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*

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Abstract

Entomopathogenic nematodes (EPNs) that are symbiotically associated with *Xenorhabdus* and *Photorhabdus* bacteria can kill target insects via direct infection and toxin action. There are limited reports identifying such organisms in the National Park of Thailand. Therefore, the objectives of this study were to identify EPNs and symbiotic bacteria from Nam Nao National Park, Phetchabun Province, Thailand and to evaluate the larvicidal activity of bacteria against *Aedes aegypti* and *Ae. albopictus*. A total of 12 EPN isolates belonging to *Steinernema* and *Heterorhabditis* were obtained form 940 soil samples between February 2014 and July 2016. EPNs were molecularly identified as *S. websteri* (10 isolates) and *H. baujardi* (2 isolates). Symbiotic bacteria were isolated from EPNs and molecularly identified as *P. luminescens* subsp. *akhurstii* (13 isolates), *X. stockiae* (11 isolates), *X. vietnamensis* (2 isolates) and *X. japonica* (1 isolate). For the bioassay, bacterial suspensions were evaluated for toxicity against third to early fourth instar larvae of *Aedes* spp. The larvae of both *Aedes* species were orally susceptible to symbiotic bacteria. The highest larval mortality of *Ae. aegypti* was 99% after exposure to *X. stockiae* (bNN112.3_TH) at 96 h, and the highest mortality of *Ae. albopictus* was 98% after exposure to *P. luminescens* subsp. *akhurstii* (bNN121.4_TH) at 96 h. In contrast to the control groups (*Escherichia coli* and distilled water), the mortality rate of both mosquito larvae ranged between 0 and 7% at 72 h. Here, we report the first observation of *X. vietnamensis* in Thailand. Additionally, we report the first observation of *P. luminescens* subsp. *akhurstii* associated with *H. baujardi* in Thailand. *X. stockiae* has potential to be a biocontrol agent for mosquitoes. This investigation provides a survey of the basic diversity of EPNs and symbiotic bacteria in the National Park of Thailand, and it is a bacterial resource for further studies of bioactive compounds.

Introduction

Xenorhabdus and *Photorhabdus* are Gram-negative bacteria of the family Enterobacteriaceae and are symbiotically associated with the entomopathogenic nematodes (EPNs), *Steinernema* and *Heterorhabditis* respectively [1]. The infective juvenile stages (IJs) of EPNs containing symbiotic bacteria in their midguts live in the soil of diverse ecological systems [2]. The nematodes can kill the target insects via direct infection through the promotion of the secondary metabolites and toxins produced by symbiotic bacteria. When the IJs of nematodes enter the mouth, anus or spiracles of insect hosts, symbiotic bacteria are released from their intestines into the hemocoel of target insects [3]. Subsequently, the symbiotic bacteria produce several toxins or secondary metabolites, killing the insects by induction of immunosuppression and pervasion of the hemolymph [4]. Finally, the symbiotic bacteria replicate rapidly and cause septicemia in insects [5]. In addition, several secondary metabolites produced by the symbiotic bacteria have been reported as bioactive compounds with activities including cytotoxic, antimicrobial, antiparasitic and insecticidal ones [6, 7].

Earlier studies on the larvicidal activity of symbiotic bacteria have demonstrated that *Photorhabdus* insect-related (Pir) protein had a high toxicity against *Aedes aegypti* and *Aedes albopictus*, a main vector of Dengue virus [8]. In addition, cell suspensions of *P. luminescens* and *X. nematophila* were orally toxic to *Ae. aegypti* [9]. The mortality rate of *Ae. aegypti* was further increased when the Cry4Ba protein from *Bacillus thuringiensis* and suspensions of *P. luminescens* and *X. nematophila* were mixed [10]. The mortality of *Ae. aegypti* larvae was 20% when fed with Cry4Ba (5 ɳg/ml) alone. At 48 h after treatment, enhancement of toxicity was observed by combining Cry4Ba toxin with *X. nematophila* (10^8 CFU) or *P. luminescens* (10^8 CFU), which showed 95% and 85% of larval mortality respectively [10]. In addition, cell suspensions of *Xenorhabdus nematophila* together with *B. thuringiensis* subsp. *israelensis* strongly promote the mortality rate of *Ae. aegypti* and *Culex pipiens pallens* [11]. Recently, the fluid cultured forms of *X. nematophila* and *P. luminescens* rapidly caused death of *Ae. aegypti* within a few hours and caused delays in the development of pupa and adults [12]. This suggests that *Xenorhabdus* and *Photorhabdus* bacteria are the natural resources for searching for bioactive compounds. However, species identifications of EPNs and their symbiotic bacteria are important fields of research for studies in agricultural and medical fields.

At present, EPNs have been identified in several geographical areas with approximately 100 species of *Steinernema* and 26 species of *Heterorhabditis* [13–27]. However, the diversity and application of both EPNs and their symbionts have not been thoroughly studied in several countries, including Thailand. In our country, six species of *Steinernema* have been found in several provinces: *S. siamkayi*, *S. websteri*, *S. minutum*, *S. khoisanae*, *S. scarabaei* and *S. kushidai*. Additionally, 6 species of *Heterorhabditis*, including *H. indica*, *H. baujardi*, *H. bacteiophora*, *H. somsookae*, *H. gerrardi* and *H. zealandica*, were recorded in Thailand [15, 28–33]. Symbiotic bacteria have been discovered worldwide, with approximately 24 species of *Xenorhabdus* and 5 species of *Photorhabdus*. In Thailand, three species of *Xenorhabdus* were reported, including *X. stockiae*, *X. miraniensis* and *X. japonica*. Additionally, 3 species with 6 subspecies of *Photorhabdus*, including *P. luminescens* subsp. *akhurstii*, *P. luminescens* subsp. *hainanensis*, *P. luminescens* subsp. *laumondii*, *P. asymbiotica* subsp. *australis*, *P. luminescens* subsp. *namnaonensis* and *P. temperata* subsp. *temperata*, were also recorded across the country [15, 33–36].

Most surveyed locations for EPNs and their associated microbes in Thailand were roadside, with minor amounts surveyed from fruit crops, rice fields and river banks [15, 31, 32]. The first novel species of EPN in Thailand, *S. siamkayi*, was isolated from soil samples in Phetchabun province [28]. Subsequently, a novel subspecies of symbiotic bacteria, *P. luminescens*

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Identification of EPNs and symbiotic bacteria and their larvicidal activity against *Aedes* spp.

subsp. *namnaonensis*, was isolated from *H. baujardi* from the Nam Nao district in Phetchabun province [36]. This suggests that EPNs and their symbionts may be diverse in Phetchabun province, a mountainous region of central Thailand. Therefore, other areas in Phetchabun province should be explored for EPNs and their symbiotic bacteria. Nam Nao National Park includes the mountainous forests of Phetchabun and Chaiyaphum provinces in Thailand. The flora consists mainly of dry dipterocarps, mixed deciduous, hill evergreens, vast bamboo groves, pine forests and some grassland areas. Despite the interesting location for the surveying of EPNs and their symbiotic bacteria, there is only one report of these organisms in the National Park (Mae Wong) of Thailand. This area is abundant with insect hosts and might lead to the recovery of several species of EPNs and symbiotic bacteria. In addition, four organisms were newly recorded in Mae Wong National Park, namely *H. zealandica*, *S. kushidai*, *X. japonica* and *P. temparata* subsp. *temperata*. Therefore, the objectives of this study were to isolate and identify EPNs and their associated *Xenorhabdus* and *Photorhabdus* from Nam Nao National Park of Thailand and to determine the larvicidal activity of these bacteria against *Ae. aegypti* and *Ae. albopictus*.

Materials and methods

Collection of soil samples

The methods for soil collection in Nam Nao National Park of Phetchabun province were approved and permitted by the Department of National Park, Wildlife and Plant Conservation, Thailand (Permission number 0907.4/8245). Overall, 940 soil samples were collected from 188 soil sites in Nam Nao National Park between February 2014 and July 2016. At each site, five soil samples (300–500 g) were collected using a hand shovel. The soil collection process was performed according to Thanwisai et al. [15]. The latitude and altitude of each soil site were determined using a GPS navigator (Garmin nüvi 1250, Taiwan). The moisture and pH of each soil sample were recorded using the soil pH and moisture meter Takemura DM15 (Takemura Electronic Works, Chiba, Japan). The soil temperature of each sample was also measured using a soil survey instrument (Yancheng Kecheng Optoelectronic Technology, Jiangsu, China). Soil texture was also noted. All soil samples were transported in ambient temperature to the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok province, Thailand for the isolation of EPNs. Soil parameters (soil temperature, pH and moisture) of samples with and without EPNs were compared statistically using the Mann-Whitney test in the SPSS Program, Version 17 ($P < 0.05$).

Identification of entomopathogenic nematodes

A baiting technique [37] using *Galleria mellonella* larvae followed by a White trap [38] was performed to isolate EPNs from soil samples. The infective juvenile stages (IJs) of EPNs emerged from the *G. mellonella* cadaver and moved to water. The IJs were collected in a culture flask and cleaned by a sedimentation technique, along with several changes of sterile distilled water. The clean IJs were kept in aerated water and stored at 13 ˚C in a refrigerator. To increase the number and confirm the entomopathogenicity of EPNs, a new *G. mellonella* larva was infected with IJs.

The genomic DNA of EPNs was extracted from the IJs as described previously [15]. The genomic DNA was dissolved in 20 μl of sterile distilled water and stored at –20 ˚C for future use.

A PCR-based analysis and DNA sequencing were conducted to identify *Steinernama* and *Heterorhabditis* species. The primers used for amplifying *Steinernama* 28S rDNA were 539_F (5ʹ`GGATTTCCTTAGTAACTGCGAGTG-3ʹ`) and 535_R (5ʹ `-TAGTCTTTCGCCCCTATACCC` `TT-3ʹ`) [39]. The PCR conditions consisted of pre-denaturation at 95 ˚C for 1 min, followed

by 35 cycles of denaturation at 94 ˚C for 1 min, annealing at 55 ˚C for 30 s, extension at 72 ˚C for 45 s, and a final extension at 72 ˚C for 7 min. The size of amplification fragment was 870 bp.

