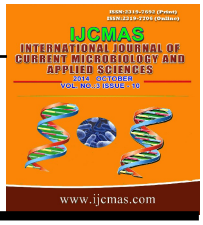
*International Journal of Current Microbiology and Applied Sciences*
***ISSN: 2319-7706*** **Volume 3 Number 10 (2014) pp. 693-699**
**http://www.ijcmas.com**




**Original Research Article**

# Isolation and Identification of Entomopathogenic Nematodes of Kodaikanal Hills of South India

**M.Razia[1*] and S.Sivaramakrishnan[2]**

[1]Department of Biotechnology, Mother Teresa Women's University, Kodaikanal, India
[2]Bharathidasan University, Tiruchirapppalli, TamilNadu, India
**Corresponding author*

**Keywords**

Entomo-pathogenic nematode, *Steinernema*, *Heterorhabditis*, Galleria, Insect pest

**A B S T R A C T**

Isolation and identification of indigenous species of Entomopathogenic nematodes are necessary for successful control of crop pest in vegetables. In the present study, different crop soils were collected and screened for occurrence of nematodes. A survey was conducted and totally 200 samples were collected in Kondikanal regions of Dindigul district of TamilNadu. *Galleria* bait method is used for isolation of nematode was performed. 7 samples were positive for entomopathogenic nematodes, with 3 containing *Heterorhabditis* and 5 *Steinernema* isolates. Morphological and molecular studies were carried out to characterize isolates. The results of morphological and molecular characterizations of isolated nematodes represent *Steinernema* species and identified as *S. siamkayai* was the most common species, which was isolated from three provinces and one *Heterorhabditis* species, *H. indica* respectively.

## Introduction

Entomopathogenic nematodes are parasites and ubiquitous in distribution throughout the world (Hominick, 2002) except Arctic and Antarctica. It search the suitable insect host with the help of leakage of root wounded plant compounds, carbon dioxide etc. Nematodes act as a biological control agents and the non-feeding infective juvenile stage (IJs) kill the insects (different stages of larva, pupa, and adult) depend upon the species of nematodes and insects; the nematode penetrates into the insect body, usually through natural body openings (mouth, anus and spiracles) or areas of thin cuticle within 24-72 h with the help of their own associated bacterial symbionts, which is present in their intestinal space (Laznik *et al*., 2011). The bacteria multiply inside the host and release a number of virulence factors, including complexes of toxins, hydrolytic enzymes, hemolysins, and antimicrobial compounds (Eleftherianos *et al*., 2010), thus providing nutrients for the nematodes development and reproduction within the insect cadaver.

Entomopathogenic nematode belongs to the genera *Steinernema* and *Heterorhabditis* and their symbiotic bacteria in the genera *Xenorhabdus* Poinar and *Photorhabdus*

respectively. In the last two decades, survey has been carried out and several species of nematode species isolated (Rosa *et al*., 2000). Environmental factors influence the nematode occurrences and distribution and their survival. Biotic and abiotic factors cause the distribution EPN to differ across different regions (KarthikRaja *et al*., 2011).

Major factors namely temperature and host availability are thought to be important in determining their distribution. Since, few commercial strains of EPN species available and isolated from North America or Europe are used worldwide (Susurluk, 2011). Nowadays, application of EPNs is developed in large scale liquid culture, production costs compact and their usage in particularly horticulture, agriculture, and forestry. Therefore, these strains may perhaps not be well adapted to local climates and their value might be reduced. Several surveys have searched for new EPN species with the intent to control important agricultural and horticultural pests under specific conditions.

Exotic EPN displace native nematodes, effects on non-target organisms (Ehlers, 2005). Isolates of EPNs are usually found in a variety of habitats, exhibit considerable variation in their respective studies such as host range, reproduction, infectivity, and survival (Laznik and Trdan, 2012).

