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Root Knot Nematode, *Meloidogyne enterolobii* in Guava (*Psidium guajava* L.) A New Record from India

K. Poornima[1], P. Suresh, P. Kalaiarasan, S. Subramanian and K. Ramaraju*
Department of Nematology
*Director, Center for Plant Protection Studies
Tamil Nadu Agricultural University, Coimbatore-641 003

Guava orchards of Tamil Nadu were facing sudden decline and sampling revealed the incidence of root knot nematode. The species was identified using morphometry and with the help of Polymerase Chain Reaction (PCR) common 18S primer as *Meloidogyne enterolobii* further confirmed by sequencing the 18s rRNA gene. The sequence had 99% similarity with *M. enterolobii* sequence previously available in NCBI database which was submitted and provided with Genebank accession number (KX611608).

Key words: Guava, Survey, Root knot nematode, *Meloidogyne enterolobii*.

Since a couple of years, Tamil Nadu farmers have been facing a unique problem of decline of guava trees leading to complete destruction of the orchards. The incidence was first reported from Ayakudi village of Dindigul district wherein 3-4 year-old trees showed sudden yellowing and wilting symptoms followed by shedding of leaves, reduction in fruit size and complete death of trees (Plate. 1). The roots of such trees were very heavily galled (Plate. 2) and sometimes showed burnt-like appearance due to complete galling caused by root knot nematode followed by rotting. On examination of infested roots under laboratory conditions the association of root knot nematode, *Meloidogyne* sp. was discernible. Considering the intensity of this nematode problem on guava and the uniqueness of galling pattern, it was thought worthwhile to conduct systematic studies on the extent of distribution of this nematode in guava growing areas of Tamil Nadu and conduct detailed study on the identification of this species of root-knot nematode based on morphological, morphometrical and molecular parameters; and the same are reported in this article.

Materials and Methods

A random survey was conducted for the presence of root knot nematode in guava crop grown in different districts of Tamil Nadu *viz.,* Chinna Ayakudi, Periya Ayakudi (Dindigul district), Manjanayakanpatty (Dindigul district), Kalipatty (Dindigul district), Kongapatty (Dindigul district), Cumbum (Theni district), Porulur (Dindigul district), Karur district, Krishnagiri district, Dharmapuri district, Usulampatti (Madurai district), TNAU, Coimbatore district, Sirumugai (Coimbatore district), Tindivanam (Villupuram district), Thamaraipakkam (Tiruvallur district) and Poolampatti, Palani (Dindigul district). Soil and root samples were collected from rhizospheric zone of diseased plants in polythene bags, brought to the laboratory and stored in a refrigerator at 10°C until processed. An aliquot of 200 cc soil from each sample was processed by Cobb's decanting and sieving method (Cobb, 1918) followed by modified Baermann's funnel method (Schindler, 1961) for extraction of vermiform stages (males and second stage juveniles). . The nematodes were fixed in Triethanolamine Formalin (TAF) (Courtney *et al.,* 1955) and temporary mounts were prepared in fixative to take measurements and other morphological observations. The males of root knot nematodes were collected by soil processing and also by teasing out the egg masses of adult females attached to the roots. Roots (5g) were stained using 0.1% acid fuchsin lacto phenol and observed for number of females within galls, egg masses and eggs. Species of root knot nematode was confirmed with the help of posterior cuticular pattern (PCP).

Measurements and microphotographs were taken with the help of image analyser (Labomed) at the Department of Nematology, Tamil Nadu Agricultural University, Coimbatore. The drawings of J2, head and tail portions of J4, male, female head and posterior cuticular pattern were made with the help of Camera Lucida.

DNA was extracted from female root knot nematodes by using Worm Lysis Buffer [WLB; 50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM $MgCl_2$, 20 µg / ml proteinase K, 0·45% Tween 20 and 0·01% gelatine] (Castagnone- Sereno *et al*., 1995). A female was picked and transferred to 1.5 ml centrifuge tube. The tubes contain 25µl worm lysis buffer and the female was crushed with a needle or micropipette tips. The tubes were centrifuged at 12,000 g for 2 min, and placed at – 80°C for 30 min., followed by freezing the sample at - 20°C (Adam *et al.,* 2007).

