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Morphological and molecular identification of root-knot nematode (*Meloidogyne graminicola*) on wheat in the Tribal Districts of Madhya Pradesh

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Abstract
A random survey of tribal areas of Madhya Pradesh *viz.*, Anuppur Dindori, Mandla, shahdol and Umaria districts was conducted during 2016-17 to find out the infestation of root knot nematode in wheat and paddy fields. The soil samples were collected from the infested fields and the samples were analysed using Baermann's funnel technique followed by Cobb sieving and decanting methods. The populations of *Meloidogyne graminicola* were morphometric and morphological identified using preparation of perineal pattern and comparisons were made for various nematode life stages of nematode populations were similar to *M. graminicola* on the basis of length and width of egg, juvenile, male and female high line tail portion, distance up to excretory pore EPST (distance of excretory pore from anterior end / stylet length female) ratio and vulval length. In the present investigation, molecular identification was conducted using ITS based techniques applying for four samples collected from *viz.*, Anuppur, Dindori, Shahdol and Umaria. All the four samples produced around 450bp intact band after PCR with ITS1 and ITS4 forward and reverse primer pairs. After sequencing of PCR product, the blast X analysis of sequences depicted 100 percent homology with *M. graminicola* in all four samples.

Keywords: *Meloidogyne graminicola*, root knot nematode, wheat and identification

Introduction
Wheat (*Triticum aestivum* L.) is one of the important cereal crops of the Madhya Pradesh and belongs to family Poaceae which includes major cereal crops such as sorghum, maize, rice, millet and barley [6]. The first cultivation of wheat**,** as per the records**,** was about 10,000 years ago as part of the "Neolithic Revolution" which saw a transmission from hunting and gathering of food to settled agriculture [14, 9]. Wheat is one of the eight food sources which provides 70-90 per cent calories and 66-90 per cent protein. Globally, wheat provides nearly 55 per cent of carbohydrate and 20 per cent calories [4]. Many factors affect yield of wheat but diseases are one of the most serious causes for low yields of irrigated wheat due to severe competition. Since the crop is cultivated in wide range of climatic conditions, *Meloidogyne graminicola* is known to infect and cause serious damage especially in rice and wheat, in many countries [2, 5, 23, 19, 19].
Soil borne pathogens and deficient root-health have been documented as the major constraint on health and productivity of rice-wheat cropping systems. The root-knot nematode (*Meloidogyne graminicola*, Golden and Birchfield, 1965) is widely distributed and is considered as a serious soil borne pathogen reducing the productivity of the rice-wheat system [17].

Material and Methods
Collection of soil and root samples
A random survey of tribal areas of Madhya Pradesh *viz.*, Anuppur, Dindori, Mandla, Shahdol and Umaria districts was conducted during 2016 -17 to find out the infestation of root knot nematode in wheat and paddy fields. The plants showing uneven patches with yellowing of foliage and stunted plant growth, reduced tillering with delayed ear head emergence and galls on the root system were uprooted with the help of spade so as to get the intect root system. The roots were wrapped in a moist paper towel and kept in a polythene bag. An amount of 500 kg soil was also collected from the vicinity of infected wheat plant after descarding top ten soil strata. The soil samples include wheat and weed roots. The samples were brought to the laboratory for further examination and extraction of nematodes.

The information on cropping history, namely type of soil, method of sowing, varieties, date of sowing, seed and soil treatment were collected from the farmers.

Extraction of nematode

The soil samples that were collected from the infested fields were mixed throughly to get homogenious mixture and a sub sample of 200 cm^3 soil was drawn and analysed to ascertain nematode population. The soil samples were analysed using Baermann's funnel technique followed by modified Cobbs Sieving and decanting method (AOAC,1969).

Extraction of nematode population

Baermann's funnel technique followed modified Cobb's Sieving and decanting method (Christie and Perry, 1957) was employed. Egg masses were collected over 400 mesh British Standard (BS) sieve and were further subjected to extraction assembly at room temperature (25°C ± 2). An extraction assembly contained PVC ring of 110 millimeter diameter holding double layered wet tissue paper supported by a guaze cloth and tightly streched with the help of a rubber band. Extraction dish was placed over a piece of sponge measuring 18x15x15 cm for providing firm support from the bottom to the tissue paper and allowing a fast passage of water suspension containing freshly washed egg masses on 400 mesh sieve. The content was poured with the help of a gentle stream of water. Assembly was later kept on a glass bowl (Yera make) holding 60 ml of aquaguard water.

