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Molecular Characterization of Mulberry Root-Knot Nematode, Caused by *Meloidogyne incognita* using Modified DNA Isolation Protocol

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A B S T R A C T

Root-knot nematodes (RKN) are notorious to cause root galls and leads to severe damage on different crops, including mulberry. RKN have very wide host range and *Meloidogyne* is a universal problem due to its polyphagous nature. Molecular diagnosis at species level is more efficient than the morphological identification of RKN. Hence, in this study, two methods were used for isolation of nematode DNA using root galls of mulberry. Between these, the modified CTAB extraction method yielded a high quantity of DNA (1244.8 to 7031.4 ng/µl), with spectrophotometer reading at A260 / A280 ratio ranged between 1.62 to 1.96 for purity. Further, the DNA was subjected to PCR amplification using *Meloidogyne* species specific markers. The best amplification was observed in DNA isolated from the modified CTAB method. The genus and species-specific markers were used for accurate detection of RKN samples collected from three mulberry growing states of South India. The amplicon size at 612 bp and 399 bp confirmed the presence of genus *Meloidogyne* and species *M. incognita*, respectively in all the samples. Further, amplified regions of 28S rDNA and SCAR were sequenced to confirm the identification of the RKN. This study revealed the association of *M. incognita* in mulberry root-knot nematode at the molecular level in South India.

Introduction

Root-knot nematodes (RKN) are extremely adaptable and cause significant damage to crop plants. *Meloidogyne* spp. was first reported by Berkeley in 1855 on cucumber roots. In mulberry, RKN was first reported from USA by Bessey during 1911 and later it was reported from various countries. In India, Swamy and Govindu (1965) observed RKN for the first time and later by Narayanan *et al.*, (1966) at Mysore on mulberry. The species like *Meloidogyne incognita*, *M. arenaria*, *M. javanica*, and *M. hapla* are found mainly in the worldwide (Saucet *et al.*, 2016). Previously, various RKN species were reported on mulberry which are *M. incognita*, *M. javanica*, *M. hapla*, *M. arenaria*, *M. arenarithamsi*, *M. mali* and *M. enterolobii* (Toida, 1984; Hida and Zhu, 1985; Ertian, 2003; Paestakahashi *et al.*, 2015; Zhang *et al.*, 2020). In India, initially *M. incognita* was found

associated and later *M. javanica* was also reported on mulberry (Sharma and Sarkar, 1998; Sujathamma *et al.,* 2014; Gnanaprakash *et al.,* 2016).

Out of these two species, *M. incognita* is very serious and persistent to the mulberry (Govindaiah *et al.,* 1991; Arunakumar *et al.,* 2021). The severity and damage depends on soil and climatic conditions of different locality (Ramkrishnan and Senthilkumar, 2003). The disease is widespread and common in sandy soils under irrigated condition (Arunakumar *et al.,* 2018). *M. incognita* is more dangerous to mulberry by severe damage to the crop and also it predisposes the plant to other soil-borne pathogens (Bhagyrathy *et al.,* 2000; Sukumar *et al.,* 2000; Nishitha Naik *et al.,* 2003).

RKN control is very difficult due to its wider host range and presence of different races of *M. incognita* (Hartman and Sasser, 1985), among them race-2 infects the mulberry in India (Govindaiah *et al.,* 1991). The severely infected plant shows the stunted growth, marginal chlorosis, necrosis and curling of leaves, formation of galls in the roots, reduced vigour of plants. Infected plants lose their ability to absorb moisture and nutrients results in reduced metabolic function which leads to deterioration in leaf quality and effects on silk worm health and quality of cocoon production (Rani and Kumari, 2017). The RKN causes leaf yield loss up to 20% in addition to deterioration of leaf quality and severity increases with age of the garden (Arunakumar *et al.,* 2021). Association of root-knot nematode with root rot disease caused by various fungal pathogens form a root disease complex (Gnanesh *et al.,* 2020).