PCR amplifications using DNA extracted from root knot female with species specific primers

*Corresponding author email: poornimamurtysista@gmail.com

were carried to total volume of 25 µl reaction which contains 2.0 µl of DNA, 1.0 µl of each 10µM primer (Forward and Reverse), 2.5 µl of 10X buffer, 2.5 µl 200mM of each dNTP and 2 units of Taq polymerase enzyme and made upto 25µl (Adam *et al.,* 2007). Two pairs of primers common 18s F and 18s R (NEM 18S F CGCGAATRGCKCATTACA, NEM 18S R GGGCGGTNTCTGAKCGCC) were used. The PCR amplification conditions used for each primer set are described in Table 2. Sequencing was done by Chromous Biotech PVT. Ltd. Bangalore, Karnataka, India.

PCR amplification conditions

Initial	- 94° C for 2 min
Denaturation	- 94° C for 30 sec
Annealing temperature	- 50°C
Extension	- 72° C for 90 sec
Final extension	- 72° C for 7 min
Hold	- 4° C
Cycle	- 45

Amplified products were resolved by 1.2 % agarose gel with Tris acetate EDTA (TAE) buffer under 80 volts for 45 min. Products were visualized with UV illuminator after ethidium bromide staining. The gel was documented with the help of Alpha imager TM 1200 documentation (Sambrook *et al.,* 1989). The PCR products were sequenced at Chromous Biotech India PVT. LTD, Bangalore, Karnataka, India.

Results and Discussion

In all of the samples collected, galling was severe with a mixture of simple and compound galls. Some galls were seen on the collar region and lower portions of the stem of the guava trees, each varying with minute size to 5 cm diameter galls. Stained roots harboured several (50-200) root-knot females within each gall, their necks reaching the stelar portion of the root. Egg masses were laid within the roots in most of the matured roots, in a well-like cavity with a brown sac-like covering. Each egg mass contained 50-200 eggs. Nematode extraction of soil samples revealed several males; overcrowding resulting in sex reversal also could be a reason for this excessive number of males (Table 1).

The body of adult female was colourless, pear-shaped and variable in size, posterior portion rounded without tail protuberance. Cuticle was finely striated at anterior neck region. Stylet knob of female was transversely elongated, distinctly indented, merging gradually with the shaft. Perineal patterns were round to dorso-ventrally ovoid. Dorsal arch was rounded, striae fine and closely spaced. Lateral lines were seldom distinguishable with break in striae or a single lateral line occurring on one side of pattern at junction of dorsal and ventral arches (Plate 3; Fig.1.). The pattern was similar to the original description of *Meloidogyne mayaguensis* as reported by Rammah and Hirschmann (1988) and now synonym of *M. enterolobii*. Tail tip area was circular, free of striae, vulva slit-like, lateral striations were present (Table 2).

Males were vermiform with variable length, tapering anteriorly, bluntly rounded posterior. Body cuticle was with faint transverse annulation. Head slightly set off, shallowly rounded to truncate, head region high without annulations and the stylet knob large, ovoid to rounded, shaft cylindrical, diameter usually uneven, curved at some points, narrowed distinctly at base, surrounded by ring. Rounded, sloping backward, dorsal knob base concave. Spicule head cylindrical well separated from shaft by indentation (Table 3). The second stage juveniles were vermiform, head slightly set off, tapering at posterior. Head region truncate, slightly set off from body. Body cuticle was with fine distinct annulations, annules were larger on posterior tail region. Stylet knob small, rounded, set off from shaft, distinctly sloping backward, bluntly rounded. Tail shape was slender, posterior part nearly straight and parallel, tapering to round. Tail was hyaline (Table 4).

Plate. 1. Wilted guava plants infested with root knot nematode, *M.enterolobii*

Plate 2. Roots of guava infested with root knot nematode, *M.enterolobii* showing galls and rotting

Table 1. Distribution of *M. enterolobii* in Tamil Nadu.