Extraction assembly was placed in such a way that the upper layer of water in glass bowl touches the stretched base of extraction dish to ensure no air bubble. The extraction was carried out at room temperature (25°C ± 2) and the second stage juveniles (J_2) were collected 24 and 48 hr after. The extraction was further continued till 72 to 96 hr and juveniles emerged within 96 hr were used for the inoculation after calibrating the population.

Identification of root-knot nematode

The specific identification of root knot nematode was carried out by following approaches.

1. Morphological and morphometric
2. Molecular methods

Morphological identification

A) Perineal Pattern

Fully developed females of root knot nematode were dissected out from the stained roots and cut into two pieces. The exterior half was trimmed further to retain head end and was mounted in drop of plain lectophenol. The posterior half was used for preparing perineal patterns following the method described by Taylor *et al.*, 1955.

B) Morphometric observations

The freshly hatched second stage juveniles of equal ages were extracted from the egg masses and relaxed by providing gentle leaf and fixed in Formeldehyde Acetic Acid (FAA) solution (2.5%).

The fixed juveniles were processed following the method of TAF fixative and permanent mounts were made as per the method described according to Seinhorst (1959). Morphometric observations were recorded under the research binocular microscope.

Females

Elongate, slite terminal protuberance present. Stylet 12-15 mm long, basal knobs ovoid, offset. Perineal pattern rounded/ oval, striae smooth, lateral field absent.

Males

Labial region not offset, labial disc not elevated. Lateral lips usually present. Stylet 15-20 mm long. Basal knobs ovoid, ofset. DGO = 3-4 mm.

Second Stage Juvenile

L = 410-480 mm, hemizonid anterior or adjacent to excretory pore, tail = 60-80 mm, tail tip finally rounded (Perry *et al*, 2010).

Dorso-ventral, oval to almost circular in shape, moderate in height of arch, no lateral incisures or gaps, tail tip marked with prominent, coarse, fairly well separated striae that sometimes form an irregular tail whorl [20].

Molecular identification

Source of Biological Material

The plants of wheat infested with root-knot nematode, (*Meloidogyne* spp.) were collected from Anuppur, Dindori, Mandla, Shahdol and Umaria districts of Madhya Pradesh during roving survey to obtain the infested root samples for isolation of nematode genomic DNA.

Chemicals

Chemicals used for DNA extraction and PCR were obtained from Sigma Aldrich Co. USA. 1 kb ladder was used from Fermentas Pvt. Ltd. India.

Molecular identification

DNA was amplified by using universal primers: ITS1 as a forward primer and ITS4 as a reverse primer targeting the ITS region.

ITS1	F	5'-TTGATTACGTCCCTGCCCTTT -3'
ITS4	R	5'-ACGAGCCCGAGTGATCCACCG-3'

Methods

DNA isolation

Genomic DNA was isolated using CTAB method with some modifications (Doyle and Doyle, 1990). The composition of DNA isolation buffer is described below. The method described below gave a good quality and quantity of DNA.

Reagents for isolation of genomic DNA:

- Extraction Buffer (100mM Tris.Cl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl,
- 10 mM 2-Mercaptoethanol)
- 20 per cent SDS
- 5M Potassium acetate, 3M Sodium acetate, pH 5.2
- TE (Tris 10 mM, EDTA 1 mM, pH 8.0)
- Phenol, Tris saturated, Bangalore Genei
- Chloroform, AR, Thomas Baker
- Isopropanol, AR, Chemcofine
- Ethanol, AR, Hayman

Procedure [24].

1. Nematode infected two gm root samples were crushed with liquid nitrogen to form fine powder.
2. The fine powder was immediately transferred into cryo-

vials and stored at - 80°C for long term storage and for maintaining genetic stability.

