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Morphological and molecular characterization of a new isolate of *Steinernema feltiae* (Filipjev, 1934) from Vancouver, Canada, with morphometrical comparison with the topotype population from Russia

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Abstract

Steinernema feltiae, strain British Columbia (BC) was collected from Vancouver, Canada. SEM, morphometrical and molecular studies were conducted and the results reported. This article presents the SEM study of different stages of *S. feltiae*. The significant characteristic found in this study is that the formula of the lateral field of *S. feltiae* is 2, 7, 8, 7, 4, 2. Morphometric comparison of the new isolate and the topotype population (strain RU from Russia) showed that the body length, distance from anterior end to excretory pore, tail length, and hyaline length are significantly different. Molecular studies of rDNA ITS regions showed that the strain BC is closely related to the topotype of *S. feltiae* (strain RU) from Russia. Sequence length of strain RU is 980 base pairs compared to 977 base pairs for strain BC. Intraspecific relationships among strains of *S. feltiae* showed that geographic distribution is associated with the genetic differences, but the differences are not sufficient to delimit new species.

Key words: Canada, *Steinernema feltiae*, entomopathogenic nematodes, morphology, molecular characterization, Greater Vancouver

Introduction

Steinernema feltiae (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 has been found from many places all over the world (Hominick, 2002). This species was originally described from a larva of *Agrotis (*syn. *Feltia) segetum* from Russia (Filipjev, 1934). Later,

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Bovien (1937) described *S. bibionis* which was synonymized with *S. feltiae* (Poinar, 1989). During late winter and early spring 2003, a search for entomopathogenic nematodes in coastal areas of the Greater Vancouver area and the Fraser Valley region was conducted. This search revealed four entomopathogenic nematode (EPN) species (Mráček and Webster, 1993; Mráček *et al.*, 2005), including one isolate, which is close to *S. feltiae and S. oregonense*. Morphological, molecular, and cross-breeding studies showed that the nematode is a strain of *S. feltiae*. Although the species was studied in more detail by Wouts (1980) in New Zealand, and by Mráček *et al*. (1982) in the former Czechoslovakia, and some SEM photographs were reported (Haukeland, 1993; Mráček & Bednarek, 1991), additional electron microscopy and molecular characterization were needed for more understanding about this important nematode species. For these reasons, the studies of this isolate were conducted and are reported herein.

Material and methods

Nematode populations

The population was recovered from a soil sample by a baiting technique (Mráček, 1980) from White Rock, a coastal area of Greater Vancouver characterized by brown, sandy soil. The nematode was named BC (British Columbia) strain, reared subsequently on *Galeria mellonella* larvae, and established as a laboratory culture at the Simon Fraser University in Burnaby. Part of this culture was moved to the Institute of Entomology in České Budějovice, Czech Republic, and kept in a refrigerator at 4–8ºC. The topotype strain Ustinow from Russia (RU) was used also for these studies.

Morphological characterization

Light microscopy

For morphometrical studies, ten *G. mellonella* were exposed to about 2000 infective juveniles in a Petri dish (100 x 15 mm) lined with two moistened filter papers. The Petri dish was kept at room temperature (25ºC). First- and second-generation adult nematodes were obtained by dissecting infected insects at 2 to 4 days and 5 to 7 days, respectively, after the insects died.

Juvenile and adult nematodes were heat-killed on glass slides in a drop of water, and mounted in aqueous mounts under a coverglass. The measurements and examination of morphology were performed with an Amplival light microscope (Carl Zeiss, Jena). A number of infective juveniles and adults of first and second generations were fixed in a hot TAF (Courtney *et al*., 1955), transferred to glycerin by a slow evaporation method (Seinhorst, 1959) and mounted in anhydrous glycerin for photographing and for deposit in the nematode collection in the Laboratory of Insect Pathology, Institute of Entomology, Czech Academy of Sciences, České Budějovice, Czech Republic. For photomicrographs,

a Zeiss Axioplan 2 photomicroscope with DIC optics was used. Coverglass supports were used in all cases to avoid flattening of specimens.

Scanning electron microscopy (SEM)

Adults of the first generation and infective juveniles were fixed in 4% formalin buffered with 0.1 M sodium cacodylate at pH 7.2 for 24 hours at 8ºC. They were post-fixed with 2% osmium tetroxide solution for 12 hours at 25ºC, dehydrated in a graded ethanol series, critical point dried with liquid CO_2, mounted on SEM stubs, and coated with gold (Nguyen & Smart, 1995). Spicules and gubernacula were prepared as suggested by Nguyen & Smart (1990).

