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RESEARCH ARTICLE

Plant Pathology

Molecular identification of the plant parasitic nematode, *Meloidogyne* species in Sri Lanka

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Abstract: Root-knot nematodes, a major type of plant pathogen of the genus *Meloidogyne*, cause substantial losses in many agricultural crops. Therefore, the correct identification of *Meloidogyne* species is essential for the application of appropriate nematode control measures for effective crop recovery. In this study, molecular identification of *Meloidogyne* species was conducted on 27 root samples with abnormal root-galls, collected from nine different types of crops. The samples were collected from 21 different agricultural fields in seven provinces of Sri Lanka, between 2014-2018. The presence of juvenile stages in 25 samples (93%) was confirmed using microscopic analysis, and DNA was isolated from purified males, eggs, and juvenile stages for molecular identification. Universal primers MF/MR, which amplified the 500 bp of ribosomal DNA, confirmed the presence of *Meloidogyne* in 23 samples (85%). Primers C2F3/1108 and 194/195 were used to confirm species-specific amplification of mitochondrial and ribosomal DNA. Species *Meloidogyne arenaria*, *Meloidogyne enterolobii,* and *Meloidogyne thailandica* were identified in 22%, 67%, and 4% of the samples collected, respectively, either as single species populations or as mixed populations. Collectively, these species were detected in several economically important crops, including tomato, cherry tomato, spinach, guava, chili, cabbage, and capsicum. *Meloidogyne enterolobii* was the most prevalent species and was detected on guava, capsicum, and tomato in the Northwestern, Central, and Western provinces. To the best of our knowledge, this is the first study on molecular identification of *Meloidogyne* species in Sri Lanka and the first report of *M. enterolobii* and *M. thailandica* in Sri Lanka. This expands the previously known worldwide distribution of these species. The presence of *Meloidogyne* species associated with economically important crops was observed in all seven provinces surveyed, indicating an urgent need for the implementation of effective nematode management strategies.

Keywords: Agricultural crops, *Meloidogyne* species, molecular identification, plant pathogens, root-knot nematode.

INTRODUCTION

Root-knot nematodes (RKNs) belonging to the genus *Meloidogyne* are economically important plant endoparasites distributed worldwide (Karssen, 2002; Perry et al., 2009; Jones et al., 2013). They are polyphagous obligate pathogens and are considered the most successful plant- parasitic nematodes in the world (Goverse et al., 2000), causing extensive economic losses (Dhandaydham et al., 2008). *Meloidogyne* spp. have a complex life cycle that includes both free-living and parasitic stages (Vanholme et al., 2004). Root-knot nematodes have a wide host range and infect almost all species of higher plants (Karssen, 2002; Perry et al., 2009).

The first discovery of *Meloidogyne* species was reported in 1855 by Reverend Miles Joseph Berkely in the roots of glasshouse-grown cucumber (*Cucumis* spp.) in England (Hunt & Handoo, 2009; Mitkowski & Abawi, 2003; Perry et al., 2009). Since then, over 100 species have been classified under the genus *Meloidogyne*. Among them, *Meloidogyne incognita*, *M. javanica, M. arenaria, M. hapla, M. chitwoodi*, and *M. graminicola* are the most widely distributed and economically important *Meloidogyne* species (Mitkowski & Abawi, 2003). The history of RKN discovery in Sri Lanka began with the identification of *M. brevicauda* from mature tea plants

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by Loose in 1953 (Hutchinson & Vythilingam, 1963), and since then, very few investigations have focused on RKN infestations. In Sri Lanka, morphological studies have reported *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, *M. brevicauda*, and *M. graminicola* on economically important crops, including vegetables, fruits, and rice, causing significant yield loss (Ekanayake et al., 2001; Ekanayake & Toida, 1997; Nishantha et al., 2025; Nugaliyedda et al., 2001). RKNs can be identified using morphological or molecular-based studies (Brito et al., 2004; Devran & Sogut, 2009; Ekanayake & Toida, 1997). However, the identification of RKNs is challenging due to their wide host range, different habitats in their life cycle, unclear species boundaries, conservative morphology, polyploidy, and sexual dimorphism (Blok & Powers, 2009). In addition, morphological identification methods for RKNs are time-consuming and comparatively difficult. At the same time, classical taxonomic knowledge and skills are continuously decreasing in many taxa, such as nematodes (Oliveira et al., 2011; Wesemael et al., 2011). Therefore, molecular methods for identifying RKNs are considered more reliable because they provide consistent results (Powers et al., 2005). These methods also have advantages, such as the ability to analyze a large number of samples and as identification is possible even in the presence of a mixture of species (Oliveira et al., 2011). Molecular identification of RKN species based on PCR-based techniques uses various target regions, such as mitochondrial DNA (Blok et al., 1997, 2002; Tigano et al., 2005), satellite DNA (Randig et al., 2009), intergenic spacer region (IGS) (Adam et al., 2007) and internal transcribed spacer regions (ITS) from ribosomal DNA (rDNA), external transcribed spacer region (ETS) (Palomares- Rius et al., 2007), and sequence-characterized amplified region (SCAR) of genomic DNA (Tigano et al., 2010).

In Sri Lanka, RKN identification has been conducted based on their morphological characteristics, and there is no available literature on their DNA-based molecular identification (Ekanayake & Toida, 1997). Hence, this study aimed to develop a DNA-based molecular approach for the accurate identification of RKN that infect economically important crops in Sri Lanka, and thereby to identify the presence and distribution of *Meloidogyne* species in different locations in Sri Lanka. The nine tested crops, including tomato, guava, long bean, cherry tomato, chili, capsicum, okra, spinach, and cabbage, are widely grown in Sri Lanka and play an important role in food security, farmer income, and local vegetable and fruit supplies. Therefore, understanding RKN diversity in these crops is essential to mitigate yield losses and support sustainable agriculture.

