DETECTION AND CHARACTERIZATION OF *MELOIDOGYNE* SPECIES ASSOCIATED WITH PEPPER IN INDIAN PUNJAB

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ABSTRACT

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A survey in the Central and Western agro climatic zones of pepper-growing areas of the Indian Punjab was conducted to identify and characterize species of root-knot nematode (RKN) infecting the pepper crop. Morphological, morphometric, and DNA sequence analysis were used to identify the nematodes. Soil and root samples were collected from farmer's fields and single egg mass progenies were maintained for identification and characterization. Progenies were identified by morphological studies and then confirmed by PCR amplification of DNA using species-specific SCAR (sequence characterized amplified region) primers, cloning, sequencing, and NCBI-BLASTn analysis of the target product. Results showed a mixed incidence of *M. incognita* and *M. javanica* in the Central agro climatic zone, whereas only *M. incognita* was detected in the Western agro climatic zone. The present study revealed that *M. incognita* and *M. javanica* are the main root-knot species infecting pepper in these regions of the Punjab. Phylogenetic analysis showed that SCAR sequences obtained in the present study were grouped closely with SCAR sequences for the respective *Meloidogyne* species identified from other parts of the world, emphasizing the reliability of the species-specific SCAR markers for quick detection of root-knot species with PCR assays.

Key words: characterization, *Meloidogyne incognita*, *Meloidogyne javanica*, morphology, morphometric, pepper, sequence analysis.

RESUMEN

Kaur Sukhjeet, S. S. Kang, N. K. Dhillon, and A. Sharma. 2016. Detección y caracterización de especies de *Meloidogyne* asociadas con el cultivo de pimienta en Indian Punjab. Nematropica 46:209-220.

Un muestreo fue realizado en zonas agroclimáticas centrales y occidentales de las zonas de cultivo de pimenton de Punjab India para identificar y caracterizar las especies de nematodos de nudo de raíz (RKN) que infectan el cultivo de pimiento, usando caracteristicas morfológicas, morfométricas y análisis de secuencias de ADN. Las muestras de suelo y raíces fueron recogidos de los campos de los agricultores y la progenies provenientes de masas de huevos se mantuvieron para la identificación y caracterización. Las progenies identificadas por los estudios morfológicos y luego confirmadas mediante amplificación de ADN aislado de estas progenies utilizando especies SCAR específica (sequence characterized amplified region), seguido de la clonación, secuenciación y análisis NCBI-BLASTn del producto. Los resultados mostraron que la incidencia mixta de *Meloidogyne incognita* y *M. javanica* en la zona climática agro central de Punjab, mientras que solo se detectó de *M. incognita* en la zona climática agro occidental. El presente estudio reveló que*M. incognita* y *M. javanica* están infectando principalmente en pimiento en Punjab. Además el uso de marcadores especificos SCAR se encontró fiable para la detección rápida de las especies de nematodos de los nudos radicales con ensayos de PCR.

Palabras clave: análisis, caracterización, *Meloidogyne incognita*, *Meloidogyne javanica*, morfologia, morfometria, pimienta, secuencias.

INTRODUCTION

Pepper (*Capsicum annuum* L.) is an important commercial crop for vegetable, spice, oleoresin, and capsaicin extraction purposes in India (Kumar and Rai, 2005). Plant-parasitic nematodes are among the important factors limiting pepper production in the country. Root-knot nematodes have been reported to cause national losses up to 23% in hot-pepper (Khan *et al*., 2014). More than 100 species of root-knot nematodes have been described (Skantar *et al.*, 2008; Hunt and Handoo, 2009; Moens *et al*., 2009), but only four major species, *Meloidogyne incognita*, *M. javanica*, *M. hapla*, and *M. arenaria*, are pests of peppers worldwide (Theis and Fery, 2002). Accurate diagnosis of root-knot nematode species is important for development of management strategies and in resistance breeding programs. Very little information is available on the identity or prevalence of root-knot species infecting pepper under Punjab conditions, and no work has been done on molecular detection and characterization of *Meloidogyne* species prevalent in the Punjab. The objectives of the present study were: i) detection and characterization of *Meloidogyne* species infecting pepper in the Punjab using morphological and molecular techniques and ii) to determine the suitability of the molecular markers for quick and reliable identification of *Meloidogyne* species.

MATERIALS AND METHODS

A population of *Meloidogyne* was collected from each major pepper-growing area of the Indian Punjab in the districts of Central (Sangrur, Patiala, Tarn Taran and Ludhiana) and Western agro climatic zone (Ferozepur). Infested pepper roots along with associated soil were put in plastic bags, tied with rubber band and brought to the laboratory for further processing. Single egg mass cultures were raised on the susceptible capsicum cultivar 'California Wonder' in pots using autoclaved soil and maintained in greenhouses.

Morphological and morphometric characterization

Twenty specimens consisting of juveniles, females, and males from each population were examined. Mature females were excised from galled capsicum roots with needle and forceps and stored in water at 4°C until observation. For morphometric analyses, females were placed on a glass slide in a water drop and measured under the microscope. Perineal patterns of females were studied by cutting the posterior end of each female and carefully removing the inner tissue with a nylon bristle. The perineal pattern was transferred to a drop lactophenol, mounted in drop of glycerin, covered with a cover slip, sealed with glyceel (Jacob and van Bezooijen, 1984) and observed under the microscope. For morphological and morphometric studies, second-stage juveniles (J2) were allowed to hatch by placing an egg mass into sterilized water in a cavity block kept at 28°C in an incubator. After 24 hr, freshly hatched J2 were collected, placed on a clean glass slide in a drop of water, killed with heat, and observed under a microscope. Males were isolated from soil taken from infected capsicum roots using the centrifugal-flotation method (Jenkins, 1964). Males were collected in a cavity block containing sterilized water and then killed with heat and observed. The following measurements were made: length, width, neck length, stylet length, and distance of dorsal esophageal gland orifice from the base of stylet for females; length, width, stylet length, distance of dorsal esophageal gland orifice from the base of stylet, anterior body length, excretory pore distance from anterior end, hyaline tail length, and tail length for J2; length, width, head length, stylet length, anterior body length, and spicule length for males. All observations were done using Leica microscope (Leica DME trinocular microscope, Wetzlar, Germany) at 4×, 10×, and 40×. Pictures were taken with a Leica digital camera (Leica EC3 digital colour camera 3.1 megapixel) attached to the microscope and measurements were made from images using LAS EZ (Leica Application Suite) software. Nematode species were identified by comparing morphological and morphometric characters with the earlier descriptions of *Meloidogyne* species (Chitwood, 1949; Orton Williams, 1972; Jepson, 1987).

