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THE USE OF DIFFERENT MOLECULAR BIOLOGY TECHNIQUES FOR ROOT-KNOT NEMATODE IDENTIFICATION

By

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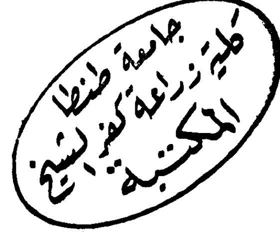

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INTRODUCTION

The genus Meloidogyne comprises a widely distributed group of plant-parasitic nematodes and serious pest problems in the agricultural areas of the world and Egypt in particular **(Ibrahim *et al.*, 1976) is** usually known as root-knot nematodes. Over 55 species have been described but only four account for more than 90% of the worldwide estimated damage caused by root-knot nematodes **(Eisenback *et al.*, 1981)**. So, the diagnosis of root-knot nematode techniques for species identification of *Meloidogyne* spp. includes morphological characters, host range tests, isozyme electrophoresis and DNA technology **(Haris *et al.*, 1990 and Eisenback and Triantaphylou, 1991)**. Morphological criteria such as the markings of the cuticle, shape of the stoma and typed of esophagus have been used for the identification and taxonomic criteria. Also, larval measurements, head shape, and stylet morphology of males, and perineal patterns of females used to differentiate the root-knot nematode. Furthermore, differential hosts with perineal pattern were used to determine the races within the same species **(Hartman and Sasser, 1985)**. These morphological methods have been problematic due to variability and the need for specialized personal to perform the species determination.

Therefore, other identification methods which have been used, isozyme electrophoresis are highly reliable, allow species identification of single female and useful for routine species of root-knot nematodes **(Cenis *et al.*, 1992)**. However, isozyme electrophoresis does not work well with single second-stage juvenile (J_2), so samples subject to isozyme analysis must contain young healthy females for successful identification. Also, isozyme analysis does not detect much intraspecific variation

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among Meloidogyne nematodes **(Cenis, 1993)**. In practice, soil samples often contain only juveniles or eggs and thus require greenhouse culture to obtain females for isozyme analysis.

The appearance of nucleic acids technology has allowed new approaches to get a reliable and precise nematode identification. So, diagnosis by using molecular methods can be a good tool for the defects of using the traditional methods.

The polymerase chain reaction (PCR) is a new development that enable the exponential amplification of DNA starting from a single molecule. Thus PCR allows the identification of some Meloidogyne stages as single eggs (**Harris *et al.*, 1990**) which are indistinguishable by any other techniques.

The use of DNA markers in genetic diagnosis has been well established. **Williams *et al.*, 1990** described genetic variations which are based on DNA amplification called RAPD markers (Random Amplified Polymorphic DNA) generated by the single primers of arbitrary nucleotide. These RAPD techniques are used for genetic diagnosis. The technique is simple, rapid (three to six hours depending on the thermal cycler) and safe because it does not involve the use of radioactive and require little amounts (micrograms of DNA), this makes it possible to analyze little materials as a single juvenile or egg which is useful in studies of genetic variation or diagnosis of mixed populations (**Cenis, 1993**).

For molecular analysis it is useful to study specific genomic regions. One such region is the ribosomal DNA (rDNA). Comparative analysis of coding and non coding regions of ribosomal DNA (rDNA) is

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becoming a popular tool for species and subspecies identification of many organisms. The advantages of this rDNA analysis are considered fast, reliable and useful for identification at any developmental stage especially when applied on individual nematode.

The aims of this study are:

- Using ITS regions (Internal transcribed spacer) as ribosomal DNA to differentiate the tested root-knot isolates.
- Using restriction enzyme analysis of ribosomal DNA ITS-RFLPs sequences to distinguish species and isolates of root-knot nematodes.
- Using multiplex-PCR to distinguish isolates of root-knot nematodes.
- Using species specific PCR primer SCARs (Sequence Characterized Amplified Regions) for identification of *M. hapla, M. javanica* and *M. arenaria*.
- Using RAPD-PCR as a DNA-based technique to differentiate the used root-knot isolates.
- Comparing the banding pattern of Egyptian isolates with the Holland isolates.
- Studying the interspecific variation between the three root-knot nematode species and the intraspecific variation within the same species.
- Drawing a phylogenetic tree for the three root-knot nematode species to estimate the relation between them.

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REVIEW OF LITERATURE

Root-knot nematode *Meloidogyne* spp. is recognized as important parasite of fields, vegetable and fruit crops in Egypt.

Meloidogyne spp. as potential serious constraint to the crop productivity were reported by **Oteifa and Elgendi (1956)**. Also, **Sohair Abd El-Hamed (1980)** studied species identification and biotypes differentiation of root-knot nematodes. She found four common species *M. incognita, M. javanica, M. arenaria* and *M. arenaria thamisi*. **Griffin and Waite (1982)** reported that root-knot nematodes *Meloidogyne chitwoodi* and *M. hapla* which are widely distributed in the pacific north west, are able to infect and reproduce on sugar beet (*Beta vulgaris* L.)

Ibrahim *et al.* (1986a,b), Ibrahim *et al.* (1994a), and El-Saedy *et al.* (1993) reported that root-knot nematodes species as *Meloidogyne incognita, M. arenaria* and *M. javanica* are highly distributed in northern Egypt, whereas *M. arenaria* was restricted in its distribution. Also, they found that *M. incognita* race 1 and *M. arenaria race* 1 were dominant in their occurrence with respect to other races within these two species.

In the sandy soil and the newly reclaimed desert land such as Nubaria, Tahrir Province, and Salhia district, the geographical distribution of root-knot nematode is most abundant such as *M. javanica* followed by *M. incognita* and other nematodes genera **(Gindi, 1980; Mohrous, 1991 and El-Shawadfy, 1997)**. **Tomeszewski *et al.* (1994)** surveyed the root-knot nematodes from peanut fields in 4 governorates in Egypt to identify the species, and found that all populations were identified as *M javanica* based on perineal patterns, stylet and body length of (J_2) esterase phenotypes and restriction fragment length

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polymorphism of mt-DNA. Therefore, accurate and reliable identification are necessary for suggesting effective control programs or IPM plans with concern of resistance development of plant culture.

Eissa (1982) reported that losses due to the root-knot nematodes in Egypt measured about 25% of the estimated total losses of plant parasitic nematodes. In the same year **Ibrahim** reported that the yield decrease of 30-40% on vegetable crops due to root-knot nematodes infestation in Beheira Governorate. Yields quantity and quality were low, resulting in a decrease of the commercial value.

In subtropical and Mediterranean climates, **Lamberti (1979)** found that seven economic important species of *M. incognita, M. javanica, M. arenaria, M. hapla, M. naasi, M. graminis* and *M. artiellia* are causing considerable losses of many crops also *M. hapla* attacks a wide range of plants during the winter months or when temperatures are relatively low.

In Morocco, **Agadr *et al.* (1979)** found that root-knot nematodes *M. incognita, M. hapla, M. javanica and M. arenaria* were damaging vegetable crops. Also, their results showed that 32% of the major crops were infested with root-knot nematodes. Collected samples showed that the four common species were *M. javanica* (61%), *M. incognita* (29%), *M. arenaria* (7%) and *M. hapla* (3%).

Eisenback (1982 and 1985) and Hirshmann (1985), used the light microscope (LM) and scanning electron microscopy (SEM) to compare the head shape and stylet morphology of juveniles of one population each of *M. incognita, M. javanica, M. arenaria* and *M. hapla,* they found differences in head morphology between *M. hapla* and other three species, the light microscopy could not accurately detect the

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differences in stylet morphology between *M. incognita* and *M. javanica.* **Hartman and Sasser (1985)** reported that root-knot nematodes can be identified or diagnosed by using their morphological characters which include, prineal patterns, stylet of females, head and stylet of males; these methods are useful in the identification.

Cenis *et al.* (1992) identified some Spanish population of root-knot nematodes using the restriction enzyme Hinf1 which was used successfully to digest the PCR product to produce patterns identical to those obtained in hybridization experiment. These results demonstrate that enzyme phenotyping and nucleic acid analysis provide consistent species identification in *Meloidogyne* spp.

The preadult stage of plant-parasitic nematodes have been difficult to identify because of their small size and lack of distinguishing morphological features identification of this stage is important since they include the infective and over wintering stages of many nematodes. So, recently many molecular techniques were successfully used for identifying single individuals of different stages which make it useful for the ecological studies and early diagnosis.

RFLP technique is considered as one of the useful DNA-based diagnostic technique to screen the DNA by using restriction endonuclease. **Curran *et al.* (1986)** digested *M. arenaria* total DNA of 11 populations of race 1, race 2, *M. incognita* race 2 and race 3, *M. javanica, M. hapla* race a and race b by using EcoR1. They successfully studied the genotypic differentiation of these root-knot nematode population of their RFLP patterns. **Curran and Robinson (1993)** found that RFLP technique could be used as a useful technique to differentiate

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population of intraspecific forms, but it needs relatively large quantities of DNA for transferring and hybridization steps.

Castagnone Sereno *et al.* (1993) investigated the hybridized BamH1 digested genomic DNA, of 18 geographical isolates belonging to six species with three homologous repeated DAN probes cloned at random from a genomic library of one population of *M. incognita*. A distinct dendrogram was drawn by using unweighted pair-group method, arithmatic average method (UPGMA) which showed the phylogenetic relationships between root-knot nematodes, *M. hapla* and parthenogenetic *M. arenaria, M. incognita,* and *M. javanica*. This phylogenetic tree showed that the parthenogenetic isolates of root-knot nematode species were clustered and the amphimictic ones were clustered together in another cluster. Their results showed that *M. arenaria* and *M. javanica* isolates are closer to each other than between them and *M. incognita* isolates.

Abad (1994) showed that the relationship between the different isolates of the two genera, *Bursaphelenchus* and *Meloidogyne* by the hybridization of different genomic libraries with total genomic DNA probes. After the selection of some probes which putatively represented moderately repeated sequences in the genome, they used it as a probe with the genomic DNA.

Molecular techniques are the optimum methods to identify nematodes because the traditional techniques aren't accurate, requires a lot of skills, also genetic variations can't be detected among Meloidogyne population and it can't use different nematode stages.

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Jennifer van Brunt, (1990) showed the PCR technique as the only technique for amplifying polymerase which gives the ability to synthesize DNA fragments in multiple rounds without having to add fresh enzyme each time. These fragments in twenty to thirty cycles, become able to amplify the original sequences by a million copies or so by using a little quantities of DNA. **Williams *et al.* (1990)** developed the new DNA polymorphism assay based on the amplification of random DNA segments with a single primer of arbitrary nucleotide sequences which can be used as a genetic marker, and also it can be used to construct genetic mapping for different species.

Harris *et al.* (1990) used the PCR technique to identify 17 population of the four common species *M. arenaria, M. incognita, M. javanica* and *M. hapla* by using single juveniles and eggs, they found that the restriction enzyme Hinfl permitted discrimination of clonal linkages of the four species and also it succeeded in cutting the PCR product (1.8 kbp) to produce differentiating bands.

Caswell *et al.* (1992) used 19 different random primers to differentiate *H. schachtii* and *H. crucifera* so as to assess genetic variability within each species. Nineteen different random primers yielded from 2 to 12 fragments whose size ranged from 200 to 1500 bp and the difference in fragment patterns allowed differentiation of the two species with each primer. The use of RAPD markers is a very useful tool for analyzing differences between population and requires minute quantities of sample DNA. Also, **Cenis (1993)** identified 18 populations as four major species *Meloidogyne incognita, M. arenaria, M. javanica* and *M. hapla*, using 22 primers, the primer OPA-1 produced amplified DNA bands whose size allowed the separation and distinction of the four

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species. Also, they found numerous polymorphism between one population of race of *M. hapla* as well as between population of *M. arenaria* but there were no clear polymorphism to differentiate between the four races of *M. incognita*.

Roosien ***et al.*** **(1993)**, tested the ability of 9 primers to amplify species-specific DNA sequences to differentiate between single juvenile and females of *Globodera rostochiensis* and *G. pallida* by RAPD technique. The amplification from single juvenile doesn't require DNA isolation, which doesn't impair the polymerase chain reaction (PCR). **Folkertsma** ***et al.*** **(1994)** evaluated the application of RAPD to asses inter and intraspecific variation of potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) by using single random primers to amplify DNA fragment from minute amounts of template DNA with the polymerase chain reaction (PCR). Results of RAPD data showed that *G. rostochiensis* populations cluster in group with similar pathotype designation and there is no clear similarity between RAPD data and pathotype classification of the *G. pallida* populations.

Costagnone-sereno ***et al.*** **(1994)** used the RAPD-PCR for ability to detect polymorphisms within and between the four major *Meloidogyne* species using 17-30 nucleotides long primers, five of them generated a total of 74 scrable markers in order to differentiate *M. hapla* from the other species, whereas *M. arenaria* was found to be closer to *M. javanica* than *M. incognita*.

Guira ***et al.*** **(1995)** used RAPD technique to estimate the genetic relationship among four species of nematodes; (*M. incognita, M.*

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arenaria, M. hapla and *M. javanica*) and found that the similarity between the species depended on the number of bands scored.

In Egypt, **Haroon and Karlovsky (1997)** analyzed by PCR using primers No. 28, 29, 53 and 58 to detect the genetic variations within and between samples of *M. incognita* which were collected from 13 fields planted with different crops in Egypt. They found that each isolates produced 3 to 10 scrable bands with each primer, particularly with primer No. 29 (5'-AAATTATTTATCGCGCTCCTTATG-3'). The values of genetic similarity were calculated using all scrable bands which were produced from all four primers, the variance among banding patterns of different populations demonstrated a high degree of genetic heterogeneity. Also they did not reveal any similarity with Egyptian populations.

Haroon and Zijlstra (1998b) collected from different vegetable fields of Fayoum governorate 47 populations of *M. incognita* to do rapid identification of genetic relationship by polymerase chain reaction RAPD markers. They evaluated 10 primers to identify the genetic variability within populations of *M. incognita* and found that only 2 amplified DNA fragments were common in all population. With primers OPA1, OPB11 and OPG1 were incompleted and weak amplification fragment which were detected with most populations. Strong fingerprinting was obtained with different degrees of population variabilities with primers OPA12, OPG2, OPG4, OPB17 and OPB10. On the other hand, some primers such as OPG6, OPB18 and OPA1 produced more complicated patterns that were hard to score. RAPD marker is very powerful for genetic mapping application as well as genetic diagnosis.

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For molecular analysis, it is useful to study specific genomic regions, one such region is the ribosomal DNA (rDNA) gene family. In most eukaryotes the rDNA consists of three ribosomal components 28S, 5.8S and 18S internal and external transcribed spaces (ITS). The ITS region does not encode for any product permitting it to evolve at a faster rate than the ribosomal coding regions. The level of variation in this region makes it suitable for detecting genetic variation among genera, species and within species.

