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Morpho-taxometrical and molecular characterization of *Steinernema abbasi* (Nematoda: Steinernematidae) and its pathogenicity and generative potential against lepidopteran pests

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Abstract

Background: An entomopathogenic nematode (EPN) was recovered by using *Galleria* baiting technique from the soils of marigold fields of Noida, Uttar Pradesh, India. Based on morphological, morphometrical and molecular characterizations, the isolated strain was identified as *Steinernema abbasi* and tagged as CS38. The isolated strain was conspecific to original description with minor deviations. Infective juveniles (IJs) of present strain were longer than original description.

Results: Molecular analysis was done using ITS1-5.8S-ITS2 and D2D3 regions. Pathogenicity and generative potential of the present strain CS38 were tested against larvae of 3 lepidopteran insect pests, namely, *Galleria mellonella* L., *Helicoverpa armigera* (Hb.) and *Spodoptera litura* (Fab.). Different concentrations of IJs/larva, *viz.* 25, 50, 100, and 200 IJs/larva, were used for bioassay trails. All experiments were repeated thrice to reach the optimum authenticity. Results of bioassays revealed that isolate CS38 was highly virulent against the 3 insect pests and caused (100%) mortality within 48 h under laboratory conditions. Generative potential of the studied *S. abbasi* CS38 was recorded high in *G. mellonella* (19×10^4 IJs/larva) at 100 IJs/larva concentration, followed by *H. armigera* (63.4×10^3 IJs/larva) and *S. litura* (60.5×10^3 IJs/larva).

Conclusions: Isolate CS38 is an indigenous, dominant and highly virulent strain that can be utilized as a biological control agent against the three studied insects. Moreover, it can be used for commercialization of the production of EPN-based biopesticide to be added under Integrated Pest Management in Indian agriculture system.

Keywords: *Steinernema abbasi*, Biological control, Entomopathogenic nematode, Pathogenicity, Generative potential

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Background

Agriculture has been facing distressing harm due to various factors worldwide and highly affected by attack of pests and diseases which decline the crop production. Insect pests are one of them causing huge yield loss (15–20%) of agricultural crops in India. The most common problem in the effective control of insect pests is that they develop resistance against chemical pesticides with time. In Integrated Pest Management (IPM), the biological control method is considered to suppress the population of insect pests in agricultural fields. Biopesticide is the effective potential measure that controls the pests by biological non-toxic means without causing environmental hazards. Entomopathogenic nematodes (EPNs) are among extraordinary biocontrol agents than others. They have been studied against various insect pests infesting agricultural crops in different biocontrol programs (Ehlers, 2001) and it has been proven that EPNs, belonging to the families Steinernematidae and Heterorhabditidae, are the most promising and highly effective biological control agents against many insect pests (Kaya and Gaugler, 1993), and they are harmless to vertebrate and plants (Burnell and Stock, 2000). They are found in various habitats from cultivated land to desert (Hominick et al., 1996). EPNs are the soil-inhabiting endoparasitoids, having a great potential to control both soil dwelling and above-ground insect pests (Kaya and Gaugler, 1993 and Ehlers, 2005). Therefore, to control a broad range of insect pests in cryptic and epigeal habitats, they can be used efficiently as a biocontrol agent against grubs, cutworms, crown borers, corn root worm, fungus gnats, thrips, cranefly, beetles, weevils, moths, butterflies, crickets, grasshoppers, and other insect pests. EPNs are effective as the highly recommended commercial products (e.g., Entonem), chemical insecticide (e.g., abamectin and thiamethoxam) that significantly decrease insect pest populations (Laznik et al. 2010). These nematodes are symbiotically associated with entomopathogenic bacteria belonging to the family enteriobacteriacae, which makes them lethal obligatory insect parasitoids that affect a variety of insects including their larval forms and kill them within a short period of time.

Infective juveniles (IJs), the only free-living stage of EPNs, has the ability to search the insect-hosts, enters them through natural openings (Poinar, 1990) or penetrates through thin sections of cuticle (Bedding and Molyneux, 1982) which releases its symbiotic bacteria into the haemolymph after entering the host. These symbionts are the primary agents which are responsible for killing the host within 24–72 h by septicaemia (Boemare and Akhurst, 1988). Two major genera *Steinernema* and *Heterorhabditis* are symbiotically associated with bacteria of genus *Xenorhabdus* and *Photorhabdus*, respectively. EPNs of both of these genera are used as biocontrol agents because they have the ability to find and kill even deep-seated insects (Bedding and Miller, 1981). Moreover, they are fast-acting, cost-effective and easy to handle. The EPNs are eco-friendly, safe to non-target organisms, have a great potential of reproduction (Kaya and Gaugler, 1993), wide host range, easy to mass produce, capable of resisting under unfavourable conditions (Askary and Ahmad, 2017), easy to apply, compatible with most of the insecticides (Chen, 2003) and sensitive to some insecticides (Laznik and Trdan, 2013a, b).

The greater wax moth, *Galleria mellonella* L., is a ubiquitous pest of honeybees and their hives. The cotton bollworm, *Helicoverpa armigera* (Hb.), and tobacco cutworm, *Spodoptera litura* (Fab.) are highly polyphagous and widely distributed insect pests.

The aim of the present study was to validate the EPN isolate *Steinernema abbasi*, on the basis of morpho-taxometrical and molecular characterizations. Furthermore, the pathogenicity and generative potential of the EPN isolate against 3 lepidopteran pests were studied.

Methods

Soil sampling

In 2017, a total of 23 soil samples were collected randomly by adopting the technique of Wallace (1971) from marigold fields of Noida (28° 32′ 7.8612″ N and 77° 23′ 27.7044″ E, 206 m above sea level), Uttar Pradesh, India, and brought to the Nematology Laboratory in a well labelled polythene bags for the isolation of EPNs. The characteristics of the positive soil sample were sandy loam with 6.5 pH.

Insect rearing and maintenance

In the present study, 3 lepidopteran insect pests, *viz*., *G. mellonella*, *H. armigera* and *S. litura*, were reared under laboratory conditions and utilised in experimental work. Larvae of *G. mellonella* were brought to the laboratory from Bio-control Lab, Sardar Vallabhbhai Patel University of Agriculture and Technology, Modipuram, and reared on semisynthetic diet as suggested by David and Kurup (1988). Eggs and larvae of *H. armigera* (National accession no. NBAII-MP-NOC-01) and *S. litura* (National accession no. NBAII-MP-NOC-02) were purchased from ICAR-National Bureau of Agriculturally Important Insects (NBAII), Bangalore. Larvae of *H. armigera* were reared under laboratory conditions on chickpea-based diet as suggested by Nagarkatti and Prakash (1974), modified by Kalia et al. (2001), while the larvae of *S. litura* were reared on fresh, properly washed and well-sterilized castor leaves. The 3rd to 4th larval instars of these insects, approximately same size and weight larvae, were used in bioassay experiments. However, the fully grown larvae have also been used for

further implications such as isolation and mass production of nematodes.

