Journal of Nematology 49(1):92–102. 2017.

First Report and Comparative Study of *Steinernema surkhetense* (Rhabditida: Steinernematidae) and its Symbiont Bacteria from Subcontinental India

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Abstract: Two populations (CS19 and CS20) of entomopathogenic nematodes were isolated from the soils of vegetable fields from Bijnor district, India. Based on morphological, morphometrical, and molecular studies, the nematodes were identified as *Steinernema surkhetense*. This work represents the first report of this species in India. The infective juveniles (IJs) showed morphometrical and morphological differences, with the original description based on longer IJs size. The IJs of the Indian isolates possess six ridges in their lateral field instead of eight reported in the original description. The analysis of ITS-rDNA sequences revealed nucleotide differences at 345, 608, and 920 positions in aligned data. No difference was observed in D2-D3 domain. The *S. surkhetense* COI gene was studied for the first time as well as the molecular characterization of their *Xenorhabdus* symbiont using the sequences of recA and gyrB genes revealing *Xenorhabdus stockiae* as its symbiont. These data, together with the finding of *X. stockiae*, suggest that this bacterium is widespread among South Asian nematodes from the "*carpocapsae*" group. Virulence of both isolates was tested on *Spodoptera litura*. The strain CS19 was capable to kill the larvae with 31.78 IJs at 72 hr, whereas CS20 needed 67.7 IJs.

Key words: D2-D3 domain, entomopathogenic nematode, ITS-rDNA, mt COI gene, *Xenorhabdus stockiae*.

Entomopathogenic nematodes (EPN) of the genus *Steinernema* Travassos, 1927, and *Heterorhabditis* Poinar, 1976, are effectual biological control agents for a wide variety of soil-dwelling insect pests (Kaya and Gaugler, 1993; Kaya et al., 2006) and in many cases have shown better performance over chemical and microbial insecticides in their ability to locate and kill even deep-seated insects (Bedding and Miller, 1981; Lewis et al., 1992, 1993; Alsaiyah et al., 2009). For a high efficiency as biological control agents against insect pests, EPN should be adapted to local environmental conditions (Gal et al., 2001; Chen et al., 2009). However, the usage of EPN in controlling pests sometimes has been limited due to the lack of information on their behavior, environmental interactions, and biology (San-Blas, 2013). Therefore, the isolation and the proper recognition of EPN species are decisive for the success of their use as biopesticide.

In India, research on EPN have been conducted since the mid-1960's (Kaya et al., 2006), and many sampling programs have been done looking for indigenous populations to be introduced as biological control agents in different crops (Divya and Sankar, 2009). Until now, six *Steinernema* species have been reported from different localities in India, *Steinernema abbasi* Elawad, Ahmad and Reid (Ganguly and Singh, 2000, 2003), which was described wrongly as *Steinernema thermophilum* (Hunt, 2007), *Steinernema bicornutum* Tallosi, Peters and Ehlers (Hussaini et al., 2001), *Steinernema riobrave* Cabanillas et al. (Ganguly et al., 2002), *Steinernema glaseri* Steiner (Kadav and Lalramliana, 2012), *Steinernema carpocapsae* Weiser (Hussaini et al., 2001), and *Steinernema siamkayai* Stock, Somsook and Reid (Ganguly et al., 2002).

During the survey of EPNs in Uttar Pradesh, India, two nematode isolates belonging to the genus *Steinernema* Travassos, 1927, were recovered from soil samples of eggplant (*Solanum melongena* L. (Solanales: Solanaceae)) and cauliflower (*Brassica oleracea* L. (Brassicales: Brassicaceae)) fields of district Bijnor. Morphological, mophometric, and molecular studies showed that these nematodes are conspecific to *Steinernema surkhetense* Khatri-Chhetri, Waeyenberge, Spiridonov, Manandhar and Moens, with larger IJs and some other differences; hence is the first report of this species in India. Furthermore, we tested virulence of this nematode and for the first time and we performed a molecular characterization of their bacterial symbiont.

Materials and Methods

Nematode isolation: Entomopathogenic nematodes were isolated from soil samples taken during the month of June in 2013 from eggplant and cauliflower fields of Bijnor district of Western part of Uttar Pradesh, India, located in between 29° 2′ and 29° 58′ North and 78° 0′ to 78° 57′ East at an altitude of 115 m using the *Galleria mellonella* L. (Lepidoptera: Pyralidae) baiting technique (Bedding and Akhurst, 1975). Cadavers of *G. mellonella* recovered from the trap were disinfected in 0.1% NaOCl solution, washed in ddH_2O, and transferred onto White trap (White, 1927). The IJs were isolated from White traps, washed twice with ddH_2O, disinfected with 0.1% NaOCl, and finally stored into tissue culture flask at 15°C ± 1°C.

Bacteria isolation and molecular characterization: The symbiotic bacterium was obtained from the hemolymph

Received for publication October 12, 2016.
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The authors thank the Department of Science and Technology (DST), New Delhi, India, for providing the financial assistance through DST INSPIRE Fellowship/2014/74. To the anonymous reviewers for their comments and suggestions.
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This paper was edited by David Shapiro-Ilan.

of *G. mellonella* 1 d after infection with *S. surkhetense* CS19 following Akhurst (1980) methodology. The hemolymph was streaked on nutrient agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride and 0.0025% (w/v) bromothymol blue (NBTA medium) and left overnight at 28°C (Akhurst, 1980). Single colonies were transferred with a sterile toothpick to YS broth (Akhurst, 1980) and cultivated on an orbital shaker (180 rpm) at 25°C.

Bacterial DNA was extracted from a 2-d-old culture using DNeasy Blood & Tissue Kit (QIAGEN) according to manufacturer's instructions. The 16S RNA was amplified using primers 10F: 5′-AGTTTGATCATGGCT CAGATTG-3′ (forward) and 1507R: 5′-TACCTTGTTAC GACTTCACCCCAG-3′ (reverse) (Sandström et al., 2001). The recombinase A gene (recA) was amplified using primers RecA1F: 5′-GCTATTGATGAAAATAAACA-3′ (forward) and RecA2R: 5′-RATTTTRTCWCCRTTRT AGCT-3′ (reverse) (Tailliez et al., 2010). The gyrase B gene (gyrB) was amplified using primers 8SF gyrB: 5′-TACACGAAGAAGAAGGTGTTTCAG-3′ (forward) and 9Rev gyrB: 5′-TACTCATCCATTGCTTCATCATCT-3′ (reverse) (Tailliez et al., 2010). The PCR was performed as described by Půža et al. (2017). All PCR products were sequenced and deposited in GenBank under the following accession numbers: KY489779 (16S sequence), KX826948 (recA sequence), KX826949 (gyrB sequence).

Morphology and morphometry: For light microscopy, nematodes were reared on *G. mellonella*. A total of 20 larvae of *G. mellonella* were infected with sterilized IJs in sterile petri plates, which were killed within 24 to 36 hr. Adults of the first and second generation and freshly emerged third-stage juveniles were recovered and killed in hot water (60°C), fixed in TAF (7ml formalin, 2 ml triethanolamine, 91 ml distilled water) (Courtney et al., 1955), processed to glycerin (Seinhorst, 1959), and mounted into a small drop of glycerin. The cover slip was placed onto the glass slide with some extra amount of paraffin wax to prevent flatting of nematodes. Morphological observations were made using light compound microscope (Magnus MLX) and phase contrast microscope (Nikon Eclipse 50i). Morphometry was done with the help of inbuilt software of phase contrast microscope (Nikon DS-L1).

Scanning electron microscopy: For the scanning electron microscope, lukewarm water killed IJs were washed three times with 0.1 M phosphate buffer (pH 7.2) followed by fixing in 4% glutaraldehyde buffered with phosphate buffer (pH 7.2) at 4°C overnight and then washed with 0.1 M phosphate buffer. Each specimen was then postfixed with a 2% osmium tetroxide solution for 12 hr at room temperature, dehydrated in a graded ethanol series 30% to 100% (20 min each), and finally washed three times in 100% ethanol, critical point dried with liquid CO_2, mounted on SEM stubs, and coated with gold (Nguyen and Smart, 1995, 1997). A total 30 IJs (15 from each isolates) were studied for the lateral field. The mounts were examined with a Neo Scope JEOL 5000 FE scanning electron microscope (JEOL, Eching, Germany).

Genomic DNA extraction, amplification, and sequencing: For phylogenetic analysis, three molecular markers were used: internal transcribed spacer (ITS) regions of rDNA; partial sequence of 28S, D2-D3 domain; and mitochondrial gene encoding cytochrome C oxidase subunit I (COI). The DNA extraction and amplification of the ITS and D2-D3 regions of the rRNA were performed according to San-Blas et al. (2016). For the COI mtDNA region, the PCR protocol included denaturation at 94°C for 3 min, followed by 37 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec, followed by a final extension at 72°C for 7 min. The products were analyzed on 1% agarose gels with TAE buffer. The amplified PCR products were purified and sequenced in both directions by Bioserve Pvt. Ltd. Hyderabad (India). Sequences were deposited under the accession numbers KP219886, KU187262, and KU721841 for ITS, D2-D3, and COI genes, respectively, for CS19 and for CS20, and KR029844, KU187263, and KU721840 for the same respective regions.

Sequence alignment and phylogenetic analyses: The sequences were edited and compared with those deposited

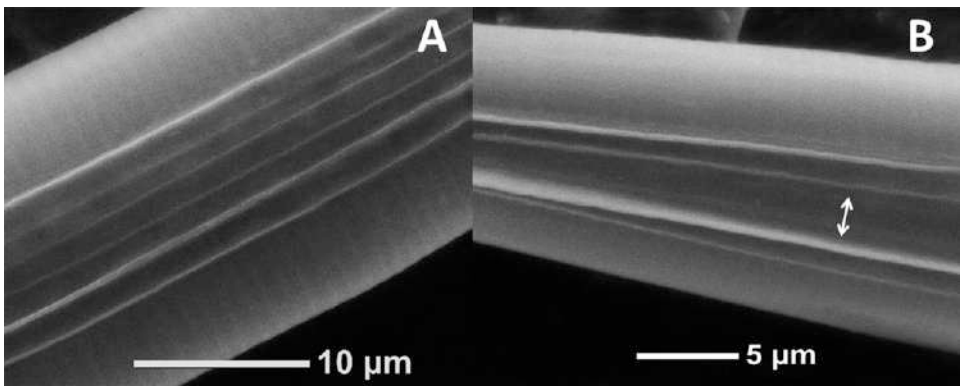

Fig. 1. Scanning electron microscope photography of infective juvenile of the Steinernema surkhetense. A. Lateral field at midbody level showing six ridges. B. Lateral field at the end of the body showing conspicuous sublateral ridges (arrows).

TABLE 1. Morphometrics of *Steinernema surkhetense* CS19. All measurements are in micrometers (except *n*, ratio, and percentage) and in the form: mean ± SD (range).

Characters	First generation		Second generation		Juvenile (IJs)
	Male	Female	Male	Female	
n	15	15	15	15	20
L	1,380 ± 220 (1,054–1,638)	5,511 ± 1,410 (3,066–8,608)	951 ± 111 (787–1,124)	2,230 ± 324 (1,553–2,795)	497 ± 17 (460–523)
a	17 ± 6.3 (10–27)	22 ± 3.1 (19–28)	15 ± 0.9 (14–17)	15 ± 3 (12–19)	19 ± 1 (17–21)
b	9 ± 1.1 (7–11)	26 ± 5 (17–38)	7 ± 0.5 (6.7–8.2)	13 ± 1.4 (11–15)	6 ± 0.7 (5–7)
c	44 ± 5 (36–51)	124 ± 35 (68–202)	36 ± 3 (29–40)	46 ± 7 (35–57)	11 ± 1.2 (10–16)
c–	1 ± 0.2 (0.6–1.3)	0.7 ± 0.1 (0.5–1.0)	0.84 ± 0.08 (0.7–0.9)	0.34 ± 0.1 (0.2–0.5)	3.5 ± 0.4 (2.6–4.4)
V	-	53 ±2.8 (48–58)	-	55 ± 1.2 (54–58)	-
Maximum body diameter	92 ± 32 (60–159)	245 ± 54 (140–345)	63 ± 7 (50–81)	150 ± 32 (109–204)	26 ± 1.1 (25–28)
Excretory pore	68 ± 9 (55–84)	87 ± 9 (73–100)	63 ± 7 (53–80)	81 ± 11 (51–99)	38 ± 3.4 (35–50)
Nerve	111 ± 12 (81–125)	160 ± 19 (121–186)	104 ± 8 (89–116)	129 ± 10 (113–155)	68 ± 8 (57 - 85)
Stoma length	146 ± 13 (120–161)	210 ± 20 (170–238)	130 ± 10 (107–145)	168 ± 16 (145–198)	91 ± 10 (72–106)
Tail length	32 ± 5 (23–41)	45 ± 6 (35–58)	25 ± 2 (21–28)	49 ± 4.4 (42–57)	47 ± 4 (33–51)
Hyaline portion	-	-	-	-	23 ± 2.4 (19–26)
Anal body width	32 ± 4 (26–41)	62 ± 13 (44–90)	30 ± 2 (26–33)	43 ± 7.9 (34–55)	13 ± 1 (11–16)
Spicule length	70 ± 4 (63–75)	-	65 ± 5 (57–73)	-	-
Spicule width	8 ±1.7 (5–11)	-	7 ± 1.1 (5–8)	-	-
Gubernaculum length	44 ± 5 (35–53)	-	38 ± 6(31–47)	-	-
Gubernaculum width	5 ± 0.6 (4–6)	-	4 ± 1 (3–7)	-	-
D%	46 ± 6 (34–59)	41 ± 5 (34–49)	48 ± 4 (42–59)	48 ± 5.6 (36–56)	42 ± 6 (36–52)
E%	215 ± 20 (168–257)	196 ± 32 (139–242)	253 ± 54 (197–344)	168 ± 24 (141–220)	82 ± 17 (70–102)
SW%	220 ± 25 (175–252)	-	223 ± 24 (185–276)	-	-
GS%	63 ± 7 (49–73)	-	58 ± 7 (49–70)	-	-
H%	-	-	-	-	49 ± 5.6 (37–59)
Mucron	3.4 ± 0.1 (2.4–4.4)	8.7 ± 0.4 (6.8–11.4)	3.9 ± 0.1 (3.4–4.8)	4.9 ± 0.3 (3.9–7.0)	-

SW = spicule length, GS = gubernaculum length/spicule width.

TABLE 2. Morphometrics of *Steinernema surkhetense* CS20. All measurements are in micrometers (except *n*, ratio, and percentage) and in the form: mean ± SD (range).

Characters	First generation		Second generation		Juvenile (IJs)
	Male	Female	Male	Female	
n	15	15	15	15	20
L	1,224 ± 128 (1,061–1,437)	5,563 ± 1,262 (2,944–7,630)	915 ± 91 (740–1,010)	1,900 ± 324 (1,502–2,453)	494 ± 23 (448–525)
a	12 ± 2 (9–15)	26 ± 4 (19–33)	15 ± 1 (14–18)	17 ± 2 (14–20)	18 ± 1 (15–21)
b	9 ± 0.9 (7–12)	28 ± 5 (18–36)	7 ± 0.5 (6–8)	12 ± 1 (10–14)	5 ± 1 (4–10)
c	46 ± 5 (39–57)	123 ± 21 (78–160)	37 ± 5 (27–45)	39 ± 6 (32–57)	10 ± 0.8 (8–11)
c′	0.8 ± 0.1 (0.6–1.0)	0.8 ± 0.1 (0.6–1.0)	09 ± 0.1 (0.6–1.1)	1.3 ± 0.2 (0.9–1.5)	4 ± 0.3 (3.3–4.4)
V	–	53 ± 1.5 (50–565)	–	57 ± 2.2 (54–61)	–
Maximum body diameter	104 ± 20 (70–142)	213 ± 45 (156–306)	61 ± 7 (51–73)	114 ± 20 (86–151)	27 ± 2 (23–32)
Excretory pore	59 ± 8 (35–71)	82 ± 12 (56–98)	60 ± 4 (52–66)	74 ± 13 (31–85)	39 ± 3 (35–44)
Nerve ring	98 ± 16 (82–145)	150 ± 13 (128–170)	98 ± 5 (90–106)	123 ± 13 (97–143)	70 ± 10 (39–91)
Stoma length	132 ± 15 (115–176)	199 ± 14 (165–216)	129 ± 8 (115–143)	157 ± 14 (133–178)	98 ± 16 (47–112)
Tail length	27 ± 3 (22–32)	45 ± 7 (26–59)	25 ± 2 (22–30)	49 ± 4 (43–57)	50 ± 5 (42–63)
Hyaline portion	–	–	–	–	22 ± 2.6 (18–26)
Anal body width	35 ± 5 (27–44)	59 ± 12 (41–78)	29 ± 3 (25–37)	39 ± 6 (31–48)	13 ± 1 (12–17)
Spicule length	73 ± 4 (66–80)	–	64 ± 6 (51–71)	–	–
Spicule width	6 ± 2 (4–9)	–	6 ± 2 (4–10)	–	–
Gubernaculum length	47 ± 6 (38–55)	–	37 ± 3 (33–43)	–	–
Gubernaculum width	5 ± 0.7 (3–6)	–	5 ± 0.7 (3–6)	–	–
D%	45 ± 8 (20–54)	41 ± 5 (32–48)	47 ± 3 (42–52)	48 ± 9 (41–60)	41 ± 9 (32–76)
E%	221 ± 34 (125–281)	183 ± 19 (149–213)	244 ± 27 (195–288)	151 ± 27 (68–185)	78 ± 11 (57–102)
SW%	214 ± 37 (160–299)	–	220 ± 27 (178–275)	–	–
GS%	65 ± 8 (50–77)	–	59 ± 7 (50–73)	–	–
H%	–	–	–	–	45 ± 5.6 (34–60)
Mucron	3.5 ± 0.1 (2.8–5.8)	9.5 ± 0.3 (7.9–12.4)	4.0 ± 0.1 (3.3–4.6)	5.1 ± 0.2 (4.1–6.8)	–

SW = spicule width, GS = gubernaculum length/spicule width.

TABLE 3. Comparative morphometrics of all generations of CS19 and CS20 with *Steinernema surkhetense*. All measurements are in micrometers (except percentage) and in the form of mean (range).

Species	L	MBD	EP	NR	ES	Tail	a	b	c	D%	E%
Infective juveniles											
CS19	497 (460–523)	26 (25–28)	38 (35–50)	68 (57–85)	91 (72–106)	47 (33–51)	19 (17–21)	6 (5–7)	11 (10–16)	42 (36–52)	82 (70–102)
CS20	494 (448–525)	27 (23–32)	39 (34–44)	70 (39–91)	98 (74 –113)	50 (42–63)	18 (15–21)	5 (4–10)	10 (8–11)	41 (32–76)	78 (57–102)
S. surkhetense	415 (393–450)	21 (18–25)	32 (28–34)	63 (57–70)	92 (84–101)	45 (38–53)	19 (16–24)	5 (4.3–4.8)	9 (8.3–10)	35 (31–40)	72 (54–84)

Species	EP	NR	ES	Tail	a	b	c	V%	D%	E%
Female I										
CS19	87 (73–100)	160 (121–186)	210 (170–238)	45 (35–58)	22 (19–28)	26 (17–38)	124 (68–202)	53 (48–58)	41 (34–49)	196 (139–242)
CS20	82 (56–98)	150 (129–170)	199 (165–216)	45 (26–59)	26 (19–33)	28 (18–36)	123 (78–160)	53 (50–56)	41 (32–48)	183 (149–213)
S. surkhetense	53 (33–86)	93 (64–114)	132 (52–190)	19 (12–30)	19 (13–25)	24 (14–49)	161 (91–309)	54 (45–59)	44 (28–117)	292 (156–453)
Female II										
CS19	81 (51–99)	129 (113–155)	168 (145–198)	49 (42–57)	15 (12–19)	13 (11–15)	46 (35–57)	55 (54–58)	48 (36–56)	168 (141–220)
CS20	74 (31–85)	123 (97–143)	157 (133–178)	49 (43–57)	17 (14–20)	12 (10–14)	39 (32–57)	57 (54–61)	48 (41–60)	151 (68–185)
S. surkhetense	137 (96–195)	159 (142–198)	193 (173–236)	103 (65–135)	13 (11–16)	5.6 (4.6–6.7)	11 (8.7–15)	50 (42–54)	71 (50–87)	137 (85–189)

Species	EP	NR	ES	Tail	SL	GL	A	b	c	D%	E%	SW%	GS%
Male I													
CS19	68 (55–84)	111 (81–125)	146 (120–161)	32 (23–41)	70 (63–75)	45 (35–53)	17 (10–27)	9 (7–11)	44 (36–51)	46 (34–59)	215 (168–257)	220 (175–252)	63 (49–73)
CS20	59 (35–71)	98 (82–145)	132 (115–176)	27 (22–32)	72 (66–80)	47 (38–55)	12 (9–15)	9 (7–12)	46 (39–57)	45 (20–54)	221 (125–281)	214 (160–299)	65 (50–77)
S. surkhetense	55 (43–78)	92 (59–140)	115 (86–146)	19 (16–23)	70 (58–78)	52 (42–63)	12 (10–14)	11 (8.0–15)	65 (43–85)	48 (37–64)	295 (205–429)	230 (168–330)	75 (66–84)
Male II													
CS19	63 (53–80)	104 (89–116)	130 (107–145)	25 (21–28)	65 (57–73)	38 (31–47)	15 (14–17)	7 (6.7–8.2)	36 (29–40)	48 (42–59)	253 (197–344)	223 (185–276)	58 (49–70)
CS20	60 (52–66)	98 (90–106)	129 (115–143)	25 (22–30)	64 (51–71)	37 (33–43)	15 (14–18)	7 (6–8)	37 (27–45)	47 (42–52)	244 (195–288)	220 (178–275)	59 (50–73)
S. surkhetense	44 (35–50)	90 (73–109)	122 (106–141)	19 (15–27)	64 (55–76)	44 (35–53)	19 (13–35)	24 (14–49)	161 (91–309)	36 (31–42)	237 (167–317)	239 (191–292)	68 (57–79)

MBD = maximum body diameter, EP = distance from the anterior end to excretory pore, NR = distance from the anterior end to nerve ring, ES = esophagus length, SL = spicule length, GL = gubernaculum length, SW = spicule width, GS = GL/SL.

in GenBank by means of a Basic Local Alignment Search Tool of the National Center for Biotechnology Information. All alignments with other relevant sequences were produced by default ClustalW parameters in MEGA 6.0 (Tamura et al., 2013). Highly variable regions of the multiple alignments were removed, and conserved regions were selected with Gblocks program (Castresana, 2000). Alignments of the bacterial recA and gyrB genes were concatenated into one dataset. Pairwise distances were computed using MEGA 6.0 (Tamura et al., 2013). Codon positions included were 1st + 2nd + 3rd + Noncoding.

The phylogenetic trees were obtained by Bayesian inference using MrBayes 3.1.1. (Huelsenbeck and Ronquist, 2001). The best-fit model was identified as the GTR + G model test using the MrModeltest 2.0 program (Nylander, 2004). Metropolis-coupled Markov chain Monte Carlo generations were run for 10,000,000 cycles and one tree was retained every 1,000 generations and a burn-in of 3,000,000 generations (Huelsenbeck and Ronquist, 2001).

All characters were treated as equally weighted and gaps as missing data. For the nematode and bacteria sequences, *Steinernema affine* Wouts, Mracek, Gerdin and Bedding and *S. abbasi* and *Xenorhabdus poinari* Akhurst and Boemare and *Xenorhabdus bovienii* Akhurst and Boemare, respectively, were used as outgroup taxa and to root the trees.

Virulence tests on Spodoptera litura*:* Virulence and reproductive potential of both *Steinernema* isolates (CS19 and CS20) against *S. litura* Fab. (Lepidoptera: Noctuidae) were performed. IJs (1 wk old from emergence) were used for larval mortality against *S. litura* in six well plates (3.5 cm diameter) lined by double Whatman filter paper No. 1. Randomly 0, 25, 50, 100, and 200 IJs were placed over the filter paper using 450 µl with distilled water. Ten insect larvae of same size and weight were used for each concentration and a single larva was set per well (repeated twice). The plates were incubated at 28°C ± 2°C. Mortality was recorded every 12-hr interval till 100% mortality was achieved. Larvae infected with 100 IJs/larva were transferred after 7 d to a modified White trap (White, 1927) to measure progeny production (18–20 d).

The insect larval mortality assay was analyzed statistically through probit analysis, and LC_{50} and LT_{50} values were calculated at 95% confidence limit. Differences between percentages of mortality depending on the isolates were assessed using analysis of variance. Data were presented as percentage ± SD. Total number of IJs/larva of the studied nematode was analyzed by *t* test analysis and presented in number of IJs ± SD (range).

Results and Discussion

Results obtained through morphology, morphometry, and molecular analysis identify both isolated nematodes (CS10 and CS20) as *S. surkhetense* (Khatri-Chetri et al., 2011). Being the first report from India, three slides of the first-generation female bearing one female on each slide, two slides of first-generation males bearing

Table 4. Sequence lengths and composition of ITS-rDNA, D2-D3 region, and COI of some closely related described *Steinernema* species from the *carpocapsae* group and the present isolate CS19 and CS20.

Species	Molecular composition							Sequence length (bp)
	ITS1 (bp)	5.8S (bp)	ITS2 (bp)	A (%)	C (%)	G (%)	T (%)	
ITS region								
S. surkhetense CS19	277	157	306	0.24	0.17	0.21	0.38	740
S. surkhetense CS20	277	157	306	0.24	0.17	0.22	0.38	740
S. surkhetense	277	157	306	0.24	0.17	0.21	0.38	740
S. nepalense	277	157	302	0.22	0.17	0.22	0.38	734
S. backanense	272	157	307	0.24	0.17	0.22	0.38	736
S. sasonense	278	157	307	0.23	0.17	0.22	0.39	742
S. carpocapsae	279	157	295	0.23	0.16	0.22	0.39	731
S. scapterisci	246	157	376	0.25	0.16	0.21	0.38	779
S. siamkayai	266	155	307	0.23	0.15	0.22	0.4	728
S. cumgarense	269	155	306	0.23	0.16	0.22	0.39	730
S. eapokense	262	155	306	0.23	0.15	0.23	0.39	723
D2-D3 region								
S. surkhetense CS19				0.25	0.17	0.30	0.28	880
S. surkhetense CS20				0.25	0.17	0.30	0.28	881
S. surkhetense				0.25	0.17	0.31	0.27	614
S. nepalense				0.25	0.17	0.31	0.27	614
S. carpocapsae				0.25	0.17	0.3	0.28	820
S. scapterisci				0.26	0.18	0.29	0.27	807
COX1								
S. surkhetense CS19				0.23	0.14	0.17	0.46	634
S. surkhetense CS20				0.25	0.14	0.17	0.45	665
S. carpocapsae				0.24	0.13	0.17	0.46	568
S. scapterisci				0.24	0.14	0.17	0.45	568

TABLE 5. Pairwise distances of the ITS region between *Steinernema* species from the "*carpocapsae*" group.

	ITS region	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*S. surkhetense* CS19 KP219886		0	5	57	35	67	50	55	66	63	40	44	51	91
2	*S. surkhetense* CS20 KR029844	100		5	57	35	67	50	55	66	63	40	44	51	91
3	*S. surkhetense* HQ190042	99.3	99.4		60	40	70	53	58	69	66	43	45	54	93
4	*S. siamkayai* AF331917	92.3	92.3	91.9		49	28	26	15	25	25	59	53	56	90
5	*S. nepalense* HQ190044	95.3	95.5	95.0	93.3		60	43	52	58	59	38	35	45	89
6	*S. huense* KF857581	91.0	91.5	91.2	96.2	92.4		30	30	31	29	59	59	64	98
7	*S. eapokense* AY487921	93.1	93.1	92.7	96.4	94.0	95.8		27	33	35	41	45	45	90
8	*S. minutum* GU647156	92.6	92.9	92.7	98.0	93.4	96.3	96.2		17	27	59	53	60	94
9	*S. tami* AY171280	91.0	91.0	90.7	96.6	92.1	95.8	95.4	97.7		34	62	58	65	99
10	*S. cumgarense* AY487920	91.4	91.3	90.9	96.5	91.9	96.0	95.2	96.3	95.3		62	60	61	98
11	*S. backanense* AY487918	94.5	94.5	94.1	91.8	94.8	91.8	94.3	91.8	91.4	91.4		38	38	88
12	*S. sasonense* AY487919	94.0	94.0	93.9	92.7	95.2	91.9	93.8	92.7	92.0	91.8	94.8		34	86
13	*S. carpocapsae* GU395621	93.2	93.5	93.2	92.4	94.4	92.7	93.7	92.6	91.1	91.6	94.8	95.4		92
14	*S. scapterisci* AY230183	87.4	87.8	87.9	87.3	88.4	88.4	86.9	88.0	86.0	85.9	87.4	87.8	89.2	

Below diagonal: percentage similarity; above diagonal: total character differences.

three males on each slide, two slides of each second-generation females and males bearing two and three specimens, respectively, on each slide were deposited in National Nematode Collection of India, IARI, New Delhi, India.

