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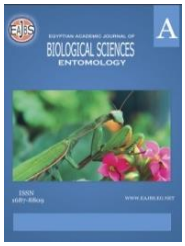

Characterisation of *Steinernema surkhetense* and Its Symbiont *Xenrorhabdus stockiae* and A Note on Its Geographical Distribution

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ABSTRACT

Two cultured populations of *Steinernema surkhetense* are described from India. Morphologically and morphometrically this material agrees with other species of the *Carpocapsae*-group (presence of short juveniles), especially with *S. surkhetense*. The morphology was somewhat similar to the original description with the distinguishing feature being the presence of anal swelling in second-generation female specimens but absent in the original specimens. The morphometric measurements were in close proximity to those in the original specimens and the PCA analysis of important morphometric characters demonstrated some variability within the test populations. Molecular studies based on 28S and ITS rDNA confirmed the Indian material is well conspecific Nepali populations of *S. surkhetense*, however, with Nepali populations, two base pair differences were observed at 376 and 713 positions only in ITS rDNA gene. We performed a molecular and biochemical characterization of the bacterial symbiont of *S. surkhetense* and the symbiont is closely related to *Xenorhabdus stockiae*, which is widespread among South Asian nematodes from the ‘‘*carpocapsae’’* group. Finally, we mapped its geographical distribution using a meta-analysis of the ITS GenBank records. The distribution of *S. surkhetense* based on a meta-analysis of the GenBank records showed its presence in the three Asian countries—India, China, and Nepal, with maximum records from the Indian subcontinent. This species is indigenous to the Indian subcontinent, thus could be tested and later used in the biocontrol of insect pests in India.

INTRODUCTION

Larval stages of many insects cause great losses to the agricultural crops. A large number of soil-dwelling insect pests are known that hamper the yield of food crops. Chemical pesticides are applied for their control which not only causes a detrimental effect on the environment, but also leads to resistance development in insect pests. Entomopathogenic nematodes (EPNs) the genera *Heterorhabditis* and *Steinernema* are exceptional parasites for soil-dwelling stages of many insect pests and are fast-acting homicide against target insect pests killing them within 24–48 hours as compared to the other biological control agents that take longer time to kill their host (Divya and Shankar, 2009). Because of the broad range of target hosts from the class Insecta, their application as biological control of crops and other plants against insect pests is so far very well known (Kaya and Gaugler, 1993).

The application of EPNs in biological control was traditionally engaged in controlling soil pests until some years ago (Ishibashi and Choi, 1991). This may be because the infective juvenile (IJ) is the only free-living stage of these nematodes found outside the insect body, which have the ability to seek and enter in their insect hosts in the soil and persist in the absence of food for longer durations. From the last two decades, researches also indicated their potential against foliar pests, but under distinct conditions (Arthurs et al., 2004). They are used commercially in European countries to control insect pests. These nematodes have a symbiotic association with bacteria of the family Enterobacteriaceae (*Photorhabdus* in *Heterorhabditis* and *Xenorhabdus* in *Steinernema*) which are the major food source of these nematodes. The mutualistic association of EPNs with specific bacteria also accelerates the rapid production of nematodes and successful pathogenicity (Ehlers *et al.*, 1988; Akhurst and Boemare 1990). Safety measure to the flora and fauna, effective insecticidal property, wide host range, mass production, ecofriendly nature, and rapid reproductive capacity added advantages for this economically important nemic fauna for the commercialization at large scale (Kaya and Gaugler 1993; Denno *et al*., 2008).

To date, a total of 98 valid species of *Steinernema* and 16 species of *Heterorhabditis* have been described throughout the globe (Bhat *et al*., 2020, Malan *et al*., 2020). So far, 17 species have been reported from India of which 14 species belong to genus *Steinernema* and 3 species to genus *Heterorhabditis* (Bhat *et al*., 2019). The objective of this study was to identify the nematode and their associated bacterial symbiont using biochemical and molecular analyses. Further, we studied the virulence of the nematodes against the *Galleria mellonella* and *Helicoverpa armigera*.

MATERIALS AND METHODS

Nematode Isolation Source, Culture, and Processing:

The soil samples were collected from agricultural and forested areas of districts Haridwar (29°91' N, 78°16' E and elevation of 359 m ASL) and Dehradun (30°51' N, 78°16' E and elevation of 372 m ASL), Uttarakhand, India. A total of 15 random soil samples were collected from each of these districts and were tested for the presence of nematodes. Nematode specimens were isolated from two soil samples by *Galleria* soil baiting technique (Bedding and Akhurst, 1975) and were designated as CS44 and CS45. The cadavers recovered from soil baits were transferred to white trap (White, 1927) after proper washing with double distilled water and sterilization with 1% NaOCl (Bhat *et al*. 2018, Suman *et al*., 2020). The nematodes that emerge in the white trap were harvested and stored in 250 ml tissue culture flasks in an incubator at 15°C as described by Bhat *et al*. (2019). For morphology and morphometry, 3rd stage juveniles (n = 200) were infected to last instar larvae of *Galleria mellonella* and larvae were killed within 24 to 36 hours at 27°C. The dead larvae were then dissected for obtaining first and second-generation adults, while 3rd stage juveniles were collected directly from the white trap which emerges into water within 10–15 days. These specimens were then killed with hot water, transferred to TAF (2 ml triethanolamine, 7 ml formaldehyde and 91 ml dH_2O) for fixation (Courtney *et al*., 1955). The fixed nematodes were dehydrated to glycerin by Seinhorst method (Seinhorst, 1959) and mounted in pure glycerine on permanent glass-slides with an extra amount of paraffin wax to prevent flattening of nematodes (Bhat *et al.*, 2017, Aasha *et al*., 2020).

Light Microscopy for Morphology and Morphometry:

Adult generations (1st and 2nd) and 3rd stage juvenile specimens were observed for morphological characters under a phase-contrast microscope (Nikon Eclipse 50i) and light microscope (Magnus MLX) while morphometric characters were measured with built-in software of the Nikon Eclipse 50i (Nikon DS–L1). Live and heat-killed adult specimens were

also observed for morphology. Demanian indices (de Man, 1880) and other morphometrical ratios were calculated. The terminology used for the morphology of stoma and spicules follows the proposals by De Ley *et al*., (1995) and Abolafia and Peña-Santiago (2017), respectively.

Statistical Analysis:

Principal component analysis (PCA) was done by XLSTAT (Addinsoft, 2007). Fourteen morphometric traits obtained from fixed nematodes, including body length, *a, b, c, c'*, excretory pore to anterior end, nerve ring to anterior end, pharynx length, tail length, spicule length, gubernaculum length, D%, E%, and V were used for PCA analysis of *Steinernema* species.

DNA Extraction, Amplification and Sequencing:

DNA was extracted from 3rd stage juveniles (n = 500) isolated from cadavers of *Galleria mellonella* infected with CS44 and CS45 using Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany). Juveniles were first washed separately with Ringer's solution followed by washing in PBS solution (Bhat *et al*., 2020). They were then transferred into a sterile Eppendorf tube (0.5 ml) and DNA was extracted following the manufacturer's instructions. The internal transcribed spacer (ITS) region was amplified using the primers 18S: 5′-TTG ATT ACG TCC CTG CCC TTT-3′ (forward) and 28S: 5′-TTT CAC TCG CCG TTA CTA AGG-3′ (reverse) (Vrain *et al.*, 1992). The flanking segment, D2-D3 regions of 28S rDNA was amplified using primers D2F: 5′–CCTTAGTAACGGCGAGTGAAA–3′ (forward) and 536: 5′–CAGCTATCCTGAGGAAAC–3′ (reverse) (Nadler *et al.*, 2006). The PCR master mix consisted of ddH2O 16.8 µl, 10x PCR buffer 2.5 µl, dNTP mix (10mM each) 0.5 µl, 1 µl of each forward and reverse primers, dream taq green DNA polymerase 0.2 µl and 3 µl of DNA extract. The PCR profiles used was:1 cycle of 94°C for 3 min followed by 40 cycles of 94°C for 30 sec, + 52°C for 30 sec for 28S rDNA or 55°C for 30 sec for ITS rDNA, + 72°C for 60 sec, 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 (Bhat *et al.*, 2018). 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: MH837094 and MH837096 for 28S of CS41 and CS44, respectively; MH822626 and MH822627 for ITS of CS41 and CS44, respectively.

