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Report of entomopathogenic nematode, *Heterorhabditis bacteriophora* from parasitised white grubs in lower Brahmaputra valley

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Abstract
Entomopathogenic nematodes are important biocontrol agent against many insect pests. For isolation of entomopathogenic nematodes a survey was conducted. Out of one hundred cadaver of *Lepidiota albistigma*, ten samples were found to be positive for entomopathogenic nematodes in Sorbhog, District Barpeta, Lower Brahmaputra valley zone, Assam, India. On the basis of morphological and morphometrical studies, the isolated nematode was identified as *Heterorhabditis bacteriophora*.

Keywords: Entomopathogenic nematodes (EPNs), white grub (*Lepidiota albistigma*), *Heterorhabditis bacteriophora*, biocontrol agent

Introduction
White grub, *Lepidiota albistigma* belonging to the Scarabaeidae family (Sub family: Melolonthinae) of the insect order Coleoptera. It is a serious pest which causes substantial yield loss to many field and horticultural crops in Assam [1]. White grub is characterized by its polaphagous feeding behaviour. 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. Synthetic insecticides, owing to their various side effects, have been widely replaced by biological insecticides. The white grub biotic environment is rich in many pathogenic fungi, bacteria, virus, and protozoa along with nematodes. Entomopathogenic nematodes (EPNs) are potential biological control agents against many insect pests. Entomopathogenic nematodes (Heterorhabditidae and Steinernematidae), offer an environmentally safe and IPM compatible alternative to chemical insecticides for the control of white grubs. 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 [2].
Several entomopathogenic nematode species have been isolated from scarabs [3]. *Heterorhabditis megidis* [4], *Steinernema anomali* [5] and *S. glaseri* [6] were all originally isolated from parasitised scarabaeid larvae. Steinernematids and Heterorhabditids have been isolated from Indian soil by various workers [7]. Devi *et al.* [8] isolated *Heterorhabditis bacteriophora* from parasitised scarabaeid larvae (*Lepidiota mansueta*) from Majuli (Upper Brahmaputra valley), Assam, India. Considering the view point, a random survey was conducted to isolate and identify EPN species from the white grub infested fields of lower Brahmaputra valley zone. Findings of this research will be helpful to search and identify the predominant species of EPNs and to develop safe, effective and economic management strategies for white grub management.

Materials and Methods
Sorbhog, Barpeta district of Assam is spread over the central part of the Lower Brahmaputra Valley region and covers an area of 1,805 km^2, is located at 26.49^0N 91.17^0E at an elevation ranging from 50 m above mean sea level. Sorbhog falls under subtropical climatic condition with a warm humid summer and cool dry winter with mean annual rainfall of 229 cm. The average maximum temperature ranges from 23 °C to 27.17 °C and minimum, from 18.8 °C to 20 °C. On an average, the relative humidity is more than 80% throughout the year. The geology of the region is of alluvial origin.

Sample collection

One hundred naturally infested third instar grubs of *Lepidiota albistigma* were collected from the white grub endemic areas of field crops as well as horticultural crops in villages of Sorbhog near to the Ramie Research Station during July to September 2018. For each location, GPS coordinates were recorded. Dead cadavers were collected individually for each sampling. Ten numbers of dead grubs were collected from each Villages, viz., Kayasta goan, Sukanjani, Buri Khamar, Puthimari, Duramari, Ahom Pathar, Kumuria Goan, Kamar goan, Pelang Bari and Pera goan of Sorbhog. The Samples were placed in a polyethylene bags and transported to the laboratory. The hand trowel was sterilized with 70% ethanol before leaving the sampling site.

Nematode isolation and culture

The dead grubs were washed with distilled water to remove dirt and placed in modified White traps [9] for EPN isolation. The emerged infective juveniles (IJs) were extracted in a beaker and cleaned two or three times with distilled water, and stored at 15 °C in distilled water and considered as one isolate. The collected IJs were transferred on to moist filter paper in Petri dishes and *Galleria mellonella* larvae were added. After their infection *G.mellonella* were dissected to isolate developmental stages of the nematode in Ringers's solution. The dissection was done 4-5 days after inoculation for first generation, 6-8 days for second generation and the infective juvenile emerged out after 10-12 days. All nematode showed the typical development pattern of heterorhabditids and designated as WG-4. Nematodes were killed and fixed in 4% hot formalin (50 to 60 °C) and kept in this solution for 48 h. Fixed nematodes were transferred to anhydrous glycerine according to Seinhorst's [10] rapid and mounted on slides using cover-glass supports. The nematodes were identified according to a selection of morphological and morphometric criteria summarized by Hominick *et al*., [11]. 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.

