CHARACTERIZATION AND DEVELOPMENT OF MOLECULAR DIAGNOSTICS FOR BURROWING NEMATODE INFECTING BLACK PEPPER

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By

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CERTIFICATE

This is to certify that the corrections suggested by the adjudicators have been incorporated and the thesis entitled "**Characterization and development of molecular diagnostics for burrowing nematode infecting black pepper**" has been revised by **Ms. Krishna P B** for submission to University of Calicut prior to the conduct of the final open defense for awarding the degree of **Doctor of Philosophy** in **Biotechnology.**

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This is to certify that the thesis entitled '**Characterization and development of molecular diagnostics for burrowing nematode infecting black pepper',** submitted by **Krishna P. B**., for the award of degree of Doctor of Philosophy in Biotechnology of the University of Calicut, is a *bona fide* record of research work done by her at ICAR-Indian Institute of Spices Research, Kozhikode under my guidance. No part of this thesis has been submitted earlier for the award of any other degree, diploma or other similar title or recognition. All sources of help received by her during the course of this investigation have been duly acknowledged.

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Date :

Dr. Santhosh J. Eapen
(Guide)

Dr. K. Nirmal babu
(Co-guide)

Dedicated with much love and affection to

my beloved parents

DECLARATION

I, **Krishna P. B.**, hereby declare that this thesis entitled '**Characterization and development of molecular diagnostics for burrowing nematode infecting black pepper'** submitted for the award of degree of Doctor of Philosophy in Biotechnology of the **University of Calicut**, is a bonafide record of research work done by me at ICAR-Indian Institute of Spice Research, Kozhikode under the guidance of **Dr. Santhosh J. Eapen**, Head, Division of Crop Protection, ICAR-Indian Institute of Spices Research, Kozhikode and Co-guidance of **Dr. K. Nirmal Babu**, Director, **ICAR-Indian Institute of Spices Research**, Kozhikode. This thesis or part of it has not been submitted to any University for the award of any degree or diploma.

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Sl. No.	Abbreviations	Expanded form
1.	µl	Microlitre
2.	µm	Micrometre
3.	AFLP	Amplified Fragment Length Polymorphism
4.	AIC	Akaike Information Criterion
5.	ANOVA	Analysis of variance
6.	BA	Bayesian analysis
7.	BLAST	Basic Local Alignment Search Tool
8.	bp	base pair
9.	COX	Cytochrome c oxidase
10.	Ct	Cycle threshold
11.	CV	Coefficient of Variation
12.	ddNTP	Dideoxy nucleotides triphosphates
13.	DNA	Deoxyribonucleic acid
14.	dNTP	Deoxy nucleotides triphosphates
15.	FAA	Formaldehyde glacial acetic acid
16.	Hsp	Heat shock protein
17.	IPTG	Isopropyl β-D-1-thiogalactopyranoside
18.	LAMP	Loop-mediated Isothermal Amplification
19.	LB	Luria Bertani
20.	LSD	Least Significant Difference
21.	MCL	Maximum Composite Likelihood
22.	ME	Minimum Evolution

23.	MEGA	Molecular Evolutionary Genetics Analysis
24.	ML	Maximum likelihood
25.	MP	Maximum parsimony
26.	NCBI	National Center for Biotechnology Information
27.	ND	NADH dehrogenase
28.	NJ	Neighbour joining
29.	PCR	Polymerase chain reaction
30.	PGI	Phosphoglucose isomerase
31.	qPCR	Quantitative Polymerase chain reaction
32.	RAPD	Random Amplification of Polymorphic DNA
33.	RFLP	Restriction Fragment Length Polymorphism
34.	RNA	Ribonucleic acid
35.	SCAR	Sequence characterized amplified regions
36.	SDS	Sodium dodecyl sulfate
37.	SE	Standard errors
38.	SEM	Scanning electron microscope
39.	Tm	Melting temperature
40.	X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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INTRODUCTION

Phylum Nematoda is highly diverse in terms of species richness and is one of the most abundant metazoan groups on earth. It is estimated that nematodes comprise nearly 90% of all multicellular organisms. Plant parasitic nematodes are recognized as a serious constraint to crop productivity in almost all countries. These nematodes continue to be amongst the most damaging and difficult-to-manage agronomic pests. Due to this reason and also due to the large scale use of highly toxic nematicides, it has become imperative for the agricultural scientists to develop alternative management strategies. Nematode rendered damages often go unnoticed or are overlooked as the damages caused by them are not easily identifiable. Further, these damages often resemble nutrient deficiency, moisture stress, etc. Out of the 15,000 nematode species described, 2200 are plant parasites. They are considered one of the most difficult organisms to identify due to their microscopic size, morphological similarity, limited number of distinguishable taxonomic characters and overlapping morphometric measurements. There is an increasing demand for developing molecular-based diagnostic protocols to identify pests and pathogens, due to decline in classical taxonomic expertise. Application of molecular techniques can be useful in species taxonomy, including nematodes, only when combined with taxonomic skills which are declining. Thus, molecular approaches should not be considered in isolation, but a balanced molecular and morphological taxonomic approach is required. (Tautz *et al*. 2003).

R. similis is a highly polyphagous parasitic nematode and is able to attack a wide range of agronomic and horticultural crops as well as many weeds (O'Bannon 1977). To date, this nematode has been observed in tropical and sub-tropical regions infecting more than 365 plant species belonging to several families. Black pepper, an

important host of *R. similis* is cultivated as a mixed crop in homesteads, trailed on several crops and is grown as a companion crop in several plantations in Kerala. Such crop combinations play an important role in building up populations of polyphagous plant parasitic nematodes like *R. similis.* As knowledge of genetic variation within *R. similis* is essential for breeding programmes for resistance to this nematode (Costa *et al*. 2008), the interest in genetic variation at the intraspecific level and the possible existence of physiological races or biotypes in *R. similis* has increased (Kaplan and Gottwald 1992; Hahn *et al.* 1994, 1996).

So far 50 species of nematodes belonging to the genus *Radopholus* have been reported. However at a later stage several species were transferred to other related genera like *Hirschmannia*, *Pratylenchoides, Radopholoides, Achlysiella* and *Zygradus* based on the morphological studies. Few other species were found to be synonymous to previously reported species. Today only 30 species are accepted as strictly belonging to the genus *Radopholus*. Among these *R. similis* is the only quarantine plant parasitic nematode which has an adverse impact on crops.

Morphological studies using light microscopy and morphometric analysis were done to understand the diversity in and among populations of *R. similis*. The molecular variations in *R. similis*, populations from many host plants and several countries have been characterised using different molecular methods like RAPD, RFLP, AFLP and sequence analysis of rDNA ITS1 and the rDNA D2/D3 expansion segments. Biochemical characterization studies were conducted using the isozymes-phosphoglucoseisomerase, lactate dehydrogenase, and malate dehydrogenase.

In India, studies on diversity of *R. similis* were done with populations collected from different localities of South India (Koshy *et al*. 1991) and Gujarat (Khan *et al*. 2016) using the morphological and morphometric methods. Single tube duplex PCR based method was developed to detect *P. capsici* and *R. similis* in infected roots of black pepper using the conserved internally transcribed spacer (ITS) region of Oomycetes pathogen, *P. capsici* and rDNA sequences of nematode pathogen, *R. similis* (Aravind *et al.* 2011).

Currently traditional methods of diagnostic assays, conventional PCR-based detection methods and LAMP method are available for the detection of *R. similis.* qPCR assay is a potentially useful alternative to the current molecular tools as it provides information about their number.

OBJECTIVE

- Morphological and molecular characterization of *Radopholus* spp. in black pepper cropping system.
- Developing Real Time PCR based diagnostic tool for the detection of *Radopholus* spp.

SIGNIFICANCE OF THE STUDY

Morphological and molecular characterization of *Radopholus* spp., the economically important plant parasitic nematode of black pepper, will help to understand its evolutionary significance and to develop better management strategies. The diversity study will help to develop a diagnostic tool for the detection of *Radopholus* spp.

REVIEW OF LITERATURE

2.1. Plant parasitic nematodes of black pepper

Several plant parasitic nematodes belonging to different groups are reported in association with black pepper. The compilation of plant parasitic nematodes associated with pepper in the major growing countries by Sundararaju *et al.* (1979) listed 48 species belonging to 29 genera, while Ramana and Eapen (1998) listed 30 genera and 54 species on pepper. In India, 17 genera of nematodes were recorded in Kerala and Karnataka, the two major pepper-growing states (Sundararaju *et al.* 1980). Plant parasitic nematodes belonging to 14 genera in association with pepper were reported in the detailed surveys conducted during 1980s in Kerala and two districts in Karnataka (Ramana and Mohandas 1989) while in Indonesia, 14 genera of plant parasitic nematodes were associated with pepper (Mustika and Zainuddin 1978, Bridge 1978). Among them *Meloidogyne* spp., *Radopholus similis, Trophotylenchulus piperis, Helicotylenchus* spp. and *Rotylenchulus reniformis* are predominant in India (Jacob and Kuriyan 1980, Ramana and Mohandas 1989). The economic damage caused by many of these species are yet to be established. However, *R. similis* and *Meloidogyne* spp. are of much economic significance as they cause severe damage to pepper and are implicated in the slow decline/yellows disease, a major production constraint in all pepper growing countries.

Slow decline disease of pepper, otherwise known as 'yellows' disease or 'slow wilt' disease is a major problem for pepper cultivation. The above ground symptoms of the disease are yellowing of leaves, defoliation, die-back, loss in vigour and productivity, leading to slow death. On roots, nematode infestation results in the formation of necrotic lesions and rotting caused by *R. similis* resulting in total loss

of feeder roots and galls due to root-knot nematodes. The disease is prevalent in most pepper growing countries like India (Nambiar and Sarma 1977), Malaysia (Kheng 1979), Brazil (Sharma and Loof 1974, Ichinohe 1975) and Thailand (Sher 1969, Bridge 1978). The disease was first observed in Bangka islands (van DerVecht 1950). The annual loss of production was estimated to be up to 32 per cent (Sitepu and Kasim 1991). Crop loss estimates due to this disease in India are not available though Menon (1949) reported about 10 per cent mortality of pepper plants in Kerala.

2.2. *Radopholus* (Thorne 1949)

The endoparasitic migratory nematodes belonging to the genus *Radopholus* are small worm-like organisms measuring between 0.4 and 0.9 mm in length, which are plant parasitic in nature. Till date there are reports of 50 species of nematodes belonging to the genus *Radopholus*. But today only 30 species are now accepted as strictly coming under the genus *Radopholus*. The different species in the genus *Radopholus*, are assumed to be native to either the Indo-Malayan or the Australasia regions (Duncan and Moens 2006).

The burrowing nematode, *R. similis* (Cobb 1893) Thorne 1949 is the only *Radopholus* species of widespread economic importance (Duncan and Moens 2006; Jones *et al.* 2013). Species identification is mainly based on the examination of morphological and morphometric characteristics. The pharyngeal glands of these nematodes overlap the intestine dorsally and laterally. Females have fully developed anterior and posterior gonads. The genus is characterized by strong sexual dimorphism. Compared with the low head, the strongly sclerotized cephalic framework and the normal stylet and pharynx in females, the males have a higher

and more off-set head and markedly reduced cephalic framework, stylet and pharynx (Siddiqi 2000; Duncan and Moens 2006).

2.2.1. Life cycle

The burrowing nematode, *R. similis*, usually reproduces sexually, but sometimes also parthenogenetically. It is a migratory endoparasite that completes its life cycle within the root cortex. All larval stages and adult females of *R. similis* are infective and capable of penetrating the roots at any point of their length, but entry is usually behind the root tip (Duncan and Moens 2006). Once entering the roots, the nematodes move intercellularly in the cortical parenchyma where they feed on the cytoplasm of nearby cells, causing lesions, cavities and root break-down (Anonymous 2014). Each female lays, on an average, four to five eggs each day (two each day in citrus species) for several weeks (Duncan and Moens 2006; Brooks 2008). At optimum temperatures, egg hatch occurs after two to three days on some hosts and up to seven days in others (Duncan and Moens 2006). After hatching from an egg, the emerging second-stage juvenile (J2) can migrate within the root and undergo a series of three moults through the third and fourth juvenile stages to reach the adult stage. The nematode completes its life cycle in about 21 days at 25 °C.

2.2.2. Symptomatology

R. similis, can parasitize root tissues of host plants causing extensive cavitation and characteristic reddish-brown to black lesions that form cankers. It causes a decline of many plant species; however, the degree of host-plant susceptibility to this nematode is very variable, as one host may be severely damaged whereas damage

to other hosts may be insignificant (Thorne 1962). Plantain (*Musa* hybrids and cultivars), citrus (*Citrus* spp.) and black pepper (*Piper nigrum*) showed typical symptoms while infected by *R. similis* (Brooks 2008).

The attack by *R. similis* on citrus trees is described as spreading decline. This disease was first recognized in Florida in the late 1920s. Symptoms in citrus species caused by burrowing nematodes include yellowing, stunting, reduction in the number and size of leaves and fruits, a delay in flowering and overall sparse foliage of fruit trees. In bananas, *R. similis* causes the so-called toppling disease (plants become uprooted and topple over), which is most dramatically expressed during banana fruiting, during strong winds or if heavy rains loosen the soil (Blake 1972; Gowen and Quénéhervé 1990; Brooks 2008).

In black pepper (*Piper nigrum* L.), the burrowing nematode causes the so-called yellows disease or slow decline disease of black pepper. This disease was first observed in the Indonesian island of Bangka (Thorne 1962). The destruction of roots leads to reduced water and nutrient uptake. This is consequently expressed as above ground symptoms of infested plants, such as pale yellow or whitish-yellow leaves that drop and then fall off from the vine, slow plant growth, flower drop and vine dieback.

2.2.3. Distribution and economic importance

R. similis is an important quarantine plant parasitic nematode which has an adverse impact on crops.It is found worldwide in tropical and sub-tropical areas of Africa, Asia, Australia, North and South America and many island regions (Sekora and Crow

2002), and is considered one of the 10 most damaging plant-parasitic nematodes worldwide (Sasser *et al*. 1987).

The species is seen in abundant numbers in majority of banana-growing areas around the world, although it seems to be low in numbers in few banana-producing countries like Israel, the Canary Islands, the Cape Verde Islands, Cyprus, Crete, Mauritius, the highlands of East Africa and Taiwan (Marin *et al*. 1998). *R. citropholus* has seen a limited presence of existence restricted to Florida, parts of the Caribbean, Cote d'Ivoire and Guyana (Brooks 2008).

The most important hosts for *R. similis* all share a common Centre of origin in the Indo-Malayan region, which suggests that this region is most probably the region of origin for this nematode species (Marin *et al.* 1998). From there, it has spread into new areas throughout the world on underground parts of infested planting material, such as rootstocks, corms and tubers, and in accompanying soil (Duncan and Moens 2006).

R. similis is a highly polyphagous nematode that can invade and feed in the cortex of roots of more than 365 plant species belonging to several families (e.g. Rutaceae, Arecaceae, Araceae, Marantaceae, Musaceae, Poaceae, Brassicaceae, Rubiaceae, Solanaceae, Piperaceae, Zingiberaceae, Theaceae, Bromeliaceae, Lauraceae, Fabaceae, Apiaceae, Dioscoraceae, Schisandraceae, Pinaceae, Rosaceae) (Brooks 2008; Moens and Perry 2009).

Although it has a broad host range, *R. similis* is probably most often disseminated to banana plantations owing to frequent exchanges of infected rhizomes, and is now widespread in tropical and sub-tropical areas (O'Bannon 1977). *R. similis* is also

present in glasshouses in some parts of Europe, where it was introduced in the 1960s with ornamental plants, in particular *Anthurium* (Anonymous 2008).

R. similis cannot move actively over very long distances. However, owing to its wide host range and virulent behaviour, the nematode has been reported to move efficiently locally (Duncan and Moens 2006) and, in citrus groves, *R. similis* has been reported to spread at a rate of 6–60 m per year, with averages of 15 m annually (Suit and DuCharme 1953). Based on the studies conducted in Honduras, the rate of spread of *R. similis* on Valery banana was approximately 2.5 m in one year (O'Bannon 1977). Locally, *R. similis* can also be disseminated through soil, water or other organisms (O'Bannon 1977). Duncan and Moens (2006) reported that this nematode can be dispersed by the movement of soil for construction purposes or on agricultural machinery. *R. similis* can survive in host-free soil for about six months (Inserra *et al.* 2005; Brooks 2008; Chabrier *et al.* 2010). Many authors (Faulkner and Bolander 1970) consider that these nematodes can mainly be spread by water. *R. similis* has been reported, in Florida and Jamaica, to be spread by water in citrus orchards and banana plantations, respectively (Chabrier *et al*. 2009).

2.2.4. Taxonomy

The genus *Radopholus* Thorne, 1949 was proposed in order to include those Pratylenchinae which closely resembling the genus *Pratylenchus* (Filip'ev 1934), but have two ovaries. The burrowing nematode was described by Cobb in 1893 from diseased banana roots collected in Fiji in 1891 (Volcy 2011). In the same publication, Cobb separately described males and females as two different new species (*Tylenchus similis* and *Tylenchus granulosus*, respectively) (Esser *et al*. 1984; Luc 1987). While studying roots of diseased sugarcane in Hawaii, Cobb in

1909, illustrated and described females and males of *Tylenchus biformis* but made no reference to his previous publications. Several years later, Cobb investigated nematodes collected from banana roots from Jamaica and concluded that *T*. *biformis* was actually *T*. *similis*. Consequently, a more detailed description of the species was published in 1915 (Thorne 1962), which allowed Cobb to transfer *T*. *biformis* and *T*. *granulosus* to *T*. *similis* (Luc 1987; Volcy 2011). Thorne in 1949 proposed, a new genus, *Radopholus*, keeping *R*. *similis* as the type species after studying specimens from sugarcane in Hawaii and two females from roots of pepper from the East Indies. (Esser *et al*. 1984; Volcy 2011). Table 1 summarizes the taxonomic investigations in burrowing nematodes and the present status of valid *Radopholus* species.

2.2.5. Morphological studies

Diversity among burrowing nematode populations has been analyzed through morphological and morphometric studies by several workers, which are summarized in table2.

Table 1: Summary of taxonomic investigations in burrowing nematodes and present status of valid *Radopholus* species.

Species	Present status	Key features	Reference
R. similis	Type species	Females: • Dome-shaped head with three to four annules, • Stylet knobs of equal size, • Lateral field with four equally spaced incisures at mid-body and three incisures between the phasmid and tail tip, • Both gonads having spermathecae of equal size and containing rod-like sperms, • Elongate-conoid tail with a narrow rounded or indented terminus (De Waele and Elsen 2002; Duncan and Moens 2006; Anonymous 2014). • The bursa is coarsely crenate, enveloping about two-thirds of the tail (Duncan and Moens 2006).	Cobb (1893)

R. oryzae	Redefined as *Hirschmannia* (Luc and Goodey1962)		Van Breda de Haan (1902)
R. gracilis	Redefined as *Hirschmannia* (Luc and Goodey 1962)		de Man (1880)
R. behningi	Redefined as *Hirschmannia* (Luc and Goodey 1962)		Micoletzky (1922)
R. zostericola	Redefined as *Hirschmannia* (Luc and Goodey 1962)		Allgen (1934)
R. gigas	Redefined as *Hirschmannia* (Luc and Goodey 1962)		Andrássy (1954)
R. lavabri	Redefined as *Hirschmannia* (Luc and Goodey 1962)		Luc (1957)
R. mucronatus	Redefined as *Hirschmannia* (Luc and Goodey 1962)		Das (1960)
R. paludosus	Considered as *nomen nudum*		Whitlock (1957)
R. neosimilis	New species	Khan and Shakil (1973) proposed the genus *Neoradopholus* for *R. neosimilis* and *R. inaequalis* based on the position of the caudal alea. Siddiqi (1986) disproved this argument and supported this taxon as a junior synonym of *Radopholus.* • More anterior position of the vulva, • Flattened lip region,	Sauer (1958)

		• Shorter hyaline area in tail terminus • Usually longer stylet and greater body length.	
R. inaequalis	New species	• Longer stylet with pointed anterior projections on the stylet knobs • Five or more lateral incisures in the lateral field	Sauer (1958)
R. williamsi	Re described as *Achlysiella* (Hunt *et al.* 1989)		Siddiqi (1964)
R. cavenessi	Considered as species *inquirenda* (Ryss 2003)		Egunjobi (1968)
R. nativus	New species	• Longer stylet and absence of sperms in the spermatheca and males compared to the closely related species *R. neosimilis* and *R. vangundyi* • Distinguished from *R. vangundyi* by the four lip region annules; • Compared to the type species the lip region is slightly or not set off, hemispherical or sometimes slightly flattened anteriorly; stylet knobs are flattened anteriorly or with anterior projecting processes; areolation of the lateral field varies from almost complete areolation to incomplete areolation only in the tail region.	Sher (1968)

R. magniglans	New species	• Long conspicuous esophageal glands, usually two lip region annules and absence of male specimens. • The face view is similar to the type species with the lateral lips distinctly set off from the other lips. The lip region usually has two annules, three annules are seen in a few specimens. • Stylet knobs are flattened anteriorly or with slightly anterior projections. • The intestine appears to extend into the tail region. • Annulation around the tail terminus is often irregular and indistinct.	Sher (1968)
R. rotundisemenus	New species	• Compared with the closely related *R. inaequalis* the sperms in the spermatheca are round; fewer incisures in the lateral field and usually shorter stylet. • The face view is similar to the type species. • Stylet knobs are flattened or usually with anterior projections (often pointed).	Sher (1968)

		• Four to six, usually irregular incisures are in the lateral field near the center of the body. • The intestine overlaps the rectum into the tail but is indistinct on most specimens. • The annulation around the tail terminus is distinct but sometimes irregular.	
R. trilineatus	New species	• Can be distinguished from the closely related *R. magniglans* by the three incisures of the lateral field in the female; more anterior position of the vulva (59-63) and the usually shorter stylet length; similar face view. • The lip region usually has two annules, some specimens without annulation. • Stylet knobs are usually round, sometimes slightly flattened anteriorly. • The intestine appears to extend into the tail region.	Sher (1968)

		• The annulation on the tail termination is irregular and usually incomplete.	
R. vangundyi	New species	• Can be distinguished from the closely related *R. neosimilis* by the hemispherical female lip region shape; more tapering female tail; usually smaller female body size; the higher lip region without annulation in the male. • In face view the female has six similar lips; the lateral lips slightly set off (similar to the type species); amphid apertures are seen on the lateral lips. • In the male face view the lips are set off with the lateral lips distinctly smaller (similar to the type species). • The female lip region has three to four annules. • The stylet knobs are round to slightly flatten anteriorly. • Incomplete aerolation of the lateral field in the tail region is seen on some of the females.	Sher (1968)

		• The tail is tapering to a rounded (often almost pointed) terminus.	
R. vertexplanus	New species	• Longer tail with annulations around the terminus. • Higher position of the vulva, the absence of sperms in the spermatheca and males and the flatter lip region. • Six similar lips without longitudinal striations are seen in face view. The lip region is slightly or not set off with two or three faint often indistinct annules. • Stylet knobs are rounded to usually flatten anteriorly. • The intestine indistinctly overlaps the rectum. • The spermatheca is often inconspicuous and without sperms. • The tail tapers to a rounded or irregularly rounded, sometimes almost pointed terminus.	Sher (1968)
R. nigeriensis	Redefined as *Zygradus* (Siddiqi 1991).		Sher (1968)

R. ritteri	New species	• Initially placed in the genus *Pratylenchoides* but Vovlas (1978) indicated that *P. ritteri* is more closely related to the genus *Radopholus* based on the unique oesophageal structure.	Sher (1970)
R. rectus	New species	• Closely resembles *R. neosimilis* but can be distinguished from this species by the shape of the sperms.	Colbran (1971)
R. crenatus	New species	• Closely resembles *R. inaequalis* but can be distinguished by the fewer incisures in the lateral fields and the dorsal stylet knob is not abnormally enlarged. • The distinctive lateral clefts on the male head have not been observed in other *Radopholus* species. • Females usually lie straight when mounted. • Sperm shape is distinct, ranging from ovate to long thick rods. • Ovaries are occasionally reflexed. • Female tails may be slightly clavate.	Colbran (1971)

