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Steinernema siamkayai n. sp. (Rhabditida: Steinernematidae), an entomopathogenic nematode from Thailand

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Abstract

Steinernema siamkayai n. sp. (Rhabditida: Steinernematidae) is a new entomopathogenic nematode isolated in Lohmsak, Thailand. Morphology, hybridisation and molecular studies indicated the distinctness of *S. siamkayai* n. sp. from other *Steinernema* spp. Diagnostic characters include: the total body length (398–495 μm) and tail length (31-41 μm) of the third-stage infective juvenile and lateral field pattern with 6-8 longitudinal ridges; the presence of a tail mucro in both first and second generation females and males; the size and shape of the spicules and gubernaculum, and the arrangement of the genital papillae of the first and second generation males; and the shape of the vulva and tail of the first generation female and second generation female.

Introduction

Entomopathogenic nematodes in the families Steinernematidae Travassos, 1927 and Heterorhabditidae Poinar, 1976 have great potential for use as biological control agents for soil-inhabiting insect pests (Gaugler & Kaya, 1990). Recent surveys have reported the presence of new isolates/species from Sri Lanka (Amarasinghe et al., 1994), Puerto Rico (Roman & Figueroa, 1994), Portugal (Rosa et al., 1994), Canada (Mracek & Webster, 1993), Argentina (Stock, 1995), Korea (Choo et al., 1995; Stock et al., 1997) and China (Jian, 1997).

During a survey of entomopathogenic nematodes in tamarind orchards in northern Thailand, a nematode belonging to the genus *Steinernema* Travassos, 1927 was recovered. Morphological, molecular and cross-breeding studies, showed that this nematode is an undescribed new species which is described and illustrated below.

Materials and methods

Origin of the isolate

Soil samples were collected during May 1996 from a sweet tamarind orchard at Lohmsak, Petchabun Province, in northern Thailand. A single composited sample (≈2 kg) was taken at a depth of 15 cm from different points in the tamarind orchard. The soil type was sandy clay loam with a moisture level of <50%. Soil samples were baited with *Galleria mellonella* (L.) larvae (Bedding & Akhurst, 1975). Dead larvae were placed into White traps (White, 1927) and infective juveniles collected and used to infect live *G. mellonella* larvae (Kaya & Stock, 1997). First and second generation adult nematodes were obtained by dissecting infected last instar *G. mellonella* larvae 2–4 and 5–7 days, respectively, after the insects died. Third stage infective juveniles were obtained upon emergence from the cadavers 8–11 days after the *G. mellonella* larvae died.

Light microscopy

First and second generation adults and third stage infective juveniles were examined either live or heat-

killed and relaxed in Ringer's solution (60°C). They were fixed in triethanolamine formalin (TAF) (Courtney et al., 1965) and processed to glycerine (Seinhorst, 1959). Quantitative measurements were made using a Leitz Ortholux II microscope equipped with an ocular micrometer and through a video digitiser using Jandel^{TM} software. Drawings were made with the aid of a camera lucida.

Scanning electron microscopy

First generation adults were dissected from *G. mellonella* larvae in Ringer's solution (pH 7.3). They were rinsed for 5 min. each in Ringer's solution three times. Five-day-old third stage juveniles were rinsed for three times 15 min. in 0.05% NaCl. All nematodes were relaxed and killed by heating in a water-bath (60°C) for 2–3 min. and were then fixed in 8% glutaraldehyde – 25% EM grade (diluted in Ringer) for 2 h at room temperature. Fixed nematodes were rinsed in distilled water three times, postfixed in OsO_4 for 1 h, rinsed in distilled water again and dehydrated at 15 min intervals through 30%, 50%, 70%, 90% , 95% and 100% ethanol. They were then critical point dried in liquid CO_2, mounted on SEM stubs, coated with gold and scanned using a SES DS-130 at 15 kV accelerating voltage.

Cross-mating study

Cross-mating studies were conducted with the following *Steinernema* spp: *S. carpocapsae* (Weiser, 1955) (ALL strain), *S. rarum* (de Doucet, 1988) (Sargento Cabral strain) and *S. monticolum* Stock, Choo & Kaya, 1997 (Chiri strain). Thirty infective juveniles were surface sterilised in 1% Hyamine (methylbenzethonium chloride) and placed in a hanging-drop of *G. mellonella* hemolymph diluted with 10 μl serum free insect tissue culture solution (SF 900 II SFM Gibco^{R}). Hanging-drop slides were placed in a Petri dish (10 cm diameter) containing a single disk of filter paper saturated with distilled water. The dishes were wrapped with Parafilm and incubated at 25°C in the dark until pre-adults formed. Three pre-adults from the Thailand isolate were then placed in hanging drop dishes with pre-adults of the opposite sex of the test species for 10 days. Each cross-mating was conducted over a period of 10 days and replicated three times. Control matings contained males and females of the same species.

