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Steinernema fabii n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa

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Summary – A new species of entomopathogenic nematode, *Steinernema fabii* n. sp., was isolated by trapping with wax moth (*Galleria mellonella*) larvae from soil in an *Acacia mearnsii* plantation in the Mpumalanga province of South Africa. The new species is morphologically characterised by the length of the infective juvenile (IJ) of 641 (590-697) μm, by a tail length of 58 (52-64) μm, ratio a = 24 (21-41), H% = 53 (37-61) and E% = 93 (83-105). The pattern of the lateral field of the IJ of the new species is 2, 5, 2 ridges (3, 6, 3 incisures). The male of the first generation can be recognised by the long spicule of 90 (79-106) μm and gubernaculum of 66 (56-77) μm; D% = 64 (52-75) and GS% = 73 (63-86). The first generation female can be recognised by a protruding vulva with a short, double-flapped epiptygmata, and the lack of a postanal swelling, while the second generation differs in having a postanal swelling and a conical, sharply pointed tail. Analysis of DNA sequences for the ITS and D2-D3 gene regions showed *S. fabii* n. sp. to differ from all other *Steinernema* species and to belong to a new monophyletic group, the 'Cameroonian' clade, consisting of *S. cameroonense*, *S. nyetense*, *S. sacchari* and *S. fabii* n. sp., all from the African continent. This group is closely related to species in the *feltiae-kraussei-oregonense* Clade III.

Keywords – D2-D3, description, ITS, molecular, morphology, morphometrics, new species, phylogeny, SEM, systematics, taxonomy.

Entomopathogenic nematodes (EPN) in the genus *Steinernema* Travassos, 1927 are obligate and lethal insect parasites that have a symbiotic relationship with bacteria in the genus *Xenorhabdus* Thomas & Poinar, 1979. These nematodes have received considerable attention for use as biological control agents because they can be mass produced in liquid culture, applied using conventional pesticide spraying equipment, control a wide range of insect pests, and are considered an environmentally preferable alternative to pesticides (see Grewal *et al*., 2005).

A report by Lewis & Clarke (2012) recognised 68 *Steinernema* species and there have been 12 additional species described since that review. A small number of these, namely *S. feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 and *S. carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982, are distributed worldwide for the use against insect pests (Hominick, 2002). Due to their biological control relevance, surveys for native EPN are important because they are often better adapted to their local environmental conditions (Kaya & Gaugler, 1993) and, therefore, may achieve a higher level of efficacy than non-native species. Furthermore, the use of native EPN negates the fears of those concerned about releases of exotic EPN into the environment; this is particularly relevant with regard to their possible non-target effects, including the displacement of native EPN species (Bathon, 1996; Millar & Barbercheck, 2001; Ehlers, 2005).

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In South Africa, EPN were first reported from the maize beetle, *Heteronychus arator* (Harington, 1953), and then later from soil samples in KwaZulu-Natal province (Spaull, 1990, 1991). More recently, several surveys have been conducted aimed at documenting the diversity of EPN in the country (Malan *et al*., 2006, 2011; Hatting *et al*., 2009). This has resulted in the description of six *Steinernema* species from South Africa, including *S. citrae* Stokwe, Malan, Nguyen, Knoetze & Tiedt, 2011 (Stokwe *et al*., 2011); *S. khoisanae* Nguyen, Malan & Gozel, 2006 (Nguyen *et al*., 2006); *S. innovationi* Çimen, Lee, Hatting, Hazir & Stock, 2014 (Çimen *et al*., 2014); *S. sacchari* Nthenga, Knoetze, Berry, Tiedt & Malan, 2014 (Nthenga *et al*., 2014); *S. tophus* Çimen, Lee, Hatting, Hazir & Stock, 2014 (Çimen *et al*., 2014); and *S. jeffreyense* Malan, Knoetze & Tiedt, 2015 (Malan *et al*., 2015). These EPN were isolated from soils in an apple and citrus orchard, a sugar cane field, a grain field, a vineyard and a guava tree, respectively.

In 2014, an apparently new species of *Steinernema* was isolated from soil samples collected in a commercial black wattle (*Acacia mearnsii* De Wild) plantation. The sampling was part of a larger survey of EPN from commercial forestry areas. The objective of this study was to characterise the apparently undescribed *Steinernema* species from South Africa using morphological and molecular characteristics to differentiate this species from described *Steinernema* species. The new species is described and illustrated herein as *S. fabii* n. sp.

Materials and methods

Nematode origin

Soil samples (*ca* 1 kg) were collected by taking five random sub-samples at a depth of 0-20 cm from black wattle plantations in the Piet Retief area, Mpumalanga province. EPN were recovered from soil samples using insect baiting with the last instars of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) following the technique described by Stock & Goodrich-Blair (2012). Infective juveniles (IJ) were maintained by recycling through *G. mellonella* larvae and stored in approximately 150 ml of sterilised distilled water in 500 ml vented tissue culture flasks at 14°C for subsequent identification and establishment of stock cultures.

Morphological observations

For observation and measurement of the different life stages, ten *G. mellonella* larvae were placed in a 9 cm diam. Petri dish lined with moistened filter paper. After inoculating with 200 IJ per *G. mellonella* larva, the larvae were kept in a growth chamber at 25°C. The larvae of *G. mellonella* were recorded as dead (no movement when prodded) 2 days after inoculation. Male and female nematodes of the first and second generations were obtained after 3-4 days and 6-7 days, respectively, by dissecting the cadavers in Ringer's solution. IJ were harvested by using a modified White trap (Woodring & Kaya, 1988). This was prepared by placing the base of the 9 cm diam. Petri dish containing infected cadavers inside a 15 cm diam. glass Petri dish, which was half-filled with filtered tap water. All the different stages were fixed in hot TAF (2% triethanolamine, 8% formalin in distilled water) at 85°C (Courtney *et al*., 1955). Water in specimens was replaced by glycerin using the modified Seinhorst (1959) technique, after which they were mounted in pure glycerin. Permanent slides were used for measurements and drawings were made by means of a Leica DM2000 compound microscope (Leica Microsystems) fitted with a digital camera, and computer with Leica Application Suite V3.5.0 software. For direct observations to confirm the morphology or the variations of specific structures the different stages were either examined live or after they had been killed with gentle heat. Exsheathed IJ were obtained by storing the nematodes in culture flasks at 14°C for 2 months.

Scanning electron microscopy (SEM)

The samples were fixed in 70% ethanol and dehydrated in an ethanol series of 80%, 90% and twice in 100% for 15 min each. After dehydration the samples were critical point dried using liquid carbon dioxide as transitional fluid. The dried samples were mounted on SEM-stubs with double-sided carbon tape and coated in a sputter coater with a 15 nm layer of gold/palladium (66/34% Au/Pd). The samples were viewed in a FEI Quanta 250 FEG SEM operating at 5 kV.

Cross-hybridisation

Reproductive compatibility of the new species was tested using the protocol suggested by Nguyen & Duncan (2002), using haemolymph of *G. mellonella* larvae. The new species was crossed with *S. sacchari*, which

is closely related on the basis of both morphology and molecular characteristics, to assess reproductive compatibility of these two species. For this purpose, a drop of *Galleria* haemolymph was placed in a sterile Petri dish (35 × 10 mm) and a single IJ was inoculated into the haemolymph from each *S. fabii* n. sp. and *S. sacchari*. As a control, crosses between the IJ of the same species were conducted. The treatment was replicated 20 times. Both the development of the inoculated IJ into adults and the reproduction of the nematodes were observed and recorded during the experimental period. The other closely related species, *S. cameroonense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 and *S. nyetense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012, were not available for this test.

Molecular characterisation and phylogenetic relationships

Total genomic DNA was isolated from pooled samples of IJ using a modified phenol chloroform protocol described by Goodwin *et al*. (1992). The internal transcribed spacer regions (ITS) and 28S (D2-D3) regions of the ribosomal DNA were PCR amplified in 25 μl final volume with the addition of 3 μl of 10 × PCR buffer + 3 mM $MgCl_2$, 1 μl of 5 mM dNTP 0.25 μl of *Taq* polymerase (Fast star), 16.75 μl of SABAX pure water (Adcock Ingram) and 1.0 μM of each primer set and 2 μl of DNA temple. The primers used in the study to amplify the ITS region were AB28 (F) and TW81 (R) as reported by Stock (2009) and Curran & Driver (1994). The primer set used for the D2-D3 region was D2F and 536R (Ntengha *et al*., 2014). The PCR cycling profile for the ITS and D2-D3 regions was the same as those described by Stock (2009) and Ntengha *et al*. (2014), respectively. Sequence data for the forward and reverse DNA strands were edited manually using CLC Main Workbench v.6 (available online at http://www.clcbio.com) and compared with those present in GenBank by means of a Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI). The ITS and D2-D3 sequences of *S. fabii* n. sp. and corresponding nucleotide sequences of other representatives of *Steinernema* available in GenBank were aligned using MAFFT (available online at http://mafft.cbrc.jp/alignment). Phylogenetic analyses (Maximum Parsimony) of sequence data were done using PAUP* v. 4.0b10 (Swofford, 2002). Heuristic tree searches were executed using the tree bisection-reconnection branch-swapping algorithm with random sequence analysis. Confidence limits for phylogenetic trees were estimated from bootstrap analyses (1000 replicates). The number of base substitutions per site between sequences was conducted using the Jukes-Cantor model (Jukes & Cantor, 1969). Base pair differences, evolutionary analysis and the resulting trees were visualised by using MEGA6 (Tamura *et al*., 2013). *Caenorhabditis elegans* (EU131007) and *Cervidellus alutus* (AF331911) were applied as outgroups in the development of the trees based on ITS and D2-D3 sequences, respectively.

