# Detection of *Meloidogyne graminicola* (RRKN) in Kumaon region of the Indian Himalayas

ASHISH KUMAR SINGH<sup>1\*</sup>, AMIT U PASCHAPUR<sup>1</sup>, AMAR KANT KUSHWAHA<sup>2</sup>, K K MISHRA<sup>1</sup> and LAKSHMI KANT<sup>1</sup>

ICAR-Vivekanada Parvatiya Krishi Anusandhan Sansthan, Almora, Uttarakhand 263 601, India

Received: 2 May 2023; Accepted: 06 July 2023

#### ABSTRACT

Rice root-knot nematode (RRKN), *Meloidogyne graminicola* is a serious pest of rice (*Oryza sativa* L.) reported globally. In India, RRKN has been reported from various parts of the rice growing region and causing 17–30% yield loss. In the present study, RRKN population infesting rice growing region of Kumaon hills of Uttarakhand, Indian Himalayan region was isolated during 2019–20, characterized using integrative taxonomy by combining molecular analysis using ITS, morphology of male, juveniles, adult female and morphometry measurements of different stages. The data presented in the comparative analysis of morphometry parameters for *Meloidogyne graminicola* isolate from the Kumaon region of the Indian Himalayas, as compared to the original description by Golden and Birchfield (1965) from Louisiana, USA, reveals some differences in the morphological characteristics of adult females, adult males, and 2nd stage juveniles (J2) of *M. graminicola*. The ITS sequence from the present study appeared as a single clade between the Fujian isolate (KM111531.1) and the Brazilian *M. graminicola* isolate (KY962651). To our knowledge, this is the first authentic characterization and report of RRKN from the Hilly region of Uttarakhand state. The detection of *M. graminicola* in the mid Indian Himalayan region provides valuable information for further studies on the natural population dynamics and distribution patterns of this nematode species. The findings highlight the need for regular monitoring and surveillance of nematode populations in the region to better understand their distribution and potential impact on rice production.

Keywords: Distribution patterns, Kumaon hills, Meloidogyne graminicola, Rice, Surveillance

Rice (Oryza sativa L.), a vital global staple crop, faces significant yield loss due to nematode infestation, particularly from the devastating Meloidogyne graminicola (RRKN) (Fanelli et al. 2017). RRKN causes economic damage in upland (20-80%) and lowland rice (16-20%) as well as wheat crops in the rice-wheat cropping system (Jain et al. 2012). The increasing spread of M. graminicola poses a threat to rice cultivation, exacerbated by climate change (Pokharel et al. 2010). Due to its damaging potential, M. graminicola is considered a quarantine pest in various countries. Kumar et al. (2020) reported economic losses of up to ₹23,272.32 million in India attributed to M. graminicola. It is an obligate sedentary endoparasite, forming knots at the root apex (Jones et al. 2013). Its life cycle, completing in 14–15 days under favourable conditions (23-30°C), allows for significant multiplication within a crop season (Jaiswal et al. 2011). The damage potential and increasing spread of M. graminicola causing rice cultivation

<sup>1</sup>ICAR-Vivekanada Parvatiya Krishi Anusandhan Sansthan, Almora, Uttarakhand; <sup>2</sup>ICAR-Central Institute for Subtropical Horticulture, Lucknow, Uttar Pradesh. \*Corresponding author email: ashish.singh1@icar.gov.in

under threat. Influence of climate change, anthropogenic activity has seriously impacted agro-ecosystem, biodiversity and creation of new crop niche for nematode like pathogen to thrive and reproduce exceptionally to the status of pest.

Detection of M. graminicola involves an integrative taxonomy approach, which includes multiple steps (Fanelli et al. 2017). The initial confirmation step is based on the observation of the hoke shape terminal gall (Fanelli et al. 2017). Subsequently, the dissection of galled roots reveals the presence of female, eggs and male stages, which are traditionally characterized using morphological features (Tian et al. 2018). DNA-based methods are employed to supplement the morphology-based detection and confirm the identity of M. graminicola (Singh et al. 2019). Additionally, the use of ribosomal DNA (rDNA) aids in understanding the phylogenetic relationship between different populations (Bogale et al. 2020). The research article focuses on investigating the presence of M. graminicola in rice fields of the Kumaon region in the Indian Himalayas. This study aims to morphologically characterize M. graminicola, conduct comparative morphometric analysis and employ molecular techniques to identify the nematode and determine its phylogenetic relationship with other populations.



Fig 1 Symptoms of rice root-knot in rice plants at the field of Hawalbagh.

