Nem1174

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

Agil KATUMANYANE[1,*], Antoinette P. MALAN[2], Louwrens R. TIEDT[3] and Brett P. HURLEY[1]

[1] *Department of Zoology and Entomology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa*

[2] *Department of Conservation Ecology and Entomology, Department of AgriSciences, Stellenbosch University, Private Bag X1, Matieland 7602, Stellenbosch, South Africa*

[3] *Laboratory for Electron Microscopy, North-West University, Potchefstroom Campus, Private Bag X6001, Potchefstroom 2520, South Africa*

Received: 1 May 2019; revised: 29 August 2019

Accepted for publication: 29 August 2019

*Corresponding author, e-mail: agil.katumanyane@fabi.up.ac.za

Summary - Two isolates of *Steinernema bertusi* n. sp. were separately recovered from Tito, Mpumalanga and Port Edward, Kwa Zulu Natal, South Africa. In this paper, we describe the isolates as a new entomopathogenic nematode (EPN) species using molecular and morphological methodologies. The new species belongs to the *cameroonense*-clade, which consists of nematodes only isolated from the African continent. *Steinernema bertusi* n. sp. is characterised by having the longest infective juvenile (IJ) for this clade at 716 (628-814) µm. The IJ is further characterised by a body diam. of 32 (28-36) µm and the pattern for the arrangement of the lateral ridges from head to tail is 2, 4, 5, 4, 2. The first-generation male spicule and gubernaculum length is 82 (72-88) µm and 63 (54-72) µm, respectively. Only 25% of the second generation males possess a mucron. The first-generation females of *S. bertusi* n. sp. have a slightly protruding vulva, with double-flapped epiptygmata and a mucron at the posterior end. The new EPN species is most closely related to *S. sacchari* and is the fifth species to be included in the *cameroonense*-clade.

Keywords - *cameroonense*-clade, molecular, morphology, morphometrics, new species, phylogeny, taxonomy.

Entomopathogenic nematodes (EPN) belonging to the order Rhabditida are known to parasitise various below- and above-ground insects (Grewal *et al.*, 2005; Lacey & Georgis, 2012). For this reason, they are used worldwide in biological control and integrated pest programs of various agricultural insect pests. EPN kill their insect hosts with the help of symbiotic bacteria, which they carry in their digestive system and sometimes in specialised bacterial chambers (Kaya *et al.*, 1993; Nobuyoshi, 2002). Death of the insect normally occurs within 48 h after infection. The advancement in *in vitro* mass production and formulation technology has allowed for a prolonged shelf life of EPN and ease of transportation over long distances. Thus, EPN are available on the market for export to different countries, in some of which, they are exempted from registration. The two families of EPN: Steinernematidae and Heterorhabditidae are easily commercialised, partly because of their ease of mass production (Shapiro-Ilan *et al.*, 2010).

In their review of EPN taxonomy, Hunt & Subbotin (2016) recognised 95 *Steinernema* and 16 *Heterorhabditis* species from around the world as being valid. In South Africa, the first EPN species description was in 2006 for *S. khoisanae* Nguyen, Malan & Gozel, 2006. To date, 13 EPN species have been described from South Africa, including recent descriptions of *S. beitlechemi* Çimen, Půža, Nermut', Hatting, Ramakuwela, Faktorová & Hazir, 2016, *S. biddulphi* Harum, Çimen, Vladimír & Půža, 2015, *S. fabii* Abate, Malan, Tiedt, Wingfield, Slippers & Hurley, 2016, and *S. nguyeni* Malan, Knoetze & Tiedt, 2016, as well as another six locally isolated species, already reported from other countries (Malan & Ferreira, 2017; Steyn *et al.*, 2017; Abate *et al.*, 2018). The discovery and description of new EPN species is important, because it allows to study the diversity among species, and the discovery of different genetic traits that can subsequently be used for genetic studies. As regards EPN as biocontrol agents of insect pests, it has also been determined that some EPN are highly host specific, thus discovering new EPN species will reassure more precise control EPN of specific insect pests in localised areas (Adams & Nguyen, 2002).

Spiridonov & Subbotin (2016) deduced from biogeographical analysis using *Steinernema* species descriptions up till 2015 that colonisation of the African continent occurred five times, with the resultant moderate diversity, for example in comparison to Asia, with the latter having repeated colonisation from other regions. They regarded the five ancestral colonisation events for Africa as: *i*) *S. citrae* Stokwe, Malan, Nguyen, Knoetze & Tiedt, 2011 (*feltiae*-clade); *ii*) *S. yirgalemense* Tallosi, Peters & Ehlers, 1995 (*bicornutum*-clade; *iii*) *S. tophus* Çimen, Lee, Hatting, Hazir & Stock, 2014, *S. innovationi* Çimen, Lee, Hatting, Hazir & Stock, 2014, *S. khoisanae* and *S. jeffreyense* Malan, Knoetze & Tiedt, 2015 (*khoisanae*-clade); *iv*) *S. karii*

Waturu, Hunt & Reid, 1997 and *S. ethiopiense* Tamiru, Waeyenberge, Hailu, Ehlers, Půža & Mráček, 2012 (*glaseri*-clade) to originate from Asia; and *v*) *S. cameroonense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 (*cameroonense*-clade) to have originated from the Americas. Since the publication of the book of Hunt & Nguyen (2016), new South African species have been added to four of the five clades, including *S. nguyeni* to the *feltiae*-clade, *S. biddulphi* to the *bicornutum*-clade, *S. fabii* to the *khoisanae*-clade, and *S. beitlechemi* to the *cameroonense*-clade.

In the current study, a new *Steinernema* species from South Africa, referred to as *S. bertusi* n. sp., is described using morphology and molecular characterisation. This will also be the twelfth *Steinernema* species described from South Africa, some 12% of the world species descriptions for this genus.

