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Development of a real-time PCR based protocol for quantifying *Radopholus similis* in field samples

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Abstract

The burrowing nematode, *Radopholus similis,* is an obligate migratory endoparasite. Currently detection of this nematode is carried out mostly by physically extracting them from soil and then observing under a light microscope. To identify this nematode, a thorough knowledge about their morphological features is quite indispensable. Developing a DNA based detection technique makes it more convenient and accurate in detection. Though PCR based methods have been reported by earlier workers, developing a real-time PCR based method will enable estimating their population in field samples. In this study, real-time PCR primers were designed using the DNA sequences from the ITS region of *R. similis.* It can detect *R. similis* up to the limit of 100 fg μL^{-1} DNA. The real-time PCR based detection serves as an efficient tool for the detection and estimation of this nematode from soil samples.

Keywords: burrowing nematode, diagnostics, ITS, *Radopholus similis*, real-time PCR, SYBR green

Introduction

The burrowing nematode, *Radopholus similis* (Cobb 1893; Thorne 1949) is an obligate migratory plant parasitic nematode, which is endoparasitic in nature. It is a highly polyphagous plant parasitic nematode and found to infect about 365 plant species including banana, black pepper, several palms and indoor decorative plants (Holdeman 1986). It enters and feeds in the cortex of the roots. This nematode occurs in tropical and subtropical areas around the world, and is seen mainly in Australia, Asia, Africa, and South America. *R. similis* is one of the ten most damaging nematodes in the world. More than 30 species are reported in the genus *Radopholus.* Out of these only *R. similis* is reported to be of economic significance. Slow decline disease of pepper, caused by *R. similis,* is a major problem for pepper cultivation (Thorne 1961). In black pepper it shows symptoms like yellowing of leaves, defoliation, necrotic lesions on the root leading to loss in productivity and slow death of the vine. It accounts for low productivity of black pepper in India.

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The damage to crops caused by plant-parasitic nematodes is related to their population densities in soil at sowing time and their reproduction potential. The management of the nematode largely depends on its early detection. Accurate identification of the nematode species is relevant for their effective management. The morphological and morphometric features allow classifying nematodes within their respective genus. The major advantage of this approach is that it is almost costless, but the drawback lies in the need for substantial expertise and training. It is very difficult to identify the species of nematodes as they are microscopic in size, similar in morphology and have very few distinguishable taxonomic characters.

PCR based molecular methods may overcome the drawbacks of traditional identification methods. Developing DNA based detection technique makes it more convenient and accurate in detecting the pathogen. Molecular based detection techniques like the conventional PCR were done on *Meloidogyne, Heterodera* and *Globodera* (Zijlstra 2000; Subbotin *et al.* 1999 & 2000). Though loop-mediated isothermal amplification (LAMP) and several PCR assays were developed for detection of *R. similis* (Ge *et al.* 2007; Wang *et al*. 2011; Aravind *et al.* 2011; Peng *et al*. 2012), they are not suitable for quantifying the nematodes in soil and plant samples.

Real-time PCR, although more expensive than conventional PCR, have the advantages of speed and sensitivity. In addition, this method provides quantitative information regarding the approximate number of nematodes present in the sample. Quantitative PCR assays have been designed for the plant-parasitic nematode genera *Bursaphelenchus* (Wang *et al.* 2005), *Meloidogyne* (Toyota *et al.* 2008), *Globodera* (Toyota *et al.* 2008; Madani *et al.* 2005), *Heterodera* (Goto *et al.* 2009), *Pratylenchus* (Berry *et al.* 2008; Sato *et al.* 2007) and *Xiphinema* (Berry *et al.* 2008). Here we describe the development of a real time PCR based detection tool for *R. similis* isolated from black pepper cropping system.

Materials and methods

Isolation of nematodes

The rhizosphere soil and root samples of black pepper vines showing symptoms of slow decline disease, like yellowing, stunted growth and black lesions on the root were collected from ten different locations of Kerala and Karnataka states of India. The samples were processed using the protocol described by Cobb and the identity of genus *Radopholus* was confirmed microscopically. The isolated nematodes were surface sterilized and cultured on carrot discs (Reise *et al.* 1987). Nematodes from these cultures were used for further studies.

