DISTRIBUTION OF ENTOMOPATHOGENIC NEMATODES IN LOWER NORTHERN THAILAND

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Abstract. Entomopathogenic nematodes (EPNs) are used successfully for biological control of subterranean larval pests leading to reduced environmental contamination if chemical control measures are employed. Their diversity and distribution in Thailand are unclear, so the present study sought to obtain a better understanding these EPN populations in the lower northern region of Thailand. We collected 930 soil samples from 186 sites of Kamphaeng Phet, Nakhon Sawan, Phetchabun, Phichit, Phitsanulok, Sukhothai, Tak, Uthai Thani, and Uttaradit Provinces, Thailand from December 2011 to November 2012. *Galleria mellonella* was used as host for isolating and propagating EPNs. Seventy soil samples (7.5%) yielded EPNs of two genera, *Steinernema* (3.0%) and *Heterorhabditis* (4.5%). The majority of the isolated EPNs were found in loam at 26ºC-33ºC and pH values of 5.0-7.0. Molecular identification from partial 28S rDNA sequences revealed *S. websteri*, isolated from soil samples from Nakhon Sawan and Uthai Thani. Phylogenetic analysis of these EPNs showed they are closely related to *S. websteri* JCI032. The identification that *S. websteri* was the predominant EPN should enable its application for biological control in the local prevailing soil conditions.

Keywords: *Steinernema websteri*, distribution, entomopathogenic nematode, Thailand

INTRODUCTION

Entomopathogenic nematodes (EPNs) refer to nematodes of genera *Steinernema* and *Heterorhabditis*, symbiotically associated with bacteria of the genus *Xenorhabdus* and *Photothabdus*, respectively. EPNs have been used for biological control of insect larvae (Smart, 1995; Divya and Sankar, 2009), which they parasitize and kill within 48 hours aided by their bacterial partners (Woodring and Kaya, 1988). Several groups of economically important insect pests can be controlled effectively using EPNs, and these include larval stages of cabbage leaf webber, fig moth, mustard sawfly, rice stem borer, termite, and white grubs (Divya and Sankar, 2009). Thus, promoting the use of EPNs in agriculture has clear implications for environmental and food safety (Smart, 1995).

EPNs are found globally in diverse geographical regions and, so far, 65 *Steinernema* spp (Maneesakorn *et al*, 2010;

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Nguyen *et al*, 2010; Cimen *et al*, 2014a,b; Mracek and Nermut, 2014) and 24 *Heterorhabditis* spp (Stock *et al*, 2009; Nguyen *et al*, 2010; Edgington *et al*, 2011) have been documented. However, many of these studies have been sporadic and have yielded little information on the diversity of EPNs, their symbiotic bacteria and their agricultural application. In Thailand, surveys of EPNs have reported their distribution based on genus only (Tangchitsomkid and Sontirat, 1998). Some studies have identified EPN species, including the novel *S. siamkayai* (Stock *et al*, 1998) and *S. minutum* (Maneesakorn *et al*, 2010), while *H. indica* hosting *Photorhabdus luminescens* was isolated from northeastern and southern Thailand (Maneesakorn *et al*, 2011). So far, the majority of species found in one study are *H. bacteriophora*, *H. baujardi*, *H. indica*, *S. khoisanae*, and *S. websteri* using sequences of 28S rDNA (Thanwisai *et al*, 2012). In this study, we isolated and identify EPNs from an extended region of the lower northern provinces of Thailand and constructed a phylogenetic tree to identify their relationships with EPNs from different geographic regions.

MATERIALS AND METHODS

Cultivation of *Galleria mellonella*

The greater wax moth, *Galleria mellonella*, was used for entomopathogenic nematode baiting and multiplication. Moths were maintained in the laboratory of the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University using a modified method of Beding and Akhurst (1975). In brief, adult moths were kept at room temperature in an aerated plastic box containing paper shavings for egg laying. Moth eggs were placed on artificial diet containing 200 g of wheat powder, 100 g of beer yeast, 100 ml of glycerol, and 100 ml of honey, and after 3-4 days larvae were hatched and were given fresh diet after 2 weeks. The late instar larvae were collected after 5-6 weeks and were stored on paper shavings for 1 month at 16°C to allow pupation.