Materials and methods

EPN SOURCE

The first isolate, called Tito 13 (Mpumalanga: 27°33.49S 30°28.85E), of the new species of EPN was obtained through baiting from soil samples from a wattle forest plantation, using the last instar larvae of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae). This isolation of the EPN in 2016, was part of a larger survey for EPNs from commercial forestry areas in South Africa (Abate *et al*., 2018). After being molecularly identified using the ITS and D2-D3 region, the isolate culture was lost as it proved impossible to be successfully maintained on *Galleria* or *Tenebrio molitor* L., 1758 larvae. During a different survey by Steyn *et al*. (2017), the isolate WS22 (Natural forest reserve, Port Edward, KwaZulu-Natal: 30°59.699' 30°15.108'E) was identified using molecular techniques, as being identical to Tito 13 and was used for the molecular and morphological description of the new *Steinernema* as *S. bertusi* n. sp.

LIGHT MICROSCOPY

Individuals belonging to different growth stages of the nematode, which included the infective juvenile (IJ), first and second-generation males and females, were obtained for morphological observations using a light microscope. A sample of IJ was stored in vented tissue culture flasks (12°C) for a period of 2 months to obtain ex-sheathed IJ. Both temporary and

permanent slides were prepared for the different growth stages of the nematode. To obtain these stages, ten *G. mellonella* larvae were placed in Petri dishes lined with moist Whatmann filter paper and inoculated with 200 IJ per larva of *G. mellonella*. The plates were placed in a plastic container lined with wet paper towels (100% moisture), and kept in a growth chamber (MRC 358, Labotec), at 25°C for a period of 48 h. Dead larvae were removed after 48 h, rinsed with distilled water, placed in different Petri dishes and returned to the growth chamber. A few individuals were removed daily and dissected in Ringers solution to observe the stage of growth of the nematodes. First generation male and female adults were obtained after 4-5 days post infection, while second generation adults were obtained after 6-7 days.

The IJ were harvested by using a modified White trap (Woodring & Kaya, 1988). The different growth stages of the nematodes were permanently fixed in hot TAF (2% triethanolamine, 8% formalin in distilled water) at 85°C (Courtney *et al*., 1955). Specimens were then processed to pure glycerin, using the modified Seinhorst technique (Seinhorst, 1959), after which they were mounted in pure glycerin to obtain permanent slides. The individual nematodes on the permanent slides were used for the morphometric studies. Measurements and drawings were made by means of a Zeiss compound microscope (Zeiss Axio Scope A1), fitted with a digital camera, and computer with ZEN lite digital imaging software (ZEN black 2.3 SP1 / blue 2.6).

SCANNING ELECTRON MICROSCOPY (SEM)

For the SEM, the samples (first and second generation adults, fresh IJ and unsheathed IJ) were fixed in TAF. They were then 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.

DNA EXTRACTION

The protocols outlined in Nguyen (2007) were used to extract DNA from a single young female nematode. The lysis buffer consisted of 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}. The first generation female was placed in

30 µl drop of the lysis buffer pipetted on the upper side of a 0.5 ml microcentrifuge tube. The nematode was cut into a few pieces, using a sterile insulin needle and the contents were immediately placed on ice and transferred to –80°C for 20 min. For total lysis of the cells and digestion of the proteins, the tubes were incubated at 65°C for 1 h and at 95°C for 10 min in a thermocycler (GeneAmp 2720). The tube was cooled on ice and centrifuged at 11,600 *g* at 10°C for 2 min and 5 µl were pipetted from the supernatant and used in the PCR amplification.

PCR AMPLIFICATION

The protocols described in Nguyen (2007) for PCR amplification of both the ITS and the D2-D3 regions were followed. The ITS regions and D2-D3 fragment of the 28S region of the ribosomal DNA were amplified in a 25 µl reaction. The PCR primers used to amplify the ITS region were the 18S primer (5-TTGATTACGTCCCTGCCCTTT-3) and the 28S primer (5-TTTCACTCGCCGTTACTAAGG-3) as described by Vrain *et al.* (1992). The primers used to amplify the D2-D3 regions of 28S rDNA were the D2F (5-CCTTAGTAACGGCGAGTGAAA-3) in Nguyen *et al.* (2007), and 536 (5-CAGCTATCCTGAGGAAAC-3) in Stock *et al.* (2001). PCR amplifications were carried out in tubes containing 5 µl nematode lysate, together with 0.5 µm of each primer and 12.5 µl KAPA2G™ Robust Hotstart ReadyMix (KAPA Biosystems). 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, with all conditions being 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 done 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). The ITS primers 18S and 28S, and additional two internal primers, KN58 (5-GTATGTTTGGTTGAAGGTC-3) and KNRV (5-CACGCTCATACAACTGCTC-3), suggested by Nguyen *et al*. (2007), were used for the sequencing of the complete ITS region. Likewise, primers 502 (5-CAAGTACCGTGAGGGAAAGTTGC-3) and 503 (5-CCTTGGTCCGTGTTTCAAGACG-3), reported by Stock *et al.* (2001) were used for sequencing of the D2-D3 regions.

SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS

Sequences were assembled, analysed and edited using the CLC DNA main Workbench ver. 8.1. The sequences of both the ITS region of the rDNA gene and that of the D2-D3 region of the 28S gene were aligned and compared with other *Steinernema* species sequences available on GenBank (NCBI). Sequence alignment was done using ClustalX 2.1 (Thompson *et al.*, 1997), while phylogenetic analyses of sequence data were done using the Maximum Parsimony (MP) method in MEGA5 (Tamura *et al.*, 2011). Support for tree branches was evaluated statistically by means of a bootstrap analysis based on 1000 re-samplings 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 (ten replicates). *Caenorhabditis elegans* (FJ589008) was used as outgroup during the calculation of the trees based on the ITS sequences, while *Cervidellus alutus* (AF331911) was used as outgroup for the calculation of the tree based on the D2-D3 sequences.

Results

***Steinernema bertusi*[*] n. sp.**

= isolate WS22 of Steyn *et al.* (2017)

=isolate Tito 13 of Abate *et al.* (2018)

(Figs 1-4)

MEASUREMENTS

See Tables 1-4.

