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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

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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

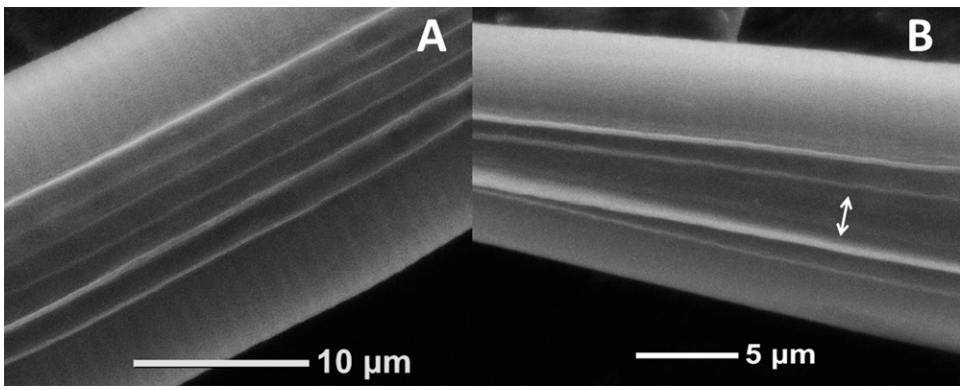

Fig. 1. Scanning electron microscope photography of infective juvenile of the Steinernema surkhetense. A. Lateral field at midbody level showing six ridges. B. Lateral field at the end of the body showing conspicuous sublateral ridges (arrows).

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.

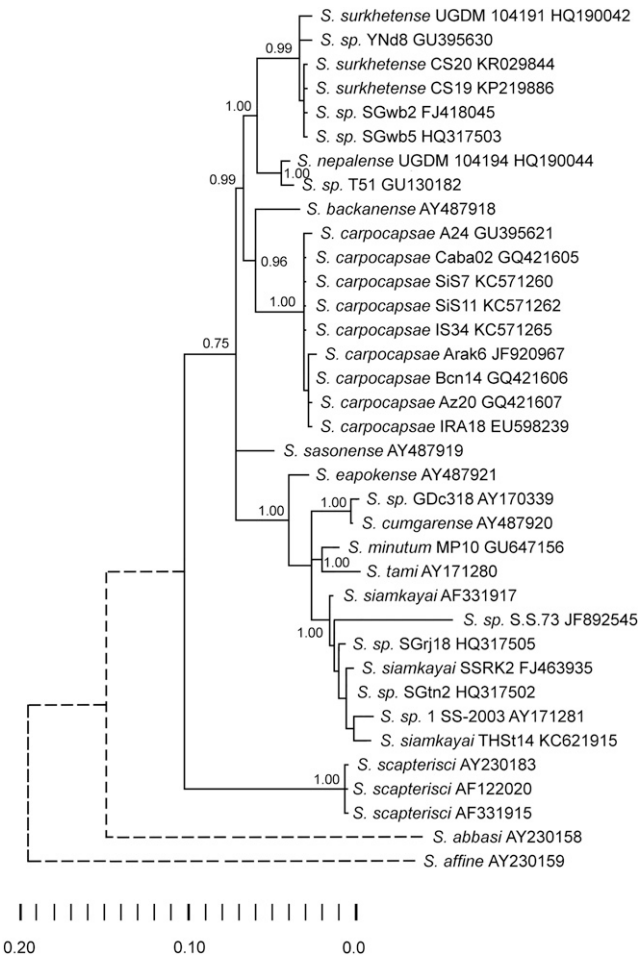

Fig. 2. Phylogenetic relationships in the "*carpocapsae*" group and other closely related species of *Steinernema* based on analysis of ITS rDNA regions. *Steinernema abbasi* and *Steinernema affine* were used as the outgroup taxon. The percentages of replicate trees in which the associated taxa clustered 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.

