supplied by the researchers who designed the primers specific for *M. enterolobii* (Tigano *et al.,* 2010; Correa & Carneiro, personal communication). The results obtained in this research indicate that the root-knot nematode present in Ahome, Sinaloa, corresponds to *M. enterolobii*. To our knowledge, this is the first report of *M. enterolobii* in chilli pepper in this region of Mexico, considered one of the vegetable crop areas of major economic importance.

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