Isolation and Molecular Characterization of Entomopathogenic Bacteria:

The endosymbiont bacterium associated with isolate CS41 was obtained from well sterilized IJs (n = 500) by crushing them in 1 ml PBS buffer (8 gm NaCl, 0.2 gm KCl, 1.15 gm Na_2HPO_4, 0.2 gm KH_2PO_4) and 100 µl of this was suspended 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 Luria broth (Akhurst, 1980) and cultivated on an orbital shaker (180 rpm) at 27°C.

Bacterial DNA was extracted from a 2-day old culture using Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. 16S rRNA was amplified using primers 10F: 59-AGTTTGATCATGGCTCAGATTG-39 (forward) and 1507R: 59-TACCTTGTTACGACTTCACCCCAG-39 (reverse) (Sandstrom *et al*., 2001). The PCR master mix consisted of nuclease-free H2O 16.8 µl, BSA 1 µl, 10x dream Taq buffer 2.5 µl, dNTPs mix (10mM) 0.5 µl, 0.75 µl of each forward and reverse primers, dream Taq DNA polymerase 0.2 µl and 2 µl of DNA extract. The PCR profile for 16S was used as 1 cycle at 94°C for 3 min followed by 33 cycles at 94°C for 60 s, 55°C for 60 s, 72°C for 2 min, and a final extension at 72°C for 10 min. All PCR products were sequenced and deposited in GenBank under the MK559716 accession number.

Phenotypic and Biochemical Characterization of Symbiotic Bacteria:

Phenotypic variations were observed in isolated 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 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 24–48h. 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 (Mumbai, 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 5ml 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 already present in GenBank using the Basic Local Alignment Search Tool (BLASTN) of the National Centre for Biotechnology Information (NCBI) (Altschul *et al*., 1990). An alignment of nematode sequences together with sequences of related steinernematid species (*carpocapsae* group) was produced for the LSU (D2-D3 rDNA) and ITS rDNA sequences using default Clustal W parameters in MEGA 7.0 (Kumar *et al*., 2016) and optimized manually in BioEdit (Hall, 1999). Pairwise distances were computed using MEGA 7.0 (Kumar et al., 2016). All characters were treated as equally weighted and gaps as missing data. *Steinernema abbasi* was used as the out-group taxa and to root the trees. The base substitution model was evaluated using jModeltest 0.1.1 (Posada, 2008). Phylogenetic trees were elaborated using the Bayesian inference method as implemented in the program MrBayes 3.2.7 (Ronquist *et al.*, 2012). 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 visualized using the Fig Tree program 1.4.3 (Rambaut, 2018).

An alignment of bacteria samples together with sequences of related bacterial species were produced for 16S genes using default Clustal W parameters in MEGA 7.0 (Kumar et al., 2016), optimized manually in BioEdit (Hall, 1999). The phylogenetic tree was obtained by the Minimum Evolution method (Rzhetsky and Nei 1992) in MEGA 7.0 (Kumar et al., 2016) and *Photorhabdus asymbiotica* were used as out-group taxa and to root the trees.

Geographic Distribution:

For studying geographical 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. surkhetense*, the BLAST search was performed with the sequence of the type isolates (MH837096) as a search query. The sequences that show 97% and higher similarity were downloaded and their identity was confirmed by phylogenetic analysis (Fig, supplementary material). The information about the site of isolation, if available, were obtained from the NCBI GenBank database, or from related publications.

RESULTS AND DISCUSSION

Based on the morphological, morphometrical and molecular studies the isolates CS41 and CS44 of *Steinernema* obtained during the present investigation were identified as *Steinernema surkhetense,* as they showed maximum resemblance with originally identified and described species *S. surkhetense* (Khatri-Chetri *et al*., 2011). The associated symbiont within this nematodes was characterized biochemically and molecularly and was found to be *Xenorhabdus stockaie*.

Morphology and Morphometry:

Morphologically the present *Steinernema* isolates CS41 and CS44 were found more or less similar to the topotype population of *S. surkhetense* (Khatri-Chetri *et al*., 2011). However, some dissimilarity was observed with the original description such as the presence of well-defined post anal swelling in first and second-generation females in present specimens vs. absent in second-generation females of Nepali populations. The spicule and gubernaculum were golden browns in colour in present strains vs. brown in the topotype population. The morphometrical data of present isolates were in close proximity with each other and with original description but little variations were also observed in body size, *a*, *b*, *c*, D%, and E% ratios. The size of IJs of present isolates CS41 and CS44 were slightly greater than the original description i.e. 450 (410-515), 436 (370-497) and 415 (393-450), respectively. The morphometric measurements and comparative morphometric of present populations with original descriptions are given in table 1-3.

Table 1. Morphometric measurements of different generations of *Steinernema surkhetense* isolate CS41 in µm (except n, ratio and percentage values) and are in the form of average ± SD (range)

Characters	First generation		Second generation		Infective juveniles
	Male	Female	Male	Female	
n	20	20	20	20	20
Body length (L)	1301 ± 293 (1026-1636)	4883 ± 1906 (2376-6985)	983 ± 85 (839-1124)	2147 ± 192 (1788-2607)	450 ± 24 (410-515)
a = L/MBD	11 ± 1.6 (9.4-13)	21 ± 1.5 (13-25)	13 ± 1.6 (11-16)	13 ± 1.3 (11-17)	18 ± 1.3 (17-22)
b = L/PS	9.2 ± 1.8 (7.3-11)	26 ± 5.4 (12-34)	7 ± 0.6 (6.3-8.3)	12 ± 1.1 (11-15)	4.2 ± 0.3 (3.7-4.8)
c = L/TL	38 ± 10 (24-47)	135 ± 57 (52-186)	34 ± 4.6 (26-43)	53 ± 7.3 (43-69)	11 ± 1.5 (9.2-15)
c' = Tail/ABW	0.8 ± 0.1 (0.6-0.9)	0.5 ± 0.2 (0.5-1.0)	0.9 ± 0.1 (0.7-1.2)	1 ± 0.1 (0.8-1.3)	3.6 ± 0.3 (2.9-4.0)
V = AV/L×100	-	53 ± 1.5 (52-55)	-	56 ± 1.7 (53-60)	-
Pharynx length (PS)	141 ± 5.9 (133-146)	193 ± 12 (181-210)	140 ± 8.3 (127-155)	180 ± 9.3 (165-195)	107 ± 5.4 (98-119)
Nerve ring to ant. End (NR)	99 ± 5.1 (98-138)	149 ± 13 (135-162)	115 ± 6.7 (102-126)	133 ± 11 (120-162)	80 ± 6.8 (71-99)
Excretory pore to ant. end (EP)	61 ± 3.1 (57-64)	79 ± 4.1 (74-84)	59 ± 5.7 (48-70)	69 ± 11 (54 - 94)	35 ± 2.3 (31-41)
Width at excretory pore (WEP)	46 ± 12 (30-59)	75 ± 10 (66-85)	28 ± 3 (24-35)	56 ± 7.6 (43-69)	14 ± 1.0 (11-16)
Bulb length (BL)	33 ± 4.2 (24-38)	44 ± 6 (38 - 52)	30 ± 4 (24 - 41)	40 ± (32 - 48)	20 ± 4.6 (13-29)
Bulb width (BW)	29 ± 1.7 (27- 30)	40 ± 4.1 (37- 45)	26 ± 2.9 (22- 33)	35 ± 3.4 (29 - 40)	9.3 ± 1.5 (7.8-12)
Tail length (TL)	35 ± 6.3 (30-44)	38 ± 4.1 (32-46)	29 ± 3.4 (21-36)	41 ±4.4 (31-50)	43 ± 4.6 (33-49)
Anal body width (ABW)	46 ± 9.9 (34-56)	56 ± 5.9 (49-73)	33 ± 3.8 (27-41)	40 ± 3.9 (35-49)	12 ± 1.3 (9.1-14)
Max. body diam. (MBD)	120 ±33 (77-158)	224 ±11 (181-279)	74 ±1.1 (53-92)	168 ± 19 (125-198)	25 ± 1.8 (21-29)
Spicule length (SL)	70 ± 6.8 (60-75)	-	63 ± 6.6 (53-73)	-	-
Gubernaculum length (GL)	48 ± 7 (40-55)	-	44 ± 9 (31-59)	-	-
D% = EP/PS×100	43 ± 2.1 (41-45)	41 ± 2.7 (37-43)	42 ± 4.4 (34-53)	38 ± 6.1 (31-54)	33 ± 1.9 (29-36)
E% = EP/TL×100	177 ± 28 (136-202)	212 ± 25 (184-248)	204 ± 31 (163-262)	168 ± 27 (132-226)	83 ± 13 (69-125)
Ant. end to vulva (AV)	-	2563 ± 954 (1311-3626)	-	1194 ± 94 (1027-1396)	-
Posterior end to vulva (PV)	-	2320 ± 951 (1065-3360)	-	954 ± 102 (1211-1211)	-
Width at vulva (WV)	-	211 ± 33 (168-251)	-	169 ± 19 (127-209)	-
Hyaline (H)	-	-	-	-	14 ± 2.9 (8.5-19)
H% = H/T×100	-	-	-	-	34 ± 6.7 (23-45)
Mucron (M)	3.6 ± 1.1 (3.1-5.2)	11 ± 0.9 (10-12)	3.3 ± 1.2 (2.4-5.3)	8.7 ± 1.3 (6.5-11)	-

