RESEARCH ARTICLE

A study on Xenorhabdus and Photorhabdus isolates from Northeastern Thailand: Identification, antibacterial activity, and association with entomopathogenic nematode hosts

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Abstract

Xenorhabdus and Photorhabdus are gram negative bacteria that can produce several secondary metabolites, including antimicrobial compounds. They have a symbiotic association with entomopathogenic nematodes (EPNs). The aim of this study was to isolate and identify Xenorhabdus and Photorhabdus species and their associated nematode symbionts from Northeastern region of Thailand. We also evaluated the antibacterial activity of these symbiotic bacteria. The recovery rate of EPNs was 7.82% (113/1445). A total of 62 Xenorhabdus and 51 Photorhabdus strains were isolated from the EPNs. Based on recA sequencing and phylogeny, Xenorhabdus isolates were identified as X. stockiae (n = 60), X. indica (n = 1) and X. eapokensis (n = 1). Photorhabdus isolates were identified as P. luminescens subsp. akhurstii (n = 29), P. luminescens subsp. hainanensis (n = 18), P. luminescens subsp. laumondii (n = 2), and P. asymbiotica subsp. australis (n = 2). The EPNs based on 28S rDNA and internal transcribed spacer (ITS) analysis were identified as Steinernema surkhetense (n = 35), S. sangi (n = 1), unidentified Steinernema (n = 1), Heterorhabditis indica (n = 39), H. baujardi (n = 1), and Heterorhabditis sp. SGmg3 (n = 3). Antibacterial activity showed that X. stockiae (bMSK7.5_TH) extract inhibited several antibiotic-resistant bacterial strains. To the best of our knowledge, this is the first report on mutualistic association between P. luminescens subsp. laumondii and Heterorhabditis sp. SGmg3. This study could act as a platform for future studies focusing on the discovery of novel antimicrobial compounds from these bacterial isolates.
Introduction

Xenorhabdus and Photorhabdus are motile, gram-negative rods, facultative anaerobes, non-sporeforming, oxidase-negative, and chemoorganotrophic heterotrophs with respiratory and fermentative metabolism. These bacteria symbiotically inhabit the intestine of the infective juvenile (IJ) stage of entomopathogenic nematodes (EPNs) belonging to the Steinernematidae and Heterorhabditidae families [1]. The IJs of EPNs enter the digestive tract of the insect larvae, penetrate the hemocoel of the insect host, and release the bacteria into the hemolymph. Together, the IJs and bacteria rapidly kill the insect larvae within 24–48 h [2]. Otherwise, the nematodes or bacteria themselves make significant contributions to pathogenesis within the insect [3–5].

Xenorhabdus and Photorhabdus can produce several secondary metabolites, including insecticidal and antimicrobial compounds, such as benzylideneacetone, phenethylamines, indole, xenocoumacins, 3,5-dihydroxy-4-isopropylstilbene [6, 7], GameXPeptide, xenoamicin, xenocoumacin, mevalagmapeptide phurealipids derivatives, and isopropylstilbene [8]. Several studies on the bioactive compounds of Xenorhabdus and Photorhabdus against various microorganisms have demonstrated their antibacterial [9], antimicrobial [10], and antiparasitic effects [11].

Xenorhabdus and Photorhabdus have been isolated from across the world, including Europe, Australia, America, and Asia. Currently, 29 species of Xenorhabdus and 20 species of Photorhabdus [12] have been reported. Over 90 confirmed species of EPNs [13] have been described from a variety of ecological habitats throughout the world, except Antarctica [14]. In Thailand, six species of Xenorhabdus: X. stockiae, X. miraniensis, X. ehlersii, X. vietnamensis, X. indica, and X. japonica [15–18], and three species of Photorhabdus: P. luminescens, P. asymbiotica subsp. australis, and P. temperata subsp. temperata have been reported [8, 17, 19, 20]. Also, at least 11 species of EPNs have been reported from several regions of the country, including Steinernema siamkayai, S. surkhetense, S. websteri (synonym S. carpocapsae), S. scarabiae, S. kushidai, S. minutum, S. khoisanae, Heterorhabditis indica (synonym H. gerrardi), H. baujardi (synonym H. somsookae), H. bacteiohora, and H. zealandica [8, 15, 17, 18, 20–22]. However, there is limited information regarding EPNs and their symbiotic bacteria from Northeastern Thailand.

The objectives of this study to isolate and identify EPNs and their symbiotic bacteria Xenorhabdus and Photorhabdus from Northeastern Thailand; we also analyzed their phylogenetic diversity. The antibacterial activity of the extracts of the identified Xenorhabdus and Photorhabdus strains against antibiotic-resistant bacteria was also evaluated using the disk diffusion method, minimum inhibitory concentration (MIC), and minimal bactericidal concentration (MBC). This study will provide information at the molecular level that can assist in taxonomy of Xenorhabdus and Photorhabdus isolates, and their EPN hosts from Thailand. These bacteria may serve as a resource for discovery a novel bioactive compound.

Materials and methods

Collection of soil samples

A total of 1,445 soil samples from 289 soil sites were collected from nine provinces in Northeastern Thailand. All soil sites belonged to public areas and no specific permission was required. For each soil site, five soil samples were randomly taken from an area of approximately 10 m² and at a depth of 10–15 cm using a spade. Approximately 500 g of each soil sample was placed in a plastic bag. Site location, latitude, longitude and altitude, soil temperature, pH, and moisture were recorded. Soil samples were maintained at 25–30°C during
transportation to the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University.

**Isolation and identification of entomopathogenic nematodes**

The IJs of EPNs were isolated by the baiting technique as previously described [17]. For each soil sample, five larvae of *Galleria mellonella* (greater wax moth) were placed on top of the soil sample stored in a plastic container. Subsequently, the container was covered with a lid, and it was turned upside down to let the larvae move into the soil. It was incubated in dark at 30˚C for 5 days. The dead larvae of *G. mellonella* were collected from the soil samples and then larval cadavers were placed on a White trap [23] to allow the IJs to emerge. The IJs were collected in a tissue culture flask, cleaned with sterile distilled water, and stored at 15˚C.

EPNs were identified by polymerase chain reaction (PCR), which was performed in an Applied Biosystems thermal cycler (Life Technologies, Carlsbad, CA, USA), and sequencing of a partial region of 28S rDNA for *Steinernema* and the internal transcribe spacer (ITS) for *Heterorhabditis*. The primers used were as follows: 539_F (5’GGATTTCCCTAGTAACGTGAGTG-3’) and 535_R (5’-TAGTCTTTGCCGCCCTATACCCTT-3’) for *Steinernema*; 18S_F (5’-TGATTACGTCCCTGCCCTT-3’) and 26S_R (5’-TTTCACTCGCCCGTTACTAAGG-3’) or TW81_F (5’-GTTTCCGTAGGTGAACCTGC-3’) and AB28_R (5’-ATATGCTTAAGTTCACGGGT-3’) for *Heterorhabditis*. The PCR reagents and conditions were as described in a previous study [17]. The PCR products were checked on 1.2% agarose gel by electrophoresis.

**Isolation and identification of symbiotic bacteria**

The infected dead larvae of the greater wax moth were surfaced sterilized with 95% ethanol before dissection. The hemolymph was collected by a sterile loop, and then streaked onto a nutrient bromothymol blue agar (NBTA). The plate was incubated at 28˚C in dark for 4 days. The bacterial isolates (blue or green colonies) were selected and stored in LB broth containing 50% glycerol (v/v) at -80˚C.

