Identification of different root-knot nematodes and detection of intraspecific and intrapopulation genetic variabilities between different nematode samples using RAPD technique

(Received: 02.06.2003; Accepted: 30.06.2003)

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ABSTRACT

Root knot nematodes (Meloidogyne species) are parasites of a multitude of host plants. Estimated yearly crop losses are approximately 5% worldwide, although the damage inflicted in certain regions of developing countries exceeds this level. The amplification of mitochondrial DNA (mtDNA) method was used to distinguish different genera and species of root knot nematodes. Single juveniles were used in a PCR reaction mixture. Primer annealing sites were located in the 3` portion of the mitochondrial gene coding for cytochrome oxidase subunit II and the 16S rRNA gene. Following PCR amplification, fragment of size 1700 bp specific for genus Meloidogyne was produced. Digestion of the amplified product with restriction endonucleases allowed discrimination among species with identically sized amplification products. Hinf I digestion of the 1700 bp fragment produced a two banded pattern in M. javanica, versus a three banded pattern in M. incognita. The random amplified polymorphic DNA (RAPD) method was used to investigate the genetic diversity in Meloidogyne species isolates and to identify molecular markers characteristic of this species, in order to develop PCR-based markers, which can be used to detect these nematode species in the field. Populations of each of M. incognita and M. javanica were easily distinguished by differences in fragment patterns with any of 10 RAPD primers. These distinctive RAPD fragments are candidates for intraspecific and intrapopulation identifications. Some primers, e.g., OPA-3, OPA-5, OPB-3 and OPB-5, yielded only a few species-specific bands that enabled differentiation of M. incognita and M. javanica. Other primers yielded more complex patterns, which were less easily interpreted.

Key words: *Molecular markers, plant parasitic nematodes, RAPD-PCR.*

INTRODUCTION

Nematodes constitute one of the largest animal phyla, consisting of over a half million species of worms. Four out of five living animals on this planet are nematodes (Platt, 1994). Nematodes can live as obligatory parasites of plants and animals; they can alternate a parasitic with a free-living life style or be strictly free-living. Despite their

and developmentally very similar.

Species of the root knot nematode *Meloidogyne* exhibit extensive cytogenetic diversity, with many parthenogenetic forms, a large proportion of which are polyploid, obligate, mitotic parthenogens demonstrating a broad geographical distribution and host range diversity (Fargette *et al.*, 1994; Hugall *et al.*, 1999).

Traditionally, species have been defined by host range tests, perineal patterns and allozyme analysis (Taylor and Sasser 1978; Eisenback *et al.*, 1981 and Esbenshade and Triantaphyllou 1990) and more recently, DNA markers, mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) diagnostics have been described (Williams *et al.*, 1993; Powers and Harris, 1993; Hugall *et al.*, 1994; Stanton *et al.*, 1997).

DNA markers have been useful for identification and phylogenetic comparison of plant-parasitic nematodes (Curran, 1991; Williamson, 1991; Jones *et al.*, 1997). Nematode genomic DNA has been investigated primarily through restriction fragment length polymorphisms (RFLPs). These RFLPs can differentiate species and, in some cases, races and populations of plant parasitic nematodes (Kaliniski and Huettel 1988; Castagnone-Sereno *et al.*, 1991). mtDNA has been more extensively investigated than genomic DNA because mtDNA evolves at rapid rate, is a relatively simple molecule and is abundant in eukaryotic cells (Powers and Sandall, 1988 and Hyman, 1989). The sensitivity of DNA analysis has been extended to allow identification of individual *Meloidogyne* juveniles through the use of the polymerase chain reaction (PCR) (Harris, 1990; Harris *et al.*, 1990).

The aim of the present work was to detect genetic variability within several races of the two major *Meloidogyne* species (*M. incognita* and *M. javanica*) that were isolated from different vegetables growing in 20 different areas of El Fayoum and Bani-Sweef governorates.

MATERIALS AND METHODS

Nematode culture and females isolation

Samples of 20 populations of root knot nematodes (*Meloidogyne* species) were collected from heavily infested spots in the fields of 9 different localities in 4 centres of Bani-Sweef governorate and 11 different localities in 5 centres of El Fayoum governorate. The samples were inspected and the associated root knot nematodes species were isolated and identified depending upon the perennial patterns and mitochondrial DNA (mtDNA) experiments.

A single egg mass from each population was maintained on tomato (*Lycopersicom esculentum* cv. Castle rock II PVPN) grown in sandy soil in 1000 cm^3 plastic pots in green house at 28°C for three months. Three pots were represented for each isolate.

After the establishment of tomato plants in the green house for three months, the root system of each plant was collected and cleaned from debris. A good technique for females isolation that provides a quantity of clean adult females of root knot nematodes from infected roots suitable for molecular studies was used (Hussey and Barker, 1973).

DNA extraction

From each nematode population, pure DNA was obtained from 30-40 mg of adult females of each sample using genomic prep cells and tissue DNA isolation kit (Amersham Pharmacia biotech, Ltd., England). Concentration of each extracted DNA was measured by using Smart Spac 3000 spectrometer (BioRad Laboratories, Inc.,

California, USA). The concentrations of the extracted DNAs of all the above samples are presented in Table (1).

Mitochondrial DNA amplification

The mitochondrial DNAs (mtDNA) of 20 populations of root knot nematodes were amplified from individual *Meloidogyne* second stage juveniles (J2) by using PCR methods, using two primers. **Primer C2F3**, 5'-GGTCAATGTTCAGAAA TTTGTGG-3', was designed by alignment of all invertebrate mitochondrial nucleotide sequences for the cytochrome oxidase subunit II (*COII*) gene available in Gene Bank. **Primer 1108**, 5'-TACCTTTGACCAATCACGCT-3', was designed by nucleotide sequencing a cloned fragment of the large subunit of ribosomal RNA gene (*IrRNA*) form *M. incognita.* These primers were sequenced as described by Harris (1990) and Powers and Harris (1993) and provided by Amersham Pharmacia biotech, Ltd., England. The amplifications process by the above 2 primers were carried out on three steps as the following:

1) Nematodes of each population were isolated from soil by a combination of sieving and density gradient centrifugation (Barker, 1985). Individuals of *Meloidogyne* J2 were handpicked and placed in 15 µl drop of sterile water on cover slip. The nematode was then ruptured by gentle pressure with a micropipet tip, which is sufficiently translucent to allow viewing the nematode to verify lysis. At this point, nematode lysate was processed immediately for PCR.
2) mtDNA amplification was carried out by using 50 µl Ready-To-Go PCR beads Kit (Amersham Pharmacia biotech, Ltd., England).A mixture of 15 µl nematode lysate, 5 µl primer C2F3 (5 pmol) and 5µl primer 1108 (5 pmol) was added to these PCR beads in 0.2 Eppendorf PCR tubes. The content of these tubes was mixed by gently vortexing. This mixture was placed in a DNA thermalcycler (Perkin-Elmer 480) already heated at 94°C for hot start. PCR amplification conditions were as follows; the reaction start by keeping the PCR tubes at 94°C for 4 minutes followed by 45 cycles of 94°C for 1 min., 48°C for 1 min. and 70°C for 2 min.
3) Following DNA amplification, the PCR products were screened on a 1 % agarose gel stained with ethidium bromide. The resulted bands were analyzed using Gel Documentation system (Gel Doc. 2000) image analysis software (BioRad Laboratories, Inc., California, USA).
4) Standard restriction digestion of the amplified products was conducted on 5 µl of the PCR amplification products and evaluated on 1.5 % agarose gels. *Hinf I* restriction enzyme (Amersham Pharmacia biotech, Ltd., England) digestion was conducted for 3 hr at 37 °C.

