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Diagnostic of new species of root knot nematode *(Meloidogyne cruciani)* associted with egg plant in babylon governorate/Iraq based on morphological characters and molecular methods

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ABSTRACT

Meloidogyne cruciani new species infecting eggplant (*Solanum melongena* L.) in Babylon governorate in Hashimia/Shomali districtis defined and demonstrated. *M. cruciani* is diagnosed based on morphological characters of perineal pattern to adult females as well as depended on molecular methods to diagnose. The results of Morphological characters of perineal pattern were showed punctuations around the anus of the female which are distinguished this species from other species that were showed in this study (*M. javanica* and *M. incognita*). Molecular methods were agreement with morphological characters when depended on partial sequence of the 18S nuclear rDNA region by using MelF /MelRuniversal primer for *Meloidogyne* species. The results of data sequences were showed present of *M. cruciani* when compared with GenBank sequenc-es that obtained to several species showed 100% maximum nucleotide identity with France isolate *M. cruciani* (HE667740.1). sequence of Iraq *M. cruciani* was deposited in GenBank database with accession number KY659414.1. The study presents the first molecular confirmation and sequence data of presence of *M. cruciani* in Iraq.

Key words *: Meloidogynecruciani, 18S nuclear rDNA region, Eggplant, MelF /MelR universal primer, Babylon Governorate/ Iraq.*

Introduction

The root-knot nematode is one of the most important pathogen affecting in many crops especially in vegetables cropsin all parts of Iraq (Stephan *et al.,* 1998). Four species of RKN, *Meloidogynejavanica*, *M. incognita*, *M. arenaria* and *M. hapla*, are major responsible for 95% of damages (Sasser *et al.,* 1983) through the past decades, a number of researches have been conducted on root-knot nematodes in Iraq (Stephan *et al.,* 1985; Stephan, 1987; Staphan, 1988; Al-Sabie and Ami, 1990). However, an inclusive work, so far, has not been done on the distribution and diagnosis of the new species of RKN that may be present in Iraq. true identification of *Meloidogyne* species is importance in terms of vegetable growing and breeding. *Meloidogyne* species have been identified based on distinct morphological characters included perineal patterns of females, and overall body sizes of juveniles, males, and females (Eisenback and

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Triantaphyllou, 1991), differential-host test (Hartman and Sasser, 1985). Since that time the identification of *Meloidogyne* species by means of perineal patterns of females became the dominant diagnostic character of the four most common *Meloidogyne* species (Chitwood, 1949) as well as, implementation of these methods only on female individuals are limited factor, difficult, and can be influenced by environmental factors (Devran and Söðüt, 2009). Therefore, most species of *Meloidogyne* are difficult to identify based on morphological characters due to their similarity to other species and poor taxonomic descriptions, or may result from morphological variations within and between populations of a same species (Kaur, 2012). accordingly, numerous research groups have been evolving molecular techniques for their identification. Protein electrophoresis was the first molecular technique to be utilized in nematology (Subbotin and Moens 2006) many studies have begun to focus on identification of *Meloidogyne* species based onmolecular techniques that depending on genetic diversity of species (Powers and Harris, 1993; Powers *et al.,* 1997; Ziljstra *et al.,* 1995; Zijlstra *et al.,* 2000; Adam *et al.,* 2007).

The aims of this study were to identify root-knot nematodes collected from the Babylon governorate Iraq using morphological characters and molecular techniques, and to select efficient primers from previous studies for *Meloidogyne* species identification in this region.

Materials and Methods

Sampling

Fourteen eggplant root samples were collected randomly from eggplant field was grown in Babylon governorate in Hashimia/Al-Shomali district during the 2017-2018 growing season to determine root-knot nematodes. Root samples were put on plastic bags and then brought to the laboratory of Nematology belongs to Dept. of Plant Protection, Faculty of Agricultural/Univ. of Baghdad.