Table 2. Morphometric measurements of different generations of *Steinernema surkhetense* isolate CS44 in µm (except n, ratio and percentage values) and are in the form of average ± SD (range)

Characters	First generation		Second Generation		
	Male	Female	Male	Female	Infective juveniles
n	20	20	20	20	20
Body length (L)	1351 ± 199 (998-1725)	3644 ± 1407 (2742 - 6236)	904 ± 125 (752-1051)	2244 ± 289 (1984-2588)	436 ± 32(370-497)
a = L/MBD	11 ± 1.4 (9-14)	19 ± 4.6(10-27)	14 ± 1.6 (12-16)	13 ± 0.7 (12-13)	18 ± 1.9 (14-22)
b = L/PS	10 ± 0.9 (7.8-11)	21 ± 6.7 (11-37)	7 ± 0.6 (6.2-8.6)	12 ± 1.2 (11-14)	5.4 ± 0.4 (4.7-6.2)
c = L/TL	38 ± 8.3 (26-53)	102 ± 35 (54-171)	33 ± 4.9 (26-38)	55 ± 10 (47-71)	11 ± 1 (9.2-13)
c' = Tail/ABW	0.8± 0.1 (0.6-1.1)	0.7 ± 0.1 (0.5-0.9)	0.9 ± 0.2 (0.7-1.2)	1 ± 0.1 (0.8-1.1)	3.8 ± 0.5 (3.0-4.7)
V = AV/L×100	-	54 ± 1.8 (51-58)	-	56 ± 3.3 (53 - 60)	-
Pharynx length (PS)	141 ±10 (127-169)	175 ± 18 (144 -203)	139 ± 12 (121-149)	180 ± 12 (167-195)	81 ± 4 (75-88)
Nerve ring to ant. End (NR)	106 ± 7.2 (97-123)	131± 14 (104-160)	103 ± 9 (92 - 111)	141 ± 17 (120-162)	64± 3.1 (58-70)
Excretory pore to ant. end (EP)	63 ± 7.4 (52-80)	72 ± 10(56-92)	55 ± 5.1 (49-61)	66 ± 13 (55-84)	53 ± 2.8 (48-58)
Width at excretory pore (WEP)	49 ± 8.4 (36 - 69)	68 ± 11 (49-86)	29 ± 3.5 (26- 33)	57 ± 8.7 (47-66)	15 ± 1.3 (13-17)
Bulb length (BL)	34 ± 8.2 (23 - 63)	42 ± 4.5(33-49)	29 ± 2.2 (26- 32)	40 ± 3.9 (36-45)	13 ± 1.7 (10-16)
Bulb width (BW)	27 ± 2.9 (20-32)	36 ± 6.4 (26-57)	25 ± 2.3 (22-28)	36 ± 4 (32-40)	10 ± 0.9 (8.2-12)
Tail length (TL)	38 ± 7.1 (30-46)	36 ± 7.6 (24-63)	27 ± 4.4 (22-33)	41 ± 4.4 (37-47)	39± 2.7 (35 - 44)
Anal body width (ABW)	48 ± 8.8 (37-66)	53 ±12 (37-79)	46 ± 9.9 (34-56)	42 ± 5.2 (37-49)	10 ± 1.2 (8.3 - 12)
Max. body diam. (MBD)	122 ± 23 (90-171)	188 ± 44 (112-249)	64 ± 15 (53-85)	178 ±16 (161-192)	25 ± 2.7 (20-33)
Spicule length (SL)	73 ± 4.1 (65-84)	-	66 ± 7.0 (60-73)	-	-
Gubernaculum length (GL)	54 ± 6.5 (40-68)	-	41 ± 6.2 (33-46)	-	-
D% = EP/PS×100	45 ± 3.4 (40-52)	42 ± 5.9 (30 - 58)	40 ± 2.5 (36-42)	36 ± 4.9 (32-43)	65 ± 4.6 (57-73)
E% = EP/TL×100	163 ± 37 (82-235)	203 ± 31 (147 - 272)	205 ± 27 (165-221)	159 ± 25 (133 - 181)	134 ± 11 (113-152)
Ant. end to vulva (AV)	-	1956 ± 745 (923-3390)	-	1263 ± 227 (1064-1563)	-
Posterior end to vulva (PV)	-	1678 ± 711 (679-2845)	-	981 ± 85 (870-1065)	-
Width at vulva (WV)	-	186 ± 40 (115-251)	-	174 ± 13 (161-192)	-
Hyaline (H)	-	-	-	-	15 ± 2 (11-19)
H% = H/T×100	-	-	-	-	38 ± 5.6 (30-48)
Mucron (M)	3.5 ± 1.1 (2.1-5.7	10 ± 2.4 (5.1-14)	4.0 ± 1.2 (2.4-5.2)	10 ± 1.0 (8.5-11)	-

Table 3. Comparative morphometrics of all generations of CS41 and CS44 with *Steinernema surkhetense*. All measurements are in µm (except ratio and percentage) and in the form of mean (range

Species	L	MBD	EP	NR	PS	Tail	a	b	c	D%
IJs										
CS41	450 (410-515)	25 (21-29)	35 (31-41)	80 (71-99)	107 (98-119)	43 (33-49)	18 (17-22)	4.2 (3.7-4.8)	11 (9-15)	33 (29-36)
CS44	436 (370-497)	25 (20-33)	35 (32-39)	64 (58-70)	81 (75-88)	39 (35-44)	18 (14-22)	5.4 (4.7-6.2)	11 (9-13)	34 (30-38)
S. surkhetense	415 (393-450)	21 (18-25)	32 (28-34)	63 (57-70)	92 (84-101)	45 (38-53)	19 (16-24)	5.5 (4.3-4.8)	9.3 (8.3-10)	35 (31-40)
Species	**EP**	**NR**	**PS**	**Tail**	**a**	**B**	**c**	**V%**	**D%**	**E%**
Female I										
CS41	79 (74-84)	149 (135-162)	193 (181-210)	38 (32-46)	21 (13-25)	26 (12-34)	135 (52-186)	53 (52-55)	41 (37-43)	212 (184-248)
CS44	72 (56-92)	131 (104-160)	175 (144-203)	36 (24-63)	19 (10-27)	21 (11-27)	102 (54-172)	54 (51-58)	42 (30-58)	203 (147-272)
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)	392 (156-453)
Female II										
CS41	69 (53-84)	133 (120-162)	180 (165-195)	41 (31-50)	13 (11-17)	12 (11-15)	53 (43-69)	56 (53-60)	38 (31-54)	168 (132-226)
CS44	66 (53-84)	141 (120-162)	157 (133-178)	49 (43-57)	13 (12-14)	12 (11-14)	55 (47-71)	56 (53-60)	36 (32-43)	159 (133-181)
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**	**PS**	**Tail**	**SL**	**GL**	**a**	**b**	**c**	**D%**
Male I										
CS41	61 (57-64)	99 (98-138)	141 (133-146)	35 (30-44)	70 (60-75)	48 (40-55)	11 (9.4-13)	9.2 (7.3-11)	38 (24-47)	43 (41-45)
CS44	63 (52-80)	106 (97-145)	141 (127-169)	38 (30-46)	73 (65-84)	54 (40-68)	11 (9-14)	10 (7.8-11)	38 (26-53)	45 (40-52)
S. surkhetense	55 (43-78)	92 (59-140)	115 (86-146)	19 (16-23)	70 (58-78)	52 (42-63)	12 (10-14)	11 (8-15)	65 (43-85)	48 (37-64)
Male II										
CS41	59 (48-70)	115 (102-126)	140 (127-155)	29 (21-36)	63 (53-73)	44 (31–59)	13 (11-16)	7.6 (6.3-8.3)	34 (26-43)	42 (34–53)
CS44	55 (49–61)	103 (92-106)	139 (121-149)	27 (22-33)	66 (60-73)	41 (33-46)	14 (12-16)	7.5 (6.2-8.6)	33 (26-38)	40 (36-42)
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)