Vrain *et al.* (1992) used to polymerase chain reaction (PCR) to amplify a region of the ribosomal gene (rDNA) and developed a sensitive method to examine sequence variability within 5.8S gene and the internal transcribed spacer (ITS) of rDNA from 19 North American populations of the *Xiphinema americanum* groups. The cluster analysis of the polymerase chain reaction and restriction fragment length polymorphisms (PCR-RFLP) allowed us to group these populations and results have the potential to clarify the status of some recently described species.

Wendt *et al.* (1993) examined the ribosomal citron of *Ditylenchus destructor, D. myceliophagus* and 7 host races of *D. dipsaci* by using restriction fragment length polymorphism (RFLPs) in the ribosomal citron 18S rDNA gene and the ribosomal internal transcribed spacer (ITS). The results showed that the mixtures of population could be detected by PCR amplification. Also, ITS fragments differentiated between two different species in mixed population.

Ferris *et al.* (1993) determined importance of the internal transcribe spacer regions of rDNA (rDNA ITS1 and ITS2) for

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phylogenetic inference in the cyst nematodes at either population or species using polymerase chain reaction (PCR). They collected sequence data for rDNA ITS1 and ITS2 following amplification.

Ibrahim *et al.* (1994b) used the PCR to amplify a fragment of the rDNA array, comprising two internal transcribed spacer and 5.8S gene to compare and differentiate species and underscribed population of *Aphelenchoides* and *D. angustus*. Amplified rDNA fragments were cut with restriction enzyme and the restriction fragment produced useful diagnostic differences between species and some undescribed populations. **Joyce (1994)** characterized the *Heterorabditis* isolates by PCR amplification of segments of rDNA genes. PCR primers set was used to amplify the internal transcribed spacer (ITS) region. Their flanking 18S and 28S coding sequences and restriction digestion of the amplification products provided a rapid method for the determination of species grouping.

Zijlstra *et al.* (1995) used restriction enzyme analysis of ribosomal DNA (rDNA) sequences to distinguish isolates of root-knot nematodes, especially *M. hapla* and *M. chitwoodi*. Digestion of the ITS regions with Alul, Dral and Hinf1, can only use small amounts of DNA from single Juvenile (J_2) or from single female.

Gour *et al.* (1996) studied the differentiation of two groups of species of the genus *Meloidogyne* by PCR-RFLP of ribosomal DNA. The digested fragments with the restriction enzyme Mps1 which was used to compare and differentiate the two groups of *Meloidogyne* species; by *M. incognita*, *M. javanica* and *M. arenaria*, in the first group, and *M.*

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graminicola, M. oryzae, M. naasi and *M. triticoryzae* in the second group.

Daniel ***et al.*** **(1996)** used two sets of primers in the PCR to make rapid identification of *M. chitwoodi, M. hapla,* and *M. fallax*. One of these two sets was used to amplify the internal transcribed spacer (ITS), and the other one to amplify the genomic rDNA intergenic spacer (IGS) with only minimal flanking sequences of the 28S and 18S rDNA. They found that direct identification of nematodes based on fragment size polymorphism of an amplified product is more efficient than the usual procedure of PCR amplification followed by digestions with endonucleases. The results were determined as restriction fragment length polymorphism (RFLP).

Allen ***et al.*** **(1997)** compared by using PCR-RFLP of ITS between cyst nematodes species (*Heteroderidae*) of agronomic significance and regulatory concern, they found that PCR-RFLP detected several restriction site differences between all isolates.

Blok ***et al.*** **(1997)** amplified the intergenic sequence (IGS) regions between the 5S and 18S genes from a single juvenile or egg masse of the ribosomal DNA of *M. incognita* and *M. javanica* of nematodes, including representatives of both Secernentea and Adenophorea, among free living insect and plant-parasitic species. The size of the amplified ITS product aids in the initial determination of group number ship and discuss ITS taxonomic implications.

Williamson ***et al.*** **(1997)** identified and distinguished single juvenile of *M. hapla* and *M. chitwoodi* by using a series of random octamer primers (MHOF, MHIR, MC3F, and MCIR). They found that a

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mixture of four primers in a single PCR reaction was abled to identify single juveniles of *M. hapla* and *M. chitwoodi* to confirm specificity. **Daniel *et al.* (1997)** identified *Meloidogyne* spp. and synthesized specific primers for several intergenic spacer (IGS) areas. They found that a multiplex-PCR amplification by using a combination of five specific and non-specific primers to amplify DNA from single juveniles or from small number of egg efficiently distinghuised *M. chitwoodii* and *M. fallax* from *M. arenaria, M. incognita, M. javanica, M. hapla* and *M. mayaguesis* without any need for restriction digest.

In differentiation among in mixtures of root-knot nematodes (*M. hapla, M. chitwoodi, M. fallax* and *M. incognita*) **Zijlstra *et al.* (1997)** described a technique using Dral, EcoR1 and Rsa1 restriction patterns of ITS-PCR products. **Zijlstra, (1997)** cloned and sequenced the amplified rDNA-ITS fragment of *Meloidogyne chitwoodi, M. fallax, M. hapla* and *M. incognita*. Alignment of sequences showed a little variation in the coding parts and some variations in the ITS region by using the primers H-18S, I-ITS and CF-ITS, when combined with reverse primer HCF1-28S in a single PCR reaction which was used to distinguish four previous species and single juveniles.

Adams *et al.* (1998) studied inter-phylogenetic relationship among the described taxa of *Heterorhabditis* using DNA sequences of the ITS region of the ribosomal tandem repeating unit. Though it appears as if some of there sister taxa were actually nonspecific, a more thorough examination of character variability within these species is required before on evolutionary species delimitation can be complished with confidence.

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Haroon and Zijlstra (1998a) in Egypt, used the internal transcribed spacer (ITS) and restriction fragment length polymorphism (RFLP) of ribosomal DNA (rDNA) to distinguish between different major species of root-knot nematode on most vegetable crops in Fayoum governorate. They used two primers (5367 and 5368) for the amplification of the ITS region which gave one major product of approximately 760 bp. Also, they used nested primers (F194 and F195) which gave a strong single band at 570 bp were shorter than ITS fragment by 190 bp. ITS regions of all isolates were digested with four restriction enzymes primers (HindIII, Hinf1, EcoR1 and Dra1). They used two clear tests to distinguish root-knot nematodes species by the size of their fragment in a single PCR reaction which indicated that only one strong band obtained from first test (Scar primer) to differentiate between *M. javanica* and *M. incognita,* and the second test (Multiplex) which revealed the size of the cloned amplified ITS regions of *M. incognita* at 760 bp.

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MATERIALS AND METHODS

1. Root-knot nematode survey:

Nematode samples were collected from root plants in Kafr El-Sheikh, El-Behera, Al-Arish, Fayoum and Mynia Governorates in 1998 (Table 1). These samples were collected from different infected crops such as: tomato, eggplant, peanut, sugar beet, okra, *Solanum* spp. Hotpepper and bean (Table 1). Isolates were maintained on tomato seedling (*Lycopersicon esculentum* L. cv. *Castle Rock Ii. pv pN*) grown in a mixture of sand peat in 1000 cm^2 clay pots at 25-28°C in a greenhouse. Identification of nematode species were carried out in Nematology Lab. IOP-DLO Research for Plant Protection. Wageningen, Holland and were compared with identified species (*M. incognita, M. javanica, M. arenaria, M. hapla,* and *M. chitwoodi).*

2. DNA extraction:

DNA extraction was carried out according to **Curran *et al.* (1986).** Twenty five females from each sample were stored frozen till the preparation time (except samples no. 4, 8, 11, 14, 16, 23, 26, 32, 33, 34, 35 and 40 only 5-12 females were used). Each population sample was transferred into 2 ml eppendorf tube. The samples were spined briefly and the supernatant was removed. The females were crushed in liquid nitrogen using mortar and pestle. Each crushed sample was transferred to sterilized eppendorf tube and 250 µl EB buffer (10.2 M tris pH 8.0, 0.1 M EDTA, 0.4 M NaCl, 2% SDS) was added. Also, 250 µl of sterilized water was added to reach the volume of 500 µl and kept on ice

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Table (1): Isolates and sources of root-knot nematodes (*Meloidogyne* spp.).

No.	DNA *	Governorate	Centre	Crop
1	42	Al-Arish	El-Sheikh Zoied	Fig
2	44	Al-Arish	El-Sheikh Zoied	Tomato
3	32	Al-Arish	El-Sheikh Zoied	Tomato
4	43	El-Behera	Nubaria	Tomato
5	49	El-Behera	Nubaria	Peanut
6	16	El-Behera	Nubaria	Eggplant
7	17	El-Behera	Nubaria	*Solanum spp.*
8	18	El-Behera	Nubaria	Eggplant
9	19	El-Behera	Nubaria	Tomato
10	20	El-Behera	Nubaria	Tomato
11	21	El-Behera	Nubaria	Tomato
12	26	El-Behera	Nubaria	Hotpapper
13	28	El-Behera	Nubaria	Tomato
14	29	El-Behera	Nubaria	Peanut
15	30	El-Behera	Nubaria	Sugar beet
16	31	El-Behera	Nubaria	Sunflower
17	1	El-Behera	Rahmania	Tomato
18	3	El-Behera	Rahmania	Eggplant
19	4	El-Behera	Rahmania	Potato
20	5	El-Behera	Rahmania	Banana
21	6	El-Behera	Rahmania	*Solanum spp.*
22	8	El-Behera	Rahmania	*Amaranthus spp.*
23	34	El-Behera	Rahmania	Tomato
24	35	El-Behera	Rahmania	Eggplant
25	38	El-Behera	Rahmania	Okra
26	39	El-Behera	Rahmania	Fig
27	40	El-Behera	Rahmania	Cucumber
28	41	El-Behera	Rahmania	*Solanum spp.*
29	45	Fayoum	Tamia	*Solanum spp.*
30	2	Kafr El-Sheikh	Dosoque	Eggplant
31	10	Kafr El-Sheikh	Balteem	Tomato
32	11	Kafr El-Sheikh	Balteem	Tomato
33	12	Kafr El-Sheikh	Balteem	Tomato
34	13	Kafr El-Sheikh	Balteem	Eggplant
35	14	Kafr El-Sheikh	Balteem	Tomato
36	15	Kafr El-Sheikh	Balteem	Tomato
37	22	Kafr El-Sheikh	Balteem	Tomato
38	23	Kafr El-Sheikh	Balteem	*Solanum spp.*
39	24	Kafr El-Sheikh	Balteem	Tomato
40	25	Kafr El-Sheikh	Balteem	Tomato
41	36	Kafr El-Sheikh	Balteem	Bean
42	46	Kafr El-Sheikh	Balteem	Tomato
43	47	Kafr El-Sheikh	Balteem	Tomato
44	48	Kafr El-Sheikh	Balteem	Tomato
45	50	Kafr El-Sheikh	Balteem	Eggplant
46	27	Kafr El-Sheikh	Motobus	Tomato
47	33	Kafr El-Sheikh	Motobus	Tomato
48	37	Kafr El-Sheikh	Motobus	*Solanum spp.*
49	9	Mynia	Matay	*Solanum spp.*

* DNA extraction number

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at least for 5 min. For each sample 50 µl proteinase K (20 mg/ml) was added and all samples were incubated at 65°C for 1 h. One µl RNase (10 µg/ml) was added to each sample. The lysate was extracted once with 500 µl equilibrated phenol, shacked well and centrifuged for 3 min. at 6500 rpm. The supernatant was transferred into new tubes and extracted with 250 µl of phenol 250 µl of chloroform: isoamyl alcohol (24:1), spined for 3 min. at 6500 rpm. The supernatant was transferred again to a new tube, the phenol/chloroform step was repeated twice then 500 µl of chloroform: isoamyl alcohol 24: 1 was added, mixed for 3 min. then centrifuged again for 3 min. at 6500 rpm.

The precipitated DNA from the supernatant using 50 µl sodium acetate (3 M) of pH 5.2 was mixed well and each sample was derived in two eppendorf tube, 700 µl of ethanol 96% was added to each tube and shaken well and then incubated for 3 h or longer at -20°C or 30 min. at -80°C. The samples were centrifuged at 15,000 rpm for 30 min at 4°C. The DNA was accumulated in the bottom of the tube as pellets. It was washed twice carefully with 70% ethanol and left to dry at room temperature for several hours. The pellet was dissolved in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The amount of extracted DNA was checked by loading 10 µl from each sample on agarose gel to be tested with (λDNA) 5, 10, 20, 50, 100 ng, the amount of DNA for each isolate was calculated as ng/µl.

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3. Multiplex PCR:

This technique was conducted for direct identification of the four common species (*M. incognita, M. javanica, M. arenaria*), *M. chitwoodi*, *M. hapla* and *M. fallax* based on length polymorphisms. This technique used three forward primers and one common reverse primer as described by **Zijlstra (1997)**. The HCFI-28S is the reverse primer as described by **Ferris *et al.* (1993)** and the three primers H-18S, CF-ITS and I-ITS are the forward primer as described by **Zijlstra, (1997)**. The reaction mixture contained 3 ng crude DNA extract; 1 unit of Taq DNA polymerase, (Sphero Q. Leiden. NL), 200 μM each of dATP, dCTP, dGTP and dTTP (Bohringer, Mannheim), 0.4 μM of each forward primers H-18S ('5-CTTGGAGACTGTTGATC-3`), CF-ITS ('5-GATTATACGCACAATT-3`) and I-ITS ('5-TGTAGGACTCTTAATG-3`); 0.4 μM of the reverse primer HCFI-28S, 3.5 mM MgCl; 1X reaction buffer (prepared by the company supplying the polymerase) and deionized water to a volume of 50 μl. The amplification performed in a perkin-Elmer Cetus DNA thermalcycler, the condition as follows: denaturation at 94°C for 4 min followed by 5 cycles at 94°C for 30 sec 55°C for 30 sec 72°C for 1 min with a decreased 1°C per cycle for the annealing temperature followed by 25 cycles at 94°C for 30 sec 50°C for 30 sec and 72°C for 1 min.

Amplification products were separated through electrophoresis in the agarose gel which was constructed by weighting out 1 gram of agarose (sigma) to which 100 milliliter of 1X TBE buffer (89 mM Tris, 89 mM Boric acid and 25 mM EDTA pH 8.0) was added. The mixture was heated up in microwave oven with regular intervals and was swirlled

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to mix the contents. Gel was cooled down to 50-60°C and 0.5 μg/ml ethidium bromide was added. Poured to submarine gel apparatus, held for 30 min for cooling down. The comb was removed and the gel tray was placed under sufficient amount of 1X TBE buffer just to cover the gel. 10 μl of samples were mixed with 3 μl blue stain (0.05% bromophenol blue, 0.05% xylene caynol, 40% sucrose) and loaded. The gel apparatus was connected to the power supply at a constant current of 60 mA, the gel was observed and visualized with a UV transillminator **(Zijlestra *et al.*, 1995)**.