Morphological identification of *Meloidogyne* species

For morphological identification of *Meloidogyne* species, ten mature laying eggmass females were extracted from each infected root system (total number of mature females were 140) based on method of Eisenback *et al.* (1981) after that mature female was put indrop of 45% lactic acid on prepper glass slide, according to Taylor and Netscher (1974) and Hartman and Sasser (1985), and then the posterior end cut off with an optical scalpel and body tissues were removed by slightly brushing the inner surface of the cuticle with a lightly supple bristle when all tissues were removed, the cuticle was carried to a drop of glycerine where it is care. Eventually the piece of cuticle with the perineal pattern is then transferred to a drop of glycerine and covered by cover slide. The slide sample was observed under compound microscope on 400x.

Molecular Methods

DNA extraction: DNA was extracted from single eggmass by using Quick-DNA™ Fungal/Bacterial Miniprep Kit Catalog No. D6005 that it was used for nematodes too. For disrupting eggmass shell a ZR Bashing Bead™ Lysis Tube (0.1 mm and 0.5 mm that it was found with Kit) was used for 10 min at maximum speed and then completely manufacturer protocol was followed for all samples. DNA quality was determined byagarose gel electrophoresis (Sambrook and Russel, 2001), and DNA concentration was determined by using a Nanodrop.

DNA amplification (Polymerase chain reaction) and DNA sequencing: DNA amplification was done in a final volume of 25 µL reaction volume containing 5 µL of1X PCR buffer(Maxime PCR PreMix kit (i-Taq) Cat. No. 25025 / iNtRON's), that containing:2.5U/µLi-Taq DNA Polymerase, 400µMdNTPs, 4 Mm $MgCl_2$, 1X Gel loading buffer,

Table 1. List of Sequences of primers and target regions to amplify of genes in *Meloidogyne* species.

Primer name	Primer sequence5'-3'	Target region	Target species	Size of bands
MelF	TACGGACTGAGATAATGGT	18S DNA	*Meloidogyne* spp.	902bp
MelR	GGTTCAAGCCACTGCGA			
JavF	GGTGCGCGATTGAACTGAGCA	SCAR marker	*M. javanica*	670bp
JavR	GGCCCTTCAGTGGAACTATAC			

*All primers were obtained from Integrated DNA Technologies (IDT) Canadian company.

and added 1µL of each primer (10Pmol/µL) and 2µL of total DNA then complete volume of reaction with 16 µL of distal water. Primer- pair of MelF/MelR was used to amplification of 18S nuclear rDNA region in *Meloidogyne* spp. (Tigano *et al.,* 2005) and primer-pair specific to *M. javanica* (Jav-F/Jav-R) was used to amplification Sequence Characterized Amplified Regions (SCAR marker), that it was developed by Zijlstra *et al.* (2000), Table 1. PCR program for MelF/MelR primer was performed at: 94 °C initial denaturation for 7 min, followed by 35 cycles of 1 min at 94 °C (denaturation), 1 min at 58 °C for primer annealing and 1min at 72 °C for primer extension and 10 min of final extension at 72 °C for 1 cycle. while PCR program for Jav-F/Jav-R primer was performed at: 94 °C initial denaturation for 5min, followed by 35 cycles of 30 sec at 94 °C (denaturation), 30 sec at 64 °C for primer annealing and 1 min at 72 °C for primer extension and 10 min of final extension at 72 °C. All PCR product were separated on a 1.5% agarose gel electrophoresis and visualized by exposure to ultra violate light (302 nm) that were stained by Red Stain staining. Samples were sent to Korean Macrogen company for sequencing by using forward primers for each DNA region. Sequence results were aligned using BioEdit Sequence Alignment Editor, and compared with previously sequenceswere deposited in the National Center for Biotechnology Information (NCBI) Gen Bank for species analysis (Zeng *et al.* 2014). The sequences of *Meloidogyne* species in this study were submitted in Gen Bank under accession numbers MG696872.1, KY659414.1 and MG682574.

