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# Characterization of New Entomopathogenic Nematodes from Thailand: Foraging Behavior and Virulence to the Greater Wax Moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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*Abstract:* Entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* and their associated bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp., respectively) are lethal parasites of soil dwelling insects. We collected 168 soil samples from five provinces, all located in southern Thailand. Eight strains of EPNs were isolated and identified to species using restriction profiles and sequence analysis. Five of the isolates were identified as *Heterorhabditis indica,* and one as *Heterorhabditis baujardi.* Two undescribed *Steinernema* spp. were also discovered which matched no published sequences and grouped separately from the other DNA restriction profiles. Behavioral tests showed that all *Heterorhabditis* spp. were cruise foragers, based on their attraction to volatile cues and lack of body-waving and standing behaviors, while the S*teinernema* isolates were more intermediate in foraging behavior. The infectivity of Thai EPN strains against *Galleria mellonella* larvae was investigated using sand column bioassays and the $LC_{50}$ was calculated based on exposures to nematodes in 24-well plates. The $LC_{50}$ results ranged from 1.99-6.95 IJs/insect. Nine centimeter columns of either sandy loam or sandy clay loam were used to determine the nematodes' ability to locate and infect subterranean insects in different soil types. The undescribed *Steinernema* sp. had the greatest infection rate in both soil types compared to the other Thai isolates and three commercial EPNs (*Heterorhabditis bacteriophora, Steinernema glaseri* and *Steinernema riobrave*).

*Key words:* Entomopathogenic Nematodes, Foraging Behavior, *Galleria mellonella*, *Heterorhabditis*, *Steinernema.*

Entomopathogenic nematodes (EPNs) in the genera *Steinernema* Travassos and *Heterorhabditis* Poinar and their symbiotic bacteria in the genera *Xenorhabdus* Thomas & Poinar and *Photorhabdus* Boemare, Akhurst & Mourant, respectively are lethal parasites of many soil-inhabiting insects worldwide (Kaya and Gaugler, 1993; Griffin et al., 2005). These EPNs serve as important regulators of insect populations and also have been developed as biological control agents for a variety of economically important insect pests (Shapiro-Ilan et al., 2002a). EPN infective juveniles (IJs), the only free-living stage, enter hosts through natural body openings or thin cuticle and penetrate into their hemocoel. The IJs release associated bacteria which secrete bacterial toxins and also produce antibiotics that prevent the growth of other micro-organisms. Normally, the symbiotic bacteria kill the infected host within 24-48 hr. The nematodes feed on the bacterial symbionts and degraded insect tissues, molting and completing one to three generations within the host cadaver. After food resources are depleted, new IJs exit the host cadaver to find new suitable hosts (Bedding et al., 1993).

EPNs have been used successfully as biological control agents to suppress insect populations (Shapiro-Ilan et al., 2002a, b; Nguyen et al., 2006). At present, mainly non-native strains have been used in nematode applications. About 10 commercially available species are normally used worldwide and most of them have been isolated from either North America or Europe (Grewal and Peters, 2005). Since there are only relatively few commercial strains, it stands to reason that most are from places other than the locations where they are used. Therefore, these strains may not be well adapted to specific local climates and environmental conditions, hence, their efficacy might be reduced (Millar and Barbercheck, 2001; Campos-Herrera and Gutiérrez, 2009), whereas native species are adapted to local climatic conditions and are therefore more likely to survive in the target area after application. Such native nematodes can be developed as new biological control agents (Grewal et al., 2002; Lewis et al., 2006) and several previous surveys have searched for new EPN species with the intent to control important agricultural and horticultural pests under specific conditions (Phan et al., 2001a, b; Nguyen et al., 2006; Campos-Herrera and Gutiérrez, 2009). In Thailand, alternatives to chemical pesticides are needed and EPNs are used as part of integrated pest management programs for several cropping systems including vegetables and sweet potato (Somsook et al., 1986). At present, six species of EPNs formerly imported from the USA are being cultured and distributed to Thai farmers. These are *Heterorhabditis indica* Poinar, Karunakar & Davis, *Heterorhabditis bacteriophora* Poinar, *Steinernema carpocapsae* Weiser, *Steinernema glaseri* Steiner, *Steinernema riobrave* Cabanillas, Poinar & Raulston, and *Steinernema feltiae* Filipjev.

As for endemic strains, the first Thai EPN, *Steinernema siamkayai* Stock, Somsook & Reid, was recovered from a soil sample collected from a tamarind orchard at Amphor

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Lomsak, Petchaboon Province, northern Thailand in 1998 (Stock et al., 1998). Later, Hotaka (2000) found a new isolate of *H. indica* in a soil sample near the Si Yok Noi water fall, Amphor Siyok, Kanchanaburi Province, western Thailand in July 1999. In April 2007, *Steinernema minutum* Maneesakorn, Grewal & Chandrapatya was isolated from a soil sample collected in a pine forest at Amphor Mueang, Chumporn Province in southern Thailand (Maneesakon et al., 2010). During our survey of EPNs in southern Thailand in 2008, EPNs were found in soil samples collected from Surat Thani and Chumporn provinces. The objectives of this study were (i) to isolate and identify EPNs native to southern Thailand, (ii) classify their foraging behaviors and (iii) determine their infectivity to *Galleria mellonella* L. larvae using a series of laboratory bioassays. This information will serve as basic knowledge to develop new biological control agents from native insect parasitic nematodes.

## Materials and Methods

*Soil samples, nematode isolations and nematode cultures:* One hundred and sixty eight soil samples were collected from undisturbed soil close to the national parks with tropical rain forest in five southern Thailand provinces (Surat Thani, Nakorn Sri Thammarat, Ranong, Pang Nga and Chumporn) between May 2008 and February 2009. For each location, GPS coordinates (Global Positioning System; Garmin®, Thailand) and environmental characteristics such as air temperature, humidity, soil temperature, pH, electrical conductivity (EC; dS/m) and % moisture content were recorded (Table 1). EPNs were baited from soil samples using *G. mellonella* larvae (Kaya and Stock, 1997). For each soil sample, 40 last-instar *G. mellonella* larvae were placed in approximately 800 g soil in a plastic container and kept at room temperature (25-28 °C). After 7-9 days, the infected larvae from each group were then placed on a layer of moistened cotton in a Petri dish for seven to 10 days. The isolated EPNs were maintained in the laboratory by recycling through *G. mellonella* larvae (Kaya and Stock, 1997) every 1-2 months. To do this, approximately 500 IJs were applied to a 4.5 cm diam Petri dish lined with Whatman No.5 filter paper containing five last-instar *G. mellonella* larvae. The Petri dishes were incubated at room temperature until IJs emerged from the cadavers and were collected in a White trap (White, 1927). The IJs were then stored for less than three weeks at 15°C in distilled water until further testing.

