Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(2): 1247-1253
© 2020 JEZS
Received: 25-01-2020
Accepted: 27-02-2020

P Kalaiarasan
Department of Nematology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Chaitra G Bhat
Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi, India

Alkesh Hada
Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi, India

Nisha Jaiswal
Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi, India

Uma Rao
Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi, India

Corresponding Author:
Uma Rao
Head and Principal Scientist
Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi, India

Molecular characterization of root-knot nematode (*Meloidogyne enterolobii*) parasitizing guava from IARI, New Delhi, India

P Kalaiarasan, Chaitra G Bhat, Alkesh Hada, Nisha Jaiswal and Uma Rao

Abstract
Guava is a tropical fruit cultivated in many tropical and subtropical regions of the world. It is the fourth important commercial fruit crop grown in India. Sustainable production of guava is facing challenges due to both above- and below-ground pests. Root-knot nematodes (RKN; *Meloidogyne* spp.) have emerged as serious belowground pests of guava in India causing severe damage leading to death of established trees. In the present study, *Meloidogyne* spp. with varied perennial patterns have been found from the guava plants for the first time from IARI, New Delhi Orchard. Morphological characterization using female perineal pattern and molecular analysis using SCAR (Sequence Characterised Amplified Region), ITS rDNA, D2-D3 expansion segments of 28S rDNA and partial Cytochrome oxidase I (*COI*) mitochondrial DNA markers confirmed the identity as *Meloidogyne enterolobii*. A confirmed South-Indian population of *M. enterolobii* collected from Coimbatore, Tamil Nadu was used as positive control for species confirmation. Phylogenetic analysis of the Indian isolate based on sequence information of ITS, D2/D3 and *COI* markers revealed that Delhi population clustered with other populations of *M. enterolobii*. This is the first report on detailed molecular characterization of an Indian isolate of *M. enterolobii* based on four markers that established the phylogentic relationship with global populations.

Keywords: *Meloidogyne enterolobii*, guava, molecular characterization, perineal pattern

Introduction

Guava, *Psidium guajava* is a tropical fruit, belongs to Myrtaceae family cultivated in many tropical and subtropical regions of the world. It is also known as poor man's apple because of its nutritive value (high level of pectin, rich in dietary fiber and vitamin C with moderate levels of folic acid). In India guava occupies an area of 2.64 lakh hectares with an annual production of 4,107 Tonnes. It is the fourth important commercial fruit grown in India after mango, banana and citrus. Major guava producing states are Uttar Pradesh, Madhya Pradesh, Bihar, Andhra Pradesh, West Bengal, Chhattisgarh, Punjab, Gujarat Tamil Nadu and Karnataka [1]. It is quite hardy, prolific bearer and a highly remunerative crop.

However, sustainable production of guava is facing challenges due to both above- and below-ground pests. Root-knot nematodes (RKN; *Meloidogyne* spp.) have emerged as serious below ground pests of guava in India. Root-knot nematodes are the most important obligate parasites of metazoan group which infest almost every crop grown worldwide. The four major species of *Meloidogyne viz.*, *M. incognita*, *M. javanica, M. arenaria* and *M. hapla* are of economic importance, known for their wide geographical distribution and wide host range. In addition, *M. enterolobii* has recently emerged as a major parasitic nematode infesting many crops worldwide [2]. RKN species, in particular *M. incognita* and *M. enterolobii* are causing severe damage leading to death of established guava trees in many orchards of India. In addition, their interactions with other soil borne plant pathogens such as *Fusarium* spp. is leading to disease complexes such as declining and wilt disease [3].

Accurate identification of different species of *Meloidogyne* is important for the management of nematodes. Morphological characterization based on perineal patterns and morphometric data of second stage juveniles, male and females of RKNs are used for species identification as a classical approach. But these approaches demand expertise and comparatively more time consuming. There is also a difficulty in differentiating closely related *Meloidogyne* species based on only morphological data.