Amplification, cloning and sequencing of ITS region

Molecular characterization of the ten isolates of nematodes was done by PCR amplification and sequencing of the internal transcribed spacer(ITS) region, using the primer pair, ITS-F (5″ - TTGATTACGTCCCTGCCCTTT -3″) and ITS-R (5″ - TTTCACTCGCCGTTACTAAGG -3″), based on the 18S rDNA and 26S rDNA sequences (Vrain *et al.* 1992). The DNA was extracted from the isolates using the protocol mentioned by Emmons *et al.* (1979). PCR amplification of the DNA was done using the amplification profile consisting of 5 min at 94 °C; 34 cycles of 30 sec at 94 °C, 1 min at 62 °C, and 1 min at 72 °C; and a final 10 min extension at 72 °C. Standard protocols were used for purifying the amplified fragments from the agarose gel by using Thermo Scientific Gene JET Gel Extraction Kit (Thermo Fisher Scientific Inc. MA USA). Eluted PCR products were cloned into pTZ57R/T vector (Thermo Scientific). Freshly prepared competent cells of *Escherichia coli* DH5α were transformed with recombinant plasmids and positive clones were selected and grown in LB medium. Plasmids were isolated and sequenced at M/s Eurofins Genomics India Pvt. Ltd., Bengaluru, India. The forward and reverse sequences obtained after sequencing were assembled using MEGA software (Kumar *et al.* 2016). The vector contamination in the assembled sequences was removed by using NCBI VecScreen. The sequences were edited using BioEdit version 7.2.5 (Hall 1999). A homology search of each sequence in FASTA format was done using BLAST-N programme (Altschul *et al.* 1997), to identify the closely related sequences from the GenBank database. All the sequences were submitted in NCBI.

Designing and optimization of real-time PCR primers

The primer set (RAD-F:AGACTTGATGAGCGCAGA and RAD-R: CGTGCCAGAGGAAGTGA) was designed from the ITS sequences obtained from the ten *R. similis* isolates. BLAST searches were used to compare the primers with other sequences in the public database to confirm the specificity of the primers. The primers were designed using the Primer 3+ software (Untergasser *et al.* 2007).

Standard graph for the real-time PCR based detection

DNA of the isolate RS01 was isolated from 2000 nematodes using phenol: chloroform (24:1) extraction method. Using this DNA sample as template, real-time PCR was done along with water and the DNA of *Meloidogyne* sp. and *Steinernema* sp. as negative controls using a qPCR thermocycler (Qiagen), Quantifast SYBR green 2x master mix (Qiagen) and 0.25 µM primer. The real time PCR profile used in the reaction for the above primer was: 95 °C for 10 min followed by 40 cycles of 95 °C for 10 sec, 60 °C annealing for 45 sec and a melt at 50–99 °C. All assays were repeated three times. A standard graph for the detection of *R. similis* was thus obtained.

Correlating Ct value to the number of R. similis

R. similis was grouped into sets of 1, 5, 10, 50, and 500 and DNA was extracted from each set. While extracting DNA from a single nematode or a set of very few nematodes, it was placed in a drop of extraction buffer placed on clean microscopic glass slide. Each nematode set mentioned above was cut into several pieces under a microscope. The nematode sample was then transferred into a small vial and DNA was extracted using the phenol: chloroform (24:1) extraction method described by Emmons *et al.* (1979). Real-time PCR was carried out using these DNA samples and the Ct values obtained were plotted on the standard graph previously created for the serial dilutions of nematode DNA obtained from 2000 nematodes. An approximate measure for the concentration and Ct values corresponding to the DNA samples extracted from a fixed number of *R. similis* was thus obtained. These values will be helpful in predicting the number of nematodes that is present in a sample with a particular Ct value.

