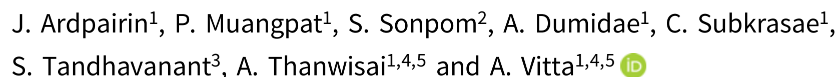
Journal of Helminthology

cambridge.org/jhl

A survey of entomopathogenic nematodes and their symbiotic bacteria in agricultural areas of northern Thailand



J. Ardpairin[1], P. Muangpat[1], S. Sonpom[2], A. Dumidae[1], C. Subkrasae[1], S. Tandhavanant[3], A. Thanwisai[1,4,5] and A. Vitta[1,4,5]

[1]Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok, 65000, Thailand; [2]Department of Agriculture Science, Faculty of Agriculture Natural Resources and Environment, Naresuan University, Phitsanulok, 65000, Thailand; [3]Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand; [4]Centre of Excellence in Medical Biotechnology (CEMB), Faculty of Medical Science, Naresuan University, Phitsanulok, 65000, Thailand and [5]Center of Excellence for Biodiversity, Faculty of Sciences, Naresuan University, Phitsanulok, 65000 Thailand

Research Paper

Cite this article: Ardpairin J, Muangpat P, Sonpom S, Dumidae A, Subkrasae C, Tandhavanant S, Thanwisai A, Vitta A (2020). A survey of entomopathogenic nematodes and their symbiotic bacteria in agricultural areas of northern Thailand. *Journal of Helminthology* **94**, e192, 1–11. https://doi.org/10.1017/S0022149X20000735

Received: 28 May 2020
Revised: 12 August 2020
Accepted: 16 August 2020

Key words:
Agriculture; entomopathogenic nematodes; phylogeny; symbiotic bacteria

Author for correspondence:
Apichat Vitta, E-mail: apichatv@nu.ac.th

Abstract

Entomopathogenic nematodes (EPNs) *Steinernema* and *Heterorhabditis* and their symbiotic bacteria, *Xenorhabdus* and *Photorhabdus*, have been successfully used for the control of insect pests. The objectives of this study were to survey the EPNs and symbiotic bacteria in the agricultural areas of the Phitsanulok province, Thailand, and to study the association between the soil parameters and presence of EPNs. We collected 200 soil samples from 40 soil sites in agricultural areas (field crops, horticulture crops and forest). The prevalence of EPNs was 8.0% (16/200). Fifteen of the EPN isolates were molecularly identified (based on 28S ribosomal DNA and internal transcribed spacer regions) as *Steinernema siamkayai*. Seven isolates of *Xenorhabdus stockiae* were identified using recombinase A sequencing. Phylogenetic analysis revealed that all the *Steinernema* and *Xenorhabdus* isolates were closely related to *S. siamkayai* (Indian strain) and *X. stockiae* (Thai strain), respectively. Significantly more EPNs were recovered from loam than from clay. Although the association between soil parameters (pH, temperature and moisture) and the presence of EPNs was not statistically significant, the elevation levels of the soil sites with and without EPNs were found to be different. Moreover, statistical comparisons between the agricultural areas revealed no significant differences. Therefore, we concluded that *S. siamkayai* is associated with *X. stockiae* in agricultural areas and that there is no association between the soil parameters of agricultural areas and presence of EPNs, except for soil texture and the elevation. *Steinernema siamkayai* may be applied as a biocontrol agent in agricultural areas.

Introduction

Entomopathogenic nematodes (EPNs) or insect-parasitic nematodes in the genera *Heterorhabditis* and *Steinernema* are symbiotically associated with bacteria *Photorhabdus* and *Xenorhabdus*, respectively (Shapiro-Ilan *et al.*, 2017). During their complex life cycle, the infective juveniles (IJs) of EPNs living in the soil penetrate the larval stage of their insect hosts via a natural opening (mouth, spiracle, anus) or the soft cuticle. Upon entering the insect host, the IJs of EPNs release symbiotic bacteria, which multiply rapidly and produce secondary metabolites. The infected insect host dies within 24–48 h (Dowds & Peters, 2002). Together, the EPNs and their symbiotic bacteria feed on the bioconverted insect host. EPNs reproduce in the insect cadaver for 2–3 generations. The IJs feed on the symbiotic bacteria; when the food is depleted, a new cohort of IJs that carry symbiotic bacteria emerges from the cadaver in search of new insect hosts. Therefore, EPNs have been used as biocontrol agents for several species of insect pests (Smart, 1995; Lacey *et al.*, 2015). These applications are safe for humans and the environment.

The presence of EPNs and their symbiotic bacteria has been globally reported at a variety of ecological habitats around the world, except Antarctica (Hominick, 2002). Biotic and abiotic characteristics are important factors for EPN survival in ecological niches. Soil parameters such as texture, pH, moisture and temperature are particularly important for the survival of EPNs. Geographical areas, habitats and soil properties are parameters that determine the diversity and distribution of EPNs. Several surveys of EPNs from different geographical ecologies have yielded variable recovery rates. To date, over 100 species of EPNs (Hunt, 2016) and approximately 30 species of their symbiotic bacteria (Tailliez *et al.*, 2010, Tailliez *et al.*, 2012; Ferreira *et al.*, 2013; Kuwata *et al.*, 2013) have been described. In Thailand, approximately ten species of EPNs have been reported in several different habitats (Stock *et al.*, 1998;

© The Author(s), 2020. Published by Cambridge University Press

CAMBRIDGE UNIVERSITY PRESS

https://doi.org/10.1017/S0022149X20000735 Published online by Cambridge University Press

