THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Entomology)

DEGREE

Entomology	Entomology
FIELD	**DEPARTMENT**

TITLE: Discovery, Identification and Molecular Phylogeny of Entomopathogenic Nematodes and Their Symbiotic Bacteria

NAME: Mrs. Patchareewan Maneesakorn

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Professor Angsumarn Chandrapatya, Ph.D.)

THESIS CO-ADVISOR

(Professor Parwinder S. Grewal, Ph.D.)

DEPARTMENT HEAD

(Associate Professor Jariya Chanpaisaeng, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

DISCOVERY, IDENTIFICATION AND MOLECULAR PHYLOGENY OF ENTOMOPATHOGENIC NEMATODES AND THEIR SYMBIOTIC BACTERIA

PATCHAREEWAN MANEESAKORN

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Entomology)
Graduate School, Kasetsart University
2010

Patchareewan Maneesakorn 2010: Discovery, Identification and Molecular Phylogeny of Entomopathogenic Nematodes and Their Symbiotic Bacteria. Doctor of Philosophy (Entomology), Major Field: Entomology, Department of Entomology. Thesis Advisor: Professor Angsumarn Chandrapatya, Ph.D. 125 pages.

Characterization and molecular phylogeny analyses of five *Steinernema* entomopathogenic nematodes based on Internal Transcribed Spacer (ITS) sequences and their obligate symbiotic bacteria based on 16S rDNA sequence suggested that four USA nematode strains (D60, D90, D98, and FC48) were closely related to *Steinernema carpocapsae* and the symbiotic bacteria of D60, D90, and D98 were closely related to *Xenorhabdus nematophila* whereas one associated with FC48 was suggested to be a new species of *Xenorhabdus*. The *Steinernema* strain (MP10) from Thailand was described as *Steinernema minuta* sp. nov. and its symbiotic bacteria was closely related to *Xenorhabdus stockiae.*

Phylogenetic relationships of sixty-seven *Heterorhabditis* entomopathogenic nematodes and their symbiotic bacteria were investigated based on ITS sequences and gyrase B gene sequence, respectively. The MP68 was sister to *H. amazonensis* clade, strains MP17 and MP111 were in *H. indica* clade. Other sixty-four strains from USA belonged to clades of four known species; *H. bacteriophora*, *H. georgiana*, *H. indica* and *H. megidis*. The symbiotic bacteria isolated from all nematode strains belonged to clades of either subspecies *Photorhabdus temperata* or *Photorhabdus luminescens.* Two bacteria strains from Thailand MP68 and MP17, were sister to each other. They are paraphyletic to strain MP111 with respect to the monophyletic sister group of *P. luminescens* subsp. *akhurstii* strain D1. Cophylogenetic tests between *Photorhabdus* and *Heterorhabditis* species using ParaFit detected a significant correlation between the nematode and bacteria trees (ParaFitGlobal = 0.001).

The virulence of four Thai entomopathogenic nematode strains was evaluated against the second instar larva of Japanese beetle, *Popillia japonica*, in laboratory conditions. *H. indica* (MP111) was the most virulent with only 136 IJs/larva were required for 50% larval mortality within 5 days whereas 199, 254 and 501 IJs/larva were required for *Heterorhabditis* sp. (MP68), *H. indica* (MP17) and *S. minuta* (MP10), respectively. The LT_{50} values at 100 IJs/larva revealed that MP111 killed larvae faster (7.4 days) than MP68 (9.4 days) followed by MP17 (10.5 days) and MP10 (15.7 days), respectively. At a concentration of 1,000 IJs/larva the MP111 strain caused the highest larval mortality (84.81%) compared with MP17 (72.15%), MP68 (72.15%), and MP10 (36.71%) at 5 days after treatment.

_____ _____ __/__/__

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

I wish to express the deepest gratitude to my advisor Professor Dr. Angsumarn Chandrapatya, who provided valuable advice, suggestion and encouragement to me throughout this study. My sincere gratitude also go to my co-advisor, Professor Dr. Parwinder S. Grewal, for teaching me entomopathogenic nematology and bacteriology, for his valuable suggestions, encouragement and enthusiasm during my research work in the USA. Without their guidance and persistent help this thesis would not have been possible.

I am indebted to the Royal Golden Jubilee Ph.D. Program (Grant: PHD/0018/2549) and the TRF Senior Research Scholar Grant #RTA 488006 under the Thailand Research Fund (TRF) and the Ohio Agricultural Research and Development Center at the Ohio State University, Wooster, Ohio, USA for financial support throughout this study.

I gratefully acknowledge Assistant Professor Dr. Weerawan Amornsak and Dr. Kriengkrai Jumroenma the representative from the Graduate School for their valuable advices. Special thanks are also given to Mrs. Vacharee Somsook, Associate Professor Dr. Suthichai Somsook, Dr. Ruisheng An and Dr. Xiaodong Bai for their helpful advices and guidance.

I would like to thank the Entomopathogenic Nematode Laboratory, Plant Protection Research and Development Office at the Department of Agriculture, Ministry of Agriculture and Co-operatives, Bangkok, for providing the materials, and all of my colleagues for their support and assistance, and my friends and colleagues in Dr. Chandrapatya's lab at the Department of Entomology, Kasetsart University, Bangkok including colleagues at Dr. Grewal's lab, Department of Entomology (OARDC), Wooster, Ohio, USA for their kindness and encouragement.

Many thanks are extended to all of my friends who have participated in my research experiments for their help, kindness and sharing their valuable times with me; Ms. Jittima Rimdusit, Ms. Sirikamol Sae Lee, Ms. Suwapat Chuklin-on, Ms. Suchonwat Wongwailikhit, Ms. Udomrat Muangyoo, Mr. Anantachai Raktham, Mr. Armit Tanpradit, Mr. Atirach Noosidum, Mr. Mongkol Santaweesuk, Mr. Paithoon Chansak, Mr. Wattana Suwannawisut, Mr. Wasan Phonphuksa and Mr. Wasan Kongsopee.

Finally, I wish to dedicate this work to my beloved parents and family (Chongchitmate) and my lovely husband (Sarayoot Maneesakorn) for their encouragement, support, and understanding during my graduate study.

Patchareewan Maneesakorn

June 2010

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

°C	=	degree Celsius
ca	=	circa
cm	=	centimeter
E	=	East
g	=	gram
h	=	hour
L	=	Liter
M	=	Molar
N	=	North
sp. nov.	=	species novel
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	milliMolar
NCBI	=	National Center for Biotechnology Information
PCR	=	Polymerase Chain Reaction
SD	=	Standard Deviation
sec	=	second
µg	=	microgram
µl	=	microliter
µm	=	micrometer
µM	=	microMolar
U	=	Unit
UTM	=	Universal Transverse Mercator

DISCOVERY, IDENTIFICATION AND MOLECULAR PHYLOGENY OF ENTOMOPATHOGENIC NEMATODES AND THEIR SYMBIOTIC BACTERIA

INTRODUCTION

Entomopathogenic nematodes (EPNs) are one of the most important biological control agents for insect pests of agriculture, horticulture, forestry, veterinary, and public health (Grewal *et al.*, 2005). At present, there is tremendous interest in developing entomopathogenic nematodes as biological control agents to reduce the use of chemical pesticides in various parts of the world. Three families of entomopathogenic nematodes, Mermithidae, Heterorhabditidae and Steinernematidae have great potential for pest control. The members of Mermithidae are used for controlling of mosquito larvae whereas the members of the other two families are used to control insect pests in a variety of ecosystems (Grewal *et al.*, 2005). Besides, EPNs have been found to have broad host range and safe to non-target organisms (Ehlers, 2005). Furthermore, there are many researchers in over 100 laboratories around the world working on these important biological control agents. Nowaday, several governmental laboratories and private companies develop the mass rearing techniques and EPN formulations to be used in agriculture, veterinary, and public health sectors (Grewal *et al.*, 2005).

EPNs have cosmopolitan distribution and have been isolated from almost all continents and on many islands, except Antarctica (Poinar, 1990). Some species have been originally found in many different habitats (Hominick, 2002). So far, 14 species of Heterorhabditidae and 61 species of Steinernematidae have been described (Nguyen and Hunt, 2007; Nguyen, 2010).

Thailand is located in the tropical region where several habitats and environments are suitable for EPNs development. *Steinernema siamkayai* Stock, Somsook and Reid is one example of EPN found in Thailand since 1998. This

nematode species is now being used throughout Thailand for controlling various insect pests. However, there is a potential for discovery of additional species and strains of Thai EPNs which can be adapted to local environmental conditions. The natural forests of Thailand which harbor great biodiversity are sites which should be targeted for isolation of EPNs. The information on diversity of EPNs in Thai forests and their effectiveness for pest control will benefit Thai agriculture and will help in reducing reliance on the use of chemical pesticides. Further, the studies on the symbiotic bacteria of new EPNs may also prove useful in pest control applications.

In this study, the nematode isolates were identified using the sequences of the Internal Transcribed Spacer (ITS) region in the mitochondria. The ITS sequences have been used successfully to identify new EPN species and to construct their phylogenetic relationships as they are capable of providing detailed information about variation within and among nematode species (Adams *et al.*, 1998; Malan *et al.*, 2008; Nguyen *et al.*, 2008). The bacteria associated with these new EPN strains were isolated and identified by using the 16S ribosomal DNA sequences and gyrase B gene in the genome which are routinely used to identify *Xenorhabdus* and *Photorhabdus* bacteria (Akhurst *et al.*, 2004; Tailliez *et al.*, 2006; Tóth and Lakatos, 2008; Stock *et al.*, 2009). Morphological characterization and cross-breeding tests were also performed to describe the newly found *Steinernema* and *Heterorhabditis* species.

Using the sequence data for *Heterorhabditis-Photorhabdus* pairs from the same strain, the assessment of the extent of co-speciation between *Heterorhabditis* and *Photorhabdus* strains was determined statistically by comparing the two phylogenetic trees.

Finally, the virulence of the new Thai nematode strains was determined against the grub of Japanese beetle *Popillia japonica* Newman (Coleoptera: Scarabaeidae) which is one of the most serious insect pests of over 400 species of plants.

OBJECTIVES

1. To collect, identify and describe the new entomopathogenic nematodes along with their symbiotic bacteria from Thailand by morphological and molecular characteristics.

2. To determine phylogenetic and cophylogenetic relationships of *Heterorhabditis* spp. and their symbiotic bacteria *Photorhabdus* spp.

3. To determine the efficacy of the newly collected Thai entomopathogenic nematodes against the Japanese beetle grub, *Popillia japonica*.

LITERATURE REVIEW

1. Taxonomy of entomopathogenic nematodes

The first entomopathogenic nematode found in Germany refers to as Steinernematidae (Filipjev, 1934). In 1923, Steiner described the entomopathogenic nematode from the larvae of the web-spinning sawfly, *Cephalcia abietis* L. collected by Dr. Krausse in 1917 as *Aplectana kraussei*. Steiner (1929) described *Neoaplectana glaseri* which was collected from the dead larvae of the Japanese beetle (*Popillia japonica* Newman) in USA. The species name was given after Rudolph Glaser and this is the first species of entomopathogenic nematode use to control the white grubs. Filipjev (1934) described *Neoaplectana feltiae* which was collected from the larvae of *Agrotis segetum* Denis and Schiffermueller (Lepidoptera: Noctuidae) and also placed *Steinernema* and *Neoaplectana* in the new subfamily Steinernematinae. Then, subfamily Steinernematinae was raised to the family Steinernematidae by Chitwood and Chitwood (1937). Later a new genus *Neosteinernema* was described by Nguyen and Smart (1994).

For decades many entomopathogenic nematodes had been found and described under *Steinernema* (=*Neoaplectana*). In 1976, Poinar erected new genus *Heterorhabditis* and described *Heterorhabditis bacteriophora* that was the first nematode species found in the body cavity of *Heliotis punctigera* Hall in Australia.

Currently, the entomopathogenic nematodes which were recovered from around the world have been described as 61 *Steinernema* sp., 1 *Neosteinernema* sp. and 14 *Heterorhabditis* sp. (Nguyen, 2010; Nguyen and Hunt, 2007).

Classification of the entomopathogenic nematodes:

Phylum: Nematoda
Class: Chromadorea
Subclass: Chromadoria
Order: Rhabditida
Suborder: Tylenchina
Infraorder: Panagrolaimomorpha
Superfamily: Strongloidoidea
Family: **Steinernematidae**
Suborder: Rhabditina
Infraorder: Rhabditomorpha
Superfamily: Strongloidea
Family: **Heterorhabditidae**
Class: Enoplea
Subclass: Dorylaimia
Order: Mermithida
Suborder: Mermithina
Superfamily: Mermithoidea
Family: **Mermithidae**

2. Biodiversity and distribution of entomopathogenic nematodes

More than 75 species of entomopathogenic nematodes had been described from many countries around the world during a past century. *Heterorhabditis* nematodes are distributed widely throughout North and South America, Australia, Europe, Asia and Africa. Fourteen species under this genus are currently recognized and various strains have been found in different parts of six continents, including some islands except Antarctica (Hominick, 2002; Nguyen and Hunt, 2007; Nguyen *et al.*, 2008; Malan *et al.*, 2008). Heterorhabditids have been isolated primarily from sandy coastal soils in the site near the sea or at the sea level. Some *Heterorhabditis* species present in more calcareous soils (i.e., *Heterorhaditis indica* Poinar, Karunakar

and David, 1992 in Guadaloupe), or more acidic soils (*H. bacteriophora* and *Heterorhabditis marelatus* Lui and Berry, 1996a), whereas other species range beyond coastal regions and are broadly distributed in turf and weedy habitats (*Heterorhaditis megidis* Poinar, Jackson and Klein, 1987) (Stuart and Gaugler, 1994; Constant *et al.*, 1998; Stock *et al.*, 1996) and tropical forests (*Heterorhabditis baujardi* Phan, Subbotin, Nguyen and Moens, 2003) (Phan *et al.*, 2003).

Intensive surveys of EPNs conducted in Europe and USA revealed habitat associations for several steinernematids (Hominick *et al.*, 1995; Stock *et al.*, 1999; Sturhan, 1999; Sturhan and Liskova, 1999). Prevalence of steinernematids seems to be the highest in woodlands (Hominick *et al.*, 1996). *Steinernema feltiae* (Filipjev, 1934) Wouts, Mrácek, Gerdin and Bedding, 1982 is commonly occurred in grasslands and woodlands (Hominick, 2002). In addition, *Steinernema kraussei* (Steiner, 1923) Travasoss, 1927 and *Steinernema intermedium* (Poinar, 1986) Mamiya, 1998 are mainly forest/woodland species. *Steinernema kraussei* has mainly been found in coniferous forests in Europe and North America (USA and Canada) on both the east and west coasts (Sturhan, 1999; Sturhan and Liskova, 1999). Presently, 61 species and lots of strains of Steinernematidae have been recovered and revealed from natural habitats whereas *Steinernema siamkayai* Stock, Somsook and Reid, 1998 was discovered from sandy clay loam in the tamarind orchard at Amphor Lomsak, Phetchabun province, in northern Thailand.

3. Mutualistic relationship between entomopathogenic nematodes and their symbiotic bacteria

Entomopathogenic nematodes in families Heterorhabditidae and Steinernematidae are mutualistically associated with bacteria *Photorhabdus* and *Xenorhabdus*, respectively. These 2 bacterial genera belong to the family Enterobacteriaceae. Both bacteria are gram-negative, rod shape, motile by peritrichous flagella or non-motile facultative anaerobic and produce the enterobacterial common antigen (Ramia *et al.*, 1982). The bacteria *Xenorhabdus* colonize a specialized vesicle in the intestine of the *Steinernema* infective juvenile (IJ)

(Bird and Akhurst, 1983) while *Photorhabdus* is located in the anterior part of the *Heterorhabditis* infective juvenile guts (Boemare *et al.*, 1996). The bacteria are transported into the susceptible insect host by their associated infective juveniles (IJs) through natural body openings and migrate into the hemolymph. The IJs release the symbiotic bacteria into the insect hemolymph via the mouth in case of *Heterorhabditis* (Ciche and Ensign, 2003) and via the anal opening in case of *Steinernema* (Martens and Goodrich-Blair, 2005). The bacteria multiply, killing insects by causing septicemia within 24 - 48 h and converting the cadaver into a food source suitable for nematode growth and reproduction. The nematodes reproduce for one to three generations and emerge as IJs when food resources are depleted. The bacteria recolonize the emerging IJs to ensure their transmission to a new insect host (Poinar, 1990).

Currently, 20 *Xenorhabdus* species and 3 *Photorhabdus* species from entomopathogenic nematodes have been described (Nguyen and Hunt, 2007). The bacteria can be grown without nematode under laboratory condition (Gaugler, 2002). This nematode-bacteria symbiotic partnership is extremely effective in killing a diverse species of insects and has been successfully implemented in biological control and integrated pest management programs worldwide (Grewal *et al.*, 2005).

4. Co-speciation between the bacterial symbionts and the nematode hosts

Although mutualistic association between *Heterorhabditis* and *Photorhabdus* species was originally thought to be strictly one-to-one in terms of co-speciation, it has been observed that some bacterial species colonize more than one recognized nematode species. For example, *Photorhabdus temperata* Fischer-Le Saux, Viallard, Brunel, Normand and Boemare, 1999 has been found associated with *H. bacteriophora*, *H. megidis*, *H. zealandica* Poinar, 1990 and *H. downesi* Stock, Griffin and Burnell, 2002 (Boemare, 2002; Adam *et al.*, 2006; Tóth and Lakatos, 2008). *Xenorhabdus bovienii* (Akhurst, 1983) Akhurst and Boemare, 1993 is symbiotic with *Steinernema affine* (Bovien, 1937) Wouts, Mrácek, Gerdin and Bedding, 1982, *S. feltiae*, *S. kraussi* and *S. intermedium*. *Xenorhabdus poinarii* (Akhurst, 1983) Akhurst

and Boemare, 1993 is symbiotic with *Steinernema cubanum* Mrácek, Hernandez and Boemare, 1994 and *Steinernema glaseri* (Steiner, 1929) Wouts, Mrácek, Gerdin and Bedding, 1982 (Gaugler, 2002). Although some bacterial species colonize more than one recognized nematode species the bacteria associated with the other many nematode species have not been described (Boemare, 2002).

5. Identification of entomopathogenic nematodes by molecular technique using ITS region

Three molecular approaches, Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and DNA sequencing, have been used for identification diagnostic delimitation species and assessment phylogenetic relationships of entomopathogenic nematodes (Stock *et al.*, 2009). RAPD was first used as a complementary method to identify the species of *Heterorhabditis* and *Steinernema* (Gardner *et al.*, 1994; Liu and Berry, 1996b). It was also used to describe the genetic variability among strains of these two nematode genera (Liu and Berry, 1995, 1996b; Hashmi *et al.*, 1996). Nevertheless, the result from RAPD method could create problems due to many factors such as primer or template and PCR cycling conditions (Stock and Hunt, 2005). RFLP method has been used as a diagnostic tools for identification of the *Steinernema* and *Heterorhabditis* (Reid and Hominick, 1992; Reid *et al.*, 1997; Anis *et al.*, 2000; Phan *et al.*, 2001). This method has also been used to interpret evolutionary relationships of these nematodes (Reid *et al.*, 1997). Currently, DNA sequencing analysis is the most suitable approach and now widely considered for assessing phylogenetic relationships in nematode systematic (Power *et al.*, 1994; Adam *et al.*, 1998; Blaxter *et al.*, 1998) and also species delimitaion (Nguyen *et al.*, 2001; Stock *et al.*, 2001; Stock and Koppenhöfer, 2003).

Several ribosomal genes have been used extensively to identify the entomopathogenic nematodes. The nuclear genes that were used to describe the nematodes include the external non-transcribed spacer (ETS), small subunit (SSU) or 18S (Liu *et al.*, 1997; Stock *et al.*, 2001), internal transcribed spacer 1 (ITS1), 5.8S,

internal transcribed spacer 2 (ITS2) (Reid *et al.*, 1997; Nguyen *et al.*, 2001), and the large subunit (LSU) or 28S (Stock *et al.*, 2001). ITS (Internal Transcribed Spacer) is a piece of non-functional RNA situated between structural ribosomal RNA (rRNA) on a common precursor transcript. The ITS region contains 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S rRNA. Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because it is easy to amplify even from small quantities of DNA, due to the high copy number of rRNA and because it has degree of variation even between closely related species. The ITS sequences are the markers commonly used to discriminate among nematode species (Power *et al.*, 1997; Nguyen *et al.*, 2001). It has been employed to assess evolutionary relationships of EPNs at different taxonomic levels (Adams *et al.*, 1998; Liu *et al.*, 1999; Stock *et al.*, 2001; Nguyen *et al.*, 2001, 2004).

Mitochondrial genes; ND3 (NADH dehydrogenase subunit 3), ND4, coxI (cytochrome c oxidase subunit 1), coxII, and 16S rDNA have been considered to describe the genetic variation within and among entomopathogenic nematode species (Bouin *et al.*, 1999; Liu *et al.*, 1999; Szalanski *et al.*, 2000).

6. Identification of symbiotic bacteria by molecular technique using 16S rDNA and gyrase B

The 16S rDNA gene in mitochondria is used for phylogenetic studies as it is highly conserved between different species of bacteria. This gene is commonly exploited for identification of bacterial strains and species. Liu *et al.* (1997) started using 16S rDNA to analyze both genera of *Xenorhabdus* and *Photorhabdus*. Then this gene has been widely used to assess evolutionary relationship in these two genera (Liu *et al.*, 2001; Lengyel *et al.*, 2005a; Tailliez *et al.*, 2006; Stock *et al.*, 2009).

Protein-coding genes, gyrase B and recA sequences, have been employed to assess the evolutionary relationship among prokaryotes and symbiotic bacteria of the entomopathogenic nematodes (Eisen, 1995; Dauga, 2002). The gyrase B gene, encoding the B subunit of DNA gyrase, is a suitable phylogenetic marker for

identification and classification of bacteria (Yamamoto and Harayama, 1996, 1999). The gyrase B that present in all bacteria regulates supercoiling of double-stranded DNA, which encodes the ATPase domain of DNA gyrase, and is necessary for DNA replication (Huang, 1996). The enzyme is conservative enough to compare among taxa that are not closely related (Yamamoto and Harayama, 1996; Huang, 1996) and it has been shown to be an excellent target for differentiating between bacteria species of the family Enterobacteriaceae (Dauga, 2002; Delmas *et al.*, 2006). Akhurst *et al.* (2004) determined gyrase B gene sequence to describe evolutionary relationship among *Photorhabdus* spp. Stock *et al.* (2009) presented the phylogenetic tree base on the gyrase B gene analysis while Dauga (2002) suggested that the gyrase B partial sequence used to be more reliable than 16S rDNA for determining the evolutionary relationships of the bacteria.

7. Identification of entomopathogenic nematodes by using morphological characters

The significant morphological characters necessary to identify an entomopathogenic nematode to new species could be performed using light microscope (LM) and scanning electron microscrope (SEM) (Nguyen and Hunt, 2007).

7.1 Family Steinernematidae

Male:

Body always curved posteriorly (C-shape or J-shape). **Cuticle** smooth under a light microscope but with striation under SEM. **Lateral field** absent or with one ridge. **Cephalic extremity** swollen or truncate with four cephalic papillae and six labial papillae. Cephalic papillae are usually larger than labial papillae. **Amphidial** aperture pore-like, but may be absent or inconspicuous. **Cephalic extremity** with or without perioral disc. **Stoma** deep, shallow or reduced, cheilorhabdions sometimes prominent, posterior part of stoma funnel-shaped, moderately cuticularised or not

cuticularised. **Pharynx** either with cylindrical corpus and swollen basal bulb, or mostly with cylindrical procorpus, somewhat swollen metacorpus, narrow isthmus surrounded by a nerve ring and a swollen basal bulb containing a valve. **Nerve ring** usually surrounding isthmus or anterior part of basal bulb, or just anterior to basal bulb. **Excretory pore** position variable, either anterior or posterior to nerve ring, or close to end of pharynx; excretory duct well (or weakly) cuticularised; excretory gland with prominent nucleus (or excretory gland not observed). **Cardia** prominent or inconspicuous. **Genital system** monarchic, reflexed. **Testis** reflexed, comprising germinal zone, growth zone and **Vas deferens**. Testis may be reduced or collapsed, the percentage of nematodes with such an atypical testis should be reported. Distance from base of pharynx to anterior end of testis may be constant or variable. **Posterior region**, with six or seven genital papillae, a single precloacal midventral papilla, two pairs adcloacal, one pair lateral, two pairs subterminal, and one pair subdorsal. The **spicule** is large, foot-shape in genus *Neosteinernema*. **Spicule head** is either longer than wide, as long as wide, or wider than long, **shaft** present, either long or short, or absent; **blade** almost straight, moderately curved or well curved; rostrum present or absent; **velum** may be prominent, thin or absent; posterior end may or may not reach tail tip. **Gubernaculum** boat-shaped in lateral view, anterior end curved, pointed or enlarged anterior or rounded. **Ventral view**, tapering anteriorly (abruptly or gradually), **Cuneus** present or absent, when present it is either V-shaped, Y-shaped, needle-shaped or arrowhead-shaped. **Gubernaculum** enlarging posteriorly, abruptly or gradually to form corpus wings. **Corpus** is either open or closed posteriorly. **Tail** coniod, without bursa, with or without phasmid. **Tail tip** either with or without mucron or digitate.

Female:

Description is similar to that of male. There are some female characters that are important in taxonomy of Steinernematidae. **Body** when heat-relaxed habitus often C-shaped or spiraled. **Cephalic region** with six partially or completely fused lips, each bearing a papilla, four cephalic papillae and two small amphidial apertures. **Cuticle** appearing smooth or annulated, lateral fields absent. **Cheilorhabdions**

sclerotised, forming a ring, posterior stoma funnel-shaped. **Pharynx** similar to male. **Vulva** in form of median transverse slit. **Epiptygmata** present or absent. **Genital system** amphididelphic, reflexed; oviparous or ovoviviparous. **Tail** short, coniod, long in first generation but longer in second generation. **Phasmids** may be inconspicuous. **Shape of tail tip**, round with mucron or papilla-like structures or bluntly pointed.

Infective Juvenile:

Body of heat-relaxed specimens slender, almost straight or slightly curved. **Cuticle** striated. **Cephalic region** truncate, continuous with body or offset. **Stoma** closed. **Head** smooth or annulated with four cephalic papillae and six labial papillae or with four cephalic papillae and no labial papillae. **Horn-like structures** on the cephalic region present or absent. Sometimes these structures cannot be seen on ensheathed infective juvenile and therefore exsheathed infective juvenile should be observed. **Amphidial apertures** prominent on SEM photographs of several species but may be obscure on others. **Lateral field pattern** the arrangement of ridges in lateral field from head to tail is important. If an electron microscope is available, this pattern should be described. A light microscope with DIC (Differential interference contrast) optics can be used to observe the lateral field pattern. **Pharynx** with thin corpus, with almost uniform diameter, or with a slightly swollen metacorpus; nerve ring surrounding isthmus, basal bulb usually elongate with a valve. **Excretory pore** at mid-pharynx, in anterior third or near basal bulb. **Cardia** small, prominent or inconspicuous. **Bacterial chamber** prominent or obscure. **Intestine lumen** narrow, **Rectum** and **Anus** distinct. **Genital primordium** prominent or obscure. **Hemizonid** (anterior to excretory pore) or **Hemizonion** (posterior to excretory pore) distinct. **Phasmid** present or absent, when present, it is small or large and located either just ventral to lateral field or interrupting the ventral-most lateral ridge and usually near mid-tail. **Hyaline** region comprises either more or less than 50% of tail length. **Tail** with dorsal or ventral depression, tail end with or without internal needle-like structure.

7.2 Family Heterorhabditidae

The description of *Heterorhabditis* species is similar to *Steinernema* with the following exceptions:

Hermaphroditic female:

Body large, plump. **Head** truncate to slightly rounded. **Lips** six, well-developed, separate, each with terminal papilla. One or two small raised structures may be present at base of each lip. **Amphidial aperture** small. **Stoma** wide, shallow, **Cheilorhabdions** forming a cuticularised refractive ring, other stoma parts fused to form collapsed posterior portion. Posterior part of stoma surrounded by pharyngeal collar. **Pharynx** lacking metacorpus, isthmus slender, basal bulb swollen with reduced valve plates. **Nerve ring** located at middle of isthmus, **Excretory pore** usually located posterior to pharynx. **Vulva** median, slit-like, surrounded by elliptical rings. **Ovotestes** two, opposed, reflexed. Oviparous at first, ovoviviparous later. **Tail** conoid, longer than anal body diameter, **Postanal** swelling usually present.

