Race profiling and phylogenetic analysis of the root knot nematode Meloidogyne incognita presages the possible emergence of virulence towards cotton in Central India

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Abstract

With a host range spanning vegetables, pulses, grains, fruits, and cash crops such as cotton, the polyphagous root knot nematode (RKN) *Meloidogyne incognita* is known globally as the most economically destructive genus of plant parasitic nematodes. During sampling efforts, severe root galling from RKN infestation was observed on cotton as well as several other vegetable crops in Nagpur, Wardha, Chandrapur, Jalgaon, Aurangabad and Yavatmal districts of the Vidarbha region of Maharashtra in Central India. *M. incognita* has been reported as the predominant nematode species in irrigated cotton growing regions of Northwestern India, but in the surveyed regions, the reniform nematode *Rotylenchulus reniformis* has been reported instead on cotton as the predominant species. As well, the sampled RKN populations were found in areas with soil characteristics not matching previously reported habitat conditions, pointing to the ability of RKN populations to tolerate and adapt to a wider variety of soil conditions than expected. Race profiling from differential host studies indicated all six populations to belong to race 3. Maximum likelihood analysis between the sampled populations and catalogued populations of *M. incognita* from other Indian states, based on the rDNA Internal Transcribed Spacer (ITS) region showed the populations to form a phylogenetic grouping, thus indicating the potential of Indian populations to exhibit increased pathogenicity towards cotton. As it stands, with cotton as the dominant crop of the region alongside other vegetable crops, the found results underscore the need for proactive surveillance of nematode infestations of cotton.

Introduction

Root-knot nematodes (RKN) belonging to the genus *Meloidogyne* are obligate endoparasites globally known as a destructive and economically costly group of plant parasitic nematodes (Sasser et al., 1987; Abad & Williamson, 2010). The nematode gets its name from the characteristic galls formed on roots due to its feeding with the size of these galls varying with host and nematode species (Dasgupta and Gaur,1986, Swarup et al., 1989) as well as being an useful indicator of infestation. Infestation-induced galling of roots results in reduced water uptake to shoots of the plant, stunting plant growth and causing yield loss (Dropkin and King 1956; Hunter, 1956; Dasgupta and Deb, 1972; Sikora & Fernandez, 2005), a primary factor in the nematode's destructiveness. The magnitude of total yield loss from RKN galling depends upon the severity of infestation, virulence of the concerned *Meloidogyne* species, crop species, season, and soil type (Dropkin,1989; Van Gundy, 1985) among other factors. Of the more than 100 species described of genus *Meloidogyne*, four species viz. *M.incognita, M.javanica, M. hapla*, and *M.arenaria* have been quantified as the most damaging to agricultural crops. (Hunt and Handoo, 2009; Moens et al, 2009). Amongst all, *M. incognita* with wide distribution among temperate, subtropical and tropical regions has distinction of the most pathogenic rootknot species (Sasser ,1979; Trudgill and Blok, 2001).

On cotton, three species, *M.acronea, M.incognita* and *M..enterolobii* have been recorded with only *M.incognita* found to be pathogenic while *M.acronea* and *M.enterolobii* are of limited non-pathogenic occurrence (Davis et al., 2018). *M.incognita* is a polyphagous nematode species with a host range spanning multiple crops including vegetables, pulses, fibre, fruits and cotton. Wide host range and

reproduction by mitotic parthenogenesis has been suggested as strategies adopted by rootknot nematode for successful parasitism (Blok et al., 2008). Darekar and Mhase (1988) have estimated the yield loss in different crops infected by root-knot nematode such as brinjal, bitter gourd and tomato at 32.73, 36.72 and 46.92 percent respectively. Cotton is an important fibre crop of considerable economic importance in India, contributing as textiles nearly 5% to the national GDP and earning 11% of the total foreign exchange through exports

(Cotton Industry - Indian Trade Portalhttps://www.indiantradeportal.in**›).***M. incognita* has been found to be the dominant nematode species affecting cotton crop in North India (Gokte-Narkhedkar & Lavhe,2000; Khan et al., 2010). *M. incognita* induced galling has been reported to account for yield losses of 16–25% (AICCIP, 2012) and on a national scale, cotton crop losses ranging between 12.3% – 20.8% have been attributed to the effects of *M. incognita* infestation (Khan et al., 2010; Kumar et al., 2020). Khan et al., 2014 recorded the race diversity of *M. incognita* across India. Of the six races documented so far (Robertson et al., 2009), races three and four are known to exhibit pathogenicity towards cotton. Race two, three and five of *M.incognita* have been reported predominantly on different crops with exclusion of cotton in Maharashtra (Darekar and Mhase, 1988; Khan, 1997; Khan et al., 2014). Race three has been reported on cotton in Karnataka and Tamil Nadu (Krishnappa, 1985) while race four has been recorded on cotton from North India (Verma & Jain, 1999).

Amongst the states of India, Maharashtra, a Central Indian state, leads in terms of both cotton production as well as acreage (www.txcindia.gov.in). Cotton soils in the state are predominantly clayey (Mandal *et al.*,2011) and reniform nematode (*R. reniformis*) is predominant on cotton (Gokte-Narkhedkar, 1999) as clayey soil is preferred by this species. Moore and Lawrence (2013) have recorded that population density of *R. reniformis* was significantly influenced by soil texture and exhibited a general decrease with increasing median soil particle size. During routine survey carried out to document the prevalence of plant parasitic nematodes, severe infestations of *M. incognita* on cotton was recorded in various districts of the Vidarbha region of Maharashtra. As RKN infestation is predominantly found in sandy loam soils (Starr et al., 1993; Ogbuji, 2004), such reports of infestations in clayey soil present a cause for concern. Additionally, the emergence of increasing virulence towards cotton in Maharashtra RKN populations raises the possibility of similar trait development in *M. incognita* populations across the country. As information on the occurrence of RKN populations in Central India is currently limited, studies were initiated for race profiling as well as morphological and molecular characterization of the sampled populations to facilitate greater understanding of patterns of pathogenicity in *Meloidogyne* and to discern phylogenetic trends that may allow for prediction of cotton virulence in other regions of India for the formulation of effective nematode management strategies.