Therefore EPNs are considered as one of the most relevant non-chemical alternatives to insect pest control due to their high reproductive potential, ease of mass production and their harmlessness to microbes, animals, humans and plants. In this study, a survey was conducted to isolate and identify EPNs from disturbed habitats in the Kodaikanal area of Dindigul region. Morphology, morphometrics and ITS region of rRNA of nematodes was studied.

## Materials and Methods

### Soil Collection

The occurrence and distribution of EPNs was surveyed in crop fields and forest area of Kodaikanal, Dindigul district of Tamil Nadu. Study area has been segregated into four areas and the soil samples were collected. The study sites namely Combai, ThandiKudi, Pumbarai, Manavannur respectively. EPNs distribution in relation to environmental changeable and soil physical–chemical characteristic from each locality soil samples were taken from vegetable crops include cabbage, cauliflower, garlic, carrot and sampling interval was about 5 km. Totally 500 soil samples were collected in the polythene bag from the depth of 10-15cm and transported to laboratory for further processing.

### Isolation and Propagation of nematodes (*Galleria* Trap Method)

In the laboratory, soil samples was processed with the insect-baiting (*Galleria* trap) method (Bedding & Akhurst,1975). 250g soil sample was placed in a plastic box and baited with five larvae of *G. mellonella.* The boxes were stored at 25ºC for five days; the dead larvae were collected and transferred to a white trap (White, 1927) to collect the infective juveniles (IJs) of nematodes. All the soil samples were baited three times with larvae to get the maximum number of positive soil samples. The collected IJs were checked for their pathogenicity against *Galleria* larvae (Pelezar and Reid, 1972).

Nematode propagation was performed by applied IJs to a 4.5 cm diam Petri dish lined with Whatman No.5 filter paper containing five *G. mellonella* larvae. The Petri dishes were incubated at room temperature until IJs

emerged from the cadavers and were collected in a White trap. The IJs were then stored for less than three weeks at 15°C in distilled water until further testing. The IJs were stored at a concentration of 1000 IJs/ ml in distilled water with 0.1% formalin in tissue culture flask, were stored at 19°C in B.O.D incubator. Nematodes were routinely cultured in *G. mellonella* larvae (Woodring and Kaya, 1988).

## Nematode Identification

### Morphological characterization

For morphological characterization of the isolate, nematodes examined heat-killed in 60°C Ringer's solution. The heat-killed nematodes place in triethanolamine–formalin (TAF) fixative and processed to anhydrous glycerine for mounting. The morphological features of males and IJs and hermaphroditic female randomly selected from different *G. mellonella* under light microscopy according to procedures described by S**einhorst** method (1959). The identity was verified by comparing its morphometrics with the data from original descriptions. A morphological feature of a representative isolate was examined using scanning electron microscopy (SEM).

### Molecular Characterization

The isolates were molecularly characterized by analysis of the internal transcribed spacer region (ITS) ribosomal DNA sequences. DNA extraction was performed according to Stock *et al*. (2001). The IJs were crushed using extraction buffer (0.05M EDTA, 1%SDS, 400µl Proteinase K) and centrifuged for 10-15 minutes for 12,000 rpm. The supernant was collected and add equal volume of phenol: chloroform: isoamylalchol at the ratio 25:24:1. Then the tubes mixed well and centrifuge 12,000 rpm for 10minutes. The upper layer was removed and to which add chloroform: isoamylalchol in the ratio of 24:1, mixed and centrifuge, 12,000rpm for 10minutes. The upper phase was separated and 3M ammomium precipitate was added for better DNA precipitation. The suspensions freeze for 2 hours and centrifuge for 30 minutes at 12,000 rpm. DNA was precipitated from aqueous phase and after drying resuspended in TE buffer. The DNA sample was run by agarose gel electrophoresis using 1kb markers of lambda DNA as standard. For polymerase chain reaction (PCR) amplification and sequencing of the ITS, primers were designed by the method of Joyce *et al*. (1994).