Molecular characterisation

Total genomic DNA was isolated as described by Reid & Hominick (1992). Purified DNA was used to produce restriction fragment length polymorphism (RFLP) profiles for *S. siamkayai* n. sp., *S. carpocapsae* (unknown isolate), *S. rarum* (Sargento Cabral isolate), *S. monticolum* (PAT-1 isolate), *S. monticolum* (MY1 isolate, Japan), *Steinernema* sp. (SSL85 isolate, Sri Lanka), and *Steinernema* sp. (Malaysia isolate). Primers used in the Polymerase Chain Reaction (PCR) were specific for the internal transcribed spacer (ITS) region of the nuclear ribosomal genes (Vrain, 1992). Primers were synthesised by Pharmacia Biotech. Amplifications were carried out in a 100 μl solution containing 50 mm KCl, 10 mm Tris (pH 9.0), 1.5 mm $mgCl_2$, 0.1% Triton X-100, 0.2 mm of each dNTP, 0.5 mm of each primer, 100 mg of purified DNA (or 5 μl of nematode lysate) and 8 units of Taq polymerase (Promega^{TM}). Amplifications were conducted out in a Teche PHC-3 thermocycler which was preheated to 95°C and incubated at 94°C for 2 min followed by 40 cycles of 94°C for 30 seconds, 45°C for 1 min and 72°C for 1 min 30 seconds. A final step of 5 min at 72°C was included to ensure that all the final amplification products were full length. Amplified products were immediately digested with 17 restriction endonucleases. Restriction enzymes were purchased from Promega and used with the buffers supplied by the manufacturer. All digestions were carried out using 4 μl of amplified products at 37°C for a minimum of 2 h. The resulting fragments were separated on 1.5% (w/w) agarose gel in TBE at 5 V/cm for 3 h. Fragments were visualized by ethidium bromide staining (Maniatis et al., 1989).

Results

Attempts to cross-hybridise *Steinernema siamkayai* n. sp. with *S. carpocapsae*, *S. rarum* and *S. monticolum* yielded no progeny. However, controls using the same species resulted in offspring.

The RFLP profiles of *S. siamkayai* n. sp proved to be distinctive from *S. rarum*, *S. monticolum* and *S. carpocapsae* (Figure 4). However, the 2 uncharacterised *Steinernema* isolates from Malaysia and Sri Lanka showed great similarity with *S. siamkayai* n. sp. As for the Malaysian isolate, 13 of 17 restriction enzymes tested were identical to the new species. Differences were observed with the following enzymes: ALU I. Hha I, Hinf I and Xba I. The Sri Lanka isolate differed in only 3 restriction enzymes (ALU I, Hha I and Hinf I (Figure 4).

