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THESIS

CHARACTERIZATION OF NEW ENTOMOPATHOGENIC NEMATODES FROM THAILAND: FORAGING BEHAVIORS AND THEIR VIRULENCE TO SELECTED AGRICULTURAL PESTS

ATIRACH NOOSIDUM

A Thesis Submitted in Partial Fulfillment of
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One hundred and sixty eight soil samples were collected from 5 provinces, all located in southern Thailand. Eight isolates of entomopathogenic nematodes (EPNs) were isolated and identified to species using restriction profiles and sequence analysis. Five of the isolates were identified as *Heterorhabditis indica,* and one as *Heterorhabditis baujardi*. Two undescribed *Steinernema* isolates were also discovered which matched no published sequences and grouped separately from the other DNA restriction profiles. Behavioral tests showed that all *Heterorhabditis* spp. were cruise foragers, based on their attraction to volatile cues and lack of body-wave behavior, while the S*teinernema* isolates were more intermediate in foraging behavior.

The LC_{50} values against *Galleria mellonella* larva ranged from 1.99-6.95 IJs/insect. Moreover, nematode virulence of new isolates were also performed against other insect pests; *Plutella xylostella* larva were killed by *H. indica* isolate K4 and showed significantly higher LC_{50} value (16.75 IJs/larva). The second and third-instar larvae of *Spodoptera litura* had the significantly highest invasion rate by *H. indica* isolate K4 (LC_{50} value = 10.27 and 16.12 IJs/larva, respectively). The LC_{50} values of 4 EPNs to second and third-instar larvae ranged from 10-34 IJs/larva. However, all nematode isolates did not infect *P. xylostella* and *S. litura* pupae whereas *Steinernema* sp. isolate K8 could invade *S. litura* pre-pupal stage for 60% at 10 d after application. In addition, *H. indica* isolate K4 had the highest virulence to *Tenebrio molitor* larvae (LT_{50} = 33.76 h), followed by *Steinernema* sp. isolate K8 (LT_{50} = 48.15 h) and *Steinernema* sp. isolate K8 was able to infect first-instar larva of *Oryctes rhinoceros* (100% mortality rate at 72 h) whereas *H. indica* isolate K4 could not kill *O. rhinoceros* larva.

Steinernema sp. isolate K8 showed potential as a biological control agent in high clay soils in nine centimeter columns of either sandy loam or sandy clay loam and the *Steinernema* sp. isolate K8 had the greatest infection rate in both soil types compared to the other Thai isolates and 3 commercial EPNs.

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Student's signature	Thesis Advisor's signature	

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Atirach Noosidum

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LIST OF ABBREVIATIONS

ºC	=	degree celcius
cm	=	centimeter
d	=	day
diam	=	diameter
EPN	=	entomopathogenic nematode
et al	=	et alia
etc	=	et cetera
g	=	gram
h	=	hour
IJ	=	infective juvenile
ITS	=	internal transcribed spacer
LC_{50}	=	lethal concentration at 50%
LT_{50}	=	lethal time at 50%
M	=	meter
mg	=	milligram
mm	=	millimeter
min	=	minute
ml	=	milliliter
µl	=	micro liter
µm	=	micrometer
µM	=	micromolar
PCR	=	polymerase chain reaction
sec	=	second
sp.	=	species
UV	=	ultra violet

CHARACTERIZATION OF NEW ENTOMOPATHOGENIC NEMATODES FROM THAILAND: FORAGING BEHAVIORS AND THEIR VIRULENCE TO SELECTED AGRICULTURAL PESTS

INTRODUCTION

Most farmers in Thailand rely on many chemical pesticides for pest control. The excessive use of insecticides resulted in insecticide resistance and environmental damage such as destruction of the natural enemies and hazards to non-target organisms including humans. Biological control was introduced to this country to control insect pests in order to reduce the use of chemical insecticides, which lead to ecological stability, economical profit ability and environmental safety.

Increased efforts in recent years have been focused on employing biological control agents such as predators, parasites, bacteria, fungi, protozoa, viruses and entomopathogenic nematodes (EPNs) (Klein, 1990, 1995; Potter, 1995, 1998; Arthur, 1996; Bell, 2000; Moore *et al.*, 2000; Marannino *et al*., 2006; Oestergaard *et al*., 2006). To date EPNs are important biological control agents in various crops (Grewal *et al*., 2005). Two families of EPNs (Heterorhabditidae and Steinernematidae) are used as bio-agents for controlling several insect pests (Gaugler and Kaya, 1990; Bedding *et al*., 1993, Kaya and Gaugler, 1993; Burnell and Stock, 2000; Gaugler, 2002; Campbell *et al.*, 2004; Grewal *et al*., 2005). Several species of EPNs within two genera: *Steinernema* and *Heterorhabditis*, and their symbiotic bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp., respectively) are obligate pathogens of insects in nature (Poinar, 1979). EPNs, which are lethal endoparasites of insects (Gaugler and Kaya, 1990; Gaugler, 2002), are found in soils and persist as non-feeding infective juveniles, seeking out a potential insect host (Poinar, 1972). The infective juveniles enter the host through natural body openings, penetrate into the host hemocoel and release their associated bacteria that kill the host within 24–48 h (Bathon, 1996; Boemare *et al*., 1996; Kaya and Gaugler, 1993). Nowadays, the infective juveniles are produced commercially and applied with conventional liquid application system to protect several economic plants (Grewal, 2002).

In the past, most commercial EPNs which are being cultured and distributed to Thai farmers were imported from the USA and only one EPN species, *Steinernema siamkayai* Stock, Somsook & Reid is of Thailand origin. At present, six endemic strains of Thai EPNs were discovered in Thailand. The first EPN strain is *S. siamkayai* which was recovered from a soil sample collected from a tamarind orchard, Amphoe Lomsak, Petchaboon province, Northern Thailand (Stock *et al*., 1998). Later, Hotaka (2000) found a new strain of *Heterorhabditis indica* Poinar, Karunakar & David from soil sample collected at a park close to Si Yok Noi water fall, Amphoe Siyok, Kanchanaburi province, western Thailand in July 1999. Then *Steinernema minutum* Maneesakorn, Grewal & Chandrapatya was isolated from a soil sample collected from a fallen pine tree at Amphoe Mueang, Chumporn Province, southern Thailand in April 2007 (Maneesakon *et al*., 2010). In addition, Maneesakon *et al*. (2010) also found 2 new strains of *H. indica* and another *Heterorhabditis* sp. in Khon Khan, Krabi and Karnchanaburi provinces, respectively (Maneesakon *et al*., 2010). Thus, there should be more entomopathogenic nematodes waiting to be discovered, some of which may have potential as biological control agents.

In this study, soil samples were collected from undisturbed area to search for new potential EPNs originated from Thailand. Nematode taxonomy, biology and behavioral ecology were investigated to classify nematode species and their foraging strategy. The virulence of the new Thai nematode strains against insect pests was also determined in this study since all basic knowledge may help to predict the outcome of field application, and all information is helpful to manage a success in field trail.

OBJECTIVES

1. To isolate new entomopathogenic nematodes native to Thailand.

2. To identify the entomopathogenic nematodes collected in Thailand.

3. To determine the virulence of entomopathogenic nematodes against selected insect pests in Thailand using both standard tests and tests of host recognition behaviors.

LITERATURE REVIEW

1. Entomopathogenic nematode

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

Entomopathogenic nematodes (EPNs) are lethal parasites of soil-dwelling insects that occur in natural and agricultural soils around the world (Hominick, 2002). They have been used for the biological control of various economic insect pests in many crops including vegetables, grasses, mushrooms, ornamentals, orchards and soft fruit production systems (Grewal *et al.*, 2005). EPNs are biological control agents that kill their hosts through a mutualistic relationship with a bacterium (*Xenorhabdus* spp. and *Photorhabdus* spp. for Steinernematidae and Heterorhabditidae, respectively) (Poinar, 1990). The success of nematode applications for insect control in soil and the survival of naturally occurring nematode populations depend on the infective juveniles (IJs) ability to disperse and persist until it can locate a host (Koppenhöfer and Fuzy, 2006). EPNs have a wide host range (Capinera and Epsky, 1992; Gaugler *et al.*, 1997; Grewal *et al.*, 2005), and they have the ability to actively seek their hosts (Campbell and Lewis, 2002).

There are 11 nematode families which could be used as biological control agent for insects. However, only 7 families are now being used which are: Mermithidae, Allantonematidae, Neotylenchidae, Sphaerularidae, Rhabditidae, Steinermatidae and Heterorhabditidae (Lacey *et al*., 2001). Nowadays, EPN application has been focused on the last 2 families which include 55 species from the genus *Steinernema* and 11 species from *Heterorhabditis* (Nguyen and Hunt, 2007).

2. Biology of Steinernematid and Heterorhabditid nematodes

2.1 Life cycle

Steinernematid and Heterorhabditid nematodes have a simple life cycle which includes an egg stage, four larval or juvenile stages and adult stage (Poinar and Leutenegger, 1968). The third-stage juvenile (J3) is the infective or the non-infective stage (Nguyen and Smart, 1992). EPNs also have a resistant stage or infective juvenile (IJ) or dauer juvenile where the third-stage nematode ensheathed while waiting for an insect host (Poinar and Leutenegger, 1968). The infective juvenile is the only free-living, non-parasitic stage of these nematodes and the purpose of this non-feeding IJ stage is to persist in the soil environment until it can infect a host via natural body openings such as mouth, anus or spiracle (Poinar, 1990; Griffin *et al*., 2005).

The IJs ultimately penetrate into the host's body cavity where they release species-specific symbiotically associated bacteria, which they have carried in their intestinal tracts. The toxins produced by the developing nematodes and bacteria actually kill the host (Burman, 1982; Akhurst and Boemare, 1990). Host death occur whithin 48–72 h after exposure to the nematodes, depending on temperature and nematode species (Kaya, 1990).

The J3 stage molts first and then feeds on the bacteria before molting in succession to the forth stage (J4) where they become adult males or females of the first generation. After mating, the females lay eggs which hatch as the first-stage juvenile (J1) and molt successively to consecutive stages (J2, J3 and J4). The J4 develops into adult female or male of the second generation. Meanwhile, the invasive nematodes complete their development and after two or three generations, lasting 7–15 d, the nematodes emerge from the dead insect as IJs and carry their bacterial symbiont to search for a new host (Kaya, 1990).

The pre-infective stage, retaining the cuticle of the second stage as a sheath, can survive for a long time under appropriate humid condition (Woodring and Kaya, 1988; Adams and Nguyen, 2002).

2.2 Symbiotic bacteria

The insect is killed by the combined action of the nematodes and their symbiotic bacteria, presumably via a combination of the toxin action and direct infection (Forst *et al*., 1997). After invading, the hemolymph which is the secondary metabolite produced by nematodes inhibit the activity of insect immune system (Simon *et al*., 1992, Hu *et al*., 1999) and affect its nervous system (Burman, 1982). Thus, this action is the initial conditions for development of a bacterial colony (Chongchitmate *et al*., 2005). In addition, symbiotic bacteria and various life stages of nematodes can release high concentrations of ammonia which can be toxic to many organisms including plant-parasitic nematodes (Grewal *et al*., 1999; Shapiro-Ilan *et al*., 2000; De Nardo *et al*., 2006). The symbiotic bacteria produce antibiotics that inhibit growth of other microorganisms in the insect cadaver and provide nutrients utilized by the nematodes (Akhurst, 1982; Gerritsen *et al*., 1992).

Xenorhabdus and *Photorhabdus* bacteria are symbiotically associated with nematodes of the families Steinernematidae and Heterorhabditidae, respectively (Boemare, 2002). These bacteria are motile gram-negative, facultatively anaerobic rods (F. Enterobacteriaceae) (Forst *et al*., 1997). They do not reduce nitrate and ferment only in a limited number of carbohydrates (Forst and Clarke, 2002). These bacteria located in the intestine of the free-living IJs also referred to as dauer juveniles, which are capable of seeking out hosts (Kaya and Gaugler, 1993). The nematode is a vector for the bacteria, protect the bacteria from the competitive environment and transport them to the haemolymph of the insect host (Sasnarukkit, 2003). On the other hand, the bacteria help the nematode to kill the insect hosts and supply the essential nutrient for nematode growth and reproduction. In addition, the bacteria also produce secondary metabolites, toxins, and antibiotics (Dutky, 1959; Webster *et al*., 2002) with bactericidal (Ji *et al*., 2004), fungicidal (Chen *et al*., 1994), and nematicidal (Grewal *et al*., 1999; Hu *et al*., 1995, 1999) properties.

3. Nematode behavioral ecology

Many studies of entomopathogenic nematode behavioral ecology provided a predictable laboratory and field efficacy result. Lewis (2002) demonstrated that nematode behaviors consist of infective juvenile dispersal, foraging strategies, host discrimination and infection. Lewis and Gaugler (1994) reported that all IJs emerging from the same infection did not have the same behavior. Male IJ of *Steinernema glaseri* (Steiner) Wouts, Mráček, Gerdin & Bedding emerged from the host before female and was more responsive to new host cues than female while *S. glaseri* female was more active to search infected host cues (Stuart *et al*., 1996). O'Leary *et al.* (1998) found that the early emerging IJs of *Heterorhabditis megidis* Poinar, Jackson & Klein were more tolerant to warm temperature than their late emerging IJs. Besides, the variation has been investigated in several studies and various different species such as *Steinernema carpocapsae* (Weiser) Wouts, Mráček, Gerdin & Bedding IJs move upward in soil column while *S. glaseri* move downward (Georgis and Poinar, 1983). Campbell *et al.* (1995) demonstrated that *S. carpocapsae* normally located in the soil surface (1-2 cm), whereas *Heterorhabditis bacteriophora* Poinar was found at the 8 cm from soil surface.

The variation of EPN foraging strategies vary along between ambush and cruise foraging (Lewis *et al*., 1992; 1993; Grewal *et al.*, 1994; Campbell and Gaugler, 1997). In addition, Lewis (2002) revealed that EPN foraging behavior has been categorized into 3 behavioral classes including ambuser, cruiser and intermediate base on its host attraction between mobile versus sedentary host.

Ambusher nematodes are usually related to mobile and soil surface dwelling host (Griffin *et al*., 2005). *Steinernema carpocapsae* and *S. scapterisci* Nguyen & Smart are ambusher nematodes since they stand on their tails during foraging (Campbell and Gaugler, 1993). On the other hand, *Heterorhabditis* spp. and *S. glaseri* are cruiser nematodes and they mostly moving through the soil. These species do not nictate and usually associate with sedentary hosts (Lewis, 2002). Moreover, *Steinernema riobrave* Cabanillas, Poinar & Raulston and *S. feltiae* (Filipjev) Wouts, Mráček, Gerdin & Bedding lift part of their bodies for a few seconds, these species belong to intermediate foraging strategy (Griffin *et al*., 2005).

Nevertheless, IJs usually discriminate directly among potential hosts by host recognition behavior. Host recognition has been measured by recording changes in several behaviors in response to host-related materials (Lewis, 2002; Griffin *et al*., 2005). Grewal *et al*. (1993) tested the responses of IJs *H. bacteriophora, S. glaseri, S. carpocapsae* and *S. scapterisci* Nguyen & Smart to gut contents of four hosts: *Acheta domesticus* (L.), *Popillia japonica* Newman, *Spodoptera exigua* (Hübner) and *Blattella germanica* (L.). The results demonstrated that these EPNs responded differently to excretory products from the hosts and nematode infectivity was correlated with their behavioral responses. Nematode infection behaviors were also studied in several experiments. Wang and Gaugler (1999) reported that *S. glaseri* penetrated primarily to the gut of *P. japonica* whereas *H. bactriophora* did not succeed in penetration. Moreover, Cui *et al.* (1993) found that *S. glaseri* followed established routes of penetration by previous nematode.

4. Distribution pattern and environmental factors affecting entomopathogenic nematodes

EPNs are widely distributed throughout the world and have been found on all continents and many islands except Antarctica (Hominick *et al*., 1996; Hominick, 2002). The distributions of EPNs in soil and the survival of naturally occurring nematode populations depend on many factors (e.g. behavioral adaptation, suitable host, temperature, soil moisture, soil texture, relative humidity, ultraviolet, radiation etc.) which have been shown to affect EPNs dispersal and persistence (Kaya, 1990; Smits, 1996).

The surveys of EPNs are conducted by using the *Galleria* bait technique (Bedding and Akhurst, 1975). In broad sense, heterorhabditids have been isolated primarily from sandy coastal soils. Some species were found in calcareous soils (*H. indica*) or acidic soils (*Heterorhaditis marelatus* Liu & Berry and *H. bacteriophora*). Besides, *H. bacteriophora* ranges beyond coastal regions and *H. megidis* is broadly distributed in tropical forests (Phan *et al*., 2003) and weedy habitats (Stuart and Gaugler, 1994; Stock *et al*., 1996; Constant *et al*., 1998).

The information on habitat preference for Steinernematid is broadly distributed in many habitats. For example, *S. feltiae* was found in grasslands and woodlands in Europe and the USA (Griffin *et al*., 1991; Hominick, 2002), while *Steinernema affinis* (Bovien) Wouts, Mráček, Gerdin & Bedding was absent from woodland (Griffin *et al*., 1991). Steiner (1994) found that *S. feltiae, S. affinis* and *S. intermedia* (Poinar) Mamiya were typical of grassland ecosystems. In addition, *Steinernema kraussei* Steiner is mainly forest species in Europe and North America (Sturhan, 1999; Sturhan and Lišková, 1999).

To date, 55 species of Steinernematid nematode have been discovered in Africa, America, Australia, Europe and Asia (Adams and Nguyen, 2002; Nguyen, 2005; Nguyen and Hunt, 2007). The surveys of the Steinernematid in Southeast Asia resulted in 23 species. These include 11 species from china (*Steinernema aciari* Qiu, Yan, Zhou, Nguyen & Pang, 2004; *S. akhursti* Qiu, Hu, Zhou, Mei, Nguyen & Pang, 2005; *S. beddingi* Qiu, Hu, Zhou, Pang & Nguyen, 2005; *S. caudatum* Xu, Wang & Li, 1991; *S. ceratophorum* Jian, Reid & Hunt, 1997; *S. guangdongense* Qiu, Fang, Zhou, Pang and Nguyen, 2004; *S. hebeiense* Chen, Li, Spiridonov & Moens, 2006; *S. leizhouense* Nguyen, Qiu, Zhou & Pang, 2006; *S. longicaudum* Shen & Wang, 1992; *S. sichuanense* Mráček, Nguyen, Tailliez, Boemare & Chen, 2006 and *S. websteri* Cutler & Stock, 2003); nine species from Vietnam (*S. backanense* Phan, Spiridonov, Subbotin & Moens, 2006; *S. cumgarense* Phan, Spiridonov, Subbotin & Moens, 2006;

S. beapokense Phan, Spiridonov, Subbotin & Moens, 2006; *S. loci* Phan, Nguyen & Moens, 2001; *S. robusticulum* Phan, Subbotin, Waeyenberge & Moens, 2005; *S. sangi* Phan, Nguyen & Moens, 2001; *S. sasonense* Phan, Spiridonov, Subbotin & Moens, 2006; *S. tami* Luc, Nguyen, Reid & Spiridonov, 2000; *S. thanhi* Phan, Nguyen & Moens, 2001); one species from Indonesia (*S. hermaphroditum* Stock, Griffin & Chaerani, 2004) and two species in Thailand that are *S. siamkayai* and *S. minutum* (Maneesakon *et al*., 2010).

The optimum temperature for growth and reproduction of EPNs is commonly in the ranges of 25-28°C (Kaya, 1977; Molyneux, 1986) where the optimum temperature for storage is in between 5-15 °C (Georgis, 1990). In addition, temporal fluctuations of EPN populations indicate the importance of temperature and moisture changes due to seasonal changes (Püza and Mráek, 2007). Kaya (1977) found that temperature above 30°C tended to inhibit nematode development in a host. Temperature had a direct effect on nematode survival. Molynex (1985) mentioned that more than 60% of *S. carpocapsae* survived for 32 weeks at 10°C. Besides, Kung *et al*. (1991) found that survival and pathogenicity of *S. carpocapsae* were significantly greater at lower temperature (5-25°C) than at the highest temperature (35°C).

Soil moisture is probably the most important factor affecting nematode performance and survival rate in the soil (Molyneux and Bedding, 1984; Kung *et al*., 1991; Koppenhöfer *et al*., 1995; Grant and Villani, 2003a, 2003b) because all nematodes are aquatic organisms and need a film of water surrounding their body in order to move (Wallace, 1958; Norton, 1978). In soil, IJs move through the water film that coats the interstitial spaces or through water-filled pores, wider in diam than the nematodes' body. As the soil dries, the water film becomes thinner and larger pores drain off water which limit nematode movements (Wallace, 1958). On the other hand, nematode movement can also be restricted if the interstitial spaces are completely filled with water or the pores' diameter is much greater than that of the nematodes body (Quénéhervé and Chotte, 1996).

Nematodes survived for 32 d at 100% relative humidity (RH) whereas survival rate decreased at lower RH (Kung *et al.*, 1991). Kamionek *et al*. (1974) reported that under environmentally conditions at 85% RH, 98% of the IJs die after 102 h and *Steinernema* survived for 2 d at 25% RH. In addition, Kamionek *et al*. (1974) found that nematode placed in moist soil survived for 20 d when the soil was slowly dried at 70% RH.

Sunlight and ultraviolet radiation had direct effect on growth and development of Steinernematid and Heterorhabditid nematodes. The IJs of *S. carpocapsae* are sensitive to short UV radiation (254 nm) and natural sunlight. Short UV radiation inhibits nematode reproduction and development, but radiation does not affect juvenile mortality (Gaugler and Boush, 1978, 1979). Moreover, Gaugler and Boush (1978) reported short UV radiation rapidly reduced nematode pathogenicity, e.g. nematodes exposed to UV light for 7 min were unable to cause lethal infection in *Galleria mellonella* (L.) larvae.

5. Identification and diagnosis of nematode species

Several techniques have been used to identify EPNs which included morphological taxonomy and molecular techniques. Identification of EPNs is based on morphological characters of the first and second generations of male, female and infective juvenile. The scanning electron microscope (SEM) is also employed to observe external morphology of EPNs (Nguyen and Smart, 1996).

Molecular characters become increasingly useful for species identification and systematic in nematology. Molecular techniques provided a tremendous amount of objective data towards EPNs systematics. However, this technique can produce spurious results, even when care is taken to use and analyze them appropriately. Kluge (2004) mentioned that several molecular techniques such as protein electrophoresis, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLPs) and Polymerase Chain Reaction (PCR) should not be used to replace traditional morphological approaches. Early studies of polygenetic relationships of EPNs included PCR and RFLP analysis of the ITS repeat unit of rDNA region, first considering both EPNs families (Reid, 1994), and later focusing only on Steinermatidae (Reid *et al*., 1997). Nucleotide sequence analysis has been proved to be a good tool not only for nematode diagnostics at different taxonomic levels, but also for providing valuable data for phylogenetic studies (Adams *et al*., 1998; Nguyen *et al*., 2001, 2004; Stock *et al*., 2001).

6. Efficacy of entomophatogenic nematodes against insect pests

Entomopathogenic nematodes had a broad host range including those found in the soil, in cryptic habitats, on foliage, in manure and aquatic habitats (Klein, 1990; Sasnarukkit, 2003). Several laboratory and field studies have shown that insects from over 17 orders and 135 families are susceptible to EPNs for some degree (Smith *et al*., 1992). EPNs had shown effectiveness against black vine weevil, cranberry girdle, mole crickets, sciariid flies, fungal gnats, armyworms and cutworms (Gaugler *et al*., 2000).

All strains of *S. carpocapsae* tested are effective against the diamondback moth, *Plutella xylostella* (L.), larvae in laboratory conditions (Baur *et al*., 1995, 1998; Ratnasinghe and Hague, 1995, 1997, 1998). Ratnasinghe and Hague (1997) reported that *S. carpocapsae* killed *P. xylostella* larvae after 6 h exposure and caused 40% mortality in immature and mature pupae. In addition, application of *S. feltiae* at 2.5×10^{10} IJs/hectare against fall armyworm, *Spodoptera frugiperda* J.E. Smith, in vegetative field corn resulted in 33-43% infection of fall armyworm larvae. *Steinernema feltiae* at 4×10^{4} IJs/ear showed up to 71% infection and up to 53% reduction of *S. frugiperda* (Richter and Fuxa, 1990). Kaya and Hara (1980, 1981) illustrated that 75% pre-pupal and 63% pupal mortality of *S. exigua* were induced by *S. capocapsae*. In the USA, Feaster and Steinkrasus (1996) demonstrated that mortalities of *Helicoverpa zea* Boddie infected with *S. riobrave* at the rate of 3.7×10^{6} and 12×10^{7} IJs/m^2 of soil were 79.2 and 91.3%, in Little River County where 78.5 and 94.8% mortalities were detected at the rate of 5.2×10^{5} and 5.3×10^{6} IJs/m^2 in Washington, USA.

Schroeder (1987) showed that 100% mortality of the citrus weevils, *Diaprepes abbreviates* L. in field plots treated with *S. carpocapsae* resulting in excellent protection of tong seedling trees and reducing the emergence of *Diaprepes* adults more than 90%. Moreover, *Steinernema* sp. and *Heterorhabditis* sp. are effective against larvae of Scarabaeidae under field condition (Klein, 1990, 1993). Simoes *et al*. (1993) found that *S. glaseri* and *S.carpocapsae* reduced larval population of white grubs and Scarabeidae by 91% and 44% respectively, when applied at the rate of 10^6 IJs/m^2 in the field.

In Thailand, the use of entomopathogenic nematodes for controlling insect pests has started in 1986 (Somsook *et al*., 1986). The first research was conducted in Chantaburi province to determine the effectiveness of *S. carpocapsae* against bark eating carterpillar, *Cossus* sp. and *Microchlora* sp., the serious pests of langsart tree, *Lansium domesticum* Corrêa in the eastern part of Thailand. Nematode suspension (200 IJs/ml) was sprayed along the twigs of langsart tree and 80% mortality of bark eating caterpillar was recorded 24 h after application. Several field trials on 1991-1993 revealed the successiveness of *S. carpocapsae* for controlling larvae of striped flea beetle, *Phyllotreta sinuata* Stephen, sweet potato weevil, *Cylas formicarius* F. and beet armyworm, *S. exigua* (Biological Control Research Group, 2000).

In addition, Sasnarukkit (2003) found that mean mortalities of diamondback moth larva cause by IJs of *S. carpocapsae* is significantly greater than that of *S. siamkayai* and *H. bacteriophora*. In addition, *S. carpocapsae* caused 67.5% mortality at 10 IJs/larva after 48 h compared with 12.5 and 10% mortality caused by *S. siamkayai* and *H. bacteriophora* at the same concentration, respectively. Malee (2003) reported the LC_{50} of *S. carpocapsae* to *Ostrinia furnacalis* (Guenèe) and *Heliothis amigera* Hübner were 4.7 and 3.2 IJs/larva, respectively, under laboratory condition. Besides, Chongchitmate *et al.* (2005) found that the pathogenicity of *S. siamkayai*, *S. carpocapsae* and *S. riobrave* against *H. amigera* larvae were 22.5, 1.2 and 1.2 IJs/larva, whereas 18.0, 0.2 and 1.8 IJs/larva were recorded for *Spodoptera litura* (F.) larvae, respectively.