MATERIALS AND METHODS

Sample collection

Root samples (n=27) infected by RKNs from nine different crop plants were collected from 21 agricultural farms in different localities in seven provinces of Sri Lanka from 2014 to 2018 (Table 1, Figure 1). The sample collection areas were decided according to information obtained from the Agriculture Departments of each province. Suspected crop plants showing above-ground symptoms such as wilting, stress, disease, poor growth, and chlorosis, in the selected geographical area were collected and examined for the presence of root galls in their root systems, which is the major below-ground symptom of RKN infection. Plants showing root galls in their root system were chosen as samples for the molecular identification of *Meloidogyne* species. The infected roots were transferred into clean polythene bags and labeled appropriately. Small amounts of water were added to the collected root samples to maintain moisture during transportation of collected samples from the agricultural farms to the Biotechnology Laboratory of the Department of Chemistry, University of Colombo. Samples were washed thoroughly with tap water and inspected for egg masses on their root surface using a hand lens. Root galls were opened with forceps and

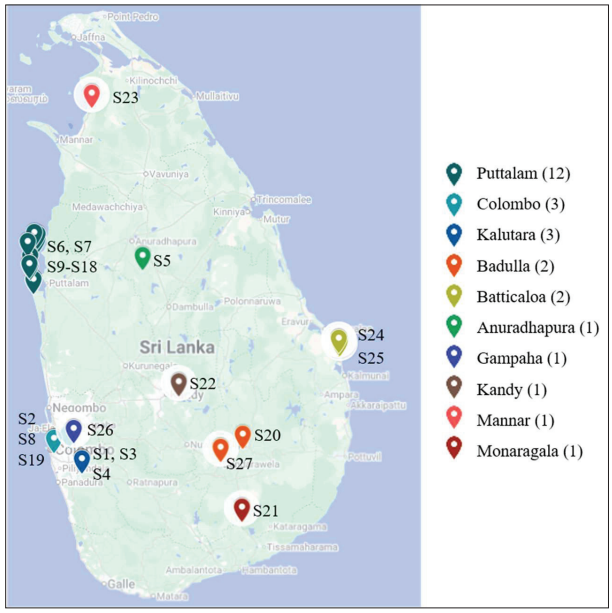

Figure 1: Sampling locations in Sri Lanka from which 27 root-knot nematode (RKN)–infected root samples were collected during 2014–2018

inspected for the presence of swollen adult females and egg masses. The females of RKNs were confirmed under a light microscope at 10×10 magnification. Roots were visually assessed to determine the gall index (GI) by counting the number of galls in each root system. The GI was scored on a scale of 0 to 5 scale, where 0 = no galls; 1 = 1-2 galls; 2 = 3-10 galls; 3 = 11-30 galls; 4 = 31-100 galls, and 5 = more than 100 galls per root system (Taylor & Sasser, 1978).

Table 1: Location and host crop of collected *Meloidogyne* infected samples.

Code	Location	District	Province	Field	Crop	Coordinates (latitude- longitude)
S1	Kananwila	Kaluthara	WP	F1	Tomato	6°45'44.9"N 80°03'43.5"E
S2	Narahenpita	Colombo	WP	F2	Tomato	6°53'60.0"N 79°52'39.5"E
S3	Kananwila	Kaluthara	WP	F1	Spinach	6°45'44.9"N 80°03'43.5"E
S4	Kananwila	Kaluthara	WP	F1	Tomato	6°45'44.9"N 80°03'43.5"E
S5	Mahailluppallama	Anuradhapura	NC	F3	Guava	8°06'42.4"N 80°28'01.1"E
S6	Kalpitiya	Puttalam	NW	F4	Tomato	8°13'41.1"N 79°45'31.0"E
S7	Kalpitiya	Puttalam	NW	F5	Guava	8°13'42.6"N 79°46'05.2"E
S8	Kaduwela	Colombo	WP	F6	Tomato	6°55'55.1"N 79°58'24.3"E
S9	Nirmalpura	Puttalam	NW	F7	Guava	7°57'15.2"N 79°44'28.7"E
S10	Kallepalliya	Puttalam	NW	F8	Guava	8°15'17.5"N 79°45'56.7"E
S11	Norochchola	Puttalam	NW	F9	Guava	8°15'53.9"N 79°44'48.4"E
S12	Eththale	Puttalam	NW	F10	Guava	8°06'55.2"N 79°43'05.4"E
S13	Nirmalpura	Puttalam	NW	F7	Long bean	7°57'15.2"N 79°44'28.7"E
S14	Nirmalpura	Puttalam	NW	F7	Tomato	7°57'15.2"N 79°44'28.7"E
S15	Palagama	Puttalam	NW	F11	Guava	8°03'42.9"N 79°42'33.5"E
S16	Illanthandiya	Puttalam	NW	F12	Guava	8°02'45.7"N 79°42'55.6"E
S17	Kandekuliya	Puttalam	NW	F13	Guava	8°12'18.4"N 79°41'58.4"E
S18	Palagama	Puttalam	NW	F11	Long bean	8°03'42.9"N 79°42'33.5"E
S19	Narahenpita	Colombo	WP	F2	Cherry tomato	6°53'60.0"N 79°52'39.5"E
S20	Passara	Badulla	UP	F14	Tomato	6°55'42.3"N 81°08'14.5"E
S21	Thanamalwila	Monaragala	UP	F15	Chili	6°26'28.6"N 81°08'03.5"E
S22	Pallekelle	Kandy	CP	F16	Capsicum	7°16'27.1"N 80°42'43.9"E
S23	Vellankulam	Mannar	NP	F17	Chili	9°11'13.2"N 80°07'52.8"E
S24	Kaluwanjikudy	Batticaloa	EP	F18	Okra	7°32'24.6"N 81°47'10.2"E
S25	Kaluwanjikudy	Batticaloa	EP	F19	Chili	7°33'56.2"N 81°46'59.6"E
S26	Biyagama	Gampaha	WP	F20	Spinach	6°57'55.9"N 80°00'19.5"E
S27	Bandarawela	Badulla	UP	F21	Cabbage	6°50'22.9"N 80°59'32.7"E

WP: Western Province, NC: North central Province, NW: Northwestern Province, CP: Central Province, UP: Uva Province, NP: Northern Province, EP: Eastern Province

Isolation of RKNs, DNA and PCR amplification

Purified males, eggs, and juvenile stages of *Meloidogyne* spp. were obtained using the incubation method (Young, 1954), which includes sieving and centrifugal flotation, to extract DNA using a mini-prep-column-based Nemato-Spin D™-Nematode DNA isolation kit (Ceygen Biotech, Sri Lanka) following the manufacturer's instructions. DNA from the isolated RKNs was used as the template for all PCR assays, which were carried out using five different primer pairs provided by New England Biolabs (UK) Ltd. Aliquots of 10 µL from the amplified PCR

products from each reaction were electrophoresed on a 1.0% (w/v) agarose gel stained with ethidium bromide at 80 V for 1 h.