The primers used for amplifying *Heterorhabditis* internal transcribe spacer (ITS) were18S_F (5'-TGATTACGTCCCTGCCCTTT-3') and 26S_R (5'-TTTCACTCGCCGTTACTAAGG-3') or TW81_F (5'-GTTTCCGTAGGTGAACCTGC-3') and AB28_R (5'-ATATGCTTAAGTTCAGCGGGT-3') [40]. The PCR conditions for *Heterorhabditis* consisted of predenaturation at 95 ˚C for 1 min, followed by 35 cycles of denaturation at 94 ˚C for 30 s, annealing at 50 ˚C for 1 min, extension at 72 ˚C for 1 min and a final extension at 72 ˚C for 7 min. The PCR product was varied between 800–1,100 bp.

A total of 30 µl of the PCR mixture of each *Steinernama* and *Heterorhabditis* consisted of 3 µl of 10x buffer (1x), 4.2 µl of 25 mM $MgCl_2$ (3.5 mM), 0.6 µl of 10 mM dNTPs (200 µM), 1.2 µl of 5 µM forward primer (0.8 µM), 1.2 µl of 5 µM reverse primer (0.8 µM), 0.3 µl of 2.5 unit Taq DNA polymerase (0.1 U/ml), 7.5 of DNA solution (20–200 ηg) and 12 µl of sterile distilled water. Thermocycling for *Steinernama* and *Heterorhabditis* was performed in the thermal cycler (Applied Biosystems, Pittsburgh, PA, USA). PCR products were verified by electrophoresis on 1.2% TBE-buffered agarose gel. The PCR products were purified using a Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., New Taipei, Taiwan) as recommended by the manufacturer. The DNA sequencing for both directions was analyzed by the Macrogen Inc. service in Korea.

Isolation and identification of symbiotic bacteria

Symbiotic bacteria were isolated from the hemolymph of dead *G. mellonella* larvae, which had been infected with the IJs of EPNs. The dead larvae of *G. mellonella* were surface-sterilized by dipping into absolute ethanol for 1 min and placed in a sterile petri dish to dry. Sterile forceps were used to open the 3rd segment from the head of *G. mellonella*. A sterile loop was used to touch hemolymph and streaked on nutrient agar supplemented with bromothymol blue and triphenyl-2,3,5-tetrazolium chloride (NBTA). Colonies of *Xenorhabdus* and *Photorhabdus* were blue and green on NBTA after four days of incubation in the dark at room temperature. A single colony of each isolate of symbiotic bacteria was inoculated in 3 ml of Luria-Bertani (LB) broth and incubated with shaking at 180 rpm overnight (approximately 18–24 h). The genomic DNA of symbiotic bacteria was extracted from bacterial pellets using the Blood/Cell DNA Mini Kit (Geneaid Biotech Ltd., New Taipei, Taiwan). The genomic DNA of symbiotic bacteria was stored at –20 ˚C for further use in PCR.

PCR-based analysis and *recA* gene sequencing was performed to identify *Xenorhabdus* and *Photorhabdus* species. Primers used were recA_F (5'-GCTATTGATGAAAATAAACA-3') and recA_R (5'-RATTTTRTCWCCRTTRTAGCT-3') to obtain an 890 pb amplicon [41]. The PCR conditions for *Xenorhabdus* and *Photorhabdus* were based on a previous report by Thanwisai el at. (2012) [15]. A total of 30 µl of the PCR reaction contained 3 µl of 10x buffer (1x), 4.2 µl of 25 mM $MgCl_2$ (3.5 mM), 0.6 µl of 10 mM dNTPs (200 µM), 1.2 µl of 5 µM forward primer (0.8 µM), 1.2 µl of 5 µM reverse primer (0.8 µM), 0.3 µl of 2.5 unit Taq DNA polymerase (0.1 U/ml), 3 µl of genomic DNA solution (20–200 ηg) and 16.5 µl of sterile distilled water. PCR was conducted in a thermal cycler (Applied Biosystems, Pittsburgh, PA, USA). The thermal profile was as previously described [15]. PCR products were visualized on a ethidium bromide-stained 1.2% agarose gel and purified using a PCR clean-up Gel Extraction Kit (Macherey-nagel, Düren, Germany). The DNA sequencing for both directions was analyzed by Macrogen Inc. service in Korea.

Sequencing and phylogenetic analysis

To identify species of EPNs (*Steinernema* and *Heterorhabditis*) and symbiotic bacteria (*Xenorhabdus* and *Photorhabdus*), comparison of the partially edited nucleotide sequences (*recA*, ITS and 28S rDNA) was performed using the BLASTN program from NCBI. A cut-off of $\geq$ 97% identity was considered for the same species. The nucleotide sequences were aligned using ClustalW, and a phylogenetic tree was constructed using the maximum likelihood method with the MEGA Version 7.0 program [42].

Bioassay for larvicidal activity

In this study, five isolates of symbiotic bacteria were selected for using in the bioassay. The symbiotic bacteria, including *P. luminescens* subsp. *akhurstii* (bNN121.4_TH), *X. stockiae* (bNN112.3_TH), *X. japonica* (bNN165.4_TH), *X. vietnamensis* (bNN167.2_TH) and *X. vietnamensis* (bNN167.3_TH), were used for determining the oral toxicity against *Ae. aegypti* and *Ae. albopictus* larvae.

The eggs of *Ae. aegypti* and *Ae. albopictus* (laboratory strain) on a filter paper were purchased from the Taxonomy and Reference Museum of the Department of Medical Sciences at the National Institute of Health of Thailand, Ministry of Public Health, Nonthaburi Province, Thailand. *Aedes* eggs were placed into distilled water to allow hatching of first instar larvae, which were fed with minced food pets. The late third and early fourth instar larvae were used in the bioassays.

Escherichia coli ATCC® 25922 and distilled water were used as controls for the bioassays. *Escherichia coli* ATCC® 25922 was inoculated in the LB broth and incubated at 37˚C for 24 h, while each isolate of *Xenorhabdus* and *Photorhabdus* was inoculated on 5YS medium (5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.05% (w/v) $NH_4H_2PO_4$, 0.05% (w/v) K_2HPO_4 and 0.02% (w/v) $MgSO_4.7H_2O$) and incubated at room temperature for 48 h. All broth cultures of bacteria were centrifuged at 10,000 x g for 10 min. The supernatant was discarded and the pellets were suspended in sterile distilled water to an OD_{600} of 0.1 with a spectrophotometer (Metertech SP-880, Taiwan).

Suspensions containing *Xenorhabdus* and *Photorhabdus* was evaluated for toxicity against the larvae (third late to early fourth instars) of *Ae. aegypti* and *Ae. albopictus*. Distilled water and a suspension of *E. coli* ATCC® 25922 were used as controls. In each bioassay, 30 larvae of each mosquito species were placed in three wells of a 24-well plate (10 larvae/well). Subsequently, 2 ml (10^7–10^8 CFU/ml) from each bacterial suspension was added to each well. This concentration of symbiotic bacteria showed the enhancement of oral toxicity against *Ae. aegypti* [10]. The larvae were fed with minced pet food during the experiment. The mortality rate of larvae was monitored after exposure to bacterial suspensions at 24, 48, 72 and 96 h. Each bioassay was tested in three replicates on different dates. The larvae were considered dead when no movement was detected when teased with a fine sterile toothpick. Larval mortality of *Ae. aegypti* and *Ae. albopictus* after exposure to symbiotic bacteria was analyzed by survival analysis (Kaplan-Meier Estimate) of SPSS Version 17 ($P < 0.05$).

Results

Isolation of entomopathogenic nematodes from soil samples

Out of the 188 soil sites from Nam Nao National Park, Phetchabun Province, Thailand, 25 tested positive for EPNs (Fig 1). Of the 940 soil samples, 27 (2.87%) were found positive for *Steinernema* or *Heterorhabditis*. This yielded 13 isolates of *Heterorhabditis* and 14 isolates of *Steinernema*. Most EPNs were isolated from loam (96.30%), with the soil pH ranging between
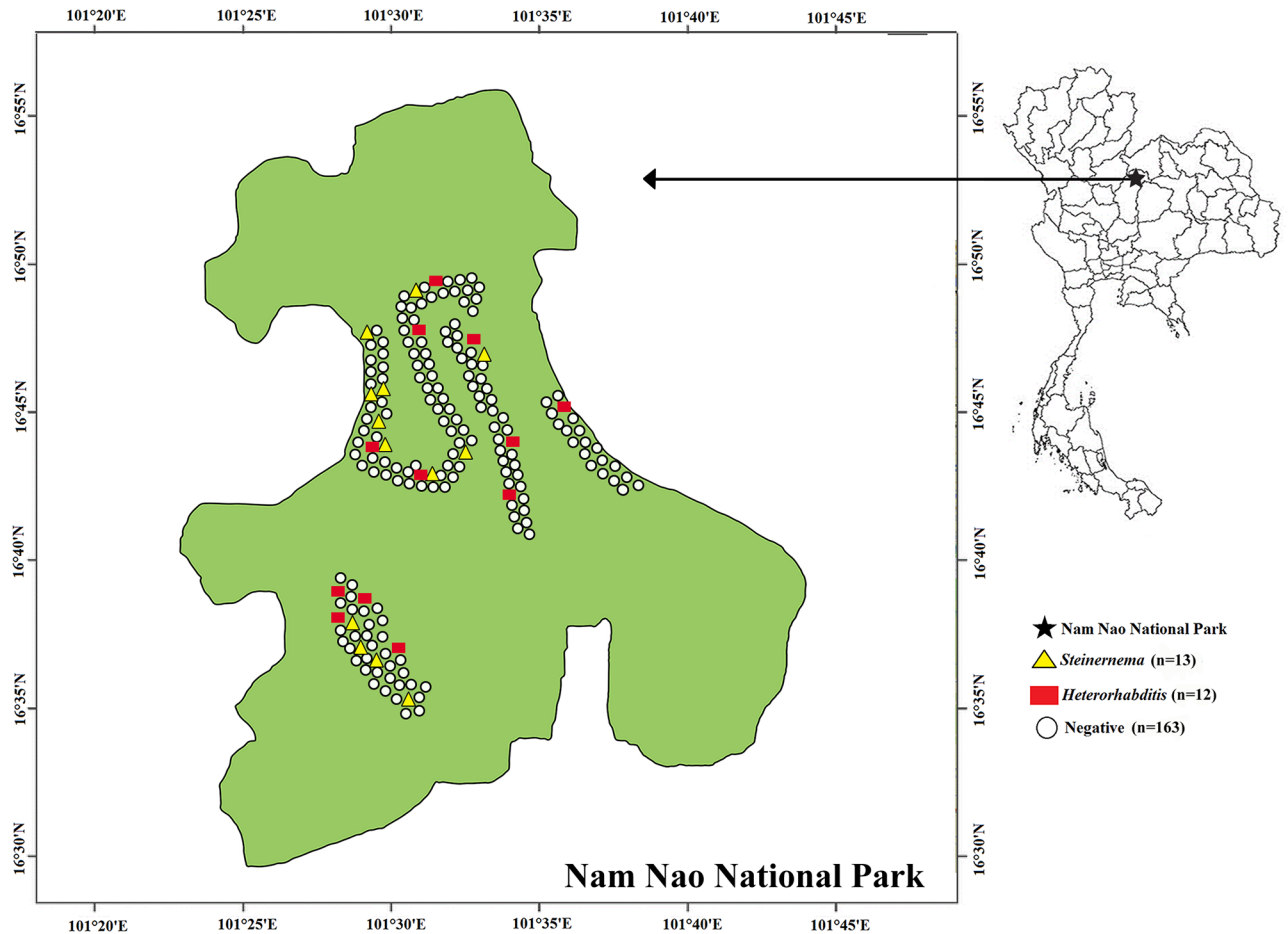
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Identification of EPNs and symbiotic bacteria and their larvicidal activity against *Aedes* spp.

