*Pakistan Journal of Nematology* (2016) 34(1): 9-24

ISSN 0255-7576 (Print)
ISSN 2313-1942 (Online)
www.pjn.com.pk

http://dx.doi.org/10.18681/pjn.v34.i01.p09

# Taxometrical and numerical characterization of an isolate of *Steinernema abbasi* (Elawad *et al.*, 1997) with larger infective juveniles comprehensive from ITS1-5.8S-ITS2 region of rRNA

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**Abstract**

In present investigation *Steinernema* sp. isolate $CS_1$ was identified with the aid of numerical taxonomy as an additional tool. Presence of two horn-like structures on head region of 3rd stage infective juveniles (IJs) placed the nematode in bicornutum group and differentiated from other compared *Steinernema* spp. The morphological characters of 3rd stage infective juveniles and 1st generation males showed close resemblance with *S. abbasi*, *S. thermophilum* and *S. pakistanense* however, the body length was varied (583mm vs 541mm, 555 mm and 683 mm, respectively). The morphometrical characters and life history events placed the present specimen near to the *S. abbasi*. Results obtained through the phylogenetic utility of maximum parsimony, maximum likelihood and neighbour joining methods and comparative account on nucleotides analyses based on ITSs study confirmed the present specimens as isolates of *S. abbasi*. The nucleotide composition resemblance within 5.8S gene with other *Steinernema* species evident the less polymorphism and highly conserved nature of this region as compared to ITS1 and ITS2 and delimit the relationship of steinernematid nematodes.

**Keyword:** *Steinernema* spp., molecular tools, polythetic divisive classificatory system, ITS region, nucleotide composition.

**E**ntomopathogenic nematodes (EPN) belonging to the families Steinernematidae and Heterorhabditidae are the lethal parasite of insects and are in the keen interest of scientific community as biological control agent from past decade (Kaya & Gagular, 1993). This can be traced by the increment in number of research publications on this group of nematodes in recent years and a large number of laboratories have engaged world-wide in EPN research. Taxonomy of these nematodes is an indispensable as the product they involved in mass production, formulation and application in insect pest management. The application of EPN has enthused frequent inspections in an effort to find new indigenous isolates and possibly also new *Heterorhabditis* and *Steinernema* species (Hominick *et al*., 1996). Members of both the families showed close intimacy with each other in terms of life cycle morphology and pathogenicity but convergent evolution are not indicative towards the close phylogenetic relationship between these two (Sudhaus, 1993; Blaxter *et al*., 1998). The present scenario shows the richness of diversity of steinernematids throughout the world. Presently the family Steinernematidae includes two genera i.e., *Steinernema* with more than 100 species globally and *Neosteinernema* Nguyen & Smart, 1994 with only one species *N. longicurvicauda* Nguyen & Smart, 1994 (Adams & Nguyen, 2002). Diversity of EPN in India is not done exhaustively so far as only 16 species

isolated from different parts of the country. Although much of the work is concentrated towards the extensive surveys and pathogenicity trials, the lack of taxonomic expertize globally and close intimacy within the genus *Steinernema* are some critical factors behind this tricky. Traditional classificatory procedure looks for the most common character for differentiation within the specimen under consideration.

Traditional methods such as morphology and morphometric were always the baseline of the taxonomy of nematodes. But now days these become supplemented with the molecular approaches to concern the identification of putative new species (Hominick *et al.*, 1997). The aim of present study was to ensure more precise identification to isolated *Steinernema* sp. isolate $CS_1$ to clear the obscure in EPN taxonomy. Here is the polythetic divisive method of classification (Malhotra *et al*., 1981) successfully used for cestode taxonomy previously and was applied to distinguish the EPN species along with morphological taxometrical and molecular analyses which give a clue to resolve the uncertainities and showed their usefulness up to some extent.

## Materials and Methods

**Isolation of entomopathogenic nematodes:** The nematode was collected from the soil of District Saharanpur from the field of *Sorghum* popularly known as Jowar through soil baiting technique of Bedding & Akhurst (1975) using *Galleria mellonella* larvae as bait. The nematode was tagged as *Steinernema* sp. isolate $CS_1$ as per the author's laboratory nomenclature.

**Nematode culture:** Population of isolated EPNs were maintained *in vivo* on the last instar larvae of *G. mellonella* at a temperature of 27±2 °C for further processing. 20 larvae of *G. mellonella* were infected with 2000 infected juveniles (IJs) in a Petri plate lined by double layer of filter paper. The cadavers were transferred on White Trap (White, 1927) and IJs were isolated from white trap, disinfected with 0.1% sodium hypochlorite and stored into tissue culture flask at 15±1°C temperature in BOD for further processing.

**Recovery of adult stages:** For recovery of adults the cadaver of *G. mellonella* were dissected 2-3 days and 4-6 days after mortality for 1st and 2nd generations males and females, respectively in Ringer's solution (Woodring & Kaya, 1988). Freshly emerged IJs were used for the identification purpose.

**Processing of nematodes for microscopy:** 1st and 2nd generation males and females along with IJs of present nematode were killed by gentle heat and fixed in TAF (Courtney *et al*., 1955) and processed to glycerol by the method adopted from Sienhorst (1959). Slides for microscopic study were prepared on glass slide where specimens were mounted in glycerine covered with cover slip. All the stages were studied under 4x, 10x, 40x and 100x magnifications using light and phase contrast microscope. Measurements were taken using phase contrast microscope inbuilt with measurement software DL1.

**Morphology and taxometry:** For morphotaxometrical comparison, size of 3rd stage juveniles was taken as base and nine species of *Steinernema* viz., *S. anatoliense* (Hazir, Stock & Keskin, 2003), *S. carpocapsae* (Weiser, 1955), *S. kushidai* (Mamiya, 1988), *S. scapterisci* (Nguyen & Smart, 1992), *S. tami* (Luc, Nguyen, Reid & Spiridonov, 2000), *S. websteri* (Cutler & Stock, 2003), *S. abbasi* (Elawad, Ahmad & Reid, 1997), *S. pakistanense* (Shahina, Anis, Reid, Rowe & Maqbool, 2001) and *S. thermophilum* (Ganguly & Singh, 2000) were chosen for comparison. The data of 3rd stage juveniles and 1st generation males was extracted from the literature and used for the comparison.

**Genomic DNA isolation:** The genomic DNA was isolated from fresh IJs culture using the DNeasy Blood and Tissue Kit (Qiagen) as per the instruction given by the manufacturer. The isolated DNA was electrophoresed with

0.7% agarose gel in TAE buffer containing EtBr. The gel was visualized under UV light for the presence of DNA.

**PCR Analysis:** Internal Transcribed Spacer (ITS) regions of the genomic DNA were used as marker to distinguish the present specimen form compared species of *Steinernema*. The amplification of ITS regions was done using the primers as suggested by Joyce *et al.*, (1994) with modifications. The composition of 25 $\mu$L of PCR reaction volume were; Dream Taq Green master mix 2x (Thermo Scientific) 12.5 $\mu$L, forward primer 0.7 $\mu$L, reverse primer $\mu$L, template DNA 2 $\mu$L, mili Q water 9.1 $\mu$L. the PCR running protocol was set for 35 cycles and it was as initial temperature 94 °C for 5 minutes, denaturation at 94 °C for 30 seconds, annealing temperature 64 °C for 30 seconds, primer extension 72 °C for 30 seconds, final extension 72 °C for 8 minutes, storage temperature 4 °C for infinity. 3$\mu$L of amplified DNA along with 100 bp DNA ladder was loaded in the well of 1% agarose gel in TAE buffer containing EtBr as visualization dye.