Isolation and culture of nematodes

EPNs were isolated from the soil samples using the *Galleria* soil baiting technique (Bedding and Akhurst, 1975). Ten last instar larvae of *G. mellonella* were placed in a 250 ml sterilized polystyrene jar containing soil sample and kept in BOD at 27 ± 1 °C. For larval mortality, samples were checked daily up to 7 days. The cadavers of *G. mellonella* were collected from the jar and washed thrice with double-distilled water (DDW), disinfected with 0.1% sodium hypochlorite and transferred on White trap (White, 1927) for IJs. Of the total 23 soil samples collected, only one sample (4.35%) was found positive for EPNs. Koch's postulate was performed for the confirmation of entomopathogenic nature of the nematodes and the isolated strain was designated as CS38. Isolated IJs were washed thrice with DDW, disinfected with 0.1% sodium hypochlorite and finally, stored in sterilized DDW into vented tissue culture flasks in BOD at 15 °C.

Morpho-taxometrical characterization

Fully grown larvae of *G. mellonella* were infected with isolated IJs (3rd stage) and adults (males and females) of first and second generations were obtained from cadavers by dissection on 3rd and 5th days after infection. In this study, 20 IJs, 15 males and 15 females of each generation were used for morphology and morphometry. Collected nematodes of different generations were killed separately with hot Ringer's solution, fixed in TAF solution (2 ml triethanolamine, 7 ml formalin, 91 ml distilled water) (Courtney et al., 1955), dehydrated by Seinhorst method (Seinhorst, 1959) and finally kept in glycerol. Then, they were mounted onto a drop of glycerine on glass slides and coverslips were sealed with paraffin wax. Morphological observations were made using a light compound microscope (Magnus MLX) and phase contrast microscope (Nikon Eclipse 50i). Morphometric measurements were taken with the help of the inbuilt software of a phase contrast microscope (Nikon DS-L1).

Molecular characterization

For molecular studies, genomic DNA was extracted from freshly emerged IJs, using a DNeasy Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and eluted DNA was stored at − 20 °C until further use. For PCR amplification, Internal Transcribed Spacers (ITS) and 28S ribosomal DNA (D2D3) were used. The Internal Transcribed Spacers regions (ITS1, 5.8S, ITS2) of the rDNA were amplified using primers 18S: 5′-TTGATTACGTCCCTGCCCTTT-3′ (forward) and 26S: 5′-TTTCACTCGCCGTTACTAAGG-3′ (reverse) (Vrain et al., 1992). The rDNA fragment containing D2D3 regions of 28S rDNA were amplified using primers (D2F): 5′-AGCGGAGGAAAAGAAA CTAA-3′ (forward) and (D2R): 5′-TCGGAAGGAA CCAGCTACTA-3′ (reverse) (Joyce et al., 1994).

The 30 μl PCR reaction mixture consisted of Dream Taq green PCR master mix (15 μl), DNA extract (3 μl), nuclease free distilled water (10 μl) and forward and reverse primers (1 μl of each). The cycling parameters were used as follows: for ITS: 1 cycle of 94 °C for 7 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min, and for D2D3 fragment of 28S rDNA: 1 cycle of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min.

PCR was followed by gel electrophoresis, in which the PCR products (5 μl) were analysed on 1% TAE (Tris–acetic acid–EDTA) buffered agarose gel (50 ml) stained with ethidium bromide (1 μl EtBr) at 70 volts for 30 min. The amplified PCR products were purified and then sequenced in both directions using ABI 3730 (48 capillary) electrophoresis instrument by Bioserve Pvt. Ltd. (Hyderabad, India). Obtained amplified forward and reverse sequences were aligned, edited, and assembled using BioEdit (Hall, 1999), and then, the sequencing results were submitted to GenBank with accession numbers MG198915 and MG198916 for ITS and D2D3 regions of *Steinernema abbasi* strain CS38 respectively.

Sequence alignment and phylogenetic analysis

The sequences were compared to other already available sequences in the GenBank using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI). Based on nucleotide similarities, the related sequences were aligned using default CLUSTALW parameters in MEGA 6.0 (Tamura et al., 2013) and optimized manually in BioEdit (Hall, 1999). Pairwise distances of ITS and D2D3 regions between isolated strain CS38 and other *Steinernema* species from 'bicornatum group' were computed in MEGA 6.0. Codon positions included were 1st + 2nd + 3rd + Noncoding.

Phylogenetic trees were constructed by Maximum Parsimony (MP) using MEGA 6.0 with bootstrap analysis based on 10,000 replicates. Evolutionary distances were also computed using *p*-distance method in MEGA 6.0 and expressed in the units of base differences per site. All the characters were treated as equally weighted. *Steinernema affine* and *S. scapterisci* and *S. nepalense* were used as outgroups for ITS and D2D3 regions, respectively.

Bioassay for pathogenicity and generative potential

Freshly emerged IJs were used to perform all bioassay experiments to evaluate the pathogenicity and generative potential of *S. abbasi* CS38 against *G. mellonella*, *H.*

armigera and *S. litura* as target hosts. For pathogenicity, bioassay trails were carried out in 6-well plates (well size 3.5 cm) lined with a double layer of Whatman Filter Paper No. 1. Four different concentrations of isolate, *viz.,* 25, 50, 100 and 200 IJs, were prepared with a final volume of 400 µl, using DDW, and were poured into each well of well-plates with the help of micropipette. Ten replicates of the 3 insect larvae of the same size and weight were used for each concentration along with control (only DDW). Larvae were placed individually into each well of above prepared well plates and then, the plates were incubated at 28 ± 2 °C in BOD. Mortality rate was recorded after every 12 h of post infection period (PIP) till 100% mortality was observed. Dead larvae of target insects were transferred onto the modified White Trap (White, 1927) to observe the persistence of infection and for emergence of IJs. Each bioassay was placed separately and to reach the optimum authenticity, all experiments were repeated thrice.

For generative potential, 10 fully grown larvae of each insect (same size and weight) were infected with 100 IJs/larva and incubated at 27 ± 1 °C in BOD. Cadavers were transferred to the White Trap and emerged IJs were collected daily in tissue culture flask for progeny count, up to 20 days till the emergence stopped. Collected nematodes were quantified under stereomicroscope (Nikon SMZ 645) with the help of counting dish in 1 ml suspension.

