Russian Journal of Nematology, 2001, 9 (1), 1 - 7

Steinernema sangi sp. n. (Rhabditida: Steinernematidae) from Vietnam

Long Phan Ke*, Ngoc Chau Nguyen* and Maurice Moens**

*Institute of Ecology and Biological Resources, National Centre for Science and Technology, Nghiado, Caugiay, Hanoi, Vietnam,
**CLO-Department for Crop Protection, Burg. Van Gansberghelaan 96, B 9820, Merelbeke, Belgium and University of Gent, Laboratory for Agrozoology, Coupure 555, 9000 Gent, Belgium
e-mail: m.moens@clo.fgov.be

Accepted for publication 21 September 2000

Summary. A description is given of *Steinernema sangi* sp. n. that was isolated from soil in Thanhhoa province in the northern part of Vietnam. The lateral field of infective juveniles (IJ) has eight ridges with the submarginal and central pair less distinct, and this characteristic makes *S. sangi* sp. n. most similar to *S. kraussei*. However, *S. sangi* sp. n. can be distinguished from *S. kraussei* by the shorter body length of the IJ (753 *vs* 951 µm), a more anterior position of the excretory pore of the IJ (51 *vs* 63 µm) and a longer spicule length (63 *vs* 49 µm). *Steinernema sangi* sp. n. can be distinguished from *S. kraussei* by RFLP patterns obtained after digestion of the ITS region by each of the enzymes: *Alu*I, *Cfo*I, *Dde*I, *Eco*RI, *Hae*III, *Msp*I, *Nde*II, *Rsa*I, and *Sal*I.

Key words: entomopathogenic nematodes, RFLPs, *Steinernema sangi* sp. n., taxonomy, Vietnam.

Species of the families Steinernematidae and Heterorhabditidae are frequently used for biological control of insects. The better adapted these entomopathogenic nematodes (epn) are to the environmental conditions under which such a control is attempted, the more efficient the insect control that can be expected. Collecting indigenous nematodes may provide isolates more suitable for inundate release against local pests because of their adaptation to local climate and population regulators (Bedding, 1990). This rationale formed the basis of an epn survey carried out by Nguyen *et al.* (1999) during which steinernematid nematodes were found in Xuanmy, Thuongxuan, Thanhhoa province (North Vietnam). A combination of morphological observations, and morphometrical and rDNA-RFLP analysis indicated that these nematode specimens represented a new species which is described here.

MATERIALS AND METHODS

Isolation of nematode. Steinernematid nematodes were isolated from organic forest soil sampled to a depth of 20 cm. The specimens were extracted from the soil samples (*ca.* 500 ml) by the Bedding & Akhurst (1975) baiting technique using *Galleria mellonella* as bait insect. Infective juveniles were collected from the *Galleria* following the method of White (1927) and stored at 15 °C in foam.

Morphological and morphometrical studies. For light microscopy, specimens were reared on *G. mellonella*. We used infective juveniles collected for a week after their first emergence from the insect cadavers, and adults of the first generation dissected from the cadavers (Nguyen & Smart, 1995). These nematodes were killed and fixed in hot formalin 4% (50-60 °C), and kept in this solution for 24 hours. Fixed nematodes were transferred to anhydrous glycerine according to Seinhorst's (1959) rapid method as modified by De Grisse (1969). All measurements were made using a drawing tube attached to a WILD M12-83435 light microscope.

For scanning electron microscopy, fixed nematodes were transferred to a drop of 4% formalin. Ultrasonic treatment for 10 minutes was used to remove any foreign particles adhering to the body of the specimens. The nematodes were dehydrated by passing them through a gradual ethanol concentration gradient 25% (overnight), 50, 75, 95% (3 hours) and 100% (overnight) at 25

°C, and then were critical point dried with liquid CO_2, mounted on SEM stubs, coated with gold, and studied using a JOEL, LSM-840 scanning electron microscope.

rDNA-RFLP analysis. Molecular analysis was done with *S. sangi* sp. n., and *S. kraussei* (Belgium). DNA was extracted from a single adult female using a modification of the methods of Joyce *et al.* (1994) and Reid *et al.* (1997). Individual nematodes were cut in 8 µl of worm lysis buffer (500 mM KCl, 100 mM Tris-Cl pH 8.3, 15 mM $MgCl_2$, 10 mM DTT, 4.5% Tween 20, 0.1% gelatin) on ice. The nematode fragments were transferred in 4 µl of the buffer to an Eppendorf tube to which 5 µl of dd. H_2O and 1 µl of proteinase K (600 µg/ml) were added. After freezing (- 70 °C for 1 h) the tubes were incubated at 65 °C for 1 h, and then at 95 °C for 10 min.