Hence, different DNA based markers such as rDNA small subunit (SSU) 18S, large subunit (LSU) 28S D2-D3 expansion segments, intergenic spacer (IGS), internal transcribed spacer (ITS) and COI region of mitochondria, random amplified polymorphic DNA (RAPD) and sequence characterized amplified regions (SCAR) markers are widely used for authentic identification of *Meloidogyne* species [4, 5]. Molecular characterization using DNA based markers for species identification is more reliable, rapid and is independent of environmental factors and life cycle stages.

During a routine survey of guava orchard in IARI, New Delhi, three to five year old tress were showing symptoms of yellowing, marginal browning, shedding of leaves, unthrifty branches and stunted growth. Examination of roots of such trees revealed the presence of small to big size multiple galls indicating root-knot nematode infestation. Hence, in the present study, morphological and molecular studies have been carried out to identify the species of root-knot nematodes infecting the guava trees in IARI, New Delhi orchards.

Materials and Methods

A routine survey was conducted for the presence of root-knot nematode infestation in guava orchard in IARI, New Delhi during October, 2019. Soil and root samples were collected from the rhizosphere of trees showing unhealthy growth. Infected roots were washed free of soil, females were carefully dissected out of galls, from which perineal patterns were cut and used for species identification [6]. Photo-microphotographs of perineal patterns were taken using Axio vision documenter-Carl Zeiss Microscope. Root galls were also examined for presence of egg mass, if any.

Molecular characterization of *Meloidogyne* spp. infesting guava

DNA was extracted separately from six single females that were dissected from guava root galls using Worm Lysis Buffer (WLB; 0.2M NaCl, 0.2M Tris pH 8.0, 1% β-mercaptoethanol, 800µg / ml of proteinase K) (modified after Castagnone- Sereno *et al*., 1995) [7]. For this, a single female was transferred to 0.5ml microfuge tube containing 25µl of nuclease free water. 25µl worm lysis buffer was added to the tube containing female and incubated at 60°C for 2-3 h followed by at 100°C for 5-10 min and stored at -20°C [8].

PCR analyses were carried out with genomic DNA extracted from root-knot females using universal primers to amplify two rDNA gene fragments (ITS rDNA, D2-D3 expansion segments of 28S rDNA), partial *COI* mtDNA gene and SCAR primers specific to both *M. incognita* and *M. enterolobii* to confirm species identity. *M. incognita* specific SCAR is used since it is very common and highly similar to *M. enterolobii.* Primer details and PCR amplification conditions used for different markers are given in Table 1 and 2. All PCR amplifications were carried out separately in 25 µl reaction volume having 2.0 µl of DNA, 1.0 µl of each 10µM primers (forward and reverse), 2.5 µl of 10X buffer,1.5 µl 200mM of each dNTP and 2 units of Taq polymerase enzyme and made upto 25µl using MilliQ water [8]. DNA extracted from a confirmed *M. enterolobii* isolate from Coimbatore, Tamil Nadu was used a positive control to confirm the identity of IARI, New Delhi isolate using all the markers used in the present study.

Table 1: Details of primers used for species identification of *Meloidogyne* species infecting Guava

S. No.	Primers
1.	Universal primers (ITS-F/R, D2D3-F/R [9] ; COI-F [10]) ITS-F: 5'- TTGATTACGTCCCTGCCCTTT 3' ITS-R: 5'- TTTCACTCGCCGTTACTAAGG 3' D2D3-F: 5'- ACAAGTACCGTGAGGGAAAGTTG 3' D2D3-F: 5'- TCGGAAGGAACCAGCTACTA 3' COI-F: 5'- TTTTTTGGGCATCCTGAG 3' COI-R: 5'- AGCACCTAAACTTAAAAC 3'
2.	*M. enterolobii* specific SCAR primers (MK7-F/R [11]) MK7-F: 5'- GATCAGAGGCGGGCGCATTGCGA 3' MK7-R: 5'- CGAACTCGCTCGAACTCGAC 3' *M. incognita* specific SCAR primers (MI-F/R [12]) MI-F: 5'- GTGAGGATTCAGCTCCCCAG 3' MI-R: 5'- ACGAGGAACATACTTCTCCGTCC 3'

