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 with a post anal swelling and conical and sharply pointed tail. Analysis of DNA sequences for the ITS and D2D3 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-oregonens*e Clade III.

Keywords - D2D3, description, ITS, molecular, morphology, morphometrics, new species, phylogeny, systematics, SEM, taxonomy

Entomopathogenic nematodes (EPNs) 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 (Grewal *et al*., 2005).

A report by Lewis and Clarke (2012) recognises 68 *Steinernema* species and there have been 12 additional species described since that review. A small number of these, namely *S. feltiae* and *S. carpocapsae* are distributed worldwide for the use against insect pests (Hominick, 2002). Due to their biological control relevance, surveys for native EPNs are important because EPNs are often better adapted to their local environmental conditions (Kaya and Gaugler, 1993) and may therefore achieve a higher level of efficacy than non-native species. Furthermore, the use of native EPNs negates the fears of those concerned about releases of exotic EPNs 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 and Barbercheck, 2001; Ehlers, 2005).

In South Africa, EPNs were first reported from the maize beetle, *Heteronychus arator* (Harington, 1953), and then later from soil samples in KwaZulu-Natal province (Spaull, 1990, 1991); in more recent years, several surveys have been conducted aimed at documenting the diversity of EPNs in the country (Malan *et al*., 2006, Hatting *et al*., 2009; Malan *et al*., 2011). 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. innovation* Cimen, Lee, Hatting, Hazir

& Stock, 2014 (Çimen *et al*., 2014); *S. sacchari* Nthenga, Knoetze, Berry, Tiedt & Malan, 2014 (Nthenga *et al*., 2014); *S. tophus* Cimen, Lee, Hatting, Hazir & Stock, 2014 (Cimen *et al*., 2014); and *S. jeffreyense* Malan, Knoetze & Tiedt, 2015 (Malan *et al*., 2015). These EPNs 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's from commercial forestry areas. The objective of this study was to characterize 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.

Material and methods

NEMATODE ORIGIN

Soil samples (*ca* 1kg) 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. EPNs were recovered from soil samples using insect baiting with the last instars of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) following the technique described by Stock and Goodrich-Blair (2012). Infective juveniles (IJs) were maintained by recycling through *G. mellonella* larvae and stored in approximately 150 ml of sterilized 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. mellonela* larvae were placed in a 9 cm diam. Petri dish lined with moistened filter paper. After inoculating with 200 IJs per *G. mellonela* larva, the larvae were kept in a growth chamber at 25°C. The larvae of *G. mellonela* were recorded as dead (no movement when prodded) two 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. IJs 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 glycerine, using the modified Seinhorst (1959) technique, after which they were mounted in pure glycerine. 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 IJs 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 2X 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 and Duncan (2002), using haemolymph of *G. mellonela* 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* Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 and *S. nyetense* Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 (Kanga *et al*., 2012) were not available for this test.

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC RELATIONSHIPS

Total genomic DNA was isolated from pooled samples of IJs using a modified phenol chloroform protocol described by Goodwin *et al*. (1992). The internal transcribed spacer regions (ITS) and of 28S (D2D3) regions of the ribosomal DNA were PCR amplified in 25 µl final volume with addition of 3 µl of 10x PCR buffer + 3 mM MgCl2, 1 µl of 5 mM dNTP's, 0.25 µl of Taq polymerase (Fast star), 16.75 µl of SABAX pour water (Adcock Ingram, Johannesburg, South Africa) 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 and Driver (1994). The primer set used for the D2D3 region was D2F and 536R (Ntengha *et al*. 2014). The PCR cycling profile for the ITS and D2D3 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 (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 D2D3 sequences of *S. fabii* n. sp. and corresponding nucleotide sequences of other representatives of *Steinernema* available in GenBank were aligned using MAFFT (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 D2D3 sequences, respectively.

Results

Steinernema fabii n. sp. (Figs. 1-4) takes its name from the Forestry and Agricultural Biotechnology Institute (FABI), University of 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: Specule. Infective juvenile. H: Anterior region; I: Tail region. G: Tail of second generation female (Scale bars: A, B, C, D = 50 µm; E, F, I = 20 µm; G, H = 10 µm).

