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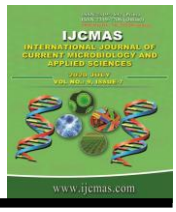

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Isolation and Identification of *Heterorhabditis bacteriophora* from Tea Plantation of Assam

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A B S T R A C T

A total 200 soil samples were collected from tea plantation areas of district, Jorhat, Assam and were assessed for entomopathogenic nematodes using the *Galleria* baiting technique. Out of 200 soil samples, EPN was found in 1 soil sample collected from Tea plantation, Chetiagaon, Jorhat and designated as EPN-H-1. Morphological and morphometrical characters were used for the identification of nematode isolate. The measurements were expressed in % ratios and Means ± SD ranges. The IJ of EPN-H-J-l showed close similarity with *H. bacteriophora* with respect to head shape, ratio b, D% and E%, but exhibited minor differences from the type measurements by having lower tail length (82 vs. 91). The males of this isolate showed close similarity with *H. bacteriophora* with respect to head shape, anal body width, gubernaculum length but exhibited minor differences from the type measurements by having higher esophagus length (107 vs. 103) and tail length (34 vs. 28). The hermaphroditic and amphimictic females of this isolate exhibited differences from the type measurements by having lower body length, lower body width and lower anal body width which are considered as intraspecific variations of *H. Bacteriophora*. Comparative analysis revealed that an EPN-H-1 isolate belong to the *Heterorhabditis bacteriophora* as earlier described by Poinar, 1976 from Brecon, South Australia in respect of body length, body width, tail length etc.

Introduction

Nematodes which are capable of killing, hampering the insect development and carrying out at least one stage of their life cycle in the host are called insect pathogenic nematodes *i.e* EPNs (entomopathogenic nematodes). Entomopathogenic nematodes contain mainly two genera *Steinernema* and *Heterorhaditis* in the family Steinernematidae and Heterorhabditidae of the order Rhabditida. They are obligate in nature and the widely disperse dispersed in the soils of different ecosystem.

They have an ability to act as best biocontrol agents against insect pests as compared to the bacterial and fungal bioagents. Recently, EPNs are considered as one of the non-chemical alternatives control strategy.

The 3rd stage juvenile act as an infective in nature and which is free-living in the soil, non-feeding, encased in a double cuticle with closed mouth and anus and capable of surviving for several weeks in the soil, before infecting a new host individual. It has ability to enter in the insect host through natural opening or actively penetrated through their cuticle. These juveniles mainly feed on the hemocoel of insect and they play strategy called as trogan horse in which they carry a *Photorhabdus* bacterium as symbionts in their intestine and the cuticle (Kaya and Gaugler, 1993; Torres-Barragan *et al.,* 2011; Zhang *et al.,* 2008, 2009, 2012).

After entering in the insect blood the juveniles released the bacteria and both overcome the insect immune system and killed the host within 24-48 hours of post infection (Adams and Nguyen, 2002). The life cycle of heterorhabditidis similar to that of steinernematids except for the fact that the IJs always develop into self-reproducing hermaphrodites (Poinar, 1990). Strauch *et al.*, (2000) observed that offspring of the first-generation hermaphrodites can either develop into amphimictic adults or into automictic hermaphrodite both can occur simultaneously. The cycle from entry of IJs into a 3rd stage until emergence of new IJs is dependent on temperature and varies for different species and strains.

Generally, life-cycle of EPNs (infective juvenile penetration to infective juvenile emergence) is completed within 12-15 days. The optimum temperature for growth and reproduction of nematodes is between 25°C and 30°C. They show their variations in respect of morphology, reproduction, infectivity, host range and conditions for survival under different environment conditions (Bedding, 1983). In the northeastern region of India, a few surveys against EPNs have been conducted in various habitats but and no survey has been conducted to document the occurrence of EPNs in tea habitats. So that a study was undertaken to isolate and identify EPNs from tea infested by *H. theivora* and *A. bipunctata* under Assam condition.