Statistical analysis

The experimental data of larval mortality bioassay was analyzed statistically, using SPSS software (version 16.0) by applying Probit analysis. To evaluate the pathogenicity, LC_{50} and LT_{50} values were also computed at 95% confidence limit. Larval mortality was recorded in the form of percentage mortality and graphical presentations were made using excel. For generative potential, the total number of produced IJs/larva of the studied nematode was analysed by descriptive analysis and presented in number of IJ ± SE (range).

Results

Morpho-taxometrical characterization

The morpho-taxometrical characterizations of the different life stages (IJs, 1st and 2nd generation males and females) of the present isolate CS38 were compared to original described species *S. abbasi* (Elawad et al., 1997). Due to the presence of horn like cephalic armature in 3rd stage IJs, the present isolate CS38 was characterized as a member of "bicornatum" group and the morphological and taxometrical studies of different life stages revealed that it was conspecific to *S. abbasi*. Hence, it is named as the same. Light microscope images of *S. abbasi* CS38 are shown in Fig. 1. Body of IJs was observed larger in size when compared with IJs of already described species. Tail is attenuated, gradually tapering and dorsally curved at tip with slight ventral depression. Female body is robust, strongly curved and C-shaped. Cuticle with fine striae, oesophagus muscular with slightly swollen rounded basal bulb, gonads reflexed containing eggs, vulva with double flapped epiptygma. Tail short, conoid with pointed tip and ventral post anal swelling present in both generations. Male body is slender, ventrally curved and J-shaped upon fixation. Cuticle with fine transverse striae, oesophagus muscular with rounded basal bulb, nerve ring located above the basal bulb and excretory pore present anterior to nerve ring. Testis reflexed, spicule paired and golden dark yellow in colour. Ventrally curved gubernaculum observed boat-shaped and slightly swollen in the middle. Tail short and conoid with a bluntly rounded terminus. However, 2nd generation females and males are smaller in size than first generation. Spicules and gubernaculum of 2nd generation male are also shorter and thinner. Detailed morphometrics of all generation of present strain CS38 and comparative morphometrics of all generations of CS38 with *S. abbasi* are presented in Tables 1 and 2, respectively.

Molecular characterization

For molecular studies, Internal Transcribed Spacer (ITS) and D2D3 region of rDNA were analysed. In the ITS sequence of rDNA, the present strain CS38 did not show any variation with the already described *S. abbasi* (AY230158). The sequence length of ITS in *S. abbasi* CS38 was 739 bp with ITS-1 268 bp, 5.8S 157 bp and ITS-2 314 bp same as found in original description. The ITS nucleotide composition of CS38 was A = 175 bp, C = 111 bp, G = 162, T = 291 bp (Table 3). The ITS sequence of CS38 was separated from the other related species of "bicornatum" group by 33 to 191 bp. The pairwise distance matrix is presented in Table 4. The sequence of the D2D3 region was 883 bp and its nucleotide composition was A = 225 bp, C = 146 bp, G = 266 bp, T = 246 bp (Table 5). The D2D3 sequences of CS38 were separated from the other related species of "bicornatum" group by 138 to 208 bp (Table 6). Pairwise distances of ITS and D2D3 regions of rDNA showed 100% similarity and 0 total character difference between present strain and original description.

Phylogenetic analysis

For phylogenetic analysis of ITS and D2D3 regions, the phylogenetic trees were constructed by the Maximum Parsimony Method, using Subtree-Pruning-Regrafting (SPR) algorithm. Both of these regions were too conservative among closely related species to resolve the

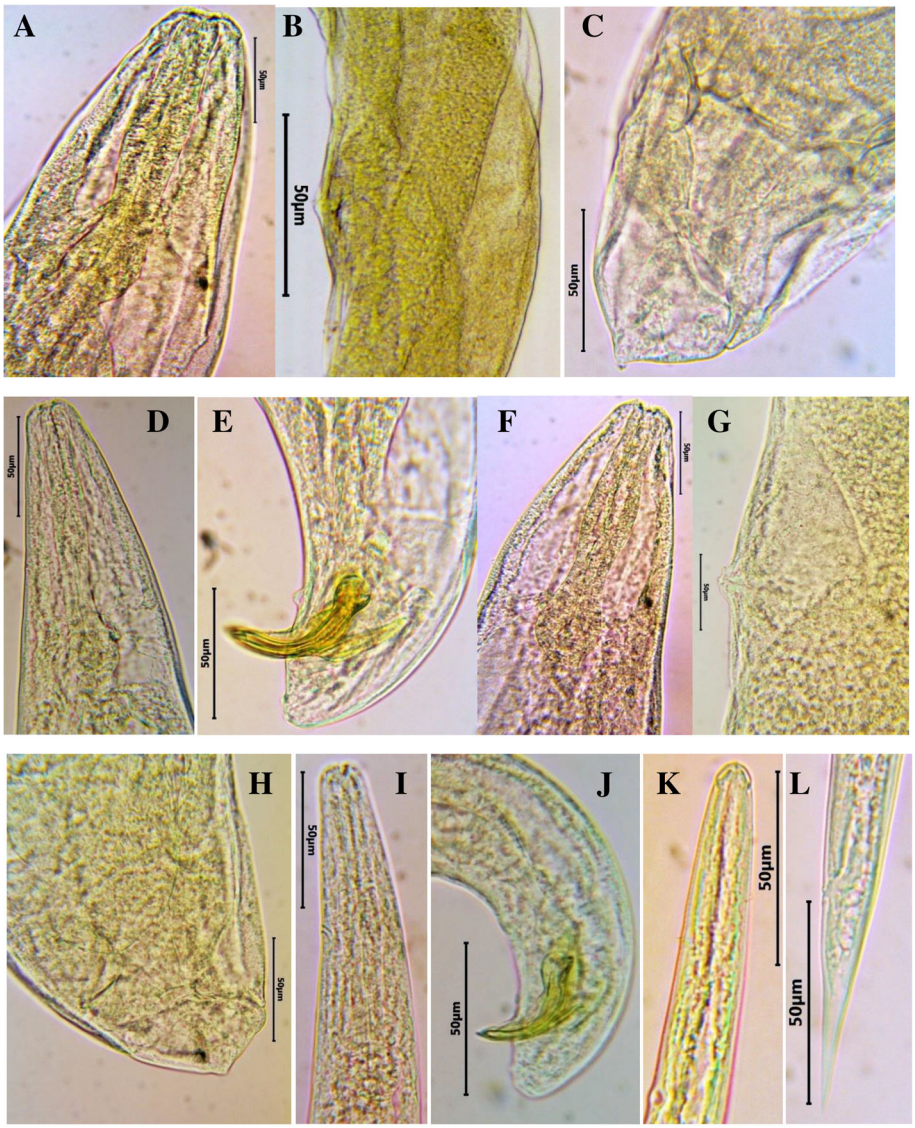

Fig. 1 Light microscope image of *Steinernema abbasi* CS38 drawn to scale at 400X. **a**–**c**: 1st generation female, **a-** anterior region, **b-** vulva region, **c-** posterior region. **d–e**: 1st generation male, **d-** anterior region, **e-** posterior region. **f**–**h:** 2nd generation female, **f-** anterior region, **g-** vulva region, **h-** posterior region. **i**–**j:** 2nd-generation male, **i-** anterior region, **j-** posterior region. **k**–**l**: Infective juveniles, **k-** anterior region, **l-** posterior region