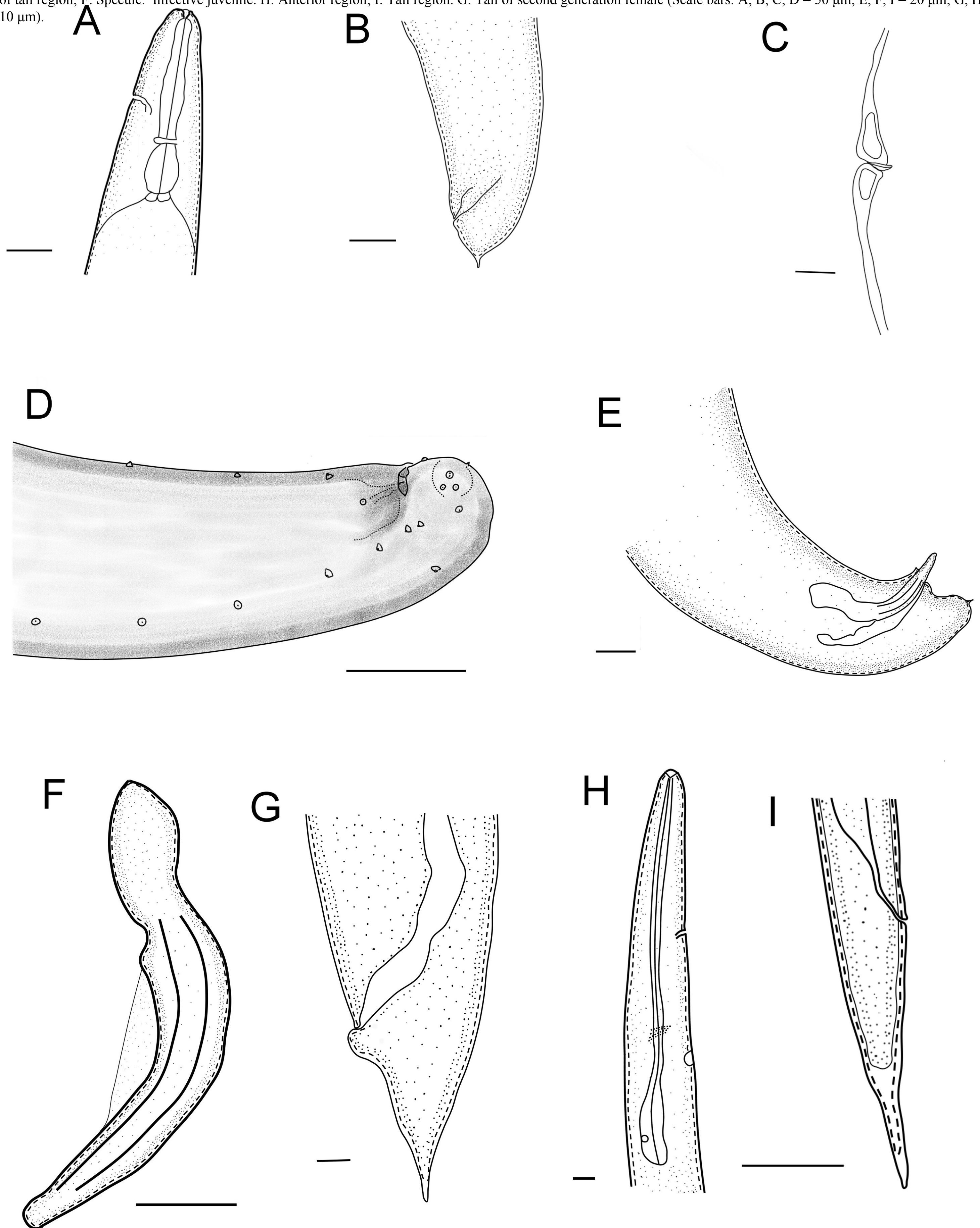
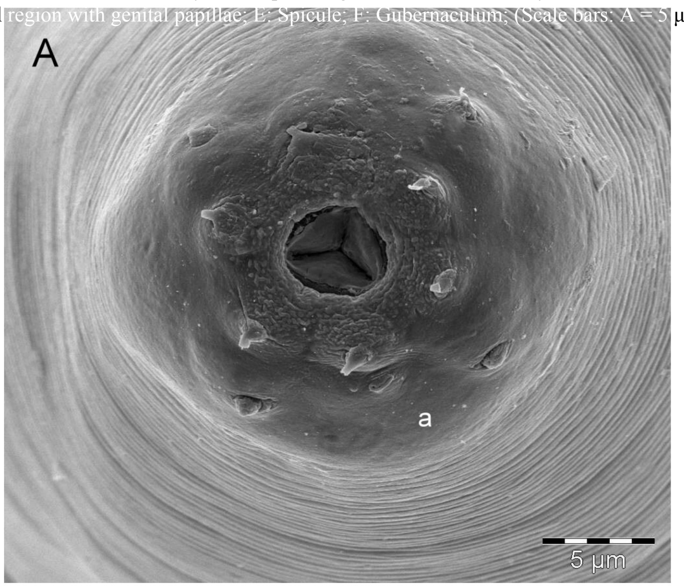
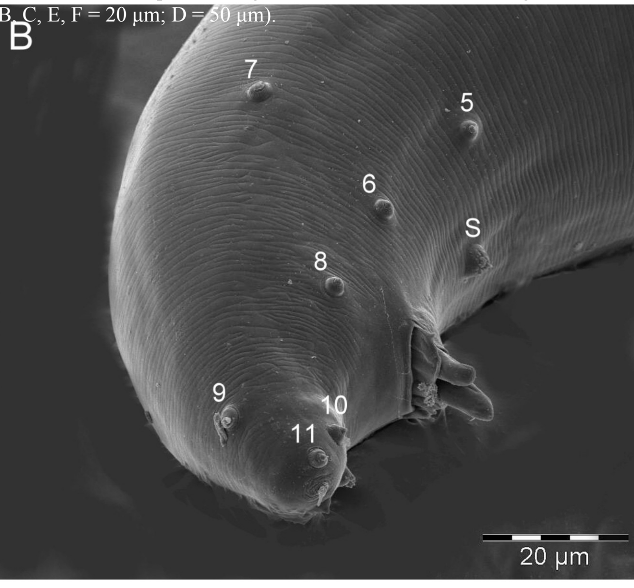
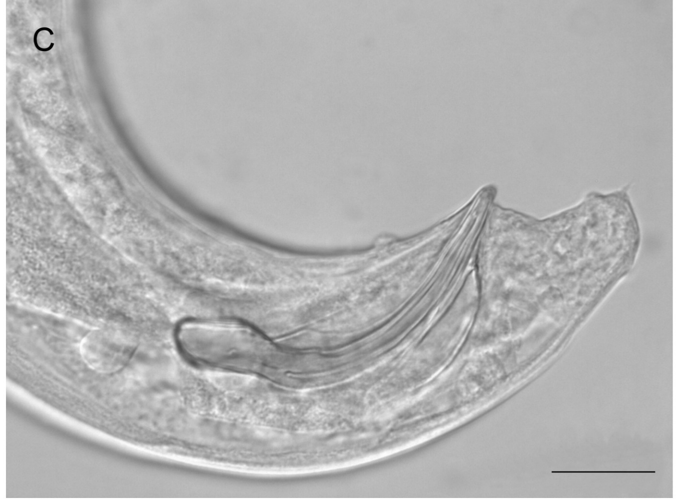


