

# Isolation and Molecular Identification of Entomopathogenic Nematodes (*Steinernema* and *Heterorhabditis*) from East Java and Bali (Isolasi dan Identifikasi Molekuler Nematoda Entomopatogen [*Steinernema* dan *Heterorhabditis*] dari Jawa Timur dan Bali)

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## ABSTRAK

Nematoda entomopatogen (NEP) dari keluarga Steinernematidae dan Heterorhabditidae adalah salah satu alternatif agen pengendali hayati terbaik untuk serangga hama. Isolat lokal bisa lebih efektif dibanding dengan yang diimpor karena daya adaptabilitasnya yang lebih tinggi terhadap kondisi lingkungan sekitarnya. Penelitian ini bertujuan mengisolasi nematoda dari Jawa Timur dan Bali dan mengidentifikasinya secara molekuler. Enam puluh delapan sampel tanah berpasir dari enam belas lokasi di daerah pantai dan pertanian diumpan dengan larva “ulat Hongkong” (*Tenebrio molitor*). Lima isolat *Heterorhabditis* dan dua isolat *Steinernema* berhasil diperoleh dari 7 sampel tanah (10% dari total sampel) dari 7 lokasi (44% dari total lokasi). Analisis sekuen nukleotida daerah *internal transcribed spacer* (ITS) 1 dan 2 dari DNA ribosomal menunjukkan bahwa kelima *Heterorhabditis* termasuk ke dalam spesies “indica” dengan kesamaan 99–100% dengan sekuen publik. Salah satu isolat *Steinernema* memiliki juvenil infektif dengan panjang tubuh rata-rata 456  $\mu\text{m}$  dan kisaran 360–547  $\mu\text{m}$  serta 99% kesamaan sekuen nukleotida dengan *S. huense* yang termasuk ke dalam kelompok “carpocapsae”. Isolat *Steinernema* lainnya (DKS1) memiliki tubuh juvenil infektif yang lebih panjang (rata-rata 672  $\mu\text{m}$  dan kisaran 548–762  $\mu\text{m}$ ) dan 95% kesamaan sekuen nukleotida dengan *S. pakistanense* yang merupakan anggota ke-lompok “bicornutum”. Kajian lebih mendalam terhadap morfologi *Steinernema* DKS1 sangat diperlukan untuk mengonfirmasi identitas spesiesnya. Bila perluasan daerah eksplorasi ke habitat yang beragam dilakukan, diduga akan ditemukan spesies NEP yang sebelumnya tidak pernah dilaporkan di Indonesia.

**Kata kunci:** *Heterorhabditis*, *Steinernema*, *internal transcribed spacer*.

## ABSTRACT

Entomopathogenic nematode (EPN) of the families Steinernematidae and Heterorhabditidae is one of the best biological control agents of insect pests. Native isolates maybe more efficacious in controlling insect pests than imported ones because they have adapted to local environment. This study aimed to isolate and identify both nematode families from East Java and Bali using DNA analysis. Sixty eight soil samples obtained from sandy soils in sixteen sites of coastal regions and agricultural fields were tested for the presence of nematodes by baiting method with mealworm larvae (*Tenebrio molitor*). Five *Heterorhabditis* and two *Steinernema* were recovered from 7 soil samples (10% of total samples) of 7 sites (44% of total sites). Sequence analysis of internal transcribed spacer (ITS) 1 and 2 regions of ribosomal DNA revealed that all *Heterorhabditis* belonged to “indica” species, with 99–100% nucleotide sequence similarities to published sequences. One of the *Steinernema* isolates had infective juveniles with short body length (mean 456  $\mu\text{m}$  and range 360–547  $\mu\text{m}$ ) and shared 99% nucleotide similarity to the sequence of *S. huense*, a member of “carpocapsae” group. The other *Steinernema* isolate (DKS1) showed longer body length of infective juveniles (mean 672  $\mu\text{m}$  and range 548–762  $\mu\text{m}$ ) and shared 95% nucleotide similarity to the sequence *S. pakistanense*, which belongs to “bicornutum” group. More detailed studies with respect to morphology are required for species confirmation of DKS1 isolate. Further exploration into diverse habitat will likely to result in more previously unrecorded EPN species in Indonesia.

**Keywords:** *Heterorhabditis*, *Steinernema*, internal transcribed spacer.

## INTRODUCTION

Entomopathogenic nematodes (EPNs) from the genus *Steinernema* and *Heterorhabditis* (Rhabditida: Steinernematidae and Heterorhabditidae) are one of the best non-chemical alternatives for the control of insect pests, mainly due to their ability to reach insects in cryptic habitats, their highly reproductive ability, and their safety for humans, other vertebrates, and environment (Kaya and Gaugler 1993). They can be mass-produced and formulated as biopesticide, are compatible with some insecticides, and have a broad spectrum of target hosts from the class Insecta in a variety of habitats (Kaya and Gaugler 1993).

These nematodes have mutualistic symbioses with bacteria from the genera *Xenorhabdus* and *Photorhabdus*, respectively (Kaya and Gaugler 1993). The third-stage infective juvenile (IJ) of these families is non-feeding, free-living stage in the soil, where they can survive for extended periods and invade their host through insect's openings to reach the hemocoel where the symbiotic bacteria are released (Kaya and Gaugler 1993). The nematodes defeat the insect immune response elicited by hemocoel several hours prior to the release of bacterial cells (Koppenhöfer and Gaugler 2009). The bacteria replicate and produce virulence factors that rapidly kill the insect host within 24–72 hours by causing septicemia and toxicemia (Burnell and Stock 2000). The bacteria thus provide nutrients for the nematode's development and reproduction within the insect cadaver. When food resources are depleted, IJs emerge from the insect cadaver and search for a new host.

