งานวิจัยและพัฒนาไส้เดือนฝอย *Steinernema* spp. Thai isolate เพื่อควบคุมศัตรูพืชโดยชีววิธี

นุชนารถ ตั้งจิตสมคิด[1] พรพิมล อธิปัญญาคม[2]
สาโรจน์ ประชาศรัยสรเดช[1] สืบศักดิ์ สนธิรัตน[1]

[1] กลุ่มงานไส้เดือนฝอย กองโรคพืชและจุลชีววิทยา
[2] กลุ่มงานวิจัยโรคไม้ผล พืชสวนอุตสาหกรรมและสมุนไพร กองโรคพืชและจุลชีววิทยา
[3] ภาควิชาโรคพืช คณะเกษตร มหาวิทยาลัยเกษตรศาสตร์

บทคัดย่อ

การสำรวจไส้เดือนฝอยในสกุล *Steinernema* spp. ระหว่างเดือนมิถุนายน 2539 ถึงมีนาคม 2541 จำนวน 306 ตัวอย่างดิน ในพื้นที่ 42 จังหวัด ของประเทศไทย สามารถแยกไส้เดือนฝอย steinernematid จากพื้นที่จังหวัดกาญจนบุรี (KB) ตาก (TK) อยุธยา (AY) กาฬสินธุ์ (KS) มหาสารคาม (MK) ขอนแก่น (KK) หนองคาย (NK) และสระแก้ว (SK) รวม 8 ไอโซเลท นำมาแบ่งแยกในเบื้องต้นโดยการทดสอบ cross mating สามารถจัดแบ่งเป็น 3 กลุ่ม คือ 1) KB TK KS MK KK และ NK 2) AY และ 3) SK เมื่อนำมาจัดจำแนกในระดับชนิด (species) โดยศึกษารูปร่างลักษณะทางสัณฐานวิทยา และตรวจสอบในระดับดีเอ็นเอ ด้วยเทคนิค PCR-RFLPs สามารถจำแนกชนิดไส้เดือนฝอยในกลุ่มที่ 1 เป็น *S. thailandensis* n. sp. กลุ่มที่ 2 *Steinernema* sp. (AY isolate) และกลุ่มที่ 3 *Steinernema* sp. (SK isolate) ทุกไอโซเลทได้จัดเก็บรวบรวมให้คงสภาพความมีชีวิตเพื่อการศึกษาวิจัยและพัฒนาสายพันธุ์ต่อไป โดยได้นำไส้เดือนฝอย *S. thailandensis* n. sp. (KB isolate) มาศึกษาชีววิทยาและศักยภาพของสายพันธุ์ จากการศึกษาพบว่า มีวงจรชีวิตสั้น (4 วัน) ที่อุณหภูมิ 30 °ซ มีความทนทานต่ออุณหภูมิสูงถึง 35 °ซ และแยกได้ symbiotic bacteria (*Xenorhabdus* sp.) บนอาหาร NBTA จากการทดสอบศักยภาพในการฆ่าแมลงด้วยวิธี insect bioassay พบว่า แมลงทดสอบตาย 50% ในเวลา 24 ชม. เมื่อใส่ไส้เดือนฝอย 10 ตัวต่อแมลง 1 ตัว การตรวจสอบการเป็น biological control agent ในห้องปฏิบัติการกับแมลง 12 ชนิด คือ หนอนกระทู้หอมดาวเรือง หนอนกระทู้ผัก หนอนใยผัก หนอนเจาะสมอฝ้าย หนอนเจาะดอกมะลิ หนอนกินรังผึ้ง หนอนในถุงเห็ด ด้วงหมัดกระโดด หนอนด้วง ปลวก เพลี้ยอ่อน และแมลงสาปพบว่า ภายในเวลา 24-48 ชม. มีเปอร์เซ็นต์การตาย 56, 60, 89, 92, 100, 100, 33, 20, 100, 42, 44 และ 57 เปอร์เซ็นต์ ตามลำดับ จากการศึกษาเพิ่มปริมาณในอาหารเทียมชนิดต่างๆ โดยใช้อาหารเทียมชนิดวุ้น 5 สูตร พบว่าสูตรที่มีองค์ประกอบของนมถั่วเหลืองให้จำนวนผลผลิตไส้เดือนฝอยสูงสุด $33x10^6$ ตัวต่ออาหาร 1 ลิตร และการเพิ่มปริมาณได้พัฒนาวิธีการเลี้ยงในอาหารชนิดแข็งกึ่งเหลว 15 สูตร พบว่าสูตรที่มีองค์ประกอบของนมถั่วเหลืองเช่นกัน ให้ผลผลิตสูงสุด $300x10^6$ ตัวต่ออาหาร 1 ลิตร

Research and Development of *Steinernema* spp. Thai Isolate For Biological Pest Control

Nuchanart Tangchitsomkid[1/] Pornpimon Atthipunyakom[2/]

Sarote Prachasaisoradate[1/] Suebsak Sontirat[3/]

[1/] Nematology Group, Plant Pathology and Microbiology Division

[2/] Fruit Tree Industrial Horticultural Crops and Herbs Pathology Group, Plant Pathology and Microbiology Division

[3/] Department of Plant Pathology, Faculty of Agriculture, Kasetsart University

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ABSTRACT

Surveys for *Steinernema* spp. were conducted during June 1996 to March 1998 in 42 provinces of Thailand. From 306 soil samples collected from the surveys, eigth isolates of steinernematid nematode were obtained from Kanchana Buri (KB), Tak (TK), Ayutthaya (AY), Kalasin (KS), Maha Sarakham (MK), Khon Kaen (KK), Nong Khai (NK), and Sakaeo (SK) provinces. They were initially separated into three groups by the cross mating test : KB, TK, KS, MK, KK and NK belonging to group 1; AY belonging to group 2; and SK belonging to group 3. On the basis of morphological characterization and DNA analysis by PCR-RFLPs technique, the isolates belonging to group 1 ,were identified as *S. thailandensis* n. sp., the isolate belonging to group 2 was identified as *Steinernema* sp. (AY isolate), and that belonging to group 3 was identified as *Steinernema* sp. (SK isolate). All isolates were maintained for further research and development. *S. thailandensis* n. sp. (KB isolate) was studied on its biological characteristics as well as its potential for practical use in controlling the insect pests. From the studies, the life cycle of this nematode was short, it was completed within four days at 30 °C. The nematode could survive at the temperature as high as 35 °C and its symbiotic bacteria (*Xenorhabdus* sp.) could be isolated on the NBTA medium. The insect bioassay showed 50 % of the insect sample died within 24 hr when 10 nematodes were used per one insect. Under laboratory conditions, mortality percentages within 24-48 hr of common leafworm, beet armyworm, diamond-back moth, American bollworm, jasmine flower borer, wax moth, mushroom worm, flea beetle, grub of scarab beetle, wet wood termite, aphids and American cockroach were 56, 60, 89, 92, 100, 100, 100, 33, 20, 42, 44, and 57 %, respectively. The nematode was multiplied in various modified artificial media. Out of five formula of the agar medium, the soybean milk formula yielded highest number of 33 x 10^6 nematodes per one liter of the medium. Out of 15 formula of the semi-solid medium, the formula containing soybean milk also yielded highest number of 300 x 10^6 nematodes per liter.

กองโรคพืชและจุลชีววิทยา กรมวิชาการเกษตร

ขอมอบเกียรติบัตรเพื่อแสดงว่างานวิจัย

เรื่อง งานวิจัยและพัฒนาไส้เดือนฝอย *Steinernema* spp. Thai isolate เพื่อควบคุมศัตรูพืชโดยชีววิธี

โดย

นุชนารถ ตั้งจิตสมคิด

พรพิมล อธิปัญญาคม สาโรจน์ ประชาศรัยสรเดช และ สืบศักดิ์ สนธิรัตน

ได้รับรางวัลที่ ๑ ในการประชุมวิชาการ ประจำปี ๒๕๔๓ กองโรคพืชและจุลชีววิทยา
ให้ไว้ ณ วันที่ ๑๐ มีนาคม พุทธศักราช ๒๕๔๓

นางประไพศรี พิทักษ์ไพรวัน
ผู้อำนวยการกองโรคพืชและจุลชีววิทยา

THESIS

A NEW ENTOMOPATHOGENIC NEMATODE (RHABDITIDA : STEINERNEMATIDAE) IN THAILAND : TAXONOMY, BIOLOGY AND ITS POTENTIAL FOR BIOLOGICAL CONTROL

MRS. NUCHANART TANGCHITSOMKID

2000

ใบรับรองวิทยานิพนธ์

บัณฑิตวิทยาลัย มหาวิทยาลัยเกษตรศาสตร์

Doctor of Philosophy (Tropical Agriculture)

ปริญญา

Tropical Agriculture — **สาขา**

Interdisciplinary Graduate Program — **ภาควิชา**

เรื่อง A New Entomopathogenic Nematode (Rhabditida : Steinernematidae) in Thailand : Taxonomy, Biology and Its Potential for Biological Control

นามผู้วิจัย Mrs. Nuchanart Tangchitsomkid

ได้พิจารณาเห็นชอบให้เป็นวิทยานิพนธ์ระดับ ดี

โดย ประธานกรรมการ Suebsak Sontirat

(Professor Suebsak Sontirat, Ph.D.)

กรรมการ Wanphen Srithongchai

(Miss Wanphen Srithongchai, Ph.D.)

กรรมการ J. Chanpaisaeng.

(Associate Professor Jariya Chanpaisaeng, Ph.D.)

หัวหน้าภาควิชา Somnuk Wongtong

(Associate Professor Somnuk Wongtong, Ph.D.)

บัณฑิตวิทยาลัย มหาวิทยาลัยเกษตรศาสตร์รับรองแล้ว

Tasnee Attanandana

(Professor Tasnee Attanandana, D.Agr.)

คณบดีบัณฑิตวิทยาลัย

วันที่ 31 **เดือน** มกุมภาพันธ์ **พ.ศ.** 2543

THESIS

A NEW ENTOMOPATHOGENIC NEMATODE (RHABDITIDA : STEINERNEMATIDAE) IN THAILAND : TAXONOMY, BIOLOGY AND ITS POTENTIAL FOR BIOLOGICAL CONTROL

MRS. NUCHANART TANGCHITSOMKID

A Thesis Submitted in Partial

Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy (Tropical Agriculture)

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Graduate School

Kasetsart University

2000

Nuchanart Tangchitsomkid 2000 : A New Entomopathogenic Nematode (Rhabditida : Steinernematidae) in Thailand : Taxonomy, Biology and Its Potential for Biological Control. Doctor of Philosophy (Tropical Agriculture), Major Field Tropical Agriculture, Interdisciplinary Graduate Program. Thesis Advisor : Professor Suebsak Sontirat, Ph.D. 105 pages.

Steinernematid (Rhabditida : Steinernematidae) is a new entomopathogenic nematode collected from Kanchana Buri province, Thailand. Based on morphological and DNA examinations, it can be distinguished from the other 23 *Steinernema* species. Diagnostic characteristics of the third-stage infective juvenile include : the body length of 404-460 (averaged 432) μm; D% of 30-37 (averaged 33); E% of 75-91 (averaged 83) and lateral field pattern with 6 longitudinal ridges, the presence of mucronate tail tip in both first and second generation males; the size of spicules 83-99 (averaged 94) and gubernaculum 61-79 (averaged 67) μm. The vulva showed a double-flapped epitygma in both first and the second generation females and the head truncate to slightly round when observed through scanning electron microscope. The restriction fragment length polymorphism within the internal transcribed spacer region of ribosomal DNA repeat unit is identified to a new species when compared to that of *S. carpocapsae* (All strain). Thus, it is proposed as a new species namely; *S. thailandensis* n. sp.

Studies on its biology and pathogenicity in the laboratory, showed that its life cycle was four days at 30 °C. Temperature ranging from 25 to 35 °C also affected the growth rate, sex ratio and infection ability of infective juveniles. A symbiotic bacterium was isolated from a drop of haemolymph of an infected *Galleria mellonella* larva. *Xenorhabdus* sp., the phase I of this bacterium was characterised by the adsorption of bromthymol blue from NBTA medium. The nematodes at a dosage of 10 infective juveniles per insect caused 53 % mortality of test insect in 24 hours. This heat tolerant isolate could kill 100 % of *G. mellonella* in 22 hours at 30 and 35 °C and was still effective even at 38 °C. This pathogenic nematode could be mass produced *in vitro* on soybean culture medium. The yield of infective juveniles was approximately 6.4 x 10^5 per 20 g culture medium. Its potential as a biological control agent was tested against 12 insect pests. Those were common leafworm (*Spodoptera litura*), beet armyworm (*S. exigua*), diamond-back moth (*Plutella xylostella*), American bollworm (*Heliothis armigera*), jasmine flower borer (*Henedecasis duplifacialia*), wax moth (*G. mellonella*), mushroom beetle (unidentified), flea beetle (*Phyllotreta sinuata*), scarab beetle (unidentified), aphid (*Myzus persicae*), and wet wood termite (*Coptotermes* sp.) and the result showed that *S. thailandensis* n. sp. caused 56, 60, 89, 92, 100, 100, 100, 33, 20, 44, and 42 % mortality, respectively at 24 hours. The American cockroach (*Periplaneta americana*) showed 57 % mortality at 48 hours.

N. Tangchitsomkid — Student's signature

Thesis Advisor's signature — 22, May 2000

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Nuchanart Tangchitsomkid

May 2000

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A New Entomopathogenic Nematode (Rhabditida : Steinernematidae) in Thailand : Taxonomy, Biology and Its Potential for Biological Control

INTRODUCTION

Entomopathogenic nematodes are obligate parasites of insects that exist in many parts of the world. Nematodes belonging to the families Steinernematidae and Heterorhabditidae are proved to be potential control agents of a number of insect pests. They consist of an invasive juvenile stage that is non-feeding and free-living which can penetrate a host either through body openings, e.g. mouth, anus and spiracle or interskeletal membranes (Bedding and Molyneux, 1982). Their symbiotic bacteria, *Xenorhabdus* or *Photorhabdus* are released as soon as they are inside the insect hosts. The bacteria proliferate, causing septicemia and rapid death of the insect host (24-48 hours) and establish suitable conditions for the reproduction of the nematodes (Thomas and Poinar, 1979). First generation develops from these initial invaders and lays eggs that give rise to a second generation. The young ones from this generation usually develop into infective juveniles which emerge from the cadaver to search for new hosts although a third generation is possible. Entomopathogenic nematodes are becoming accepted as a biological control agent, especially against insects in the soil environment. They are tested for mass production, application, field efficacy, and safety standards. A number of commercial enterprises worldwide are now producing entomopathogenic nematodes (Poinar, 1990).

The search of entomopathogenic nematodes becomes interesting in many countries which successfully use the nematodes to control agricultural and horticultural insect pests. Thailand is situated in the tropical region of the world, in

which insect is one of the most damaging pests causing losses in many crops. The control of the insects is undertaken by using hazardous chemicals adversely affect human and environment. However, entomopathogenic nematode, *Steinernema carpocapsae*, All strain imported from the United States more than 12 years ago proves highly effective to control several insect pests in Thailand and its acceptance is increasing. *S. carpocapsae* could be produced both by *in vivo*and *in vitro* methods, easy to use and inexpensive compared with the chemicals. The nematodes are currently commercialized in sponge package of 4 million nematodes/package. However, the search and related studies of indigenous strains of entomopathogenic nematodes are of great interest to both government and private sector since the native nematodes could serve as resource for use in the country. In the tropics, the local nematode isolates could be more effective to control native insect pests compared with the exotic isolates.

OBJECTIVES

1. To collect and identify native isolates of entomopathogenic nematodes in Thailand by morphological taxonomy and molecular techniques.

2. To study biology of a new Thai entomopathogenic nematode isolate including its life cycle, sex ratio, infection, symbiotic bacteria and pathogenicity.

3. To mass rearing the new Thai entomopathogenic nematode isolate on modified artificial media in the laboratory.

4. To study the potential of the new Thai entomopathogenic nematode isolate as a biological control agent of insect pests under the laboratory conditions.

LITERATURE REVIEW

1. Historical aspects

The Swiss-born scientist, Gotthold Steiner (1886-1961) described the first steinernematid nematode isolated from Germany in 1923 under the name *Aplectana kraussei* (Steiner, 1923). Steiner came to U.S. and remained to work under N.A. Cobb. He could well be considered the "Father of Entomogenous Nematodes" in America because of his contributions to the systematics of many insect-parasitic nematode groups (Poinar, 1990). Rudolf William Glaser (1888-1947) isolated a parasitic nematode from the Japanese beetle *Popillia japonica,* in New Jersey in May 1929, which was described by Steiner as a new genus and species, *Neoaplectana* (*Steinernema*) *glaseri* (Steiner, 1929). Glaser became the first to cultivate an entomopathogenic species on solid media and axenically, and the first to conduct field experiments with cultured nematodes against an insect pest, the Japanese beetle (Poinar, 1990).

Significant developments have continued over the last 75 years. Many species and strains of entomopathogenic nematodes have been isolated worldwide. The development in classical and molecular taxonomy of nematodes, relationship between nematodes and their symbiotic bacteria and ecological studies have often been proven. Finally, field experiments have shown that these nematodes have great potentials as biological insecticides against a range of soil and other insects. A number of research work have been conducted leading to the commercialization of entomopathogenic nematodes.

2. Global distributions of steinernematid nematode

In the broadest sense, steinernematid nematodes are widespread. The only continent where they have not been found is Antarctica (Griffin *et al.*, 1990). The search for potential biocontrol agents has led to worldwide surveys of naturally occurring entomopathogenic nematodes. The surveys are conducted by using the *Galleria* bait technique (Bedding and Akhurst, 1975).

Europe is the most intensively surveyed region on earth for entomopathogenic nematodes and is the region with most of the available quantitative data (Hominick *et al.*, 1995). The prevalences of steinernematids that have been reported (37-49 %) are highest documented so far in Northern Europe and are at least partly explained by the method whereby the soil in a negative assay is exposed to *Galleria* larvae a second time because nematodes frequently do not infect in the first bioassay (Hominick and Briscoe, 1990a, b). Other Northern European surveys varied widely in the prevalence of steinernematids, ranging from 5.8 % in Finland (Vanninen *et al.*, 1989) to 10.4 % in the Republic of Ireland (Griffin *et al.*, 1991), 18.3 % in Norway (Haukeland, 1993), 25 % in Sweden (Burman *et al.*, 1986), 26.5 % in the Swiss Alps (Steiner, 1994) and 36.8 % in Czechoslovakia (Mracek, 1980). Boag *et al.* (1992) found only 2.2 % of 1,014 sites in Scotland positive and felt that this low recovery was a reflection of the cold climate of Scotland. Such a conclusion is supported by data from a recent survey in Western Canada (Mracek and Webster, 1993). Blackshaw (1988) reported a prevalence of 3.8 % in Northern Ireland because the majority of the sample sites contained clay type soils which consistently yielded low numbers of entomopathogenic nematodes (Hominick and Briscoe, 1990a). It thus appears that the climate and soil types of England, Wales and the Netherlands provide excellent conditions for steinernematid populations in particular.

Within the steinernematids, two species appear to have a global distribution, namely *S. feltiae* and *S. carpocapsae*. These two species have been isolated from soils in Europe (Ehlers *et al.*, 1991), North and South America (Poinar, 1992), Australia (Poinar, 1990) and China (Yang *et al.*, 1990). However, some species of the nematodes occur in rather restricted area. For example, *S. affinis* and *S. kraussie* only appear to be present in Europe (Hominick *et al.*, 1995) while *S. rara* and *S. ritteri* are restricted to South America (Hominick *et al.*, 1995).

In Asia, the surveys of the steinernematid nematodes were conducted in some countires. They were recovered from Japan (Mamiya, 1988; Yoshida *et al.*, 1998)), India (Poinar *et al.*, 1992), China (Han, 1994), Sri Lanka (Amarasinghe *et al.*, 1994), Korea (Stock *et al.*, 1997) and Oman (Elawad *et al.*, 1997).

3. Habitat

The information on habitat preference for steinernematids is by and large contradicting. Griffin *et al.* (1991) found *S. feltiae* in woodland, tilled fields, pasture and roadside verge, while *S. affinis* was absent from woodland. However, the prevalences were low and showed no significant association between nematode occurrence and habitat. Boag *et al.* (1992) found mostly in pasture and absent from heathland. As their prevalences were less than 5 %, it was difficult to draw firm conclusions. Steiner (1994) found no relationship between vegetation and the prevalence of *S. kraussei*, but did conclude that *S. feltiae, S. affinis* and *S. intermedia* were typical of grassland ecosystems. Hominick *et al.* (1995) demonstrated that few steinernematids showed a distinct habitat preference, *S. feltiae, S. affinis* were found in fields and verges, species B3 was exclusive to woodlands, while *S. kraussei* was most frequently found in woodland.

4. Taxonomy and systematics of steinernematid nematodes

The classification of family Steinernematidae has been outlined by Poinar and Thomas (1984).

Phylum Nematoda

Class Secernentea

Order Rhabditida

Sub-order Rhabditina

Superfamily Rhabditoidea

Family Steinernematidae

The genus *Steinernema* was erected by Travassos in 1927 for the species *Aplectana kraussei* which Steiner had described in 1923 from a sawfly, *Cephalaeia abietis*. Another genus in the family, *Neoaplectana*, described by Steiner (1929) is considered a junior synonym of *Steinernema* by Wouts *et al.* in 1982 (Poinar, 1990).

Steinernema is an obligate entomopathogenic nematode capable of infecting a wide variety of insects. The infective-stage juvenile contains cells of a symbiotic bacterium (*Xenorhabdus* spp.) in its alimentary tract. The infective juvenile is capable of surviving in the environment, entering the body cavity of a host, and then developing into a male or female. One, two, or more generations are possible in the host.

4.1 Morphological taxonomy Morphology is at present the basis for taxonomy and classification. The morphological characters show that there is a great deal of scopes for their use for the determination of species, genera into groups and systems (Siddiqi, 1986). Identification of entomopathogenic nematodes is based on morphometric and morphological characters of the first and second generation

of female, male and infective juvenile. These differences complicate identification in some cases. Also, structures observed with the scanning electron microscope (SEM) and the description of additional species have necessitated modification of family and generic diagnoses (Nguyen and Smart, 1996).

Female : Large, size variable. Cuticle smooth or annulated. Head rounded or truncate, rarely offset. Six lips present, partly or completely fused, each lip with one labial papilla, four cephalic papillae. Amphids present, small. Stoma collapsed; cheilorhabdions pronounced, forming a ring resembling two large sclerotized dots in lateral view. Excretory pore distinct. Esophagus rhabditoid with metacorpus slightly swollen, narrow isthmus surrounded by nerve ring, and large basal bulb with reduced valve. Reproductive system didelphic, amphidelphic, reflexed. Vulva at midbody, sometimes on a protuberance with and without epiptygma. Females are oviparous or ovoviviparous with juveniles developing up to the infective stage before emerging from the body of the female. Tail longer or shorter than anal body width, with or without prominent phasmids (Nguyen and Smart, 1996).

