JOURNAL OF NEMATOLOGY
Research Paper | DOI: 10.2478/jofnem-2022-0049
e2022-1 | Vol. 54

Steinernema africanum n. sp. (Rhabditida, Steinernematidae), a New Entomopathogenic Nematode Species Isolated in the Republic of Rwanda

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Abstract

Alternatives to hazardous insecticides are urgently needed for an environmentally friendly and effective management of insect pests. One such option is the use of entomopathogenic nematodes (EPN). To increase the availability of EPN with potential for biocontrol, we surveyed agricultural soils in the Republic of Rwanda and collected two *Steinernema* isolates. Initial molecular characterization showed that they represent a new species, for which we propose the name *S. africanum* n. sp. To describe this new species, we reconstructed phylogenetic relationships, calculated sequence similarity scores, characterized the nematodes at the morphological level, conducted crossing experiments, and isolated and characterized their symbiotic bacteria. At the molecular level, *S. africanum* n. sp. is closely related to *S. litorale* and *S. weiseri*. At the morphological level, *S. africanum* n. sp. differs from closely related species by the position of the nerve ring and also because the stoma and pharynx region is longer. The first-generation males have ventrally curved spicules with lanceolate manubrium and fusiform gubernaculum and the second-generation males have rounded manubrium and anteriorly hook-like gubernaculum. *Steinernema africanum* n. sp. does not mate or produce fertile progeny with any of the closely related species.

Keywords

biocontrol agents, nematode morphology, phylogenetics, species description, taxonomy, *Xenorhabdus*

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This paper was edited by
David Shapiro-Ilan.

Received for publication
June 7, 2022.

The genus *Steinernema* Travassos, 1927 is one of two major genera of entomopathogenic nematodes (EPNs) used to control insect pests in agriculture. The members of this genus infest and kill numerous insects aided by their symbiotic, entomopathogenic bacteria of the genus *Xenorhabdus*. Together, they constitute a highly valuable pest management tool in sustainable and eco-friendly agriculture (Smart, 1995).

An important step for the use of EPNs in agriculture is the proper description and characterization of the nematode isolates with promising biocontrol traits. In the case of *Steinernema*, there are hundreds of isolates in different laboratories around the globe, which have been assigned to one of the more than 100 *Steinernema* species described so far (Bhat *et al*., 2020). Many isolates still await being assigned to formal taxonomic studies, and it is very likely that they also represent new, undescribed species.

The species of the genus *Steinernema* are phylogenetically grouped into 12 clades according to the sequences of the internal transcribed spacer (ITS) region of the rRNA (Spiridonov and Subbotin, 2016). There are nine multiple species clades: "*Affine*," "*Bicornutum*," "*Cameroonense*," "*Carpocapsae*," "*Costaricense*," "*Feltiae*," "*Glaseri*," "*Karii*," "*Khoisanae*," "*Kushidai*," "*Longicaudum*," and "*Monticolum*"; and three monospecies clades: *S. neocurtillae*, *S. unicornum*, and *S. rarum* (Spiridonov and Subbotin, 2016). The "*feltiae*-clade" currently contains at least 16 species, and many of them are closely related to the novel *S. africanum* n. sp., described in this study (Spiridonov and Subbotin, 2016). These are *S. citrae* Stokwe *et al*., 2011, *S. feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982; *S. hebeiense* Chen *et al*., 2006; *S. ichnusae* Tarasco *et al*., 2008; *S. litorale* Yoshida, 2005; *S. nguyeni* Malan *et al*., 2016; and *S. weiseri* Mráček *et al*., 2003.

During a survey of agricultural soils in the Republic of Rwanda in 2014, two nematode isolates, *Steinernema* sp. RW14-M-C2b-1 and RW14-M-C2a-3, were recovered (Yan *et al*., 2016). Initial molecular characterization suggested that they represent a new species. In this study, we describe this new EPN species through the reconstruction of phylogenetic relationships based on nuclear and mitochondrial genes, sequence similarity calculations, morphological and morphometric characterizations, self-crossing and cross-hybridization experiments, and the isolation and characterization of their symbiotic bacteria. This study contributes to a better understanding of the biodiversity and phylogenetic relationships of an important group of biological control agents, which is essential for the establishment of biocontrol programs in sustainable and eco-friendly agriculture.

Materials and Methods

Nematode origin

Steinernema africanum n. sp. RW14-M-C2b-1 and RW14-M-C2a-3 nematodes were isolated from soils of a banana, pumpkin, and sorghum intercrop in a valley of the Republic of Rwanda (GPS coordinates: 1°28′11.1"S 29°41′36.2"E; 1,865 m. s. n. m.) (Yan *et al*., 2016). Nematode isolation was achieved by baiting mixed soil samples with *Galleria mellonella* (Lepidoptera: Pyralidae) larvae, and placing the infected larvae in White traps (White, 1927).

Nematode morphological and morphometrical characterization, light and scanning electron microscopy

First- and second-generation adult nematodes were obtained by dissecting infested *G. mellonella* cadavers in Ringer's solution after 5 d to 6 d and 8 d to 9 d of post infestation, respectively. Infective juveniles (IJs) were collected after their emergence from *G. mellonella* larvae in White traps (White, 1927). Nematodes were killed with water at 60°C, fixed in 4% formalin solution (4 mL formaldehyde, 1 mL glycerol, and 95 mL ddH_2O) and transferred to anhydrous

glycerin by the Seinhorst method (Seinhorst, 1959). Nematodes were then mounted on permanent glass slides with a thicker layer of paraffin wax to prevent the flattening of the nematodes (Grisse, 1969). Morphological measurements were taken using the Olympus BX51 software built into the ZEISS Axio Lab. A1 light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Fifteen specimens of *S. africanum* n. sp. RW14-M-C2b-1 at each developmental stage were measured. To obtain light microscopy (LM) and scanning electron microscopy (SEM) photographs, specimens were processed following techniques described in detail by Abolafia (2022). Briefly, the nematodes, fixed in 4% formalin solution, were processed to anhydrous glycerin with Siddiqi's method using lactophenol-glycerin solutions (Siddiqi, 1964). Then, the nematodes were permanently mounted on glass microscope slides using the glycerin-paraffin method (Maeseneer and d'Herde, 1963; Siddiqi, 1964). Light microscopy photographs were taken using a Nikon Eclipse 80i microscope (Olympus, Tokyo, Japan) equipped with differential interference contrast optics (DIC) and a Nikon Digital Sight DS-U1 camera. For scanning electron microscopy, nematodes preserved in glycerin were taken from the permanent microscope slides by removing the cover glass, re-hydrated in distilled water, dehydrated in a graded ethanol-acetone series, critical point dried with liquid CO_2, mounted on SEM stubs with copper tape, coated with gold in a sputter coater, and finally observed with a Zeiss Merlin microscope (5 kV) (Zeiss, Oberkochen, Germany) (Abolafia, 2015). Light microscopy micrographs, obtained at different levels for each structure, were processed and combined using Adobe® Photoshop®CS (Microsoft Corporation, Redmond, WA). Morphological characters of closely related species were taken from the original publications (Hunt and Nguyen, 2016).

Self-crossing and cross-hybridization experiments

Self-crossing and cross-hybridization experiments were conducted as described by Kaya and Stock (1997) with some minor modifications (Kaya and Stock, 1997). Briefly, drops of hemolymph obtained from surface-sterilized *G. mellonella* larvae were placed in sterile Petri dishes (35 mm x 10 mm). A few micrograms of phenylthiourea were added to hemolymph drops to prevent melanization. Then 40 to 60 surface-sterilized juvenile nematodes (IJs) were added to the hemolymph drops. Nematodes were surface sterilized by immersing them in 0.1% NaOCl, and then washed thrice with autoclaved double distilled water. Then, Petri dishes were wrapped in moistened paper tissue and kept in plastic bags at 25°C. Petri plates were observed daily until IJs developed into adults. Then, male and female adults were separated by observing them under a light microscope. For self-crossing experiments, three males and three females of the same species were transferred to fresh hemolymph drops as described above. For cross-hybridization experiments, three males and three females of different species were transferred to fresh hemolymph drops as described above. Females without males were also included to confirm their virginity. Petri plates were observed daily to determine the production of offspring. For each crossing type, 10 independent Petri plates were included. Experiments were conducted twice under the same conditions. The following species were included in these experiments: *Steinernema africanum* n. sp. RW14-M-C2b-1 and RW14-M-C2a-3, *S. feltiae* Jakub, *S. ichnusae* Sardinia, *S. litorale* Aichi, and *S. weiseri* 1025 (Yoshida, 2004; Tarasco *et al*., 2008; Půža *et al*., 2021).

Nematode molecular characterization and phylogenetic relationships

Genomic DNA from about 20 females was extracted using the genomic DNA isolation kit from QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The following genes/genomic regions were amplified by polymerase chain reaction (PCR): the D2–D3 expansion segments of the 28S rRNA, the ITS region of the rRNA, the mitochondrial 12S rRNA, and the cytochrome oxidase subunit I (COI). To amplify the ITS rRNA, the following primers were used: 18S (5′-TTGATTACGTCCCTGCCC TTT-3′) and 26S (5′-TTTCACTCGCCGTTACTAAGG-3′) (Joyce *et al*. 1994). To amplify the D2–D3 region, the following primers were used: D2F (5′- CCTTAGTAAC GGCGAGTGAAA-3′) and 536 (5′-CAGCTATCCTGA GGAAAC-3′) (Subbotin *et al*., 2006). To amplify the 12S mitochondrial rRNA gene, primers 505F: 5′-GTTCCAG AATAATCGGCTAGAC-3′ and 506R: 5′-TCTACTTTACT ACAACTTACTCCCC-3′ were used (Nadler *et al*., 2006). Primers LCO-1490 (5′-GGTCAACAAATCATAAA GATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGT GACCAAAAAATCA-3′) were used to amplify the COI (Folmer *et al*., 1994). PCR reactions consisted of 12.5 µL of DreamTaq Green PCR Master Mix (Thermo Scientific, Waltham, MA USA), 0.5 µL of each forward and reverse primers at 10 µM, 1 µL of genomic DNA, and 10.5 µL of nuclease free distilled water. The PCR reactions were performed using a thermocycler with

the following settings. For ITS and D2–D3: 1 cycle of 5 min at 94°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 50°C, 1 min 30 sec at 72°C, and by a single final elongation step at 72°C for 10 min. For the 12S gene, the PCR protocol included initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec, followed by a final extension at 72°C for 15 min. For the COI gene, the PCR program was as follows: 1 cycle of 94°C for 2 min, followed by 37 cycles of 94°C for 30 sec, 51°C for 45 sec, 72°C for 2 min, and a final extension at 72°C for 12 min. PCR was followed by electrophoresis (45 min, 100 V) of 10 µL of PCR products in a 1% TBA (Tris–boric acid–EDTA) buffered agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA). PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced using reverse and forward primers by Sanger sequencing (Microsynth AG, Balgach, Switzerland). Obtained sequences were manually curated and trimmed and deposited in the National Center for Biotechnology Information (NCBI) under the accession numbers given on the phylogenetic trees. To obtain genomic sequences of nematodes that belong to all the validly described species of the "*feltiae*-clade," we searched the database of the NCBI using the Basic Local Alignment Search Tool (Altschul *et al*. 1990). The resulting sequences were used to reconstruct phylogenetic relationships by the maximum likelihood method based on the following nucleotide substitution models: Hasegawa–Kishino–Yano model (HKY + G) (ITS), General Time Reversible model (GTR + G + I) (COI), Kimura 2-parameter (K2 + G + I) (D2–D3), and Tamura 3-parameter model (T92) (12S) (Kimura, 1980; Hasegawa *et al*., 1985; Tamura, 1992; Nei and Kumar, 2000). To select the best substitution models, best–fit nucleotide substitution model analyses were carried out in MEGA 7 (Kumar *et al*., 2016). Sequences were aligned with MUSCLE (v3.8.31) (Edgar, 2004). The trees with the highest log likelihood are shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. In some cases, a discrete Gamma distribution (+G) was used to model evolutionary rate differences among sites and the rate variation model allowed for some sites to be evolutionarily (+I). The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Graphical representation and edition of the phylogenetic trees were performed with Interactive Tree of Life (v3.5.1) (Chevenet *et al*., 2006; Letunic and Bork, 2016).

Symbiotic relationships

The entomopathogenic *Xenorhabdus* bacteria associated with *S. africanum* n. sp. RW14-M-C2b-1 nematodes were isolated as described (Machado *et al*., 2018, 2019). To establish their taxonomic identities, we reconstructed phylogenetic relationships based on whole genome sequences of the isolated bacteria and all the different species of the genus *Xenorhabdus*. Genomic sequences were obtained as described (Machado *et al*., 2021b, 2021c). Genome sequences were deposited in the National Centre for Biotechnology Information. Accession numbers are listed in Table S1 in Supplementary Material. Phylogenetic relationships were reconstructed based on the assembled genomes and the genome sequences of all validly published species of the genus with publicly available genome sequences as described (Machado *et al*., 2021a). Whole genome sequence similarities were calculated by the digital DNA–DNA hybridization (dDDH) method using the recommended formula 2 of the genome-to-genome distance calculator (GGDC) web service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Auch *et al*., 2010a, 2010b; Meier-Kolthoff *et al*., 2013, 2014).

Life cycle

The life cycle of *S. africanum* n. sp. was studied by infesting wax moth larvae (*G. mellonella*) with either 50 or 150 *S. africanum* n. sp. RW14-M-C2b-1 IJs per larva ($n = 30$). Larvae were individually placed in Petri plates lined with a sheet of moist filter paper and incubated at 24°C. Upon infection, a few cadavers were dissected daily to collect and observe the number of nematodes at each developmental stage.

Results and Discussion

Two populations of *Steinernema* nematodes, RW14-M-C2b-1 and RW14-M-C2a-3, were isolated from agricultural soils in the Republic of Rwanda (Yan *et al*., 2016). Initial molecular characterization showed that they are identical, belong to the "*feltiae*-clade," are closely related to *S. feltiae*, *S. citrae*, *S. litorale*, *S. nguyeni*, and *S. weiseri*, and represent a new species, which we named *S. africanum* n. sp. To describe this new species,

we compared it with other closely related species at the molecular and morphological level, and conducted cross-hybridization and self-crossing experiments. As both populations are identical at the molecular level, we selected RW14-M-C2b-1 for detailed morphological characterization.

Steinernema africanum n. sp.