Results

Steinernema fabii* n. sp.
(Figs 1-4)

Measurements

See Table 1.

Description

First-generation male

Habitus J-shaped when heat-killed. Cuticle smooth under light microscope, but finely striated under SEM. Head region smooth, rounded and not offset from rest of body. Six labial and four cephalic papillae present. Amphids slit-like and located laterally between labial and cephalic circle of papillae. Stoma tri-radiate, 6-9 μm in length and 7-12 μm in diam. Pharynx muscular, procorpus cylindrical, metacorpus slightly swollen. Nerve ring usually surrounding anterior part of basal bulb. Excretory pore located anterior to nerve ring at *ca* 64-68% of length from anterior end to pharynx base; excretory duct well cuticularised. Cardia inconspicuous. Genital system monorchic, ventrally reflexed (testis reflexion 293-589 μm). Twenty-three genital papillae, comprising 11 pairs and a single mid-ventral papilla, located just anterior to cloacal opening. Spicules paired, symmetrical, well curved, bright yellow in colour. Manubrium of spicules longer than wide (21 μm/16 μm), short shaft present, blade well curved with spicule terminus blunt. Velum prominent, posterior end not reaching spicule tip. Gubernaculum boat shaped in lateral view, cuneus needle shaped and Y-shaped posteriorly, not reaching tip of corpus wings, which are

* Specific epithet derived from the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, where the DST-NRF Centre of Excellence in Tree Health Biotechnology is based.

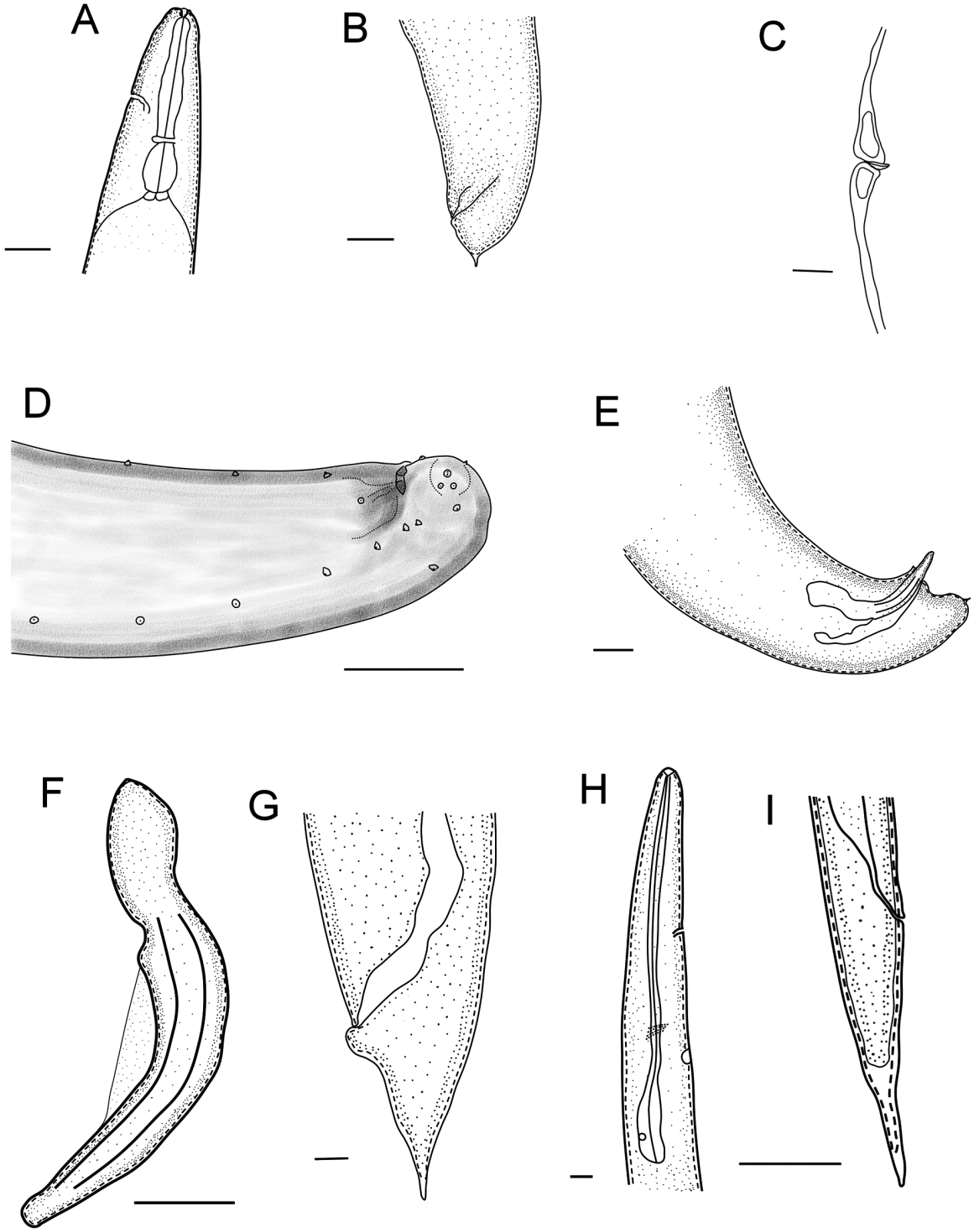

Fig. 1. *Steinernema fabii* n. sp. A-C, first generation female. A: Anterior region; B: Tail region; C: Vulva. First generation male (D-F). D: Ventral view of tail; E: Lateral view of tail region; F: Spicule. G: Tail of second generation female. Infective juvenile. H: Anterior region; I: Tail region. (Scale bars: A-D = 50 μm; E, F, I = 20 μm; G, H = 10 μm.)