The genomic DNA of 113 isolates of the symbiotic bacteria was extracted with the Genomic DNA Mini Kit (Blood/Cultured cell) (Geneaid Biotech Ltd., Taiwan) according to the manufacturer’s instructions. A partial sequence of recA gene was amplified from the genomic DNA by PCR using forward and reverse primers (5’-GCTATTGATGAAAATAAACA-3’ and 5’-RATTTTRTCWCCRTTRTAGCT-3’) to obtain an 890 bp amplicon (24). The PCR mixture (50μL) consisted of 10 μL of 5X buffer, 7 μL of 25 mM MgCl₂, 1 μL of 200 mM dNTPs, 2 μL of 5 mM of each primer, 0.5 μL of 5 unit Taq polymerase (Sigma, USA), 2.5 μL of DNA template, and 25 μL distilled water. PCR cycling parameters for the recA gene of *Xenorhabdus* were as follows: an initial denaturing step of 94˚C for 5 min, followed by 30 cycles of denaturation at 94˚C for 1 min, annealing at 50˚C for 1 min, and extension at 72˚C for 2 min, and a final extension at 72˚C for 7 min. PCR parameters for *Photorhabdus* were as follows: an initial denature step of 94˚C for 5 min, followed by 30 cycles of denaturation at 94˚C for 1 min, annealing temperature of 50˚C for 45 s and extension of 72˚C for 1.5 min, and a final extension at 72˚C for 7 min. The PCR products were checked on 1.2% agarose gel by electrophoresis and purified using the Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., Taiwan).

**PCR for 16S rDNA, gyrB, dnaN, gltX, and infB**

recA analysis revealed that one *Xenorhabdus* (KK9.1_TH) isolate had lower than 96% similarity in the BLASTN search; this isolate was selected for further analysis, and sequencing of its additional nucleotide regions, including 16S rDNA, gyrB, dnaN, gltX, and infB, was performed.
Primers and PCR reagents used for 16S rDNA, gyrB, dnaN, gltX, and infB were as previously described [24, 25]. PCR was performed in a Biometra TOne Thermal cycler (Analytik Jena AG, Jena, Germany). The PCR products were verified on 1.2% agarose gel by electrophoresis.

**Sequence and phylogenetic analysis**

The sequencing of the PCR products was done at Macrogen Inc. Service (Korea) (http://www.macrogen.com). The nucleotide sequences were edited and merged with the SeqMan™ II software (DNASTAR Inc., Wisconsin, USA). The recA sequences of the bacteria from the present study were deposited in the Genbank accession numbers KY809276 to KY809337, MT160765 to MT160768, and MT158222 for *Xenorhabdus* spp., and KY809338 to KY809388 for *Photorhabdus* spp. The 28S rDNA sequences of *Steinernema* isolates were deposited in the NCBI database under the Genbank accession numbers KY809389 to KY809425, and the ITS sequences of the *Heterorhabditis* isolates were deposited in NCBI database under the Genbank accession numbers KY809426 to KY809468.

The consensus sequences of each species were used for multiple sequence alignment using Clustal W [26] in the MEGA software version 6.0 [27]. Species identification was performed using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Similarity ≥ 97% was considered as the same species. The known nucleotide sequences of EPNs and their symbiotic bacteria in the NCBI database were downloaded and used as the reference species. For EPNs, maximum likelihood (ML) trees of the entire gene (28S rDNA, and ITS) were constructed based on Tamura 3-parameter with 1,000 bootstrap replicates model using MEGA 6.0 software [27]. For symbiotic bacteria, maximum likelihood (ML) trees of the entire gene (16S rDNA, recA, gyrB, dnaN, gltX, and infB) and the concatenation of truncated sequences of recA, gyrB, dnaN, gltX, and infB were constructed based on Tamura 3-parameter model using MEGA 6.0 software [27]. Also neighbor-joining trees (NJ) were constructed based on a Kimura 2-parameter with 1,000 bootstrap replicates using MEGA 6.0 software [27]. Bayesian analysis was performed based on Markov chain Monte Carlo method in MrBayes v3.2 [28].

**Preparation of antibiotic-resistant bacteria**

Fifteen strains of antibiotic-resistant bacteria, including *Acinetobacter baumannii* (four clinical strains), *Escherichia coli* (two clinical strains), *E. coli* ATCC35218, *Klebsiella pneumoniae* (two clinical strains), *K. pneumoniae* ATCC700603, *Enterococcus faecalis* ATCC51299, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (two clinical strains), and *S. aureus* ATCC20475, were used as the pathogens for testing the antibacterial activity of the extracts of the symbiotic bacteria. These bacteria were streaked on Mueller-Hinton agar (MHA) and incubated at 37°C for 24 h. A single colony was resuspended in 0.85% sodium chloride (NaCl), and the turbidity was adjusted to 0.5 McFarland standards. Then, 100 μL of the bacterial suspension was swabbed on MHA plate for disk diffusion test [29].

**Screening of Xenorhabdus and Photorhabdus isolates**

*Xenorhabdus* and *Photorhabdus* isolates were cultured on NBTA at 28°C in dark for four days. A single colony from each isolate was transferred to a 15-ml tube containing 5 mL of LB broth and incubated at room temperature for 48 h under shaking conditions. A paper disk (6 mm with diameter) with a 20 μL drop of the whole cell culture was placed on MHA plated with antibiotic-resistant bacteria. The plates were placed in an incubator at 37°C for 24 h. The inhibition zone (clear zone) was checked and measured (millimeter). The most effective isolates of *Xenorhabdus* and *Photorhabdus* were selected for crude compound extraction.
Bacterial extracts

A single colony of *Xenorhabdus* and *Photorhabdus* on NBTA medium was inoculated to 1000 mL flask containing 500 mL of LB. The culture flask was shaken at 180 rpm for 72 h. The bacteria cultured was added with 1000 mL ethyl acetate and mixed well. All solvents were removed from bacterial extracts by a rotary vacuum evaporator (Buchi, Flawil, Switzerland). Dimethyl sulfoxide (DMSO) was added to bacterial extracts to make a final concentration of 500 mg/mL and stored at -20˚C until used.

Disk diffusion method

Cultured drug resistant bacteria were spread on MHA agar. A sterile 6 mm disc was put onto MHA agar plate and then 10 μL of each bacterial extract was dropped onto a sterile disc. Negative control was DMSO and Positive control was antibiotic disks. The plates were incubated at 37˚C for 24 h. The inhibition zone was measured in millimeter. The most effective results of bacterial extracts were further evaluated by MIC and MBC.

MIC and MBC assays

Bacterial extracts were diluted in two-fold serial dilutions in a 96-well micro titer plate. The suspension of drug resistant bacteria (1 × 10⁸ cell/ml) was added into each well and mixed well. Cultured drug resistant bacteria, cultured drug resistant bacteria mixed with DMSO, and sterile Mueller-Hinton (MH) broth were used as controls. Plates were incubated at 37˚C for 24 h. No visible growth of drug resistant bacteria in the well was considered as MIC. In addition, 10 μL from each well from the MIC assay was dropped onto MHA plates. The plates were then incubated at 37˚C for 24 h. The lowest concentration of bacterial extract without growth of drug resistant bacteria was considered as MBC.

