Agr. Nat. Resour. 54 (2020) 453–462

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Genetic variation of *Heterorhabditis indica* Poinar, Karunakar & David (Nematoda, Rhabditida) population in Thailand

Achariya Nitjarunkul, Atsalek Rattanawannee, Atirach Noosidum*

Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.

Article Info

Article history:
Received 29 May 2019
Revised 30 November 2019
Accepted 2 March 2020
Available online 31 August 2020

Keywords:
Entomopathogenic nematode,
Haplotype,
Heterorhabditis,
Thailand

Abstract

Entomopathogenic nematodes (EPNs) in the genus *Heterorhabditis* are widely used to control many soil-borne insects. Thus, studies on the biodiversity of the local EPNs are needed to develop their suitable use in insect pest management programs. The phylogeny, base difference and haplotype network of the EPNs were investigated using morphology and a molecular technique to compare the genetic information on EPNs among regions in Thailand. In total, 27 isolates of EPNs were recovered from 221 soil samples collected from six regions of Thailand. All isolates of EPNs were identified as *Heterorhabditis indica* based on internal transcription spacer regions (98% identity) and morphological data. Within the 27 sequences, 28 polymorphic sites were defined to 13 distinct haplotypes of *H. indica* in Thailand and the percentage sequence divergence was in the range 0.12–2.12% (1–24 bp). The comparison of migration rate at 0–∞ nematodes per generation could distinguish among provinces from different regions of Thailand. The demographic history and the dispersal of the nematode population had a low frequency of mutation. The phylogenetic tree of nematode haplotype was monophyletic and separated into three clades. These results suggested that using the internal transcription spacer region would be successful in describing these EPNs in detail at the species level.

Introduction

Nematodes belonging to the Phylum Nematoda and can be found around the world in various habitats, including freshwater, marine and soil environments (Andrássy, 1976; Shaw et al., 2016). They are either free-living or parasitic, acting as parasites to plants, invertebrates and vertebrates (Andrássy, 1976; De Ley and Blaxter, 2004). One important group is the entomopathogenic nematodes (EPNs) that cause lethal parasitism to various soil-dwelling insects such as caterpillars and immature beetles (Andrássy, 1976; Boemare et al., 1993; Forst and Nealson, 1996; Reid et al., 1997; Hominick, 2002). Beside Antarctica, EPNs exist in different climatic and environmental conditions on all continents of the world (Hominick, 2002; Nguyen and Hunt, 2007; Maneesakorn et al., 2010; Noosidum et al., 2010).

Two families of the EPNs (Steinernematidae and Heterorhabditidae) have proved to be effective biological control agents for controlling many insect pests worldwide (Kaya and Gaugler, 1993, Grewal et al., 2005; Griffin et al., 2005). EPNs killed the insect hosts with their own mutualistic bacteria in the genus *Xenorhabdus* and *Photorhabdus* for Steinernematid and Heterorhabditid nematodes, respectively (Boemare et al., 1993; Kaya and Gaugler, 1993; Forst and Nealson, 1996; Reid et al., 1997). After the nematode had entered the insect's hemocoel, septicemia toxin produced by mutualistic bacteria can later digest the insect tissues to provide food for the nematode (Griffin et al., 2005).

Numerous species of EPNs in the families Steinernematidae (over 100 species) and Heterorhabditidae (16 species) have been described in many places around the world (Andrássy, 1976; Shaw et al., 2016; Shapiro-Ilan et al., 2017; Al-Zaidawi et al., 2019). However, more information is still required on the diversity of EPNs in Thailand.

* Corresponding author.
E-mail address: fagrarn@ku.ac.th (A. Noosidum)

https://doi.org/10.34044/j.anres.2020.54.4.15

Many novel techniques have been used to identify nematodes to the species level (Stock and Goodrich-Blair, 2012; Nguyen and Hunt, 2007). Since the 19th century, techniques for morphological characterization have been used to describe a nematode species (Porazinska et al., 2009; Griffiths et al., 2018). However, a superficial analysis of the morphological characterization can occur and result in inaccurate conclusions in the case of two related nematode species (De Ley and Blaxter, 2004; Porazinska et al., 2009; Griffiths et al., 2018). Molecular techniques are a conventional method that is a very effective tool for discrimination between close species of nematodes, providing specific information such as gene pattern, protein pattern, phylogeny, genetic distance and haplotype network of the nematode (Adams et al., 2006; Stock, 2009). Various molecular techniques such as random amplification of polymorphic DNA, restriction fragment length polymorphism and DNA sequencing have been applied and have shown that the internal transcribed spacer (ITS) region and DNA sequences from the nucleus (18S and 28S rDNA) can be effectively used to identify nematode species (Reid et al., 1997; Stock, 2009).

Based on the effectiveness of molecular techniques, the aims of this study were to investigate the phylogeny, base difference and haplotype network of the EPNs in Thailand and compare the genetic information on EPNs among regions within the country.

Materials and Methods

Soil sampling and isolation of entomopathogenic nematodes

In total, 221 soil samples were collected from undisturbed areas close to national parks in six regions (Northern, Central, Western, Northeastern, Eastern and Southern) in Thailand (5° 37–20° 28' N and 97° 21'–105° 37 E) as shown in Fig. 1. About 10–20 soil samples were taken using a hand shovel within the top 20 cm of soil in each province. Each sample (approximately 1 kg) was placed in a polyethylene bag, closed tightly, kept in a cooler box (15–20°C) (Noosidum et al., 2010) and transported to the laboratory at the Department of Entomology, Kasetsart University, Bangkok, Thailand.

In the laboratory, EPNs were recovered from soil samples using a modified insect baiting technique (Stock and Goodrich-Blair, 2012). Four soil sub-samples (150 g each) from each soil sample were put into a plastic container (7.5 cm × 10 cm × 5 cm) containing 20 last instar larvae of honeycomb moth, *Galleria mellonella* L. Each container of soil sub-sample was covered with a lid and kept in the darkroom at 25 ± 3°C for 3–7 d.

Parasitized cadavers of *G. mellonella* larvae were transferred into the modified White trap (White, 1927). The insect cadavers were placed on a moistened or wet filter paper (Whatman® No.1 filter paper) in a small Petri dish (5.5 cm diameter) which was placed in a large Petri dish (9 cm diameter), filled with 0.001% formaldehyde. The large Petri dish was covered with a lid and kept in a dark room at 25 ± 3°C until the infective juveniles (IJs) emerged. After they emerged and had moved from the small Petri dish to the 0.001% formaldehyde in the large Petri dish, the IJs of EPNs were collected. Each IJ was cleaned with distilled water (DI) and stored at 15 ± 3°C.

