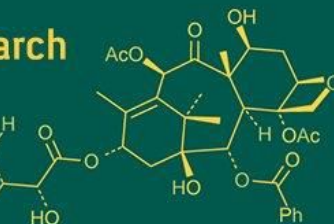
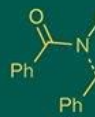
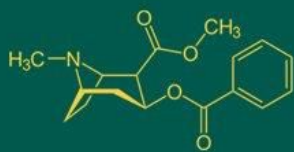


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Morpho-molecular characterization of cereal cyst nematode, *Heterodera avenae* from Hisar region of Haryana

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Abstract

Cereal cyst nematode (CCN, *Heterodera avenae*) holds the notorious reputation in wheat and barley, causing more problem in light sandy soil. Due to increasing number of species, accurate identification of *H. avenae* group are critical for developing effective control measures, particularly when identifying sources of host-plant resistance against *Heterodera* species. Rapid and precise identification is also essential for quarantine and regulatory purposes. To address these needs, molecular methods have been widely explored for identifying species within the group. The ITS-rDNA have proven to be highly effective for differentiating species among *H. avenae* group. Comparing ITS-rDNA sequences of unidentified species with published sequences in GenBank has further facilitated the rapid and accurate identification of most cyst nematodes.

Keywords: Haryana, morphology, nematode, sequencing, wheat

1. Introduction

Wheat production faces constraints due to a combination of biotic and abiotic factors. Among biotic factors, the crop remains prone to attack by more than 30 pests and pathogens, which have the potential to modify the chemical properties and overall quality of wheat. Amongst these, plant-parasitic nematodes (PPNs) are one of the major contributors to yield reduction in crops. The nematode causes about 10% decrease in their presence (Whitehead, 1998) [20]. About 8 to 9 genera of PPNs infect and damage the wheat crop. Among these, the cereal cyst nematode (CCN), *Heterodera avenae* Wollenweber, 1924 holds the notorious reputation of being the most destructive soil-borne pathogen affecting wheat (Nicol *et al.* 2009, Duggal and Mann 2019) [12, 4], leading to substantial yield losses worldwide (Nicol *et al.*, 2003; Peng *et al.*, 2007) [11, 13]. In India, the first record of CCN was from Sikar district of Rajasthan on wheat by Vasudeva (1958) [19], inciting *Molya* disease. Subsequently the nematode was reported from Jammu and Kashmir, Haryana, Punjab, Delhi, Himachal Pradesh, Uttar Pradesh and Madhya Pradesh. The annual loss caused by this nematode in wheat has been estimated to the tune of Rs. 66 crores in Haryana alone (Kanwar *et al.* 2007) [6] while avoidable losses due to CCN in major *Molya* affected states (Rajasthan and Haryana) were calculated as Rs. 8967.52 million (Kumar *et al.* 2020) [8]. Successive cropping of wheat in the main producing areas has created favourable conditions for *H. avenae* infection and survival. The symptoms appear on roots as busy appearance with light swelling in roots at the site of infection (Plate 1), yellowing and wilting of upper part of the plants and stunted growth in patches. The presence of white female on 3-4 months old plants is the confirmatory sign of this nematode (Kort, 1972) [7].

Heterodera avenae group taxonomy has been extensively reviewed in several studies. This group forms a complex of species that exclusively invade and reproduce in the roots of cereals and grasses. The species within this group are distinguished by only minor morphological and morphometric differences (Subbotin *et al.* 2003) [17]. The increasing number of species has made identification based on morphology and morphometry increasingly challenging, as it is time-consuming and requires specialized expertise. Despite these challenges, accurate identification of *H. avenae* group members are critical for

developing effective control measures, particularly when identifying sources of host-plant resistance against *Heterodera* species (Dababat *et al.* 2015) [2]. Rapid and precise identification is also essential for quarantine and regulatory purposes. To address these needs, molecular methods have been widely explored for identifying species within this group. The internal transcribed spacer (ITS) regions of ribosomal DNA (ITS-rDNA) have proven to be

highly effective for differentiating species within the *H. avenae* group (Zheng *et al.* 2015) [22]. Comparing ITS-rDNA sequences of unidentified species with published sequences in GenBank has further facilitated the rapid and accurate identification of most cyst-forming nematodes. The present study focuses on the morpho-molecular characterization of cereal cyst nematodes from Hisar, Haryana region.



Plate 1: *Heterodera avenae* cysts and their symptoms on wheat roots

2. Materials and Methods

2.1 Collection of nematode populations

The infested soil was collected from farmer's field of Hisar district of Haryana (India). Cysts were extracted from this soil by Cobb's sieving and decanting technique (Cobb, 1918) [1]; on 60 mesh sieves (pore size 250 μ m). Cysts retained on 250 μ m sieve were then hand-picked under a stereomicroscope and stored at 4 °C for further use. Identification of cysts was done on the basis of cone top structure, and separate pure culture of *H. avenae* was maintained on wheat susceptible variety (WH 1105) in screen house in 5 kg earthen pots filled with steam sterilized sandy soil for further use in experimentations.

