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Morphological and molecular characterization of *Meloidogyne* species (Root Knot Nematodes) Associated with the *Sorghum bicolor* crop

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Abstract
Background: Though nematode damage to *Sorghum bicolor* crops in Telangana has occasionally been collected and suspected, no research on the morphology and molecular characterization of *Meloidogyne* species (root-knot nematodes) has yet been published. The aim of the research was to describe the phenotypic and genetic characteristics of *Meloidogyne* species related to the *Sorghum bicolor* crop in Telangana, India.
Results: From 60 different fields, a total of 60 rhizosphere soil samples with roots were taken, and composite soil samples were processed. Morphological characteristics like head shape, stylet morphology, perennial patterns, and body shape were used for identification. Eight (24.24%) samples out of the 33 *Meloidogyne*-like species detected by optical microscopy demonstrated amplification of a 1.7 kb (kilobase) fragment by polymerase chain reaction, confirming the presence of *M. incognita* and *M. javanica*. A two-banded pattern was created in *M. javanica* following *Hinf I* digestion of the 1.7-kb fragment as opposed to a three-banded pattern in *M. incognita*. However, no fragments representing other *Meloidogyne* species were observed in our study.
Conclusions: We recommend the integrative taxonomic approach for the precise diagnosis of *Meloidogyne* species as well as morphological and molecular data must be shared to improve the resolution and dependability of studies on Etiology and phylogenetics.

Keywords: *Sorghum* crop, Root-Knot Nematode (RKN) species, *Meloidogyne* species, Morphological approach, Polymerase Chain Reaction (PCR)

Introduction
The maturity of root-knot nematodes belongs to the genus *Meloidogyne*. It has about 100 species that have been described (Karssen *et al.*, 2013) [23]. All the plant-parasitic nematodes, its members are without a doubt the most extensively dispersed (Sasser, 1977) [35]. The genus has entered more disquisition than any other plant-parasitic nematodes including the cyst-forming nematodes, primarily because of its expansive range and economic significance (Sasser & Carter, 1982) [13]. The capability of the pestilent larva to enter the root tip and move across cells to the vascular cylinder is a major factor in the success of parasitism. Due to the different climates of the Telangana region, the root-knot nematodes (*Meloidogyne* spp.) constitute a major aboriginal problem for numerous crops tended in Telangana, India. *Meloidogyne* incognita, M. *javanica*, M. *arenaria*, and M. *hapla* account for the maturity of crop losses caused by root-knot nematodes. A comprehensive study of roughly 1300 *Meloidogyne* populations from 70 countries representing major food-producing regions of the world, set up at least one of these four species in 95% of the samples (Carter & Sasser., 1982) [13]. All four species have a universal distribution, but the first three are generally temperature confined to an area between latitudes 40° N and 33°S.
Several writers have argued the relevance of *Sorghum bicolor* as a host of *Meloidogyne* spp. (Rodríguez-Kabana & Touchton., 1984; Birchfield., 1983) [33, 8]. One of the ten *Sorghum* cultivars grown in Louisiana is only relatively resistant to *M. incognita*, while the other nine are highly vulnerable (Birchfield, 1983) [8]. Others demonstrated that, despite some reproduction, *Sorghum* was a poor host for *M. incognita* (Orr & Morey, 1978) [29]. The response of *Sorghum* to populations of *Meloidogyne* spp. may vary by region due to variations in the host compatibility of graminaceous crops (Baldwin & Barker., 1970) [38].

In Kolar and Bagepalli, Karnataka, India, cucumber fields, the root-knot nematode *M. incognita* was assessed for its impact on yield losses. Both areas had significant levels of root-knot nematode incidence and severity (Nagesh *et al.*, 2005) [26]. Numerous researchers have observed that the host plant species as well as the quantity and species of nematodes play a role in the size and general appearance of galls (Dropkin., 1955) [15]. Their identification at the species level continues to be extremely difficult for many diagnosticians despite the numerous studies about their biology and taxonomy (Blok & Powers., 2009) [9], mostly due to their extremely low inter-specific morphological variation (Jepson, 1987) [22]. A key area of taxonomic study in nematology has been the identification of species. Nematode taxonomic investigations typically involve counting, measuring, and examining various traits under various types of microscopes. Differential host testing and microscopic examination of the perineal patterns of adult females are frequently used to try and identify the most prevalent and agriculturally significant species. Characterization of a species is mostly based on the morphological characteristics of males, adult females, and second-stage juveniles. Juveniles are often identified through a combination of meticulously examining morphological traits (Eisenback *et al.*, 1981) [17] and time-consuming reproduction on a variety of host plants. The biochemical and molecular approach for taxonomic studies involves techniques borrowed from chemists and molecular biologists, e.g., enzymatic assays, determination of the composition of various building blocks, and DNA technology. Researchers observed qualitative changes when comparing the lipid and fatty acid composition of *M. incognita* and *M. arenaria* females and eggs (Krusberg *et al.*, 1973) [25]. *Meloidogyne* species have been the subject of numerous investigations involving a variety of hosts and geographical areas. Most of these techniques result in a poor percentage of detection and frequently take a lot of time and money to complete. As an alternative to or addition to these processes, species-specific DNA hybridization probes have been sought after (Powers., 1992, Burrows., 1990) [30, 11].