## Results and Discussion

### Isolation of Entomopathogenic nematodes

A total of 500 soil samples from the Kodaikanal region of Dindigul district were surveyed for EPNs, of which 7 resulted in positive nematode isolates (Table 1). Two of the samples were positive for *Heterorhabditis* species and 5 were positive for *Steinernema species*. Compare to forest area, occurrence of EPNs was present in crop lands and absence in forest area. This was due to presence of insect pest was rare in forest, because only found long tree trunks only seen, low number of weed plants there(Razia *et al*., 2011).

### Morphological Characterization

The morphometric measurements of *Heterorhabditis* sp. collected from two places was identified as *H. indica* based on the length of IJs less than 600µm, Head to excretory pore 98µm and Tail length 101µm (Table 2). The male and IJs coincide well with the description given by Poinar *et al*. (1992).

**Table.1** Occurrence of EPNs in different agroecosystem of Kodaikanal Region of Dindigul

| **Sampling Area** | ***Steinernema* spp.** | ***Heterorhabditis* spp.** |
|---|---|---|
| Combai | **2** | **1** |
| ThandiKudi | **1** | **-** |
| Pumbarai | **1** | **1** |
| Mannavanur | **1** | **1** |

**Table 2.** Morphomertric characters of infective juveniles of *H. indica* (n=25) (Mean and Range, all measurements in µm)

| **Character** | **MTU06** | **MTU07** | **Poinar *et al*. (1992)** |
|---|---|---|---|
| Total Length (L) | 512.4 (500 - 560) | 510.7 (480 - 570) | 528 (479 - 573) |
| Greatest Width (W) | 19.9 (19 - 21) | 19.7 (19 - 20) | 20 (19 - 22) |
| Anterior end to Excretory pore (EP) | 93.2 (90 -100) | 97.3 (93 -105) | 90 (88 - 107) |
| Esophagus (ES) length | 105.8 (100 - 119) | 104.3 (100 - 120) | 115 (109 -123) |
| Tail length (L) | 98.5 (95 -108 ) | 99.0 (95 -106) | 101 (93 - 109) |

**Table 3.** Morphomertric characters of first generation male of *H.indica* (n=25) (Mean and Range, all measurements in µm)

| **Character** | **MTU06** | **MTU07** | **Poinar *et al*. (1992)** |
|---|---|---|---|
| Total Length (L) | 582.6 (575 - 685) | 591.3 (580 -780) | 721 (573 -788) |
| Greatest Width (W) | 39.9 (36 - 45) | 41.7 (38 - 45) | 42 (35 - 46) |
| Excretory pore (EP) | 110.5 (110 -130) | 119.7 (115 -135) | 123 (109 - 138) |
| Esophagus (ES) | 96.2 (93 - 100) | 97.8 (95 -105) | 101 (93 - 109) |
| Tail length (L) | 24.6 (24 - 30) | 26.8 (25 - 31 ) | 28 (24 -32) |
| Spicule Length (SpL) | 36.9 (35 - 45) | 37.9 (36 - 46) | 43 (35 - 48) |
| Gubernaculum length (GuL) | 18.5 (18 - 21) | 19.6 (18 - 20) | 21 (18 - 23) |

**Table 4.** Morphometric characters of infective juveniles of *S.siamkayai* (n=25) (Mean and Range, all measurements in µm)

| Nematode Isolates | Total Length (L) | Greatest Width (W) | Excretory pore (EP) | Esophagus length (ES) | Tail length (TL) |
|---|---|---|---|---|---|
| MTU01 | 459.9 (447- 470) | 21.2 (18 - 23) | 30.8 (30 - 37) | 97.9 (94 -100) | 35.9 (31 - 41) |
| MTU02 | 473.1 (435 - 488) | 23.2 (19 - 24) | 38.3 (31 - 38) | 106.2 (92 - 107) | 41.3 (30 - 40) |
| MTU03 | 486.8 (474 - 492) | 21.5 (18 - 23) | 35.2 (32 - 38) | 101.5 (89 - 105) | 40.7 (35 - 41) |
| MTU04 | 489.6 (456 - 490) | 20.6 (19 - 24) | 36.7 (34 - 38) | 105.6 (90 - 107) | 41.9 (31 - 41) |
| MTU05 | 469.7 (428 -483) | 20.9 (19 - 23) | 34.6 (32 - 36) | 102.5 (90 -105) | 42.3 (31 - 40) |
| *S. siamkayai* | 446 (398 - 495) | 21 (18 - 24) | 35 (29 - 38) | 94.5 (80 - 107) | 35.5 (31 - 41) |