Male:

Bursa usually with nine papillae but some variation is normal. The distribution of **bursa rib** is as follow: pair 1 well anterior, extending to, or beyond the bursa rib, pair 2 and 3 in a group just posterior to cloaca, pair 4 usually curved laterally. **The arrangement of pairs** 1-6 is not variable in *Heterorhabditis*. Pairs 7, 8 and 9, forming the terminal group, are the most variable and can be used for species differentiation. Several species such as *H. megidis*, *H. zealandica*, *H. marelatus* and *H. bacteriophora* have three pairs of ribs in the terminal group; *Heterorhabditis mexicana* Nguyen, Shapiro-Ilan, Stuart, McCoy, James and Adams, 2004, *Heterorhabditis floridensis* Nguyen, Gozel, Koppenhöfer and Adams, 2006, have either one pair, two pairs, or three ribs on one side, two ribs on the other side; for *H. indica* Poinar, Karunakar and David, 1992, the arrangement of the bursa ribs is varied and the occurrence of three pairs in the terminal group is very rare. At least ten bursas

should be observed and the variation, if any, should be reported, e.g. nine bursas with three pairs of rib, one with two pairs in the terminal group. **Spicules** and **ventral view of gubernaculums** can be used as diagnostic characters.

Female:

Head region with six anteriorly-directed papillae; almost similar for all species expect that labial papillae are curved outward in *H. indica*. **Amphidial apertures** present, but **Cephalic papillae** not observed with SEM. The **form of the vulva** and **surrounding pattern** appear to be good diagnostic characters.

Infective Juvenile:

Morphological and morphometrical characteristics of *Heterorhabditis* IJ, are insufficient to describe or identify a new species of *Heterorhabditis*. Molecular characterization is always necessary to confirm species identity.

The measurement parameters that are used for *Steinernema* and *Heterorhabditis* species are listed in Appendix Tables 1 and 2.

8. Cross hybridization studies

There are several ways of doing cross hybridization study in nematode. One of which is using the hemolymph of tested organisms. Kaya and Stock (1997) called this technique as “Hanging blood drop”. Approximately 30 - 50 IJs were placed in a drop of *Galleria mellonella* L. (Lepidoptera: Pyralidae) hemolymph mixed with serum-free medium to prevent drying of hemolymph in a well slide. Pre-adult male and female of tested species were separated and matched with the opposite sex of control species. Nguyen and Duncan (2002) used a drop of *G. mellonella* hemolymph placed in a sterile Petri dish. A single IJ of the tested nematode in the opposite species are added and the same species of IJs serve as control. Whilst Mrácek *et al.* (2006) reported that the study on cross hybridization between *Steinernema sichuanese*

Mrácek, Nguyen, Tailliez, Boemare and Chen, 2006 and other species was failed because most of the IJs died in a drop of hemolymph indicating that *Galleria* may not be the suitable host for this new species. Insect cadaver can also be used to study the cross hybridization. The cadavers are prepared by injecting the symbiotic bacteria of the female partner in the cross into the haemocoel of *Galleria* larvae for 14 days before running the test. Five virgin females and five males of the appropriate strains are injected into each cadaver. After one week, dissecting cadavers are observed for the offspring (Kaya and Stock, 1977). In lipid agar plates method, the bacteria within the nematode female partner in the cross was inoculated before 10 virgin females and 10 males of the appropriate species were released and then the progeny are indicated (Kaya and Stock, 1977).

9. Efficacy of entomopathogenic nematodes against the Japanese beetle grub

Popillia japonica is commonly known as the Japanese beetle. This beetle, 15 mm long and 10 mm wide, has a shiny, metallic-green body and bronze-colored outer wings. The beetle has six small tufts of white hair along the sides and back of its body under the edges of wings. The male is slightly smaller than female. The larvae are typical white grubs that are C-shaped when disturbed. First instar larvae are 1.5 mm long while the mature third instars are 32 mm long.

History:

Japanese beetle is native to Japan. The larvae entered the USA in a shipment of iris bulbs prior to 1912 when inspections of commodities entering the country was started. This insect was first found in the USA in 1916 in a nursery near Riverton, New Jersey.

Unfortunately, the beetle entered country without its natural enemies and found a favorable climate and an abundant food supply. The Japanese beetle becomes a serious pest and a threat to American agriculture and is now established throughout the eastern United States and spreading westwards (Vittum *et al.*, 1999).

Life cycle:

The life cycle of the beetle is typically once a year. The females emerge and also release a sex pheromone to attract males. After feeding and mating for a few days, the female burrows into the soil to lay 1 to 5 eggs at a depth of 2 to 4 inches before returning to plants to feed and mate, and continues until the female laid up 40 to 60 eggs. The eggs are laid by mid-August and hatched within 8 to 14 days then the first instar larvae dig to the soil surface to feed on roots and organic material. The first instars molt in 17 to 25 days while the second instars take 18 to 45 days to mature and molt again. Most of the grubs reach the third larval instar by late September and started digging deeper into the soil to overwinter in October. The grubs return to the surface in the spring when the soil temperature warms up, usually in mid-April. The grubs continue their development and form a pupa in May and emerging to be adult in June - July (Potter, 1998; Vittum *et al.*, 1999).

Damage:

The adult beetles feed on leaves and flowers of over 400 plant species, preferably the tissue between the veins, a type of feeding is called skeletonizing. The larvae feed on plant roots and organic matter in the soil (Potter, 1998).

Entomopathogenic nematodes against Japanese beetle:

Since the Japanese beetle entered in the USA without its natural enemies, several control strategies were conducted to suppress both adults and grubs. The chemical products commonly used to kill the beetle are trichlorofon, chlorpyrifos, carbaryl, diazinon, imidacloprid and halofenozide (George *et al.*, 2007; Oliver *et al.*, 2009).

Tiphia vernalis Rohwer, a parasite of Japanese beetle grub and *Istocheta aldrichi* Mesnil, a parasite of the adult Japanese beetle have been brought from Asia, mass reared but are not commercially available (Oliver *et al.*, 2005).

The milky disease bacteria, *Paenibacillus popilliae* Dutky can be used against the grub as a long term control. This pathogen required two to three years to build up its population in the soil to be effective against the grub (Koppenhöfer *et al.*, 2000).

Steinernema glaseri, *S. scarabaei* Stock and Koppenhöfer, 2003 and *H. megidis* were originally collected and described from naturally infected Japanese beetles (Wouts *et al.*, 1982; Poinar *et al.*, 1987; Stock and Koppenhöfer, 2003). The nematodes *Steinernema* and *Heterorhabditis* have been used to control white grubs successfully. The commercially available nematode products against Japanese beetle grubs are *H. bacteriophora* and *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mrácek, Gerdin and Bedding, 1982 (Grewal *et al.*, 2005). The virulence of different nematode species and strains against the Japanese beetle grubs differs substantially (Grewal *et al.*, 2002, 2004; Koppenhöfer *et al.*, 2006). The first and second instars of the Japanese beetle have been found to be more susceptible to the nematodes than the third instar, both in the laboratory and in the field conditions (Klein, 1990; Koppenhöfer and Fuzy, 2004; Power *et al.*, 2009).

MATERIALS AND METHODS

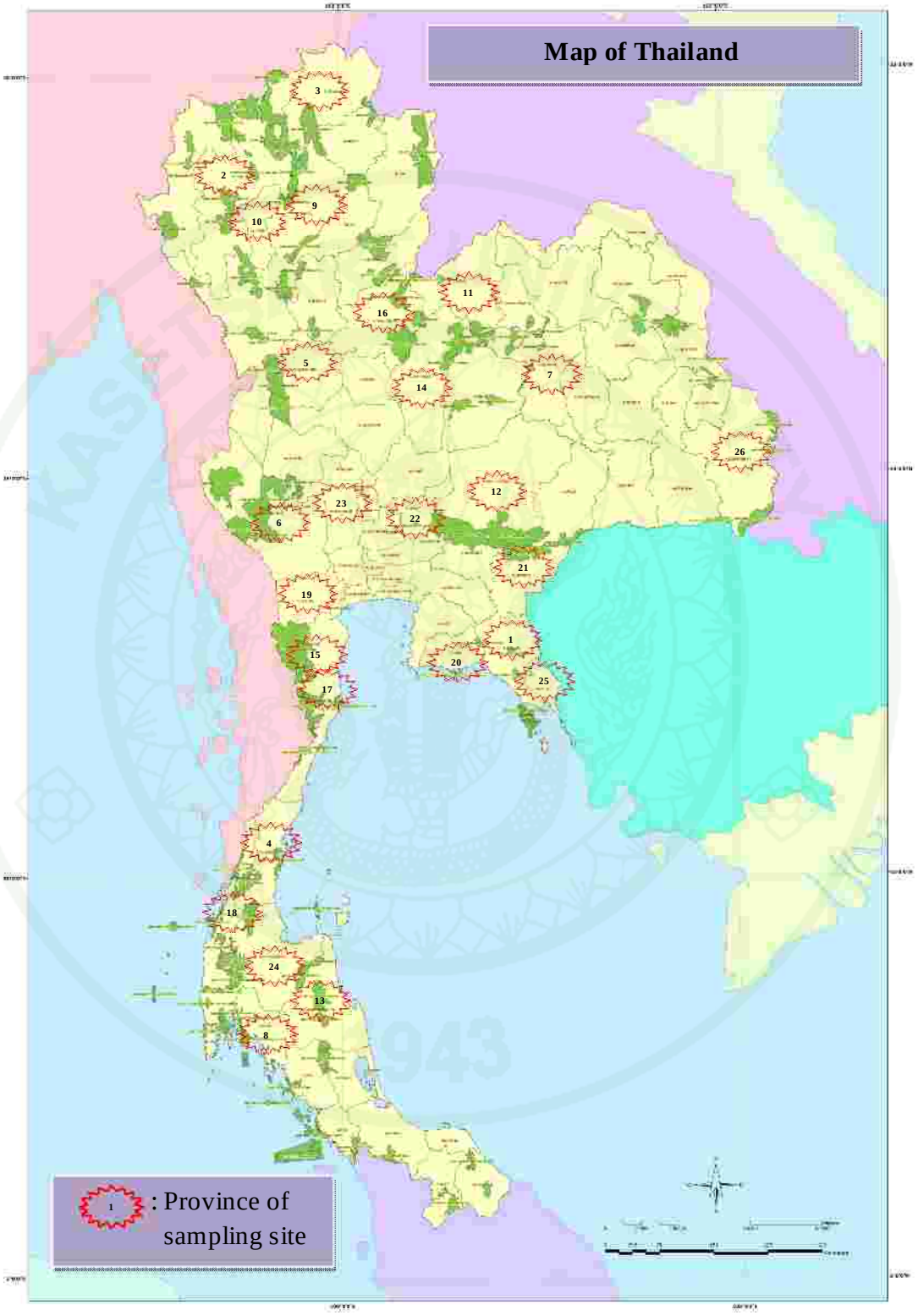
1. Soil sampling and nematode isolation

One hundred and fifteen soil samples were collected from 48 forest habitats in 26 provinces of Thailand between March 2007 and February 2008 (Fig. 1, Appendix Table 3). Three sampling sites, 100 m apart from each other were chosen based on accessibility to the area. Each sample (ca. 2 kg of soil), consisted of a composite of 5 sub-samples taken along a 1 m transect. Samples were taken with a hand shovel to a depth of 20 cm from the soil surface. Each sample was placed in a polyethylene bag and closed tightly to prevent water loss, kept in coolers (ca. 15°C) and transported to the laboratory.

EPNs were recovered from the soil samples using the insect baiting technique (Bedding and Akhurst, 1975). Soil samples were thoroughly mixed and moistened with tap water. Ten last instar *G. mellonella* larvae were placed on the top of 150 g of soil sample in plastic container (5 containers/sample). Containers were covered with a lid, turned upside down, and kept at room temperature (25°C). After 7 - 9 days, all insects were recovered, and the EPN parasitized cadavers, recognized by their characteristic color, smell and consistency were placed in a modified White trap (White, 1927) to allow for the emergence of infective juveniles (IJs). Emerging IJs were pooled from the traps and thoroughly rinsed to remove insect debris before being used to infect fresh *G. mellonella* larvae to confirm Koch's postulates for pathogenicity and for future identification and establishment of cultures. All nematode cultures were stored in 100 ml tissue culture flasks at 10 - 15°C.

Figure 1 Map showing the sampling sites (provinces) in Thailand

1. Chantaburi
2. Chiang Mai
3. Chiang Rai
4. Chumphon
5. Kampangphet
6. Kanchanaburi
7. Khon Kaen
8. Krabi
9. Lampang
10. Lamphun
11. Loei
12. Nakhon Ratchasima
13. Nakornsrithamarat
14. Phetchabun
15. Phetchaburi
16. Phitsanulok
17. Prachuap Khiri Khan
18. Ranong
19. Ratchaburi
20. Rayong
21. Sa Kaeo
22. Saraburi
23. Suphanburi
24. Surat Thani
25. Trat
26. Ubon Ratchathani



In addition, 4 *Steinernema* and 64 *Heterorhabditis* strains were included in these studies of which sixty-one were from the continental USA, two from the Midway Islands (USA), one each from New Zealand, Argentina, Hungary, Trinidad and Tobago and the United Kingdom. The original localities and sources of all the strains are listed in Tables 1 and 2. All nematodes were cryo-preservated in liquid N_2 and recycled through *G. mellonella* larvae once a month.

Table 1 The strains, origins and sources of 4 *Steinernema* strains from USA used in this study

Strain	Origin	Source
D60	Ohio, USA	Parwinder S. Grewal
D90	Ohio, USA	Parwinder S. Grewal
D98	Ohio, USA	Parwinder S. Grewal
FC48	Ohio, USA	Parwinder S. Grewal

Table 2 The strains, origins and sources of 64 *Heterorhabditi*s strains from USA used in this study

Strain	Origin	Source
GPS1	Ohio, USA	Parwinder S. Grewal
GPS11	Ohio, USA	Parwinder S. Grewal
GPS12	Ohio, USA	Parwinder S. Grewal
GPS13	Ohio, USA	Parwinder S. Grewal
GPS14	Ohio, USA	Parwinder S. Grewal
GPS15	Ohio, USA	Parwinder S. Grewal
GPS16	Ohio, USA	Parwinder S. Grewal
GPS17	Ohio, USA	Parwinder S. Grewal
GPS18	Ohio, USA	Parwinder S. Grewal
GPS19	Ohio, USA	Parwinder S. Grewal
GPS20	Ohio, USA	Parwinder S. Grewal
GPS21	Ohio, USA	Parwinder S. Grewal
GPS22	Ohio, USA	Parwinder S. Grewal
GPS23	Ohio, USA	Parwinder S. Grewal
GPS24	Ohio, USA	Parwinder S. Grewal
GPS25	Ohio, USA	Parwinder S. Grewal
GPS26	Ohio, USA	Parwinder S. Grewal
GPS27	Ohio, USA	Parwinder S. Grewal
GPS28	Ohio, USA	Parwinder S. Grewal
GPS29	Ohio, USA	Parwinder S. Grewal
GPS30	Ohio, USA	Parwinder S. Grewal
GPS31	Ohio, USA	Parwinder S. Grewal
GPS32	Ohio, USA	Parwinder S. Grewal
GPS33	Ohio, USA	Parwinder S. Grewal
GPS34	Ohio, USA	Parwinder S. Grewal
GPS35	Ohio, USA	Parwinder S. Grewal
KMD1	Ohio, USA	Michael D. Klein
KMD10	Ohio, USA	Michael D. Klein
KMD19	Ohio, USA	Michael D. Klein
KMD37	Ohio, USA	Michael D. Klein
KMD41	Ohio, USA	Michael D. Klein
KMD42	Ohio, USA	Michael D. Klein

Table 2 (Continued)

Strain	Origin	Source
KMD60	Ohio, USA	Michael D. Klein
KMD61	Ohio, USA	Michael D. Klein
KMD62	Ohio, USA	Michael D. Klein
KMD63	Ohio, USA	Michael D. Klein
KMD64	Ohio, USA	Michael D. Klein
KMD65	Ohio, USA	Michael D. Klein
KMD69	Ohio, USA	Michael D. Klein
KMD70	Ohio, USA	Michael D. Klein
KMD81	Ohio, USA	Michael D. Klein
KMD82	Ohio, USA	Michael D. Klein
KMD84	Ohio, USA	Michael D. Klein
OB1	Ohio, USA	Michael D. Klein
OB2	Ohio, USA	Michael D. Klein
ACOWS	Nebraska, USA	Byron J. Adams
Mar	Oregon, USA	Byron J. Adams
OH25	Oregon, USA	Byron J. Adams
Riwaka	New Zealand	Byron J. Adams
RDS109	Indiana, USA	Douglas S. Richmond
RDS123	Indiana, USA	Douglas S. Richmond
RDS96	Indiana, USA	Douglas S. Richmond
BF2	California, USA	Edwin E. Lewis
SPCM3	Indiana, USA	James A. Cate
UK76	Site of 76, UK	Jeremy D. Pearce
KMD24	Virginia, USA	Michael D. Klein
KMD74	Hungary	Michael D. Klein
KMD83	Tennessee, USA	Michael D. Klein
MID09	Midway Island, USA	Michael D. Klein
MID10	Midway Island, USA	Michael D. Klein
TN2	Tennessee, USA	Michael D. Klein
NC1	North Carolina, USA	Raymond J. Akhurst
Arg	Argentina	S. Patricia Stock
TTO1	Trinidad and Tobago	Todd A. Ciche

2. Identification of nematodes and their symbiotic bacteria by molecular techniques

2.1 Internal Transcribed Spacer (ITS) region sequences of *Steinernema* and *Heterorhabditis*

Genomic DNA extraction:

To extract nematode genomic DNA, *G. mellonella* larvae were infected with 100 IJs. After 96 h, a single female adult nematode was dissected from *G. mellonella* cadaver using a platinum needle and placed into a PCR tube with 20 µl lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% [w/v] gelatin and 60 µg/ml proteinase K). The mixture was incubated at -80°C for 15 min and then incubated at 60°C for 1 h and 95°C for 15 min in a BIO-RAD DNAEngine® thermocycler (Lissemore *et al.*, 2005). The resulting DNA samples were examined by agarose gel electrophoresis.

PCR amplification and cloning:

PCR amplification of ITS DNA was carried out in 50 µl reactions containing 10 µl 5X GoTaq® buffer (Promega), 1 µl 10 µM dNTP (Promega), 1.25 U GoTaq® DNA Polymerase (Promega), 2 µl each of 10 µM primers, 32.75 µl sterilized water and 2 µl of template DNA. The primers used for ITS amplification were ITS Forward: 5'-TTG AAC CGG GTA AAA GTC G-3' and ITS Reverse: 5'-TTA GTT TCT TTT CCT CCG CT-3' (Nadler *et al.*, 2000). The PCR reactions were incubated at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec and a final extension at 72°C for 10 min in a BIO-RAD DNA Engine® thermocycler. PCR products were separated in 1.2% agarose gel in 0.5X Tris-borate-EDTA (TBE) buffer. The bands corresponding to the desired product sizes were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were cloned into pGEM®-T Easy Vector (Promega) according to the manufacturer's instructions. The ligated products were transformed into *Escherichia coli* TOP10

competent cells. The transformants were selected on LB plate supplemented with 100 mg/ml ampicillin and 50 mg/ml X-Gal (Promega). The plasmid DNA was isolated from positive colonies using the QIAprep Spin Miniprep Kit (Qiagen) and digested with *Eco*RI (Promega) to confirm the presence of inserts with desired sizes.

Gene sequence and phylogeny analysis:

The plasmids were sequenced from both ends using the universal primers T7 and SP6 with an Applied Biosystems 3730 DNA Analyzer. Low quality and vector sequences were removed from the analysis. Ambiguities in the sequence were addressed by examining the chromatogram. The high quality sequences were aligned with ClustalW algorithm (Thompson *et al.*, 1994). Phylogeny tree was built by Maximum Parsimony (MP) with a bootstrap 100 replications for *Steinernema* sp. using PHYLIP (PHYLogeny Inference Package) and visualized in TreeView (Page, 1996). Separate trees were constructed with or without the USA strains and with all the *Steinernema* strains in the database (Table 3) or with only "small IJ" (Table 4) and *H. bacteriophora* GPS11 was used as the outgroup. For *Heterorhabditis* all strains with the available sequence data in the GenBank were included (Table 5). For phylogenetic analysis, the unaligned sequence ends were deleted, gaps in the aligned domains were treated as either missing data or a fifth base, and *Caenorhabditis elegans* (X03680) was used as the outgroup taxon and to root the trees for *Heterorhabditis* (Malan *et al.*, 2008; Tóth and Lakatos, 2008). Initially, all 67 strains were included in maximum parsimony phylogenetic analyses which were performed with bootstrap (500 replications) using Mega software (Tamura *et al.*, 2007). However, as many new strains formed a big monophyletic group, the datasets were pruned in each phylogenetic analysis to include just representatives for each reciprocally monophyletic group in order to simplify searches of tree space. In addition to MP analysis, a maximum likelihood (ML) phylogenetic reconstruction was implemented in PHYML (Guindon and Gasquel, 2003) using the model of nucleotide substitution estimated from log-likelihood parameters following the Akaike Information Criterion (AIC) 1 with 4 gamma categories in ModelGenerator (Keane *et al.*, 2006). ML trees were inferred using a neighbor-joining starting tree

with NNI (Nearest Neighbor Interchange) tree search algorithm, and support for the topology was tested using 100 bootstrap pseudoreplicates.

Table 3 *Steinernema* species recognized with available Internal Transcribed Spacer sequences deposited in the GenBank used in this study

Steinernema species	Locality	Accession no.
S. abbasi	UK	AY248749
S. aciari	China	AY787660
S. affine	UK	AY230159
S. akhursti	China	DQ375757
S. arenarium	Russia	AY230160
S. ashiunense	Japan	DQ354694
S. backanense	Viet Nam	AY487918
S. beddingi	China	AY603397
S. bicornutum	Yugoslavia	AY230163
S. carpocapsae	Iran	EU122951
S. ceratophorum	China	AY230165
S. cholashanense	China	EF431959
S. cubanum	Cuba	AY230166
S. cumgarense	Viet Nam	AY487920
S. diaprepesi	USA	AF122021
S. eapokense	Viet Nam	AY487921
S. feltiae	Jordan	EU200354
S. glaseri	USA	AY230171
S. guangdongense	China	AY170341
S. hebeiense	China	DQ105794
S. intermedium	USA	AY230172
S. karii	Kenya	AY230173
S. khoisanae	South Africa	DQ314287
S. kraussei	Italy	AY230174
S. loci	Viet Nam	AY355443
S. longicaudum	China	AY230177
S. monticolum	Korea	AF122017
S. neocurtillae	USA	AF122018
S. oregonense	USA	AY230180
S. pakistanense	Pakistan	AY230181
S. rarum	Argentina	AY275273
S. riobrave	USA	AY230182
S. robustispiculum	Viet Nam	AY355442
S. sangi	Viet Nam	AY355441
S. sasonense	Viet Nam	AY487919
S. scapterisci	Uruguay	AY230183
S. siamkayai	Thailand	AF331917
S. sichuanense	China	DQ884965
S. tami	Viet Nam	AY171280
S. texanum	USA	EF152568
S. thanhi	Viet Nam	AF355444
S. thermophilum	India	DQ665651
S. yirgalemense	Ethiopia	AY748450

Table 4 The recognized small infective juvenile *Steinernema* species with available Internal Transcribed Spacer sequences deposited in the GenBank used in this study

Steinernema species and strain	Locality	Accession no.
S. abbasi	UK	AY248749
S. anatoliense Al-Jubiha	Jordan	EU200356
S. backanense	Viet Nam	AY487918
S. carpocapsae All	USA	AY230164
S. carpocapsae Al-Balka-Arida-1	Jordan	EU200352
S. carpocapsae C101	Slovenia	EU914854
S. carpocapsae IRA18	Iran	EU598239
S. carpocapsae Iran4	Iran	EU122951
S. carpocapsae SGIB	China	AY170334
S. carpocapsae Breton	USA	AF121049
S. cumgarense	Viet Nam	AY487920
S. eapokense	Viet Nam	AY487921
S. rarum J1-USA	USA	DQ221116
S. sasonense	Viet Nam	AY487919
S. scapterisci	Uruguay	AY230183
S. siamkayai T9	Thailand	AF331917
S. siamkayai SSRK2	India	FJ463935
S. siamkayai SSRK4	India	FJ463937
S. tami	Viet Nam	AY171280
S. thermophilum	India	DQ665651

Table 5 *Heterorhabditis* species recognized with available Internal Transcribed Spacer sequences deposited in the GenBank used in this study

Heterorhabditis species	Locality	Accession no.
H. amazonensis	Brazil	DQ665222
H. bacteriophora	Australia	AY321477
H. bacteriophora	Australia	EF043438
H. downesi	Ireland	AY321482
H. downesi	Ireland	EF043442
H. floridensis	USA	DQ372922
H. georgiana	USA	EU099032
H. indica	India	AY321483
H. indica	India	AY170329
H. marelatus	USA	AY321479
H. marelatus	USA	EF043441
H. megidis	USA	AY293284
H. megidis	USA	AY321480
H. mexicana	Mexico	AY321478
H. mexicana	Mexico	EF043444
H. safricana	South Africa	EF488006
H. tayearae	Egypt	EF043443
H. zealandica	New Zealand	EF530041
H. zealandica	New Zealand	AY170330

2.2 16S rDNA and gyrase B gene sequence of *Xenorhabdus* and *Photorhabdus*

Bacteria isolation:

The symbiotic bacteria were isolated from the nematode strains according to the following procedure. Within 48 h post nematode infection, a drop of *G. mellonella* hemolymph was collected by bleeding a foreleg of the surface sterilized *G. mellonella* (Poinar, 1990) and spread on NBTA indicator plates (5 g/L peptone, 3 g/L beef extract, 15 g/L agar, 0.025 g/L bromothymol blue, and 0.04 g/L triphenyl tetrazolium chloride) (Akhurst, 1980). After 48 h incubation at 28°C, the bacterial colonies were analyzed for catalase activity using 3% hydrogen peroxide treatment (Boemare, 2002). Only bacterial colonies showing negative catalase activity were selected for further studies. For *Photorhabdus*, the colonies were tested for bioluminescence (Boemare, 2002), and only those colonies showing positive luminescent activity were selected for further studies.

Bacterial genomic DNA extraction:

The bacterial cells were collected by centrifugation and resuspended in 400 µl sterile salt homogenizing buffer (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, and 2 mM EDTA pH 8.0). Then, 40 µl of 20% SDS (2% final concentration) and 8 µl of 20 mg/ml proteinase K (400 µg/ml final concentration) were added and mixed well. The bacterial samples were incubated at 60°C for at least 1 h or overnight, after which 300 µl of 6M NaCl (NaCl saturated H_2O) was added to each isolate. Each bacterial sample was vortexed for 30 sec at maximum speed, and spun for 30 min at 10,000 g. The supernatant was transferred to fresh tubes and an equal volume of isopropanol was added to each tube, mixed well, and incubated at -20°C for 1 h. Bacterial samples were then centrifuged at 10,000 g for 20 min at 4°C. The pellets were washed with 70% ethanol, air-dried and finally resuspended in 200 µl elution buffer (Aljanabi and Martinez, 1997). The DNA samples were examined by agarose gel electrophoresis.

PCR amplification and cloning:

The primer used for amplification 16S ribosomal DNA of *Xenorhabdus* sp. sequence were 11F-NH: (5'-GTT TGA TCM TGG CTC AG-3') and 907R-nf: (5'-CCG TCA ATT CMT TTR AGT TT-3') (universal primer). The PCR reactions were incubated at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 1 min and a final extension at 72°C for 10 min in a BIO-RAD DNA Engine® thermocycler. PCR products were separated in 1.2% agarose gel in 0.5X Tris-borate-EDTA (TBE) buffer. The bands corresponding to the desired product sizes were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified 16S rDNA PCR product for each strain was separately cloned into pGEM®-T Easy Vector (Promega) according to the manufacturer's instructions. The plasmids containing 16S rDNA PCR products were isolated using the QIAprep Spin Miniprep Kit (Qiagen). The ligated products were transformed into *Escherichia coli* TOP10 competent cells. The transformants were selected on LB plate supplemented with 100 mg/ml ampicillin and 50 mg/ml X-Gal (Promega). The plasmid DNA was isolated from positive colonies using the QIAprep Spin Miniprep Kit (Qiagen) and digested with *Eco*RI (Promega) to confirm the presence of inserts with desired sizes.

