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New Distributional Record of *Steinernema hermaphroditum* (Rhabditida: Steinernematidae) from Kerala, India

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ABSTRACT: A total of 141 soil samples were collected from coconut gardens in various tracts of Alappuzha, Kollam, Pathanamthitta, Idukki and Eranakulam districts of Kerala for the natural occurrence of EPN using soil-baiting techniques with greater wax moth larvae, *Galleria mellonella*. Among these samples, 13.5% were found positive for EPN, which included three steinernematids and 16 heterorhabditids identified based on the characteristic colour of the infected cadavers. Three heterorhabditids and two steinernematid isolates were further subjected to molecular characterization by sequencing ITS region of the ribosomal DNA using 18s and 26s primers. All the three heterorhabditid species intercepted in the survey, were found to have 99% sequence similarity with *Heterorhabditis indica*. Both the steinernematid isolates were found to be belonging to the *glaseri* group. The steinernematid isolate CPCRI0804 was found non-homologous with any of the described species and, therefore, presumed to be a new species for identification. The presence of long and highly curved spicule is the striking feature of this unidentified species. One of the steinernematids (CPCRI0905) was 99% homologous with *Steinernema hermaphroditum* and is the first report of this species from South India. The identity of this isolate was confirmed based on the morphological and morphometric characters as well as the presence of first generation hermaphroditic females, which is a unique characteristic feature of *S. hermaphroditum*.

Key words: Entomopathogenic nematodes, Biological control, Hermaphroditism

The members of nematode families, Steinernematidae and Heterorhabditidae, which are lethal insect pathogens, are commonly referred as entomopathogenic nematodes (EPN) (Gaugler, 2002). The bacteria, *Xenorhabdus* sp. and *Photorhabdus* sp. respectively are symbiotically associated with these nematodes. While most of the bio-control agents are slow acting in terms of host mortality, EPNs with the help of their symbiotic bacteria are extremely quick to induce insect mortality within one or two days attracting them in wider use in pest management. Third stage juveniles, commonly known as infective juveniles (IJs) are non-feeding and only functional survival stage in the life cycle of EPN. They are naturally present in almost all types of soils and when they intervene with the suitable insect host, they enter the body either through natural openings like mouth, anus or spiracles or by direct penetration through thin cuticle. Once IJs reach the insect haemocoel, they release the bacteria, where the bacteria proliferate and induce septicemia by the production of various toxins and enzymes, which ultimately leads to host mortality. The nematodes complete its life cycle by feeding on the metabolites produced by the bacteria. EPNs are absolutely safe for human, animal or plant health and devoid of any environmental or ecological ill effects and can be used without any registration procedure. In this context EPN forms an excellent candidate in biological pest suppression especially against soil and cryptic pests (Lacey and Georgis, 2012).

Rao and Manjunath (1966) initiated the work on steinernematids in India, by importing DD-136 strain of *S. carpocapsae* for insect pest management in rice and sugarcane. Initially many researchers used exotic strains of *S. glaseri* (NC 34), *S. feltiae* and *Heterorhabditis bacteriophora* and reported bio-control potential against a wide spectrum of insect pests (Ganguly *et al*., 2006). Extensive and exploratory searches through soil-baiting technique were undertaken across the country to identify indigenous strains of EPN, as they could have enhanced adaptability, survival and virulence invading insect pests

of national importance (Ganguly *et al*., 2006; Banu *et al*., 1998). Keeping this in view, an elaborate probing for the detection of EPN was undertaken in coconut plantations of Kerala, India a state enjoying a wide distribution of rainfall and undisturbed forest niche to ascertain the presence of any new virulent species of EPN that could be commercially exploited for mass production and successfully employed in biological control of coconut pests.