EPN is distributed in all continents except Antarctica (Hominick 2002). There are 84 validated species of *Steinernema* (Nguyen 2014), 19 of *Heterorhabditis* (Nguyen 2014), and 1 of *Neosteinernema* that have been described (Vitta et al. 2017). High EPN species diversity was found in China, with 15 and 4 species of *Steinernema* and *Heterorhabditis*, respectively (Wang et al. 2014) and in Pakistan with 10 and 2 species of *Steinernema* and *Heterorhabditis*, respectively (Shahina et al. 2017). With the aid of molecular techniques, the number of identified new species of EPNs and their symbiotic bacteria will likely increase, especially from Africa and Asia (Nikdel et al. 2010; Noosidum et al. 2010; Kanga et al. 2012; Yan et al. 2016; Shahina et al. 2017). While many surveys have been conducted in other parts of the world, only few surveys that produced valid results have been conducted in Indonesia (Griffin et al. 2000; Chaerani et al. 2007; Baliadi 2011; Rahardjo et al. 2014). Due to its diverse ecological

systems, the chance of finding new species, especially *Steinernema*, is high in Indonesia. This is because *S. hermaphroditum*, the first known self-fertile hermaphrodite of steinernematid, was first discovered in Indonesia (Griffin et al. 2000; Stock et al. 2004).

The collection of indigenous EPNs is a major interest from biological control and biodiversity aspects (Ansari et al. 2007). Native EPNs have already adapted to local environment, which could help increase the success rate of biological control program of local insect pests (Tangchitsomkid and Sontirat 1998; Campos-Herrera et al. 2007; Noosidum et al. 2010; Malan et al. 2011; Yan et al. 2016). Good examples of successful searches for and subsequent use of EPNs for biological control in the field were shown by the isolation of *S. scapterisci* and *S. riobraves*, which were later sold for controlling mole crickets and citrus root weevils, respectively, in the USA (Nguyen et al. 2007).

Identification of EPNs is a prerequisite for proper classification, biodiversity studies, and their potential use in pest management programs (Nasmith et al. 1996). Because overlapping morphological characters are common among species within each genus, molecular tools are needed to identify and distinguish species. Sequencing of different regions of the genome has become the most useful approach for species delimitation and also for assessing phylogenetic relationship (Valadas et al. 2014). Among nuclear genes, ribosomal DNA (rDNA) genes have been used extensively for species identification of nematode and phylogenetic inference (Valadas et al. 2014). Ribosomal DNA contains internal transcribed spacer (ITS) array, which consists of the entire ITS1, 5.8S rRNA gene, and ITS2 regions of the nuclear rDNA cistron. The 5.8S gene sequence is highly conserved, whereas the ITS1 and ITS2 sequences are more variable and highly polymorphic, but are more similar within species and more divergent between species (Valadas et al. 2014).

This study aimed to isolate EPNs in East Java and Bali Provinces and identify their species by means of sequencing the ITS1 and ITS2 regions of rDNA. The study was a part of the efforts to develop biological control program of local insect pests.

## MATERIALS AND METHODS

### Soil Sampling and Nematode Isolation

This study used the same sampling strategy implemented by Chaerani et al. (2007). Soil with

sandy textures and vegetation covers in coastal regions of north-western and eastern East Java Province and of northern and southern Bali Province was collected in March 2013 (Table 1). With one exception, an agricultural field in inland of East Java with sandy soils was also surveyed. Each sample was a composite of 5–10 random sub-samples collected from the same location and spaced about 10 m away from each other. Soils were taken by using a hand trowel to a depth of 10–15 cm, where insect hosts were suspected to dwell. Between sampling sites, the trowel was rinsed with water and dried with tissue paper to prevent cross contamination. Samples were placed in plastic bags and about 1 kg of soils was transported to the laboratory.

### Nematode Isolation and Culture Establishment

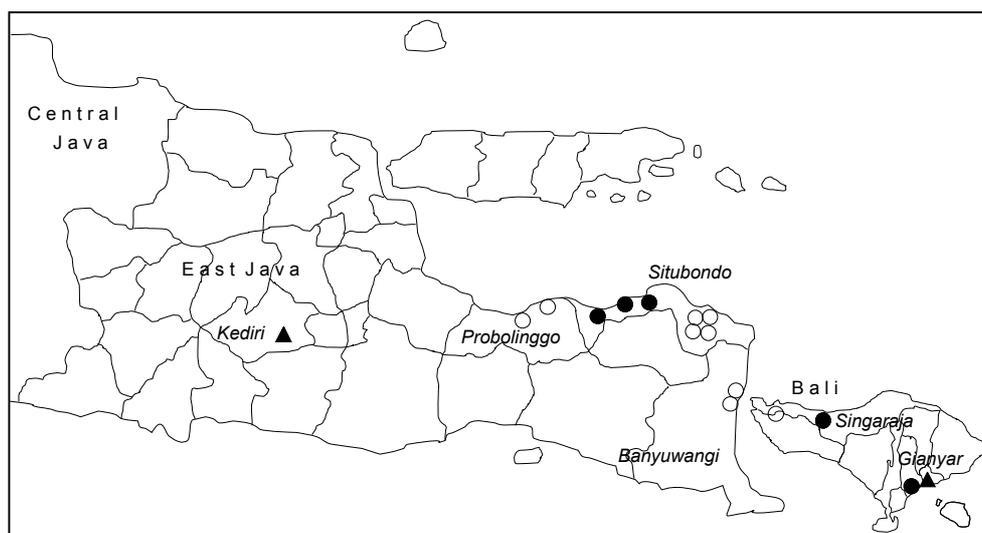
Each soil sample was shaken to uniformly mix the particles and make soils friable. Each sample was further divided into 3–4 sub-samples for nematode isolation using the insect baiting technique (Bedding and Akhurst 1975). Five to ten mealworm larvae