Entomopathogenic nematode preparation

EPNs were maintained in the laboratory by rearing on *G. mellonella* larvae (Stock and Goodrich-Blair, 2012) every 4 wk. A nematode suspension at 500 IJs/700 µL of DI water of each EPN was applied to a 5 cm diameter Petri dish, lined with a Whatman® No.1 filter paper. Then, six last instar larvae of *G. mellonella* were placed into the Petri dish. After 3 d, the cadavers were transferred to the modified White trap (Stock and Goodrich-Blair, 2012). The emerging IJs were collected and stored in a 200 mL culture flask at 15 ± 3°C.

DNA amplification and DNA sequencing

For the preparation of DNA extract, *G. mellonella* larvae were infected by 100 IJs of EPNs for 96 hr. To collect one couple (adult male and amphimictic female) of the EPNs, the infected cadaver was dissected using a platinum needle and then placed into a drop of *G. mellonella* hemolymph (30 µL) on the sterilized box lid (30 × 30 mm). Two mL of Ringer's solution was filled in the box and the lid was closed. This method was modified from the hanging blood drop method of Stock and Goodrich-Blair (2012). The hanging blood case was wrapped with paraffin and incubated at 25 ± 2°C until it had 30 adult nematodes and these were used for DNA extraction.

Genomic DNA was extracted from 27 isolates of EPNs using a GenUP™ gDNA Kit (Biotechrabbit Company: Germany). The ITS region was amplified using standard polymerase chain reaction (PCR). Universal primers number 18S 5'TT GAT TAC GTC CCT GCC CTT T3' (forward) and number 28S 5'TT TCA CTC GGC CGT TAC TAA GG3' (reverse) were used (Vrain et al., 1992; Stock, 2009). PCR amplification was performed in a reaction volume of 40 µL, containing 20 µL of 2 × Multiplex PCR Master Mix

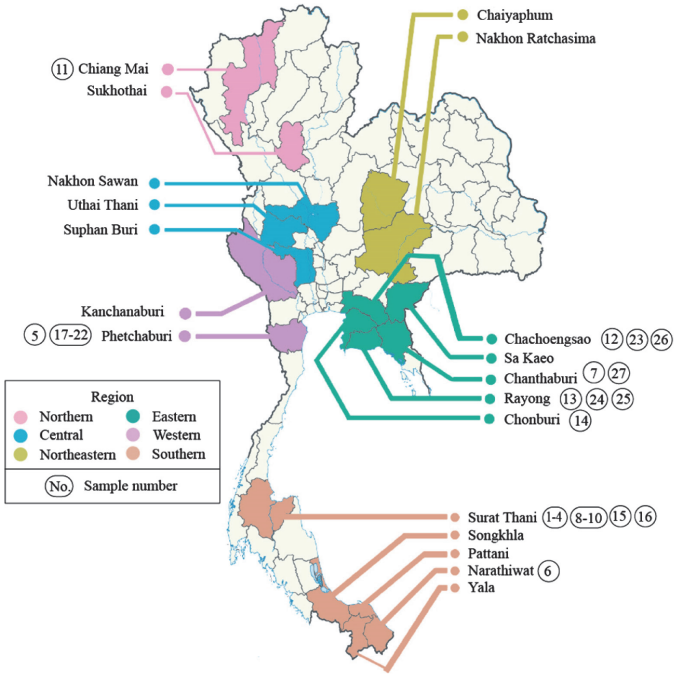

Fig. 1 Soil sampling locations for entomopathogenic nematodes in six regions of Thailand

(Biotechrabbit Company, Germany), 6.4 µL of H_2O, 0.8 µL of forward primer (10 mmol), 0.8 µL of reverse primer (10 µmol), and 12 µL of genomic DNA (300 ng). The thermocycler profiles of the ITS region consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of the denaturation step at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1.5 min and the last step of the initial extension was at 72°C for 5 min. The amplification products were purified using a GenUP™ PCR Cleanup Kit (Biotechrabbit Company, Germany). Gel electrophoresis was used to confirm product quality. The purified DNA products were sequenced by AITbiotech (Singapore).

All EPNs sequences were blasted based on the NCBI database and then aligned using MEGA7 (Tamura et al., 2013). DnaSp version 4.5.0.3 (Rozas et al., 2003) was used to analyze the number of polymorphic sites (S), number of haplotypes (No), haplotype diversity (*h*) (Nei and Kumar, 2000), nucleotide diversities (*Pi*) (Tajima, 1989) and mean number of pairwise nucleotide differences (k). The Kakusan 4 program (Tanabe, 2007) was used to analyze the best fit model for phylogenetic tree construction. The programs MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001) and Treefinder (Jobb et al., 2004) were used to analyze the phylogenetic tree using the methods of maximum likelihood (ML) and Bayesian inference (BI), respectively. All sequences were then deposited in the NCBI GenBank.

Morphological and morphometric characters

The IJs, the first generation of hermaphroditic females, the second of amphimictic females, and males were used for morphometric study. Adults were obtained by dissecting *G. mellonella* larvae in two different time ranges, namely days 3–4 for hermaphroditic females and days 5–7 for amphimictic females and males. Based on the molecular identification, a representative of each species was selected and considered for the determination of qualitative and quantitative data.

All stages of the EPNs were killed in Ringer's solution at 60°C and placed in triethanolamine-formalin fixative (7 mL of 40% formaldehyde, 2 mL of triethanolamine, and 91 mL of DI water) for 12 hr (Stock and Goodrich-Blair, 2012). After the first fixative was applied, the second fixative method was accomplished by these steps: 1) transferred the specimens into Solution I (20 mL of 95% ethanol, 1 mL of glycerine, and 79 mL of DI water) for 12 hr; 2) moved the specimens to solution II (95 mL of 95% ethanol, 5 mL of glycerine) for 3 hr; 3) moved the specimens to solution III (50 mL of 95% ethanol, 50 mL of glycerine) for 3 hr; and 4) moved the specimens to solution IV (100% glycerine). In steps 1–3, the specimen containers were kept in a 95% ethanol desiccator at 35°C, 40°C and 40°C, respectively (Seinhorst, 1959). The fixed specimens were used for measurements with the aid of an Olympus CH-2 camera fitted with a Dino-eye AM423X.

Results

Soil samples and entomopathogenic nematode isolation

In total, 27 EPNs (13.12%) were isolated from 221 soil samples collected from Northern, Eastern, Western and Southern Thailand (Fig. 1).

