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Characterization of Guava Root Knot Nematode, *Meloidogyne enterolobii* Occurring in Tamil Nadu, India

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A B S T R A C T

Root knot nematodes (*Meloidogyne* spp.) are the most common and destructive plant parasitic nematode group with wide host range of crops, including guava (*Psidium guajava* L.). *Meloidogyne enterolobii* (Syn:*M. mayaguensis*), the root knot nematode of the guava tree, belongs to the group of tropical root knot nematodes and is considered as one of the most damaging species, due to its wide host range, pathogenicity and ability to develop and reproduce on several crops carrying resistance genes. The present study was aimed at identifying *Meloidogyne* species attacking guava orchards in three districts such as Dindigul, Coimbatore and Thiruvannamalai districts of Tamil Nadu. The morphological study based on perineal patterns of the females confirmed the presence of *Meloidogyne enterolobii.* In addition, the molecular identification was carried out based on the partial sequence of internal transcribed spacer (ITS1-ITS2) regions. The amplicon size was approximately 600 bp. The sequences were compared with those of *M. enterolobii* in the GenBank database with high similarity (98%). Sequences were submitted in NCBI and obtained gene accession numbers of Dindigul populations (MK940246 and MK955348), Coimbatore populations (MK955350 and MN006626) and Thiruvannamalai populations (MN381161 and MN381109) were obtained. Phylogenetic studies placed present study population with other *Meloidogyne* species retrieved from the GenBank database.

Introduction

Root knot nematodes, *Meloidogyne* spp. constitute one of the major important group of plant parasitic nematodes causing severe damage to both agricultural and horticultural crops. They are sedentary endoparasites and their parasitic life depends on the success to induce feeding sites in the roots of host plants (Perry and Moens, 2006). They are dispersed worldwide and parasitizes nearly every species of both cultivated and uncultivated plants. The four major species of *Meloidogyne viz., M. incognita*, *M. javanica*,*M. arenaria*and *M. hapla*are of immense economic importance because of their wide geographical distribution and wide host range (Perry *et al*., 2009). The most common species are

estimated to be able to infest more than 5500 plant species (Trudgill and Blok, 2001) and estimated yield losses of 25-50% over cultivated plants (Taylor and Sasser, 1978).

A large number of crops worldwide are affecting by root knot nematodes and so far more than 100 species have been described (Hunt and Handoo, 2009). Infested plants show poor growth and wilt due to nutrient partitioning alterations and limited water uptake due to deformations of conducting vessels (Kaloshian *et al*., 1996). Damage is more pronounced in tropical climates than in temperate climates because of the favourable conditions for nematode survival and multiplication (De Waele and Elsen, 2007 and Kaloshian*et al*., 1996).

Root knot nematode, *M. enterolobii* (Syn: *M. mayaguensis*) Yang & Eisenback (1983) is one of the most important nematode causing severe problems in guava orchards of India especially in Tamil Nadu. Moreover it is associated with other soilborne plant pathogens such as *Fusarium* and cause disease complex on guava (Poornima *et al*., 2016). *M. enterolobii* became economically important nematode and they are emerged as major parasitic nematode in many crops worldwide (Moens *et al.*, 2009). Similar to *M. incognita*, *M. enterolobii*is also considered to be highly polyphagous species with wide host range (Yang & Eisenback, 1983). The many hosts include vegetables, tomato, pepper, watermelon (Yang & Eisenback, 1983; Rammah & Hirschmann, 1985), guava (Gomes *et al.*, 2012), ornamental plants (Brito *et al*., 2010) and weeds (Rich *et al.*, 2009).

M. enterolobii was considered as a highly aggressive species and induce severe root galls than other species of root knot nematodes. Moreover, heavy infested field became unviable for guava cultivation in Brazil (Carneiro *et al.*, 2007).In addition, the ability of *M. enterolobii* in breaking resistance to the major *Meloidogyne* species including resistant cotton, sweet potato, tomatoes (Mi-1 gene), potato (Mhgene), soybean (Mir1 gene), bell pepper (N gene), sweet pepper (Tabasco gene) and cowpea (Rkgene) is major concern (Yang &Eisenback, 1983; Fargette & Braaksma, 1990: Brito *et al*., 2007:Cetintas *et al*., 2008).

