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

Isaiah NTHENGA [1], Rinus KNOETZE [2], Shaun BERRY [3], Louwrens R. TIEDT [4] and Antoinette P. MALAN [5,*]

[1] *Department of Biology, Nematology Research Group, University of Ghent, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium*
[2] *Directorate Inspection Services, Department of Agriculture, Forestry and Fisheries, Private Bag X5015, Stellenbosch, 7599, South Africa*
[3] *South African Sugar Research Institute, Private Bag X02, Mount Edgecombe 4300, South Africa*
[4] *Laboratory for Electron Microscopy, North-West University, Potchefstroom Campus, Private Bag X6001, Potchefstroom 2520, South Africa*
[5] *Department of Conservation Ecology and Entomology, Department of AgriSciences, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa*

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Summary – A new species of entomopathogenic nematode, *Steinernema sacchari* n. sp., was isolated by trapping with the sugar cane borer, *Eldana saccharina*, from soil of a sugar cane field in the KwaZulu-Natal province of South Africa. The new species is morphologically characterised by the length of the infective juvenile (IJ) of 680 (630-722) μm, tail length of 64 (51-74) μm, ratio a = 19 (14-23), H% = 49 (43-57) and E% = 82 (70-109). The pattern of the lateral field of the IJ of the new species is 2, 5, 2 ridges (3, 6, 3 lines or incisures). The male of the first generation can be recognised by the long spicule of 83 (73-89) μm, gubernaculum of 61 (50-68) μm, D% = 67 (54-88) and GS% = 73 (66-81). The first generation male lacks a mucron, while the second generation male always has one. The first generation female can be recognised by the vulval lips not being raised, the possession of long double-flapped epiptygmata and the lack of a postanal swelling. Analysis of the ITS and D2D3 regions showed *S. sacchari* 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* and *S. sacchari* n. sp. This group is closely related to the *feltiae-kraussei-oregonens*e Clade III.

Keywords – D2D3, description, *Eldana saccharina*, ITS, molecular, morphology, morphometrics, new species, phylogeny, SEM, *Steinernema monticolum* group, systematics, taxonomy.

Entomopathogenic nematodes (EPN) are obligate insect parasites. They work in close association with a specific bacterium to be effective natural biocontrol agents against the soil stages of insects. EPN have proved to be effective pathogens against many soil-dwelling insect pests and have the potential to be used in an Integrated Pest Management system in order to reduce the need for toxic chemicals (Clausi *et al.*, 2011). In Africa, the amount of information that is available on their taxonomy is currently limited. Thus, there is a need for the isolation and for the correct identification of indigenous EPN strains, which is important for the realisation of their use as biocontrol agents for controlling insect pests (Conlong, 1994; Ganguly *et al.*, 2011; Kanga *et al.*, 2012). Currently, approximately 84 species of *Steinernema* and 20 species of *Heterorhabditis* have been described worldwide.

In South Africa, the first recording of an EPN was on the maize beetle, *Heteronychus sanctae-helenae* Blanch, in Grahamstown, Eastern Cape province, by Harrington (1953). In 1988, during a survey four isolates of EPN, three *Steinernema* and one *Heterorhabditis*, were found in KwaZulu-Natal. They were subsequently investigated for the management of the sugar cane stalk borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae), in laboratory and field trials (Spaull, 1988, 1990). Spaull (1991) conducted another survey to obtain effective EPN against *E.*

* Corresponding author, e-mail: apm@sun.ac.za

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saccharina, in which 15 *Steinernema* isolates and seven *Heterorhabditis* isolates were discovered. However, these isolates were not identified to species level.

According to extensive surveys conducted by Malan *et al.* (2006, 2011) and Hatting *et al.* (2009), *Heterorhabditis bacteriophora* Poinar, 1976 was found to be the most abundant species in South Africa. Two new species, which were discovered during the 2006 survey, were subsequently described as *Steinernema khoisanae* Nguyen, Malan & Gozel, 2006 and *H. safricana* Malan, Nguyen, De Waal & Tiedt (Nguyen *et al.*, 2006; Malan *et al.*, 2008). *Steinernema yirgalemense* was also reported during a survey that was undertaken to establish the diversity of EPN in citrus orchards (Malan *et al.*, 2011). Currently, two more new species from citrus orchards in South Africa have been described: *S. citrae* Stokwe, Malan, Nguyen, Knoetze & Tiedt, 2011 and *H. noenieputensis* Malan, Knoetze & Tiedt, 2013 (Stokwe *et al.*, 2011; Malan *et al.*, 2013).

The objective of this study was to characterise a third new *Steinernema* species from South Africa, and it is described and illustrated herein as *S. sacchari* n. sp. Morphological and molecular characteristics were used to differentiate between the new species and other species of *Steinernema* already described. This work also adds to existing knowledge on the distribution and diversity of EPN.

Materials and methods

Nematode source

Soil (1.5 kg) was collected by taking ten subsamples from a sugar cane field in Gingindlovu, KwaZulu-Natal (29°01′37″S, 31°35′37″E). The nematode isolate SB10 was isolated in the laboratory from 500 ml soil in three glass containers with ten larvae of laboratory-cultured *E. saccharina*, according to the method described by Bedding & Akhurst (1975), in each, closed with a lid and left for 7 days at room temperature. The infective juveniles (IJ) were maintained by recycling through *Galleria mellonella* L. (Lepidoptera: Pyralidae) (Dutky *et al.*, 1964) and were stored in 140 ml of water in 500 ml, horizontally placed, vented culture flasks at 14°C.

Morphological observations

For observation and measurement of the different life stages, ten *G. mellonella* larvae were placed in 9 cm diam. Petri dishes lined with moistened filter paper and, after inoculating with 200 IJ per larva of *G. mellonella*, were kept in a growth chamber at 25°C. Two days after inoculation, the *Galleria* larvae were dead. Males and females of the first and second generations were obtained after 4-5 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) that was prepared by placing the base of the 9 cm Petri dish containing infected cadavers inside a 15 cm 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). Specimens were then processed to 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 with Leica Application Suite V3.5.0. software. For direct observations to confirm the morphology or the variations of specific structures, different stages were either examined live or after they had been killed with gentle heat. Exsheathed IJ were obtained by storing in culture flasks at 14°C for 2 months.

Scanning electron microscopy (SEM)

For the SEM, males and females of the first and second generation, as well as IJ, were fixed in TAF for a minimum of 3 days, washed three times in 0.05 M cacodylate buffer for 15 min each, and then washed three times in distilled water for 15 min each, after which they were dehydrated in a graded ethanol series (70, 80, 90 and 2 × 100%). The samples were critical point dried with liquid CO_2, mounted on SEM stubs and sputter coated with 20 nm gold/palladium (66/33%). The samples were viewed with a FEI Quanta 200 ESEM, operating at 10 kV under high-vacuum mode.

Molecular characterisation and phylogenetic relationships

DNA was extracted from a single female, using a modification of a method reported by Nguyen (2007). The nematode was placed in 30 μl of lysis buffer (50 mM $MgCl_2$, 10 mM DTT, 4.5% Tween-20, 0.1% gelatine and 1 μl of proteinase K at 60 μg m^{-1}) on the side of an Eppendorf tube, where it was cut into pieces with the aid of the sharp side of a sterile insulin needle. The tube was

immediately stored at −80°C for at least 15 min. The tube was then incubated at 65°C for 1 h and then at 95°C for 10 min in order to lyse the cells completely, as well as to digest the proteins. The tube was cooled on ice and centrifuged at 11 600 *g* at 10°C for 2 min. Twenty μl of the supernatant containing the DNA were collected and stored at −80°C for further use.