After centrifugation (1 min; 13000 g) of the tubes, 10 µl of the DNA suspension were added to a PCR reaction mixture containing 4 µl of 10X PCR buffer, 1 µl of $MgCl_2$ (25 mM), 1 µl of dNTP mixture (10 mM each), 0.2 µl (500 nM) of each primer, 1.5U *Taq* polymerase and 33.3 µl of dd. H_2O to a final volume of 50 µl. Primers 18S (5'-TTGATTACGTCCCTGCCCTTT-3') and 26S (5'-TTTCACTCGCCGTTACTAAGG-3') as described by Vrain *et al.* (1992) were used in the PCR reaction. The PCR amplification temperature/time conditions were: 2 min 92 °C; 35 cycles of 30 sec 92 °C, 30 sec 54 °C, and 2 min at 72 °C. After the final cycle a 10 min polymerisation period at 72 °C was done. Following DNA amplification, 5 µl product were loaded on a 1% agarose gel for electrophoresis in 1X TAE buffer. After electrophoresis, DNA was visualised and photographed under UV light. The product remaining was stored at − 20 °C.

Five µl of PCR product were digested with 17 restriction enzymes in buffer stipulated by the manufacturer. The digested DNA was loaded on a 1.5% agarose gel, separated by electrophoresis at 130V for 3 hours, stained with ethidium bromide, visualised, and photographed under UV light.

DESCRIPTION

Steinernema sangi sp. n. (Figs. 1 & 2, Table 1)

Holotype (Male, first generation): L = 1590 µm; body width = 166 µm; distance from anterior end to excretory pore = 90 µm; total oesophagus = 168 µm; anal body width = 45 µm; tail length = 39 µm; spicule length = 65 µm; gubernaculum = 38 µm; a = 9.6; b = 9.5; c = 41; D = 0.5; E = 2.3; SW = 1.4, GS = 0.6.

Paratype measurements are given in Table 1.

Male (first generation). Body wide (Fig. 1A), ventrally curved, J-shaped or C-shaped when heat-killed. Cuticle smooth. Head rounded, offset from the body with distinct labial papillae. SEM face view with six outer labial papillae and 4 cephalic papillae (Fig. 2A). Amphids inconspicuous. Mouth opening usually funnel-shaped with two sclerotised elliptical rings on the walls. Oesophagus muscular with cylindrical procorpus; metacorpus distinct, swollen and non-valvate; isthmus distinct; basal bulb pyriform, valve not observed. Nerve ring surrounding isthmus just above basal bulb. Cardia prominent and protruding into intestine lumen. Excretory pore anterior to nerve ring at middle of oesophagus. Excretory duct less cuticularised than excretory pore, excretory glands swollen. Monorchic gonad reflexed. Spicules paired, curved (Figs. 1G, 1H, 2B & 2C) and golden dark yellow in colour. Spicule head short about 1/5 of spicule length (Fig. 2C). Velum large. Gubernaculum about 65% of spicule length, boat shaped, swollen at middle, proximal end with knob, ventrally curved (Fig. 1G, H). A single ventral precloacal and twelve pairs of genital papillae, seven pairs preanal situated subventrally in two rows and one pair laterally close to the level of second preanal papillae from anus. Three pairs caudal, subventral and one pair caudal, subdorsal. Phasmids inconspicuous. Tail shorter than anal body width with a small mucron (Fig. 2B) not always observed in permanent mounts.