## MATERIALS AND METHODS

Sample collection and extraction of nematode: A random survey of rice planting sites ICAR-VPKAS Experimental Farm, Hawalbagh, located in Kumaon region, Uttarakhand Indian mid Himalayan region was conducted in the July, 2019–20. The site was regularly being planted with rice crop by following standard recommended practices. Rice plant (cultivar: VL Dhan 86) showing yellowing and stunting symptoms were uprooted and adhering soil sample were collected. The infestation has been confirmed based on the above ground symptom as yellowing and stunting of foliar parts and typical hoke shape gall on terminal part of the root (Fig 1) which most conspicuous to ensure M. graminicola infestation. The geo-coordinates of infested site were recorded using GPS data smart android Examobile application. The roots showing hoke shaped terminal swelling were collected in polythene bag and carried to lab. For identification purpose, J3, J4, female and male stages were directly collected by dissecting galled roots with needle and forceps. Egg masses collected from dissection of galled root and placed over modified Baermann funnel to collect juveniles.

Morphological characterization: The harvested eggs were incubated over wire mess wrapped thin tissue paper layered over petridish. The eggs were allowed to hatch in growth chamber 25±2°C for 3 days. The hatched juveniles were fixed in TAF (triethanolamine 2 ml, formalin 7 ml and water 91 ml) in a vial. The fixed juveniles of M. graminicola were processed into Seinhorst I for 12 h (20 parts of 95%) ethanol, 1 part of glycerin, and 79 parts of distilled water) and Seinhorst II solutions (95 parts of 95% ethanol and 5 parts of glycerin) for 4 h to clear the internal body organs and better visualization under the light microscope. The individual specimen was mounted on separate glass slides  $(75 \text{ mm} \times 25 \text{ mm} \times 1.4 \text{ mm})$  and labeled with ink proof marker. Nematode specimen mounted glass slides were visualized with Olympus laboratory light microscope. Morphometric measurement was recorded using Olympus light microscope equipped with measurement software

application. Morphometric variable measured for male, juvenile, (including body length, greatest body width, dorsal esophageal gland orifice (DEGO), stylet length (SL), tail length (TL), spicule length (SP), 'a' (total body length/greatest body width) 'c' (total body length/tail length)), female and eggs (length, width) were considered. The female perennial pattern was prepared following procedure of Hartman and Sasser (1985).

Molecular characterization and phylogenetic study: For molecular diagnostic, total genomic DNA were extracted using Holterman lysis buffer (0.2M NaCl, Tris base 0.2M, 10 µl Beta-mercaptoethanol and proteinase K) from juvenile. For DNA sample preparation, 4-6 juveniles were placed in 25 µl sterile water and 25 µl of lysis buffer were added. Specimen was crushed with sterile micropestle in a 1.5 ml Eppendorf tube. The resulting lysates incubated at 56°C in a hot water bath for 3 h and at 95°C for 5 min with repeated tapping of tube and centrifugation at 15 min interval. The isolated lysates were stored at -20°C till further use. DNA concentration quantified at 260/280 in Thermo Scientific NanoDrop. PCR performed for amplification of extracted sample DNA with universal r-DNA ITS primer set V5367/26S (5'-TTTCACTCGCCGTTACTAAGG-3'; 5'-TTGATTACGTCCCTGCCCTTT-3') (Vrain et al. 1992) using PCR in a thermocycler programme of 94°C for 5 min, followed by 35 cycles of 92°C for 1 min, 58°C for 30 s and 72°C for 1 min, followed by the final extension of 72°C for 10 min. In PCR components of 50 µl reactions (25 µl Himedia *Taq* polymerase master mix, 1 µl each primer, 8 μl template DNA, 15 μl PCR grade water) were prepared and amplified in thermocycler programme with the set of 35 cycle. The amplified products were separated and visualized using 5 µl of PCR products under 1.5% agarose gel electrophoresis in TAE buffer stained with EtBr. The obtained result compared with gene ruler marker of (100 bp) showed nearly 700 bp PCR product (Fig 2).

Sequencing and reconstruction of phylogenetic tree: The PCR product were purified and sequenced using Sanger platform. The obtained reads imported in BLASTn showed significant similarity to Meloidogyne graminicola. The sequence submitted to NCBI accession number OP550212. For the phylogenetic tree, Pratylenchus penetrans (accession: FJ799117) were chosen as outgroup. Multiple sequence alignment and phylogenetic tree of 21 M. graminicola populations were constructed in MEGA 10 software (Kumar et al. 2018). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura et al. 1993) with bootstrap consensus of 1000 replicates (Felsenstein et al. 1985).

