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Intraspecies variation studies of Indian populations of *Heterodera avenae* Wollenweber, 1924

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Abstract
Indian populations of cereal cyst nematode from Delhi, Kangra (Himachal Pradesh), Leh (Jammu and Kashmir) were studied for their intraspecies characters of morphological and morphometric through light, scanning electron microscopy, sequences of Internal transcribed spacer (ITS)-ribosomal rDNA. Delhi populations of cyst nematode were morphological; morphometric characteristics and Sequence of ITS region showed more than 98% similarity with the *H. avenae* accession no KC152906. Well-developed underbridge of cone top and the small tail followed by hyaline tail terminus in juveniles was present in the Kangra population as similar to *H. filipjevi.* Leh population was characterized by comparatively short juveniles (489 µm) with a small stylet (25 µm), anterior end to median bulb length (67 µm), tail (57 µm) and Hyaline region (35 µm); small cysts (L=669 µm) and vulval slit length (5.8 µm). ITS region was much small compared to other populations. Based on morphological, morphometric, and molecular shreds of evidence Leh population was suggested to be a new variant of *H. avenae* complex. COI sequences were highly unpredictable and BLAST results were erratic w.r.t. its taxonomic importance in this nematode. In silico analysis suggested that restriction enzymes AgeI, BcgI, BsrFI, and BsaWI; Tth111I, PflFI, NciI, and BstXI; and AhdI and FauI had unique restriction sites for ITS sequences of Kangra, Delhi (IARI), and Leh populations, respectively.

Keywords: Cyst nematode, *Heterodera avenae*, ITS-rDNA, morphology and morphometric

Introduction
The wheat cereal cyst nematodes (CCN) *Heterodera avenae* group reported globally as a biotic constraint for wheat production. It causes the damage of almost $78 billion worldwide (1). It can cause about 40-50 percent yield loss that can reach up to 60-75 percent in case of severe infection (14). In isolated areas losses in Wheat up to 100 percent have been reported in India (23). Since 2003, an infestation of CCN was recorded in rice-wheat rotation fields (11).*H. avenae* group presently contains at least thirteen valid species: *H. avenae* Wollenweber, 1924 [24], *H. arenaria* Cooper, 1955 [3], *H. aucklandica* Wouts & Sturhan, 1995 [25], *H. australis* Subbotin, Sturhan, Rumpenhorst & Moens, 2002 [17], *H. bifenestra* Cooper, 1955 [3], *H. filipjevi* Madzhidov, 1981 [12], *H. mani* Mathews, 1971 [13], *H. pratensis* Gäbler, Sturhan, Subbotin & Rumpenhorst, 2000 [19], and. Several undescribed species of this group are also suggested by different authors (25, 15, 17 & 18). In India, it has been reported from the states of Rajasthan, Haryana, Delhi, Himachal Pradesh, Jammu and Kashmir, Madhya Pradesh, Punjab, and Uttar Pradesh (9). Accurate identification of nematode species and awareness of high population density in affected fields are essential not only for taxonomic purposes but also for designing effective control measures. Population in Leh, India (hilly region having common border area with China) was suspected to maybe differ from *H. avenae*. Therefore, the objective of this study was to evaluate the intraspecies variation studies of Indian populations of *H. avenae* from Delhi, Kangra (Himachal Pradesh), and Leh (Jammu and Kashmir).

Materials and Methods
Nematode Populations
Soil samples were collected from the wheat-growing cyst-nematode infested fields of IARI (Delhi), Village Tihri, Kangra (Himachal Pradesh), and Leh (Jammu and Kashmir). Cobb's decanting and sieving method was used to separate nematodes from the soil. The suspension was sieved through a set of sieves 20 and 60 mesh sieves.

A residue left on a 60 mesh sieve was collected in a beaker and was used for cysts examination. Cysts were handpicked under a Wild Heerbrugg stereo-zoom microscope. Freshly emerged larvae from the cysts were hand-picked and processed for taxonomic studies.

Field sandy loam soil and sand mixture (3:1) ratio was steam sterilized in an autoclave at 15 lbs pressure for 30 minutes, filling the 15cm earthen pots. Pure culture of *H. avenae* was maintained on Wheat variety HD 3967 at Division of Nematology, Indian Agricultural Research Institute, and New Delhi throughout the investigations. Soil samples were collected between 2½ and 3 months after the t inoculations. The soil was washed with Cobb's decanting and sieving technique using a set of 20 and 325 mesh sieves. The residue from the 325 mesh sieve was filtered over wire-gauze support having a double-layered tissue paper for 48 h. The suspension was collected into a beaker transferred to a counting dish and observed under a stereoscopic binocular microscope. Males were hand-picked for further processing and taxonomic observations.

Killing, fixing, and preparation of permanent mounts

Juveniles and males were collected in 2 ml Eppendorf tubes containing a small quantity of water. Nematode suspensions were subjected to 60 ^{0}C in a dry bath for 2 mins. An equal volume of double strength formalin (8%) was added to the suspension for fixing the nematodes in a 4% formalin solution. Processing of the nematodes for the permanent mount was done after exposure of at least 8h in the fixative. Permanent mounts of males and juveniles were prepared by the glycerol-ethanol method of Seinhorst (1959) [16]. Cysts were cut with the help of a small surgical blade and trimmed small square shape. These cones were washed twice in 70% alcohol followed by dehydration in absolute ethanol for 3-5 min. The cone-top was then cleaned in clove oil and mounted on a glass-slide in Canada balsam. Four very fine pieces of crushed glass slide were placed in the Canada balsam for providing support to the coverslip.

Light and scanning microscopy

The morphological and morphometric characters of various stages of *H. avenae* were studied and photos were taken by using the compound research Olympus BX-50 (withdrawing tube attachment) and Zeiss AxioImager M2m microscopes and scanning electron Tescan-Vega3 microscope.