Cross hybridization

The two isolates, BC (British Columbia) strain, and the topotype strain, Ustinow from Russia (RU) were used. The cross hybridization test method described by Nguyen & Duncan (2002) was used in this study.

Molecular characterization

Extraction of DNA

DNA was extracted from a single female using a DNeasy Tissue kit (QIAGEN Inc., Valencia, CA). Methods in the instruction handbook were followed in this study.

PCR amplification

The ITS regions of rDNA were amplified using the methods reported by Nguyen *et al*. (2001), except all PCR reactions were run in a PTC-200 Peltier Thermo Cycler (MJ Research, Inc., Waltham, MA) with the cycling profile: 1 cycle of 94ºC for 7 min followed by 35 cycles of 94ºC for 60 sec, 50ºC for 60 sec, 72ºC for 60 sec. The last step was 72ºC for 10 min.

Sequencing

PCR products were purified with a QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA). Purified DNA was sequenced in both directions (with four primers) on automated sequencers as reported previously (Nguyen *et al*., 2001). Sequencher™, version 4.2 (Gene Code Corporation, Ann Arbor, MI) was used for sequence editing and verifying base-calls.

Multiple alignments

Sequences of studied species were aligned using the default parameters of Clustal X (Thompson *et al*., 1997), then optimized manually in MacClade 4.0 (Maddison & Maddison, 2002).

Phylogenetic relationships

Sequences of the internal transcribed spacer regions (ITS) of *Steinernema* species have been used by different authors (Stock *et al*., 2001; Nguyen *et al*., 2001; Nguyen & Duncan, 2002; Nguyen & Adams, 2003; Spiridonov *et al*., 2005) in taxonomic and phylogenetic studies. In this paper, sequences of these regions were used.

Sequences of the following taxa were used in this study: *Steinernema feltiae* strain A1 (AY230169), A2 (AY230170) from England, MY9 (AY230178) from Japan, T92 (AY230185) from Indonesia (Spiridonov *et al*., 2004), Microbio (AY170335) from China (GenBank), SN (AF121050) from France (Nguyen *el al.*, 2001). All of these sequences were reported by Spiridonov *et al*. (2005). *Steinernema feltiae* BC strain (DQ310470) from Canada, and RU (DQ310469) from Russia. These strains were selected because they were from different geographic and climatic conditions (four from Europe, three from Asia and one from America). Sequences reported by Nguyen & Adams (2003) also were used for phylogenetic analysis.

Molecular phylogenetic relationships were obtained by maximum parsimony (MP) using PAUP, 4.0b8 (Swofford, 2002). All data were assumed to be unordered, all characters were treated as equally weighted, and gaps were treated as missing data. MP was performed with a heuristic search (simple stepwise addition, tree-bisection-recombination branch swapping). For the phylogenetic analysis *Steinernema intermedium* was treated as the outgroup taxon for resolving relationships among the rest of the *Steinernema* species (Nguyen *et al*., 2001; Nguyen & Adams, 2003).

Results and discussion

First generation male

Head truncate, four cephalic and six labial papillae prominent, but cephalic papillae are not as prominent as those of *S. glaseri* (Fig. 1A, Nguyen & Smart, 1995). Posterior region of the body curved ventrally (Fig. 1C,D), mucron very long, usually straight or curved dorsally (Fig. 1D–F). One single, ventral, precloacal papilla, and eleven pairs of papillae (Fig. 1B). Six pairs are precloacal, subventral, one pair adanal, one pair lateral, two pairs subterminal subventral, and one pair post cloacal, subventral (Fig. 1D,E). Spicules are similar to those of other isolate of *S. feltiae* and *S. oregonense* (Fig. 2) with spicule head much longer than width (length/width ratio = 1.4–2.1), shaft very short or absent, spicule blade not well curved (Fig. 2C,D), velum obscure. Gubernaculum thin, almost straight, anterior end curved ventrally.

First generation female

Face view of the female is similar to other *Steinernema* species: four cephalic and six labial papillae prominent (Fig. 3B). Occasionally (5%), an abnormal face view was observed (Fig. 3A). Amphids small, close to lateral papillae. Vulva protruding (Fig. 3C, Fig. 4E,F), epiptygma absent. Tail always with long, pointed terminus which is different from female tail of other species.