Previously, identification of root-knot nematode at species level was largely dependent on perineal pattern which is difficult for non-specialists. At present, many molecular diagnostic tools have facilitated easy and rapid identification of different species of root-knot nematode (Ye *et al.,* 2015). As evident, this strategy was successful in identification of *M. enterollobii* from South China in mulberry (Zhang *et al.,* 2020). And hence, molecular diagnosis by using specific markers to different species provides a quick and easy diagnostic technique for RKN of mulberry. Thus, in the present study, a modified the CTAB DNA extraction method was followed to extract DNA from root galls and attempted to characterize RKN at species level in mulberry.

Materials and Methods

Collection of nematode samples

The galled roots of mulberry infected by RKN were collected from four mulberry cultivation locations of South India (Table 1). The collected samples were kept in plastic covers, brought to laboratory and stored under refrigerated condition (4^{o} C) for further use.

Modified DNA extraction method

The DNA of root-knot nematodes was isolated using root galls as a source material. The presence of 90-150 juveniles per root gall was confirmed using microscope. The root-knot nematodes DNA extraction method was modified as given originally by Tesarova *et al.,* (2003). The modified protocol used in this study is described below;

Preheated the extraction buffer of pH 8.0 with 50 mM EDTA, 250mM TrisHCl, 2.5 M NaCl, 3% (W/V) CTAB, 0.2% mercaptoethanol and 2.5% (W/V) PVP (Polyvinylpyrrolidone).

Galled root samples were thoroughly washed with sterile distilled water, 2 gm of sample was crushed to fine powder using liquid nitrogen in pestle and mortar.

Added 1.5 ml of pre-warmed CTAB extraction buffer with 0.37 mg of PVP (2.5%) and vortexed thoroughly in a new tube (2.0 ml).

Then tubes were incubated for 30 min at 65°C and centrifuged for 10 min at 10,000 rpm. The supernatant was shifted to a two ml centrifuge tube, added with equal volume of isoamyl alcohol:

chloroform (1:24) and mixed robustly by inverting 5 - 10 times and centrifuged at 12,000 rpm for 10 min and added equal volume of frozen iso-propanol to clear supernatant and kept for 2 hrs at -20°C.

Afterwards the samples were centrifuged for 10 minutes at 12,000 rpm.

DNA pellet was washed twice with 40 µl of 70% ethanol, pellet was air dried and suspended in 100 µl of TE buffer.

Qualitative and quantitative estimation of extracted DNA

The quantity of isolated DNA was estimated by using a spectrophotometer (Nanodrop 2000, Thermofisher scientific) at 260 nm. Purity of isolated DNA was obtained by calculating the absorbance ratio A260/280. Assessment of polysaccharides contamination was done by calculating the absorbance ratio A260/230 (Wilson and Walker, 2005). DNA quality and yield was assessed by electrophoresis at 0.8% agarose and documented in gel documentation system (Gene genius, Syngene). The data was analyzed using IBM SPSS Statistics 23.0.

PCR using genus and species specific root-knot nematode diagnostic markers

PCR amplification size of 612 bp was produced using a universal primer RK28SF/MR (Hu *et al.,* 2011) for the *Meloidogyne* identification. A SCAR primer Inc-K14-F / Inc-K14-R was used to identify *M. incognita* with the amplification size of 399 bp (Randig *et al.,* 2002). Similarly, *M. javanica* and *M. arenaria* species specific primers were also used (Table 2). PCR was conducted in a 25 µl reaction mixture consisting of ten picomolar of each primer (Eurofins Pvt. Ltd., Bengaluru), 2X PCR Amplicon master mix and 40 ng of sample DNA. The PCR program consisted of 94°C for 4 min, followed by 35 cycles of 94°C for 30 sec, temperature specific to annealing of 52-54°C for 30 sec, for extension 72°C for 45 sec, and final elongation phase at 72°C for 8 min using a thermal cycler (GeneAmp 9700, Applied Biosystems, USA; Supplementary table 1). Gel electrophoresis was carried out in 1.5 % agarose gel stained with ethidium bromide and buffered with 1X TBE for separation of amplified products. Gel images were documented in gel documentation systems (Gene genius, Syngene, UK).