Fig. 2. Male of *Steinernema fabii* n. sp., first generation male. A: *En face* view; B: Lateral view of tail; C: Spicule and gubernaculum of tail of second generation male; D: Lateral view of tail region with genital papillae; E: Spicule; F: Gubernaculum; (Scale bars: A = 5 μm; B, C, E, F = 20 μm; D = 50 μm).







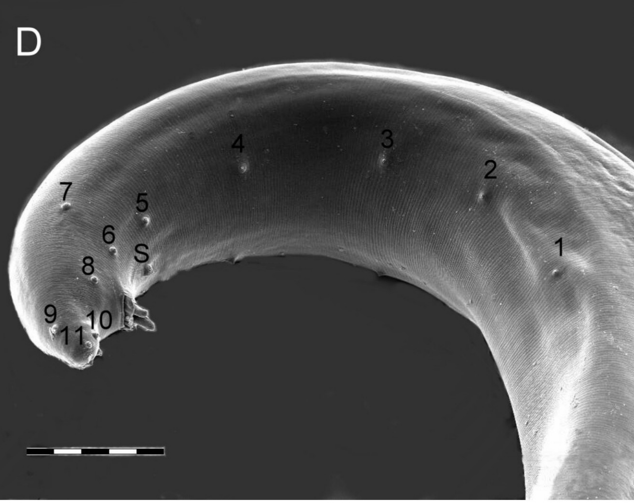
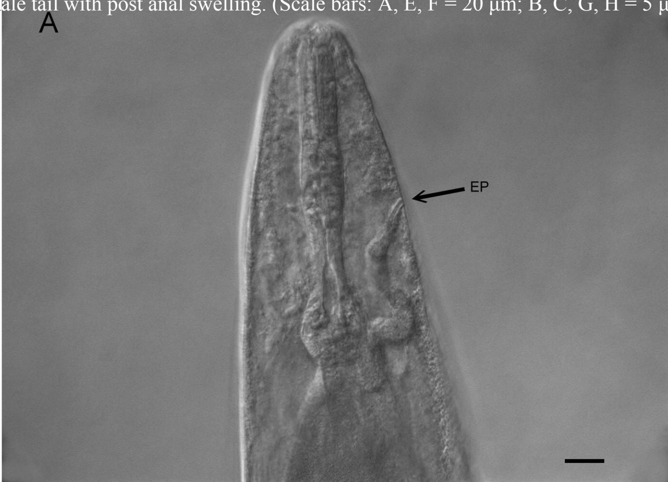
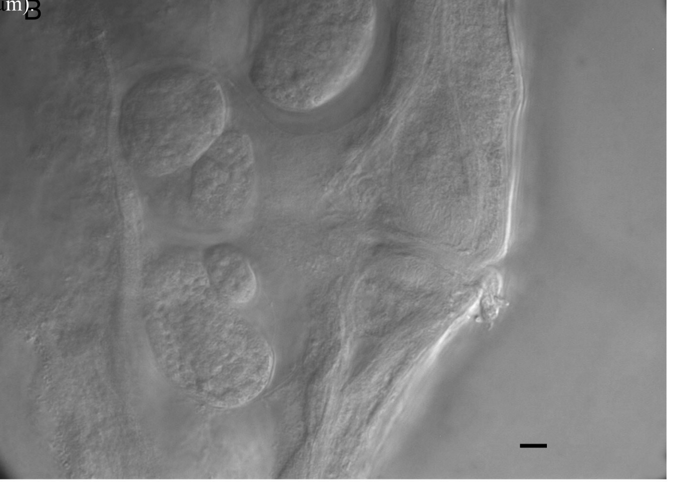
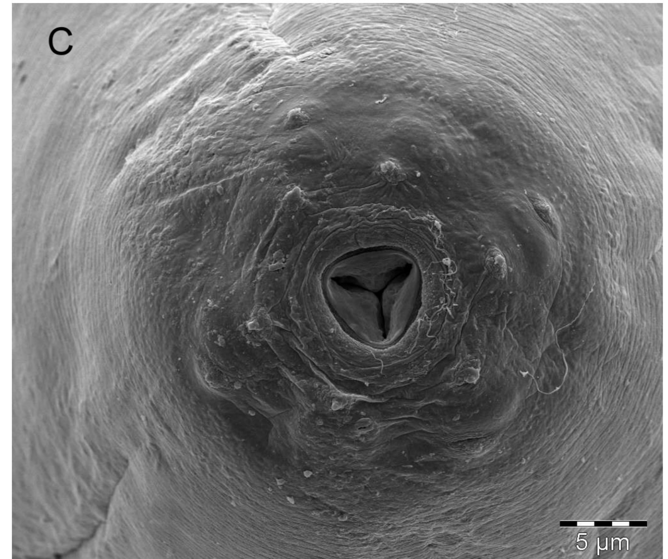
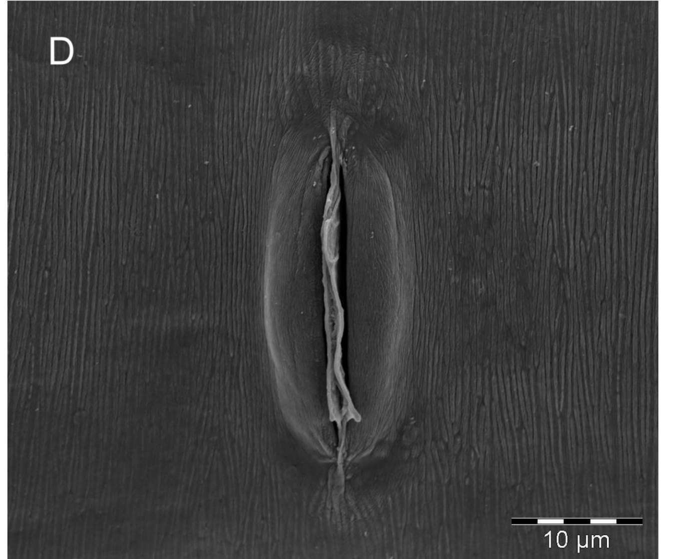