MATERIAL AND METHODS

Survey and soil sample collection

Entomopathogenic nematodes occur naturally in all soil types, but they can reproduce only in the haemocoel of infected insect hosts. Soil sampling followed by baiting of samples with *Galleria mellonella* larvae (greater wax moth) is the method followed for the isolation of infective stage juveniles of EPNs (Bedding and Akhurst, 1975, Mracek, 1980). Survey was conducted in various tracts of coconut gardens in Alappuzha, Kollam, Pathanamthitta, Idukki and Eranakulam districts of Kerala for the natural occurrence of EPN during August and September 2017. Soil samples were ccollected at a depth of 5 to 15 cm using a soil auger and transferred to the laboratory in a plastic bag with appropriate labeling. All the debris from the collected soil samples were removed off before being bagged. Around 100 cc of soil was baited with three larvae of the greater wax moth, *G. mellonella* in a clean plastic container with a lid. These plastic containers were turned upside down up for a period of 15 days and regularly observed for any EPN infection every 2-3 days, Dead insects showing symptoms of EPN infection if any, were removed, cadavers rinsed in sterile distilled water and placed them on a modified White trap for recovery of nematode progeny from infected cadavers.

In vivo multiplication and storage of EPN

The recovered isolates of EPN from Kerala soil were multiplied in greater wax moth larvae and harvested using modified White trap method (Kaya and Stock, 1997). The EPN was stored in sterile double distilled water at 15 °C.

Morphological characterization

The nematode isolate intercepted from Pathiyoor, Alappuzha district, Kerala, India was subjected to morphological characterization. Twenty specimens from each life stages (Infective juveniles and adult males and females of first generation) were randomly collected from ten *G. mellonella* cadavers. Nematodes were examined live as well as after heat-relaxed in Ringer's solution at 60°C. Nematodes were fixed in triethanolamine formalin (TAF) (Courtney *et al.*, 1955) and processed to anhydrous glycerine for mounting (Seinhorst, 1959). Observations were made from live and mounted specimens using Nikon Eclipse Ni microscope equipped with differential interference contrast optics. Specimen measurements were made with a stage micrometer. Selection of morphometric characters was done according to Hominick *et al.* (1997).

Molecular characterization and phylogenetic analysis

Extraction of DNA

DNA was extracted from infective juveniles using the method reported by Hominick *et al*. (1997). The nematodes were crushed with micro pestle in 20 µl of lysis buffer (50 mM KCL, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% Tween 20 and 60 µg ml^{-1} proteinase K) in a sterilized 1 ml micro-centrifuge tube on ice. The tube was frozen at -20°C for 10 min, incubated at 65°C for 60-90 min, followed by 95°C incubation for 8 min. The tube was cooled on ice and centrifuged at 11600 g for 2 min. The supernatant containing the DNA was collected and kept at -20°C (Nguyen and Hunt, 2007).

PCR amplification

PCR amplification was carried out based on the method described by Nguyen and Hunt (2007). The amplification of ITS region of the ribosomal DNA was carried out in a 25 µl reaction tube by the polymerase chain reaction (PCR). Tubes were set up on ice and each tube was added with: 2.5 µl of 10X PCR buffer, 1 µl of dNTP mixture (10 mM each), 1 µl of 10 pM forward primer, 1 µl of 10 pM reverse primer, 1.5 µl $MgCl_2$ (50

mM), 0.02 µl of Taq DNA polymerase (5 U μl^{-1}), 15 µl of distilled water, and 2.5 µl of DNA. The 18S: 5'-TTGATTACGTCCCTGCCCTTT-3' (forward) and 26S: 5'-TTTCACTCGCCGTTACTAAGG-3' (reverse) primers were used (Vrain *et al*. 1992). All PCR reactions were run in a Thermocycler with the cycling profile suggested by Nguyen *et al*. (2004): 1 cycle of 94°C for 7 min followed by 35 cycles of 94°C for 60 s, 50°C for 60 s, 72°C for 60 s. The last step is 72°C for 10 min. The presence of DNA was confirmed by running 5 µl of the PCR product on a 1% agarose gel in 0.5X TBE buffer for 30-40 min and visualized by ethidium bromide staining (Maniantis *et al*., 1989).

Sequencing and Phylogenetic analysis

PCR products are purified with a QIAquick PCR purification kit. Purified DNA was sequenced in both directions using 18S and 26S primers. Molecular characterization was done by analysis of ITS region of ribosomal DNA sequences. An existing library of more than 30 *Steinernema* spp. was used for sequence comparisons and phylogenetic interpretation. Multiple sequence alignments were made using ClustalW. A phylogenetic tree was constructed with the ITS sequences using Mega ver. 6.0 by the neighbor joining method with 1000 replications for bootstrap analysis.