Table 2: PCR amplification conditions used for different primer sets

PCR conditions	Primers				
	ITS	D2/D3	COI	MK7	MI
Initial denaturation	95 ºC for 5 min	95 ºC for 10 min	94 ºC for 5 min	95 ºC for 10 min	94 ºC for 4 min
			* 5 cycles (94 ºC for 30 sec; 54 ºC for 30 sec 72 ºC for 30 sec)		
Denaturation	95 ºC for 45 sec	94 ºC for 30 sec	94 ºC for 30 sec	94 ºC for 40 sec	94 ºC for 30 sec
Annealing	56 ºC for 45 sec	60 ºC for 45 sec	50 ºC for 45 sec	66 ºC for 40 sec	64 ºC for 45 sec
Extension	72 ºC for 90 sec	72 ºC for 45 sec	7 2ºC for 30 sec	72 ºC for 1 min	68 ºC for 1 min
Final extension	72 ºC for 10 min	72 ºC for 10 min	72 ºC for 10 min	72 ºC for 8 min	72 ºC for 10 min
Hold	4 ºC	4 ºC	4 ºC	4 ºC	4 ºC
Number of cycle	40	40	35	35	35

5 µl of amplified products were resolved on 1.0% agarose gel prepared in 1X Tris acetate EDTA (TAE) buffer (pH 8.0) containing Ethidium bromide. Electrophoresis was performed at 5V/cm for 45 min. The gels were visualised using Alpha image analyser. The amplified PCR products were sequenced by Sanger dideoxy sequencing method.

The sequence information obtained for each marker was analysed by BLASTN analysis at NCBI. Aligned

Meloidogyne sequences were retrieved from NCBI and used for phylogenetic analysis. The phylogenetic analysis was performed by MEGA7 software [13]. Maximum Likelihood analysis was used for generating the evolutionary history using a suitable model as determined by MEGA7. The bootstrap consensus tree was inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates were collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated.

Results and Discussion

Unhealthy trees exhibited symptoms like stunting, yellowing of leaves with marginal browning. Examination of roots of unhealthy trees revealed presence of many simple and compound root galls. Most of the galls harboured 10-15 females with short to elongated neck. Galls also revealed the presence of several of egg masses that were embedded inside the gall along with the females. There were about 50-200eggs/egg mass. Further, the adult females were colourless or milky white, pear shaped and variable in size having round to flat bottom of perineal area without tail protuberance (Figure1).

To identify the *Meloidogyne* species, adult females (n = 25) were picked from galled roots and used for perineal pattern preparation. Examination of perineal patterns showed round to dorso-ventrally ovoid pattern. Dorsal arch was round to slightly squarish, striae fine and closely spaced. Lateral lines were seldom distinguishable with break in striae at junction of dorsal and ventral arches. Tail tip area was circular, free of striae, vulva slit-like, lateral striations were present (Figure 2). The female perineal patterns were similar to the original description of *M. mayaguensis* as reported by Rammah and Hirschmann (1988) [14] and presently a synonym of *M. enterolobii.* However, some samples deviated from the original description by showing a moderately high to high dorsal arch.

Therefore, molecular approach using specific DNA markers was undertaken to arrive at unequivocal species confirmation. PCR amplification with MK7-F/MK7-R primers (*M.enterolobii* specific SCAR primers) produced an amplicon of 520 bp. However no amplification was obtained with species specific SCAR primer (MIF/MIR) for *M. incognita.* PCR amplification of ITS, D2D3 and *COI* regions produced 780bp, 760bp and 410 bp respectively (Figure 3). All the amplicons of different markers were sequenced and analysed using NCBI. BLAST analysis of sequence information of ITS, 28S (D2/D3), and *COI* unequivocally confirmed that species under investigation is *M. enterolobii*. Sequence information of each of the markers analysed was submitted to GenBank database; their accession numbers from the GenBank are presented in Table 3.