*Nematode identification:* Nematodes were identified by species-distinctive PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) bands of the ribosomal DNA internal transcribed spacer (ITS) region. Representatives of each restriction profile were then sequenced and matched with known sequences using the Genbank search engine BLAST. DNA extractions and PCR conditions followed Nadler et al. (2000), the ITS-1 and ITS-2 regions of ribosomal DNA were amplified using 18S and 28S primers (no. 93, 5'TTGAACCGGGTAAAAGTCG and no. 94, 5'TTAGT TTCTTTTCCTCCGCT) and the restriction profiles were compared to the profiles of known isolates as well as restriction digest patterns generated from GENBANK sequences. Amplified products were purified using enzymatic treatment with exonuclease I and shrimp alkaline phosphatase (PCR product pre-sequencing kit, USB Corporation). Internal primers used for sequencing included no. 264 5'CGTTTTTCATCGATACG and no. 389 5'TGCAGACGCTTAGAGTG (Nadler et al., 2000) for heterorhabditid species and no. 533, 5'CAAGTCTTA TCGGTGGAT and no. 534, 5'GCAATTCACGCCAAA TAA (Stock et al., 2001) for steinernematid species. Contigs were assembled using Aligner (Version 3.6.1) and a BLAST search performed on the final consensus sequence.

Table 1. Collecting sites for Thai entomopathogenic nematodes in the southern Thailand (May 2008 and February 2009).

| Species | Locations | Provinces | GPS conditions | E | AT | RH |
|---|---|---|---|---|---|---|
| *H. indica* isolate K1 | Krom Cave 2 | Surat Thani | 0846174E 9922072N | 51 | 29 | 58 |
| *H. indica* isolate K2 | Tai Rom Yen National Park 1 | Surat Thani | 0840550E 9928372N | 430 | 26 | 80 |
| *H. indica* isolate K3 | Tai Rom Yen National Park 2 | Surat Thani | 0840433E 9928264N | 359 | 26 | 82 |
| *H. indica* isolate K4 | Tai Rom Yen National Park 3 | Surat Thani | 0840281E 9928198N | 341 | 25 | 80 |
| *H. indica* isolate K5 | Kha Min Cave 1 | Surat Thani | 0849797E 9922799N | 131 | 29 | 70 |
| *H. baujardi* isolate K6 | Kha Min Cave 2 | Surat Thani | 0849802E 9922750N | 85 | 30 | 68 |
| *Steinernema* sp. isolate K7 | Somdej Phrasrinakarin Park 1 | Chumporn | 0956847E 9902478N | 51 | 30 | 60 |
| *Steinernema* sp. isolate K8 | Somdej Phrasrinakarin Park 2 | Chumporn | 0956842E 9902470N | 51 | 30 | 60 |

E = Elevation (m), AT = Air temperature (°C), RH = relative humidity (%).

*Behavioral observations:* Nematode behavior was investigated by classifying each isolate's foraging strategy and measuring host recognition with *G. mellonella*. We examined nematode foraging behavior by measuring the difference in movement rate on smooth agar versus agar with sand sprinkled on the surface. We hypothesized that ambushing nematodes would show a significant decrease in net movement rate on sand-sprinkled agar compared with smooth agar because the presence of sand would allow them to body-wave. Agar (2%) was prepared and approximately 60 ml was poured into each Petri dish (9 cm diam) and cooled for 1 hr. The sandy dishes were prepared by sprinkling sand particles onto the top of the cooled agar. Four concentric circles of 1, 2, 3 and 4 cm diam were drawn from the center of the lid and two perpendicular lines were drawn on the lid to make four equal quadrants. Approximately 300 IJs were then placed into the center of the experimental plate and covered with the prepared lid. The numbers of IJs in each circle from opposite quadrants were counted every 10 min after they were introduced into the arena for 30 min. The net movement rate for each isolate was calculated as

$$\frac{[(2 * A) + (3 * B) + (4 * C)]}{N} * 100$$

where A, B, C were the numbers of IJs in second, third and fourth circles from the center, respectively; 2, 3 and 4 were the distances in cm from the center (2, 3 and 4 cm); and N was the total number of IJs in the opposite quadrants (Glazer and Lewis, 2000). Five replications were performed for each treatment.

In the volatile attraction assays, approximately 60 ml of 2% water agar was poured into a plastic Petri dish (nine cm diam) and cooled for one hr. Lids were marked as above. A small hole (two mm diam) was made on the lid at the edge of one quadrant and a 0.5 cm diam hole was also made at the center of the first circle of the lid. Two last-instar *G. mellonella* larvae were put into a one ml plastic pipette tip and sealed with parafilm. The lid was sealed to the plate with parafilm and modeling clay was used to affix the pipette tip to a small hole in the lid. The center hole was closed with paper tape and the arena was kept at room temperature for one hr before starting the experiment. Approximately 300 IJs were then placed into the center of experimental plate and the hole was closed with tape. The numbers of IJs in each arc in the quadrant containing the pipette tip and the one opposite were counted every 10 m after introduction for 30 m. Movement was calculated as:

$$\frac{[(2 * A) + (3 * B) + (4 * C)] - [(2 * D) + (3 * E) + (4 * F)]}{N} * 100$$

where A, B and C were the number of IJs in second, third and fourth arcs from the center in the quadrant with the pipette tip, respectively; D, E and F were the number of IJs in the second, third and fourth arcs from the center in the opposite quadrant, respectively; each number is multiplied by the distance in cm from the center for each arc, and N was the total number of IJs in the opposite quadrants (Glazer and Lewis, 2000). Five replications were performed for each treatment and their attraction rates were record.

*Virulence bioassays:* We selected four isolates; *H. indica* isolates K1 and K4, *H. baujardi* isolate K6 and *Steinernema* sp. isolate K8 to investigate nematode virulence against *G. mellonella* larvae in both filter paper assays and sand column bioassays. A 50µl drop containing 1, 3, 5, 10, 25 or 50 IJs was applied to 24 well-plates lined with Whatman No.1 filter paper. One last-instar *G. mellonella* was placed into each well after which the dish was covered and held at 25 °C, and 20 *G. mellonella* larvae were tested per concentration. Mortality rates were assessed after 24 hr.

