Occurrence of entomopathogenic nematode (Rhabditida: Heterorhabditidae, Steinernematidae) in white grub infested areas of Majuli, Assam, India

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ABSTRACT

Entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* and their associated bacteria such as *Xenorhabdus* spp. and *Photorhabdus* spp. are lethal parasites of soil dwelling insects. A survey was conducted to collect the entomopathogenic nematodes from *Lepidiota mansueta* endemic field of Majuli river island, Assam, India. During the survey, a total of 140 soil samples (1 kg each) and 80 dead grubs of *L. mansueta* were collected from different locations from cultivated as well as uncultivated fields of Majuli. Out of the total collection, 8 numbers of soil samples and 35 grubs were positive for entomopathogenic nematodes with 5.7 and 43.7 per cent respectively. Soil samples recorded 2.1 and 3.5 per cent *Heterorhabditis* and *Steinernema* isolates whereas the grubs registered 15 and 28.7 per cent of *Heterorhabditis* and *Steinernema* isolates respectively. Based on morphometric and cross-breeding studies, the *Heterorhabditis* isolates were identified as *H. bacteriophora* and this species was recovered from diverse habitats with different soil characteristics. Being a river island, the soil of Majuli may be the reservoir of many more species of EPNs which needs further study.

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INTRODUCTION

Lepidiota mansueta Burmeister (Coleoptera: Scarabidae) is a notorious root feeder which severely infests many field crops in Majuli river island. The severely affected crops were potato, sugarcane, *Colocasia* and green gram. The extent of damage was found to vary between 42-48, 15-20, 35-40 and 25-30 per cent respectively. The damage becomes evident only when the entire plant dries up due to the grub infestation. The infestation is mainly because of extensive feeding by third instar grubs. Being subterranean in habit it is very difficult and laborious to manage this species with chemical insecticides.

Currently Entomopathogenic Nematodes (EPNs) are an important part of the spectrum of potentially available biological control agents against insect pests. Species of *Steinernema* and *Heterorhabditis* are considered as potential biocontrol agents because of their association with parasitic symbiotic bacteria, *Xenorhabdus* spp. and *Photorhabdus* spp. These EPNs are environmentally safe and there is no problem of insect resistance. Different species often exhibit variation in relation to host range, infectivity and environmental tolerance. Indigenous entomopathogenic nematodes may be more suitable for innundative release against local insect pests because of their adaptability to local climate (Stock *et al*., 1999). Steinernematids and Heterorhabditids have been isolated from Indian soil by various workers (Ganguli *et al*., 2006). Considering the viewpoint, a random survey was conducted to isolate and identify EPN species from the white grub infected fields of Majuli. Findings of this research will be helpful to search and identify the predominant species of endemic EPNs and to develop safe, effective and economic management strategies for white grub management.

MATERIALS AND METHODS

Majuli district of Assam covers an area of 92,460 hectares, lying between 26°45′N - 27°15′N latitude and 93°45′E - 94°30′ E longitude, at an elevation ranging from 60 to 85 m above mean sea level. Majuli falls under subtropical climatic condition with a warm humid summer and cool dry winter with mean annual rainfall of 1900 mm. The average maximum temperature ranges from 23.6 °C to 31.7 °C and minimum, from 10.0°C to 24.2 °C. On an average, the relative humidity is more than 80% throughout the year.

Sample collection

One hundred and forty soil samples and eighty dead white grubs were collected from cultivated and non-cultivated areas of Majuli during July to September 2014 and 2015. For each location, GPS coordinates and soil physico-chemical characteristics such as soil texture, soil pH, organic matter (%), soil moisture (%), and soil temperature (^{0}C) were recorded (Table1). Each soil sample (1kg) was a composite of 5 random sub-samples taken distantly located (2 m) from each other in an area of 10m^2 and at a depth of 10–30 cm. Samples were taken with a hand shovel, and filled up in polyethylene bags to prevent water loss. Between each collection of the samples, the shovel was thoroughly cleaned and rinsed with 70% ethanol to prevent contamination of the next sampling unit.