Table 3: GenBank accession numbers for Indian *Meloidogyne enterolobii* isolates IARIDELHI_G1 and TNCBEK-1

Marker	Accession number	
	***M. enterolobii*, isolate IARIDELHI_G1**	***M.enterolobii*, isolate TNCBEK-1**
SCAR sequence-*Meloidogyne enterolobii*	MN728678	-
ITS rDNA	MT062610	MN752201
D2-D3 of 28S rRNA	MT062611	MN752205
COI of Mitochondria	MT075847	-

Phylogenetic comparison of *M. enterolobii* isolate IARIDELHI_G1 with other *M. enterolobii* isolates from different parts of the world and with other *Meloidogyne* species was performed considering ITS, 28S (D2/D3), and COI markers. In all the three cases, IARI isolate of *M. enterolobii* clustered with other global isolates of *M. enterolobii* (Figure 4-6). Further, comparison of ITS and D2D3 sequence of *M. eneterolobii* IARIDELHI_G1 isolate with corresponding sequence of *M. enterolobii* isolate TNCBEK-1from Tamil Nadu revealed >98% similarity between them.

In India, *M. enterolobii* infecting guava has been reported for the first time from Dindigul district of Tamil Nadu [15]. Recently, *M. enterolobii* infestation has been reported from other Indian states like Uttarakhand [16], Karnataka [17], Madhya Pradesh, Rajasthan and Uttar Pradesh [18]. Very few attempts have been made for detailed molecular characterization of Indian populations of *M. eneterolobii* using widely used molecular markers. Molecular characterization of *M. enterolobii* populations from three different districts of Tamil Nadu was carried out based on the partial sequence of internal transcribed spacer regions (600 bp amplicon size) [19] using different set of primers as compared to present study. Another *M. eneterolobii* population from Lucknow, UP was characterised using only 18S region of rDNA (638 bp) and 63V repeat region of mitochondria (322 bp). Phylogenetic analysis of this isolate based on the sequences of these two markers was made taking five populations for 18s marker while only two populations were considered for 63V marker [20]. Hence, the phylogenetic analysis based on a few populations in both the markers is not very informative as it could not properly resolve the relationship of the Indian isolate with that of global populations. In addition to this, the region of the markers used in these analyses was different compared to the markers employed in the present study. Therefore, existing sequence information for these Indian populations of *M. enterolobii* could not be considered for comparison of IARI population in the present study.

In the present investigation SCAR marker along with ITS rDNA, D2D3 expansions of 28S rDNA and COI mtDNA marker have been used to arrive at unambiguous identification of an Indian isolate of *M. enterolobii* from IARI, New Delhi and also to resolve phylogenetic relationship with global isolates. To the best of our knowledge, this is the first report of *M. enterolobii* from Delhi. Molecular approach helps in rapid and authentic species identification and also aids in monitoring the spread of the species.

Fig 1: A) Unhealthy guava plants showing symptoms like yellowing and marginal browning of leaves B) *Meloidogyne eneterolobii* infested root system of guava C) *M. eneterolobii* females dissected from the infested guava roots

Fig 2: Variations in the perineal patterns of *Meloidogyne enterolobii* infesting guava plants at IARI orchard