The primers used for amplification of 16S ribosomal DNA of *Heterorhabditis* sp. sequences were 16S-F2 (5'-CAG ACT CCT ACG GGA GGC AGC A-3') and 16S-R2 (5'-CTC ACG GTT CCC GAA GGC ACT-3'). The primers used for amplification of gyrase B gene were gyrB-F (5'-GAA GTC ATC ATG ACC GTT CTG CAY GCN GGN GGN AAR TTY GA-3') and gyrB-R (5'-AGC AGG GTA CGG ATG TGC GAG CCR TCN ACR TCN GCR TCN GTC AT-3') according to Yamamoto and Harayama (1995). The PCR reactions were incubated at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min and a final extension at 72°C for 10 min in a BIO-RAD DNA Engine® thermocycler. PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen).

Gene sequence and phylogeny analysis:

The plasmids of *Xenorhabdus* spp. were sequenced from both ends using the universal primers T7 and SP6 with an Applied Biosystems 3730 DNA Analyzer. The purified gyrase B gene PCR products were sequenced from both ends using the oligonucleotides with the same sequences primers gyrB-F and gyrB-R as described by Yamamoto and Harayama (1995). Site polymorphisms in the sequenced products were recorded as described previously (Nadler *et al.*, 2006). After removing low quality and vector sequences, the resulting high quality sequences were used to produce alignments of 16S rDNA and gyrase B with other known nematodes or bacteria for comparative purposes using ClustalW algorithm (Thompson *et al.*, 1994). Phylogeny tree was built by Maximum Parsimony (MP) with a bootstrap 100 replications for *Xenorhabdus* using PHYLIP (PHYLogeny Inference Package) and visualized in TreeView (Page, 1996). The phylogenetic tree was built using recognized *Xenorhabdus* spp. with available 16S rDNA and *Photorhabdus* spp. with available gyrase B gene sequences deposited in the GenBank (Tables 6 and 7).

For phylogenetic analysis, the unaligned sequence ends were deleted, gaps in the aligned domains were treated as either missing data or a fifth base. *P. luminescens* (AY278508) was used as the outgroup for *Xenorhabdus* and *X. nematophila* (AY322431) was used as the outgroup taxon and to root the trees for *Photorhabdus* according to previous studies (Malan *et al.*, 2008; Tóth and Lakatos, 2008). Initially, all 67 strains were included in MP phylogenetic analyses which were performed with bootstrap (500 replications) using Mega software (Tamura *et al.*, 2007). However, as many new strains formed a big monophyletic group, the datasets were pruned in each phylogenetic analysis to include just representatives for each reciprocally monophyletic group in order to simplify searches of tree space. In addition to MP analysis, a maximum likelihood (ML) phylogenetic reconstruction was implemented in PHYML (Guindon and Gasquel, 2003) using the model of nucleotide substitution estimated from log-likelihood parameters following the Akaike Information Criterion (AIC) 1 with 4 gamma categories in ModelGenerator (Keane *et al.*, 2006). ML trees were inferred using a neighbor-joining starting tree

with NNI tree search algorithm, and support for the topology was tested using 100 bootstrap pseudoreplicates.

Table 6 The recognized *Xenorhabdus* species with available 16S rDNA sequences deposited in the GenBank used in this study

Xenorhabdus species and strain	Nematode host	Locality	Accession no.
X. beddingii DSM4764	*Steinernema* sp.	DSM	AY278675
X. budapestensis DSM16342	*S. bicornutum*	Serbia and Montenegro	AJ810293
X. cabanillasii USTX62	*S. riobrave*	USA	AY521244
X. doucetiae FRM16	*S. diaprepesi*	Martinique	DQ211709
X. ehlersii CN01	*S. serratum*	China	DQ208307
X. griffiniae ID10	*S. hermaphroditum*	Indonesia	DQ211710
X. hominickii KE01	*S. karii*	Kenya	DQ211719
X. indica OM01	*S. abbasi*	Oman	DQ211718
X. innexi DSM16336	*S. scapterisci*	Uruguay	AJ810292
X. japonica DSM16522T	*S. kushidai*	Japan	DQ202310
X. koppenhoeferi USNJ01	*S. scarabaei*	USA	DQ205450
X. kozodoii SaV	*S. arenarium*	Russia	DQ211716
X. mauleonii VC01	*Steinernema* sp.	Saint Vincent	DQ211715
X. miraniensis Q1	Steinernematidae	Australia	DQ211713
X. nematophila ATCC19061	*S. carpocapsae*	ATCC	D78009
X. poinarii G1	*S. glaseri*	USA	NR_025875
X. romanii PR06-A	*S. puertoricense*	Puerto Rico	DQ211717
X. stockiae TH01	*S. siamkayai*	Thailand	DQ202309
X. szentirmaii DSM16338	*S. rarum*	Argentina	AJ810295

ATCC = American Type Culture Collection

Table 7 *Photorhabdus* species with available gyrase B gene sequences deposited in the GenBank used in this study

Photorhabdus species and strain	Accession no.
P. asymbiotica subsp. *australis* 1216-79	AY278492
P. luminescens subsp. *akhurstii* D1	AY278499
P. luminescens subsp. *kayaii* FR33	EU930349
P. luminescens subsp. *laumondii* HP88	AY278508
P. luminescens subsp. *luminescens* Hb	AY278501
P. luminescens subsp. *thracensis* H3210	EU053173
P. temperata subsp. *temperata* BE09	EU930354
P. temperata C1	AY278497
P. temperata Habana	AY278503
P. temperata MEG1	AY278512
P. temperata NZH3	AY278513
P. temperata subsp. *cinerea* H3107	EU053168
P. temperata subsp. *stackebrandtii* GPS11	GU249303
P. temperata T327	EU930356

2.3 Cophylogenetic analysis of *Heterorhabditis* and *Photorhabdus*

Most of the *Photorhabdus* strains were identified as *H. bacteriophora*. Hence, the study was designed to evaluate whether there is an evidence for cophylogeny between *Photorhabdus* and *Heterorhabditis*. One or two representatives from each monophyletic group were selected in order to avoid possible over-representation in the cophylogeny tests. The strains included in this test are listed in Table 8. The distance matrices among the nematodes or the bacteria were computed from the ML tree using the best model as described above (Keane *et al.*, 2006). The congruence between the phylogenetic trees of nematodes and bacteria was first visualized using TreeMap 1.0, a tree topology based program (Page, 1994) and then statistically assessed by ParaFit analysis (Legendre *et al.*, 2002) as incorporated in the CopyCat platform (Meier-Kolthoff *et al.*, 2007). In the ParaFit analysis, probabilities were based on 999 permutations and the correlation was considered significant at $p < 0.02$ (Meier-Kolthoff *et al.*, 2007). The null hypothesis of the global test is that the associations between *Heterorhabditis* and *Photorhabdus* are randomly distributed on the phylogeny. In the test of individual links, the null hypothesis is that a given *Heterorhabditis* - *Photorhabdus* association is established at random.

Table 8 *Heterorhabditis* and their corresponding *Photorhabdus* strains used for cophylogeny evaluation. Data for nematode and bacterial strains D1, HF85, K122 and NZH3 were obtained from the NCBI database while the rest were from this study

Strain	Nematode		Bacteria	
	Species	Accession no.	Species	Accession no.
ACOWS	*H. georgiana*	not submitted yet	*Photorhabdus* sp.	not submitted yet
D1	*H. indica*	AY170329	*P. luminescens*	AY278499
GPS11	*H. bacteriophora*	not submitted yet	*P. temperata*	GU249303
GPS15	*H. bacteriophora*	not submitted yet	*P. luminescens*	not submitted yet
GPS30	*H. bacteriophora*	not submitted yet	*P. temperata*	not submitted yet
HF85	*H. megidis*	EF043439	*P. temperata*	AY278502
K122	*H. downesi*	EF043442	*P. temperata*	EU930355
KMD60	*H. bacteriophora*	not submitted yet	*P. temperata*	not submitted yet
KMD65	*H. bacteriophora*	not submitted yet	*P. temperata*	not submitted yet
KMD81	*H. bacteriophora*	not submitted yet	*Photorhabdus* sp.	not submitted yet
KMD82	*H. georgiana*	not submitted yet	*Photorhabdus* sp.	not submitted yet
KMD83	*H. bacteriophora*	not submitted yet	*P. luminescens*	not submitted yet
KMD84	*H. bacteriophora*	not submitted yet	*P. luminescens*	not submitted yet
MID10	*H. indica*	not submitted yet	*P. luminescens*	not submitted yet
MP111	*H. indica*	not submitted yet	*P. luminescens*	not submitted yet
MP17	*H. indica*	not submitted yet	*P. luminescens*	not submitted yet
MP68	*Heterorhabditis*	not submitted yet	*P. luminescens*	not submitted yet
NZH3	*H. zealandica*	EF530041	*P. temperata*	AY278513
OB2	*H. bacteriophora*	not submitted yet	*P. luminescens*	not submitted yet
OH25	*H. georgiana*	not submitted yet	*Photorhabdus* sp.	not submitted yet
RDS96	*H. bacteriophora*	not submitted yet	*P. luminescens*	not submitted yet
SPCM3	*H. georgiana*	not submitted yet	*P. temperata*	not submitted yet
UK76	*H. megidis*	not submitted yet	*P. temperata*	not submitted yet

3. Identification of new Thai entomopathogenic nematodes by morphological characters

3.1 Morphological characters

All EPNs were reared in the laboratory on *G. mellonella* larvae. A total of 200 IJs were exposed to ten larvae in a 10 cm diam. Petri dish lined with a filter paper. First and second generation adult nematodes were obtained by dissecting infected larvae 72 h and 144 h after infection for *Steinernema* and 96 h and 168 h for *Heterorhabditis*, respectively. The IJs were obtained when they emerged from the cadavers 10 - 12 days after the *G. mellonella* larvae died. Specimens were fixed in triethanolamine formalin (TAF) (Courtney *et al.*, 1955) and processed in anhydrous glycerin (Seinhorst, 1959). Nematodes were mounted on glass slides with a small piece of wire coil as the cover glass support to avoid flattening of the specimens. The pictures and measurements were made using program SPOT (Diagnostic Instruments, Inc., Sterling Heights, Michigan) with an ocular micrometer. Drawings were made with the aid of a camera lucida.

Adults of the first generation were dissected from *G. mellonella* larvae in Ringer's solution (pH 7.3). Five day-old IJs and fresh adults dissected from the cadavers were rinsed for 15 min in Ringer's solution and three times in 0.05% NaCl. All nematodes were relaxed and killed instantly by plunging in a heated water-bath at 60°C for 2 - 3 min and were fixed in 8% glutaraldehyde (diluted in Ringer) for 2 h at room temperature. Fixed nematodes were rinsed in distilled water three times, post-fixed in osmium tetroxide (OsO_4) for 1 h, rinsed in distilled water again and dehydrated at 15 min intervals in 30%, 50%, 70%, 90% and three times in 100% ethanol. They were critical point dried in liquid CO_2, mounted on SEM stubs, coated with gold and scanned (Stock *et al.*, 1998) using a Jeol JEM 5410 LV scanning electron microscope.

3.2 Cross hybridization test

Cross-hybridization studies between the new Thai *Steinernema* sp. and *Steinernema siamkayai* were conducted using the hanging blood drop method (Kaya and Stock, 1997). One hundred IJs were surface sterilized in 0.2% Thimerosal (sodium ethylmercurithiosalicylate) and placed in agar plates which were incubated at 25°C in the dark until pre-adults were formed. Five pre-adult females of *Steinernema* sp. with five males of *S. siamkayai*, and vice versa, were placed in a hanging blood drop of *G. mellonella* hemolymph on a glass slide. Hanging blood drop slides were placed on a small Petri dish (60 mm x 15 mm) which was placed in a larger Petri dish (100 mm x 15 mm) containing distilled water to prevent the hemolymph from drying. The dishes were carefully wrapped with Paraffin film and incubated at 25°C in the dark. Each cross-breeding test was conducted over a period of 10 days and replicated three times. Control mating contained males and females of the same species.

4. Virulence of Thai EPN strains against second instar larva of the Japanese beetle

Grubs, Nematodes and Soil:

The second instar larvae of *P. japonica* were collected twice for two experiments (late August and late September 2008) in turf areas at the Wooster campus of the Ohio State University. Only second instar larvae were carefully taken from each collected batch to run the experiments. Larvae were kept in the soil mixed with grass seeds at room temperature (22 ± 1°C) for three days before use in the experiments. Only actively moving grubs were used in all bioassays.

All four nematode strains used in this study were recently isolated from Thailand. There were two strains of *H. indica*, one of undescribed *Heterorhabditis* species and one of a new species of *Steinernema*, *S. minuta* (Maneesakorn *et al.*, 2010). All nematodes were reared in the last instar *Galleria mellonella* L. at 25°C. Nematodes were harvested from the White traps (White, 1927) and stored in tap water at 10°C for 3 to 30 days prior to use in the experiments.

The soil used in this experiment was identified as silty clay (61.2% silt, 26.2% clay, 12.6% sand) with 3.6% organic matter and 7.1 pH. The soil was pasteurized for 10 h at 121°C and air-dried before use. The soil moisture was adjusted to 24% (w/w) by adding sterilized tap water immediately before setting up the experiments.

Virulence bioassays:

Virulence bioassays were conducted at room temperature (22 ± 1°C). Individual larvae were placed at the bottom of a 30-ml plastic cup which was filled with 20 g of 24% moisture soil mixed with perennial ryegrass seed (0.2 g/20 g soil) as a food source. The nematode concentrations were 0 (control), 10, 33, 100, 330, and 1,000 IJs/larva. Treatments were replicated three times with 10 cups/replication. The entire experiment was also repeated once using a different batch of grubs but the same batch of nematodes. Larval mortality was assessed at 5, 10, and 15 days after treatment (DAT).

Statistical analyses:

The larval mortalities at different nematode concentrations and DAT were analyzed using Probit analysis. Lethal concentration for 50% mortality (LC_{50}) at 5 days after treatment and lethal time in days to 50% mortality (LT_{50}) at 100 IJs/larva for each nematode strain was calculated. The differences in the LC_{50}s and LT_{50}s were considered significant if there was no overlap between the 95% fiducial limits. Percentage grub mortalities were corrected for control mortality using Henderson-Tilton's formula (Henderson and Tilton, 1955) and arcsine transformed before subjecting to analysis of variance (ANOVA). Minitab statistical software, version 15.1.20.0, 2007 (Minitab Inc., State College, PA, USA) was used for data analyses.

Place and Duration

1. Department of Entomology, Faculty of Agriculture, Kasetsart University and Plant Protection Research and Development Office, Department of Agriculture, Ministry of Agriculture and Co-operatives, Bangkok, Thailand, November 2006 – February 2008 and March 2009 – November 2009.

2. Department of Entomology, Ohio Agricultural Research and Development Center, the Ohio State University, Wooster, Ohio, USA, March 2008 – February 2009 and December 2009 – March 2010.

Study Support

This study was fully supported by the Royal Golden Jubilee Ph.D. Scholarship (Grant: PHD/0018/2549), TRF Senior Research Scholar #RTA 4880006 under Thailand Research Fund (TRF) and the research grants to Prof. Dr. Parwinder S. Grewal at the Ohio State University (OARDC, Wooster) of USA.

RESULTS AND DISCUSSION

1. Soil sampling and nematode isolation

One *Steinernema* and three *Heterorhabditis* were found from 115 soil samples collected from 48 forests of 26 provinces in Thailand (Table 9).

Table 9 The nematode strains were recovered from different areas in Thailand

Strain	Nematode	Locality	Date
MP10	*Steinernema* sp.	Chumphon	8 April 2007
MP17	*Heterorhabditis* sp.	Khon Kaen	10 May 2007
MP68	*Heterorhabditis* sp.	Kanchanaburi	18 December 2007
MP111	*Heterorhabditis* sp.	Krabi	20 January 2008

Steinernema:

One *Steinernema* was recovered from soil sample in the forest at 10°23.060’ N and 99°17.042’ E, on the slope at 36 m from the base of steeply mountain in Chumphon province, Southern Thailand (Fig. 2). This site is located in tropical rain forest comprised with the pine tree forest. The *Steinernema* nematode used its tail for standing, waving and jumping. This character is called ambusher (Fig. 3) and the nematode can be recognized by black color of the wax moth cadaver (Fig. 4).

Heterorhabditis:

Three *Heterorhabditis* were collected from soil samples in different forests at Khon Kaen province (MP17) which is the mixed deciduous forest, Kanchanaburi province (MP68) which is the dry evergreen forest, and Krabi province (MP111) which is the tropical rain forest (Fig. 2). All nematode species can be recognized by the red color of wax moth cadaver (Fig. 4).

Figure 2 Four collecting sites where the entomopathogenic nematodes are found

A: Chumphon province

B: Khon Kaen province

C: Kanchanaburi province

D: Krabi province

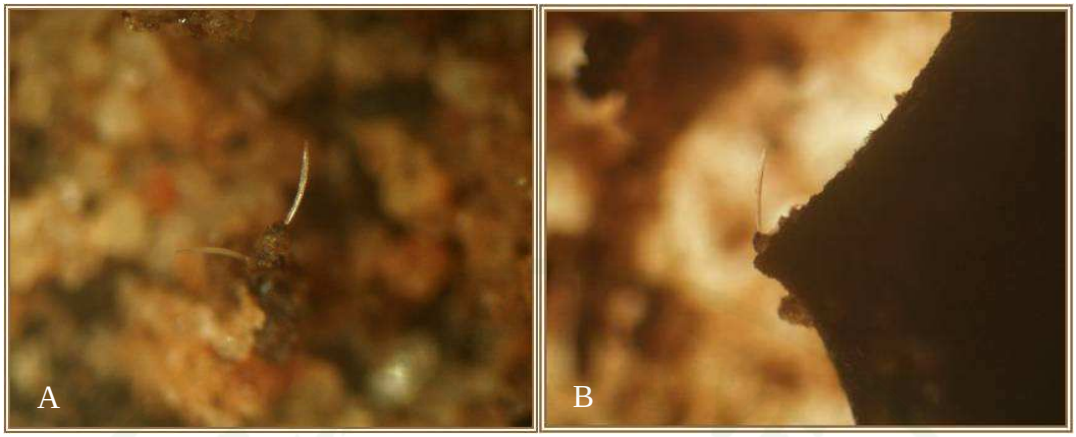


Figure 3 The ambush-type character (A and B) of *Steinernema* MP10 strain from Thailand

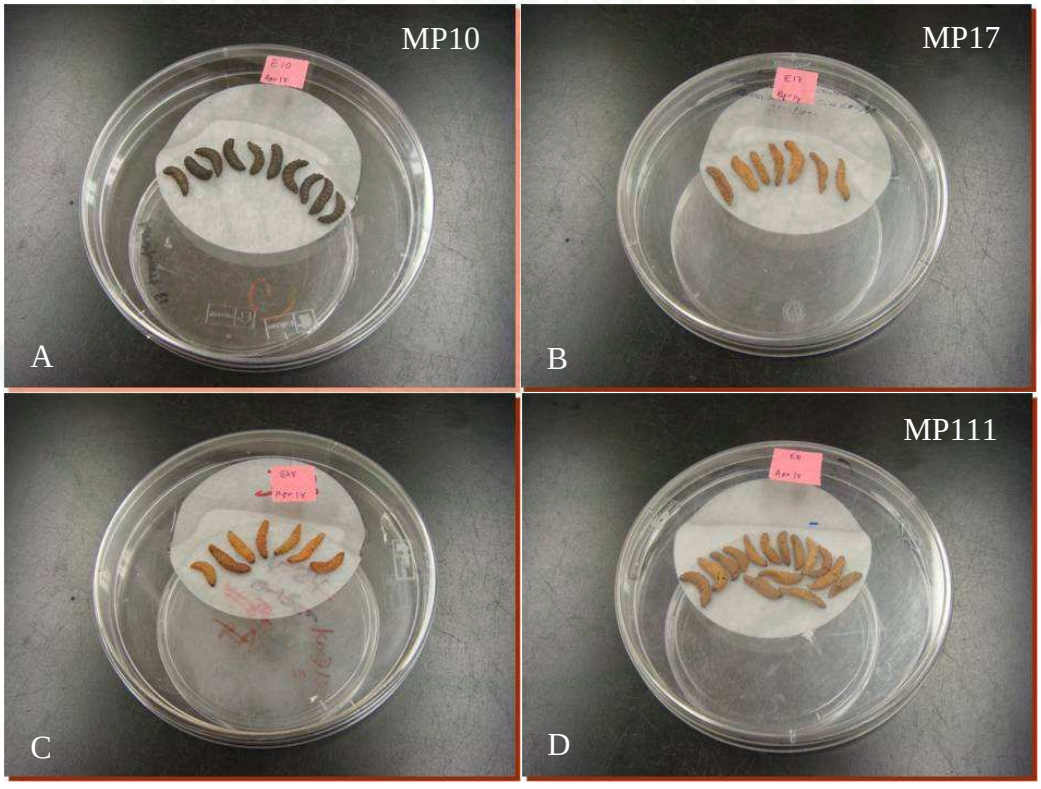


Figure 4 The cadavers of wax moth after infected by different entomopathogenic nematodes

A: *Steinernema* sp. MP10 B: *Heterorhabditis* sp. MP17

C: *Heterorhabditis* sp. MP68 D: *Heterorhabditis* sp. MP111

2. Identification of nematodes and their symbiotic bacteria by molecular techniques

2.1 Internal Transcribed Spacer (ITS) region sequences of *Steinernema* and *Heterorhabditis*

Steinernema:

The nematode ITS sequences with 852 base pairs for all five nematode strains showed high similarity to that of *Steinernema* species in the NCBI GenBank database. The ITS sequences of four USA strains D60, D90, D98, and FC48 exhibited high similarity (>99%) to that of the deposited sequences of *S. carpocapsae*. The Thai strain MP10 sequence showed 97% similarity to *Steinernema tami* Luc, Nguyen, Reid and Spiridonov, 2000.

The phylogenetic trees based upon the ITS sequences showed that D60, D90 and D98 strains were grouped with *S. carpocapsae* but FC48 was not grouped with *S. carpocapsae* whilst the MP10 strain grouped with *S. tami* (Fig. 5). Since all five *Steinernema* strains had IJ body length close to 600 µm that represents the "small IJ" group (sensu Stock *et al*., 2001), another phylogenetic tree was using available ITS sequences of all the *Steinernema* strains with the body length of less than 600 µm. The relationships among different species were better resolved in this "small IJ" group tree. MP10 strain again grouped with *S. tami* and was placed as sister taxa to *Steinernema cumgarense* Phan, Spiridonov, Subbotin and Mones, 2006, *Steinernema eapokense* Phan, Spiridonov, Subbotin and Mones, 2006 and *S. siamkayai* (Fig. 6). Hence, the FC48 which was not grouped with the other species might be placed as a new species.

Figure 5 Phylogenetic tree of *Steinernema* species based on sequences of Internal Transcribed Spacer (ITS) region. The ITS sequence of *Heterorhabditis bacteriophora* GPS11 is used as the outgroup. Bootstrap values that are greater than 50 are indicated at the branch

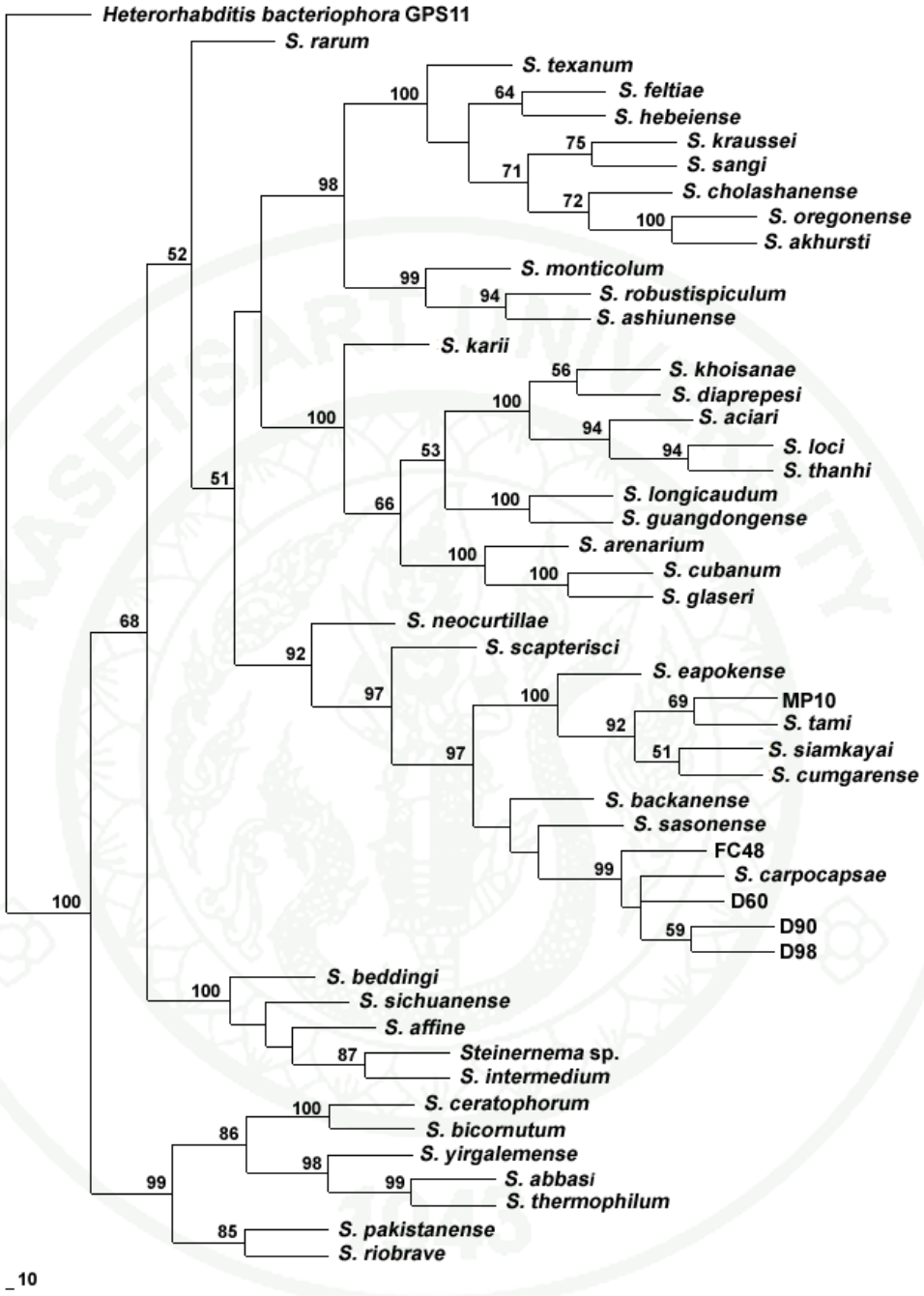
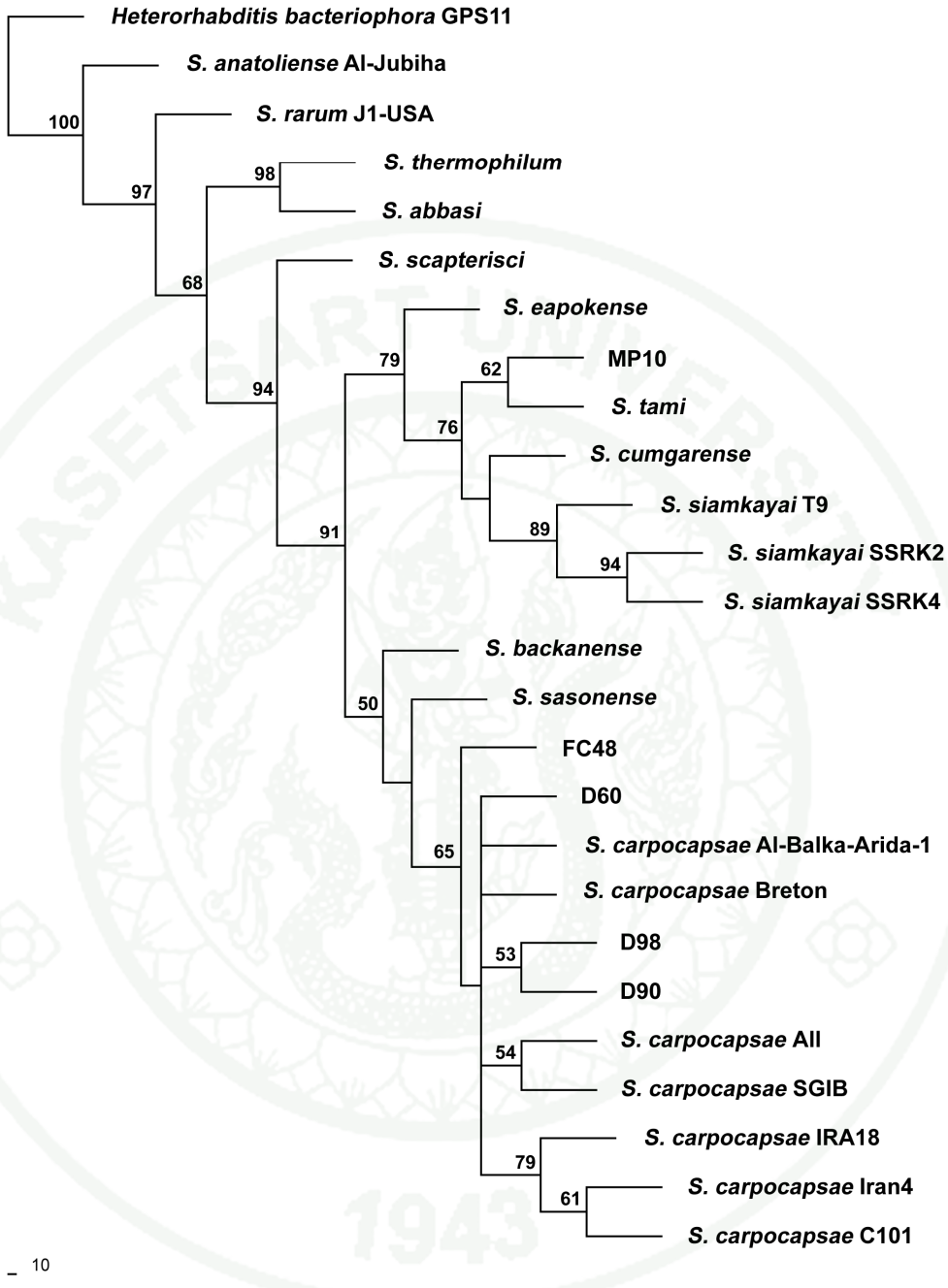


Figure 6 Phylogenetic tree of *Steinernema* species belonging to the small infective juvenile (IJ length less than 600 μm) group based on sequences of Internal Transcribed Spacer (ITS) region. The ITS sequence of *Heterorhabditis bacteriophora* GPS11 is used as the outgroup. Bootstrap values that are greater than 50 are indicated at the branch



Heterorhabditis:

The nematode ITS sequences for all 67 strains showed high similarity to that of *Heterorhabditis* species in the NCBI GenBank database. Out of the 67 strains, 52 exhibited high similarity (99% or higher) to deposited sequences of *H. bacteriophora*. Nine (GPS29, ACOWS, GPS30, KMD82, KMD1, OH25, SPCM3, KMD37 and KMD64) were highly similar (99% or higher) to that of *Heterorhabditis georgiana* Nguyen, Shapiro-Ilan and Mbata, 2008. One strain (UK76) showed high similarity (99%) to *H. megidis*. Four strains (MID09, MID10, MP17 and MP111) had high similarity (99% or higher) to *H. indica*. One strain (MP68) was 98% similar to *Heterorhabditis amazonensis* Andaló, Nguyen and Moino, 2007. The ITS sequences of the nematode strains varied in the length of the aligned region. For strains with similarity to *H. bacteriophora*, *H. georgiana*, *H. megidis*, *H. indica* and *H. amazonensis*, the length of the aligned ITS region excluding the gap was 901, 901, 885, 869 and 890 base pairs, respectively.

