

Genetic Fingerprinting of Root Knot Nematode as important Pest in EGYPT

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ABSTRACT

Root knot nematode is considered as one of the most important nematode all over the world in Egypt it was found out that these nematode attack the root system of most vegetables, fruits and Feld crops causing significance damages and economic loss. Using the morphological characterization isn't enough for nematode identification to species. So different molecular technology was used to identify different isolated of these nematode for fast and more accurate results. The internal transcript spacer (ITS) and restriction fragment length polymorphism (RFLP) of Ribosomal DNA (rDNA) sequences were used to distinguish between different major species of root knot nematode. DNA fragments containing the internal transcribed spacer (ITS) rDNA were amplified from DNA genome of different *Meloidogyne* spp. isolates from different governorate in Egypt. Ten females were used to amplify PCR product. When primers 5368 and 5367 were used for amplification of the ITS region, every isolate from the *Meloidogyne* spp. gave one major product of approximately 760 bp.

When ITS regions of all isolates were digested with restriction enzymes, by using RFLP test (Restriction fragment length polymorphisms), the size of the DNA fragment using Hind III was strong band at 560 bp and another weak band at 200 bp that is typical for *M. incognita* and *M. javanica* with Hinf I, bands of 440 and 320 pb were obtained while 520, 240 bp bands were cleared when EcoRI are used. Four bands of 220, 200, 180 and 160 bp were obtained when I restriction enzyme was used. In multiplex test, a fragment of 415 bp was obtained when *M. incognita* was used as template (positive control), that confirming the fact that most nematodes isolates in Egypt are *M. incognita*

Keywords: *Meloidogyne*, multiplex, PCR, ribosomal DNA, ITS, RFLP, Scar, restriction enzymes.

Introduction

During the past few years, rapid advances in molecular biology have affected our understanding of plant diseases and revealed many powerful tools that help in many areas of nematology. The new tools include species, race and patliotype identification, development of resistant cultivars, population dynamics, development of useful biological control agents and the design of new nematicides.

This review will highlight the area of biotechnology for the prospects of future advances in applied nematology that is extremely useful nowadays.

In the beginning, it is very important to reach an accurate identification of nematode species, it is the fundamental of nemato-logical research, quarantine enforcement for regulatory purposes and nematode management, especially management that does not rely on nematicides. An immediate objective of new molecular biology techniques in nematology is to increase the accuracy and speed of genus and species identification.

A few years ago, root-knot nematodes (*Meloidogyne* spp.) are sometimes difficult to be identified using traditional morphological characterization, so alternative identification methods that are easily interpreted are desirable. It is now possible to distinguish among root knot nematodes by the use of DNA markers, the big advantage of using DNA that it does not change with environmental conditions during a nematode's lifetime. Consequently, DNA markers are stable and useful when applied for identifying nematode species and

populations. Various DNA polymorphisms that allow identification of the major root-knot nematode species have been described also polymerase chain reaction (PCR) is a recently developed technique that has been of particular consequence to molecular genetics because it allows amplification of specific sequences from minute quantities of DNA. In PCR two oligonucleotide primers are used to hybridize to opposite DNA strands flanking the desired sequence in the target DNA, so that DNA polymerase can create a copy of the intervening target DNA. The exponential amplification mediated by cycles of heating and cooling can produce 10⁶ - 10⁹ copies of the target DNA sequence. The technique is effective with samples containing little biomass; for example, it has been used to amplify DNA from single human sperm or from blood stains.

Restriction fragment length polymorphisms (RFLPs) generated after PCR amplification of ribosomal DNA can be used for identification of single root-knot nematode (2nd Juvenile J2). The technique uses PCR and primers to generate DNA fragments that are subsequently subjected to restriction endonuclease digestion, yielding species-specific RFLPs that allow discrimination of different root knot nematode species such as *M. arenaria*, *M. chitwoodi*, *M. incognita*, *M. javanica*, and *M. hapla*.