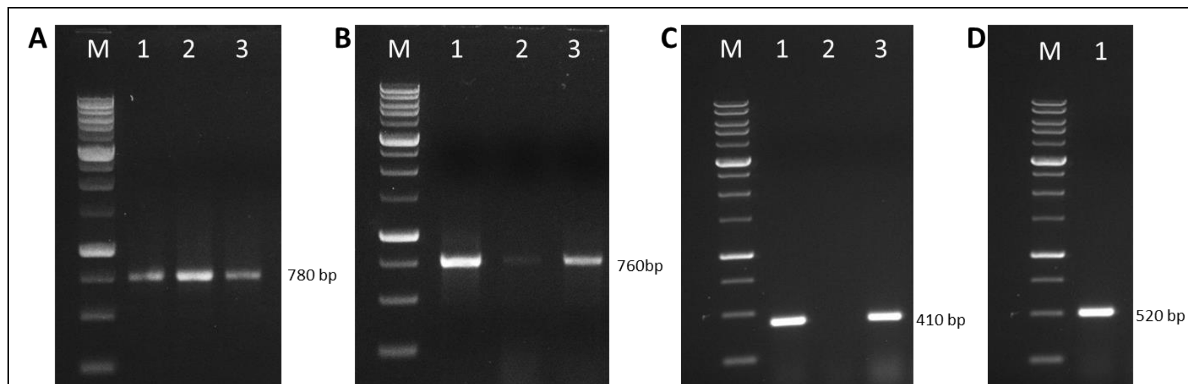

Fig 3: PCR amplification of different molecular markers from *M. enterolobii,* isolate IARIDELHI-G1
A) M: 1 kb DNA ladder; Lane 1-3: Amplification of ITS marker; B) M: 1 kb DNA ladder; Lane 1-3: Amplification of 28S (D2D3) marker; C) M: 1 kb DNA ladder; Lane 1-3: Amplification of COI marker; D) Lane 1: Positive amplification using *M. enterolobii* specific SCAR marker (MK7F/MK7R)

KT936633 Meloidogyne enterolobii isolate YAT1
MH128530 Meloidogyne enterolobii isolate P210072
JX683714 Meloidogyne enterolobii isolate HN1
MG816470 Meloidogyne enterolobii isolate 5349
KU372161 Meloidogyne enterolobii voucher T337
KY084502 Meloidogyne enterolobii isolate 4930
99
MH399805 Meloidogyne enterolobii isolate TIG39
MK387170 Meloidogyne enterolobii isolate D12
MH399804 Meloidogyne enterolobii isolate YAT12
MH128520 Meloidogyne enterolobii isolate P179070
KY203704 Meloidogyne enterolobii isolate R6B
MF673765 Meloidogyne enterolobii isolate R22
MF673764 Meloidogyne enterolobii isolate R19A
MT075847 Meloidogyne enterolobii isolate IARIDELHI-G1
MH399802 Meloidogyne hapla isolate THK4
KU174206 Meloidogyne haplanaria isolate Florida
MH332667 Meloidogyne arenaria isolate 17
99
MH743221 Meloidogyne incognita isolate M
77
JX683706 Meloidogyne javanica isolate BE1
KU517179 Meloidogyne fallax isolate NEMBAR1145
KU517170 Meloidogyne naasi isolate NEMBAR070
KU726971 Globodera ellingtonae

Fig 4: Phylogenetic analysis of *COI* sequences of *Meloidogyne eneterolobii* and other *Meloidogyne* sp. by Maximum Likelihood method; Hasegawa-Kishino-Yano model was used and analysis involved 22 nucleotide sequences.

