Steinernema schliemanni sp. n. (Steinernematidae; Rhabditida) – a new species of steinernematids of the '*monticolum'* group from Europe

Sergei E. Spiridonov[1], Lieven Waeyenberge[2] and Maurice Moens[2,3]

[1] Centre of Parasitology of A.N. Severtsov Institute of Ecology and Evolution of Russian Academy of Sciences, Leninskii prospect, 33, Moscow, 119071, Russia
e-mail: s_e_spiridonov@rambler.ru
[2]Institute of Agriculture and Fisheries Research, Burg. Van Gansberghelaan 96, 9820 Merelbeke, Belgium;
[3]Laboratory of Agrozoology, Department of Crop Protection, Ghent University, Coupure Links 653, 9000 Ghent, Belgium.

Accepted for publication 28 August 2010

Summary. *Steinernema schliemanni* sp. n. found on the surface of a cadaver of *Osmoderma ceremita* (Coleoptera) in Germany in April 2003 is described. The new species belongs to the group of species related to *S. monticolum* ('*monticolum'*-group). It differs from the other species of this group in the structure of the lateral field of infective juveniles, *viz.* 8 equal ridges at mid-body in *S. schliemanni* sp. n. *vs* unequal ridges in *S. monticolum* and *S. robustispiculum* and five ridges in *S. ashiuense*. Infective juveniles of *S. schliemanni* sp. n. are longer (842-1008 μm) than those of all other species of the '*monticolum'* group. *Steinernema schliemanni* sp. n. differs from the other species of the '*monticolum'*-group in at least 115 positions of the ITS rDNA sequence (18%). The nucleotide difference of the D2D3 region of the LSU rDNA between *S. schliemanni* sp. n. and the closest species, *S. monticolum*, is 22 bp (3.9% of the compared sequences), which is higher than differences between species such as *S. feltiae, S. kraussei* and *S. oregonense*. The 16S rDNA sequence of the bacterium associated with *S. schliemanni* sp. n. demonstrates close relationships with several *Xenorhabdus* species; the nucleotide difference is the lowest with *X. hominickii* (28 bp or 2%), isolated from *S. monticolum*.

Key words: bacterial symbionts, molecular phylogeny, rDNA, SEM, Steinernematids, taxonomy.

The geographical distribution of at least some species of entomopathogenic steinernematid nematodes does not demonstrate clear patterns. When some cosmopolite species, like *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982, can be found in a wide variety of habitats, the 'endemism' of other steinernematids might be explained by inadequate sampling. For example, *S. glaseri* (Steiner, 1929) Wouts, Mráček, Gerdin & Bedding, 1982, was described as a characteristic component of North American soil ecosystems but was later also reported from continental Europe, Iran and the Azores (Rosa *et al*., 2000; Ansari *et al*., 2005; Karimi *et al*., 2009). Some groups of steinernematid species demonstrate more or less pronounced patterns of geographical distribution. *Steinernema monticolum* Stock, Choo & Kaya, 1997 was described from Korea (Stock *et al*., 1997) and later reported from Japan (Kuwata *et al*., 2006). Two related species, *S. robustispiculum* Phan, Subbotin, Waeyenberge & Moens, 2005 and *S. ashiuense* Phan, Takemoto & Futai, 2006, were described from Vietnam and Japan, respectively (Phan *et al*., 2005, 2006). These three species can be considered as forming a separate evolutionary line or sub-clade in the genus *Steinernema* ('*monticolum*' group within the '*feltiae-kraussei-oregonense*' group). A strain closely resembling *S. ashiuense* was found during a survey in the south of the Russian Far East (Vladivostok area), whereas another strain similar to *S. monticolum* was reported from Kamchatka peninsula (Spiridonov & Moens, 2005). Thus, until now all species and strains of the '*monticolum'* group were only reported from Eastern Asia.

In 2003 a larva of the genus *Osmoderma* Lepeletier & Serville, 1828 (Coleoptera) naturally infected with entomopathogenic nematodes (EPNs) was found on the soil surface in Mecklenburg (North-East Germany). The cadaver with nematodes

was sent by Prof. Walter Sudhaus to the BBA Institute of Nematology in Münster, where a living culture was established on *Galleria mellonella* L. The morphology of these nematodes was studied under both the light and scanning electron microscope and sequences of the ITS rDNA and the D2D3 region of the LSU rDNA were obtained through direct sequencing or cloning of corresponding PCR-products with consecutive sequencing. It was found that the strain belonged to the '*monticolum'* group and constituted a new *Steinernema* species. The morphology, morphometrics and molecular features of this European representative of the '*monticolum'* group are herein described.

MATERIAL AND METHODS

Nematode culture and fixation. The juvenile suspension obtained in 2003 in Münster was used for re-inoculation of *Galleria* last-instar larvae. Initially, a stable laboratory culture could only have been established through injections in last-instar *Galleria* larvae. The nematodes obtained were used to infect *Galleria* caterpillars in Petri dishes lined with moist filter paper. Since then the nematodes were kept alive in *G. mellonella*. Adult stages of nematodes were recovered from insect cadavers at 3 (first generation) and 7 days (second generation) post inoculation. They were fixed in hot formaldehyde (4-6% formaline) and processed into glycerol for morphological study (Seinhorst, 1959). Drawings and measurements were made using a Karl Zeiss Jenaval light microscope.

Cross breeding. For cross-breeding experiments the nematodes were cultured in blood drops of *Galleria* haemolymph (Nguyen & Duncan, 2002). Only *S. monticolum* was available for cross-breeding tests. In a first experiment pairs of infective juveniles of both species were formed by carrying two juveniles into the hanging drop. A total of 60 pairs included 20 pairs of juveniles from both species, and 20 pairs of conspecific juveniles of each species. In a second experiment fourth-stage juveniles were dissected from *Galleria* cadavers and after identification of their sex were transferred to hanging drops (n=32). Conspecific pairs were also established (n=25). Twenty five single forth-stage female juveniles were also cultured in drops for each species. The formation of first- and second-stage juveniles in haemolymph or inside females was considered as positive results of mating.

SEM study. For scanning electron microscopy the nematodes were fixed in 4-6% formalin, dehydrated in ethanol solutions with gradient concentrations (30%, 50%, twice 96% 1 h each at room temperature). They were critical point dried with liquid CO_2 (HCP-2 HITACHI), mounted on aluminium stubs, coated with gold (BIO-RAD SC502) and studied using a scanning electron microscope JEOL-100 CX. Spicules and gubernacula were prepared as suggested by Nguyen and Smart (1994).

Molecular observations. DNA was obtained in two ways. Single juveniles were cut in two parts which were transferred to 8 µl of worm-lysis buffer (100 mM KCl, 20 mM Tris-HCl pH 8.3, 3 mM MgCl2, 2 mM DDT and 0.9% Tween 20). This mixture was incubated for 1 h under 65ºC followed by 10 min at 95ºC. Alternatively, suspensions of living juveniles and adults were washed several times with sterile water through centrifugation in 1.5 ml Eppendorf tubes. DNA was then extracted with the use of columns from Wizard® SV Genomic DNA Purification System according to the producer's protocol (Promega Benelux, Leiden, The Netherlands).

Either 1-4 µl of the homogenate in worm-lysis buffer or 1-2 µl of DNA obtained from Wizard® columns, were used as template in a PCR reaction. For amplification of ITS rDNA two primer pairs were used: 18S (5'-TTG ATT AGG TCC CTG CCC TTT-3') and 26S (5-TTT CAC TCG CCG TTA CTA AGG-3') (Vrain *et al.*, 1992), or TW81 (5'-GTT TCC GTA GGT GAA CCT GC-3') and AB28 (5'-ATA TGC TTA AGT TCA GCG GGT-3') (Curran & Driver, 1994). For amplification of the D2D3 region of the LSU rDNA the pair of primers D2A (5'-ACA AGT ACC GTG AGG GAA AGT -3') and D3B (5'-TGC GAA GGA ACC AGC TAC TA-3') was used (He *et al*., 2005). The PCR cycling parameters included a primary denaturation step at 94ºC for 5 min followed by 34 cycles of 94°C for 1 min, 55°C (for amplification of ITS rDNA) or 50°C (for amplification of D2D3 region of LSU rDNA) for 1 min and 72°C for 1 min, followed by a post-amplification extension step at 72°C for 6 min. Following electrophoresis (100V, 10mA) on a 0.8% agarose gel, PCR products were cleaned using Promega® Wizard SV Gel and PCR Clean-Up System, ligated into pGEM-T vector (both Promega Benelux) and used to transform JM 109 *Escherichia coli* competent cells (Promega Benelux) according to Promega protocols and application guide, third edition. Transformed colonies were subcultured in 1.5 ml of LB medium and incubated 16-22 h at 37°C. Plasmid DNA was obtained from transformed *E. coli* using Promega® SV Miniprep DNA purification system (Promega Benelux), according to the manufacturer's instructions and sent for

sequencing by commercial company with vector primers T7 and SP6.