Random amplification of polymorphic DNA (RAPD) assaying the entire genome for DNA markers is a powerful way to obtain markers linked to characters of interest. The RAPD assay refers to PCR amplification of target DNA with single primer, and hence produces DNA fragments distributed over the

Table (1)

Governorate	No. of regions	Major crops in the area
Beni Suief	4	Tomato, cucumber, lettuce
Al Gharbya	4	Banana, Grape, Apple, vegetables, Israeli Banana
Al Qaliubya	6	Tomato, cucumber, squash
AL BUHEIRA	6	Cucumber, Peach, Tomato, Eggplant, Beans
SUHAG	10	Grape, Cucumber, Tomato, Banana, Horticultural Crops, banana
ASSUIT	6	Pomegranate, Banana, Eggplant, Tomato
Al Minya	9	Okra, Eggplant, banana, tomato
Al Sharqya	7	Eggplant, Tomato, Cucumber
Al Ismailia	5	Tomato, Black nightshade
Al Monofya	1	Grape

Preparation of pure culture from root knot nematode samples: One adult female with one egg mass from each sample were propagated in pots filled with sterilized sands under greenhouse condition to insure the purity of the population for each sample that will be used for DNA extraction (fig 2). Preparation of root explant cultures was accomplished according to Haroon et al., (1993). Seeds were sinicated for 3 min. in 95% ethanol followed by a 10-min soak in 0.5% sodium hypochlorite and were transferred to sterile 1.5% water agar plates where they were maintained for 3 days at 25°C. A 5-mm-long root tip was excised from each seed-plate (one root tip per plate) containing Gamborg's B-5 medium (Gibco, Grand Island, N.Y.). Each plate was inoculated with one egg mass that was extracted from one population of each genera of root knot nematode. All the plates were stored in an incubator at 28°C and three months later 25 females from each dish were isolated for DNA extraction (Fig.3)



(Fig 2)

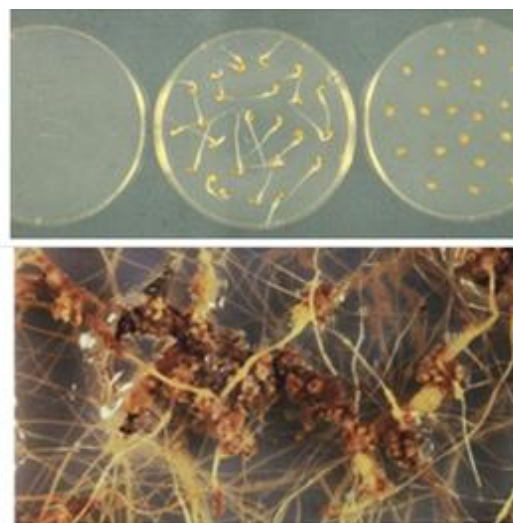


Fig (3): Tomato seeds in water agar and plates after 3 months of inoculation with RKN

FIRST: DNA isolation for Root Knot Nematode molecular identification: DNA

isolated from different samples was done using Genomic prep™ Cells and Tissue DNA Isolation kit (Amersham Pharmacia Biotech, ltd, England). **Isolated DNA was measured to determine the purity and quality to be used for further experiments as following:**

Step 1: Ribosomal DNA amplification (ITS) Primers 5367 (5' - TIGATI

ACGTCCTGCC CTTT-3') and primer 5368 (5' TTICACTCGCCGTIACAGG3') were sequences as described by Vrain et al.,(1992) and Vrain (1993). The DNA fragments containing the ITS regions were amplified by PCR. The reaction mixtue contained 10 mM Tris, pH 9.0; 1.5 mM MgCl₂; 50mM KCl; 0.01 % (wt/vol) gelatin; 0.1% Triton X-100; 100 uM each of dATP dTP dGTP and dTTP (Boehringer GmbH, Mannheim, Germany); 0.6uM each primer; 0.1 to 10 fig of total DNA or the crude DNA extract from 25 females of each isolate; 0.6 units of Taq DNA polymerase (Sphaero Q. Leiden, the Netherlands); and deionized water to a volume of 50 pi. The amplification was carried out in a thermal cycler (PCR) DNA

Initially PCR amplification conditions were as follows: denaturation at 94°C for 2 min, repeated for 20 cycles. A 5-min incubation period at 72.C° followed the last cycle to complete any partially synthesized strands. The fastest available transition between each temperature was used (Zijlestra et al., 1995).

