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MOLECULAR DIAGNOSIS AND GENETIC VARIABILITY OF NEW ISOLATES OF ROOT – KNOT NEMATODES *MELOIDOGYNE* SPP.ON CUCUMBER PLANTS

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ABSTARACT

Results of the amplified DNA products of the ITS revealed the presence of two bands among the six amplified products,in position at about 500 bp as illustrated by agarose gel electrophoresis,then sequencing of the amplification products showed the presence of two species out of a total of six nematode isolates including *Meloidogyne incognita* and *M. javanica* which displayed some nucleotide differences through comparing the DNA sequence alignment of their ITS and emphasized by a DNA Dot plot for each species against the other,where a genetic similarity was 98.84%. Phylogenetic tree analysis for *M.incognita* showed that the closest species to it is *M.incognita* isolate CN 36 from China with 99.55% genetic similarity whilst more nucleotide differences were observed by two isolates (A-1and B-2) of *M.incognita* from Iraq with a genetic similarity of 97.99%%. As for *M.javanica,*the closest species to it is a group of three nematode species including: *M.javaniac* voucher M J- 21, *M.incognita* isolate Nem Mi TG2 and *Meloidogyne* sp.Mj – c3b from Brazil, India and New Zealand respectively, after which nucleotide differences increased more,as it reached its maximum level with *M. incognita* isolate C N 36 with a genetic similarity of 98.84%.

KEYWORDS: Meloidogyne spp. Molecular diagnosis, Phylogenetic analysis, Cucumber

INTRODUCTION

Root-knot disease is severe disease on family of cucurbitaceae including cucumber plant that are affected by *Meloidogyne* for example *M.incognita and M.javanica* (Nickle,1984).The important species are *M.arenaria, M.javanica, M.hapla* and *M.incognita* which cause high damages in various plants (Khalil,2013). Root-knot nematodes cause formationof galls on plant root known as root-knot disease.They are invisible enemy for farmer and the most commonly encountered parasitic nematode of vegetables (Kumar *et al.*,2014**).** *Meloidogyne* pecies can be recognized depending on morphological properties (Eisenback and Triataphyllou,1991), response of host-plants (Hartman and Sasser,1985),analysis of Isozyme (Esbenshade and Triataphyllou, 1990) also methods of molecular (Ziljistra *et al.*,1995).

There are several reasons that confirms why molecular information is more reliable phylogenetic study than morphological features. DNA sequence is more accurate than morphological characters.In addition, molecular features are generated in shorter time as compared to the morphological characteristics (Subbotin and Moens, 2006 and Karajeh *et al.* (2010).Molecular technique that used in nematode diagnosis are generally depends on nucleic acid. Most of these techniques are known to be reliable, sensitive, robust, and specific in distinguishing and detecting numerous *Meloidogyne* species as compared with* morphological or biochemical techniques (Powers *et al.*,2005 and Berry *et al.*,2007).DNA extraction using various nematode stage including many or single J2s,eggs and females (Karajeh *et al.*,2010).The purpose of this study was to diagnose new isolates of *Meloidogyne* species on cucumber plants using molecular technique in addition to determine the genetic