Location	No. of galls/ root	No. of females/ gall	No. of eggs/ Egg mass	Age of the s/s (months)	Type of gall	Type of planting material	Symptoms
Chinna Ayakudi, Dindigul	120	25	97	72	Compound	Ground layer	Witling with abscised fruits
Periya Ayakudi, Dindigul	66	52	132	30	Compound	Ground layer	Wilting and death of plants
Manjanayakanpatty, Dindigul	20	13	120	12	Simple	Ground layer	Wilting and total death of plants with intact fruits
Kalipatty, Dindigul	33	32	123	36	Compound	Ground layers	Wilting of plants
Kongapatty, Dindigul	42	40	76	30	Compound	Rooted cuttings	Wilting and total death of plants with intact fruits
Cumbum, Theni	12	3	32	1	Simple	Grafts	Wilting and yellowing
Porulur, Dindigul	26	43	192	36	Compound	Rooted cuttings	Pale green leaves with wilting
Karur	53	32	130	18	Simple, compound	Ground layers and rooted cuttings	Chlorotic leaves , plants showing unthrifty growth
Krishnagiri	32	20	133	24	Simple	Ground layers	Bronzing of leaves
Dharmapuri	30	23	56	24	Simple, compound	Grafts	Yellowing and wilting
Usulampatti, Madurai	32	22	132	36	Compound	Grafts and ground layers	Marginal necrosis in leaves and wilting
TNAU, Coimbatore	45	25	33	120	Compound	Ground layers	Bronzing of leaves, no flower
Sirumugai, Coimbatore	43	32	45	42	Compound	Budded plants	Yellowing of leaves
Villupuram	30	33	153	48	Compound	Ground layers	Wilting of plants
Poolampatti, Palani	32	40	32	18	Simple	Rooted cuttings	Wilting of plants

As a preliminary work, only one population (Dindigul) was used for molecular identification of the root knot nematode population, and further populations are proposed to be investigated by molecular means. In this investigation, specific and common 18s primers were used for identification of the root knot nematode population from Ayakudi, Dindigul district of Tamil Nadu. An 18S F and 18S R primer set was produced at 859 bp amplicon (Plate 4.). No amplification was obtained from any of the species specific SCAR primers such as Fjav/ Rjav, Fin/ Rin and Far/ Rar.

Fig. 1. Posterior Cuticular Pattern (PCP) of *M.enterolobii*

The identity was further confirmed by sequencing with 18s rRNA gene (KX611608) Kiewnick et al, 2009 observed that no difference was found between *M.mayaguensis* and *M.enterolobii* at DNA level. Hence, the two species were synonimised as *M.enterolobii* (syn. *M.mayaguensis*).

Our study sequence was subjected to similarity searches in National Centre for Biotechnology Information (NCBI) through Blast. It had 99% similarity with *M. enterolobii* sequence previously available in NCBI database (Ye *et al*., 2015. Based on this data, a phylogenetic tree was derived using Mega 6 software (Fig. 2). Hence, it is confirmed that the Ayakudi, Dindigul, Tamil Nadu isolate may be *M.enterolobii* as per the above results.

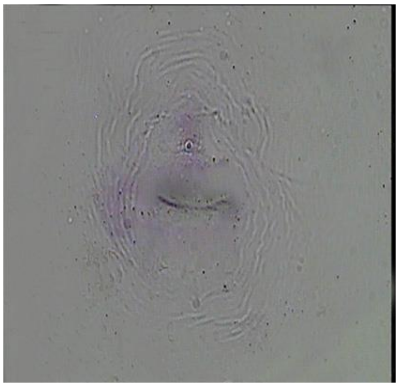

Plate 3. Posterior Cuticular Pattern (PCP) of *M.enterolobii*

Table. 2. Dimensions of two populations of females of *M. enterolobii*

Dimensions (µm)	Original description (µm) *	Locations					
		Villupuram			Dindigul		
		Mean	SD	CV %	Mean	SD	CV %
Body length	735.0 (541.3- 926.3)	592 (567 -710)	189.47	21	697 (579 - 795)	172.52	24
Body width	606.8 (375.7-809.7)	544 (395 - 529)	224.25	17	652.33 (385 - 675)	248.03	18
Stylet length	15.1 (13.2-18.0)	17.16 (14 - 19)	2.75	16	18 (14 - 19)	3.60	15
Stylet knob height	2.4 (1.9-3.1)	2.63 (2 -3)	0.55	15	3 (2.5 – 3.0)	0.5	16
Stylet knob width	4.9 (4.1-5.6)	5.16 (4 - 5)	1.04	10	5.4 (4.7- 6)	0.65	12
DOGO	4.9 (3.7-6.2)	5.83 (4 -7)	1.60	9	6.33 (5.5 -7)	0.70	12
Vulval slit length	28.7 (25.3-32.4)	31.66 (26 33)	5.13	11	29.66 (26 – 33.6)	3.51	11
Vulva to anus distance	22.2 (19.7-26.6)	23.83 (20 – 23.5)	3.40	14	27 (25 - 28)	2	7