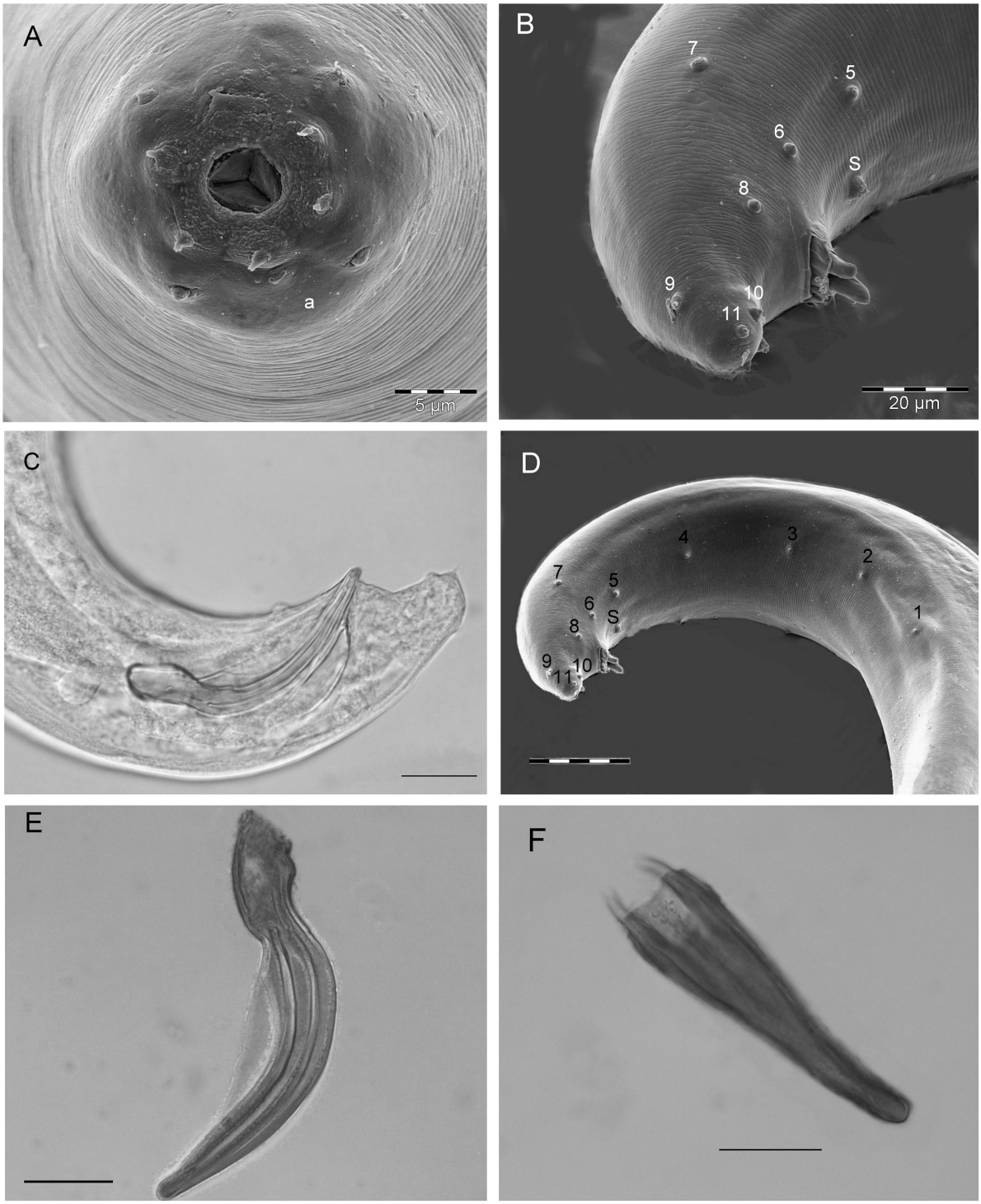

Fig. 2. Male of *Steinernema fabii* n. sp., first generation male. A: *En face* view (a = amphidial aperture); B: Lateral view of tail; C: Spicule and gubernaculum of tail of second generation; D: Lateral view of tail region with genital papillae; E: Spicule; F: Gubernaculum. The papillae in B and D are numbered; S = single mid-ventral papilla. (Scale bars: A = 5 μm; B, C, E, F = 20 μm; D = 50 μm.)

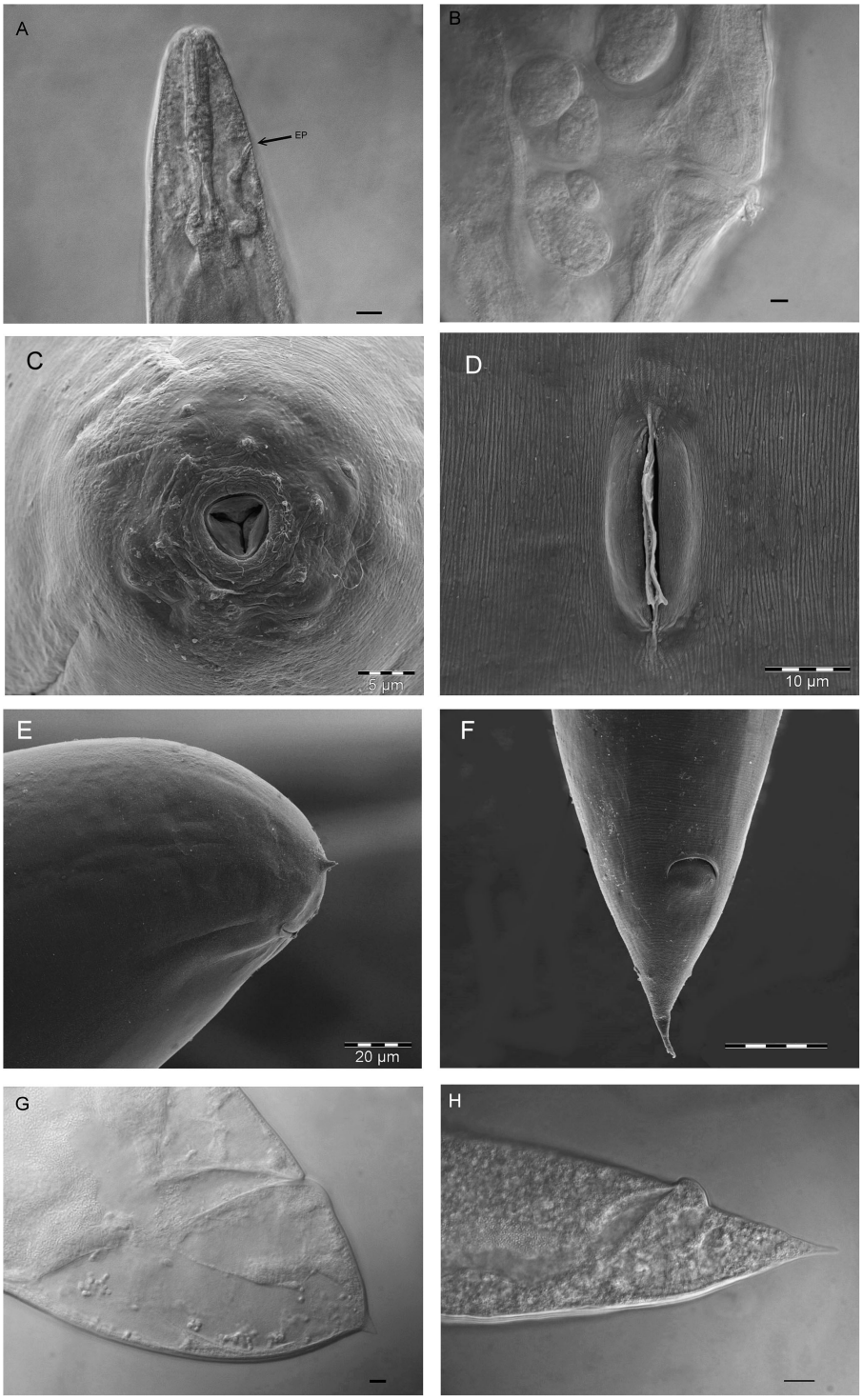

Fig. 3. Female *Steinernema fabii* n. sp. First generation. A: Anterior region showing pharynx and excretory pore (ep); B, D: Vulva; C: *En face* view; E, G: Tail. F, H: Second generation female tail with postanal swelling. (Scale bars: A, E, F = 20 μm; B, C, G, H = 5 μm; D = 10 μm.)