Morphological and morphometric characters of nematodes

The morphological and morphometric characters were studied for Cyst, second-stage juveniles, and males of cereal cyst nematode. For Cyst, color, shape, length, width, length/Width, fenestral length, fenestral width, vulval bridge, underbridge length, vulval slit length, and fenestra to anus distance. For second-stage juveniles (J2); L, a, b, c, stylet length, labial region height, labial region diameter, DGO, anterior end to median bulb valve, excretory pore, esophagus-intestinal junction; Body diameter at mid-body, anus; tail length, hyaline region. And for male adults; L, a, b, c, stylet, labial region height, labial region diameter, DGO, anterior end to median bulb valve, excretory pore, esophagus-intestinal junction; -spicule length, body diameter at the anus, and tail length characters were taken.

Scanning electron microscope (SEM) studies

Nematodes (cysts/ J2s / males) were collected and killed as described in 2.1 and 2.2. Two fixatives viz. 2.5% gluteraldehyde and 2% osmium tetraoxide were prepared in 0.2M sodium cacodylate buffer (pH 7.0). Nematodes were left in gluteradehyde overnight, rinsed with 0.2M sodium cacodylate buffer, transferred to osmium tetroxide for 4-6h, and again rinsed with the buffer. Dehydration of nematodes was done gradually (for 10 min each) in the serial dilutions of 30, 40, 50, 60, 70, 80, 90%, and absolute ethanol. Two more changes were given in absolute ethanol and the nematodes were then subjected to critical point drying in liquid CO2. Dry nematodes were transferred to double-adhesive carbon tape on an aluminum stub. Sputter coating of nematodes was done with gold-pallidium in Quorum technologies SC7620 Mini Sputter Coater set at 18mA plasma current for 60 sec. Nematodes were observed in Tescan Vega3 SEM.

Molecular characterization of nematodes

Isolation of Genomic DNA and Polymerase Chain Reaction (PCR)

The Genomic DNA from eggs (collected from 10-15 mature cysts) was isolated using Qiagen 1042611 Puregene buccal cell core kit and amplified in a PCR using primers described by Subbotin *et al*., 2003 [18]. The amplification of DNA following primers were used, for Forwarding primer – TW81 (18s), 5'-GTTTCCGTAGGTGAACCTGC-3' and Reverse primer – AB28 (28s), 5'-ATATGCTTAAGTTCAGCGGGT-3'. For master mix of 25ul reaction was set up consisting of 10 µl of the DNA suspension was added to the PCR reaction mixture containing 0.2 mM dNTP's, 0.2 µM of each of the primer (reverse and forward) and 2.5 µl 10× buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2), 1 unit of Taq polymerase, and double distilled water to a final volume of 25 µl.

For ITS amplification DNA, thermal cycler settings consisted of 4 min 94 ˚C, 44 cycles of 1 min 94 ˚C, 1 min 30 sec. 55 ˚C, 2 min 72 ˚C; and 10 min 72 ˚C. After PCR amplification, 5 µl of each PCR product were examined in a 1.5% standard agarose gel stained with ethidium bromide (0.1 µg/ml).

PCR product purification

After the PCR reaction of genomic DNA, it was purified for sequencing. DNA binding condition was adjusted, 1volume of the sample with 2volume of buffer (NTI) mixed.

Binding of DNA nucleon spin gel and PCR clean-up column was done, followed by centrifugation for 30sec at 11000rpm, flow-through was discarded and the Column was placed back into the collection tube. Silica membrane was washed and 700 µl buffer NT3 was added to nucleon spin centrifuge for 35sec at 1000rpm. Silica membrane was dried, centrifuged for 1min at 11000 rpm to remove buffer NT3. DNA was eluted, 15-30 µl buffer NE was added and centrifuged for 1min at 11000 rpm. The PCR amplified products were gel purified and sent to a commercial company for the genomic ITS region of ribosomal DNA sequencing.

Analysis

Multiple sequence alignment (MSA) was done by MUSCLE, alignment curation by Gblock (www.phylogeny.fr). A restriction map was generated by NEBcutter. Morphometric characters of J2, mature cysts, cone top, and males were analyzed statistically to compute their respective mean and SD.

Results

Cysts: Lemon shaped with prominent neck and vulval cone. Cysts of the Delhi population were dark to moderately brown, whereas those from Leh and Kangra were light to dark brown. Cysts of the Leh population were much smaller 669.7 than the cysts (≥739.44) from the other two areas (Table 1). Length (without neck)/ width ratio was lowest in Leh followed by Kangra and Delhi populations. Cysts were covered with a sub-crystalline layer which got detached at many places in due course of time Vulva smaller than fenestral width (Fig 4, 5, 6). Bullae present (Fig 1). Underbridge was present in Kangra (Fig 1d) with bifurcated ends and was without underbridge but certain cone topes a delicate underbridge was seen whereas absent in Delhi population but in case of Leh population cyst length and width ratio were lowest as compared to Kangra and Delhi population, bullae and underbridge were also present (Table 1), ((Fig 6).

Juveniles: Body curved slightly on the ventral side when second-stage juveniles were relaxed with heat. Juveniles from Leh were smaller (489.83 ± 38.14 µm) in size than Delhi (593.7 ± 34.78 µm) and Kangra (593.25 ± 13.94µm) populations (Table 2 Fig 2). Midbody diameter was comparatively small in the Leh population. The labial region was offset. Labial annules were more prominent in the Delhi population (Fig 4). The labial framework is strongly sclerotized. Amphidial apertures pore-like. Oral opening round or oval (Fig 4, 5, 6).