Sequencing and phylogenetic analysis

The amplified PCR products were sequenced (Eurofins Pvt. Ltd., Bangalore, Karnataka, India) after purification. The BioEdit software (Hall 2014) was used to edit nucleotide sequences obtained and similar sequences were found at NCBI database in a BLAST analysis. The phylogenetic analysis of sequences obtained in this study was carried out using MEGA X software (Kumar *et al.,* 2018) and alignment of sequences were done by using the default parameters of clustalw. The Maximum Likelihood method based on the Tamura 3-parameter model was used to study the evolutionary history. Different species of *Meloidogyne* sequences were used with sequenced data of this study and an out-group was obtained in a phylogenetic tree using 28S rDNA region of *Pratylenchus penetrans*.

Results and Discussion

Assessment of DNA isolated from root galls

In this study, a modified CTAB buffer with chemical alteration was used to extract DNA from RKN infected root galls. The quantity and quality of DNA samples extracted by common and modified CTAB methods were assessed spectrophotometrically and agarose gel electrophoresis method (Table 3).

The DNA quality isolated by modified CTAB was found to be the best and evidenced by the spectrophotometer values at A260 / A280 ratio (1.62 to 1.96). Whereas, the purity ratio of DNA isolated from common CTAB method was observed between 0.66 to 2.10. Totally, 10 replications for each method was maintained although nanodrop reading

was shown error in few replication of DNA isolated from common CTAB method (Supplementary fig. 1) and smeared nucleic acid bands appeared in 0.8% agarose gel (Supplementary fig. 2). The modified CTAB method yielded high quantity of DNA which was found from 1244.8 to 7031.4 ng/µl. While, DNA isolated using common CTAB method was found low purity and less quantity (Table 3).

PCR amplification

The selected primers showed to be a potential tool for RKN diagnosis. They were observed to be sensitive, specific, and the only crucial prerequisite was isolation of good quality DNA and sufficient concentration for PCR. This could be resolved by using a modified CTAB DNA isolation method which gives adequate quantities of target DNA in each sample (Table 4). Common CTAB method produced faint DNA bands resulted in ambiguity which showed error at sequencing work.

The genus specific diagnostic marker RK28SF/MR for targeted gene 28S identified *Meloidogyne* which produced the amplicon size of 612 bp. Amplification at 399 bp was produced by primer set Inc-K14-F / Inc-K14-R, for *M. incognita* in all the four samples collected from four locations of South India (Fig. 1). But, it was observed that there was no amplification with other species specific primers (FJav/RJav, DJF/DJR and DIF/DIR) in all the four samples tested (Fig. 1). In mulberry, for the first time using species specific molecular markers it was evidenced that *M. incognita* is the species associated in the root-knot nematode of mulberry.

Sequence homology of the gene sequences of root-knot nematode

A genus-specific primers used to amplify a unique sequence in the root-knot nematode gene that detect *Meloidogyne* sp. The PCR product obtained from primer pairs set RK28SF/MR and Inc-K14 were sequenced at Eurofins Pvt. Ltd., Bangalore. The obtained sequences were deposited in GenBank (Table 5).

Phylogeny assessment

The BLAST analysis of obtained sequences revealed that similarity of the sequences of *Meloidogyne* sp. was found between 92.38 to 100 %. The phylogenetic analysis of the *Meloidogyne* sequences from RK28S (Fig. 2) revealed that MW187117, MW187113, MW187115 and MW187116 are in the same clade of *Meloidogyne* species, while other species of *Meloidogyne* are also sub clustered in different groups with the same genus. Other genus of root-knot nematode *Pratylenchus penetrans* was used for analysis and found out-grouped in the cluster.