Material And Methods

Collection and Maintenance of Root-Knot Nematode Populations

Populations of root-knot nematodes (RKN) were collected from Nagpur (Cotton *Gossypium hirsutum)*, Brinjal (*Solanum melongena* L.), Tomato (*Solanum lycopersicum* L)., Wardha (Tomato), Yavatmal (Cotton),

Jalgaon (okra *Abelmoschus esculentus* L.), Aurangabad (Okra) and Chandrapur (Cotton) districts of Maharashtra. Individual RKN stock cultures of populations were raised from single egg masses and proliferated on tomato plants belonging to *Lycopersicon esculentum* cv. Pusa ruby in 30 cm diameter pots in sterilized soil.

Morphological Characterization of Root-Knot Nematode

For morphological characterization female root knot nematodes were teased from infected galled roots roots and maintained in water at 4°C until observation. For taking morphometric measurements females were placed on a glass slide with a drop of sterile tap water, covered with 0 number glass coverslip supported with glass wool rods and measured under the microscope. To observe the perineal pattern, the posterior end of each female was cut with a razor blade and mounted in lactophenol solution (Franklin and Goodey, 1949). Extraction of second-stage juveniles was done by separation of egg masses from the roots and incubation in sterile distilled water at room temperature (28-30^0 C) for 24h. Male specimen were obtained from egg masses as well as by washing the soil adhering to roots as per Cobb's sieving and decanting technique followed by modified Baerrmann funnel technique (Cobb, 1918, Christie & Perry, 1951). Nematodes were killed by pouring in an equal volume of hot water (>80^{o}C) to nematode suspension, fixed in hot 4% formalin (50-60°±2^{o}C) and mounts were prepared in glycerine according to Seinhorst's (1959) rapid method. The morphological features considered in the adult females were the perineal pattern, body length, body width, stylet length, neck length, and neck width. Juveniles had body length, stylet length, head to median bulb length, median bulb to excretory pore and tail length measured. Male body length, stylet length, distance to DGO, spicule length and gubernaculum length were also measured. All observations were done under Leica DBLB microscope at X100 and X400 .

DNA Extraction from female nematode

20 white gravid females were selected by random sampling for DNA extraction for molecular characterization and crushed to fine powder in liquid nitrogen. (Christoforou *et al.,* 2017). Lysis buffer (1M Nacl, 1MTris, 0.5M EDTA, 10% SDS) was added to the pooled nematode homogenate for a total volume of 3 ml. The homogenate was incubated at 37^0 C for 30 minutes after addition of Proteinase K solution 20mg/ml (150 µl). DNA extraction was done with buffer saturated phenol in a 1:1 phenol:chloroform followed by chloroform:iso-amyl alcohol mixture. Quantification of DNA was done through spectrophotometry and electrophoresis on 1.5% agarose gel.

PCR amplification, Cloning and phylogenetic analysis

PCR amplification of the internal transcribed spacer (ITS) region of the ribosomal RNA genes was done using forward (5'TTTCACTCGCCGTTACTAAGG3') and reverse (5'TTGATTACGTCCCTGCCCTTT 3') primers (Vrain *et al*., 1992) with the purified gravid female DNA as a template. The master mix for the 25µl PCR reactions contained sterile distilled water (13.8 µl), 10X reaction buffer (5.0 µl), MgCl2 50mM (1.5µl), dNTPs 10mM (1.0 µl), forward primer (1.0 µl), reverse primer (1.0 µl), Taq polymerase (0.2 µl) and template (1.0 µl). Negative and positive controls were kept. Amplification reactions were performed in a Biorad

thermal cycler with a PCR programme as follows: Initial denaturation - 94^0C for 2 minutes; 35 cycles of 94^0C for 30s (denaturation), 60^0C for 45s (primer annealing), and 72^0C for 45s (primer extension) then a single 5 minute cycle at 72^0C for final extension. Post-amplification, 5µl of the amplified product was resolved on 1% agarose gel and DNA fragments were visualized through ethidium bromide staining using the Bio-Rad Gel Documentation System. Subsequently, cloning was done in a pGEM-T vector from Promega (Madison, USA) and transformed into *Escherichia coli,* strain JM109 ((Cat.# L2001)procured from Promega following the manufacturer's protocol. Transformants were identified with blue-white colony selection. LB/ampicillin plates were prepared by adding ampicillin to a final concentration of 100µg/ml using sterile filters to autoclaved and cooled LB medium. LB/ampicillin plates were stored at 4°C. 100µl of 100mM IPTG and 20µl of 50mg/ml X-Gal was spread over the surface of an LB-ampicillin plate with a sterile spreader and allowed to absorb for 30 minutes at 37°C prior to use. The plates were left to dry in laminar flow chamber with lids slightly open. 10-100 µL of transformed *E. coli* cells were spread onto the LB agar plates using sterile spreader and plates were incubated at 37°C for 24-48 hours. Blue and white colonies appeared after due incubation and recombinant white colonies were picked and cultured. Colony PCR was performed to confirm the presence of the insert and plasmid preparation was done using Pureyield plasmid miniprep system from Promega (New Delhi, India) after which sequencing was done through SciGenom (Kochi, India). Chromatographic quality of sequences was verified with the Applied Biosystems Sequence scanner (v1.0). The nucleotide sequences were then compared with catalogued data using NCBI BLAST to determine similarities and establish identity. The contig of partial assembled sequence were made by using CAP3: A DNA sequence assembly program (Huang and Madam,1999). These assembled sequences were submitted to NCBI through BankIt and deposited in the Nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore) .