R. intermedius	New species	• Slender body, which is strongly curved when relaxed. • Reduced posterior gonad • Short stylet • Relatively long tail with short hyaline area and unstriated terminus. • Resembles *R. laevis* in overall appearance but differs in having a posterior ovary with spermatheca and more anteriorly placed vulva.	Colbran (1971)
R. serratus	New species	• Closely resembles *R. vertexplanus*, from which it is distinguished by the shape of the sperms and stylet knobs. • In some paratypes the sperms are thick, rod-like (much shorter and thicker than in *R. similis*), whereas in others they vary from round to short, ellipsoidal. • In some paratypes the caudal alae envelop little more than half the tail.	Colbran (1971)
R. megadorus	New species	• Very large stylet knobs and small unstriated lip region with heavy internal sclerotization.	Colbran (1971)

		• The male lip region is larger and more spherical.	
R. inanis	New species	• Resembles *R. vacuus* and *R. intermedius* in having a smooth tail terminus with little thickening. • Distinguished from *R. vacuus* by the shorter oesophageal overlap and from *R. intermedius* by the greater development of the posterior gonad. • The swelling at the base of the male stylet is smaller than in other *Radopholus* species.	Colbran (1971)
R. clarus	New species	• Most closely resembles *R. vangundyi* but can be distinguished from this species by the shorter oesophageal overlap, four annules on the lip region and broader tail.	Colbran (1971)
R. vacuus	New species	• Resembles *R. brevicaudatus* and *R. magniglans* in having a relatively long oesophagus but distinguished from them by the small hyaline area in the tail terminus.	Colbran (1971)

R. capitatus	New species	• Closely resembles *R. magniglans* but can be distinguished from this species by the shape of the lip region and the anterior and posterior spermathecae are approximately equal in size.	Colbran (1971)
R. brevicaudatus	New species	• Closely resembles *R. magniglans*, but can be distinguished from this species by the shorter body and number of annules on the lip region. • The tail is shorter in relation to the stylet length.	Colbran (1971)
R. ferax	Described as a junior synonym of *R. neosimilis* (Luc 1987) and subsequently as a junior synonym of *R. natives.* (Ryss and Wouts 1997)		Colbran (1971)
R. colbrani	New species	• Has the longest tail in the female (75-98 µm) and the absence of the bursa in the male.	Kumar (1980)
R. citrophilus	Junior synonym of *R. similis* (Vallete *et al*. 1998)		Huettel *et al*. (1984)
R. megalobatus	Transferred to *Pratylenchoides* (Ryss1988)		Bernard (1984)
R. sanoi	New species	• Characteristically shortened posterior ovary. • Differs from *R. triversus* by annulated tail tip, four lateral incisures.	Mizukubo (1989)

		• Differs from *R. litoralis* by longer spear and shape of head (elevated, dome-shaped). • Differs from *R. laevis* by size and thickness of spear, shape of head (not flattened anteriorly), annulated tail terminus and long hyaline part of tail.	
R. bridgei	New species	• Differs from *R. similis* and *R. citrophilus* in having a smaller stylet (15.5 µm), dorsal gland orifice located closer to the stylet knobs and an indistinct terminal hyaline portion of the tail.	Siddiqi (1995)
R. citri	New species	• Relatively strongly developed conus of the male stylet and less well developed but distinct knobs.	Machon and Bridge (1996)
R. allius	Transferred to *Pratylenchoides* (Siddiqi2000)		Shahina and Maqbool (1996)
R. brassicae	Transferred to *Pratylenchoides* (Siddiqi 2000)		Shahina and Maqbool (1996)

R. nelsonensis	New species	• c = 19-33 (highest value in the genus) • c' = 1.0-1.8 (lowest value in the genus) • The stylet length = 25.28 µm (longest value in the genus) • The ratio tail length to stylet length = 0.66-1.07 (lowest value in the genus).	Ryss and Wouts (1997)
R. kahikateae	New species	• Females differ from *R. rotundisemenus* females by the 4 incisures in the lateral field by midbody (*vs*. 5 or 6), the 4 or 5 cephalic annules (*vs*. 3), the narrowly rounded tail terminus (*vs*. round to broadly rounded), the length of the hyaline part of the tail being 11-15 µm (*vs*. 7-11 µm) and the continuous lip region (*vs*. set off). • Differs from *R. similis* females by the incisures of the lateral field ending at midtail (*vs*. 3 postphasmidial incisures reaching the hyaline tail part), the annulated tail tip (*vs*. mostly smooth), round sperm (*vs*. rod-like), the continuous lip region (*vs*. set off) and the length of the tail (c = 9.9-19 *vs* 7-13).	Ryss and Wouts (1997)

		• Males differ from those of *R. similis* by the conical to hemispherical shape of the lip region (vs. knob-like) and by the distinctly separated stylet knobs (*vs*. amalgamated in one round base).	
R. antoni	New species	• Larger dorsal stylet knob • Reduced posterior ovary, V= 66-69 • A 16-17 µm-long stylet • A 25.5 to 44 µm-long tail • Males with a reduced stylet	Berg (2000)
R. musicola	New species	• Female lateral lines with deep folds on the outer edge • Males with a uniquely long thin tail (73.6-92.8 μm) having a pointed terminus; a smaller stylet (8.8-12 vs 12-17 μm) • Differs from *R. bridgei* by well-developed separated stylet knobs *vs* tiny or obscure knobs	Stanton *et al*. (2001)

R. duriophilus	New species	• Differs from *R. similis* by the position of the excretory pore located posterior to pharyngo-intestine junction (*vs* at level of pharyngo-intestine junction). • Oval shape of sperms • The lateral field incisures terminated far behind position of phasmid are four in number. • The bursa in male never reaches the tail terminus. • Females differ from *R. natives* females by stylet length (16.5-19 *vs*19-23 [1]m), oval or kidney-shaped sperm (*vs* rod-like), four incisures at level of phasmid (*vs* three) and their areolated lateral field (*vs* not areolated). The position of excretory pore of both female and male is located posterior to pharyngo-intestine junction (*vs* at level or anterior to pharyngo-intestine junction). • Females differ from *R. clarus* females by stylet length (16.5-19 vs19-21 µm) and areolated lateral field (*vs* no areolation).	Trinh (2003)

		• Females differ from *R. musicola* females by their lateral field with equidistant incisures at mid-body (*vs* two deep outer folds and two faint shallow inner incisures), oval or kidney-shaped sperm (*vs* rod-like), and rounded terminus tail (*vs* sharply pointed). The species also differ in male stylet length (11.5-15 *vs* 8.8-12 µm). • Females differ from *R. bridge* females by stylet length (16.5-19 *vs* 15-17.5 µm), median bulb length (11-16.5 *vs* 11-13 µm), length of hyaline tail (3-11 *vs* not more than 4 µm), and lateral field areolated for entire body (*vs* not areolated except irregularly on neck and tail). The male differs by stylet length (11.5-15 *vs* 10-12 µm) and length of the hyaline portion (4-9 *vs* 1-4 µm).	
R. arabocoffeae	New species	• Females having broad amphidial apertures with prominent margins and long tail • Males are characterized by the bursa extending to one third, rarely middle, of the tail.	Karssen (2004)

		• Differs from *R. bridgei* by the lateral field having three bands of equal width and completely areolated over whole body; distinctiveness of hemizonid; four lateral field incisures terminating far behind phasmid and the lateral lines fuse at two thirds of tail; a longer spicule length of 18-21 µm; • Differs from *R. colbrani* by the rod-like vs round sperm, spicule length (18-21 *vs* 13-16 µm), tail length to stylet ratio (4.1-4.9 *vs* more than 5.1) and presence of a bursa. • Differs from *R. duriophilus* by the shape of sperm; shorter stylet length, smaller distance between dorsal pharyngeal gland orifice and stylet base, shorter hyaline tail, and bursa extending to one third of tail in males. • In males of *R. arabocoffeae* the stylet base is rudimentary and amalgamated and the inner lateral lines fuse at two thirds of the tail whereas in *R. musicola* the stylet base is with knobs and the inner lateral lines fuses just posterior to the phasmid.	

R. daklakensis	New species	• The distinguishing feature of *R. daklakensis* and *R. arabocoffeae* is in the differences in female tail shape and the direction in which the stylet knob is projected. • Differs from *R. duriophilus* by the stylet knobs, which are always, directed posteriorly vs rounded, and dorsal knob sometimes projected anteriorly, and sperm rod-shaped vs oval and kidney-shaped. • Differs from *R. similis* in the absence of a postrectal intestinal sac, four incisures of the lateral fields terminating far posterior to the position of the phasmid, the bursa in male reaching one-third of tail to half, never reaching tail terminus and smaller stylet length in females.	Trinh (2012)

Table 2: Major taxonomical studies carried out on burrowing nematodes (*Radopholus* spp.).

Details of burrowing nematode species/isolated studied			**Technique used**	**Key findings**	**Reference**
Species/ isolates	**Geographical region**	**Host plant**			
Radopholus spp.	USA	Anthurium	Karyotyping, isozyme patterns and mating behavior	• Reported a new *R. citrophilus* isolate not infecting citrus	Huettel *et al.* (1986)
R. similis and *R. citrophilus*	USA	*Anthurium andreaenum*, *Citrus autrantium* and banana	Morphometrics; SEM studies	• Provided additional support for distinctiveness of *R. citrophilus* and *R. similis* • Reported several diagnostic differences between *R. citrophilus* and *R. similis*	Huettel and Yaegashi (1988)
R. similis (12)	India	Banana	Morphometrics	• Except for few characters most of the measurements recorded in these studies fall within the range of measurements of *R. similis* reported previously.	Koshy *et al*. (1991)

R. similis (2)	Africa	*Vigna unguiculata* and one unknown	Morphometrics	• Proposed *R. citrophilus* as a junior synonym of *R. similis*	Valette *et al.* (1998)
R.similis (10)	Africa	Banana	Morphometrics	• Proposed *R. cítrophilus* as a synonym of *R. similis*.	Elbadri *et al.* (1999)
R. similis (8)	Australia, Belgium, Costa Rica, Cuba, Germany, Indonesia and Panama	Banana, *Calathea makoyana*, *Anthurium andreanum, Philodendron* sp. and black pepper	Morphological and morphometric characterization	• Confirmed *R. citrophilus* as a synonym of *R. similis*	Elbadri *et al.* (1999)
R. similis (20)	Netherlands, China, Malaysia and Singapore	Ornamental plants and ginger	Morphological characters and karyotyping	• Supported that *R. citrophilus* is a synonym of *R. similis*	Xu *et al*. (2014)

R. similis (29)	India	Banana	Morphological and morphometric characterization	• Studied the morphological and morphometric variability of *R. similis* in Gujarat State	Khan *et al.* (2016)
R.similis (3)	Philippines	Banana	Morphological characteristics, reproductive fitness and pathogenicity	• Studied correlation of nematode morphology with pathogenicity and reproductive fitness	Pinili *et al.* (2018)
R. similis	India	Banana	Morphological and morphometric charecterization	• Intra-specific morphological and morphometric variability of *Radopholus similis* from Kerala state.	Roy *et al.* (2018)

2.2.6. Molecular studies

In order to study the genetic variations in *R. similis*, populations from many host plants and several countries have been characterized using different molecular and biochemical methods (Hahn *et al.* 1994; Fallas *et al*. 1996; Hahn *et al.* 1996; Kaplan *et al*. 1996; Kaplan and Opperman 1997; Kaplan *et al*. 2000; Elbadri *et al*. 2002; Costa *et al*. 2008; Tan *et al*. 2010 and Plowright *et al*. 2013). The details of the population used and the methods used in these studies are summarised in chronological order in table 3.

Table 3: Different molecular studies on *R. similis.*

Details of burrowing nematode species/isolated studied			**Technique used**	**Key findings**	**Reference**
Location	**Isolates used**	**Host plant**			
Sri Lanka and East Java	*R. similis* (15)	Tea, arecanut, coffee, banana, Guatemala, grass, *Anthurium*, clove and citrus	RAPD	• Arranged most of the 15 populations into three putative groups and revealed three divergent isolates which are monotypic	Hahn *et al.* (1994)
Uganda, Nigeria, the Ivory Coast, Cameroon, Guinea, Costa Rica, Guadeloupe, Queensland, Sri Lanka and Indonesia	*R. similis* (9*)* and *R. bridge* (1)	Banana and turmeric	RFLP, RAPD and isoenzyme analysis	• Indicated existence of two gene pools within the *R. similis* isolates. • Could not detect any correlation between the genomic diversity and either geographic distribution of the isolates or differences in their pathogenicity	Fallas *et al*. (1996)

Indonesia, Uganda, SriLanka, Kenya, Fiji, Cameroon, Guadeloupe, Ivory coast, Nigeria, Cook islands and Guinea.	*R. similis* (13), *R. bridgei* (1) and *R. citri* (1)	*Citrus* sp., banana, arecanut, tea, ginger, turmeric, black pepper and clove	RAPD	• Arranged the 15 populations into two clusters. • Highlighted the distinctiveness of *R. citri* and *R. bridgei*	Hahn *et al.* (1996)
Florida, Hawaii and Central America.	*R. similis* (14)	Citrus	RAPD	• Indicated that a 2.4 kb fragment appeared to be associated with citrus parasitism	Kaplan *et al*. (1996)
Central America, Dominican Republic, Florida, Guadeloupe, Hawaii and Puerto Rico	*R. similis* (37) and *R. Citrophilus* (1)	*Anthurium* and *Citrus*	RAPD, isoenzyme analysis	• Did not support assignment of sibling species status to burrowing nematodes that differ in citrus parasitism	Kaplan and Opperman (1997)

Australia, Cameroon, Central America,Cuba, Dominican Republic, Florida, Guadeloupe, Hawaii, Nigeria, Honduras, Indonesia, Ivory Coast,Puerto Rico, South Africa, and Uganda	*R. similis* (57)	-	ITS and D2/D3 sequencing	• Substantiated that the *R. similis* genome is highly conserved across geographic regions • Supported the concept that *R. similis* is comprised of two pathotypes-one that parasitizes citrus and one that does not	Kaplan *et al*. (2000)
Belgium, Australia, S. Africa,Sudan, Costa Rica, Cuba, Ghana, Guinea, Indonesia, Panama, Germany and Uganda	*R. similis* (19) and *R. citrophilus* (1).	Banana, *Anthurium andreanum,* *Calathea makoyana,* *Piper nigrum,* and *Philodendron* sp.	RFLP and ITS sequencing	• Further supported the synonymy of *R. similis* and *R. citrophilus*	Elbadri *et al*. (2002)

Brazil, Cuba, Costa Rica and Australia	*R. similis* (12)	Banana	RAPD	• *R. similis* populations were separated into five similarity groups. • Observed no correlation between geographic proximity and genetic similarity among *R. similis* populations, except for one population. • Proposed evidence for a close association between the level of aggressiveness on bananas and a short genetic distance in most of the populations	Costa *et al*. (2008)
Australia and Costa Rica	*R. similis* (16)	Banana	ITS sequencing	• Suggested the lack of genetic diversity of Australian *R. similis*, populations	Tan *et al*. (2010)

Uganda	*R. similis* (7)	Banana	RAPD and AFLP	• Indicated the close association of Ugandan populations with populations from Sri Lanka on separate occasions from different sources	Plowright *et al.* (2013)

2.3. Management measures

2.3.1. Agro technical control methods

R. similis is able to survive for more than six months without the roots of host plants or live rhizome tissue (Whitehead 1997). It can also survive on volunteer bananas that grow during the fallow period between cropping cycles, and this was found to be a major cause of carry-over of these nematodes to the next crop (Stirling and Pattison 2008). Crop rotation with non-susceptible plants, such as *Crotalaria*, and some other management practices, including the elimination of banana regrowth and weeds (and cultivation of a nematode-resistant crop for at least one year), affect populations of *R. similis* and reduce them to levels at which at least six nematicide applications can be saved in each crop cycle (Stirling and Pattison 2008).

Bare fallowing, crop destruction using the herbicide glyphosate to eliminate regrowth from the previous crop, or growing of fully resistant cover crops was found to decrease heavy infestations of soil to trace populations (Whitehead 1997; Stirling and Pattison 2008). Flood fallowing may also affect *R. similis* populations dramatically. Many other phytosanitary approaches have also been implemented to control *R. similis*. These include the use of clean propagative material and approved growing media (sterile peat, clean sand, vermiculite etc.), the use of clean irrigation water, and weed control.

2.3.2. Physical control methods

In 1954, Birchfield discovered that immersing citrus roots in water at 50°C for 10 minutes killed all *R. citrophilus* (Whitehead 1997). After heat treatment, the plants had to be transferred immediately to cold water for 10 minutes to prevent tissue damage (Whitehead 1997). Hot water treatment of banana rhizomes at 55 °C for 20 minutes has been extensively used in Cuba against *R. similis.* Immersing ornamental plants infested with *R. similis* into hot water at 50°C for 10–16 minutes has also been reported

to be a highly effective method of controlling burrowing nematodes (Arcinas *et al*. 2004).

2.3.*3. Chemical control methods*

In the past, soil fumigants such as 1,3-dichlorpropene, ethylene-dibromide and 1,2-dibromo-3-chlorpropane were intensively used in citrus and banana orchards to control the burrowing nematode and to increase citrus and banana yields (Anonymous 1997). It has now been recognised that excessive use of fumigants leads to serious environmental and health problems (Duncan and Moens 2006). Granular non-fumigant nematicides that were reported to be effective against *R*. *similis* were organophosphates (fenamiphos, fensulphothion, ethoprophos and isazophos) and carbamates (aldicarb, oxamyl and carbofuran) (Whitehead 1997). Owing to environmental concerns, there is a need to develop effective alternative and complementary management methods.

2.3.4. Biological control methods

Interest in biological control of nematodes was elicited as soon as plant parasitic nematodes were recognized as pests. Several bacteria were reported to have inhibitory effects on nematodes, but species belonging to *Pasteuria* are the only obligate parasites of nematodes. (Poinar and Hansen 1986; Sayre and starr 1988). Several root colonizing bacteria like *Serratia*, *Psuedomonas* spp. (Oostendorp and sikora 1989, 1990; Spiegel *et al*. 1991; Kloepper *et al*. 1992; Kluepfel *et al*. 1993) and *Bacillus* spp. (Becker *et al*. 1988) were reported to inhibit the development of plant parasitic nematodes. Several reviews have been published exclusively on the fungal antagonists of nematodes (Barron 1977; Kerry 1984; Gray 1987, 2017; Jansson and Nordbring-Hertz 2017; Morgan-Jones and Rodriguez-Kabana 1988). Fungi belonging

to widely divergent orders and families from Phycomycetes to Basidiomycetes were reported as antagonists of nematodes (Mankau 1980).

Even though these methods are efficient in nematode management, once the nematodes multiply into large number, it is difficult to control them by any of these management methods. Hence it is important to detect the presence of the nematode at an early stage, before it spreads at an alarming rate.

2.4. Diagnostic studies in *Radopholus*

Developing a diagnostic assay for the accurate identification and quantification of plant parasitic nematode is critical for making plant management decisions. Sampling and extracting nematodes from roots, corms, tubers or the surrounding soil of host plants by Bearmann's sieving and decantation method can establish the presence of burrowing nematodes. Traditionally the extracted nematodes are diagnosed by microscopic observation and quantification. However, the traditional methods of diagnosis are not ideal as they are time consuming and require expertise in identifying *R. similis*.

Ge *et al*. (2007) designed primers based on duplex PCR to identify *R. similis*; the minimum amount of DNA of *R. similis* that could be detected was 100 pg. Wang *et al*. (2011) reported a PCR assay for detection of *R. similis* ITS-rDNA equivalent to 4×10^{-3} nematodes. Single tube duplex PCR based method was developed to detect *P. capsici* and *R. similis* in infected roots of black pepper using the conserved internally transcribed spacer (ITS) region of Oomycetes pathogen, *P. capsici* and rDNA sequences of nematode pathogen, *R. similis*. (Aravind *et al*. 2011). The LAMP based detection method developed by Peng *et al*. (2012) was also very effective in detecting *R. similis.* However, they are not suitable for quantifying the nematodes in soil and plant samples as they do not provide any information about their number. The *R. similis* qPCR assay

is a potentially useful alternative to the current molecular tools as it provides quantitative information about the nematode.

To date, 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*, *M. fallax*, *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 and 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).

While most papers published using real-time PCR and nematodes are based on the non-coding internal transcribed spacer regions ITS-1 or ITS-2 or on the intergenic spacer regions IGS1 or IGS2 located between the ribosomal genes including the intragenic spacers, a few papers focus on coding genes or gene regions. Coding genes used to design real-time PCR assays for nematode identification are for instance the heat shock protein (Hsp90) (Madani *et al.* 2011), the β-1,4 endoglucanase gene (Mokrini *et al*. 2013), the MspI satellite DNA (François *et al.* 2007), sequence characterized amplified (SCAR) based genes or the cytochrome oxidase subunit I (COI) gene (Kiewnick *et al*. 2015). Furthermore, Gamel *et al.* (2017) established new

molecular markers based on microsatellite loci for simultaneous detection of three nematode species across two genera.

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. Min (2007) could detect a single *P. penetrans* individual in a sample with an abundant number of free-living nematodes using a SYBR Green I-based qPCR method. 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.

Min (2007) reported a higher variation in numbers of *P. penetrans* when introducing a range of nematode individuals into soil aliquots before extraction than using simply diluted DNA samples, likely due to variation in the extraction and purification of DNA. Berry *et al.* (2008) constructed standard curves using different numbers of *M. javanica, P. zeae*, and *X. elongatum* and discussed that the estimated numbers appeared to represent the variation that could exist among replicate samples of the same number of nematodes and, thus, tended to be more representative of the variation that could occur in native samples.

2.5. Studies in Indian populations

The Indian sub-continent is the largest producer of banana, with an estimated annual production of 10.4 million tonnes from an area of 0.4 million ha (Chadha 1995). The burrowing nematode *R. similis*, is a major hindrance to banana production wherever the crop is grown in India. A total infestation percentage of 31% to 41% has been found (Anonymous 1979; Reddy *et al*. 1992).

The infestations have been recorded in several parts of the country like Kerala (Nair *et al.* 1996), Tamil Nadu (Rajagopalan and Chinnarajan 1976), Karnataka (Venkitesan 1976), Maharashtra (Reddy and Singh 1980), Gujarat (Sethi *et al.* 1981), Central Madhya Pradesh (Tiwari and Dave 1985), Southern Andhra Pradesh (Reddy and Khan 1987), West Coastal region of Goa (Koshy and Sosamma 1988), etc. The nematode is present in north eastern states of India as well, including Manipur (Anandi and Dhanachand 1992), Orissa (Mohanty *et al*. 1992), Chotanagpur - Bihar (Khan and Kumar 1993), Tripura (Mukherjee *et al.* 1994), etc. Even far reaching island states like Lakshwadeep (Sundararaju 1995) have not been spared by this nematode, which prevails as a major infestation to the crop species like coconut, arecanut, cardamom and pepper (Koshy *et al*. 1978) in this region. Further, a study of the occurrence of *R. similis* infestation in banana plantations of India, where the study focused primarily on the Northeastern states (Khan 1999) suggested regions like Nagaland followed by Uttar Pradesh, West Bengal, Bihar and Assam displayed a relatively high rate of infestation by the species. In India studies to understand the morphological and morphometric diversity of *R. similis* were done using populations collected from different localities of South India (Koshy *et al*. 1991) and Gujarat (Khan *et al*. 2016).

METHODOLOGY

3.1. Sample collection

A survey was conducted during the year, 2014-2015 in the black pepper and banana growing areas of Kerala and Karnataka to isolate the plant parasitic nematode, *Radopholus* spp. from the black pepper vines trailed on different standards like polyalthia, silver oak, arecanut palm, glyricidia, mango tree, oil palm, coconut palm and jackfruit tree. 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 roots (Figure1), were collected from different locations.

Fig. 1: Slow decline infected black pepper vine.

The nematodes were collected following the protocol described by Cobb (1918) and were observed at 25x and 80x magnifications under the stereomicroscope for the characteristic features of *Radopholus* spp. (Figure 2).

