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Notes on *Steinernema abbasi* (Rhabditida: Steinernematidae) strains and virulence tests against lepidopteran and coleopterans pests

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Abstract
Three populations (DS4, DS6 and DS7) of entomopathogenic nematodes were isolated from the agricultural lands of district Meerut of western Uttar Pradesh, India. Morphological characters especially presence of horn like structures on labial region indicated that the strains were closely related to the "*bicornutum*" group' of *Steinernema* spp. The nematodes were conspecific to *Steinernema abbasi* based on morphology, morphometric and molecular analysis. The morphology was similar to the type population with only difference being the presence of mucron in second generation male not observed in original population. The analysis of ITS rDNA sequences revealed that at positions, 211 and 407, T and A are present in studied strains while in the type species, AY230158 two unambiguous sequences Y and R are present at same locations. No difference was observed in D2-D3 domain of 28S rRNA. The Indian strains were also tested positively for its virulence against four major pests, namely, *Galleria mellonella*, *Helicoverpa armigera*, *Spodoptera litura* and *Holotrichia serrata* with good efficacy on the virulence except *H. serrata*. Strain DS7 was more pathogenic compared with other two strains with LD50 values of 7.28, 5.65 and 17.65 IJs, respectively against *G. mellonella*, *H. armigera* and *S. litura*.

Keywords: *Bicornutum*, 28S rRNA, entomopathogenic nematode, ITS rDNA

Introduction
India is agriculture diverse country and every year there are huge losses to the agricultural crops by larval stages of many above ground and soil dwelling insect pests. Chemical pesticides although help in bringing down pest population, but simultaneously they take a heavy toll on our environment and human health and resistance development in insect pests. A recent United Nations report (2017) assessed that 2 lakh people across the world die per year from toxic exposure of pesticides and cancer problems are increasing from past few years which are directly or indirectly linked to pesticide poisoning (https://www.aljazeera.com/news/2017/03/ 200000 -die-year-pesticide-poisoning-170308140641105.html). Entomopathogenic nematodes (EPNs) being eco-friendly and safe to other non-target organisms are exceptional parasites for soil-dwelling stages of many insect pests and are fast acting (within 24–48 h) homicide target insect pests as compared to the other biological control agents that took longer time to kill their hosts (Istkhar *et al.*, 2016; Kaya and Gaugler, 1993; Denno *et al.*, 2008; Chaubey *et al.*, 2016) [23, 24, 26, 14, 11]. Because of broad spectrum of target hosts from the class Insecta, their application as a way of biological control of plants against insect pests is so far very well known (Kaya and Gaugler, 1993) [26].
EPNs especially *Steinernema* and *Heterorhabditis* species are engaged as biocontrol agents of insect pests due to their mutualistic association with enteriobacteriace bacteria, *Xenorhabdus* and *Photorhabdus*, respectively and together are lethal duo (Gaugler & Kaya, 1990; Burnell& Stock, 2000; Kaya *et al.*, 2006) [19, 8]. They have a broad host range, are easy to apply and compatible with some insecticides (Keshari *et al.*, 2019; Istkhar *et al.*, 2016) [23, 24]. Large-scale application and demonstration of EPNs as biological control agents has been dominated in the western countries covering thousands hectares of land, but in India, no species is commercialized up to this level. Native species of EPNs that are adapted to local environmental and climatic conditions are especially good candidates for use as biological control agents as they may provide outstanding results.
In India, little EPN diversity has been reported during the surveys and till date only 17 valid species (14 *Steinernema* and 3 *Heterorhabditis*) have described from a list 113 species known globally (Bhat *et al.*,2017, 2018) [7, 5].

Discovery of new species and strains of EPN is a continuous process and it is assumed that lots of species are waiting for their discovery. Identification of nematodes on the basis of morphological features and their measurements along with molecular tools has proven helpful in resolving ambiguities among the existing and newer species of EPNs (Bhat *et al.*, 2016) [4]. Therefore, the present study was taken to isolate the local strains of EPNs and to test their virulence against insect pests so that they may be implemented in Indian IPM system in future.

Materials and methods

Insect Culture

Four insect pests were used in the study. Larvae of *Galleria mellonella* (Fabricius, 1798) (Lepidoptera: Pyralidae) were taken from Bio-control Lab, Sardar Vallabhbhai Patel University of Agriculture and Technology, Modipuram and were fed on semisynthetic diet as described by David and Kurup (1988). Larvae and eggs of *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae) (National accession no. NBAII-MP-NOC-01) and *Spodoptera litura* (Fabricius, 1775) (Lepidoptera: Noctuidae) (National accession no. NBAII-MP-NOC-02) were purchased from ICAR-National Bureau of Agriculturally Important Insects (NBAII) Bangalore, the former insect were fed on chickpea based diet as described Nagarkatti and Prakash (1974) [31], modified by Kalia *et al*., (2001) [1], while later were fed on properly washed and well sterilized castor leaves. The *Holotrichia serrata* (Hope, 1837) (Coleoptera: Scarabaeidae) were taken from agricultural fields and reared in lab. Larvae of these insects were used for bioassay experimentation; however, *G. mellonella* larvae were also used as a bait insect for nematode isolation.

Isolation and culture of nematodes

Soil samples (n=80) were collected from agricultural lands of three districts, Baghpat (28°94' N, 77°23' E and 253 m ASL), Meerut (28°59′N, 77°42′E and 225 m ASL) and Muzaffarnagar (29°47' N, 77°71 ' E and 248 m ASL) of Western Uttar Pradesh, India. The soil samples were processed for presence of EPNs by *Galleria* baiting technique (Bedding and Akhurst, 1974) and eight samples were found positive for EPNs, three of which belong to *Steinernema* and rest were found to belong to genus *Oscheius*. The *Steinernema* species were processed for further studies and were designated as DS4, DS6 and DS7. They were respectively collected from Baghpat *(Saccharum officinarum*), Meerut *(Saccharum officinarum*) and Muzaffarnagar (*Saccharum officinarum*). Their live cultures were maintained by recycling through *G. mellonella* larvae and stored in 150 ml of sterilized distilled water in 500 ml vented tissue culture flasks at 15 °C.

Morpho-taxometrical characterisation

For morphology and morphometric studies, the 3rd stage juveniles (about 500) were infected to last instar larvae of *G. mellonella* and first and second generation adults (♂ and ♀) were collected by dissecting cadavers on 3rd and 5th day after infection. However, the infective juveniles (IJs) were obtained from White trap (White, 1927) [43]. The different generations were killed separately with hot Ringer's solutions, fixed in tri-ethanol amine formalin solution for fortnight (Courtney *et al*., 1955) [12], dehydrated by Seinhorst method (Seinhorst, 1959) [36] and finally kept in glycerol. They were mounted on a glass slide in glycerine drop using paraffin wax. Morphological observations were made using a light compound microscope (Magnus MLX) and phase contrast microscope (Nikon Eclipse 50i). Morphometrics was done with the help of the inbuilt software of a phase contrast microscope (Nikon DS-L1).