Sequence analysis

After alignment, the DNA sequences comprising 885 base pairs (bp) were obtained from the 27 isolates of EPNs. The alignment was able identify 848 (95.83%) conserved sites, 10 (1.13%) singleton informative sites, 18 (2.03%) parsimony-informative sites, four (0.45%) insertion sites and five (0.56%) deletion sites. The ITS nucleotide frequencies indicated a similar content for all base groups [adenine (A) = 25.37%, thymine (T) = 29.70%, cytosine (C) = 19.95%, guanine (G) = 24.98%]. All 27 samples of EPNs in Thailand matched with *Heterorhabditis indica* DH2 (MK273195) from the NCBI database (98% identity). In addition, the phylogenetic relationships of ITS regions among EPNs were strongly grouped as *H. indica* DH2 by 100% of BI and 1.0 of ML (Fig. 2A). In total, 13 haplotypes (Hap1–Hap13) were grouped (Table 1) and 28 polymorphic sites were found, consisting of A/G transitions, T/C transitions, G/T transversions and G/C transversions for 6 (21.43%), 5 (17.86%), 5 (17.86%) and 12 (42.85%) sites, respectively (Table 1).

Confirming the divergence of sequences, the pairwise distance comparisons of the 13 haplotypes (Hap) were in the range 0.12–2.12% (1–24 bp). The lowest sequence divergence was for the comparison of Hap2 with Hap5, Hap2 with Hap6, Hap3 with Hap4, Hap4 with Hap9 and Hap8 with Hap11. The maximum sequence divergence was in Hap3 with Hap10 (Table 2).

Genetic diversity indices and a summary of the demographic history indices of the six regions are presented in Table 3. The results identified 13 haplotypes and 28 polymorphic sites. The average number of nucleotide differences was 5.34 and haplotype diversity was 0.8860 ± 0.0370. The nucleotide diversity was 0.0062 ± 0.0010. The samples collected from Eastern Thailand had a greater polymorphic site than the other regions. The number of nucleotide differences was 7.55. The haplotype diversity was 0.9170 ± 0.0920 and nucleotide diversity was 0.0087 ± 0.0019. Overall demographic history indices of the EPNs showed that the values of Tajima's D (*D*), Fu's Fs (*Fs*) and Ramos-Onsins and Rozas' R_2 (R_2) were -0.9686 ($p = 0.52$), -1.8250 ($p = 0.23$) and 0.0883 ($p = 0.12$), respectively.

Pairwise comparisons of genetic distances among populations of Thai *H. indica* are shown in Table 4. The pairwise genetic distance (F_{ST}) was in the range 0.0000–0.7037. The F_{ST} between the Western and Southern regions was significantly difference to the other pairs. The pairwise per-generation migration rate (*Nm*) was low to high values at 0.1052–∞.

Monophyly was detected from 13 haplotypes of the *H. indica* up to 100% BI and 1.00 ML. The 13 haplotypes were classed into three clades, most of which were strongly supported up to 100% of BI and 1.00 of ML. On the other hand, some of the sub-clades in each clade had low support (Fig. 2B).

The 13 haplotypes were grouped into a single connected network. The main haplotypes were Hap2 and Hap8 which had the most abundant sequences with six individuals. These two haplotypes were connected with the other 11 haplotypes between one and 14 mutation steps. The most varied haplotypes were Hap2 (in samples from Eastern, Western and Southern Thailand) and Hap6 (in samples from Northern, Eastern and Western Thailand) as shown in Fig. 3.

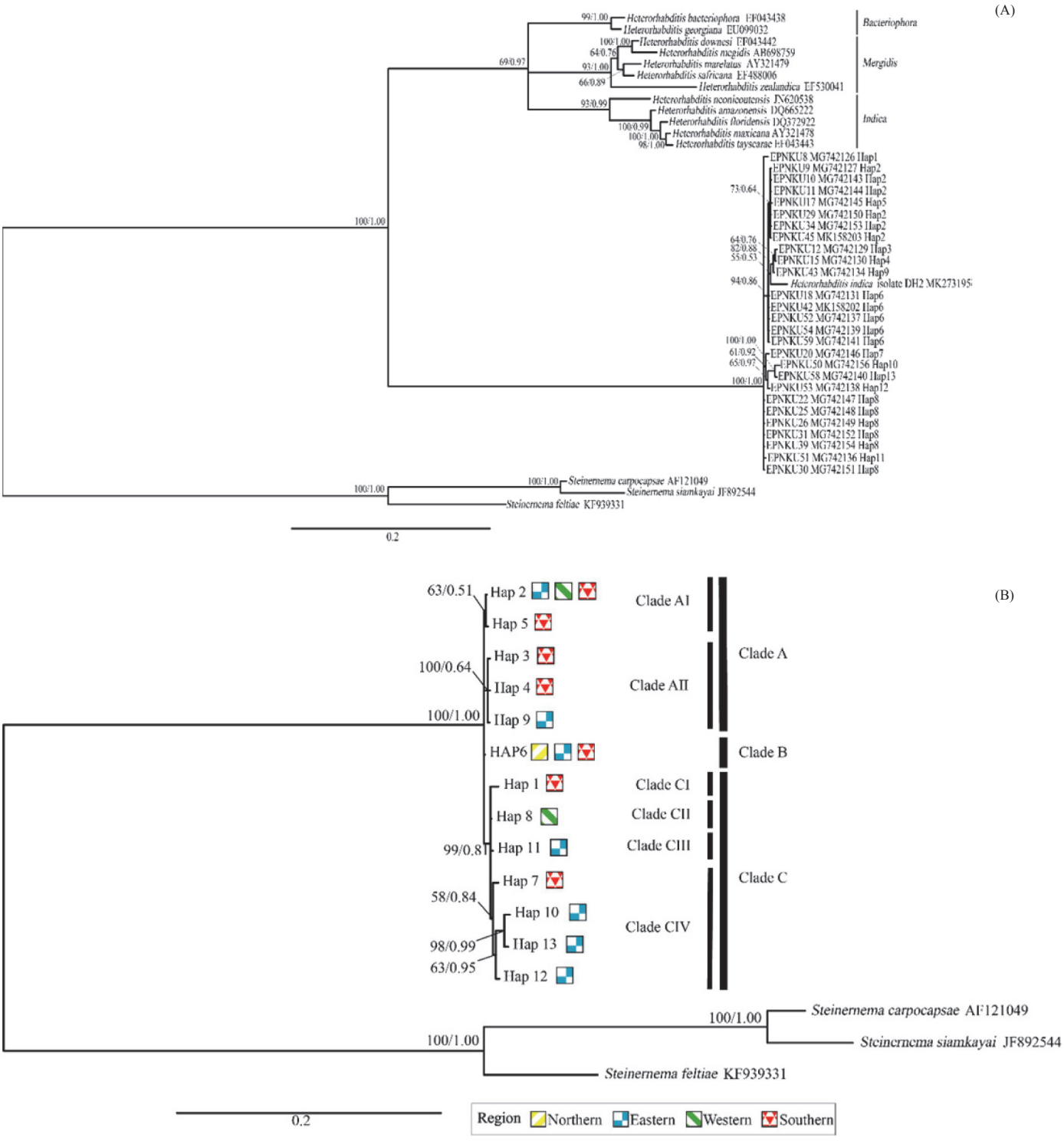

Fig. 2 Phylogenetic relationships: (A) between Thai *Heterorhabditis indica* and other *Heterorhabditis* sp. from National Center for Biotechnology Information database; (B) Thai *H. indica* inferred from the internal transcription spacer fragment based on maximum likelihood, where *Steinernema carpocapsae* (AF121049), *Steinernema siamkayai* (JF892544), and *Steinernema feltiae* (KF939331) are utilized as an outgroup and characteristic of node supports was inferred from Bayesian posterior probability and bootstrap value for maximum likelihood