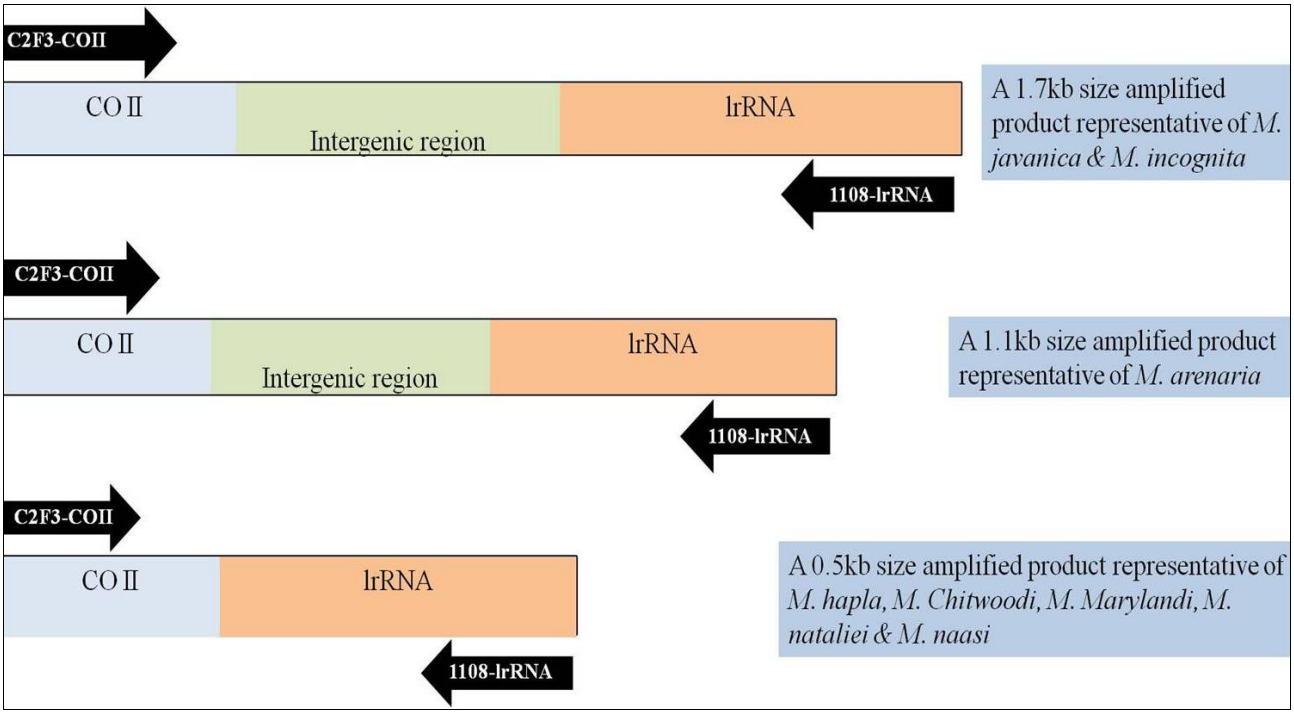

Fig 1: The *Meloidogyne* mitochondrial genome's PCR primer-binding regions are presented schematically. Primer #1108 anneals about 450 base pairs (bp) downstream of the large subunit of the ribosomal RNA (lrRNA) gene, while primer #C2F3 anneals to the coding strand of the cytochrome oxidase subunit II gene (COII).

Several polymerase chain reaction (PCR) or sequence-dependent molecular biology approaches and spectroscopic techniques have been developed for nematode taxonomic investigations to get over these limitations in conventional morphology-based analysis (Abebe *et al.*, 2011; Barthès *et al.*, 2011) [1, 6]. The PCR, which was developed more subsequently, offers a highly sensitive approach to DNA amplification and detection. To identify the five main *Meloidogyne* species, Powers and Harris (1993) [31] used PCR to amplify mitochondrial DNA (mtDNA) from individual *Meloidogyne* J_2 and eggs (Harris et al,, 1990). Studies on the morphology and molecular characterization of *Meloidogyne* species (root-knot nematodes) related to the *Sorghum bicolor* crop are extremely rare. To comprehend the morphological and molecular characterization of *Meloidogyne* species (root-knot nematodes) connected with the *Sorghum bicolor* crop, further thorough investigations are required. Moreover, no attempt has been made to understand the *Meloidogyne* species associated with the *Sorghum* crop in India, more specifically in the Telangana region. As varieties of *Meloidogyne* species are affecting crops globally, understanding the morphological and molecular characteristics in association with different crops in different regions is needed. Therefore, regional-specific studies are very important to understand the damage caused to the *Sorghum* crop by a variety of *Meloidogyne* species. Therefore, this study aims to evaluate the morphological and molecular characteristics of *Meloidogyne* species associated with *Sorghum bicolor* (L.) Moench crop in Telangana, India. Further, we report the efficacy of PCR and conventional methods in the identification of *Meloidogyne* species associated with the *Sorghum* crop.

Table 1: The distribution of fields by District, the average number of *Meloidogyne*-like species and other nematode species per field, and the *Meloidogyne* species confirmed by PCR.

Agro-climatic Districts	Field Distribution (N, %; 60, 100)	Other nematode species (N=56)		*Meloidogyne* like species (n=33)		
		Mean	SEM	Mean	SEM	Species confirmed by PCR (%)
Mahabubabad	21 (35)	0.67	0.210798	0.52	0.202506	3 (37.5%) isolates with *M. incognita* and *M. javanica*
Ranga Reddy	20 (33.3)	1.05	0.40048	0.9	0.289347	5 (62.5%) isolates with *M. incognita* and *M. javanica*
Khammam	17 (28.3)	1.12	0.341733	0.24	0.136305	Nil
Sericella	2 (3.3)	1	0.999849	0	0	Nil
Overall observations		0.93	0.180868	0.55	0.12897	8 (24.24%)
One-way ANOVA (Between the Districts)						
Sum of squares		2.352		4.753		
DF Value		3		3		
Mean square		0.784		1.584		
F value		0.387		1.640		
Significance		0.763		0.190		

Note: The values were represented as Mean ± SEM, Abbreviations: N - Number, % - percentage, SEM - Standard Error of the Mean

Table 2 Details of morphological and morphometric variations of second stage juveniles (J_2) and mature *M. incognita* and *M. javanica* [N=8]

Characteristics of each stage	As per previous reports (Lengths in µm)		Current report (Lengths in µm)	
	M. incognita†	*M. javanica*§	*M. incognita*	*M. javanica*
Second stage juveniles (J_2)				
♀ body	NA	NA	671±130	674±110
♂ body	337-403 (371)	387-459 (417)	390±120	420±112
♀ stylet	13-16 (14)	14-18 (15)	20.5±0.5	18±3
♂ stylet	23.0-32.7 (25.0)	20.0-23.0 (21.2)	26±7	23±2
♀ tail	NA	NA	17±2	19±3
♂ tail	38-55 (46)	36-56 (49)	56±5	61±8
♀ tail hyaline	NA	NA	9±4	14±3
♂ tail hyaline	6.3-13.5 (8.9)	9-18 (13.7)	14±3	15±5
Adult stage				
♀ body	NA		702±124	701±112
♂ body	NA		345±130	437±120
♀ stylet	NA		22.5±0.5	20±2
♂ stylet	NA		30±7	24±2
♀ tail	NA		18±3	21±4
♂ tail	NA		50.5±4.5	55.5±17.5
♀ tail hyaline	NA		11±3	16±4
♂ tail hyaline	NA		13±4	17±5

Note: The data was represented in mean±standard deviation (SD) of second stage juvenile-like male and female, NA- Not available. †Williams (1973). §Williams (1972). Source: EPPO Bulletin, Volume: 46, Issue: 2, Pages: 190-201, First published: 19 July 2016 DOI: (10.1111/epp.12293). The mean was given in brackets.

Results

The study relied on 60 *Sorghum* fields from 4 agro-climatic districts of Telangana. A total of 56 other root-knot nematode species and 33 *Meloidogyne*-like species were noted. Out of 33 *Meloidogyne*-like species, a greater number were recorded from the fields of Ranga Reddy District (0.90±0.289) compared to other districts. A lower number of *Meloidogyne*-like species were recorded from the fields of Khammam (0.24±0.136) and Sericella (0.000±0.000) districts. Out of 56 other nematode species, high numbers were recorded in the fields of Khammam district (1.12±0.342) compared to other districts. A lower number of other nematode species were recorded in the agro-climatic fields of Mahabubabad district compared to other districts. The distributions of various types of nematodes between different districts were statistically insignificant. The district-wise distribution of fields, the mean number of *Meloidogyne*-like species and other nematode species per field, and the *Meloidogyne* species confirmed by PCR are given in Table 1. Schematic details of primer designing were given in Figure 1. A total of eight (n-8) *Meloidogyne*-like specimens were measured for morphological characteristics of female, male, and juvenile in this study. The details of morphological and morphometric variations of second-stage juveniles (J_2) and mature *M. incognita* and *M. javanica* are given in Table 2 **(**EPPO Bulletin, 2016) [18].