## RESULTS AND DISCUSSION

In present study, Rice root-knot nematode, *M. graminicola* is being reported for the first time from Kumaon region, Uttarakhand of Indian Himalayan region. Earlier this nematode has been reported from other states of the countries including Karnataka (Prasad *et al.* 2001) West Bengal, Orissa, Assam, UP (Khan and Ahamad 2020) and



Fig 2 Morphological observations of 2nd and 3rd stage juvenile, and 4th stage, female and perennial pattern.

Himachal Pradesh (Jain *et al.* 2012). In the brief survey of rice nursery field grown at Hawalbagh farm we have found presence of RRKN in the rice field. Rice plants at the field of Hawalbagh showed characteristic symptoms of typical root-knot nematode infection caused by *M. graminicola*. Infected plants exhibited stunted growth, reduced tillering, and yellowing of leaves. Galls, which are characteristically swelling or knots on roots, were observed at the terminal ends of the roots. Similar symptoms-based diagnosis has been confirmed by Jain *et al.* (2012) and Dutta *et al.* (2012). Dissection of root showed presence of male, female and egg masses embeded in the root tissues. The light microscopic photographs of different life stages have been given in

Fig 1. The isolated females were typical pear shaped, perennial pattern was with round dorsal arches, oval and fine striation matching with the original description of *M. graminicola* (Golden and Birchfield 1965) (Fig 1). The recorded morphometric measurement of different life stages has been given in the Table 1 along with other Indian isolates.

The morphological and morphometric observation of isolated *M. graminicola* were in correspondence to original description of *M. graminicola* (Fig 2). The data presented in the comparative analysis of morphometry parameters for *M. graminicola* isolate from the Kumaon region of the Indian Himalayas, as compared to the original description by Golden and Birchfield in 1965 from Louisiana, USA,

Table 1 Comparative analysis of morphometry data among adult female, adult male, 2<sup>nd</sup> stage juvenile of *M. graminicola* isolate of present study and original description

Morphometric parameter*	Original description of Golden and Birchfield (1965)	Present study
Reported place	Louisiana USA	Hawalbagh, Almora
Host plant	Echinocloa crusgalli	Oryza sativa L.
Adult female		
Body length (L)	573 (445–765)	619.67 (492.6–812)
Body width	419 (275–520)	367.7 (281.7–482.9)
a	1.37 (1.2–1.8)	1.684 (1.107–2.559)
Stylet length	11.08 (10.60–11.20)	10.04 (8.5–12.1)
Adult male		
Body length (L)	1222 (1020–1428)	1369.7(1210.8–1532.5)
Body width	29.8 (24–34.7)	26.6 (21.8–31.2)
a	NA	51.44 (41.361–66.191)
Stylet length	16.8 (16.2–17.3)	17.08 (15.5–18.5)
Tail length	11.1 (6.1–15.1)	9.18 (8.2–10.1)
Spicule length	28.1 (27.4–29.1)	20.95 (20.4–21.5)
Second stage juvenile (J2)		
Body length (L)	441 (415–484)	484.42 (460.5–505.8)
Body width	NA	15.94 (13.5–19)
a	24.8 (27.3–22.3)	30.39 (25.99–35.68)
Stylet length	11.38 (11.20–12.32)	11.82 (10.6–13.1)
Tail length	70.9 (67.0–76.0)	19.65 (16.7–22.5)

<sup>\*</sup>All the measurements represented are in  $\mu m$  in pattern of means (range), n refers to number of specimens examined, a refers to body length/body width.

reveals some differences in the morphological characteristics of adult females, adult males, and 2nd stage juveniles (J2) of M. graminicola. Adult females from the present study showed slightly larger body length (619.67 µm) compared to the original description (573 µm), with a less wide body width (367.7 μm) compared to the original description (419 μm). The 'a' value, representing the shape of the female body, was also slightly higher (1.684) in the present study compared to the original description (1.37). However, the stylet length of adult females in the present study (10.04 μm) was slightly shorter compared to the original description (11.08  $\mu$ m). For adult males, the body length (1369.7  $\mu$ m) and body width (26.6 µm) in the present study were larger compared to the original description (1222 µm and 29.8 μm, respectively). The 'a' value, in the male body, was not reported in the original description, but in the present study it was found to be 51.44, indicating a different body shape in the isolates from the Kumaon region compared to the Louisiana isolates. The stylet length of adult males in the present study (17.08 µm) was slightly longer compared to the original description (16.8 µm), while the tail length (9.18 µm) and spicule length (20.95 µm) were shorter compared to the original description (11.1 µm and 28.1 μm, respectively). For 2nd stage juveniles (J2), the body length (484.42 µm) in the present study was slightly longer compared to the original description (441 µm), while the body width (15.94 µm) was narrower compared to the original description (not reported). The 'a' value, of the J2 body, was higher (30.39) in the present study compared to

the original description (24.8). The stylet length (11.82  $\mu$ m) of J2 in the present study was slightly longer compared to the original description (11.38  $\mu$ m), while the tail length (19.65  $\mu$ m) was significantly shorter compared to the original description (70.9  $\mu$ m).