Materials and Methods

Survey and sample collection

A survey was undertaken in the tea plantation areas of Jorhat district of Assam for the presence of entomopathogenic nematodes (EPNs) during the year 2017-18. A total of 200 soil samples were collected randomly during the period Nov 2017 to Nov 2018. Each soil sample, weighing approximately 1 kg, was a composite of five random subsamples collected at least 100m apart at each site at a depth of 10–20 cm in an area of $20m^2$. Information regarding date of sampling, and soil type along with GPS (Global Positioning System) location was recorded. Samples were packed in polythene bags and maintained at refrigerated conditions in the laboratory for further processing. The soil was thoroughly mixed on a plastic sheet and half of each sample was used for extraction of EPNs.

Rearing of bait insect

Greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae) larvae is used as a bait insect for the trapping of EPNs. *G. mellonella* was collected from the Department of Entomology, AAU, Jorhat. The culture of *G. mellonella* was maintained on a semi-synthetic diet in the P.G laboratory, Department of Nematology, AAU, Jorhat (Plate 4).All solid ingredients, *viz.*, corn flour (400 gm), wheat bran (150 gm), wheat flour (200 gm), wheat germ (50 gm), yeast and milk powder (200 gm) were mixed thoroughly in a clean flat plastic tray.

Honey and glycerine (200 gm) dissolved in lukewarm water (200 gm) was added slowly to the solid mixture followed by the addition of streptomycin sulphate (100 gm). The mixture was kneaded thoroughly until semisolid light yellow coloured dough was obtained. This diet was transferred to 2 L capacity wide mouth jars and filled up to 1/4th of its volume. They were inoculated with 20-25 egg masses (each containing 500 eggs).

The jars were covered with white muslin cloth, secured by rubber band and incubated at 25ºC in the BOD incubator. Incubated eggs hatched within a week, emerging neonates fed voraciously and underwent 5 moults before pupation in 25 days. Pupae were transferred to a clean adult rearing cage. Adult moths emerged from pupae within 10-12 days. The emerging moths were collected every morning, sorted into males and females based on their size and shape and released into the egg laying chamber in the ratio of 1:6 (male : female).

Tissue papers made into folds were hanged inside the chamber for egg laying. Adults were feed on 20-30% honey solution through cotton wick in a small plastic dish. Within few days of mating, females laid eggs on the tissue paper. These eggs were then transferred to a fresh culture medium kept at 28°C in the BOD incubator for next generation. The 4th instar larvae were used for baiting.

Extraction of entomopathogenic nematodes (EPNs) from soil samples

Entomopathogenic nematodes were isolated from the soil samples by the method described by Fan and Hominick (1991) with larvae of the greater wax moth (*Galleria mellonella*). Before processing, soil samples were homogenized and then baited. Ten last instar larvae of *G. mellonella* were released into the plastic container containing 200g of soil sample. Baited samples were stored in the dark at room temperature. Samples were inverted at regular intervals and monitored for mortality up to 7-8 days. Insect cadavers from each soil sample were taken out and examined for infection. Collected *G. mellonella* larvae were transferred to White traps (White, 1927) and infected cadavers were placed on a 9 cm Whatman No.1 filter paper over a small Petri dish (50 mm × 17 mm) which was then placed in bigger Petri dish (100 mm × 20 mm) containing water. IJs recovered for the 5-12 following days were collected. IJs that emerged were pooled from each sample and used to infect fresh last instars of G. *mellonella* larvae to verify their pathogenicity and allow the production of progeny for identification at the genus level, considering the characteristic colour of the G. *mellonella* cadavers (Kaya and Stock, 1997).

Isolation of adults

In nature, the adults of first and second generation are found only in the haemocoel of cadaver; hence they were extracted by dissection in Ringer's solution. The dissection was done at 2-4 and 4-5 days after inoculation (DAI) for recovering the first generation and second-generation adults, respectively. The recovered nematodes were kept in clean ringer's solution for further processing.

Processing of nematodes

Third stage infective juveniles in sterile distilled water and freshly dissected out first and second-generation adults in Ringer's solution were killed and fixed by pouring equal volumes of hot tri-ethanolamine formalin (TAF) fixative over the EPN suspension (Kaya and Stock,1997). After 24 hrs, the specimens were handpicked individually and transferred to 100% TAF and fixed for a week.

Killed and fixed nematodes were further processed with Seinhorst's slow glycerol dehydration method (Seinhorst, 1959). Permanent mounts were prepared by transferring the nematodes to a drop of anhydrous glycerin on a clean glass slide supported by radially placed 3 small pieces of glass wool supports with thickness approximately equal to the diameter of the nematode to prevent flattening of specimens.