Results

Isolation of EPNs

A total of 1,445 soil samples from 289 sites were collected from the Northeastern region of Thailand, including Kalasin [30], Khon Kaen, Chaiyaphum, Nakhon Ratchasima, Maha Sarakham, Loei, Nong Khai, Nong Bua Lamphu, and Udon Thani provinces. The recovery rate of EPNs was 7.82% (113/1,445) of the total soil samples collected. We isolated 62 strains belonging to *Xenorhabdus* spp. and 51 strains belonging to *Photorhabdus* spp. from the EPNs (Table 1). Most of the soil samples were positive with only one of the two genera of EPNs (*Steinernema* spp. and *Heterorhabditis* spp.). In contrast, few soil samples (two samples from Maha Sarakham province and one sample from Nong Khai province) were positive with both *Steinernema* and *Heterorhabditis*. Most of the EPNs were isolated from loam, and the mean pH, temperature, and moisture of the soil samples were 6.6, 28.4˚C, and 1.5%, respectively (Table 2). These soil parameters were not significantly different between soil samples with and without EPNs (Mann-Whitney test).

Identification and phylogenetic tree of *Xenorhabdus* and *Photorhabdus* isolates

Sixty-two isolates belonging to the genus *Xenorhabdus* were identified using BLASTN search of partial sequence of the *recA* gene. Most *Xenorhabdus* isolates (n = 60) were identified as *X. stockiae* (97–100% identity). One isolate (bKK26.2_TH) was identified as *X. indica* (97% identity), but the remaining one isolate (bKK9.1_TH) was unidentified due to low similarity of its
recA sequence to that of X. thuongxuanensis (95% identity) and X. eapokensis (94% identity). To confirm the species of Xenorhabdus bKK9.1_TH, five additional genes (16S rDNA, gyrB, dnaN, gltX, and infB) were amplified and sequenced. Nucleotide sequences of four genes revealed high similarity to that of X. eapokensis: 16S rDNA and infB (99% identity), dnaN and gltX (98% identity). gyrB sequence of Xenorhabdus bKK9.1_TH showed low similarity with that of X. eapokensis (90%).

The ML tree derived from all the sequences of recA among the Thai Xenorhabdus isolates and reference strains from GenBank database is shown in Fig 1. The Thai Xenorhabdus isolates were distributed in three groups. Group 1 was the majority group (60 isolates), which was closely related to X. stockiae. Group 2 contained one isolate (bKK26.2_TH), which was closely related to X. indica. Group 3 also contained one isolate (bKK9.1_TH), which fell in the clade of X. thuongxuanensis, X. ishibashii, and X. eapokensis. The ML tree derived from 16S rRNA, gyrB, dnaN, gltX, and infB genes are shown in S1–S5 Figs. The ML tree of concatenation of the five truncated genes (recA, gyrB, dnaN, gltX, and infB) is shown in Fig 2. All phylogenetic trees supported that Xenorhabdus bKK9.1_TH was closely related to X. eapokensis.

Fifty-one isolates of Photorhabdus were identified using BLASTN search of partial sequences of the recA gene. Twenty-nine isolates were identified as P. luminescens subsp. akhurstii (97–100% identity) and 18 isolates were identified as P. luminescens subsp. hainanensis (98–100% identity). Two isolates were identified as P. asymbiotica subsp. australis (99–100% identity). The remaining two Photorhabdus isolates were identified as P. luminescens subsp. laumondii (98% identity). ML analysis of 51 recA sequences of Photorhabdus distributed the isolates in three groups. Group 1 contained 47 isolates closely related to P. luminescens subsp. akhurstii and P. luminescens subsp. Group 2 contained two Photorhabdus isolates, which were closely related to P. luminescens subsp. laumondii. Group 3 contained the remaining two isolates of Photorhabdus, which were most closely related to P. asymbiotica subsp. australis (Fig 3).

Table 1. Number of symbiotic bacteria isolated from soil samples from the Northeastern region of Thailand.

| Province          | No. of soil sites | No. of sampling sites with EPNs (%) | No. of soil samples | No. of soil samples with EPNs (%) |
|-------------------|------------------|-------------------------------------|---------------------|-----------------------------------|
|                   | Xenorhabdus      | Photorhabdus                        | Total               | Xenorhabdus                      | Photorhabdus                        | Total               |
| Kalasin           | 24               | 6                                   | 3                   | 9 (37.50%)                        | 9                                  | 3                   | 12 (10.00%)          |
| Khon Kaen         | 29               | 5                                   | 5                   | 10 (34.88%)                       | 5                                  | 6                   | 11 (7.58%)           |
| Chaiyaphum        | 60               | 9                                   | 8                   | 17 (28.33%)                       | 11                                 | 11                  | 22 (7.33%)           |
| Nakhon Ratchasima | 66               | 11                                  | 12                  | 23 (34.85%)                       | 17                                 | 14                  | 31 (9.39%)           |
| Maha Sarakham     | 26               | 3                                   | 6                   | 9 (34.62%)                        | 4                                  | 7                   | 11 (8.46%)           |
| Loei              | 22               | 0                                   | 2                   | 2 (9.09%)                         | 2                                  | 0                   | 2 (1.82%)            |
| Nong Khai         | 20               | 2                                   | 2                   | 4 (20.00%)                        | 3                                  | 2                   | 5 (5.00%)            |
| Nong Bua Lamphu   | 22               | 7                                   | 5                   | 12 (54.55%)                       | 7                                  | 5                   | 12 (10.91%)          |
| Udon Thani        | 20               | 3                                   | 3                   | 6 (30.00%)                        | 4                                  | 3                   | 7 (7.00%)            |
| **Total**         | **289**          | **48 (16.60%)**                     | **44 (15.22%)**     | **92 (31.82%)**                   | **62 (4.29%)**                     | **51 (3.53%)**       | **113 (7.82%)**      |

Table 2. pH, temperature, and moisture content of the soil samples (n = 1,445) in the presence and absence of EPNs.

| Soil parameter | Soil with EPNs (n = 113) | Soil without EPNs (n = 1,332) | P-value (Mann-Whitney test) |
|----------------|---------------------------|--------------------------------|-----------------------------|
| Range          | Mean                      | Range                          | Mean                        |                              |
| pH             | 5.2–7.0                   | 6.6                            | 3.4–7.5                     | 6.6                          | 0.2144                        |
| Temperature (°C)| 25–34                     | 28.4                           | 22–39                       | 28.6                         | 0.8401                        |
| Moisture (%)   | 0.0–8.0%                  | 1.5%                           | 0.0–8.0%                    | 1.7%                         | 0.7039                        |
Fig 1. Maximum likelihood tree of 62 Xenorhabdus isolates (black bold letter) based on recA gene (588 bp) compared with Xenorhabdus strains downloaded from GenBank. Escherichia coli was used as an out-group. Bootstrap values are reported out of 1000 replicates. The numbers shown above the branches are support values of Maximum likelihood/Neighbor-joining/Bayesian posterior probabilities for clades supported above the 50% level. The bar indicates 5% sequence divergence. The EPN species from which they were isolated are also shown. https://doi.org/10.1371/journal.pone.0255943.g001

Fig 2. Maximum likelihood tree of concatenated sequences of Xenorhabdus sp. (KK9.1_TH) (shown in bold letter) based on truncated recA (588 bp), gyrB (846 bp), dnaN (828 bp), gltX (1,057 bp), and infB (1,052 bp) compared with the sequences of Xenorhabdus strains from GenBank. P. luminescens subsp. luminescens is included as an out-group. Bootstrap values are reported out of 1000 replicates. The numbers shown above the branches are support values of Maximum likelihood/Neighbor-joining/Bayesian posterior probabilities for clades supported above the 50% level. The bar indicates 2% sequence divergence. https://doi.org/10.1371/journal.pone.0255943.g002
Xenorhabdus and Photorhabdus against antibiotic-resistant bacteria