Nematode isolation and culture

Entomopathogenic nematodes were recovered from soil samples using the insect baiting method described by Bedding and Akhurst (1975). Insect baits (larvae of *Galleria mellonella*) were placed in 250 ml plastic containers with moistened soil obtained from each sample. Containers were covered with a lid, turned upside down and kept at room temperature.Dead larvae were taken out on the basis of their visual appearance.The dead larvae were thoroughly rinsed in distilled water and placed in modified White traps (Kaya and Stock, 1997) until the emergence of third-stage infective juveniles. The emerged infective juveniles (IJs) were extracted in a beaker and cleaned two or three times with distilled water by sedimentation, followed by decantation. The extracted nematodes were stored at 15° C in distilled water for future processing. To obtain first and second generation male and female, the cadavers were dissected in Ringers's solution. The dissection was done 2- 4 or 4-5 days after inoculation for first generation, 5-7 or 6-8 days for second generation and the infective juvenile emerged out after 10-12 days.The adults of both generations along with infective juveniles were killed by gentle heat and fixed in TAF and then processed with anhydrous glycerin by the method of Seinhorst (1959). The adults and the IJs (20 each) were then mounted in dehydrated glycerin using appropriate sized glass support. The mounted specimens were used for detailed microscopic studies (Poinar, 1990) using a compound microscope (Magnus) equipped with an ocular micrometer. In addition to the deMan formula, the other characters studied were: D% (Distance from Head to Excretory pore/ oesophageal length x 100), E% (Distance from Head to Excretory pore/tail length x 100), F% (Body width/tail length x 100), SW (Spicule length / cloacal body width), GS (gubernaculums length / spicule length). Morphological and morphometrical data of the isolates were compared with the original description of the type species. As *G.mellonella* was used as the laboratory bait insect, it was reared on artificial diet as per the procedure described by David and Kurup (1988).

The cross hybridization technique of *Heterorhabditis* species was tested using *G. mellonella* hemolymph by the method reported by Dix *et al*. (1992). Isolation of EPN symbiotic bacteria was done following the procedure described by Akhurst (1980). Conventional phenotypic criteria were used to verify generic identity (*Xenorhabdus* and *Photorhabdus*) of bacterial isolates (Boemare and Akhurst, 1988; Akhurst, 1993). The cellular morphology was assessed by microscopic examination of 24hr old nutrient broth cultures. Dye adsorption of bromothymol blue was tested on nutrient agar supplemented with 0.004 per cent

triphenyltetrazolium chloride and 0.0025 bromothymol blue (NBTA medium) for *Xenorhabdus* isolates. Dye adsorption of neutral red was tested on MacConkey agar for *Photorhabdus* isolates. Soil samples which contained EPN were analyzed for different soil physico-chemical parameters with standard procedures.

RESULTS AND DISCUSSIONS

Out of the 140 soil samples, 8 (5.7%) samples and 35 (43.7%) grubs of *L. mansueta* were found to be positive for EPN (*Heterorhabditis* sp., *Heterorhabditis* sp. and *Steinernema* sp., *Steinernema* sp.. One heterorhabditid strain was isolated by soil baiting technique (designated as H-M-1) and from white grub cadavers (designated as WG-H-M-1) from green gram cultivated areas. Another two heterorhabditid strains were isolated by soil baiting technique (designated as H-M-2, H-M-3) and from white grub cadavers (designated as WG-H-M-2, WG-H-M-3) from uncultivated areas. Steinernematid strains (designated as S-M-1, S-M-2, S-M-3) were isolated by soil baiting technique and white grub cadavers (designated as WG-S-M-1, WG-S-M-2, WG-S-M-3) from sugarcane and *Colocasia* cultivated areas respectively (Table 1).

In the survey, the majority of positive results were found from sandy clay loam soil followed by silty loam, sandy loam and silty clay loam with acidic soil (pH 5.5 to 6.5). The organic matter content of the positive samples varied from 1.59 to 1.77 per cent. Soil temperature and soil moisture ranged from 20° C to 22° C and 13.25 to 33.49 per cent respectively. Steinernematids were isolated from sandy to silty clay loam with acidic soil (pH 5.5 to 5.9), organic matter (1.65-1.69%), soil temperature (20.0° C) and moisture content (23.83 to 34.18%) respectively. Similarly heterorhabditids were isolated from sandy to silty loam with moderately acidic soil to slightly acidic (pH 5.8 to 6.5), organic matter content (1.59-1.77%), soil temperature (20-22° C) and moisture contents from 13.25 to 25.27 per cent respectively. Both heterorhabditids and steinernematids distribution was influenced by soil texture. However heterorhabditids have adapted to thrive at different soil physico-chemical parameters. Males and females of each isolate of *Heterorhabditis* sp. (H-M-1), (H-M-2) and (H-M-3) and *Heterorhabditis* sp. (WG-H-M-1), (WG-H-M-2) and (WG-H-M-3) were mated and offsprings were recorded which indicated that they belong to the same species. No offsprings were produced by crosses with the above mentioned isolates and the strain (S-M-1) of *Steinernema sp*. (Table 2).