Morphology and morphometry: The present two *Steinernema* isolates (CS19 and CS20) were identified as *S. surkhetense*; however, some differences with the original description can be notated. Postanal swellings in the first- and second-generation females are present (rarely seen in original description) and the number of lateral ridges observed in IJs was six (in all studied specimens) instead of eight in the original description (Fig. 1). Different number of lateral ridges from the same species has been described in the "*carpocapsae*" group, however, specimens came from the same studied populations (Stock et al., 1998; Hazir et al., 2003); the rest of the species within the group have either six or eight ridges (but not both). The variation of the number of lateral ridges in the Indian populations compared with the Nepali can be attributed to differences in their geographical origin or by an inaccuracy in the original description.

Morphometrically, body size of IJs *S. surkhetense* CS19 (Table 1) was comparatively longer than the original description 497 (460–523) vs. 415 (393–450) μm. Apart from this, distance from anterior end to excretory pore 38 (35–50) vs. 32 (28–34) μm showed variation. The first-generation males of both the specimens showed difference in gubernaculum length 45 (35–53) vs. 52 (42–63) μm, and GS% 63 (50–73) vs. 75 (66–84); however, the spicule length was in near vicinity 70 (63–75) vs. 70 (58–78) μm. Abovementioned character differences were also notified in isolate CS20 (Table 2) when compared with original species showing also longer IJs, but the ranges were slightly overlapping with the original description (494 ± 23 [448–525] vs. 415 [393–450] μm). Nevertheless, the IJ size could be sufficient to distinguish the Nepalese and Indian populations. Males of the second generation also showed variations in the morphometry. Highly large body sizes were observed in females of both generations of Indian isolates and were almost double to the Nepali isolates and highly varied with other morphometrical parameters such as pharynx length, excretory pore, and nerve ring position in first generation and vice versa in second-generation females. Tail lengths were also found varied in both generations in Indian and Nepali isolates. A comparison in morphometrical parameters in all generations is shown in Table 3.

TABLE 6. Pairwise distances of the D2-D3 regions between *Steinernema* species of the "*carpocapsae*" group.

	D2-D3 region	1	2	3	4	5	6	7	8	9	10
1	*S. surkethense* CS19		0	0	6	4	9	7	9	7	42
2	*S. surkethense* CS20	100		0	6	4	9	7	9	7	42
3	*S. surkhetense* HQ190043	100	100		3	4	5	4	6	4	27
4	*S. simakayai* CS33 KX871218	99.3	99.3	99.5		7	8	10	10	10	44
5	*S. nepalense* HQ190045	99.3	99.3	99.3	98.7		7	8	10	8	29
6	*S. huense* KF857582	98.9	98.9	99.1	99.0	98.8		12	14	12	45
7	*S. websteri* AY841762	99.1	99.1	99.3	98.8	98.6	98.5		2	0	44
8	*S. anatoliense* AY841761	98.9	98.9	98.9	98.7	98.2	98.3	99.8		2	46
9	*S. carpocapsae* HM140688	99.1	99.1	99.3	98.8	98.6	98.5	100.0	99.8		44
10	*S. scapterisci* GU395646	94.8	94.8	95.2	94.5	94.8	94.4	94.5	94.3	94.5	

Below diagonal: percentage similarity; above diagonal: total character differences.

Molecular characterization: From the original ITS sequence of *S. surkhetense*, both Indian strains differ by 5 bp, while differ from each other by 2 bp. In the present study, we sequenced for the first time the full-length D2-D3 region of the 28S rDNA of *S. surkhetense*. The only D2-D3 sequence of *S. surkhetense* (HQ190043) available so far has only 614 bp (Tables 4-6). No variation in the D2-D3 sequence was found between CS19 and CS20 strains and the original *S. surkhetense*. For the first time, we sequenced the COI gene of *S surkhetense* and the two Indian strains differ from each other by 7 bp.

Phylogenetic analysis: Phylogenetic analyses of the "*carpocapsae*" group based on ITS region showed a clear monophyly of the group formed by the isolates CS19 and CS20 and original *S. surkhetense* and several other, probably conspecific isolates (Fig. 2). Sequences of *S. surkhetense* formed a monophyletic group with *S. nepalense* Khatri-Chhetri, Waeyenberge, Spiridonov, Manandhar and Moens, and this pair was sister to the pair of *Steinernema backanense* Phan, Spiridonov, Subbotin and Moens and *S. carpocapsae*.

In the D2-D3 tree, *S. surkhetense* formed a monophyletic group with *S. carpocapsae*, *Steinernema anatoliense* Hazir, Stock and Keskin and *Steinernema websteri* Cutler and Stock (Fig. 3). However, the D2-D3 region is too conservative to resolve the relationships among these closely related species. In general, molecular data accompanied by morphological and morphometrical confirmed the status of two isolates as species of *S. surkhetense* according to the phylogenetic and evolutionary species concept (Adams, 1998).

For the COI region, there were not enough sequences within "*carpocapsae*" group to construct any useful phylogenetic tree. However, both resulting sequences were added to GENBANK with accession numbers of KU721840 (CS20) and KU721841 (CS19).

Symbiotic bacterium: The molecular characterization of the symbiotic bacterium of *S. surkhetense* was performed for the first time. Based on the sequences of the 16S, recA, and gyrB genes, the bacterium *Xenorhabdus* sp. CS19 is very close to *Xenorhabdus stockiae* Tailliez, Pagès, Ginibre and Boemare (similarity 99%, 96%, and 97%, respectively, data not shown). The phylogenetic tree based on the concatenated recA and gyrB sequences shows a highly supported group of the *Xenorhabdus* sp.

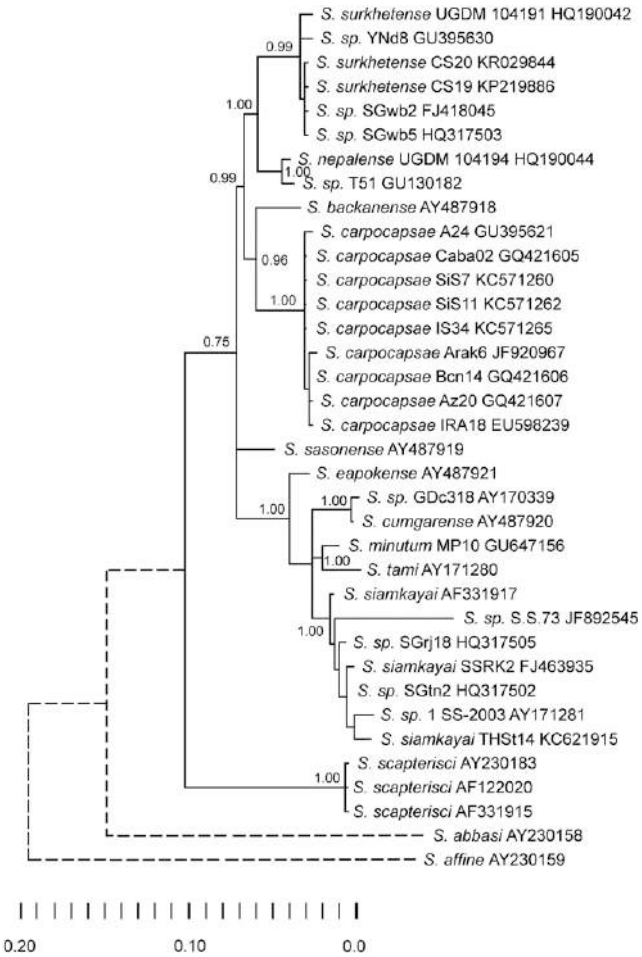

FIG. 2. Phylogenetic relationships in the "*carpocapsae*" group and other closely related species of *Steinernema* based on analysis of ITS rDNA regions. *Steinernema abbasi* and *Steinernema affine* were used as the outgroup taxon. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of number of base differences per site.

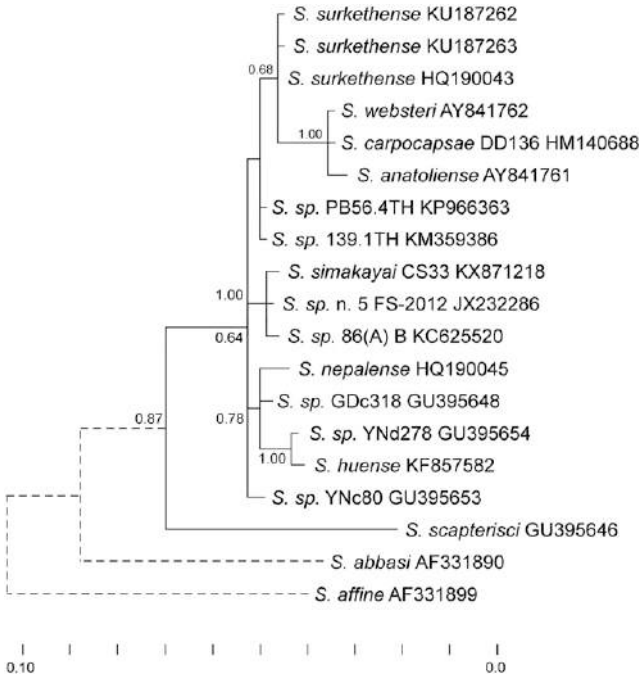

FIG. 3. Phylogenetic relationships in the "*carpocapsae*" group and other closely related species of *Steinernema* based on analysis of D2-D3 expansion segments of the 28S rDNA. *Steinernema abbasi* and *Steinernema affine* were used as the outgroup taxon. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of number of base differences per site.

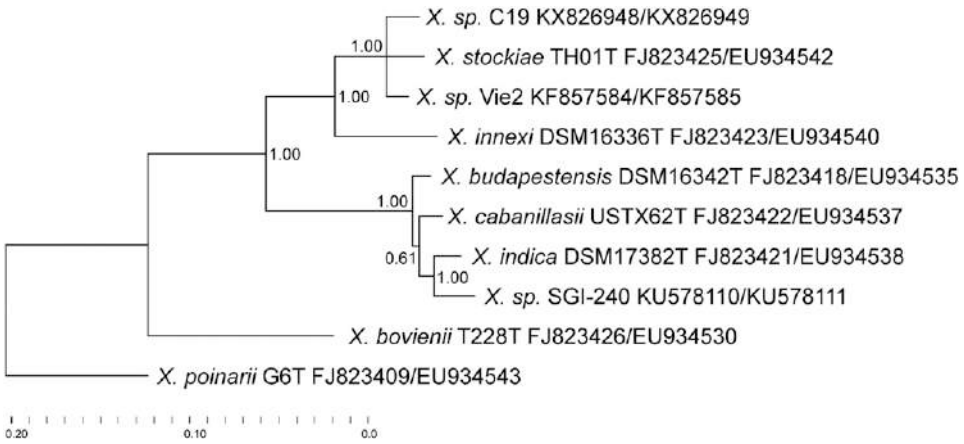

FIG. 4. Phylogenetic relationships of *Xenorhabdus* sp. isolated from *Steinernema surkhetense* (CS19) with other closely related species of *Xenorhabdus* based on analysis of recA and gyrB gene sequences. *Xenorhabdus bovieni* and *X. poinari* were used as the outgroup taxa. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of number of base differences per site.

CS19 with *X. stockiae* and *Xenorhabdus* sp. VIE2 (Fig. 4). So far, *X. stockiae* was isolated from closely related nematodes *S. siamkayai* (Tailliez et al., 2006), *S. minutum* (Maneesakorn et al., 2010), and *S. huense* (Phan et al., 2014). These data, together with finding of *X. stockiae* in *S. surkhetense*, suggest that this bacterium is widespread among "*carpocapsae*" group nematodes occurring in South Asia.

Virulence tests: The parameters measured when the nematodes were applied to *S. litura* larvae favored the strain CS19. The LD_{50} at 72 hr, showed that the strain CS19 was capable to kill the larvae with 31.78 IJs, whereas at the same time, CS20 the number of IJs need to kill 50% of the *S. litura* larvae was doubled (LD_{50} = 67.7 IJs). Progeny production was greater in the strain CS19 (61,440 ± 3,817 IJs per larva) than the progeny produced by the strain CS20 (27,990 ± 3,187 IJs per larva) ($T = 5.8$; $P \leq 0.001$; $\alpha = 0.05$). The dynamics of the infection according to different doses also showed that the strain CS19 achieved higher mortality rates in less time than the strain CS20 when the target was *S. litura* (Fig. 5A); the best example of this could be observed when 25 IJs were applied to the larva, where at 48 hr, the percentage of mortality was 15 ± 5% *vs* 5%; at 72 hr, the percentage of mortality increased to 65 ± 5% *vs* 35± 5% and after 84 hr, strain CS19 was capable to kill 85 ± 5% of the larvae and strain 20 killed 65 ± 5% of the offered larvae (Fig. 5B). This variation of differences in the virulence strains of the same species has previously been reported. Shapiro-Ilan et al. (2003) found large differences in infectivity and mortality of *Curculio caryae* Horn (Coleoptera: Curculionidae) using different strains of *S. carpocapsae*; similarly, Campos-Herrera et al. (2006) also found differences in virulence tests of *S. feltiae* in three different hosts: These mentioned differences could be related to metapopulation theory, where diverse populations of the same species coming from different geographic sites could behave differently due to natural changes such as niche, presence-absences of determined host, adaptation to abiotic factors, etc. (Harris et al., 2013).

India is the second largest producer of vegetables in the world (after China) with an annual production of 101.43 million tons from 6.76 million ha of land (Rai

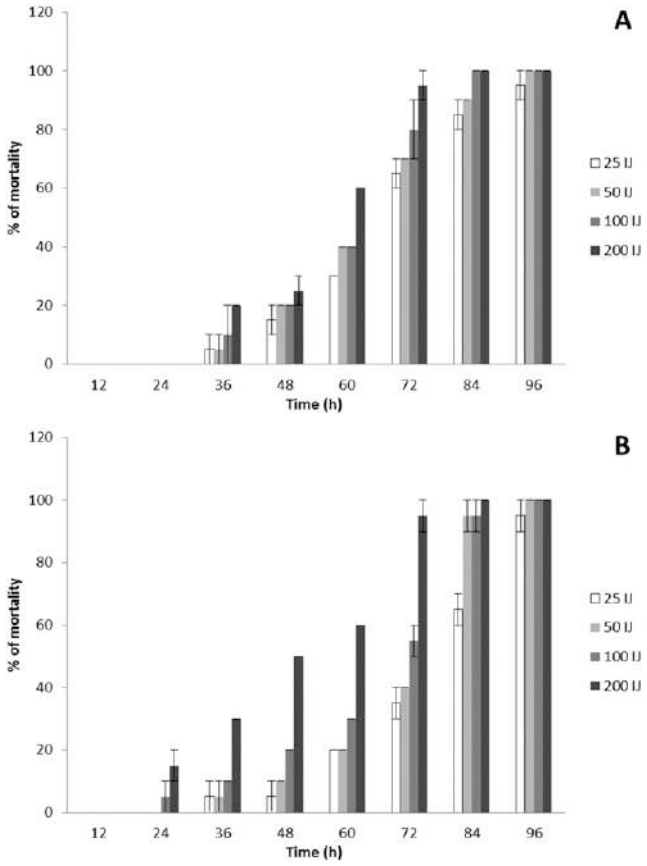

FIG. 5. Percentage of mortality of *Spodoptera littura* larvae with different doses of *Steinernema surkhetense*. A) Strain CS19. B. Strain CS20.

and Pandey, 2007). It is also the most important producer of cauliflowers and the second of eggplants. The most important pests of these crops include *S. litura*, *Plutella xylostella* L. (Lepidoptera: Plutellidae), *Crocidolomia binotalis* Zeller (Lepidoptera: Pyralidae), *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae), *Leucinodes orbonalis* Guenee (Lepidoptera: Crambidae), *Pieris brassicae* L. (Lepidoptera: Pieridae), *Hellula undalis* Fab. (Lepidoptera: Crambidae), *Spilosoma obliqua* Walker (Lepidoptera: Erebidae), and *Brevicoryne brassicae* L. (Hemiptera: Aphididae) all widely distributed in different agroclimatic conditions in India. *Steinernema surkhetense* is an indigenous species to Indian subcontinent; efforts should be made to evaluate its virulence and pathogenicity against the mentioned agricultural pests throughout the country. This may lead to incorporate *S. surkhetnese* as a regular biological control agent in integrated pest management programs in the future.

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The first report of *Xenorhabdus indica* from *Steinernema pakistanense*: co-phylogenetic study suggests co-speciation between *X. indica* and its steinernematid nematodes

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Research Paper

Cite this article: Bhat AH, Chaubey AK, Půža V. The first report of *Xenorhabdus indica* from *Steinernema pakistanense*: co-phylogenetic study suggests co-speciation between *X. indica* and its steinernematid nematodes. *Journal of Helminthology* https://doi.org/10.1017/S0022149X17001171

Received: 29 September 2017
Accepted: 29 November 2017

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Abstract

During a survey in agricultural fields of the sub-humid region of Meerut district, India, two strains of entomopathogenic nematodes, labelled CS31 and CS32, were isolated using the *Galleria* baiting technique. Based on morphological and morphometric studies, and molecular data, the nematodes were identified as *Steinernema pakistanense*, making this finding the first report of this species from India. For the first time, we performed a molecular and biochemical characterization of the bacterial symbiont of *S. pakistanense*. Furthermore, a co-phylogenetic analysis of the bacteria from the monophyletic clade containing a symbiont of *S. pakistanense*, together with their nematode hosts, was conducted, to test the degree of nematode–bacteria co-speciation. Both isolates were also tested in a laboratory assay for pathogenicity against two major pests, *Helicoverpa armigera* and *Spodoptera litura*. The morphology of the Indian isolates corresponds mainly to the original description, with the only difference being the absence of a mucron in first-generation females and missing epiptygmata in the second generation. The sequences of bacterial *recA* and *gyrB* genes have shown that the symbiont of *S. pakistanense* is closely related to *Xenorhabdus indica*, which is associated with some other nematodes from the '*bicornutum*' group. Co-phylogenetic analysis has shown a remarkable congruence between the nematode and bacterial phylogenies, suggesting that, in some lineages within the *Steinernema* / *Xenorhabdus* complex, the nematodes and bacteria have undergone co-speciation. In the virulence assay, both strains caused a 100% mortality of both tested insects after 48 h, even at the lowest doses of 25 infective juveniles per insect, suggesting that *S. pakistanense* could be considered for use in the biocontrol of these organisms in India.

Introduction

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae are important biocontrol agents of a broad range of insects that damage agricultural crops throughout the world (Shapiro-Ilan *et al.*, 2014; Lacey *et al.*, 2015; Kergunteuil *et al.*, 2016). They are used as an alternative for insect control due to their ability to locate host insects, great reproductive capacity and virulence, and association with bacteria of the genera *Xenorhabdus* spp. (Steinernematidae) and *Photorhabdus* spp. (Heterorhabditidae) (Gaugler & Kaya, 1990; Kaya & Gaugler, 1993; Burnell & Stock, 2000; Kaya *et al.*, 2006). Steinernematids are cosmopolitan and, to date, 95 species (Hunt & Nguyen, 2016) have been described in all the continents except Antarctica, and this number is growing every year.

In India, little EPN diversity has been found during surveys of agricultural soils. Currently, a total of nine EPN species (seven steinernematids and two heterorhabditids) have been reported from India, of which only one new species (*Heterorhabditis indica* Poinar, Karunakar and David, 1992) has been described from the country. Among steinernematids, no new species have been reported to date, but two previously described new species have been synonymized with existing ones, namely *Steinernema thermophilum* (Gaungly & Singh, 2000) was synonymized with *S. abbasi* (Hunt, 2007) and *Steinernema dharanai* (Kulkarni, Rizvi, Kumar, Paunikar & Mishra, 2012) was recognized as a junior synonym of *S. hermaphroditum* (Stock, Griffin & Chaerani, 2004).

For the commercial and successful establishment of EPNs as biological control agents, it is important to isolate and identify the effective EPN strain. In the search for EPN populations, a survey was conducted in the agricultural fields of the Meerut District of Western Uttar Pradesh, India during the month of June, when temperatures can reach up to 40°C, to assess their presence during such high temperatures. Two nematode populations belonging to the genus *Steinernema* Travassos, 1927, were recovered from soil samples of pepper (*Capsicum*

annuum L. (Solanales: Solanaceae)) and pearl millet (*Pennisetum glaucum* L. (Poales: Poaceae)) fields. The third-stage infective juveniles display two horn-like structures on the labial region, and thus belong to the '*bicornutum*' group. The morphology, morphometry and molecular characterization of these isolates revealed that they are conspecific to *S. pakistanense* Shahina, Anis, Reid, Rowe and Maqbool, 2001; hence it is the first report of *S. pakistanense* from the Indian subcontinent.

For many steinernematids, the identity of the bacterial symbiont is unknown, even though such information is necessary for our better understanding of the diversity of these nematode–bacteria complexes. In the only study focused on the symbiont of *S. pakistanense,* Shahina *et al.* (2004) reported this nematode to be associated with *Xenorhabdus nematophila*. This bacterium is known to be associated with *Steinernema carpocapsae*, and its presence in *S. pakistanense*, a nematode from a different phylogenetic clade, seems surprising, although host switches in the *Steinernema–Xenorhabdus* complex have been reported to be frequent (Lee & Stock, 2010). However, in their study, Shahina *et al.* (2004) identified the bacterium based solely on phenotypic data, which are now considered insufficient for the precise identification of bacteria. Thus we have performed the first molecular characterization of the bacterium associated with *S. pakistanense*.

Because the isolated bacterium was found to be related to the symbionts reported from other related nematode species, suggesting a possible co-evolutionary history for the nematodes and bacteria, we have also performed a co-phylogenetic analysis, to test the degree of nematode–bacteria co-speciation.

Finally, the isolates were tested in laboratory bioassays for pathogenicity against *Spodoptera litura* (Fabricius, 1775) (Lepidoptera: Noctuidae) and *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae).

Materials and methods

Nematode origin and culture

A total of 25 soil samples were collected by taking five random sub-samples at a depth of 0–20 cm from different agricultural fields of the Meerut district (28°59′N, 77°42′E and 225 m above sea level) of Western Uttar Pradesh. EPNs were recovered from only two soil samples using the insect-baiting technique (Bedding & Akhurst, 1974) with the last instars of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), and the remaining soil samples were found to be negative for EPN infection. Infective juveniles (IJs) were maintained by recycling through *G. mellonella* larvae, washed properly with double-distilled water (DDW), disinfected with 0.1% sodium hypochlorite and stored in approximately 150 ml of sterilized distilled water in 500-ml vented tissue-culture flasks at 15°C for subsequent identification and the establishment of stock cultures.

Morphological and morphometric characterization of the nematodes

For taxonomic studies, first- and second-generation adults were obtained by dissecting the dead *G. mellonella* cadavers in Ringer's solution on the third and fifth days after infection, respectively, while fresh IJs were collected from a White trap (White, 1927). They were killed in hot water (60°C), fixed in TAF (7 ml formalin, 2 ml triethanolamine, 91 ml distilled water) (Courtney *et al.*, 1955), processed to glycerin (Seinhorst, 1959) and mounted in a small drop of glycerin on a glass slide. The cover slip was placed on to the glycerin drop with some extra paraffin wax to prevent the flattening of the nematodes. Morphological observations were made using a light compound microscope (Magnus MLX; Olympus, New Delhi, India) and phase-contrast microscope (Nikon Eclipse 50i; Nikon, Tolyo, Japan). Morphometric details were gathered with the help of the inbuilt software of a phase-contrast microscope (Nikon DS-L1).

DNA extraction, amplification and sequencing of the nematodes

The total genomic DNA was extracted from the pool of infective juveniles using a Quiagen DNA Blood and Tissue Kit (Qiagen, Hilden, Germany) (Bhat *et al.*, 2017) followed by agarose gel electrophoresis (AGE) for the detection of DNA in the collected eluate. Molecular characterizations were based on internal transcribed spacer (ITS) regions of rDNA and a partial sequence of 28S rDNA (D2–D3 domain). These regions were amplified using the primers suggested by Vrain *et al.* (1992) and Joyce *et al.* (1994), respectively. The amplifications of the ITS and D2–D3 regions of the rRNA were performed according to Bhat *et al.* (2017). The products were analysed on 1% agarose gels with TAE (Tris–acetic acid–EDTA) buffer. The amplified polymerase chain reaction (PCR) products were purified and sequenced in both directions by Bioserve Pvt. Ltd (Hyderabad, India). Amplified regions were annotated and submitted to the National Center for Biotechnology Information (NCBI) under accession numbers MF289980 and MF289982 for ITS and D2–D3 genes, respectively, for CS31; and for CS32, MF289981 and MF289983 for the same respective regions.

Isolation and molecular characterization of entomopathogenic bacteria

The endosymbiont bacterium associated with *S. pakistanense* CS31 was obtained from the haemolymph of *G. mellonella* 1 day after infection with CS31, by adopting the method of Akhurst (1980). The haemolymph was streaked on nutrient agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride and 0.0025% (w/v) bromothymol blue (NBTA medium) and left overnight at 28°C (Akhurst, 1980). Single colonies were transferred with a sterile toothpick to YS broth (Akhurst, 1980) and cultivated on an orbital shaker (180 rpm) at 25°C.

Bacterial DNA was extracted from a 2-day-old culture using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. The 16S RNA was amplified using primers 10 F: 59-AGTTTGATCATGGCTCAGATTG-39 (forward) and 1507R: 59-TACCTTGTTACGACTTCACCCCAG-39 (reverse) (Sandstrom *et al.*, 2001). The recombinase A gene (*recA*) was amplified using primers RecA1 F: 59-GCTATTGATGAAAA TAAACA-39 (forward) and RecA2R: 59-RATTTTRTCWCC RTTRTAGCT-39 (reverse) (Tailliez *et al.*, 2010). The gyrase B gene (*gyrB*) was amplified using primers 8SF gyrB: 59-TACA CGAAGAAGAAGGTGTTTCAG-39 (forward) and 9Rev gyrB: 59-TACTCATCCATTGCTTCATCATCT-39 (reverse) (Tailliez *et al.*, 2010). The PCR master mix consisted of 7.2 ml ddH_2O, 0.5 ml bovine serum albumin (BSA), 1.25 ml 103 PCR buffer, 1 ml deoxyribonucleoside triphosphates (dNTPs), 0.75 ml of each forward and reverse primer, 0.1 ml polymerase and 1 ml of

DNA extract. The PCR profiles were used as follows. For 16S: one cycle at 94°C for 1 min; followed by 33 cycles at 94°C for 60 s, 55°C for 60 s and 72°C for 2 min; and a final extension at 72°C for 3 min. For *recA*: one cycle at 94°C for 2 min; followed by 35 cycles at 94°C for 30 s, 49.5°C for 35 s and 72°C for 60 s; and a final extension at 72°C for 2 min. For *gyrB*: one cycle at 94°C for 2 min; followed by 35 cycles at 94°C for 30 s, 66°C for 35 s and 72°C for 60 s; and a final extension at 72°C for 2 min. All PCR products were sequenced and deposited in GenBank under the following accession numbers: MF592490 (*recA* sequence) and MF592491 (*gyrB* sequence).

Phenotypic and biochemical characterization of symbiotic bacteria

Phenotypic variations were observed in symbiotic bacteria on the basis of adsorption properties towards bromothymol blue (BTB) and neutral red. The adsorption of BTB was examined on NBTA agar (Akhust, 1980). Bacteria were present in two phases, namely phase I and phase II. Phase II bacteria reduced tetrazolium chloride (TTC) to formazan and produced red-coloured colonies. In the case of phase I bacteria, the reduction of TTC was hidden as they adsorbed BTB and produced blue-green colonies. The production of a blue-green or red colour was taken as the characteristic mark of phase I or phase II bacterial colonies.

For neutral red adsorption, the bacteria were grown by streaking on MacConkey agar and were incubated for 24–48 h. Phase I variants of bacteria showed adsorption of neutral red and appeared as red/reddish-brown while the phase II variants appeared as light yellow/off-white.