**Table 5.** Morphometric characters of first generation male of *S.siamkayai* (n=25) (Mean and Range, all measurements in µm)

| Isolates | Total Length(L) | Greatest Width (W) | Excretory pore(EP) | Esophagus length (ES) | Tail length (TL) | Spicule length (SpL) | Gubernaculum (GuL) |
|---|---|---|---|---|---|---|---|
| MTU01 | 1141.3 (1100 -1190) | 123.8 (110 -150) | 55.6 (48 - 61) | 135.2 (128 -140) | 24.1 (22 - 30) | 76.9 (75- 80) | 53.8 (48 - 60) |
| MTU02 | 1133.5 (1130 - 1210) | 123.5 (121 - 155) | 56.8 (49 - 63) | 138.1 (130 - 140) | 24.8 (27 - 31) | 75.8 (75 - 79) | 55.2 (50 -65) |
| MTU03 | 1140.4 (1095 - 1195) | 123.8 (120 - 148) | 57.9 (48 - 59) | 136.5 (129 - 141) | 25.2 (22 - 30) | 74.9 (75 -79) | 52.8 (50 - 61) |
| MTU04 | 1131.3 (1092 - 1188) | 128.9 (110- 145) | 59.1 (49 - 61) | 138.2 (130 - 140) | 24.8 (22 - 32) | 75.2 (75 -78) | 54.6 (49 - 62) |
| MTU05 | 1143.8 (1092 - 1188) | 124.8 (110- 145) | 55.4 (49 - 61) | 136.5 (130-140) | 23.8 (22 - 30) | 74.1 (75 - 79) | 55.1 (50 - 61) |
| *S. siamkayai* | 1035 -1278 (1135) | 107-159 (139.5) | 47.5 - 67 (57) | 123 -141 (134) | 22-32 (27.5) | 75 - 80 (77.5) | 47 – 65 (53.5) |

In male, first generation male average length was 721µm whereas in the 596µm in the present study, spicule 40µm and gubernaculum 20µm (Table 3). Head truncate to slightly round. Nerve ring near the basal bulb. Spicules paired and separate, with pointed tips. Gubernaculum flat, narrow, approximately half the spicule length.

Among *Steinernema* spp. five was identified as *S. siamkayai* based on their length of IJs was less than 600µm, 486µm in the present study, Head to excretory pore 35µm and Tail length 40µm (Table 4). The third stage of Juveniles is slender, tapering regularly from base of oesophagus to anterior end and from anus to terminus. Oesophagus is long, and narrow. The first generation of male curved posteriorly, "J"-shaped when heat-killed. The first generation of male, total length average was 1,135 µm where as 1120µm in the present study, spicule 77µm and gubernaculum 53µm (Table 5).

## Molecular Characterization

The isolates were identified as 2 *H. indica* and 5 *S. siamkayai* confirming morphometric and morphological data. DNA sequences analysis was considered as the first approach to identify the EPN isolates in this survey. The isolates of *Heterorhabditis* and *Steinernema* yielded 1 kb fragment upon PCR amplification with the ITS primers. Ribosomal DNA (ITS region of nematodes) sequences generated for the isolate were individually matched and aligned with sequences available in GenBank.

Survey has been conducted in different crop ecosystem of Kodaikanal region of Dindigul District,mainly sampling was focused in the agriculture practices (Tillage, pesticides, fertilizer) involved and human interference area. The study was determined to know that the key indicator their distribution and impact of environmental stress response due to agriculture practices. In the present study 7 isolates (*Heterorhabditis* sp. and *Steinernema* sp.) were recovered. Morphometric measurements and molecular characterization of isolated EPNs were attained. Based on the morphometric characters studies variation between the strains observed. Further, molecular characterization was performed by using ITS region of rDNA

## Acknowledgement

The authors would like to acknowledge the UGC, NewDelhi for providing Fund for this research work.