Infective juvenile

Head not annulated, longer than that of most other species. Lateral field begins with

one line from the first annule (Fig. 5A). At annules 8–10, 2 additional lines appear to form two ridges (Fig. 5B). Between excretory pore and base of esophagus, the lateral field changes from two ridges to seven ridges (Fig. 5C). A short distance posteriorly, the central ridge divides into two, making a total of eight ridges, the maximum number of ridges in lateral field (Fig. 5C). In posterior third of body, the lateral field reduces gradually to seven, then to four ridges near anus (Fig. 5E), and finally to two ridges at phasmid (Fig. 5F). The formula of lateral field of *S. feltiae* is 2, 7, 8, 7, 4, 2. Phasmid located anterior to mid-tail, right on the ventral lateral line (Fig. 5F).

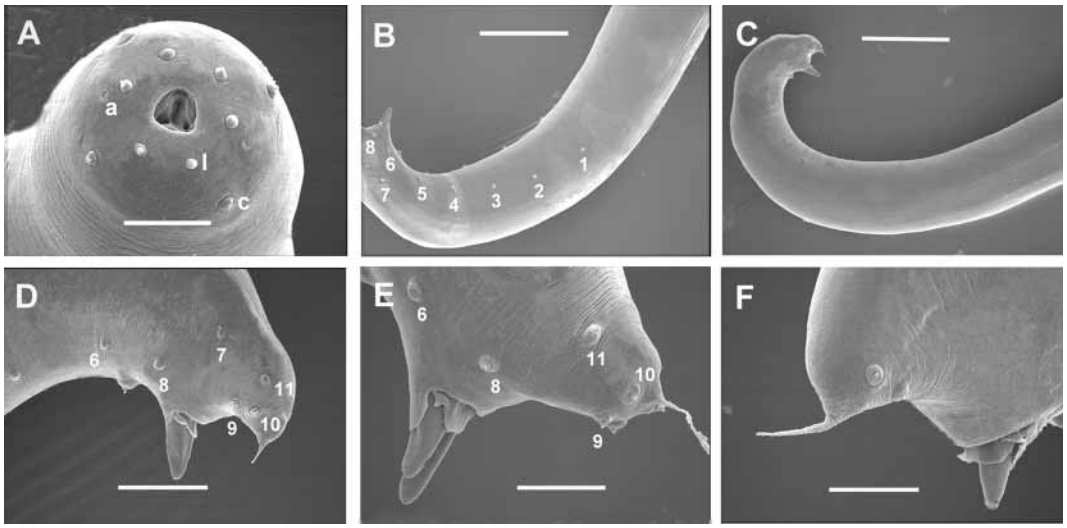

FIGURE 1. SEM of male of *S. feltiae* strain BC. A, face view. B, C, posterior regions with prominent genital papillae in B and not very prominent in C. D–F, variation of mucron and tail tip. Scale bars: A=7.5 µm, B=50.0 µm, C=85.7 µm, D=23.1 µm, E=10 µm, F=8.57 µm.

Morphometrical comparison with topotype

In general, for infective juvenile, morphometrics of *S. feltiae* strain BC and strain RU are similar except the body length, distance from anterior end to excretory pore, tail length and hyaline portion; these differences are statistically significant (Table 1). For male, spicule length, gubernaculum length, D% are similar. Interestingly, the mucron in the second generation male in both isolates is much longer than that in the first generation (Table 2).

Cross hybridization

Cross hybridization test showed that the male and female of the two strains mated and offspring developed indicating that the two isolates are in the same species.