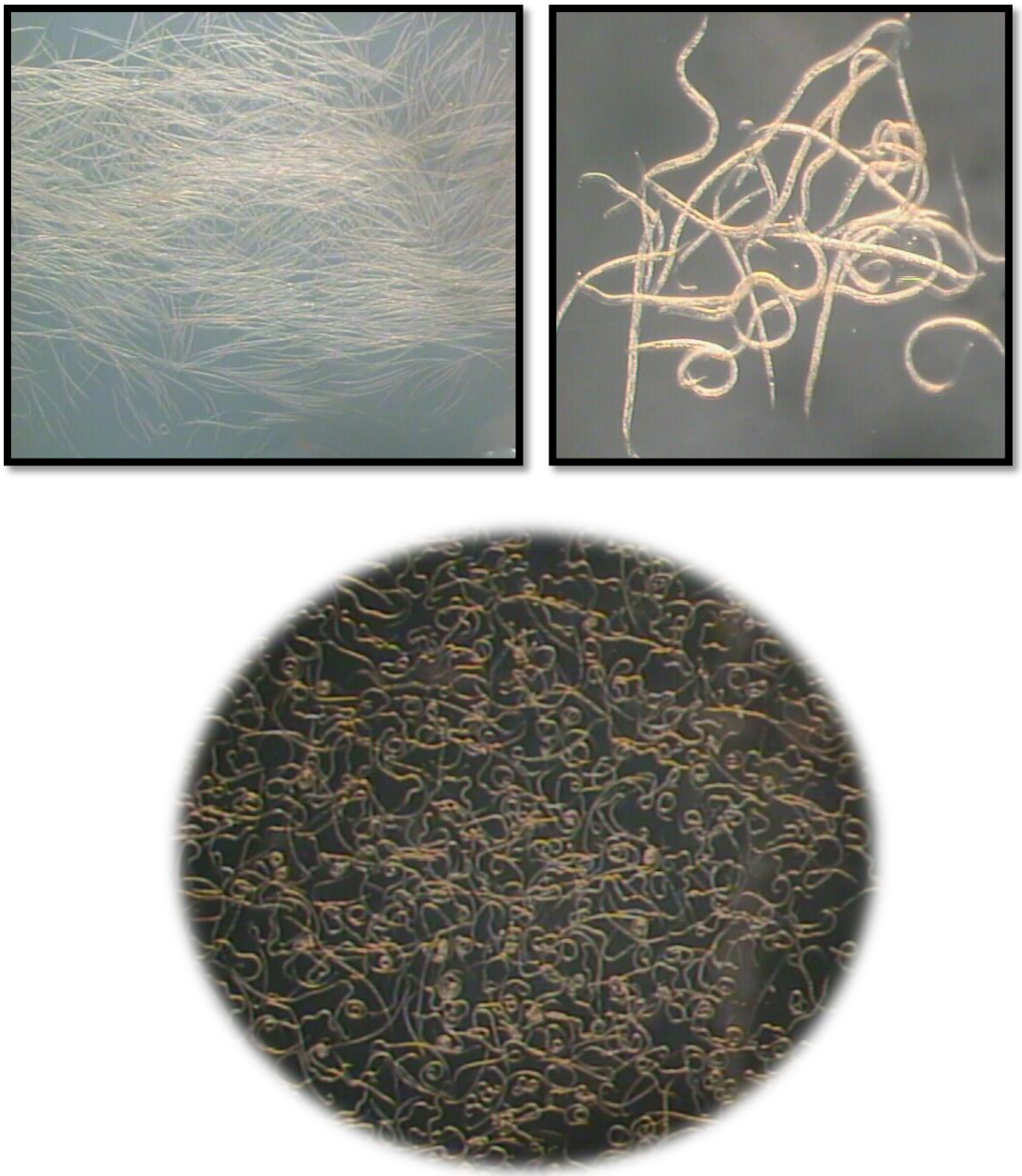


Fig. 2: *Radopholus* sp. isolates observed under stereomicroscope at 25x and 80x

3.2. Culturing of *Radopholus* spp.

The isolated nematodes were surface sterilized using 6mg/ml streptomycin sulphate, washed in sterile distilled water and inoculated on to carrot callus disc (Figure 3). The plates were incubated at 26^{o}C for two months (Reise *et al*. 1987). To collect nematodes from these cultures, the carrot discs were teased and kept for sieving using Cobb's sieving and decantation method.

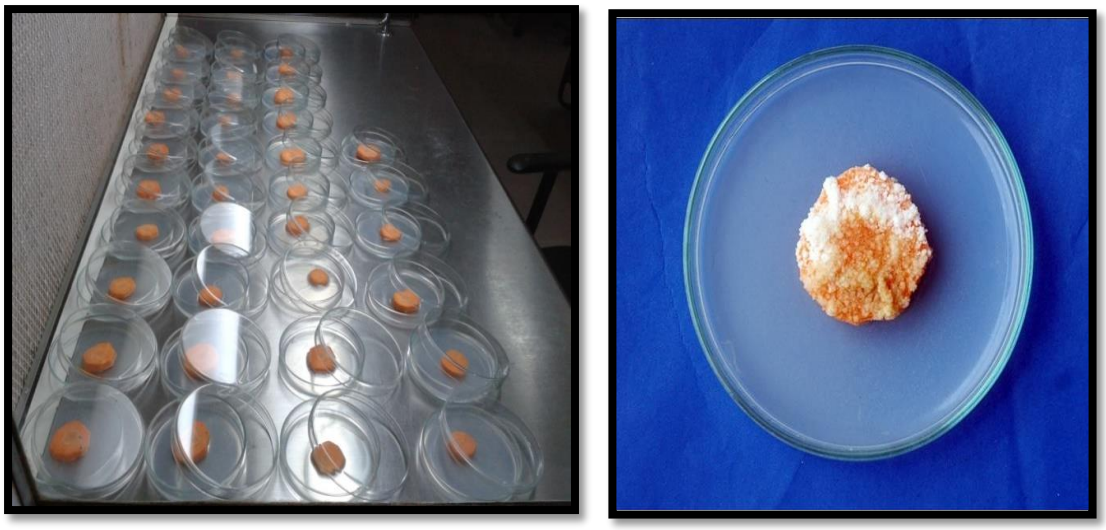


Fig. 3: Pure culture of *Radopholus* sp. isolates maintained on carrot callus disc

3.3. Morphological Studies

3.3.1. Light Microscopy

Twenty females and twenty males of the above isolates were fixed using formaldehyde glacial acetic acid (FAA) fixation method for two days. The fixed specimens were dehydrated using glycerin alcohol for 21 days and mounted on a clean glass slide (Seinhorst1962).

The morphometric parameters of all the isolates were recorded using light microscopy following De' mans formula (De Man 1880).

Morphometric parameters used

L = overall body length

a = body length / greatest body diameter

b = body length / distance from anterior to esophago-intestinal valve

b' = body length / distance from anterior to base of esophageal glands

c = body length / tail length

c' = tail length / tail diameter at anus or cloaca

T/S= tail length /stylet length

o = % distance of dorsal esophageal gland opening from stylet knobs in relation to stylet length

V = % distance of vulva from anterior

G_1 = % length of anterior female gonad in relation to body length

G_2 = % length of posterior female gonad in relation to body length

3.3.2. Statistical analysis:

All the measured morphological parameters were recorded and an ANOVA analysis was carried out. The general mean, p-value, CV (%), SE (d), LSD at 5% of each character for all the ten isolates were calculated.

3.4. Molecular Studies

Molecular characterization of all the ten isolates was done by PCR amplification and sequencing of its genomic and mitochondrial DNA, using the primer pairs listed in table 4.

Table 4: Primers used for molecular characterization

PRIMER	SEQUENCE	PRODUCT SIZE	REFERENCE
18S FP	5′TTGATTACGTCCCTGCCCTTT3′	920 bp	(Vrain *et al.* 1992)
26S RP	5′TTTCACTCGCCGTTACTAAGG3′.		
COX FP	5I TTTTGGTTTTTACCTGCTGCT3I	899 bp	(Blessy *et al.* 2017)
COX RP	5I TCCTTGTAAACCAGCAAAATG3I		
ND FP	5I CTTTTAGCTTTAAGTCAGAATCGTC3I	701 bp	(Blessy *et al.* 2017)
ND RP	5ICGATAACGAGGTAAAACACTACGA3I		

3.4.1. Total DNA extraction

The DNA was extracted from the ten isolates using the protocol described by Emmons *et al.* (1979). Around 2000 nematodes were suspended in 500 µl of extraction buffer (200 mM TrisHC1 (pH 8.0), 10 mM EDTA, 400 mM NaC1, and 2% SDS (sodium dodecyl sulfate) containing 10mg/ml of proteinase K. After incubation for 1 hour at 65^0C, an additional 5 mg/ml Proteinase K was added to the digest and the incubation was continued for 30 minutes. Nucleic acid was extracted from the nematode digest by the addition of two volumes of phenol saturated with 50 mM

Tris-HCl containing 1 mM EDTA (pH 8.0). The aqueous phase was recovered and the phenol extraction repeated. One volume of buffer saturated phenol plus one volume of chloroform was then added to the aqueous phase. Residual phenol was removed from the aqueous phase by one extraction with two volumes of chloroform: isoamyl alcohol (19:1). Total nucleic acid was then precipitated with 2.5 volumes of 100% ethanol. The nucleic acid pellet was washed twice with 70% ethanol and suspended in 0.5 ml of a solution containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 150 mM NaCl. RNA was then digested by incubation of the nucleic acid with 100 µg/ml pancreatic RNase for 30 minutes at 37^0C. DNA was extracted and precipitated with 100% ethanol. Purified DNA was suspended in sterile distilled water.

3.4.2. Quantification of Genomic DNA

One micro liter of all the DNA samples was diluted 50 times using nuclease free water and absorbance was taken at 260nm. The concentration of DNA in the sample was directly obtained from the biophotometer (µg/ml). This reading was multiplied using the dilution factor (50) to get the actual concentration in nanograms and was assumed to contain that much of double stranded DNA per µl, free of proteins, RNA and polysaccharide contaminants.

3.4.3. PCR amplification of the target sequence

All the components required for the PCR amplification of the DNA were mixed in the concentration as given in the table 4. It was then placed in a Thermocycler (Eppendorf Master Cycler) after spinning it at low speed for a few seconds. The DNA amplification profile consisted of conditions mentioned in table 5 and table 6.

Table 5: Components used for PCR amplification

Components of PCR	Concentration of stocks	Quantity used in PCR (µl)
Nuclease free water	1x	17.63
Taq buffer	10x	2.3
Magnesium chloride	25mM	2.0
dNTPs	10mM	0.2
Taq Polymerase	3U/µl	0.17
Forward primer	10µM	0.5
Reverse primer	10µM	0.5
Template DNA	-	1.5
	Total	25

Table 6: PCR amplification profile

Step	Temperature	Time
Initial denaturation	94^0C	5 min
Denaturation	94^0C	30 min
Annealing	62^0C	1 min
Synthesis	72^0C	1 min
Final synthesis	72^0C	10 min

The PCR was held at 4^0C, after the completion of cycles and was taken for further analysis by agarose gel electrophoresis.

3.4.4. Preparation of agarose gel electrophoresis tank

- Prepare sufficient electrophoresis buffer (1XTAE) (Appendix I) to fill the electrophoresis tank and to prepare the gel. To prepare the gel 0.8 % agarose was dissolved in required volume of electrophoresis buffer in an Erlenmeyer flask. The solution was cooled to 60^0C and ethidium bromide (Appendix II) was added to a final concentration of 0.5 µg/ml. It was mixed gently without forming air bubbles.
- The comb was placed at one end of the casting tray, positioning it at 0.5 -1.0 cm from the edge. The gap between the teeth base of the comb and surface of the tray was adjusted so as to form wells to hold the samples. The prepared gel was poured into the casting tray. It was allowed to solidify for 25-30 min.
- The prepared gel was placed in the electrophoresis tank and 1XTAE buffer was poured into the tank so that the gel is completely immersed.

3.4.5. Sample preparation and loading

The required volume of PCR product was thoroughly mixed with 6x gel loading dye (Appendix III). The samples were carefully loaded into the wells without allowing cross contamination of samples. The samples were run to one third of the total gel length, at 5V/cm using 1x TAE buffer (Sambrook and Russel 2001). A DNA size standard (1kb DNA ladder, Thermo Scientific, Bangalore) was also loaded in one of the wells to determine the size of the amplified products.

3.4.6. Gel documentation

The DNA gels were visualized under UV, on a transilluminator and documented using Gel documentation system (Alpha imager 2220, Alpha Innotech Corporation).

3.4.7. Gel extraction of the amplified DNA

The amplified fragments were excised from agarose gel and purified using Thermo Scientific GeneJET Gel Extraction Kit (Thermo Fisher Scientific Inc., MA USA) as described below.

- Excise gel slice containing the DNA fragment using a clean scalpel or razor blade. Cut as close to the DNA as possible to minimize the gel volume. Place the gel slice into a pre-weighed 1.5 mL tube and weigh. Record the weight of the gel slice.
- Add 1:1 volume of Binding Buffer to the gel slice (volume: weight).
- Incubate the gel mixture at 50-60°C for 10 min or until the gel slice is completely dissolved. Mix the tube by inversion every few minutes to facilitate the melting process. Ensure that the gel is completely dissolved. Vortex the gel mixture briefly before loading on the column.
- Transfer up to 800 µL of the solubilized gel solution to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
- Add 100 µL of Binding Buffer to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
- Add 700 µL of Wash Buffer (diluted with ethanol) to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
- Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove residual wash buffer.

- Transfer the GeneJET purification column into a clean 1.5 mL microcentrifuge tube. Add 50 µL of Elution Buffer to the center of the purification column membrane. Centrifuge for 1 min.
- Discard the GeneJET purification column and store the purified DNA at -20 °C.

3.4.8. Ligation to vector

The concentration of the eluted PCR product was estimated using a biophotometer (Eppendorf, Hamburg, Germany). The eluted products were ligated separately into pTZ57R/T vector (Fermentas, Vilnius, Lithuania) (Fig. 4) according to the manufacturer's instructions. A 15µl ligation reaction was set up in 3:1 molar ratio of insert and vector DNA (Table 7). For 3:1 insert to vector ratio the amount of eluted PCR product required is calculated using the formulae

$$\text{Amount of eluted PCR product required} = \frac{\text{Size of the PCR product (Kb) X 55ng vector}}{\text{Size of the vector (Kb)}} \text{ X 3}$$

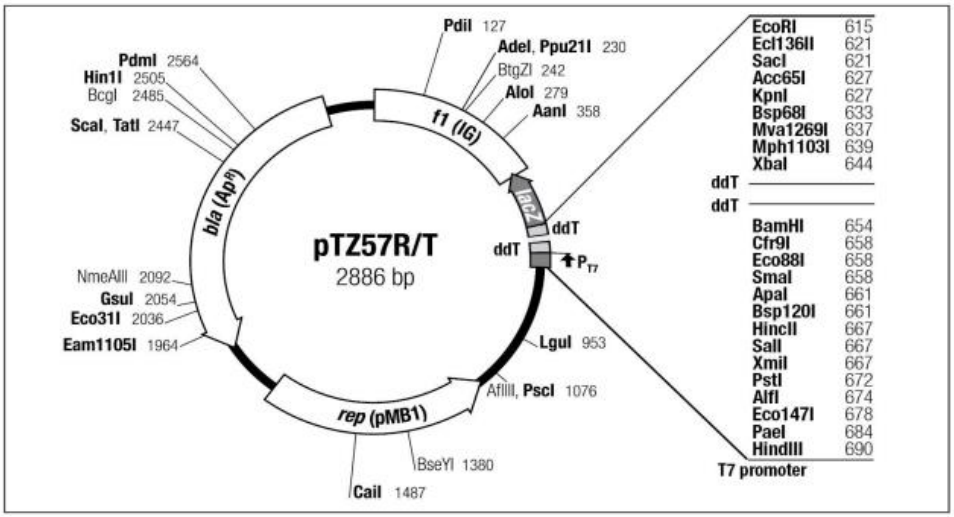


Fig. 4: Vector map of pTZ57R/T

Table 7: Components used for ligation reaction was set as follows

Components	**Volume**
PCR product (25ng/µl)	2µl
Ligation buffer (5x)	3µl
Linearized vector pTZ57R/T (55ng/ µl)	1.5µl
T4 DNA ligase (5U/ µl)	0.5µl
Water	8.0µl
Total volume	15µl

The ligation mixture was incubated at 25^0C for one hour. The ligated product was later kept in ice until the transformation experiment starts.

3.4.9. Preparation of competent cells (E. coli)

The competent cells for the introduction of recombinant plasmids were prepared using the protocol (Sambrook and Russel 2001) given below.

- 5ml of Luria Bertani (LB) broth was inoculated with a single colony of *E. coli* strain DH5α and incubated overnight in an orbital shaker at 37^0C with agitation at 200rpm.
- 500µl of this overnight culture (12-16h) was used to inoculate 50ml of LB broth on the next day morning and incubated at 37^0C with moderate agitation (˜200rpm) until the A_{595} =0.375 (initial inoculum should have A_{595}‹ 0.1).
- Transferred the culture to sterile, pre-chilled Falcon tubes and incubated for 10 min in ice.
- Cetrifuged the cells for 10 min at 3000g at 4^0C and then resuspended the pellet in 1ml of 0.1M $CaCl_2$ solution. Left the cells on ice for another 1h.

- Made 200µl aliquots of final preparation in micro-centrifuge tubes and allowed to be there on ice or used immediately. The remaining cells were also stored at -70^{0}C by mixing with equal volume of 40% glycerol.

3.4.10. Transformation

- The ligated product was mixed with 200µl of prepared competent cells, and incubated in ice for 30min without disturbing.
- A heat shock was given to the ligation + competent cell mixture at 42^{0}C for 2 min.
- Just after the 2 min of heat shock, a quick chilling was given to the mixture in ice and incubated for 2-3 min.
- Added 1 ml of LB broth to the mix and placed the tube in an orbital shaker maintained at 37^{0}C for 1 h with an agitation of ~200rpm.
- During this incubation period IXA plates were prepared. For preparing the IXA plates, 50ml of LB agar was melted in a conical flask using a microwave oven. This was allowed to cool to 40^{0}C. Ampicillin, X-gal, and IPTG was added to a final concentration of 50µg/ml, 80µg/ml and 80µg/ml respectively in 50ml of molten LB agar. Mixed the molten agar properly without forming air bubbles and poured on to the sterile petri plates allowed solidifying for 10-15min and plates were incubated at 37^{0}C until plating.
- After 1 h incubation in orbital shaker the tubes containing cells were centrifuged at 1000rpm for 10min at room temperature and resuspended the pellet in 100µl of fresh LB broth. The suspension was then plated on IXA plate by spread plating using a bend glass rod.
- After completing spreading, plate was covered and were incubated overnight at 37^{0}C keeping it upside down.

3.4.11. Identification of positive recombinant clones

For the purpose of confirmation of recombinant clones, blue white selection was done followed by PCR. The plasmid vectors used in the studies carried coding information for the first 146 amino acids of the β- galactosidase gene. Embedded in this coding region is the polycloning site (does not disrupt the reading frame of the gene) into which insert DNA is cloned. When expressed, this 146 amino acid fragment of β- galactosidase protein is capable of acting on the chromogenic substrate (X-gal). But when expressed in appropriate host cells which expresses the carboxyl terminal fragment of the β- galactosidase, these two protein fragments can associate to form an enzymatically active protein and such cells turn blue when plated on plates containing X- gal. But if the insert DNA gets cloned in the polycloning site, it invariably results in production of an amino terminal fragment that is not capable of complementation and hence those colonies remain white (Ullmann *et al*. 1967).LB agar plates containing ampicillin, X-gal and IPTG in the required concentrations were prepared as described earlier, before which grids were drawn at the bottom plate on its outer side. Each white and light blue tinged bacterial colony were picked up using fine inoculation loop and carefully streaked inside the grid. Each of the colonies was numbered consecutively. After the inoculation, the plates were incubated overnight at 37^0C.

3.4.12. Colony PCR

The colony PCR protocol in combination with the appropriate primers provides a rapid means to determine the length and orientation of a cloned insert (Gussow and Clackson1989). This method was adopted when the colony number obtained on the spread plate was large. The protocol for colony PCR was as follows.

- Picked up an isolated colony with a sterile toothpick and resuspended in 50µl of 10mM EDTA in a microfuge tube.
- Kept the bacterial suspension in boiling water bath for 5min and then vortexed for a few seconds. The suspension was centrifuged briefly at maximum speed.
- 2 µl of the supernatant was used as template for PCR.

3.4.13. Preparation of plasmid for sequencing

The plasmid was isolated using GeneJET Plasmid Miniprep Kit. The protocol is as follows.

- Suspend the pelleted cells in 250 µL of the Resuspension Solution. Transfer the cell suspension to a microcentrifuge tube. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
- Add 250 µL of the Lysis Solution and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear.
- Add 350 µL of the Neutralization Solution and mix immediately and thoroughly by inverting the tube 4-6 times.
- Centrifuge for 5 min to pellet cell debris and chromosomal DNA.
- Transfer the supernatant to the supplied GeneJET spin column by decanting or pipetting. Avoid disturbing or transferring the white precipitate.
- Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.

- Add 500 µL of the Wash Solution (diluted with ethanol) to the GeneJET spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.
- Repeat the wash procedure using 500 µL of the Wash Solution.
- Discard the flow-through and centrifuge for an additional 1 min to remove residual Wash Solution. This step is essential to avoid residual ethanol in plasmid preps.
- Transfer the GeneJET spin column into a fresh 1.5 mL microcentrifuge tube. Add 50 µL of the Elution Buffer to the center of GeneJET spin column membrane to elute the plasmid DNA. Take care not to contact the membrane with the pipette tip. Incubate for 2 min at room temperature and centrifuge for 2 min.
- Discard the column and store the purified plasmid DNA at -20°C.

3.4.14. Automated sequencing

The isolated plasmids were sequenced at Eurofins Genomics India Pvt. Ltd., Bangalore, India. The automated sequencing utilizes the principle of Sanger's method of DNA sequencing (dideoxy sequencing or chain termination method), that utilizes the dideoxy nucleotides (ddNTP's) in addition to the normal nucleotides (NTP's) found in DNA. With the automated procedures the reactions are performed in a single tube containing all four ddNTP's, each labeled with a different colour dye. Since the four dye fluoresce at different wavelengths, a laser then reads the gel to determine the identity of each band according to the wavelengths at which it fluoresces. The results are then depicted in the form of a chromatogram, which is a diagram of colored peaks that correspond to the nucleotide in that location in the sequence (Russell and Gordey 2002).

3.4.15. *Sequence analysis:*

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). All the sequences were submitted in NCBI BankIt. 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 Gen Bank database. These sequences were downloaded in FASTA format. For phylogenetic analysis, the ten ITS sequences along with ITS sequences of *R. similis* from different geographical locations were used as one set of data. Since COX and ND sequences are adjacent regions on the mitochondrial genome sequence, they were taken together as the second set of data. Both these sets of sequences of the ten isolates were used for further studies.

The sequences were edited and aligned with accessions from GenBank using Clustal X version 2.1 (Larkin *et al*. 2007) to correct obvious sequencing errors and code ambiguous sites in order to produce a consensus sequence. The alignment was edited in BioEdit version 7.2.5 (Hall 1999) to trim aligned concatenated sequences to an equal size and set missing data to question marks. The edited alignment was then analyzed in jModelTest version 2.1.7 (Posada 2008) to select the most appropriate model for the following phylogenetic analyses. This provides a rank of models according to the "Akaike Information Criterion" (AIC) (Akaike 1973).

3.4. 16. Phylogenetic analysis

Phylogenetic analysis was conducted using five different methods (Bayesian analysis, Maximum likelihood, Maximum parsimony, Minimum Evolution and Neighbour

joining methods). A concatenated tree was constructed to get a combination of phylogenetic analysis conducted using all the five methods.

Bayesian analysis (BA): Bayesian analysis was performed using MrBayes version 3.2.6 (Ronquist *et al.* 2012) for five lakh generations with K80+a model. The Standard Deviation of the split frequencies were always less than 0.01.

Maximum likelihood (ML): Analysis was performed using PhyML version 3.0 (Guindon and Gascuel 2003) with the selected model K80+a, with 1000 bootstrap replicates.

Maximum parsimony (MP): Analysis was conducted using PAUP version 4.0 (Swofford 2002), Bootstrap method with Heuristic search with 1000 replicates and maximum trees set to 1000.