DNA extraction, amplification, and sequencing

For molecular studies, DNA was extracted from 50 infective juveniles using a DNA extraction buffer (ddH_2O 17.7 µl, 10X PCR buffer 2 µl, 1% tween 0.2 µl and proteinase K 0.1 µl). The IJs were disinfected by placing them in 0.1 % NaOCl for 1 hour, washed thrice with ddH_2O and transferred into a sterile 0.5 ml Eppendorf tubes containing 20 µl extraction buffers. The buffer with IJs were first frozen at -80°C for 10 minutes and then immediately incubated in water bath at 65°C for 1.2 hr followed by incubation at 95°C for 10 minutes. The lysates were cooled on ice and centrifuged at 13000 rpm for 2 minutes and 3 µl of this was used for PCR amplification.

For PCR amplification, a fragment of rDNA containing the internal transcribed spacer regions (ITS1, 5.8S, ITS2) was amplified using primers 18S: 5'-TTGATTACGTCCCTGCCCTTT-3' (forward), and 28S: 5'-TTTCACTCGCCGTTACTAAGG-3' (reverse) (Vrain *et al*. 1992) [42]. (Vrain *et al.*, 1992) [42]. The rDNA fragment containing D2D3 regions of 28S rDNA was amplified using primers D2F: 5'-CCTTAGTAACGGCGAGTGAAA-3' (forward) and 536: 5' -CAGCTATCCTGAGGAAAC-3' (reverse) (Nadler *et al.*, 2006). The 25 µl PCR reaction mixture consisted of Dream Taq green PCR master mix 12.5 µl, 1 µl of each forward and reverse primers, nuclease free dH_2O 7.5 µl and 3 µl of DNA-extract. The PCR profiles were used as follows. For ITS: 1 cycle of 94 °C for 3 min followed by 40 cycles of 94°C for 30 s, 55 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 7 min. For D2-D3 fragment of 28S rDNA: 1 cycle of 94 °C for 3 min followed by 40 cycles of 94°C for 30 s, 52 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 10 min. PCR was followed by electrophoresis (45 min, 100 V) of 5 µl of PCR product in a 1% TAE (Tris–-acetic acid–EDTA) buffered agarose gel stained with ethidium bromide (2µl EtBr per 100 ml of gel). All PCR-products were sequenced using ABI 3730 (48 capillary) electrophoresis instrument by Bioserve Pvt. Ltd (Hyderabad, India) and sequencing results were submitted to NCBI with accession numbers MK641593, MF927779 and MK273198 for ITS rRNA of *Steinernema* strains DS4, DS6 and DS7 respectively and MK643331 for D2D3 rRNA of DS7 strain.

Sequence alignment and phylogenetic analysis

The sequences were edited and compared with those deposited in GenBank by means of a Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI). All alignments with other relevant sequences were produced by default ClustalW parameters in MEGA 7.0 (Kumar *et al.*, 2016) [28]. The Phylogenetic trees of the ITS and 28S rRNA were obtained by the minimum evolution method in MEGA 7.0 (Kumar *et al.*, 2016) [28]. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) [32] and are expressed as the number of base differences per site. Pairwise distances were computed using MEGA 7.0 (Kumar *et al*., 2016) [28]. Codon positions included were 1st + 2nd + 3rd + Noncoding.

All characters were treated as equally weighted and gaps as missing data. *C. elegans* was used as out-group taxa and to root the trees.

Virulence tests on *Spodoptera litura* and *Helicoverpa armigera*

Laboratory bioassay trails of the *Steinernema* isolates viz., DS4, DS6 and DS7 against four pests, *Spodoptera litura*, *Helicoverpa armigera*, *Galleria mellonella* and *Holotrichia serrata* were carried out in six well plates (well size 3.5 cm) lined with a double layer of Whatman Filter Paper No. 1. For bioassay experimentation, 1 week old IJs were used and four different concentrations viz. 0, 25, 50, 100 and 200 IJs were placed over the filter paper using 450 µl with distilled water in case of above insect pests except *H. serrata*. For each concentration, ten insect larvae of the same size and weight were used and a single larva was set per well. The plates were incubated at 27 ± 2 °C. Mortality was recorded at every 12-hr interval till complete mortality was achieved.

For *H. serrata*, soil bioassay experiments were carried out in 100 ml beakers using concentrations 250, 500, 1000, 2000 IJs/beaker. Autoclaved soil was used and moistened with ddH_20 and here also for each concentration, ten insect larvae of the same size and weight were used and a single larva was set per beaker. However, mortality was recorded at every 24 h interval and continued for 5 days.

Larvae infected with 100 IJs/larva doses (preferred optimum dose for counting progeny) were transferred after 7 days to a modified White trap (White, 1927) [43] to observe the persistence of infection and emergence of IJs (18–20 days). The counting of the nematodes was done with the help of counting dish in 1 ml suspension as described by Bhat *et al.*, (2016) [4]. Each bioassay was placed separately and all experiments repeated twice along with control.

The insect larval mortality assay was analysed statistically through probit analysis, and LD_{50} values were calculated at a 95% confidence limit. Differences between percentages of mortality depending on different isolates were assessed using SPSS and PRISM8 (https://www.graphpad.com/guides/prism/8/user-guide/index.htm?new-organization.htm). Data was presented as a percentage ± SD. Nematode progeny production was analysed by analysis of variance in the same programs and presented in the form of mean ± SD.

Results and Discussion

Morphology, morphometry and molecular data of the strains DS4, DS6 and DS7 of *Steinernema abbasi* as observed during the present investigation was, in general, similar to the type population in the original species description (Elawad, Ahmed & Reid, 1997) [15] and hence described as the same. The slides as well as live cultures of these strains have been deposited in the depository of Department of Zoology, Chaudhary Charan Singh University, Meerut. This species was first reported from soils of alfalfa fields in the south of the Sultanate of Oman by Dr. M. A. Hubeis. The species has later also been found in agricultural soils of Pakistan, Nepal and Egypt and some other Asian countries like Philippines, China, Palestine (GenBank records). In India, it has, however, been reported from agricultural soils of almost all the states and is found to be dominant and abundant species of *Steinernema* in Indian subcontinent. *Steinernema thermophilum* previously a new species by Ganguly and Singh (2000) [18] has been synonymized to be a species of *S. abbasi*.