The biochemical characterization was examined using a KB003 Hi25 Enterobacteriaceae Identification Kit from Hi-media (Mumbia, India), designed for the identification of Gram-negative Enterobacteriaceae species. A total of 13 conventional biochemical tests and 11 carbohydrate utilization tests were performed using this kit. For biochemical characterization, bacteria were cultured on NBTA media and blue-green colonies were shifted into 5 ml heart infusion broth (Hi-media). The culture was grown overnight and 50-µl aliquots were then inoculated into each of 24 wells of the kit. The kit was incubated according to the manufacturer's instructions and changes in the colour of media were recorded as 'plus' and 'minus'.

Sequence alignment and phylogenetic analyses

The sequences were edited and compared with those present in GenBank by means of a Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) of the National Center for Biotechnology Information. An alignment of nematode samples together with sequences of related steinernematid species was produced for the ITS and 28S regions using default Clustal W parameters in MEGA 6.0 (Tamura *et al.*, 2013) and optimized manually in BioEdit (Hall, 1999). Pairwise distances were computed using MEGA 6.0 (Tamura *et al.*, 2013). The phylogenetic trees of the ITS and 28S genes were obtained by the Minimum Evolution method (Rzhetsky & Nei, 1992) in MEGA 6.0 (Tamura *et al.*, 2013). *Steinernema nepalense* and *Steinernema scapterisci* were used as outgroup taxa. The Minimum Evolution tree was searched using the Close-Neighbour-Interchange (CNI) algorithm (Nei & Kumar, 2000). The neighbour joining algorithm (Saitou & Nei, 1987) was used to generate the initial tree. The evolutionary distances were computed using the p-distance method (Nei & Kumar, 2000) and are expressed as the number of base differences per site.

Alignments of the bacterial *recA* and *gyrB* genes were concatenated into one dataset. The tree was constructed in the same way as the nematode trees and *Xenorhabdus bovienii* was used as the outgroup taxon.

Co-phylogenetic analysis

The bacteria from the monophyletic clade of *X. indica*, *Xenorhabdus stockiae* and *Xenorhabdus innexi*, and several unidentified bacterial strains, together with their nematode hosts were used in the co-phylogenetic analysis. For the analysis concatenated datasets of the ITS and D2–D3 regions, and *recA* and *gyrB* genes, were used to infer the nematode and bacterial trees, respectively. The trees were constructed using the Minimum Evolution algorithm, as described above. A comparison of the nematode and bacterial phylogenies was made and the significance of the observed co-divergence was assessed using TreeMap version 3.0 (Charleston & Page, 2002). The randomization tests were used to test the null hypothesis that the topological similarities could be accounted for by chance.

Virulence tests on Spodoptera litura *and* Helicoverpa armigera

Mortality bioassays of both the *Steinernema* isolates, CS31 and CS32, were performed against two pests, *S. litura* and *H. armigera*. Six-well plates (well size 3.5 cm) lined with a double layer of Whatman Filter Paper No. 1 were used for bioassay experimentation using 1-week-old IJs. Concentrations of 0, 25, 50, 100 and 200 IJs in 450 µl distilled water were placed over the filter paper. 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 28 ± 2°C. Mortality was recorded at 12-h intervals until 100% mortality was achieved. Larvae infected with 100 IJs/larva (preferred optimum dose for counting progeny) were transferred after 7 days to a modified White trap (White, 1927) to observe the persistence of infection and emergence of IJs (18–20 days). Each bioassay was placed separately and all experiments repeated twice.

The insect larval mortality assay was analysed statistically through probit analysis, and median lethal time (LT_{50}) values were calculated at a 95% confidence limit. Differences between percentages of mortality depending on different isolates were assessed using Wald statistics in the STATISTICA program v. 10 (StatSoft Inc., Tulsa, Oklahoma, USA). Data were presented as a percentage ± SD. Nematode progeny production was analysed by analysis of variance in the same program, and presented in the form of mean ± SD.

Results and discussion

The morphological, morphometric and molecular analysis identified both of the isolated nematodes, CS31 and CS32, as *S. pakistanense* (Shahina *et al.*, 2001). This is the first report of this species from India, and three slides of IJs, each bearing seven specimens, three slides of first-generation females bearing one female on each slide, two slides of first-generation males bearing three males on each slide, two slides each of second-generation females and males bearing two and three specimens, respectively, on each slide were deposited in the National Nematode Collection of

Table 1. Comparative morphometrics of all generations of CS31 and CS32 with the original description of *Steinernema pakistanense*. All measurements are in micrometres (except percentage) and in the form of mean (range).

	Infective juveniles			Male I			Male II			Female I			Female II		
	CS31	CS32	*pakistanense*	CS31	CS32	*pakistanense*	CS31	CS32	*pakistanense*	CS31	CS32	*pakistanense*	CS31	CS32	*pakistanense*
L	695 (649–733)	592 (548–627)	683 (649–716)												
BD	28 (27–33)	25 (21–29)	27 (24–29)												
EP	58 (51–66)	53 (48–63)	54 (49–58)	75 (64–85)	70 (58–84)	81 (72–92)	70 (55–82)	66 (57–79)	72 (64–86)	82 (69–96)	76 (64–87)	81 (72–94)	77 (69–91)	70 (61–77)	72 (64–80)
NR	82 (76–89)	70 (63–82)	80 (76–83)	101 (83–116)	89 (74–100)	99 (88–107)	84 (74–90)	87 (78–100)	82 (80–88)	120 (106–135)	115 (94–32)	112 (96–126)	98 (88–108)	99 (81–112)	10 4(97–110)
ES	109 (103–116)	94 (84–108)	113 (108–122)	133 (120–156)	125 (103–143)	132 (126–146)	109 (92–121)	119 (106–132)	121 (110–137)	160 (141–176)	167 (141–198)	179 (165–194)	132 (119–145)	136 (117–146)	146 (140–152)
Tail	63 (55–68)	58 (46–66)	58 (53–62)	26 (22–31)	27 (21–30)	25 (24–27)	22 (19–25)	26 (23–30)	24 (21–30)	43 (29–73)	45 (35–55)	49 (35–63)	50 (40–60)	58 (51–66)	61 (57–64)
a	25 (21–26)	24 (19–29)	24 (21–27)	12 (11–16)	12 (10–15)	13 (11–16)	16 (14–19)	16 (14–17)	18 (14.-18)	22 (13–37)	26 (17–39)	37 (25–43)	16 (14–21)	14 (12–16)	19 (18–22)
b	6.4 (6.0–6.9)	6.3 (5.6–7.4)	6.0 (5.0–6.0)	9.3 (7.8–11.8)	9.4 (8.3–11.9)	10.4 (8.7–11)	8.5 (7.2–10)	6.5 (5.6–7.2)	7.4 (5.8–8)	26 (17–38)	37 (25–52)	57 (37–62)	15 (12–20)	8.6 (7.5–10)	12 (10–15)
c	11 (10–12)	10 (8.7–13)	11 (10–12)	47 (36–63)	43 (38–54)	55 (52–62)	42 (33–49)	30 (26–37)	37 (31–43)	118 (67–174)	139 (86–228)	200 (170–236)	41 (30–60)	20 (16–25)	29 (23–38)
D%	53 (47–61)	57 (48–70)	47 (42–53)	56 (47–64)	57 (49–67)	60 (50–60)	64 (49–73)	55 (49–63)	60 (40–60)	51 (42–58)	45 (39–52)	40 (30–40)	58 (53–64)	52 (48–63)	50 (40–50)
E%	92 (83–115)	92 (76–113)	91 (87–102)	286 (237–361)	263 (222–331)	310 (210–370)	315 (227–400)	257 (200–305)	300 (250–370)	199 (117–283)	170 (133–206)	160 (130–200)	157 (120–200)	121 (99–142)	110 (100–130)
SL				68 (59–80)	67 (60–74)	68 (62–73)	58 (44–67)	57 (50–66)	52 (47–59)						
GL				43 (35–53)	39 (30–45)	41 (36–45)	32 (27–36)	26 (21–30)	29 (25–32)						
SW%				179 (147–229)	180 (148–217)	180 (100–220)	185 (151–228)	203 (166–244)	167.7						
GS%				64 (54–79)	58 (46–70)	60 (50–60)	56 (45–72)	45 (3.8–55)	55.8						
Mucron				Absent	Absent	Present	Absent	Absent	Absent	Absent	Absent	Absent	Present	Present	Present
V%										53 (49–58)	51 (48–54)	49 (47–52)	54 (48–59)	56 (47–71)	52 (47–55)

L, Total body length; BD, max. body diameter; EP, anterior to excretory pore; NR, anterior to nerve ring; ES, pharynx length; *a*, total body length/maximum body diameter (*L*/BD); *b*, total body length/pharynx length (*L*/ES); *c*, total body length/tail length (*L*/*T*); D%, excretory pore position/ pharynx length*100 (EP/ES*100); E%, excretory pore position/tail length (EP/T*100); GL, gubernaculum length; SW%, spicule length/ anal or cloacal body width*100 (SL/ABD*100); GS%, gubernaculum length/ spicule length (GL/SL*100); V%, vulva position from anterior end expressed as percentage of L (AV/L*100).

Table 2. Pairwise distances of the ITS region between *Steinernema* species from the '*bicornutum*' group.

ITS region	*CS31	*CS32	pak	bif	bid	abb	rio	yir	ral	cer	gow	pap	bic
MF289980 *S. pakistanense* *CS31		0	1	9	99	199	207	212	213	213	222	230	234
MF289981 *S. pakistanense* *CS32	100		1	9	99	199	207	212	213	213	222	230	234
AY748449 *S. pakistanense* (pak)	100	100		8	98	200	209	213	215	212	224	232	233
JX989267 *S. bifurcatum* (bif)	100	100	100		95	201	209	215	213	211	224	230	232
KT373857 *S. biddulphi* (bid)	97	97	97	97		168	186	201	190	175	201	208	188
AY230158 *S. abbasi* (abb)	92	92	92	92	94		200	145	190	172	186	199	189
DQ835613 *S. riobrave* (rio)	91	91	91	91	92	92		202	109	172	144	125	188
AY748450 *S. yirgalemense* (yir)	91	91	91	91	92	95	92		193	182	195	208	206
KP036472 *S. ralatorei* (ral)	91	91	91	91	92	93	96	92		155	132	72	180
AY230165 *S. ceratophorum* (cer)	91	91	91	91	93	94	93	93	95		166	159	87
KR781038 *S. goweni* (gow)	91	91	91	91	92	93	95	92	96	94		146	184
KJ913707 *S. papillatum* (pap)	90	90	90	90	91	92	95	92	100	94	95		191
AY171279 *S. bicornutum* (bic)	90	90	90	90	92	93	93	92	94	98	94	93	

Below diagonal, percentage similarity; above diagonal, total character differences.

India, IARI, New Delhi, India. *Steinernema pakistanense* has been reported from Karachi, Sindh, and near Sonmiani beach and Mubarak village, Balochistan, Pakistan. It has also been reported from a horticultural farm at Hisar, India and several other places but only on a morphological and morphometric basis, which is not valid proof of its existence in India.

Morphology and morphometry

Steinernema isolates CS31 and CS32, obtained during the present investigation, were identified as *S. pakistanense*; however, some disparity with the original report may be noted. The mucron was found to be absent in all generations except the second-generation females, while in the original description it was observed in second-generation females and 10% of first-generation males. Epiptygmata were absent in the females of the present specimens while weakly developed, double-flapped epiptygmata were reported originally. A post-anal swelling was visible in second-generation females but this was not the case in the original description.

Morphometrical measurements of all the generations of isolates CS31 and CS32 were similar to the topotype population of *S. pakistanense* (Shahina *et al.*, 2001) but little divergence is seen when they are compared with each other or with the original description. A comparison in morphometrical parameters in all generations is given in table 1.

Molecular characterization

ITS sequences of *S. pakistanense* of both the Indian strains (CS31 and CS32) differ from the original ITS sequences by 1 bp at position 268, which is T in the present specimens and C in the type

Table 3. Pairwise distances of the D2–D3 regions between *Steinernema* species of the '*bicornutum*' group.

D2-D3 region	*CS31	*CS32	pak	bid	yir	bic	abb	bif	ral	cer	rio	gow	pap
MF289982 *S. pakistanense* *CS31		1	2	30	57	60	63	64	68	70	72	74	77
MF289983 *S. pakistanense* *CS32	100		1	29	56	59	62	63	67	69	71	73	76
JX068824 *S. pakistanense* (pak)	100	100		28	55	58	61	62	66	68	70	72	77
KT580950 *S. biddulphi* (bid)	95	95	95		52	55	49	49	53	63	64	63	65
AY748451 *S. yirgalemense* (yir)	89	89	89	90		50	33	32	50	54	55	51	59
AF331904.1 *S. bicornutum* (bic)	88	89	89	90	91		47	48	40	20	46	41	55
AF331890 *S. abbasi* (abb)	88	88	88	91	94	91		1	52	52	60	53	61
JQ838179 *S. bifurcatum* (bif)	88	88	88	91	94	91	100		52	53	61	54	60
KP036475 *S. ralatorei* (ral)	87	87	87	90	91	93	90	90		50	17	25	16
AF331888 *S. ceratophorum* (cer)	86	87	87	88	90	96	90	90	91		55	47	63
AF331893 *S. riobrave* (rio)	86	86	86	88	90	91	89	88	97	90		31	28
KR781039 *S. goweni* (gow)	85	86	86	88	90	92	90	90	95	91	94		34
KJ913708 *S. papillatum* (pap)	85	85	85	88	89	90	88	89	97	88	95	94	

Below diagonal, percentage similarity; above diagonal, total character differences.

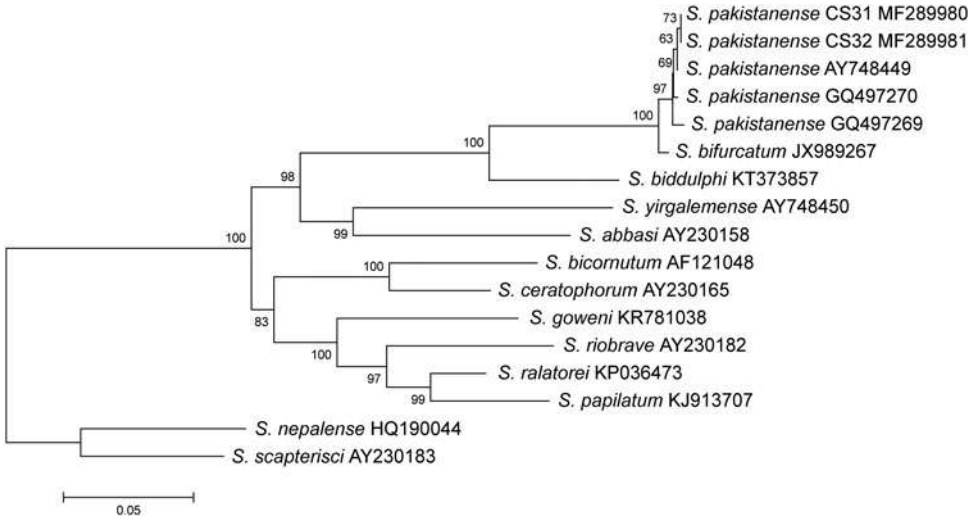

Fig. 1. Phylogenetic relationships in the '*bicornutum*' group of *Steinernema*, based on analysis of ITS rDNA regions. *Steinernema scapterisci* and *S. nepalense* were used as outgroup taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000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

population, while they show no nucleotide differences between each other. The ITS sequence of CS31 and CS32 is separated from those of other related species by 9–234 bp (table 2). No sign of intra-individual variability in the ITS sequence was observed.

No variation in the D2–D3 sequence was found between the CS31 and CS32 strains, but a variation was seen within *S. pakistanense*. The D2 and D3 expansion fragments of the 28S rRNA gene of both the Indian isolates were separated by 30–77 bp from other related species (table 3).

Phylogenetic analysis

The phylogenetic analyses of the '*bicornutum*' group based on ITS regions and the 28S rRNA gene showed the apparent monophyly of the group formed by the isolates of CS31 and CS32 and the original *S. pakistanense*, thus confirming their identification (figs 1 and 2). Both phylogenetic analyses have shown that *S. pakistanense* is a sister species to the well-supported monophyletic clade containing *S. biddulphi*, *S. bifurcatum*, *S. abbasi* and *S. yirgalemense*. Within this group, *S. pakistanense* clusters with another Asian species, *S. bifurcatum* (fig. 1) and the African species, *S. biddulphi* (figs 1 and 2). However, our results suggest that, based on molecular data, the status of *S. bifurcatum* is problematic. The ITS sequence of this species shows only 12 bp differences from *S. pakistanense*, corresponding to a 98.5% similarity. Such a similarity, however, falls well within the range for intraspecific variation in steinernematids (Půža *et al.*, 2015). On the other hand, the analysis based on the D2–D3 sequence places *S. bifurcatum* as a sister species to *S. abbasi*. In the analysis of pairwise distances, the sequences of these two species differ by 37 bp; however, the differences occur only in the first *c.* 150 bp, while in the

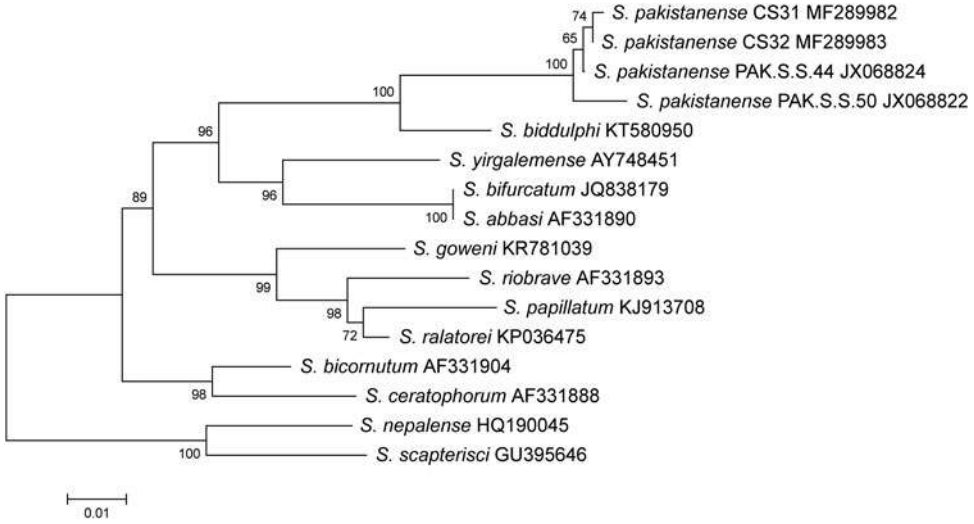

Fig. 2. Phylogenetic relationships in the '*bicornutum*' group of *Steinernema*, based on the analysis of D2–D3 regions of the 28S rDNA. *Steinernema scapterisci* and *S. nepalense* were used as outgroup taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap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

Table 4. Biochemical characteristics of *X. indica* CS31 isolates confirmed by the Hi-media Enterobacteriaceae Identification Kit.

Sample number	Tests	Result	Sample number	Tests	Result
1	ONPG	–	14	Arabinose	–
2	Lysine utilization	–	15	Xylose	–
3	Orthine utilization	–	16	Adonitol	–
4	Urease	–	17	Rhamnose	–
5	Phenylalanine deamination	–	18	Cellobiose	–
6	Nitrate reduction	–	19	Melibiose	–
7	H_2S production	–	20	Saccharose	–
8	Citrate utilization	–	21	Raffinose	–
9	Voges Proskauer's	–	22	Trehalose	–
10	Methyl red	–	23.	Glucose	+
11	Indole	–	24	Lactose	–
12	Malonate utilization	–	25	Oxidase	–
13	Esculin hydrolysis	+			

ONPG, *O*-nitrophenyl-beta-d-galactopyranoside; –, negative; +, positive.

rest of the alignment, the sequence of *S. bifurcatum* is identical to that of *S. abbasi*. It thus seems that the differences were created by an incorrect editing of the JQ838179 sequence. Our results are in agreement with the work of Cimen *et al.* (2016), who state that the 28S rDNA sequence attributed to *S. bifurcatum* (JQ838179) obviously belongs to *S. abbasi*.

Endosymbiont bacteria: molecular, phenotypical and biochemical diagnosis

Bacteria isolated from *S. pakistanense* CS31were grown on NBTA media, producing both phase I and II variants, confirmed by their dye-absorbance ability. Gram staining differentiation showed that the bacterial isolates were Gram-negative rods. The culture grown overnight on heart infusion broth, when transferred and incubated on the KB003 Hi25 Enterobacteriaceae Identification Kit of Hi-media, produced mostly negative reactions (table 4). On nutrient agar, colonies have a brownish pigmented centre, appear shiny and opaque, and are circular to irregular and convex. Phase I colonies adsorb neutral red, forming red colonies on MacConkey agar, and adsorb bromothymol blue forming blue colonies on NBTA. Esculin was weakly hydrolysed. Acids were weakly assimilated (table 4). Most of the phenotypic and biochemical results of symbiotic bacteria reported by Shahina *et al.* (2004) were identical to the results found here. Only one difference was noted in that phenylalanine deamination was found to be negative in the present investigations, while Shahina *et al.* (2004) reported it as 26–75% positive. The secondary forms of *Xenorhabdus* species were reported as stable colony variants that have lost their ability to produce pigment, antimicrobial agents and secondary metabolites, are not able to take up dye, and have sometimes lost their luminescent properties, as in the case of *Photorhabdus luminescens* (Boemare *et al.*, 1993).

The sequences of the *recA* and *gyrB* genes of *Xenorhabdus* sp. CS31 exhibited the highest similarity to the sequences of *X. indica* 157-C (98.4 and 99.1%, respectively) and *Xenorhabdus* sp. SGI-246 (98.1 and 99.1%, respectively) (fig. 3). Thus *Xenorhabdus* sp. CS31 probably represents another strain of *X. indica*. This result is in contradiction to those of Shahina *et al.* (2004), who identified the symbiont as *X. nematophila,* but only based on

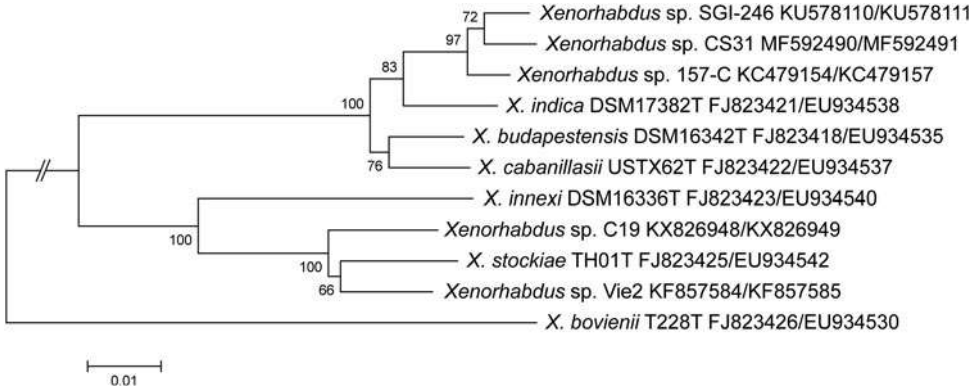

Fig. 3. Phylogenetic relationships of *Xenorhabdus* sp. isolated from *Steinernema pakistanense* (CS31) with other closely related species of *Xenorhabdus*, based on analysis of *recA* and *gyrB* gene sequences. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of number of base differences per site.

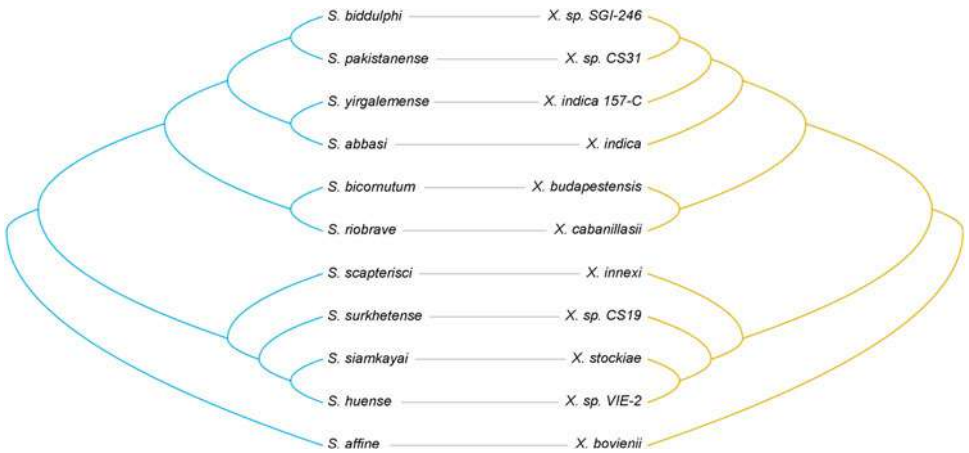

Fig. 4. Tanglegram showing correspondence between *Xenorhabdus* sp. CS31 and related *Xenorhabdus* spp. (right) and their nematode partners (left). Associated pairs are linked by a grey line.

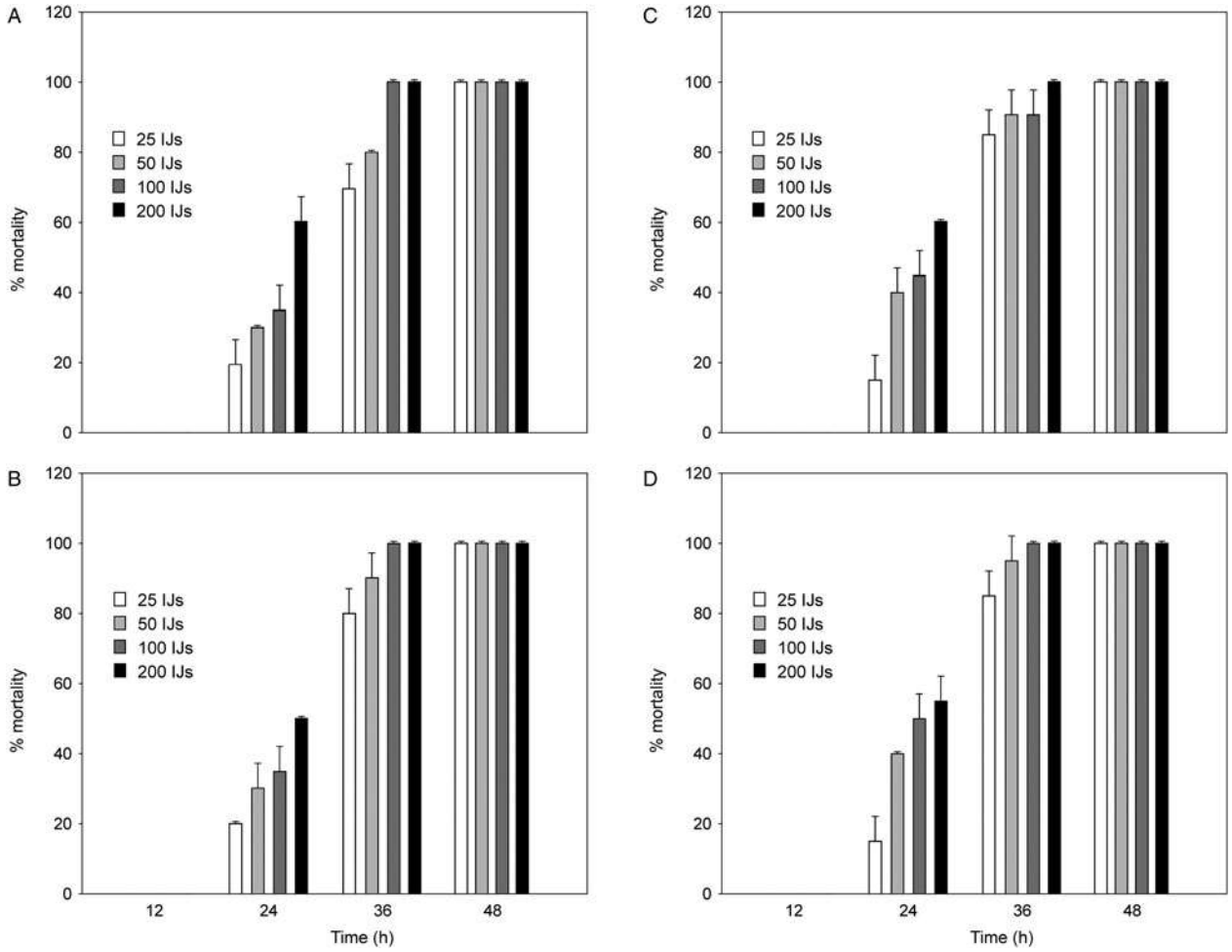

Fig. 5. Percentage mortality (mean and SD) of *Spodoptera littura* (A and B) and *Helicoverpa armigera* (C and D) larvae with different doses of *Steinernema pakistanense* strains CS31 (A and C) and CS32 (B and D).

the phenotypic characters. In the light of our results, their identification can be regarded as incorrect.