The frequency of occurrence of (F) (%) of Entomopathogenic nematodes from the white grub endemic areas was calculated using the following formula.

$$F = \frac{\text{No. of EPN positive samples}}{\text{Total no. of samples}} \times 100$$

Results and Discussions

Survey data revealed that out of 100 samples, 10 samples were positive for entomopathogenic nematodes (10% frequency of occurrence). The heterorhabditid strain (WG-4) was isolated from white grub cadavers from green gram cultivated areas of Kamar goan (N 26^0 31713' & E 91^0 005135'), Sorbhog, district Barpeta, Assam, India.

Nematode identification

Morphological and morphometrical studies of different life stages (infective juveniles, males of second generation, hermaphroditic female, amphimictic female) of WG-4 revealed that it is corresponded to *H. bacteriophora* [11, 12]. 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 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 IJs of WG-4 strain showed close similarity with *H. bacteriophora* with respect to head shape, position of nerve ring, position of excretory pore but exhibited minor differences from the type measurements by having higher body length(586 vs.570), lower body width (21 vs.24), esophageal length (116 vs 125). The males of this strain showed close similarity to *H. bacteriophora* with respect to head shape, tail length, anal body width, spicule length, gubernaculum length but exhibited minor differences from the type measurements by having lower body length (752 vs.820), higher body width (47 vs.43), esophagus length (96 vs.103), position of excretory pore (92 vs. 121). The hermaphroditic and amphimictic females of this isolate showed close similarity with *H. bacteriophora* with respect to head shape, tail length and vulval position but 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*. The strain WG-4 thus identified as *Heterorhabditis bacteriophora*.

Nguyen and Smart [13] observed variations 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 605µm (579µm-634µm) on 3rd day of harvest where as body length 565µm (524 µm -604 µm) on 15th day of harvest. Sivakumar *et al*., [14] observed the difference in the size of the second-generation females of Indian population of *H. bacteriophora*. The second-generation females was very pronounced when compared with the original description (L = 0.99 - 1.70 mm as against 3.18-3.85 mm) and this reflected on the other body dimensions also. Stock *et al*., [15] reported that the morphological differences between the populations of *S. kraussei* could be related to their geographic origins.

Poinar [12] 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 distributed in America, Southern and Central Europe, Australia and East Asia [16, 17]. In Europe it has been reported from Spain, Italy, Moldova, Hungary, Southern France [18], the Azores, Germany, Switzerland, South Russia [19], the European part of Turkey [20, 21] and Slovenia [22]. *H. bacteriophora* was recorded from Caribbean area, particularly in Guadeloupe islands and neighbouring islands (Marie-Galante, La Desirade, Petite Terre, Les Saintes, Saint-Barthelemy, Saint-Martin) with 12% frequency of occurrence [23]. *Heterorhabditis* is distributed predominantly along the coastal fringe, as observed were noted in Hawaiian Island and its occurrence was positively correlated with ocean beaches [24]. In Sri Lanka, *Heterorhabditis sp.* was reported to be restricted to sandy