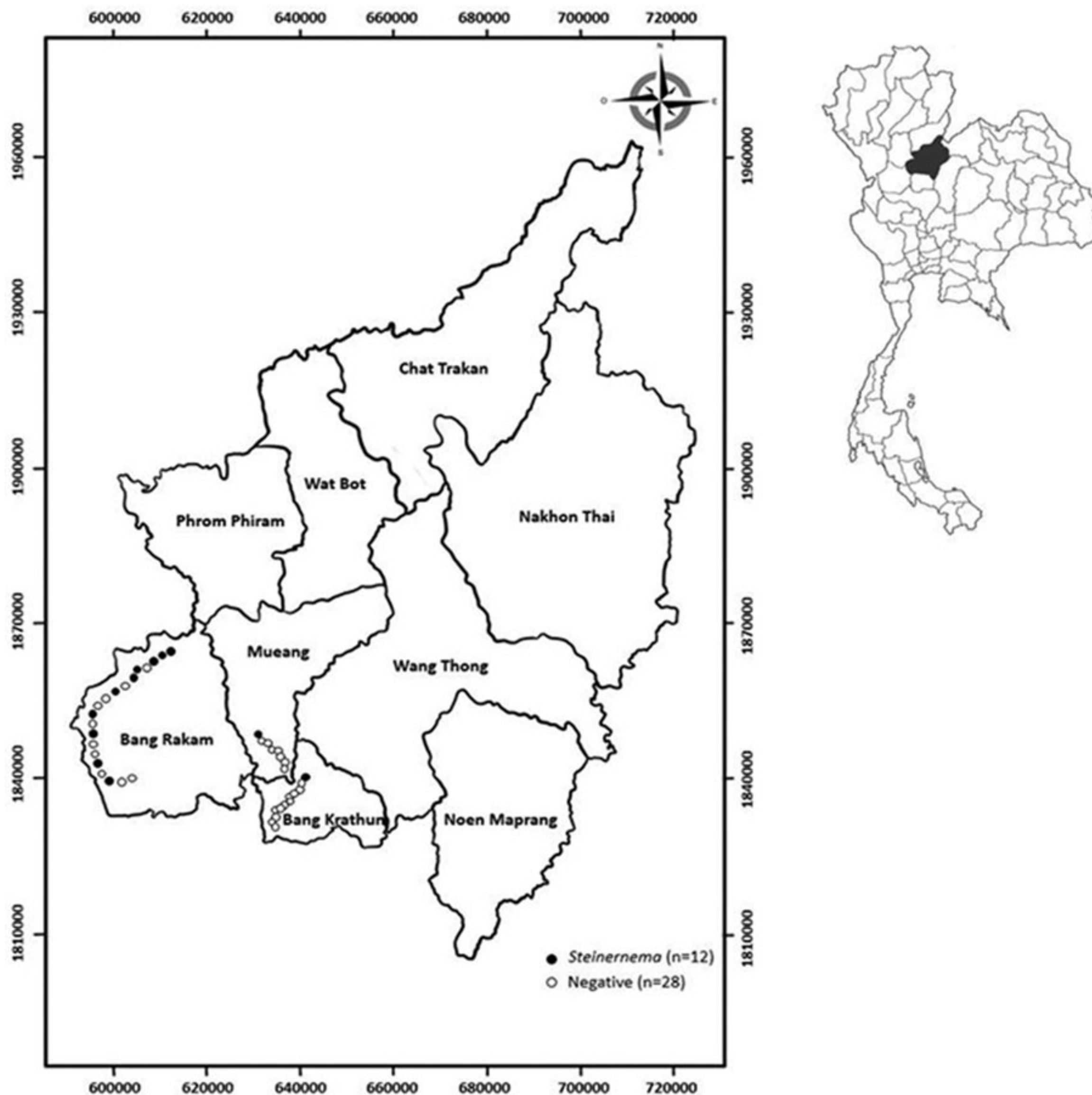


Fig. 1. Map of the Phitsanulok province in Thailand where samples from 40 soil sites in agriculture areas were collected to search for EPNs. Twelve soil sites are positive for *Steinernema* (filled circle), and 28 sites are negative for the EPNs (blank circle).

Maneesakorn *et al*., 2010; Thanwisai *et al*., 2012; Vitta *et al*., 2015, 2017; Muangpat *et al*., 2017; Yooyangket *et al*., 2018; Suwannaroj *et al*., 2020).

The identification of indigenous EPNs and symbiotic bacteria is ideal for the use of EPNs as biocontrol agents in local areas. Several attempts have been made to use indigenous EPNs in controlling insect pests such as the Caribbean fruit fly (Heve *et al*., 2017), termite (Al-Zaidawi *et al*., 2020) and house fly (Arriaga & Cortez-Madrigal, 2018). The areas surveyed for EPNs and symbiotic bacteria in Thailand were mainly on roadside verges and banks of ponds or rivers. Although surveys of EPNs and their associated bacteria have been conducted in several regions, the EPNs in the agricultural areas of Thailand had not yet been studied. Accordingly, we determined that information regarding the relationship between ecological factors and soil-dwelling EPNs would be useful for the application of EPNs as biological control agents in these specific areas. Therefore, we conducted a survey of EPNs in the agricultural areas of the Phitsanulok province in lower northern Thailand. Molecular identification of the EPNs and symbiotic bacteria was performed. In addition, the association between soil parameters and soil samples based on the presence or absence of EPNs was evaluated. The present study may help further the efforts in basic science for the further application of EPNs in local areas of Thailand.

Materials and methods

Soil collection

A total of 200 soil samples from 40 sites were collected from agricultural areas in the Phitsanulok province between February and March 2018 (fig. 1). Soil collection was performed using methods previously described by researchers in the field (Yooyangket *et al*., 2018). Soil sites were randomly selected from several agricultural areas. The agricultural areas were defined into three main groups according to the species of plants present: (1) field crops, (2)

https://doi.org/10.1017/S0022149X20000735 Published online by Cambridge University Press

Table 1. Prevalence of entomopathogenic nematodes in the agricultural areas of Phitsanulok province, Thailand.

Agricultural areas	Number of soil sites collected	Number of soil sites positive for EPNs	Prevalence	Number of soil samples collected	Number of soil samples positive for EPNs	Prevalence
Field crop						
Corn fields	10	5	50	50	8	16
Rice fields	4	1	25	20	1	5
Cassava plantation	1	1	100	5	1	20
Bonavista bean plantation	2	2	100	10	3	30
Sugarcane plantation	5	0	0	25	0	0
Taro plantation	1	0	0	5	0	0
Sun hemp garden	1	0	0	5	0	0
Horticulture crop						
Mixed fruit garden	1	0	0	5	0	0
Ivy gourd plantation	3	1	33.33	15	1	6.66
Banana plantation	5	2	40	25	2	8
Marigold garden	2	0	0	10	0	0
Grapefruit garden	1	0	0	5	0	0
Pandan garden	1	0	0	5	0	0
Lemongrass garden	1	0	0	5	0	0
Yardlong bean garden	1	0	0	5	0	0
Forest						
Teak plantation	1	0	0	5	0	0
Total	40	12	30	200	16	8

horticultural crops and (3) forest area (table 1). Approximately 300–500 g of each soil sample was collected using a hand shovel. Soil parameters, including pH, temperature, texture and moisture, were recorded. The altitude, latitude and elevation of each soil site were determined using a GPS navigator (Garmin nüvi 1250, Garmin, New Taipei, Taiwan). Soil samples were transported under ambient temperature to the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Thailand.

Isolation and identification of EPNs

The IJ EPNs were isolated from the soil samples using the *Galleria mellonella* baiting technique. *Galleria mellonella*, the greater wax moth, was maintained with artificial food (wheat flour 200 g, honey 100 ml, glycerol 100 ml and instant dry yeast 50 g) in the laboratory. The White trap technique was used to obtain the emerging IJs from the *G. mellonella* cadaver (White, 1927). The IJs were collected and cleaned with sterile distilled water and then kept at −20°C for genomic DNA extraction.

Genomic DNA from the IJ nematodes was extracted using Phire Tissue Direct PCR Master Mix (ThermoFisher Scientific, Carlsbad, California, USA), according to the manufacturer's instructions but with some modifications. The dilution and storage protocol was performed via reduction steps for nucleotide extraction. Approximately 200–500 IJs of EPN in a 1.5 ml microcentrifuge tube were mixed with 20 µl dilution buffer, and the DNARelease additive (0.5 µl) was added to the tube. To break the cells, a 200 µl tip was used to crush the nematode, and the reaction was mixed by vortexing. The tubes were incubated at room temperature for 2–5 min. Subsequently, the tubes were placed in a 95°C water bath for 5 min. The tubes were then centrifuged at 12,000 g for 1 min. The supernatant containing genomic DNA was collected and kept at −20°C prior to the polymerase chain reaction (PCR).

