*Pakistan Journal of Nematology* (2017) 35 (2): 157-173

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

http://dx.doi.org/10.18681/pjn.v35.i02.p157-173

# Two new records of entomopathogenic nematodes (Nematoda: Steinernematidae) from Gilgit-Baltistan, Pakistan

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**Abstract**

A survey of District Hunza, Gilgit and Nager, Gilgit-Baltistan, Pakistan was conducted during January, 2017. Out of 20 soil samples, 6 samples were found positive for *Steinernema* species. One sample of *Prunus avium* had *Steinernema affine* from Hunza, two samples had *S. cholashanense* from *Juglans nigra* and *Malus pumila* from Gilgit, and three samples harboured *S. feltiae* from *Prunus armeniaca*, *Prunus persica* and *Malus pumila* from Nager. This paper deals with re-description of *S. affine* based on comparative study of morphological and molecular characteristics with brief study of *S. cholashanense* and *S. feltiae*. *S. affine* Pak. G.S.356 deposited in NCBI with accession number MF150033; *S. cholashanense* Pak.G.S.350 and Pak. G.S.355 with accession numbers MF125282 and MF0339642, respectively; *S. feltiae* Pak. G.S. 354, 357, 358 deposited with accession numbers MF150034, MF158314 and MF144570, respectively.

**Keywords:** Taxonomy, *Steinernema affine, S. cholashanense, S. feltiae*, new records, Gilgit - Baltistan.

National Nematological Research Centre (NNRC), University of Karachi, Pakistan, has conducted many surveys in Pakistan to isolate EPNs for effectively utilized to control important insect pests. Studies of EPN in Pakistan have discovered six new species along with seven known species (Shahina *et al*., 2017).

This paper deals with re-description of *S. affine* with brief study of *S. cholashanense* and *S. feltiae*. According to Nguyen & Hunt, 2007 *Steinernema affine* was described in 1937 by Bovien as *Neoplactana affinis* from diseased fly larvae of bibionid fly in Denmark. In 1971, *N. affinis* re-isolated from larvae of Danish bibionid by Poinar & Lindhardt and deposited in the Davis Nematode Collection at University of California, Davis, CA, USA. Both aforesaid scientists studied *N. affinis* and *N. binionis*, they play the same role as parasites of bibionid flies in Denmark. Where as in 1979, Poinar re-described and differentiated both species on morphological basis. In 1988, Poinar designated lectotypes and two paralectotypes, one with male and one female. After that it was re-described by Mracek *et al*., (2005a) as *S. affine* from British, Columbia and Canada. *S. affine* is widely distributed species and found throughout Europe (Sturhan & Liskova, 1999; Sturhan & Mracek, 2000; Mracek *et al*., 2005a), UK and Ireland (Griffen *et al*., 1991; Hominick *et al*., 1995; Hominick, 2002), Vancouver area, British Columbia, Canada (Mracek *et al*., 2005b). Hazir *et al*., (2003) reported *S. affine* from Turkey and Tarasco *et al*., (2015) from Italy. *S. cholashanense* was described by Nguyen *et al*., (2008) collected from Chola Shan Mountain covered by xerophytic vegetables, Sichuan Province, China. *S. feltiae* originally described from the larvae of *Agrotis* (syn. *Feltia*) *sgetum* by Filipjev (1934). From Pakistan, it was reported by Anis *et al*., (2002) from Turbella

Dam, Khyber Pakhtunkhwa after that this species was re-described by Tabassum & Shahina in 2004. During the present study, EPN were further explored from Gilgit-Baltistan, Pakistan. The samples were collected from three districts: Gilgit (35.9197°N. 74.3881°E), Hunza (36.3082°N. 74.6195°E) and Nager (35.24°N. 73.48°E), Gilgit-Baltistan having minimum temperature -1-10 ºC during January, 2017. The climate of all three districts varies from region to region, in District Gilgit, maximum temperature ranges between 20-30 ºC whereas minimum temperature ranges -0.4- 1 ºC (www. wikipedia. org), in Hunza 10-26 Max., -1 to -10 Min. (www. tiptop gleb.com) whereas in Nager 10-15 Max., and-1 to -14 Min. (www. wikipedia.org) have notified.

## Materials and Methods

**Nematodes collection and isolation:** Soil samples were collected from different areas of Gilgit-Baltistan by removing 6-10 cm layer of ice at the depth of 6 cm in the rhizosphere of different fruits (Fig. 1). Nematodes were isolated from soil samples using the insect baiting with the last instars of *Galleria mellonella* (L.) following the technique described by Bedding & Akhurst (1975) and kept at 5-10 $^{o}$C. For the emergence of infective juveniles (IJs) dead larvae were placed in White traps (White, 1927). Nematodes have been maintained through soil assays.

**Morphology and morphometrics of the isolated nematodes:** Only IJ collected from White traps on the fourth day of emergence were used for measurement. The first and second generation adults were obtained at 3-4 and 6-7 days, respectively. All the different stages were killed by hot water (85 ºC), then fixed in TAF (Courtney *et al*., 1955), water in specimens replaced by glycerin using the Seinhorst technique (Seinhorst, 1959), after which they were mounted in pure glycerin. Permanent slides were made for measurements. Measurements and Photomicrographs were taken using a Nikon E-400 and Nikon DS-Fil, respectively.

**Molecular characterization and phylogenetic relationship:** DNA was extracted from a single female using the modified method reported by Joyce *et al*., (1994). Primers TW81 and AB28 were used for amplification of internal transcribed regions ITS1-5.8S-ITS2. For D2D3 expansion segment of the 28S rRNA region, the primer 501 and 391 were used as reported by Nguyen *et al*., 2006. The method reported by Khatri-Chhetri *et al*., 2011 were used for PCR amplification. PCR products were purified using DNA purification kit (Promega purification kit) and sequenced. The resulting sequences were edited and analyzed using software packages Chromas 2.23 (Technelysium, Tewantin, QLD, Australia) and Bio Edit 7.0.4.1 (Hall, 1999) and deposited in GenBank with accession numbers MF116307 and MF125282 for *S. cholashanense* isolate by using ITS; MF150034, MF144570 and NF158314 for *S. feltiae*, whereas MF150033 for *S. affine* using D2D3 28S RNA region. The resulting sequences of other species of *Steinernema* available in GenBank were aligned using Clustal X1.83 (Thompson *et al*., 1997). Phylogenetic analyses based on Maximum parsimony or pair wise distance of sequences, were done by using PAUP* v 4.0b10 (Swofford, 2002). Through *MrEnt* 2.0 (Zuccon & Zuccon, 2010) the resulting trees were visualized. *Caenorhabditis elegans* (EU131007) was applied as an out-group during calculation of the trees based on ITS regions. *Cervidellus alutus* (AF331911), together with *Panagrellus redivivus* (AF331910) were used as out group for calculation of the trees based on D2D3 region.