Interestingly, *X. indica* 157-C and *Xenorhabdus* sp. SGI-246 are associated symbiotically with *S. yirgalemense* (Ferreira *et al.*, 2014) and *S. biddulphi* (Cimen *et al.*, 2016) and both these nematode species are closely related to *S. pakistanense*.

The co-phylogenetic analysis suggests that there are significantly more co-divergence events between the nematodes and

bacteria ($P = 0.0001$) than would be expected by chance if their phylogenies were independent (fig. 4). Reconciling the nematode and bacteria phylogenies indicates remarkable topological congruence, with 18 co-divergence events out of a possible 23, with two duplications, two losses and no host switch.

In the *Steinernema* and *Xenorhabdus* complex, the only study addressing co-speciation (Lee & Stock, 2010) found no evidence for co-speciation and suggested that there have been at least 17 host switches of strains of *Xenorhabdus* spp. within clades and even between clades. Recent reports of switches of symbionts between nematodes of distantly related clades (Cimen *et al*., 2016; Dreyer *et al*., 2017) suggest that switches can be frequent in the *Steinernema* and *Xenorhabdus* complex. In the light of these facts, our results are surprising. Our analyses suggest that in some steinernematid lineages the nematodes have undergone co-speciation with their bacterial symbionts. This fact suggests that the specificity of the *Steinernema* spp. / *Xenorhabdus* spp. pairs might differ in different lineages. On the other hand, our analysis did not include *S. carpocapsae*, which is a member of the '*carpocapsae*' clade, but is associated with the symbiont *X. nematophila,* belonging to a different clade within the *Xenorhabdus* phylogeny. The switch of *X. nematophila* to *S. carpocapsae* was documented by Stock (2015).

Virulence tests

Both strains of *S. pakistanense* were able to kill and reproduce in both of the insects tested. The mortality of *S. litura* and *H. armigera* caused by strains CS31 and CS32 did not differ (Wald stat. = 0.0339, $P = 0.854$ and Wald stat. = 0.086, $P = 0.768$, respectively) and reached 100% after 48 h at all doses (fig. 5). The median lethal time (LT_{50}) for the lowest dose of 25 IJs/larva was *c*. 30 h in both strains and both hosts. The onset of mortality is very rapid. With the same insects, and with comparable doses of *S. abbasi*, Kalia *et al.* (2014) observed that 100% mortality occurred no earlier than 96 h in *H. armigera* and 192 h in *S. litura*.

The production of progeny in both of the tested hosts did not differ significantly between strains ($F = 1.5$, $P = 0.23$), and the mean production of progeny by the strains CS31 and CS32 in *S. litura* was 54,980 ± 22,245 IJs and 60,680 ± 20,826 IJs, respectively. In *H. armigera*, the production of nematode progeny was significantly lower ($F = 7.63$, $P = 0.008$) and reached 39,240 ± 5598 IJs (strain CS31) and 46,780 ± 13,855 IJs (strain CS32). In conclusion, our data show that *S. pakistanense* is very virulent towards the insect pests tested, and thus this species could be considered for use in the biocontrol of these organisms in India.

Acknowledgement. We thank Mr Christopher Steer, University of South Bohemia, Czech Republic, for language correction.

Financial support. The authors thank the Department of Science and Technology (DST), New Delhi, India, for providing financial assistance through DST INSPIRE Fellowship/2014/76.

Conflict of interest. None.

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Acta Parasitologica
https://doi.org/10.2478/s11686-019-00061-9

ORIGINAL PAPER

Study of *Steinernema hermaphroditum* (Nematoda, Rhabditida), from the West Uttar Pradesh, India

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Received: 29 January 2019 / Accepted: 24 April 2019

Abstract

Introduction The entomopathogenic nematodes have been reported from all continents (except Antarctica) and almost all regions of the world. Surveys of EPNs in India has resulted in the recovery of several isolates of *Steinernema*. Among one of them, isolate CS34 was identified as *S. hermaphroditum* Stock, Griffin & Chaerani, 2004. We investigated the identification and the pathogenicity of *S. hermaphroditum* in District Meerut of Western Uttar Pradesh, India.

Materials and methods The *Steinernema* was examined for its pathogenicity and accurate identification by the mean of morphological and molecular technique and its geographical distribution was mapped based on meta-analysis of the ITS GenBank records.

Results The surveys of agricultural soils of district Meerut, India, resulted in the isolation of one strain from entomopathogenic nematode labelled CS34 through *Galleria* baiting technique. Morphological characters and morphometrical analysis indicated that the strain CS34 was closely related to the "*glaseri*" group of *Steinernema* spp. The Nblast results indicated that ITS rDNA sequence had no nucleotide differences in comparison with the *S. hermaphroditum* (JQ687355). However, one variation in the D2–D3 segment of 28S rDNA was observed in comparison with the AY598358. The phylogenetic analysis using ITS and 28S rDNA indicated that the Indian *S. hermaphroditum* could be placed together with other *S. hermaphroditum*, with strong posterior probability. Besides, the PCA analysis demonstrated some variability within the test populations. The distribution of *S. hermaphroditum* based on meta-analysis of the GenBank records showed its presence in the three Asian countries—India, Thailand and Indonesia. The Indian strain of *S. hermaphroditum* also tested positively for its virulence against three major pests, namely, *Galleria mellonella*, *Helicoverpa armigera*, and *Spodoptera litura*, with resultant which showed good efficacy on the mortalities.

Conclusions In conclusion, the economy of India is agriculture-based, but there are huge losses due to different insect pests infesting different crops. *Steinernema hermaphroditum* CS34 is an indigenous species to Indian subcontinent and efforts should be made to evaluate its virulence and pathogenicity against the other agricultural pests hampering productivity throughout the country. This may lead to incorporate *S. hermaphroditum* strain CS34 as a regular biological control agent against important lepidopteran pest in integrated pest management programs in the future.

Keywords 28S rDNA · Biocontrol · Entomopathogenic nematode · ITS rDNA · Phylogeny

Introduction

Entomopathogenic nematodes (EPNs) are considered as being useful biological control agents against diverse insect groups and have a more active role in contributing to the mortality of the nematode–bacterium complex than merely relying on their symbiotic bacteria [47]. Also, EPNs are an excellent model system for investigating vertebrate- and human-parasitic nematodes, especially with regard to the function of excretory/secretory metabolites [10, 11, 47]. When infective third-stage juveniles (IJs) penetrate

Electronic supplementary material The online version of this article (https://doi.org/10.2478/s11686-019-00061-9) contains supplementary material, which is available to authorized users.

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the insect body, they undergo quantifiable morphological changes during transition from free-living to feeding-stage [31, 17]. Transition from free-living IJ to an active parasitic lifestyle requires dramatic morphological and physiological changes, referred to as IJ activation rather than recovery, as the process was previously referred to in *Caenorhabdus elegans* dauers [27, 48].

The entomopathogenic nematodes have been reported from all continents (except Antarctica) and almost all regions of the world [32]. However, few surveys have been done regarding the EPNs in India previously. At the time of writing this paper, only 17 species (14 steinernematids and 3 heterorhabditids) were described, with *Heterorhabditis indica* Poinar et al. [57] described as new species [14, 44]. Previously, several reports of new EPN species were described from the Indian subcontinent, but were either included as species *inquirenda* or synonymized with existing species. *Steinernema masoodii* [6, 8], *S. seemae* [6, 8], *S. sayeedae* [5], *S. qazii* [7], were considered as species *inquirendae*. *S. mushtaqi* [53] was considered to be a *nomen nudum*, while *S. meghalayense* [24] was regarded as a junior synonym of *S. carpocapsae* [73, 75], *S. thermophilum* [25] was regarded as a junior synonym of *S. abbasi* [21] and *S. dharanai* [41] was accepted as a junior synonym of *S. hermaphroditum* [68, 34]. The other steinernematids reported from India included *S. bicornutum* [70, 35], *S. riobrave* [26], *S. carpocapsae* [73, 35], *S. tami* [49, 35], *S. glaseri* [67, 36], *S. siamkayai* [69, 9], *S. monticolum* [62], *S. feltiae* [22, 75] (Ganguly and Sosamma, unpublished data), *S. sangi* [54, 44], *S. surkhetense* [40, 16], *S. cholashanense* [51, 45] and *S. pakistenense* [14]. Among above indigenous isolates described so far from India, there are no authentic data of *S. feltiae* and *S. riobrave*, simply described on few morphometrical characters which are not a valid proof of their existence from India. Among heterorhabditids, apart from *H. indica* the other indigenous species from India include *H. bacteriophora* [56, 66] and *H. baujardi* [55, 71].

Surveys of EPNs in the agricultural fields of District Meerut of Western Uttar Pradesh has resulted in the recovery of several isolates; among one of them, isolate CS34 was identified morphologically and molecularly as *S. hermaphroditum* [68]. This isolate was recovered from the soil samples of sugarcane fields with soil pH 7.7. The isolates of this species were first recovered from soil samples under different vegetation covers (i.e., scrub and trees) along the coastal fringe of the islands of Seram (Kamal) and Ambon (Waai) in the Indonesian archipelago [28]. Stock et al. [68] initially described *S. hermaphroditum* from Indonesia based on RFLP profiles of ITS rDNA and 28S rRNA sequence analyses plus with the cross-hybridization and morphometrical data. Apart from above type locality, the species has also been isolated from soil samples around banana/herbs and grass/herbs/coconut habitats in Waai, Ambon Island, Indonesia [28, 68]. This species was later described from India by Kulkarni et al. [41] as new species of *Steinernema* (*S. dharanai*) but its ITS rRNA gene sequence (KC252604) revealed only one nucleotide difference with already described *S. hermaphroditum* (JQ687355). Thus, based on molecular evidence, *S. dharanai* was considered a junior synonym of *S. hermaphroditum* [33], a conclusion also supported by Puža V. (pers. comm.).

Further, isolate CS34 was tested in the laboratory condition for its pathogenicity against *Spodoptera litura* (Fabricius, 1775) (Lepidoptera: Noctuidae) and *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae). The natural occurrence of EPN species facilitates its use in particular areas; we also attempted to map the distribution of *S. hermaphroditum* based on meta-analysis of the GenBank records.

Materials and Methods

Nematode Isolation and Culture

Twenty soil samples were collected from different agricultural fields of District Meerut (28°59′N, 77°42′E and 225 m above sea level) of Western Uttar Pradesh in May, 2016. One of the soil samples from sugarcane (*Saccharum officinarum* L. (Poales: Poaceae) fields near Poothi region yielded a *Steinernema* isolate using insect baiting technique [12] with the last instars of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). Cadavers of *G. mellonella* recovered from the trap were disinfected in 0.1% NaOCl solution, washed in ddH2O, and transferred onto White trap [74].

Entomopathogenic nematodes were propagated in *G. mellonella* at 28–30 °C. Next, emerging IJs were harvested in modified White traps (Bhat et al. [13], disinfected with 0.1% NaOCl, washed twice with ddH_2O, and finally stored into tissue culture flask at 15 °C for subsequent studies and establishment of stock cultures.

Morphological and Morphometric Characterization of the Nematodes

For light microscopic studies, 20 individuals of males and females (1st and 2nd generation) were collected by dissecting *G. mellonella* cadavers in Ringer's solution on days 3 and 5 after infection, respectively, whereas 20 infective juveniles were collected from a White trap [74]. Each generation was examined alive or heat-killed in Ringer's solution at 60 °C, fixed in triethanolamine formalin (TAF) [19] and processed to anhydrous glycerin for mounting [63]. Specimens were mounted on glass slides, with cover slip placed onto a glycerin drop with some extra paraffin wax to avoid flattening. Observations were made from live and mounted

specimens using a light compound microscope (Magnus MLX) and phase-contrast microscope (Nikon Eclipse 50i; Nikon). Morphometric measurements were made with the help of the inbuilt software of a phase-contrast microscope (Nikon DS-L1).

Principle Component Analysis (PCA)

Principal component analyses (PCA) were performed on morphometric variables of fixed specimens representing data from populations of *S. hermaphroditum* and its junior synonym *S. dharanai* from different localities using XLSTAT program Addinsoft, [4]. Twelve variables, including body length, *a*, *b*, *c*, excretory pore, nerve ring, pharynx length, tail length, anal body diameter, D%, spicule length, gubernaculum length, SW% and GS% were considered.

DNA Extraction, Amplification and Sequencing of Nematodes

The DNA was extracted from the pool of IJs (100 individuals) collected from the White trap by Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the instructions in the manual with slight modifications [16]. A fragment of rDNA containing the internal transcribed spacer regions (ITS1, 5.8S, ITS2) was amplified using primers 18S: 5′-TTGATTACGTCCCTGCCC TTT-3′ (forward) and 28S: 5′-TTTCACTCGCCGTTA CTAAGG-3′ (reverse) [72]. The other rDNA fragment containing D2D3 regions of 28S rDNA was amplified using primers D2F: 5´-CCTTAGTAACGGCGAGTG AAA-3´ (forward) and 536: 5´-CAGCTATCCTGAGGA AAC-3´ (reverse) [50]. The PCR master mix consisted of nuclease free dH_2O 16.8 µl, 10 × PCR buffer 2.5 µl, dNTP mix (10 mM each) 0.5 µl, 1 µl of each forward and reverse primers, dream taq DNA polymerase 0.2 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, 50 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 15 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 15 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 (3 µl ETB per 100 ml of gel). All PCR-products were sequenced using ABI 3730 (48 capillary) electrophoresis instrument by Bioserve Pvt. Ltd. (Hyderabad, India). The sequences were annotated and deposited with the Genbank under accession numbers MF663703 and MF693228 for the ITS and D2D3 regions, respectively.

Sequence Alignment and Phylogenetic Analyses

The sequences were edited and compared with those already present in GenBank using the Basic Local Alignment Search Tool (BLASTN) of the National Center for Biotechnology Information (NCBI). The ribosomal LSU and ITS rDNA sequences were analysed and aligned using BioEdit [29]. The length of the alignment was 787 bp and the base substitution model was evaluated using jModeltest 0.1.1 [58]. Phylogenetic trees were elaborated using the Bayesian inference method as implemented in the program Mr Bayes 3.1.2 (Ronquist and Huelsenbeck, [61]. The HKY + Γ (gamma distribution of rate variation with a proportion of invariable sites) model was selected. The selected model was initiated with a random starting tree and run with the Markov Chain Monte Carlo (MCMC) for 10^6 generations. The Bayesian tree was ultimately visualised using the TreeView program [52]. Genetic pairwise distance was estimated using options implemented in Mega V.7.0.14 (Kumar et al. [42, 43]. All characters were treated as equally weighted and gaps as missing data. For the nematode sequences, *Steinernema yirgalamense* was used as outgroup taxa and to root the trees.

Geographic Distribution

The ITS sequence was selected for the analysis, as it enables a clear distinction of the species in steinernematids, unlike another frequently sequenced marker D2D3 region of the 28S rDNA. The search for the sequences of *S. hermaphroditum*, the BLAST search was performed with the sequence of the type isolate (JQ687355) as a search query. The sequences that show 97% and higher similarity were downloaded and their identity was confirmed by phylogenetic analysis (Fig. 5, supplementary material). The information about the site of isolation, if available, were obtained from the NCBI Genbank database, or from related publications.

Efficacy and Virulence Tests on *Spodoptera litura* and *Helicoverpa armigera*

Virulence and efficacy of *Steinernema* isolate CS34 was carried out against larvae of three harmful insect pests, *G. mellonella* Fabricius (Lepidoptera: Pyralidae), *S. litura* Fabricius (Lepidoptera: Noctuidae) and *H. armigera* Hübner (Lepidoptera: Noctuidae). Bioassay experiments were carried in six-well plates (well size 3.5 cm) lined with a double-layered Whatmann Filter Paper No. 1 using 1-week-old IJs (Bhat et al. [15]. Four concentrations, 25, 50, 100 and 200 IJs in 450 µl distilled water were placed over the filter paper along with the untreated control. Larvae of the same size and same weight (10 larvae for each concentration) were used, and one larva was set up per well. Plates were incubated at 28 ± 2 °C and mortality recorded at 12-h interval till

100% mortality was achieved. Larvae infected with 100 IJs/larva (preferred optimum dose for counting progeny) were transferred after 7 days to a modified White trap [74] to observe the persistence of infection and emergence of IJs (18–20 days).

Larval mortality assay was analysed statistically through probit analysis and LD_{50} values were calculated at 95% confidence limit. Differences between percent mortalities, depending on the isolates, were assessed further using analysis of variance. Data were presented as per cent ± SD (Standard Deviation).

Results

Morphological and Morphometric Characters

First-Generation Males

Body ventrally curved, C shaped when killed by gentle heat, much smaller and slenderer than females. Anterior part similar to females. Cuticle appearing smooth under light microscope. Head rounded and continuous with the body. Amphidial apertures narrow, slightly rounded, located posterior to lateral labial papillae. Buccal cavity funnel-shaped, stoma shallow, cheilorhabdions distinct. Pharynx muscular, procorpus cylindrical, metacorpus slightly swollen, nonvalvate, isthmus distinct, basal bulb pyriform and valvate. Nerve ring often surrounding anterior portion of isthmus, in some cases close to basal bulb. Secretory-excretory pore located at isthmus level, close to the basal bulb, but always anterior to nerve ring. Testis monorchic and reflexed. Spicules paired, symmetrical, slightly brownish in color, curved, length/width ratio = 10.45, manubrium rounded, calomus separating manubrium from lamina, lamina slightly curved, with two internal ribs, velum present, extending from beginning of lamina to distal part of lamina, not covering spicule tip, spicule tip rounded. Gubernaculum ca 59% of spicule length, curved, slightly swollen at middle. Genital papillae disposed as single mid-ventral, pre-cloacal papilla and ten pairs arranged as follows: six pairs ventral pre-cloacal (three pairs lateral pre-cloacal, three pairs sub-ventral pre-cloacal), one subdorsal precloacal pair, one postcloacal subdorsal pair, one subventral postcloacal pair, one terminal postcloacal pair. They occur in less number (< 1% of the females).

Second Generation Males

Morphologically similar to the first generation males but shorter and most of the morphometric measurements smaller; for example, tail length and body diameter ca half that of the first generation males (Table 1). Testis flexure extending more posteriorly, mucron absent. Just like the first generation males, they occur in less number (< 1%).

First-Generation Females

Hermaphrodite. Body robust, habitus C shaped when killed by gentle heat, variable in length, ranging from 3.9 to 7.8 mm. Cuticle appearing smooth under light microscopy, lateral fields and phasmids not visible. Head rounded, continuous with the neck. Amphids distinct and located at cephalic papillae level. Buccal cavity funnel-shaped, stoma shallow. Pharynx with cylindrical procorpus, metacorpus slightly swollen and non-valvate, isthmus distinct, basal bulb pyriform and non-valvate. Nerve ring located just anterior to isthmus. Secretory-excretory pore usually located at isthmus level with cuticularised duct. Cardia prominent, protruding into intestinal lumen. Ovaries opposed, reflexed in dorsal position; oviduct well developed; glandular spermatheca filled with spermatozoa; uterus in ventral position. Vagina short, with muscular walls. Vulva close to mid-body with asymmetric vulvar lips (anterior lip larger than the posterior one). Epiptygma absent, vagina short, oblique with muscular walls. Tail dome-shaped, shorter than anal body diameter and with a terminal peg.

Second-Generation Females

Similar to the first generation in general morphology, but shorter (Table 1). Body length 33–34% less than the first generation. Vulval opening close to mid-body. Tail conoid with mucron at its end. Post-anal swelling not developed.

Infective Third-stage Juveniles

Body slender, slightly curved after heat relaxation, tapering regularly from base of pharynx to anterior end and from anus to body terminus. Mouth closed, and intestine collapsed. Lip region rounded, smooth and labial papillae not seen, but four projected cephalic papillae are visible. Transverse slit-like amphidial aperture situated posterior to labial disc, but at the level of cephalic papillae. Pharynx long, narrow, isthmus distinct, surrounded by nerve ring, basal bulb elongated and valvate. Secretory-excretory pore located close to isthmus level, hemizonid distinct, positioned anterior to metacorpus-isthmus junction. Bacterial vesicle relatively elongated. Lateral field not visible. Deirids not observed. Tail conical with ventral depression, no spine like structure was seen on tail tip, hyaline portion distinct, ca 52% of tail length. Phasmids indistinct.

Table 1 Morphometrics of *S. hermaphroditum* CS34. All measurements are in µm (except n, ratio, and percentage) 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)	1676 ± 249 (1323-2172)	5920 ± 1051 (3947–7862)	1103 ± 99 (945–1261)	1989 ± 312 (1334–2566)	852 ± 57 (752–962)
L′ (L–T)	1641 ± 246 (1293–2131)	5861 ± 1051 (3947–7862)	1080 ± 97 (925–1234)	1927 ± 306 (1288–2488)	773 ± 56 (673–879)
a (L/BD)	16 ± 1.8 (12–19)	21 ± 2.6 (17–26)	15 ± 1.7 (14–17)	17 ± 1.7 (14–21)	24 ± 2.0 (20–28)
b (L/ES)	11.9 ± 1.3 (9.3–13.3)	30 ± 6.2 (19–46)	8.5 ± 0.9 (7.6–9.2)	14 ± 1.9 (11–18)	7.1 ± 0.9 (5.6–8.8)
c (L/T)	48 ± 3.6 (41–54)	102 ± 18 (62–127)	45 ± 3.0 (42–47)	32 ± 4.7 (25–44)	10.8 ± 0.7 (9.4–11.9)
c′ (T/ABW)	0.8 ± 0.1 (0.6–0.8)	1.0 ± 0.1 (0.7–1.3)	0.7 ± 0.1 (0.6–0.9)	1.9 ± 0.2 (1.5–2.5)	3.9 ± 0.2 (3.4–4.2)
V% (AV/L × 100)		50 ± 3.0 (41–56)		54 ± 1.5 (51–57)	
Body diam.(BD)	108 ± 19 (79–145)	275 ± 34 (224–324)	71 ± 2.8 (63–78)	115 ± 17 (71–155)	35 ± 2.8 (31–43)
Excretory pore (EP)	112 ± 15 (90–136)	129 ± 17 (84–153)	91 ± 4.1 (85–97)	94 ± 8.3 (74–106)	74 ± 3.8 (63–81)
Width at excretory pore	50 ± 7.3 (41–68)	97 ± 13 (71–114)	40 ± 5.7 (35–54)	48 ± 8.4 (33–70)	22 ± 2.4 (20–30)
Nerve ring (NR)	101 ± 14 (83–125)	142 ± 11 (111–160)	94 ± 1.8 (91–102)	105 ± 9.1 (83–24)	93 ± 8.3 (81–110)
Pharynx length (PL)	141 ± 17 (115–165)	197 ± 21 (138–223)	130 ± 4.2 (124–138)	146 ± 12 (115–171)	121 ± 12 (107–141)
Bulb length	32 ± 3.9 (23–38)	48 ± 6.0 (38–59)	26 ± 2.4 (22–33)	34 ± 3.2 (28–41)	18 ± 2.8 (13–24)
Bulb width	25 ± 3.0 (20–30)	39 ± 3.3 (32–43)	21 ± 1.9 (17–27)	27 ± 2.9 (20–33)	11.7 ± 1.3 (8.8–13.8)
Testis reflection	206 ± 30 (142–261)		169 ± 24 (136–194)		
Tail length (T)	35 ± 3.9 (30–42)	59 ± 5.5 (50–70)	24.5 ± 1.6 (20–27)	63 ± 9.3 (46–82)	79 ± 3.5 (73–86)
Anal body diam. (ABD)	46 ± 3.2 (42–51)	61 ± 7.3 (50–79)	35 ± 2.1 (26–37)	33 ± 4.8 (23–43)	20 ± 1.6 (18–24)
Spicule length (SL)	82 ± 7.1 (71–97)		60 ± 2.1 (54–62)		
Gubernaculum length (GL)	48 ± 4.8 (41–57)		35 ± 1.6 (37–46)		
D % (EP/ES × 100)	79 ± 5.2 (71–94)	66 ± 8.4 (45–91)	70 ± 4.7 (68–72)	64 ± 5.0 (54–76)	62 ± 6.0 (52–71)
E % (EP/T × 100)	318 ± 37 (250–384)	222 ± 27 (166–278)	316 ± 62 (187–349)	159 ± 22 (132–210)	93 ± 6.4 (80–103)
SW % (SL/ABD × 100)	178 ± 15 (152–206)		171 ± 13 (163–175)		
GS % (GL/SL × 100)	58 ± 5.7 (47–66)		56 ± 5.3 (42–63)		
Width at vulva		291 ± 35 (223–342)		116 ± 17 (75–154)	
Anterior to vulva (AV)		2983 ± 559 (1812–3895)		1081 ± 167 (729–1339)	
Posterior to vulva		2936 ± 532 (1718–3967)		909 ± 150 (604–1227)	
Hyaline as % of TL					52 ± 8.6 (48–52)
Mucron	–	5.4 ± 1.2 (3.3–7.8)	–	–	

Remarks on the *S. hermaphroditum*

Morphologically, the isolate CS34 of *Steinernema* obtained during the present investigation was identified as *S. hermaphroditum*. However, some dissimilarity might, with the original description and with junior synonym *S. dharanai*, be noted. The mucron was absent in males when compared with the strains, except for the first male generation in *S. dharanai* that bear the mucron. Similarly, the mucron was absent in the second female generation of the present isolate, whereas it was present in the analogized strains. The morphometry of the present strain is shown in Table 1. The comparison of morphometric measurements of isolate CS34 in all generations when compared with those of strains (*S. hermaphroditum* and *S. dharanai*) is given in Table 2.

PCA Analysis

Morphologically, males of the genus *Steinernema* were significant for the species identification. Therefore, its PCA analysis is presented. The results for the females and IJs yielded the same results and showed the variation within the three isolates of *S. hermaphroditum*. Morphometric variation in females of strains was observed within the populations of *S. hermaphroditum*. The *S. hermaphroditum* isolate 1, isolate 2 and the population studied as CS34 were separated on the basis of the male characters. An accumulated variability of 100% was detected in the male by the F1 (63.51%) and F2 (36.49%). All characters exhibited positive correlations among the strains and were responsible for the variability of the F1, except for *a* value and nerve ring from anterior end. Some characters such as *b* value and pharynx length had the most contribution to the variability and had a positive and high correlation with the F1 (Fig. 1).

DNA Characters and Phylogenetic Analysis

Analyses of ITS sequences showed that no variation existed between the Indian strain CS34 and the isolate (JQ687355) studied by Ogier et al. (unpublished). However, both isolates differed from the *S. dharanai* (AY598358) by one bp at position 162, which is T and G, respectively. The ITS sequence of CS34 is separated from those of other related species by 35–111 bp (Table 3).

One variation in the D2–D3 sequence occured between CS34 and the sequence (JQ687356) studied by Ogier et al. (unpublished) and with the original topotype (AY598358) population. The D2–D3 segment of 28S rRNA gene in the Indian isolate differed in 20–66 bp from other related species (Table 4). Sequence length and composition of isolate CS34 and other species belonging to the '*glaseri*' group are presented in Table 5.

Phylogenetic analyses of the "*glaseri*" group based on ITS sequences showed clear monophyly of the group formed by isolate CS34, *S. hermaphroditum* and its junior synonym *S. dharanii*, thereby confirming its identification (Fig. 2). The above three sequences of *S. hermaphroditum* formed a monophyletic group with *S. lamjungense* [39], which together formed a sister clade with *S. pui* [60], *S. guangdongense* [59] and *S. longicaudum* [65].

In the phylogenetic tree based on D2-D3 sequences also, isolate CS34 formed a monophyletic clade with original *S. hermaphroditum* (AY598358) and the sequence (JQ687356) studied by Ogier et al. (unpublished) (Fig. 3). Analyses of both phylogenetic trees have shown that the isolate CS34 belonged to *S. hermaphroditum*.