PCR was performed to amplify the 28S ribosomal DNA (rDNA) region using a primer pair: 539_F (5′GGATTTCCTTA-GTAACTGCGAGTA-3′) and 535_R (5′-TAGTCTTTCGCCCC-TATACCCTT-3′) (Stock *et al*., 2001). The PCR reaction (30 µl) contained 15 µl of 2X Phire Tissue Direct PCR Master Mix (1X),

https://doi.org/10.1017/S0022149X20000735 Published online by Cambridge University Press

1.2 µl of 5 µM forward primer (0.8 µM), 1.2 µl of 5 µM reverse primer (0.8 µM), 1.8 µl of DNA template (100–200 ηg) and 10.8 µl of sterile distilled water. Thermocycling was done in a Biometra TOne thermal cycler (Analytik Jena AG, Jena, Germany) as follows: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec; and a final extension at 72°C for 7 min. In addition, a partial sequence of the internal transcribed spacer (ITS) was amplified using a pair of primers: TW81_F (5′- GTTTCCGTAGGTGAACCTGC -3′) and AB28_R (5′- ATATGCTTAAGTTCAGCGGGT -3′) (Hominick *et al.*, 1997). The PCR reaction (30 µl) was of the same volume and concentration as that of the 28S rDNA with the exception of the primers. Thermocycling was performed using a Biometra TOne Thermal cycler (Analytik Jena AG, Jena, Germany) as follows: initial denaturation at 98°C for 5 min; followed by 35 cycles of denaturation at 98°C for 5 sec, annealing at 55°C for 5 sec, and extension at 72°C for 30 sec; and a final extension at 72°C for 1 min. The 1.2% agarose gel electrophoresis was performed at a constant current of 100 V. Subsequently, the gel was stained with ethidium bromide, destained with distilled water and visualized and photographed under ultraviolet light. The PCR products were purified using a NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. For nucleotide sequencing in both the forward and reverse directions, the purified PCR products were transported to Macrogen Inc., Seoul, Korea.

Isolation and identification of symbiotic bacteria

Isolation of symbiotic bacteria was performed as previously described in the literature (Yooyangket *et al.*, 2018). The symbiotic bacteria were isolated from the haemolymph of a *G. mellonella* cadaver, which had been infected with the IJs. A sterile loop was used to touch the haemolymph, and it was streaked on nutrient agar supplemented with bromothymol blue and triphenyl-2,3,5-tetrazolium chloride (NBTA). Colonies of symbiotic bacteria were observed on the NBTA plates after four days of incubation in the dark at room temperature. A single colony of each isolate of symbiotic bacteria was inoculated into 3 ml of Luria-Bertani broth and incubated with shaking at 180 rpm overnight (approximately 18–24 h). The genomic DNA of the symbiotic bacteria was extracted from the bacterial pellets using the Blood/Cell DNA Mini Kit (Geneaid Biotech Ltd., New Taipei, Taiwan). The genomic DNA of the symbiotic bacteria was stored at −20°C for further use in PCR. Analysis of the recombinase A (*recA*) gene sequence was performed to identify the symbiotic bacteria. The pair of primers used were recA_F (5′-GCTATTG-ATGAAAATAAACA-3′) and recA_R (5′–RATTTTRTCWCCR-TTRTAGCT-3′) (Tailliez *et al.*, 2010). A total volume of 30 µl of the PCR reagents was used, containing 15 µl of EconoTaq® PLUS 2X Master Mix (1X) (Lucigen, Middleton, Wisconsin, USA), 1.5 µl of 5.0 µM recA_F primer (1.0 µM), 1.5 µl of 5.0 µM recA_R primer (1.0 µM), 1.5 µl of DNA template (100–200 ηg) and 10.5 µl of sterile distilled water. The PCR parameters for symbiotic bacteria were followed based on a previous description in Yooyangket *et al.* (2018). Thermocycling was performed using a Biometra TOne Thermal cycler (Analytik Jena AG, Jena, Germany). Agarose gel electrophoresis was performed with constant 100 V current. The PCR products were visualized on ethidium-bromide-stained 1.2% agarose gel. The method used for the purification of the PCR products was similar to that used to identify the EPNs. The nucleotide sequencing for each direction (forward and reverse) was also analysed by Macrogen Inc. in Korea.

Analysis of sequences and construction of phylogeny

All the sequences were edited by viewing the peak of the chromatogram in SeqMan II software (DNASTAR, Madison, Wisconsin, USA). The nucleotide sequences were aligned using ClustalW. The identified EPN and symbiotic bacteria species were confirmed by a BLASTN search in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), with a nucleotide similarity above 97% being considered significant. Maximum likelihood (ML) and neighbour-joining (NJ) trees were constructed based on the Tamura–Nei and Kimura two-parameter models, respectively, and run on MEGA version 7.0 (Kumar *et al.*, 2016). In addition, Bayesian analyses were performed using the Markov chain Monte Carlo method in MrBayes version 3.2 (Ronquist *et al.*, 2012). Although three methods were followed to construct a phylogeny, only ML topology is shown in the present study. The bootstrap values from two methods and the percentage of Bayesian posterior probabilities are indicated on the branch of the ML tree.

Statistical analysis

The soil parameters (soil temperature, soil pH and soil moisture) and elevation level of each agricultural area were statistically analysed for the groups with positive or negative indications for the presence of EPNs, and SPSS version 17 was used for the same (SPSS Inc., Chicago, IL). Multivariable (soil parameters and elevation) logistic regression was carried out to calculate the odds ratios (ORs) and 95% confidence interval (CI), considering the presence of EPNs as the main outcome. In addition, the association between soil texture and presence of EPNs was statistically analysed using the Fisher's exact test. Each agricultural area was also statistically analysed using the Fisher's exact test. The differences between the two groups were considered to be statistically significant if the P-value was <0.05.

Results

Prevalence and molecular identification of EPNs

A total of 40 soil sites tested; 12 sites were positive (30%) and 16 out of 200 soil samples (8.0% prevalence) were positive for EPNs (table 1). All the isolates were primarily identified as belonging to the genus *Steinernema*. Based on the 549 bp of the nucleotide in the 28S rDNA region, 15 of the *Steinernema* isolates were identified as *Steinernema siamkayai*, with 99.82% identity to *S. siamkayai* strain CS33 (accession number MN194613). The one remaining sequence was not included for analysis due to its short length. The sequences of *Steinernema* in the present study were deposited in the NCBI database with accession numbers MT478151–MT478165. Based on the ML tree, all the sequences in the present study were grouped with *S. siamkayai*, *S. capocapsae*, *S. huense* and *S. surkhetense* (fig. 2). In addition, nine isolates of *Steinernema* (accession numbers MT872205–MT872213) were analysed based on 671 bp of the nucleotide in the ITS region. These ITS sequences showed the highest similarities (99.55–99.70%) with *S. siamkayai* (accession number GQ377414). Also, the ML tree based on the ITS region showed similar topology with the 28S rDNA region, due to which all the nine sequences

https://doi.org/10.1017/S0022149X20000735 Published online by Cambridge University Press