2.2 Vulval cone preparation

From pure culture, the vulval cone part of the ten similar looking cysts were cut and mounted in glycerine jelly for some morphological features like semi fenestra shape, underbridge and bullae development. Depending on these structures, the identifications of cysts were done (Handoo, 2002) [5]. Among the selected cysts, five cysts were observed for morphological characters and measured using Zeiss Upright Axio Imager M2 Fluorescence Microscope in Dept. of Plant Pathology, CCSHAU, Hisar. For the preparation of vulval cone, cysts were soaked in water for 24 h. After placing the cyst on a Perspex plate and cut off the posterior end with the help of a small surgical blade and trimmed to small squarish shape to keep fenestral area is in the centre of the piece. After removing the adhering body contents, the portions were transferred to melted glycerine jelly for providing support, on to a glass slide.

2.3 Killing, fixing of nematodes and preparation of slides

Juveniles were collected in 100 ml beaker containing a known quantity of water. An equal amount of double strength 8% boiling formalin as fixative, was added to the suspension followed by putting the suspension in a vial and closing the lid tightly. Processing of the cyst nematodes for the permanent mount was done after 24 hours using the glycerol-ethanol method of Seinhorst (1959) [16]. The morphological characters of cyst and juvenile of *H. avenae* were studied.

2.4 DNA extraction

Genomic DNA was extracted from cyst nematodes using a method based on the protocol established by Reid and Pickup (2005) [15]. Eggs and second-stage juveniles (J2s) were extracted by puncturing the cyst and transferred into a sterile 200 μ L PCR tube. Next, 20 μ L of deionized water (ddH₂O) and 2 μ L of 10 $\times$ PCR buffer containing Mg²⁺ were added. The tube was stored overnight at -80 °C to ensure stabilization. After thawing, 2 μ L of protease K (20 mg/mL) was introduced to the mixture, which was incubated at 65 °C for 90 minutes to promote cell lysis. This step was followed by an incubation at 95 °C for 30 minutes to deactivate the protease K enzyme. To remove debris, the sample was centrifuged at 14,000 rpm for 3 minutes. The resulting genomic DNA was stored at -20 °C for future use in polymerase chain reaction (PCR) analysis.

2.5 PCR amplification

PCR amplification of the COX 1 region was done with following JB3-JB5 primers:

1. Forward primer: JB3 (5'-TTT TTT GGG CAT CCT GAG GTT TAT-3')

2. Reverse primer: JB5 (5'-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3')

The PCR thermal cycler program consisted of following steps:

- 5 min at 95 °C
- 40 cycles of 94 °C for 30 sec
- 48 °C for 45 sec, and 72 °C for 45 sec
- Final extension for 8 min at 72 °C

After PCR amplification, 5 μ L of each PCR product was mixed with 1 μ L of 6X loading buffer (Fermentas Life Sciences) and loaded on a 1.5% standard TAE buffered agarose gel. After electrophoresis at 100 V for 40 min, the gel was stained with ethidium bromide (0.1 μ g/mL) for 15 min and visualized and photographed under UV light. The remaining PCR product was stored at -20 °C.

2.6 PCR product purification

PCR products were purified using a PCR purification kit to remove any residual primers, nucleotides, and other impurities. The purified products, along with appropriate sequencing primers, were then sent to SOLS sequencing facility, Cheeka, Kaithal (Haryana) for Sanger sequencing. The obtained sequences were compared to known sequences in public databases to identify the closest matching pieces by performing a BLAST (Basic Local Alignment Search Tool) search using the GenBank NCBI database.

3. Results and discussion

Cysts were predominantly lemon-shaped, dark brown with a protruding neck and posterior protuberance (Plate 2). Earlier the cyst colour was white which changes to brown. The cyst wall was dark brown to black in colour. Vulval cone of cysts was bifenestrate with a vulval slit smaller than the fenestral width. Numerous dark brown bullae were prominent (Plate 3). Total length without neck was $653.115 \pm 30.597 \mu\text{m}$, neck length as $72.439 \pm 2.592 \mu\text{m}$, $512.410 \pm 14.449 \mu\text{m}$ width. Fenestral length was $50.032 \pm 1.372 \mu\text{m}$, fenestral width $23.017 \pm 1.087 \mu\text{m}$, while vulval slit length $10.444 \pm 0.278 \mu\text{m}$ and vulval bridge width as $9.960 \pm 0.216 \mu\text{m}$ (Table 1).