Female (first generation). Female larger than male. Body robust, spiral shaped or C-shaped when heat-killed (Fig. 1E). Anterior end rounded, not offset from the body. SEM face view with six labial papillae and 4 cephalic papillae. Pore-like amphids located behind outer labial papillae (Fig. 2D). Mouth opening cup-shaped to funnel-shaped. Cuticle smooth, lateral field not observed. Excretory pore anterior to nerve ring at middle of oesophagus. Oesophagus muscular; procorpus cylindrical, metacorpus swollen non-valvate, isthmus distinct, basal bulb pyriform, valve not observed. Nerve ring surrounds isthmus or above basal bulb (Fig. 1C). Cardia prominent and protruding into intestine lumen. Didelphic, amphidelphic gonads, reflexed; filled with eggs (Fig. 1E). Vulva a transverse slit (Fig. 2E) at mid-body. Vulva lips protruding from the body (Fig. 1F). Epiptygma absent. Vagina short, straight, with muscular walls.

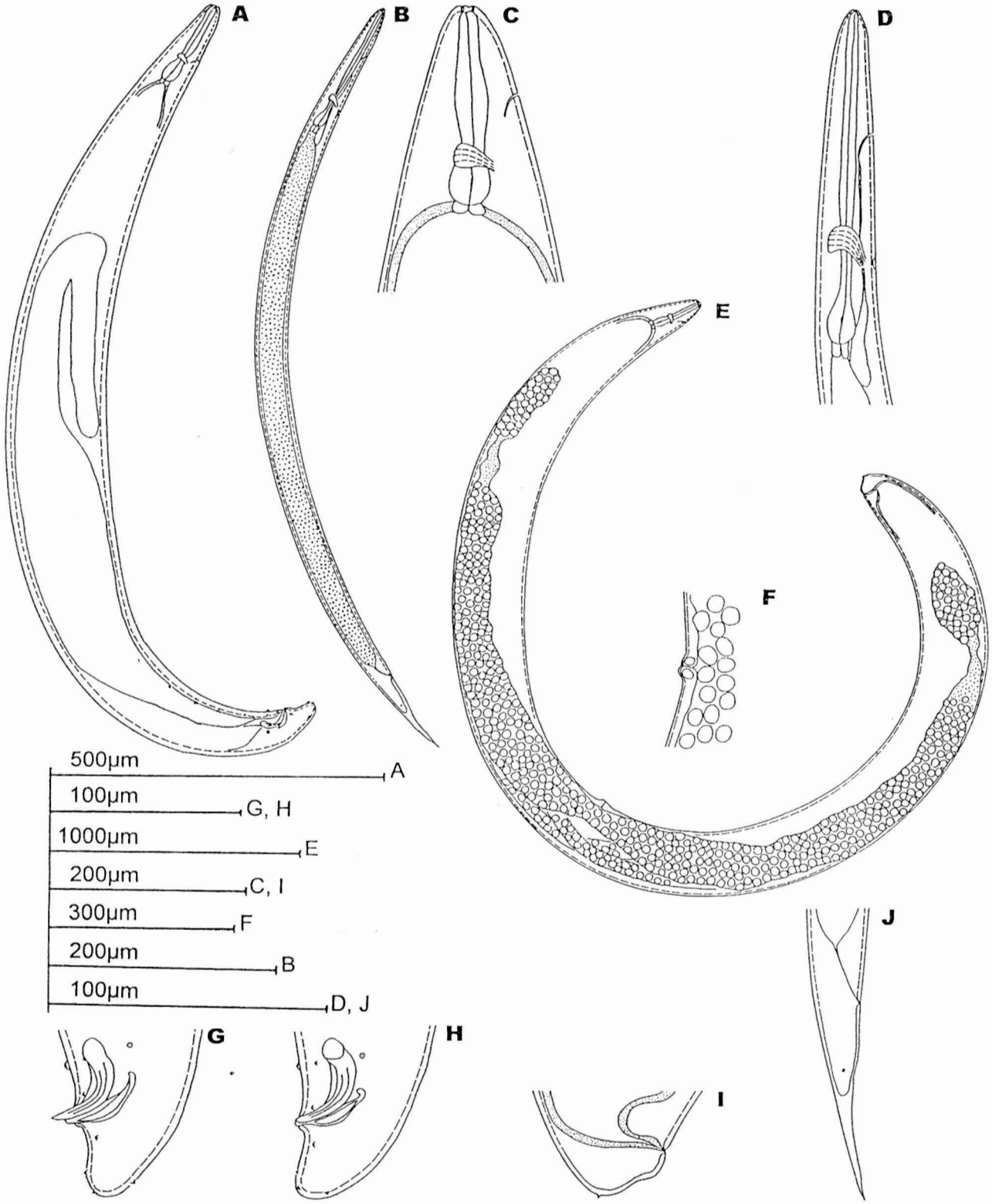

Fig. 1. *Steinernema sangi* sp. n. A, G & H: Male first generation. A: Entire view; G & H: Tail in lateral view. C, E, F, I: Female first generation. C: Oesophagus region; E: Entire view; F: Vulva region; I: Tail in lateral view. B, D & J: Third stage juvenile. B: Entire view; D: Oesophagus region; J: Tail in lateral view.