3. About 200 mg finely powder was added to 500 µl extraction buffer. The micro centrifuge tubes were left open for some time till the powder thawed; mixed by tapping and then by vigorous vortexing.
4. 35 µl of 20 per cent SDS was added, mixed thoroughly with gentle tapping and by inversion.
5. Tubes were incubated at 65^0C on a heat block for 10 minutes.
6. 170 µl of five M potassium acetate was added to the tube and mixed by inversion. The tubes were incubated on ice for 20 minutes.
7. 500 µl chloroform was added, mixed by inversion and centrifuged at 14000 rpm for 10 minutes at room temperature
8. The supernatant was transferred to a fresh tube. 500 µl isopropanol was added, mixed gently by inversion and centrifuged at 10000 rpm for 2 minutes.
9. The supernatant was discarded and the pellet was washed with one ml 70 per cent ethanol. Tubes were again centrifuged at 10000 rpm for one min at room temperature.
10. The pellet was air-dried in the laminar flow and dissolved in 250 µl TE, pH 8.0 by incubating for ten minutes at 37^0C followed by five minutes at 65^0C. The contents were mixed by gently tapping the tube.
11. The contents of two such tubes were pooled together in a fresh tube to make the final volume 500 µl and incubated with five µl RNase A (10 mg/ml, Roche) at 37^0C for 30 minutes, to remove RNA.
12. 500 µl phenol: chloroform (1:1) (Tris saturated, pH 8.0, Bangalore Genei) was added to the tube, mixed by tapping and centrifuged at 14000 rpm for ten minutes at room temperature.
13. Top aqueous layer was transferred to a fresh microcentrifuge tube. 500µl chloroform was added, mixed by tapping and centrifuged at 14000 rpm for ten minutes at room temperature.
14. Top aqueous layer was transferred to a fresh tube and then 1/10th volume (50 µl) of three M sodium acetate (pH 5.2) was added. Tube mixed by tapping followed by adding two volumes (1 ml) of 100% ethanol.
15. The contents were mixed by inversion and centrifuged at 10000 rpm for 10 minutes at room temperature.
16. The supernatant was discarded and the pellet was washed with one ml 70% ethanol.
17. The pellet was dried and dissolved in 100 µl TE (pH 8.0) by incubating for ten minutes at 37^0C followed by five minutes at 65^0C.

Agarose gel electrophoresis

Reagents for gel electrophoresis

- Agarose, SRL
- TAE (Tris-acetate-EDTA) - 1X from 50X stock (1 liter TAE 50X stock solution
- Tris Base (SRL)- 242 g, Glacial acetic acid- 57.1 ml, 0.5M EDTA (pH 8.0)-100 ml)
- Loading dye, 6X: Bromophenol blue in 50% glycerol, or ready-made Fermentas 6X loading dye)
- Ethidium bromide (HIMEDIA), 10 mg/ ml stock
- Ladders: Lambda DNA *Hind* III / *Eco* RI O-Gene Ruler Ladder (Fermentas).

Procedure

Agarose gel was prepared by dissolving 1% (w/v) agarose (Bangalore Genei) in 1x TAE [24] in a microwave oven. For visualization of DNA 1 µl of ethidium bromide (10 mg/ml) per 50 ml gel (cooled to about 40^0C) was added prior to pouring. Samples were mixed with 1/5 th volume of 6X loading dye (Fermentas) before loading. Genomic DNA was run in 1x TAE buffer at 80 volts and visualized on a gel doc (Syngene).

DNA quantification

Isolated DNA was quantified according following formula:

Concentration of DNA = OD_{260} x 50µg/ml x Dilution fact

Dilution of DNA

The quantified DNA was diluted according to the DNA quantity in each sample for PCR amplification in sterile double distilled water. Dilutions were carried out according to the following formula (Edward, 2000).

$$\text{Dilution} = \frac{\text{Required concentration of DNA (ng/µl) X Total volume required (µl)}}{\text{Available concentration of DNA (ng/µl)}}$$

Polymerase Chain Reaction (PCR)

The polymerase chain reactions (PCR) were carried out using the extracted root-knot nematode DNA as the template. The master mix for 30 µl reactions was prepared using the following ingredients:

Sterilized distilled water (17.8 µl),	10X reaction buffer (3.0 µl),
MgCl2 50mM (3.0 µl),	dNTPs 2mM (3.0 µl),
Forward Primer (1.0 µl),	Reverse Primer (1.0 µl),
Taq polymerase (0.2 µl) and	DNA (1.0 µl).

The reaction mixture was prepared for 4 samples and the DNA was amplified for ITS region using following programme. The samples were coded as MG1, MG2, MG3 and MG4 respectively for Anuppur, Dindori, Shahdol and Umaria locations sample. 5 ml of amplified PCR Product was visualized on 1.5 per cent Agarose gel and remaining amount of each sample was sent for sequencing.