Molecular characterization

Total DNA was extracted from nematodes using a Proteinase K enzyme method (Williamson *et al*., 1997). Juveniles and egg masses were picked, transferred to a 1.5-ml micro tube containing 50 µl nematode lysis buffer (1X PCR buffer with 100 µg/ml proteinase K), and crushed using a conical micro pestle before freezing at -20°C for 1 hr. Incubation was performed at 60°C for 1 hr, followed by inactivation of proteinase K by incubation at 94°C for 10 min. Centrifugation was done at 13,000 rpm for 2 min. and supernatants (DNA extracts) were taken in separate sterile tubes. Two volumes of cold absolute ethanol (-20°C) were added to the supernatants and left in the freezer at -20°C for 1 hr. The precipitated DNA was pelleted by centrifugation at 14,000 rpm for 3 min., washed with 70% ethanol, air dried for 2 hr at room temperature (25°C) and re-suspended in

50 µl of tris extraction (1xTE) buffer and stored at -20°C until used as PCR template.

Confirmation of root-knot nematode species was done by PCR amplification of DNA with SCAR (sequence characterised amplified regions) primers specific to three main *Meloidogyne* species, *M. incognita*, *M. javanica*, and *M. arenaria* (Table 1). Total DNA from *M. incognita* single-egg mass progenies was subjected to PCR amplification with *M. incognita* specific MiF/MiR primers (Meng *et al*., 2004) and Finc/Rinc (Zijlstra *et al*., 2000) primers. DNA from *M. javanica* progenies was subjected to PCR amplification with *M. javanica* specific SCAR primer Fjav/Rjav (Zijlstra *et al*., 2000).

In order to check the specificity of the primers, DNA from some of the *M. javanica* progenies (Mj_1, Mj_2, Mj_3, and Mj_5) was also subjected to PCR amplification with *M. incognita* specific MiF/MiR primers (Meng *et al*., 2004) along with *M. incognita* progenies. Similarly, DNA of some of the *M. incognita* progenies (Mi_3, Mi_4, and Mi_6) was also subjected to PCR amplification with *M. javanica* specific SCAR primers Fjav/Rjav (Zijlstra *et al*., 2000) along with DNA of *M. javanica* progenies. DNA of *M. incognita* and *M. javanica* progenies was also subjected to PCR amplification with *M. arenaria* SCAR (Zijlstra *et al*., 2000) and *M. arenaria* MarF/R (Dong *et al*., 2001) primers in order to check the primer specificity.

The PCR amplification was done with reaction mixture (25 µl) consisting of 2 µl of template DNA (50 ng/µl), 0.25 µl (500 unit) of taq polymerase (Promega, Madison, WI, USA), 1.5 µl (25 mM) MgCl2, 0.5µl (2 mM) dNTPs, 1.0 µl (20 picomol/µl)) of each forward and reverse primers in 5 µl of reaction buffer (5×) (Green Go taq, Promega, Madison, WI, USA), and the volume was made to 25 µl with nuclease-free water. The amplification was carried out using an Eppendorf PCR system (Mastercycler pro, Eppendorf, Hamburg, Germany). The amplification program consisted of one initial cycle of denaturation at 94°C for 2 min., and 35 cycles of denaturation at 94°C for 30 sec., annealing at specific temperatures (Table 1), extension at 68°C for 1 min., then finishing the amplification program with final extension for 10 min. at 72°C.

The amplified PCR products were analyzed using 1.0% agarose gel with ethidium bromide (10 mg/ml) stain. The gel was viewed under UV light using an Alpha Imager HP gel documentation system (Alpha Innotech, San Jose, CA, USA). The expected size amplified products were visualized and extracted from the gel using a Nucleospin Gel and PCR clean-up kit (Macherey-Nagel GmbH & Co., Duren, Germany) as per the manufacturer's instructions. These cleaned PCR products were then cloned in pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* strain DH5aplha competent cells using heat shock method. Six recombinant plasmids for *M. incognita* and six for *M. javanica* were selected for sequencing. Plasmid isolation from positive clones was done using a Nucleospin Plasmid Isolation Kit (Macherey-Nagel GmbH & Co., Duren, Germany) as per the manufacturer's protocol and out-sourced for sequencing to Xcelris Lab Ltd., Ahmadabad, India. The sequences were analyzed by NCBI-BLASTn analysis (Altschul *et al*., 1990). After confirming the identity, these sequences were submitted to GenBank.

For the phylogenetic analysis, BLASTn searches were performed for all the partial genomic SCAR

Table1. List of primers used in this study.

Primers	Primer sequence	Annealing temperature	Product size	Reference
Meloidogyne incognita				
MiF/MiR	F : GTGAGGATTCAGCTCCCCAG R: ACGAGGAA CATACTTCTCCGTCC	64°C (30 sec.)	955bp	Meng *et al.*, 2004
SCAR Finc/Rinc	F:CTCTGCCCAATGAGCTGTCC R: CTCTGCCTCACATTAAG	54°C (2 min.)	1200bp	Zijlstra *et al*., 2000
Meloidogyne javanica				
SCAR Fjav/Rjav	F: GGTGCGCGATTGAACTGAGC R:CAGGCCCTTCAGTGGAACTATAC	63°C (1 min.)	670bp	Zijlstra *et al*., 2000
Meloidogyne arenaria				
SCAR Far/Rar	F: TCGGCGATAGAGGTAAATGAC R: TCGGCGATAGACACTACAACT	61°C (45 sec.)	420bp	Zijlstra *et al.*, 2000
Mar F/R	F: TCGAGGGCATCTAATAAAGG R: GGGCTGAATATTCAAAGGAA	50°C (1 min.)	950bp	Dong *et al*., 2001