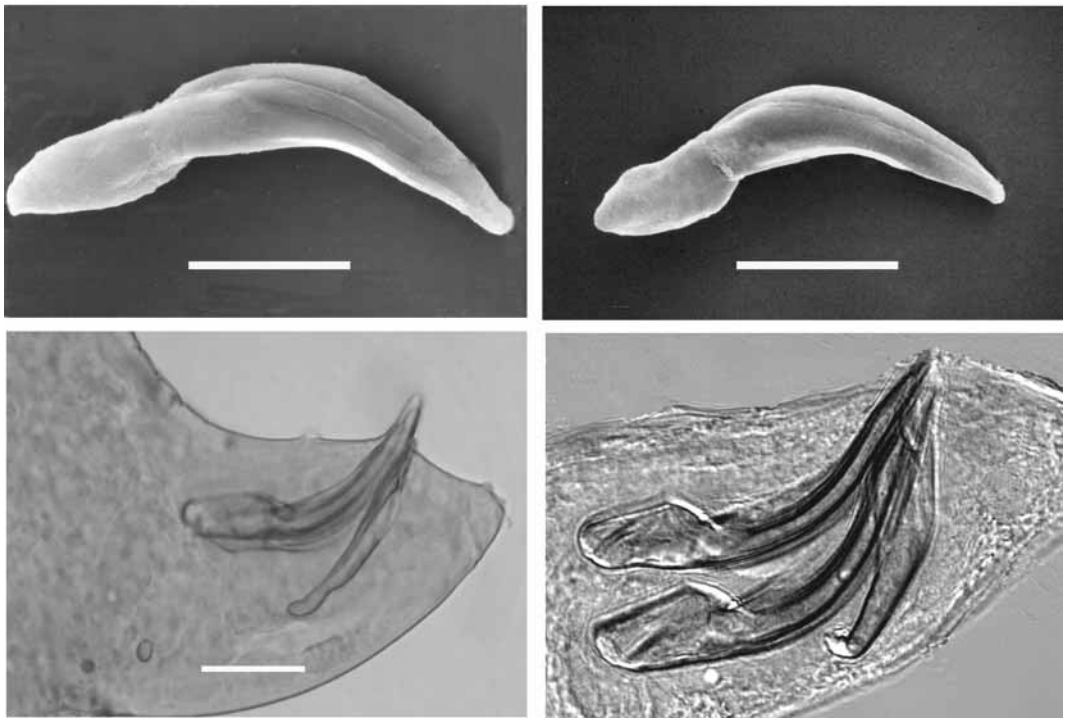

FIGURE 2. SEM and light microscopy. Comparative morphology of spicules *S. feltiae* and *S. oregonense.* A, spicule of *S. feltiae* strain SN. B, spicule of *S. oregonense.* C,D, variation of spicule of *S. feltiae* strain BC. Scale bars: A=21 µm, B=24 µm, C=24 µm, D=16 µm (in C).

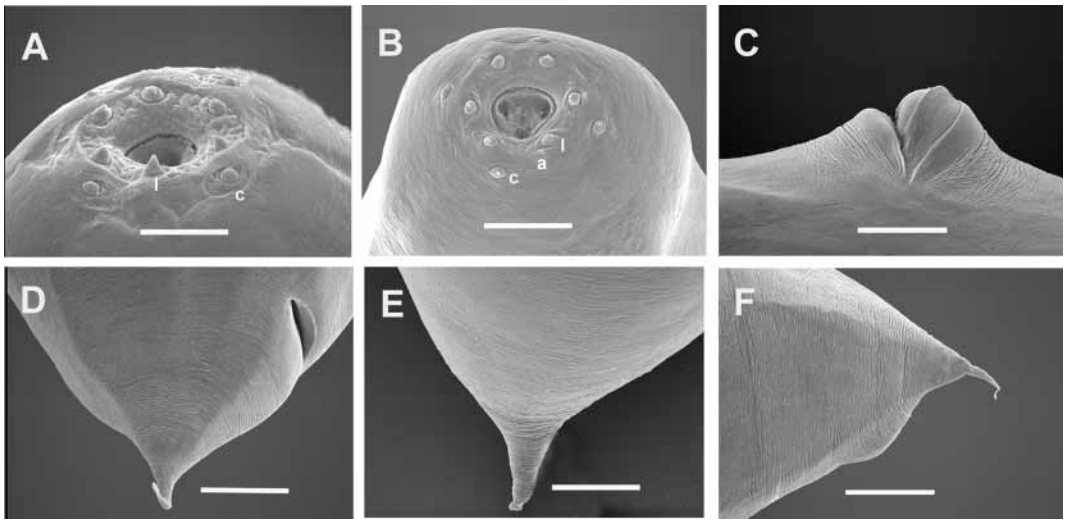

FIGURE 3. SEM of female of *S. feltiae* strain BC. A, an abnormal face view. B, a normal face view. C, vulva. D–E, variations of female tail tip. Scale bars: A=6.67 µm, B=10.0 µm, C=15 µm, D=12 µm, E=15 µm, F=16.7 µm.