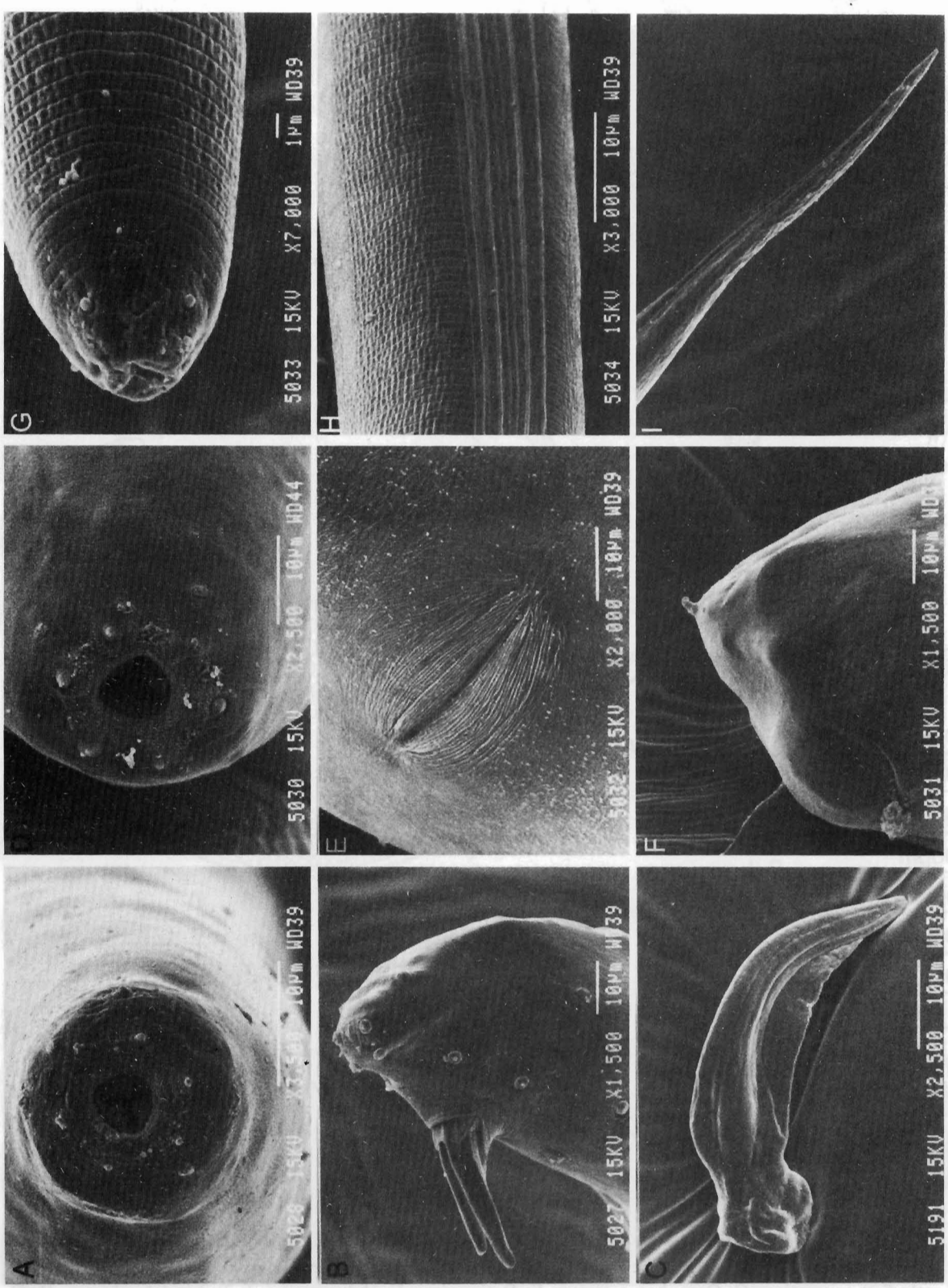

Fig. 2. SEM photographs of *Steinernema sangi* sp. n. A-C: Male first generation. A: Head; B: Tail; C: Spicules. D-F: Female first generation. D: Head; E: Vulva; F: Tail; G-I: Third stage juveniles; G: Head; H: Lateral field; I: Tail.

Table 1. Morphometrics (in µm) of *Steinernema sangi* sp. n.

Character	Infective juvenile	1st generation female	1st generation male
n	50	20	20
Body length	753±18 (704-784)	6030±679.8 (4830-7200)	1674±220.5 (1440-2325)
Width	35±2.3 (30-40)	336±22.9 (270-360)	159±27.8 (120-225)
Stoma length	–	16±1.2 (14-18)	9±1.8 (6-14)
Stoma width	–	14±1.4 (12-17)	8±1 (6-10)
EP	51±1.8 (46-54)	101±10.4 (80-121)	82±9.2 (67-99)
NR	91±3 (78-97)	158±8.2 (140-170)	126±13 (109-166)
ES	127±3.9 (120-138)	229±7.8 (216-240)	166±15 (150-221)
Testis flexure	–	–	332±50.2 (244-428)
Tail	81±3 (76-89)	49±7.3 (36-62)	32±3.9 (27-42)
H%	49±2.6 (44-52)	–	–
ABW	18± 0.6 (17-19)	111±13.6 (84-140)	43±2.6 (40-50)
Mucron length	–	6±1.2 (5-8)	Opaque
Spicule length (SP)	–	–	63±4.4 (58-80)
Spicule width	–	–	12±1.1 (10-14)
Gubernaculum length (GU)	–	–	40±3.1 (34-46)
Gubernaculum width	–	–	7±0.8 (5-9)
Vulva (%)	–	51±2.2 (43-53)	–
a	22±1.3 (19-25)	18±2 (14-21)	10.6±1.2 (8.7-12.5)
b	5.9± 0.2 (5.6-6.3)	26±2.9 (20-31)	10.1±1 (8.8-12.0)
c	9.3±0.3 (8.7-10.2)	125±18.9 (99-170)	52±7.7 (40-75)
D% = EP/ES x 100	40±1.7 (36-44)	44±4.7 (35-51)	49±5.8 (42-63)