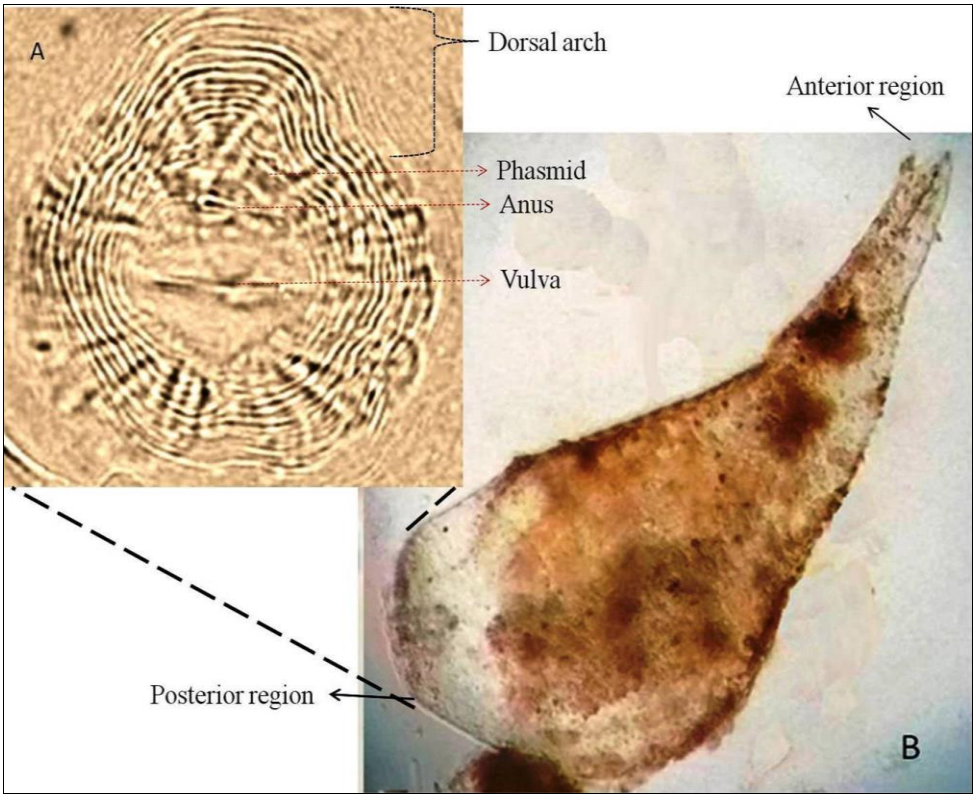

Fig 2: *Meloidogyne incognita* female whole body (B) perineal pattern (A)

***Meloidogyne* incognita**

Female: The body had an annulated cuticle and was formed like a pear with a circular posterior portion. The body was 702±124 µm in size. The head was properly positioned to the body. The stylet was 22.5±0.5 µm in length and was straight and slender. Transversely ovoid stylet knobs were present. The stylet knobs were 3-5 µm posterior to the entrance of the dorsal esophageal gland. With an oval valve, the median bulb measured 67-80 µm from the anterior end. As it approached the lining of the median bulb, the esophageal lumen lining grew and shrank. The neck measured 140-190 µm. The dorsal arch was medium-high to high, the apex of the dorsal arch was broadly rounded to square, and the junction between lateral lines was typically y-shaped (Figure 2). The perineal pattern exhibited a circular to oval shape. Phasmids had an indeterminate shape and a diameter of 1-2 µm. There was a 19-30 µm interphasmidial gap. The punctuation in the rectus was small and infrequent. Inconspicuous or forked striae served as indicators of the lateral lines. The tail end measured 18±3 µm. The anus was 10-27 µm posterior to the vulva, and the vulval slit measured 20-31 mm in length.

Fig 3: Second stage juvenile-like *M. incognita* male with a blunt tail. Note: Identify any internal structure or head shape.

Male: The body (345±130 µm) was vermiform. The head areas weren't distinguished by a high, spherical cap, but rather were in line with the body and correspond to those of a second stage juvenile. Stylets had enormous, ovoid knobs that sloped backward and were straight and 30±7 µm long. The stylet knobs were 3-6 µm posterior to the entrance of the dorsal esophageal gland. Between the median bulb and the tail, the lateral fields were isolated. A narrow isthmus followed a narrow medial bulb, which was narrow to elongate. From the anterior end, the excretory hole was 115-184 µm away. The tail was blunt and not annulated, measuring 50.5±4.5 µm in length (Figure 3). 24-34 µm long spicule arcuate were present. The gubernaculum short measured 6-9 µm. Unlike cloacals, phasmids had pore-like characteristics.

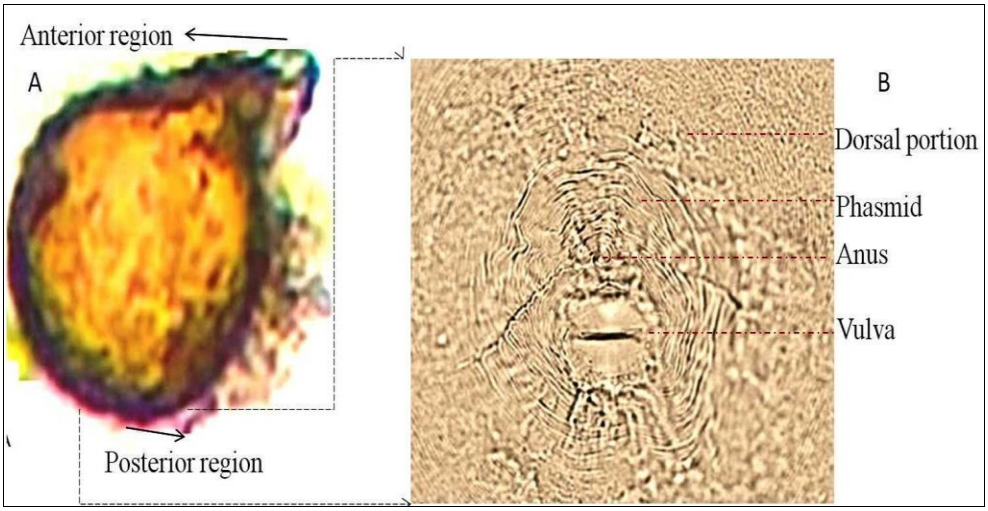

Fig 4: *Meloidogyne javanica* female whole body (A) and perineal pattern (B)

Second-stage juvenile: The body was vermiform, 390±120 µm long, narrow, tapering anteriorly and posteriorly, and straight ventrally. The head wasn't protruding and was hemispherical. The stylet was conical and 26±7 µm long. Stylet knobs had a big, backward-sloping design. The stylet knobs were 2-5 µm posterior to the entrance of the dorsal esophageal gland. An oval valve causes the medial bulb to expand. From the anterior end, the excretory hole was 63-91 µm away. The tail was conical in shape and 56±5 µm long, with a tapered termination.