Virulence Test

The isolate CS34 was tested for its virulence against three insect pests, with the results showing the existence of significant pathogenic properties against *G. mellonella*, *H. armigera* and *S. litura* (Fig. 4a–c). The strain was able to kill and reproduce in all three insects. The mortality in all three cases was observed after 24 h. The isolate showed high virulence against *G. mellonella* and killed all larvae within 48 h, whereas in the other two, total mortality was achieved at 60 h post-infection. At 36 h, isolate CS34 had killed all *G. mellonella* larvae with 50, 100 and 200 IJs/larva doses. However, similar observations occurred at 48 h in *H. armigera* and *S. litura*. At 36 h, the strain CS34 was capable of killing *G. mellonella* larvae with LD_{50} being at 7.67 IJs, *H. armigera* larvae at 42.17 IJs and *S. litura* larvae at 46.76 IJs. At 48 h, the number of IJs needed to kill 50% of *H. armigera* and *S. litura* were 12.29 and 7.67 IJs, respectively. The progeny production was more in *G. mellonella* ($91,816 \pm 32,841$) followed by *S. litura* ($52,640 \pm 11,816$), with the least being in *H. armigera* ($46,780 \pm 13,143$) (Fig. 4d) ($F = 14.22$; $P < 0.0001$; $\alpha = 0.05$).

Discussion

The morphological, morphometric and molecular analyses identified the isolated nematode, CS34 as *S. hermaphroditum* [68]. The species was originally reported from soil samples under different vegetation covers (i.e., scrub and trees) along the coastal fringe of the islands of Seram (Kamal) and Ambon (Waai) in the Indonesian archipelago [28], with limited information on its existence in the Indian subcontinent. Previously, *S. dharanai* was reported from soil sampled around teak (*Tectona grandis*) plantation, about 27 km from Dharana (Madhya Pradesh, Central India), which was later proposed on the basis of sequence analysis as a junior synonym of *S. hermaphroditum* [33]. The current study,

Table 2 Comparative morphometrics of all generations of CS34 with the original description of *Steinernema hermaphroditum*. All measurements are in µm (except percentage and ratio) and in the form of mean (range)

	Infective Juveniles			Male I			Male II			Female I			Female II		
	CS34	*hermaph-roditum*	*dharanai*	CS34	*hermaph-roditum*	*dharanai*	CS34	*hermaph-roditum*	*dharanai*	CS34	*hermaphrodi-tum*	*dharanai*	CS34	*hermaph-roditum*	*dharanai*
L	852 (752–962)	928 (700–1100)	999 (890–1100)	1676 (1323–2172)	1481 (1115–1560)	1271 (1200–1372)	1103 (945–1261)	845 (698–975)	1054 (945–1165)	5920 (3947–7862)	6550 (5843–7154)	5390 (4730–6050)	1989 (1334–2566)	5129 (3874–5623)	3938 (3400–4500)
MBD	35 (31–43)	28.5 (25–32.5)	35 (31–40)	108 (79–145)		66 (30–37)	71 (63–78)		61 (58–64)	275 (224–324)		169 (150–190)	115 (71–155)		169 (150–190)
EP	74 (63–81)	65 (62.5–68)	79.5 (70–85)	112 (90–136)	75 (65–80)	100 (90–110)	91 (85–97)	53 (49–60)	90 (85–95)	129 (84–153)	124 (110–134)	129 (125–133)	94 (74–106)	87 (79–98)	128 (125–132)
NR	93 (81–110)	102 (95–112.5)	103 (95–113)	101 (83–125)	119.5 (114–125)	103 (98–110)	94 (91–102)	119.5 (114–125)	94 (92–96)	142 (111–160)	147 (134–154)	141 (135–147)	105 (83–124)	126 (115–133)	121 (118–124)
ES	121 (107–141)		140 (130–151)	141 (115–165)		140 (130–150)	130 (124–138)		130 (125–135)	142 (111–160)	147 (134–154)	141 (135–147)	146 (115–171)		153 (135–175)
T	79 (73–86)	77 (65–82.5)	89 (83–94)	35 (30–42)	33.5 (28–35)	26.5 (25–28)	24.5 (20–27)	21.5 (19–25)	24 (23–25)	59 (50–70)	69 (65–72)	56 (52–60)	63 (46–82)	58 (50–62)	47 (40–50)
a	24 (20–28)	29 (23–35)	29.5 (25–38)	16 (12–19)			15 (14–17)			21 (17–26)		25 (21–36)	17 (14–21)		22.5 (20–25)
b	7.1 (5.6–8.8)	6.2 (5.6–7.1)	7.2 (6.9–7.6)	11.9 (9.3–13.3)			8.5 (7.6–9.2)			30 (19–46)		28 (26.5–30)	14 (11–18)		21.5 (18–26)
c	10.8 (9.4–11.9)	10.6 (9.3–11.7)	11.6 (11–12.1)	48 (41–54)			45 (42–47)			102 (62–127)		99 (87–115)	32 (25–44)		86 (80–90)
SL				82 (71–97)	68 (65–70)	69.5 (60–81)	60 (54–62)	60 (55–64)	59 (57–62)						
GL				48 (41–57)	48 (47–50)	39.5 (38–42)	35 (37–46)	41 (39–44)	37.5 (35–40)						
SW %				178 (152–206)	150 (140–160)	205 (130–260)	171 (163–175)	180 (170–190)	193 (140–250)						
GS %				58 (47–66)	71 (70–75)	55 (50–60)	56 (42–63)	65 (60–70)	55 (50–60)						
D %	62 (52–71)	50 (47–55)	56 (53–59.5)	79 (71–94)	48 (44–50)	74 (64–83)	70 (68–72)	40 (39–43)	67.5 (60–75)	66 (45–91)			64 (54–76)		
E %	93 (80–103)	85 (76–100)	85 (84–88.5)	318 (250–384)		385 (321–430)	316 (187–349)		360 (300–425)	222 (166–278)			159 (132–210)		
H %	52 (48–52)	51 (49–53)	52 (47–58)												
V %										50 (41–56)	55.5 (51–61)	51 (46–56)	54 (51–57)	52 (50–53)	46.5 (43–50)
Mucron				Absent	Absent	Present	Absent	Absent	Absent	5.4 (3.3–7.8)	2 (1.5–2.5)	Present	Absent	Present	Present

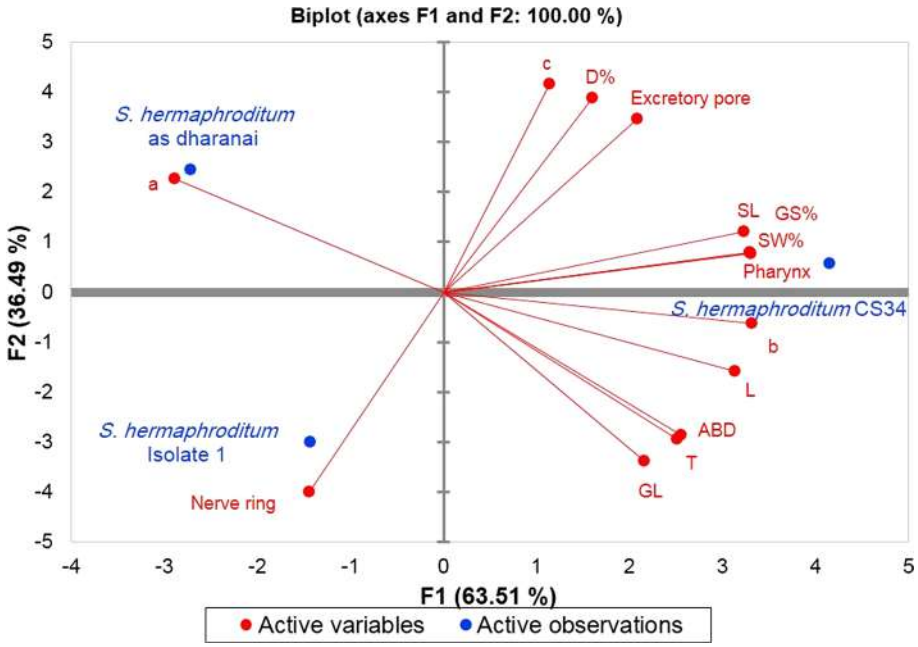

Fig. 1 PCA result from different populations of *Steinernema hermaphroditum* based on male characters

therefore, describes the first valid proof of the existence of *S. hermaphroditum* from India. The strain CS34 showed a very less occurrence of first- and second-generation males (< 1%), confirming previous observations [68].

The general characters of IJs, males and females for strain CS34 were similar to those *S. hermaphroditum* [68], but with some deviations. Morphometrics of CS34 were analogous to those of the topotype populations of S. *hermaphroditum* [68] and *S. dharanai* [41]. Similarly, there were also a few discrepancies when compared with the original descriptions. Variations in morphology and morphometry of strains of the same *Steinernema* species had been reported on various occasions. For example, Bhat et al. [16] reported morphological and morphometrical differences in Indian and Nepali strains of *S. surkhetense.* Similarly, Bhat et al. [14] observed differences in morphology and morphometry of Indian and Pakistani strains of *S. pakistanense*. Campos-Herrera et al. [18] also observed morphometric differences in nematode strains isolated from different sites and hosts. Aasha et al. [1] noted morpho-taxometric varitions in Indian and Sultane of Oman *S. abbasi* strains. Geographical origin and prevailing environmental conditions such as climate, soil type, soil salinity and chemicals in the soil could induce morphological and morphometrical differences.

Results of PCA analysis for morphometric characters within the populations of *S. hermaphroditum* in the current study suggested the existence of some variations. Variations in measurements of male and IJ characters were suggested as the most suitable for the distinction among steinernematid and heterorhabditid populations [3, 20], which had been resolved using PCA approaches. The PCA analysis has been used for the Argentinian species of *Heterorhabditis* [2], which correctly categorized the species into different group. The same result was obtained in our study. However, the variation within the isolates of *S. hermaphroditum* using PCA had not been established.

Differences in virulence against the three pests might be due to host-specificity, with such differences previously noted by other investigators [16, 64]. Campos-Herrera et al. [18] reported differences in virulence tests of *S. feltiae* to three different hosts; Shapiro-Ilan et al. [64] observed considerable variation in infectivity and mortality of *Curculio caryae* Horn (Coleoptera: Curculionidae) using different strains of *S. carpocapsae*. Bhat et al. [16] 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 [14]. Using the same insects and comparable doses of *S. abbasi*, Kalia et al. [37] observed that 100% mortality occurred in at least 96 h in *H. armigera* and 192 h in *S. litura*. 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. [23, 30, 38, 46].

In conclusion, the economy of India is agriculture-based, but there are huge losses due to different insect pests infesting different crops. *Steinernema hermaphroditum* CS34 is an indigenous species to Indian subcontinent and efforts should be made to evaluate its virulence and pathogenicity against the other agricultural pests hampering productivity throughout the country. This may lead to incorporate *S. hermaphroditum* strain CS34 as a regular biological control agent

Table 3 Pairwise distances of the ITS rDNA regions between *Steinernema* species from the "*glaseri*" group

	ITS region	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	CS34 MF663703		**0**	**1**	**35**	**39**	**40**	**41**	**47**	**79**	**80**	**84**	**85**	**86**	**87**	**87**	**90**	**90**	**91**	**93**	**96**	**96**	**97**	**97**	**98**	**100**	**102**	**105**	**111**
2	*S. hermaphroditum* JQ687355	**100**		1	35	39	40	41	47	79	80	84	85	86	87	87	90	90	91	93	96	96	97	97	98	100	102	105	111
3	*S. dharanai* KC252604	**100**	100		35	39	40	40	46	80	81	84	85	86	88	88	90	90	91	93	97	97	97	97	98	100	102	106	111
4	*S.lamjungense* HM000101	**94**	94	94		71	69	82	96	145	118	135	127	137	147	157	136	141	162	161	152	156	161	164	153	163	161	164	184
5	*S. longicaudum* AY230177	**93**	93	93	89		26	58	107	134	99	120	110	116	121	135	121	122	144	143	134	130	137	146	145	141	144	137	183
6	*S. guangdongense* AY170341	**93**	93	93	90	96		52	111	137	118	125	124	126	138	144	125	125	150	154	151	149	146	151	152	149	150	155	183
7	*S. pui* GU395618	**93**	93	93	88	92	93		117	135	114	133	126	127	137	143	135	130	152	148	158	151	151	151	144	148	156	158	186
8	*S. khoisanae* DQ314287	**92**	92	92	87	84	85	84		159	131	151	140	132	171	165	150	151	180	171	167	166	181	192	177	186	179	173	196
9	*S. arenarium* AY230160	**86**	86	85	78	80	80	80	77		158	156	172	159	158	51	145	143	103	94	152	155	157	135	136	125	160	164	193
10	*S. tophus* KJ701241	**86**	86	86	83	85	83	83	82	77		165	47	158	177	160	156	155	170	189	169	176	184	182	180	176	184	187	205
11	*S. pwaniense* KT358811	**84**	84	84	79	81	80	79	77	76	75		169	148	139	153	63	63	162	173	128	135	144	175	164	174	141	151	162
12	*S. innovationi* KJ578793	**86**	86	86	82	84	82	82	81	75	94	74		171	188	178	167	164	196	202	181	187	184	194	188	189	191	197	212
13	*S. jeffreyense* KC897093	**85**	85	85	80	83	82	81	82	77	78	78	77		177	150	154	163	164	170	167	161	181	176	172	173	177	177	192
14	*S. diaprepesi* AF122021	**85**	85	85	79	82	80	80	77	77	75	79	74	75		159	147	140	168	176	149	133	144	184	177	185	144	141	199
15	*S. vulcanicum* MF491477	**84**	84	84	76	79	79	79	76	93	76	76	74	78	77		136	144	112	102	152	160	158	140	133	131	167	166	189
16	*S. ethiopiense* JN651414	**83**	83	83	79	81	80	79	77	78	76	91	75	77	78	79		38	145	168	122	135	142	174	166	171	139	147	169
17	*S. karii* AY230173	**83**	83	83	78	80	80	79	77	78	76	91	75	75	80	78	94		145	171	119	135	149	173	162	172	144	146	164
18	*S. apuliae* HQ416968	**84**	84	84	76	78	78	77	74	86	75	75	73	76	76	84	78	78		99	149	155	172	173	164	162	180	166	203
19	*S. aciari* AY787660	**83**	83	83	78	79	78	77	77	78	76	81	74	76	79	77	82	82	78		167	175	171	165	155	153	187	184	203
20	*S. boemarei* FJ152414	**84**	84	84	77	79	78	78	76	87	73	74	72	76	76	86	75	75	87	76		108	156	175	161	174	147	108	183
21	*S. leizhouense* AY170340	**84**	84	84	78	80	78	78	78	78	76	80	75	78	82	77	80	80	78	85	76		170	189	184	189	163	38	196
22	*S. austral* FJ235125	**83**	83	83	77	79	79	78	75	77	74	78	75	75	81	77	79	78	76	78	76	77		186	165	176	94	179	186
23	*S. cubanum* AY230166	**82**	82	82	75	78	77	77	72	81	73	73	72	75	74	80	74	74	76	74	77	73	73		98	34	185	191	205
24	*S. phyllophagae* FJ410327	**82**	82	82	76	77	77	78	74	80	73	74	72	74	74	80	74	75	76	75	78	73	76	86		93	166	183	198
25	*S. glaseri* AY171288	**82**	82	82	75	79	78	78	73	82	74	73	73	75	74	81	74	74	77	74	79	73	75	95	87		175	196	204
26	*S. brazilense* FJ410325	**82**	82	82	76	78	78	77	75	77	74	79	73	75	80	76	79	78	75	79	74	77	87	73	75	75		172	192
27	*S. loci* AY355443	**82**	82	82	77	79	78	77	77	77	74	78	73	76	81	76	79	79	76	85	75	95	76	73	73	72	76		205
28	*S. akhursti* DQ375757	**80**	80	80	72	72	73	72	72	72	70	75	70	72	71	72	74	75	70	73	71	72	73	70	70	70	72	70	

Below diagonal: percentage similarity; above diagonal: total character differences

Table 4 Pairwise distances of the D2-D3 rDNA regions between *Steinernema* species of the "*glaseri*" group

	D2D3 domain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	CS34 MF693228		**1**	**20**	**23**	**24**	**25**	**27**	**28**	**30**	**33**	**32**	**33**	**34**	**35**	**39**	**39**	**40**	**46**	**49**	**49**	**50**	**51**	**60**	**60**	**62**	**66**
2	*S. hermaphroditum* JQ687356	**100**		20	24	24	25	27	29	31	29	33	29	34	35	37	38	37	41	45	44	42	46	55	58	60	60
3	*S. lamjungense* HM0001021	**97**	96		17	18	13	20	20	21	13	30	14	21	20	18	24	27	26	25	26	20	31	36	30	29	32
4	*S. guangdongense* AY169558	**95**	95	96		24	19	9	24	29	19	29	20	21	20	25	26	29	29	28	27	26	34	40	31	30	33
5	*S. jeffreyense* KP164866	**95**	95	97	94		7	24	18	26	9	31	10	23	23	22	24	28	26	28	28	26	34	40	32	31	35
6	*S. khoisanae* GU569052	**97**	97	98	96	99		29	16	26	10	26	10	22	25	35	26	32	40	38	39	37	45	52	49	51	55
7	*S. longicaudum* GU569054	**97**	96	97	98	96	97		27	30	37	31	37	36	39	39	40	43	47	39	45	40	52	67	59	61	64
8	*S. aciari* AY787661	**94**	94	96	95	96	97	94		9	18	34	17	24	21	30	29	29	32	27	25	24	37	37	31	31	33
9	*S. leizhouense* AY169557	**94**	93	95	94	94	94	93	98		26	39	25	28	26	38	32	30	35	29	28	29	40	34	37	37	39
10	*S. innovation* KJ578794	**96**	96	98	96	98	99	96	96	94		26	4	28	31	39	34	39	38	38	44	42	41	54	54	55	60
11	*S. ethiopiense* JN651409	**94**	94	95	94	94	96	95	93	92	95		29	33	32	30	34	37	11	35	36	39	6	45	37	36	40
12	*S. tophus* KJ701240	**96**	96	98	96	98	99	96	96	95	100	95		28	31	39	34	39	42	38	44	42	45	58	56	57	60
13	*S. diaprepesi* GU569048	**96**	95	96	95	96	97	96	95	94	97	94	97		17	49	22	26	46	38	37	39	55	59	48	51	51
14	*S. puertoricense* AF331903	**96**	95	97	96	96	97	96	95	94	96	94	96	98		55	15	17	43	40	33	39	52	54	48	48	51
15	*S. pui* GU395636	**95**	95	97	95	96	96	95	94	92	95	95	95	94	93		56	56	52	56	62	58	57	76	76	77	76
16	*S. australe* FJ235126	**95**	95	96	94	96	97	95	94	93	96	94	96	97	98	93		14	51	36	33	37	59	56	45	47	53
17	*S. brazilense* FJ410326	**95**	95	96	94	95	96	95	94	94	95	94	95	97	98	93	98		50	37	39	41	60	61	49	50	57
18	*S. pwaniensis* KT358813	**94**	94	96	94	96	95	94	93	92	95	98	95	94	95	93	94	94		56	56	58	17	65	70	72	72
19	*S. boemarei* GU569046	**94**	94	96	94	95	95	95	94	94	95	94	95	95	95	93	96	96	93		17	26	60	68	45	49	53
20	*S. arenarium* AF331892	**94**	94	96	94	95	96	95	95	94	95	94	95	96	96	92	96	95	93	98		32	61	65	41	45	51
21	*S. apuliae* GU569044	**94**	94	97	94	95	96	95	95	94	95	93	95	95	95	93	95	95	93	97	96		64	65	46	50	55
22	*S. karii* AF331902	**94**	94	95	93	94	95	94	92	91	95	99	95	94	94	93	93	93	98	93	93	92		70	72	72	77
23	*S. akhursti* KF289902	**93**	93	94	91	93	94	92	92	93	94	92	93	93	94	91	93	93	92	92	92	92	92		71	71	84
24	*S. glaseri* AF331908	**93**	92	95	93	94	94	93	93	92	94	94	93	94	94	91	95	94	91	95	95	94	92	92		7	19
25	*S. cubanum* AF331889	**93**	92	95	94	94	94	93	93	92	94	94	93	94	94	91	94	94	91	94	95	94	92	92	99		19
26	*S. phyllophagae* FJ666054	**92**	92	95	93	94	94	93	93	92	93	93	93	94	94	91	94	94	92	94	94	93	91	91	98	98	

Below diagonal: percentage similarity; above diagonal: total character differences

Table 5 Sequence lengths and composition of ITS-rDNA (ITSl + 5.8S + ITS2) and D2D3 region of 28S rRNA gene of some closely related described *Steinernema* species from the "*glaseri*" group and the present isolate CS34

	Molecular composition							Sequence length (bp)
	ITS-1 (bp)	5.8S (bp)	ITS-2 (bp)	A (%)	C (%)	G (%)	T (%)	
ITS region								
S. hermaphroditum **CS34**	**285**	**158**	**172**	**0.23**	**0.18**	**0.26**	**0.33**	**615**
S. hermaphroditum	285	158	172	0.23	0.18	0.26	0.33	615
S. dharanai	285	158	172	0.23	0.18	0.26	0.33	615
S. guangdongense	285	158	293	0.22	0.18	0.27	0.33	736
S. diaprepesi	301	158	313	0.23	0.2	0.24	0.32	772
S. austral	293	157	315	0.24	0.19	0.24	0.34	765
S. longicaudum	255	157	291	0.23	0.18	0.25	0.33	703
S. aciari	284	157	307	0.22	0.2	0.25	0.33	748
S. apuliae	385	155	470	0.24	0.2	0.26	0.3	1010
S. brazilense	284	157	319	0.25	0.19	0.23	0.32	760
S. arenarium	307	136	302	0.23	0.19	0.26	0.32	745
S. vulcanicum	318	136	287	0.23	0.2	0.26	0.31	741
S. leizhouense	300	157	325	0.24	0.18	0.24	0.33	782
S. glaseri	278	157	302	0.22	0.21	0.27	0.3	738
S. pui	284	154	289	0.22	0.19	0.26	0.33	727
S. pwaniense	258	157	269	0.25	0.17	0.24	0.33	684
S. phyllophagae	268	157	284	0.24	0.19	0.25	0.32	709
S. lamjungense	293	157	298	0.22	0.19	0.26	0.33	748
S. loci	309	160	323	0.24	0.18	0.25	0.33	792
S. jeffreyense	277	157	324	0.24	0.21	0.24	0.32	758
S. karii	260	157	278	0.24	0.18	0.25	0.33	695
S. ethiopiense	262	157	275	0.24	0.19	0.25	0.33	694
S. tophus	275	157	340	0.24	0.18	0.25	0.33	772
S. innovation	315	157	346	0.25	0.19	0.24	0.32	818
S. cubanum	278	157	304	0.22	0.21	0.26	0.3	739
S. boemarei	322	136	317	0.23	0.2	0.25	0.32	775
S. akhursti	275	157	291	0.24	0.18	0.23	0.35	723
D2-D3 region								
S. hermaphroditum **CS34**				**0.24**	**0.19**	**0.31**	**0.25**	**857**
S. hermaphroditum				0.24	0.19	0.31	0.25	741
S. guangdongense				0.24	0.2	0.32	0.24	463
S. diaprepesi				0.24	0.2	0.31	0.25	870
S. longicaudum				0.24	0.2	0.31	0.25	869
S. aciari				0.25	0.19	0.31	0.26	463
S. apuliae				0.24	0.2	0.31	0.24	838
S. brazilense				0.24	0.21	0.31	0.24	915
S. arenarium				0.24	0.21	0.32	0.24	876
S. leizhouense				0.24	0.19	0.32	0.26	466
S. glaseri				0.24	0.21	0.32	0.23	874
S. pui				0.24	0.21	0.31	0.25	813
S. pwaniense				0.24	0.2	0.3	0.25	913
S. phyllophagae				0.24	0.21	0.31	0.24	857
S. lamjungense				0.24	0.19	0.32	0.24	621
S. jeffreyense				0.23	0.2	0.32	0.25	586
S. karii				0.24	0.2	0.31	0.24	871
S. ethiopiense				0.25	0.2	0.32	0.23	594

Table 5 (continued)

	Molecular composition							Sequence length (bp)
	ITS-1 (bp)	5.8S (bp)	ITS-2 (bp)	A (%)	C (%)	G (%)	T (%)	
S. tophus				0.25	0.2	0.31	0.25	856
S. innovation				0.24	0.2	0.31	0.25	856
S. cubanum				0.24	0.21	0.32	0.24	875
S. boemarei				0.24	0.22	0.32	0.23	837
S. akhursti				0.24	0.2	0.31	0.26	891
S. puertoricense				0.25	0.19	0.31	0.25	870
S. khoisanae				0.25	0.2	0.31	0.25	871

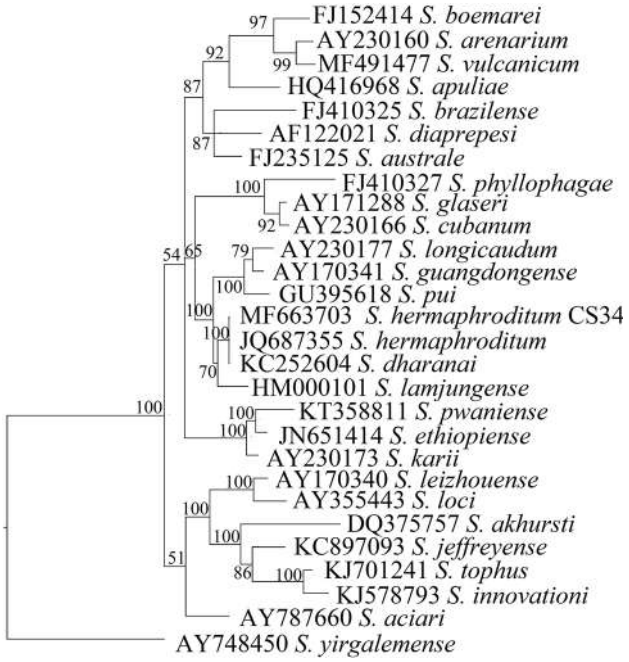

Fig. 2 The Bayesian Inference tree from known and the newly sequenced *Steinernema hermaphroditum* based on sequences of the ITS rDNA region

against important insect pests in integrated pest management programs in the future.

Geographic Distribution

The assessment of the species distribution using the records in Genbank has naturally many limits. The absence of the record from some area does not mean the absence of the organism. On the other hand, the existing record means the presence of the species in the locality.

Interestingly, even though *S. hermaphroditum* was described from Indonesia, the type sequence (JQ687355) is the only one in Genbank from Indonesia. The GenBank records of this species are very less. The three sequences originate from the strains isolated in India (MF663703, MH802516 and KC252604). Based on the NCBI Genbank records, the species seems to be present in India, having been isolated from three states of the subcontinent, India. The records originate from North India (Uttar Pradesh), South India (Kerala) and Central India (Madhya Pradesh). One strain labelled *Steinernema* sp. K8 EEL-2011 (JF834533) was isolated in Thailand, which further extends the known range of *S. hermaphroditum* to Thailand.

The number of the sequences in Genbank from a particular region reflects not only the abundance of the organism within the area, but also the actual sampling effort. However, the species seems to be present in some parts of Asia, whereas no reports of this species exist in other continents.

The Genbank records of *S. hermaphroditum* ($n=5$) are less like other species of the "*glaseri*" group (Table 6) except *S. glaseri* ($n=32$) and *S. arenarium* ($n=20$). Based on the records in NCBI Genbank database, species of "*glaseri*" group seem less frequently sequenced members. Till date, 22 species of "*glaseri*" group are known worldwide, of which one species *S. caudatum* lacks molecular data and the other one, *S. apuliae* is described only on RFLP profiles and cytochrome c oxidase subunit I. The GeneBank records of members of the "*glaseri*" group from different geographical areas are shown in Table 6. Most species are endemic in certain areas only while the other was found in many areas. The explanation for these differences is unclear, and further research in this field could bring interesting results.

These findings have implications for biocontrol. As *S. hermaphroditum* seems to be indigenous in the tropical Asia; the biocontrol product based on this species could be used for biocontrol purposes throughout this region.