Two PCR primers were used to amplify the ITS regions, including the 5.8S ribosomal gene, as well as short parts of the 18S and 28S ribosomal genes. The 18S primer (5′-TTGATTACGTCCCTGCCCTTT-3′) and 28S primer (5′-TTTCACTCGCCGTTACTAAGG-3′) have been described by Vrain *et al.* (1992) for purposes of amplification of the ITS regions. To amplify the D2D3 regions of 28S rDNA, primers D2F (5′-CCTTAGTAACGGCGAGTGAAA-3′), reported by Nguyen *et al.* (2006), and 536 (5′-CAGCTATCCTGAGG AAAC-3′), reported by Stock *et al.* (2001), were used.

PCR amplification reactions contained 5 μl of nematode lysate, together with 0.5 μM of each primer, dATP, dCTP, dGTP and dTTP, each at 200 μM final concentration, 1× *Taq* reaction buffer, 1.5 mM $MgCl_2$ and 1 U *Taq* polymerase. The final reaction volume was 25 μl.

The cycling conditions were as follows: denaturation at 94°C for 20 s, annealing at 50-55°C for 30 s, and extension at 72°C for 45 s, repeated for 35 cycles. A 2-min incubation period at 72°C followed the last cycle in order to complete any partially synthesised strands. The PCR product was then run on 1% agarose gel in a 1× TBE buffer, and visualised by means of ethidium bromide staining.

Post-PCR purification was undertaken using the NucleoFast Purification System (Macherey Nagel). Sequencing was performed with the BigDye Terminator V1.3 sequencing kit (Applied Biosystems), followed by electrophoresis on the 3730 × 1 DNA Analyser (Applied Biosystems) at the DNA Sequencing Unit (Central Analytical Facilities, Stellenbosch University). Two internal primers, KN58 (5′-GTATGTTTGGTTGAAGGTC-3′) and KNRV (5′-CACGCTCATACAACTGCTC-3′), suggested by Nguyen *et al.* (2004), were used in addition to the ITS primers 18S and 28S to enable the sequencing of the complete ITS regions. Likewise, primers 502 (5′-CAAGTACCGTGAGGGAAAGTTGC-3′) and 503 (5′-CCTTGGTCCGTGTTTCAAGACG-3′), reported by Stock *et al.* (2001), were used for sequencing of the D2D3 regions. Sequence assembly and editing was performed on the CLC DNA Workbench (see http://www.clcbio.com).

The sequences generated of the ITS region of the 18S rDNA gene and of the D2D3 region of the 28S gene of *S. sacchari* n. sp. were compared with those of the *Steinernema* species available on GenBank (NCBI). The alignment was done using ClustalX 2.1 (Thompson *et al.*, 1997). Phylogenetic analyses of sequence data were done using the Maximum Parsimony (MP) method in MEGA5 (Tamura *et al.*, 2011). Trees were evaluated statistically by means of a bootstrap analysis based on 1000 resamplings of the dataset. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1, in which the initial trees were obtained by means of the random addition of sequences (10 replicates). *Caenorhabditis elegans* (EU131007) was used as the outgroup during the calculation of the trees based on the ITS sequences and *Cervidellus alutus* (AF331911) was used as the outgroup for the calculation of the tree based on the D2D3 sequences.

Results

Steinernema sacchari[*] n. sp.
(Figs 1-4)

Measurements

See Table 1.

Description

First generation male

Body curved posteriorly, mostly J-shaped when heat-relaxed. Cuticle smooth under light microscope, but with striation under SEM. Lateral field absent. Cephalic region with four cephalic and six labial papillae. Amphidial apertures and perioral disk not observed. Stoma shallow, funnel-shaped, cheilorhabdions prominent, moderately cuticularised. Pharynx with cylindrical procorpus and slightly swollen metacorpus, isthmus narrow, surrounded by nerve ring, basal bulb swollen, with valve. Excretory pore anterior to nerve ring, well cuticularised, excretory gland not observed. Cardia prominent. Bacterial chamber obscure. Genital system monorchic, reflexed, comprising germinal zone, growth zone, *vas deferens*. Spicules paired, light brown in colour. Head (manubrium) of spicules somewhat curved, shaft (calomus) short, blade

[*] Specific epithet derived from the sugar cane (*Saccharum officinarum*) field from which the species was collected.

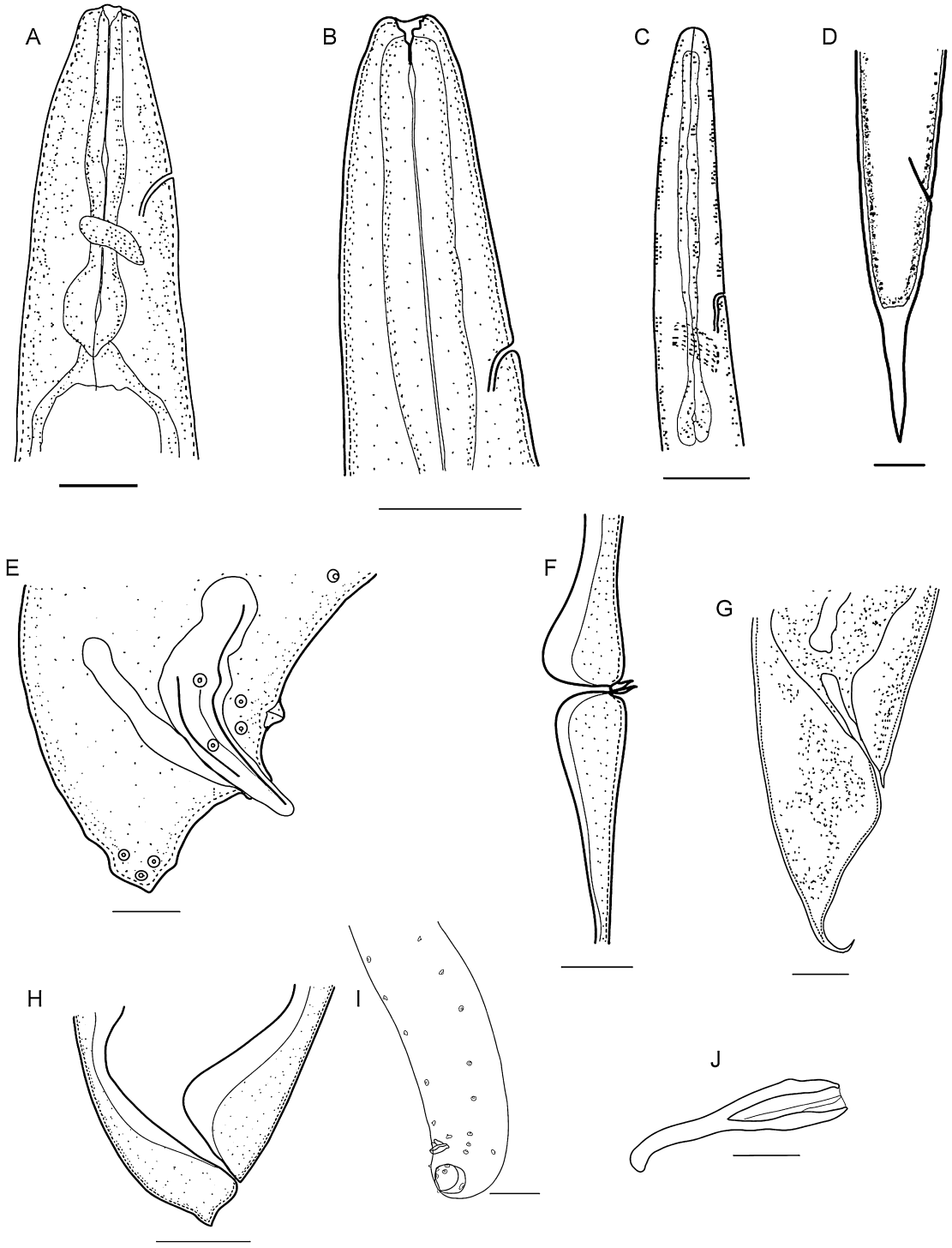

Fig. 1. *Steinernema sacchari* n. sp. First generation male. A: Anterior region; E: Lateral view of tail region; I: Ventral view of tail; J: Gubernaculum. First generation female. B: Anterior region; F: Vulva with double-flapped epiptygmata; H: Tail region. Infective juvenile. C: Anterior region; D: Tail region. G: Tail of second generation female. (Scale bars: A = 50 μm; B-H, J = 20 μm; I = 25 μm.)