Fig 1. Map of sites for soil collection in Nam Nao National Park, Phetchabun Province, Thailand. A total of 188 soil sampling sites with their statuses of positive or negative for EPNs, *Steinernema* and *Heterorhabditis*, in Nam Nao National Park, Phetchabun Province, Thailand.

4.8 and 7.0, soil temperature ranging between 19 ˚C and 30 ˚C and soil moisture ranging between 1.0 and 8.0 (Table 1). One isolate of the EPNs was isolated from sandy loam and none of the EPNs were isolated from soil with a clay texture.

Identification and phylogeny of entomopathogenic nematodes

To identify *Heterorhabditis* and *Steinernema* species, PCR-based analysis and sequencing of the ITS and 28S rDNA regions were performed together with a BLASTN search of the edited sequences. Two isolates of *Heterorhabditis* (accession no. MG209260 and MG209261) were identified as *Heterorhabditis baujardi* with 99% identity after BLASTN search using 542 nucleotides

Table 1. Temperature, pH and moisture from soil samples (n = 940) with and without EPNs in Nam Nao National Park, Phetchabun Province, Thailand.

Soil parameter	Soil with EPNs (n = 27)	Soil without EPNs (n = 913)	*P-value* (Mann-Whitney test)
Temperature (˚C)	20–25	19–30	0.055
pH	6.2–7.0	4.8–7.0	0.165
Moisture	1.0–3.0	1.0–8.0	0.972

of the ITS region. For the genus *Steinernema* (accession no. MG209262, MG209263, MG209264, MG209265, MG209266, MG209267, MG209268, MG209269, MG209270 and MG209271), 10 isolates of *S. websteri* were identified with 99% identity after BLASTN search using 502 nucleotides of the 28S rDNA region. *S. websteri* was the most recovered species in soil samples from the studied area. The remaining 15 EPN isolates were lost through protozoa and fungal contamination. A phylogenetic tree based on the maximum likelihood method revealed two isolates of the genus *Heterorhabditis* grouped in *H. baujardi* (accession no. AF548768.1) ([Fig 2](#)) and 10 isolates of the genus *Steinernema* grouped in *S. websteri* (accession no. AY841762.1) ([Fig 3](#)).

Isolation, identification and phylogeny of *Xenorhabdus* and *Photorhabdus*

Based on colony morphology on NBTA agar, 27 isolates of symbiotic bacteria were identified as *Photorhabdus* (13 isolates) and *Xenorhabdus* (14 isolates). A partial sequence of the *recA* gene was amplified and sequenced to identify the *Xenorhabdus* and *Photorhabdus* species. A BLASTN search of the edited sequences was performed to find the identity. A total of 13 isolates of *Photorhabdus* (accession no MG209233, MG209234, MG209235, MG209236, MG209237, MG209238, MG209239, MG209240, MG209241, MG209242, MG209243, MG209244 and MG209245) were identified as *P. luminescens* subsp. *akhurstii* with 97–100% identity. Also, 14 isolates of *Xenorhabdus* (accession no MG209246, MG209247, MG209248, MG209249, MG209250, MG209251, MG209252, MG209253, MG209254, MG209255, MG209256, MG209257, MG209258 and MG209259) were identified as *X. stockiae* (11 isolates), *X. vietnamensis* (2 isolates) and *X. japonica* (1 isolate) with an identity ranging from 97–99%. [Fig 4](#) shows the phylogenetic tree based on the maximum likelihood method, which revealed that only one group of *Photorhabdus* (13 isolates in the present study) was closely related to *P. luminescens* subsp. *akhurstii* (accession no. FJ862005.1) derived from the NCBI database. Two isolates of *Photorhabdus* (bNN64.4_TH and bNN77.4_TH) were hosted by *H. baujardi* ([Table 2](#)). In all, 11 isolates of *P. luminescens* subsp. *akhurstii* were associated with *Heterorhabditis* sp. ([Table 2](#)). [Fig 5](#) shows the phylogenetic tree based on the maximum likelihood method of 14 *Xenorhabdus* isolates in the present study. The group of isolates was divided into 3 groups. Group 1 contained 2 isolates of *Xenorhabdus* (bNN167.3_TH and bNN167.3_TH) and the referenced *X. vietnamensis* (accession no. GU481051.1). Group 2 contained only 1 isolate of *Xenorhabdus* (bNN165.4) in the present study and *X. japonica* (accession no. FJ823400.1) isolated from *Steinernema kushidai*. Group 3 was composed of 11 isolates of *Xenorhabdus*, which were closely related to *X. stockiae* strain TH01 (accession no. FJ823425.1). Most *Xenorhabdus* isolates in the present study were associated with *S. websteri* ([Table 2](#)). Two *Xenorhabdus vietnamensis* isolates and one isolate of *Xenorhabdus japonica* were hosted by *Steinernema* sp. ([Table 2](#)).

Larvicidal activity of symbiotic bacteria against *Aedes* larvae

The symbiotic bacteria used for the bioassay were selected based on their locations in different groups on the phylogenetic tree. Both, the *Xenorhabdus* and *Photorhabdus* isolates in the present study were assumed to be pathogenic when ingested by *Ae. aegypti* and *Ae. albopictus* larvae. [Fig 6](#) and [Table 3](#) demonstrate that the highest mortality (99%) of *Ae. aegypti* larvae was after exposure to *X. stockiae* bNN112.3_TH for 96 h. A significant difference (Kaplan-Meier Estimate, *P-value* = 0.000) was observed when comparing the mortality rate of *A. aegypti* larvae between the tested group and the control groups (distilled water and *E. coli* ATCC® 25922). The mortality rate in the control groups was low at 0% at 24, 48, and 72 h and 7% for 96 h.

For the bioassay of *Ae. albopictus*, the highest mortality of the larvae was 98% after exposure to *P. luminescens* subsp. *akhurstii* (bNN121.4_TH) for 96 h, while the control groups
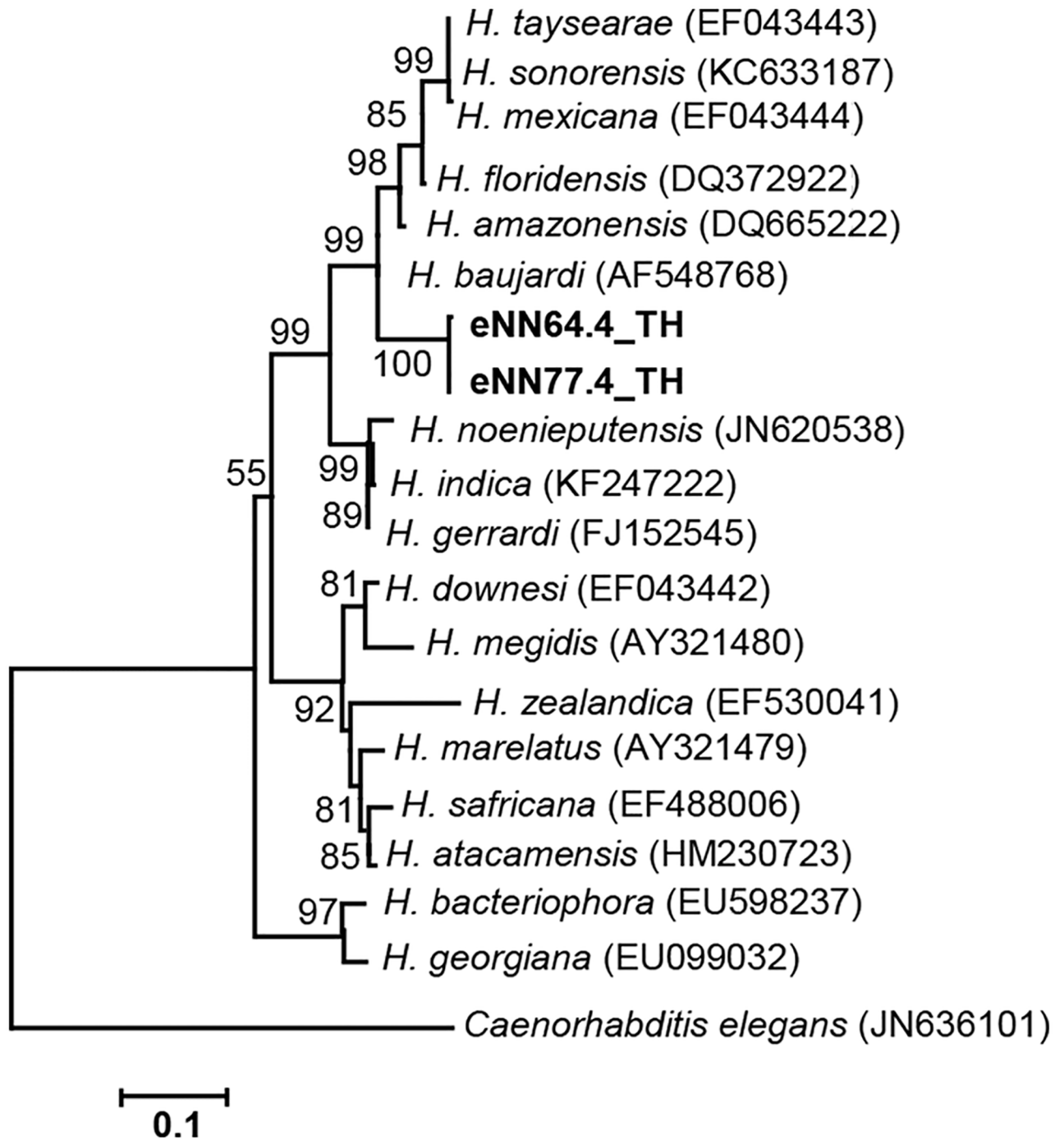
Fig 2. Phylogenetic tree of *Heterorhabditis*. Maximum likelihood tree of a 542 bp of the ITS region from two *Heterorhabditis* isolates from Nam Nao National Park, Phetchabun Province, Thailand (codes ending with TH), together with 18 *Heterorhabditis* sequences downloaded from the GenBank database. *Caenorhabditis elegans* (accession no. JN636101.1) was used as the outgroup. Bootstrap values are based on 1,000 replicates.