Based on the morphological characters, the second-stage juveniles (J2s) were vermiform in shape, slender shaped

strong stylet with large anteriorly flattened to concave basal knobs and hemispherical head region, sharply pointed tail (Plate 4). Tail was conoid with hyaline portion. Body length was $620.626 \pm 8.481 \mu\text{m}$, max. body width $20.510 \pm 1.063 \mu\text{m}$, stylet length $24.80 \pm 0.47 \mu\text{m}$, robust, exhibiting moderately concave stylet knobs. Hyaline length and tail length were $39.45 \pm 1.23 \mu\text{m}$ and $62.26 \pm 2.84 \mu\text{m}$, respectively (Table 1).

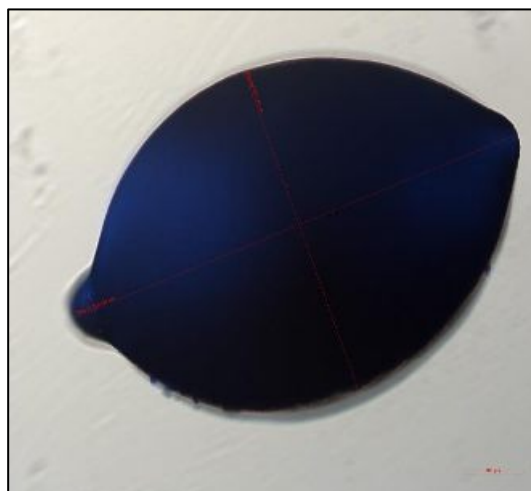


Plate 2: *Heterodera avenae* cyst

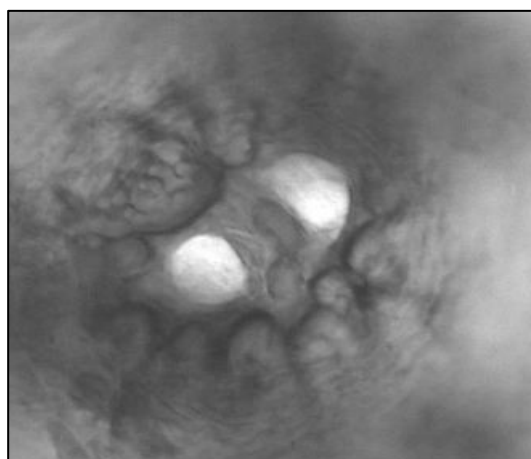


Plate 3: *Heterodera avenae* vulval cone

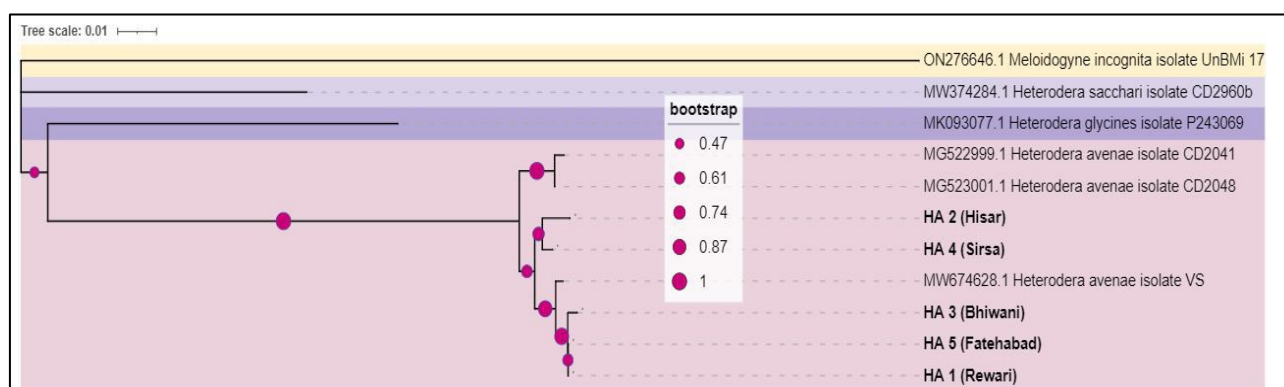


Plate 4: *Heterodera avenae* J2s and eggs

Table 1: Morphometric of cyst along with its vulval cone and juveniles (mean in $\mu\text{m} \pm \text{S.E.}$)