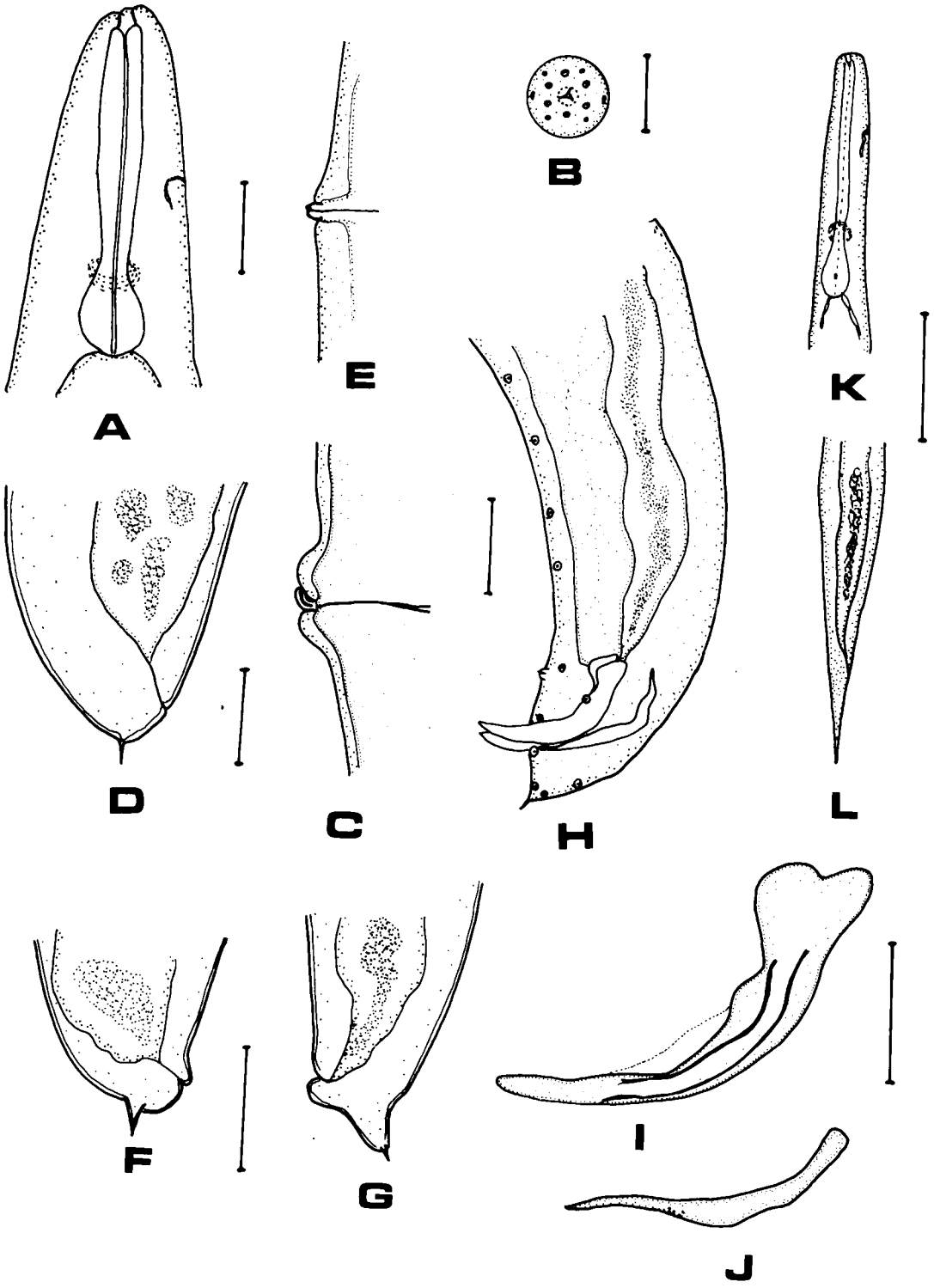

Figure 1. Steinernema siamkayai n. sp. A. Anterior end of first generation female; B. *En face* view of first generation female; C. Vulva of first generation female; D. Tail of first generation female; E. Vulva of second generation female; F–G. Tail of second generation female; H. Tail of first generation male, lateral view; I. Spicule, lateral view; J. Gubernaculum, lateral view; K. Anterior end of third stage infective juvenile; L. Posterior end of third stage infective. *Scale-bars*: A, C–J, 50 μm; B, 10 μm; K–L, 40 μm.