Table 1 Sequence analysis presenting the 38 polymorphic sites of Thai *Heterorhabditis indica*

Haplotype	Nucleotide position																																						
	11	12	13	44	166	293	302	304	305	306	307	308	309	210	348	362	372	374	377	378	383	384	385	387	389	392	398	407	530	593	617	619	623	665	744	858	860	872
Hap1	A	G	A	T	G	T	C	T	T	–	–	C	G	A	C	C	T	T	C	T	–	–	C	G	G	G	G	G	G	G	G	C	G	G	G	C	A	C
Hap2	*	*	*	*	*	G	T	C	–	–	–	–	–	–	*	*	C	*	*	C	–	–	*	T	*	*	*	*	*	*	*	*	T	*	A	G	G	*
Hap3	*	A	*	C	T	G	T	C	–	–	–	–	–	–	*	*	C	*	*	C	–	–	*	T	*	*	*	*	*	*	*	*	T	*	A	G	G	*
Hap4	G	A	-	C	*	G	T	C	–	–	–	–	–	–	*	*	C	*	*	C	–	–	*	T	*	*	*	*	*	*	*	*	T	*	A	G	G	*
Hap5	*	*	*	*	T	G	T	C	–	–	–	–	–	–	*	*	C	*	*	C	–	–	*	T	*	*	*	*	*	*	*	*	T	*	A	G	G	*
Hap6	*	*	*	*	*	G	T	C	–	–	–	–	–	–	*	*	C	*	*	C	T	G	*	*	*	*	*	*	*	*	*	*	T	*	A	G	G	*
Hap7	*	*	*	*	*	*	*	*	*	–	–	*	*	*	*	*	*	*	G	*	–	–	*	*	*	C	*	*	C	*	*	*	T	*	A	G	G	*
Hap8	*	*	*	*	*	*	*	C	*	T	T	*	*	*	*	*	*	*	*	*	–	–	*	*	*	*	*	*	*	*	*	*	T	*	A	G	G	*
Hap9	*	A	*	C	*	G	T	C	–	–	–	*	*	*	*	*	C	*	*	C	T	G	*	*	*	*	*	*	*	*	*	*	T	*	A	G	G	*
Hap10	*	*	*	*	*	*	*	C	*	T	T	*	*	*	G	G	*	*	*	*	–	–	G	*	*	C	*	T	*	C	C	G	T	C	A	G	G	G
Hap11	*	*	*	*	*	*	*	C	*	T	T	*	*	*	*	*	*	G	*	*	–	–	*	*	*	*	G	*	*	*	*	*	T	*	A	G	G	*
Hap12	*	*	*	*	*	*	*	C	*	T	T	*	*	*	*	*	*	*	*	*	–	–	*	*	*	C	G	T	*	*	*	*	T	*	A	G	G	*
Hap13	*	*	*	*	*	*	*	C	*	T	T	*	*	*	G	G	*	*	*	*	–	–	G	*	A	C	*	T	*	*	*	G	T	C	A	G	G	*

[1]A = adenine, C = cytosine, G = guanine, T = thymine,

* positions that are different from haplotype, –gap positions

Table 2 Pairwise comparisons of nucleotide sequences of Thai *Heterorhabditis indica*

Haplotype	Hap1	Hap2	Hap3	Hap4	Hap5	Hap6	Hap7	Hap8	Hap9	Hap10	Hap11	Hap12	Hap13
Hap1		10	13	13	11	11	7	7	13	17	9	10	15
Hap2	0.0117		3	3	1	3	10	7	4	16	9	10	15
Hap3	0.0153	0.0035		2	6	16	8	14	6	24	16	17	22
Hap4	0.0141	0.0023	0.0012		5	7	17	15	8	21	17	18	23
Hap5	0.0129	0.0012	0.0023	0.0035		4	16	12	9	22	14	15	18
Hap6	0.0105	0.0012	0.0047	0.0035	0.0023		14	12	5	22	12	15	20
Hap7	0.0082	0.0105	0.0141	0.0129	0.0117	0.0093		6	13	14	8	7	12
Hap8	0.0058	0.0058	0.0093	0.0082	0.0070	0.0047	0.0047		11	10	2	3	8
Hap9	0.0129	0.0035	0.0023	0.0012	0.0047	0.0023	0.0117	0.0070		21	13	14	19
Hap10	0.0177	0.0176	0.0212	0.0200	0.0188	0.0165	0.0141	0.0117	0.0188		12	9	4
Hap11	0.0070	0.0070	0.0105	0.0093	0.0082	0.0058	0.0058	0.0012	0.0082	0.0129		3	10
Hap12	0.0105	0.0105	0.0141	0.0129	0.0117	0.0093	0.0070	0.0047	0.0117	0.0117	0.0035		6
Hap13	0.0153	0.0153	0.0188	0.0176	0.0165	0.0141	0.0117	0.0094	0.0164	0.0047	0.0105	0.0094	

The above-diagonal numbers are absolute distance values and the below-diagonal numbers are mean distance values.

Table 3 Summary of molecular diversity indices and population expansion for Thai *Heterorhabditis indica*

Region	N	No.	S	k	*h*±SD	*Pi*±SD	*D*	*Fs*	R_2
Northern	1	1	-	-	-	-	-	-	-
Eastern	9	7	21	7.55	0.9170±0.0920	0.0087±0.0019	-0.1092	0.3080	0.1415
Western	7	2	5	1.42	0.2860±0.1960	0.0016±0.0011	-1.4861	2.5080	0.3499
Southern	10	7	16	4.71	0.8670±0.1070	0.0054±0.0018	-0.7731	0.8810	0.1362
All samples	27	13	28	5.34	0.8860±0.0370	0.0062±0.0010	-0.9686	-1.8250	0.0883

N = number of individuals; No. = number of haplotypes; S = number of polymorphic (segregation) sites; k = average number of nucleotide differences; *h* = haplotype diversity; *Pi* = nucleotide diversity; *D* = Tajima's D, *Fs* = Fu's Fs, R_2 = Ramos-Onsins Rozas' R_2.