Cyst morphology		Cyst and vulval cone measurements (Means of 5 replicates)	
Color	Dark brown to black	Length without neck (μm)	653.115 $\pm$ 30.597 (617.142-765.560)
		Neck length (μm)	72.439 $\pm$ 2.592 (65.23-79.562)
Shape	Lemon shaped	Width (μm)	512.410 $\pm$ 14.449 (475.193-559.338)
		Fenestral length (μm)	50.032 $\pm$ 1.372 (45.458-53.111)
Neck	Medium	Fenestral width (μm)	23.017 $\pm$ 1.087 (20.735-26.478)
		Vulval slit length (μm)	10.444 $\pm$ 0.278 (9.712-11.125)
Bullae	Numerous	Vulval bridge width (μm)	9.960 $\pm$ 0.216 (9.405-10.701)
Juveniles' morphology		Juveniles' measurements (Means of 5 replicates)	
Color	Transparent	Body length (μm)	620.626 $\pm$ 8.481 (598.22-642.25)
Shape	Vermiform	Max. body width (μm)	20.510 $\pm$ 1.063 (18.259-23.562)
Curve	Slightly ventral side	Stylet length (μm)	24.80 $\pm$ 0.47 (24.33-26.67)
Stylet	Strong	Hyaline portion length (μm)	39.45 $\pm$ 1.23 (35.61 to 42.43)
Tail	With hyaline portion	Tail length (μm)	62.26 $\pm$ 2.84 (54.02-71.08)

* Figures in parenthesis are range

**Fig 1:** Phylogenetic relationship inferred from neighbour joining (NJ) analysis of cereal cyst nematode (*H. avenae*) based on *COX 1* sequencing. The percentage of replicate tree in the bootstrap test is 1000 replicates. The evolutionary distances were computed using the Kimura 2-parameter method.

The tree (Fig. 1) includes an outgroup, *Meloidogyne incognita* (ON278646.1), which helps root the phylogeny and confirm the evolutionary divergence of the studied *Heterodera* isolates. *Heterodera avenae* isolate (HA 2) from different regions form a distinct clade with reference isolates of *Heterodera avenae*, with high bootstrap values (0.87 and above), signifying high genetic similarity. Distant relationships, such as with *H. glycines* and *H. sacchari*, are supported by moderate bootstrap values (0.47). Devindrappa *et al.* (2020) [3] studied Indian CCN populations from three location, Delhi, Kangra and Leh for their intraspecies characters. Delhi populations showed >98% similarity with the *H. avenae* (accession no KC152906) on the basis morphology; morphometric characteristics and Sequence of ITS region while Leh population was suggested as a new variant of *H. avenae* complex. Mokrini (2016) [10] investigated 11 CCN populations from wheat growing areas in Morocco, on morphological and molecular basis using light microscopy along with species-specific primers and ITS-rDNA sequences. Mostly population were found as *H. avenae* on morphological basis while only one isolate

showed morphometrics similar to *H. latipons*. Rao *et al.* (2013) [14] used sequencing and PCR-RFLP of rDNA to confirm the cyst species identity and genetic homogeneity of an Indian population for whole genome and transcriptome sequencing. The Sequence of ITS1 and ITS2 including 5.8S showed approximately 99% similarity with the existing sequences of *H. avenae* from different locations. Further, about 200 individual cysts were analysed separately by PCR-RFLP of rDNA with five restriction enzymes that could distinguish *H. avenae* from other closely related species within the group. This analysis did not reveal any variation within the population indicating it is genetically homogeneous. Kumari (2017) [9] studied seven populations of *H. avenae* molecularly and one population was described morphologically using 18S, 28S, ITS 1-rDNA in which 18S gene of six populations did not reveal any variation, whereas the ITS 1 and 28S sequences of six populations differed by only two nucleotides from a population in Žilina. Yan and Smiley (2010) [21] differentiated *H. avenae*, *H. filipjevi* using PCR-RFLP of ITS-DNA with up to six restriction endonucleases i.e. TaqI, HinfI, PstI, HaeIII, RsaI, and AluI.

The method was validated by inspecting underbridge structures of cyst vulval cones. The molecular analysis proved to be a robust tool for species identification and differentiation. The *COX 1* sequences obtained from Haryana populations were compared with existing sequences in GenBank, confirming their identity as *H. avenae*. The results align with previous studies that highlighted the utility of conserved regions for resolving taxonomic ambiguities within cyst-forming nematodes (Subbotin *et al.* 2000) ^[18]. The molecular data also facilitated the detection of genetic variability, which could have implications for understanding the adaptability and virulence of *H. avenae* in different agro-climatic zones.

4. Conclusion

The present study provides the morpho-molecular characterization of *Heterodera avenae* populations in Haryana, addressing a significant knowledge gap in understanding this economically important nematode in the region. The combination of morphological and molecular approaches enabled precise identification of the cyst nematode species, which is crucial for developing effective management strategies. Morphologically, the populations studied showed typical characteristics consistent with the *H. avenae* group, including minor morphological variations that are commonly observed among geographically distinct populations. However, the reliance on morphological features alone can be challenging due to the subtle differences within the group and the influence of environmental factors on nematode morphology.

5. Conflict of Interest

The authors declare no conflicts of interest.

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