RAPD reactions

In the present work, the following 10 random arbitrary primers were used:

OPK-1: 5`-GGTGCGGGAA-3`
OPK-2: 5`-GTTTCGCTCC-3`
OPK-3: 5`-GTAGACCCGT-3`
OPK-4: 5`-AAGAGCCCGT-3`
OPK-5: 5`-AACGCGCAAC-3`
OPK-6: 5`-CCCGTCAGCA-3`
OPA-3: 5`-AGTCAGCCAC-3`
OPA-5: 5`-AGGGTCTTTG-3`
OPB-3: 5`-CATCCCCCTG-3`
OPB-5: 5`-TGCGCCCTTC-3`

The amplification process was carried out by using 50 µl Ready-To-Go RAPD analysis Beads kit (Amersham Pharmacia biotech, Ltd., England). The samples were

introduced to a Perkin Elmer 480 thermocycler (Norwalk, CT) where the PCR program used for the amplification was as following; one cycle of 95°C for 5 min followed by 10 cycles of 95 °C for 1 min, 36°C for 30 sec and 72°C for 30 sec, then followed by 30 cycles of 95°C for 10 sec, 35°C for 30 sec and 72°C for 30 sec.

After the amplification, the banding pattern of the randomly amplified DNA was visualized and analyzed. RAPD analysis was done on 2 % agarose gel and the electrophoresis reactions were run at 120 volt for 2 hr. The resulted bands were analyzed using Gel Documentation System (Gel Doc., 2000) image analysis software (BioRad Laboratories, Inc., California, USA) and the sizes of the bands were detected by 100 bp marker.

RESULTS AND DISCUSSION

Most methods of nematode diagnostics have some limitations. Nematode morphology has been the basis of nematode taxonomy and hypotheses of nematode phylogeny; however, the homology of morphological characters that appear to be similar may be uncertain (Hyman and Powers, 1991). Species identification based on differences in morphological characters (Hartman and Sasser, 1985) requires a lot of skill and is often inconclusive for individuals.

Species identification in *Meloidogyne* has been a component of taxonomic research in nematology. Isozyme analysis is relatively a fast way to identify species of the genus *Meloidogyne* (Esbenshade and Triantaphyllou, 1985; Esbenshade and Triantaphyllou, 1990). However, for clear reliable results, the Isozyme analysis can only be done with females of a specific developmental stage.

DNA based diagnosis provides attractive solutions to the problems associated with these identification methods, because they do not rely on the expressed products of the genome and are independent of environmental influence or developmental stage. Direct comparison of genotypes eliminates environmentally induced phenotypic variation; consequently, RFLP and DNA equence analysis have proliferated in systematics, with comparisons of mtDNA and rDNA sequences playing a particularly important role (Moritz and Hillis, 1990; Hillis and Dixon, 1991; Doly, 1993). Benefits of these methods are that they require no radioactive isotopes and small amounts of template DNA.

Nematode identification by mitochondrial DNA (mtDNA)

PCR primers C2F3 and 1108 were used for amplification of mtDNA from individual J2 of each examined population. Figure (1), illustrates the sizes of the amplified products of the 20 different populations. All isolates produced one major product approximately at 1700 bp specific for the genus *Meloidogyne*.

Restriction digestion of the 1700 bp amplification products with *Hinf I* produced diagnostic patterns for all isolates of *M. incognita* and *M. javanica* isolates (Fig. 2). One restriction site in the *M. javanica* products resulted in cleavage into 700 and 1000 bp bands, while in case of *M. incognita* products, an additional restriction site resulted in cleavage of the 700 bp fragment to yield two fragments of 400 and 300 bp.

In the present study, the nematode genus and species of the cultured populations were identified by perennial patterns and mtDNA amplifications as ten populations of *M. javanica* and ten populations of *M. incognita*. The geographical origin and species identification of each population were

summarized in Table (1). The mtDNA amplification experiments in the present work indicate that all El Fayoum and Bani-Sweef isolates belong to *M. incognita* and *M. javanica*. The primers C2F3 and 1108 amplified single mtDNA fragment approximately 1700 bp with all examined isolates. These results agree with those of Powers and Harris (1993) which indicate different size classes of amplification products in reactions with five *Meloidogyne* species. All isolates of both *M. incognita* and *M. javanica* produced a 1700 bp fragment, which are different from the isolates of *M. arenaria* that produced a 1100 bp fragment and all isolates of *M. chitwoodi* and *M. hapla* which produced 520 bp products.

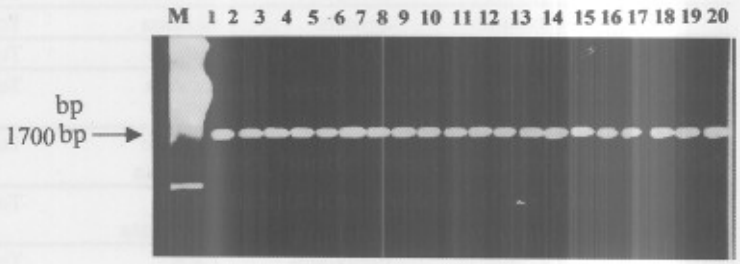

Fig. (1): Typical separation on 1 % agarose gel of products from PCR amplification of mtDNA with two primers C2F3 and 1108 from single Meloidogyne second-stage juveniles (J2) of 20 different populations. The 1.7 Kb product is characteristic for all examined populations. M is 100 bp ladder.

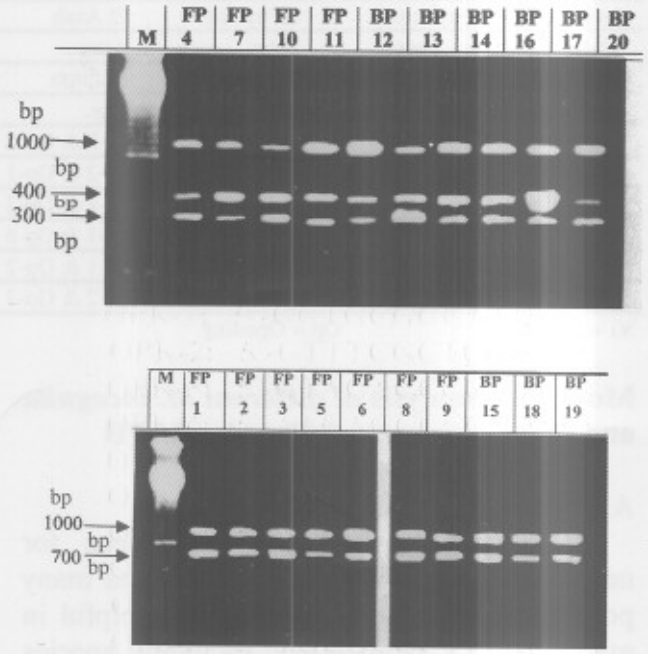

Fig. (2): Hinf I digestion products of the amplified 1.7 kb fragment separated on 1.5 % agarose gel. (A) expose digestion products from 10 Meloidogyne incognita populations, while (B) expose digestion products from 10 Meloidogyne javanica populations. The 1.0 kb digestion product is shared by both species, but an additional Hinf I restriction site in the 0.7 kb fragment products two restriction fragments of 0.4 and 0.3 kb in M. incognita populations. M is 100 bp ladder.