Symbiotic bacteria. Symbiotic bacteria were isolated from a suspension of infective juveniles after 2 h incubation in 0.1% mertiolate. Surface-sterilised juveniles were homogenized in a glass micro-mortar; the mortar content was spread over the surface of NBTA agar (Koppenhöfer, 2007). Bacteria from blue colonies were transferred to LB broth, which was shaken during 2-3 days at room temperature. After the centrifugation of the bacterial culture (2000 *g*, 5 min), the sediment of bacterial cells was transferred to 500 µl of lysis buffer (10mM Tris-HCl, pH: 8.0; 1mM EDTA; 1% Triton X-100) and incubated at 95ºC for 30 min. After centrifugation (7000 *g*, 2 min) 2 µl of the supernatant was used for PCR (Babic *et al.*, 2000) with following primer pair: sense - (5'-GAA GAG TTT GAT CAT GGC TC-3'), antisense - (5'-AAG GAG GTG ATC CAG CCG CA-3'). The obtained PCR product was directly sequenced using the same primers.

Phylogenetic analysis. For comparative purposes and construction of the phylogeny, the set of nematode and bacterial rDNA sequences deposited in GenBank was used. BLAST option was used to trace out related forms (Altschul *et al.*, 1990). The NCBI accession numbers of used sequences are indicated on the trees near the appropriate specific names. Bootstrap values are presented near nodes. Sequence alignments were generated using Clustal X under default values for gap opening and gap extension penalties. Alignments were analysed using PAUP* 4.0b10 (Swofford, 1998) for maximum parsimony (MP), and maximum likelihood (ML). *Steinernema affine* (Bovien, 1937) Wouts, Mráček, Gerdin & Bedding, 1982 and *S. intermedium* (Poinar, 1985) were used as outgroups in all cladograms. The GTR+G+I model was selected for maximum likelihood analysis with the use of ModelTest 5.0 (Posada & Crandall, 1998). The programme MtGui by Pablo Nuin (2005) was used as an interface to prepare Modeltest results for ML analysis in PAUP* 4.0b10.

Table 1. Morphometrics (mean ± SD (range), in µm of *Steinernema schliemanni* sp. n.

Character	1st generation male (n=15)	2nd generation male (n=13)	1st generation female (n=10)	2nd generation female (n=10)	Infective juvenile (n=25)
Body length	1463±368 (896-2213)	1143±71 (1059-1273)	6408±1269 (4431-8340)	2497±278 (2112-2991)	934±54 (842-1008)
Maximal body diameter	87±13 (76-120)	59±5.6 (50-70)	176±38 (134-261)	139±12.4 (121-169)	35±2.4 (30-38)
Pharynx total length	152±13 (121-170)	137±4.5 (125-142)	180±23 (152-221)	177±17.5 (156-209)	148±9.2 (127-162)
Distance from anterior end to excretory pore	85±13 (68-106)	87±5.8 (79-98)	114±3.8 (106-117)	106±4.1 (102-112)	72±5.3 (61-80)
Anal body width	36±3.6 (31-42)	35.7±3.7 (30-42)	60±5.6 (54-69)	42±2.7 (37-47)	17±0.8 (16-18)
Spicule length along chord	72±5.4 (61-81)	53±3.8 (50-61)	–	–	–
Gubernaculum length	53±5.4 (43-64)	38±4.8 (31-48)	–	–	–
Tail length	28±3.7 (22-32)	28±7.6 (23-51)	46±6.1 (36-55)	35±4.4 (31-46)	88±5.4 (76-95)
Hyaline portion of tail	–	–	–	–	55±7.3 (40-70)
Vulva (%)	–	–	54±2.5 (50.8-59.6)	56±1.2 (54.1-58.1)	–
a	15.5±2.7 (10.3-20.5)	19.2±1.5 (17.3-21.4)	37.5±9.4 (25.6-50.4)	17.9±1.5 (16.2-20.9)	26±1.8 (23-30)
b	8.9±1.2 (7.4-11.8)	8.3±0.7 (7.7-9.7)	35.8±7.1 (24.3-44.4)	14.1±1.0 (13.1-16.1)	6.3±0.3 (5.6-6.7)
c	49.3±14.8 (35.9-87.2)	42.5±7.8 (24.9-53.3)	141.3±39.9 (91.4-204.6)	71.2±11.3 (54.9-88.5)	10.6±0.6 (9.6-12.2)
h%	–	–	–	–	66±0.2 (51-74)
D% (EP/Pharynx x 100)	54.4±3.1 (50-58)	63.3±6.2 (58-74)	61.4±4.9 (52-70)	59.6±5.2 (50-66)	47.6±3.4 (42-55)

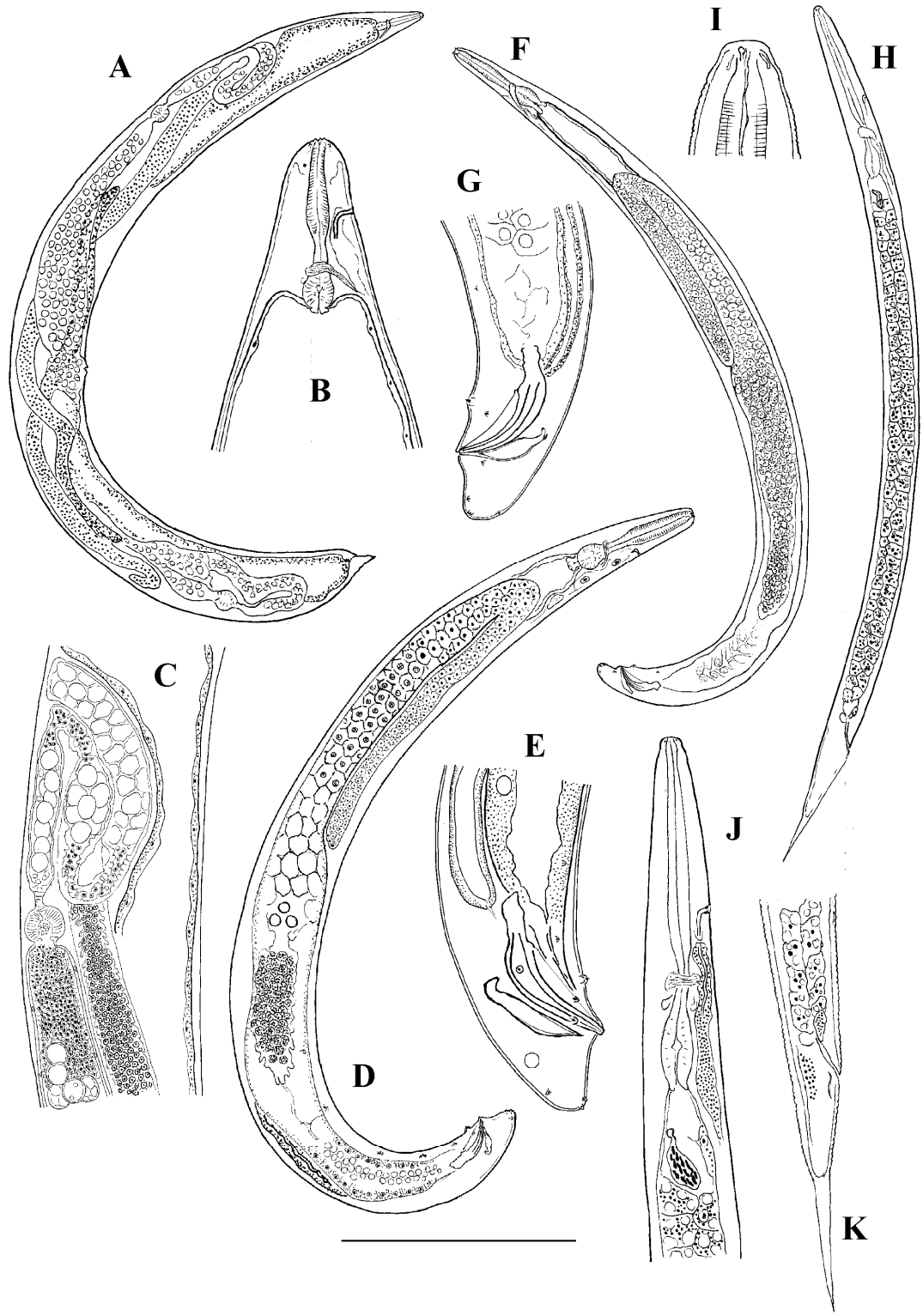

Fig. 1. *Steinernema schliemanni* sp. n. morphology, lateral aspect. A-C: First generation female. A: total view; B: anterior end, C: spermatozoa in oviduct and uterus. D, E: first generation male. D: total view; E: posterior end; F, G: Second generation male. F: total view; G – posterior end; H-K: Infective juveniles. H: total view; I: head end; J: anterior end (vesicle in the intestine); K: posterior end. Scale bars: A = 1000μm, B, C = 250 μm; D, F = 300 μm; E, G, J = 100 μm; I = 50 μm; K = 90 μm.