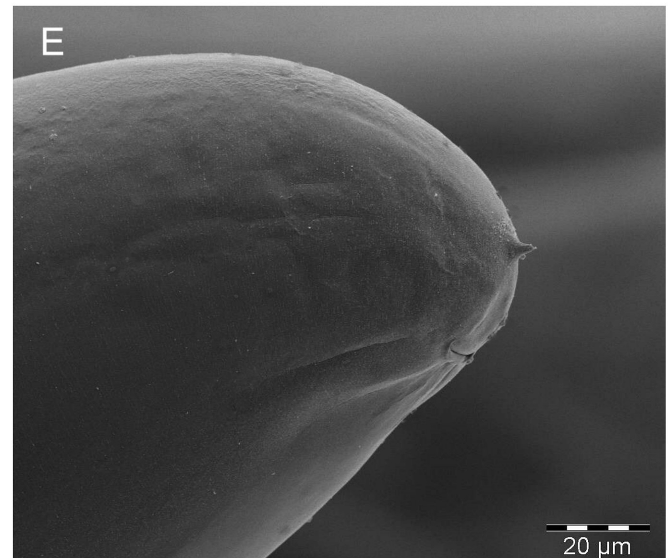
Fig. 3. Female Steinernema fabii n. sp. First generation. A: Anterior region showing pharynx and excretory pore; B, D:Vulva; C: En face view; E, G: Tail. F, H: Second generation female tail with post anal swelling. (Scale bars: A, E, F = 20 μm; B, C, G, H = 5 μm; D =10 μm).