Nematode infection rates were also determined in sand column bioassays. Two soil types, sandy loam (80% sand + 20% clay) and sandy clay loam (65% sand + 35% clay), as described by USDA (Singer and Munns, 2006), were prepared from standard sand and clay flour (No. X -23, AMACO® USA) which were 10% w/w moist. Two hundred g of soil were added to PVC columns (nine cm long, five cm diam). Three last-instar *G. mellonella* larvae were held in a small aluminum cage to restrict movement, and were then placed at the bottom of each column. Five hundred IJs in 500 µl of nematode suspension were then applied to the soil surface and the column was covered with a plastic lid. All columns were kept at room temperature and the test was replicated 10 times. Dead insects were removed daily and all cadavers were dissected to determine the number of IJs inside the infected hosts. Three commercially available EPNs (*H. bacteriophora*, *S. glaseri* and *S. riobrave*) were used as standards for comparison throughout this experiment. There were two exposure periods; one for the first 48 hr and the second for 72 hr after exposure.

*Nematode dispersal in different sand particle sizes:* Nematode motility in sand was evaluated in another sand column assay, this time without the possibility of nematodes infecting the bait insects. Sand samples were sieved through 0.5 mm (35 µm sieve) and sieved again through 0.25 mm (60 µm sieve). Coarse sand (>0.5 mm diam), medium sand (0.25-0.5 mm diam) and fine sand (<0.25 mm diam) were cleaned and baked in a forced air oven at 180 °C until dry. PVC columns (five cm diam, 10 cm length) were divided four times into 2.5 cm thick rings (depths of soil in the columns were 0-2.5, 2.5-5, 5-7.5 and 7.5-10 cm) and joined with paper tape. The last rings of each column were put on another plastic ring sealed with polyethylene nets (less than 500 mesh size) at the top side. Columns were filled with prepared sands which were moistened with distilled water (10% w/w) and covered with a Petri dish lid. Three last-instar

TABLE 2. Soil characteristics of nematode habitat (May 2008 and Febuary 2009).

| Species | ST | pH | EC | %MC |
|---|---|---|---|---|
| *H. indica* isolate K1 | 25.2 | 5.64 | 0.58 | 34 |
| *H. indica* isolate K2 | 22.5 | 5.56 | 0.43 | 24 |
| *H. indica* isolate K3 | 22.5 | 5.55 | 0.44 | 24 |
| *H. indica* isolate K4 | 23.0 | 5.56 | 0.44 | 26 |
| *H. indica* isolate K5 | 23.9 | 5.55 | 0.51 | 42 |
| *H. baujardi* isolate K6 | 24.8 | 5.80 | 0.51 | 20 |
| *Steinernema* sp. isolate K7 | 23.5 | 6.08 | 0.34 | 36 |
| *Steinernema* sp. isolate K8 | 24.2 | 6.78 | 0.37 | 32 |

ST = Soil temperature, EC = electrical conductivity (dS/m), MC = soil moisture content (%).

*G. mellonella* larvae were placed underneath the polyethylene nets, which excluded the nematodes, and approximately five hundred IJs in 500 µl of nematode suspension were then applied to the soil surface. We prevented the nematodes from infecting the hosts in this assay because IJs respond to infected insects differently than they respond to uninfected ones (Grewal et al., 1997), and this would likely affect the distribution of the nematodes in an unpredictable way. The column was covered with a plastic lid and kept at room temperature for three days. After the incubation period, the sections of the column were separated, and the sand was pushed out of each ring into a 500 ml beaker. Infective juveniles were extracted from each sample by filling the beaker that contained the sand plus nematodes, then quickly pouring the water and sand between two beakers four times. After allowing the sand to settle for a few seconds, the supernatant was poured slowly through a 400 µm sieve, and the nematodes were rinsed from the sieve into a 50 ml plastic centrifuge tube in which they were stored until counting. This test was conducted with *Steinernema* sp. isolate K8, *S. glaseri* and *H. indica* isolate K4 and the test was replicated 10 times.

*Statistical analyses:* Analyses were performed on the data collected from each set of experiments. Nematode movement rates on smooth versus sandy agar were compared using a Student Paired sample *t*-test. Nematode attraction to *G. mellonella* was compared with a one-way analysis of variance and where appropriate, means were separated using the LSD test (SPSS® Program version 15.0 and significant differences were reported when $P \leq 0.05$). Probit analysis was used to calculate $LC_{50}$ values and to calculate the respective 95% confidence intervals. The numbers of nematodes inside infected cadavers in the sand column bioassays were subjected to one-way analysis of variance and the LSD test was used to separate means where appropriate. Finally, the nematode dispersal rates in sand columns were compared among treatments by contingency table analysis. The distribution of IJs found in different sections of the columns was compared with a four column by nine row analysis. Because the distribution was not the same among treatments in the overall analysis, pairwise comparisons were made among treatments. An alternate analysis of the same data compared the nematode dispersal rates (number of IJs inside each ring) by analysis of variance followed by the LSD test. Arcsine transformation was carried out on the proportion of IJs in each section before analyses.

## RESULTS

*Nematode identification and locations:* In our survey for EPNs, eight isolates were extracted from 168 soil samples collected in southern Thailand. The EPN isolates were found in four locations; Krom Cave (8°46'E 99°22'N), Tai Rom Yen National Park (8°40'E 99°28'N), Ka Min Cave (8°49'E 99°22'N) in Surat Thani province and Somdej Phrasrinakarin Park (9°56'E 99°02'N) in Chumporn Province (Table 1). All collecting sites were located in tropical rain forest habitat; the air temperature and relative humidity at the collecting sites ranged from 25 to 30°C and 58 to 82%, respectively.

Restriction digests revealed three pattern groupings. Group 1 included isolates K1, K2, K3, K4 and K5. Group 2 included isolates K7 and K8 while isolate K6 was grouped separately. Sequence analyses of all isolates revealed that Group 1 (isolates K1, K2, K3, K4 and K5) was a 100% match for *H. indica* while K6 (Group 3) a 99% match for *Heterorhabditis baujardi* Phan, Subbotin, Nguyen & Moens (Phan et al., 2003) which is a new species record in Thailand. Isolates K7 and K8 (*Steinernema* spp.*)* are the same species but did not match any sequences closely (Table 1).