7. Interaction between entomophatogenic nematodes and other biocontrol agents

Conceivably, combining entomopathogenic nematodes with other biocontrol agents may result in synergistic interactions that would enhance the potential for biological control of insect pests (Jaques and Morris, 1981; Thurston *et al*., 1993, 1994; Koppenhöfer and Kaya, 1997; Koppenhöfer *et al*., 1999; Shapiro-Ilan *et al*., 2003; Ansari *et al*., 2004). Synergistic effects resulting from combination of entomopathogenic nematodes with other entomopathogens have been reported in a number of studies. For examples, synergistic virulence to *Cyclocephala* spp. was observed in combinations of entomopathogenic nematodes with bacterium, *Paenibacillus popilliae* (Dutky) (Thurston *et al*., 1993, 1994). Koppenhöfer and Kaya (1997) observed that a synergistic effect occurred against the white grub *Cyclocephala hirta* LeConte with a combined application of EPN and *Bacillus thuringiensis* subsp. *japonensis.* A combination of *H. bacteriophora* and *Beauveria bassiana* (Vuill) Balsamo against *S. exigua* and *S. carpocapsae* with *Beauveria brongniartii* (Saccardo) Petch against the white grubs resulted in a higher mortality than that caused by either the nematode or fungus alone (Barbercheck and Kaya, 1991). Koppenhöfer *et al*. (1999) also demonstrated that combining *H. bacteriophora* and *S. glaseri* with *B. thuringiensis* subsp. *japonensis* strain caused additive and synergistic effects against masked chafers, *C. hirta* and *C. pasadenae* Casey, in the greenhouse and under field conditions. However, interactions between entomopathogenic nematodes and other entomopathogens can also be antagonistic (Jaques and Morris, 1981; Koppenhöfer and Kaya, 1997; Baur *et al*., 1998; Brinkman and Gardner, 2000; Shapiro-Ilan *et al*., 2003).

8. Diamondback moth

Phylum: Arthropoda
Class: Insecta
Order: Lepidoptera
Family: Plutellidae
Genus: *Plutella*
Spcies: *xylostella*

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is one of the serious pests of cruciferous crop in Thailand (Areekul, 1966; Rushtapakornchai and Vattanatangum, 1986; Miyata *et al.*, 1988; Rushtapakornchai *et al.*, 1989; Roongsook, 1992) and in many parts of the world (Robertson, 1939; Bonnemaison, 1965; Salinas, 1967; Talekar *et al.*, 1985; Lim, 1992; Verkerk and Wright, 1996; Cheramakara, 2002). The diamondback moth has been recorded beyond latitude 60°N in Iceland, in the tropics zone and temperate zone and it prefers a warm environment for its development (Cheramakara, 2002). Its adaptability to a wide range of conditions, high reproductive rate and development of insecticide resistance has all contributed to the insect becoming increasingly difficult to control (Talekar *et al.*, 1985; Tabashnik *et al.*, 1990; Talekar, 1992). In addition, diamondback moth has the distinction of being the first insect to develop resistance to *B. thuringiensis* or BT (Kirsch and Schmutterer, 1988; Tabashnik *et al.*, 1990; Hama, 1992; Shelton and Wyman, 1992; Sun 1992; Shelton *et al.*, 1993; Pérez and Shelton, 1997).

8.1 Life cycle

Most adults of diamondback moth mate at dusk, on the day of emergence (Pivnick *et al.*, 1990; Kim and Lee, 1991). Female moths start laying eggs soon after mating and the oviposition period take place in the evening and during the night (Harcourt, 1985; Pongprasert, 1985). The female moth generally laid single or few eggs on the underside of leaves, mid-rib and principal veins (Bhalla and Dubey, 1986). After 3 to 6 d, the neonate larvae initiate feeding on foliage and the first-instar mines in the spongy mesophyll tissue, whereas older larvae feed from the lower leaf surface and usually consume all tissue except the wax layer on the upper surface (Harcourt, 1985; Sasnarukkit, 2003). When the fourth instar has completed its feeding, it constructs an open network cocoon on the lower surface of leaves and spends 2 d period of quiescence marking the pre-pupal stage (Harcourt, 1957, 1985; Pongprasert, 1985; Bhalla and Dubey, 1986). Adults feed on water drops or dew and short-lived.

8.2 Host plants

The host range of diamondback moth is presented on the family Cruciferae such as broccoli, brussel sprouts, cabbage, cauliflower, collards, edible rap, kale, kohlrabi, leaf mustard, radish, turnip and watercress (Chermakara, 2002). All cruciferous vegetables contain mustard oil and glycosides (Gupta and Thorsteinson, 1960; Hillyer and Thorsteinson, 1971)

In Thailand, diamondback moth is prevalent from February to April when optimum climatic conditions and food plants are more readily available (Vattanatangum, 1988; Sasnarukkit, 2003). However, diamondback moth can be observed throughout the year because cruciferous vegetables are planted all year round, especially in the central part of Thailand (Rushtapakornchai and Vattanatangum, 1986; Vattanatangum, 1988; Thongsungvorn, 1998; Sasnarukkit, 2003). In addition, cruciferous vegetables grown at high elevation in the northern part of Thailand (mostly cabbage, Chinese cabbage and cauliflowers) also serve as food sources for diamondback moth as well (Thongsungvorn, 1998; Chermakara, 2002).

8.3 Controlling of the diamondback moth

Controlling diamondback moth in the past normally based on chemical application. Chemical control of diamondback moth consisted of lead arsenate, nicotine, pyrethroid, organochlorine, organophosphate, carbamate, synthetic pyrethoids and insect growth regulators. Talekar *et al.* (1985) reported that botanical insecticide and *B. thuringiensis* were also employed to control the diamondback moth.

In Thailand, synthetic pyrethoids, fenvalerate, permethrin, cypermethrin and deltamethrin gave impressive control of diamondback moth in urban areas of Bangkok in 1976, hence, they were used extensively for 3 consecutive years, then diamondback moth started to develop resistance to these chemicals (Rushtapakornchai and Vattanatangum, 1986; Cheramakara, 2002). In 1989, abamectin was introduced into Thailand and this insecticide continuously gave good control in both laboratory and field tests (Sinchaisri *et al*., 1989, 1990, 1992; Rushtapakornchai *et al*., 1989, 1990, 1991). In addition, Sombatsiri and Temboonkeat (1987) reported that neem (*Azadirachta indica* Juss. *var. siamensis* Valeton) seed kernel extract gave a good insecticidal activity against the diamondback larvae. Moreover, *B. thuringiensis* has been used in diamondback moth control since 1972 and the use of 30% *B. thuringiensis* at 7 d interval or 4 d in severe infestation condition could reduce the damage caused by the diamondback moth (Vattanatangum, 1982; Rushtapakornchai *et al*., 1989, 1990).

The diamondback moth has several natural enemies and pathogens. Many parasites and predators have been recorded for diamondback moth larvae. Goodwin (1979) found that the diamondback moth had more than 90 species of parasitoid and most of diamondback moth larvae were parasitized by *Cotesia plutellae* Kurdjumov and *Diadegma* sp. Large larvae, pre-pupae, and pupae are often killed by the parasitoids *Microplitis plutellae* (Muesbeck), *Diadegma insulare* (Cresson), and *Diadromus subtilicornis* (Gravenhorst). Fungi, granulosis virus, and nuclear polyhedrosis virus sometimes occur in high density of diamondback moth larval populations. All strains of *S. carpocapsae* had shown effectiveness against the diamondback moth larvae in laboratory studies (Baur *et al.*, 1995 1998; Ratnasinghe and Hague, 1995, 1997, 1998) and Ratnasinghe and Hague (1997) reported that *S. carpocapsae* killed 100% *P. xylostella* larvae after 6 h exposure.

9. Common cutworm

Phylum: Arthropoda
Class: Insecta
Order: Lepidoptera
Family: Noctuidae
Genus: *Spodoptera*
Spcies: *litura*

The common cutworm, *Spodoptera litura* (F.) (Lepidoptera: Noctuidae), is the serious cosmopolitan pests of various economic plants in many parts of the world (USDA, 1982). This species also has a wide geographical range over the Asia continent and southern Australia (IIE, 1993; Zhang, 1994; Pogue, 2002). This insect has a number of common names including armyworm, rice cutworm, cotton leafworm or tobacco cutworm (USDA, 1982). The damage is caused by seriously defoliate and outbreak in a crop generally occurs within a warm weather condition as 25-30ºC (Boardman, 1977; Hill, 1983). The outbreak of this species depends upon amount of host plants, resistance to insecticides and favorable weather conditions (Rattanapan, 2007). The increasing of insecticide resistance in this species to many synthetic insecticides caused by inappropriate and indiscriminate uses, leaded to the development of resistance in *S. litura* and also contaminated in the environments (Surendra and Reddy, 1994). Thus, the development of other alternative methods such as biological control should be considered.

9.1 Life cycle

The female of *S. litura* lays eggs underneath the leaves in batches of 200-300 eggs which are usually covered with brownish-yellow scales. Eggs hatch within 3-5 d after oviposition. The newly hatched larvae are tiny, blackish-green, black head capsule and quickly disperse over the host plant. The young larvae can hang on a tiny silk produced from saliva for moving to other leaves and they become solitary in late instar. Normally, there are six larval instars, feed only at night and hide in the soil during the day. Pupation takes place in the soil next to host plant and pupa is red-brown in color. The adults emerge after 6-7 d, nocturnal moths are active at night and mate several times. Mature moths have a 30 mm wingspan, 15-20 mm in length, grayish-brown moths with wavy markings on upper wings. The male is distinguished from female moth by having blue-grey band on forewings and slender abdomen. The whole life cycle requires 25-30 d depending upon time of the year (Areekul *et al*., 1963; Hill, 1983; Rattanapan, 2007).

9.2 Host plant

The host range of this species covers over 120 species of economically importance crop species such as apple, asparagus, groundnut, banana, bean, beet, broccoli, cabbage, carrot, castor bean, citrus, cocoa/cacao, coffee, corn, cotton, derris, eggplant, ginger, lettuce, lotus, mango, mint, mulberry, mung bean, onion, orange, orchid, pak choi, papaya, pea, peanut, pepper, pineapple, potato, pumpkin, radish, rice, rose, rubber tree, sorghum, soybean, strawberry, sugarcane, sunflower, taro, tea, tobacco, tomato and weed (Thomas *et al*. 1969, USDA 1982, Balasubramanian *et al*., 1984; Sharma, 1994; Zhang, 1994; CAB, 2003; Pogue, 2003; Venette *et al*., 2003).

9.3 Controlling of the common cutworm

Numerous insecticides have been reported to control *S. litura* under laboratory and field conditions (Issa *et al*., 1984; Abo-El-Ghar *et al*., 1986). Singh and Nath (1998) tested the efficacy of 17 synthetic insecticides on *S. litura* in groundnut field and the effective insecticides were cypermethrin (0.016%), deltamethrin (0.003%), endosulfan (0.05%), fluvalinate (0.05%) and phosphamidon (0.05%). In addition, Natesan and Balasubramauian (1981) reported that 0.02% diflubenzuron and 0.06% chlorpyrifos caused high mortality on *S. litura* larvae and also had additive effect when both insecticides were combined. Besides, other insecticides have been reported as effective insecticides to control this species such as phoxim, methamidophos, quinalphos, fenvalerate and diflubenzuron (Ramzan and Singh, 1982). Moreover, numerous studies showed the possibility of biological control such as parasites, predators, microorganism and nematodes (Whitcomb and Godfrey, 1991; Evans and England, 1996; Yasuda, 1998; Yeargan, 1998). Plant extracts can suppress the pest population by anti-feedant, anti-growth and toxicity activities (Ruamthum, 2002; Bullangpoti, 2004; Noosidum, 2007).

10. Greater wax moth

Phylum: Arthropoda
Class: Insecta
Order: Lepidoptera
Family: Pyralidae
Genus: *Galleria*
Sepcies: *mellonella*

Greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), is associated wih honey bees, damages beehives and injures combs during larval stage (Smith, 1965; Burges, 1978; Chang and Hsieh, 1992, Haewoon *et al*., 1995). Normally, the moths attack only abandoned beehives or active ones in which the bee colony has been weakened (Caron, 1992). This species is a serious pest of economic importance in bee rearing and world-wide in distribution (Roversi *et al*., 2007). The larvae of *G. mellonella* have been shown in several studies to be a well-suited model organism for in-vivo toxicology, insect immune system study and pathogenicity testing (McCaleb and Kumaran, 1979; Pryce *et al*., 1990; Dunphy and Webster, 1991; Kavanagh and Reeves, 2004; Scully and Bidochka, 2006; Kavanagh and Fallon, 2010; Mak *et al*., 2010). Furthermore, phosphine gas, methyl bromide (Charriere and Imdorf, 1997), paradichlorobenzene, phestoxin (Goodman *et al.*, 1990), male sterile technique (MST) (Hornitzky, 1994; Walker *et al*., 1975) have been commonly used to control greater wax moth.

The females lay their eggs in the crevices of beehives. The newly hatched larvae tunnel into the comb, leaving and making the punctured comb while causing honey leakage. The larvae feed on the honeycomb inside bee nests resulting in the most damage to a hive (Chang and Hsieh, 1992). The mature larvae make trails of web to spin cocoons before pupation take place. The adult moths have brownish front wings, with wing-spans of 30–41 mm (Chang and Hsieh, 1992; Jarfari *et al*., 2010).

11. Meal worm

Phylum: Arthropoda
Class: Insecta
Order: Coleoptera
Family: Tenebrionidae
Genus: *Tenebrio*
Sepcies: *molitor*

Meal worm or darkling beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), is considered a pest of grain products and distributed world-wide (Anonymous, 1967). The immature stages are typically used as a food source (Ramos-Elorduy *et al*., 2002) for several pets such as wild birds and fishs. Fisher commonly use *T. molitor* larvae for fishing bait. This species is also used in biological research. Besides, *T. molitor* was also used as a model system for studies in biology, biochemistry, evolution, immunology and physiology (Hernández 1987, 1988; Ramos-Elorduy and Pino, 1990; Tsybina *et al*., 2004).

The eggs of *T. molitor* hatch in 4-19 d after female oviposition. Larvae typically measure about 2.5 cm and they undergo repeated molting 9-20 times until pupate. The young pupa in creamy white color, changes slowly to brown. Adults are generally between 1.25 and 1.8 cm in length (Anonymous, 1967).

12. Rhinoceros beetle

Phylum: Arthropoda

Class: Insecta

Order: Coleoptera

Family: Scarabaeidae

Genus: *Oryctes*

Sepcies: *rhinoceros*

Rhinoceros beetle, *Oryctes rhinoceros* MacLeay (Coleoptera: Scarabaeidae), is a serious pest of coconut and oil palm, occurring from India through South-East Asia and South Pacific (Bedford, 1973, 1980, 1986; Young 1986). *Oryctes rhinoceros* adults normally mate in their feeding and breeding sites. The oviposition usually takes place where the young beetles left (Cumber, 1957; Zelazny, 1975). Adult females lay their eggs in rotting palm, compost and decay (Bedford, 1980; Young 1986; Hocherg and Waage, 1991). Annually, *O. rhinoceros* larvae develop in the top of the dead coconut palm, compost or decaying wood. The beetle bores into the tender part, biting the fibrous portion (Bedford, 1973). This insect can delay its larval development during unfavorable climatic or nutritional conditions up to 14 months (Bedford, 1973, 1980). This pest is controlled by treating the breeding places (manure dumps or compost pits) with fungus, *Metharizium anisopliae* Metschnikoff and virus, *Rhabdionvirus oryctes* Hüger (Bedford, 1973).

MATERIALS AND METHODS

1. Nematode collection and isolation

1.1 Soil sample

One hundred and sixty eight soil samples were collected from undisturbed soil close to the national parks of tropical rain forest in five southern Thailand provinces (Surat Thani, Nakorn Sri Thammarat, Ranong, Pang Nga and Chumporn) between May 2008 and February 2009 (Fig. 1). Samples were taken by a hand shovel with a depth of 20 cm from soil surface. Each sample was placed in polyethylene bag and closed tightly to prevent water loss. Samples were kept in coolers (15-20°C) and transported to the laboratory at the Department of Entomology, Kasetsart University). For each location, GPS coordinates (Global Positioning System; Garmin®, Thailand) and environmental characteristics such as air temperature, humidity, soil temperature, pH, EC (dS/m) and percentage of moisture content were recorded (Fig. 2A).

1.2 Nematode isolation

EPNs were recovered from soil samples by the modified insect baiting technique (Kaya and Stock, 1997). Soil samples were thoroughly mixed with tap water to moisten the soil. Twenty last-instar larvae of *G. mellonella* were placed on the trap of 200 g of soil sample in plastic containers (4 containers/soil sample). Each container was covered with a lid, turned upside down and kept at room temperature (Fig. 2B). After 7-9 d, insects were recovered for parasitized cadavers, recognized by the change of their color (Fig. 2C). Then, the cadavers were placed in modified white traps (White, 1927). Each group of infected larvae was placed on a layer of moisten cotton on a small Petri dish (5.5 cm diam). The small dish holding the cadavers were floated in 0.1% formaldehyde inside plastic box (7×10×5 cm) with lid and kept in the dark at room temperature. After 7 d, the infective juveniles emerged from the cadavers surrounding the Petri dish and were surface sterilized with 0.1% hyamine and rinsed three times with 0.1% formaldehyde. Infective juveniles then were stored in 0.1% formaldehyde (Fig. 2D).

1.3 Nematode culture

The isolated EPNs were maintained in the laboratory by recycling through *G. mellonella* larvae (Kaya and Stock, 1997) every 1-2 months. Approximately 500 IJs were applied to 4.5 cm diam Petri dish lined with Whatman #5 filter paper containing 5 last-instar *G. mellonella* larvae. The Petri dishes were incubated at room temperature for 3 d. The infected larvae were transferred on the modified white trap (Lewis and Gaugler, 1994). Infective juveniles emerging from the larval cadavers were collected in distilled water and stored at 15-20°C. The IJs were used for subsequent studies within 2–4 weeks (Figs. 2E and F).

Figure 1 Map showing the sampling sites form 5 provinces in southern Thailand.

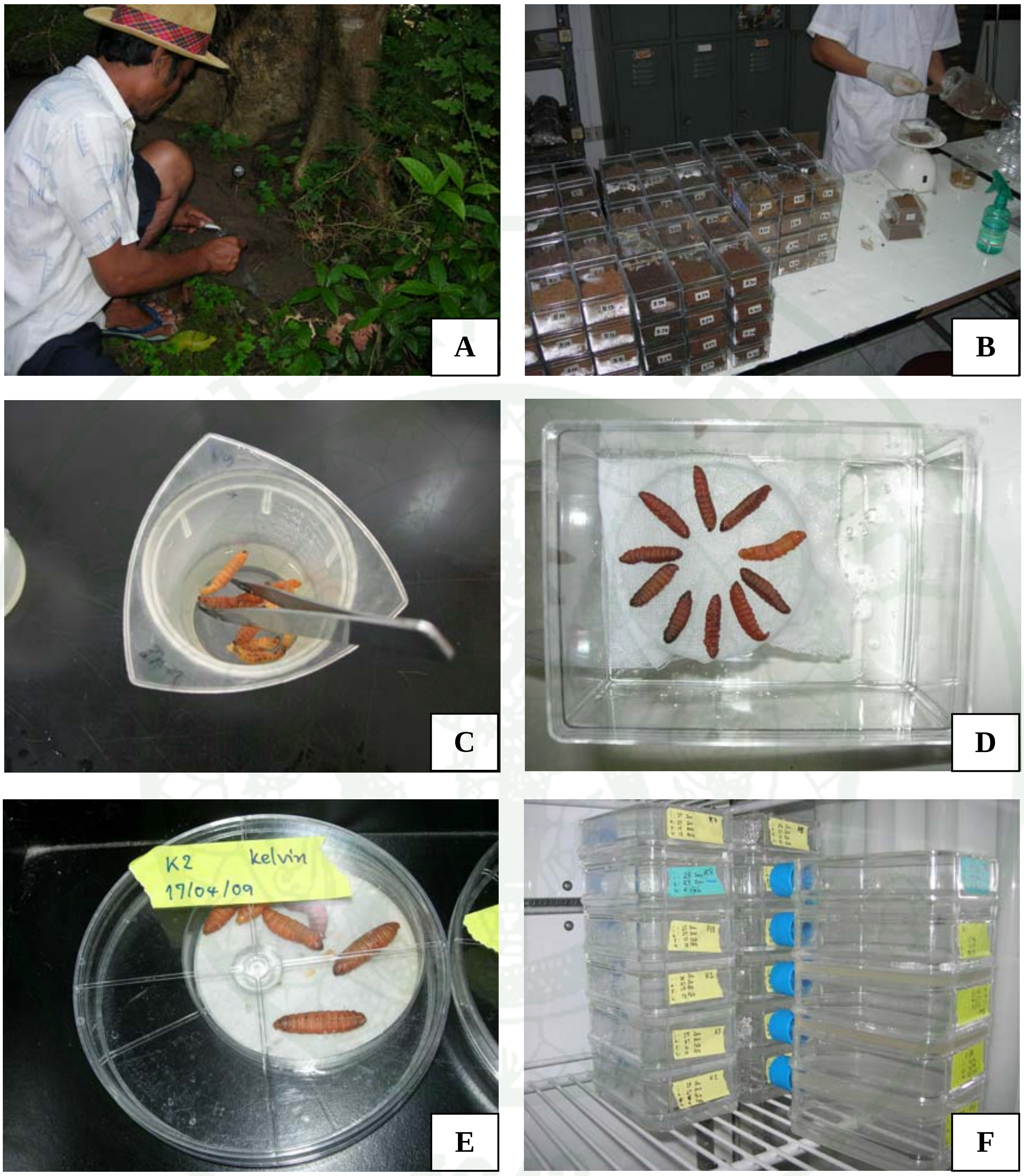

Figure 2 Nematode isolation and nematode culture method. A: soil collecting; B: nematode isolation by *Galleria mellonella* baiting technique; C: infected cadaver cleaning; D: infected cadavers were placed in white trap; E: nematode culture by modified white trap; F: fresh IJs were stored in an incubator.

2. Nematode identification

2.1 Light microscopy

First and second generation *Steinernema* sp. adults (isolate K7 and K8) were obtained by dissecting infected larvae at 48 and 96 h after infection, respectively (Fig. 3A). Infective juveniles (IJs) were obtained from emerging nematodes after infection for 6 d. All selected nematodes were cleaned and then heat killed at 60°C in Ringer's solution (Kaya and stock, 1997). Nematodes were fixed in triethanolamine formalin (TAF) for 24 h (Courtney *et al.*, 1955) and then transferred to anhydrous glycerine (Seinhorst, 1959). Quantitative measurements and examination were made using a microscope equipped with an ocular micrometer (Dino capture ver. 2.0) and drawings were made with the aid of a camera lucida (Stock *et al.*, 1998) using an Olympus BH-2 light microscrope.

2.2 Scanning electron microscopy

First generation *Steinernema* sp. adults (isolate K7 and K8) were dissected from *G. mellonella* larvae in Ringer's solution and rinsed for 5 min each in Ringer's solution three times. Third-stage juveniles (IJs) were rinsed for 15 min in Ringer's solution three times. All nematodes were relaxed and killed by heating in a water-bath (60°C) for 2–3 min and fixed in 2% glutaraldehyde (diluted in Ringer's solution) over night at room temperature. Fixed nematodes were rinsed in distilled water three times and then post-fixed in osmium tetraoxide solution (OsO_4) for 1 h. Nematode samples were rinsed in distilled water again and dehydrated at 10 min intervals through 10%, 20%, 30%, 50%, 70%, 90% and tree times in 100% ethanol. They were then critical point dried in liquid CO_2, mounted on SEM stubs, coated with gold and scanned under a Jeol JEM 5410 LV scanning electron microscrope (Figs. 3B and C).

2.3 Cross hybridization test

Cross hybridization studies were conducted using the hanging blood drop method as described by Kaya and stock (1997). *Steinernema glaseri, S. siamkayai S. carpocapsae* and *S. riobrave* were used for assessing reproductive compatibility of the new species. All IJs were sterilized in 0.1% hyamine for 20 min and rinsed three times in distilled water. Sterilized IJs were placed in agar plate and incubated at 25°C in dark room until pre-adults were formed. A drop of *G. mellonella* larva haemolymph was placed on a cover glass and 5 pre-adult females *Steinernema* sp. isolate K8 with five males of another *Steinernema* sp. and vice versa were placed in the hanging drop. The hanging drop slides were then placed on a small Petri dish (60 mm×15 mm) which was placed in a larger Petri dish (100 mm×15 mm) lined with Whatman #5 filter paper that was saturated with distilled water. The dishes were wrapped with parafilm and incubated at 25°C in the dark room. There were 10 replicates/cross breeding tests and tests were repeated twice (Fig. 3D).

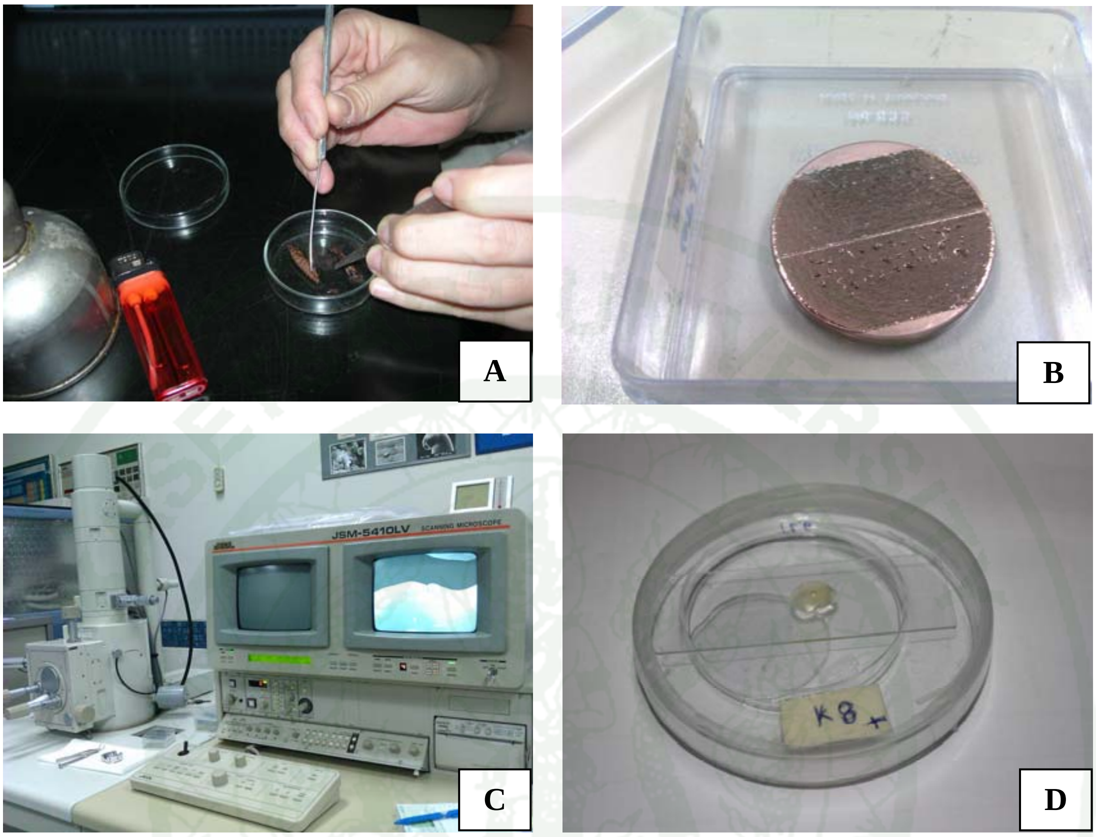

Figure 3 Nematode identification. A: nematode dissection; B: gold coated nematode sample for SEM; C: scanning electron microscope (SEM); D: an experimental disc for cross hybridization test.

2.4 Molecular characterization and phylogeny analysis

Nematodes were identified by species-distinctive PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) bands of the ribosomal DNA internal transcribed spacer (ITS) region. Representatives of each restriction profile were then sequenced and matched with known sequences using the Genbank search engine BLAST. Individual adult nematodes were dissected from infected *G. mellonella* cadavers, cut in half and placed in a mix of 20 µl 'Chelex 100' resin (5%, Bio-Rad, Hercules, CA), and 1 µl proteinase K solution (20 mg/ml; Sigma, St. Louis, Missouri). The mix was incubated at 56°C for 1 h, 100°C for 8 min, and then cooled to 40°C for 30 sec. After vortexing for 30 sec, samples were stored at -17°C. The ITS-1 and ITS-2 regions of ribosomal DNA were amplified using universal 18S and 28S primers (no. 93, 5' TTGAACCGGGTAAAAGTCG and no. 94, 5'TTAGTTTCTTTTCCTCCGCT) designed by Nadler *et al.* (2000). PCR amplification was performed in a reaction volume of 25 µl, containing 2.5 µl of 10xPCR-buffer, 14.6 µl of H_2O, 3 µl of $MgCl_2$ (25mM), 0.5 µl of dNTPs (10 mM), 0.4 µl Taq polymerase (5 units/µl, Gene choice T-18), 1.25 µl of Primer forward (10 µM), 1.25 µl of Primer reverse (10 µM), and 1.5 µl of DNA. All PCR reactions were conducted in an Applied Biosystems 2720 thermocycler with a profile of: 1 cycle at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 50 sec. The last step was a post-amplification extension at 72°C for 4 min. Digestion of amplification products was performed by adding 5 µl PCR product in 25 µl reaction buffer (enzymes Alu I, Dde I, and Sau 3AI, Promega: Madison, WI). Ten µl of the digested product was loaded on a 1.8% agarose gel and electorphoresed in 1 X TBE at 100 V for 5 h. Restriction fragments were visualized by GelRed stain (Biotium, Hayward, CA) and the restriction profiles were compared to the profiles of known isolates as well as restriction digest patterns generated from GenBank sequences.

