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Steinernema tami sp. n. (Rhabditida: Steinernematidae) from Cat Tien Forest, Vietnam

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Summary. *Steinernema tami* sp. n. from Cat Tien Forest, Vietnam has characteristics of species in the "*carpocapsae*" group (IJ3 body length < 600 μm). It can be distinguished from species in this group by the mean IJ3 body length (530 μm), which is shorter than that of *S. kushidai*, *S. scapterisci*, *S. carpocapsae*, and *S. abbasi* (589, 572, 558, and 541 μm) but longer than that of *S. rarum*, *S. ritteri*, and *S. siamkayai* (511, 510, and 446 μm). In the male, the spicule of the new species (77 μm) is shorter than that of *S. scapterisci* (83 μm) but longer than that of all other species in the group (< 75 μm); ratio SW (spicule length/anal body width) of the first generation male (2.0) is smaller than that of *S. scapterisci* (2.5) but greater than that of other species (< 1.8). *Steinernema tami* sp. n. is further differentiated from other species in the group by the profiles of at least 3-4 enzymes in rDNA-RFLP analysis.
Key words: "*carpocapsae*" group, entomopathogenic nematodes, South East Asia, *Steinernema tami* sp. n., taxonomy, Vietnam.

Several steinernematid species described during the last decade were found in tropical and subtropical regions, e.g. *Steinernema scapterisci* from Uruguay (Nguyen & Smart, 1990), *S. neocurtillae* from Florida, USA (Nguyen & Smart, 1992), *S. puertoricense* from Puerto Rico (Roman & Fugueroa, 1994), *S. abbasi* from Oman (Elawad *et al.*, 1997), *S. karii* from Kenya (Waturu *et al.*, 1997), *S. monticolum* from Korea (Stock *et al.*, 1997) and *S. siamkayai* from Thailand (Stock & Kaya, 1998). As the number of species increases, the differentiation of species based on morphological characters becomes more complicated. Molecular methods have established in taxonomy and have been used successfully to provide additional information for separating steinernematid species (Reid *et al.*, 1997). Several steinernematid isolates from tropical regions were found to be new species, and their descriptions are under preparation in different laboratories. A new species of steinernematids from tropical lowland forest in Vietnam is described here.

MATERIALS AND METHODS

Isolation. *Steinernema tami* sp. n. was isolated from the "Cat Tien Forest", a National Park in Vietnam. Soil samples of approximately 1 kg each were collected during the dry season in the forest which mainly comprised of *Lagerstroemia, Afzelia, Ficus* and *Dipterocarpus* trees growing in the central area of the national park. Last instar larvae of the greater wax moth, *Galleria mellonella,* were used to bait entomopathogenic nematodes (EPN) from an aliquot of 100 g of soil. Infected larvae were placed on White traps (White, 1927) to collect infective juveniles. The nematode population collected is maintained in the laboratory on *G. mellonella* at the Institute of Ecology and Biological Resources, National Center for Science and Technology, Hanoi, and at the Institute of Parasitology, Russian Academy of Sciences, Moscow.

Light microscopy. Isolated steinernematids were reared in the laboratory on *Galleria mellonella* larvae.

Adult nematodes of different generations were obtained by dissecting infected *Galleria* larvae, and infective juveniles were collected from a White trap 13-16 days after the *Galleria* died. Specimens collected were fixed in 6% formalin at 80 °C, and processed to glycerine by a slow evaporation method (Seinhorst, 1959) at 37 °C. Measurements were obtained with the aid of a drawing tube. All drawings and photographs were taken from specimens in lateral position unless otherwise specified, using a "Zeiss" photomicroscope III with differential-interference contrast (DIC).

Scanning electron microscopy. Nematodes were fixed in 4% formalin, washed in 5 changes of 0.1 M sodium cacodylate buffer, and postfixed in 2% osmium tetroxide for 12 hours. All other steps were as described by Nguyen & Smart (1990, 1994, 1995).

Molecular analysis. Nematodes used for this study were *S. tami* sp. n., *S. carpocapsae* (strain All), *Steinernema* sp. "Malaysia", *Steinernema* sp. "SSL 1" (Sri Lanka site 107) and *Steinernema* sp. "SSL 2" (Sri Lanka site 85) and *S. siamkayai.* The extraction of DNA, the PCR amplification of the internal transcribed spacer (ITS) region, and the restriction fragment length polymorphism (RFLP) profiles were obtained using the methods described by Hominick *et al.* (1997).

DESCRIPTION
Steinernema tami sp. n.
(Figs. 1-6, Table 1-2)

Holotype (Male, first generation): L = 1377 μm; body width = 125 μm; distance from anterior end to excretory pore = 63 μm; total oesophagus = 153 μm; anal body width = 38 μm; tail length = 26 μm; spicule (along chord) = 75 μm; spicule (along arc) = 82 μm; gubernaculum = 52 μm; a = 11.0; b = 9.0; c = 53.0; D = 0.4; E = 2.4, SW = 1.97.

Paratype measurements are given in Table 1.