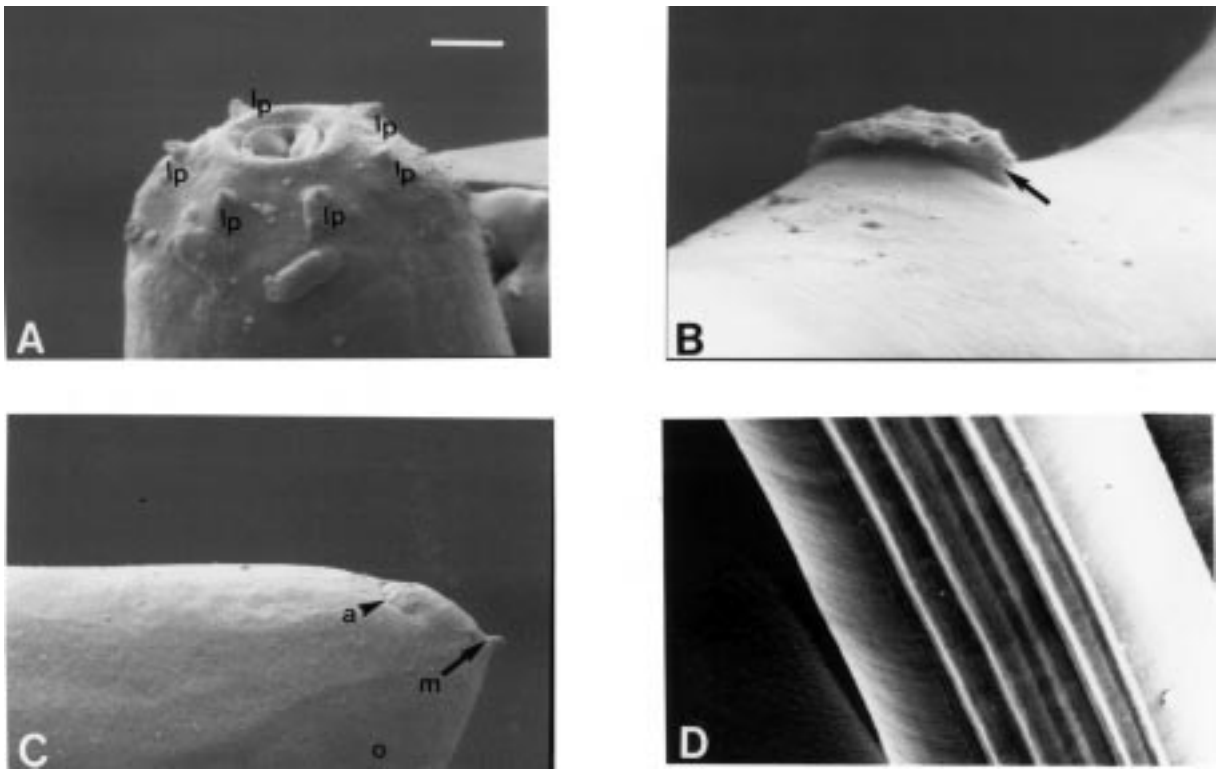

Figure 2. Steinernema siamkayai n. sp. A. *En face* view of first generation female showing six labial papillae (lp); B. Vulva of second generation female, with double-flapped epiptygma; C. Tail of first generation female in lateral view showing anus (a) and mucro(m); D. Lateral field of third-stage infective juvenile showing six longitudinal ridges. *Scale-bars*: 10 μm.

These results together with some unique morphological features indicated that this nematode is a new species, and its description follows.

***Steinernema siamkayai* n. sp.** (Figures 1–3)

Type-host: Unknown in nature, from bait-insect *Galleria mellonella* (L.) in sandy-clay loam soil under sweet tamarind *Tamarindus indicus* L.
Type-locality: Lohmsak district (16.4°N, 101.2°E), Petchabun Province, Thailand.
Type-specimens: Holotype male first generation (UCDNC 3645), allotype female first generation, (UCDNC 3646), 5 paratype males first generation (UCDNC 3647), 5 paratype females first generation (UCDNC 3648), 5 paratype third stage infective juveniles (UCDNC 3649) deposited in the University of California Nematode Collection, Davis, California, USA; 5 paratype males first generation, 5 paratype females first generation; 5 paratype third-stage infective juveniles deposited in Division of Entomology and Zoology, Department of Agriculture, Bangkok, Thailand. Field collection number: T9.
Etymology: The specific epithet derives from Siam, the old designation of the country of origin and *kayai* after Harry K. Kaya, a leading scientist in insect pathology and biocontrol.

Description

Male. Body curved posteriorly, "J"-shaped when heat-killed. First generation male larger (average: 1,135 μm) than second generation male (average: 830 μm). Cuticle smooth under light microscopy, but with fine transverse striae visible under SEM. Lateral field and phasmid inconspicuous. Head truncated to slightly round, continuous with the body. Six lips united but tips distinct, and with one labial papilla each (Figures 1B, 2A). Four cephalic papillae (Figure 1B). Amphids small, located posterior to lateral labial papillae. Stoma reduced, short and wide, with inconspicuous sclerotised walls. Cheilorhabdion and telorhabdion vestigial. Oesophagus muscular; procorpus cylindrical; metacorpus slightly swollen and