DNA sequences of *M. incognita* and *M. javanica* obtained in the present study. The representative sequences were downloaded from the GenBank database so that comparisons could be made between the sequences obtained in this study and similar sequences of these species from different hosts and geographical regions available in the GenBank database. Apart from this, SCAR genomic DNA sequences of other *Meloidogyne* species viz., *M. arenaria* and *M. graminicola* were retrieved from the NCBI database for comparison along with SCAR sequences of genus *Heterodera*, which were used for outgrouping (Table 2). The sequences were aligned using clustalW (with gap opening penalty for multiple alignment of 15 and extension of 6.66) using Mega 6.0 software (Tamura *et al*., 2013). The alignment was analysed to get the base substitution model for these sequences using Mega 6 software. Out of maximum likelihood fits of 24 different nucleotide substitution models, T92 model (Tamura, 1992) with lowest BIS score (Bayesian Information Criterion) was selected and considered to describe the substitution pattern best. The phylogram was generated using base substitution model T92 and running Maximum Likelihood Model with 1,000 bootstrap replicates.

Table 2. GenBank, NCBI SCAR sequence used for phylogenetic analysis.

Species name	GenBank accession no.	Country
Meloidogyne incognita	KF041328.1	Malaysia
Meloidogyne incognita	KF041338.1	Malaysia
Meloidogyne incognita	KF041334.1	Malaysia
Meloidogyne incognita	KF481971.1	China
Meloidogyne incognita	JN005841.1	South China
Meloidogyne incognita	JN005840.1	South China
Meloidogyne javanica	JN005834.1	South China
Meloidogyne javanica	KF041326.1	Malaysia
Meloidogyne javanica	KF041289.1	Malaysia
Meloidogyne javanica	KP253751.1	Indonesia
Meloidogyne javanica	KF041327.1	Malaysia
Meloidogyne javanica	KF041320.1	Malaysia
Meloidogyne arenaria	KP253748.1	Indonesia
Meloidogyne arenaria	KP234264.1	Indonesia
Meloidogyne graminicola	KF499563.1	Philippines
Heterodera filipjevi	KC529338.1	China
Heterodera avenae	JQ405270.1	China

RESULTS

Morphological and morphometric analyses

Perineal pattern of the mature females was used as basic criteria for initial detection followed by morphometric measurements of the different developmental stages of the population. Morphometric characters play a significant role in defining nematode species and populations. These characters can show significant differences not only between species but among populations within a species (Carneiro *et al.*, 2008; Skantar *et al*., 2008). Morphometrics of females, second-stage juveniles, and males of the root-knot nematode under study were compared with typical descriptions of the *Meloidogyne* species. Incidence of *M. incognita* was recorded in both Central and Western agro climatic zone of Punjab, but incidence of *M. javanica* was not recorded from the Western zone.

Description of Meloidogyne incognita *populations from different districts of Punjab*

Females:

The females were pear shaped with body length ranging from 482.4-746.2 µm and body width ranging from 289-487 µm (Fig.1A) which was greater than the typical *M. incognita* descriptions given by Jepson, 1987 (Table 3). The neck protruded anteriorly 175-311 µm. Stylet length was 12.2-17.4 µm with the stylet cone having the anterior half distinctly curved dorsally. Stylet knobs offset, rounded to transversely elongated. Distance of dorsal esophageal gland from base of stylet ranged from 2.6-4.2 µm (Fig.1B). Stylet length and distance of dorsal esophageal gland from base of stylet was slightly higher than the type descriptions of the species. Esophagus had a large muscular bulb with conspicuous valve plate. Perineal pattern showed characteristic high dorsal arch with distinct wavy forked striae and no lateral lines (Fig.1C).

Variation was also observed among the *M. incognita* populations collected from different districts. Morphometric comparison of five populations from different districts of Punjab showed that the maximum female body length (746.2 µm) and width (487 µm) was recorded in Patiala populations. Minimum body length (482.4 µm) and width (289 µm) was recorded in Ferozepur and Tarn Taran populations, respectively. Stylet length was shortest (12.2 µm) in Patiala populations and longest (17.4 µm) in Tarn Taran populations.

Second-stage juveniles (J2):

Second-stage juveniles ranged from 335-428 µm in length (Fig. 2A) and 10.1-13 µm in width

Fig. 1: Micrographs of *Meloidogyne incognita* female (A) Whole body of a female; (B) Anterior region of a female; (C) Perineal pattern with high squarish dorsal arch without lateral lines.