Precise identification of different species of *Meloidogyne* is important for the management of nematodes (Cenis, 1993). The accurate identification based on a combination of several methods such as morphological characteristics and morphometrics, host preferences biochemical and molecular techniques are essential methods for confirming the species (Eisenback *et al.*, 1981). Earlier, root knot nematodes were characterized mainly based on morphological features, such as perineal patterns and morphometric data of second stage juveniles, male and females (Jepson, 1987; Carneiro and Cofcewicz, 2008). However, morphological and morphometric data require skilled person and thus may not be sufficient to differentiate closely related *Meloidogyne* species (Hirschmann, 1986; Zijlstra *et al*., 2000).

DNA based identification of species is an attractive solution as it does not rely on expressed gene products, and is independent of environmental influence and life cycle stages and has high discriminating power (Zijlstra*et al*., 2000). Different regions DNA markers that aid for identification of *Meloidogyne* species include the rDNA small subunit (SSU) 18S (Powers, 2004), large subunit (LSU) 28S D2-D3 expansion segments (Chen *et al*., 2003), intergenic spacer (IGS) (Blok *et al*., 1997), internal transcribed spacer (Powers and Harris, 1993) and mitochondrial DNA (Powers and Harris, 1993 and Xu *et al*., 2004), random amplified polymorphic DNA (RAPD) (Cenis, 1993) and sequence characterized amplified regions (SCAR) markers (Zijlstra *et a*l., 2000).

Therefore, the main goal of the present study was to identify the root knot nematode, *Meloidogyne enterolobii* collected from different guava orchards of various places in Tamil Naduthrough morphological, morphometric and by using molecular tools.

Materials and Methods

Survey and occurrence of *Meloidogyne enterolobii*

Soil and root samples were collected from guava orchards of different places (Table 1) such as Ayakudi and old Ayakudi of Dindigul district, Vettavalam and Aavur of Thiruvannamalai district and Karamadai and Thondamuthur of Coimbatore district of Tamil Nadu. Collected disease materials (galled roots) were brought to Department of Nematology, Tamil Nadu Agricultural University Coimbatore for examination. Infested root were examined in stereo zoom microscope and then stained with hot acid fuchsin and destained with lactophenol for detailed examination.

Morphological identification of *M. enterolobii*

Generally**,** identification of *Meloidogyne* spp. was done by studying perineal pattern of females. Perineal patterns of ten egg laying matured females from each location were prepared as per the procedure described by Eisenback *et al*. (1981).

Second stage juveniles were collected from soil by Cobb's decanting and sieving (Cobb, 1918) method followed by Baermann's funnel techniques (Schindler, 1961). Photomicrograph of perineal patterns, anterior and posterior regions of male and females and second stage juveniles were made by camera attached to compound microscope.

Molecular identification of *M. enterolobii*

DNA was extracted from females of root knot nematodes as per the method described by Cenis (1993) with slight modifications. Ten to twenty females were collected from guava roots (originated from single eggmass) and placed in 1.5 ml of Eppendorf tube, disinfested with 500 µl of 0.5 % sodium hypochlorite for 3-5 minutes and centrifuged at 10000 rpm for 10 minutes. The pellet was washed with sterile water. Then, 400 µl of extraction buffer (250 mMTris-HCl, pH 8.0; 250 mM sodium chloride; 50 mM EDTA and 0.5 % Sodium DodicylSulfate, SDS and 10 µl of β-Mercaptoethanol) were added and crushed with the help of micro pestle and mortarmanually for 10-15 minutes. Then, the homogenate were kept at -20º C for 30 minutes after the addition of 0.5 volume of 3M sodium acetate (pH 5.2). Tubes were then centrifuged at 10,000 rpm for 5 minutes and supernatant was transferred to another sterile Eppendorf tube. Two volumes of cold isopropanol were added into tubes and incubated at -20º C for overnight for precipitation of DNA. After incubation, the tubes were centrifuged at 12000 rpm for 10 minutes and discarded the supernatant. The pellet was washed with 70% ethanol, air dried for one hour at room temperature and resuspended in 50 µl of nuclease free water for further use. The quality of the DNA was analysed by 0.8 % of agarose gel electrophoresis.