(Figures 1–7 and Tables 1–4)

First-generation male

Body slender, ventrally curved posteriorly, C- or J-shaped when heat-killed. Cuticle with transversal incisures scarcely marked, with annuli appearing slightly visible. Lateral fields and phasmids inconspicuous under LM. Lip region truncate to slightly round, continuous with body. Six lips amalgamated, with one acute labial papilla and one low cephalic papilla each, except lateral lips. Amphidial apertures small, located at lateral lips

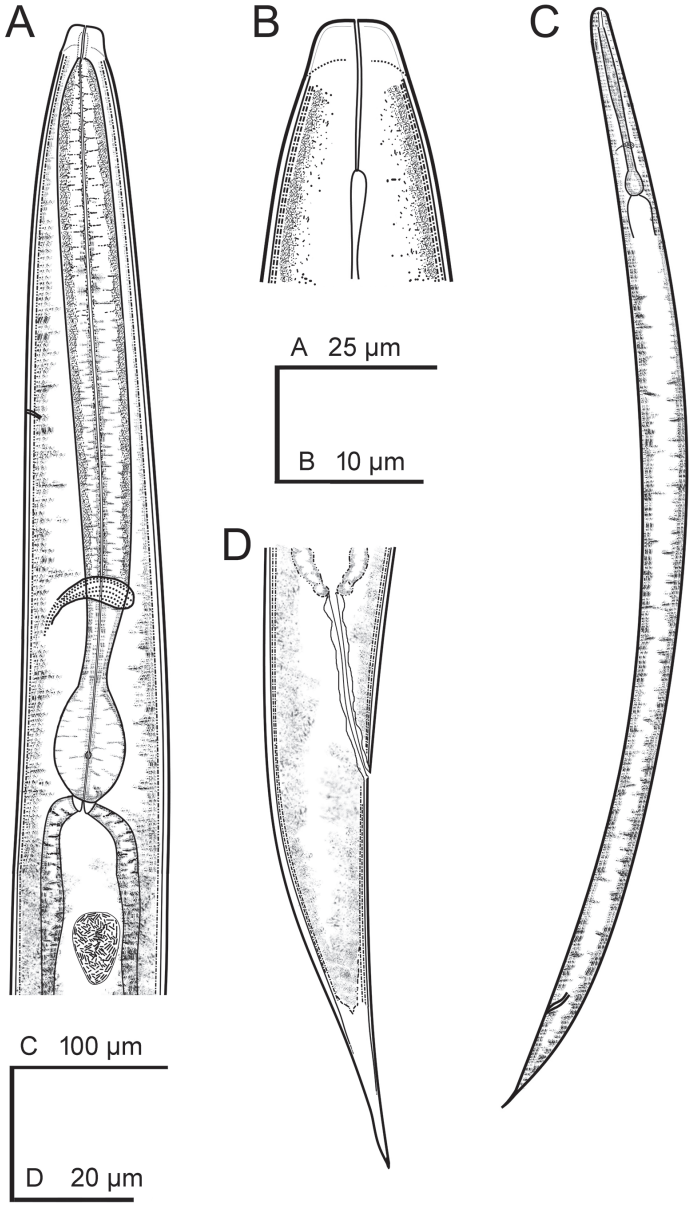

Figure 1: Line drawings of *Steinernema africanum* n. sp. IJ. **(A)** Stoma and pharynx region. **(B)** Anterior end. **(C)** Entire IJ. **(D)** Posterior end. IJ, infective juvenile.

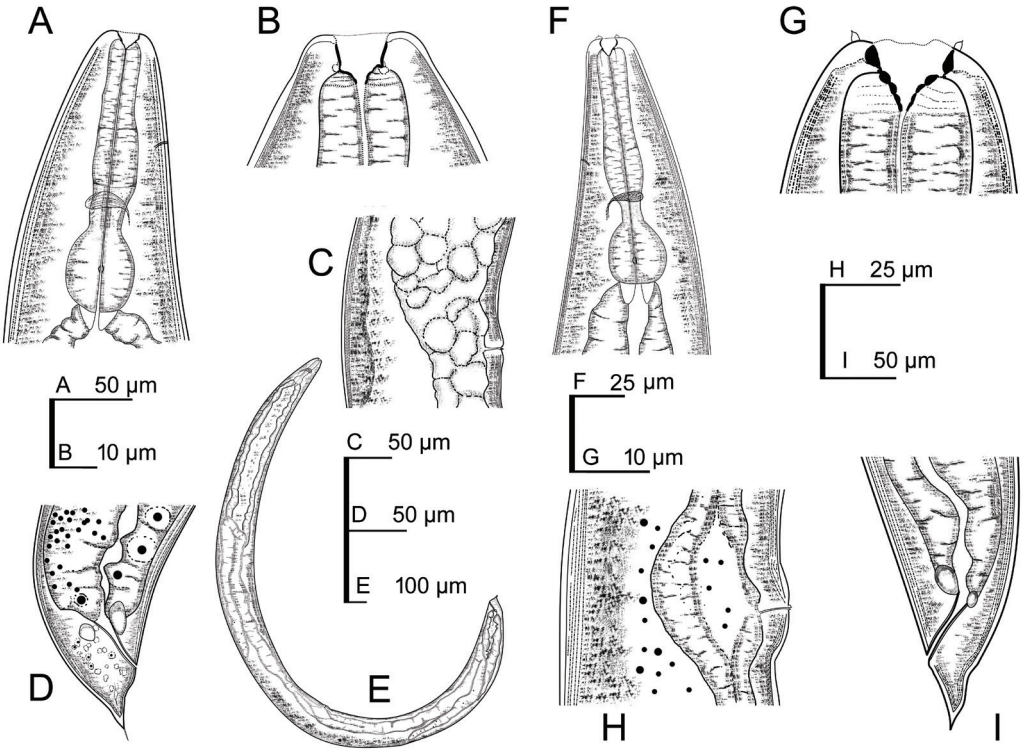

Figure 2: Line drawings of first- and second-generation *Steinernema africanum* n. sp. females. **(A–E)** First-generation female: **(A)** Stoma and pharynx region; **(B)** Lip region and stoma; **(C)** Vagina region; **(D)** Posterior end; **(E)** Entire female. **(F–I)** Second-generation female: **(F)** Stoma and pharynx region; **(G)** Lip region and stoma; **(H)** Vagina region; **(I)** Posterior end.

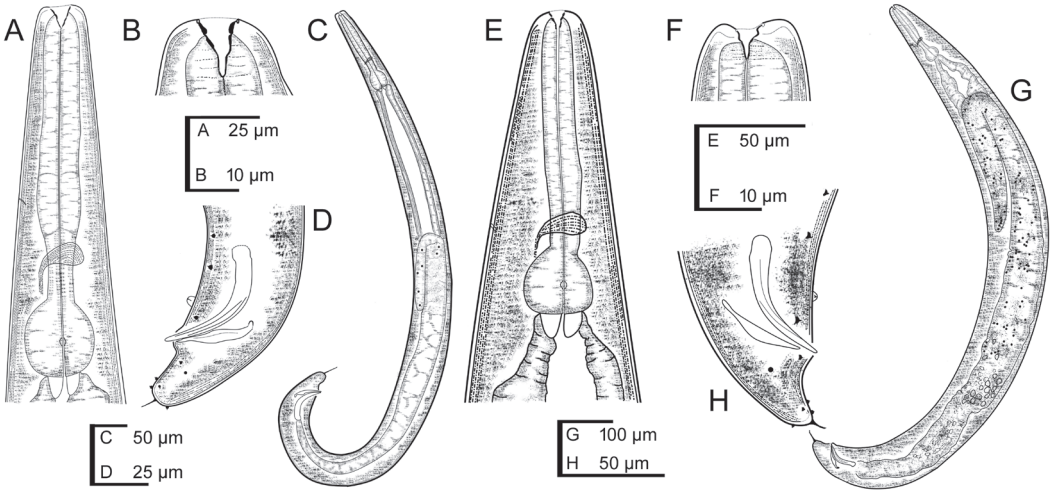

Figure 3: Line drawings of first- and second-generation *Steinernema africanum* n. sp. males. **(A–D)** First-generation male: **(A)** Stoma and pharynx region; **(B)** Lip region and stoma; **(C)** Entire male; **(D)** Posterior end. **(E–H)** Second-generation male: **(E)** Stoma and pharynx region; **(F)** Lip region and stoma; **(G)** Entire male; **(H)** Posterior end.

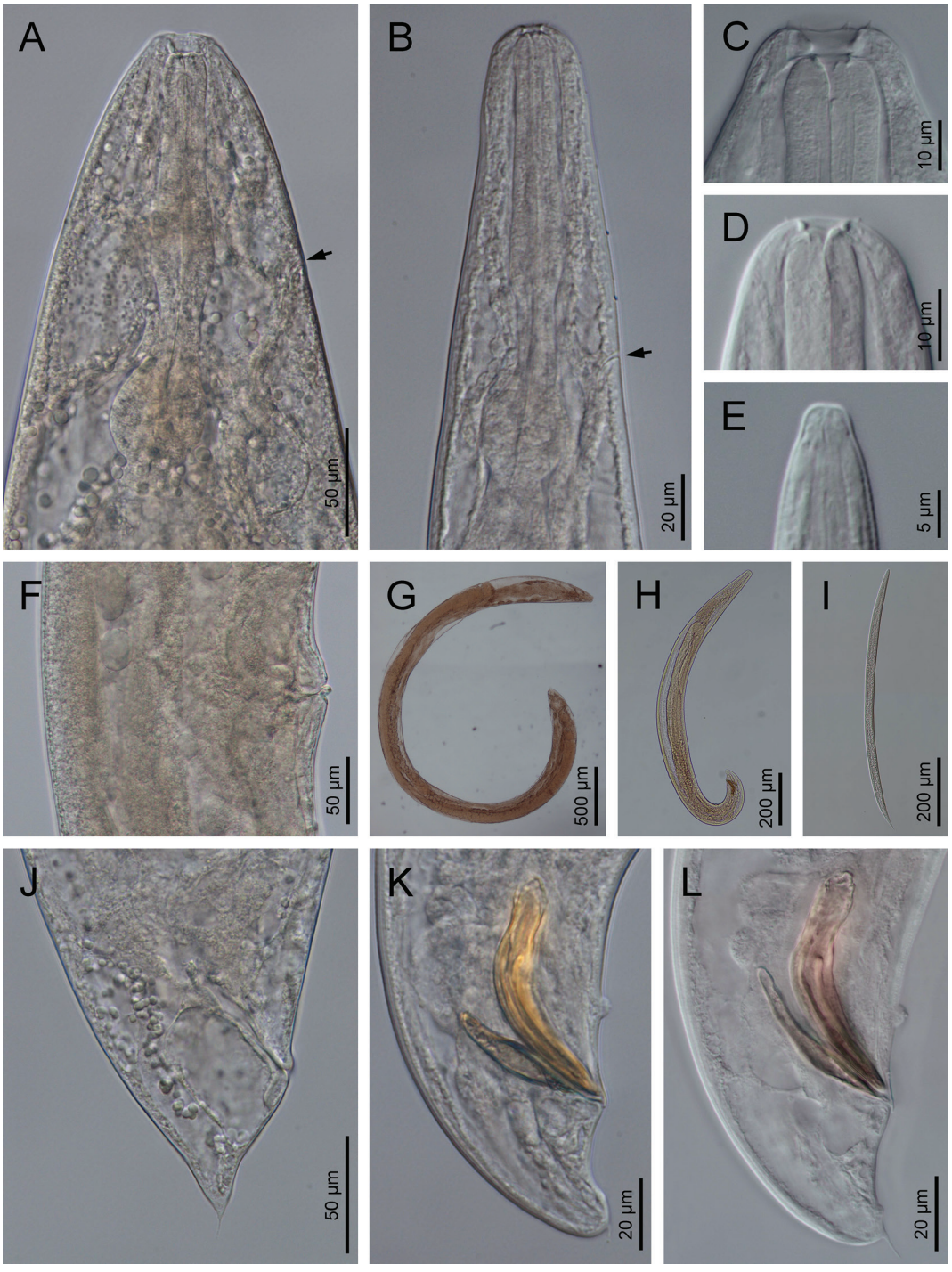

Figure 4: Light microscope micrographs of first-generation adults and IJ of *Steinernema africanum* n. sp. **(A, B)** Stoma and pharynx region of female and male, respectively. **(C–E)** Lip region and stoma of female, male and IJ, respectively. **(F)** Vagina region. **(G–I)** Entire female, male and IJ, respectively. **(J)** Posterior end of a female. **(K, L)** Posterior end of males. Pictures are in right lateral view. IJ, infective juvenile.

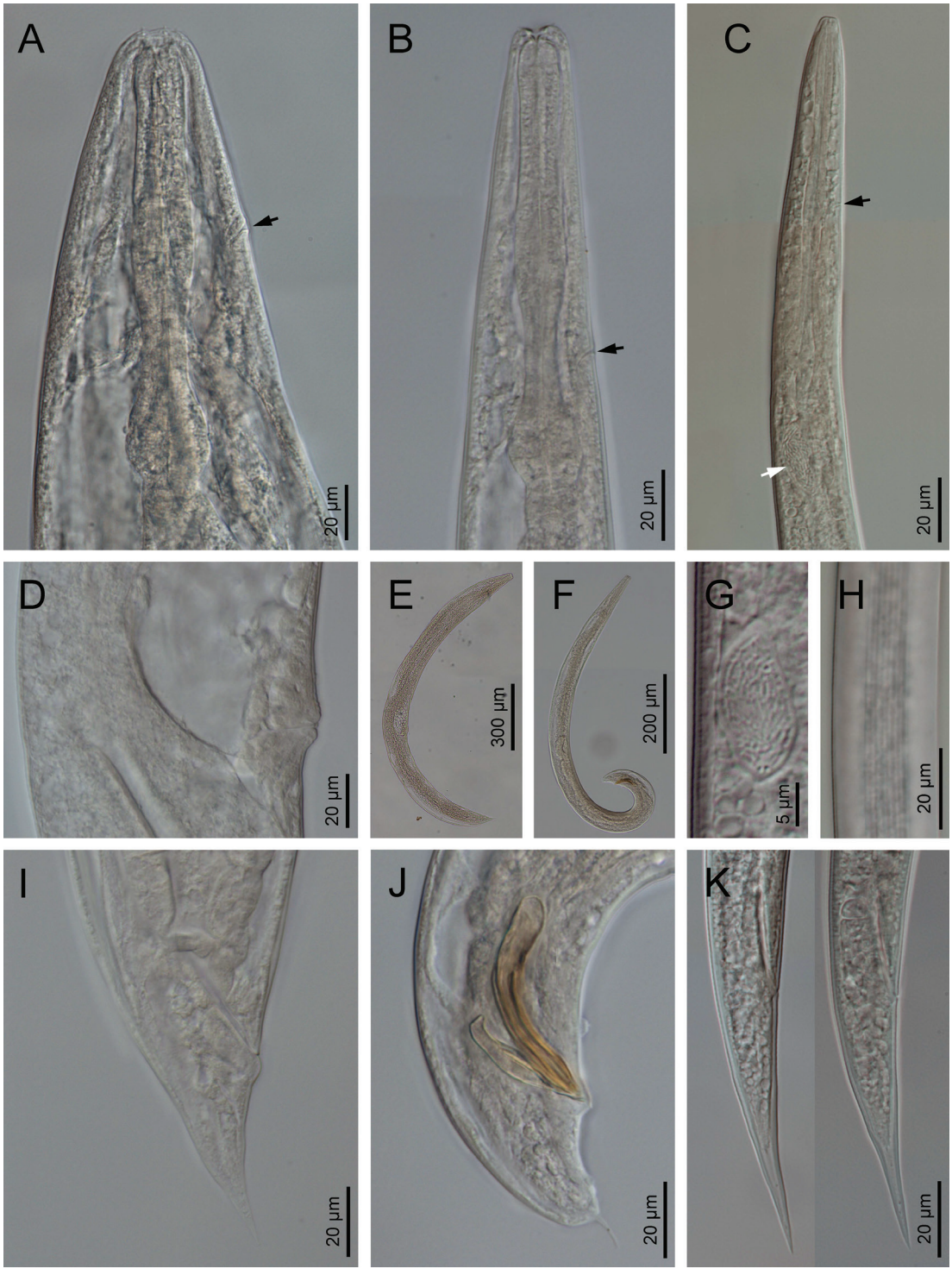

Figure 5: Light microscope micrographs of second-generation adults and IJ of *Steinernema africanum* n. sp. **(A–C)** Stoma and pharynx region of female, male and IJ, respectively. Black arrow pointing the EP, white arrow pointing the bacteria sac. **(D)** Vagina region. **(E, F)** Entire female and male, respectively. **(G, H)** IJ bacterial sac and lateral field, respectively. **(I–K)** Posterior end of female, male, and IJ, respectively. Pictures are in right lateral view. EP, excretory pore; IJ, infective juvenile.