Validation of real-time PCR based detection using field sample

In order to validate the real-time PCR method developed for the detection of *R. similis*, 30 soil samples from black pepper rhizosphere were collected from different regions of Kerala. To examine the reliability of the method, especially to detect the nematode in symptomless plants, samples were taken from apparently healthy plants around the infected plants. About 100 g of field soil sample was taken uniformly from each of the sample sites and the nematodes were extracted from the soil by Cobb's sieving and decanting method. The nematode suspension so obtained was subjected to microscopic observations. After quantifying the *R. similis* by direct microscopic examination, all the nematodes present in each sample was pelleted in Eppendorf tubes by centrifugation, the DNA was extracted and a qPCR assay was conducted by adopting the corresponding standardized protocols. The specificity of the qPCR product was also checked by subjecting to melt curve analysis from 60 ^{0}C to 95 ^{0}C. The Ct values of the reactions were calculated using the Rotor-Gene Q system software and the corresponding number of nematodes was calculated using the standard curve. A correlation analysis was conducted from the number of nematodes obtained using microscopic method and the values obtained from real time PCR based method using the software SAS 9.3.

Results and discussion

Accurate identification and quantification of plant parasitic nematodes are critical for making any plant management decision. However, the currently available traditional methods of diagnostic assays are not ideal as they are time consuming and require expertise in nematode identification. The conventional PCR-based detection methods and LAMP method for detection of *R. similis* do not provide any information about their population level in soil.

Several studies have clearly demonstrated that real-time PCR assays are well suited for detection, identification and even quantification of plant-parasitic nematodes. Real-time PCR assays have been developed for the root-knot nematodes *Meloidogyne javanica* (Berry *et al.* 2008), *M. chitwoodi*

and *M. fallax* (Zijlstra & Van Hoof 2006), *M. minor* (De Weerdt *et al.* 2011). They have been developed for the lesion nematodes *Pratylenchus zeae* (Berry *et al.* 2008), *P. thornei* (Yan *et al.* 2012), *P. scribneri* (Huang & Yan 2017), and for *P. penetrans* (Mokrini *et al.* 2013); for species specific identification and differentiation of *Ditylenchus dipsaci*, *D. destructor*, and *D. gigas* (Jeszke*et al.* 2015), for the identification of the dagger nematode *Xiphinema elongatus* (Berry *et al.* 2008), as well as *X. index*, *X. diversicaudatum*, *X. vuittenezi*, *X. italiae* (Van Ghelder *et al.* 2015). Real-time PCR assays have also been developed for species level identification of cyst nematodes of the genus *Globodera* and *Heterodera* (Gamel *et al.* 2017; Madani *et al.* 2005 & 2011), for the reniform nematode *Rotylenchus reniformis* (Sayler *et al.* 2012) and the pinewood nematode *Bursaphelenchus xylophilus* (François *et al.* 2007). So it was felt that a qPCR assay is a potentially useful alternative to the current molecular tools as it provides quantitative information about the nematode.

DNA isolation, PCR amplification and sequencing of ITS region

For developing a qPCR protocol for *R. similis*, ten different isolates of *R. similis* were obtained from infected black pepper soils in Kerala and Karnataka. DNA from these isolates was extracted using phenol: chloroform (24:1) extraction method and then the ITS region of each of these DNA was amplified and sequenced. PCR amplification of ten isolates of *R. similis* DNA using the universal primers of the rDNA-ITS region generated approximately 920 bp long products. These sequences showed similarity to the ITS regions of *R. similis* isolates reported worldwide. Nucleotide sequences of PCR products from these ten isolates were deposited in NCBI (Table 1).