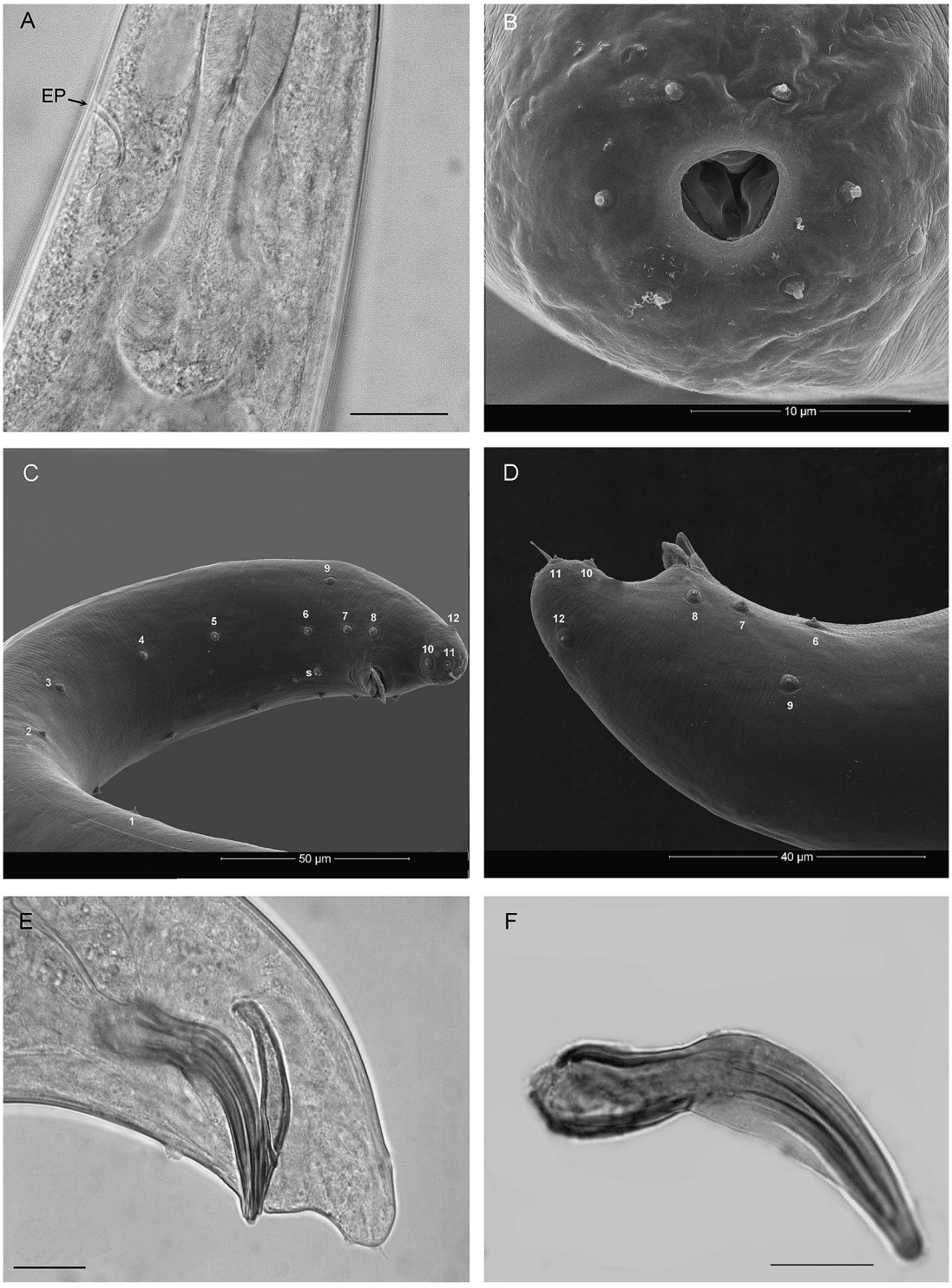

Fig. 2. Male of *Steinernema sacchari* n. sp. A-C, first generation. A: Pharyngeal region showing excretory pore (EP); B: *En face* view; C: Tail region, showing 12 pairs of papillae and one midventral papilla (s); D: Lateral view of tail of second generation; E: Spicula and gubernaculum of tail of first generation; F. Spicule shape. (Scale bars: A, E, F = 20 μm; B = 10 μm; C = 50 μm; D = 40 μm.)

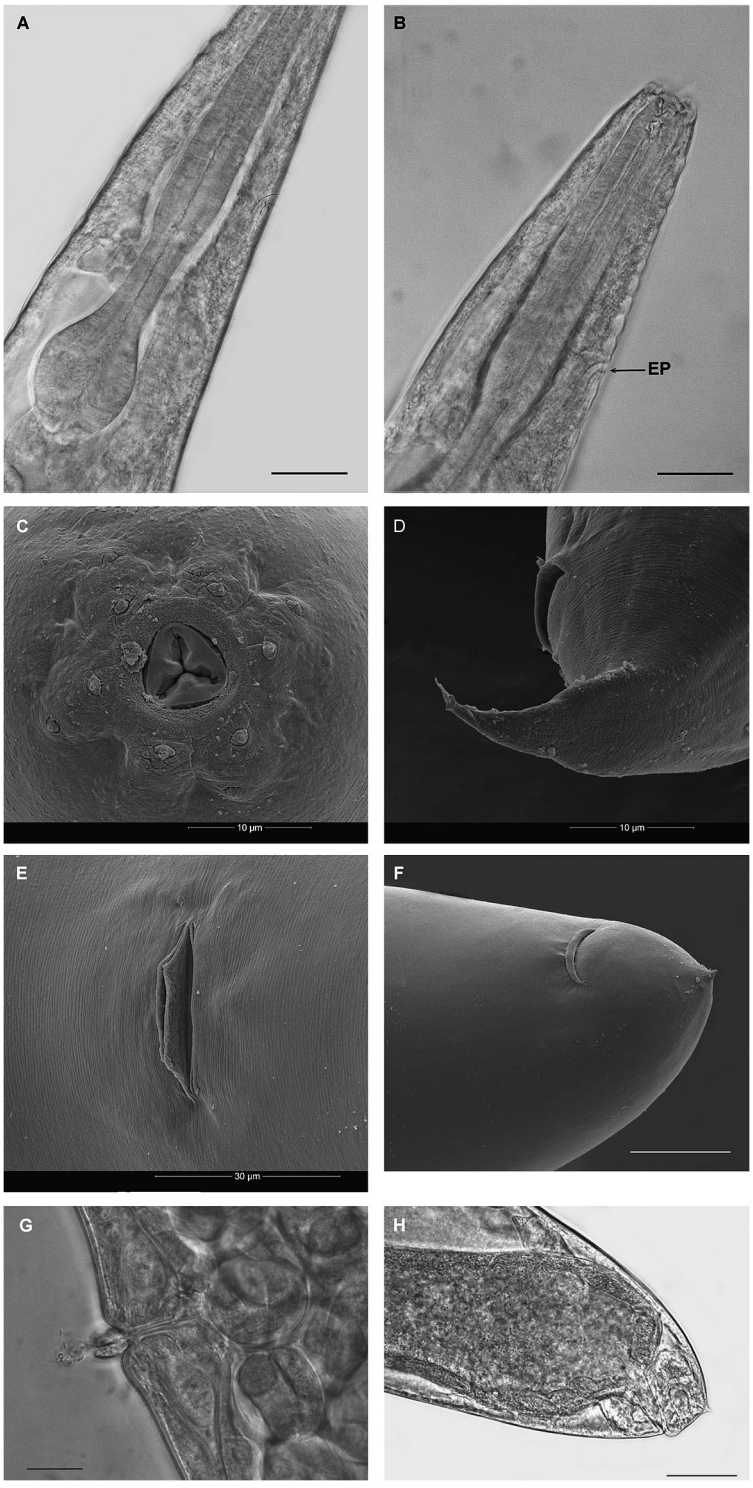

Fig. 3. Female *Steinernema sacchari* n. sp. A-C and E-H, first generation. A: Anterior region showing pharynx; B: Excretory pore position and stoma; C: *En face* view. D: Second generation tail; E: Double-flapped epiptygmata; F: First generation tail; G: Vulva with epiptygmata; H: Tail of first generation. (Scale bars: A, B, G, H = 20 μm; C, D = 10 μm; E = 30 μm; F = 40 μm.)