4. Internal Transcribed Spacer (ITS) amplification:

The rDNA amplification Figure (1) showed the location of the primers used for amplifying segments. It presents the location of the internal transcribed spacer (ITS) primers which were used in test ITS-RFLP.

The primers 5367 ('5-TTGATTAGGTCCCTGCCCTTT-3') and 5368 ('5-TTTGATCCGCCGTTATTAAGG-3') were the forward and backward primers, respectively as described by **Vrain *et al.* (1992) and Vrain (1993)**.

The DNA fragments containing the ITS regions were amplified by PCR which were performed in 50 μl of reaction mix containing 3 ng of template DNA. 1 unit of Taq polymerase (Sphaero Q. Leiden, NL) and 200 μM each of dATP, dCTP, dGTP and dTTP) (Boehringer. Mannheim). 1.5 mM $MgCl_2$, 0.6 μM from each primer, 10X reaction buffer (prepared by the Company supplying the polymerase) and

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completed the volume by deionized water to 50 µl the amplification was carried out in a perkin-Elmer Cetus DNA thermalcycler.

PCR amplification condition was carried out at 94°C for 1 min annealing at 55°C for 1 min and extension at 72°C for 2 min., repeated for 20 cycle, incubation period for 5 min at 72°C, followed the last cycle to complete any partially synthesizes strands.

Amplification products were separated through electrophoresis as Multiple x PCR.

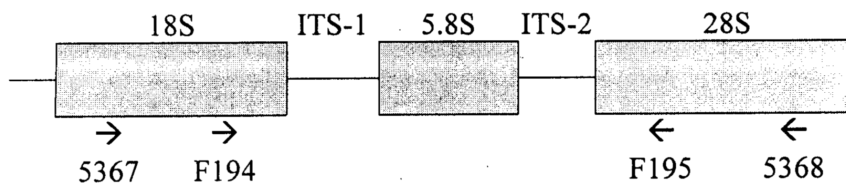

Fig. (1): Diagram of ribosomal DNA gene showing the location of the internal transcribed spacer (ITS) primers which were used in ITS test and ITS-RFLP.

5. ITS-RFLP:

Restriction sites were predicted from sequence data with **(Zijlstra *et al.*, 1995).** Amplified DNA of ITS fragments were digested with Hinf1 BamH1 and EcoR1 restricted enzymes. A master mix was prepared using one µl of selected restriction enzyme, 1.5 µl restriction enzyme buffer and 10.5 µl 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 overnight. The digested DNA was loaded on agarose gel as Multiplex PCR.

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6. PCR protocols for SCARs primers:

These techniques used a one specific forward primer on one hand and specific reverse primer for each on the other (*M. hapla, M. arenaria* and *M. javanica*) which were identified directly.

6.1. SCAR test for *M. hapla*:

The reaction mixture contained 3 ng crude DNA extract, 1 unit Taq DNA polymerase, 200 μM each of dATP, dCTP, dGTP and dTTP), 3 μM each reverse primer and 3 μM forward primer, 10X reaction buffer (prepared by the company supplying the polymerase), and deionized water to a 50 μl volume. The amplification was carried out using a Perkin-Elmer. The amplification condition (non published) as personal communication with Dr. Caroline Zijlstra at DLO Research Institute for Plant Protection (IPO-DLO), Holland. Amplification products were loaded on agarose gel as Multiplex-PCR.

6.2. SCAR test for *M. arenaria*:

The same conditions described in the previous test were used except for two different specific primers which were used for *M. hapla*.

6.3. SCAR test for *M. javanica*:

All conditions were used as in previous scar test for *M. arenaria* and *M. hapla* except for that kind of primers and program of PCR.

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7. Random Amplified Polymorfic DNA (RAPD):

To perform a RAPD assay, a single oligonucletide (primer) mixed with genomic DNA in the presence of a thermostable DNA polymerase and suitable buffer, and then subjected to temperature cycling conditions typical of the polymerase chain reaction. The products of the reaction depend on the sequence length of the oligonucleatide and on the reaction condition often a single primer can be used to identify several polymorphisms.

Table (2): Primers tested for RAPD analysis of *Meloidogyne* spp.

Primer	Sequence (5 → 3)
OPA-1	CAGGCCCTTC (Cenis, 1993)
OPA-11	CAATCGCCGT (Cenis, 1993)
OPA-12	TCGGCGATAG (Cenis, 1993)
OPG-2	GGCACTGAGG (Roosien *et al.*, 1993)

Four primers of random sequence were provided by DLO research Institute for Plant Protection IPO-DLO. Holland,These primers are listed

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in Table (2). PCR was carried out in a final volume of 50 μl containing 10 ng of genomic DNA, 2μM primer, 200 μM for each of dATP, dCTP, dGTP and dTTP (Boehringer), 10X reaction buffer (prepared by the company supplying the polymerase) ; 1 unit Taq DNA polymerase (Sphero Q. Leiden. NL) and deionized water to a volume 50 μl. Amplification was performed on a Perkin-Elmer Cetus DNA thermal cycler, optimum amplification conditions were proceeded by 1 min at 98 °C followed by 35 cycles of denaturation at 94 °C for 20 sec, annealing at 36°C for 30 sec. and extension at 70°C for 2 min a final incubation of 70°C, 10 min.

After the end of the cycles, 10 μl of the PCR product was taken from the tube, loaded in agrose gel and electrophoresed as Multiplex-PCR. A negative control without DNA was included in all the reactions. Also as a comparison with the Egyptian populations, sample of each *M. chitwoodi, M. incognita, M. javanica* and *M. hapla* from Holland were used as positive control. The experiment was repeated two times for each primer.

7.1. Method of data analysis for RAPD-PCR:

The banding patterns generated by 4 primers analysis were compared to determine the genetic relationship of *Meloidogyne* isolates. All bands were scored as present (1) or absent (0). The similarity matrix was performed using 64 bands, from this matrix and estimator F = 2 Nxy/Nx + Ny of similarity **Nei and Li (1979)** was computed where Nxy

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is the number of bands shared by the isolates x and y, Nx and Ny being the total number of bands of the two isolates. The formula of Dice coefficient was used to calculate a similarity coefficient for pairs of isolates.

The similarity matrix was used to perform the phylogenetic tree by using NTSys. ver. 1.4 package **(Ralf, 1988)**.

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RESULTS

1. The amounts of DNA

The obtained amounts of the extracted DNA from 49 isolates root knot nematode *Meloidogyne* spp. from different Egyptian locations were presented in Table (3).

2. Multiplex-PCR:

Multiplex-PCR was performed by using four primers H-18S, CF-ITS, I-ITS, and HCFI-28S in a single PCR reaction. Results presented in Fig. (2) and Table (3) show that multiplex-PCR early differentiates the species *M. hapla*, (*M. incognita* or *M. javanica*) and *M. chitwoodi* (the last one was from Holland). The two isolates of *M. hapla* (No 29 and 30) gave one major band at 660 bp. This is the first record for *M. hapla* in Egypt on Peanut and sugar beet. *M. incognita* or *M. javanica* produced one major band at 416 bp. *M. chitwoodi* gave major band at 525 bp. Twenty three isolates of *M. incognita* or *M. javanica* presented a strong band at 416 bp which was similar to positive control for *M. incognita* and *M. javanica* populations from Holland.

3. ITS-rDNA amplification:

The PCR amplified rDNA ITS fragments product was approximately 760 bp in 26 samples on using the primers of the ITS region from 49 samples of root-knot from different governorates, (Table 3 and Fig. 3). Also, isolates *M. incognita*, *M. javanica* and *M. hapla* from Holland gave the same results.

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Table (3): Conclusion for all results from rDNA technique that were used in this study.

No. of population	DNA ng/ul	ITS-result	Multiplex PCR	SCAR test
1	1.0	reaction	javanica /incognita	javanica
2	1.5	reaction	javanica /incognita	javanica
3	1.0	reaction	javanica /incognita	javanica
4	0.1	no reaction	no reaction	no reaction
5	0.3	no reaction	javanica /incognita	incognita
6	2.0	reaction	javanica/incognita	javanica
8	0.1	no reaction	no reaction	not tested
9	2.0	reaction	javanica /incognita	javanica
10	5.0	reaction	javanica /incognita	javanica
11	0.1	no reaction	no reaction	no tested
12	0.5	reaction	javanica /incognita	incognita
13	0.3	no reaction	no reaction	incognita
14	0.1	no reaction	no reaction	no tested
15	2.0	reaction	javanica /incognita	javanica
16	0.1	reaction	no reaction	javanica
17	0.5	no reaction	javanica /incognita	incognita
18	0.5	no reaction	javanica /incognita	incognita
19	2.0	reaction	javanica /incognita	javanica
20	0.5	no reaction	javanica /incognita	incognita
21	1.0	reaction	javanica /incognita	javanica
22	0.5	reaction	javanica /incognita	javanica
23	0.1	no reaction	no reaction	no reaction
24	0.3	no reaction	no reaction	incognita
25	0.5	reaction	javanica /incognita	javanica

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Table (3): Cont.

No. of population	DNA ng/ul	ITS-result	Multiplex PCR	SCAR test
26	0.1	reaction	no reaction	not tested
27	0.5	reaction	javanica /incognita	incognita
28	1.0	reaction	javanica /incognita	javanica
29	0.5	reaction	hapla	hapla
30	1.0	reaction	hapla	hapla
31	0.1	no reaction	no reaction	not tested
32	-	no reaction	no reaction	not tested
33	-	no reaction	no reaction	not tested
34	-	no reaction	no reaction	not tested
35	-	no reaction	no reaction	not tested
36	2.5	reaction	javanica /incognita	incognita
37	0.5	no reaction	javanica /incognita	incognita
38	0.2	reaction	no reaction	incognita
39	0.1	no reaction	no reaction	incognita
40	0.1	no reaction	no reaction	no reaction
41	0.1	no reaction	no reaction	incognita
42	0.5	reaction	javanica /incognita	incognita
43	0.5	reaction	javanica /incognita	javanica
44	0.9	reaction	javanica /incognita	javanica
45	0.6	no reaction	javanica /incognita	javanica
46	0.6	reaction	javanica /incognita	javanica
47	0.5	reaction	javanica /incognita	javanica
48	0.6	reaction	javanica /incognita	javanica
49	0.2	no reaction	javanica /incognita	javanica
50	0.1	no reaction	javanica /incognita	javanica

No reaction = not appeared band.

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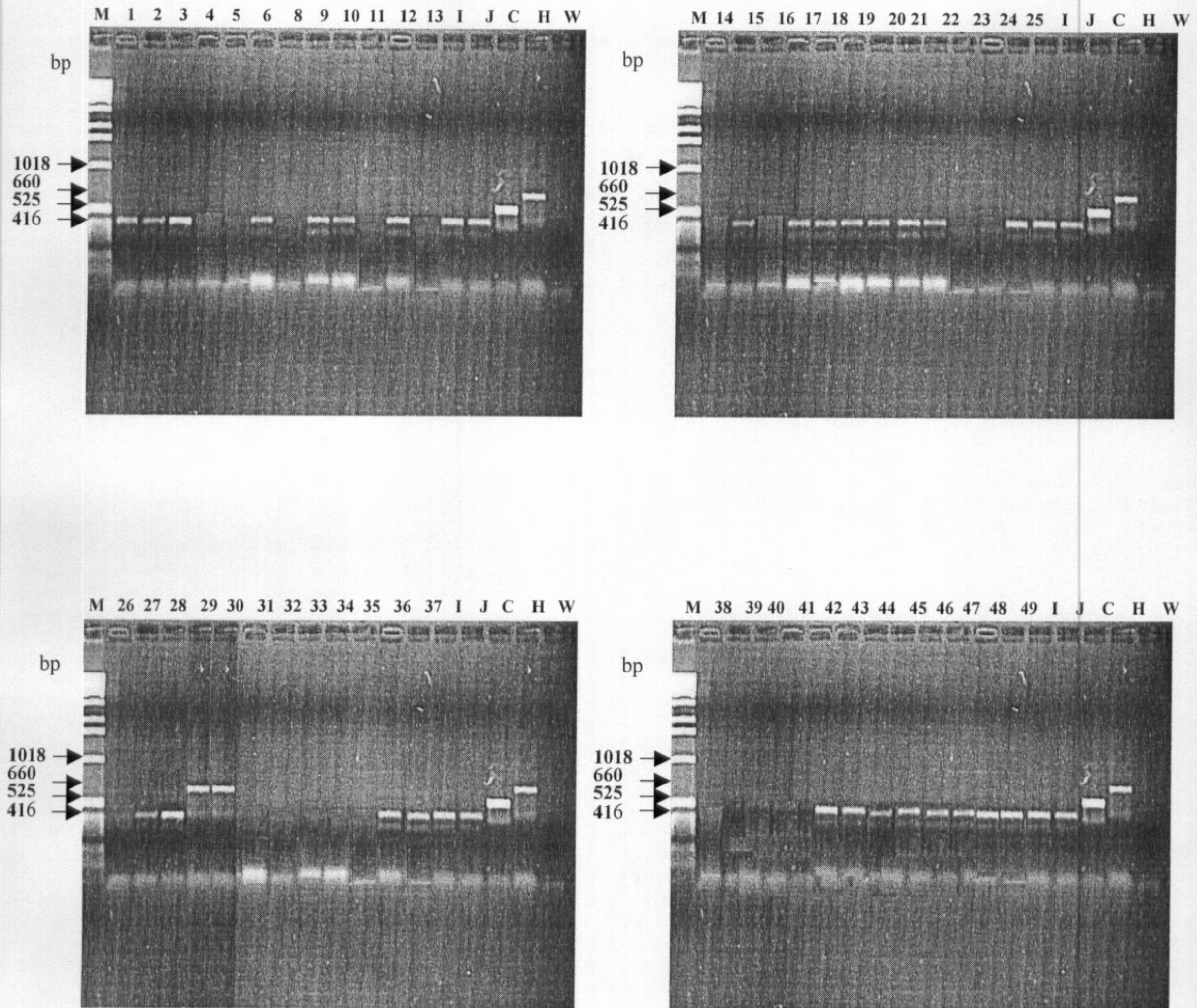

Fig. (2) : Typical amplification products of Multiple-PCR reaction using forward primers and reverse primer in a single PCR reaction with 3 ng of template DNA of *M. incognita* (I), *M. javanica* (J), *M. chitwoodi* (C) and *M. hapla* (H) as positive control from Holland. W= control without template DNA. M = Size marker DNA.