Primer designing and quantitative real-time PCR

A species-specific real-time PCR primer pair (Rad F and Rad R) was designed using these sequences to specifically amplify a 227 bp product. It showed similarity to all the *R. similis* nematode ITS sequences deposited in GenBank. Real-time PCR analysis was carried out with the DNA isolated from *R. similis* along with water and negative control. The primers specifically amplified *R. similis* DNA (Fig. 1). A standard curve was generated using DNA isolated from 2000 *R. similis* and serially diluted with water in a 1:1 dilution series (Fig. 2). This standard curve would later help in identifying the approximate concentration of *R. similis* nematode(s) in any unknown sample based on the cycle threshold (Ct) value of that sample. The Ct values were correlated with the DNA template concentration (R^2 = 0.996), indicating the validity of the assay and its potential for quantification of target DNA (Table 2). The potential of the RT-qPCR technique in detection of the number of nematodes present in a sample was determined. This data provided

Table 1. Details of GenBank accession numbers for the ITS sequences of the ten isolates of *R. similis*

Isolate No.	Location	Host plant	GenBank Accn. No.
IISR_RS01	Kozhikode, Kerala	Black pepper	MF197883
IISR_RS02	Idukki, Kerala	Black pepper	MF197884
IISR_RS03	Kozhikode, Kerala	Banana	MF197885
IISR_RS04	Malappuram, Kerala	Banana	MF197886
IISR_RS05	Sirsi, Karnataka	Black pepper	MF197887
IISR_RS06	Wayanad, Kerala	Black pepper	MF197888
IISR_RS07	Malappuram, Kerala	Black pepper	MF197889
IISR_Rs08	Thiruvananthapuram, Kerala	Black pepper	MF197890
IISR_RS09	Thrissur, Kerala	Black pepper	MF197891
IISR_RS10	Kozhikode, Kerala	Black pepper	MF197892

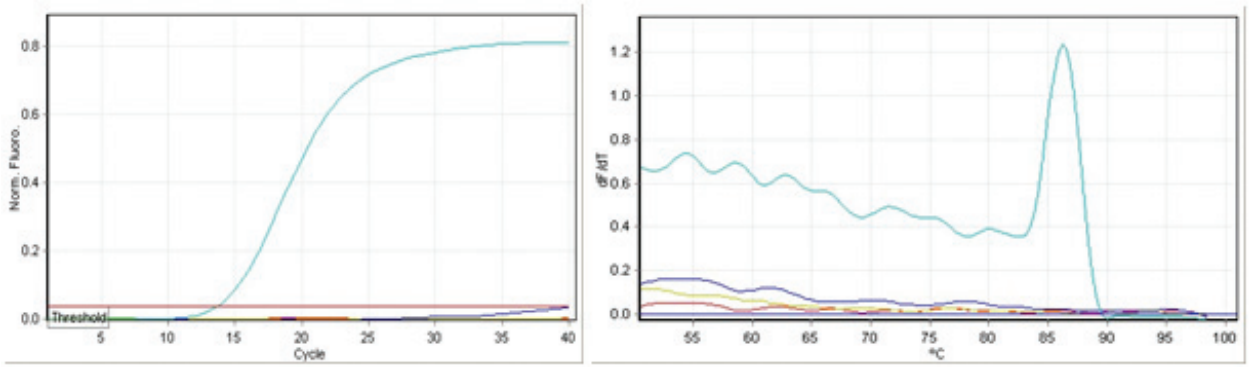

Fig. 1. Real-time PCR detection of *R. similis* (A) Amplification curves of real-time PCR obtained with positive, negative and water control; (B) Melt curve analysis of real-time PCR product