RESULTS

Soil sample collection and isolation of EPN

Soil samples were collected from various tracts of Alappuzha, Kollam, Pathanamthitta, Idukki and Eranakulam districts of Kerala and were processed using soil-baiting techniques with greater wax moth larvae, *G. mellonella*. Out of 141 soil samples baited, 19 (13.5%) were found positive for EPN, which included three steinernematids and 16 heterorhabditids identified based on the characteristic colour of the infected cadavers. Soil samples from Alappuzha and Pathanamthitta districts yielded EPNs with a recovery of 22.5% and 4.8%, respectively. However, steinernematids were isolated only from Pathiyoor and Krishnapuram of Alappuzha districts. All the steinernematids isolated were characterised by the presence of long infective juveniles with mean body length in the range 800 to 1000µ, which is the characteristic feature of members of *feltiae* group. The nematode isolate retrieved from Pathiyoor, Alappuzha district was identified to be *S. hermaphroditum* CPCRI0905 based on the molecular and morphological characteristics. Steinernematid obtained from ICAR-CPCRI, Regional Station, Krishnapuram, Kayamkulam (*Steinernema* CPCRI0805) did not match with any of the identified species. The details of the sampling location and isolates recovered are given in table 1.

Morphology and morphometrics

Infective juveniles

Long and slender body, gradually tapering anteriorly from the pharynx posteriorly from the anal region. Long and narrow pharynx. Average body length is 920 µ. with 73 µ long tail.

First generation male

Posteriorly curved body which took J shape when heat relaxed. Testis was single and reflexed. Paired,

Table 1. Details on the location of soil sample collection and recovery of EPNs

Sl No.	Location	No. of samples	Positive samples	EPN Species
1.	Alappuzha	80	18 (22.5%)	*Heterorhabditis* sp. (15), *Steinernema* sp. (3)
2.	Pathanamthitta	21	1 (4.8%)	*Heterorhabditis* sp. (1)
3.	Kollam	30	0 (0.0%)	
4.	Ernakulam	5	0 (0.0%)	
5.	Idukki	5	0 (0.0%)	
	Total	**141**	**19 (13.5%)**	*Heterorhabditis* sp. (16), *Steinernema* sp. (3)

brown colored and curved spicule with rectangular manubrium, short calomus, wide lamina and short velum. Gubernaculam is two third of spicule length.

Second generation male

Body length and diameter, spicule and guberanculum length were less than first generation males. Excretory pore was more anteriorly located as compared to the first generation.

First generation hermaphroditic females

Body assumed C-shape when heat relaxed. Cephalic region was truncate to slightly round and continuous with body. Stoma was short and broad with inconspicuous sclerotised walls. Excretory pore located just anterior to nerve ring. Pharynx was set off from the intestine with cylindrical procorpus, slightly swollen metacorpus, distinct isthmus and pyriform basal bulb with reduced valve. Nerve ring surrounded the isthmus or anterior part of the basal bulb. Opposed and reflexed ovaries had a well developed oviduct. Glandular spermatheca was filled with spermatozoa. Vulva located almost middle of the body with asymmetric and protruding vulval lips. Digitate tail with mucron and post anal swelling.

Second generation amphimictic females

Shorter than first generation hermaphroditic females. Spermatheca was absent and vulva with slightly protruding vulval lips. Round conoid tail without mucron and post anal swelling. All other characters were same as in first generation.

Molecular characterization

Three heterorhabditids and two steinernematid isolates were subjected to molecular characterization by sequencing ITS region of the ribosomal DNA using 18*s* and 26*s* primers. All the three heterorhabditid species intercepted in the survey, were found to have 99% sequence similarity with *Heterorhabditis indica*. Phylogenetic reconstruction of both the steinernematid isolates indicated to be belonging to the *glaseri* group. One of the steinernematids (CPCRI S0905) showed 99% identity with *Steinernema hermaphroditum* (Genbank Acc. No. JQ687355, MF663703). Ribosomal sequences for *S. hermaphroditum* (CPCRI S0905) was deposited in GenBank under the accession number MH802516.