First generation males. Body slightly swollen in the middle. Heat-killed specimens C- or J-shaped (Figs. 2A & 3B, C). Cuticle with faint transverse annulation, inconspicuous under light microscope. Head rounded with six low lips, 6 labial papillae (Figs. 1B & 3A) and four prominent cephalic papillae. Mouth opening more or less circular. Stoma about 5-6 μm deep with two sclerotized rings. Anterior ring lightly sclerotized, almost rounded, posterior ring more sclerotized, smaller, elliptical in shape and located on frontal border of oesophageal tissue. Oesophagus with procorpus, slightly swollen metacorpus (Fig. 2B), indistinct isthmus and pyriform basal bulb. Nerve ring surrounding isthmus. Cardia prominent. Excretory pore located slightly anterior to mid-oesophagus, excretory duct about 20 μm long, cuticularized (Fig. 2C). Excretory glands swollen, 12-18 μm wide sometimes displacing basal bulb to dorsal side. Pseudocoelom in oesophageal region filled with granular material. Distance from anterior end to testis flexure ranging from 280 to 350 μm. Different spermatogenesis stages visible in testis (Fig. 1A). In mid-testis region, the small spermatocytes disappeared and were replaced by two-three dozens of 40-50 μm large cells, variable in appearance, and often with wart-like structures on the cell membrane. Closer to the *vas deferens* these structures appear almost separated from the membrane, producing groups of refractive granules around large cells. *Vas deferens* tightly filled with both large cells and granules. Spicules curved, yellow-brownish in colour. Oblique muscles well developed in precloacal zone (Fig. 1C). Spicule head (manubrium) length about 1-1.5, rarely 2 times as long as wide (Figs. 2F & 3D-F). Spicule shaft (calomus) prominent. Blade (lamina) moderately curved bearing ribs reaching spicule tip. Velum thin. Spicula distal end bluntly pointed (Fig. 3D-F). Gubernaculum boat-shaped, proximal end slightly curved (Figs. 1C & 3G-I), in ventral view, corpus tapering gradually anteriorly, cuneus Y-shaped (Fig. 3G). Eleven pairs and a single genital papillae present, 6 pairs subventral, precloacal, a single larger precloacal midventral papilla,one pair sublateral precloacal, at level of mid-lamina, one pair subventral adcloacal, two pairs subventral postcloacal, and one pair subdorsal postcloacal. Tail dorsally convex, conoid with a bluntly rounded terminus (Fig. 3B, C). Mucron 3-4 μm long (Fig. 2E),sometimes not observed. Phasmids inconspicuous.

Second generation males. Heat killed specimens C-shaped. Anterior region similar to that of first generation males, but smaller; lips relatively more prominent. Excretory duct cuticularization discernible only for 3-4 μm from excretory pore. Testis reflexion usually behind mid-body. Spermatogenesis similar to that of first generation males, with up to 18 large cells with granules on the surface in *vas deferens.* Spicules and gubernaculum of the same colour and shape as in first generation males. Distribution of genital papillae resembling that in first generation males, but often postcloacal subventral papillae displaced on the tail terminus with a small, 2-3 μm long, mucron protruding between posteriormost pair of papillae.

First generation females. Body robust, obese, usually C-shaped when heat relaxed. Anterior end

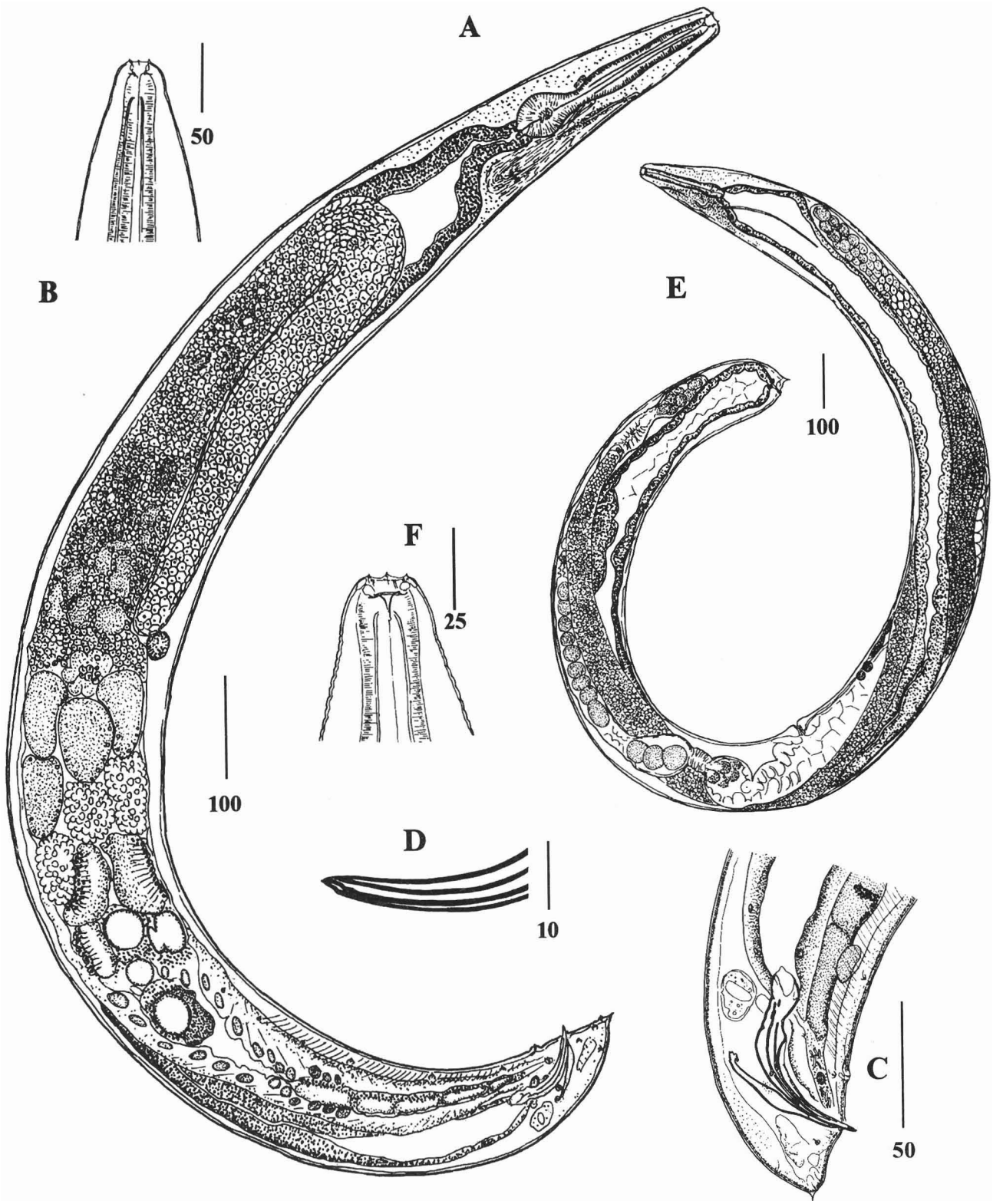

Fig. 1. *Steinernema tami* sp. n. first generation adults. A: Male, entire body; B: Male cephalic end; C: Male tail; D: Spicule, distal end; E: Female, entire body; F: Female, cephalic end. Scale bars in µm.