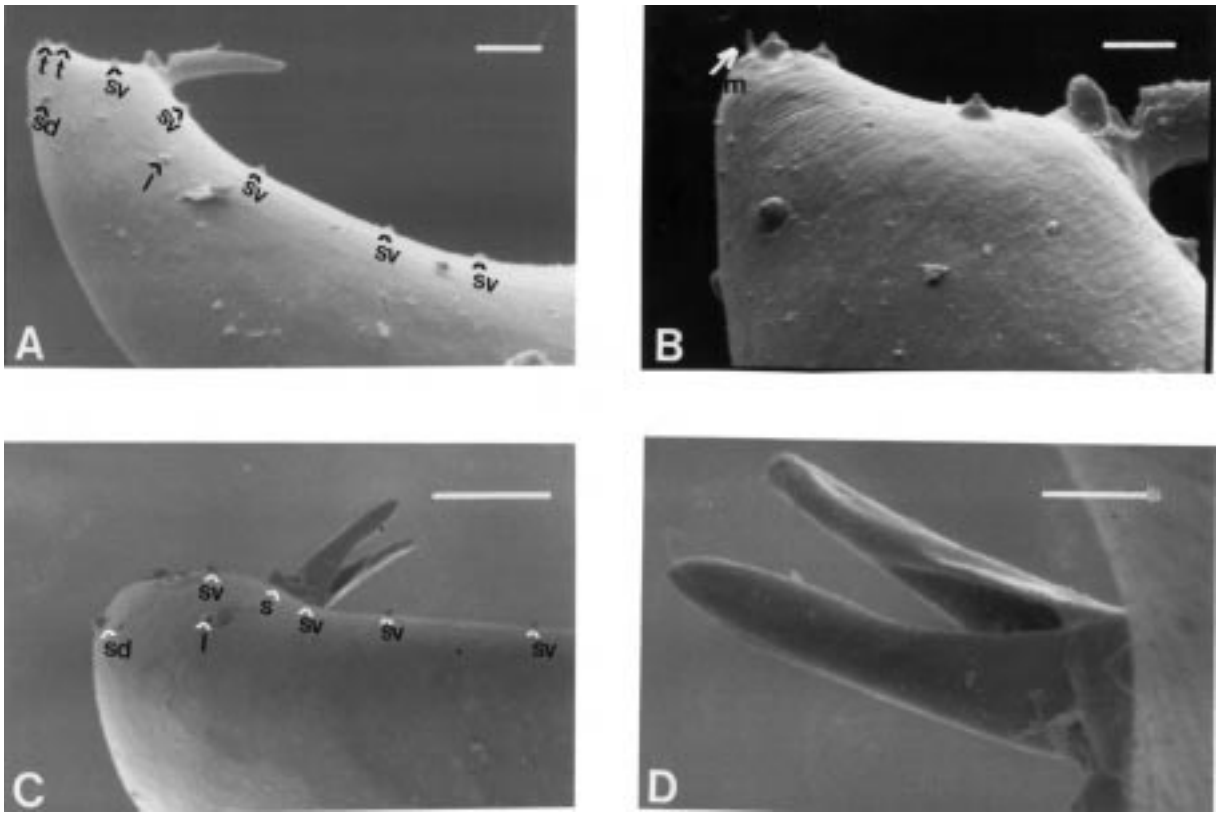

Figure 3. Steinernema siamkayai n. sp. A. Tail of first generation male, in lateral view, showing five of the paired precloacal papillae: four subventral (sv) and one lateral (l); four paired postcloacal papillae: one pair subventral (sv), one pair subdorsal (sd), two pairs terminal (t) and the extended spicules; B. Tail of second generation male showing mucro (m); C. Tail of first generation male showing pre and postcloacal papillae; D. Extended spicules of first generation male showing rounded tips. *Scale-bars*: A, 15 μm; B, 7 μm; C, 4 μm; D, 20 μm.

non-valvated; indistinct isthmus followed by pyriform basal bulb containing reduced valve. Oesophagus set off from intestine. Nerve-ring usually surrounding isthmus or anterior part of basal bulb. Excretory pore opening circular, located at posterior 1/3 of metacorpus, just anterior to nerve-ring (Figure 1A). Single reflexed testis, consisting of germinal growth zone leading to seminal vesicle. Vas deferens with inconspicuous walls. Spicules paired, symmetrical, curved, with orange-brown coloration. Manubrium romboid. Lamina with rostrum or retinaculum and 2 internal ribs. Velum present (Figure 1I). Gubernaculum large and slender, c. 3/4 length of spicules, arcuate, distally forked in ventral or dorsal view (Figure 1J). Tail conoid and mucronated. Second generation males with mucro longer than first generation. There are 23 genital papillae (11 pairs and one single) arranged as follows: 6 precloacal subventral pairs, one precloacal lateral pair; one single precloacal papilla (located between precloacal pairs 5 and 6); one pair postcloacal subventral; one pair postcloacal subdorsal, 2 subdorsal pairs near tail tip (Figures 1H, 2 A–C).

Female. Cuticle, lip region, stoma and oesophageal region as in male. Body 'C'-shaped when heat-killed. First generation females larger (average 3,937 μm) than second generation females (average 1,836 μm). Ovaries opposed, reflexed in dorsal position; oviduct well developed; glandular spermatheca and uterus in ventral position. Vagina short, with muscular walls. Vulva located near middle of body. First generation females with protruding vulval lips (Figure 1C). First and second generation females with a double-flapped epiptygma (Figures 1E, 3B). Tail of first and second generation females conoid, blunt, with mucro (Figure 3C). First generation females without post-anal swelling (Figures 1F, 3C). Post-anal swelling present in second generation females (Figure 1G).