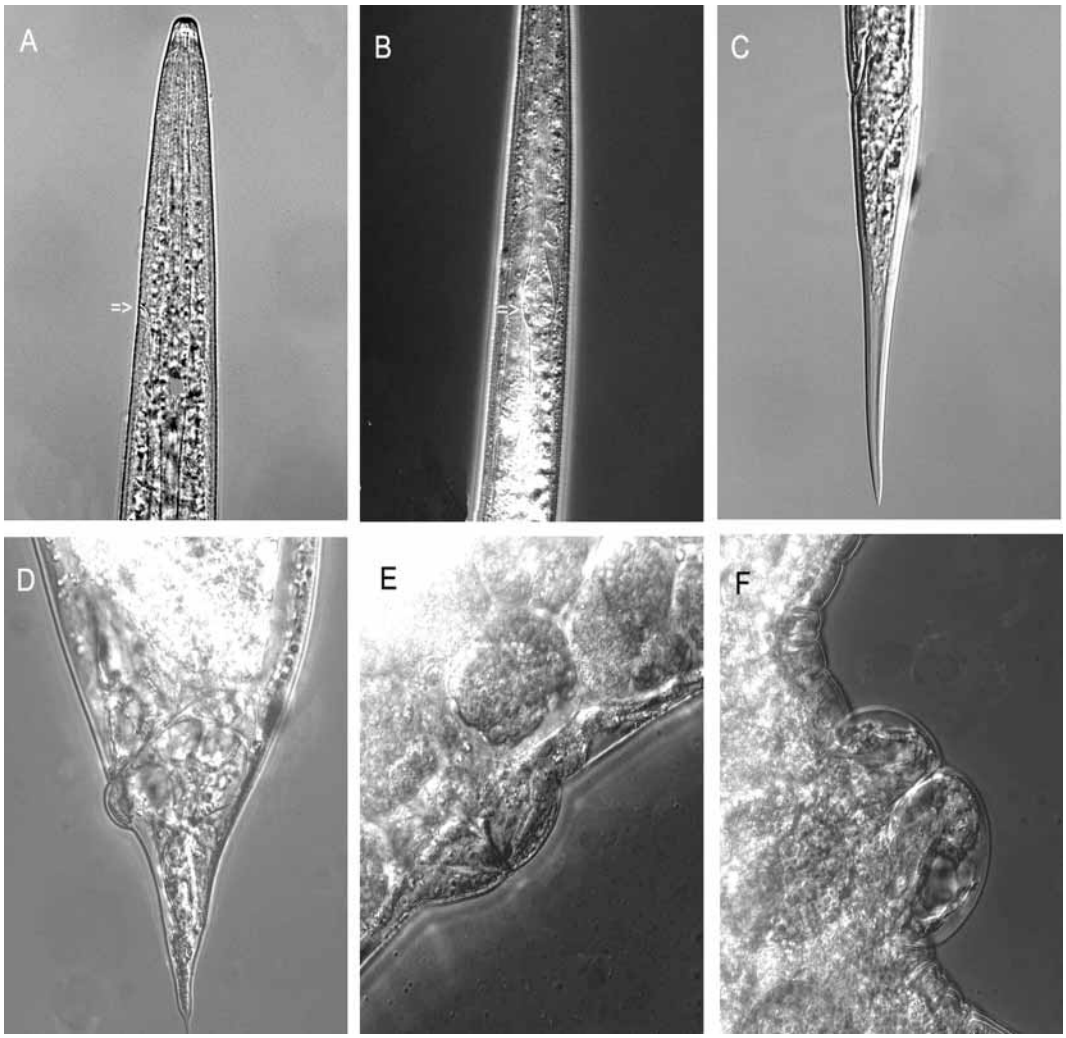

FIGURE 4. Light microscope photographs of *S. feltiae* strain BC. A–C, infective juvenile showing excretory pore, basal bulb and long tail . D–F, second generation female, D, tail, E, young , F, old specimen. Scale bars: A–C=14 µm, D=32 µm, E, F=20 µm.

DNA characterization

Sequences and multiple alignments: The sequence lengths, flanked by the two primers 18S and 26S of the ITS regions of *S. feltiae* strain BC, are 977 base pairs (bp), ITS1 = 272 bp, ITS2 = 298 bp, and those of the topotype strain RU are 980 bp, ITS1 = 275 bp, ITS2 = 298 bp. The strain BC has only four base pairs different from the topotype population RU (Table 4). The alignment of the sequences of the ITS regions of eight strains of *S. feltiae* (not reported here) showed that most differences come from the numbers of base pairs in ITS1. One important difference found in this alignment is that the 5.8S region of the China

strain has two insertions making its length become 159 bp (compared to 157 bp for most *Steinernema* species) (Table 3). The presence of these two characters needs to be confirmed.