Minimum Evolution (ME): Analysis was conducted using PAUP version 4.0 (Swofford 2002), Bootstrap method with Heuristic search with 1000 replicates and maximum trees set to 1000.

Neighbour joining (NJ): Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.

Concatenated tree: Phylogenetic trees were viewed and edited in FigTree version 1.4 (Rambaut and Drummond 2012). Concatenated tree from all the methods was created using Treefinder (Jobb 2011).

3.4.17. Haplotype analysis

Haplotype analysis was done using the ITS sequences using the software NETWORK V.5.0. Statistical parsimony network was done to further confirm the accuracy of the phylogenetic analysis. Different taxon was represented as circle. The area of the circle indicated the number of individuals present in that particular haplotype.

3.5. Real Time PCR based detection of *R.similis*

3.5.1. Designing of specific primers

The primer set was designed from the ITS region of the ten *R. similis* isolates, submitted to the GenBank/EMBL database. Primers were compared to other sequences in the database through BLAST and FASTA searches to confirm specificity, and their design was optimized using the Primer 3+ software (Untergasser 2007).

3.5.2. Synthesis of primers

Desalted oligonucleotide primers were synthesized at IDT-USA (Imperial Biomedical Lab). Every oligo was desalted to remove small molecule impurities. The oligonucleotides were supplied in 25nM with an OD of 3. Quality control for each primer was checked by MALDI-TOF-Mass spectrophotometry.

3.5.3. *Standard graph for the Real Time PCR based detection*

DNA of the isolate IISR RS01 was isolated from 2000 nematodes using phenol chloroform extraction method. The stock DNA sample was diluted 8 times in 1:2ratios. Using these DNA samples as template, Real time PCR was done along with water and DNA of *Meloidogyne* sp. and *Steinernema* sp. as negative controls. Real time PCR was performed using, quantifast SYBR green 2X master mix (Qiagen), and

0.25µM primer concentration on a qPCR thermocycler (Qiagen).The cycling conditions for the designed primers with SYBR green were as follows: 95^{o}C for 10 min followed by 40 cycles of 95^{o}C for 10sec, 60^{o}C annealing for 45sec and a melt at 50-99^{o}C. All assays were performed with three technical replications and all experiments were repeated. A standard graph for the detection of *R. similis* was thus obtained.

3.5.4. Correlating Ct value to the number of R. similis

DNA extraction from 1, 5, 10, 50, and 500 *R. similis* was done using phenol chloroform extraction method as mentioned earlier. They were subjected to Real time PCR and the results obtained along with corresponding results of water and negative controls, were plotted on the standard graph previously obtained.

3.5.5. Detection limits of R. similis specific primer using Real time PCR and LAMP method

The DNA isolated from single nematode was diluted 10-fold and it was detected using the newly developed Real time PCR based method and previously reported LAMP method. It was done to find the detection limit and to understand the efficiency of the newly designed primer over the LAMP technique. Real time PCR was done as previously mentioned.The LAMP reaction was performed according to the method described by Notomi *et al.* (2000). The optimized LAMP reaction mixture contained 40 pmol each of inner primers FIP and BIP, 5 pmol each of outer primers F3 and B3, 20 pmol of LF primer, 1.4 mM of dNTP mix, 1.6 M betaine (Sigma-Aldrich, St Louis, MO, USA), 4.5 mM MgSO4, 8U of Bst DNA polymerase (New England Biolabs, Ipswich, MA, USA), 1× Thermopol Reaction buffer (20 mMTris-HCl, pH 8.8, 25°C, 10 mMKCl, 10 mM (NH4)2SO4, 2 mM $MgSO_4$, 0.1% Triton X-100), 1 μl of extracted DNA solution

and PCR grade water to 25 μl. The reaction mixture was incubated at 60-65°C for 45 min and then heated to 85°C for 5 min to terminate the reaction. Reaction mixes without DNA template was included as a negative control. Sensitivity of both the methods for detection of *R. similis* DNA was determined by testing serial ten-fold dilutions of the genomic DNA of single *R. similis*. This provided the sensitivity comparison of qPCR with LAMP.

3.5.6. Validation of the 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 rhizhosphere were collected from different regions of Kerala. To examine the reliability of the method, especially to detect the nematode in symptomless plants, samples taken from apparently healthy plants around the infected plants were used. These samples were subjected to direct microscopic examination and qPCR by adopting the corresponding standardised protocols along with known positive and negative controls. The specificity of the qPCR product was also checked by subjecting to melt curve analysis from 60^0C to 95^0C. 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.

RESULT

4.1. Sample collection

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 roots, were collected from different locations as mentioned in Table 8.

Table 8: Details of samples collected for isolation of burrowing nematodes

DISTRICT	SAMPLE COLLECTED	INFECTED SAMPLE	CROP
KOZHIKODE	114	76	Black pepper
IDUKKI	53	47	Black pepper
UTTARA KANNADA	1	1	Black pepper
WAYANAD	72	66	Black pepper
MALAPPURAM	74	52	Black pepper
THIRUVANANTHAPURAM	86	17	Black pepper
THRISSUR	46	27	Black pepper
KOZHIKODE	17	3	Banana
MALAPPURAM	14	6	Banana

4.2. Culturing of *Radopholus* spp.

The isolated nematodes were cultured on carrot callus disc. The nematode population was collected in the filtrate. Bulk population of pure culture of *R. similis* was obtained. One samples each from all these different locations were used for further studies. The details of the isolates selected for further study are listed in the table 9.

Table 9. Details of the burrowing nematode isolates used for the study

SLNO	SAMPLE CODE	LOCATION	HOST	STANDARD	LATITUDE	LONGITUDE
1	IISR RS01	IISR, KOZHIKODE	BLACK PEPPER	POLYALTHIA	11°17 '38 "N	75°49'13"E
2	IISR RS02	PAMPADUMPARA, IDUKKI	BLACK PEPPER	SILVER OAK	9°79'67"N	77°15' 86"E
3	IISR RS03	THAMARASSERY, KOZHIKODE	BANANA	-	11 °15'30"N	75°55'13"E
4	IISR RS04	PULAMANTHOLE, MALAPPURAM	BANANA	-	10°90'63"N	76°19'43"E
5	IISR RS05	SIRSI, UTTARA KANNADA	BLACK PEPPER	ARECANUT	14°61'71"N	74°84'49"E
6	IISR RS06	KALPETTA, WAYANAD	BLACK PEPPER	GLYRICEDIA	11°61'03"N	76°08'28"E
7	IISR RS07	PULAMANTHOLE, MALAPPURAM	BLACK PEPPER	MANGO TREE	10°90'63"N	76°19'43"E
8	IISR RS08	PALLODE, THIRUVANANTHAPURAM	BLACK PEPPER	OIL PALM	8°72'44"N	77°02'48"E
9	IISR RS09	MANNUTHY, THRISSUR	BLACK PEPPER	COCONUT	10°53'32"N	76°26'56"E
10	IISR RS10	MUTHAPPANPUZHA, KOZHIKODE	BLACK PEPPER	JACK TREE	11°44'38"N	76°55'13" E

4.3. Morphological characterization

4.3.1. Morphological measurements

Several morphological characters required to calculate the DeMan's formulae were measured and several distinguishing characters of *Radopholus* genera were observed

in the study. All the isolates had a total body length in the range of 553 to 849 µm (Figure 5a and 5b).

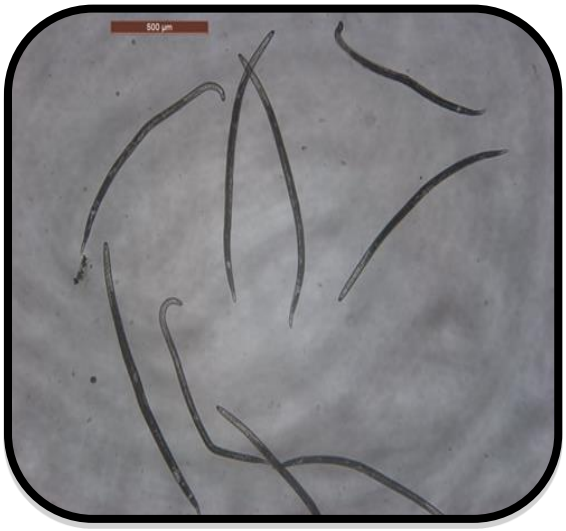


Fig.5a: Female *R. similis*

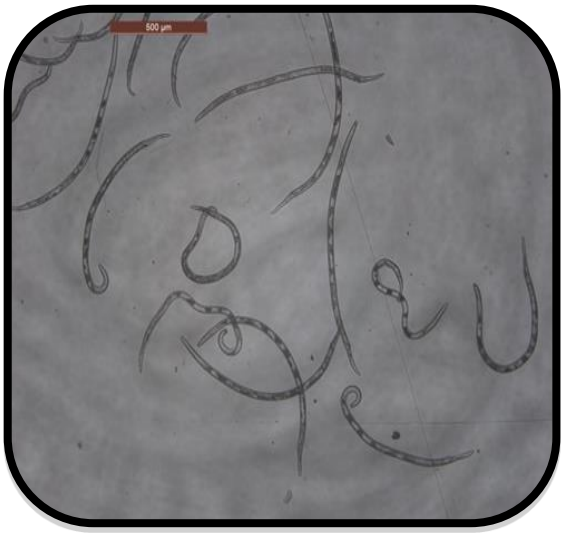


Fig. 5b: Male *R. similis*

The length of the females of all the isolates varied broadly. Few of the females were as small as 553 µm whereas few (mainly the gravid females) were as big as 849 µm. The males showed length of a narrow range 602 to 698 µm, compared to the females. Even though the average body length of males and females of the isolates were similar, the body diameter of most of the females were greater than the body diameter of males, hence the females had a lower 'a' value (body length / greatest body diameter) than males. The body diameters of females ranged from 19.8-32.3 and the body diameter of males from 14.6-23.2µm. In females the, 'a' value range from 24-30 µm and in males they ranged from 30-44µm. This gives a long slender body shape to males when compared to females.

The cephalic region of females of all the isolates are dome shaped and slightly set off. The male cephalic region is knob like and clearly set off. The number of cephalic

annules for males and females of the ten isolates studied show 3-4 annules on their cephalic region.

Females of all the isolates showed the presence of a prominent stylet, and their length ranged from 17.1-20.4 µm and the stylet of males are not quite well developed and their length ranged from 12.3-17.2 µm. The stylet knobs of all the ten female isolates studied were well-developed. In males the stylet knob got amalgamated in one round base. The 'o' value (% distance of dorsal oesophageal gland opening from stylet knobs in relation to stylet length) depicting the distance from the stylet base to DGO (dorsal oesophageal gland orifice) was prominent in females and it ranged from 2.3-4.0µm. The 'o' value of all the female isolates ranged from 12.2-20.3.

Further, the position of excretory pore has been found to be at the pharyngo intestinal junction in all the isolates. Females of all the isolates showed a 'b' value (body length/ distance from anterior to esophago-intestinal valve) in the range 4.7 to 7.5 and males had a 'b' value in the range of 6.1-6.6.

The esophageal gland overlapped the intestine dorsally in all males and females of the ten isolates. All the female nematodes have a prominent esophagus, with distinct esophageal regions, but the esophagus of males has not been as prominent as females in all isolates. In females the distance from anterior to base of esophageal glands ranged from 147-178 µm and in males it ranged from 126-170µm. The females of different isolates have a b' value (body length/ distance from anterior to base of esophageal gland) in the range of 3.3-5.2 and the males had a b' value in the range of 4.1-4.9. The pharynx was 125 to 156 µm long in all the females.

As for the male reproductive features, the bursa, in all the isolates was found to be either extended to mid tail or to the tail terminus. Further, the spicule length, for

these isolates was found to be in the range of 18 to 22 µm and the gubernaculum, which is the tissue that holds the spicule in its position, measured 8-11 µm (which is almost half the length of the spicule) in all the isolates.

The reproductive system of all females of the ten isolates has been found to be didelphic, extended, with oocytes in a single row. The length of the anterior female gonad and the posterior female gonad was almost of equal length, but the anterior gonad was slightly longer than the posterior female gonad in few nematodes of all the isolates. But that difference is negligible. Hence the G1 (% length of anterior female gonad in relation to body length) and G2 (% length of posterior female gonad in relation to body length) values of all nematodes of the isolates are found to be almost equal. The G1 and G2 values ranged from 20 to 28. Further, the vulva has been found to be flat or slightly projecting. The 'V' value given by the % distance of vulva from anterior ranged from 55-60.

The isolates depict diverse tail terminus shapes in males and females. The tails of most nematodes are conoid, tapering and rounded in females and almost pointed in males. Further, the tail terminus sculpture in females and males of the ten isolates studied has been found to be annulated. The c value (body length / tail length) of males and females of all the isolates have been found to be very small because of their characteristic long tail. c value of females of all the isolates ranged from 7.7-12.5while it ranged from 7.1-10.8 in males.

The females have a moderate tail length and a moderate anal body width, but the males have longer tail and their anal body width is quite small giving it a characteristic narrow long appearance. The tail diameter at anus or claoca in females ranged from 13.9 to 29.6 µm and in males it ranged from 9.5-18.3µm. The c' value

(ratio of the tail length to the anal body width) of females ranged from 3.0 to 3.9 and that of males from 5.0 to 6.9. Also, females of all the isolates have a hyaline length of 8 to 16 µm and the males have a hyaline length of 6 to 11 µm.

The females have moderate tail length and long stylet. This gave the females a T/S ratio (tail length/stylet length) of 2.7 to 5.7. The males have long tail and shorter stylet giving them a larger T/S ratio. The T/S value in males ranged from 3.7 to 7.2. Figure 6a and 6b, shows the light microscopy images of the females and male *R. similis* isolates.

4.3.2. *Statistical analysis*

The ANOVA analysis on morphological characters did not show any significant difference in most of the characters (Table 10a and 10b). However, the length of stylet, spicule and gubernaculum in males and o value and V value in females showed significant differences.

Table 10 (a): Detailed morphometric variability of *R. similis* females associated with black pepper in Kerala and Karnataka (all measurements are in µm).

	Length	a	b	b'	Tail length	C	c'	Hyaline	Stylet	T/S	o	V	G1	G2	Distance from anterior to DGO	Pharynx
RS01	706 ± 64.40	28± 1.43	6.3± 0.70	4.3± 0.38	73±11.86	9.9±1.18	3.7±0.22	12±2.10	18.9±0.71	3.9±0.71	18±1.55	58±1.12	25±1.98	25±2.01	23±0.88	143±5.78
	(587-838)	(25-30)	(4.9-7.4)	(3.5-4.9)	(55-103)	(8.2-12.2)	(3-4)	(8-16)	(17.6-20)	(3-5.8)	(15-21)	(56-59)	(20-27)	(20-27)	(21-24)	(132-154)
RS02	714±53.15	28± 1.44	6.4± 0.66	4.4± 0.39	75±11.79	9.8±1.17	3.7±0.13	12±2.21	19±0.86	4±0.67	17±1.87	58±1.39	25±2.00	25±1.86	23±1.14	142±8.31
	(595-821)	(24-30)	(4.8-7.4)	(3.5-5)	(56-98)	(8.5-12.6)	(3.5-4)	(8-16)	(17.4-20.1)	(2.9-5.5)	(14-19)	(55-60)	(21-28)	(21-28)	(20-24)	(128-155)
RS03	700±67.72	28± 1.57	6.4± 0.59	4.3± 0.33	73±9.23	9.7±1.02	3.7±0.16	13±1.79	19.1±0.64	3.9±0.47	17±1.52	58±1.24	25±1.51	25±1.51	23±0.88	142±8.93
	(553-845)	(25-30)	(5.1-7.3)	(3.6-4.8)	(57-91)	(8.4-11.7)	(3.4-4)	(8-15)	(17.1-19.8)	(3-4.8)	(14-19)	(56-61)	(22-28)	(22-27)	(20-24)	(128-154)
RS04	702± 55.04	28± 1.59	6.3± 0.64	4.5± 0.30	74±7.44	9.6±1.09	3.7±0.16	13±1.84	19±0.63	3.9±0.43	18±1.92	57±1.09	26±2.00	25±2.00	23±0.78	137±5.61
	(605-838)	(24-30)	(4.9-7.3)	(3.7-5)	(61-89)	(8.3-12)	(3.4-4)	(9-15)	(18-20.1)	(3.2-4.8)	(13-20)	(55-59)	(22-29)	(22-28)	(22-24)	(126-148)
RS05	695±52.34	28± 1.50	6.3± 0.55	4.3± 0.41	72±9.11	9.9±0.96	3.7±0.15	13±2.09	18.8±0.69	3.9±0.52	18±1.62	58±1.16	25±1.62	25±1.65	23±0.89	141±7.56
	(583-799)	(25-30)	(4.9-7.5)	(3.4-4.9)	(60-87)	(8.4-11.8)	(3.4-3.9)	(9-16)	(17.4-19.8)	(3.1-4.8)	(15-20)	(56-60)	(21-28)	(21-27)	(21-24)	(126-155)
RS06	693± 74.13	28± 1.43	6.3± 0.59	4.3± 0.47	73±13.35	9.7±1.12	3.6±0.14	13±2.06	19±0.53	3.9±0.72	17±1.52	57±0.96	25±0.97	25±1.80	23±0.63	141±7.91
	(576-834)	(25-30)	(4.8-7.3)	(3.5-5.1)	(56-107)	(7.8-11.9)	(3.4-3.9)	(8-16)	(18.1-20)	(3-5.6)	(13-19)	(55-59)	(22-28)	(21-27)	(22-24)	(125-152)
RS07	707± 43.09	28± 0.97	6.2± 0.52	4.3± 0.30	73±7.99	9.9±1.00	3.7±0.13	13±1.74	19±0.70	3.9±0.43	18±1.30	58±1.06	25±2.04	24±2.12	23±0.86	145±8.37
	(610-813)	(26-29)	(4.9-7.4)	(3.8-4.9)	(61-93)	(7.9-11.9)	(3.5-4)	(9-15)	(17.8-20.2)	(3.1-4.8)	(17-21)	(56-60)	(22-29)	(21-28)	(22-24)	(127-157)
RS08	717± 60.17	28± 1.43	6.3± 0.61	4.4± 0.38	77±10.40	9.5±0.76	3.7±0.16	12±1.85	18.9±0.58	4.1±0.51	19±1.18	57±0.78	25±1.70	25±1.75	23±0.68	145±8.05
	(593-849)	(25-30)	(4.7-7.5)	(3.6-5.2)	(61-100)	(7.7-10.5)	(3.4-4)	(9-15)	(18.1-19.9)	(3.2-5.1)	(17-21)	(55-58)	(22-28)	(22-28)	(22-24)	(131-155)
RS09	731± 37.75	28± 1.55	6.5±0.44	4.5±0.27	75±8.91	10±0.97	3.7±0.15	13±1.79	19±0.75	4±0.56	18±1.26	58±1.33	25±1.80	25±1.88	23±0.92	144±7.56
	(669-820)	(25-30)	(5.3-7.1)	(4-4.9)	(61-90)	(8.4-12)	(3.4-4)	(10-15)	(17.3-20.1)	(3.1-4.9)	(15-20)	(55-60)	(21-28)	(21-27)	(21-24)	(131-156)
RS10	710± 63.13	28± 1.54	6.4± 0.58	4.3± 0.41	73±11.21	10±1.00	3.7±0.15	13±2.07	19.2±0.74	3.8±0.63	18±1.51	58±0.93	25±1.97	25±1.96	23±0.91	145±9.49
	(574-824)	(25-30)	(5.2-7.5)	(3.5-5.2)	(50-98)	(8.2-11.6)	(3.4-4)	(8-16)	(18.1-20.4)	(2.7-5)	(15-21)	(56-60)	(22-29)	(22-28)	(21-25)	(125-156)
Mean±SD	707.01±18.36	27.27±0.46	6.3±0.188	4.31±0.117	73.43±3.254	9.74±0.327	3.65±0.049	12.2±0.62	18.9±0.217	3.88±0.182	17.34±0.487	57.15±0.354	24.68±0.65	24.34±0.65	22.26±0.274	141.95±2.481
	(553-849)	(24-30)	(4.7-7.5)	(3.32-5.16)	(49.9-106.9)	(7.7-12.5)	(3.0-3.9)	(8-16)	(17.1-20.4)	(2.7-5.7)	(12-20)	(55-60)	(20-28)	(20-28)	(20-24)	(125-156)
CV (%)	8.21	5.33	9.41	8.56	14.01	10.63	4.28	16.07	3.62	14.83	8.89	1.96	7.50	7.66	3.89	5.53
LSD at 5%	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.96	0.70	NS	NS	NS	NS

Table 10(b): Detailed morphometric variability of *R. similis* males associated with black pepper in Kerala and Karnataka (all measurements are in µm).