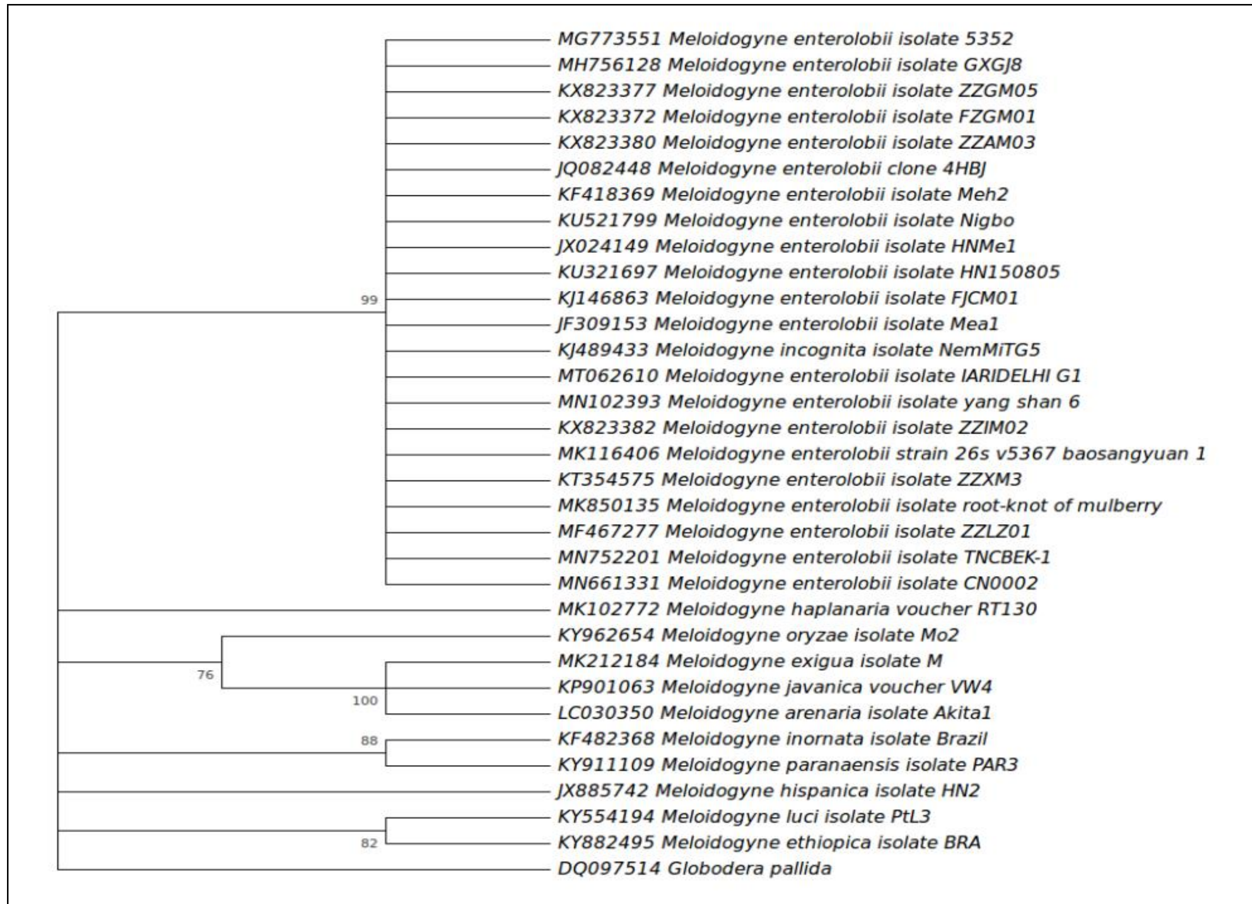

Fig 5: Phylogenetic analysis of ITS sequences of *Meloidogyne eneterolobii* and other *Meloidogyne* sp. by Maximum Likelihood method; Kimura 2-parameter model was used and analysis involved 33 nucleotide sequences.