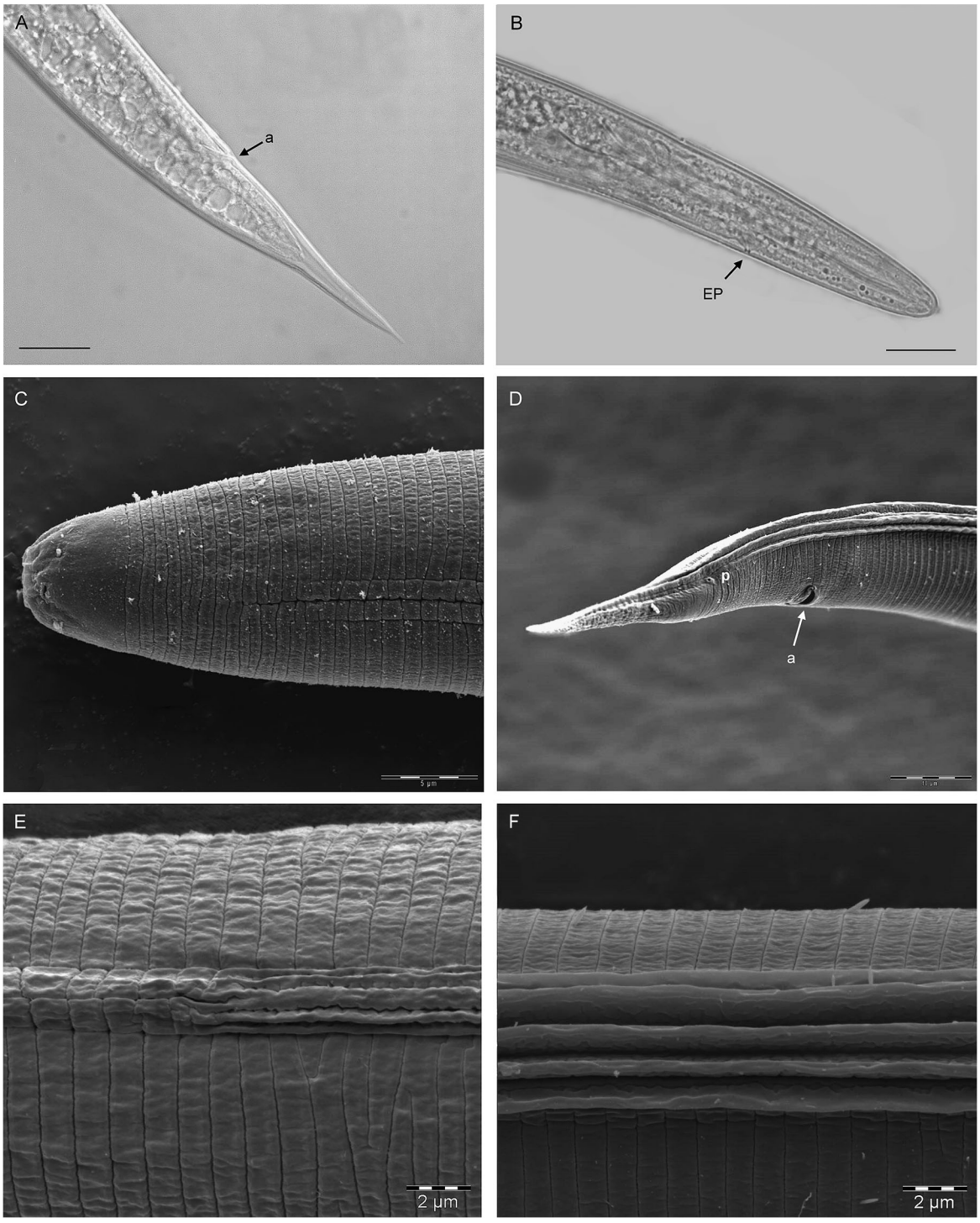

Fig. 4. Infective juvenile of *Steinernema sacchari* n. sp. A: Tail region showing shape of tail and anus (a); B: Anterior end showing excretory pore (EP); C: Pharynx showing start of two lateral ridges; D: Tail with anus (a) and phasmid (p); E: Splitting of ridges in lateral field from two to five (from anterior); F: Five ridges in lateral field in mid-body. (Scale bars: A, B = 20 μm; C, F = 5 μm; D = 10 μm; E = 2 μm.)

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

Character	First generation			Second generation		Infective juvenile
	Male		Female	Male	Female	
	Holotype	Paratypes	Paratypes	Paratypes	Paratypes	
n	–	20	20	20	20	25
L	1867	1829 ± 228 (1406-2181)	4316 ± 623 (3163-5600)	1101 ± 134 (877-1441)	1394 ± 150 (1244-1849)	680 ± 27 (630-722)
a	12	14 ± 1.6 (9.3-16)	15 ± 2.6 (11-20)	15 ± 0.9 (14-18)	13 ± 0.7 (11-14)	19 ± 2.1 (14-23)
b	11	11 ± 1.0 (9.0-13)	20 ± 2.7 (15-26)	8.3 ± 0.6 (7.0-9.1)	8.0 ± 0.6 (7.4-9.7)	6.0 ± 0.2 (5.6-6.5)
c	58	54 ± 7.1 (38-68)	160 ± 35 (102-218)	45.2 ± 6.4 (34-56)	33 ± 7.1 (26-57)	10.5 ± 0.8 (9.6-12.3)
c′	–	0.7 ± 0.1 (0.6-0.9)	0.4 ± 0.1 (0.28-0.64)	– –	– –	– –
V	–	– –	54 ± 1.8 (50-57)	– –	56 ± 1.1 (54-58)	– –
Body diam. (MBD)	153	145 ± 27 (86-205)	290 ± 25 (243-347)	72 ± 6.4 (61-82)	109 ± 7.4 (100-130)	37 ± 4.1 (30-47)
Excretory pore (EP)	107	110 ± 16 (84-158)	103 ± 21 (76-150)	92 ± 5.8 (81-102)	86 ± 5.4 (78-97)	53 ± 2.3 (49-58)
Nerve ring (NR)	113	116 ± 10 (97-135)	144 ± 14 (126-182)	96 ± 8.2 (82-119)	130 ± 6.4 (119-145)	84 ± 3.8 (78-97)
Pharynx length (ES)	168	164 ± 10 (149-183)	216 ± 10 (199-244)	133 ± 11 (119-169)	175 ± 7.6 (163-192)	113 ± 4.5 (104-127)
Hemizonion	–	– –	– –	– –	– –	99 ± 5.6 (86-101)
Testis reflex	435	419 ± 75 (262-541)	– –	299 ± 58 (208-405)	– –	– –
Tail length (T)	32	34 ± 3.1 (29-40)	28 ± 4.1 (21-37)	25 ± 2.4 (19-29)	44 ± 4.6 (33-51)	64 ± 5 (51-74)
Anal body diam. (ABD)	47	49 ± 5.3 (40-57)	66 ± 9.6 (50-83)	35 ± 3.2 (28-40)	36 ± 1.8 (33-39)	19 ± 1.1 (17-22)
Hyaline region (H)	–	– –	– –	– –	– –	32 ± 3.4 (25-39)
Spicule length (SL)	89	83 ± 4.6 (73-89)	– –	70 ± 3.5 (64-78)	– –	– –
Spicule width (SW)	14	14 ± 1.6 (12-18)	– –	12 ± 0.8 (11-14)	– –	– –
Gubernaculum length (GL)	65	61 ± 5.3 (50-68)	– –	46 ± 3.9 (39-53)	– –	– –
Gubernaculum width	10	9 ± 0.9 (7.7-10.6)	– –	7.6 ± 1.0 (6.0-9.7)	– –	– –
D% = (EP/ES) × 100	63	67 ± 7.2 (54-88)	48 ± 10 (35-70)	69 ± 5.8 (51-78)	49 ± 3.3 (43-56)	47 ± 2.6 (41-54)
E% = (EP/T) × 100	228	225 ± 22 (185-285)	372 ± 90 (236-604)	376 ± 39 (318-442)	201 ± 24 (167-255)	82 ± 8.7 (70-109)
SW% = (SL/ABD) × 100	191	171 ± 18 (146-210)	– –	205 ± 20 (172-244)	– –	– –
GS% = (GL/SL) × 100	73	73 ± 5.0 (66-81)	– –	65 ± 5.1 (58-77)	– –	– –

Table 1. (Continued.)