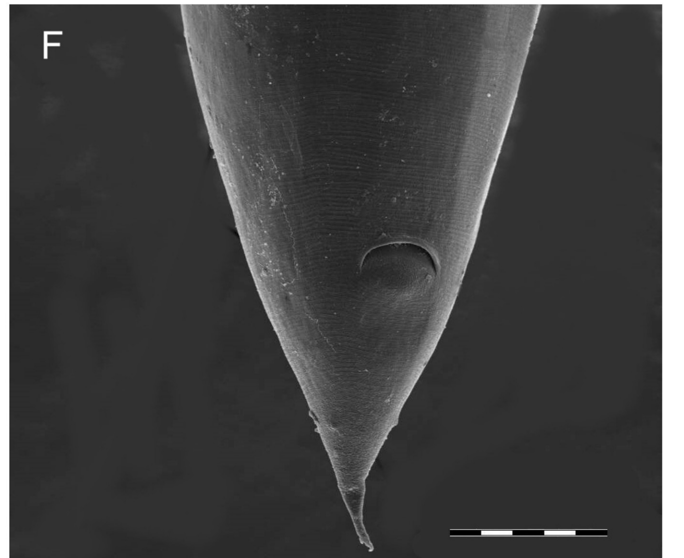


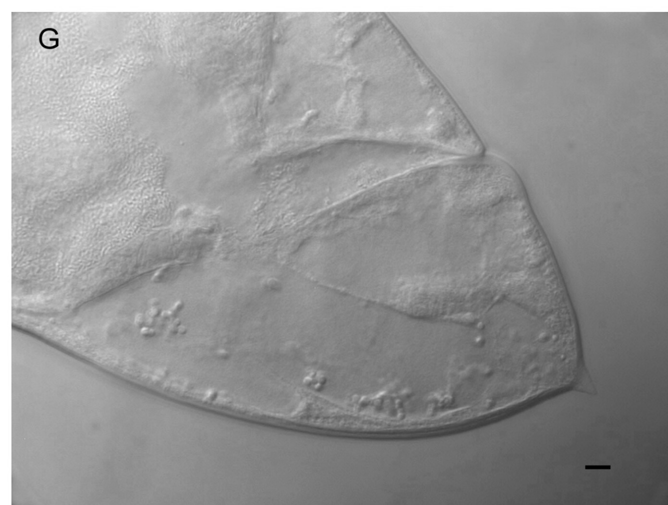












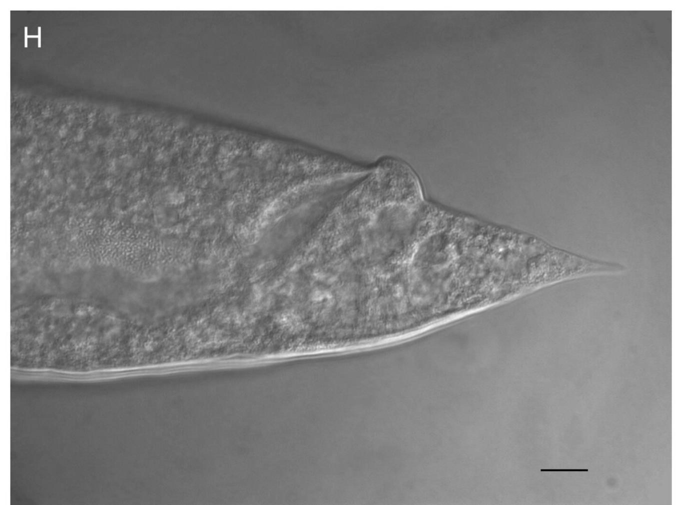
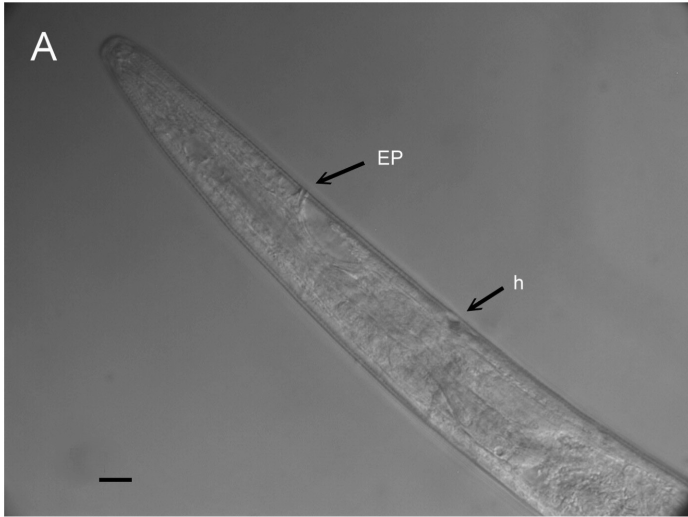
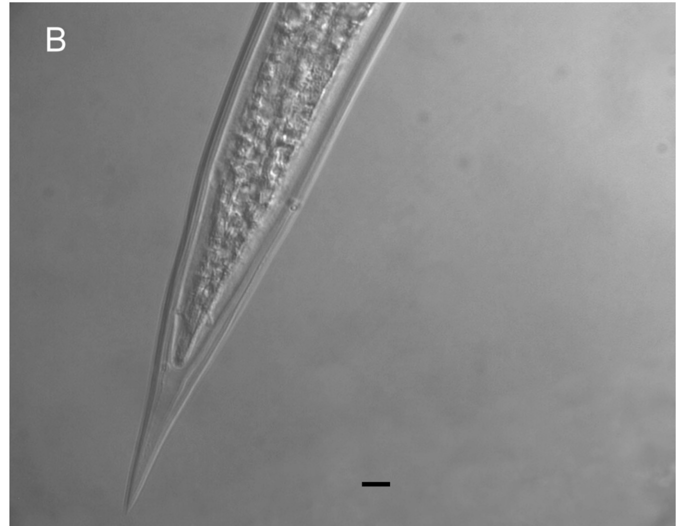
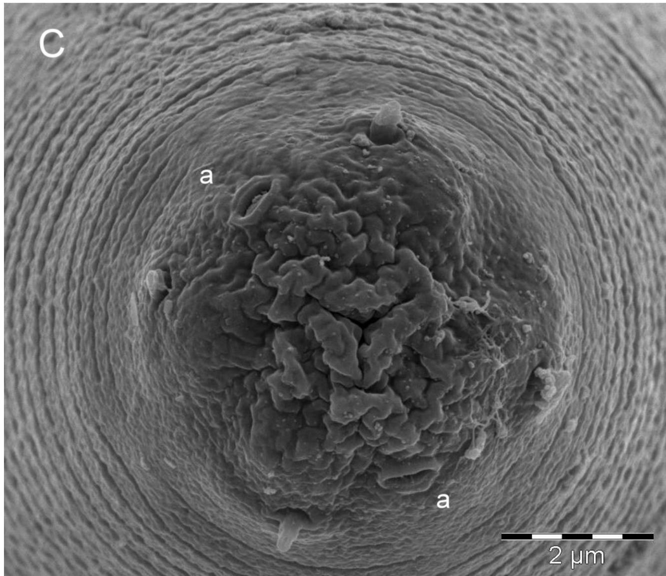
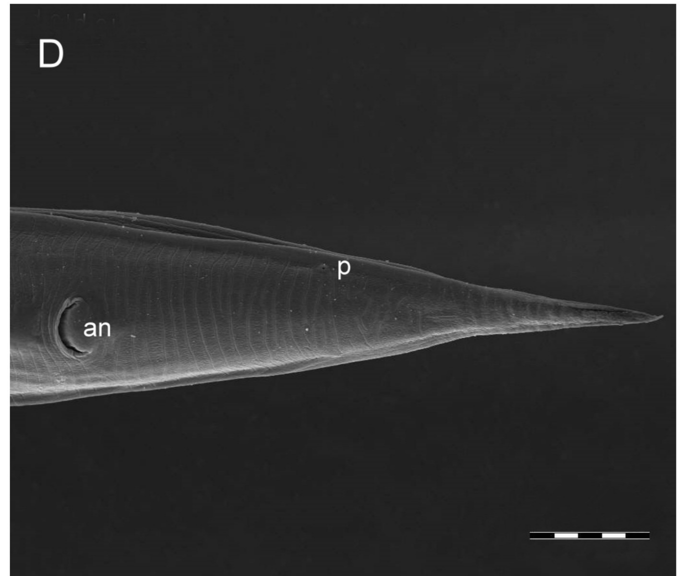