Amplified products were purified using enzymatic treatment with exonuclease I and shrimp alkaline phosphatase (PCR product pre-sequencing kit, USB Corporation). Internal primers used for sequencing included no. 264 5' CGTTTTTCATCGATACG and no. 389 5'TGCAGACGCTTAGAGTG (Nadler *et al*., 2000) for heterorhabditid species and no. 533, 5' CAAGTCTTATCGGTGGAT and no. 534, 5' GCAATTCACGCCAAATAA for steinernematid species. Contigs were assembled using Aligner (Version 3.6.1) and a BLAST search performed on the final consensus sequence (Table 1). Phylogeny tree was built by Maximum Parsimony (MP) with a bootstrap 100 replications for *Steinernema* sp. using CLC sequence viewer Program Ver. 6.5.1 to visualize in tree viewer.

Table 1 *Steinernema* species recognized with available Internal Transcribed Spacer sequences deposited in the GenBank used in this study.

Steinernema species	Locality	Accession number
S. abbasi	UK	AY248749
S. aciari	China	AY787660
S. affine	UK	AY230159
S. akhursti	China	DQ375757
S. anatoliense	Jordan	EU200356
S. anomali	USA	AF192985
S. apuliae	Italy	HQ416968
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. hermaphroditum	Indonesia	AY598358
S. intermedium	USA	AY230172
S. jollieti	USA	GU569051
S. karii	Kenya	AY230173
S. khoisanae	South Africa	DQ314287
S. kraussei	Italy	AY230174

Table 1 (Continued)

Steinernema species	Locality	Accession number
S. kushidai	Japan	AB243441
S. loci	Viet Nam	AY355443
S. longicaudum	China	AY230177
S. minutum	Thailand	GU647156
S. monticolum	Korea	AF122017
S. neocurtillae	USA	AF122018
S. oregonense	USA	AY230180
S. pakistanense	Pakistan	AY230181
S. puntauvense	Costa Rica	FJ381665
S. puertoricense	USA	AF331903
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. scarabaei	Netherlands	FJ040424
S. siamkayai	Thailand	AF331917
S. sichuanense	China	DQ884965
S. silvaticum	China	DQ399663
S. tami	Viet Nam	AY171280
S. texanum	USA	EF152568
S. thanhi	Viet Nam	AF355444
S. thermophilum	India	DQ665651
S. unicornum	Chile	GQ497167
S. websteri	Costa Rica	FJ381666
S. weiseri	France	FJ165549
S. xueshanense	China	FJ666052
S. yirgalemense	Ethiopia	AY748450

3. Behavioral observations

Nematode behaviors were investigated by classifying each isolate's foraging strategy and ability to recognize hosts. Nematode foraging behavior was examined by measuring the difference in movement rate in a smooth agar dish versus a sand-sprinkled dish. The hypothesis was ambushing nematodes would show a significant decrease in net movement rate on sand-sprinkled agar compared with smooth agar because they would be able to nictate. Water agar (2%) was prepared and approximately 60 ml was poured into each Petri dish (9 cm diam) and cooled for 1 h. The sandy dishes were prepared by sprinkling sand particles onto the top of the cooled agar. Four concentric circles of 1, 2, 3 and 4 cm diam were drawn from the center of the lid and 2 perpendicular lines were also drawn on the lid to make 4 equal quadrants. Approximately 300 IJs were then placed into the center of each experimental plate and covered with the prepared lid. The numbers of IJs in each circle from 2 opposite quadrants were counted every 10 min after exposure for 30 min. The net movement rate for each isolate was calculated as:

$$\frac{[(2\times A)+(3\times B)+(4\times C)]\times 100}{N}$$

where A, B, C were the numbers of IJs in second, third and fourth circles from the center, respectively; 2, 3 and 4 were the distance in cm from the center as 2, 3 and 4 cm; and N was the total number of IJs in 2 opposite quadrants (Glazer and Lewis, 2000). Five replications were performed for each treatment (Figs. 4A-C).

In the host recognition assays, approximately 60 ml of 2% water agar was poured into a plastic Petri dish (9 cm diam) and cooled for 1 h. Lids were marked as above. A small hole was made on the lid at the edge of one quadrant and a 0.5 cm diam hole was also made at the center of the first circle of the lid. Two last-instar *G. mellonella* larvae were put into a 1 ml plastic pipette tip and sealed with parafilm. The lid was sealed to the plate with parafilm and modeling clay was used to affix the pipette tip to a small hole in the lid. The center hole was closed with paper tape and the arena was kept at room temperature for 1 h before starting the experiment. Approximately 300 IJs were then placed into the center of each experimental plate and the hole was closed with tape. The numbers of IJs in each arc in the quadrant containing pipette tip and the one opposite were counted every 10 m after exposure for 30 m. Movement was calculated as:

$$\frac{[(2\times A)+(3\times B)+(4\times C)]-[(2\times D)+(3\times E)+(4\times F)]}{N}\times 100$$

where A, B and C were the number of IJs in second, third and fourth arcs from the center in the quadrant with the pipette tip, respectively; D, E and F were the number of IJs in the second, third and fourth arcs from the center in the opposite quadrant, respectively; each number is multiplied by the distance in cm from the center for each arc, and N was the total number of IJs in 2 opposite quadrants (Glazer and Lewis, 2000). Five replications were performed for each treatment (Fig. 4D).

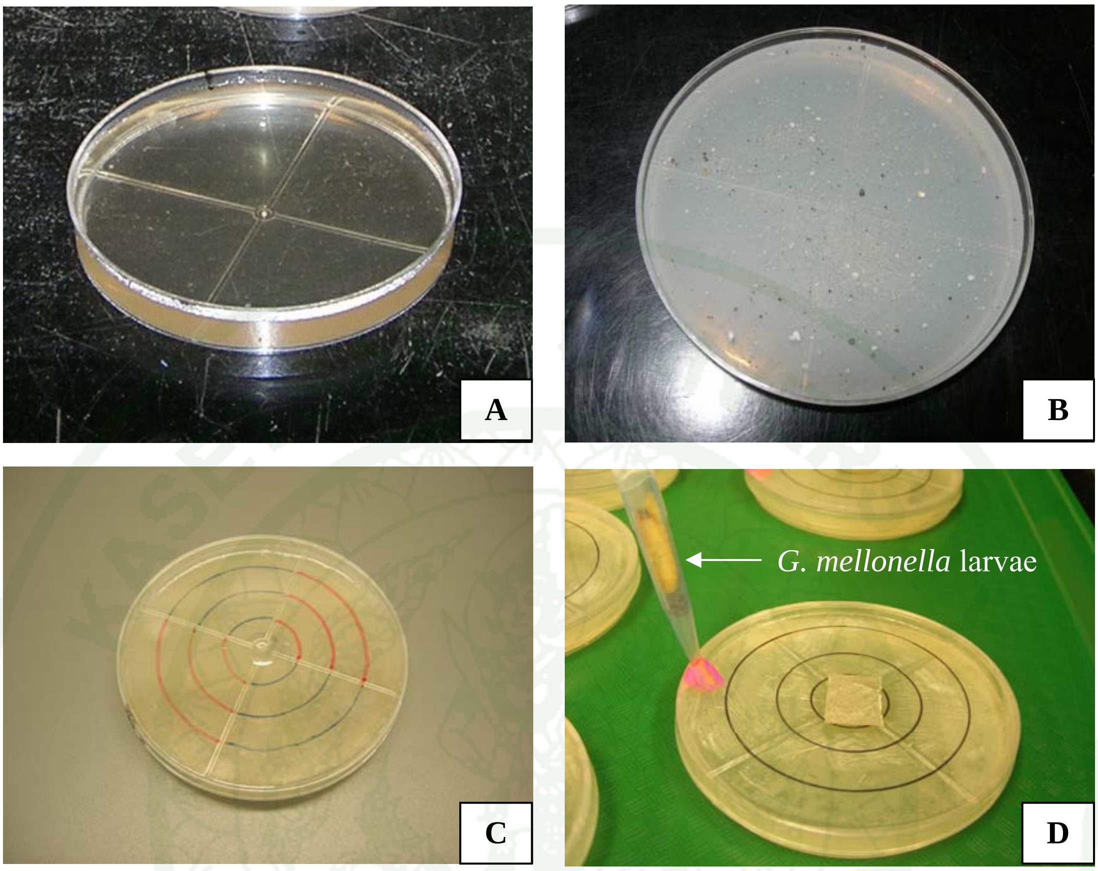

Figure 4 Behavioral observations. A: an water agar plate; B: sand-sprinkled plate; C: an experimental disc and counting lid for foraging behavior test; D: an experimental disc for host recognition assay.

4. Nematode horizontal dispersion

Dispersal behavior was examined in a square plastic container (19×19 cm) (Rubbermaid, Wooster, OH) containing 700 g of sand sample (<2 mm diam). Two sand samples including pure sand (100% sand) and sandy clay loam (70% sand+30%clay) were used as the experimental arena. The sand was moistened with distilled water to 10% moisture. Five thousand IJs in 1000 µl of nematode suspension were pipetted onto the sand in the center of the container and incubated at 25°C for 72 h. After incubation, sand in each container was divided into 25 squares (3.8×3.8 cm) with a spatula. The center square and the squares on the edge of the arena were placed individually into 250 ml beakers. The sand was carefully washed 3 times with distilled water to extract the IJs. The number of non dispersed IJ extracted from a center section and the furthest dispersed IJ from the squares at the edge were counted. The IJ from the squares from the edges were combined, and the proportion of dispersed and non-dispersed IJ was calculated.

5. Virulence of EPNs against selected insect pest in the laboratory.

5.1 Virulence of various nematode isolates on *P. xylostella* larva and pupa.

Cultures:

Plutella xylostella larvae were collected from the cabbage filed in Nonthaburi province, Thailand. The colony were maintained on cabbage leaves under laboratory conditions at 25°C and a photoperiod of 12:12 (L:D) for more than 5 generations. The adults were fed 20% glucose solution through a cotton wick and eggs laid by the adults were transferred to a separate rearing container. The larvae were provided with fresh cabbage leaves daily. The third-instar larvae were used for inoculation in the experiments (Figs 5A and B).

Larval test:

Test was conducted in 24 well-plates (17 mm diam) lined with filter paper (Whatman #1). Kale leaf discs (10 mm diam) were placed into the well and then 30 µl of predetermined concentrations of nematode suspension were applied. One third-instar *P. xylostella* larva was placed in each well, covered with lid and sealed with parafilm. Larval mortalities were recorded at 48 h after treatment (20 replications/treatment). Six dosages were tested (0, 15, 30, 50, 100 and 300 IJs/larva) and 4 nematode species including *H. bacteriophora, S. siamkayai, H. indica* isolate K4 and *Steinernema* sp. isolate K8 were used in this experiment (Figs. 6A and B).

Pupal test:

Test was conducted in 24 well-plates lined with filter paper as aboved mention and 30 ml of nematode suspension were then applied into the well. One *P. xylostella* pupa was placed in each well and covered with its lid. Pupal mortalities were recorded at 48 h after treatment (20 replications/treatment). A predetermined concentration at 300 IJs/larva was tested and 4 nematode species including *H.*

bacteriophora, S. siamkayai, H. indica isolate K4 and *Steinernema* sp. isolate K8 were used in this experiment (Figs. 6A and B).

5.2 Virulence of various nematode isolates on *S. litura* larva and pupa.

Culture:

Spodopterla litura larvae were collected from the cabbage filed in Nonthaburi province, Thailand. The colony were maintained on artificial diet under laboratory conditions at 25°C and a photoperiod of 12:12 (L:D) for more than 5 generations. The adults were fed 20% glucose solution through a cotton wick and eggs laid by the adults were transferred to a separate rearing container (Figs. 5C and D).

Larval test (second and third-instar larvae):

Test was conducted in 24 well-plates (17 mm diam) lined with filter paper (Whatman #1). Kale leaf sections (10 mm diam) were placed into the well and then 30 µl of predetermined concentrations of nematode suspension were applied. One *S. litura* larva was placed in each well, covered with lid and sealed with parafilm. Larval mortalities were recorded at 48 h after treatment (20 replications/treatment). Six dosages were tested (0, 15, 30, 50, 100 and 300 IJs/larva) and 4 nematode species including *H. bacteriophora, S. siamkayai, H. indica* isolate K4 and *Steinernema* sp. isolate K8 were used in this experiment. This method was also conducted for third-instar test (Figs. 6A and B).

Pre-pupal and pupal tests:

Test was conducted in insect rearing cup (4.5 cm diam) with 10 g of sterile soil. One *S. litura* pre-pupa was then placed in each cup and 300 IJs in 30 µl of nematode suspension were applied. Soil moisture was 15% and incubation temperature was 25°C for all nematode dosage treatments. Mortalities were recorded every 24 h after treatment until the pupa turned to adult (20 replications/treatment). At the end of the incubation period, adults were dissected and infection incidences were recorded. Four nematode species including *H.bacteriophora, S. siamkayai, H. indica* isolate K4 and *Steinernema* sp. isolate K8 were used in this experiment and this method was also conducted with *S. litura* pupa (Figs. 6C and D).

5.3 Virulence of various nematodes on *T. molitor* and *O. rhinoceros* larvae

Insect samples:

Last-instar *T. molitor* larvae were received from Professor Lewis's laboratory at the University of California, Davis, USA. The colony of *O. rhinoceros* larvae were collected from golf course at Ram Inthra distrinct, Bangkok, Thailand

Tenebrionid test:

Three Thai isolates; *H. indica* isolate K4, *H. baujardi* isolate K6 and *Steinernema* sp. isolate K8 were investigated their virulence against *T. molitor* larvae in filter paper assay. A 50 µl drop containing 300 IJs was applied to 24 well-plates lined with Whatman #1 filter paper. One last-instar *T. molitor* was placed into each well, covered and held at 25ºC (20 *T. molitor* larvae/concentration). Mortality rates were calculated after 24, 48, 72 and 96 h after exposure (Fig. 6A).

Coconut root weevil test:

Four nematodes isolates; *H. indica* isolate K4, *S. glaseri*, *S. siamkayai* and *Steinernema* sp. isolate K8 were selected to investigate nematode virulence against *O. rhinoceros* larvae in filter paper assay. A 50 µl drop containing 300 IJs was applied to an insect rearing cup (4.5 cm diam) lined with Whatman #1 filter paper. One first-instar *O. rhinoceros* was placed into each rearing cup after which the cup was covered and held at 25ºC (20 *O. rhinoceros* larvae/concentration). Mortality rate were calculated after 24, 48, 72 and 96 h exposure (Fig. 6E).

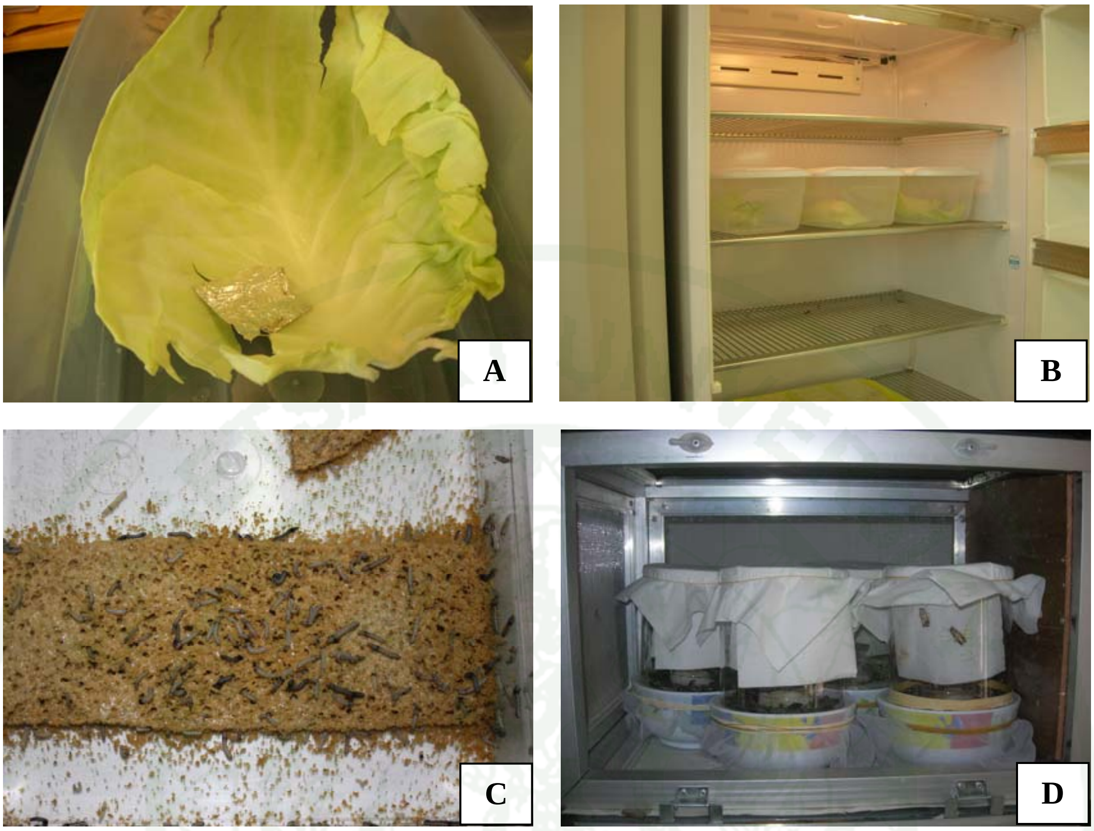

Figure 5 Insect rearing. A: *Plutella xylostella* eggs on cabbage leaf; B: insect rearing container maintained in an incubator; C: *Spodoptera litura* larvae in artificial diet; D: mating cage for *S. litura* adults.

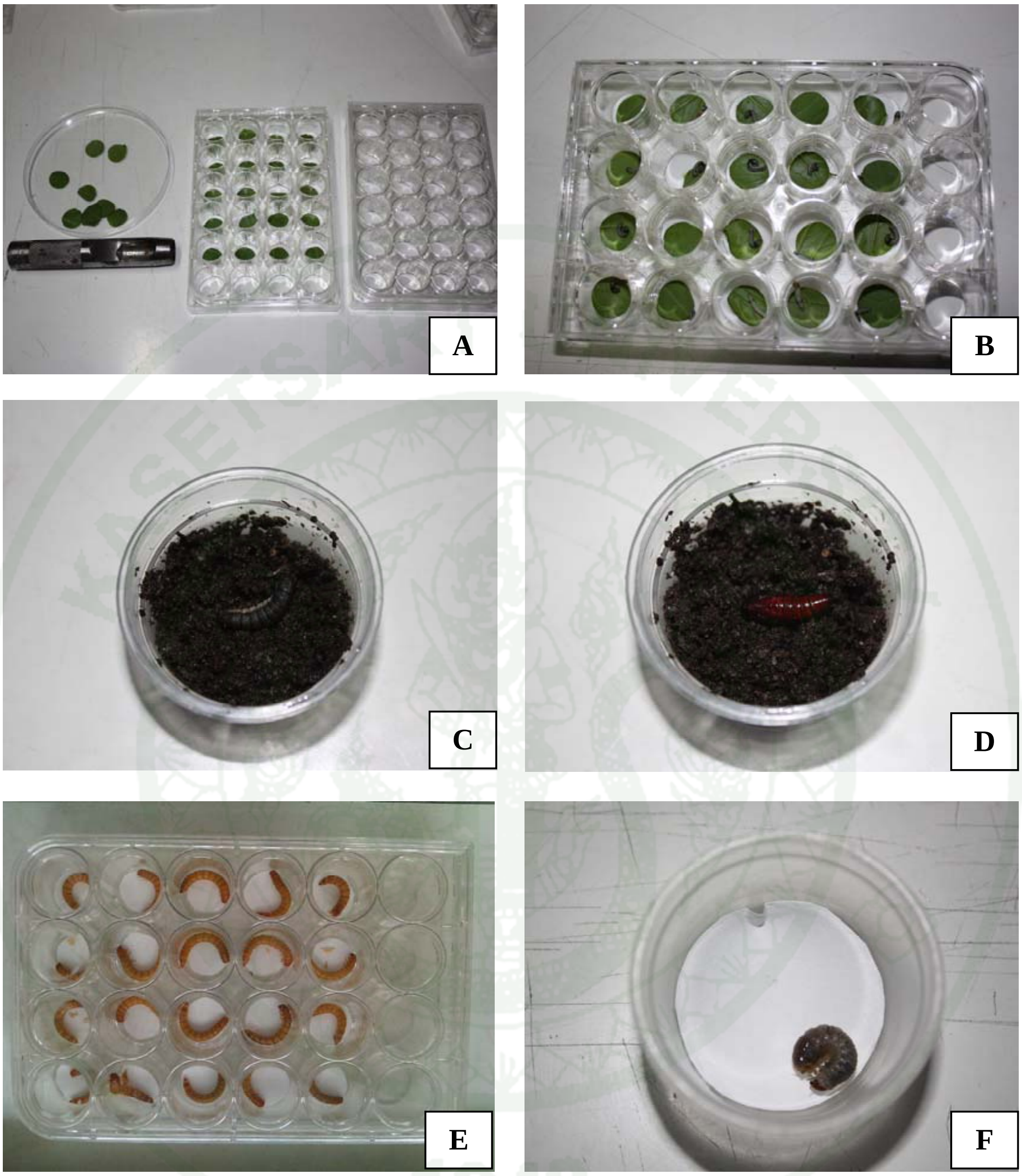

Figure 6 Nematode virulence tests. A and B: leaf discs bioassays; C: pre-pupal test in sandy cup; D: pupal test in sandy cup; E: filter paper test for *Tenebrio* larvae; F: filter paper test for *Oryctes* larva.

5.4 Virulence of various nematode isolates on *G. mellonella* larva

Culture:

Adult of *G. mellonella* were fed with 10% honey in plastic boxes (11×11×6.5 cm) containing a piece of paper and covered with a lid with ventilation screen drilled on the top. Egg masses were appeared on the paper after 3-4 d. Paper containing egg masses was cut and placed on the artificial diet in a new plastic box (13×17×6.5 cm) (Somsook, 1997; Tangchitsomkid, 2000). The eggs hatched within 6-7 d and the larvae fed on the diet for 3 weeks. The last-instar larvae were collected to be used in the experiments. Larvae were stored in plastic containers at 10°C for 2 weeks (Figs. 7A-F).

Virulence bioassay:

Four nematode strains; *H. indica* isolate K1, *H.indica* isolate K4, *H. baujardi* isolate K6 and *Steinernema* sp. isolate K8 were selected to investigate nematode virulence against *G. mellonella* larvae in filter paper assays. A 50 µl drop containing 1, 3, 5, 10, 25 or 50 IJs was applied to 24 well-plates lined with Whatman #1 filter paper. One last-instar *G. mellonella* was placed into each well after which the dish was covered and held at 25ºC, (20 larvae/concentration). Mortality rates were calculated after 24 h exposure (Figs. 8A and B).

Sand column bioassay:

Nematode infection rates were determined in column bioassays. Two soil types, sandy loam (80% sand + 20% clay) and sandy clay loam (65% sand + 35% clay), were prepared from standard sand and clay flour (No. X -23, AMACO® USA). Two hundred g of soil were added to PVC columns (9 cm long, 5 cm diam). Three last-instar *G. mellonella* larvae were held in a small aluminum cage to restrict movement, and were then placed at the bottom of each column. Five hundred IJs were then applied to the soil surface and the column was covered with a plastic lid. All columns were kept at room temperature and the test was replicated 10 times for each nematode strain. Dead insects were removed daily and all cadavers were dissected to determine the number of IJs inside the infected host. Three commercially available EPNs (*H. bacteriophora*, *S. glaseri* and *S. riobrave*) were used as standards for comparison throughout this experiment. There were two exposure periods; one for the first 48 h and the second from 48–72 h (Figs. 8C and D).

Nematode dispersal in different sand sizes:

Nematode motility in sand was evaluated in another sand column assay. Sand samples were sieved through 0.5 mm (35 µm sieve) and sieved again through 0.25 mm (60 µm sieve). Coarse sand (>0.5 mm diam), medium sand (0.25-0.5 mm diam) and fine sand (<0.25 mm diam) were cleaned and baked in forced air oven at 180ºC until dried. PVC columns (5 cm diam, 10 cm length) were divided four times in 2.5 cm length per ring (containing 0-2.5, 2.5-5, 5-7.5 and 7.5-10 cm length) and joined with paper tape. The last ring of each column was put on another plastic ring sealed with polyethylene net (less than 500 mesh size) at the top side. Columns were filled with prepared sands which were moistened with distilled water (10% w/w) and cover with Petri dish. Three last-instar *G. mellonella* larvae were placed underneath the polyethylene nets and approximately five hundred IJs in 500 µl of nematode suspension were then applied to the soil surface. The column was covered with a plastic lid and kept at room temperature for 3 d. After the incubation period, the sand was pushed out of the column and divided into four layers: 2.5, 5, 7.5 and 10 cm depth, started from the top. Each sand layer was placed into a 500 ml beaker. IJs were extracted from each sand sample by rinsed with distilled water into another beaker, then quickly mixed between two beakers four times (let settle a few sec) and poured slowly into 400 µm sieve. All samples were repeated once, then rinsed sediment into 50 ml tube before all samples were stored sample in cold room and numbers of IJs were counted. *Steinernema* sp. isolate K8, *S. glaseri* and *H. indica* isolate K4 were performed in this test and the test was replicated 10 times (Figs. 8E and F).

Figure 7 *Galleria mellonella* rearing technique. A: artificial diet preparation; B: crushed diet for *G. mellonella* larva rearing; C: *G. mellonella* egg masses on paper stripes on diet container; D: *G. mellonella* larvae rearing; E: *G. mellonella* adult in mating cage; F: last-instar *G. mellonella* larvae for experiment.