DESCRIPTION

First generation male

Body curved ventrally posteriorly. Body J-shaped when heat relaxed. Cuticle smooth under light microscope, but striations visible with SEM. Head rounded, two amphidial apertures

[*] Patronym in honour of Albertus J. Meyer, or 'Bertus' as he was affectionately known, a pioneer of Nematology in South Africa.

observed. Posterior part of stoma funnel shaped. Testis monorchic, reflexed posteriorly. Head rounded with four cephalic papillae and six labial papillae. Pharynx with cylindrical procorpus, metacorpus slightly swollen, excretory pore located anterior to nerve ring and close to metacorpus. Testis reflex variable. Each spicule with two internal ribs, narrow velum running from rostrum to spicule tip. Gubernaculum boat-shaped in lateral view, anterior end curved. Copulatory papillae totalling 21 and comprising a single precloacal, midventral papillae and ten pairs arranged as: six pairs precloacal subventral, one pair adcloacal, one pair lateral and two pairs subterminal. Anal body diam. larger than tail length, no mucron observed in first generation males.

Second generation male

Six labial papillae more prominent than cephalic papillae, which occur further back on head. Labial region less rounded and continuous with body shape. Body more slender than first generation, with smaller spicules and gubernaculum. Mucron observed in 25% of individuals.

First generation female

Body stout, C-shaped when heat-relaxed and when fixed with TAF. Body cuticle smooth under light microscope, but faint annules observed under SEM. Labial region rounded and continuous with body, six labial papillae and four cephalic papillae. Cheilorhabdions large, well sclerotised. Stoma prominent, posterior part funnel shaped. Amphidial apertures not observed. Pharynx with cylindrical procorpus, metacorpus slightly swollen, isthmus distinct, basal bulb enlarged. Nerve ring surrounding isthmus and just anterior to basal bulb. Excretory pore anterior to nerve ring in mid-pharynx region. Cardia prominent. Gonads amphidelphic, reflexed. Vulva a median transverse slit, slightly protruding from body surface, situated in mid-body region, with long double-flapped epiptygmata. Tail length shorter than body anal diam. Tail terminus short and tapering with mucron.

Second generation female

Morphology much like first generation but body much shorter and smaller. Tail tapering, no mucron observed.

Infective juvenile

Cuticle (second-stage cuticle) present but lost in storage. Cuticle marked with prominent transverse striations. Body elongate and tapering posteriorly at both ends. Body slightly curved from middle towards both ends when heat relaxed. Visible striations on body under light microscope. Ensheathed juvenile with six labial and four cephalic papillae. Cephalic region smooth and continuous with body. Amphidial apertures prominent. Pharynx with a thin corpus and a slightly swollen metacorpus. Excretory pore located at mid-pharynx level. Excretory pore anterior to nerve ring. Bacterial chamber prominent and located in anterior intestine. Lateral field beginning anteriorly with two ridges and forming the following formula: 2, 4, 5, 4, 2. Portion with five ridges forming greatest part of lateral field. Ridges more prominent towards posterior end up to end of tail.

TYPE HOST AND LOCALITY

Natural host unknown. The isolate was collected by baiting with larvae of *Galleria mellonella*, although this was not a preferred host as the population declined after each re-cycling. Isolated from the Natural Forest Reserve in Port Edward, KwaZulu-Natal: 30°59.699'S 30°15.108'E (Steyn *et al*., 2017).

TYPE MATERIAL

Holotype first generation male, paratype males and paratype females and IJ are deposited in the National Collection of Nematodes, Biosystematics Division, Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa. Accession numbers NCN50711-NCN50728 are allocated to the slides containing the first generation male holotype and first and second generation male and female paratypes. Additionally, vials containing first and second generation adults and IJ fixed in TAF are deposited under accession numbers NCN39413-NCN39417. Other samples have been sent to the United States Department of Agriculture Nematode Collection (USDANC), Beltsville, MD, USA, and we await accession numbers.

DIAGNOSIS AND RELATIONSHIPS

Steinernema bertusi n. sp. is characterised by differences in the morphology and the morphometrics of the IJ and adults, as shown in Tables 1-4. The IJ of the new species can be recognised by a body length of 716 (628-814) µm, a body diam. of 32 (28-36) µm and a tail length of 66 (58-74) µm. The lateral field ridges corresponds to a 2, 4, 5, 4, 2 pattern. The first-generation male has a spicule and gubernaculum of 82 (72-88) µm and 63 (54-72) µm, respectively, and lacks a mucron. The second-generation males have similar morphological characters to the first generation, except only 25% of the tails have a mucron. The first-generation females of *S. bertusi* n. sp. have a slightly protruding vulva, with double-flapped epiptygmata and a mucron at the posterior end. The first generation female tail is shorter than the anal body diam. and possesses a mucron. The new species has the longest IJ body length of the six EPN species in the *cameroonense*-clade. Of the *cameroonense*-clade, only *S. bertusi* n. sp. and *S. sacchari* have no mucron in the first generation males.

Steinernema bertusi n. sp. differs from *S. nyetense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 by having a longer IJ body length of 716 (628-814) *vs* 648 (565-708) µm, and a slightly longer mean pharynx length of 123 (112-137) *vs* 114 (104-128) µm, although the ranges overlap. The first-generation male of *S. bertusi* n. sp. has a longer mean gubernaculum length of 63 (54-72) *vs* 53 (40-62) µm (Fig. 2; Tables 3, 4), although the ranges overlap, a tail mucron is lacking *vs* present, the spicules are brown with a narrow velum *vs* yellowish-brown spicule with a large velum, and the genital papillae have a 20 + 1 arrangement *vs* 22 + 1 (Table 4). The tail of the first generation female of *S. bertusi* n. sp. is dome shaped *vs* conical and pointed (Table 4).