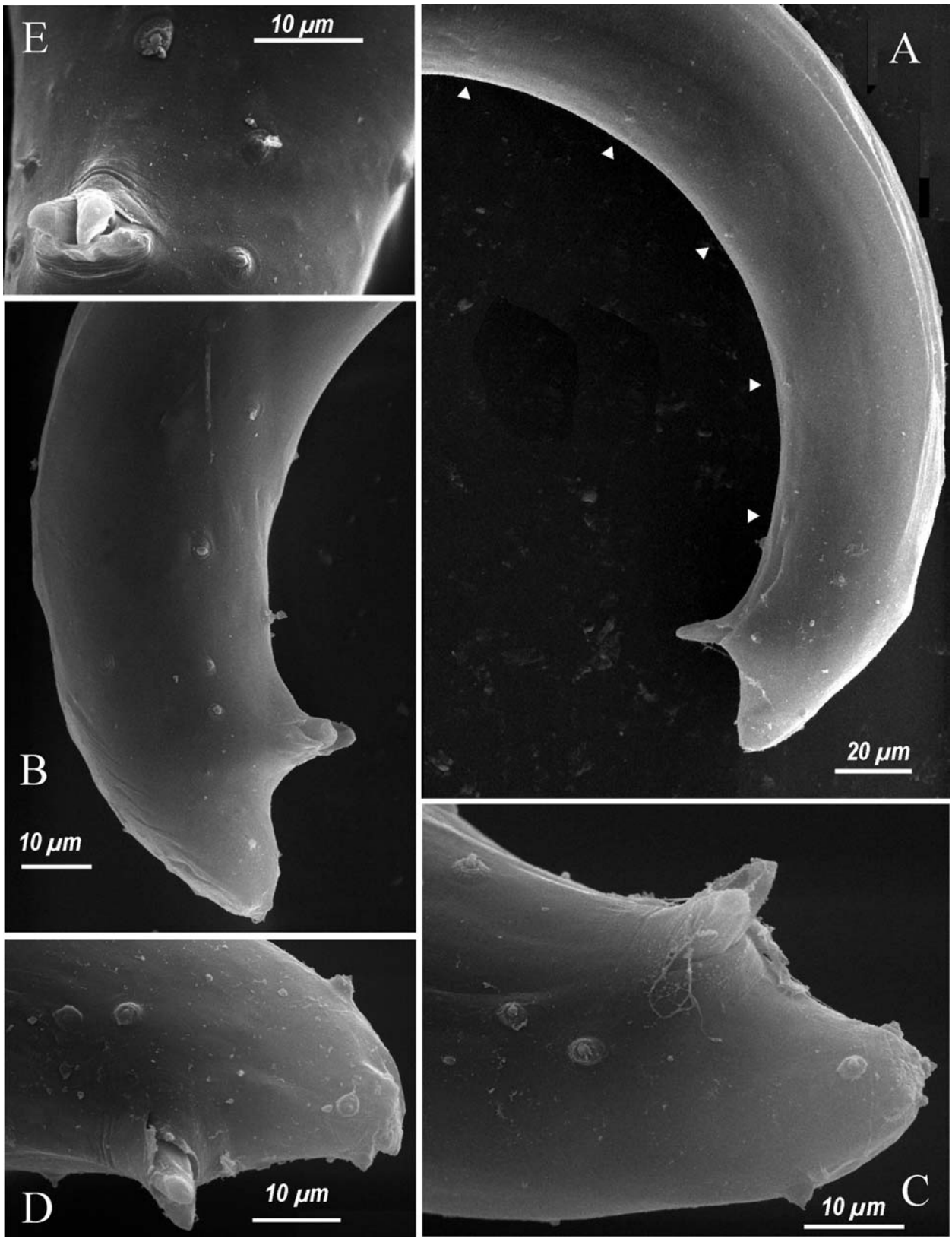

Fig. 2. *Steinernema schliemanni* sp. n. Male morphology, SEM. A: posterior end, lateral view, (white arrowheads indicating position of genital papillae); B: posterior end of second specimen, lateral view; C: posterior end of third specimen, lateral view, D: posterior end, subventral view, E: posterior end, ventral view.

DESCRIPTION

Steinernema schliemanni sp. n. (Figs 1-7)

Measurements (Table 1).

Holotype (first generation male): Body length = 1140 μm; Maximal body diameter = 77 μm; Pharynx total length =139 μm; Tail length = 26 μm; Spicule length along the chord = 65; Gubernaculum length = 49 μm.

First generation male. Body C- or J-shaped after fixation. Anterior end hemispherical, 20-25 μm in diameter, with broad funnel-shaped buccal cavity. Strongly cuticularised stoma walls 2-3 μm long, stegostom at least 5 μm long. Pharynx 12-15 μm wide at stegostom level, widening gradually up to 16-19 μm at level of excretory pore, then narrowing to isthmus (11-13 μm). Basal bulb 25-30 μm wide, without conspicuous valves. Cardium round, 6-8 μm long lobes protruding into intestinal lumen. Prominent deirids at cardium level. Excretory duct 1-1.5 μm wide, curving backward 5 μm from surface. Flexure of testis 50-60 μm posterior to basal bulb. Prominent pseudoceolomocytes (15 μm diam.) anterior to flexure. Testis filled with spermatocytes with round 4 μm wide nuclei at level of flexure. Nuclei in spermatocytes disappear at mid-body level, with broader testis lumen posteriorly filled with 7-10 μm spermatids with granular cytoplasm. Strongly vacuolated *vas deferens*, walls narrow to ejaculatory duct starting at level of spicule heads. Narrow posterior part of intestine squeezed between *vas deferens* and body wall. Spicules moderately curved (approx. 120º), with 12 μm long and 10 μm wide head and 7-8 μm wide shaft (Fig. 3A). Anteriorward projection on ventral edge of spicule proximal end (Fig. 3B). Proximal end of spicules covered with thin cuticular membrane (Fig. 3D). Velum 4 μm wide, ending at 2-12 μm from rounded tip of spicules (Fig. 3A-C). Gubernaculum with protruding crurae, conspicuous median hole and proximal beak-like projection, directed toward ventral body surface. Cuneus absent (Fig. 3E).

Two pairs of postloacal papillae on ventral side near tail terminus. Tiny (1 μm high) peg-like mucron. Pair of dorsolateral papillae near tail terminus. One pair of papillae at cloaca level and one pair situated in 8-10 μm anterior to cloaca (Fig. 2C-E). Unpaired median papilla as flattened elevation (4 μm in diam.) in 20 μm anterior to cloaca. Five pairs of genital papillae discernible in precloacal area anterior to unpaired median papilla. A pair of post-deirids in dorsolateral position in front of anteriormost genital papillae. Total count of genital papillae: 22+1.

First generation female. Cephalic end rounded, diameter approx. 30 μm. Bowl-shaped buccal cavity 12-15 μm wide. Corpus of pharynx increasing in diameter from 20 μm at stoma to 30 μm before isthmus. Isthmus 20 μm wide. Basal bulb 40 μm wide, with transparent 10 μm long lobes of cardium. Excretory duct 1-2 μm wide. Excretory channel lumen in lateral chords traced up to vulva, its walls collapsing at posterior. Intestinal cells at proventriculus level large, more than 50 μm in size. Ovary flexures 150-200 μm posterior to basal bulb and in 20-50 μm before the anus level. Sometimes ovary forms additional loop. Spherical chamber at mid-oviduct, composed of radially arranged cells. Numerous spermatozoa (diameter of nucleus containing end: 7-9 μm) forming chains of 3-8 cells scattered in lumen of spherical chamber and neighbouring parts of ovary and uterus. Anterior vulva lip 20-30 μm high overhanging smaller (10 μm high) posterior lip. Tail end cupola-shaped with acute 20 μm long terminus.

Second generation males. Body rarely C-shaped, more often J-shaped after fixation. Main morphological features similar to those of first generation males. Strongly cuticularised stoma walls 2-3 μm long.

Second generation female. No pronounced difference between first and second generation females in morphology and even in morphometrics of cephalic end, buccal cavity and pharynx. Basal bulb usually narrower (30-35 μm wide), lobes of cardium of same size. Intestinal cells with thinner walls. Ovary flexures situated at longer distance from basal bulb or anal opening (300-400 and 100-150 μm, respectively). No morphologically distinct chambers or widened parts of oviduct. Vulva lips equal, 10 μm high, usually covered with transparent substance. Tail end conical with rounded terminus.