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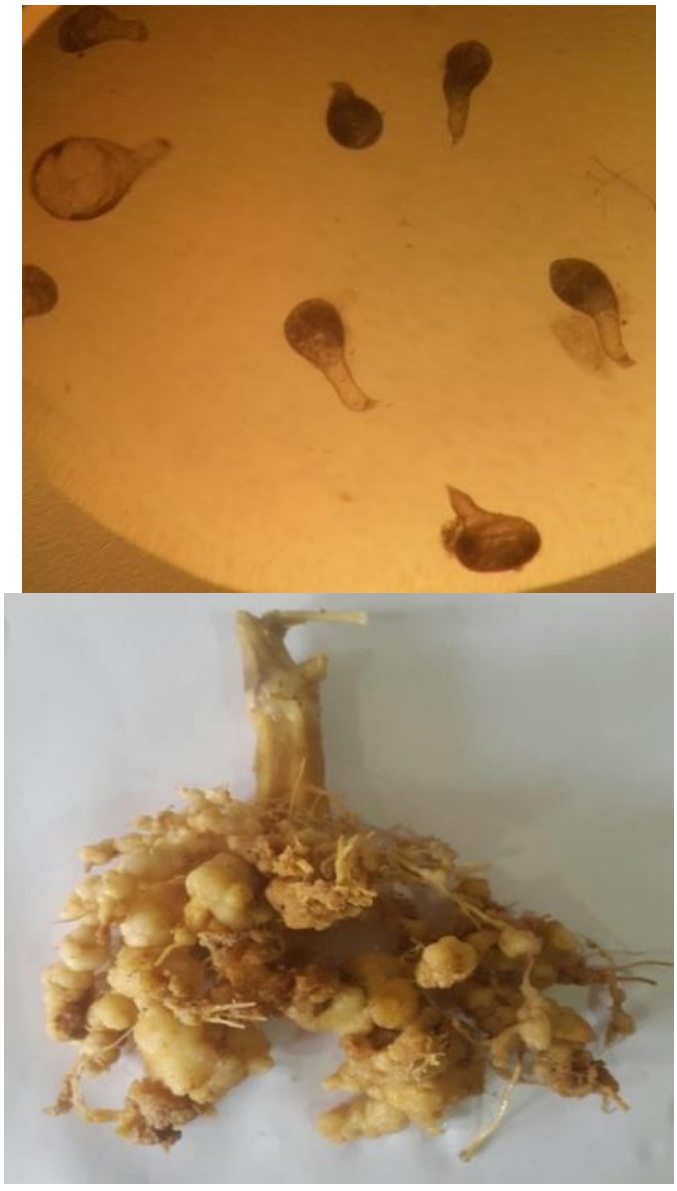
variability between the diagnosed species.that were extracted from infected cucumber collected from plastic greenhouses in Duhok province, Kurdistan Region-Iraq.

Materials and Methods

1-Extraction of root-knot nematode females: Mature females (Figure 1A) of root-knot nematodes extracted from roots of infected cucumber (Figure1 B) collected during a field survey of plastic greenhouses in six locations (6 isolates) in Duhok province, Kurdistan Region-Iraq.



A **B**

Fig. (1): Mature females of *Meloidogyne* spp. (A).Galling of cucumber roots infected with *Meloidogyne* spp. (B).

2- DNA extraction and purification: Several females of *Meloidogyne* spp.for each of the six isolates were extracted separately from infected cucumber roots and placed on a slide that contained 15µl of WLB (worm lysis buffer) which consisted of 50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM MgCl2, 60µg mL1proteinase K (Roche), 0.45% NP4O (Fisher scientific), 0.45% Tween 20 (Sigma) and 0.01% gelatin as described by (Castagnone-Sereno *et al.*, 1995) and then they were cut into pieces under stereomicroscope.10µl of WLB was added to

female pieces then another 10µl of WLB was added to female pieces after being transferred to a special tube, Female pieces were placed in the block heater at 60ºC for one hr. followed by 90 ºC for 10 minutes. Then kept in the frozen at -20ºC as used by Onkendi and Moleleki (2013).

3-Amplification of the target DNA: Two universal primers were used for the amplification of the *ITS-rRNA* gene included:TW81-F(5'-GTTTCCGTAGGTGAACCTGC-3') and AB28 Actin-R(5'-ATATGCTTAAGTTCAGCGGGT-3') provided by Germany company (Jena Bioscience).The amplification was done in 25 µl reactions containing PCR Mater Mix 2x Red Load Taq Master/high yield (12.5 µl), Distilled water DNA-free, RNA-free (8.5µl) ,Forward primer: TW81-F (1 µl) ,Reverse primer: AB28Actin-R (1µl) , DNA template(2 µl). The PCR reaction conditions enclosed Initial denaturation 94 °C for 3min., tailed by 30 cycles of Denaturation 94 °C for 30 sec., Annealing 53 °C (30 sec.) , Elongation 72°C (1min.), then a final elongation 72 °C for 5min. The amplified products wee separated on a 1.5% agarose gel stained with Gel Red in 1× TBE and then examined under UV light.