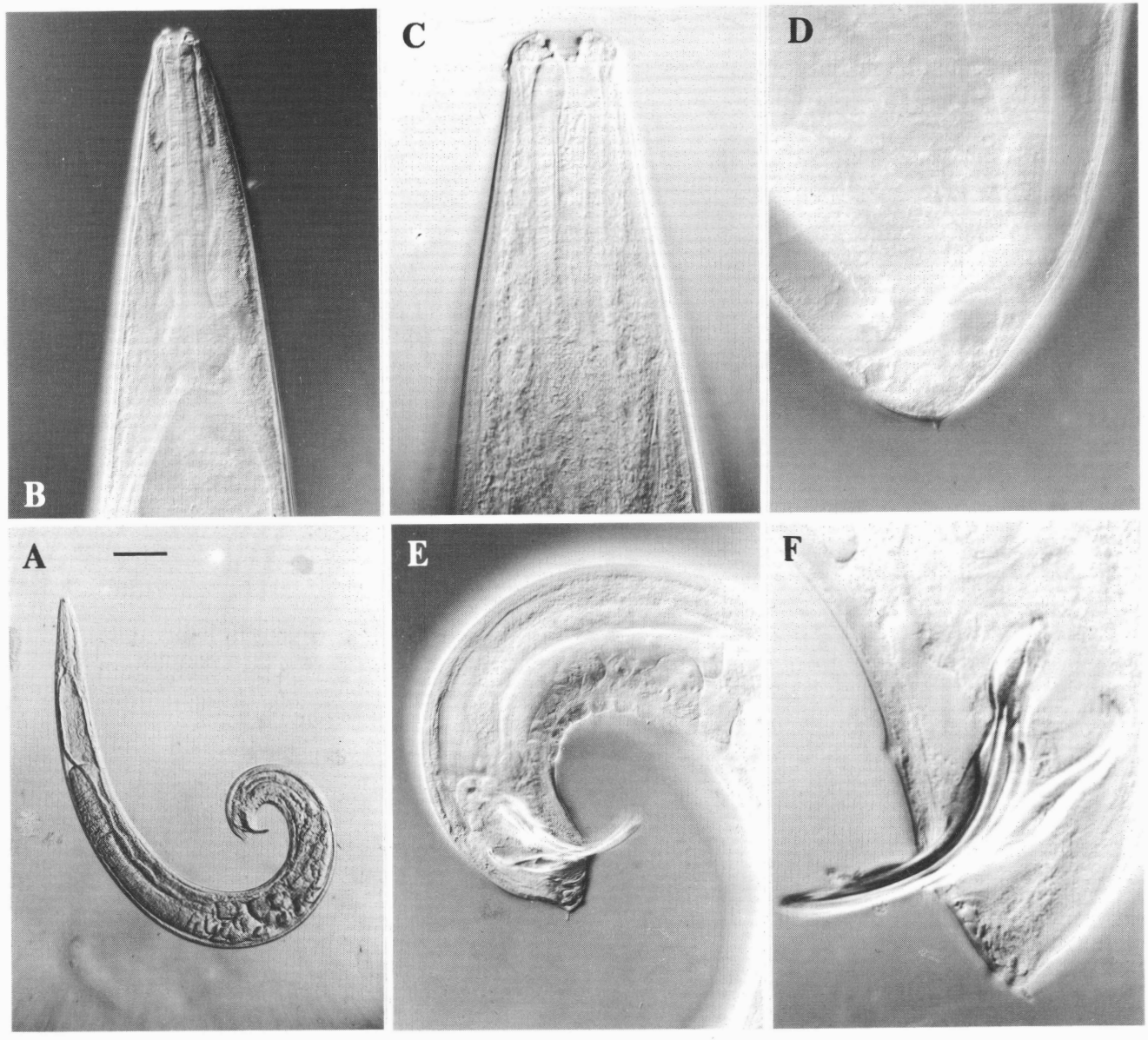

Fig. 2. *Steinernema tami* sp. n. first generation adults, DIC. A: Male, total view; B: Male oesophagus; C: Male head end; D: Female tail; E: Male posterior end; F: Copulatory apparatus, tail terminus with mucron. Bar (in A): A - 100 μm, B - 22 μm., C, D, F -10 μm, E - 26 μm.