phylogenetic relations. For ITS region, phylogenetic analysis involved 15 nucleotide sequences and the relationships among those 15 *Steinernema* species, obtained with MP Tree length = 757; Consistency index = 0.665094; Retention index = 0.742754 and Composite index = 0.533762 (0.494001). For D2D3 region, phylogenetic analysis involved 14 nucleotide sequences and the relationship between those 14 *Steinernema* species were obtained with MP Tree length = 266; Consistency index = 0.630137; Retention index = 0.736156; Composite index = 0.511988 (0.463879). Phylogenetic analysis of the "bicornatum group" showed a clear monophyly of the group formed by the isolates CS38 and original *S. abbasi* isolates (Figs. 2 and 3). All positions containing gaps and missing data were eliminated in both ITS and D2D3 regions.

Bioassay for pathogenicity and generative potential

To test the pathogenicity, different IJ concentrations were applied to all target host larvae and different parameters were measured. The data revealed that the present strain CS38 was highly virulent against all the studied insects. Mortality was initiated in all the target insects after 24 h of post infection period (PIP) and recorded as 80, 80, 90 and 100% mortality in *G. mellonella*, followed by 30, 50, 50 and 60% mortality in *H. armigera*, and 0, 10, 10 and 20% mortality in *S. litura*, with 25, 50, 100 and 200 IJs/larva concentrations. At 36

Table 1 Morphometrics of all generations of *Steinernema abbasi* CS38. All measurements are in μm (except *n*, ratio and percentage) and in ± SD (range)

Characters	Male		Female		Infective juveniles
	First generation	Second generation	First generation	Second generation	
n	15	15	15	15	20
Body length (L)	1270 ± 111 (1082–1441)	883 ± 69 (761–1006)	5041 ± 792 (3731–6314)	1631 ± 237 (1287–2071)	581 ± 20 (520–610)
Excretory pore (EP)	72 ± 8.6 (57–83)	62 ± 3.5 (54–68)	80 ± 7.6 (70–96)	55 ± 4.0 (46–60)	46 ± 3.3 (39–53)
Width EP (WEP)	51 ± 6.4 (41–62)	30 ± 1.2 (29–33)	81 ± 6.4 (70–88)	43 ± 4.9(34–52)	18 ± 1.0 (16–20)
Nerve ring (NR)	91 ± 8.3(81–114)	72 ± 5.1 (63–85)	102 ± 10 (92–127)	75 ± 3.2 (71–81)	57 ± 6.4 (42–67)
Pharynx length (ES)	138 ± 9.1 (121–162)	118 ± 5.9 (107–126)	169 ± 8.0 (159–190)	134 ± 4.5 (125–140)	88 ± 7.3 (69–98)
Esophageal bulb length	29 ± 3.3(23–35)	25 ± 2.5(20–31)	43 ± 4.4(35–54)	31 ± 3.5(26–39)	16 ± 1.4 (14–19)
Esophageal bulb width	24 ± 1.8 (20–27)	21 ± 1.7 (19–25)	33 ± 2.9 (28–38)	26 ± 1.9 (23–30)	11 ± 1.4 (8–13)
Tail (T)	22 ± 2.7 (16–27)	18 ± 1.4 (15–20)	47 ± 11 (29–72)	44 ± 8.2(26–56)	55 ± 4.9 (42–63)
Anal body width (ABW)	35 ± 3.4 (29–41)	28 ± 1.5 (26–30)	67 ± 16 (41–95)	35 ± 5.8 (28–48)	14 ± 1.3 (12–17)
Body dia. (BD)	135 ± 14 (101–156)	60 ± 5.2 (54–73)	205 ± 20 (169–239)	95 ± 19 (69–130)	26 ± 1.6 (22–30)
Testis reflection (TR)	241 ± 26 (186–282)	74 ± 6.3 (63–84)			
Spicule length (SL)	72 ± 4.5 (62–80)	53 ± 7.7 (33–63)			
Gubernaculum length (GL)	46 ± 2.8 (42–50)	37 ± 2.6 (30–40)			
Anterior to vulva			2676 ± 428 (2136–3299)	891 ± 124 (710–1080)	
Posterior to vulva			2386 ± 413 (1571–3015)	740 ± 128 (540–996)	
Width at vulva (WV)			194 ± 24 (161–229)	102 ± 19 (72–136)	
a = L/GBW	9.5 ± 1.1(7.5–11)	15 ± 1.6 (12–17)	25 ± 5.6 (17–37)	17 ± 1.4 (15–19)	23 ± 1.7 (20–25)
b = L/ES	9.2 ± 0.8 (8.1–11)	7.5 ± 0.6 (6.2–8.6)	30 ± 4.2 (22–37)	12 ± 1.6 (10–16)	6.7 ± 0.4 (6.0–7.5)
c = L/Tail	58 ± 10 (46–83)	50 ± 3.4 (43–54)	113 ± 29 (55–173)	38 ± 6.9 (27–50)	11 ± 1.1 (9.2–13.6)
c' = Tail/ABW	0.6 ± 0.1 (0.5–0.7)	0.6 ± 0.0(0.5–0.7)	0.7 ± 0.1 (0.5–0.9)	1.3 ± 0.3 (0.9–1.8)	3.8 ± 0.4 (3.2–4.8)
D% = EP/ES × 100	52 ± 4.9 (44–60)	53 ± 2.1 (48–56)	47 ± 3.6 (42–55)	41 ± 3.0 (34–46)	53 ± 3.7 (45–58)
E% = EP/Tail×100	325 ± 42 (248–403)	350 ± 36 (294–438)	180 ± 42 (97–237)	129 ± 29 (89–201)	85 ± 8.7 (70–102)
F% = GBW/Tail×100	619 ± 111 (427–875)	338 ± 39 (285–409)	457 ± 103(321–677)	218 ± 45 (154–305)	47 ± 5.5 (36–64)
V = V'/L × 100			53 ± 2.7 (47–58)	55 ± 2.6 (50–59)	
SW%	210 ± 25 (159–245)	191 ± 26 (114–220)			
GS%	65 ± 6.3 (58–80)	70 ± 9.2 (57–91)			
EP/WEP	1.4 ± 0.2 (0.9–1.7)	2.1 ± 0.1 (1.8–2.3)	1.0 ± 0.1 (0.8–1.2)	1.3 ± 0.2 (1.1–1.6)	2.5 ± 0.2 (2.1–3.0)
EBL/EBW	1.2 ± 0.1 (1.1–1.4)	1.2 ± 0.1 (1.0–1.3)	1.3 ± 0.1 (1.1–1.5)	1.2 ± 0.1 (1.0–1.4)	1.6 ± 0.2 (1.1–2.1)