## Results and Discussion

***Steinernema affine*** **(Bovien, 1937) Wouts, Mracek, Gerdin & Bedding, 1982**
**(Fig. 2. A-F)**

**Measurements: Table 1**

**Female (First Generation)**: Body variable in shape, usually C shaped sometimes coiled on


**Fig.1.** Surveyed localities and collection of samples from Gilgit, Baltistan.

heat relaxation. Head rounded some time offset from body, cuticle smooth, six lips each bearing a single labial papilla. Four cephalic papillae present. Amphids distinct, crescent shaped located between labial and cephalic papilla. Lateral field and phasmid inconspicuous. Stoma short shallow, cheilorhabdions distinct. Excretory pore located mostly at the base of metacorpus and always anterior to nerve ring.

Pharynx muscular with cylindrical procorpus extending into a slightly swollen metacorpus, a narrower isthmus surrounded by nerve ring and large valvated basal bulb. Genital tract amphidelphic with reflexed ovaries. Vulva transverse slit like with protruding lips. Narrow rectum, distinct anal opening, postanal swelling reduced or slightly present. Tail usually with a terminal knob at base.

**Female (Second Generation):** Similar to first generation females in general morphology but smaller. Vulval aperture located posterior to mid body, lips protruding. Tail straight conoid which often ends in a fine mucron. Post anal swelling absent or slightly present.

**Males**: Cephalic region, cuticle and pharynx similar to females, except nerve ring usually located at anterior portion of basal bulb. Testis single, well developed, ventrally reflexed. Spicules paired, moderatelly curved and colorless. Spicule head longer than wide, shaft short, velum present. In ventral view, gubernaculum was forked at proximal portion. The distal portion varied from simple to hook shaped. There are 23 genital papillae comprises eleven pairs and a single ventral preanal papillae. Five pairs subventral, anterior to cloaca, three pairs in the region of spicule and gubernaculum, two pairs ventral subterminal, one pair postcloacal, subdorsal.

**Infective juveniles**: Third stage juvenile is enclosed in the second stage cuticle. Cephalic region finely rounded and offset from body. The mouth and anus are closed, intestine collapsed. Bacterial cells are filled in a modified vesicle in the anterior portion of the intestinal lumen. Lateral field with six ridges (7 lines), having prominent sub-marginal pair. Excretory pore located anterior to nerve ring or at middle of pharynx. Tail sharply pointed, hyaline portion short.

**Remarks:** *Steinernema affine* was found from cherry *Prunus avium* from Village Murtazabad District Hunza, Gilgit-Baltistan, characterized genetically by D2D3 region of rDNA. The sequence, having the accession number MF150033 was deposited in GenBank. The sequence length of *S. affine* is 598 bp having nucleotide composition, A = 0.23810, C = 0.17619, G=0.33810, and T = 0.24762. For D2D3 sequence, MP trees were calculated based on 1059 characters, of which 102 are constant, 183 are variable characters are parsimony un-informative and 774 characters (included) are parsimony informative. The phyogenetic relationships between 46 species of *Steinernema* are presented in Fig. 3 (For MP, tree length = 3083, Cl = 0.5800, RI = 0.7673, RC = 0.4450, HI = 0.4520). The sequence length composition and pair wise distance have shown in Table 3 and 4, respectively.

The population of S. *affine* Pak.G.S.356 has some variation in morphometric data. In male shorter body in length (1362 vs 1400-1733) µm; greater distance from head to excretory pore (100-125 vs 82-114) µm; shorter anal body width (53-60 vs 52-72) µm; shorter gubernaculum in length (37-45 vs 37-56); lower D % (61 vs 69); higher SW (151 vs 117). In IJs and female slight differences have been found (Table 1). *S. affine* is widely distributed throughout Europe, UK, Ireland, Coloumbia, and Canada. From Asian countries *S. affine* was reported only from Italy and now this species has recovered from Gilgit-Baltistan, Pakistan.

**Table 1. Morphometric values of *Steinernema affine* (n = 20 each). All measurements are in μm and in the form of mean ± SD (range).**

| Characters | First Generation | | Second Generation | | Infective stage juveniles |
|---|---|---|---|---|---|
| | Male | Female | Male | Female | |
| TL | 1489 ± 123 (1362-1733) | 3824 ± 328 (3117-4212) | 1293 ± 120 (1150-1500) | 1646 ± 126 (1460-1820) | 675 ± 35 (610-835) |
| Lip width | 14.5 ± 1.2 (14-16) | 33.5 ± 2.7 (28-35) | 12.5 ± 1.1 (12-13) | 21 ± 1.6 (19-25) | 10 ± 0.8 (9-11) |
| MBW | 93 ± 5.2 (80-105) | 179 ± 37.8 (140-255) | 64 ± 15.4 (60-72) | 91.8 ± 5.3 (85-100) | 29 ± 2.4 (27-33) |
| EP | 112 ± 10 (100-125) | 122.4 ± 15.3 (107-150) | 94.8 ± 46 (85-106) | 98.3 ± 4.6 (90-105) | 61 ± 7.5 (50-68) |
| NR | 124 ± 5.5 (115-145) | 158.7 ± 9.1 (150-175) | 117 ± 35 (108-123) | 112 ± 8.4 (98-123) | 95 ± 12 (85-100) |
| ES | 161 ± 4.8 (155-168) | 238 ± 29 (213-317) | 159 ± 55 (149-169) | 162 ± 9.8 (150-179) | 128 ± 6.5 (110-140) |
| TL (Ijs with sheath) | 47 ± 3.0 (45-50) | 66.4 ± 8 (50-75) | 35.7 ± 23.6 (30-42) | 54 ± 6 (48-64) | 67 ± 7 (60-75) |
| Anal body width | 55 ± 2.7 (53-60) | 60.9 ± 6.2 (55-72) | 45 ±17.2 (40-51) | 40 ± 1.5 (38-42) | 16.5 ± 2.3 (15-19) |
| V% | -- | 51.7 ± 3.7 (46-59) | -- | 53.7 ± 2 (51.5-57.6) | -- |
| a | 18.4 ± 1.3 (16.5-20.7) | 22.2 ± 4.8 (16-30) | 20 ± 3.8 (18.5-24.5) | 17.7 ± 0.68 (17-19) | 26 ± 1.8 (20-28) |
| b | 9.0 ± 0.8 (8-10.2) | 16 ± 1.8 (12.8-18.7) | 8 ± 0.6 (6.8-9.6) | 10 ± 0.79 (8.3-11) | 5.8 ± 1.2 (5.1-6.0) |
| c | 30 ± 3.0 (27-34) | 59.4 ± 11.6 (52-70) | 36.3 ± 11.5 (30-40.6) | 30.6 ± 3.2 (25-35.7) | 10 ± 1.5 (9-11.5) |
| c' | 0.78 ± 0.01 (0.75-0.89) | 1.08 ± 0.13 (0.9-13) | 0.78 ± 0.07 (0.72-0.9) | 1.34 ± 0.11 (1.2-1.5) | 0.9 ± 0.07 (0.7-1.0) |
| D % | 69 ± 6.4 (61-78) | 52 ± 9.6 (50.8-64.7) | 59.5 ± 5.1 (50-68) | 60.5 ± 5 (51-66) | 48 ± 3.2 (41-50) |
| E % | 234 ± 22 (200-250) | 189.5 ± 27 (147-214) | 267 ± 27 (232-306) | 183.4 ± 21.7 (154-210) | 93 ± 8 (70-110) |
| Spicules | 81 ± 3.6 (75-88) | -- | 69 ± 4.6 (65-72) | -- | -- |
| Gubernaculum | 41 ± 5 (37-45) | -- | 37.8 ± 18.6 (30-42) | -- | -- |
| SW % | 151 ± 39 (131-166) | -- | 152 ± 10.4 (139-172) | -- | -- |
| GS % | 60 ± 6.3 (51-66) | -- | 55 ± 5.2 (44-58.5) | -- | -- |
| H% | -- | -- | -- | -- | 39 ± 0.7 (35-44) |