(*Tenebrio molitor*, Coleoptera: Tenebrionidae) were placed in the bottom of 250-ml jam jars or plastic cups and filled with soils. Each jar or cup was covered with a lid and incubated in the dark at room temperature. Where necessary, soil was moistened with 7–15 ml tap water prior to baiting. After 5–7 days, dead larvae (cadaver) showing color changes, i.e. light brown or cream color and brick red or brown, which indicates infection by *Steinernema* and *Heterorhabditis*, respectively (Figure 2), were collected and washed under running tap water, dried with tissue paper, and placed in modified White traps (Woodring and Kaya 1988). Larvae with bad odor or larvae with multiple colors were discarded. Soil samples were re-baited with fresh larvae to maximize EPN recovery from soils (Banu et al. 2005). IJs emerged from the insect cadavers were tested for its pathogenicity on 10–20 mealworm larvae using filter paper assay in 5-cm-diameter petri dish lined with two filter papers (Whatman No. 1) (Woodring and Kaya 1988). Isolates that satisfied Koch's Postulate were stored at 10°C in 9-cm petri dishes containing

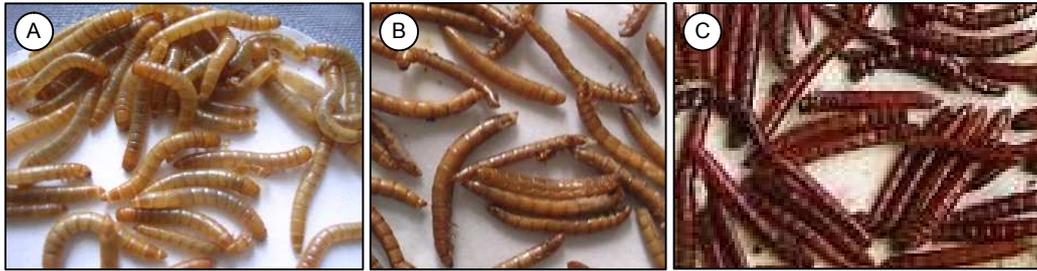
**Table 1.** Percentage of recovery and abundance of entomopathogenic nematodes (EPNs) *Steinernema* and *Heterorhabditis* in East Java and Bali Provinces during a survey in March 2013.

Province	No. of sampled soil (and sites)				Percentage of recovery frequency and abundance*
	Sampled	With <i>Steinernema</i>	With <i>Heterorhabditis</i>	Total with EPNs	
East Java	50 (11)	1 (1)	3 (3)	4 (4)	8.0 (36.4)
Bali	18 (5)	1 (1)	2 (2)	3 (3)	16.7 (60.0)
Total	68 (16)	2 (2)	5 (5)	7 (7)	10.3 (43.8)

\*Percentage of recovery frequency = (no. positive samples/no. total samples) × 100, percentage of abundance = (no. positive sites/no. total sites) × 100 (Campos-Herrera et al. 2007).



**Figure 1.** Occurrence of entomopathogenic nematodes (EPNs) *Steinernema* (▲) and *Heterorhabditis* (●) in East Java and Bali Provinces during a survey in March 2013. Sites where EPNs were not found are marked with “○”. Details of sampling sites are given in Table 2.



**Figure 2.** Healthy and infected mealworm larvae (*Tenebrio molitor*). (A) Healthy mealworm larvae. (B) Mealworm larvae infected by *Steinernema*. (C) Mealworm larvae infected by *Heterorhabditis*.

**Table 2.** Vegetation type of sampled sites in East Java and Bali Provinces during the survey of entomopathogenic nematodes (EPNs) in March 2013.

Hamlet or village and district	Regency and Province	Vegetation cover	No. of soil sample	EPN and isolate code
Plosokidul, Plosoklaten	Kediri, East Java	Sugarcane	1	<i>Steinernema</i> AGS1
Tanjung Tembaga	Probolinggo, East Java	Grasses, corn flower	6	-
Paiton	Probolinggo, East Java	Grasses	7	-
Banyuglugur, Mlandingan	Probolinggo, East Java	Grasses	3	<i>Heterorhabditis</i> NH1
Duwet, Pasir Putih	Situbondo, East Java	Shrubs	3	<i>Heterorhabditis</i> PH1
Banyuputih, Asembagus	Situbondo, East Java	Coconut, grasses, cover crops	5	-
Klatakan, Pasir Putih	Situbondo, East Java	Shrubs	3	<i>Heterorhabditis</i> PH2
Bayeman, Asembagus	Situbondo, East Java	Grasses, woodland	7	-
Talang, Asembagus	Situbondo, East Java	Grasses, woodland	3	-
Experiment Station (Banyuputih, Asembagus)	Situbondo, East Java	Cotton, sesame, physic nut, cashew nut	9	-
Watudodol, Ketapang, Banyuwangi	Banyuwangi, East Java	Grasses	3	-
Ketapang	Banyuwangi, East Java	Grasses, maize	7	-
Saba, Blahbatuh	Gianyar, Bali	Pandanus	1	<i>Heterorhabditis</i> DKH1
Keramas, Blahbatuh	Gianyar, Bali	Pandanus	1	<i>Heterorhabditis</i> DKH2
Banyupoh, Grokgak	Singaraja, Bali	Grasses, shrubs	6	<i>Steinernema</i> DKS1
Labuhan	Singaraja, Bali	Grasses	3	-

water supplemented with few drops of 4% formaldehyde. All isolates were maintained by routine sub-culture on mealworm larvae at regular intervals (2 or 3 mos). In this study, an “isolate” refers to nematodes obtained from one soil sample. Occurrence of EPNs was assessed as recovery frequency (no. positive samples/no. total samples) and abundance (no. positive sites/no. total sites) expressed as percentage (Campos-Herrera et al. 2007).