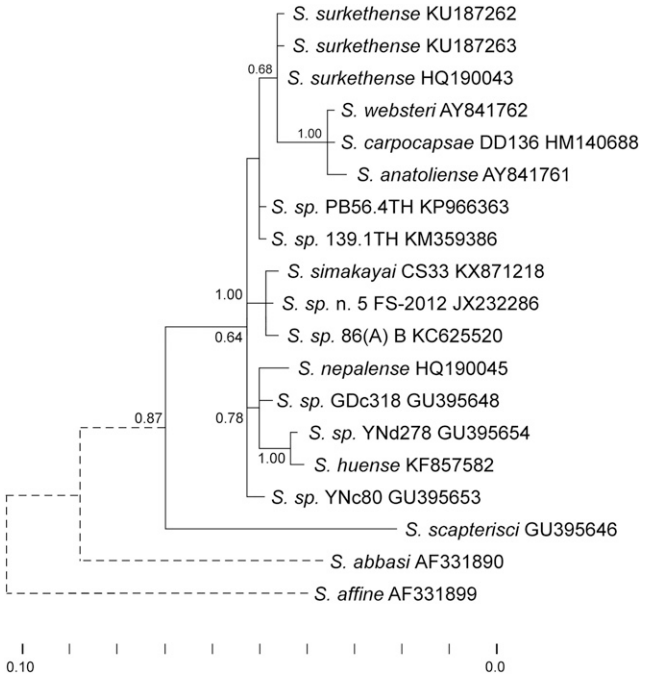

Fig. 3. Phylogenetic relationships in the "*carpocapsae*" group and other closely related species of *Steinernema* based on analysis of D2-D3 expansion segments of the 28S rDNA. *Steinernema abbasi* and *Steinernema affine* were used as the outgroup taxon. The percentages of replicate trees in which 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.

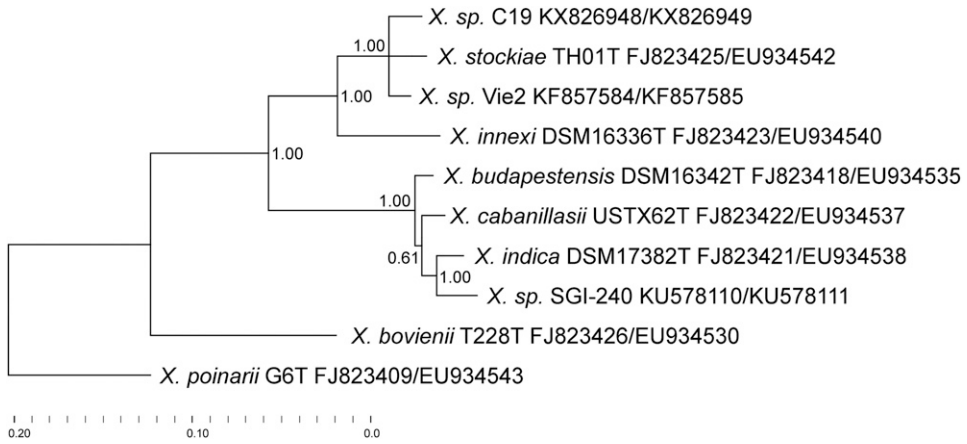

FIG. 4. Phylogenetic relationships of *Xenorhabdus* sp. isolated from *Steinernema surkhetense* (CS19) with other closely related species of *Xenorhabdus* based on analysis of recA and gyrB gene sequences. *Xenorhabdus bovieni* and *X. poinari* were used as the outgroup taxa. The percentages of replicate 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

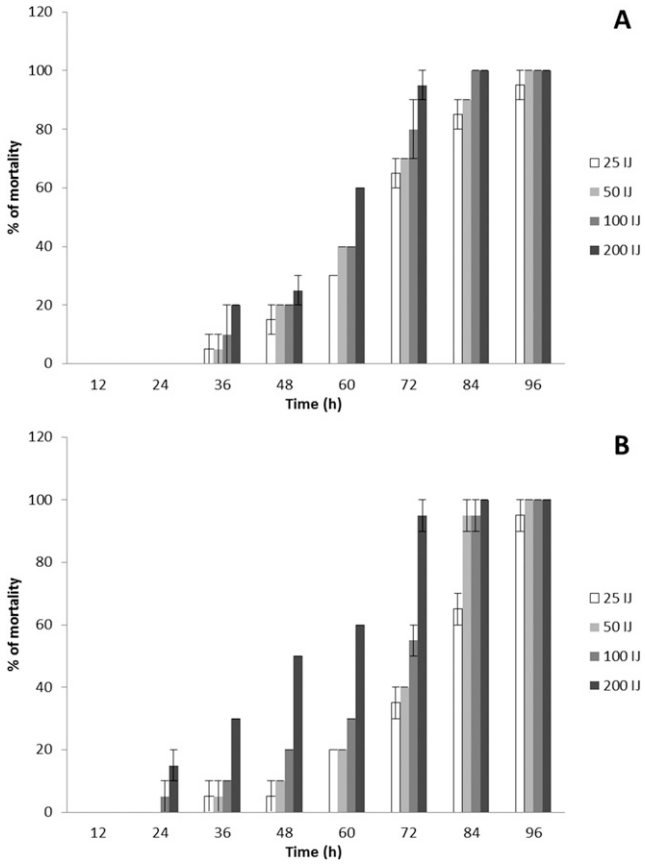

FIG. 5. Percentage of mortality of *Spodoptera littura* larvae with different doses of *Steinernema surkhetense*. A) Strain CS19. B. Strain CS20.

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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