Infective juveniles. Heat relaxed body broadly C-shaped. Anterior end slightly set-off from body contours (Fig. 4B). Amphidial opening near labial circle. Four cephalic papillae in 4-5 μm from anterior end, just posterior to amphids. Lateral field starts in 60-70 μm from anterior end (Fig. 4A and C) as four ridges. At 10 μm from the beginning, the lateral field contains six ridges (Fig. 2C). Eight equal ridges discernible at mid-body (addition of two marginal ones). This pattern continues to mid-tail level.

Cross-breedings tests. The pairs of males and females of *S. monticolum* and *S. schliemanni* sp. n. were unable to produce progeny either the pair was composed of single infective juveniles of each species, or fourth-stage individuals of opposite sex from each species were combined. In conspecific pairs of juveniles two pairs produced

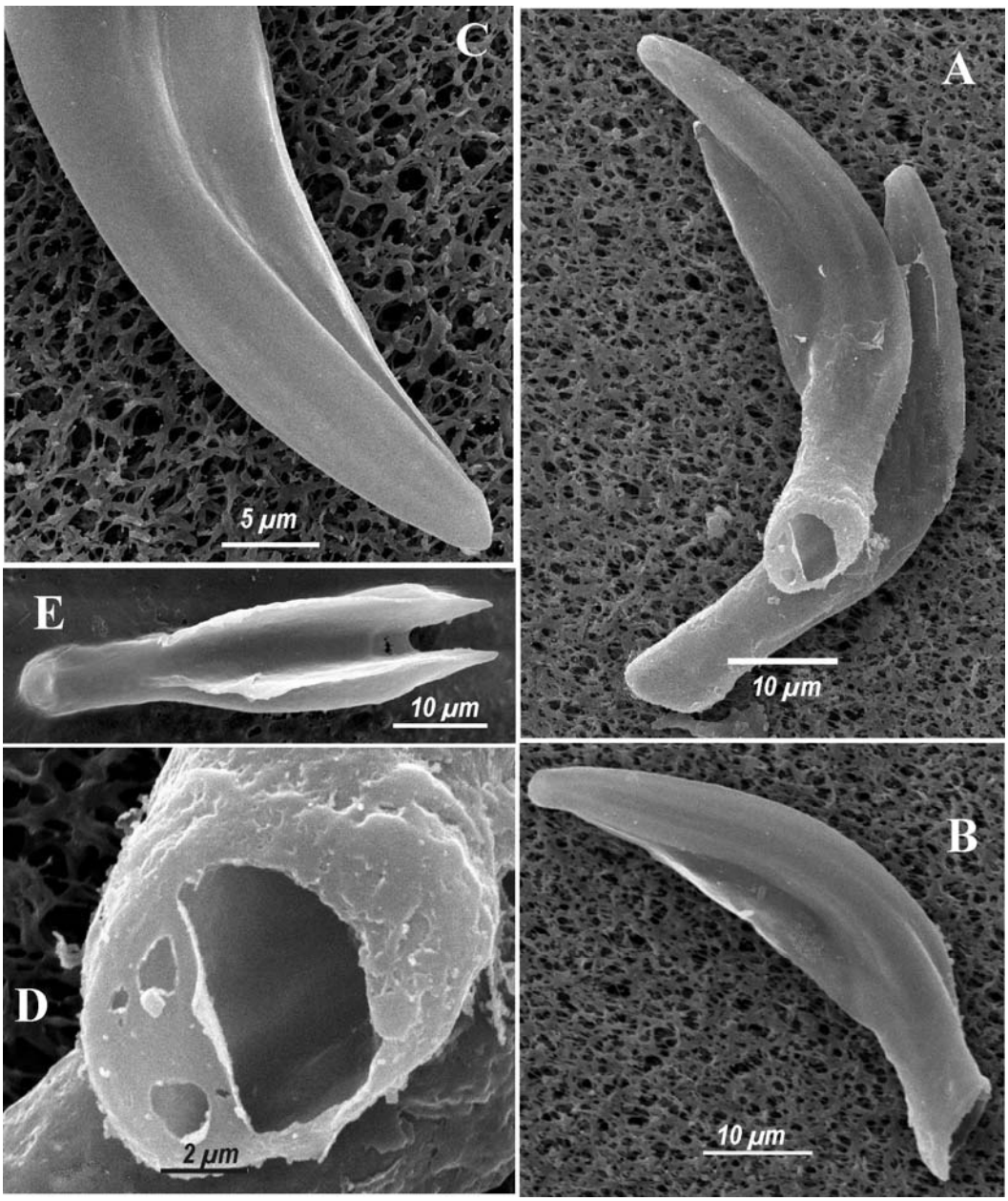

Fig. 3. *Steinernema schliemanni* sp. n. Morphology of male copulatory apparatus, SEM. A: pair of spicules; B: separate spicule with different shape of shaft and velum; C: ending of velum; D: proximal part of spicule, E: gubernaculum.

Table 2. Pairwise differences in ITS rDNA region between *Steinernema schliemanni* sp. n. and related species. Below diagonal: total character differences, above diagonal: mean character differences.

	Species (accession number in NCBI GenBank)	1	2	3	4	5	6
1	*Steinernema schliemanni* sp. n. (HM778112)	–	18.5%	20.5%	20.1%	19.9%	23.6%
2	*Steinernema monticolum* (AF122017)	115	–	6.0%	4.5%	4.9%	20.8%
3	*Steinernema ashiuense*, (DQ354694)	128	39	–	2.7%	0.9%	21.2%
4	*Steinernema robustispiculum*, (AY355442)	126	30	18	–	1.8%	21.0%
5.	*Steinernema* sp. MY5 (AB243437)	124	33	6	12	–	21.1%
6	*Steinernema feltiae*, (AY171246)	151	135	137	136	137	–