P. luminescens subsp. hainanensis (FJ862004.1)  H. indica
bCP48.1_TH

P. luminescens subsp. akhurstii (FJ862005.1)  H. baujardi
bNSM32.4_TH

P. luminescens subsp. carboniana (KM357904.1)  H. indica

P. luminescens subsp. caribbeanensis (FJ862003.1)  H. indica

bNC11.5_TH

P. luminescens subsp. noaepeuteus (JG424881.1)  H. indica

bKK14.3_TH

P. luminescens subsp. laumondii (FJ861999.1)  H. indica

bKK23.2_TH

P. luminescens (KM357901.1)  H. indica
bKK23.2_TH

P. temperata subsp. cinerea (KF740654.1)  H. indica
bCP25.2_TH

P. temperata subsp. stackebrandii (KF740655.1)  H. indica
bNSM43.1_TH

P. temperata subsp. khanii (FJ862010.1)  H. indica
bUDT3.4_TH

Group 1 (47 isolates)

Group 2 (2 isolates)  Heterorhabditis sp. SGm3

Group 3 (2 isolates)  H. indica

P. temperata subsp. tasmaniens (FJ862008.1)  H. indica

Escherichia coli str. K-12 substr.MG1655
Identification and phylogenetic tree of entomopathogenic nematodes

A total of 113 EPNs were isolated from the soil samples. EPNs (80 isolates) were identified using BLASTN search of a partial sequence of 28S rDNA for Steinernema and internal transcribed spacer for Heterorhabditis. The remaining 33 isolates of EPNs were lost due to fungal contamination. Thirty-seven isolates were identified as Steinernema and the remaining 43 isolates were identified as Heterorhabditis. Steinernema (35 isolates) were identified as S. surkehtense (97–99% identity). One isolate was identified as S. sangi with 98% similarity. Species of the remaining one isolate Steinernema eKK26.2_TH was unidentified due to its low identity with S. abbas (90%).

The phylogenetic relationships among the Steinernema isolates and reference strains from GenBank database are shown in Fig 4. The ML analysis of the 37 sequences distributed them into three groups. Group 1 contained 35 isolates of Steinernema, which were closely related to S. surkehtense and S. anatoliense. Group 2 contained only one isolate, which was closely related to S. abbas. Group 3 contained one isolate of Steinernema, which was closely related to S. sangi.

For Heterorhabditis nematodes, 39 isolates were identified as H. indica (98–100% identity), one isolate was identified as H. baujardi (99% identity), and three isolates were identified as Heterorhabditis sp. SGmg3 (97–99% identity). ML analysis of the 43 sequences of Heterorhabditis distributed them into three groups (Fig 5). Group 1 was the majority group (39 isolates), which was closely related to the clade of H. indica. Group 2 contained only one isolate, which was closely related to H. baujardi. Group 3 contained three isolates, which were closely related to Heterorhabditis sp. SGmg3.

Maximum association was observed between X. stockiae and the nematode host S. surkehtense (35 isolates). A single isolate of X. indica was associated with Steinernema sp., and one isolate of Xenorhabdus sp. (bKK9.1_TH), closely related to X. eapokensis, was associated with S. sangi. In addition, 39 isolates of P. luminescens were associated with H. indica. A single isolate of P. luminescens subsp. akhurstii was associated with H. baujardi. Two isolates of P. asymbiotica subsp. australis were associated with H. indica. A single isolate of P. luminescens subsp. luamondii was associated with Heterorhabditis sp. SGmg3.

Antibacterial activity

We found that whole cell extracts of four (X. stockiae, n = 3 and X. indica, n = 1) out of 113 isolates could inhibit the growth of at least one antibiotic-resistant bacterial strain. The extract from these bacterial isolates was tested against the antibiotic-resistant bacteria by disk diffusion method. Two isolates of X. stockiae (bMSK7.5_TH and bKS8.5_TH) and one isolate of X. indica (bKK26.2_TH) showed potential inhibition of the growth of the antibiotic-resistant bacteria (Table 3). X. stockiae (bMSK7.5_TH) could inhibit A. baumannii strain AB320 (extensively drug resistant; XDR), A. baumannii strain AB321, AB322 (multi drug resistant; MDR), A. baumannii strain AB324 (XDR), S. aureus ATCC20475, S. aureus strain PB36 (methicillin resistance Staphylococcus aureus; MRSA), E. coli ATCC35218, E. coli strain PB1 (extended spectrum beta-lactamase; ESBL and MDR), E. coli strain PB231 (ESBL and carbapenem-resistant Enterobacteriaceae; CRE), P. aeruginosa strain PB30 (MDR), K. pneumoniae ATCC700603, K. pneumoniae strain PB5 (ESBL and MDR), and K. pneumoniae strain PB21.
(ESBL and CRE). *X. stockiae* (bKS8.5_TH) and *X. indica* (bKK26.2_TH) could inhibit *S. aureus* strain PB36 (MRSA). However, *X. stockiae* (bUDT18.2_TH) was unable to inhibit any antibiotic-resistant bacteria by the disk diffusion method.

We also evaluated the MIC and MBC of *X. stockiae* (bMSK7.5_TH) extract against 13 antibiotic-resistant bacterial strains, including *A. baumannii* strain AB320 (XDR), *A. baumannii* strain AB321, AB322 (MDR), *A. baumannii* strain AB324 (XDR), *S. aureus* ATCC20475, *S. aureus* strain PB36 (MRSA), *E. coli* ATCC35218, *E. coli* strain PB1 (ESBL and MDR), *E. coli* strain PB231 (ESBL and CRE), *P. aeruginosa* strain PB30 (MDR), *K. pneumoniae* ATCC700603, *K. pneumoniae* strain PB5 (ESBL and MDR), and *K. pneumoniae* strain PB21 (ESBL and CRE). MIC and MBC of *X. stockiae* (bMSK7.5_TH) extract against these antibiotic-resistant bacteria were 3.90 mg/mL and 7.81 mg/mL, respectively, whereas *X. stockiae* (bKS8.5_TH) and *X. indica* (bKK26.2_TH) showed potential efficacy only against *S. aureus* strain PB36 (MRSA). MIC and MBC of *X. stockiae* (bKS8.5_TH) and *X. indica* (bKK26.2_TH) extracts against *S. aureus* strain PB36 were 62.5 mg/mL and 15.62 mg/mL, respectively (Table 4).

**Discussion**

The overall recovery rate of the EPNs (*Steinernema* and *Heterorhabditis*) from soil samples of Northeastern region of Thailand was 7.82%. This result was similar to that reported by Brodie [31] from Fiji Islands (7.3%), Valadas [32] from Portugal (6.7%), and Hatting [33] from South Africa (5%). However, this rate was higher than those reported by Caoili [34] from the Philippines (2.5%), Majić [35] from Croatia (2.0%), and Noujem [36] from Lebanon (1%). Higher prevalence of EPNs in soil from that determined in the present study was observed by Kanga [37] from Southern Cameroon (10.4%), Khatri-Chhetri [38] in Nepal (10.5%), and Malan [39] in South Africa (17%). This suggests that global prevalence of EPNs is variable. Distribution of *Steinernema* and *Heterorhabditis* has been reported from several ecological niches in USA, Australia, Europe, and Asia, including Thailand [8, 15–18, 24, 40, 41]. Biotic and abiotic characteristics influence the distribution of the EPNs; however, in our study, soil temperature, moisture, and pH of the soil samples with and without EPNs were not significantly different. Nevertheless, our data supported previous reports from Thailand, which showed that EPNs were able to survive in a diverse soil environment and various soil types with a wide range of pH (3.2–6.9), temperature (20°C–32°C), and moisture (0–8%) [17, 18, 22, 23]. Soil moisture, temperature, and rainfall affect the distribution of the insects that could be probable hosts for the EPNs [42]. This could also affect the distribution of EPNs.