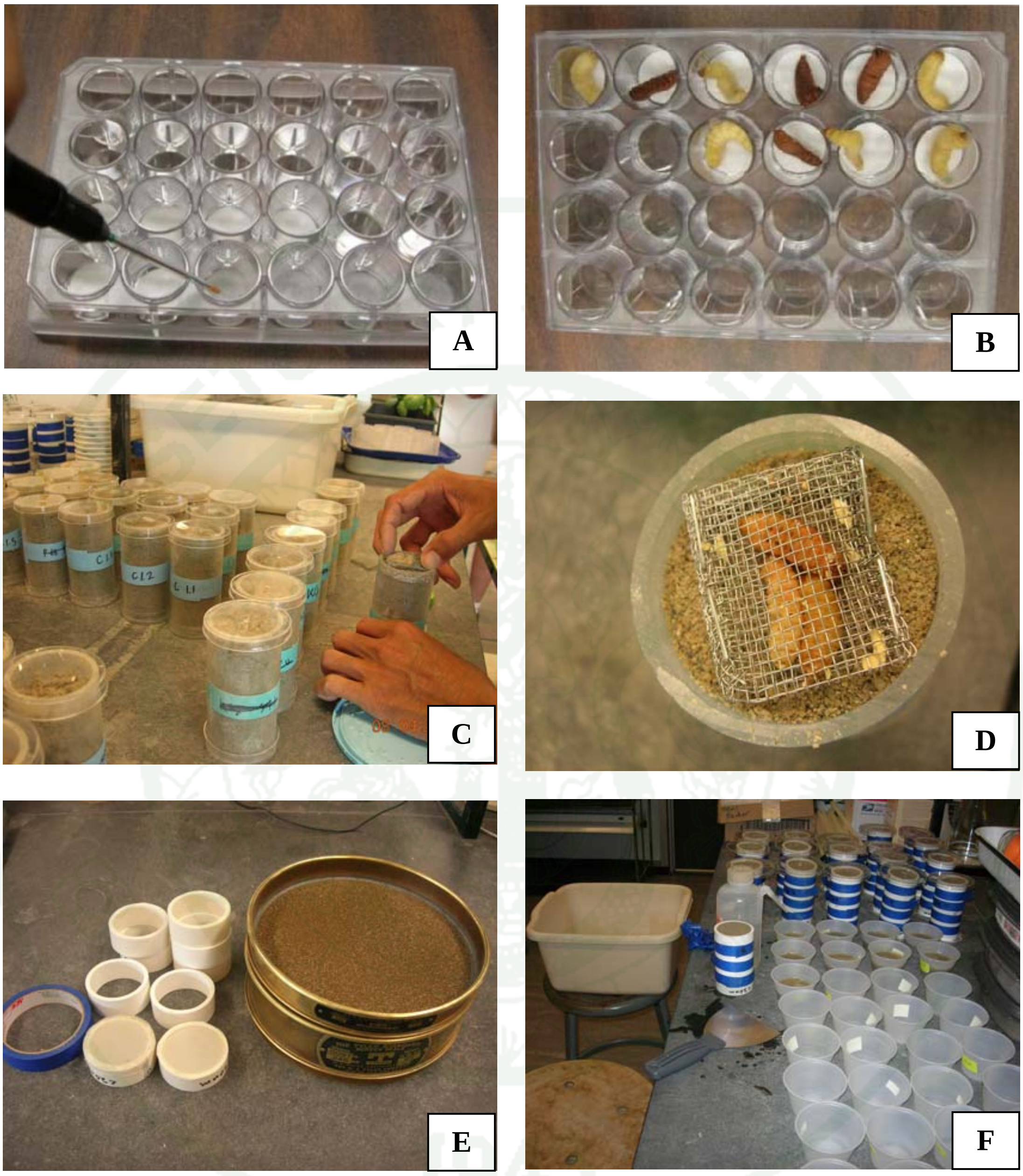

Figure 8 Nematode virulence against *Galleria mellonella* larva. A and B: filter paper bioassays; C and D: sand column bioassays; E and F: nematode dispersal in different sand sizes test.

6 Data analysis

Three separate analyses were performed depending on the experiments. Student Paired sample *t*-test was used to compare means of the nematode movement rate between agar plate and sandy plate where analysis of variance (one-way ANOVA) with the Least Significant Difference Test (LSD's test) was used to compare means in nematode attraction rates and sand column bioassay. Probit analysis was employed to calculate the lethal concentrations causing 50% mortality (LC_{50}) and the lethal time causing 50% mortality (LT_{50}) for the filter paper bioassays. In sand column bioassays, contingency table analysis and analysis of variance (ANOVA) with the LSD's test were used in both nematode dispersal experiments. Arcsine transformation was carried out on numbers of IJs before analyses.

Place and Duration

1. Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand, November 2008 – March 2009 and April 2010 – March 2011 .

2. Department of Nematology, University of California, Davis, California, USA, April 2009 – March 2010.

Study Support

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RESULTS

1. Soil sampling and nematode isolation

A total of 168 soil samples were collected from 5 provinces located in southern Thailand during May 2008 to February 2009. The EPNs were baited from each soil saples using *G. mellonella* larvae and only 8 EPNs strains were founded in 4 habitats; Krom Cave (Amphoe Ban Nasarn; 8°46’E 99°22’N), Tai Rom Yen National Park (Amphoe Weing Sra; 8°40’E 99°28’N), Kha Min Cave (Amphoe Ban Nasarn; 8°49’E 99°22’N) in Surat Thani province and Somdej Phrasrinakarin Park (Amphoe Lang Suan; 9°56’E 99°02’N) in Chumporn province. All collecting sites were located in tropical rain forest habitat where the air temperature and relative humidity at the collecting sites ranged from 25 to 30°C and 58 to 82%, respectively (Table 2).

Cadaver of *G. mellonella* harboring *Heterorhabditis* nematode can be regcognized by its red color whereas the cadaver invaded by *Steinernema* turn brown (Fig. 9). All *Heterorhabditis* isolates (K1, K2, K3, K4, K5 and K6) were found in Surat Thani province where soil temperature ranged from 22.5-25.2°C and their moisture contents varied between 20-42%. All *Heterorhabditis* nematodes were isolated from acidic soil (pH 5.55 to 5.80) and the EC varied from 0.43 to 0.58 dS/m. Both *Steinernema* isolates (K7 and K8) were recovered from Chumporn province where soil temperature varied between 23.5 and 24.2°C and moisture contents of 32 to 36% were recorded. Both Steinernematids were isolated from neutral pH soil (6.08 to 6.78), and the EC values of the soil were 0.34 and 0.37 dS/m, respectively (Table 3).

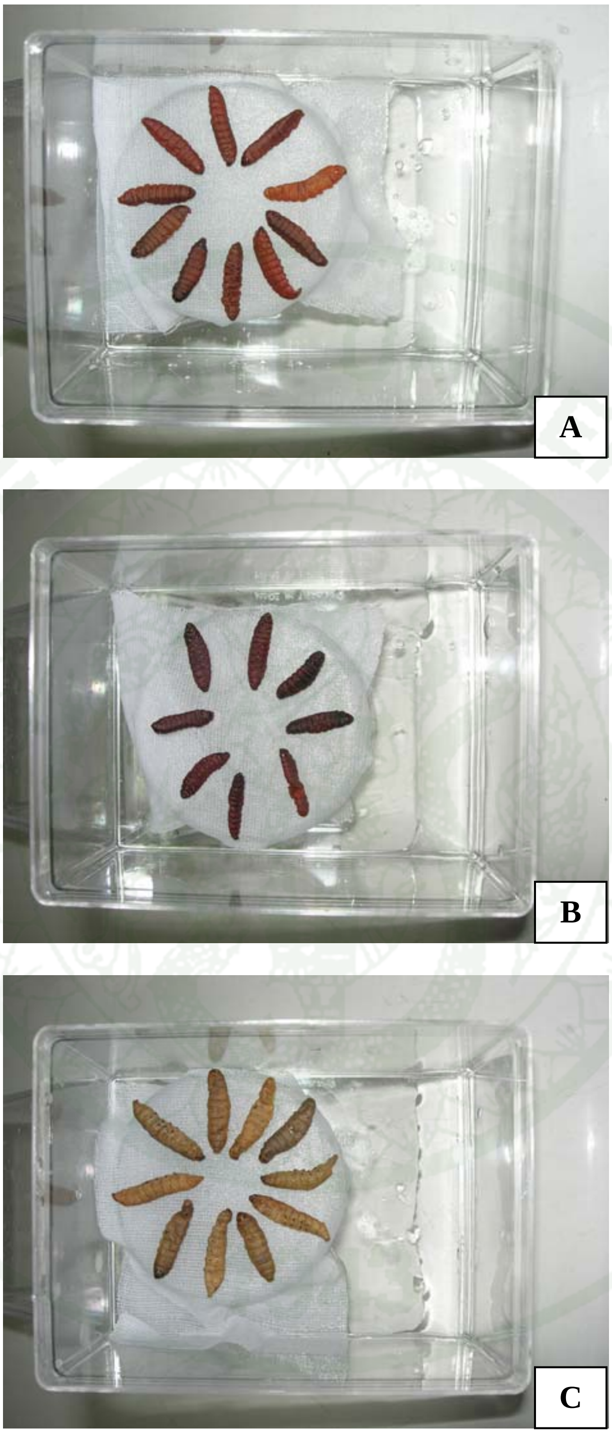


Figure 9 Cadavers of *Galleria mellonella* larvae harboring different strains of nematode. A: *Heterorhabditis* isolates K1-K5; B: *Heterorhabditis* isolate K6; C: *Steinernema* isolates K7-K8.

Table 2 Collecting sites and local conditions for Thai entomopathogenic nematodes in southern Thailand (May 2008-February 2009).

Isolates	Locations	Provinces	Collecting dates	GPS conditions		E	AT	RH
K1	Krom Cave 2	Surat Thani	June 4, 2008	0846174E	9922072N	51	29	58
K2	Tai Rom Yen National Park 1	Surat Thani	June 5, 2008	0840550E	9928372N	430	26	80
K3	Tai Rom Yen National Park 2	Surat Thani	June 5, 2008	0840433E	9928264N	359	26	82
K4	Tai Rom Yen National Park 3	Surat Thani	June 5, 2008	0840281E	9928198N	341	25	80
K5	Kha Min Cave 1	Surat Thani	June 4, 2008	0849797E	9922799N	131	29	70
K6	Kha Min Cave 2	Surat Thani	June 4, 2008	0849802E	9922750N	85	30	68
K7	Somdej Phrasrinakarin Park 1	Chumporn	February 18, 2009	0956847E	9902478N	51	30	60
K8	Somdej Phrasrinakarin Park 2	Chumporn	February 18, 2009	0956842E	9902470N	51	30	60

E= Elevation (m), AT= Air temperature (°C), RH= relative humidity (%).

Table 3 Soil characteristics for each nematode habitat in the Surat Thani and Chumporn Provinces, southern Thailand during May 2008-February 2009.

Isolates	ST	pH	EC	%MC
K1	25.2	5.64	0.58	34
K2	22.5	5.56	0.43	24
K3	22.5	5.55	0.44	24
K4	23.0	5.56	0.44	26
K5	23.9	5.55	0.51	42
K6	24.8	5.80	0.51	20
K7	23.5	6.08	0.34	36
K8	24.2	6.78	0.37	32

ST= Soil temperature, pH= Potential of Hydrogen ion, EC=electrical conductivity (dS/m), MC= soil moisture content.

2. Nematode identification

2.1 Molecular Identification

Restriction digests revealed 3 different pattern groupings. Group 1 included isolates K1, K2, K3, K4 and K5. Group 2 included isolates K7 and K8 while isolate K6 was grouped separately. Sequence analyses of all isolates reveled that group 1 (isolate K1, K2, K3, K4 and K5) was a 100% match for *H. indica* while K6 (group 3) a 99% match for *Heterorhabditis baujardi* Phan, Subbotin, Nguyen & Moens (Phan *et al*., 2003) which was a new species record in Thailand. Isolates K7 and K8 (*Steinernema* spp.*)* were the same species but did not match any sequences closely.

The phylogenetic trees base on the ITS sequences deposited in GenBank indicated that new *Steinernema* isolates (K7 and K8) grouped with *Steinernema loci*, *S. xueshanense, S. hebeiense, S. robustispiculum* and *S. sangi* (Fig. 10). Another phylogenetic analysis with IJ body length of more than 800 µm revealed that the presence of several distinct clades within *Steinernema*, which are similar to *S. loci* and both species were placed as sister taxa to *S. diaprepesi*, *S. khoisanae* and *S. aciari* base on ITS sequences (Fig. 11).

2.2 Cross hybridization

Cross-mating studies were conducted with four *Steinernema* species: *S. glaseri*, *S. carpocapsae*, *S. riobrave* and *S. siamkayai*. Males and females of *Steinernema* sp. isolate K8 did not interbreed with those of *S. glaseri*, *S. carpocapsae*, *S. riobrave* or *S. siamkayai*. In the control, males and females of each species mated and progeny were produced. In addition, control using only *Steinernema* sp. isolate K8 female also produced offspring.

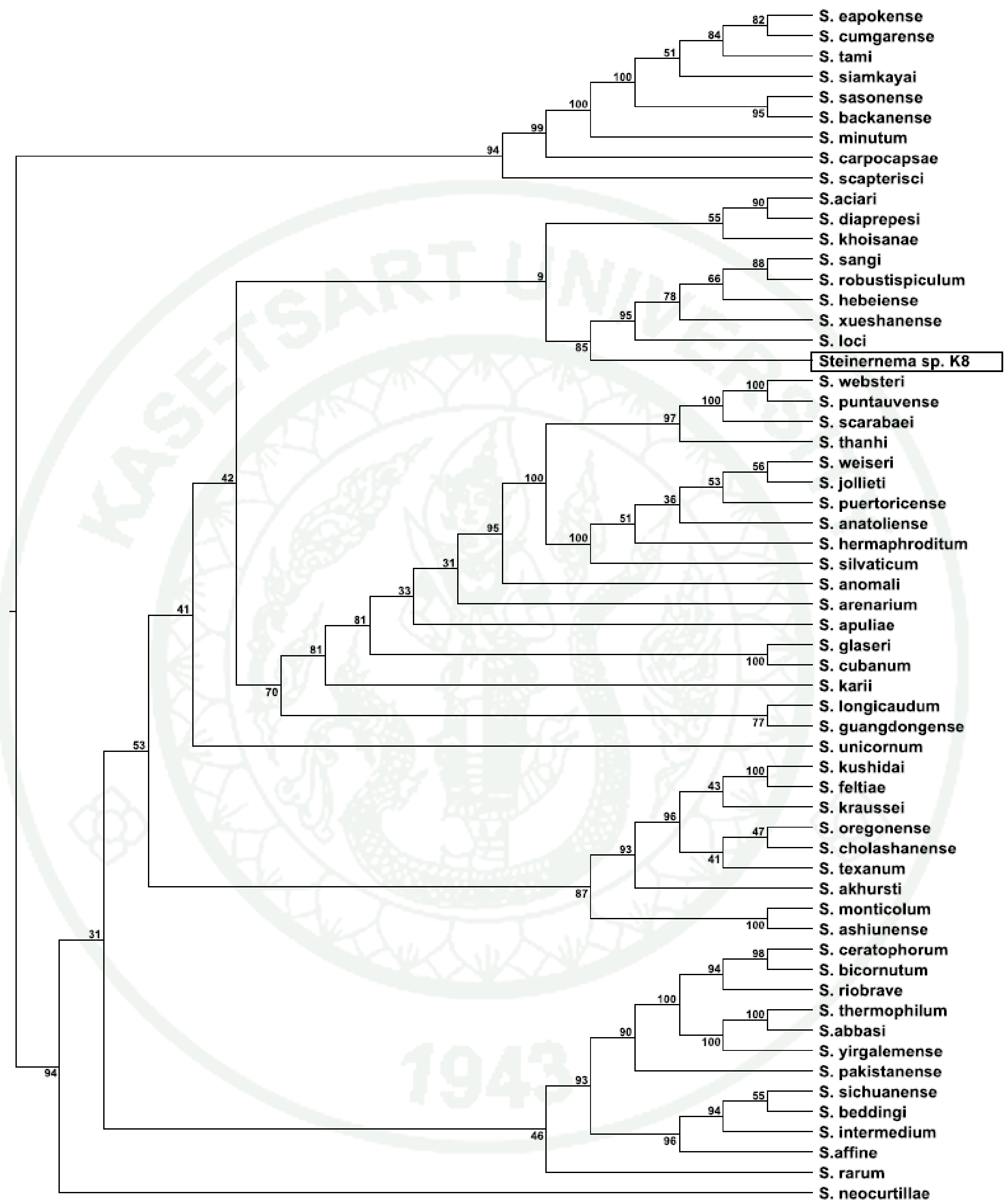


Figure 10 Phylogenetic tree of *Steinernema* species based on sequences of Internal Transcripbed Spacer (ITS) region.

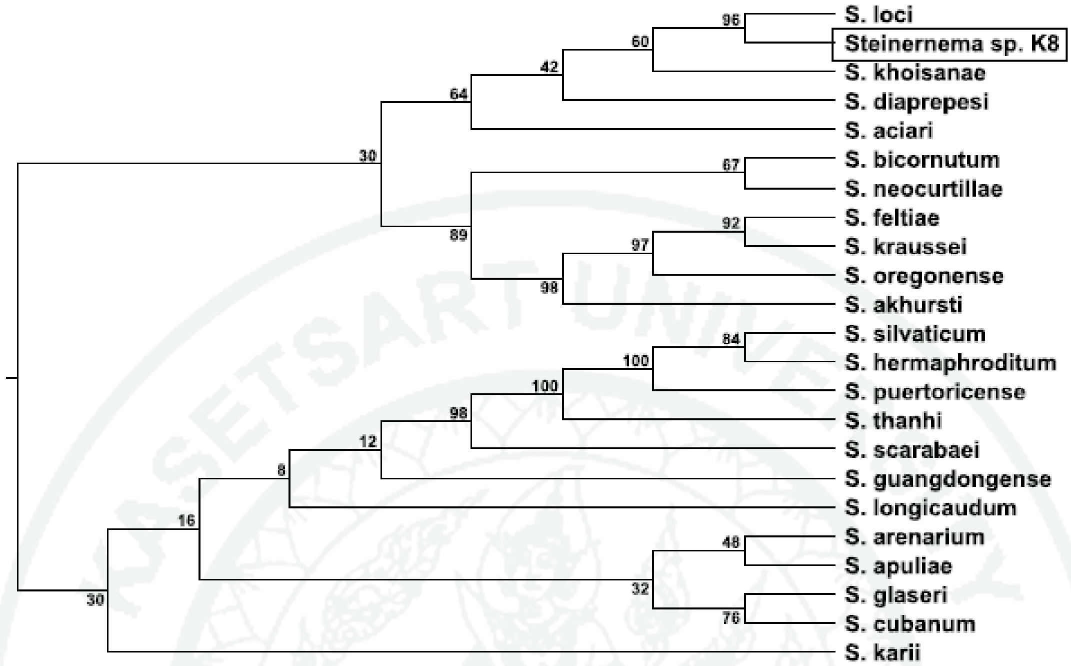


Figure 11 Phylogenetic tree of *Steinernema* species based on sequences of Internal Transcripbed Spacer (ITS) region (body size; larger than 800 µm).

2.3 Morphology identification of *Steinernema* sp. isolates K7 and K8

First generation male:

Body curved ventro-posteriorly, J-shaped when heat-killed (Figs. 12A and 13A). Cuticle appears smooth under LM and fine transverse striae visible under SEM. Head truncate to slightly rounded, slightly offset from body. SEM en face view with 6 prominent labial papillae, 4 cephalic papillae and 2 small amphidial apertures (Fig. 14A). Stoma funnel-shaped or cup-shaped and shallow. Cheilorhabdions prominent, oesophagus muscular; procorpus cylindrical; metacarpus slightly swollen non-valvate; isthmus distinct; basal bulb pyriform containing reduced valve. Nerve-ring surring isthmus just anterior to basal bulb. Cardia prominent and protruding into intestinal lumen. Excretory pore at level of middle of oesophagus and excretory duct cuticularised (Fig. 12C). Testis reflexed. Spicules 2, yellow-brownish in color, strongly curved, large (Figs. 13C-F); head (manubrium) as long as wide; blade arcuate, with straight tip and 3 well defined lobes; antero-dorsal lobe enlarged dorsally and distinctly curved, terminates posterior to spicule tip; lateral lobe prominent, usually enlarged anteriorly in width, terminate at spicule tip; ventral lobe enlarged anteriorly on ventral side to form prominent rostrum, terminate at spicule tip; velum present (Figs. 12A and B). Gubernaculum 69% of spicule length, boat-shaped in lateral view, swollen in middle, with ventrally curved knob at proximal end (Fig. 13F); in ventral view, cuneus long, bifurcate, not reaching end of corpus; corpus separated posteriorly (Fig. 13C). Single ventral precloacal papilla and 11 pairs of genital papillae; latter arranged as follows: 5 pairs subventral pre-anal, 1 pair lateral, 1 pair subdorsal and 2 pairs terminal. Tail bluntly conoid, without mucron (Figs. 14B and C).

Second generation male:

Male body similar to the first generation but smaller in body length, body width and other measurements (Fig. 13B).

First generation female:

Body robust, C shaped when heat-killed (Fig. 15A) Cuticle smooth under LM but with fine transverse striae visible under SEM (Figs. 16A-D). Head truncate to slightly rounded, continuous with body. Six lips amalgamated but tips distinct, and with one labial papilla each. Four cephalic papillae and small 2 amphids located behind lateral labial papillae (Figs. 16A and B). Stoma shallow and cup-shaped, short and wide, with inconspicuous sclerotised walls. Cheilorhabdion present, oesophagus muscular; procorpus cylindrical; metacarpus slightly swollen and non-valvated; distinct isthmus followed by pyriform basal bulb containing reduced valve and cardia present. Oesophagus set off from intestine. Nerve-ring usually surrounding isthmus or anterior part of basal bulb (Figs. 12C and 15C). Excretory pore opening circular, located in 2/3 of metacorpus. Ovaries opposed, reflexed in dorsal position; oviduct well developed, glandular spermatheca and uterus in ventral position. Vagina short, with muscular walls. Vulva located near middle of body (Figs. 12D-E and 16C). Anal prominent and short tail (Figs. 15D-F and 16C-D).

Second generation female:

Similar to first generation female but smaller and shorter (Fig. 15B)

Third-stage infective juvenile:

Body slender, tapering regularly from base of oesophagus to anterior end and from anus to terminus (Figs. 17 A-D). Lip region smooth, mouth closed. Lips indistinct, with six labial papillae. Amphids circular, small. Cuticle with transverse striae. Lateral field distinct with 8 longitudinal ridges in the mid-body region, with central ridges closer to each other than the marginal and submarginal ridges (Figs. 18A-D). Oesophagus long and narrow, with a slender isthmus and pyriform basal bulb. Cardia present. Nerve-ring located at level of isthmus. Excretory pore located in anterior 2/3 of oesophagus (Fig. 12E). Anterior portion of intestine with dorsally displaced pouch containing symbiotic bacterium. Lumen of intestine narrow, rectum long; anus distinct. Genital primordium evident. Tail conoid with pointed terminus. Hyaline portion about half of the tail length (Fig. 17D).

Type host, locality and type material:

Steinernema sp. isolates K7 and K8 were collected from soil samples at Somdej Phrasrinakarin Park, Chumporn province in southern Thailand in February 2009. The natural host is unknown in nature. Holotype male first generation, allotype female first generation, 5 paratype males first generation, 5 paratype females first generation, 5 paratype third-stage infective juveniles were deposited at the University of California Nematode Collection, Davis, California, USA; 5 paratype males first generation, 5 paratype females first generation; 5 paratype third-stage infective juveniles were deposited at the USDA Nematode Collection, Beltsville, Maryland, USA; 5 paratype males first generation, 5 paratype females first generation; 5 paratype third-stage infective juveniles were deposited at the Department of Entomology, Kasetsart University, Bangkok, Thailand.

Differential diagnosis:

Steinernema sp. isolates K7 and K8 were characterised by an IJ body length of 1166.7 (883.2-1448.4) μm, a distance from the anterior to the excretory pore of 65.8 (54.0-86.0) μm, a tail length of 81.1 (72.0-94.0) μm and an E% (distance from anterior end to excretory pore/tail length×100) of 81.3% (65.1–93.2). In addition, the lateral fields at mid-body have eight ridges (submarginal and central pair less distinct) and the males have large spicules (Table 4). The morphometrics of the IJs of *Steinernema* sp. isolates K7 and K8 are close to those of *S. kraussei*. Moreover, the new isolates can be distinguished from *S. kraussei* by male characters, such as a longer spicule length (104.4 vs 55 μm) and bigger gubernaculum (71 vs 33 μm). Both isolates have very long IJ length (1166.7 vs 951 μm), but the new Thai starin can be separated by male tail showing mucron (Table 5).

Table 4 Morphometrics of type population of *Steinernema* sp. isolate K8. Measurement are in µm and in the form of mean±SD (range), (n=20).

Character	First generation Male	First generation Female	Second generation Male	Second generation Female	Infective juvenile
body length (L)	1995.4±307.5 (1465.0-2633.7)	8015.3±1977.3 (5431.0-11012.2)	1266.6±311.7 (910.4-1489.4)	2239.3±362.9 (1666.3-2925.5)	1166.7±208.4 (883.2-1448.4)
a (L/MBD)	-	-	-	-	29.4±4.8 (20.2-40.8)
b (L/ES)	-	-	-	-	8.4±1.5 (6.6-10.8)
c (L/T)	-	-	-	-	14.4±2.5 (11.2-18.1)
Maximum body diam. (MBD)	127.7±16.9 (102.0-155.0)	291.6±84.8 (194.0-476.6)	79.4±20.7 (56.1-95.8)	140.0±33.2 (75.9-206.7)	40.6±9.8 (28.9-69.5)
EP	131.6±29.6 (84.0-174.6)	166.1±33.4 (124.0-225.3)	101.3±13.6 (86.0-112.0)	100.8±25.6 (80.2-173.3)	65.8±6.2 (54.0-76.0)
NR	150.9±27.6 (110.0-188.1)	194.9±39.5 (143.0-261.5)	125.3±2.9 (122.8-128.5)	128.2±28.4 (103.6-197.4)	99.0±4.4 (89.0-105.0)
ES	182.2±33.8 (135.0-241.9)	246.0±44.1 (190.0-305.9)	166.7±13.3 (153.1-179.7)	177.5±36.6 (145.4-250.9)	138.7±5.5 (131.0-153.0)
Tail length	34.8±9.1 (20.0-49.1)	62.9±16.4 (40.0-95.0)	32.6±3.5 (28.9-35.9)	61.3±9.0 (45.3-76.4)	81.1±5.1 (72.0-94.0)
Anal body diam. (ABD)	46.8±8.2 (32.0-63.0)	71.8±16.7 (46.0-101.4)	44.9±3.4 (41.3-48.1)	40.5±5.4 (29.1-49.5)	23.3±3.6 (19.0-32)
Spicule leagth (SL)	104.4±21.9 (70.0-143.4)	-	73.8±19.0 (52.7-89.7)	-	-
Spicule width (SW)	16.4±4.6 (9.0-25.5)	-	14.8±6.1 (8.4-20.6)	-	-
Gubernaculum leagth (GL)	71.0±11.8 (52.0-93.2)	-	46.5±8.4 (38.4-55.1)	-	-
Gubernaculum width (GW)	9.3±1.2 (7.0-11.4)	-	7.2±3.4 (3.3-9.5)	-	-
D% (EP/ES×100)	71.9±6.0 (54.9-80.4)	67.5±5.1 (60.5-76.5)	60.6±3.9 (56.2-63.4)	56.8±6.8 (44.5-69.1)	47.5±4.9 (37.2-55.5)
E% (EP/T×100)	-	-	-	-	81.3±8.3 (65.1-93.2)
SW% (SL/ABD ×100)	223.6±30.7 (179.5-300.0)	-	163.2±35.2 (127.6-198.0)	-	-
GS% (GL/SL×100)	68.9±7.8 (57.1-88.5)	-	64.2±7.7 (58.3-72.9)	-	-
H% (H/T×100)	-	-	-	-	54.6±4.2 (47.1-62)

EP=distance from anterior end to excretory pore; NR=distance from anterior end to nerve ring; ES=distance from anterior end to end of pharynx.