	Length	a	b	b'	Tail length	C	c'	Hyaline	Stylet	T/S	Gubernaculum	Spicule
RS01	655±27.10	36± 3.40	6.4± 0.13	4.6± 0.27	75±6.62	8.8±0.62	5.8±0.43	9±1.21	14.5±1.00	5.3±0.53	9±0.85	19±1.04
	(604-694)	(31-44)	(6.1-6.6)	(4.2-5)	(64-85)	(8.1-10)	(5.2-6.8)	(7-11)	(12.3-16.4)	(4.3-6.1)	(8-11)	(18-22)
RS02	644± 31.73	37± 2.97	6.4± 0.11	4.6± 0.29	74±7.64	8.9±0.88	5.9±0.43	9±1.50	14.8±1.46	5±0.58	9±1.39	19±0.86
	(602-695)	(30-42)	(6.1-6.5)	(4.1-5)	(63-92)	(7.5-10.8)	(5.3-6.7)	(6-11)	(12.3-17.2)	(3.8-6)	(8-10)	(18-21)
RS03	651± 28.21	37± 2.87	6.3± 0.13	4.6± 0.27	77±7.96	8.6±0.80	6.1±0.46	9±1.19	14.4±0.75	5.4±0.63	10±0.75	19±0.86
	(609-698)	(32-42)	(6.1-6.5)	(4.2-4.9)	(61-97)	(7.2-10.1)	(5.3-6.6)	(7-11)	(13.2-15.9)	(4.5-7.2)	(9-11)	(18-21)
RS04	650± 27.57	36± 2.17	6.3± 0.13	4.6± 0.26	76±7.20	8.7±0.66	6±0.41	9±1.15	15±1.21	5.2±0.75	9±0.92	20±1.15
	(606-697)	(31-40)	(6.1-6.5)	(4.2-5)	(62-91)	(7.5-10)	(5.3-6.5)	(7-11)	(13.1-17.2)	(3.7-6.7)	(8-11)	(18-22)
RS05	653± 27.53	36± 3.30	6.3± 0.13	4.6± 0.27	77±6.14	8.6±0.62	6±0.40	9±0.88	13.9±1.02	5.6±0.57	9±0.79	19±0.79
	(609-697)	(31-43)	(6.1-6.5)	(4.1-5)	(66-91)	(7.5-10.2)	(5.3-6.5)	(7-10)	(12.3-15.6)	(4.6-6.8)	(8-10)	(18-20)
RS06	649±29.47	36± 2.07	6.4± 0.12	4.6± 0.29	76±6.39	8.7±0.55	5.9±0.41	9±1.15	14.9±0.90	5.1±0.58	10±0.83	20±0.97
	(609-698)	(33-41)	(6.1-6.5)	(4.2-5)	(66-89)	(7.7-9.7)	(5.3-6.5)	(7-11)	(13.6-16.2)	(4.4-6.2)	(9-11)	(19-22)
RS07	660± 26.52	37± 2.28	6.4± 0.13	4.6± 0.23	76±7.27	8.8±0.81	6±0.45	9±1.05	14.6±0.95	5.3±0.64	9±0.79	19±0.76
	(617-698)	(32-40)	(6.1-6.5)	(4.1-5)	(65-91)	(7.4-10.5)	(5.2-6.5)	(7-11)	(12.7-15.8)	(4.1-6.4)	(8-10)	(18-20)
RS08	656± 28.58	36± 2.44	6.3±0.12	4.6±0.26	77±7.00	8.6±0.67	6±0.46	9±1.23	14.8±1.24	5.3±0.68	9±0.97	19±1.08
	(618-698)	(32-41)	(6.1-6.5)	(4.1-5)	(63-89)	(7.2-10)	(5.2-6.5)	(6-11)	(12.7-16.7)	(4.3-6.7)	(8-11)	(18-22)
RS09	658±27.46	36± 3.17	6.3± 0.12	4.6± 0.26	77±6.69	8.7±0.80	5.9±0.67	9±1.31	14.4±0.82	5.4±0.55	9±0.93	20±1.23
	(610-694)	(31-43)	(6.1-6.5)	(4.1-5)	(61-89)	(7.2-10.3)	(5.2-7)	(6-11)	(13.2-15.7)	(4.5-6.3)	(8-11)	(18-22)
RS10	659± 27.30	36± 3.59	6.3± 0.13	4.6± 0.25	77±6.26	8.7±0.70	5.8±0.49	9±0.99	14.8±0.81	5.3±0.60	9±0.73	19±0.79
	(603-695)	(31-44)	(6.1-6.5)	(4.1-4.9)	(67-89)	(7.3-10.2)	(5-6.5)	(7-11)	(13.5-16.1)	(4.2-6.4)	(8-10)	(18-20)
Mean±SD	653.11±8,912	35.89±0.909	6.29±0.04	4.55±0.084	75.95±2.195	8.66±0.227	5.9±0.147	8.6±0.372	14.61±0.329	5.23±0.194	9.14±0.266	19.3±0.306
	(602-698)	(30-44)	(6.1-6.6)	(4.1-4.9)	(60.9-96.9)	(7.1-10.8)	(5.0-6.9)	(6-11)	(12.3-17.2)	(3.7-7.2)	(8-11)	(18-22)
CV (%)	4.32	8.01	2.01	5.83	9.14	8.3	7.9	13.69	7.11	11.72	9.21	5.01
LSD at 5%	NS	NS	NS	NS	NS	NS	NS	NS	0.65	NS	0.52	0.6

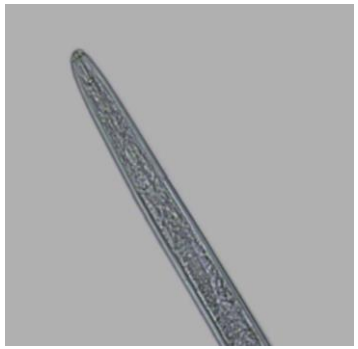
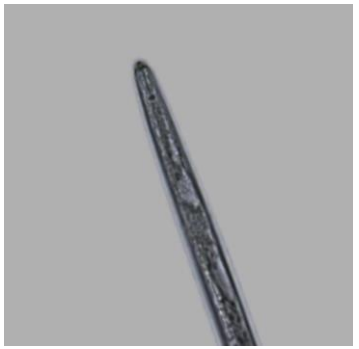
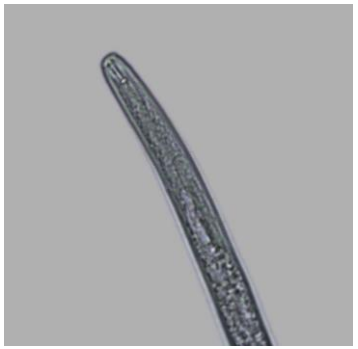
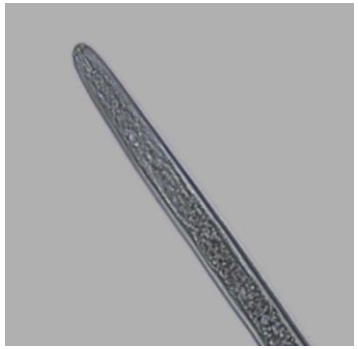
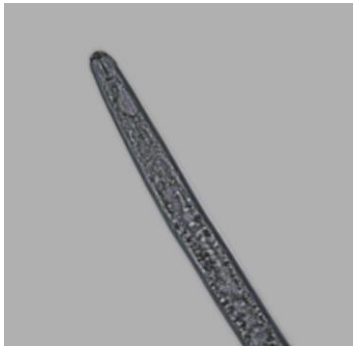
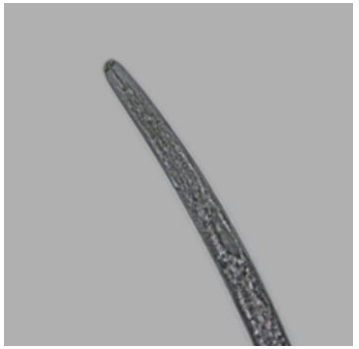
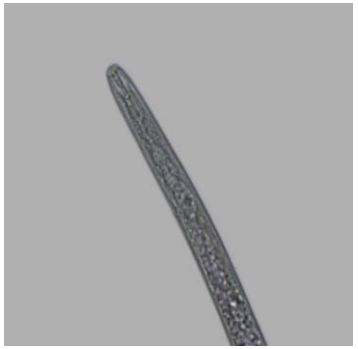
				
RS01 FEMALE HEAD	RS02 FEMALE HEAD	RS03 FEMALE HEAD	RS04 FEMALE HEAD	RS05 FEMALE HEAD
				
RS06 FEMALE HEAD	RS07 FEMALE HEAD	RS08 FEMALE HEAD	RS09 FEMALE HEAD	RS10 FEMALE HEAD

Fig. 6a: Cephalic region of females of the ten isolates (RS01-RS10).

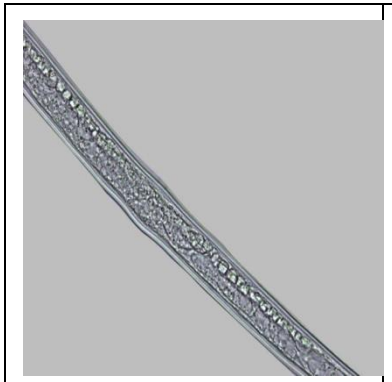
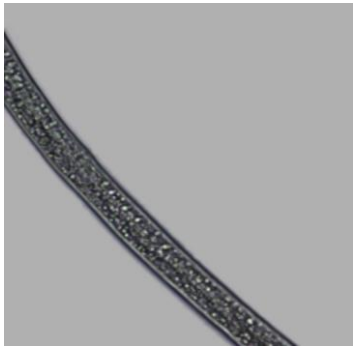
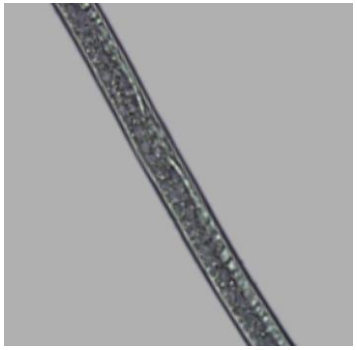
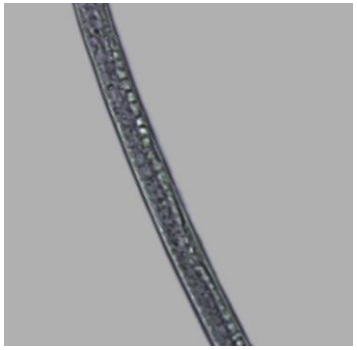
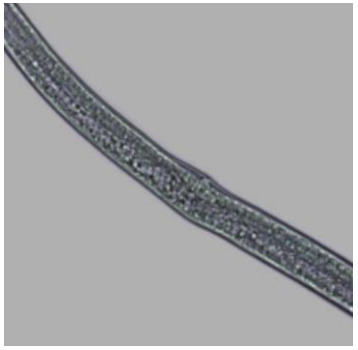
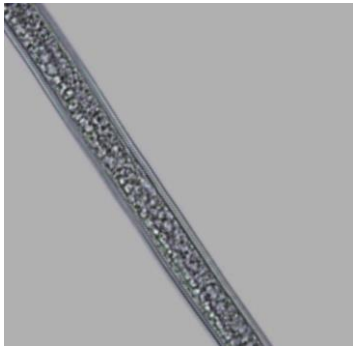
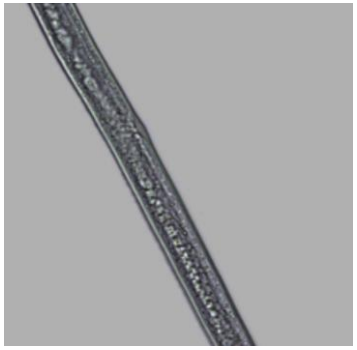
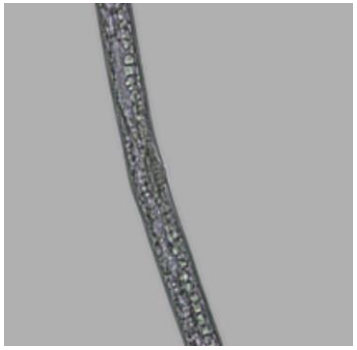
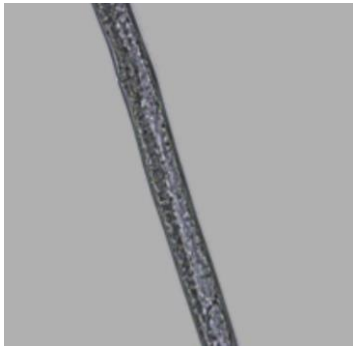
				
RS01 FEMALE MID BODY	RS02 FEMALE MID BODY	RS03 FEMALE MID BODY	RS04 FEMALE MID BODY	RS05 FEMALE MID BODY
				
RS06 FEMALE MID BODY	RS07 FEMALE MID BODY	RS08 FEMALE MID BODY	RS09 FEMALE MID BODY	RS10 FEMALE MID BODY

Fig.6b: Mid body of females of the ten isolates (RS01-RS10).

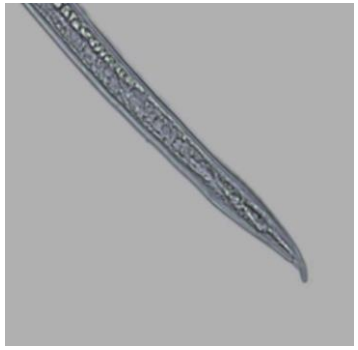
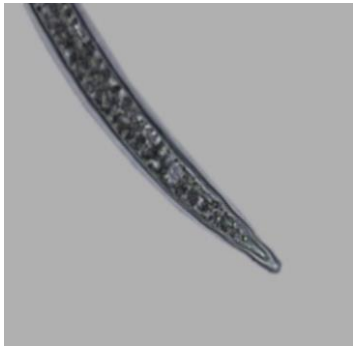
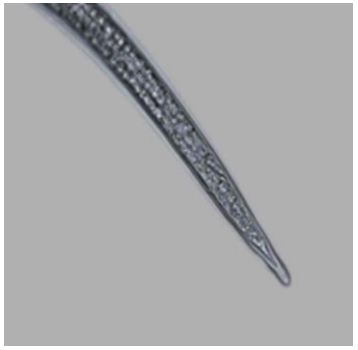
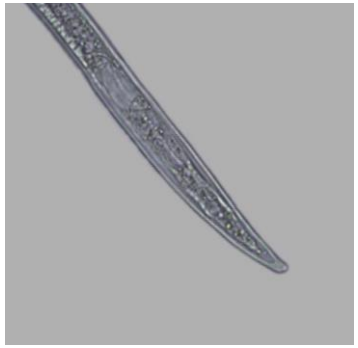
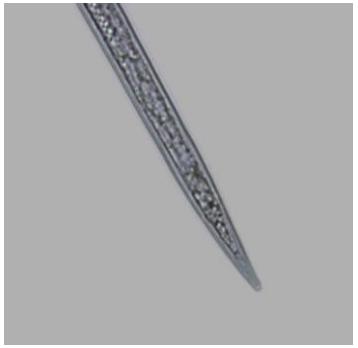
				
RS01 FEMALE TAIL	RS02 FEMALE TAIL	RS03 FEMALE TAIL	RS04 FEMALE TAIL	RS05 FEMALE TAIL
				
RS06 FEMALE TAIL	RS07 FEMALE TAIL	RS08 FEMALE TAIL	RS09 FEMALE TAIL	RS10 FEMALE TAIL

Fig.6c: Tail region of females of the ten isolates (RS01-RS10).

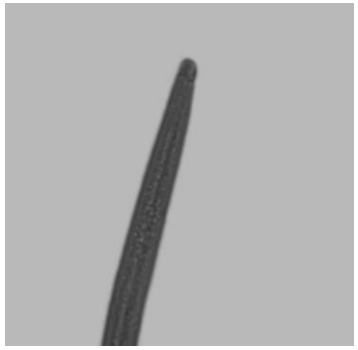
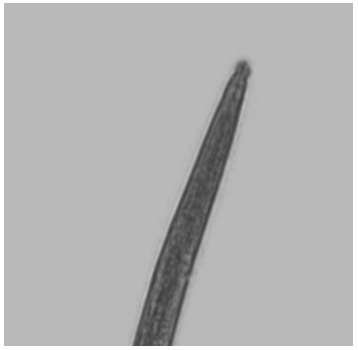
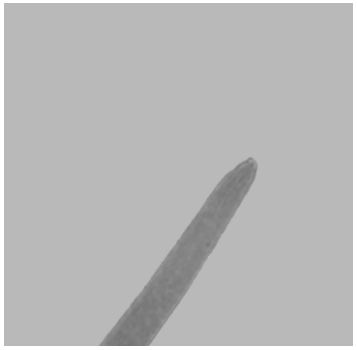
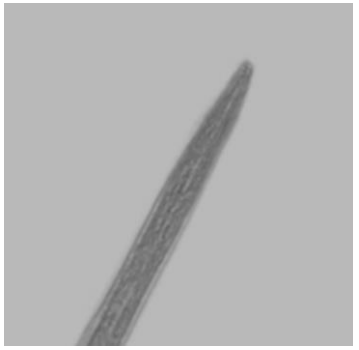
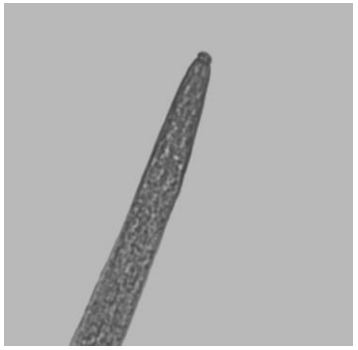
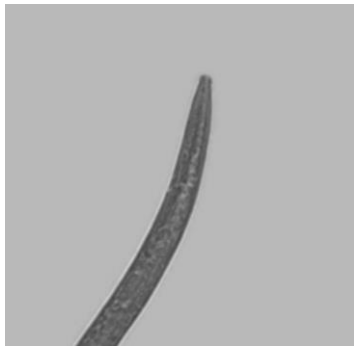
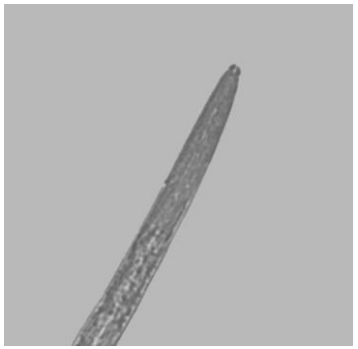
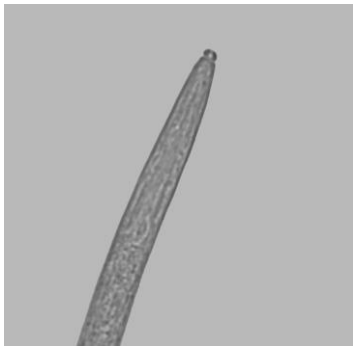
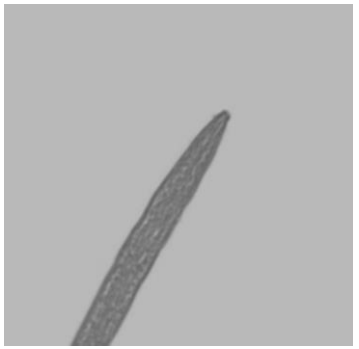
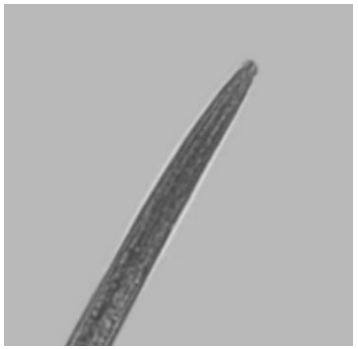
				
RS01 MALE HEAD	RS02MALE HEAD	RS03MALE HEAD	RS04MALE HEAD	RS05MALE HEAD
				
RS06MALE HEAD	RS07MALE HEAD	RS08MALE HEAD	RS09MALE HEAD	RS10MALE HEAD

Fig.6d: Cephalic region of males of the ten isolates (RS01-RS10).

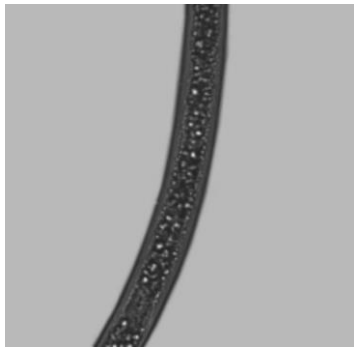
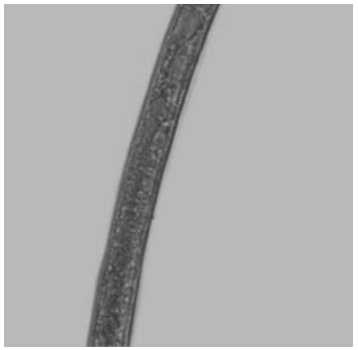
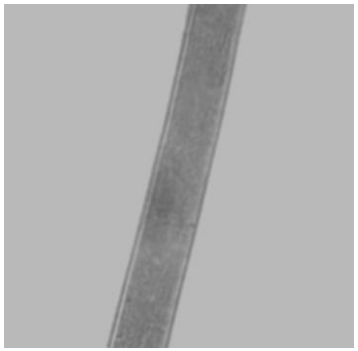
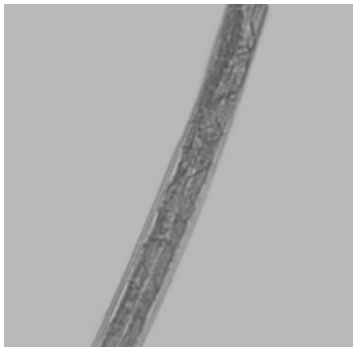
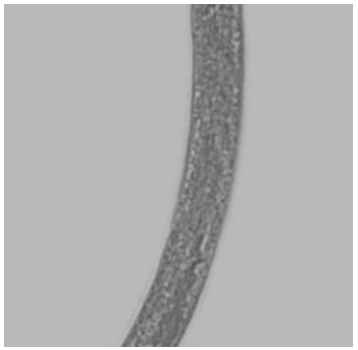
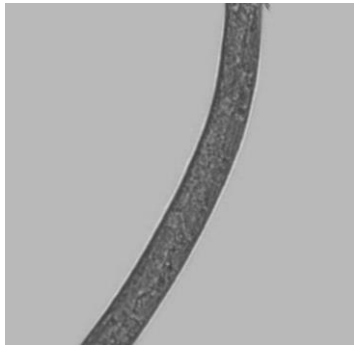
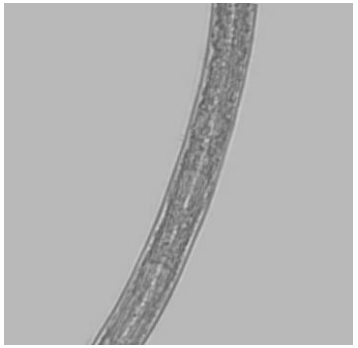
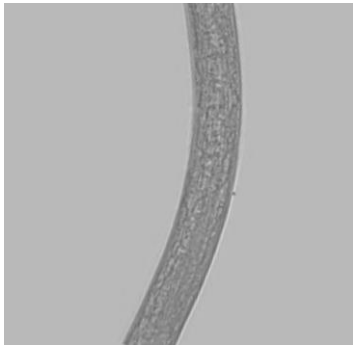
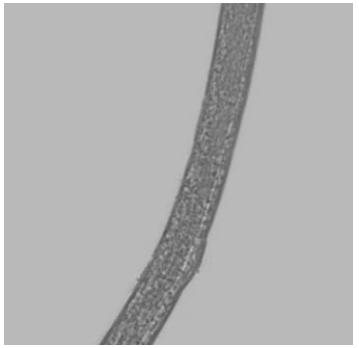
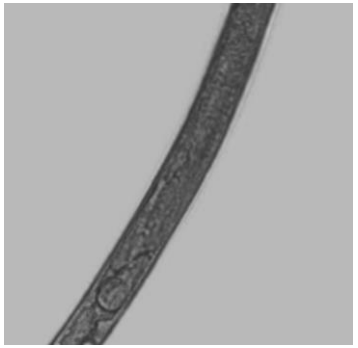
				
RS01 MALE MID BODY	RS02MALE MID BODY	RS03MALE MID BODY	RS04MALE MID BODY	RS05MALE MID BODY
				
RS06MALE MID BODY	RS07MALE MID BODY	RS08MALE MID BODY	RS09MALE MID BODY	RS10MALE MID BODY

Fig.6e: Mid body of males of the ten isolates (RS01-RS10).

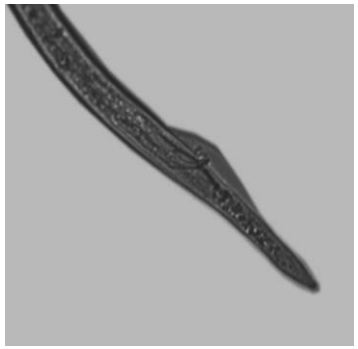
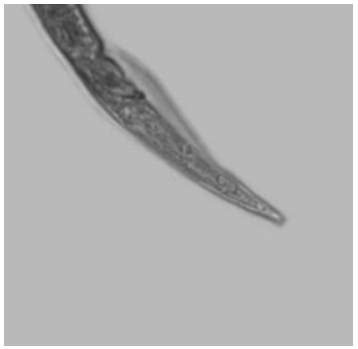
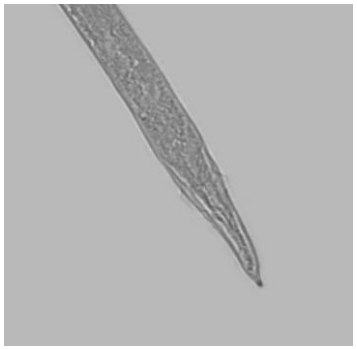
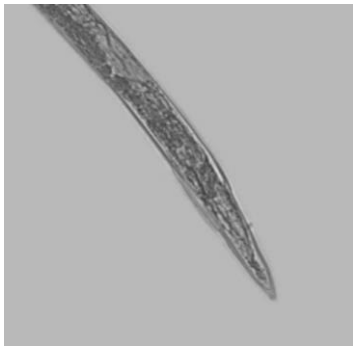
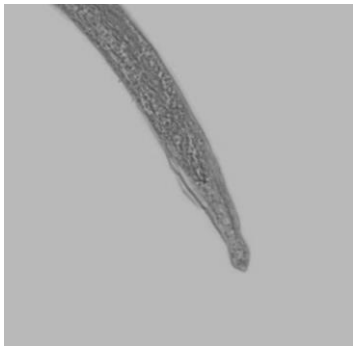
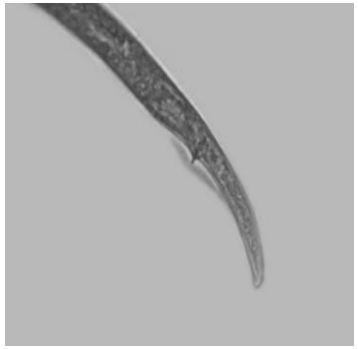
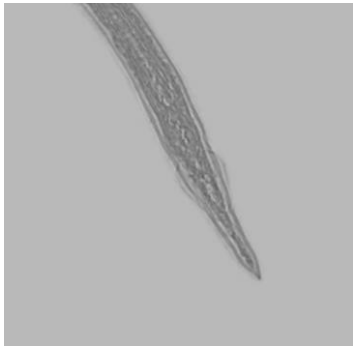
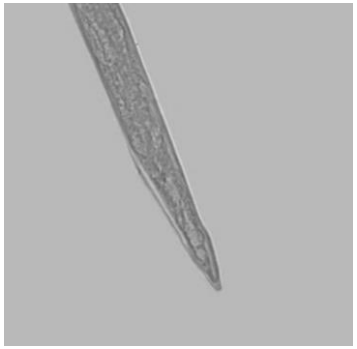
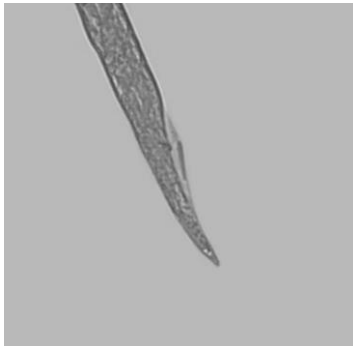
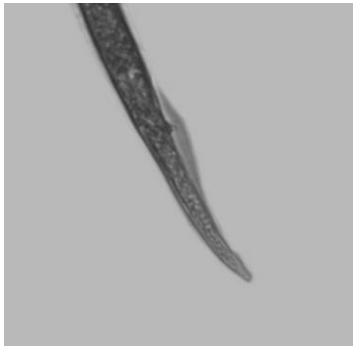
				
RS01 MALE TAIL	RS02MALE TAIL	RS03MALE TAIL	RS04MALE TAIL	RS05MALE TAIL
				
RS06MALE TAIL	RS07MALE TAIL	RS08MALE TAIL	RS09MALE TAIL	RS10MALE TAIL

Fig.6f: Tail region of males of the ten isolates (RS01-RS10).