These differences may indicate some level of variation in the *M. graminicola* population in the Kumaon region, which could be attributed to geographical and environmental factors, as well as host plant differences (*Oryza sativa* L.) in the present study compared to *Echinocloa crusgalli* in the original description). Hence, molecular analyses conducted to confirm the identity and genetic diversity of *M. graminicola*.

The sequencing of ITS region resulted 766 bp nucleotide length and deposited to GenBank under accession number OP550212. The BLAST result of amplified ITS sequence showed 93.53% sequence similarity to GenBank accession KM236560.1, M. graminicola isolate and other similar sequence of NCBI. Phylogenetic tree based on ITS (Fig 3) were reconstructed using 21 sequences of M. graminicola with Pratylencus penetrans as outgroup (FJ99117) (Fig 2). M. graminicola population under this study formed supporting group with other M. graminicola isolates. The ITS sequence of present study appeared as single clade between Fujian isolate (KM111531.1) (Zhou et al. 2015) and Brazilian M. graminicola isolate (KY962651) (Da Silva et al. 2017). Our sequence does not cluster with any of Indian population suggesting intraspecific variation among M. graminicola population from Indian isolates. Similar

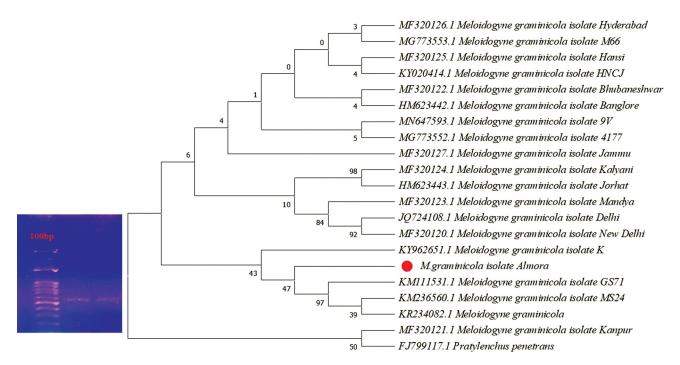


Fig 3 Agarose gel electrophoresis of PCR amplified product of *M. graminicola* isolated DNA. rDNA ITS region was amplified with universal primer.

Maximum likelihood phylogenetic tree showing relationship between *M. graminicola* isolate Almora and other isolates. The phylogenetic tree reconstructed based on rDNA ITS region. Number on nodes indicates the bootstrap value. The red label indicates the *M. graminicola* isolate Almora of present study.

result of intraspecific variation among Chinese populations of *M. graminicola* have been reported by Tian *et al.* (2018). Phylogenetic relationship study indicates the independent origin of Almora isolate and does not form groups with the other Indian isolates. However, there are chances of spread from place of occurrence to new place through the infected transplanting material, soil movement and irrigation (Torrini *et al.* 2020, Gaur 2023). Agarose gel electrophoresis of the PCR amplified product of *M. graminicola* isolated DNA from Almora showed a clear band on the agarose gel (Fig 3), indicating successful amplification of the rDNA ITS region using universal primers.

The occurrence of Meloidogyne graminicola in the hilly region of Uttarakhand, India, as reported in this study, represents the first documented record of this nematode species in the region. The findings highlight the need for regular monitoring and surveillance of nematode populations in the region to better understand their distribution and potential impact on rice production. The geographical spatiotemporal distribution of M. graminicola in the Indian Himalayan region is of significant interest. The phylogenetic analysis of the detected populations showed that they did not match with any known Indian populations, indicating that the occurrence of M. graminicola in this region may be a result of natural dispersal or introduction from other regions. Although the population of M. graminicola in the hilly region of Uttarakhand was found to be low, it is important to consider the potential factors that could influence their distribution. Altitudinal variation, temperature effects, crop cycle, rainfall patterns, and dispersal through planting material are some of the factors that may affect the population dynamics of M. graminicola in this region. The detection of M. graminicola in the mid Indian Himalayan region provides valuable information for further studies on the natural population dynamics and distribution patterns of this nematode species. Under the influence of climate change the information about distribution of nematode pest in hilly region expands our undemanding and update in the occurrence of nematode species. The molecular data along with phylogenetic study will serve as comparator for identification of other M. graminicola population.