Polymerase chain reaction and DNA sequencing

After DNA extraction, PCR was performed for 25µlcontains, 1 µl genomic DNA (50 ng), 0.5 µM primers (forward and reverse) , 0.5 mM of each dNTPs, 2.5 µl 1× PCR buffer, 2.5 mM MgCl2, and 1.25µl Taq DNA polymerase (Sigma, USA) for amplification of Internal transcribed spacer regions of root knot nematodes.

The internal transcribed spacer 1 (ITS1) -5.8S gene was amplified using the forward primer TW81 (5′ GTTTCCGTAGGTGAACCTGC-3′) and reverse primer AB28 (5′ - ATATGC TTAAGTTCAGCGGGT-3’) Subbotin *et al*. (2001).

The PCR cycles began with an initial denaturation at 94°C for 5 min(35 cycles with denaturation for 60 sec at 94°C, primer annealing for 30 sec at 60°C and extension for 60 sec at 72°C) and final extension at 72°C for 5 min. PCR products were visualized with UV illumination after staining with ethidium bromide of 1.2 % agarose gels under a UV transilluminator (Alpha imager EC 1200) (Sambrook *et al*., 1989). Amplified PCR products were sequenced at Chromous Biotech PVT Ltd, Bangalore, Karnataka.

DNA sequences were aligned using CLUSTAL W followed by manual adjustment. Sequences were deposited in NCBI and accession numbersobtained. Phylogenetic tree was constructed using MEGA 7 from aligned sequences of the present study and retrieved from the GenBankdatabase.

Results and Discussion

Survey and occurrence of *Meloidogyne enterolobii*

All the collected samples were found to be highly infested by root knot nematode, *M. enterolobii*. Totally six isolates were isolated from guava orchards in Dindigul, Coimbatore and Thiruvannamalai districts. Females and second stage juveniles were extracted from root and soil and identified as *M. enterolobii* with the help of morphological characters.

Morphological characterization of *M. enterolobii*

The female, perineal patterns were round to high dorsal arch with absence of lateral fields and absence of wings. However, dorsal arch in perineal patterns were quite variable (Fig 2). Perineal pattern from some females showed a high trapezoidal dorsal arch similar to that of *M. incognita* (Fig 2). In the present study, observed characters coincided with original description given by Yang & Eisen back (1983). Moreover, Thiruvannamalai population slightly differed from the original description (Fig.2), which might be due to change in varietal (of the host) and edaphic conditions. Morphology of perineal patterns have been the typical characteristic used for the identification of the most common *Meloidogyne* species since 1949 (Chitwood 1949). In the present study, *M. enterolobii* were identified based on perineal patterns and other morphological characters.

The morphometrics of *M. enterolobii* females recorded an average body length and stylet length of all isolates as590 (554-690) µm and 14.37 (12.50–16.00) µm (Table 1).The average body length and stylet length of males were 1593 (1175-1742) and 23.33 (22 - 24.5µm). The average body length of the second stage juveniles was 448.33 (415 – 495) and stylet length was 13 (12-13.5µm). Those observed measurements were similar to the original descriptions given by Rammah and Hirschmann, 1988. *Meloidogyne enterolobii* was first reported in roots of *Enterolobium contortisiliquum* L. in South China (Yang and Eisenback, 1983), while *M. mayaguensis* was first reported on eggplants (*Solanum melongena* L.) in Puerto Rico (Rammah andHirschmann, 1988).

The nematode, *M. enterolobii* was first reported in 2004 in the Paluma variety of guava, which is the most common variety in commercial orchards at Limoeiro do NorteCounty (Torres *et al*., 2005) in the eastern region of the state.