The loose consensus trees built based upon the ITS sequences using maximum parsimony (MP) analysis indicated that the strains were divided into five species, *H. bacteriophora*, *H. georgiana*, *H. megidis*, *H. indica* and a potentially new *Heterorhabditis* species comprising the MP68 strain collected from Thailand (Fig. 7). The MP tree was well resolved for strains UK76, MP68, MP17, MP111, MID09, MID10, KMD1, KMD37, KMD64, KMD82, GPS29, GPS30, ACOWS, SPCM3 and OH25 with high bootstrap support. The MP17, MP111, MID09 and MID10 strains were sister to *H. indica*, forming a monophyletic group with strong (99%) bootstrap support, UK76 is part of the *H. megidis* clade (99% bootstrap support), and MP68 was sister to *H. amazonensis* and a paraphyletic group formed by *H. mexicana*, *Heterorhabditis tayearae* Shamseldean, El-Sooud, Abd-Elgawad and Saleh, 1996 and *H. floridensis*. This result suggests that MP68 may represent a new *Heterorhabditis* species.

This prediction was also supported by comparison of currently available *Heterorhabditis* ITS sequences deposited in the NCBI GenBank which show that

partial ITS sequences differ from 89 to 98% between species and shared 99% or higher similarity within species. Strains KMD1, KMD37, KMD64, KMD82, GPS29, GPS30, ACOWS, SPCM3 and OH25 formed a *H. georgiana* monophyletic group with high bootstrap support (96%). For the rest of the strains forming *H. bacteriophora* group, bootstrap MP analysis received weak clade support (< 70%), which may be due to the overwhelming number of strains in this clade. By reducing the number of strains that were part of *H. bacteriophora* clade to perform the MP and ML analyses, this clade received high bootstrap support (> 90%) as shown in one of the representative examples (Fig. 8). Also the topology of the trees showed high similarity between ML and MP analyses.

Figure 7 Loose consensus of maximum parsimony trees inferred from sequences of Internal Transcribed Spacer (ITS) region in *Heterorhabditis* species and some of 67 strains used in this study. Maximum parsimony bootstrap clade frequency values ≥70% mapped above the internal nodes

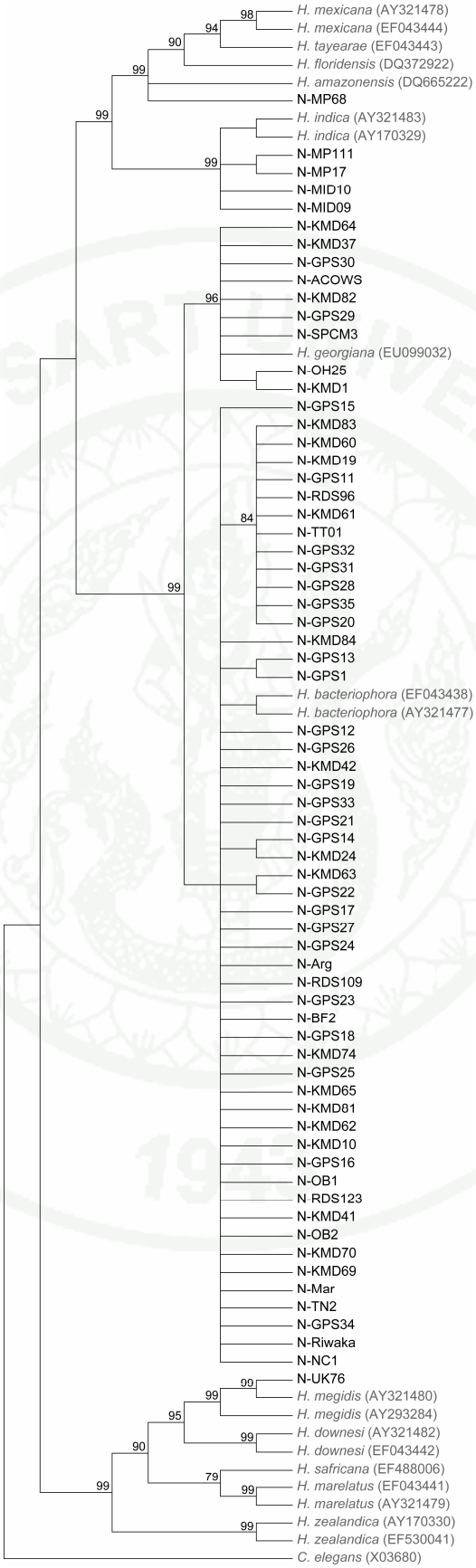
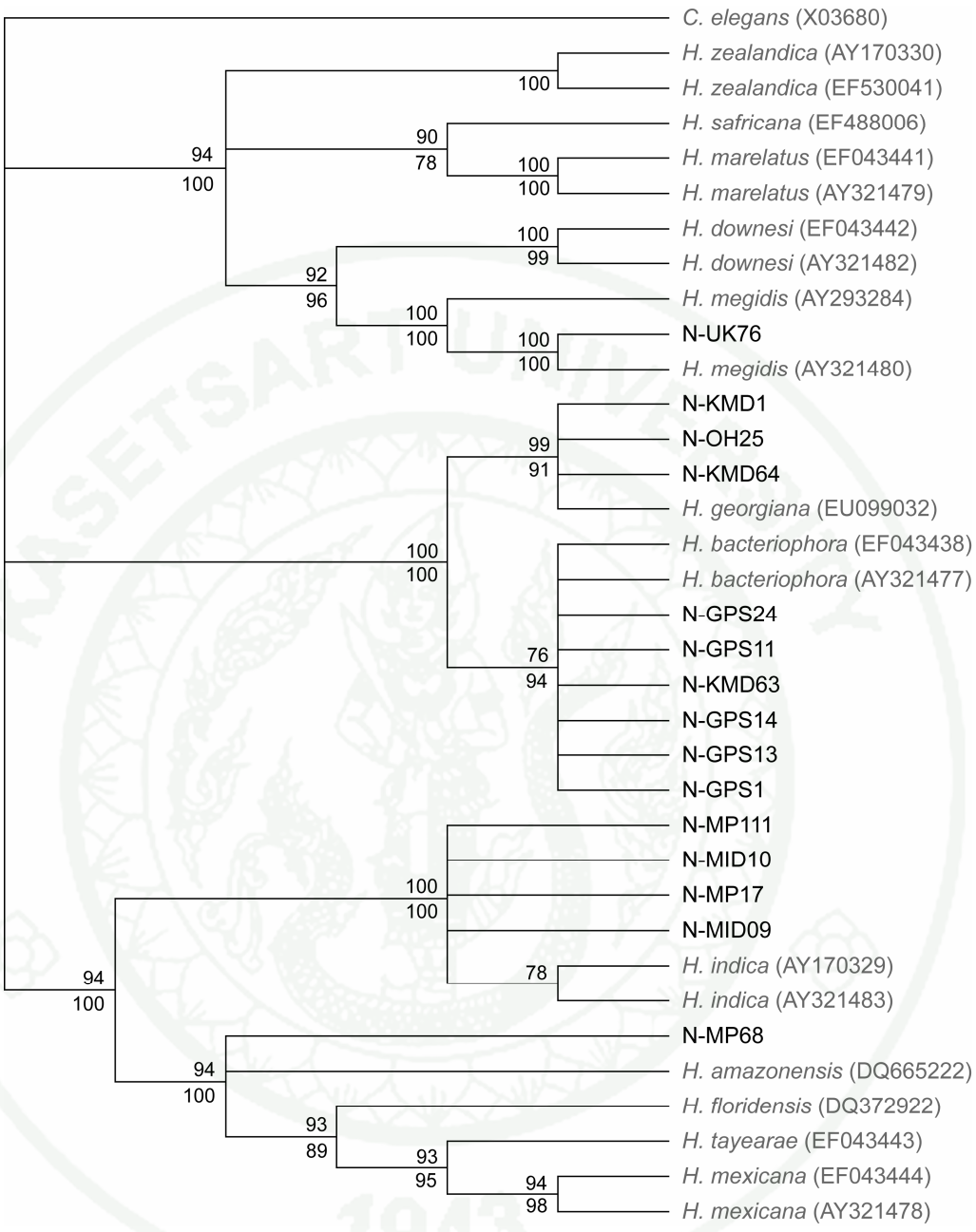


Figure 8 Maximum likelihood tree inferred from sequences of Internal Transcribed Spacer (ITS) region in *Heterorhabditis* species and some of 67 strains used in this study. Maximum likelihood bootstrap values ≥70% shown above the internal nodes. Maximum parsimony bootstrap clade frequency values ≥70% mapped below the internal nodes

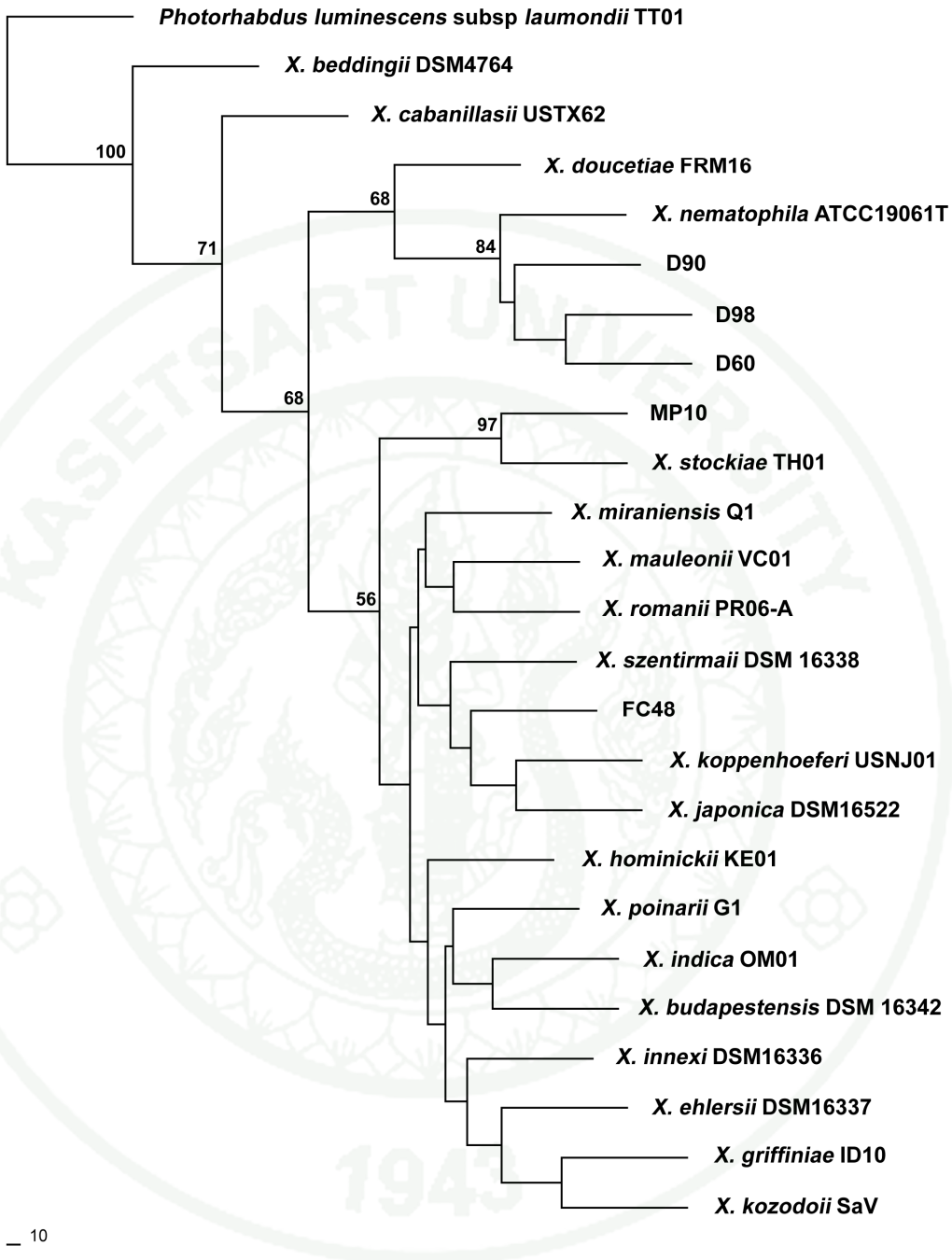


2.2 16S rDNA and gyrase B gene sequence of *Xenorhabdus* and *Photorhabdus*

16S rDNA of *Xenorhabdus* spp.:

The 16S rDNA sequences with 916 base pairs for the bacteria of all five *Steinernema* strains showed high similarity to that of *Xenorhabdus* in the NCBI GenBank. The bacteria isolates D60, D90 and D98 shared high similarity (>99%) to *Xenorhabdus nematophila* (Poinar and Thomas, 1965) Thomas and Poinar, 1979 in 16S rDNA sequences. The 16S rDNA sequence of FC48 symbiotic bacterium had 98% similarity to that of *X. szentirmaii* Lengyel, Lang, Fodor, Szallas, Schumann and Stackebrandt, 2005(b). Phylogeny of the symbiotic bacteria determined by 916 base pairs of the 16S rDNA showed that bacteria isolates D60, D90 and D98 were closely grouped with *X. nematophila*. For MP10 strain, the bacteria closely grouped with *X. stockiae* (Fig. 9) which is the recognized symbiont of *S. siamkayai* that was originally isolated from Thailand. However, the symbiotic bacterium of FC48 did not closely group with any other *Xenorhabdus* species, indicating that it is potentially represent a new species.

Figure 9 Phylogenetic tree of *Xenorhabdus* species based on partial 16S ribosomal DNA (rDNA) sequences. The 16S rDNA sequence of *Photorhabdus luminescens* subsp. *laumondii* TT01 is used as the outgroup. Bootstrap values that are greater than 50 are indicated at the branch



gyrase B gene:

The symbiotic bacteria of all 67 nematode strains were confirmed to be *Photorhabdus* species based on the analysis of their 16S rDNA sequences (data not shown) which have been typically used to identify bacterial species. Their gyrase B gene sequences also showed high similarity to that of *Photorhabdus* in the NCBI GenBank. The strict (70%) consensus trees determined by 766 base pairs of the gyrase B gene sequences using MP analysis showed that all strains were divided into two clades, *P. temperata* and *Photorhabdus luminescens* (Thomas and Poinar, 1979) Boemare, Akhurst and Mourant, 1993 (Fig. 10). Within *P. temperata* clade, five monophyletic groups were identified with 97% bootstrap support, which are *P. temperata* subsp. *temperata* including UK76 strain, *P. temperata* subsp. *cinerea*, *P. luminescens* subsp. *thracensis*, *P. temperata* subsp. *stackebrandtii* (An and Grewal, 2010) and one *P. temperata* subgroup awaiting recognition.

According to the MP tree, the bacterial strain H3210 should belong to a *P. temperata* subspecies rather than a *P. luminescens* subspecies which has been recognized previously (Hazir *et al.*, 2004). Within *P. luminescens* clade, the monophyletic group comprising the symbiotic bacteria of KMD81, KMD37, KMD82, ACOWS and OH25 was sister to the clade formed by currently recognized *P. luminescens* subspecies, indicating that they may belong to a new subspecies of *P. luminescens*. Also while gyrase B gene sequences of these five bacterial strains were identical to each other, they showed only 90 - 93% similarity to the currently recognized *Photorhabdus* species. The bacterial strains MP17, MP68, MP111, MID09 and MID10 were part of a clade including *P. luminescens* subsp. *akhurstii* which is sister to the clades represented by three of the currently recognized *P. luminescens* subspecies. The strain KMD74 belonged to the clade represented by *P. luminescens* subsp. *kayaii* with 100% MP bootstrap support. The remaining strains belonged to *P. luminescens* subsp. *laumondii* clade within which two monophyletic groups were identified.

Also, as most of bacterial strains belonged to *P. luminescens* subsp. *laumondii* or the new *P. temperata* subgroup clades, the number of bacterial strains were reduced to perform the MP and ML analyses. Similarly, the strains belonging to *P. temperata* subsp. *stackebrandtii* clade were further divided into two monophyletic groups (Fig. 11).

The topology of the MP tree was similar to the ML tree, with the exception of differences in *P. luminescens* clades. In MP tree, clades representing five currently recognized *P. luminescens* subspecies are sister to each other, forming a big sister group of the clade comprising the symbiotic bacteria of KMD81, KMD37, KMD82, ACOWS and OH25. In ML tree, the monophyletic group including *P. luminescens* subsp. *akhurstii* formed a sister group to *P. luminescens* subsp. *luminescens* first. In either case, the monophyletic group including the symbiotic bacteria of KMD81, KMD37, KMD82, ACOWS and OH25 may potentially represent a new *P. luminescens* subspecies.

Photorhabdus luminescens is currently divided into five subspecies (Hazir *et al*., 2004), four of which, *P. luminescens luminescens, P. luminescens laumondii*, *P. luminescens kayaii* and *P. luminescens thracensis* were isolated from *H. bacteriophora*, and one *P. luminescens akhurstii* from *H. indica*. Association of *P. temperata* appears to be even more diverse and complex. This species has been found associated with *H. bacteriophora, H. megidis*, *H. zealandica*, and *H. downesi* Stock, Griffin and Burnell, 2002.

This results showed that both *P. luminescens* and *P. temperata* can also associate with *H. georgiana*. Out of eight *H. georgiana* associated bacterial strains, four (KMD37, KMD82, ACOWS and OH25) were recognized as a potentially new *P. luminescens* subspecies, and four (GPS29, SPCM3, KMD1, KMD64) belonged to *P. temperata* subsp. *stackebrandtii*.

Figure 10 Loose consensus of maximum parsimony trees inferred from gyrase B gene sequences in *Photorhabdus* subspecies and some of 67 strains used in this study. Maximum parsimony bootstrap clade frequency values ≥70% mapped above the internal nodes

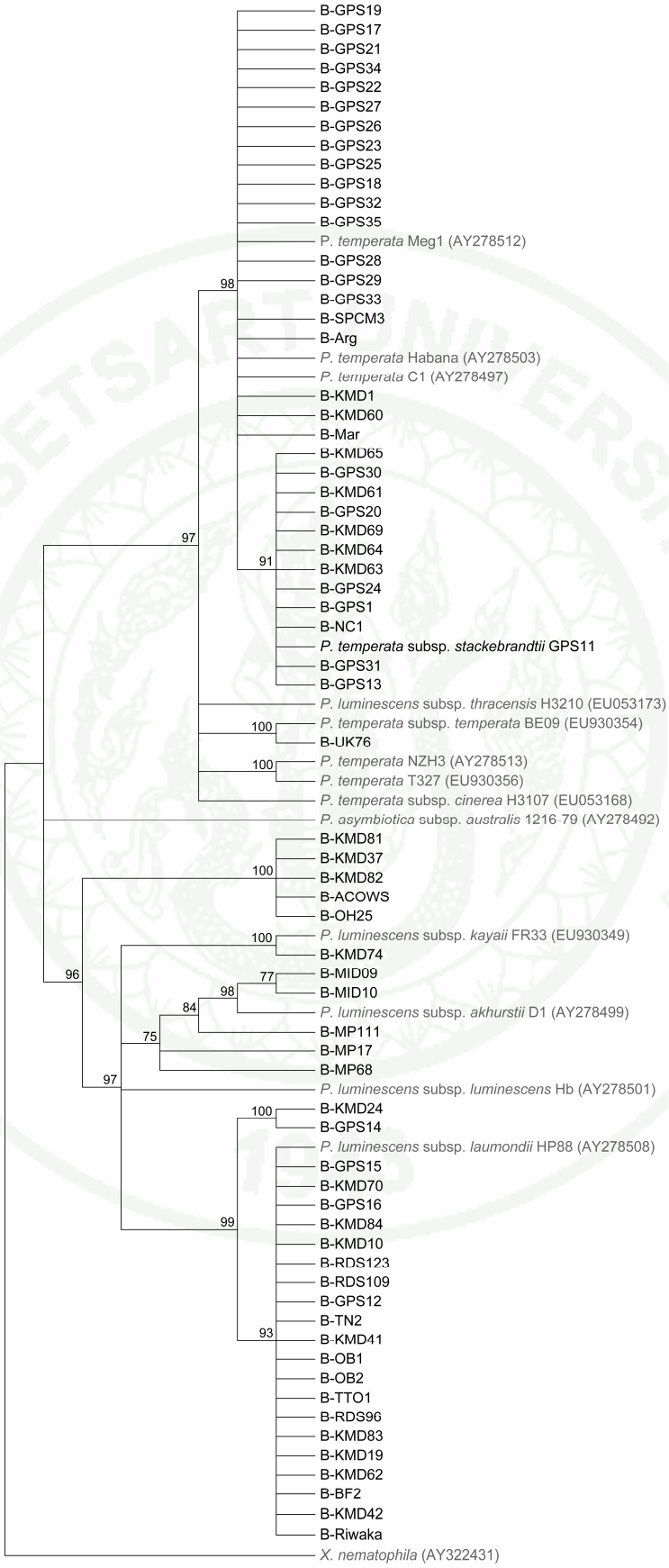
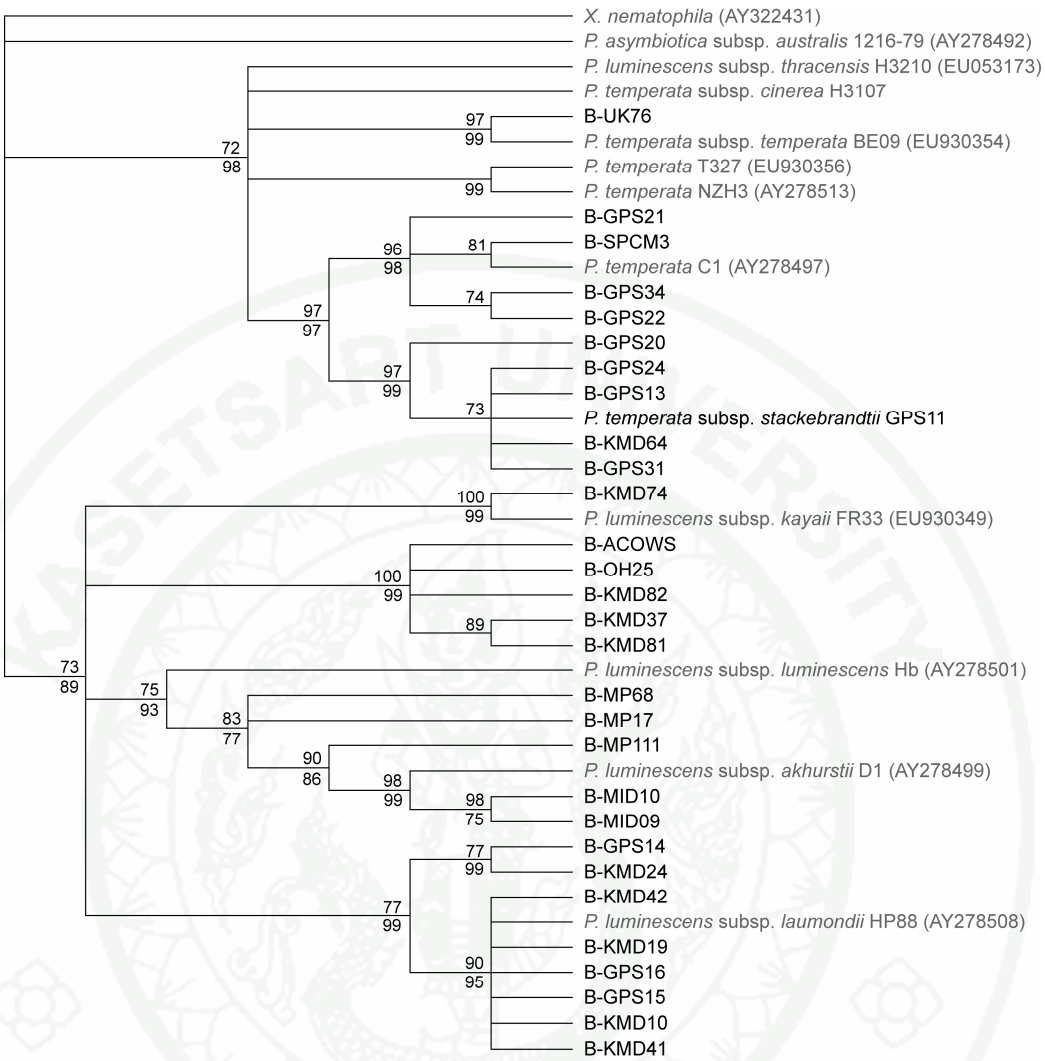


Figure 11 Maximum likelihood tree inferred from gyrase B gene sequences in *Photorhabdus* subspecies and some of 67 strains used in this study. Maximum likelihood bootstrap values ≥70% shown above the internal nodes. Maximum parsimony bootstrap clade frequency values ≥70% mapped below the internal nodes



2.3 Cophylogenetic analysis of *Heterorhabditis* and *Photorhabdus*:

While such outcomes may challenge the well established concept of one-to-one species association between entomopathogenic nematodes and their symbiotic bacteria, cophylogenetic tests using ParaFit rejected the null hypothesis that *Photorhabdus* bacteria and *Heterorhabditis* nematodes have evolved independently ($p = 0.001$), indicating there is significant cophylogenetic history. Further, although ParaFit detected a significant correlation between the nematode and bacteria trees (ParaFitGlobal = 0.001), the test of individual links indicated that not all *Heterorhabditis-Photorhabdus* associations equally contribute to the global fit between the two phylogenies.