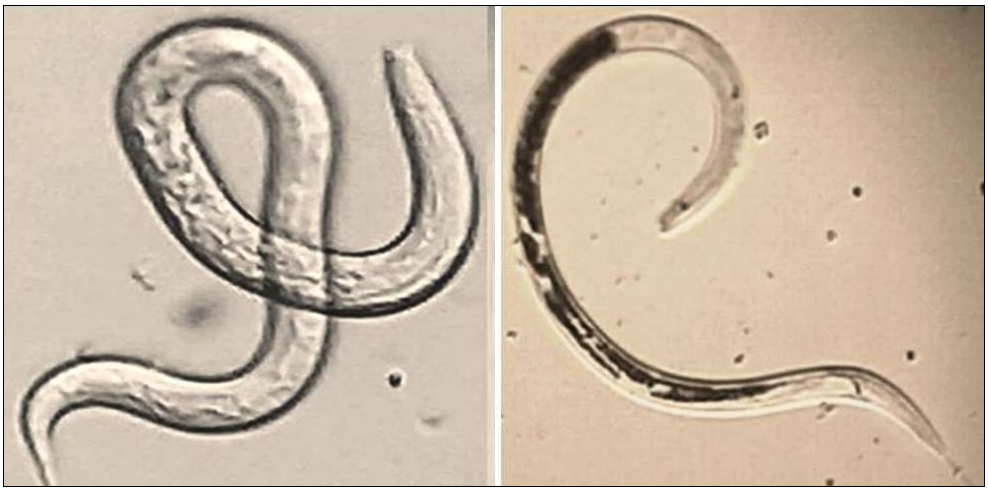

Fig 5: Second stage juvenile-like *M. javanica* male with sharp tail

Meloidogyne javanica

Female: The body had an oblong form. The body was 701±112 µm in size. The stylet measured 20±2 µm in length, with oval, backward-sloping basal knobs. The aperture of the dorsal oesophagus gland was 3-4 µm behind the stylet knobs. The medial bulb was spherical, muscular, and had an oval valve. It measured 66-87 µm from the anterior end. In certain instances, the oval median bulb lumen lining was directly in front of an expansion of the oesophageal lumen lining. The neck measured 135-196 µm. Round to oval perineal pattern, low dorsal arch, square to broadly rounded apex, and low to high inner striae above tail termination. Two lateral lines served as distinct lateral lines (Figure 4). Phasmids were tiny, with diameters of 1-2 µm. The interphasmidial distance ranged from 20 to 41 micrometers. The vulval slit was 12-25 µm anterior to the anus and 19-32 µm long.

Male: The vermiform body had a tapering posterior end and an anterior end that was rounded. The annulation of the cuticle was seen. The head region was continuous, rounded, and not asymmetrical. The body was 437±120 µm in size and correspond to those of a second stage juvenile. The stylet measured 24±2 µm in length, was straight, had a cone-shaped shaft, a knobbed tip, and was robust and transversely ovoid (Figure 5). The entrance of the dorsal

esophagus gland is located 3-5 µm behind the stylet knobs. A longer medial bulb was present. From the anterior end, the excretory hole was 112-179 µm away. The tail had a length of 55.5±17.5 µm, was tapering, and wasn't annular. The ventrally curved, 25-30 µm long spicule had a length. The gubernaculum was very small (7-8 µm). Unlike cloacals, phasmids had pore-like characteristics. Second stage juvenile: The body was vermiform in shape, slender, primarily ventrally curled, and 420±112 µm long. The head kept going and was rounded. The stylet measured 23±2 µm in length. The aperture of the dorsal oesophagus gland was 2-4 µm behind the stylet knobs. The medial bulb had an oval valve and was ovoid. From the anterior end, the excretory hole was 69-91 µm away. The tail was tapering, conical-shaped, long, and narrow, measuring 61±8 µm (Table 2).

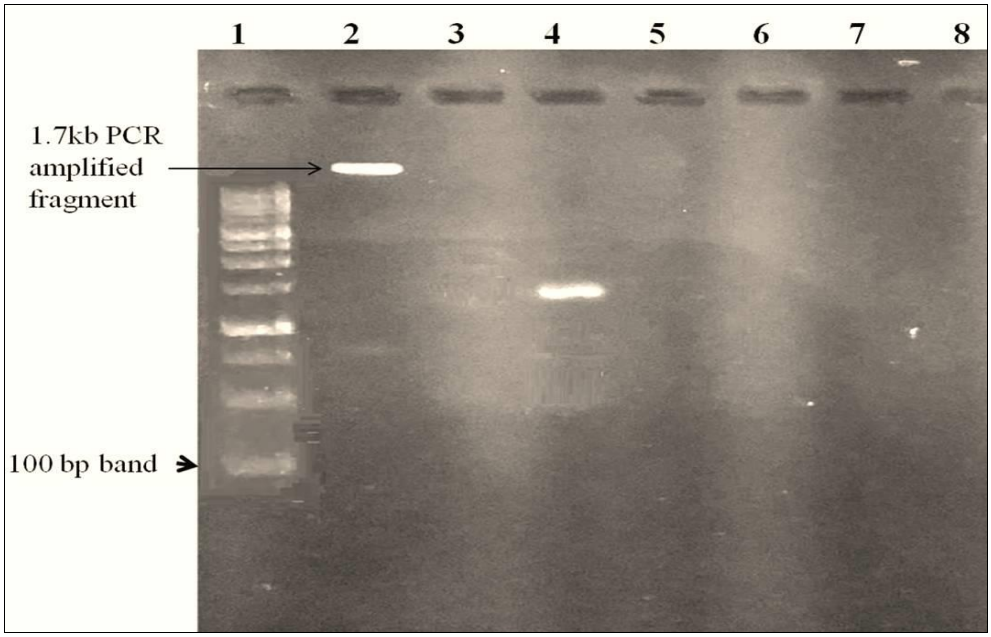

Figure 6: Typical separation of PCR amplified DNA using a lysate of root-knot nematodes on 1.5% agarose gel electrophoresis. A 100 base pair (bp) DNA molecular weight marker is represented by the first well. The 1.7 kb amplified DNA in the 2nd well represents *M. javanica & M. incognita*. For internal control, the 4th well-human beta-actin gene was used. Wells 3, 5, 6, and 7 represent the absence of PCR amplification from the samples of root-knot nematode DNA samples. 8th well: Negative control, double distilled water.