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(distilled water and *E. coli* ATCC® 25922) showed the lowest mortality at 0% at 24 and 48 h, and 7% at 72 h (Fig 6 and Table 3). Moreover, *Ae. aegypti* and *Ae. albopictus* larvae exhibited a low susceptibility to *X. japonica* (bNN165.4_TH) and *X. vietnamensis* (bNN167.2_TH and bNN167.3_TH) as indicated by the 22–61% mortality after exposure for 96 h.
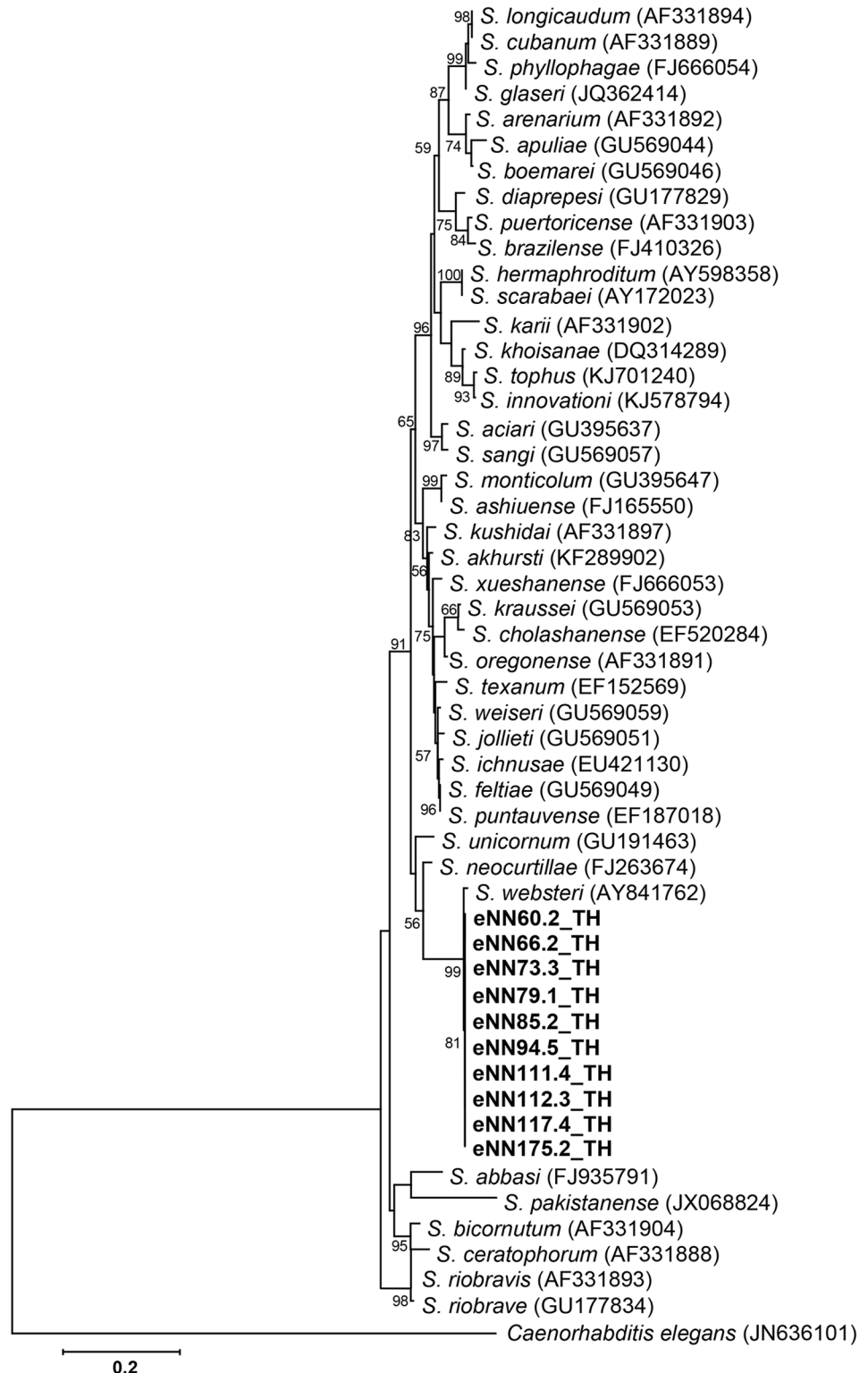
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Identification of EPNs and symbiotic bacteria and their larvicidal activity against *Aedes* spp.

Fig 3. Phylogenetic tree of *Steinernema*. Maximum likelihood tree of a 502 bp of the 28S rDNA region from 10 *Steinernema* isolates from Nam Nao National Park, Phetchabun Province, Thailand (codes ending with TH), together with 41 *Steinernema* sequences downloaded from the GenBank database. *Caenorhabditis elegans* (accession no. JN636101.1) was used as the outgroup. Bootstrap values are based on 1,000 replicates.
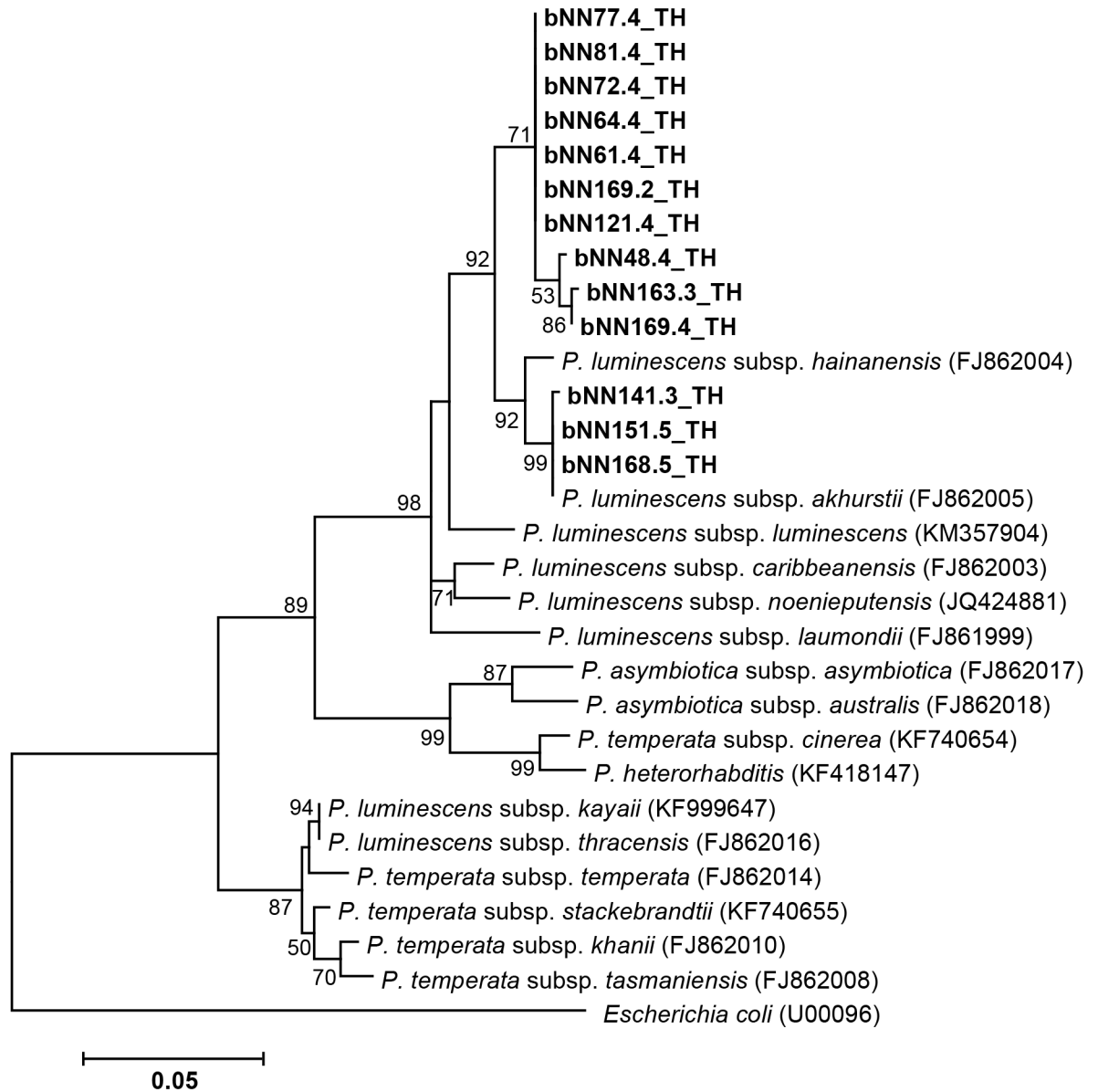
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Identification of EPNs and symbiotic bacteria and their larvicidal activity against *Aedes* spp.

Fig 4. Phylogenetic tree of *Photorhabdus*. Maximum likelihood tree of a 588 bp of the *recA* region for 13 *Photorhabdus* isolates from Nam Nao National Park, Phetchabun Province, Thailand (codes ending with TH), together with 16 *Photorhabdus* sequences downloaded from the GenBank database. *Escherichia coli* (accession no. U00096.3) was used as an outgroup. Bootstrap values are based on 1,000 replicates.