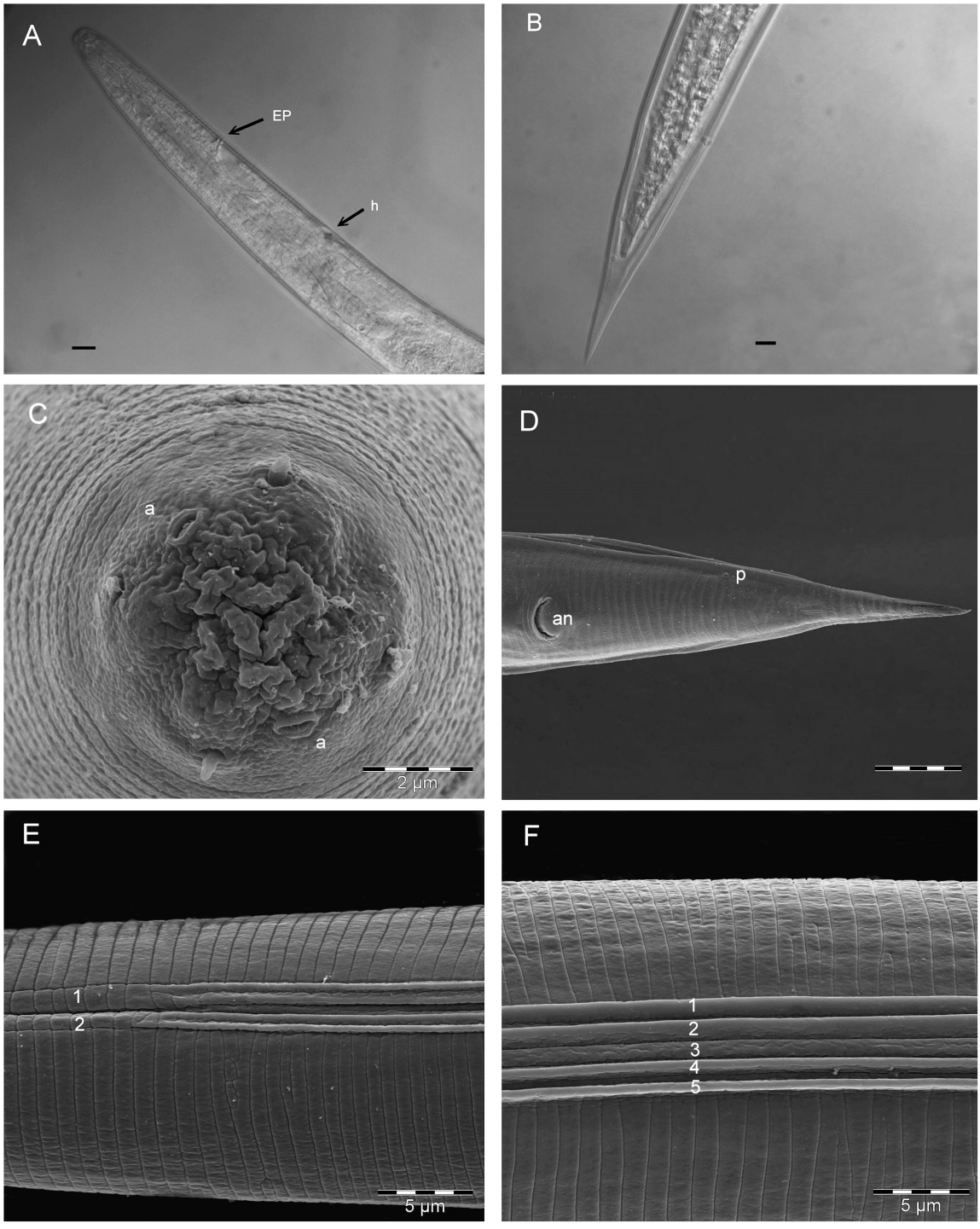

Fig. 4. Infective juvenile of *Steinernema fabii* n. sp. A: Anterior end showing excretory pore (EP) and hemizonid (h); B: Tail region showing hyaline region; C: Anterior region showing four cephalic papillae and two amphidial apertures (a); D: Tail with anus (an) and phasmid (p); E: Splitting of ridges in lateral field from two to five (from anterior; ridges numbered); F: Five ridges in lateral field at mid-body (ridges numbered). (Scale bars: A, B, E, F = 5 μm; C = 2 μm; D: 10 μm.)

Table 1. Morphometrics of *Steinernema fabii* n. sp. All measurements are in μm and in the form: mean ± s.d. (range).

Character	First generation			Second generation		
	Male		Female	Male	Female	Infective juvenile
	Holotype	Paratype	Paratypes	Paratypes	Paratypes	Paratypes
n		20	20	20	20	25
L	2005	1976 ± 294 (1499-2435)	4599 ± 476 (3552-5388)	1121 ± 44 (1034-1206)	1508 ± 123 (1316-1687)	641 ± 28 (590-697)
a	15	16 ± 1.7 (13-19)	22 ± 3.6 (15-30)	17 ± 0.8 (16-18)	16 ± 1.3 (13-19)	24 ± 3.7 (21-41)
b	13	13 ± 1.6 (11-16)	23 ± 3.1 (18-28)	8.1 ± 0.4 (7.4-8.9)	9.2 ± 0.7 (8.2-10)	4.8 ± 0.2 (4.4-5.3)
c	53	52 ± 11 (29-71)	129 ± 24 (101-186)	36 ± 5.3 (29-45)	20 ± 3.0 (15-28)	11 ± 0.4 (10-12)
c′	0.78	0.7 ± 0.1 (0.5-0.9)	0.6 ± 0.1 (0.4-0.8)	1.0 ± 0.1 (0.8-1.2)	1.3 ± 0.2 (0.6-1.6)	3.7 ± 0.2 (3.4-4.2)
V	–	– –	53 ± 1.9 (51-57)	– –	53 ± 2.5 (48-58)	– –
Body diam. (BD)	137	126 ± 16 (97-153)	215 ± 36 (162-295)	65 ± 2.6 (61-70)	93 ± 5.2 (85-102)	28 ± 1.0 (26-31)
Stoma length	8	7.4 ± 0.8 (6.1-9.0)	13 ± 2.0 (9.9-17)	5.7 ± 0.6 (4.7-6.9)	5.1 ± 1.3 (3.4-8.5)	– –
Stoma diam.	11	8.8 ± 1.4 (6.7-12.0)	13 ± 1.3 (11-15)	7 ± 0.6 (5.6-8.0)	6.1 ± 1.3 (3.3-8.7)	– –
Excretory pore (EP)	109	96 ± 11 (80-116)	107 ± 9.4 (92-125)	74 ± 3.5 (68-81)	114 ± 10 (95-138)	53 ± 1.9 (49-57)
Nerve ring (NR)	104	103 ± 8.9 (91-121)	124 ± 14 (87-138)	86 ± 6.2 (75-101)	120 ± 5.8 (110-128)	65 ± 7.9 (55-84)
Pharynx length (ES)	159	150 ± 12 (128-171)	198 ± 13 (181-222)	138 ± 5.4 (128-147)	163 ± 5.3 (155-172)	132 ± 7.8 (120-146)
Hemizonid	– –	– –	– –	– –	– –	93 ± 3.5 (87-99)
Testis reflexion	556	437 ± 88 (293-589)	– –	230 ± 33 (164-287)	– –	– –
Tail length (T)	38	39 ± 7.3 (28-54)	36 ± 5.1 (27-45)	32 ± 4.7 (25-39)	78 ± 7.8 (52-88)	58 ± 2.7 (52-64)
Anal body diam. (ABD)	49	53 ± 9.0 (37-68)	65 ± 12 (46-88)	32 ± 3.0 (29-37)	62 ± 6.6 (54-85)	15 ± 0.89 (14-18)
Hyaline region (H)	– –	– –	– –	– –	– –	30 ± 3.0 (21-37)

Table 1. (Continued.)

Character	First generation			Second generation		
	Male		Female	Male	Female	Infective juvenile
	Holotype	Paratype	Paratypes	Paratypes	Paratypes	Paratypes
Spicule length (SL)	96	90 ± 6.3	–	77 ± 3.5	–	–
		(79-106)	–	(68-84)	–	–
Spicule width	18	18 ± 1.9	–	13 ± 0.5	–	–
		(13-21)	–	(12-14)	–	–
Spicule head length	–	21 ± 2.9	–	–	–	–
	–	(15-27)	–	–	–	–
Spicule head width	–	16 ± 2.1	–	–	–	–
	–	(11-19)	–	–	–	–
Gubernaculum length (GL)	–	66 ± 5.9	–	44 ± 3.3	–	–
	–	(56-77)	–	(39-51)	–	–
Gubernaculum width	–	8.3 ± 1.0	–	7.2 ± 0.9	–	–
	–	(6.3-11)	–	(5.8-8.9)	–	–
D % = EP/ES × 100	68	64 ± 6.9	54 ± 5.1	54 ± 4.6	70 ± 7.3	41 ± 4.6
		(52-75)	(46-65)	(47-65)	(57-85)	(35-60)
E% = EP/T × 100	286	258 ± 59	299 ± 48	235 ± 34	148 ± 21	93 ± 5.4
		(149-395)	(230-412)	(172-299)	(124-223)	(83-105)
SW% = SL/ABD × 100	196	177 ± 30	–	240 ± 26	–	–
		(126-224)	–	(198-273)	–	–
GS% = GL/SL × 100	66	73 ± 6.5	–	57 ± 4.2	–	–
		(63-86)	–	(50-65)	–	–
H% = H/T × 100	–	–	–	–	–	53 ± 5
	–	–	–	–	–	(37-61)

open posteriorly. Tail dorsally convex, terminus bluntly rounded, terminal mucron present.

Second-generation male

Similar to first generation, but smaller in size. Spicules and gubernaculum shorter and thinner. Tail terminus with mucron, usually subterminal subventral in position.

First-generation female

Body shape of heat-relaxed specimens coiled in a closed 6-shape. Fine annulations visible on cuticle with SEM. Head region smoothly tapering, not offset from rest of body. Face view with six labial and four cephalic papillae. Amphidial apertures absent or inconspicuous. Cephalic region with perioral disc. Stoma tri-radiate, 10-17 μm in length and 11-15 μm in diam. Pharynx muscular, procorpus cylindrical, metacorpus slightly swollen. Nerve ring in vicinity of isthmus. Excretory pore located anterior to nerve ring, *ca* 51-56% of length from anterior end to pharynx base, excretory duct well cuticularised. Cardia prominent. Gonads amphidelphic, reflexed dorsally. Vulva a median transverse slit, located 53% from anterior end of body, slightly protruding, with short double flapped epiptygmata. Tail shorter than anal body diam., bluntly rounded with a terminal peg.

Second-generation female

Similar to first generation in general morphology, but shorter and narrower. Body slightly curved when heat relaxed. Tail length longer than first generation (78 (52-88) *vs* 36 (27-45) μm). Tail conical and sharply pointed. Terminal mucron absent. Postanal swelling present.

Infective juvenile

Body of heat-relaxed specimens slightly curved, slender, slightly tapering towards anterior and posterior ends. Body cuticle with fine annulations. Sheath (second-stage cuticle) present after harvesting, but usually lost during storage. Cephalic region slightly truncated. Four distinct cephalic papillae. Amphidial apertures pore-like. Lip region smooth, continuous, stoma closed. Pharynx long, narrow, with a slightly expanded procorpus, narrower isthmus and pyriform basal bulb with nuclei of dorsal pharyngeal and two subdorsal glands clearly visible. Excretory pore anterior to nerve ring. Hemizonid distinct, located towards middle of isthmus. Nerve ring at level of isthmus anterior to basal bulb. Lateral field starting with two ridges (three lines) from anterior end, further posteriorly each ridge dividing into two with four equal ridges (five lines), in mid-body with five equal ridges (six lines) and ridges remaining unchanged until phasmid. Only two prominent ridges observed posterior to phasmid. Lateral field formula: 2, 4, 5, 2. Bacterial pouch obscure. Cardia indistinct. Tail conoid with pointed terminus. Hyaline portion occupying *ca* 37-61% of tail length.