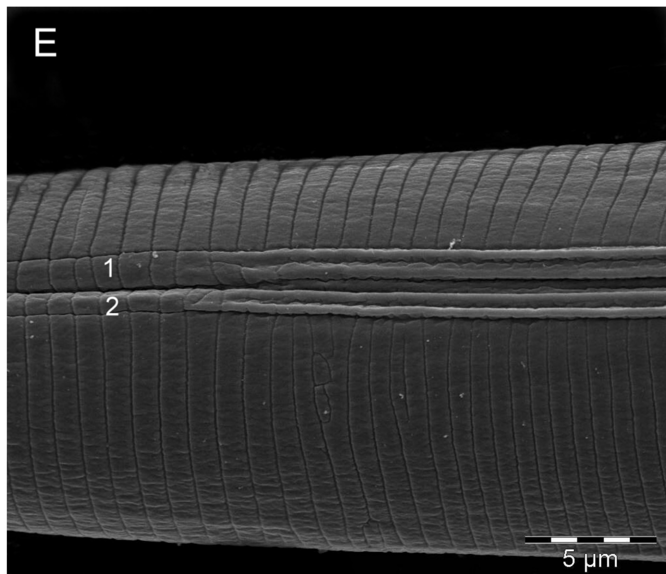
Fig. 4. Infective juvenile of *Steinernema fabii* n. sp. A: Anterior end showing excretory pore (EP) and hemizonid (h); B: Tail region showing hyaline region; C: Anterior region showing four cephalic papillae and two amphidial apertures (a); D: Tail with anus (an) and phasmid (p); E: splitting of ridges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

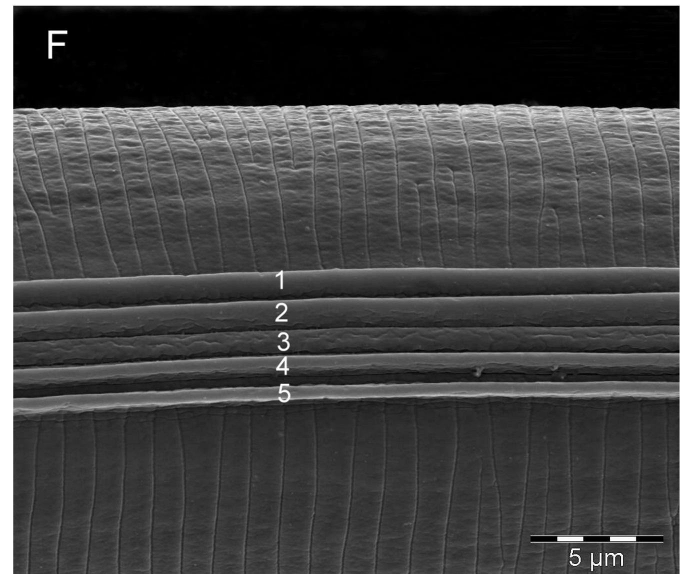












MEASUREMENTS

See Table 1.

DESCRIPTION

First generation male

The habitus of the first generation male is J-shaped when heat-killed. Cuticle smooth under light microscope, but finely striated under SEM. Head region smooth, rounded and not offset from the rest of the 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 the anterior part of basal bulb. Excretory pore located in front of the nerve ring approximately 64-68% of the length from anterior end to pharynx base; excretory duct well cuticularised. Cardia inconspicuous. Genital system monorchic and ventrally reflexed (testis reflexion 293-589 μm). Twenty-three genital papillae, comprising of 11 pairs and a single mid-ventral papilla, located just anterior to cloacal opening. Spicules paired, symmetrical, well curved and 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 and posterior end does not reach spicule tip. Gubernaculum boat shaped in lateral view, cuneus needle shaped and Y-shaped posteriorly, not reaching the tip of the corpus wings, which are 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 close 6-shape. Fine annulations visible on cuticle with SEM. Head region smoothly tapering and not off-set from the rest of the 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 the vicinity of the isthmus. Excretory pore located in front of the nerve ring, approximately 51-56% of the length from anterior end to pharynx base; excretory duct well cuticularised. Cardia prominent. Gonads amphidelphic, reflexed dorsally. Vulva median transverse slit, 53% from the anterior end of the body, slightly protruding, with short double flapped epiptygmata. Tail shorter than the anal body diameter, 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 78 (52-88) μm than second generation 36 (27-45) μm. Tail conical and sharply pointed. Terminal mucron absent. Post anal 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 and stoma closed. Pharynx long, narrow, with a slightly expanded procorpus, narrower isthmus and pyriform basal bulb with nuclei of the dorsal oesophageal and two sub dorsal glands clearly visible. Excretory pore anterior to nerve ring. Hemizonid distinct, locate towards the middle of