The IJ of *S. bertusi* n. sp. differs from *S. cameroonense* by a longer body length of 716 (628-814) *vs* 622 (490-694) µm (Table 2). Although the IJ is longer, the relative position of the excretory pore and the nerve ring of *S. bertusi* n. sp. is similar to that of *S. cameroonense* at 59 (53-65) *vs* 54 (45-64) µm and 91 (76-110) *vs* 85 (69-100) µm, respectively (Table 2). The spicule and gubernaculum of the first-generation male of *S. bertusi* n. sp. are longer at 82 (72-88) *vs* 69 (51-85) µm and 63 (54-72) *vs* 45 (37-57) µm, respectively (Table 3). The genital papillae of first generation males of *S. bertusi* n. sp. have a 20 + 1 arrangement *vs* 22 + 1 (Table 4) and the first-generation male of *S. bertusi* also differs from that of *S. cameroonense* in lacking a mucron *vs* present (Tables 3, 4). First generation females of *S. bertusi* n. sp. have a dome-shaped *vs* conoid tail (Table 4).

Steinernema bertusi n. sp. differs from *S. sacchari* Nthenga, Knoetze, Berry, Tiedt & Malan, 2014 by its longer IJ length of 716 (628-814) *vs* 680 (630-722) µm (Table 2). The spicules of the first generation males of *S. bertusi* n. sp. do not have prominent rostrum whereas it is so in *S. sacchari* (Table 4). The males of *S. bertusi* n. sp. are also narrower in body diam. at 116 (84-152) *vs* 145 (86-205) µm (Table 3). The genital papillae of *S. bertusi* n. sp. are in a 20 + 1 (Fig. 2C, D) arrangement *vs* 24 + 1. The vulva of *S. bertusi* n. sp. is slightly protruding, while that of *S. sacchari* does not protrude (Table 4).

Steinernema bertusi n. sp. differs from *S. fabii* by the longer IJ at 716 (628-814) *vs* 641 (590-697) µm (Table 2). The first generation males of *S. bertusi* n. sp. lack a mucron *vs* present, the spicules are shorter at 82 (72-88) *vs* 90 (79-106) µm, and the body diam. is slightly narrower at 116 (84-152) *vs* 138 (102-196) µm (Table 3). The genital papillae of *S. bertusi* n. sp. first generation males have a 20 + 1 arrangement in *vs* 22 + 1. The first generation females of *S. bertusi* n. sp. and *S. fabii* have double-flapped epiptygmata, although the epiptygmata are more protruding in *S. fabii* than in *S. bertusi* n. sp. (Table 4).

Steinernema bertusi n. sp. differs from *S. beitlechemi* by the IJ having the lateral field comprising 2, 4, 5, 4, 2 ridges *vs* 2, 5, 6, 5, 2 (Table 4). The first generation males of *S. bertusi* n. sp. lack a mucron *vs* present, the spicules are shorter at 82 (72-88) *vs* 93 (88-110) µm (Table 3), and the genital papillae have a 20 + 1 arrangement *vs* 22 + 1. The first generation females of *S. bertusi* n. sp. have slightly protruding double-flapped epiptygmata *vs* small and indistinct epiptygmata (Table 4).

MOLECULAR CHARACTERISATION

Steinernema bertusi n. sp. clustered with *S. nyetense*, *S. sacchari*, *S. cameroonense*, *S. sacchari*, *S. fabii*, and *S. beitlechemi* in both the ITS and D2-D3 phylogenetic analyses. All these species belong to the *cameroonense*-clade, a clade only reported from the African continent. According to Spiridonov & Subbotin (2016), the ancestors of the *cameroonense*-clade have their origins in the Americas.

Steinernema bertusi n. sp. is characterised genetically by the sequences of the ITS (ITS1 + 5.8S + ITS2) (KY082902) and the D2-D3 (KY08290) rDNA regions (Figs 5, 6). Pairwise distances using the ITS region show that the new species differs from its closest relatives *S. nyetense* by 16 bp and *S. cameroonense* by 26 bp and from *S. sacchari* by 28 bp, while differing from all other closely related species by larger differences in bp (Table 5). Pairwise comparison

using the D2-D3 regions is presented in Table 6. The bp difference between *S. bertusi* n. sp. and its closest relative *S. sacchari* was 4 bp and 6 bp between *S. beitlechemi*, *S. cameroonense*, *S. fabii*, and *S. nyetense*. This small bp difference can be attributed to the conserved nature of the 28S (D2-D3) region of EPN.

Conclusion

According to molecular and morphological observations, evidence is presented to confirm *S. bertusi* n. sp. as a new EPN species from South Africa. The species belongs to the *cameroonense*-clade, with its origin proposed to be from the Americas (Spiridonov & Subbotin, 2016). The IJ of *S. bertusi* n. sp. has the longest body length of all the described species in this clade. The first generation males of *S. bertusi* n. sp. have a long spicule and gubernaculum, with no mucron, while 25% of the second generation males have a mucron. The first-generation females of *S. bertusi* n. sp. have a slightly protruding vulva, with double-flapped epiptygmata and with a mucron at the posterior end. Molecularly it differs in the ITS region with 16 bp and 26 bp from its closest relatives, *S. nyetense* and *S. cameroonense* respectively. This new description adds a fifth species to the *cameroonense*-clade and becomes the fourteenth EPN descried from South Africa.

Acknowledgements

The authors wish to thank the Members of the Tree Protection Cooperative Programme (TPCP) National Research Foundation of South Africa (TP14062571871 and ITR150119112367) for the financial support made available during the study.

References

Abate, B., Malan, A.P., Tiedt, R.L., Wingfield, M.J., Slippers, B. & Hurley, B.P. (2016). *Steinernema fabii* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. *Nematology* 18, 235-255. DOI: 10.1163/15685411-00002956

Abate, B., Slippers, B., Wingfield, M.J., Malan, A.P. & Hurley, B.P. (2018). Diversity of entomopathogenic nematodes and their symbiotic bacteria in south African plantations and indigenous forests. *Nematology* 20, 355-371. DOI: 10.1163/15685411-00003144

Adams, B.J. & Nguyen, K.B. (2002). Taxonomy and systematics. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 1-33.