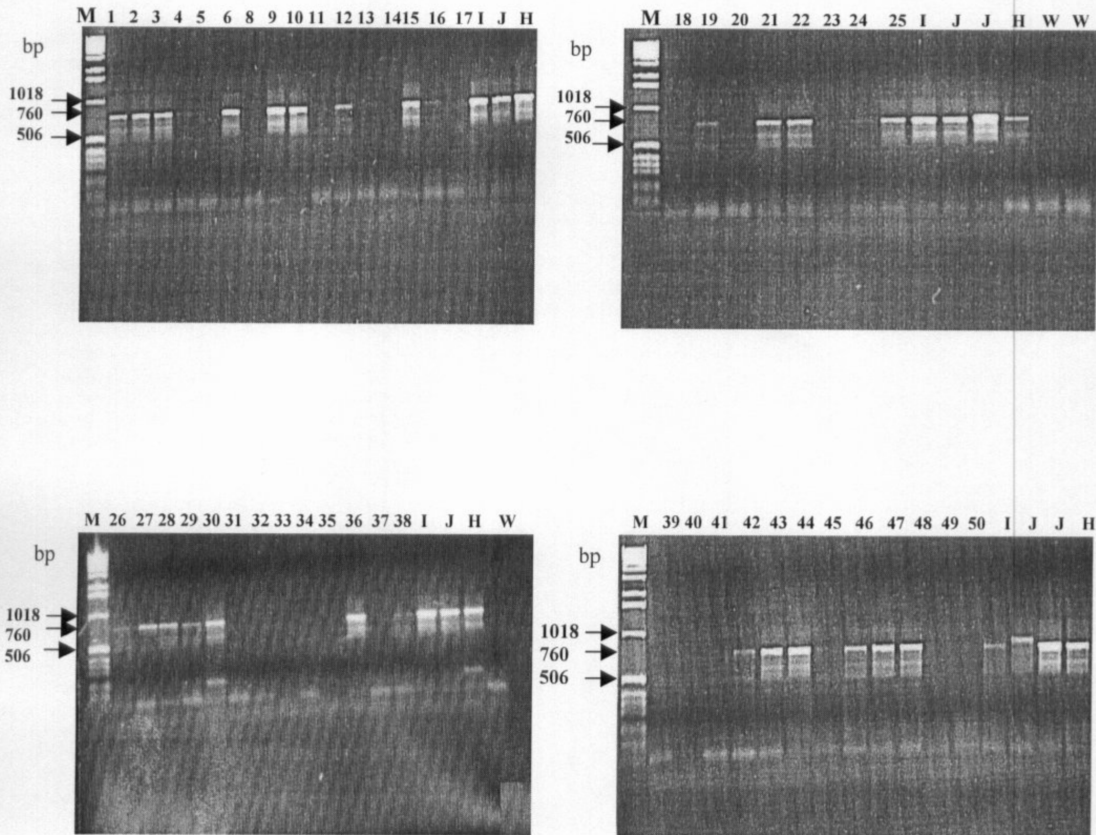

Fig. (3) : Typical amplification of 760 bp polymerase chaine reaction (PCR) Product from template of total DNA extracted from *Meloidogyne* spp. with internal transcribed spacer (ITS) with primers 5367 and 5368. (J, I, H,) = *M. javanica, M. inognita,* and *M. hapla*) positive control from Holland. W = Control without template DNA. M = Size Marker DNA.

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4. Restriction enzyme results:

Digestion of the 760 bp amplification product of ITS region of certain isolates were digested with restriction enzymes (Hinf1, EcoR1, and BamH1), which distinguished between *Meloidogyne hapla*, *M. incognita* or *M. javanica* (Table 4).

Table (4): Sizes of the DNA fragment (bp) obtained after restriction enzyme digestion of the 760 bp internal transcribed spacer regions *Meliodogyne hapla*, *M. incognita* and *M. javanica.*

Restriction enzyme	**DNA fragment (bp)**	
	M. hapla	***M. incognita/M. javanica***
Hinf1	**490, 270**	**440, 320**
EcoR1	**760**	**520, 240**
BamH1	**430, 330**	**760**

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No restriction enzyme has been found to distinguish between *M. incognita* and *M. javanica* up till now. Some of the typical restriction pattern of the 760 bp amplification product from isolates of *M. incognita*, *M. javanica* and two isolates of *M. hapla* show identical restriction pattern. Digestion with BamH1 is specific for *Meloidogyne hapla* and shows fragments 430 bp, and 330 bp and does not digest fragments with *Meloidogyne incognita* or *M. javanica* (Fig. 4). Also, the fragment which is the product of EcoR1 presented two digested bands at 520 and 240 bp with *M. incognita* or *M. javanica,* it doesn't digest the ITS region with *M. hapla* (Fig. 5). Another restriction enzyme Hinfl was used with *M. incognita/M. javanica* cut ITS region into two fragments (440 and 320 bp) and with *M. hapla* gave two fragments (490 and 270 bp) (Fig. 6).

5. SCARS primer test:

The results from *M. hapla* SCAR primers test indicated that one major strong band, especially for *M. hapla* as similar to the positive control *M. hapla* from Holland. Two samples were obtained from Nubaria Province, sample no. 29 on peanut and sample no 30 on sugar beet (Fig. 7), which gave the same results (Table 3 and Fig. 8) while *M. incognita, M. javanica* and *M. arenaria* with this test did not give any result.

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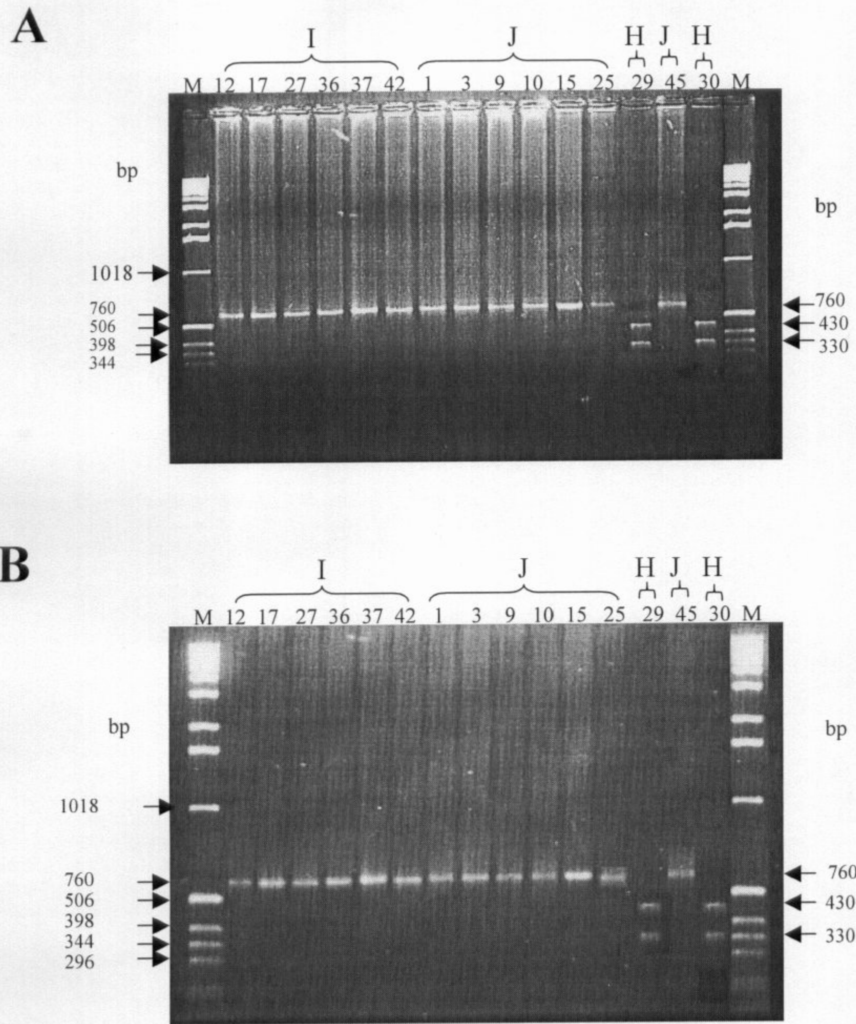

Fig. (4) : Typical digestion of the 760 bp polymerase chain amplified internal transcribed spacer region with restirction enzyme BamH1, (A) the agarose gel after 45 min, (B) the agarose gel after 120 min. M = size marker DNA.

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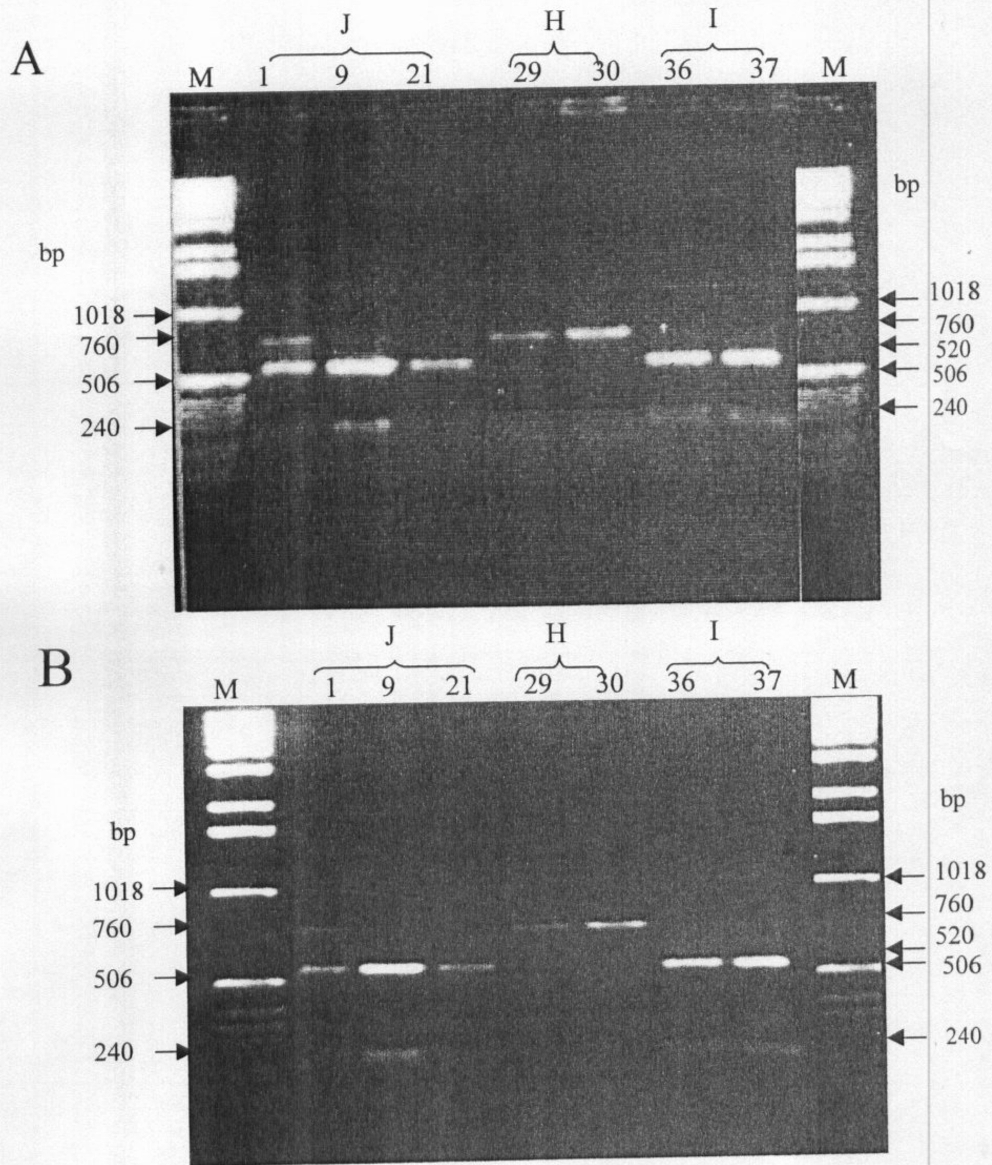

Fig. (5) : Typical digestion of the 760 bp polymerase chain amplified internal transcribed spacer region with restirction enzyme EcoR1, (A) the agarose gel after 45 min, (B) the agarose gel after 120 min. M = Size marker DNA

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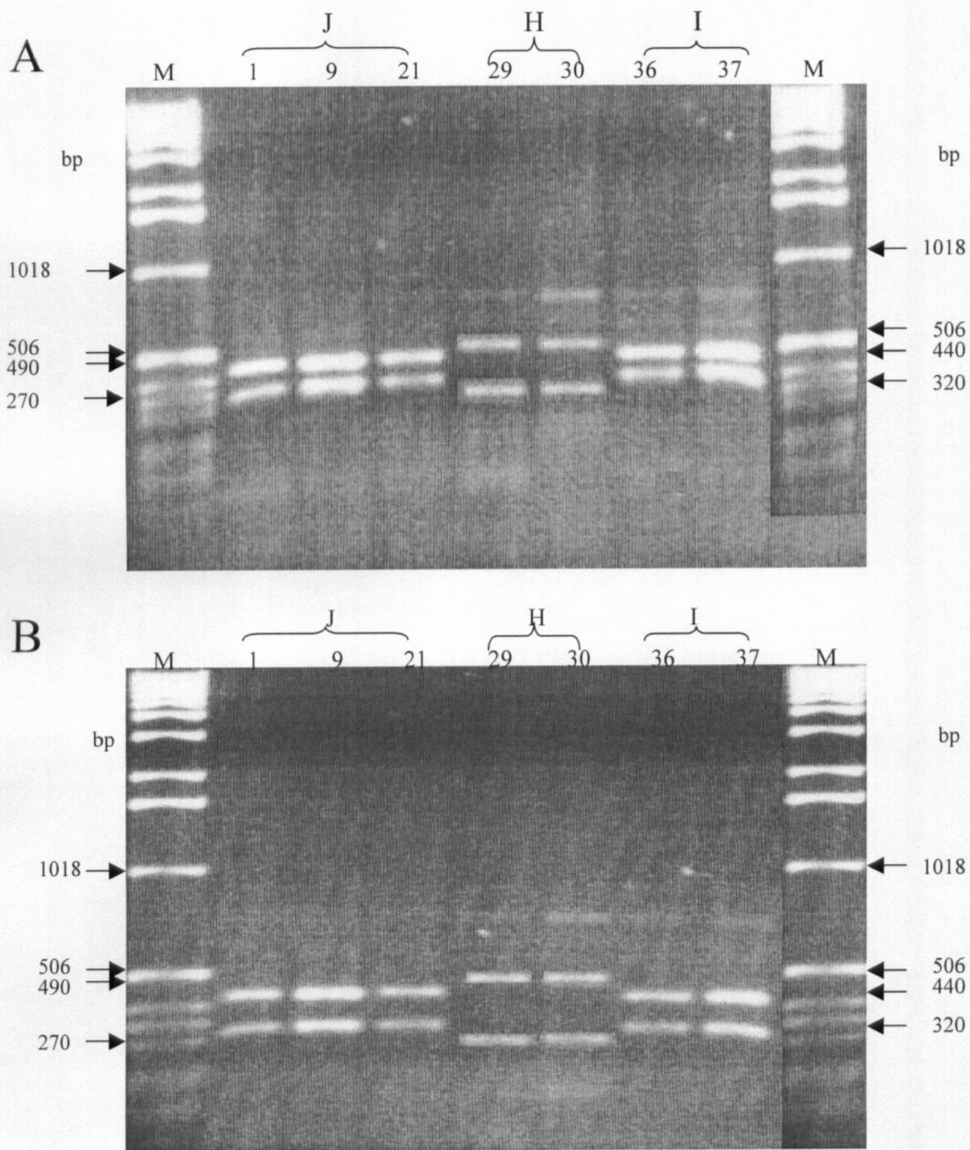

Fig. (6) : Typical digestion of the 760 bp polymerase chain amplified internal transcribed spacer region with restirction enzyme Hinf1, (A) the agarose gel after 45 min, (B) the agarose gel after 120 min. M=size marker DNA.