the isthmus. Nerve ring at the level of the isthmus above the 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, because *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 (S27^{0}12'30", E31^{0}1'4") 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 IJs, (four slides with 30 specimens) isolated from *G. mellonella* were 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 were deposited in the same place (50 IJs; 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 IJs; 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 IJs and adults. The IJs of the new species can be recognized 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 measurement of 90 (79-106) μm and 66 (56-77) μm, respectively. Other diagnostic characters include D% = 64 (52-75) μm, E% = 258 (149-395) μm, SW% = 177 (126-224) and GS% = 73 (63-86). The second generation males have similar morphological characters with the first generation, but smaller in size and narrower in body width. Both generations of males have 11 pairs of genital papillae, a single mid-ventral papillae and mucron at the tip of the tail. The first generation females have 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 D2D3 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, 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 longer than *S. cameroonense* and *S. rarum*. 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, *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 its longer body length of the first generation male, 1976 (1499-2435) μm *vs* 1331 (1019-1718) μm. The body diameter 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; however, first generation females of *S. cameroonense* and *S. nyetense* both have mucrons. The vulvas of the first and second generation females of the new species are protruding and have epiptygmata, in contrast to *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 D2D3 (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 bp *vs* 311 bp and 291 bp, respectively) and

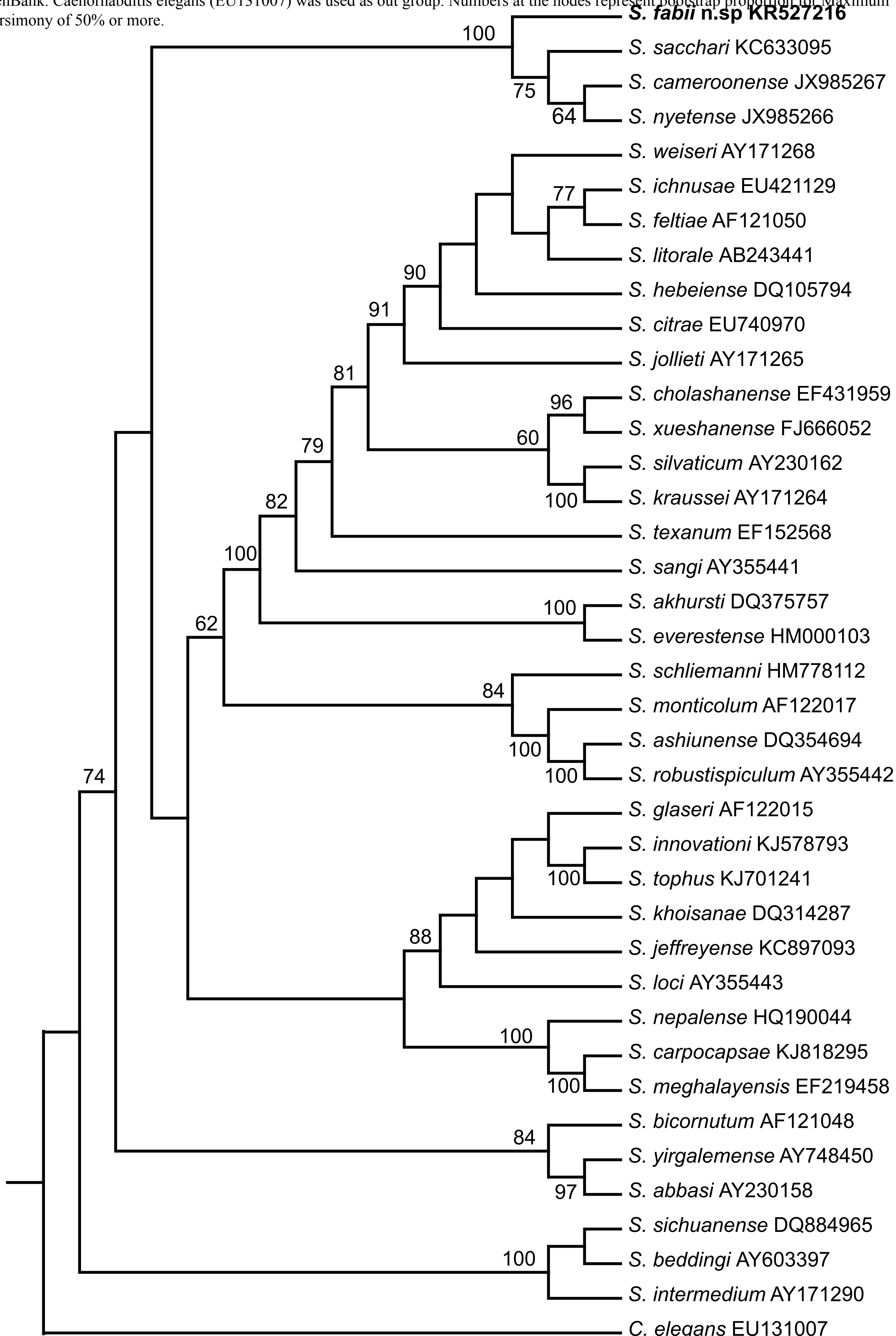
the ITS2 (312 bp 296 bp and 284 bp, respectively) (Table 5). Pairwise distances using the ITS region show that the new species differs from its closest relatives *S. sacchari* and *S. cameroonense* by 22 bp and from *S. nyetense* by 26 bp while differing from all other closely related species with higher numbers of bp. These bp 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* differ from *S. weiseri*, *S. ichnusae* and *S. feltiae* with eight, nine and ten bps respectively (Table 6).