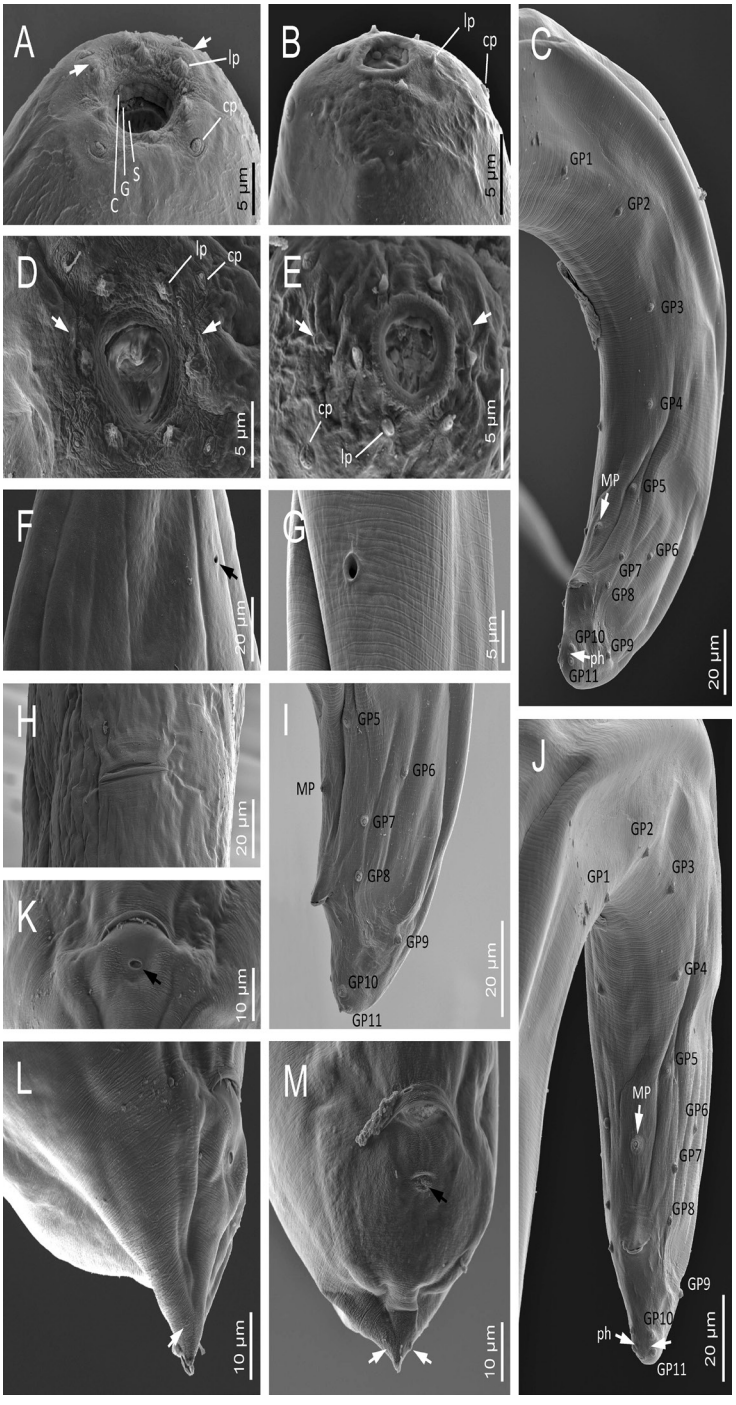

Figure 6: Scanning electron microscope micrographs of first-generation adults of *Steinernema africanum* n. sp. **(A, B)** Lip region of a female and a male, respectively, in lateral view. Arrows pointing the amphids (C: cheilostom, G: gymnostom, S: stegostom, cp: cephalic papillae, and lp: labial papillae). **(C)** Male posterior end in sub-ventral view – GP, MP, ph. **(D, E)** Lip region of a female and a male, respectively, in frontal view. **(F, G)** EP of a female and a male, respectively. **(H)** Vulva. **(I)** Male posterior end in sub-ventral view – GP, MP, ph. **(J)** Male posterior end in sub-ventral view – GP, MP. **(K)** Female anus. Arrow pointing a post-anal pore. **(L, M)** Female tail in lateral and ventral views, respectively. Black arrow pointing a post-anal pore, white arrows pointing the phasmids. EP, excretory pore; GP, genital papillae; MP, mid-ventral papilla; ph, phasmid.

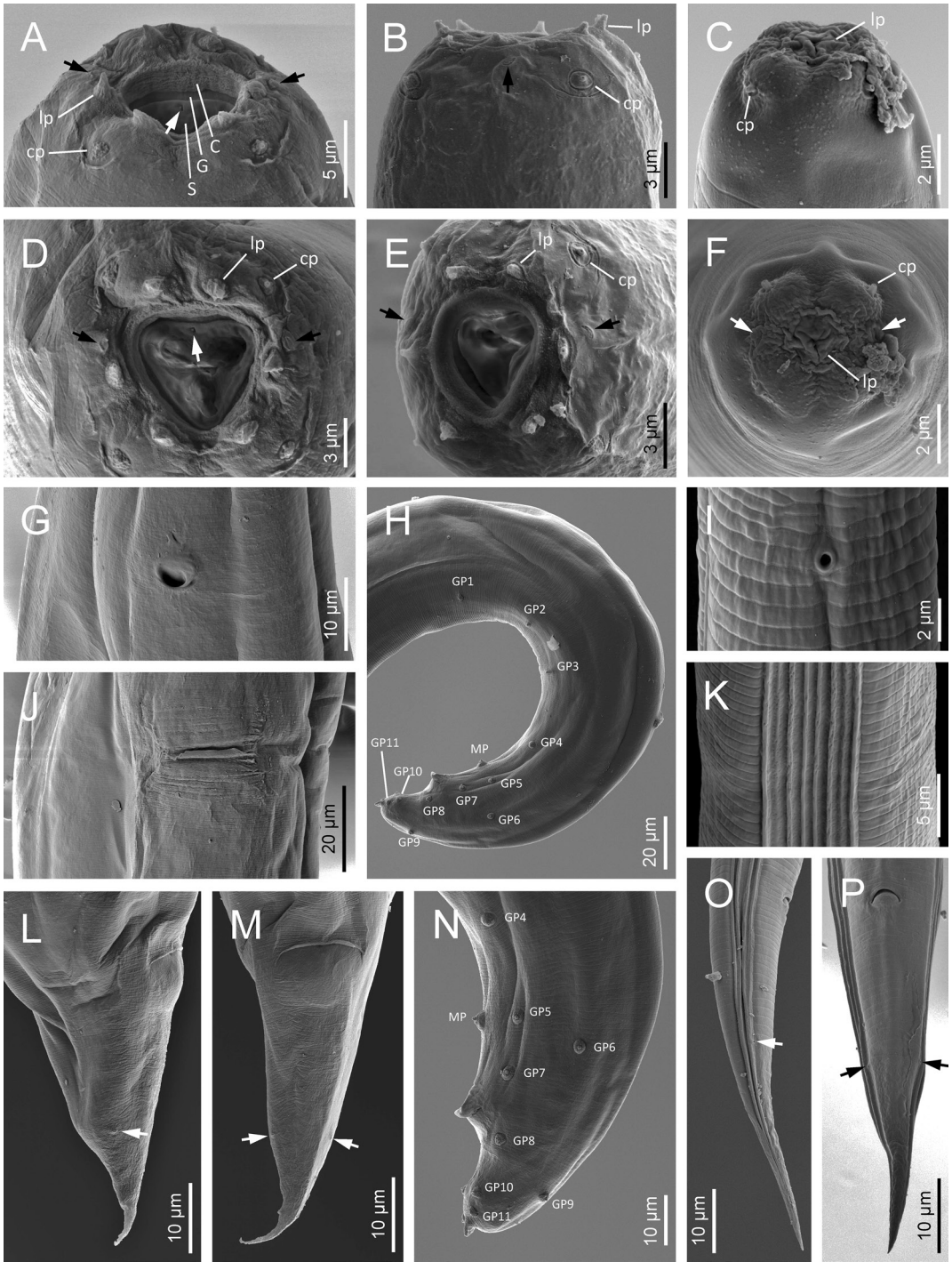

Figure 7: Scanning electron microscope micrographs of second-generation adults and IJ of *Steinernema africanum* n. sp. **(A–C)** Lip region of female, male, and IJ in lateral view, respectively. Arrows pointing the amphids (C: cheilostom, G: gymnostom, S: stegostom, cp: cephalic papillae, lp: labial papillae). **(D–F)** Lip region of female, male, and IJ in frontal view, respectively. Black arrows point to the amphids, white arrow points to the dorsal gland opening. **(G)** EP of female. **(H)** Male posterior region in lateral view – GP, MP, ph. **(I)** EP of IJ. **(J)** Vulva. **(K)** IJ lateral field. **(L, M)** Female tail (arrows pointing the phasmids). **(N)** Male posterior region in lateral view. **(O, P)** IJ tail in lateral and ventral views, respectively. Arrows pointing the phasmids. EP, excretory pore; GP, genital papillae; MP, mid-ventral papilla; ph, phasmid.

Table 1. Morphometrics of IJs and adult generations of *Steinernema africanum* n. sp.

Characters	Male first generation		Male second generation	Female first generation	Female second generation	Infective juvenile
Specimen type	Holotype	Paratype	Paratype	Paratype	Paratype	Paratype
n	1	15	15	15	15	15
Body length (L)	1,300	1,202.2 ± 141.4 (977–1,400)	955.4 ± 92.8 (831–1,213)	3,319.1 ± 763.2 (2,469–5,033)	1,620.0 ± 487.6 (924–2,311)	750.9 ± 39.8 (690–802)
a (L/BD)	9.9	10.3 ± 0.9 (9.1–11.6)	17.0 ± 1.6 (14.3–19.0)	18.0 ± 3.9 (13.2–27.0)	13.5 ± 1.0 (12.1–15.7)	28.0 ± 2.4 (23.2–30.4)
b (L/NL)	8.8	8.4 ± 0.8 (7.2–9.6)	7.3 ± 0.6 (6.6–8.5)	17.4 ± 3.7 (12.8–23.9)	10.1 ± 2.1 (7.2–12.9)	4.9 ± 0.5 (4.3–6.3)
c (L/TL)	28.3	29.0 ± 3.3 (24.5–33.9)	29.7 ± 2.9 (25.8–35.7)	74.1 ± 17.6 (50.6–103.9)	33.5 ± 11.7 (16.5–50.9)	11.9 ± 1.3 (10.3–14.9)
c´ (TL/ABW)	0.9	1.0 ± 0.1 (0.9–1.1)	1.0 ± 0.1 (0.7–1.1)	0.9 ± 0.1 (0.7–1.0)	1.5 ± 0.3 (1.2–2.3)	3.7 ± 0.4 (2.9–4.2)
T (MR/L × 100), V (VA/L × 100)	94	87.3 ± 11.8 (65–99)	71.6 ± 13.4 (58–96)	52.0 ± 2.7 (50–57)	56.0 ± 1.9 (53–58)	–
Lip region diameter	21	19.5 ± 3.2 (13–25)	15.1 ± 1.6 (11–17)	24.9 ± 3.3 (19–31)	22.3 ± 6.5 (14–34)	6.1 ± 0.7 (5–7)
Stoma length	9	7.5 ± 2.1 (5–11)	6.7 ± 1.2 (5–9)	12.8 ± 2.9 (8–19)	11.1 ± 2.4 (8–16)	17.6 ± 4.4 (10–23)
Corpus length	84	83.5 ± 5.2 (72–90)	69.9 ± 3.3 (65–76)	91.9 ± 8.1 (77–108)	83.0 ± 14.0 (62–101)	85.5 ± 8.1 (72–97)
Isthmus length	26	20.8 ± 3.3 (14–26)	22.4 ± 2.7 (18–28)	27.6 ± 5.6 (21–40)	23.8 ± 7.1 (11–35)	27.6 ± 5.4 (19–37)
Bulb length	28	29.2 ± 3.7 (25–38)	26.9 ± 2.6 (22–32)	45.1 ± 6.6 (37–58)	33.3 ± 4.7 (24–42)	22.8 ± 2.0 (18–26)
Cardia length	11	8.7 ± 1.5 (6–11)	5.8 ± 1.8 (4–11)	11.5 ± 3.1 (8–19)	7.7 ± 2.7 (4–12)	–
NR to anterior end	95	91.5 ± 8.1 (79–104)	83.5 ± 7.6 (74–101)	105.4 ± 14.9 (79–130)	96.4 ± 19.8 (69–124)	117.7 ± 11.5 (87–132)
EP to anterior end	105	93.4 ± 12.4 (69–109)	91.2 ± 6.7 (83–104)	89.4 ± 14.3 (67–111)	83.6 ± 11.6 (61–104)	59.3 ± 4.2 (54–68)
PL	138	134.7 ± 5.4 (123–142)	123.5 ± 5.6 (117–135)	169.6 ± 8.8 (158–188)	140.9 ± 19.7 (113–167)	137.2 ± 9.2 (113–153)
NL (Stoma+PL)	147	142.5 ± 4.4 (132–147)	131.1 ± 5.6 (124–143)	185.3 ± 7.9 (170–201)	152.6 ± 21.6 (122–181)	154.4 ± 12.0 (123–167)
BD at neck base	73	65.7 ± 10.8 (41–76)	44.7 ± 11.4 (37–73)	124.4 ± 9.3 (113–153)	77.5 ± 17.4 (47–105)	26.1 ± 1.6 (21–29)
BD at midbody	131	114.8 ± 17.9 (65–131)	54.1 ± 5.2 (39–59)	181.0 ± 14.8 (154–208)	114.7 ± 37.3 (59–167)	26.9 ± 1.6 (25–32)
Anal BD (ABW)	50	41.9 ± 5.5 (33–50)	34.8 ± 5.7 (30–48)	52.4 ± 8.2 (37–70)	33.9 ± 5.8 (26–43)	17.2 ± 1.1 (16–20)
Vagina length	–	–	–	20.8 ± 6.3 (11–33)	24.2 ± 11.9 (10–49)	–
VA end	–	–	–	1,767.1 ± 445.4 (1,284–2,411)	905.9 ± 266.0 (516–1,217)	–
Rectum length (RL)	–	–	–	46.4 ± 8.8 (33–63)	37.3 ± 14.3 (20–64)	28.3 ± 3.9 (23–38)

(Continued)

Table 1: Continued

Characters	Male first generation		Male second generation	Female first generation	Female second generation	Infective juvenile
Specimen type	Holotype	Paratype	Paratype	Paratype	Paratype	Paratype
Tail length (TL)	46	41.1 ± 4.0 (34–46)	33.9 ± 4.8 (28–46)	44.6 ± 5.9 (35–55)	49.7 ± 5.7 (40–60)	64.0 ± 7.1 (52–72)
Tail hyaline length (H)	–	–	–	7.4 ± 2.6 (4–11)	12.2 ± 3.8 (6–19)	22.5 ± 3.7 (17–27)
SL	72	71.1 ± 3.6 (65–76)	59.5 ± 4.8 (53–68)	–	–	–
GL	42	42.5 ± 4.2 (32–49)	32.6 ± 5.7 (25–46)	–	–	–
Stoma length/lip region width	0.4	0.4 ± 0.1 (0.2–0.7)	0.4 ± 0.1 (0.3–0.5)	0.5 ± 0.1 (0.3–0.6)	0.5 ± 0.1 (0.3–0.7)	2.9 ± 0.6 (1.5–3.7)
NR % (NR/NL × 100)	65	63.9 ± 4.9 (56–71)	63.7 ± 3.5 (60–71)	56.9 ± 7.9 (43–65)	63.2 ± 8.5 (43–69)	76.3 ± 5.8 (69–90)
D % (EP/NL × 100)	71	65.1 ± 7.2 (52–74)	69.6 ± 2.7 (67–75)	48.3 ± 8.4 (36–62)	55.5 ± 9.7 (49–79)	38.1 ± 2.7 (34–46)
E % (EP/TL × 100)	228	224.6 ± 22.7 (197–261)	284.3 ± 34.5 (243–345)	204.8 ± 44.9 (132–281)	170.9 ± 33.4 (122–221)	94.7 ± 17.4 (79–129)
Rectum % (RL/ABD × 100)	–	–	–	90.4 ± 19.7 (58–112)	108.1 ± 29.3 (74–163)	164.3 ± 17.1 (128–211)
H % (H/TL × 100)	–	–	–	15.7 ± 5.1 (8–20)	21.6 ± 11.7 (12–40)	35.2 ± 3.4 (28–39)
SW % (SL/ABD×100)	144.0	172.3 ± 21.3 (144–197)	177.2 ± 19.8 (142–217)	–	–	–
GS % (GL/SL × 100)	58	59.9 ± 5.9 (49–68)	54.7 ± 7.1 (46–68)	–	–	–
Male reproductive system (MR)	1,218	1,044.3 ± 240.9 (639–1,371)	692.9 ± 172.4 (553–1,166)	–	–	–
Testis reflexion	281	280.2 ± 66.3 (116–352)	159.7 ± 64.6 (89–273)	–	–	–
Mucron	5.1	4.8 ± 0.7 (4.0–5.3)	7.3 ± 2.5 (5–10)	4.4 ± 0.7 (3.7–5.0)	Absent	–

All characters are in µm (except ratios and percentages) and given as mean ± s.d. (range).