Male : Smaller than female. Anterior end usually with six labial papillae, four large cephalic papillae, and usually with perioral disc. Testis single, reflexed; spicule paired; gubernaculum long; bursa absent. Tail tip rounded, digitate, or mucronate. One single and 10 to 14 pairs of genital papillae present with 7 to 10 pairs precloacal (Nguyen and Smart, 1996).

Infective juvenile (third-stage infective juvenile) : Body slender. Cuticle annulated. Lateral fields present with 4 to 9 incisures and 3 to 8 smooth ridges. Mouth and anus closed. Excretory pore anterior to nerve ring. Esophagus and intestine appearing reduced. Tail conoid or filiform. Phasmids, located about mid-tail (Nguyen and Smart, 1996).

Currently, the genus *Steinernema* containing 23 species. Species of *Steinernema* is listed below in chronological order :-

S. kraussei (Steiner, 1923) syn. *Aplectana kraussei* Steiner, 1923.

S. glaseri (Steiner, 1929)

S. feltiae (Filipjev, 1934)

S. affinie (Bovien, 1937)

S. carpocapsae (Weiser, 1955)

S. arenarium (*anomalae*) (Kozodoi, 1984)

S. intermedium (Poinar, 1985)

S. rarum (De Doucet, 1986)

S. kushidai (Mamiya, 1988)

S. ritteri (Doucet & Doucet, 1990)

S. scapterisci (Nguyen & Smart, 1990)

S. caudatum (Xu *et al.*, 1991)

S. neocurtillae (Nguyen & Smart, 1992)

S. longicaudum (Shen, 1992)

S. cubanum (Mracek *et al.*, 1994)

S. puertoricense (Roman & Figueroa, 1994)

S. riobrave (Cabanillas *et al.*, 1994)

S. bicornutum (Tallosi *et al.*, 1995)

S. oregonense (Liu & Berry, 1996)

S. monticolum (Stock *et al.*, 1997)

S. karii (Waturu *et al.*, 1997)

S. abbasi (Elawad *et al.*, 1997)

S. ceratophorum (Jian *et al.*, 1997)

Identification criteria to species of the genus *Steinernema* are based on infective-stage juvenile and first-generation male characters. Diagnostic characters

of 23 *Steinernema* species showed the species names arranged in descending order of the infective-stage juvenile length.

Infective-stage juvenile length greater than 1,000 micrometers are as follows :

Steinernema cubanum

IJ length = 1283 μm (1149-1508), EP = 106 μm (101-114), T = 67 μm (61-77), E% = 160, D% = 70. Spicule length = 58 μm (50-67), SW = 1.41, spicule head continuous with spicule blade dorsally, spicule tip hookless, in ventral view, cuneus of gubernaculum V-shaped, velum narrow, mucron absent.

Steinernema puertoricense

IJ length = 1171 μm (1057-1238), EP = 95 μm (90-102), T = 94 μm (98-107), E% = 101 (88-108). Spicule length = 78 μm (71-88), spicule well curved with tip without a hook, D% = 77, mucron absent. Female double-flapped epiptygma.

Steinernema glaseri

IJ length = 1130 μm (864-1448), EP = 102 μm (87-110), T = 78 μm (62-87), E% = 102 (87-110). Spicule length = 77 μm (64-90), spicule with prominent shaft and hooked tip, in ventral view, gubernaculum tapering gradually anteriorly, cuneus Y-shaped, D% = 70 (60-78), mucron absent. Female epiptygma absent.

Steinernema longicaudum

IJ length = 1063 μm , EP = 81 μm, T = 95 μm, E% = 85. Spicule length = 77 μm, hookless spicule tip, spicule narrows suddenly to form tip with flattened terminus, mucron absent. Female epiptygma absent.

Steinernema arenarium (*anomalae*)

IJ length = 1034 μm (724-1408), EP = 83 μm (76-86), T = 75 μm (64-84), E% = 119 (106-130). Spicule length = 84 μm (81-91), hookless spicule with ball-like tip, D% = 93 (88-102), in ventral view, cuneus of gubernaculum V-shaped, mucron absent.

Infective-stage juvenile length less than 1,000 micrometers are as follows :

Steinernema oregonense

IJ length = 980 μm (820-1110), EP = 66 μm (60-72), T = 70 μm (64-78), E% = 100 (90-110), D% = 73 (64-75). Spicule length = 71 μm (65-73), spicule moderately curved, spicule head enlarged, tapering anteriorly, mucron absent.

Steinernema kraussei

IJ length = 951 μm (797-1102), EP = 63 μm (56-66), T = 79 μm (63-86), E% = 80. Spicule length = 49 μm (42-53), spicule well or moderately curved, mucron small, D% = 53, SW = 1.10.

Steinernema karii

IJ length = 932 μm (876-982), EP = 74 μm (68-80), T = 74 μm (64-80), E% = 96. Spicule length = 83 μm (73-91), mucron absent, spicule well curved with blunt tip, spicule head twice as long as wide, velum thin. gubernaculum with posterior end bifurcate, cuneus present.

Steinernema neocurtillae

IJ length = 885 μm (741-988), EP = 18 μm (14-22), E% = 23 (18-30), EP extremely short, T = 80 μm (64-97), D% = 12 (10-15). Spicule length = 58 μm

(52-64), GS% = 89 (82-93), spicule short, spicule tip with a prominent depression on ventral side; mucron present, short.

Steinernema feltiae (= bibionis)

IJ length = 849 µm (736-950), EP = 62 µm (53-67), T = 81 µm (70-92), E% = 78 (69-86). Spicule length = 70 µm (65-77), mucron long, GS% = 59 (52-61), spicule slightly curved, spicule head twice as long as wide.

Steinernema bicornutum

IJ length = 769 µm (648-873), EP = 61 µm (53-65), T = 72 µm (63-78), E% = 84 (80-100), D% = 52 (50-60). Spicule length = 65 µm (53-70), spicule curved, spicule head typified by ventral projection and a width greater than length, blade well curved anteriorly or at mide region, posterior part slightly curved to straight, velum present; in ventral view, cuneus arrowhead-shaped, mucron absent.

Steinernema monticolum

IJ length = 706 µm (612-821), EP = 58 µm (54-62), T = 77 µm (71-95), E% = 76 (63-86), D% = 55 (49-51), excretory pore at the level of the posterior third of metacorpus. Spicule length = 70 µm (61-80).

Steinernema ceratophorum

IJ length = 706 µm (591-800), EP = 55 µm (47-70), T = 66 µm (56-74), E% = 84. Spicule length = 71 µm (54-90), mucron absent in first and second generation, spicule curved with blunt tip, velum large extending nearly to spicule tip, gubernaculum boat-shaped with posterior end bifurcate, cuneus small.

Infective-stage juvenile length less than 700 micrometers are as follows :

Steinernema affine

IJ length = 693 μm (608-880), EP = 62 μm (51-69), T = 66 μm (64-74), E% = 94 (74-108), internal spine in IJ tail. Spicule length = 70 μm (67-86), mucron present, small.

Steinernema intermedium

IJ length = 671 μm (608-800), EP = 65 μm (59-69), T = 66 μm (53-74), E% = 96 (89-108), dorsal depression of IJ tail. Spicule length = 91 μm (84-100), mucron absent, spicule well curved with blunt tip, rostrum prominent, velum large.

Steinernema riobrave

IJ length = 622 μm (561-701), EP = 56 μm (51-64), T = 54 μm (46-59), E% = 105 (93-111). Spicule length = 67 μm (63-75), spicule sickle-shaped, spicule head tapering anteriorly, in ventral view, width of neck of gubernaculum large, cuneus Y-shaped, mucron absent, D% = 71 (60-80), SW = 1.14.

Infective-stage juvenile length less than 600 micrometers are as follows :

Steinernema kushidai

IJ length = 589 μm (524-662), EP = 46 μm (42-50), T = 50 μm (44-59), E% = 92 (84-95). Spicule length = 63 μm (48-72), D% = 51, SW = 1.50, spicule head truncate or rounded anteriorly, almost as long as wide, blade with large anterior end, rostrum prominent, velum present; mucron absent.

Steinernema scapterisci

IJ length = 572 μm (517-609), EP = 39 μm (36-48), T = 54 μm (48-60), E% = 73 (60-80). Spicule length = 83 μm (72-92), SW = 2.52 (2.04-2.80), mucron present. Female with large double-flapped epiptygma, and excretory duct with elliptically-shaped structure.

Steinernema carpocapsae

IJ length = 558 μm (438-650), EP = 38 μm (30-60), T = 53 μm (46-61), E% = 60 (54-66). Spicule length = 66 μm (58-77), D% = 41 (27-55), SW = 1.72 (1.40-2.00), spicule head wider than long, ventrally projected, spicule tip pointed or bluntly pointed; in ventral view, cuneus arrowhead-shaped or Y-shaped, mucron present.

Steinernema abbasi

IJ length = 541 μm (496-579), EP = 48 μm (46-51), T = 56 μm (52-61), E% = 86. Spicule length = 65 μm (57-74), mucron absent, spicule curved, velum large, gubernaculum boat-shaped.

Steinernema rarum

IJ length = 511 μm (443-573), EP = 38 μm (32-40), T = 51 μm (44-56), E% = 72 (63-80), morphometrics of this nematode are less than those of other steinernematids. Spicule length = 47 μm (42-52), D% = 50 (44-51), SW = 0.94 (0.91-1.05), mucron present.

Steinernema ritteri

IJ length = 510 μm (470-590), EP = 38 μm (32-40), T = 51 μm (44-56), E% = 88 (79-97). Spicule length = 69 μm (58-75), D% = 47 (44-50), SW = 1.56 (1.44-1.57), mucron absent. Female with epiptygma.

Remarks : Abbreviations used in diagnoses, IJ = infective-stage juvenile; EP = distance from anterior end to excretory pore; T = IJ tail length; E% = EP of IJ divided by tail length x 100; D% = EP of male divided by esophagus length x 100; SW = spicule divided by anal body width; μm = micrometers.

4.2 Molecular taxonomy Molecular characters are becoming increasingly useful for species identification and systematics in nematology. Recent developments in methodology resulting in more convenient, rapid, and specific assays have opened the door to greater use of these tests for detecting and identifying plant parasitic nematodes and entomopathogenic nematodes. Proteins and DNA are particularly useful characters for species identification because they are less subject to environmental effects and investigator interpretation than are many other characters. DNA is the genetic code of the organism and thus its properties are a direct reflection of genetic identity of that organism. Proteins are products of the genetic codes and because of the availability of techniques to directly measure their properties, are also particularly useful as characters (Williamson, 1990).

The rapid development of DNA-based taxonomic techniques provides a fresh impetus to the use of molecular techniques in systematics in the 1980s, and these technologies are being increasingly applied to the resolution of taxonomic problems in economically important nematode groups (Curran and Webster, 1987).

In the Rhabditoidea, within *Steinernema,* species have been recognized by morphological differences, supplemented by cross breeding tests and despite some problems of misidentification and nomenclature, these have been little difficulty in species recognition (Curran, 1990).

5. Biology of steinernematid nematodes

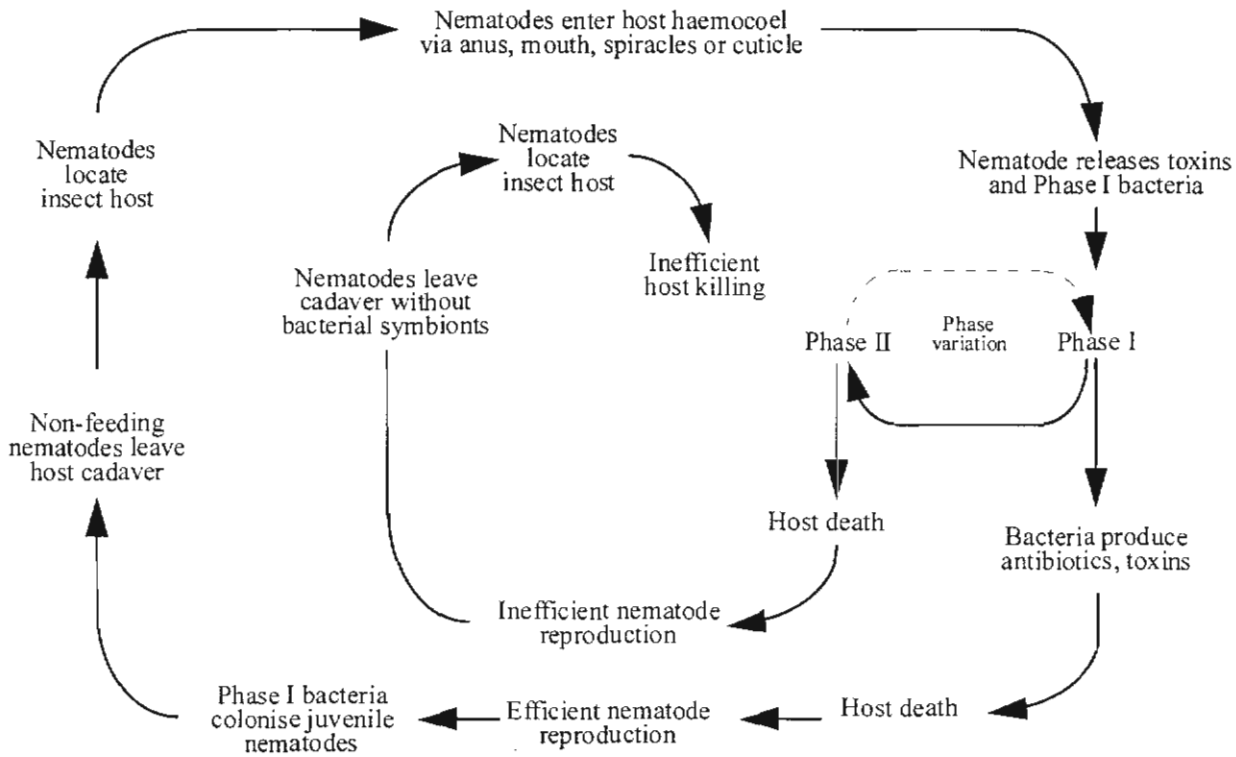
5.1 Infection The infective juveniles (IJs) of the family Steinernematidae are non-feeding, free living stage in the soil. The mouth and the anus of IJs are closed and the digestive tract is non-functional. IJs contain large amounts of energy reserve stored as lipids which supports their prolonged survival in the soil. The third-stage juvenile ensheathed by the second stage cuticle (Poinar, 1979), carries up to 0-250 cells of its bacterial symbiont in the anterior part of intestine (Spiridonov *et al.,* 1991).

The infective juveniles are actively attracted to a host (Bedding and Akhurst, 1975) through gradients of temperature (Byers and Poinar, 1982) and carbon dioxide (Gaugler *et al.,* 1980), and by chemicals and microorganisms associated with the excretory system. The infective juveniles actively invade the host through natural openings-mouth, anus, and sometimes spiracles (Wouts, 1980). IJs of different entomopathogenic nematode species differ in their ecological and behavioral traits. For example, *S. carpocapsae* nictates (Kondo and Ishibashi, 1986), tends to stay near the soil surface and does not disperse far (Moyle and Kaya, 1981), is unresponsive to host cues (Lewis *et al.,* 1992), and is adapted to infecting mobile hosts on the soil surface (sit and wait strategist or ambusher) (Campbell and Gaugler, 1993). *S. glaseri* occurs deeper in the soil and travels much farther (Georgis and Poinar, 1983), responds strongly to host cues (Lewis *et al.,* 1992), and is adapted to infecting sedentary hosts (active foraging strategist or cruiser) (Campbell and Gaugler, 1993).

Once a suitable host is found, the IJs enter the host and penetrate into the haemocoel. There are indications that the penetration process is supported by proteasic factors produced by the exsheathed infective juvenile (Roque *et al.,* 1994).

Once the IJs entering the insect body, they release the bacterial associate into the haemolymph. Providing the insect's defense mechanisms does not succeed in eliminating the nematode-bacterium complex, the insect dies in 2-4 days after infection. Several authors have summarized the current knowledge of the pathogenicity mechanism of the nematode-bacterium complex and its interactions with the defence system of a insect host (Dunphy and Thurston, 1990).

The life cycle of the nematode-bacterium infection complex is shown below.



From : Connor Thomas (http:/www.microbiology.adelaide.edu.au/)

5.2 Symbiotic bacteria The enterobacteria are mutualistically associated with steinernematids belonging in the genus *Xenorhabdus* (Thomas and Poinar, 1979). These bacteria are medium to long motile rod with peritrichous flagellae. They are Gram-negative, facultative anaerobes and form spheroplasts (x = 2.6

microns diameter) in older cultures. Non-spore formers, *Xenorhabdus* spp. do not have an environmentally resistant stage and have not been naturally found except in the nematode vectors or insect hosts (Woodring and Kaya, 1988). The bacteria may be isolated in primary and secondary forms that are usually distinguished by their colony morphology and absorption of dyes. Primary colonies are generally smaller and more convex than the secondary ones. The most reliable indicator is the primary form's absorption of neutral red from MacConkey agar. Bromthymol blue in NBTA is also often absorbed by colonies in the primary phase (Woodring and Kaya, 1988). The primary form of *Xenorhabdus* differs biochemically from the secondary, supports greater nematode production for both *in vivo* and *in vitro* cultures (Akhurst, 1980), and produces antibiotics (Akhurst, 1982). The primary form is isolated from infective juvenile nematodes and recently infected insects and converts to the more stable secondary form while growing in insects, pure culture and *in vitro* nematode culture. Both forms are equally infectious when injected intrahaemocoelically into insect hosts (Akhurst, 1980).

Xenorhabdus, on the other hand, cannot enter the insect alone; it needs the nematode to serve as a vector. Feeding studies have shown that *Xenorhabdus* is unable to infect insects. (Poinar and Thomas, 1966). The bacterium does not have an environmentally resistant stage. Not surprisingly, *Xenorhabdus* has never been isolated from the soil. The nematodes have also been demonstrated to produce a factor that inhibits the antibacterial enzymes of the insect (Gotz *et al.*, 1981). If axenic nematodes (i.e., without their associated bacterium) are inoculated into the haemocoel of an acceptable host, they are generally unable to kill the host and may develop to adults (Poinar and Thomas, 1966).

5.3 Life cycle The life cycle of species in the genus *Steinernema* as described by previous researcher consists of an egg stage, four juvenile stages, and

an adult stage (males and females). The J2 (second-stage juvenile) may be the pre-infective stage or the non-preinfective stage, and the J3 (third-stage juvenile) may be the infective stage or the non-infective stage (Nguyen and Smart, 1992). Generally two complete generations occur in an insect host but smaller insects may permit only a single generation or perhaps only an incomplete generation to develop (Jackson, 1985). Most researchers agree that some of the eggs produced by the first-generation females develop into IJ (infective J3), but the majority of the egg develop into second-generation adults. The egg produced by the second-generation females develop into IJ. Wouts (1980) presented an updated life cycle and a redescription of *S. feltiae*. He reported that in a fresh host with a low population density, the J1 (first-stage juvenile) developed directly to the J4 (fourth-stage juvenile) without going through the J2 and J3 stages. When the population density increased, however, the J1 developed to J2 and then to IJ.

Nguyen and Smart (1992) showed that life cycle of *S. scapterisci* from IJ to IJ might proceed by one of two routes. If the nutrient supply is sufficient and the population is not overcrowded, the IJ develops to adult males and females of first-generation. Most eggs from these adult females hatch and the juveniles develop through each life stage to become adult males and females of the second-generation. Eggs produced by these females develop to IJ. This cycle takes 8-10 days (long cycle) and takes 6-7 days (short cycle) at 24 °C, but the cycle described requires 10-14 days for *S. feltiae* (Woodring and Kaya, 1988).

5.4 Persistence Natural populations of steinernematid has patchy distribution and nematode numbers are often very low (Stuart and Gaugler, 1994). Occasionally they rise to several millions per m^{-2} when epizootics occur. A single insect cadaver may produce over 100,000 juveniles so very high local densities do occur in nature (Smith, 1996). Persistence is solely a measure of number of live

nematodes present in the soil and is distinct from infectivity which is a measure of the ability of these live nematodes to enter an insect host (Curran, 1993). The persistence of applied nematodes differs between strains, species and conditions, such that no two situations are identical or comparable.

Factors of major importance are age and lipid reserves of infective juveniles. These characteristics directly influence the ability of nematodes to survive a period of time without a host and their ability to find and infect a host (Womersley, 1990). If applied as biocontrol agent, the nematodes can also be influenced by its pre-application conditions, such as storage temperatures and storage periods (Fan and Hominick, 1991).

Many studies mainly in laboratories have tested the persistence of nematodes under various conditions in sterile soil. The data generally indicated a survival of weeks, rather than months and a gradual decline in the numbers of living nematodes recovered. Their infectivity potential in some cases, however, did not follow the same pattern because at least some nematode individuals were capable of entering a quiescent stage and then became active again (Womersley, 1990).

Behavioral adaptations and phase of anhydrobiosis or quiescence will influence the pattern of persistence (Womersley, 1990). Most of the data showed a rapid decline in viable nematodes in the first few days after application but after that period the rate of population decline rapidly (Molyneux, 1985). Several factors such as soil type, humidity levels in soil, temperatures and soil pH affect their persistence and infectivity in soil.

6. Steinernematid nematodes as a biological control agent

6.1 Safety The wide host range of steinernematid has given rise to concern about their safety to vertebrates and non-target invertebrates. They are environmentally safe and show no evidence of mammalian pathogenicity (Ehlers and Peters, 1995). Long-term effects on non-target organisms or other environmental impacts following the application of nematodes have not been reported. On the contrary, the environmental safety of nematodes has been exceptionally documented under field conditions (Bathon, 1996). Under experimental conditions, very high dosages of nematodes caused mortality in only young tadpoles of frogs and toads (Poinar, 1990).

Entomopathogenic nematodes are beneficial organisms and although they are symbiotically associated with bacteria, they are not placed in the category of micro-organisms for pest control in most countries. They are exempted from registration in the United States by the Environmental Protection Agency (Gaugler and Kaya, 1990). Many other countries have also exempted nematodes from registration, but some have restrictions or require registration. In the United Kingdom, for example, only indigenous nematodes can be released for biological control (Miduturi, 1997).

6.2 Commercialization

6.2.1 Mass production Large scale commercial yields of entomopathogenic nematodes require economies of scale (Friedman, 1990). Mass production of entomopathogenic nematodes has evolved from the first large-scale *in vitro* solid media production by Glaser *et al.* (1940). He is the first to develop an axenic culture process for *S. glaseri* and later *S. carpocapsae*. Dutky *et al.* (1964)

are referring to the "nematode-bacterium complex" which they maintained on peptone-glucose agar and pork kidney and found that a medium with the bacterial associate, could support the reproduction of *S. carpocapsae* DD136 strain. Bedding, (1981 and 1984) increased the yield of the solid phase monoxenic process from 1 million per Petri dish to 40 millions per flask. Pace *et al.* (1986) reported that ox kidney homogenate-yeast extract or various homogenized offals can serve as media in liquid monoxenic processes. In kidney-yeast medium, they reported yields as high as 90,000 nematodes per milliliter in fermenters in approximately 3 weeks and yields of 190,000 nematodes per milliliter in shake flasks.

A commercial process is economy of scale. Therefore, methods of producing entomopathogenic nematodes have been reviewed and analyzed from the perspective of scale-related costs. In models described, liquid monoxenic culture proves to be the most robust of the current production methods for development to large scale (Friedman, 1990).