Character	First generation			Second generation		Infective juvenile
	Male		Female	Male	Female	
	Holotype	Paratypes	Paratypes	Paratypes	Paratypes	
H% = (H/T) × 100	–	–	–	–	–	49 ± 3.7
		–	–	–	–	(43-57)

–, measurement not available.

(lamina) thick, tapering slightly posteriorly, with blunt terminus, velum present. Each spicule with two internal ribs. Gubernaculum boat-shaped in lateral view, anterior end curved. Copulatory papillae with single precloacal mid-ventral papilla and 12 pairs of genital papillae arranged as follows: seven pairs precloacal subventral, one pair cloacal, one pair lateral, one pair subdorsal and two pairs subterminal. Tail conoid, hair-like mucron usually absent, but observed in 5% of first generation males.

Second generation male

Body length shorter and narrower than that of first generation. Averages for different morphometric measurements of second generation are 80% of those of first generation, except for T, ABW, SL, SW and GL (pooled *t*-test). Tail length and ABW of second generation are both 72% of those of first generation. However, SL of second generation male = 85% of that of first generation, SW = 91% and GL = 76%, respectively. Tail same shape as first generation, but always with a mucron.

First generation female

Body C-shaped when heat-relaxed and fixed with TAF. Body cuticle smooth under a light microscope, but with annules under SEM. Lateral field not observed. Head rounded and continuous with body. Six labial papillae more prominent than four cephalic papillae, amphidial apertures not observed. Stoma, well sclerotised, funnel-shaped, cheilorhabdions prominent. Pharynx with cylindrical, muscular procorpus, swollen metacorpus, distinct isthmus, basal bulb enlarged, valvated. Excretory cell obscure, but duct cuticularised. Deirid not observed. Nerve ring surrounding isthmus just anterior to basal bulb. Pharyngo-intestinal valve prominent. Excretory pore position variable, from near mid-pharynx to mid-basal bulb. Genital system amphidelphic, ovaries reflexed. Vulva a median transverse slit, not protruding from body surface, situated in mid-body region, with double-flapped epiptygmata. Postanal swelling absent. Tail bluntly conical to dome-shaped, shorter than anal body diam., peg-like mucron (8-10 μm) present.

Second generation female

One-third shorter and narrower in body diam. than first generation. Two lateral lines with one ridge present. Vulva non-protruding from body surface, with double-flapped epiptygmata. No postanal swelling on posterior lip. Tail differing from first generation in being longer than anal body diam., tapering gently to a sharp point, without a mucron.

Infective juvenile (IJ)

Body straight when heat-relaxed, elongate and tapering both posteriorly and anteriorly. Cephalic region gently rounded, continuous with body shape. Retained second-stage cuticle not usually observed after harvesting. Stoma closed. Amphidial apertures prominent. Pharynx with a thin corpus of almost uniform diam., slightly swollen metacorpus, narrow isthmus and distinct, valvated basal bulb. Nerve ring encircling isthmus, immediately anterior to basal bulb. Excretory pore at mid-pharynx level. Deirid not observed. Hemizonid distinct, just anterior to basal bulb. Excretory pore prominently situated anterior to nerve ring in isthmus region. Lateral field starting with two ridges (three lines) at ninth annule from head, with five equal ridges (six lines) through mid-body and ending in two ridges (three lines) in vicinity of phasmid on mid-tail, almost disappearing at end of tail. Bacterial pouch obscure. Phasmid prominent in mid-tail region just ventral to lateral field. Tail long conoid, evenly attenuated, *ca* four anal body diam. long. Hyaline region comprising 49% of tail length.

TYPE LOCALITY

Steinernema sacchari n. sp. isolate SB10 was collected from a soil sample taken from a sugar cane field at coordinates 29°01′37″S, 31°35′37″E, in Gingindlovu, KwaZulu-Natal, South Africa. Nematodes were isolated by means

of laboratory trapping with a sugar cane borer, *Eldana saccharina*. The natural host is unknown.

TYPE MATERIAL

Holotype (male first generation), isolated from haemocoel of *G. mellonella*, deposited in the University of Ghent, Belgium. Paratype males (11) and females (19) of the first generation and third-stage IJ (42) in TAF deposited in the same collection. Permanent slides with each of the different stages are deposited in the National Collection of Nematodes, Biosystematics Division, Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa (six IJ, four first generation males; three first generation females; five second generation males and six second generation females). Paratype males, females and IJ are also deposited in the United States Department of Agriculture Nematode Collection (USDANC), Beltsville, MD, USA (18 IJ, five first generation males; three first generation females; four second generation males and five second generation females).

DIAGNOSIS AND RELATIONSHIPS

Steinernema sacchari n. sp. is characterised by differences in the morphology and the morphometrics of the IJ and adults. The IJ of the new species can be recognised by the pattern of the lateral field of 2, 5, 2 ridges (3, 6, 3 lines), the body length of 680 (630-722) μm, body diam. of 37 (30-47) μm, distance from anterior end to excretory pore of 53 (49-58) μm, distance from anterior to nerve ring of 88 (78-97) μm, distance from anterior to end of pharynx of 113 (104-127) μm, tail length of 64 (51-74) μm, anal body diam. of 19 (17-22) μm, D% = 47 (41-54), E% = 82 (70-109) and H% = 49 (43-57) (Table 1). The first generation male has a long spicule measuring 83 (73-89) μm and gubernaculum of 61 (50-68) μm, while other diagnotic characters include D% = 67 (54-88), E% = 255 (185-285), SW% = 171 (146-210) and GS% = 73 (66-81). The first generation male lacks a mucron, while the second generation male always has one. Both generations of males have 24 pairs of genital papillae and a single midventral papilla. Females have a non-protruding vulva with double flapped epiptygmata, no postanal swelling and the first generation female tail is dome shaped with a terminal peg.

Steinernema sacchari n. sp. is similar to two African species from Cameroon, namely *S. cameroonense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 and *S. nyetense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 (Kanga *et al.*, 2012), and the '*monticolum* group', including *S. ashiuense* Phan, Takemoto & Futai, 2006 (Japan), *S. monticolum* Stock, Choo & Kaya, 1997 (Korea), *S. robustispiculum* Phan, Subbotin, Waeyenberge & Moens, 2005 (Vietnam), *S. schliemanni* Spiridonov, Waeyenberge & Moens, 2010 (Germany) and *S. rarum* (de Doucet, 1986) Mamiya, 1988 (Argentina) (Doucet *et al.*, 2003).