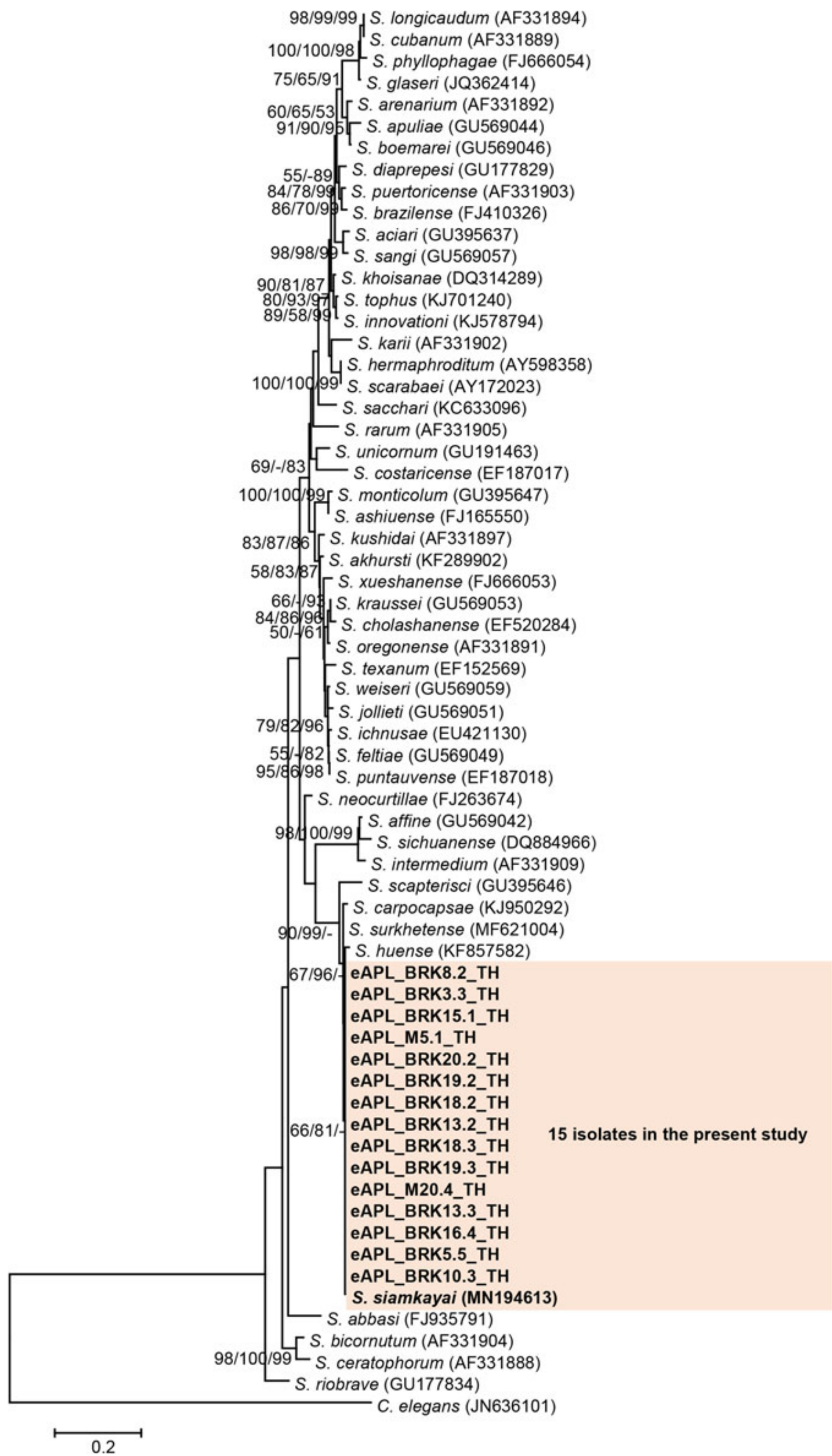


Fig. 2. Topology of maximum-likelihood phylogenetic tree based on 549 nucleotides of a partial region of the 28S rRNA gene from 15 *Steinernema* isolates in the present study together with several *Steinernema* species retrieved from the NCBI database. Support values (ML bootstrap/NJ bootstrap/Bayesian posterior probabilities) are shown above the branches, and a dash (–) instead of a numerical support value indicates that a certain grouping was not seen by that method of analysis. Bold letters indicate the sequences obtained in the present study. *Caenorhabditis elegans* or *C. elegans* (accession number JN636101) was included in the phylogeny as the outgroup. Scale bar shows 20% sequence divergence.

in the present study were grouped with *S. siamkayai*, *S. capocapsae*, *S. huense* and *S. surkhetense* (fig. 3).

Isolation and molecular identification of symbiotic bacteria

Seven isolates of *Xenorhabdus* bacteria were identified based on their colony morphology on the NBTA agar. All the isolates in the present study were identified, based on 577 bp of a partial *recA* sequence, as *Xenorhabdus stockiae*. This showed that identity ranged from 98.96 to 99.83% with the *X. stockiae* strain TH01 (accession number FJ823425) and *X. stockiae* strain CS33 (accession number MK401900) after a BLASTN search. All the nucleotide sequences of *Xenorhabdus* in the present study were deposited in the NCBI database with accession numbers MT465733–MT465739. The ML tree represents all *Xenorhabdus* isolates closely related to *X. stockiae* (accession number FJ823425) (fig. 4).

https://doi.org/10.1017/S0022149X20000735 Published online by Cambridge University Press