4. DNA sequencing: This process was performed by preparation of samples to be sent to Macrogene (Korea),where samples of amplified DNA products were sent for determination of the order of the four nitrogenous base, including: Adenine(A),),Cytosine (C), Guanine (G and Thymine (T) in the strand of DNA.The BioEdit and NCBI programs were used for sequence scanner and alignment to check the sequence quality.Sequences of amplified product for each nematode isolate was submitted to GenBank in NCBI to determine their accession number. **5-Extraction of genetic variability and phylogenic tree:** Genetic variability amoung nematode species that have been diagnosed in this study and Phylogenetic tree for each one was constracted using the NCBI BLAST program (https://www.ncbi.nlm.nih.gov/BLAST/),in addition, percent identity and genetic affinity was rxtracted between each nematode species with some of the other species that are themselves species of the same genus..

RESULTS AND DISCUSSION

1- Diagnosis of root – knot nematode species : Amplification results of DNA products for the Internal Transcribed Spacer ITS via Polymerase Chain Reaction (P C R) revealed the presence of two bands among the six amplification products,which were isolate 3 and 6, where they Visualized in position at ~500 bp as agarose gel electrophoresis illustrated by lane 3 and 6 (Figure.2) through the use of two universal primers TW81-F and AB28 Actin-R.This result is out of a total of six nematode isolates on infected cucumber roots in Duhok province This result is agreement with what has been found by AL–Sinjary (2017) for *M. javanica* using the same universal primer pairs.

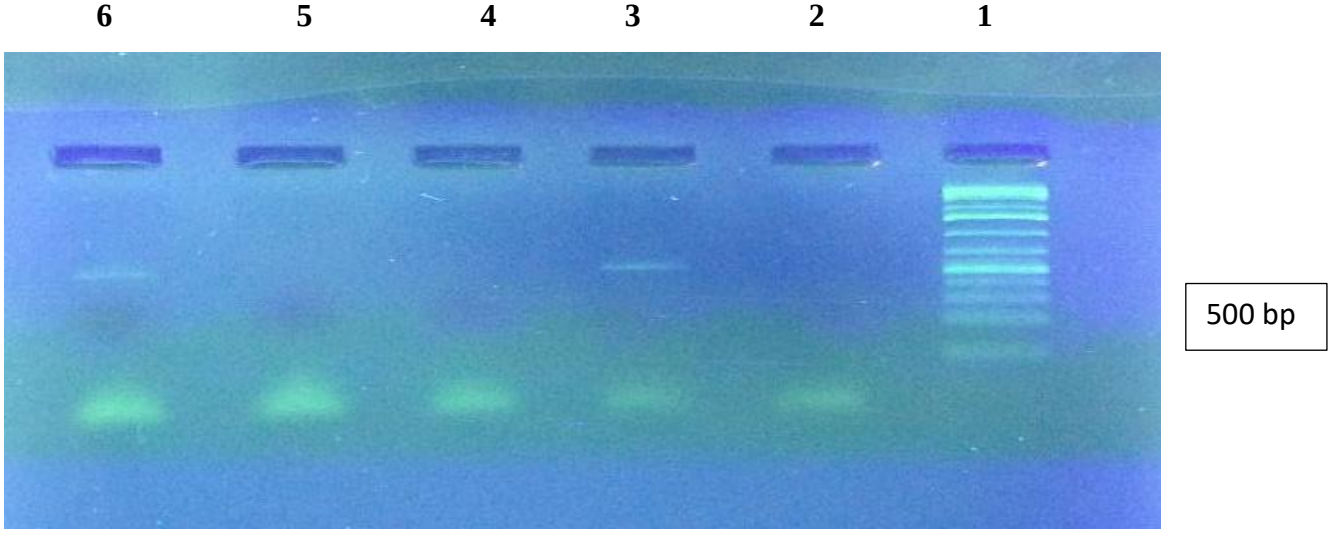


Fig. (2) : Agarose gel electrophoresis of the P C R products, Lane 1 is a Marker ladder, Lane 3 and 6 are positive products for pair of primers (TW81-F with AB28Actin-R) for two nematode isolates which showed after sequencing that they are: *M. incognita* isoalte CN36 (lane 3) and *M. javanica* isolate MJ- 32 (lane 6).