6.2.2 Formulation and storage Formulation of nematodes into a stable product has played a significant role in commercialization of these biological control agents. Active nematodes must be immobilized to prevent depletion of their food reserves. This has been accomplished by maintenance of the nematode in aqueous suspensions at low temperature (5-15 °C). This approach, however, is not commercially accepable. Currently, gel polymers or clay are used in nematode formulations (Georgis and Poinar, 1994) and also as water dispersible granules (Hirsch and Georgis, 1996). Steinernematids, especially *S. carpocapsae,* can be maintained for up to 5 months at room temperature or up to 12 months under refrigeration (Georgis, 1990).

6.2.3 Application The most common application method is to apply nematodes onto the soil surface. Application is carried out using spraying equipment or irrigation systems. No adverse effects of different pumps, strainers or nozzles at standard application pressure have been determined (Klein and Georgis, 1994). Entomopathogenic nematodes are sensitive to ultra violet (UV) light (Gaugler *et al.*, 1992). That is why they are usually applied at low UV radiation onto moist soil surface. Entomopathogenic nematodes are compatible with conventional insecticides and can be integrated into standard chemical control practices (Rovesti and Deseo, 1990).

6.2.4 Field efficacy Entomopathogenic nematodes in the family Steinernematidae possess many qualities that make them excellent biological control agents (Gaugler, 1988). They have a broad host range, can be easily mass produced, possess the ability to seek out their hosts, kill their host rapidly, and are environmentally safe. Availability of commercial entomopathogenic nematodes offer more opportunities to use against wide variety of insects. Targets for nematodes are pest insects in cryptic habitats. Much of the efficacy data have been summarised (Klein, 1990). Entomopathogenic nematodes are also effective against white grubs, larvae of Scarabaeidae, are major pests of turfgrass, pastures, sugarcane and forests throughout the world. They are the most serious pests of turf in the north-eastern U.S. The nematode in genus *Steinernema* has shown that it can be effective biological control agents against these insects (Klein, 1990). Excellent results have been obtained against the blackvine weevil *Otiorhynchus sulcatus* larvae. This summary showed that moderate (50-80 %) to high (> 80%) control occurred when temperatures are above 16 °C (Georgis and Poinar, 1989). Klein (1990) indicated that *S. carpocapsae, S. glaseri* and *S. feltiae* provided excellent control of the banana root borer, *Cosmopolites sordidus,* in Puerto Rico. The tropical environment appears to be a logical situation for successfully using these nematodes.

Using *S. carpocapsae* for control of termites (*Reticulitermes* spp.) in buildings has been achieved a success rate of 80-87 % (Poinar and Georgis, 1989). *S. scapterisci* from Uruguay shows its potential for control mole crickets, *Scapteriscus* spp. which are major pests on golf courses where it has been applied, and appears to be reducing population of mole crickets (Smart *et al.,* 1991).

Production and storage capabilities of the most efficacious nematode species and strains need to be developed so that consistent field results can be obtained. As new species and strains are discovered through exploration and developed through genetic manipulation, they can be combined with advances in application techniques to provide reliable pest control to a broad range of consumers (Klien, 1990).

MATERIALS AND METHODS

Materials

1. Standard equipment for soil-sampling, cylindrical soil-core sampling tube (dia. 8 cm, height 80 cm), plastic bucket, plastic bag, cooler, etc.

2. Plastic container (capacity 300 ml)

3. Wax moth, *Galleria mellonella* L.

4. Insect culture

5. Light microscope

6. Chemicals for Light microscopy

7. Scanning electron microscope, JSM-35 CF model (SEM)

8. Chemicals for SEM study

9. DNA thermal cycler

10. Equipments for DNA analysis laboratory, centrifuge, deep freezer, incubator, etc.

11. Equipments for aseptic technique, laminar flow, autoclave, etc.

12. Chemicals for DNA analysis and restriction enzymes

13. Nutritions of the artificial media

Methods

1. Discovery of steinernematid nematodes in Thailand

1.1 Collection sites Sites were chosen to represent ecologically diverse habitats, including fruit crops, field crops, woodland and beach. Soil samples were taken from different parts (13 locations) in the country between June, 1996 to December, 1996 in Rayong, Chantha Buri, Chon Buri, Chiang Mai, Lampang, Chiang Rai, Phetchabun, Ratcha Buri, Ranong, Phangnga, Krabi, Chumphon and Kanchana Buri provinces.

1.2 Soil collection Soil samples representing of each site were collected to a depth of 10-15 cm by using a cylindrical soil-core sampling tube. At each site, five random samples were taken over an area of approximately 10 m^2. They were placed in a plastic bucket and mixed thoroughly. One kilogram of a soil sample was taken from the plastic bucket and placed in a plastic bag. Samples were placed in 18-20 °C coolers during transit to the laboratory and they were stored at 10±1 °C until processed. Location, soil temperature, associated vegetation and insects presence were recorded.

1.3 Isolation Presence of entomopathogenic nematodes in soil samples were tested by baiting with wax moth larvae *Galleria mellonella* L. (Lepidoptera : Noctuidae) (Bedding and Akhurst, 1975). Ten last instars of *G. mellonella* were placed at the bottom of each 300 ml plastic container, the soils were added and the lids with ventilation holes were positioned. The *Galleria* trap bioassays were incubated at 25 °C, 85 % relative humidity and kept in the dark. After 7 days, the larvae were removed from the soil. The dead larvae with characteristic signs and symptoms of entomopathogenic nematode infection were either dissected or placed

on a wet filter paper which was on a Petri dish cover 55 mm in diameter, then kept in a Petri dish (100 x 15 mm) with 20 ml of sterile distilled water (White, 1927). The emerging infective juveniles were collected, 8 to 10 days after infection. These juveniles were reexposed to *G. mellonella* in Petri dishes to confirm pathogenicity. Soil samples positive to entomopathogenic nematodes were analyzed for their texture and pH.

1.4 Storage The isolated nematode was stored at a concentration of approxi- mately 5,000 infective juveniles per ml distilled water in culture flasks at 25±2 °C. It was recultured every 3 months using *G. mellonella* larvae for culturing. The insect culture was maintained in aerated plastic containers (32.5 x 17.6 x 10 cm) at 25-28 °C on an artificial media containing 100 g rice powder, 200 g soybean milk, 100 ml honey, 5 ml formalin, 20 ml vitamin, 100 ml glycerin, 100 g wax honey and 375 ml distilled water. Approximately 200-300 eggs were placed on a piece of artificial media in plastic containers, then kept at 25-28 °C. The eggs hatched in 3-4 days. After 2 weeks, the larvae were given new media. After 3 weeks, late instar larvae were ready to be collected. Larvae could then be stored on wood shavings for 2-3 weeks at 10±1 °C. Nematodes needed for other experiments were stored no longer than 10 days before being used in the experiment.

2. Identification

2.1 Morphological characters The nematode recovered from the *Galleria* trap was identified to genera and species with its morphological descriptions. First and second generation adults were obtained by dissecting cadavers of *G. mellonella* larvae 4 and 7 days, respectively after infection and infective juveniles were obtained by collecting from the water trap 10 days after infection of the larvae.

Light microscopy **:** First and second generation adults and infective-stage juveniles were killed by heating in a water bath at 60 °C for 2 minutes. Once the nematodes were killed, add the fixative, TAF (7 ml of 40 % formaldehyde, 2 ml triethanolamine and 91 ml distilled water) and remain in this solution for 12 hours. Fixed nematodes were transferred to a Syracuse watchglass containing 0.5 ml of solution I (20 parts 95 % ethanol, 1 part glycerin, 79 parts distilled water). The watchglass was then placed in a desiccator and enough 95 % ethanol was added to the desiccator to half fill the space below the holding shelf. The desiccator should be placed in an oven at 35 °C for at least 12 hours to allow slow evaporation of the water from solution I in the watchglass. After this, the watchglass was removed from the desiccator and filled with solution II (5 parts glycerin, 95 parts ethanol). The watchglass was placed in a Petri dish, which was partially covered to allow slow evaporation of the ethanol, and was put back in an oven at 40 °C for 3 hours. The ethanol evaporated, leaving the nematodes in pure glycerin, and ready for mounting. The nematodes were mounted in a drop of glycerin under a coverglass and sealed with permount. The examination of morphology and microphotography were performed with an Olympus microscope equipped with differential interference contrast optics.

The following morphometric characters of third-stage infective juveniles of *Steinernema* species were used :- L (body length); W (greatest width); EP (distance from anterior end to excretory pore), ES (distance from anterior end to esophagus); T (tail length). The ratios were calculated using De Man's formula modified by Poinar (1990) and identification criteria proposed by Nguyen (1993) as follows :

a = body length divided by width (L/W)

b = body length divided by esophagus length (L/ES)

c = body length divided by tail length (L/T)

D% = EP/ES x 100

E% = EP/T x 100

The following morphometric characters of adults in the first and second generations were used :- L; W; EP; ES; T; anal body width and vulva % in female. The criteria of males used for identification were spicule and gubernaculum lengths (Nguyen, 1993), followed by the ratios :- D% = EP/ES x 100; SW = spicule length/ body width at cloaca; GS = gubernaculum length/spicule length.

Scanning electron microscopy : First generation adults were dissected from *G. mellonella* larvae in Ringer's solution (9 g NaCl, 0.4 g KCl, 0.4 $CaCl_2$, 0.2 g NaH_2CO_3, 1 liter distilled water). They were rinsed in Ringer's solution for three times (5 minutes each). Infective-stage juveniles were rinsed for three times (15 minutes each) in 0.05 % NaCl. All nematodes were relaxed and killed by heating in water at 60 °C for 2 minutes and then fixed in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 24 hours at 8 °C. Fixed nematodes were rinsed with distilled water three times, post-fixed in 2 % osmium tetroxide for 6-12 hours at 25 °C, rinsed in distilled water again, and dehydrated at 15 minutes 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 JSM-35 CF model SEM.

2.2 <u>Molecular taxonomy</u> The polymerase chain reaction (PCR) technique (Joyce *et al.*, 1994) was used and the primers used in this study were specific for the internal transcribed spacer (ITS) region within the ribosomal DNA

(rDNA) repeat (Vrain *et al.*, 1992). The DNA product was digested with 17 restriction enzymes and analyzed by the restriction fragment length polymorphism (RFLP) technique.

DNA extraction : Individual infective juvenile of the Thai nematode isolate and *S. carpocapsae* All strain (Nematodik-Thailand Co., Ltd.) was placed in 10 µl drop of worm lysis buffer, WLB (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45 % tergitol, 0.45% Tween 20, 0.01 % gelatin, 60 µg/ml proteinase K) on a glass slide under a dissecting microscope. The nematodes were then cut into two or more pieces with a sterile needle. The nematode pieces along with the WLB were immediately transferred into a sterile 0.5 ml Eppendorf tube. The tube contents were then frozen at -80 °C for 10 minutes and rapidly thawed. This freezing and thawing cycles disrupt cell membranes and facilitates the release of DNA. The lysis mix were then incubated at 65 °C for 1 hour, boiled at 95°C for 10 minutes, and centrifuged for 1 minute at 12,000 rpm speed to remove debris. Up to 5 µl of the supernatant were then used in PCR reaction.

PCR amplification : PCR reactions were carried out in 0.5 ml Eppendorf tubes in a volume of 50 µl. The reactions were set up on ice and the following added; 5 µl 10x PCR buffer, 0.5 µl dNTP mixture (20mM each), 0.5 µl 18S forward primer (1,000 µg/ml), 0.5 µl 26S reverse primer (1,000 µg/ml), 2U *Taq* Polymerase, distilled water to 45 µl and 5 µl worm lysate.

The primers used in this study were specific to the internal transcribed spacer (ITS) region within the rDNA repeat. Sequences corresponding to the ITS of rDNA from Vrain *et al.* (1992) were amplified and the length polymorphisms among nematode isolates were analysed. The sequences of 18S forward primer and 26S reverse primer proposed by Vrain *et al.* (1992) were :

18S (5'- TTGATTACGTCCCTGCCCTTT - 3')

26S (5'- TTTCACTCGCCGTTACTAAGG - 3')

All reactions were topped with two drops of mineral oil and run in a thermocycler pre-heated at 95°C. PCR reaction conditions were as described for ITS primer by Vrain *et al.* (1992), 2 minutes at 94°C, 40 cycles of 30 seconds at 94°C, 60 seconds at 45°C, 90 seconds at 72 °C and a final extension cycle of 5 minutes at 72°C.

The amplified products were tested by running an aliquot of 5 µl of PCR product on a 1 % (w/v) agarose gel in 0.5 x TBE (0.9 M Tris-borate, 0.646 M boric acid, 0.025 M EDTA) at 5 V/cm for 1 hour to determine the presence and the size of the PCR product.

Restriction enzyme digestion and electrophoresis : Five µl of amplified product at 37°C was digested for a minimum of 12 hours with 10 µl of each 17 restriction enzyme. The enzymes used were *Alu* I, *Dde* I, *Eco*R I, *Eco*R II, *Hae* III, *Hha* I, *Hind* III, *Hinf* I, *Hpa* I, *Kpn* I, *Pst* I, *Pvu* II, *Rsa* I, *Sal* I, *Sau*3A I, *Sau*96 I and *Xba* I. Digested products were run on a 1.5 % (w/v) agarose gel in 0.5 x TBE at 5 V/cm for 3.5 hours. Fragments were visualized by ethidium bromide staining, viewed on UV transilluminator, and photographed. The new Thai isolate nematode was identified by comparing its RFLP to that of *S. carpocapsae* (All strain).

3. Biology of the nematode

3.1 Life cycle A piece of 1 x 1.5 cm filter paper (Whatman # 2) was placed into a 1.5 ml microtube having center-punctured lid. 300±50 infective juveniles of the nematode isolate in 25 µl of water were inoculated onto the filter paper in the microtube. *G. mellonella* was placed singly in the microtube and then incubated at 30 °C. The development of the nematode was observed every 24 hours by dissecting 5 nematode cadavers until the completion of the nematode's life cycle. There were 35 microtubes prepared for this experiment.

3.2 Effect of temperature on the life cycle To determine the influence of temperature on the life cycle within *G. mellonella,* the nematodes were placed in incubation chambers at different temperatures. Three insects were placed in each of 15 Petri dishes (100 x 15 mm) and exposed to 300±50 infective juveniles. Two of the Petri dishes were placed in incubation chambers at 15, 20, 25, 30, 35 and 38 °C. After 24 hours, 3 insects were dissected daily and the life stages of the nematode determined.

3.3 Effect of temperature on sex ratio To determine the influence of temperature on female/male sex ratio, 10 insects were placed in each of 3 Petri dishes and exposed to 300±50 infective juveniles. Each of the dishes was placed in an incubator at 25, 30 and 35 °C. After 3 days, all insects were dissected and the number of females and males in each insect were counted. The female/male ratio was analyzed with Duncan's Multiple Range Test.

3.4 Effect of temperature on nematode infection To determine the influence of temperature on the infection of infective juveniles of the Thai nematode isolate in *G. mellonella,* 10 insects were placed in the Petri dish and

exposed to 300±50 infective juveniles. Each of the dishes was placed in an incubator at 15, 20, 25, 30, 35 and 38 °C. After 24 hours, 5 insects of each temperature were dissected. The number of nematodes in each insect was counted and its mean number was estimated. Insect mortality was observed in 10 *G. mellonella* 24 hours after inoculation.

3.5 Symbiotic bacteria The bacterium was isolated from a drop of haemolymph of the nematode-infested *G. mellonella* larva. Ten insects were put into a Petri dish with moist filter paper and infected with approximately 1,000 infective juveniles. After 48 hours, cadaver was rinsed with 70 % alcohol for 5-10 minutes. The cadaver was cut through with a scissor or needle and a drop of haemolymph was streaked on NBTA (37 g standard-I-nutrient agar, 25 mg bromthymol blue, 1,000 ml distilled water, 4 ml sterile filtrated 1 % 2,3,5 triphenyl-tetrazolium chloride solution) with sterile loop. The Petri dishes were maintained in an incubator at 28 °C. After 48 hours, single colonies of the bacteria with any morphological differences such as size, shape, growth rate, or color were subcultured by streaking them onto a nutrient agar (8 g nutrient broth, 15 g agar, 1,000 ml distilled water) and left at 28 °C for approximately 48 hours. Single colonies were examined to compare general characteristics, colony morphology and absorption of bromthymol blue.

4. Pathogenicity

4.1 Insect bioassay A piece of 1 x 1.5 cm filter paper was placed into a 1.5 ml microtube having center-punctured lid. The infective juveniles of the Thai nematode isolate at different numbers in 25 µl of water then dropped onto the filter paper in the microtube and one *G. mellonella* was placed on the filter paper. The

Completely Randomized Design with 5 treatments and 3 replications were applied. The treatments were 4, 6, 8, and 10 nematodes per one insect. Control treatment contained the test insect without the nematode. The data were calculated in percentages, then subjected to analysis of variance. If significant differences were detected among treatment effects they were separated using Duncan's Multiple Range Test at $P = 0.05$.

4.2 Heat tolerance A piece of 1 x 1.5 cm filter paper was placed into a 1.5 ml microtube having center-punctured lid. 300±50 infective juveniles of the Thai nematode isolate as compared with the isolate from the U.S. (*S. carpocapsae*) in 25 µl of water, then inoculated onto a filter paper in the microtube. *G. mellonella* was placed singly in the microtube. Fifteen microtubes were placed in the incubation chambers at 15, 20, 25, 30, 35 and 38 °C. The mortality check was carried out 10 hours after inoculation and every two hours until 48 hours.

5. Mass rearing

Mass rearing of this nematode was in artificial media. The medium formulae and techniques were developed in the laboratory at Nematology Section, Plant Pathology and Microbiology Division, Department of Agriculture. The cost-benefit parameter of maximal harvest of infective nematodes and low cost could be adjusted simultaneously and reliably. The Thai nematode isolate was reared on monoxenic culture and on a modified medium when its yield was required.

Nematode inoculum : The nematode was obtained by exposing the larvae of *G. mellonella* to 5,000 infective juveniles of nematode from Thailand in 9 cm Petri dishes lined with two filter papers. Twenty insects were released in each Petri dish and all dishes were maintained in an incubator at 30 °C. Five days after

exposure, the cadavers were transferred onto the White's water trap. Five days later, infective-stage juveniles were extracted from the cadavers into the water trap. The infective juveniles were surface-sterilized with 0.1 % hyamine for 15 minutes and gravity washed for 3 times with distilled water by using high speed micro-centrifuge at 8,000 rpm.

Bacterial inoculum : The primary form of *Xenorhabdus* sp. was isolated from a Thai nematode isolate on NBTA and maintained in an incubator at 28 °C. After 48 hours, single colonies of the bacteria were subcultured by streaking onto nutrient agar at 28 °C for approximately 48 hours. All single colonies were streaked one more time on nutrient agar to ensure its purity. These colonies were transferred onto glycerol medium (4 g nutrient broth, 150 ml glycerin, 500 ml distilled water) in 2 ml cryogenic vials and stored at -70 °C.

Culture media : All modified media containing 10 g of either soybean milk (SM), wheat flour (WF), soybean flour (SF), corn syrup (CS) and corn flour (CF) were mixed in 5 g yeast extract, 1 ml sunflower oil, 10 g agar and 1 liter distilled water. The lipid agar was prepared, according to Dunphy and Webster (1989) ; 10 g corn syrup, 5 g yeast extract, 25 g nutrient agar, 2.5 ml cod liver oil, 2 g $MgCl_2$ and 1 liter distilled water. The media were then autoclaved at 121°C for 20 minutes and then poured aseptically into 9 cm Petri dishes.

Experiment procedure : The bacterial colonies were removed from storage and subcultured to 125 ml Erlenmeyer flasks filled with 20 ml nutrient broth (8 g Bacto nutrient broth, 1,000 ml distilled water) under continuous agitation for 24 hours. Each culture dish was inoculated with 1 ml of the broth. The Petri dishes were incubated at 30°C for 24 hours and then inoculated with 5,000 infective juveniles per dish. All cultures were incubated at 30°C for 10 days.

Harvesting and data analysis : All nematode cultures were harvested 10 days post inoculation by flooding the dishes with 10 ml of distilled water for 10 minutes to dislodge juvenile nematodes from the agar-bacterial matrix. The total collective number of nematodes per dish was estimated based upon the average yield of nematodes in three samples of suspension per dish. Each culture was replicated five times.

6. Potential for biological control

Test insect pest species were : Larvae of Lepidoptera ; common leafworm (*Spodoptera litura*), beet armyworm (*S. exigua*), diamond-back moth (*Plutella xylostella*), American bollworm (*Heliothis armigera*), jasmine flower borer (*Henedecasis duplifacialia*) and wax moth (*G. mellonella)*. Grubs and adults of Coleoptera; mushroom beetle (unidentified), flea beetle (*Phyllotreta sinuata*) and scarab beetle (unidentified). The nymph of Homoptera, aphid (*Myzus persicae*). Larva and adult of Isoptera, wet wood termite (*Coptotermes* sp.). Adult of Blattoidea, American cockroach (*Periplaneta americana*).

Each insect pest species was tested in 5 cm Petri dishes each containing two pieces of Whatman # 2 filter paper. Approximately 1,000 infective juveniles of the Thai nematode isolate in 500 µl water were added to each dish. The nematodes were used within 10 days only after they were produced. Control treatment contained the test insects without the nematode. The tests were not conducted simultaneously, and the number of test insect pests varied as follows : 10 : 10 (treated : control), 10 : 10, 45 : 30, 25 : 25, 12 : 10, 50 : 50, 10 : 10, 15 : 15, 5 : 5, 35 : 35, 30 : 30, and 14 : 14, respectively. Dishes were stored in the dark at 25±3 °C. Numbers of dead test insects were counted after 24 hours, except the

American cockroach which were examined after 48 hours. Data analysis was percentage of mortality corrected by Abbott's formula (1925).

7. Places

The experiments were conducted at the Nematology Section, Plant Pathology and Microbiology Division, Department of Agriculture, Chatuchak, Bangkok and at the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkhen Campus, Chatuchak, Bangkok. The part of molecular taxonomy was carried out at the Nematology and Soil Zoology, National Institute of Agro-Environmental Sciences, Tsukuba, Ibaraki 305-8604, Japan.

8. Duration

The studies were carried out from June, 1996 to January, 1999.

RESULTS

1. Discovery of a steinernematid nematode in Thailand

The soil survey of entomopathogenic nematode was conducted in 13 locations (provinces) of Thailand during June - December 1996. A total of 98 soil samples were extracted using *Galleria* baiting technique (Table 1). A steinernematid nematode was isolated from 1 out of 12 soil samples collected at a coconut plantation in Kanchana Buri province. It represented 1.0 % of the total number of soil samples. The soil type was sandy loam, pH 7.2. The nematode was recovered from the soil sample which had the soil temperatures of 27 °C at the depth of 10-15 cm below the soil surface. The global position of the nematode discovery was latitude 14 °N, longitude 99 °E and altitude 400 m above mean sea level (Figure 1). It was shown that this nematode could survive well in the dry and hot environments in Kanchana Buri province.