The present mtDNA protocol was able to differentiate between the two *Meloidogyne* species by the endonuclease digestion of the amplified products. Digestion by *Hinf I* restriction enzyme recognized one restriction site in the *M. javanica* product resulted in cleavage products of the 700 and 1000 bp, while in case of *M. incognita*, an additional

restriction site was recognized and resulted in cleavage of the 700 bp fragment to yield two fragments of 400 and 300 bp. Such results are in a good agreement with the results of Powers and Harris (1993).

Table (1): Sources of 20 different populations of M. incognita and M. javanica isolated from 20 different locations.

Sample Number	Governorate	Center	Location	Host plant	Nematode Species	DNA Conc. µg/ml	Sample Code
1	El Fayoum	ElFayoum	El Amria	Okra	*M. javanica*	204.5	FP-1
2	El Fayoum	Senoris	El Edoa	Tomato	*M. javanica*	84.0	FP-2
3	El Fayoum	Atsa	Garado	Tomato	*M. javanica*	90.0	FP-3
4	El Fayoum	ElFayoum	Hawara Adlan	Tomato	*M. incognita*	145.0	FP-4
5	El Fayoum	Senoris	Sanhore ElKablia	Tomato	*M. javanica*	360.0	FP-5
6	El Fayoum	ElFayoum	Zaoit ElKerdasa	Tomato	*M. javanica*	65.0	FP-6
7	El Fayoum	ElFayoum	Zaoit ElKerdasa	Tomato	*M. incognita*	160.0	FP-7
8	El Fayoum	Abshawai	AboDenkash	Tomato	*M. javanica*	85.0	FP-8
9	El Fayoum	Tamia	El Roda	Tomato	*M. javanica*	90.0	FP-9
10	El Fayoum	Atsa	Garado	Tomato	*M. incognita*	100.0	FP-10
11	El Fayoum	ElFayoum	El Azab	Okra	*M. incognita*	120.0	FP-11
12	Bani-Sweef	Beba	Beba	Tomato	*M. incognita*	108.5	BP-12
13	Bani-Sweef	El Wasta	El Ziton	Tomato	*M. incognita*	63.5	BP-13
14	Bani-Sweef	Ehnasia	Kay	Tomato	*M. incognita*	102.2	BP-14
15	Bani-Sweef	Somosta	Vi-2 & Op-8	Egg plant	*M. javanica*	255.0	BP-15
16	Bani-Sweef	El Fashn	Vi-3 & Op-1	Tomato	*M. incognita*	55.0	BP-16
17	Bani-Sweef	El Fashn	Vi-2 & Op-2	Tomato	*M. incognita*	185.0	BP-17
18	Bani-Sweef	Somosta	Vi-1 & Op-6	Tomato	*M. javanica*	250.0	BP-18
19	Bani-Sweef	Somosta	Vi-1 & Op-2	Egg plant	*M. javanica*	55.0	BP-19
20	Bani-Sweef	El Fashn	Vi-2 & Op-7	Cucumber	*M. incognita*	95.0	BP-20

Vi = Village Op = Opening

Molecular analysis of different *M. incognita* and *M. javanica* populations by RAPD

A) RAPD analysis

Biotechnology is not a panacea for nematological problems, but it provides many powerful tools. These tools will be helpful in many areas of nematology, including species identification, race and pathotype identification, development of resistant cultivars, definition of nematode-host interactions, nematode population dynamics, establishment of optimal rotations, the ecology of biological control and development of useful biological control agents and the design of new nematicides (Chet, 1993).

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

distributed over the entire target DNA pool (Williams *et al.*, 1990).

RAPD-PCR technique is uncomplicated and reproducible. It is also simple, rapid and safe because it does not involve the use of radioactive isotopes. In addition, this technique is clearly useful for the detection of genetic variability. Amplification with RAPD primers is extremely sensitive to single base changes in the primer target site. This feature suggests that RAPD technique should be highly useful for phylogenetic analysis among closely related individuals, but less useful for analysis of genetically diverse individuals (Edward *et al.*,1992, Cenis, 1993 and Baum *et al.*, 1994).

Ten oligonucleotid primers were used to generate RAPD fingerprinting of 10 *M. incognita* and 10 *M. javanica* populations. The total number of amplified polymorphic DNA fragments obtained were 422 and 416 for *M. incognita* and *M. javanica* populations, respectively. Under the reaction conditions described before, the size of the amplified polymorphic DNA fragments ranged approximatly from 200 to 1300 base pair (bp). Three kinds of polymorphic DNA fragments could be distinguished, DNA fragments common to all genotypes, DNA fragments amplified in all populations of one species (intraspecific DNA fragments) and DNA fragments amplified in not all but at least two genotypes (intrapopulation DNA fragments).

RAPD analysis of total DNA extracted from 10 different populations of *M. incognita* and 10 different populations of *M. javanica* revealed common markers and differences among the populations (Figs. 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12).

All populations examined with primer OPK-1 (Figs. 3 and 4) yielded complex patterns, with major bands of approximately 300, 350, 600 and 900 bp common at all populations of the two *Meloidogyne* species (*M. incognita* and *M. javanica*). Intraspecific variations occurred among the two *Meloidogyne* species were detected with major bands approximately 500 bp specific for *M.incognita* populations and 1300 bp specific for *M. javanica* populations. Intrapopulation variations of the 20 examined populations with primer OPK-1 has been observed in several bands.

Primer OPK-2 amplified common bands approximately 300 and 700 bp observed in all examined populations. The intraspecific polymorphic DNA fragments that were amplified in all populations of one *Meloidogyne* species were observed. The DNA fragment at 500 bp was intraspecific fragment for *M. incognita* populations, while DNA fragment at 400 bp was intraspecific fragment for *M. javanica* populations (Figs. 5, 6 and 10). This primer amplified several polymorphic DNA fragments specific to only a few populations within each *Meloidogyne* species.

Figs. 4 and 6, represent typical fingerprints obtained with primer OPK-3 of all populations of each of *M. incognita* and *M. javanica* and show all types of polymorphisms. The major polymorphic DNA fragments amplified in all examined populations were observed at band sizes, 300 bp and 800 bp. While the intraspecific polymorphic DNA fragments of populations of each *Meloidogyne* species were observed in association with the primer OPK-3. The recorded band size was 200 bp specific to *M. incognita* populations and there is no intraspecific DNA fragments amplified by this primer specific to *M. javanica* populations.