The results (Table 10) showed that some individual associations, such as those between *H. zealandica*, *H. indica* and *H. megidis* strains and their symbiotic bacteria ($p < 0.02$) significantly contribute to the overall cophylogenetic structure, which can also be observed visually by comparison of the *Heterorhabditis-Photorhabdus* phylogenies (Fig. 12). However, other individual associations including those between the symbiotic bacteria and their respective nematode hosts *H. bacteriophora* and *H. georgiana* strains appear not to contribute significantly to the overall cophylogenetic structure. These results indicated that while *Heterorhabditis* and *Photorhabdus* phylogenies were consistent with a global cophylogenetic pattern, there were also cases of apparent mismatch between the two trees.

One explanation for the mismatch of associations between *H. bacteriophora* strains and the symbiotic bacteria may be the wide distribution of this species (Boemare and Akhurst, 2006), which may present more host switching opportunities to *Photorhabdus* bacteria. The possibility of host switching in *Heterorhabditis-Photorhabdus* association has been implied in previous studies that although *H. bacteriophora* nematodes grown on the symbiotic bacteria of *H. megidis* typically produce germfree infective juveniles, occasionally some *H. bacteriophora* infective juveniles can retain the symbiotic bacterium (Gerritsen *et al*., 1998).

Grewal *et al*. (1997) showed that *Steinernema scapterisci* Nguyen and Smart, 1990 retained and reproduced *X. nematophila*, the bacterial symbiont of *S. carpocapsae*. Indeed, studies on other symbiotic systems have shown that host switching has evolved through evolutionary processes that can confound cophylogenetic patterns (Charleston and Robertson, 2002; Weckstein, 2004; Huyse and Volckaert, 2005). In addition, the result showed that the potentially new *P. temperata* subspecies associate with both *H. georgiana* and *H. bacteriophora*. This may be due to the closer phylogenetic relationship of *H. georgiana* with *H. bacteriophora* than with other species (Fig. 12) as it has been previously suggested that association with closely related host species by the same symbiont may be parsimonious in the cophylogenetic history (Hugot *et al*., 2001). Taken together, the cophylogenetic analysis revealed a significant coevolutionary relationship between *Photorhabdus-Heterorhabditis* association despite few mismatches.

Table 10 The results of ParaFit analysis showing the cophylogenetic relationship between *Heterorhabditis* and *Photorhabdus*

	Bacterial strain	Nematode strain	Probability
	B-MP68	N-MP68	0.00100*
	B-D1	N-D1	0.00100*
	B-MID10	N-MID10	0.00100*
	B-MP17	N-MP17	0.00100*
	B-MP111	N-MP111	0.00100*
	B-NZH3	N-NZH3	0.00400*
	B-HF85	N-HF85	0.00801*
	B-UK76	N-UK76	0.00901*
	B-K122	N-K122	0.01902*
	B-GPS15	N-GPS15	0.78078
	B-RDS96	N-RDS96	0.79279
	B-KMD84	N-KMD84	0.80581
	B-OB2	N-OB2	0.73574
	B-KMD83	N-KMD83	0.78879
	B-KMD81	N-KMD81	0.57357
	B-KMD82	N-KMD82	0.46146
	B-OH25	N-OH25	0.44444
	B-ACOWS	N-ACOWS	0.44044
	B-SPCM3	N-SPCM3	0.30430
	B-KMD60	N-KMD60	0.25245
	B-GPS11	N-GPS11	0.24825
	B-KMD65	N-KMD65	0.22422
	B-GPS30	N-GPS30	0.31231

* significance at ≤ 0.02.

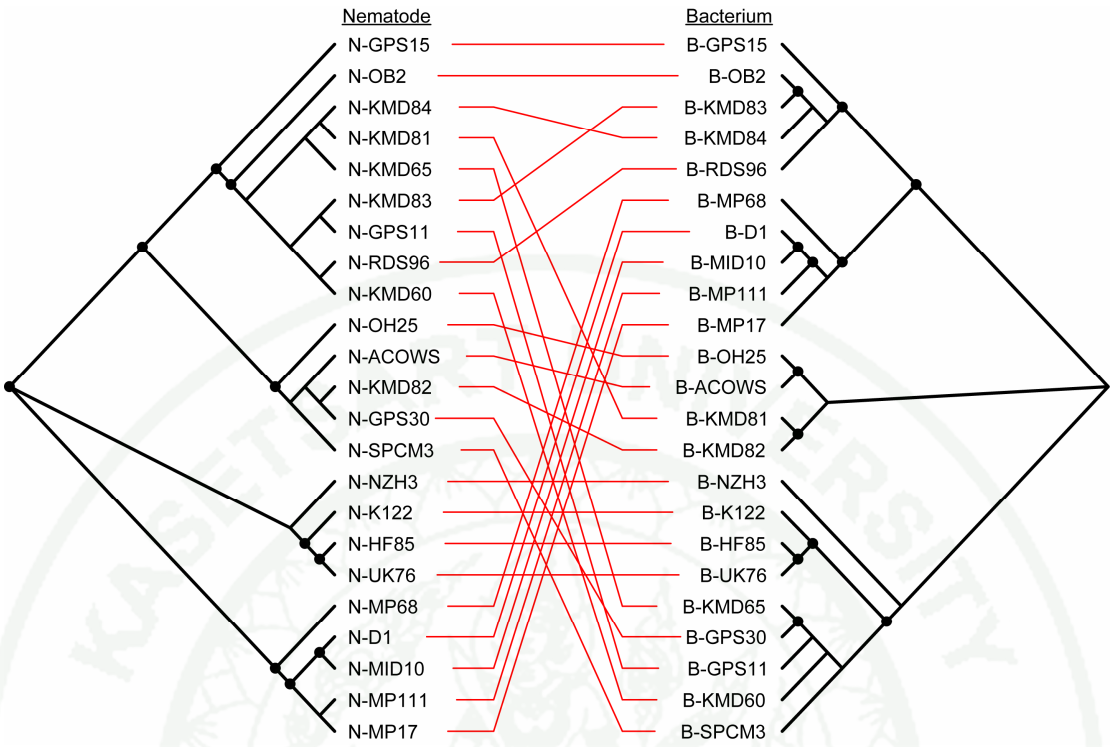


Figure 12 Phylogenetic trees for *Heterorhabditis* (left) and *Photorhabdus* (right) strains with information on their association. The nematode tree is inferred from the Internal Transcribed Spacer (ITS) sequences and the bacterial tree is from gyrase B gene sequences. Cospeciating nodes as inferred from the TreeMap are indicated with closed circles

3. Identification of new Thai entomopathogenice nematodes

3.1 Morphological characterization

Steinernema:

One strain of *Steinernema* (MP10), was isolated from the soil that was collected from Chumphon province, Thailand. The position of soil sampling site including important information are shown in the Appendix Table 3. The morphological characterization was used to examine and confirm the species of MP10 strain. The result indicated that this nematode strain, MP10, is a new species and it was described as *Steinernema minuta* sp. nov. (Maneesakorn *et al*., 2010)

Descriptions:

First generation male

Body slender, ventrally curved posterior, J shaped when heat-relaxed. Cuticle smooth with very faint transverse striae. Lateral field and phasmids inconspicuous. Head truncate to slightly rounded continuous with body. Six lips fused at base, each lip bearing a labial papilla, 4 cephalic papillae, amphidiales small, distinct, located laterally between labial and cephalic circle of papillae. Stoma reduced, short, shallow, cheilorhabdions distinct. Mouth funnel-shaped. Oesophagus muscular, procorpus cylindrical, metacorpus slightly swollen, non-valvate, isthmus distinct, basal bulb pyriform, valve distinct. Nerve ring usually surrounding isthmus or anterior part of basal bulb. Cardia prominent and protruding into intestinal lumen. Excretory pore opening circular at anterior half of oesophagus. Testis single, reflexed, distance from anterior end about 350 µm, consisting of germinal growth zone leading to seminal vesicle. Vas deferens with inconspicuous walls. Spicules paired, symmetrical, strongly curved, ochre-brown in color, head (manubrium) length (46.3 µm), longer than wide (27.3 µm), calomus (shaft) distinct, lamina with rostrum or retinaculum and two internal ribs, velum present. Gubernaculum arcuate, ca 77%

of spicule length, boat-shaped in lateral view, swollen in middle with ventrally curved knob at proximal end, cuneus long, bifurcate, not reaching end of corpus, corpus separated posteriorly. Tail conoid with an inconspicuous mucron (Figs. 13, 15). Single ventral precloacal papilla and nine pairs of genital papillae. The latter are arranged as follows: five pairs subventral pre-anal, one pair lateral, one pair subdorsal, and two pairs terminal (Fig. 14A).

Second generation male

Similar to the first generation male, but smaller in the body length, much smaller in width and other measurements (Table 11). Tail mucron prominent (3-4 µm long) (Fig. 15).

First generation female

Body robust, C-shaped when heat-killed. Cuticle appears smooth under light microscrope. Head broadly rounded. SEM *en face* and lateral view (Figs. 14C, D) with 6 pointed labial papillae and 4 cephalic papillae, amphids inconspicuous. Mouth funnel-shaped, stoma shallow, cheilorhabdions distinct. Oesophagus with cylindrical procorpus, metacorpus slightly swollen and non-valvate, isthmus indistinct, basal bulb pyriform. Excretory pore cuticularised and excretory gland swollen. Cardia prominent, protruding into intestinal lumen. Amphidelphic, gonad reflexed and filled with eggs. Vulva a transverse slit, protruding from body with a double epiptygma near the middle of body. Vagina short, oblique with muscular walls. Body slightly enlarged postanally. Tail shorter than anal body width, mucron always present (Figs. 13 and 16).

Second generation female

Similar to first generation female in general morphology, but smaller (Table 11). Vulva symmetrical and almost slightly protruding with a double epiptygma.

Excretory pore position more anterior. Conical and sharply pointed tail, postanal not swollen (Fig. 16).

Third stage infective juvenile

Body almost straight when heat killed, tapering regularly from base of oesophagus to anterior end and from anus to terminus. Mouth and anus closed. Cuticle striated. In SEM *en face* view (Fig. 13B), head annulated with 6 labial papillae and 4 cephalic papillae, amphidial apertures inconspicuous. Oesophagus long, narrow, isthmus distinct, surrounded by nerve ring, basal bulb elongate with valve. Cardia prominent. Excretory pore located at anterior 36% of oesophagus. Hemizonid distinct, anterior to basal bulb. Tail long, constricted in hyaline region, especially on dorsal side. Hyaline portion occupying about 49% of tail length (Figs. 13 and 17).

Type host and locality:

The natural host is unknown. *Steinernema minuta* sp. nov. was collected from a soil sample under pine tree (10°23.060' N, 99°17.04' E, elevation 36) at Amphor Mueang, Chumphon province in southern Thailand in April 2007. Soil in this area is sandy-loam and acidic (~pH 4.4).

Type material:

Holotype, first generation male, five first generation paratype males, five first generation paratype females and five paratype third stage infective juveniles were deposited in the USDA Nematode Collection at Beltsville, Maryland. Five first generation paratype males, five first generation paratype females and five paratype third stage infective juveniles were deposited in the University of California Nematode Collection in Davis, California, USA. Five first generation paratype males, five first generation paratype females and five paratype third stage infective juveniles

were deposited in the Department of Entomology, Kasetsart University, Bangkok, Thailand.

Differential diagnosis:

Steinernema minuta sp. nov. belongs to the group of species related to *S. tami* which are in the "small IJ" group with IJ body length of less than 600 µm. This new species can be distinguished from the species of this group by morphometric traits and several important morphological features of the IJ, first generation male, and female. The *S. minuta* sp. nov. IJs can be separated from *S. tami* by the value of D (35-46 vs 28-34%) and also by the length of pharynx (85-99 vs 110-123 µm) and from *S. eapokense* by the location of excretory pore from the anterior end (31-36 vs 26-31 µm). The value of SW of the first generation male in *S. minuta* sp. nov. can be separated from *S. cumgarense* (134-231 vs 250-313%) (Table 12). Vulval lips of the first generation female slightly protruding with a double epiptygma in *S. minuta* sp. nov. but has not protruding and no epiptygma in *S. tami*. The new species also differs from *S. siamkayai* which has feather shaped epiptygma. Furthermore, there is no postanal swelling in the second generation female of *S. minuta* sp. nov. which is present in both *S. siamkayai* and *S. tami*.

3.2 Cross hybridization test:

Cross hybridization tests between males and females of *S. minuta* sp. nov. and *S. siamkayai* yielded no progeny. In controls, offspring were produced in all the self-crosses. Unfortunately, that the other closely-related species *S. tami*, *S. cumgarense* and *S. eapokense* were not available for this study.

Table 11 Morphometrics of *Steinernema minuta* sp. nov. from Thailand, measurements are in µm and in the form of mean ± SD (range) (n = 20)

Character	Male paratypes		Female paratypes		IJ paratypes
	1st gen	2nd gen	1st gen	2nd gen	
L (body length)	1566.5 ± 100.1 (1393.8 – 1737.2)	815.6 ± 54.5 (737.3 – 909.5)	6390.3 ± 763 (5252 – 8039.6)	1599.8 ± 144.6 (1414 – 1979.6)	403 ± 22.2 (363.6 – 454.5)
MBD (maximum body diam.)	123.9 ± 10.3 (103.7 – 150.3)	57 ± 5.9 (47.7 – 67.7)	215.2 ± 20.1 (184.9 – 258.4)	100.9 ± 15.3 (80.4 – 139.4)	21 ± 1 (19.6 – 22.7)
EP (distance from anterior end to excretory pore)	71 ± 4.6 (65 – 81)	54.1 ± 3.9 (45.3 – 61.9)	83 ± 7.6 (67.4 – 96)	53.2 ± 5.6 (39.8 – 62.5)	33.8 ± 1.4 (31.3 – 36.3)
NR (distance from anterior end to nerve ring)	102.5 ± 7.2 (87.1 – 115.9)	84.4 ± 4 (78.7 – 92.7)	154.6 ± 10.1 (139.9 – 171.3)	102.4 ± 6.4 (89.3 – 113.3)	66.3 ± 2.7 (61.1 – 73.2)
ES (pharynx length)	140.1 ± 6.7 (122.3 – 151.7)	114.2 ± 3.1 (108.1 – 118.6)	209.3 ± 13 (190.4 – 234.2)	144.8 ± 6.5 (131.3 – 155.3)	94.4 ± 3.4 (84.9 – 99.2)
T (tail length)	24.7 ± 1.9 (21.3 – 27.8)	16.8 ± 1.3 (14.3 – 19.1)	23.5 ± 4.3 (18.3 – 35.4)	26.7 ± 3.1 (20.9 – 31)	40.9 ± 2.6 (36.7 – 46.5)
ABD (anal body diam.)	40.2 ± 4 (31.9 – 46.4)	31 ± 3.5 (22.7 – 35.1)	68 ± 7.9 (55.7 – 83.4)	34.8 ± 5 (24.7 – 44.7)	11.5 ± 0.6 (10.4 – 12.9)
a (L/MBD)	-	-	-	-	19.2 ± 1.1 (17.4 – 21.6)
B (L/ES)	-	-	-	-	4.3 ± 0.3 (3.9 – 4.9)
c (L/T)	-	-	-	-	9.9 ± 0.8 (8.5 – 11.2)
D% (EP/ES x 100)	50.7 ± 3.4 (46.7 – 61.2)	47.4 ± 3.7 (40.7 – 54.4)	39.7 ± 3.7 (32.2 – 46.2)	36.8 ± 4 (28.7 – 45.7)	35.8 ± 1.4 (33.3 – 39.1)
E% (EP/T x 100)	-	-	-	-	82.8 ± 5 (76.4 – 94.6)
SL (Spicule length)	75.4 ± 4 (67.7 – 83.6)	64.9 ± 5.2 (57 – 78.4)	-	-	-
SW (Spicule width)	11.5 ± 1.2 (8.4 – 13.2)	9.5 ± 1.4 (7.8 – 12.9)	-	-	-
GL (Gubernaculum length)	58.1 ± 4.2 (49.6 – 65.9)	46.3 ± 5.1 (37.3 – 53.8)	-	-	-
GW (Gubernaculum width)	6.4 ± 0.7 (5.2 – 7.5)	5.4 ± 0.9 (3.7 – 6.7)	-	-	-
SW% (SL/ABD x100)	189.1 ± 20.9 (156.3 – 232)	211.9 ± 27.3 (171.5 – 260.1)	-	-	-
GS% (GL/SL x 100)	77.2 ± 6 (66.1 – 87.1)	71.6 ± 7.4 (59.2 – 85.1)	-	-	-
H% (H/T*100)	-	-	-	-	49.4 ± 4.8 (37.9 – 57.9)

Table 12 Comparison of infective juvenile and first generation male and female of *Steinernema minuta* sp. nov. with the other similar species by morphometric and morphology. (All measurements are in µm and in the form of mean (range).

Species	Infective juvenile							First generation male					First generation female		
	L	MBD	Tail	EP	ES	D%	E%	SL	GL	D%	SW%	GS%	M	EPI	VL
S. minuta sp. nov.	403 (364-455)	21 (19-24)	40 (35-47)	34 (31-36)	95 (85- 99)	40 (35-46)	93 (73-117)	79 (71-95)	61 (52-73)	45 (41-52)	185 (134-231)	77 (64-94)	P	P	SP
S. tami	530 (400-600)	23 (19-29)	50 (42-57)	36 (34-41)	117 (110-123)	31 (28-34)	73 (67-86)	77 (71-84)	48 (38-55)	44 (30-60)	200 (140-300)	62 (-)	P	A	NP
S. siamkayai	446 (398-495)	21 (18-24)	36 (31-41)	35 (29-38)	95 (80-107)	37 (31-43)	96 (85-112)	78 (75-80)	54 (47-65)	42 (35-49)	170 (140-220)	70 (60-80)	P	P	PR
S. cumgarense	402 (384-432)	22 (21-25)	36 (34-38)	35 (33-38)	86 (80-91)	41 (38-43)	99 (87-103)	76 (68-81)	58 (48-66)	45 (35-52)	277 (250-313)	76 (71-83)	P	P	PR
S. eapokense	402 (370-434)	19 (18-21)	39 (35-45)	29 (26-31)	84 (79-88)	34 (32-37)	73 (66-80)	69 (63-74)	50 (47-56)	43 (37-48)	246 (221-282)	73 (69-80)	P	P	PR

L = length; MBD = maximum body diameter; EP = excretory pore from the anterior end; ES = pharynx length; D% = EP/ES x 100; E% = EP/T x 100; SL = spicule length; GL = gubernaculum length; SW% = SL/anal body width x 100; GS% = GL/SL x 100; M = mucron; EPI = epiptygma; VL = vulva; A = absent; P = present; PR = protruding; NP = not protruding; SP = slightly protruding.

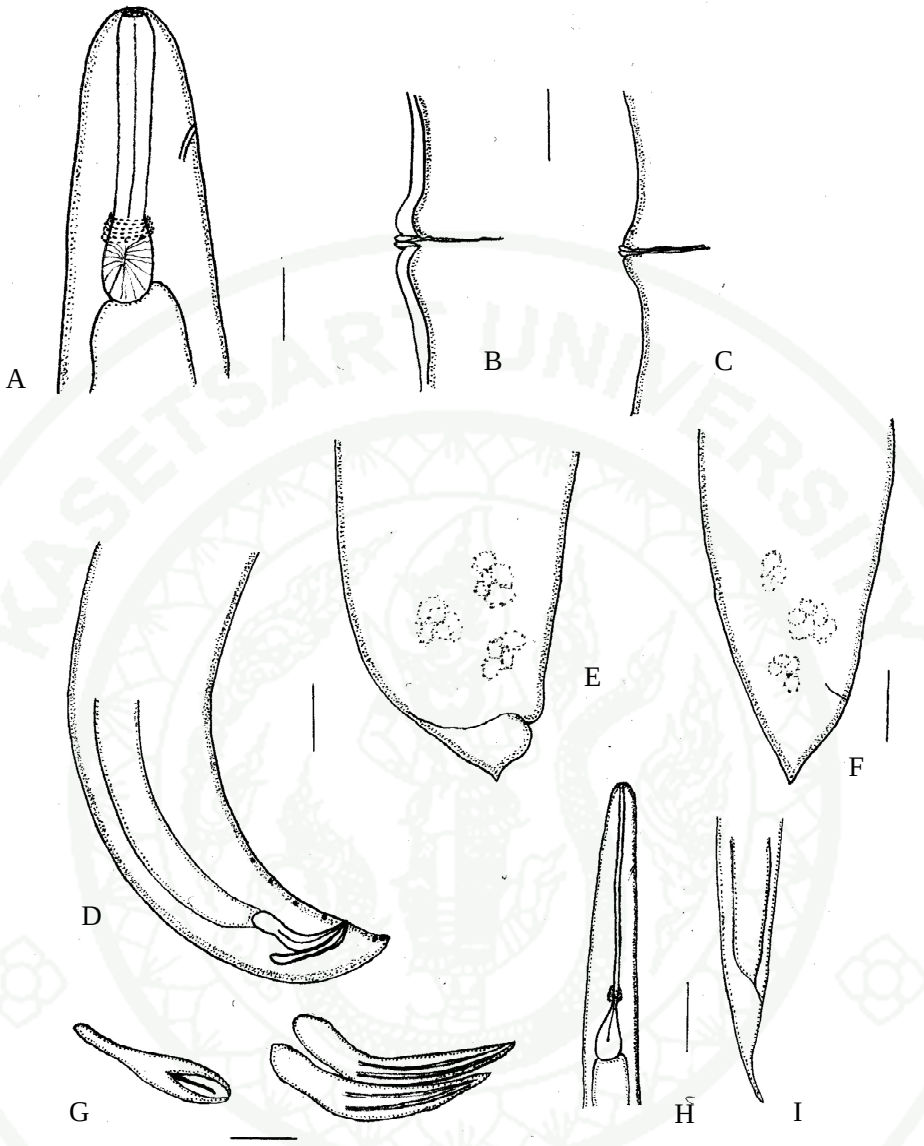


Figure 13 *Steinernema minuta* sp. nov. A: Anterior of first generation female; B and C: Vulva of first and second generation female; D: Posterior of first generation male; E and F: Posterior of first and second generation female; G: Spicule and gubernaculum of first generation male; H and I: Anterior and posterior of third stage infective juvenile. Scale-bar: A, D, F: 50 µm; B, C, E, G, H, I: 25 µm

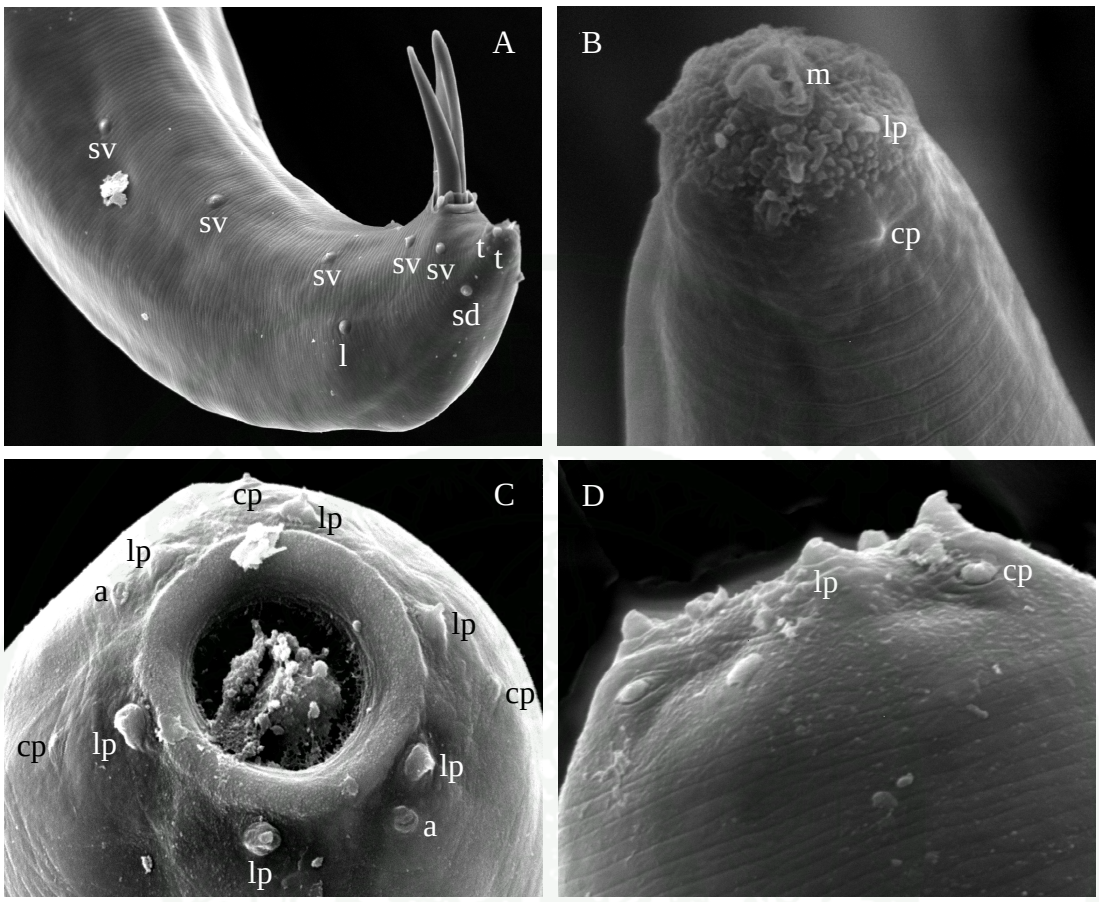


Figure 14 SEM of *Steinernema minuta* sp. nov. A: Posterior of first generation male showing five pairs subventral (sv), one pair subdorsal (sd), one pair lateral (l), two pairs terminal (t) and spicules; B: *En face* view of third stage infective juvenile showing mouth (m), labial (lp) and cephalic (cp) papillae; C and D: *En face* and lateral view of first generation female showing four cephalic (cp), six labial papillae (lp) and two amphids (a)