Results and Discussion

Morphological characters of perineal pattern to identify *Meloidogyne* species: The results of perineal pattern were appeared present *M. javanica* in eggplant field in the rate of 79.28%. under the light microscope characterized of the perineal patterns of *M. javanica* were ideal for *M. javanica* with a rounded to flatten dorsal arch and apparent lateral lines that visibly separated the dorsal and ventral regions of the patterns (Fig. 1. A, B). While the results were showed present *M. incognita* in 16.43% eggplant field. Characterized of Perineal pattern to *M. incognita* was showed high dorsal arch with wavy striae that curvature toward the laterial incisures (Fig.1. C, D). also recorded present new species of *Meloidogyne* which called *M. cruciani* that perineal pattern of it was described by Garcia-Martinez *et al.* (1982) that characterized in subcuticular stippling ahnost surrounding anus on lateral and posterior sides Striae deep, wavy, sometimes broken. Lateral field fairly deep. Vulva lips faintly serrated, margins with very fine striae. Tail terminus indistinct (Fig. 1.E, F). Garcia-Martinez *et al.* (1982) were who the first described and illustrated *M. cruciani* infecting tomato (*Lycopersicon esculentum* Mill.) in the U.S. Virgin Islands and they depended on morphological characterized of perineal pattern to distinguish front other species moreover they were mentioned perineal patterns of *M.cruciani* similar with *M. javanica* in having eminent lateral lines but differ in tile pronounced lateral lines of *M. cruciani* do not expand as far as of *M. javanica* therefore in this study depending on molecular methods in addition to morphological charachtraized of perineal pattern to adult females. Molecular methods were based on PCR band fragments analysis of 18S rDNA sequencing of the RKN species (Ye *et al.,* 2015; Zeng *et al.,* 2014). The results were showed when used Mel F/R primer, universal primer that detection for 18S DNA region, all sample were given single band was 902bp (Fig. 2a). These results were agreement with results of Tigano *et al.* (2005) and Herrera *et al.* (2011). While when used SCAR-PCR marker, (Jav F/R) specific primer, only seven samples were given a single fragment of 670bp as expected (Fig.2b). These results correspond with Zijlstra *et al.* (2000). Whilst the samples that don't given any fragment when used SCAR-PCR marker but they were given single band in universal primer (Mel F/R primer) PCR products of them were sent to Macrogen company for DNA sequencing analyses. The results of sequence of Mel F/R, that detected of 18S rDNA region, were showed present *M. incognita* which compatibility ratio was 96% with *M.incognita* (MH983020.1). The results of size and specificity of PCR product for SCAR-PCR marker (F/R javprimer) were agreed with studies ofHasan and Abood (2018) that were showed present *M.javanica* and *M. incognita* in Iraq when used SCAR-PCR marker and 18S rDNA universal primer and they deposited sequences of these species in GenBank database with accession numbers MG682574.1, MG696872.1, respectively. Moreover, new species of *Meloidogyne* called *M. cruciani* was recoded in our study when used 18S rDNA universal primer and then the sequence of this species deposited in GenBank database with accession num-