Collection of soil samples

Soil samples were randomly collected from areas of roadside verges, orchards, fields growing rice, maize, sugar cane, fruit, grassland, woodland, river and pond banks in Kamphaeng Phet, Nakhon Sawan, Phetchabun, Phichit, Phitsanulok, Sukhothai, Tak, Uthai Thani, and Uttaradit Provinces located in the lower northern regions of Thailand. A total of 930 soil samples from 186 sites were collected between December 2011 and November 2012. At each site, 5 soil samples (300-600 g each) were removed from approximately 10 m^2 area at a depth of 5-10 cm and transferred to individual plastic bags. Temperature, pH and moisture content were recorded using a soil survey instrument (KC-300 model, Yancheng Kecheng Optoelectronic Technology, Jiangsu, China). Site location (longitude, latitude and altitude using a Nüvi 1250 GPS Navigator; Garmin, New Taipey City, Taiwan) and soil type were recorded.

Isolation of entomopathogenic nematodes from soil samples

EPNs were isolated from soil samples using *G. mellonella* as bait as previously described (Bedding and Akhurst, 1975). In brief, 300-600 g soil samples were placed in a plastic box followed by five last instar larvae onto the soil sample, and the box was covered with a lid. Then the box was inverted to allow the moth larvae to migrate into the soil from below and stored at room temperature for 5 days. *G. mellonella* cadavers were collected and placed into a White trap (White, 1927) which maintained at 20ºC-25ºC to allow emergence of infective EPN juveniles. All soil samples were re-baited using fresh insect larvae to maximize EPN recovery. Emergent nematodes were re-exposed to insect larvae to confirm entomopathogenicity and to increase EPN yields (Thanwisai *et al*, 2012). A 500-1,000 µl aliquot of water containing 100-200 infective juvenile nematodes were added to 3 insect larvae, which were kept in darkness at room temperature (25ºC-30ºC). Insect larvae were observed daily and dead *G. mellonella* were collected. Infective juvenile nematodes were recovered as described above and kept in distilled water at 13ºC until analyzed.

Morphological and molecular identifications of EPN

EPNs were classified by genera according to the following criteria: 1) position of excretory pore of infective juvenile stage: anterior to nerve ring distance in *Steinernema* and posterior to nerve ring distance in *Heterorhabditis*, 2) presence of bursa in male *Heterorhabditis* and bursa absence in *Steinernema* (Kaya and Stock, 1988; Nguyen and Smart, 1996), and 3) color of cadavers: yellow-brown or black in *Steinernema* and red, brick-red or orange in *Heterorhabditis* (Emelianoff *et al*, 2008). Nematodes were mounted in water under a cover glass and were photographed (40x magnification).

For molecular identification, a partial fragment of 28S rDNA was PCR amplified and sequenced (Hominick *et al*, 1997; Stock *et al*, 2001). In short PCR was performed using extracted nematode DNA as described previously (Thanwisai *et al*, 2012). Amplicons were analyzed by agarose gel-electrophoresis and visualized with ethidium bromide staining. Amplicons were purified using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, New Taipei City, Taiwan) and directly sequenced (Macrogen Seoul, Korea). BLASTN search against a nucleotide database of EPN 28S rDNA was performed (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Phylogenetic analysis

Phylogenetic analysis based on partial 28S rDNA sequences was performed using MEGA software version 5.05. Sequences of known species from NCBI database were used to align with sequences of this study and trimmed to 605 bp region using ClustalW program (Thompson *et al*, 1994). Neighbor Joining trees were reconstructed using Kimura-2-parameter model with a MEGA software version 5.05. Bootstrap analysis was performed with 1,000 datasets.