h of PIP, 100% mortality was recorded in *G. mellonella* with all concentrations, while, in *H. armigera* and *S. litura* 100% mortality was observed only in 50, 100 and 200 IJs/larva concentrations. In 25 IJs/larva, complete mortality was observed at 48 h of PIP in *H. armigera* and *S. litura*. In control group, no mortality was observed during the experiment even after 60 h. Graphical presentations of percentage mortality in larvae of *G. mellonella*, *H. armigera* and *S. litura* are shown in Fig. 4a–c. Lethal concentration (LC_{50}) was calculated at 24 and 36 h PIP and showed the requirement of 7.806 IJs/larva and 86.032 IJs/larva of the isolate at 24 h to kill the half population of *G. mellonella* and *H. armigera*, respectively, which were only 7.67 IJs/larva and 16.202 IJs/larva at 36 h in *H. armigera* and *S. litura*, respectively. The lowest LT_{50} was found at 100 IJs/larva concentration recorded as 19.4 h in *G. mellonella*, while in *H. armigera*, the LT_{50} values were 26.96, 23.17 and 24.32 h at 25, 200 and combined IJs/larva, respectively. However, in *S. litura* LT_{50} values were 26.53, 26.53 and 25.74 h at concentrations of 50, 100 and 200 IJs/larva, respectively.

To evaluate the generative potential of present isolate, the cadavers of target hosts, infected with 100 IJs/larva concentration, were used for progeny production. The highest progeny count at 100 IJs/larva concentration was recorded as 24.9×10^4, 92×10^3 and 90×10^3 IJs/larva, while the lowest progeny count was 14.8×10^4, 37×10^3 and 31×10^3 IJs/larva in *G. mellonella*, *H. armigera* and *S. litura*, respectively. The mean IJs/larva production count was 19×10^4 IJs/larva in case of *G. mellonella*

Table 2 Comparative morphometrics of all generations of present strain CS38 with *Steinernema abbasi*. All measurements are in micrometers (except percentage) and in the form of mean (range)

Characters	Male				Female				Infective juveniles	
	First generation		Second generation		First generation		Second generation			
	CS38	*Steinernema abbasi*	CS38	*Steinernema abbasi*	CS38	*Steinernema abbasi*	CS38	*Steinernema abbasi*	CS38	*Steinernema abbasi*
Total body length (L)	1270 (1082–1441)	1252 (999–1534)	883 (761–1006)	861 (606–1035)	5041 (3731–6314)	3510 (2453–4477)	1631 (1287–2071)	2609 (1897–3917)	581 (520–610)	541 (496–579)
Greatest body width (GBW)	135 (101–156)	87 (82–98)	60 (54–73)	70 (64–80)	205 (169–239)	159 (143–181)	95 (69–130)	130 (123–148)	26 (22–30)	29 (27–30)
Excretory pore (EP)	72 (57–83)	80 (68–89)	62 (54–68)	66 (62–79)	80 (70–96)	71 (62–79)	55 (46–60)	66 (61–73)	46 (39–53)	48 (46–51)
Nerve ring (NR)	91 (81–114)	103 (99–123)	72 (63–85)	99 (93–106)	102 (92–127)	125 (120–137)	75 (71–81)	114 (110–118)	57 (42–67)	68 (64–72)
Pharynx length (ES)	138 (121–162)	133 (121–144)	118 (107–126)	121 (112–130)	169 (159–190)	165 (155–176)	134 (125–140)	146 (136–157)	88 (69–98)	89 (85–92)
Tail	22 (16–27)	26 (20–31)	18 (15–20)	21 (17–24)	47 (29–72)	37 (31–40)	44 (26–56)	36 (32–39)	55 (42–63)	56 (52–61)
Spicule length (SPL)	72 (62–80)	65 (57–74)	53 (33–63)	61 (51–69)	-	-		-	-	-
Gubernaculum length (GL)	46 (42–50)	45 (33–50)	37 (30–40)	43 (35–48)	-	-		-	-	-
a	9.5 (7.5–11)	-	15 (12–17)	-	25 (17–37)	-	17 (15–19)	-	23 (20–25)	18 (17–20)
b	9.2 (8.1–11)	-	7.5 (6.2–8.6)	-	30 (22–37)	-	12 (10–16)	-	6.7 (6.0–7.5)	6.0 (5.5–6.6)
c	58 (46–83)	-	50 (43–54)	-	113 (55–173)	-	38 (27–50)	-	11 (9.2–13.6)	9.8 (8.1–10)
D%	52 (44–60)	60 (51–68)	53 (48–56)	56 (50–70)	47 (42–55)	42 (36–48)	41 (34–46)	45 (43–47)	53 (45–58)	53 (51–58)
E%	325 (248–403)	-	350 (294–438)	-	180 (97–237)	-	129 (89–201)	-	85 (70–102)	-
SW%	210 (159–245)	156 (107–187)	191 (114–220)	159 (128–185)	-	-	-	-		-
GS%	65 (58–80)	70 (58–85)	70 (57–91)	70 (58–81)	-	-	-	-		-

Table 3 Nucleotide composition of *Steinernema abbasi* isolate CS38 compared with the other known species based on Internal Transcribed Spacers (ITS) region