BD, body diameter; EP, excretory pore; GL, gubernaculum length; IJs, infective juveniles; NL, neck length; NR, nerve ring; PL, pharynx length; SL, spicule length; VA, vulva–anterior end.

Table 2. Comparative morphometrics of *Steinernema africanum* n. sp. IJs.

Species	Reference	L	BD	EP	NR	NL	TL	a	b	c	c'	D%	E%	H%
S. africanum	Present study	751 (690–802)	27 (25–32)	59 (54–68)	117 (87–132)	154 (123–167)	64 (52–72)	28 (23–30)	4.9 (4.3–6.3)	12 (10–15)	3.7 (2.9–4.2)	38 (34–46)	94 (79–129)	35 (28–39)
S. akhursti	Qiu *et al*. (2005)	812 (770–835)	33 (33–35)	59 (55–60)	90 (83–95)	119 (115–123)	73 (68–75)	24 (23–26)	6.8 (6.6–7.2)	11 (10–12)	3.5 (3.3–3.7)	47 (45–50)	77 (73–86)	52 (49–56)
S. cholashanense	Ma *et al*. (2012)	843 (727–909)	30 (26–35)	62 (59–65)	87 (72–97)	125 (110–138)	73 (60–80)	28 (24–34)	6.8 (6.1–7.2)	12 (10–14)	4.3 (3.5–5.0)	49 (46–53)	81 (76–91)	39 (33–47)
S. citrae	Stokwe *et al*. (2011)	754 (623–849)	26 (23–28)	56 (49–64)	98 (83–108)	125 (118–137)	71 (63–81)	30 (25–34)	6.0 (5.1–7.1)	15 (13–17)	NA	44 (39–58)	110 (85–132)	43 (37–50)
S. feltiae	Nguyen *et al*. (2007)	849 (766–928)	29 (22–32)	63 (58–67)	113 (108–117)	136 (130–143)	86 (81–89)	30 (27–34)	6.4 (5.8–6.8)	10 (9.4–11)	4.8 (4.5–5.1)	46 (44–50)	74 (67–81)	44 (37–51)
S. hebeiense	Chen *et al*. (2006)	658 (610–710)	26 (23–28)	48 (43–51)	78 (73–83)	107 (100–111)	66 (63–71)	26 (24–28)	6.2 (5.7–6.7)	10 (9.4–11)	NA	45 (40–50)	72 (65–80)	43 (32–50)
S. ichnusae	Tarasco *et al*. (2008)	866 (767–969)	31 (27–35)	63 (59–68)	102 (94–108)	138 (119–148)	81 (76–89)	28 (24–32)	6.3 (5.6–6.9)	11 (9–12)	4.6 (4.2–5.1)	46 (42–49)	77 (68–83)	48 (44–50)
S. jollieti	Spiridonov *et al*. (2004)	711 (625–820)	23 (20–28)	60 (53–65)	NA	123 (115–135)	68 (60–73)	31 (25–34)	5.7 (4.9–6.4)	10.5 (9–12)	4.5 (NA)	48 (46–50)	88 (NA)	55 (46–60)
S. kraussei	Nguyen *et al*. (2007)	951 (797–1,102)	33 (30–36)	63 (50–66)	105 (99–111)	134 (119–145)	79 (63–86)	29 (NA)	7.1 (NA)	12.1 (NA)	3.9 (NA)	47 (NA)	80 (NA)	38 (35–40)
S. kushidai	Mamiya (1998)	589 (424–662)	26 (22–31)	46 (42–50)	76 (70–84)	111 (106–120)	50 (44–59)	23 (19–25)	5.3 (4.9–5.9)	11.7 (10–13)	NA	41 (38–44)	92 (NA)	NA
S. litorale	Yoshida, (2004)	909 (834–988)	31 (28–33)	61 (54–69)	96 (89–104)	125 (114–133)	83 (72–91)	30 (27–31)	7.3 (6.7–7.9)	11 (10–11.9)	4.5 (3.8–5.4)	49 (44–56)	73 (68–84)	33 (NA)
S. nguyeni	Malan *et al*. (2016)	737 (673–796)	25 (22–28)	52 (47–58)	80 (74–86)	110 (101–121)	67 (61–73)	29 (27–33)	6.7 (6.2–7.4)	11 (10–12)	4.3 (2.8–4.8)	48 (43–57)	79 (70–86)	27 (20–31)
S. oregonese	Liu and Berry, (1996)	980 (820–1,110)	34 (28–38)	66 (60–72)	NA	132 (116–148)	70 (64–78)	30 (24–37)	7.6 (6–8)	14 (12–16)	4.7 (NA)	50 (40–60)	100 (90–110)	31 (30–33)
S. populi	Tian *et al*. (2022)	1,095 (973–1,172)	36 (33–41)	77 (70–86)	106 (98–113)	149 (134–159)	64 (55–72)	30 (24–33)	7.4 (6.8–8.5)	17 (15–20)	2.8 (2.4–3.3)	52 (47–61)	121 (105–140)	35 (26–44)
S. puntauvense	Uribe-Lorío *et al*. (2007)	670 (631–728)	33 (31–38)	25 (20–30)	54 (46–69)	94 (81–103)	54 (51–59)	20 (17–23)	6.1 (7.1–7.9)	12 (11–13)	NA	42 (25–50)	44 (35–56)	54 (52–55)
S. sandneri	Lis *et al*. (2021)	843 (708–965)	27 (23–32)	56 (44–64)	103 (83–118)	138 (123–151)	75 (64–86)	31 (27–34)	6.1 (5.5–6.9)	11.2 (11–13)	NA	40 (36–45)	74 (63–86)	34 (23–40)
S. sangi	Phan *et al*. (2001)	753 (704–784)	35 (30–40)	52 (46–54)	91 (78–97)	127 (120–138)	81 (76–89)	22 (19–25)	5.9 (5.6–6.3)	9.3 (9–10)	4.5 (NA)	40 (36–44)	62 (56–70)	49 (44–52)

(*Continued*)

Table 2. Continued

Species	Reference	L	BD	EP	NR	NL	TL	a	b	c	c'	D%	E%	H%
S. silvaticum	Sturhan *et al.* (2005)	860 (670–975)	30 (26–35)	62 (51–73)	96 (75–109)	121 (100–141)	75 (63–86)	29 (23–33)	7.3 (6.3–7.7)	11.4 (10–13)	4.0 (3.1–4.9)	50 (46–56)	–	46 (37–53)
S. tielingense	Ma *et al.* (2012)	915 (824–979)	35 (32–38)	69 (64–73)	98 (90–105)	128 (120–135)	81 (74–85)	295 (27–31)	73 (67–79)	11 (10–12)	4 (3.5–4.6)	49 (44–56)	23 (68–84)	58 (53–64)
S. texanum	Nguyen *et al.* (2007)	756 (732–796)	30 (29–34)	59 (52–62)	92 (84–102)	115 (111–120)	73 (60–79)	25 (22–27)	6.5 (6.2–7.0)	10.4 (10–13)	3.3 (3.3–4.6)	51 (46–53)	81 (76–88)	59 (53–69)
S. xinbinense	Ma *et al.* (2012)	694 (635–744)	30 (28–31)	51 (46–53)	86 (75–90)	116 (109–125)	73 (65–78)	24 (21–25)	6.1 (5–7)	9.7 (8–11)	4.2 (3–5)	44 (40–47)	71 (65–78)	35 (30–42)
S. xueshanense	Mráček *et al.* (2009)	860 (768–929)	30 (29–33)	67 (60–72)	91 (81–96)	135 (130–143)	87 (80–92)	28 (26–32)	6.4 (5.8–7.0)	9.9 (9–11)	4.6 (3.8–5.1)	50 (46–52)	78 (70–90)	51 (46–55)
S. weiseri	Mráček *et al.* (2003)	740 (586–828)	25 (24–29)	57 (43–65)	84 (72–92)	113 (95–119)	60 (49–68)	29 (25–33)	6.6 (5.7–7.2)	12 (10–14)	3.7 (3.2–4.1)	51 (44–55)	95 (NA)	22 (18–24)

All measurements are in µm (except ratios and percentages).
BD, body diameter; EP, excretory pore; IJs, infective juveniles; NL, neck length; NR, nerve ring.

posterior to lateral labial papillae. Stoma shallow, funnel-shaped, short and wide, with inconspicuous sclerotized walls; cheilostom short with small rhabdia; gymnostom scarcely developed with minute rhabdia stegostom robust with funnel-shaped lumen and walls with minute rhabdia. Deirids inconspicuous. Pharynx muscular with a cylindrical procorpus, a slightly swollen and non-valvate metacorpus, narrower isthmus and basal bulb spheroid with reduced valves. Nerve ring (NR) usually located about mid-isthmus level or on the anterior part of the basal bulb. Secretory-EP opening circular, located posterior to NR, close to isthmus-basal bulb junction. Cardia prominent, conoid. Intestine tubular without differentiations. Reproductive system monorchic, ventrally reflexed. Spicules paired, symmetrical, ventrally curved with manubrium rhomboidal, calamus narrower and lamina ventrad curved at anterior part, bearing two longitudinal ribs, and ending in a blunt terminus, with scarcely developed velum not reaching the spicule tip, without rostrum or retinaculum. Gubernaculum fusiform with elongate tip, about one-half of the length of spicules. Tail conoid with rounded terminus bearing a fine mucron. Bursa absent. There are 23 GP (11 pairs and one single) arranged as follows: five pairs sub-ventral precloacal, one pair lateral precloacal, one single mid-ventral papilla, two pairs sub-ventral ad-cloacal, one pair subdorsal post-cloacal, and two pairs of terminal papillae. Phasmids terminal, located between the last pair of GP.

Second-generation male

General morphology similar to that of first-generation males, but smaller in size and slenderer. Tail with mucron robust, dorsally curved. Spicules ventrally curved, with manubrium rounded, calamus slightly narrower than manubrium, and lamina ventrally curved at anterior part, lanceolate posterior part with finely rounded tip, reduced ventral velum, and two longitudinal lateral ribs. Gubernaculum slenderer than that of first-generation male, with manubrium ventrad bent, corpus robust, and narrow and slender terminus. Genital papillae and phasmids with arrangement similar to that in first-generation male.

First-generation female

Body C-shaped when heat-relaxed and fixed. Cuticle with transversal incisures marked, appearing poorly visible annuli. Lateral fields not observed. Deirids inconspicuous, difficult to observe even under SEM. Labial region rounded, continuous with the adjacent part of body. Stoma and pharynx region similar to males. Excretory pore located at level of the

Table 3. Comparative morphometrics of first-generation *Steinernema africanum* n. sp. males. All measurements are in micrometer (except ratios and percentages).

Species	L	BD	EP	NR	NL	TL	SL	GL	a	b	c	c'	D%	SW%	GS%
S. africanum	1,202 (977–1,400)	115 (65–131)	93 (69–109)	92 (79–104)	143 (132–147)	41 (34–46)	71 (65–76)	43 (32–49)	10 (9–12)	8 (7–12)	29 (25–34)	1.0 (0.9–1.1)	65 (52–74)	172 (144–197)	60 (49–68)
S. akhursti	1,589 (1,350–1,925)	131 (115–150)	102 (93–113)	136 (120–163)	182 (168–205)	35 (30–40)	90 (85–100)	64 (58–68)	NA	NA	NA	NA	56 (52–61)	180 (140–200)	71 (65–77)
S. cholashanense	1,428 (1,070–1,778)	137 (73–204)	99 (75–135)	106 (91–126)	152 (135–173)	35 (29–43)	66 (60–71)	39 (32–45)	11 (9–24)	9.3 (8–11)	41 (36–51)	0.7 (0.6–0.9)	64 (50–85)	115 (92–144)	71 (61–85)
S. citrae	1,154 (1,028–1,402)	103 (87–113)	81 (64–92)	106 (92–119)	139 (123–155)	25 (17–31)	65 (57–80)	44 (32–59)	NA	NA	NA	NA	58 (47–67)	198 (156–233)	68 (48–89)
S. feltiae	1,612 (1,414–1,817)	75 (60–90)	115 (110–126)	69 (55–87) [a]	170 (164–180)	89 (37–43)	70 (65–77)	41 (34–47)	11.5 (NA)	9.5 (NA)	41.3 (NA)	0.8 (NA)	60 (51–64)	113 (99–130)	59 (52–61)
S. hebeiense	1,177 (1,036–1,450)	86 (74–98)	64 (58–73)	84 (78–93)	126 (118–132)	30 (24–35)	57 (51–63)	46 (38–50)	14 (12–17)	9 (8–11)	39 (30–49)	0.8 (0.6–0.9)	51 (48–59)	140 (120–170)	80 (60–90)
S. ichnusae	1,341 (1,151–1,494)	137 (73–204)	101 (94–108)	NA	165 (135–173)	40 (33–48)	66 (64–67)	44 (43–46)	22 (20–29)	8.2 (7–9)	34 (29–39)	0.8 (0.8–0.9)	62 (59–65)	139 (120–162)	67 (64–69)
S. jollieti	1,662 (1,296–1,952)	115 (98–135)	98 (83–110)	NA	156 (110–168)	33 (24–38)	64 (55–70)	54 (45–60)	15 (12–19)	11 (8–14)	51 (53–86)	0.8 (NA)	64 (53–83)	145 (NA)	84 (NA)
S. kraussei	1,400 (1,200–1,600)	128 (110–144)	81 (73–99)	105 (95–122)	153 (137–178	39 (36–44)	49 (42–53)	33 (29–37)	11	9 (NA)	37 (NA)	0.9 (NA)	53 (NA)	110 (NA)	67 (NA)
S. kushidai	1,400 (1,200–1,900)	97 (75–156)	84 (71–105)	129 (120–137)	167 (156–189)	33 (30–40)	63 (48–72)	44 (39–60)	NA	NA	NA	NA	51 (42–59)	150 (NA)	70 (NA)
S. litorale	1,360 (1,230–1,514)	96 (82–111)	96 (77–107)	114 (94–128)	147 (133–163)	34 (26–41)	75 (67–89)	53 (44–64)	14 (12–16)	9.3 (8–10)	41 (33–56)	0.8 (0.6–0.9)	40 (34–56)	174 (154–200)	71 (62–81)
S. nguyeni	997 (818–1,171)	82 (58–106)	59 (47–71)	91 (70–103)	124 (112–144)	21 (18–25)	66 (58–75)	43 (30–55)	12 (11–15)	8 (7–10)	46 (38–53)	0.7 (0.6–0.8)	48 (38–57)	215 (185–279)	66 (46–81)
S. oregonense	1,680 (1,560–1,820)	138 (105–161)	112 (95–139)	111 (101–133)	154 (139–182)	29 (24–32)	71 (65–73)	56 (52–59)	NA	NA	NA	0.6 (NA)	73 (64–75)	151 (NA)	79 (NA)
S. puntauvense	1,591 (1,010–1,931)	119 (101–139)	94 (68–114)	115 (104–128)	140 (130–159)	33 (28–40)	77 (71–81)	34 (30–40)	NA	NA	NA	NA	67 (45–85)	170 (140–200)	65 (55–75)
S. sandneri	1,461 (1,206–1,635)	155 (124–178)	80 (64–92)	126 (112–138)	157 (148–170)	41 (35–46)	60 (53–65)	44 (39–50)	10 (9–11)	9.3 (8–10)	37 (31–42)	NA	51 (42–59)	111 (97–127)	79 (61–83)
S. sangi	1,774 (1,440–2,325)	159 (120–225)	82 (67–99)	126 (109–166)	166 (150–221)	32 (27–42)	63 (58–80)	40 (34–46)	NA	NA	NA	NA	49 (42–63)	150 (120–160)	60 (50–70)