The slides were sealed with paraffin wax and labelled with adequate information including locality, slide number, sex and stage of nematode.

Light microscopic studies

The permanent slides were examined for detailed morphological characters and body dimensions were studied using de Man's formula (De Man, 1880) and additional ratios to establish their taxonomic identity.

The morphological identification was performed on the basis of characters of third stage infective juveniles and male individuals (Poinar, 1976; Poinar and Georgis, 1990; Nguyen and Smart, 1995; Stock *et al.,* 2002).

Morphological characters

The following morphological characters were taken into consideration for identification at species level.

(a) Shape of head
(b) Presence or absence of epiptygma
(c) Shape and size of spicules
(d) Shape and size of gubernaculum
(e) Presence or absence of post anal swelling in adult females
(f) Tail shapes of both adults and infective juveniles
(g) Presence or absence of mucron in adults of both sexes

Morphometrical measurements

In addition to the morphological characters, morphometric measurements also have taxonomic significance in differentiation of species. Morphological characters of 20 specimens each of infective juveniles, males and females of first and second generation were observed. Morphological observations and quantitative measurements were made by advanced stage compound microscope (Olympus).

Liner body dimensions recorded were as follows

Body length (L)
Body width (W)
Oesophageal length (ES)
Distance from anterior end to excretory pore (EP)
Distance from anterior end to nerve ring (NR)
Spicule length (SL)
Gubernaculums length (GL)
Anal body width (ABW)
Tail length (T)

The following ratios were computed

Ratio a = Body length/Greatest body width
Ratio b = Body length/Oesophageal length
Ratio c = Body length/Tail length
V = Distance of vulva from anterior end/Body length × 100
D% = Distance from anterior end to excretory pore / Oesophageal length × 100
E%= Distance from anterior end to excretory pore/Tail length × 100
SW% = Spicule length/anal body with × 100
GS% = Gubernaculum length/ Spicule length × 100

Comparison with known species

The morphological character and body

dimension of *Heterorhabditis* sp. identified in the present study were compared with the original descriptions of known species. Variations in the morphometrical characters of the existing species were recorded and described.

Results and Discussion

A random survey was conducted for the natural occurrence of EPNs during 2017-18 from tea plantation area of the district, Jorhat, Assam. A total of 200 samples were collected from Experimental farm for tea plantation crops, Section-4, 10, 19 AAU, Experimental farm for plantation crops, Section-14 AAU, Jorhat, Experimental Farm, Tocklai Tea Research Station and Chetiagaon, Jorhat. Survey data revealed that out of 200 samples, 1 sample was positive for EPN with 1 sample containing *Heterorhabditis* sp. (0.5%) (Table.1). One heterorhabditid isolate designated as EPN-H-1 was isolated from rhizosphere of tea from Chetiagaon, Jorhat. Morphological and morphometrical characters were used for the identification of nematode isolates.

Morphological and morphometrical studies of different life stages (infective juveniles, adults) of EPN-H-J-1 revealed that it is closely resemble with *Heterorhabditis bacteriophora* (Poinar, 1975) in most of the characters.The head of the third-stage infective juvenile (IJ) bears dorsal tooth with mouth and anus is closed. Stoma appears as a closed chamber. The head is with sheath (cuticle of second-stage juvenile). Esophagus and intestine are reduced. The excretory pore is posterior to nerve ring. The tail is long, pointed and covered with a sheath. The male of second generation had slightly round head. They possess a tubular stoma and pharynx with a cylindrical corpus. The isthmus is distinct with a globose basal bulb and a prominent valve. The nerve ring surrounding the isthmus is located near the basal bulb. The excretory pore is located near the middle of the basal bulb. The reproductive structure is monarchic and anteriorly reflexed. The spicules are paired, symmetrical and separate, with pointed tips, slightly curved ventrally. The gubernaculum is flat and narrow. Bursa is peloderan, open, with nine pairs of genital papillae. Tail is pointed. The hermaphroditic female of first generation body curved ventrally when heat-killed. Head region a slightly rounded.