Step 2: ITS-RFLP: Amplified DNA of ITS fragments were digested with Hind I, Hinf I, EcoRI and Dra 1 restriction enzymes. A master mix was prepared by using 1 ul of selected enzyme, 1.5 ul of matching buffer and 12.5 ul of DNA template (obtained from ITS test). The mixture for each sample was placed in incubator at 37°C for at least 3 hours or until the second day. The digested DNA was loaded on a 2.5% agarose gel, separated by electrophoresis and detected by ethidium bromide staining.

Step 3: Species specific (Multiplex) PCR: For conducting the species “specific (multiplex) PCR test, amplifying segments of the ITS region of each isolate was used. This technique was used to identify root knot nematode species by the size of their amplified fragments in a signal PCR reaction. In other words the test was used for direct identification of the four species based on length polymorphism. The reverse primer HCFI-28S is the backward primer as described by Ferris et al. (1993).

The reaction mixture contained 10 mM Tris pH 9.0, 3.5mM MgCl₂, 50 mM KCl, 200 uM each of dATP, dCTP, dGTP, and dTTP, 0.4 uM of each forward primers H-18S (5'-CTTGGAGACfGTTGATC3'), CF-ITS (5'-GATTATACGC ACAATT), and I-ITS (5'^TGTAGGACTCTTTAATG 3') and 0.4 uM of the reverse primer HCFI-28S. One unit of Taq DNA polymerase (Pharmacia), 3 ng of total DNA or the crude DNA extract from 25 female, was mixed with deionized water to a volume of 50ul. PCR cycle was denaturation at 94C° for 4 min. followed by five cycles at 94C° for 30s, 55C° for 30 s, 72.C° for 1 min. with a decrease of 1 C° per cycle for the annealing temperature followed by 25 cycles at 94C° for 30 s, 50C° for 30 s, and 72C° for 1 min.

2. Results:

Five thousand samples of nematode populations were collected from different agriculture sectors of each governorates that were considered as its important in crop production in Egypt. The survey covers 47 different crops. Results presented show the prominence values of the eleven plant parasitic nematode genera, including ectoparasitic, endoparasitic and semiendoparasitic forms, which were recovered from the soil samples. These genera were *Meloidogyne* spp., *Pratylenchius*, *Helicotylenchius*, *Hemicycliophora*, *Hoplolaimus*, *Paratylenchus*, *Rotylenchius*, *Tylenchorhynchus*, *trichadorus*, *longidorus*, *tylenchulus* and *Xiplinema*. The root-knot nematode, *Meloidogyne*; the lesion nematode, *Pratylenchius*; the reniform nematode,

Rotylenchius; the stunt nematode, *Tylenchorhynchus* and the lance nematode, *Hoplolaimus* were the major nematodes distributed. Data revealed that *Meloidogyne*, *Pratylenchius* and *Rotylenchius* were found with relatively higher frequency of occurrence (fig 4), (fig 5), (fig 6A&B)

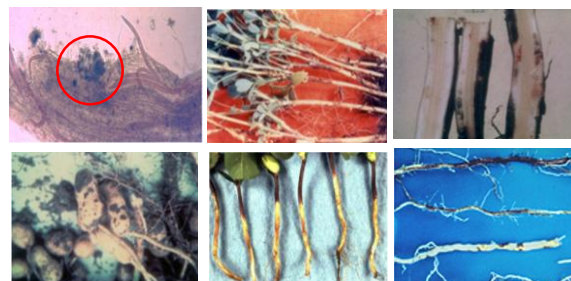


Fig (4) : lesion nematode in clover, Banana, Peanut, strawberry and roses respectively



Fig (5) : cotton Nematode in cotton plants

It was very important to identify all the isolated of Root Knot Nematode, to species , races and population as we found out that the majority of crop s is seriously damaged with this group of nematodes, so using this molecular techniques is important to design effective and suitable program of IPM for control this pest