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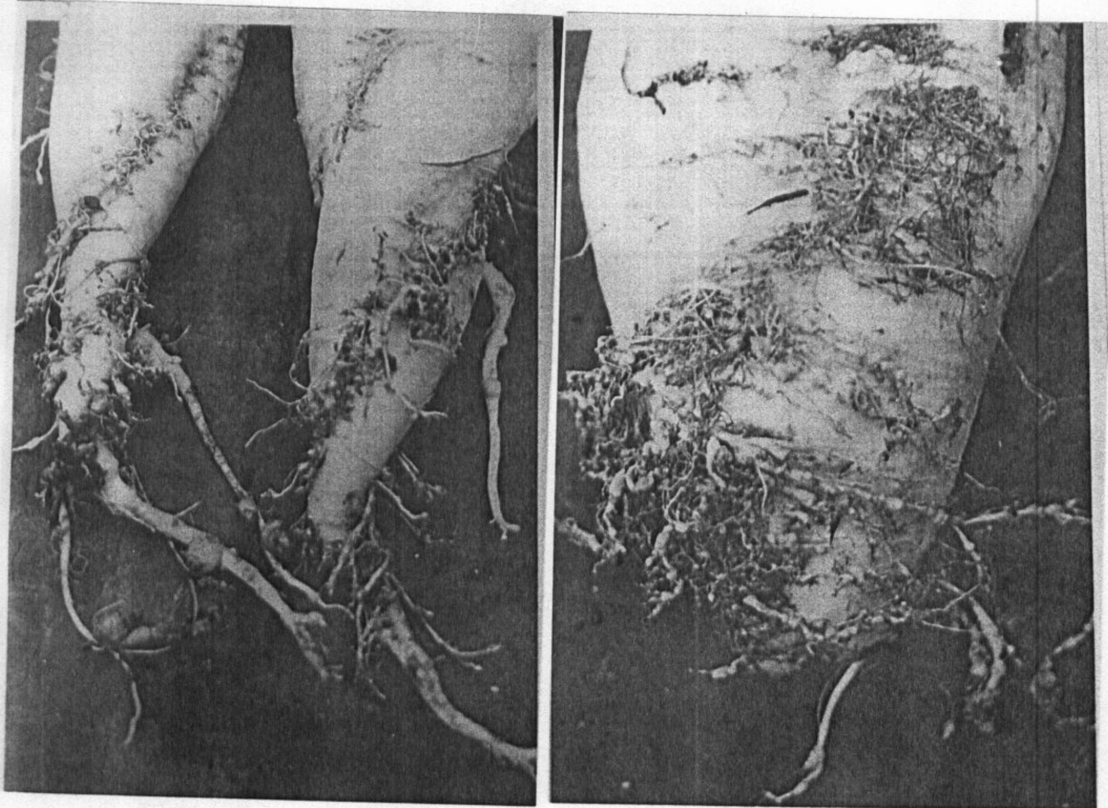

Fig. (7): The symptoms of root-knot nematode on sugar beet from Nubaria province which is identified as *Meloidogyne hapla.*

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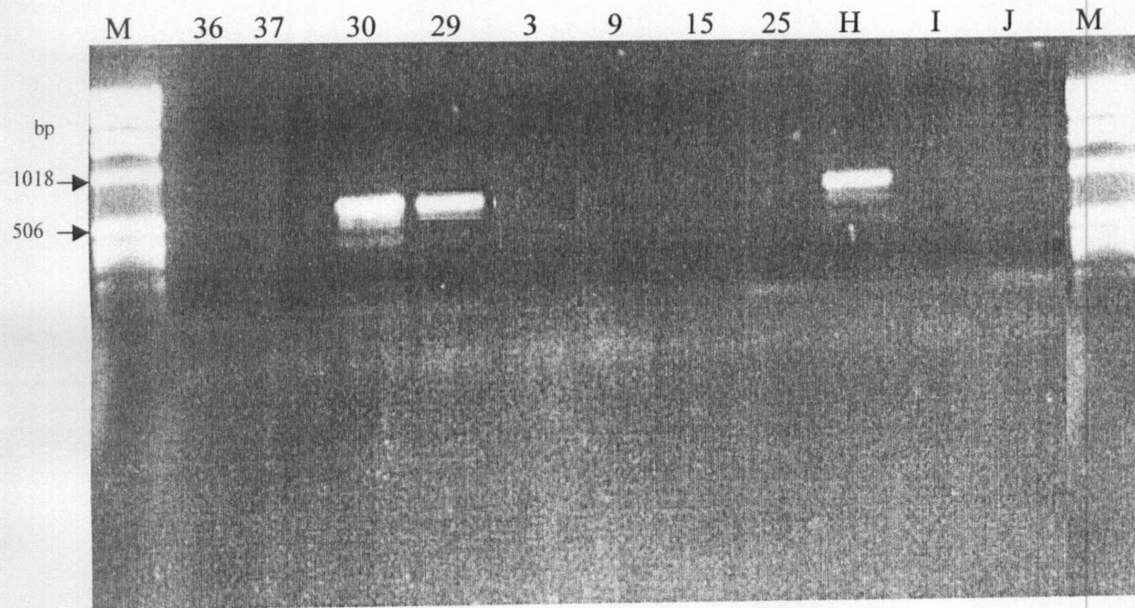

Fig. (8) : SCAR primer for *Meloidogyne hapla.* (H, I, J,) = Positive control for (*M. hapla, M. incognita* and *M. javanica*) respectively from Holland M = Size marker DNA .

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When scar primers of *M. arenaria* were used for amplifying *M. arenaria* isolate (obtained from Holland as a positive control), it gave one major band. In our isolates no major bands were observed (Fig. 9). So, no *Meloidogyne arenaria* in our Egyptian isolates were obtained from different areas as described in Table (3).

SCAR primers for identify *M. javanica* gave only one strong band in 21 samples (Table 3 and Fig. 10). Similar strong band was observed of positive control isolates from Holland (Fig. 10) and did not give any band with *M. incognita* and *M. hapla*.

From the previous techniques 21 isolates were identified as *N. javanica*, 14 isolates as *M. incognita* and 2 isolates as *M. hapla*. Another 12 isolates was not identified because the amounts of females and cycles of PCR were not sufficient to produce good amplification.

6. RAPD-PCR identification:

Four primers (OPA-01, OPO-11, OPA-12 and OPG-02 were examined with 24 Meloidogyne isolates (Table 5) which represent the three different species (*Meloidogyne javanica, M. incognita* and *M. hapla*). The resulted banding patterns amplification products using OPA-01 primer in reaction with tested root-knot nematode isolates were illustrated in Fig. (11). The bands were ranged at size from 398 and 2000 bp. Two strong species-specific bands are 1550 bp and 2000 bp band which appeared only with all of *M. javanica* isolates. All isolates of *M. incognita* produced 540 bp and 1000 bp bands. One isolates of *M. hapla* produced polymorphic bands at 850 bp and 1020 bp. These polymorphic bands appeared in the three isolates which obtained from Holland.

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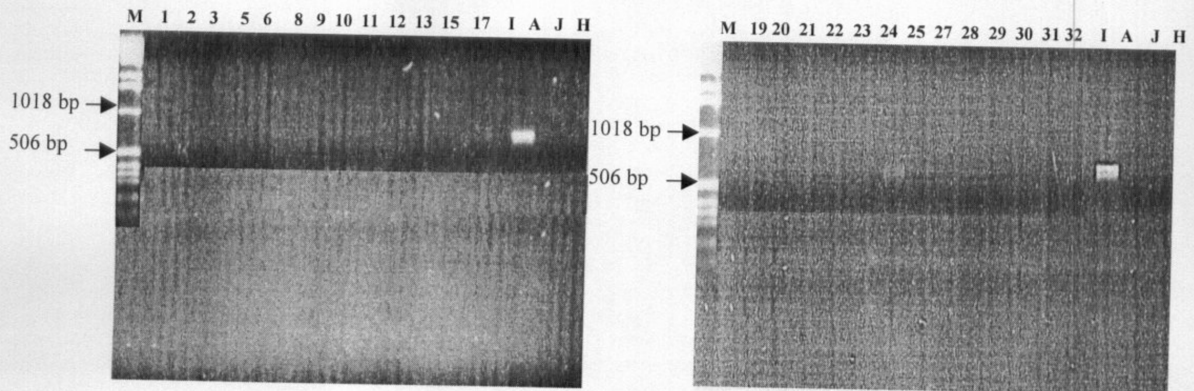

Fig. (9) : SCAR primer for *Meloidogyne arenaria.*(H, I, J,) = Positive control for (*M. hapla, M. incognita* and *M. javanica*) respectively from Holland M = Size marker DNA

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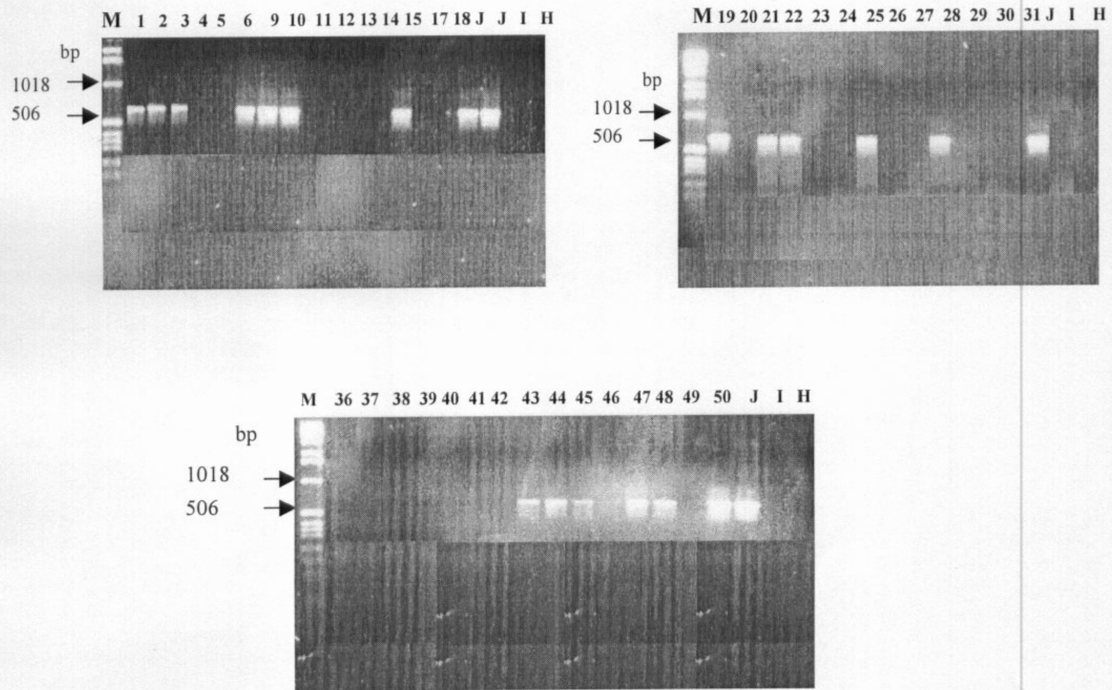

Fig. (10) : SCAR primer for *Meloidogyne javanica.* (H, I, J,) = Positive control for (*M. hapla, M. incognita* and *M. javanica*), respectively from Holland. M = Size marker DNA

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Table (5): The result of rDNA test identification and code of root-knot nematode isolates for RAPD test.

No	*	Governorate	Centre	Result of rDNA	Code	Plant source
1	1	Behera	Rahmania	*M. javanica*	JBR1	Tomato
2	3	Behera	Rahmania	*M. javanica*	JBR2	Egg plant
3	6	Behera	Rahmania	*M. javanica*	JBR3	*Solanium* spp.
4	19	Behera	Nubaria	*M. javanica*	JBN1	Tomato
5	21	Behera	Nubaria	*M. javanica*	JBN2	Tomato
6	28	Behera	Nubaria	*M. javanica*	JBN3	Tomato
7	43	Behera	Nubaria	*M. javanica*	JBN4	Tomato
8	49	Behera	Nubaria	*M. javanica*	JBN5	Peanut
9	2	Kafr El-Sheikh	Desoque	*M. javanica*	JKD1	Egg plant
10	10	Kafr El-Sheikh	Balteem	*M. javanica*	JKB1	Tomato
11	15	Kafr El-Sheikh	Balteem	*M. javanica*	JKB2	Tomato
12	9	Menia	Matay	*M. javanica*	JMM1	*Solanium* spp.
13	17	Behera	Nubaria	*M. incognita*	IBN5	*Solanium* spp.
14	20	Behera	Nubaria	*M. incognita*	IBN7	Tomato
15	27	Kafr El-Sheikh	Motobs	*M. incognita*	IKM9	Tomato
16	37	Kafr El-Sheikh	Motobs	*M. incognita*	IKM10	*Solanium* spp.
17	12	Kafr El-Sheikh	Balteem	*M. incognita*	IKB11	Tomato
18	36	Kafr El-Sheikh	Balteem	*M. incognita*	IKB14	Bean
19	42	Al Aresh	El-Sheikh Zoid	*M. incognita*	IAS2	Fig
20	30	Behea	Nubaria	*M. hapla*	HBN9	Sugar beet
21	29	Behera	Nubaria	*M. hapla*	HBN10	Peanut
22		Holland		*M. incognita*	IH	Tomato
23		Holland		*M. javanica*	JH	Tomato
24		Holland		*M. hapla*	HH	Tomato

* DNA extraction number.