PCA Analysis:

Twelve Eigenvalues were noted for ♀, ♂ and IJs of *S. surkhetense* populations for the measured morphometric parameters. High variability was observed for the components (F1

and F2) compared to the other components. The cumulative variability for F1 and F2 for ♀, ♂ and IJ populations are given in table 4. The eigenvectors contribute to the coefficient of variables either negatively or positively. The majority of the eigenvectors contributed positively to F1 and F2 (Table 4). Both ♀ and ♂ *S. surkhetense* populations had D% and *c* contributing negatively to F1. However, pharynx length and tail length contributed negatively to F2 for both ♀ and♂ of *S. surkhetense* populations. The eigenvectors for IJs are presented in table 5.

Table 4. Eigen values for female, male and infective juvenile *S. surkhetense* nematode populations

Characters	1st generation ♀		1st generation ♂		Infective Juvenile	
	F1	F2	F1	F2	F1	F2
Eigenvalue	9.041	1.645	7.629	2.400	4.366	3.352
Variability (%)	75.343	13.711	63.577	20.002	39.690	30.472
Cumulative %	75.343	89.054	63.577	83.579	39.690	70.162

Table 5. Eigenvectors for 1st generation female, 1st generation male and infective juvenile *S. surkhetense* populations

Characters	1st generation ♀		Characters	1st generation ♂		Characters	Infective Juvenile	
	F1	F2		F1	F2		F1	F2
L	0.325	0.155	L	0.312	0.074	L	0.138	0.293
a	0.268	0.311	a	0.167	0.556	a	-0.249	-0.355
b	0.202	0.614	b	-0.315	0.033	b	0.221	-0.280
c	-0.233	0.544	c	-0.325	0.277	c	0.334	0.327
c'	0.103	-0.144	c'	0.289	0.280	c'	-0.150	-0.276
Excretory pore to ant. end	0.327	-0.078	Excretory pore to ant. end	0.348	0.124	Excretory pore to ant. end	0.472	-0.017
Nerve ring to ant. end	0.326	-0.074	Nerve ring to ant. end	0.338	0.089	Nerve ring to ant. end	-0.049	0.536
Pharynx length	0.327	-0.069	Pharynx length	0.358	-0.079	Pharynx length	-0.289	0.434
Tail length	0.329	-0.110	Tail length	0.317	-0.273	Tail length	-0.132	-0.174
D%	-0.326	0.080	D%	-0.259	0.349	D%	0.459	-0.134
E%	-0.306	0.239	Spicule length	0.088	-0.433	E%	0.446	-0.044
V%	-0.298	-0.307	Gubernaculum length	-0.218	-0.340			

Variables were either positively or negatively correlated in ♀, ♂ and IJs of *S. surkhetense* populations (Table 6). Highly correlated variables (positive) with F1 were total body length (r = 0.98), excretory pore (r = 0.98), nerve ring (r = 0.98), pharynx length (r = 0.98), tail length (r = 0.99) and *a* (r = 0.81) for ♀ populations. In case of ♂ populations, total body length (r = 0.86), excretory pore (r = 0.96), nerve ring (r = 0.93), pharynx length (r = 0.99), tail length (r = 0.99), and *c* ' (r = 0.8) were highly correlated with F1. Negatively correlated variables with F1 were D% (r = − 0.98), E% (r = − 0.92) and V% (r = − 0.9) in case ♀ populations, and *b* (r = − 0.87) and *c* (r = − 0.9) in ♂ populations. Other sets of variates were highly correlated with F2 for both ♀ and ♂ *S. surkhetense* populations. Highly correlated variables (positive) with F2 were *b* (r = 0.79) and *a* (r = 0.86) for ♀ and ♂ populations (Table 6). For IJ *S. surkhetense* populations, highly correlated variables (positive) with F1 were total body length (r = 0.99), D% (r = 0.96) and E% (r = 0.93) and with F2, nerve ring (r = 0.98) and pharynx length (r = 0.80). Negatively correlated variables with F1 and F2 are given in table 6. Pearson Correlation among variables for ♀, ♂ and IJs of *S. surkhetense* nematode populations are given in table 7.

Table 6. Correlations between variables and components for 1st generation female, 1st generation male and infective juvenile *Steinernema surkhetense* populations

Characters	1st generation ♀		Characters	1st generation ♂		Characters	Infective Juvenile	
	F1	F2		F1	F2		F1	F2
L	0.976	0.198	L	0.862	0.115	L	0.288	0.536
a	0.806	0.399	a	0.460	0.862	a	-0.521	-0.650
b	0.607	0.787	b	-0.869	0.051	b	0.463	-0.512
c	-0.701	0.697	c	-0.898	0.429	c	0.698	0.599
c'	0.311	-0.185	c'	0.798	0.434	c'	-0.314	-0.506
Excretory pore to ant. end	0.983	-0.100	Excretory pore to ant. end	0.961	0.192	Excretory pore to ant. end	0.986	-0.030
Nerve ring to ant. end	0.980	-0.095	Nerve ring to ant. end	0.932	0.137	Nerve ring to ant. end	-0.102	0.981
Pharynx length	0.984	-0.088	Pharynx length	0.989	-0.122	Pharynx length	-0.604	0.795
Tail length	0.988	-0.141	Tail length	0.876	-0.423	Tail length	-0.276	-0.319
D%	-0.979	0.102	D%	-0.716	0.541	D%	0.959	-0.246
E%	-0.921	0.306	Spicule length	0.243	-0.671	E%	0.932	-0.081
V%	-0.897	-0.394	Gubernaculum length	-0.603	-0.526			

Table 7. Pearson correlation among variables for 1st generation female, 1st generation male and infective juvenile of *Steinernema surkhetense* nematode populations.

Variables ♀	L	a	b	c	c'	EP	NR	ES	TL	D%	E%	V%
L	1											
a	0.847	1										
b	0.743	0.846	1									
c	-0.540	-0.340	0.116	1								
c'	0.260	0.397	0.126	-0.342	1							
Excretory pore to ant. end	0.951	0.693	0.502	-0.744	0.251	1						
Nerve ring to ant. end	0.946	0.675	0.501	-0.734	0.217	0.998	1					
Pharynx length	0.954	0.696	0.512	-0.736	0.244	1.000	0.999	1				
Tail length	0.941	0.742	0.486	-0.793	0.323	0.989	0.982	0.988	1			
D%	-0.933	-0.690	-0.500	0.746	-0.202	-0.985	-0.991	-0.987	-0.976	1		
E%	-0.820	-0.683	-0.330	0.885	-0.322	-0.907	-0.906	-0.906	-0.946	0.937	1	
V%	-0.959	-0.795	-0.836	0.330	-0.107	-0.866	-0.874	-0.873	-0.827	0.868	0.685	1
Variables ♂	**L**	**a**	**b**	**c**	**c'**	**EP**	**NR**	**ES**	**TL**	**D%**	**SL**	**GL**
L	1											
a	0.498	1										
b	-0.506	-0.353	1									
c	-0.712	-0.028	0.821	1								
c'	0.706	0.719	-0.661	-0.520	1							
Excretory pore to ant. end	0.936	0.629	-0.743	-0.770	0.828	1						
Nerve ring to ant. end	0.941	0.594	-0.676	-0.753	0.795	0.986	1					
Pharynx length	0.860	0.343	-0.852	-0.945	0.715	0.932	0.904	1				
Tail length	0.812	0.022	-0.684	-0.970	0.501	0.790	0.792	0.932	1			
D%	-0.436	0.205	0.786	0.914	-0.313	-0.512	-0.452	-0.786	-0.836	1		
Spicule length	0.038	-0.343	-0.298	-0.453	-0.014	0.129	0.238	0.274	0.399	-0.333	1	
Gubernaculum length	-0.302	-0.714	0.789	0.346	-0.677	-0.577	-0.466	-0.522	-0.196	0.341	0.205	1
Variables IJs	**L**	**a**	**b**	**c**	**c'**	**EP**	**NR**	**ES**	**TL**	**D%**	**E%**	
L	1											
a	-0.263	1										
b	0.375	0.433	1									
c	0.562	-0.557	0.172	1								
c'	-0.473	0.082	-0.228	-0.837	1							
Excretory pore to ant. end	0.175	-0.583	0.359	0.619	-0.185	1						
Nerve ring to ant. end	0.410	-0.558	-0.580	0.560	-0.549	-0.133	1					
Pharynx length	0.228	-0.234	-0.722	0.035	-0.172	-0.610	0.839	1				
Tail length	-0.431	0.693	0.165	-0.055	-0.414	-0.339	-0.149	-0.120	1			
D%	0.057	-0.414	0.470	0.481	-0.089	0.976	-0.339	-0.767	-0.241	1		
E%	-0.055	-0.534	0.246	0.588	-0.209	0.962	-0.135	-0.616	-0.145	0.951	1	