On the other hand, results of the sequence quality observed the presence of two *Meloiodogyne* species,that are *M. incognita* (Kofoid and White,1919) Chitwood, 1949 and *M. javanica* (Treub,1885) Chitwood,1949 due to recording of some nucleotide differences between both nematode species, then the accession number for each species was detrermined by NCBI to be MW152160 for *M.incognita* and MW168165 for *M.javanica*. Nucleotide differences between both nematode species were emphasized by comparing the DNA sequence alignment of the internal transcribed spacer (ITS) region as shown in Figure .3 and Fig.ure 4 for *M. incognita* and *M. javanica* respectively,and by A DNA Dot plot extraction (A graphical method for comparing two biological sequences) for each species against the other (Figure 5 and Figure.6).Thus, according to these results it is not possible to rely on this universal primer pairs and agarose gel electrophoresis in diagnosing both nematode species together and therefore it is necessary to use species specific primers or sequencing for the amplified DNA products to achieve the aforementioned goal.

Score	Expect	Identities	Gaps	Strand
614 bits(332)	2e-180	340/344(99%)	0/344(0%)	Plus/Plus

```
Query  1
TGCTGATACGGTTGTGAACGTCCGTGGCTGTATATGTGGTGACATGTTAGGACTCTAATG  60
Sbjct  105
TGCTGATACGGTTGTGAACGTCCGTGGCTGTATATGTGGTGACATGTTAGGACTTTAATG  164
Query  61
AGTTTAAGACCTAATGAGCCTCTTACGTGAGGCCGCCAGCAACCtttttttttCTCTACAT  120
Sbjct  165
AGTTTAAGACCTAATGAGCCTTTTAAGTGAGGCCGCCAGCAACCTTTTTTTTCTCTACAT  224
Query  121
TTTaaaaaaaaaaCTAAAATTCTATCCTTATCGGTGGATCACTAGGCTCGTGGATCGATG  180
Sbjct  225
TTTAAAAAAAAAACTAAAATTCTATCCTTATCGGTGGATCACTAGGCTCGTGGATCGATG  284
Query  181
AAGAACGCAGCAAACTGCGATAATTATTGCGAACTGCAAAAGTATTGAGCACAAAAGTTT  240
Sbjct  285
AAGAACGCAGCAAACTGCGATAATTATTGCGAACTGCAGAAGTATTGAGCACAAAAGTTT  344
Query  241
TGAACGCAAATGGCCGCATTGAGGTCAAACTCTTTGCAACGTCTGGTTCAGGGTCATTTT  300
Sbjct  345
TGAACGCAAATGGCCGCATTGAGGTCAAACTCTTTGCAACGTCTGGTTCAGGGTCATTTT  404
Query  301  CTCTTATAGCGGAAGCTTTAATTTCTATAATGATGTTGTTGCTT  344
Sbjct  405  CTCTTATAGCGGAAGCTTTAATTTCTATAATGATGTTGTTGCTT  448
```

Fig.(.3) : *Meloidogyne incognita* isolate C N 36 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence. Sequence ID: MW152160.1 Length: 448 Number of Matches:1

Score	Expect	Identities	Gaps	Strand
614 bits(332)	2e-180	340/344(99%)	0/344(0%)	Plus/Plus

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Query  105
TGCTGATACGGTTGTGAACGTCCGTGGCTGTATATGTGGTGACATGTTAGGACTTTAATG  164
Sbjct  1
TGCTGATACGGTTGTGAACGTCCGTGGCTGTATATGTGGTGACATGTTAGGACTCTAATG  60
Query  165
AGTTTAAGACCTAATGAGCCTTTTAAGTGAGGCCGCCAGCAACCtttttttttCTCTACAT  224
Sbjct  61
AGTTTAAGACCTAATGAGCCTCTTACGTGAGGCCGCCAGCAACCTTTTTTTTCTCTACAT  120
Query  225
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Sbjct  121
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Sbjct  181
AAGAACGCAGCAAACTGCGATAATTATTGCGAACTGCAAAAGTATTGAGCACAAAAGTTT  240
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Sbjct  241
TGAACGCAAATGGCCGCATTGAGGTCAAACTCTTTGCAACGTCTGGTTCAGGGTCATTTT  300
Query  405  CTCTTATAGCGGAAGCTTTAATTTCTATAATGATGTTGTTGCTT  448
Sbjct  301  CTCTTATAGCGGAAGCTTTAATTTCTATAATGATGTTGTTGCTT  344
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Fig. (4):*Meloidogyne javanica* isolate M J -32 internal transcribed spacer 1, partial sequence; 5.8S ribosomal
RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene,partial sequence Sequence ID: MW168165.1 Length: 430 Number of Matches: 1