4.4. Molecular characterization

4.4.1. PCR amplification of the Radopholus spp. isolates

The DNA was isolated from mass multiplied pure cultures of the ten isolates of *Radopholus* spp and was quantified to get the required concentration of DNA. Products of 920 bp,899 bp and 701bp, respectively, were obtained when genomic DNA of the isolates was amplified using the primer pair 18S-26S (Figure 7), COX (Figure 8) and ND (Figure9).

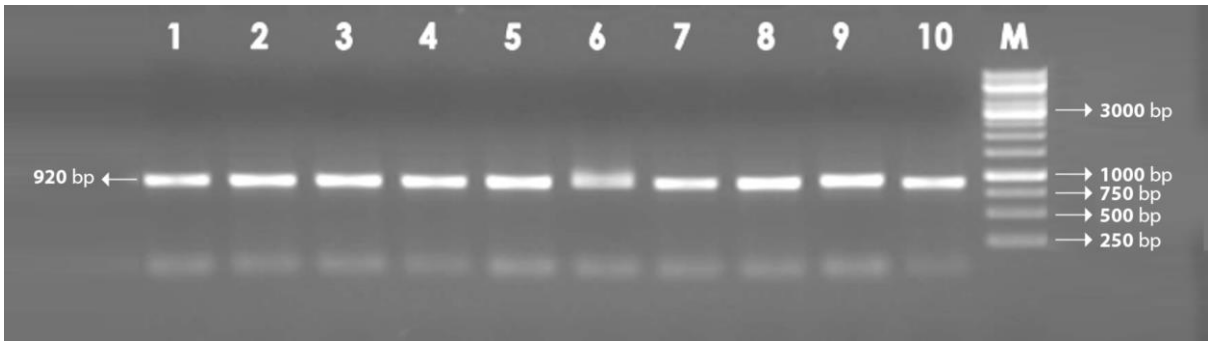


Fig. 7: PCR amplification of the *Radopholus* spp. isolates using ITS (18S-26S) primer pair. Lane 1=RS 01; Lane 2: RS 02; Lane 3: RS03, Lane 4: RS04, Lane 5: RS05, Lane 6: RS06, Lane 7: RS07, Lane 8: RS08, Lane 9: RS09, Lane 10: RS10 and Lane 11: Marker.

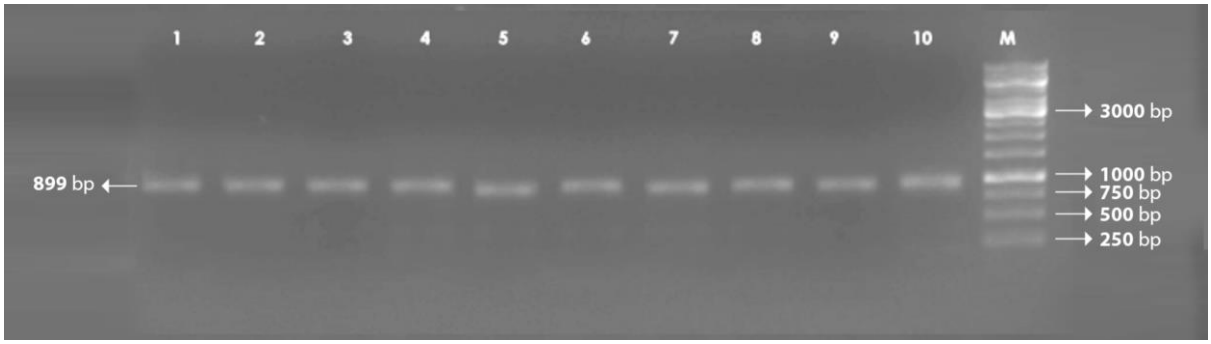


Fig. 8: PCR amplification of the *Radopholus* spp. isolates using COX primer pair. Lane 1=RS 01; Lane 2: RS 02; Lane 3: RS03, Lane 4: RS04, Lane 5: RS05, Lane 6: RS06, Lane 7: RS07, Lane 8: RS08, Lane 9: RS09, Lane 10: RS10 and Lane 11: Marker.

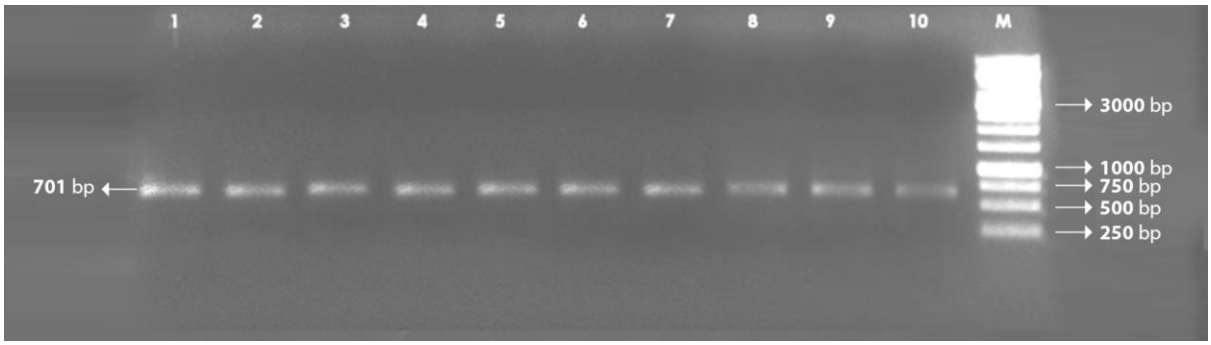


Fig. 9: PCR amplification of the *Radopholus* spp. isolates using ND primer pair. Lane 1=RS 01; Lane 2: RS 02; Lane 3: RS03, Lane 4: RS04, Lane 5: RS05, Lane 6: RS06, Lane 7: RS07, Lane 8: RS08, Lane 9: RS09, Lane 10: RS10 and Lane 11: Marker.

The amplified fragments were purified and cloned into pTZ57R/T vector using standard protocol. The positive clones developed from transformed *E. coli* DH5α (Figure 10) were selected for plasmid isolation and sequencing.

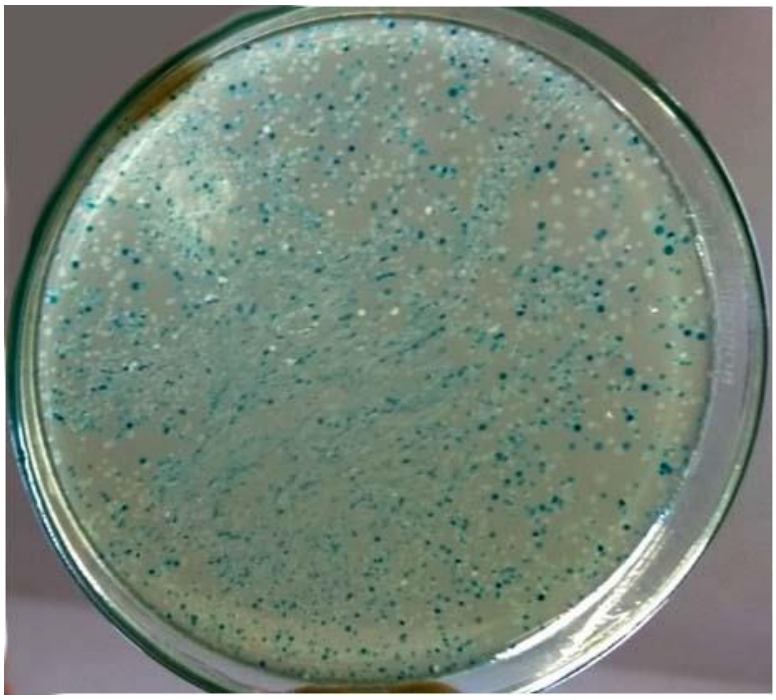


Fig. 10: Blue white colonies containing the transformed *E. coli*

4.4.2. Sequence analysis

Post DNA sequencing of the ITS, COX and ND regions, the respective sequences of the ten isolates were obtained. These sequences where then subjected to BLAST search in NCBI. All the ITS sequences showed homology to several *R. similis* ITS regions reported from different geographical regions worldwide. The COX and ND sequences of all the 10 isolates showed high level of similarity to their respective regions on the complete mitochondrial genome sequence of *R. similis*. The COX sequences also showed similarity to one of the COX sequence of *R. similis* reported previously in the public database. All these sequences were then deposited in NCBI (Table 11).

Table 11: ITS, COX and ND gene sequences amplified from different isolates of *Radopholus similis* collected from India and their corresponding NCBI accession numbers.

Sequence	Gen Bank accession number		
	ITS	**COX**	**ND**
RS01	MF197883	MK682607	MK732481
RS02	MF197884	MK682608	MK732482
RS03	MF197885	MK682609	MK732483
RS04	MF197886	MK682610	MK732484
RS05	MF197887	MK682611	MK732485
RS06	MF197888	MK682612	MK732486
RS07	MF197889	MK682613	MK732487
RS08	MF197890	MK682614	MK732488
RS09	MF197891	MK682615	MK732489
RS10	MF197892	MK682616	MK732490

4.4.3. Phylogenetic analysis:

The sequences were edited and aligned with accessions from GenBank using Clustal X version 2.1 (Larkin *et al*. 2007) and the alignment was edited in BioEdit sequence alignment editor version 7.2.5 (Hall 1999) (Figure 11 and 12).

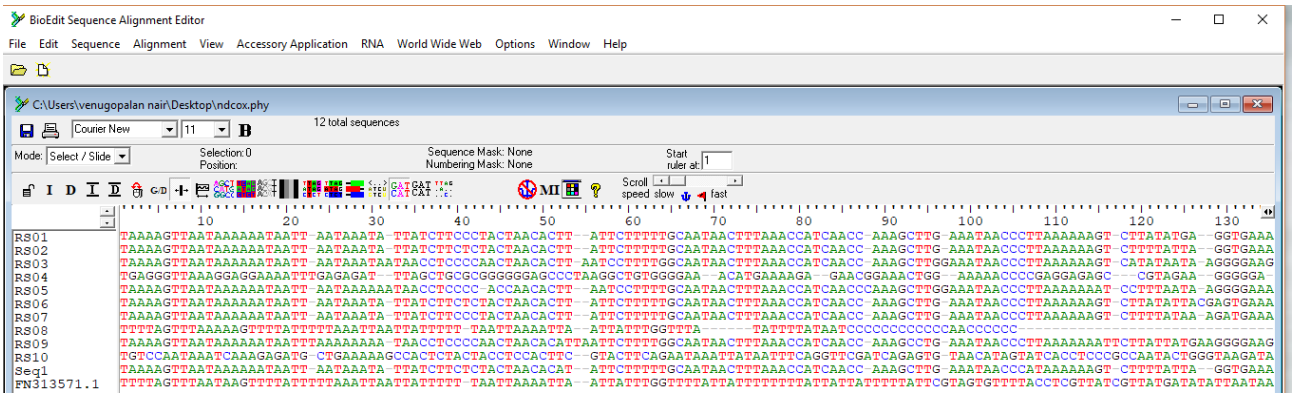


Fig. 11: Multiple sequence alignment of mitochondrial sequence COX and ND

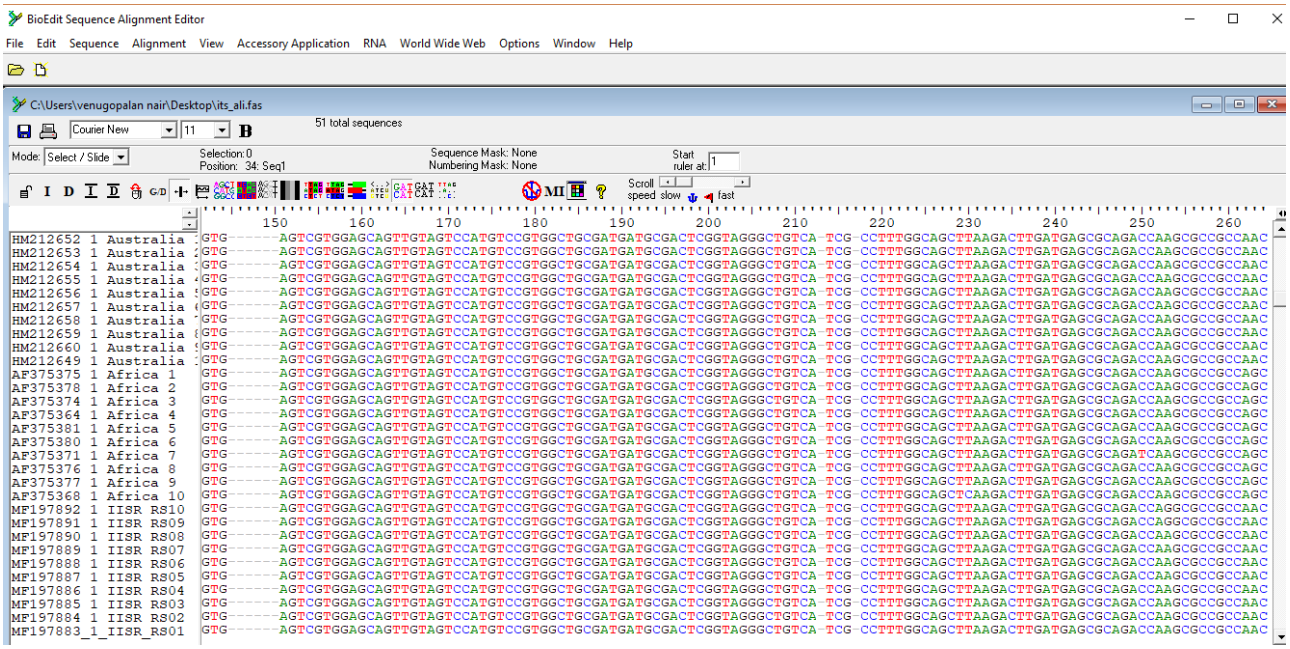


Fig. 12: Multiple sequence alignment of ITS sequence.

Phylogenetic analysis was conducted using the five methods (Bayesian analysis, Maximum likelihood, Maximum parsimony, Minimum Evolution and Neighbour joining methods). The concatenated tree constructed to get a combination of these five methods, were used to make inferences regarding the molecular diversity of the isolates using mitochondrial sequences (COX-ND) and ITS.

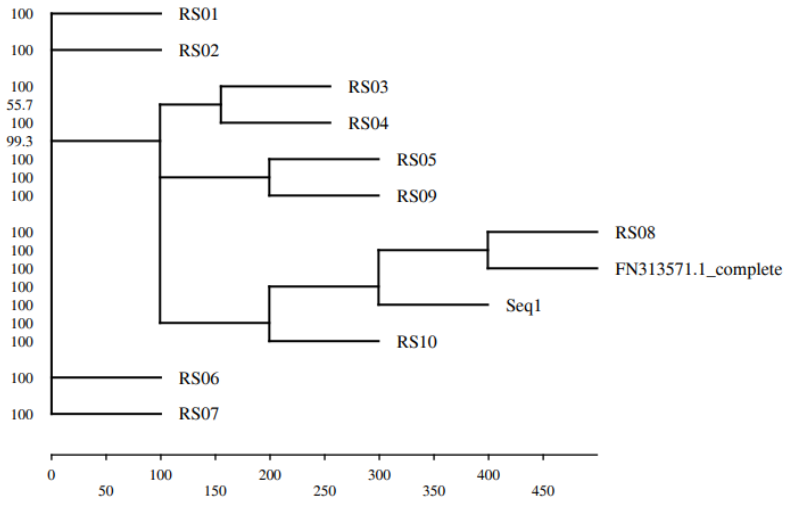


Fig. 13: Concatenated tree obtained by combining the five methods using the COX-ND sequences of the ten isolates.

Figure 13 shows the concatenated tree obtained by combining these five methods using the COX-ND sequences of the ten isolates. All the five phylogenetic analysis methods, resulted in similar tree topologies. The topology and branch lengths of the inference are shown in the figure. As per the concatenated tree drawn for the mitochondrial gene sequences (COX-ND) for the ten isolates in the phylogenetic analysis, all the mitochondrial sequences were observed in the similar branch length. Isolates RS01, RS02, RS06 and RS07 showed maximum variation from one another. Isolates RS03, RS04, RS05, RS09, RS08 and RS10 formed a cluster with three branches. RS03 and RS04 were observed in the same node and formed a clade,

while RS05 and RS09 were observed in other. RS08 and reference FN313571.1 showed another clade. Most nodes in this tree have high bootstrap values which indicate the robustness of the tree computed.

Figure 14 shows the concatenated tree obtained by combining these five methods using the ITS sequences of the ten isolates. In the concatenated tree obtained from the phylogenetic analysis using ITS sequences, two sequences of *R. duriophilus* and one sequence of *R. arabocoffeae*, were taken as outgroups for comparing the results. They formed a separate branch outside the main cluster of sequences. All the rest of sequences formed a single cluster of *R. similis.* The sequences in this cluster formed two groups. One group consisting of all the isolates from Africa and another group consisting of all the rest of the sequences. The African isolates formed a different clade and was very distinct from the Australian and American isolates. However three Asian isolates showed similarity to the African isolates. High level of diversity was observed between the African isolates indicating that *R. similis* was introduced to Africa in different occasions most likely from Asia.

In the other group, which consisted of all the isolates of Australia, America, the ten isolates used in this study and the remaining Asian isolates, all the Australian isolates were found to be highly conserved. The American population showed high level of resemblance to the set of Australian populations. All the *R. similis* isolates used for our study was found to be similar to the Asian, Australian and American isolates. It did not show any resemblance to the African isolates. The isolates RS09 and RS10 formed a separate sub-clade. Isolate RS03, RS04, RS05, RS06 and RS07 were part of a single branch. Both these groups along with the isolates RS01, RS02 and RS08 showed high level of resemblance to the Australian and American population.

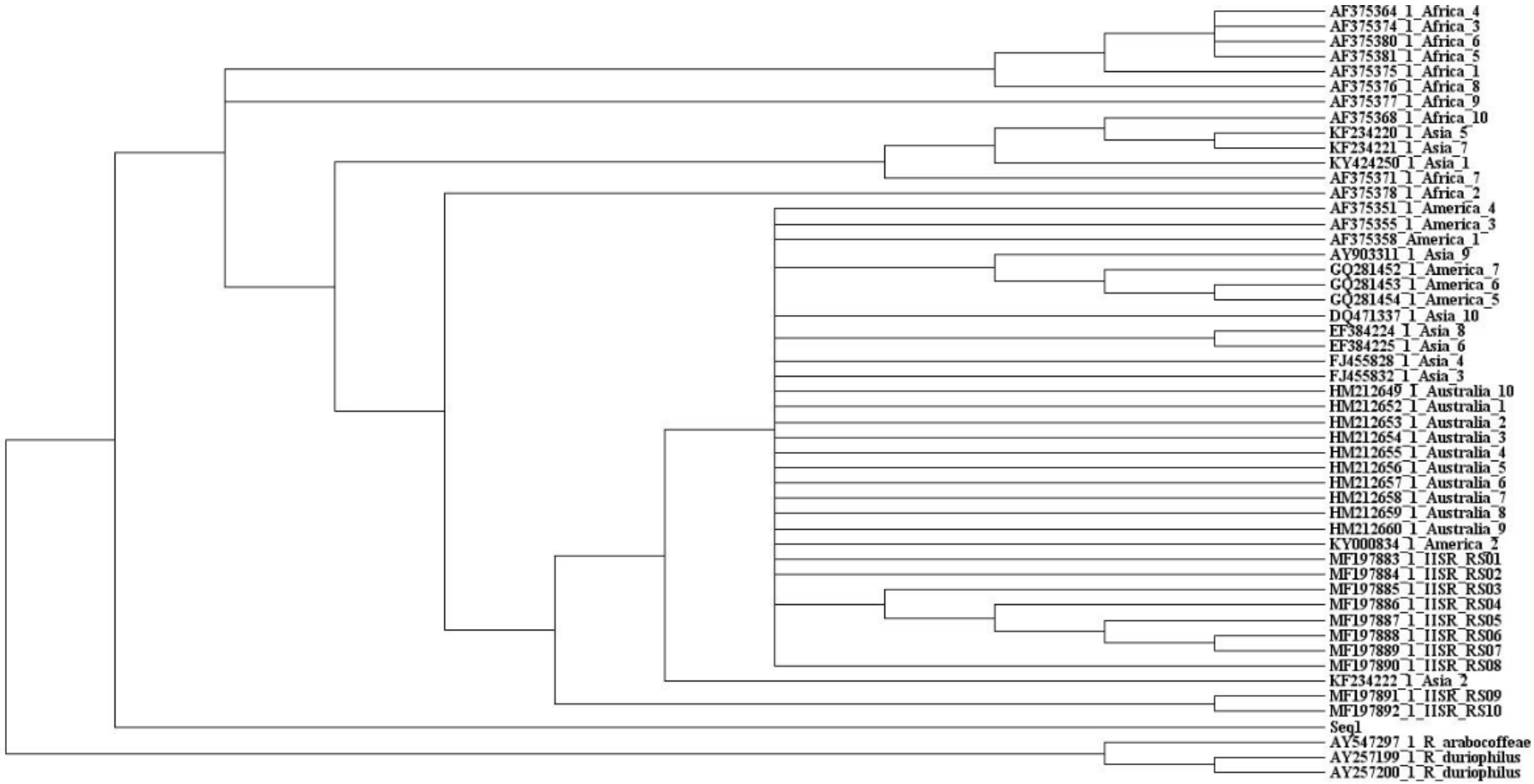


Fig. 14: Concatenated tree obtained by combining the five methods using the ITS sequences of the ten isolates

4.4.4. Haplotype analysis:

A statistical parsimony network was done to further confirm the accuracy of the phylogenetic analysis (Figure 15). Each circle represents the different taxon. The area of the circle indicates the number of individuals present in that particular haplotype**.** It was observed that the haplotype analysis also yielded the similar results as obtained from the phylogenetic study. Outgroups formed a different cluster. A node separated the complete *R. similis* populations into two clusters. One cluster consisted of only African populations with several branches. Near to the node there was branch with African as well as Asian isolates. In the other cluster all the Australian and most of the American isolates were found in a large circle representing a taxon. The ITS sequences of these isolates were highly conserved. Asian isolates were found scattered amidst isolates from all the locations indicating their high diversity. Among the isolates used for this study, isolates RS09 and RS10 showed maximum variation followed by RS02. All the rest of the ten isolates got clustered in a single circle. None of the isolates showed similarity to the African isolates. All the isolates were similar to its Australian, American and Asian counterparts.