Principal Component Analysis (PCA) was used in grouping and distinguishing among five populations of *S. surkhetense*. An accumulated variability of 83.58% was reached by the components F1 (63.58%) and F2 (20%) in the case of ♂ populations. Except for gubernaculum length, *b*, *c*, and D% value, all other important morphometric characters displayed high positive correlations among the *S. surkhetense* isolates and were responsible for the variability of the F1. All positively correlated variates had the most contribution to the variability and showed a high correlation with the F1 (Fig.1). In the case of females, and accumulated variability of 87.05% was reached by the components F1 (75.34%) and F2 (13.71%). Here except *c*, D%, E%, and V% values, all other variants were positively correlated with F1; excretory pore, nerve ring, tail length, total body length, and pharynx length had the most contribution to the variability and showed a high correlation with the F1 (Fig. 2). Further, in the case of IJs, an accumulated variability of 70.16% was reached by the components F1 (39.69%) and F2 (30.47%). Here half variates were positively correlated with

F1, while rests were negatively correlated (Fig. 3). These findings where in accordance with the results of Nyaku *et al*., (2016) who distinguished and grouped populations of reniform nematodes. PCA analysis was also proved useful in characterization entomopathogenic nematode populations (Adams *et al*., 1998; Dolinski *et al*., 2008). Bhat et al., (2019) used PCA analysis of male and IJ characters to distinguish within the populations of *S. hermaphroditum* and the same was found in the present study. The PCA analysis has previously been used for the Argentinian species of *Heterorhabditis* (Achinelly *et al*., 2017), which correctly categorized the species into different groups. The same result was obtained in our study. However, the variation within the isolates of *S. surkhetense* using PCA had not been established.

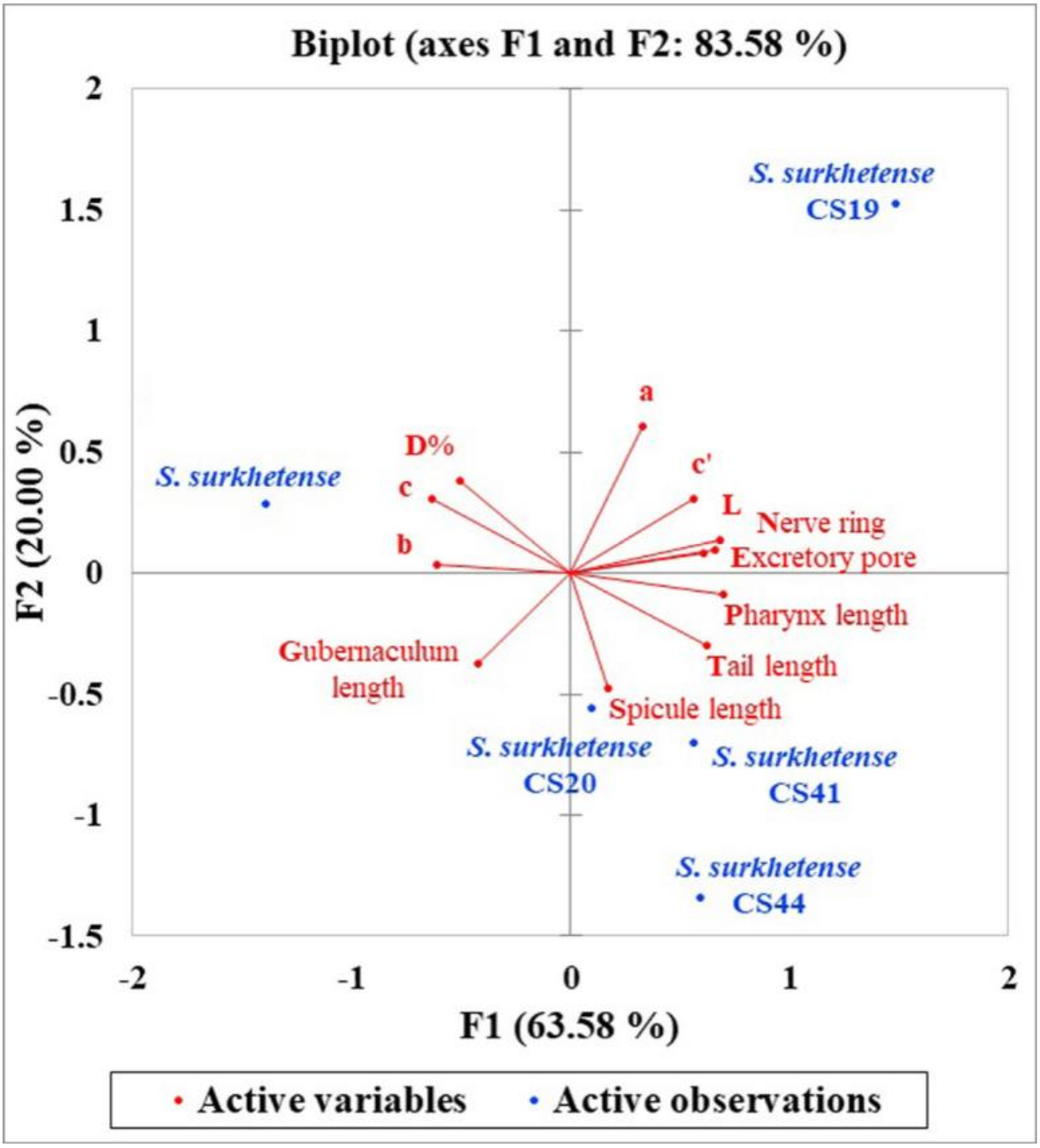

Figure 1. PCA of different populations of *Steinernema surkhetense* based on male characters.

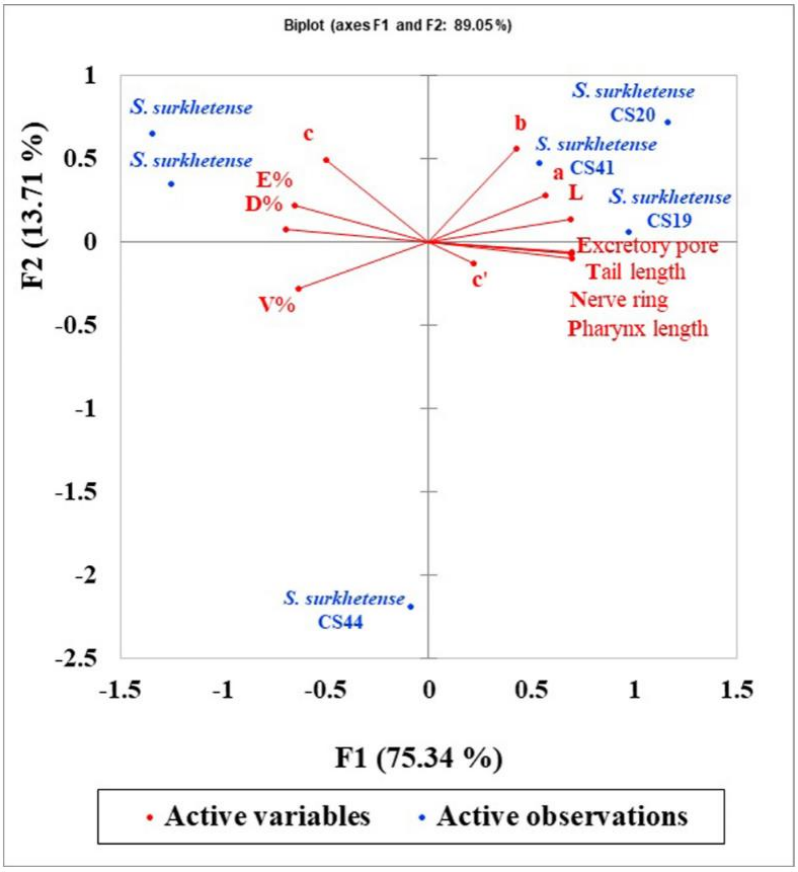

Figure 2. PCA of different populations of *Steinernema surkhetense* based on hermaphroditic female characters.