Morphology and morphometry

Steinernema isolates DS4, DS6 and DS7, obtained during the present investigation, were identified as *S. abbasi*; however, some disparity with the original report may be noted. The morphology was similar to the type population with only difference being the presence of mucron in second generation male which was not observed in original description. The tail was more sharply pointed with anal swelling, thus confirming the previous observations.

Morphometric measurements of all the generations of isolates DS4, DS6 and DS7 were similar to the topotype population of *S. abbasi* (Shahina *et al.*, 2001) [37] but little divergence is seen when they are compared with each other or with the original description (tables 1-3). A comparison in morphometric parameters in all generations is given in table 4.

Molecular characterisation

The internal transcribed spacer rRNA sequences of the *Steinernema* strains DS4, DS6 and DS7 do not have any difference with the ITS sequence of already described *S. abbasi* (AY230158) and also with each other. However, two unambiguous sequences Y and R are seen in the type species, AY230158 at positions, 211 and 407, in place of T and A in present three strains. ITS sequence of DS4, DS6 and DS7 were separated from other closely related species of "*bicornutum*" group by 19–231 bp (table 5).

In the D2-D3 rRNA sequences also, the present strain DS7 do not show any variation with type populations of *S. abbasi* (AF331890). The D2 and D3 expansion fragments of the 28S rRNA gene of the Indian strain were separated by 12–122 bp from other closely related species of "*bicornutum*" group (table 6).

Phylogenetic analysis

The phylogeny of the present three strains DS4, DS6 and DS7 based on ITS rRNA showed a clear monophyly of the group formed by the present three isolates and described *S. abbasi* (AF331890) (Fig. 1). Sequences of *S. abbasi* formed a monophyletic group with *Steinernema kandii* Godjo,Afouda, Baimey, Couvreur, Zadji, Houssou, Bert, Willems and Decraemer (2018) and this pair was sister to the *Steinernema yirgalemense* Gaugler, Tesfamariam, Adams, Gozel and Nguyen (2004) [33].

In the D2-D3 tree, strain DS7 formed a monophyletic group with type population, *S. abbasi* and formed a sister clad with *S. kandii* Godjo, Afouda, Baimey, Couvreur, Zadji, Houssou, Bert, Willems and Decraemer (2018) and *Steinernema bifurcatum* Fayyaz, Yan, Qui, Han, Gulsher, Khanum and Javed (2014) (Fig. 2). However, the D2-D3 region is too conservative to resolve the relationships among these closely related *Steinernema* species. In general, molecular data accompanied by morphology and morphometric data confirmed the status of studied three strains as species of *S. abbasi* according to the phylogenetic and evolutionary species concept (Adams, 1998) [1].

Virulence tests

All three strains of *S. abbasi* were able to kill and reproduce in all four of the insects tested. All the three strains led to complete mortality of the tested insects except for *H. serrata*, in which not more than 50-60% mortality was recorded even after six days of infection.

In case of *H. armigera* larvae, the parameters measured when

the nematodes were applied favoured all the three strains, *viz*., DS4, DS6 and DS7 and caused complete mortality within 48 hours of infection with all the doses applied (Fig. 3 A-C). All the strains started the mortality only after 12 h of infection with 100 and 200 IJs/larva doses while with 25 and 50 IJs/larva dose, death was noticed after 24 h except DS4. In case of *S. litura* larvae, all the strains started larval mortality at 12 h with 50, 100 and 200 IJs/larva doses, and lead to complete death of insect pests after 36 h post infection period with these three doses, however, with 25 IJs/larva dose, death started at 24 h and complete mortality was recorded after 48 h (Fig. 3D-F). With the same insects, and with comparable doses of *S. abbasi*, Kalia *et al.* (2014) [22] observed that 100% mortality occurred no earlier than 96 h in *H. armigera* and 192 h in *S. litura*.

When the IJs of the strains were applied to larvae of *G. mellonella*, mortality was found to begin at 12 h with all doses in case of DS4; however, in DS6 and DS7, it began with only 50, 100 and 200 IJs/larva doses. In all the three cases, complete mortality was seen after 48 h (Fig. 3 G-I). With same insect and doses of *S. abbasi*, Istkhar *et al.*, (2016) [23, 24] and Bhat *et al.*, (2015) [6] observed that complete mortality occurred no earlier than 48 h and 60 h respectively. Also, Chaubey *et al.*, (2016) [11] and Bhat *et al.*, (2016) [4] with same insects and doses of *H. indica* noticed that insect mortality completed after 60 h of infection.

The mortality of *S. litura*, *H. armigera* and *G. mellonella* caused by present strains showed differences, however, the onset of mortality was very rapid in all the three tested insects. At 36 h, the strain DS4, DS6 and DS7 were capable of killing *G. mellonella* larvae with LD_{50} being at 12.35, 10.37 and 7.28 IJs, respectively; for *H. armigera* larvae at 18.01, 15.98 and 5.65 IJs; for *S. litura* larvae at 34.5, 14.9 and17.53 IJs. From these above findings, DS7 was found pathogenic against tested insects. These virulence differences against the tested entomic pests might be due to host-specificity, with such differences previously noted by other investigators (Bhat *et al.*, 2017; Shapiro-Ilan *et al.*, 2003) [7, 38, 41]. Campos-Herrera *et al.*, (2006) [29] reported differences in virulence tests of *S. feltiae* to three different hosts; Shapiro-Ilan *et al.*, (2003) [38, 41] observed considerable variation in infectivity and mortality of *Curculio caryae*, Horn (Coleoptera: Curculionidae) using different strains of *S. carpocapsae*. Bhat *et al.*, (2017) [7] also noted differences in efficacy tests of two *S. surkhetense* strains against larvae of two different insect pests. Two *S. pakistanense* strains were also reported to show differences in their virulence against different larvae of insect pests (Bhat *et al.*, 2018) [5]. Such differences in virulence of EPN species/strains depend on factors such as the rate of penetration, reproductive potential, type of host, bacterium complex and some other biotic and abiotic factors, niche, presence-absence of determined host, adaptation to abiotic factors, etc. (Kaya & Gaugler, 1993; Lewis *et al.*, 1992; Forschler & Nordin, 1988) [26, 30, 17].

The higher progeny production was observed in case *G. mellonella* in all the three strains, maximum in DS6 strain (147122 ± 8445) followed by DS7 (142280 ± 9528) and DS6 (134488 ± 6182). In case of *H. armigera* and *S. litura*, IJ production was in close proximity to all the strains and very low when compared to *G. mellonella* (Fig. 4).