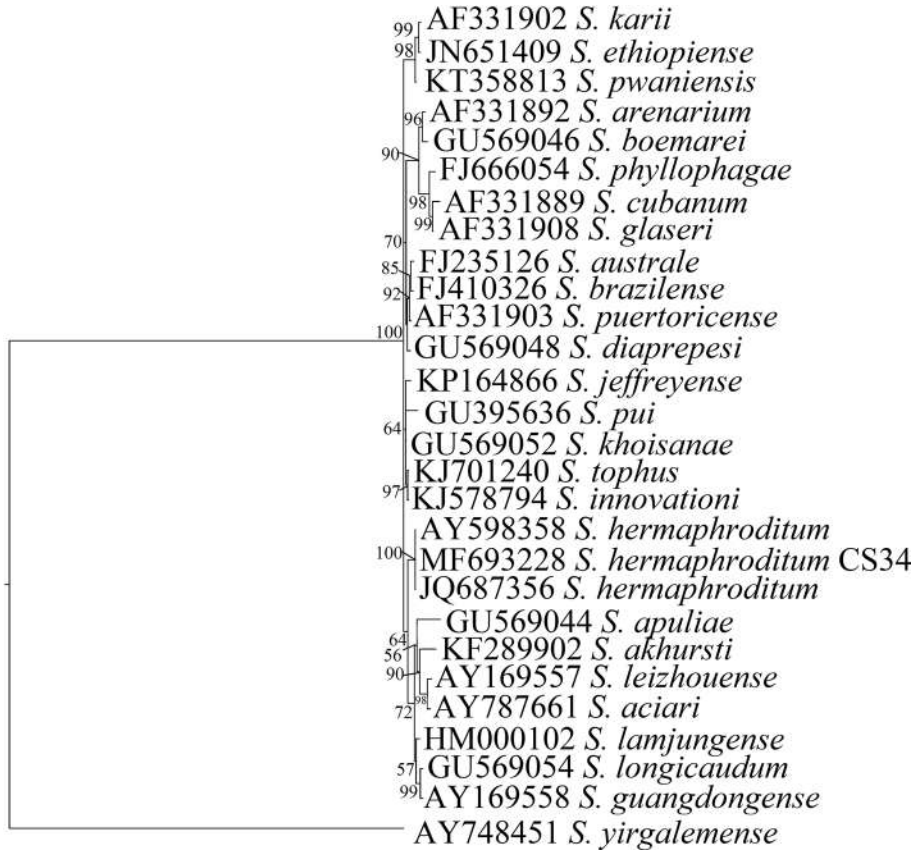

Fig. 3 The Bayesian Inference tree from known and the newly sequenced *Steinernema hermaphroditum* based on sequences of the 28S rDNA region

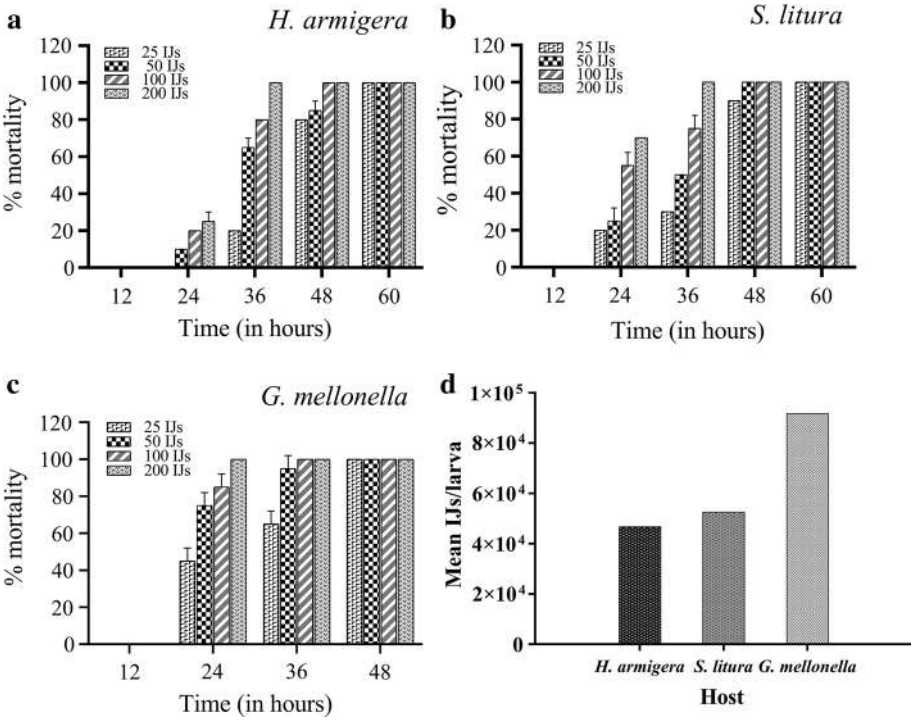

Fig. 4 Percentage mortality of *Spodoptera littura* (**a**), *Helicoverpa armigera* (**b**) and *Galleria mellonella* (**c**) larvae with different doses of *Steinernema hermaphroditum* strain CS34. Mean IJs production of *S. hermaphroditum* CS34 in *H. armigera*, *S. litura* and *G. mellonella* at 100 IJ Larva^{-1} doses (**d**)

Table 6 GeneBank records of *S. hermaphroditum* and other members of the "*glaseri*" group from different geographical areas

S. no.	Species	Isolation source	Accession number	Country	Submission in GenBank
1	*S. hermaphroditum*	Soil	MF663703	Uttar Pradesh, India	2017
		Soil	JQ687355	Indonesia	2012
		Soil	MH802516	Kerala, India	2018
		Soil	KC252604	Madhya Pradesh, India	2012
		Soil	JF834533	Thailand	2011
2	*S. arenarium*	*M. hippocastani*	DQ314288	South Africa	2005
		Alluvial soil	HM160094	Bulgaria	2010
		Alluvial soil	HM160095	Bulgaria	2010
		Soil	KU194608	Ukraine	2015
		Soil	KR058347	Ukraine	2015
		Soil	KU194607	Ukraine	2015
		Soil	KU194609	Ukraine	2015
		Soil	KR058349	Ukraine	2015
		Soil	KU194611	Ukraine	2015
		Soil	KU194610	Ukraine	2015
		Soil	KC633190	Palestine	2013
		Soil	KR058348	Ukraine	2015
		Soil	KU194615	Ukraine	2015
		Soil	KU194613	Ukraine	2015
		Soil	KF134911	Russia	2013
		Soil	KU194612	Ukraine	2015
		Soil	KF134912	Russia	2013
		Soil	KU194614	Ukraine	2015
		Soil	KY818704	Poland	2017
		Soil	AY230160	Russia	2003
2	*S. caudatum*	Soil	No DNA data	Guangdong, China	1991, 1994
3	*S. cubanum*	Soil	AY230166	Cuba	2003
4	*S. glaseri*	Soil	AF192986	USA	1999
		Soil	GQ497262	Tamil Nadu, India	2009
		Soil	AY171288	Portugal	2002
		Soil	FJ860037	Iran	2009
		H. philanthus	DQ082737	Belgium	2005
		Soil	FJ463939	Tamil Nadu, India	2008
		Soil	GU173999	Florida, USA	2009
		Soil	GU173998	Florida, USA	2009
		Soil	KM016403	USA	2014
		Soil	KM016410	USA	2014
		Soil	KM016415	USA	2014
		Soil	KM016414	USA	2014
		Soil	KM016399	USA	2014
		Soil	KM016401	USA	2014
		Soil	KM016413	USA	2014
		Soil	KM016417	USA	2014
		Soil	KM016412	USA	2014
		Soil	KM016407	USA	2014
		Soil	KM016409	USA	2014
		Soil	KM016418	USA	2014
		Soil	KM016406	USA	2014
		Soil	KM016411	USA	2014

Table 6 (continued)

S. no.	Species	Isolation source	Accession number	Country	Submission in GenBank
		Soil	KM016400	USA	2014
		Soil	KM016402	USA	2014
		Soil	KM016404	USA	2014
		Soil	KM016405	USA	2014
		Soil	KM016416	USA	2014
		Soil	MK503713	Tamil Nadu, India	2019
		Soil	GU395635	China	2010
		Soil	AY230171	USA	2003
		P. olivieri	EU048543	Iran	2007
		P. japonica	AF122015	USA	1999
5	*S. longicaudum*	Soil	AY170338	Guangdong, China	2002
		Soil	AY230177	Guangdong, China	2003
		Soil	AY170337	Guangdong, China	2002
		Soil	MH656898	South Korea	2018
6	*S. puertoricense*	Sandy soil	AF331903	Puerto Rico	2000
7	*S. diaprepesi*	*D. abbreviates*	AF122021	Florida, USA	1999
		Soil	GU173995	Florida, USA	2009
		Soil	GU173994	Florida, USA	2009
		Soil	GU173996	Florida, USA	2009
		Soil	AY230187	Venezuela	2003
		Soil	GU173997	Florida, USA	2009
		Soil	AF440764	Florida, USA	2001
8	*S. apuliae*	Soil	RFLP data	Bari, Italy	2004
9	*S. gaungdongense*	Soil	AY170341	Guangdong, China	2002
10	*S. aciari*	Soil	AY787660	Guangdong, China	2004
12	*S. khoisanae*	Soil	DQ314287	South Africa	2005
		Soil	EU683802	South Africa	2008
		Soil	EU727170	South Africa	2008
		Soil	EU727169	South Africa	2008
		Soil	EU727168	South Africa	2008
		Soil	FJ175378	South Africa	2008
13	*S. leizhouense*	Soil	AY170340	Guangdong, China	2002
14	*S. australe*	Soil	FJ235125	Chile	2008
		Soil	MH453881	South Africa	2018
15	*S. phyllophagae*	White grubs	FJ410327	Florida, USA	2008
16	*S. vulcanicum*	Soil	GU929442	Italy	2010
		Soil	MF491477	Italy	2017
17	*S. brazilense*	Soil	FJ410325	Brazil	2008
18	*S. boemarei*	Soil	FJ152414	France	2008
19	*S. pui*	Soil	GU395618	China	2010
20	*S. pwaniense*	Soil	KT358811	Tanzania	2015
		Soil	KT358812	Tanzania	2015
		Soil	JQ687354	Tanzania	2012
21	*S. khuongi*	Soil	GU174002	Florida, USA	2009
		Soil	GU174003	Florida, USA	2009
		Soil	GU174004	Florida, USA	2009
22	*S. taiwanensis*	Soil	KX853101	Taiwan	2016
		Soil	MF161467	Vietnam	2017
		Soil	KX40517	Vietnam	2016

Acknowledgements We thank the Head of the Department of Zoology, Chaudhary Charan Singh University, Meerut, India, for providing necessary facilities for conducting the experiments.

Funding The authors thank the Department of Science and Technology (DST), New Delhi, India, for providing financial assistance through DST INSPIRE Fellowship/2014/76.

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Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(2): 954-964

Received: 12-01-2019
Accepted: 15-02-2019

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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.

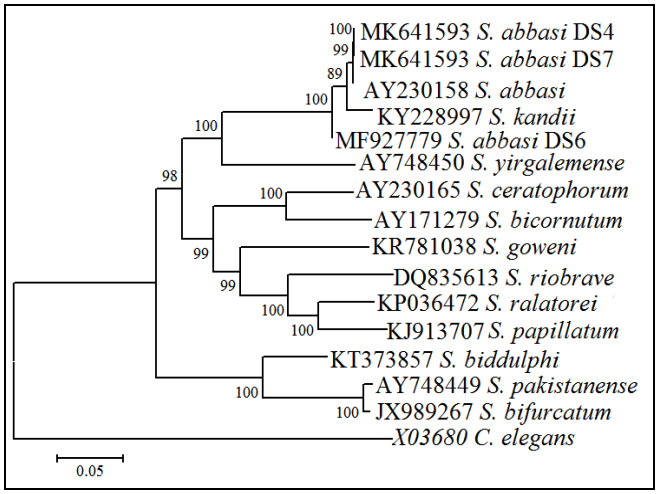

Fig 1: Phylogenetic relationships in the '*bicornutum'* group of the *Steinernema* based on analysis of ITS rRNA regions. *Caenorhabditis elegans* was used as out-group taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is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

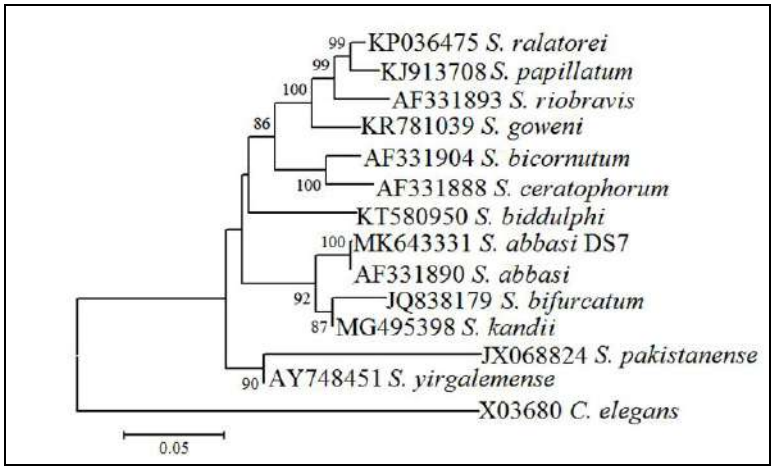

Fig 2: Phylogenetic relationships in the '*bicornutum'* group of the *Steinernema* based on analysis of D2–D3 regions of the 28S rRNA regions. *Caenorhabditis elegans* was used as out-group taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000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

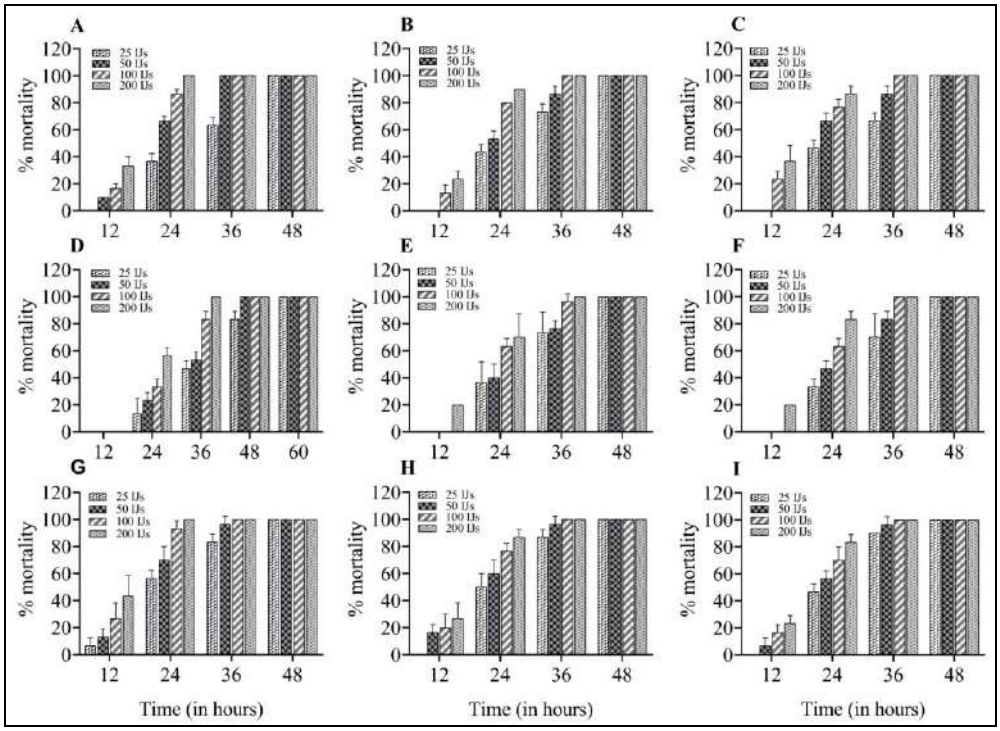

Fig 3: Percentage mortality of *Helicoverpa armigera* (A-C), *Spodoptera litura* (D-F) and *Galleria mellonella* (G-H) larvae with different doses of *Steinernema abbasi* strains DS4 (A, D, G), DS6 (B, E, H) and DS7 (C, F, I).

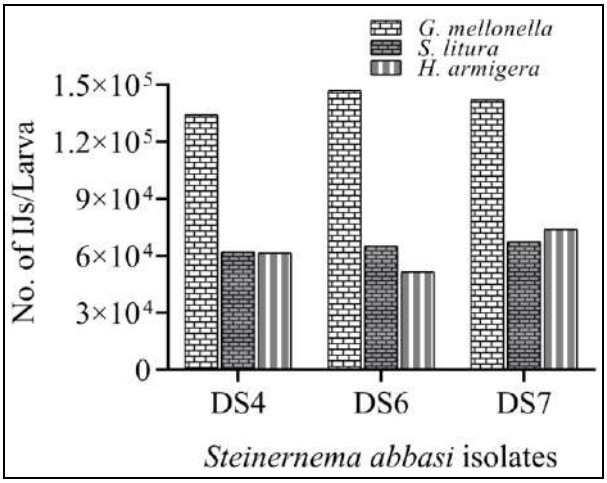

Fig 4: Mean IJs production of *Steinernema abbasi* strains DS4, DS6 and DS7 in *Helicoverpa armigera*, *Spodoptera litura* and *Galleria mellonella* at 100 IJ/Larva doses (D).

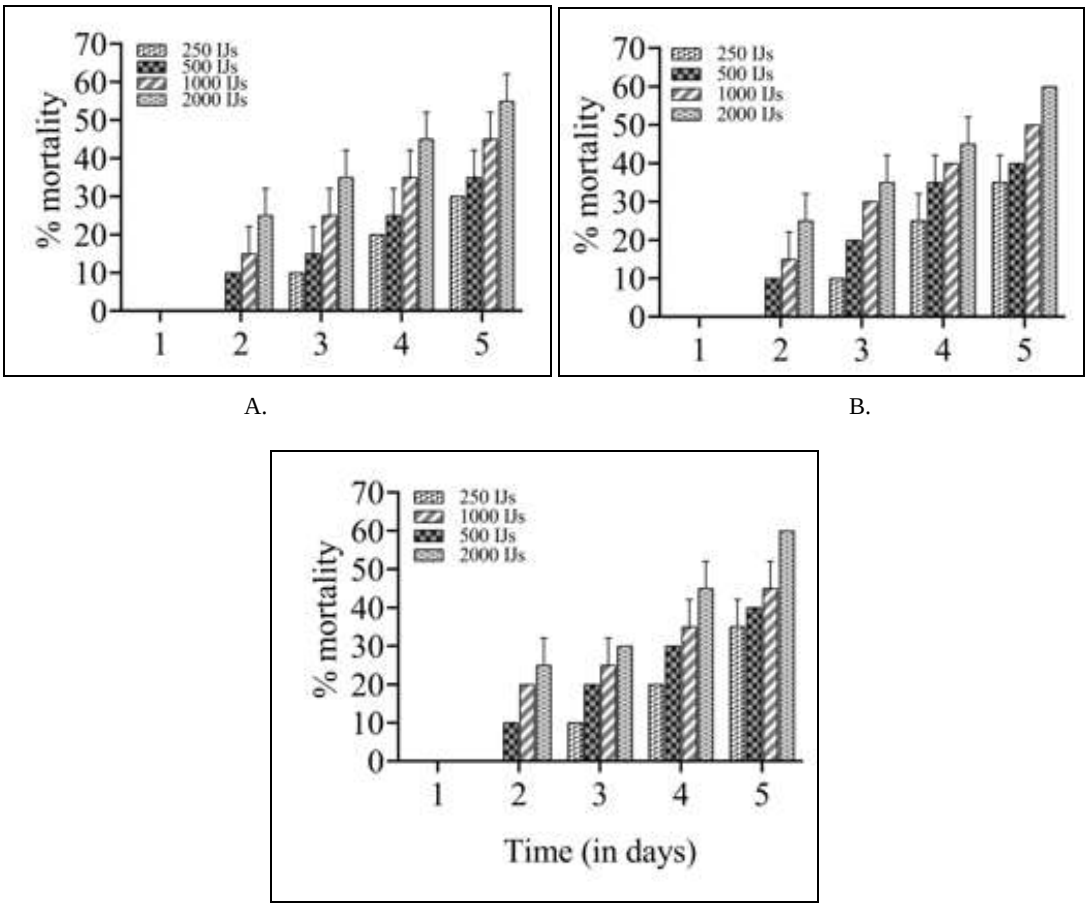

Fig 5: Percentage mortality of *Holotrichia serrata* (A-C), larvae with different doses of *Steinernema abbasi* strains DS4 (A), DS6 (B) and DS7 (C).

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	

Financial support
Department of Science and Technology (DST), New Delhi, India, is highly acknowledged for providing the financial assistance to Aasha through DST WOS-A (SR/WOS-A/LS-1083/2014).

Acknowledgement
The authors thank the Head of the Department of Zoology, Chaudhary Charan Singh University, Meerut, India, for providing necessary facilities.

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Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2015; 4(1): 27-32

Received: 13-11-2015
Accepted: 16-12-2015

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Virulence and recycling potential of entomopathogenic nematodes (Nematoda: Steinernematidae, Heterorhabditidae) from Saharanpur district, western Uttar Pradesh, India

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Abstract
Entomopathogenic nematodes have been developed as the new hope for insect pest management. Four isolates of entomopathogenic nematodes tagged as CS_1, CS_{21}, CH_5 and CH_6 were recovered from the soil of Saharanpur District of Uttar Pradesh, India, two of each genus *Steinernema* and *Heterorhabditis* respectively and were tested for their pathogenicity and recycling potential against *Galleria mellonella* larvae. Results revealed that isolate CH_6 was highly virulent and caused 100% mortality within 48 h followed by CS_{21} and CH_5 within 60 hours. A positive correlation was found between LC_{50} and LT_{50} values where increment in LT_{50} lead to the increment in LC_{50} values. The recovery of IJs/larva was $3.1\pm0.13\times10^5$, $2.16\pm0.1\times10^5$, $1.68\pm0.08\times10^5$and $1.44\pm0.1\times10^5$ with CS_1, CH_5, CH_6 and CS_{21} respectively. Results revealed that all the isolates are highly virulent with an excellent reproductive potential in laboratory condition and can be applied further for pest management and commercialization.

Keywords: Entomopathogenic nematodes, *Steinernema*, *Heterorhabditis*, reproductive potential, pest management.

1. Introduction
Development of resistance against chemical pesticides in agricultural insect pests is a common problem for their effective control. Hazardous nature and toxic environmental effects also lead for the development of efficacious bio-formulation with safe application and recycling nature within environment. Entomopathogenic nematodes (EPN), of the Families Steinernematidae and Heterorhabditidae, are free living soil dwelling insect parasitizing nematodes. These nematodes are in keen interest as they do not have detrimental effect on environment and yet to be safe for humans, flora and fauna, and can therefore be used as biocontrol representatives [1, 2]. They are ubiquitously distributed all over the world and most species are polyphagous, highly virulent, and kill their hosts rapidly. These traits have created extreme interest in the development of bio-pesticides against insect pests [1, 3]. Further, these nematodes have a unique mutualistic relationship with bacteria which reside in their alimentary canal and produce a large number of toxins which help nematodes to kill their hosts within very short time [4, 5]. For the development of a suitable formulation, the primary requirement is to use the appropriate indigenous EPN isolate, as they are more adapted to the local environment, high virulent and having a high recycling potential within the insect host. Keeping these concepts, the present study was planned in search of the locally available potential EPN isolates and to test them on larvae of *Galleria mellonella* because of their ideal susceptibility so that they would be applicable in Indian climatic conditions against agricultural pests.

2. Materials and Methods
Site and Soil Sampling: Sugarcane fields of district Saharanpur, Western Uttar Pradesh were selected for soil sampling. Fifty soil samples were collected from different sites and brought to the laboratory in well labelled polythene bags for isolation and extraction of nematodes.

Insect Culture: Larvae of *G. mellonella* were fed on semi-synthetic diet. 3rd to 4th stage larvae with approximate same size and weight were used in each bioassay experiments. Fully grown larvae were also used for isolation and mass production of nematodes for further implications.

Nematode Isolation and Culture: Entomopathogenic Nematodes were recovered from soil by adopting the method of Bedding and Akhurst [6]. Ten last instar larvae of *G. mellonella* were placed in polystyrene jar containing fine and moist soil and placed in BOD at 27±1 ºC. Samples were examined daily for insect mortality. The cadaver of *G. mellonella* were disinfected with 0.1% sodium hypochlorite, washed with double distilled water (DDW) and transferred on White trap [7] for isolation of Infective Juveniles (IJs). Isolated IJs were further disinfected with 0.1% sodium hypochlorite solution and washed properly before storing in culture flask using DDW.

Bioassay: Koch's postulate was performed for the confirmation of nematodes as entomopathogenic and they were identified upto genera level based on morphological characters. Well plate bioassay was performed for pathogenicity testing [8]. Four different concentrations of each isolate viz. 25, 50, 100 and 200 IJ were prepared in 350 µl DDW and were released in the wells of the well plates lined by double layer of filter paper. Ten replicates of larvae for each experiment along with control (only DDW) were placed in above prepared well plates. Observations were taken after each 12 hours post infection period (PIP) to check the mortality of insect larvae. Dead larvae of *G. mellonella* were placed to the modified White Trap [7] to observe the persistence of infection and emergence of IJ. Each bioassay was placed separately and all experiments were repeated thrice to reach the optimum authenticity.

Recycling Potential: Ten larvae of *G. mellonella* (same size and weight), were infected with 100 IJ/larva and incubated in BOD at 27±1 ºC till they reach their mortality. They were then placed on white trap and IJ, which migrated into distilled water, were collected daily till the emergence stopped and stored in BOD at 15±1 ºC. The period of collection of IJ was 15-17 days. The IJ were counted in counting dish under stereomicroscope (Nikon SMZ 645).

Statistical analysis: The experimental data was analysed statistically through probit analysis to calculate LC_{50} and LT_{50} and the reproductive potential was presented in mean ± standard error of mean (SEM).

3. Results

Bioassay

Four samples were found positive for the presence of nematodes and were named as isolate CS_1 and CS_{21} for *Steinernema* species and isolate CH_5 and CH_6 for *Heterorhabditis* species. All the four different concentrations (25, 50, 100 and 200 IJ) were applied to infect the larvae and were believed to be equal to the rate of penetration of IJ /larva. All the isolates tested were strongly virulent (Fig 1). Both the isolates of *Heterorhabditis* viz. CH_6 and CH_5 killed all the larvae within 48 and 60 h respectively. *Steinernema* isolate, CS_{21} also caused 100% mortality in 60 h but CS_1 was little bit less pathogenic and killed 50%, 60%, 60% and 90% larvae with all respective concentrations applied at this stage, but it was the only isolate observed which initiated mortality within 12 h in 200IJ /larva. Mortality initiation within 24 h with all the isolates with their respective concentrations showed that lowest dose of IJ (25IJ/larva) was sufficient to produce mortality. At 24 h PIP, the highest mortality was observed in CH_6 followed by CH_5, CS_{21} and CS_1. After 36 h, 100% mortality was recorded in CH_6 following the penetration of 50, 100 and 200 IJ/larva, whereas CH_5 showed same mortality in 100 and 200 IJ/larva and CS_{21} in 200 IJ/larva at 48 h PIP. In CS_1, 100% mortality was recorded at 72 h in 100 and 200 IJ/larva; lower dose caused 70% and 80%mortality up to this period of infection.