#### Morphometric of Infective Juveniles

About 100 IJs of the first generation, i.e. the first IJs emerging from infected mealworm larvae, were placed on a glass slide, heat killed, and mounted on slides in lactophenol (10 ml lactic acid, 10 ml glycerol, 10 ml phenol, and 10 ml H<sub>2</sub>O). Specimens were covered with a thin glass slip supported with glass fibers to avoid flattening the nematodes. Total body length was measured under a light microscope (Olympus BX51) equipped with a digital camera and software (DP2-BSW).

#### Molecular Identification

Total genomic DNA was extracted from IJs using sodium dodecyl sulfate (SDS) extraction method (Cenis 1992). The ITS1-5.8S-ITS2 regions of rDNA cistron were amplified using the forward primer TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and the reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') (Joyce et al. 1994). DNA was amplified in a 25- $\mu$ l reaction containing 1 $\times$  PCR buffer, 0.2 mM of dNTPs mixture, 0.4  $\mu$ M of each forward and reverse primers, 1 U of DreamTaq, and 5  $\mu$ l of DNA extract. PCR was performed in a Bio-Rad thermocycler with the following cycling profile: 1 cycle of initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 50°C for 30 sec, and extension at 72°C for 45 sec, and finalized with 7 min incubation at 72°C. A portion of the amplification product (5  $\mu$ l) was electrophoresed in a 1.0 % agarose gel in 0.5 $\times$  TBE buffer at 50 V for 40 min. Images were taken in UV-transilluminator (ChemiDoc, Bio-Rad) after gel staining in ethidium bromide (0.5  $\mu$ g/l). The size of

amplified products was determined by comparison with a 100 bp DNA ladder (Vivantis). Samples were purified by gel extraction method and sequenced using both primers at 1st Base (Singapore). To identify EPN species, the resulting sequences were compared to those published nucleotide sequence database using BLASTn tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) after edited using BioEdit ver. 7.2.6 (Hall 1999).

### Phylogenetic Analysis

Multiple sequences of ITS1-5.8S-ITS2 regions of rDNA of nine *Heterorhabditis* species representing two broad monophyletic groups “indica” and “megidis” and at least two species from five *Steinernema* groups (“glaseri” with IJ body length >1,000 µm, “feltiae” with IJ body length between 700–1000 µm, “intermedium” with IJ body length between 600–700 µm, “carpocapsae” with IJ body length <600 µm, and “bicornutum” with two horn-like structure in the head of IJ) (Nguyen 2014) were downloaded from the NCBI database (<https://www.ncbi.nlm.nih.gov/nucleotide/>) and aligned with the sequences obtained in this study using Muscle (Edgar 2004) integrated in MEGA software ver. 7.05 (Kumar et al. 2016) with default parameters. The evolutionary history among species was inferred using Neighbor-Joining method with evolutionary distances computed using the Kimura 2-parameter model in MEGA software. Bootstrap analysis was performed with 1,000 replicates. *Caenorhabditis elegans* accession FJ589008 was used as the outgroup in both phylogeny analyses.

## RESULTS AND DISCUSSION

### Isolation of EPN

Fifty soil samples from 11 sites in East Java Province and 18 samples from 5 sites in Bali Province were examined for the presence of EPNs in 2013. A total of 7 isolates were recovered from 7 soil samples (10% recovery frequency rate) and from 7 sites (44% abundance rate; Table 1, Figure 1). Two steinernematids were obtained from two sampling sites (one in each province), whereas five heterorhabditids were obtained from three and two sampling sites in East Java and Bali, respectively. The percentage of EPN recovery and abundance in Bali were higher than those in East Java. There was no EPN that could be detected in the second baiting, whether the soil samples were tested negative or positive for EPN in the first baiting. The two nematode genera were never found in the same soil sample or

the same site in this study. During the survey, natural infection of insect by EPN was never encountered in the sampled sites.

All EPN isolates passed Koch’s Postulate when tested for their pathogenicity on mealworm larvae. Infected larvae showed uniform color change for the respective nematode genus (Figure 2). They also reproduced fairly well on this larva species, with the exception of *Steinernema* AGS1. The majority of AGS1 IJs had smaller body sizes after several times of rejuvenation on mealworm larvae, indicating that this insect species may not be a suitable host for this isolate.

EPNs were recovered from both natural and managed ecosystems (Table 2). The vegetation types where EPNs were found included sugarcane, grasses, shrubs, and pandanus.