RESULTS

EPNs isolated from soil samples

From 930 soil samples collected from 186 sites in 9 provinces of lower northern Thailand (Table 1), 70 (7.5%)soil samples yielded EPNs, identified based on morphology as belonging to two genera: *Heterorhabditis* (42 isolates) and *Steinernema* (28 isolates) (Fig 1). The nematodes were recovered from 51 roadside verges (5%), 10 rivers or ponds (1%), 4 fruit crops (0.5%), 2 grasslands (0.1%), 2 woodlands (0.1%), and 1 field crop (0.1%). EPN isolates (n = 53) were found in loam (76%) with pH range of 5-7 and soil temperature of 26-35ºC, and in sandy loam (16%), sand (4%), and clay (4%).

EPN identification and phylogenetic analysis

As the majority of EPNs were contaminated with fungi during their collection, only two isolates (139.1 TH and 155.1 TH) were identified based on partial 28S

Table 1
Isolation of entomopathogenic nematodes (EPNs) from soil samples in lower northern Thailand.

Province	Total sites	Total soil samples	No. of positive soil samples (%)		
			Steinernema sp	*Heterorhabditis* sp	All EPN spp
Phitsanulok	24	120	0	2	2 (1.7)
Phichit	20	100	2	7	9 (9.0)
Uttaradit	19	95	3	8	11 (11.6)
Phetchabun	20	100	3	1	4 (4.0)
Kamphaeng Phet	20	100	8	4	12 (12.0)
Sukhothai	20	100	1	4	5 (5.0)
Nakhon Sawan	21	105	6	3	9 (8.6)
Tak	21	105	2	7	9 (8.6)
Uthai Thani	21	105	3	6	9 (8.6)
Total	186	930	28 (3.0)	42 (4.5)	70 (7.5)

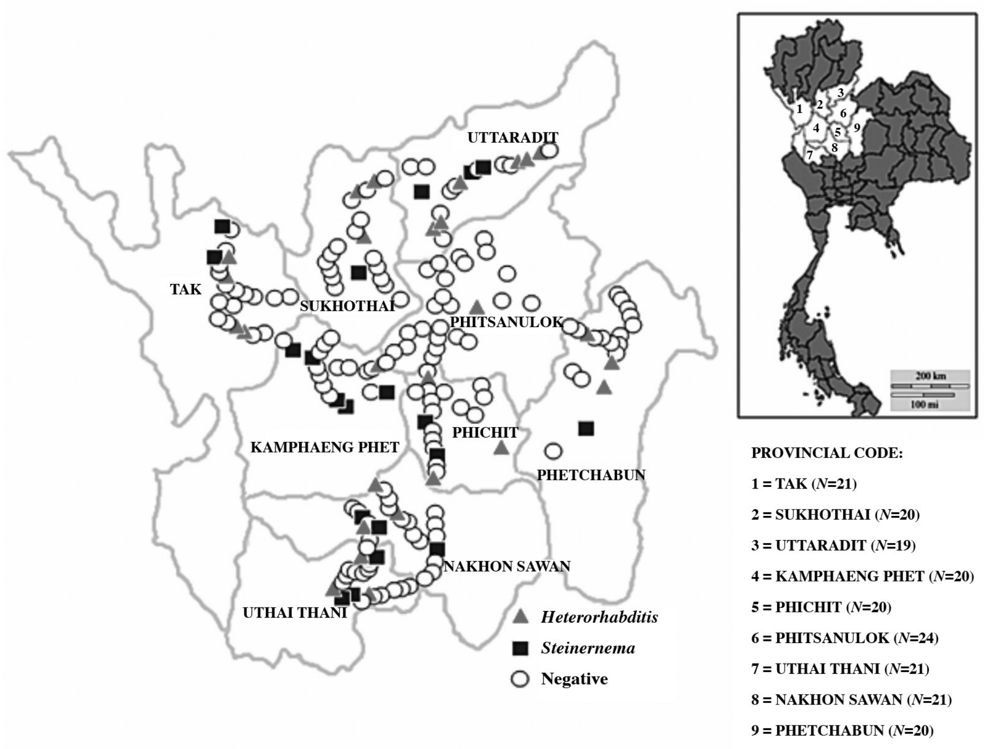

Fig 1–Map of soil sampling sites for entomopathogenic nematodes in lower northern Thailand.