The tail length of the IJ of *S. sacchari* n. sp. at 64 (51-74) μm differs from that of *S. cameroonense* (76 (52-107) μm) and *S. nyetense* (82 (54-113) μm) (Table 2). The pharynx of *S. sacchari* n. sp., at 113 (104-123) μm, is shorter in comparison to those of *S. monticolum* which 124 (120-131) μm (Table 2). The first generation male of *S. sacchari* n. sp. differs from that of *S. monticolum* in the SW%, being 171 (146-210) *vs* 140 (120-150) and in the GS%, being 73 (66-81) *vs* 60 (50-60) (Table 3). The gubernaculum of *S. sacchari* n. sp. is boat-shaped, with a

Table 2. Comparative morphometrics of the third-stage infective juveniles of *Steinernema sacchari* n. sp. and similar *Steinernema* spp. (in descending order of body length). All measurements are in μm and in the form: mean (range).

Species	Morphometric character												Reference
	L	W	EP	NR	ES	T	a	b	c	D%	E%	*n*	
S. schliemanni	934 (842-1008)	35 (30-38)	72 (61-80)	– –	148 (127-162)	88 (76-95)	26 (23-30)	6 (6-7)	11 (10-11)	48 (42-55)	– –	25	Spiridonov *et al.*, 2010
S. ashiuense	768 (720-800)	30 (28-33)	55 (51-59)	86 (77-91)	119 (113-128)	71 (66-76)	25 (24-27)	6 (6-7)	11 (10-12)	46 (53-50)	78 (70-85)	20	Phan *et al.*, 2006
S. robusti-spiculum	712 (642-778)	28 (26-35)	56 (50-68)	84 (80-100)	120 (115-152)	75 (68-92)	25 (18-29)	6 (4-6)	10	46 (43-59)	75 (67-87)	25	Phan *et al.*, 2005

Table 2. (Continued.)

Species	Morphometric character												Reference
	L	W	EP	NR	ES	T	a	b	c	D%	E%	*n*	
S. monticolum	706 (612-821)	37 (32-46)	58 (54-62)	88 (81-93)	124 (120-131)	77 (71-95)	19 (14-22)	6 (5-6)	9 (7.6-11.1)	47 (44-50)	76 (63-86)	–	Stock *et al.*, 1997
S. sacchari n. sp.	680 (630-722)	37 (30-47)	53 (49-58)	84 (78-97)	113 (104-127)	64 (51-74)	19 (14-23)	6 (6-7)	11 (10-12)	47 (41-54)	82 (70-109)	25	
S. nyetense	648 (565-708)	32 (25-37)	52 (46-57)	85 (72-102)	114 (104-128)	82 (54-113)	21 (19-26)	6 (5-6)	8 (6-11)	46 (37-50)	66 (44-89)	20	Kanga *et al.*, 2012
S. cameroonense	622 (490-694)	30 (24-35)	54 (45-64)	85 (69-100)	113 (105-125)	76 (52-107)	21 (17-25)	6 (5-6)	9 (6-12)	48 (42-56)	75 (48-116)	20	Kanga *et al.*, 2012
S. rarum	510 (446-578)	32 (25-37)	37 (33-40)	67 (62-76)	99 (88-117)	48 (42-52)	20 (18-23)	5 (4-6)	10 (9-12)	41 (36-43)	81 (67-91)	20	Nguyen *et al.*, 2007

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

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

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

Abbreviations as in Table 1 and references as in Table 2; – measurement not available.

long cuneus, whereas in *S. monticolum* it is arcuate, large and with the posterior end forked (Table 4).

The IJ of *S. sacchari* n. sp. differs from that of *S. ashiuense* in body length of 680 (630-722) *vs* 768 (720-800) μm (Table 2). The lateral lines of the IJ of *S. sacchari* n. sp. begin and end with two ridges and have five equal ridges (six lines) at the mid-body (Fig. 4), which differs from all similar species, except for *S. ashiuense*, which has the same arrangement. *Steinernema sacchari* n. sp. can be differentiated from *S. rarum* and *S. robustispiculum* by the uniquely shaped spicules of the latter two species.

The first generation male differs from all closely related species in the length of the spicule at 83 (73-89) μm and of the gubernaculum at 61 (50-68) μm (see Table 3).

Table 4. Comparative morphology of *Steinernema sacchari* n. sp. and similar 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. 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 the 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 epiptygmata	Dome shaped with terminal peg	P
S. sacchari n. sp.	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 epiptygmata	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 D2D3 regions of species of *Steinernema* closely related to *Steinernema sacchari* n. sp.

Species	ITS1 (bp)	ITS2 (bp)	A (%)	C (%)	G (%)	T (%)	Sequence length (bp)
ITS regions							
S. sacchari n. sp.	311	296	22.51	19.63	23.82	34.03	764
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. 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
D2D3 regions							
S. sacchari n. sp.			24.06	19.50	30.79	25.66	877
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. schliemanni			23.01	19.48	31.91	25.60	539
S. sichuanense			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. Pairwise differences between the ITS region of *Steinernema sacchari* n. sp. and 22 species of *Steinernema*. The number of base differences per sequences from between sequences is shown below the diagonal. The number of base substitutions per site from between sequences, according to the Jukes-Cantor model, is shown above the diagonal.

No.	Species	ITS region																						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	*S. sacchari* n. sp. KC633095		0.044	0.059	0.212	0.212	0.215	0.215	0.215	0.215	0.217	0.220	0.225	0.225	0.228	0.230	0.233	0.233	0.236	0.236	0.238	0.244	0.277	0.376
2	*S. nyetense* JX985266	22		0.042	0.236	0.247	0.238	0.238	0.244	0.241	0.233	0.247	0.252	0.249	0.255	0.244	0.247	0.257	0.252	0.263	0.255	0.252	0.300	0.379
3	*S. cameroonense* JX985267	29	21		0.233	0.247	0.236	0.236	0.247	0.238	0.238	0.244	0.247	0.244	0.255	0.244	0.255	0.255	0.255	0.257	0.255	0.263	0.294	0.386
4	*S. cholashanense* EF431959	95	104	103		0.044	0.054	0.010	0.050	0.065	0.179	0.063	0.054	0.036	0.046	0.109	0.233	0.050	0.187	0.061	0.197	0.063	0.100	0.348
5	*S. kraussei* AY171264	95	108	108	22		0.071	0.054	0.038	0.069	0.194	0.067	0.069	0.042	0.063	0.120	0.230	0.069	0.202	0.080	0.215	0.076	0.106	0.363
6	*S. jollieti* AY171265	96	105	104	27	35		0.054	0.074	0.078	0.167	0.078	0.063	0.065	0.046	0.125	0.230	0.048	0.174	0.052	0.182	0.065	0.098	0.341
7	*S. xueshanense* FJ666052	96	105	104	5	27	27		0.057	0.071	0.177	0.065	0.061	0.042	0.048	0.109	0.238	0.050	0.184	0.063	0.194	0.065	0.098	0.357
8	*S. silvaticum* AY230162	96	107	108	25	19	36	28		0.084	0.204	0.076	0.076	0.052	0.071	0.127	0.247	0.076	0.212	0.087	0.223	0.080	0.115	0.366
9	*S. sangi* AY355441	96	106	105	32	34	38	35	41		0.194	0.067	0.084	0.063	0.071	0.111	0.247	0.071	0.192	0.080	0.204	0.080	0.122	0.338
10	*S. monticolum* AF122017	97	103	105	82	88	77	81	92	88		0.199	0.189	0.189	0.170	0.207	0.207	0.179	0.034	0.172	0.046	0.192	0.228	0.354
11	*S. texanum* EF152568	98	108	107	31	33	38	32	37	33	90		0.087	0.067	0.078	0.134	0.247	0.078	0.197	0.091	0.207	0.091	0.127	0.357
12	*S. citrae* EU740970	100	110	108	27	34	31	30	37	41	86	42		0.063	0.048	0.120	0.236	0.044	0.197	0.052	0.207	0.052	0.100	0.360
13	*S. oregonense* AF122019	100	109	107	18	21	32	21	26	31	86	33	31		0.048	0.115	0.230	0.054	0.194	0.065	0.207	0.067	0.095	0.360
14	*S. weiseri* AY171268	101	111	111	23	31	23	24	35	35	78	38	24	24		0.129	0.230	0.024	0.177	0.028	0.187	0.036	0.087	0.354
15	*S. kushidai* AB243440	102	107	107	52	57	59	52	60	53	93	63	57	55	61		0.247	0.127	0.215	0.136	0.215	0.143	0.157	0.345
16	*S. schliemanni* HM778112	103	108	111	103	102	102	105	108	108	93	108	104	102	102	108		0.233	0.220	0.238	0.230	0.255	0.266	0.405
17	*S. ichnusae* EU421129	103	112	111	25	34	24	25	37	35	82	38	22	27	12	60	103		0.187	0.038	0.197	0.038	0.093	0.357
18	*S. robustispiculum* AY355442	104	110	111	85	91	80	84	95	87	17	89	89	88	81	96	98	85		0.182	0.022	0.199	0.230	0.351
19	*S. litorale* AB2434S41	104	114	112	30	39	26	31	42	39	79	44	26	32	14	64	105	19	83		0.194	0.040	0.095	0.357
20	*S. ashiuense* DQ354694	105	111	111	89	96	83	88	99	92	23	93	93	93	85	96	102	89	11	88		0.210	0.244	0.354
21	*S. feltiae* AF121050	107	110	114	31	37	32	32	39	39	87	44	26	33	18	67	111	19	90	20	94		0.091	0.357
22	*S. hebeiense* DQ105794	119	127	125	48	51	47	47	55	58	101	60	48	46	42	73	115	45	102	46	107	44		0.389
23	*S. rarum* DQ221116	152	153	155	143	148	141	146	149	140	145	146	147	147	145	142	161	146	144	146	145	146	156	