L = Total body length; MBW= Maximum body width; EP= Excretory pore; ES= Distance from anterior end to oesphagus; TL= Tail length; D%= Distance from anterior end to excretory pore divided by pharynx length × 100; E % = Distance from anterior end to excretory pore divided by tail length× 100; SW = Spicule length divided by anal body diam.; GS = Gubernaculum length divided by spicule length; H = Hyaline portion × 100/tail length.

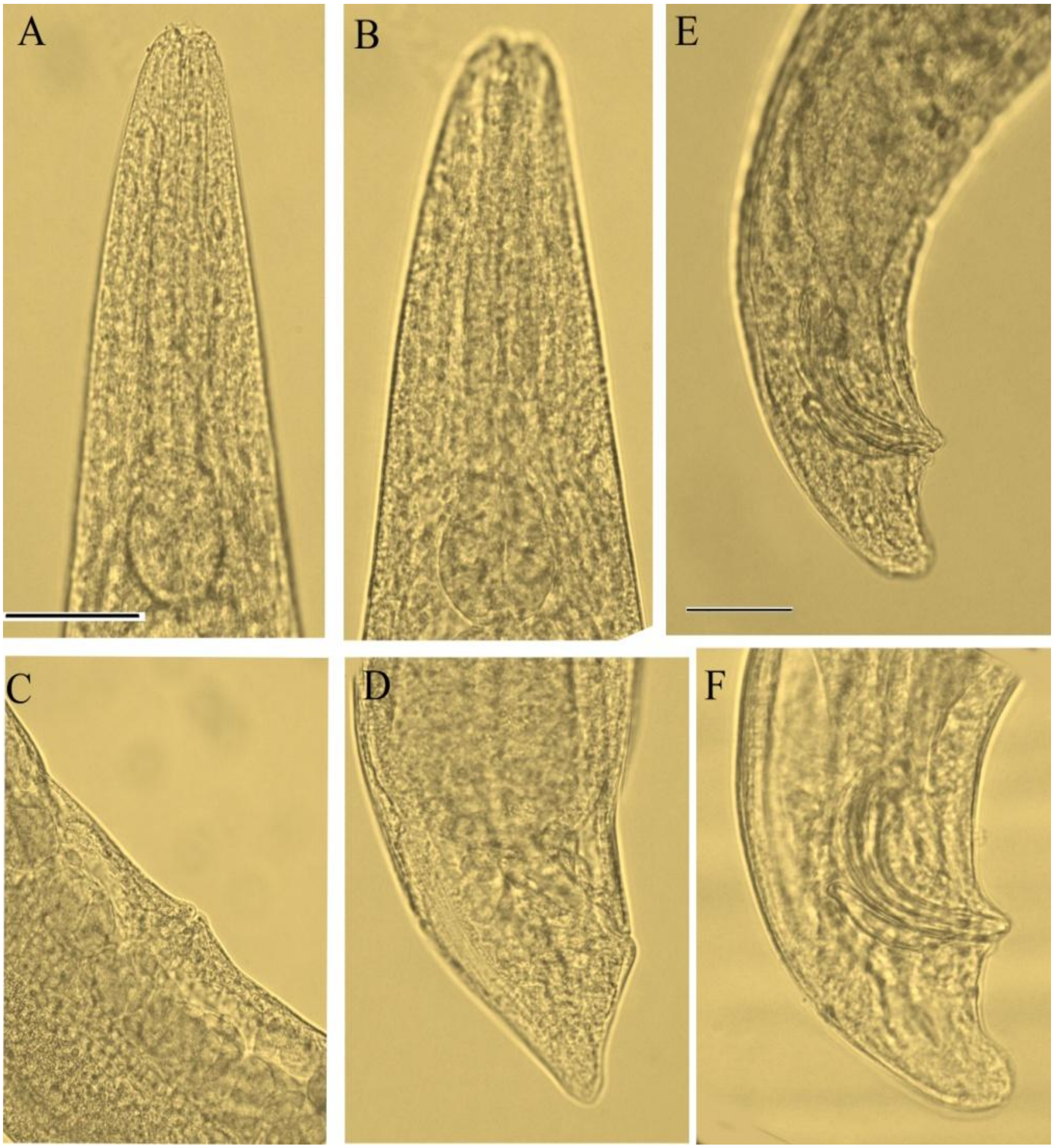


**Fig. 2.** Light micrograph of *Steinernema affine*. Female first generation A-D: A, B. Anterior region; C. Vulval region; D. Tail region; Male first generation: E, F. Tail region (Scale bars: A-D, F= 10 µm, E = 20 µm).