DNA Sequencing and phylogenetic analyses

Randomly selected PCR-amplified products were sent for custom sequencing (Macrogen Inc, Korea). The raw sequences obtained were carefully observed, aligned using multAlin version 5.4.1 (Corpet, 1988), and manually edited using BioEdit version 7.0.9 (Hall, 1999) by correcting mismatches. The consensus sequences obtained were used to search and compare the sequences available in the GenBank, National Center of Biotechnology Information (NCBI) database, based on homology using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), to determine the identity of RKN isolates. In the phylogenetic analysis, the consensus sequences obtained for regions between COII and the large subunit of the rRNA gene (lrRNA) of the identified *Meloidogyne* species and homologous sequences retrieved from the GenBank database were aligned in ClustalX 2.1 (gap opening – 15; gap extension – 6.66). A total of 17 sequences, including five from the current study, were aligned for phylogenetic analysis. Some sequences of Sri Lankan *M. enterolobii* identified in this study were compared with each other and with those available in the GenBank to evaluate intra- and interspecies variations. The phylogenetic tree was constructed using COII sequences based on the Maximum Parsimony (MP) analysis (Tigano et al., 2005) in PAUP* version 4 software (Swofford, 2002) with a heuristic search performed by adding 100 random replicates and Tree Bisection-Recombination branch swapping (TBR). Gaps were considered as missing data. Bootstrap analysis was conducted with 1000 replicates to evaluate the support for each phylogenetic branch on the tree (Landa et al., 2008). For each dataset, both unweighted and weighted MP analyses were performed using PAUP* 4.0b 10 software, and support for each cluster was assessed using MP analysis with 1000 replicates. Consensus sequences obtained for the IGS/rDNA regions of the identified *Meloidogyne* species and homologous sequences retrieved from the GenBank database were aligned using ClustalW/MUSCLE MEGA version 5.0 (Tamura et al., 2011). A total of 15 sequences, including five from the current study, were aligned for phylogenetic analysis. Phylogenetic analysis was conducted using MEGA5 software with the Tamura-Nei model as the base substitution method and the Maximum Likelihood (ML) model as the statistical method with 1000 bootstrap replicates.

RESULTS AND DISCUSSION

Evaluation of RKN infestation in root samples collected from different crop fields

Galls were an integral part of the infected roots (Figure 2A). Hyaline to brown colored egg masses were detected on the surface of the galls, and when the galls were dissected, one to several whitish masses corresponding to adult females of RKNs embedded in the inner firmer tissues were detected either with the naked eye or using a hand lens. Direct microscopic examination of the collected root samples revealed the presence of females and egg masses of RKNs in 25 samples (93 %) of the 27

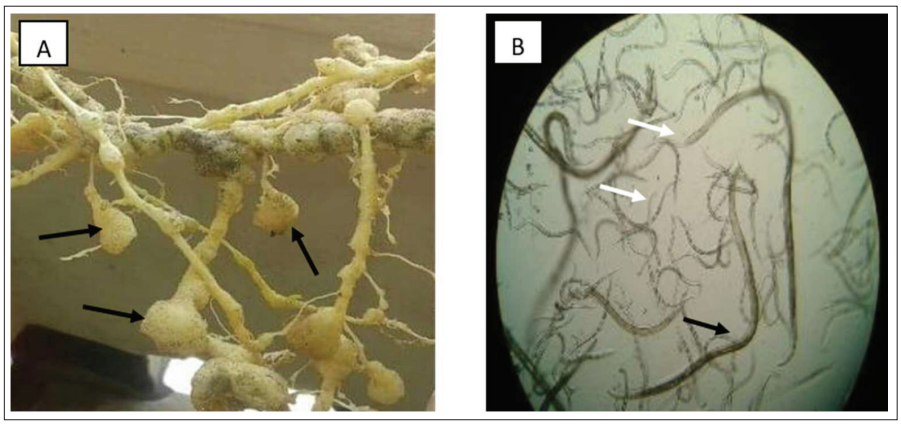

Figure 2: A. The gall morphology on the collected tomato plant roots. Galls indicated with arrows appear as integral parts of the tomato roots. B. Light micrograph of infected roots showing male (10 x 10) (arrowed in black) and juvenile stages (arrowed in white) of RKN.

samples collected. Microscopic analysis of RKN isolates also revealed the presence of juvenile stages, males, and eggs of RKNs in these samples (Figure 2B). Most of the collected root samples had a higher GI and clear large galls (Table 2). Crop-wise variations in the same field and area-based variations in the same crop were observed. The highest GI (5) was observed on guava, capsicum, and tomato. The microscopic observation of these samples showed that RKN infestations are scattered in all provinces tested.

Table 2: Gall indices of the *Meloidogyne*-infected root samples according to the method described by Taylor and Sasser (1978)

Gall index (GI)	RKN isolates
1	S14, S25
3	S2, S4, S5, S19, S20, S24, S27
4	S1, S3, S6, S7, S11, S16, S17, S21, S23, S26
5	S8, S9, S10, S12, S15, S22

GI: Gall index (0 = 0; 1 = 1-2; 2 = 3-10; 3 = 11-30; 4 = 31-100 and 5 = more than 100 galls per root system.

Molecular identification of the *Meloidogyne* genus

In Sri Lanka, several studies on RKN identification have been conducted (Ekanayake et al., 2001; Ekanayake & Toida, 1997; Nishantha et al., 2025; Nugaliyedda et al., 2001). However, all previous identifications were based on morphological characteristics. This may lead to the misidentification of *M. enterolobii* because of the overlap of its morphological characteristics with those of the dominant tropical species, since the perineal patterns of *M. enterolobii* are similar to *M. incognita* (Brito et al., 2004; Brito et al., 2015). Therefore, molecular methods have been utilized in most countries to identify *M. enterolobii* (Blok et al., 2002; Fargette & Braaksma, 1990). In this study, a simple, sensitive, and accurate diagnostic assay based on PCR was developed to identify *Meloidogyne* species collected from different crops in the region.

Amplification of the D2-D3 expansion domain of RKNs 28S ribosomal DNA with MF/MR universal primers resulted in a 500 bp fragment specific to the genus *Meloidogyne.* Out of 27 samples examined, 23 gave positive results for the amplification of the 500 bp fragment. The 500 bp amplified products for isolates S1 to S17 are shown in Figure 3.