All 20 populations examined with primer OPK-4 yielded three kinds of polymorphic DNA fragments (Figs. 4 and 7), a band of approximately 1100 bp is common to all

populations of both *Meloidogyne* species, an intraspecific band of 200 bp is specific to *M. incognita* populations and there is no intraspecific bands specific to *M. javanica* populations. However, several intrapopulation bands at different sizes were amplified among all examined populations.

Primer OPK-5 amplified complex patterns of polymrphic DNA fragments with three major bands of approximately 250, 400 and 700 bp common to all populations tested and it amplified only one intraspecific DNA fragment at band size of 600 bp specific for 10 *M. incognita* populations but failed to amplify intraspecific fragments specific to *M. javanica* populations (Figs. 4 and 8). However, the same primer (OPK-5) amplified several polymorphic DNA fragments specific to only a few populations. These plymorphic DNA fragments detected the intrapopulation variations that occurred among the populations of each *Meloidogyne* species (*M. incognita* and *M. javanica*).

Figs. 9 and 10, illustrate typical amplified complex patterns of polymorhpic DNA fragments obtained with primer OPK-6 of all populations of each of *M. incognita* and *M. javanica* and show all types of polymrphisms. DNA fragments at sizes of 300, 400, 500 and 700 bp were amplified at all examined populations. Such fragments indicate intrapopulation variations among populations of both *Meloidogyne* species. However, this primer failed to differentiate between *M. incognita* and *M. javanica* populations (there is no intraspecific amplified DNA fragments).

Primers OPA-3 and OPA-5 amplified complex patterns of polymorphic DNA fragments, with major fragments common to all populations examined with these primers. These polymorphic DNA fragments were approximately 400, 450 and 500 bp with primer OPA-3 and 1000 bp with primer OPA-5. These two primers yielded both intraspecific and intrapopulation polymorphic DNA fragments. The interested intraspecific DNA fragments, specific to single species, were observed in association with primer OPA-3, DNA fragments at band sizes of 200, 230 and 250 bp were amplified only in *M. incognita* populations, while a DNA fragment at a band size of 300 bp was amplified only in *M. javanica* populations. On the other hand, in case of intraspecific DNA fragments amplified in association with primer OPA-5, a DNA fragment at a band size of 800 bp was specific to *M. incognita* populations, while in *M. javanica* populations, the observed DNA fragment was at the band size of 500 bp. However, both primers (OPA-3 and OPA-5) yielded several intrapopulation DNA fragments specific to only a few populations (Figs. 11 and12).

Primers OPB-3 and OPB-5 amplified complex patterns of DNA fragments, with major fragments common to all populations examined with these primers. The common polymorphic DNA fragments were approximately 500 and 1000 bp with primer OPB-3 and 500, 550 and 600 bp with primer OPB-5. In *M. javanica* populations, primer OPB-3 yielded intraspecific DNA fragments at 450, 600 and 1100 bp, while by using the primer OPB-5, intraspecific DNA fragment at a band size of 400 bp was observed. However, there is no intraspecific DNA fragments observed in *M. incognita* populations in association with both primers (OPB-3 and OPB-5). The intrapopulation DNA fragments of both *Meloidogyne* species in association with both primers were detected and illustrated in (Fig. 12).

Populations of each of *M. incognita* and *M. javanica* were easily distinguished by differences in fragment patterns with any of

the 10 RAPD primers. These distinctive RAPD fragments are candidates for intraspecific and intrapopulation identifications. Some primers, e.g., OPA-3, OPA-5, OPB-3 and OPB-5, yielded only a few, species-specific bands that enabled differentiation of *M. incognita* and *M. javanica*. Other primers yielded more complex patterns, which were less easily interpreted. However, all 10 RAPD primers used in the present work allowed differentiation between populations within both studied *Meloidogyne* species (*M. incognita* and *M. javanica*). The results indicated that the RAPD technique is a useful diagnostic tool for nematode species and intrapopulation variations identifications.

The present results demonstrated that RAPD is a powerful method for the characterization of intraspecific and intrapopulation polymorphism among populations of *M. Incognita* and *M. javanica*. These results confirm those of Cenis (1993) who showed that it is possible to use RAPD-PCR to identify the four major species of the genus *Meloidogyne*.

A noteworthy result of the present study indicated that RAPD displayed a higher rate of polymorphism among root knot nematode populations compared to that obtained with amplified fragment length polymorphism (AFLP). These results are in agreement with that previously demonstrated by Powers and Harris, (1993); Cines, (1993); El-Ashery, (1998); and Haroon and Zijlistra, (1998) and Semblat *et al,* (1998) on the root knot nematode species *M. incognita, M. arenaria* and *M. Javanica*, while these results are in a close agreement with RAPD data of six California populations of *Heterodera schachtii* (Edward *et al.,* 1992) and nine populations of *Globodera rostochiensis* (Flokertsma *et al.,* 1994), which assessed the ability of RAPD markers to detect genetic variations and relatedness among different populations.

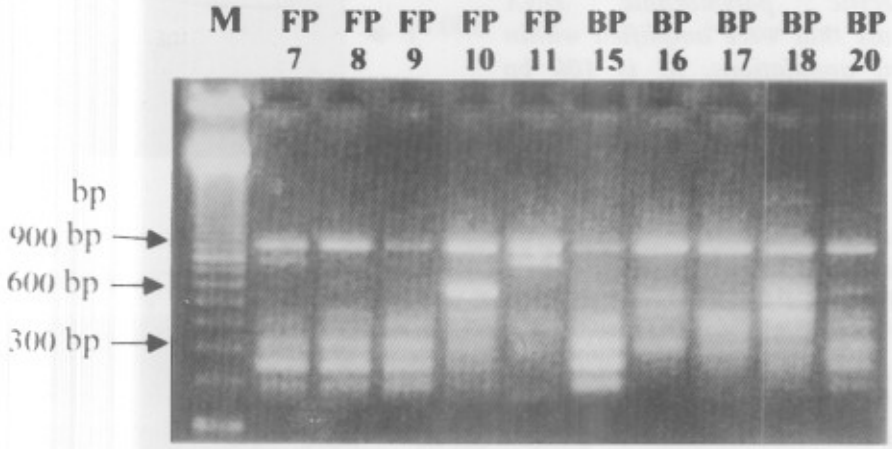

Fig. (3): Agarose gel pictures of random amplified polymorphic DNA. Bands generated from genomic DNA of M.incognita and M. javanica populations expose intraspecific and intrapopulation variations within the examined populations. Polymorphic DNA bands produced with primer OPK-1 and populations FP7, FP8, FP9, FP10, FP11, BP15, BP16, BP17, BP18 and BP20. Arrows indicate bands size of molecular size standard 100 bp ladder in Lane 1.