Meloidogyne incognita also known as the southern root-nematode and takes 37 days at 21°C for completion of its life cycle (Perry *et al.*, 2013). The root-knot nematode is polyphagous and *Meloidogyne* is highly damaging genus (Li and Chen 2017). Nevertheless, four common species of this genus are *Meloidogyne incognita*, *M. arenaria*, *M. hapla* and *M. javanica* have been reported as dangerous (Dong *et al.*, 2014). Out of four species, *M. incognita* is the highly dangerous due to its high reproduction rate, large host range and ability to produce disease complex with other pathogens (Vos *et al.*, 2013).

Morphological identification of *Meloidogyne* spp. required highly expertise nematologist due to morpho-similarity of different species. Novel technology in molecular characterization have allowed for better identification of RKN at species level.

Chemical composition of CTAB buffer modification

Different researchers identified nematode DNA extraction from different source but they are not simple and amenable for easy extraction due to nematode size, load and elastic nature. The common CTAB method developed by Tesarova *et al.*, (2003) is the simple method but couldn't succeed in our experiment due to woody nature of the crop. From

all the samples very low quantity and quality of the DNA was obtained from common CTAB method. In our study, the modified method yielded enhanced DNA in terms of quality and quantity, due to availability of more juveniles in the root galls, also, some alteration in CTAB chemical composition like Tris-HCL, CTAB, NaCl, and PVP has helped in increasing the yield of the RKN DNA.

Murray and Thomsan (1980) developed universal CTAB methodology for extraction of DNA from different tissues but it was revised to decrease contaminants like polysaccharides and polyphenols there in the plant tissues (Peterson *et al.,* 1997). Extraction of DNA from nematodes encapsulated within the root zones of mulberry is inherently more difficult because mulberry root zone have higher lignin content 0.35–0.50 mm fraction as described in Hatfield *et al.,* (1999). It was found difficult for separation of DNA from hard tissues and succeeding reactions in common CTAB. DNA isolated from this method formed highly viscous, brownish, and sticky pellets, indicating the presence of phenolic compounds (Moreira and Oliveira, 2011). In most of the DNA isolation protocols 2% of CTAB and 1.4 M of NaCl have been used (Murray and Thompson, 1980). The ionic potency needed for CTAB to precipitate polysaccharides is provided by more than 0.5 M NaCl concentration (Paterson *et al.,* 1993). However, Heikrujam *et al.,* (2015) also recommended to use higher concentration of the NaCl to precipitate polysaccharides in CTAB; therefore in the present study considered the same strategy and increased the concentration of NaCl and CTAB for obtaining high-quantity and quality of genomic DNA from root galls. The high quality of DNA isolated from the root gall samples could also be accredited by PVP (2.5%) with the higher concentration and with low molecular weight (10,000). Similarly, Couch and Fritz (1990) and Chaudhry *et al.,* (1999) have solved the problem of phenolics by using PVP with low molecular weight. Enough quantity of polyphenol-free DNA can be isolated by using PVP with low molecular weight which is less affinity of precipitating with the nucleic acids (Zhang *et al.,* 2000). In this study, a major modification was made in the CTAB to isolate nematode DNA suitable for PCR amplification.

The PCR performance can be greatly improved by using nanodrop absorbance profile to detect contaminants (Holden *et al.,* 2009). The high quality DNA with no contamination was obtained by the 260/280 nm ratio of 1.8, more than 1.9 designate the presence of RNA and less than 1.7 show the presence of proteins (Abouseadaa *et al.,* 2015; Latif and Osman, 2017). Extracted DNA of quality and quantity using modified CTAB method has been best as confirmed by the spectrophotometer reading and the modified CTAB method proved to be a superior for nematode DNA extraction.