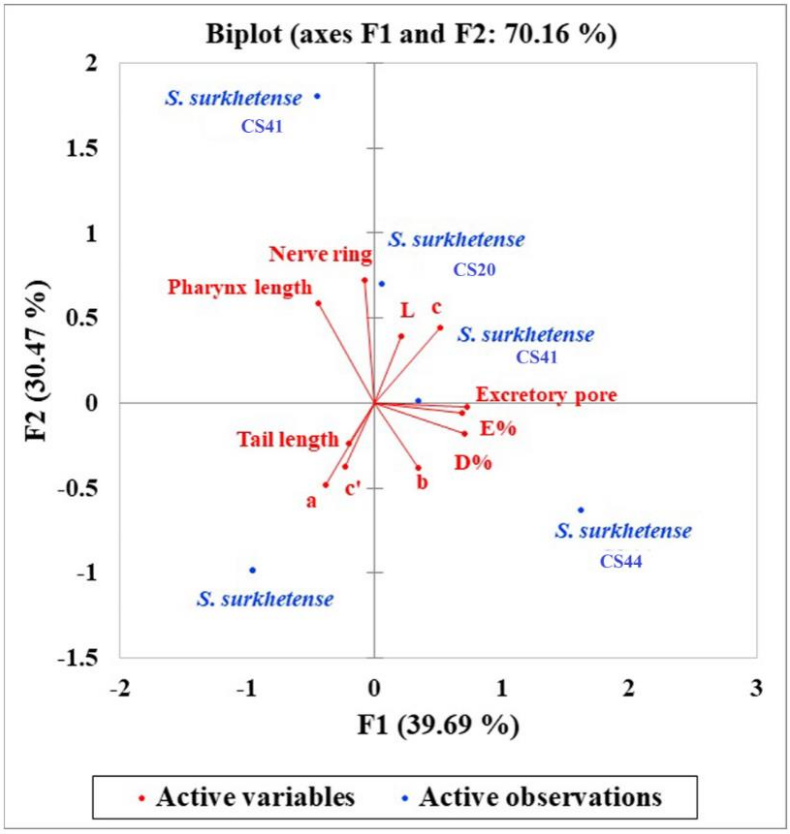

Figure 3. PCA of different populations of *Steinernema surkhetense* based on infective juvenile characters.

Molecular Characterization:

Present two populations CS41 and CS44 were characterized genetically by sequences of ITS (MH822626 and MH822627, respectively) and D2D3 (MH837094 and MH837096, respectively) sequences. The ITS region is 740 base pairs long (ITS1, 277 bp; 5.8S, 157 bp; ITS2, 306 bp). The sequence length and nucleotides composition of ITS gene in both Indian isolates showed 100% similarity with each other, however, with Nepali populations two base pair differences were observed at 376 and 713 positions. However, we sequenced the full-length D2D3 region of Indian populations CS41 and CS44 which is 805 bp long vs. 640 bp in originally described species. No nucleotide difference in D2D3 region was observed between present populations and with topotype populations. The BlastN analysis of ITS and D2D3rDNA of CS41 and CS44 showed maximum 100% similarity with Nepali isolates of *S. surkhetense* (Khatri-Chhetri *et al*., 2011).

Phylogenetic Relationships:

Phylogenetic scrutiny of the species of the ‘‘*carpocapsae’’* group based on ITS rDNA sequences showed clear monophyly of the group formed by the Indian populations CS41 and CS44 and Nepali population of *Steinernema surkhetense* and several other, probably conspecific isolates (Fig. 4). Sequences of populations of *S. surkhetense* formed a monophyletic group with *Steinernema nepalense* (Khatri-Chhetri *et al*., 2011). The ITS sequence of Indian isolates CS41 and CS4 are separated from those of other related species by 34–92 bp (Table 8).

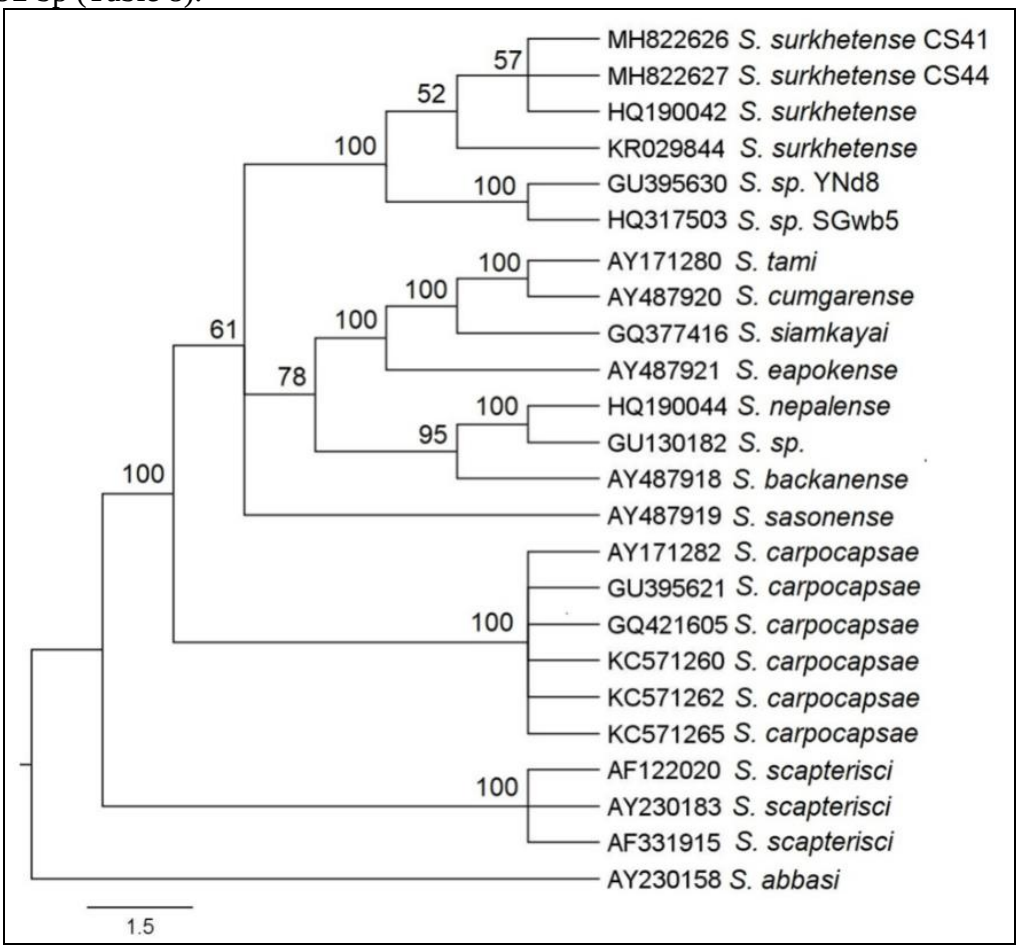

Figure 4. Bayesian Inference tree of *Steinernema* strains (CS41 and CS444)) with other known species of *carpocapsase* group based on ITS rDNA region. Bayesian posterior probabilities (%) are given for each clade. Scale bar shows the number of substitutions per site.

Table 8. Pairwise distances of the **ITS region** between *Steinernema* species from the '*carpocapsae'* group.