TABLE 3. Movement rate of Thai entomopathogenic nematodes in movement test between smooth agar plate versus sandy agar plate.

| Species | Average movement rates ($\bar{X}$±SE) between smooth plate and sandy plate (cm) | | |
|---|---|---|---|
| | 10 min | 20 min | 30 min |
| *H. indica* isolate K1 | 0.09±0.03 / 0.19±0.02ns | 0.11±0.02 / 0.17±0.03ns | 0.23±0.03 / 0.28±0.02ns |
| *H. indica* isolate K2 | 0.10±0.03 / 0.06±0.01ns | 0.18±0.04 / 0.09±0.03ns | 0.42±0.07 / 0.21±0.06ns |
| *H. indica* isolate K3 | 0.15±0.03 / 0.13±0.06ns | 0.19±0.02 / 0.15±0.06ns | 0.28±0.04 / 0.19±0.04ns |
| *H. indica* isolate K4 | 0.41±0.04 / 0.31±0.01ns | 0.70±0.08 / 0.41±0.08ns | 0.74±0.08 / 0.54±0.06ns |
| *H. indica* isolate K5 | 0.17±0.04 / 0.10±0.03ns | 0.31±0.06 / 0.20±0.04ns | 0.39±0.04 / 0.31±0.05ns |
| *H. baujardi* isolate K6 | 0.49±0.06 / 0.53±0.04ns | 0.73±0.05 / 0.77±0.03ns | 0.83±0.04 / 0.89±0.05ns |
| *Steinernema* sp. isolate K7 | 0.26±0.04 / 0.21±0.04ns | 0.38±0.02 / 0.48±0.01ns | 0.56±0.04 / 0.69±0.05ns |
| *Steinernema* sp. isolate K8 | 0.27±0.05 / 0.35±0.07ns | 0.34±0.04 / 0.54±0.05ns | 0.50±0.05 / 0.70±0.06ns |

ns = non significant different between two results ($P \leq 0.05$; *t*-test).

TABLE 4. Movement rate of Thai EPNs in behavioral test.

| Species | Nematode net movement rates ($\bar{X}$ ±SE) in host attraction test (cm) | | |
|---|---|---|---|
| | 10 min | 20 min | 30 min |
| *H. indica* isolate K1 | 0.004±0.01bA | 0.043±0.02bAB | 0.071±0.02aB |
| *H. indica* isolate K2 | 0.004±0.02bA | 0.049±0.06bA | 0.027±0.02aA |
| *H. indica* isolate K3 | 0.024±0.02bA | 0.080±0.02abA | 0.062±0.03aA |
| *H. indica* isolate K4 | 0.035±0.03bA | 0.083±0.03abA | 0.090±0.03aA |
| *H. indica* isolate K5 | 0.004±0.04bA | 0.003±0.05bA | 0.020±0.10aA |
| *H. baujardi* isolate K6 | 0.141±0.04aA | 0.164±0.03aA | 0.113±0.03aA |
| *Steinernema* sp. isolate K7 | 0.013±0.01bA | 0.012±0.01bA | 0.045±0.03aA |
| *Steinernema* sp. isolate K8 | 0.003±0.01bA | 0.001±0.03bA | 0.014±0.04aA |

Means followed by the same letters are not significantly different at the 5% level as determined by LSD ($\alpha = 0.05$). Lowercase letters compare means in columns, uppercase letters compare means in rows.

*Soil characteristics:* All *Heterorhabditis* spp. were found in Surat Thani province where soil temperature ranged from 22.5-25.2°C and soil moisture contents varied between 20-42%. All nematodes were isolated from acidic soil (pH 5.55 to 5.80) and the EC varied from 0.43 to 0.58 dS/m. Both *Steinernema* spp. (isolates K7 and K8) were recovered from Chumporn province where soil temperature varied between 23.5 and 24.2°C and moisture contents from 32 to 36% were recorded. All steinernematids were isolated from neutral pH soil (6.08 to 6.78), and the EC values of the soil from which isolates K7 and K8 were isolated were 0.34 and 0.37 dS/m, respectively (Table 2).

*Nematode Behavioral tests:* All *Heterorhabditis* spp. isolates (K1, K2, K3, K4, K5 and K6) in this study were highly attracted to volatile cues with positive movement rates of 0.003-0.164 cm/IJ (Table 4). Their movement on surfaces with different characteristics (sandy versus smooth agar) did not significantly differ ($P > 0.05$) (Table 3). We did not observe body-waving, standing or jumping behaviors in any of these isolates (Table 5). S*teinernema* spp. isolates (K7 and K8) were also attracted to host volatiles (0.001-0.045 cm/IJ), and like the *Heterorhabditis* species, showed no difference in movement rates between sandy and smooth surfaces. Neither body-waving nor jumping was observed (Tables 3-5).

*Nematode virulence:* The $LC_{50}$ values of four EPN isolates (K1, K4, K6 and K8) to *G. mellonella* ranged from 1.99-6.95 IJs/larva. All *Heterorhabditis* spp. had lower $LC_{50}$ values than *Steinernema* spp. *Heterorhabditis indica* isolate K4 required of the fewest IJs of any isolate (1.99 IJs/larvae) to induce 50% mortality of last-instar *G. mellonella* larvae in the filter paper assays (Table 6).

In sand column bioassays, *Steinernema* sp. isolate K8 showed the greatest infection rate in both soil types at 48 hr after exposure when compared with the other Thai isolates and three commercially produced EPNs (*H. bacteriophora, S. glaseri* and *S. riobrave*) (Figs. 1 and 2). In sandy loam (80% sand + 20% clay), isolate K8 had a significantly higher number of IJs inside the infected host (150.40±43.42 IJs/column) compared to the other Thai isolates and commercial isolates ($F=26.37$; df = 7, 80; $P < 0.001$), where the number of IJs ranged from only 20-77 IJs/column. On the other hand, the *Heterorhabditis* spp. did not differ significantly in the number of IJs inside the hosts compared to the commercial strains ($P > 0.05$) (Fig. 1). In sandy clay loam (65% sand + 35% clay), isolate K8 had the greatest infection rate after 48 hr (95.10±23.32 IJs/column) ($F=31.77$; df = 7, 80; $P < 0.001$) (Fig. 2). All Thai *Heterorhabditis* spp. failed to invade *G. mellonella* after 48 hr of exposure, but two of the commercial strains (*H. bacteriophora* and *S. riobrave*) achieved an infection rate of 3-28 IJs/column. Infection rates did not differ among strains when the exposure duration was 72 hr (Figs. 1 and 2).