**Molecular characterization:** The obtained sequence was submitted to GenBank (accession no. KP0369180).10 sequences of ITS regions of already described *Steinernema* species showing close similarity with the sequence of present specimen based on BLAST analysis included some of those were compared morphologically downloaded from NCBI. ITS region of *Caenorhabditis elegans* was used as an outgroup. The sequences were aligned using the default parameters (gap opening penalty 15, gap extension penalty 6.66) in Clustal W (Tamura *et al.*, 2004) programme in MEGA software version 6 (Tamura *et al.*, 2013). Phylogenetic trees were constructed by neighbour joining (Saitou & Nei, 1987), maximum likelihood (Tamura & Nei, 1993) and maximum parsimony (Nei & Kumar, 2000) methods. The pair wise distance between all the sequences was also calculated using the same software. Nucleotide compositions of the sequences were calculated through Bio Edit software.

**Polythetic divisive classificatory system:** Total body length (L), Greatest body width (GBW), Position of excretory pore from anterior end (EP), Nerve ring (NR), Oesophagus (ES), Tail length (Tail) Ratio a (L/GBW), b (L/ES), c (L/T), D% (EP/ES×100) and E% (EP/Tail×100) were statistically compared where the method of polythetic Divisive classificatory system (Malhotra *et al.*, 1981) was applied.

## Results

### Morphological characters of *Steinernema* sp. isolate $CS_1$ (Fig. 1-2; Table 1-3.)

**First generation males:** Body ventrally curved when hot killed. Cuticle smooth, lateral fields and phasmid not observed under light and phase contrast microscope. Stoma reduced, cheilorhabdions prominent, posterior part of the stoma funnel shaped, moderately cuticularized or not cuticularized. Pharynx muscular, divided into cylindrical procorpus with slightly swollen metacorpus and distinct isthmus surrounded by nerve ring, basal bulb prominent, length of basal bulb is slightly more than width. Excretory pore located on isthmus, anterior to nerve ring above the basal bulb. Distance from the anterior to excretory pore always more than body width at excretory pore but less than greatest body width. Width at excretory pore is about 59% of the oesophagus length. Oesophagus muscular. Cardia present and prominent. Genital system monorchic and clearly visible with reflexed testis makes 15-20% of total body. Spicules golden yellow in view, paired, heavy, curved with internal ribs.

Capitulum rounded. Spicule tip somewhat conoid, manubrium short with 9-15$\mu$m almost similar length and width, velum prominent extending for more than 2-3$^{rd}$ of the blade. The spicule at its posterior 3$^{rd}$ straight and a depressed on the ventral side before the spicule tip. Calomus (shaft) present and the blade or lamina moderately curved; rostrum usually present. Gubernaculum 70% of the spicule length, boat-shaped, tapering anteriorly, slightly swollen in the middle to a somewhat ventrally curved end. Corpus separated posteriorly while cuneus rod-like. Tail is 70% of its width, conoid, without bursa. Mucron not visible.

**Second generation males:** Morphologically similar to 1$^{st}$ generation but approximately 69% of its size. Size of excretory pore, oesophagus, nerve ring and tail are 82-86% of the 1$^{st}$ generation. Spicules and gubernaculum smaller and thinner in size possessing 86% and 66% size of 1$^{st}$ generation. Shape of the spicule and gubernaculum not different from 1$^{st}$ generation males, but slightly variable between individuals of same generation. Mucron like process observed rarely on tail.

**First generation females:** Similar to other species of *Steinernema*. Body spiral when heat killed. Giant females 40-50% larger to normal 1$^{st}$ generation females, however, similar in morphology. Cuticle smooth, lateral fields and phasmids inconspicuous. Labial region rounded with six labial palp in light and phase contrast microscopy. Excretory pore anterior to nerve ring, length slightly less than the width and 31% of oesophagus length in average. Pharynx muscular; procorpus, metacorpus and isthmus prominent. Basal bulb enlarged. Nerve ring usually surrounding anterior portion of basal bulb or posterior part of isthmus. Cardia present. Genital system amphidelphic usually reflexed and occupied with eggs, endotokiametricida common. Vulva in the form of transverse slit protruding from the body surface, double-flapped epiptygmata present. Vagina short and muscular. Vulval aperture located posterior to mid-body. Postanal swelling usually present. Tail conoid to dome shaped, shorter than anal body, mucronate process absent.

**Second generation females**: Body C shaped when heat killed, similar to 1$^{st}$ generation females in common features but smaller (about 29%) in size and diameter (about 39% at vulva). Tail with prominent swelling, longer than anal body diameter and 1$^{st}$ generation female's tail, tapering or pointed with prominent mucron.

**Infective juveniles:** Body shape ventrally curved or J shaped or straight when heat killed, slender, elongate, tapering at both ends, labial region continuous with body. Cuticle with fine striae. Labial region smooth, usually continuous. Head bearing two horn like structure, Oral apertures and anus closed. Excretory pore poorly developed located posterior to nerve ring, 8% of the total body and 36% longer to its width. Nerve ring distinct located above the middle of excretory pore and basal bulb. Pharynx long and narrow, distinctly narrower at the level of nerve ring, terminating in a dorsally displaced, valvate bulb. Nerve ring distinct, situated anterior to basal bulb. Cardia present. Tail 26% greater of its anal body width, conical in shape and terminally pointed.

**Molecular characterization and phylogenetic relationship:** The amplified region of the studied specimen (KP036918) yielded a 800 nucleotide long sequence containing 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, complete sequence and internal transcribed spacer 2 partial sequence. For ITS regions, maximum parsimony analysis showed that the alignment resulted in 1017 characters of which 185 are constant, 449 are parsimony uninformative and 383 are parsimony-informative.