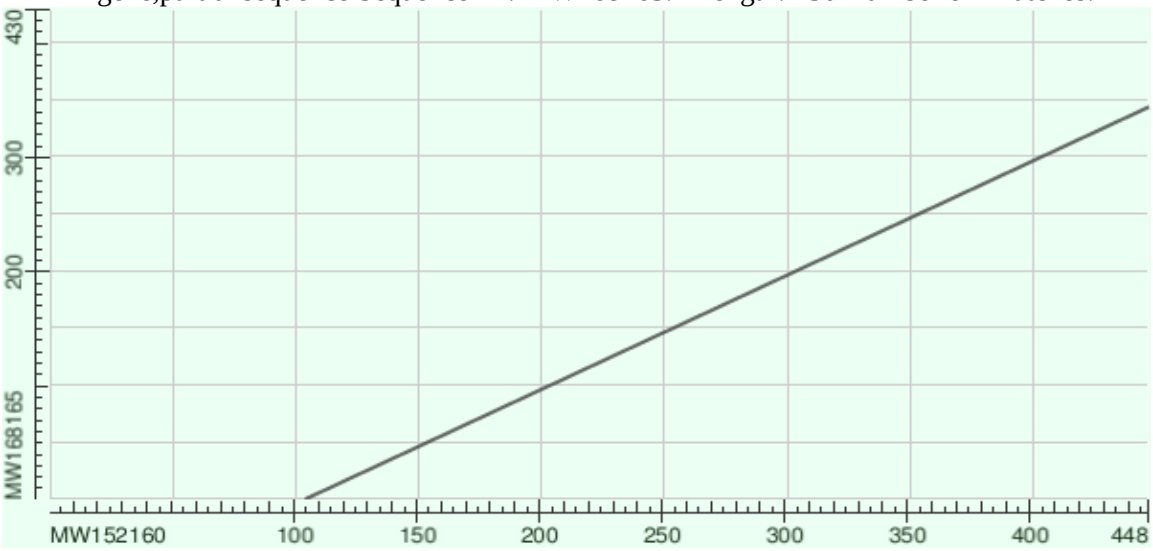

Fig. (5) : A DNA Plot of *M.incognita* (MW152160.1) vs *M.javanica* (MW168165.1)