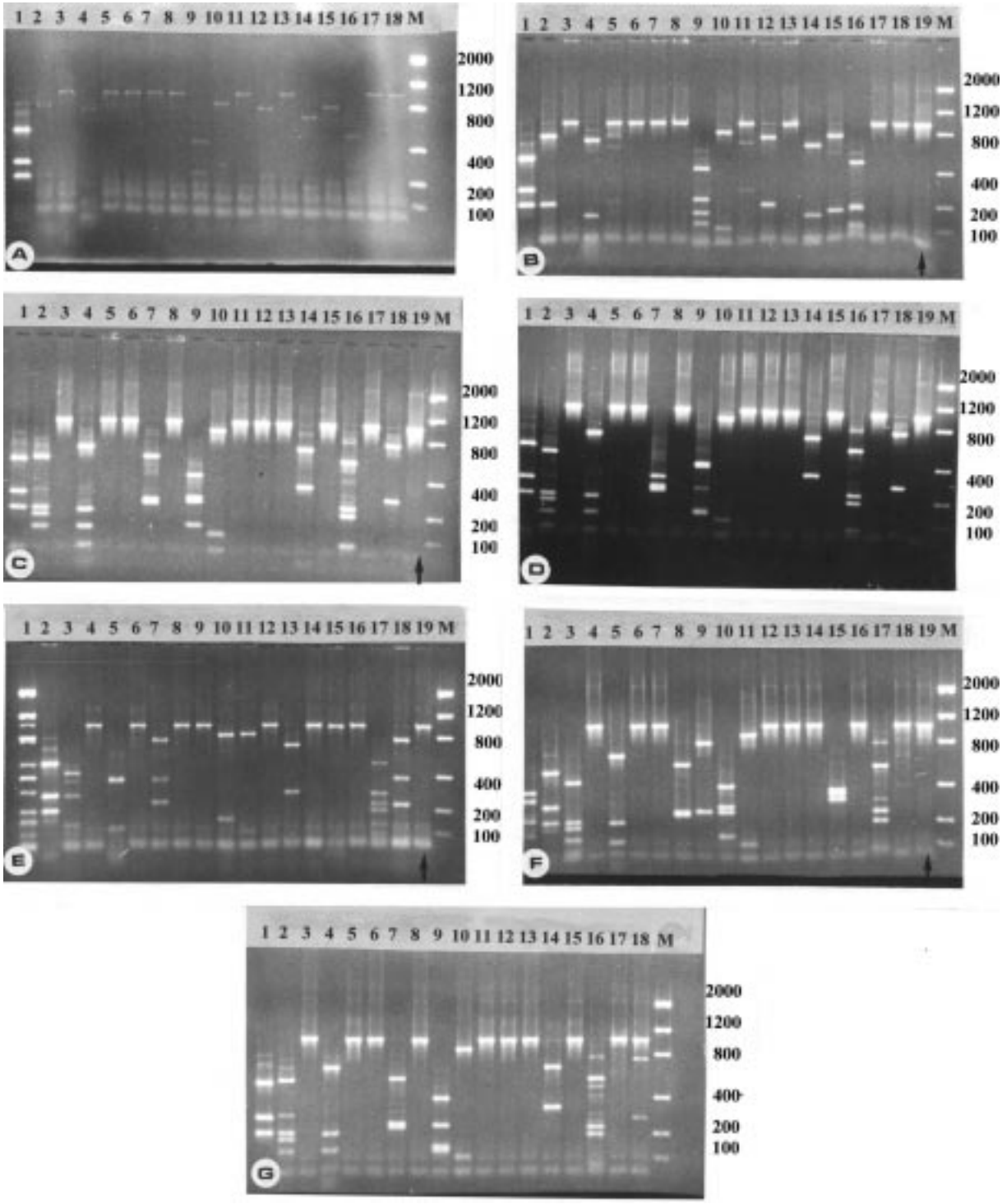

Figure 4. PCR amplified products from the internal transcribed spacer (ITS) digested with 17 different restriction enzymes. Fragments were separated with ethidium bromide stained 1.5% (w/v) agarose gels. Samples are: A. *Steinernema monticolum* (PAT-1 isolate, Korea); B. *S. monticolum* (isolate MY1, Japan); C. *Steinernema* sp. (Malaysia isolate); D. *Steinernema* sp. (isolate SSL85, Sri Lanka); E. *S. rarum* (isolate Sargento Cabral, Argentina); F. *S. carpocapsae* (unknown isolate); and G. *S. siamkayai* n. sp (T9 isolate). In each gel lane one is a digest of *S. feltiae* (UK, site 76) with *Alu* I. Lanes 2–18 are individual digests of the respective species for that gel with the following restriction enzymes: 2, Alu I; 3, BstO I; 4, Dde I; 5, EcoR I; 6, Hae III; 7, Hha I; 8, Hind III; 9, Hinf I; 10, Hpa II; Kpn I; 12, Pst I; 13, Pvu II; 14, Rsa I; 15, Sal I; 16, Sau3 A I; 17, Sau96; 18, Xba I; 19, uncut ITS product (↑). Lane M is the molecular weight marker and the band sizes are shown in base pairs.