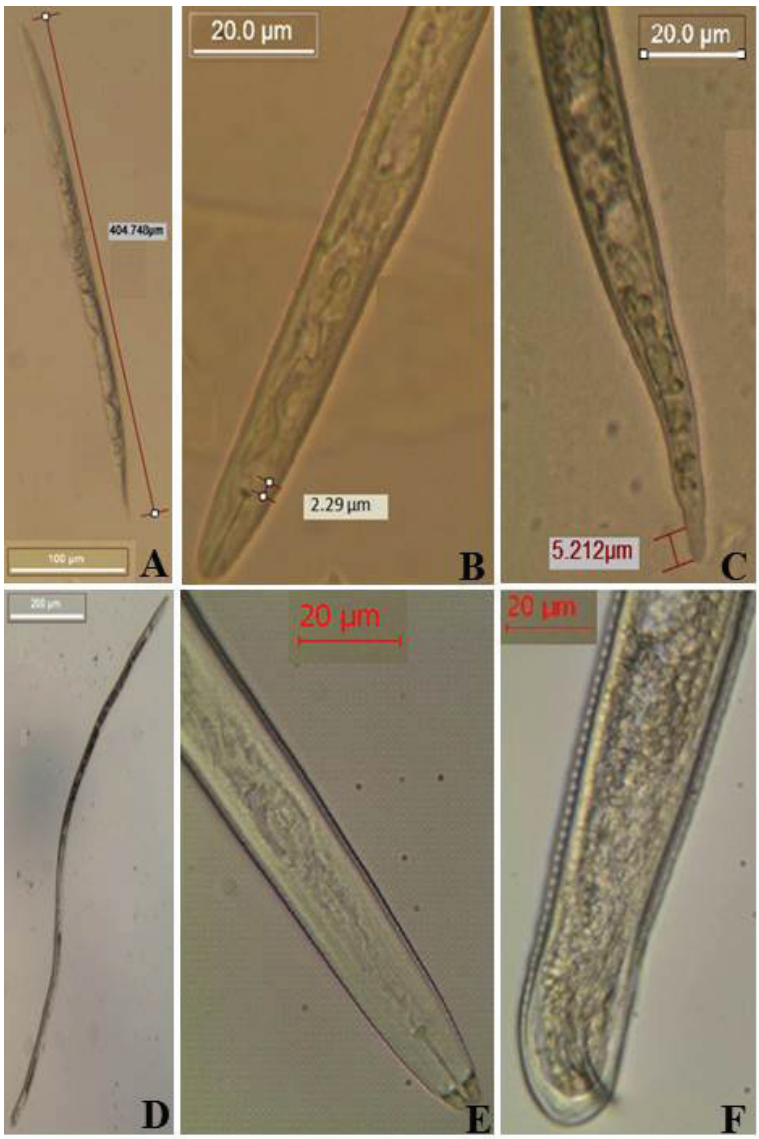

Fig. 2: Micrographs of *Meloidogyne incognita* second-stage juveniles (A-C) and Males (D-F). (A) Whole body of a juvenile; (B) Anterior region of a juvenile; (C) Juvenile tail with slightly blunt tail end and shorted hyaline tail length; (D) Whole body of a male (E) Anterior region of a male (F); Male tail with spicules.

Table 3. Comparison of measurements of *Meloidogyne incognita* females and juveniles with Jepson,1987. Measurements [in micrometers (µm)] are in the format of mean ± standard deviation (ranges).

Characters (µm)	Sangrur (Mi_1)	Tarn Taran (Mi_2)	Ludhiana (Mi_3)	Ferozepur (Mi_4)	Patiala (Mi_5)	Gross range of population under study	Jepson (1987)
FEMALES							
Length	577 ± 47.9 (502-646)	570 ± 58.21 (524-646)	570.2 ± 44.12 (554-626)	568.7 ± 78.2 (482.4-694.5)	581.8 ± 87.1 (514-746.2)	482.4-746.2	510-690
Width	369.9 ± 59.9 (289.7-480)	351.2 ± 37.4 (289-480)	365.2 ± 38.6 (318-410)	377.0 ± 56.12 (343-474.8)	384.8 ± 60.7 (333-487)	289-487	300-430
Neck length	212 ± 30.7 (175-260)	220.4 ± 47.8 (175-311)	204 ± 28.15 (175-240)	236.5 ± 41.9 (175-286)	229 ± 39.05 (178-291)	175-311	-
Stylet length	15.3 ± 0.76 (14.2-16.4)	15.0 ± 2.1 (13-17.4)	15.0 ± 0.52 (14.2-16)	14.3 ± 0.98 (13.3-15.8)	13.4 ± 1.41 (12.2-15.2)	12.2-17.4	15-16
DGO[y]	3.9 ± 0.25 (3.5-4.2)	4.0 ± 0.16 (3.8-4.2)	3.7 ± 0.3 (3.3-4)	3.2 ± 0.49 (2.7-4)	3.5 ± 0.97 (2.6-4.2)	2.6-4.2	-
JUVENILES							
Length	357.0 ± 30.14 (340-400)	347.5 ± 1.5 (335-360)	408.8 ± 15.83 (388-428)	395.8 ± 12.42 (376-412)	356.7 ± 16.1 (338.1-382.2)	335-428	337-403
Width	11.9 ± 0.76 (10.9-13)	11.5 ± 0.75 (10.5-12.1)	11.1 ± 0.36 (10.1-12.6)	11.4 ± 0.71 (10.6-12.6)	11.3 ± 0.56 (10.2-12.2)	10.1-13	
Stylet length	10.7 ± 0.58 (9.7-11.2)	10.6 ± 0.53 (9.8-11.2)	11.2 ± 1 (10.1-12.4)	10.6 ± 0.54 (9.8-11.2)	10.7 ± 0.65 (9.6-11.3)	9.6-12.4	9.6-11.7
DGO[y]	2.5 ± 0.32 (2.4-3)	2.6 ± 0.15 (2.4-2.7)	2.4 ± 0.25 (2.1-2.8)	2.3 ± 0.1 (2.2-2.5)	2.3 ± 0.14 (2.1-2.5)	2.1-3	-
Anterior body length[z]	14.2 ± 0.81 (13.0-15.2)	13.80 ± 0.37 (13.5-14.5)	14.56 ± 0.57 (14.0-15.3)	13.63 ± 0.66 (12.5-14.2)	14.03 ± 0.27 (13.5-14.3)	12.5-15.3	-
Excretory pore from anterior end	68.2 ± 14.75 (59.0-83)	75.1 ± 3.05 (72.0-78.2)	83.2 ± 1.25 (81.1-84.6)	80.5 ± 2.3 (78.4-84)	69.5 ± 1.94 (67.1-72.3)	59-84.6	-
Hyaline tail length	8.7 ± 1.88 (5.6-11)	9.3 ± 1.7 (6.7-10.8)	10.4 ± 1.09 (8.4-11.7)	8.8 ± 1.45 (6.6-10)	8.2 ± 0.65 (7-8.7)	5.6-11.7	6.3-13.5
Tail length	47.4 ± 4.2 (42.9-54)	46.4 ± 3.3 (42.1-15.3)	53.4 ± 3.9 (47-58.7)	49.4 ± 2.25 (47-52.2)	44.3 ± 38 (40.1-51)	40.1-58.7	45-52.2

[y]Dorsal esophageal gland orifice distance from stylet base.
[z]Distance from head to the base of stylet.

(Table 3). Stylet length ranged from 9.6-12.4 µm and anterior body length (distance from head to the base of stylet) varied from 12.5-15.3 µm. Distance of the dorsal esophageal gland from the base of the stylet ranged from 2.1-3 µm (Fig. 2B). The excretory pore was 59-84.6 µm from the anterior body end. Tail length was 40.1-58.7 µm with rounded tip (Fig. 2C). Hyaline tail length ranged from 5.6-11.7 µm. Second-stage juveniles of the population under study showed slight increase in morphometric measurements over the type descriptions of the species in terms of body length and stylet length, whereas there was a slight decrease in hyaline tail length compared with the type description given by Jepson, 1987.