Species	ITS-1	5.8S	ITS-2	G + C%	A + T%	A	C	G	T	Total length
ITS Regions										
MG198915 *Steinernema abbasi* CS38	**268**	**157**	**314**	**36.94**	**63.06**	**175**	**111**	**162**	**291**	**739**
EF469773 *Steinernema abbasi*	269	159	310	36.94	63.06	175	111	162	290	738
AY748450 *Steinernema yirgalomense*	270	157	284	35.72	64.28	191	93	161	266	711
AY230165 *Steinernema ceratophoram*	243	157	341	36.17	63.83	192	117	151	281	741
AY171279 *Steinernema bicornatum*	281	157	330	37.5	62.5	201	123	165	279	768
KR781038 *Steinernema goweni*	283	157	324	38.5	61.5	196	118	178	272	764
KJ913707 *Steinernema papillatum*	314	157	323	35.14	64.86	223	110	169	292	791
DQ835613 *Steinernema riobrave*	281	157	316	34.75	65.25	204	98	164	288	754
AY748449 *Steinernema pakistanense*	291	157	300	36.9	63.1	216	118	158	256	748

Table 4 Pairwise distances of the ITS region between *Steinernema* species from the "*bicornatum*" group

S. No.	ITS Region	1	2	3	4	5	6	7	8	9	10	11	12
1	***Steinernema abbasi* CS38**		*0*	*33*	*35*	*87*	*93*	*93*	*93*	*98*	*101*	*110*	*191*
2	AF331890 *Steinernema abbasi*	*100*		31	33	87	90	91	93	96	99	110	188
3	JQ838179 *Steinernema bifurcatum*	*96*	96		39	109	109	104	113	119	110	131	193
4	AY748451 *Steinernema yirgalemense*	*94*	94	93		51	59	54	56	50	61	55	65
5	AF331904.1 *Steinernema bicornutum*	*89*	89	86	91		87	67	36	79	82	90	188
6	KT580950 *Steinernema biddulphi*	*88*	88	86	90	89		97	92	89	100	107	151
7	KR781039 *Steinernema goweni*	*88*	88	86	91	91	87		73	40	52	52	187
8	AF331888 *Steinernema ceratophorum*	*88*	88	85	90	96	88	90		87	88	97	199
9	KP036475 *Steinernema ralatorei*	*88*	88	85	91	90	88	95	89		19	34	199
10	KJ913708 *Steinernema papillatum*	*87*	87	85	89	89	87	93	88	98		42	196
11	AF331893 *Steinernema riobrave*	*86*	86	82	90	89	86	93	88	96	95		204
12	JX068824 *Steinernema pakistanense*	*72*	72	72	89	72	79	72	69	70	70	69	

Note: Data of present strain shown in italics. Below diagonal: percentage similarity; above diagonal: total character differences

Table 5 Nucleotide composition of *Steinernema abbasi* isolate CS38 compared with the other known *Steinernema* species based on D2D3 region

Species D2D3 Regions	G+C%	A+T%	A	C	G	T	Total length
MG198916 *Steinernema abbasi* CS38	**46.66**	**53.34**	**225**	**146**	**266**	**246**	**883**
AF331890 *Steinernema abbasi*	46.67	53.33	220	143	263	244	870
AY748451 *Steinernema. yirgalomense*	48.61	51.39	159	116	198	173	646
AF331888 *Steinernema. ceratophoram*	45.69	54.31	230	146	252	243	871
KT580950 *Steinernema biddulphi*	45.59	54.41	218	138	250	245	851
AF331904 *Steinernema bicornatum*	46.9	53.1	221	145	263	241	870
KR781039 *Steinernema goweni*	48.03	51.97	208	149	253	227	837
KP036475 *Steinernema ralatorei*	47.58	52.42	222	155	267	243	887
KJ913708 *Steinernema papillatum*	47.25	52.75	210	143	252	231	836
AF331893 *Steinernema riobrave*	47.99	52.01	217	153	264	235	869
JQ838179 *Steinernema bifurcatum*	46.82	53.18	222	151	268	254	895
JX068824 *Steinernema pakistanense*	48.2	51.8	219	157	271	241	888

Table 6 Pairwise distances of the D2D3 region between *Steinernema* species from the "bicornatum" group

S. No.	D2D3 Region	1	2	3	4	5	6	7	8	9	10	11	12
1	***Steinernema abbasi* CS38**		*0*	*138*	*160*	*173*	*176*	*177*	*186*	*192*	*195*	*206*	*208*
2	AY230158 *Steinernema abbasi*	*100*		150	167	181	183	187	194	202	209	214	216
3	AY748450 *Steinernema yirgalemense*	*76*	74		181	205	195	195	193	200	201	220	219
4	AY230165 *Steinernema ceratophorum*	*71*	70	65		164	87	185	165	172	183	197	197
5	KT373857 *Steinernema biddulphi*	*68*	67	61	70		179	210	193	207	202	95	98
6	AY171279 *Steinernema bicornutum*	*68*	68	64	87	69		196	196	202	207	216	216
7	KR781038 *Steinernema goweni*	*68*	66	64	67	60	79		132	156	166	229	230
8	KP036472 *Steinernema ralatorei*	*65*	64	63	70	63	75	79		72	99	218	219
9	KJ913707 *Steinernema papillatum*	*64*	62	62	69	60	73	75	90		120	238	239
10	DQ835613 *Steinernema riobrave*	*62*	60	62	66	61	57	73	85	81		227	227
11	JX989267 *Steinernema bifurcatum*	*60*	59	58	63	85	56	57	58	53	56		8
12	AY748449 *Steinernema pakistanense*	*60*	59	58	63	85	100	56	58	53	56	99	

Notes: Data of present strain shown in italic, Below diagonal: percentage similarity; above diagonal: total character differences