Third stage infective juvenile. Body slender, tapering regularly from base of oesophagus to anterior end and from anus to terminus. Cuticle with transverse striae; lateral field distinct with 6 or 8 longitudinal ridges in mid-body region (Figure 2D). Lip region smooth; mouth closed. Oesophagus long, narrow. Nerve-ring located at level of isthmus. Excretory pore located in anterior 1/3 of oesophagus (Figure 1K). Basal bulb valvate. Cardia present. Anterior portion of intestine with dorsally displaced pouch containing symbiotic bacterium. Lumen of intestine narrow; rectum long; anus distinct. Genital primordium evident. Tail conoid with pointed terminus (Figure 1L).

Dimensions (all measurements are in micrometres)
Male, first generation (n = 20). Total length 1,035–1,278 (1,135). Greatest width 107–159 (139.5). Stoma length 3–4 (3.5), width 3–5 (4). Distance from anterior end to excretory pore 47.5–67 (57), to nerve-ring 81–101 (91), to oesophagus base 123–141 (134). Testis reflexion 296–355 (341). Tail length 22–32 (27.5). Mucro length 2.5-3.5 (3). Body width at cloaca 37–54 (45). Spicule length 75–80 (77.5), width 7.5–9 (8). Gubernaculum length 47–65 (53.5), width 4–7 (5). SW (spicule length divided by body width at cloaca) 1.4–2.2 (1.7). GS (gubernaculum length divided by spicule length) 0.6–0.8 (0.7). D% (ratio D) 0.35–0.49 (0.42). E% (ratio E) 1.66–2.57 (2.07).

Female, first generation (n = 20). Total length 3,161–5,172 (3,937). Greatest width 170–280 (198). Stoma length 6–10 (8), width 7.5–12 (9.5). Distance from anterior end to excretory pore 50–84 (66), to nerve-ring 116–147 (133), to oesophagus base 152–199 (177). V% (distance from anterior end to end vulva as percentage of length) 48–53 (51). Tail length 22–37.5 (31). Mucro length 4.5–11 (7). Body width at anus 48–53 (51).

Male, second generation (n = 20). Total length 716–952 (830). Greatest width 47–75 (57). Stoma length 7–10 (8.5), width 5–8 (6). Distance from anterior end to excretory pore 54–68 (58), to nerve-ring 80–91 (85), to oesophagus base 108.5–121 (112.5). Testis reflexion 90–120 (110). Tail length 19–23 (22). Mucro length 2–4 (3). Body width at cloaca 25–31 (29). Spicule length 55–73 (62), width 5–6.5 (5.5). Gubernaculum length 37–55 (41.5), width 3–4.5 (3.5). SW 0.97–1.17 (1.08). GS 0.66–0.75 (0.69). D% 49–56 (51). E% 26–29 (27).

Female, second generation (n = 20). Total length 1,410–2,560 (1,836). Greatest width 83–128 (105). Stoma length 6–8 (7), width 7–10 (8). Distance from anterior end to excretory pore 65–70.5 (68), to nerve-ring 113–130 (123), to oesophagus base 141–169 (151). V% 53–58 (56). Tail length 28–52 (41). Mucro length 3–6 (4.5). Body width at anus 30–49 (38).

Infective juveniles (third-stage juvenile) (n = 20). Length 398–495 (446). Greatest width 18–24 (21). Distance from anterior end to excretory pore 29–38 (35), to nerve-ring 68–80 (72), to oesophagus base 80–107 (94.5). Tail length 31–41 (35.5). Body width at anus 9–15.5 (11.5). Ratio a 19–23 (21). Ratio b 4.0–6.1 (4.7). Ratio c 10.3–14.8 (11.3). D% 31-43 (37). E% 85–112 (96).