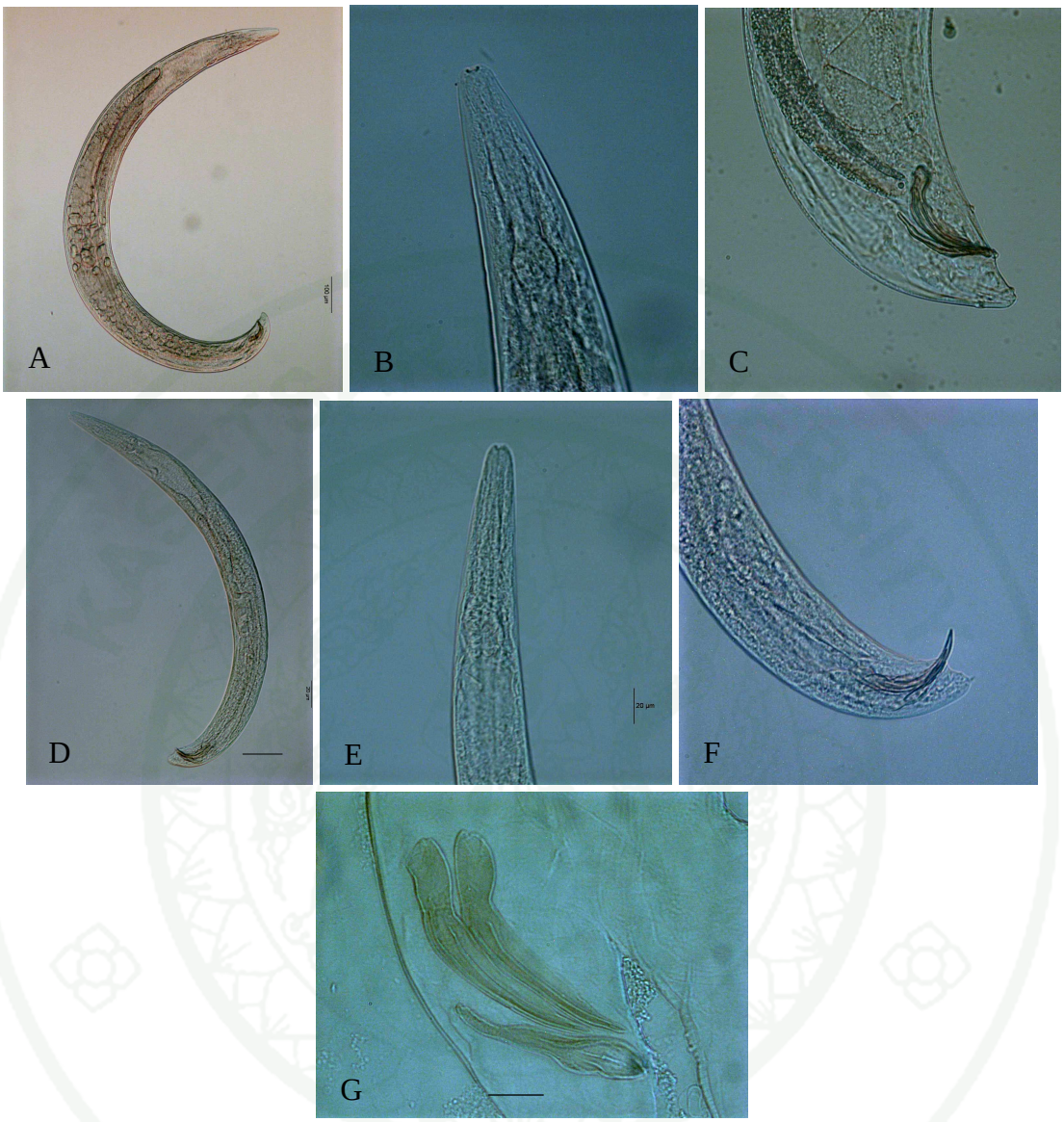


Figure 15 *Steinernema minuta* sp. nov. A: First generation male entire body; B: Anterior of first generation male; C: Posterior of first generation male; D: Second generation male entire body; E: Anterior of second generation male; F: Posterior of second generation male; G: Spicules and gubernaculum of first generation male. Scale-bar: A: 100 µm; B, C, E, F: 20 µm; D: 60 µm; G: 10 µm

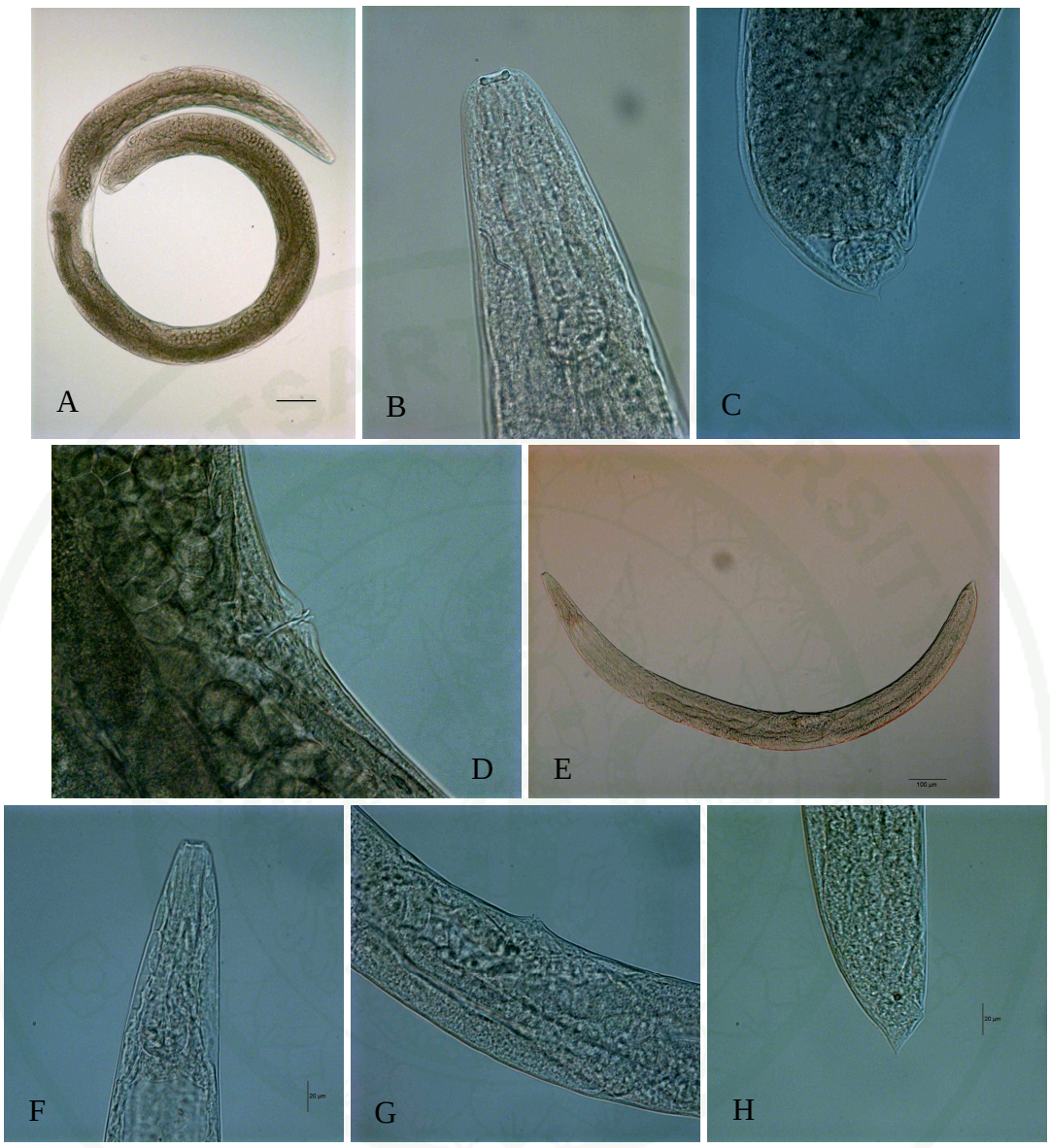


Figure 16 *Steinernema minuta* sp. nov. A: First generation female entire body; B: Anterior of first generation female; C: Posterior of first generation female; D: Vulva of first generation female; E: Second generation female entire body; F: Anterior of second generation female; G: Vulva of second generation female; H: Posterior of second generation female. Scale-bar: A: 80 µm; E: 100 µm; B-D, F-H: 20 µm

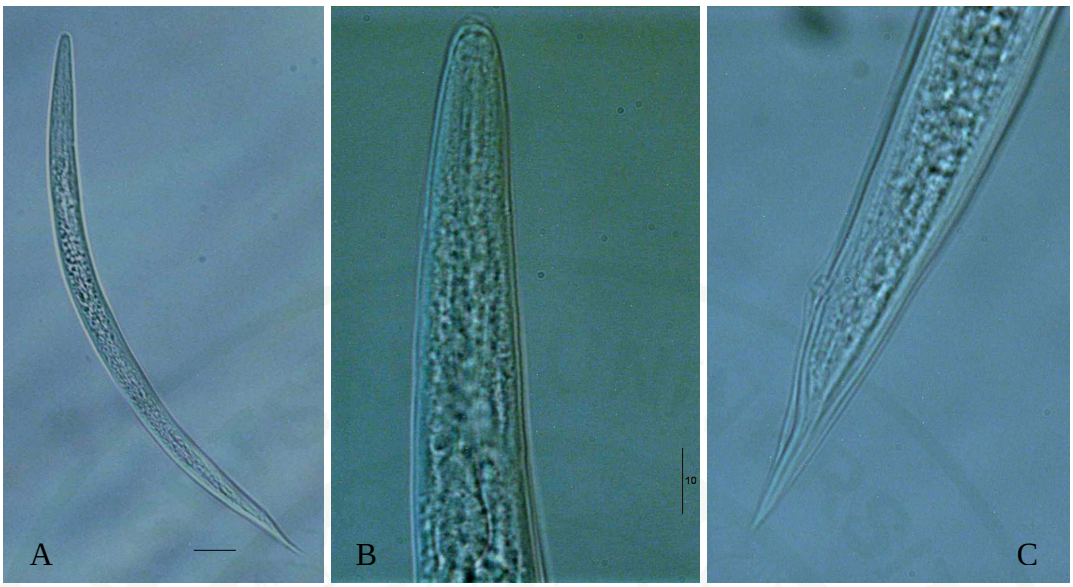


Figure 17 *Steinernema minuta* sp. nov. A: Entire body of third stage infective juvenile; B: Anterior of third stage infective juvenile; C: Posterior of third stage infective juvenile. Scale-bar: A: 60 µm; B and C: 10 µm

Heterorhabditis:

Three strains of *Heterorhabditis* (MP17, MP68 and MP111) were isolated from the soil at Khon Kaen, Kanchanaburi and Krabi provinces, respectively in Thailand. The position of soil sampling sites including important informations were shown in the Appendix Table 3. Preceding identification by molecular characteristics indicated that the nematode strains MP17 and MP111 were *H. indica* and MP68 may potentially represent a new species. Therefore, in this study, the *Heterorhabditis* strain MP17 was observed and studied by morphometric measurement (Table 13). SEM pictures on the important portions to characterize of three strains were also illustrated in Figures 18, 19, 20 and 21.

Table 13 Morphometrics of *Heterorhabditis indica* strain MP17 from Thailand, measurements are in µm and in the form of mean ± SD (range) (n = 20)

Character	Hermaphroditic female	2nd generation		IJ paratypes
		Female	Male	
L (body length)	2572.5 ± 346.4 (2040.2 – 3353.2)	1859.4 ± 154 (1595.8 – 2242.2)	908.9 ± 52.6 (740.9 – 980.2)	589.8 ± 24.2 (545.4 – 626.2)
MBD (maximum body diam.)	130.5 ± 20.5 (106.1 – 192.5)	111.9 ± 10.8 (95.5 – 135.4)	52.7 ± 2.9 (48.6 – 59.1)	21.4 ± 0.9 (19 – 22.4)
EP (distance from anterior end to excretory pore)	164.5 ± 12.6 (135.9 – 181.6)	114.5 ± 6.5 (99.4 – 124.3)	114.2 ± 8 (99.6 – 126.2)	100.2 ± 6.1 (94.3 – 120)
NR (distance from anterior end to nerve ring)	116.8 ± 9.4 (102.6 – 134.6)	80.9 ± 7.2 (66.7 – 90.4)	74.7 ± 4.6 (66.4 – 84.4)	84.8 ± 2.4 (80.5 – 88.8)
ES (pharynx length)	175.9 ± 16.6 (156 – 212.2)	122.2 ± 6.8 (112.9 – 139.3)	100.4 ± 6 (91.7 – 109.9)	120 ± 3.2 (113.2 – 127)
T (tail length with sheath)	96.6 ± 13.2 (77.2 – 132.5)	76.5 6.4 (64.5 – 85.1)	32.1 ± 3.2 (26 – 38.5)	108 ± 4.2 (100.7 – 115.4)
T (tail length without sheath)	-	-	-	86 ± 5.9 (76.5 – 97.1)
ABD (anal body diam.)	47.7 ± 5.7 (41.1 – 62.5)	32.6 ± 3 (27.6 – 38.9)	27.2 ± 1.8 (24.2 – 30.6)	14.6 ± 0.5 (13.7 – 15.6)
a (L/MBD)	-	-	-	27.6 ± 1.4 (25.8 – 30.5)
b (L/ES)	-	-	-	4.9 ± 0.2 (4.5 – 5.3)
c (L/T)	-	-	-	5.5 ± 0.4 (4.8 – 6.1)
D% (EP/ES x 100)	93.8 ± 5.8 (84.6 – 103.4)	93.8 ± 4 (85.2 – 101)	113.8 ± 6.7 (105.3 – 129.8)	83.5 ± 4.9 (76.3 – 99.8)
E% (EP/T x 100)	-	-	-	92.9 ± 7.4 (84.9 – 115.7)
SL (Spicule length)	-	-	41.2 ± 2.9 (37 – 45.8)	-
GL (Guberbnaculum length)	-	-	19.7 ± 2.5 (16.4 – 24.3)	-
SW% (SL/ABD x100)	-	-	152.2 ± 14.4 (125.1 – 174.7)	-
GS% (GL/SL x 100)	-	-	47.7 ± 4.5 (39.5 – 54.7)	-

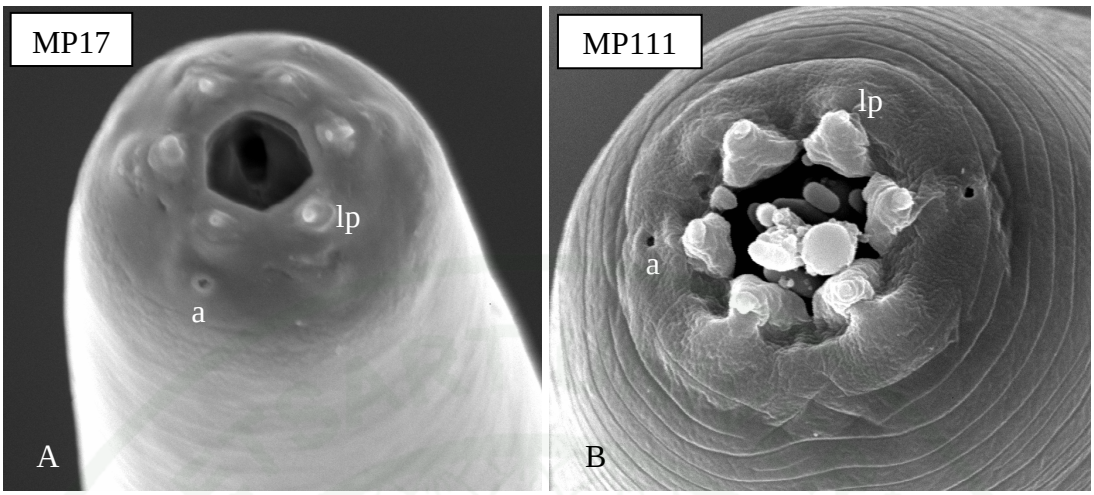


Figure 18 SEM of *Heterorhabditis indica* strains MP17 and MP111. A: *En face* of male strain MP17 and B: *En face* of a hermaphroditic female showing mouth opening, six labial papillae (lp) and amphids (a)

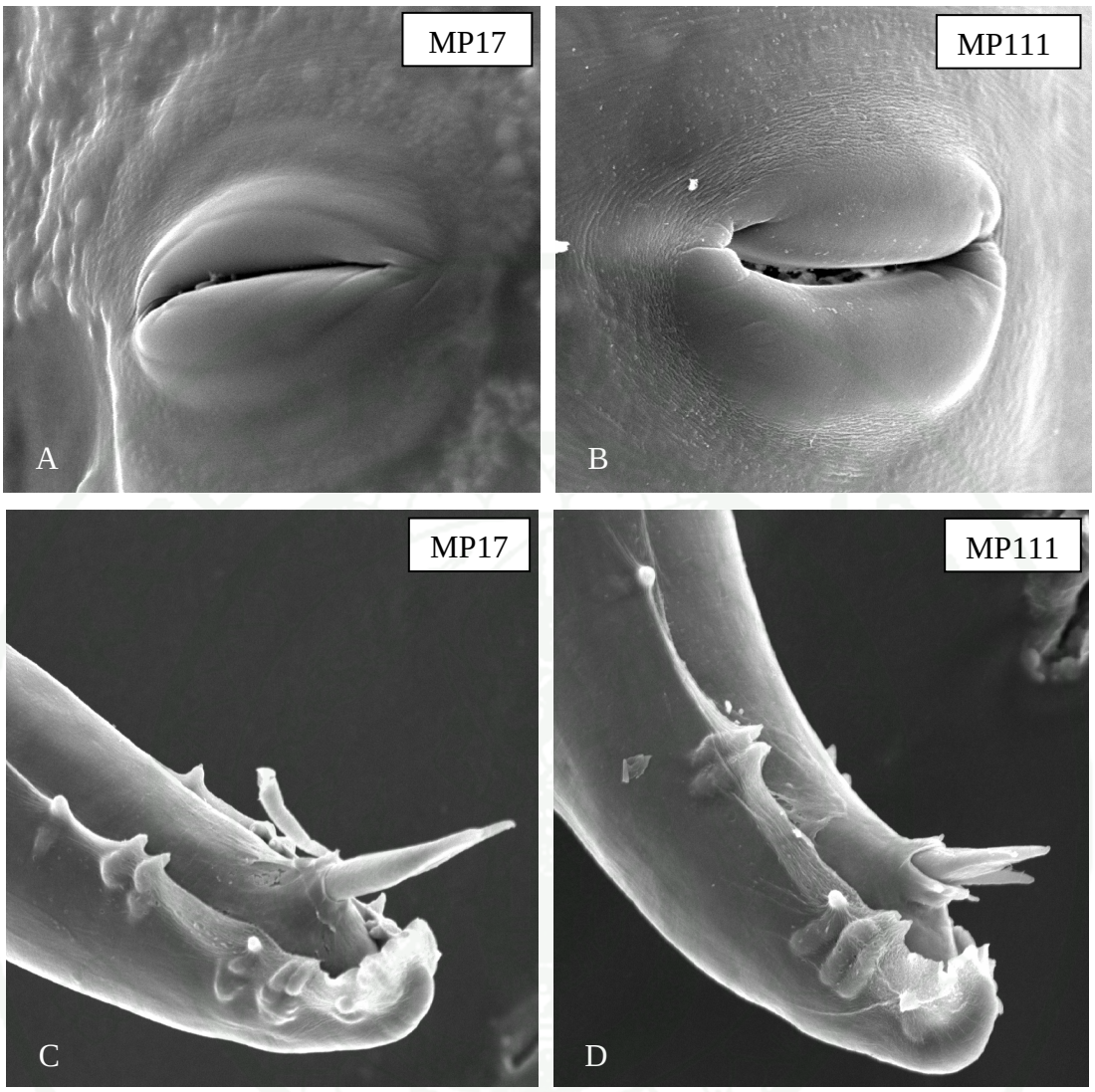


Figure 19 SEM of *Heterorhabditis indica* strains MP17 and MP111. A and B: Different feature vulvae of hermaphroditic female; C and D: Posterior of male showing protruding spicule and bursa with papillae

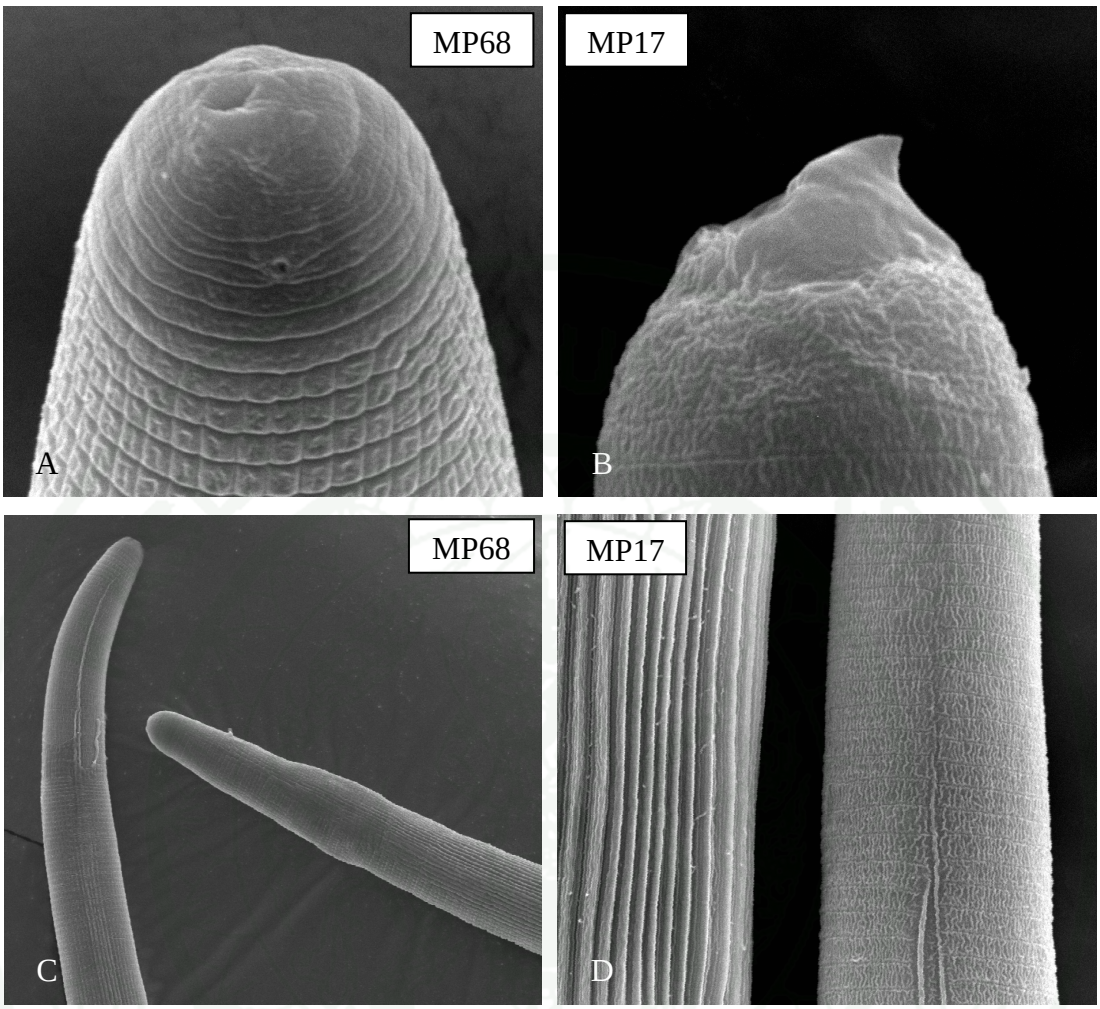


Figure 20 SEM of third stage infective juvenile of *Heterorhabditis* sp. strain MP68 and *Heterorhabditis indica* strain MP17. A: Anterior region showing head with six annules and amphid; B: Anterior region showing dorsal tooth; C and D: Portion of body showing tessellate pattern and longitudinal ridges and lateral field in anterior region with one ridge, then changing in two ridges

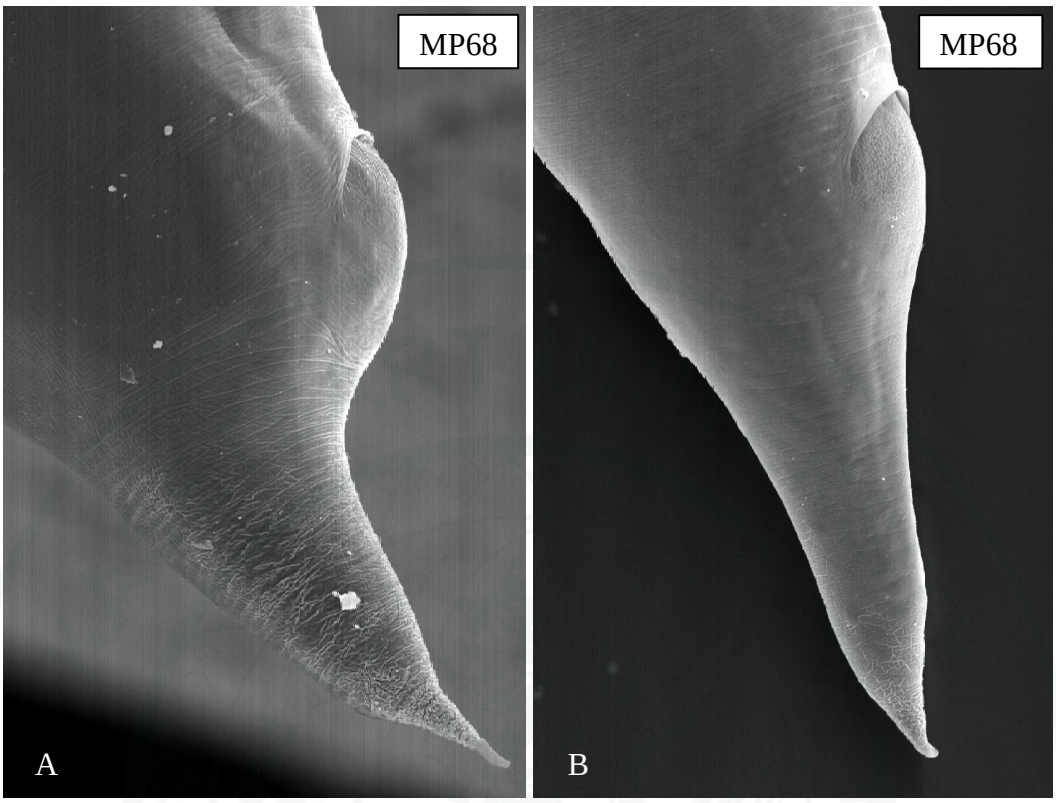


Figure 21 *Heterorhabditis* sp. strain MP68. Variation of hermaphroditic female tail (A and B)

4. Virulence of Thai EPN strains against second instar larva of the Japanese beetle

The three *Heterorhabditis* strains, *H. indica* strains MP17 and MP111, and *Heterorhabditis* sp. strain MP68 were more virulent than *S. minuta* strain MP10 against the Japanese beetle second instar larva in both experiments. Since there was no difference in the two trials, the data were pooled and reanalyzed. Among the *Heterorhabditis* strains, MP111 was the most virulent with LC_{50} of 136 IJs/larva (95% Confident Interval (CI): 77 - 199) which was lower than that of the MP68 (199, 95% CI: 123 - 287) and MP17 (254, 95% CI: 176 - 353). *S. minuta* MP10 was the least virulent with the mean LC_{50} of 501 IJs/larva (95% CI: 332 - 917). The MP111 strain was significantly more virulent than MP10 but not from MP17 and MP68 at 5 days after treatment (Fig. 22). These results collaborated the finding of Grewal *et al*. (2002) who reported that the pathogenicity of different nematode species and strains can vary substantially toward the white grub species. Power *et al*. (2009) reported that 127 IJs of *H. bacteriophora* strain GPS11 caused 50% mortality of the second instar *P. japonica* at 5 DAT in laboratory tests. Thus, the MP111 strain is similar in virulence to the commercially available GPS11 strain of *H. bacteriophora*.

The nematode strains also differed significantly in the speed of kill (Fig. 23). The LT_{50} values at 100 IJs/larva against the second instar *P. japonica* were lowest (7.4 days) for *H. indica* (MP111) followed by *Heterorhabditis* sp. (MP68), *H. indica* (MP17), and *S. minuta* (MP10), respectively. The MP111 strain killed the larvae significantly faster than MP10 but not from MP17 and MP68.

Significant differences in total mortality were recorded among the four nematode strains at 5 DAT ($P < 0.05$, $df = 3$, $F = 3.10$). At the concentration of 1,000 IJs/larva, *H. indica* (MP111) was the most virulent toward *P. japonica* resulting in 84.8% mortality and it differed significantly from all other strains. There was no significant difference between MP68 (72.2%) and MP17 (72.2%), but the two strains differed significantly from MP10 (36.7%). Furthermore, *H. indica* (MP111) caused the highest mortality of the Japanese beetle larva at each concentration (Fig. 24). There was a significant difference in larval mortality at 1,000 IJs/larva compared with

100 IJs/larva at 5 DAT ($P < 0.05$, $df = 4$, $F = 2.76$). At 100 IJs/larva the MP111, MP68 and MP17 strains caused 34.2, 21.5, and 19% mortality of the second instar *P. japonica* at 5 DAT. According to Koppenhöfer and Fuzy (2004), *H. bacteriophora* TF strain caused about 30% mortality of the second instar of *P. japonica* at 100 IJs/larva at 7 DAT. *S. minuta* strain MP10 at 100 IJs/larva caused only 16.5 and 43.9% mortality of the second instar of *P. japonica* at 5 and 15 DAT whereas Koppenhöfer and Fuzy (2004) reported that *S. scarabaei* at 20 IJs/larva caused about 43 and 65% mortality of the second instar *P. japonica* at 7 and 14 DAT, respectively. Therefore, *S. minuta* is significantly less virulent to *P. japonica* compared with *S. scarabaei*. There was no interaction between nematodes strains and concentrations ($P > 0.05$, $df = 15$, $F = 1.71$) (Fig. 25).