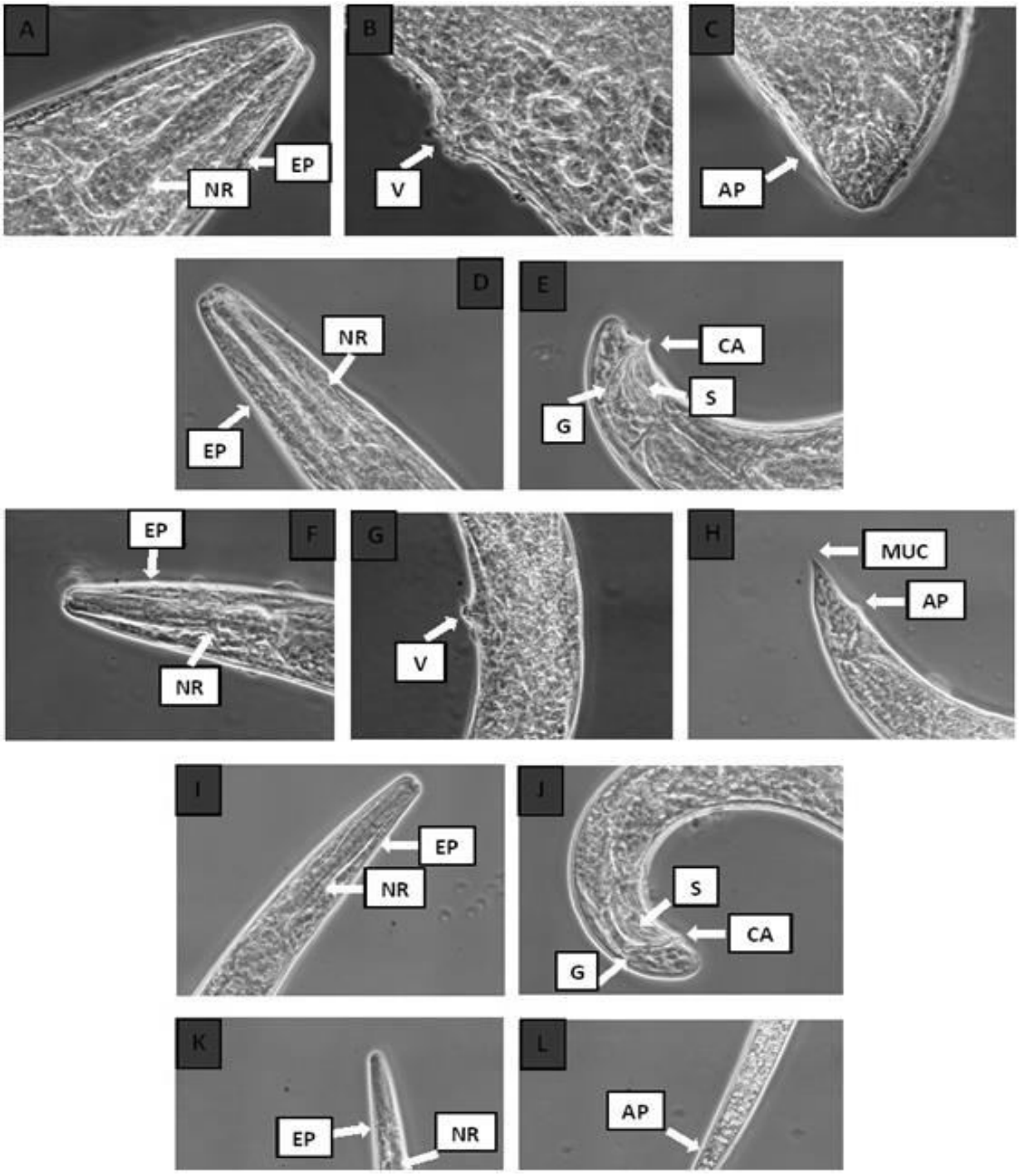


**Fig. 1 (A-L).** Microphotographs of *Steinernema* sp. isolate $CS_1$. A-C: First generation female, D-E: First generation male, F-H: Second generation female, I-J: Second generation male, K-L: Third stage juvenile. A, D, F, I, K: Anterior region, B, G: Middle region, C, E, H, J, L: Posterior region.

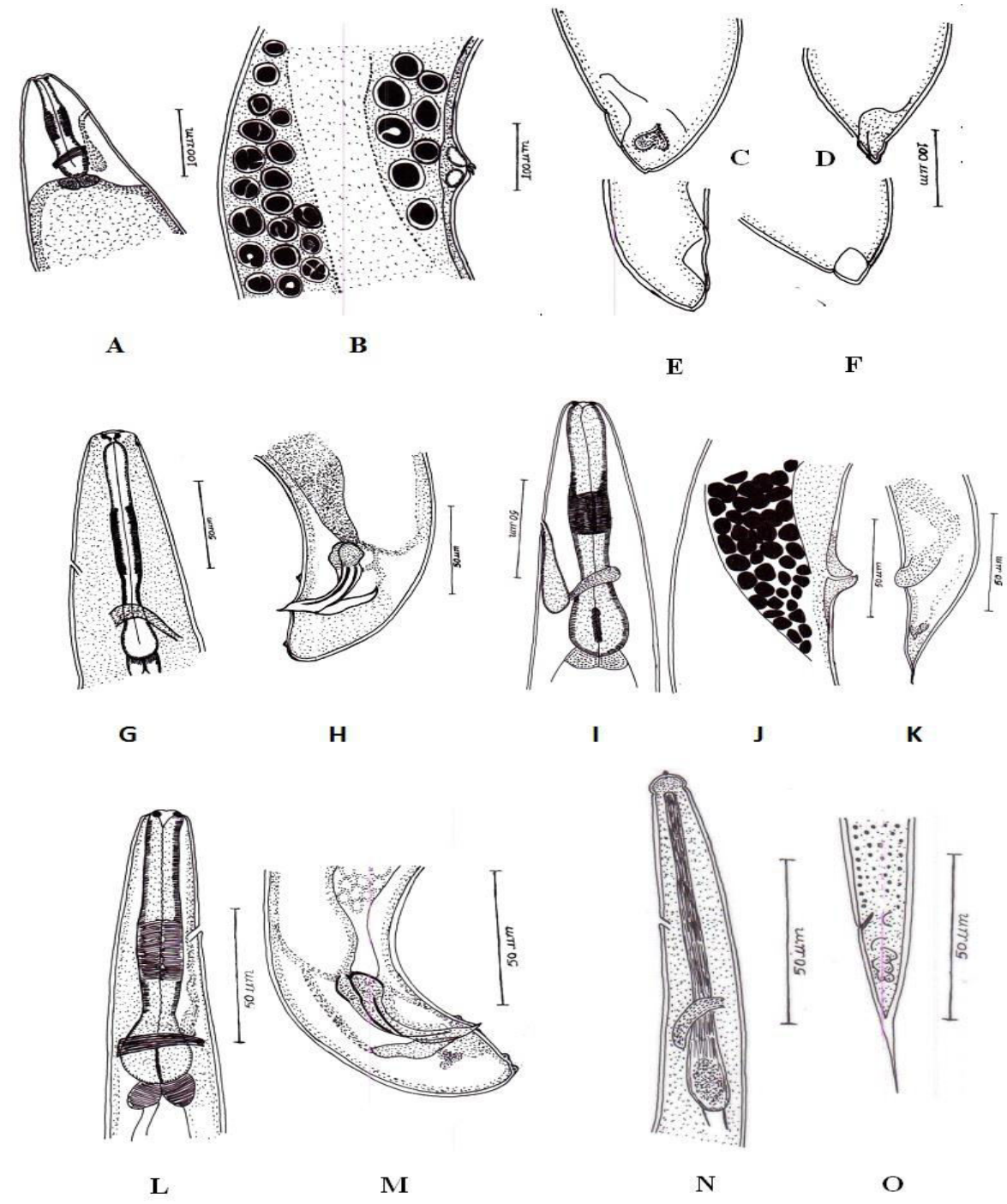


**Fig. 2.** Camera Lucida sketch of *Steinernema* sp. isolate $CS_1$. A-F: First generation female, G-H: First generation male, I-K: Second generation female, L-M: Second generation male, N-O: Third stage juvenile, A, G, I, L, N: Anterior region, B, J: Middle region, C-F, H, K, M, O: Posterior region; C-F: Variation in tail region of 1st generation female.