There are several advantages of PCR method in molecular diagnosis of *Meloidogyne* spp. (Williamson *et al.,* 1997). Different species of *Meloidogyne* can also be detected using RAPD, but this is not always reproducible since this technique is very sensitive to varying reaction conditions (Ellsworth *et al.,* 1993; Munthali *et al.,* 1992). ITS markers but they were found to be less suitable for differentiating *M. arenaria*, *M. javanica* and *M. incognita*, due to same PCR product size (436 bp) and hampering unambiguous determination at species level (Kiewnick *et al.,* 2014). Zijlstra *et al.,* (2000) solved this problem by identifying SCAR markers to differentiate species of nematodes. However, with the effort of Dong *et al.,* (2001), Randig *et al.,* (2002) and Hu *et al.,* (2011) published data set gave reliable results for identifying different species of nematodes. For the identification of *M. incognita,* species specific markers are the better option compared to highly conserved ITS regions (Tesarova *et al.,* 2003).

Table.1 Details of root-knot nematode samples collected for this study

Sample Code	Latitude (N)	Longitude (E)	Altitude (m)	Place and District	State
RKN_01	14°22'13.8"	77°21'52.6"	423	Ananthapur	Andhra Pradesh
RKN_02	12°16'13.1"	77°11'08.9"	692	Sathanur, Ramanagar	Karnataka
RKN_03	11°15'21.2"	77°20'06.7"	313	Bhavani, Erode	Tamil Nadu
RKN_04	12°14'57.9"	76°37'23.8"	770	CSRTI, Mysore	Karnataka

Table.2 Details of primers used in the present study

Genus/ species of RKN	Target for amplification	Fragment size (bp)	Primer sequence	References
Meloidogyne	RK28SF	612	CGGATAGAGTCGGCGTATC	Hu et al. (2011)
	RK28SR		GATGGTTCGATTAGTCTTTCGCC	
Meloidogyne incognita	Inc-K14-F	399	CCCGCTACACCCTCAACTTC	Randig et al. (2002)
	Inc-K14-R		GGGATGTGTAAATGCTCCTG	
Meloidogyne javanica	FJav	670	GGTGCGCGATTGAACTGAGC	Zilstra et al. (2000)
	RJav		CAGGCCCTTCAGTGGAACTATAC	
Meloidogyne javanica	DJF	1650	CCTTAATGTCAACACTAGAGCC	Dong et al. (2001)
	DJR		GGCCTTAACCGACAATTAGA	
Meloidogyne arenaria	DIF	950	TCGAGGGCATCTAATAAAGG	Dong et al. (2001)
	DIR		GGGCTGAATATTCAAAGGAA	

Table.3 Root-knot nematode DNA quality and yield, isolated using different methods

	Modified CTAB Method (This study)		**Common CTAB method (Tesarova *et al.*, 2003)**	
DNA Code	**Quality (A260/A280)**	**Yield (ng µl^{-1})**	**Quality (A260/A280)**	**Yield (ng µl^{-1})**
RKN_01	1.62 ± 0.10	4226.1± 18.6	1.20 ± 0.34	223.3 ± 18.7
RKN_02	1.96 ± 0.12	7031.4 ± 31.2	2.10 ± 0.18	365.9 ± 22.3
RKN_03	1.75 ± 0.08	1244.8 ± 22.1	0.66 ± 0.21	100.7 ± 15.4
RKN_04	1.81 ± 0.14	5054.8 ± 16.9	1.08 ± 0.16	318.4± 12.8
Mean	1.78	4389.27	1.26	252.07
SD	0.078	195.725	0.172	76.406

Table.4 Comparison of concentration used in the extraction buffer chemical composition

Chemicals/materials	Concentration used in different methods	
	CTAB method by Tesarova *et al.*, (2003)	This study
Tris HCL	50mM	250mM*****
NaCl	0.7M	2.5 M*****
EDTA	10mM	50 mM*
CTAB (wt/vol)	1 %	3 %*****
Polyvinylpyrrolidone	**	2.5 %*****
2-mercaptoethanol (v/v)	**	0.2 %
Plant material used	Tomato root galls	Mulberry root galls