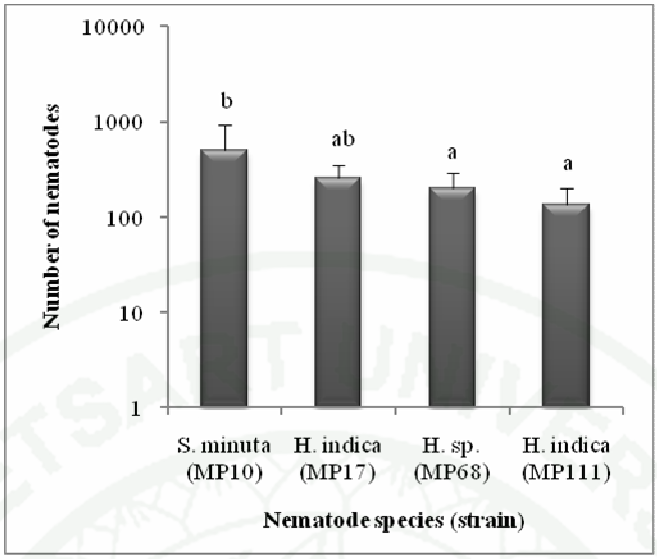


Figure 22 LC_{50} value of four Thai entomopathogenic nematode strains against the second instar larva of *Popillia japonica* at 5 DAT at 22 ± 1°C. Bars with different letters show significant differences among nematode strains

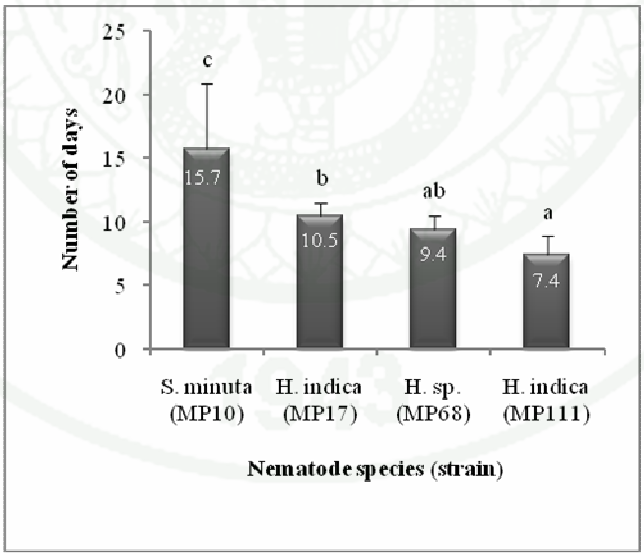


Figure 23 LT_{50} value of four Thai entomopathogenic nematode strains against the second instar larva of *Popillia japonica* at 100 IJs/larva at 22 ± 1°C. Bars with different letters show significant differences among nematode strains

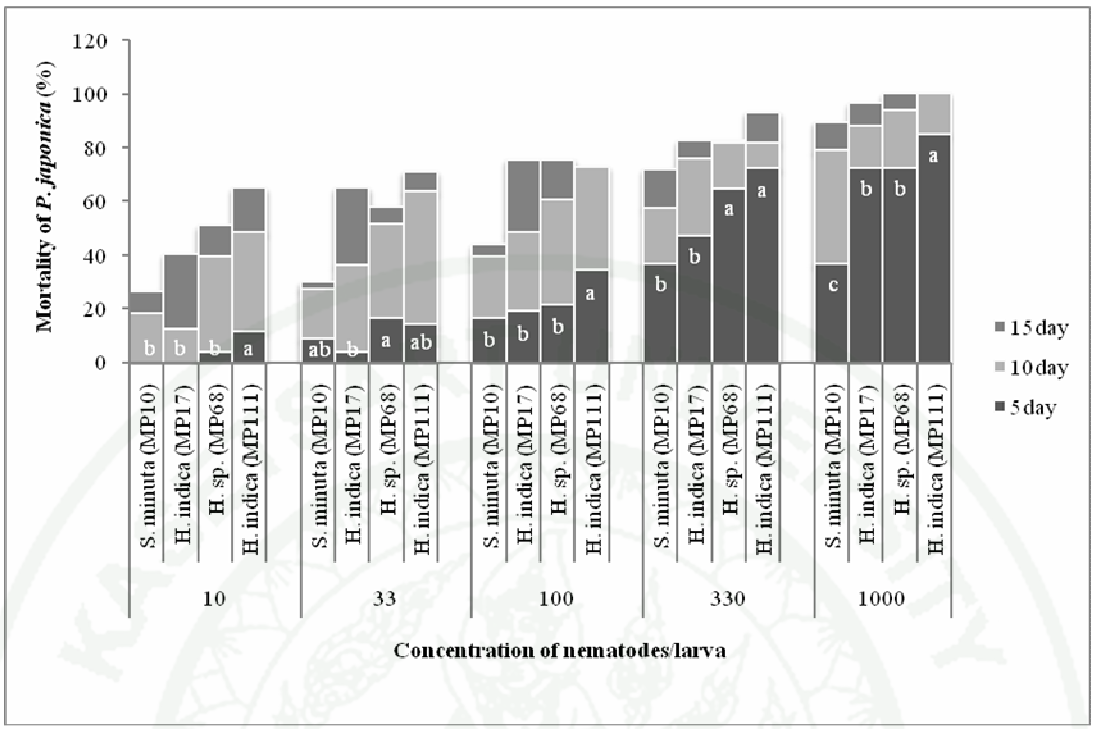


Figure 24 Percent mortality of the second instar larva of *Popillia japonica* caused by four Thai entomopathogenic nematode strains at different concentrations at 5, 10, and 15 days after treatment at 22 ± 1°C. Bars with different letters show significant differences among nematode strains at each concentration at 5 DAT. Data shown are corrected for control mortality

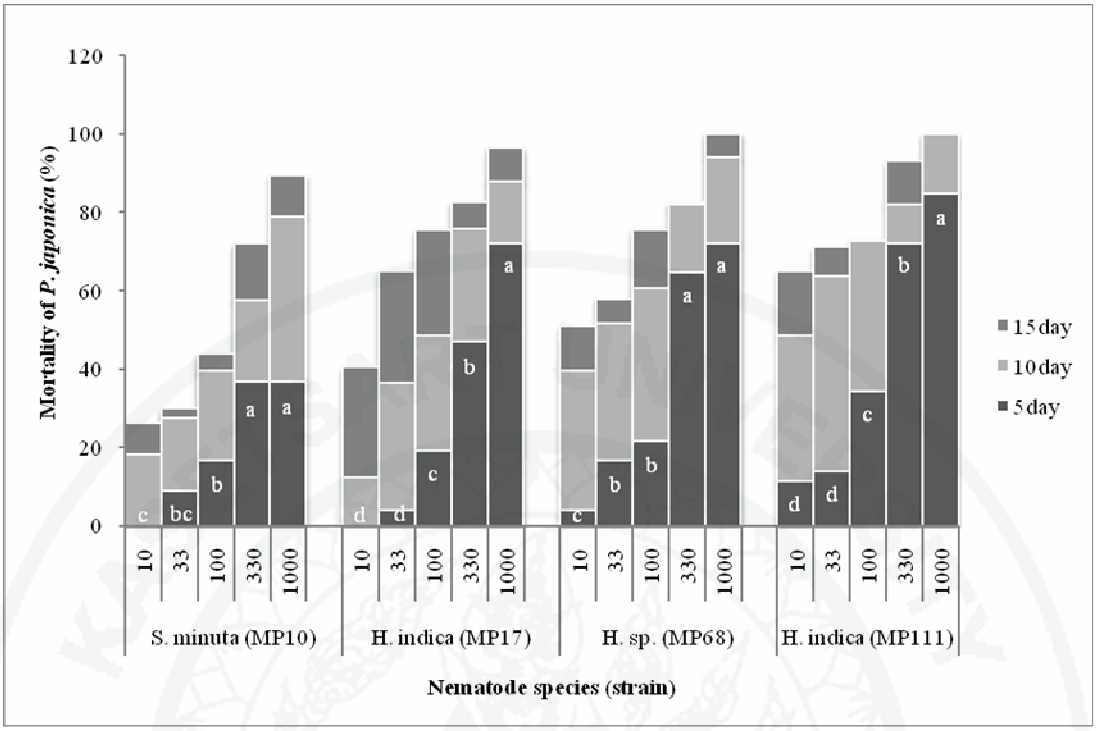


Figure 25 Percent mortality of the second instar larva of *Popillia japonica* at different concentrations of four Thai entomopathogenic nematode strains at 5, 10, and 15 days after treatment at 22 ± 1°C. Bars with different letters show significant differences among nematode concentrations for each nematode strain at 5 DAT. Data shown are corrected for control mortality

CONCLUSION AND RECOMMENDATION

Thailand is located in the tropical region where diverse soil types and various forests could be found. Hence, there is a good chance to discover new EPNs to be used as biological control agent in the future.

In this study, a new species of entomopathogenic nematode, *Steinernema* (MP10), was recovered from the soil at Chumphon province, and three new record of *Heterorhabditis indica* (MP17, MP111) and *Heterorhabditis* sp. (MP68) were isolated from the soil at Khon Kaen, Krabi and Kanchanaburi provinces Thailand, respectively.

Molecular techniques were employed to identify unknown *Steinernema* nematode revealed that the strains D60, D90 and D98 from USA are *S. carpocapsae* while FC48 from USA is potentially a new species. In addition, one *Steinernema* strain from Thailand (MP10) was described as *Steinernema minuta* based on their molecular and morphological characteristics.

All 67 strains of *Heterorhabditis* sp. were classified into 5 group species; 52 strains belong to *H. bacteriophora*, 9 strains belong to *H. georgiana*, 4 strains belong to *H. indica*, 1 strain belong to *H. megidis* and 1 strain closely related to *H. amazonensis*. MP17 and MP111 *Heterorhabditis* strains from Thailand were grouped with *H. indica* and MP68 *Heterorhabditis* strain may represent a new species.

The symbiotic bacteria of D60, D90 and D98 strains were *X. nematophila* where that of MP10 strain from Thailand was *X. stockiae*. Symbiotic bacteria of FC48 may represent a new species. The symbiotic bacteria of 67 strains of *Heterorhabditis* were divided into *P. temperata* and *P. luminescens*. However, symbiotic bacteria of KMD81, KMD37, KMD82, ACOWS and OH25 strains may belong to a new subspecies of *P. luminescens*.

Cophylogenetic analysis revealed a few mismatches in coevolutionary relationship between *Heterorhabditis* and *Photorhabdus*. This result provided insights into the diversity and cophylogeny of the nematode-bacterium partnership. The symbiotic bacteria of most currently recognized species and strains of *Heterorhabditis* have not been described. This is also true for species of *Steinernema* and *Xenorhabdus* species. Therefore, future research on the descriptions of entomopathogenic nematode species should also include descriptions of their bacterial partners from the same strain and vice versa to facilitate robust coevolutionary studies and to discover novel nematode and bacteria associations for using in biological control.

All Thai EPN strains are pathogenic to the Japanese beetle second instar larva and *H. indica* strain MP111 was the most virulent. Future studies should focus on field efficacy of this nematode strain against other insect pests in Thailand.

LITERATURE CITED

Adams, B.J., A.M. Burnell and T.O. Powers. 1998. A phylogenetic analysis of *Heterorhabditis* (Nemata:Rhabditidae) based on internal transcribed spacer 1 DNA sequence data. **J. Nematol.** 30: 22-29.

_____, A. Fodor, H.S. Koppenhöfer, E. Stackebrandt, S.P. Stock and M.G. Klein. 2006. Biodiversity and systematics of nematode-bacterium entomopathogens. **Biol. Cont.** 37: 32-49.

Akhurst, R.J. 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes, *Neoaplectana* and *Heterorhabditis*. **J. Gen. Microbiol.** 121: 303-309.

_____. 1983. Taxonomic study of *Xenorhabdus*, a genus of bacteria symbiotically associated with insect pathogenic nematodes. **Int. J. Syst. Bact.** 33: 38-45.

_____, N.E. Boemare. 1993. In: Validation list no. 47. Validation of publication of new names and new combinations previously effectively published outside the IJSB. **Int. J. Syst. Bact.** 43: 864-865.

_____, _____, P.H. Janssen, M.M. Peel, D.A. Alfredson and C.E. Beard. 2004. Taxonomy of Australian clinical isolates of the genus *Photorhabdus* and proposal of *Photorhabdus asymbiotica* subsp. *asymbiotica* subsp. nov. and *P. asymbiotica* subsp. *australis* subsp. nov. **Int. J. Syst. Evol. Microbiol.** 54: 1301-1310.

Aljanabi, S.M. and I. Martinez. 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. **Nucle. Acids Res.** 25: 4692-4693.

An, R. and P.S. Grewal. 2010. *Photorhabdus temperata* subsp. *stackebrandtii* subsp. nov. (Enterobacteriales: Enterobacteriaceae). **Curr Microbiol.** DOI 10.1007/s00284-010-9610-9.

Andoló, V., K.B. Nguyen and A. Moina. 2007. *Heterorhabditis amazonensis* n. sp. (Rhabditida: Heterorhabditidae) from Amazonas, Brazil. **Nematology.** 8: 853-867.

Anis, M., F. Shahina, A.P. Reid and M.A. Maqbool. 2000. Redescription of *Heterorhabditis indica*, Poinar et al., 1992 (Rhabditida: Heterorhabditidae) from Pakistan. **Pakistan. J. Nematol.** 12: 220-231.

Bedding, R.A. and R.J. Akhurst. 1975. A simple technique for the detection of insect parasitic rhabditid nematodes in soil. **Nematologica.** 21: 109-110.

Bird, A.F. and R.J. Akhurst. 1983. The nature of the intestinal vesicle in nematodes of the family Steinernematidae. **Int. J. Parasitol.** 13: 599-606.

Blaxter, M.L., P. De Ley, J.R. Garey, L.X. Liu, P. Scheldeman, A. Vierstraete, J.R. Vanflateran, L.Y. Mackey, M. Dorris, L.M. Frisse, J.T. Vida and W.K. Thomas. 1998. A molecular framework for the phylum Nematoda. **Nature.** 392: 71-75.

Boemare, N. 2002. **Biology, taxonomy and systematics of *Photorhabdus* and *Xenorhabdus***, pp. 35-56. *In* R. Gaugler, ed. Entomopathogenic Nematology. Wallingford, UK: CAB international.

_____ and R.J. Akhurst. 2006. The genera *Photorhabdus* and *Xenorhabdus*. **Prokaryotes.** 6: 451-494.

_____, C. Laumond and H. Mauleon. 1996. The entomopathogenic nematode-bacterium complex: biology, life cycle and vertebrate safety. **Bioc. Sci. Technol.** 6: 333-345.

Boemare, N.E., R.J. Akhurst and R.G. Mourantl. 1993. DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes and a proposal to transfer *Xenorhabdus luminescens* to a new genus. *Photorhabdus* gen. nov. **Int. J. Syst. Bacteriol.** 43: 249-255.

Bouin, M.S., J. Liu and R.E. Berry. 1999. Life variation and genetic structure of nematode populations. **Heredity.** 83: 253-259.

Bovien, P. 1937. Some types of association between nematodes and insects. Videnskabelige Meddelelser fra Dansk Naturhistorisk. **Forening.** 101: 1-114.

Charleston, M.A. and D.L. Robertson. 2002. Preferential host switching by primate lentiviruses can account for phylogenetic similarity with the primate phylogeny. **Syst. Biol.** 51: 528-535.

Chitwood, B.G. and M.B. Chitwood. 1937. **An Introduction to Nematology. Section 1, Anatomy**. Baltimore, MD, USA, Monumental Printing Co., 213 pp.

Ciche, T.A. and J.C. Ensign. 2003. For the insect pathogen *Photorhabdus luminescens*, which end of a nematode is out? **Appl. Environ. Microbiol.** 69: 1890-1897.

Constant, P., L. Marchay, M. Fischer-Le Saux, S. Briand and H. Mauleon. 1998. Natural occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Guadaloupe islands. **Fundam. Appl. Nematol.** 21: 667-672.

Courtney, W.D., D. Polley and V.L. Miller. 1955. TAF, an improved fixative in nematode techniques. **Plant. Dis. Rep.** 39: 570-571.

Dauga, C. 2002. Evolution of the *gyrB* gene and the molecular phylogeny of Enterobacteriaceae: a model molecule for molecular systematic studies. **Int. J. Syst. Evol. Microbiol.** 52: 531-547.

Delmas, J., F. Breysse, G. Devulder, J.P. Flandrois and M. Chomarat. 2006. Rapid identification of Enterobacteriaceae by sequencing DNA gyrase subunit B encoding gene. **Diagn. Microbiol. Infect Dis.** 55: 263-268.

Ehler, R.U. 2005. **Forum on safety and regulation**, pp. 107-114. *In*: P.S. Grewal, R.U. Ehlers and D.I. Shapiro-Ilan, eds. Nematodes as Biocontrol Agents. Wallingford, UK: CAB international.

Eisen, J.A. 1995. The RecA protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of RecAs and 16S rRNAs from the same species. **J. Mol. Evol.** 41: 1105-1123.

Filipjev, I.N. 1934. Miscellanea Nematologica 1. Eine neue Art der Gattung Neoaplectana Steiner nebst Bermerkungen über die systematische Stellung der letzteren. **Parazitologichesky.** 4: 229-240.

Fischer-Le Saux, M., V. Viallard, B. Brunel, P. Normand and N.E. Boemare. 1999. Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. **Int. J. Syst. Bact.** 49: 1645-1656.

Gardner, S.L., S.P. Stock and H.K. Kaya. 1994. A new species of *Heterorhabditis* from the Hawaiian islands. **J. Parasitol.** 80: 100-106.

Gaugler, R. 2002. **Entomopathogenic Nematology.** Wallingford, UK: CAB international. 388 pp.

George J., C.T. Redmond, R.N. Royalty and D.A. Potter. 2007. Residual effects of imidacloprid on Japanese beetle (Coleoptera: Scarabaeidae) oviposition, egg hatch, and larval viability in turf grass. **J. Econ. Entomol.** 2: 431-439.

Gerritsen, L.J.M., G.L. Wiegers and P.H. Smits. 1998. Pathogenicity of new combinations of *Heterorhabditis* spp. and *Photorhabdus luminescens* against *Galleria mellonella* and *Tipula oleracea*. **Biol. Control.** 13: 9-15.

Grewal, P.S., M. Matsuura and V. Converse. 1997. Mechanisms of specificity of association between the nematode *Steinernema scapterisci* and its symbiotic bacterium. **Parasitology.** 114: 483-488.

_____, R.U. Ehlers and D.I. Shapiro-Ilan. 2005. **Nematodes as Biocontrol Agents.** Wallingford, UK: CAB international. 505 pp.

_____, S.K. Grewal, V.S. Malik and M.G. Klein. 2002. Difference in susceptibility of introduced and native white grub species to entomopathogenic nematodes from various geographic localities. **Biol. Cont.** 24: 230-237.

_____, K.T. Power, S.K. Grewal, A. Suggars and S. Haupricht. 2004. Enhanced consistency in biological control of white grubs (Coleoptera: Scarabaeidae) with new strains of entomopathogenic nematodes. **Biol. Cont.** 30: 73-82.

Guindon, S. and O. Gasquel. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by Maximum Likelihood. **Syst. Biol.** 52: 696-704.

Hashmi, G., I. Glazer and R. Gaugler. 1996. Molecular comparisons of entomopathogenic nematodes using randomly amplified polymorphic DNA (RAPD) markers. **Fund. Appl. Nematol.** 19: 399-406.

Hazir, S., E. Stackebrandt, E. Lang, P. Schumann, R.U. Ehlers and N. Keskin. 2004. Two new subspecies of *Photorhabdus luminescens*, isolated from *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae): *Photorhabdus luminescens* subsp. *kayaii* subsp. nov. and *Photorhabdus luminescens* subsp. *thracensis* subsp. nov. **Syst. Appl. Microbiol.** 27: 36-42.

Henderson, C.F. and E.W. Tilton. 1955. Tests with acaricides against the brow wheat mite. **J. Econ. Entomol.** 48: 157-161.

Hominick, W.M. 2002. **Biogeography**, pp. 115-143. *In* R. Gaugler, ed. Entomopathogenic Nematology. Wallingford, UK: CAB international.

_____, A.P. Reid and B.R. Briscoe. 1995. Prevalence and habitat specificity of steinernematid and heterorhabditid nematodes isolated during soil surveys of the UK and the Netherlands. **J. Helminthol.** 69: 27-32.

_____,_____, D.A. Bohan and B.R. Briscoe. 1996. Entomopathogenic nematodes-biodiversity, geographical distribution and the convention on biological diversity. **Bioc. Sci. Technol.** 6: 317-331.

Huang, W.M. 1996. Bacterial diversity based on type II DNA topoisomerase genes. **Annu. Rev. Genet.** 30: 79-107.

Hugot, J.P., J.P. Gonzalez and C. Denys. 2001. Evolution of the old world Arenaviridae and their rodent hosts: generalized host-transfer or association by descent? **Infect. Genet. Evol.** 1: 13-20.

Huyse, T. and F.A.M. Volckaert. 2005. Comparing host and parasite phylogenies: Gyrodactylus flatworms jumping from goby to goby. **Syst. Biol.** 54: 710-718.

Kaya, H.K. and S.P. Stock. 1997. **Techniques in insect nematology**, pp. 281-324. *In* L.A. Lacey, ed. Manual of Techniques in Insect Pathology. Biological Techniques Series, Academic Press, San Diego.

Keane, T.M., C.J. Creevey, M.M. Pentony, T.J. Naughton and J.O. Mclnerney. 2006. Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. **BMC Evol. Biol.** 6: 29.

Klein, M.G. 1990. **Efficacy against soil-inhabiting insect pests**, pp. 195-214. *In* R. Gaugler and H.K. Kaya, eds. Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, Florida.

Koppenhöfer, A.M. and E.M. Fuzy. 2004. Effect of white grub developmental stage on susceptibility to entomopathogenic nematodes. **J. Econ. Entomol.** 97: 1842-1849.

_____, P.S. Grewal and E.M. Fuzy. 2006. Virulence of the entomopathogenic nematodes *Heterorhabditis bacteriophora*, *Heterorhabditis zealandica*, and *Steinernema scarabaei* against five white grub species of economic importance in turf grass in North America. **Biol. Cont.** 38: 397-404.

_____, M. Wilson, I. Brown, H.K. Kaya and R. Gaugler. 2000. Biological control agents for white grubs (Coleoptera: Scarabaeidae) in anticipation of the establishment of the Japanese beetle in California. **J. Econ. Entomol.** 93: 71-80.

Legendre, P., Y. Desdevises and E. Bazin. 2002. A statistical test for host-parasite coevolution. **Syst. Biol.** 51: 217-234.

Lengyel, K., E. Lang, A. Fodor, E. Szallas, P. Schumann and E. Stackebrandt. 2005a. Description of four novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus budapestensis* sp. nov., *Xenorhabdus ehlersii* sp. nov., *Xenorhabdus innexi* sp. nov., and *Xenorhabdus szentirmaii* sp. nov. **Syst. Appl. Microbiol.** 28: 115-122.

_____, _____, _____, _____, _____ and _____. 2005b. In: Validation list no. 104. Validation of publication of new names and new combinations previously effectively published outside the IJSEM. **Int. J. Syst. Bact.** 55: 1395-1397.

Lissemore, J.L., L.L. Lackner, G.D. Fedoriw and E.A. De Stasio. 2005. Isolation of *Caenorhabditis elegans* genomic DNA and detection of deletions in the unc-93 gene using PCR. **Biochem. Mol. Bio. Edu.** 33: 219-226.

Liu, J. and R.E. Berry. 1995. Determination of PCR conditions for RAPD analysis in entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae). **J. Invertebr. Pathol.** 65: 79-81.

_____ and _____. 1996a. *Heterorhabditis marelatus* n. sp. (Rhabditida: Heterorhabditidae) from Oregon. **J. Invertebr. Pathol.** 67: 48-54.

_____ and _____. 1996b. Phylogenetic analysis of the genus *Steinernema* by morphological characters and randomly amplified polymorphic DNA fragments. **Fundam. Appl. Nematol.** 19: 463-469.

_____, _____ and A.F. Moldenke. 1997. Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae, Steinernematidae) inferred from partial 18S rDNA gene sequences. **J. Invertebr. Pathol.** 69: 246-252.

_____, _____ and M.S. Blouin. 1999. Molecular differentiation and phylogeny of entomopathogenic nematodes (Rhabditida: Heterorhabditidae) based on ND4 gene sequences of mitochondrial DNA. **J. Parasitol.** 85: 709-715.

_____, _____ and _____. 2001. Identification of symbiotic bacteria (*Photorhabdus* and *Xenorhabdus*) from the entomopathogenic nematodes *Heterorhabditis marelatus* and *Steinernema oregonense* based on 16S rDNA sequence. **J. Invertreb. Pathol.** 77: 87-91.

Luc, P.V., K.B. Nguyen, A.P. Reid and S.E. Spiridonov. 2000. *Steinernema tami* sp. n. (Rhabditida: Steinernematidae) from Cat Tien Forest, Vietnam. **Russ. J. Nematol.** 8: 33-43.

Malan, A.P., K.B. Nguyen, J.Y. deWaal and L. Tiedt. 2008. *Heterorhabditis safricana* n. sp. (Rhabditida: Heterorhabditidae) an entomopathogenic nematode from South Africa. **Nematology.** 10: 381-396.

Mamiya, Y. 1988. *Steinernema kushidai* n. sp. (Nematoda: Steinernematidae) associated with scarabaeid beetle larvae from Shizuoka, Japan. **Appl. Ento. Zool.** 23: 313-320.

Maneesakorn, P., P.S. Grewal and A. Chandrapatya. 2010. *Steinernema minuta* sp. nov. (Rhabditida: Steinernematidae): a new entomopathogenic nematode from Thailand. **Int. J. Nematol**. 20: 19-34.

Martens, E.C. and H. Goodrich-Blair. 2005. The *Steinernema carpocapsae* intestinal vesicle contains a subcellular structure with which *Xenorhabdus nematophila* associates during colonization initiation. **Cell. Micro.** 7: 1723-1735.

Meier-Kolthoff, J.P., A.F. Auch, D.H. Huson and M. Göker. 2007. COPYCAT : cophylogenetic analysis tool. **Bioinformatics.** 23: 898-900.

Minitab. Release 15.1.20.0. Minitab Inc. 2007.

Mráček, Z., E.A. Hernandez and N.E. Boemare. 1994. *Steinernema cubana* sp. n. (Nematoda: Rhabditida: Steinernematidae) and the preliminary characterization of its associated bacterium. **J. Invertebr. Pathol.** 64: 123-129.

_____, K.B. Nguyen, P. Tailliez, N. Boemare and S. Chen. 2006. *Steinernema sichuanense* n. sp. (Rhabditida, Steinernematidae), a new species of entomopathogenic nematode from the province of Sichuan, east Tibetan Mts., China. **J. Invertebr. Pathol.** 93:157-169.

Nadler, S.A., E. Bolotin and S.P. Stock. 2006. Phylogenetic relationships of *Steinernema* Travassos, 1927 (Nematoda: Cephalobina: Steinernematidae) based on nuclear, mitochondrial and morphological data. **Syst. Parasitol.** 63: 159-179.

_____, E.P. Hoberg, D.S.S. Hudspeth and L.G. Rickard. 2000. Relationships of *Nematodirus* species and *Nematodirus battus* isolates (Nematoda: Trichostrongyloidea) based on nuclear ribosomal DNA sequences. **J. Parasitol.** 86: 588-601.

Nguyen, K.B. 2010. **Species of *Steinernema*.** Available Source: http://nematology.ifas.ufl.edu/nguyen/morph/steinsp1.htm, February 1, 2010.

_____ and G.C. Jr. Smart 1990. *Steinernema scapterisci* n. sp. (Steinernematidae: Nematoda). **J. Nematol.** 22: 187-199.

_____ and _____ 1994. *Neosteinernema longicurvicauda* n. gen., n. sp. (Rhabditia: Steinernematidae), a parasite of the termite *Reticulitermes flavipes* (Koller). **J. Nematol.** 26: 162-174.