G. mellonella parasitized by this nematode isolate turned black with no luminescence in the dark which indicated a form of steinernematid (Figure 2). The insects killed by heterorhabditids turned red, brick-red, purple, orange and sometimes green with luminescence in the dark.

Table 1 Locations, soil textures, and associated habitats or vegetations in which samplings for the steinernematid nematodes were conducted in Thailand during June - December 1996 and the nematode isolated.

No.	Province	No. of samples	Sampling time (month)	Soil texture	Habitat or vegetation	No. of samples with nematode
1	Rayong	15	June	Sandy loam	Fruit crop	0
				Sand	Beach	0
2	Chantha Buri	3	June	Sand	Beach	0
3	Chon Buri	5	June	Sand	Beach	0
4	Chiang Mai	5	August	Sandy loam	Field crop	0
5	Lampang	5	September	Sandy loam	Woodland	0
6	Chiang Rai	5	October	Clay loam	Woodland	0
7	Phetchabun	5	October	Clay loam	Woodland	0
8	Ratcha Buri	3	November	Clay loam	Fruit crop	0
9	Ranong	4	November	Sandy clay loam	Fruit crop	0
10	Phangnga	17	November	Sandy loam	Fruit crop	0
11	Krabi	3	November	Sandy loam	Guava	0
12	Chumphon	16	November	Sandy loam	Fruit crop	0
13	Kanchana Buri	12	December	Sandy loam	Coconut	1
Total	13	98				

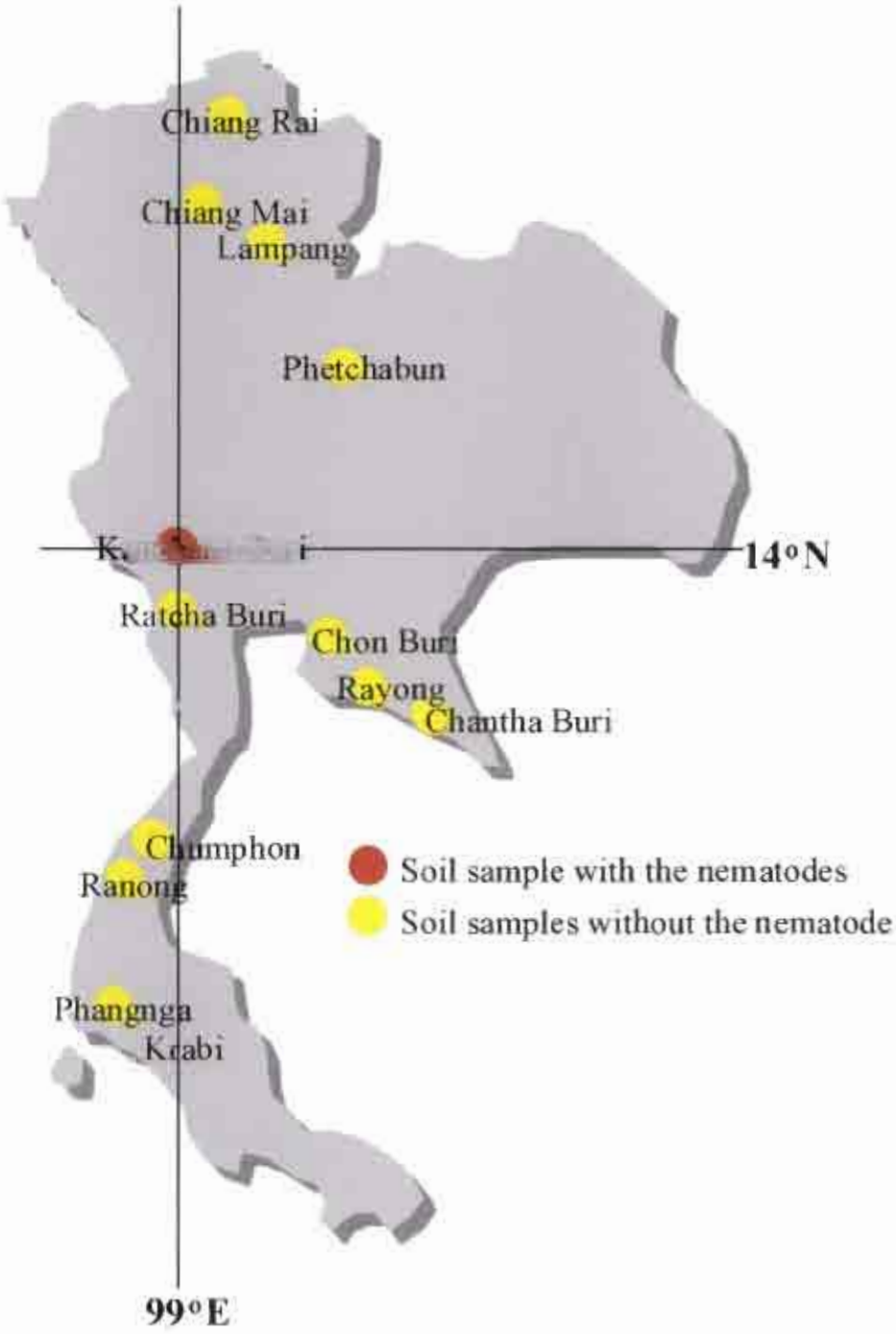


Figure 1 Locations and global position where samplings for the steinernematid nematodes were conducted.

Figure 2 Coloration of *Galleria mellonella* cadavers infected by steinernematids (S) and heterorhabditids (H).

2. Identification

2.1 Morphological characters

The results from morphological studies indicated that the *Steinernema* nematode found in Kanchana Buri province is a new species. It was named *S. thailandensis* n. sp. and its descriptions are as follows :

Type host : unknown in nature; from bait-insect (*G. mellonella*).

Type locality : in sandy loam soil under a coconut tree (*Cocos nucifera* L.) at Kanchana Buri Horticultural Experiment Station, in Kanchana Buri province, Thailand.

Type specimens :

Holotype (male, first-generation) : Recovered from haemocoel of *G. mellonella*. Slide number KU-01.

Allotype (female, first-generation) : Recovered from haemocoel of *G. mellonella*. Slide number KU-02.

Paratype (first-generation males and females and infective-stage juveniles) : Recovered from haemocoel of *G. mellonella*. Twenty males, 20 females and 10,000 IJs fixed in TAF in Vial number KU-03.

All specimens were deposited at the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Chatuchak, Bangkok 10900, Thailand.

SYSTEMATICS : *Steinernema thailandensis* n. sp.

Infective juveniles (third-stage juvenile) (Figure 3-5)

Measurements : (n = 20). All measurements showed total length of range (mean ± SD) in micrometers.

Total body length 404-460 (432 ± 13). Greatest width 22-24 (22 ± 0.6). Distance from anterior end to excretory pore 34-39 (35 ± 2). Distance from anterior end to esophagus base 99-112 (106 ± 4). Tail length 37-47 (42 ± 2). a 18-21 (20 ± 0.8). b 3.8-4.3 (4 ± 0.1). c 9-11 (10 ± 0.4). D % 30-37 (33 ± 1.7). E % 75-91 (83 ± 5).

Description : Body slender and small size, 404 to 460 µm long. Body straight and a gradual reduction in breadth of an elongate body (Figure 3A). Cuticle smooth, slightly annulated. Cuticle of second-stage juvenile, as a sheath, present or absent (may be lost). Excretory pore in anterior 1/3 of esophagus. Esophagus long and narrow, distinctly narrower at level of nerve ring, terminating in a valvate basal bulb (Figure 3B). Cardia present. Anterior portion of intestine with dorsally displaced pouch containing symbiotic bacteria. Lumen of intestine narrow, rectum long, narrow, anus distinct. Tail conical with pointed terminus (Figure 3C). Lip region smooth, generally not set off. Head non-annulated, four cephalic papillae and amphids distinct, labial papillae not developed (Figure 4A). Mouth openings closed. Lateral fields with six incisures (Figure 4B). The phasmids are located about 35 % of the tail length, posterior to the anus (Figure 5A). Posterior region of a living third-stage juvenile, the typical ventral curvature of the tail region forming an angle 110 degrees with body (Figure 5B).

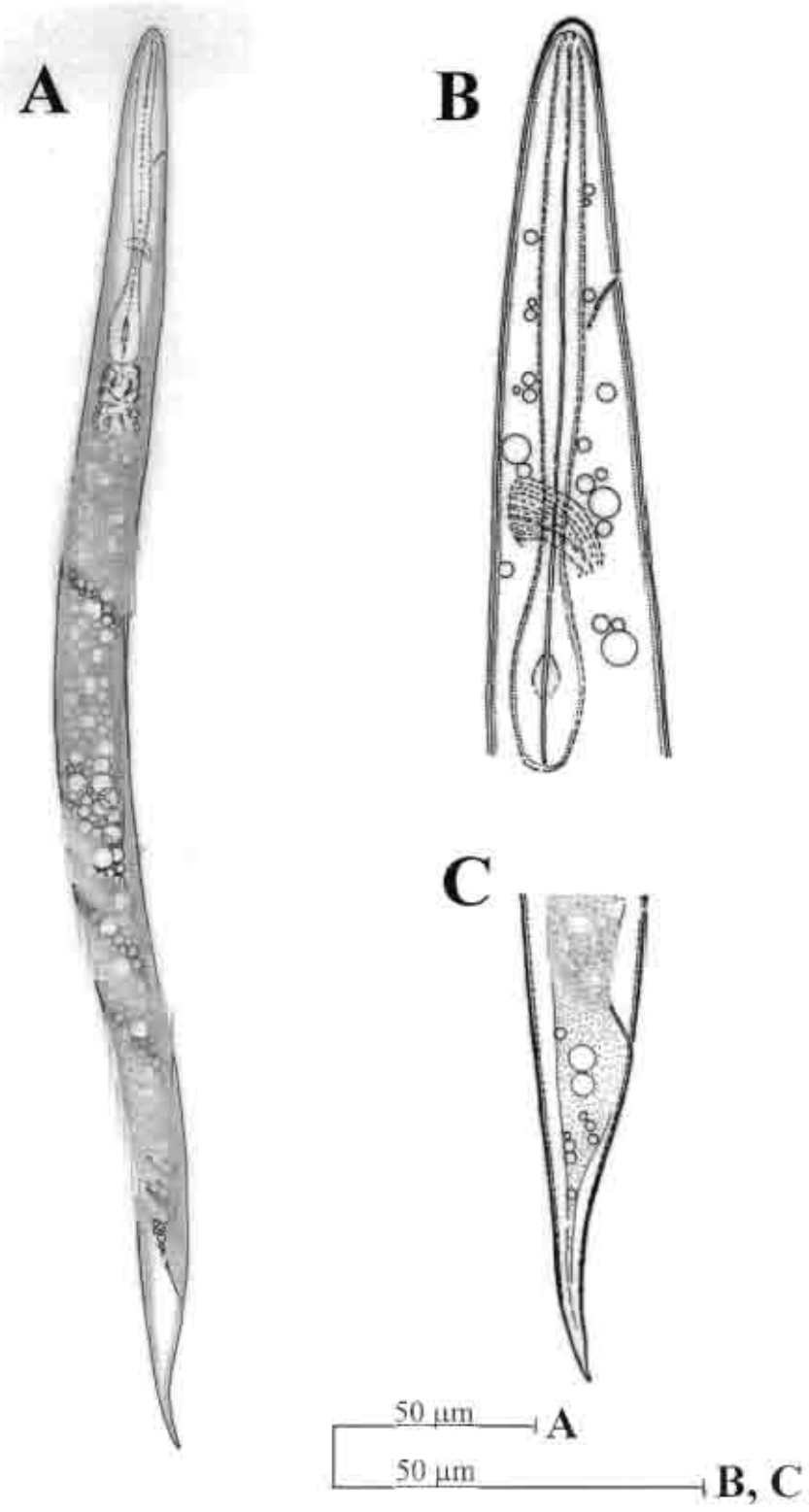


Figure 3 *Steinernema thailandensis* n. sp., third-stage infective juvenile. A) Entire body. B) Anterior end. C) Posterior end.

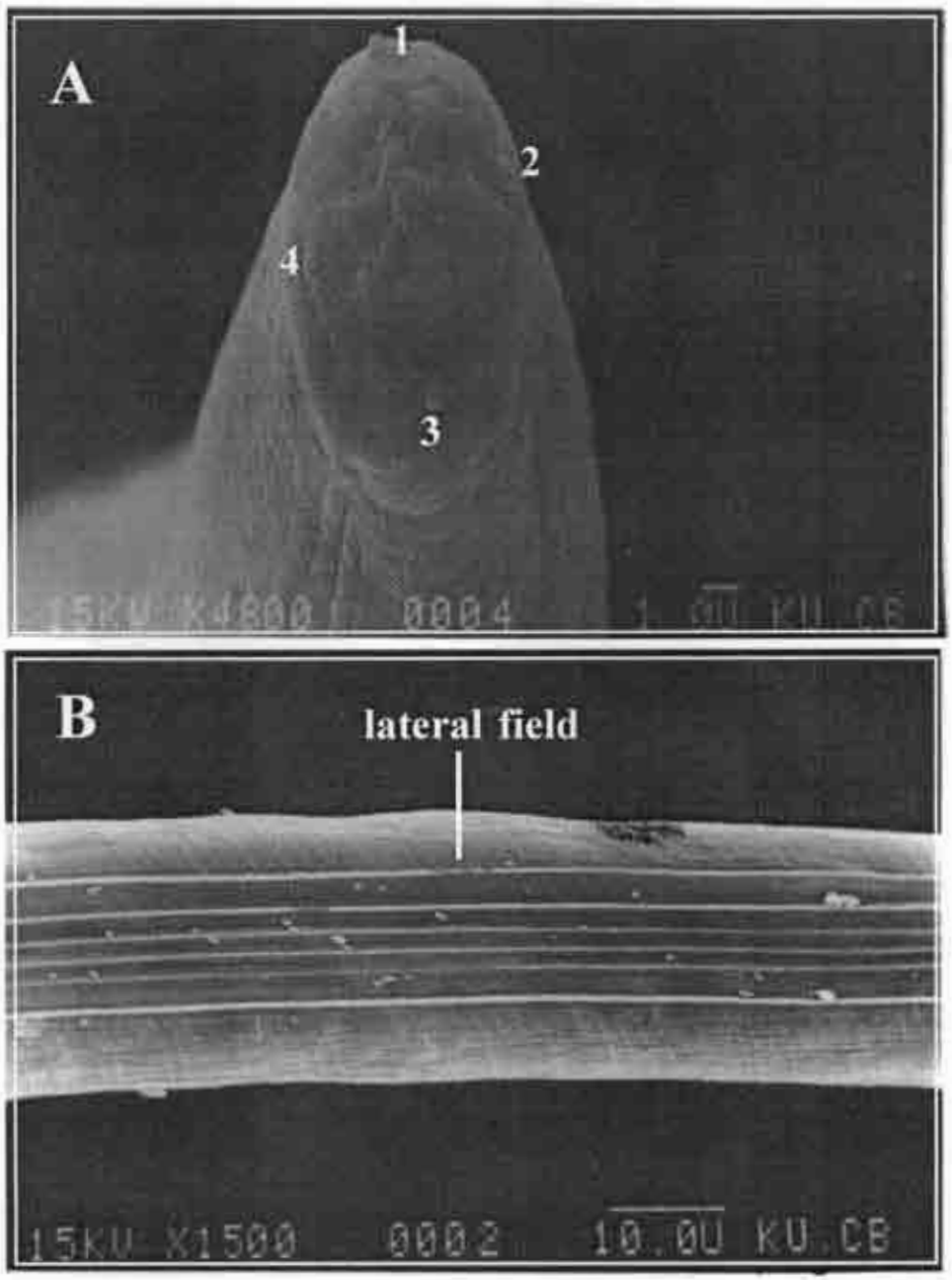


Figure 4 SEM micrographs of *Steinernema thailandensis* n. sp., infective-stage juvenile. A) Anterior region showing four cephalic papillae. B) Lateral field with six ridges.

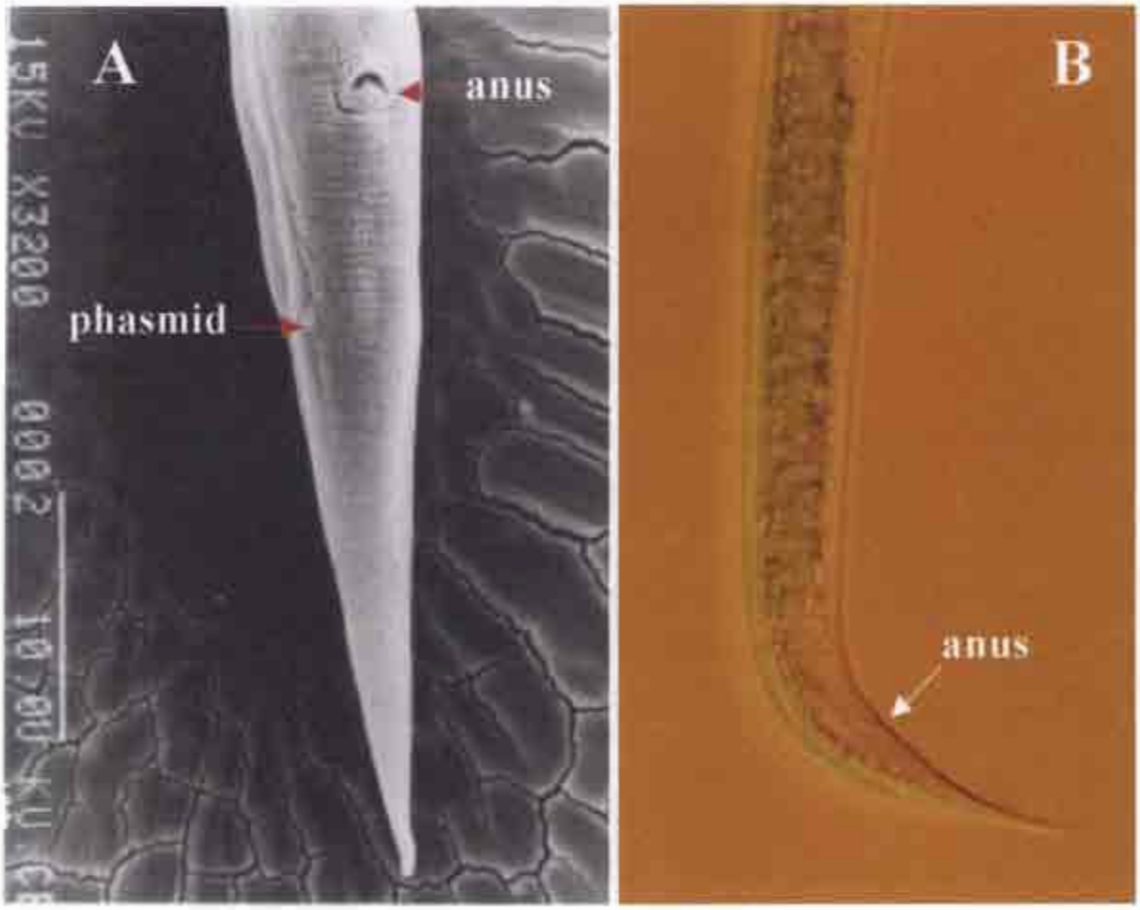


Figure 5 SEM and light micrographs of *Steinernema thailandensis* n. sp., infective-stage juvenile. A) Phasmid. B) Posterior region of a living but inactive nematode. Typical ventral curvature of the tail region.

Male (Figure 6-7)

Measurements : All measurements showed total length of range (mean ± SD) in micrometers.

Male, first generation (n = 10). Total body length 1,784-2,260 (2,008 ± 120). Greatest width 145-172 (155 ± 8). Distance from anterior end to excretory pore 85-103 (93 ± 6). Distance from anterior end to esophagus base 175-197 (188 ± 7). Anal body width 39-63 (51 ± 7). Tail length 24-41 (33 ± 4). Spicule length 83-99 (94 ± 5). Gubernaculum length 61-79 (67 ± 4). D % 44-56 (50 ± 4). SW (spicule length divided by anal body width) 1.3-2.5 (1.9 ± 0.3). GS (gubernaculum length divided by spicule length) 0.6-0.8 (0.7 ± 0.05).

Male, second generation (n = 10). Total body length 1,047-1,530 (1,360 ± 130). Greatest width 70-94 (85 ± 8). Distance from anterior end to excretory pore 67-98 (87 ± 8). Distance from anterior end to esophagus base 130-159 (147 ± 8). Anal body width 27-33 (29 ± 2). Tail length 17-27 (20 ± 3). Spicule length 78-92 (85 ± 4). Gubernaculum length 43-69 (58 ± 7). D % 42-64 (59 ± 6). SW 2.4-3.3 (2.9 ± 0.3). GS 0.5-0.8 (0.7 ± 0.01).

Description : Body enlarged and curved posteriorly, first generation males larger than second generation males (1st generation, 2,008 μm and 2nd generation males, 1,360 μm), C-shaped when heat-killed (Figure 6A). Head truncate to slightly round, continuous with the body. Cuticle smooth, lateral field not observed. Lip region with six fused lips, with a characteristic rhabditoid. Mouth opening triangular, stoma reduced, short and wide. Esophagus muscular cylindrical procorpus; metacorpus slightly swollen; an indistinct isthmus and basal bulb enlarged. Cardia well developed. Excretory pore located at posterior 1/2 of esophagus length. Nerve ring crossing isthmus (Figure 6A). Testis single and

reflexed. Manubrium (head) of spicules short, shaft distinct, manubrium comprised about 30 % of the spicule length (Figure 6B). Laminar (blade) long and narrow bearing with two ridges. Velum present. Gubernaculum slender and long about 3/4 length of spicules. Tail conoid and mucronate, 23 genital papillae (11 pairs and a single ventral preanal) (Figure 7A). Spicules paired, light brown in color (Figure 7B).