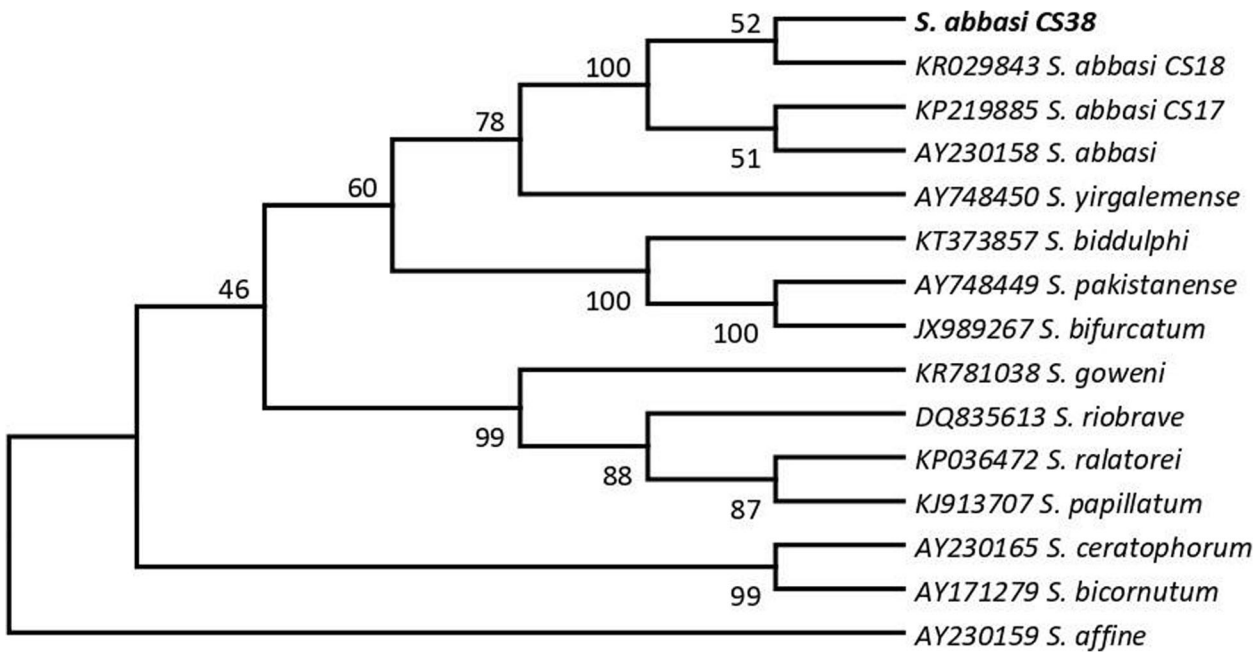

Fig. 2 Phylogenetic relationships in the "*bicornatum*" group of *Steinernema* based on analysis of ITS rDNA regions. *Steinernema affine* were used as outgroup taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in the units of number of base differences per site

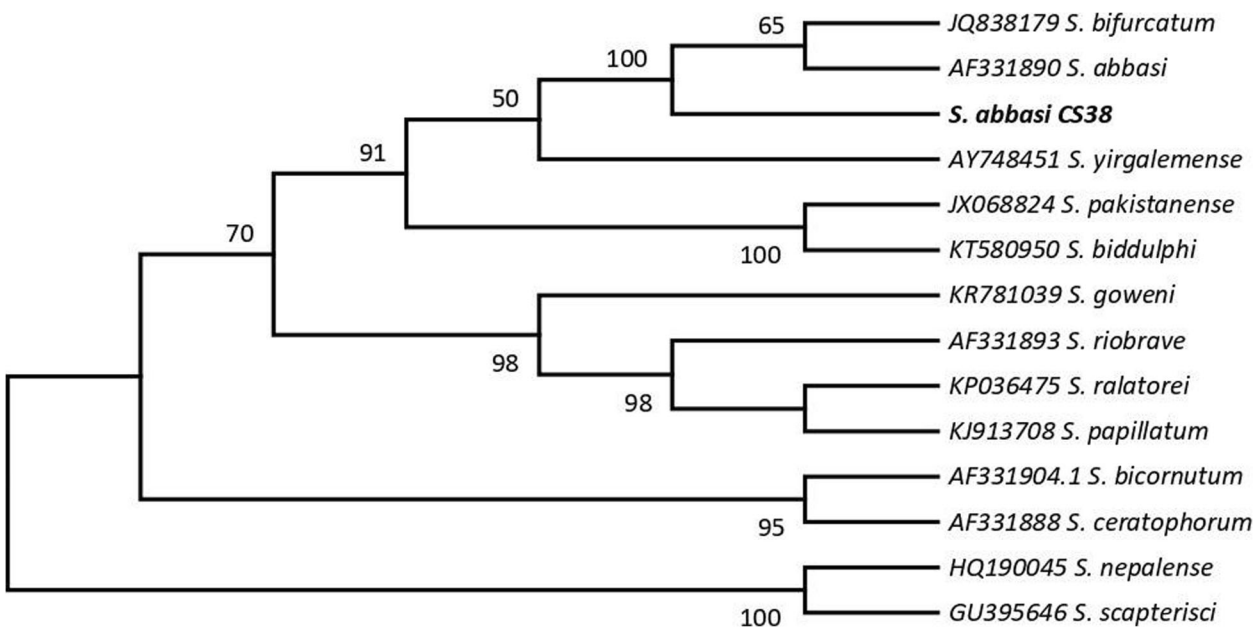

Fig. 3 Phylogenetic relationships in the "*bicornatum*" group of *Steinernema* based on analysis of D2D3 regions. *Steinernema scapterisci* and *Steinernema nepalense* were used as outgroup taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in the units of number of base differences per site

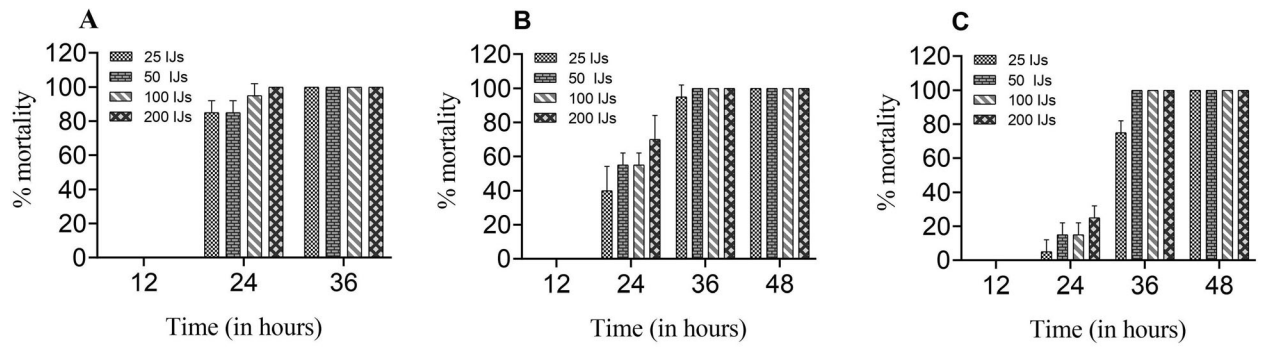

Fig. 4 **a** Percentage mortality in larvae of *Galleria mellonella* infected with different doses of *Steinernema abbasi* isolate CS38. **b** Percentage mortality in larvae of *Helicoverpa armigera* infected with different doses of *Steinernema abbasi* isolate CS38. **c** Percentage mortality in larvae of *Spodoptera litura* infected with different doses of *Steinernema abbasi* isolate CS38

63.4 × 10^3 IJs/larva in *H. armigera* and 60.5 × 10^3 IJs/larva in *S. litura* (Fig. 5).