Table 7. Pairwise comparison of the D2D3 region of *Steinernema sacchari* n. sp. with 17 *Steinernema* spp. 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.

No.	Species	D2D3																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*S. sacchari* n. sp. KC633096		0.008	0.008	0.074	0.081	0.083	0.088	0.090	0.090	0.093	0.093	0.093	0.095	0.095	0.098	0.098	0.107	0.112
2	*S. cameroonense* JX985265	4		0.008	0.079	0.086	0.086	0.090	0.093	0.088	0.095	0.095	0.095	0.098	0.098	0.095	0.100	0.107	0.114
3	*S. nyetense* X985264	4	4		0.074	0.081	0.081	0.086	0.088	0.083	0.090	0.090	0.090	0.093	0.093	0.095	0.095	0.109	0.109
4	*S. schliemanni* HM778113	34	36	34		0.045	0.047	0.052	0.054	0.054	0.056	0.056	0.054	0.056	0.056	0.065	0.054	0.100	0.072
5	*S. kushidai* AF331897	37	39	37	21		0.054	0.032	0.036	0.054	0.036	0.036	0.041	0.045	0.038	0.045	0.038	0.107	0.056
6	*S. monticolum* EF439651	38	39	37	22	25		0.052	0.049	0.054	0.056	0.056	0.061	0.061	0.058	0.070	0.058	0.109	0.077
7	*S. silvaticum* DQ399663	40	41	39	24	15	24		0.013	0.025	0.004	0.004	0.025	0.021	0.006	0.021	0.019	0.114	0.032
8	*S. oregonense* AF331891	41	42	40	25	17	23	6		0.023	0.013	0.013	0.023	0.023	0.015	0.034	0.017	0.119	0.034
9	*S. texanum* EF152569	41	40	38	25	25	25	12	11		0.025	0.025	0.030	0.034	0.023	0.043	0.032	0.117	0.045
10	*S. feltiae* AF331906	42	43	41	26	17	26	2	6	12		0.000	0.025	0.021	0.006	0.025	0.019	0.119	0.032
11	*S. puntauvense* EF187018	42	43	41	26	17	26	2	6	12	0		0.025	0.021	0.006	0.025	0.019	0.119	0.032
12	*S. xueshanense* FJ666053	42	43	41	25	19	28	12	11	14	12	12		0.030	0.025	0.047	0.023	0.109	0.043
13	*S. cholashanense* EF520284	43	44	42	26	21	28	10	11	16	10	10	14		0.028	0.038	0.021	0.112	0.045
14	*S. ichnusae* EU421130	43	44	42	26	18	27	3	7	11	3	3	12	13		0.028	0.021	0.122	0.034
15	*S. citrae* GU004534	44	43	43	30	21	32	10	16	20	12	12	22	18	13		0.041	0.114	0.054
16	*S. kraussei* AF331896	44	45	43	25	18	27	9	8	15	9	9	11	10	10	19		0.119	0.034
17	*S. rarum* AF331905	48	48	49	45	48	49	51	53	52	53	53	49	50	54	51	53		0.139
18	*S. hebeiense* DQ399664	50	51	49	33	26	35	15	16	21	15	15	20	21	16	25	16	61	

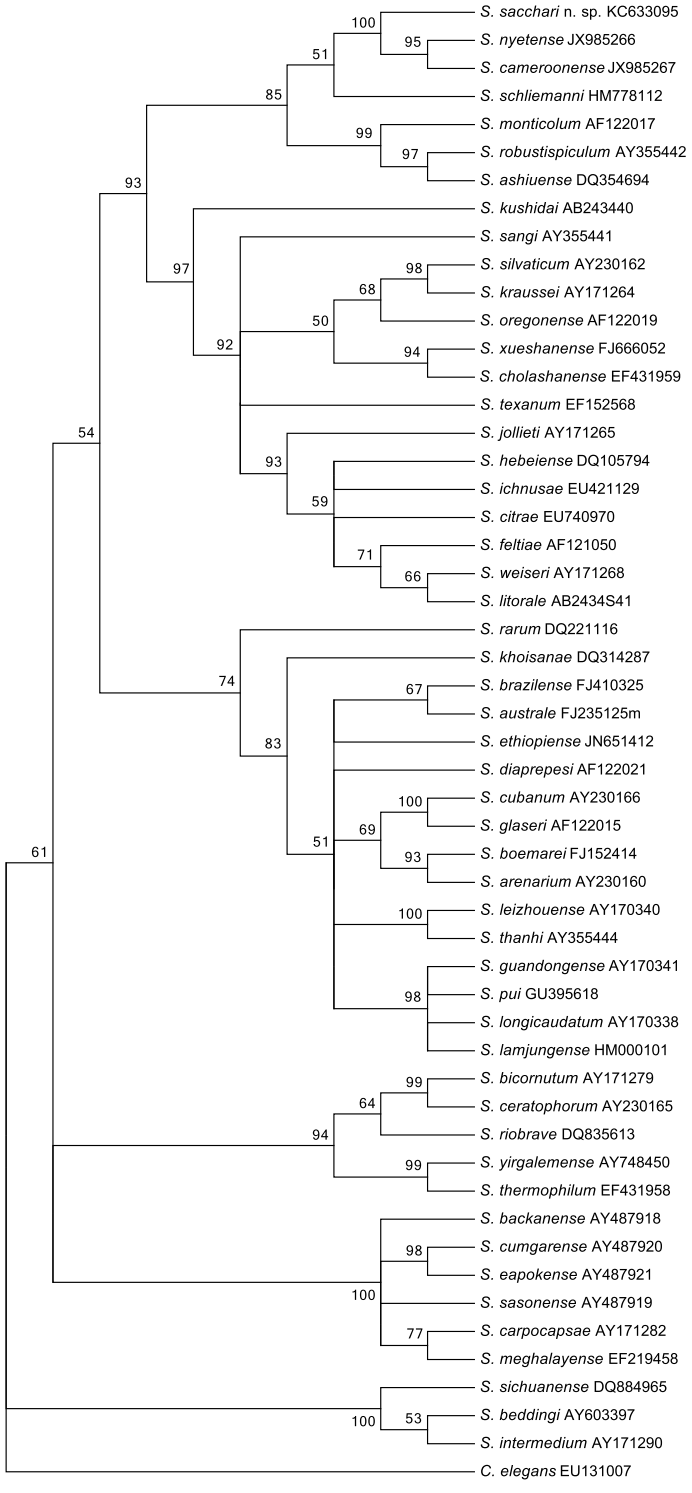

Fig. 5. Phylogenetic relationships of *Steinernema sacchari* n. sp. Fifty three species of *Steinernema* based on the ITS-rDNA sequences from GenBank. *Caenorhabditis elegans* (EU131007) was used as outgroup. Numbers at the nodes represent bootstrap proportion for Maximum Parsimony of 50% or more.