Çimen, H., Půža, V., Nermuť, J., Hatting, J., Ramakuwela, T., Faktorová, L. & Hazir, S. (2016). *Steinernema beitlechemi* n. sp., a new entomopathogenic nematode (Nematoda: Steinernematidae) from South Africa. *Nematology* 18, 439-453. DOI: 10.1163/15685411-00002968

Courtney, W.D., Polley, D. & Miller, V.I. (1955). TAF, an improved fixative in nematode technique. *Plant Disease Reporter* 39, 570-571.

Grewal, P.S., Koppenhöfer, A.M. & Choo, H.Y. (2005). Lawn, turfgrass and pasture applications. In: Grewal, P.S., Ehlers, R.-U. & Shapiro-Ilan, D.I. (Eds). *Nematodes as biocontrol agents*. Wallingford, UK, CABI Publishing, pp. 115-146.

Hunt, D.J. & Subbotin, S.A. (2016). Taxonomy and systematics. In: Hunt, D.J. & Nguyen, K.B. (Eds) (2016). *Advances in entomopathogenic nematode taxonomy and phylogeny*. *Nematology Monographs and Perspectives, 12* (Series Editors: Hunt, D.J. & Perry, R.N.). Leiden, The Netherlands, Brill, pp. 13-58.

Kanga, F.N., Trinh, Q.P., Waeyenberge, L., Spiridonov, S.E., Hauser, S. & Moens, M. (2012). Two new species of *Steinernema* Travassos, 1927 from the humid forest of southern Cameroon. *Russian Journal of Nematology* 20, 15-36.

Kaya, K.H., Bedding, A.R. & Akhurst, J.R. (1993). An overview of insect-parasitic and entomopathogenic nematodes. In: Bedding, A.R., Akhurst, J.R. & Kaya, K.H. (Eds). *Nematodes and the biological control of insect pests*. Melbourne, Australia, CSIRO Publications, pp. 1-15.

Lacey, L.A. & Georgis, R. (2012). Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. *Journal of Nematology* 44, 218-225.

Ma, J., Chen, S., De Clercq, P., Han, R. & Moens, M. (2012). *Steinernema changbaiense* sp. n. (Rhabditida: Steinernematidae), a new species of entomopathogenic nematode from Northeast China. *Russian Journal of Nematology* 20, 97-112.

Malan, A.P. & Ferreira, T. (2017). Entomopathogenic nematodes. In: Fourie, H., Spaull, V.W., Jones, R.K., Daneel, M.S. & De Waele, D. (Eds). *Nematology in South Africa: a view from the 21st century*. Cham, Switzerland, Springer International Publishing, pp. 459-480.

Nguyen, K.B. (2007). Methodology, morphology and identification. In: Nguyen, K.B. & Hunt, D.J. (Eds). *Entomopathogenic nematodes: systematics, phylogeny and bacterial symbionts*.

Nematology Monographs and Perspectives, 5 (Series Editors: Hunt, D.J. & Perry, R.N.). Leiden, The Netherlands, Brill, pp. 59-119. DOI: 10.1163/ej.9789004152939.i-816.25

Nguyen, K.B., Tesfamariam, M., Gozel, U., Gaugler, R. & Adams, B.J. (2004). *Steinernema yirgalemense* n. sp. (Rhabditida: Steinernematidae) from Ethiopia. *Nematology* 6, 839-856. DOI: 10.1163/1568541044038605

Nobuyoshi, I. (2002). Behaviour of entomopathogenic nematodes. In: Lee, D.L. (Ed.). *The biology of nematodes*. London, UK, Taylor & Francis, pp. 511-520.

Nthenga, I., Knoetze, R., Berry, S., Tiedt, L.R. & Malan, A.P. (2014). *Steinernema sacchari* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. *Nematology* 16, 475-494. DOI: 10.1163/15685411-00002780

Phan, L.K., Subbotin, S.A., Waeyenberge, L. & Moens, M. (2005). A new entomopathogenic nematode, *Steinernema robustispiculum* n. sp. (Rhabditida: Steinernematidae), from Chumomray National Park in Vietnam. *Systematic Parasitology* 60, 23-32. DOI: DO00001373

Phan, L.K., Takemoto, S. & Futai, K. (2006). *Steinernema ashiuense* sp. n. (Nematoda: Steinernematidae), a new entomopathogenic nematode from Japan. *Nematology* 8, 681-690. DOI: 10.1163/156854106778877901

Seinhorst, J.W. (1959). A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. *Nematologica* 4, 67-69.

Shapiro-Ilan, D., Byron, G. & Gaugler, R. (2010). Nematodes (Rhabditida: Steinernematidae and Heterorhabditidae). Online at: https://biocontrol.entomology.cornell.edu/pathogens/nematodes.php (accessed 10 September 2018).

Spiridonov, S.E. & Subbotin, S.A. (2016). Phylogeny and phytogeography of *Heterorhabditis* and *Steinernema*. In: Hunt, D.J. & Nguyen, K.B. (Eds). *Advances in entomopathogenic nematode taxonomy and phylogeny*. *Nematology Monographs and Perspectives, 12* (Series Editors: Hunt, D.J. & Perry, R.N.). Leiden, The Netherlands, Brill, pp. 413-427.

Spiridonov, S.E., Waeyenberge, L. & Moens, M. (2010). *Steinernema schliemanni* sp. n. (Steinernematidae; Rhabditida) – a new species of steinernematids of the 'monticolum' group from Europe. *Russian Journal of Nematology* 12, 175-190.

Steyn, W.P, Malan, A.P., Daneel, M.S. & Slabbert, R.M. (2017). Entomopathogenic nematodes from north-eastern South Africa and their virulence against the false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae). *Biocontrol Science and Technology* 27, 1265-1278.

Stock, S.P., Choo, H.Y. & Kaya, H.K. (1997). An entomopathogenic nematode, *Steinernema monticolum* sp. n. (Rhabditida: Steinernematidae) from Korea with a key to other species. *Nematologica* 43, 15-29.