They possess a tubular stoma and pharynx with a cylindrical corpus.The isthmus is distinct and short. Nerve ring surrounding isthmus is just anterior to basal bulb. Basal bulb often surrounded by anterior portion of intestine. Execratory pore is posterior to the basal bulb. Gonads are amphidelphic and reflexed. Vulva is near to the mid-body. Tail is pointed. Tail is longer than anal body width and conoid with pointed terminus. Anal region is slightly protruding.

The amphimictic females of second generation body are ventrally curve when heat-killed, smaller in size than hermaphroditic female. Head region is sub conical. They possess a tubular stoma and with a cylindrical corpus. The vulva is not protrude outward and is surrounded by a hard deposit. Anal region is slightly protruding.

The IJ of EPN-H-J-l showed close similarity with *H. bacteriophora* with respect to head shape, ratio b, D% and E%, but exhibited minor differences from the type measurements by having lower tail length (82 vs. 91) (Table 3). The males of this isolate showed close similarity with *H. bacteriophora* with respect to head shape, anal body width, gubernaculum length but exhibited minor differences from the type measurements by having higher esophagus length (107 vs. 103) and tail length (34 vs.

28). The hermaphroditic and amphimictic females of this isolate exhibited differences from the type measurements by having lower body length, lower body width and lower anal body width which are considered as intraspecific variations of *H. bacteriophora* (Table 3).

A total of one isolate of entomopathogenic nematodes from 200 soil samples collected from Tea plantation areas of district Jorhat, Assam with a per cent recovery of 0.5%. EPN distribution depends on temperature, precipitation and soil type and is closely related to vegetation type and presence of insect host (Nielsen and Philipsen, 2003 and Puza and Mracek, 2005). The soil is sandy or sandy loam with a good amount of organic matter. The nematode presence and abundance were low in different tea fields of most of the sampling site. Although EPN was recorded at a low rate in present study, one isolate of *Heterorhabditis bacteriophora* (0.5%) was recorded.

It may be resulted due to condition of the crop land in terms of irrigation of the field, where the temperature and the soil moisture was suitable for their persistence. One reason for the low recovery rate obtained in the present study, could be the fact that only one insect, *Galleria mellonella*, was used as bait insect may not be the appropriate host for all EPN species (Kary *et al.*, 2009).

Table.1 Occurrence of Entomopathogenic nematodes in tea plantation areas of district, Jorhat, Assam

Locality	**No. of samples**	**No. of +ve samples for EPN**	**Crop**	**EPN isolate**		**Latitude, Longitude**
				Heterorhabditis sp.	Frequency of occurrence (%)	
Experimental farm for plantation crops, Section-4,10,19 AAU, Jorhat	50	0	Tea			
Experimental farm for plantation crops, Section-14 AAU, Jorhat	50	0	Tea			
Experimental Farm, Tocklai Tea Research Station	50	0	Tea			
Chetiagoan	50	1		EPN-H-J-1	0.5	26°71'36.59"N 94°19'79.81"E
Total	200	1			0.5	

Table.2 Morphometrics of *Heterorhabditis sp*. (EPN-H-J-1) infective juveniles and second generation male in comparison with original description of *Heterorhabditis bacteriophora*

Character	*Heterorhabditis sp.* (EPN-H-J-1) (IJ) (n=40)	Type measurement *H. bacteriophora* (IJ) (Poinar,1976) (n=15)	Type measurement *H. bacteriophora* (IJ) (Poinar, 1990) (n=25)	*Heterorhabditis sp.* (EPN-H-J-1) (Male) (n=20)	Type Measurement of *H. bacteriophora* (Male) (Poinar, 1976) (n=15)
Body length (L)	542.57±42.339 (483-624)	570 (520-600)	558 (512–671)	842.4±63.9 (760-980)	820 (780-960)
Body width (W)	23.75±5.278 (14-30)	24 (21-31)	23 (18–31)	46.85±3.498 (42-53)	43 (38-46)
Anterior end to excretory pore (EP)	98.2±6.680 (86-108)	104 (94-109)	103 (87–110)	119.25±9.64 (106-132)	121 (114-130)
Anterior end to nerve ring (NR)	84.7±5.816 (76-94)	83 (81-88)	85 (72–93)	75.6±3.73 (71-82)	72 (65-81)
Anterior end to esophagus base (ES)	118.325±13.00 (96-135)	125 (119-130)	125 (100–139)	107.35±6.68 (97-118)	103 (99-105)
Testis reflection	-	-	-	81.2±5.094 (74-90)	79 (59-87)
Tail length(T)	82.05±8.661 (64-92)	91 (83-99)	98 (83–112)	34.2±2.44 (31-38)	28 (22-36)
Anal body width (ABW)	16.475±3.219 (9-21)	-	-	22.05±1.09 (19-23)	23 (22-25)
Ratio a= (L/W)	24.089±6.303 (16-40)	25 (17-30)	25 (17–30)	-	-
Spicule length (SL)	-	-	-	42.75±3.66 (38-50)	40 (36-44)
Gubernaculum length (GL)	-	-	-	23.05±2.45 (28-19)	20 (18-25)
D%= (EP/ES)×100	-	-	84 (76-92)	111.38±10.28 (96.4-127.8)	117
SW%=SL/ABW×100	-	-	-	194.35±19.44 (165-238)	174
GS%=GS/SL×100	-	-	-	54.2±6.90 (44-68)	50