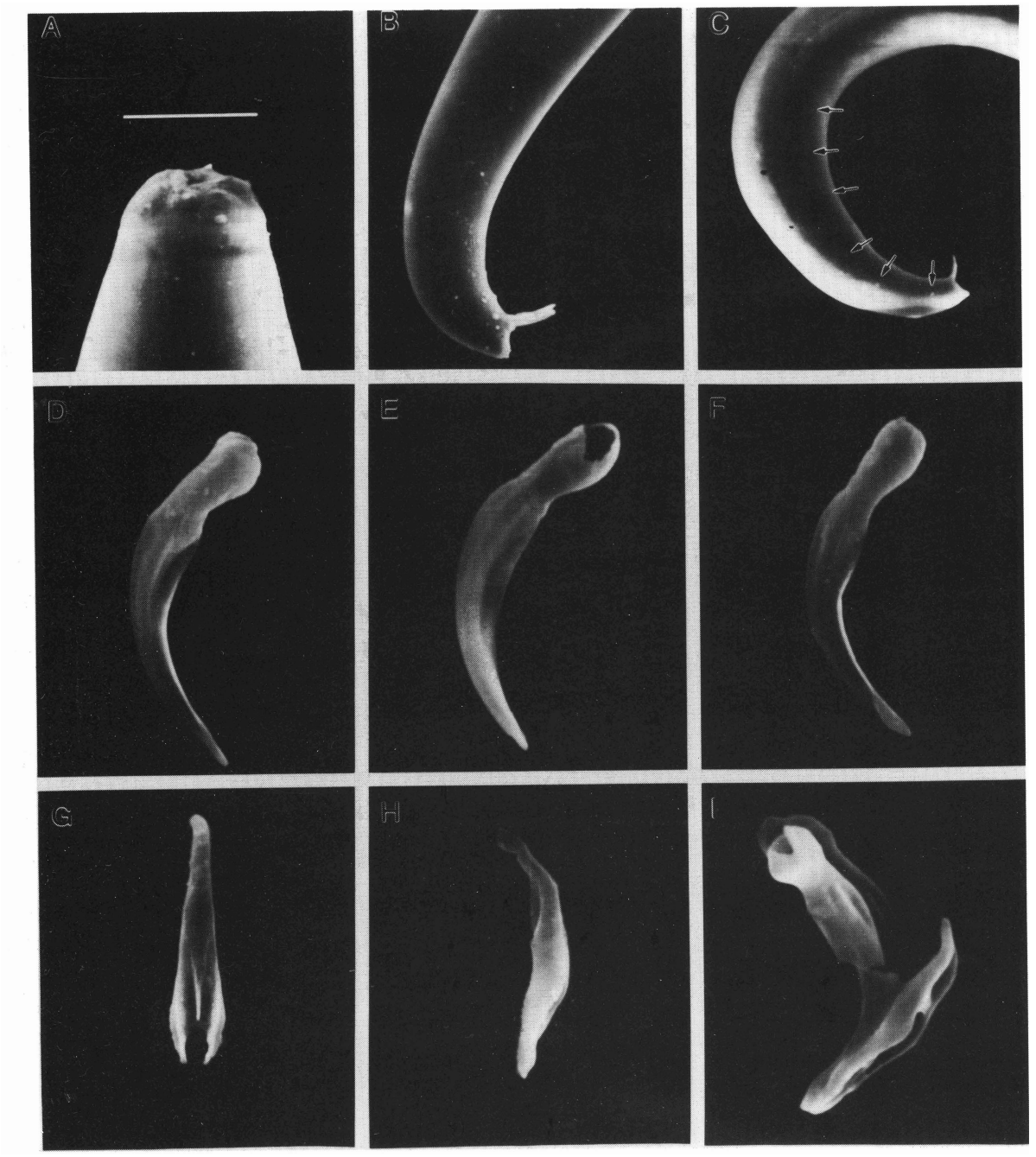

Fig. 3. *Steinernema tami* sp. n. males of 1st generation. SEM. A: Anterior region of 1st generation male showing labial and cephalic papillae; B,C: Posterior region of 1st generation males showing genital papillae and spicules; D-F: Spicules showing head, shaft and blade; G: Gubernaculum, ventral view with Y-shaped cuneus, ventrally curved anterior end and forked posterior end. H: Gubernaculum lateral view. I: Spicules (left one was broken) and gubernaculum. Scale bar (in A): A - 17.6 μm, B - 61 μm, C - 120 μm, D - I - 25 μm.