Table 5 Comparison of morphometrics of *Steinernema* sp. isolate K8 with other *Steinernema* species (body size; larger than 800 µm)

	Infective juvenile											First generation male						
	L	MBD	EP	NR	ES	T	a	b	c	D%	E%	SL	GL	MBD	D%	SW%	GS%	MUC
Steinernema sp. K8	1166	41	**66**	99	139	**81**	29	8.4	14.4	48	**81**	**104**	71	128	**72**	224	69	A
S. glaseri	1130	43	**102**	120	162	**78**	29	7.3	14.7	65	**131**	**77**	55	72	**70**	210	70	A
S. aciari	1113	47	**95**	114	146	**78**	24	7.6	14.4	65	**123**	**86**	56	104	**76**	204	65	A
S. khoisanae	1062	31	**95**	109	140	**85**	35	7.6	12.5	68	**112**	**85**	56	108	**85**	203	70	A
S. diaprepesi	1002	34	**74**	102	138	**83**	30	7.3	12.1	54	**90**	**79**	54	-	**80**	180	69	A
S. loci	986	37	**80**	102	141	**75**	27	7.0	13.0	57	**107**	**71**	46	-	**73**	190	70	A
S. oregonense	980	34	**66**	-	132	**70**	30	7.6	14.0	50	**100**	**71**	56	138	**73**	151	79	A
S. kraussei	951	33	**63**	105	134	**79**	29	7.1	12.1	47	**80**	**55**	33	128	**53**	110	71	P
S. hermaphroditum	928	-	**65**	102	-	**77**	29	6.2	10.6	50	**85**	**68**	48	-	**48**	150	71	A
S. litorale	909	31	**61**	96	125	**83**	30	7.3	11.0	49	**73**	**75**	53	96	**65**	174	71	P
S. silvaticum	860	30	**62**	96	121	**75**	29	7.1	11.4	50	**83**	**51**	37	65	**60**	155	73	P
S. feltiae	849	26	**62**	99	136	**81**	31	6.0	10.4	45	**78**	**70**	41	75	**60**	113	59	P
S. akhursti	812	33	**59**	90	119	**73**	24	6.8	11.0	47	**77**	**90**	64	131	**56**	180	71	P

L=Length; MBD=maximum body diameter; EP=distance from anterior end to excretory pore; NR=distance from anterior end to nerve ring; ES=distance from anterior end to end of pharynx; T=tail; a=L/MBD; b=L/ES; c=L/T; D%=(EP/ES×100); E%=(EP/T×100); SL=spicule length; GL=gubernaculum length; SW%=(SL/ABD×100); GS%=(GL/SL×100); MUC=mucron

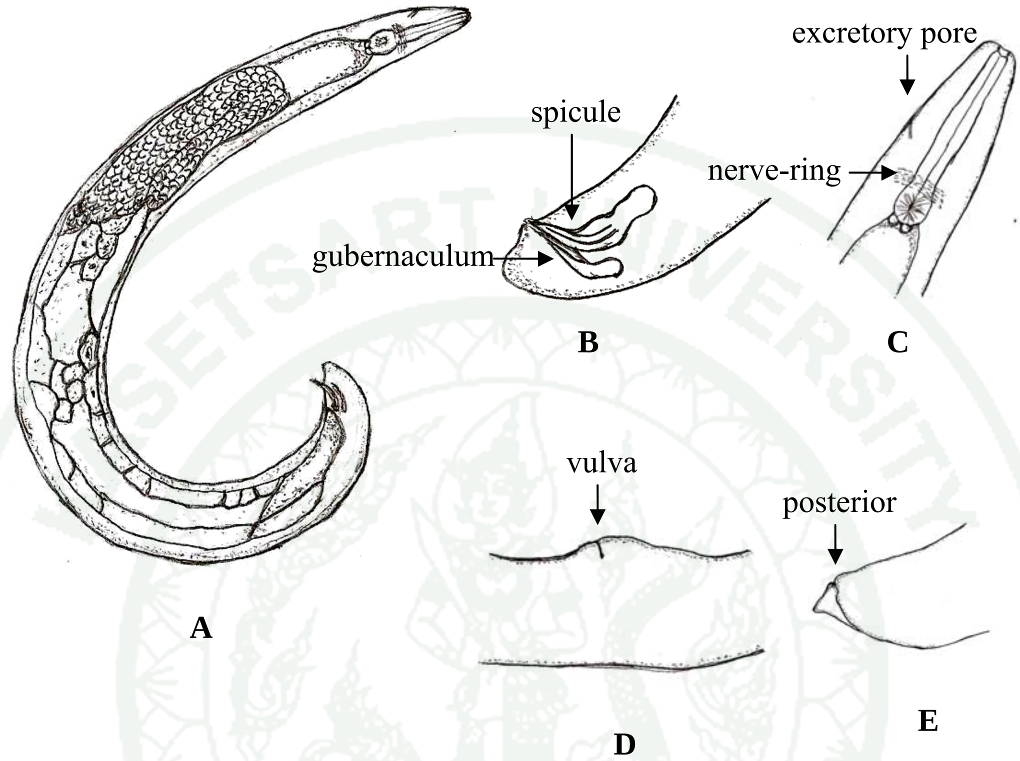


Figure 12 Drawing of *Steinernema* sp. isolate K8 A: first generation male J shaped in heat-relaxed; B: spicule and gubernaculum of first generation male; C: anterior of first generation male showing pharynx; D: vulva of first generation female; E: posterior of female showing anal and tail (Scale bars: A, = 200 µm; B-E = 20 µm).

Figure 13 LM of *Steinernema* sp. isolate K8 A: lateral view of first generation male; B: second generation male; C: posterior of male; D: spicule and gubernaculum; E: spicule; F: gubernaculum (Scale bars: A, B = 200 μm; C-E = 100 μm).

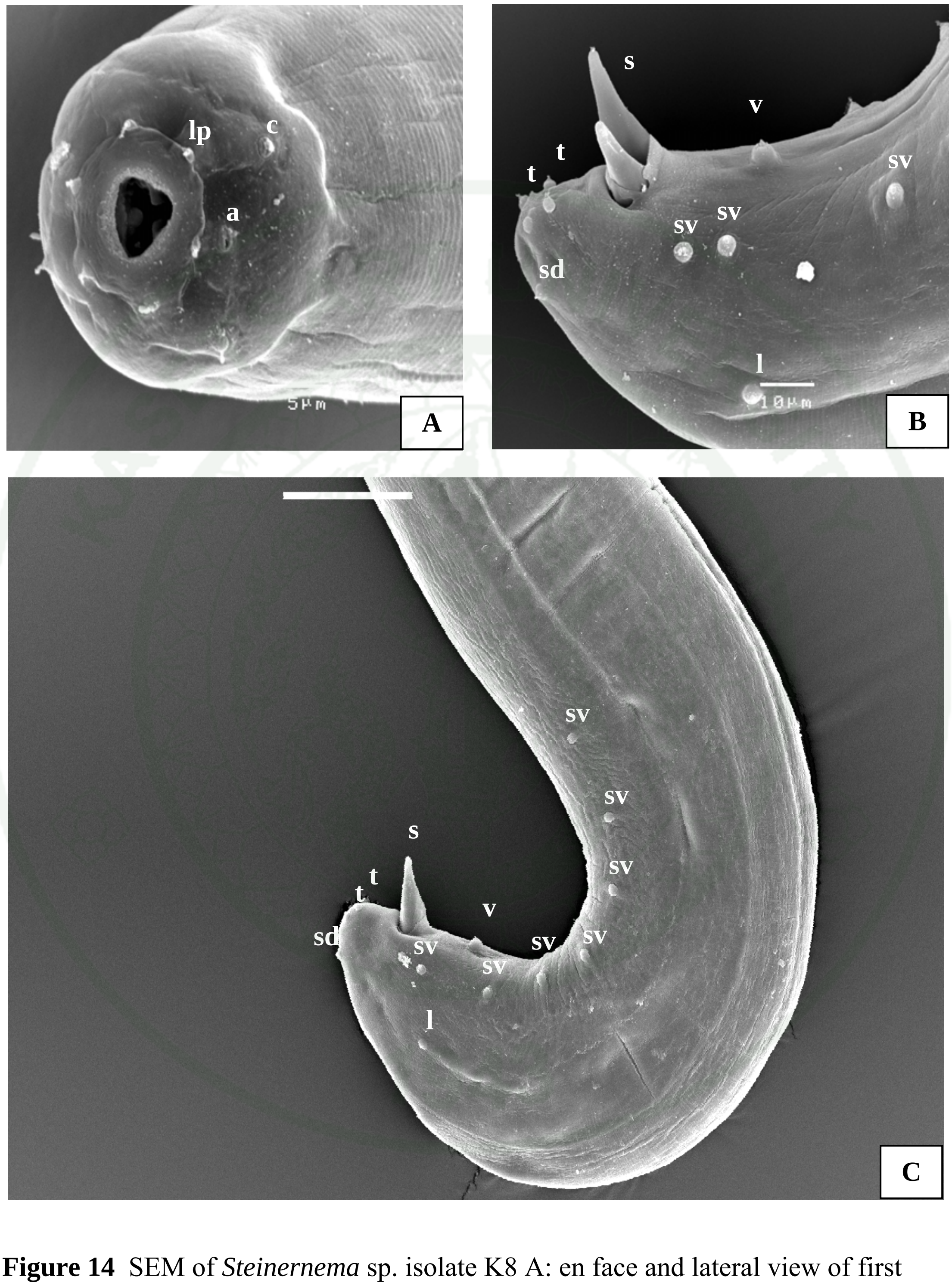


Figure 14 SEM of *Steinernema* sp. isolate K8 A: en face and lateral view of first generation male showing four cephalic (cp), six labial papillae (lp) and two amphids (a); B and C: posterior of first generation male showing eight pairs subventral (sv), one pair lateral (l), one pair subdorsal (sd), two pairs terminal (t) and spicules (s).

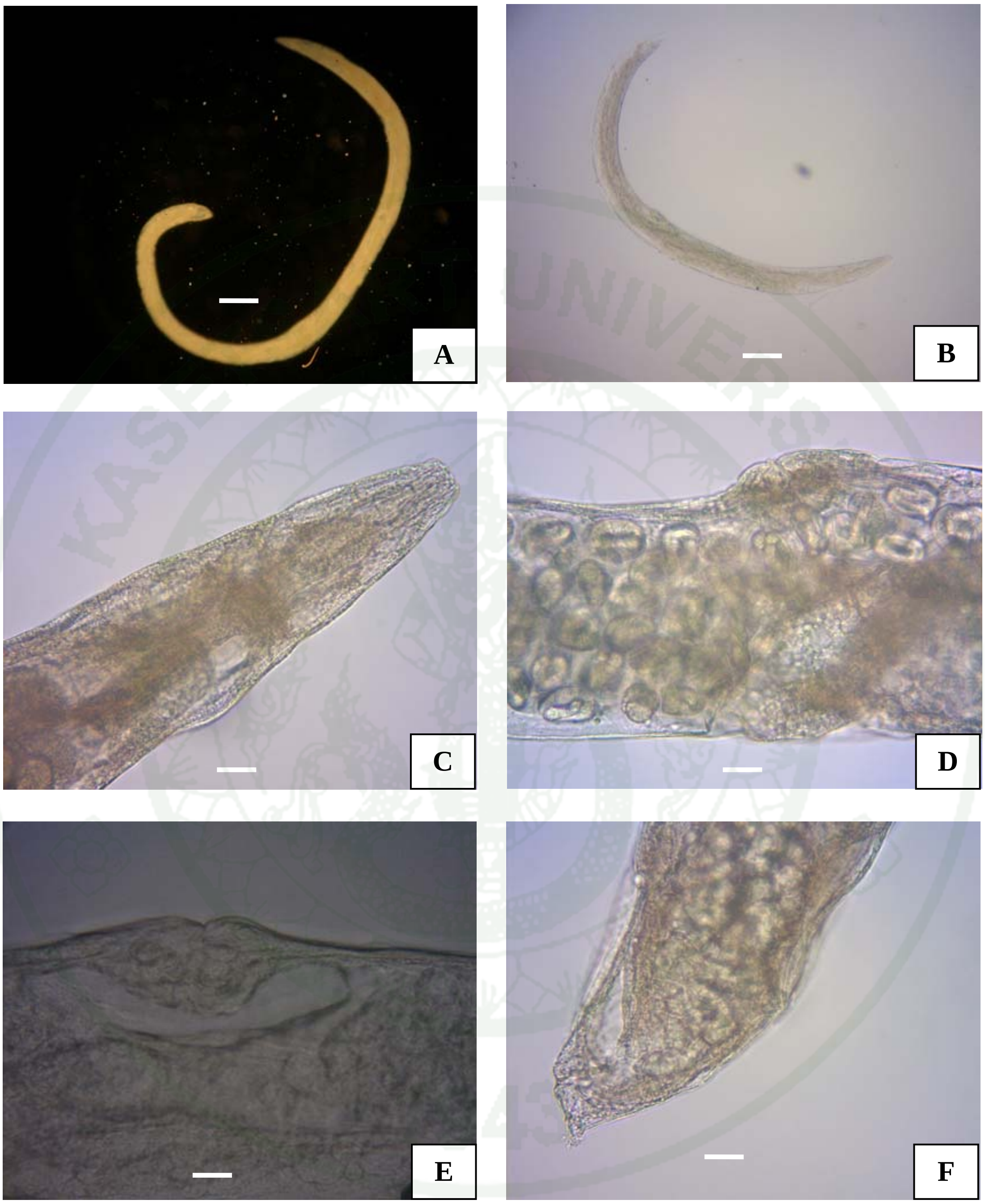


Figure 15 LM of *Steinernema* sp. isolate K8 A: first generation female; B: second generation female; C: anterior end of female body; D: vulva of first generation female; E: vulva of second generation female; F: posterior of first generation female. (Scale bars: A = 600 µm; B = 200 µm; C-F = 80 µm).

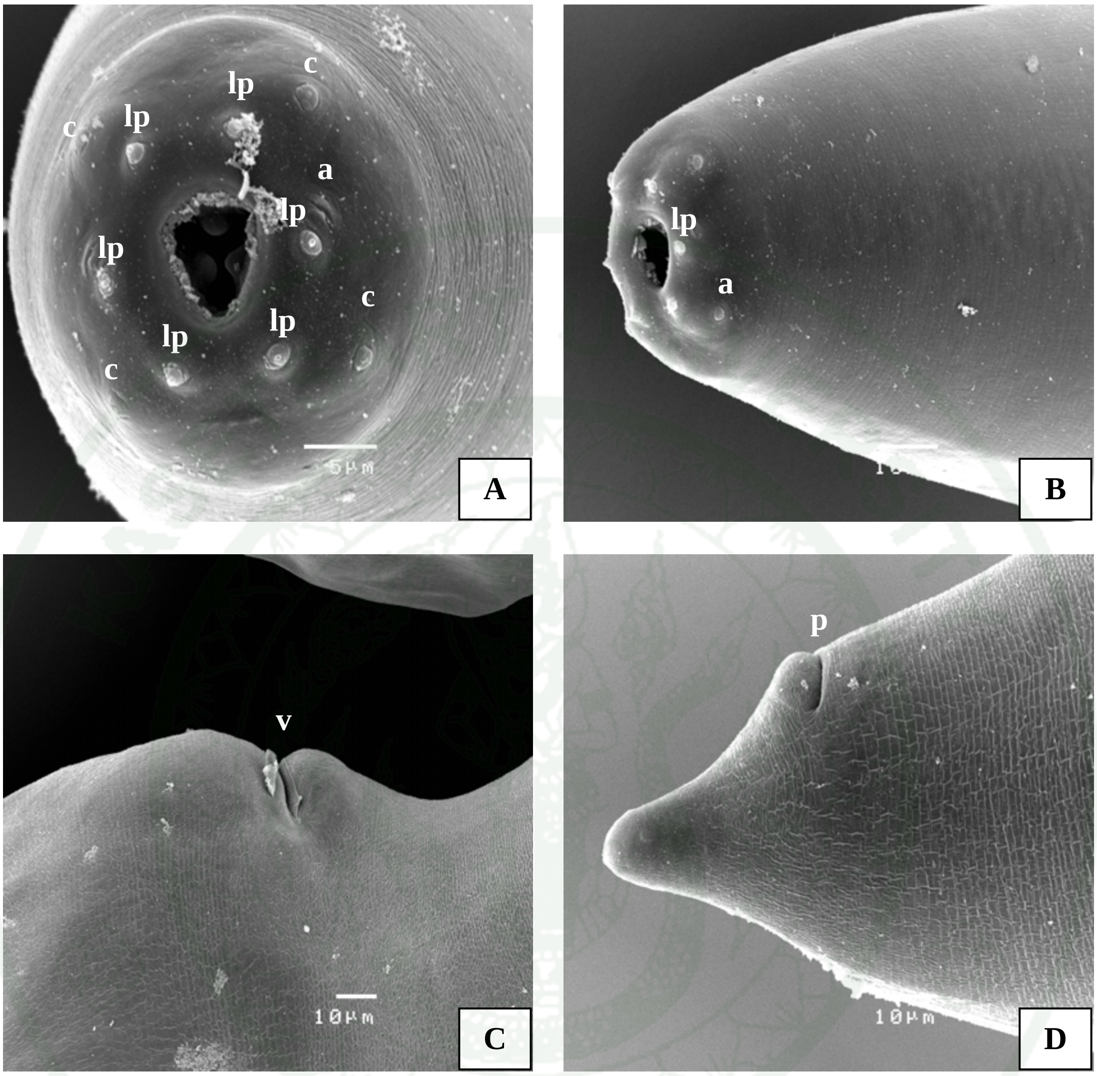


Figure 16 SEM of *Steinernema* sp. isolate K8 A and B: en face and lateral view of first generation female showing four cephalic (cp), six labial papillae (lp) and two amphids (a); C: vulva of first generation female (v); D: posterior of first generation female showing tail and post anal (p).

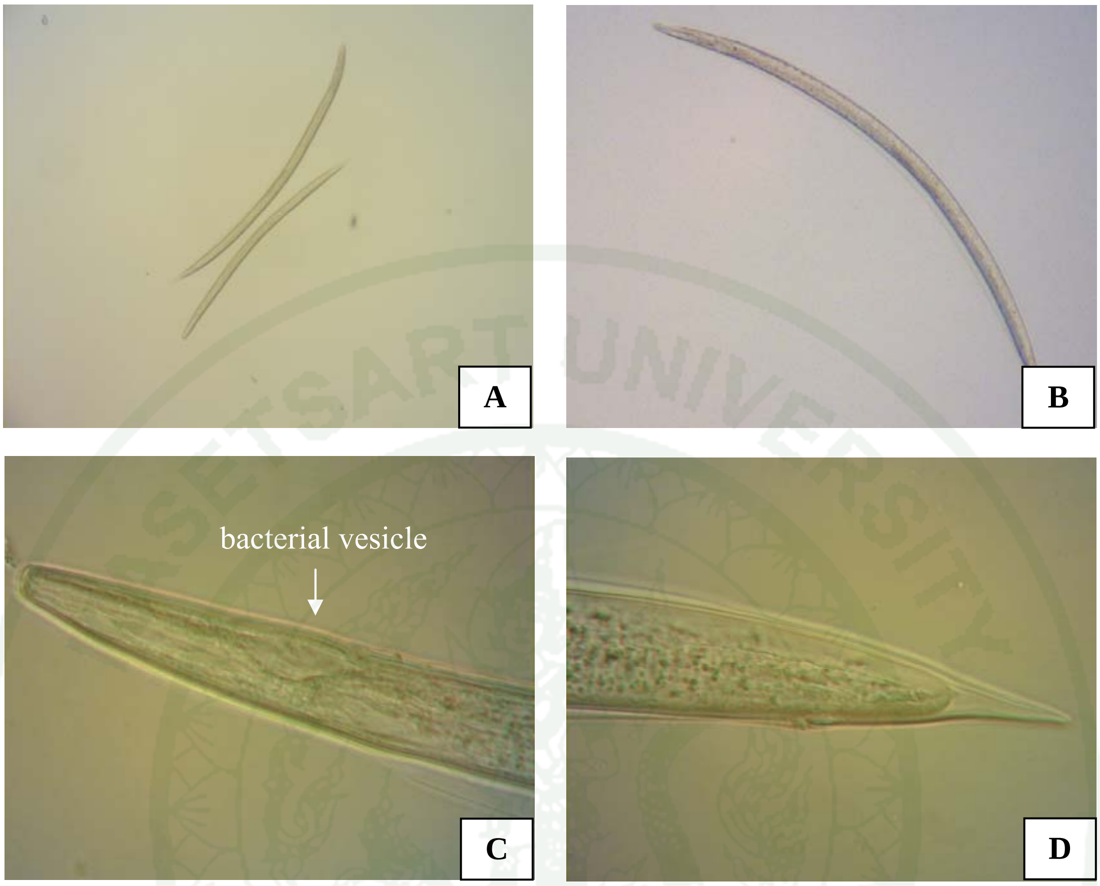


Figure 17 LM of *Steinernema* sp. isolate K8 A and B: lateral view of third-stage infective juvenile; C: rounded head, pharynx and bacterial vesicle; D: tail with hyaline layer. (Scale bars: A, B = 200; C, D = 20 µm).

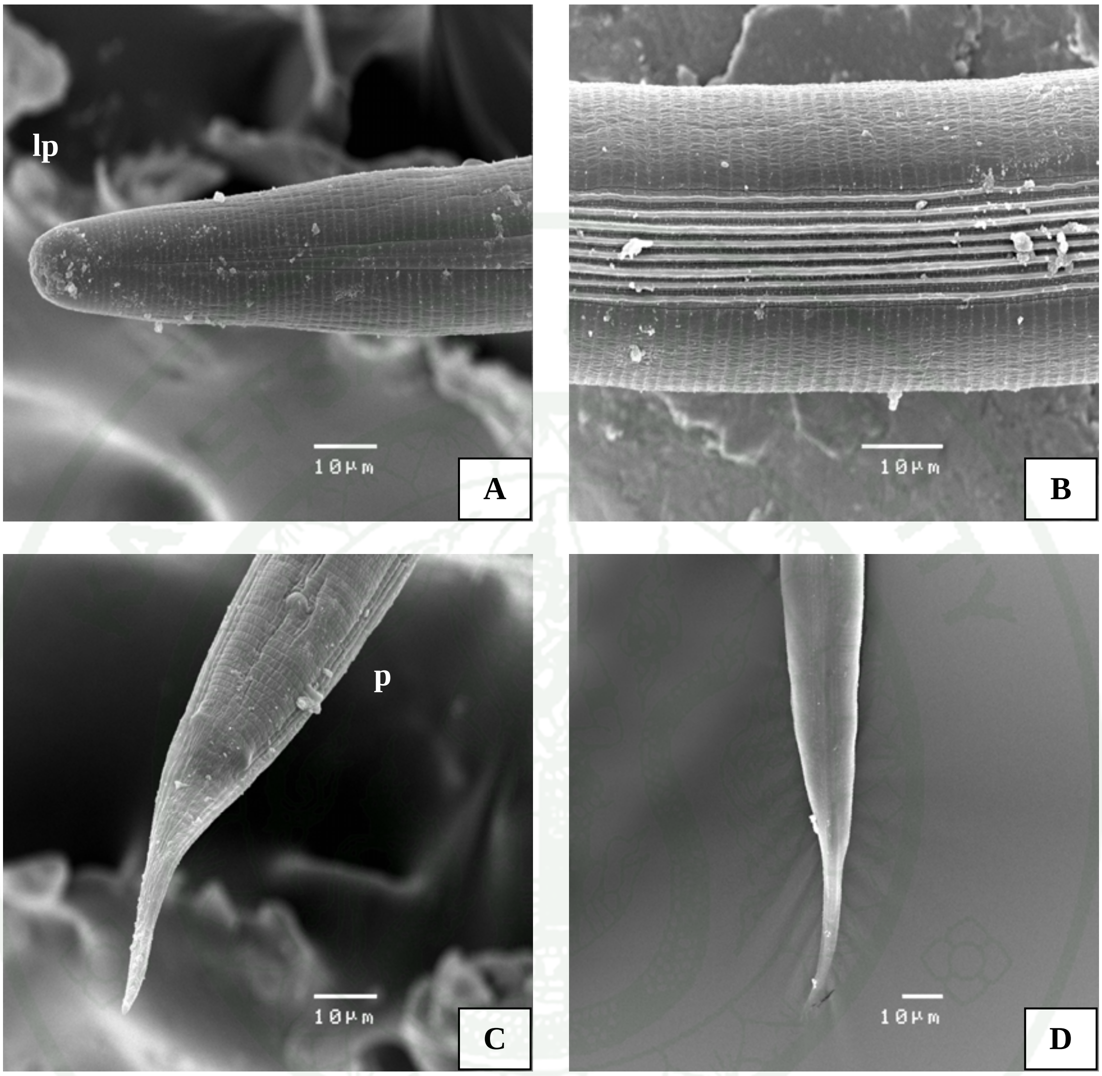


Figure 18 SEM of *Steinernema* sp. isolate K8 infective juvenile A: en face and lateral view; B: lateral field ridges; C and D: posterior region of third-stage infective juvenile showing a tail.

3. Nematode Behavioral tests

All *Heterorhabditis* spp. (*H. indica* isolates K1, K2, K3, K4, K5 and *H. baujardi* isolate K6) in this study were highly attracted to volatile cues with positive movement rates of 0.003-0.164 cm (Table 6). Their movement on surfaces with different characteristics (sandy versus smooth agar plates) did not significantly differ ($P > 0.05$) (Table 7). The body-wave or jumping behaviors were not observed in any of these isolates (Table 8). *Steinernema* sp. isolates K7 and K8 were also attracted to host volatiles (0.001-0.045 cm), and like the *Heterorhabditis* species, showed no difference in movement rates between sandy and smooth surfaces. Neither body-wave nor jumping were observed (Tables 6-8).

Table 6 Movement rates of Thai EPNs in behavioral test.

Isolates	Nematode net movement rates (mean±SE) in host attraction test (cm)*		
	10 min	20 min	30 min
H. indica isolate K1	0.004±0.01 Ba	0.043±0.02 Bab	0.071±0.02 Ab
H. indica isolate K2	0.004±0.02 Ba	0.049±0.06 Ba	0.027±0.02 Aa
H. indica isolate K3	0.024±0.02 Ba	0.080±0.02 ABa	0.062±0.03 Aa
H. indica isolate K4	0.035±0.03 Ba	0.083±0.03 ABa	0.090±0.03 Aa
H. indica isolate K5	0.004±0.04 Ba	0.003±0.05 Ba	0.020±0.10 Aa
H. baujardi isolate K6	0.141±0.04 Aa	0.164±0.03 Aa	0.113±0.03 Aa
Steinernema sp. isolate K7	0.013±0.01 Ba	0.012±0.01 Ba	0.045±0.03 Aa
Steinernema sp. isolate K8	0.003±0.01 Ba	0.001±0.03 Ba	0.014±0.04 Aa

*Means followed by the same letters are not significantly different at the 5% level as determined by LSD (α=0.05). Capital letters compare means in columns, small letters compare means in rows.

Table 7 Movement rates of Thai EPNs in movement test between smooth agar plate versus sandy agar plate.

Isolates	Average movement rates (mean±SE) between smooth plate and sandy plate (cm)*		
	10 min	20 min	30 min
H. indica isolate K1	0.09±0.03 / 0.19±0.02 ns	0.11±0.02 / 0.17±0.03 ns	0.23±0.03 / 0.28±0.02 ns
H. indica isolate K2	0.10±0.03 / 0.06±0.01 ns	0.18±0.04 / 0.09±0.03 ns	0.42±0.07 / 0.21±0.06 ns
H. indica isolate K3	0.15±0.03 / 0.13±0.06 ns	0.19±0.02 / 0.15±0.06 ns	0.28±0.04 / 0.19±0.04 ns
H. indica isolate K4	0.41±0.04 / 0.31±0.01 ns	0.70±0.08 / 0.41±0.08 ns	0.74±0.08 / 0.54±0.06 ns
H. indica isolate K5	0.17±0.04 / 0.10±0.03 ns	0.31±0.06 / 0.20±0.04 ns	0.39±0.04 / 0.31±0.05 ns
H. baujardi isolate K6	0.49±0.06 / 0.53±0.04 ns	0.73±0.05 / 0.77±0.03 ns	0.83±0.04 / 0.89±0.05 ns
Steinernema sp. isolate K7	0.26±0.04 / 0.21±0.04 ns	0.38±0.02 / 0.48±0.01 ns	0.56±0.04 / 0.69±0.05 ns
Steinernema sp. isolate K8	0.27±0.05 / 0.35±0.07 ns	0.34±0.04 / 0.54±0.05 ns	0.50±0.05 / 0.70±0.06 ns

*ns= non significant different between two results ($P \leq 0.05$; *t*-test)

Table 8 Summary of nematode behavioral test for all Thai isolates.