Table 4 Genetic distance and gene flow among populations of Thai *Heterorhabditis indica*

Region	Northern	Eastern	Western	Southern
Northern		∞	0.1052	∞
Eastern	0.0000		1.1348	2.4762
Western	0.7037	0.1805		0.2787
Southern	0.0000	0.0917	0.4728*	

* significant difference at $p < 0.05$

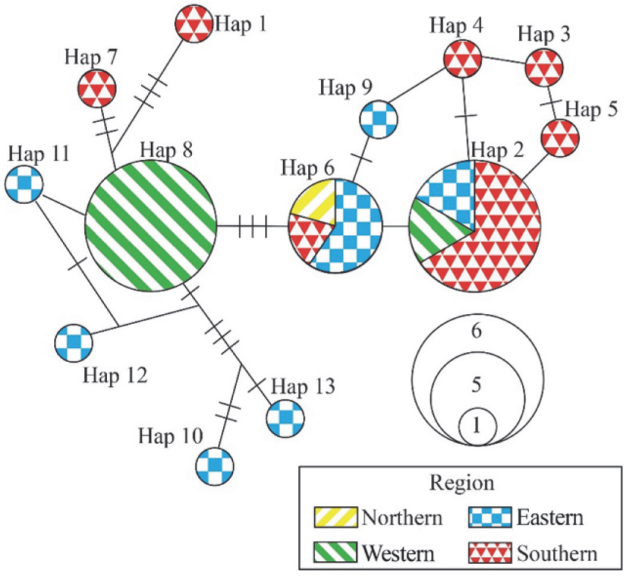

Fig. 3 Haplotype network of Thai *Heterorhabditis indica* based on internal transcription spacer fragment, where the size of circles is proportional to haplotype frequency, with the pie slices showing proportions of haplotype frequency found in different regions of Thailand and the line connecting two haplotypes indicates the haplotype relationship, while the number of perpendicular lines on the connected lines indicates the number of mutation steps.

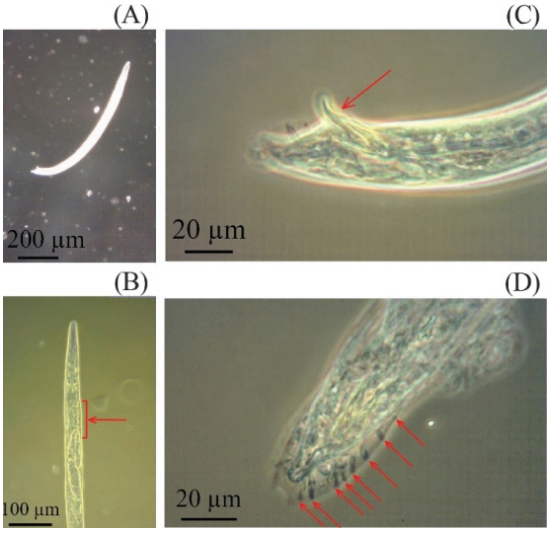

Fig. 4 Light microscopy photographs of *Heterorhabditis indica* EPNKU53 male: (A) entire worm; (B) testis reflection; (C) lateral view of posterior end and spicule, and (D) bursal papillae

Morphological identification

Based on the molecular identification, all Thai isolates were conspecific to *H. indica* Poinar, Karunakar & David. The morphological information of a representative (*H. indica* EPNKU53), all adults and IJs presented with truncated and slightly rounded head. In males, the testis was monarchic and reflexed anteriorly. Spicules were paired and separated with a pointed tip. The gubernaculum was approximately 60–70% of a spicule length. The bursa contained 8 pairs of papillae, while pair 9 was absent. The papillae formation was divided into 4 groups; the first group was the bursa (pair 1 solitary), the second group was bursa pairs 2 and 3, the third group was bursa pairs 4, 5, and 6 and the last group was bursa pairs 7 and 8 (Fig. 4). The hermaphroditic female and amphidelphic female both had a tail longer than the anal body length diameter. In addition, the anus was swollen and prominent. The lengths of these two females were similar. Lastly, the body of IJs was short and covered by a second-stage cuticle as a sheath (Fig. 5) (Table 5).

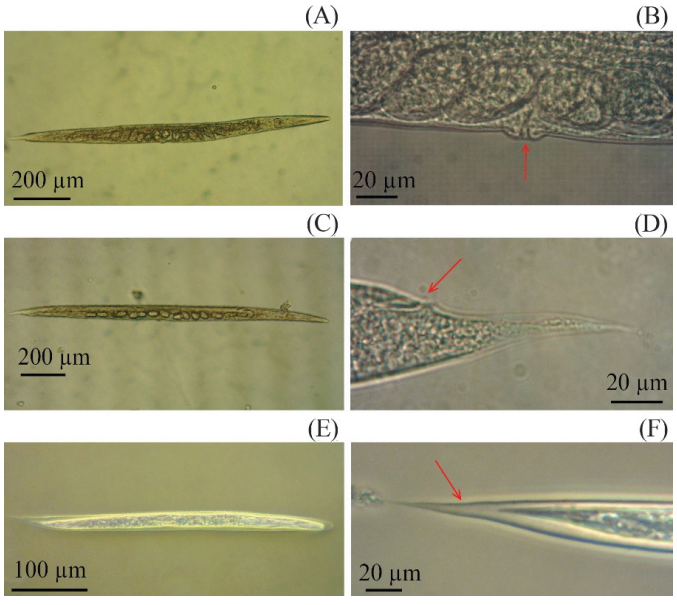

Fig. 5 Light microscopy photographs of *Heterorhabditis indica* EPNKU53: (A–B) hermaphroditic female; (A) entire worm; (B) vulva region; (C–D) amphimictic female; (C) entire worm; (D) lateral view of posterior end and prominent anal swelling; (E–F) infective juvenile; (E) entire worm; (F) lateral view of posterior end and cuticle sheath