EPN recovery rate was lower with intermediate abundance rate compared to earlier studies in Indonesia (Griffin et al. 2000; Chaerani et al. 2007). *Steinernema* was less prevalent (2 isolates from 16 sites [12.5%]) than *Heterorhabditis* (5 isolates from 16 sites [31.3%]); these figures are congruent with the result of Chaerani et al. (2007). Surveys in several tropical regions in the world found that *Heterorhabditis* was more prevalent than *Steinernema*, which led some investigators to hypothesize that heterorhabditid is more adapted to tropical environment than steinernematid (Banu et al. 2005). However, this may not always be the case. With a larger number of sites (79) and soil (223) sampled in 5 islands, Griffin et al. (2000) found that *Steinernema* and *Heterorhabditis* were equally prevalent in Indonesia.

Surveys around the world found EPN in 0.7–50% soil samples (Bruck 2004; Pillay et al. 2009). Even large-scale surveys detected only 2–11% EPN from >1,000 soil samples (Yoshida et al. 1998; Hazir et al. 2003; Hatting et al. 2009; Ma et al. 2010). Varying EPN recovery rate can result from variation in sampling method, insect bait used, and soil type sampled (Cheruiyot et al. 2013). EPN prevalence may also vary with time because of edaphic factors such as soil moisture, soil texture, pH, temperature, and biotic factors (Cheruiyot et al. 2013). Negative detection does not mean that EPN is absent. Since they are highly dependent on insect aggregation, EPNs tend to be aggregated in soil rather than randomly distributed (Hominick 2002; Campos-Herrera et al. 2013). Larger sample number that covers more sites may increase EPN detection and the chance to obtain higher EPN diversity (Hominick 2002; Erbaş et al. 2014). This

study and the previous one (Griffin et al. 2000; Chaerani et al. 2007) prove that EPN is relatively easy to be found in sandy, coastal areas of Indonesia. Positive association of EPN recovery with sandy soil was repeatedly demonstrated elsewhere (Prasad et al. 2001; Hatting et al. 2009; Ma et al. 2010; Abd-Elbary et al. 2012; Kanga et al. 2012; Valadas et al. 2014; Vashisth et al. 2015). Sand content is important for nematode mobility and survival, and both are lower in soil with higher clay and silt contents due to decreased pore size and reduced oxygen availability (Barbercheck and Kaya 1991). Although sandy soils are rich in EPNs, clay, silty clay, loam, and silty clay loam soils also harbor EPNs (Nyasani et al. 2008; Ma et al. 2010; Abd-Elbary et al. 2012; Kanga et al. 2012). In Indonesia, silty loam soil in mountainous regions

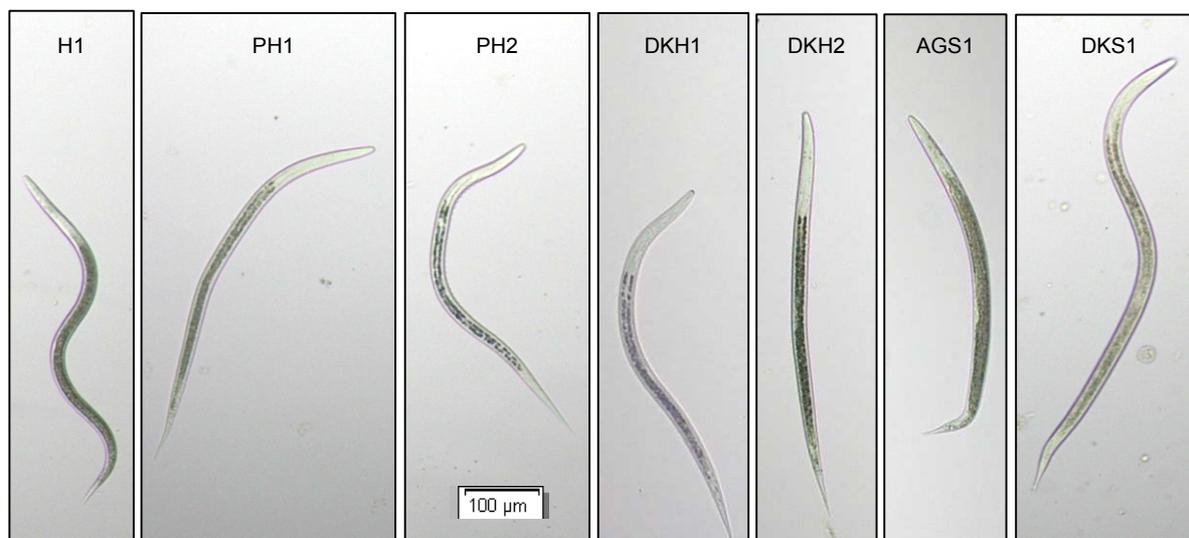
were also reported to contain *Steinernema* (Chaerani et al. 2007; Baliadi 2011; Rahardjo et al. 2014). Given that mountainous regions with such light soil texture are common in Indonesia, a thorough sampling in such areas will be our target in the future.

Our study and the previous ones (Griffin et al. 2000; Chaerani et al. 2007) targeted surveys on uncultivated areas. It is not known whether the percentage of EPN recovery will be larger if samplings were done in cultivated areas. Nyasani et al. (2008) proposed that intensively cultivated land, which is characterized by frequent tillage, high agrochemical input, and frequent fluctuation in environmental conditions, imposes detrimental effects on EPNs. However, large scale surveys by Hazir et al. (2003) in

**Table 3.** Total body length of infective juveniles of *Steinernema* and *Heterorhabditis* isolated from soils in East Java and Bali Provinces during a survey in March 2013 compared to previous measurements from various species. Details of the locations where isolates were obtained are given in Table 2.