Stock, S.P., Campbell, J.F. & Nadler, S.A. (2001). Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology* 87, 877-889.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, N. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28, 2731-2739. DOI: 10.1093/molbev/msr121

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876-4882. DOI: 10.1093/nar/25.24.4876

Vrain, T.C., Wakarchuck, D.A., Lèvesque, A.C. & Hamilton, R.I. (1992). Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563-573.

Woodring, J.L. & Kaya, H.K. (1988). *Steinernematid and heterorhabditid nematodes: a handbook of biology and techniques. Southern Cooperative Bulletin 331*. Fayettville, USA, Arkansas Agricultural Experiment Station.

List of figures

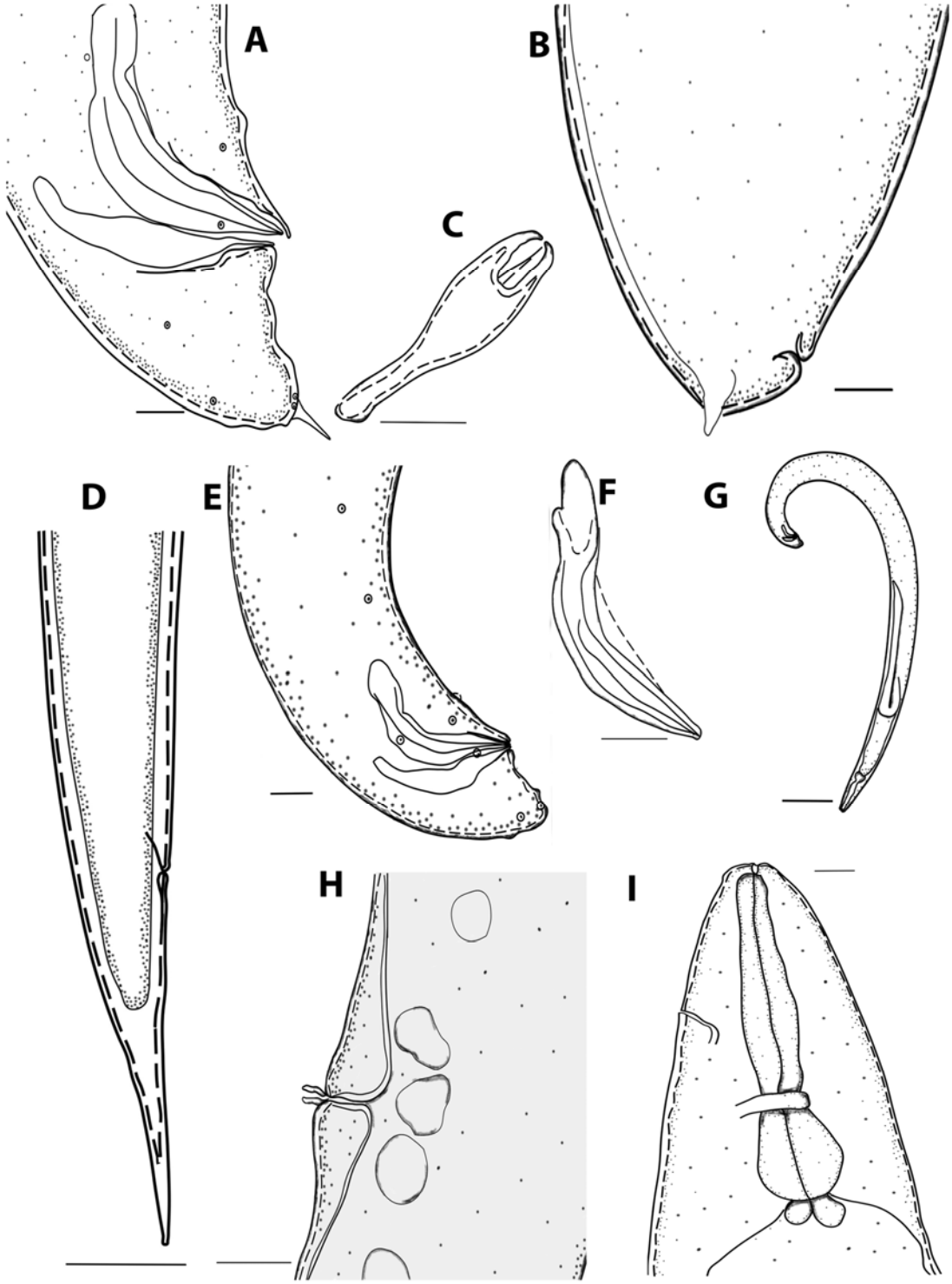

Fig. 1. *Steinernema bertusi* n. sp. A: Tail region of second generation male. Female: B, H, I. B: Tail region; H: Vulva and epiptygmata; I: Anterior region. Infective juvenile. D: Tail region. First generation male: C, E-G. C: Gubernaculum; E: Tail region; F: Spicule; G: Body shape. (Scale bars: A = 10 µm; B, D, E, I: 20 µm; C, F, H: 1000 µm; G: 200 µm.)