Furthermore, pathogenicity bioassays of the three strains against *H. serrata* showed that the complete mortality was not observed even after 6 days of infection with all the doses (250, 500, 1000, 2000 IJs/larvae) applied. Mortality was found to occur on day 2 with all doses except 250 IJs/larva which began on day 3. All the three strains show somewhat equal results and the dynamics of the infection according to different doses also showed that the strains achieved somewhat near mortality rates when the target was *H. serrata* (Fig. 5.A-C); the best example of this could be observed when 250 IJs of DS6 were applied to the larvae, where at day 4, the overall percentage of mortality was 30%; at day 5, the percentage of mortality increased to 40% (Fig. 5 B), however, did not reach to 100% mortality even after 6th day. With highest doses (2000 IJs/larva), the mortality at day 5 was recorded 50% with DS4 and DS6, while 60% with DS7. These findings are in accordance with the results of Shelter *et al.* (1988) [39], Ansari *et al.* (2003) [2], Pillay *et al.* (2009) [35], Glazer *et al.* (2007) [20], Supekar and Mohite (2015) [40] and Chandel *et al.* (2005) [10] which too do not noticed complete mortality in white grubs with *Steinernema* species, and found good pathogenicity results of *Heterorhabditis* species against white grubs.

These findings concluded that these strains can be used for control of above three pests, however may not be good for control of white grubs.

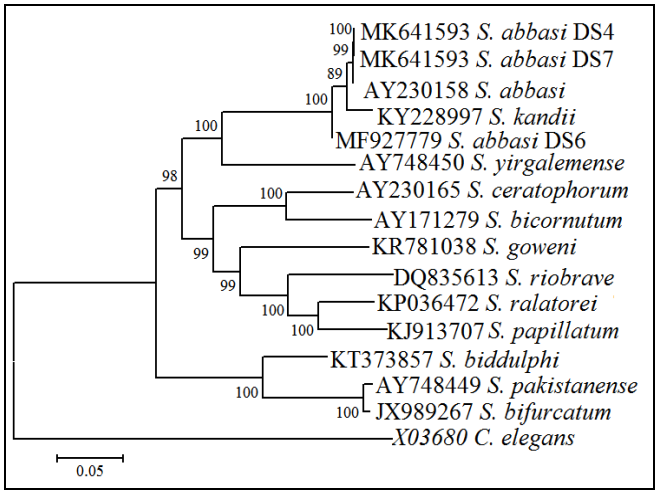

Fig 1: Phylogenetic relationships in the '*bicornutum'* group of the *Steinernema* based on analysis of ITS rRNA regions. *Caenorhabditis elegans* was used as out-group taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of number of base differences per site.

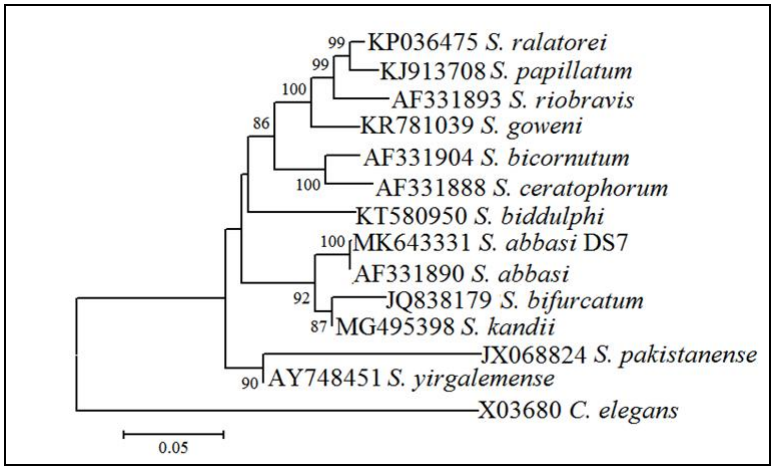

Fig 2: Phylogenetic relationships in the '*bicornutum'* group of the *Steinernema* based on analysis of D2–D3 regions of the 28S rRNA regions. *Caenorhabditis elegans* was used as out-group taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of number of base differences per site.

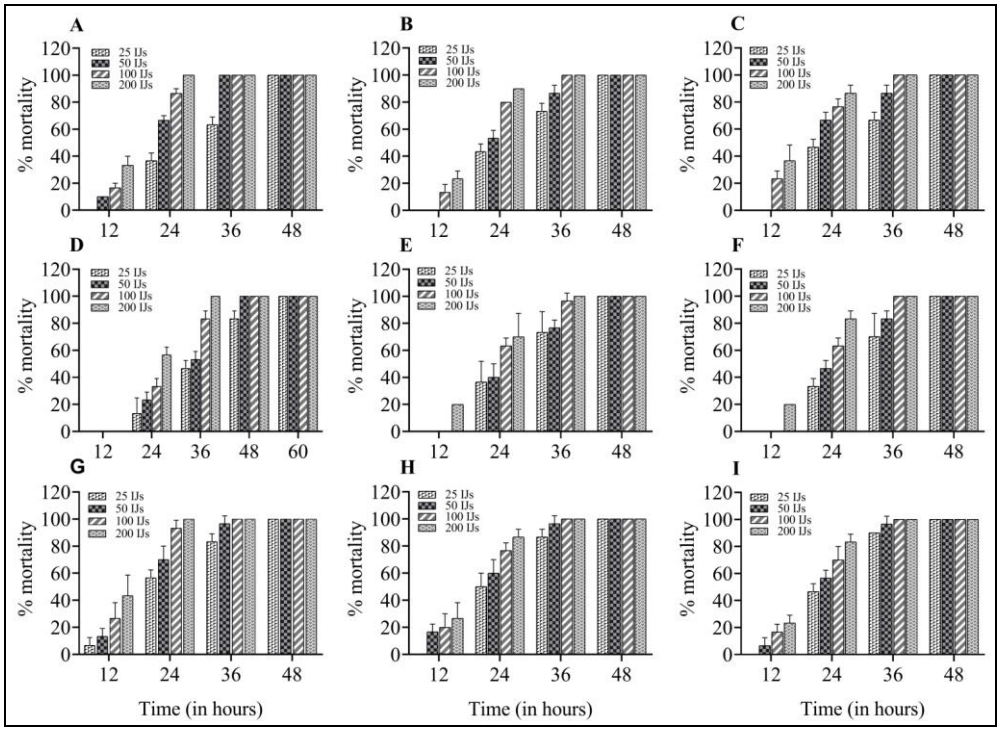

Fig 3: Percentage mortality of *Helicoverpa armigera* (A-C), *Spodoptera litura* (D-F) and *Galleria mellonella* (G-H) larvae with different doses of *Steinernema abbasi* strains DS4 (A, D, G), DS6 (B, E, H) and DS7 (C, F, I).