Identification and phylogenetic analysis of 62 *Xenorhabdus* isolates revealed that *X. stockiae* was the predominant species. This bacterium was hosted by *S. surkhetense*, which has been previously described from India [43]. *X. stockiae* has also been isolated from *S. siamkayai* and *S. minutum* in Thailand [16, 24, 44, 45]. It was reported as a bacterial symbiont with *S. huense* in Vietnam [46]. One isolate of *X. indica* was found to be associated with *Steinernema* sp. (90% similar with *S. abbsi*). *X. indica* was first reported to be associated with *S. thermophilum* [47]. Subsequently, the association between *X. indica* and *S. abbsi* was reported from Taiwan [48]. In a previous study, *X. indica* was associated with *S. yirgalemense* [49], and in the current
Therefore, we identified reported to produce isopropylstilbene [44, 60], which has multiple biological activities, including antibiotic activity against 10 antibiotic-resistant bacterial strains, and all inhibition. In contrast, higher similarity of this isolate was found with X. eapokensis when 16S rDNA and infB (99% identity), and gltX and dnaN (98% identity) sequences were analyzed. In addition, multilocus sequence analysis (MLSA) based on concatenated partial gene sequences of recA, gyrB, dnaN, gltX, and infB revealed that Xenorhabdus (bKK9.1_TH) was closely related to X. eapokensis. Therefore, we identified Xenorhabdus (bKK9.1_TH) as X. eapokensis. This suggests that analysis of multiple genes may aid in the identification of this bacterium. Also, whole genome sequencing of this bacterium may assist in confirmation of its identity at the species level. We found that X. eapokensis was associated with S. safricana, which has been reported as a host for X. vietnamensis and X. thuongxuanensis [25, 51].

For the genus Photorhabdus, in the current study, the following four species were identified: P. luminescens subsp. akhurstii (n = 29), P. luminescens subsp. hainanensis (18 isolates), P. luminescens subsp. luamondii (n = 2), and P. asymbiotica subsp. australis (n = 2). P. luminescens subsp. akhurstii and P. luminescens subsp. hainanensis were associated with H. indica and H. sp. SGmg3. These associations have been previously reported from Thailand [15, 17, 18]. In addition, P. luminescens subsp. hainanensis has also been isolated from H. baujardi in Thailand [8]; however, P. luminescens subsp. akhurstii has been found in association with H. bacteriophora in Iran, Hungary, Argentina, USA, and in association with H. indica in China [19]. To the best of our knowledge, this is the first report on mutualistic association between P. luminescens subsp. luamondii and Heterorhabditis sp. SGmg3. However, P. luminescens subsp. luamondii has also been reported to be associated with H. bacteriophora in Thailand, USA, and Argentina [17, 52] and with H. safricana from South Africa [53]. P. asymbiotica subsp. australis was also found in the present study, which was in association with H. indica. This association has been found in Thailand previously [17, 54]. P. asymbiotica is an emerging pathogen that has been reported to cause locally invasive soft tissue infection and disseminated bacteremia; clinical cases have been identified in both Australia and USA [55, 56]. This suggests that P. asymbiotica could also cause these diseases in the residents of Thailand. Although no clinical case of P. asymbiotica infection has been reported in the country, management and healthcare strategies should be prepared in advance.

In the current study, X. stockiae (bMSK7.5_TH) extract showed the highest inhibitory effect against several antibiotic-resistant bacteria. Previous studies have shown that Xenorhabdus produces xenocoumacin [57] and amicoumacin derivatives [58], which are potent against S. aureus [59]. All Photorhabdus extracts from Mae Wong national park could inhibit S. aureus ATCC20475, S. aureus strain PB36 (MRSA), and S. aureus strain PB57 (MRSA) (8). In addition, P. luminescens subsp. akhurstii (bSBR11.1_TH) extract from Saraburi province could inhibit up to 10 antibiotic-resistant bacterial strains, and all Photorhabdus isolates showed the potential to inhibit the growth of S. aureus strain PB36 (MRSA) [41]. P. luminescens has been reported to produce isopropylstilbene [44, 60], which has multiple biological activities, including antibiotic activity against E. coli, B. subtilis, S. pyogenes, and S. aureus RN4220 (drug resistant and clinical isolate) [61, 62]. The bio-activity of isopropylstilbene has been extended to...
Table 3. Antibacterial activity of *Xenorhabdus* and *Photorhabdus* extracts against antibiotic-resistant bacteria as assessed by disk diffusion.

| Bacteria (code) | Inhibit the growth of drug resistant bacteria |
|-----------------|-----------------------------------------------|
|                 | A. baumannii strain AB320 (XDR)\(^a\) | A. baumannii strain AB321 (MDR)\(^b\) | A. baumannii strain AB322 (XDR)\(^c\) | A. baumannii strain AB324 (XDR)\(^d\) | S. aureus ATCC20475 | S. aureus strain PB56 (MRSA)** | S. aureus strain PB57 (MRSA)** | E. coli ATCC35218 | E. coli strain PB1 (ESBL and MDR)** | E. coli strain PB313 (ESBL and CRE)** | P. aeruginosa strain PB50 (MDR)** | E. faecalis ATCC515299 | K. pneumoniae ATCC700603 | K. pneumoniae strain PB5 (ESBL and MDR)** | K. pneumoniae strain PB21 (ESBL and CRE)** |
| X. stockiae (bMSK7.5_TH) | + | + | ++ | + | ++ | ++ | - | + | + | + | + | - | + | + | + | + |
| X. stockiae (bKS8.5_TH) | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| X. stockiae (bKDT18.2_TH) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| X. indica (bKK26.2_TH) | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |

- No activity (6 mm), + weak inhibition (7–10 mm.), ++ moderate/average inhibition (11–15 mm.)

\(^a\)extensively drug resistant
\(^b\)multidrug resistant
\(^c\)methicillin resistance *Staphylococcus aureus*, and
\(^d\)extended spectrum beta-lactamase, Carbapenem-resistant *Enterobacteriaceae*

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inhibit the growth of fungi [10]. We found that the bioactivity (MIC and MBC) of the crude extracts emphasized that the *X. stockiae* (bMSK7.5_TH) extracts were active against both gram-positive and gram-negative bacteria. The MIC values exhibited by all the extracts in this study ranged between 3.90–62.5 mg/mL, and the MBC ranged between 7.81–15.62 mg/ml. This may be due to the ability of each symbiotic bacterial isolate to produce different effective metabolites to kill drug resistant bacteria. This suggests that *Xenorhabdus* and *Photorhabdus* isolates are potential agents for the inhibition of the growth of MDR bacteria. Therefore, both *Xenorhabdus* and *Photorhabdus* isolates are a potential source for novel antibiotics.

**Conclusions**

In summary, 113 isolates of EPNs were obtained from a total of 1,445 soil samples collected from 289 sites in Northeastern region of Thailand. *S. surkhetense* and *H. indica* were the two most common EPN species found in the soil samples. For symbiotic bacteria, *X. stockiae*, *X. indica*, *X. eapokensis*, *P. luminescens* subsp. *akhurstii*, *P. luminescens* subsp. *hainanensis*, and *P. asymbiotica* subsp. *australis* were found in the studied area, and *X. stockiae* and *P. luminescens* subsp. *akhurstii* were found to be predominant. The common associations observed between EPN hosts and their symbiotic bacteria were *S. surkhetense*-*X. stockiae* and *H. indica*-*P. luminescens*. EPN host of *X. eapokensis* was *S. angi* and that of *X. indica* was unidentified *Steiner- nema*. In addition, the crude extract from *X. stockiae* (bMSK7.5_TH) showed a broad-spectrum inhibitory activity against several antibiotic-resistant bacterial strains. Thus, this study will be useful in further drug discovery from natural resources.