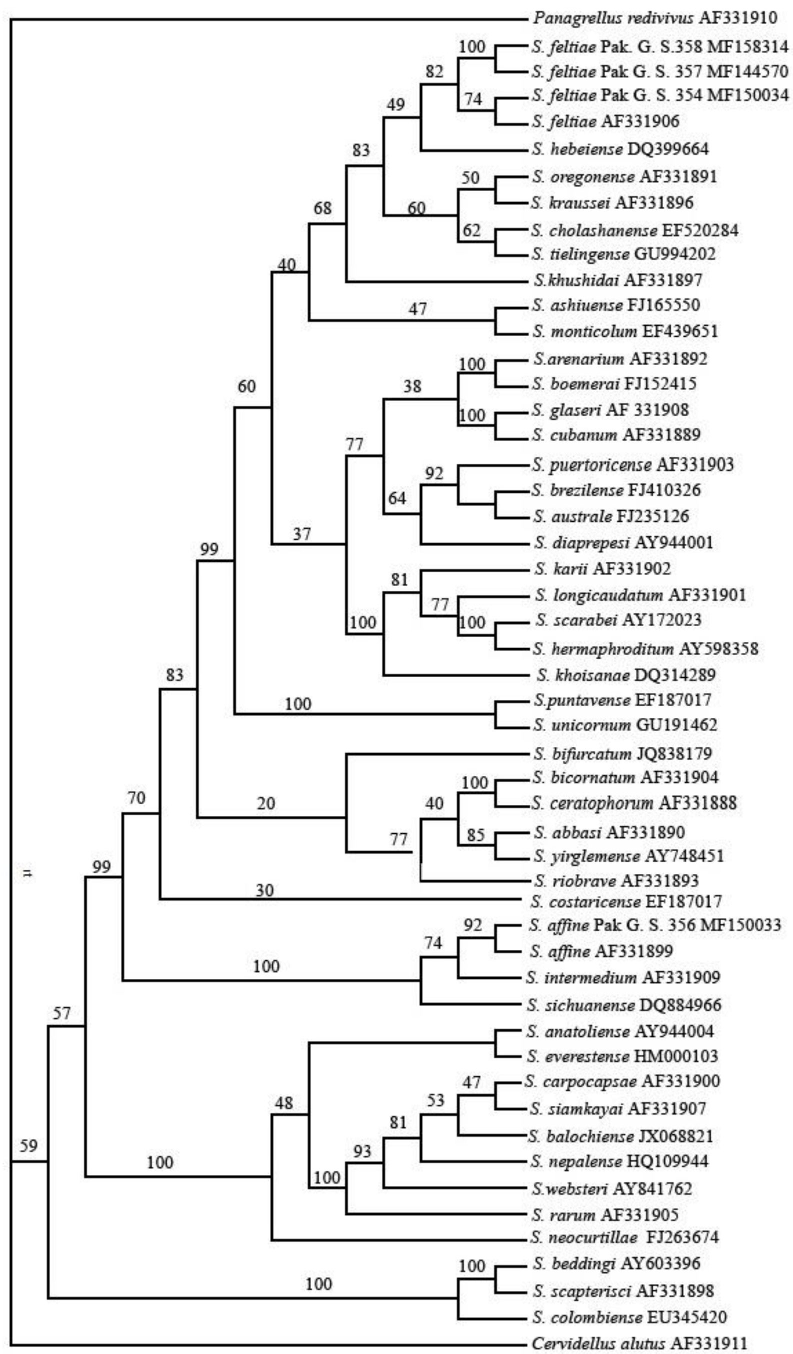


**Fig. 3.** Phylogenetic relationships of new records of *Steinernema* species, 1 isolate of *S. affinae* and 3 isolates of *S. feltiae* with 46 species of *Steinernema* based on the D2-D3 rDNA sequences from GenBank. *Cervidellus alutus* (AF331911) was used as out group. Numbers at the nodes represent bootstrap proportion for Maximum Parsimony of 50% or more.

***S. cholashanense*** **Nguyen, Puza & Mracek, 2008 (Fig. 4. A-F)**

**Measurements: Table 2**

During the survey of Gilgit-Baltistan, Pakistan a new record *S. cholashanense* Pak.G.S.350 and Pak.G.S.355 were isolated from apple (*Malus pumila*) of village Nasirabad, District Hunza and from walnut (*Juglans nigra*) of Village Danyour, District Gilgit, respectively. These isolates were morphologically and genetically characterized by ITS rDNA region.

**Remarks:** The sequences were deposited in GenBank under accession numbers MF125282 and MF039642. Maximum parsimony of ITS regions shows that the alignment resulted in 1094 characters, 91 are constant, 196 are variable characters are parsimony-uninformative and 807 characters (included) are parsimony-informative. The phylogenetic relationships between 46 species of *Steinernema* are presented in Fig. 5 (for MP, tree length = 4812, Cl = 0.4362, RI = 0.6342, RC = 0.2766, HI = 0.5638). The sequence length composition (Table 3) and pair wise distance have shown in Table 4. *S. cholashanense* was molecularly identified by utilizing ITS rDNA region.

***S. feltiae*** **Filipjev, 1934**

Three isolates of *S. feltiae* Pak.G.S.354 (MF150034), Pak.G.S.357 (MF144570) and Pak.G.S.358 (MF158314) were also found during survey of *Prunus armeniaca*, *Prunus persica* and *Malus pumila* from Village Hoper and Thole, District Nager, Gilgit-Baltistan. These isolates were identified through morphologically and molecularly with D2D3 rDNA regions (Table 5).