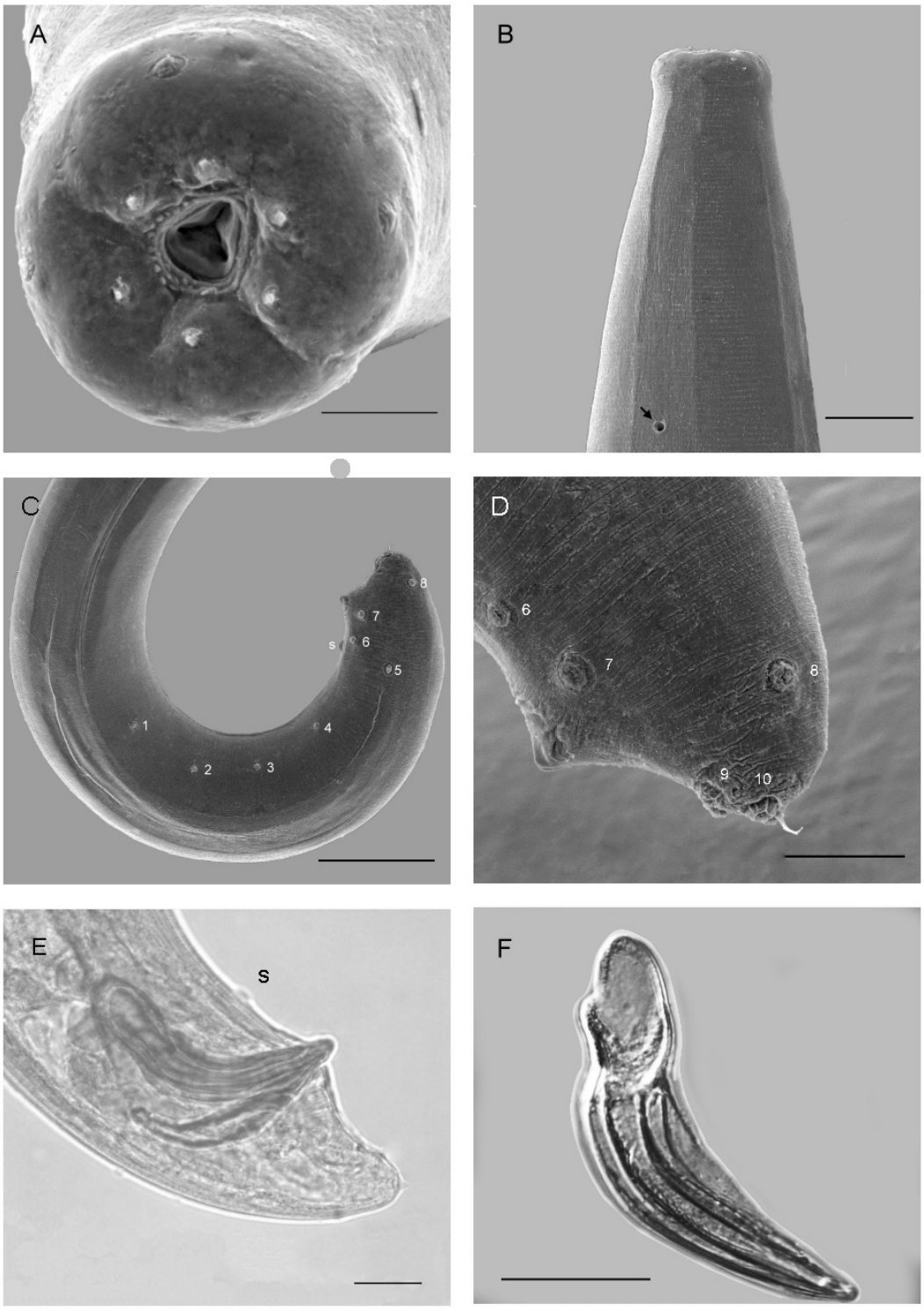

Fig. 2. Adult male of *Steinernema bertusi* n. sp. A: *En face* view showing four cephalic papillae and six labial papillae; B: Excretory pore (arrow); C: First generation male tail region showing papillae (numbered) and midventral papilla (S); D: Second generation tail region showing shape of tail and papillae (numbered); E: Tail region of second generation male (midventral papilla = S); F: Spicule. (Scale bars: A = 10 µm; B, D-F= 20 µm; C = 100 µm.)