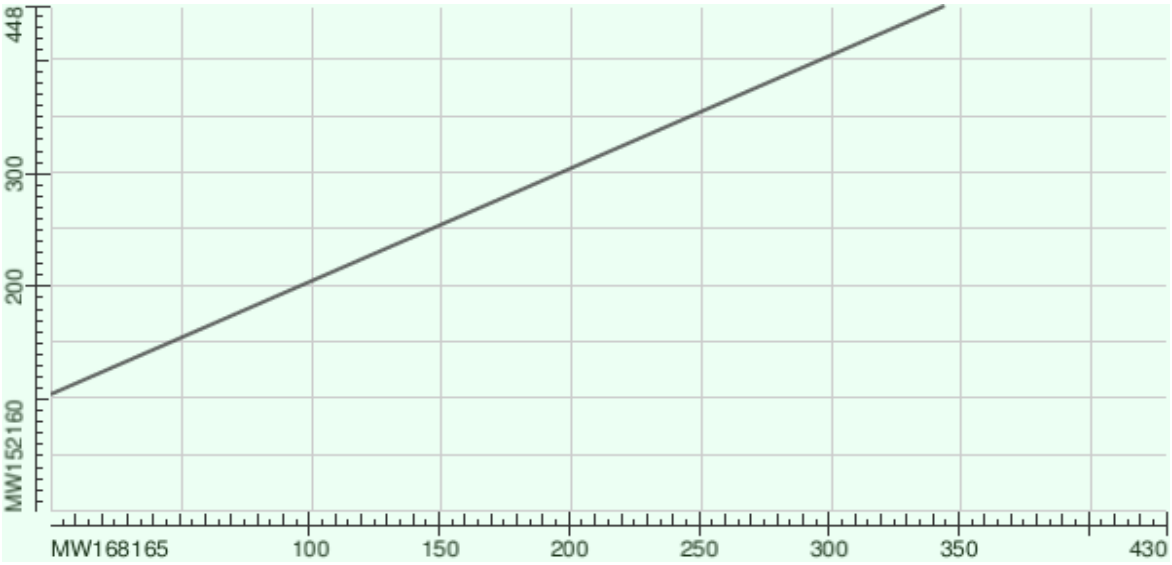

Fig. (6) : A DNA Plot of *M. javanica* (MW168165.1) vs *M.incognita* (MW152160.1)

2- Genetic similarity and phylogenetic tree of both root – knot nematode isolates :

Results of genetic similarity as illustrated by description of the Query ID (according to the blast program)for both nematode species diagnosed in this study showed a similarity of 98.84%,while it does not increased more than 99.33% and 99.53 % as a percent identity of *M.incognita* and *M.javanica* respectively with the other root – knot nematode species (Table,1 and 2),

Table (1): Percentage identity of *M. incognita* isoalte CN36 (MW152160.1) with some of the other root – knot nematode species.

Nematode species and its isolate	Percent identity (%)	Sequence ID	Country
M. incognita isolate C N 36	99.55	MT490912.1	China
M. sp.Mj-c3b on tamarillo	99.33	JX465575.1	New Zealand
M. javanica voucher VW4 18S	99.1	KP901063.2	USA
M. konaensis isolate KON-B	99.1	KY911100.1	Brazil
M. polycephannulata isolate POLY	99.1	KY882491.1	Brazil
M. arenarai isolate Tuku	99.33	KJ572384.1	Taiwan
M. sp.Mi-c2a	99.1	JX465577.1	New Zealand
M. incognita isolate CN12	98.44	MT490895.1	China
M. javanica isolate MJ – 32	98.84	MW168165.1	Iraq
M. incognita isolate CN7	97.99	MT490891.1	China
M. incognita isolate A-1	97.99	ON677753.1	Iraq
M. incognita isolate B-2	97.99	ON677754.1	Iraq

Table (2) : Percentage identity of *M. javanica* isolate MJ- 32 **(** MW168165.1) with some of the other species of root – knot nematode .

Nematode species	Percent identity (%)	Sequence ID	Country
Meloidogyne javaniac voucher M J- 21	99.53	MF168971.1	Brazil
Meloidogyne incognita isolate Nem Mi T G 2	99.53	KJ451618.1	India
Meloidogyne sp.Mj – c3b	99.53	JX465575.1	New Zealand
Meloidogyne arenaria isolate G 0337	99.30	MK188474.1	South Korea
Meloidogyne javanica voucher M J -33	99.30	MF168970.1	Brazil
Meloidogyne konaesis isolate KON – B	99.30	KY911100.1	Brazil
Meloidogyne phaseoli isolate PHA	99.30	KY882498.1	Brazil
Meloidogyne polycephannulata isolate POLY	99,30	KY882491.1	Brazil
Meloidogyne incognita Isolate CN19	98.60	MT490900.1	China
Meloidogyne incognita isolate C N 36	98.84	MW152160.1	Iraq

Phylogenetic tree for both nematode species (Figure.7 and 8) which was constructed to quantify the amount of genetic change per time unit between each species of nematode and some other species and isoltes of root –knot nematodes .