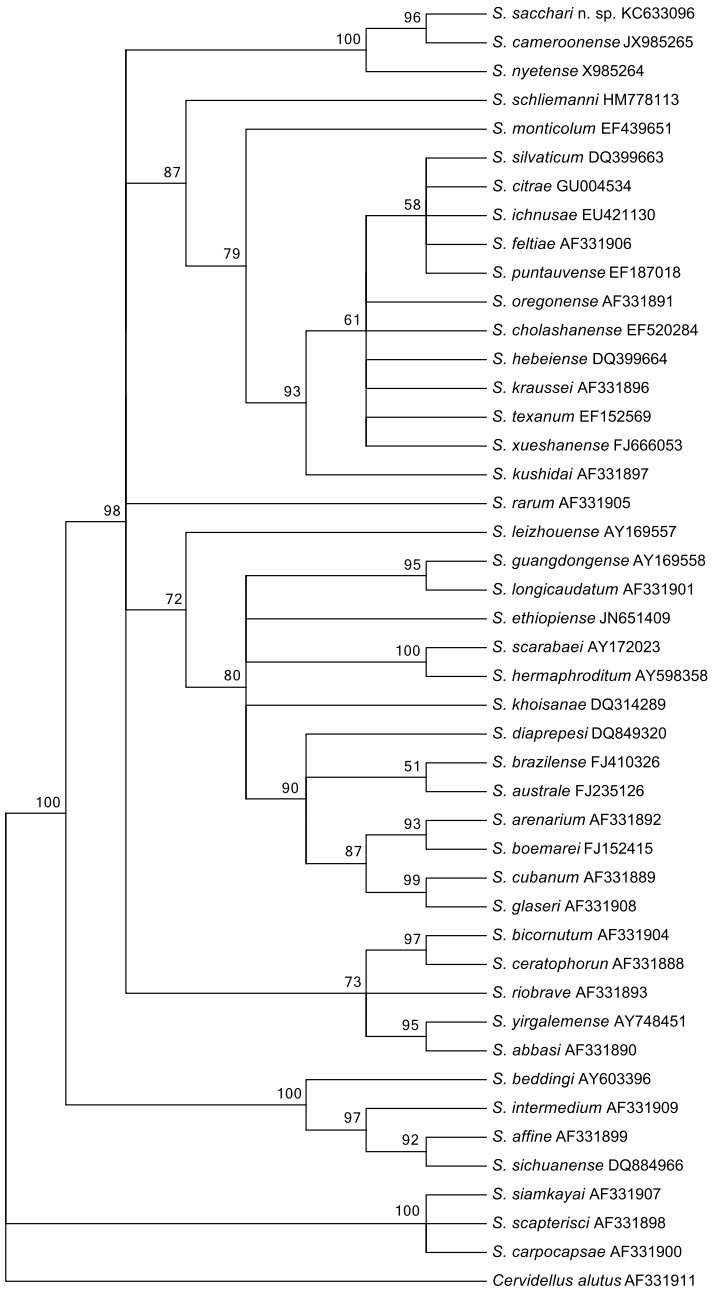

Fig. 6. Phylogenetic relationships of *Steinernema sacchari* n. sp. with 19 species of *Steinernema* based on the D2D3-rDNA sequences from GenBank. *Cervidellus alurus* (AF331911) was used as outgroup. Numbers at the nodes represent bootstrap proportion for Maximum Parsimony of 50% or more.

The tail of the first generation male of *S. sacchari* n. sp. differs from all similar species in the absence of a mucron, although some individuals may have a hair-like projection seen with SEM. The body diam. of the first generation male of *S. sacchari* n. sp. is 145 (86-205) μm and is wider than that of all similar species (Table 3). The genital papillae of the male tail differ from those of similar species with a total of 25, comprising 12 pairs and a single mid-ventral papilla (24 + 1) (Table 4). The tail of the first-generation female of *S. sacchari* n. sp. is

short, conical and ends in a sharp peg, with no postanal swelling in the first or second generation female. The vulva of the first and second generation female of the new species differs from that of all closely related species in being non-protruding, except for *S. monticolum*, which also has a non-protruding vulva, and *S. schliemanni*, with a slightly protruding vulva. The first generation female of *S. sacchari* n. sp. differs from that of *S. ashiuense*, *S. monticolum* and *S. rarum* in having a vulva with long epiptygmata (Fig. 3E, G).

Cross-breeding with such closely related species as *S. cameroonense* and *S. nyetense* would support the separation of species. However, according to the authors, no living specimens of the two *Steinernema* species from Carmeroon were available.

Molecular characteristics

Steinernema sacchari n. sp. is characterised genetically by sequences of the ITS (KC633095) and the D2D3 (KC633096) rDNA regions. The sequence of ITS regions of *S. sacchari* n. sp., including ITS1 + 5.8S + ITS2, can be recognised by its length of 764 bp (ITS1 = 311 bp; ITS2 = 296 bp) with a composition of A = 0.2251, C = 0.1963, G = 0.2383, T = 0.3403. The sequence lengths and frequencies of nucleotide distribution for closely related species are shown in Table 5. *Steinernema sacchari* n. sp. is different from the closest species, *S. cameroonense* and *S. nyetense*, in terms both of the ITS1 length (311 bp *vs* up to 291 bp and 282 bp, respectively) and ITS2 length (296 bp *vs* 284 bp for both species). Pairwise distances using the ITS regions (Table 6) show that the new species differs from its closest relatives, *S. nyetense*, by 22 bp, and from *S. cameroonense* by 29 bp, as well as from its most divergent species, *S. rarum*, by 152 bp.

The sequence of the D2D3 region of *S. sacchari* n. sp. is 877 bp long and its base composition is: A = 0.2406, C = 0.1950, G = 0.3079 and T = 0.2566 (Table 5). Pairwise comparison using the D2D3 regions (Table 7) reveals that the closest relative to *S. sacchari* n. sp., with respect to mean character difference calculated by means of the Jukes-Cantor method, are *S. nyetense* and *S. cameroonense*, with 0.8% dissimilarity, and differing in terms of only four nucleotides compared to *S. rarum*, whose dissimilarity is 14%, differing by 61 nucleotides in the sequence.

Phylogeny

Phylogenetic relationships of *S. sacchari* n. sp. with other *Steinernema* species, inferred from ITS-rRNA sequences by using the Maximum Parsimony method, are shown in Figure 5 (tree length = 1069; CI = 0.4134; RI = 0.7223). Analysis of the most parsimonious tree indicates the presence of a monophyletic group consisting of *S. sacchari* n. sp., *S. cameroonense* and *S. nyetense*, with 100% bootstrap support. *Steinernema schliemanni* is also closely related to this group, forming a separate group with these three species within the '*monticolum*' clade, but with a low bootstrap support of 51%.

Phylogenetic relationships of *S. sacchari* n. sp. with other *Steinernema* species, inferred from D2D3 sequences of 28S rRNA by using the Maximum Parsimony method (Fig. 6) (tree length = 581; CI = 0.529963; RI = 0.778269), reveal the same monophyletic group, consisting of *S. sacchari* n. sp., *S. cameroonense* and *S. nyetense*, with 100% bootstrap support.

Morphological and molecular data confirmed *S. sacchari* n. sp. as belonging to a new monophyletic group, the 'Cameroonian' clade, consisting of *S. cameroonense*, *S. nyetense*, and low support for *S. schliemanni*, also belonging to this group. This group is closely related to the *feltiae-kraussei-oregonens*e Clade III (Spiridonov *et al.*, 2004b).

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