#### REFERENCES

- Bogale M, Baniya A and DiGennaro P. 2020. Nematode identification techniques and recent advances. *Plants* 9(10): 1260
- Da Silva Mattos V, Cares J, Gomes C, Almeida M R, Gomes A C, Monteiro J and Carneiro R. 2017. First report of, and additional information on, *Meloidogyne oryzae* (Nematoda: Meloidogyniae) parasitizing rice crop in South of Brazil. NCBI KY962651.1
- Dutta T K, Ganguly A K and Gaur H S. 2012. Global status of rice root-knot nematode, *Meloidogyne graminicola*. *African Journal of Microbiology Research* **6**(31): 6016–21.
- Fanelli E, Cotroneo A, Carisio L, Troccoli A, Grosso S, Boero C and De Luca F. 2017. Detection and molecular characterization of the rice root-knot nematode *Meloidogyne graminicola* in Italy. *European Journal of Plant Pathology* **149**(2): 467–76.

- Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783–91.
- Gaur H S. 2023. Root-knot nematode disease: an expanding problem in rice-based cropping systems. *Integrated Pest Management in Diverse Cropping Systems*, pp. 183–208. Apple Academic Press.
- Golden A M and Birchfield W. 1965. *Meloidogyne graminicola* (Heterodera), a new species of root knot nematode from grass. *Proceedings of the Helminthological Society of Washington* **32**: 228–31.
- Hartman K M and Sasser J N. 1985. Identification of *Meloidogyne* species on the basis of differential host test and perineal pattern morphology. *An advanced treatise on Meloidogyne* 2: 69–77.
- Jain R K, Khan M R and Kumar V. 2012. Rice root-knot nematode (*Meloidogyne graminicola*) infestation in rice. *Archives of Phytopathology and Plant Protection* **45**(6): 635–45.
- Jaiswal R K, Singh K P and Srivastava A N. 2011. Pathogenic effect of *Meloidogyne graminicola* on growth of rice seedlings and susceptibility of cultivars. *Annals of Plant Protection Sciences* 19(1): 174–77.
- Jones J T, Haegeman A, Danchin E G, Gaur H S, Helde J and Jones M G. 2013. Top 10 plant-parasitic nematodes in molecular plant pathology. *Molecular Plant Pathology* 14(9): 946–61.
- Khan M R and Ahamad F. 2020. Incidence of root-knot nematode (*Meloidogyne graminicola*) and resulting crop losses in paddy rice in northern India. *Plant Disease* **104**(1): 186–93.
- Kumar S, Stecher G, Li M, Knyaz C and Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35**: 1547–49.
- Kumar V, Khan M R and Walia R K. 2020. Crop loss estimations due to plant-parasitic nematodes in major crops in India. *National Academy Science Letters* 43: 409–12.
- Prasad J S, Vishakanta and Gubbaiah. 2001. Outbreak of root knot nematode, *Meloidogyne graminicola* in Mandya district, Karnataka state and farmers' perceptions. (*In*) *National Congress on Centenary of Nematology in India Appraisal and Future Plans*, New Delhi, December 5–7, 2001.
- Pokharel R R, Abawi G S, Duxbury J M, Smat C D, Wang X and Brito J A. 2010. Variability and the recognition of two races in *Meloidogyne graminicola*. *Australasian Plant Pathology* **39**: 326–33.
- Singh P, Mishra K K and Patnaik A. 2019. Harnessing the powerful potential of genomics in nematology. (In) National Seminar on "Recent Advances in Agriculture for Sustainable Rural Development, BAU Bihar, March, ISBN: 978-93-85675-11-9.
- Tamura K and Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10: 512–26
- Tian Z L, Maria M, Barsalote E M, Castillo P and Zheng J W. 2018. Morphological and molecular characterization of the rice root-knot nematode, *Meloidogyne graminicola*, Golden and Birchfield, 1965 occurring in Zhejiang, China. *Journal of Integrative Agriculture* 17(12): 2724–33.
- Torrini G, Roversi P F, Cesaroni C F and Marianelli L. 2020. Pest risk analysis of rice root-knot nematode (*Meloidogyne graminicola*) for the Italian territory. *EPPO Bulletin* **50**(2): 330–39.
- Zhou X, Liu G K, Xiao S and Zhang S S. 2015. First report of *Meloidogyne graminicola* infecting banana in China. *Plant Disease* **99**(3): 420.