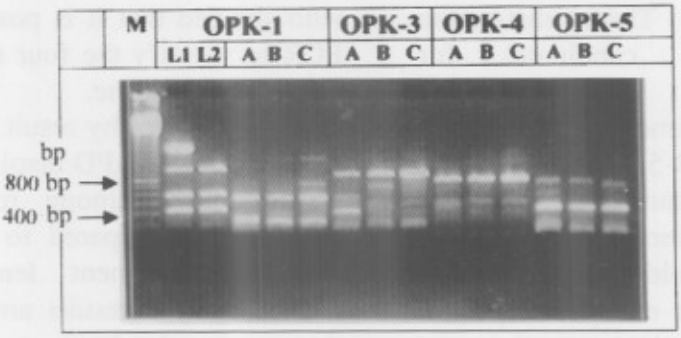

Fig. (4): Agarose gel pictures of random amplified polymorphic DNA bands generated from genomic DNA of M. javanica populations expose intrapopulation variations within the examined populations. These polymorphic DNA bands produced with primers OPK-1, OPK-3, OPK-4 and OPK-5 and populations FP1, FP2 and FP3 (A, B and C respectively). Arrows indicate bands size of molecular size standard 100 bp ladder in Lane 1. Lanes 2 and 3 indicate RAPD analysis of control DNA of E. coli (races BL21 and C1a).

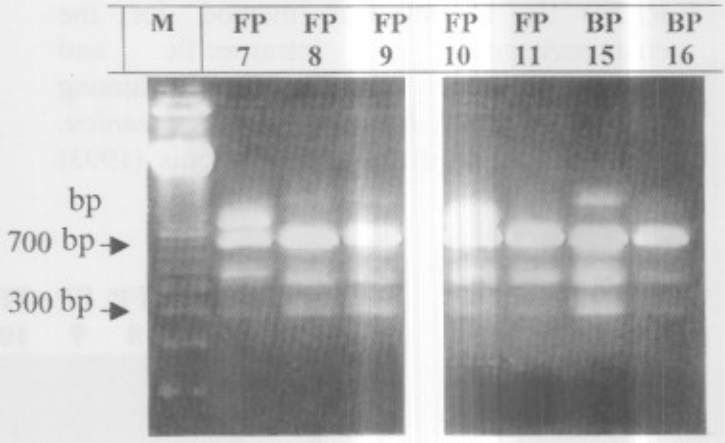

Fig. (5): Polymorphic bands of amplified DNA produced in FP7, FP8, FP9, FP10, FP11, BP15 and BP16 of M. incognita and M. javanica with primers OPK-2. Arrows indicate intrapopulation and intraspecific polymorphic DNA fragments that were amplified within different populations. M is 100 bp ladder.

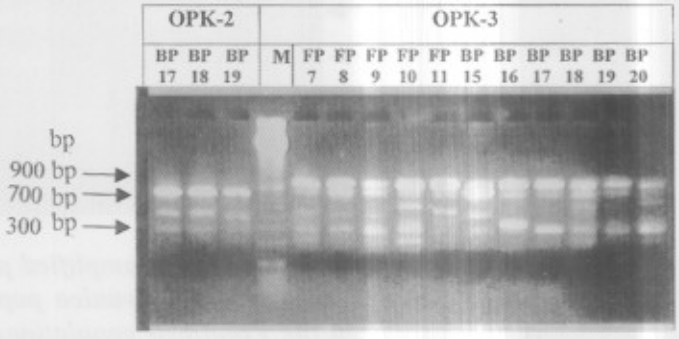

Fig. (6): Polymorphic bands of amplified DNA produced in FP7, FP8, FP9, FP10, FP11, BP15, BP16, BP17, BP18, BP19 and BP20 populations of M. incognita and M. javanica with primers OPK-2 and OPK-3. Arrows indicate intrapopulation and intraspecific polymorphic DNA fragments that were amplified within different populations. M is 100 bp ladder.

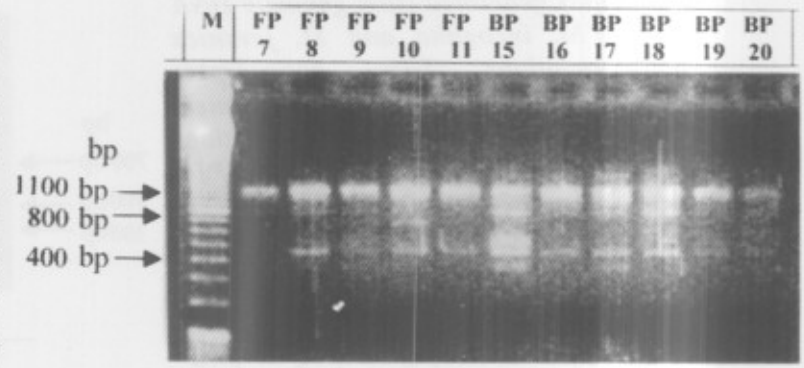

Fig. (7): Agarose gel picture of random amplified polymorphic DNA fragments generated from DNA of 11 different populations FP7, FP8, FP9, FP10, FP11, BP15, BP16, BP17, BP18, BP19 and BP20 of M. incognita and M. javanica originating from different geographic locations in El Fayoum and Bani-Sweef governorates with random decamer primer OPK-4. Arrows indicate band sizes of intraspecific and intrapopulation DNA fragments. M is 100 bp ladder.

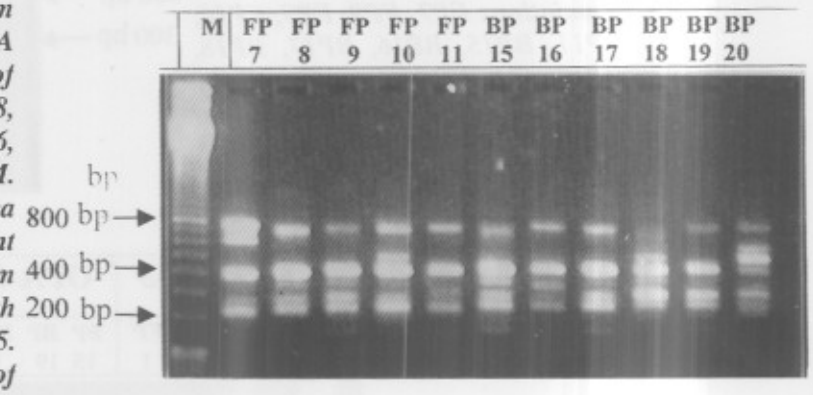

Fig. (8): Agarose gel picture of random amplified polymorphic DNA fragments generated from DNA of 11 different populations FP7, FP8, FP9, FP10, FP11, BP15, BP16, BP17, BP18, BP19 and BP20 of M. incognita and M. javanica originating from different geographic locations in El Fayoum and Bani-Sweef governorates with random decamer primer OPK-5. Arrows indicate band sizes of intraspecific and intrapopulation DNA fragments. M is 100 bp ladder.