However it should be noted that even though there was high diversity between the mitochondrial (COX-ND) and the ITS region of the ten isolates, all the isolates were present within the *R.similis* cluster in the phylogenetic and haplotype analysis. Moreover when the sequences from the ten isolates from both the regions were subjected to BLAST analysis, all the ten isolates showed maximum resemblance to their respective gene sequences of *R.similis* present in NCBI. Thus all the isolates taken for the study were characterised as *R. similis* and can now be used for developing an accurate diagnostic tool for the detection of *R.similis.*

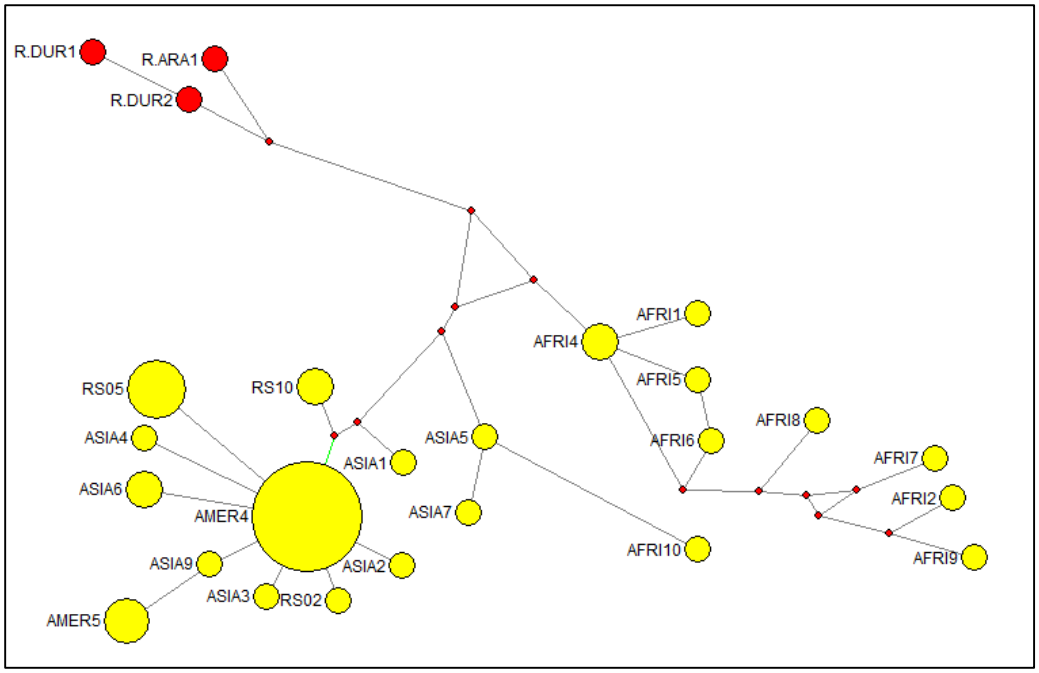


Fig. 15: Haplotype analysis conducted using the ITS region RS01-RS10 as well as the ITS region of *R. similis* isolates from different geographical regions using NETWORK V.5.0.

4.5. Real Time PCR based detection of *R. similis*

4.5.1. Designing of specific primers

The *R. similis* specific qPCR primers (Rad F and Rad R) were designed from the conserved region of the ITS sequences of the ten isolates using the Primer3+ software (Table 12) to give a 227bp product(Figure 16). Primers were compared to other sequences in the database through BLAST and FASTA searches to confirm specificity, and their design was optimized. The primer pair showed similarity to the *R. similis* nematode ITS sequences deposited in GenBank.

4.5.2. *Standard graph for the Real Time PCR based detection*

Quantitative Real-time PCR (qPCR) analysis was carried out with the DNA isolated from *R. similis*. The primers specifically amplified *R. similis* DNA (Figure 17). The

sensitivity of the method for nematode detection was further measured with a 1:2 dilution series of DNA from 2000 *R. similis* (Figure 18). 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 13). A standard curve was thus generated using 2000 *R. similis* DNA serially diluted with water. This standard curve would later help in identifying the approximate concentration of *R. similis* nematode(s) in any unknown sample based on the Ct value of that sample.

4.5.3. Correlating Ct value to the number of R. similis

Real time PCR based method was effectively used in the detection of single plant parasitic nematode. In this study, the potential of the qPCR technique in detecting the number of nematodes present in a sample was determined (Figure 19). The DNA from nematodes ranging from 1-500 was correlated to Ct values (Table 14). The qPCR assay also detected DNA from a single nematode of *R. similis*, which was 0.003 ng/µl.

4.5.4. Detection limits of R. similis specific primer using Real time PCR and LAMP method

Further, the DNA isolated from single nematode was diluted 10-fold and a comparison of sensitivity of the method with the previously reported LAMP method was done. The minimum detection concentration required for the Real time PCR was $1X\ 10^{-2}$, i.e. as little as 100fg of *R. similis* DNA (Figure 20) and for LAMP test it was found to be $1X\ 10^{-1}$of single nematode genomic DNA (Figure 21) The concentration of these diluted DNA samples were further correlated to their Ct values (Table 15), to obtain the detection limit of the designed primer pair. No amplification was observed in any template controls in both the cases.

4.5.5. 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 rhizhosphere soil were validated using 30 field samples collected from different regions of Kerala. The DNA from these samples was subjected to qPCR according to the standardised conditions. 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 few samples taken from yellow vines did not show the presence of *R. similis* and few vines which appeared to be healthy were infected by the nematode. Further, based on the Ct value obtained, the number of nematodes in each sample was estimated using real time PCR. There was a direct correlation between the number of nematodes found using direct microscopic observation and that estimated from the Ct value obtained by real time PCR based method. The details are given in Table 16 and Figure 22below. 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 either 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. Hence Real time PCR based method could be effectively used for the detection of *R. similis* in soil.

Table 12: Details of the real time PCR primer

Primer	sequences	length	TM	GC%	Product size
RAD -F	AGACTTGATGAGCGCAGA	18	55.4	50	227
RAD-R	CGTGCCAGAGGAAGTGA	17	56.7	58.8	

TTTCACTCGCCGTTACTAAGGGAATCCTTGTTAGTTCTTTTCCTCCGCTTACTGATATGCTTAAGTTCAGCGGGTATTCACGTCTGAGTTCAGGTCGAATGATT
TGGAAAAGCTGCCAATTTGCACAATGAACATACGTGAATTTCTTTCGCCACACGGCAAATACTCACGAAATTCATGTCCAAGTAAAAAATTAGCAAACTCTGAA
TTTCCATTGCATGAAAATATTAATTATCACGCATTCATGTACGCATTTGTCTTGCGTTTTTGGTAAACGACCCTGAACCAGGCGTGCCAGAGGAAGTGACTCCA
ATGGCGCAATGTGCATTCGAATGTTTAGTGTTCAAAGGTTCTGCAGTTCACACTAGATATCGCAGCTGGCTGCGTTCTTCATCGACCTACGAGCCGAGTGATCC
ACCGATAAGGCTAGAATTCTTGTTTGCCTTGAATGGCATTTGAAAAAGTTTATTGGAAAAAAATGGTTGTTGGCGGCGCTTGGTCTGCGCTCATCAAGTCTTAA
GCTGCCAAAGGCGATGACAGCCCTACCGAGTCGCATCATCGCAGCCACGGACATGGACTACAACTGCTCCACGACTCACAGACGCCAACCCAGCCGTTCGCGTC
ACATTTGATGGGTTTCGCCAATGCCTGAGGGGCACTGCCCACCAAAGGACGGAATCGTATTGGATAGGTTCACTTGTAAGTAATGATCCTGCAGCAGGTTCACC
TACAGCAACCTTGTTACGACTTTTGCCCGGTTCAAACTGATGCGATTAAGCCGGTTCTCACCAGAGAGACGCCCGAAGACATCAGACCGGCAGATCCCCGGATT
TCTCGAATCGGCTCAGTCCCGGAGCGATTGTTCGACCCCCCGGTGTGTACAAAGGGCAGGGACGTAATCAA

Fig.16: ITS region of *R. similis* showing the newly designed Real time PCR product.

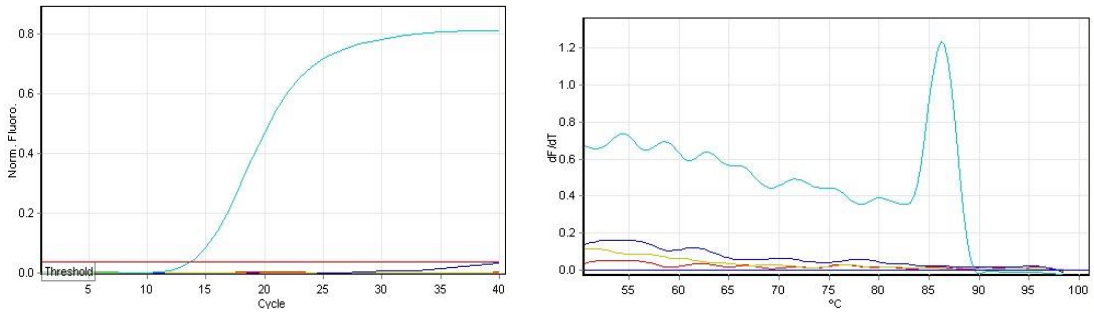


Fig. 17: Standardization of real-time PCR for the detection of *Radopholus similis:* (A) Amplification curves of real-time PCR obtained with positive, negative and water control. *(B) M*elt curve analysis of real-time PCR.

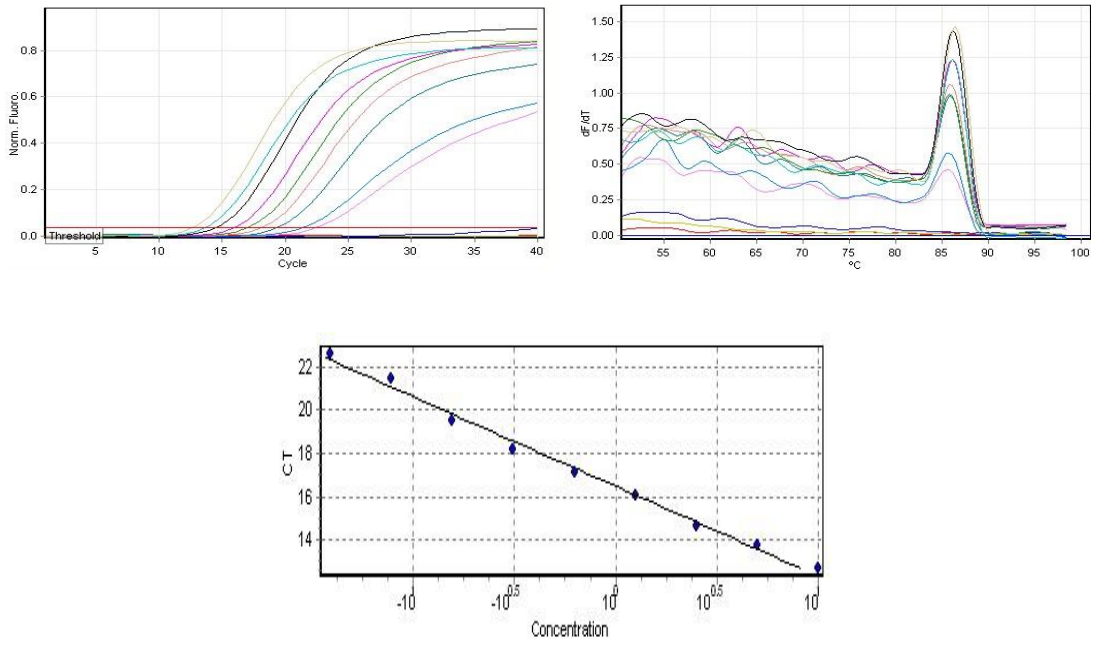


Fig. 18: Standard curve of Real-Time PCR obtained with a 1:2 dilution series of *R. similis* DNA: (A) Amplification curve. (B) Melt curve analysis. (C) Standard graph.

Table 13: Correlating the Ct values to the concentration of *R.similis* DNA

Sample	Ct value	Concentration (ng/µl)
NTC	-	-
EPN (NEGATIVE	-	-
RKN (NEGATIVE	-	-
1	12.71	8.266
2	13.74	4.655
3	14.62	2.823
4	16.04	1.2812
5	17.13	0.6979
6	18.22	0.3795
7	19.49	0.1854
8	21.41	0.0642
9	22.58	0.03314

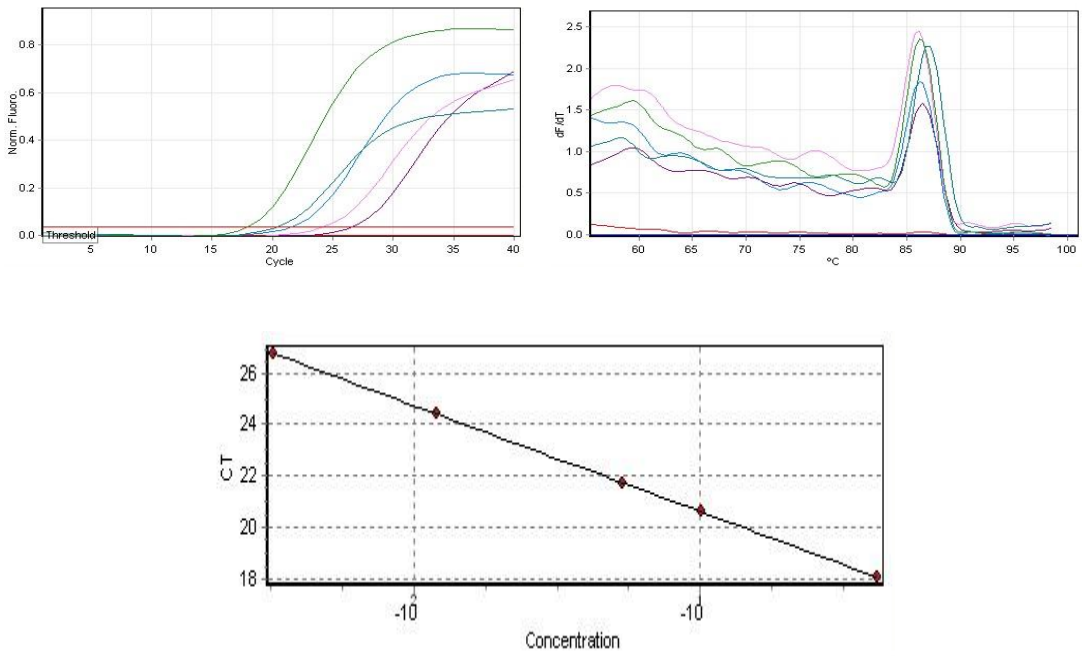


Fig. 19: Real-time PCR amplification obtained with fixed number of *R. similis*: (A) Amplification curve (B) Melt curve analysis (C) Standard graph.

Table 14: Correlating the Ct values to fixed number of *R. similis* DNA

Sl. No.	Number of nematodes	Ct value	Concentration(ng/µl)
1	500	18.05	0.41
2	50	20.59	0.10
3	10	21.73	0.05
4	5	24.39	0.01
5	1	26.75	0.003

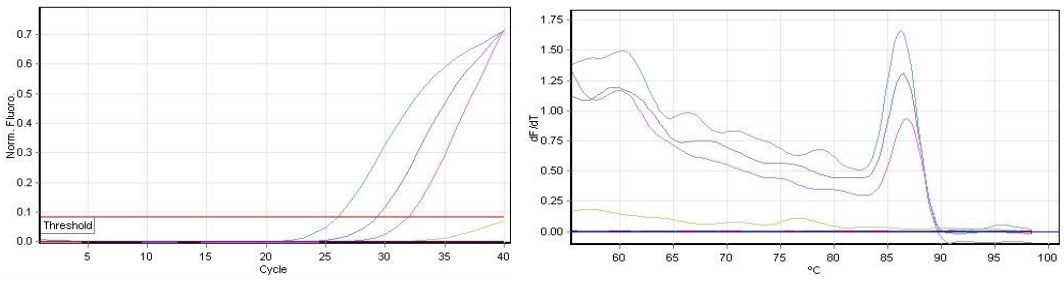


Fig. 20: Detection limits of *Radopholus similis* DNA using Real-Time PCR:

(A) Amplification curve (B) Melt curve analysis.

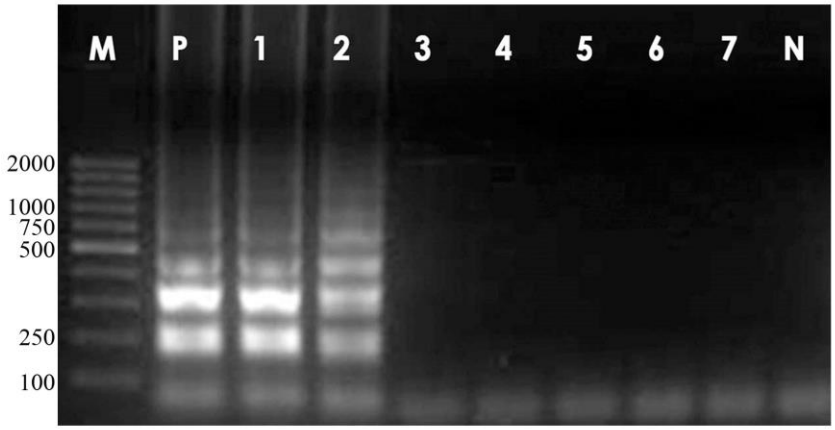


Fig. 21: Detection limits of *Radopholus similis* DNA using LAMP method. M= Marker P=Positive control; 1= Single nematode DNA sample; 2-7: Serial dilution of sample 1 in the ratio 1:10. N= Water control.

Table 15: Detection limit of *R. similis* specific Real Time PCR primer

Sl. No.	Number of nematodes	Ct value	Concentration (ng/µl)
1	1	26.75	0.003
2	10^{-1} of single nematode	29.30	0.0008
3	10^{-2} of single nematode	31.93	0.0001
NTC	0	-	-

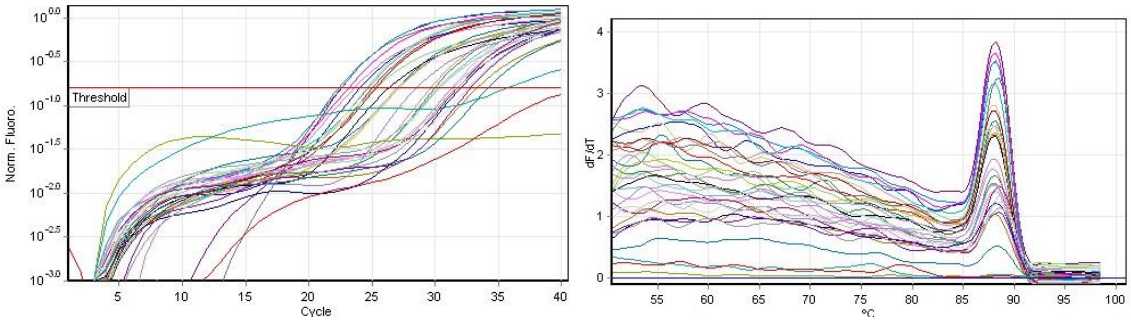


Fig. 22: Detection and quantification of *R. similis* in field samples using the qPCR. (a) Amplification curve and (b) Melt curve showing the Ct values of amplified samples.

Table 16: Field validation of Real time PCR Primer

By correlating the Ct value to the number of nematodes we know the approximate number of nematodes present in a sample which gives a certain Ct value.

Number of nematodes	**Ct value**
50-500	18.05-20.59
10-50	20.59-21.73
5-10	21.73-24.39
1-5	24.39-26.75
1X 10^{-1} - 1	26.75-29.30
1X 10^{-2}- 1X 10^{-1}	29.30-31.93
0 (Below detection limit)	>31.93

				Real Time PCR	
	Black pepper sample	**Visible infection on Blackpepper vine**	**Microscopic count**	**Ct value**	**Number of nematode**
1	Kozhikode	+	3	25.02	1 - 5
2	Kozhikode	+	1	27.14	$1X\ 10^{-1}$ - 1
3	Kozhikode	+	5	24.03	5-10
4	Palakkad	+	9	22.59	5-10
5	Malappuram	+	0	31.42	$1X\ 10^{-2}$- $1X\ 10^{-1}$
6	Palakkad	+	9	22.53	5-10
7	Kozhikode	+	1	26.99	$1X\ 10^{-1}$ - 1
8	Malappuram	+	0	29.69	$1X\ 10^{-2}$- $1X\ 10^{-1}$
9	Kozhikode	+	0	29.99	$1X\ 10^{-2}$- $1X\ 10^{-1}$
10	Palakkad	+	8	23.11	5-10
11	Kozhikode	+	2	26.32	1-5
12	Palakkad	+	4	24.70	1-5
13	Malappuram	+	5	24.56	1-5
14	Palakkad	+	4	25.32	1-5
15	Palakkad	+	0	30.40	$1X\ 10^{-2}$- $1X\ 10^{-1}$
16	Kozhikode	-	0	30.27	$1X\ 10^{-2}$- $1X\ 10^{-1}$
17	Kozhikode	-	0	28.48	$1X\ 10^{-1}$ - 1
18	Malappuram	-	1	27.02	$1X\ 10^{-1}$ - 1
19	Malappuram	-	6	23.58	5-10
20	Palakkad	-	4	25.27	1-5
21	Malappuram	-	0	33.30	0
22	Malappuram	-	0	-	0

23	Palakkad	-	0	35.77	0
24	Palakkad	-	0	34.15	0
25	Malappuram	-	0	31.98	0
26	Malappuram	-	0	32.80	0
27	Kozhikode	-	0	31.77	$1X\ 10^{-2}$- $1X\ 10^{-1}$
28	Kozhikode	-	0	32.60	0
29	Palakkad	-	0	31.28	$1X\ 10^{-2}$- $1X\ 10^{-1}$
30	Malappuram	-	0	31.69	$1X\ 10^{-2}$- $1X\ 10^{-1}$
	P		-	22.37	-
	N		-	-	-

DISCUSSION

Burrowing nematode, *Radopholus similis*, is an obligate migratory endoparasite. It is a highly polyphagous plant parasitic nematode attacking several plant species and distributed worldwide, mostly in tropical and sub-tropical areas. It is one of the 10 most damaging plant-parasitic nematodes worldwide (Jones *et a*l. 2013).

Survey and distribution of burrowing nematodes

Survey conducted during July to December months of 2014 to 2015 revealed the presence of *R. similis* at varying levels in different districts of Kerala. Survey of plant parasitic nematodes associated with black pepper in Kerala has reported *Meloidogyne incognita, Radopholus similis, Rotylenchulus reniformis* and *Helicotylenchus* sp. as the major nematode species associated with the crop (Ramana 1987). In our study we found high level of *R. similis* infection in samples collected from Idukki, Wayanad, Malappuram and Kozhikode. Large populations of root knot nematode were seen in samples collected from Wayanad and Thrissur. This is in agreement with the results of Koshy *et al.* (2005) who observed association of root-knot nematodes (*Meloidogyne* spp.) with black pepper plants in India. *Rotylenchulus* and *Helicotylenchus* were found in lesser number in all the locations. Thiruvananthapuram was least affected by *R. similis*. Ramana and Mohandas (1987), in his study, observed high incidence of *M. incognita* in Kozhikode, Kannur and Wyanad districts and high *R. similis* infection in Kozhikode, Kannur and Idukki districts of Kerala. He also observed very low incidence of *R. similis* in Kollam, Thiruvananthapuram and Wyanad districts. During rainy seasons most of the vines showed black lesions on leaves, apart from severe yellowing mainly because of *Phytophthora* infection. Ramana *et al.* (1994) and Anandaraj *et al.* (1994) reported

that black pepper plants showed wilting symptoms faster when *Meloidogyne* spp. and *R. similis* were inoculated first followed by *Phytophthora capsici.*

There are more than 30 species in the *Radopholus*, genus and among them *R. similis* is the only species of widespread occurrence and economic importance. Even though there are efficient methods for managing this nematode, the management becomes difficult once they multiply into large numbers. Hence it is important to detect their presence at an early stage, before it attains alarming levels. Accurate identification of nematodes is highly relevant for their effective management. The morphological features allow identification and classification of nematodes. This requires substantial expertise because of their microscopic size, limited number of distinguishable taxonomic characters and overlapping morphology.

Morphological studies on burrowing nematodes

The morphological studies of the ten isolates used in this study showed a high similarity within as well as among the populations. Based on the morphometric analysis carried out, most of these characters were stable. Significant morphological diversity was manifested in the length of stylet, spicule and gubernaculum in males and 'o' and 'V' values in females. However all the values measured were found to be within the range of values reported earlier for *R. similis* isolates (Taylor 1969, Sher 1968, Koshy *et al.* 1991, Esser *et al*. 1984, Elbadri *et al.* 1999, Roy *et al.* 2018, Xu *et al.* 2014, Pinili *et al.* 2018). Three of the parameters that showed much variation viz. length of stylet, spicule and gubernaculum in males were remarkably consistant according to Taylor (1969), however Koshy *et al.* (1991) found a narrow range of variation for these characters.