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Discussion

Here, we report a low recovery of EPNs (2.87%) from Nam Nao National Park of Thailand. Our findings revealed two genera of EPNs, which consisted of 10 isolates of *Steinernema* and two isolates of *Heterorhabditis*. Most EPNs were isolated from loam, which is consistent with the study by Thanwisai et al. (2012) [15]. In addition, EPNs were not found in clay in the present study.

Soil parameters including temperature, pH, and moisture are important for EPN survival and infectivity. In the present study, the soil moisture was 1.0–3.0% (average 1.8%) for the EPN-positive samples and 1.0–8.0% (average 2.0%) for the EPN-negative samples. This

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Identification of EPNs and symbiotic bacteria and their larvicidal activity against *Aedes* spp.

Table 2. Association between EPN hosts and symbiotic bacteria in Nam in Nam Nao National Park, Phetchabun Province, Thailand.

Association between EPN and their symbiotic bacteria	No. of Associations (isolates)	Code
Heterorhabditis associated with *P. luminescens* subsp. *akhurstii*	11	NN48.4_TH, NN61.4_TH, NN72.4_TH, NN81.4_TH, NN121.4_TH, NN141.3_TH, NN151.5_TH, NN163.3_TH, NN168.5_TH, NN169.2_TH, NN169.4_TH
Heterorhabditis baujardi associated with *P. luminescens* subsp. *akhurstii*	2	NN64.4_TH, NN77.4_TH
Steinernema websteri associated with *Xenorhabdus stockiae*	10	NN60.2_TH, NN66.2_TH, NN70.5_TH, NN73.3_TH, NN79.1_TH, NN85.2_TH, NN94.5_TH, NN111.4_TH, NN112.3_TH, NN117.4_TH
Steinernema associated with *Xenorhabdus stockiae*	1	NN175.2_TH
Steinernema associated with *Xenorhabdus vietnamensis*	2	NN167.2_TH, NN167.3_TH
Steinernema associated with *Xenorhabdus japonica*	1	NN165.4_TH

indicated that EPNs, especially *S. websteri* and *H. indica* (the most common species in Thailand), could live in a restricted range of moisture contents. In addition, soil moisture contents between 4.0–8.0% may not suitable for the survival of EPNs because none were recovered from this range of moisture contents. Although the difference in soil moisture contents found in the present study between the EPN-positive and EPN-negative samples was not significantly great (*P-value* = 0.972), soil moisture is an important factor that supports the ability of EPNs to infect hosts and its survival in different environments [43, 44]. High and low moisture content could affect the movement of EPNs to find a new host; thus, this soil parameter might affect survival rates. The appropriate moisture content for survival is 8–18% for *H. indica*, 6–20% for *S. themophilum* and 8.0–25.0% for *S. glaseri* [45]. This indicates that each species of EPN responds to different levels of moisture. Soil temperatures between EPN-positive samples (20–25 ˚C) and EPN-negative samples (19–30 ˚C) in the present study was not significantly great (*P-value* = 0.055). In general, temperatures under 0 ˚C and above 40˚C would lead to EPN death. Temperatures between 10 and 15 ˚C could limit the movement of EPNs, while temperatures between 30 and 40 ˚C could result in low infectivity of the EPNs [46]. In the present study, the soil pH between EPN-positive samples (6.2–7.0) and EPN negative samples (4.8–7.0) was not significantly great (*P-value* = 0.165). Soil pH is one of the factors that is important for the infectivity and survival of EPNs. For *Steinernema*, infection and survival rates showed a low level of decrease when the soil pH was between 4 and 8. Rapid decreases in EPN infectivity and survival rates were observed with pH = 10 [47].

Herein, we isolated and identified two isolates of *H. baujardi* and 10 isolates of *S. websteri*. The most common species found in Nam Nao National Park was *S. websteri*, which has previously been isolated from soil samples collected from the roadside areas of several provinces of Thailand [15,31,32]. This EPN was also found in Mae Wong National Park, Kampheang Phet province and central Thailand [33]. This confirmed that *S. websteri* was abundant in natural and cultivated fields across the country. *H. baujardi*, a less common species that was found in the present study, is also recovered in a low number of isolates across the country [16]. In the early studies of EPNs in Thailand, Tangchitsomkid and Sontirat reported the first isolation of the *Steinernema* and *Heterorhabditis* genera from soil samples from Kanchanaburi, Kalasin, Maha Sarakham, Khon Kaen, Nong Khai, Phichit and Sa Kaeo provinces in Thailand [48]. *Steinernema saimkayi*, a novel species, has been isolated from a soil sample collected from the land used to cultivate tamarind in Phetchabun province [28]. *Steinernema minutum*, a second novel species reported in our country, was recovered from soil samples in Chumphon province, which is in south Thailand [29]. Our finding is consistent with previous studies that the
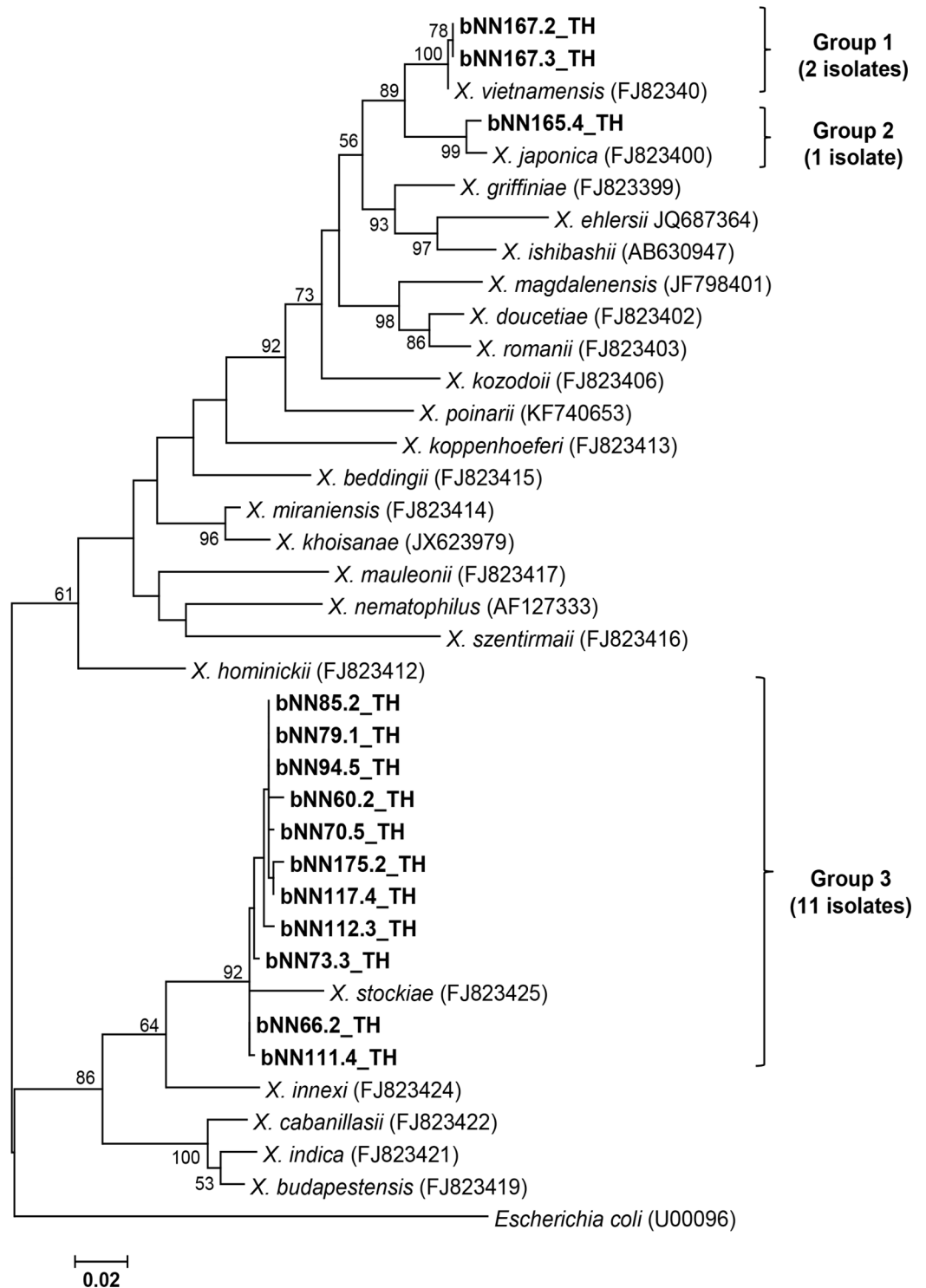
Fig 5. Phylogenetic tree of *Xenorhabdus*. Maximum likelihood tree of a 588 bp of the *recA* gene for 14 *Xenorhabdus* isolates from Nam Nao National Park, Phetchabun Province, Thailand (codes ending with TH), together with 23 *Xenorhabdus* sequences downloaded from the GenBank database. *Escherichia coli* (accession no. U00096.3) was used as an outgroup. Bootstrap values are based on 1,000 replicates.