*Nematode dispersal in different sand particle sizes:* Nematode motility was affected by the size distribution of sand particles in the columns when they were prevented from infecting the hosts. After the incubation period, the highest numbers of IJs were recorded in the bottom layer of the coarse sand columns in all three nematode isolates (Fig. 3). However, in columns with medium and

TABLE 5. Summary of nematode behavioral tests for all Thai isolates.

| Species | Foraging strategies | body-wave | Jumping | Dispersal decreased by sand | Attraction increase by host |
|---|---|---|---|---|---|
| *H. indica* isolate K1 | cruiser | No | No | No (p>0.05) | Yes (+) |
| *H. indica* isolate K2 | cruiser | No | No | No (p>0.05) | Yes (+) |
| *H. indica* isolate K3 | cruiser | No | No | No (p>0.05) | Yes (+) |
| *H. indica* isolate K4 | cruiser | No | No | No (p>0.05) | Yes (+) |
| *H. indica* isolate K5 | cruiser | No | No | No (p>0.05) | Yes (+) |
| *H. baujardi* isolate K6 | cruiser | No | No | No (p>0.05) | Yes (+) |
| *Steinernema* sp. isolate K7 | Intermediate | Lift their bodies | No | No (p>0.05) | Yes (+) |
| *Steinernema* sp. isolate K8 | Intermediate | Lift their bodies | No | No (p>0.05) | Yes (+) |

TABLE 6. $LC_{50}$ of *Heterorhabditis* spp. and *Steinernema* sp. against last instar *Galleria mellonella* larvae in filter paper bioassays at 48 hr after application.

| Isolates | % mortality* Concentrations (IJs/larva) 1 | 3 | 5 | 10 | 20 | 50 | $LC_{50}$ (IJs/larva) |
|---|---|---|---|---|---|---|---|
| *H. indica* isolate K1 | 0 Aa | 40 Bb | 80 Bc | 80 Ac | 100 Ac | 100 Ac | 3.75 AB |
| *H. indica* isolate K4 | 30 Aa | 40 Ba | 100 Bb | 100 Ab | 100 Ab | 100 Ab | 1.99 A |
| *H. baujardi* isolate K6 | 0 Aa | 10 Aa | 80 Bb | 80 Ab | 90 Ab | 100 Ab | 4.99 AB |
| *Steinernema* sp. isolate K8 | 0 Aa | 40 Bb | 30 Ab | 90 Ac | 70 Bc | 80 Ac | 6.95 B |

*Means followed by the same letters are not significantly different at the 5% level as determined by LSD ($\alpha = 0.05$). Capital letters compare means in columns, small letters compare means in rows.

fine grain sand, the highest numbers of IJs remained in the upper layer at 5-7.5 cm depth (Fig. 4 and 5). The overall contingency table analysis showed that among all treatments of nematode species, that the vertical distribution of nematodes in the sand columns was influenced by the size of the sand grains ($\chi^2 = 4000.93$; df = 24; $P < 0.001$). The nematode strains also had different vertical distributions from each other ($\chi^2 = 254.98$; df = 6; $P < 0.001$) and different sand particle sizes affected species differently ($\chi^2 = 3030.85$; df = 6; $P < 0.001$). The overall analysis of variance showed significant differences among the various treatment combinations (F = 21.51; df = 35, 360; $P < 0.001$) with respect to IJ vertical distribution within sand columns.

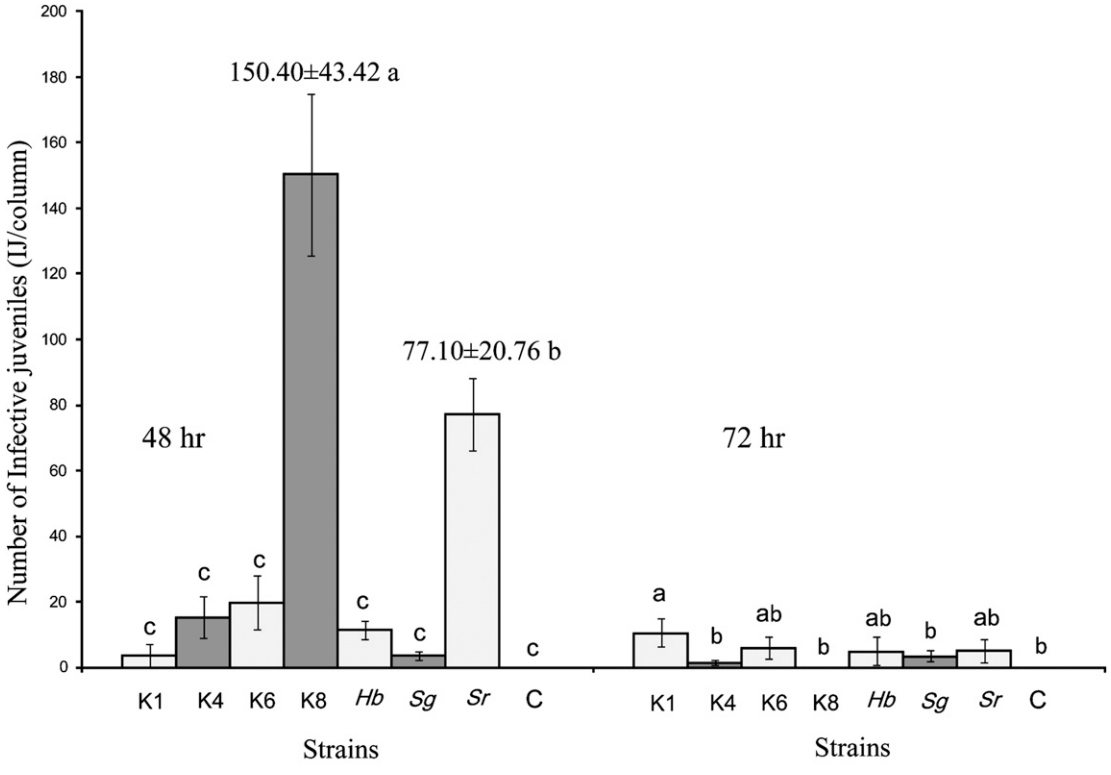



FIG. 1. Number of infective juveniles in sand column bioassays of *Heterorhabditis* spp. and *Steinernema* spp. infecting last-instar *Galleria mellonella* larvae in sandy loam (80% sand + 20% clay) at 48 and 72 hr after exposure. The same letters above the bars (within the same exposure period) indicate no significant difference among means at $P = 0.05$. Bars show standard error of the means.