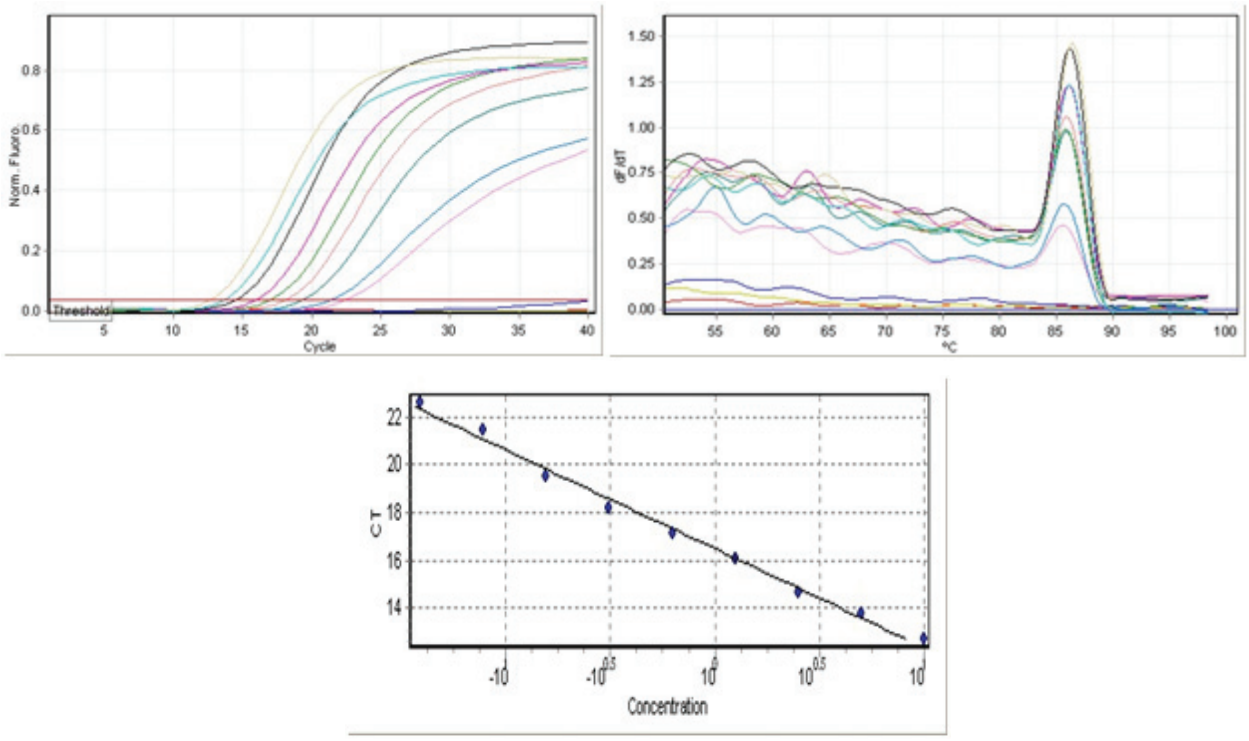

Fig. 2. Standard curve of real-time PCR obtained with a 1:1 dilution series of *R. similis* DNA: (A) Amplification curve, (B) Melt curve analysis and (C) Standard graph

the information regarding the approximate number of *R. similis* present in any test sample based on their Ct value. The DNA from nematodes ranging from 1–500 was correlated to Ct values (Table 3). The PCR assay also detected DNA from a single nematode of *R. similis*, which was 0.003 ng μL^{-1}. Further, on 1:10 dilution of the DNA isolated from single nematode, the Ct values obtained were 29.30 and 31.93, respectively. The target DNA was detectable in suspensions diluted in water containing as little as 100 fg of *R. similis* DNA.

R. similis was quantified directly in DNA extracts from its pure culture using a species specific qPCR. The assay was sensitive and detected 10^{-2} dilution of genomic DNA of a single juvenile nematode. The sensitivity of our *R. similis* assay was comparable with that observed from other qPCR assays. Wang *et al*. (2011) reported a PCR assay for detection of *R. similis* ITS-rDNA equivalent to 4×10^{-3} nematodes. Madani *et al.* (2005) was able to detect a single second-stage juvenile of the cyst-forming nematodes *Globodera pallida* and *H. schachtii* using qPCR with SYBR Green I dye. Sato *et al.* (2007) could detect a single *P. penetrans* individual in a sample with abundant number of free-living nematodes using qPCR.