Note:*****Concentration of the chemicals was increased; **: Not mentioned

Table.5 NCBI accession numbers of four different isolates of root-knot nematode

Sample code	Primer used	RKN isolate	Accession number
RKN_01	RK28SF/MR	*Meloidogyne* sp.	MW187117
RKN_02	RK28SF/MR	*Meloidogyne* sp.	MW187113
RKN_03	RK28SF/MR	*Meloidogyne* sp.	MW187115
RKN_04	RK28SF/MR	*Meloidogyne* sp.	MW187116
RKN_01	Inc-K14	*Meloidogyne incognita*	MW181659
RKN_02	Inc-K14	*Meloidogyne incognita*	MW187114
RKN_03	Inc-K14	*Meloidogyne incognita*	MW193121
RKN_04	Inc-K14	*Meloidogyne incognita*	MW183247

Supplementary Table.1 PCR amplification conditions for root-knot nematode diagnostic primers

Name of primer	Amplification conditions	No. of cycles
RK28SF, Jav*, DJ * & DI*	94°C 4 min 94°C 30 sec 52°C 30 sec 72°C 45 sec 72°C 8 min	35
Inc–K14	94°C 4 min 94°C 30 sec 54°C 30 sec 72°C 45 sec 72°C 8 min	35

Note: *Gradient PCR (51.6 to 59.2°C) has been used for PCR amplification

Fig.1 PCR amplification of four DNA samples from different mulberry gardens of South India. L: 100 bp ladder; 1-4; RK28SF/MR_*Meloidogyne*, 5-8; Inc-K14_*M. incognita*, 9-12; Jav _*M. javanica*, 13-16; DJ_*M. javanica*, 17-20; DJ_*M. arenaria*, 21 and 22; controls