Measurements in µm and in the form: mean± SD (range)

Table.3 Morphometrics of *Heterorhabditis sp*. (EPN-H-J-1) hermaphroditic and amphimictic female in comparison with original description of *Heterorhabditis bacteriophora*

Character	*Heterorhabditis sp.* (EPN-H-J-1) (Hermaphroditic females) (n=20)	Type measurement *H. bacteriophora* (Hermaphroditic females) (Poinar, 1976) (n=15)	*Heterorhabditis sp.* (EPN-H-J-1) (Amphimictic females) (n=20)	Type Measurement of *H. bacteriophora* (Amphimictic females) (Poinar, 1976) (n=15)
Body length (L)	2136.2±300.35 (1460-2469)	4030 (3630-4390)	1972.9±257.3 (1560-2495)	3500 (3180-3850)
Body width (W)	158.9±9.727 (146-178)	165 (160-180)	153.2±11.13 (132-168)	190 (160-220)
Anterior end to excretory pore (EP)	168.4±21.08 (132-204)	209 (189-217)	151.9±16.64 (124-182)	192 (174-214)
Anterior end to nerve ring (NR)	123.05±8.55 (104-132)	126 (121-130)	114.2±5.23 (99-120)	103 (93-118)
Anterior end to esophagus base (ES)	174.75±8.77 (162-198)	197 (189-205)	166.65±16.60 (189-133)	-
Tail length (T)	86.05±6.605 (74-96)	90 (81-93)	81.85±3.66 (76-88)	82 (71-93)
Anal body width (ABW)	32.35±5.30 (24-41)	46 (40-53)	23.45±3.10 (18-29)	28 (22-31)
V%= distance from anterior end to vulva as percentage of length	49.4±2.707 (43.05-53.02)	44 (41-47)	49.53±3.90 (43.04-56.04)	47 (42-53)

Measurements in µm and in the form: mean± SD (range)