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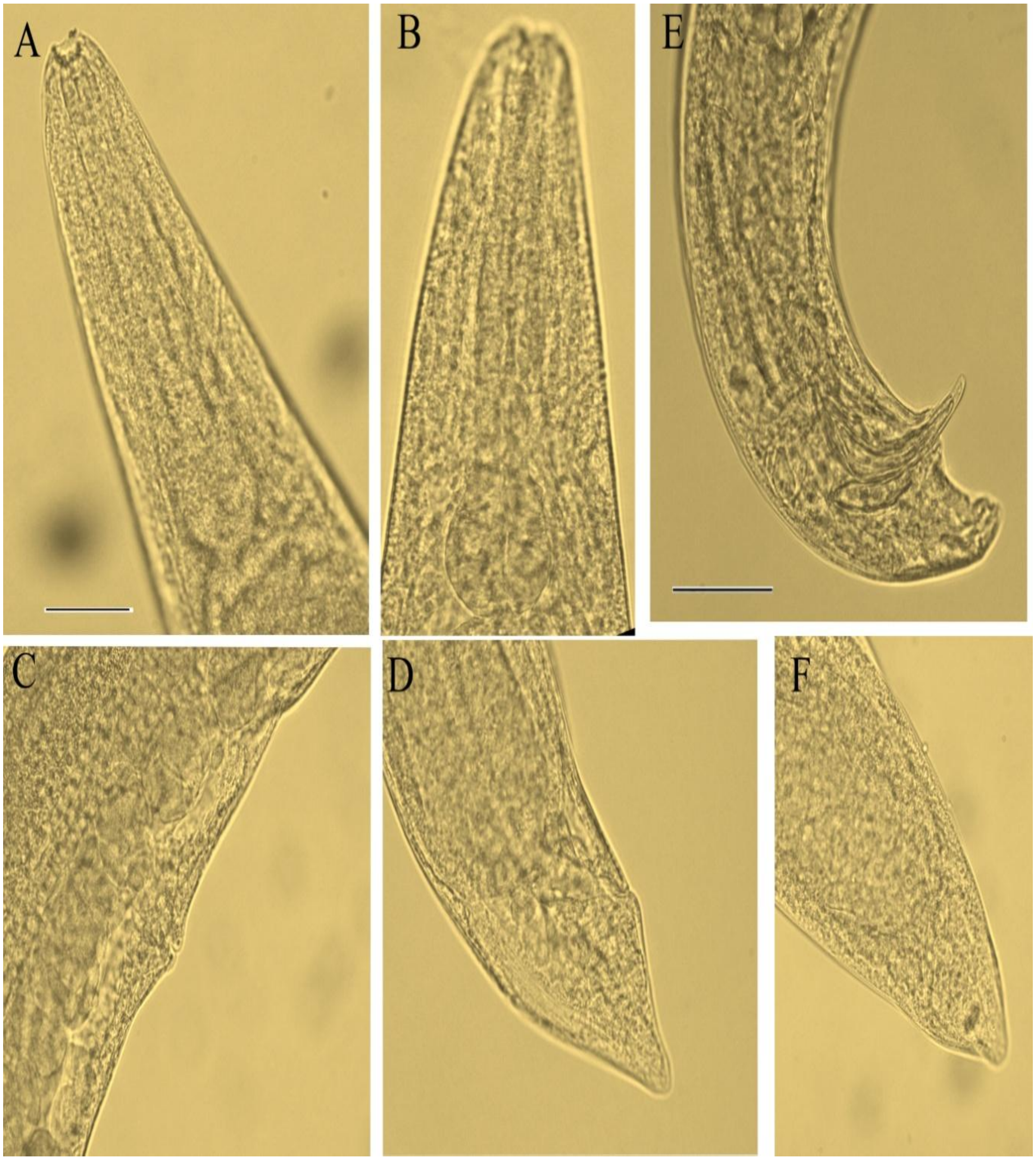


**Fig. 4.** Light micrograph of *Steinernema cholashanense*: Female first generation A-D, F: A, B. Anterior region; C. Vulval region; D, F. Tail region; Male first generation: E. Tail region. (Scale: A-D, F= 10 µm, E = 20 µm).

**Table 2. Morphometric values of *S. cholashanense* (n=25 each). All measurements are in µm and in the form of mean ± SD (range).**

| Characters | First Generation | | Second Generation | | Infective stage juveniles |
|---|---|---|---|---|---|
| | Male | Female | Male | Female | |
| TL | 1536 ± 206 (1185-2047) | 4280 ± 549 (1302-4975) | 830 ± 28 (712-950) | 1982 ± 195 (1435-2758) | 815 ± 45 (700-875) |
| MBW | 143 ± 35. (85-178) | 235 ± 25 (133-262) | 71 ± 8.8 (50-89) | 125 ± 18 (95-156) | 26 ± 1.5 (24-30) |
| EP | 110 ± 18 (90-125) | 99 ± 11 (75-125) | 59 ± 5.3 (45-72) | 86 ± 5.3 (65-105) | 65 ± 1.3 (53-68) |
| NR | 115 ± 11 (90-130) | 132 ± 12 (125-150) | 94 ± 6.4 (82-104) | 112 ± 8.8 (90-142) | 79 ± 2.1 (65-94) |
| ES | 162 ± 14 (125-184) | 185 ± 10 (158-225) | 119 ± 5.8 (108-129) | 176 ± 12 (145-200) | 121 ± 3.6 (105-129 ) |
| Testis reflection | 450 ± 58 (300-680) | -- | 212 ± 9.3 (200-256) | -- | -- |
| TL (Ijs with sheath) | 37 ± 4 (32-45) | 55 ± 5.3 (45-62) | 39 ± 4.1 (27-48) | 61 ± 3.8 (45-72) | 103 ± 6.3 (95-115) |
| Anal body width | 45 ± 5.6 (35-56) | 73 ± 12.5 (62-87) | 40 ± 3.5 (35-47) | 40 ± 1.5 (33-47) | 16 ± 0.8 (14-18) |
| V% | -- | 50 ± 3.2 (49-52) | -- | 51 ± 2.3 (45-56) | -- |
| a | 13 ± 3.6 (8.2-21) | 21 ± 1.2 (20-24.5) | 12 ± 2.1 (9-14) | 14.3 ± 1.5 (11-16) | 26.5 ± 1.5 (22-31) |
| b | 8.3 ± 1.0 (6.5-9.5) | 19 ± 1.8 (18-21.3) | 5.9 ± 1.2 (5.5-6.8) | 11.1 ± 1.4 (9-12.5) | 6.3 ± 0.5 (6.2-7.5) |
| c | 39.7 ± 4.2 (34-48) | 63 ± 9 (49-79) | 23 ± 1.8 (17-30) | 35 ± 2.4 (25-43) | 11 ± 1.0 (10-13) |
| c' | 0.7 ± 0.1 (0.5-0.85) | 0.7 ± 0.1 (0.6-0.9) | 1.0 ± 0.1 (0.8-1.3) | 1.1 ± 0.1 (1-1.5) | 4.1 ± 0.2 (3.2-4.9) |
| D % | 67 ± 8.5 (51-79) | 51 ± 7.2 (33-60) | 49 ± 3.7 (45-57) | 48 ± 3.1 (45-56) | 47 ± 2.3 (42-51) |
| E % | 274 ± 35 (193-350) | 183 ± 22 (125-240) | 187 ± 18 (130-210) | 153 ± 9.6 (130-170) | 84 ± 4.3 (75-95) |
| Spicules | 63 ± 3.7 (58-70) | -- | 45 ± 2.1 (40-50) | -- | -- |
| Gubernaculum | 36 ± 2.5 30-40 | -- | 29 ± 1.8 (25-37) | -- | -- |
| SW % | 120 ± 13 (90-140) | -- | 107 ± 7.3 (85-130) | -- | -- |
| GS % | 69 ± 4.5 (55-79) | -- | 53 ± 2.1 (51-68) | -- | -- |
| H% | -- | -- | -- | -- | 35 ± 4.8 (33-49) |