Isolate	N	Total body length and range (µm)	Greatest width and range (µm)
<i>Heterorhabditis</i> NH1	49	536 (476–584)	25 (16–34)
<i>Heterorhabditis</i> PH1	50	520 (386–579)	22 (12–30)
<i>Heterorhabditis</i> PH2	52	553 (521–595)	24 (14–38)
<i>Heterorhabditis</i> DKH1	51	539 (482–599)	25 (16–35)
<i>Heterorhabditis</i> DKH2	49	478 (429–534)	25 (18–37)
<i>H. indica</i> <sup>a</sup>	25	528 (479–573)	20 (19–22)
<i>H. amazonensis</i> <sup>b</sup>	20	589 (567–612)	23 (20–24)
<i>H. bacteriophora</i> <sup>c</sup>	15	562 (537–587)	27 (25–31)
<i>H. megidis</i> <sup>c</sup>	-	768 (736–800)	29 (27–32)
<i>Steinernema</i> AGS1	59	456 (360–547)	32 (20–49)
<i>S. huense</i> <sup>d</sup>	-	527 (444–571)	-
<i>S. siamkaya</i> <sup>e</sup>	-	446 (398–495)	21 (18–24)
<i>Steinernema</i> DKS1	49	672 (548–762)	33 (24–51)
<i>S. pakistanense</i> <sup>f</sup>	20	683 (649–716)	27 (24–29)

<sup>a</sup>After Poinar et al. (1992) and Dolinski et al. (2008), <sup>b</sup>After Dolinski et al. (2008), <sup>c</sup>After Sagun et al. (2015), <sup>d</sup>After Phan et al. (2014), <sup>e</sup>After Stock et al. (1998), <sup>f</sup>After Shahina et al. (2001).



**Figure 3.** Infective juveniles of *Heterorhabditis* (DKH1, DKH2, NH1, PH1, and PH2) and *Steinernema* (AGS1 and DKS1) isolates recovered from soils in East Java and Bali Provinces during a survey in March 2013.

Turkey and Yan et al. (2016) in Rwanda detected that EPN recovery rate were higher in intensively cultivated or managed habitat. Yoshida et al. (1998) proposed that differences in habitat preference by EPN is due to differences in the distribution of suitable host.

Increase in positive samples and sites containing EPN can be expected when the standard Lepidopteran insect bait, *Galleria mellonella*, is used for EPN isolation (Bedding and Akhurst 1975), although its usefulness is limited when EPN is a host-specific species (Nguyen et al. 1990; Atakan et al. 2009; Hatting et al. 2009). Given the patchy

distribution of EPN, intensive sampling within sites can increase EPN recovery rate (Hominick 2002; Kanga et al. 2012).

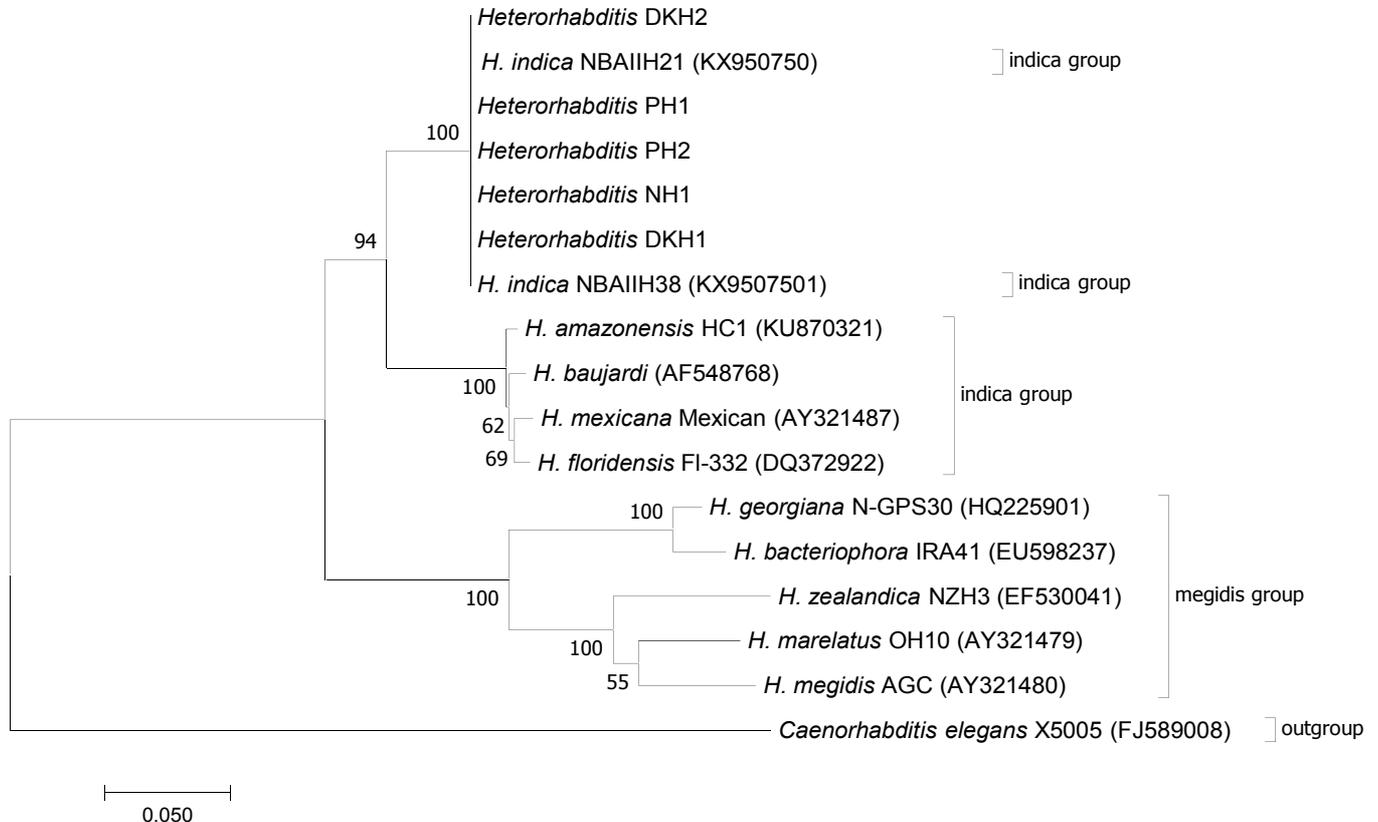
#### Identification of EPN and Phylogenetic Analysis