**Supporting information**

S1 Fig. Maximum likelihood phylogenetic tree of *Xenorhabdus* (KK9.1_TH) based on a partial 16S rDNA sequence (1,401 bp) compared with *Xenorhabdus* strains downloaded from GenBank. *P. luminescens* subsp. *luminescens* was used as an out-group. Bootstrap values are reported out of 1000 replicates. The numbers shown above the branches are support values of Maximum likelihood/Neighbor-joining/Bayesian posterior probabilities for clades supported above the 50% level. The bar indicates 5% sequence divergence.

(DOCX)

S2 Fig. Maximum likelihood phylogenetic tree of *Xenorhabdus* (KK9.1_TH) based on a partial *gyrB* sequence (846 bp) compared with *Xenorhabdus* strains downloaded from GenBank. *P. luminescens* subsp. *luminescens* was used as an out-group. Bootstrap values are reported out of 1000 replicates. The numbers shown above the branches are support values of Maximum likelihood/Neighbor-joining/Bayesian posterior probabilities for clades supported above the 50% level. The bar indicates 5% sequence divergence.

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Table 4. Antibacterial activity of *Xenorhabdus* extracts against antibiotic-resistant bacteria as assessed by minimum inhibitory concentration and minimal bactericidal concentration.

| Bacterial list (Code) | Concentration of inhibition (mg/mL) |
|-----------------------|-------------------------------------|
|                       | *S. aureus* strain PB36 (MRSA)       |
|                       | *K. pneumoniae* strain PB5 (ESBL+MDR) |
|                       | *A. baumannii* strain AB320 (XDR)    |
|                       | *P. aeruginosa* strain PB30 (MDR)    |
|                       | *E. coli* strain PB1 (ESBL+MDR)      |
|                       | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** |
| *X. stockiae* (bMSK7.5_TH) | 3.9 | 7.81 | 3.9 | 7.81 | 3.9 | 7.81 | 3.9 | 7.81 | 3.9 | 7.81 |
| *X. stockiae* (bKS8.5_TH) | 62.5 | 62.5 | - | - | - | - | - | - | - | - |
| *X. indica* (bKK26.2_TH) | 15.62 | 15.62 | - | - | - | - | - | - | - | - |

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above the 50% level. The bar indicates 2% sequence divergence.

S3 Fig. Maximum likelihood phylogenetic tree of *Xenorhabdus* (KK9.1_TH) based on a partial *dnaN* sequence (828 bp) compared with *Xenorhabdus* strains downloaded from GenBank. *P. luminescens* subsp. *luminescens* was used as an out-group. Bootstrap values are reported out of 1000 replicates. The numbers shown above the branches are support values of Maximum likelihood/Neighbor-joining/Bayesian posterior probabilities for clades supported above the 50% level. The bar indicates 5% sequence divergence.

S4 Fig. Maximum likelihood phylogenetic tree of *Xenorhabdus* (KK9.1_TH) based on a partial *gltX* sequence (1,057 bp) compared with *Xenorhabdus* strains downloaded from GenBank. *P. luminescens* subsp. *luminescens* was used as an out-group. Bootstrap values are reported out of 1000 replicates. The numbers shown above the branches are support values of Maximum likelihood/Neighbor-joining/Bayesian posterior probabilities for clades supported above the 50% level. The bar indicates 2% sequence divergence.

S5 Fig. Maximum likelihood phylogenetic tree of *Xenorhabdus* (KK9.1_TH) based on a partial *infB* sequence (1,052 bp) compared with *Xenorhabdus* strains downloaded from GenBank. *P. luminescens* subsp. *luminescens* was used as an out-group. Bootstrap values are reported out of 1000 replicates. The numbers shown above the branches are support values of Maximum likelihood/Neighbor-joining/Bayesian posterior probabilities for clades supported above the 50% level. The bar indicates 2% sequence divergence.

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References

1. Akhurst RJ. Neoapectana species: specificity of association with bacteria of the genus Xenorhabdus. Exp Parasitol. 1983; 55(2):258–63. https://doi.org/10.1016/0014-4894(83)90020-6 PMID: 6832284

2. Poinar GO Jr, Thomas GM. Significance of Achromobacter nematophilus Poinar and Thomas (Achromobacteriaceae: Eubacteriales) in the development of the nematode, DD-136 (Neoapectana sp. Steinernematidae). Parasitology. 1966; 56(2):385–90. https://doi.org/10.1017/s0031182000070980 PMID: 4960247

3. Lewis EE, Clarke DJ. Nematode parasites and entomopathogens. In: Vega FE, Kaya HK, editors. Insect Pathology. 2nd ed. Elsevier; 2012. p. 395–424

4. Lu D, Macchietto M, Chang D, Barros MM, Baldwin J, Mortazavi A, et al. Activated entomopathogenic nematode infective juveniles release lethal venom proteins. PLoS Pathog. 2017; 13(4):e1006302. https://doi.org/10.1371/journal.ppat.1006302 PMID: 28426766

5. Chang DZ, Serra L, Lu D, Mortazavi A, Dillman AR. A core set of venom proteins is released by entomopathogenic nematodes in the genus Steinernema. PLoS Pathog. 2018; 15(5):e1007626. https://doi.org/10.1371/journal.ppat.1007626 PMID: 31042778

6. Eleftherianos I, Boundy S, Joyce SA, Aslam S, Marshall JW, Cox RJ, et al. An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. Proc Natl Acad Sci U S A. 2007; 104(7):2419–24. https://doi.org/10.1073/pnas.0610525104 PMID: 17294598

7. McInerney BV, Taylor WC, Lacey MJ, Akhurst RJ, Gregson RP. Biologically active metabolites from Xenorhabdus spp., Part 2. Benzopyran-1-one derivatives with gastrophoric activity. J Nat Prod. 1991; 54(3):785–95. https://doi.org/10.1021/np50075a006 PMID: 1955881

8. Muangpat P, Yotuyangk T, Fukraka C, Suwanna M, Yimthin T, Srithnak S, et al. Screening of the antimicrobial activity of Xenorhabdus spp. against antibiotic-resistant bacteria of Photorhabdus and Xenorhabdus. J Nat Prod. 2014; 77(4):779–83. https://doi.org/10.1021/np4007525 PMID: 24673206

9. Akhurst RJ. Antibiotic activity of Xenorhabdus spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. J Gen Microbiol. 1982; 128(12):3061–5. https://doi.org/10.1099/00221287-128-12-3061 PMID: 7183749

10. Grundmann F, Kaiser M, Schiell M, Batzer A, Kurz M, Thanwisai A, et al. Antiparasitic chalophamine from entomopathogenic Xenorhabdus spp. PhB61.4. J Nat Prod. 2014; 77(4):779–83. https://doi.org/10.1021/np4007525 PMID: 24673206

11. Gen M. Antimicrobial Activity of the Nematode Symbionts , Xenorhabdus spp. and Their Symbiotic Entomopathogenic Nematodes from Thailand. Front Microbiol. 2017; 8:1142. https://doi.org/10.3389/fmicb.2017.01142 PMID: 28702004

12. Euzéby JP. List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. Int J Syst Bacteriol. 1997 Apr; 47(2):590–2. https://doi.org/10.1099/00221287-47-2-590 [cited 2021 July 23]. Available from: http://www.bacterio.cict.fr/ PMID: 9103655

13. Hominick WM. Biogeography. In: Gaugler R, editor. Entomopathogenic nematode taxonomy and phylogeny. Nematology Monographs and Perspectives 12: Brill; 2016. p. 1–11

14. Hominick WM. Biogeography. In: Gaugler R, editor. Entomopathogenic nematode taxonomy and phylogeny. Nematology Monographs and Perspectives 12: Brill; 2002. p. 115–44.