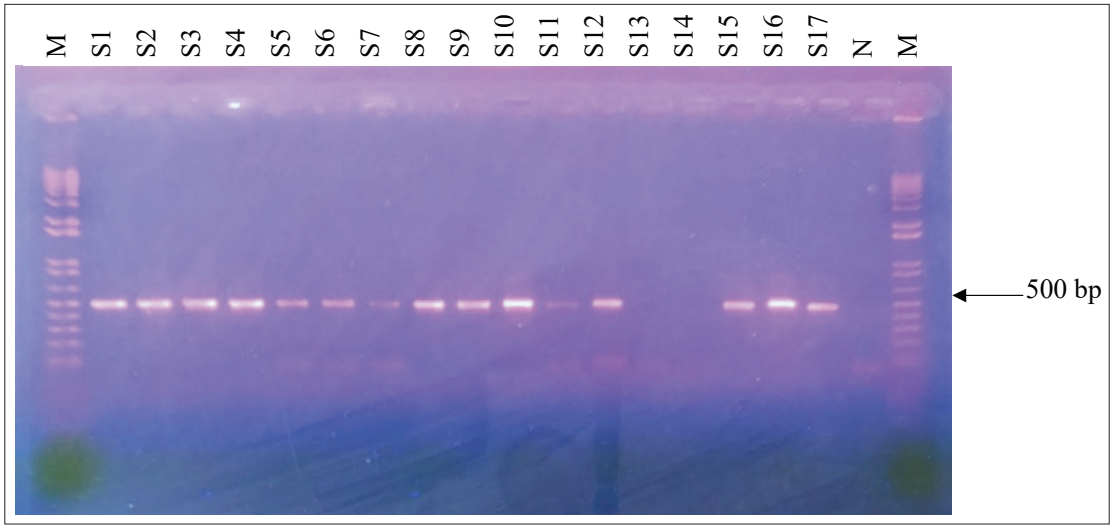

Figure 3: Gel image showing PCR amplification of nematode DNA samples with MF/MR primers. M, 1kb DNA ladder marker; S1-S17, amplified products of RKN DNA samples; N, Negative control.

However, isolates S13, S14 (Figure 3), S18, and S24 (data not shown) did not show any PCR amplification with *Meloidogyne* genus-specific primers. DNA sequencing of a 500 bp fragment of S1 (GenBank accession number MT254734.1) showed 100% similarity to the most common *Meloidogyne* species available in the GenBank database (accession numbers MN847618.1, MN661334.1, MN072431.1, etc.). Thus, samples

collected from all the districts surveyed were infected with RKNs, demonstrating the widespread occurrence of these RKN pathogens in Sri Lanka. These results further confirmed that, except for long bean and okra, all the other infected crops collected, including tomato, cherry tomato, spinach, guava, chili, cabbage, and capsicum were infected with RKNs, and need good management control of the pathogen.

Molecular identification of species of *Meloidogyne* based on mitochondrial DNA

Mitochondrial DNA is a potential source of genetic markers for species-level diagnostics of RKNs (Thomas & Wilson, 1991). *M. enterolobii* can be initially detected by producing a unique size of ~700 bp using the primers C2F3/1108 (Blok et al., 2002; Brito et al., 2004; Brito et al., 2015 Jeyaprakash et al., 2006; Powers, 2004; Tigano et al., 2005). According to previous studies, the 1100 bp amplicon for the COII/lrRNA region of the mitochondrial DNA is specific for *M. arenaria* (Powers & Harris, 1993).

The RKN DNA of 23 *Meloidogyne* genus positive isolates was amplified using primers C2F3/1108 for the region between COII/16S genes in mitochondrial DNA. Amplification of mitochondrial DNA with C2F3/1108 primers successfully discriminated the important species of RKNs in most of the infected samples. This resulted in three different amplified products in the 1100 bp, 700 bp and 520 bp for identification of *M. arenaria, M. enterolobii* and *M. daklakensis*, respectively (Figure 4A & B). *M. arenaria* was identified in isolates S1 & S2 with ~1100 bp DNA fragment (Figure 4A). *M. enterolobii* was identified in 18 isolates (S1, S5-S12, S15-S17, S19, S21-S23, S25, and S27) using the ~700 bp DNA fragment (amplification of S8 to S19 are shown in Figure 4B). Although the samples S3, S4, S20, and S26 were confirmed for the presence of *Meloidogyne* genus, no PCR amplification was obtained for species confirmation with C2F3/1108 primers. The RKN DNA of S6 and S19 isolates obtained from tomato and cherry tomato plants, respectively, showed ~520 bp band amplification, in addition to 700 bp amplified products (amplification of S19 is shown in Figure 4B).

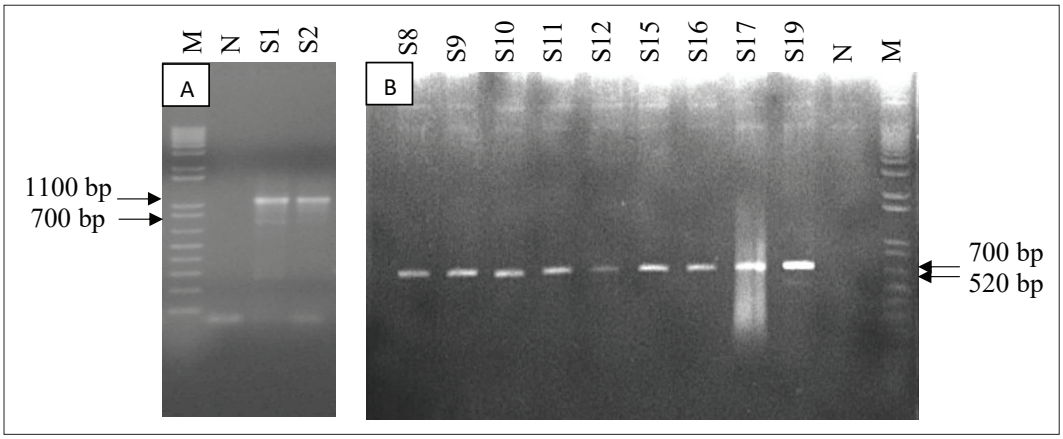

Figure 4: Gel image sAhowing amplified products of RKN DNA samples with C2F3/1108 primers. A. PCR amplification of nematode DNA samples S1 and S2. B. PCR amplification of nematode DNA samples S8-S12, S15-S17, S19. M, 1kb DNA ladder marker; N, Negative control.

The results obtained were further confirmed by DNA sequencing, and the sequencing result of the ~520 bp amplification product of the isolate S19 showed more than 70% similarity with the COII sequence of *M. daklakensis* (GenBank accession number. KU243337.1) (Table 3). The sequencing results of the 700 bp amplification products of isolates S21, S22, and S23 showed 100% similarity to *M. enterolobii* (Table 3). Moreover, the sequence of a 1100 bp amplicon of the S1 isolate showed more than 95% similarity to *M. arenaria* (Table 3).

Table 3: Identification of *Meloidogyne* species based on COII/16S rRNA BLAST results.

Isolate	Amplicon size (bp)	GenBank accession no. of the closest match	Closest match *Meloidogyne* spp.	Max score	e-value	Max identity (%)	GenBank accession no. of the isolate
S1	1100	KX983450.1	*M. arenaria*	729	0	95.65	MT741800
S19	520	KU243337.1	*M. daklakensis*	226	1e-54	71.22	MT741801
S21	700	MN269947.1	*M. enterolobii*	1081	0	100	MT741794
S22	700	MN269947.1	*M. enterolobii*	1157	0	100	MT741795
S23	700	MN269947.1	*M. enterolobii*	1139	0	100	MT741796

Globally, two genotypes of *M. arenaria* have been discovered, as reflected by amplicons of two different sizes from the analysis of the COII/lrRNA region. For example, Han et al. (2004) reported 1100 bp amplicons for RKN collected from the USA, 1700 bp amplicons for RKN collected from Japan and Korea, and both sizes of amplicons for samples collected from China, indicating the presence of both *M. arenaria* genotypes. However, in the present study, only one PCR product of 1100 bp was obtained with C2F3/1108 primers for the DNA of two isolates (S1 and S2) (Figure 4A), reflecting the presence of only one genotype of *M. arenaria* in Sri Lanka. This result agrees with previous studies that reported a 1100 bp amplicon for the COII/lrRNA region of *M. arenaria* (Powers & Harris, 1993).