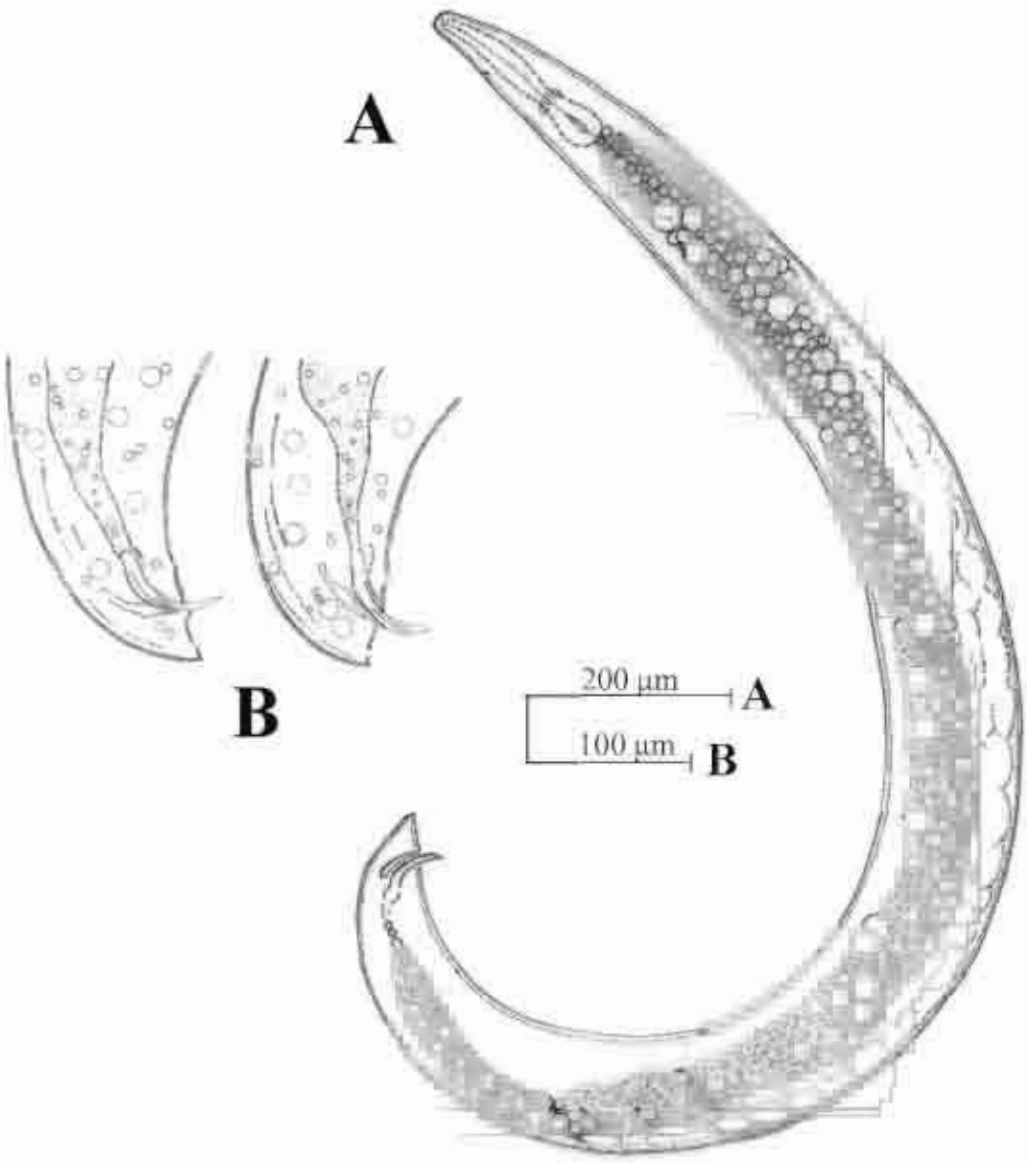


Figure 6 *Steinernema thailandensis* n. sp., first-generation male. A) Entire body. B) Tail shape.

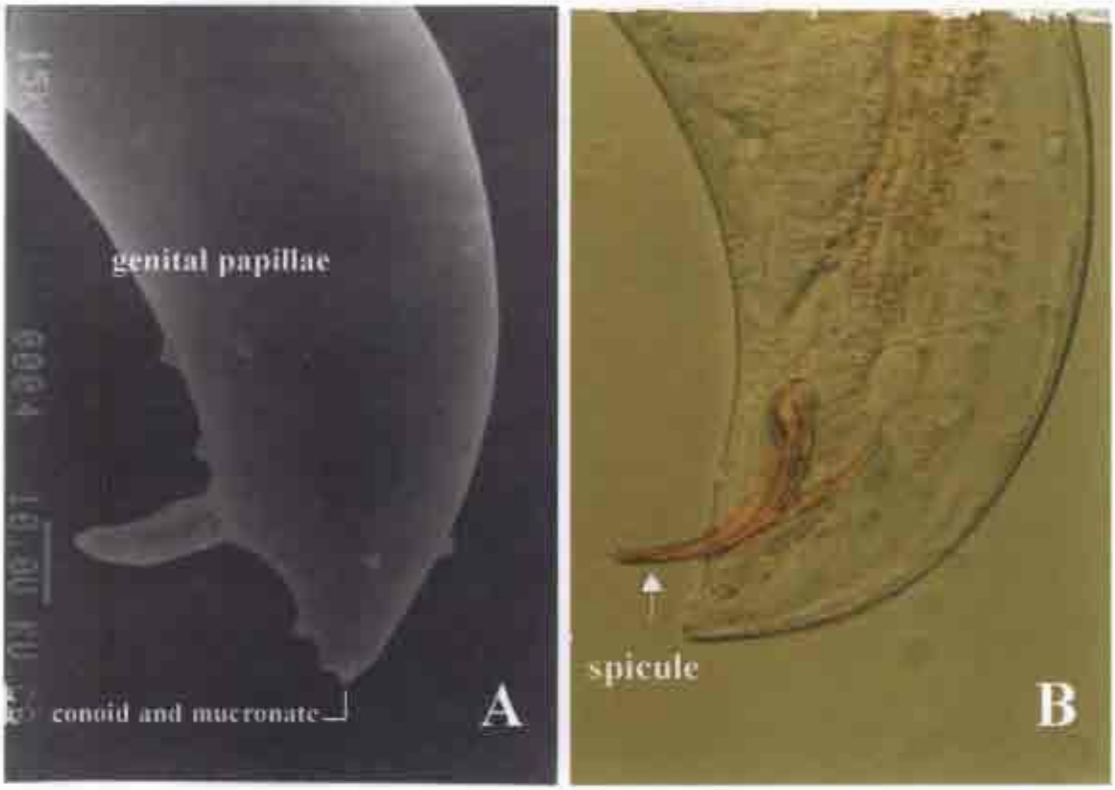


Figure 7 SEM and light micrographs of *Steinernema thailandensis* n. sp., male. A) Posterior region showing shape, genital papillae and conoid and mucronate. B) Spicule paired, light brown in color.

Female (Figure 8-10)

Measurements : All measurements showed total length of range (mean ± SD) in micrometers.

Female, first generation (n = 10). Total body length 5,332-8,332 (6,926 ± 871). Greatest width 206-259 (230 ± 16). Distance from anterior end to excretory pore 94-153 (129 ± 16). Distance from anterior end to esophagus base 255-294 (275 ± 11). Anal body width 75-106 (91 ± 11). Tail length 20-35 (28 ± 5). Vulva % 52-55 (53 ± 0.9).

Female, second generation (n = 10). Total body length 2,857-3,854 (3,318 ± 332). Greatest width 204-243 (219 ± 10). Distance from anterior end to excretory pore 71-94 (86 ± 7). Distance from anterior end to esophagus base 255-290 (271 ± 11). Anal body width 39-59 (51 ± 7). Tail length 36-47 (42 ± 4). Vulva % 53-58 (56 ± 1.6).

Description : Body elongate, spiral shaped when heat-killed (Figure 8A and 10A). First generation females larger than second generation females (1st generation, 6,926 μm and 2nd generation, 3,318 μm). Cuticle slightly and easily to crack in water. Cuticle smooth under light microscopy, but annules faint under SEM. Esophagus typical of family, procorpus cylindrical, muscular; metacorpus slightly swollen, non-valvate; isthmus distinct; basal bulb muscular, with small size (Figure 8B). Cardia well developed. Nerve ring surrounding isthmus. Esophago-intestinal junction large, prominent (Figure 8B). Excretory pore anterior to mid-metacorpus. The first and second generation females had protruding vulval lips and both with double-flapped epiptygmas (Figure 8C). Vagina short, muscular; genital tracts amphididelphic, Filling much of the body. Vulva located near middle of body. Head truncated to slightly round, continuous with the body. Six labial,

four cephalic. Six lips, distinct, each with one papilla (Figure 9A). Cephalic papillae sometimes obscure. Amphids not observed. Stoma shallow, stoma circular anteriorly (Figure 9A). Cheilorhabdions prominent, heavily thickened (Figure 9B). Tail of female first generation, blunt tail tip with mucronate (Figure 10B), but female second generation, pointed tail tip (Figure 10C).

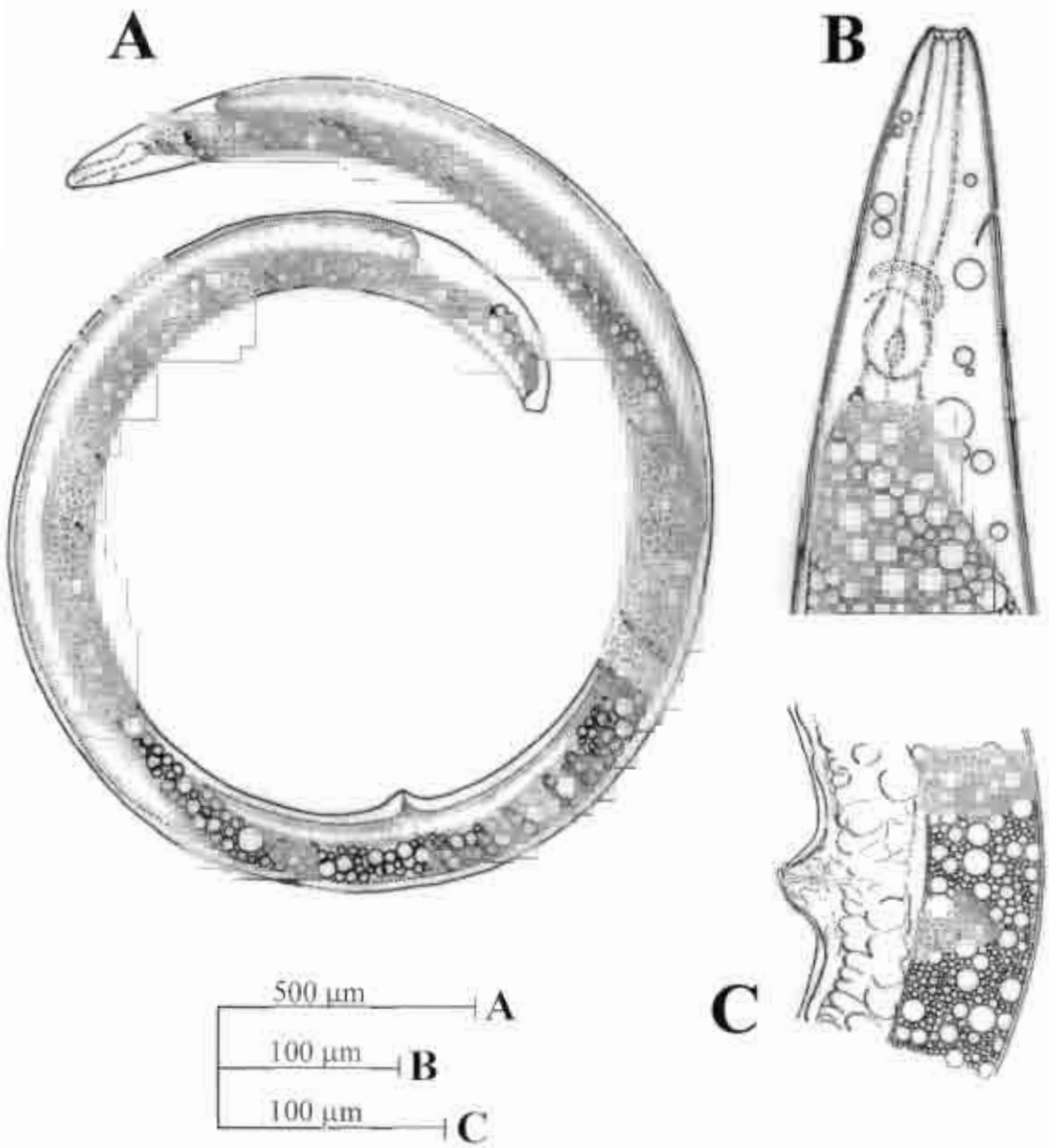


Figure 8 *Steinernema thailandensis* n. sp., first-generation female. A) Entire body. B) Anterior end. C) Double-flapped epiptygma on vulva.

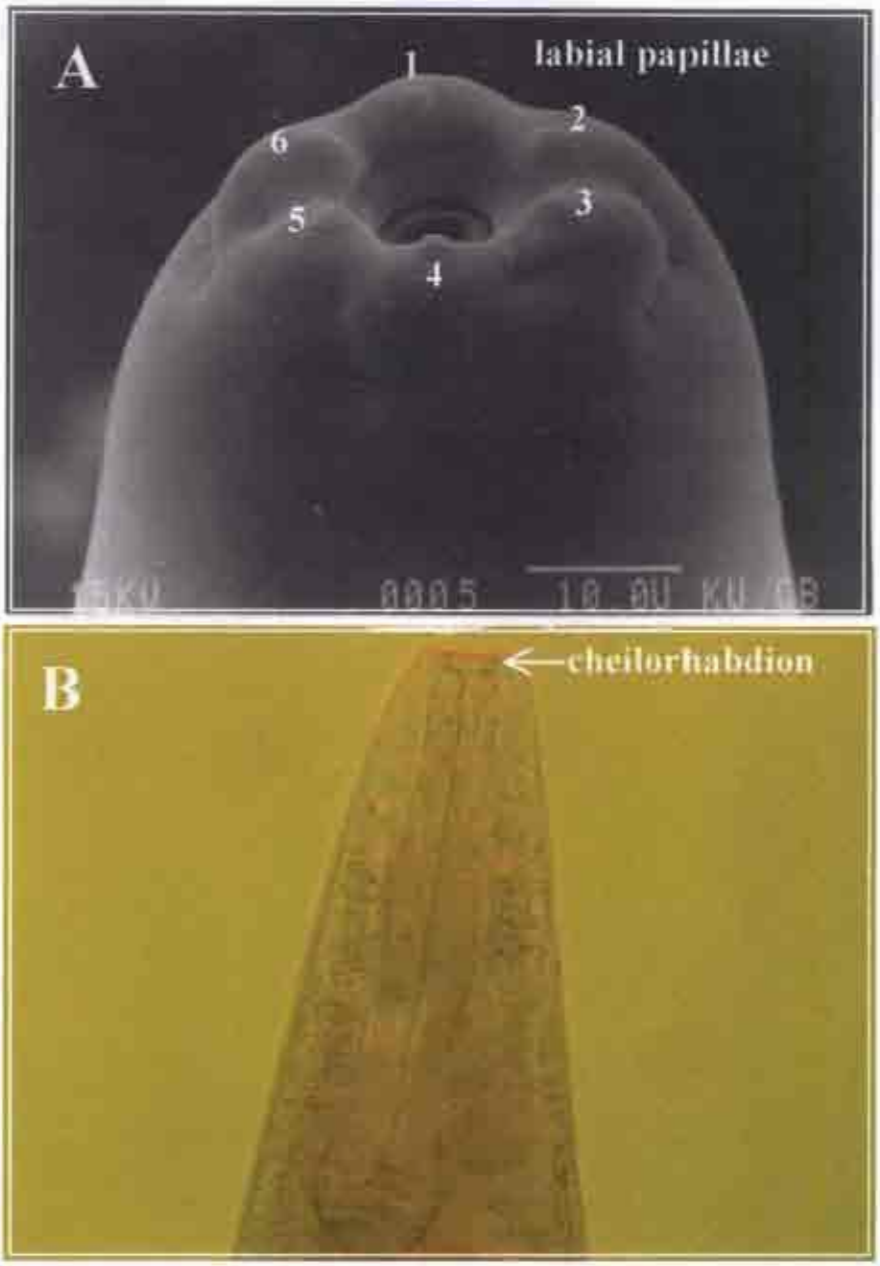


Figure 9 SEM and light micrographs of *Steinernema thailandensis* n. sp., female. A) Anterior region showing six labial papillae. B) Head of the first-generation female showing thickened cheilorhabdions.

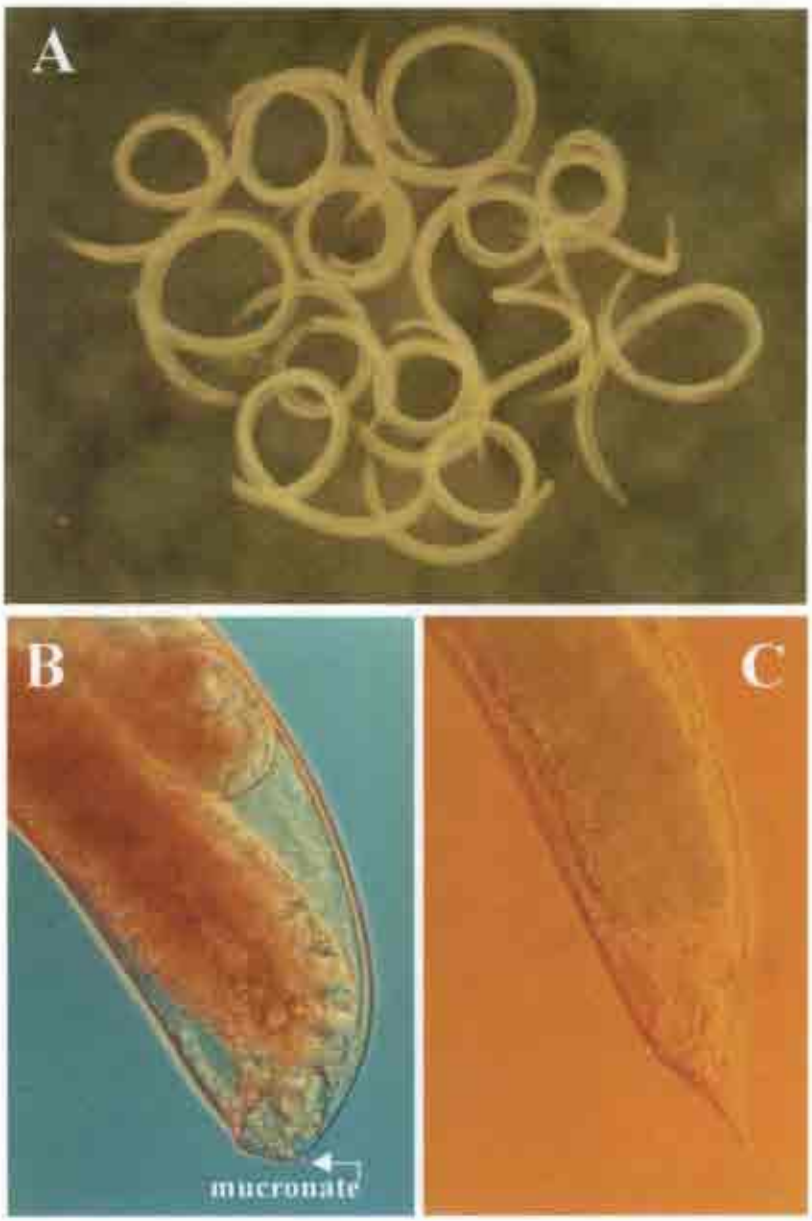


Figure 10 Light micrographs of *Steinernema thailandensis* n. sp., female. A) Spiral-shaped bodies when heat-killed. B) Tail of the first generation female. C) Tail of the second generation female.

2.2 Molecular taxonomy

The restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region of the rDNA repeat produced restriction fragment bands unique to the new species when compared to that of *S. carpocapsae* (Figure 11). PCR amplified products from the ITS region digested with 17 restriction enzymes showed that the RFLP pattern of *S. thailandensis* n. sp. was obviously different from that of *S. carpocapsae*.

Although the majority of the restriction enzyme profiles for *S. thailandensis* n. sp. were identical to that of the *S. carpocapsae*, its *Alu* I, *Dde* I, *Hind* III, *Rsa* I, and *Xba* I profiles were distinct. The Thai steinernematid nematode was similar to *S. feltiae* in its profile yielded by digestion with *Alu* I, while that of *S. carpocapsae* was different.

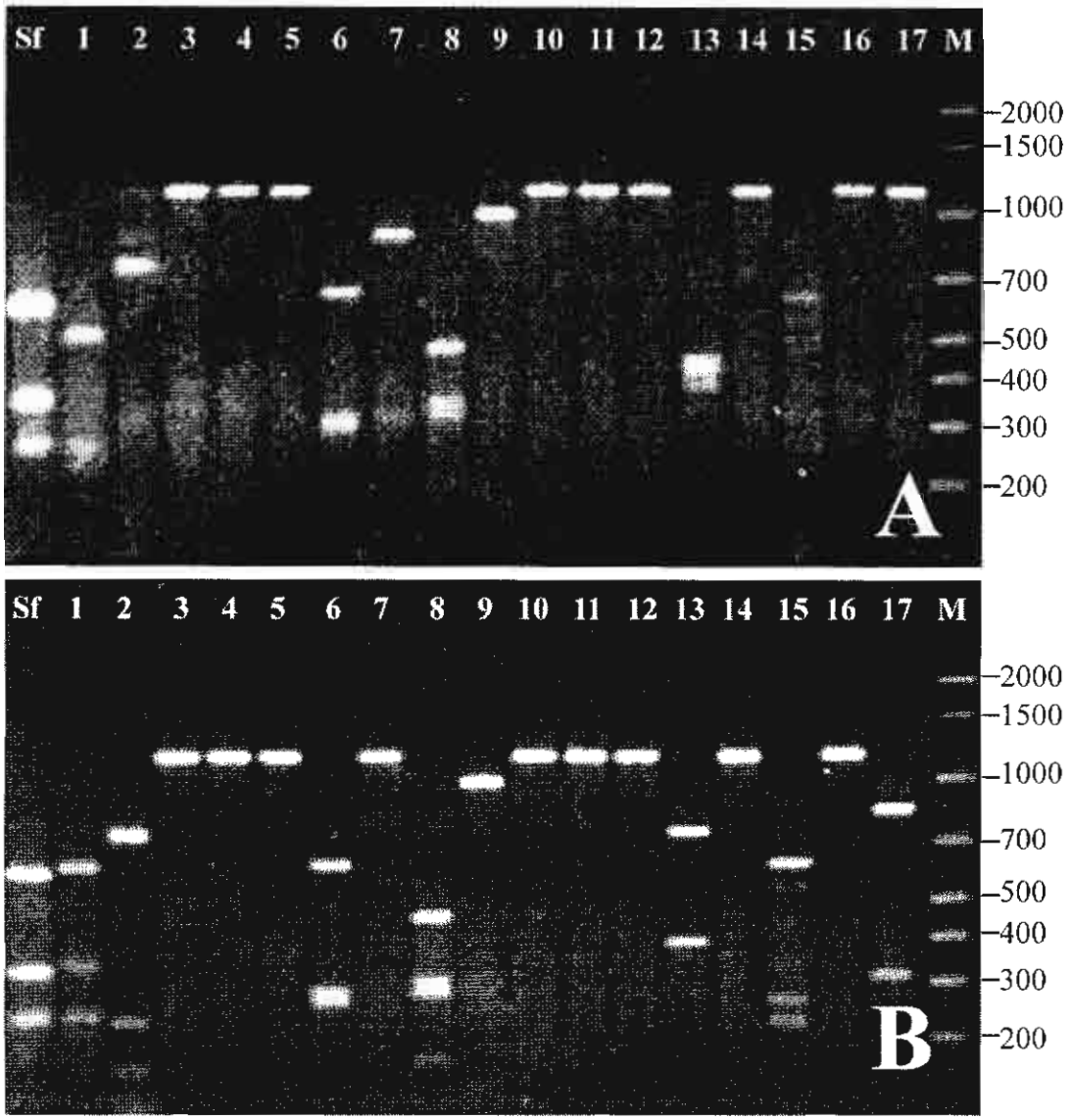


Figure 11 RFLP profiles of two steinernematid isolates digested with 17 different restriction enzymes. (A) *Steinernema carpocapsae.* (B) *S. thailandensis* n. sp. Sf lane is the digest of *S. feltiae* with *Alu* I. Lane 1-17 are digested of each isolate with the following restriction enzymes : 1, *Alu* I; 2, *Dde* I; 3, *Eco*R I; 4, *Eco*R II; 5, *Hae* III; 6, *Hha* I; 7, *Hind* III; 8, *Hinf* I; 9, *Hpa* II; 10, *Kpn* I; 11, *Pst* I; 12, *Pvu* II; 13, *Rsa* I; 14, *Sal* I; 15, *Sau*3A I; 16, *Sau*96 I; and 17, *Xba* I. M, molecular weight marker. A band sizes are shown in base pairs.