Nematode identification

Morphological and morphometrical studies of different life stages (infective juveniles, males of second generation) of H-M-1 revealed that it closely resembles *H. bacteriophora* (Poinar, 1975) in most of the characters. The head of the third-stage infective juvenile (IJ) bears dorsal tooth with mouth and anus 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 IJs of this strain showed close similarity with *H.bacteriophora* with respect to head shape, ratio b, ratio c, D% and E%, but exhibited minor differences from the type measurements by having higher body length (572 vs.570), body width (26 vs.24) position of nerve ring (84 vs. 83), which were considered as intraspecific variations of *H. bacteriophora* (Table 3). 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; anteriorly reflexed. The spicules are paired, symmetrical and separate, with pointed tips, slightly curved ventrally. The gubernaculum is flat and narrow, bursa peloderan, open, with nine pairs of genital papillae, tail pointed. The males of this strain showed close similarity to *H. bacteriophora* with respect to head shape, tail length, anal body width,

Table 1. Characterization of the soil physico-chemical parameters and enumeration of entomopathogenic nematodes from Majuli river island, Assam, India.(**Mean±SD)**

Sampling site	Sampling Type (Habitats)	Soil texture	pH	Organic matter (%)	Soil Moisture (%)	Soil temperature (°C)	Total number of samples	Number of samples positive for EPN	
								Steinernema spp. (Designation of isolates)	*Heterorhabditis spp.* (Designation of isolates)
Samuguri ($N27^0 01.659'$ $E94^0 22.144'$)	Cultivated (Green gram)	Sandy loam	5.8±0.01	1.59±0.01	13.25±0.02	22±0.01	5 (soil) 5 (dead insect)	-	1 (H-M-1) 5 (WG-H-M-1)
Major chapori ($N26^0 90.936'$ $E93^0 97.132'$)	Uncultivated	Silty loam	6.5±0.02	1.76±0.01	24.68±0.01	20±0.01	10 (soil) 10 (dead insect)	-	1 (H-M-2) 5 (WG-H-M-2
Majgaon ($N27^0 01.085'$ $E94^0 22.907'$)	Cultivated (sugarcane)	Sandy clay loam	5.9±0.01	1.69±0.01	33.49±0.02	20±0.01	10 (soil) 10 (dead insect)	1 (S-M-1) 10 (WG-S-M-1)	-
Kargil chapori ($N27^0 01.674'$ $E94^0 22.434'$)	Cultivated (colocasia)	Silty clay loam	5.7±0.01	1.65±0.02	23.83±0.01	20±0.01	10 (soil) 10 (dead insect)	2 (S-M-2) 3 (WG-S-M-2)	-
Kuli chapori ($N26^0 55.065'$ $E94^0 07.237'$)	Uncultivated	Silty loam	6.3±0.02	1.77±0.02	25.27±0.01	22±0.02	10 (soil) 10 (dead insect)	-	1 (H-M-3) 2 (WG-H-M-3)
Mohorichuk ($N26^0 57.413'$ $E94^0 08.232'$)	Cultivated (sugarcane)	Sandy clay loam	5.5±0.01	1.68±0.01	34.18±0.02	20±0.02	10 (soil) 10 (dead insect)	2 (S-M-3) 10(WG-S-M-3)	-

Total number of location = 20; Total number of soil sample collected=140; Total number of dead white grub collected= 80; not found

Table 2. The results of cross-breeding experiments of ***Heterorhabditis*** *and* ***Steinernema*** isolates from Majuli river island, Assam

FEMALE	MALE						
	H-M-1	H-M-2	H-M-3	WG-H-M-1	WG-H-M-2	WG-H-M-3	S-M-1
H-M-1	+	+	+	+	+	+	_
H-M-2	+	+	+	+	+	+	_
H-M-3	+	+	+	+	+	+	_
WG-H-M-1	+	+	+	+	+	+	_
WG-H-M-2	+	+	+	+	+	+	_
WG-H-M-3	+	+	+	+	+	+	_
S-M-1	_	_	_	_	_	_	+

+ cross resulting in fertile progeny; - no progeny detected.

Table 3. Comparative measurements (µm) of infective juveniles and males of ***Heterorhabditis* sp. (H-M-1)** from Majuli river island, Assam and those of its type measurements. **(Mean ± SD)**