(*Continued*)

Table 3. Continued

Species	L	BD	EP	NR	NL	TL	SL	GL	a	b	c	c'	D%	SW%	GS%
S. silvaticum	1,090 (975–1,270)	65 (52–78)	79 (71–92)	119 (90–126)	142 (116–168)	34 (20–47)	51 (42–64)	37 (30–43)	17 (14–20)	7.7 (8–9)	34 (24–55)	1.0 (0.8–1.4)	60 (45–63)	155 (NA)	73 (NA)
S. tielingense	1,778 (1,430–2,064)	129 (111–159)	114 (94–133)	112 (96–132)	160 (145–173)	26 (22–33)	88 (79–98)	62 (49–70)	11 (11–18)	11 (9–13)	70 (57–85)	0.5 (0.3–0.6)	71 (64–78)	191 (176–212)	73 (59–82)
S. texanum	1,296 (1,197–1,406)	99 (81–116)	90 (79–100)	104 (94–114)	135 (123–147)	23 (19–30)	60 (55–66)	45 (39–53)	NA	NA	NA	NA	67 (58–73)	157 (127–203)	75 (62–84)
S. xinbinense	1,265 (1,133–1,440)	103 (90–126)	68 (57–75)	106 (91–120)	149 (138–159)	37 (30–41)	56 (49–62)	35 (30–41)	12 (11–13)	8.5 (7–9)	34 (31–39)	0.9 (0.7–1.0)	45 (41–50)	137 (114–156)	63 (54–72)
S. xueshanense	1,589 (1,313–2,040)	144 (97–159)	128 (113–137)	NA	160 (151–175)	38 (29–48)	76 (66–91)	49 (41–60)	NA	NA	NA	NA	80 (73–87)	152 (93–172)	64 (58–95)
S. weiseri	1,180 (990–1,395)	112 (84–138)	70 (57–84)	99 (94–115)	141 (134–154)	25 (19–32)	68 (62–72)	53 (46–57)	11 (9–12)	8 (7–10)	48 (36–64)	0.7 (0.6–0.9)	49 (39–60)	180 (150–240)	80 (70–85)

[a]According to Aksary *et al.* (2020).

BD, body diameter; EP, excretory pore; GL, gubernaculum length; NR, nerve ring; NL, neck length; SL, spicule length.

metacorpus-isthmus junction. Nerve ring surrounding the isthmus. Reproductive system didelphic, amphidelphic. Ovaries reflexed in dorsal position; oviducts well developed with glandular spermatheca, and uteri tubular with numerous uterine eggs; vagina short, with muscular walls; vulva protruding, in the form of transverse slit located slightly post-equatorial with lips slightly protruding, asymmetrical, with small epiptygma. Rectum 0.6 to 1.1 times the BD, with three rectal glands. Tail conoid, shorter than body anal diameter, with terminus bearing a fine mucron. Phasmids located at anterior part of tail, at 23% to 34% of tail length.

Second-generation female

Similar to first-generation female but smaller. Tail conoid, longer than the first-generation female, lacking mucron. Phasmid located at the posterior part of the tail, at 58% to 59% of tail length.

Third-stage infective juvenile

Body straight or slightly curved when heat-killed, tapering gradually from the base of pharynx to the anterior end and from anus to the distal end. Cuticle with transverse incisures, appearing well-developed annuli. Lateral fields begin as a single line close to the anterior end, increasing to eight ridges, posteriorly gradually reduced to four (anus level) and two (phasmid level). Lip region slightly narrower than the adjacent part of body, with six lips, the lateral ones smaller with six reduced labial and four prominent cephalic papillae. Amphidial apertures pore-like. Stoma reduced, with small cheilostom and elongate gymno-stegostom. Pharynx reduced with narrow corpus, slightly narrower isthmus, and pyriform basal bulb with reduced valves. Nerve ring surrounding the isthmus. Excretory pore located at metacorpus level. Hemizonid present, between NR and pharynx base. Cardia conoid. Deirids inconspicuous. Intestine bears a bacterial sac at its anterior part. Rectum long, almost straight, with very short cuticular part and elongate cellular part. Anus distinct. Genital primordium located equatorial. Tail conoid, tapering gradually with pointed terminus; cellular part longer than hyaline part, which comprises 28% to 39% of tail length; cellular-hyaline junction irregular. Phasmids located at 34% to 43% of tail length.

Life cycle

Steinernema africanum n. sp. readily infests and develops in *G. mellonella* larvae. However, the development of *S. africanum* is unusually slow at

Table 4. Comparative morphometrics of first-generation *Steinernema africanum* n. sp. females. All measurements are in micrometer (except ratios and percentages).

Species	L	BD	EP	NR	NL	TL	a	b	c	c'	V	ABD	D%	Mucron
S. africanum	3,205 (2,469–4,215)	180 (154–194)	89 (67–111)	105 (79–130)	185 (170–201)	45 (35–55)	18 (13–27)	17 (13–24)	74 (51–104)	0.9 (0.7–1.0)	52 (50–57)	52 (37–70)	48 (36–62)	Present
S. akhursti	7,283 (5,625–9,000)	239 (200–270)	126 (113–138)	164 (150–175)	239 (213–258)	49 (38–63)	29[b] (NA)	31[b] (NA)	141[b] (NA)	0.6[b](NA)	51 (48–53)	86 (68–100)	53 (NA)	Present
S. cholashanense	4,692 (3,232–6,363)	255 (156–332)	129 (111–148)	190 (176–223)	196 (181–231)	57 (46–70)	13 (13–23)	25 (18–32)	83 (62–119)	0.8 (0.6–1.0)	53 (50–57)	77 (54–105	50 (29–65)	Present
S. citrae	3,087 (2,038–4,019)	175 (137–212)	75 (54–90)	151 (130–179)	215 (189–220)	44 (33–60)	NA	NA	NA	NA	54 (50–59)	62 (43–79)	37 (27–46)	Present
S. feltiae	3,380 (3,095–3,774)	204 (170–254)	82 (68–97)[a]	84 (70–97)[a]	237 (197–304)	52 (39–70)	17 (14–20)	14 (12–17)	65 (49–88)	1.0 (0.7–1.2)[a]	56 (44–57)	52 (47–62)	46 (40–54)[a]	Present
S. hebeiense	3,465 (3,972–4,254)	167 (142–245)	65 (48–95)	104 (88–123)	147 (133–158)	35 (25–50)	21 (17–25)	24 (21–29)	103 (67–129)	0.7 (0.5–0.9)	54 (50–57)	53 (45–65)	45 (36–66)	Absent
S. ichnusae	5,514 (4,547–6,186)	269 (242–323)	126 (106–156)	NA	239 (215–262)	60 (51–79)	21 (17–24)	23 (21–26)	93 (68–113)	0.8 (0.6–1.0)	53 (51–57)	80 (70–94)	53 (47–63)	Present
S. jollieti	5,148 (3,746–6,030)	259 (219–298)	111 (96–136)	NA	214 (184–310)	43 (31–55)	20 (15–24)	26 (19–31)	128 (72–185)	NA	51 (44–56)	NA	52	Present
S. kraussei	4,200 (2,500–5,400)	240 (153–288)	87 (66–99)	137 (127–146)	192 (178–205)	48 (33–59)	17	22	88	NA	54	45 (39–50)	45	Present
S. kushidai	3,500 (2,100–4,700)	175 (54–59)	91 (78–105)	124 (111–144)	227 (204–255)	38 (30–45)	NA	NA	NA	NA	56 (54–59)	64 (54–84)	40 (37–46)	Absent
S. litorale	4,462 (3,930–5,048)	191 (175–215)	88 (65–105)	146 (130–165)	196 (185–213)	39 (25–60)	23 (21–26)	23 (20–26)	117 (78–157)	0.6 (0.5–0.9)	56 (0.5–0.9)	62 (55–75)	45 (33–57)	Present
S. nguyeni	4,775 (2,290–5,361)	178 (130–216)	72 (49–98)	113 (84–139)	169 (137–194)	32 (20–67)	21 (15–30)	22 (15–30)	119 (53–165)	0.7 (0.6–1.1)	56 (52–63)	178 (130–216)	43 (30–56)	Absent
S. oregonense	5,200 (4,400–6,200)	242 (217–268)	103 (217–268)	147 (129–162)	210 (186–220)	37 (28–46)	NA	NA	NA	0.7 (NA)	52 (46–56)	56 (42–79)	49 (43–57)	Absent
S. puntauvense	6,198 (3,687–8,335)	198 (181–221)	70 (51–85)	135 (123–146)	192 (141–206)	49 (41–66)	NA	NA	NA	NA	53 (51–55)	76 (57–102)	37 (25–45)	Present
S. sandneri	4,628 (4,244–5,014)	210 (181–261)	84 (61–102)	147 (132–158)	185 (173–194)	147 (32–61)	22 (17–25)	25 (24–27)	102 (75–140)	NA	54 (49–57)	94 (62–122)	46 (36–54)	Present
S. sangi	6,030 (4,830–7,200)	336 (270–360)	101 (80–121)	158 (140–170)	229 (216–240)	49 (936–62)	NA	NA	NA	NA	51 (43–530)	111 (84–140)	44 (35–51)	Present

(*Continued*)

Table 4. Continued

Species	L	BD	EP	NR	NL	TL	a	b	c	c'	V	ABD	D%	Mucron
S. silvaticum	2,150 (1,520–3,290)	116 (50–175)	69 (50–175)	113 (50–175)	146 (121–188)	45 (33–79)	19 (15–41)	15 (10–18)	49 (34–80)	1.3 (1.0–1.8)	52 (44–57)	35 (26–53)	47 (33–79)	Absent
S. texanum	3,058 (2,720–3,623)	163 (130–202)	88 (78–107)	122 (111–135)	172 (160–189)	40 (30–52)	NA	NA	NA	NA	52 (50–55)	60 (50–71)	NA	Absent
S. tielingense	6,190 (4,028–8,538)	251 (200–307)	84 (82–103)	136 (111–144)	202 (186–263)	45 (40–69)	23 (17–32)	29 (21–45)	117 (72–158)	0.6 (0.5–0.9)	51 (49–54)	69 (56–92)	41 (32–49)	Absent
S. xinbinense	4,037 (3,025–5,121)	176 (159–200)	80 (70–87)	126 (106–141)	186 (167–192)	40 (30–53)	22 (19–25)	22 (17–26)	106 (79–123)	0.6 (0.5–0.8)	52 (46–57)	61 (50–67)	40 (38–45)	Present
S. xueshanense	5,092 (4,181–8,181)	257 (182–343)	148 (117–148)	NA	230 (196–274)	55 (43–66)	NA	NA	NA	NA	54 (52–62)	84 (38–72)	NA	Present
S. weiseri	4,610 (3,780–5,940)	223 (202–263)	80 (75–86)	125 (108–154)	184 (162–226)	42 (38–59)	21 (17–29)	25 (22–31)	111 (87–156)	0.7 (0.5–0.8)	53 (50–58)	63 (51–80)	NA	Present

[a]According to Aksary *et al.* (2020).
[b]Calculated from Allotype
BD, body diameter; EP, excretory pore; NR, nerve ring; NL, neck length.

24°C compared to several other *Steinernema* species including *S. feltiae*, *S. weiseri*, *S. ischunanense*, *S. litorale*, *S. surkhetense*, *S. hermaphroditum*, *S. akhursti*, *S. cholashanense*, and *S. xueshanense*. We typically observe that *G. mellonella* larvae infested with 50 to 150 IJs of the above-mentioned species die within 2 d to 3 d, but insects take 5 d to 6 d to die when infested with *S. africanum.* Nematode adults of the first and second generations are found in insect cadavers within 5 d to 6 d and 8 d to 9 d, respectively, when infested by the above-mentioned species, while they are found after 8 d to 9 d and 11 d to 12 d, respectively, when infested with *S. africanum*. Pre-IJs emerge from insect cadavers after 12 d to 15 d upon infestation by the above-mentioned species, but only after 18 d to 21 d when infested by *S. africanum*.

Type host and locality

The type hosts are unknown as the nematodes of this genus can be hosted by different insect species (Yan *et al*., 2016; Kajuga *et al*., 2018; Fallet *et al*., 2022) and were isolated from mixed soil samples by the *Galleria* baiting technique (White, 1927; Bedding and Akhurst, 1975). Briefly, *S. africanum* n. sp. RW14-M-C2b-1 and RW14-M-C2a-3 nematodes were isolated, using the "*Galleria* baiting" method, from soil samples collected in a banana, pumpkin, and sorghum intercrop in a valley in the Republic of Rwanda (GPS coordinates: 1°28'11.1"S 29°41'36.2"E; 1,865 m. s. n. m.) (Yan *et al*., 2016). Cultures of this species are maintained in the Institute of Biology, University of Neuchatel (Switzerland), in the Rwanda Agriculture and Animal Resource Development Board (Rubona, Rwanda), and in CABI Swiss laboratories in Hungary.

Type material

RW14-M-C2b-1 nematodes are the type material for *S. africanum* n. sp. Three slides of each stage, including first-generation adults (males and females), second-generation adults (males and females), and IJs, were deposited in the Nematology Collection of the Aquaculture Research Unit of the University of Limpopo, South Africa with the accession numbers ULRS-N1 to ULRS-N15. Additional specimens were deposited at the nematode collection of the Department of Animal Biology, Plant Biology and Ecology of the University of Jaén, Spain with the following accession numbers: RWA004-01 to RWA004-20 and RWA005-01 to RWA005-15. Slides will be made available upon reasonable request. Nematode cultures are maintained in the Institute of Biology, University of Neuchatel, Switzerland and

in the Rwanda Agriculture and Animal Resource Development Board, Rubona, Rwanda.