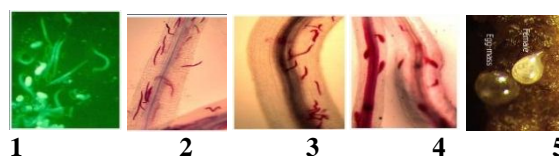
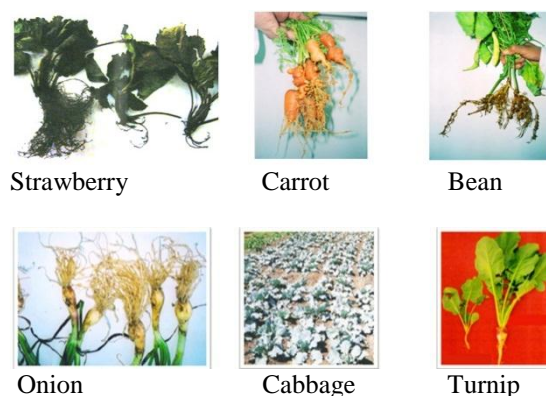


Fig (6 A): Development of Root knot nematode



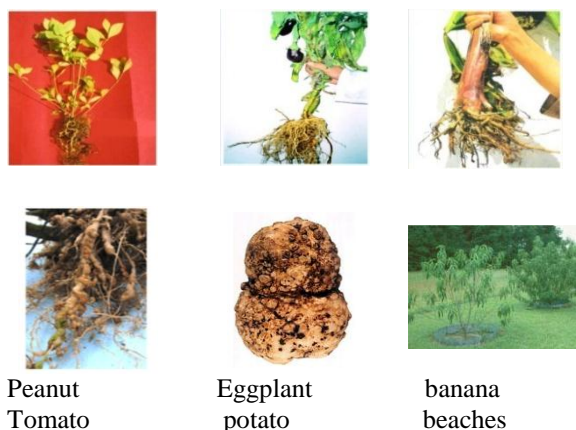


Fig (6 B): Root knot nematode symptoms in different plants

Table 2. The Distribution of different Root knot nematode different species all over Egypt.

Governorate	Major crops in the area	Root Knot nematode genus
Beni Suief	Tomato, cucumber, lettuce	<i>M. incognita</i> , <i>M. Javanica</i>
Al Gharbya	Banana, Grape, Apple, vegetables, Israeli Banana	<i>M. Javanica</i> <i>M.arenaria</i>
Al Qaliubya	Tomato, cucumber, squash	<i>M. incognita</i>
AL BUHEIRA	Cucumber, Peach, Tomato, Eggplant, Beans	<i>M. incognita</i> , <i>M. Javanica</i> , <i>M. arenaria</i> , <i>M.hapla</i>
SUHAG	Grape,Cucumber, Tomato, Banana, Horticultural Crops, banana	<i>M. Javanica</i>
ASSUIT	Pomegranate, Banana, Eggplant, Tomato	<i>M. incognita</i> , <i>M. Javanica</i>
Al Minya	Okra, Eggplant, banana, tomato	<i>M. incognita</i>
Al Sharqya	Eggplant, Tomato, Cucumber	<i>M. incognita</i> , <i>M. Javanica</i>
Al Ismailia	Tomato, Black nightshade	<i>M. incognita</i>
Al Monofya	Grape	<i>M. incognita</i> , <i>M. Javanica</i>

A isolation for Root Knot Nematode molecular identification:

Step: ITS Test:

After the measurement of all obtained pure DNA from each isolate of root knot nematode, the first test (ITS) was conducted for each isolate separately to insure that the entire DNA is for root knot nematode. So, primer 5368 and 5367 were used for amplification of the ITS region, all the root knot isolates from different governorates and districts gave one main product of approximately 760bp. This 760 bp PCR product obtained from the amplified ITS region of *Meloidogyne* was shorter than the product reported for other nematodes (Vrain et al., 1992; Zijlestra et al., 1997).(Fig. 7)

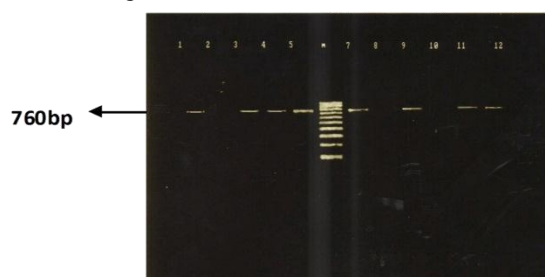


Fig (7): Typical amplification of 760bp polymerase chain reaction — PCR product from template of total DNA extracted from *Meloidogyne* spp. of different isolates from different governorates and districts with internal transcribed spacer (ITS) with primers 5367 and 5368.