L = Total body length; MBW= Maximum body width; EP= Excretory pore; ES= Distance from anterior end to oesphagus; TL= Tail length; D%= Distance from anterior end to excretory pore divided by pharynx length × 100; E % = Distance from anterior end to excretory pore divided by tail length× 100; SW = Spicule length divided by anal body diam.; GS = Gubernaculum length divided by spicule length; H = Hyaline portion × 100/tail length.

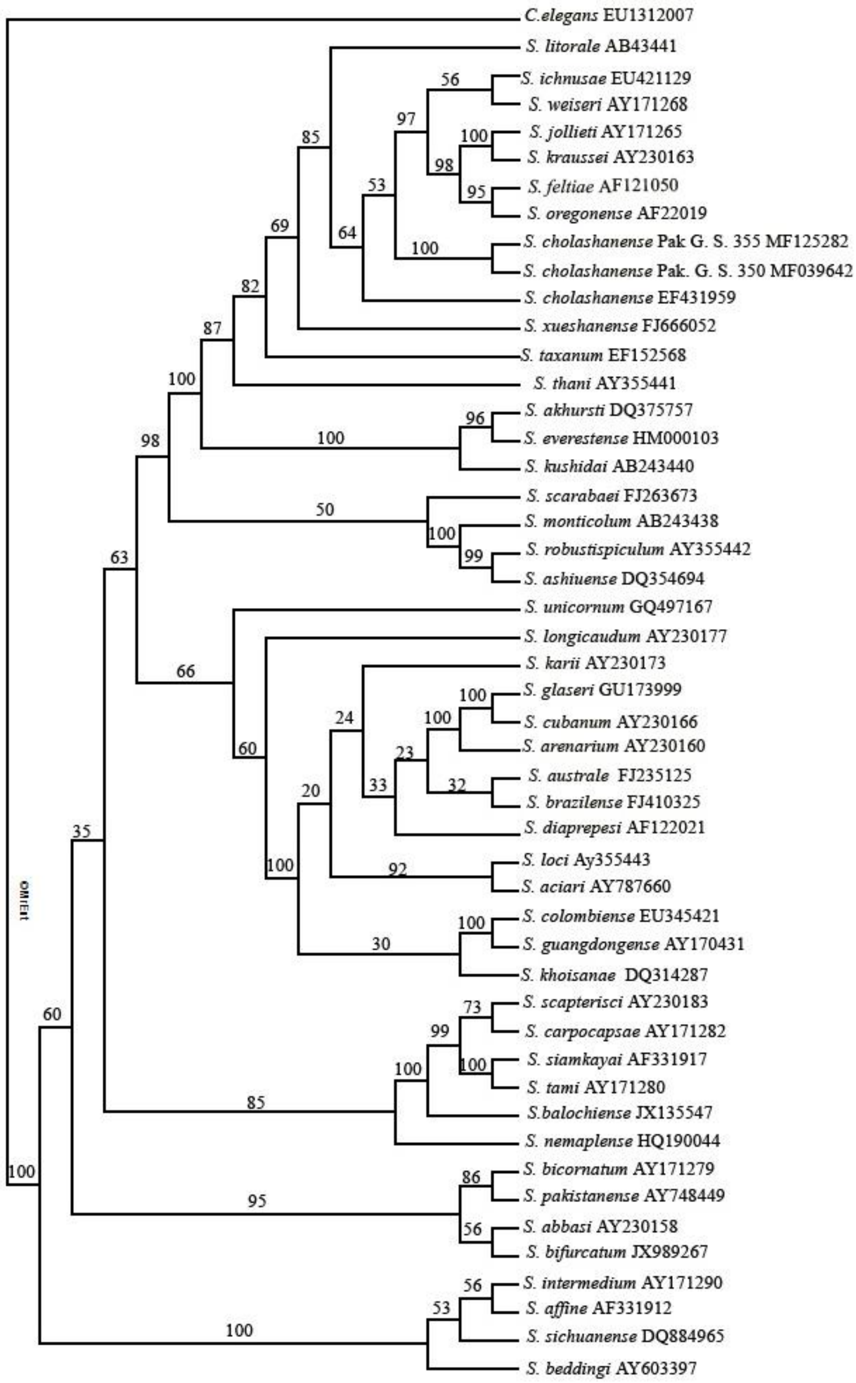


**Fig. 5.** Phylogenetic relationships of new record of *S. cholashanense* with 47 species of *Steinernema* based on the ITS- rDNA sequences from GenBank. *Cervidellus elegans* (EU131007) was used as out group. Numbers at the nodes represent bootstrap proportion for Maximum Parsimony of 50% or more.