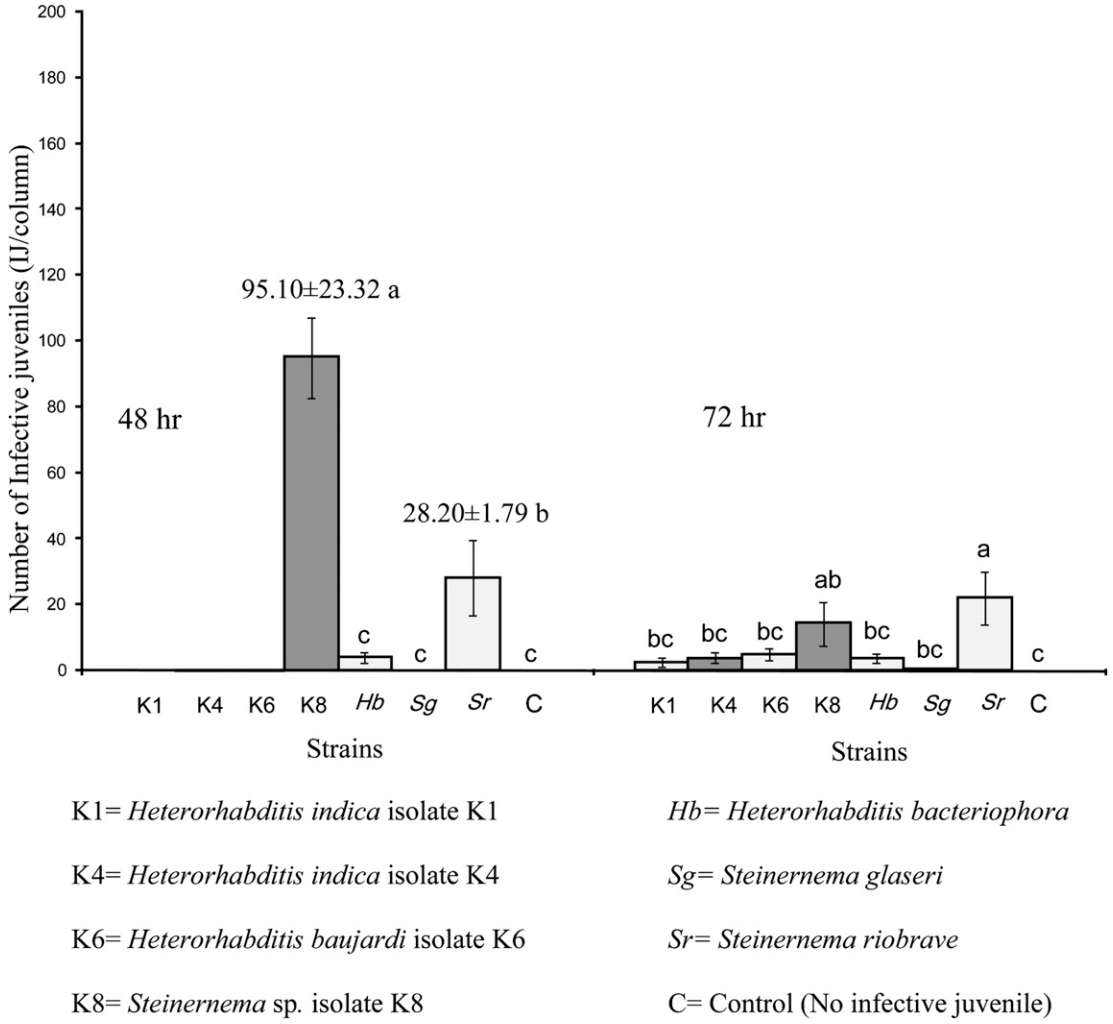



FIG. 2. Number of infective juveniles in sand column bioassays of *Heterorhabditis* spp. and *Steinernema* spp. infecting last-instar *Galleria mellonella* larvae in sandy clay loam (65% sand + 35% clay) at 48 and 72 hr after exposure. The same letters above the bars (within the same exposure period) indicate no significant difference among means at $P = 0.05$. Bars show standard error of the means.

The result also showed a significant interaction between the main effects within the comparisons of nematode strains and different sand particle sizes (Fig. 3–6).

## DISCUSSION

The effectiveness of EPNs typically depends on a combination of nematode foraging strategy, insect host species, host location, soil conditions (soil type, pH, soil moisture, etc.), climate (Glazer and Lewis, 2000) and application methods (Shapiro-Ilan et al., 2006). Ecological studies are recommended to develop hypotheses about the abilities and limitations of new EPN species (Shapiro-Ilan et al., 2006). The new EPN species here were found in very diverse habitats and local conditions. We isolated *H. indica,* which has already been found in Thailand, *H. baujardi,* which is a new record for Thailand, and an undescribed species of steinernematid nematode (isolates K7 and K8, same species). The degree of nematode diversity we observed is similar to that reported in other surveys conducted in Southeast Asia (Phan et al., 2003; Mráček et al., 2006). For example, Phan et al., 2003 isolated *H. indica* and *H. baujardi* in Vietnam.