The outcome of molecular methods

Eight (24.24%) samples out of the 33 *Meloidogyne*-like species discovered by optical microscopy demonstrated amplification of the mitochondrial genes encoding for cytochrome oxidase subunit II and 16S rRNA by PCR. The presence of *M. incognita* and *M. javanica* is indicated by the amplification of a 1.7 kb segment. Figure 6 shows an example of PCR-amplified DNA being separated using a lysate of root-knot worms on 1.5% agarose gel electrophoresis. No *Meloidogyne* species PCR amplification from the isolates of other nematode species was found. *Hinf I* restriction enzymes were used to digest each sample that tested positive for the 1.7 kb PCR fragment. There was a clear diagnostic pattern that corresponded to *M. javanica* and *M. incognita.* In contrast, no fragments from other *Meloidogyne* species were found throughout our investigation. With the *Hinf I* enzyme, all isolates of *M. javanica* generated 1 kb and 0.7 kb digested products on a single restriction digestion. The amplified products of *Meloidogyne*-like species had an extra restriction digestion site, which caused the 0.7 kb fragment to be split into two smaller fragments of 0.4 kb and 0.3 kb. Figure 7 shows an example of a restriction digestion separation of PCR-amplified DNA of *Meloidogyne*-like species (1.7 kb) on 1.5% agarose gel electrophoresis. In contrast, no isolates of the *M. chitwoodi, M. hapla, M. nataliei, M. naasi,* or *M. marylandi* species were discovered in our study using PCR. Therefore, in our study, no *Dra I* digestion was carried out. The districts of Ranga Reddy (62.5%) and Mahabubabad (37.5%) were responsible for the majority of isolates of both *M. incognita* and *M. javanica*, resulting in notably severe damage to *Sorghum* crops.

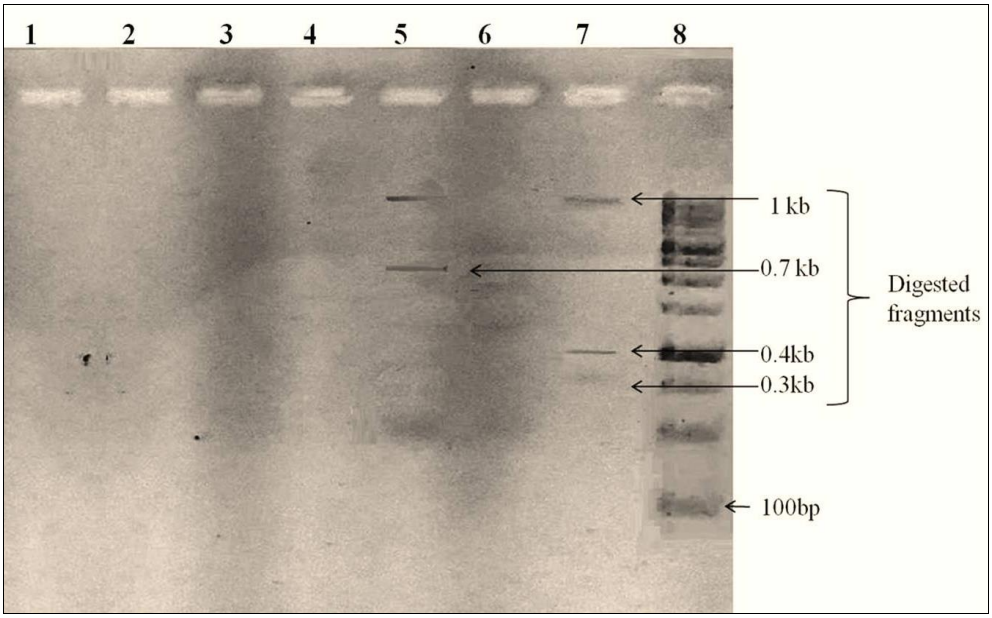

Figure 7: Typical separation of restriction digestion of PCR amplified DNA (1.7kb) of *Meloidogyne*-like species on 1.5% agarose gel electrophoresis. Wells 1 to 4 and 6 represent the absence of PCR amplification from the samples of other nematodes. *M. javanica* is represented by the 1 kb and 0.7 kb *Hinf I* digested products in the fifth well, and *M. incognita* is represented by the 1 kb, 0.4 kb, and 0.3 kb digested products in the seventh well. A 100 base pair (bp) DNA molecular weight marker is represented by the eighth well. Both species shared the 1.0 kb digestion result, whereas *M. incognita* creates two restriction fragments of 0.4 and 0.3 kb from the 0.7 kb fragment due to an additional *Hinf I* restriction site.

Discussion

At present, our study reported the morphological and morphometric similarities of different root-knot nematodes along with the molecular characterization of *Meloidogyne* species associated with the *Sorghum bicolor* crop in Telangana, India. In this study, the common root-knot nematode species were identified by light microscopy using morphological traits such as head form, stylet morphology, perennial patterns, and body shape. Despite some slight physical variations between populations, *Meloidogyne*-like species share several fundamental traits that set them apart from other root-knot nematode species. The ability to recognize the crop-infecting species is crucial for farmers. To diagnose specimens, they must be observed in the lateral position. Some species, such *M. enterolobii* and *M. incognita*, can be distinguished based on the distance from the dorsal esophageal gland orifice to the base of the stylet in the males (Almeida *et al.*, 2008) [2]. The major findings of our study focused on notable traits specific to *M. javanica* and *M. incognita*. Other root-knot nematode species have knobs that are undefined and eventually meld with the tile shaft, unlike *M. javanica*, which has stylet knobs that are low, wide, and set out from the tile shaft. The skull forms and stylet morphology were the same across all groups. In light microscopy, the distinctively formed stylet cone of *M. incognita* cannot always be seen clearly, and in some specimens, the stylet knobs seem as wide as in *M. javanica*. As a result, the styler morphology of *M. incognita* mimics that of *M. javanica*. However, based on the shape of the skull, these two species may be easily separated. Striae are smooth, wavy, and occasionally zigzag; lateral lines are absent; *M. incognita* has a squarish high dorsal arch with a conspicuous whorl at the tail terminus (Eisenback, 1985b) [16]. Although the female stylet knobs were bigger, both sexes of *M. incognita* had a distinct stylet cone form, and the overall shape of the knobs was identical. Striae are smooth and slightly wavy; the dorsal arch is commonly low and rounded, occasionally high and squarish, and frequently has a whorl towards the tail terminus (Eisenback, 1985b) [16]. *M. javanica* has distinctive lateral ridges that run across the pattern, fading away near the tail terminus. The stylet cone of *M. javanica* was straight, and both sexes had knobs that were small, broad, and anteriorly indented. Due to their apparent morphological variety and rarity, the relevance of males in root-knot nematode taxonomy has not previously been highlighted. Nevertheless, the majority of the field, however, was populated by males, and it appears that their head morphology and stylet morphology serve as additional taxonomic features. However, with the discovery of novel disease species, morphological approaches based on a microscope have proven to be insufficiently precise to detect some species. As a result of the similarity in their perineal patterns, *M. enterolobii* and *M. inornata* have frequently been mistaken for *M. incognita* (Carneiro *et al.*, 2016) [12].