**Table 3. Sequence length and composition of ITS and D2D3 regions of species of *Steinernema* species.**

| Species<br>ITS region | A | C | G | T |
|---|---|---|---|---|
| *S. cholashanense* Pak. G. S.350 | 0.26667 | 0.13333 | 0.25000 | 0.35000 |
| *S. cholashanense* Pak. G. S.355 | 0.36667 | 0.23333 | 0.13333 | 0.21667 |
| *S. jollieti* | 0.26667 | 0.12500 | 0.25000 | 0.35833 |
| *S. weiseri* | 0.26250 | 0.13333 | 0.25000 | 0.35833 |
| *S. ichnuase* | 0.26667 | 0.13750 | 0.24583 | 0.35000 |
| *S. feltiae* | 0.26667 | 0.12917 | 0.25000 | 0.34167 |
| *S. xueshanense* | 0.26617 | 0.12500 | 0.25000 | 0.35833 |
| *S. litorale* | 0.26250 | 0.12917 | 0.25000 | 0.35417 |
| *S. cholashanense* | 0.26667 | 0.12917 | 0.24167 | 0.36250 |
| *S. kraussei* | 0.26667 | 0.14583 | 0.25417 | 0.36250 |
| *S. oregonense* | 0.26667 | 0.12917 | 0.25000 | 0.35417 |
| *S. taxanum* | 0.26667 | 0.12917 | 0.24167 | 0.36250 |
| *S. thani* | 0.27500 | 0.17350 | 0.24583 | 0.34167 |
| *S. akhursti* | 0.27500 | 0.12917 | 0.24583 | 0.35000 |
| *S. everstense* | 0.27500 | 0.12917 | 0.24583 | 0.35000 |
| *S. kushidai* | 0.27500 | 0.13333 | 0.24583 | 0.34583 |
| *S. monticolum* | 0.28750 | 0.12500 | 0.23750 | 0.35000 |
| *S. ashiuense* | 0.29583 | 0.11667 | 0.22917 | 0.35833 |
| *S. scarabaei* | 0.26667 | 0.13750 | 0.25833 | 0.33750 |
| *S. unicornum* | 0.30000 | 0.13750 | 0.22917 | 0.33333 |
| *S. longicoedum* | 0.28750 | 0.14167 | 0.25417 | 0.31667 |
| *S. karrii* | 0.28333 | 0.15000 | 0.25417 | 0.31250 |
| *S. carpocapsae* | 0.27917 | 0.14583 | 0.25000 | 0.32500 |
| *S. balochiense* | 0.27500 | 0.14167 | 0.24583 | 0.33750 |
| *S. siamkayai* | 0.28750 | 0.13750 | 0.24583 | 0.32917 |
| *S. tami* | 0.29167 | 0.15000 | 0.24167 | 0.31667 |

| **Species**<br>**D2D3 region** | **A** | C | G | T |
|---|---|---|---|---|
| *S. feltiae* Pak. G. S. 358 | 0.29848 | 0.16190 | 0.32381 | 0.22381 |
| *S. affine* Pak. G. S. 356 | 0.23810 | 0.17619 | 0.33810 | 0.24762 |
| *S. feltiae* Pak. G. S. 357 | 0.25238 | 0.30000 | 0.21905 | 0.22857 |
| *S. feltiae* Pak. G. S. 354 | 0.24762 | 0.31429 | 0.20952 | 0.22857 |
| *S. feltiae* | 0.24286 | 0.17619 | 0.33333 | 0.24762 |
| *S. hebeiense* | 0.24268 | 0.17619 | 0.33333 | 0.24762 |
| *S. oregonense* | 0.24286 | 0.17619 | 0.33810 | 0.24286 |
| *S. cholashanense* | 0.24286 | 0.17619 | 0.33333 | 0.24762 |
| *S. tielingense* | 0.23810 | 0.17619 | 0.33333 | 0.25238 |
| *S. kushidai* | 0.25238 | 0.6190 | 0.32857 | 0.25714 |
| *S. ashiuense* | 0.23333 | 0.17143 | 0.33810 | 0.25714 |
| *S. glesseri* | 0.23810 | 0.19524 | 0.33810 | 0.22857 |
| *S. cubanum* | 0.23810 | 0.19048 | 0.33810 | 0.23333 |
| *S. diaprepsi* | 0.24286 | 0.17619 | 0.33333 | 0.24762 |
| *S. citrai* | 0.24762 | 0.17619 | 0.33333 | 0.24762 |
| *S. scarabei* | 0.25238 | 0.16667 | 0.32857 | 0.30476 |
| *S. unicornum* | 0.29524 | 0.14762 | 0.33810 | 0.33333 |
| *S. affine* | 0.26667 | 0.14286 | 0.30000 | 0.29048 |
| *S. intermedium* | 0.26667 | 0.14762 | 0.30476 | 0.28095 |
| *S. sichuanense* | 0.27619 | 0.13810 | 0.30476 | 0.28095 |
| *S. everstense* | 0.29524 | 0.16667 | 0.20476 | 0.33333 |
| *S. carpocapsae* | 0.25238 | 0.14762 | 0.29524 | 0.30476 |
| *S. siamkayai* | 0.24286 | 0.17619 | 0.33333 | 0.24762 |
| *S. balochiense* | 0.22857 | 0.19048 | 0.34286 | 0.23810 |
| *S. websteri* | 0.21857 | 0.16762 | 0.29524 | 0.24762 |
| *S. rarum* | 0.23810 | 0.14286 | 0.32384 | 0.2462 |

**Table 4. Pairwise distance of ITs regions of *Steinernema* species.**

| S.# | Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *S. cholashanense* Pak. G. S.350 | - | | | | | | | | | | | | | | | | | | | | | |
| 2 | *S. cholashanense* Pak. G. S.355 | 145 | - | | | | | | | | | | | | | | | | | | | | |
| 3 | *S. jollieti* | 96 | 2 | - | | | | | | | | | | | | | | | | | | | |
| 4 | *S. weiseri* | 96 | 2 | 130 | - | | | | | | | | | | | | | | | | | | |
| 5 | *S. ichnuase* | 96 | 3 | 130 | 17 | - | | | | | | | | | | | | | | | | | |
| 6 | *S. feltiae* | 96 | 5 | 132 | 17 | 19 | - | | | | | | | | | | | | | | | | |
| 7 | *S. xueshanense* | 96 | 2 | 131 | 14 | 16 | 32 | - | | | | | | | | | | | | | | | |
| 8 | *S. litorale* | 96 | 3 | 130 | 15 | 17 | 33 | 19 | - | | | | | | | | | | | | | | |
| 9 | *S. cholashanense* | 95 | 3 | 132 | 13 | 15 | 31 | 17 | 15 | - | | | | | | | | | | | | | |
| 10 | *S. kraussei* | 96 | 9 | 134 | 15 | 20 | 31 | 22 | 18 | 17 | - | | | | | | | | | | | | |
| 11 | *S. oregonense* | 96 | 1 | 131 | 15 | 17 | 34 | 19 | 17 | 15 | 25 | - | | | | | | | | | | | |
| 12 | *S. taxanum* | 95 | 6 | 130 | 16 | 18 | 33 | 22 | 20 | 18 | 27 | 29 | - | | | | | | | | | | |
| 13 | *S. thani* | 96 | 9 | 134 | 17 | 19 | 35 | 21 | 19 | 17 | 26 | 28 | 19 | - | | | | | | | | | |
| 14 | *S. akhursti* | 96 | 7 | 131 | 13 | 15 | 29 | 15 | 11 | 9 | 19 | 21 | 12 | 18 | - | | | | | | | | |
| 15 | *S. everstense* | 95 | 61 | 131 | 13 | 15 | 29 | 15 | 11 | 9 | 19 | 21 | 12 | 18 | 13 | - | | | | | | | |
| 16 | *S. kushidai* | 95 | 10 | 131 | 13 | 14 | 18 | 30 | 18 | 14 | 12 | 22 | 24 | 13 | 21 | 16 | - | | | | | | |
| 17 | *S. monticolum* | 97 | 10 | 131 | 18 | 21 | 34 | 21 | 19 | 17 | 27 | 29 | 20 | 26 | 22 | 25 | 21 | - | | | | | |
| 18 | *S. ashiuense* | 98 | 13 | 132 | 21 | 21 | 34 | 23 | 21 | 19 | 29 | 31 | 22 | 28 | 25 | 27 | 23 | 22 | - | | | | |
| 19 | *S. scapterisci* | 98 | 35 | 131 | 35 | 31 | 41 | 38 | 34 | 33 | 39 | 41 | 32 | 36 | 33 | 37 | 36 | 33 | 34 | - | | | |
| 20 | *S. carpocapsae* | 94 | 36 | 132 | 33 | 32 | 44 | 38 | 34 | 35 | 41 | 41 | 34 | 36 | 35 | 40 | 37 | 34 | 34 | 36 | - | | |
| 21 | *S. siamkayai* | 96 | 38 | 129 | 37 | 36 | 48 | 44 | 38 | 39 | 45 | 45 | 40 | 39 | 38 | 45 | 41 | 38 | 39 | 36 | 38 | - | |
| 22 | *S. tami* | 97 | 38 | 133 | 39 | 38 | 51 | 45 | 40 | 41 | 47 | 45 | 42 | 42 | 41 | 46 | 42 | 39 | 43 | 38 | 40 | 41 | - |
| 23 | *S. balochiense* | 98 | 34 | 131 | 34 | 33 | 34 | 40 | 34 | 35 | 42 | 42 | 36 | 36 | 35 | 41 | 32 | 34 | 36 | 33 | 32 | 34 | 35 |