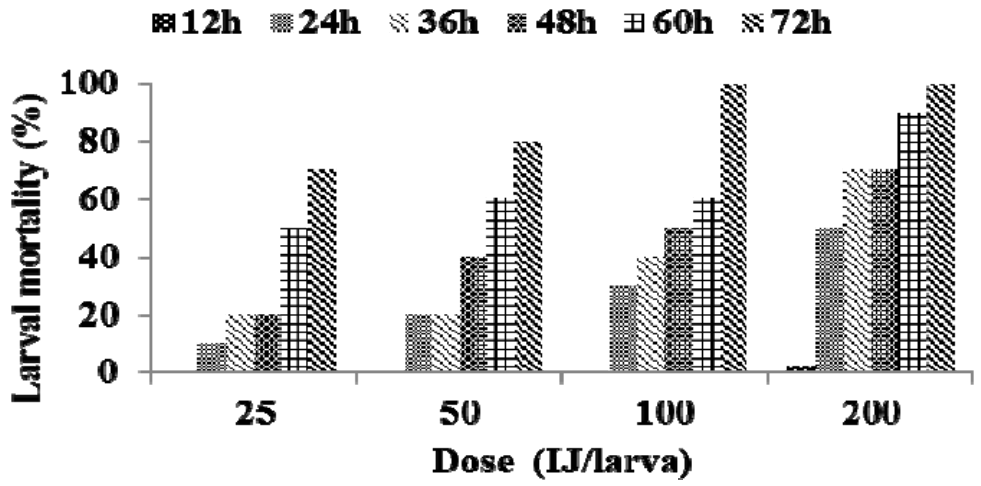
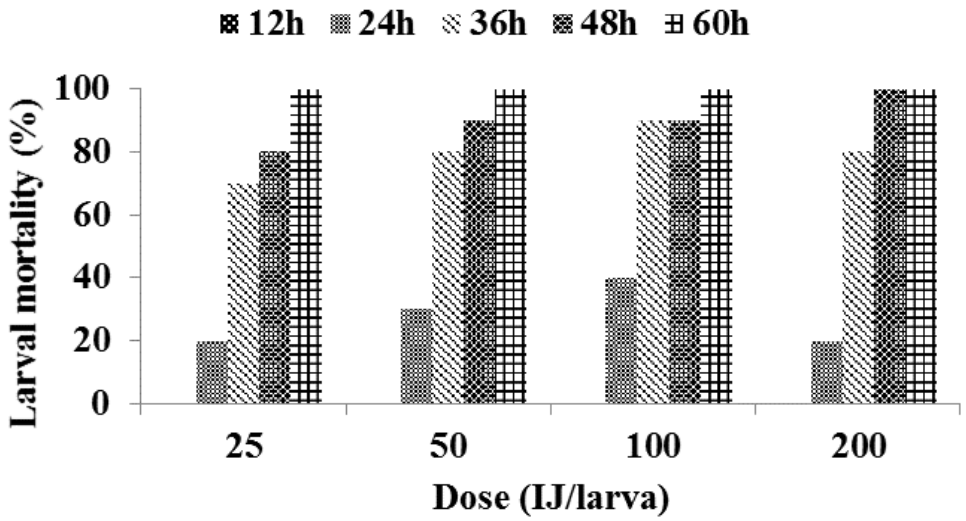


Fig 1.1



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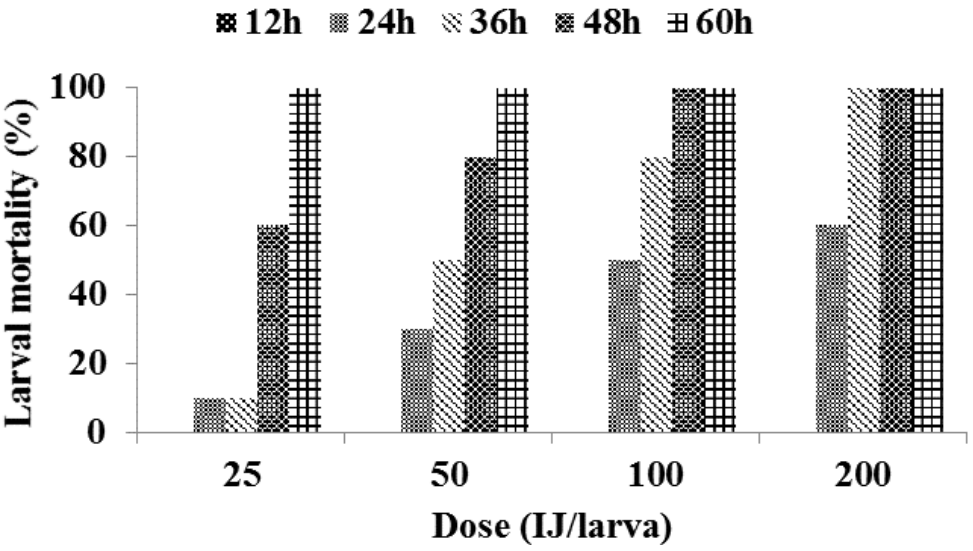

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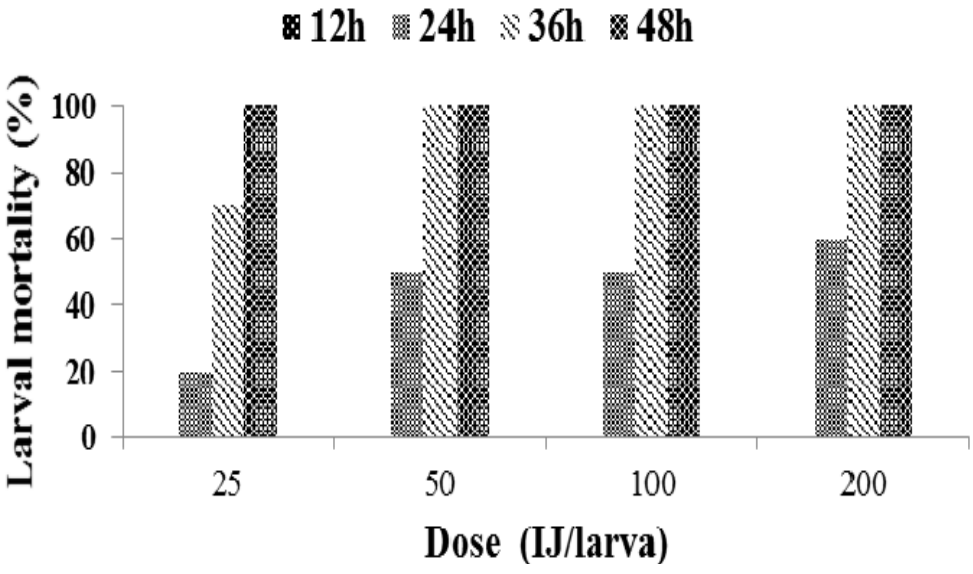

Fig 1.4

Fig 1: The percentage mortality of *G. mellonella* larvae at the exposure to different doses of infective juveniles (IJ) of nematodes. **1.1**-CS_1, **1.2**-CS_{21}, 1.3-CH_5, 1.4-CH_6.

LC_{50} values in number of IJ/larva were calculated at three different time intervals viz., 24, 48 and 72 h, through probit analysis which decreased with time increment (Table. 1). At 24 h, the LC_{50} values were 95.19 IJ/larva, 119.22 IJ/larva, 211.89 IJ/larva and 357.51 IJ/larva in CH_6, CH_5, CS_1 and CS_{21} respectively. LC_{50} value at 48 h was not calculated in CH_6 due to 100% mortality achievement. The lowest LC_{50} value was calculated in $CS_{21}$with 6 IJ/larva followed by CH_5 (22.29 IJ/larva) and CS_1 (88.67 IJ/larva). At 72 h PIP, the LC_{50} value was 17.73 IJ/larva in CS_1 and was not calculated in other isolates due to 100% mortality within 60 h. LT_{50} values (in h) of all the isolates were also calculated followed by combined mortality in all the doses (Fig. 2). The pattern of LT_{50} values from highest to lowest were as CH_6> CS_{21}>CH_5>CS_1 with 24.91, 28.94, 29.87 and 42.39 h respectively.

Table 1: LC_{50} values (in terms of number of IJ/larva) of all the isolates at different time intervals

Isolate	LC_{50} @ 24h	LC_{50} @ 48h	LC_{50} @ 72h
CS_1	211.89	88.67	17.726
CS_{21}	357.51	6.00	*
CH_5	119.22	22.29	*
CH_6	95.19	*	*

* 100% mortality achieved upto this time hence, statistics was not computed

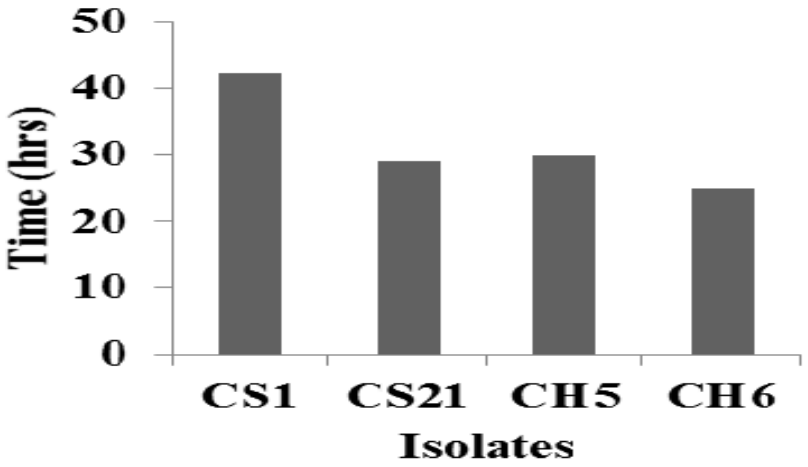

Fig 2: LT_{50} values (in hrs) following the total mortality with all the respective concentrations**.**

Recycling potential

The descriptive statistics of number of IJ/larva was performed for all the isolates and presented in mean ± SEM. The data revealed that the Infective Juveniles production was maximum in CS_1 with $3.1\pm0.13\times10^5$ IJ/larva (260000-372000) followed by CH_5 with $2.16\pm0.1\times10^5$ IJ/larva (159000-269000), CH_6 with $1.68\pm0.08\times10^5$ IJ/larva (108000-198000)and CS_{21} with $1.44.\pm0.1\times10^5$ IJ/larva (90000-193000) (Fig. 3).

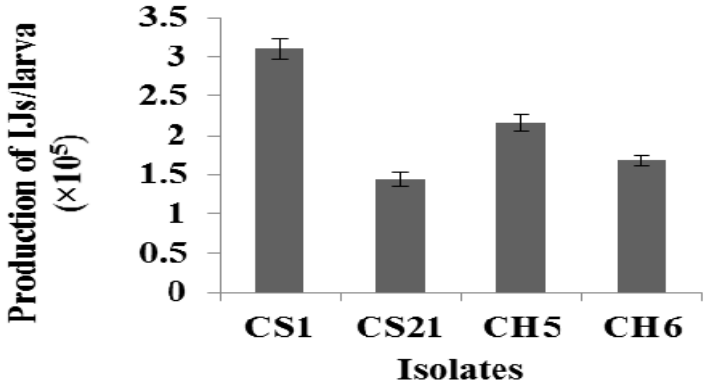

Fig 3: Mean infective juveniles production in larvae of *G. mellonella* exposing with 100 IJ/larva**.**

4. Discussion

In the present study, all the four isolates tested were found enough virulent to kill the larvae within short duration with high progeny productions. The pathogenicity of nematodes was varied within isolates which was previously demonstrated by different workers [9, 10]. A positive correlation was found in rate of penetration and mortality which was not initially observed by Caroli *et al.* [11] and Ricci *et al.* [12] but was clearly indicated by several other workers [8, 13, 14, 15]. This theory suggested that high infection rate increases the production of toxins produced by developing bacteria associated with nematodes [5]. *Heterorhabditis* isolate CH_6 took least time to cause 100% mortality and killed all the larvae within 48 h following all the concentrations whereas isolate CH_5 of the same genus killed all the larvae in only 100 and 200 IJ/larva doses. In *Steinernema* isolate CS_{21} only 200 IJ/larva dose

killed all the larvae and the result can be corroborated with the findings of Phan *et al.* [15] and Caroli *et al.* [11] with other *Steinernema* species/strains. Except CS_1, all the nematode isolates were able to kill all the larvae within 60 h duration following all the concentrations. Isolate CS_{21} of *Steinernema* sp. showed lowest LC_{50} values at 48 h followed by least pathogenic *Steinernema* isolate CS_1 but CH_6 was judged to be most pathogenic isolate among all, as at 48 h LC_{50} value was unpredictable because of its high mortality aptitude. LT_{50} values ranges from 25.09-42.39 h where highly virulent *Heterorhabditis* isolate CH_6 took least time followed by ceiling time taken by *Steinernema* CS_{21} isolate. Decrease in LC_{50} with time increment suggested that longer time lead to the multiplication of more bacteria result in more production of toxins to kill insect host by septicaemia. Positive correlation was found between LC_{50} and LT_{50} values where increment in LT_{50} lead to the increment in the LC_{50} values, this also indicated the higher virulence of CH_6 with minimal time and IJs required.

Entomopathogenic nematodes have numerous attributes that make them an admirable bio-control driving force. The genera *Steinernema* and *Heterorhabditis* of these nematodes have gained much importance for commercialization and their application [14, 16]. *In vivo* mass production of EPNs is easy where *G. mellonella* is a most widely used insect species. In present study, the IJs production rate from larvae of *G. mellonella* was very high as was proposed by Elawad *et al.* [17]. The least pathogenic isolate CS_1 showed highest recycling potential whereas the highest pathogenic isolate CH_6 produced lower number of Infective juveniles. This could be because of longer residing time within the host for production of more generations. The same result was obtained with CH_5 as it also showed less progeny count as compared to CS_{21} but more than CH_6. However, recovery of IJs from $CS_{21}$was lowest which was strong virulent after CH_6, could lead a controversy in above said hypothesis. The IJs production of *Steinernema* isolates was comparable with the results of some other workers with different *Steinernema* species [17, 18]. Shapiro-Ilan *et al.* [19] reported the production of *H. bacteriophora* from single *G. mellonella* upto 300,000. Comparatively to the author, the production of IJs of studied unknown *Heterorhabditis* isolates was similar.

5. Conclusion

Based on the findings of present study it can be concluded that all the four isolates of EPN were virulent to larvae of *G. mellonella* and produced good number of IJ. However *Steinernema* isolate CS_1 was less pathogenic to *Steinernema* isolate CS_{21} and *Heterorhabditis* isolate CH_6 and CH_5, but its progeny production rate was high in comparison to all. Further work is required to identify the isolates and study the host range, as well as the virulence of its symbiotic bacteria. It may therefore, be concluded that these all EPN isolates have good bio-control potential for future applications on insect pests and commercialization against them.

6. Acknowledgement

Authors are also thankful to Head of the Department for providing necessary facilities to carry out the work. AK Chaubey & Istkhar is thankful to Council of Scientific and Industrial Research (CSIR), New Delhi, India for providing the financial assistance through its EMR project (37(1550)/12-EMR-II) and fellowship under the scheme respectively.

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Article *in* International Journal of Pharmacy and Biological Sciences · August 2016

DOI: 10.21276/ijpbs.2016.6.3.7

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International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 6 | Issue 3| JUL-SEP | 2016 | 60-73
Original Research Article – Biological Sciences

PHYLOGENIC, PATHOGENIC AND REPRODUCTIVE CHARACTERIZATION OF *HETERORHABDITIS INDICA* FROM DISTRICT MEERUT, INDIA

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ABSTRACT

Heterorhabditis indica isolate CH_{18} was isolated from District Meerut of Uttar Pradesh, India and characterized by short juveniles and the arrangement of the terminal group of bursal ribs. The values of E and D ratio differentiate it from other described species of Heterorhabditis. It can be recognised by; L = 534 (514-548) µm, pharynx = 107 (99-111) µm; ratio 'a' = 24 (22-27); ratio 'c' = 5.4 (5-6); D% = 84 (75-91) and E%= 92 (83-101). It was further recognised by the male characters viz. L = 721 (592-884) µm, SL = 41 (32-55) µm; GL = 19 (13-27) µm; GBW = 46 (33-54) µm. H. indica isolate CH_{18} was genetically recognised by sequence lengths of the ITS region (737 bp), D2-D3 region (942 bp) and COX1 (596 bp). Pathogenicity trial based on different IJ concentration viz. 25, 50, 100, 200 IJ/larva showed that the degree of susceptibility to nematode infection varied from different dose infection and also on the exposure time. A positive relation was observed in between the doses of infective juvenile and larval mortality where increments in doses also lead to increment in mortality rate. With a maximum count of progeny ($1.79\pm0.8\times10^5$ IJs/ Larva) in lowest larval dose, a strong negative correlation was observed where the number of IJ production decreased significantly with increment in the dose of infection.

KEY WORDS

ITS region, D2-D3 region, COXI gene, pathogenicity, IJs production, negative correlation.

INTRODUCTION

Entomopathogenic nematodes, (*Heterorhabditis* and *Steinernema*) are used as potential biological control agents as they are efficient parasites of several economically important insect pests[1, 2, 3] being symbiotically associated with entomopathogenic bacteria *Photorhabdus*[4] and *Xenorhabdus*[5]. To date, twenty five species have been described for *Heterorhabditis* and more than hundred for *Steinernema*. There has been considerable debate about the proper identification of entomopathogenic nematodes[6,3,7]. Taxonomic relationships of these nematodes are usually based on morphological characters for *Heterorhabditis* species while for *Steinemema* species morphological characters are combined with cross breeding data[8,9,7]. Morphological characters cannot be used unambiguously to place new isolates into a particular species. The feasibility of using these nematodes as biological control agents depends upon the resources required for a rapid and accurate means to determine the genetic diversity among existing populations of entomopathogenic nematode species. These methods can also be used for the development of identification tools. The use of molecular approaches to study systematic relationships among entomopathogenic nematodes have been emphasized[10]. Currently regions of taxonomic importance include the internal transcribed spacer (ITS) and D2D3 region of the ribosomal DNA (rDNA). Both of these regions have been widely used for the identification of few nematodes [11, 12] and will make major contributions to the identification of entomopathogenic nematode species.

During a survey of sampling in cultivars of Meerut region, an isolate of Heterorhabditid nematode tagged as CH_{18} was isolated and based on morphological and molecular characteristics was

identified as *Heterorhabdtis indica*. In the present investigation, three regions viz. ITS, D2D3 and COX1 were used to delimit its species status in addition morpho-taxometrical investigation. The pathogenicity and reproductive prospective of this isolate was also observed in the present study.

MATERIALS AND METHODS

Nematode collection

The infective juvenile (IJ) stages of EPN mostly reside in the moist soils and have been reported in almost all type of soils except dry soils. Therefore, to get hold of these pristine indigenous, local EPN, soils were collected randomly from different agricultural fields of District Meerut using appropriate sampling methods. Recovery of EPN from soil samples was scrutinized using insect baiting with the last instars of *Galleria mellonella* (L.) (Lepidoptera:Pyralidae) [13]. Maintenance of IJ were done by recycling through *G. mellonella* larvae and stored in approximately 150 ml of sterilized distilled water in 500 ml tissue culture flasks at 15°C for subsequent identification and establishment of stock cultures.

Morphological Observations

To study the morphology and morphometry, adult stages and 3rd stage juveniles were recovered from the cadaver of *G. mellonella*. For recovery of adults, the cadaver of *G. mellonella* were dissected 2-3 days and 4-6 days after mortality for hermaphroditic females and subsequent generation of amphimictic males and females respectively in Ringer's Solution[14] while freshly emerged IJ were obtained from white trap[15] for the identification purpose. These all stages were killed with lukewarm water, fixed in TAF[16] and subsequently processed to glycerine by the Seinhorst method[17]. Nematodes were transferred to the fresh drop of glycerine on a microscope slide, crystals of paraffin wax placed around the glycerin drop and covered with a cover glass and placed on hot plate for melting of wax for sealing.

Morphometric analysis of the nematode specimens was done for 15 individuals of the adult stages of generations and infective juveniles (IJ), using light microscopy and the image analyzing software DL-S1 (Phase contrast, Japan).

Molecular Characterization and Phylogenetic Relationships

Genomic DNA was extracted from 3rd stage infective juveniles through Quigen DNA Blood and Tissue Kit as per manufacturer's instruction with slight modifications. Agarose Gel Electrophoresis (AGE) was performed to detect the presence of DNA in the eluted solution and the primers suggested by Joyce et al.[18] were used for PCR. The amplification of the ribosomal and mitochondrial regions was carried out using Dream Taq Green PCR Master Mix 2X (Thermo Scientific) in 100 µl eppendorf (AB Scientific) with a final reaction volume of 30 µl. Amplifications were carried out using Verti 96 well fast thermocycler with heated lid pre-set at 95 ^{0}C and subjected to the following cycling profile: For the ITS rDNA region, PCR conditions included initial denaturation at 94 ^{0}C for 5 min, followed by 33 cycles of 94 ^{0}C for 1 min, 55 ^{0}C for 1 min 30 sec and 72 ^{0}C for 2 min, followed by a final extension at 72 ^{0}C for 5 min to ensure all of the amplification products are full length.. For the 28S (D2D3) rDNA region, the parameters included denaturation at 94 ºC for 5 min, followed by 33 cycles of 94 ºC for 30 sec, 52 ^{0}C for 30 sec and 72 ^{0}C for 1 min, followed by a final extension at 72 ºC for 7 min. For the COX1 mtDNA region, the PCR protocol included denaturation at 94 ^{0}C for 3 min, followed by 37 cycles of 94 ^{0}C for 30 sec, 50 ^{0}C for 30 sec and 72 ^{0}C for 45 sec, followed by a final extension at 72 ^{0}C for 7 min. Electrophoresis of the PCR products was carried in a 1% TAE buffered agarose gel stained with EtBr. The products were analyzed on 1% agarose gel with TAE buffer.

The amplified PCR products were purified and sequenced in both directions by Bioserve Biotechnologies Pvt. Ltd, Hyderabad, India. The contig sequences were deposited in NCBI GenBank database under the following accession numbers; ITS regions (KU176726), D2-D3 regions (KU176127) of rDNA and COX1 (KU306237) for mtDNA. The sequences were edited and compared with those present in the Genbank by means of a Basic Local Alignment Search Tool (BLAST). An alignment of samples together with sequences of the closely related species of the *Heterorhabditis* was produced for each amplified DNA region using default Crustal W parameters in MEGA

6.0 (Tamura et al., 2013) and optimized manually in BioEdit. Pairwise distances and phylogenetic trees (Maximum Parsimony) were computed using MEGA 6.0[19]. Codon positions included were first + second + third + non-coding.

Bioassay experimentation for Pathogenicity and Reproductive Potential

Pathogenicity and reproductive potential of the present species was carried out at different inoculums viz. 25, 50, 100 and 200 IJ/Larvae. For evaluating pathogenicity, freshly emerged IJ were used for larval mortality against *G. mellonella* in 6 well plates (3.5 cm diameter) lined by double Whatman filter paper No. 1. Four different concentrations viz. 25, 50, 100 and 200 IJ were prepared separately in DDW. The total moisture with each IJ dose was maintained 450 µl with distilled water and was poured into each well with the help of micro pipette in the well plates separately. IJ viability was confirmed under stereo microscope (NIKON SMZ 645) before pouring into wells. Larvae of same size and weight were infected in 6 well plates and all experiments were replicated 10 times along with control for each treatment and incubated at 28±2^{0}C in BOD till the mortality of larvae. After the death, all the larvae were transferred onto the white trap for the emergence of IJ to confirm that the mortality was due to IJ infection. Mortality was recorded after each 12 h interval till 100% mortality was achieved and the same cadavers were utilized to compute the progeny production of IJ. The IJ started to emerge from the cadavers into the white trap after 5-7 days of transfer and the emerged IJ from each larva were collected till their emergence (18-20 days) stopped and were transferred from production dish to a culture flask separately in DDW. The amount of DDW was maintained to 50 ml and the nematodes were counted under stereomicroscope (NIKON SMZ 645) in 1ml suspension with the help of counting dish. The total progeny production was determined with the help of statistical tools.

Statistical Analysis

Data obtained through measurements of De Man Indices was analyzed statistically where the descriptive analysis was performed and data was presented in measurement in µm ± SD (range) except the ratios and percentage values. The insect larval mortality assay was analyzed statistically through probit analysis and LC_{50} and LT_{50} values were calculated at 95% confidence limit. All the mortality recorded in the form of percentage mortality and graphical presentations were made using excel.

Total No. of IJ/Larva of the studied nematode was analyzed by descriptive analysis and presented in number of IJ ± SE (range). Analysis of variance (One way ANOVA) was performed to find out the significance relationship between IJ penetration and production with a significance level of $P<0.05$.

RESULTS AND DISCUSSION

Morphological Observations

The *H.* indica isolate CH_{18} was isolated from the soils of sugarcane fields (*Saccharum officinarum L.*) of Baksar area of district Meerut, Uttar Pradesh, India. Present isolate was characterized by the short juveniles almost similar to the original description and the varied arrangement of the terminal group of bursal ribs. The values of E and D ratio differentiate it from other described species of *Heterorhabditis*. It can be recognised by IJ body diam. = 22 (20-24) µm, L = 534 (514-548) µm, pharynx = 107 (99-111) µm; ratio 'a' = 24 (22-27); ratio 'c' = 5.4 (5-6); D% = 84 (75-91) and E%= 92 (83-101). It was further recognised by the male characters viz. L = 721 (592-884) µm, SL = 41 (32-55) µm; GL = 19 (13-27) µm; GBW = 46 (33-54) µm.

H. indica isolate CH_{18} was compared with six closely related species of *Heterorhabditis*, and it differed in some morphological and morphometric characters with compared species but showed much similitude with *H. indica*. The comparison of the morphometrics with other species is presented in the Tables (Table 1 & 2) and is described as follows:

H. indica isolate CH_{18} was separated from *H. taysearae* by longer body of IJ 534 (514-548) vs. 418 (332-499) µm; ratio 'a' =24 (22-27) vs. 21 (18-27); ratio 'b' = 5 (4.74-5.47) vs. 3.8 (3.4-4.2) and shorter ratio 'c' = 5.46 (4.97-5.95) vs. 7 (6.5-8.7) µm; E% = 92 (83-101) vs. 180 (110-230) and tail 98 (89-106) vs. 55 (44-70) µm . Males of the present specimen can be differentiated from *H. taysearae* by SL = 41 (32-55) vs. 39 (30-42) µm; GBW = 46 (33 -54) vs. 43 (38-48) µm; EP = 74 (65-86) vs. 95 (78-120) µm; ES = 88 (78-96) vs. 112 (85-

123) µm; TR = 101 (75-131) vs. 122 (100-146) µm and SW% = 200 (113-299) vs. 156.

Table 1. Comparative morphometrics of infective juveniles of *H. indica* CH_{18} and related *Heterorhabditis* spp. (in ascending order of body length). Measurements are in µm (expect n, ratio and percentage) and in the form: mean (range). Data for *H. indica* CH_{18} in bold.

Characters	*taysearae*	*indica*	CH_{18}	*baujardi*	*floridensis*	*mexicana*	*bacteriophora*
n	30	25	**15**	25	25	25	15
l	418 (332-499)	528 (479-573)	**534 (514 -548)**	551 (497-595)	562 (554-609)	578 (530-620)	588 (512-617)
a	21 (18-27)	26 (25-27)	**24 (22-27)**	28 (26-30)	27 (25-32)	25 (23.6-28.4)	25 (17-30)
b	3.8 (3.4-4.2)	4.5 (4.3-4.8)	**5 (4.7-5.5)**	4.8 (4.5-5.1)	4.3 (3.9-4.9)	4.6 (4.2-5.1)	4.5 (4.0-5.1)
c	7.7 (6.5-8.7)	5.3 (4.5-5.6)	**5.4 (5-6)**	6 (6-6.7)	5.6 (5.3-6.6)	5.9 (5.5-6.3)	6.2 (5.5-7.0)
GBW	20 (17-23)	20 (19-23)	**22 (20-24)**	20 (18-22)	21 (19-23)	23 (20-24)	23 (18-31)
EP	90 (74-113)	98 (88-107)	**90 (82- 95)**	97 (91-103)	109 (101-122)	102 (83-109)	103 (87-110)
NR	64 (58-87)	82 (72-85)	**78 (70- 85)**	81 (75-86)	86 (68-107)	81 (74-88)	85 (72-93)
ES	110 (96-130)	117 (109-123)	**107 (99-111)**	115 (107-120)	135 (123-142)	122 (104-142)	125 (100-139)
Tail with sheath	55 (44-70)	101 (93-109)	**98 (89-106)**	90 (83-97)	103 (91-113)	99 (91-106)	98 (83-112)
ABW	-	-	**15 (13-17)**	13 (11-14)	14(12-16)	15 (12-17)	-
D%	82 (71-96)	84 (79-90)	**84 (75 -91)**	84 (78-88)	81 (71-90)	81 (72-86)	84 (76-92)
E%	180 (110-230)	94 (83-103)	**92 (83 -101)**	108 (98-114)	105 (95-134)	104 (87-111)	112 (103-130)

–, Data not available.

Table 2. Comparative morphometrics of first-generation males of *H. indica* CH_{18} and related *Heterorhabditis* spp. Measurements are in µm (expect n, ratio and percentage) and in the form: mean (range). Data for *H. indica* CH_{18} in bold.

Characters	*taysearae*	*Indica*	CH_{18}	*baujardi*	*floridensis*	*mexicana*	*bacteriophora*
N	20	12	**15**	14	20	20	15
L	703 (648-736)	721 (573-788)	**721 (592-884)**	889 (818-970)	862 (785-924)	686 (614-801)	820 (780-960)
GBW	43 (38-48)	42 (35-46)	**46 (33-54)**	49 (45-53)	47 (43-50)	42 (38-47)	43 (38-46)
EP	95 (78-120)	123 (109-138)	**74 (65-86)**	81 (71-93)	117 (104-128)	124 (108-145)	121 (114-130)
NR	65 (54-88)	75 (72-85)	**65 (56-74)**	65 (54-77)	80 (73-90)	71 (61-83)	72 (65-81)
ES	112 (85-1230	101 (93-109)	**89 (78-96)**	116 (105-132)	105 (97-111)	96 (89-108)	103 (99-105)
TR	122 (100-146)	91 (35-144)	**101 (75-131)**	91 (28-38)	93 (78-116)	96 (65-130)	79 (59-87)
SL	39 (30-42)	43 (35-48)	**41 (32S-55)**	40 (33-45)	42 (36-46)	41 (30-47)	40 (36-44)
GL	18 (14-21)	21 (18-23)	**19 (13-27)**	20 (18-22)	23 (17-30)	23 (18-32)	20 (18-25)
D%	88	121	**84 (68-93)**	-	112 (105-119)	129 (114-149)	117
SW	156	187	**200 (113-299)**	182 (138-208)	157 (133-209)	167 (130-196)	174
GS	46	49	**46 (38-56)**	50 (44-61)	53 (47-65)	56 (43-70)	50

–, Data not available.