Etymology

The specific name refers to the continent where the species was isolated.

Cross-hybridization experiments

No progeny was observed when *S. africanum* n. sp. (isolates RW14-M-C2b-1 or RW14-M-C2a-3 nematodes) were allowed to interact with specimens of *S. feltiae*, *S. ichnusae*, *S. litorale*, or *S. weiseri*. No progeny was observed in the single-female control plates. When *S. africanum* n. sp. (isolates RW14-M-C2b-1 or RW14-M-C2a-3 nematodes) were allowed to interact, fertile progeny was observed. Fertile progeny was also observed when all nematode strains were self-fertilized. Hence, *S. africanum* n. sp. (RW14-M-C2b-1 or RW14-M-C2a-3) are conspecific and reproductively isolated from closely related species such as *S. feltiae*, *S. ichnusae*, *S. litorale*, and *S. weiseri*.

Diagnosis of Steinernema africanum n. sp.

Steinernema africanum n. sp. adults have short stoma with rounded cheilorhabdia, pharynx robust with rounded basal bulb; males monorchid with ventrally curved spicules having lanceolate manubrium in the first generation and rounded manubrium in the second generation, gubernaculum fusiform in the first generation and anteriorly hook-like in the second generation, tail conoid and slightly ventrally curved with fine mucron in the first generation (34–46 µm, c = 25–34, c´ = 0.9–1.1, mucron = 4.0–5.3 µm) and with more robust mucron in the second generation (28–46 µm, c = 26–36, c´ = 0.7–1.1, mucron = 5–10 µm); females didelphic–amphidelphic with shorter conoid tail bearing a fine mucron in the first generation (35–55 µm, c = 51–104, c´ = 0.7–1.0, mucron =3.7–5.0 µm) and longer conoid, tail-lacking mucron in the second generation (40–60 µm, c = 17–51, c´ = 1.2–2.3); and IJs with short body (0.69–0.80 mm), poorly developed pharynx (132–153 µm), H% (28–39), D% (79–105), and E% (135–290), lateral fields with eight longitudinal wings, and tail conoid-elongate (52–72 µm, c = 10–15, c´ = 2.9–4.2).

Morphological relationships of S. africanum n. sp. with other species

Based on morphological and morphometric traits, *S. africanum* n. sp. belongs to the "*feltiae*-clade." Nematodes of this group are characterized by having third-stage IJs between 700 µm and 1,000 µm long. The lateral fields of IJs in the "*feltiae*-clade" are characterized by eight ridges arranged evenly in the mid-body region. *Steinernema africanum* n. sp., a member of the "*feltiae*-clade," presents several traits common to this group. Specifically, the IJs have a large body size (690–802 µm), and they have eight ridges in the mid-body region of the lateral field. Several of the morphometric traits of the IJs overlap with those of other species in the "*feltiae*-clade" (Tables 2 and 3).

Steinernema africanum n. sp. IJs and first-generation adults are morphologically similar to *S. citrae*, *S. feltiae*, *S. ichnusae*, *S. litorale*, *S. nguyeni*, *S. weiseri*, *S. jollieti*, and *S. puntauvense*. The IJs of *S. africanum* n. sp. can be distinguished from the IJs of these latest species and other species of the "*feltiae*-clade" because the position of the NR is more posterior in *S. africanum* n. sp. and the stoma and pharynx regions are longer. In addition, *S. africanum* n. sp. IJs differ from *S. citrae* IJs in hyaline region occupying posterior (28%–39% vs 37%–50%) of tail length, hemizonid (present vs not observed), and cardia (conoid vs inconspicuous). *Steinernema africanum* n. sp., *S. feltiae, and S. ichnusae* IJs differ in tail length (52–72 µm vs 81–8 µm vs 76–89 µm), c´ (2.9–4.2 vs 4.5–5.1 vs 4.2–5.1), D% (34–46 vs 44–50 vs 42–49), hyaline region comprising (28–39% vs 37–51% vs 44–50%) of tail length, and E% (79–129 vs 67–81 vs 68–83). *Steinernema africanum* n. sp. and *S. litorale* IJs differ in body length (690–802 µm vs 834–988 µm), tail length (52–72 µm vs 72–91 µm), b ratio (4.3–6.3 vs 6.7–7.9), and location of hemizonid (between NR and pharynx base vs basal bulb). *Steinernema africanum* n. sp. and *S. nguyeni* IJs differ in b ratio (4.3–6.3 vs 6.2–7.4), neck length (NL) (123–167 µm vs 101–121 µm), and hyaline region comprising (28–39% vs 20–31%) of tail length. *Steinernema africanum* n. sp. and *S. weiseri* IJs differ in deirids (inconspicuous vs located in center of lateral fields at level of pharyngeal bulb or slightly posterior), location of hemizonid (between NR and pharynx base vs at level of basal bulb or immediately posterior), hyaline region comprising (28–39% vs 18–24%) of tail length, and D% (34–46 vs 44–55). *Steinernema africanum* n. sp. and *S. jollieti* IJs differ in the number of structures of the lateral field at mid-body (eight vs six), D% (34–46 vs 46–50), and hyaline region comprising (28–39% vs 46–60%) of tail length. *Steinernema africanum* n. sp. and *S. puntauvense* IJs differ in hemizonid (present vs not observed), hyaline region comprising (28–39% vs 52–55%) of tail length, BD (25–32 µm vs 31–38 µm), distance from anterior region to EP (54–68 µm vs 20–30 µm), distance from

anterior region to NR (87–132 µm vs 46–69 µm), a ratio (23–30 vs 17–23), and D% (79–129 vs 35–56) (Table 2).

First-generation males of *S. africanum* n. sp. differ from the males of *S. citrae* in lateral field (inconspicuous vs present in mid-body, with one narrow ridge) and tail length (34–46 µm vs 17–31 mm); from the males of *S. feltiae* in body length (0.98–1.40 mm vs 1.41–1.81 mm), the position of the EP (69–109 µm vs 110–126 µm), NL (132–147 µm vs 164–180 µm), and SW% (144–197 vs 99–130); from the males of *S. ichnusae* in the presence of prominent mucron vs absent, spicule manubrium rhomboidal vs always oblongate, body size (0.98–1.40mm vs 1.15–1.49 mm), and a ratio (9–12 vs 20–29); from the males of S. litorale in shape of spicule manubrium (rhomboidal vs oval to somewhat angular, rectangular), c (25–34 vs 33–56), D% (52–74 vs 34–56), and GS% (49–68 vs 62–81); from the males of *S. nguyeni* in presence of amphidial apertures (small vs inconspicuous), lateral field inconspicuous vs with one ridge, the position of the EP (69–109 µm vs 47–71

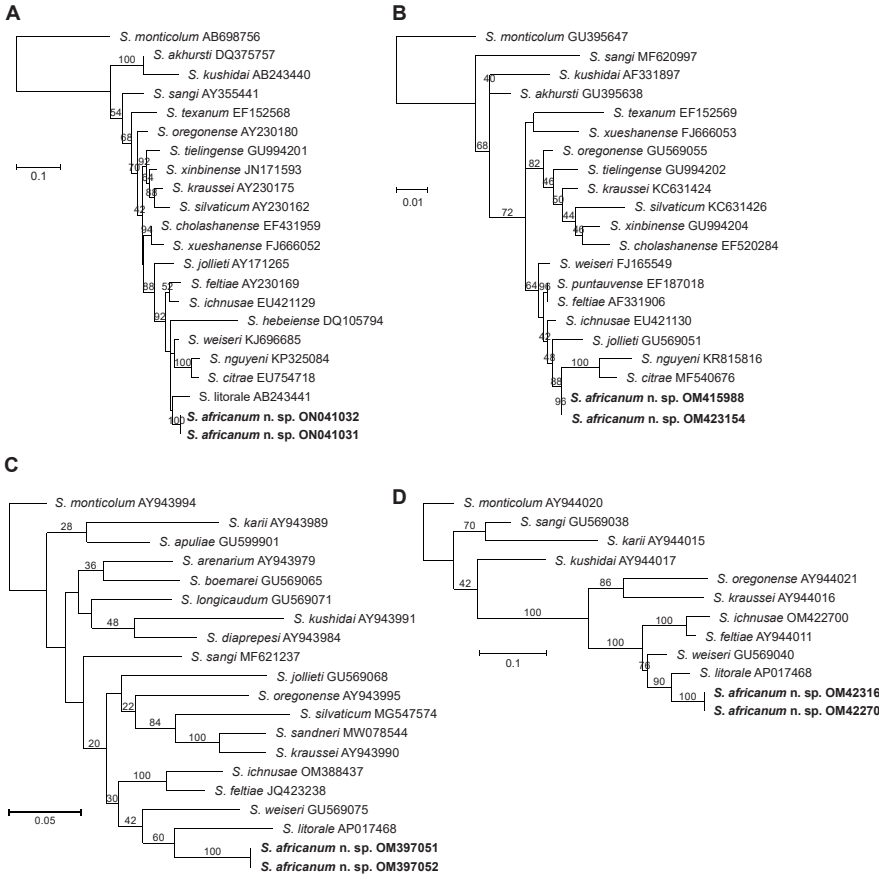

Figure 8: Phylogenetic relationships between the newly described *Steinernema africanum* n. sp. and other *Steinernema* species. Maximum-likelihood phylogenetic tree reconstructed from: **(A)** the nucleotide sequences of the ITS rRNA. A total of 705 nucleotide positions, flanked by primers 18S and 26S, were analyzed; **(B)** the nucleotide sequences of the D2–D3 expansion segments of the 28S rRNA. A total of 786 nucleotide positions, flanked by primers D2F and 536, were analyzed; **(C)** the nucleotide sequences of the COI gene. A total of 567 nucleotide positions, flanked by primers LCO-1490 and HCO-2198, were analyzed; and **(D)** the nucleotide sequences of the mitochondrial 12S rRNA gene. A total of 467 nucleotide positions, flanked by primers 505F and 506R, were analyzed. Numbers at nodes represent bootstrap values based on 100 replications. Bars represent average nucleotide substitutions per sequence position. NCBI accession numbers of the nucleotide sequences used for the analyses are shown next to the species names. COI, cytochrome oxidase subunit I; ITS, internal transcribed spacer; NCBI, National Center for Biotechnology Information.

µm), tail length (34–46 µm vs 18–25 µm), c (25–34 vs 38–53), and D% (52–74 vs 38–57); from the males of *S. weiseri* in the presence of mucron vs absent, spicule manubrium rhomboidal vs distinctly elongated, tail length (34–46 µm vs 19–32 µm), gubernaculum length (GL) (32–49 µm vs 46–57 µm), c (25–34 vs 36–64), and GS% (49–68 vs 70–85); from the males of *S. jollieti* in the presence of prominent mucron vs absent, shape of spicule manubrium (rhomboidal vs elongated), shape of gubernaculum (fusiform vs boat-shaped), deirids inconspicuous vs located posterior to level of pharyngo-intestinal junction, and a ratio (9–12 vs 12–19*);* from the males of *S. jollieti* in the distance of anterior end NR (79–104 µm vs 104–128 µm), presence of spicule calomus (narrower vs inconspicuous), lamina with rostrum or retinaculum (present vs absent), and velum (scarcely developed velum not reaching the spicule tip vs extending from rostrum to spicule terminus) (Table 3).

Nematode molecular characterization and phylogenetic relationships

Phylogenetic reconstructions based on the nucleotide sequences of the D2–D3 expansion segments of the 28S rRNA, the ITS region of the rRNA, the mitochondrial 12S rRNA, and the COI show that *S. africanum* n. sp. belongs to the "*feltiae*-clade" (Fig. 8). Based on sequence similarities, *S. africanum* n. sp. is closely related to *S. citrae*, *S. ichnusae*, *S. litorale*, *S. nguyeni*, and *S. weiseri* (Figs. S1 and S2 in Supplementary Material). These species share between

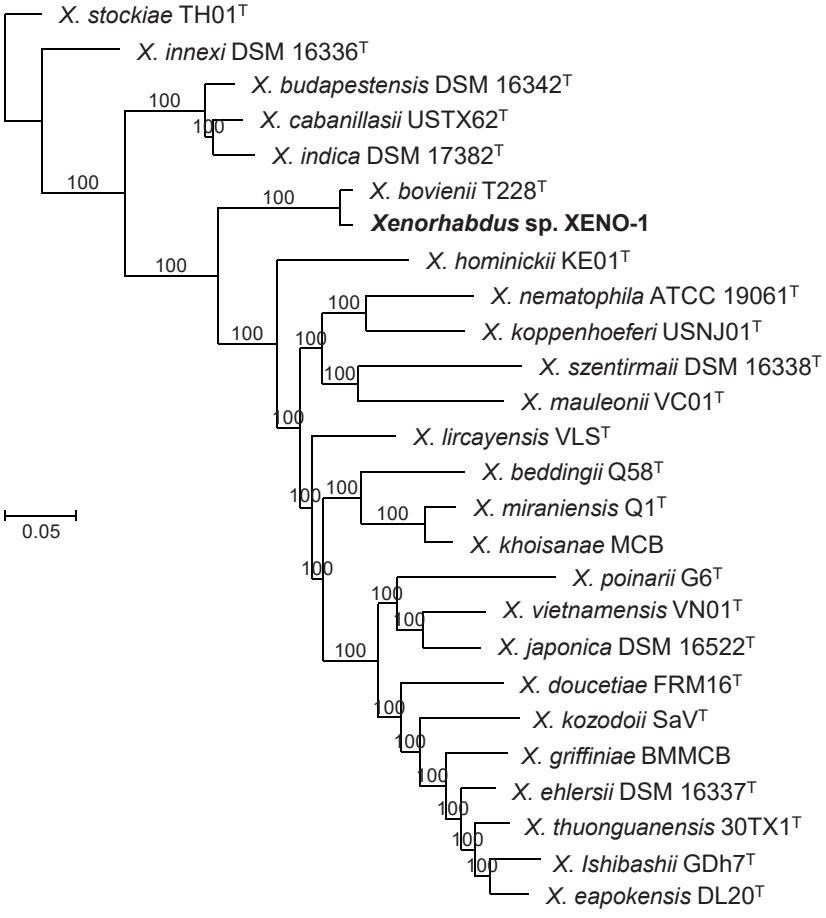

Figure 9: Phylogenetic relationships between the *Xenorhabdus* symbiont isolated from *Steinernema africanum* n. sp. and other *Xenorhabdus* species. Phylogenetic trees were built based on core genome sequences. A total of 1,719,910 nucleotide positions were used in the analyses. Numbers at the nodes represent SH-like branch supports. Bar represents 0.05 nucleotide substitutions per sequence position. Accession numbers of the genome sequences used for the reconstruction are shown in Table S1 in Supplementary Material.

92.4% and 95.9% and differ in 24 to 58 nucleotides with *S. africanum* n. sp. in the ITS sequences flanked by primers 18S and 26S (Fig. S1 in Supplementary Material). Less sequence similarities were observed between *S. africanum* n. sp. and all the other species of the "*feltiae*-clade," supporting its novel taxonomic status (Figs. S1 and S2 in Supplementary Material).