Isolates	Foraging strategies (ambusher/cruiser)	Body-wave	Jumping	Dispersal decreased by sand	Host attraction response
H. indica isolate K1	cruiser	No	No	No (p>0.05)	Yes (+)
H. indica isolate K2	cruiser	No	No	No (p>0.05)	Yes (+)
H. indica isolate K3	cruiser	No	No	No (p>0.05)	Yes (+)
H. indica isolate K4	cruiser	No	No	No (p>0.05)	Yes (+)
H. indica isolate K5	cruiser	No	No	No (p>0.05)	Yes (+)
H. baujardi isolate K6	cruiser	No	No	No (p>0.05)	Yes (+)
Steinernema sp. isolate K7	Intermediate	Lift their bodies	No	No (p>0.05)	Yes (+)
Steinernema sp. isolate K8	Intermediate	Lift their bodies	No	No (p>0.05)	Yes (+)

4. Nematode dispersal in horizontal movement

Nematode motility on soil surface was investigated under different sand particle conditions. All IJs populations moved from the center of the experimental arena through the edge of arena. After the incubation period, the higher numbers of IJs were recorded in the edge area. The contingency table analysis showed that the distance was dependable on different sand particles (χ^2 = 195.93; df = 40; P < 0.001). The different exposure times were related with nematode dispersion (χ^2 = 7265.20; df = 40; P < 0.001). The overall analysis of variance showed significant differences among the various treatment combinations (F = 53318.51; df = 7, 40; P < 0.001) with respect to IJ dispersal. The result also showed a significant interaction between the main effects within the comparisons of distances and different sand particles. In addition, *Steinernema* sp. isolate K8 showed the highest number of IJ in sand sample of 70% sand + 30% clay at 72 h after exposure. During 24 h after application, nematode movement in both soil types did not differ in the same area. On the other hand, the differences between two sand types were found at 72 h after exposure in the edge area (Fig. 19).

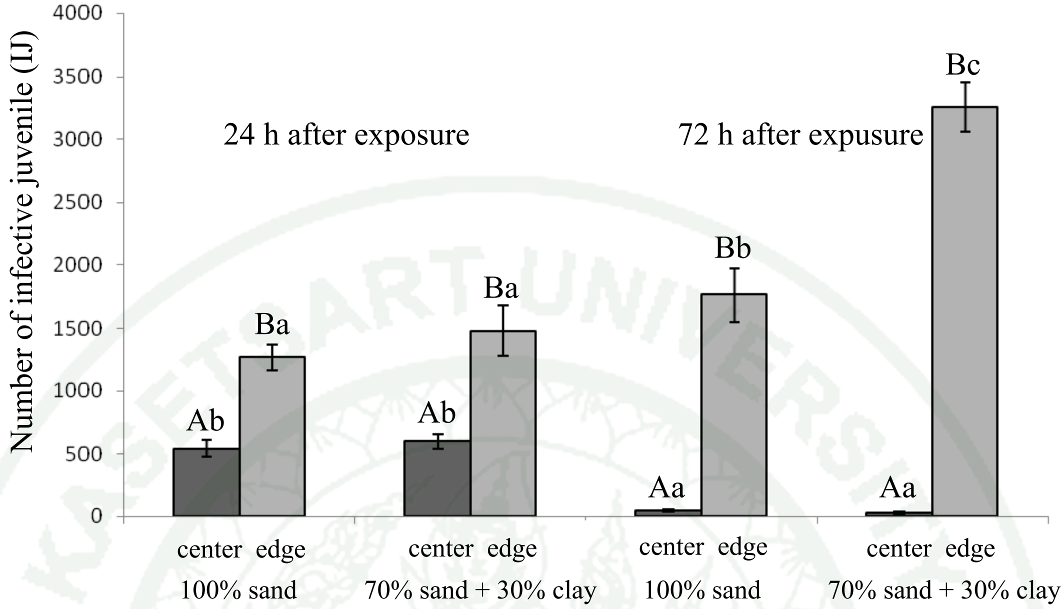


Figure 19 Number of IJs of *Steinernema* sp. isolate K8 in nematode horizontal dispersal test in 100% sand and 70% sand + 30% clay at 24 and 72 h after exposure. The same letters above the bars indicate no significant difference among means at P = 0.05. Bars show standard error of the means. Capital letters compares means between partner bars, small letters compare means in all same distant area.

5. Virulence of EPNs on selected insect pest in the laboratory.

5.1 Virulence of various nematode isolates on *P. xylostella* larva and pupa.

Mean mortality of third-stage *P. xylostella* larvae caused by *H. indica* isolate K4 and *Steinernema* sp. isolate K8 infective juveniles was significantly greater than two commercial nematode isolates (*H. bacteriophora* and *S. siamkayai*). Most nematodes killed *P. xylostella* larva more than 50% from the concentration at 30 IJs/larva and killed 100% *P. xylostella* larva at the dosages of 300 IJs, except *S. siamkayai* caused 90% mortality to *P. xylostella* larva at the same dosage. The infectivity of all nematodes at the concentration of 50 IJs/larva did not differ from 100 and 300 IJs/ larva. However, all nematode isolates could not infect *P. xylostella* pupa (Table 9).

Probit analyses showed that the LC_{50} values of 4 EPN isolates to *P. xylostella* larvae ranged from 17-34 IJs/larva. *H. indica* isolate K4 had the highest virulence (lowest LC_{50} value) on *P. xylostella* larva (LC_{50} = 16.75 IJs/larva), followed by *Steinernema* sp. isolate K8 (21.55), *H. bacteriophora* (27.43) and *S. siamkayai* (33.54) (Table 9).

Table 9 LC_{50} of *Heterorhabditis* spp. and *Steinernema* spp. against third-instar *Plutella xylostella* larvae in leaf disc bioassays at 48 h after application.

Isolates	% Mortality*					LC_{50} (IJs/larva)	Pupal test
	Concentrations (IJs/larva)						
	15	30	50	100	300		
H. indica isolate K4	45 Aa	70 Ab	95 Cc	95 Ac	100 Ac	16.75 A	-
H. bacteriophora	30 Aa	55 Aab	75 Bbc	75 Abc	100 Ac	27.43 B	-
Steinernema sp. isolate K8	35 Aa	65 Ab	80 Bbc	90 Ac	100 Ac	21.55 A	-
S. siamkayai	25 Aa	55 Ab	60 Ab	75 Abc	90 Ac	33.54 C	-

*Means followed by the same letters are not significantly different at the 5% level as determined by LSD (α=0.05). Capital letters compare means in columns, small letters compare means in rows.

5.2 Virulence of various nematode isolates on *S. litura* larva and pupa.

Both mean mortalities of second and third-instar larval of *S. litura* caused by *H. indica* isolate K4 Infective juvenile was significantly greater than any other nematode isolates and killed 100% second-instar larva at only 30 IJs/larva (Tables 10 and 11). Most nematodes killed second and third-instar larvae of *S. litura* more than 50% starting from the concentration of 50 IJs/larva and killed 95-100% *S. litura* larvae at the dosages of 300 IJs. Moreover, only *Steinernema* sp. isolate K8 was able to infect pre-pupal stage of *S. litura* with 60% pupal mortality. However, all nematode isolates did not infect *S. litura* pupa whereas the nematode infection reached 100% in adult stage (Table 12).

Probit analyses showed that the LC_{50} values of 4 EPN isolates to second-instar larvae ranged from 10-32 IJs/larva whereas the LC_{50} values of 4 EPNs in third-instar larvae ranged from 16-34 IJs/larva. Moreover, *H. indica* isolate K4 had the highest virulence to *S. litura* larva on both stages (second and third-instars) with the LC_{50} values of 10.27 and 16.12 IJs/larva at 48 h after exposure, respectively. Most EPN isolates exhibited significantly higher efficacy against second-instar *S. litura* larva than third-instar larva. In contrast, *S. siamkayai* showed similar LC_{50} values for both second and third-instar larvae experiments (Tables 10 and 11).

Table 10 LC_{50} of *Heterorhabditis* spp. and *Steinernema* spp. against second-instar *Spodoptera litura* larvae in filter paper bioassays at 48 h after application.

Isolates	% Mortality*					LC_{50} (IJs/larva)
	Concentrations (IJs/larva)					
	15	30	50	100	300	
H. indica isolate K4	60 Ba	100 Cb	95 Cb	100 Ab	100 Ab	10.27 A
H. bacteriophora	45 ABa	40 Aa	70 ABb	85 Ac	100 Ad	25.72 B
Steinernema sp. isolate K8	30 Aa	60 Bb	80 BCbc	90 Ac	95 Ac	23.15 B
S. siamkayai	40 ABa	35 Aa	50 Aa	90 Ab	100 Ab	32.45 C

*Means followed by the same letters are not significantly different at the 5% level as determined by LSD (α=0.05). Capital letters compare means in columns, small letters compare means in rows.

Table 11 LC_{50} of *Heterorhabditis* spp. and *Steinernema* spp. against third-instar *Spodoptera litura* larvae in filter paper bioassays at 48 h after application.

Isolates	% Mortality*					LC_{50} (IJs/larva)
	Concentrations (IJs/larva)					
	15	30	50	100	300	
H. indica isolate K4	45 Ba	85 Bb	95 Bb	100 Ab	100 Ab	16.12 A
H. bacteriophora	40 ABa	30 Aa	65 Ab	80 Abc	95 Ac	32.58 B
Steinernema sp. isolate K8	30 Aa	55 ABb	60 Ab	70 Ab	95 Ac	31.87 B
S. siamkayai	35 ABa	40 Aa	50 Aa	85 Ab	100 Ab	33.86 B

*Means followed by the same letters are not significantly different at the 5% level as determined by LSD (α=0.05). Capital letters compare means in columns, small letters compare means in rows.

Table 12 *Heterorhabditis* spp. and *Steinernema* spp. against pre-pupa, pupa and adult of *Spodoptera litura* in various bioassays at 10 d after application.

Isolates	Pre-pupa	Pupa	Adult
H. indica isolate K4	-	-	100% infected
H. bacteriophora	-	-	100% infected
Steinernema sp. isolate K8	60% infected	-	100% infected
S. siamkayai	-	-	100% infected

5.3 Virulence of various nematodes on *T. molitor* and *O. rhinoceros* larvae.

Virulence of 2 *Heterorhabditis* and 1 *Steinernema* isolates collected in Thailand were investigated on *T. molitor* larvae using filter paper bioassays at the concentration of 300 IJs/ larva. The results indicated that mean mortality of last-instar *T. molitor* larvae caused by IJs of *H. indica* isolate K4 was significantly greater than two other isolates (*Steinernema* sp. isolate K8 and *H. baujardi* isolate K6), where 100% mortality was observed at 48 h after exposure. The mortalities cause by *Steinernema* sp. isolate K8 and *H. baujardi* isolate K6 tremendously increased, reaching 90-95% at 96 h after exposure. In addition, all nematodes were able to kill more than 50% of *T. molitor* larvae at 48 h after exposure (Fig. 20). Probit analyses showed that *H. indica* isolate K4 had the highest infectivity rate to *T. molitor* larvae (LT_{50} = 33.76 h), followed by *Steinernema* sp. isolate K8 and *H. baujardi* isolate K6 where their LT_{50} values were 48.15 and 49.75 h, respectively (Table 13).

Three nematodes; *S. glaseri*, *Steinernema* sp. isolate K8 and *S. siamkayai*, were able to infect only first-instar larva of *O. rhinoceros* whereas third-instar larvae were not invaded by any nematode isolates (Table 14). The mean mortalities of *O. rhinoceros* larvae caused by *S. glaseri* and *Steinernema* sp. isolate K8 reaching 100% at 72 h after application. Both mortality rates induced by *S. glaseri* and *Steinernema* sp. isolate K8 drastically increased to 100% at 72 h. In contrast, *S. siamkayai* caused only 30% mortality to *O. rhinoceros* larva whereas *H. indica* isolate K4 could not kill *O. rhinoceros* larva at 96 h after application (Fig. 21).

Table 13 LT_{50} of *Heterorhabditis* spp. and *Steinernema* sp. against last-instar *Tenebrio molitor* larvae in filter paper bioassays at various times after application.

Isolates	% Mortality* Exposure time (h)				LT_{50} (h)
	24	48	72	96	
H. indica isolate K4	0 Aa	100 Bb	100 Bb	100 Ab	33.76 A
H. baujardi isolate K6	0 Aa	55 Ab	85 Ac	90 Ac	49.75 A
Steinernema sp. isolate K8	0 Aa	55 Ab	90 ABc	95 Ac	48.15 A

*Means followed by the same letters are not significantly different at the 5% level as determined by LSD (α=0.05). Capital letters compare means in columns, small letters compare means in rows.

Table 14 *Heterorhabditis* spp. and *Steinernema* spp. against first and third-instar larvae of *Oryctes rhinoceros* in filter paper bioassays at various times after application.

Isolates	% Mortality of first-instar larvae* Exposure time (h)				Third-instar larvae
	24	48	72	96	
H. indica isolate K4	0 Aa	0 Aa	0 Aa	0 Aa	non infected
Steinernema sp. isolate K8	0 Aa	25 Ab	100 Cc	100 Cc	non infected
S. glaseri	0 Aa	30 Ab	100 Cc	100 Cc	non infected
S. siamkayai	0 Aa	0 Aa	25 Bb	30 Bb	non infected

*Means followed by the same letters are not significantly different at the 5% level as determined by LSD (α=0.05). Capital letters compare means in columns, small letters compare means in rows.

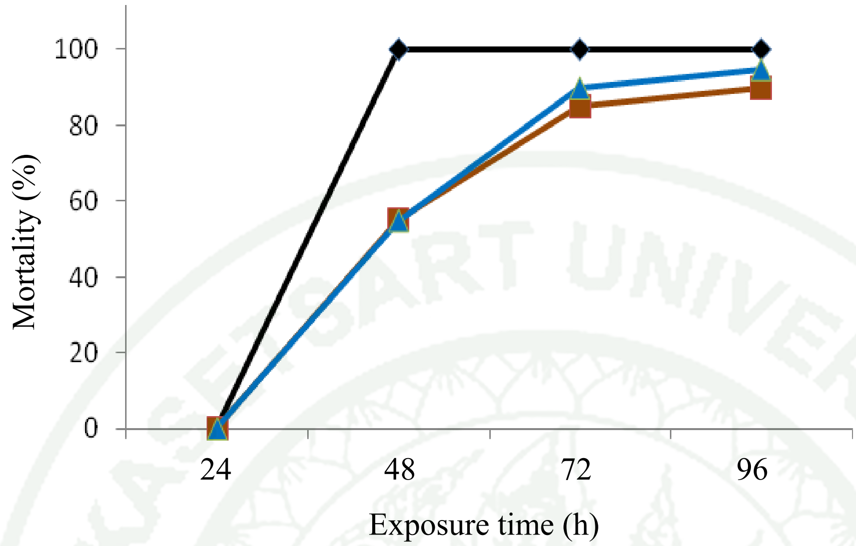


Figure 20 Mean % mortality of *Tenebrio molitar* larvae cause by *Heterorhabditis indica* isolate K4 (♦), *Heterorhabditis baujardi* isolate K6 (■) and *Steinernema* sp. isolate K8 (▲) at different exposure times.

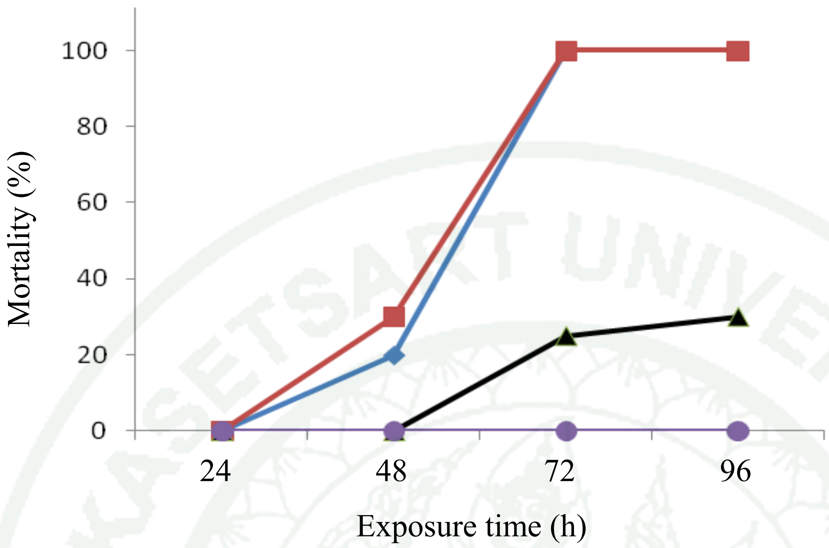


Figure 21 Mean % mortality of *Oryctes rhinoceros* larvae cause by *Heterorhabditis indica* isolate K4 (●), *Steinernema glaseri* (■), *Steinernema siamkayai* (▲) and *Steinernema* sp. isolate K8 (♦) at different exposure times.

5.4 Virulence of various nematode isolates on *G. mellonella* larva.

Virulence bioassay:

The LC_{50} values of 4 EPN isolates; *H. indica* isolate K1, *H. indica* isolate K4, *H. baujardi* isolate K6 and *Steinernema* sp. isolate K8 to *G. mellonella* larvae ranged from 1.99-6.95 IJs/ larva. All *Heterorhabditis* spp. had lower LC_{50} values than *Steinernema* spp. *Heterorhabditis indica* isolate K4 required the fewest IJs (1.99 IJs/larvae) as compared to other isolates to induce 50% mortality of last-instar *G. mellonella* larvae in the filter paper assays. All nematodes killed last-instar *G. mellonella* larvae 80-100% at the concentration of 50 IJs. (Table 15).

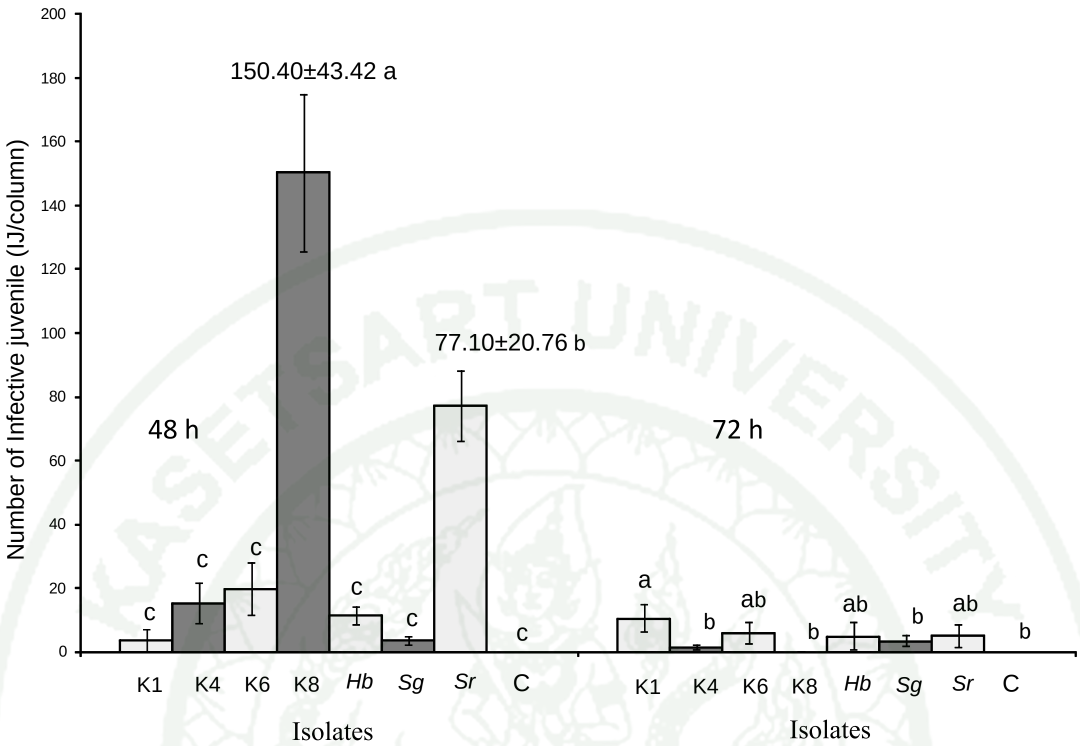
Table 15 LC$_{50}$ of *Heterorhabditis* spp. and *Steinernema* sp. against last-instar *Galleria mellonella* larvae in filter paper bioassays at 48 h after application.

Isolates	% Mortality*						LC$_{50}$ (IJs/larva)
	Concentrations (IJs/larva)						
	1	3	5	10	20	50	
H. indica isolate K1	0 Aa	40 Bb	80 Bc	80 Ac	100 Ac	100 Ac	3.75 AB
H. indica isolate K4	30 Aa	40 Ba	100 Bb	100 Ab	100 Ab	100 Ab	1.99 A
H. baujardi isolate K6	0 Aa	10 Aa	80 Bb	80 Ab	90 Ab	100 Ab	4.99 AB
Steinernema sp. isolate K8	0 Aa	40 Bb	30 Ab	90 Ac	70 Bc	80 Ac	6.95 B

*Means followed by the same letters are not significantly different at the 5% level as determined by LSD (α=0.05). Capital letters compares means in columns, small letters compare means in rows.

Sand column bioassay:

In sand column bioassays, *Steinernema* sp. isolate K8 showed the greatest infection rate in both soil types at 48 h after exposure when compared with the other Thai isolates and 3 commercial EPNs (*H. bacteriophora, S. glaseri* and *S. riobrave*) (Figs. 22 and 23). In sandy loam (80% sand + 20% clay), *Steinernema* sp. isolate K8 had a significantly higher number of IJs inside the infected host (150.40±43.42 IJs/column) compared to the other Thai isolates ($F = 26.37$; df = 7, 80; $P < 0.001$), where the number of IJs ranged from only 20-77 IJs/column for other Thai isolates and commercial isolates 48 h after application. On the other hand, the *Heterorhabditis* spp. did not differ significantly in the number of IJs inside the hosts (14.20-25.80 IJs/column) comparing to the commercial isolates ($P > 0.05$), except for *S. riobrave* where higher number of IJs (77.10±20.76) was observed (Fig. 22). In Sandy clay loam (65% sand + 35% clay), *Steinernema* sp. isolate K8 had the greatest infection rate of 95.10±23.32 IJs/column ($F = 31.77$; df = 7, 80; $P < 0.001$) within 48 h (Fig. 23). All Thai *Heterorhabditis* spp. failed to invade *G. mellonella* within 48 h after exposure while 2 commercial isolates (*H. bacteriophora* and *S. riobrave*) achieved an infection rate of 3-28 IJs/column. Infection rates did not differ among isolates during the period of 72 h in both column bioassays (Figs. 22 and 23).



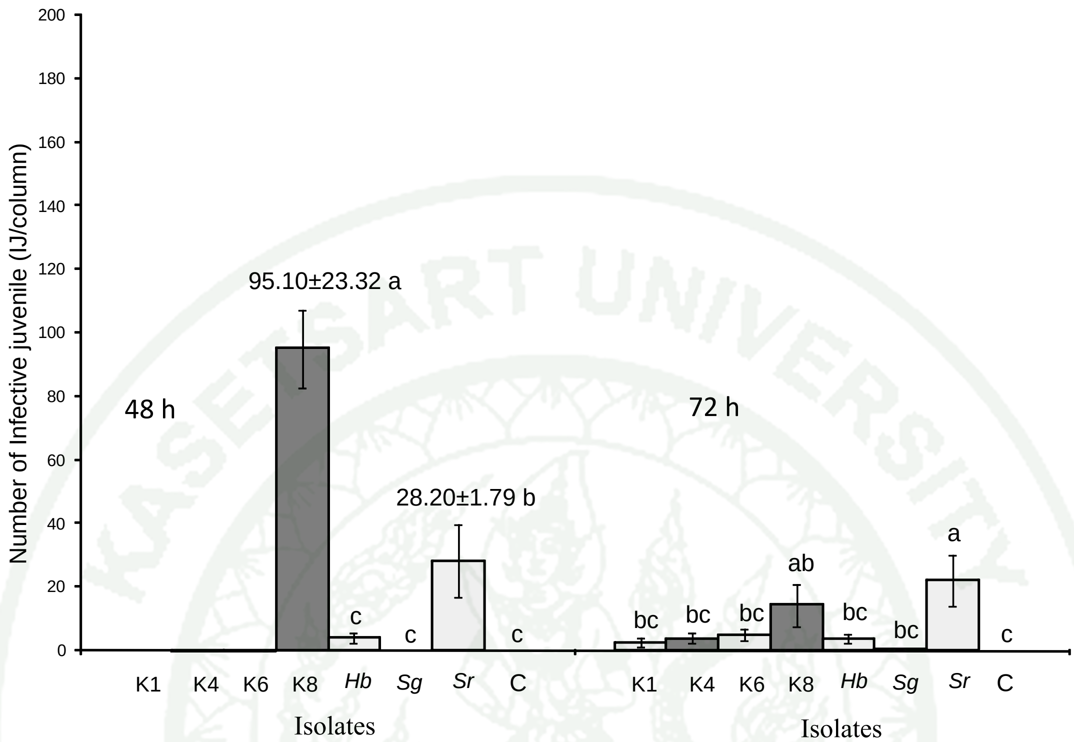
K1= *Heterorhabditis indica* isolate K1 *Hb*= *Heterorhabditis bacteriophora*

K4= *Heterorhabditis indica* isolate K4 *Sg*= *Steinernema glaseri*

K6= *Heterorhabditis baujardi* isolate K6 *Sr*= *Steinernema riobrave*

K8= *Steinernema* sp. isolate K8 C= Control (No IJ)

Figure 22 Number of IJs in sand column bioassays of *Heterorhabditis* spp. and *Steinernema* spp. infecting last-instar *Galleria mellonella* larvae in sandy loam (80% sand + 20% clay) at 48 and 72 h after exposure. The same letters above the bars (within the same exposure period) indicate no significant difference among means at P = 0.05. Bars show standard error of the means.



K1= *Heterorhabditis indica* isolate K1 *Hb*= *Heterorhabditis bacteriophora*

K4= *Heterorhabditis indica* isolate K4 *Sg*= *Steinernema glaseri*

K6= *Heterorhabditis baujardi* isolate K6 *Sr*= *Steinernema riobrave*

K8= *Steinernema* sp. isolate K8 C= Control (No IJ)

Figure 23 Number of IJs in sand column bioassays of *Heterorhabditis* spp. and *Steinernema* spp. infecting last-instar *Galleria mellonella* larvae in sandy clay loam (65% sand + 35% clay) at 48 and 72 h after exposure. The same letters above the bars (within the same exposure period) indicate no significant difference among means at P = 0.05. Bars show standard error of the means.

Nematode dispersal in different sand sizes:

Nematode motility in different sand particles indicated that IJs populations moved from the surface of the columns through the entire column layer in order to penetrate into *G. mellonella* larvae which were presented at the bottom of the column. After the incubation period, the highest numbers of IJs were recorded in the bottom layer of the coarse sand columns in all three nematode isolates (Fig. 24). However, in medium and fine sand columns, the highest numbers of IJs remained in the upper layer at 5-7.5 cm depth (Figs. 24 and 25). The overall contingency table analysis showed that among all treatments of nematode species, column depth and different sand particles were dependent (χ^2 = 4000.93; df = 24; P < 0.001). The different nematode isolates were relative to nematode dispersion in different depths (χ^2 = 254.98; df = 6; P < 0.001) and different sand particles were also relative to nematode dispersion in different depths (χ^2 = 3030.85; df = 6; P < 0.001). The overall analysis of variance showed significant differences among the various treatment combinations (F = 21.51; df = 35, 360; P < 0.001) with respect to IJs dispersal. The results also showed a significant interaction between the main effects within the comparisons of nematode isolates and different sand particles (Figs. 24-27).