Although the primer pair C2F3/1108 was stated as good enough to identify various RKN species in previous studies (Powers et al., 2005; Powers & Harris, 1993), no PCR amplification was obtained for samples S3, S4, S20, and S26, which were previously confirmed for the presence of the *Meloidogyne* genus. Several studies have previously reported the absence of PCR products with C2F3/1108 for species *M. arenaria*, *M. javanica*, and *M. incognita* (Devran & Sogut, 2009; Joseph et al., 2016; Mwesige et al. 2016). Therefore, in previous surveys, to distinguish between these sibling RKN species and to minimize false identifications, multiple molecular characteristics have been examined using different primer sets. In the present study, pale ~520 bp bands were obtained with the primer set C2F3/1108 for the S6 and S19 RKN isolates (Figure 4B). Sequencing results of these amplification products were not satisfactory despite repeated trials, which prompted the use of the primer set 194/195 for the exact identification of RKN present in these samples.

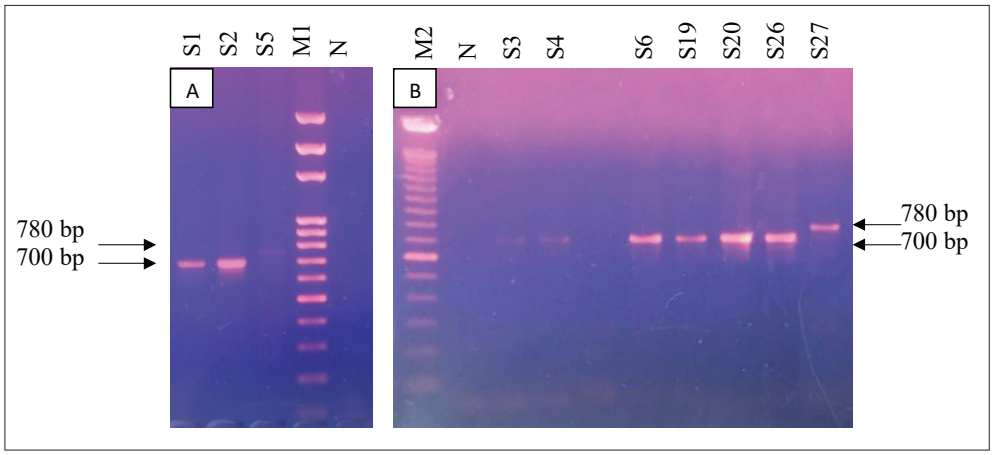

Figure 5: Gel image showing amplified products of RKN DNA samples with 194/195 primers. A. PCR amplification of nematode DNA samples S1, S2 and S5. B. PCR amplification of nematode DNA samples S3, S4 and S6, S19, S20, S26 and S27. M, 1kb DNA ladder marker; N, Negative control; M2, 100 bp ladder marker.

Molecular identification of the *Meloidogyne* species based on ribosomal DNA

The primer set 194/195 utilizes the sequence divergence exhibited by RKNs between the 5S-18S genes of the IGS-2 region of rDNA for species discrimination. Adam et al. (2007) reported an amplification product of 650-700 bp for *M. hapla*, 780 bp for *M. enterolobii* and 720 bp for the closely related tropical species *M. arenaria*, *M. incognita*, and *M. hapla.* Amplification of the IGS-2 region of ribosomal DNA with 194/195 primers yielded ~700 bp amplicons specific for *M. arenaria* and *M. hapla* for S1 and S2 isolates (Figure 5A), which produced 1100 bp size amplicons with C2F3/1108 primers. Samples S3, S4, S20 and S26, which showed no PCR amplifications with C2F3/1108 primers, showed 700 bp amplicons with 194/195 primers (Figure 5B). S6 and S19 isolates which produced multiple PCR products with C2F3/1108 primers also produced ~700 bp amplicons with 194/195 primers (Figure 5B). The 780 bp amplicon specific for *M. enterolobii* was obtained for the DNA of isolate S5 (Figure 5A) and S27 (Figure 5B). However, the 780 bp amplicons expected for the S1, S6, and S19 isolates were not discernible from the 700 bp amplicons. Selected PCR amplified products with 194/195 primers were sequenced and sequencing results revealed that the species responsible for samples S2, S3, S4 and S6 with ~700 bp products is *M. arenaria*. However, the S20 sample with ~700 bp product showed 100% identity for *M. thailandica* and ~780 bp product obtained for S5 showed 100% identity to *M. enterolobii* (Table 4).

Table 4: Identification of *Meloidogyne* species based on based on rDNA-IGS2 BLAST results.

Isolate	Amplicon size (bp)	GenBank accession no. of the closest match	Closest match *Meloidogyne* spp.	Max score	e-value	Maximum identity (%)	GenBank accession no. of the isolate
S2	700	U423442.1	*M. arenaria*	1175	0	100	MT741790
S3	700	GQ395518.1	*M. arenaria*	1130	0	100	MT741791
S4	700	U423442.1	*M. arenaria*	1047	0	100	MT741792
S6	700	GQ395518.1	*M. arenaria*	541	2e-154	97.82	MT741793
S20	700	HF568829.1	*M. thailandica*	1119	0	100	MT741789
S5	780	KP411228.1	*M. enterolobii*	1304	0	100	MT741788

Most of the RKN species relevant to the 520 bp size amplicon for the COII/lrRNA region displayed a length polymorphism between 5S-18S genes with the primer set 194/195 enabling their differentiation. Adam et al. (2007) reported a 700 bp amplicon for *M. hapla* and 1600-1700 bp products for *M. fallax* and *M. chitwoodi* with the primer set 194/195. Therefore, in the present study, the ~700 bp amplicon obtained for the DNA of isolate S6 with the primer set 194/195 was expected to be due to *M. hapla*. However, Adam et al. (2007) stated that the primer set 194/195 produces very similar sizes of PCR products for tropical species (*M. arenaria*, *M. javanica*, and *M. incognita*) and *M. hapla* (720 bp and 700bp, respectively) often leading to misidentification. In accordance with this statement, sequencing analysis of the ~700 bp amplicon obtained for the DNA of isolate S6 with the primer set 194/195 showed that the species was *M. arenaria.* These results indicated the importance of utilizing multiple gene targets to accurately identify *Meloidogyne* species.