Umarao *et al.* (2003) characterized races of *M. incognita* based on their rDNA ITS sequences and reported that variation in the sequences of the four catalogued races of *M. incognita* resulted in variance in their restriction sites, thus allowing for differentiation of the races based on restriction site distributions. The restriction enzymes SwaI, Bsa1, BsmA1 and EcoR1 were reported for this purpose. Therefore, in our studies 10 individual colonies were tested for the restriction site unique to each enzyme with the sequences for these populations. Sequence data for *M.incognita* available on NCBI was also analyzed for the presence of restriction sites for *EcoR1, SwaI, Bsa1* and *BsmA1* (Table 5). Sequences were incorporated into the BioEdit Sequence Alignment Editor v.7.2.5 and compared with catalogued sequence data for *M. incognita* available at the National Center for Biotechnology Information (NCBI) GenBank nucleotide database using a standard BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was done using the MEGA X (Molecular Evolutionary Genetic Analysis) v10.1 (Kumar *et al.*, 2018) program under maximum likelihood parameters and the resultant phylogenetic tree was generated.

Race Characterization by Differential host test.

Six differential hosts viz. *Nicotiana tabacum* cv. NC 95 (tobacco); *Gossypium hirsutum* cv. Deltapine 16 (cotton); *Capsicum frutescens* cv. California Wonder (pepper); *Citrullus vulgaris* cv. Charleston Grey (watermelon), *Arachis hypogaea* cv. Florunner (peanut) and *Solanum lycopersicum* cv. Rutgers (tomato)

were used for race characterization as per Hartman & Sasser (1985; Robertson *et al*., 2009). Three seeds of each differential host were sown in sterilized soil in 10 cm diameter earthen pots in glass house. Plants were later thinned to retain one seedling per pot. Tomato, pepper and tobacco seedlings were transplanted. After two weeks, a prepared inoculum containing 400 second-stage juveniles in 3 ml of water was gently applied near the base of each plant using sterile pipette tips, with four replicates per differential host. After 60 days, plants were gently uprooted and number of egg masses/galls on each root system were counted and assigned a rating index number according to the following scale : 0 = no gall/egg masses, 1 = 1-2 galls/egg masses; 2 = 3-10 egg masses/galls; 3 = 11-30 egg masses/galls; 4 = 31-100 egg masses/galls and 5 = over 100 egg masses/galls. Plants with index values of 2 or less were designated as non-hosts (-) while plants with index values greater than 2 were designated as hosts (+).

Results And Discussion

Morphology:

All eight populations were found to belong to *Meloidogyne incognita* as evinced by the collected morphological and morphometric data of the mature females, second stage juveniles and males, given in Tables 1, 2 & 3. Perineal pattern of the females in all populations were '*incognita* type' (Mulvey *et al*., 1975) with a distinct high dorsal arch made of smooth to wavy *striae*. The morphological measurements are in conformity to that of the typed species and differences recorded are within bounds for intra-specific variation within *M. incognita (*Kaur & Attri (2013).

Table 1
Morphometric characterization of female populations of *M. incognita*

Nematode population	Female Measurements (µm) mean ± SD (range). N = 10							
	Length	Body width	Stylet length	Neck length	Neck width	Vulva length	Vulva-anus distance	Anus to tail terminus
Nagpur (Cotton)	700 ± 29 (690–723)	550 ± 31 (510–620)	19.5 ± 0.4 (16.9–22.0)	251 ± 36 (230–269)	100 ± 12; (89–110)	20 ± 0.9 (16.8–22)	18.3 ± 0.4 (17.4–23.3)	15.3 ± 1.2 (13.8–17.3)
Nagpur (Brinjal)	710 ± 19 (695–730)	545 ± 29 (522–590)	19.4 ± 0.3 (17.0–21.0)	230 ± 33 (225–265)	98 ± 11; (88–105)	20 ± 0.8 (17.8–21)	18.6 ± 0.4 (17.8–23.8)	15.1 ± 1.1 (13.6–17.1)
Nagpur (Tomato)	690 ± 30 (680–725)	530 ± 30 (508–590)	19.2 ± 0.3 (16.5–21.5)	234 ± 31 (228–260)	97 ± 11; (83–109)	19.9 ± 0.8 (17.1–21.9)	18.1 ± 0.3 (16.8–22.3)	15.3 ± 1.1 (13.6–17.0)
Jalgaon (Okra)	705 ± 35 596–760 um	510 ±24 490–640	19.0 ± 0.3 16.1–22.4	210 ±26 200–290	92 ± 10 79–121	20 ± 0.7 16–23	18.0 ± 0.3 16.1–24	14.8 ± 1.0 12 -18.3
Aurangabad (Okra)	680 ± 40 (650–770)	523 ± 31 (510–610)	18.8 ± 0.3 (17.0–20.0)	228 ± 22 (210–243)	97 ± 8; (82–114)	19.9 ± 0.9 (17.0-23.5)	18.8 ± 0..3 (17.7–22)	14.9 ± 1.1 (137 – 16.9)
Wardha (Tomato)	610 ± 31 (596–680)	500 ± 28 (490–586)	17.0 ± 0.3 (16.1–20.4)	214 ± 38 (200–239)	90 ± 9; (79–104)	18 ± 0.7 (16.0–21)	17.3 ± 0.3 (16.1–22.0)	13.3 ± 1.0 (12.0-14.5)
Yavatmal (Cotton)	690 ± 40 (600–760)	530 ± 35 (500–590)	18.5 ± 0.4 (17.1–21.0)	238 ± 21 (220–250)	96 ± 10; (80–104)	18.9 ± 0.8 (17.0-22.5)	18.0 ± 0..3 (17.2–22.8)	14.8 ± 1..1 (13.0-16.8)