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Identification of EPNs and symbiotic bacteria and their larvicidal activity against *Aedes* spp.
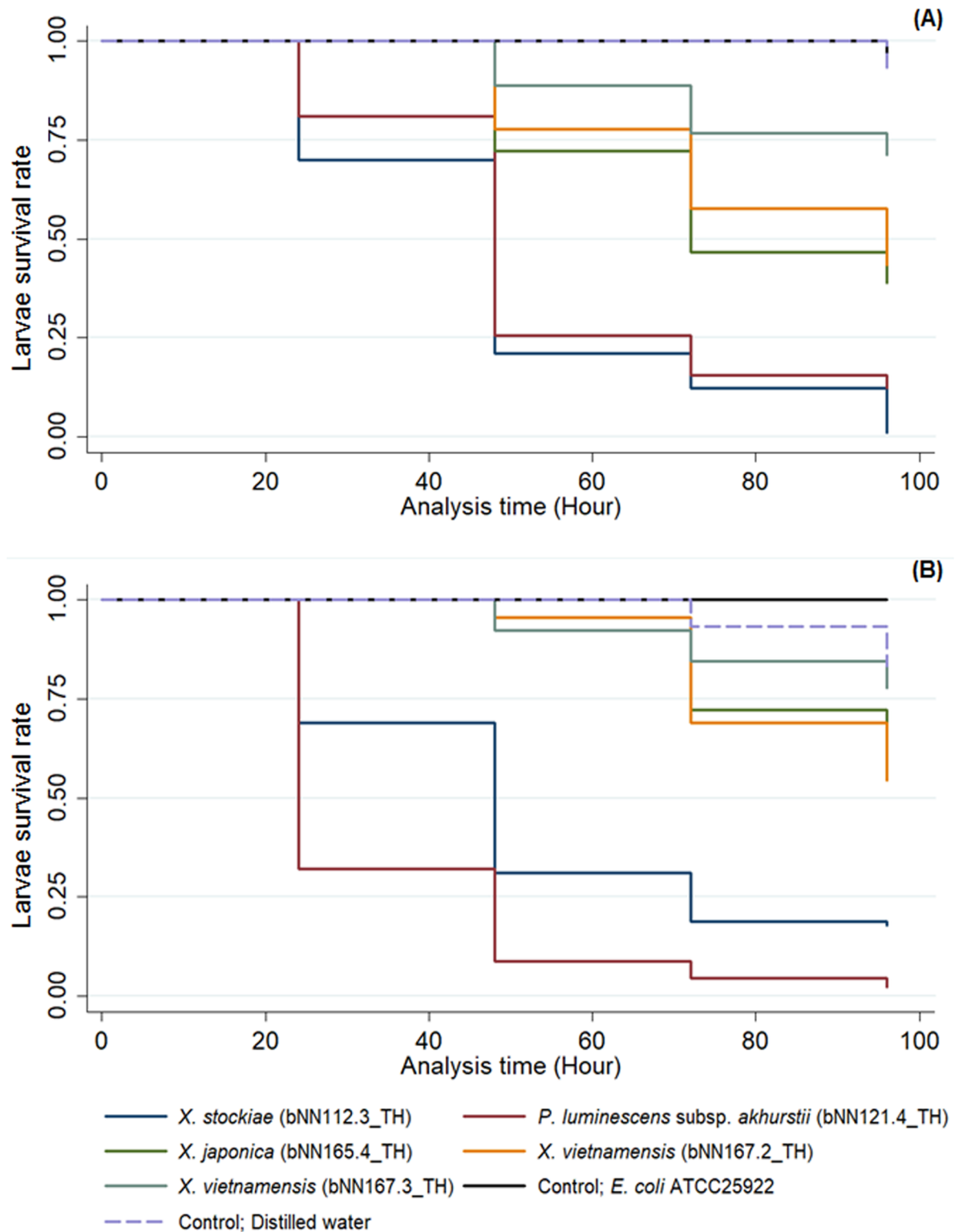
Fig 6. Kaplan-Meier overall survival curves comparing the mortality rate of *Ae. aegypti* larvae (A) and *Ae. albopictus* larvae (B) after exposure to suspensions of *Xenorhabdus* and *Photorhabdus* bacteria isolated from entomopathogenic nematodes in Nam Nao National Park, Phetchabun Province, Thailand.

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Identification of EPNs and symbiotic bacteria and their larvicidal activity against *Aedes* spp.

Table 3. Mortality rate of *Ae. aegypti* and *Ae. albopictus* larvae after exposure to suspensions of *Xenorhabdus* and *Photorhabdus* isolated from entomopathogenic nematodes in Nam Nao National Park, Phetchabun Province, Thailand.

Bacteria (Code)	Mortality rate (Mean ± SD)							
	Ae. aegypti				*Ae. albopictus*			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
X. stockiae (bNN112.3_TH)	30 ± 26.03	79 ± 11.71	88 ± 15.40	99 ± 1.92	31 ± 53.89	69 ± 45.26	81 ± 24.11	82 ± 25.02
P. luminescens subsp. *akhurstii* (bNN121.4_TH)	19 ± 32.72	74 ± 30.97	84 ± 26.94	88 ± 21.17	68 ± 36.63	91 ± 5.09	96 ± 5.09	98 ± 3.85
X. japonica (bNN165.4_TH)	0 ± 0.00	28 ± 12.62	53 ± 21.86	61 ± 13.47	0 ± 0.00	8 ± 5.09	28 ± 7.70	36 ± 9.62
X. vietnamensis (bNN167.2_TH)	0 ± 0.00	22 ± 1.92	42 ± 3.84	57 ± 8.82	0 ± 0.00	4 ± 1.92	31 ± 8.39	46 ± 16.78
X. vietnamensis (bNN167.3_TH)	0 ± 0.00	11 ± 8.39	23 ± 15.28	29 ± 15.03	0 ± 0.00	8 ± 5.09	16 ± 6.94	22 ± 9.62
E. coli ATCC® 25922	0 ± 0.00	0 ± 0.00	0 ± 0.00	7 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Distilled water	0 ± 0.00	0 ± 0.00	0 ± 0.00	7 ± 0.00	0 ± 0.00	0 ± 0.00	7 ± 0.00	17 ± 0.00

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most common species of EPNs in Nam Nao National Park was *S. websteri*. However, when comparing our findings with a previous study in Mae Wong National Park, we did not isolate *H. zealandica* and *S. kushidai*, which may be due to the low distribution of these two EPNs in the National Park.

Of the symbiotic bacteria, all 13 isolates of *Photorhabdus* were molecularly identified as *P. luminescens* subsp. *akhurstii*. *Xenorhabdus* species (14 isolates) were molecularly identified as *X. stockiae* (11 isolates), *X. vietnamensis* (2 isolates) and *X. japonica* (1 isolate). In the present study, we found *X. stockiae* was associated with *S. websteri*, which was consistent with the previous research in Thailand [15, 33]. However, *S. websteri* was also symbiotically associated with *X. nematophila* [49]. Two isolates of *X. vietnamensis* were first identified in Nam Nao National Park of Thailand. Unfortunately, the EPN host *X. vietnamensis* was only identified to the genus level in the present study. A previous study in Vietnam reported *X. vietnamensis* was associated with *S. sangi* [50,51]. The finding of one isolate of *X. japocina* in Nam Nao National Park is similar to the finding of Muangpat, who first recorded *X. japonica* associated with *S. kushidai* in Mae Wong National Park of Thailand [33]. For the genus *Photorhabdus*, *P. luminescens* subsp. *akhurstii* was associated with *H. baujardi*. Elsewhere, *P. luminescens* subsp. *hainanensis*, *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *laumondii* were associated with *H. indica* [15]. The relationship found in this study between *P. luminescens* subsp. *akhurstii* and *H. baujardi* is a new observation in Thailand.

We demonstrated the toxicity of Thai *Xenorhabdus* and *Photorhabdus* isolates against *Ae. aegypti* and *Ae. albopictus* larvae. *Xenorhabdus stockiae* bNN112.3_TH and *P. luminescens* subsp. *akhurstii* bNN121.4 isolated in the present study have potential to be control agents for *Ae. aegypti* and *Ae. albopictus*. We suppose that the symbiotic bacteria are pathogenic when ingested by *Aedes* larvae. In general, the diet of *Aedes* larvae are dead and living organic material. These are bacteria, fungi and protozoans that grow on container or are suspended in fluid [52]. These bacteria may produce toxins and secondary metabolites that kill the *Aedes* larvae. To support this scenario, *Xenorhabdus* spp. might produce insecticidal compounds including toxin complexes (Tcs), which play an important role in the pathogenicity of immunosuppression by inhibiting eicosanoid biosynthesis in the target insect [4,53]. *Xenorhabdus nematophila* produces at least eight bacterial metabolites that play crucial roles in suppressing the immune responses of target insects [54]. *X. stockiae* PB09 showed miticidal activity against mushroom

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Identification of EPNs and symbiotic bacteria and their larvicidal activity against *Aedes* spp.

mites [55]. Several species of *Photorhabdus* have been reported to produce several toxins including toxin complexes (Tcs), make caterpillars floppy (Mcf), *Photorhabdus* insect-related (Pir) proteins and *Photorhabdus* virulence cassettes (PVC) [56]. The Tcs destroy the epithelial cells of the insect midgut and are similar to the δ-endotoxin from *Bacillus thuringiensis* (Bt) [5]. The Mcf is active upon injection and disrupts the insect hemocytes by promoting their apoptosis [57]. The *Photorhabdus Pir* toxin is composed of *Pir*A and *Pir*B, which have been found to be effect against mosquito larvae especially the dengue vectors, *Ae. aegypti* and *Ae. albopictus* [8, 58]. *Xenorhabdus nematophila* and *P. luminescens* showed oral toxicity against *Ae. aegypti* [9]. Recently, an enhanced mortality rate of *Ae. aegypti* was demonstrated by mixing the Cry4Ba protein of *Bacillus thuringiensis* with *X. nematophila* and *P. luminescens* [10]. The PVC causes toxicities against a variety of insects such as *Manduca sexta* and *Gallaria mellonella* [59]. Moreover, culture fluids from *X. nematophila* caused a delay in pupation and the emergence of adults. The fluids were lethal to larvae, with a cumulative mortality higher than 90% by day 14 [11]. In addition, cell suspensions of *X. ehlersii* isolated from *S. scarabaei* from Mae Hong Son Province in northern Thailand showed a potential efficacy in killing *Ae. aegypti* with 100% mortality [60]. In contrast, *Ae. aegypti* and *Ae. albopictus* larvae had low mortality rates (4–61%) after exposure to *X. japonica* NN165.4_TH, *X. vietnamensis* NN167.2_TH and *X. vietnamensis* NN167.3_TH. This may have been due to their inability to produce very toxic metabolites to *Aedes*. In addition, different strains of symbiotic bacteria may produce different secondary metabolites. This may give a variable effectiveness in killing the target organisms. Also, the test of the mortality rate of the insects may be related to the variable number of bacterial cells ingested by each larva. It is possible that the symbiotic bacteria are alive in the water and may produce some secondary metabolites in the water. This may be the cause of the larval death.