## References

Arkansas Agricultural Experiment Station, Fayetteville, Arkansas, pp. 30.

Bedding, R.A., and Akhurst R.J. 1975. A simple technique for the detection of insect parasitic rhabditid nematodes in soil. Nematologica 21: 109 - 110.

Ehlers, R-U. 2001. Mass production of entomopathogenic nematodes for plant protection. Appl Microbiol and Biotechnol 56: 623 - 633.

Eleftherianos, I., and Joyce, S., Ffrench-Constant, R.H., Clarke, D.J., Reynolds, S.E., 2010. Probing the tri-trophic interaction between insects, nematodes and *Photorhabdus*. Parasitol 137: 1695–1706.Hill,NewYork.

Hominick, W.M. 2002. Biogeography. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 115-143.

Joyce, S.A., Burnell, A.M., and Powers, T.O. 1994. Characterization of *Heterorhabditis* isolates by PCR amplification of segments of mtDNA and rDNA. Journal of Nematology 26: 260 - 270.

Laznik ,Z., and Trdan, S. 2012. Entomopathogenic nematodes (Nematoda: Rhabditida) in Slovenia: from tabula rasa to implementation into crop production systems. In: Perveen F,editor. Insecticides: Advances in Integrated Pest Management. Rijaka, Croatia: In Tech, pp. 627–656.

Laznik, Z., Znidarcic D., and Trdan, S. 2011. Control of Trialeurodes vaporariorum (Westwood) adults on glasshouse-grown cucumbers in four different growth substrates: an efficacy comparison of foliar application of Steinernema feltiae (Filipjev) and spraying with thiamethoxamn. Turk J Agric For 35: 631–640.

Pelezer, M.J. and Reid, R.D.1972. Microbiology, Third Edition. Mc Graw

Poinar, G. O. 1992. *Steinernema feltiae* (Steinernematidae: Rhabditida) parasitizing adult fungus gnats (Mycetophilidae: Diptera) in California. Fundamental and Applied Nematology 15: 427 - 430.

Raja, R.K., Sivaramakrishnan, S., Hazir, S. 2011. Ecological characterization of Steinernema siamkayai (Rhabditida: Steinernematidae), a warm adapted entomopathogenic nematode isolate from India. BioControl 56:789–798.

Razia, M., Raja, R.K., Padmanaban, K., Chellapandi, P., Sivaramakrishnan, S. 2011. Monitoring Entomopathogenic Nematodes as Ecological Indicators in the Cultivated Lands of Karur District, Tamil Nadu: a Survey Report. Electronic Journal of Biology 7: 16-19.

Rosa,J.S., Bonifassi, E., Amaral,J., Lacey, L.A., Simoes,N. and Laumond,C. 2000. Natural Occurrence of Entomopathogenic Nematodes (Rhabditida: *Steinernema*, *Heterorhabditis*) in the Azores. Journal of Nematology 32: 215 -222.

Stock, S.P., Campbell, J.F. and Nadler, S.A. 2001. Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae). Journal of Parasitology 87: 877 - 889.

Susurluk İA (2011). Potential of Steinernema feltiae (Rhabditida: Steinernematidae) as a biological control agent against the cabbage maggot Delia radicum (Diptera: Anthomyiidae) in oilseed rape. Turk J Agric For 35: 413–419.

Susurluk, A. and Ehlers, R-U. 2008. Field persistence of the entomopathogeni nematode *Heterorhabditis bacteriophora* in different crops. Biological Control 53: 627- 641.

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

Woodring, J.L. and Kaya, H.K. 1988. *Steinernematid* and *heterorhabditid* nematodes. A handbook of techniques. Southern Cooperative Series Bulletin 331,