Nematode population	Female Measurements (µm) mean ± SD (range). N = 10							
	Length	Body width	Stylet length	Neck length	Neck width	Vulva length	Vulva-anus distance	Anus to tail terminus
Chandrapur (Cotton)	715 ± 25 (650–740)	550 ± 21 (535–640)	19.8 ± 0.5 (17.9–22.4)	271 ± 43 (246–290)	116 ± 20; (95–121)	21 ± 0.9 (17.8–23)	19.1 ± 0.4 (18.5–24.0)	16.0 ± 1.4 (13.9–18.3)

Table 2

Morphometric characterization of Second stage juveniles populations of M. incognita

Nematode population	**Second stage juveniles Measurements (µm) mean ± SD (range). N = 20**					
	Length,	tail length	head to median bulb length	Median bulb to excretory pore	Stylet length	Anal body width
Nagpur (Cotton)	320 ± 52 (290–356)	23-±3.2 (17–25)	55 ± 11 (47–59)	24.0 ± 7.1 (19.0–28)	19.6 ± 5.2 (15–22.0)	15.4 ± 2.1 (14–17.0)
Nagpur (Brinjal)	308 ± 50 (287–350)	22.7-±3.1 (16.8–24)	53 ± 10 (48–56)	23.0 ± 6.8 (19.1–25)	19.1 ± 4.8 (14.6–21.0)	15.0 ± 2.0 (13.6–16.8)
Nagpur (Tomato)	300 ± 48 (294–350)	23-±3.0 (16–24)	55 ± 10 (48–60)	23.0 ± 6.9 (18.0-28.8)	19.1 ± 5.0 (15.2–22.0)	15.2 ± 2.0 (13.8–16.9)
Wardha (Tomato)	290 ± 40 (276–306)	20-±2 – 0 (16–23)	52 ± 6 (42–61)	21.0 ± 5.1 (17.0–25.0)	18.0 ± 4.1 (14–21)	13.0 ± 2.0 (12–16.0)
Yavatmal (Cotton)	304 ± 42 (286–341)	21-±3.0 (17–24)	53 ± 10 (49–59)	23.0 ± 6.0 (18.3–28.2)	18.6 ± 4.8 (15–21.0)	14.4 ± 1.9 (13–17.0)
Chandrapur(Cotton)	329 ± 48 (300–370)	23.6-±3.1 (18–25)	59 ± 8 (51–62)	25.0 ± 7.9 (20.1–29.1)	19.0 ± 5.0 (15–23.0)	16.3 ± 2.2 (14–18.0)
Jalgaon (Okra)	296 ± 32 (270–316)	21-±1 – 0 (17–21)	51 ± 6 (42–60)	22.0 ± 5.6 (18.2–26.1)	18.4 ± 4.0 (15–20.0)	13.1 ± 2.1 (12.0-11.7)
Aurangabad(Okra)	280 ± 28 (277–318)	22-±2 – 0 (17–23)	52 ± 8 (44–60)	23.0 ± 5.7 (18.0-24.2)	18.6 ± 4.2 (15–22.0)	13.8 ± 2.1 (13.1–16.4)

Table 3
Morphometric characterization of Males from different populations of *M. incognita*

Nematode population	Males Measurements (µm) mean ± SD (range). N = 10			
	length	Stylet length	Spicule length	Gubernaculum length
Nagpur (Cotton)	1300 ± 17.2 (1229–1850)	23.9 ± 0.2(19–25)	27.5 ± 0.4 (20.4–29.9)	9.3 ± 0.2. (8.0-11.2)
Nagpur (Brinjal)	1290 ± 16.8 (1229–1850)	22.9 ± 0.1(19–23)	26.8 ± 0.3 (20.2–28.0)	8.9 ± 0.1 (8.0-11.8)
Nagpur (Tomato)	1298 ± 17.0 (1230–1890)	21.9 ± 0.2(18–25)	26.7 ± 0.3 (21.3–28.7)	9.1 ± 0.2. (9.0-11.5)
Wardha (Tomato)	1210 ± 14.2 (1170–1600)	21.8 ± 0.2(19–23)	25.5 ± 0.3 (19.6–28.2)	9.0 ± 0.1. (8.0-11.2)
Yavatmal (Cotton)	1410 ± 16.2 (1090–1770)	22.1 ± 0.1(18–24)	26.1 ± 0.4 (21.4–27.3)	9.0 ± 0.2. (9.0-11.2)
Chandrapur (Cotton)	1560 ± 15.2 (1539–1670)	24.2 ± 0.2(20–25)	27.9 ± 0.4 (22.1–30.0)	9.1 ± 0.2. (8.0-11.2)
Jalgaon (Okra)	1260 ± 11.2 (1100–1500)	22.4 ± 0.2(19–22)	24.5 ± 0.4 (20.1–25.2)	9.0 ± 0.1 (8.0–11.0)
Aurangabad(Okra)	1290 ± 17.1 (1070–1700)	22.7 ± 0.2(20–23)	26.5 ± 0.3 (20.6–28.4)	9.2 ± 0.2. (9.0-11.3)