At present, 2.5 billion people in 100 countries are considered living in areas with a risk infection of the Dengue virus [61,62]. It is transmitted by female mosquitoes mainly of the species *Ae. aegypti* and, to a lesser extent, *Ae. albopictus*. This mosquito also transmits chikungunya, yellow fever and Zika infection [63,64]. At present, there are no particular treatments or vaccines available to combat infections by these viruses; the only effective method to prevent infection is to avoid mosquito bites [65]. Control measures for *Ae. aegypti* and *Ae. albopictus* can be achieved using repellents, by cleaning the water source where larvae breed, or applying synthetic insecticides such as organophosphates (temephos and fenthion). The insect growth regulators (diflubenzuron and methoprene) are generally used for the control of mosquito larvae [66–68]. The constant use of such insecticides induces resistance in populations of *Aedes* species [69]. Furthermore, biological control might be an alternative approach to avoid these effects. The advantage of using *Xenorhabdus* and *Photorhabdus* bacteria is that they rapidly kill both *Ae. aegypti* and *Ae. albopictus* larvae within 48 h. These bacteria also may be non-toxic to humans. *Xenorhabdus* and *Photorhabdus* may thus be effective in all stages of *Aedes* larvae.

Conclusion

We report a diversity of EPNs and symbiotic bacteria from Nam Nao National Park, Phetchabun Province, Thailand, with the most commonly isolated EPN species being *Steinernema websteri*. The most commonly isolated species of symbiotic bacteria was *X. stockiae*. *Xenorhabdus vietnamensis* is the first recorded member of this species of symbiotic bacteria in Thailand. *P. luminescens* subsp. *akhurstii* associated with *H. baujardi* is a new observation in Thailand. *Xenorhabdus stockiae* and *P. luminescens* subsp. *akhurstii* are potential bio-control agents for *Ae. aegypti* and *Ae. albopictus*. Our findings provide information regarding the diversity of EPNs and symbiotic bacteria in Nam Nao National Park, Phetchabun Province, Thailand and

provide a natural resource for further studies of bioactive compounds for controlling mosquitoes. Further investigations of the structure and isolation of the metabolic compounds from *Xenorhabdus* and *Photorhabdus* are needed to update the basic knowledge of the secondary metabolites found in these symbiotic bacteria.

Acknowledgments

Author Contributions

References

1. Boemare NE, Akhurst RJ, Mourant RG. DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. Int J Syst Bacteriol. 1993; 43:249–255.

2. Dillman AR, Guillermin ML, Lee JH, Kim B, Sternberg PW, Hallem EA. Olfaction shapes host–parasite interactions in parasitic nematodes. Proc Natl Acad Sci. 2012; 109(35):E2324–33. https://doi.org/10.1073/pnas.1211436109 PMID: 22851767

3. Kaya HK, Gaugler R. Entomopathogenic nematodes. Annu. Rev. Microbiol. 1993; 38:181–206.

4. Park Y, Kim Y. Eicosanoids rescue *Spodoptera exigua* infected with *Xenorhabdus nematophilus*, the symbiotic bacteria to the entomopathogenic nematods *Steinernema carpocapsae*. J Insect Physiol. 2000; 11:1467–1476.

5. Forst S, Dowds B, Boemare N, Stackebrandt E. *Xenorhabdus* and *Photorhabdus* spp. Bugsthat Kill Bugs. Annu Rev Microbiol. 1997; 51:47–72. https://doi.org/10.1146/annurev.micro.51.1.47 PMID: 9343343

6. Bode Hb. Entomopahogenic bacteria as a source of secondary metabolites. Curr Opin Chem Biol. 2009; 13(2):224–230. https://doi.org/10.1016/j.cbpa.2009.02.037 PMID: 19345136

PLOS | ONE

Identification of EPNs and symbiotic bacteria and their larvicidal activity against *Aedes* spp.

7. Grundmann F, Kaiser M, Schiell M, Batzer A, Kurz M, Thanwisai A, et al. Antiparasitic Chaiyaphumines from entomopathogenic *Xenorhabdus* sp. PB61.4. J. Nat Prod. 2014; 77: 779–83. https://doi.org/10.1021/np4007525 PMID: 24673206

8. Ahantarig A, Chantawat N, Waterfield NR. PirAB Toxin from *Photorhabdus asymbiotica* as a larvicide against dengue vectors. J Appl Environ Microbiol. 2009; 75(13):4627–4629.

9. de Silva OS, Prado GR, Silva CE, Costa M, Heermann R. Oral toxicity of *Photorhabdus luminescens* and *Xenorhabdus nematophila* (Enterobacteriaceae) against *Aedes aegypti* (Diptera: Culicidae). Parasitol Res. 2013; 112:2891–2896. https://doi.org/10.1007/s00436-013-3460-x PMID: 23728731

10. Park Y. Entomopathogenic bacterium, *Xenorhabdus nematophila* and *Photorhabdus luminescens*, enhances *Bacillus thuringiensis* Cry4Ba toxicity against yellow fever mosquito, *Aedes aegypti*. J Asia Pac Entomol. 2015; 18:459–463.

11. Park Y, Kyo JJ, Kim Y. A Mixture of *Bacillus thuringiensis* subsp. israelensis With *Xenorhabdus nematophila*-cultured broth enhances toxicity against mosquitoes *Aedes albopictus* and *Culex pipiens* pallens (Diptera: Culicidae). J Econ Entomol. 2016; 109(3):tow063.

12. de Silva JL, Schwalm UF, Silva EC, Costa-da M, Heermann R, Silva-da SO. Larvicidal and growth-inhibitory activity of entomopathogenic bacteria culture fluids against *Aedes aegypti* (Diptera: Culicidae). J Econ Entomol. 2017; 110(2):378–385. https://doi.org/10.1093/jee/tow224 PMID: 28062794

13. Mráček Z, Liu QZ, Nguyen KB. *Steinernema xueshanense* n. sp. (Rhabditida, *Steinernematidae*), a new species of entomopathogenic nematode from the province of Yunnan, southeast Tibetan Mts., China. J Invertebr Pathol. 2009; 102(1):69–78. https://doi.org/10.1016/j.jip.2009.05.006 PMID: 19460386

14. Thanwisai A. Isolation of entomopathogenic nematodes and associated Xenorhabdus/Photorhabdus spp. In Thailand. Doctural dissertation, Ph. D., Mahidol University. Bangkok. 2012.

15. Thanwisai A, Tandhavanant S, Saiprom N, Waterfield NR, Ke Long P, Bode HB, et al. Diversity of *Xenorhabdus* and *Photorhabdus* spp. and their symbiotic entomopathogenic nematodes from Thailand. PLoS One. 2012; 7(9):e43835. https://doi.org/10.1371/journal.pone.0043835 PMID: 22984446

16. Cimen H, Lee MM, Hatting J, Hazir S, Stock SP. *Steinernema innovationi* n. sp. (Panagrolaimomorpha: Steinernematidae), a new entomopathogenic nematode species from South Africa. J Helminthol. 2014; 3:1–4.

17. Cimen H, Lee MM, Hatting J, Hazir S, Stock SP. *Steinernema tophus* sp. n. (Nematoda: Steinernematidae), a new entomopathogenic nematode from South Africa. Zootaxa. 2014; 3821(3):337–353. https://doi.org/10.11646/zootaxa.3821.3.3 PMID: 24989748

18. Phan KL, Mrácek Z, Puza V, Nermut J, Jarosova A. *Steinernema huense* sp. n., a new entomopathogenic nematode (Nematoda: Steinernematidae) from Vietnam. Nematology. 2014; 16:761–775.

19. Nthenga I, Knoetze R, Berry S, Tiedt LR, Malan AP. *Steinernema sacchari* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. Nematology. 2014; 16(4):475–494.

20. Nikdel M, Niknam G, Ye W. *Steinernema arasbaranense* sp. n. (Nematoda: Steinernematidae), a new entomopathogenic nematode from arasbaran forests, Iran. Nematol Mediterr. 2011; 39:17–28.

21. Abate AB, Malan AP, Tiedt LR, Winghfield MJ, Slippers B, Hurley B. *Steinernema fabii* n. sp. (Rhabditida: *Steinernematidae*), a new entomopathogenic nematode from South Africa. Nematology. 2016; 18(2):235–255.

22. Cimen H, Půža V, Nermut J, Hatting J, Ramakuwel T, Hazir S. *Steinernema biddulphi* n. sp., a New Entomopathogenic Nematode (Nematoda: *Steinernematidae*) from South Africa. J Nematol. 2016; 48(3):148–158. PMID: 27765988

23. Cimen H, Půža V, Nermut J, Hatting J, Ramakuwel T, Hazir S. *Steinernema beitlechemi* n. sp., a new entomopathogenic nematode (Nematoda: Steinernematidae) from South Africa. J Nematol. 2016; 18(4):439–453.

24. Půža V, Nermut J, Maráčk Z, Gengler S, Haukeland S. *Steinernema pwaniensis* n. sp., a new entomopathogenic nematode (Nematoda: Steinernematidae) from Tanzania. J Helminthol. 2016; 91(1):20–34. https://doi.org/10.1017/S0022149X15001157 PMID: 26765335

25. de Bride AL, Rosa JMO, de Oliveira CMG, de Castro e Castro BM, Serrao JD, Zanuncio JC, Leite LG, Wilcken SRS. Entomopathogenic nematodes in agricultural areas in Brazil. Sci Rep. 2017; 7:45254. https://doi.org/10.1038/srep45254 PMID: 28382937

26. Li XY, Liu Q, Nermuť J, Půža V, Mráček Z. *Heterorhabditis beicherriana* n. sp. (Nematoda: Heterorhabditidae), a new entomopathogenic nematode from the Shunyi district of Beijing, China. Zootaxa. 2012; 3569:25–40.

27. Malan AP, Knoetze R, Tiedt L. *Heterorhabditis noenieputensis* n. sp. (Rhabditida: Heterorhabditidae), a new entomopathogenic nematode from South Africa. J Helminthol. 2014; 88(2):139–51. https://doi.org/10.1017/S0022149X12000806 PMID: 23232041

PLOS | ONE

Identification of EPNs and symbiotic bacteria and their larvicidal activity against *Aedes* spp.

28. Stock SP, Somsook V, Reid AP. *Steinernema siamkayai* n. sp. (Rhabditida: Steinernematidae), anento-mopathogenic nematode from Thailand. Syst Parasitol. 1998; 41(2):105–113.

29. Maneesakorn P, Grewal PS, Chandrapatya A. *Steinernema minutum* sp. nov. (Rhabditida: Steinerna-matidae): A new entomopathogenic nematode from Thailand. J Nematol. 2010; 42(4):281–291.