Diagnosis and relationships

Steinernema thailandensis n. sp. can be separated from *S. kushidai* (Mamiya), *S. scapterisci* (Nguyen and Smart), *S. carpocapsae* (Weiser), *S. abbasi* (Elawad, Ahmad and Reid), *S. rarum* (De Doucet) and *S. ritteri* (Doucet & Doucet) which are members of a group with the length less than 600 μm by morphological characters.

Morphologically, the average length of *S. thailandensis* n. sp. (432 μm) infective juveniles was different from those of *S. kushidai* (589 μm), *S. scapterisci* (572 μm), *S. carpocapsae* (558 μm), *S. abbasi* (541 μm), *S. rarum* (511 μm), and *S. ritteri* (510 μm) (Table 2). It was the shortest of all the currently described *Steinernema* spp. E% (distance from the anterior end to the excretory pore divided by the tail length x 100) of the infective juveniles of *S. thailandensis* n. sp. did not overlap those of *S. carpocapsae*, *S. rarum* and *S. kushidai*, but it somewhat overlapped those of *S. scapterisci* and *S. abbasi*. The juvenile of *S. thailanensis* n. sp. was short-tailed and its lateral field composing of 6 longitudinal ridges. The male can be separated from those of the other *Steinernema* species by the long spicule lengths of 84 and 74 μm in first and second generations, respectively, and by the strongly curved spicules. Its gubernaculum was large and slender, 3/4 of the length of the spicule. The first and second generation females had protruding vulval lips and both with double-flapped epiptygmas.

Morphological characterization results of the infective-stage juveniles and adults of the Thai nematode isolate did not fit the descriptions of currently recognized species of steinernematid nematodes.

Thus conclusively, identification of species of the Thai steinernematid nematode was determined on the basis of the following characteristics : 1) average length of infective-stage juveniles, 2) tail length of infective-stage juveniles, 3) the D% (the distance from the anterior end to the excretory pore divided by the esophagus x 100) and E% (the distance from the anterior end to the excretory pore divided by the tail length x 100) of infective juveniles, 4) spicule length of males, 5) spicule shape, and 6) double-flapped epiptygma of first and second generation females.

From the studies, this nematode species could be identified as a new species on morphological characters as well as on the DNA characters. DNA examination indicated the distinctness of *S. thailandensis* n. sp. when compared with populations of *S. carpocapsae.*

Table 2 Comparison of morphometric characters of infective-stage juveniles and males of *Steinernema thailandensis* n. sp. and other *Steinernema* spp.

Species	Infective-stage juveniles				Males		Reference
	Body length (μm)	Tail length (μm)	D%	E%	Spicule length (μm)	Gubernaculum (μm)	
S. kushidai	589 (524-662)	50 (44-59)	41 (38-44)	92 (84-95)	63 (48-72)	44 (39-60)	Mamiya, 1988
S. scapterisci	572 (517-609)	54 (48-60)	31 (27-40)	73 (60-80)	83 (72-92)	65 (59-75)	Nguyen & Smart, 1990
S. carpocapsae	558 (438-650)	53 (46-61)	26 (23-28)	60 (54-66)	66 (58-77)	47 (39-55)	Weiser, 1955
S. abbasi	541 (496-579)	56 (52-61)	53 (51-58)	86 (79-94)	65 (57-74)	45 (33-50)	Elawad *et al.* , 1997
S. rarum	511(443-573)	51 (44-56)	35 (30-39)	72 (63-80)	47 (42-52)	34 (23-38)	De Doucet, 1986
S. ritteri	510 (470-590)	49 (44-54)	46 (44-50)	88 (79-97)	69 (58-75)	44 (33-50)	Doucet & Doucet, 1990
S. thailandensis n. sp.	432 (404-460)	42 (37-47)	33 (30-37)	83 (75-91)	84 (73-89)	67 (61-79)	-

Notes : D%, distance from anterior end to excretory pore divided by distance from anterior end to esophagus x 100; E%, distance from anterior end to excretory pore divided by the tail length x 100.

3. Biology of the nematode

3.1 Life cycle

The life cycle of *S. thailandensis* n.sp. is comparable to existing species of *Steinernema,* including an egg, four juvenile stages, and adults (male and female). It took four days at 30°C for the infective juvenile (IJ) to enter the haemocoel of *G. mellonella,* release its associated bacterium, complete its first generation and develop to become the IJ again (Figure 12).

The IJ invaded the host through the mouth or spiracles, and penetrated into the haemocoel. The body of the IJ became wider, and its stoma were open. It secreted toxin which inhibited the insect inducible immune system. It also released its insect-pathogenic bacterial symbiont through the anus into the haemocoel of the host. This process took less than 12 hours after the IJ entered the host. The insect died 16-20 hours after infection. After 24 hours, IJ molted to the fourth-stage juvenile (J4). The body width of the J4 increased much faster than its length did until it became almost as wide as the young male and female. Then the young male and female developed to become the first generation adult males and females in 48 hours after entering the host. These adults mated and the female laid eggs initially, but later the eggs were retained and hatched in her body. Some 12-24 hours later, the first-stage juvenile (J1) molted and broke out of female body and moved into the haemocoel of the insect. Some of the J1 molted to second-stage juvenile (J2), which later became preinfective J2 and to the IJ in 12-24 hours.

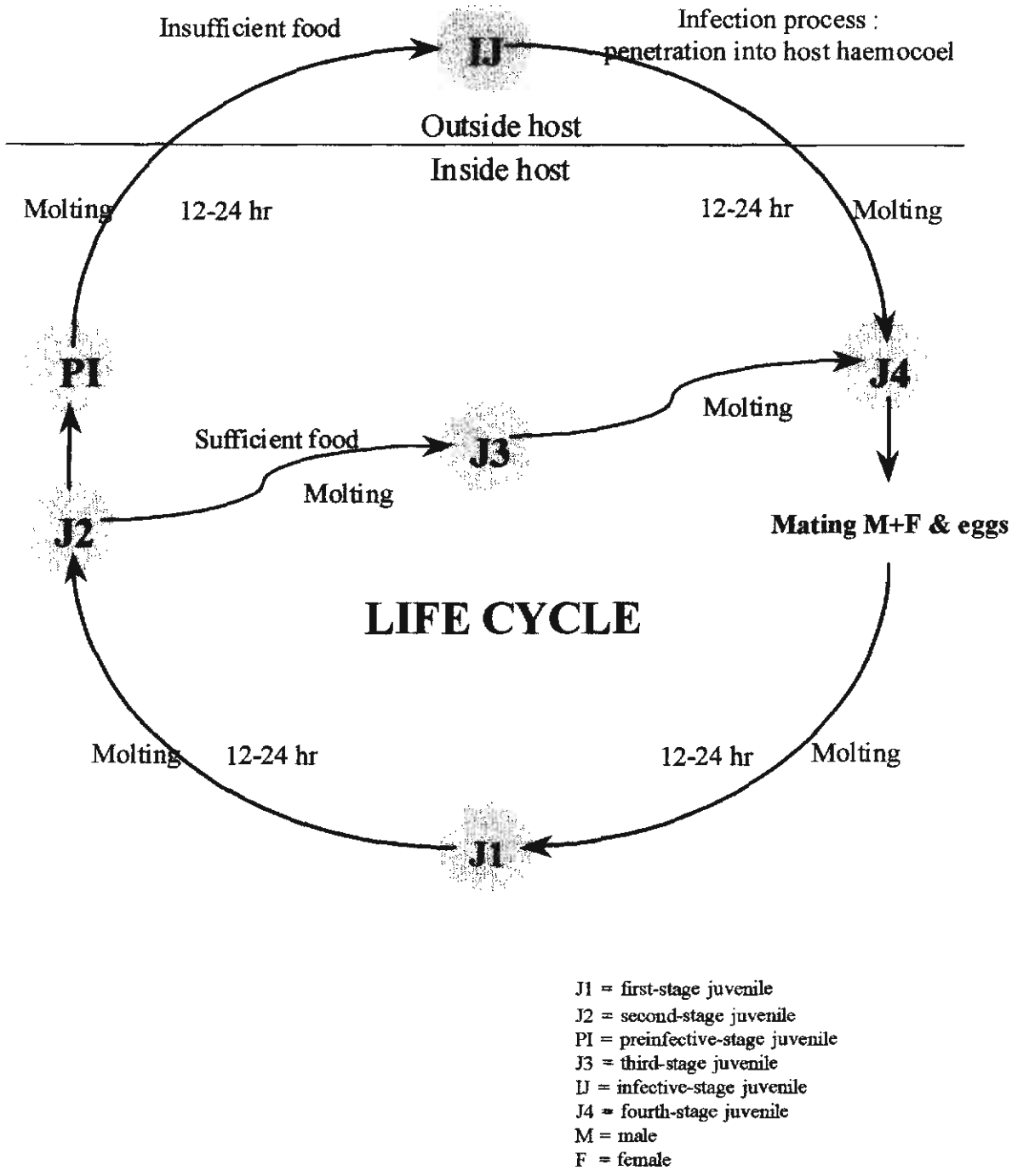


Figure 12 Life cycle of *Steinernema thailandensis* n. sp. at 30 °C.

3.2 Effect of temperature on the life cycle

Temperature was found effecting the growth rate and maturation of *S. thailandensis* n. sp. in *G. mellonella* (Table 3).

At 15 °C, the IJs poorly developed into the J4 stages. The J4 first appeared at 3.6 % after 48 hours and they never reached the adult stage because all died within 144 hours after exposure to the hosts. The infected host did not die after 144 hours.

At 20 °C, the IJs developed into the J4, and the first-generation males and females at 85.8 % and 14.2 %, respectively, in 48 hours. After 96 hours, most of the first-generation adults had already mated, and laid some eggs . The J1 emerged from the female bodies, and the J2 and J3 were observed inside the *Galleria* cadavers within 120 hours after exposure.

At 25 °C, the IJs developed into the J4 at 52.5 % within 24 hours and then developed into the first-generation males and females in 48 hours after exposure. In 144 hours, the second-generation adults were observed at 1.7 %.

At 30 °C, the IJs developed into adult males and females at 4.3 % in 24 hours after exposure to the hosts. J1 and J2 from the first-generation females first appeared in 72 hours and J3 were found in 96 hours. In 144 hours, 2.9 % of the second-generation adults were present and some of these females contained J1.

At 35 °C, the first-generation males and females first appeared in 24 hours at 2.6 %. Adults started mating and produced eggs in 72 hours after exposure. The J1, J2 and J3 were found inside the *Galleria* cadavers within 96 hours. The

Table 3 Development of *Steinernema thailandensis* n. sp. in *Galleria mellonella* at different temperatures.

Temperatures °C	Stages of nematode	Development percentage at different durations (hours)					
		24	48	72	96	120	144
15	IJ	100	96.4	80.0	72.2	66.7	63.3
	J4	0	3.6	20.0	27.8	33.3	36.7
20	IJ	84.2	0	0	0	0	0
	J4	15.8	85.8	53.6	27.0	23.0	17.4
	Adults (G1)	0	14.2	46.4	73.0	97.4	52.2
	J1 & J2	0	0	0	0	2.4	13.0
	J3	0	0	0	0	0.2	17.4
25	IJ	47.5	0	0	0	0	0
	J4	52.5	11.1	17.7	6.7	11.0	12.3
	Adults (G1)	0	88.9	82.3	93.3	85.4	13.4
	J1 & J2	0	0	0	0	2.4	60.0
	J3	0	0	0	0	1.2	12.6
	J4	0	0	0	0	0	0
	Adults (G2)	0	0	0	0	0	1.7
30	IJ	18.8	9.9	0	0	0	0
	J4	76.9	23.2	0	0	0	0
	Adults (G1)	4.3	66.9	78.8	5.2	2.0	0
	J1 & J2	0	0	21.2	93.0	88.2	72.6
	J3	0	0	0	1.8	5.8	20.0
	J4	0	0	0	0	4.0	4.5
	Adults (G2)	0	0	0	0	0	2.9
35	IJ	62.1	21.7	0	0	0	0
	J4	35.5	0	0	0	0	0
	Adults (G1)	2.6	78.3	100.0	40.0	30.8	30.9
	J1 & J2	0	0	0	50.7	42.3	55.6
	J3	0	0	0	9.3	7.7	10.3
	J4	0	0	0	0	19.2	2.2
	Adults (G2)	0	0	0	0	0	1.0
38	IJ	100.0	42.8	16.7	22.8	34.6	20.0
	J4	0	28.6	60.3	58.6	38.9	45.7
	Adults (G1)	0	38.6	23	18.6	26.5	34.3

Notes : G1 = first-generation; G2 = second-generation.

second-generation adults were present in 144 hours at 1.0 % and some of these females contained J1.

At 38 °C, the IJs did not develop in 24 hours after exposure to the hosts. This temperature affected the development cycle of the nematodes. However, the nematodes reached the adult stage but males and females became adults without mating and producing eggs. Some of the dead adults were found in dissected *Galleria*.

It was shown from the results that 30 °C was the optimum temperature for the enhancement of the growth rate and maturation of *S. thailandensis* n. sp.

3.3 Effect of temperature on the sex ratio

Temperature also affected the sex ratio of *S. thailandensis* n.sp. in *G. mellonella*. The ratios of the total number of first-generation females to that of the males developed in dissected *Galleria* at different temperatures were significantly different according to Duncan's Multiple Range Test (Table 4).

At 20, 25, 30 and 35 °C, the first-generation female/male ratio was 2.2 : 1 (13/6), 1.8 : 1 (83/46), 4.6 : 1 (106/23) and 3.2 : 1 (71/22), respectively. The total number of females was highest at the 30 °C. From microscopic observations, it was shown that one male could mate with more than one female. Consequently, the greater number of females at 30 °C could produce more of the IJ.

At lower temperature, the total number of adults was found to be increased when the temperature was increased, i.e. from 20 °C to 25 °C, the total number of adults increased from 19 to 129 per one dissected *Galleria*. On the

Table 4 Number of first-generation females and males of *Steinernema thailandensis* n. sp. at 20, 25, 30 and 35 °C in *Galleria mellonella*.

Temperature °C	Total adults/insect[1/]	No. of first generation adults ± SE[1/] Range[2/]	
		Female	Male
20	19	13 ± 3 d[3/] (9-19)	6 ± 3 c (2-12)
25	129	83 ± 11 b (59-101)	46 ± 7 a (36-58)
30	129	106 ± 17 a (76-132)	23 ± 6 b (15-33)
35	93	71 ± 10 c (56-86)	22 ± 4 b (17-30)

[1/] = mean, averaged from 10 insects; [2/] = a range of numbers found in 10 insects; [3/] = numbers with the same letters in the same column are not significantly different at 5 % level, according to Duncan's Multiple-Range Test.

other hand, the total number of adults decreased when the temperature was increased at higher temperature, i.e. from 30 °C to 35 °C, the total number of adults decreased from 129 to 93 per one dissected *Galleria.*

3.4 Effect of temperature on the nematode infection

At the temperatures of 15, 20, 25, 30, 35, and 38°C, the number of infective juveniles per insect found were 7.1, 7.4, 43.4, 65.7, 39.2 and 12.7 %, respectively. The mortality percentages at those temperatures after 24 hours exposure were 0, 0, 80, 100, 100 and 0 %, respectively. At 38 °C, the infection percentage was decreased. The nematode infected well at 25, 30 and 35 °C as shown by the average numbers of infective juveniles of 130, 197 and 118, respectively in the dissected *Galleria* within 24 hours. The greatest average number of the infective juveniles was found at 30 °C at 65.7 % of infective juveniles per insect (Figure 13).

Laboratory studies indicated that *S. thailandensis* n. sp. caused high mortality against the test insect under different temperature ranges. At lower temperatures of 15 and 20 °C and at higher temperature of 38 °C, the nematode could not kill the insect within 24 hours but at 25, 30 and 35 °C it caused 80, 100 and 100 % mortality, respectively (Figure 13).

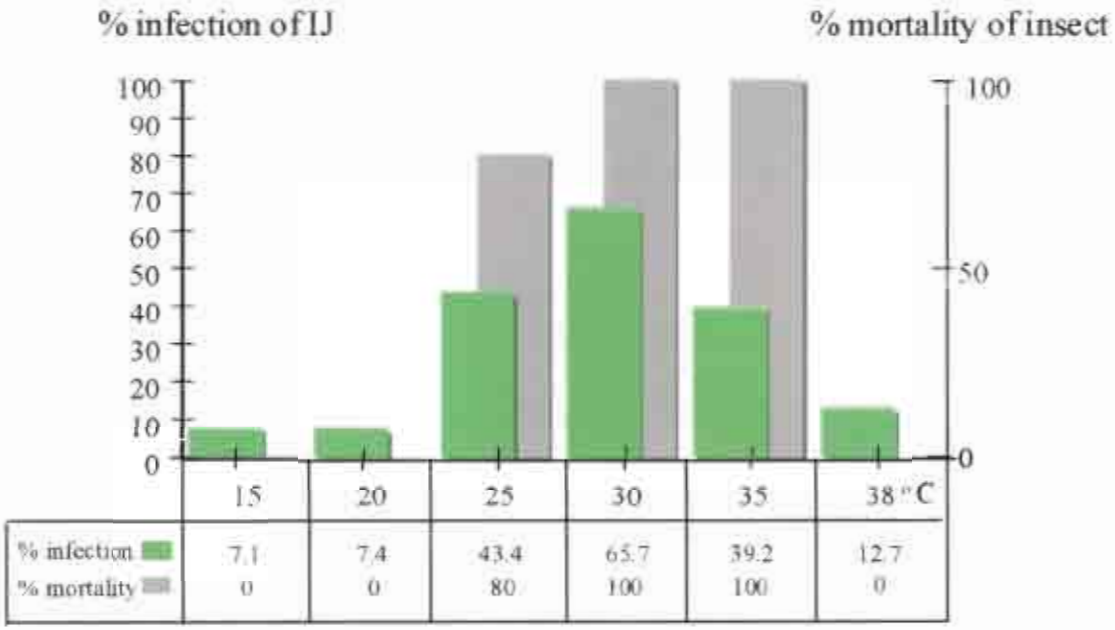


Figure 13 The infection percentage of the infective juvenile (*Steinernema thailandensis* n. sp.) and mortality of the test insect (*Galleria mellonella*) after 24 hours at different temperatures.

3.5 Symbiotic bacteria

The bacteria symbiotically associated with the insect pathogenic nematode, *S. thailandensis* n. sp. was examined. The third-stage infective juveniles of *S. thailandensis* n. sp. contained the cells of a symbiotic bacterium in the intestine lumen. The bacterium was isolated from a drop of haemolymph of nematode-infected *G. mellonella* larvae. It was found similar to *Xenorhabdus* sp. The morphological characters of the colony were granulate, convex, opaque and circular with irregular margin. The primary form of the symbiotic bacterium of *S. thailandensis* n. sp. was characterized by the absorption of bromthymol blue (BTB) from NBTA medium and with clear zone around the colony (Figure 14). The secondary form performed no BTB absorption, red colony and no clear zone. Insect cadavers parasitized by this nematode-bacterium complex presented a black coloration.

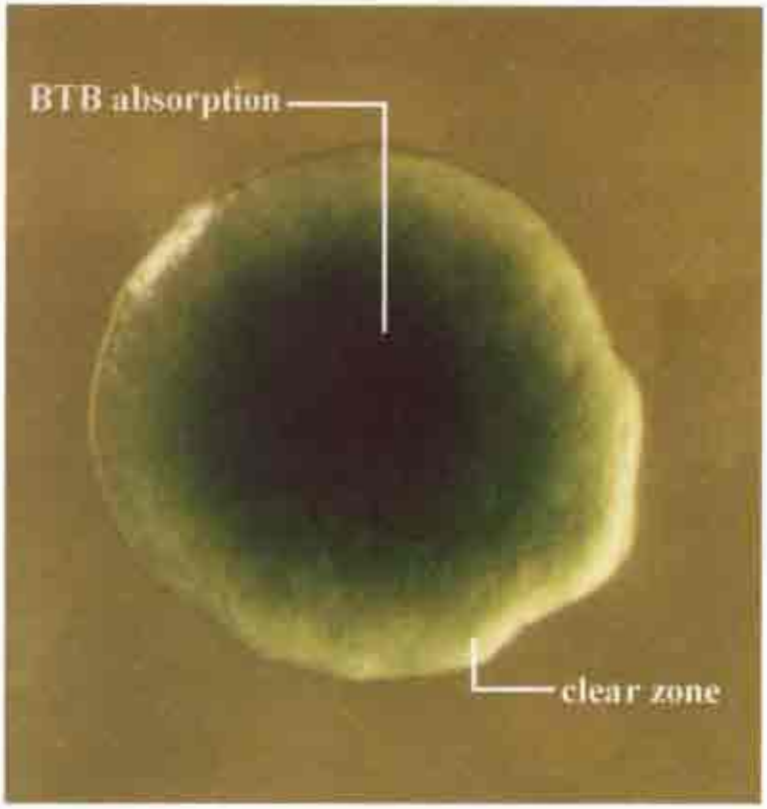


Figure 14 Colony of the symbiotic bacterium (*Xenorhabdus* sp.) isolated from *Steinernema thailandensis* n. sp. showing bromthymol blue (BTB) absorption and clear zone around the colony.

4. Pathogenicity

4.1 Insect bioassay

Pathogenicity test indicated that *S. thailandensis* n. sp. with the symbiotic bacterium was highly virulent against *G. mellonella*. The effect of different nematode dosages on the insect mortality was significant, as summarized in Table 5.

A new nematode isolate from Thailand was highly pathogenic in its host. At a dosage of 10 : 1 (IJs/insect), most number of nematodes were moving towards the opening of the test insect and killed 53 % of the insect in the shortest time of 24 hours. This treatment was significantly different from the other treatments. From the treatments with 4 : 1, 6 : 1 and 8 : 1 IJs per insect, the insects were killed at least 50 % within 36 hours. Figure 15 showed the relationships among time, insect mortality and nematode population. The nematode could kill the insect more rapidly when its number was increased. The mortality percentage was also increased when the time exposed to the insect was increased.

Table 5 Mortality of *Galleria mellonella* infected with *Steinernema thailandensis* n. sp. at different doses and times.

Doses	Mortality of *Galleria mellonella* (%)			
(IJ : insect)	12 hours	24 hours	36 hours	48 hours
0 : 1 (control)	0 c[1]	0 d	0 c	0 d
4 : 1	6.7 c	13.3 c	50.0 b	53.3 c
6 : 1	20.0 b	26.7 b	56.7 b	63.3 b
8 : 1	23.3 b	33.3 b	56.7 b	63.3 b
10 : 1	43.3 a	53.3 a	73.3 a	83.3 a
CV. (%)	24.0	26.3	13.4	9.8

[1] Numbers with the same letters in the same column are not significantly different at 5 % level, according to Duncan's Multiple-Range Test.