E% = EP/tail length x 100	62±3.1 (56-70)	209±23.9 (162-249)	255±31.5 (209-341)
SW = SP/ABW	–	–	1.5±0.1 (1.2-1.6)
GS = GU/SP	–	–	0.6±0.04 (0.5-0.7)

Tail shorter than anal body width with mucron (Figs. 1I & 2F). Phasmids inconspicuous.

Infective juveniles. Body ventrally curved when heat-killed (Fig. 1B), annulated. Sheath (second-stage cuticle) present, sometimes lost. Head narrow. SEM face view with 4 prominent cephalic papillae (Fig. 2G). Pore-like amphids before cephalic papillae. Mouth and anus closed. Oesophagus narrows with slender isthmus surrounded by nerve ring. Basal bulb distinct, pyriform; valve not observed. Cardia prominent. Excretory pore anterior to middle of oesophagus. Hemizonid distinct at isthmus level (Fig. 1D). Lateral field with eight ridges (at mid-body), submarginal and central pair less distinct (Fig. 2H). Tail elongated, conoid and straight without mucron (Fig. 2I). Hyaline portion well pronounced, about 39% of tail length. Phasmids distinct in anterior half of tail (Fig. 1J).

Type locality. The forest of Xuanmy, Thuongxuan, Thanhhoa province, Vietnam (longitude 105°14'E, latitude 19°57'N, altitude 600 m above sea level). Type host cannot be attributed; the species was isolated using *Galleria mellonella* as bait. Symbiotic bacterium present has not been identified, and emits a characteristic aroma.