Table 5 Morphometrics (mean ± SD) of *Heterorhabditis indica* EPNKU53

	Hermaphroditic females	Amphimictic females	Males	Infective juveniles
n	20	20	10	20
L	1091.19 ± 82.35 (916.27 – 1275.97)	1056.55 ± 123.00 (775.06 – 1189.70)	759.49 ± 75.38 (655.33 – 892.09)	375.90 ± 23.99 (306.97 – 412.23)
a	-	-	-	23.72 ± 1.42 (21.73 – 27.05)
b	-	-	-	3.79 ± 0.38 (3.38 – 5.17)
c	-	-	-	11.58 ± 1.88 (8.01 – 14.33)
V	33.20 ± 4.79 (25.67 – 46.90)	32.07 ± 6.09 (17.81 – 41.34)	-	-
MBD	65.49 ± 28.34 (5.01 – 168.74)	58.80 ± 8.34 (37.61 – 67.58)	35.90 ± 6.02 (28.16 – 48.93)	15.87 ± 1.07 (13.51 – 17.51)
EP	149.66 ± 11.81 (130.30 – 175.77)	130.36 ± 12.10 (104.39 – 149.88)	133.25 ± 18.81 (112.82 – 162.11)	78.52 ± 8.48 (58.83 – 92.99)
NR	134.99 ± 11.54 (121.32 – 157.72)	111.06 ± 10.74 (94.61 – 139.93)	115.02 ± 17.79 (94.87 – 143.52)	69.20 ± 9.12 (52.75 – 86.57)
ES	182.01 ± 8.67 (161.57 – 194.13)	157.64 ± 9.17 (135.77 – 169.77)	154.09 ± 24.43 (102.25 – 188.02)	99.91 ± 8.72 (73.08 – 113.90)
TR	-	-	113.33 ± 34.06 (74.07 – 184.89)	-
T	106.52 ± 15.19 (76.17 – 133.95)	72.43 ± 9.94 (49.28 – 83.23)	35.16 ± 9.50 (26.90 – 59.52)	33.33 ± 6.19 (24.50 – 46.26)
TS	-	-		59.59 ± 16.93 (6.31 – 93.21)
ABD	21.36 ± 3.13 (15.14 – 26.62)	22.32 ± 4.45 (13.67 – 28.35)	26.26 ± 4.30 (19.02 – 32.48)	7.71 ± 1.77 (5.53 – 12.17)
SL	-	-	38.35 ± 9.27 (27.92 – 57.96)	-
GL	-	-	20.60 ± 4.09 (16.09 – 28.12)	-
D%	82.22 ± 4.98 (71.32 – 94.95)	82.63 ± 4.97 (66.91 – 88.28)	87.30 ± 10.47 (78.18 – 115.52)	78.59 ± 4.60 (72.13 – 90.68)
E%	-	-	-	242.01 ± 45.64 (184.18 – 316.42)
SW%	-	-	158.43 ± 65.49 (89.92 – 281.91)	-
GS%	-	-	13.91 ± 4.60 (7.66 – 19.38)	-

L = total body length; a = L/MBD; b = L/ES; c = L/TL; V = vulva; MBD = maximum body diameter; EP = distance from anterior end to excretory pore; NR = distance from anterior end to nerve-ring position; ES = distance from anterior end to base of esophagus; TR = testis-reflection length; T = tail length without sheath; TS = tail length with sheath; ABD = anal body diameter; SL = spicule length; GL = gubernaculum length; D% = (EP/ES) × 100; E = (EP/TL) × 100; SW = SL/ABD × 100; GS = (GL/SL) × 100

Discussion

Morphometric and molecular analysis were used to determine that the 27 nematode samples were *H. indica*. This EPN species has been recorded in various kinds of habitat throughout the world including Thailand (Andrássy, 1976; Shaw et al., 2016). From the morphometric study, Thai *H. indica* isolates were smaller in size than the Indian isolate of *H. indica* (Nguyen and Hunt, 2007). This might be explained by the influence of geological origin on morphological characteristics of EPNs (Nguyen and Hunt, 2007; Al-Zaidawi et al., 2019). However, a common characteristic of the spicule in males was similarly expressed between Thai EPNs and the original isolate of *H. indica* (Nguyen and Hunt, 2007).

The percentage of discovered EPNs in this study was similar to the normal percentage of EPNs found in the soil at 2–45% but based on the *Galleria* trap (Hominick, 2002). In addition to previous study, discovery rates of 2.89–7.17% have been reported for various new *Heterorhabditis* isolates in Thailand (Maneesakorn et al., 2010; Noosidum et al., 2010). It was suggested that the current result was remarkable since the EPNs were generally aggregated or clumped rather than randomly dispersed in the soil (Hominick, 2002). The small number of nematodes recovered from the soil samples in this study

was caused by various rigid factors such as the soil sampling method, the low population in a natural habitat and the aggregated living form (Griffin et al., 1991).

However, repeating the *Galleria* baiting technique on the initially negative soil samples did not significantly increase the number of EPNs (Hominick and Briscoe, 1990). Likewise, in Germany, repeating the soil collection in the same area cannot ensure that EPNs would be discovered once again in the same area (Sturhan, 1999). In the current study, all *H. indica* were mostly recovered from the Eastern and Southern regions where were the coastal areas of Thailand, supporting that *Heterorhabditis* nematodes are frequently found in the nearby sea locations (Griffin et al, 2000; Hominick, 2002).

The G + C content (44.93%) of the EPNs was lower than of the A + T content (55.07%). This ratio is normally in nuclear DNA in accordance with Malan et al. (2016), who reported that the G + C content and A + T content in nuclear DNA of *Steinernema kraussei* Steiner were 39.61% and 60.39%, respectively. Therefore, the nucleotide sequences in this study were positively extracted from nuclear DNA supported by a small difference in percentage between G + C and A + T content. Since the richness of the A + T content was normally shown in mitochondrial DNA, it was high (up to 80%) in mitochondrial DNA of *Meloidogyne hapla* Chitwood (Hugall et al., 1997).

In the current study, the percentages of conserved sites, singleton informative sites, parsimony-informative sites, insertion sites and deletion sites were similar to Schenk et al. (2016) who studied the nucleotide sequences of *Plectus parientinus* Gerlach, *P. cirratus* Bastian and *Chiloplectus andrassyi* Timm in the small sub-unit (*SSU*) and the large sub-unit (*LSU*) in nuclear DNA. They found 0.20% singleton informative sites and 0.40% parsimony-informative sites in the *SSU* and 1.70% singleton informative sites and 0.60% parsimony-informative sites in the *LSU*. The nucleotide sequences in mitochondrial DNA showed 8.00% singleton informative sites and 27.40% parsimony-informative sites. The sequence divergence (0.12–2.12%) was normally found in nematode molecular studies since the sequence divergences in ITS region found were at 1–5% in the family Steinernematidae (Ali and Wharton, 2017) and Hoplolaimidae (Palomares-Rius et al., 2018). Although the percentages of sequence divergence in the ITS region were lower than the percentages of sequence divergence in the mitochondrial DNA, the percentages of sequence divergence in the ITS region were higher than the sequence divergence in the *SSU* and *LSU* (Schenk et al., 2016). This suggested that the nuclear DNA was more conservative than mitochondrial DNA (Schenk et al., 2016; Ali and Wharton, 2017; Palomares-Rius et al., 2018).