rDNA sequences. Isolate 139.1 TH from Uthai Thani Province was collected from sand, pH 7.0 and 31ºC at 12º 21′ 48.2″ N 099º 58′ 46.1″ E, and 155.1 TH, Nakhon Sawan Province, from loam, pH 6.5 and 33ºC at N 15º 35′ 35.3″ E 099º 51′ 21.3″. The two sequences (Genbank accession nos. KM359386 (for 139.1 TH) and KM359387 (155.1 TH) were identical, with 98% and 99% identity to *S. anatoliensis*

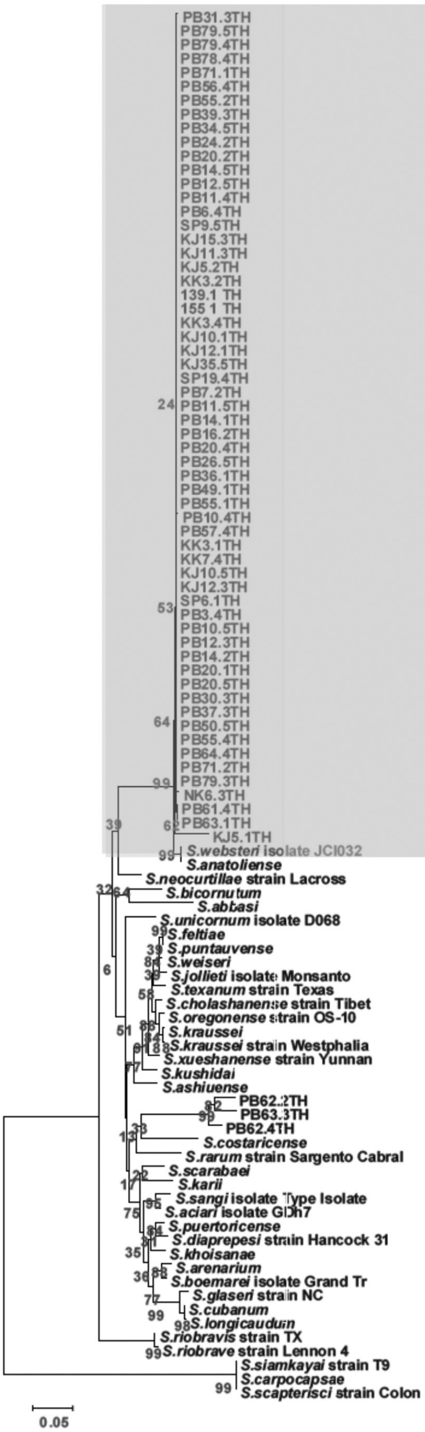

Fig 2–Neighbor joining phylogenetic tree based on 605 bp 28S rDNA of 2 entomopathogenic nematodes (139.1TH and 155.1TH) together with 58 *Steinernema* isolates from Thailand (codes ending with TH) and sequences downloaded from GenBank database. Bootstrap values are 1,000 replicates. The bar indicates 5% sequence divergence.