Table 2. Ct value vs. DNA concentration obtained using real-time PCR with a 1:1 dilution series of *R. similis* DNA

Sample	Ct value	DNA concentration (ng μL^{-1})
No template control (Water)	-	-
Negative control (DNA of *Meloidogyne* sp.)	-	-
Negative control (DNA of *Steinernema* sp.)	-	-
Sample 1 (DNA isolated from 2000 *R. similis*)	12.71	8.266
Sample 2 (1:2 dilution of sample 1)	13.74	4.655
Sample 3 (1:2 dilution of sample 2)	14.62	2.823
Sample 4 (1:2 dilution of sample 3)	16.04	1.2812
5 Sample 5 (1:2 dilution of sample 4)	17.13	0.6979
6 Sample 6 (1:2 dilution of sample 5)	18.22	0.3795
7 Sample 7 (1:2 dilution of sample 6)	19.49	0.1854
8 Sample 8 (1:2 dilution of sample 7)	21.41	0.0642
9 Sample 9 (1:2 dilution of sample 8)	22.58	0.03314

Table 3. Ct value Vs Concentration of DNA obtained using real-time PCR with different numbers of *R. similis*

Number of nematodes	Ct nematodes	Concentration (ng μL^{-1})
500	18.05	0.41
50	20.59	0.10
10	21.73	0.05
5	24.39	0.01
1	26.75	0.003

Toyota *et al.* (2008) sensitively detected a single second-stage juvenile of *G. rostochiensis* in mixed nematode communities of 1,000 free-living individuals. Yan *et al.* (2012) developed a qPCR assay for *P. thornei* and detected one second-stage juvenile in 1 g of sterilized soil.

Validation of the real-time PCR based detection using field sample

The real-time PCR method developed for detection of *R. similis* in infected black pepper rhizosphere soil was validated using 30 field samples collected from different regions of Kerala. Among the 30 samples collected, 15 vines showed typical yellowing and 15 vines appeared to be uninfected with *R. similis.* However, when these soil samples were processed for nematode extraction and were subjected to direct microscopic count, a few samples taken from vines showing yellowing symptoms did not show the presence of *R. similis* and a few vines which appeared to be healthy were infected by the nematode. Further, the DNA from these samples was subjected to qPCR and the number of nematodes in each sample was estimated based on the Ct value obtained. The details are given in Table 4. In the correlation analysis conducted using the number of nematodes obtained using microscopic method and the estimated population obtained from the real-time PCR based method, a positive correlation was observed (correlation coefficient = 0.94 at $P<0.01$). However, few samples which did not show the presence of any *R. similis* when observed microscopically showed a Ct value which corresponds to 1:100 dilution of a single *R. similis* DNA. This could be due to the presence of one by hundredth fraction of a degraded nematode, which was present in the soil and escaped through the pores of the sieve during nematode extraction process.

In this study, we developed a qPCR assay for *R. similis* based on the ITS region. The technique

Table 4. Field validation of real-time PCR protocol for estimating burrowing nematodes

Location	Yellowing and wilting	No. of *R. similis* observed through sieving and counting	Real-time PCR	
			Ct value	Estimated range of *R. similis* population
Kozhikode	+	3	25.02	1–5
Kozhikode	+	1	27.14	0–1
Kozhikode	+	5	24.03	5–10
Palakkad	+	9	22.59	5–10
Malappuram	+	0	31.42	0–1
Palakkad	+	9	22.53	5–10
Kozhikode	+	1	26.99	0–1
Malappuram	+	0	29.69	0–1
Kozhikode	+	0	29.99	0–1
Palakkad	+	8	23.11	5–10
Kozhikode	+	2	26.32	1–5
Palakkad	+	4	24.70	1–5
Malappuram	+	5	24.56	1–5
Palakkad	+	4	25.32	1–5
Palakkad	+	0	30.40	0–1
Kozhikode	-	0	30.27	0–1
Kozhikode	-	0	28.48	0–1
Malappuram	-	1	27.02	0–1
Malappuram	-	6	23.58	5–10
Palakkad	-	4	25.27	1–5
Malappuram	-	0	33.30	0
Malappuram	-	0	-	0
Palakkad	-	0	35.77	0
Palakkad	-	0	34.15	0
Malappuram	-	0	31.98	0
Malappuram	-	0	32.80	0
Kozhikode	-	0	31.77	0–1
Kozhikode	-	0	32.60	0
Palakkad	-	0	31.28	0–1
Malappuram	-	0	31.69	0–1

developed could be effectively used for the detection of *R. similis* from field samples.

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