Stylet strong with well-developed basal knobs. Average stylet length low in Leh population. Basal knobs were sometimes pointed giving anchor shape to the stylet knobs in the Leh population. Lateral lines four, though two outer lateral lines were less prominent in Leh population. Outer lateral lines were areolated. Excretory pore near or anterior in Leh and Kangra whereas posterior in Delhi population to the esophagus-intestinal junction (Table 2, Fig 3 D, E, F). Tail and hyaline region smaller in Leh (57.88 ± 5, 35.08±3.42 µm) population compared to Delhi (73.71 ± 4.97, 44.93±5.68 µm) and Kangra (70.24 ± 2.56, 42.32±2.79 µm) populations (Fig 3).

Males: Labial region setoff, dome-shaped with four to five annules (Fig 4, 5, 6). Amphidial aperture oval to slightly oblong above the anterior-most labial annule (Table 3, Fig 7). The cephalic framework was strong. The stylet is well developed with a strong shaft and knobs. Conus was about ½ the length of stylet. DGO was less than ⅓rd stylet length behind the knobs. The excretory pore was posterior to an esophageal-intestinal junction (Fig 7). The tail ca ≤ ½ the body diameter at the anus.

ITS region sequenced from all the nematodes yielded a fragment size of around 1090bp in Delhi (IARI) populations, 1021bp in Kangra (Himachal Pradesh), and 669bp in Leh (Jammu & Kashmir) populations. Nematode DNA sequence deposited in Gen Bank under accession number was (HG738844.1, HG738845.1 & HG738846.1) Delhi (IARI), Kangra (H.P), and Leh (J & K) populations, respectively. The 5.8S gene showed a minimum variation. The ITS2 region was small compared to ITS1 and it showed less sequence divergence compared to ITS 1. The restriction map generated by NEBcutter showed unique restriction enzyme cleavage sites in the three populations. Enzymes AgeI, BcgI, BsrFI, and BsaWI; Tth111I, PflFI, NciI and BstXI; and AhdI and FauI had unique restriction sites for Kangra (H.P.), Delhi (IARI), and Leh (J&K) ITS sequences respectively and hence could be useful for differentiating these populations.

Discussion

The *Heterodera avenae* group has 13 valid species. Many variations in this group of species have been observed and with the growing number of described *Heterodera* species distinction and reliable identification of species based on morphological characters alone are becoming increasingly difficult. Due to this difficulty, many species of this group have wrongly been placed. In terms of resolving relationships at the species level, internal transcribed spacers (ITS1 and ITS2) situated between the small nuclear ribosomal subunit (18S gene) and the large subunit (28S gene) have been used by several authors for accurate species identification. Ferris and co-authors (4, 5, 6 &7) demonstrated that nucleotide sequences of ITS1 and ITS2 were useful and practical for phylogenetic analysis of the cyst-forming nematodes. Three populations of cereal cyst nematode from wheat grown areas of Delhi, Kangra and Leh were similar in characters of cysts, juveniles, and males in the *H. avenae* complex. Well-developed bullae were present in all the populations studied which differentiated them from *H. fenestra, H. hordicalis, H. latipon*, and *H. spinicauda*. Morphological and morphometric characteristics revealed the Delhi population to be *H. avenae.* The sequence of ITS region of Delhi population showed more than 98% similarity with the *H. avenae* (accession no KC152906) as reported recently by Rao *et al.* (2013) [22]. Therefore, the Delhi population was clearly identified as *H. avenae.*

Prominent under a bridge was present in the Kangra (H.P) population, morphological and morphometric characters of Kangra and Delhi populations were similar to each other. Compared to *H. iri* (Tail=94 µm Hyaline terminal length = 62 µm) tail and the hyaline region was much smaller in Kangra population (Tail=70 µm Hyaline terminal length = 42 µm). Underbridge is also present in *H. spinicauda, H. latipons,* and *H. hordecalis* however these species don't have well-developed bullae, have a wide vulval bridge (>16 µm) and shorter second-stage juveniles (mean <500 µm).

Taxonomic studies of 8 populations of cereal cyst nematode from Rajasthan, Punjab, and Himachal Pradesh were carried out by Bishnoi and Bajaj (2004) [2]. Major differences in juvenile (J2), males, and cyst characters were observed in Rajasthan, Punjab, and Himachal Pradesh populations. They also reported that a conspicuous under-bridge was present in the Himachal Pradesh population and it was identified *H. filipjevi* based on morphological and molecular differentiation. Kaushal (1989) [10] reported *H. iri* from the wheat grown in Village Tihri, Kangra based on the presence of underbridge and concave stylet knobs. In our studies (morphological, morphometric, and molecular) population from Village Tihri, Kangra was found closest to and identified as *H. filipjevi.* That *H. iri* does not infect wheat and wheat is a host of *H. filipjevi* further supported our findings.

Amongst three populations studied the Leh population was characterized by comparatively shorter juveniles (489 µm) with a small stylet (25 µm), MB (67 µm), tail (57 µm) and Hyaline region (35 µm); small cysts (L=669 µm) and vulval slit length (5.8 µm). In Delhi populations are juveniles (593 µm) with a small stylet (27µm), MB (23 µm), tail (73 µm) and Hyaline region (44 µm); cysts (L=736 µm) and vulval slit length (6.84 µm), In Kangra populations, are juveniles (593 µm) with a small stylet (26µm), MB (23 µm), tail (70 µm) and

Hyaline region (42 µm); cysts (L=742µm) and vulval slit length (7.5 µm). Underbridge was often absent in Leh population however sometimes weak underbridge was observed in a few cysts. Like *H. pratensis* the inner two lateral lines were more prominent in the juveniles of the Leh population but their outer ones were less clean. However, males of Delhi Kangra and Leh populations were morphologically very similar. Removal of primer sequences in the flanking 18S and 28S gave 566 and 215 bp ITS 1 and 2 sequences respectively for Delhi and Kangra populations. Whereas sequencing of the ITS revealed a smaller total length (626 characters; ITS1+5.8S+ITS2) of ITS region of Leh population. Most of the shortening was found in the ITS1 region. Therefore, the Leh population appeared to be a new variant of *the H. avenae* complex only loosely matching in characters with the other two populations. Studied herein as also with species of *H. avenae* complex.