**Table 5. Pairwise distance of D2 D3 regions of *Steinernema* species.**

| S. # | **Species** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *S. feltiae* Pak. G. S. 358 | - | | | | | | | | | | | | | | | | | | | | | |
| 2 | *S. affine* Pak. G. S. 356 | 90 | - | | | | | | | | | | | | | | | | | | | | |
| 3 | *S. feltiae* Pak. G. S. 357 | 144 | 144 | - | | | | | | | | | | | | | | | | | | | |
| 4 | *S. feltiae* Pak. G. S. 354 | 144 | 141 | 142 | - | | | | | | | | | | | | | | | | | | |
| 5 | *S. feltiae* | 82 | 2 | 34 | 8 | - | | | | | | | | | | | | | | | | | |
| 6 | *S. litorale* | 128 | 124 | 120 | 144 | 141 | - | | | | | | | | | | | | | | | | |
| 7 | *S. hebeiense* | 82 | 2 | 34 | 144 | 141 | 0 | - | | | | | | | | | | | | | | | |
| 8 | *S. oregonense* | 81 | 5 | 34 | 144 | 142 | 4 | 144 | - | | | | | | | | | | | | | | |
| 9 | *S. cholashanense* | 81 | 8 | 31 | 144 | 141 | 6 | 125 | 6 | - | | | | | | | | | | | | | |
| 10 | *S. tielingense* | 82 | 4 | 33 | 144 | 140 | 2 | 121 | 2 | 121 | - | | | | | | | | | | | | |
| 11 | *S. kushidai* | 82 | 10 | 32 | 145 | 142 | 9 | 124 | 9 | 124 | 9 | - | | | | | | | | | | | |
| 12 | *S. ashiuense* | 85 | 16 | 39 | 143 | 140 | 15 | 126 | 16 | 126 | 15 | 13 | - | | | | | | | | | | |
| 13 | *S. arenarium* | 85 | 20 | 40 | 143 | 140 | 19 | 127 | 19 | 127 | 19 | 20 | 18 | - | | | | | | | | | |
| 14 | *S. boemeri* | 84 | 19 | 40 | 146 | 143 | 18 | 127 | 18 | 127 | 18 | 17 | 17 | 16 | - | | | | | | | | |
| 15 | *S. glesseri* | 86 | 19 | 38 | 142 | 139 | 19 | 127 | 19 | 127 | 19 | 20 | 18 | 19 | 19 | - | | | | | | | |
| 16 | *S. affine* | 90 | 35 | 00 | 135 | 142 | 34 | 120 | 34 | 120 | 34 | 35 | 34 | 32 | 33 | 30 | - | | | | | | |
| 17 | *S. intermedium* | 90 | 35 | 2 | 145 | 142 | 34 | 120 | 34 | 120 | 34 | 35 | 34 | 32 | 33 | 30 | 120 | - | | | | | |
| 18 | *S. sichuanense* | 88 | 35 | 4 | 143 | 140 | 34 | 119 | 34 | 119 | 34 | 35 | 34 | 32 | 33 | 30 | 119 | 119 | - | | | | |
| 19 | *S. carpocapsae* | 96 | 87 | 90 | 147 | 146 | 88 | 134 | 88 | 134 | 88 | 86 | 88 | 84 | 89 | 86 | 134 | 134 | 87 | - | | | |
| 20 | *S. siamkayai* | 84 | 18 | 37 | 145 | 142 | 17 | 125 | 17 | 125 | 17 | 16 | 16 | 17 | 17 | 17 | 125 | 125 | 18 | 21 | - | | |
| 21 | *S. balochiense* | 85 | 21 | 37 | 144 | 141 | 21 | 127 | 21 | 127 | 21 | 22 | 20 | 19 | 21 | 19 | 127 | 127 | 16 | 25 | 24 | - | |
| 22 | *S. websteri* | 82 | 29 | 37 | 144 | 141 | 28 | 126 | 28 | 126 | 28 | 29 | 26 | 27 | 26 | 28 | 126 | 126 | 32 | 25 | 32 | 29 | - |
| 23 | *S. rarum* | 81 | 20 | 34 | 143 | 140 | 20 | 125 | 20 | 125 | 21 | 20 | 19 | 18 | 20 | 18 | 125 | 125 | 15 | 24 | 23 | 22 | 22 |

*COST 812 Biotechnology*: *Genetics of Entomopathogenic Nematodes-Bacterium Complexes*, Proceedings of Symposium & Workshop. St. Patrick's College, Maynooth, Co., Kildare, Ireland. European Commission, DGXII, Luxembourg, pp. 178-187.

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(Received: 10 March, 2017)