The haplotype diversity and nucleotide diversity identified in the current study were similar to that reported by Holguin et al. (2016) of low haplotype diversity (0.0000–0.1000) and nucleotide diversity in the ITS region (0.0000–0.9000) of *Hoplolaimus stephanus* Sher. It demonstrated that the genetic diversity in the ITS region was lower than in the *COI* region (Holguin et al., 2016).

The current results detected negative values of Tajima's D and Fu's Fs, but a positive value of Ramos-Onsins and Rozas' R_2. The results were in agreement with other nematode populations, such as *Haemonchus contortus* Rudolphi (*D* = -1.6100; *Fs* = -24.2000), *H. placei* Place (*D* = -1.6600; *Fs* = -16.1600), *Mazamastrongylus ocoidei* (*D* = -1.6100; *Fs* = -24.2900) and *Teladorsaqia circumcincta* Stadelman (*D* = -1.8300; *Fs* = -24.5700) (Mes, 2003). The negative values of Tajima's D and Fu's Fs in the current study indicated that the *Heterorhabditis* population had a low frequency of mutation since they had been crossing over the genetic drift (Tajima, 1989). This might have been because of the strict selective neutrality of variants, the constant population size, the lack of subdivisions or the lack of gene flow (Tajima, 1989).

In addition, the highest gene flow was between the two *Heterorhabditis* populations in the Eastern and Southern regions of Thailand because that per-generation migration rate was farthest from 1.0000. It was similar to Zhou et al. (2017) who reported that the per-generation migration rate (2.1510) of *Bursaphenlenchus xylopphilus* Xu was very high and also found the highest gene flow of *B. xylopphilus* from China. On the other hand, a lower gene flow was recorded in the population of *B. mucronatus* Mamya and Enda since its per-generation migration rate (1.0910) was close to 1.0000 (Zhou et al., 2017). Because there was more non-restricted genetic exchange and rapid spread between districts of the nematode population, there was a higher per-generation migration rate which was over 1.0000 (Robinet et al., 2009). Moreover, the current haplotype network analysis result showed that all 13 haplotypes were in a completely interconnected network supported by 1) the result of the phylogenetic tree since all EPNs were closely grouped in *H. indica* and 2) the recombination genetic or maternally inherited genetic ITS region or nuclear DNA (Schenk et al., 2016).

In conclusion, the haplotype diversity correspondingly proved that all 27 isolates of *H. indica* were the same species, exposed by a monophyletic pattern. Although, the ITS region could be used successfully to identify EPNs in the current study, other regions (ex, *COI*, *LSU* and *SSU*) should be studied. In addition, this discovery could be the starting point of future research into developing a biological agent to control insect pests.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This study was funded by The Kasetsart University Research and Development Institute (KURDI), Bangkok, Thailand, the Thailand Research Fund (MRG6080195) and the Graduate School, Kasetsart University. Dr. Ratchadawan Ngoen-Klan, Dr. Watcharapong Chumchuen and Dr. Shiela Villamor Chumchuen provided valuable assistance.

References

Adams, B.J., Fodor, A., Koppenhöfer, H.S., Stackenbrandt, E., Stock, S.P., Klein, M.G. 2006. Biodiversity and systematic of nematode-bacterium

entomopathogens. Biol. Control 37: 32–49. doi.org/10.1016/j.biocontrol.2005.11.008

Al-Zaidawi, J.B., Karimi, J., Moghadam, E.M. 2019. Molecular characterizations of the entomopathogenic nematodes, *Heterorhabditis bacteriophora* and *Oscheius myriophilus* from Iraq. Egypt. J. Biol. Pest Co. 29: 38. doi.org/10.1186/s41938-019-0136-6

Ali, F., Wharton, D.A. 2017. A survey of entomopathogenic nematodes from Otago, New Zealand, with the first record of *Steinernema kraussei* (Steiner, 1923) (Rhabditida: Steinernematidae) from the Southern Hemisphere. New Zeal. J. Zool. 44: 245–255. doi.org/10.1080/03014223.2017.1322620

Andrássy, I. 1976. Evolution as a Basis for the Systematization of Nematodes. Pitman. London, UK.

Boemare, N.E., Akhurst, R.J., Mourant, R.G. 1993. 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. 43: 249–255. doi.org/10.1099/ 00207713-43-2-249

De Ley, P., Blaxter, M. 2004. A new system for Nematoda: combining morphological characters with molecular trees, and translating clades into ranks and taxa. In: Cook, R., Hunt, D.J. (Eds.). Nematology Monographs and Perspectives. Brill Academic. Leiden, the Netherlands, pp. 633–653.

Forst, S., Nealson, K. 1996. Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. Microbiol. Rev. 60: 21–43. doi: 10.1128/M MBR.60.1.21-43.1996

Grewal, P.S., Ehlers, R-U., Shapiro-Ilan, D.I. 2005. Nematodes as Biocontrol Agents. CABI Publishing. Wallingford, UK.

Griffin, C.T., Moore, J.F., Downes, M.J. 1991. Occurrence of insect parasitic nematodes (Steinernematidae, Heterorhabditidae) in the Republic of Ireland. Nematologica 37: 92–100. doi.org/10.1163/187529291X00097

Griffin, C.T., Chaerani, R., Fallon, D., Reid, A.P., Downes, M.J. 2000. Occurrence and distribution of the entomopathogenic nematodes *Steinernema* spp. and *Heterorhabditis indica* in Indonesia. J. Helminthol. 74: 143–150. doi.org/10.1017/ S0022149X00000196

Griffin, C.T., Boemare, N.E., Lewis, E.E. 2005. Biology and behaviour. In: Grewal, P.S., Ehlers, R.-U., Shapiro-Ilan, D.I. (Eds.), Nematodes as Biocontrol Agents. CABI Publishing. Wallingford, UK, pp. 47–75.

Griffiths, B.S., De Groot, G.A., Laros, I., Stone, D., Geisen, S. 2018. The need for standardisation: exemplified by a description of the diversity, community structure and ecological indices of soil nematodes. Ecol. Indic. 87: 43–46. doi.org/10.1016/ j.ecolind.2017.12.002

Holguin, C.M., Ma, X., Mueller, J.D., Agudelo, P. 2016. Distribution of *Hoplolaimus* species in soybean fields in South Carolina and North Carolina. Plant Dis. 100: 149–153. doi.org/10.1094/PDIS-12-14-1332-RE

Hominick, W.M. 2002. Biogeography. In: Gaugler, R. (Ed.). Entomopathogenic Nematology. CABI Publishing. Wallingford, UK, pp. 115–144.