Type host and locality

Natural host unknown as *S. fabii* n. sp. (isolate ML15) was isolated by baiting a soil sample from an *A. mearnsii* plantation with *G. mellonella* larvae in the Mpumalanga Province (27°12′30″S, 31°1′4″E) of South Africa. The soil texture was sandy-loam, acidic (pH 4) with low organic matter (3%) content.

Type material

Holotype first generation male, paratype males (five slides with 20 specimens), paratype females (six slides with 18 specimens) and IJ (four slides with 30 specimens) isolated from *G. mellonella* deposited in the National Collection of Nematodes, Biosystematics Division, Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa. In addition to permanent slides, paratypes of males, females and IJ fixed in TAF deposited in the same place (50 IJ; 20 first generation males; 25 first generation females; 35 second generation males and 20 second generation females). Paratypes of males, females and IJ fixed in TAF were also deposited in the USDA Nematode Collection (40 IJ; 20 first generation males; 25 first generation females; 33 second generation males and 20 second generation females).

Diagnosis and relationships

Steinernema fabii n. sp. is characterised by differences in the morphology and the morphometrics of the IJ and adult stages. The IJ of the new species can be recognised by the pattern of the lateral field of 2, 4, 5, 2 ridges, the body length of 641 (590-697) μm, body diam. of 28 (26-31) μm, distance from anterior end to excretory pore of 53 (49-57) μm, distance from anterior end to nerve ring of 65 (55-84) μm, distance from anterior end to base of pharynx of 132 (120-146) μm, tail length of 58 (52-64) μm, anal body diam. of 15 (14-18) μm, D% = 41 (35-60), E% = 93 (83-105) and H% = 53 (37-61) (Table 1). The first generation male has a long spicule and gubernaculum with a length of 90 (79-106) μm and 66 (56-77) μm, respectively. Other diagnostic characters include D% = 64 (52-75), E% = 258 (149-395), SW% =

177 (126-224) and GS% = 73 (63-86). The second generation males have similar morphological characters to the first generation, but are smaller in size and narrower in body diam. Both generations of males have 11 pairs of genital papillae, a single mid-ventral papillae and mucron at the tail tip. The first generation females have a protruding vulva with double flapped epiptygmata. The second generation female can be distinguished by the presence of postanal swelling and conical and sharply pointed tail, whereas the first generation female tail is dome-shaped with a terminal peg.

Steinernema fabii n. sp. clustered separate from, but most closely to, *S. sacchari*, *S. cameroonense* and *S. nyetense* both in ITS and D2-D3 phylogenetic analysis. It also shares some morphological similarity with *S. monticolum* Stock, Choo & Kaya, 1997 and *S. rarum* Doucet, 1986 based on IJ body length and spicule length. However, the new species can be differentiated from all closest *Steinernema* species by a number of features (Tables 2-4), including its longer IJ pharynx length 132 (120-146) μm, and the length of the first generation male spicule 90 (79-106) μm and gubernaculum 66 (56-77) μm.

The IJ body length of *S. fabii* n. sp. is shorter than *S. nyetense*, *S. monticolum* and *S. sacchari*, but is longer than *S. cameroonense* and *S. rarum* (see Table 2). The first generation males of *S. fabii* n. sp. differ from those of *S. sacchari* in the number of genital papillae, with the new species having 11 pairs and *S. sacchari* 12 pairs. In addition, the *S. fabii* n. sp. first generation male has a mucron at the tail tip whereas *S. sacchari* lacks a mucron.

Steinernema fabii n. sp. can be differentiated from *S. cameroonense* by the longer body length of the first generation male, 1976 (1499-2435) *vs* 1331 (1019-1718) μm. The body diam. of 126 (97-153) μm of the first generation male of *S. fabii* n. sp. is narrower than *S. monticolum* and *S. sacchari* and wider than that of *S. cameroonense*, *S. nyetense* and *S. rarum*.

The first generation female of *S. fabii* n. sp. lacks a mucron at the tail tip whereas first generation females of *S. cameroonense* and *S. nyetense* both have a mucron. The vulva of the first and second generation females of the new species are protruding and have epiptygmata, the opposite of the condition in *S. monticolum*, *S. sacchari* and *S. rarum*.

Molecular characteristics

Steinernema fabii n. sp. is characterised genetically by the sequences of the ITS (KR527216) and the D2-D3 (KR527217) rDNA regions. The sequences of the ITS regions of *S. fabii* n. sp. include the ITS1 + 5.8S + ITS2, can be recognised by being 781 bp long (ITS1 = 321 bp; ITS2 = 313 bp) with a percentage composition of A = 22.79, C = 19.72, G = 23.05, T = 34.44. The sequence lengths and frequencies of nucleotide distribution for closely related species are shown in Table 5. *Steinernema fabii* n. sp. is different from the closest related species *S. sacchari* and *S. cameroonense*, in terms of both the ITS length (312 *vs* 311 and 291 bp, respectively) and the ITS2 (312, 296 and 284 bp, respectively) (Table 5). Pairwise distances using the ITS region show that the new species differs from its closest relatives *S. sacchari* and *S. cameroonense* by 22 bp and from *S. nyetense* by 26 bp while differing from all other closely related species by higher numbers of bp. These basepair differences are significant as compared to differences with other previously described species; for example, the bp difference between *S. everestense* and *S. akhursti* is three and, in addition, *S. litorale* differs from *S. weiseri*, *S. ichnusae* and *S. feltiae* by eight, nine and ten bp respectively (Table 6).

The sequence of the D2-D3 region of *S. fabii* n. sp. is 801 bp and its base percentage composition is: A = 23.50, C = 19.38, G = 30.88 and T = 26.25 (Table 5). Pairwise comparison using the D2-D3 regions is presented in Table 7. Due to the relatively short sequences available for *S. cameroonense* (593 bp) and *S. nyetense* (592 bp), coupled with the conserved nature of the 28S (D2-D3) region, there were two, four and one base pair differences between the new species and the closest related *Steinernema* species, *S. cameroonense*, *S. nyetense* and *S. sacchari*, respectively.

Phylogeny

Maximum Parsimony (MP) analyses of the aligned data for the ITS regions resulted in 1020 characters, of which 480 variable characters were parsimony uninformative and 540 characters were parsimony-informative. The phylogenetic relationship of *S. fabii* n. sp. with the other 37 *Steinernema* species and the outgroup *C. elegans*, inferred from the ITS rRNA sequences using the MP method, is shown in Figure 5 (CI = 0.443, RI = 0.653 and HI = 0.557). The most parsimonious tree indicates that *S. fabii* n. sp. forms a clade with three species: *S. sacchari*, *S. cameroonense and S. nyetense* with bootstrap support of 100%.

For the D2-D3 region, MP analysis of the aligned data resulted in 604 characters of which 348 variable characters were parsimony uninformative and 256 characters

Table 2. Comparative morphometrics of infective juveniles of *Steinernema fabii* n. sp. and related *Steinernema* spp. (in descending order of body length). Measurements are in μm and in the form: mean (range).

Species	Morphometric character												References
	L	BD	EP	NR	ES	T	a	b	c	D%	E%	n	
S. schliemanni	934	35	72	–	148	88	26	6	11	48	–	25	Spiridonov *et al.*, 2010
	(842-1008)	(30-38)	(61-80)	–	(127-162)	(76-95)	(23-30)	(6-7)	(10-11)	(42-55)	–		
S. ashiuense	768	30	55	86	119	71	25	6	11	46	78	20	Phan *et al.*, 2006
	(720-800)	(28-33)	(51-59)	(77-91)	(113-128)	(66-76)	(24-27)	(6-7)	(10-12)	(53-50)	(70-85)		
S. robustispiculum	712	28	56	84	120	75	25	6	10	46	75	25	Phan *et al.*, 2005
	(642-778)	(26-35)	(50-68)	(80-100)	(115-152)	(68-92)	(18-29)	(4-6)	(6-11)	(43-59)	(67-87)		
S. monticolum	706	37	58	88	124	77	19	6	9	47	76		Stock *et al.*, 1997
	(612-821)	(32-46)	(54-62)	(81-93)	(120-131)	(71-95)	(14-22)	(5-6)	(7.6-11.1)	(44-50)	(63-86)	–	
S. sacchari	680	37	53	84	113	64	19	6	11	47	82	25	Malan *et al.*, 2014
	(630-722)	(30-47)	(49-58)	(78-97)	(104-127)	(51-74)	(14-23)	(6-7)	(10-12)	(41-54)	(70-109)		
S. nyetense	648	32	52	85	114	82	21	6	8	46	66	20	Kanga *et al.*, 2012
	(565-708)	(25-37)	(46-57)	(72-102)	(104-128)	(54-113)	(19-26)	(5-6)	(6-11)	(37-50)	(44-89)		
S. fabii n. sp.	641	28	53	65	132	58	24	4.8	11	41	93	25	–
	(590-697)	(26-31)	(49-57)	(55-84)	(120-146)	(52-64)	(21-41)	(4.4-5.3)	(10-12)	(35-60)	(83-105)		
S. cameroonense	622	30	54	85	113	76	21	6	9	48	75	20	Kanga *et al.*, 2012
	(490-694)	(24-35)	(45-64)	(69-100)	(105-125)	(52-107)	(17-25)	(5-6)	(6-12)	(42-56)	(48-116)		
S. rarum	510	32	37	67	99	48	20	5	10	41	81	20	Doucet, 1986
	(446-578)	(25-37)	(33-40)	(62-76)	(88-117)	(42-52)	(18-23)	(4-6)	(9-12)	(36-43)	(67-91)		

Abbreviations as in Table 1. –, measurement not available.