Although there are some variations in morphological characters these differences exist not only among the populations but also between the individuals within the same populations, so based on these morphological characters, we cannot separate different geographic populations of *R. similis.* It is possibly due to these overlapping morphological characters of *R. similis* that *R. citropholus* was initially assigned a new species designation erroneously.

The present study clearly proved that the differences in the morphological parameters occurring between the *R. similis* isolates of black pepper and banana collected from Kerala and Karnataka do not qualify for erecting a new species. These results are in conformity with the findings of Koshy *et al*. (1991), who studied the morphological features of *R. similis* isolated from South India. The findings of the study were also in agreement with the observations made by Elbadri *et al*. (1999), in black pepper isolates of *R. similis* obtained from Indonesia. Morphological variations were observed among African (Elbadri *et al.* 1999) and Philippine (Pinili *et al.* 2018) populations of *Radopholus*. However, the morphological characteristics observed from those studies fell within the range of morphological measurements reported by other authors (Huettel *et al.* 1986).

In this study all the isolates had a total body length in the range of 553 to 849 µm. Males normally did not go beyond a range of 602 to 698 µm, but few of the females were quite larger in size. This observation was supported by previous findings that females with eggs in one or both of the uteri average a little longer and wider than developing female (Taylor 1969, Koshy *et al.* 1991). The body diameters of females ranged from 19.8-32.3 µm and the body diameter of males from 14.6-23.2 µm. The females had a lower 'a' value (body length / greatest body diameter) than males.

Taylor (1969) reported 23-34 µm as the body width of female and 16-17 µm as the body width of males. Koshy *et al.* (1991) reported a female body width of 18-28 µm and 13-21 µm for males. The body diameter of most of the females was greater than the body diameter of males. In females the, 'a' value ranged from 24-30 µm and in males they ranged from 30-44 µm. This gives a long slender body shape to males when compared to females.

The cephalic region of females of all the isolates are dome shaped and slightly set off. The male cephalic region is knob like and clearly set off. The number of cephalic annules for males and females of the ten isolates studied showed 3-4 annules on their cephalic region. Elbadri *et al.* (1999) found that the number of head annuli varied from 2–5. Colbran (1971), when describing 11 new *Radopholus* species, reported the number of annules for *R. rectus* as being 3-4, whereas *R. intermedius, R. inanis,* and *R. brevicaudatus* had 3, *R. ferax* had 4, *R. vacuus* and *R. capitatus* had only 2, and *R. crenatus* and *R. megadorus* did not have distinct annules. Ryss and Wouts (1997) reported two new species of the genus *Radopholus, R. nelsonensis* and *R. kahikateae,* as having 4-6 and 4-5 annules, respectively. Elbadri *et al.* (1999) found that variation in the number of head annules exists both within and between the species of *Radopholus.* This is in contrast to the closely related genus *Pratylenchus,* where the number of head annules is considered a good taxonomic character (Sher & Allen, 1953).

Females of all the isolates showed the presence of a prominent stylet and their length ranged from 17.1-20.4 µm and the stylet of males are not quite well developed and their length ranged from 12.3-17.2 µm. *R. musicola* is reported to have characteristic small stylet length of 8.8-12 µm and *R. nelsonensis* the longest stylet of 25-28 µm. The

stylet knobs of all the ten female isolates studied were well developed and were anteriorly pointed. In males the stylet knob got amalgamated in one round base. The 'o' value (% distance of dorsal oesophageal gland opening from stylet knobs in relation to stylet length) depicting the distance from the stylet base to DGO (dorsal oesophageal gland orifice) was prominent in females and it ranged from 2.3-4.0 µm. The 'o' value of all the female isolates ranged from 12.2-20.3.

Further, the position of excretory pore has been found to be at the pharyngo intestinal junction in all the isolates. Females of all the isolates showed a 'b' value (body length/ distance from anterior to esophago-intestinal valve) in the range 4.7 to 7.5 and males had a 'b' value in the range of 6.1-6.6.

The esophageal gland overlapped the intestine dorsally in all males and females of the ten isolates. All the female nematodes have a prominent esophagus, with distinct esophageal regions, but the esophagus of males has not been as prominent as females in all isolates. In females the distance from anterior to base of esophageal glands ranged from 147-178 µm and in males it ranged from 126-170 µm. The females of different isolates have a b' value (body length/ distance from anterior to base of esophageal gland) in the range of 3.3-5.2 and the males had a b' value in the range of 4.1-4.9. The pharynx was 125 to 156 µm long in all the females.

As for the male reproductive features, the bursa, in all the isolates was found to be either extended to mid tail or to the tail terminus. Absence of bursa is a distinguishing character of *R. colbrani* (Kumar 1980). Further, the spicule length, for these isolates was found to be in the range of 18 to 22 µm and the gubernaculum, which is the tissue that holds the spicule in its position, measured 8-11 µm (which is almost half the length of the spicule) in all the isolates. Elbadri *et al.* (1999) reported a shorter spicule length

of 16±1.4 µm in a black pepper isolate from Indonesia. Van Weerdt (1958) reported a spicule length of 17.3±1.06 µm from a citrus population and 15±1.3 µm from a banana population. However, Sher (1968) reported a spicule length of 21 µm in a *R. similis* isolate from a sugarcane population from Hawaii.

The reproductive system of all females of the ten isolates has been found to be didelphic, extended, with oocytes in a single row. The length of the anterior female gonad and the posterior female gonad was almost of equal length, but the anterior gonad was slightly longer than the posterior female gonad in few nematodes of all the isolates. But that difference is negligible. Hence the G1 (length of anterior female gonad vs. body length) and G2 (length of posterior female gonad vs. body length) values of all nematodes of the isolates are found to be almost equal. The G1 and G2 values ranged from 20 to 28. The length of the posterior ovary is slightly shorter than the anterior ovary and this confirms previously reported data obtained from African populations (Elbadri *et al.* 1999) and Philippines population (Pinili *et al.* 2018) of *R. similis*. Reduced posterior gonad is a characteristic feature in *R. intermedius* (Colbrani 1971) and *R. sanoi* (Mizukubo 1989).

Further, the vulva has been found to be flat or slightly projecting. The 'V' value given by the % distance of vulva from anterior ranged from 55-60. This parameter distinguishes few species like *R. antoni* (Berg 2000) from the remaining species by their larger value (66-69).

The tails of most nematodes were conoid, tapering and rounded in females and almost pointed in males. This observation confirms the findings of Siddiqi (1986). Elbadri *et al.* (1999) observed that 47% of female and 27% of male tail ends had forked ends in an Indonesian population and this was the first report of this type of

tail end for *R. similis.* Xu *et al.* (2014) showed much more variations in tail shape - conoid, widely cylindrical or bearing a pointed end and only one intercepted population showed forked ends. Further, the tail terminus sculpture in females and males of the ten isolates studied has been found to be annulated.

The length of female tail was 49.9-106.9 21 µm and that of male was 60.9-96.921 µm. Kumar *et al.* (1980) reported a new species *R. colbrani* which was reported to have the longest tail in the genus. *R. musicola* is yet another species with long thin tail (73.6-92.821 µm). However recent studies by Xu *et al.* (2014) reported a tail length up to 163.8 µm in isolates of *R. similis.* The c value (body length / tail length) of males and females of all the isolates has been found to be very small because of their characteristic long tail. c value of females of all the isolates ranged from 7.7-12.5 while it ranged from 7.1-10.8 in males. The highest c value in the genus was observed in *R. nelsonensis* (Ryss and Wouts 1997).

The females have a moderate tail length and a moderate anal body width, but the males have longer tail and their anal body width is quite small giving it a characteristic narrow long appearance. The tail diameter at anus or claoca in females ranged from 13.9 to 29.6 µm and in males it ranged from 9.5-18.3 µm. The c' value (ratio of the tail length to the anal body width) of females ranged from 3.0 to 3.9 and that of males from 5.0 to 6.9. In *R. nelsonensis* this value was found to be 1.0-1.8 (lowest value in the genus). Also, females of all the isolates have a hyaline length of 8 to 16 µm and the males have a hyaline length of 6 to 11 µm.

The females have moderate tail length and long stylet. This gave the females a T/S ratio (tail length/stylet length) of 2.7 to 5.7. The males have long tail and shorter

stylet giving them a larger T/S ratio. The T/S value in males ranged from 3.7 to 7.2. The lowest T/S ratio was observed in *R. nelsonensis* with a value of 0.66-1.07.

The presence of diversity within and among populations of *R. similis* has been confirmed previously through morphology and morphometric studies (Huettel *et al.* 1986, Huettel and Yaegashi 1988, Elbadri *et al.* 1999, Xu *et al.* 2014). Variations of about 10 per cent (Koshy *et al.* 1991) and 15 per cent (Taylor 1969) from the average was common for most dimensions. Koshy *et al.* (1991) found no specific and significant morphological variation in twenty-eight populations of *R. similis* isolated from banana, coconut, arecanut, and black pepper from India. These observations stem the hypothesis that the species represented by the ten isolates from India used in the present study belongs to the species *R. similis*.

Molecular characterization of burrowing nematodes

Developing a DNA based detection technique makes it more convenient and accurate in detecting the pathogen. Conventional PCR is currently being used by many diagnostic laboratories because of its low cost and ease of availability in most plant pathology laboratories. Several PCR and loop-mediated isothermal amplification assays were developed for detection of *R. similis*. Real time PCR, although more expensive than conventional PCR, can be used to quantify the pathogens and has the advantage of speed and sensitivity. Application of molecular techniques is useful in species taxonomy, including nematodes, only when combined with taxonomic skills which are declining. Thus, molecular approaches should not be considered in isolation, but a balanced molecular and morphological taxonomic approach is required.

The greatest advantage of molecular data is the extent of the data set. Because all heritable information of an organism is encoded in DNA the set of morphological data with a genetic basis is a small subset of molecular information. The need for a supplementary analysis in order to identify the *Radopholus* species accurately warrants the use of molecular characterization as well. Historically the ribosomal genome sequence has been more widely adopted for molecular characterization and a heavy reliance has not been placed on the mitochondrial genome sequence. However, recent studies that took into account the several regions of the mitochondrial genome have proven to be effective in providing good results during diversity studies. This study engages the ITS (18S-26S) region of the ribosomal DNA in tandem with the COX and ND regions of the mitochondrial genome sequence.

The DNA isolated from the ten isolates of *Radopholus* spp. used in this study gave the expected product size of 920 bp, 899 bp and 701 bp, respectively, when amplified using the primer pair for ITS (18S-26S) region, cytochrome c oxidase gene 1 (COX) and NADH dehydrogenase gene 1 (ND) regions.

Initially most of the molecular diversity studies of *R. similis* focused on RAPD markers (Hahn *et al.* 1994, 1996, Fallas *et al*. 1996, Kaplan *et al*. 1996, Kaplan and Opperman 1997, Costa *et al*. 2008, Plowright *et al.* 2013). Using this method, Hahn *et al.* (1994, 1996) arranged 15 populations of *R. similis* into three putative groups and revealed three divergent isolates which are monotypic. Fallas *et al*. (1996) also made some of his studies using RAPD and isoenzyme analysis and indicated the existence of two gene pools within the *R. similis* isolates. Kaplan *et al*. (1996), Kaplan and Opperman (1997), indicated that a 2.4 kb fragment appeared to be associated with citrus parasitism using RAPD primers. However they did not support assignment of sibling

species status to burrowing nematodes that differ in citrus parasitism. Costa *et al*. (2008) separated *R. similis* populations into five similarity groups using RAPD analysis. Plowright *et al.* (2013) indicated the close association of Ugandan populations with populations from Sri Lanka on separate occasions from different sources using RAPD and AFLP study.

In several previous studies (Kaplan *et al*. 2000, Elbadri *et al*. 2002, Tan *et al*. 2010), PCR amplification was carried out using the ITS primers, which was sequenced and analyzed. Kaplan *et al*. (2000) studied 57 isolates of *R. similis*, by amplifying and sequencing the ITS (TW81 -AB28 primer) and D2/D3 (D2A-D3B primers) region and substantiated that the *R. similis* has a highly conserved genome. Elbadri *et al.* (2002) carried out direct sequencing of the internal transcribed spacer rDNA region (18S-26S) with a product size of 920 bp of 19 isolates of *R. similis* which yielded significant diversity, both among isolates and within some populations. Tan *et al*. (2010) suggested the lack of genetic diversity of Australian *R. similis* populations by ITS (18S-26S) sequencing.

In few studies (Fallas *et al*. 1996, Elbadri *et al*. 2002), these 920 bp rDNA ITS (18S-26S) sequences were further subjected to restriction digestion using various enzymes and conclusions were drawn based on the RFLP pattern obtained. Fallas *et al.* (1996) digested the 920 bp rDNA ITS (18S-26S) fragment with six restriction enzymes and could find little variation between the *R. similis* isolates, except for some differences with two enzymes: *Alu I* and *Hae III*. However, Elbadri *et al*. (2002) found significant diversity upon digestion of the 920 bp product and further supported the synonymy of *R. similis* and *R. citrophilus* using RFLP studies.

There are a number of conceptually distinct methodologies used to reconstruct phylogenetic trees using sequence data along with numerous phylogenetic analysis software packages. The phylogenetic literature is full of debates regarding which of these methods is the best, and there exist vigorously entrenched camps in favour of one method or another method over the others. However, when applied carefully to a reliable data set (i.e. a correct multiple sequence alignment), any of the widely used methods for phylogenetic reconstruction should prove to be largely accurate for inferring evolutionary relationships. As such, agreement between multiple methods can be taken as a measure of support for an inferred evolutionary relationship of interest, and therefore the use of multiple methods of reconstruction, where appropriate to the data being used. In this study we used five different methods for phylogenetic analysis and inferences were drawn based on a concatenated tree constructed by combining the trees drawn using the five methods.

A novel method of species delimitation was proposed by Adams (1998) that uses the phylogenetic species concept to recover species strictly defined by the evolutionary species concept. It is important to select appropriate genetic loci for species delimitation, but solely relying on them can lead to erroneous conclusions. If species delimitation was based on a single genetic locus that had multiple alleles, or heterozygous, or was non recombining, such as mitochondrial DNA, gene trees need not necessarily reflect phylogenetic relationships among species (Doyle 1992, 1995). A range of traits have to be considered together for obtaining the most accurate inference of phylogenetic relationships (Eernisse and Kluge 1993; Fitch, 1997; Thomas *et al.* 1997).

In the research reported herein, attempts were made to characterize two variable regions - one within the rDNA repeat and the other mitochondrial region - to define the taxonomic/phylogenetic relationship of burrowing nematodes *R. similis* from different geographical regions of Kerala and Karnataka.

The rDNA ITS1 sequence was compared because it is considered to be a non-coding, homozygous, nuclear DNA, useful for understanding nematode phylogeny (Adams 1998; Baldwin *et al*. 1995). It has been used to estimate phylogeny and to identify several nematode species including animal, plant and insect parasites (Campbell *et al*. 1995; Chilton *et al*. 1995; Ferris *et al*. 1993, 1994, 1995; Hoste *et al.* 1995; Thiery and Mugniery 1996; Vrain *et al.* 1992). However, intra-individual variability in ITS1 regions can complicate analyses and possibly lead to erroneous conclusions.

Phylogeny of ascaridoid nematodes was deciphered by comparing 18S and 26S rRNA sequences as the available morphological characteristics were inadequate (Nadler 1992). In this case, ascaridoid phylogeny was estimated by treating heterologous nucleic acids in rDNA sequences as autapomorphic characters. Though the results tallied with a prior classification based on evolutionary trends, it differed from the more widely accepted classification based on a set of physical traits. For burrowing nematodes that were morphologically similar, we determined that regardless of collection site, the nucleic acid sequences for the ITS1 of rDNA and the mitochondrial sequences COX and ND were identical to DNA sequences of *R. similis* available in NCBI.

Molecular studies typically use the 18S rDNA, mainly because of the availability of universal nematode primers and its phylogenetic resolution at the genus and higher taxon level. Unfortunately, the 18S gene has low resolution when it comes to

distinguishing closely related species. The mitochondrial cytochrome oxidase c subunit 1 (COI) gene is one of the most popular markers for population genetic and phylogeographic studies across the animal kingdom. Its popularity has increased even more since it appears that the M1-M6 partition of the COI gene (Folmer region) is an efficient identification tool for Metazoan species, turning it into the core fragment for DNA barcoding. Nevertheless, in COI based DNA barcoding, the Folmer region sometimes shows little resolution at the species level so that other COI regions such as I3-M11 or other genes such as the nuclear ribosomal ITS have been proposed for barcoding purposes, and the occurrence of nuclear copies of the COI gene (so-called 'numts') may confuse DNA barcoding results and may lead to an overestimation of taxonomic diversity (Derycke *et al*. 2010).

Molecular diagnostics for detecting and quantifying burrowing nematodes

Developing a diagnostic assay for the accurate identification and quantification of plant parasitic nematode is critical for making plant management decisions. The traditional methods of diagnosis are not ideal as they are time consuming and require expertise in identifying *R. similis*. Loop-mediated isothermal amplification (LAMP) and several PCR assays were developed for detection of *R. similis* (Ge *et al.* 2007, Wang *et al*. 2011, Peng *et al*. 2012). Compared to conventional PCR, real-time PCR and LAMP assays are more specific and sensitive and are less labor intensive. Real-time PCR can detect the amplification of products, as the products are synthesized. With the development of technology, PCR has become a very popular technique, especially for the detection and identification of bacteria. The real-time PCR uses a florescent dye system and thermocycler equipped with fluorescent- detection capability. The LAMP assay is a nucleotide acid amplification method that features high sensitivity,

specificity, and rapidity under isothermal conditions (Notomi *et al*. 2000). LAMP requires a set of four primers to react with six distinct regions in the target. The reaction can be accelerated by adding two loop primers. The LAMP assay utilizes a water bath or heat block for amplification thus avoiding the dependency on a thermocycler or electrophoresis equipment. The interpretation of the reaction can be observed by the naked eye with visual fluorescence. However, they are not suitable for the quantification of the nematodes in soil samples, as they do not provide any information about its number. Lin (2012) in his study compared the LAMP and Real time PCR based diagnostic and reported real time PCR as a better tool for detection purpose. Here we have developed a qPCR assay for the detection of *R. similis*.

In the present study *R. similis* was quantified directly from DNA extracts from its pure culture using a species specific qPCR. The target DNA was detectable in suspensions diluted in water containing as little as 100 fg of *R. similis* DNA. 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. Min *et al*. (2007) could detect a single *P. penetrans* individual in a sample with abundant number of free-living nematodes using qPCR. 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.

Based on our observations made in the present study we conclude that the morphological and molecular diversity observed in different black pepper isolates of burrowing nematodes present in Kerala and Karnataka are of minor importance as they do not qualify for delineating any new species. As reported earlier, the burrowing nematodes infecting black pepper belong to *Radopholus similis* only. Further the molecular studies also confirmed the identity as *R. similis* on subjecting to BLAST analysis. However, the haplotype analysis using ITS sequences clearly indicated the Asian lineage of Indian population of *R. similis*. As the intraspecific variability in Indian population is minimal, molecular detection techniques like PCR, LAMP etc. can be easily employed for their rapid and reliable identification in the field. Further the utility of real-time PCR in detecting and quantifying burrowing nematodes was clearly established in this study. This technique is accurate and more reliable and can detect *R. similis* to the limit of 10^{-2} dilution of genomic DNA of a single juvenile nematode. Application of such reliable techniques for early detection and estimation of *R. similis* in soil and root samples will pave way for the effective management of this serious nematode pest.

SUMMARY

Burrowing nematode, *Radopholus similis* (Cobb, 1893) is an obligate migratory endoparasite found worldwide in tropical and sub-tropical areas. It is a highly polyphagous plant parasitic nematode which infects about 365 plant species and is one of the 10 most damaging plant-parasitic nematodes worldwide. There are 30 species in the genus *Radopholus*, among them *R. similis*, is the only species of widespread economic importance. Slow decline disease of pepper, toppling disease of banana, spreading decline disease of citrus etc are some of the serious diseases caused by *R. similis*.

The accurate identification and characterization of nematodes infecting a crop is a pre-requisite for designing effective control strategies. So, a combined morphological and molecular characterization study was undertaken for the accurate identification of *Radopholus* spp. isolates infecting black pepper and banana roots collected from different regions of Kerala and Karnataka. Further a real time PCR based diagnostic tool was developed for its detection.

Several morphological characters prescribed for nematode taxonomy were studied in ten isolates of *Radopholus* spp. Collected from different parts of Kerala and Karnataka. Except for a few characters, most of the measurements fall within the range of measurements for *R. similis* reported by previous workers. These observations substantiate the hypothesis that the burrowing nematode isolates infesting black pepper in South India have the closest resemblance to *R. similis*.

To support this hypothesis molecular characterization of these isolates was also carried out as well. For this, the internal transcribed spacer region (ITS1-5.8S-ITS2) region of the ribosomal DNA in tandem with the cytochrome c oxidase gene 1 (COX) and NADH dehydrogenase gene 1 (ND) regions of the mitochondrial genome sequence were used. The results obtained through phylogenetic analysis of ITS

region showed a high level of similarity between the ten isolates and the ITS region of *R. similis* reported worldwide. The COX and ND regions also showed higher similarity to the *R. similis* complete mitochondrial genome sequence counterparts for COX and ND. Hence, the sequence analysis of these three regions, along with the previously conducted morphological characterization studies confirmed that all the 10 isolates used in this study belong to the species, *R. similis*.

Besides phylogenetic study, a phylogeographical analysis was also conducted to understand the likely origin of Indian *R. similis* populations. In this analysis the Asian African populations assembled differently. The ten isolates used in this study grouped with the Asian isolates. However, the Asian group has a scattered presence among the rest of the world population, which indicates that the Asian population might have been the original species to spawn the rest of the *R. similis* population in other parts of the world.

Developing a diagnostic assay for the accurate identification and quantification of plant parasitic nematode is critical for making plant management decisions. The traditional methods of diagnosis are not ideal as they are time consuming and require expertise in identifying *R. similis*. Loop-mediated isothermal amplification (LAMP) and several PCR assays have already been developed for detection of *R. similis*. However, these techniques are not suitable for quantifying nematodes in soil samples. Hence a qPCR assay was standardized for the detection and estimation of *R. similis* population in field samples. For this, species specific real-time PCR primer was designed using the conserved region of the ITS sequences and the target DNA could be detected in suspensions containing as little as 100 fg of *R. similis* DNA.

The real time PCR method developed for detection of *R. similis* in infected black pepper rhizhosphere soil was validated using field samples collected from different regions of Kerala. The technique developed is accurate and reliable and can be deployed for early detection and estimation of *R. similis* in soil and root samples which will pave way for the effective management of this serious nematode pest.

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APPENDICES

APPENDIX 1

Preparation of electrophoresis buffer (1XTAE)

To prepare 50x TAE Electrophoresis Buffer add the Tris free base (242 g) and Disodium EDTA (18.61 g) to approximately 700 ml DDI H2O and stir until the Tris and EDTA are dissolved. Add the acetic acid (57.1 ml) and adjust the volume to 1 litre. The 1x TAE solution is 40mM Tris, 20mM Acetate and 1mM EDTA and typically has a pH around 8.6 (do not adjust). Depending on the volume of 1x TAE required the 50xTAE buffer is diluted with water.

APPENDIX 2

Preparation of ethidium bromide stock (10mg/ml)

- Weigh out 100 mg ethidium bromide conical flask / beaker / 15-ml polypropylene centrifuge tube. Add 7 – 8 ml water.
- Mix until all ethidium bromide dissolves completely. This may take long time.
- Adjust the volume to 10 ml with Deionized / Milli-Q water.
- Ethidium bromide solution is ready for use.

APPENDIX 3

Preparation of 6X DNA loading dye

To prepare 10 ml of 6X DNA loading dye, weigh out 25 mg bromophenol blue, 25 mg xylene cyanol FF and 1.5 gram Ficoll 400. Transfer them to a screw-capped tube (graduated polypropylene centrifuge tube). Add 7 ml deionized / Milli-Q water. Mix until all ingredients are dissolved completely. Adjust the volume to 10 ml with deionized / Milli-Q water. Mix it again. Store the solution at room temperature or 4°C for long time.