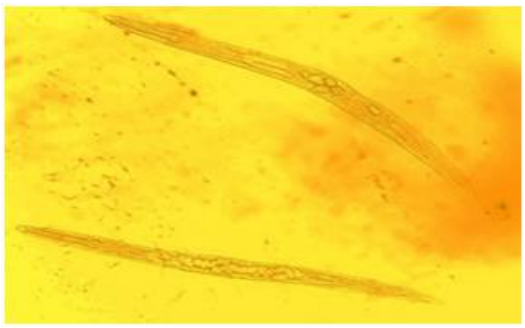

Figure.1 Hermaphroditic female of *Heterorhabditis bacteriophora*

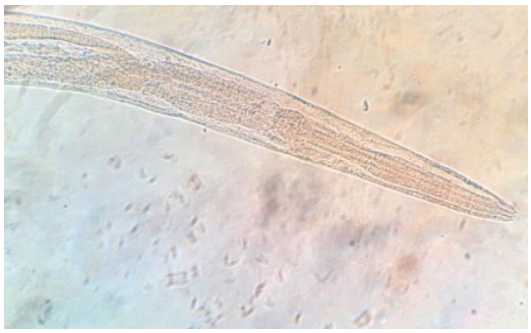

Figure.2 Oesophagus of Hermaphroditic female of *Heterorhabditis bacteriophora*

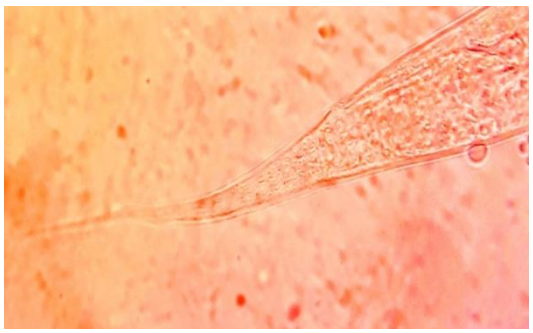

Figure.3Tail region of Hermaphroditic female of *Heterorhabditis bacteriophora*

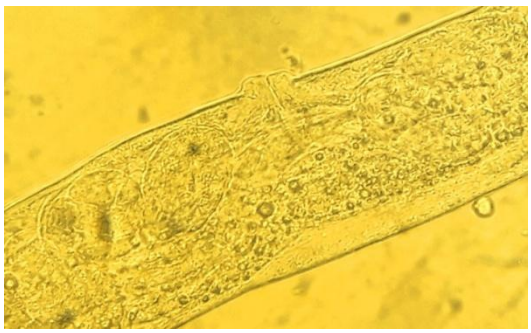

Figure.4 Vulva of Hermaphroditic female of *Heterorhabditis bacteriophora*

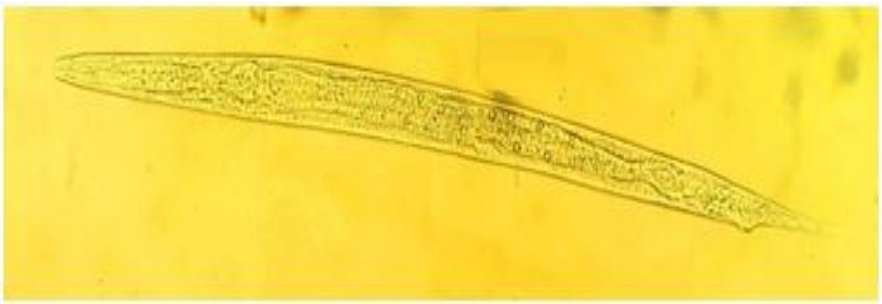

Figure.5 Infective juveniles of *Heterorhabditis bacteriophora*

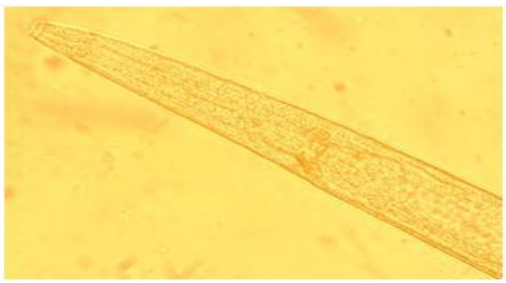

Figure.6 Anterior part of Amphimictic female of *Heterorhabditis bacteriophora*

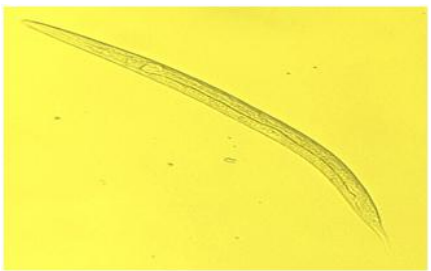

Figure.7 Amphimictic female of *Heterorhabditis bacteriophora*

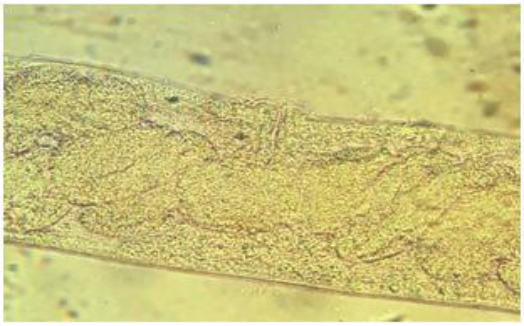

Figure.8 Vulva of Amphimictic female of *Heterorhabditis bacteriophora*

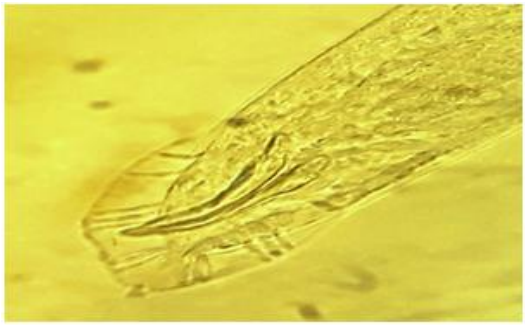

Figure.9 Spicule of *Heterorhabditis bacteriophora*

Figure.10 Male of *Heterorhabditis bacteriophora*

Furthermore, the choice of sampling sites may contribute to difference in EPN recovery percentage (Mracek *et al.,* 2005). Lower percentage of EPNs probably also due to chemical control of insect pest in tea fields which partially reduces the abundance of natural biocontrol agents. However this low recovery percentage is not unusual, and it has already been reported from other surveys (Hazir *et al.,* 2003; Kary *et al.,* 2009).

The EPN isolate positive soil samples were from sandy loam soil and this finding was in agreement with findings of the surveys conducted by Ambika and Sivakumar (2000), which revealed that the occurrence of EPNs was more in light soils like sandy loam, sandy, loamy sand, and loam soil rather than in heavy soils. However, EPNs are present in heavy soils like clay soil also as recorded by Shyamprasad *et al.,* (2001) and Sosamma and Rasmi (2002) in the South Andaman and Kerala, respectively. In Sri Lanka, *Heterorhabditis* sp. was reported to be restricted to sandy soils within 100 m of the sea (Amarsinghe *et al.,* 1994). The heterorhabditid isolate was similar to *H. bacteriophora* in original description with respect to third stage infective juvenile in characters like greatest width; distance from anterior end to excretory pore; distance from anterior end to pharynx base; body width at anus; ratio a; ratio b; ratio c; D% ; E%. However, the isolate showed variation in body length of IJs (542 vs.570) and tail length (82 vs. 91), Variation also observed with respect to adult stage of both male and female generations in some characters like body length, position of pharynx, position of excretory pore, tail length, spicule length and gubernaculum length, etc.