_____ and L.W. Duncan. 2002. *Steinernema diaprepesi* n. sp. (Rhabditida: Steinernematidae), a parasite of the citrus root weevil *Diaprepes abbreviates* (L.) (Coleoptera: Curculionidae). **J. Nematol.** 34: 159-170.

_____ and D.J. Hunt. 2007. **Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts.** Leiden, Netherlands: Brill Academic, 816 pp.

_____, J. Maruniak and B.J. Adams. 2001. Diagnostic and phylogenetic utility of the rDNA internal transcribed spacer sequences of *Steinernema*. **J. Nematol.** 33: 73-82.

_____, D.I. Shapiro-Ilan and G. Mbata. 2008. *Heterorhabditis georgiana* n. sp. (Rhabditida: Heterorhabditidae) from Georgia, USA. **Nematology.** 10: 433-448.

_____, U. Gozel, H.S. Koppenhöfer and B.J. Adam. 2006. *Heterorhabditis floridensis* n. sp. (Rhabditida: Heterorhabditidae) from Florida. **Zootaxa.** 1177: 1-19.

_____, _____, R.J. Stuart, C.W. McCoy, R.R. James and B.J. Adams. 2004. *Heterorhabditis mexicana* n. sp (Rhabditida: Heterorhabditidae) from Tamaulipas, Mexico, and morphological studies of the bursa of *Heterorhabditis* spp. **J. Nematol.** 6: 231-244.

Oliver J.B., C.M. Mannion, M.G. Klein, J.J. Moyseenko and B. Bishop. 2005. Effect of insecticides on *Tiphia vernalis* (Hymenoptera: Tiphiidae) oviposition and survival of progeny to cocoon stage when parasitizing *Popillia japonica* (Coleoptera: Scarabaeidae) larvae. **J. Econ. Entomol.** 98: 694-703.

_____, M.E. Reding , N.N. Youssef, M.G. Klein, B.L. Bishop and P.A. Lewis. 2009. Surface-applied insecticide treatments for quarantine control of Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae), larvae in field-grown nursery plants. **Pest Manag. Sci.** 6: 381-390.

Page, R.D.M. 1994. Maps between trees and cladistic analysis of historical associations among genes, organisms, and areas. **Syst. Biol.** 43: 58-77.

_____. 1996. TREEVIEW: an application to view phylogenetic trees on personal computer. **CABIOS.** 12: 357-358.

Phan, K.L., N.C. Nguyen and M. Moens. 2001. *Steinernema loci* sp. n. and *Steinernema thanhi* sp. n. (Rhabditida: Steinernematidae) from Vietnam. **Nematology.** 3: 503-514.

_____, S.A. Subbotin, N.C. Nguyen and M. Moens. 2003. *Heterorhabditis baujardi* sp. n. (Rhabditida: Heterorhabditidae) from Vietnam and morphometric data for *H. indica* populations. **Nematology.** 5: 367-382.

_____, S.E. Spiridonov, S.A. Subbotin and M. Moens. 2006. Four new species of *Steinernema* Travassos, 1928 with short infective juveniles from Vietnam. **Russ. J. Nematol.** 14: 11-29.

Poinar, Jr. G.O. 1976. Description and biology of a new insect parasitic rhabditoid, *Heterorhabditis bacteriophora* n. gen. n. sp. (Rhabditida: Heterorhabditidae n. fam.) **Nematologica.** 21: 463-470.

_____. 1986. *Neoaplectana intermedia* n. sp. (Steinernematidae: Nematoda) from South Carolina. **Revue de Nematologie.** 8: 321-327.

_____. 1990. **Taxonomy and biology of Steinernematidae and Heterorhabditidae**. pp. 23-60. *In* R. Gaugler and H.K. Kaya eds. Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, FL.

_____ and G.M. Thomas. 1965. A new bacterium, *Achromobacter nematophilus* sp. nov. (Achromobacteriaceae: Eubacteriales), associated with a nematode. **Int. Bull. Bacteriol. Nomencl. Taxon.** 15: 249-252.

_____, T. Jackson and M. Klein. 1987. *Heterorhabditis megidis* sp. n. (Heterorhabditidae: Rhabditida), parasitic in the Japanese beetle, *Popillia japonica* (Scarabaeidae: Coleoptera), in Ohio. **Proceedings of the Helminthological Society of Washington.** 54: 53-59.

_____, G.K. Karunakar and H. David. 1992. *Heterorhabditis indicus* n. sp. (Rhabditida: Nematoda) from India: separation of *Heterorhabditis* spp. by infective juvenile. **Fund. Appl. Nematol.** 15: 467-472.

Potter, D.A. 1998. **Destructive Turfgrass Insects: Biology, Diagnosis, and Control**. Ann. Arbor Press, Chelsea, Michigan. 344 pp.

Power, K.T., R. An and P.S. Grewal. 2009. Effectiveness of *Heterorhabditis bacteriophora* strain GPS11 applications targeted against different instars of the Japanese beetle *Popillia japonica*. **Biol. Cont.** 48: 232-236.

Power, T.O., T.S. Harris and B.C. Hyman. 1994. Mitochondrial DNA sequence divergence among *Meloidogyne incognita*, *Romanomermis culicivorax*, *Ascaris suum* and *Caenorhabditis elegans*. **J. Nematol.** 25: 564-572.

_____, T.C. Todd, A.M. Burnell, P.C.B. Murray, A.L. Zsalanski, B.J. Adam and T.S. Harris. 1997. The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. **J. Nematol.** 29: 2441-2450.

Ramia, S., E. Neter and D.J. Brenner. 1982. Production of enterobacterial common antigen as an aid to classification of newly identified species of the families Enterobacteriaceae and Vibrionaceae. **Int. J. Syst. Bacteriol.** 32: 395-398.

Reid, A.P. and W.M. Hominick. 1992. Restriction fragment length polymorphism within ribosomal DNA repeat unit of British entomopathogenic nematodes (Rhabditida: Steinernematidae). **J. Parasitol.** 105: 317-323.

_____, _____ and B.R. Briscoe. 1997. Molecular taxonomy and phylogeny of entomopathogenic nematode species (Rhabditida: Steinernematidae) by RFLP analysis of the ITS region of rDNA repeat unit. **Syst. Parasitol.** 37: 187-193.

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

Shamseldean, M.M., A.B. Abou El-Sooud, M.M. Abd- Elgawad and M.M. Saleh. 1996. Identification of a new heterorhabditid species from Egypt, *Heterorhabditis taysearae* n. sp. (Rhabditida: Heterorhabditidae). **Egyptian J. Biol. Pest Cont.** 6: 129-138.

Steiner, G. 1923. *Aplectana kraussei* n. sp., eine in der Blattwespe Lyda sp. parasitierende Nematodenform, nebst Bemerkungen uber das Seitenorgan der parasitischen Nematoden. **Zentralblatt für Bakteriologie, Parasitenkunde, Infektions krankheiten and Hygiene, Abteilung II.** 59: 14-18.

_____. 1929. *Neoaplectana glaseri* n. g., n. sp. (Oxyuridae), a new nemic parasite of the Japanese beetle (*Popillia japonica* Newman.). **J. Wash. Acad. Sci.** 19:436-440.

Stock, S.P. and A.M. Koppenhöfer. 2003. *Steinernema scarabaei* n. sp. (Rhabditida: Steinernematidae), a natural pathogen of scarab beetle larvae (Coleoptera: Scarabaeidae) from New Jersey, USA. **Nematology.** 5: 191-204.

_____ and D.J. Hunt. 2005. **Nematode morphology and systematic**, pp. 3-43. *In* P. S. Grewal, R.U. Ehlers and D.I. Shapiro-Ilan, eds. Nematodes as Biocontrol Agents. Wallingford, UK: CAB international.

_____, D. Strong and S.L. Gardner. 1996. Identification of *Heterorhabditis* (Nematoda: Heterorhabditidae) from California with a new species isolated from the larvae of the ghost moth *Hepialis californicus* (Lepidoptera: Hepialidae) from the Bodega Bay Natural Reserve. **Fundam. Appl. Nematol.** 19: 585-592.

_____, V. Somsook and A.P. Reid. 1998. *Steinernema siamkayai* n. sp. (Rhabditida: Steinernematidae), an entomopathogenic nematode from Thailand. **Syst. Parasitol.** 41: 105-113.

_____, B.M. Pryor and H.K. Kaya. 1999. Distribution of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) in natural habitats in California. **Biodivers. Conserv.** 8: 535-549.

_____, J.F. Campbell and S.A. Nadler. 2001. Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. **J. Parasitol.** 87: 877-889.

_____, C.T. Griffin and A.M. Burnell. 2002. Morphological characterization of three isolates of *Heterorhabditis* Poinar, 1976 from the “Irish group” (Nematoda: Rhabditida: Heterorhabditidae) and additional evidence supporting their recognition as a distinct species, *H. downesi* n. sp. **Syst. Parasitol.** 51: 95-106.

_____, J. Vandenberg, I. Glazer and N. Boemare. 2009. **Insect Pathogens Molecular Approaches and Techniques**. Wallingford, UK: CAB international. 417 pp.

Stuart, R.J. and R. Gaugler. 1994. Patchiness in populations of entomopathogenic nematodes. **J. Invertebr. Pathol.** 64: 39-45.

Sturhan, D. 1999. **Prevalence and habitat specificity of entomopathogenic nematodes in Germany**, pp. 123-132. *In* R.L. Gwynn, P.H. Smits, C.T. Griffin, R.U. Ehlers, N. Boemare and J.P. Masson, eds. COST 819. Entomopathogenic Nematodes. Application and Persistence of Entomopathogenic Nematodes, XII. European Commission, Luxemburg, DG.

_____ and M. Liskova. 1999. Occurrence and distribution of entomopathogenic nematodes in the Slovak Republic. **J. Nematol.** 1: 273-277.

Szalanski, A.L., D.B. Taylor and P.G. Mullin. 2000. Assessing nuclear and mitochondrial DNA sequence variation within *Steinernema* (Rhabditida: Steinernematidae). **J. Nematol.** 32: 229-233.

Tailliez, P., S. Pages, N. Ginibre and N. Boemare. 2006. New insight into diversity in the genus *Xenorhabdus*, including the description of ten novel species. **Int. J. Syst. Evol. Microbiol.** 56: 2805-2818.

Tamura, K., J. Dudley, M. Nei and S. Kumar. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. **Mol. Biol. Evol.** 24: 1596-1599.

Thomas, G.M. and G.O. Poinar Jr. 1979. *Xenorhabdus* gen-nov, a genus of entomopathogenic, nematophilic bacteria of the family Enterobacteriaceae. **Int. J. Syst. Bacteriol.** 29: 352-360.

Thompson, J.D., D.G. Higgins and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. **Nucle. Acids Res.** 22: 4673-4680.

Tóth, T. and T. Lakatos. 2008. *Photorhabdus temperata* subsp. *cinerea* subsp. nov., isolated from *Heterorhabditis* nematodes. **Int. J. Syst. Evol. Microbiol.** 58: 2579-2581.

Travassos, L. 1927. Sobre o genero *Oxysomatium*. **Boletim Biologico, Sao Paulo.** 5: 20-21.

Vittum P.J., M.G. Villani and H. Tashiro. 1999. **Turfgrass Insects of the United States and Canada**. Cornell University Press, Ithaca, NY.

Weckstein, J.D. 2004. Biogeography explains cophylogenetic patterns in toucan chewing lice. **Syst. Biol.** 53: 154-164.

Weiser, J. 1955. *Neoaplectana carpocapsae* n. sp. (Angullulinata: Steinernematidae) novy, cizopasnic housenik obalece jableneho, *Carpocapsa pomonella* L. **Westnik Ceskoslovenske Zoologicke Spolecnosti.** 19: 44-52.

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

Wouts, W.M., Z. Mráček, S. Gerdin and R.A. Bedding. 1982. *Neoaplectana* Steiner, 1929 a junior synonym of *Steinernema* Travassos, 1927 (Nematoda: Rhabditida). **Syst. Parasitol.** 4: 147-154.

Yamamoto, S. and S. Harayama. 1995. PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of Pseudomonas-putida strains. **Appl. Environ. Microbiol.** 61: 1104-1109.

_____ and_____. 1996. Phylogenetic analysis of *Acinetobacter* strains based on the nucleotide sequence of *gryB* genes and on the amino acid sequences of their products. **Int. J. Syst. Bacteriol.** 46: 506-511.

APPENDIX

Appendix Table 1 Morphometric characters recommended (*) for describing *Steinernema* species. Measurements should be in the form of mean ± SD (range)

Character	First generation		Second generation		Infective Juvenile
	Male	Female	Male	Female	
Body length (L)	*	*	*	*	*
a = L/MBD					*
b = L/ES					*
c = L/T					*
V		*		*	
Body diam.	*	*	*	*	*
EP	*	*	*	*	*
NR	*	*	*	*	*
ES	*	*	*	*	*
Testis reflexion	*		*		
Tail length (T)	*	*	*	*	*
Anal body diam. (ABD)	*	*	*	*	*
Spicule length (SL)	*		*		
Spicule width	*		*		
Gubernaculum length (GL)	*		*		
Gubernaculum width	*		*		
D% = EP/ES x 100	*	*	*	*	*
E% = EP/T x 100					*
SW% = SL/ABD x 100	*		*		
GS% = GL/SL x 100	*		*		
H% = Hyaline/T x 100					*

Distance from anterior end to end of pharynx (ES)
Distance from anterior end to nerve ring (NR)
Distance from anterior end to excretory pore (EP)

Appendix Table 2 Morphometric characters recommended (*) for describing *Heterorhabditis* species. Measurements should be in the form of mean ± SD (range)

Character	Male	Hermaphrodite	Female	IJ
Body length (L)	*	*	*	*
a = L/MBD				*
b = L/ES				*
c = L/T				*
Body diam.	*	*	*	*
EP	*	*	*	*
NR	*	*	*	*
ES	*	*	*	*
Testis reflexion	*			
Tail length with sheath (T)				*
Tail length without sheath	*	*	*	*
Anal body diam. (ABD)	*	*	*	*
Spicule length (SL)	*			
Gubernaculum length (GL)	*			
D% = EP/ES x 100	*	*	*	*
E% = EP/T x 100				*
SW% = SL/ABD x 100	*			
GS% = GL/SL x 100	*			

Distance from anterior end to end of pharynx (ES)
Distance from anterior end to nerve ring (NR)
Distance from anterior end to excretory pore (EP)

Appendix Table 3 The locality and position of sampling sites in Thailand

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP1	14/3/2007	Rayong	Sandy-loam	30.9	47P 0806513 1427433	12°53.882'	101°49.483'
MP2	14/3/2007	Rayong	Sandy-loam	46.4	47P 0806162 1426023	12°53.119'	101°49.286'
MP3	14/3/2007	Rayong	Sandy-loam	-	-	-	-
MP4	15/3/2007	Chantaburi	Silt-loam	36	48P 0193572 1386074	12°31.466'	102°10.811'
MP5	15/3/2007	Chantaburi	Silt-loam	-	-	-	-
MP6	15/3/2007	Chantaburi	Silt-loam	-	-	-	-
MP7	15/3/2007	Chantaburi	Silt-loam	-	-	-	-
MP8	15/3/2007	Chantaburi	Silt-loam	-	-	-	-
MP9	8/4/2007	Chumphon	Clay-loam	9	47P 0525711 1144629	10°21.325'	099°14.093'
MP10	8/4/2007	Chumphon	Sandy-loam	36	47P 0531088 1147829	10°23.060'	099°17.042'

Appendix Table 3 (Continued)

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP11	8/4/2007	Chumphon	Sandy-loam	64	47P 0531170 1147765	10°23.025'	099°17.087'
MP12	28/4/2007	Ubon Ratchathani	Sandy-loam	163	48P 0556262 1702739	15°24.129'	105°31.463'
MP13	28/4/2007	Ubon Ratchathani	Silt-loam	138	48P 0552522 1691154	15°17.850'	105°29.357'
MP14	28/4/2007	Ubon Ratchathani	Silt-loam	-	-	-	-
MP15	10/5/2007	Khon Kaen	Silt-loam	187	48Q 0206085 1846319	16°40.952'	102°14.633'
MP16	10/5/2007	Khon Kaen	Silt-loam	241	48Q 0206109 1846330	16°40.959'	102°14.646'
MP17	10/5/2007	Khon Kaen	Silt-loam	239	48Q 0206161 1846331	16°40.960'	102°14.675'
MP18	10/5/2007	Khon Kaen	Silt-loam	246	48Q 0206065 1846265	16°40.920'	102°14.735'
MP19	10/5/2007	Khon Kaen	Silt-loam	289	48Q 0180348 1853269	16°44.516'	102°00.107'
MP20	10/5/2007	Loei	Silt-loam	280	47Q 0804030 1867241	16°52.210'	101°51.223'

Appendix Table 3 (Continued)

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP21	16/6/2007	Trat	Sandy-loam	52	48P 0212314 1328472	12°00.349'	102°21.462'
MP22	16/6/2007	Trat	Sandy-loam	66	48P 0212950 1329261	12°00.780'	102°21.808'
MP23	17/6/2007	Trat	Sandy-loam	14	48P 0213810 1336083	12°04.483'	102°22.247'
MP24	17/6/2007	Trat	Sandy-loam	9	48P 0207431 1334550	12°03.618'	102°18.741'
MP25	1/7/2007	Sa Kaeo	Silt-loam	205	48P 0198517 1550495	14°00.604'	102°12.524'
MP26	1/7/2007	Sa Kaeo	Silt-loam	133	48P 0198621 1548670	13°59.615'	102°12.593'
MP27	21/7/2007	Rayong	Sandy-loam	37	47P 0766827 1389645	12°33.618'	101°27.358'
MP28	21/7/2007	Rayong	Sandy-loam	34	47P 0767131 1389735	12°33.665'	101°27.526'
MP29	12/11/2007	Ratchaburi	Silt-loam	-	-	-	-
MP30	12/11/2007	Ratchaburi	Silt-loam	202	47P 0560019 1466242	13°15.819'	99°33.248'

Appendix Table 3 (Continued)

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP31	12/11/2007	Ratchaburi	Silt-loam	193	47P 0559974 1466238	13°15.317'	99°33.233'
MP32	13/11/2007	Ratchaburi	Sandy-loam	-	-	-	-
MP33	14/11/2007	Phetchabun	Silt-loam	163	47Q 0727227 1842341	16°39.239'	101°07.844'
MP34	14/11/2007	Phetchabun	Silt-loam	192	47Q 0727052 1842091	16°39.104'	101°07.744'
MP35	14/11/2007	Phetchabun	Silt-loam	161	47Q 0728138 1843778	16°40.013'	101°08.365'
MP36	14/11/2007	Phitsanulok	Silt-loam	411	47Q 0697942 1863080	16°50.641'	100°51.484'
MP37	14/11/2007	Phitsanulok	Silt-loam	412	47Q 0697833 1863224	16°50.719'	100°51.423'
MP38	14/11/2007	Phitsanulok	Silt-loam	473	47Q 0698442 1862584	16°50.369'	100°51.762'
MP39	14/11/2007	Phitsanulok	Silt-loam	-	-	-	-
MP40	14/11/2007	Phitsanulok	Silt-loam	-	-	-	-

Appendix Table 3 (Continued)

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP41	15/11/2007	Phitsanulok	Silt-loam	1259	47Q 0713359 1879957	16°59.708'	101°00.259'
MP42	15/11/2007	Phitsanulok	Silt-loam	1261	47Q 0714722 1879773	16°59.601'	101°01.026'
MP43	15/11/2007	Phitsanulok	Silt-loam	1550	47Q 0721225 1870066	16°54.302'	101°04.632'
MP44	18/11/2007	Kanchanaburi	Silt-loam	106	47P 0515837 1588230	14°22.040'	099°08.814'
MP45	18/11/2007	Kanchanaburi	Silt-loam	104	47P 0515915 1588109	14°21.974'	099°08.858'
MP46	18/11/2007	Kanchanaburi	Silt-loam	-	-	-	-
MP47	22/11/2007	Kamphangphet	Silt-loam	-	-	-	-
MP48	22/11/2007	Kamphangphet	Silt-loam	-	-	-	-
MP49	23/11/2007	Kamphangphet	Sandy-loam	-	-	-	-
MP50	23/11/2007	Kamphangphet	Sandy-loam	-	-	-	-

Appendix Table 3 (Continued)

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP51	23/11/2007	Kamphangphet	Sandy-loam	-	-	-	-
MP52	29/11/2007	Saraburi	Sandy-loam	-	-	-	-
MP53	29/11/2007	Saraburi	Sandy-loam	-	-	-	-
MP54	29/11/2007	Saraburi	Sandy-loam	-	-	-	-
MP55	29/11/2007	Saraburi	Sandy-loam	34	47P 0711114 1597891	14°26.795'	100°57.522'
MP56	29/11/2007	Saraburi	Sandy-loam	43	47P 0710629 1598666	14°27.218'	100°57.256'
MP57	8/12/2007	Chumphon	Sandy-loam	65	47P 0531045 1147849	10°23.070'	099°17.019'
MP58	8/12/2007	Ranong	Sandy-loam	87	47P 0463182 1113614	10°04.488'	098°39.836'
MP59	8/12/2007	Ranong	Sandy-loam	105	47P 0463291 1113732	10°04.552'	098°39.896'
MP60	8/12/2007	Ranong	Sandy-loam	100	47P 0463508 1114066	10°04.734'	098°40.014'

Appendix Table 3 (Continued)

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP61	15/12/2007	Chantaburi	Silt-loam	-	-	-	-
MP62	15/12/2007	Chantaburi	Silt-loam	-	-	-	-
MP63	18/12/2007	Suphanburi	Silt-loam	255	47P 0550251 1653552	14°57.453'	099°28.043'
MP64	18/12/2007	Suphanburi	Silt-loam	236	47P 0550522 1653615	14°57.487'	099°28.195'
MP65	18/12/2007	Suphanburi	Sandy-loam	209	47P 0552356 1654176	14°57.789'	099°29.219'
MP66	18/12/2007	Kanchanaburi	Silt-loam	301	47P 0533165 1620239	14°39.396'	099°18.483'
MP67	18/12/2007	Kanchanaburi	Silt-loam	-	-	-	-
MP68	18/12/2007	Kanchanaburi	Silt-loam	-	-	-	-
MP69	20/12/2007	Loei	Silt-loam	280	47Q 0804030 1867241	16°52.210'	101°51.223'
MP70	20/12/2007	Loei	Silt-loam	-	-	-	-

Appendix Table 3 (Continued)

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP71	22/12/2007	Prachuap Khiri Khan	Silt-loam	48	47P 0610045 1346265	12°10.642'	100°00.699'
MP72	22/12/2007	Prachuap Khiri Khan	Silt-loam	20	47P 0610058 1346253	12°10.636'	100°00.706'
MP73	23/12/2007	Prachuap Khiri Khan	Sandy-loam	24	47P 0567549 1285042	11°37.491'	099°37.185'
MP74	23/12/2007	Prachuap Khiri Khan	Sandy-loam	19	47P 0567589 1285030	11°37.484'	099°37.207'
MP75	23/12/2007	Prachuap Khiri Khan	Sandy-loam	33	47P 0567604 1285013	11°37.475'	099°37.215'
MP76	23/12/2007	Prachuap Khiri Khan	Sandy-loam	11	47P 0576640 1286321	11°38.173'	099°42.191'
MP77	23/12/2007	Prachuap Khiri Khan	Sandy-loam	14	47P 0576585 1286380	11°38.166'	099°42.160'
MP78	23/12/2007	Prachuap Khiri Khan	Sandy-loam	17	47P 0576571 1286273	11°38.147'	099°42.153'
MP79	5/1/2008	Nakhon Ratchasima	Sandy-loam	447	47P 0757244 1601894	14°28.729'	101°23.210'
MP80	5/1/2008	Nakhon Ratchasima	Silt-loam	698	47P 0757970 1601145	14°28.318'	101°23.610'

Appendix Table 3 (Continued)

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP81	5/1/2008	Nakhon Ratchasima	Silt-loam	767	47P 0756126 1597194	14°26.187'	101°22.561'
MP82	14/1/2008	Chiang Mai	Silt-loam	684	47Q 0492219 2080254	18°48.934'	098°55.569'
MP83	14/1/2008	Chiang Mai	Silt-loam	761	47Q 0492226 2080359	18°48.991'	098°55.572'
MP84	14/1/2008	Chiang Mai	Silt-loam	717	47Q 0492488 2080265	18°48.940'	098°55.722'
MP85	14/1/2008	Chiang Mai	Silt-loam	388	47Q 0481635 2069563	18°43.132'	098°49.547'
MP86	14/1/2008	Chiang Mai	Sandy-loam	299	47Q 0481614 2069602	18°43.153'	098°49.535'
MP87	14/1/2008	Chiang Mai	Sandy-loam	357	47Q 0481706 2069332	18°43.007'	098°49.587'
MP88	14/1/2008	Chiang Mai	Silt-loam	665	47Q 0458111 2049839	18°32.415'	098°36.182'
MP89	14/1/2008	Chiang Mai	Silt-loam	496	47Q 0463295 2047676	18°31.248'	098°39.132'
MP90	14/1/2008	Chiang Mai	Clay-loam	414	47Q 0464870 2045690	18°30.172'	098°40.029'

Appendix Table 3 (Continued)

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP91	15/1/2008	Chiang Rai	Silt-loam	846	47Q 0491346 2399231	21°41.894'	098°54.979'
MP92	15/1/2008	Lampang	Sandy-loam	-	-	-	-
MP93	15/1/2008	Lampang	Sandy-loam	-	-	-	-
MP94	15/1/2008	Lampang	Sandy-loam	433	47Q 0528613 2040530	18°27.380'	099°16.262'
MP95	15/1/2008	Lamphun	Sandy-loam	465	47Q 0527626 2048245	18°31.565'	099°15.707'
MP96	17/1/2008	Kanchanaburi	Silt-loam	91	47P 0484327 1595368	14°25.912'	098°51.274'
MP97	17/1/2008	Kanchanaburi	Clay-loam	34	47P 0484574 1595641	14°26.061'	098°51.411'
MP98	17/1/2008	Kanchanaburi	Clay-loam	92	47P 0484651 1595627	14°26.053'	098°51.455'
MP99	19/1/2008	Nakhonsrihamarat	Silt-loam	-	-	-	-
MP100	19/1/2008	Nakhonsrihamarat	Silt-loam	-	-	-	-

Appendix Table 3 (Continued)

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP101	19/1/2008	Nakhonsrihamarat	Sandy-loam	-	-	-	-
MP102	19/1/2008	Nakhonsrihamarat	Silt-loam	-	-	-	-
MP103	19/1/2008	Nakhonsrihamarat	Silt-loam	-	-	-	-
MP104	19/1/2008	Nakhonsrihamarat	Clay-loam	112	47P 0585235 0996064	09°00.651'	099°46.535'
MP105	19/1/2008	Nakhonsrihamarat	Clay-loam	87	47P 0585292 0996054	09°00.645'	099°46.566'
MP106	19/1/2008	Nakhonsrihamarat	Clay-loam	84	47P 0585323 0996004	09°00.619'	099°46.583'
MP107	19/1/2008	Surat Thani	Silt-loam	189	47P 0553036 0979399	08°51.636'	099°28.944'
MP108	19/1/2008	Surat Thani	Silt-loam	-	-	-	-
MP109	19/1/2008	Surat Thani	Silt-loam	191	47P 0553173 0979338	08°51.603'	099°29.019'
MP110	20/1/2008	Krabi	Sandy-loam	-	-	-	-

Appendix Table 3 (Continued)

Code	Date	Locality	Soil type	Position			
				elevation	UTM	N	E
MP111	20/1/2008	Krabi	Sandy-loam	-	-	-	-
MP112	20/1/2008	Krabi	Sandy-loam	-	-	-	-
MP113	20/1/2008	Krabi	Clay-loam	-	-	-	-
MP114	20/1/2008	Krabi	Clay-loam	-	-	-	-
MP115	27/1/2008	Phetchaburi	Silt-loam	109	47P 0568214 1427569	12°54.826'	099°37.734'

Note (-) There are no signal.

CURRICULUM VITAE

NAME : Mrs. Patchareewan Maneesakorn

BIRTH DATE : Feb 25, 1975

BIRTH PLACE : Bangkok, Thailand

EDUCATION :

YEAR	INSTITUTE	DEGREE/DIPLOMA
1997	Kasetsart Univ.	B.Sc.(Integrated Pest Management)
2005	Kasetsart Univ.	M.S.(Entomology)

SCHOLARSHIP/AWARDS : Royal Golden Jubilee Ph.D. Program 2006 - 2010