Among the different populations collected, the maximum body length of juveniles (428 µm) was recorded in the Ludhiana population and minimum (355 µm) in the Tarn Taran population. The longest stylet (12.4 µm) was observed in the Ludhiana population. Tail length (58.7 µm) and hyaline tail length (11.7 µm) were also longest in the Ludhiana population. The shortest tail length (40.1 µm) was observed in the Patiala population.

Males:

Populations of males from Sangrur, Ludhiana, and Patiala districts were recovered (Fig. 2 D-F). Sizes of males varied from 1727.5-2128 µm in length and 20.4-39.6 µm in width, which was higher than the type description of the species as given by Chitwood, 1949 (Table 4). The male head was 5.9-8 µm long. Head cap was high with a concave labial disc. Stylet was robust with a stylet cone longer than the stylet shaft. Stylet length ranged from 18.6-20.9 µm, which was shorter than the type description. Distance of dorsal esophageal gland orifice from base of stylet was consistent with the typical *M. incognita*

Table 4. Comparison of measurements of *Meloidogyne incognita* males with Chitwood, 1949 and Jepson, 1987. Measurements [in micrometers (µm)] are in the format of mean ± standard deviation (ranges).

Characters (µm)	Sangrur (Mi_1)	Ludhiana (Mi_3)	Patiala (Mi_5)	Gross range of population under study	Chitwood (1949)	Jepson (1987)
Length	1962.2 ± 113.7 (1870-2128)	1888 ± 45.9 (1856-1921)	1867.8 ± 126.2 (1727.5-1972)	1727-2128	1200-2000	-
Width	37.7 ± 1.76 (35.7-39.6)	35.7 ± 1.6 (34.6-36.8)	23.9 ± 33 (20.4-27)	20.4-39.6	-	-
Head length	7.3 ± 0.37 (6.9-7.8)	7.6 ± 0.54 (7.3-8)	6.2 ± 0.2 (5.9-6.3)	5.9-8	-	-
Stylet length	19.3 ± 0.95 (18.6-20.6)	20.4 ± 0.63 (20-20.9)	19.6 ± 0.56 (18.9-20.3)	18.6-20.9	23-26	23-26
DGO[y]	3.2 ± 0.57 (2.4-3.7)	3.1 ± 0.38 (2.8-3.4)	3.5 ± 0.38 (3.0-3.9)	2.4-3.9	1.75-3.5	2.7-3.15
Anterior body length[z]	25.5 ± 0.60 (27-28.3)	27.7 ± 0.38 (27.5-28.2)	26.8 ± 1.7 (24.8-28.1)	24.8-28.3	-	-
Excretory pore from anterior end	159.7 ± 36.8 (117.6-186.4)	179 ± 4.24 (176-182)	153.5 ± 35.4 (121-202)	117.6-202	-	-
Spicules (length of arc) length	34.0 ± 4.94 (28.2-39.8)	38.8 ± 0.97 (37.8-39.6)	23.9 ± 3.3 (20.4-27)	20.4-39.8	34-36	-

[y]Dorsal esophageal gland orifice distance from stylet base.
[z]Distance from head to the base of stylet.

descriptions and ranged from 2.4-3.9 µm. Excretory pore distance was 117.6-202 µm from anterior end. Tail bluntly rounded with spicules slightly curved, 20.4-39.8 µm long.

Description of Meloidogyne javanica *populations from different districts of Punjab*

Females:

Meloidogyne javanica females were pear-shaped, perineal pattern showed distinct lateral ridges that divide the dorsal and the ventral striae, lateral lines extended on both sides of tail terminus (Fig. 3A).The size of females varied from 519-790 µm in length and 287-517 µm in width. Neck length, 129-300 µm. Stylet was slender, 13.8-18.3 µm long with rounded knobs. Distance of dorsal esophageal gland opening from stylet base ranged from 2.9-4.8 µm. All the measurements of the females of *M. javanica* populations in this study were in agreement with the typical *M. javanica* descriptions per Jepson, 1987 (Table 5).

Body size of the *M. javanica* females varied among the different populations collected from Punjab. Maximum body length (790 µm) was recorded in the population from Sangrur District and minimum body length (519 µm) occurred in the Patiala population. Neck length was maximum (300 µm) in the Tarn Taran population and minimum (129 µm) in the Ludhiana population. The longest stylet (18.3 µm) was observed in the Ludhiana population.

Second-stage juveniles (J2):

Juveniles were 360-440 µm long (consistent with the typical *M. javanica* descriptions) and 11-13.6 µm wide (Table 5). Stylet length ranged from 9.6-11.7 µm. The anterior end of the juveniles was 12.8-15.4 µm from the lip region. Distance of dorsal esophageal gland from stylet base, 2.0-3.2 µm. Tail length ranged between 44.8-59.9 µm. Tail end was tapering with 9.3-13 µm hyaline tail terminus (Fig. 3B).

Maximum body length (440 µm) was recorded in the Ludhiana population and the shortest (360 µm) was in the Patiala population. Stylet length was also maximum (11.7 µm) in the Ludhiana population. The longest tail (59.9 µm) was observed in the Tarn Taran population and the shortest hyaline tail length (9.3µm) was observed in the Sangrur population.

Males:

Meloidogyne javanica males were recovered only from the Patiala population (Fig. 3C). Sizes from this population varied from 1,200-1,287 µm (length) and 26.8-36.6 µm (width). Head length ranged from 4.2-5.5 µm and stylet was 18.4-20.8 µm long. The body length and stylet length were in agreement with the type descriptions of *M. javanica*, but the head was shorter than the type description