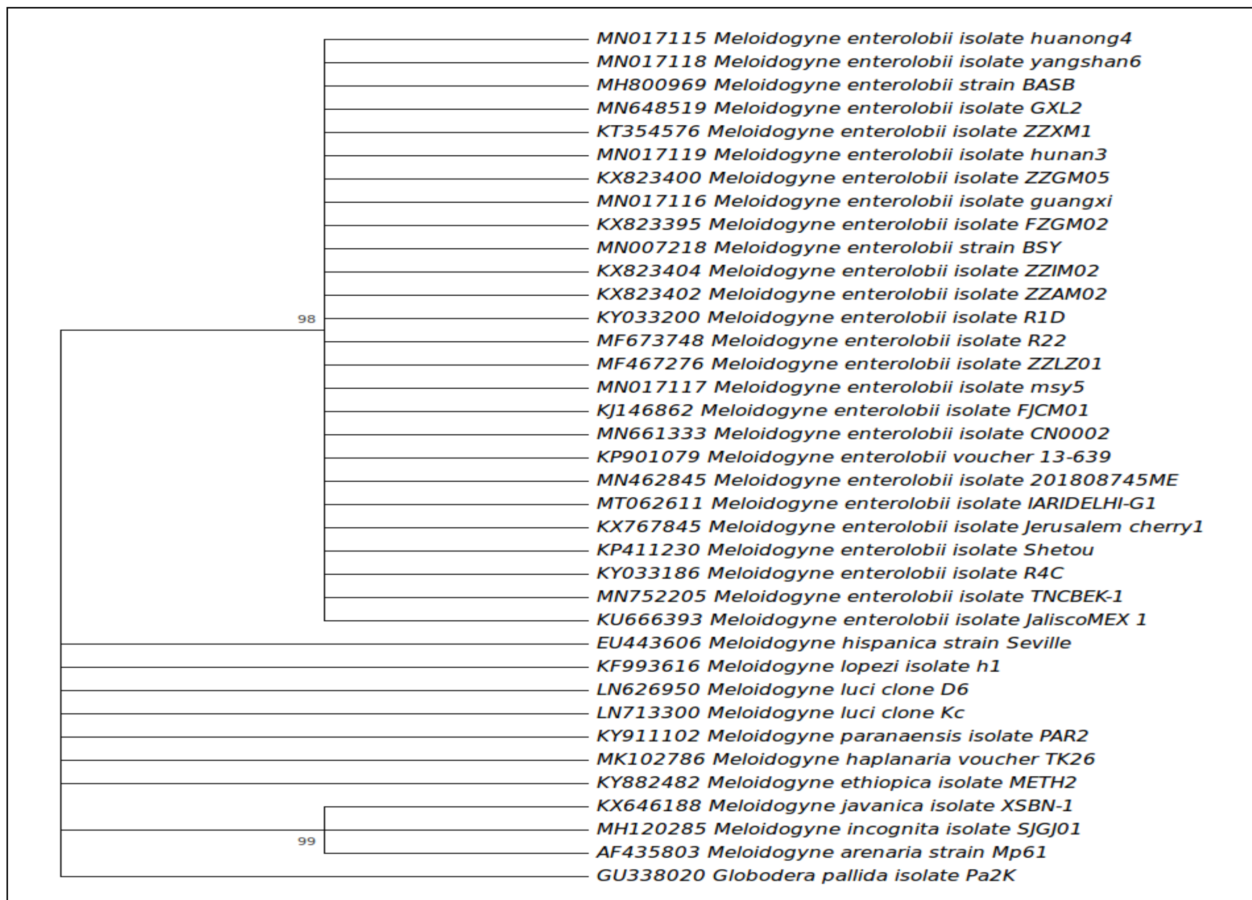

Fig 6: Phylogenetic analysis of 28S (D2 and D3 region) sequences of *Meloidogyne eneterolobii* and other *Meloidogyne* sp. by Maximum Likelihood method; Kimura 2-parameter model was used and analysis involved 37 nucleotide sequences.