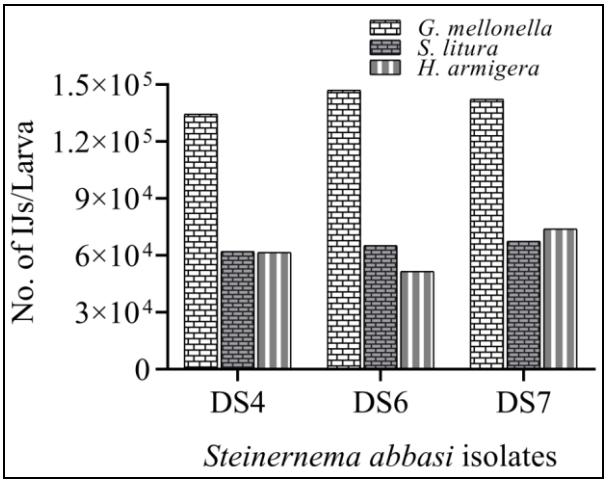

Fig 4: Mean IJs production of *Steinernema abbasi* strains DS4, DS6 and DS7 in *Helicoverpa armigera*, *Spodoptera litura* and *Galleria mellonella* at 100 IJ/Larva doses (D).

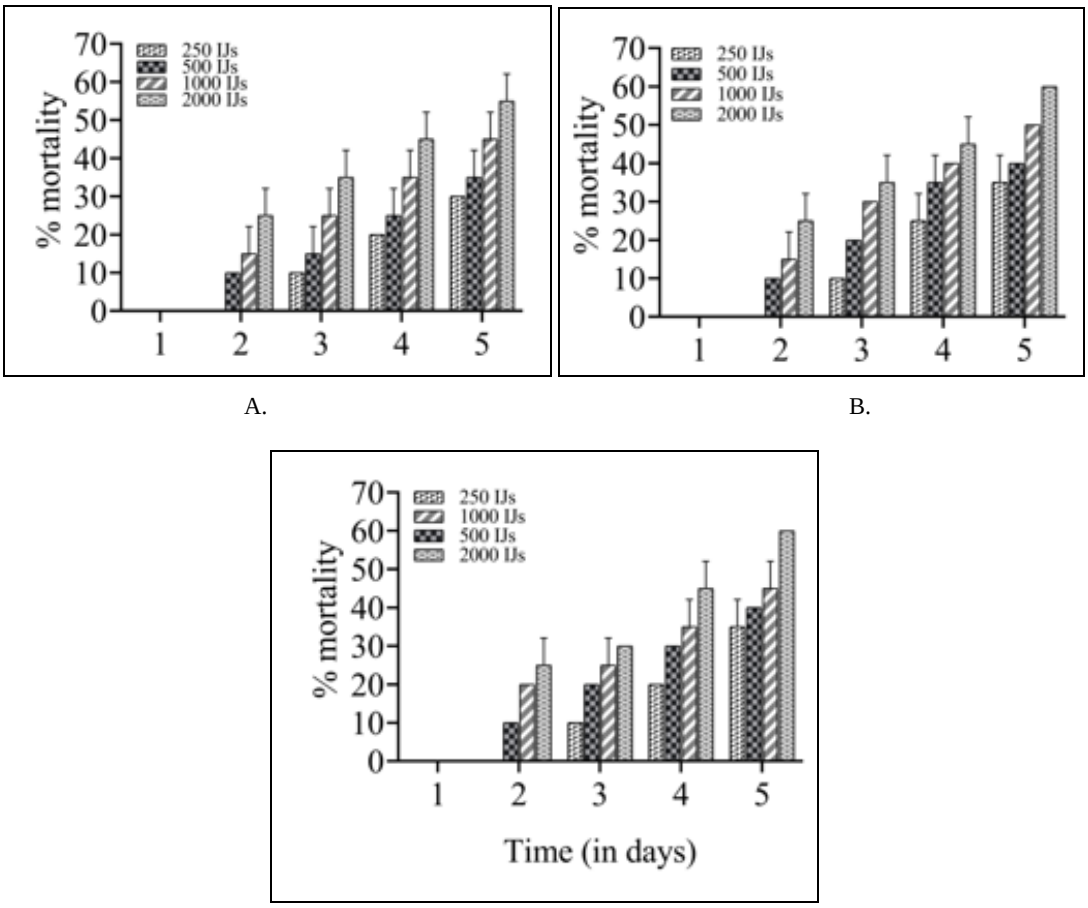

Fig 5: Percentage mortality of *Holotrichia serrata* (A-C), larvae with different doses of *Steinernema abbasi* strains DS4 (A), DS6 (B) and DS7 (C).

Table 1: Morphometrics of *Steinernema abbasi* DS4. Measurements are in µm and in the form: mean ± SD (range).

Characters	First Generation		Second Generation		Infective Juveniles
	Male	Female	Male	Female	
N	20	20	20	20	25
Body Length (L)	1370±133 (1149-1617)	7195±1049 (5351-9537)	934±99 (555-1027)	1085±107 (908-1269)	593± 43 (456-653)
L' ((L-T)	1347±133 (1123-1592)	7148±1052 (5310-9484)	910±98 (534-1006)	1036±108 (855-1218)	537± 42 (401-601)
a (L/BD)	12±1.3 (11-15.4)	27±6.9 (3.1-36)	16±2 (10-20)	56±1.9 (52-59)	25± 1.9 (20-28)
b (L/ES)	9.5±0.8 (8.4-11)	33±4.9 (25-42)	8.8±1.2 (4.8-10)	151 ±3 (13-18)	7.1± 0.8 (5.5-8.6)
c (L/T)	60±10.3 (44-67)	165±45 (60-256)	40±5 (26-49)	8.1± 0.7 (6.9-9.1)	7.1± 0.8 (8.3-13)
c' (T/ABW)	0.8±0.2 (0.6-1.6)	0.6±0.2 (0.01-0.9)	1.4±0.2 (1-1.8)	23± 5.7 (17-40)	4.5± 0.4 (3.8-5.7)
V (V'/L)100		51±2.7 (46-55)		56±1.9 (52-59)	
Body Diameter (BD)	111±15 (91-135)	364±514 (222-2546)	57±4.6 (50-69)	70±7.7 (51-83)	23± 1.3 (20-28)
Excretory Pore (EP)	81 ± 8.1 (63-94)	89±7.1 (72-100)	55±5.5 (46-65)	58±7.7 (46-73.6)	47± 9.3 (36-85)
Width at EP (WEP)	46±5.2 (37-57)	92±8 (79-104)	37±57 (22-278)	36±5.3 (26-47)	15±1.9 (10-19)
Nerve Ring (NR)	82±6.9 (66-96)	120±9.3 (104-145)	58±5.9 (46-70)	76±5.3 (70-90)	54± 3.9 (49-63)
Pharynx Length (ES)	144±8.3 (130-169)	218±11 (202-240)	107±6.4 (97-121)	134±5.9 (124-142)	84± 8.3 (70-105)
Bulb Length (EBL)	33±3.8 (24-39)	56±5.7 (46-66)	27±2.8 (23-34)	32±2.9 (27-36)	13± 2.1 (8.1-17)
Bulb Width (EBW)	24±1.9 (21-29)	40±3.1 (34-48)	20±1.5 (17-22)	23±1.8 (21-27)	8.2± 1.1 (6.1-11)
Testis Reflection (TR)	242±48 (137-327)		215±28 (172-283)		
Tail	23±3.6 (16-31)	47±17 (33-111)	23±2.2 (21-27)	49±8.5 (27-65)	57± 3.7 (50-66.6)
Anal Body Width (ABW)	31±5.3 (17-42)	415±1524 (52-6887)	17±2.5 (14-26)	29±5.7 (24-51)	13± 1.1 (10-15)
Spicule Length (SPL)	68±6 (57-80)		60±4.9 (53-72)		
Spicule Width (SPW)	9.1±1.6 (6.6-12)		11±1.8 (6.3-13)		
Gubernaculum Length (GL)	39±5 (32-54)		30±2.4 (23-34)		
Gubernaculum Width (GW)	7.2±1.2 (5.5-9.6)		6.8±1 (3.7-8.2)		
D% (EP/ES×100)	56±5.9 (44-67)	41±4 (32-47)	51±5.7 (40-60)	1.7± 0.3 (1.1-2)	56± 8 (41-80)
E% (EP/T×100)	355±57 (268-476)	201±41 (87-258)	238±35 (179-310)	43± 6.3 (33-56)	84± 17 (54-154)
SW% (SL/ABD*100)	225±47 (160-376)		362±50 (215-473)		
GS% (GL/SL×100)	58±9.5 (41-85)		50±4.1 (41-60)		
Width at Vulva (WV)		268±22 (218-328)		78±8.5 (59-96)	
Anterior to Vulva (AV)		3654±590 (2763-5044)		604±63 (511-720)	
Posterior to Vulva (PV)		3541±528 (2588-4495)		682±896 (393-4482)	