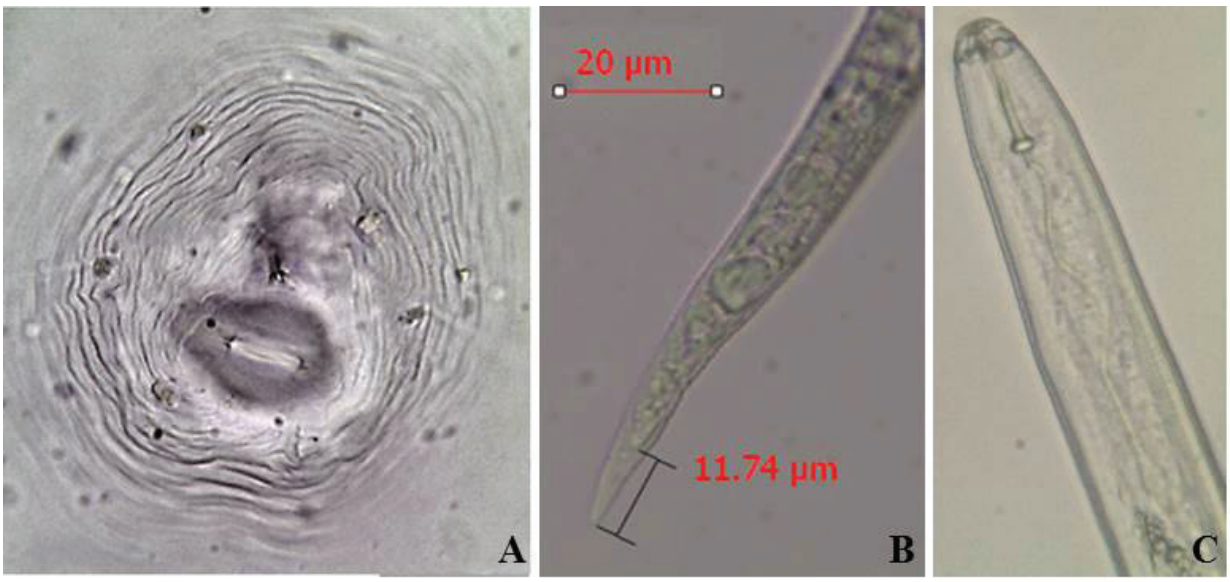

Fig. 3: Micrographs of *Meloidogyne javanica* species. (A) Perineal pattern of *M. javanica* female showing distinct lateral lines; (B) Tail of *M. javanica* second-stage juvenile with pointed tail terminus and longer hyaline tail length; (C) Anterior region of a male.

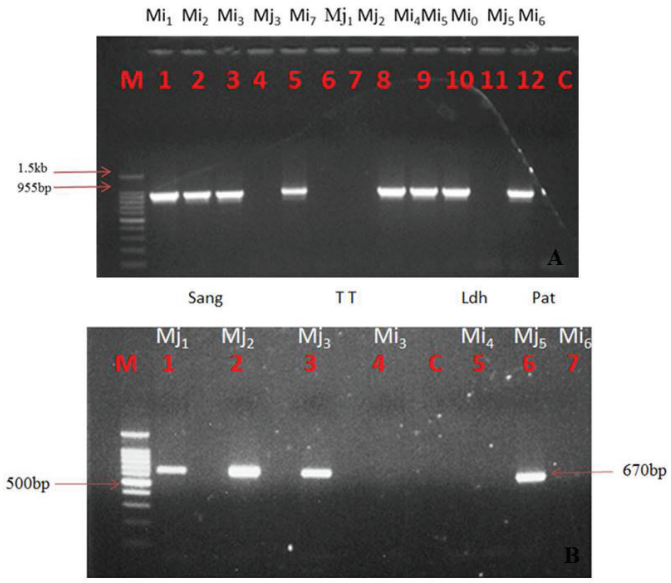

Fig.4: Agarose gel (1%) showing amplification of *Meloidogyne incognita* and *M. javanica* with species specific primers. (A) Amplicon of 955bp with MiF/MiR primers. Samples:1-2 (Sangrur-Mi_1 and Mi_2), 3-4 (Tarn Taran-$Mi_3$and Mj_3), 5(Ferozpur-Mi_7), 6-7 (Sangrur-$Mj_1$and Mj_2), 8-10 (Ludhiana Mi_4, Mi_5, and Mi_0),11-12 (Patiala-Mj_5 and Mi_6) ; (B) Amplicon of 670bp with Fjav/Rjav primers. Samples: 1-2 (Sangrur - Mj_1 and Mj_2), 3-4 (Tarn Taran - Mj_3 and Mi_3), 5(Ludhiana -Mi_4), 6-7(Patiala-Mj_5 and Mi_6), C- control, M-marker (100bp). Abbreviations are given as; Sang- Sangrur, TT-Tarn Taran, Frz – Ferozepur, Ldh – Ludhiana, Pat-Patiala ;Mi- *M. incognita*, Mj –*M. javanica*.

(Table 6). The dorsal esophageal gland opening from stylet base was 2.6-4.4 µm that is within the range of the type description. Excretory pore distance from the head was 148-150 µm. Spicule arc length ranged from 26.8-30.9 µm, which was also consistent with the type *M. javanica*. Overall, morphometric measurements of *M. javanica* were comparable with the previously reported type descriptions for the species by Orton Williams, 1972 and Jepson, 1987.

Molecular analysis

All the *M. incognita* single-egg-mass progenies, Mi_1 and Mi_2 (Sangrur), Mi_3 (Tarn Taran), Mi_0, Mi_4, and Mi_5 (Ludhiana), Mi_6 (Patiala), and Mi_7 (Ferozepur), showed an expected size amplicon of 955 bp with MiF/MiR primer (Fig. 4A) and amplicon of 1,200 bp with Finc/Rinc primer pairs. There was no amplification with DNA from *M.*