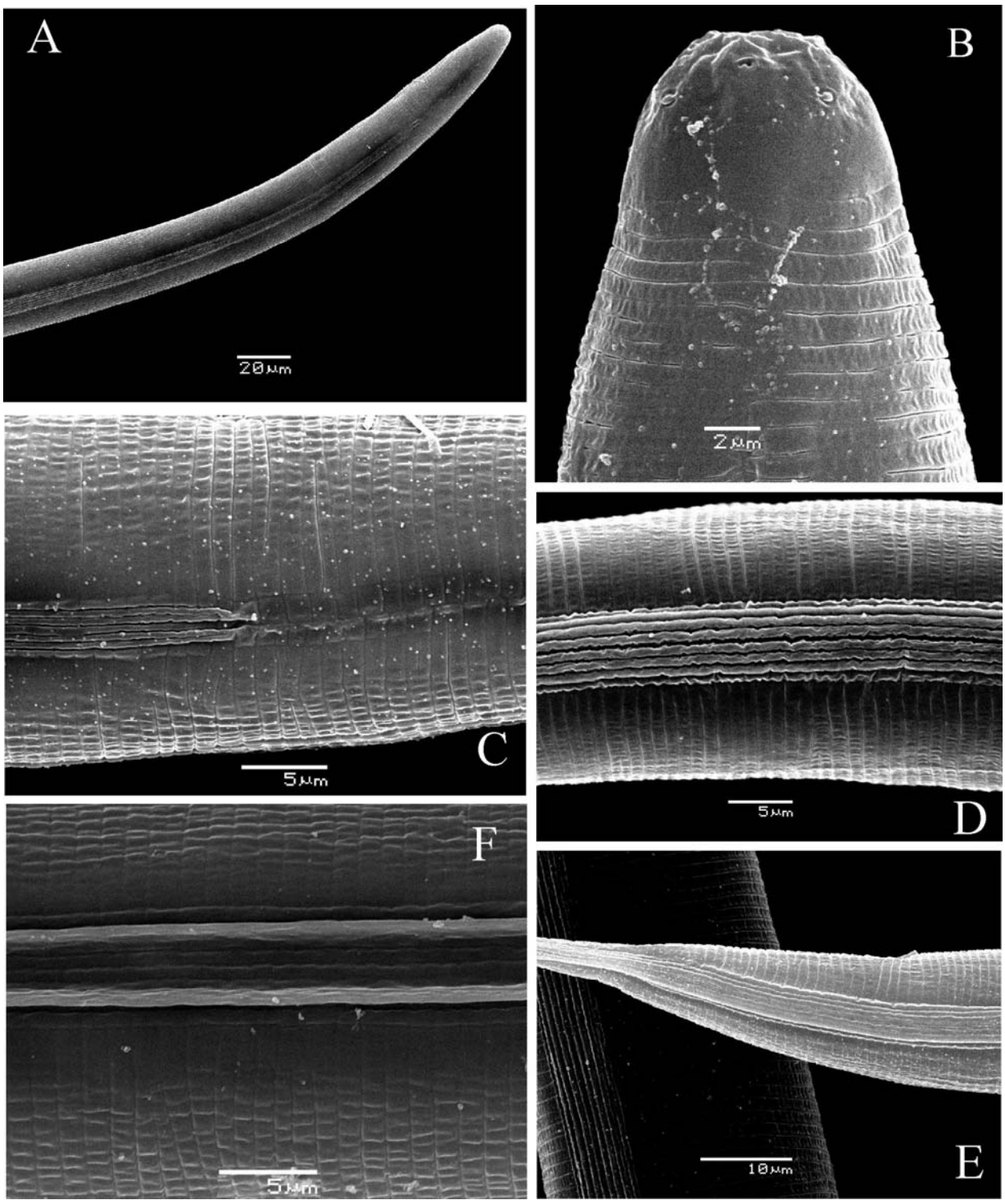

Fig. 4. *Steinernema schliemanni* sp. n. and *S. monticolum*. A-E: *S. schliemanni* sp. n. Cuticle of infective juvenile in lateral view, SEM. A: Anterior end; B: Head end; C: beginning of lateral field; D: Lateral field at mid-body; E: Lateral field at tail end; F: *S. monticolum*. Lateral field at mid-body.

progeny for *S. schliemanni* sp. n. and one for *S. monticolum* (n=50 for both species). Single nematodes of both isolates did not produce progeny. Conspecific pairs of pre-adult stages produced progeny in about 8-12% of mating experiments (2 and 3 cases for these two species correspondingly; n=25 for both species).

DISCUSSION

Molecular characterisation. Sequences of ITS rDNA for *S. schliemanni* sp. n. were obtained through direct sequencing in 2003 of nematodes originating from the first inoculation of *Galleria* caterpillars with newly obtained infective juveniles. Since then ITS rDNA sequences were obtained through cloning of PCR product in 2007 and in 2009. Sequences of the D2D3 region of LSU rDNA were obtained in 2007. Obtained sequences were deposited in GenBank (for *S. schliemanni* sp. n. - 862 bp long ITS1-5.8S-ITS2 sequence: HM778112; partial 541 bp long D2D3 LSU rDNA sequence: HM778113; partial 510 bp long D2D3 LSU rDNA sequence for type culture of *S. monticolum*: HM778114). Phylogenetic relationships of *S. schliemanni* sp. n. with related *Steinernema* spp. are presented in Figs 5-7. In both the MP and ML ITS rDNA cladograms the new species is related to *S. monticolum, S. ashiuense* and *S. robustispiculum* (Figs 5-6) composing the '*monticolum*' clade. Bootstrap support for this clade is moderate (89%) or high (100%) in MP and ML trees, respectively. The inner topology of the '*monticolum*' clade is the same in both cladograms (*S. schliemanni* sp. n. (*S. monticolum* (*S. ashiuense* + *S. robustispiculum*))). The new species is positioned in the basal position in this lineage. The entire clade is a sister group for the '*feltiae-kraussei-oregonense'* clade. The cladograms obtained from the MP and ML analysis of partial D2D3 LSU rDNA *S. schliemanni* sp. n. and *S. monticolum* form a single clade (Fig. 7) which is the sister one for the '*feltiae-kraussei-oregonense* clade'. The nominal LSU rDNA sequence obtained for *S. monticolum* by Stock *et al*. (2002) and deposited in GenBank under AF 331895 is linking inside '*carpocapsae'*-clade of *Steinernema* genus.

Molecular characterisation of symbiotic bacteria. The partial 16S sequence of symbiotic bacteria of *S. schliemanni* sp. n. was deposited in NCBI GenBank under accession number HQ122653. In the MP analysis, the *Xenorhabdus* sp. from *S. schliemanni* sp. n. clusters with a group composed of *X. bovienii* (Akhurst, 1983) Akhurst & Boemare, 1993, *X. hominickii* Tailliez, Pagès, Ginibre & Boemare, 2006, *X. japonica* Niushimura, Hagiwara, Suzuki & Yamanaka, 1995, and *X. koppenhoeferi* Tailliez, Pagès, Ginibre & Boemare, 2006. *Xenorhabdus bovienii* demonstrates a stable but weakly supported relationship with the *Xenorhabdus* sp. isolated from *S. schliemanni* sp. n. (Fig. 8A). Inner phylogenetic nodes of this *Xenorhabdus* group were not resolved in our analysis; in the strict consensus trees they were collapsing (data not shown). In the NJ tree, inner nodes of this group were collapsing in 50% majority rule consensus tree (Fig. 8B). *Xenorhabdus hominickii* is the closest *Xenorhabdus* species to the *Xenorhabdus* sp. from *S. schliemanni* sp. n. with only 28 bp difference (Table 4).

Differential diagnosis. In all phylogenetic trees, *S. schliemanni* sp. n. clusters with the species of the '*monticolum*'-group. Apart of three described species (*S. monticolum, S. ashiuense* and *S. robustispiculum*), a strain identified as *Steinernema* sp. MY5 (Yoshida *et al*., 1998) is a permanent member of this lineage. The lateral field of infective juveniles of *S. schliemanni* sp. n. consists of eight ridges at mid-body (Fig. 4D). In two species of the aforementioned group, the lateral field has a completely different appearance at mid-body, *viz.* two well pronounced submarginal lines and two lower ridges in the centre of the field. Such lateral field morphology was described for both *S. robustispiculum* (Phan *et al*., 2005) and *S. monticolum* (Fig. 4F). The lateral field of *S. ashiuense* was described as containing five ridges (Phan *et al*., 2006). Inside the '*monticolum*' clade, *S. schliemanni* sp. n. can be easily distinguished from the three other species by the morphometrical characters of infective juveniles. Infective juveniles of *S. schliemanni* sp. n. are significantly longer (842-1008 μm) than those of all three other species. The ranges of the body length of infective juveniles in *S. monticolum, S. ashiuense* and *S. robustispiculum* are 612-821 μm, 720-800 μm and 642-778 μm, respectively. The EP (distance from anterior end to excretory pore) and tail length are proportionally higher in the longer juveniles of *S. schliemanni* sp. n. The ratios D% and 'c' of infective juveniles are virtually the same in all four species under comparison. The size of spicules of first generation males of *S. schliemanni* sp. n. resembles that of the spicules of *S. monticolum* (64-81 *vs* 61-80); spicules of first generation males of *S. ashiuense* and *S. robustispiculum* are shorter (not longer than 65 μm). The nucleotide differences between the three known species of the '*monticolum*'