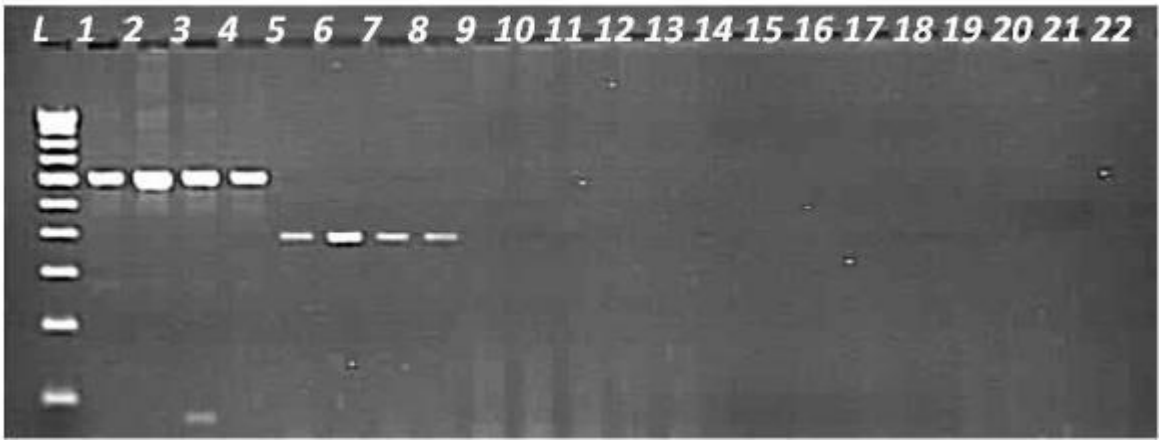
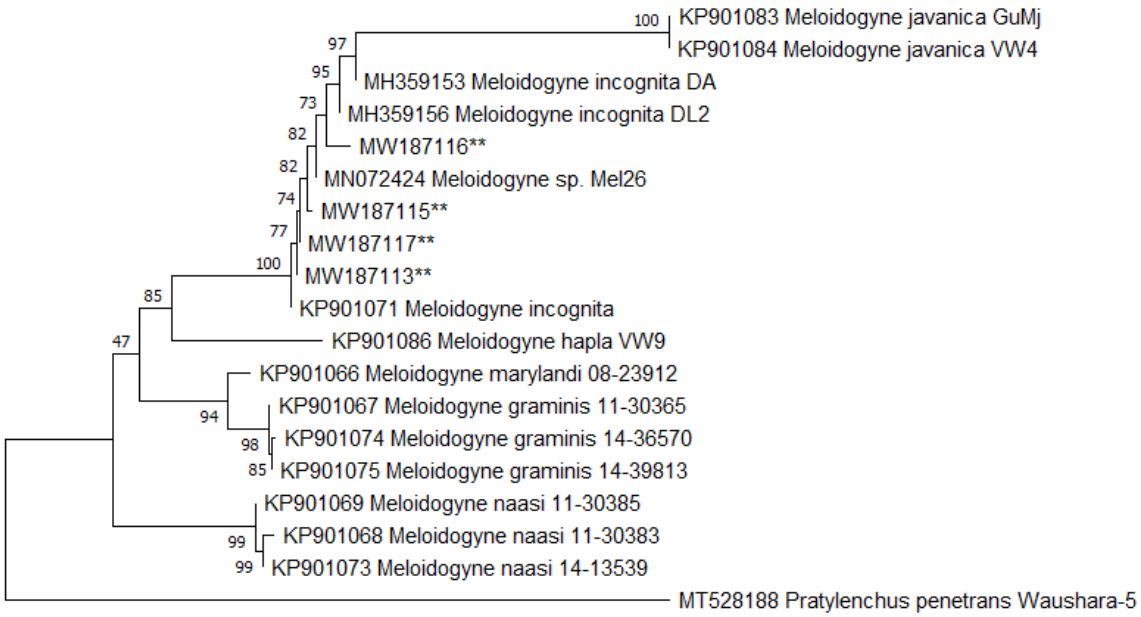
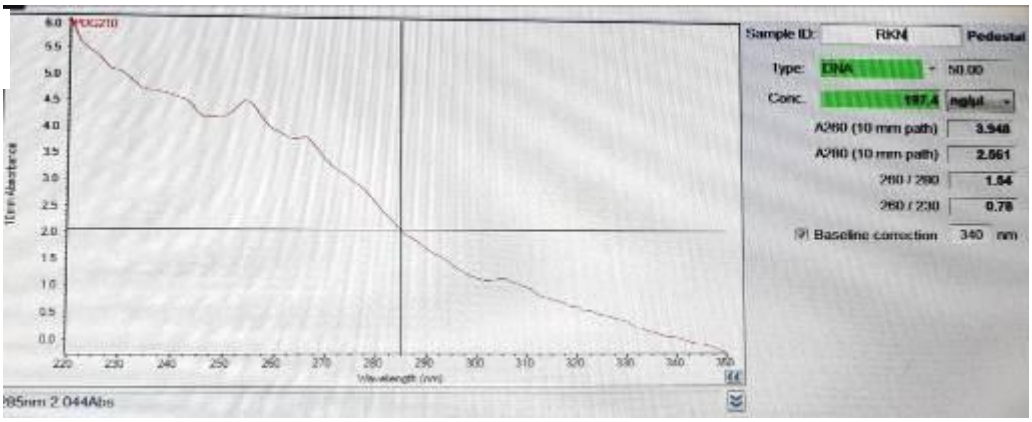


Fig.2 Phylogenetic relationship of root-knot nematodes infecting mulberry concluded by Tamura-Nei model of the 28S gene sequences. **sequences of this study.