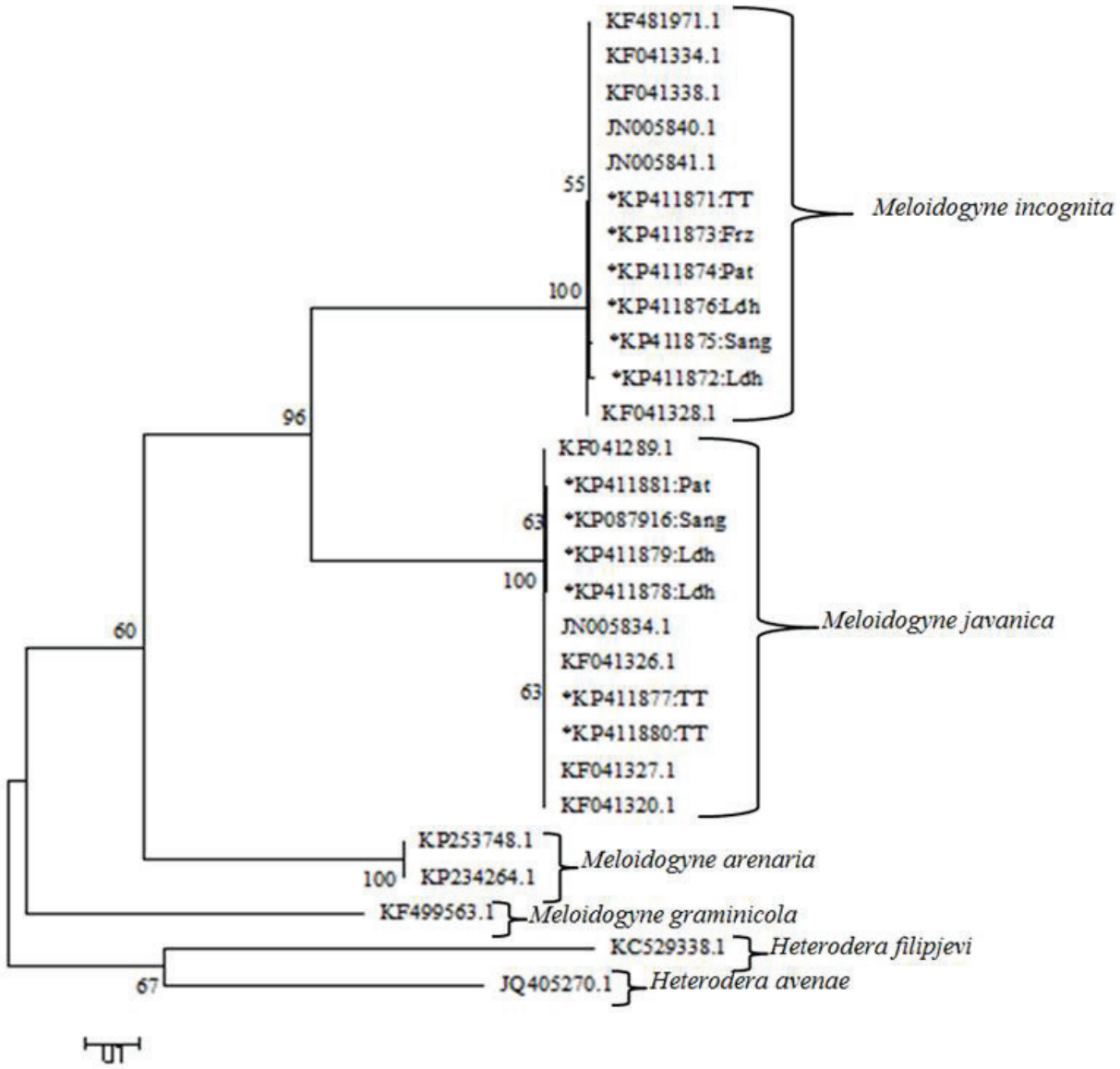

Fig. 5. Maximum likelihood tree was inferred from partial *Meloidogyne* species SCAR genomic DNA sequences under T92 model (BIC = 5420.861; INL = -2460.512; AIC = 5031.724; freqA = 0.359; freqT = 0.359; freqC = 0.141; freqG = 0.141; R = 1.08). The analysis was done using 1,000 bootstrap replicates. The bootstrap value for each clade is indicted on the nodes. Out-group SCAR sequences from genus *Heterodera* were used for alignment as well for the construction of the phylogram. The sequences obtained in the current study are shown with (*) and locality abbreviations are provided as follows; Sang-Sangrur; TT-Tarn Taran; Frz – Ferozepur; Ldh – Ludhiana; Pat-Patiala.

javanica progenies (Mj_1, Mj_2, Mj_3, and Mj_5) and negative control. Similarly, SCAR primer Fjav/Rjav amplified an expected 670 bp amplicon with DNA of morphologically identified *M. javanica* populations Mj_1 and Mj_2 (Sangrur), Mj_3 (Tarn Taran), and Mj_5 (Patiala), whereas no amplification was observed with *M. incognita* progenies (Mi_3, Mi_4, and Mi_6) and the negative control (Fig. 4B). None of the populations showed amplification with *M. arenaria* SCAR (Zijlstra *et al*., 2000) and *M. arenaria* MarF/R (Dong *et al*., 2001) primers, indicating high specificity of these species-specific SCAR primers for reliable detection of *Meloidogyne* species.

Table 5. Comparison of measurements of *Meloidogyne javanica* females and juveniles under present study with Jepson,1987. Measurements [in micrometers (µm)] are in the format of mean ± standard deviation (ranges).