Furthermore, this study clearly showed that amplicon sizes alone are incapable of differentiating closely related tropical RKN species *M. incognita*, *M. javanica*, and *M. arenaria*, which produce ~720 bp size amplicons, and *M. hapla*, which produces 650-700 bp amplicons. Therefore, the incorporation of additional genetic markers is important to differentiate sibling RKN species.

Confirmation of *M. arenaria* species with Far/Rar species specific SCAR primers

The isolates S1, S2, S3, S4, S6, S19, S20, and S26, which showed *M. arenaria* specific amplification with 194/195 primers, were further analyzed with *M. arenaria* specific Far/Rar SCAR primers. Except for S20, all isolates checked with Far/Rar confirmed the presence of *M. arenaria* producing a 420 bp amplification. PCR amplification of the 420 bp specific for the S26 *M. arenaria* isolate is shown in Figure 6 and for further confirmation, sequencing was carried out. The sequencing analysis

also showed 100% identity to *M. arenaria* isolates from Indonesia (Accession number. KP234264) in the GenBank database.

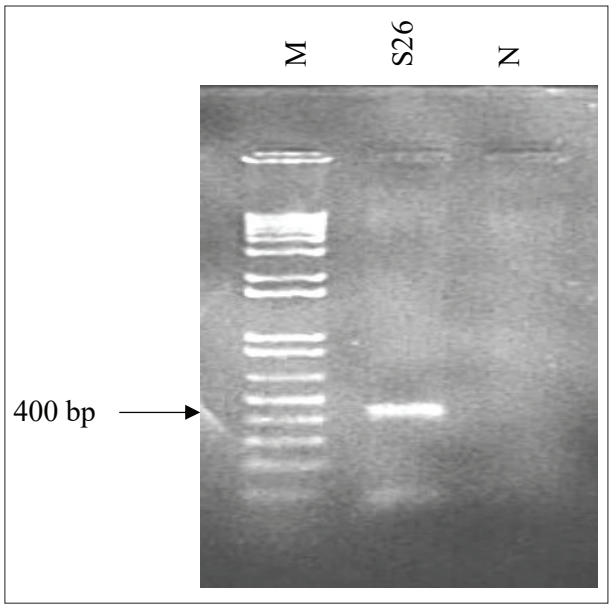

Figure 6: Gel image showing amplified product of RKN DNA sample S26 with *M. arenaria* specific Far/Rar primers. M, 1kb DNA ladder marker; N, Negative control.

No PCR amplification was observed for the S20 isolate with SCAR primers. However, isolate S20 produced an ~700 bp amplification product with 194/195 primers (Figure 5). Although isolates S1, S2, S3, S4, S6, S19, and S26 produced an ~700 bp fragment with 194/195 primers confirming the presence of *M. arenaria* with the sequencing results, isolate S20 showed the presence of *M. thailandica* (Table 4). Altogether, our results emphasize the importance of considering both PCR amplification and sequencing results to confirm the identification of different species in the *Meloidogyne* genus**.**

Confirmation of the *M. enterolobii* species with MeF/MeR species specific primers

The 18 RKN DNA samples (S1, S5-S12, S15-S17, S19, S21-S23, S25, and S27) that produced ~700 bp amplification products with C2F3/1108 primers, were further analyzed with *M. enterolobii* specific MeF/MeR primers that amplify a portion of the rDNA-IGS2 region. The presence of *M. enterolobii*, was confirmed by ~240 bp PCR products for the DNA of all RKN isolates tested. Isolates S7, S8 and S9 were sequenced and the sequencing analysis of the ~240 bp PCR products (Figure 7) obtained for S7 (GenBank accession number. MT741798), S8 (GenBank accession number. KY551570.1), and S9 (GenBank accession number. MT741797) showed 100% homology for the intragenic spacer region 2 (IGS2) of *M. enterolobii* isolates from Taiwan (Accession number. KP411228.1), Oman (Accession number. KM008548.1), China (Accession number. JN005846.1), and Switzerland (Accession number. GQ395524.1) in the GenBank database.

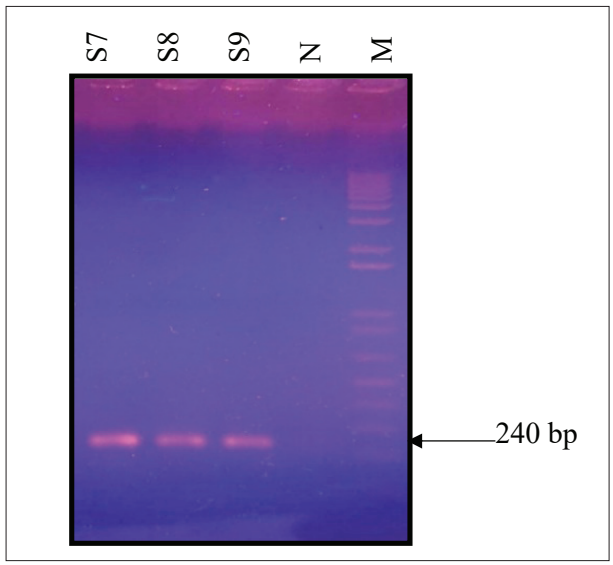

Figure 7: Gel image showing amplified products of RKN DNA samples S7, S8 and S9 with *M. enterolobii* specific MeF/MeR primers. N, Negative control; M, 1kb DNA ladder marker.

M. enterolobii was the most prevalent species, detected in 18 of 23 isolates (78.3%) positive for *Meloidogyne* genus-specific primers, occurring alone in 15 isolates and in mixed populations in 3 isolates. This is the first report of detecting *M. enterolobii* within Sri Lanka and our results further confirmed the presence of this very aggressive species in all the studied provinces in Sri Lanka. However, previous surveys conducted by Lamberti et al. (1987) and Ekanayake & Toida (1997), based on morphological characters, identified them as *M. incognita* and *M. arenaria*, which they reported as the dominant species infesting several vegetable crops in Sri Lanka. Morphological characters can always lead to misidentification of *M. enterolobii* due to the overlap of

its morphological characters with those of the dominant tropical species, i.e., perineal patterns of *M. enterolobii* are similar to *M. incognita* (Brito et al., 2004; Brito et al., 2015). *M. enterolobii* is considered the most aggressive emerging species (Brito et al., 2004) primarily due to its virulence against several root knot nematode resistance genes, such as the *Mi-1* gene in resistant tomato (Fargette, 1987). Due to this resistance breaking ability, this nematode displays a higher pathogenicity and reproductive potential than any other tropical species (Brito et al., 2004).