The horizontal lines or branches in each tree represent an evolutionary descent that changes over time and the longest the branch in the horizontal dimension, the largest the amount of alteration. The scale bar at the base of the left side for each figure dsipalys a scale for this. In this case the line segment with the number 0.008 or 0.003 for phylogenetic tree of *M.incognita and M. javanica* respectively shows branch length representing an amount of genetic change by 0.008 or 0.0-03.Branch length units are the nucleotide substitutions per site, which is the number of changes or substitutions divided by the length of the sequence.Vertical lines are used to outline the visual tree, thus simply showing how the horizontal lines connect to each other and how long they are not pertinent.are non pertinent.It seems from the phylogenetic tree analysis for *M.incognita* diagnosed in this study (Figure.7) and Genetic similarity percentage (table,1) that the closest species to *M. incognita* is *M.incognita* isolate CN36 (MT490912.1) from China with a genetic similarity of 99.55%, Followed by two grouping of different nematode species with a genetic similarity range 98.44 – 99.1%,while the nucleotide differences increased with the other species of nematode where more differences were noticed with two isolates [A-1(ON677753.1) and B-2 (ON677754.1)] of *M.incognita* from Iraq with a genetic similarity of 97.99%% (Table 1).

As for *M.javanica,* and according to the phylogenetic tree analysis (Fig.ure 8) and percent identity (table.2) the closest species to it is a group consisting of three nematode species that included: *M. javaniac* voucher M J- 21(MF168971.1), *M. incognita* isolate Nem Mi TG2 (KJ451618.1) and *Meloidogyne* sp.Mj – c3b (JX465575.1) from Brazil, India and New Zealand respectively, with a genetic similarity of 99.53% with each species, then the nucleotide differences started to increase slightly with the 2nd group of nematode species that consisted of *M.arenaria* isolate G 0337 (MK188474.1), *M. konaesis* isolate KON – B (KY911100.1), M. *javanica* voucher M J -33 (MF168970.1) , *M. phaseoli* isolate PHA (KY882498.1) and *M. polycephannulata* isolate POLY (KY882491.1) from Brazil with a genetic similarity of 99.30%

after which nucleotide differences increased more, as it reached its maximu level with *M. incognita* isolate CN 19 (MT490900.1) from China with a genetic similarity of 98.60 %.Thus, the results showed a high percentage of genetic similarity, whether it is between the both species of root – knot nematodes diagnosed in this study or between each species and other root - knot nematode species extracted from the genetic bank, and this is certainly due to their belonging to the same genus..

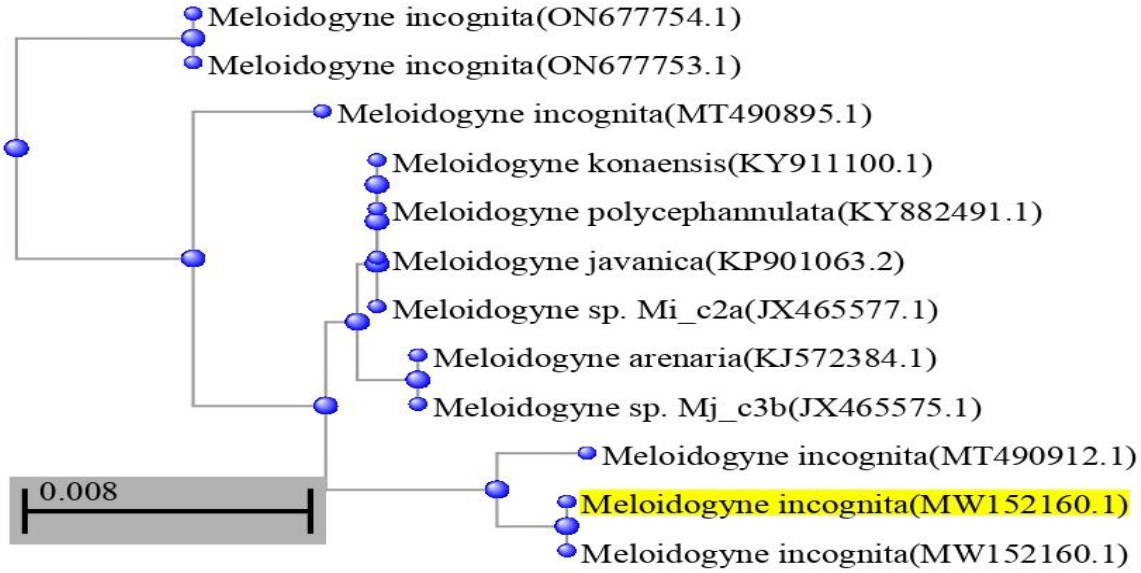

Fig.(7): Phylogenetic tree produced by Neighbor joining method using Blast Pairwise alignments showing phylogenetic positioning of *M.incognita* diagnosed in this study with some nematode species of the same genus. Branch lengthes are proportional to the number of inferred changes