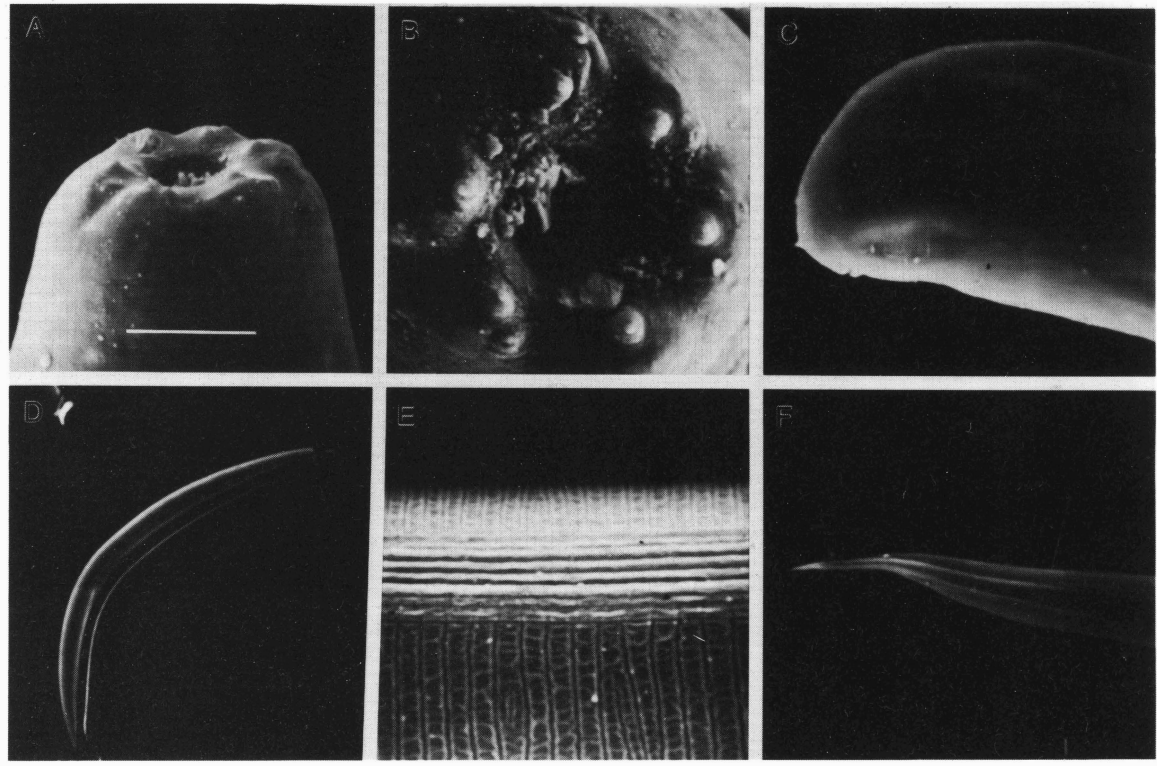

Fig. 4. *Steinernema tami* sp. n. first generation female and infective juveniles. SEM. A: Anterior region of a female showing mouth, lips, labial and cephalic papillae; B: Face view of a female showing 6 prominent labial papillae; C: Rounded tail of a female with a mucron at the end. D: Entire infective juvenile; E: Lateral field showing 8 ridges with 4 central ridges raised and 4 marginal ones lower; F: Infective juvenile showing 2 prominent submarginal ridges, marginal ridges disappeared gradually posteriorly. Bar (in A): A - 15 μm, B - 7.5 μm, C - 50 μm, D - 120 μm, E - 4.3 μm, F - 17.6 μm.

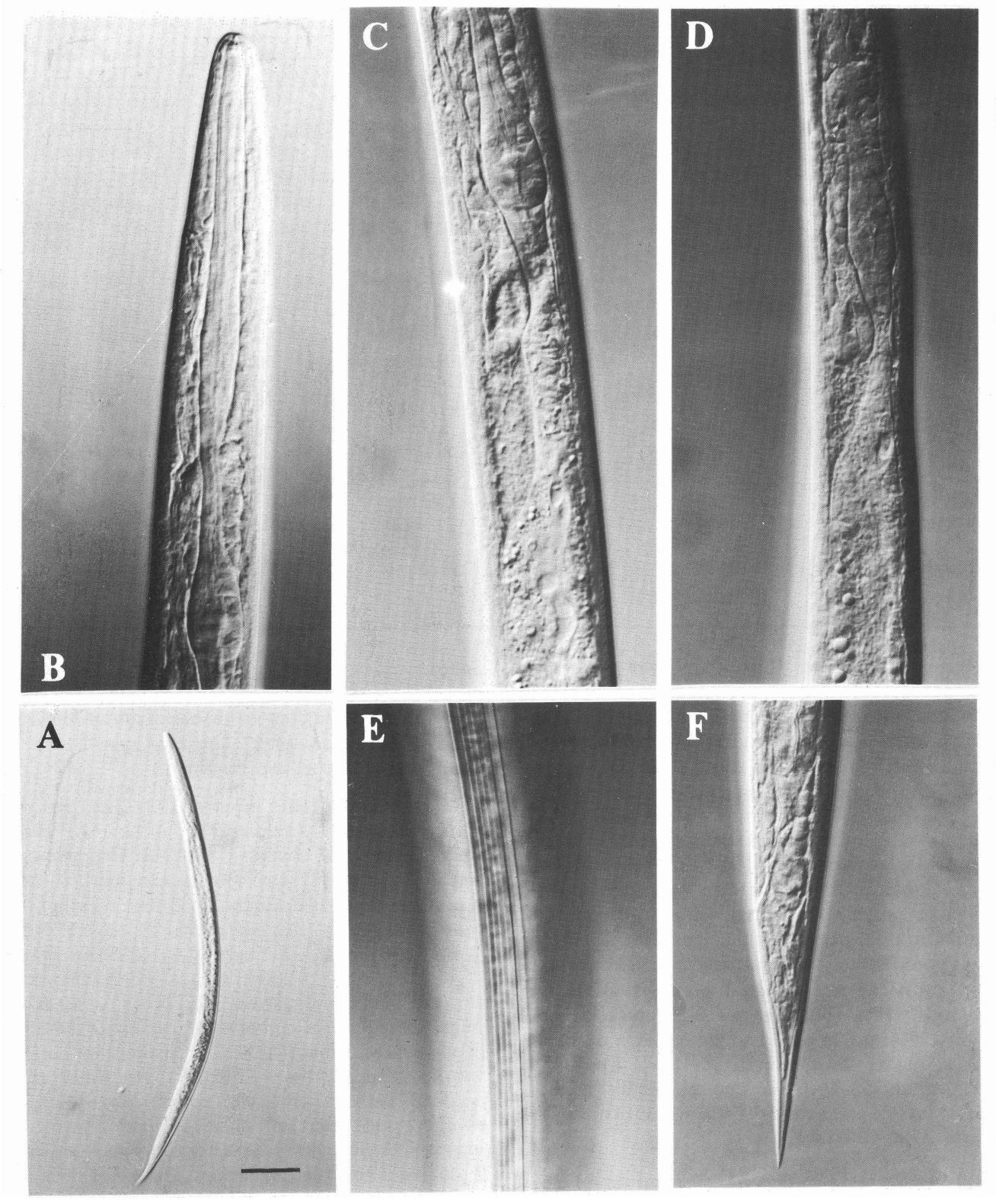

Fig. 5. *Steinernema tami* sp. n. infective juvenile. A: Entire body; B: Anterior end showing oesophagus; C: Basal portion of oesophagus showing excretory gland and bacterial pouch; D: Basal portion of oesophagus showing excretory gland but without bacterial chamber; E: Lateral field; F: Tail. Scale bar (in A): A - 66 μm, B-F - 10 μm.

Table 1. Morphometrics of *Steinernema tami* sp. n. (All measurements in μm, except for L).