Symbiotic relationships

Phylogenetic reconstructions based on whole genome sequences show that the bacterial symbiont isolated from *S. africanum* n. sp. RW14-M-C2b-1, named here XENO-1, is closely related to *X. bovienii* T228^T (Fig. 9). The dDDH value between *X. bovienii* T228^T and XENO-1 is 71.2%, suggesting that the symbiont of *S. africanum* n. sp. represents a novel subspecies within the *X. bovienii* species (Fig. S3 in Supplementary Material). This subspecies will be formally described elsewhere.

Acknowledgments

The authors thank the Institute of Biology of the University of Neuchatel (Switzerland), the University of Limpopo (South Africa), and the Swiss National Science Foundation for their support. SEM pictures were obtained with the assistance of technical staff (Amparo Martínez-Morales) and equipment of the Centro de Instrumentación Científico-Técnica (CICT) of the University of Jaén (Spain). The authors thank the Rwanda Agriculture and Animal Resource Development Board for its support during nematode collection, which includes benefit sharing, material transfer agreements, and research permits.

Conflicts of Interest

The authors declare that no conflict of interest exists. The newly described entomopathogenic nematodes were discovered through public funds. There are no financial dependencies of authors with regard to the described nematode species.

Funding

The work of RARM is supported by the Swiss National Science Foundation (Grant No. 186094 to RARM). The work of AHB is supported by a Swiss Government Excellence Scholarship (Grant No. 2021.0463 to AHB). Light microscopy and scanning electron microscopy studies were performed thanks to the financial support of the Research Support Plan "POAIUJA 2021/2022: EI_RNM02_2021" of the University of Jaén, Spain. The surveys in Rwanda were financed by the AgriTT Research Challenge Funds of DFID of UK (Project 1301) and by DGIS of the Netherlands within the Action on Invasives program of CABI. The current nematode work in Rwanda is supported by the National Research and Innovation Fund (NRIF) via the International Development Research Center (IDRC) under the National Council for Science and Technology of Rwanda (Grant NCST-NRIF-IDRC/SSR-AGR/002/2021). CABI is grateful for the financial support provided by its donors and member countries detailed under: https://www.cabi.org/about-cabi/who-we-work-with/key-donors/

Author Contributions

Conceptualization: RARM; Formal analysis: RARM, AHB, JA, ES; Funding acquisition: RARM, JA, ES, JK, ST; Investigation: RARM, AHB, JA, ES; Methodology: RARM, AHB, JA, ES, XY; Project administration: RARM, JK; Resources: RARM, JA, ES, PF, TCJT, ET, VP, JK, XY, ST; Supervision: RARM; Visualization: RARM, AHB, JA, ES; Writing – original draft: RARM, AHB, JA, ES; Writing – review & editing: RARM, AHB, JA, ES, ST, PF, TCJT, ET, VP, JK, XY, ST.

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Supplementary Table and Figures

Table S1. National Center for Biotechnology Information (NCBI) accession numbers of the sequences used in this study.

Strain	Genome
***Xenorhabdus sp.* XENO-1**	JAMGSK01
X. beddingii Q58^T	MUBK01
X. bovienii T228^T	JANAIF01
X. budapestensis DSM 16342^T	NIBS01
X. cabanillasii USTX62^T	QTUB01
X. doucetiae FRM16^T	FO704550
X. eapokensis DL20^T	MKGQ01
X. ehlersii DSM 16337^T	NIBT01
X. griffiniae BMMCB	LDNM01
X. hominickii KE01^T	NJAI01
X. indica DSM 17382T	NKHP01
X. innexi DSM 16336^T	NIBU01
X. ishibashii GDh7^T	NJAK01
X. japonica DSM 16522^T	FOVO01
X. khoisanae MCB	LFCV01
X. koppenhoeferi USNJ01^T	FPBJ01
X. kozodoii SaV^T	NJCX01
X. lircayensis VLS^T	JACOII01
X. mauleonii VC01^T	NITY01
X. miraniensis Q1^T	NITZ01
X. nematophila ATCC 19061^T	FN667742
X. poinarii G6^T	FO704551
X. stockiae TH01^T	NJAJ01
X. szentirmaii DSM 16338^T	NIBV01
X. thuongxuanensis 30TX1^T	MKGR01
X. vietnamensis VN01^T	MUBJ01

A

	S. monticolum AB698756	*S. akhursti* DQ375757	*S. kushidai* AB243440	*S. sangi* AY355441	*S. texanum* EF152568	*S. oregonense* AY230180	*S. tielingense* GU994201	*S. xinbinense* JN171593	*S. kraussei* AY230175	*S. silvaticum* AY230162	*S. cholashanense* EF431959	*S. xueshanense* FJ666052	*S. jollieti* AY171265	*S. feltiae* AY230169	*S. ichnusae* EU421129	*S. hebeiense* DQ105794	*S. weiseri* KJ696685	*S. nguyeni* KP325084	*S. citrae* EU754718	*S. litorale* AB243441	***S. africanum* n. sp. ON041032**	***S. africanum* n. sp. ON041031**
S. monticolum AB698756	ID	69.2	66.4	67.8	69.2	69.7	70.0	70.2	70.1	68.6	70.6	70.2	70.2	67.9	69.7	67.1	70.7	69.3	69.3	70.6	70.9	70.9
S. akhursti DQ375757	69.2	ID	91.5	82.2	81.7	80.2	80.5	81.0	80.0	79.4	82.0	80.9	81.5	79.2	80.3	76.7	80.1	78.9	79.9	79.4	80.0	80.0
S. kushidai AB243440	66.4	91.5	ID	80.0	77.2	78.1	77.8	78.9	78.3	77.5	79.5	78.6	78.5	75.6	77.7	74.5	77.2	76.2	77.2	76.9	77.8	77.8
S. sangi AY355441	67.8	82.2	80.0	ID	84.3	84.1	85.0	84.7	84.6	83.1	85.7	84.3	83.3	81.2	83.7	78.3	83.0	81.8	82.5	82.0	83.2	83.2
S. texanum EF152568	69.2	81.7	77.2	84.3	ID	87.1	88.6	88.2	88.1	86.6	89.2	86.8	86.6	85.5	88.5	83.4	88.3	87.7	87.9	87.0	88.8	88.8
S. oregonense AY230180	69.7	80.2	78.1	84.1	87.1	ID	91.4	91.1	91.2	90.1	92.9	93.0	86.5	86.4	89.3	83.9	89.0	87.5	88.1	87.2	88.4	88.4
S. tielingense GU994201	70.0	80.5	77.8	85.0	88.6	91.4	ID	94.3	93.8	92.0	93.8	90.5	87.3	86.7	88.2	83.9	89.3	87.9	88.6	86.8	88.8	88.8
S. xinbinense JN171593	70.2	81.0	78.9	84.7	88.2	91.1	94.3	ID	94.9	93.9	94.6	91.2	87.9	87.5	89.2	84.6	90.1	88.7	89.1	88.2	89.8	89.8
S. kraussei AY230175	70.1	80.0	78.3	84.6	88.1	91.2	93.8	94.9	ID	94.6	94.2	90.9	87.7	86.7	88.8	84.0	89.1	88.5	89.2	87.2	88.8	88.8
S. silvaticum AY230162	68.6	79.4	77.5	83.1	86.6	90.1	92.0	93.9	94.6	ID	92.9	89.8	87.4	85.7	88.2	83.0	87.8	87.7	88.1	86.2	88.1	88.1
S. cholashanense EF431959	70.6	82.0	79.5	85.7	89.2	92.9	93.8	94.6	94.2	92.9	ID	95.7	89.2	88.2	91.0	84.5	91.1	89.9	90.5	89.0	91.0	91.0
S. xueshanense FJ666052	70.2	80.9	78.6	84.3	86.8	93.0	90.5	91.2	90.9	89.8	95.7	ID	86.6	86.3	89.4	83.8	89.1	87.9	88.3	87.2	88.7	88.7
S. jollieti AY171265	70.2	81.5	78.5	83.3	86.6	86.5	87.3	87.9	87.7	87.4	89.2	86.6	ID	87.4	89.6	84.0	88.7	87.8	88.2	88.9	89.7	89.7
S. feltiae AY230169	67.9	79.2	75.6	81.2	85.5	86.4	86.7	87.5	86.7	85.7	88.2	86.3	87.4	ID	92.8	85.0	91.6	90.0	90.6	90.8	91.7	91.7
S. ichnusae EU421129	69.7	80.3	77.7	83.7	88.5	89.3	88.2	89.2	88.8	88.2	91.0	89.4	89.6	92.8	ID	87.6	94.9	92.9	93.4	94.1	95.9	95.9
S. hebeiense DQ105794	67.1	76.7	74.5	78.3	83.4	83.9	83.9	84.6	84.0	83.0	84.5	83.8	84.0	85.0	87.6	ID	87.8	87.2	87.1	87.9	87.2	87.2
S. weiseri KJ696685	70.7	80.1	77.2	83.0	88.3	89.0	89.3	90.1	89.1	87.8	91.1	89.1	88.7	91.6	94.9	87.8	ID	93.1	94.1	95.3	96.6	96.6
S. nguyeni KP325084	69.3	78.9	76.2	81.8	87.7	87.5	87.9	88.7	88.5	87.7	89.9	87.9	87.8	90.0	92.9	87.2	93.1	ID	96.6	91.8	92.4	92.4
S. citrae EU754718	69.3	79.9	77.2	82.5	87.9	88.1	88.6	89.1	89.2	88.1	90.5	88.3	88.2	90.6	93.4	87.1	94.1	96.6	ID	92.7	93.1	93.1
S. litorale AB243441	70.6	79.4	76.9	82.0	87.0	87.2	86.8	88.2	87.2	86.2	89.0	87.2	88.9	90.8	94.1	87.9	95.3	91.8	92.7	ID	95.3	95.3
***S. africanum* n. sp. ON041032**	70.9	80.0	77.8	83.2	88.8	88.4	88.8	89.8	88.8	88.1	91.0	88.7	89.7	91.7	95.9	87.2	96.6	92.4	93.1	95.3	ID	100
***S. africanum* n. sp. ON041031**	70.9	80.0	77.8	83.2	88.8	88.4	88.8	89.8	88.8	88.1	91.0	88.7	89.7	91.7	95.9	87.2	96.6	92.4	93.1	95.3	100	ID

B

S. monticolum AB698756	ID	231	254	241	225	225	225	223	223	234	219	221	221	240	224	243	218	226	226	218	215	215
S. akhursti DQ375757	231	ID	62	133	141	148	145	142	148	153	134	143	137	155	146	173	147	155	148	153	149	149
S. kushidai AB243440	254	62	ID	150	176	164	166	158	162	168	153	161	161	183	167	191	170	178	171	173	166	166
S. sangi AY355441	241	133	150	ID	120	116	109	112	112	123	104	116	122	140	119	159	124	133	128	132	123	123
S. texanum EF152568	225	141	176	120	ID	88	77	82	80	92	72	91	100	108	85	120	87	89	87	97	84	84
S. oregonense AY230180	225	148	164	116	88	ID	63	65	64	72	51	50	98	100	77	116	80	90	85	93	84	84
S. tielingense GU994201	225	145	166	109	77	63	ID	41	45	58	45	69	92	98	85	116	78	87	82	96	81	81
S. xinbinense JN171593	223	142	158	112	82	65	41	ID	37	44	39	64	88	92	77	111	72	81	78	86	74	74
S. kraussei AY230175	223	148	162	112	80	64	45	37	ID	39	42	66	89	98	80	115	79	82	77	93	81	81
S. silvaticum AY230162	234	153	168	123	92	72	58	44	39	ID	51	74	91	105	84	122	88	88	85	100	86	86
S. cholashanense EF431959	219	134	153	104	72	51	45	39	42	51	ID	31	78	86	64	111	64	72	67	79	65	65
S. xueshanense FJ666052	221	143	161	116	91	50	69	64	66	74	31	ID	97	100	77	118	79	88	85	92	81	81
S. jollieti AY171265	221	137	161	122	100	98	92	88	89	91	78	97	ID	92	75	116	82	88	85	80	74	74
S. feltiae AY230169	240	155	183	140	108	100	98	92	98	105	86	100	92	ID	52	109	61	72	68	67	60	60
S. ichnusae EU421129	224	146	167	119	85	77	85	77	80	84	64	77	75	52	ID	88	35	50	47	42	29	29
S. hebeiense DQ105794	243	173	191	159	120	116	116	111	115	122	111	118	116	109	88	ID	86	91	92	86	91	91
S. weiseri KJ696685	218	147	170	124	87	80	78	72	79	88	64	79	82	61	35	86	ID	48	41	33	24	24
S. nguyeni KP325084	226	155	178	133	89	90	87	81	82	88	72	88	88	72	50	91	48	ID	24	58	54	54
S. citrae EU754718	226	148	171	128	87	85	82	78	77	85	67	85	85	68	47	92	41	24	ID	52	49	49
S. litorale AB243441	218	153	173	132	97	93	96	86	93	100	79	92	80	67	42	86	33	58	52	ID	33	33
***S. africanum* n. sp. ON041032**	215	149	166	123	84	84	81	74	81	86	65	81	74	60	29	91	24	54	49	33	ID	0
***S. africanum* n. sp. ON041031**	215	149	166	123	84	84	81	74	81	86	65	81	74	60	29	91	24	54	49	33	0	ID

Figure S1: Pairwise comparisons of the ITS nucleotide sequences. **(A)** Sequence similarities (%). **(B)** Number of nucleotide differences (bp). A total of 808 nucleotide positions, flanked by primers 18S and 26S, were analyzed. NCBI accession numbers of the nucleotide sequences used for the analyses are shown next to the species names. ITS, internal transcribed spacer; NCBI, National Center for Biotechnology Information.