Type material. Holotype male (first generation) and paratypes deposited in the University of Gent, Institute for Zoology, Belgium. Paratype slides with 6 males, 6 females of first generation and 10 infective juveniles deposited in the Department of Nematology, Institute of Ecology and Biological Resources, National Centre for Science and Technology, Nghiado, Caugiay, Hanoi, Vietnam.

Molecular diagnosis. Eight (*Bsiz*I, *Hin*dIII, *Hin*fI, *Kpn*I, *Mva*I, *Pst*I, *Pvu*II, and *Xba*I) out of 17 enzymes did not restrict the PCR amplified product of the two species. The other 9 enzymes each produced RFLP patterns that were different

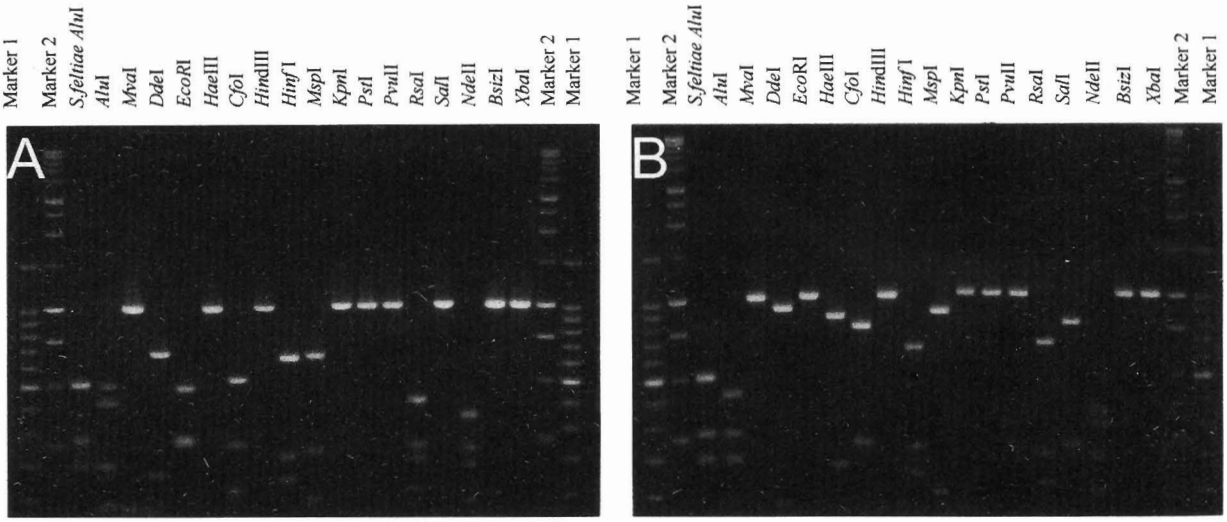

Fig. 3. RFLP patterns of PCR products of the ITS regions of two *Steinernema* species digested by 17 restriction enzymes with a positive control (*Alu*I restriction digest of the ITS region from *S. feltiae*). A: *Steinernema sangi* sp. n. ; B: *S. kraussei*. Marker 1 – 1 Kb, marker 2 – 100 bp.

for the two species (Fig. 3).

Differential diagnosis. *Steinernema sangi* sp. n. is characterised by a combination of morphological, mophometrical and rDNA-RFLP features. The infective juveniles have a lateral field with eight ridges, with the submarginal and central pair less distinct. Consequently, the isolate is morphologically most similar to *S. kraussei* (Hominick *et al.*, 1997), however, it can be easily distinguished from this species by the shorter body length of the infective juvenile (753 *vs* 951 μm), a more anterior position of the excretory pore of the infective juvenile (51 *vs* 63 μm) and a longer spicule length (63 *vs* 49 μm).

Steinernema sangi sp. n. can be distinguished from *S. kraussei* by RFLPs obtained after digestion of the ITS-region by each of the enzymes: *Alu*I, *Cfo*I, *Dde*I, *Eco*RI, *Hae*III, *Msp*I, *Nde*II, *Rsa*I, and *Sal*I (Fig. 3).