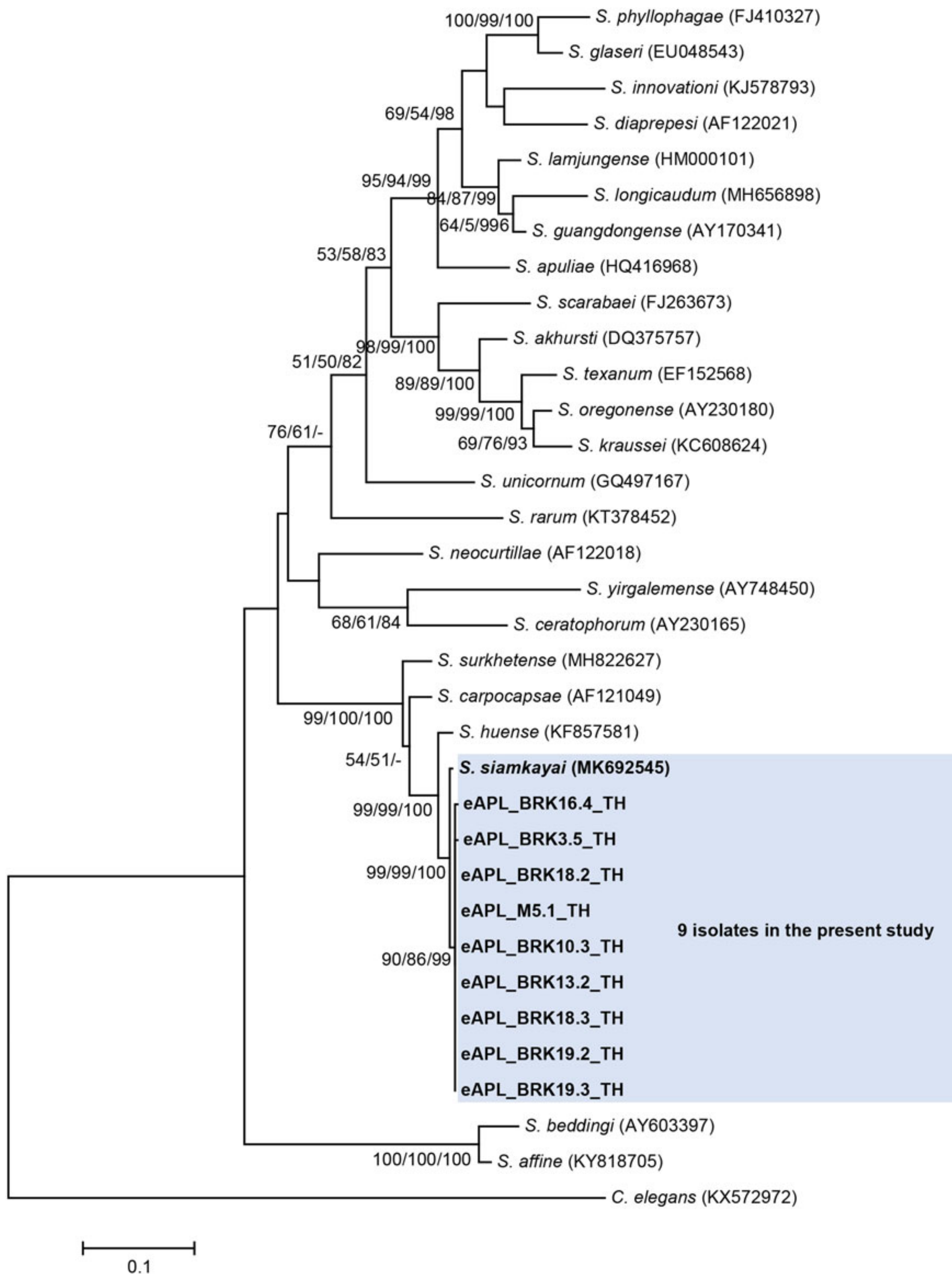


Fig. 3. Topology of maximum-likelihood phylogenetic tree based on 671 nucleotides of a partial ITS region from nine *Steinernema* isolates in the present study together with several *Steinernema* species retrieved from the NCBI database. Support values (ML bootstrap/NJ bootstrap/Bayesian posterior probabilities) are shown above the branches, and a dash (–) instead of a numerical support value indicates that a certain grouping was not seen by that method of analysis. Bold letters indicate the sequences obtained in the present study. *Caenorhabditis elegans* or *C. elegans* (accession number KX572972) was included in the phylogeny as the outgroup. Scale bar shows 10% sequence divergence.

Association between soil parameters and EPNs

The soil samples that were positive for the presence of EPNs were found in loam, sandy loam and clay loam soil types. In contrast, clay soil samples collected in the present study were found to be negative for EPNs (table 2). Significantly more EPNs were recovered from loam than from clay (Fisher's exact test; $P = 0.038$) and from clay loams than from clay (Fisher's exact test; $P = 0.04$). The parameters for the soil samples (temperature, pH and moisture)

https://doi.org/10.1017/S0022149X20000735 Published online by Cambridge University Press

Fig. 4. Topology of a maximum-likelihood phylogenetic tree based on 577 nucleotides of a partial region of the *recA* gene of 7 *Xenorhabdus* isolates in the present study as well as several *Xenorhabdus* species retrieved from the NCBI database. Support values (ML bootstrap/NJ bootstrap/Bayesian posterior probabilities) are shown above the branches, and a dash (–) instead of a numerical support value indicates that a certain grouping was not seen by that method of analysis. Bold letters indicate the sequences obtained in the present study. *Escherichia coli* (accession number U00096), a bacterial species in the same family (Enterobacteriaceae) as *Xenorhabdus*, was included in the phylogeny as the outgroup. Scale bar shows 2% sequence divergence.

showed no significant difference between the positive and negative EPNs (table 3). In contrast, the elevation levels of the soil samples with EPNs ($n = 16$, mean ± standard deviation (SD) = 46.5 ± 3.57, range = 40–55) or without EPNs ($n = 184$, mean ± SD = 42.4 ± 6.4, range = 22–55) were significantly different (logistic regression test; $P = 0.02$, OR = 1.13, 95% CI = 1.01–1.27).

Association between agricultural areas and EPNs

Table 4 presents the relationship between agricultural areas and the presence of EPNs in the Phitsanulok province of Thailand. Most of the EPNs were recovered from field crop areas (10.8%), and a few samples positive for EPNs were recovered from horticultural crops (4.0%). Among the field crop sites, soil samples collected from the corn field, rice field, cassava plantation and bonavista bean plantation were positive for EPNs. Of the horticultural crops, soil samples from the ivy gourd and banana plantations were positive for EPNs. Half of the soil samples positive for EPNs were found in corn field areas (table 1). The soil samples from the horticultural and field crop areas could not be correlated to the presence or absence of EPNs (Fisher's exact test; $P = 0.073$) (table 4).

https://doi.org/10.1017/S0022149X20000735 Published online by Cambridge University Press

Table 2. Prevalence of entomopathogenic nematodes by soil texture.

Soil texture	Number of soil samples positive for EPNs	Number of soil samples negative for EPNs	Total	Prevalence (%)	*P*-value (Fisher's exact test)
Loam	12	115	127	9.45	0.035
Sandy loam	2	13	15	13.33	
Clay loam	2	10	12	16.66	
Clay	0	46	46	0	
Total	16	184	200	8.0	

Table 3. The association between soil parameters and the presence of EPNs.

	Soil samples positive for EPNs ($n = 16$)		Soil samples negative for EPNs ($n = 184$)		Multivariable logistic regression		
Soil parameters	Min–max	Mean ± SD	Min–max	Mean ± SD	*P*-value	OR	95% CI
Temperature (°C)	25–33	27.81 ± 2.04	22–33	27.26 ± 2.31	0.45	1.09	0.85–1.39
pH	4–7	6.28 ± 0.88	4–7	6.42 ± 0.60	0.49	0.68	0.22–2.06
Moisture (1–8%)	1–8	2.66 ± 2.02	1–8	2.80 ± 2.23	0.63	0.91	0.63–1.31
Elevation (meter)	40–55	46.56 ± 3.57	22–55	42.41 ± 6.40	0.02	1.13	1.01–1.27

OR, odds ratio; 95% CI, 95% confidence interval; SD, standard deviation.

Discussion

In 1998, *S. siamkayai* was initially isolated from soil samples of sweet tamarind orchards in the Phetchabun province of Thailand (Stock *et al.*, 1998). Subsequently, this species was reported in India and Nepal also (Banu *et al.*, 2005; Khatri-Chhetri *et al.*, 2010; Raja *et al.*, 2011). Herein, we have reported that *S. siamkayai* was isolated from agricultural areas, and none of the genus *Heterorhabditis* was recovered. This may be because there are more species of *Steinernema* than *Heterorhabditis* nematodes, and, therefore, the former species is distributed in several habitats. In 2016, over 90 species of *Steinernema* were formally described, while approximately 15 species of *Heterorhabditis* were recorded across the world (Hunt, 2016). The prevalence of *S. siamkayai* in the present study was 8.0% in the agricultural areas of Thailand. Further, *S. siamkayai* have previously been isolated from agricultural areas in Nepal (Khatri-Chhetri *et al.*, 2010). To support this status, the abundance of the EPNs was associated with ecological habitats in which the human impact is considerable, such as agricultural fields (Mráček & Webster, 1993; Shahina *et al.*, 1998). In previous studies, more isolates of *Steinernema* (than *Heterorhabditis*) were reported in Thailand (Vitta *et al.*, 2017; Yooyangket *et al.*, 2018); however, some EPN surveys found more *Heterorhabditis* isolates than *Steinernema* isolates (Vitta *et al.*, 2015; Muangpat *et al.*, 2017). Therefore, the occurrence of *Heterorhabditis* and *Steinernema* may be associated with several factors, although these factors have not been delineated. Up to ten species of EPNs were reported from several ecological habitats (roadside verge, riverbank and national park) of Thailand. These EPNs were *Steinernema surkhetense*, *Steinernema websteri* (synonym *Steinernema carpocapsae*), *Steinernema scarabiae*, *Steinernema kushidai*, *Steinernema minutum* and *Steinernema khoisanae* and *Heterorhabditis indica* (synonym *Heterorhabditis gerrardi*), *Heterorhabditis baujardi* (synonym *Heterorhabditis somsookae*), *Heterorhabditis bacteriophora* and *Heterorhabditis zealandica* (Maneesakorn *et al.*, 2010; Thanwisai *et al.*, 2012; Fukruksa *et al.*, 2017; Muangpat *et al.*, 2017; Vitta *et al.*, 2017; Yooyangket *et al.*, 2018). *Heterorhabditis zealandica* and *S. kushidai* are isolates from the soil of the forest native to Mae Wong National Park in Thailand. The most common species of EPNs found in the country were *S. surkhetense* and *H. indica*. However, these two species were not found in the present study. This may be due to differences in several factors such as life cycle, insect host abundance and habitat. The preferential survey of EPNs in agricultural areas may be one factor which results in more positive samples. Therefore, *S. siamkayai* may be frequently found in the agricultural areas. In Portugal, *Steinernema feltiae* was mostly recovered in agricultural land (Valadas *et al.*, 2014). Also noted in Italy were *S. feltiae* and *H. bacteriophora*, which were found to be related with vegetation habitat (Tarasco *et al.*, 2015). In Brazil, *Heterorhabditis amazonensis*, *Metarhabditis rainai*, *Oscheius tipulae* and *Steinernema rarum* were isolated from soil in agricultural areas (de Brida *et al.*, 2017). *Oscheius onirici* was also isolated from a wild cranberry marsh in Jackson County, Wisconsin, USA (Ye *et al.*, 2018). In addition, *S. khoisanae*, *Steinernema yirgalemense*, *Steinernema citrae*, *H. bacteriophora*, *H. zealandica* and *Heterorhabditis* sp. were recovered from citrus orchards in South Africa (Malan *et al.*, 2011). *Steinernema abbasi, S. minutum, Steinernema tami* and *H. indica* were found to be present in the agricultural and forested areas in the Philippines (Caoili *et al.*, 2018). Including in the present study, *S. siamkayai* was isolated from the field and horticulture crops. This indicates that EPNs have global abundance in agricultural areas. Further research on the application of these EPNs in specific agricultural areas will be performed to achieve a reduction of chemical use in the control of insect pests.