In our studies, we found that the full length of the ITS region was 976 characters in Delhi and 971 characters in Kangra populations. Subbotin *et al* (2003) [18] reported that the length for the full data set of the ITS region was 998 characters in *H. avenae* group. They found that the ITS region length was shorter in the outgroup taxa (961, 962), and varied within in group taxa from 964-965 (*H. pratensis*) to 975- 976 (*H. filipjevi* and *H. ustinovi*) which explained slight differences in the size of ITS region of Delhi and Kangra populations. However, to our surprise, with the use of the same primes, an ITS region of 626 characters was obtained from the Leh population. Much of it was due to the shorter ITS1 region which was 597 characters long in Kangra and Delhi and 280 characters long in Leh population.

Tanha *et al* (2003) [21] found that the results of RFLP and sequences of ITS-rDNA of 45 populations of cyst forming nematodes from Iran matched with the morphological and morphometric features. They also reported that restriction of ITS-rDNA by enzymes HinfI and TaqI allowed discrimination of the species within the Avenae group: HinfI distinguished unknown *Heterodera* sp. 1. (817, 187, 41 bp) from *H. avenae* (499, 318, 191, 41 bp) and TaqI differentiated *H. filipjevi* from the other Avenae group species.

Rao *et al* (2013) [22] used sequencing and PCR-RFLP of rDNA to determine the genetic homogeneity of Indian populations of *H. avenae* for whole-genome and transcriptome sequencing. Their study indicated that the population was genetically homogeneous and suitable for next-generation sequencing. Morphological and morphometric characters of the Delhi population in our study matched with *H. avenae*. The ITS sequence of the Delhi population aligned best with the sequence deposited (accession no. KC152906) by Rao *et al* (2013) [22].

Within *H. avenae*, intraspecific polymorphism in the ITS region of rDNA was reported by Subbotin *et al* (1999) [20]. They grouped the Indian population with *H. filipjevi* from Spain. A shorter J2 body length, tail length, and hyaline part of tail length were reported to distinguish the Indian population from the other *H. avenae* populations. They identified at least two types of ITS regions: type A for most European populations, type B for the Indian population, and their combination (type A+B) for three French populations. These three genetic types generally corresponded to morphological types analogous to the same populations. In our studies, differences in morphology, morphometric, and ITS region of three populations were also observed. Subbotin *et al* (1999) [20] reported that the Indian population differed from all other populations. *Alu*I and *Rsa*I digested the ITS region and permitted differentiation of this population from the other *H. avenae* populations. Restriction enzymes *Alu*I and *Rsa*I did not digest PCR amplified products of European populations.

Differences in morphological and molecular data of Leh population from other species of *H. avenae* group were observed in our study. Subbotin *et al* (2003) [18] reported that morphologically cereal cyst nematode populations were distributed within four clades in two main groups. The first main group had three clades representing populations from Africa and Asia, populations from France, and other populations from Europe. Populations from China formed the second main group representing another evolutional branch of the cereal cyst nematodes. A close relationship of the 'Chinese cereal cyst nematode' with the European *H. pratensis* and the unidentified Iranian *Heterodera* sp. from grassland was observed by these authors. They also found that the single cyst contained two types of ITS clones belonging to two different groups. Their data also revealed that cysts from some Asian populations contained recombinant sequences. The presence of sequences belonging to different groups was suggested to be a result of gene flow between populations. Observations of Subbotin *et al* (2003) [18] and geographical contiguity Leh region of India with China supports the differences seen in morphological and ITS sequence data in our observations.

Present results were further supported by Ganguly and Rao (2009) [8], random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis on 7 populations of *H. avenae* (from Delhi, Sirsa, Jhansi, Tikamgarh (MP), Jaipur, Udaipur, and Ludhiana) indicated that populations of Sirsa, Tikamgarh, Udaipur, and Ludhiana were more closely related forming one cluster. They concluded that there was a tremendous amount of genetic variability among the different geographical populations of India with a possibility of more variability within a population hitherto unknown.

Table 1: Morphometric of cyst and cone top structures (mean in µm ± S.E.) of three populations of cereal cyst nematode from Delhi, Kangra and Leh

Characters	Delhi (IARI)	Kangra (H.P)	Leh (J & K)
Cysts	**N= 15**	**N=15**	**N=15**
Length without neck (L)	7369 ± 66.55 (600-840)	742 ± 38.81 (690-800)	669.70 ± 34.75 (610-720)
Neck length	85.56 ± 9.84 (70.0-100.0)	74.5 ± 7.61 (60-85)	78.52 ± 8.61 (60-90)
Width (W)	526.11 ± 75.08 (310.0-580.0)	537 ± 21.10 (510-560)	500.58 ± 53.08 (400-570)
L/W ratio	1.43 ± 0.19 (1.24-1.94)	1.38 ± 0.10 (1.50-1.23)	1.34 ± 0.13 (1.12-1.67)
Fenestral length	44.62 ± 5.22 (36.48-53.58)	45.86 ± 7.42 (37.95-55.55)	47.41 ± 2.88 (42.90-51.49)
Fenestral width	19.71 ± 3.12 (15.94-23.55)	21.43 ± 3.97 (15.77-25.30)	21.33 ± 3.81 (14.42-26.04)
Vulval bridge width	5.06 ± 0.75 (4.11-6.53)	5.70 ± 1.08 (3.96-7.04)	5.97 ± 0.69 (5.03-7.0)
Vulval slit length	6.84 ± 0.76 (5.71-8.10)	7.50 (5.82-7.04)	6.0 ± 0.71 (5.10-7.07)
Anus to Fenestra distance	30.41 ± 5.86 (23.95-38.12)	31.23 ± 4.85 (26.02-39.80)	27.58 ± 5.94 (20.11-39.67)