Differential host test. The eight RKN populations under study were tested on the six differential hosts as per Hartman & Sasser (1985) to assess their colonization patterns. On cotton only two races, 3 and 4 have been reported. Race 3 is known to multiply on *Gossypium hirsutum* cv. Deltapine 16 (cotton); *Capsicum frutescens* cv. California Wonder (pepper); *Citrullus vulgaris* cv. Charleston Grey (watermelon), *Arachis hypogaea* cv. Florunner (peanut) and *Solanum lycopersicum* cv. Rutgers (tomato) but not *Nicotiana tabacum* cv. NC 95 (tobacco); Race 4 is reported to be virulent against cotton, pepper; watermelon, peanut and (tomato and also *Nicotiana tabacum* cv. NC 95 (tobacco). All six populations under study proliferated on cotton, but not on tobacco, thus indicating their race identity as race 3 (Hartman & Sasser (1985) (Table 4).

Table 4
Response of *Meloidogyne incognita* populations with differential hosts

Response	Locations of Rootknot nematode population	Response of Host Differentials					
		Pepper cv. California Wonder	Cotton cv. Deltapine 16	Tobacco cv. NC 95	Watermelon cv. Charleston Grey	Peanut cv. Florunner	Tomato cv. Rutgers
Host Reaction	Nagpur (Cotton)	+	+	-	+	-	+
	Nagpur (Tomato)	+	+	-	+	-	+
	Nagpur (Brinjal)	+	+	-	+	-	+
	Wardha (Tomato)	+	+	-	+	-	+
	Yavatmal (Cotton)	+	+	-	+	-	+
	Chandrapur (Cotton)	+	+	-	+	-	+
	Jalgaon (Okra)	+	+	-	+	-	+
	Aurangabad (Okra)	+	+	-	+	-	+
Host Rating index	Nagpur (Cotton)	3	4	0	3	0	4
	Nagpur (Tomato)	2	3	0	4	0	3
	Nagpur (Brinjal)	2	3	0	3	0	4
	Wardha (Tomato)	2	3	0	2	0	3
	Yavatmal (Cotton)	3	3	0	3	0	3
	Chandrapur (Cotton)	3	2	0	4	0	4

	Jalgaon (Okra)	2	3	0	2	0	3
	Aurangabad (Okra)	2	3	0	2	0	3

Molecular and Phylogenetic analysis

PCR of all eight populations amplified a single 1 kb amplicon (18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence). Alignment and analysis of sequences using the NCBI BLAST software tool confirmed the identity of all populations to that of *M. incognita* with reference to pre-existing sequence data. The sequences of the three populations collected from Nagpur were identical and therefore aggregated as a single population and the collected rDNA sequences were submitted to NCBI (Nagpur-KC342236; Wardha- KJ913700; Yavatmal-KP233824; Chandrapur-KP233823, Jalgaon-KT696493, Aurangabad- KT869139).

Identification of unique restriction sites was done to verify race determination of the *M. incognita* populations. The sequence of the aggregated Nagpur population had two restriction sites for *EcoR1* at positions 209 and 687, but not for *Swal, Bsa1* and *BsmA1.* The other three populations viz. Wardha, Yavamal and Chandrapur had one site for *EcoR1* at position 55. These results further confirmed the results of the differential host test for the four populations of *M.incognita* to belong to race 3.

Umarao *et al.*(2003) characterized races of *M. incognita* based on their rDNA ITS sequences and reported that variation in the sequences of the four catalogued races of *M. incognita* resulted in variance in their restriction sites, thus allowing for differentiation of the races based on restriction site distributions. The restriction enzymes Swal, Bsa1, BsmA1 and EcoR1 were reported for this purpose. The sequence of *M. incognita* race 4 had restriction sites for Bsa1 and BsmA1 while Swal was reported to cut at restriction sites only for the race 1 sequence. Similarly, EcoR1 was shown to have restriction sites for races 1, 3 and 4 but not for race 2. In our studies all six populations under study recorded restriction site for EcoR1 and none for Swal, Bsa1and BsmA1. Nagpur population had two restriction sites for enzyme Eco R1 while other five had only one for Eco R1 (Table 5)

Table 5. Sites for restriction enzymes Swal, Bsa1, BsmA1 and EcoR1 in gene sequences of Indian populations of root-knot nematode *Meloidogyne incognita*

Location of Root-knot nematode population		Site for restriction enzyme	Position of site
Andaman	Andaman	EcoR1	212
Gujarat	Gujarat	EcoR1	211
Haryana	Hisar	EcoR1	167
Himachal Pradesh	Palampur	EcoR1	166
Karnataka	Bangalore	EcoR1	180
Kerala	Kerala	EcoR1	211
Madhya Pradesh	Jabalpur	EcoR1	172
Maharashtra	Nagpur	EcoR1	209, 687
	Chandrapur	EcoR1	55
	Wardha	EcoR1	55
	Yavatmal	EcoR1	55
	Jalgaon	EcoR1	55
	Aurangabad	EcoR1	55
	Rahuri	BsmA1	142
Manipur	Manipur	EcoR1	145
Mizoram	Mizoram	EcoR1	203
Odisha	Odisha	BsmA1	717
Rajasthan	Rajasthan	EcoR1	211
Rajasthan	Rajasthan	RcoR1	134
Tamil Nadu	Coimbatore	EcoR1	177
	Ooty	EcoR1	144
Telanganana	Hyderabad	EcoR1	55
Tripura	Tripura	EcoR1	159
West Bengal	Siliguri	EcoR1	166