Table.1 Morphometrical measurements of *Meloidogyne enterolobii* populations

S. No	Characters	Dindigul population*			Coimbatore population*			Thiruvanamalai population*		
		J_2	Male	Female	J_2	Male	Female	J_2	Male	Female
1	Body length	448.33 (415 -495)	1564.00 (1426-1650)	590 (567 -710)	434.00 (405 - 476)	1585.33 (1448-1750)	596 (577 -740)	457.33 (435 - 465)	1484.33 (1396-1650)	589 (560 -713)
2	Stylet length	12.00 (11 - 14)	46.00 (40 – 46.00)	544 (395 - 529)	11. (11 - 13)	45 (40 - 47)	540 (405 - 519)	11.50 (10 - 16)	46.33 (39 - 47.00)	546 (395 - 609)
3	Body width		23.33 (22 -24.5)	17.00 (14 - 19)		22.00 (22 -25)	17.16 (14 - 19)		20.33 (19 -24)	16.00 (13 - 19)
4	Tail length	52.8 (42 - 62.5)	-	-	50.8 (45-62)	-	-	48.33 (40-66)	-	-
5	Vulval slit length (PCP)	-	-	31.66 (26 -33)	-	-	29.00 (22-39)	-	-	30.33 (26 -33)

*Values are mean of five replications

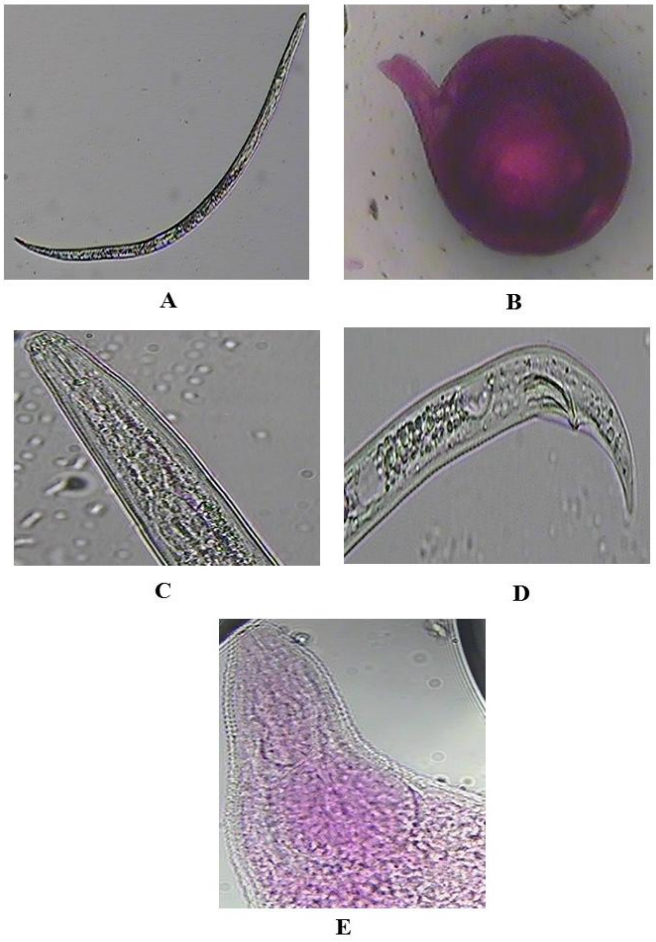


Fig. 1. different parts of *Meloidogyne enterolobii*. A) View of second stage juvenile, B) View of matured female, C) Male head portion, D) Male tail portion and E) Anterior portion of female