Previous studies have demonstrated that endemic EPNs can be employed as biological control agents (Phan et al., 2001a, b; Nguyen et al., 2006; Campos-Herrera and Gutiérrez, 2009). However, native EPNs may be less or more efficacious than exotic strains depending on the foraging strategy of each EPN and which hosts are targeted (Shapiro-Ilan and Cottrell, 2005; Berry et al., 1997). EPNs have been categorized into 3 behavioral classes; ambusher, cruiser and intermediate foragers, based on a suite of behavioral characteristics (Lewis, 2002). All *Steinernema* spp. in our study were intermediate foragers according to the criteria discussed by Lewis (2002). Both *Heterorhabditis* spp. isolated in this study employed the cruiser strategy, and our data were similar to previously reported foraging behaviors for *H. bacteriophora* (Lewis et al., 1992; Kaya and Gaugler, 1993). We did not find any isolates that had characteristics of ambushers. In our experiments, the two *Heterorhabditis* species isolated from Thailand

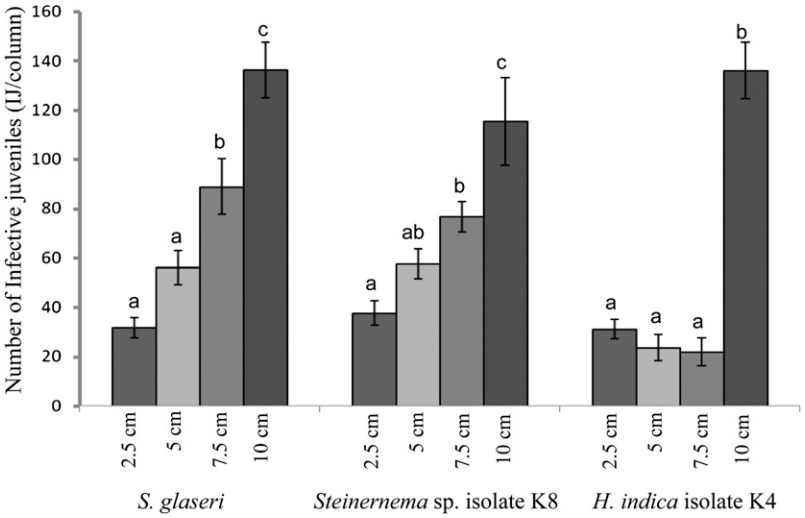



FIG. 3. Number of infective juveniles in nematode dispersal observation of *Steinernema* spp. and *Heterorhabditis* sp. in different depths of sand column within coarse sand at 72 hr after exposure. The same letters above the bars (within the same species) indicate no significant difference among means at $P = 0.05$. Bars show standard error of the means.

showed high infection rates in *G. mellonella* larvae. In addition, *Steinernema* sp. isolate K8 generally tended to be moderate in virulence to *G. mellonella* relative to other isolates from Thailand.

EPN virulence can be measured by several different methods including the one-on-one bioassay, dose-response tests providing a calculated $LC_{50}$ value, establishment efficiency, invasion rate and the sand column bioassay (Glazer and Lewis, 2000; Grewal, 2002). Two bioassay methods, the dose-response test and the sand column bioassay, were used to evaluate Thai nematode virulence against last-instar *G. mellonella* in this study. The $LC_{50}$ value of *H. indica* isolates K1 and K4, *H. baujardi* isolate K6 and *Steinernema* sp. isolate K8 against *G. mellonella* larvae ranged from 1.99-6.95 IJs/larva. These values are similar to Ricci et al. (1996) who reported that 15 and 50 IJs of *S. riobrave* s and *S. feltiae* respectively caused 100% insect mortality to *G. mellonella* larvae after 48 hr of incubation and Ansari et al. (2003) revealed that the $LC_{50}$ values of *S. glaseri* and *Heterorhabditis megidis* Poinar, Jackson & Klein for *Hoplia philanthus* Füessly were 4.6 and 9.7 IJs/larvae, respectively. In contrast, one or just a few IJs of *S. carpocapsae, S. feltiae or S. riobrave* kill 50% *G. mellonella* larvae (Converse and Miller, 1999; Grewal, 2002). Moreover, Ehlers et al. (1997) reported that 20 IJs of *S. feltiae* caused 90% mortality of *Tipula oleracea* L. The low $LC_{50}$ values obtained in this study may be partly due to the small arena and the use of the extremely susceptible host, *G. mellonella*.

The effect of soil type on nematode infectivity also varied among nematode species. Isolate K8 showed the highest infectivity in both soil types, but had lower

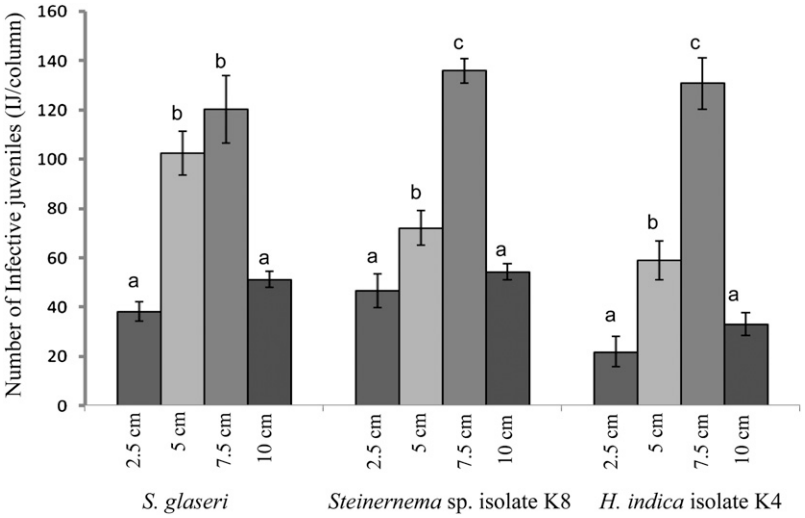



FIG. 4. Number of infective juveniles in nematode dispersal observation of *Steinernema* spp. and *Heterorhabditis* sp. in different depths of sand column within medium sand at 72 hr after exposure. The same letters above the bars (within the same species) indicate no significant difference among means at $P = 0.05$. Bars show standard error of the means.