M.incognita populations available on NCBI dataset with the exception of Odisha and Rahuri also showed site for enzyme EcoR1 (Table 5). Odisha and Rahuri populations showed site for restriction enzyme BsmA1 only and not for EcoR1, Swal and Bsa1 further alluding to the diversity of races of *M. incognita* and the dynamicity of their population genetics. Although race 3 of *M. incognita* has been reported to colonize

cotton in the southern states of Karnataka and Tamil Nadu, the predominant hosts have been found to be vegetable crops (Krishnappa, 1985) while race 4 of RKN infestations has been reported to infest cotton in North India (Verma & Jain, 1999). Confirmation of the race identity of the four *M.incognita* populations from Maharashtra ties into the results of the MEGA-X phylogenetic analysis which confirms RKN populations from central India to be in a grouping with race 3 populations from South India (Figs. 1, 2 & 3). *M. incognita* race 3 has been reported to occur extensively throughout the cotton belt of USA and its damaging effects on cotton were first reported by Atkinson, 1892. Though RKN host races 3 and 4 are able to parasitize cotton in the USA (Veech and Starr, 1986) most populations encountered on cotton in the United State have been assigned to race 3 (Robbins *et al*.,1989; Robinson et al., 1999; Davis et al., 2018). In Brazil races 3 and 4 of *M.incognita* have been recorded as highly aggressive to the susceptible control FM966 and virulent to the accessions LA-887 (da Silva *et al*.,2014). *M.incognita* which thrives in warm/temperate to tropical climes (Sasser and Carter,1985) can be highly pathogenic to cotton with damage thresholds in the range of 1–9 nematodes/500cc soil (Roberts et al., 1985, Starrr et al., 1989). Average temperature of the coldest month of the year is the principal limiting factor in the occurrence of *M. incognita*, *M. javanica* and *M. arenaria*, none occurring where the coldest month average temperature is < 3 deg C. Other ecological factors examined include precipitation and soil analysis. (Taylor *et al*.,1982). Cotton growing areas in India harbor temperatures well within the ecological range of rootknot nematodes. In Central India even at peak winter months soil temperatures average well above this threshold temperature for RKN. Starr, 1993 recorded that low soil moisture inhibits egg hatch indicating extended longevity of egg masses of *M. incognita* under rainfed cotton cultivation which is being practiced in surveyed cotton areas of vidarbha. As this particular nematode species is primarily a pest of cotton in regions with coarsely textured soil, with infestations of cotton in areas of heavy textured soil with higher clay contents being quite rare, (Robinson *et al*., 1987, Starr *et al*., 1930), the discovery of RKN populations that exhibit virulence towards cotton in the latter described soil conditions presents as a possible expansion of their pathogenic range. Cotton is a commercial crop grown largely in monoculture, allowing for buildup of nematode populations (Starr *et al*.,1993). In Maharashtra cotton is largely grown rainfed and recent emphasis on micro-irrigation in rainfed cotton areas is likely to accentuate nematode problems as in rainfed agriculture lack of water keeps nematode population in check as water is crucial for nematode movement, host finding and survival in soil. From the above described phylogenetic grouping, predictions as to the possible emergence of increased virulence of RKN populations of Central India to cotton may be made. Race profile specific breeding for resistance against RKN infestation appears crucial for effective management of this pest and strategies to monitor and treat RKN infestations would be well-advised to take the above factors into consideration (Starr et al., (2007).

Declarations

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cotton by RNAi mediated silencing of parasitism genes of *Meloidogyne incognita'* under which this work was carried out.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for Publication

All authors have given consent for publication of the paper.

Availability of data and material

The data underlying this article is available in Crop Protection Division, ICAR-Central Institute for Cotton Research, Nagpur, India and will be shared on reasonable request to the corresponding author..

Competing interests

The authors declare that they have no conflict of interest.

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Author contributions

NGN wrote the manuscript,, NGN and SS conducted the experiments, DTN and SPG analyzed the data and NGN, PKC and SPG contributed to the final version of the manuscript. All authors read and approved the manuscript.