Morphologically *H. indica* CH_{18} was separated from *H. baujardi* by the shape of gubernaculum proximal end bearing knob vs. lacking, ventrally straight vs. curved. Males of *H. indica* CH_{18} differs from *H. baujardi* by ES = 89 (78-95) vs. 116 (105-132) µm, TR = 101 (75-131) vs. 91 (28-38) µm, Tail = 27 (18-42) vs. 33 (28-38) µm, SW% = 200 (113-299) vs. 182 (138-208) and GS% = 46 (38-56) vs. 50 (44-61). The IJ of the present isolate differs from *H. baujardi* by having shorter body length 534 (514-548) vs. 551 (497-595) µm, ratio 'a' = 24 (22-27) vs. 28 (26-30), EP = 90 (82-95) vs. 97 (91-103) µm, E% = 92 (83-101) vs. 108 (98-114) and tail 98 (89-106) vs. 90 (83-97) µm.

H. indica CH_{18} males were differentiated from *H. floridensis* males by a EP =74 (65-86) vs. 117 (104-128) µm, NR = 65 (56-74) vs. 80 (73-90) µm, ES = 89 (78 -96) vs. 105 (97-111) µm, GL = 19 (13-27) vs. 23 (17-30) µm, SW% = 200 (113-299) vs. 157 (133-209), GS%.= 46 (38-56) vs. 54 (47-65) and D% = 84 (68-93) vs. 112 (105-119). IJ were shorter than *H. floridensis* 534 (514-548) vs. 562 (554-609) µm, Tail = 98 (89-106) vs. 103 (91-113) µm and ratio 'a' = 24 (22 -27) vs. 27 (25-32), E% = 92 (83-101) vs. 101 (95-134).

Male of *H. indica* CH_{18} was separated from *H. mexicana* by GBW= 46 (33-53) vs. 42 (38-47) µm, EP = 74 (65-86) vs. 124 (108-145) µm, GL = 19 (13-27) vs. 23 (18-32) µm, D% = 83 (68-93) vs. 129 (114-149), SW% = 200 (113-299) vs. 167 (133-196), GS%.= 46 (38-56) vs. 56 (43-70). IJ can be distinguished by the body length = 534 (514-548) vs. 578 (530-620) µm, EP = 90 (81-94) vs. 102 (83-109) µm, ES = 107 (99-111) vs. 122 (104-142) µm, D% = 84 (75-91) vs. 81 (72-86) and E% = 92 (83-101) vs. 104 (87-111). The vulval pattern in

females is different in both specimens which also separated them from one another.

Males of *H. indica* CH_{18} was differentiated from *H. bacteriophora* males by SL = 41 (32-55) vs. 40 (36-44) µm, GL = 19 (13-27) vs. 20 (18-25) µm, D% = 84 (68-93) vs. 117, ABW= 21 (17-29) vs. 23 (22-25) µm, SW% = 200 (113-299) vs. 174 and GS% = 46 (38-56) vs. 50. IJ can be characterised by the shorter body length 534 (514-548) vs. 588 (512-617) µm, NR = 78 (70-85) vs. 85 (72-93) µm, EP = 90 (81-94) vs. 103 (87-110) µm, ratio 'a' = 24 (21-26) vs. 25 (17-30) µm, ES = 107 (99-111) vs. 125 (100-139) µm and E% = 92 (83-101) vs. 112 (103-130).

Molecular Characterization and Phylogenetic Relationships

H. indica isolate CH_{18} was characterised genetically by sequences of ITS (KU176726), D2-D3 regions (KU176127) of rDNA and COX1 (KU306237) mt DNA. The quality of PCR products of said genes was confirmed by agarose gel electrophoresis. The length of the internal transcribed spacer region was 737 bp with ITS1 = 369 bp, 5.8S = 154 bp and ITS2 = 214 bp and its nucleotide composition was: A = 26.46%, C = 19.95%, G = 24.83%, T = 28.77%. The length of the total sequence is the smallest among the 14 related species of *Heterorhabditis* (Table 3). *H. indica* isolate CH_{18} differed from *H. baujardi*, *H. bacteriophora*, H. *floridensis* and *H. mexicana* from its closest taxon by 25 bp, 34 bp, 23 bp and 24 bp, respectively while with already described *H. indica* (AY321483) by only 2 bp and thus not much variation, hence considered same. Pairwise distance of ITS and D2D3 regions of rDNA are shown in Table 4-5. Both the regions of taxonomic importance showed 100% percentage similarity with the original specimen.

Table 3. Sequence lengths and nucleotide composition of ITS (ITS1 + 5.8S + ITS2) and D2-D3 regions of species of *Heterorhabditis* closely related to *H. indica* CH_{18}. Data of *H. indica* CH_{18} in bold.

Species		Molecular markers							ITS(bp)	Seq. (bp)
ITS	Acc. No.	ITS1(bp)	5.8S(bp)	ITS2(bp)	A	C	G	T		
***H. indica* CH_{18}**	**KU176126**	**369**	**154**	**214**	**26.46**	**19.95**	**24.83**	**28.77**	**739**	
H. indica	AY321483	370	154	215	20.78	21.43	28.57	29.22	739	
H. noenieputensis	JN620538	371	154	216	20.78	21.43	28.57	29.22	741	
H. amazonens	DQ665222	395	154	211	21.43	21.43	28.57	28.57	760	
H. baujardi	AF548768	397	153	212	20.92	21.57	28.76	28.76	762	
H. floridens	DQ372922	393	154	213	21.43	21.43	28.57	28.57	760	
H. Mexicana	AY321478	394	154	213	22.08	21.43	28.57	27.92	761	
H. bacteriophora	AY321477	389	154	228	21.43	20.78	28.57	29.22	771	
H. Georgiana	EU099032	389	154	228	22.08	20.13	28.57	29.22	771	
H. atacamensis	HM230723	350	154	211	20.78	21.43	29.22	28.57	715	
H. safricana	EF488006	379	154	211	22.08	19.48	29.22	29.22	744	
H. marelatus	AY321479	379	154	211	21.43	21.43	28.57	28.57	744	
H. zealandica	AY321481	387	154	212	21.43	22.08	27.92	28.57	753	
H. downesi	AY321482	374	154	212	21.43	21.43	28.57	28.57	740	
H. megidis	AY321480	384	154	220	22.08	21.43	28.57	27.92	758	
D2D3										
***H. indica* CH_{18}**	**KU176127**				**27.49**	**19.43**	**28.24**	**24.84**		**942**
H. indica	JQ178379				25.85	19.39	29.13	25.63		913
H. amazonens	EU099036				26.07	19.55	29.06	25.32		936
H. floridens	EU099034				25.56	19.79	29.3	25.35		935
H. Mexicana	EU100414				25.48	19.81	29.44	25.27		934
H. bacteriophora	JQ178377				25.49	19.89	29.45	25.16		910
COX I										
***H. indica* CH_{18}**	**KU306237**				**23.83**	**12.75**	**19.3**	**44.13**		**596**
H. indica	AB355853				22.81	9.81	19.89	47.48		377

H. taysearae	EF043421	24.42	11.06	19.47	45.05	606
H. Mexicana	EF043422	24.92	10.4	18.81	45.87	606
H. bacteriophora	JQ423214	23.77	8.62	18.76	48.85	917

The sequence of the D2-D3 region was 942 bp long with nucleotide composition as: A = 27.49%, C = 19.43 %, G = 28.24%, T = 24.84%. The sequence of the COX1 mtDNA region was 596 bp with nucleotide configuration: A = 23.83%, C = 12.75%, G = 19.30%, T = 44.13% (Table 3).

For ITS regions, maximum parsimony analysis showed that the alignment resulted in 1019 characters, of which 267 were constant, 467 variable characters were parsimony uninformative and 285 characters were parsimony informative. Parsimony and distance based tree building approaches produced almost identical trees. The phylogenetic relationships between 16 *Heterorhabditis* species are presented in Fig. 1, with MP Tree length = 710, Consistency Index = 0.74498, Retention Index = 0.875, Composite Index = 0.718486 (0.651857). In this consensus tree, two species, *H. indica* (AY321483) and *H. indica* isolate CH_{18} form a monophyletic group with bootstrap support of 98%. All positions containing gaps and missing data were eliminated. There were a total of 574 positions in the final dataset.

For D2-D3 region, maximum parsimony analysis showed the alignment resulted in 1034 characters, of which 174 are constant, 530 variable characters are parsimony uninformative and 330 characters are parsimony informative. The phylogenetic relationships between 17 *Heterorhabditis* species are presented in Fig. 2, with MP Tree length = 687, Consistency Index = 0.814126, Retention Index = 0.862637, Composite Index 0.799854 (0.702296). There were a total of 574 positions in the final dataset. In the consensus tree, *H. indica* and the present specimen along with two undescribed species form a monophyletic group.

For the COX1 gene, maximum parsimony analysis showed that the alignment resulted in 1034 characters, of which 105 are constant, 609 variable characters are parsimony-uninformative and 217 characters are parsimony-informative. The phylogenetic relationships between 17 *Heterorhabditis* species are presented in Fig. 3, with MP Tree length = 555, Consistency Index = 0.679878, Retention Index = 0.562500, Composite Index = 0.456081 (0.382431). There were a total of 354 positions in the final dataset. The present species showed deviation from the above results which might be due to non-utility of CO1 gene for identification purpose of EPN.

Table 4. Pairwise distances of the ITS regions between *H.indica* CH_{18} and closely related species of *Heterorhabditis*. Below diagonal: % age similarity; above the diagonal: no. of base substitutions per site between sequences, according to the Jukes-Cantor model. Data for isolate CH_{18} in bold.

ITS region	CH_{18}	*ind*	*bac*	*bau*	*noe*	*ama*	*flo*	*mex*	*geo*	*ata*	*saf*	*mar*	*zea*	*dow*	*meg*
CH18		**0.003**	**0.031**	**0.023**	**0.008**	**0.021**	**0.024**	**0.024**	**0.031**	**0.033**	**0.034**	**0.036**	**0.046**	**0.039**	**0.040**
H. indica	**100**		0.032	0.023	0.008	0.021	0.024	0.025	0.031	0.034	0.035	0.037	0.047	0.039	0.041
H. bacteriophora	**77**	77		0.034	0.032	0.033	0.035	0.035	0.006	0.024	0.024	0.026	0.035	0.027	0.028
H. baujardi	**86**	86	74		0.024	0.008	0.009	0.011	0.034	0.035	0.036	0.037	0.044	0.039	0.038
H. noenieputensis	**98**	98	76	86		0.022	0.024	0.025	0.031	0.033	0.034	0.036	0.045	0.039	0.040
H. amazonensis	**88**	87	75	98	87		0.010	0.012	0.033	0.035	0.036	0.037	0.044	0.040	0.039
H. floridensis	**85**	85	73	97	85	97		0.008	0.035	0.037	0.038	0.039	0.047	0.043	0.042
H. Mexicana	**85**	84	73	96	84	96	98		0.035	0.038	0.039	0.040	0.048	0.044	0.043
H. Georgiana	**78**	77	99	74	77	75	73	98		0.025	0.025	0.027	0.035	0.028	0.029
H. atacamensis	**74**	73	85	71	74	72	69	98	85		0.007	0.010	0.029	0.016	0.018
H. safricana	**73**	72	86	71	73	71	68	97	85	98		0.010	0.029	0.016	0.018
H. marelatus	**70**	70	84	69	71	70	66	99	83	96	97		0.029	0.017	0.019
H. zealandica	**61**	60	73	62	61	62	58	98	73	79	79	79		0.030	0.030
H. downesi	**68**	68	82	68	68	68	64	99	81	93	93	91	79		0.014
H. megidis	**66**	65	79	68	66	67	64	99	78	90	90	89	78	94	

Table 5. Pairwise distances of the D2D3 regions between *Heterorhabditis indica* CH_{18} and closely related species of *Heterorhabditis*. Below diagonal: %age similarity; above the diagonal: no. of base substitutions per site between sequences, according to the Jukes-Cantor model. Data for isolate CH_{18} in bold.

D2D3 egion	CH_{18}	*ind*	*ama*	*mex*	*noe*	*flo*	*saf*	*mar*	*bac*	*ata*	*geo*	*zea*	*meg*
CH18		**0.002**	**0.008**	**0.008**	**0.002**	**0.008**	**0.011**	**0.012**	**0.011**	**0.011**	**0.011**	**0.297**	**0.012**
H. indica	**100**		0.008	0.008	0.002	0.008	0.011	0.012	0.010	0.011	0.011	0.296	0.012
H. amazonensis	**96**	96		0.003	0.008	0.003	0.011	0.011	0.010	0.011	0.010	0.288	0.012
H. Mexicana	**96**	96	99		0.008	0.003	0.012	0.012	0.010	0.011	0.011	0.287	0.012
H. noenieputensis	**100**	100	96	96		0.008	0.011	0.012	0.010	0.011	0.011	0.295	0.012
H. floridensis	**96**	96	99	99	96		0.012	0.012	0.010	0.011	0.010	0.287	0.012
H. safricana	**94**	94	93	93	94	93		0.004	0.009	0.004	0.009	0.272	0.006
H. marelatus	**93**	93	93	93	93	93	99		0.009	0.004	0.009	0.273	0.006
H. bacteriophora	**93**	94	94	94	94	94	95	95		0.008	0.003	0.303	0.010
H. atacamensis	**93**	93	94	94	93	93	99	99	96		0.008	0.272	0.006
H. Georgiana	**93**	93	94	94	93	94	95	95	99	96		0.305	0.010
H. zealandica	**49**	50	48	48	48	50	41	42	53	42	55		0.273
H. megidis	**92**	92	93	25	92	92	98	98	94	98	94	42	

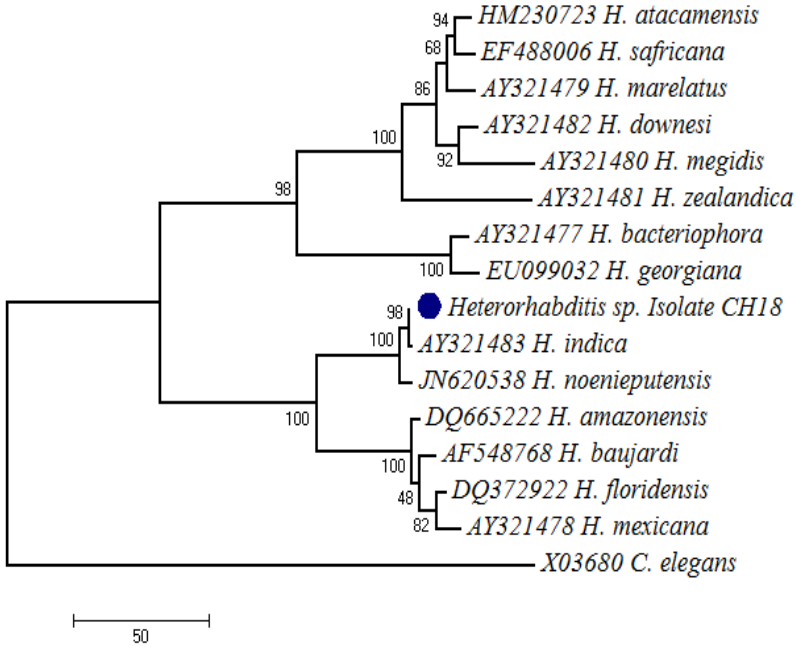

Fig. 1. Phylogenetic analyses of ITS region of isolate CH_{18} and other species of *Heterorhabditis*. The dendrogram was constructed by Maximum Parsimony method. Bootstrap values are included.

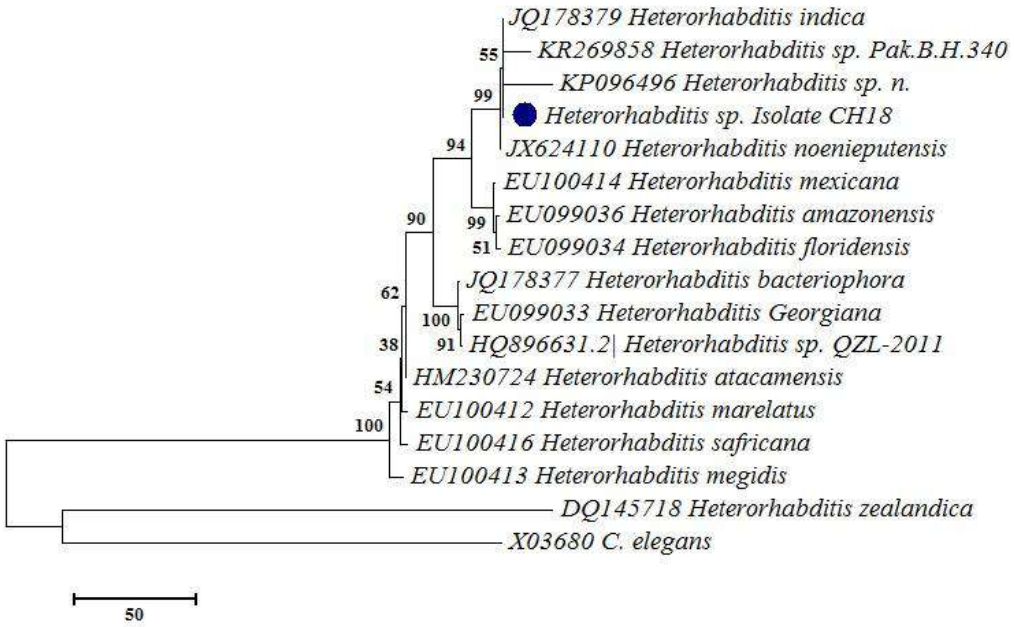

Fig. 2. Phylogenetic relationships based on Maximum Parsimony between 17 *Heterorhabditis* species and isolate CH_{18} with bootstrap analysis of D2D3 regions.

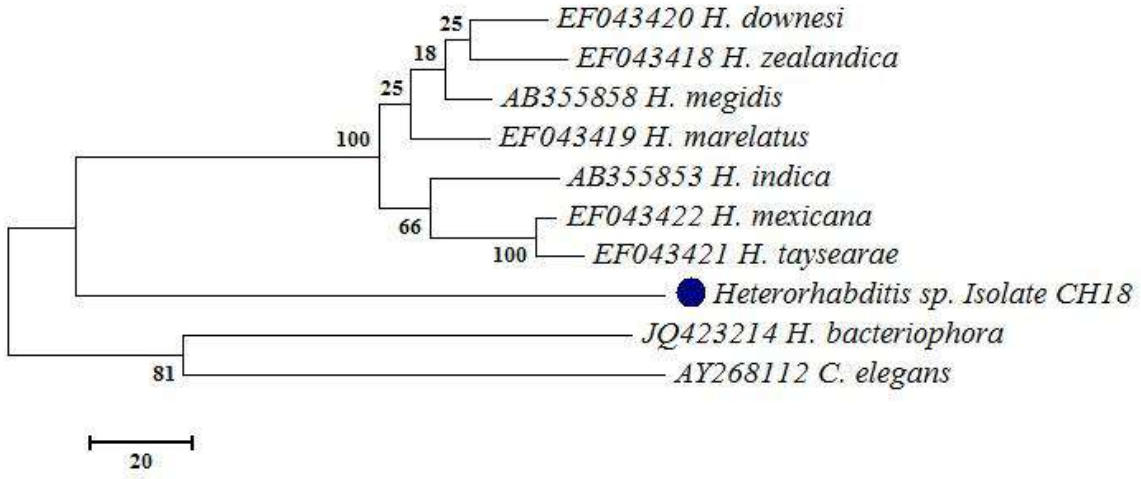

Fig. 3. Phylogenetic relationships based on Maximum Parsimony between 10 *Heterorhabditis* species and isolate CH_{18} with bootstrap analysis of COI regions.

Pathogenicity

The evaluation of the pathogenicity and reproductive potential of *H. indica* isolate CH_{18} was carried out in laboratory condition against *G. mellonella* as target host using different IJ concentration viz. 25, 50, 100, 200 IJ/larva. At 12 h post infection period, no mortality was recorded in all infected larva against the applied doses, while 10, 30, 30 and 90% mortality was observed in 25, 50, 100, 200 IJ/larva, respectively after 24 h post infection period (Fig. 4). At 36 h post infection period 100% mortality was noticed in 200 IJ/larva dose followed by 70, 60, and 40% respectively in 100, 50 and 25 IJ/larva. At 48 h post infection period, 90% mortality was observed in 100 IJ/larva while 80% was shown in 25 and 50 IJ/larva. At 60 h post infection period, 100% was discerned in all the concentrations. However, the degree of susceptibility to nematode infection varied from different dose infection and also on the exposure time. No mortality was recorded in control group even after 60 h during the experiment. All the dead larvae were transferred on the modified white trap to confirm their death was due to EPN where the emergence of IJ from the cadaver was the sign of nematode infection. A positive relation was observed in between the doses of infective juvenile and larval mortality where increments in doses also lead to increment in mortality rate.

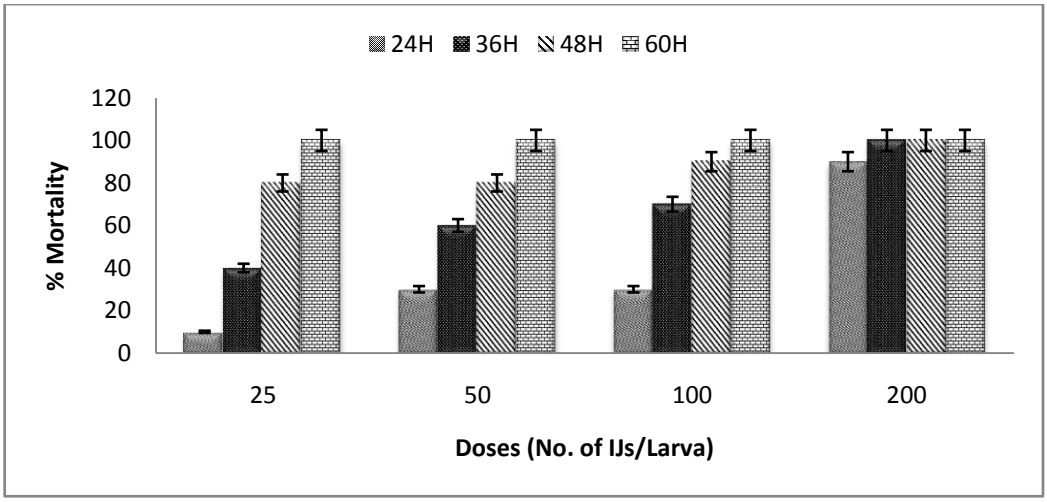

Fig. 4. Percentage mortality of *G. mellonella* larvae treated with *H. indica* isolate CH_{18}.

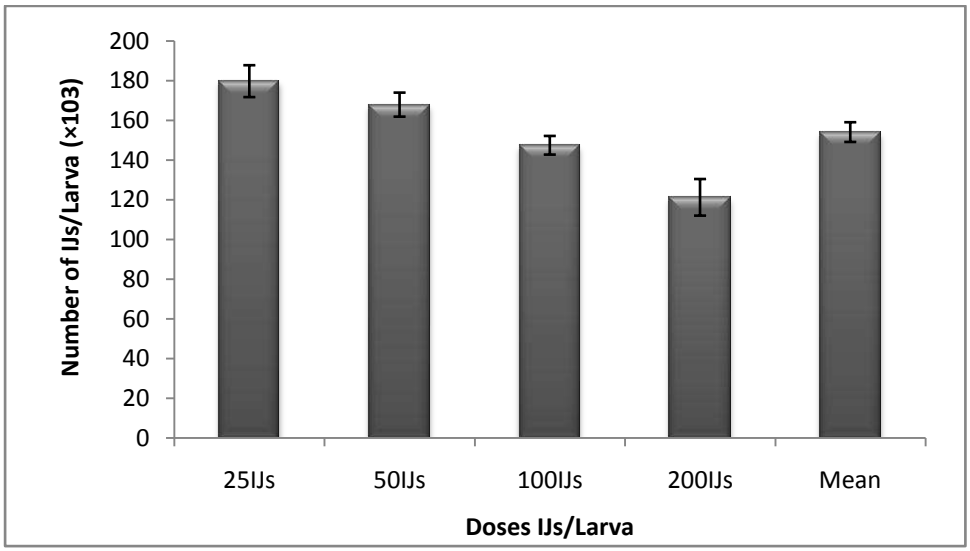

Fig. 5. Progeny productions *H. indica* isolate CH_{18} in *G. mellonella* larvae at different doses of IJ.

LC_{50} and LC_{90} value were also calculated to judge the pathogenicity of isolate by probit analysis at three different post infection periods viz. 24, 36 and 48 h and presented in IJ/Larva. Higher LC_{50} value was recorded at 24 h post infection period which was about 96 IJ/Larva followed by 37 IJ/Larva at 36 h post infection period where as lowest LC_{50} value was reported after 48 h which was only 8 IJ/Larva confirming the high pathogenicity of present isolate.

LT_{50} and LT_{90} values were also figured out at different concentrations viz. 25, 50, 100 and 200 IJ/Larva and for mean doses too through same analysis. The lowest time (LT_{50}) was taken with high dose which was only 19.3 h followed by 29.5, 31.4 and 36.8 h with 100, 50 and 25 IJ/Larva respectively. The mean LT_{50} calculated for all the doses applied was 25.7 hours.

The above findings showed similitude with that of the Sankar et al.[20] and with that of Hara and Kaya[21] which reported that nematodes are symbiotically associated with mutualistic bacteria, which allow them to kill their hosts quickly (36 to 48 h) and thus give them an advantage over other parasitoids and pathogens. Divya et al.[22] revealed highest death rate of the greater wax moth larvae at concentration levels of 300 IJ/larva within 24 h exposure of *H. indica* but the present *Heterorhabditis* isolate showed 100% mortality with different concentrations viz., 25, 50, 100 and 200 only after 36 h of exposure. This may be due to the fact the pathogenicity being complex process, depends upon many biotic and abiotic factors as was reported by Kaya and Gaugler[23].

Reproductive potential

In vivo progeny production of infective juveniles was also performed to assess the reproductive potential at different concentrations for same larvae used in pathogenicity bioassay. The IJ production was recorded as number of IJ/Larva. Descriptive analysis for IJ production revealed that the population count of IJ decreased with increase in increment of dose. The highest progeny production was recorded 1.79×105 (140800-217600) at 25 IJ/Larva infection whereas lowest was recorded at 200 IJ/Larva 1.21×105 (86400-172800). At 50 and 100 IJ/Larva, progeny count calculated were 1.68×10^5 (147200-211200) and 1.47×10^5 (121600-169600), respectively (Fig. 4).

The overall effectiveness of pest control program is directly related to the reproductive capability of EPN within the host and determines the time and dose applied in the field[24,25]. In conjugation with this, it is far too expensive to rear EPN by in vitro media as they required a lot of care, instruments and subsequent labor practices. Hence the species/strain of high yielding progeny is a requisite of the biological control program. Reproductive capability of different nematode species to produce infective juveniles was different[26,27]. Apart from this, the size of the nematode and their behaviour directly influenced the reproduction of nematode species within the body of

host[28] as reported by Bhatnagar et al.[29] in *H. bacteriophora*. Availability of food also influenced the number of eggs in the uterus of hermaphrodite by delaying the *endotokia matricida*[30].

Analysis of variance (ANOVA) indicated that there was negative correlation in between the production of IJ and doses applied where the significance value was zero. The positive relationship between the dose of IJ and host mortality found in the present examination has also been documented in several other investigations[31,32,33]. Gupta et al.[34] reported gradual increase in progeny production up to 160 IJ of *S. carpocapsae* per larva of *Pieris brassicae*, after which a sudden decrease from 2.20 ×105 larva to 1.70 ×105 was noticed. Small size of *H. indica* isolate CH_{18} and hermaphroditism could be the reason of the high yield of progeny as compared to others and supported by several findings[7,35,36]. However, in the present study, the highest reproduction was in low dose (25 IJ/Larva dose with 1.79 lacs IJ/Larva) as compared to other researchers with different/ same species in different/ same insect host.

ACKNOWLEDGEMENTS

Authors are grateful to the Department of Science and Technology (DST), New Delhi, India for providing the financial assistance through DST INSPIRE Fellowship/2014/76 and University Grants Commission (UGC), New Delhi, India for providing support via Rajeev Gandhi National Fellowship (F1-17.1/2015-16/RGNF-2015-17-SC-UTT-183610).

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