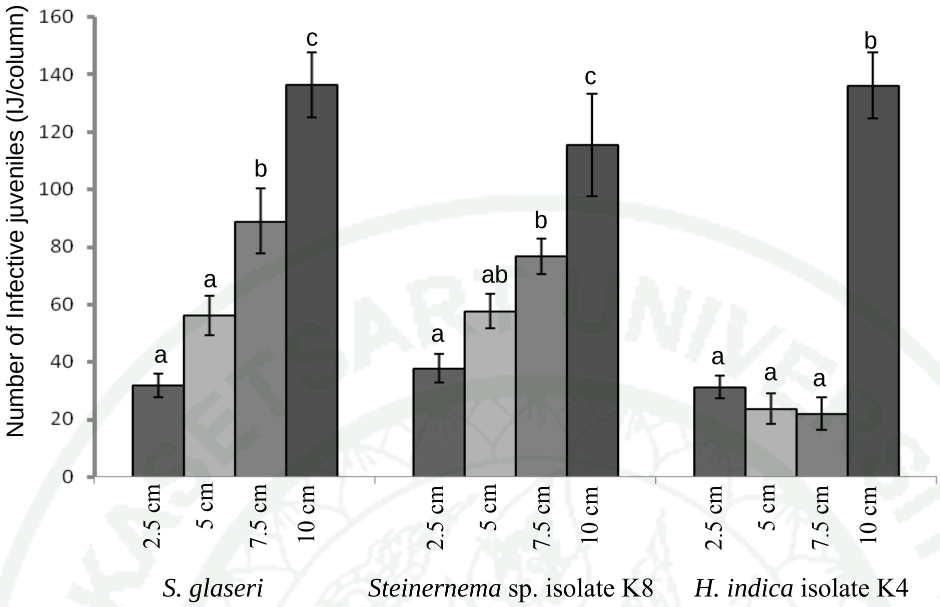


Figure 24 Number of IJs in nematode dispersal observation of *Steinernema* spp. and *Heterorhabditis* sp. in different depths of sand column within coarse sand at 72 h after exposure. The same letters above the bars (within the same species) indicate no significant difference among means at P = 0.05. Bars show standard error of the means.

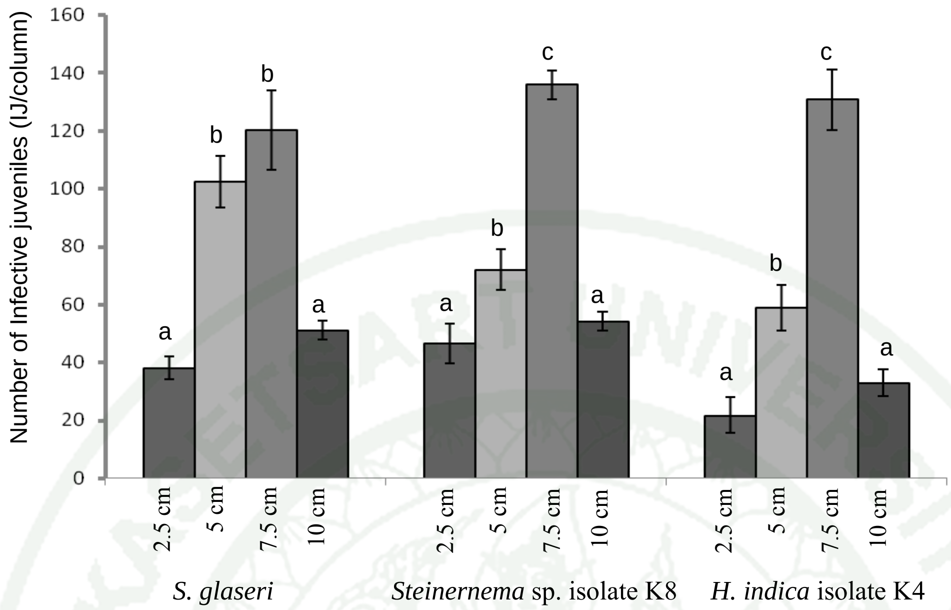


Figure 25 Number of IJs in nematode dispersal observation of *Steinernema* spp. and *Heterorhabditis* sp. in different depths of sand column within medium sand at 72 h after exposure. The same letters above the bars (within the same species) indicate no significant difference among means at P = 0.05. Bars show standard error of the means.

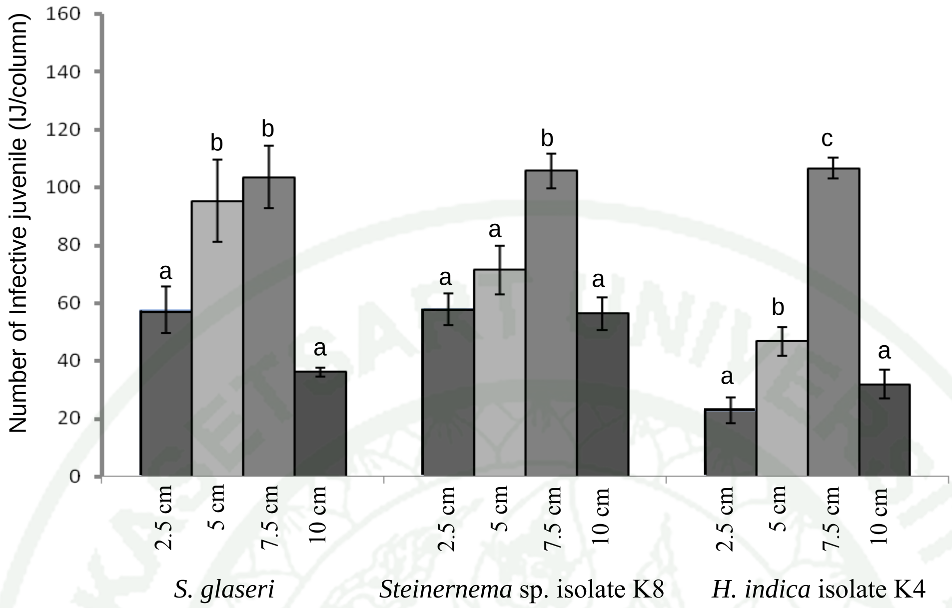
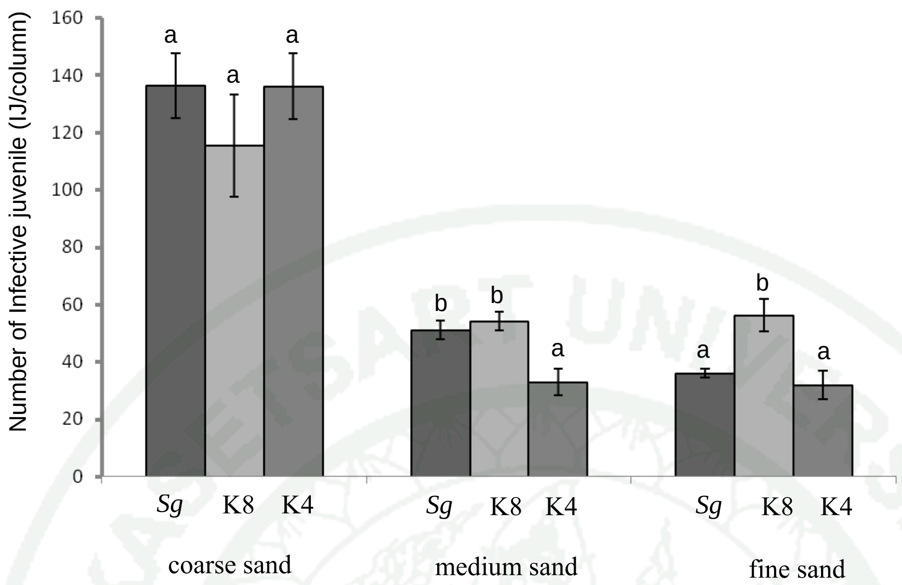


Figure 26 Number of IJs in nematode dispersal observation of *Steinernema* spp. and *Heterorhabditis* sp. in different depths of sand column within fine sand at 72 h after exposure. The same letters above the bars (within the same species) indicate no significant difference among means at $P = 0.05$. Bars show standard error of the means.



Sg= *Steinernema glaseri* K4= *Heterorhabditis indica* isolate K4

K8= *Steinernema* sp. isolate K8

Figure 27 Number of IJs in nematode dispersal observation of *Steinernema* spp. and *Heterorhabditis* sp. in 10 cm depth of sand column within coarse, medium and fine sand at 72 h after exposure. The same letters above the bars (within the same sand type) indicate no significant difference among means at P = 0.05. Bars show standard error of the means.

DISCUSSION

1. Nematode isolation

EPNs are widely distributed throughout the world and have been found in all continents except Antarctica (Hominick *et al*., 1996; Hominick, 2002). The surveys of EPNs are normally conducted by using the *Galleria* bait technique (Bedding and Akhurst, 1975). In this study, new eight EPN isolates were extracted from 168 soil samples collected in various locations and all collecting sites were located in tropical rain forest. All *Heterorhabitis* isolates were extracted from acidic soil (pH 5.55 to 5.80) whereas *Steinernema* isolates were extracted from neutral pH soil (6.08 to 6.78). The EC values varied from 0.43 to 0.58 dS/m and 0.34 to 0.37 dS/m for *Heterorhabitis* isolates and *Steinernema* isolates, respectively.

In broad sense, heterorhabditids have been isolated from sandy coastal soils; *H. indica* was found in calcareous soils whereas *H. bacteriophora* and *H. marelatus* were found in acidic soils. Besides, *H. bacteriophora* can be found beyond coastal regions and *H. megidis* are broadly distributed in tropical forests (Phan *et al*., 2003) and weedy habitats (Stuart and Gaugler, 1994; Stock *et al*., 1996; Constant *et al*., 1998). For Steinernematid, *S. feltiae, S. affinis* and *S. intermedia* were typically found in grassland (Griffin *et al*., 1991; Hominick, 2002), while *S. affinis* was absent from woodland (Griffin *et al*., 1991).

Temperature had a direct effect on nematode survival and the optimal temperature for growth and reproduction of EPN commonly varies from 25-28°C (Kaya, 1977; Molyneux, 1986). Soil moisture is the important factor affecting nematode performance and survival in the soil (Molyneux and Bedding, 1984; Kung *et al*., 1991; Koppenhöfer *et al*., 1995; Grant and Villani, 2003a, 2003b) because all nematodes require water film surrounding their body for their living or moving (Wallace, 1958; Norton, 1978). Besides, lower RH can directly decrease nematode survival as well (Kung *et al.,* 1991).

The effectiveness of EPNs typically depends on the nematode foraging strategy, insect species, host location, soil conditions (soil type, pH, soil moisture, etc.), climate (Glazer and Lewis, 2000) and application methods (Shapiro-Ilan *et al.*, 2006). Hence, ecological studies are recommended to develop hypotheses about the abilities and limitations of new EPN species (Shapiro-Ilan *et al.*, 2006). The new EPN species here were found in very diverse habitats and local conditions. In this study, *H. indica* has already been found in Thailand, *H. baujardi* is a new record whereas two steinernematid nematodes (isolates K7 and K8, same species) are considered new to species. The degree of nematode diversity is similar to that reported in other surveys conducted in Southeast Asia (Phan *et al*., 2003; Mráček *et al*., 2006). For example, Phan *et al*. (2003) isolated *H. indica* and *H. baujardi* in Vietnam.

2. Nematode behavior

Previous studies have demonstrated that endemic EPNs can be employed as biological control agents (Phan *et al.*, 2001a, b; Nguyen *et al*., 2006; Campos-Herrera and Gutiérrez, 2009). However, native EPN's may be either less or more efficacious than exotic isolates depending on the foraging strategy of each EPN and which hosts were targeted (Berry *et al.*, 1997; Shapiro-Ilan and Cottrell, 2005). EPNs have been categorized into 3 behavioral classes; ambusher, cruiser and intermediate foragers based on a suite of behavioral characteristics (Lewis, 2002). All *Steinernema* spp. in this study were intermediate foragers according to the criteria discussed by Lewis (2002). *Heterorhabditis* spp. employed the cruiser strategy, and were similar to previously reported strategies for *H. bacteriophora* (Lewis *et al*., 1992; Kaya and Gaugler, 1993) but did not find any isolates that had characteristics of ambushers.

3. Nematode virulence

EPN virulence can be measured by several different methods including the one-one bioassay, dose-response tests providing a calculated LC_{50} value, establishment efficiency, invasion rate and the sand column bioassay (Glazer and Lewis, 2000; Grewal, 2002). Two bioassay methods, the dose-response test and the sand column bioassay, were used to evaluate Thai nematode virulence against last-instar *G. mellonella* in this study. The LC_{50} values of *H. indica, H. baujardi* and *Steinernema* sp. against *G. mellonella* larvae ranged from 1.99-6.95 IJs/larvae. These values are relatively similar to Ansari *et al.* (2003) who reported that the LC_{50} values of *S. glaseri* and *H. megidis* for *Hoplia philanthus* Füessly were 4.6 and 9.7 IJs/larvae, respectively. In contrast, one or just a few IJs of *S. carpocapsae, S. feltiae* or *S. riobrave* is sufficient to kill 50% *G. mellonella* larvae (Converse and Miller, 1999; Grewal, 2002). Moreover, Ehlers *et al.* (1997) reported that 20 IJs of *S. feltiae* caused 90% mortality of *Tipula oleracea* L. The low LC_{50} values obtained in this study may be partly due to the small arena and the use of a susceptible host as *G. mellonella*. Two EPN isolates isolated from Thailand showed high infection rates in *G. mellonella* larvae. In addition, *Steinernema* sp. isolate K8 generally tended to be moderate in virulence to *G. mellonella* relative to other isolates from Thailand.

Moreover, virulences of Thai nematode isolates were tested against other insect pests. Nematode dosage, nematode isolate and insect species influenced the nematode virulences. *Plutella xylostella* larvae were killed by *H. indica* isolate K4. This particular isolate showed significantly lower LC_{50} (16.75 IJs/larva) than other nematode isolates, followed by *Steinernema* sp. isolate K8, *H. bacteriophora* and *S. siamkayai* and their LC_{50} values were 21.55, 27.43 and 33.54 IJs/larva, respectively. Most nematodes killed *P. xylostella* larva more than 50% at the concentration of 30 IJs/larva and killed 90-100% *P. xylostella* larvae at the dosages of 300 IJs. However, all nematode isolates did not infect *P. xylostella* pupa. The results obtained on invasion efficacy of *S. siamkayai* where *H. bacteriophora* to *P. xylostella* agreed with Sasnarukkit (2003) who reported that the LC_{50} of *S. siamkayai* to *P. xylostella* larvae ranged from 37-82 IJs/larva and the LC_{50} of *H. bacteriophora* to *P. xylostella* larvae ranged from 44-90 IJs/larva.

The second and third-instar larvae of *S. litura* had significant highest invasion rate by *H. indica* isolate K4 (LC_{50} value = 10.27 and 16.12 IJs/larva, respectively). The LC_{50} values of 4 EPN isolates to second-instar larvae ranged from 10-32 IJs/larva whereas the LC_{50} values of 4 EPNs for the third-instar larva ranged from 16-34

IJs/larva. This result agreed with Baur *et al.* (1998) who stated that early instars were more susceptible than late instar. Most nematode killed second and third-instar larvae of *S. litura* more than 50% from the concentration of 50 IJs/larva and killed 95-100% *S. litura* larva at the dosages of 300 IJs. Moreover, only *Steinernema* sp. isolate K8 was able to infect pre-pupal stage of *S. litura* when 60% pupal mortality was recorded. However, all nematode isolates did not infect *S. litura* pupa whereas the nematode infection appeared again in adult stage. Most EPN isolates showed higher significant efficacy against *S. litura* larva in the second than third-instar lavae when compared their LC_{50} values, except for *S. siamkayai* where similar LC_{50} values for both experiments were recorded. This result agreed with other lepidopterus researches that the LC_{50} of *S. siamkayai*, S. *carpocapsae* and *S. riobrave* against *S. litura* larva were 18.0, 0.2 and 1.8 IJs/larva at 72 h after application, respectively (Chongchitmate *et al.,* 2005).

Campos-Herrera and Gutiérrez (2009) indicated that *S. feltiae* induced 70±4.5% and 80.3±9.5% larval mortality at 2 IJs/cm^2 of *Spodoptera littoralis* Boisduval and *Trichoplusia ni* Hübner, respectively. Mortality rate increased when concentration was raised where 95% mortality was recorded at the concentrations of 25 and 75 IJs/cm^2. The LC_{50} of *S. feltiae* for *S. littoralis* and *T. ni* were 0.69 and 0.27 IJs/cm^2. Significant differences in larval mortality depended on insect species and dosages. *Spodoptera littoralis* and *T. ni* were sensitive organisms and died within a few days (3.5±0.22 and 3.0±0.10 d, respectively). Park *et al*. (2001) studied the effect of *S. carpocapsae* PC, *H. bacteriophora* HY, *S. longicaudum* GJ and *S. glaseri* MK at the concentration of 300 IJs/larva against second-instar larvae of *S. litura* and reported 100% mortality within 73 h after application. For the third-instar larvae, *S. carpocapsae* PC, *H. bacteriophora* HY caused 100% mortality in 47 h, and after 73 h all nematodes produced 90-100% mortality (Park *et al*., 2001). Moreover, Garcia *et al*. (2008) reported that mortality of fall armyworm larvae treated with 400 IJs of *H. indica* was 75%. In contrast, *S. kushidai* failed to kill *S. litura* larvae or causing change in the insect development (Yamanaka *et al*., 1992). In addition, Chongchitmate *et al*. (2005) found that LC_{50} values of *S. siamkayai*, S. *carpocapsae* and *S. riobrave* against *H. armigera* were 22.5, 1.2 and 1.2 IJs/larva at 72 h after application, respectively.

In pupal test, all nematodes failed to induce pupal infection to both *P. xylostella* and *S. litura* pupae whereas *Steinernema* sp. isolate K8 could invade 60% of *S. litura* pre-pupal at 10 d after application. This result contrasted to Ratnasinghe and Hauge (1995, 1997) who reported that *S. carpocapsae* killed 100% *P. xylosstella* larva at 6 h after exposure and caused 40% mortality in immature and mature pupae. Moreover, Kaya and Hara (1980) stated that pre-pupa of *G. mellonella*, *S. exigua* and *Mythimna unipuncta* Haworth were highly susceptible to *S. carpocapsae* infection whereas *G. mellonella* pupae were highly susceptible, *S. exigua* pupae were moderately and *M. unipuncta* pupae were least susceptible to *S. carpocapsae*.

Although *P. xylostella* and *S. litura* larvae are sensitive to all nematodes isolates, the habitat of both species probably unsuitable for EPN application because their larvae are foliar feeding and EPN activities were decreased in foliar application due to desiccation, temperature, and ultraviolet light (Georgis, 1990; Glazer *et al*., 1992). However, the protective formulations and antidesiccants have been developed (Grewal, 2002) such as *S. carpocapsae* Mexican strain was formulated for within canopy applications in cotton plants against *Earias insulana* Boisduval, *H. armigera* and *S. littoralis* (Glazer *et al*., 1992). Due to the limitations in foliar control, EPN application for foliar pests has been largely unsuccessful in field trial (Grewal and Georgis, 1999; Shapiro-Ilan *et al*., 2006). Therefore, Thai EPNs should be formulated with the addition of surfactants to improve their survival and efficacy in foliar application.

Virulence of nematode to late-instar larva of *T. molitor* was shown in filter paper bioassays at a concentration of 300 IJs/ larva. The results indicated that *H. indica* isolate K4 showed the highest virulence to *T. molitor* larvae (LT_{50} = 33.76 h), followed by *Steinernema* sp. isolate K8 and H. *baujardi* isolate K6 where their LT_{50} values were 48.15 and 49.75 h, respectively. All nematodes killed *T. molitor* larvae more than 50% when 300 IJs/larva was applied for 48 h. Shapilo-Ilan *et al.* (2009)

revealed that *T. molitor* mortality was higher in 4 nematode isolates; *Steinernama rarum* Mamiya, *S. riobrave*, *H. indica* and *Heterorhabditis georgiana* Nguyen, Shapiro-Ilan & Mbata when it was exposed to 100 IJs/larva 1 d after exposure except for the *Heterorhabditis mexicana* Nguyen, Shapiro-Ilan, Stuart, McCoy, James & Adams and *Heterorhabditis floridensis* Nguyen, Gozel, Koppenhöfer & Adams. Once the concentration was increased to 500 IJs/larva, *T. molitor* mortality was higher in all treatments except in the *H. mexicana* treatment at 1 d after application. For 2 of 100 IJs/insect, all nematodes caused higher *T. molitor* mortality and *H. georgiana* and *H. mexicana* treatments resulted in lower mortality than other nematodes. In 500 IJs application, *T. molitor* mortality was higher in all nematodes relative to the control (Shapilo-Ilan *et al.*, 2009).

Steinernema glaseri, *Steinernema* sp. isolate K8 and *S. siamkayai* were able to infect first-instar larva of *O. rhinoceros* whereas *H. indica* isolate K4 could not kill this larva. The maximum mortality of *O. rhinoceros* larva caused by *S. glaseri* and *Steinernema* sp. isolate K8 were 100% at 72 h after application whereas *S. siamkayai* induced only 30% mortality. This study agreed with Zelanzy (1985) who reported that 50 IJs of *S. feltiae* induced over 50% mortality on first-instar larva of *O. rhinoceros* at 5 d after exposure. In addition, all *Steinernema* spp. showed higher efficacy than *Heterorhabditis* sp. which also agreed with Koppenhöfer and Kaya (1997) who indicated that *S. kushidai* was the most pathogenic nematode causing 87±6, 97±3 and 97±3% mortality of *Cyclocephala hirta* LeConte at 4, 7, and 14 d after exposure, respectively, followed by *S. glaseri* with 47±9, 75±3, and 78±3% mortality, and *H. bacteriophora* with 8±5, 61±14, and 67±11% mortality whereas *H. marelatus* Liu & Berry could not complete their life cycle in *L. decemlineata* (Armer *et al.* 2004).

In contrast, Shapilo-Ilan *et al.* (2009) indicated that *S. riobrave*, *S. rarum*, *H. indica*, *H. Mexicana*, *H. floridensis* and *H. georgiana* caused higher mortality against *D. abbreviates* larvae at 7 d after application, and the highest mortality was observed in the *H. indica* and *S. riobrave* treatments whereas the lowest was observed in *S. rarum*. Grewal *et al*. (2002) reported that *Heterorhabditis zealandica* Poinar was significantly more virulent towards *P. japonica* than any other *Heterorhabditis* isolates, the LC_{50} = 272 IJs/grub.

4. Sand column bioassay

Sand column bioassay demonstrated that there are significant differences in virulence among the Thai isolates, with the isolate K8 being significantly more virulent to last-instar *G. mellonella* larva than Thai *Heterorhabditis* spp. and commercial isolates. Different bioassays can lead to different conclusion. The results from the sand column bioassay differed from the filter paper bioassay. In the filter paper test, most heterorhabditid nematodes showed higher efficacy (lower LC_{50}) than steinernematid nematodes. In contrast, *Steinernema* spp. caused the highest efficacy in sand column bioassay whereas *Heterorhabditis* spp. did not do as well.

The effect of soil type on nematode infectivity also varied among nematode species. *Steinernema* sp. isolate K8 showed the highest infectivity in both soil types, but had lower infectivity in the sandy clay loam. Moreover, the nematode dispersal assay evaluated the ability of the nematodes to move vertically through the sand profile and to locate a target host. These results indicated that, most numbers of IJs for all isolates remained in the bottom layer of the column in coarse sand. On the other hand, all nematode isolates moved slower in medium and fine sand, whereas the greatest proportions of three isolates were found in the middle layer.

Results from nematode dispersal assay were similar to results in sand column bioassays. The highest nematode infection rate was found in large sand particle and the rate decreased when nematode move in more heavily textured sand. These results agreed with many reports that nematode efficacy was significantly influenced by soil type (Molyneux and Bedding, 1984; Kaspi *et al*., 2010). Most EPNs are not effective in heavily textured soils (Georgis and Poinar, 1983; Choo and Kaya, 1991) and the virulence of EPNs increased with soil sand content (Molyneux and Bedding, 1984; Kung *et al.,* 1990; Portillo-Aguilar *et al.,* 1999; Koppenhöfer and Fuzy, 2006). Laboratory, greenhouse and field experiments demonstrated that virulence was positively correlated with the percentage of sand, silt and organic matter (Choo and Kaya, 1991; Koppenhöfer and Fuzy, 2006, 2007; Campos-Herrera *et al.*, 2008) and negatively correlated with the percentage of clay and electrical conductivity (Georgis and Poinar, 1983; Choo and Kaya, 1991; Campos-Herrera *et al*., 2008). This is probably because nematode movement is restricted in finer textured soils due to smaller soil pores causing poor aeration. Hence, smaller soil pores resulted in the reduction of nematode respiration, nematode survival and their efficacy (Burman and Pye, 1980; Portillo-Aguilar *et al.,* 1999; Koppenhöfer and Fuzy, 2007).

Deciding on the best test of nematode virulence depends on the objective of each project because EPNs need different types of attributes depending on the intended use in the field. The results from the filter paper bioassay and sand column bioassay differed; *Heterorhabdiits* spp. out-performed *Steinernema* sp. isolate K8 in the filter paper bioassay whereas *Steinernema* sp. isolate K8 had greater infection rates in sand column bioassay. This re-emphasizes that a single bioassay does not often supply sufficient information to develop hypotheses about nematode efficacy in the field (Glazer and Lewis, 2000; Grewal, 2002). The filter paper bioassay is a rapid and simple method to screen for nematode virulence, but removes any environmental barriers to infection. The sand column bioassays are closer to field conditions. Hence, these results agreed with several previous studies and indicated that the sand column bioassay is a better standard tool for predicting EPN efficacy in a field trial, especially when soil-dwelling insect pest are considered (Molyneux, 1986; Mannion and Janssan, 1993; Grewal, 2002).

CONCLUSION AND RECOMMENDATION

The conclusion and recommendation of all experiments in this study can be shown as follows:

1. In this study, 168 soil samples were collected from undisturbed area close to the national park in southern Thailand. Two new isolates of *Steinernema* sp., 5 new isolates of *H. indica* and one new record of *H. baujardi* were recovered.

2. The undescribed species (*Steinernema* sp. isolate K8) showed potential as a biological control agent because it was effective even in high clay soils. Moreover, this species out performed 3 commercial nematodes in sand column bioassays. Therefore, the possibility for using this species in biological control program is of considerable interest. However, its virulence and effectiveness remain to be confirmed against agricultural pests, both in the greenhouse and in field trials.

3. The virulence of the new Thai nematode isolates was determined against the larvae and pupae of *S. litura* and *P. xylostella* for several bioassays. Both insect larvae were highly susceptible to Thai isolates (*H. indica* isolate K4 and *Steinernema* sp. isolate isolate K8). Since the habitats of both species are unsuitable for EPN application because their larvae are foliar feeding, therefore the suitable formulation and antidesiccants are needed to be developed for foliar application.

4. The *Steinernema* sp. isolate K8 shows high virulence as a bio-agent to suppress both *T. molitor* and *O. rhinoceros* larvae in filter paper bioassay and its virulence is closed to the larval mortality rate caused by *S. glaseri* (100% mortality at 72 h after exposure). Therefore, the possibility for using this species in soil-insect pest control should be considered.

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APPENDICES

Appendix A

Soil collecting information

Appendix Table A1 Collecting sites, local conditions and soil characteristics for all soil samples from southern Thailand.