The identification of RKN species has mostly relied on the classical taxonomy, based on morphological and morphometric research, and biochemical approaches. The development of molecular-based diagnostics is receiving more attention as a result of the dearth of taxonomic traits and the waning interest in classical taxonomy. The existence of polymorphisms in DNA sequences between groups of worms, particularly in nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA), is a prerequisite for molecular approaches. To differentiate between the main RKN species, the multicopy base of the rDNA comprises variation and stability (Roberts *et al.*, 2016) [32]. Phylogenetic analyses and RKN identification can both be done using mtDNA genes (Kiewnick *et al.*, 2014) [24]. Each cell contains many copies of the mtDNA, which aids in its amplification. It also has variable areas surrounded by conserved domains and is largely conserved across species (Roberts *et al.*, 2016) [32]. Despite these benefits, mtDNA has not been investigated as an alternative to rDNA for the

diagnosis of RKN linked to the *Sorghum* crop in Telangana. The molecular approach relies on DNA that has been collected from fetuses, eggs, and adults, as well as dead animals. When compared to isozymes phenotypes and perineal pattern analyses, which both need mature females, the molecular technique is more beneficial, accurate, and quick (Oliveira *et al.*, 2011) [28]. According to our reports, Telangana has no expert knowledge of nematode taxonomy (Berry *et al.*, 2008) [7]. *Ostertagia ostertagi*, a nematode parasite of cattle, and a region of multiple nucleotide repeating units in *Meloidogyne* have both been found to exhibit intraspecific mitochondrial variation *(*Burrows, 1990) [11]. The repeating units are not in the amplified region of the mitochondrial genome as described in the current work; nonetheless, intraspecific variation suggests that sub specific discrimination in *Meloidogyne* may be provided by more quickly evolving mitochondrial genome regions. Sequencing the amplified products can be used to examine the degree of genetic divergence and viability of host race differentiation among *Meloidogyne* species.

The molecular technique involves PCR amplification of DNA using universal primers, followed by restriction endonuclease digestion of the amplification products. As nucleotide sequences differ between species, restriction sites are distributed differently along the genome, resulting in different-sized pieces and separated using agarose gel electrophoresis. The identification of *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla* is possible by differentiating from varied sizes of restriction fragments (Seessao *et al.*, 2017; Han *et al.*, 2004) [36, 20]. At present, a 1.7 kb DNA band has been found in samples of *M. incognita* and *M. javanica*. Further, the PCR products were cut with the restriction endonucleases *Hinf I* and *Dra I* to distinguish between the two species, and the resultant fragments of varied sizes between *M. incognita* and *M. javanica* allowed their identification (Han *et al.*, 2004) [20]. With the *Hinf I* enzyme, all isolates of *M. javanica* generated 1 kb and 0.7 kb digested products on a single restriction digestion. The amplified products of *Meloidogyne*-like species had an extra restriction digestion site, which caused the 0.7 kb fragment to be split into two smaller fragments of 0.4 kb and 0.3 kb. In our analysis, a diagnostic pattern for *M. incognita* and *M. javanica* was clear. We did not use another molecular technique to support our results nor the results of the positive controls and sequencing data. Furthermore, we only processed five to six nematodes per population and a maximum of eight populations from 33 *Meloidogyne* sample population. A drawback of PCR-RFLP is the potential for false positive results from organisms that are not the target (Roberts *et al.*, 2016) [32]. These could be the limiting factors of our findings. Although the figures were of moderate quality, however they represent our efforts to show juvenile stages and adult stages of *Meloidogyne*. The molecular approach is routinely employed in all laboratories throughout the world. No radioactive isotopes were required, and simple nematode lysis before PCR was all that was needed instead of laborious DNA extraction. Species determinations can be carried out before planting thanks to the capacity to recognize soil J_2. With just one PCR amplification, the assay's specificity might make it possible to track species transitions in populations of mixed *Meloidogyne*. This set of primers can be used to amplify at least three additional *Meloidogyne* species, which can then be distinguished from the other five species by endonuclease digestion of the amplified product. However, it will be necessary to look at a variety of isolates from the "Minor" species before species specificity can be established.

Conclusions

Amplification of a 1.7 kb fragment by PCR was seen in 8 (24.24%) of the samples from the 33 *Meloidogyne*-like species identified by optical microscopy, showing the presence of *M. incognita* and *M. javanica*. *Hinf I* and *Dra I* restriction enzymes were used to break down all PCR-positive samples, but only the *M. incognita* and *M. javanica* fragments were found. The majority of these positive samples were from the districts of Ranga Reddy and Mahabubabad. These findings show that *M. javanica* and *M. incognita* are present in these two districts and are linked to *Sorghum* crop loss. The study has a local focus, but the knowledge and technique it produced may be of interest to people all around the world. To confirm the method's broad applicability, a survey of isolates from throughout the world should be carried out. With this procedure, no resolution below the species level has yet been attained. The integrative taxonomic method, however, is what we advise for a precise diagnosis of RKN species. Studies on phylogenetics and Etiology should include molecular, biochemical, and morphological data to improve their resolution and dependability. Accurate identification allows for the recommendation of control measures and the reduction of RKN losses.

List of Abbreviations

Kb, kilobase, RKN, root-knot nematode, *M. javanica*, *Meloidogyne javanica*, PCR, polymerase chain reaction, DNA, Deoxy nucleic acid, lrRNA, large subunit of the ribosomal RNA, COII, cytochrome oxidase subunit II gene, mtDNA, mitochondrial DNA, SEM, standard error of the mean; SD, standard deviation, NA, Not available, bp, base pair; rRNA, ribosomal ribonucleic acid%, percentage, RFLP, restriction enzyme length polymorphism.

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Supplementary Files

Methods

Collection and processing of soil samples: A total of 60 rhizosphere soil samples, together with root samples, were gathered from 60 *Sorghum* fields of the *S. bicolor*-producing sites throughout Telangana's four agroclimatic districts (Supplementary Figure S1). The samples were mixed and transferred for examination after being collected to a depth of 15 cm from three locations at the same site during the years 2015 and 2022. The normal techniques were applied to all such composite samples taken from each field and season to isolate root-knot nematodes. Plant and root samples were brought to the lab and rigorously checked for the presence of galls while being stored in polythene bags with the required labels. Each sample was divided into four equal portions and carefully mixed after being sieved through a screen with 6-mm holes. Samples were processed 24 hours after they were collected. The plant's roots were examined for galls, if any were found, and their number was recorded. Every count was changed to nematodes per 500 cc of soil.

Method for dissecting roots: Plant materials were properly cleansed and put in water-filled Petri plates. The roots were separated using forceps and dissecting needles, then examined under a microscope with a 15-50x magnification. With the help of a handling needle or paintbrush, the developing nematodes, egg masses, etc. were removed from the suspension and examined under a microscope (Boda *et al.*, 2023).

Baermann Funnel Method: Water was added to infected plant matter. After 48 hours, the active nematodes had wriggled out of the material and into the sink (Baermann, 1917) [3].

Cobb's sieving, also known as the gravity technique, is a technique for removing active nematodes from soil and sediments by utilizing many characteristics of nematodes and soil particles, including size, shape, sedimentation rate, and nematode mobility.

Identification of the species by inverted light microscope: For the study of morphology, the isolates containing nematodes were placed in the nematode counting chamber and observed under the inverted biological microscope (BLM-290, Best Scope, China). Photomicrographs and morphometric measurements of the nematodes were taken using the inbuilt camera of the microscope. Some species can be distinguished by measuring the distance between the males' stylet base and the dorsal esophagus gland orifice (DGO). Even though some RKN species share identically sized stylets, the size and form of the stylet also have a balanced taxonomical value for recording RKN species. The same property was used in this study.