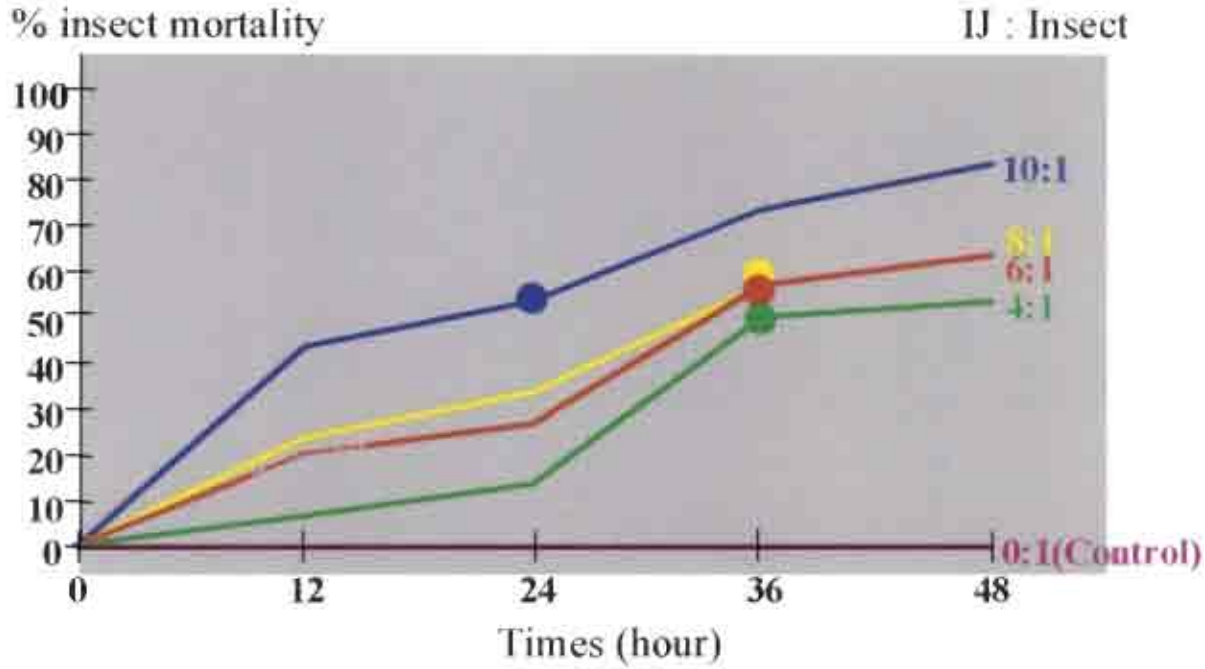


Figure 15 Relationships among time, mortality percentage of *Galleria mellonella*, and population of *Steinernema thailandensis* n. sp.

4.2 Heat tolerance

Heat tolerance test of *S. thailandensis* n. sp. and *S. carpocapsae* from the U.S. was investigated against *G. mellonella* at various temperatures. From the results, temperature seemed to have high effect on insect death. At both 30 and 35 °C, *S. thailandensis* n. sp. caused insect death of 100 % within 22 hours, while *S. carpocapsae* caused insect death 100 % at 30 °C within 30 hours. At both 35 and 38 °C, none of *S. carpocapsae* died within 48 hours. At 15 °C, none of both *S. thailandensis* n. sp. and *S. carpocapsae* died within 48 hours. At the highest temperature of 38 °C, the infective juveniles of *S. thailandensis* n. sp. still could kill the insect at 30 hours, but no 100 % mortality at 48 hours (Figure 16).

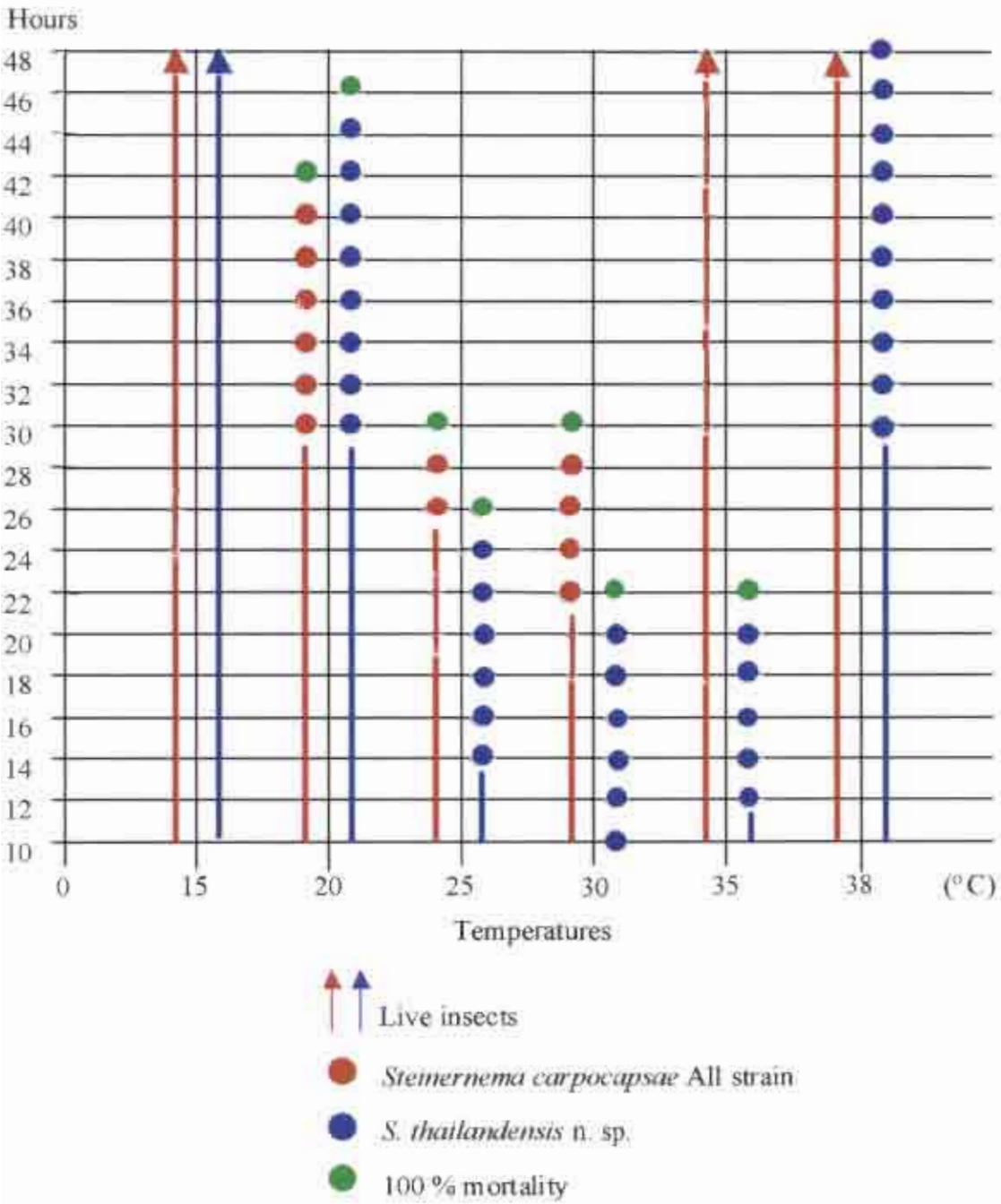


Figure 16 Mortality of *Galleria mellonella* exposed to *Steinernema carpocapsae* All strain and *S. thailandensis* n. sp. at different temperatures.

5. Mass rearing

Nematode counts of infective-stage juveniles were conducted in five samples collected from modified culture media compared with those in lipid agar (LP). The numbers of infective juveniles produced in 20 g medium of soybean milk (SM), wheat flour (WF), soybean flour (SF), corn syrup (CS) and corn flour (CF) were shown in Figure 17.

The yield of infective juveniles was very high in the media containing soybean, reaching approximately 6×10^5 per 20 g medium. The major components of SM and SF were proteins and lipids which were necessary for the production of nematode progeny. Soybean milk and soybean flour at a concentration of 10 g per liter were supplemented to the media. Both culture media supported greater nematode reproduction than did the wheat or corn medium.

The bacterial symbionts are essential for successful mass culture of the nematodes. These bacteria multiplied on artificial media and the nematodes feed on the bacterial cells and other necessary nutrients to reproduce large numbers of offsprings. *S. thailandensis* n. sp. could be reared in soybean media. However, the yield of this nematode in lipid agar was higher than that in modified media. The nematode yield in SM and SF media were 661,400 and 615,000 per 20 g medium, respectively while in the lipid agar, the yield was 1,215,200 (Figure 17).

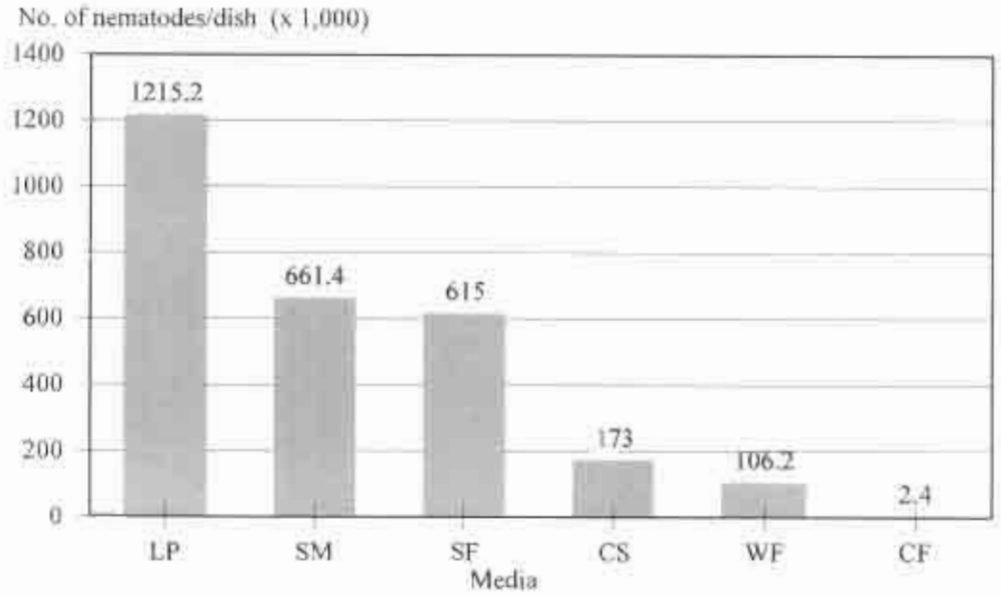


Figure 17 Population count of *Steinernema thailandensis* n. sp. reared on modified culture media compared to lipid agar medium (LP). The modified culture media are soybean milk (SM), soybean flour (SF), corn syrup (CS), wheat flour (WF), and corn flour (CF).

6. Potential for biological control

The infective juveniles of entomopathogenic nematodes have been observed to destroy a wide range of insect pests under laboratory conditions. The results of this experiment indicated that *S. thailandensis* n. sp. and its symbiotic bacterium had high potential to be a biological control agent of seven species of Lepidoptera, three species of Coleoptera, and one species each of Isoptera, Homoptera and Blattoidea. The nematode was effective in killing all of these insect pests species in the laboratory tests (Table 6).

S. thailandensis n. sp. exhibited a higher degree of controlling insects in the order Lepidoptera than controlling those in the other orders. The percentage of mortality in an insect was achieved after exposure to the infective juveniles for 24 hours. One hundred percent mortality was individually found in the larvae of *H. duplifacialia* and *G. mellonella*. The larvae of *S. litura*, *S. exigua*, *P. xylostella* and *H. armigera* died at 55.6, 60.0, 88.9 and 91.7 %, respectively. In Lepidoptera, dissection of infected insect larva showed that the first generation of nematode could develop within 3-4 days. In dead or infected insect larva, it could be seen in all parts of the cadaver when the host cuticle was partially removed to expose the nematodes (Figure18). In Coleoptera, the mortality percentage of an unidentified mushroom beetle, the flea beetle (*P. sinuata*) and an unidentified scarab beetle were approximately 100, 33.0 and 20.0 %, respectively. The cadavers of unidentified scarab beetle when parasitized by this nematode produced wrinkled and black coloration in its body (Figure 19). In Homoptera, the mortality percentage of the aphid, *Myzus persicae* was 44.4 %. The infective juveniles of this nematode entered the natural openings and penetrated into the body cavity of the aphid, and caused insect death. The household affecting insects, *Coptotermes* sp. and *P. americana* also died at 42.1 and 57.1 % at 24 and 48 hours, respectively (Figure 20).

Table 6 Mortality of various insect species when exposed to *Steinernema thailandensis* n. sp.

Insect species	Treated			Control			Mortality (%)[1/]
	Tested (n)	Dead (n)	Alive (n)	Tested (n)	Dead (n)	Alive (n)	
Lepidoptera							
Spodoptera litura	10	6	4	10	1	9	55.56
S. exigua	10	6	4	10	0	10	60.00
Plutella xylostella	45	42	3	30	3	27	88.89
Heliothis armigera	25	23	2	25	1	24	91.67
Henedecasis duplifacialis	12	12	0	10	2	8	100.00
Galleria mellonella	50	50	0	50	0	50	100.00
Coleoptera							
mushroom beetle	10	10	0	10	0	10	100.00
Phyllotreta sinuata	15	5	10	15	0	15	33.33
scarab beetle	5	1	4	5	0	5	20.00
Homoptera							
Myzus persicae	35	20	15	35	8	27	44.44
Isoptera							
Coptotermes sp.	30	19	11	30	11	19	42.11
Blattoidea							
Periplaneta americana	14	8	6	14	0	14	57.14

[1/] Percentage of mortality corrected by Abbott's formula (1925) which is (X - Y) / X x 100, where X = % insects alive in the control treatment; Y = % insects alive in the nematode treatment; (n) = number of test insect.

Lepidoptera

Figure 18 Mortality of some Lepidopterous insects infected by *Steinernema thailandensis* n. sp.

Coleoptera

Figure 19 Mortality of unidentified mushroom beetle and scarab beetle, and *Phyllotreta sinuata* infected by *Steinernema thailandensis* n. sp.

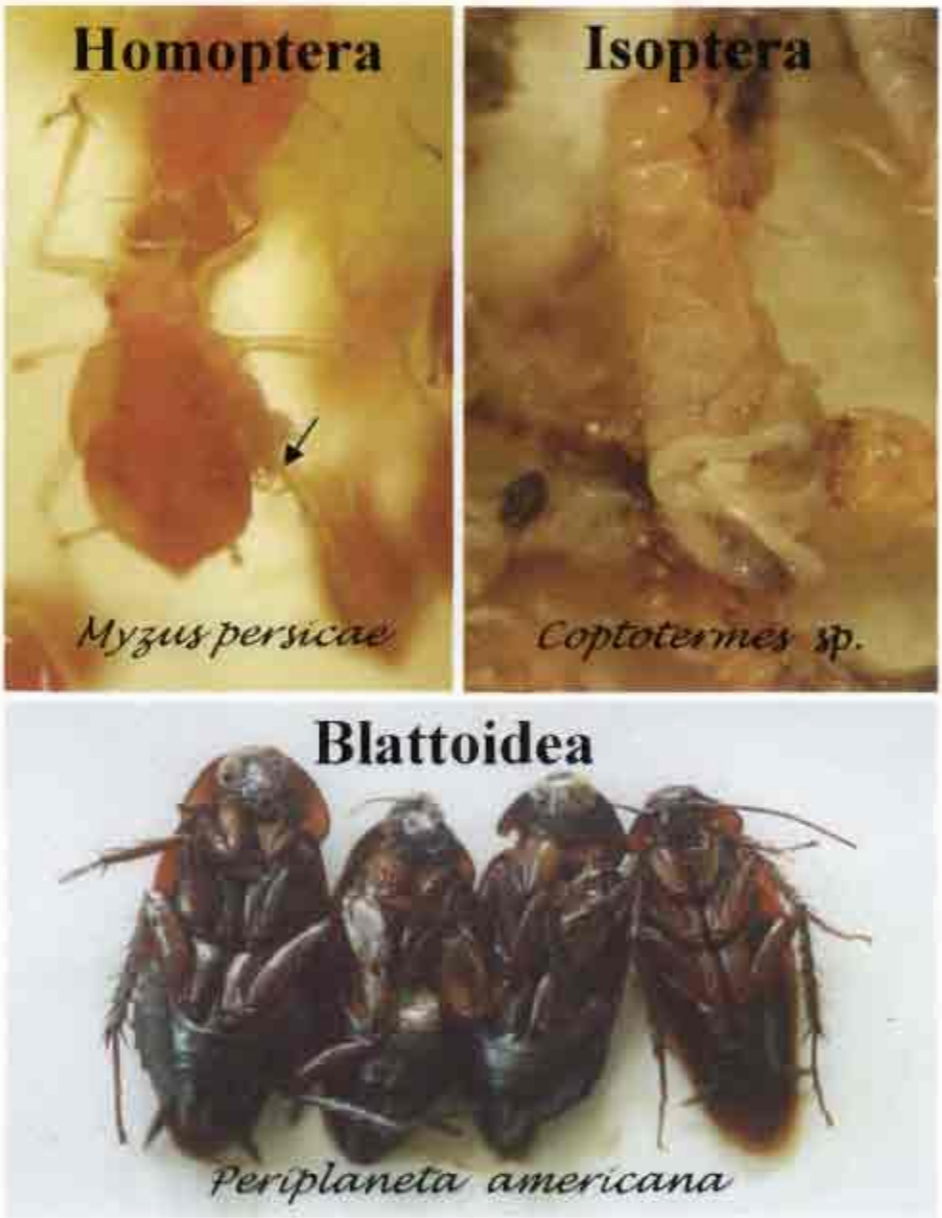


Figure 20 Mortality of *Myzus persicae*, *Coptotermes* sp. and *Periplaneta americana* infected by *Steinernema thailandensis* n. sp.

DISCUSSION

The aim of this study was to obtain a tropically adapted entomopathogenic nematode from Thailand. It was the first extensive survey in Thailand and the first record of the Thai species of *Steinernema* which proved to be a potential biological control agent of certain insect pests in the tropical region. Occurrence of this entomopathogenic nematode was low and restricted to a coconut field in which it was recovered from 1 out of 98 soil samples. The recovery rate of this entomopathogenic nematode was considerably lower than those of 37-48 % which were obtained from the surveys conducted in the European temperate zone (Hominick *et al.*, 1995). The steinernematid was recovered from the high temperature soil and it could survive well in the dry and hot environments in Kanchana Buri province, western Thailand. Soil temperature was one of the important factors determining the abundance of nematodes in the soil. Since the nematode was recovered from the high temperature soil (33 °C at soil surface and 27 °C at 10-15 cm soil depth), this collected isolate may be considered as a heat tolerant strain. It is similar to *S. riobrave* from Texas, USA where its global positions were latitude 26 degrees 08.155'N, longitude 97 degrees 57.366'W, and altitude 21.7 m above mean sea level (Cabanillas *et al.*, 1994).

The new nematode described here was named *S. thailandensis* n.sp. after the country where the species was discovered. It belongs to the same steinernematid as *S. kushidai*, *S. scapterisci*, *S. carpocapsae*, *S. abbasi*, *S. rarum*, and *S. ritteri*, which can be easily distinguished from the other species by the length of the infective-stage juveniles (Nguyen and Smart, 1996). Also, they were separated as a new group because of morphological differences in the body length, D% and E% of the third-stage infective juvenile.

S. thailandensis n. sp. was most similar to *S. carpocapsae* (Weiser, 1955) and *S. scapterisci* (Nguyen and Smart, 1990) in the general morphology of the infective juveniles, adult males and females, but it can be separated from these species by a combination of certain morphological characters. The average length of the infective juveniles of *S. thailandensis* n.sp. (432 μm) was different from those of *S. carpocapsae* (558 μm) and *S. scapterisci* (572 μm). However, range of the total length of *S. thailandensis* n. sp. (404-460 μm) overlaped more or less with *S. carpocapsae* (438-650 μm) and *S. rarum* (443-573 μm).

The third-stage juveniles of *S. thailandensis* n. sp. differed from *S. carpocapsae* in the tail length (37-47 μm vs 46-61 μm), by the ratio b (3.8-4.3 vs 4.0-4.8), D% (30-37 vs 23-28) and E% (75-91 vs 54-66) values. The third-stage infective juveniles of this Thai species could be separated from *S. scapterisci* by the tail length (37-47 μm vs 48-60 μm). Males could also be distinguished from both *S. carpocapsae* and *S. scapterisci* by the shape and size of the spicule and gubernaculum. The first and second generation females of the new species had protruding vulval lips and both with double-flapped epiptygmas which is different from females of *S. carpocapsae.*

S. thailandensis n. sp. is morphologically similar to *S. siamkayai,* a Thai steinernematid previously recovered from Phetchabun province, northern Thailand (Stock *et al.,* 1997). *S. thailandensis* n. sp. differed from *S. siamkayai* in the tail length of the infective-stage juvenile (37-47 μm vs 31-41 μm) and the E% (75-91 vs 85-112). Morphological characters are not always reliable because many characters have wide ranges in size, especially when dealing with very small samples (Reid, 1994).

DNA examination indicated the distinctness of a new species. Obviously, the RFLP profiles for *S. thailandensis* n. sp. were distinct from those of *S. carpocapsae* (All strain), *S. siamkayai* (Stock *et al.*, 1997), *S. monticolum* (Yoshida *et al.*, 1998) and those of the uncharacterized *Steinernema* sp. from Malaysia (Stock *et al.*, 1997). From the comparison among the five steinernematids, *Steinernema* sp. (Malaysian isolate) revealed the most similarlity of its RFLP profiles to those of *S. thailandensis* n. sp. Only its profiles yielded by those digestion with *Alu* I, *Hinf* I, and *Xba* I were distinct from those of *S. thailandensis* n. sp. *S. carpocapsae* was the second-most similar to *S. thailandensis* n. sp. in its profiles yielded by digestion with *Alu* I, *Dde* I, *Hind* III, *Rsa* I, and *Xba* I.

However, the new Thai steinernematid was different from *S. siamkayai* and *S. monticolum* when their DNAs were both digested with eight enzymes. The enzymes which showed different profiles of *S. siamkayai* were *Alu* I, *Dde* I, *Eco*R I, *Hha* I, *Hinf* I, *Rsa* I, *Sau*96 I, and *Xba* I (Stock *et al.*, 1997) while those showed different profiles of *S. monticolum* were *Alu* I, *Dde* I, *Hha* I, *Hinf* I, *Pst* I, *Sal* I, *Sau*3A I, and *Xba* I (Yoshida *et al.*, 1998).

Results from this study indicated that there is a natural distribution of steinernematids in Thailand the same as in Japan (Yoshida *et al.*, 1998) due to the climatic and geographic variations. Since it is shown that the genomic DNA of *S. thailandensis* n. sp. which was recovered from the soil under a coconut tree in the hot and dry Kanchana Buri province is greatly different from that of *S. siamkayai* which was recovered from the soil under a sweet tamarind tree in the cooler Phetchabun province.