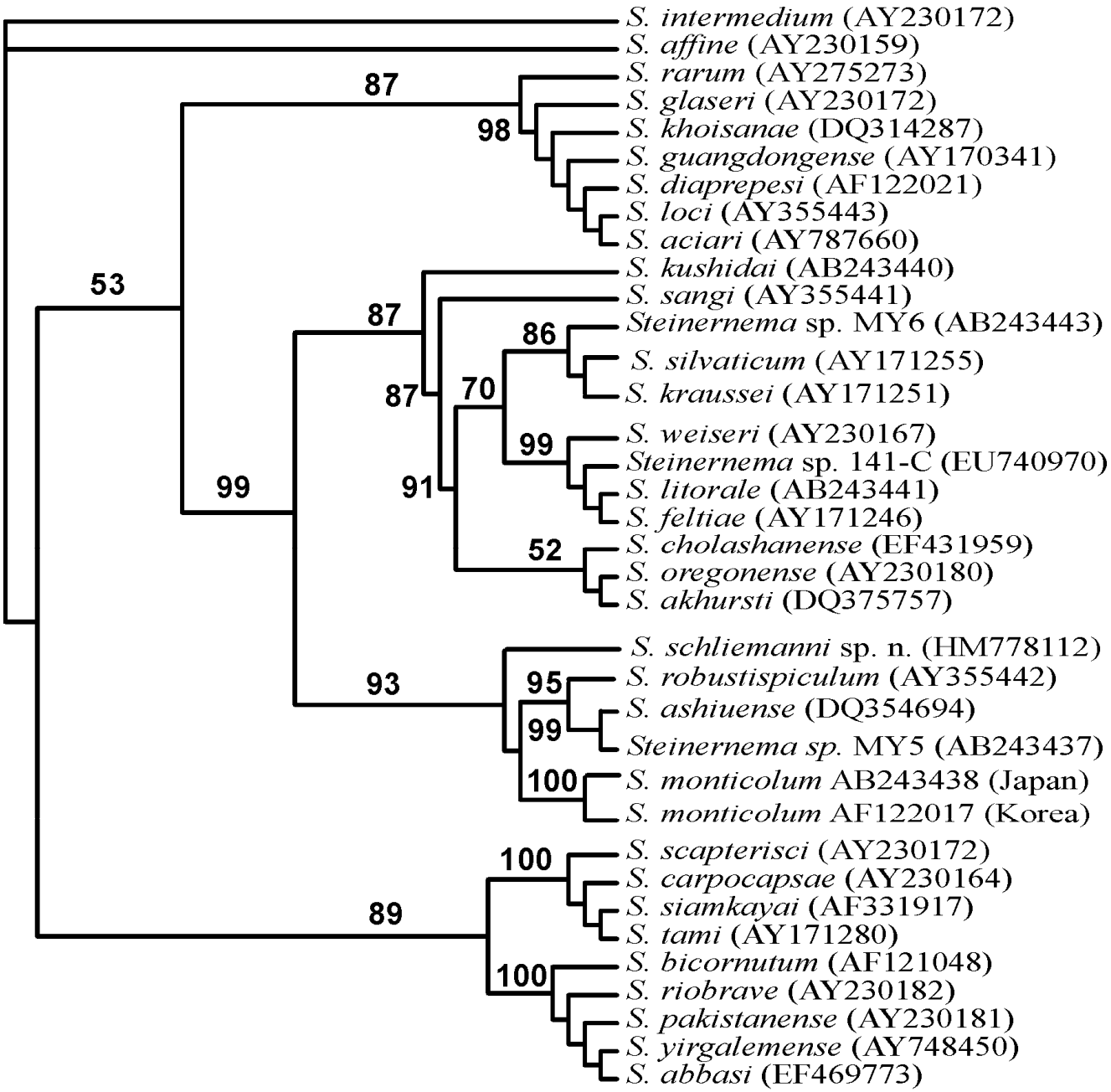

Fig. 5. *Steinernema schliemanni* sp. n. phylogenetic position inferred from analysis of ITS rDNA sequences. Bootstrap 50% majority-rule consensus tree. Criterion = parsimony. Of 892 total characters 570 are parsimony-informative. Gaps are treated as "missing". 10000 bootstrap replicates.

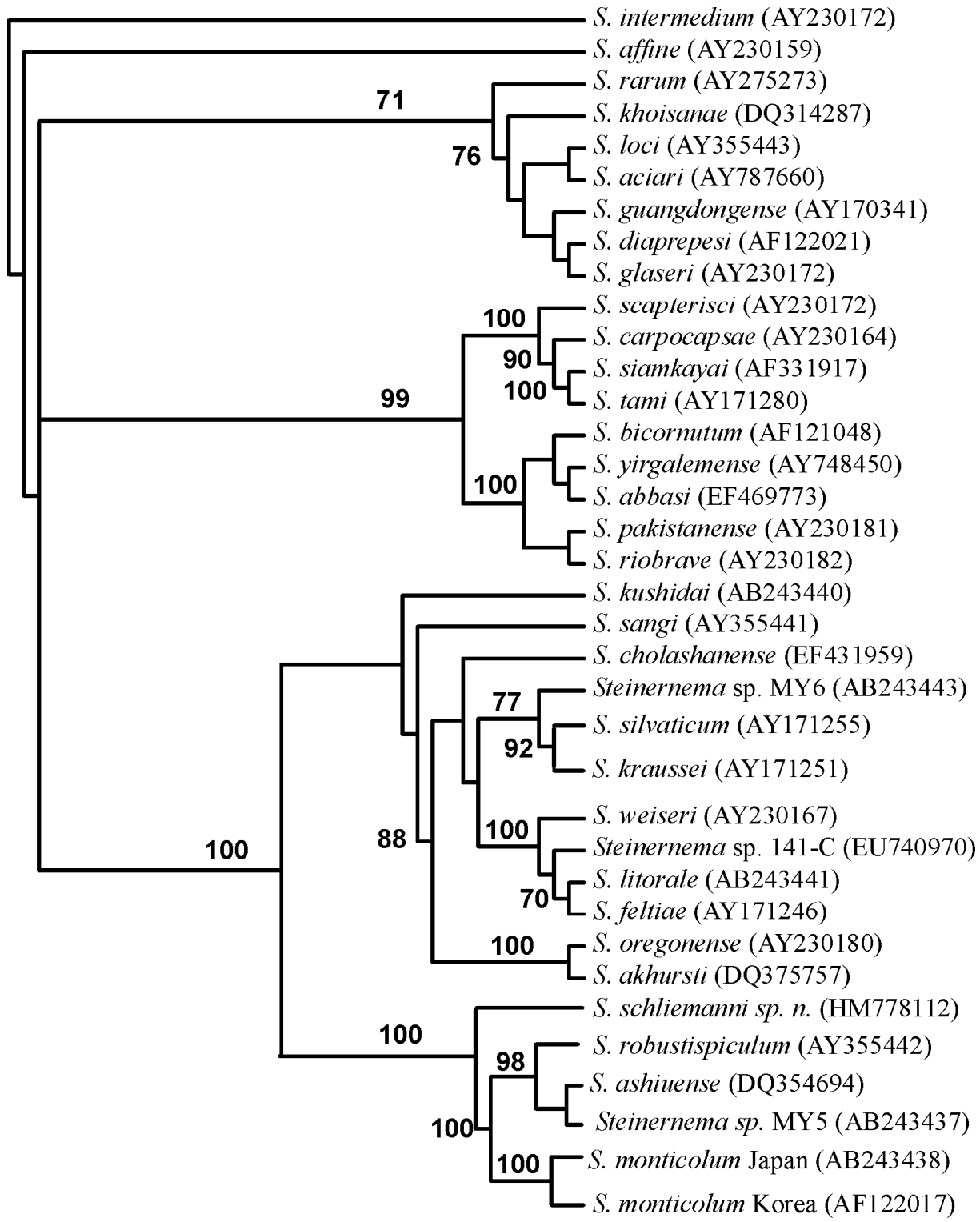

Fig. 6. *Steinernema schliemanni* sp. n. phylogenetic position inferred from analysis of ITS rDNA sequences. Bootstrap 50% majority-rule consensus tree. Criterion = likelihood. GTR+G+I model. 100 bootstrap replicates.