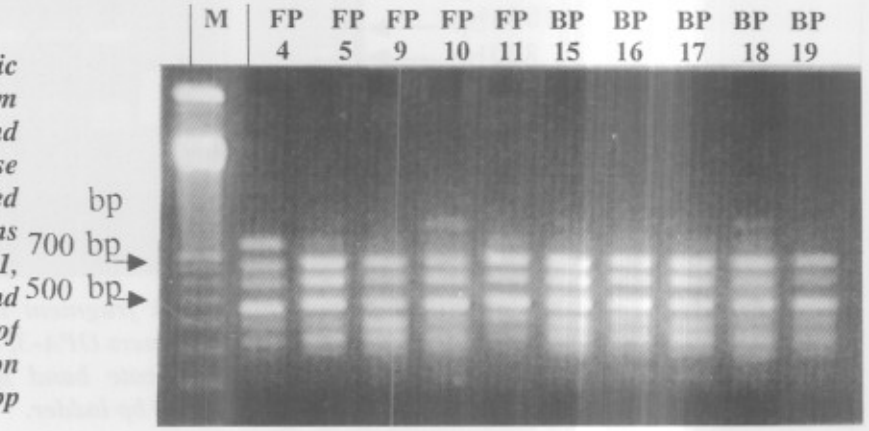

Fig. (9): Random amplified polymorphic DNA bands generated from genomic DNA of M. incognita and M. javanica populations. These polymorphic DNA bands produced with primer OPK-6 and populations FP4, FP5, FP9, FP10, FP11, BP15, BP,16, BP17, BP18 and BP19. Arrows indicate band sizes of intraspecific and intrapopulation DNA fragments. M is 100 bp ladder.

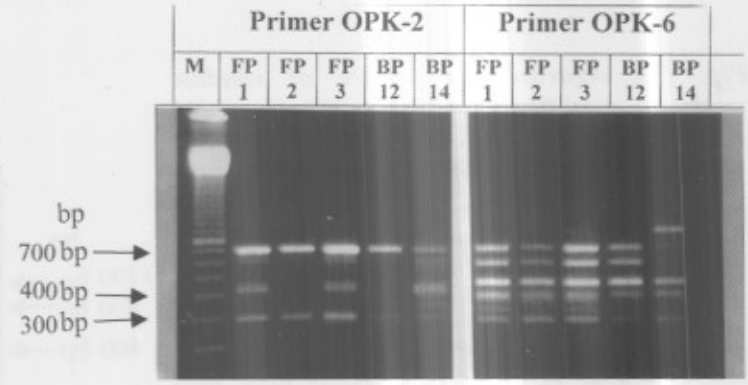

Fig. (10): Random amplified polymorphic DNA bands generated from genomic DNA of M. incognita and M. javanica populations. These polymorphic DNA bands produced with primers OPK-2 and OPK-6 and populations FP1, FP2, FP3, BP12 and BP14. Arrows indicate band sizes of intraspecific and intrapopulation DNA fragments. M is 100 bp ladder.

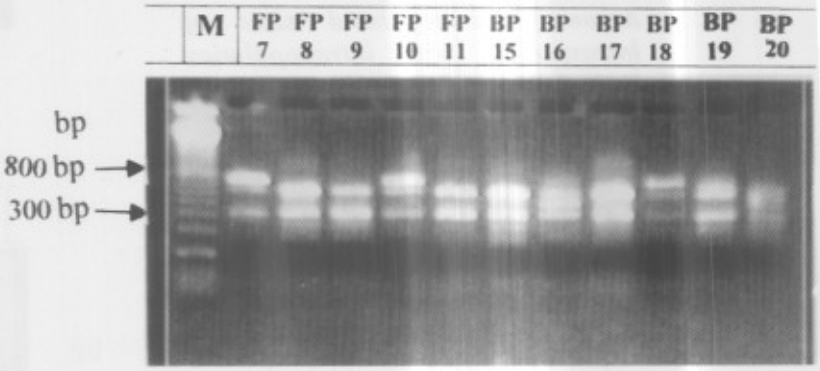

Fig. (11): Random amplified polymorphic DNA fragment markers from different populations of M. incognita and M. javanica produced with primer OPA-5 and populations FP7, FP8, FP9, FP10, FP11, BP15, BP16, BP17, BP18, BP19 and BP20. Arrows indicate band sizes of intraspecific and intrapopulation polymorphic DNA fragments. M is 100 bp ladder.

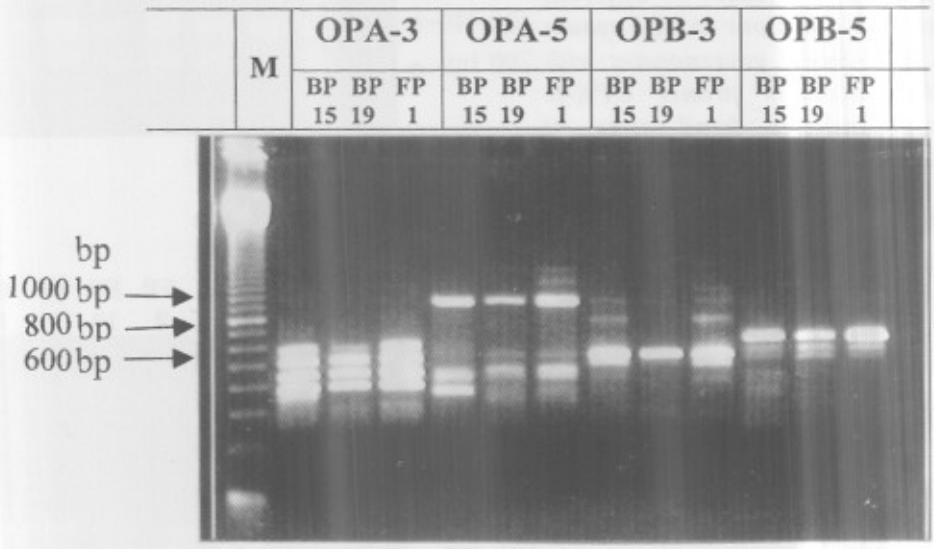

Fig. (12): Random amplified polymorphic DNA fragment markers from different populations of M. javanica produced with 4 decamer primers OPA-3, OPA-5, OPB-3 and OPB-5 and populations BP15, BP19 and FP1. Arrows indicate band sizes of intraspecific and intrapopulation polymorphic DNA fragments. M is 100 bp ladder.