Measurements of IJs showed that the average total body length of all *Heterorhabditis* isolates was between 478–539  $\mu\text{m}$  long (Table 3). These figures fell within the range of the description for *H. indica* (Poinar et al. 1992; Dolinski et al. 2008). The two *Steinernema* isolates showed contrasting morphometric. The IJs of *Steinernema* AGS1 was shorter (456  $\mu\text{m}$ ) than *Steinernema* DKS1 (672  $\mu\text{m}$ )

**Table 4.** Nucleotide sequence similarity of entomopathogenic nematodes.

Isolate	Closely related species, origin and accession	Similarity rate (%)*
<i>Heterorhabditis</i> NH1	<i>H. indica</i> strain NBAlIH38 India (KX950751)	100
<i>Heterorhabditis</i> PH1	<i>H. indica</i> strain NBAlIH21 India (KX950750)	99
<i>Heterorhabditis</i> PH2	<i>H. indica</i> strain NBAlIH21 India (KX950750)	100
<i>Heterorhabditis</i> DKH1	<i>H. indica</i> strain NBAlIH38 India (KX950751)	100
<i>Heterorhabditis</i> DKH2	<i>H. indica</i> strain NBAlIH38 India (KX950751)	100
<i>Steinernema</i> AGS1	<i>S. huense</i> strain Vie2 Vietnam (KF857581)	99
<i>Steinernema</i> DKS1	<i>S. pakistanense</i> SSRK31 Pakistan (GQ258656)	95

\*Similarity rate compared to sequence database.



**Figure 4.** Phylogenetic relationships of *Heterorhabditis* isolate DKH1, DKH2, NH1, PH1, and PH2 based on the analysis of ITS1-5.8S-ITS2 rDNA regions using Neighbor-Joining method. Numbers higher than 70% on branches indicate the percentage of 1,000 bootstrap replicates.

and even shorter than the average total body length of *Heterorhabditis* (Table 3, Figure 3). Both *Steinernema* isolates were shorter than those previously obtained in Indonesia (Griffin et al. 2000). The total body length of AGS1 IJs was within the range of that of *S. huense* (Phan et al. 2014) and *S. siamkayai* (Stock et al. 1998), although the average length was closer to that of *S. siamkayai*. The total body length of DKS1 IJs was close to that of *S. pakistanense* (Shahina et al. 2001).

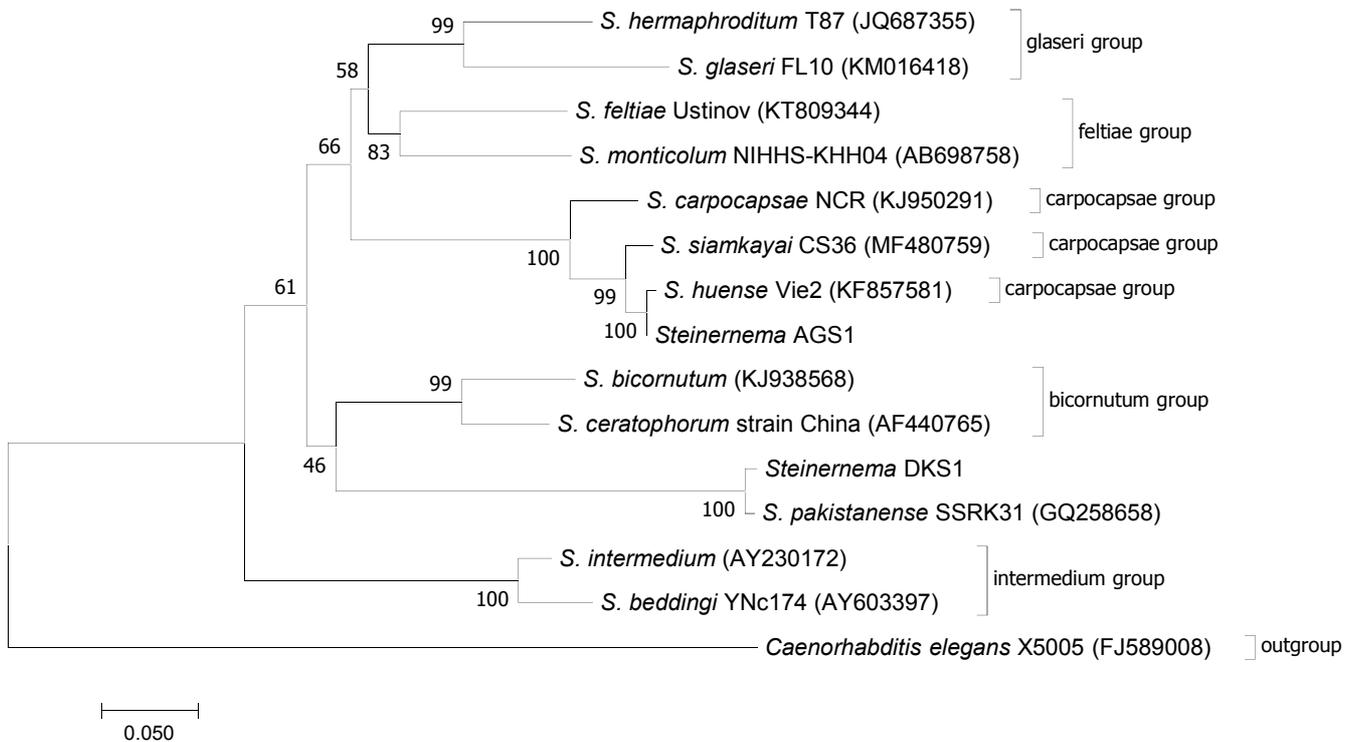
PCR of the entire ITS region amplified a single band of ~850 bp in all isolates. After a BLASTn search of the trimmed nucleotide sequences of 795–809 bp in length against sequence database, all sequences of *Heterorhabditis* isolates showed sequence similarity of 99–100% to that of *H. indica* strain NBAIIH21 and NBAIIH38 from India (Table 4). Further analysis by construction of phylogenetic tree based on published *Heterorhabditis* spp. sequences downloaded from GenBank placed all five *Heterorhabditis* in one group with *H. indica* (Figure 4). These sequence data along with the measurement of IJ body length concluded with high confidence that the five heterorhabditids belong to *H. indica*.