Original description* Yang and Eisenback (1983)
DOGO-Dorsal Oesophageal Gland Orifice

Identification based on morphological features is time consuming and requires expertise (Blok *et al.* 2002*). M. enterolobii* is regarded as the most aggressive species in comparison to other tropical species of root knot nematode (Brito *et al*., 2004) in view of its high reproduction rate, induction of large galls and a very wide host range, and their combinations, *and* has become a threat to guava production worldwide leading to decimation of several

Lane 1 500 bp ladder

Lane 2 859 bp amplified PCR product

Plate 4. PCR amplification of 18s primer in *M.enterolobii* isoate from guava

guava orchards. As against other root-knot nematodes, *M. enterolobii* is capable of inducing severe root galling and plant decline). This is primarily due to its ability to overcome resistance genes, such as *Mi*- 1 gene in tomato (Kiewnick *et al*., 2009). Khan *et al*. (2001) reported several plant parasitic nematodes in the rhizosphere of healthy and wilted guava plants from India. Concomitant inoculation

of *Helicotylenchus dihystera* and *Fusarium oxysporum* showed synergistic interaction, particularly at 500 and 2000 nematodes/plant (along with fungus) as initial inoculum densities. In the studies on the characterization of *Meloidogyne* species from China, with isozymes and mtDNA, Meng *et al.* (2000) included two *M. enterolobii* populations from Hailan Island isolated from guava. This nematode species has also been identified from various parts of the world, such as France, USA (Florida), two greenhouses in Switzerland, Brazil and China (Blok *et al*., 2002; Brito *et al*., 2004; Kiewnick *et al*., 2009; Hu *et al*., 2011). Kiewnick *et al*., (2009) observed no differences at DNA level M. *enterolobii* and *M. mayaguensis*, hence the two species were synonymized, *M. enterolobii* (syn. *M. mayaguensis*).

Nematodes by themselves cause slow decline just as in dieback of citrus, however, occurrence of sudden death in guava must be due to association of rot/ wilt causing pathogens causing disease complex. Being an endoparasite, root knot nematode can be easily disseminated from infested areas (nursery sites) to nematode-free areas through soil and plant root material, soil and growing medium and infested planting material. Incidentally it was observed that guava root stocks used for grafting purchased from several nurseries located at Pochampalli, Periyakulam, Theni, Pudukkottai (Tamil Nadu) and

Table. 3. Dimensions of different populations of males of *M. enterolobii*

Dimensions (µm)	Original description (µm) *	Locations					
		Villupuram			Dindigul		
		Mean	SD	CV %	Mean	SD	CV %
Body length	1599.8 (1348.6-1913.3)	1584.33 (1408 - 1750)	316.74	19	1558.67 (1295- 1871)	361.69	9.0
Body width	42.3 (37.0-48.3)	44 (40 - 47.5)	3.96	8	43 (41- 45)	2	5.6
Stylet length	23.4 (21.2-25.2)	23.33 (22 -24.5)	1.25	5	23,6 (22- 25)	1.52	6.2
Stylet knob height	3.3 (2.6-3.9)	3.36 (2.6 -4)	0.70	11	3.6 (2.6-4.7)	1.05	2.5
Stylet knob width	5.4 (4.5-5.8)	5.1 (4.5 -5.8)	0.65	12	6.16 (4.5- 6.5)	1.52	3.5
DOGO	4.7 (3.7-5.3)	4.6 (4 - 5.5)	0.76	11	5 (4 -6.5)	1.32	2.8
Spicule length	30.4 (27.3-32.1)	30.3 (27 -33)	2.51	8	31 (28-33)	2.64	11

Original description* - Yang and Eisenback (1983)

DOGO-Dorsal Oesophageal Gland Orifice

Table 4. Dimensions of different populations of second stage juveniles of *M. enterolobii*

Dimensions (µm)	Original description (µm) *	Locations					
		Villupuram			Dindigul		
		Mean	SD	CV %	Mean	SD	CV %
Body length	436.6 (405.0-472.9)	448.33 (415 – 495)	41.63	16	443 (419 – 475)	28.84	15
Body width	15.3 (13.9-17.8)	12.83 (11 – 14)	1.60	12	12.66 (11 – 14)	1.52	15
'a'	28.6 (24.0-32.5)	25.5 (20 -32)	6.06	13	24.33 (22 - 26)	2.08	9.2
Stylet length	11.7 (10.8-13.0)	13 (12-13.5)	2.32	12	12 (11.5 - 14)	2.10	11
DOGO	3.4 (2.8-4.3)	3 (3 - 5.5)	1.20	8	3.5 (3.7- 4.5)	1.43	6.6
Tail length	56.4 (41.5-63.4)	52.8 (42 - 62.5)	10.29	11	48.66 (41 - 55)	7.09	15