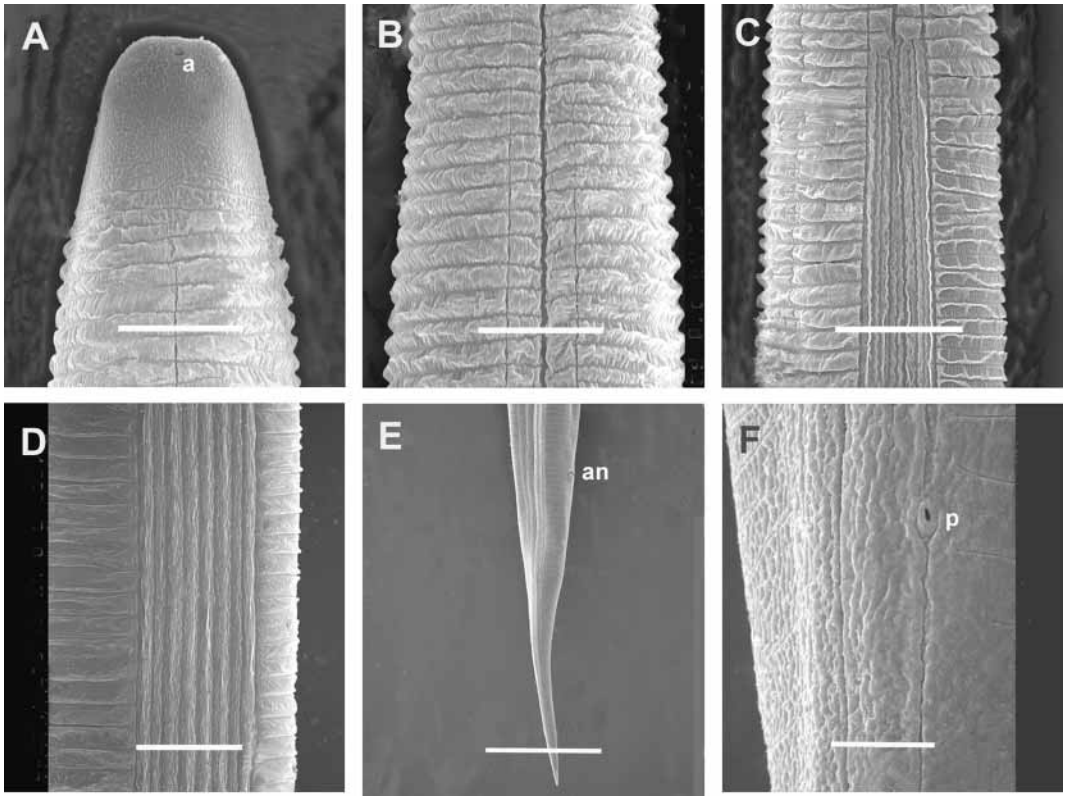

FIGURE 5. SEM of infective juvenile of *S. feltiae* strain BC showing lateral field pattern. A, anterior region showing smooth head, amphid, and one line in lateral field. B, lateral field with two ridges. C, lateral field with two ridges anteriorly, seven ridges posteriorly. D, lateral field with eight ridges. E, lateral field in posterior region showing four ridges near anus. F, lateral field with two ridges and phasmid. Scale bars: A=4.29 µm, B=5.0 µm, C=7.5 µm, D=7.5 µm, E=23.1 µm, F=1.67 µm.

For intra-specific relationship, BC strain is different from strain A1 by three bp, from T29, SN, and China strains by four bp, from strain MY9 by 14 bp and from strain A2 by 21 bp (Table 4). These differences make the sequence lengths of ITS regions of the eight studied strains variable from 970 to 985 bp. Sequences of four strains, A1, SN, RU and T29 are identical, 980 bp. Among these eight strains, strain A2 from United Kingdom is the most divergent from others with eleven diagnostic characters and 15–21 bp differences from others (Tables 3, 4); the next one is MY9 from Japan with seven diagnostic characters and 10–15 bp differences from others. These differences show that the two strains MY9 from Japan, and A2 from United Kingdom are the most divergent from other

strains. All six other strains are close to each other, 0 to 4 bp apart, in which no distances were found between the three strains T92, RU, and A1. The above results indicate that geographic distances (France, Indonesia, Russia and United Kingdom) contribute to the genetic differences (Tables 3, 4), but those differences do not create new species (Strain A2, and MY9 were confirmed as strains of *S. feltiae* by Reid and Hominick [1992, 1993], and Yoshida [2003]).