PCR programme

94 °C for 2 min

94 °C for 30 sec
55 °C for 30 sec
72 °C for 30 sec
} X 34 Cycles

72 °C for 7 min

4 °C Hold

Sequencing

Amplified PCR product were sent to SciGenome Labs Private Ltd. Plot no: 43A, SDF, 3rd floor A Block, CSEZ, Kakkanad Cochin, Kerala-682037, INDIA for sequencing using with ITS1& ITS4 primer pairs.

Sequence analysis

The sequence information obtained from SciGenome Labs Private Ltd. Plot no: 43A, SDF, 3rd floor A Block, CSEZ, Kakkanad Cochin, Kerala-682037, INDIA was further analysed using BLAST (Basic Local Allignment Search Tool) on www.ncbi.nlm.nih.gov and nucliotide blast was performed for sequence homology & molecular identification.

Results and discussion

Morphometric identification of *Meloidogyne graminicola*

The length and width of different stages of *Meloidogyne graminicola* were measured using Inverted microscope. The experiment was conducted under pot conditions and the data on morphometric observations are presented in Table 9. It was observed from the data that length ranged from 80 to 98 µm and width of egg ranged from 41 to 46 µm. The juvenile was also measured and it was observed that length of juveniles ranged from 374 to 393 µm and width ranged from 15 to 21 µm. The male of *Meloidogyne graminicola* ranged from 924 to 1220 µm and 26 to 36 µm in width. The length of female of *M. graminicola* ranged from 429 to 522 and width ranged from 253 to 287 µm.

Female

Body pearly white, globular to pear shaped with relatively small neck situated anteriorly on mediun plane with vulva. Body cuticle annotated often with fine irregular punctations. Stylet small and delicate with rounded knobs sloping posterierly. Esophagus well developed and three in number, obscure (Plate 3 fig A).

Perineal pattern

Perineal pattern is prominent and dorso-ventral oval to almost circular in shape with distinct striations moderate in height of arch, no lateral incisures or gaps, tail tip marked with prominent, coarse fairly well separated striae formally on irregular tail whorl (Plate 3 fig B).

Table 1: Morphometric identifications of *Meloidogyne graminicola*

Stage	Length (µm)	Width ((µm)
Egg	80-98	41-46
Juvenile	314-393	15-21
Male	924-1220	26-38
Female	429-522	253-287

Molecular identification of *Meloidogyne graminicola*

The sequence information obtained from SciGenome Labs Private Ltd. Cochin, Kerala, INDIA was further analysed on NCBI (www.ncbi.nlm.nih.gov). In total four samples from Anuppur (MG1), Dindori (MG2), Shahdol (MG3) and Umaria (MG4) were sent for sequencing. The raw sequences were trimmed for poor quality peaks and trimmed good quality sequences were further used for BLASTX analysis. The first and second sample comprised respectively 317 bp and 366 bp good quality sequence length. However, third and fourth sample comprised respectively 368 bp and 364 bp good quality sequences after trimming. After BLASTX analysis, all the four samples showed 100 per cent similarity with the *M. graminicola* and confirmed the molecular identity of all the four samples as *M. graminicola*. The sequence information and of all the four samples has been presented in Plate 3 and Fig C The BLASTX analysis of all four samples has been depicted in Fig. 4,5,6,7 and 8.

On the basis of morphology and morphometric observation (Table1) and on the basis of perineal pattern the nematode is diagnosed as *Meloidogyne graminicola* [19].

Species determination of *M. graminicola* is complex and usually based on the symptoms (hook-like galls production). The applications of molecular methods have been used with success to identify the species in particular sequences of nuclear ribosomal (rDNA) and mitochondrial DNA (mtDNA) have been largely used [19, 13, 11]. Recently, two molecular methods were developed one used a diagnostic SCAR marker [3] for rapid and reproducible identification of *M. graminicola* and the second one used real-time PCR primers for the quantification of this nematode in soil [12]. In the present investigation, ITS based identification was conducted for four samples. All the four samples produced around 450bp intact band after PCR with ITS1 and ITS4 forward and reverse primer pairs. After sequencing of PCR product, the blast X analysis of sequences depicted 100 percent homology with *M. graminicola* in all the four samples.