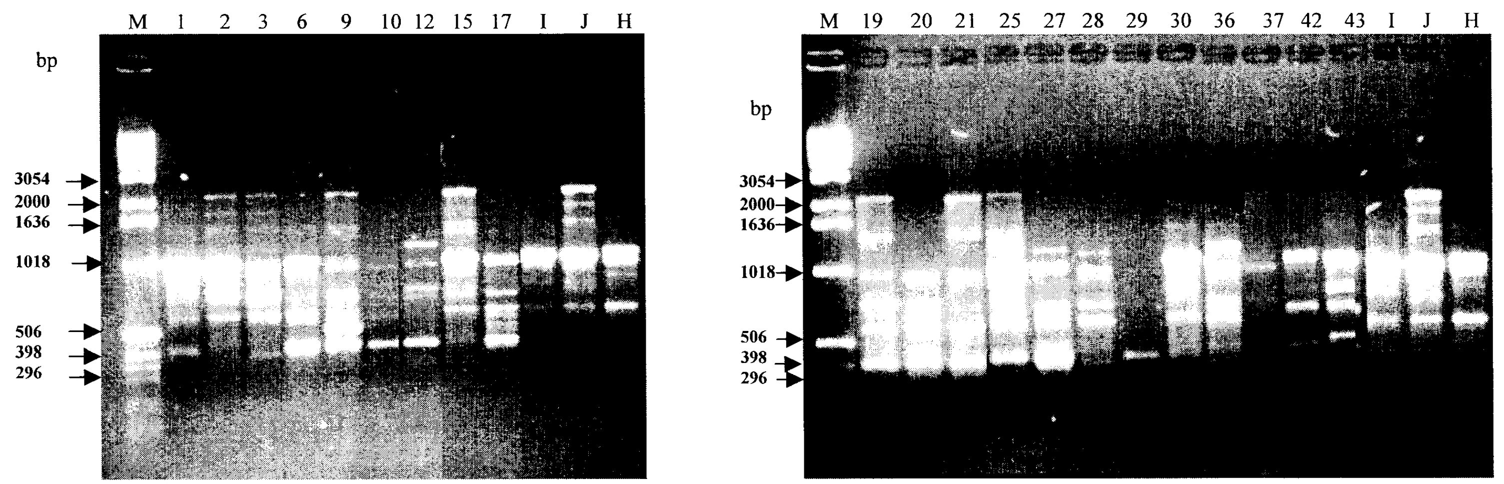

Fig. (11) : RAPD-PCR banding patterns of the 24 root- knot nematode isolates tested with primer OPA-O1. (I), *M. incognita* (J), *M. javanica* and (H) *M. hapla* as positive control from Holland . M = Size marker DNA.

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Figure (12) shows the banding patterns of the 24 root knot nematode isolates which were subjected to primer OPA-11. The results indicated the ability of these primer to detect some polymorphisms between *M. hapla* isolates and other isolates. The resulted bands were ranged between 300-3500 bp, two polymorphic bands 700 and 1000 bp appeared with two isolates of *M. hapla* (HBN9 and HBN10), these bands were observed in *M. hapla* (HH) isolate from Holland. Two polymorphic bands 1550 and 2000 bp appeared with *M. javanica*. There were two monomorphic bands 400 and 620 bp.

Using OPA-12 primer didn't give good banding patterns (Fig. 13). Generally, there was no specific banding patterns for any of the tested species. Only, there was a monomorphic band (760 bp) but this band did not appear in *M. hapla* isolate (HBN_{10}).

Using OPG-02 indicated that bands were ranged between 300 and 1700 bp (Fig. 14). This primer (OPG-02) didn't give species-specific banding patterns. There were polymorphic bands but they were not species specific bands because of their absence with some isolates from the same species or their presence with same isolates from different species. Also, the isolates from Holland were different from the Egyptian isolates.

6.1. RAPD-data analysis:

All bands resulted from using the 4 primers were scored as present (1) or absent (0) as Table (6a and b).

The similarity matrix showed different degrees of inter-and intra-specific variations between each pairs of isolates (Fig. 15). The isolates of *M. javanica, M. incognita* and *M. hapla* showed the level of similarity

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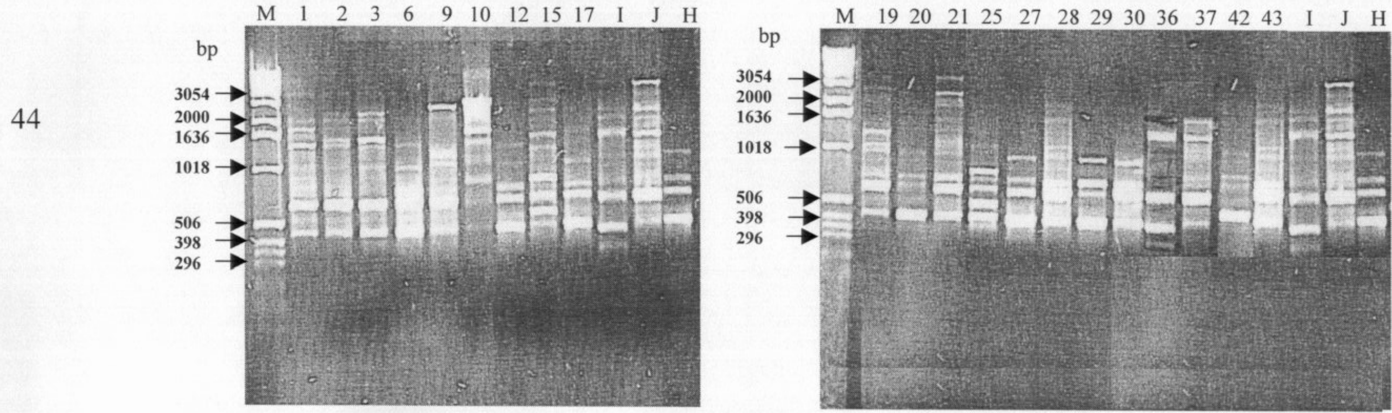

Fig. (12) : RAPD-PCR banding patterns of the 24 root- knot nematode isolates tested with primer OPA-11. (I), *M. incognita* (J), *M. javanica* and (H) *M. hapla* as positive control from Holland. M = Size marker DNA.

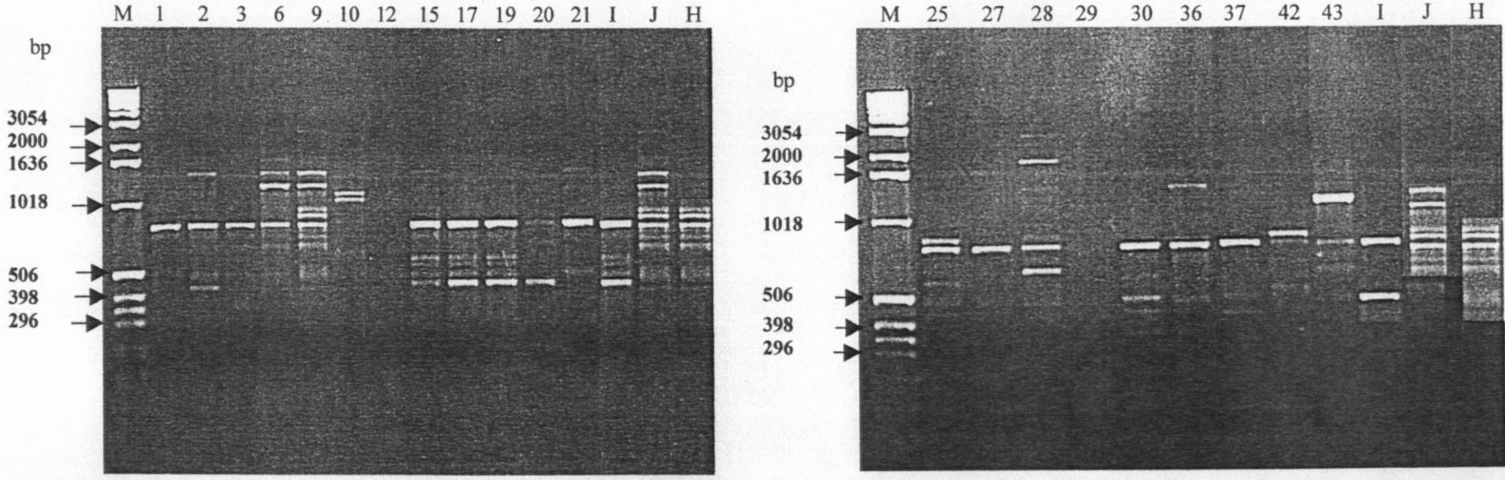

Fig. (13) : RAPD-PCR banding patterns of the 24 root-knot nematode isolates tested with primer OPA-12. (I), *M. incognita* (J), *M. javanica* and (H) *M. hapla* as positive control from Holland . M = Size marker DNA.

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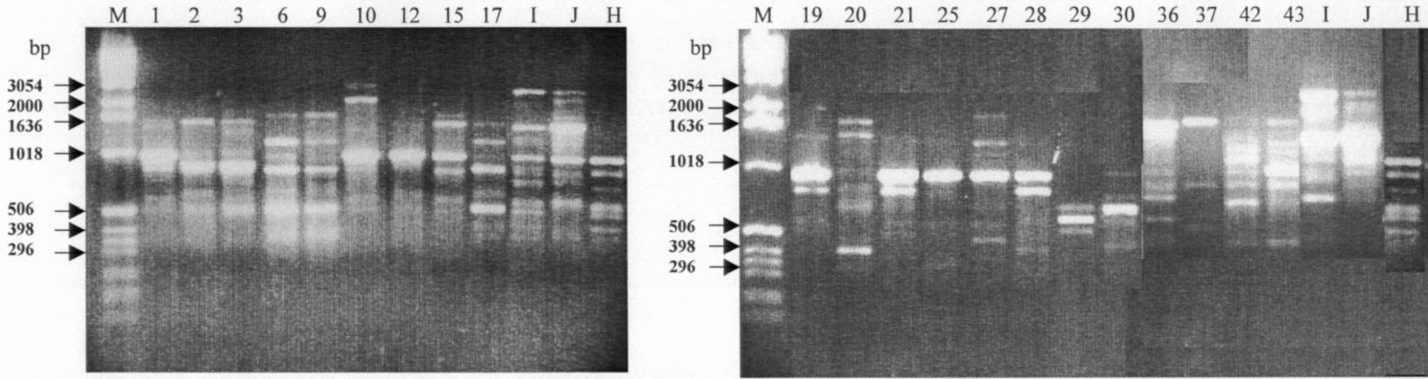

Fig. (14) : RAPD-PCR banding patterns of the 24 root- knot nematode isolates tested with primer OPG-O2. (I), *M. incognita* (J), *M. javanica* and (H) *M. hapla* as positive control from Holland . M = Size marker DNA.

Table (6) (a and b) : The scored bandes of the four primers with the 24 root-knot nematode isolates. 0, absent; 1 , present.

(a)

Isolates / Band	C	JBR1	JKD1	JBR2	JBR3	JMM1	JKB1	IKB11	JKB2	JBN5	JBN1	JBN7	JBN2	JKB4	IKM9	JBN3	HBN10	HBN9	IKB14	IKM10	IAS2	JBN4	IH	JH	HH
		1	2	3	6	9	10	12	15	17	19	20	21	25	27	28	29	30	36	37	42	43	I	J	H
OPA1-(01)		0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
OPA1-(02)		1	1	1	1	1	0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	1	0	1	0
OPA1-(03)		0	1	1	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0
OPA1 -(04)		0	1	1	0	1	0	0	1	0	1	0	1	1	0	0	1	1	1	0	0	1	1	1	1
OPA1-(05)		0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
OPA1-(06)		0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0
OPA1-(07)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
OPA1-(08)		1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	0	1	1	1	1	1	1	1
OPA1-(09)		1	1	1	1	0	0	0	1	1	1	1	1	1	0	1	0	1	0	1	1	0	0	0	0
OPA1-(10)		1	0	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0
OPA1-(11)		0	1	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	1	1	1	0	1	0
OPA1-(12)		0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	1	0	0	0	0	0	0
OPA1-(13)		0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0
OPA1-(14)		1	1	1	1	1	0	0	1	1	0	1	1	0	1	1	0	1	1	0	1	1	1	1	1
OPA1-(15)		0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
OPA1-(16)		0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
OPA1-(17)		1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
OPA11-(01)		0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OPA11-(02)		1	1	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0
OPA11-(03)		0	1	1	0	1	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
OPA11-(04)		1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
OPA11-(05)		1	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	1	1	0
OPA11-(06)		0	1	1	0	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
OPA11-(07)		1	1	1	1	0	1	1	1	1	1	0	1	1	0	0	0	0	0	0	1	0	0	1	0
OPA11-(08)		0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0
OPA11-(09)		0	0	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	0	0	0	0	0	1	1
OPA11-(10)		1	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
OPA11-(11)		0	0	0	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
OPA11-(12)		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
OPA11-(13)		0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
OPA11-(14)		1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

(b)

Isolates / Band	C	JBR1	JKD1	JBR2	JBR3	JMM1	JKB1	IKB11	JKB2	JBN5	JBN1	JBN7	JBN2	JKB4	IKM9	JBN3	HBN10	HBN9	IKB14	IKM10	IAS2	JBN4	IH	JH	HH
		1	2	3	6	9	10	12	15	17	19	20	21	25	27	28	29	30	36	37	42	43	I	J	H
OPA11-(15)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0
OPA12-(01)		0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
OPA12-(02)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
OPA12-(03)		0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
OPA12-(04)		0	0	0	1	1	0	0	1	0	1	0	1	0	0	0	0	0	1	1	1	1	0	0	0
OPA12- (05)		0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0
OPA12- (06)		0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OPA12- (07)		0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OPA12- (08)		0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OPA12 -(09)		0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
OPA12-(10)		1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
OPA12-(11)		0	1	1	1	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0
OPA12-(12)		0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OPA12-(13)		0	1	1	1	1	0	0	1	1	1	0	1	1	1	1	0	1	1	0	0	0	0	0	1
OPA12-(14)		0	1	0	0	0	0	0	1	1	1	0	0	1	0	0	0	1	0	0	0	0	0	1	1
OPA12-(15)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
OPG2 - (01)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
OPG2- (02)		0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
OPG2- (03)		0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0
OPG2- (04)		0	1	0	0	1	0	0	1	1	0	1	0	0	1	1	0	0	0	1	1	1	0	1	0
OPG2-(05)		1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0
OPG2- (06)		0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0
OPG2- (07)		0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1
OPG2- (08)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
OPG2- (09)		1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1
OPG2- (10)		1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	1	0
OPG2- (11)		0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
OPG2- (12)		0	0	0	1	1	0	1	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	1
OPG2- (13)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
OPG2- (14)		1	0	0	0	0	1	1	0	0	1	0	1	0	1	0	1	0	1	1	0	0	1	0	0
OPG2- (15)		0	1	1	1	1	0	0	0	1	0	0	0	0	1	1	0	1	0	1	0	0	1	0	0
OPG2- (16)		0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	1	0	0
OPG2- (17)		0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0

* (C) The code of the isolates.