The steinernematid isolate CPCRI S0804 showed only 92% identity with the described species and, therefore, presumed to be a new species for identification. The phylogenetic relationships between 21 species of *Steinernema* with new isolates are presented in Fig. 2. The species in the *glaseri*-group (*S. apuliae, S. arenarium, S. glaseri, S. hermaphroditum, S. guangdongense, S. longicaudum, S. lamjungense, S. khoisanae, S. diaprepesi, Steinernema* S0905 CPCRI and *Steinernema* S0804 CPCRI*)* form a monophyletic group. However, *Steinernema* S0804 CPCRI did not group with any other species, but showing more relation with members of *glaseri* group.

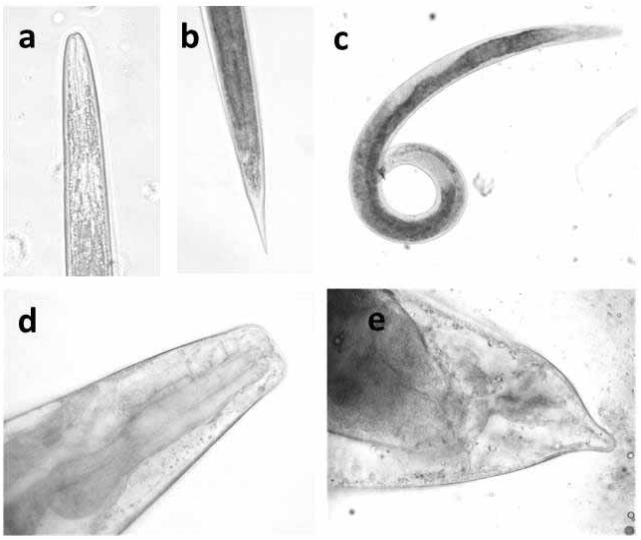

Fig. 1. a & b: Infective juvenile (a-anterior region; b-tail); c - first generation male; d & e - first generation hermaphroditic female (d-anterior region; e-tail).

DISCUSSION

Extensive surveys have been conducted worldwide as well as in India and a spectrum of EPN belonging to Steinernematids and Heterorhabditids have been reported (Josephrajkumar and Sivakumar, 1997; Nguyen *et al*., 2007; Hatting *et al*., 2009). Interception of EPN could be observed in different soil types as well as in soils with

Table 2. Morphometrics of *Steinernema hermaphroditum* CPCRI S0905. Measurements are in µm and in the form: mean ± SD (Range).

Character	Male (First Generation)	Hermaphrodite female (First Generation)	Infective juveniles
Length	2368 ± 71	8365 ± 211	922 ± 76
a (L/MBD)	-	-	24 ± 4
b (L/ES)	-	-	6.3 ± 1
C (L/T)	-	-	11.7 ± 1
c' (T/ABD)	-	-	4.0 ± 0.3
Vulval aperture	-	58 ± 4	-
Max. body diam.	130 ± 8	289 ± 11	34 ± 3
Excretory pore	86 ± 6	135 ± 9	72 ± 3
Nerve ring	128 ± 4	152 ± 5	102 ± 2
Esophagus	182 ± 9	217 ± 12	134 ± 5
Tail length	40 ± 3	68 ± 3	73 ± 2
Anal body diam.	49 ± 2	69 ± 2	18 ± 1
Spicule length	65 ± 2	-	-
Spicule width	14 ± 1	-	-
Gubernaculum length	49 ±1	-	-
Gubernaculum width	7.4 ± 1	-	-
D% (EP/ES X 100)	47 ± 4	-	53 ± 3
E% (EP/T X 100)	-	-	99 ± 6
SW% (SL/ABD X 100)	133 ± 30	-	-
GS% (GL/SL X 100)	75 ± 2	-	-
Hyaline tail	-	-	37 ± 2
H% (H/T X 100)	-	-	51 ± 1