Phylogenetic analysis of *Meloidogyne* populations based on COII/16S rRNA sequences

The phylogenetic trees were constructed to study the diversity among RKNs in Sri Lanka. The phylogram obtained through maximum parsimony analysis contained several clades and sub-clades, which were separated based on Bootstrap Support (BS) values such as (i) *M. incognita*, *M. arenaria*, *M. javanica*, *M. thailandica*, and *M. floridensis* populations (BS=100%), (ii) *M. chitwoodi* and *M. fallax* populations (BS = 100%), (iii) *M. hapla* and *M. haplanaria* populations (BS = 100%), (iv) *M. enterolobii* populations (BS = 100%), and (v) *M. daklakensis* population (BS=82%) (Figure 8A). The outgroup sequence (GU177865.1) from the closely related *Bursaphelenchus* genus formed a separate clade. Putatively identified nematode populations from the current study grouped correctly with those identified from several parts of the world. The isolate S1 previously identified as *M. arenaria* was grouped with the species of clade (i). As shown in the phylogram, GenBank and Sri Lankan *M. enterolobii* populations (S21, S22, and S23) formed a monophyletic clade with a high bootstrap value (100%). Moreover, the sequence from the S19 isolate clustered with *M. daklakensis* in clade (v), but with a low bootstrap value of 57%.

The phylogenetic tree based on the COII-16S rRNA region showed no significant differences between the COII sequences of the Sri Lankan population and those retrieved from the GenBank, NCBI database for *M. enterolobii*, proving the lack of diversity among *M. enterolobii* populations from various geographic regions (Figure 8A). The homogenous nature of *M. enterolobii* populations identified through phylogenetic analyses suggested the applicability of similar control methods in farms infected with *M. enterolobii.* As evidenced by the bootstrap values, the clade containing *M. enterolobii* (100%) was closer to the tropical species clade comprising *M. javanica*, *M. incognita*, *M. arenaria*, *M. thailandica*, and *M. floridensis* (100%) than to the temperate species clade including *M. hapla*, and *M. haplanaria* (84%). This result is consistent with McClure et al., (2012) who reported that *M. enterolobii* is closely related to tropical species than to temperate ones based on topology of the phylogenetic tree constructed using mitochondrial DNA sequences. This close relationship can be explained by the mode of reproduction as both *M. enterolobii* and the majority of tropical species are mitotically parthenogenetic *Meloidogyne* species (Tigano et al., 2005).

Phylogenetic analysis of *Meloidogyne* populations based on IGS/rDNA sequences

Since ML has been previously proven in several studies as the ideal model that gives the best phylogenetic tree for IGS analysis, it was chosen to construct phylogenetic relationships based on IGS-rDNA region (Onkendi & Moleleki, 2013). Putatively identified nematode populations from the current study grouped correctly with those identified from several parts of the world (Figure 8B).

The phylogram obtained through maximum likelihood analysis contained several clades and sub-clades which were separated based on BS values: (i) *M. incognita*, *M. arenaria*, *M. javanica*, *M. thailandica*, and *M. floridensis* populations (BS=78%), (ii) *M. chitwoodi* and *M. fallax* populations (BS = 100%), (iii) *M. hapla* population (BS = 100%), (iv) *M. enterolobii* populations (BS = 100%), and (v) *M. haplanaria* population (BS=82%).

The isolates S2, S3, and S4 previously identified as *M. arenaria* and S20 previously identified as *M. thailandica* were grouped with the species of clade (i), and the isolate S5, which was identified as *M. enterolobii*, was in clade (iv).

Phylogenetic analysis based on the IGS-rDNA region resulted in grouping most of the tropical species into one clade that is distinct from the clade that grouped the temperate species. The clade with tropical species contained closely related apomictic species such as *M. arenaria*, *M. javanica*, and *M. incognita*, along with *M. thailandica* and *M. floridensis*, which are common in warmer climates. Clades with temperate species contained the automictic species *M. chitwood*i and *M. fallax* while *M enterolobii* formed an independent clade that is closer to the clade with tropical species than to the temperate ones. Phylogenetic analysis clearly separated the facultative parthenogenetic species *M. hapla* by forming an independent group between the automictic and apomictic species, but closer to the clade with automictic

ones. This agrees with previous studies that suggested *M. hapla* is closely related to automictic species than to apomictic species based on the percentage nucleotide base substitution model using total genomic DNA (Castagnone-Sereno et al., 1999; Onkendi & Moleleki, 2013). According to the phylogram constructed based on the IGS-rDNA, all the *Meloidogyne* species identified and used in the analysis were grouped closely into the clades with their respective *Meloidogyne* species, which were identified from several parts of the world. Altogether, the results presented in this study validate a basis to formulate alternative methods for RKN control.