Nguyen *et al.,* (1995) observed variation in body length, position of excretory pore, tail length and value of E% of *H. bacteriophora* in relation to time of harvest. It was observed that body length of infective juvenile was 605um (579µm-634µm) on 3rd day of harvest whereas body length 565nm (524 µm 604 µm) on 15th day of harvest. In the present

investigation the third stage IJs were obtained when they emerged from the cadavers after 7 to 10 days. Devi *et al.,* (2016) reported occurrence of *H. bacteriophora* from white grub infested areas of Majuli, Assam.

They exhibited minor differences in morphometrical studies of *H. bacteriophora* from the type measurements by having higher body length of IJs (572 vs. 570), body width (26 vs. 24) position of nerve ring (84 vs. 83).Identification was confirmed using infective juveniles and males because morphology of females and hermaphrodites vary with the nematode generation in the insect (Gaugler, 1990; Wright, 1990). Poinar (I976) isolated and described *H. bacteriophora* from Brecon, South Australia.

The nematode was isolated from the body cavity of *Heliothis punctigera* Hall (Noctuidae: Lepidoptera). *H. bacteriophora* is highly mobile, responding to chemical signals from the host, and being adapted to infect less mobile insect that are found in lower soil layers (Ishibashi, 2002). *H. bacteriophora* is distributed in America, Southern and Central Europe, Australia and East Asia (Hominick *et al.,* 1996). In Europe it has been reported from Spain, Italy, Moldova, Hungary, Southern France (Smits *et al.,* 1991), the Azores, Switzerland (Hominick, 2002), South Russia (Ivanova *et al.,* 2000), the European part of Turkey (Hazir *et al.,* 2003) and Slovenia (Laznik *et al.,* 2009), *H. bacteriophora* isolates were found in neutral (vertisol) or acidic (oxysol) soils incrop lands, orchards, and woodland habitats in Guadeloupe (Grande Terre, Basse Terre). *H. bacteriophora* was reported from India by Sivakumar *et al.,* (1989) and Hussaini *et al.,* (2001).

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