Table 2: Morphometric of *second stage juveniles* (mean in µm ± S.E.) of three Populations of cereal cyst nematode from Delhi, Kangra and Leh

Characters	Delhi (IARI)	Kangra (H.P.)	Leh (J & K)
Second stage larvae	**N=15**	**N=15**	**N=15**
Length (L)	593.70 ± 34.78 (538.73 - 638.32)	593.25 ± 13.93 (550.10-614.73)	489.83 ± 38.14 (436.36-567.85)
Lip height	4.53 ± 0.46 (3.77 - 5.32)	4.72 ± 0.39 (4.02-5.40)	(4.46 ± 0.37) (3.88-5.08)
Lip diameter	10.08 ± 0.48 (9.24 - 10.59)	10.27 ± 0.32 (9.71-10.97)	9.29 ± 1.40 (4.52-10.22)
Stylet length	27.15 ± 1.00 (25.63 - 28.80)	26.80 ± 1.05 (24.25-28.40)	25.40 ± 0.56 (24.61-26.59)
DGO	8.73 ± 3.21 (6.15 - 16.39)	7.85 ± 0.87 (6.14-9.11)	5.15 ± 1.40 (2.29-7.72)
Body diameter at mid Body	23.77 ± 1.97 (20.93 - 27.49)	23.25 ± 0.62 (22.16-24.25)	20.73 ± 0.62 (19.77-21.90)
Anterior end to Excretory pore	120.86 ± 8.85 (128.60-104.74)	121.07 ± 3.41 (113.51-127.07)	98.08 ± 6.09 (90.50-109.98)
Width at Excretory pore	21.44 ± 1.55 (19.80 -23.29)	21.95 ± 1.30 (19.69-24.05)	19.14 ± 1.06 (17.52-20.75)
Anterior end to median bulb (MB)	79.49 ± 6.28 (71.16 -90.42)	84.45 ± 3.62 (78.37-92.03)	67.80 ± 4.52 (60.94-77.25)
Anterior end to esophago-intestinal junction	113.51 ± 9.20 (95.37-121.27)	138.07 ± 5.27 (133.28-154.05)	109.33 ± 8.20 (96.40-123.69)
Body Diameter at anus (BDA)	17.10 ± 1.01 (15.24-18.39)	16.87 ± 0.60 (16.23-18.55)	14.85 ± 0.62 (13.89 -15.80)
Tail length	73.71 ± 4.97 (66.16-80.11)	70.24 ± 2.56 (62.36-73.69)	57.88 ± 5.00 (52.19-70.45)
Hyaline part of tail length (H)	44.93 ± 5.68 (35.79 -52.57)	42.32 ± 2.79 (37.20-48.50)	35.08 ± 3.42 (31.53-43.40)
A	25.91 ± 1.20 (24.27-28.64)	25.48 ± 0.79 (23.95-27.12)	23.65 ± 2.02 (21.41-28.18)
B	5.44 ± 0.39 (5.09-6.25)	4.30 ± 0.19 (3.90-4.56)	4.45 ± 0.17 (4.08-4.65)
C	8.27 ± 0.42 (7.75-8.84)	8.45 ± 0.28 (7.99-8.82)	8.42 ± 0.58 (7.48-9.25)
Tail/BDA	4.35 ± 0.32 (3.92-4.90)	4.17 ± 0.25 (3.70-4.50)	3.90 ± 0.32 (3.59-4.66)
H/stylet	1.62 ± 0.20 (1.32-1.90)	1.58 ± 0.13 (1.39-1.87)	1.38 ± 0.12 (1.23-1.63)
L/MB	7.46 ± 0.40 (6.76-8.25)	7.02 ± 0.41 (6.35-7.82)	7.23 ± 0.37 (6.50-7.83)

Figures in parentheses show the range

Table 3: Morphometric of *males* (mean in µm ± S.E.) of three populations of cereal cyst nematode from Delhi, Kangra and Leh