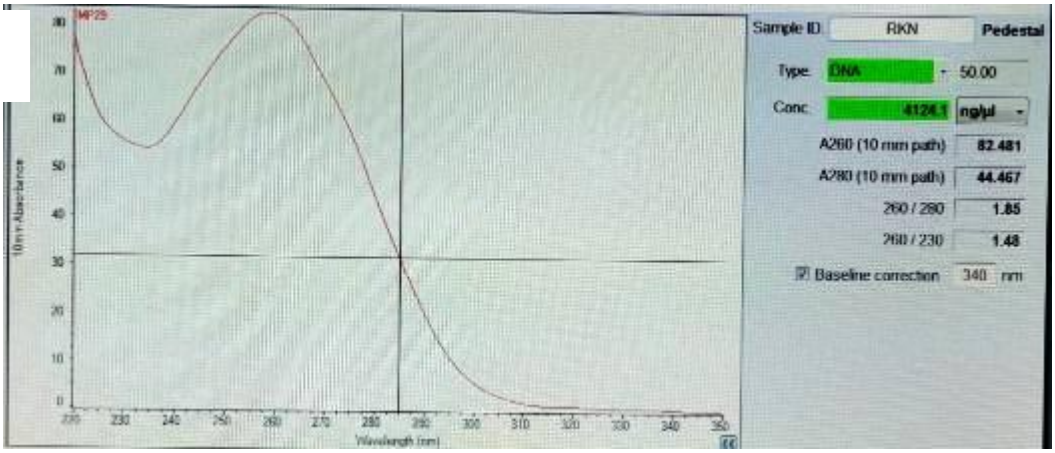


Supplementary Fig.1 Representative graph of DNA samples purity assessment.
(A) Common method of DNA isolation
(B) Modified method of DNA isolation.

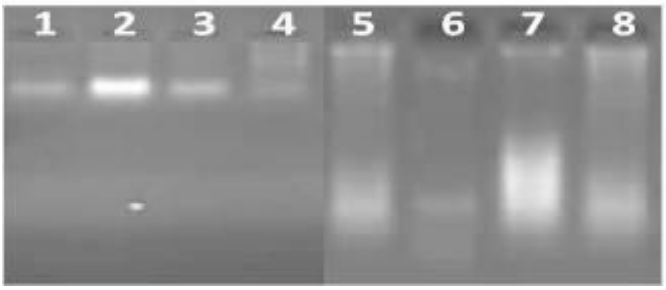
A



B



Supplementary Fig.2 Gel (0.8 %) pictures of genomic DNA isolated from nematodes using two methods. Lane 1-4, Modified CTAB; 5-8, Common CTAB method.



Similarly, Ye *et al.,* (2015) used the same set of primers to detect RKNs of turf grass. Compared with other molecular diagnosis (Adam *et al.,* 2005; Powers *et al.,* 2005; Berry *et al.,* 2008; McClure *et al.,* 2012; Holterman *et al.,* 2012; Zeng *et al.,* 2015), this assay requires only PCR and gel electrophoresis. However, in the present study for rapid detection of RKN, species-specific primers were used.

In Mulberry, several RKN species were reported based on the morphological characters (Toida, 1984; Ertian, 2003). *M. incognita* was found to be a common and *M. javanica* was rarely detected in mulberry (Swamy and Govindu, 1965, Sharma and Sarkar, 1998). Hence in this study, different sets of primers were used to differentiate the genus *Meloidogyne* and species *M. incognita*.

Finally, modified CTAB method for DNA extraction from galled roots of mulberry was found successful with high quantity and quality DNA which was efficiently amplified using PCR with RKN diagnostic markers. Hence, the modified CTAB method is found to be a simpler, effective and accurate.

Thus, this method could be useful to extract nematode DNA for further RKN characterization in mulberry. The RKN species *M. incognita* was found associated with mulberry in South India.

It was characterized for the first time using molecular markers in India to our knowledge. The phylogenetic analysis of the sequences also confirmed the results to a greater extent.

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Abbreviations

RKN - Root-knot nematodes
CTAB - Cetyl trimethylammonium bromide
PVP - Polyvinylpyrrolidone
NCBI - National Center for Biotechnology Information
DNA - Deoxyribonucleic acid
RNA - Ribonucleic acid
PCR - Polymerase Chain Reaction

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