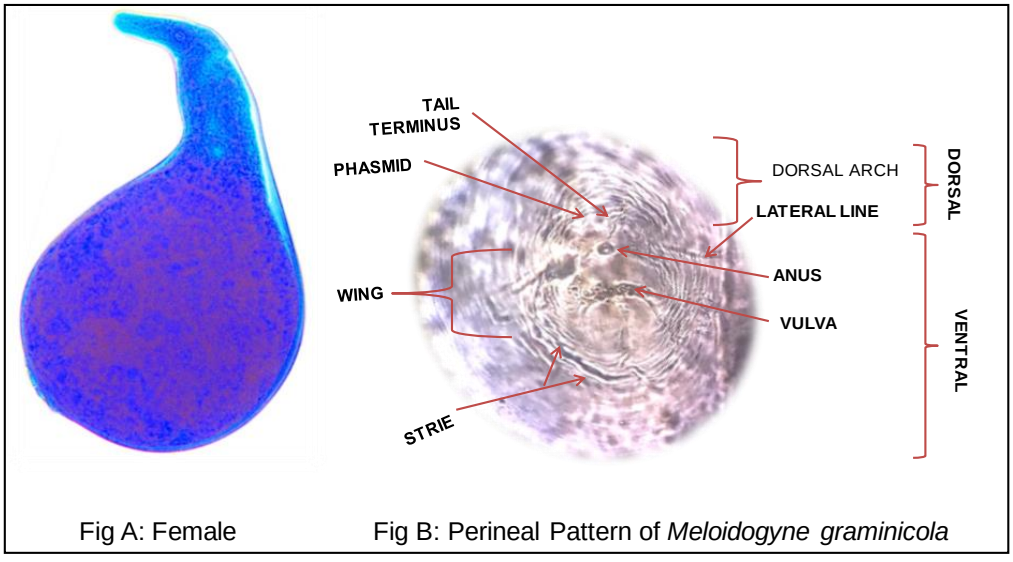

Fig A: Female Fig B: Perineal Pattern of *Meloidogyne graminicola*

Plate 1: Morphological identification of *M. graminicola.*

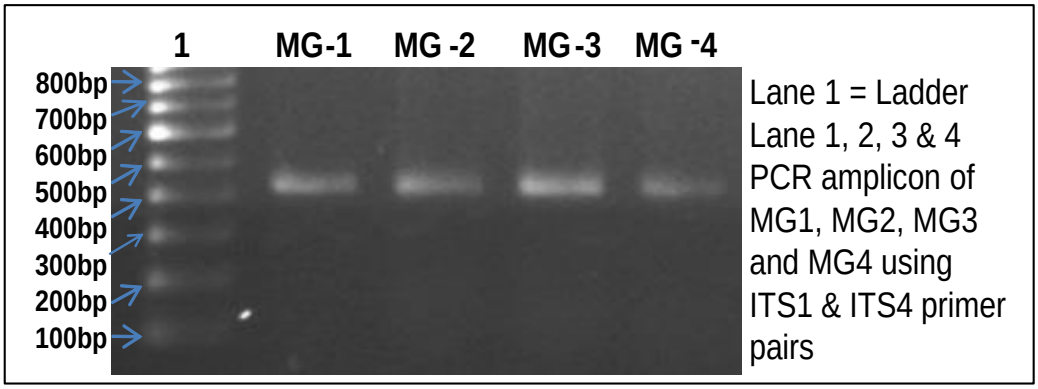

Fig 1: PCR amplification using ITS1 & ITS4 primer pair and genomic DNA as template provided = 450 bp size amplicon was obtained. The amplified PCR product was sent for sequencing and sequenced using the same set of primer pairs. The amplified PCR product of all the 4 samples has been depicted in plate

>MG-1 (317 bp)
TTGGGGACCGTTGATTAAACATTTATTTGTTTTTTTGATGGA
AACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCG
TAACAAGGTAGCTGTAGGTGAACCTGCTGCTGGATCATTAC
TTTTTATGTAATGCTTTACATTTGAATTTATCGCATCATTTCAT
TTGATGTGTAACGGCTCTCACTGGTGTCTAGGTGTTGCTGA
TTCAGCTGTCTTCGTCCGTGGCTGAATATGAGGTGACATGT
TAGGATTCTATTGAATCGTAAGACTTAATGAGCCTCTTAAGT
GAGGACGCCAGCAATTTTTTTTTTCT