Original description* - Yang and Eisenback (1983)
'a' – Length / maximum body width; DOGO-Dorsal Oesophageal Gland Orifice

Raipur (Chhattisgarh); grafted seedlings, and layered cuttings (ground layers) purchased by farmers from various nurseries harbored large numbers of root knot nematodes which were confirmed to be *M. enterolobii.* Hence it can be inferred that the planting material of guava (root stocks, grafts and ground layers) themselves are the carriers of this nematode which are in turn getting introduced into new orchards.

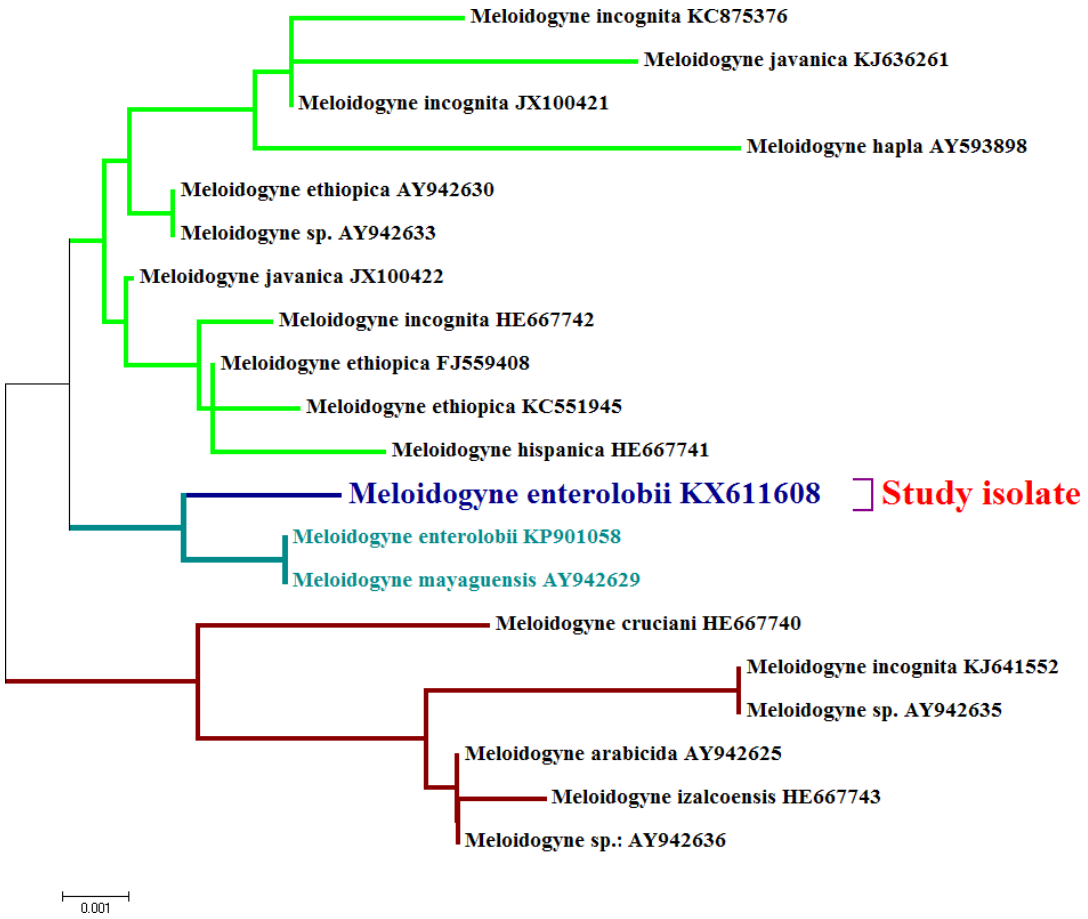

Fig.2. Phylogenetic tree

Further studies on etiology, pathogenicity, biology of this nematode and its interaction studies with other microorganism (*Fusarium* spp.) in guava are in progress at Tamil Nadu Agricultural University, Coimbatore.

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