Table 2: Morphometrics of *Steinernema abbasi* DS6. Measurements are in µm and in the form: mean ± SD (range).

	First generation		Second generation		
Characters	**Male**	**Female**	**Male**	**Female**	**Juveniles**
N	**20**	20	20	20	20
Body Length (L)	1348 ± 125 (1197-1668)	5679 ± 1073 (4222-8949)	890 ± 70 (801-1085)	1218 ± 83 (1042-1358)	438 ± 28 (383-488)
L' ((L-T)	1324 ± 126 (1168-1665)	5636 ± 1072 (4182-8907)	867 ± 69 (779-1059)	1168 ± 82 (1001-1307)	394 ± 26 (343-446)
a (L/BD)	12 ± 1.16 (10-14)	23 ± 3.8 (18-34)	15 ± 1.9 (12-20)	17 ± 1.5 (14-20)	19 ± 1.3 (17-22)
b (L/ES)	10 ± 0.9 (8.6-12)	32 ± 5.4 (25-46)	8.2 ± 0.83 (7.2-10)	9.0 ± 0.7 (7.7 -10)	6.0 ± 0.8 (4.4-7.2)
c (L/T)	58 ± 9.2 (40-71)	133 ± 28 (90-213)	38 ± 3.7 (32-44)	24 ± 3.5 (19-32)	10.0 ± 0.8 (8.2-11.2)
c' (T/ABW)	0.71 ± 0.07 (0.61-0.9)	0.6 ± 0.09 (0.5-0.8)	1.3 ± 0.16 (1.0-1.6)	1.7 ± 0.2 (0.9-2.2)	3.4 ± 0.4 (2.2-4.0)
V (V'/L)×100		53 ± 3.7 (42 - 57)		54 ± 2.3 (52-60)	
Body Diameter (BD)	108 ± 13 (83-143)	238 ± 17 (201-267)	56 ± 4.4 (49-67)	68 ± 3.0 (62-72)	22 ± 1.4 (20-25)
Excretory Pore (EP)	88 ± 4.4 (80-88)	91 ± 10 (72-112)	53 ± 3.9 (48-61)	58 ± 5.3 (49-69)	33 ± 4.6 (26-47)
Width at EP (WEP)	47 ± 4.2 (40-50)	83 ± 11 (49-97)	25 ± 1.9 (21-29)	37 ± 4.1 (29-48)	13 ± 1.0 (11-15)
Nerve Ring (NR)	84 ± 3.2 (77-89)	90 ± 4.8 (81-99)	58 ± 4.8 (48-68)	75 ± 5.3 (61-85)	62 ± 9.6 (49-84)
Pharynx Length (ES)	132 ± 12 (108-163)	177 ± 11 (156-197)	108 ± 5.6 (95-115)	134 ± 4.6 (126-141)	73 ± 9.0 (62-92)
Bulb Length (EBL)	28 ± 4.3 (18-36)	46 ± 3.4 (41-53)	27 ± 2.5 (23-31)	32 ± 2.7 (26-36)	12 ± 2.2 (8.6-18)
Bulb Width (EBW)	23 ± 3.1 (14-28)	34 ± 2.5 (28-38)	21 ± 2.2 (18-25)	23 ± 2.6 (21-29)	8.4 ± 1.4 (6.0-11)
Testis Reflection (TR)			212 ± 18 (189-267)		
Tail	23 ± 3.0 (19-31)	43 ± 6.8 (33-58)	23 ± 2.1 (19-27)	50 ± 6.4 (36-62)	44 ± 4.1 (35-53)
Anal Body Width (ABW)	33 ± 3.5 (27-39)	64 ± 12 (45 - 93)	17 ± 1.6 (15-21)	30 ± 5.2 (25-50)	10 ± 1.46 (35-53)
Spicule Length (SPL)	70 ± 5.7 (56-82)		59 ± 2.9 (55-69)		
Spicule Width (SPW)	9.8 ± 1.6 (5.7-11)		9.2 ± 1.0 (7.3-10)		
Gubernaculum Length (GL)	43 ± 3.9 (37-48)		29 ± 1.9 (24-31)		
Gubernaculum Width (GW)	6.0 ± 1.4 (3.7-8.3)		6.5 ± 0.9 (4.6-8.0)		
D% (EP/ES×100)	67 ± 5.7 (55- 75)	52 ± 6.0 (36-60)	1.3 ± 0.6 (42-58)	43 ± 4.6 (35-53)	46 ± 8.6 (33-75)
E% (EP/T×100)	385 ± 47 (265- 462)	206 ± 63 (2.2-285)	230 ± 27 (189-293)	117 ± 15 (99-143)	77 ± 13 (54-122)
SW% (SL/ABD×100)	215 ± 23 (175-268)		350 ± 37 (271-413)		
GS% (GL/SL×100)	61 ± 7.2 (44- 73)		49 ± 3.7 (41-54)		
Width at Vulva (WV)		254 ± 29 (199-297)		76 ± 6.6 (63-91)	
Anterior to Vulva (AV)		3019 ± 633 (2269-4876)		696 ± 46 (611-793)	
Posterior to Vulva (PV)		2660 ± 511 (1951-4072)		522 ± 522 (411-589)	

Table 3: Morphometrics of *Steinernema abbasi* DS7. Measurements are in µm and in the form: mean ± SD (range).