**Table 1. Morphometrical characters of *Steinernema* sp. isolate $CS_1$. All measurements in $\mu$m except n (number), M±SD followed by range.**

| Characters | (Male) | | (Female) | | | |
|---|---|---|---|---|---|---|
| | 1st Generation | 2nd Generation | 1st Generation | Giant Female | 2nd Generation | Juveniles |
| n | 15 | 15 | 15 | 11 | 15 | 20 |
| L | 1432.1±260.8 (978.4-1964.9) | 839.2±83 (653.3-990.2) | 4985±710.9 (3200-5930.7) | 8664.7±1671.1 (6884.1-11419.4) | 1432.1±237.4 (1021.2-1732.8) | 583.1±39.2 (512.9-675.1) |
| a | 14.4±2.2 (10.2-18.6) | 16.5±1.5 (13.2-19.1) | 21.5±3.8 (12.8-26.5) | 33.6±5.4 (24.7-43) | 18.2±1.5 (15.2-20.3) | 23.8±0.9 (22.6-25.6) |
| b | 10.1±1.5 (7.6-12.1) | 7±0.7 (5.7-8.6) | 29±4.4 (18.3-33.9) | 46.5±7.3 (39.3-58.7) | 22.3±3.2 (17-28.1) | 5.9±0.3 (5.3-6.60) |
| c | 57.4±8.9 (40.8-74) | 41±3.9 (36.4-49.1) | 150.2±31.9 (105.2-242) | 184.2±58.7 (113-282.2) | 26.4±3.2 (21.9-33.2) | 10.4±0.7 (9.1-11.8) |
| c' | 0.3 (0.1-0.3) | 0.4 (0.3-0.5) | 0.1 (0.1-0.2) | 0.2±0.1 (0.1-0.3) | 0.7±0.1 (0.6-0.8) | 2.3±0.2 (2-2.6) |
| V | | | 53.5±1.9 (49.8-56.1) | 51.8±1.6 (49.5-54.4) | 54.6±2 (52.9-60.7) | |
| GBW | 99.3±8.9 (89.2-121.2) | 51.1±4.4 (45-59.6) | 235.2±33.4 (153.5-294.3) | 264.3±66.6 (185-365.4) | 78.9±11.9 (65.9-102) | 24.5±1.5 (20.2-26.5) |
| EP | 81.1±7.1 (63.5-89.5) | 66.4±4.3 (54.9-75.1) | 53.7±8.9 (40.7-77.9) | 88.4±13 (75.5-121.6) | 64.2±4.8 (57.8-77.6) | 48.1±3.2 (39.8-52) |
| NR | 108.5±10.7 (94.4-133.4) | 93.4±4.5 (86.7-101.7) | 127.8±5.5 (119.7-138) | 140.7±9.5 (129.3-159.4) | 97.4±5.8 (88.2-107.7) | 77.5±4.7 (64.1-84.8) |
| ES | 138.5±10.3 (124.3-161.5) | 119.3±3.8 (114-127.7) | 172.5±9.7 (157.5-196.2) | 185.6±13 (170.5-215) | 134.8±4.1 (129-142.9) | 99.3±3.5 (92.3-105.1) |
| TR | 255.5±55.1 (196.4-383.6) | 175.8±31.2 (118.3-225.3) | | | | |
| Tail | 24.9±1.8 (21.6-28.1) | 20.5±2 (17.9-25.1) | 33.8±5.2 (21.8-42.8) | 50.8±17.2 (30.9-86.1) | 54.1±4.2 (46.1-60.6) | 56.3±3.7 (48.3-62.3) |
| ABW | 35.6±3.5 (31.8-40.7) | 29.3±2.3 (24.9-33.2) | 47.5±10.2 (31.8-66.7) | 76.9±27.9 (53.8-138.6) | 33.8±4.8 (24.2-41.4) | 14.7±1.3 (11.7-16.6) |
| SL | 65.7±4.5 (58.9-73.4) | 55±4.6 (46.6-61.9) | | | | |
| SW | 11.1±1.7 (8.9-15.2) | 6.6±0.9 (4.7-7.9) | | | | |
| GL | 44.1±5 (35.4-52.3) | 29.4±3.7 (23.7-37.2) | | | | |
| GW | 7.2±0.9 (5.8-9.3) | 6.3±0.8 (4.9-7.6) | | | | |
| D% | 58.6±4.6 (50.9-69.6) | 55.7±2.9 (47.5-58.8) | 31.3±5.7 (20.8-44.6) | 47.5±4.9 (41.7-56.5) | 47.6±2.8 (42.6-55.4) | 48.5±2.4 (42.1-51.8) |
| E% | 325.8±24.72 (64.6-366.5) | 327.8±40.1 (226.5-383.6) | 161±26.9 (114-198.7) | 193.9±77.2 (99.6-338.8) | 119.1±9.6 (102.1-134.4) | 85.7±5.8 (71.1-92) |
| F% | 399.4±36.8 (342.1-500.7) | 251.5±31.9 (190.7-326.9) | 724.1±222.9 (416.5-1348.9) | 576±254.1 (325.9-989.8) | 145.7±16.1 (122.9-182.9) | 43.7±3.2 (38.3-50.6) |
| SW% | 186.2±23.1 (151.6-220) | 189±21.5 (142.8-221.6) | | | | |
| GS% | 67.1±5.9 (55.9-80.5) | 53.5±5.1 (45.6-62.8) | | | | |

**Table 2. Comparison table of 3rd stage juveniles of *Steinernema* species. All measurements in $\mu$m except n (number), M±SD followed by range.**

| Characters | *S. anatoliense* | *S. carpocapsae* | *S. kushidai* | *S. scapterisci* | *S. tami* | *S. websteri* | *S. abbasi* | *S. pakistanense* | *S. thermophilum* | *Steinernema* sp. CS |
|---|---|---|---|---|---|---|---|---|---|---|
| n | 20 | | | | | | | 20 | 20 | 20 |
| L | 545 ± 21 (507-580) | 558 (438-650) | 589 ± 39.8 (424-662) | 572 ± 27 (517-609) | 530 ± 4 (400-600) | 584 ± 13 (553-631) | 541±24 (496-579) | 683±21 (649-716) | 555±34 (480-620) | 583.1±39.2 (512.9-675.1) |
| a | 22 ± 2 (19-27) | 21 (19-24) | 22.5 ± 1.6 (19.3-25.2) | 24 (20-31) | 23 ± 2 (19-28) | 28 ± 2.7 (24-35) | 18±0.9 (17-20) | 24±1.5 (21-27) | 26±0.9 (24-28) | 23.8±0.9 (22.6-25.6) |
| b | 5.1 ± 0.3 (4.6-5.7) | 4.4 (4-4.8) | 5.3 ± 0.3 (4.9-5.9) | 4.5 (4-4.6) | 5 ± 0.3 (3.7-5.1) | 5.1 ± 0.2 (4.8-5.6) | 6±0.3 (5.5-6.6) | 6±0.3 (5-6) | 6.4±0.4 (5.8-7.1) | 5.9±0.3 (5.3-6.60) |
| c | 10.5 ± 0.5 (9.4-11.8) | 10 (9-11) | 11.7 ± 0.6 (9.9-12.9) | 10.7 (9.2-11.7) | 11 ± 0.5 (9-11) | 12.6 ± 1.3 (11-15.5) | 9.8±0.8 (8.1-10.8) | 11±0.5 (10-12) | 12.3±0.4 (11.5-12.8) | 10.4±0.7 (9.1-11.8) |
| c' | - | - | - | - | 4.2 | - | - | - | 3.4±0.3 (3-3.9) | 2.3±0.2 (2-2.6) |
| GBW | 24.5 ± 2 (21-28) | 25 (20-30) | 26 ± 2.5 (22-31) | 24 ± 4 (18-30) | 23 ± 2 (19-29) | 21 ± 17 (17-25) | 29±1 (27-30) | 27±1.2 (24-29) | 21±0.7 (21-23) | 24.5±1.5 (20.2-26.5) |
| EP | 37 ± 1 (36-39) | 38 (30-56) | 46 ± 2 ( 42-50) | 39 ± 4 (36-48) | 36 ± 2 (34-41) | 36 ± 3.1 (29-40) | 48±1.5 (46-51) | 54±2.2 (49-58) | 40±2 (37-46) | 48.1±3.2 (39.8-52) |
| NR | 76 ± 3 (71-82) | 85 (76-99) | 76 ± 3.5 (70-84) | 97 ± 1.1 (83-106) | - | 88 ± 3.6 (83-95) | 68±2.4 (64-72) | 80±2.1 (76-83) | 71±4 (65-79) | 77.5±4.7 (64.1-84.8 |
| ES | 107 ± 6 (97-124) | 120 (103-190) | 111 ± 4.1 (106-120) | 127 ± 6 (113-134) | 117 ± 4 (110-123) | 115 ± 4.4 (107-122) | 89±1.8 (85.92) | 113±4.2 (108-122) | 87±6 (80-100) | 99.3±3.5 (92.3-105.1) |
| Tail | 53 ± 3 (46-58) | 53 (46-61) | 50 ± 3.4 (44-59) | 54 ± 3 (48-60) | 50 ± 4 (42-57) | 47 ± 4.5 (37-56) | 56±3.2 (52-61) | 58±2.1 (53-62) | 45±3 (40-52) | 56.3±3.7 (48.3-62.3) |
| Hyaline (H) | 22.5 ± 1 (19-26) | - | - | - | - | 11 ± 0.5 (10-14) | - | - | - | - |
| ABW | 12 ± 0.7 (11-14) | - | - | 31 ± 3 (27-40) | 12 ± 1 (11-13) | 12 ± 1.2 (10-14) | - | - | 13±1 (12-15) | 14.7±1.3 (11.7-16.6) |
| D% | 35 ± 2.5 (31.5-39) | 26 (23-28) | 41 ± 1.4 (38-44) | 73 ± 6 (60-80) | 31 ± 0 (28-34) | 31 ± 2.7 (24-34) | 53±2 (51-58) | 47±2.7 (42-53) | 46±3.5 (42-53) | 48.5±2.4 (42.1-51.8) |
| E% | 72 ± 3.9 (64-81.5) | 60 (54-66) | 92 | - | 73 ± 0 (67-86) | 77 ± 11 (62-102) | 86±5 (79-94 | 91±5 (87-102) | 90±6 (81-102) | 85.7±5.8 (71.1-92) |
| F% | - | - | - | - | - | - | - | - | - | 43.7±3.2 (38.3-50.6) |
| H% | 43 ± 0.8 (41-45) | - | - | - | 40 ± 0.1 (30-50) | 33 ± 0.6 (30-34) | - | - | - | - |