Character	Male paratypes		Female paratypes		IJ3 paratypes
	1st generation (n=20)	2nd generation (n=20)	1st generation (n=20)	2nd generation (n=20)	6 month old (n=20)
Body length (mm)	1.6±0.2 (1.2-1.9)	0.9±0.05 (0.8-1.0)	5.1±1.0 (2.8-6.3)	1.6±0.1 (1.4-1.9)	0.53±0.04 (0.4-0.6)
Body width	129±17 (98-161)	59±3 (55-65)	196±23 (145-240)	93±10 (70-107)	23±2 (19-29)
Anterior to excretory pore	68±12 (43-92)	64±4 (58-72)	81±17 (50-115)	70±4 (64-77)	36±2 (34-41)
Total oesophagus	153±8 (137-166)	142±6 (131-155)	221±16 (187-245)	166±9.6 (150-184)	117±4 (110-123)
Anal body width	37±6 (25-40)	29±2 (25-32)	62±13 (45-85)	31±5 (25-42)	12±1 (11-13)
Tail length	23±5 (10-32)	18±2 (15-22)	32±10 (19-48)	28±6 (21-40)	50±4 (42-57)
Spicule length (along the chord)	72±4 (63-78)	59±3 (52-63)	–	–	–
Spicule length (along the arc)	77±4 (71-84)	67±4 (62-76)	–	–	–
Gubernaculum length	48±5 (38-55)	37±3 (31-45)	–	–	–
a	12.5±1.5 (9.6-15.0)	15±1 (13-19)	25±3 (19-31)	18±2 (15-22)	23±2 (19-28)
b	10.4±1.2 (7.2-12.5)	6.4±0.4 (5.7-7.1)	23±4 (15-29)	10±1 (9-12)	5±0.3 (3.7-5.1)
c	74±23 (53-164)	50±7 (39-63)	178±67 (58-304)	62±11 (47-79)	11±0.5 (9-11)
H (hyaline part/tail)	–	–	–	–	0.4±0.1 (0.3-0.5)
V	–	–	52±2 (47-55)	54±3 (48-58)	–
D (excretory pore/ oesophagus)	0.44±0.1 (0.3-0.6)	0.45±0.0 (0.4-0.5)	0.37±0.1 (0.2-0.5)	0.42±0.0 (0.4-0.5)	0.31±0.0 (0.28-0.34)
E (excretory pore/tail)	3.2±1.1 (2.2-7.3)	3.5±0.5 (2.9-4.6)	2.9±1.3 (1.0-6.0)	2.6±0.5 (1.7-3.5)	0.73±0.0 (0.67-0.86)
SW (spicula length/ anal body width)	2.0±0.4 (1.4-3.0)	2.1±0.2 (1.7-2.5)	–	–	–

truncate to rounded (Fig. 4A). Face view with rounded mouth, six labial papillae (Fig. 4B), and four cephalic papillae immediately posterior. Cervical part of body with coarse annulation. Lateral field inconspicuous. Stoma about 10-15 μm long and 8-10 μm wide, with two cuticular rings in the walls: anterior ring thick, posterior one smaller, more refractive and located on the oesophageal tissue (Fig. 1F). Oesophagus more or less cylindrical with isthmus. Cardia prominent, protruding into intestine lumen. Excretory pore anterior to mid-oesophagus. Excretory duct heavily cuticularized up to 30 μm from pore. Excretory gland large, elongated. Anterior ovary flexure situated about 100-200 μm posterior to basal bulb. About 10-20 amoeboid cells 20-25x30-45 μm in gonad lumen between ovary and uterus. Vagina short, sclerotized. Vulval lips usually not protruding. Developing oocytes in multiple rows. Tail short, convex-conoid with a peg-like mucron (Figs. 2D & 4C).

Second generation females. General morphology of anterior end similar to that of first generation females. Excretory duct cuticularization visible up to 5-8 μm posterior to excretory pore. Cardia relatively more developed than in first generation females, especially when gonads are less developed. Spermatozoa present in oviduct having the same morphology as in first generation females. Tail relatively longer than that in first generation females, conoid, terminus with a 2 μm long spike-like mucron. Post-anal swelling prominent.

Third stage infective juvenile (IJ3). Heat relaxed specimens ventrally arcuate (Fig. 4D & 5A). Body smoothly tapering towards both ends. Cephalic end rounded (Figs. 4D & 5B). Cuticle marked with prominent transverse annulation approximately 1 μm wide at mid-body (Figs. 4E & 5E, F). Lateral fields at mid-body with 6-8 ridges (7-9 incisures) (Figs. 4E & 5E). At mid-body, central ridges are more elevated than marginal ones (Fig. 4F). On tail, most ridges disappearing gradually, the submarginal ones become prominent (Fig. 4F). Oesophagus narrow with a slender isthmus (Fig. 5B). Basal bulb subpyriform, usually displaced towards dorsal side by overdeveloped excretory gland. Hemizonid distinct, located at isthmus level. Excretory pore cuticularized, 2 μm in diameter in lateral view. Excretory duct less cuticularized than pore. Bacterial pouch rarely seen. *Xenorhabdus* cells, when found, with poorly visible walls, tightly compressed in bacterial vesicle. Tail conoid, tapering to a finely pointed terminus. No refractive inclusion in the tip. Hyaline portion well