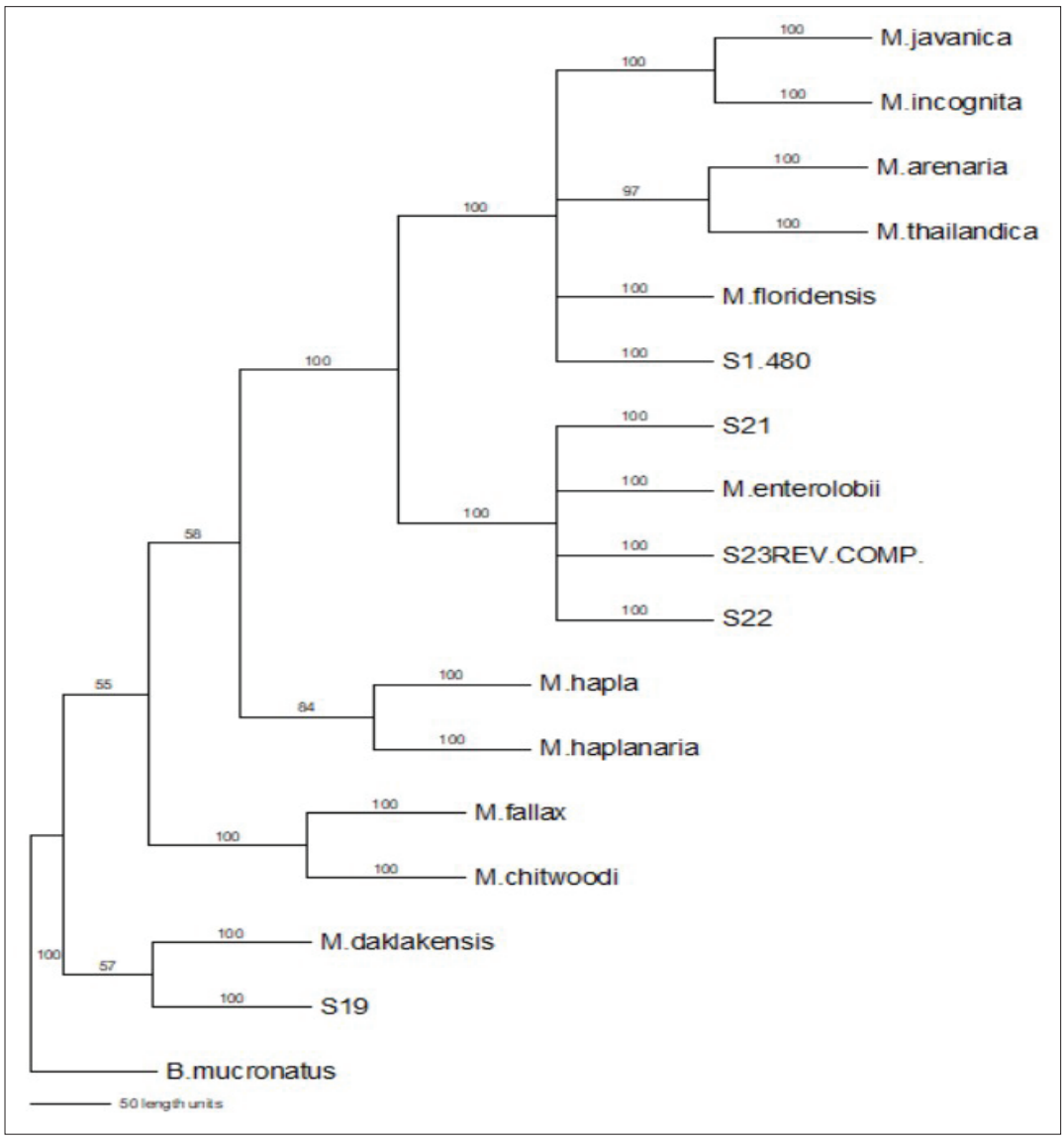

Figure 8A: Maximum parsimony tree based on mitochondrial COII-16S rRNA sequences of *Meloidogyne* species, preliminarily identified in the present study, and reference sequences obtained from the GenBank, NCBI database. S1, S19, S21, S22 and S23 represent the consensus sequences obtained in this study; the remaining sequences were retrieved from the GenBank/NCBI database as references. The analysis included 1,000 bootstrap replicates. The bootstrap support value of each clade is indicated on the nodes. *Bursaphelenchus mucronatus* (GU177865.1) was used as the outgroup in the alignment and construction of the phylogram.

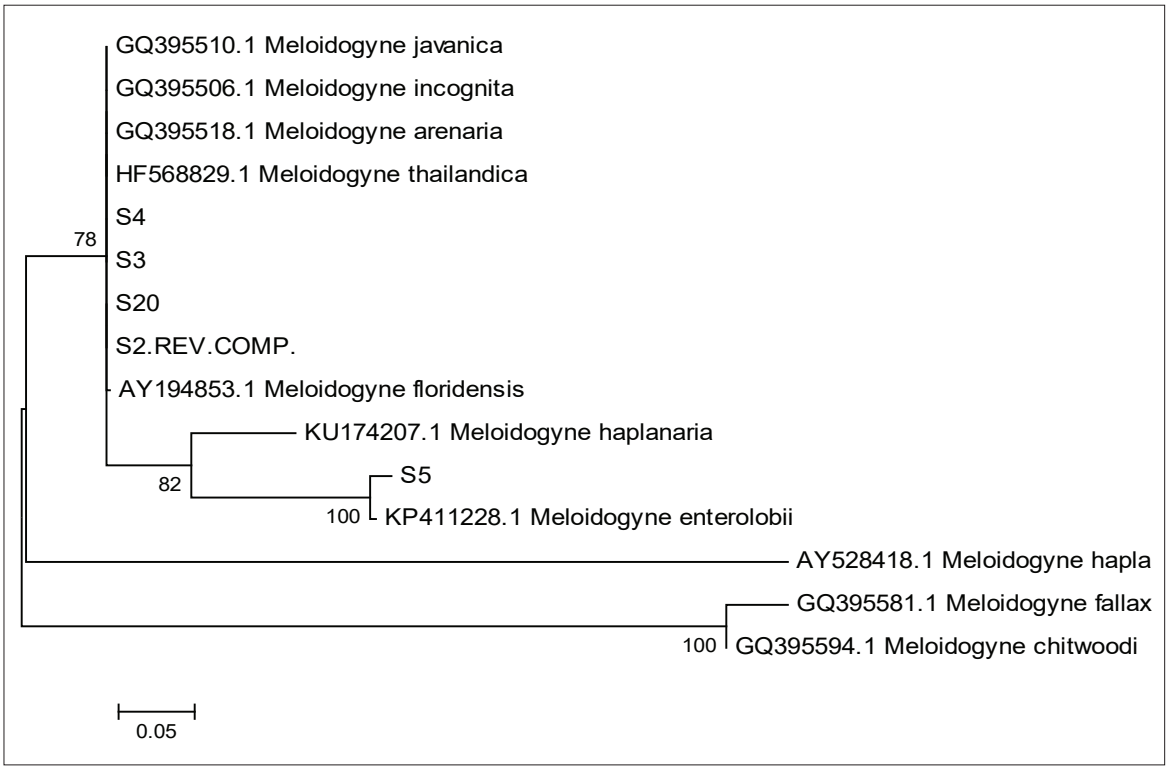

Figure 8B: Maximum likelihood tree constructed using the Tamura-Nei model based on the alignment of IGS/rDNA sequences of *Meloidogyne* species identified in this study, along with reference sequences from the GenBank, NCBI database. The sequences S4, S3, S20, S2 and S5 were obtained in this study. The analysis involved 1,000 bootstrap replicates and the bootstrap support value for each clade is indicated on the nodes. Since the 194/195 primers used in the IGS/rDNA analysis are specific to *Meloidogyne* species, no outgroup was included in the phylogram.

CONCLUSION

To the best of our knowledge, this is the first study of molecular identification of *Meloidogyne* species in Sri Lanka. In this study, *Meloidogyne* species *M. arenaria*, *M. enterolobii*, and *M. thailandica* were identified across multiple economically important crops including, tomato, cherry tomato, spinach, guava, chili, cabbage, and capsicum. Furthermore, *M. enterolobii* and *M. thailandica* were recorded for the first time in Sri Lanka through this study. The distribution of root-knot nematodes across all the provinces included in this investigation shows their threat to agriculture. These findings indicate the importance of accurate molecular identification of plant-pathogenic *Meloidogyne* species to support the implementation of effective root-knot nematode management strategies in Sri Lanka. Internal quarantine, varietal screening and prophylactic treatments are important in safeguarding the economically important crops susceptible to RKN species through in-depth studies.

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