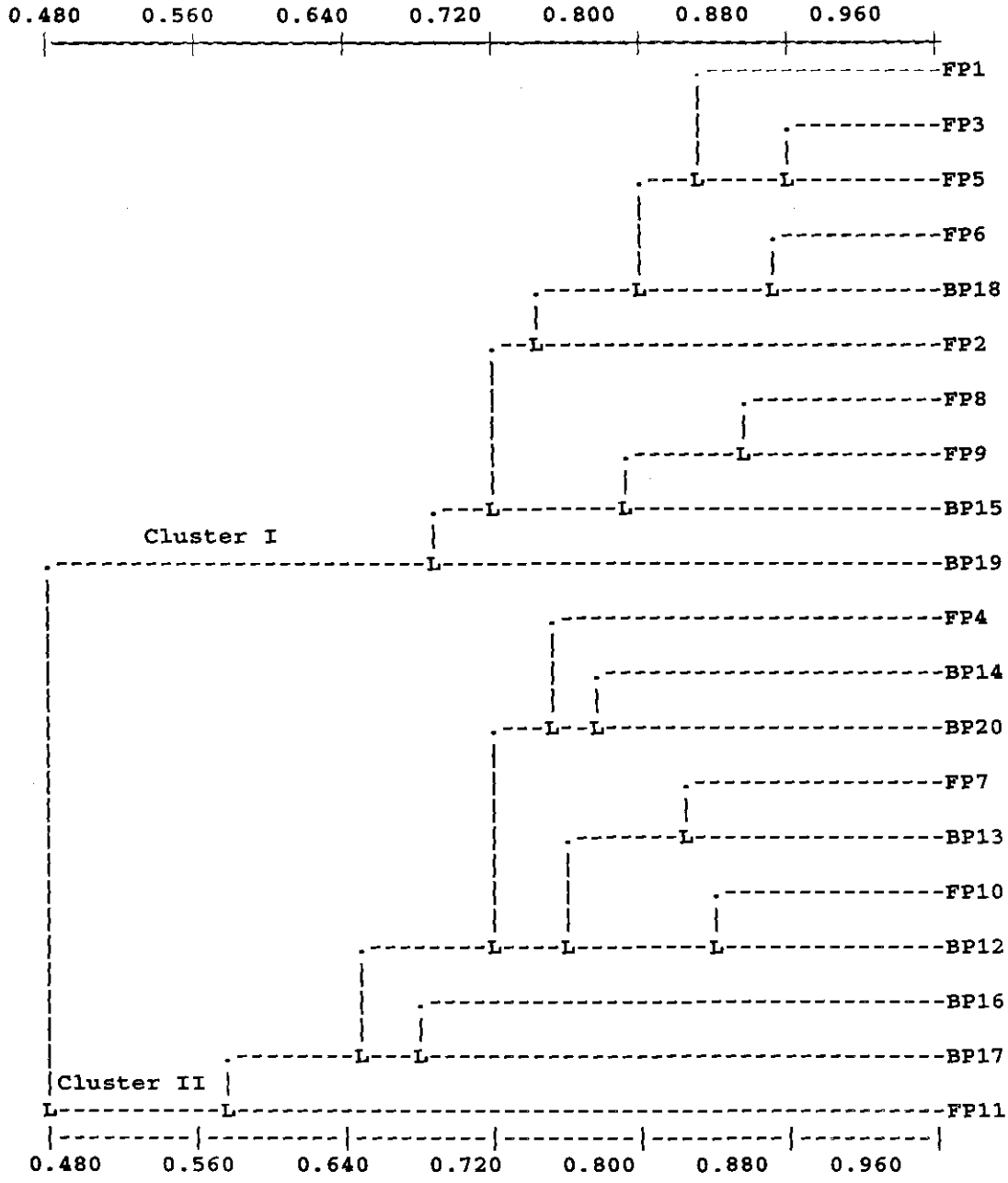

Fig. (13): Similarity dendrogram of 20 Meloidogyne species (M. incognita and M. javanica) populations constructed from the genetic distance based on 77 random amplified polymorphic DNA fragments, using the unweighted pair group method with arithmetic mean (UPGAMA) in the Clustan 3.2 VAX-VMS program.

B) Dendrogram analysis

For the quantitative evaluation of the RAPD results, the value of genetic similarities were calculated using all scorable bands that amplified with all primers. The distance matrix was constructed and cluster analysis was made using UPGAMA method.

Various similarity measures have been used to determine the genetic divergence between organisms on the basis of RAPD data (Caswell-Chen *et al.,* 1993; Demek *et al.,* 1992; Kambhampati *et al.,* 1992). Chapco *et al.* (1992) who stated that until a theoretical frame work is developed for the variations generated by the RAPD assay, the statistic similarity coefficient F=2Nxy/(Nx+Ny) (Nei and Li, 1979) is at present the most unambiguous formula to express intraspecific and intrapopulation similarity.

From the number of common bands in the fingerprinting pattern of isolates, the formula of simple matching (SM) coefficient was used to calculate a similarity coefficient for each pair of isolates. Pairs, which have a higher coefficient, are more closely related than those with a lower coefficient. In the construction of the matrix, it is assumed that corresponding band arise by amplification of the same genetic locus (Duncan *et al.,* 1993). NTSys program (Rolf, 1988) was used to calculate the genetic similarity matrix to evaluate the genetic similarity between the isolates as distance of each pair of them. The degree of similarity was shown as numbers from (0), for the most dissimilar pair, to (1), for the most similar pair of isolates.

The isolates (FP11) and (BP15) showed the lowest similarity (0.3438).Very low level of similarities between populations of both species (*M. incognita* and *M. javanica*) was observed and ranged from 0.3438 to 0.5385. Generally, the intrapopulation genetic variation of the same species is very low, which can be considered as an indicator of high level of homogeneity.

The similarity matrix was used to perform the phylogenetic tree by using NTSys ver. 1.4 program. Drawing the phylogenetic tree was done by SAHN program (complete link method) as shown in Fig. (13). The resulted phylogenetic tree scaled as F-value which ranged from 0.000 to 1.200. The dendrogram phylogenetic tree that was produced from the 10 primers (OPK-1, OPK-2, OPK-3, OPK-4, OPK-5, OPK-6, OPA-3, OPA-5, OPB-3 and OPB-5) separated the 20 isolates of *Meloidogyne* into two distinct clusters (I and II) and show many similarities between the populations within each cluster (Fig. 13).

The perennial patterns and cluster analysis obtained with the 10 different primers showed that the nematode populations in cluster I (FP1, FP3, FP5, FP6, BP18, FP2, FP8, FP9, BP15 and BP19) were determined as *M. javanica,* while in cluster-II the nematode populations FP4, BP14, BP20, FP7, BP13, FP10, BP12, BP16, BP17 and FP11 were determined as *M. incognita.*

According to similarity coefficient values between populations of *M. incognita* and *M. javanica* and the dendrogram results, cluster-I was divided into three subclusters, subcluster one includes populations FP1, FP3 and FP5, subcluster two includes populations FP6, BP18 and FP2 and subcluster three includes populations FP8, FP9, BP15 and BP19. However, cluster-II was divided into three subclusters, subcluster one includes populations FP4, BP14 and BP20, subcluster two includes populations FP7, BP13, FP10 and BP12 and subcluster three include populations BP16, BP17 and FP11.

In the present study, Similarity coefficients calculated from pair wise comparisons of shared and non-shared markers

for different geographic populations, indicated close affinity, though not identity, among these populations.

The intrapopulation similarity among *M. incognita* and *M. javanica* populations studied in the present work ranged from 0.658 to 0.903 and from 0.739 to 0.955, respectively. At the specific level, the genetic relationships obtained from this study are in close agreement with other RAPD data which concluded Low genetic variability within *M. incognita* and *M. javanica* populations compared to that observed among *M. hapla* and *M. arenaria* populations (Esbenshade and triantaphyllou, 1985 and Castagnone-Sereno *et al.*, 1994). The low intrapopulation genetic variations detected among the ten populations of each of *M. incognita* and *M. javanica* is common because of the reproduction of these species by mitotic parthenogenesis. Nevertheless, the existence of variation in the form of mixtures of clones originated by mutation can not be discarded. In contrast, more intrapopulation genetic variations are to be expected in field populations of *M. hapla* race A, which reproduces by facultative parthenogenesis (Cenis, 1993).