>MG-2 (366 bp)
TGGGGACCGTTGATTAAACATTTATTTGTTTTTTTGATGGAA
ACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGT
AACAAGGTAGCTGTAGGTGAACCTGCTGCTGGATCATTACT
TTTTATGTAATGCTTTACATTTGAATTTATCGCATCATTTTATT
TGATGTGTAACGGCTCTCACTGGTGTCTAGGTGTTGCTGAT
TCAGCTGTCTTCGTCCGTGGCTGAATATGAGGTGACATGTT
AGGATTCTATTGAATCGTAAGACTTAATGAGCCTCTTAAGTG
AGGACGCCAGCAATTTTTTTTTTCAATAAATTTTTTTTTAAAA
AACATTAATAAAAATTAACCTTACCGGTGGAT

Fig 2: Nucleotide sequencing of MG1 and MG2.

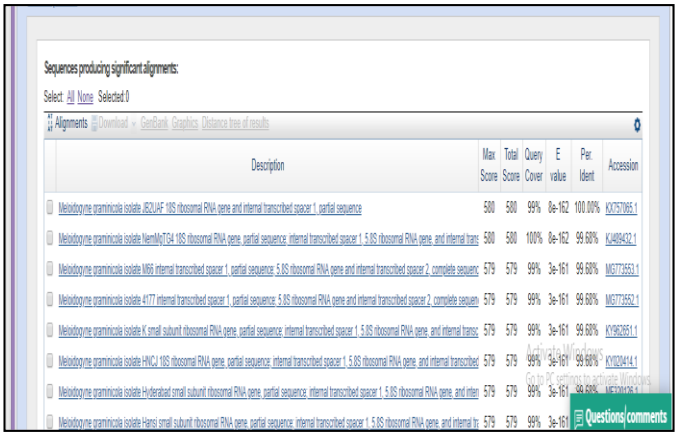

Fig 3: BLAST X analysis of nucleotide sequence of sample MG-1

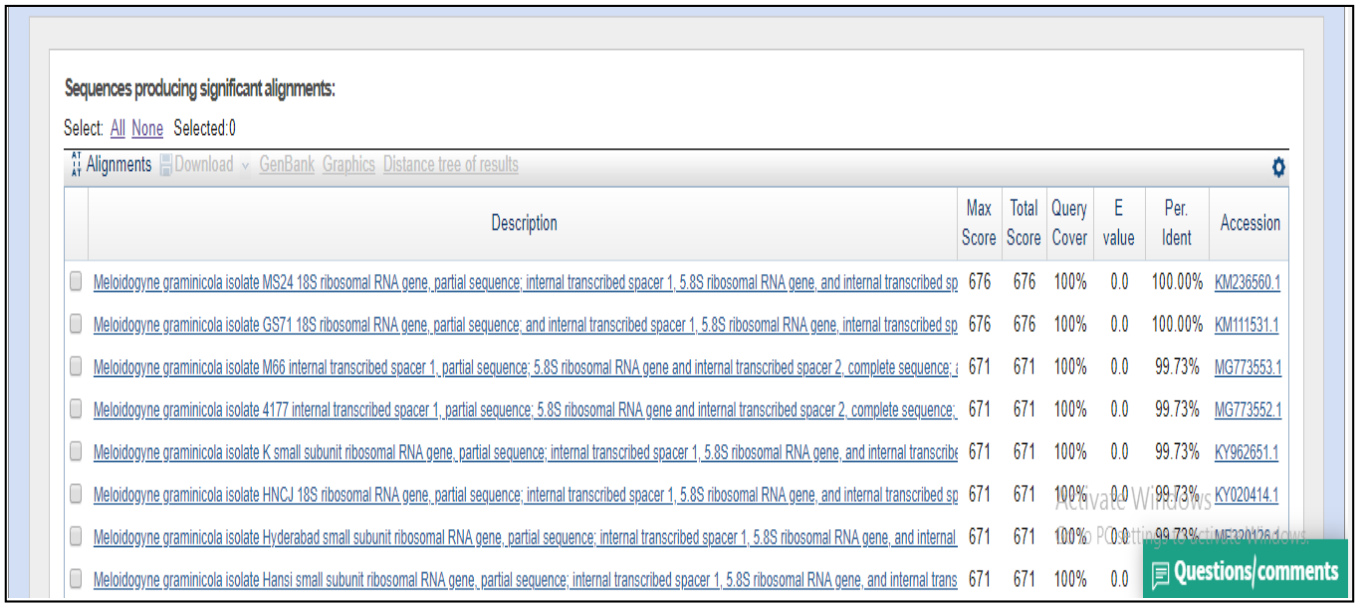

Fig 4: BLAST X analysis of nucleotide sequence of sample MG-2

>>MG-3 (368 bp)
TGGGGACCGTTGATTAAACATTTATTTGTTTTTTTGATGGAAA
CCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTAA
CAAGGTAGCTGTAGGTGAACCTGCTGCTGGATCATTACTTT
TTATGTAATGCTTTACATTTGAATTTATCGCATCATTTTATTTG
ATGTGTAACGGCTCTCACTGGTGTCTAGGTGTTGCTGATTC
AGCTGTCTTCGTCCGTGGCTGAATATGAGGTGACATGTTAG
GATTCTATTGAATCGTAAGACTTAATGAGCCTCTTAAGTGAG
GACGCCAGCAATTTTTTTTTTCAATAAATTTTTTTTAAAAGA
CATTAATAAAAATTAACCTTACCGGTGGATCA