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
1	Klong Mai Dang Forest 1	Surat Thani	May 2, 08	- -	-	30	70	24.1	-	-	-
2	Klong Mai Dang Forest 2	Surat Thani	May 2, 08	- -	-	29	62	24.3	-	-	-
3	Klong Mai Dang Forest 3	Surat Thani	May 2, 08	- -	-	28	70	24.2	-	-	-
4	Wipawadee Water fall 1	Surat Thani	May 2, 08	0909631E 9852943N	134	30	65	25.9	-	-	-
5	Wipawadee Water fall 2	Surat Thani	May 2, 08	0909593E 9852913N	180	30	70	24.7	-	-	-
6	Wipawadee Water fall 3	Surat Thani	May 2, 08	0909583E 9852905N	187	30	69	24.6	-	-	-
7	Krom Cave 1	Surat Thani	June 4, 08	0846196E 9922116N	44	29	58	25.7	-	-	-
8	Krom Cave 2	Surat Thani	June 4, 08	0846174E 9922072N	51	29	58	25.2	5.64	0.58	34
9	Krom Cave 3	Surat Thani	June 4, 08	0846207E 9922111N	35	29	58	25.8	-	-	-

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
10	Kha Min Cave 1	Surat Thani	June 4, 08	0849797E 9922799N	146	29	70	23.9	5.55	0.51	42
11	Kha Min Cave 2	Surat Thani	June 4, 08	0849802E 9922750N	131	30	68	24.8	5.80	0.51	20
12	Kha Min Cave 3	Surat Thani	June 4, 08	0849794E 9922755N	85	30	70	21.8	-	-	-
13	Muang Tuod Water fall 1	Surat Thani	June 4, 08	0845082E 9926090N	155	29	60	25.3	-	-	-
14	Muang Tuod Water fall 2	Surat Thani	June 4, 08	0845048E 9926204N	167	29	60	24.9	-	-	-
15	Muang Tuod Water fall 3	Surat Thani	June 4, 08	0844893E 9936090N	165	30	62	24.7	-	-	-
16	Chulaporn Research Park 1	Surat Thani	June 6, 08	0849526E 9926047N	444	26	80	23.4	-	-	-
17	Chulaporn Research Park 2	Surat Thani	June 6, 08	0849194E 9926244N	392	30	60	23.8	-	-	-
18	Chulaporn Research Park 3	Surat Thani	June 6, 08	0849469E 9925848N	412	30	56	23.1	-	-	-

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
19	Dadfha Water fall 1	Surat Thani	June 6, 08	0851734E 9928840N	275	30	60	24.7	-	-	-
20	Dadfha Water fall 2	Surat Thani	June 6, 08	0851577E 9929081N	264	29	65	23.8	-	-	-
21	Dadfha Water fall 3	Surat Thani	June 6, 08	0851762E 9928780N	265	28	70	23.5	-	-	-
22	Pueng Cave 1	Surat Thani	June 5, 08	0847203E 9852365N	95	28	75	24.5	-	-	-
23	Pueng Cave 2	Surat Thani	June 5, 08	0847155E 9852343N	94	29	75	24.3	-	-	-
24	Pueng Cave 3	Surat Thani	June 5, 08	0847156E 9852289N	98	29	78	25.3	-	-	-
25	Ban Nam Dood 1	Surat Thani	June 5, 08	0845694E 9852306N	94	27	75	24.4	-	-	-
26	Ban Nam Dood 2	Surat Thani	June 5, 08	0845689E 9852278N	90	27	75	24.6	-	-	-
27	Ban Nam Dood 3	Surat Thani	June 5, 08	0845728E 9852281N	90	27	74	24.7	-	-	-

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
28	Tai Rom Yen National Park 1	Surat Thani	June 5, 08	0840550E 9928372N	430	26	80	22.5	5.56	0.43	24
29	Tai Rom Yen National Park 2	Surat Thani	June 5, 08	0840433E 9928264N	359	26	82	22.5	5.55	0.44	24
30	Tai Rom Yen National Park 3	Surat Thani	June 5, 08	0840281E 9928198N	341	25	80	23.0	5.56	0.44	26
31	Khao Panom National Park 1	Surat Thani	June 6, 08	0852737E 9840548N	91	27	92	26.2	-	-	-
32	Khao Panom National Park 2	Surat Thani	June 6, 08	0852711E 9840504N	103	27	93	28.1	-	-	-
33	Khao Panom National Park 3	Surat Thani	June 6, 08	0852681E 9840485N	96	27	95	25.6	-	-	-
34	Khao Kok Cave 1	Surat Thani	June 6, 08	0841550E 9922751N	60	28	74	26.1	-	-	-
35	Khao Kok Cave 2	Surat Thani	June 6, 08	0841575E 9922730N	60	29	70	25.1	-	-	-
36	Khao Kok Cave 3	Surat Thani	June 6, 08	0841609E 9922716N	50	29	70	32.2	-	-	-

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
37	Khao Sok National Park 1	Surat Thani	June 7, 08	0854760E 9830449N	115	29	80	25.9	-	-	-
38	Khao Sok National Park 2	Surat Thani	June 7, 08	0854804E 9830834N	93	27	80	25.2	-	-	-
39	Khao Sok National Park 3	Surat Thani	June 7, 08	0854841E 9831243N	85	27	85	25.3	-	-	-
40	Khao Tha Pet National Park 1	Surat Thani	June 7, 08	0905838E 9921081N	190	29	65	25.8	-	-	-
41	Khao Tha Pet National Park 2	Surat Thani	June 7, 08	0905885E 9921110N	199	30	62	26.1	-	-	-
42	Khao Tha Pet National Park 3	Surat Thani	June 7, 08	0905717E 9920910N	127	31	60	25.6	-	-	-
43	Suan Moke Wanaram Park 1	Surat Thani	June 7, 08	0921471E 9910234N	6	28	71	25.8	-	-	-
44	Suan Moke Wanaram Park 2	Surat Thani	June 7, 08	0921422E 9910188N	3	29	71	25.8	-	-	-
45	Suan Moke Wanaram Park 3	Surat Thani	June 7, 08	0921494E 9910156N	5	28	71	25.6	-	-	-

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
46	Khao Wang Thong Cave 1	Nakorn Sri Tammarat	July 14, 08	0912347E 9946489N	77	29	68	25.3	6.15	382	35
47	Khao Wang Thong Cave 2	Nakorn Sri Tammarat	July 14, 08	0912292E 9946469N	73	28	69	25.3	6.26	488	42
48	Khao Wang Thong Cave 3	Nakorn Sri Tammarat	July 14, 08	0912280E 9946445N	71	28	70	24.8	6.39	438	43
49	Si Keet Water fall 1	Nakorn Sri Tammarat	July 14, 08	0900808E 9946264N	79	31	70	25.7	6.44	175	20
50	Si Keet Water fall 2	Nakorn Sri Tammarat	July 14, 08	0900823E 9946224N	89	29	70	25.2	6.22	435	27
51	Si Keet Water fall 3	Nakorn Sri Tammarat	July 14, 08	0900821E 9946206N	86	29	73	24.8	6.27	296	22
52	Sunantha Water fall 1	Nakorn Sri Tammarat	July 14, 08	0846048E 9948139N	117	30	69	25.4	6.19	337	19
53	Sunantha Water fall 2	Nakorn Sri Tammarat	July 14, 08	0846035E 9948086N	118	29	75	24.8	6.25	153	18
54	Sunantha Water fall 3	Nakorn Sri Tammarat	July 14, 08	0846045E 9948103N	105	29	80	25.0	6.10	187	23

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
55	Hin Thoo Water fall 1	Nakorn Sri Tammarat	July 14, 08	0847115E 9947154N	91	29	76	25.3	6.00	293	20
56	Hin Thoo Water fall 2	Nakorn Sri Tammarat	July 14, 08	0847091E 9947547N	81	27	84	25.4	5.97	318	24
57	Hin Thoo Water fall 3	Nakorn Sri Tammarat	July 14, 08	0847155E 9947602N	82	29	84	25.3	6.14	94	30
58	Klong Jung Water fall 1	Nakorn Sri Tammarat	July 15, 08	0816277E 9938671N	88	26	83	24.1	6.09	132	24
59	Klong Jung Water fall 2	Nakorn Sri Tammarat	July 15, 08	0816275E 9938688N	180	29	76	24.3	5.93	227	21
60	Klong Jung Water fall 3	Nakorn Sri Tammarat	July 15, 08	0816245E 9938667N	178	34	59	24.6	5.96	216	23
61	Tha Pae Water fall 1	Nakorn Sri Tammarat	July 15, 08	0821760E 9941406N	170	30	70	23.5	6.00	145	6
62	Tha Pae Water fall 2	Nakorn Sri Tammarat	July 15, 08	0821752E 9941408N	200	31	60	23.7	5.73	321	29
63	Tha Pae Water fall 3	Nakorn Sri Tammarat	July 15, 08	0821734E 9941243N	171	31	66	24.1	5.62	402	33

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
64	Ka Rome Water fall 1	Nakorn Sri Tammarat	July 15, 08	0822548E 9944177N	224	31	60	24.4	5.67	243	18
65	Ka Rome Water fall 2	Nakorn Sri Tammarat	July 15, 08	0822475E 9944164N	192	31	60	24.9	5.53	253	20
66	Ka Rome Water fall 3	Nakorn Sri Tammarat	July 15, 08	0822427E 9944163N	163	30	59	24.1	5.55	300	24
67	Keaw Sura Kan Cave 1	Nakorn Sri Tammarat	July 15, 08	0821675E 9947099N	48	29	64	24.4	5.59	453	12
68	Keaw Sura Kan Cave 2	Nakorn Sri Tammarat	July 15, 08	0821660E 9947140N	49	33	60	25.5	5.67	462	26
69	Keaw Sura Kan Cave 3	Nakorn Sri Tammarat	July 15, 08	0821647E 9947127N	34	31	60	25.1	5.74	584	26
70	Phrom Loke Water fall 1	Nakorn Sri Tammarat	July 16, 08	0831555E 9946948N	211	29	63	25.5	5.87	354	33
71	Phrom Loke Water fall 2	Nakorn Sri Tammarat	July 16, 08	0831543E 9946956N	225	29	65	25.1	5.88	270	23
72	Phrom Loke Water fall 3	Nakorn Sri Tammarat	July 16, 08	0831469E 9946934N	236	30	69	24.8	5.77	336	31

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
73	Ai Keaw Water fall 1	Nakorn Sri Tammarat	July 16, 08	0833480E 9946618N	173	29	75	24.0	6.05	66	74
74	Ai Keaw Water fall 2	Nakorn Sri Tammarat	July 16, 08	0833444E 9946600N	182	29	74	24.2	5.74	250	16
75	Ai Keaw Water fall 3	Nakorn Sri Tammarat	July 16, 08	0833473E 9946694N	174	30	76	24.8	5.70	274	30
76	Yod Leung Water fall 1	Nakorn Sri Tammarat	July 16, 08	0837781E 9943745N	150	31	67	25.4	5.77	218	21
77	Yod Leung Water fall 2	Nakorn Sri Tammarat	July 16, 08	0837696E 9943666N	130	34	54	24.4	5.70	241	18
78	Yod Leung Water fall 3	Nakorn Sri Tammarat	July 16, 08	0837695E 9943618N	130	33	62	25.6	5.70	357	31
79	Krung Ching Water fall 1	Nakorn Sri Tammarat	July 16, 08	0843321E 9940168N	215	30	65	24.4	5.71	738	41
80	Krung Ching Water fall 2	Nakorn Sri Tammarat	July 16, 08	0843293E 9940197N	183	31	67	24.4	5.68	770	48
81	Krung Ching Water fall 3	Nakorn Sri Tammarat	July 16, 08	0843205E 9940208N	215	32	72	24.2	5.78	656	95

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
82	Hong Cave 1	Nakorn Sri Tammarat	July 17, 08	0844025E 9938150N	118	33	60	24.8	5.90	348	35
83	Hong Cave 2	Nakorn Sri Tammarat	July 17, 08	0844006E 9938148N	116	31	63	25.0	5.91	453	31
84	Hong Cave 3	Nakorn Sri Tammarat	July 17, 08	0844011E 9938156N	123	30	66	24.5	5.79	907	51
85	Krung Nang Water fall 1	Nakorn Sri Tammarat	July 17, 08	0846349E 9935473N	236	29	65	24.6	6.14	327	9
86	Krung Nang Water fall 2	Nakorn Sri Tammarat	July 17, 08	0846323E 9935507N	210	29	71	24.5	6.03	405	18
87	Krung Nang Water fall 3	Nakorn Sri Tammarat	July 17, 08	0846329E 9935584N	217	30	72	24.5	5.93	545	40
88	Klong Paw Water fall 1	Nakorn Sri Tammarat	July 17, 08	0848189E 9934348N	186	30	68	27.0	6.09	306	44
89	Klong Paw Water fall 2	Nakorn Sri Tammarat	July 17, 08	0848142E 9934375N	181	32	75	25.7	6.11	312	32
90	Klong Paw Water fall 3	Nakorn Sri Tammarat	July 17, 08	0848132E 9934355N	184	31	70	25.8	6.10	313	32

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
91	Somdej Phrasrinakarin Park 1	Pang Nga	October 27, 08	0826134E 9831037N	60	26	87	24.6	-	585	77
92	Somdej Phrasrinakarin Park 2	Pang Nga	October 27, 08	0826128E 9831035N	54	26	86	24.7	-	400	47
93	Somdej Phrasrinakarin Park 3	Pang Nga	October 27, 08	0826045E 9831151N	50	25	85	25.1	-	697	78
94	Ta Pan Cave 1	Pang Nga	October 27, 08	0827296E 9831676N	49	29	74	24.5	-	619	50
95	Ta Pan Cave 2	Pang Nga	October 27, 08	0827199E 9831681N	45	28	74	24.7	-	807	59
96	Ta Pan Cave 3	Pang Nga	October 27, 08	0827261E 9831632N	53	26	80	25.1	-	641	40
97	Tone Water fall 1	Pang Nga	October 27, 08	0829432E 9829131N	125	28	78	25.5	-	56	34
98	Tone Water fall 2	Pang Nga	October 27, 08	8294633E 9829156N	120	28	76	25.2	-	84	36
99	Tone Water fall 3	Pang Nga	October 27, 08	0829410E 9829145N	111	28	75	24.8	-	66	29

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
100	Ra Man Water fall 1	Pang Nga	October 28, 08	0826964E 9826680N	106	28	77	24.6	-	50	38
101	Ra Man Water fall 2	Pang Nga	October 28, 08	0826999E 9826705N	90	28	79	24.3	-	103	41
102	Ra Man Water fall 3	Pang Nga	October 28, 08	0827067E 9826723N	90	28	76	25.3	-	-	-
103	Suwan Kuha Cave 1	Pang Nga	October 28, 08	0825789E 9828278N	34	28	76	25.3	-	719	42
104	Suwan Kuha Cave 2	Pang Nga	October 28, 08	0825789E 9828278N	34	28	75	25.1	-	867	102
105	Suwan Kuha Cave 3	Pang Nga	October 28, 08	0825777E 9828279N	34	28	75	25.4	-	805	54
106	Ma No Ra Water fall 1	Pang Nga	October 28, 08	0830634E 9832491N	80	26	85	23.5	-	72	36
107	Ma No Ra Water fall 2	Pang Nga	October 28, 08	0830669E 9832470N	83	26	83	24.5	-	223	38
108	Ma No Ra Water fall 3	Pang Nga	October 28, 08	0830691E 9832414N	85	26	82	23.8	-	60	31

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
109	Tone Pairwat Water fall 1	Pang Nga	October 28, 08	0836860E 9833038N	386	25	85	22.9	-	100	58
110	Tone Pairwat Water fall 2	Pang Nga	October 28, 08	0836887E 9833032N	382	25	85	23.4	-	78	40
111	Tone Pairwat Water fall 3	Pang Nga	October 28, 08	0836842E 9832982N	387	25	84	22.9	-	71	49
112	Kha Nim Water fall 1	Pang Nga	October 29, 08	0829797E 9817036N	13	23	76	24.6	-	134	19
113	Kha Nim Water fall 2	Pang Nga	October 29, 08	0829815E 9817028N	20	23	87	24.6	-	66	21
114	Kha Nim Water fall 3	Pang Nga	October 29, 08	0829824E 9817002N	25	24	90	24.2	-	473	22
115	Lam Pee Water fall 1	Pang Nga	October 29, 08	0827860E 9816937N	66	25	94	24.0	-	101	24
116	Lam Pee Water fall 2	Pang Nga	October 29, 08	0827334E 9816938N	113	25	95	24.4	-	143	32
117	Lam Pee Water fall 3	Pang Nga	October 29, 08	0827857E 9816916N	71	25	94	24.1	-	80	36

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
118	Lam Roo Water fall 1	Pang Nga	October 29, 08	0839130E 9826880N	100	24	80	24.1	-	-	-
119	Lam Roo Water fall 2	Pang Nga	October 29, 08	0839120E 9826873N	104	24	85	23.8	-	-	-
120	Lam Roo Water fall 3	Pang Nga	October 29, 08	0839074E 9826887N	99	25	90	23.5	-	-	-
121	Tam Nhung Water fall 1	Pang Nga	October 30, 08	0859804E 9828268N	101	25	85	23.6	-	-	-
122	Tam Nhung Water fall 2	Pang Nga	October 30, 08	0859815E 9828214N	101	25	87	24.0	-	-	-
123	Tam Nhung Water fall 3	Pang Nga	October 30, 08	0859804E 9828159N	100	25	89	24.0	-	-	-
124	Tone Dang Water fall 1	Pang Nga	October 30, 08	0859684E 9827991N	38	25	90	24.2	-	80	53
125	Tone Dang Water fall 2	Pang Nga	October 30, 08	0859687E 9828018N	40	25	91	23.5	-	128	34
126	Tone Dang Water fall 3	Pang Nga	October 30, 08	0859711E 9828021N	48	25	93	23.5	-	109	49

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
127	Suan Mai Water fall 1	Pang Nga	October 30, 08	0918071E 9825029N	193	26	85	23.1	-	91	29
128	Suan Mai Water fall 2	Pang Nga	October 30, 08	0918086E 9820012N	190	25	84	23.1	-	50	38
129	Suan Mai Water fall 3	Pang Nga	October 30, 08	0918090E 9824337N	110	25	82	23.1	-	76	29
130	Khao Kreab Cave 1	Chumporn	February 18, 09	0949173E 9902335N	158	26	77	23.4	5.45	382	28
131	Khao Kreab Cave 2	Chumporn	February 18, 09	- -	-	26	84	23.3	5.63	407	32
132	Khao Kreab Cave 3	Chumporn	February 18, 09	0949174E 9902347N	158	25	85	23.2	5.87	369	20
133	Somdej Phrasrinakarin Park 1	Chumporn	February 18, 09	0956847E 9902478N	51	30	60	23.5	6.08	0.34	36
134	Somdej Phrasrinakarin Park 2	Chumporn	February 18, 09	0956842E 9902470N	51	30	60	24.2	6.78	0.37	32
135	Somdej Phrasrinakarin Park 3	Chumporn	February 18, 09	0956844E 9902490N	58	29	62	24.5	6.82	376	43

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
136	Klong Rhung Water fall 1	Chumporn	February 18, 09	0950915E 9844900N	123	33	50	25.9	7.16	268	18
137	Klong Rhung Water fall 2	Chumporn	February 18, 09	0950925E 9844911N	101	32	52	24.0	7.16	318	15
138	Klong Rhung Water fall 3	Chumporn	February 18, 09	0950933E 9844921N	120	32	51	24.3	7.33	224	26
139	Hell Loam Water fall 1	Chumporn	February 18, 09	0943726E 9840926N	119	32	55	26.1	7.34	213	12
140	Hell Loam Water fall 2	Chumporn	February 18, 09	0943731E 9840967N	92	32	60	23.6	7.39	206	16
141	Hell Loam Water fall 3	Chumporn	February 18, 09	0943710E 9840909N	81	32	62	23.8	6.78	236	18
142	Tone Pet Water fall 1	Ranong	February 19, 09	0943155E 9836978N	123	30	70	23.2	6.78	230	14
143	Tone Pet Water fall 2	Ranong	February 19, 09	0943187E 9836977N	93	29	70	22.9	6.85	191	12
144	Tone Pet Water fall 3	Ranong	February 19, 09	0943172E 9836984N	90	29	70	23.0	6.79	233	6

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
145	Ngaw Water fall 1	Ranong	February 19, 09	0951305E 9837844N	92	26	80	24.3	6.98	198	28
146	Ngaw Water fall 2	Ranong	February 19, 09	0951297E 9837837N	100	25	82	24.6	6.85	249	16
147	Ngaw Water fall 3	Ranong	February 19, 09	0951345E 9837846N	99	26	82	24.4	6.78	240	30
148	Pun Ya Ban Water fall 1	Ranong	February 19, 09	1003790E 9840276N	91	29	69	23.1	6.91	204	27
149	Pun Ya Ban Water fall 2	Ranong	February 19, 09	1003943E 9840198N	126	29	75	23.1	7.04	160	26
150	Pun Ya Ban Water fall 3	Ranong	February 19, 09	1003955E 9840200N	112	28	78	23.5	6.69	140	23
151	Suwan Kee Ree Water fall 1	Ranong	February 19, 09	1013603E 9845458N	130	30	65	23.3	6.90	185	16
152	Suwan Kee Ree Water fall 2	Ranong	February 19, 09	1013578E 9845457N	88	29	70	23.1	6.91	223	20
153	Suwan Kee Ree Water fall 3	Ranong	February 19, 09	1013561E 9845459N	80	29	70	23.5	6.90	241	20

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
154	Bok Rai Water fall 1	Ranong	February 20, 09	1022440E 9851490N	88	30	62	23.8	6.80	244	15
155	Bok Rai Water fall 2	Ranong	February 20, 09	1022444E 9851433N	124	29	65	23.7	6.90	185	16
156	Bok Rai Water fall 3	Ranong	February 20, 09	1022473E 9851443N	160	29	65	23.7	6.70	346	21
157	Tan Lod Cave 1	Chumporn	February 20, 09	1014111E 9856778N	38	28	80	25.5	6.77	366	21
158	Tan lod Cave 2	Chumporn	February 20, 09	1014120E 9856779N	42	29	80	25.3	6.72	446	14
159	Tan lod Cave 3	Chumporn	February 20, 09	1014118E 9856790N	30	28	82	25.3	6.92	322	25
160	Kang Krome Water fall 1	Chumporn	February 20, 09	1013932E 9857605N	31	30	70	24.1	7.11	227	9
161	Kang Krome Water fall 2	Chumporn	February 20, 09	1013933E 9857600N	33	30	75	24.6	7.00	270	12
162	Kang Krome Water fall 3	Chumporn	February 20, 09	1013944E 9857610N	39	30	74	24.7	7.11	300	10

Appendix Table A1 (Continued)

Soil samples	Locations	Provinces	Collecting dates	GPS conditions	E	AT	RH	ST	pH	EC	%MC
163	Khao Peep Cave 1	Chumporn	February 20, 09	1008731E 9906240N	13	34	54	27.3	6.77	517	16
164	Khao Peep Cave 2	Chumporn	February 20, 09	1008741E 9906246N	13	32	55	27.3	6.70	557	56
165	Khao Peep Cave 3	Chumporn	February 20, 09	1008763E 9906265N	12	31	55	26.0	7.03	344	14
166	Khao Pra Cave 1	Chumporn	February 20, 09	0943741E 9306494N	50	30	50	25.8	6.93	520	23
167	Khao Pra Cave 2	Chumporn	February 20, 09	0943742E 9306494N	50	30	50	24.9	7.19	325	23
168	Khao Pra Cave 3	Chumporn	February 20, 09	0943823E 9906543N	15	32	52	27.4	7.18	442	19

Appendix B

Solution and Molecular detail

Composition of artificial diet used in *Spodoptera litura* larva rearing**.**

Ingredients	Quanlity
Mungbean flour	130 g
Yeast	10 g
Wheat germ	10 g
Methyl parahydroxybenzoate	2.5 g
Sorbic acid	1.5 g
Ascorbic acid	3 g
Casein	3 g
Choline chloride	0.5 g
Formaline	2 g
Agar	13 g
Vitamin stock	10 g
Distilled water	700 ml
Vitamin stock	
Niacin	1.2 g
Inositol	1 g
Calcium panthothenate	0.6 g
Thiamine	1 g
Riboflavin	0.6 g
Pyridoxine	0.3 g
Folic acid	0.3 g
Biotin	1 g
Vitamin B12	4 g
Chloline chloride	2.5 g
Distilled water	100 ml

Fixative preparation

TAF fixative

Formalin	7 ml
Triethanolamine	2 ml
Water	91 ml

Lactophenol

Lactic acid	100 ml
Phenol	100 ml
Glycerin	200 ml
Water	100 ml

Ringer's solution

Nacl	9 g
KCl	0.4 g
$CaCl_2$	0.4 g
NaH_2CO_3	0.2 g
Distilled water	1 litre

All the sequences are for the ITS ribosomal region (ITS I and II). All external primers were

F 93:TTGAACCGGGTAAAAGTCG

R 94:TTAGTTTCTTTTCCTCCGCT

Internal primers for K7 and K8 were

F 533: CAAGTCTTATCGGTGGATCAC

R 534: GCAATTCACGCCAAATAACGG

Internal primers for K6 were

F 389: TGCAGACGCTTAGAGTGGTG

R 264: CGTTTTTCATCGATACGCG

>>Seq1 [Organism=*Heterorhabditis baujardi* isolate K6] *Heterorhabditis baujardi* isolate K6, 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 2 and 28S ribosomal RNA gene, partial sequence.

TCCGCTTTAGTTTCTTTTCCTCCGCTGAATGATATGCTTAAGTTCGGCGG

GTAGTCACGACTGAGCTCAGGTTGCATAATGAGACTTATAGGAAACACGA

TTTATGTATATAACCACGTCGTATTTCATACATGGCATATCCATAAGTGA

AACCTAATATTTCTCCGGCGTTAAGAGAAATTCATTTAACCACCATTCTC

CCCTGTAACGGGGCATGCACCACATTTTAGGCACTATCACCGTTTGGTTC

ACGGTATTGCCGCCTTCCTAGCAATACCGAAGTCTATTAAACAACCCTGA

GCCAGACGTGCCGAAGGGAAAACCCAACGGCGCTGTGCGTTCAAAATTTC

ACCACTCTAAGCGTCTGCAATTCGTGGCAAATAACGCAGCTTGCTGCGTT

TTTCATCGATACGCGAATCAACCGATCCATCGCTAAGGCTAATGTTCCAC

TAACACTCAATAGCCTTCACTAGAAACAAGTTAATTGGGAAGAGATTTAC

CGATACTGGCGGTTGTCTCGGTCAGGTGACACGAACAACTCATTAAGGCT

CCCCGCATAGCAGATTAGTGATTTTGACAAAGTCTCATCACTTAGCCACC

AACTATCGATGGGTTTTTGGTGAGGCGACAGACCTAGATCAGCTCATTAA

GTCTTATGCTCTACCCTTACGGGGAGCATACCCAATTATCATTGCCGTCA

CTCAAATCGGCTCCAATCAAGAATAGACACCTCTCCGAGAGCTTAGATGG

GGTGAGACACCGACTGAAATCAAGAACAAGCTTGATTCCATGATTATCAG

CATCTCGTGACCAAAGCATATACCTATAAGGTATCGACGATGATCCATCT

GCAGGTTCACCTACAGATACCTTGTTACGACTTTTACCCGGTTCAAAGNN

>Seq1 [Organism=*Steinernema* sp. isolate K8] *Steinernema* sp. isolate K8, 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 2 and 28S ribosomal RNA gene, partial sequence.

AAGTCTTTGAACCGGGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCT
GCGGAAGGATCATTATTGAGCTTATCATTTCATATGATGATTGTTCGGAA
CGGCACTGCTTCGTTTCTAGGTGTCGATTTCGTTCGCAAACGGCTTTGAA
TGGTTTCTATAGGTGTCTGGAGCAGCTGTATGAGCGTGGCTGTGGTGAAG
GACATTTGACATCCTATGCCAGACGTGGTCTGTTTCTAGCGTTTGGTGAT
GTAGAATTGAAGAGGTCAGTCGGAGACCCGCCGTTCACAAACCCTACCAT
TAACATTTTACTTGATGATGCTCCATGCGAATGGTGCAAATAACAATTAT
CAAGTCTTATCGGTGGATCACTCGGTTCGTAGTTCGATGAAAAACGGGGC
AAAAACCGTTATTTGGCGTGAATTGCAGACATATTGAACGCTAAAATTTT
GAACGCAAATGGCACTATCAGGTTTATATCTGTTAGTATGTTTGGTTGAG
GGTCGATTAACTCGTGACTTGCAGTCAGCTGAGACTGTTCTTTCGATTAG
CTACTTGAAAGAGTACCTTTTCGGTGTGGACGCCGTCATTGGCGATAGTT
TAATGGAGGTTTTAGGCGAGCGTCTCTTTCGCTAAAACTTCTGCTATCAT
ATCGGTTCTGTGCGTTAGTGGTTTGGCGCGTCTCTTGCCAACTGACTTGT
GCGGACAGCGTTTCGTGCGTAAGTTTCTAGAAGTCAGCAGCCACGTGGTG
ACTCAGCGTGTTTCCGTTGGTCAACGAGCGTACTTGGAACTATGAATTCG
ATGTTTTCGAATTACGACCTCAACTCAAGCAAGACTACCCGCTGAACTTA
AGCATATCAGTAAGCGGAGGAAAAGAAACTAAAGCGGAGGAAAAGAAA
CTAAAGCGGAGGA

CURRICULUM VITAE

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