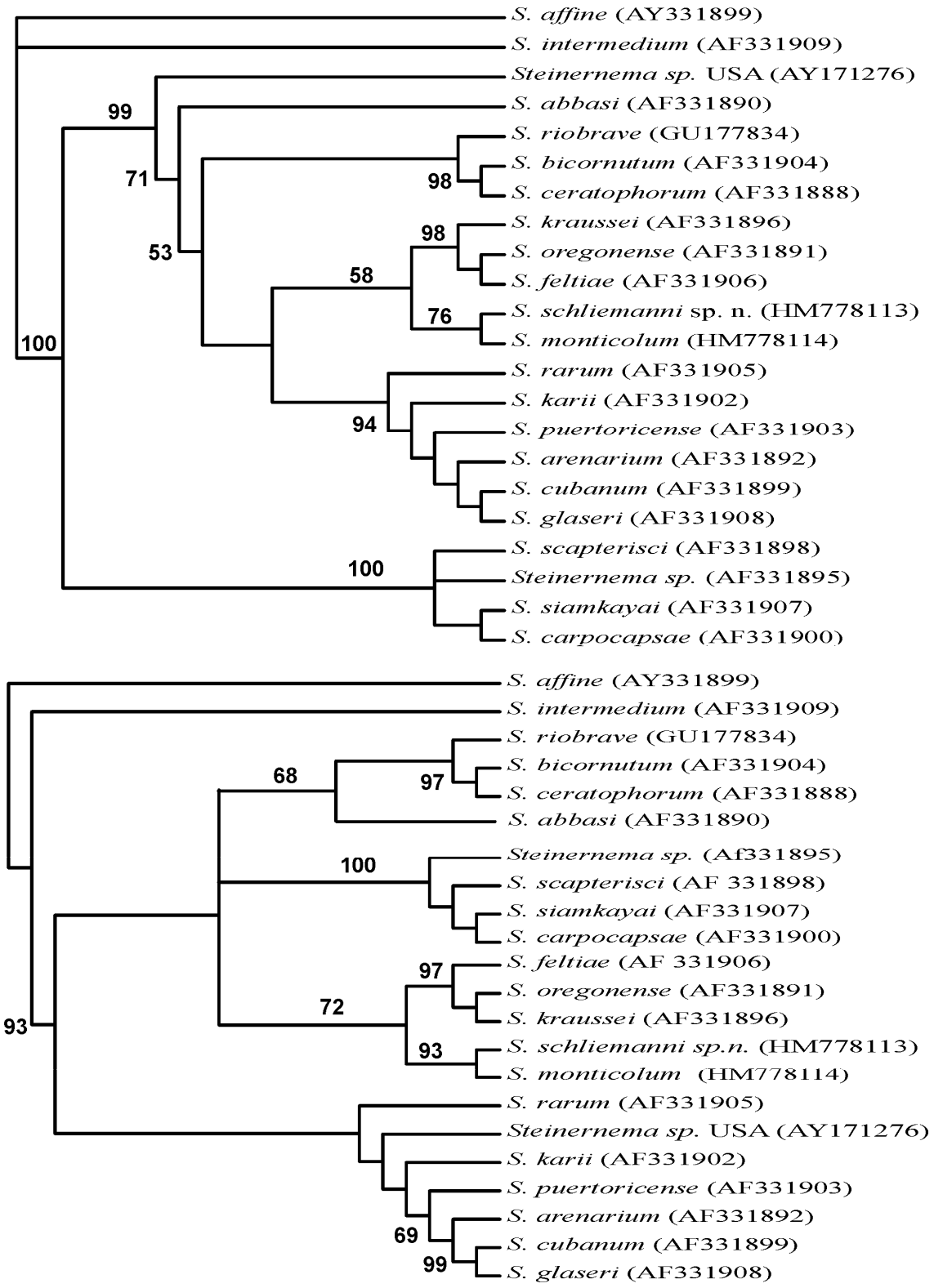

Fig. 7. *Steinernema schliemanni* sp. n. phylogenetic position inferred from analysis of D2D3 expansion segment sequences of LSU rDNA. A: bootstrap 50% majority-rule consensus tree. Criterion = parsimony. Of 595 total characters 232 are parsimony-informative. Gaps are treated as "missing".100 bootstrap replicates. B: bootstrap 50% majority-rule consensus tree. Criterion = likelihood. GTR+G model. 100 bootstrap replicates.

Table 3. Pairwise differences in 16S DNA region between *Xenorhabdus* sp. bacteria isolated from *Steinernema schliemanni* sp. n. and related species. Below diagonal: total character differences, above diagonal: mean character differences.

	Species	1	2	3	4	5	6	7
1	*Xenorhabdus sp. ex Steinernema schliemanni* sp. n.	–	2.4%	2.7%	2.2%	2.8%	2.0%	2.3%
2	*Xenorhabdus bovienii*	34	–	4.0%	3.7%	3.8%	4.1%	3.5%
3	*Xenorhabdus budapestensis*	39	56	–	2.1%	3.0%	3.0%	3.0%
4	*Xenorhabdus ehlersii*	31	52		–	%	2.8%	3.3%
5	*Xenorhabdus japonica*	40	53	34	37	–	2.4%	2.4%
6	*Xenorhabdus hominickii*	**28**	42	42	39	42	–	2.4%
7	*Xenorhabdus koppenhoeferi*	32	49	42	47	34	34	–

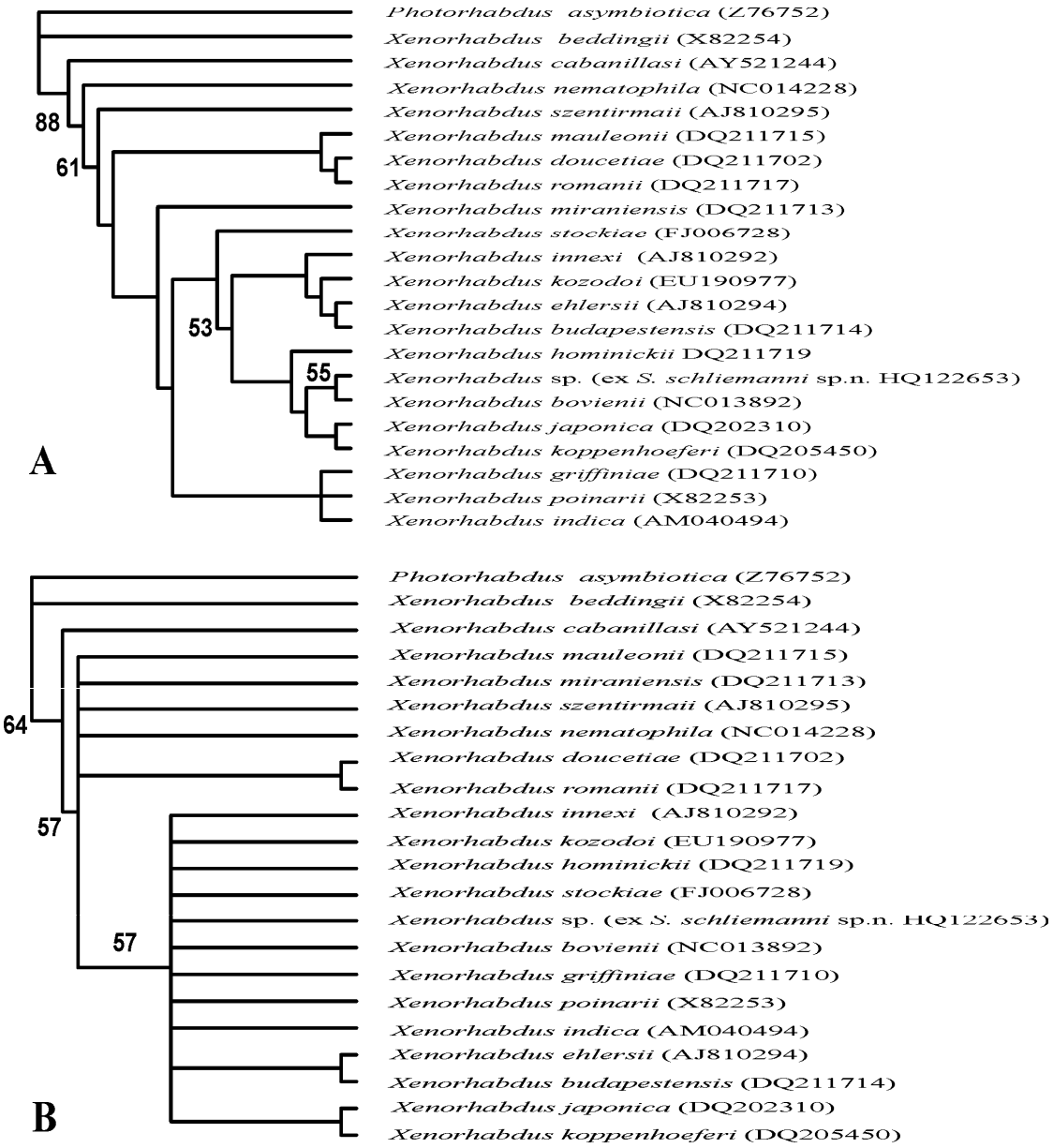

Fig. 8. Phylogenetic position of *Xenorhabdus* sp. isolated from infective juveniles of *Steinernema schliemanni* sp. n. inferred from analysis of 16S DNA. 1000 bootstrap replicates. A: Bootstrap 50% majority-rule consensus tree. Criterion = parsimony. Of 1402 total characters 116 are parsimony-informative. Gaps are treated as "missing". B: Neighbour-joining bootstrap 50% majority-rule consensus tree. Tree length = 522.

clade varies between 30-39 bp; this corresponds to 4-6% of the ITS region. The nucleotide differences between the three species and *S. schliemanni* sp. n. are much greater (115 bp or 18%) (Table 2). Though very few sequences of the D2D3 region of the LSU rDNA are deposited in NCBI GenBank for species related to *S. schliemanni* sp. n., it is obvious that the level of nucleotide differences between the new species and the closest *S. monticolum* is higher than that between species of '*feltiae-kraussei-oregonense*' group (Table 3). Remarkably, the difference with the sequence of the nominal *S. monticolum* from GenBank (AF 331895) is much higher, reaching 31% of the compared sequences.

Type locality. The forest in Mecklenburg-Vorpommern province of Germany. Numerous juveniles and non-mature second generation individuals were found around the cadaver of *Osmoderma eremita* Scopoli, 1763 beetle larva on the soil surface in April, 2003.

Deposition of type material: Holotype - male (N°1109) and paratype - 1st generation female (N° 1110) are deposited in the collection of the Centre of Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow. Paratype male is present in the collection of Julius Kühn-Institut in Münster, Germany. Paratypes (male and female) are also deposited in the collection of University of Ghent (Belgium).

Ethymology. The species is named after entrepreneur and scientist Heinrich Schliemann (1822-1890) who was born in Neubukow (Mecklenburg-Vorpommern) and used his fortune gained in Russia to conduct the famous excavations of Troy.