Step 2: Multiplex Test:

Sequencing data reveal that the sizes of the cloned amplified ITS regions of *M. incognita* is 760 bp. First, PCR reactions were performed with HC FI — 285 and one of the three forward primers. The sizes of the fragments obtained after amplification could be estimated from the sequences.

The combination of primers H-18S and HCFI - 28S resulted in amplified fragment of 660 bp. this fragment is typical for *M. hapla*. No reaction with any other governorates isolates was shown the amplified fragment of 660bp was recognized. PCR with primers ITS and HCFI - 28S resulted in amplification of a 415 bp fragment when *M. incognita* was used as template. The majority from all governorates isolates react with this combination of the primers. (Fig. 8)

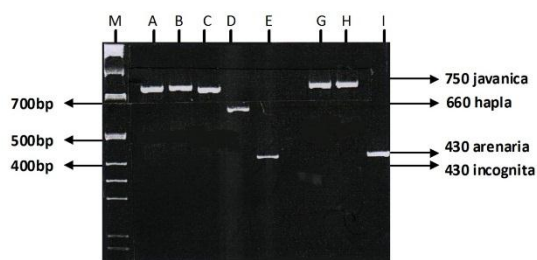


Fig (8): Amplification products of multiplex PCR reactions using forward primers H- 18S, I — ITS, EF — ITS and the reverse primer in a single PCR reaction with 3ng of template DNA of root knot nematode population collected from different governorate of Egypt, when ITS primer sets H-18S/HCFI-28C used with *M. hapla* the result was 660bp, when ITS Prime sets I-ITS/HCFI-28S used with *M.incognita* the result was 415bp, and when ITS Prime sets H-18S/CF-ITS/HCFI-28S used with *M.javanica* the result was 750bp and when ITS primer sets ITS / HCF1, 28C used in *arenaria*

ITS regions of all isolates were digested with restriction enzymes. When Hind III was used in RFLP test (Restriction fragment length polymorphisms), the size of the DNA fragment obtained was not clear while 520, 200 bp bands were cleared when EcoRI are used with isolates from *M.incognita* and *M.javanica*, also two bands were recognized at 440 bp and 320bp when Hind I was used with isolates from the same nematodes species. These results were typical for *M. incognita*, and *M.javanica* confirming the previous findings by Zijlstra et al., (1995 and 1997). (Fig. 9)

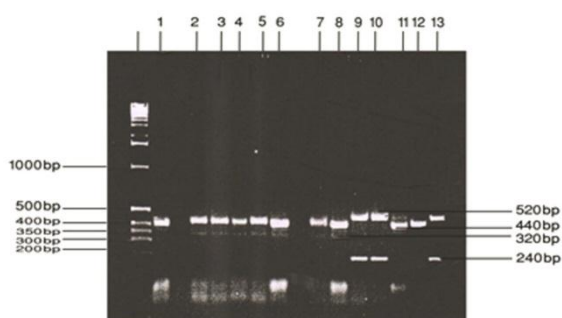


Fig (9) Restriction product OF 760 bp polymerase chain reaction amplified internal transcribed region of *Meloidogyne spp* upon restriction with Hind I, EcoRI

Discussion and Conclusion:

This research confirms that Root-knot nematode (*Meloidogyne spp.*) is the major pests in Egypt, causing yield losses to many economic crops table . There are many research opportunities in basic nematology that utilize the new approaches and tools of molecular biology. The tools will facilitate research on previously intractable questions in nematode biology; also current nematode control options include the use of nematicides, cultural practices, resistant cultivars, and crop rotations. In the future,

successful integrated nematode management will depend on combinations of control tactics to reduce nematode numbers. Application of biotechnology to nematode control tactics will influence applied nematology in diverse ways, from nematode identification to the development of resistant cultivars, and will improve effectiveness and increase the number of management options available. Increased communication and cooperation between researchers with expertise in applied science is necessary to enlarge our understanding of nematode biology and ecology. The outcome will enhance development of new nematode management strategies and allow refinement of old technique.

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