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139.1_TH                  TGGTGCGAAT TCTCTTT-GA CTAGGG---- -ATCCAAAGA GGGTGCTAGA CCCTTACGCA TTGTTGACTT TTCGTACGCG
155_1_TH                  .......... .......-.. ......---- -......... .......... .......... .......... ..........
NK6.3TH                   .......... .......-.. ......---- -......... .......... .......... .......... ..........
KJ5.1TH                   G.C....C.. ......C-.. ...A.T---- -......TT. ........C. G.....T.G. .......... ....GC.A..
PB10.4TH                  .......... .......-.. ......---- -......... .......... .......... .......... ..........
PB31.3TH                  .......... .......-.. ......---- -......... .......... .......... .......... ..........
PB61.4TH                  .......... .......-.. ......---- -......... .......... .......... .......... ..........
PB63.1TH                  .......... .......-.. ......---- -......... .......... .......... .......... ..........
S.anatoliense             .AA....... .TC....T.. ......---- -......... .......... .......... .......... ..........
S.websteri_isolate_JC1032 .AA....... .TC....-.. ......---- -......... .......... .......... .......... ..........

139.1_TH                  TTC-GTTTCT TGGAGTAGGG TTGTTTTGGA TCGCAGCCCA AAGTAGG-TG GTATACTTCA TCTAAA-GCT AAATACGACT
155_1_TH                  ...-...... .......... .......... .......... .......-.. .......... ......-... ..........
NK6.3TH                   ...-...... .......... .......... .......... .......-.. .......... ......-... ..........
KJ5.1TH                   ...TAA.... .......... .......... .......... .......-.. .......... ......-... ..........
PB10.4TH                  ...-...... .......... .......... .......... .......-.. .......... ......-... ......C...
PB31.3TH                  ...-...... .......... .......... .......... .......-.. .......... ......-... ..........
PB61.4TH                  ...-...... .......... .......... .......... .......-.. .......... ......-... ..........
PB63.1TH                  ...-...... .......... .......... .......... .......-.. .......... ......-... ..........
S.anatoliense             ...-...... .......... .......... .......... .......G.. .......... ......A... ..........
S.websteri_isolate_JC1032 ...-...... .......... .......... .......... .......-.. .......... ......-... ..........

139.1_TH                  ACGAATCCGA TAGCAAACAA GTACCGTGAG GGAAAGTTGC AAAGTACTTT GAAGAGAGAG TTCAAGAGGA CGTGAAACCG
155_1_TH                  .......... .......... .......... .......... .......... .......... .......... ..........
NK6.3TH                   .......... .......... .......... .......... .......... .......... .......... ..........
KJ5.1TH                   .......... .......... .......... .......... .......... .......... .......... ..........
PB10.4TH                  .......... .......... .......... .......... .......... .......... .......... ..........
PB31.3TH                  .......... .......... .......... .......... .......... .......... .......... ..........
PB61.4TH                  .......... .......... .......... .......... .......... .......... .......... ..........
PB63.1TH                  .......... .......... .......... .......... .......... .......... .......... ..........
S.anatoliense             .......... .......... .......... .......... .......... .......... .......... ..........
S.websteri_isolate_JC1032 .......... .......... .......... .......... .......... .......... .......... ..........

139.1_TH                  GTAGGGTGGA AGCAGATAAA GTTGACG-AA CGTGT--GTC GTATTC---A GAACTACA-A TTTGTG---- --GTTTGTTT
155_1_TH                  .......... .......... .......-.. .....--... ......---. ........-. ......---- --........
NK6.3TH                   .......... .......... .......-.. .....--... ......---. ........-G ......---- --........
KJ5.1TH                   .......... .......... .......-.. .....--... ......---. ........-. ......---- --........
PB10.4TH                  .......... .......... .......-.. .....--... ......---. S.......-. ......---- --........
PB31.3TH                  .......... .......... .......-.. .....--... ......---. ........-. ......---- --........

PB61.4TH                  .....A.... .......... .......-.. .....--... ......---. ........-. ......---- --........
PB63.1TH                  .....A.... .......... .......-.. .....--... ......---. ........-. ......---- --........
S.anatoliense             .......... .......... ...-...-.. .....--... ......---. ........-. ......---- --........
S.websteri_isolate_JC1032 .......... .......... .......-.. .....--... ......---. ........-. ......---- --........