The trimmed nucleotide sequence of 781 in length of *Steinernema* isolate AGS1 shared 99% sequence similarity with *S. huense* Vie2 (KF857581)

from Vietnam, whereas the trimmed sequence of 783 bp in length of *Steinernema* isolate DKS1 shared lower sequence similarity (95%) with *S. pakistanense* SSRK31 (GQ258658) from Pakistan (Table 4). Phylogenetic analysis placed AGS1 in “carpocapsae” group with *S. carpocapsae*, *S. siamkayai*, and *S. huense* (Figure 5). This group is characterized by IJs with short body length (<600 µm).

*Steinernema* DKS1 was placed in the same group with *S. pakistanense* SSRK31 (GQ258656) from India. Both *Steinernema* were placed close to “bicornutum” group in the phylogeny tree. Member of this group is characterized by IJs with horn-like structure in the head. *S. pakistanense* IJ is also characterized by two prominent horn-like structures on labial region of IJs (Shahina et al. 2001). Close examination of IJs of *Steinernema* DKS1 morphology would be needed to determine the presence of horn-like structures in this isolate.

Thus far, *H. indica* is the only heterorhabditid reported in this country. The detection of *H. indica* in our survey was expected, as this species is more adapted to warm conditions and therefore it is commonly found in tropical climate, especially in coastal areas (Constant et al. 1998; Kanga et al. 2012; Yan et al. 2016). In contrast, the two *Steinernema*



**Figure 5.** Phylogenetic relationships of *Steinernema* isolates AGS1 and DKS1 and published sequence of selected *Steinernema* based on the analysis of ITS1-5.8S-ITS2 rDNA regions using Neighbor-Joining method. Numbers higher than 70% on branches indicate the percentage of 1,000 bootstrap replicates.

found in this study have not been recognized previously in Indonesia. Although molecular characterization is useful for species delimitation, taxonomy studies must be undertaken for detailed characterization of their morphology, especially for DKS1 which showed lower DNA sequence similarity (95%) compared to AGS1. However, morphological studies were beyond the resources of our work. Our aim was to find candidate biocontrol agent and once EPNs and their basic information were obtained, screening for efficacy against important insect pest will be performed. *In vitro* test against white sugarcane grub (*Lepidiota stigma*) showed that *Steinernema* DKS1 and *Heterorhabditis* PH1 were the most efficacious nematodes from each genus by causing 80–90% and 70–80% mortality of third instar grub, respectively (Indrayani et al. 2018).

It is highly likely that the number of EPNs recovered so far does not represent the full range of EPNs that occur in Indonesia because exploration has been conducted mostly in coastal areas of some regions of Java, Bali, South Sulawesi, and Moluccan islands. As Indonesia is located along equatorial line and it is divided by Wallace line into two biogeographical regions that have diverse habitats, more new EPN species (especially *Steinernema*) is predicted to be found in this country. Additional investigations with regard to the occurrence, distribution, and diversity of EPNs related to soil type and characteristic, habitat type, and seasonal change in temperature and rainfall are envisaged.

### CONCLUSION

Seven EPNs consisted of five *Heterorhabditis* and two *Steinernema* isolates were successfully recovered from soil samples collected in East Java and Bali Provinces. Morphometric analyses of IJs indicated that the total body length of all *Heterorhabditis* isolates (478–539) were similar to *H. indica*, whereas the total body length of one of the *Steinernemas* (456  $\mu\text{m}$ ) was within the range of *S. huense* or *S. siamkayai* and that of the other *Steinernema* (672  $\mu\text{m}$ ) resembled to *S. pakistanense*. Sequencing of ITS1-5.8S-ITS2 regions of rDNA revealed that all *Heterorhabditis* isolates belonged to *H. indica* (99–100% sequence similarity), whereas *Steinernema* isolate with the shorter IJs' body length shared 99% sequence similarity to *S. huense* and the other *Steinernema* isolate had 95% sequence similarity to *S. pakistanense*. With a diverse ecology, it is predicted that more EPN species will likely to be found in Indonesian soils.

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