Within each of the two root knot nematode species studied, the RAPD analysis did not reveal any correlation between genomic similarity and geographical distribution of the populations. Such result is in a good agreement with a recent RAPD and AFLP work on tropical *Meloidogyne* species, which showed that grouping of lines within a species did not reflect their geographical provenance (Block *et al.*, 1997b and Semblat *et al.*, 1998). Even though, the number of populations tested in the present study is rather low and should be increased to strengthen the informative value of our data. This study nevertheless provides arguments for the hypothesis that root knot nematodes may not be indigenous throughout their current geographical distribution (Block *et al.*, 1997a and Trudgill, 1995).

Results of dendrogram analysis cleared the ability of RAPD markers to detect genetic variations and relatedness among the 20 populations of *M. incognita* and *M. javanica*. These results agree to, a great extent, with those of Castagnone-Sereno *et al.* (1994) who reported genetic analyses conducted on root-knot nematode populations belonging to the four major species of the genus *Meloidogyne* and originating from many countries throughout the world. The authors used discrete genetic markers which were random genomic DNA sequences amplified by the polymerase chain reaction (RAPD-markers). Using RAPD patterns alone or in combination, they reported that all the *Meloidogyne* species and populations studied could be unambiguously discriminated. Based on the presence or absence of bands, maximum-parsimony analysis of the data resulted in clustering of species and populations congruent with previous iso-enzymatic and molecular data.

The obtained data showed that despite reproduction among the studied species is by mitotic parthenogenesis, intrapopulation variations in different populations in El Fayoum and Bani-Sweef governorates have occurred. The presence of populations with low genomic similarity in the same region could be the result of random dissemination from a number of centers of origin and juxtaposition through agronomical practices rather than extreme genetic drift from a common and local ancestors. That is very important to detect the risk of this potentially dangerous nematodes and to implement appropriate quarantine measures.

From a practical point of view, the ability to accurately detect polymorphisms

between root knot nematode populations is of outstanding importance for the design of effective integrated control of these parasites. Plant resistance is currently the most efficient and environmentally sound method of controlling root- knot nematodes, but the implementation of crop rotations including resistant cultivars requires specific and subspecific identification of these pests. The fact that, most *Meloidogyne* species (including *M. incognita* and *M. javanica*) reproduce by parthenogenesis precludes any Mendelian genetic approach with these nematodes (i.e. crossing experiments), making it sometimes difficult to inter relationships between groups within this genus (Semblat *et al.*, 1998).

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الملخص العربي

تعريف أجناس و أنواع نيماتودا تعقد الجزور و إستكشاف الإختلافات الوراثية بين الأنواع المختلفة و بين أفراد النوع الواحد داخل المجتمعات المختلفة بإستخدام تقنية RAPD

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خلال هذه الدراسة تم عمل حصر لأنواع النيماتودا تعقد الجذور الشائعة الأنتشار فى منطقة مصر الوسطى وخاصة محافظتى الفيوم وبنى سويف فقد قمنا بتجميع العينات من ٢٠ منطقة مختلفة فى هاتين المحافظتين. وتم التعرف على أجناس وأنواع النيماتودا لتلك المجتمعات الجغرافية بإستخدام النموذج العجانى (Perennial pattern) وقد أوضحت النتائج أن هناك ١٠ عينات نيماتودا من نوع *M. incognita* والعشر عينات الأخرى من النوع *M. javanica* ولتأكيد النتائج المتحصل عليها لتحديد الجنس والنوع بإستخدام الطريقة التقليدية السابقة فقد تم إستخدام إحدى طرق البيولجيا الجزيئية وخاصة التى تعتمد على إستخدام جهاز التفاعل المتسلسل (PCR) وهى طريقة Mitochondrial DNA amplification حيث أنه تم إستخدام الدنا المستخلص من فرد واحد من الطور اليرقى الثانى **J2** لكل عينة من ال٢٠عينة المستخدمة و ذلك حيث تم إكثار منطقة ال Mitochondrial DNA بإستخدام فرد الطور اليرقى الواحد بجهاز التفاعل المتسلسل. و بإستخدام بادئات (Primers) معينة تم تحديدها من قبل بواسطة بعض العلماء وهذه البادئات تأخذ الأرقام ١١٠٨ و C2F3 وبعد عملية الأكثار لوحظ أن كل العينات المختبرة قد أعطت قطعة واحده من الدنا (DNA band) على مسافة ١٧٠٠ قاعدة نيتروجينية وهى مميزة لجنس النيماتودا *Meloidogyne* ولكى يتم تحديد الأنواع قمنا بعملية هضم لناتج الPCR السابق بإستخدام إحدى إنزيمات الهضم البيولوجية Restriction Enzymes وهو إنزي م *Hinf* I و لوحظ أن هناك تباين بين العينات المختبرة حيث أنه فى حالة النيماتودا من نوع *M. javanica* يتم تقطيع قطعة الدانا (١٧٠٠ قاعدة نيتروجينية) إلى قطعتين عند المسافة ١٠٠٠ و المسافة ٧٠٠ قاعدة نيتروجينية ولكن فى حالة النوع *M. incognita* يتم التقطيع إلى ثلاث قطع عند المسافات ١٠٠٠ و ٤٠٠ و ٣٠٠ قاعدة نيتروجينية وبذلك تم التعرف على جنس ونوع كل العينات التى تم تجميعها و قد أوضحت تلك الدراسة أن المجتمعات FP-4, BP-14, BP-16, Bp-17, Bp-20 FP-7, FP-10, FP-11, BP-12, BP-13, هى من نوع *M. incognita* و المجتمعات FP-1, FP-2, FP-3, FP-5, FP-6, FP-8, FP-9, BP-15, BP-18, BP-19 هى من نوع *M. javanica*
وبعد الخطوة السابقة من جمع العينات و تحديد الجنس و النوع لكل عينة قمنا بالكشف عن التنوع الوراثى الموجود داخل و فيما بين المجتمعات الجغرافية للنيماتودا وذلك بإستخدام عشرة بادئات عشوائية Oligonucleotid primers و هذه الطريقة تعرف بإسم Random Amplified Polymorphic DNA (RAPD) . وقد تم هذا التفاعل بإستخدام الدنا المستخلص من إناث النيماتودا لكل عينة و تم إجراء تلك التفاعلات فى جهاز التفاعل المتسلسل بإستخدام الخليط المعد سابقا والذى يعرف تجاريا بإسم PCR Ready-to-Go amplification kit و قد نجحت هذه الطريقة فى تحديد الإختلافات الوراثية بين المجتمعات المختلفة لنيماتودا تعقد الجزور. و من خلال دراسة تلك القرائات على إنها صفات ثنائية العد التى عن طريقها يمكن تحويل هذه النتائج إلى شبكة من قيم التشابة F-Value و أيضا تمكنا من الحصول على الشجرة الوراثية Dendrogram و التى تعطى نسب التقارب و التباعد بين المجتمعات المختلفة داخل الجنس و النوع الواحد وقد تم الحصول على هذا التحليل الرياضى بإستخدام نظام إحصائى يسمى UPGAMA . ومن خلال هذه الدراسة وضح أن الحصول على Molecular characterization بين المجتمعات المختلفة داخل الجنس و النوع الواحد بإستخدام تقنية ال RAPD-PCR هى من أحسن التقنيات المستخدمة فى هذا المجال .