30. Maneesakorn P, An R, Grewal PS, Chandrapatya A. *Heterorhabditis somsookae* sp. novo (Rhabditida: Heterorhabditidae): a new entomopathogenic nematode from Thailand. Int J Nematol. 2015; ISSN:1368-8774.

31. Vitta A, Yimthin T, Fukruksa C, Wongpeera W, Yotpanya W, Polseela R, et al. Distribution of entomo-pathogenic nematodes in lower northern Thailand. Southeast Asian J Trop Med Public Health. 2015; 46 (4):564–573. PMID: 26867375

32. Vitta A, Fukruksa C, Yimthin T, Deelue K, Sarai C, Polseela R, Thanwisai A. Preliminary survey of ento-mopathogenic nematodes in upper northern Thailand. Southeast Asian J Trop Med Public Health. 2017; 48(1):18–26.

33. Muangpat P, Yooyangket T, Fukruksa C, Suwannaroj M, Yimthin T, Sitthisak S, Chantratita N, Vitta A, Thanwisai A. 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. Front Microbiol. 2017; 8:1142.

34. Tailliez P, Pages S, Ginibre N, Boemare N. New insight into diversity in the genus *Xenorhabdus*, includ-ing the description of ten novel species. Int J Syst Evol Microbiol. 2006; 56:2805–2818. https://doi.org/10.1099/ijs.0.64287-0 PMID: 17158981

35. Maneesakorn P, An R, daneshvar H, Taylor K, Bai X, Adam BJ, et al. Phylogenetic and cophylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae: Rhabditida) and their symbiotic bac-teria (*Photorhabdus*: Enterobacteriaceae). Mol Phylogenet Evol. 2011; 59(2):271–280. https://doi.org/10.1016/j.ympev.2011.02.012 PMID: 21335093

36. Glaeser SP, Tobias NJ, Thanwisai A, Chantratita N, Bode HB, Kämpfer P. *Photorhabdus luminescens* subsp. *namnaonensis* subsp. nov., isolated from *Heterorhabditis baujardi* nematodes in Nam Nao dis-trict of central Thailand. Int J Syst Evol Microbiol. 2016; 67:1046–1051.

37. Bedding RA, Akhurst RJ. A simple technique for the detection of insect parasitic rhabditid nematodes in soil. Nematologica. 1975; 21(1):109–110.

38. White GF. A method for obtaining infective nematode larvae from cultures. Science. 1927; 66 (1709):302–303.

39. Stock SP, Campbell JF, Nadler SA. Phylogeny of *Steinernema* Travassos 1927 (Cephalobina: Steiner-nematidae) inferred from ribosomal DNA sequences and morphological characters. J Parasitol Res. 2001; 87(4):877–889.

40. Hominick WM, Briscoe BR, del Pino FG, Heng J, Hunt DJ, Kozodoy E, et al. Biosystematics of entomo-pathogenic nematodes: Current status, protocols and definitions. J Helminthol. 1997; 71(4):271–298. PMID: 9443947

41. Tailliez P, Laroui C, Ginibre N, Paule A, Pages S, Boemare N. Phylogeny of *Photorhabdus* and *Xenor-habdus* based on universally conserved proteincoding sequences and implications for the taxonomy of these two genera. Int J Syst Evol Microbiol. 2010; 60(pt8):1921–1937.

42. Kumar S, Steche G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Big-ger Datasets. Mol Biol Evol. 2016; 33(7):1870–1874. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

43. Kung SP, Gaugler R, Kaya HK. Effects of soil temperature, moisture, and relative humidity on entomo-pathogenic nematode persistence. J Invertebr Pathol. 1991; 57:242–249.

44. Grant JA, Villani MG. Soil moisture effects on entomopathogenic nematodes. Environ Entomol. 2003; 32:80–87.

45. Yadav AK, Lalramliana. Evaluation of the efficacy of three indigenous strains of entomopathogenic nematodes from Meghalaya, India against mustard sawfly, Athalia lugens proxima Klug (Hymenoptera: Tenthredinidae). J Parasit Dis. 2012; 36(2):175–180. https://doi.org/10.1007/s12639-012-0099-y PMID: 24082523

46. Rohde C, Moino A Jr, Silva MA, Carvalho FD, Ferreira CS, 2010. Influence of soil temperature and moisture on the infectivity of entomopathogenic nematodes (Rhabditida: Heterorhabditidae, Steinerne-matidae) against larvae of *Ceratitis capitata* (Wiedeman) (Diptera: Tephritidae). Biol Control. 2010; 39:608–611.

47. Molyneux AS. Heterorhabditis spp. and Steinernema spp. temperature and aspects of behavior and infectivity. Exp Parasitol. 1986; 62(2):169–180.

48. Tangchitsomkid N, Sontirat S. Occurrence of Entomopathogenic Nematodes in Thailand. Kasetsart J (Nat. Sci.). 1998; 32:347–354.

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Identification of EPNs and symbiotic bacteria and their larvicidal activity against *Aedes* spp.

49. Lee MM, Stock SP. A multigene approach for assessing evolutionary relationships of *Xenorhabdus* spp. (γ-Proteobacteria), the bacterial symbionts of entomopathogenic *Steinernema* nematodes. J Invertebr Pathol. 2010; 104(2):67–74. https://doi.org/10.1016/j.jip.2010.01.005 PMID: 20102721

50. Ke LP, Nguyen CN, Moens M. *Steinernema sangi* Sp. n. (Rhabditida: *Steinernematidae*) from Vietnam. Russ. J. Nematol. 2001; 9(1):1–7.

51. Tailliez P, Laroui C, Ginibre N, Paule A, Pages S, Boemare N. 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. temperate* 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. Int J Syst Evol Microbiol. 2010; 60:1921–1937. https://doi.org/10.1099/ijs.0.014308-0 PMID: 19783607

52. Merritt RW, Dadd RH, Walker ED. Feeding behaviour, natural food, and nutritional relationships of larval mosquitoes. Annu Rev Entomol. 1992; 37:349–376. https://doi.org/10.1146/annurev.en.37.010192.002025 PMID: 1347208

53. Dunphy GB, Webster JM. Interaction of *Xenorhabdus nematophilus* subsp. *nematophilus* with the hemolymph of *Galleria mellonella*. J Insect Physiol. 1984; 30:883–889.

54. Eom S, Park Y, Kim Y. Sequential immunosuppressive activities of bacterial secondary metabolites from the entomopahogenic bacterium *Xenorhabdus nematophila*. J Microbiol. 2014; 529(2):161–168.

55. Namsena P, Bussaman P, Rattanasena P. Bioformulation of *Xenorhabdus stockiae* PB09 for controlling mushroom mite, *Luciaphorus perniciosus* Rack. Bioresour Bioprocess. 2016; 3(19):1–7.

56. Rodou A, Ankrah DO, Stathopoulos C. Toxin and secretion systems of *Photorhabdus luminescens*. Toxins. 2010; 2(6):1250–1264. https://doi.org/10.3390/toxins2061250 PMID: 22069636

57. Jallouli W, Zouari N, Jaoua S. Involvement of oxidative stress and growth at high cell density in the viable but nonculturable state of *Photorhabdus temperata* ssp. *temperata* strain K122. Process Biochem. 2010; 45:706–713.

58. Ffrench-Constant RH, Dowling A, Waterfield NR. Insecticidal toxin from *Photorhabdus* bacteria and their potential use in agriculture. Toxin. 2006; 49:436–451.

59. Forst S, Nealson K. Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. Microbiol Rev. 1996; 60:21–43. PMID: 8852894

60. Fukruksa C, Yimthin T, Suwannaroj M, Muangpat P, Tandhavanant S, Thanwisai A, et al. Isolation and identification of *Xenorhabdus* and *Photorhabdus* bacteria associated with entomopathogenic nematodes and their larvicidal activity against *Aedes aegypti*. Parasit Vectors. 2017; 10(1):440. https://doi.org/10.1186/s13071-017-2383-2 PMID: 28934970

61. Villabona-Arenas CJ, Zanotto PM. Worldwide spread of Dengue virus type 1. PLoS One. 2013;13; 8(5): e62649. https://doi.org/10.1371/journal.pone.0062649 PMID: 23675416

62. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyer CL, et al. The global distribution and burden of dengue. Nature. 2013; 496(7446):504–507. https://doi.org/10.1038/nature12060 PMID: 23563266

63. Benelli G, Mehlhorn H. Declining malaria, rising of dengue and Zika virus: insights for mosquito vector control. Parasitol Res. 2016; 115(5):1747–1754. https://doi.org/10.1007/s00436-016-4971-z PMID: 26932263

64. Mint Mohamed Lemine A, Ould Lemrabott MA, Hasni Ebou M, Mint Lekweiry K, Ould Ahmedou Salem MS, Ould Brahim K, et al. Mosquitoes (Diptera: Culicidae) in Mauritania: a review of their biodiversity, distribution and medical importance. Parasit Vectors. 2017; 10(1):35. https://doi.org/10.1186/s13071-017-1978-y PMID: 28103955

65. WHO [Internet]. Zika virus. c2016- [cited 2016 Dec 27]. http://www.who.int/mediacentre/factsheets/zika/en/

66. Silva JJ, Mendes J. Susceptibility of *Aedes aegypti* (L) to the insect growth regulator diflubenzuron and methoprene in Uberlândia, State of Minas Gerais. Rev Soc Bras Med Trop. 2007; 40(6):612–616. PMID: 18200410

67. Lucia A, Harburgue L, Licastro S, Zerba E, Masuh H. Efficacy of a new combined larvicidal-adulticidal ultralow volume formulation against *Aedes aegypti* (Diptera: Culicidae) vector of dengue. Parasitol Res. 2009; 104(5):1101–1107. https://doi.org/10.1007/s00436-008-1294-8 PMID: 19082625

68. Salokhe SG, Deshpands SG, Mukherjee SN. Evaluation of the insect growth regulator lufenuron (Match®) for control of *Aedes aegypti* by simulated field trials. Parasitol Res. 2012; 111(3):1325–1329. https://doi.org/10.1007/s00436-012-2968-9 PMID: 22638920

69. Naqqash MN, Gokce A, Bakhsh A, Salim M. Insecticide resistance and its molecular basis in urban insect pests. Parasitol Res. 2016; 115(4):1363–1373. https://doi.org/10.1007/s00436-015-4898-9 PMID: 26758450