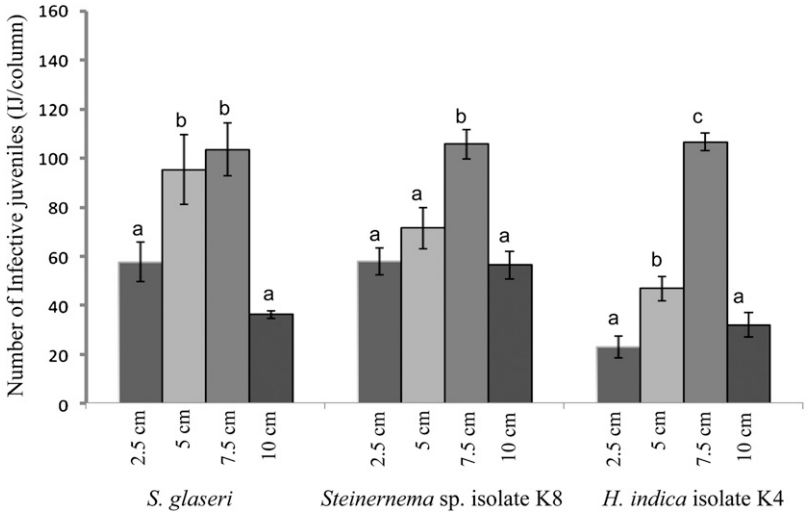
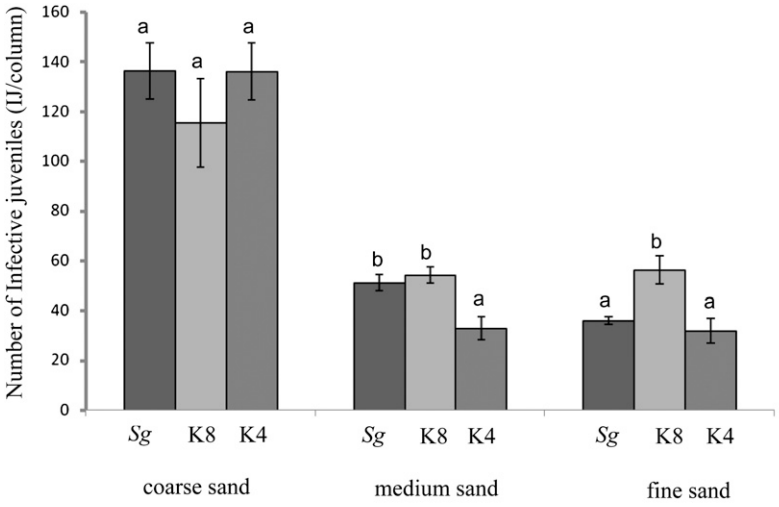



FIG. 5. Number of infective juveniles in nematode dispersal observation of *Steinernema* spp. and *Heterorhabditis* sp. in different depths of sand column within fine sand at 72 hr after exposure. The same letters above the bars (within the same species) indicate no significant difference among means at $P = 0.05$. Bars show standard error of the means.

infectivity in the soil with the higher clay content. The nematode dispersal assay was designed to evaluate the ability of the nematodes to move vertically through the sand profile. Our results indicate that more IJs of all strains were found in the bottom layer of the column filled with coarse sand. On the other hand, all nematode strains moved less in medium and fine sand, and the greatest proportions of all three strains were found in the middle layer. Results from the nematode dispersal assay were in agreement with results from the sand column bioassays, in that finer particle sizes in soil inhibit nematode movement. Our results agree with many reports showing that nematode efficacy is significantly influenced by soil type in general and particle size in particular (Molyneux and Bedding, 1984; Kaspi et al., 2010). Most EPN species are not effective in heavily textured soils (Georgis and Poinar, 1983; Choo and Kaya, 1991) and the infectivity of EPNs increased with soil sand content (Molyneux and Bedding, 1984; Kung et al., 1990; Portillo-Aguilar et al., 1999; Koppenhöfer and Fuzy, 2006). Laboratory, greenhouse and field experiments have shown that infectivity was positively correlated with the percentage of sand, silt and organic matter (Choo and Kaya, 1991; Koppenhöfer and Fuzy, 2006, 2007; Campos-Herrera et al., 2008) and negatively correlated with the percentage of clay and electrical conductivity (Georgis and Poinar, 1983; Choo and Kaya, 1991; Campos-Herrera et al., 2008). This is probably




*Sg*= *Steinernema glaseri*; K4= *Heterorhabditis indica* isolate K4; K8= *Steinernema* sp. isolate K8

FIG. 6. Number of infective juveniles in nematode dispersal observation of *Steinernema* spp. and *Heterorhabditis* sp. in 10 cm depth of sand column within coarse, medium and fine sand at 72 hr after exposure. The same letters above the bars (within the same sand type) indicate no significant difference among means at $P = 0.05$. Bars show standard error of the means.

because nematode movement is restricted in finer textured soils due to smaller soil pores causing poor aeration. Hence, smaller soil pores resulted in the reduction of nematode respiration, nematode survival and their efficacy (Burman and Pye, 1980; Portillo-Aguilar et al., 1999; Koppenhöfer and Fuzy, 2007).

However, our results from the sand column bioassay differed from what would be predicted by the filter paper bioassay. In the filter paper test, most heterorhabditid nematodes showed higher efficacy (lower $LC_{50}$) than steinernematid nematodes. In the sand column bioassay, the *Steinernema* species were more efficient at infecting *G. mellonella* larvae than were *Heterorhabditis* species. Deciding on the best test of nematode virulence depends on the intended use in the field. Our results from the filter paper bioassay and sand column bioassay differed; *Heterorhabditis* spp. out-performed *Steinernema* sp. isolate K8 in the filter paper bioassay whereas *Steinernema* sp. isolate K8 had greater infection rates in the sand column bioassay. This reemphasizes that a single bioassay does not often supply sufficient information to develop hypotheses about nematode efficacy in the field (Glazer and Lewis, 2000; Grewal, 2002). The filter paper bioassay is a rapid and simple method to screen for nematode virulence, but removes any environmental barriers to infection, while the sand column bioassays are closer to field conditions. Several previous studies have indicated that the sand column bioassay is a better standard tool for predicting EPN efficacy in a field trial, especially when soil-dwelling insect pests are considered (Molyneux, 1986; Mannion and Janssan, 1993; Grewal, 2002). For example, Ricci et al. (1996) reported that the results from a sand column assay differed from the results from tests conducted in multi-well plates. In multi-well tests, mortality of *G. mellonella* larvae due to IJs of *S. riobrave* and *S. feltiae* was high after 48 hrs exposure compared with the mortality rates caused by *H. bacteriophora* and *S. scapterisci*. On the other hand, the same study found that the highest mortality of *G. mellonella* larvae in a sand column assay was caused by *S. feltiae* followed by *H. bacteriophora*, whereas *S. riobrave* and *S. scapterisci* caused low mortality after the same exposure.

In summary, we discovered one potential new nematode species (*Steinernema* sp. isolate K8) from Thailand and established the presence of *H. indica and H. baujardi*. The undescribed species (*Steinernema* sp. isolate K8) shows potential as a biological control agent because it was effective, even in high clay soils. Moreover, this species out performed three commercial nematodes in our assays. Therefore, the possibility for using this species in biological control programs is of considerable interest. However, its effectiveness remains to be confirmed against agricultural pests both in the greenhouse and in field trails.

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