Character	*Heterorhabditis sp.* H-M-1(IJ) (n=20)	Type measurement *H. bacteriophora* (IJ) (Poinar,1976) (n=15)	*Heterorhabditis sp.* H-M-1(male) (n=20)	Type measurement *H. bacteriophora* (male) (Poinar,1976) (n=15)
Body length	572±4 (520-675)	570 (520-600)	831±55 (750-970)	820 (780-960)
Body width	26±4.6 (19-35)	24 (21-31)	48±3.9 (44-50)	43 (38-46)
Anterior end to excretory pore	103±4.9 (93-110)	104 (94-109)	117±6.2 (109-130)	121 (114-130)
Anterior end to nerve ring	84±6.9 (80-100)	83 (81-88)	77±3.1 (74-84)	72 (65-81)
Anterior end to esophagus base	128±6.3 (115-140)	125 (119-130)	110±8.5 (99-130)	103 (99-105)
Testis reflexion	-	-	82±5.2 (76-90)	79 (59-87)
Anal body width	-	-	22±1.9 (20-25)	23 (22-25)
Tail length	92±5.4 (83-100)	91 (83-99)	32±2.8 (30-36)	28 (22-36)
Spicule length	-	-	43±2.6 (40-50)	40 (36-44)
Gubernaculum length	-	-	21±1.5 (20-25)	20 (18-25)
Ratio a	23±3.4 (17-28)	25 (17-30)	-	-
Ratio b	4.4±0.35 (4-5.2)	4.5 (4.0-5.1)	-	-
Ratio c	6.1±0.5 (5.7-7.1)	6.2 (5.5-7.0)	-	-
D%	0.8±0.03 (0.78-0.88)	0.84 (0.76-0.92)	106±5.9 (90-118)	-
E%	1.1±0.05 (1-1.2)	1.12 (1.03-1.20)	-	-
F%	0.28±0.04 (0.22-0.35)	0.25 (0.22-0.36)	-	-
SW	-	-	2.1±0.2 (1.8-2.5)	-
GS	-	-	0.45±0.4 (0.4-0.5)	-

- : Not applicable

gubernaculum length but exhibited minor differences from the type measurements by having higher body length (831 vs.820), body width (48 vs.43), esophagus length (110 vs. 103), position of excretory pore (117 vs. 121), which are considered as intraspecific variations of *H. bacteriophora*. The strain H-M-1 thus identified as *Heterorhabditis bacteriophora*. *H. bacteriophora* was reported from India by Sivakumar *et al*. (1989) and Hussaini *et al*. (2001). *H. bacteriophora* is distributed in America, Southern and Central Europe, Australia and East Asia (Hominick *et al*., 1996).

Bacterial characterization

The bacterial symbionts were identified as *Xenorhabdus* sp. and *Photorhabdus sp*. for *Steinernema* sp. and for *Heterorhabditis* sp. The present study recorded for the first time the occurrence of *Heterorhabditis sp*. and *Steinernema sp*. in Majuli. Nematodes were recovered from both the cultivated as well as uncultivated soils. Rosa *et al*. (2000) reported that most of the surveys showed their recovery rate from soil to vary between 6 to 35 per cent. Raj Kumar *et al*. (2001) showed that out of 105 soil samples collected from Rajasthan, 5 were found to be positive for steinernematids and heterorhabditids (4.76%). Bruck (2004) reported that recovery frequency of EPNs may vary from 0.7 to 70.1 per cent. In Sri Lanka, *Heterorhabditis sp.* was reported to be restricted to sandy soils within 100 m of the sea (Amarsinghe *et al*., 1994). Surveys have revealed natural occurrence of several species/strains of *Steinernema* and *Heterorhabditis* in Andaman and Nicobar islands (Prasad *et al*., 2001), Gujarat (Vyas, 2003), Kerala (Banu *et al*., 1998), New Delhi (Ganguly and Singh, 2000), and Tamil Nadu (Bhaskaran *et al*., 1994).

Soil physiological parameters are important factors which affect the distribution of EPN in the soil. *Heterorhabditis* is distributed predominantly along the coastal fringe, as observed were noted in Hawaiian Island and its occurrence was positively correlated with ocean beaches (Hara *et al*., 1991). *Heterorhabditis* species have been reported from tropical and subtropical regions (Hominick *et al*., 1996). This species has been reported from sandy soils at coastal sites in other subtropical and tropical regions of the world (Burnell and Stock, 2000). In the Azores, *H. bacteriophora* displayed no habitat preference and was recorded from cropland, woodland, pasture, orchard and native vegetation (Rosa *et al.,* 2000). Similarly, in New Jersey, *H. bacteriophora* was found broadly distributed in turf and weedy habitats (Stuart and Gaugler, 1994). Although, EPN was recovered only from 8 (5.7%) soil samples and 35 (43.7%) grubs of *L. mansueta*, the recovery of *Heterorhabditis bacteriophora* and *Steinernema* spp. highlights the importance of conducting a more intensive survey in the other areas of this river island. The area has fertile and sandy clay loam to silty loam soil and good vegetation cover. These conditions have supported the survival and prevalence of host insects and ultimately has high occurrence of EPNs.

This indicates their potential role in the natural control of soil inhabiting insect pests which are of immense economic importance. Further studies on characterization and host ranges of these EPN species are necessary to explore and ascertain their possible utilization in biological control programme of this economically important pest.

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