S. No.	ITS Regions	1	2	3	4	5	6	7	8	9	10	11	12	13
1	**MH822626 *S. surkhetense* CS41**		**0**	**2**	**34**	**42**	**44**	**51**	**52**	**57**	**68**	**63**	**66**	**92**
2	**MH822627 *S. surkhetense* CS44**	**100**		**2**	**34**	**42**	**44**	**51**	**52**	**57**	**68**	**63**	**66**	**92**
3	HQ190042 *S. surkhetense*	**100**	**100**		36	44	46	53	54	59	70	65	68	94
4	HQ190044 *S. nepalense*	**95**	**95**	95		37	35	42	41	50	57	50	59	88
5	AY487918 *S. backanense*	**94**	**94**	93	95		39	40	44	63	66	62	67	88
6	AY487919 *S. sasonense*	**94**	**94**	93	95	94		35	44	52	57	54	61	87
7	AY171282 *S. carpocapsae*	**92**	**92**	92	94	94	95		43	59	65	58	61	86
8	AY487921 *S. eapokense*	**92**	**92**	92	94	93	93	93		27	33	27	35	91
9	GU647156 *S. minutum*	**91**	**91**	91	92	90	92	91	96		17	19	27	95
10	AY171280 *S. tami*	**89**	**89**	89	91	90	91	90	95	98		29	34	101
11	GQ377416 *S. siamkayai*	**90**	**90**	90	92	90	92	91	96	97	96		28	94
12	AY487920 *S. cumgarense*	**90**	**90**	89	91	89	91	90	95	96	95	96		100
13	AF122020 *S. scapterisci*	**84**	**84**	84	85	85	85	86	84	83	82	84	82	

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

In the D2-D3 tree also, Indian populations CS41 and CS44 formed a monophyletic group with the already described populations of *Steinernema surkhetense* (Fig. 5). The D2D3 expansion fragments of the 28S rRNA gene of present *S. surkhetense* isolates were separated by 4–42 bp from other related species (Table 9). Thus, molecular studies supplemented by morphological and morphometrical data confirmed the identification of present Indian specimens as species of *S. surkhetense* according to the phylogenetic and evolutionary species concept (Adams, 1998) and hence considered the same.

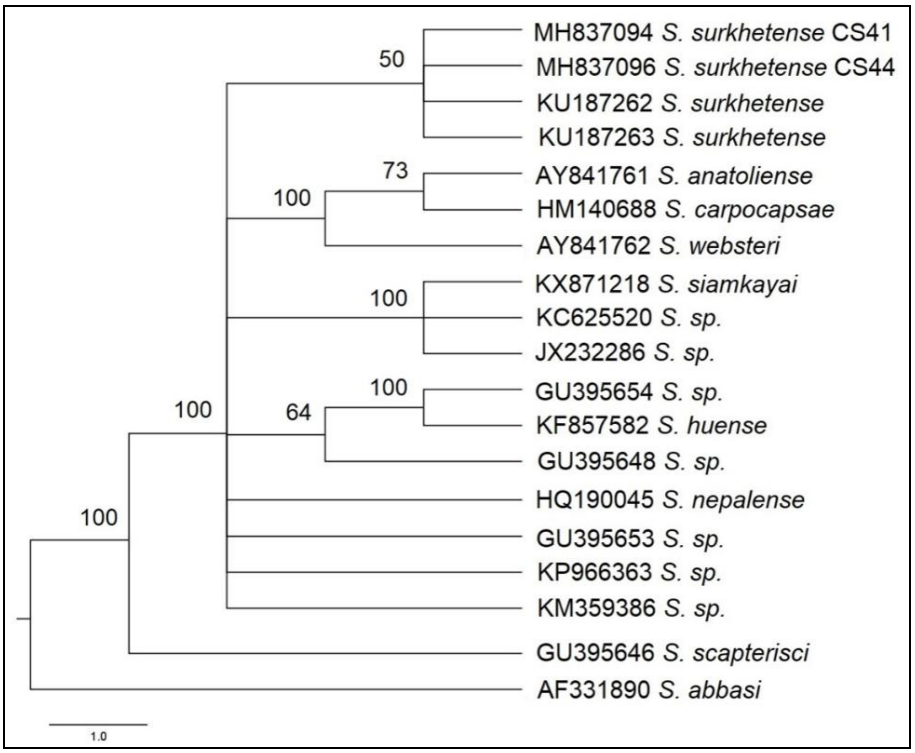

Figure 5. Inference tree of *Steinernema* strains (CS41 and CS444)) with other known species of *carpocapsase* group based on the analysis of flanking regions of D2–D3 regions of the 28S rDNA. Bayesian posterior probabilities (%) are given for each clade. Scale bar shows the number of substitutions per site.

Table 9. Pairwise distances of the D2D3 region between *Steinernema* species from the *carpocapsae'* group.

S. No.	D2D3 Regions	1	2	3	4	5	6	7	8	9
1	**MH837094 *S. surkhetense* CS41**		**0**	**0**	**4**	**6**	**8**	**8**	**9**	**42**
2	**MH837096 *S. surkhetense* CS41**	**100**		**0**	**4**	**6**	**8**	**8**	**9**	**42**
3	HQ190043 *S. surkhetense*	**100**	**100**		4	3	5	11	10	27
4	HQ190045 *S. nepalense*	**99**	**99**	99		7	9	15	12	29
5	KX871218 *S. siamkayai*	**99**	**99**	99	99		11	11	8	44
6	AY841762 *S. websteri*	**99**	**99**	99	98	99		10	16	45
7	HM140688 *S. carpocapsae*	**99**	**99**	98	97	99	99		22	46
8	KF857582 *S. huense*	**99**	**99**	98	98	99	98	97		45
9	GU395646 *S. scapterisci*	**95**	**95**	95	95	94	94	94	94	

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

Endosymbiont Bacteria:

Phenotypical, Biochemical and Molecular Diagnosis:

Bacteria isolated from two Indian populations of *S. surkhetense* were grown on NBTA media and they produced both phase I and II variants, which was confirmed by their dye-absorbance capacity. Gram staining differentiation showed that the bacterial isolates were Gram-negative rods. The culture that grown overnight on heart infusion broth, when transferred and incubated on the KB003 Hi25 Enterobacteriaceae Identification Kit of Hi-Media, revealed mostly negative reactions (Table 10). On nutrient agar, bacterial colonies formed a brownish pigmented center, appeared shiny and opaque, and were circular to irregular and convex. Phase I bacterial colonies adsorb neutral red dye and appeared as reddish colonies on MacConkey agar; however, on NBTA plates, they formed blue or blue-green colonies as they adsorb bromothymol blue. Esculin was weakly hydrolyzed. Acids were weakly assimilated (Table 10). Urease and Glucose were positively assimilated. There is no previous study of biochemical and phenotypic characterization of endosymbiont of *Steinernema surkhetense*. 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 the dye, and have sometimes lost their luminescent properties, as in the case of *Photorhabdus luminescens* (Boemare *et al*., 1993).

The molecular categorization of the symbiotic bacterium associated Indian population of *S. surkhetense* CS41 was performed. Based on the sequences of the 16S rRNA, the bacterium *Xenorhabdus* sp. C41 was found very close to *Xenorhabdus stockiae* Tailliez *et al*. (2006) and the same species was also reported by Bhat *et al*. (2017) from the same species. The phylogenetic tree based on the 16 sequences shows a highly supported group of the *Xenorhabdus* sp. C41 with *X. stockiae* strains (Fig. 6) and hence was considered as the same. This bacterial symbiont has been isolated from some closely related nematodes viz. *Steinernema siamkayai* (Tailliez *et al*., 2006), *Steinernema minutum* (Maneesakorn *et al*., 2010), and *Steinernema huense* (Phan *et al*., 2014). These discoveries, together with finding of *X. stockiae* in Indian *S. surkhetense* populations, suggest that this symbiont is widespread among ‘‘*carpocapsae''* group nematodes occurring in South Asia.

Table 10. Biochemical characterization of *Xenorhabdus* species associated with CS19

S. No.	Tests	Results	S. No.	Tests	Results
1.	O-Nitrophenyl-β-D-galactopyranoside (ONPG)	-	18.	Arabinose	-
2.	Lysine utilization	-	19.	Xylose	-
3.	Ornithine utilization	-	20.	Adonitol	-
4.	Urea hydrolysis	+	21.	Rhamnose	-
5.	Phenylalanine deaminase	-	22.	Cellobiose	-
6.	Nitrate reduction	-	23.	Melibiose	-
7.	H_2S production	-	24.	Saccharose	-
8.	Citrate utilization	-	25.	Raffinose	-
9.	Voges Proskauer's	-	26.	Trehalose	-
10.	Methyl red	-	27.	Glucose	+
11.	Indol	-	28.	Lactose	-
12.	Malonate utilization	-	29.	Oxidase	-
13.	Esculin hydrolysis	Weakly +	30.	Ribose	-
14.	Myo-inositol		31.	Bioluinescence	-
15.	Dye absorption BTB from NBTA	+	32.	Pigmentation	Brownish
16.	Neutral red MaConkey agar	Red	33.	Motility	+
17.	Tryptophan deaminase	-	34.		