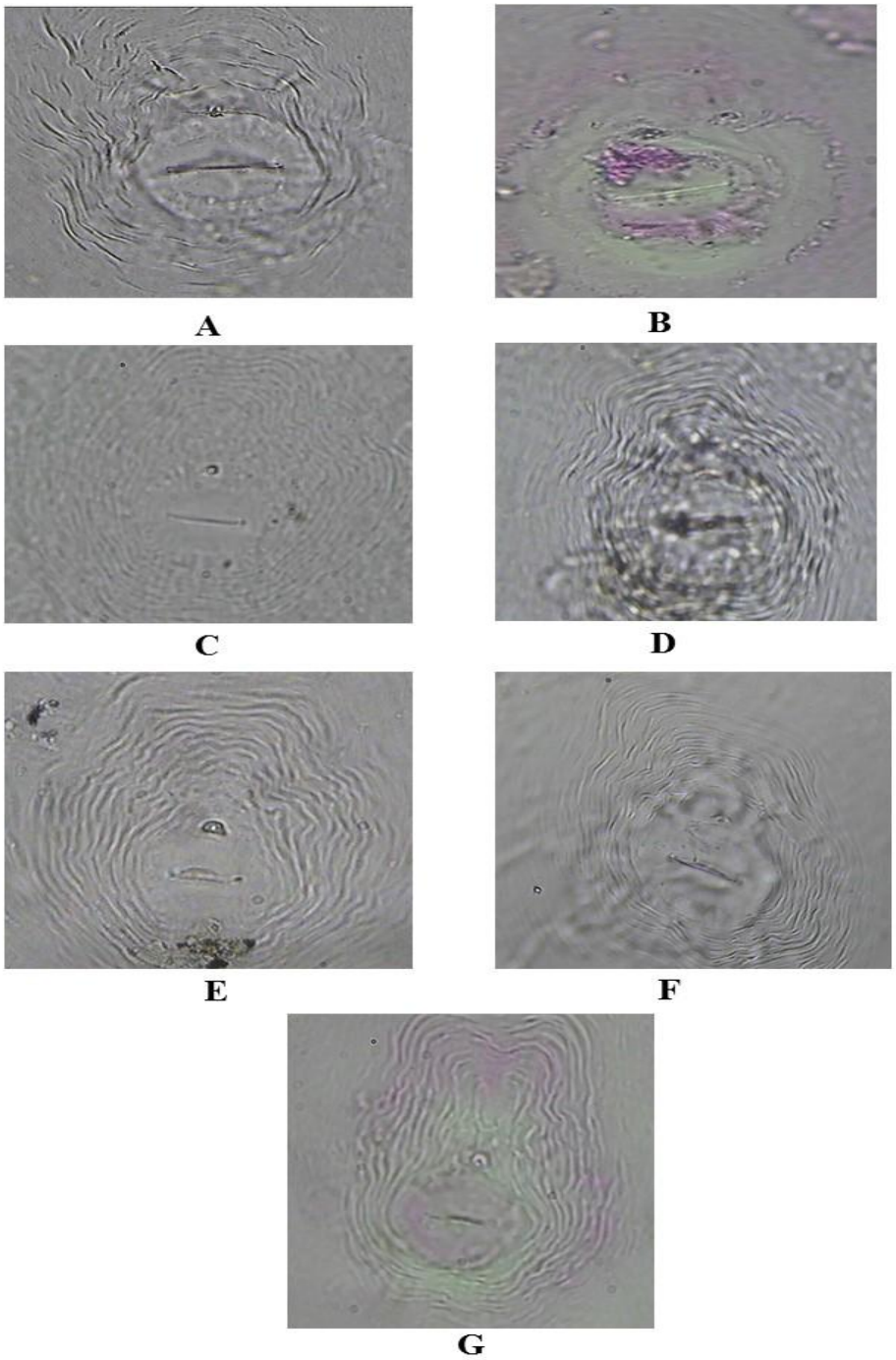


Fig. 2 perineal patterns of *Meloidoogyne enterolobii* isolates from different districts of Tamil Nadu. A&B) Dindigul isolates, B&C) Coimbatore isolates, D&E) Thiruvannamalai isolates and G) *M.incognita* isolate