Table 3. Comparative morphometrics of first-generation males of *Steinernema fabii* n. sp. and related *Steinernema* spp. (in descending order of spicule length). Measurements are in μm and in the form: mean (range).

Species	Morphometric character (n = 20)					
	Spicule	Gubern.	BD	D%	SW%	GS%
S. fabii n. sp.	90	66	126	64	177	73
	(79-106)	(56-77)	(97-153)	(52-75)	(126-224)	(63-86)
S. sacchari	83	61	145	67	171	73
	(73-89)	(50-68)	(86-205)	(54-88)	(146-210)	(66-81)
S. nyetense	80	53	106	55	199	66
	(67-98)	(40-62)	(62-159)	(40-70)	(125-283)	(51-77)
S. schliemanni	72	53	87	54	–	–
	(61-81)	(43-64)	(76-120)	(50-58)	–	–
S. monticolum	70	45	160	55	140	60
	(61-80)	(35-54)	(117-206)	(49-61)	(120-150)	(50-70)
S. cameroonense	69	45	90	64	170	64
	(51-85)	(37-57)	(65-124)	(48-76)	(131-201)	(47-76)
S. ashiuense	59	37	106	50	149	63
	(50-65)	(25-43)	(80-125)	(44-56)	(128-167)	(43-73)
S. robustispiculum	58	41	127	56	129	70
	(51-65)	(36-44)	(105-150)	(50-63)	(111-150)	(64-79)
S. rarum	47	34	50	50	94	71
	(42-52)	(23-38)	(44-51)	(44-51)	(91-105)	(55-73)

Abbreviations as in Table 1 and references as in Table 2.

were parsimony-informative. Phylogenetic relationships of *S. fabii* n. sp. with the other 41 *Steinernema* species and the outgroup of *Cervidellus alutus*, inferred from sequences for the D2-D3 region of the 28S rRNA based on MP are shown in Figure 6 (tree length = 751, CI = 0.523, RI = 0.761 and HI = 0.477). The new species could thus be placed in the same monophyletic group as using the ITS region, namely the Cameroonian clade including *S. cameroonense*, *S. nyetense* and *S. sacchari*, with 100% bootstrap support.

Both morphological and molecular data showed that *S. fabii* n. sp. resides in the Cameroonian clade (Ntengha *et al*., 2014) that includes *S. sacchari*, *S. cameroonense* and *S. nyetense*. The nematodes residing in this clade are known only from the African continent, specifically from Cameroon and South Africa. This group is closely related to the *feltiae-kraussei-oregonense* Clade III (Spiridonov *et al*., 2004).

Cross-hybridisation

Cross-hybridisation assays testing for reproduction compatibility between the new species and *S. sacchari* yielded no progeny. In the self-cross controls, offspring were produced normally. Additional cross-hybridisation with related species, in this case with *S. cameroonense* and *S. nyetense*, would have provided further support for the separation of the species, but living specimens of these latter two species from Cameroon were not available for study.

Bionomics

Steinernema fabii n. sp. has a life cycle comparable to that of other described EPN species. *Galleria mellonella* larvae were killed after 2 days and first generation adults developed after 3-4 days at 25°C. Second generation adults developed after 6 days. It usually required more than 12 days for IJ to emerge from the insect cadavers.

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Table 4. Comparative morphology of *Steinenernema fabii* n. sp. and closely related species.

Species	IJ	Male 1st generation				Male 2nd generation	Female 1st generation		
	Lateral line	Spicule	Gubernaculum	Genital papillae	Mucron	Mucron	Vulva	Tail	Post-anal swelling
S. fabii n. sp.	5 equal ridges in mid-body	Bright yellow, velum prominent and posterior end does not reach spicule tip	Boat-shaped, cuneus needle-shaped, not reaching tip of wings of corpus	22 + 1	P	P	Protruding, with epiptygmata	Dome-shaped, with terminal peg	A
S. ashiuense	5 equal ridges in mid-body	Slightly yellowish, velum large, not covering spicule tip	Boat-shaped, cuneus long, needle-shaped, wing of corpus expanding laterally	20 + 1	P	P	Protruding, no epiptygmata	Dome-shaped, with terminal peg	–
S. cameroonense	2, 4, 5, 4, 3, 2	Yellow, brown, velum present	Boat-shaped in lateral view, cuneus needle shaped	22 + 1	P	P	Protruding with epiptygmata	Conical pointed, with micron	P
S. monticolum	8 unequal ridges in mid-body	Brown-orange, velum present, spicule tip pointed	Arcuate, large, posterior end forked	21/23 + 1	P	P	Not protruding, no epiptygmata	Short, blunt, with mucron	P
S. nyetense	2, 4, 5, 4, 3, 2	Yellow brown, velum large	Boat-shaped in lateral view, cuneus needle-shaped	22 + 1	P	P	Protruding with epiptygmata	Conoid and pointed, mucron on tip	P
S. rarum	2, 8, 10, 6, 2	Velum thin, spicule tip usually blunt	Cuneus rod-like	21/23 + 1	P	P	Protuding, no epiptygmata	Conoid to dome shaped, terminal peg	P
S. robustispiculum	8 unequal ridges in mid-body	Yellow-brown, prominent rostrum, velum large	Boat-shaped, cuneus long	22 + 1	P	–	Protruding, with epiptygma	Dome shaped with terminal peg	P
S. sacchari	5 equal ridges in mid-body	Yellow-brown, prominent rostrum, velum not reaching spicule tip, spicule tip blunt	Boat-shaped, cuneus long	24 + 1	A	P	Not protruding, with epiptygma	Dome shaped with terminal peg	A
S. schliemanni	8 equal ridges at mid-body	Anteriorward projection on ventral edge of spicule proximal end	Cuneus absent	22 + 1	P	P	Slightly protruding	Conical with rounded terminus	A

A: absent; P: present; –: information not available.

Table 5. Sequence lengths and nucleotide composition of ITS (ITS1 + 5.8S + ITS2) and D2-D3 regions of species of *Steinernema* closely related to *Steinernema fabii* n. sp.

Species	ITS1 (bp)	ITS2 (bp)	A (%)	C (%)	G (%)	T (%)	ITS length (bp)	Sequence length (bp)
ITS regions								
S. fabii n. sp.	312	312	22.790	19.720	23.050	34.440	781	
S. ashiuense	261	245	26.40	14.87	22.16	36.57	662	
S. cameroonense	291	284	22.54	19.40	25.14	32.92	732	
S. cholashanense	265	303	24.80	17.52	22.35	35.33	725	
S. citrae	265	292	25.35	15.27	21.71	37.68	730	
S. everestense	271	299	23.93	18.16	23.25	34.66	727	
S. feltiae	275	298	24.80	16.44	21.64	37.12	730	
S. hebeiense	265	290	25.98	15.31	21.63	37.08	725	
S. ichnusae	265	318	24.13	17.16	21.76	36.96	717	
S. jollieti	266	289	25.42	16.15	21.91	36.52	712	
S. khoisanae	227	331	24.20	18.74	23.50	33.56	715	
S. kraussei	264	314	24.80	16.67	21.55	36.99	737	
S. kushidai	279	304	23.11	18.24	24.05	34.60	740	
S. litorale	264	290	25.88	16.60	21.38	36.15	711	
S. monticolum	264	245	26.58	15.17	22.82	35.44	666	
S. nyetense	282	284	22.27	19.64	24.21	33.89	723	
S. oregonense	267	298	24.21	17.70	22.27	35.82	723	
S. sacchari	311	296	22.51	19.63	23.82	34.03	764	
S. schliemanni	232	262	27.95	15.67	20.43	35.95	651	
S. rarum	240	312	26.81	18.33	22.22	32.64	270	
S. robustispiculum	262	249	26.79	14.82	22.31	36.08	668	
S. sangi	255	308	23.16	18.72	23.44	34.67	721	
S. silvaticum	264	304	25.45	17.24	22.28	35.03	728	
S. texanum	236	286	24.22	17.00	21.53	37.25	706	
S. weiseri	265	297	25.17	16.55	22.03	36.25	731	
S. xueshanense	264	293	23.81	17.09	22.55	36.55	729	
D2-D3 regions								
S. fabii n. sp.			23.50	19.38	30.88	26.25		801
S. cholashanense			24.56	19.62	30.32	25.50		851
S. citrae			24.46	19.39	29.76	26.38		887
S. everestense			24.35	19.81	31.98	23.86		616
S. feltiae			24.52	19.52	30.36	25.60		840
S. ichnusae			24.82	19.74	30.26	25.18		853
S. intermedium			26.36	17.61	28.72	27.30		846
S. kraussei			25.00	19.33	30.09	25.58		864
S. kushidai			24.68	18.45	30.33	26.53		867
S. monticolum			24.60	18.70	30.63	26.08		813
S. oregonense			24.77	19.59	30.42	25.23		868
S. sacchari			24.06	19.50	30.79	25.66		877
S. schielemanni			23.01	19.48	31.91	25.60		539
S. sichaunense			26.71	16.78	28.42	28.08		876
S. texanum			24.91	19.53	30.29	25.26		855
S. xueshanense			24.71	19.49	30.28	25.52		862