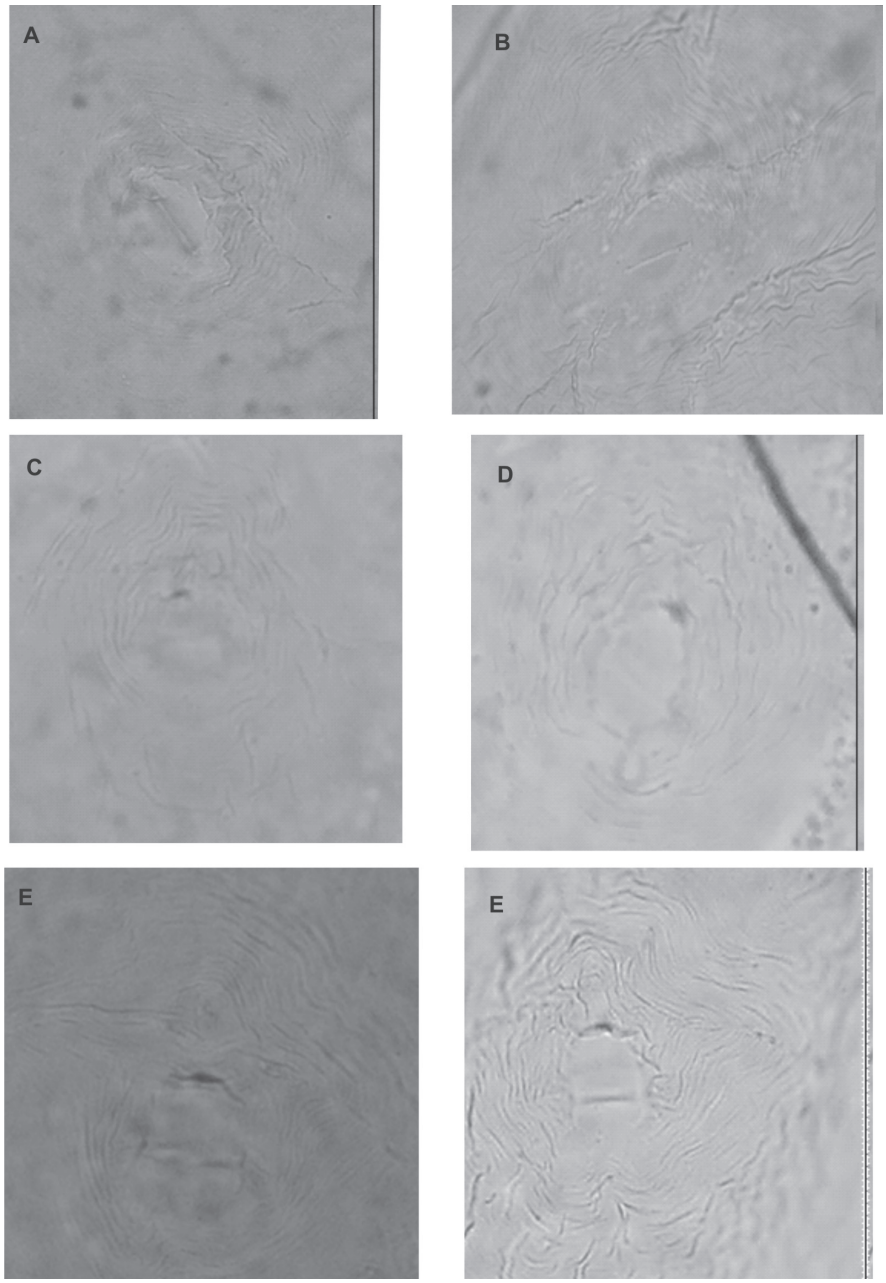

Fig. 1. Perineal pattens of *Meloidogyne* species in eggplant field **A-B,** *Meloidogynejavanica* **: C-D,** *M. incognita*: **E-F**, *M. cruciani*

ber KY659414.1. *Meloidogyne cruciani* was first recorded in tomato Rutgers *(Lycopersicon esculentum* Mill.) in the U.S. Virgin Islands (Garcia-Martinez *et al.,* 1982). *M.cruciani* was also recorded in France based on genetic methods (Tomalova *et al.,* 2012). As a result of this study, *M. cruciani* was identified in Babylon -Iraq, which is the first report of this species in Iraq, specifically Babylon governorate in Hashimia/Shomali district. Phylogenetic tree of the Iraqi isolation (*M. cruciani*) was drawnto illustrate the relationship between species that deposited on GenBank database based on the 18S rDNA gene region by using Mega 6 program. Fig. 3 illustrated relationship of *M. cruciani* with nineteen sequences of global isolates in GenBank were divided into two main nodes (A and B) node A included three inter-

nal nodes, first internal nodes were included four isolations (KP901046.1, KJ641552.1, KP90106.1 and KP9016064.1) that identity of99%. Second internal nodes were included Iraqi isolation (*M. cruciani*) KY659414.1 which was most similarity to French isolation HE667740.1 with asequence identity of 100% while similarity was 99% with Brazilian isolation AY942626.1. Finally, the third internal nodesin main node (A) included American isolation AF442193.1 which similarity with Pakistan isolation JQ806343.1 and JQ806345.1 was 99%. While second main nodes (B) were divided into two internal nodes. First internal nodes were included three isolations (KF993644.1, KM100868.1 and KP901060.1) which similarity was 100% between them. While second internal nodes were included two branches, the first was included three Brazilian isolations (AY9426224.1, AY9426223.1 and AY9426234.1) that similarity between them was reached 99%. While second branches included four isolations. Two Taiwan isolations (XJ100422.1 and XJ100421.1) that similarity with Brazilian isolation (AY942632.1) was reached 98% as well as these three isolations were similarity with Taiwan isolation (JX100420.1) reached 99% Fig. 3. The results of this study was first report that recorded present of *M. cruciani* in