Characters	**First generation**		**Second generation**		**Infective Juveniles**
	Male	**Female**	**Male**	**Female**	
N	20	20	20	20	20
Body Length (L)	1463 ± 49 (1389-1561)	6632 ± 535 (6012-7969)	884 ± 29 (807-934)	1297 ± 59 (1335-2424)	436 ± 30 (395-495)
L' ((L-T)	1440 ± 49 (1365-1532)	6592 ± 535 (5967-7928)	860 ± 29 (783-909)	1246 ± 59 (1179-1375)	395 ± 31 (353-459)
a (L/BD)	13 ± 1.0 (11-15)	26 ± 2.3 (22-30)	15 ± 1.14 (13-18)	9.5 ± 0.58 (8.3 - 10.6)	24 ± 2.1 (20-27)
b (L/ES)	11 ± 0.68 (9.8-12)	36 ± 3.15 (32-43)	8.2 ± 0.3 (7.-.8.9)	25 ± 2.6 (20-32)	6.1 ± 0.6 (4.7-7.5)
c (L/T)	63 ± 7.0 (45-73)	154 ± 20 (115-195)	36 ± 3.7 (31-42)	1.7 ± 0.26 (1.05-2.09)	8.7 ± 0.9 (6.2-10)
c' (T/ABW)	0.7 ± 0.1 (0.54-1.1)	0.67 ± 0.08 (0.5-0.8)	0.8 ± 0.1 (0.7-1.0)	55 ± 1.8 (51-59)	5.7 ± 1.0 (4.2-9.1)
V (V'/L)×100		52 ± 2.6 (48 - 61)		68 ± 3.9 (59-73)	
Body Diametre (BD)	111 ± 7.4 (95-123)	247 ± 17 (211-289)	57 ± 3.6 (48-63)	57 ± 3.8 (49-64)	23 ± 1.3 (20-26)
Excretory Pore (EP)	90 ± 2.49 (86-95)	93 ± 6.4 (77-102)	53 ± 2.6 (49-56)	62 ± 11 (47-77)	36 ± 3.4 (29-41)
Width at EP (WEP)	49 ± 3.6 (41-53)	85 ± 5.4 (72-95)	25 ± 1.2 (23-28)	71 ± 15 (28-736)	13 ± 1.3 (11-15)
Nerve Ring (NR)	102 ± 5.68 (91-113)	90 ± 3.5 (85-99)	57 ± 3.3 (48-62)	107 ± 14 (69-713)	61 ± 1.7 (54-75)
Pharynx Length (ES)	133 ± 6.9 (119-142)	179 ± 6.7 (166-189)	107 ± 3.5 (98-111)	135 ± 3.2 (128-140)	92 ± 7.4 (78-105)
Bulb Length (EBL)	31 ± 2.25 (27-35)	46 ± 3.2 (40-51)	28 ± 2.6 (24-1.9)	34 ± 3.04 (29-39)	13 ± 1.0 (11-15)
Bulb Width (EBW)	23 ± 2.4 (18-29)	34 ± 2.2 (30-39)	21 ± 2.2 (18-25)	23 ± 2.03 (24-45)	7.8 ± 0.8 (6.6-9.6)
Testis Reflection (TR)	345±42 (278-441)		206±14 (192-250)		
Tail	25 ± 2.8 (20-31)	43 ± 4.7 (36-55)	24 ± 1.9 (21-27)	51 ± 4.4 (39-59)	41 ± 2.9 (35-45)
Anal Body Width (ABW)	32 ± 3.2 (26-41)	66 ± 9.8 (55-89)	17 ± 1.8 (14-22)	30 ± 4.5 (24-45)	22 ± 1.2 (20-25)
Spicule Length (SPL)	67 ± 5.8 (58-78)		55 ± 2.4 (51-60)		
Spicule Width (SPW)	7.2±0.9 (4.6-8.9)				
Gubernaculum Length (GL)	41 ± 2.5 (34-45)		30 ± 3.0 (25-36)		
Gubernaculum Width (GW)	3.8±0.65 (2.7-5.7)				
D% (EP/ES×100)	68 ±3.571 (63- 74)	51 ± 4.4 (42-58)	50 ± 2.8 (44-54)	42 ± 3.9 (35-49)	65 ± 10 (46-83)
E% (EP/T×100)	392 ± 40 (295- 440)	216 ± 29 (149-261)	221 ± 19 (185-265)	113 ± 10.6 (95-127)	93 ± 18 (60-128)
SW% (SL/ABD×100)	208 ± 29 (139-269)		327± 35 (243-378)		
GS% (GL/SL×100)	61 ± 5.9 (48- 70)		53 ± 4.6 (40-58)		
Width at Vulva (WV)		266 ± 15 (227-299)		79 ± 5.2 (68-86)	
Anterior to Vulva (AV)		3481 ± 392 (3098-4876)		722 ± 39 (621-794)	
Posterior to Vulva (PV)		3155 ± 259 (2769-3827)		575 ± 36 (511-686)	

Table 4: Comparative morphometrics (µm) of *Steinernema abbasi* strains with type population. Measurements are in the form: mean (range).