15. Fukruna C, Yimthin T, Suwanna 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

16. Tailliez P, Pages S, Ginibre N, Boemare N. New insight into diversity in the genus Xenorhabdus, including 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

17. Thanwisai A, Tandhavanant S, Sajipon N, Waterfield NR, Long PK, Bode HB, et al. Diversity of Xenorhabdus and Photorhabdus spp. and Their Symbiotic Entomopathogenic Nematodes from Thailand. PLoS ONE. 2012; 7: e43835. https://doi.org/10.1371/journal.pone.0043835 PMID: 22984446

18. Yotuyangk T, Muangpat P, Polseela R, Tandhavanant S, Thanwisai A, Vitta A. 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. 2018; 13(4): e0195681. https://doi.org/10.1371/journal.pone.0195681 PMID: 29641570

19. Maneesakom P, An R, Daneshvar H, Taylor K, Bai X, Adams BJ, et al. Phylogenetic and cophylogenetic relationships of entomopathogenic nematodes (Heterorhabditis: Rhhabditida) and their symbiotic bacteria (Photorhabdus: Enterobacteriaceae). Mol Phylogenet Evol. 2011; 59(2):271–80. https://doi.org/10.1016/j.ympev.2011.02.012 PMID: 21335093
20. Hunt DJ, Subbotina SA. Taxonomy and Systematics. In: Hunt DJ, Nguyen KB, editors. Advances in entomopathogenic nematode taxonomy and phylogeny. Nematology Monographs and Perspectives 12: Brill; 2016. p. 13–58.

21. Stock SP, Somsok V, Reid AP. Steinerema siamkayai n. sp. (Rhabditida: Steinernematidae), an entomopathogenic nematode from Thailand. Systematic Parasitology. 1998; 41(2):105–13. https://doi.org/10.1023/A:1006078171957

22. Vitta A, Fukrukusa C, Yimthin T, Deelues K, Sarai C, Poliseelra R, et al. Preliminary Survey of Entomopathogenic Nematodes in Upper Northern Thailand. Southeast Asian J Trop Med Public Health. 2017; 48(1):18–26. PMID: 29644816.

23. White GF. A Method for Obtaining Infective Nematode Larvae from Cultures. Science. 1927; 66(1709); 302–3. https://doi.org/10.1126/science.66.1709.302-a PMID: 17749713

24. Tailliez P, Larouci 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. viennensis 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. Int J Syst Evol Microbiol. 2010; 60: 1921–37. https://doi.org/10.1099/ijs.0.014308-0 PMID: 19783607

25. Kamps P, Tobias NJ, Ke LP, Bode HB, Glaesser SP. Xenorhabdus thuongxiuensis sp. nov. and Xenorhabdus eapokensis sp. nov., isolated from Steinernema species. Int J Syst Evol Microbiol. 2017; 67(5):1107–14. https://doi.org/10.1099/ijs.0.001770 PMID: 28906225

26. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994; 22(22):4673–80. https://doi.org/10.1093/nar/22.22.4673 PMID: 7984417

27. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013; 30(12):2725–30. https://doi.org/10.1093/molbev/mst197 PMID: 24132122

28. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, et al. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 2012; 61(3):539–42. https://doi.org/10.1093/sysbio/sys029 PMID: 22357727

29. Seier-Petersen MA, Jasni A, Aarestrup FM, Vigne H, Mullaney P, Roberts AP, et al. Effect of subinhibitory concentrations of four commonly used biocides on the conjugative transfer of Tn916 in Bacillus subtilis. J Antimicrob Chemother. 2014; 69(2):343–8. https://doi.org/10.1093/jac/dkt370 PMID: 24092655

30. Yimthin T, Fukrukusa C, Loetjaratkuntaworn, N, Phumdoing K, Vitta A, Thanwisai A. Identification of Xenorhabdus spp. and Photorhabdus spp. isolated from Entomopathogenic nematodes of Kalasin province, Thailand. M. Sc. Thesis, Naresuan University. 2014.

31. Brodie G. Natural occurrence and distribution of entomopathogenic nematodes (Steinernematidae, Heterorhabditidae) in Viti Levu, Fiji Islands. J Nematol. 2020; 52; 1–17. https://doi.org/10.21307/jofnem-2020-017 PMID: 32191017

32. Valadas V, Laranjo M, Mota M, Oliveira S, A survey of entomopathogenic nematode species in continental Portugal. J Helminthol. 2014; 88(3):327–41. https://doi.org/10.1017/S0022149X13000217

33. Hatting J, Patricia Stock S, Hazir S. Diversity and distribution of entomopathogenic nematodes (Steinernematidae, Heterorhabditidae) in South Africa. J Invertebr Pathol. 2009; 102(2):120–8. https://doi.org/10.1016/j.jip.2009.07.003 PMID: 19615373

34. Caolli BL, Latina RA, Sandoval RFC, Orajay JL. Molecular Identification of Entomopathogenic Nematode Isolates from the Philippines and their Biological Control Potential Against Lepidopteran Pests of Corn. J Nematol. 2018; 50(2):99–110. https://doi.org/10.21307/jofnem-2018-024 PMID: 30451431

35. Majic I, Sarajlic A, Lakatos T, Toth T, Rasputic E, Zebec V, et al. First Report of Entomopathogenic Nematode Steinernema Feltiae (Rhabditida: Steinernematidae) from Croatia. Helminthologia. 2018; 55(3):256–60. https://doi.org/10.2478/helm-2018-0024 PMID: 31662653

36. Noujeim E, Khater C, Pages S, Ogier JC, Tailliez P, Hamze M, et al. The first record of entomopathogenic nematodes (Rhabditidae: Steinernematidae and Heterorhabditidae) in natural ecosystems in Lebanon: A biogeographic approach in the Mediterranean region. J Invertebr Pathol. 2011; 107(1):82–5. https://doi.org/10.1016/j.jip.2011.01.004 PMID: 21241704

37. Kanga FN, Waeyenberge L, Hauser S, Moens M. Distribution of entomopathogenic nematodes in Southern Cameroon. J Invertebr Pathol. 2012; 109(1):41–51. https://doi.org/10.1016/j.jip.2011.09.008 PMID: 21983478
38. Khatri-Chhetri HB, Waeyenberge L, Manandhar HK, Moens M. Natural occurrence and distribution of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) in Nepal. J Invertebr Pathol. 2010; 103(1):74–8. https://doi.org/10.1016/j.jip.2009.10.007 PMID: 19836397

39. Malan AP, Knoetze R, Moore SD. Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth. J Invertebr Pathol. 2011; 108(2):115–25. https://doi.org/10.1016/j.jip.2011.07.006 PMID: 21839086

40. Hominick WM, Reid AP, Bohan DA, Briscoe BR. Entomopathogenic Nematodes: Biodiversity, Geographical Distribution and the Convention on Biological Diversity. Biocontrol Science and Technology. 1996; 6(3):317–32. https://doi.org/10.1080/09583159631307107