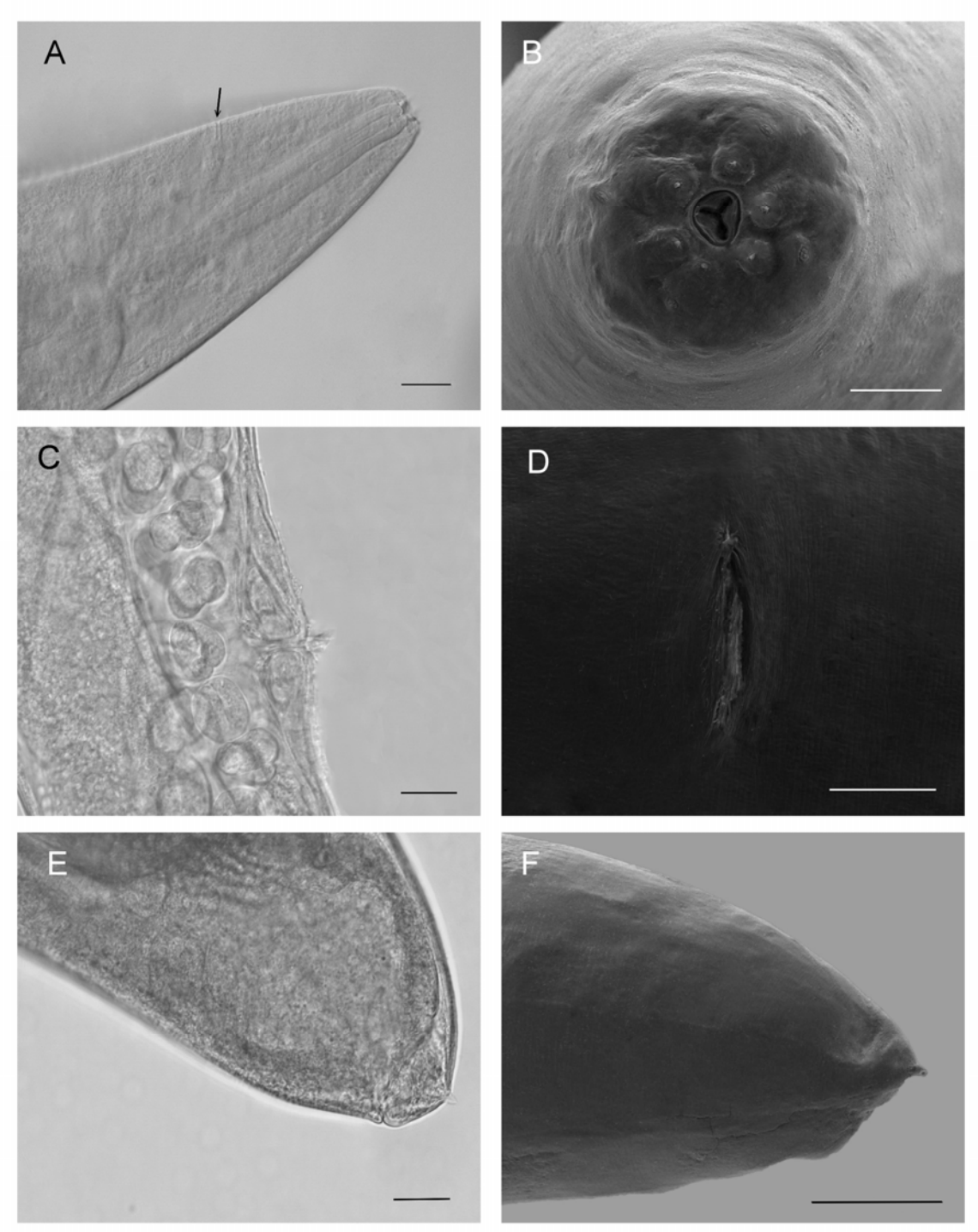

Fig. 3. Adult female of *Steinernema bertusi* n. sp. A: Pharyngeal region showing mouth and excretory pore (arrow); B: *En face* showing four cephalic papillae and six labial papillae; C, D: Vulva with double-flapped epiptygmata; E, F: Anal region of first generation female showing mucron. (A, C, E = 50 µm; B, D, F = 20 µm.)

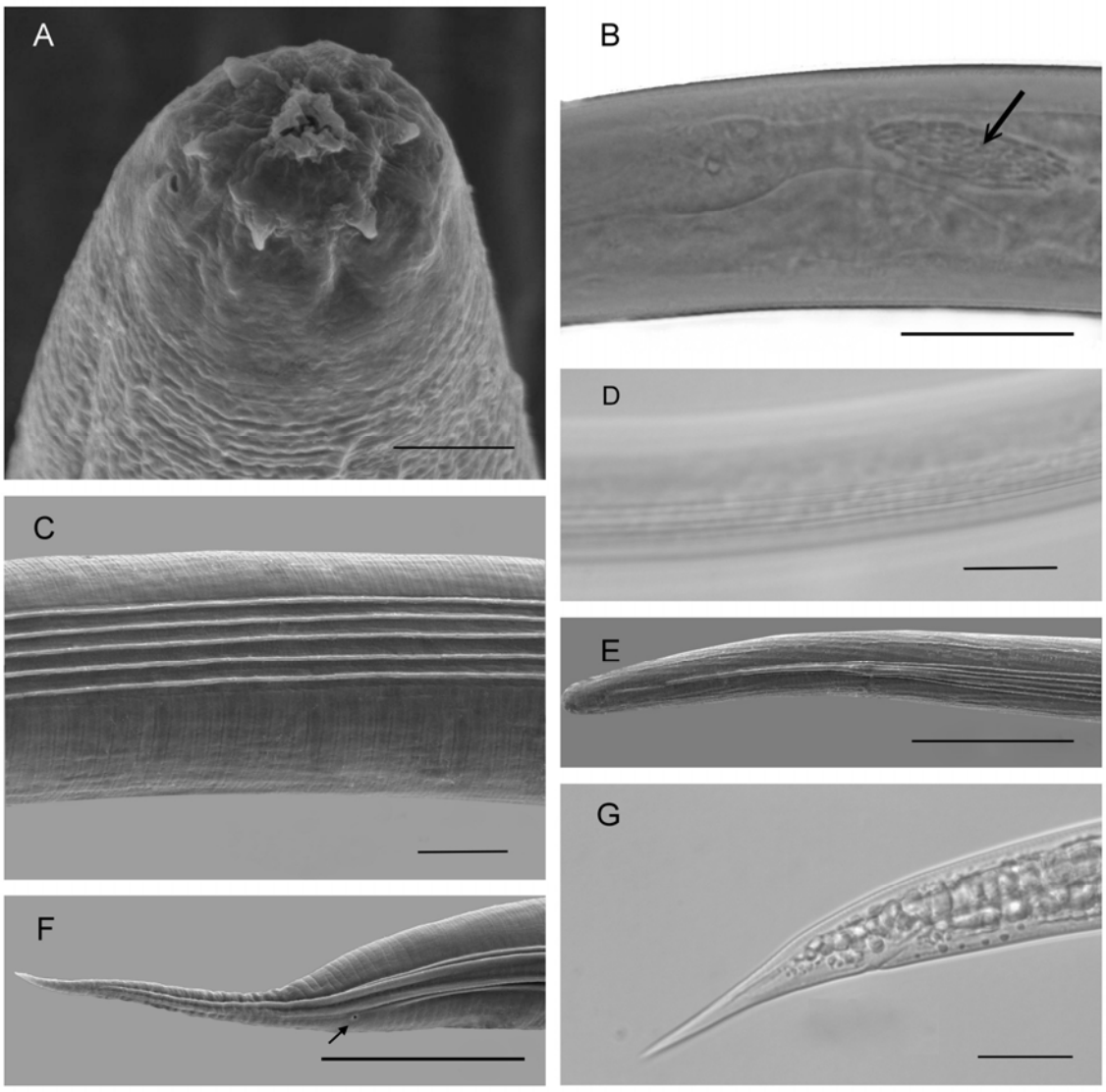

Fig. 4. Infective juvenile of *Steinernema bertusi* n. sp. A: *En face* view; B: Bacterial chamber (arrow); C-E: Lateral field; F, G: Tail region showing excretory pore (arrow). (A = 5 µm, B, C, D, F, G = 20 µm, E = 50 µm.)

List of tables