The perineal pattern of females: A total of eight (n-8) perennial patterns using female *Meloidogyne*-like specimens were analyzed in this study. The anterior parts of the nematodes were carefully cross-sectioned and fixed on the clean glass slides with glycerol. The slides prepared from each sample or localities were inspected under a microscope for their perineal patterns and features (Eisenback *et al.*, 1981) [17]. The documentation of females and males employed the shape and graphical elements of the complete perineal area, dorsal arch, dorsal striae, lateral lines, and phasmid features.

Molecular identifications of species

Isolation of DNA from nematodes (Floyd *et al.*, 2002) [19]

Each nematode was isolated by a combination of sieving and density gradient centrifugation from agricultural field soils and the respective root homogenates were numbered. Five to six individually pooled nematodes were kept in the labeled sterile 1.5 mL Eppendorf tubes from each sample and centrifuged at 3000 rpm for five minutes on a REMI centrifuge. After carefully discarding the supernatant, 20 µL of 0.25 M NaOH (base) was added, and it was then left to incubate for 3 to 16 hours at room temperature (Stanton *et al.*, 1998) [37]. The sample was then heated at 95 °C for three minutes. To neutralize the base, 10 µL of 0.5 M Tris-HCl buffer (pH 8.0) and four (4) µl of HCl (acid) were added. After that, 5 µL of 2% Triton X-100 and spent 3 minutes heating at 95 °C. After 10 minutes of centrifugation at 4000 rpm with the lysate, 40 µL of DNA-containing supernatant was obtained. The pellet with debris was discarded. The 200 µl PCR tubes containing the DNA samples were kept at -20 °C until the next experiment.

Primer designing

The following primers were used to amplify the intergenic cytochrome oxidase subunit II (COII-16S) region of mitochondrial DNA (mtDNA). Powers & Harris (1993) [31] described the design of the PCR primer #C2F3 (5'-GGTCAATGTTCAAATTTGTGG-3') for the cytochrome oxidase subunit II gene (COII) available in GenBank, as well as primer #1108 (5'-TACCTTTGACCAATCACGCT-3') for the large subunit of the ribosomal RNA gene (lrRNA) from *M. incognita* (Figure 1). At Bangalore Genie in Bangalore, India, primers were created.

PCR analysis

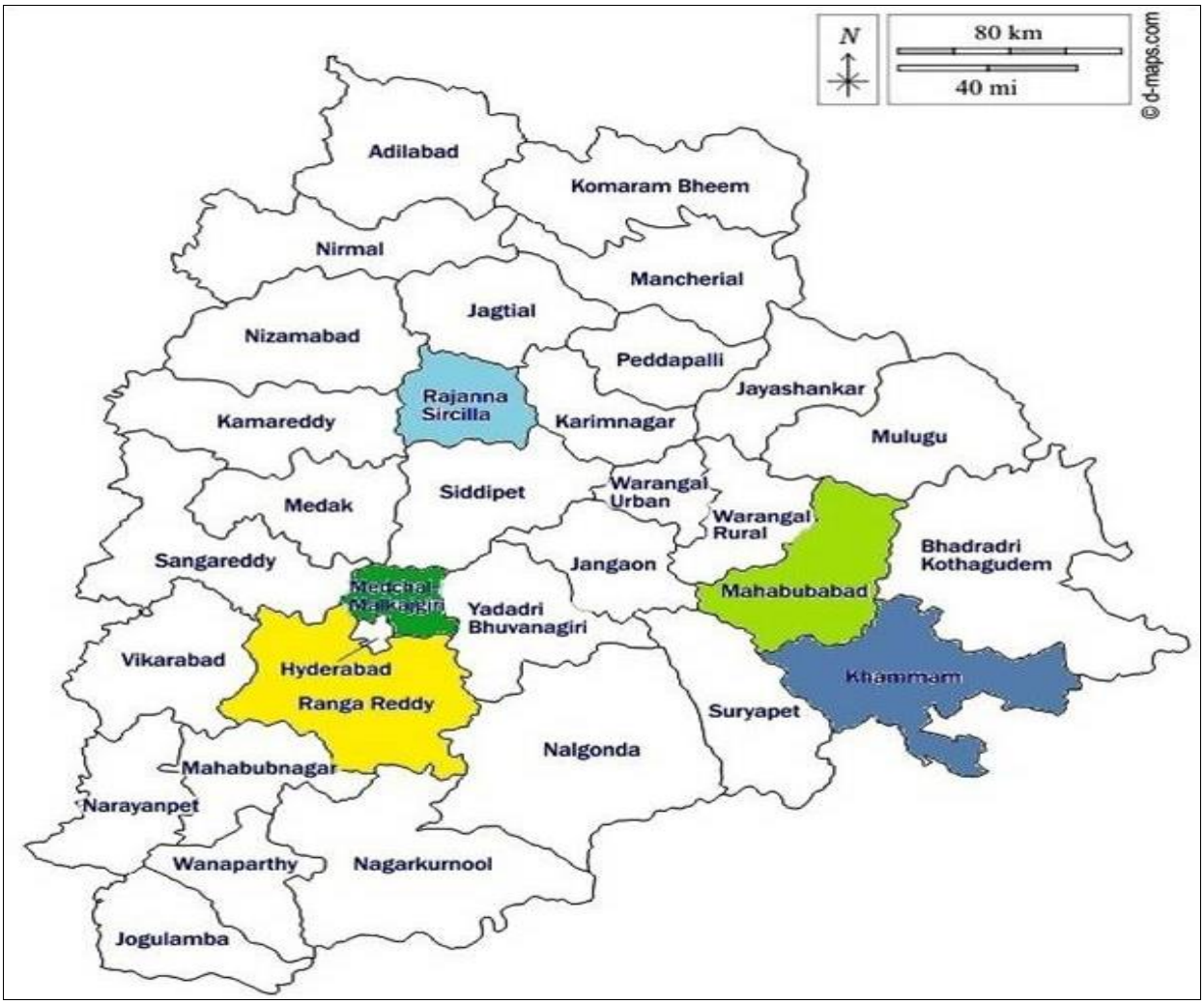
Before beginning the experiment, all reaction components were maintained in an ice bath. Following Supplementary Table 1, the components were added to 200µl sterile microfuge tubes (PCR tubes) in the specified order. The reaction mixtures were gently vortexed and subsequently all of the components were rapidly pelleted at the bottom of the tube through centrifugation. To start the thermocycling process, these PCR tubes were moved from the ice onto Eppendorf PCR equipment in Hamburg, Germany. The template DNA was first denatured at 94 ºC for 5 minutes, followed by 45 seconds at that temperature, 45 seconds at 48 ºC, 45 seconds at 72 ºC, and so on for 30 cycles. Finally, to finish the partially produced second strands, a 5-minute incubation period at 72 ºC was carried out. Samples were kept at 4° C after PCR was finished (Supplementary Figure S2). Electrophoresis on a 1.5% agarose gel containing ethidium bromide was performed with a constant voltage (110 V) and at room temperature for one hour by loading the Gene Rule 100 bp DNA ladder/molecular weight marker, PCR amplified DNA solution combined with 1 µl of 6X DNA loading dye in different well on 1X TAE buffer (Amresco, Amresco LLC, USA). Under UV light, the gel's

bands could be seen and measured using Gel Doc-XRT and image lab software. For scoring the banding patterns, 10 µl of the amplified product was put onto the agarose gel. The relative intensities of the bands and their migration distance from the DNA molecular weight marker (standards) were compared to estimate the purity and size of the DNA. Five *Meloidogyne* species were used in PCR experiments, and different sizes of amplified products were found. All *M. incognita* and *M. javanica* isolates generated a 1.7-kb fragment. Molecular techniques allow for the observation of the 1.1-kb fragment for *M. arenaria* as well as the 0.52-kb fragments for *M. Chitwoodi*, *M. hapla*, *M. marylandi*, *M. nataliei*, and *M. naasi*. However, no isolates were found that belonged to the *M. chitwoodi*, *M. arenaria*, *M. hapla*, *M. nataliei*, *M. naasi*, or *M. marylandi* species.