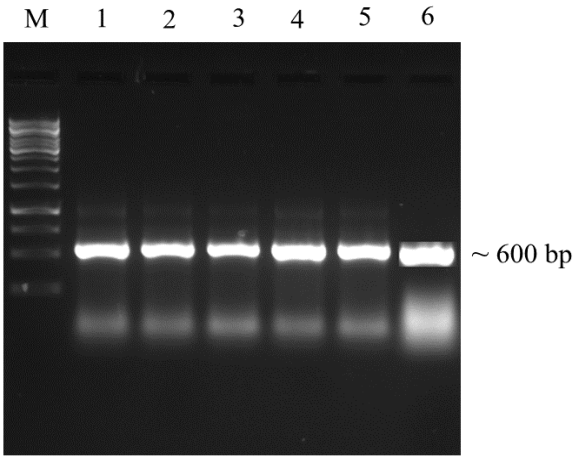
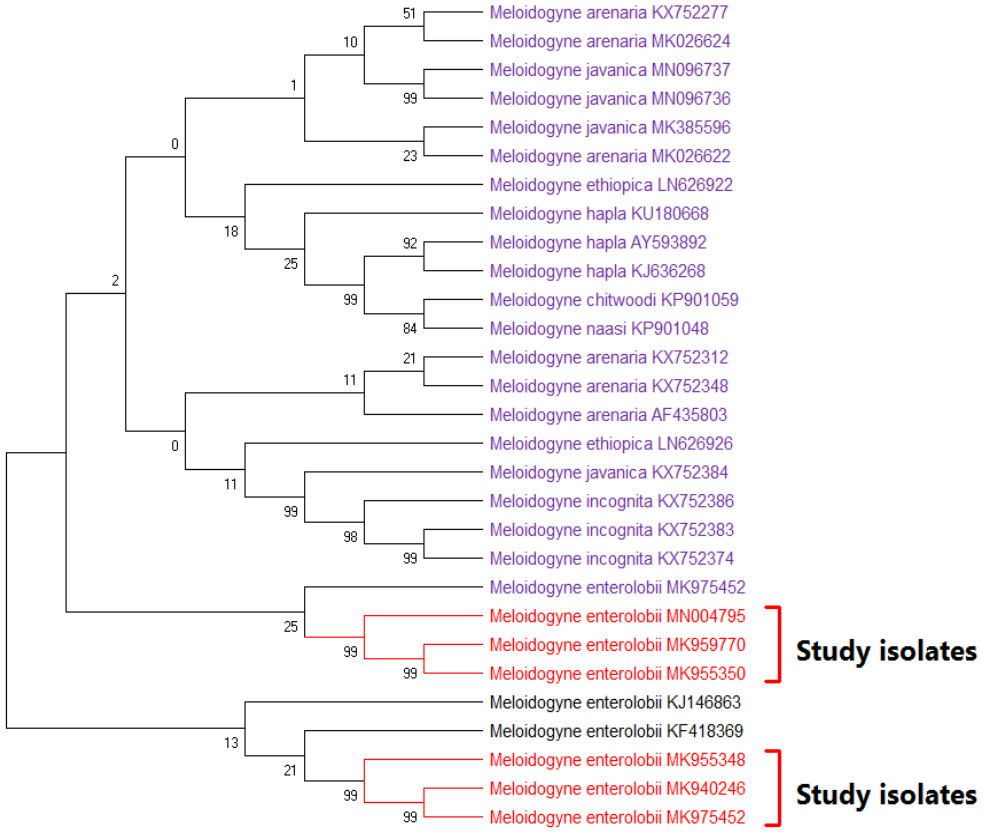


Fig 3. PCR amplifications of ITS regions *Meloidogyne enterolobii*. M- 1 Kb ladder, lane 1&2) Dindigul isolates, lane 3&4) Coimbatore isolates and lane 5&6) Thiruvannamalai isolates.

Fig.4 Maximum likelihood tree after an alignment of consensus sequences based on the ITS region of various *Meloidogyne enterolobii* identified in this study. Newly obtained sequences in this study are in labelled as study isolates. Analysis was done using 1000 bootstrap replicates. The bootstrap support value for each clade is indicated on the nodes.