139.1_TH                  TTACGATCGA T--GTGGGCT GGCGTCTTTG GTTAACTTAG ----TGTCTG GCGGCAATGG TGACCCTGCG GAGGGATAAT
155_1_TH                  .......... .--....... .......... .......... ----...... .......... .......... ..........
NK6.3TH                   .......... .--....... .......... .......... ----...... .......... .......... ..........
KJ5.1TH                   .......... .--....... .......... .......... ----...... .......... .......... ..........
PB10.4TH                  .......... .--....... .......... .......... ----...... .......... .......... ..........
PB31.3TH                  .......... .--....... .......... .......... ----...... .......... .......... ..........
PB61.4TH                  .......... .--....... .......... .......... ----...... .......... .......... ..........
PB63.1TH                  .......... .--....... .......... .......... ----...... .......... .......... ..........
S.anatoliense             .......... .--....... .......... .......... ----...... .......... .......... ........C.
S.websteri_isolate_JC1032 .......... .--....... .......... .......... ----...... .......... .......... ........C.
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139.1_TH                  CGGTTGT--- CGTGCGATGC TTGGTATGGC TAGAGGTTCG CT---GGTCT ---TATA-GT CATCGCTTTA TCTGACCCGT
155_1_TH                  .......--- .......... .......... .......... ..---..... ---....-.. .......... ..........
NK6.3TH                   .......--- .......... .......... .......... ..---..... ---....-.. .......... ..........
KJ5.1TH                   .......--- .......... .......... .......... ..---..... ---....-.. .......... ..........
PB10.4TH                  .......--- .......... .......... .......... ..---..... ---....-.. .......... ..........
PB31.3TH                  .......--- .......... .......... .......... ..---..... ---....-.. .......... .T........
PB61.4TH                  .......--- .......... .......... .......... ..---..... ---....-.. .......... ..........
PB63.1TH                  .......--- .......... .......... .......... ..---..... ---....-.. .......... ..........
S.anatoliense             .......--- .......... .......... .......... ..---...T. ---....-.. .......... ..........
S.websteri_isolate_JC1032 .......--- .......... .......... .......... ..---...T. ---....-.. .......... ..........

139.1_TH                  CTTGAAACAC GGACCAAGGA GTGTAGCGCT TACGCG-AGT CTTAGAGTGT GTCAAAACTT TGAGGCGTAA CGAAAGTAAA
155_1_TH                  .......... .......... .......... ......-... .......... .......... .......... ..........
NK6.3TH                   .......... .......... .......... ......-... .......... .......... .......C.. ..........
KJ5.1TH                   .......... .......... .......... ......-... .......... .......... .......... ..........
PB10.4TH                  .......... .......... .......... ......-... .......... .......... .......... ..........
PB31.3TH                  .......... .......... .......... ......-... .......... .......... .......... ..........
PB61.4TH                  .......... .......... .......... ......-... .......... .......... .......... ..........
PB63.1TH                  .......... .......... .......... ......-... .......... .......... .......... ..........
S.anatoliense             .......... .......... .....C.... ......G... .......... .......... .......... ..........
S.websteri_isolate_JC1032 .......... .......... .....C.... ......-... .......... .......... .......... ..........

139.1_TH                  TGTGGATTTA --TTCACTGA CTTGGGATGC -GTTGTCTT- TTTTGGATAG CGTT-GGACC ATGGTTTTAT CGTAATCGCT
155_1_TH                  .......... --........ .......... -........- .......... ....-..... .......... ..........
NK6.3TH                   .......... --........ .......... -........- .......... ....-..... .......... ..........
KJ5.1TH                   .......... --........ .......... -........- ........TC ....-..... .......... A........A
PB10.4TH                  .......... --........ .......... -........- .......... ....-..... .......... ..........
PB31.3TH                  .......... --........ .......... -........- .......... ....-..... .......... ..........
PB61.4TH                  .......... --........ .......... -........- .......... ....-..... .......... ..........
PB63.1TH                  .......... --........ .......... -........- .......... ....-..... .......... ..........