ACKNOWLEDGEMENTS

The authors thank ABOS/VLIR for the scholarships enabling Phan Ke Long to study in Belgium; Dr S.E. Spiridonov for critical reading of the manuscript; Mrs. Rita Van Driessche for assistance in preparing SEM photographs; and Dr Nguyen Van Sang, Zoology Department of the Institute of Ecology and Biological Research, Hanoi, Vietnam for collecting and providing the soil sample.

REFERENCES

Bedding, R.A. & Akhurst, R.J. 1975. A simple technique for the determination of insect parasitic rhabditid nematodes in soil. *Nematologica* 2: 109-110.

Bedding, R.A. 1990. Logistics and strategies for introducing entomopathogenic nematodes technology into developing countries. In: *Entomopathogenic nematodes in biological control* (R. Gaugler & H.K. Kaya. Eds.). pp. 233-245. Florida, CRC press.

De Grisse, A.T. 1969. Redescription ou modifications de quelques techniques utilisées dans l'étude de nématodes phytoparasitaires. *Mededelingen van de Rijksfaculteit van Landbouwetenschappen-Gent.* 34: 315-359.

Hominick, W.M., Briscoe, B.R., del-Pino, F.G., Heng, J., Hunt, D.J., Kozodoy, E., Mracek, Z., Nguyen, K.B., Reid, A.P., Spiridonov, S., Stock, P., Stuhan, D., Waturu, C. & Yoshida, M. 1997. Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. *Journal of Helminthology* 71: 271-298

Joyce, S.A., Reid, A., Driver, F. & Curran, J. 1994. Application of polymerase chain reaction (PCR) methods to identification of entomogenic nematodes. In: *COST 812 Biotechnology: Genetics of entomopathogenic nematode bacterium complexes.* Proceeding of Symposium & Workshop, St. Patrick's College, Maynooth, Co. Kildare, Ireland. (A.M. Burnell, R.U. Ehlers, & J.P. Masson. Eds.) pp. 178-187. Luxembourg, European Commission, DG XII.

Nguyen K.B. & Smart, Jr.,G.C. 1996. Identification of entomopathogenic nematodes in the Steinernematidae and Heterorhabditidae (Nemata: Rhabditida). *Journal of Nematology* 28: 286-300.

Nguyen, N.C., Nguyen, V.T., Phan, K.L. & Lai, P.H. 1999. Preliminary surveys on natural occurrence of entomopathogenic nematodes in Vietnam. *Biology* 21: 94-100.

Reid, A.P., Hominick, W.M. & Briscoe, B.R. 1997. Molecular taxonomy and phylogeny of entomopathogenic nematodes species (Rhabditida: Steinernematidae) by RFLP analysis of the ITS region of the ribosomal DNA repeat unit. *Systematic Parasitology* 37: 187-193.

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

Vrain, T.C., Wakarchuk, D.A., Levesque, A.C. & Hamilton, R.I. 1992. Intraspecific rDNA restriction fragment length polymorphisms in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15: 563-574.

White, G.F. 1927. A method for obtaining infective nematodes larvae from culture. *Science* 66: 302-303.

Phan Ke Long, Nguyen Ngoc Chau, Moens M. *Steinernema sangi* sp. n. (Rhabditida: Steinernematidae) из Вьетнама.

Резюме. Приводится описание нового вида *Steinernema sangi* sp. n., изолированного из почвы в провинции Тхань Хоа в северной части Вьетнама. Латеральные поля инвазионных личинок с 8-ю кутикулярными ребрами. Субмаргинальные и центральные ребра латерального поля менее заметны, что сближает *S. sangi* sp. n. с *S. kraussei*. Однако, *S. sangi* sp. n. отличается от *S. kraussei* более коротким телом инвазионных личинок (753 мкм), смещеной кпереди экскреторной порой (51 мкм) и более длинными спикулами (63 мкм). *Steinernema sangi* sp. n. отличается также от *S. kraussei* по результатам RFLP-анализа ITS-участка рибосомального гена при использовании рестриктаз *Alu*I, *Cfo*I, *Dde*I, *Eco*RI, *Hae*III, *Msp*I, *Nde*II, *Rsa*I, и *Sal*I.