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	JBR$_1$	JKD$_1$	JBR$_2$	JBR$_3$	JMM$_1$	JKB$_1$	JKB$_1$	JKB$_2$	IBN$_5$	JBN$_1$	JBN$_7$	JBN$_2$	JKB$_4$	IKM$_3$	JBN$_3$	HBN$_{10}$	HBN$_9$	IKB$_{14}$	IKM$_{10}$	IAS$_2$	JBN$_4$	IH	JH	HH
JBR$_1$	1.00																							
JKD$_1$	0.57	1.00																						
JBR$_2$	0.58	0.77	1.00																					
JBR$_3$	0.55	0.65	0.71	1.00																				
JMM$_1$	0.43	0.60	0.61	0.75	1.00																			
JKB$_1$	0.49	0.43	0.42	0.44	0.38	1.00																		
JKB$_1$	0.61	0.41	0.46	0.59	0.45	0.58	1.00																	
JKB$_2$	0.63	0.77	0.68	0.58	0.61	0.50	0.50	1.00																
IBN$_5$	0.49	0.60	0.56	0.67	0.54	0.48	0.53	0.57	1.00															
JBN$_1$	0.58	0.73	0.70	0.62	0.49	0.56	0.57	0.68	0.60	1.00														
JBN$_7$	0.49	0.51	0.56	0.59	0.54	0.38	0.53	0.46	0.71	0.51	1.00													
JBN$_2$	0.71	0.67	0.68	0.61	0.49	0.43	0.46	0.67	0.47	0.73	0.51	1.00												
JKB$_4$	0.59	0.55	0.56	0.53	0.46	0.43	0.58	0.68	0.52	0.65	0.48	0.55	1.00											
IKM$_3$	0.53	0.55	0.50	0.57	0.49	0.47	0.63	0.49	0.70	0.50	0.67	0.55	0.47	1.00										
JBN$_3$	0.51	0.57	0.53	0.64	0.56	0.42	0.50	0.55	0.58	0.44	0.64	0.53	0.46	0.67	1.00									
HBN$_{10}$	0.30	0.18	0.34	0.39	0.32	0.38	0.58	0.29	0.31	0.41	0.36	0.36	0.31	0.41	0.35	1.00								
HBN$_9$	0.42	0.50	0.55	0.57	0.45	0.28	0.46	0.49	0.60	0.55	0.56	0.50	0.47	0.60	0.58	0.55	1.00							
IKB$_{14}$	0.59	0.51	0.42	0.53	0.54	0.48	0.58	0.54	0.48	0.56	0.52	0.64	0.48	0.65	0.58	0.44	0.51	1.00						
IKM$_{10}$	0.59	0.55	0.50	0.68	0.53	0.46	0.65	0.49	0.56	0.61	0.57	0.55	0.46	0.61	0.59	0.48	0.50	0.62	1.00					
IAS$_2$	0.56	0.57	0.53	0.70	0.55	0.44	0.55	0.51	0.68	0.58	0.65	0.52	0.49	0.53	0.51	0.30	0.42	0.63	0.71	1.00				
JBN$_4$	0.60	0.61	0.52	0.59	0.67	0.44	0.54	0.65	0.58	0.57	0.59	0.57	0.62	0.52	0.51	0.32	0.43	0.67	0.58	0.75	1.00			
IH	0.53	0.50	0.40	0.48	0.49	0.51	0.46	0.38	0.47	0.45	0.46	0.55	0.37	0.60	0.49	0.34	0.45	0.74	0.56	0.53	0.57	1.00		
JH	0.53	0.62	0.51	0.53	0.57	0.52	0.43	0.69	0.48	0.47	0.44	0.62	0.48	0.47	0.54	0.25	0.39	0.48	0.43	0.49	0.60	0.63	1.00	
HH	0.34	0.44	0.38	0.51	0.43	0.30	0.44	0.44	0.55	0.43	0.39	0.39	0.40	0.49	0.48	0.54	0.81	0.50	0.36	0.40	0.46	0.43	0.42	1.00

Fig.(15): The similarity matrix of the 24 root-knot nematode isolates by using NTSys program based on the Dice similarity coefficient.

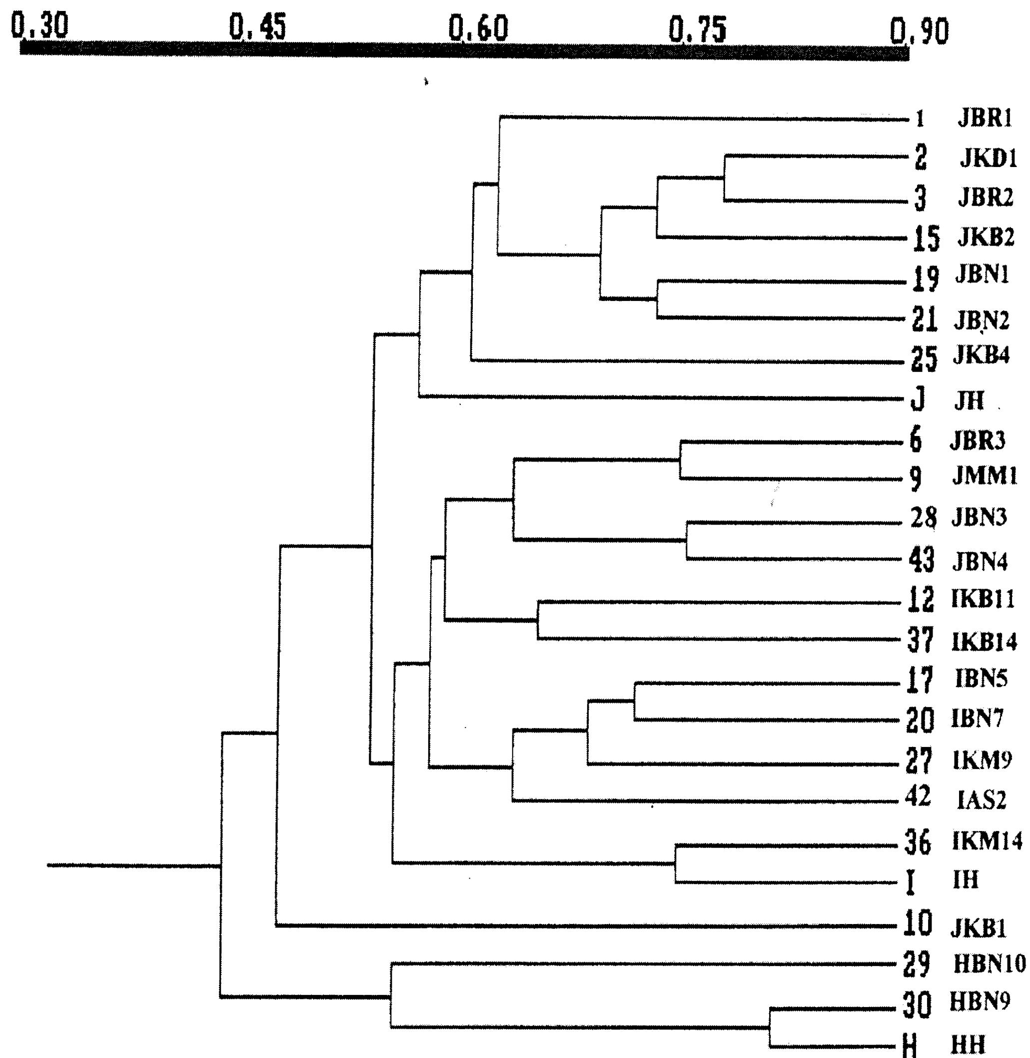

Fig.(16): The similarity dendrogram of the 24 different root-knot nematode isolates by using four primers of RAPD-PCR, analyzed with NTSys program using the Dice coefficient for similarity.

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among isolates within species as follows: 0.42-0.77, 0.46-0.74 and 0.54-0.81, respectively. The intra-specific variations among species of *M. incognita-M. javanica, M. hapla-M. javanica* and *M. incognita-M. hapla*, were 0.38-0.71, 0.18-0.60 and 0.31-0.60, respectively.

The resulted phylogenetic tree scaled as (F) value ranged from 0.30-0.90. The tree included two main clusters, one of them included *M. hapla* (HH, HBN9 and HBN10) and the other one included the rest of the used isolates which were divided into two sub cultures, one of them included the isolate JKB10, and the other one included the rest of the *M. javanica* and *M. incognita*. The isolates of the *M. javanica* located in a separate sub-sub-cluster were close to the isolates of *M. incognita* which located in a another sub sub-cluster. The four isolates (JBR3, JMM, JBN3 and JBN4) located in sprat cluster were close to *M. incognita*. Also, the *M. hapla*, from Holland (HH) located under the same cluster with the isolates, *M. hapla* (HBN9 and HBN10) from Egypt. Also, *M, javanica* and *M. incognita* from Holland were located under sub-sub cluster with Egyptian isolates *M. javanica* and *M. incognita,* respectively (Fig. 15).

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DISCUSSION

Modern molecular techniques has been developed in recent years, and it could be argued that molecular systematic will have its greatest impact among the nematode taxa. Nematode molecular systematies is in its infancy, using nucleotide sequence data to assess the relationships among nematodes. Also, molecular systematies can put nematodes on the same footing as better understood organisms (Power and Adams, 1994).

The present results show that it is possible to use many molecular techniques as valuable tools for the identification of root-knot nematodes. Therefore, we used multiplex-PCR which easily differentiates the species *M. hapla* and *M. incognita* from each and from *M. chitwoodi* and *M. fallax*, even in mixtures. The size of the IT'S-PCR products of *M. hapla, M. chitwoodi, M. fallax, M. incognita* and, *M. javanica* that measured by using ITS-specific primers 5367 and 5368 was approximately 760 bp.

The sequences, described by **Zijlstra (1997)** were very useful for providing sequences of species-specific primers (H-18S, CH-ITS, I-ITS, and HCFI-28S) and show that there are slight size differences between the species ranging from 766 bp (*M. incognita/M. javanica*) and 784 bp (*M. chitwoodi)*. More variations are present in the IT'S-1 region. The ITS-1 sequences of *M. hapla* and *M. incognita* which differ from each other and from *M. chitwoodi* and *M. fallax*. More variations have been found in the IT'S-2 region indicating that the primers H-18S and I-ITS allow species-specific amplification of *M. hapla* and *M. incognita* respectively, resulting in a PCR product of 660 bp for *M. hapla* when

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reverse primer HCFI-28S was used and a fragment 416 bp for *M. incognita* or *M. javanica*.

The IT'S region of Meloidogyne which produced 760 bp considerably shorter than PCR amplification on other nematode genera using the same set of primers resulted in different size fragments with slight variations e.g. 1400 bp for the *Caenorhabditis elegans* (**Vrain, 1993**), 1500 bp for the *X. americanum* group (**Vrain *et al.*, 1992**), 900 and 1200 bp for the *D. dispaci* and *D. destructor*, respectively (**Wendt *et al.*, 1993**). Similar results were obtained by **Zijlstra *et al.*, (1995)**. Also, **Gour *et al.* (1996)** obtained the IT'S fragments at 800 bp for *M. arenaria, M. incognita* and *M. javanica*.

Comparison of restriction patterns derived from amplified ITS regions proved to be a useful molecular approach to separate the species *M. hapla, M. incognita* or *M. javanica,* since the last two species could not be distinguished from each other (**Xue *et al.*, 1992**). In our results EcoRI restriction enzyme patterns clearly separated *M. hapla* (760 bp) from *M. incognita* and *M. javanica* (520 and 240 bp). Also, Hinf1 restriction patterns clearly separated *M. hapla* 490 and 270 bp from *M. incognita* and *M. javanica*. With BamH1 restriction enzyme patterns contained bands of 430 and 330 bp with *M. hapla* and didn't digest *M. incognita* or *M. javanica* (760 bp). Thereby distinguishing *M. hapla* from *M. incognita,* on the other hand **Xue *et al.* (1992)** digested of IT'S fragments of *M. hapla*, *M. incongnita, M. javanica* and *M. arenaria* with EcoR1 and Hinf1 which didn't show differences between species.

So ITS-RFLP technique has been considered to be a valuable tool to accurately differentiate species in a mixed population. So, the

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intensities of the bands can be quantified. This method could be applied routinely, preferably using crude extracts of juveniles, females and eggs to determine the species composition of a field population.

The advantages of rDNA analysis are that the method is fast and reliable, it is useful for identification at any developmental stage because of the abundance of rDNA in the genome. Also, it can even be applied on individual nematodes. Multiplex technique becomes widely used in routine diagnostic tests to identify species and to sensitively differentiate them from each other in field populations **(Zijlstra, 1997)**.

In their original paper describing RAPD technology, **Williams *et al.* (1990)** reported the use of 10-nucleotide random primers to detect genomic polymorphisms. This methodology allowed the separation of the four major species but failed to detect any intraspecific polymorphisms within *M. incognita* and *M. javanica* populations **(Cenis, 1993)**. The present data show the ability of four primers (OPA-01, OPA-11, OPA-12 and OPG-02) to amplify of DNA from the three root-knot nematodes species *M. javanica*, *M. incognita* and *M. hapla* and also three identified isolates from Holland. Most of primers produced species-specific patterns. *M. javanica, M. incognita* and *M. hapla* RAPD patterns often shared one or more bands of the same size. In most cases *M. hapla* didn't share bands with the other two species. these agree with the currently accepted grouping of this species **(Cenis, 1993)**.

Our results on the OPA-01 primer can be used as a tool to distinguish the three root-knot nematodes species. Two species-specific bands are 1550 bp and 2000 bp appeared with *M. javanica,* but *M. incognita* were produced 540 bp and 1000 bp bands, while two bands

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850 and 1020 bp were produced by *M. hapla.* These polymorphic bands appeared in the three isolates from Holland. Similar results were obtained by **Cenis (1993)** who got 540 bp and 1000 bp specific band for *M. incognita* and 850 bp for *M. hapla.* **Williamson *et al.* (1997)** and **El-Ashry (1998)** got similar results. **Cenis (1993)** found that an additional band at 1020 bp was present in the population race A of *M. hapla* but was not present in the population from race B while 1550 bp and 1100 bp bands were found specific for *M. javanica.* These results for *M. javanica* were contradictory with our results. The reason for this discrepancy with results of **Cenis (1993)** has not been known yet, but could be due to differences in reagent sources and the kind of enzyme.