Although *S. siamkayai* was not evaluated for its biological activity in the present study, several reports on insecticidal activities of this EPN were experimentally tested against insect pests.

https://doi.org/10.1017/S0022149X20000735 Published online by Cambridge University Press

Table 4. The association between agricultural areas and the presence of EPNs.

Agricultural area	Number of soil samples			Prevalence (%)	*P*-value (Fisher's exact test)
	Positive for EPNs	Negative for EPNs	Total		
Horticulture crop	3	72	75	4.0	0.073
Field crop	13	107	120	10.8	
Forest	0	5	5	0	
Total	16	184	200	8.0	

Previous studies have shown that *S. siamkayai* has the potential to control *Aedes aegypti*, *Anopheles stephensi*, *Culex quinquefasciatus* (Dilipkumar *et al.*, 2019), pulse beetle *Callosobruchus chinensis* (Fayyaz & Javed, 2009) and *Spodoptera litura* Fabricius (Wetchayunt *et al.*, 2009). In general, the effective bioactivity of this organism against insect hosts is not restricted to its being an EPN, but also includes its bacterial symbionts. *Steinernema siamkayai* was associated with *X. stockiae* (Tailliez *et al.*, 2006), and our findings demonstrate that *X. stockiae* is also hosted by *S. siamkayai*. This may be a symbiont duo with high specificity. However, *X. stockiae* was also reported as a bacterial symbiont with *S. surkhetense* (Bhat *et al.*, 2017), *S. minutum* (Maneesakorn *et al.*, 2010) and *S. huense* (Phan *et al.*, 2014). These EPNs were closely related in terms of evolution (as indicated in the phylogeny), and *X. stockiae* could be symbiotically associated with these EPNs. Several strains of *X. stockiae* were reported as being potential microbial agents to control *Ae. aegypti*, *Aedes albopictus*, mushroom mites and cow Mastitis-causing bacteria (Bussaman *et al.*, 2012; Namsena *et al.*, 2016; Fukruksa *et al.*, 2017; Bussaman & Rattanasena, 2016; Vitta *et al.*, 2018; Yooyangket *et al.*, 2018). This indicates that EPNs and their symbiotic bacteria are effective against insect pests. Therefore, *S. siamkayai* and its symbiont *X. stockiae* have broadly effective bioactivity and may be alternative bio-agents for the control of insect pests.

Xenorhabdus stockiae was identified by *recA* sequencing. Several housekeeping genes have been reported as the genetic markers for taxonomic purposes. The 16S rRNA and 50S ribosomal protein L2 genes cannot discriminate at the species level, but they are useful for identification at the genus level (Tailliez *et al.*, 2010). It is possible that the glutamyl-tRNA synthetase is gained from lateral gene transfer (Tailliez *et al.*, 2010). In contrast, the DNA polymerase III subunit beta (*dnaN*) and *recA* sequences demonstrated correct clustering in the phylogenetic analysis. Therefore, the *dnaN* and *recA* sequences may be good genetic markers to differentiate the *Xenorhabdus* and *Photorhabdus* species (Tailliez *et al.*, 2010). In addition, *recA* is the most widely used marker for identification and phylogenetic analysis in Thailand (Thanwisai *et al.*, 2012; Fukruksa *et al.*, 2017; Muangpat *et al.*, 2017; Yooyangket *et al.*, 2018).

The presence or absence of EPNs in the soil samples could be affected by several factors such as the sampling method and isolation techniques used. Accordingly, the limitations of the present study in analysing the association between the soil factors and presence of EPNs could be due to, in part, the small number of samples and low recovery rate of the EPNs. We found only *S. siamkayai* (15 isolates) in the present study. Most of the positive samples with *S. siamkayai* were isolated from loam. Also, *S. siamkayai* (in Nepal) was recovered from silt loam and sandy loam (Khatri-Chhetri *et al.*, 2010). This is consistent with other reports showing that the IJ of EPNs prefer soil with high sand content for their movements and survival (Hazir *et al.*, 2003; Kary *et al.*, 2009). In the present study, the soil parameters (moisture, pH and temperature) were not significantly associated with the presence of EPNs in the samples. This could also be due to the small number of samples. *Steinernema siamkayai* was recovered at high pH (4–7), temperature (25–33°C) and moisture (1–8%) ranges. Similarly, *S. siamkayai* in Nepal was recovered from warm agricultural areas with soil pH of 4.1–7 (Khatri-Chhetri *et al.*, 2010). This suggests that *S. siamkayai* in warm temperatures may widely occupy several niches. In addition, the elevation of soil sites was associated with the presence of EPNs (logistic regression test; $P = 0.02$, $OR = 1.13$, 95% $CI = 1.01–1.27$). Elevation could affect the distribution of EPN (Rosa *et al.*, 2000). At lower altitudes, *Heterorhabditis* was most abundant in soil samples. *Steinernema* became more abundant above 300 m. The prevalence of *Heterorhabditis* at sea level and *Steinernema* above 300 m was high (Hara *et al.*, 1991). This might be the reason for our finding of low prevalence of *S. siamkayai*, which was found at 20–55 above mean sea level. However, several factors may affect the distribution of the EPNs. Soil moisture, temperature and rainfall also affect the distribution of the insects that could possibly be hosts for the EPN (Kung *et al.*, 1990; Garcia del Pino & Palomo, 1996).