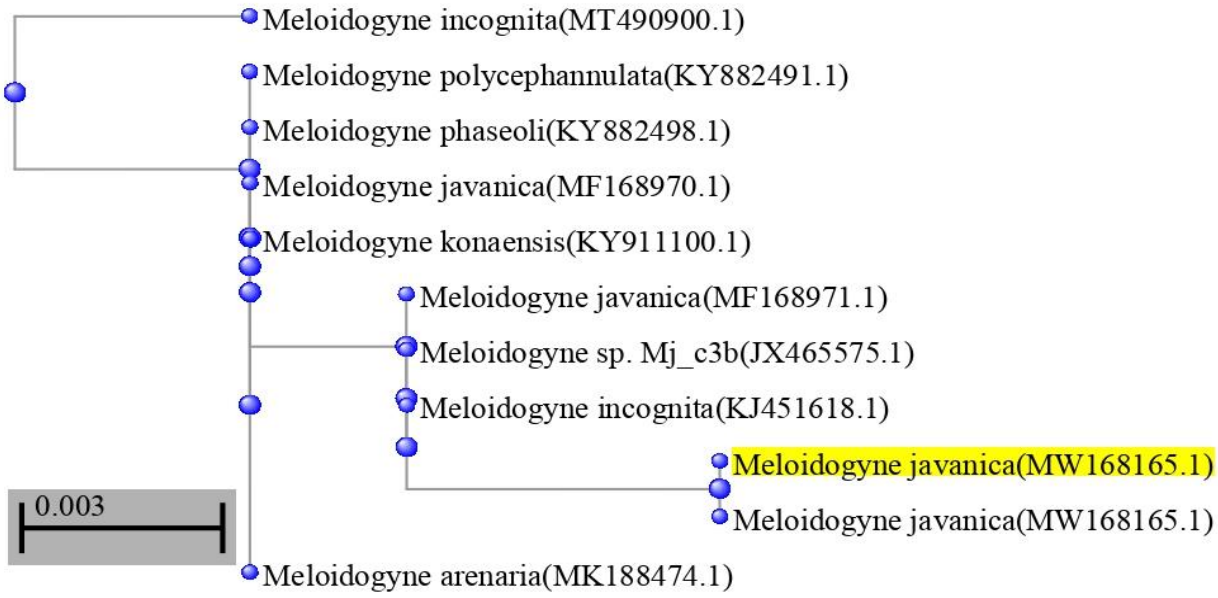

Fig. (8): Phylogenetic tree produced by Neighbor joining method using Blast Pairwise alignments showing phylogenetic positioning of *M.javanica* diagnosed in this study with some nematode species of the same genus. Branch lengthes are proportional to the number of inferred.changes

CONCLUSIONS

According to the results, researchers concluded that sequencing is necessary for diagnosing nematode species by molecular method. Both nematode species diagnosed in this study (*M. incognita and M. javanica*) genetically differed ,with a percent identity of 98.84%. Phylogenetic tree analysis showed that the most closest species to *M. incognita* is *M.incognita* isolate CN 36 from China, whilst nucleotide differences augmented with the other species of nematode were found with two isolates [A-1(ON677753.1) and B-2 (ON677754.1)] of *M.incognita* from Iraq. As for *M. javanica*, the most closest species to it is a group of three nematode species included: *M .javaniac* voucher M J- 21, *M. incognita* isolate Nem Mi and *Meloidogyne* sp.Mj – c3b from Brazil, India and New Zealand respectively,whilst more ucleotide differences was observed with *M. incognita* isolate CN19 from China.

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