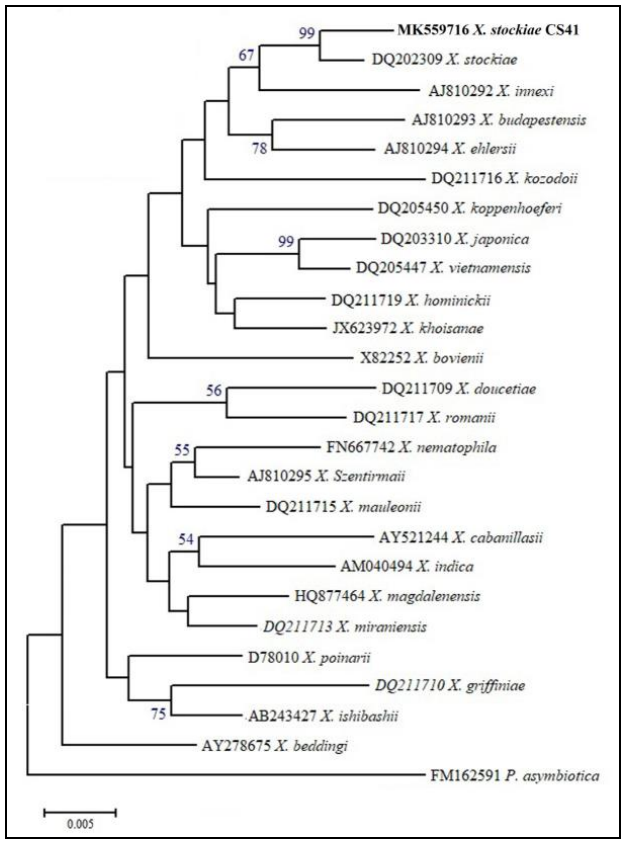

Figure 6. Phylogenetic relationships in *Photorhabdus* species based on analysis of 16S rRNA regions. *Photorhabdus asymbiotica* was used as the out-group taxon. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of the number of base differences per site.

Geographical Distribution:

The geographical distribution of *Steinernema surkhetense* was assessed using the records in GenBank. 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.

Table 11. GenBank records of *Steinernema surkhetense* and other species of "*carpocapsae*" group of *Steinernema* from different regions of the globe.

Species	Accession No.	Country	Submission year	Species	Accession No.	Country	Submission year
S. sukhetense	HQ190042	Nepal	2010	*S. carpocapsae*	AF121049	France	2003
	MH822627	Uttarakhand, India	2018		GQ421604	France	2012
	MH822626	Uttarakhand, India	2018		MH204152	India	2019
	MF919618	Uttarakhand, India	2017		GU395621	China	2012
	FJ418045	West Bengal, India	2008		GQ421607	Portugal	2012
	MG976890	Assam, India	2018		KC571262	Israel	2013
	MF919613	Punjab, India	2017		KC571265	Israel	2013
	KP219886	Uttar Pradesh, India	2014		GQ421606	Portugal	2012
	KR029844	Uttar Pradesh, India	2014		KC571260	Israel	2013
	HQ317503	West Bengal, India	2010		AY170334	China	2006
	MF618311	Mizoram, India	2017		EU077232	Iran	2007
	MF618310	Mizoram, India	2017		EU200353	Jordan	2008
	MF618309	Mizoram, India	2017		MK530239	South Korea	2019
	MF618308	Mizoram, India	2017		MG551679	Mexico	2017
	MF618312	Mizoram, India	2017		MG551678	Mexico	2017
	GU395630	Guangdong, China	2010		KJ950291	Russia	2015
S. kushidai	AB243440	Japan	2007		KY363935	Iran	2016
S. rarum	AY275273	Argentina	2003		EU122951	Iran	2007
	AY275272	Argentina	2003		GQ438788	India	2009
	KT378451	Argentina	2015		GU130182	Pakistan	2009
	KT378452	Argentina	2015		JF920967	Iran	2011
	KJ938570	Switzerland	2014		AY230164	USA	2004
	AY171300	Argentina	2002		HQ406729	India	2011
S. scapterisci	AF122020	USA	2003		EF219458	India	2009
	AY230183	UK	2004		MF919612	India	2017
	AF331915	USA	2001		LN624759	Italy	2014
	HM140695	Uruguay	2011		LN624758	Italy	2014
S. tami	KY807716	Philippines	2017		EU345421	Colombia	2009
	AY171280	Vietnam	2013		FJ860033	Iran	2009
S. backanense	AY487918	Vietnam	2004		KJ818295	Switzerland	2014
S. cumgarense	AY487920	Vietnam	2004		MH231235	Colombia	2018
	AY170339	China	2006		KC621916	India	2013
S. sasonense	AY487919	Vietnam	2004		MH231236	Colombia	2018
S. colombiense	EU345421	Colombia	2009		EU598239	Iran	2008
S. minutum	GU647156	Thailand	2011		HM140694	USA	2011
	GU395623	China	2012		AF331913	USA	2001
	MG742108	Thailand	2018		MF187619	Iran	2018
	GU130185	Pakistan	2009		MF187617	Iran	2018
	KX405166	Vietnam	2017		MF187616	Iran	2018
	KX405165	Vietnam	2017		AY171283	Russia	2013
	MK182971	India	2018		AY171282	Russia	2013
	KY807715	Philippines	2018		KX462931	Turkey	2016
	KY807711	Philippines	2018		KX462907	Turkey	2016
	KY807717	Philippines	2018		MF187618	Iran	2018
	KY807712	Philippines	2018		MK530238	South Korea	2019
S. nepalense	HQ190044	Nepal	2011		EU914854	Slovenia	2008
S. huense	KF857581	Vietnam	2015		AF036604	UK	1998
	GU395620	P.R. China	2012		KJ636405	Netherlands	2015
	KX405167	Vietnam	2017		FJ381666	La Selva	2008
	KX405166	Vietnam	2017		KJ636404	Netherlands	2015
	KX405165	Vietnam	2017		GU569043	Jordan	2010
S. eapokense	AY487921	Vietnam	2004				

Steinernema surkhetense was first described from Nepal; type sequence (HQ190042) is the only one in GenBank from Nepal deposited in 2010. Most of the records originate from India (n = 14). Based on the NCBI GenBank records, the species seems to be present in India, having been isolated from 6 states of the subcontinent, India. The records originate from North India (Uttar Pradesh, Uttarakhand, and Punjab), Northeast India (Mizoram, Assam) and East India (West Bengal). One strain labeled Steinernema sp. YNd8 (GU395630) was isolated from soil samples in Guangdong, the Republic of China, which further extends the known range of *S. surkhetense* to China (Table 11).

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 abundance of *S. surkhetense* is less in comparison with other species of the "*carpocapsae*" group (Table 11). Based on the records in NCBI GenBank database, *Steinernema surkhetense* seems the less frequently sequenced member of the "*carpocapsae*" group. The species with a worldwide distribution *Steinernema carpocapsae* has 45 records. Other closely related species have a much lower number of records. The explanation for

these differences is unclear, and further research in this field could bring interesting results. Till date, 17 species of "*carpocapsae*" group are known worldwide; of one species *Steinernema caudatum* lacks molecular data and the other one, *Steinernema apuliae* is described only on RFLP profiles and cytochrome c oxidase subunit I. The GenBank records of members of the "*carpocapsae*" group from different geographical areas are shown in table 11. Most species are endemic in certain areas only while others found in many areas. The explanation for these differences is unclear, and further research in this field could bring interesting results.

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List of abbreviations

ITS rDNA = internal transcribed spacer ribosomal deoxyribonucleic acid, PCA = principal component analysis, ASL = above sea level, TAF = triethanol amine formaldehyde, PCR = polymerase chain reaction, LSU = large subunit, BTB = bromothymol blue, TTC = tetrazolium chloride, MBD = maximum body diameter, EP = distance from the anterior end to excretory pore, NR = distance from the anterior end to nerve ring, PS = pharynx length, SL = spicule length, GL = gubernaculum length.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals.

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