Steinernema siamkayai was recovered in soil samples from field and horticultural crop areas, with its presence being mostly found in corn fields. A few isolates of *S. siamkayai* were recovered from the rice fields, cassava plantation, bonavista bean plantation, ivy gourd plantation and banana plantation. This suggests that *S. siamkayai* may be used as a biocontrol agent for the control of insect pests in these areas, especially corn fields. A previous study used *H. bacteriophora* for controlling the larvae of western corn rootworm in maize crops (Modic *et al.*, 2020). Therefore, the application of *S. siamkayai* to control insect pests in corn fields may be feasible and potentially lead to the reduction of chemical insecticide use.

In summary, we identified *S. siamkayai* and their symbiotic bacteria *X. stockiae* in agricultural areas of Thailand. This EPN species was recovered from loam in field and horticultural crop areas with high pH, temperature and moisture ranges. Although the soil parameters and agricultural areas were not correlated with the presence or absence of EPNs, *S. siamkayai* has a potential application as a biocontrol agent in fields or horticultural crop areas.

Acknowledgements. We would like to thank the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan

https://doi.org/10.1017/S0022149X20000735 Published online by Cambridge University Press

University, Thailand, for provision of facilities. We would also like to thank Miss Buchita Samritnok, Miss Benchawan Ratsamee and Miss Prapasiri Waranuch for their help in soil collection.

Financial support. This work was supported by the Naresuan University Fund (grant number R2563C015).

Conflicts of interest. None.

Ethical standards. This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Al-Zaidawi JB, Karimi J and Mahdikhani ME (2020) Entomopathogenic nematodes as potential biological control agents of subterranean termite, *Microcerotermes diversus* (Blattodea: Termitidae) in Iraq. *Environmental Entomology* **49**, 412–442.

Arriaga A and Cortez-Madrigal H (2018) Susceptibility of *Musca domestica* larvae and adults to entomopathogenic nematodes (Rhabditida: Heterorhabditidae, Steinernematidae) native to Mexico. *Journal of Vector Ecology* **43**, 312–320.

Banu JG, Nguyen KB and Rajendran G (2005) Occurrence and distribution of entomopathogenic nematodes in Kerala, India. *International Journal of Nematology* **15**, 9–16.

Bhat S, Singh J and Vig A (2017) Instrumental characterization of organic wastes for evaluation of vermicompost maturity. *Journal of Analytical Science and Technology* **8**, 2.

Bussaman P and Rattanasena P (2016) Additional property of *Xenorhabdus stockiae* for inhibiting cow mastitis-causing bacteria. *Biosciences Biotechnology Research Asia* **13**, 1871–1878.

Bussaman P, Sa-Uth C, Rattanasena P and Chandrapatya A (2012) Acaricidal activities of whole cell suspension, cell-free supernatant, and crude cell extract of *Xenorhabdus stokiae* against mushroom mite (*Luciaphorus* sp.). *Journal of Zhejiang University-Science* **13**, 261–266.

Caoili BL, Latina RA, Sandoval RFC and Orajay JI (2018) Molecular identification of entomopathogenic nematode isolates from the Philippines and their biological control potential against lepidopteran pests of corn. *Journal of Nematology* **50**, 99–110.

de Brida AL, Rosa JM, Oliveira CM, Castro BM, Serrão JE, Zanuncio JC, Leite LG and Wilcken SR (2017) Entomopathogenic nematodes in agricultural areas in Brazil. *Scientific Reports* **7**, 45254.

Dilipkumar A, Raja Ramalingam K, Chinnaperumal K, Govindasamy B, Paramasivam D, Dhayalan A and Pachiappan P (2019) Isolation and growth inhibition potential of entomopathogenic nematodes against three public health important mosquito vectors. *Experimental Parasitology* **197**, 76–84.

Dowds BCA and Peters A (2002) Virulence mechanisms. pp. 79–98 *in* Gaugler R (*Ed*) *Entomopathogenic nematology*. Wallingford, CABI Publishing.

Fayyaz S and Javed S (2009) Laboratory evaluation of seven Pakistani strains of entomopathogenic nematodes against a stored grain insect pest, pulse beetle *Callosobruchus chinensis* (L.). *Journal of Nematology* **41**, 255–260.

Ferreira T, van Reenen CA, Endo A, Sproer C, Malan AP and Dicks LMT (2013) Description of *Xenorhabdus khoisanae* sp. nov., the symbiont of the entomopathogenic nematode *Steinernema khoisanae*. *International Journal of Systematic and Evolutionary Microbiology* **63**, 3220–3224.

Fukruksa C, Yimthin T, Suwannaroj M, Muangpat P, Tandhavanant S, Thanwisai A and Vitta A (2017) Isolation and identification of *Xenorhabdus* and *Photorhabdus* bacteria associated with entomopathogenic nematodes and their larvicidal activity against *Aedes aegypti*. *Parasites and Vectors* **10**, 440.

Garcia del Pino F and Palomo A (1996) Natural occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Spanish soils. *Journal of Invertebrate Pathology* **68**, 84–90.

Hara AH, Gaugler R, Kaya HK and LeBeck LM (1991) Natural populations of entomopathogenic nematodes (Rhabditida: Heterorhabditidae, Steinernematidae) from the Hawaiian Islands. *Environmental Entomology* **20**, 211–216.

Hazir S, Keskin N, Stock SP, Kaya HK and Özcan S (2003) Diversity and distribution of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Turkey. *Biodiversity and Conservation* **12**, 375–386.

Heve WK, El-Borai FE, Carrillo D and Duncan LW (2017) Biological control potential of entomopathogenic nematodes for management of Caribbean fruit fly, *Anastrepha suspensa* Loew (Tephritidae). *Pest Management Science* **73**, 1220–1228.

Hominick WM (2002) Biogeography. pp. 115–144 *in* Gaugler R (*Ed*) *Entomopathogenic nematology*. Wallingford, CABI Publishing.

Hominick WM, Briscoe B, Del Pino F, *et al.* (1997) Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. *Journal of Helminthology* **71**, 271–298.

Hunt DJ (2016) Introduction. pp. 1–11 *in* Hunt DJ and Nguyen KB (*Eds*) *Nematology monographs and perspectives volume 12: advanced in entomopathogenic nematode taxonomy and phylogeny*. Leiden, Brill.

Kary EN, Gholamreza N, Christine G, Seyed M and Vahed MM (2009) A survey of entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in the north-west of Iran. *Nematology* **11**, 107–116.

Khatri-Chhetri HB, Waeyenberge L, Manandhar HK and Moens M (2010) Natural occurrence and distribution of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) in Nepal. *Journal of Invertebrate Pathology* **103**, 74–78.

Kumar S, Stecher G and Tamura K (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**, 1870–1874.

Kung S, Gaugler R and Kaya HK (1990) Influence of soil, pH, and oxygen on persistence of *Steinernema* spp. *Journal of Nematology* **22**, 440–445.

Kuwata R, Qiu LH, Wang W, Harada Y, Yoshida M, Kondo E and Yoshiga T (2013) *Xenorhabdus ishibashii* sp. nov., isolated from the entomopathogenic nematode *Steinernema aciari*. *International Journal of Systematic and Evolutionary Microbiology* **63**, 1690–1695.

Lacey L, Grzywacz D, Shapiro-Ilan D, Frutos R, Brownbridge M and Goettel M (2015) Insect pathogens as biological control agents: back to the future. *Journal of Invertebrate Pathology* **132**, 1–41.

Malan AP, Knoetze R and Moore SD (2011) Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth. *Journal of Invertebrate Pathology* **108**, 115–125.