varied content of organic matter. However, the prevalence of EPN in coastal sandy soil as compared to soil with high clay content is reported by several workers (Hara *et al*., 1991; Amarasinghe *et al*., 1994; Mason *et al*., 1996; Griffin *et al*., 2000; Jawish *et al*., 2015). The output of the present survey was in agreement with these findings as 18 out of 19 isolates were from coastal sandy soil. The exact reason for the prevalence of EPNs in coastal sandy soil is not completely understood. It is presumed to be the coastal location or sandy texture or both of them (Griffin *et al*., 2000). Sand fraction of the soil favours the survival and optimum movement of nematodes, where as clay content restricts mobility (Stock *et al*., 1999; Molyneux and Bedding 1984; Kung *et al*., 1990). Thus the host finding process becomes comparatively easier in sandy soil. Prevalence of higher insect population in washed up marine detritus which ensures availability of hosts for the survivability of EPN could favour the prevalence in marine habitat (Hominick *et al*., 1996; Griffin *et al*., 2000).

During the present survey, a total of 16 heterorhabditids and only 3 steinernematids were isolated. Predominance of Heterorhabditids over steinernematids

were reported from different parts of the world (Hara *et al*., 1991; Roman and Figueroa, 1995; Shamseldean and Abd-Elgawad, 1994; Amarasinghe *et al*., 1994; Rosa *et al*., 2000). However the dominance of Steinernematids were also documented (Garcia del Pino, 1996; Griffin *et al*., 1991; Hominick *et al*., 1995; Steiner, 1996; Sturhan and Liscova, 1999; Yoshida *et al*., 1998). The relative prevalence of any one of the genera over the other is attributed to the various soil and environmental parameters as well as the altitude of the sampling site (Rosa *et al*., 2000). Heterorhabitids were reported to be more abundant at lower altitudes, where as steinernematids prefers higher elevations (Hara *et al*., 1991). Coastal sandy habitat is reported to be most preferred habitat for heterorhabditids (Hominick *et al*. 1996; Griffin *et al*., 2000).

Over all EPN recovery (13.5%) was comparatively on higher side and most of the positive sites were from the coastal sandy belt. Natural occurrence of EPN in this part of the world could one of the reasons that the soil borne white grubs are scanty and these EPN could naturally suppress the white grub population beyond certain level. In addition, the weather factors prevailing would encourage survival of IJs in soil for a long period of time. Organic farming popularized by the Government policy adds further boon for the survival and prevalence of such natural bio-agents in the system and Kerala is known for one of the Biodiversity hotspots. Unscientific application of soil insecticides in coconut gardens is another advantageous feature to mark this accomplishment.