**Table 3. Comparison table of 1st generation males of *Steinernema* species. All measurements in µm except n (number), M±SD followed by range.**

| Characters | *S. anatoliense* | *S. carpocapsae* | *S. kushidai* | *S. scapterisci* | *S. arvi* | *S. websteri* | *S. abbasi* | *S. pakistanense* | *S. thermophilum* | $CS_1$ |
|---|---|---|---|---|---|---|---|---|---|---|
| n | 19 | 25 | 20 | 10 | 20 | 19 | - | 20 | 20 | 15 |
| L | 1532±195 (1236-1841) | 1450 (1090-1710) | 1400±16 (1200-1900) | 1728±358 (1319) | 1600±20 (1200-1900) | 1712±92 (1523-1865) | 1252±189 (999-1534) | 1357±89 (1163-1505) | 1197±236 (990-1780) | 1432±261 (978-1965) |
| a | - | - | - | - | - | - | - | 13.4±1.8 (11-16) | 15.2±1.6 (12.8-17.8) | 14.4±2.2 (10.2-18.6) |
| b | - | - | - | - | - | - | - | 10.4±0.6 (8.7-11) | 9.7±1.4 (8-12.5) | 10.1±1.5 (7.6-12.1) |
| c | - | - | - | - | - | - | - | 55±3.3 (52-62) | 53±4 (48-63) | 57.±8.9 (40.8-74) |
| c' | - | - | - | - | - | - | - | - | 0.6±0.1 (0.5-0.7) | 0.3 (0.1-0.3) |
| GBW | 109±15 (87-150) | 102 (77-131) | 97±19 (75-156) | 156±49 (97-231) | 129±17 (89-161) | 147±17 (119-175) | 87±6.7 (82-98) | 102±10.2 (80-128) | 77±15.2 (60-100) | 99±8.9 (89.2-121.2) |
| EP | 67±0.5 (61-88) | 61 (47-74) | 84±7.9 (71-105) | 71±11 (63-98) | 68±12 (43-92) | 62±6 (54-73) | 80±7.8 (68-89) | 81±4.8 (72-92) | 75±8.5 (64-92) | 81±7.1 (64-90) |
| NR | 107±15 (87-131) | 110 (93-124) | 129±5.3 (120-137) | 136±11 (120-152) | - | 119±10 (100-139) | 103±6.5 (99-123) | 99±6.3 (88-107) | 93±8.2 (80-110) | 108.5±10.7 (94-133) |
| ES | 144±8 (129-160) | 155 (136-167) | 167±7.9 (156-189) | 187±21 (164-216) | 153±8 (137-166) | 163±12 (135-180) | 133±6 (121-144) | 132±5.8 (126-146) | 125±20 (97-165) | 139±10.3 (124-162) |
| TR | - | 563 (400-808) | - | 374±52 (306-447) | - | - | 274±33 (234-319) | - | - | 256±55 (196.4-383.6) |
| Tail | 30±3 (26-64) | 30 (23-39) | 33±3 (30-40) | 25±3 (21-30) | 23±5 (10-32) | 29±2 (25-33) | 26±3 (20-31) | 25±0.8 (24-27) | 22.4±4.7 (19-34) | 25±1.8 (22-28) |
| ABW | 52.5±3.5 (47-60) | 43 (33-55) | 42±5 (36-54) | 33±5 (31-45) | 37±6 (25-40) | 37.5±2 (34-41) | 43±5 (37-55) | 36±2.3 (32-40) | 37±7 (28-49) | 36±3.5 (32-41) |
| SPL | 74±3.5 (68-84) | 65 (59-72) | 63±5.5 (48-72) | 83±5 (72-92) | - | 68±2 (64-72) | 65±5.7 (57-74) | 68±3.6 (62-73) | 61±7 (44-72) | 66±4.5 (59-73) |
| SPW | - | 11 (9-13) | - | 13±4 (13-14) | 77±4 (71-84) | - | 12±1.3 (10-14) | - | - | 11±1.7 (9-15) |
| GL | 47±3.5 (42-59) | 47 (39-56) | 44±4.9 (39-60) | 65±5 (59-75) | 48±5 (38-55) | 49±3.2 (42-56) | 45±4.3 (35-50) | 41±3.2 (36-45) | 36±4 (30-42) | 44±5 (35-52) |
| GW | - | 5 (4-7) | - | 8±0.5 (8-9) | - | - | 7±0.1 (6-8.5) | - | - | 7.2±0.9 (5.8-9.3) |
| D% | 48.5±3.5 (46.5-55) | 39 | 51±4.8 (42-59) | 36±2 (32-39) | 4410 (30-60) | 40±10 (30-50) | 60±5 (51-58) | 60±3 (50-60) | 63±10 (50-87) | 59±4.6 (51-70) |
| E% | - | - | - | - | - | 210±20 (180-250) | - | 310±40 (210-370) | 343±43 (269-400) | 326±25 (65-367) |
| F% | - | - | - | - | - | - | - | - | - | 399±37 (342-501) |
| SW | 175±10 (160-190) | 151 | 150 | 252 (204-280) | 20040 (140-300) | 180±10 (160-210) | 156±22 (107-187) | 189 | 1.7±0.4 (1.2-2.8) | 1.9±0.2 (1.5-2.2) |
| GS | 55±4 (40-65) | 72 | 70 | 78 (69-84) | 62 | 70±10 (60-80) | 70±7 (58-85) | 60.3 | 0.6-0.1 (0.5-0.7) | 0.7±0.1 (0.6-0.8) |
| MUC | + | + | - | + | + | + | - | + | - | - |