Hominick, W.M., Briscoe, B.R. 1990. Occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in British soils. Parasitology 100: 295–302. doi.org/10.1017/S0031182000061308

Huelsenbeck, J.P., Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755. doi.org/10.1093/bioinformatics/17.8.754

Hugall, A., Stanton, J., Moritz, C. 1997. Evolution of the AT-rich mitochondrial DNA of the root knot nematode*, Meloidogyne hapla*. Mol. Biol. Evol. 14: 40–48. doi.org/10.1093/ oxfordjournals.molbev.a025700

Jobb, G., Haeseler, A.V., Strimmer, K. 2004. Treefinder: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol. Biol. 4: 18. doi:10.1186/1471-2148-4-18

Kaya, H.K., Gaugler, R. 1993. Entomopathogenic nematodes. Annu. Rev. Entomol. 38: 181–206.

Malan, A.P., Knoetze, R., Tiedt, L.R. 2016. *Steinernema nguyeni* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. Nematology 18: 571–590. doi.org/10.1163/15685411-00002978

Maneesakorn, P., An, R., Grewal, P.S., Chandrapatya, A. 2010. Virulence of four new strains of entomopathogenic nematodes from Thailand against second instar larva of the Japanese beetle, *Popillia japonica* (Coleoptera: Scarabaeidae). Thai J. Agric. Sci. 43: 61–66.

Mes, T.H. 2003. Demographic expansion of parasitic nematodes of livestock based on mitochondrial DNA regions that conflict with the infinite-sites model. Mol. Ecol. 12: 1555–1566. doi: 10.1046/j.1365-294x.2003.01846.x

Nei, M., Kumar, S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press. New York, NY, USA.

Nguyen, K.B., Hunt, D.J. 2007. Entomopathogenic Nematode: Systematics, Phylogeny and Bacterial Symbionts Nematology Monographs and Perspectives. Brill Academic. Boston, MA, USA.

Noosidum, A., Hodson, A.K., Lewis, E.E., Chandrapatya, A. 2010. Characterization of new entomopathogenic nematodes from Thailand: Foraging behaviors and virulence to the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae). J. Nematol. 42: 281–291.

Palomares-Rius, J.E., Cantalapiedra-Navarrete, C., Archidona-Yuste, A., Tzortzakakis, E.A., Birmpilis, L.G., Vovla, N., Subbotin, S.A., Castillo, P. 2018. Prevalence and molecular diversity of reniform nematodes of the genus *Rotylenchulus* (Nematoda: Rotylenchulinae) in the Mediterranean Basin. Eur. J. Plant Pathol. 150: 439–455. doi: 10.1007/s10658-017-1292-8

Porazinska, D.L., Giblin-Davis, R.M., Farmerie, W., et al. 2009. Evaluating high-throughput sequencing as a method for metagenomic analysis of nematode diversity. Mol. Ecol. Resour. 9: 1439–1450. doi.org/10.1111/j.1755-0998.2009.02611.x

Reid, A.P., Hominick, W.M., Briscoe, B.R. 1997. Molecular taxonomy and phylogeny of entomopathogenic nematode (Rhabditida: Steinernematidae) by RFLP analysis of the ITS region of the ribosomal DNA repeat unit. Syst. Parasitol. 37: 187–193. doi :10.1023/A:1005843320923

Robinet, C., Roques, A., Pan, H., Fang, G., Ye J., Zhang Y., Sun, J. 2009. Role of human-mediated dispersal in the spread of the pinewood nematode in China. PLoS ONE 4: e4646. doi.org/10.1371/journal.pone.0004646

Rozas, J., Sanchez-delbarrio, J.C., Messeguer, X., Rozas, R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496–2497. doi.org/10.1093/bioinformatics/btg359

Schenk, J., Traunspurger, W., Ristau, K. 2016. Genetic diversity of widespread moss-dwelling nematode species in German beech forests. Eur. J. Soil Biol. 74: 23–31. doi.org/10.1016/j.ejsobi.2016.03.002

Seinhorst, J.W. 1959. A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. Nematologica 4: 67–69. doi.org/10.1163/187529259X00381

Shapiro-Ilan, D., Hazir, S., Glazer, I. 2017. Basic and applied research: entomopathogenic nematodes. In: Lacey, L.A. (Ed.). Microbial Control of Insect and Mite Pests. Elsevier. London, UK, pp. 91–105.

Shaw, E.A., Denef, K., De Tomasel, C.M., Cofruto, M.F., Wall, D.H. 2016. Fire affects root decomposition, soil food web structure, and carbon flow in tallgrass prairie. Soil 2: 199–210. doi: 10.5194/soil-2-199-2016

Stock, S.P., Goodrich-Blair, H. 2012. Nematode parasites, pathogens and associates of insects and invertebrates of economic importance. *In*: Lacey, L.A. (Ed.). *Manual of Techniques in Invertebrate Pathology.* Elsevier. Yakima, WA, USA, pp. 373–426.

Stock, S.P. 2009. Molecular approaches and the taxonomy of insect-parasitic and pathogenic nematodes. In: Stock, S.P., Vandenberg, J., Glazer, I., Boemare, N. (Eds.). Insect Pathogenic: Molecular Approaches and Techniques. CABI Publishing. Wallingford, UK, pp. 71–100.

Sturhan, D. 1999. Prevalence and habitat specificity of entomopathogenic nematodes in Germany. In: Gwynn, R.L., Smits, P.H., Griffin, C., Ehlers, R-U., Boemare, N., Masson, J.P. (Eds.). Entomopathogenic Nematodes: Application and Persistence of Entomopathogenic Nematodes**.** European Commission. Luxembourg, pp. 123–132.

Tajima, F. 1989. Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. Genetics 123: 585–595.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30: 2725–2729. doi: 10.1093/molbev/mst197.

Tanabe, A.S. 2007. Kakusan: A computer program to automate the selection of a nucleotide substitution model and the configuration of a mixed model on multilocus data. Mol. Ecol. Notes. 7: 962–964. doi.org/10.1111/j.1471-8286.2007.01807.x

Vrain, T.C., Wakarchuk, D.A., Levesque, A.C., Hamilton, R.I. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. Fund. Appl. Nematol. 15: 563–573.

White, G.F. 1927. A method for obtaining infective nematode larvae from cultures. Science 66: 302–303.

Zhou, L.F., Chen, F.M., Xie, L.Y., Pan, H.Y., Ye, J.R. 2017. Genetic diversity of pine-parasitic nematodes *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* in China. Forest Pathol. 47: e12334. doi.org/10.1111/efp.12334