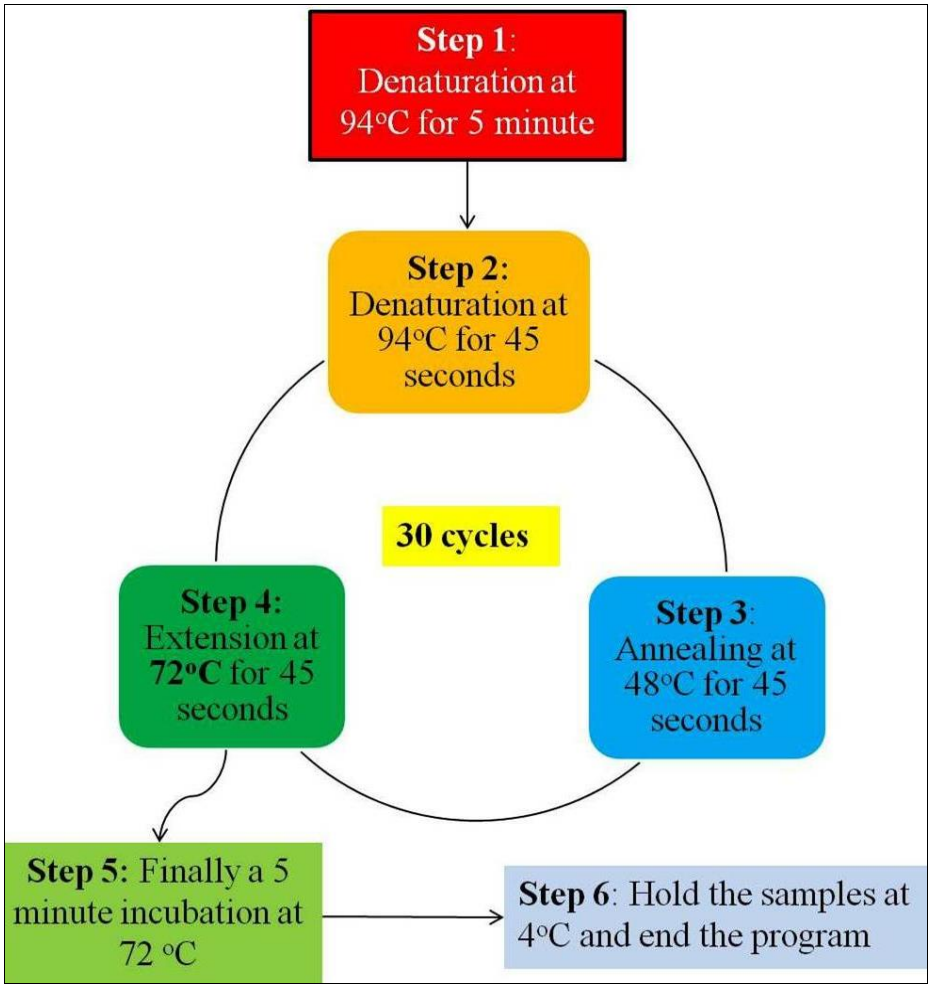
Standard restriction digestion of PCR products for species confirmation: The generated products were subjected to standard restriction digestion utilizing 15 µl of the PCR product with *Hinf I* and *Dra I* digestions carried out for 2 hours at 37 ºC and analyzed on 2.0% agarose gels (Supplementary Table S2). For all *M. incognita* and *M. javanica* isolates, restriction digestion of the 1.7-kb amplification product with *Hinf I* produced diagnostic patterns. Products from one restriction site on *M. javanica* were 1.0 and 0.7 kb in size. The *M. incognita* product contained an extra restriction site, which caused the 0.7-kb fragment to be split into two fragments of approximately 0.4 and 0.3 kb. The restriction enzyme *Dra I* can distinguish between the two species by digesting the products of *Meloidogyne chitwoodi* (290 pb) and *M. hapla* (230 bp). Two smaller segments of 130 bp and 100 bp could be seen because each *M. chitwoodi* isolate's 230-bp fragment had an extra restriction site. *Dra I* can spot distinct patterns for *M. nataliei*, *M. naasi*, and *M. marylandi*. The species *M. chitwoodi*, *M. hapla*, *M. nataliei*, *M. naasi*, and *M. marylandi* were not represented by any isolates, though. It was impossible to assess the patterns' specificity and consistency.

Statistical Analysis: The distribution of fields by district, the average number of *Meloidogyne*-like species and other nematode species per field, and the *Meloidogyne* species confirmed by PCR were statistically analyzed by using SPSS Version 20 for Windows (Table 1). Simple frequency distribution with measures of central tendency (mean) and dispersion (standard errors of the mean, SEM) were determined. The data were presented as Mean ± SEM and percentage (%). One-way ANOVA (between the districts) was performed using the number of other nematode species and *Meloidogyne*-like species. A p-value of 0.05 or less was regarded as statistically significant for data.

Supplementary Figures



Supplementary Figure S1: Sample collection districts of Telangana (Coloured)



Supplementary Figure S2: PCR amplification program

Supplementary Tables

Supplementary Table S1: PCR reaction (Volume: 50µl)

S. No	Component	Volume in µl	Final concentration
1	Autoclaved Nuclease-free water	30	0.05 milliporee
2	10X Taq Buffer A (Tris with 15 mM Magnesium Chloride, pH-8.0), (Bangalore Genie, Bangalore, India)	5	1X (Tris with 1.5 mM $MgCl_2$)
3	Template DNA (Nematode lysate)	5	1.8 ng
4	Deoxyribonucleoside triphosphates (dNTPs) Mix, 10 mM (Bangalore Genie)	1	200 µM
5	IrRNA FWD (10 µM), Bangalore Genie	4	0.8 µM
	COIIR REV (10 µM), Bangalore Genie	4	0.8 µM
6	Taq DNA Polymerase (3U/µl), 250 Units, Bangalore Genie	1	0.05 U
Total Volume		50	

Supplementary Table S2: Reaction for standard restriction digestion of PCR products

Components of reaction	Vol. in µl	Final concentration
Autoclaved Nuclease-free water	2	0.05 millipore
10 X Digestion Buffer with BSA (Fermentas)	2	1X
PCR Amplicons	15	Detectable by agarose gel electrophoresis
Hinf I / Dra I (1500 Units) Enzyme (Fermentas)	1	0.5U
Total Volume	20	-