References

1. Anonymous, Welfare F. Horticultural statistics at a glance. OUP Catalogue, 2018, 146.
2. Castagnone-Sereno P. *Meloidogyne enterolobii* (*M. mayaguensis*): profile of an emerging, highly pathogenic, root-knot nematode species. Nematology. 2012; 14(2):133-138.
3. Singh N. Emerging problem of guava decline caused by *Meloidogyne enterolobii* and *Fusarium oxysporum* f. sp. *psidii.* Indian Phytopathology. 2020. 1-2.
4. Ye W, Zeng Y, Kerns J. Molecular characterisation and diagnosis of root-knot nematodes (*Meloidogyne* spp.) from turf grasses in North Carolina, USA. PLoS One, 2015, 10(11).
5. Donkers-Venne DT, Fargette M, Zijlstra C. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays. Nematology. 2000; 2(8):847-853.
6. Yang B, Eisenback JD. *Meloidogyne enterolobi*i n. sp. (*Meloidogynidae*), a root-knot nematode parasitizing pacara earpod tree in China. Journal of Nematology. 1983; 15(3):381.
7. Castagnone-Sereno P, Esparrago G, Abad P, Leroy F, Bongiovanni M. Satellite DNA as a target for PCR-specific detection of the plant-parasitic nematode *Meloidogyne hapla*. Current Genetics. 1995; 28(6):566-570.
8. Adam MAM, Phillips MS, Blok VC. Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). Plant Pathology. 2007; 56:190-197.
9. Cunha TGD, Visotto LE, Lopes EA, Oliveira CMG, God PIVG. Diagnostic methods for identification of root-knot nematode species from Brazil. Ciencia Rural. 2018; 48(2).
10. Derycke S, Vanaverbeke J, Rigaux A, Backeljau T, Moens T. Exploring the use of cytochrome oxidase c subunit 1 (COI) for DNA barcoding of free-living marine nematodes. PLoS One. 2010; 5(10).
11. Tigano M, De Siqueira K, Castagnone-Sereno P, Mulet K, Queiroz P, Dos Santos M, Carneiro RMDG. Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and development of a SCAR marker for this guava-damaging species. Plant pathology. 2010; 59(6):1054-1061.
12. Meng QP, Long H, Xu JH. PCR assays for rapid and sensitive identification of three major root-knot nematodes, *Meloidogyne incognita*, *M. javanica* and *M. arenaria*. Acta Phytopathologica Sinica. 2004. 34(3):204-210.
13. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution. 2016; 33(7):1870-1874.
14. Rammah A, Hirschmann H. *Meloidogyne mayaguensis* n. sp. (Meloidogynidae), a root-knot nematode from Puerto Rico. Journal of Nematology. 1988; 20(1):58-69.
15. Poornima K, Suresh P, Kalaiarasan P, Subramanian S, Ramaraju K. Root Knot Nematode, *Meloidogyne enterolobii* in Guava (*Psidium guajava* L.) A New Record from India. Madras Agricultural Journal. 2016; 103(10-12): 359-365.
16. Kumar S, Rawat S. First Report on the Root-Knot Nematode *Meloidogyne enterolobii* (Yang and Eisenback, 1988) Infecting Guava (*Psidium guajava*) in Udham Singh Nagar of Uttarakhand, India. International Journal of Current Microbiology and Applied Sciences. 2018; 7(4):1720-1724.
17. RavichandraNG. New Report of Root-Knot Nematode (*Meloidogyne enterolobii*) on Guava from Karnataka, India". EC Agriculture. 2019; 5(9):504-506.
18. Khan M, Ahmad I, Kumar KH, Singh A, Shukla PK. Infestation of *Meloidogyne enterolobii* in newly established/old guava orchards and nurseries in Madhya Pradesh, Rajasthan and Uttar Pradesh. Annals of Plant Protection Sciences. 2019; 27(1):170-171.
19. Suresh P, Poornima K, Kalaiarasan P, Nakkeeran S, Vijayakumar RM. Characterization of Guava Root Knot Nematode, *Meloidogyne enterolobii* occurring in Tamil Nadu. International Journal of Current Microbiology and Applied Sciences. 2019; 8(09):1987-1998.
20. Khan M, Ahmad I, Kumar KH, Singh A. Identification of *Meloidogyne enterolobii* infesting Guava using mitochondrial DNA based analysis and host status. Annals of Plant Protection Sciences. 2019; 27(2):282-284.