The sequence of the D2D3 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 D2D3 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 (D2D3) 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%.

Fig. 5. Phylogenetic relationships of Steinernema fabii n. sp. thirty seven 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.



For the D2D3 region, the MP analysis of the aligned data resulted in 604 characters of which 348 variable characters were parsimony uninformative and 256 characters 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 D2D3 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 with 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-hybridization 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-hybridization 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 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

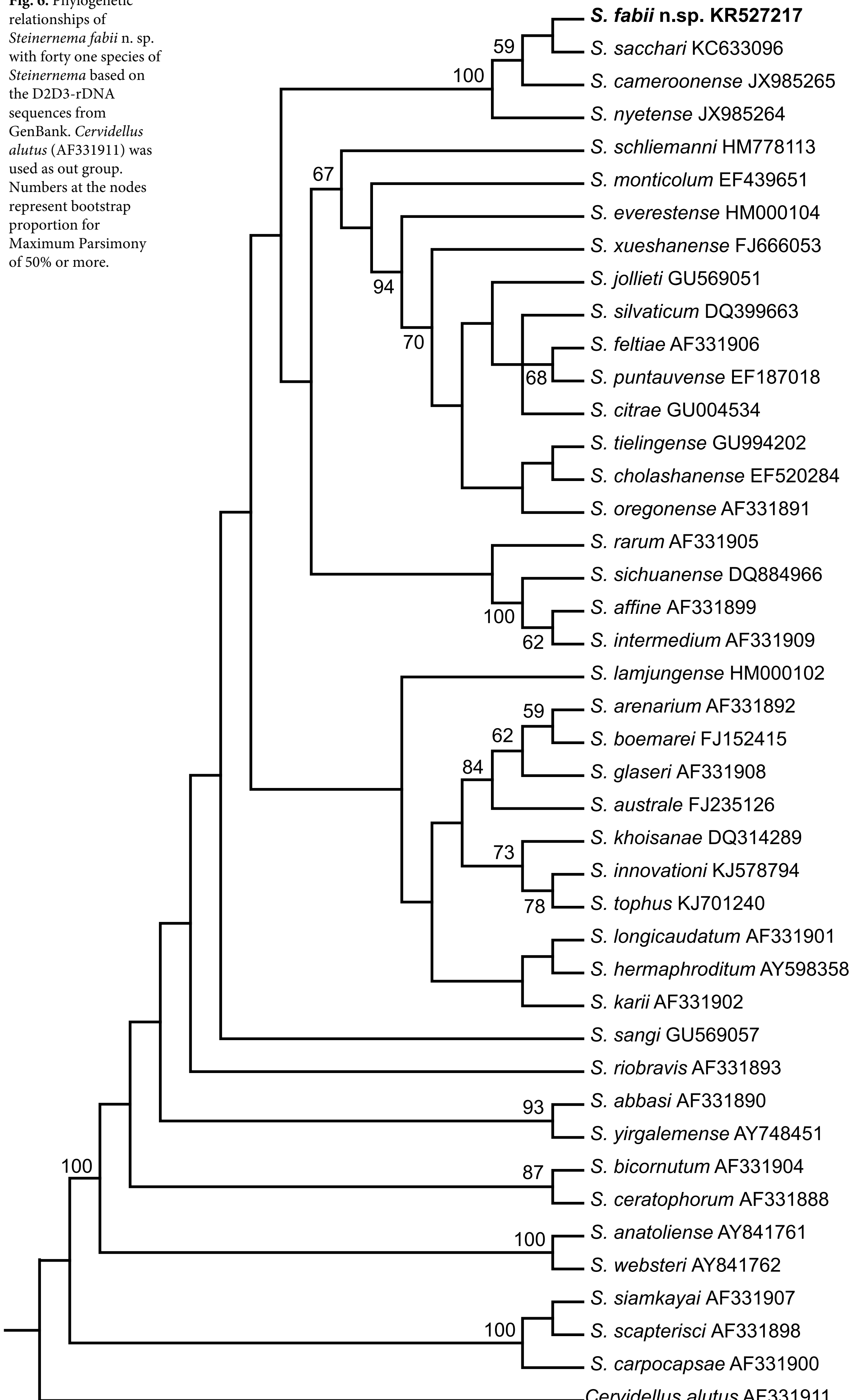


Fig. 6. Phylogenetic relationships of *Steinernema fabii* n. sp. with forty one species of *Steinernema* based on the D2D3-rDNA sequences from GenBank. *Cervidellus alutus* (AF331911) was used as out group. Numbers at the nodes represent bootstrap proportion for Maximum Parsimony of 50% or more.

days at 25°C. Second generation adults developed after 6 days. It usually required more than 12 days for IJs to emerge from the insect cadavers.

ACKNOWLEDGEMENTS

We thank members of the Tree Protection Cooperative Programme (TPCP), the University of Pretoria for funding this research. The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF. We are grateful to Hardus Hatting for assistance in the field, Reinaert van der Merwe for technical assistance and Tito Vorster for allowing us to sample from his farm.

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