41. Muangpat P, Suwannaraj M, Yimthirin T, Fukruksa C, Sithisak S, Chantraklin N, et al. Antibacterial activity of *Xenorhabdus and Photorhabdus* isolated from entomopathogenic nematodes against antibiotic-resistant bacteria. *PLoS* One. 2020; 15(6):e0234129. https://doi.org/10.1371/journal.pone.0234129 PMID: 32502188

42. García del Pino F, Palomo A. Natural Occurrence of Entomopathogenic Nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Spanish Soils. *J Invertebr Pathol*. 1996; 68(1):84–90. https://doi.org/10.1006/jipp.1996.0062 PMID: 8812576

43. Bhat AH, Istkhar, Chaubey AK, Puza V, San-Blas E. First Report and Comparative Study of *Steinernema surkhetense* (Rhabditida: Steinernematidae) and its Symbiont Bacteria from Subcontinental India. *J Nematol*. 2017; 49(1):92–102. https://doi.org/10.21307/jofnem-2017-049 PMID: 28512381

44. Buscato E, Buttner D, Bruggerhoff A, Klingler FM, Weber J, Scholz B, et al. From a multipotent stilbene to soluble epoxide hydrolase inhibitors with antiproliferative properties. *ChemMedChem*. 2013; 8(6):919–23. https://doi.org/10.1002/cmdc.201300057 PMID: 23596124

45. Maneesakorn P, Grewal PS, Chandrapaty A. *Steinernema minutum* sp. nov. (Rhabditida: Steinernematidae): a new entomopathogenic nematode from Thailand. International Journal of Nematology. 2010; 20(1):27–42.

46. Phan KL, Mráček Z, Půža V, Nermut J, Jarosová A. *Steinernema huense* sp. n., a new entomopathogenic nematode (Nematoda: Steinernematidae) isolated from Vietnam. *J Nematol*. 2014; 16: 761–775. https://doi.org/10.1163/15685411-00002806

47. Somvanshi VS, Lang E, Ganguly S, Swiderski J, Saxena AK, Stackebrandt E. A novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus indica* sp. nov., symbiotically associated with entomopathogenic nematode *Steinernema thermophilum* Ganguly and Singh, 2000. *Syst Appl Microbiol*. 2006; 29(7):519–25. https://doi.org/10.1016/j.syapm.2006.01.004 PMID: 16459045

48. Tsai MH, Tang LC, Hou RF. The bacterium associated with the entomopathogenic nematode *Steinernema abbasii* (Nematoda: Steinernematidae) isolated from Taiwan. *J Invertebr Pathol*. 2008; 99(2):242–5. https://doi.org/10.1016/j.jip.2008.04.002 PMID: 18486948

49. Ferreira T, van Reenen CA, Talillez P, Pages S, Malan AP, Dicks LM. First report of the symbiotic bacterium *Xenorhabdus indica* associated with the entomopathogenic nematode *Steinernema yirgalemense*. *J Helminthol*. 2016; 90(1):108–12. https://doi.org/10.1017/S0022149X14000583 PMID: 25119819

50. Bhat AH, Askary TH, Ahmad MJ, Suman, Aasha, Chaubey AK. Description of *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae) isolated from hilly areas of Kashmir Valley. *Egyptian Journal of Biological Pest Control* volume. 2019; 29(1). https://doi.org/10.1186/s41938-019-0197-6

51. Lailramngkhai HC, Vanlahlimpuija, Vanramlima, Lalramlima. Characterization of a new isolate of entomopathogenic nematode, *Steinernema sangi* (Rhabditida, Steinernematidae), and its symbiotic bacteria *Xenorhabdus vietnemensis* (gamma-Proteobacteria) from Mizoram, northeastern India. *J Parasit Dis*. 2017; 41(4):1123–31. https://doi.org/10.1007/s12639-017-0945-z PMID: 29114152

52. Fischer-Le Saux M, Viaillard V, Brunel B, Normand P, Boemare NE. Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens*, *P. luminescens* subsp. *akhurstii*, *P. luminescens* subsp. *laumondii*, *P. temperata* subsp. *luminescens*, *P. temperata* subsp. *temperata*, *P. temperata* subsp. *asymbiotica*. *Int J Syst Bacteriol*. 1999; 49 Pt 4:1645–56. https://doi.org/10.1099/00221076-49-4-1645 PMID: 10553346

53. Geldenhuys J, Malan AP, Dicks LM. First Report of the Isolation of the Symbiotic Bacterium *Photorhabdus luminescens* subsp. *laumondii* Associated with *Heterorhabditis safricana* from South Africa. *Curr Microbiol*. 2016; 73(6):790–5. https://doi.org/10.1007/s00284-016-1116-7 PMID: 27567899

54. Suwannaraj M, Yimthirin T, Fukruksa C, Muangpat P, Yoyoyangket T, Tanthavanant S, et al. Survey of entomopathogenic nematodes and associate bacteria in Thailand and their potential to control *Aedes aegypti*. *J. appl Entomol*. 2020; 144(3):212–23. https://doi.org/10.1111/jen.12726 PMID: 29641570

55. Gerrard JG, Joyce SA, Clarke DJ, ffrench-Constant RH, Nimmo GR, Looke DF, et al. Nematode symbiont for *Photorhabdus asymbiotica*. *Emerg Infect Dis*. 2006; 12(10):1562–4. https://doi.org/10.3201/ eid1210.060646 PMID: 17167572
56. Weissfeld AS, Halliday RJ, Simmons DE, Trevino EA, Vance PH, O’Hara CM, et al. *Photorhabdus asymbiotica*, a pathogen emerging on two continents that proves that there is no substitute for a well-trained clinical microbiologist. J Clin Microbiol. 2005; 43(8):4152–5. https://doi.org/10.1128/JCM.43.8.4152-4155.2005 PMID: 16081963

57. Reimer D, Luxenburger E, Brachmann AO, Bode HB. A new type of pyrrolidine biosynthesis is involved in the late steps of xenocoumacin production in *Xenorhabdus nematophila*. Chembiochem. 2009; 10(12):1997–2001. https://doi.org/10.1002/cbic.200900187 PMID: 19598185

58. Park HB, Perez CE, Perry EK, Crawford JM. Activating and Attenuating the Amicoumacin Antibiotics. Molecules. 2016; 21(7):824. https://doi.org/10.3390/molecules21070824 PMID: 27347911

59. Bode HB. Entomopathogenic bacteria as a source of secondary metabolites. Curr Opin Chem Biol. 2009; 13(2):224–30. https://doi.org/10.1016/j.cbpa.2009.02.037 PMID: 19345136

60. Li J, Chen G, Wu H, Webster JM. Identification of two pigments and a hydroxystilbene antibiotic from *Photorhabdus luminescens*. Appl Environ Microbiol. 1995; 61(12):4329–33. https://doi.org/10.1128/aem.61.12.4329-4333.1995 PMID: 8534100

61. Derzelle S, Duchaud E, Kunst F, Danchin A, Bertin P. Identification, characterization, and regulation of a cluster of genes involved in carbapenem biosynthesis in *Photorhabdus luminescens*. Appl Environ Microbiol. 2002; 68(8):3780–9. https://doi.org/10.1128/AEM.68.8.3780-3789.2002 PMID: 12147472

62. Shi D, An R, Zhang W, Zhang G, Yu Z. Stilbene Derivatives from *Photorhabdus temperata* SN259 and Their Antifungal Activities against Phytopathogenic Fungi. J Agric Food Chem. 2017; 65(1):60–5. https://doi.org/10.1021/acs.jafc.6b04303 PMID: 27960253