Table 6. Parwise differences between the ITS region of *Steinernema fabii* n. sp. and 20 species of *Steinernema.*

ITS region	Species	ITS region																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*S. fabii* n. sp.		0.050	0.050	0.060	0.173	0.170	0.179	0.176	0.176	0.159	0.170	0.167	0.165	0.181	0.162	0.204	0.176	0.176	0.173	0.201	0.184
2	*S. sacchari*	22		0.067	0.062	0.176	0.173	0.187	0.179	0.179	0.162	0.165	0.162	0.167	0.187	0.165	0.210	0.179	0.179	0.176	0.207	0.181
3	*S. cameroonense*	22	29		0.046	0.184	0.184	0.198	0.187	0.179	0.167	0.181	0.181	0.173	0.193	0.176	0.204	0.167	0.173	0.184	0.201	0.184
4	*S. nyetense*	26	27	20		0.196	0.190	0.193	0.198	0.196	0.179	0.193	0.190	0.184	0.204	0.181	0.225	0.198	0.193	0.184	0.204	0.198
5	*S. weiseri*	70	71	74	78		0.025	0.027	0.018	0.029	0.029	0.053	0.048	0.034	0.053	0.020	0.072	0.065	0.069	0.055	0.165	0.125
6	*S. ichnusae*	69	70	74	76	11		0.025	0.020	0.032	0.029	0.050	0.053	0.027	0.053	0.022	0.077	0.069	0.069	0.057	0.162	0.130
7	*S. feltiae*	72	75	79	77	12	11		0.022	0.038	0.041	0.055	0.060	0.041	0.065	0.029	0.082	0.082	0.077	0.067	0.173	0.138
8	*S. litorale*	71	72	75	79	8	9	10		0.029	0.036	0.057	0.055	0.041	0.060	0.025	0.077	0.072	0.077	0.060	0.162	0.127
9	*S. citrae*	71	72	72	78	13	14	17	13		0.032	0.060	0.055	0.041	0.060	0.032	0.082	0.065	0.069	0.062	0.167	0.125
10	*S. cholashanense*	65	66	68	72	13	13	18	16	14		0.036	0.027	0.009	0.034	0.020	0.077	0.046	0.046	0.036	0.154	0.125
11	*S. silvaticum*	69	67	73	77	23	22	24	25	26	16		0.032	0.038	0.057	0.046	0.091	0.067	0.062	0.060	0.165	0.140
12	*S. kraussei*	68	66	73	76	21	23	26	24	24	12	14		0.036	0.057	0.043	0.094	0.069	0.069	0.055	0.159	0.138
13	*S. xueshanense*	67	68	70	74	15	12	18	18	18	4	17	16		0.038	0.025	0.077	0.055	0.055	0.046	0.154	0.125
14	*S. texanum*	73	75	77	81	23	23	28	26	26	15	25	25	17		0.043	0.099	0.067	0.067	0.048	0.176	0.146
15	*S. jollieti*	66	67	71	73	9	10	13	11	14	9	20	19	11	19		0.069	0.053	0.053	0.050	0.143	0.114
16	*S. hebeiense*	81	83	81	88	31	33	35	33	35	33	39	40	33	42	30		0.104	0.109	0.101	0.193	0.165
17	*S. akhursti*	71	72	68	79	28	30	35	31	28	20	29	30	24	29	23	44		0.007	0.065	0.162	0.127
18	*S. everestense*	71	72	70	77	30	30	33	33	30	20	27	30	24	29	23	46	3		0.065	0.165	0.130
19	*S. sangi*	70	71	74	74	24	25	29	26	27	16	26	24	20	21	22	43	28	28		0.176	0.151
20	*S. schliemanni*	80	82	80	81	67	66	70	66	68	63	67	65	63	71	59	77	66	67	71		0.148
21	*S. monticolum*	74	73	74	79	52	54	57	53	52	52	58	57	52	60	48	67	53	54	62	61	

The number of base pair differences between sequences is shown below the diagonal. The number of base substitutions per site between sequences, according to the Jukes-Cantor model, is shown above the diagonal.

Table 7. Parwise comparison of the D2-D3 region of *Steinernema fabii* n. sp. with 20 *Steinernema* spp.

D2-D3	Species	D2-D3																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*S. fabi*i n. sp.		0.004	0.002	0.009	0.049	0.068	0.066	0.059	0.066	0.054	0.054	0.056	0.077	0.059	0.073	0.070	0.070	0.077	0.080	0.077	0.077
2	*S. cameroonense*	2		0.002	0.009	0.049	0.066	0.063	0.061	0.066	0.054	0.054	0.056	0.077	0.059	0.070	0.068	0.070	0.077	0.075	0.077	0.077
3	*S. sacchari*	1	1		0.007	0.047	0.066	0.063	0.061	0.063	0.052	0.052	0.054	0.075	0.056	0.070	0.068	0.068	0.075	0.077	0.075	0.075
4	*S. nyetense*	4	4	3		0.045	0.066	0.066	0.059	0.061	0.049	0.049	0.052	0.073	0.049	0.070	0.070	0.066	0.073	0.075	0.073	0.073
5	*S. lamjungense*	22	22	21	20		0.038	0.042	0.042	0.033	0.018	0.018	0.020	0.042	0.031	0.042	0.047	0.056	0.063	0.056	0.059	0.061
6	*S. arenarium*	30	29	29	29	17		0.013	0.026	0.049	0.038	0.038	0.040	0.056	0.042	0.056	0.026	0.073	0.090	0.066	0.066	0.068
7	*S. boemarei*	29	28	28	29	19	6		0.029	0.045	0.042	0.042	0.045	0.066	0.052	0.054	0.026	0.082	0.099	0.075	0.075	0.077
8	*S. australe*	26	27	27	26	19	12	13		0.056	0.040	0.038	0.040	0.063	0.052	0.061	0.038	0.077	0.092	0.068	0.070	0.077
9	*S. longicaudatum*	29	29	28	27	15	22	20	25		0.042	0.042	0.045	0.052	0.033	0.059	0.059	0.082	0.094	0.077	0.085	0.082
10	*S. khoisanae*	24	24	23	22	8	17	19	18	19		0.007	0.009	0.040	0.031	0.042	0.042	0.056	0.068	0.052	0.059	0.061
11	*S. innovationi*	24	24	23	22	8	17	19	17	19	3		0.007	0.038	0.031	0.045	0.045	0.056	0.066	0.049	0.054	0.056
12	*S. tophus*	25	25	24	23	9	18	20	18	20	4	3		0.045	0.033	0.042	0.047	0.059	0.073	0.056	0.061	0.063
13	*S. karii*	34	34	33	32	19	25	29	28	23	18	17	20		0.040	0.073	0.061	0.075	0.077	0.061	0.075	0.070
14	*S. hermaphroditum*	26	26	25	22	14	19	23	23	15	14	14	15	18		0.061	0.056	0.061	0.077	0.061	0.059	0.061
15	*S. sangi*	32	31	31	31	19	25	24	27	26	19	20	19	32	27		0.059	0.073	0.090	0.066	0.070	0.077
16	*S. glaseri*	31	30	30	31	21	12	12	17	26	19	20	21	27	25	26		0.082	0.090	0.066	0.075	0.077
17	*S. schliemanni*	31	31	30	29	25	32	36	34	36	25	25	26	33	27	32	36		0.042	0.040	0.040	0.045
18	*S. monticolum*	34	34	33	32	28	39	43	40	41	30	29	32	34	34	39	39	19		0.038	0.049	0.047
19	*S. everestense*	35	33	34	33	25	29	33	30	34	23	22	25	27	27	29	29	18	17		0.024	0.018
20	*S. xueshanense*	34	34	33	32	26	29	33	31	37	26	24	27	33	26	31	33	18	22	11		0.020
21	*S. jollieti*	34	34	33	32	27	30	34	34	36	27	25	28	31	27	34	34	20	21	8	9	

The number of base pair differences between sequences is shown below the diagonal. The number of base substitutions per site between sequences, according to the Jukes-Cantor model, is shown above the diagonal.