A

	S. monticolum GU395647	*S. sangi* MF620997	*S. kushidai* AF331897	*S. akhursti* GU395638	*S. texanum* EF152569	*S. xueshanense* FJ666053	*S. oregonense* GU569055	*S. tielingense* GU994202	*S. kraussei* KC631424	*S. silvaticum* KC631426	*S. xinbinense* GU994204	*S. cholashanense* EF520284	*S. weiseri* FJ165549	*S. puntauvense* EF187018	*S. feltiae* AF331906	*S. ichnusae* EU421130	*S. jollieti* GU569051	*S. nguyeni* KR815816	*S. citrae* MF540676	***S. africanum* n. sp. OM423154**	***S. africanum* n. sp. OM415988**
S. monticolum GU395647	ID	93.7	94.2	94.7	93.7	93.8	94.7	93.9	93.7	93.2	93.6	93.8	94.8	94.1	94.5	94.5	94.2	93.1	93.2	94.7	94.7
S. sangi MF620997	93.7	ID	95.8	96.5	94.8	94.7	95.7	95.3	95.1	94.7	95.0	95.1	95.8	95.7	96.1	95.8	95.5	94.1	94.4	96.0	96.0
S. kushidai AF331897	94.2	95.8	ID	97.6	95.2	96.6	96.9	96.6	96.5	95.6	96.2	96.4	97.1	96.6	97.0	96.7	96.2	95.7	96.0	97.1	97.1
S. akhursti GU395638	94.7	96.5	97.6	ID	96.4	97.1	97.9	97.1	97.0	96.4	96.2	96.6	97.9	97.4	97.8	97.8	97.3	95.7	96.4	97.5	97.5
S. texanum EF152569	93.7	94.8	95.2	96.4	ID	96.6	97.0	96.6	96.2	95.1	95.7	96.4	97.1	96.9	97.3	97.3	96.7	95.3	95.6	96.9	96.9
S. xueshanense FJ666053	93.8	94.7	96.6	97.1	96.6	ID	97.8	97.4	97.3	96.4	97.1	97.4	98.0	97.5	97.9	97.8	97.1	95.7	96.0	97.4	97.4
S. oregonense GU569055	94.7	95.7	96.9	97.9	97.0	97.8	ID	98.7	98.8	97.5	98.2	98.3	98.4	98.2	98.5	98.3	97.8	96.2	96.6	98.0	98.0
S. tielingense GU994202	93.9	95.3	96.6	97.1	96.6	97.4	98.7	ID	98.5	97.4	98.4	98.4	98.3	98.3	98.7	98.2	97.6	96.6	97.0	98.2	98.2
S. kraussei KC631424	93.7	95.1	96.5	97.0	96.2	97.3	98.8	98.5	ID	97.8	98.4	98.2	97.8	97.8	98.2	97.9	97.1	95.8	96.2	97.6	97.6
S. silvaticum KC631426	93.2	94.7	95.6	96.4	95.1	96.4	97.5	97.4	97.8	ID	97.8	97.5	96.9	96.9	97.3	96.7	96.0	94.7	95.1	96.5	96.5
S. xinbinense GU994204	93.6	95.0	96.2	96.2	95.7	97.1	98.2	98.4	98.4	97.8	ID	98.7	97.5	97.5	97.9	97.4	97.1	95.6	96.0	97.4	97.4
S. cholashanense EF520284	93.8	95.1	96.4	96.6	96.4	97.4	98.3	98.4	98.2	97.5	98.7	ID	97.6	97.7	98.0	97.5	97.0	96.1	96.4	97.5	97.5
S. weiseri FJ165549	94.8	95.8	97.1	97.9	97.1	98.0	98.4	98.3	97.8	96.9	97.5	97.6	ID	98.9	99.3	99.1	98.8	97.1	97.5	99.1	99.1
S. puntauvense EF187018	94.1	95.7	96.6	97.4	96.9	97.5	98.2	98.3	97.8	96.9	97.5	97.7	98.9	ID	99.6	99.1	98.3	96.9	97.3	98.8	98.8
S. feltiae AF331906	94.5	96.1	97.0	97.8	97.3	97.9	98.5	98.7	98.2	97.3	97.9	98.0	99.3	99.6	ID	99.4	98.7	97.3	97.6	99.2	99.2
S. ichnusae EU421130	94.5	95.8	96.7	97.8	97.3	97.8	98.3	98.2	97.9	96.7	97.4	97.5	99.1	99.1	99.4	ID	98.7	97.3	97.6	99.2	99.2
S. jollieti GU569051	94.2	95.5	96.2	97.3	96.7	97.1	97.8	97.6	97.1	96.0	97.1	97.0	98.8	98.3	98.7	98.7	ID	96.9	97.3	98.8	98.8
S. nguyeni KR815816	93.1	94.1	95.7	95.7	95.3	95.7	96.2	96.6	95.8	94.7	95.6	96.1	97.1	96.9	97.3	97.3	96.9	ID	98.4	97.9	97.9
S. citrae MF540676	93.2	94.4	96.0	96.4	95.6	96.0	96.6	97.0	96.2	95.1	96.0	96.4	97.5	97.3	97.6	97.6	97.3	98.4	ID	98.3	98.3
***S. africanum* n. sp. OM423154**	94.7	96.0	97.1	97.5	96.9	97.4	98.0	98.2	97.6	96.5	97.4	97.5	99.1	98.8	99.2	99.2	98.8	97.9	98.3	ID	100
***S. africanum* n. sp. OM415988**	94.7	96.0	97.1	97.5	96.9	97.4	98.0	98.2	97.6	96.5	97.4	97.5	99.1	98.8	99.2	99.2	98.8	97.9	98.3	100	ID

B

S. monticolum GU395647	ID	49	45	41	49	48	41	47	49	53	50	48	40	46	43	43	45	54	53	41	41
S. sangi MF620997	49	ID	32	27	40	41	33	36	38	41	39	38	32	33	30	32	35	46	43	31	31
S. kushidai AF331897	45	32	ID	18	37	26	24	26	27	34	29	28	22	26	23	25	29	33	31	22	22
S. akhursti GU395638	41	27	18	ID	28	22	16	22	23	28	29	26	16	20	17	17	21	33	28	19	19
S. texanum EF152569	49	40	37	28	ID	26	23	26	29	38	33	28	22	24	21	21	25	36	34	24	24
S. xueshanense FJ666053	48	41	26	22	26	ID	17	20	21	28	22	20	15	19	16	17	22	33	31	20	20
S. oregonense GU569055	41	33	24	16	23	17	ID	10	9	19	14	13	12	14	11	13	17	29	26	15	15
S. tielingense GU994202	47	36	26	22	26	20	10	ID	11	20	12	12	13	13	10	14	18	26	23	14	14
S. kraussei KC631424	49	38	27	23	29	21	9	11	ID	17	12	14	17	17	14	16	22	32	29	18	18
S. silvaticum KC631426	53	41	34	28	38	28	19	20	17	ID	17	19	24	24	21	25	31	41	38	27	27
S. xinbinense GU994204	50	39	29	29	33	22	14	12	12	17	ID	10	19	19	16	20	22	34	31	20	20
S. cholashanense EF520284	48	38	28	26	28	20	13	12	14	19	10	ID	18	18	15	19	23	30	28	19	19
S. weiseri FJ165549	40	32	22	16	22	15	12	13	17	24	19	18	ID	8	5	7	9	22	19	7	7
S. puntauvense EF187018	46	33	26	20	24	19	14	13	17	24	19	18	8	ID	3	7	13	24	21	9	9
S. feltiae AF331906	43	30	23	17	21	16	11	10	14	21	16	15	5	3	ID	4	10	21	18	6	6
S. ichnusae EU421130	43	32	25	17	21	17	13	14	16	25	20	19	7	7	4	ID	10	21	18	6	6
S. jollieti GU569051	45	35	29	21	25	22	17	18	22	31	22	23	9	13	10	10	ID	24	21	9	9
S. nguyeni KR815816	54	46	33	33	36	33	29	26	32	41	34	30	22	24	21	21	24	ID	12	16	16
S. citrae MF540676	53	43	31	28	34	31	26	23	29	38	31	28	19	21	18	18	21	12	ID	13	13
***S. africanum* n. sp. OM423154**	41	31	22	19	24	20	15	14	18	27	20	19	7	9	6	6	9	16	13	ID	0
***S. africanum* n. sp. OM415988**	41	31	22	19	24	20	15	14	18	27	20	19	7	9	6	6	9	16	13	0	ID

Figure S2: Pairwise comparisons of the nucleotide sequences of the D2–D3 expansion segments of the 28S rRNA. **(A)** Sequence similarities (%). **(B)** Number of nucleotide differences (bp). A total of 786 nucleotide positions, flanked by primers D2F and 536, were analyzed. NCBI accession numbers of the nucleotide sequences used for the analyses are shown next to the species names. NCBI, National Center for Biotechnology Information.

	X. stockiae TH01^T	*X. innexi* DSM 16336^T	*X. indica* DSM 17382^T	*X. budapestensis* DSM 16342^T	*X. cabanillasii* USTX62^T	***Xenorhabdus* sp. XENO-1**	*X. bovienii* T228^T	*X. szentirmaii* DSM 16338^T	*X. mauleonii* VC01^T	*X. koppenhoeferi* USNJ01^T	*X. nematophila* ATCC 19061^T	*X. hominickii* KE01^T	*X. lircayensis* VLS^T	*X. beddingii* Q58^T	*X. miraniensis* Q1^T	*X. khoisanae* MCB	*X. poinarii* G6^T	*X. vietnamensis* VN01^T	*X. doucetiae* FRM16^T	*X. japonica* DSM 16522^T	*X. kozodoii* SaV^T	*X. griffiniae* BMMCB	*X. ehlersii* DSM 16337^T	*X. thuongxuanensis* 30TX1^T	*X. ishibashii* GDh7^T	*X. eapokensis* DL20^T
X. stockiae TH01^T	ID	34.2	27.2	27.1	26.4	23.6	23.6	22.2	22.2	23.2	22.8	23.5	22.9	22.9	23.6	23.1	22.5	23.5	22.6	23.4	22.3	22.5	23.3	22.5	22.9	22.9
X. innexi DSM 16336^T	34.2	ID	26.2	26.5	27.2	23.1	23.3	22.8	23.1	23.2	23.5	22.7	23.1	22.5	22.6	22.8	22.5	22.7	22.8	22.5	22.0	22.3	22.4	22.3	22.2	22.4
X. indica DSM 17382^T	27.2	26.2	ID	51.1	49.8	24.3	24.1	22.4	22.7	23.8	23.3	24.2	23.6	23.3	24.7	24.4	22.9	23.9	23.0	24.0	23.6	24.4	24.2	23.7	24.1	23.8
X. budapestensis DSM 16342^T	27.1	26.5	51.1	ID	53.1	24.7	24.5	22.8	22.9	24.0	23.8	24.6	23.8	23.2	24.5	24.4	22.7	23.9	23.0	24.2	23.3	24.2	24.5	24.1	24.1	24.1
X. cabanillasii USTX62^T	26.4	27.2	49.8	53.1	ID	24.0	24.4	23.7	24.1	24.6	25.2	23.9	24.2	23.2	24.1	23.9	23.4	23.5	23.7	23.8	22.9	23.6	23.9	23.6	24.0	23.6
***Xenorhabdus* sp. XENO-1**	23.6	23.1	24.3	24.7	24.0	ID	71.2	23.1	23.2	24.8	24.2	25.5	25.7	24.7	25.0	25.1	23.0	24.5	24.1	24.5	23.8	24.0	24.6	24.1	24.3	24.3
X. bovienii T228^T	23.6	23.3	24.1	24.5	24.4	71.2	ID	23.3	23.2	25.1	24.7	26.0	25.7	24.6	25.1	25.1	23.3	24.7	24.3	24.6	24.0	23.9	24.7	24.2	24.5	24.4
X. szentirmaii DSM 16338^T	22.2	22.8	22.4	22.8	23.7	23.1	23.3	ID	26.4	25.7	26.4	24.1	26.3	25.0	24.7	24.5	24.2	24.2	24.6	24.4	23.9	24.0	24.2	24.2	24.3	24.3
X. mauleonii VC01^T	22.2	23.1	22.7	22.9	24.1	23.2	23.2	26.4	ID	26.5	26.4	24.2	26.6	24.9	25.2	25.1	24.3	24.7	24.9	24.9	24.3	24.4	24.8	24.7	24.7	24.7
X. koppenhoeferi USNJ01^T	23.2	23.2	23.8	24.0	24.6	24.8	25.1	25.7	26.5	ID	34.1	26.7	30.5	27.0	27.5	27.7	25.3	27.0	26.9	26.9	25.9	26.2	26.7	26.3	26.5	26.4
X. nematophila ATCC 19061^T	22.8	23.5	23.3	23.8	25.2	24.2	24.7	26.4	26.4	34.1	ID	26.1	29.1	26.2	26.8	26.9	25.6	26.0	26.3	26.4	25.3	25.6	25.9	25.6	26.3	25.8
X. hominickii KE01^T	23.5	22.7	24.2	24.6	23.9	25.5	26.0	24.1	24.2	26.7	26.1	ID	28.7	26.2	27.2	27.1	24.8	27.0	25.7	27.0	25.5	25.8	26.6	26.2	26.2	26.3
X. lircayensis VLS^T	22.9	23.1	23.6	23.8	24.2	25.7	25.7	26.3	26.6	30.5	29.1	28.7	ID	29.6	30.3	30.7	26.5	28.7	28.3	29.6	27.5	28.4	29.3	29.0	28.8	28.7
X. beddingii Q58^T	22.9	22.5	23.3	23.2	23.2	24.7	24.6	25.0	24.9	27.0	26.2	26.2	29.6	ID	31.8	31.4	25.2	27.0	26.6	27.3	26.1	26.9	27.0	26.7	26.9	27.0
X. miraniensis Q1^T	23.6	22.6	24.7	24.5	24.1	25.0	25.1	24.7	25.2	27.5	26.8	27.2	30.3	31.8	ID	53.6	25.5	27.9	26.8	28.5	27.0	27.7	27.9	27.9	27.8	27.8
X. khoisanae MCB	23.1	22.8	24.4	24.4	23.9	25.1	25.1	24.5	25.1	27.7	26.9	27.1	30.7	31.4	53.6	ID	25.4	27.7	26.8	28.2	27.0	28.8	27.8	27.8	27.6	27.7
X. poinarii G6^T	22.5	22.5	22.9	22.7	23.4	23.0	23.3	24.2	24.3	25.3	25.6	24.8	26.5	25.2	25.5	25.4	ID	28.8	27.5	29.1	26.7	27.1	27.6	27.7	27.6	27.4
X. vietnamensis VN01^T	23.5	22.7	23.9	23.9	23.5	24.5	24.7	24.2	24.7	27.0	26.0	27.0	28.7	27.0	27.9	27.7	28.8	ID	29.3	41.6	29.4	30.8	31.9	31.7	31.3	31.3
X. doucetiae FRM16^T	22.6	22.8	23.0	23.0	23.7	24.1	24.3	24.6	24.9	26.9	26.3	25.7	28.3	26.6	26.8	26.8	27.5	29.3	ID	30.6	30.9	31.8	32.5	32.1	31.8	31.9
X. japonica DSM 16522^T	23.4	22.5	24.0	24.2	23.8	24.5	24.6	24.4	24.9	26.9	26.4	27.0	29.6	27.3	28.5	28.2	29.1	41.6	30.6	ID	30.0	31.3	32.8	32.3	32.0	31.9
X. kozodoii SaV^T	22.3	22.0	23.6	23.3	22.9	23.8	24.0	23.9	24.3	25.9	25.3	25.5	27.5	26.1	27.0	27.0	26.7	29.4	30.9	30.0	ID	34.0	34.6	34.1	33.3	33.6
X. griffiniae BMMCB	22.5	22.3	24.4	24.2	23.6	24.0	23.9	24.0	24.4	26.2	25.6	25.8	28.4	26.9	27.7	28.8	27.1	30.8	31.8	31.3	34.0	ID	45.7	41.7	39.0	39.9
X. ehlersii DSM 16337^T	23.3	22.4	24.2	24.5	23.9	24.6	24.7	24.2	24.8	26.7	25.9	26.6	29.3	27.0	27.9	27.8	27.6	31.9	32.5	32.8	34.6	45.7	ID	53.7	46.3	48.0
X. thuongxuanensis 30TX1^T	22.5	22.3	23.7	24.1	23.6	24.1	24.2	24.2	24.7	26.3	25.6	26.2	29.0	26.7	27.9	27.8	27.7	31.7	32.1	32.3	34.1	41.7	53.7	ID	48.3	51.9
X. ishibashii GDh7^T	22.9	22.2	24.1	24.1	24.0	24.3	24.5	24.3	24.7	26.5	26.3	26.2	28.8	26.9	27.8	27.6	27.6	31.3	31.8	32.0	33.3	39.0	46.3	48.3	ID	51.2
X. eapokensis DL20^T	22.9	22.4	23.8	24.1	23.6	24.3	24.4	24.3	24.7	26.4	25.8	26.3	28.7	27.0	27.8	27.7	27.4	31.3	31.9	31.9	33.6	39.9	48.0	51.9	51.2	ID

Figure S3: Pairwise comparison of dDDH scores (%) of *Xenorhabdus* strains. A total of 1,719,910 nucleotide positions were used in the analyses. Accession numbers of gene sequences used are shown in Table S1 in Supplementary Material. dDDH, digital DNA–DNA Hybridization.