Characters (µm)	Sangrur (Mj_1)	Ludhiana (Mj_3)	Tarn Taran (Mj_2)	Patiala (Mj_5)	Gross range of population under study	Jepson (1987)
FEMALES						
Length	677 ± 59.1 (620-790)	610.7 ± 44.27 (554-685)	602.14 ± 40.4 (549.7-645)	600 ± 71.72 (519-744)	519-790	545-800
Width	414 ± 18.1 (374-432)	416.9 ± 43.54 (364-507)	356 ± 17.65 (328-376)	376.6 ± 79.7 (287-517)	287-517	300-545
Neck length	238 ± 24 (200-282)	183.5 ± 64.21 (129-295)	244.2 ± 48.8 (204-300)	217.6 ± 11.81 (200-233)	129-300	-
Stylet length	16.2 ± 1.3 (14.7-17.9)	16.3 ± 1.81 (13.8-18.3)	16.4 ± 0.74 (15.6-17.3)	17.2 ± 1.2 (15-18.2)	13.8-18.3	14-18
DGO[y]	3.8 ± 0.75 (2.9-4.8)	3.8 ± 0.45 (3.3-4.5)	4.1 ± 0.43 (3.6-4.6)	3.7 ± 0.5 (3.2-4.4)	2.9-4.8	-
JUVENILES						
Length	426.8 ± 6.64 (418-435)	412 ± 20.8 (386-440)	418.8 ± 5.07 (412-425)	374.7 ± 9 (360-385.4)	360-440	387-459
Width	11.6 ± 0.61 (11-12.6)	12.9 ± 0.41 (12.4-13.6)	11.4 ± 0.44 (11.5-12.6)	12 ± 0.51 (11-12.3)	11-13.6	-
Stylet length	10.8 ± 0.53 (10.1-11.5)	10.8 ± 0.78 (9.6-11.7)	10.6 ± 0.48 (10-11.3)	11 ± 0.35 (10.3-11.3)	9.6-11.7	9.4-11.4
DGO[y]	2.6 ± 0.36 (2.2-3.2)	2.5 ± 0.25 (2.2-3)	2.3 ± 0.37 (2-3.1)	2.4 ± 0.32 (2-3)	2-3.2	-
Anterior body length[z]	14.8 ± 0.36 (14.4-15.4)	13.8 ± 0.56 (12.8-14.5)	14 ± 0.35 (13.4-14.4)	13.7 ± 0.34 (13.2-14.1)	12.8-15.4	-
Excretory pore from anterior end	79.3 ± 2.91 (75.3-83.5)	77.9 ± 8.04 (62.9-85)	79.4 ± 2.74 (76.5-84.5)	75.78 ± 4.31 (70.8-82)	62.9-85	-
Hyaline tail length	10.7 ± 1.19 (9.3-13)	11.8 ± 0.89 (10.1-13)	10.9 ± 0.46 (10.5-11.7)	10.5 ± 0.45 (9.8-11)	9.3-13	9-18
Tail length	54.2 ± 3.66 (48.4-58.2)	55.9 ± 1.2 (54.2-57.9)	54.3 ± 3.87 (47.4-59.7)	47.9 ± 2.65 (44.8-52.5)	44.8-59.9	46.8-59.8

[y]Dorsal esophageal gland orifice distance from stylet base.
[z]Distance from head to the base of stylet.

Partial characterization of root-knot nematode species

Further confirmation of results was done by cloning and sequencing of desired amplicon of 955 bp for *M. incognita* obtained using MiF/MiR primer pair and 670 bp for *M. javanica* amplified using Fjav/Rjav primer from different populations. An expected 1-kb band from *M. incognita* and 800 bp band from *M. javanica* positive clones was observed. Six recombinant plasmid clones each for *M. incognita* (Tarn Taran: OP5, Ludhiana: OP1, Ludhiana: OP2, Ferozepur: OP Frz, Patiala: OP3, Sangrur: OPsh3) and *M. javanica* (Tarn Taran: MjTT, Ludhiana: Mj Ldh1and Mj Ldh2, Tarn Taran: Mj TT, Patiala: Mj Pat and Sangrur: Mj Sang) were selected for sequencing. These clones were sequenced, analysed, and submitted to GenBank under accession numbers: KP411871, KP411872, KP411876, KP411873, KP411874, KP411875 for M. incognita and KP411877, KP411878, KP411879, KP411880, KP411881, KP087916 for *M. javanica*, respectively.

Phylogenetic analysis

For the phylogenetic analysis, a total of 29 sequences were aligned, 12 of which were from the current study (Fig. 5). The phylogram obtained after the Maximum Likelihood Analysis grouped the sequences in two major clusters belonging to *M. incognita* and *M. javanica*. *Meloidogyne*

arenaria sequences and *M. graminicola* sequences formed a separate clade. The out-group sequences, (KC529338.1 and JQ405270.1) from the closely-related genus, *Heterodera*, formed a different clade. These results showed that both the *Meloidogyne* species identified in the present study were able to group closely with respective *Meloidogyne* species identified from the other parts of the world.

DISCUSSION

The root-knot nematode is a major limiting factor in successful vegetable production and very limited information is available on the root-knot nematode species infecting different vegetable crops in the Punjab. Accurate identification of root-knot nematodes is important so growers can choose an appropriate management strategy as each root-knot nematode species has specific biological characters and host range. Studies and descriptions of nematode populations are therefore essential for understanding the variability among the populations and to recognize stable characters for their diagnosis (Skantar *et al*., 2008; Hunt and Handoo, 2009). In the present study, we have identified both *M. incognita* and *M. javanica* infecting peppers being grown in Indian Punjab using morphological and molecular tools.

Perineal pattern of females was historically considered as a basic morphological character for distinguishing the *Meloidogyne* species. Female perineal patterns of the root-knot nematode population in this study were typical of *Meloidogyne* species. For example, *M. incognita* population showed high dorsal arch with no visible lateral lines in the perineal pattern whereas, the perineal pattern of *M. javanica* populations showed a high dorsal arch with visible lateral lines. The SCAR marker analysis helped in further confirmation by specifically amplifying the genomic DNA for *M. incognita* and *M. javanica* species.

Morphological variation is common in *Meloidogyne* populations, making species discrimination relatively difficult. In our study, *Meloidogyne incognita* were characterized by slightly larger females, males, and juveniles as compared to the earlier reported descriptions (Chitwood, 1949; Orton Williams, 1972; Jepson, 1987). However, *M. incognita* populations reported by Kaur and Attri in 2013 from Punjab were somewhat larger than ones in the present study. The variation among the measurements might be due to collection of *M. incognita* from different host crops (okra, banana, sunflower, brinjal, and bottle gourd) by Kaur and Attri (2013) and direct observation of the field population. In the present study, nematodes came only from single host, and single egg mass progeny from each population was observed, which could have resulted in comparatively less variation in the morphometric observations of our populations.

SCAR markers used in the present study were highly species-specific and thus can be used for quick diagnosis of *Meloidogyne* species. Phylogenetic analysis of the *Meloidogyne* species identified in the present study were able to group closely with respective *Meloidogyne* species identified from the other parts of the world, which further shows that SCAR markers are able to consistently identify and confirm the identity of various *Meloidogyne* species SCAR-PCR methods have the potential to be used in routine diagnostic applications using DNA extracts from single juveniles, soil samples, or even infected plant materials (Zijlistra, 2000; Randing *et al.*, 2002; Adam *et al.*, 2007). Species identifications based on morphological and morphometric characters require a considerable degree of skill (Hooper *et al*., 2005) and are time consuming. These species-specific markers appear to be reliable for quick and correct detection of *Meloidogyne* species, and should be helpful in planning future root-knot nematode resistance breeding programs and developing nematode management strategies in the Punjab.

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