>MG-4 (364 bp)
GGGGACCGTTGATTAAACATTTATTTGTTTTTTTGATGGAAA
CCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTA
ACAAGGTAGCTGTAGGTGAACCTGCTGCTGGATCATTACTT
TTTATGTAATGCTTTACATTTGAATTTATCGCATCATTTTATTT
GATGTGTAACGGCTCTCACTGGTGTCTAGGTGTTGCTGATT
CAGCTGTCTTCGTCCGTGGCTGAATATGAGGTGACATGTTA
GGATTCTATTGAATCGTAAGACTTAATGAGCCTCTTAAGTGA
GGACGCCAGCAATTTTTTTTTTCAATAAATTTTTTTTAAAAG
ACATTAATAAAAATTAACCTTACCGGTGGA

Fig 5: Nucleotide sequencing of MG3 and MG4.

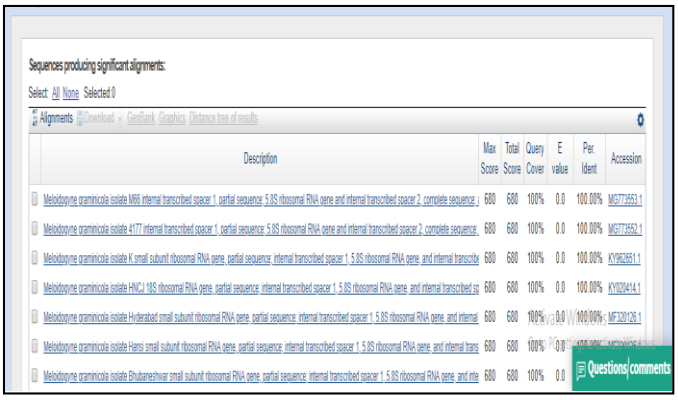

Fig 6: BLAST X analysis of nucleotide sequence of sample MG-3.

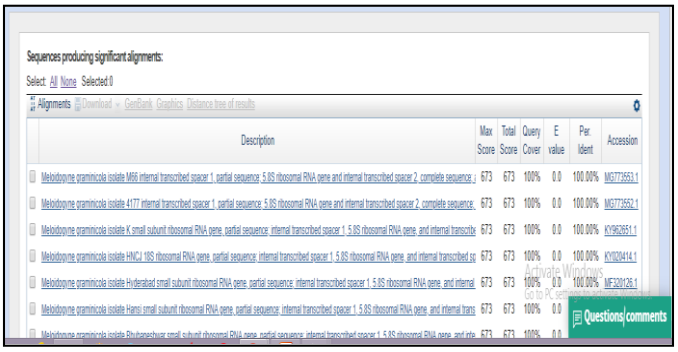

Fig 7: - BLAST X analysis of nucleotide sequence of sample MG-4.

Conclusion

In this investigation root knot nematode (*M. graminicola*) of wheat was identified morphological and morphometric using preparation of perineal pattern and ITS sequenced based techniques were employing for molecular identification and after blast analysis 100 percent homology with *M. graminicola* in all four samples.

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