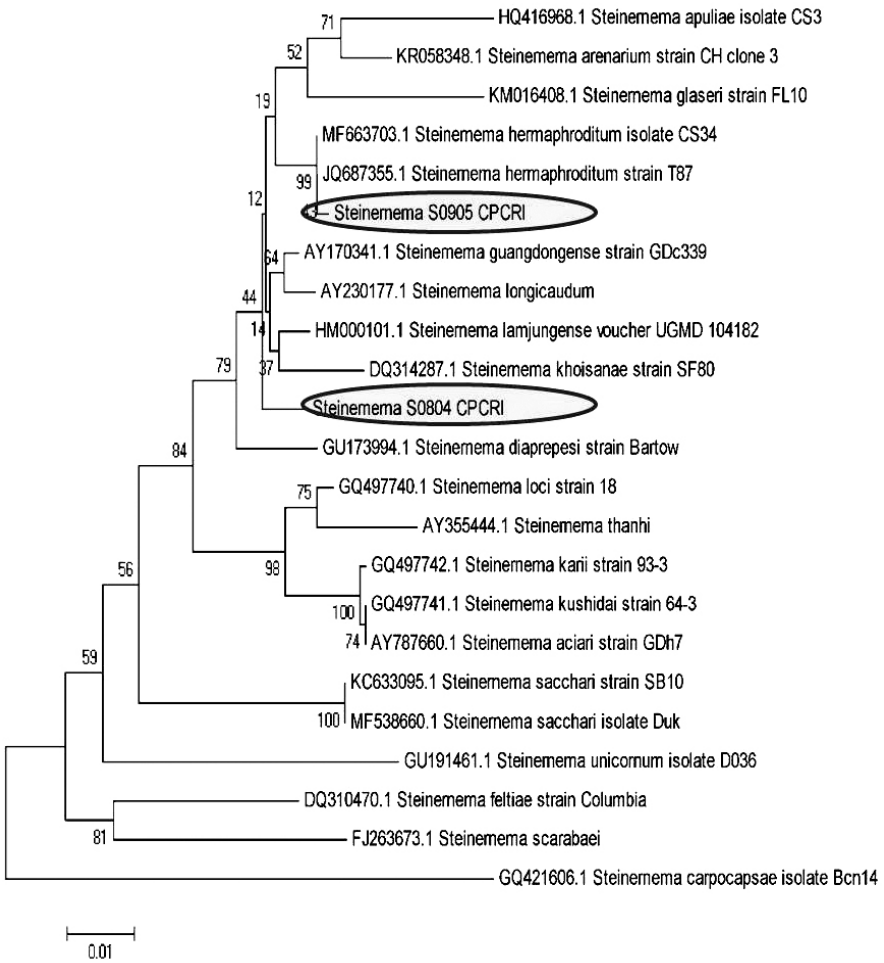

Fig. 2. Phylogenetic relationships of 23 species of Steinernema based on analysis of ITS rDNA regions. Numbers at the nodes represent bootstrap proportion for MP (below) and neighbour joining (above) (50% or more).

One of the most important achievement of this survey was the isolation and identification of *S. hemaphroditum* from coconut garden at Pathiyoor, Alappuzha district. Surveys conducted in India could not report the occurrence of this species so far and this marks the first report in the country based on morphological and molecular characterization. The identity of the isolate was initially indicated by the presence of hermaphroditic females and very rare frequency of males in the first adult generation. The morphometric characters of infective juveniles of new isolate showed more closeness with the members of feltiae group which are characterised by the length of IJs in the range between 800 to 1000µ. However, morphometrics of other stages of this isolate could delineate it from this group. Nevertheless, phylogenetic analysis indicated the closeness of this species with the members of *glaseri* group. Since all the morphological, morphometric and molecular characteristics of this isolate were in agreement with the original description, the identity of the new isolate was confirmed as *S. hermaphroditum*. This species was originally described by Patricia Stock *et al*. (2004) from Moluccan islands, Indonesia. This species is characterized by the presence of hermaphrodites in the first adult generation. Approximately 1% of the IJ developed into males and males were also present in the second adult generation, but at a very low level (1-6%) (Griffin *et al.*, 2001). Because of the hermaphroditism in this species, even the entry of a single individual in to the host insect can lead to progeny production and host mortality, which is otherwise a common characteristic feature among Heterorhabditids. Key morphological diagnostic characters of this species are: a digitate tail with a mucro and a glandular spermatheca filled with sperm in the first generation hermaphrodite; the value of D%; the morphology of the male spicules and gubernaculum and the number and arrangement of the genital papillae; the values of D%, E% and the pattern of the lateral field of the third-stage infective juvenil (Patricia Stock *et al*., 2004).

CONCLUSION

The steinernematid isolated from coconut garden of Pathiyoor, Alappuzha district was identified as *S. hermaphroditum* (CPCRI 0905) based on the molecular, morphological and morphometric characters. The sequence information of ITS region of ribosomal DNA of this isolate exhibited 99% homology with two available sequences of the species in NCBI. The presence of first generation hermaphroditic females and very rare frequency of males in both the generations is unique characteristic feature of this species. Morphological and morphometric characters are in agreement with the original description of the species. This forms the first report of this species from Peninsular India. Widespread natural occurrence of EPN from Kerala indicates the favourable niche prevailing in the region for biological suppression of soil pests in a sustainable manner. Organic Farming policy adopted by the Government adds further positive tone to this approach. All the local isolates of EPN encountered were found to be effective against red palm weevil infesting coconut, which can be effectively employed for their management.

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