S.anatoliense             .......... --........ ........A. C........- .......... ....-..... .......... ..........
S.websteri_isolate_JC1032 .......... --........ ........A. -........- .......... ....-..... .......... ..........
139.1_TH                  TGCGATGCG
155_1_TH                  .........
NK6.3TH                   .........
KJ5.1TH                   .TT.GG...
PB10.4TH                  .........
PB31.3TH                  .........
PB61.4TH                  .........
PB63.1TH                  .........
S.anatoliense             .........
S.websteri_isolate_JC1032 .........
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Fig 3–Alignment of partial 28S rDNA sequences from entomopathogenic nematodes 139.1_TH and 155.1_TH, and *S. websteri* isolates from Thailand (NK6.3TH, KJ5.1TH, PB10.4TH, PB31.3TH, PB61.4TH, and PB63.1TH), *S. websteri* isolate JCI032 and *S. anatoliense*. Dot represents agreement with the consensus base at that position and dash indicates alignment gap.

and *S. websteri*, respectively. Phylogenetic tree analysis showed that the two isolates were closely related to *S. websteri* (58 isolates) from Thailand, *S. websteri* and *S. anatoliense* (Fig 2). The partial 28S rDNA sequences of 52 *S. websteri* Thai isolates were identical to 139.1TH and 155.1TH, but 6 isolates, *S. websteri* NK6.3TH, KJ5.1TH, PB10.4TH, PB31.3TH, PB61.4TH and PB63.1TH, were 96%-99% identical to these two isolates (Fig 3).

DISCUSSION

Our result showed low recovery (7.5%) of EPN in soil samples, mostly from soil samples of roadside verges. In European surveys successful isolation ranged from 2.2%-36.8% (Hominick *et al*, 1995; 1996). Four species of *Steinernema* have been reported from Thailand, namely, *S. khoisanae* (Thanwisai *et al*, 2012), *S. minutum* (Maneesakorn *et al*, 2010), *S. siamkayi* (Stock *et al*, 1998), and *S. websteri*, and 3 species of *Heterorhabditis*, namely, *H. indica*, *H. baujardi* and *H. bacteriophora* along with *Heterorhabditis* sp. SGmg3 and *Heterorhabditis* sp SGgi (Maneesakorn *et al*, 2011; Thanwisai *et al*, 2012). A previous survey in Thailand recorded recovery of 28% for *Steinernema* (dominated by *S. websteri*) and 27% for *Heterorhabditis* (*H. indica* being prominent) (Thanwisai *et al*, 2012). We also found a predominance of *S. websteri* in different regions of lower northern Thailand.

S. websteri was first recovered from soil, Beijing, China (Cutler and Stock, 2003) and subsequently found in Peru and Thailand (Lee and Stock, 2012; Thanwisai *et al*, 2012). It is used for the control of common cutworm, *Agrotis segetum*, in Turkey. Mortality rate of *A. segetum* is 100% when 500 infective juvenile stages/g of dry sand is applied for 5 days under laboratory conditions (Gokce *et al*, 2015). *S. websteri* was found to be associated with *Xenorhabdus nematophila*, used for control of *Plutella xylostella* larvae (Park *et al*, 2012) and *Aedes aegypti* larvae (Silva *et al*, 2013). In Thailand, *S. websteri* has been found associated with *X. stockiae*, used for control of mushroom mite (Bussaman *et al*, 2012). *S. websteri* is common mostly in wild areas and largely absent in cultivated soil. Whether this reflects pesticide sensitivity or present of suitable hosts is unclear.

Because of its greater abundance, *S. websteri* is likely to thrive best in light loamy soil and high soil temperature, which exist in the sampling regions and to have the greatest impact on subterranean larval populations. Thus, this species should be the first choice in biological control and/or integrated insect pest management programs in this region of Thailand, with the aim of reducing chemical pesticide usage. In addition, *S. websteri* symbiotic bacteria may yield novel bio-pesticides, which are already optimized for use under these environmental conditions.

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