Maneesakorn P, Grewal PS and Chandrapatya A (2010) *Steinernema minutum* sp. nov. (Rhabditida: Steinernema): a new entomopathogenic from Thailand. *International Journal of Nematology* **20**, 27–42.

Modic Š, Žigon P, Kolmanič A, Trdan S and Razinger J (2020) Evaluation of the field efficacy of *Heterorhabditis Bacteriophora* Poinar (Rhabditida: Heterorhabditidae) and synthetic insecticides for the control of Western corn rootworm larvae. *Insects* **11**, 202.

Mráček Z and Webster JM (1993) Survey of heterorhabditidae and steinernematidae (Rhaditida. Nematoda) in Western Canada. *Journal of Nematology* **25**, 710–717.

Muangpat P, Yooyangket T, Fukruksa C, Suwannaroj M, Yimthin T, Sitthisak S and Thanwisai A (2017) Identification and characterization of the antimicrobial activity against drug resistant bacteria of *Photorhabdus* and *Xenorhabdus* associated with entomopathogenic nematodes from Mae Wong National Park, Thailand. *Frontiers in Microbiology* **8**, 1142.

Namsena P, Bussaman P and Rattanasena P (2016) Bioformulation of *Xenorhabdus* stockiae for controlling mushroom mite, *Luciaphorus perniciosus* Rack. *Bioresources and Bioprocessing* **3**, 19.

Phan KL, Mráček Z, Půža V, Nermut J and Jarošová A (2014) *Steinernema huense* sp. n., a new entomopathogenic nematode (Nematoda: Steinernematidae) from Vietnam. *Nematology* **16**, 761–775.

Raja RK, Sivaramakrishnan S and Hazir S (2011) Ecological characterisation of *Steinernema siamkayai* (Rhabditida: Steinernematidae), a warm-adapted entomopathogenic nematode isolate from India. *Biocontrol* **56**, 789–798.

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA and Huelsenbeck JP (2012) MrBayes 3.2:

https://doi.org/10.1017/S0022149X20000735 Published online by Cambridge University Press

Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. *Systematic Biology* **61**, 539–542.

Rosa JS, Bonifassi E, Amaral J, Lacey LA, Simões N and Laumond C (2000) Natural occurrence of entomopathogenic nematodes (Rhabditida: *Steinernema*, *Heterorhabditis*) in the Azores. *Journal of Nematology* **32**, 215–222.

Shahina F, Anis M, Zainab S and Maqbool MA (1998) Entomopathogenic nematodes in soil samples collected from Sindh, Pakistan. *Pakistan Journal of Nematology* **16**, 41–50.

Shapiro-Ilan D, Hazir S and Glazer I (2017) Basic and applied research: entomopathogenic nematodes. pp. 91–105 *in* Lacey LA (*Ed*) *Microbial control of insect and mite pests*. Cambridge, Academic Press.

Smart GC (1995) Entomopathogenic nematodes for the biological control of insects. *Journal of Nematology* **27**, 529–534.

Stock SP, Somsook V and Reid AP (1998) *Steinernema siamkayai* n. sp. (Rhabditida: Steinernematidae), an entomopathogenic nematode from Thailand. *Systematic Parasitology* **41**, 105–113.

Stock SP, Campbell JF and Nadler SA (2001) Phylogeny of *Steinernema* Travassos 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology* **87**, 877–889.

Suwannaroj M, Yimthin T, Fukruksa C, Muangpat P, Yooyangket T, Tandhavanant S, Thanwisai A and Vitta A (2020) Survey of entomopathogenic nematodes and associate bacteria in Thailand and their potential to control *Aedes aegypti*. *Journal of Applied Entomology* **144**, 212–223.

Tailliez P, Pagès S, Ginibre N and Boemare N (2006) New insight into diversity in the genus *Xenorhabdus*, including the description of ten novel species. *International Journal of Systematic and Evolutionary Microbiology* **56**, 2805–2818.

Tailliez P, Laroui C, Ginibre N, Paule A, Pages S and Boemare N (2010) Phylogeny of *Photorhabdus* and *Xenorhabdus* based on universally conserved protein-coding sequences and implications for the taxonomy of these two genera. Proposal of new taxa: *X. vietnamensis* sp. nov., *P. luminescens* subsp. *caribbeanensis* subsp. nov., *P. luminescens* subsp. *hainanensis* subsp. nov., *P. temperata* subsp. *khanii* subsp. nov., *P. temperata* subsp. *tasmaniensis* subsp. nov., and the reclassification of *P. luminescens* subsp. *thracensis* as *P. temperata* subsp. *thracensis* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **60**, 1921–1937.

Tailliez P, Pages S, Edgington S, Tymo LM and Buddie AG (2012) Description of *Xenorhabdus magdalenensis* sp. nov., the symbiotic bacterium associated with *Steinernema australe*. *International Journal of Systematic and Evolutionary Microbiology* **62**, 1761–1765.

Tarasco E, Clausi M, Rappazzo G, *et al.* (2015) Biodiversity of entomopathogenic nematodes in Italy. *Journal of Helminthology* **89**, 359–366.

Thanwisai A, Tandhavanant S, Saiprom N, Waterfield NR, Ke Long P, Bode HB and Chantratita N (2012) Diversity of *Xenorhabdus* and *Photorhabdus* spp. and their symbiotic entomopathogenic nematodes from Thailand. *PLoS ONE* **7**, 43835.

Valadas V, Laranjo M, Mota M and Oliveira S (2014) A survey of entomopathogenic nematode species in continental Portugal. *Journal of Helminthology* **88**, 327–341.

Vitta A, Yimthin T, Fukruksa C, Wongpeera W, Yotpanya W, Polseela R and Thanwisai A (2015) Distribution of entomopathogenic nematodes in lower northern Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health* **46**, 564–573.

Vitta A, Fukruksa C, Yimthin T, Deelue K, Sarai C, Polseela R and Thanwisai A (2017) Preliminary survey of entomopathogenic nematodes in upper northern Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health* **48**, 18–26.

Vitta A, Thimpoo P, Meesil W, Yimthin T, Fukruksa C, Polseela R and Thanwisai A (2018) Larvicidal activity of *Xenorhabdus* and *Photorhabdus* bacteria against *Aedes aegypti* and *Aedes albopictus*. *Asian Pacific Journal of Tropical Biomedicine* **8**, 31–36.

Wetchayunt W, Rattanapan A and Phairiron S (2009) Temperature effect on novel entomopathogenic nematode *Steinernema siamkayai* Stock, Somsook and Reid (n. sp.) and its efficacy against *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae). *Communications in Agricultural and Applied Biological Sciences* **74**, 587–592.

White GF (1927) A method for obtaining infective nematode larvae from cultures. *Science* **66**, 302–303.

Ye W, Foye S, MacGuidwin AE and Steffan S (2018) Incidence of *Oscheius onirici* (Nematoda: Rhabditidae), a potentially entomopathogenic nematode from the marshlands of Wisconsin, USA. *Journal of Nematology* **50**, 9–26.

Yooyangket T, Muangpat P, Polseela R, Tandhavanant S, Thanwisai A and Vitta A (2018) Identification of entomopathogenic nematodes and symbiotic bacteria from Nam Nao National Park in Thailand and larvicidal activity of symbiotic bacteria against *Aedes aegypti* and *Aedes albopictus*. *PLoS ONE* **13**, 0195681.

https://doi.org/10.1017/S0022149X20000735 Published online by Cambridge University Press