The use of RFLP analysis or DNA-band molecular technique was proved to be helpful to first determine the unidentified nematode isolate in this study. This technique is most reliable because it allows the examination of any stages of the life cycle as the genomic DNA of an organism remains constant throughout its life (Reid, 1994).

The life cycle of *S. thailandensis* n. sp. was similar to those of all other *Steinernema* species already described including eggs, four juvenile stages and adults. The third-stage infective juvenile entered the haemocoel of the insect to deliver the associated bacterium and completed at least two generations before emerging from the cadaver as infective juveniles. Its life cycle was temperature-dependent, it was not completed at lower temperature (15 °C) or at higher temperature (38 °C). The optimum temperature was approximately 30 °C, particularly in the *G. mellonella* adult where it developed within 36 hours and completed its life cycle in 4 days after initial infection. The optimum temperature and time for *S. arenarium* to complete its life cycle in both *G. mellonella* and *Otiorhynchus sulcatus* were 23 °C and 132 hours (Poinar and Kozodoi, 1988; Kakouli, 1995). Those for *S. carpocapsae* in the larch sawfly, *Cephalcia lariciphila* were 25 °C and 11 days (Georgis, 1981). Elawad *et al.* (1999) reported that the optimum temperature range for establishment of *S. abbasi* and *S. riobrave* was 25-30 °C. *S. abbasi* completed its development at 30 °C in 108 hours after initial infection. It was the shortest life cycle so far reported for a steinernematid. *S. riobrave* completed its life cycle after 5 days. Both species were the only steinernematids isolated from the semi-arid tropics.

S. thailandensis n. sp. appeared to be more active at higher temperature than *S. carpocapsae*. This nematode species was more high temperature tolerable and less low temperature tolerable than was *S. carpocapsae*. Kaya (1977) reported that *S. carpocapsae* strain DD-136 did not reproduce at 10, 15, or 30 °C and above.

At 15 °C, he found all stages of the nematode in dissected infected *Galleria* larvae, but no infective juvenile emerged up to 21 days. It reproduced well at 20 and 25 °C, with 25 °C was the optimum temperature. Danilov (1976) reported that the life cycle of *S. carpocapsae* strain Agriotos was completed at temperatures from 15 to 28 °C, but the cycle took 60 days to complete at 15 °C. The optimum temperature was 25 °C.

The greatest average number of the first generation females and males of *S. thailandensis* n. sp. produced in *Galleria* at both 25 and 30 °C was 129. It was approximately seven and one fold of the numbers produced at 20 and 35 °C, respectively.

This experiment studied the effect of different temperatures on infection ability of infective juveniles of *S. thailandensis* n. sp. Temperatures affected the number of infecting nematodes and the mortality of the test insect (*G. mellonella*). The numbers of infective juveniles which were found in the test insects at 15 and 20 °C were less than those found at the other temperatures. At 38 °C, the infection percentage was decreased. The infective juveniles infected well at 25, 30 and 35 °C. The greatest average number of infective juveniles in one insect was 197 at 30 °C. Under laboratory conditions, this new nematode species caused high mortality up to 100 % in the test insect at the temperature over 25 °C.

The reported bacterial symbionts of all known species of the family Steinernematidae are in the genus *Xenorhabdus* (Akhurst and Boemare, 1990). The symbiotic bacteria obtained are highly pathogenic to the host insects when released into the haemolymph by the nematodes. They supported nematode reproduction by producing nutrients and antimicrobial agents that inhibited the growth of a wide range of organisms (Akhurst, 1982). Four species have been described : *X. nematophilus, X. bovienii, X. poinarii* and *X. beddingii* (Akhurst and Boemare,

1990). The *Steinernema* has a specific natural association with only one *Xenorhabdus* species. But, the *Xenorhabdus* sp. may be associated with more than one nematode species, *X. bovienii* associated with *S. feltiae, S. kraussei, S. intermedia,* and *S. affinis* (Akhurst and Boemare, 1988). The bacterium from *S. thailandensis* n. sp. was isolated and cultured in the laboratory. It was identified as *Xenorhabdus* sp. by morphological characterization of its colony on NBTA agar-medium. Each steinernematid species has its own specific symbiotic bacterial strain or subspecies (Akhurst, 1982). Further studies on *Xenorhabdus* sp., associated with *S. thailandensis* n. sp. will be conducted to provide a more specific identification.

To determine the potential of a nematode as a biological control agent, it is essential to determine its bio-control level. Although 0-250 cells of the bacteria were found in the foregut of the nematode (Ehlers and Peters, 1995) and the entomopathogenic nematode at infective-stage juvenile is associated with the bacteria in inducing disease in the insect, the rapidity in killing the insect is not dependent solely upon the bacteria. It also depends upon the ability of the nematode to search the host insects as well as how fast the nematode can carry the bacteria into the insect blood system. From the laboratory bioassay, the least number of the infective juveniles of *S. thailandensis* n. sp. that could kill a *Galleria* at 50 % in 24 hours was 10. This capability was consistent in the three reported experiments. However, further bioassays on the methods for determining control quality of this nematode species is needed prior to field trials.

Bioassays on the selection of a heat tolerant Thai steinernematid were conducted to search an effective biological control agent of tropical insect pests. Temperature was one of the criteria affecting its success. *S. thailandensis* n. sp. is a heat-tolerant isolate which is similar to the species from Texas (*S. riobrave*) and Oman (*S. abbasi*) (Elawad *et al.*, 1999). It is a high temperature nematode, effective in killing

insects at the temperature above 35 °C. Other species from Europe, North USA, Japan, China, Russia, Australia, and New Zealand are not heat-tolerant because they are found in the temperate zone.

Steinernematids can be cultured on synthetic diet (Dutky *et al.*, 1964). Monoxenic mass culture techniques with the goal of producing, cost effectively, large numbers of infective nematodes have been used for several years (Dunphy and Webster, 1989). However, the effects of dietary components on nematode culture under monoxenic conditions were insufficiently known to enable optimum culture of nematodes. Throughout the study, as exemplified by the data from the modified media, monoxenic culture of the new Thai entomopathogenic nematode exhibited qualitative and quantitative differences in its response to the modified media. This may reflect species differences in nematode and/or bacteria metabolism and differences in the nematode-bacterium metabolic association.

The cost of the production of nematodes on larvae of *G. mellonella* or on dog biscuit was 1 USS per million nematodes (Poinar, 1972). This experiment demonstrated that the cost of nematode production by using soybean milk and soybean flour was less than 41 baht per 50 dishes (1 liter). The total nematode yield was 33×10^6 and 31×10^6 in soybean milk and soybean flour, respectively. Consequently, the nematodes must be produced in very large number and at low cost.

The potential of *S. thailandensis* n. sp. against larvae and adults of 12 insect pests was tested under laboratory conditions. From the results, this nematode species could kill the insects in the order Lepidoptera better than those in the other orders. This ability is accounted by the nematode's own capable of causing disease towards the insects, and by its association with the symbiotic bacterium, *Xenorhabdus* sp., which

was usually found in the intestinal lumen of the infected insect species. Generally, the use of entomopathogenic nematodes has been against the feeding stages of various insect pests. These results indicate that this nematode will kill larvae and adults of insects. Thus, utilization of *S. thailandensis* n. sp. against various kinds of insect pests is feasible for the field control. Further research is necessary to be able to use *S. thailandensis* n. sp. more practically and effectively as a biocontrol agent against harmful insect pests in the tropics.

CONCLUSION

A new entomopathogenic nematode was collected from a coconut field in Kanchana Buri province, Thailand. Studies had shown that the nematode did not fit nominal species, and it is described herein as *S. thailandensis* n. sp. This nematode species can be distinguished from the other species in its unique characteristics as follows : The body length of the third-stage juvenile was the shortest of all the currently described *Steinernema* spp. The juvenile was short-tailed and its lateral field comprised 6 longitudinal ridges. The spicules of male had long and strongly curved spicules. Its gubernaculum was large and slender. The first and second generation females had protruding vulval lips and both with double-flapped epiptygma. The RFLP banding pattern was also unique when compared to that of *S. carpocapsae* (All strain).

The life cycle at the optimum temperature of 30 °C was four days. The growth rate, sex ratio and infection ability of this nematode were influenced by the temperature. Its life cycle was not completed at 15 and 38 °C. At 20-35 °C, the number of females in the population was greater than that of males, but at 30 °C, the number of females constituted 82 % of the population. At lower (15 and 20 °C) and high temperatures (38 °C), the nematode could not kill the test insects within 24 hours but at 25, 30 and 35 °C, it caused 80, 100 and 100 % mortality in the insects, respectively.

A symbiotic bacterium was isolated from a drop of haemolymph of a nematode infected *G. mellonella* larva. Like other *Xenorhabdus* sp., the phase I of this bacterium was characterised by the adsorption of bromthymol bule from NBTA medium. Insect cadavers parasitized by this nematode-bacterium complex presented a black coloration.

Pathogenicity test indicated that *S. thailandensis* n. sp. with its symbiotic bacterium was highly virulent against *G. mellonella*. The nematode at a dosage of 10 infective juveniles per test insect caused 53 % mortality of the insect in 24 hours. This nematode was a heat-tolerant isolate, still effective in killing insects even at soil temperature above 35 °C. It could kill 100 % of *G. mellonella* in 22 hours at 30 and 35 °C and was still effective even at 38 °C.

This pathogenic nematode could be mass produced *in vitro* on culture medium containing soybean under the laboratory condition. The yield of infective juveniles was approximately 6.4×10^5 per 20 g culture medium.

Its potential as a biological control agent was tested against 12 insect pests. Those were common leafworm (*S. litura*), beet armyworm (*S. exigua*), diamond-back moth (*P. xylostella*), American bollworm (*H. armigera*), jasmine flower borer (*H. duplifacialia*), mushroom beetle (unidentified), wax moth (*G. mellonella)*, flea beetle (*P. sinuata*), grub of scarab beetle (unidentified), aphid (*Myzus persicae*), wet wood termite (*Coptotermes* sp.) and American cockroach (*P. american*). The mortality percentage of 12 insects were 20-100 %, depending on species of test insect. *S. thailandensis* n. sp. exhibited a higher degree of controlling the insects in the order Lepidoptera than the insects in the other orders. One hundred percent insect mortality was found in the larvae of jasmine flower borer as well as in wax moth. In the insect pests of vegetable crops such as aphid, leaf worm, beet armyworm, diamond-back moth and American bollworm, the percentages of mortality were 44, 56, 60, 89 and 92 %, respectively. The mortality of the Coleoptera such as mushroom beetle, scarab beetle and flea beetle were 100, 20 and 33 %, respectively. Also, some household insects such as termite and cockroach died at 42 and 57 %, respectively.

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ตารางเปรียบเทียบวัตถุประสงค์ของโครงการ

วัตถุประสงค์เดิม	วัตถุประสงค์ใหม่
1. เพื่อทราบการแพร่กระจายของไส้เดือนฝอยศัตรูแมลงในประเทศไทย	1. เพื่อการสำรวจและค้นหาไส้เดือนฝอยในกลุ่มที่เป็นศัตรูธรรมชาติของแมลง ครอบคลุมทุกภาคของประเทศไทย
2. เพื่อเก็บรวบรวมไส้เดือนฝอยศัตรูแมลงที่พบในประเทศไทย	2. เพื่อการจัดเก็บรวบรวมไส้เดือนฝอยสายพันธุ์พื้นเมืองในแต่ละพื้นที่แบ่งแยกเป็นไอโซเลทและกำหนดรหัสไส้เดือนฝอย
3. เพื่อการจัดจำแนกชนิดและสายพันธุ์ โดยศึกษารูปร่างลักษณะทางสัณฐานวิทยาภายใต้กล้อง LM และการศึกษา protein เพื่อการจัดจำแนก	3. เพื่อการจัดจำแนกชนิดและสายพันธุ์ โดยวิธีการผสมข้ามชนิด (cross mating) ศึกษารูปร่างลักษณะทางสัณฐานวิทยาภายใต้กล้อง LM และ SEM การตรวจสอบสายพันธุ์ในระดับยีนด้วยเทคนิคทางอณูชีวโมเลกุล
4. เพื่อศึกษาศักยภาพของไส้เดือนฝอยสายพันธุ์ไทยในการเป็น obligate parasite ในแมลง โดยวิธี pathogenicity test	4. เพื่อศึกษาความสามารถของไส้เดือนฝอย Thai isolate ในการทำให้เกิดโรคกับแมลง โดยวิธี pathogenicity test และ heat tolerance test
	5. เพื่อศึกษาชีววิทยาของไส้เดือนฝอย Thai isolate โดยศึกษาวงจรชีวิต ผลของอุณหภูมิต่อวงจรชีวิต sex ratio และการเข้าทำลาย ศึกษาการแยกเชื้อบริสุทธิ์ของ symbiotic bacteria จากไส้เดือนฝอย Thai isolate
	6. เพื่อศึกษาการขยายปริมาณไส้เดือนฝอย Thai isolate ในอาหารเทียมในสภาพ monoxenic culture
	7. เพื่อทดสอบศักยภาพในการกำจัดแมลงสำคัญใน order Lepidoptera, Coleoptera, Homoptera, Isoptera และ Blattoidea

กิจกรรมที่วางแผน/ที่ได้ดำเนินการ และผลที่ได้รับตลอดโครงการ

กิจกรรมที่วางแผนไว้	กิจกรรมที่ได้ดำเนินการ	ผลที่ได้รับตลอดโครงการ
1. การสำรวจเก็บตัวอย่างดิน - ในพื้นที่ต่างๆ ได้แก่ ทุ่งหญ้า ป่าไม้ พื้นที่เพาะปลูก - ไม่ได้ระบุจำนวนตัวอย่างดิน	1. การสำรวจเก็บตัวอย่างดิน - ในพื้นที่ต่างๆ ได้แก่ ป่าไม้ ชายทะเล พื้นที่เพาะปลูกพืชต่างๆ และพื้นที่ไม่ทำการเกษตร ครอบคลุมทุกภาคของประเทศ - จำนวน 306 ตัวอย่างดิน 42 จังหวัด (location)	1. การสำรวจเก็บตัวอย่างดิน - แยกได้ไส้เดือนฝอยจากดินชนิดร่วนปนทราย ในพื้นที่ไม่ได้ทำการเกษตร และแปลงปลูกไม้ผล มีอุณหภูมิดิน ณ จุดเก็บ เท่ากับ 24-29 °ซ มีระดับ pH เท่ากับ 6.8-7.9 - แยกได้จากดินในพื้นที่จังหวัดกาญจนบุรี พิจิตร อยุธยา กาฬสินธุ์ มหาสารคาม ขอนแก่น หนองคาย ร้อยเอ็ด และสระแก้ว - มีการกระจายตัวสูงในเขตตอนกลางของประเทศ ไม่พบในเขตตอนเหนือและใต้
2. การแยกไส้เดือนฝอยโดยวิธี *Galleria* bait technique	2. การแยกไส้เดือนฝอยโดยวิธี *Galleria* bait technique	2. แยกได้ไส้เดือนฝอยจำนวน 9 ไอโซเลท แบ่งเป็น 2 สกุลคือ *Steinernema* sp. 8 ไอโซเลท และ *Heterorhabditis* sp. 1 ไอโซเลท
3. การเก็บรวบรวมไส้เดือนฝอยในน้ำกลั่นใน culture flask และกำหนดรหัสไส้เดือนฝอยตามแหล่งที่พบ	3. การเก็บรวบรวมไส้เดือนฝอยในน้ำกลั่นใน culture flask และกำหนดรหัสไส้เดือนฝอยตามแหล่งที่พบ - กำหนดรหัสไส้เดือนฝอยโดยใช้ตัวพิมพ์ใหญ่ภาษาอังกฤษจำนวน 2 ตัวอักษร ของชื่อจังหวัดที่ค้นพบ - ศึกษาการเก็บรักษาไส้เดือนฝอย *Steinernema* และ *Heterorhabditis* ให้คงความมีชีวิตรอดได้นาน	3. เก็บรวบรวมไส้เดือนฝอย - *Steinernema* Thai isolate ในน้ำกลั่นใน culture flask จำนวน 8 ไอโซเลท คือ KB, PC, AY, KS, MK, KK, NK และ SK code และ *Heterorhabditis* Thai isolate จำนวน 1 ไอโซเลท คือ RE code - การเก็บรักษาให้คงความมีชีวิตนาน ที่อุณหภูมิเหมาะสมของ *Steinernema* คือ อุณหภูมิ 25 °ซ และ *Heterorhabditis* ที่ 15 °ซ

กิจกรรมที่วางแผน/ที่ได้ดำเนินการ และผลที่ได้รับตลอดโครงการ

กิจกรรมที่วางแผนไว้	กิจกรรมที่ได้ดำเนินการ	ผลที่ได้รับตลอดโครงการ
4. การจัดจำแนกชนิดไส้เดือนฝอย - การศึกษารูปร่างลักษณะทางสัณฐานวิทยาโดยใช้กล้อง light microscope (LM) เปรียบเทียบกับ key มาตรฐาน - การศึกษา protein เพื่อการจัดจำแนกชนิด	4. การจัดจำแนกชนิดไส้เดือนฝอย - โดยวิธีผสมข้ามชนิด (cross mating) - การศึกษารูปร่างลักษณะทางสัณฐานวิทยาโดยใช้กล้อง LM และ SEM เปรียบเทียบกับ key มาตรฐาน - การตรวจสอบชนิดและสายพันธุ์โดยการวิเคราะห์ดีเอ็นเอ ด้วยวิธี PCR-RFLP	4. การจัดจำแนกชนิดไส้เดือนฝอย - จัดแบ่งชนิดของไส้เดือนฝอย *Steinernema* spp. Thai isolate เป็น 3 กลุ่ม คือ กลุ่มที่ 1 คือ KB, KS, MK, KK และ NK code กลุ่มที่ 2 คือ AY code และกลุ่มที่ 3 คือ SK code - จัดจำแนกได้ไส้เดือนฝอยชนิดใหม่สายพันธุ์ไทยของกลุ่มที่ 1 ให้ชื่อคือ *Steinernema thailandensis* n. sp. - ไส้เดือนฝอย *Heterorhabditis* sp. RE code มีความใกล้เคียงกับ *H. indica* ที่แยกได้จากประเทศอินเดีย
5. การศึกษาชีววิทยาของไส้เดือนฝอย Thai isolate - ไม่ได้วางแผน	5. การศึกษาชีววิทยาของไส้เดือนฝอย Thai isolate - การศึกษาวงจรชีวิต - การศึกษาผลของอุณหภูมิต่อวงจรชีวิต ต่อ sex ration และการเข้าทำลาย - การแยกเชื้อบริสุทธิ์ของ symbiotic bacteria	5. การศึกษาชีววิทยาของไส้เดือนฝอย *S. thailandensis* n. sp. Thai isolate - ไส้เดือนฝอย *S. thailandensis* n. sp. มีวงจรชีวิตสั้น (4 วัน) ที่อุณหภูมิ 30 °ซ - อุณหภูมิที่เหมาะสมต่อวงจรชีวิตในแมลงอาศัย อัตราการเป็นเพศผู้-เพศเมีย และความสามารถในการเข้าทำลายแมลงเหยื่อ อยู่ในช่วงระหว่าง 25-35 °ซ - แยกได้เชื้อบริสุทธิ์ของ symbiotic bacteria จากไส้เดือนฝอย *S. thailandensis* n. sp. จำแนกเป็นแบคทีเรียในสกุล *Xenorhabdus* sp.

กิจกรรมที่วางแผน/ที่ได้ดำเนินการ และผลที่ได้รับตลอดโครงการ

กิจกรรมที่วางแผนไว้	กิจกรรมที่ได้ดำเนินการ	ผลที่ได้รับตลอดโครงการ
6. การศึกษาความสามารถของไส้เดือนฝอย Thai isolate ในการทำให้เกิดโรคในแมลง - การทดสอบ pathogenicity test ในอัตราไส้เดือนฝอย 5 ตัวต่อหนอนทดสอบ 1 ตัว	6. การศึกษาความสามารถของไส้เดือนฝอย Thai isolate ในการทำให้เกิดโรคในแมลง - การทดสอบ pathogenicity test ในอัตราไส้เดือนฝอย 4 6 8 และ 10 ตัวต่อหนอนทดสอบ 1 ตัว - การทดสอบความทนทานต่ออุณหภูมิเปรียบเทียบกับสายพันธุ์จากต่างประเทศ	6. การศึกษาความสามารถของไส้เดือนฝอย Thai isolate ในการทำให้เกิดโรคในแมลง - ไส้เดือนฝอยมีความสามารถในการฆ่าแมลงทดสอบตาย 50 % ภายในเวลา 24 ชม. ในอัตราไส้เดือนฝอย 10 ตัวต่อหนอน 1 ตัว - ไส้เดือนฝอย Thai isolate มีความทนทานต่ออุณหภูมิสูง 35 °ซ จัดเป็นสายพันธุ์ทนร้อน (heat tolerant isolate) ที่มีศักยภาพในการฆ่าแมลง
7. การขยายปริมาณไส้เดือนฝอย Thai isolate ในอาหารเทียม - ไม่ได้วางแผน	7. การขยายปริมาณไส้เดือนฝอย Thai isolate ในอาหารเทียม - ศึกษาการขยายปริมาณในอาหารเทียมชนิดวุ้นในสภาพการเพาะเลี้ยงแบบ monoxenic culture จำนวน 5 สูตรอาหารดัดแปลง เปรียบเทียบกับสูตรอาหารสังเคราะห์ (lipid agar)	7. การขยายปริมาณไส้เดือนฝอย Thai isolate ในอาหารเทียม - ไส้เดือนฝอย *S. thailandensis* n. sp. สามารถเพาะเลี้ยงขยายปริมาณได้ในอาหารเทียมที่มีองค์ประกอบของนมถั่วเหลือง ให้ผลผลิตเท่ากับ 6.6×10^5 ตัวต่ออาหาร 20 กรัม หรือ 33×10^6 ตัวต่ออาหาร 1 ลิตร
8. การทดสอบ pathogenicity กับแมลงศัตรูสำคัญ - ไม่ได้วางแผน	8. การทดสอบ pathogenicity กับแมลงศัตรูสำคัญ - ทดสอบกับแมลง 12 ชนิด คือ หนอนกระทู้ผัก หนอนกระทู้หอม หนอนใยผัก หนอนเจาะสมอฝ้าย หนอนเจาะดอกมะลิ หนอนกินรังผึ้ง หนอนด้วงในถุงเท้า ด้วงหมัดกระโดด หนอนด้วงกินรากสตรอเบอรี่ เพลี้ยอ่อน ปลวก และแมลงสาบ ในระดับห้องปฏิบัติการ	8. การทดสอบ pathogenicity กับแมลงศัตรูสำคัญ - ไส้เดือนฝอย *S. thailandensis* n. sp. สามารถฆ่าแมลงในกลุ่มหนอนผีเสื้อได้ตั้งแต่ 55-100 % และกลุ่มหนอนด้วงเท่ากับ 20-100 % ในเพลี้ยอ่อน 44 % และปลวก 42 % ภายในเวลา 24 ชม. นอกจากนั้น ฆ่าแมลงสาบตาย 57 % ภายในเวลา 48 ชม.