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Figures

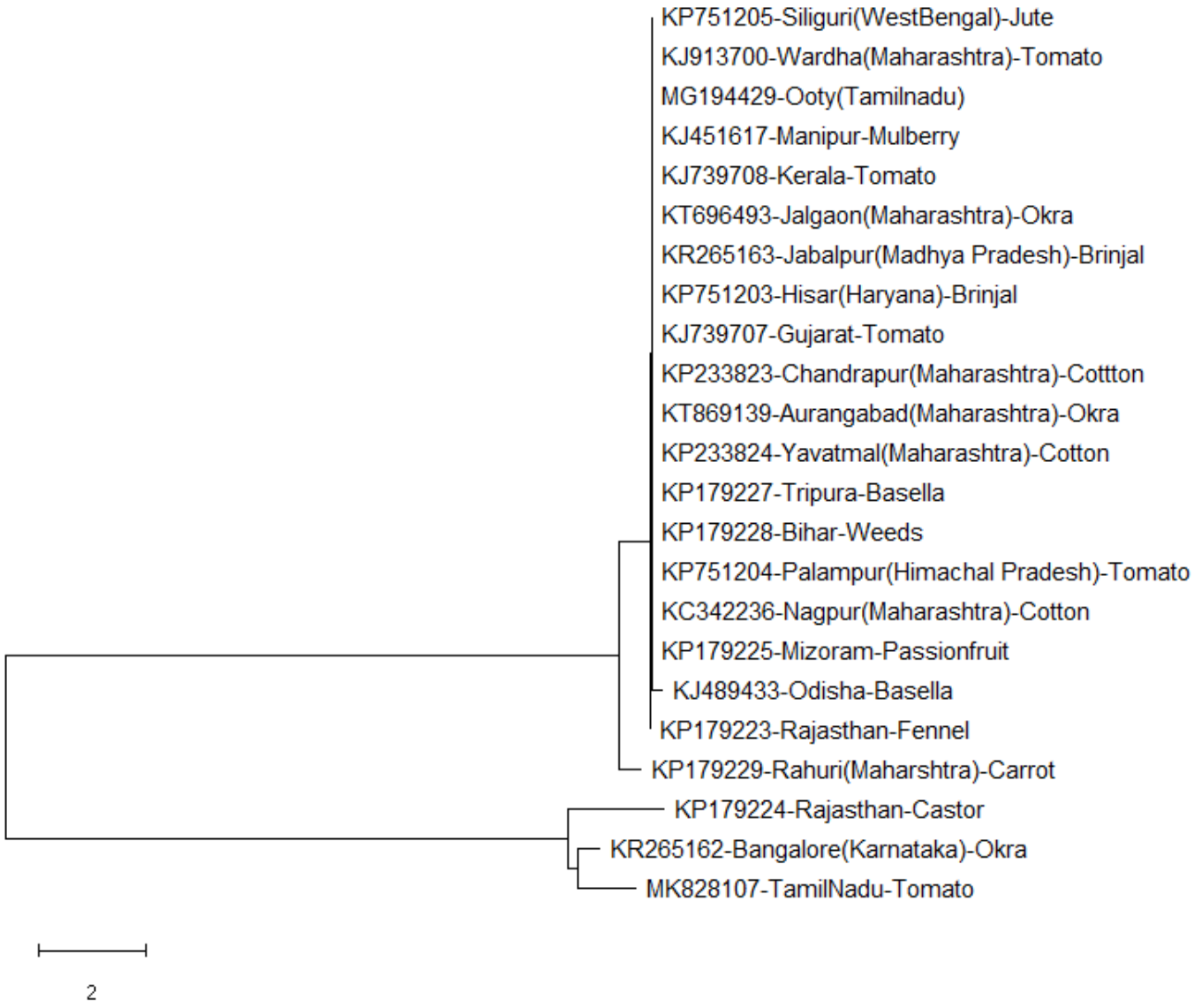

Figure 1

Maximum Likelihood analysis of Indian isolates of *Meloidogyne incognita* based on ITS1 rDNA.