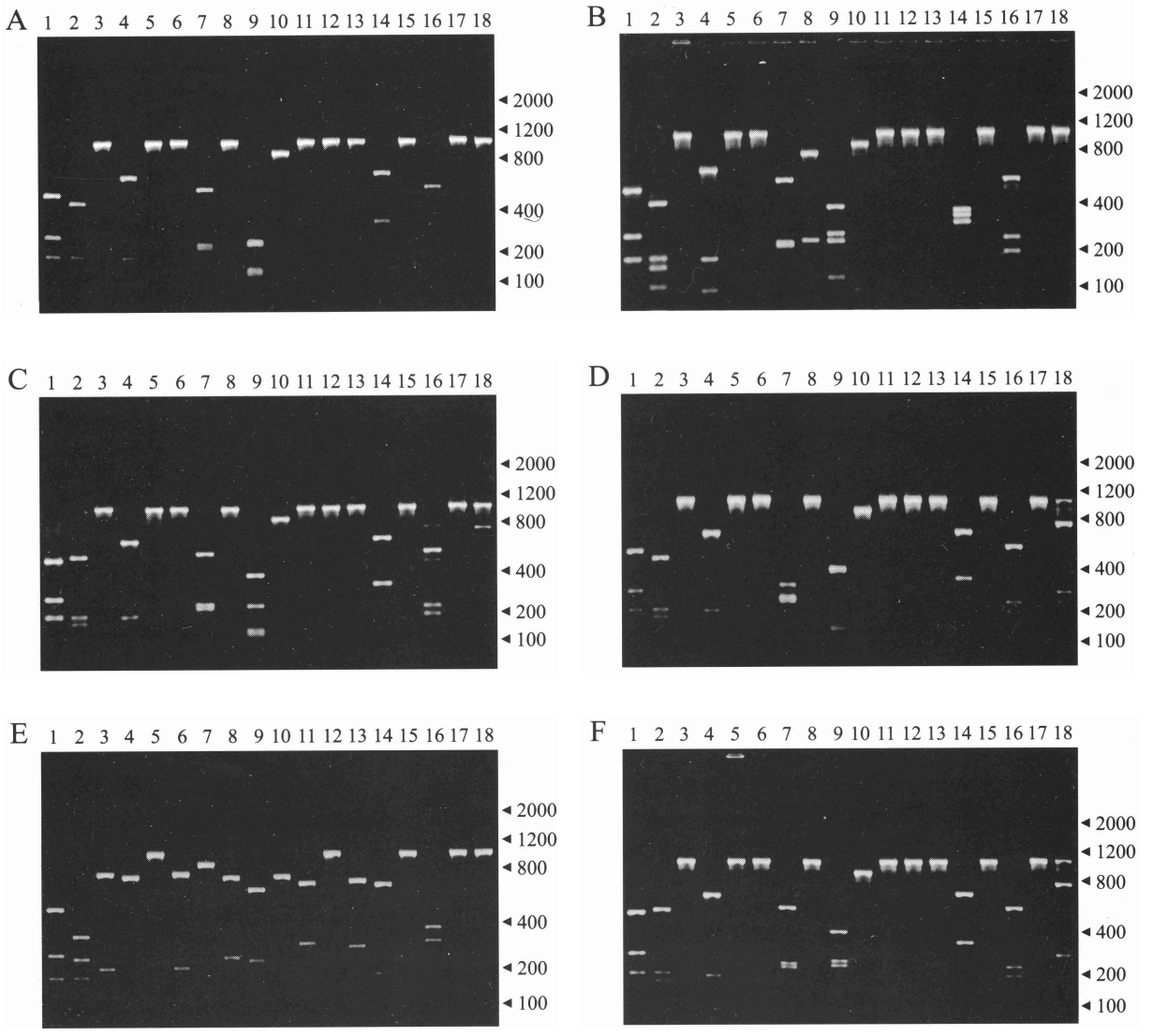

Fig. 6. RFLP patterns of PCR products of ITS (internal transcribed spacer) region of 6 nematodes digested by seventeen restriction enzymes. Fragments were separated on 1.5 % agarose gel stained with ethidium bromide. A: *S. tami* sp. n.; B: *S. carpocapsae*; C: *S. siamkayai*; D: *Steinernema* sp. "Malaysia"; E: *Steinernema* sp."SSL" 1; F: *Steinernema* sp. "SSL 2". For each species lane 1 is the ITS region of *S. feltiae* (UK site 76) cut with Alu I. The other lanes are the ITS region of that species cut by the following restriction enzymes: lane 2, *Alu*I; 3, *Bst*OI; 4, *Dde*I; 5, *Eco*RI;6, *Hae*III; 7, *Hha*I; 8, *Hin*dIII; 9, *Hin*fI; 10, *Hpa*II; 11, *Kpn*I; 12, *Pst*I; 13, *Pvu*II; 14, *Rsa*I; 15, *Sal*I; 16, *Sau*3AI;17, *Sau*96I; 18, *Xba*I. The position of the molecular weight markers is shown as base pairs.

pronounced, occupying about 39% of tail length. Phasmids distinct, located in anterior half of tail.

Type host and locality. Natural host unknown. Type locality is the primary forest in Cat Tien National Park along the Dong Nai river, Vietnam. The nematodes were recovered by *Galleria* baiting using soil samples collected on 6-12 April 1997.

Type specimens. Holotype male (first generation) and paratypes deposited in the Collection of Nematodes of Invertebrates of the Institute of Parasitology, Russian Academy of Sciences, Moscow. Slides with one male and one female of first generation deposited in the Hanoi Institute of Ecology and Biological Resources, Vietnam; German Nematode Collection in the Institute of Nematology and Vertebrate Studies in Münster, Germany and in Nematode Collection of USDA, Beltsville, USA. Living cultures maintained in Hanoi, Moscow, and at CABI Bioscience, Egham, England.

Features of *Steinernema tami* sp. n. life cycle. Optimal temperatures for the infection of *Galleria mellonella* with infective juveniles of *Steinernema tami* sp. n. were between 22 and 27 °C. Between 22

Table 2. RFLP patterns of six *Steinernema* species obtained using 17 restriction enzymes (For each restriction enzymes species with the same letter show the same RFLP pattern).