In India, it was first reported by Poornima *et al* (2016) from guava orchards of Ayakudi villages of Dindigul district of Tamil Nadu. Various authors reported *M. enterolobii* from various locations of guava orchards in the states of Pernambuco and Bahia (Carneiro *et al*., 2001), Rio de Janeiro (Lima *et al*., 2003), Ceará (Torres *et al*., 2005), São Paulo (Almeida *et al*., 2006), Paraná (Carneiro *et al*., 2006), Piauí (Silva *et al*., 2006) and Espírito Santo (Lima *et al.*, 2007). However, earlier it was misidentified as *M. incognita* (Fargette *et al.*, 1994 and Brito *et al*., 2004). The present study used a different combination (Perineal pattern, morphometrics and DNA bases) tools for identification of *M. enterolobii* in guava orchards of Tamil Nadu. Analysis of perineal patterns requires considerable skill because of significant intraspecific variations (Carneiro *et al*., 1996). In this study, minor variations were observed in perineal pattern of three district population isolates when compared with previously published *M. enterolobii* perineal patterns, which could due to influence of varietal (of the host) or edaphic factors.

Molecular characterization of *M. enterolobii*

Root knot nematode, *M. enterolobii* was confirmed by DNA based methods. The primers (TW81 and AB28) led to positive amplifications and sequencing for all the isolates. The amplicon size were approximately 600 bp of ITS region of *Meloidogyne* spp. (Fig. 3). Amplified PCR products were purified and partially sequenced at Chromous Biotech PVT Ltd. Bangalore, India. The sequences were deposited in BLAST and Gene accession numbers *viz*., Dindigul populations (MK940246 & MK955348), Coimbatore populations (MK955350, MN006626) and Thiruvannamalai populations (MN381161 and MN381109) were obtained. Various molecular approaches have been designed for identification of root knot nematode species. This is primarily because DNA based methods are rapid and reliable when compared to morphological or biochemical methods (Powers *et al*., 2005).

The most popularly used DNA based methods are mitochondrial DNA (mtDNA), sequence characterized amplified region markers (SCAR), amplified fragment length polymorphisms (AFLP), random amplified polymorphism DNA (RAPD), ribosomal DNA (rDNA), restriction fragment length polymorphisms (RFLPs), microsatellite DNA (satDNA), microarrays and real time PCR (qPCR). In the present study, *M. enterolobii* from guava was identified through PCR using ITS region of Nematodes.

The method for identification of root knot nematode species using PCR was first reported by Harris *et al*. (1990), who amplified the mt DNA from single second stage juvenile and this method was further developed by Powers & Harris (1993) who designed primers for amplifying the intervening region between the mt DNA gene coding for cytochrome oxidase subunit II and the 16S rRNA primers and used them to identify five major *Meloidogyne* species *viz*., *M. incognita* , *M. javanica*, *M. arenaria*, *M. hapla*and *M. chitwoodi*.

Regions of the nuclear genomes of 18Sand 28S rDNA have been used for characterization of root knot nematode species. Ribosomal DNA has been very informative regions for diagnostic and phylogenetic studies of plant parasitic nematodes (Blok, 2005).

Kiewnick *et al*. (2009) reported that, no differences were found at DNA level between two species *M. enterolobii* and *M. Mayaguensis*, and they concluded *M. enterolobii* as synonyms of *M. mayaguensis* and *M. mayaguensis* asjunior synonym of *M. enterolobii.*

Phylogenetic analysis

DNA sequences of the internal transcribed spacer (ITS) have been useful for identification of nematode species (Zijlstra*et al*., 1997). In the present study, an approximately 600 bp DNA fragment of internal transcribed spacer (18S-28S) of *M. enterolobii* nematode population were amplified and partially sequenced. Sequences were aligned with other *Meloidogyne* species obtained from GenBank database and the phylogenetic tree was constructed.

The Maximum Likelihood tree (Fig 4)was highly resolved and showed that in the present study, populations of *M. enterolobii* formed a clade together with sequences other *M. enterolobii* (MK975452 and KF418369) populations retrieved from GenBank database. Separate clades were formed for other species of *Meloidogyne*with high bootstrap support (Tamura *et al*., 2011).

In summary, results of this study demonstrated clearly the presence of *M. enterolobii* in all the six isolates collected from three districts of guava growing orchards of Tamil Nadu, identified based on morphological and molecular methods.

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