Results also indicated that the primer OPA-11 is considered as a one of the primers which can give the best amplification between *M. hapla* and *M. javanica.* These results are in contrast with **Cenis (1993)** who considered that this primer would give the best amplification between *M. hapla* and *M. incognita.* He also indicated that OPA-12 primer gave the best amplification between *M. incognita* and *M. arenaria*, which differed from our results, since this primer didn't give any polymorphisms.

The phylogenetic relationships within the genus Meloidogyne inferred from this RAPD analysis are consistent with existing phylogenetics deduced from both isoenzyme surveys (**Esbenshed and Triantaphyllou, 1987**) and molecular studies based on total genomic DNA (**Xue *et al.*, 1992; Castagnone Sereno *et al.*, 1993 and 1994).** Our results confirm the early divergence of *M. hapla* from the other species and also show that *M. incognita* is closer to *M. javanica.* RAPD fingerprinting can easily be used for phylogentic evaluations between

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species. Even if the validity of using RAPD markers for estimating genetic similarities between isolates was sometimes questionable (**Block, 1993**), the technique looked accurate at least under experimental conditions in the case of root-knot nematodes which is in accordance with the results obtained by **Castagnone-Sereno *et al.* (1994)**.

The pattern of genetic variation in a species depends on a number of factors, including the genetic population structure, geographical genetic variation, amount of gene flow and the effect of evolutionary forces on the history of the population such as selection and genetic drift **(Power and Adams, 1994).**

Phylogenetic inference has been made by using nematode nucleotide sequences **(Beckenbach *et al.*, 1992; Ferris *et al.*, 1993; Hugall *et al.*, 1994)** which indicate that there was a such sequence divergence between linkages of *M. arenaria* as there was between *M. arenaria* and *M. javanica* (**Hugall *et al.*, 1994**). This study helps explain the different discordance, between nuclear and organelle phylgenies in *Meloidogyne.*

rDNA and RAPD-PCR should be useful tools for diagnostic purposes. the techniques are simple, rapid and safe because they don't involve the use of radioactive isotopes which require micrograms amounts of DNA. This makes it possible to analyze little material as a single juvenile, eggs or females which are useful in studies of genetic variation or diagnosis in mixed isolates RAPD-PCR should be useful tool for addressing many unresolved questions of genetic variation and isolates genetics of root-knot nematodes.

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SUMMARY

Nematode species belonging to the genus *Meloidogyne* are pests of major food crops, vegetables, fruits and ornamental plants. Certain species are widely distributed and able to cause great losses in production and quality.

Identification of *Meloidogyne* spp. is important for the design of an efficient control plan in the IPM program. The identification of nematode species was always based on morphological characteristics that require a lot of skill and experience, but it is not very accurate. Recently, the appearance isozyme electrophoresis and nucleic acid technology have allowed new approaches to get reliable and precise nematode identification.

Comparative analysis of coding and non coding regions of ribosomal DNA has become a popular tool for nematode species identification.

This study aimed to use some advanced techniques ITS-RFLPs, multiplex-PCR, SCARs and RAPD-PCR to identify the different isolates of root-knot nematodes by using DNA fragments.

In this study, samples collected from different locations, different hosts and identified isolates from Holland were used. The results of different DNA techniques can be summarized as follows:

1- Amplification of ITS-region by using 5368 and 5367 primers gave a strong band at 760 bp in 26 samples of tested *Meloidogyne* isolates. Digestion of 760 bp fragment with restriction enzyme

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Hinfl produced two distinguished bands at 440 and 320 bp which are reliable identification to *M. incognita* or *M. javanica*, whereas, *M. hapla* produced two bands at 490 and 270 bp. On concerning restriction enzyme EcoR1, produced two bands at 520 and 240 bp with *M. incognita* or *M. javanica* and not bands were observed for *M. hapla*. Restriction enzyme BamH1 is specificity for *M. hapla* and produced clear bands at 430 and 330 bp. .

Results of ITS-RFLPs techniques could be considered as a differentiating tool for *M. incognita* or *M. javanica* and *M. hapla* identification. The nematode species *M. hapla* is the first record for diagnosing and identification of this species on peanut and sugar beet plants in Nubaria province, Egypt in samples No. 29 and 30, respectively.

2- Multiplex-PCR was performed by using four primers H-18S, CF-ITS, I-ITS and HCFI-28S in a single PCR reaction, which gave a strong distinguished band for *M. javanica* or *M. incognita* at 415 bp. and *M. hapla* at 660 bp. This technique is accurate for distinguish *M. hapla*.

3- SCARs primers: used two specific species primers for accurate distinguishable technique for *M. hapla*, *M. javanica* and *M. arenaria*, one strong band for *M. javanica* was obtained in twenty isolates and two isolates for *M. hapla*, whereas no major band was observed with *M. arenaria* which ensure that there is no *M arenaria* in our collected isolates.

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4- Four primers (OPA-01, OPA-11, OPA-12 and OPG-02) were examined with twenty four *Meloidogyne* isolates to differentiate the three *Meloidogyne* species (*M. incognita, M. javanica* and *M. hapla*). Results indicated that the best efficient primer was OPA-01 which was able to differentiate the three root-knot nematode species.

5- Scoring of the banding patterns which resulted from using four primers were analyzed by using the NTSys. program. The analysis of similarity matrix of data emphasizes the relationship between each pair of isolates.

6- Using the similarity matrix to perform the phylogenetic tree (Dendrogram) by graphically showing indicating that *M. javanica* and *M. incognita* are close to each other than *M. hapla*.

Actually, this study clarified the fact that using advanced techniques ITS-RFLPs, multiplex-PCR, SCARs and RAPD can gave a good accurate distinguishable differentiation between the three root-knot nematode isolates, *M. javanica*, *M. incognita* and *M. hapla* which are considered faster and more reliable than that of the traditional methods. Moreover, they become a popular tool for species of root-knot nematode identification.

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الملخص العربى

استخدام بعض طرق البيولوجيا الجزيئية المختلفة فى تعريف نيماتودا تعقد الجذور

رباب ممدوح احمد العماوى

تعتبر النيماتودا المتطفله على النباتات عامة ونيماتودا تعقد الجذور خاصة من المسببات المرضية لكثير من الأنواع النباتية سواء المحاصيل الحقلية أو البستانية أو محاصيل الخضر. وتعد الأنواع *M. incognita, M. javanica, M. arenaria* and *M. hapla* أهم الأنواع التى تنتشر فى معظم دول العالم ومنها مصر.

والهدف من إجراء هذه الدراسة هو استخدام بعض التكنيكات الحديثة لتعريف العزلات المختلفة من النيماتودا تعقد الجذور باستخدام الـ DNA ومن هذه الطرق:

١- ITS-RFLPs technique والتى تعتمد على استخدام إنزيمات قطع لهضم منطقة internal transcribed spacer (ITS) ومن هذه الإنزيمات BamH1 & EcoR1 & Hinf1 وهذه الإنزيمات تستخدم للتمييز بين نيماتودا تعقد الجذور *M. javanica/M. incognta, M. hapla*.

٢- Multiplex-PCR technique يستخدم هذا الاختبار للتفريق بين *M. hapla , M. chitwoodi, M. javanica/M. incognita* ويعتمد هذا التكنيك على استخدام ثلاث بادئات Forward primers وبادئ واحد Reverse primer وهى تعطى حزمه مميزه لكل نوع فى اختيار واحد.

٣- SCARs primers technique ويستخدم لتفريق *M. hapla , M. javanica, M. arenaria* وفيها يستخدم زوج من البادئات المتخصصه لكل نوع من النيماتودا السابق ذكرها فى اختبار مستقل عن الآخر (وهذا التكنيك تم أخذه عن طريق الاتصال الشخصى بالدكتوره كارولين جيسلترا بمعهد وقاية النبات فاجنن ـ هولندا (بحث لم ينشر بعد).

٤- استخدام طريقة الاكثار العشوائى للحامض النووى Random Amplified Polymorphic DNA (RAPD) والتى تعتمد على التفاعل المتسلسل للبلمرة Polymerase Chain Reaction (PCR) وذلك للتفريق بين العزلات المصرية من نيماتودا تعقد الجذور ومقارنتها بثلاث عزلات هولندية بحيث يمكن استخدام هذه الطرق مستقبلا فى التمييز بين الأنواع المختلفة من نيماتودا تعقد الجذور وتحديد العينات النقية أو المختلطة منها والتعريف السريع والدقيق للعزلات.

رات

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ويمكن تلخيص أهم النتائج المتحصل عليها على النحو التالى:

١- استخدام البادئ رقم 5368 والبادئ رقم 5367 وذلك لاكثار منطقة ITS من كل عزلة من عزلات النيماتودا وقد أعطت حزمه على مسافة 760 bp وهى حزمه مميزه لكل أنواع نيماتودا تعقد الجذورعلى السواء وقد تم استخدام ناتج ITS لهضمه بأحد إنزيمات القطع BamHI, EcoRI, HinfI وقد أسفرت نتائج هذا الاختبار إنه عند استخدام الإنزيم المتخصص HinfI اعطى حزمه مميزه عند 440 bp وحزمه أحرى عند 320 bp وهذه النتيجة مميزه ومحدده *M. incognita/M. javanica* وقد أعطى مع *M. hapla* حزمتين واضحتين هما 490 bp & 270 bp أما إنزيم EcoRI فإنه لا يعمل على *M. hapla* وبالتالى فإنه لم يتم قطع منطقة ITS وتبقى 760 bp كما هى ولكنه يعطى حزمتين واضحتين مع *M. javanica/M. incognita* عند 520 bp واضحة أما عند 240 bp فيعطى حزمه ضعيفة وأيضا باستخدام الإنزيم BamHI فانه يؤدى إلى قطع *M. hapla* ويعطى حزمتين واضحتين عند 330 bp, 430 bp ولا يقطع عزلات *M. javanica/M. incoginta* وتبقى 760 bp ومن هذا الاختبار يتم التفريق بوضوح بين العزلات *M. javanica/M. incognita* , *M. hapla* وتعتبر *M. hapla* أول تسجيل لها على بنجر السكر والفول السودانى بمنطقة النوبارية بالبحيرة وذلك فى العينتين رقم ٢٩ و ٣٠.

٢- تم استخدام تكنيك Multiplex-PCR وقد استخدمت البادئات HCFI-28S, H-18S, ITS-18S, CF-ITS وذلك فى اختبار واحد وهذا الاختبار يعطى حزمه واحدة مميزه لكل نوع وقد أعطت عزلات *M. javanica/M. incognita* حزمه واضحة عند 415 bp وقد أعطت *M. hapla* حزمه عند 660 bp وهذا اختبار أخر تأكيدى للحكم على وجود *M. hapla* أما العزلات الهولندية فقد أعطت نفس الحزمه (عند 660 bp).

٣- عند استخدام الاختيار المسمى SCARs primers وهو عبارة عن استخدام زوج من الـ primers المتخصص لكل من *M. hapla, M. incognita, M. javancia* أعطت عشرون عينة نتيجة مع *M. javanica* وثلاثة عشر عينة مع *M. incoginta* وعينتان فقط مع *M. hapla* من النوبارية على الفول السودانى وبنجر السكر وخمسة عشر عينة لم تعطى تفاعل مع الاختيارات السابقة.

٤- تم اختبار اربعة بادئات هـــى OPA-01, OPA-11, OPA-12, OPG-02 فـــى اختبارRAPD-PCR مع عزلات نيماتودا تعقد الجـــذور التـــى تـم تعريفــها مــن الاختبارات السابقة والتأكد من صحة تعريفها وقد أعطـــت نتـــائج جيـــدة فـــى كـــل الاختبارات.

أ- كانت أفضل البادئات (Primers) المستخدمة فى التفريق بين الثلاثة أنواع هو البادئ OPA-01 فقد أمكن عن طريقة التمييز بين عزلات *M. hapla* , *M. javanica*., و *M. incognita*.

ب- أظهر البادئ OPA-11 إمكانية التمييز بين عـــزلات *M. hapla* عــن العـــزلات الخاصة بالنوعين الآخرين.

جـ- أما البادئات OPG-02 و OPA-12 لم يمكنهما بدرجة كافية التمييز بيـــن العـــزلات للأنواع المختلفة ، ولكن البادئ OPG-02 أعطى اختلافات واضحة بين الحزم الأمـــر الذى لم يمكن الحكم والتفريق بين الأنواع المستخدمة.

د- عند تسجيل الأشكال الناتجة من استخدام الاربعة بادئات وتحليلها باســـتخدام برنـــامج التقسيم الرقمى (NTsys. progrm.) أوضحت مصفوفة التشابه الناتجة درجة القرابـــة بين كل زوج من العزلات المستخدمة فى هذا البحث.

هـ- استخدمت مصفوفة التشابه لرسم (Dendrogram) Phylogenetic-tree لإظـــهار صله القرابة بين الأنواع المختلفة ، حيث اتضح بشكل عام صله القرابة بدرجة عاليـــة بين النوعين *M. javanica* و *M. incognita* ولكن لم تكن صلــــه القرابـــة بـــهذا الوضوح بين كل من هذين النوعين السابقين والنوع *M. hapla* حيث وقعت الأخيرة فى عنقود Cluster منفصل عن العنقود الذى وقعت به عزلات النوعيـــن الآخريـــن السابق ذكرهم.

وبذلك يتضح أنه باستخدام الطرق الســـابقة SCARs, Multiplex-PCR, ITS-RFLP, و RAPD أمكن من السهل عمليا التعريف والتمييـــز بيـــن الأنـــواع الثلاثة من نيماتودا تعقد الجذور *M. hapla* , *M. javanica*, *M. incognita* والتى وجدت فى العزلات التى تم حصرها من المناطق المختلفة تحت الدراسة وذلـــك فـــى وقت قصير وبدقة أعلى منها فى حالة استخدام الطرق التقليدية المستخدمة فى التعريف والتمييز بين الانواع المختلفة لنيماتودا تعقد الجذور.

إستخدام بعض طرق البيولوجيا الجزيئية المختلفة فى تعريف نيماتودا تعقد الجذور

رسالة مقدمة من

رباب ممدوح احمد العماوى

بكالوريوس العلوم الزراعية ـــ شعبة أمراض نبات

كلية الزراعة بكفرالشيخ ـــ جامعة طنطا ١٩٩٥

للحصول على درجة

الماجستير فى العلوم الزراعية

أمراض النبات (نيماتودا)

قسم النبات الزراعى

كلية الزراعة بكفرالشيخ

جامعة طنطا

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