The maximum parsimony generated 3 parsimoneous tree with a length of 927 positions. The consistency index, retention index and the composite index were 0.652, 0.654 and 0.426, respectively for all sites and parsimony-informative sites. Phylogenetic analysis through neighbour joining (Fig. 4), maximum likelihood (Fig. 5) and maximum parsimony (Fig. 6) yielded a total of 575 positions in the final dataset. Pair wise distance analysis (Fig. 3) showed no difference between *Steinernema* sp. isolate $CS_1$, *S. abbasi* and *S. thermophilum*. Analysis of nucleotide composition were also indicative for the same results (Table 4).

| | $CS_1$ | Sab | Sth | Spa | Sbi | Sce | Syi | Sca | Sku | Ssc | Sta | Cle |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Steinernema* sp. $CS_1$** | | | | | | | | | | | | |
| ***S. abbasi*** | 0.000 | | | | | | | | | | | |
| ***S. thermophilum*** | 0.000 | 0.000 | | | | | | | | | | |
| ***S. pakistanense*** | 0.348 | 0.348 | 0.348 | | | | | | | | | |
| ***S. bicornutum*** | 0.281 | 0.281 | 0.281 | 0.345 | | | | | | | | |
| ***S. ceratophorum*** | 0.277 | 0.277 | 0.277 | 0.350 | 0.111 | | | | | | | |
| ***S. yirgalemense*** | 0.239 | 0.239 | 0.239 | 0.346 | 0.324 | 0.321 | | | | | | |
| ***S. carpocapsae*** | 0.392 | 0.392 | 0.392 | 0.530 | 0.380 | 0.399 | 0.443 | | | | | |
| ***S. kushidai*** | 0.453 | 0.453 | 0.453 | 0.550 | 0.431 | 0.422 | 0.516 | 0.336 | | | | |
| ***S. scapterisci*** | 0.395 | 0.395 | 0.395 | 0.532 | 0.374 | 0.391 | 0.452 | 0.108 | 0.343 | | | |
| ***S. tami*** | 0.396 | 0.396 | 0.396 | 0.523 | 0.396 | 0.422 | 0.443 | 0.083 | 0.388 | 0.137 | | |
| ***C. elegans*** | 0.978 | 0.978 | 0.978 | 1.084 | 0.955 | 0.936 | 1.040 | 1.015 | 1.132 | 1.063 | 1.019 | |

**Fig. 3.** Distance matrix of compared *Steinernema* species.

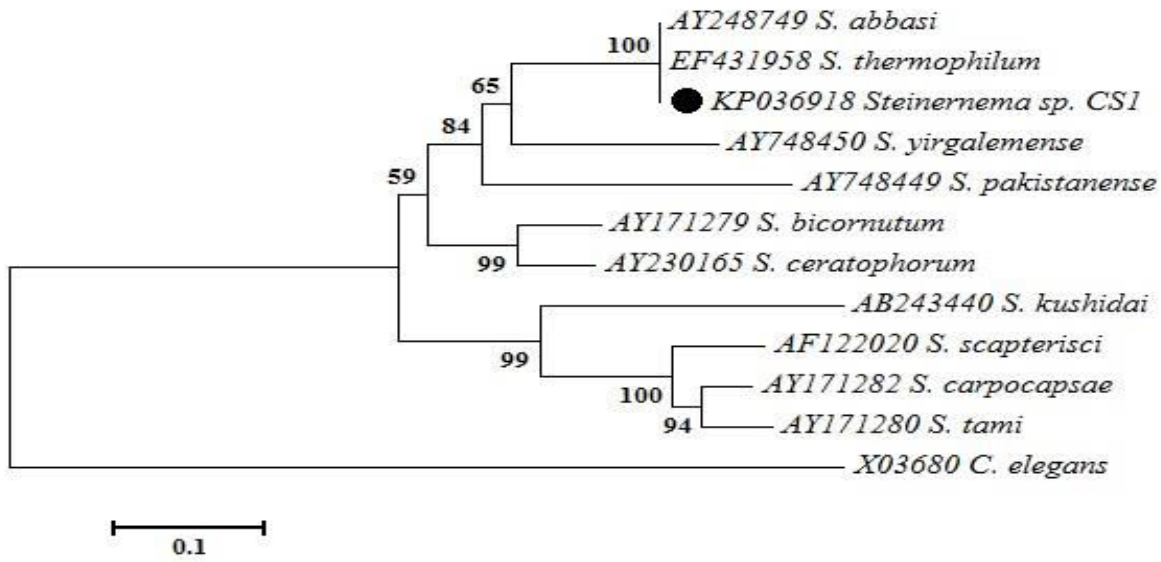


**Fig. 4.** Phylogenetic analysis of internal transcribed spacer region (ITS) of ribosomal DNA based on neighbour joining analysis.

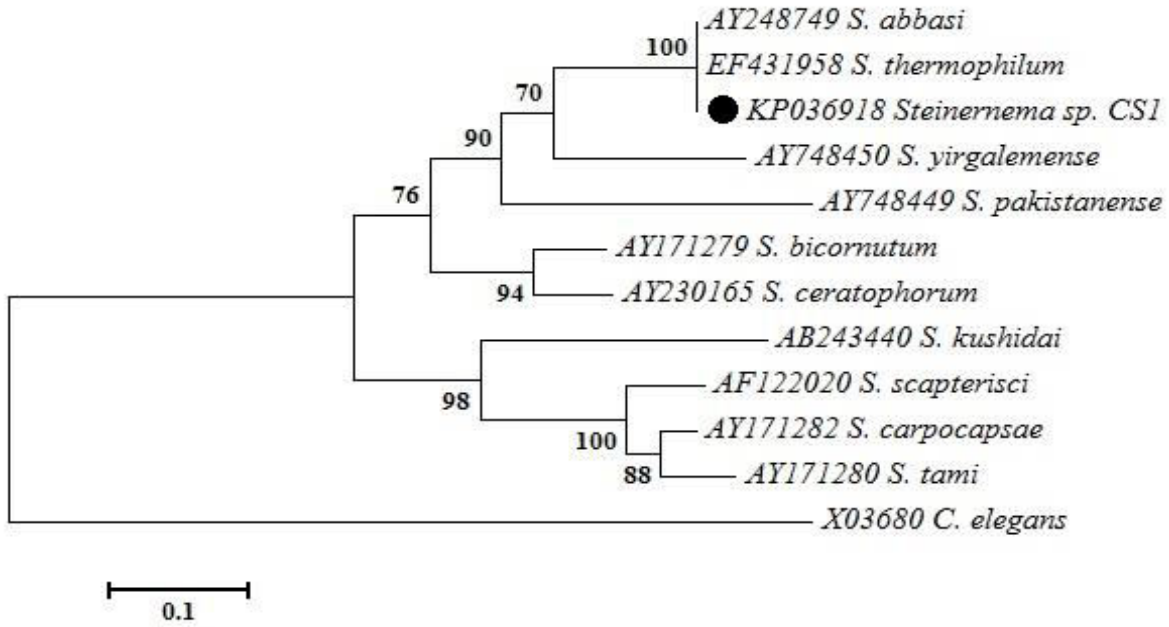


**Fig. 5.** Phylogenetic analysis of internal transcribed spacer region (ITS) of ribosomal DNA. based on maximum likelyhood analysis.