Characters	Delhi (IARI)	Kangra (H.P.)	Leh (J & K)
Males	N=15	N=15	N=15
Length (L)	1452.51± 130.23 (1164.30-1719.75)	1457.33 ± 100.26 (1271.34-1588.21)	1449.15 ± 81.57 (1304.17-1569.66)
Lip height	6.47± 0.38 (5.72-7.02)	6.78± 0.58 (5.85-7.87)	(6.81 ± 0.97) (5.32-9.13)
Lip diameter	11.51 ± 0.41 (10.66- 12.46)	11.51± 0.46 (10.66-12.46)	11.73 ± 0.43 (11.06-12.43)
Stylet length	30.09± 0.90 (29.14 – 32.14)	29.98 ± 0.5 (29.00-30.73)	29.67± 0.72 (11.06-12.43)
DGO	7.55 ± 0.48 7.02-8.60	8.35 ± 1.02 (7.02-9.38)	7.56±0.72 (7.07-9.57)
Body diameter at mid Body	29.81 ± 1.90 (26.00 – 31.91)	29.38± 1.20 (28.41-31.42)	29.74 ± 1.05 (27.91-30.95)
Anterior end to Excretory pore	189.91 ± 10.99 (166.70-200.54)	191.11±11.77 (172.90-207.70)	183.18 ±18.35 (143.59-200.26)
Width at Excretory pore	25.78± 1.42 (23.83 -27.63)	27.63± 1.12 (23.68-27.00)	25.28± 1.37 (22.30-27.23)
Anterior end to median bulb (MB)	114.35± 4.42 (103.98-120.04)	113.67± 2.98 (108.89-119.04)	114.325± 6.91 (105.76-126.67)
Anterior end to esophago-intestinal junction	143.00± 8.43 (122.98-153.90)	134.92± 5.71 (128.43-145.38)	136.40± 9.91 (122.26-155.28)
Anterior end to Dorsal gland base	244.45± 33.61 (167.90-286.78)	262.91± 7.23 (255.71-270.17)	277.79± 14.92 (259.14-59.14)
Body Diameter at anus	15.25±2.26 (2.26-18.63)	15.23± 1.55 (12.80-17.73)	15.32± 1.17 (13.37-17.65)
Tail	7.51± 0.95 (5.57-9.09)	7.04± 0.97 (4.86-9.09)	7.97± 0.89 (5.31-8.87)
Spicule length	28.21 ± 2.57 (22.45 -32.38)	34.53 ± 2.46 (30.00-36.80)	31.93± 3.75 (25.75-38.85)
Gubernaculum length	8.97 ± 0.71 (7.60-9.58)	8.80 ± 0.60 (7.76-9.45)	8.44 ± 0.99 (7.13-9.57)
A	49.95± 3.78 (56.00-45.16)	49.81± 3.56 (44.53-54.35)	49.40± 3.11 (45.67-54.38)
B	10.18 ± 0.99 (8.01-11.99)	10.87 ± 0.83 (11.70-9.53)	10.65± 0.82 (9.76-11.76)
b’	6.04 ± 0.93 (4.65-8.18)	5.79±.32 (5.56-6.15)	5.29± 0.36 (4.76-5.56)
C	195.90± 26.97 (151.80-247.04)	211.74± 37.76 (171.76-323.83)	184.06± 32.26 (157.24-282.15)

Figures in parentheses show the range

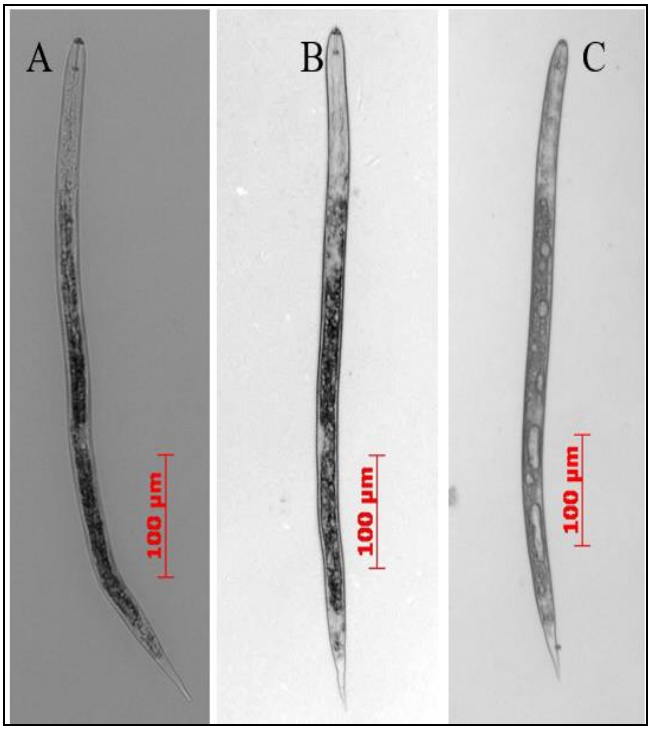

Fig 1: Second stage juveniles of cereal cyst nematode from Delhi (A), Kangra (B) and Leh (C) population

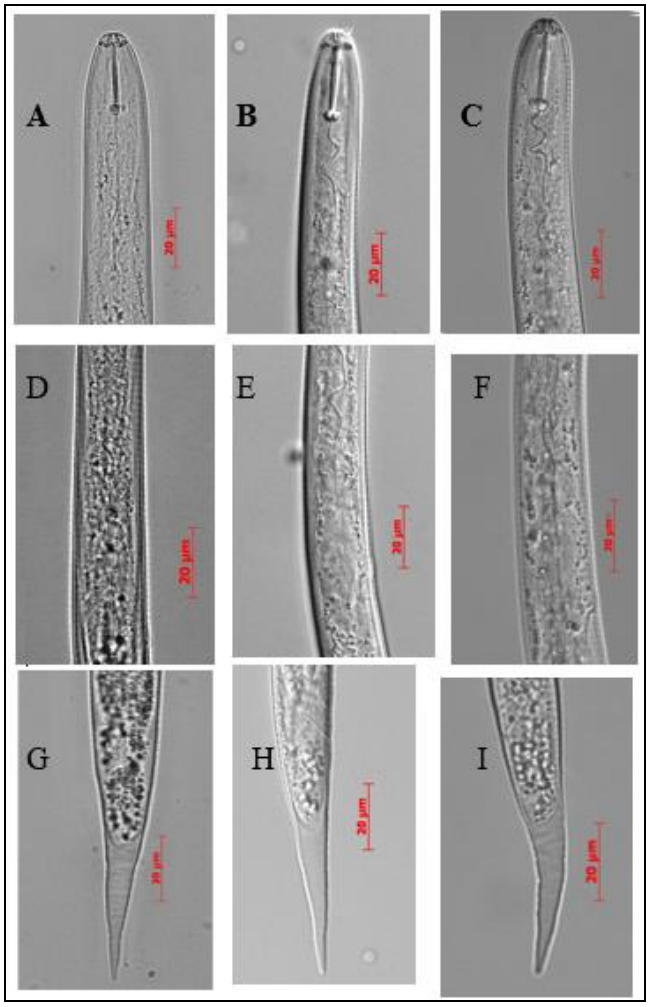

Fig 2: Head, esophagus and tail second stage juveniles of cereal cyst nematode from Delhi (A, D, G), Kangra (B, E, H) and (C, F, I) Leh population