DISCUSSION

An analysis of the phylogenetic relationships of the species of the '*monticolum*' sub-clade of the genus *Steinernema* inferred from the available ITS rDNA (ITS1+5.8S+ITS2) sequences and partial sequences of LSU rDNA, reveals contradiction in the position of *S. monticolum*. In the trees based on ITS rDNA sequences (Stock, 2001; Nguyen *et al.*, 2001), *S. monticolum* clusters with the '*feltiae-kraussei-oregonense*' clade; however, in the trees based on LSU rDNA the species is a member of '*carpocapsae'* clade (Stock, 2001). In the course of our study we obtained a partial sequence of the D2D3 expansion segment of the LSU rDNA for the *S. monticolum* type strain. When this sequence was used for phylogenetic analysis, this species clustered with *S. schliemanni* sp. n. and showed relationships with the '*feltiae-kraussei-oregonense*' clade (Fig. 7). When using the sequence deposited previously under accession number AF 331895 for *S. monticolum*, the species always clustered with *S. carpocapae* and related species. It seems that the LSU rDNA sequence for *S. monticolum* deposited in NCBI GenBank represents a species of the '*carpocapsae'* group, rather then *S. monticolum,* as was already reported by Nguyen *et al.* (2007). The congruence between ITS rDNA and LSU rDNA trees based on the newly obtained sequence for *S. monticolum* looks more logical.

Obviously, *S. schliemanni* sp. n. is a member of the '*monticolum*" clade, i.e. a group of three described species and several isolates of unknown taxonomic status. The newly described species always occupied a basal position inside this clade; species found in Asia formed a strongly supported subgroup. The 16S rDNA sequence of bacteria associated with *S. schliemanni* sp. n. demonstrates close relationships with several *Xenorhabdus* species; the nucleotide difference is the lowest with *X. hominickii* (28 bp or 2%). This latter species was earlier isolated from both *S. karii* and *S. monticolum* (Koppenhöfer, 2007). A level of nucleotide differences around 2% was reported for some pairs of independent *Xenorhabdus* species, but usually interspecific difference is higher (Table 3). Additional studies are needed to elucidate the taxonomic status of the *Xenorhabdus* species from *S. schliemanni* sp. n. In the meantime it can be concluded that the molecular characteristics of both the nematode and its associated bacterium confirm the affiliation of the nematode with the '*monticolum*' group.

ACKNOWLEDGEMENT

We are grateful to Dr Nguyen Ba Khuong (University of Gainesville, Florida) for providing the *S. monticolum* type culture and his kind attention toward this study, and to Dr Dieter Sturhan, who provided the primary nematode culture and for his critical comments during the manuscript preparation.

REFERENCES

ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W. & LIPMAN, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215: 403-410.

ANSARI, M.A., WAEYENBERGE, L. & MOENS, M. 2005. First record of *Steinernema glaseri* Steiner, 1929 (Rhabditida: Steinernematidae) from Belgium: a natural pathogen of *Hoplia philanthus* (Coleoptera: Scarabaeidae). *Nematology* 7: 953-956.

BABIC, I., FISHER-LE SAUX, M., GIRAUD, E. & BOEMARE, N. 2000. Occurrence of natural dixenic associations

between the symbiont *Photorhabdus luminescens* and bacteria related to *Ochrobactrum* spp. in tropical entomopathogenic *Heterorhabditis* spp. (Nematoda, Rhabditida). *Microbiology* 146: 709-718.

CURRAN, J. & DRIVER, F. 1994. Molecular taxonomy of *Heterorhabditis*. Pages 41–48 *in* A.M. Burnell, R.-U. Ehlers and J.P. Masson (Eds), COST 812 Biotechnology: Genetics of Entomopathogenic Nematode-Bacterium Complexes. European Commission, DG XII, Luxembourg, Belgium.

HE Y., SUBBOTIN, S.A., RUBTSOVA T.V., LAMBERTI F., BROWN, D.J.F. & M. MOENS. 2005. A molecular phylogenetic approach to Longidoridae (Nematoda: Dorylaimida). *Nematology*: 7: 111-124.

KARIMI, J., KHARAZI-PAKDEL, A., YOSHIGA, T. & KOOHI-HABIBI, M**.** 2009. Report of entomopathogenic nematode, *Steinernema glaseri*, from Iran, *Russian Journal of Nematology* 17: 83-86.

KOPPENHÖFER, H.S. 2007. Bacterial symbionts of *Steinernema* and *Heterorhabditis*. (pp. 735-788). In: Entomopathogenic nematodes: systematics, phylogeny and bacterial symbionts. K.B. Nguyen and D.J. Hunt (Eds), Nematology Monographs and Perspectives, vol. 3, Brill, Leiden, 816 pp.

KUWATA, R., SHIGEMATSU, M., YOSHIGA, T., YOSHIDA, M. & KONDO, E. 2006. Phylogenetic analyses of Japanese steinernematid nematodes and their associating *Xenorhabdus* bacteria. *Nihon Senchu Gakkai Shi (Japanese Journal of Nematology)* 36: 75-85.

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.

PHAN, L.K., TAKEMOTO, S. & FUTAI, K. 2006. *Steinernema ashiuense* sp. n. (Nematoda: Steinernematidae), a new entomopathogenic nematode from Japan. *Nematology* 8: 681-690.

POSADA, D. & CRANDALL, K.A. 1998. *Modeltest*: testing the model of DNA substitution. *Bioinformatics* 14: 817–818.

NGUYEN, K.B., MARUNIAK, J. & ADAMS, B.J. 2001. Diagnostic and phylogenetic utility of the rDNA internal transcribed spacer sequences of *Steinernema*. *Journal of Nematology* 33: 73-82.

NGUYEN, K. B., DUNCAN, L. W. 2002. *Steinernema diaprepesi* n. sp. (Rhabditida: Steinernematidae), a parasite of the citrus weevil *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae). *Journal of Nematology* 34: 159-170.

NGUYEN, K.B., STUART R. J., ANDALO V., GOZEL U.& ROGERS M.E. 2007. *Steinernema texanum* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from Texas, USA *Nematology* 9: 379-396.

ROSA, J.S., BONIFASSI, E., AMARAL, J., LACEY, L.A., SIMÕES, N. & C. LAUMOND. 2000. Natural occurrence of entomopathogenic nematodes (Rhabditida: Steinernema, Heterorhabditis) in the Azores. *Journal of Nematology* 32: 215–222.

SEINHORST, J.W., 1959. A rapid method for transfer of nematodes from fixative to anhydrous glycerine. *Nematologica* 4: 67-69.

SPIRIDONOV, S.E. & MOENS, M. 2005. New strains of entomopathogenic nematodes from Kamchatka peninsula, Russian Far East. *Russian Journal of Nematology* 13: 162-163.

STOCK, S.P., CHOO, H.Y. & KAYA, H.K. 1997. *Steinernema monticolum* sp. n. (Rhabditida: Steinernematidae), an entomopathogenic nematode 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.

VRAIN, T.C., WAKARCHUK, D.A., LEVESQUE, A.C. & HAMILTON, R.J. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15: 563-573.

Yoshida, M., Reid, A.P., Briscoe, B.R. & Hominick, W.M. 1998. Survey of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Japan. *Fundamental and Applied Nematology* 21: 185-198.

S. E. Spiridonov, L.Waeyenberge, M. Moens. *Steinernema schliemanni* sp. n. (Steinernematidae; Rhabditida) – новый вид штейнернематид группы ‘*monticolum’* из Европы.

Резюме. Приводится описание *Steinernema schliemanni* sp. n. , личинки которых были обнаружены на поверхности трупов личинок жуков *Osmoderma ceremita* (Coleoptera) в Германии в апреле 2003. Новый вид относится к группе видов, близких к виду *S. monticolum* (т.н. группа ‘*monticolum’*). Новый вид отличается от других видов этой группы строением латеральных полей инвазионных личинок: наличием восьми равных ребер в поле, в отличие от неравных по высоте ребер у *S. monticolum* и *S. robustispiculum* и пяти ребер у *S. ashiuense*. Инвазионные личинки *S. schliemanni* sp. n. имеют более длинное тело (842-1008 мкм), чем у остальных представителей группы ‘*monticolum’*. *Steinernema schliemanni* sp. n. отличается от других видов группы ‘*monticolum’* как минимум по 115 позициям в последовательности ITS rDNA (18%). Различия в нуклеотидном составе участка D2D3 домена LSU rDNA между *S. schliemanni* sp. n. и ближайшим видом *S. monticolum* составляет 22 пары нуклеотидов (3,9% сравниваемых последовательностей), что превышает различия по этому участку между такими видами, как *S. feltiae, S. kraussei* и *S. oregonense*. Последовательность 16S rDNA ассоциированной с *S. schliemanni* sp. n. бактерии близка по нуклеотидному составу к последовательностям нескольких видов *Xenorhabdus.* При этом наименьшие различия отмечены с *X. hominickii* (28 пары нуклеотидов или 2%), выделенными от *S. monticolum*.