	Infective Juveniles			**First Generation Male**			**Second Generation Male**			**First Generation Female**			**Second Generation Female**		
	DS6	**DS7**	***S. abbasi***	**DS6**	**DS7**	***S. abbasi***	**DS6**	**DS7**	***S. abbasi***	**DS6**	**DS7**	***S. abbasi***	**DS6**	**DS7**	***S. abbasi***
L	438 (383-488)	436 (395-495)	541 (496-579)	1348 (1197-1668)	1463 (1389-1561)	1252 (999-1534)	890 (801-1085)	884 (807-934)	861 (2453-4477)	5679 (4222-8949)	6632 (6012-7969)	3510 (606-1035)	1218 (1042-1358)	1297 (1335-2424)	2069 (1897-3917)
BD	22 (20-25)	23 (20-26)	29 (27-30)	108 (83-143)	111 (95-123)	87 (82-98)	56 (49-67)	57 (48-63)	70 (64-80)	238 (201-267)	247 (211-289)	159 (143-81)	68 (62-72)	57 (49-64)	130 (123-48)
EP	33 (26-47)	36 (29-41)	48 (46-51)	88 (80-88)	90 (86-95)	80 (68-89)	53 (48-61)	53 (49-56)	66 (62-79)	91 (72-112)	93 (77-102)	71 (62-79)	58 (49-69)	62 (47-77)	66 (61-73)
NR	62 (49-84)	61 (54-75)	68 (64-72)	84 (77-89)	102 (91-113)	103 (99-123)	58 (48-68)	57 (48-62)	99 (93-106)	90 (81-99)	90 (85-99)	125 (120-137)	75 (61-85)	107 (69-713)	114 (110-118)
ES	73 (62-92)	92 (78-105)	89 (85-92)	132 (108-163)	133 (119-142)	133 (121-144)	108 (95-115)	107 (98-111)	121 (112-130)	177 (156-197)	179 (166-189)	165 (155-176)	134 (126-141)	135 (128-140)	146 (136-157)
TL	44 (35-53)	41 (35-45)	56 (52-61)	23 (19-31)	25 (20-31)	26 (20-31)	23 (19-27)	24 (21-27)	21 (17-24)	43 (33-58)	43 (36-55)	37 (31-40)	50 (36-62)	51 (39-59)	36 (32-39)
a	19 (17-22)	24 (20-27)	18 (17-20)	12 (10-14)	13 (11-15)		15 (12-20)	15 (13-18)		23 (18-34)	26 (22-30)		17 (14-20)	9.5 (8.3 - 10.6)	
b	6.0 (4.4-7.2)	6.1 (4.7-7.5)	6.0 (5.5-6.6)	10 (8.6-12)	11 (9.8-12)		8.2 (7.2-10)	8.2 (7.-.8.9)		32 (25-46)	36 (32-43)		9.0 (7.7 - 10)	25 (20-32)	
C	10 (8.2-11.2)	8.7 (6.2-10)	9.8 (8.1-10)	58 (40-71)	63 (45-73)		38 (32-44)	36 (31-42)		133 (90-213)	154 (115-195)		24 (19-32)	1.7 (1.05-2.09)	
SL				70 (56-82)	67 (58-78)	65 (57-74)	59 (55-69)	55 (51-60)	61 (51-69)						
GL				43 (37-48)	41 (34-45)	45 (33-50)	29 (24-31)	30 (25-36)	43 (35-48)						
SW%				215 (175-268)	208 (139-269)	156 (107-187)	350 (271-413)	327 (243-378)	159 (128-185)						
GS%				61 (44- 73)	61 (48- 70)	70 (58-85)	49 (41-54)	53 (40-58)	70 (58-81)						
D%	46 (33-75)	65 (46-83)	53 (51-58)	67 (55- 75)		60 (51-68)	1.3 (42-58)	50 (44-54)	56 (50-70)	52 (36-60)	51 (42-58)	42 (36-48)	43 (35-53)	42 (35-49)	45 (43-47)
E%	77 (54-122)	93 (60-128)	86 (79-94)	385 (265- 462)	385 (265- 462)		230 (189-293)	221 (185-265)		206 (2.2-285)	216 (149 -261)		117 (99-143)	113 (95-127)	
V%									55 (52-60)	53 (42 - 57)	52 (48 - 61)		54 (52-60)	68 (59-73)	55 (50-77)

Table 5: Pairwise distances of the ITS region between *Steinernema* species from the '*bicornutum'* group. Below diagonal, percentage similarity; above diagonal, total character differences.

	ITS regions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	MK641593 DS4		0	0	0	19	146	181	196	196	197	201	203	214	231	231
2	MF927779 DS6	100		0	0	15	84	126	142	129	136	149	149	146	155	155
3	MK273198 DS7	100	100		0	19	146	181	196	196	197	201	203	214	231	231
4	AY230158 *S. abbasi*	100	100	100		18	145	176	189	190	190	197	196	208	223	223
5	KY228997 *S. kandii*	97	97	97	98		151	181	191	194	196	197	194	212	222	222
6	AY748450 *S. yirgalemense*	75	81	75	75	74		180	188	201	202	194	189	201	222	221
7	AY230165 *S. ceratophorum*	68	70	68	68	67	66		168	190	87	161	150	167	219	221
8	KR781038 *S. goweni*	65	66	65	66	66	65	71		212	178	156	132	166	228	230
9	KT373857 *S. biddulphi*	63	68	63	64	63	61	63	60		201	220	204	217	95	98
10	AY171279 *S. bicornutum*	64	67	64	65	64	62	87	70	62		195	180	180	231	233
11	KJ913707 *S. papillatum*	63	63	63	63	63	64	72	75	58	67		72	120	244	248
12	KP036472 *S. ralatorei*	63	63	63	64	64	64	74	79	61	69	90		99	220	223
13	DQ835613 *S. riobrave*	60	64	60	61	60	62	71	73	58	68	81	85		236	238
14	JX989267 *S. bifurcatum*	56	61	56	57	57	56	57	58	85	55	53	58	54		289
15	AY748449 *S. pakistanense*	55	61	55	57	57	56	57	57	85	55	51	57	54	99	

Table 6: Pairwise distances of the D2–D3 regions between *Steinernema* species from the '*bicornutum'* group. Below diagonal, percentage similarity; above diagonal, total character differences.

	D2D3 Region	1	2	3	4	5	6	7	8	9	10	11	12	13
1	MK643331 DS7		0	12	34	47	90	96	96	96	103	104	112	182
2	AF331890 *S. abbasi*	100		10	32	45	90	93	96	94	101	102	112	179
3	MG495398 *S. kandii*	98	98		40	11	53	55	62	60	68	61	69	64
4	AY748451 *S. yirgalemense*	94	94	93		37	50	60	55	54	61	50	55	65
5	JQ838179 *S. bifurcatum*	94	94	98	94		123	107	126	100	106	131	146	176
6	AF331904 *S. bicornutum*	89	89	90	91	84		88	36	67	81	81	89	174
7	KT580950 *S. biddulphi*	87	88	90	90	86	88		93	97	100	89	107	140
8	AF331888 *S. ceratophorum*	88	88	88	90	83	96	88		73	87	89	96	188
9	KR781039 *S. goweni*	87	87	89	91	87	91	87	90		52	40	52	177
10	KJ913708 *S. papillatum*	86	86	87	89	86	89	87	88	93		19	42	185
11	KP036475 *S. ralatorei*	87	87	89	91	83	90	88	89	95	98		39	188
12	AF331893 *S. riobrave*	86	86	87	90	80	89	86	88	93	95	95		194
13	JX068824 *S. pakistanense*	73	73	88	89	75	74	80	72	74	72	72	70	

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