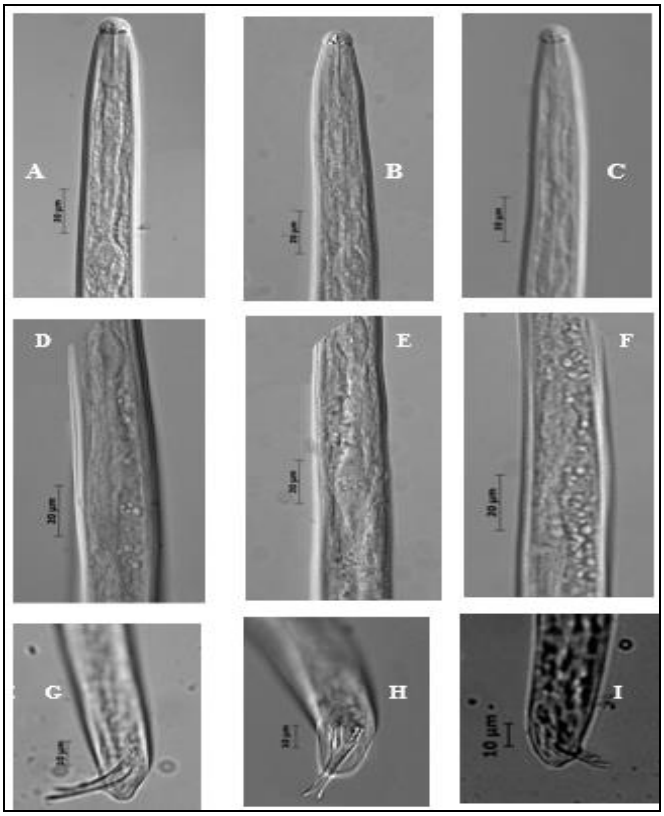

Fig 3: Head, esophagus and tail region of males of cereal cyst nematode from Delhi (A, D, G), Kangra (B, E, H) and (C, F, I) Leh population

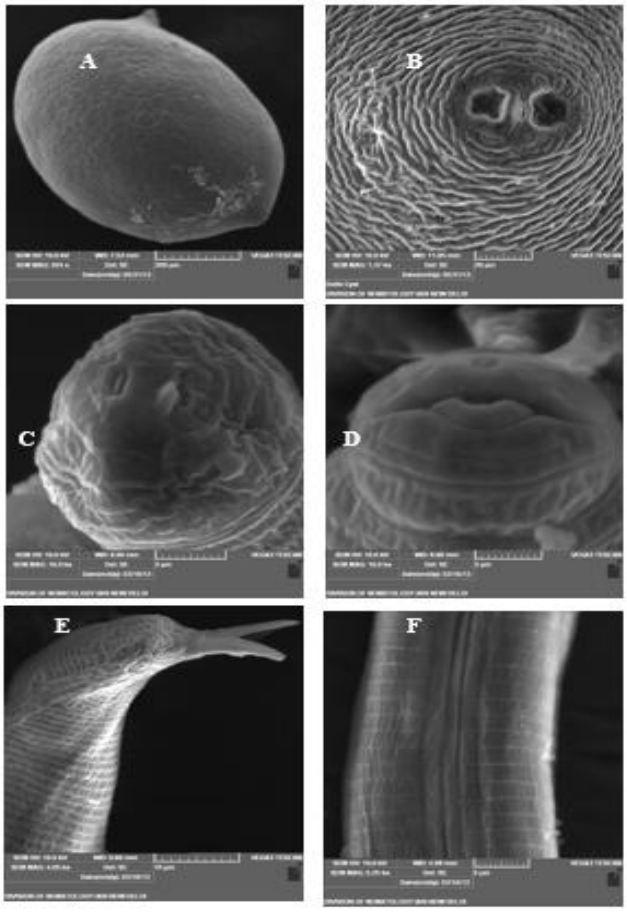

Fig 4: SEM images of cyst shape, anterior portion of cyst, Labial region, spicule and annules morphology of cereal cyst nematode from Delhi (A, B, C, D, E, & F), population

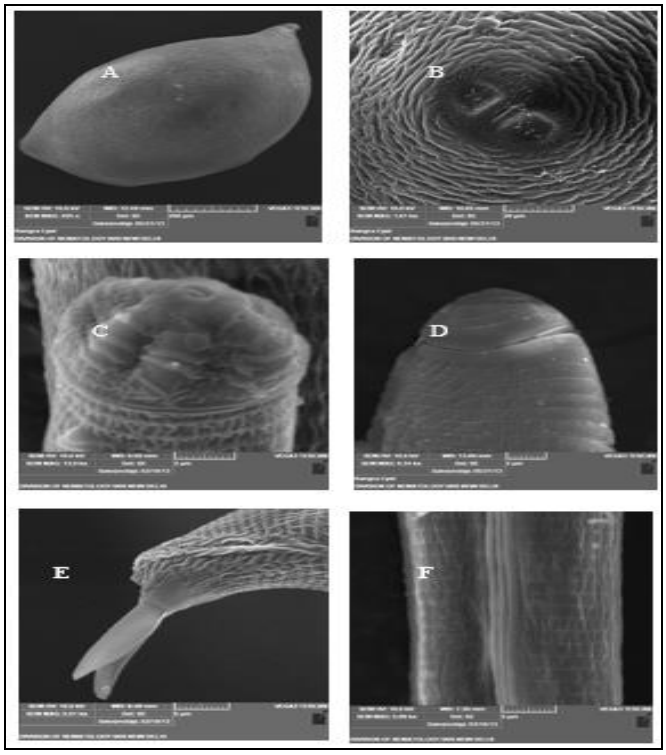

Fig 5: SEM images of cyst shape, anterior portion of cyst, Labial region, spicule and annules morphology of cereal cyst nematode from Kangra (A, B, C, D, E, & F), population