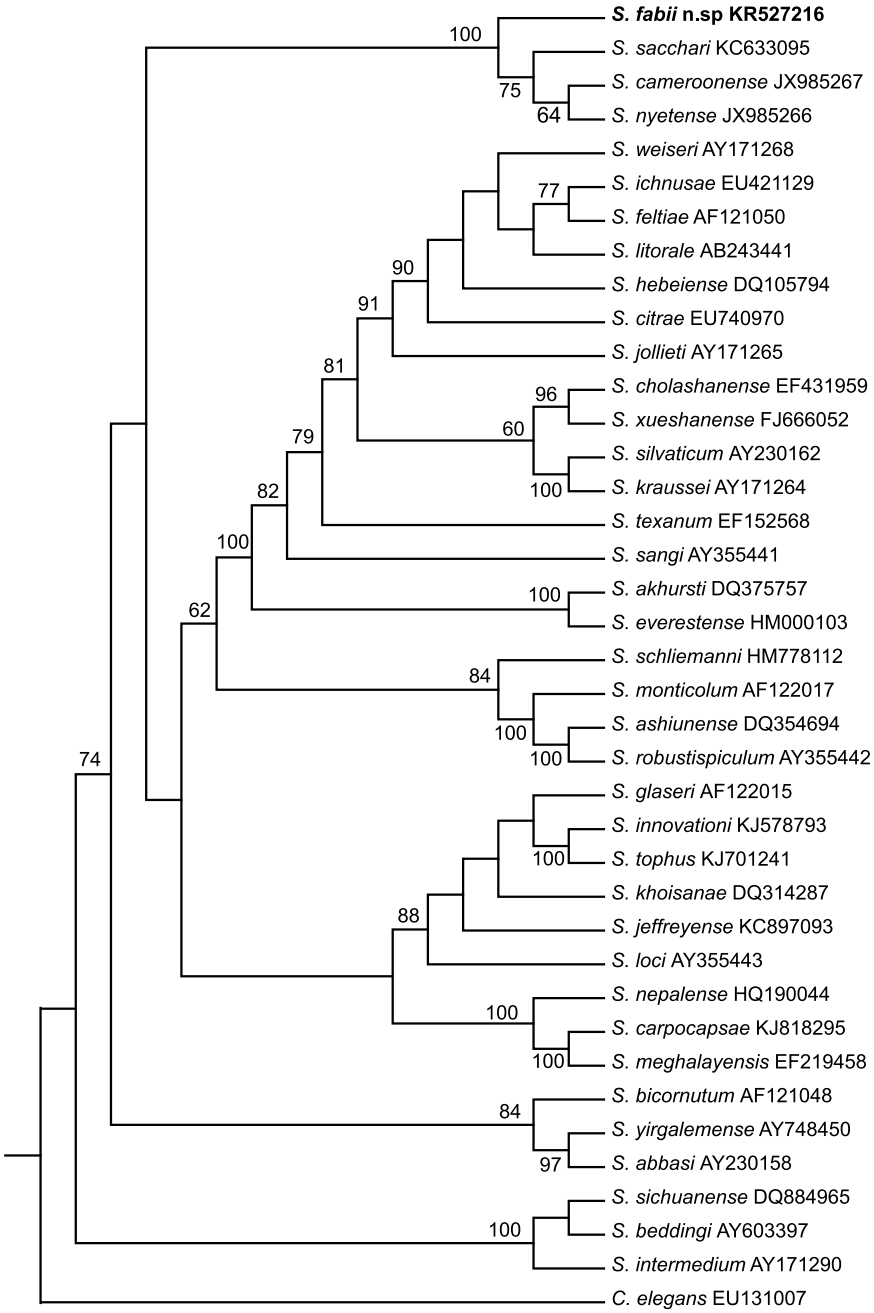

Fig. 5. Phylogenetic relationships of *Steinernema fabii* n. sp. with 37 species of *Steinernema* based on the ITS-rDNA sequences from GenBank. *Caenorhabditis elegans* (EU131007) was used as out group. Numbers at the nodes represent bootstrap proportion for Maximum Parsimony of 50% or more.

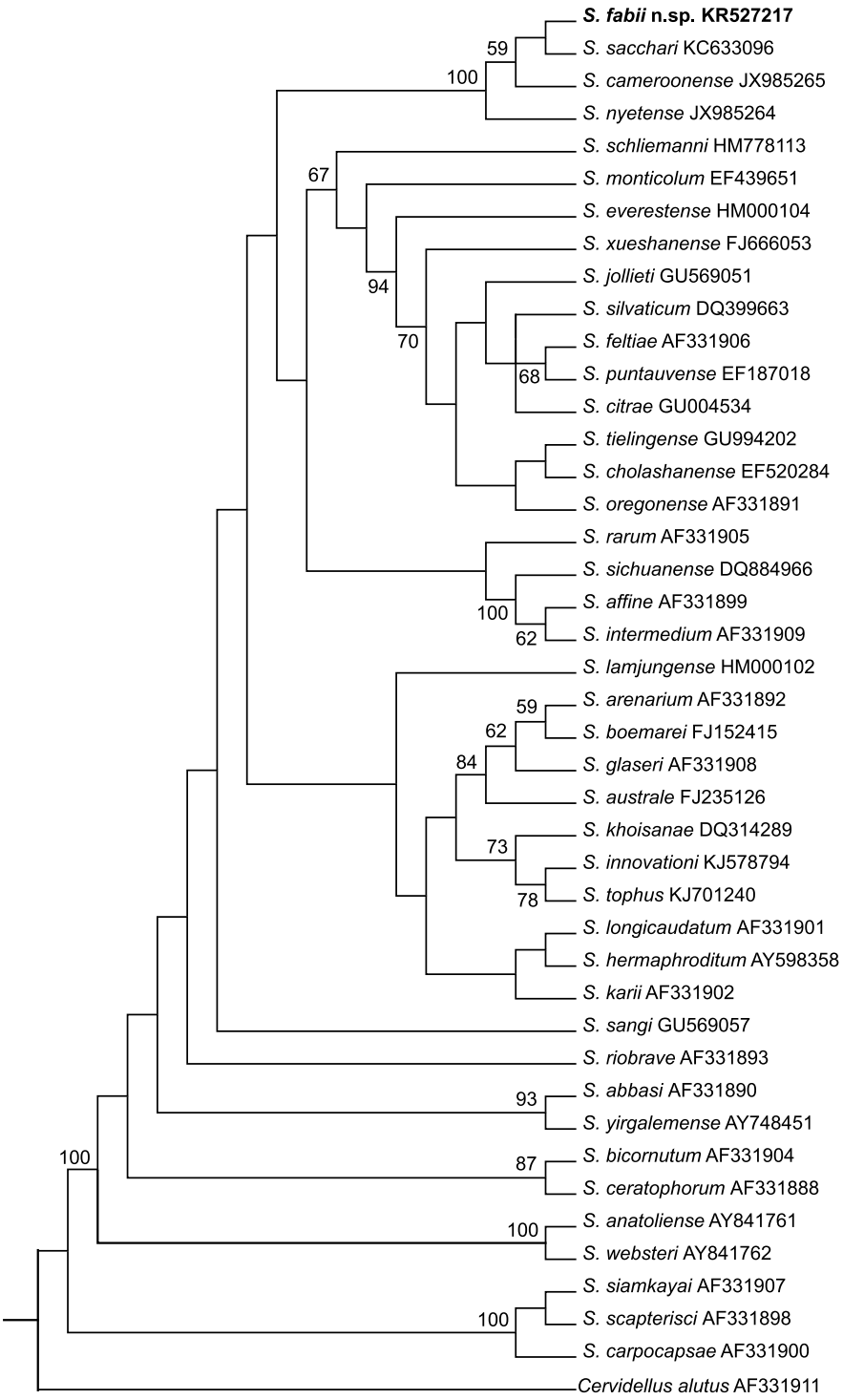

Fig. 6. Phylogenetic relationships of *Steinernema fabii* n. sp. with 41 species of *Steinernema* based on the D2-D3 rDNA sequences from GenBank. *Cervidellus alutus* (AF331911) was used as out group. Numbers at the nodes represent bootstrap proportion for Maximum Parsimony of 50% or more.

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