Diagnosis

Steinernema siamkayai n. sp. is characterised by the following combination of morphological features: third-stage infective juvenile with a body length c. 445 (398–495) μm; juvenile's tail short, c. 35.5 (31–41) μm, and the lateral field with 6–8 longitudinal ridges; tail of both first and second generation adults with a cuticular mucro; males with long spicules, c. 77.5 and 73 μm in first and second generation, respectively; gubernaculum large and slender, c. 3/4 of the length of the spicule; 23 genital papillae; first generation female with protruding vulval lips; second generation female without protruding vulval lips; first and second generation female with a double-flapped epiptygma; first and second generation female tail conoid with a cuticular mucro of c. 7 and 4.5 μm, respectively; first generation female without post-anal swelling; and second generation female with a post-anal swelling. The RFLP banding pattern is also unique when compared to other *Steinernema* spp. (Figure 4).

The life-cycle of *Steinernema siamkayai* n. sp. is similar to other *Steinernema* spp. The third-stage infective juveniles enter the insect host through the mouth and spiracles (entrance through the anus was not observed) and then penetrate into the haemocoel. Once in the haemocoel, the infective juveniles release the symbiotic bacterium killing the insect within 36–48 hours. Two amphimictic generations occur inside the host, and progeny of the second generation emerge from the insect cadaver as infective juveniles. Observations done in blood drops of *G. mellonella* showed that the infective juveniles are able to reach the adult

Table I. Comparison of morphometrics (range and mean) of infective juveniles of *Steinernema siamkayai* n. sp. and other *Steinernema* spp.

Species	Body length (μm)	Greatest width (μm)	AE-EP	Tail length (μm)	Ratio a	D%	E%
S. siamkayai n. sp.	(308–495) 446	(18–24) 21	(29–38) 35	(31–41) 35.5	(19–23) 21	(31–43) 37	85–112 96
*S. carpocapsae**	(438–650) 558	(20–30) 25	(30–56) 38	(46–61) 53	(19–24) 21	(23–28) 26	(54–66) 60
*S. rarum***	(443–573) 511	(18–26) 23	(32–40) 38	(44–56) 51	(20–26) 23	(30–39) 35	(63–80) 72
*S. monticolum****	(612–821) 706	(32–46) 37	(54–62) 58	(71–95) 77	(14–22) 19	(44–50) 47	(63–85) 76

AE–EP, anterior end to excretory pore.
* After Poinar (1990).
** After de Doucet (1988).
*** After Stock et al. (1997).

stage in 24–32 hours at 22°C, second generation adults appear in 5–6 days and infective juveniles emerge 8–10 days after infection.

Symbiotic bacterium

The third-stage infective juveniles of *Steinernema siamkayai* n. sp. carry the cells of a symbiotic bacterium in the ventricular portion of the intestine. The bacterium was isolated from a drop of haemolymph of a nematode infected *G. mellonella* larva (Kaya & Stock, 1997). Like other *Xenorhabdus* spp., the Phase I of this bacterium was characterised by the adsorption of neutral red and bromothymol blue from McConkey agar and NBTA, respectively. Insect cadavers parasitised by this nematode-bacterium complex present a black coloration. Further studies on this *Xenorhabdus* sp. will be conducted to provide a more specific identification.

Discussion

Steinernema siamkayai n. sp. is most similar to *S. rarum* (De Doucet) *S. carpocapsae* (Weiser) and *S. monticolum* Stock, Choo & Kaya in the general morphology of the infective juveniles, males and females, but it can be separated from these species by a combination of morphological, DNA and hybridisation techniques. The average length of the infective juveniles of *S. siamkayai* n. sp. (446 μm) is the shortest of all the currently described *Steinernema* spp. (Table I).

Males of the new species can be separated from *S. carpocapsae*, *S. rarum* and *S. monticolum* by the shape and size of the spicules and gubernaculum.

The third-stage infective juveniles of *S. siamkayai* n. sp. differ from *S. carpocapsae* by the tail length (31–41 μm *vs* 46–61 μm), by the D% (31–43 *vs* 25–28) and E% (85–112 *vs* 54–66) values. The infective juveniles of the new species can be separated from *S. rarum* by the tail length (31–41 *vs* 44–56 μm); value of ratio c (10.3–14.8 *vs* 8.7–11) and E% (85–112 *vs* 63–80) (Table I).

The juveniles of *S. siamkayai* n. sp. can be separated from *S. monticolum* by the location of the excretory pore (29–38 μm *vs* 54–62 μm); the tail length (31–41 *vs* 44–50 μm); the value of D% 31–43 *vs* 44–50 and E% 85–112 *vs*. 63–85).

The similarities between *S. siamkayai* and the two uncharacterised *Steinernema* spp. from Malaysia and Sri Lanka in their RFLP profiles suggest that they might be conspecific. However, morphological characterisation as well as hybridisation tests should be carried out to support this.

Acknowledgements

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