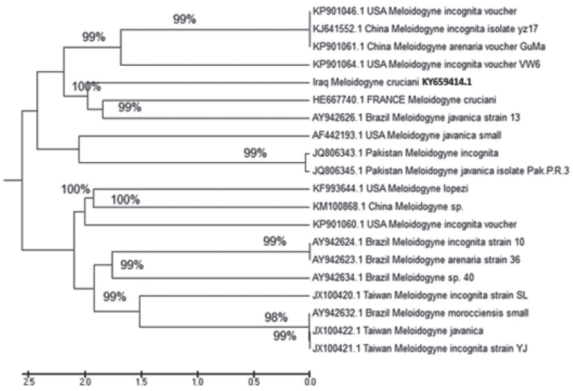

Fig. 3. A neighbor-joining phylogenetic tree of *Meloidogynecruciani* in this study was built based on 18S rDNA sequences. Numbers upper the branches are bootstrap values 50% majority rule agreement tree. The texts near of the species names are indicated to the accession number of Iraq isolate and those were selected from the GenBank. This tree was created by MEGA6 software.

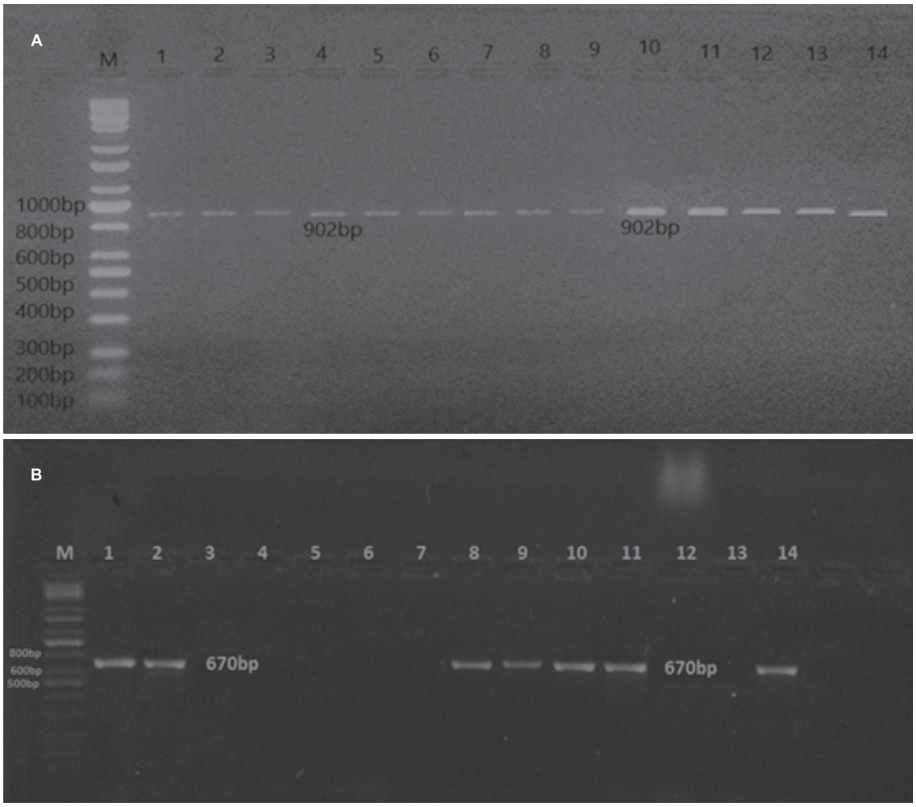

Fig. 2. A- amplification products of PCR reaction to the partial sequence of the 18S nuclear rDNA region by using MelF /MelR unversial primer for *Meloidogyne* species with a 10000 bpmarker, B- amplification products of PCR reaction to SCAR marker by using JavF /JavR primer for *M. javanica*with a 10000 bpmarker.

Iraq and in the Arab world that mentioned deposition database sequence of this species in GenBank database.

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