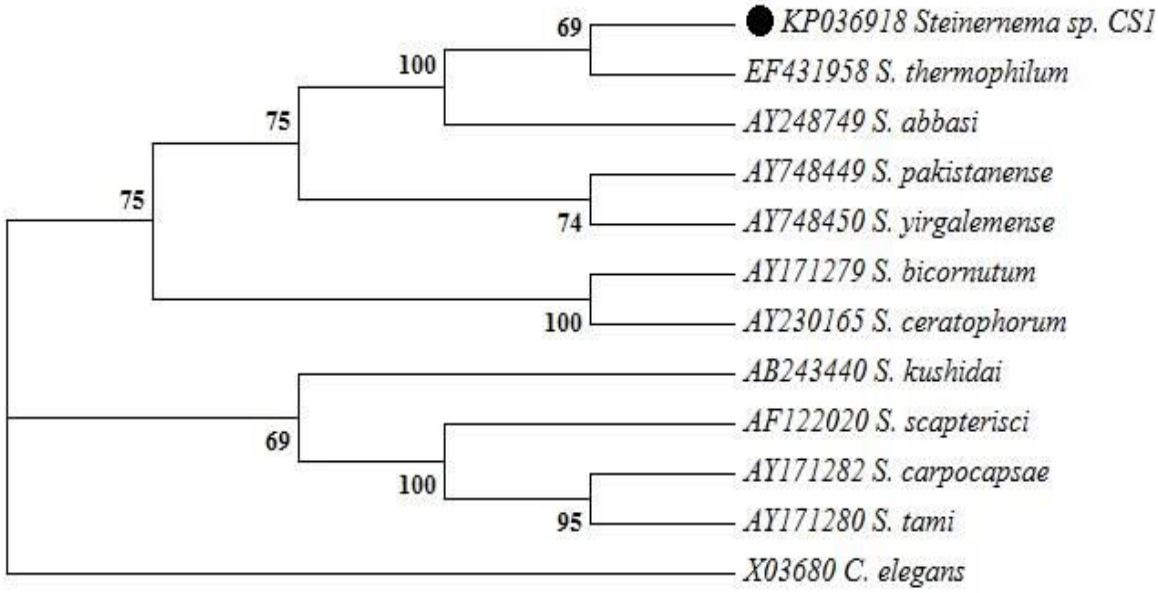


**Fig. 6.** Phylogenetic analysis of internal transcribed spacer region (ITS) of ribosomal DNA.based on maximum parsimony analysis.

**Table 4. A comparative account of nucleotide compositions.**

| S. No. | Species | Accession number | Length (bp) | ITS1 | 5.8S | ITS2 | G+C% | A+T% | A | C | G | T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Isolate $CS_1$ | KP036918 | 739 | 268 | 157 | 314 | 36.94 | 63.06 | 175 | 111 | 162 | 291 |
| 2 | *S. tami* | AY171280 | 728 | 269 | 155 | 304 | 38.87 | 61.13 | 163 | 120 | 163 | 282 |
| 3 | *S. carpocapsae* | AY171282 | 731 | 279 | 157 | 295 | 37.89 | 62.11 | 170 | 117 | 160 | 284 |
| 4 | *S. scapterisci* | AF122020 | 710 | 247 | 157 | 306 | 36.76 | 63.24 | 169 | 108 | 153 | 280 |
| 5 | *S. pakistanense* | AY748449 | 748 | 291 | 157 | 300 | 36.9 | 63.1 | 216 | 118 | 158 | 256 |
| 6 | *S. bicornutum* | AY171279 | 768 | 281 | 157 | 330 | 37.5 | 62.5 | 201 | 123 | 165 | 279 |
| 7 | *S. ceratophorum* | AY230165 | 741 | 243 | 157 | 341 | 36.17 | 63.83 | 192 | 117 | 151 | 281 |
| 8 | *S. yirgalemense* | AY748450 | 711 | 270 | 157 | 284 | 35.72 | 64.28 | 191 | 93 | 161 | 266 |
| 9 | *S. abbasi* | AY248749 | 739 | 268 | 157 | 314 | 36.99 | 62.74 | 175 | 111 | 162 | 291 |
| 10 | *S. thermophilum* | EF431958 | 739 | 268 | 157 | 314 | 36.94 | 63.06 | 175 | 111 | 162 | 291 |
| 11 | *S. kushidai* | AB243440 | 741 | 279 | 157 | 305 | 42.24 | 57.76 | 172 | 135 | 178 | 256 |
| 12 | *C. elegans* | X03680 | 1001 | 464 | 153 | 384 | 47.55 | 52.45 | 229 | 220 | 256 | 296 |

**Comparative and polythetic divisive analysis:** The *Steinernema* sp. isolate $CS_1$ was placed in bicornutum clade that includes species bearing two horn like structure on head region of 3rd stage infective juveniles. Available data of 9 closely related species viz., *S. anatoliense*, *S. carpocapsae*, *S. kushidai*, *S. scapterisci*, *S. tami*, *S. websteri*, *S. abbasi*, *S. pakistanense* and *S. thermophilum* were used for the comparison. Taxometrical characters of 3rd stage juveniles are given in Table 2 and 3. MCD, CD, C-Dis and CS values were calculated for total body length, greatest body width, position of excretory pore and nerve ring from anterior end, oesophagus length; a,b,c ratio and D% and E% based on the data available, as shown in Table 5.

**Discussion:** Adams (2001) and Sites & Marshall (2004) explored the difficulties of species differentiations in their studies. Morphological and reproductive diversity of nematodes are especially heavy tasks for the accurate description of a new nematode species (Nadler, 2002). The present specimen varied morphologically with *S. anatoliense*, *S. carpocapsae*, *S. kushidai*, *S. scapterisci*, *S. tami* and *S. websteri* based on the presence of 2 horn like structure on the head region of unsheathed 3rd stage infective juveniles hence was placed in bicornutum group together with *S. abbasi*, *S. pakistanense* and *S. thermophilum* with whom it was compared in detail. The length of juveniles is an indicative character of classification of different groups of *Steinernema* species. Total body length of *Steinernema* sp. isolate $CS_1$ placed the specimen in between the *S. abbasi*, *S. thermophilum* and *S. pakistanense*. Other characters were also showing the divergence from all the compared species along with the species of bicornutum group. However, morphological observations were almost similar and presence of mucronate process in tail region only in 2nd generation female divert the specimen from *S. pakistanense* and placed near to *S. abbasi* and *S. thermophilum*.

Maximum parsimonious tree of ITS regions with 575 positions in final dataset showed closest resemblance of present specimen with *S. abbasi* and *S. thermophilum*. Similar results were obtained from neighbour joining and maximum likelihood method. The distance matrix revealed no difference among the present specimen and *S. abbasi* and *S. thermophilum.* Composition of nucleotides (the GC%, AT% and number of A, C, G, T) were also similar in ITS1-5.8.ITS2 region of the amplified region.