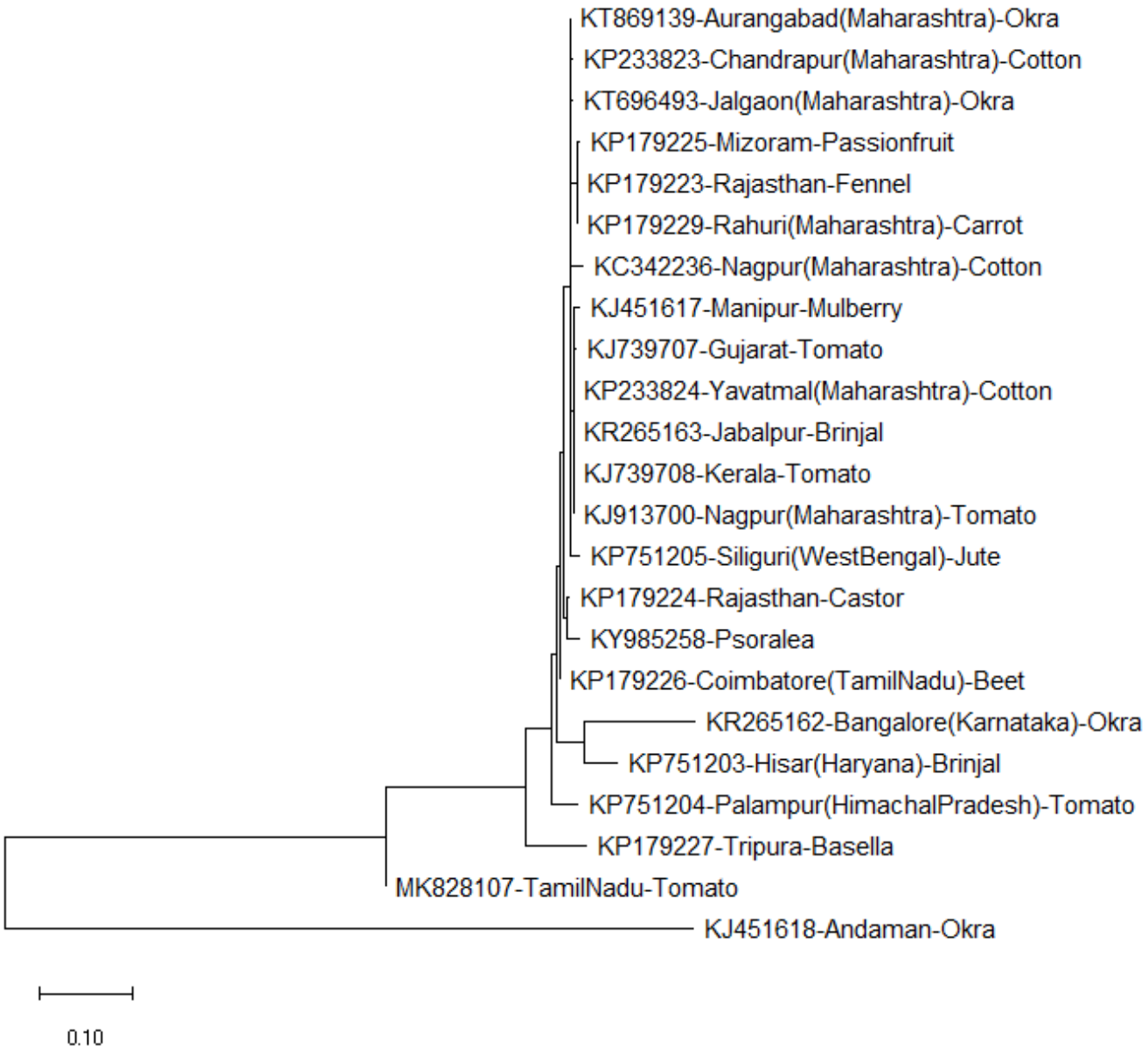

Figure 2

Maximum Likelihood analysis of Indian isolates of *Meloidogyne incognita* based on 5.8 rDNA.

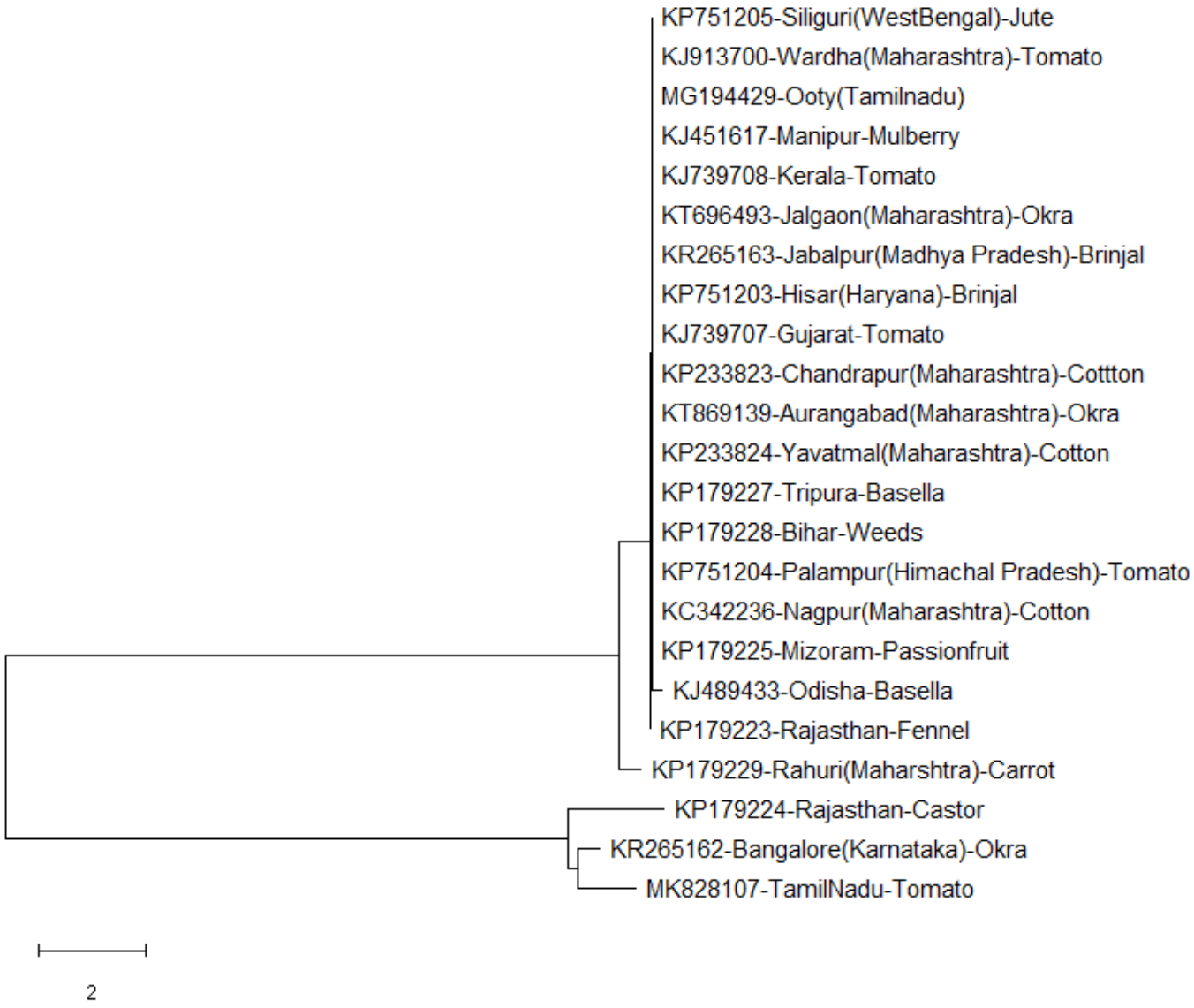

Figure 3

Maximum Likelihood analysis of Indian isolates of *Meloidogyne incognita* based on ITS1 rDNA.