Discussion

This is the first valid report of the existence of *S. abbasi* in Noida, Uttar Pradesh, India. Based on the morpho-taxometrical and molecular studies, the present isolate CS38 was identified as *Steinernema abbasi* (Elawad et al., 1997). Morphologically, the body of IJs was thin, elongated and larger in size than already described species. Intra-individual variability in the ITS sequence was not found in molecular studies. In the D2D3 sequence of rDNA, there was also no difference between CS38 and the original *S. abbasi* (AF331890). For ITS region, sequences of *S. abbasi* formed a monophyletic group with *S. yirgalemense* (Nguyen et al., 2004) and this pair was sister to the pair of *S. biddulphi* (Cimen et al., 2016), *S. pakistanense* (Fayyaz et al., 2001) and *S. bifurcatum* (Fayyaz et al., 2014). The phylogenetic analysis also showed that the alignment resulted in 1633 characters, of which 289 were constant, 579 variable characters, 375 characters were parsimony uninformative and 390 characters were parsimony informative. A total of 589 positions were present in final dataset. For D2D3 region, *S. abbasi* CS38 formed a monophyletic group with *S. bifurcatum* (Fayyaz et al., 2014), *S. yirgalemense* (Nguyen et al., 2004), *S. pakistanense* (Fayyaz et al., 2001) and *S. biddulphi* (Cimen et al., 2016) (Fig. 3). The phylogenetic analysis showed that the alignment resulted in 1309 characters, of which 547 constant, 377 variable characters, 188 parsimony uninformative and 197 characters are parsimony informative. A total of 555 positions were present in the final dataset.

Efficacy of the *S. abbasi* CS38 was tested on larvae of all the studied insects, i.e., *G. mellonella*, *H. armigera* and *S. litura*, and was found to be excellent biocontrol agent against all 3 target lepidopteran pests under the laboratory conditions. Bhat et al. (2015) also tested 3 isolates of *Steinernema* sp. against *G. mellonella* and recorded 100% mortality after 60 h of PIP. Istkhar et al. (2016) observed that the mortality initiated in 24 h of PIP and one strain of *Steinernema* showed 100% mortality within 60 h in all concentrations, but in case of second strain of *Steinernema*, the complete mortality was not observed even after 72 h of PIP in 25 and 50 IJs/larva concentrations. Chaubey et al. (2019) studied 3 isolates of *S. abbasi* and observed that the mortality initiated in larvae of *G. mellonella*, *H. armigera* and *S. litura* only after 12 h of PIP and complete mortality was recorded within 48 h of infection. Gorashi et al. (2014) recorded a complete mortality rate with the same species after 96 and 192 h of PIP in *H. armigera* and *S. litura*, respectively. These virulence alterations showed by different species or strains of EPNs against insect pests depend on some factors such as locality and climate, type of insect host, foraging strategy, penetration rate, bacterium complex, generative potential, and other abiotic and biotic factors (Kaya and Gaugler, 1993). IJs are also attracted by roots damaged by insect larvae, showing a chemotactic response to volatile compounds and influenced by different factors and their interactions, which is a strain-specific characteristic of EPN (Laznik and Trdan, 2013a, b). Chaubey et al. (2019) observed the differences in generative potential of different strains of *S. abbasi* against larvae of the 3 insect pests and recorded a high progeny production in case of *G. mellonella*. Patil et al. (2020) also noted the difference in generative potential of 3 strains of *Steinernema* sp. against larval and pupal stages of *Mythimna separata* (oriental armyworm). The variations found in generative potential of different species or stains of EPNs might be due to host-specificity, larval stage of insect, size of larvae, concentration of nematode and other factors. In the present study, *S. abbasi* CS38 produced

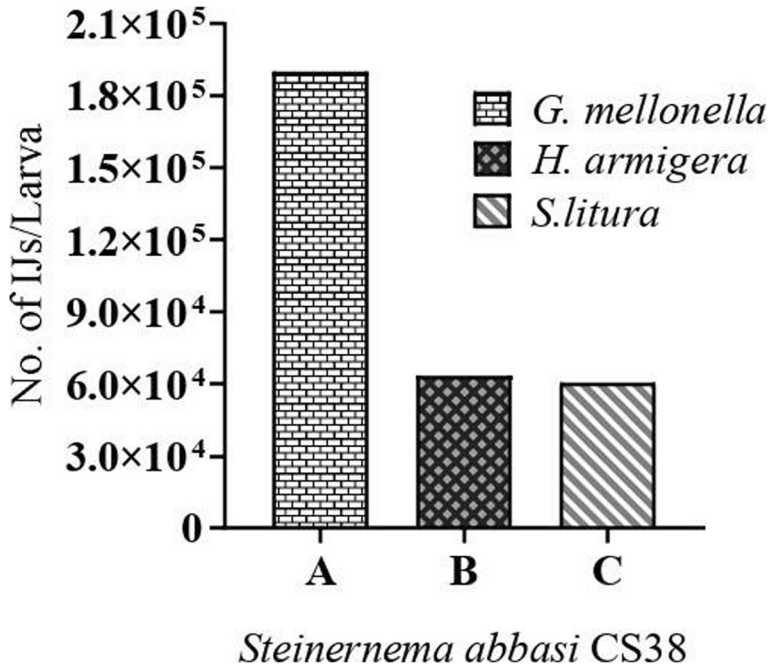

Fig. 5 Production of IJs in *G. mellonella*, *H. armigera* and *S. litura* at 100 IJs/Larva doses of *Steinernema abbasi* isolate CS38

significantly more progeny in *G. mellonella* than *H. armigera* and *S. litura*.

Conclusions

Based on the findings, the present isolate *S. abbasi* CS38 was characterized as highly virulent to the larvae of *G. mellonella*, *H. armigera* and *S. litura*, which shows that the isolate has good biocontrol potential on insect pests. In comparison to all target insect pest, the rate of generative potential of isolate was high in *G. mellonella*. The above findings concluded that *S. abbasi* can be utilized as a good biocontrol agent against all three insect pests. Further studies are still needed before recommending the strain to be applied.

Abbreviations

EPN: Entomopathogenic nematode; IJs: Infective juveniles; DDW: Double-distilled water; TAF: Triethanolamine formalin; ITS: Internal Transcribed Spacer; PCR: Polymerase chain reaction; PIP: Post infection period; LC: Lethal concentration; LT: Lethal time; IPM: Integrated Pest Management

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Authors' contributions

The study was designed by all authors. H and AR calculated the morphometric measurements. H and AHB performed the molecular analysis. Interpretation of the data, manuscript writing and reference settings were done by the first author (H). Errors and grammatical mistakes in manuscript were removed and edited by AKC. Then, final manuscript was read and approved by all authors.

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The authors declare that they have no competing interests.

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