Lane	Restriction enzyme	*S. tami* sp. n.	*S. carpocapsae*	*S. siamkayai*	*Steinernema* sp. Malaysia	*Steinernema* sp. SSL 1	*Steinernema* sp. SSL 2
2	*Alu*I	A	A	B	A	C	D
3	*Bst*OI	A	A	A	A	B	A
4	*Dde*I	A	B	A	A	C	A
5	*Eco*RI	A	A	A	A	A	A
6	*Hae*III	A	A	A	A	B	A
7	*Hha*I	A	A	A	B	C	A
8	*Hin*dIII	A	B	A	A	B	A
9	*Hin*fI	A	B	C	C	D	B
10	*Hpa*II	A	A	A	A	A	A
11	*Kpn*I	A	A	A	A	B	A
12	*Pst*I	A	A	A	A	A	A
13	*Pvu*II	A	A	A	A	B	A
14	*Rsa*I	A	B	A	A	C	A
15	*Sal*I	A	A	A	A	A	A
16	*Sau*3AI	A	A	A	A	B	A
17	*Sau*96I	A	A	A	A	A	A
18	*Xba*I	A	A	B	B	A	B

and 24 °C the first generation males and females were observed inside *Galleria* cadavers on days 3 or 4 after inoculation. First generation adults not present on days 6 or 7, however numerous feeding juvenile stages of second generation were found in *Galleria* bodies. Young males and females of the 2nd generation were present in cadavers on days 8 or 9. Mass migration of the infective juveniles occured on days 13-16 depending on humidity. Infective juveniles were stored at room temperature (18-24 °C). Prolonged storage at 3-5 °C substantially increased juvenile mortality.

Molecular diagnosis. Table 2 shows the RFLP patterns yielded from each of the six species with seventeen different restriction enzymes. For each restriction enzyme, species with the same letter show the same RFLP pattern. The RFLP for all species (except SSL 1) are similar, the only restriction enzyme differentiating *S. tami* sp. n. from the other five species being *Hin*fI (Table 2). From the above results, it is evident that *S. tami* sp. n., *S. carpocapsae*, *S. siamkayai*, *Steinernema* spp. Malaysia and SSL 2 are genetically similar (Fig. 6).

Differential diagnosis. Morphological data indicate that *S. tami* sp. n. belongs to the group of species related to *S. carpocapsae*, referred to as the "*carpocapsae*" group (IJ3 body length < 600 µm). The new species can be distinguished from other species of the group by the average body length of infective juveniles, 530 µm, which is shorter than that of *S. kushidai* (589 µm), *S. scapterisci* (572 µm), *S. carpocapsae* (558 µm), *S. abbasi* (541 µm); but longer than that of *S. rarum*, *S. ritteri*, and *S. siamkayai* (511, 510 and 446 µm).

The prominent difference between *S. tami* sp. n. and *S. siamkayai* is the infective juvenile tail length, 35.5 (31-41) µm in *S. siamkayai vs* 50 (42-57) µm in *S. tami* sp. n. Other clearly distinguishing characters are: E% in juveniles: 96 (85-112)% in *S. siamkayai vs* 73 (67-86)% in *S. tami* sp. n., in juveniles the position of the excretory pore from the anterior end: 57 (48-67) µm in *S. siamkayai vs* 36 (34-41) µm in *S. tami* sp. n. The position of the excretory pore in infective juvenile expressed as a percentage of the oesophagus length (D%) distinguishes the *S. tami* sp. n. [D% = 31 (28-34)] from other steinernematids that have short infective stage juveniles: *S. abbasi*, D% = 53 (51-58), *S. riobrave* D% = 49 (45-55), and *S. ritteri* D% = 46 (44-50). Infective juveniles of *S. tami* sp. n. are quite different from those of *S. kushidai*, but similar to *S. carpocapsae* and *S. scapterisci*, with D% ranging between 31-34%, tail length about 50 µm and hyaline portion expressed as a percentage of total tail length H% = 38-39. The E% of *S. tami* sp. n. juveniles is about 73, similar to that of *S. scapterisci*, but larger than that of *S. carpocapsae* (E% = 60). The new species can be distinguished from *S. carpocapsae* and *S. scapterisci* by SEM observation of spicules and the gubernacula of these two species (Hominick *et al.*, 1997, Fig. 4) and those of *S. tami* sp. n. (Fig. 3).

Steinernema tami sp. n. also differs from other species in the group by male characteristics. Except for *S. scapterisci*, in which the spicule length (SL)

= 83 μm, spicule length of *S. tami* sp. n. (77 μm) is longer than that of all other species in "*carpocapsae*" group: *S. siamkayai* with SL = 75 μm, *S. ritteri* with SL = 69 μm; *S. carpocapsae*, SL = 66 μm; *S. abbasi*, SL = 65 μm; *S kushidai*, SL = 63 μm; *S. rarum*, SL = 47 μm. The ratio SW also can be used to distinguish *S. tami* sp. n. from other species. SW of *S. tami* sp. n. (2.0) is smaller than that of *S. scapterisci* (2.5) but larger than that of all other species of the group: *S. carpocapsae* with SW = 1.7; *S. siamkayai*, SW = 1.7; *S. abbasi*, SW = 1.6; *S. ritteri*, SW = 1.6; *S. kushidai*, SW = 1.5, and *S. rarum*, SW = 0.94 (Nguyen & Smart, 1996).

The new species can be also distinguished from closely related species by rDNA-RFLP analysis. RFLP profiles show that *S. tami* sp. n. differs from other species of this group in the profiles of at least 3 or 4 enzymes.

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Фам Ван Лык, Нгуен Ба Хыонг, Рейд А.П., Спиридонов С.Э. *Steinernema tami* sp. n. (Rhabditida: Steinernematidae) из леса Кат Тиен, Вьетнам.

Резюме. Описывается новый вид *Steinernema tami* sp. n. из леса Кат Тиен, Вьетнам из группы видов *carpocapsae*. Новый вид отличается от других видов этой группы средней длиной инвазионных личинок. Средняя длина спикул самцов нового вида, измеренная по дуге (77 мкм) меньше, чем у *S. scapterisci* (83 мкм), но больше чем у всех остальных видов этой группы. *Steinernema tami* sp. n. отличается от других видов по спектрам рестрикции участка рибосомальной ДНК по 3-4 ферментам.