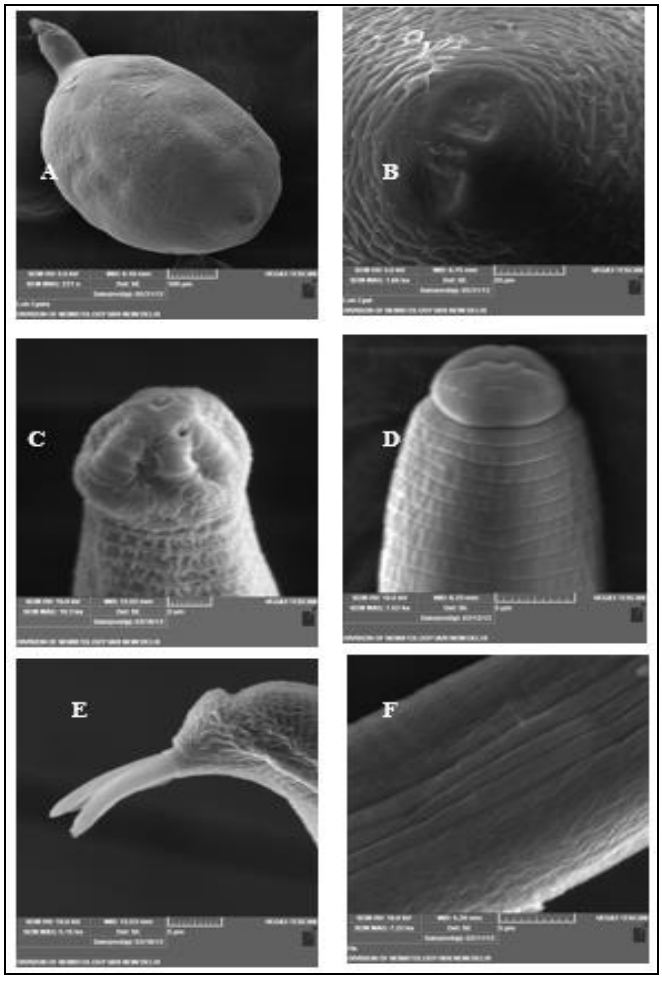

Fig 6: SEM images of cyst shape, anterior portion of cyst, Labial region, spicule and annules morphology of cereal cyst nematode from Leh (A, B, C, D, E, & F), population

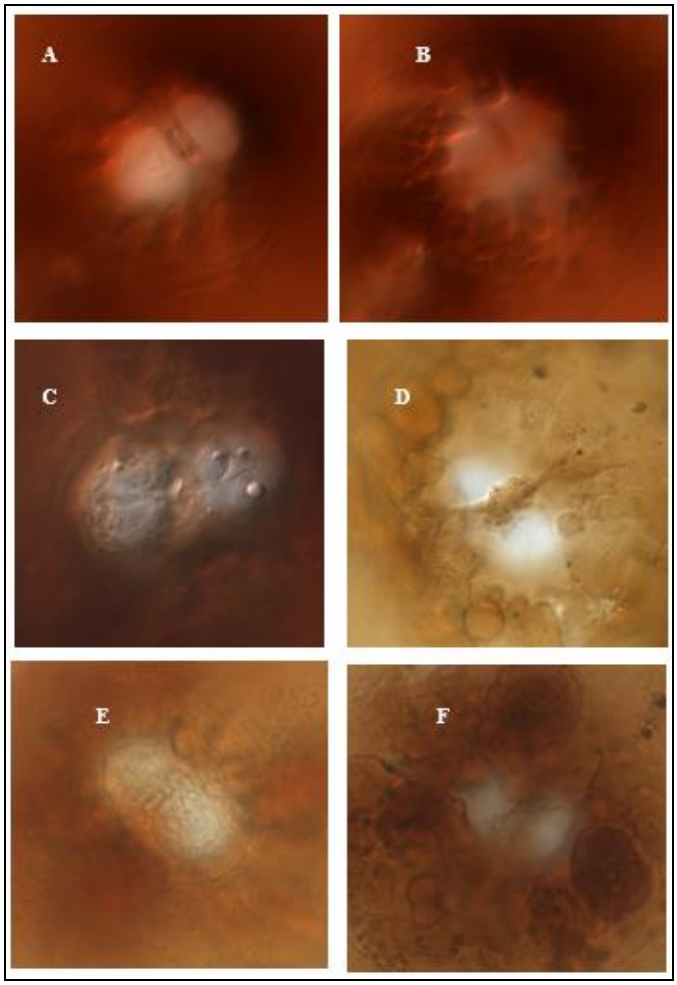

Fig 7: Cone top structure of cereal cyst nematode from Delhi (A, B), Kangra (C, D) and Leh (E, F) population.

Conclusion

Amongst the three populations of cereal cyst nematode studied Delhi's population was undoubtedly *H. avenae.* Kangra population from Tihri village was *H. filipjevi* and not *H. iri* as reported earlier. Morphological, morphometric and ITS sequence data of Leh population was suggested to be a new variant of (species?). Further detailed studies required for confirmation of new species of *H. avenae* complex in Leh population

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