**Table 5.** A comparative account of MCD, CD, C-Dis and CS values of infective juveniles of compared species.

| Characters | *S. anatoliense* | *S. carpocapsae* | *S. kushidai* | *S. scapterisci* | *S. tami* | *S. websteri* | *S. abbasi* | *S. pakistanense* | *S. thermophilum* |
|---|---|---|---|---|---|---|---|---|---|
| **MCD Values** | | | | | | | | | |
| L | 0.18 | 0.28 | 0.30 | 0.20 | 0.29 | 0.19 | 0.19 | 0.17 | 0.23 |
| a | 0.21 | 0.16 | 0.18 | 0.24 | 0.22 | 0.26 | 0.13 | 0.17 | 0.13 |
| b | 0.19 | 0.18 | 0.18 | 0.16 | 0.23 | 0.18 | 0.18 | 0.18 | 0.19 |
| c | 0.22 | 0.21 | 0.23 | 0.22 | 0.21 | 0.30 | 0.24 | 0.20 | 0.17 |
| GBW | 0.24 | 0.28 | 0.26 | 0.32 | 0.29 | 0.26 | 0.17 | 0.20 | 0.16 |
| EP | 0.16 | 0.35 | 0.20 | 0.25 | 0.21 | 0.25 | 0.17 | 0.20 | 0.22 |
| NR | 0.19 | 0.24 | 0.21 | 0.23 | - | 0.19 | 0.18 | 0.16 | 0.21 |
| ES | 0.17 | 0.29 | 0.12 | 0.14 | 0.11 | 0.12 | 0.10 | 0.12 | 0.16 |
| Tail | 0.22 | 0.23 | 0.24 | 0.21 | 0.24 | 0.28 | 0.19 | 0.18 | 0.23 |
| D% | 0.19 | 0.18 | 0.16 | 0.22 | 0.18 | 0.24 | 0.15 | 0.20 | 0.20 |
| E% | 0.22 | 0.20 | - | - | 0.22 | 0.35 | 0.19 | 0.19 | 0.22 |
| **CD Values** | | | | | | | | | |
| L | 0.07 | 0.04 | 0.01 | 0.02 | 0.10 | 0.00 | 0.07 | 0.16 | 0.05 |
| a | 0.08 | 0.12 | 0.06 | 0.01 | 0.03 | 0.16 | 0.28 | 0.01 | 0.09 |
| b | 0.14 | 0.29 | 0.10 | 0.27 | 0.16 | 0.14 | 0.02 | 0.02 | 0.09 |
| c | 0.01 | 0.04 | 0.12 | 0.03 | 0.06 | 0.19 | 0.06 | 0.06 | 0.17 |
| GBW | 0.65 | 0.63 | 0.60 | 0.67 | 0.71 | 0.78 | 0.50 | 0.56 | 0.78 |
| EP | 0.41 | 0.43 | 0.61 | 0.46 | 0.38 | 0.38 | 0.65 | 0.75 | 0.48 |
| NR | 0.02 | 0.09 | 0.02 | 0.22 | - | 0.13 | 0.13 | 0.03 | 0.09 |
| ES | 0.07 | 0.19 | 0.11 | 0.25 | 0.16 | 0.15 | 0.11 | 0.13 | 0.13 |
| Tail | 0.06 | 0.06 | 0.12 | 0.04 | 0.12 | 0.18 | 0.00 | 0.03 | 0.22 |
| D% | 0.32 | 0.60 | 0.17 | 0.40 | 0.44 | 0.44 | 0.09 | 0.03 | 0.05 |
| E% | 0.17 | 0.35 | 0.07 | 0.00 | 0.16 | 0.11 | 0.00 | 0.06 | 0.05 |
| **C-Dis Values** | | | | | | | | | |
| L | 0.20 | 0.33 | 0.36 | 0.22 | 0.34 | 0.20 | 0.21 | 0.19 | 0.26 |
| a | 0.24 | 0.18 | 0.20 | 0.28 | 0.25 | 0.25 | 0.14 | 0.19 | 0.14 |
| b | 0.21 | 0.20 | 0.20 | 0.18 | 0.27 | 0.18 | 0.20 | 0.20 | 0.21 |
| c | 0.25 | 0.23 | 0.26 | 0.25 | 0.23 | 0.30 | 0.27 | 0.22 | 0.19 |
| GBW | 0.28 | 0.33 | 0.30 | 0.38 | 0.34 | 0.32 | 0.19 | 0.23 | 0.18 |
| EP | 0.18 | 0.44 | 0.22 | 0.28 | 0.23 | 0.30 | 0.19 | 0.22 | 0.25 |
| NR | 0.21 | 0.27 | 0.23 | 0.26 | - | 0.21 | 0.20 | 0.18 | 0.24 |
| ES | 0.19 | 0.36 | 0.13 | 0.15 | 0.12 | 0.13 | 0.10 | 0.13 | 0.18 |
| Tail | 0.24 | 0.27 | 0.27 | 0.24 | 0.28 | 0.33 | 0.21 | 0.20 | 0.26 |
| D% | 0.21 | 0.20 | 0.18 | 0.25 | 0.20 | 0.28 | 0.17 | 0.22 | 0.22 |
| E% | 0.25 | 0.23 | - | - | 0.25 | 0.37 | 0.21 | 0.21 | 0.24 |
| **CS Values** | | | | | | | | | |
| L | 0.80 | 0.67 | 0.64 | 0.78 | 0.66 | 0.80 | 0.79 | 0.81 | 0.74 |
| a | 0.76 | 0.82 | 0.80 | 0.72 | 0.75 | 0.75 | 0.86 | 0.81 | 0.86 |
| b | 0.79 | 0.80 | 0.80 | 0.82 | 0.73 | 0.82 | 0.80 | 0.80 | 0.79 |
| c | 0.75 | 0.77 | 0.74 | 0.75 | 0.77 | 0.70 | 0.73 | 0.78 | 0.81 |
| GBW | 0.72 | 0.67 | 0.70 | 0.62 | 0.66 | 0.68 | 0.81 | 0.77 | 0.82 |
| EP | 0.82 | 0.56 | 0.78 | 0.72 | 0.77 | 0.70 | 0.81 | 0.78 | 0.75 |
| NR | 0.79 | 0.73 | 0.77 | 0.74 | - | 0.79 | 0.80 | 0.82 | 0.76 |
| ES | 0.81 | 0.64 | 0.87 | 0.85 | 0.88 | 0.87 | 0.90 | 0.87 | 0.82 |
| Tail | 0.76 | 0.73 | 0.73 | 0.76 | 0.72 | 0.67 | 0.79 | 0.80 | 0.74 |
| D% | 0.79 | 0.80 | 0.82 | 0.75 | 0.80 | 0.72 | 0.83 | 0.78 | 0.78 |
| E% | 0.75 | 0.77 | - | - | 0.75 | 0.63 | 0.79 | 0.79 | 0.76 |

*Blank columns indicate the absence of non-availability of data.

The nucleotide composition resemblance within 5.8S gene with other *Steinernema* species evident the less polymorphism and highly conserved nature of this region as compared to ITS1 and ITS2 delimit the relationship of steinernematid nematodes, results correlated to the study of other researchers (Nguyen *et al*., 2001; Szalanski *et al*., 2000). 100% similarity obtained through molecular analysis showed no difference in *S. abbasi*, *S. thermophilum* and *Steinernema* sp. isolate $CS_1$ and support the synonymizing of *S. thermophilum* as a junior synonym of *S. abbasi* (Hunt, 2007). Previously, the study on the same bacterial symbiont was an indicative of the similarity of the *S. thermophilum* with *S. abbasi* (Tailliez *et al.*, 2006).

Close resemblance based on C-Dis, CS, CD and MCD values could also found some important part of taxonomical identification where total body length, position of excretory pore and nerve ring from anterior end, oesophagus length; a, b ratio and D% and E% in 3rd stage juveniles also support the resemblance of *Steinernema* sp. isolate $CS_1$ with *S. abbasi*.

Based on the results obtained through morphology, life history events, taxometry, molecular characterization and numerical taxonomy, the present specimen was concluded as an another isolate of *S. abbasi* with larger infective juveniles form Saharanpur District of Western part of Uttar Pradesh, India.

## Acknowledgements

Authors are thankful to the Head, Department of Zoology, Chaudhary Charan Singh University, Meerut for providing necessary facilities to carrying out the work. Council of Scientific and Industrial Research (CSIR), New Delhi is highly acknowledged for the financial assistance through the EMR project (37(1550)/12-EMR-II).

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(Accepted: November 25, 2015)