For inter-specific relationship, all distances between species are much higher than those between strains (at least 117 bp between *S. feltiae* and the closest species *S. oregonense*, and 101 bp between *S. carpocapsae*, and *S. siamkayai* compared to at most 21 bp, between strain A2 and strain BC of S. *feltiae*) (Table 4).

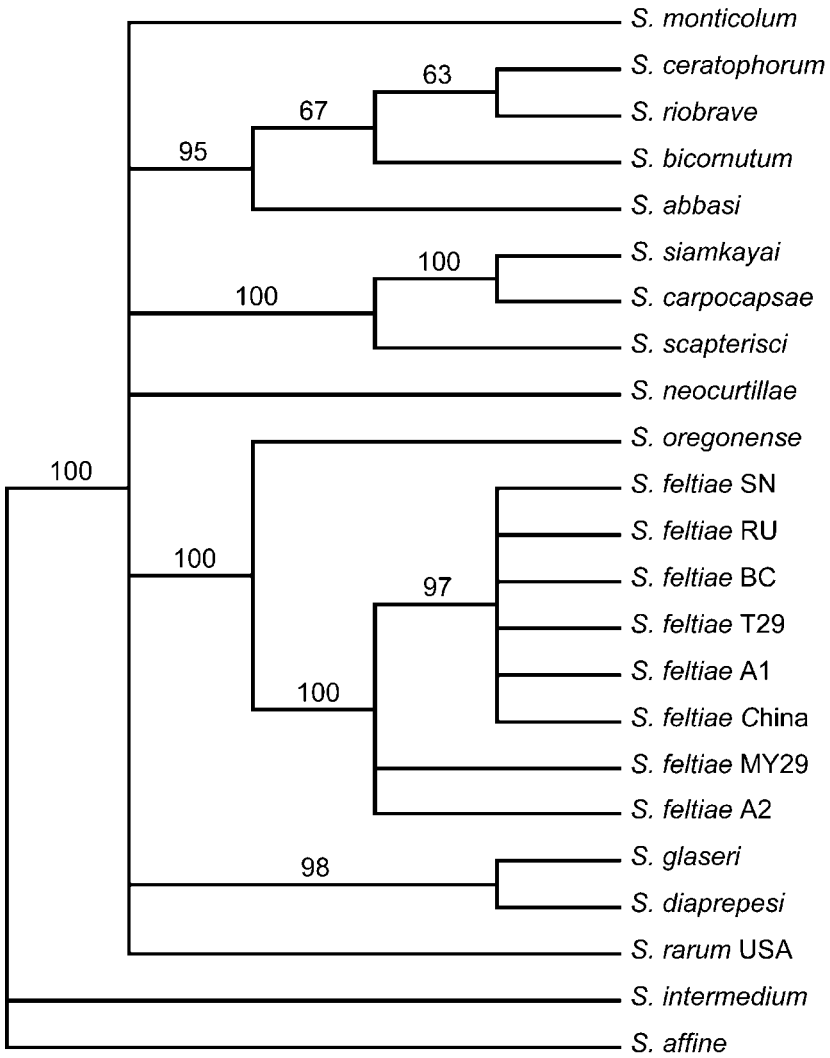

FIGURE 6. Phylogenetic relationships between 23 species and strains of *Steinernema* with bootstrap analysis of ITS regions. The eight strains of *S. feltiae* from a monophyletic group. Numbers at the nodes represent bootstrap proportion.

Phytogenetic relationships: The phylogenetic relationships among the 23 *Steinernema* species and strains are presented in Figure 6 (tree length = 2511, CI = 0.5472, RI = 0.5251). The eight strains of *S. feltiae* comprise a monophyletic group by analysis of the ITS region. In this clade, the six strains, A1, China, BC, SN, T29, and RU form a monophyletic group representing the sister group to strain MY9 and A2. This topology shows that the two nematode isolates MY9 and A2 are genetically different from other strains of *S. feltiae*, but the difference is not enough for new species.

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