soils within 100 m of the sea [25]. *Heterorhabditis* species has been reported from sandy soils at coastal sites in other subtropical and tropical regions of the world [26]. In the Azores, *H. bacteriophora* displayed no habitat preference and was recorded from cropland, woodland, pasture, orchard and native vegetation [27]. Similarly, in New Jersey, *H. bacteriophora* was found broadly distributed in turf and weedy habitats [28]. Gradinarov *et al*., [29] recorded *H. bacteriophora* in calcareous soils and at altitudes from 0 to 1175 m, in habitats both along the Black Sea coast and inland in Bulgaria. *H. bacteriophora* was found in a wide range of habitats including: mixed forest, transitional woodland-shrub, vineyards, broad leaved forest, land occupied by agriculture with natural vegetation and permanently irrigated land in continental Portugal [30]. *H. bacteriophora* was reported from India by Sivakumar *et al*. [14] and Hussaini *et al*., [31]. *H. bacteriophora* was reported from uncultivated as well as forest soil of Maharajganj, Tarai region of the Indo-Gangetic Plain,UP, India [32]. Bhat *et al*., [33] isolated and described *H. bacteriophora* from hilly areas of Kashmir valley. Rosa *et al*. [27] reported that most of the surveys showed their recovery rate from soil to vary between 6 to 35 per cent. Raj Kumar *et al*. [34] showed that out of 105 soil samples collected from Rajasthan, 5 were found to be positive for steinernematids and heterorhabditids (4.76%). Bruck [35] reported that recovery frequency of EPNs may vary from 0.7 to 70.1 per cent. *H. bacteriophora* was isolated from 6 samples out of 320 soil samples from the potato fields of North-West of Iran [36-38]. Kalita *et al*., [39] recorded 1% frequency of occurrence of *H. bacteriophora* in the experimental fields of Assam Agricultural University, Jorhat. Bharath *et al*., [40] recorded 0.5% frequency of occurrence of *H. bacteriophora* in tea plantation areas of Jorhat district of Assam. Although, EPN was recovered only from 10(10%) grubs of *L*., the recovery of *H. bacteriophora* highlights the importance of conducting a more intensive survey in the other areas. The area has fertile and good vegetation cover. These conditions have supported the survival and prevalence of host insects and ultimately has high occurrence of EPNs. 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 economically important pests.

Conclusion

Recovery of *H. bacteriophora* highlights the importance of more survey for isolation of local strains of entomopathogenic nematodes. Further work emphasis on molecular identification of the isolate and the determination of its efficacy against other insect pests in the field.

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Table 1: Morphometrics of infective juveniles and second generation male of *Heterorhabditis* sp. from fields of Sorbhog, Assam in comparison with original description of *Heterorhabditis bacteriophora.* Measurements in µm and in the form: mean± SD (range)

Character	*Heterorhabditis sp.* (IJ) (n=40)	Type measurement *H. bacteriophora* (IJ) (Poinar, 1976) (n=15)	*H. bacteriophora* (IJ) (Adams & Nguyen, 2002) [41]	*Heterorhabditis* sp. (male) (n=20)	Type measurement *H. bacteriophora* (male) (Poinar, 1976) (n=15)
Body length (L)	586.50±21.98 (548-621)	570 (520-600)	588 (512-671)	752.20±59.21 (691-912)	820 (780-960)
Body width (W)	21.00±1.22 (17-22)	24 (21-31)	23 (18-31)	47.25±5.09 (36-53)	43 (38-46)
Anterior end to excretory pore (EP)	98.80±4.51 (92-108)	104 (94-109)	103 (87-110)	92.00±4.87 (82-101)	121 (114-130)
Anterior end to nerve ring (NR)	84.18±4.02 (77-90)	83 (81-88)	85 (72-93)	80.20±7.17 (68-97)	72 (65-81)
Anterior end to esophagus base (ES)	116.60±4.09 (110-123)	125 (119-130)	125 (100-139)	96.85±5.16 (92-110)	103 (99-105)
Tail length(T)	99.13±5.52 (89-107)	91 (83-99)	98 (83-112)	31.20±3.34 (25-36)	28 (22-36)
Anal body width (ABW)	13.30±1.13 (11-15)	-	-	19.35±3.46 (16-28)	23 (22-25)
Testis reflexion				84.50±8.42 (71-98)	79 (59-87)
Ratio a= (L/W)	28.01±1.83 (24-33)	25 (17-30)	25 (17-30)	16.05±1.70 (13-19)	
Ratio b= (L/ES)	5.03±0.13 (4.6-5.3)		4.5 (4.0-5.1)	7.78±0.61 (7.1-9.7)	
Ratio c= (L/T)	5.92±0.19 (5.6-6.2)		6.2 (5.5-7.0)	24.30±2.48 (19-28)	
D%= (EP/ES)*100	84.76±3.31 (78-94)		84 (76-92)	95.01±1.75 (89-96)	
E%= (EP/T)*100	99.81±11.16 (90.47-109.27)		112 (103-130)		
Spicule length (SL)				39.40±3.37 (32-43)	40 (36-44)
Gubernaculum length(GL)				20.05±1.79 (17-22)	20 (18-25)
SW(SL/ABW*100)				208.30±33.30 (153-268)	
GS(GL/SL*100)				50.94±2.47 (46-55)	

Table 2: Morphometrics of hermaphroditic female and amphimictic female of *Heterorhabditis* sp. from fields of Sorbhog, Assam in comparison with original description of *Heterorhabditis bacteriophora*. Measurements in µm and in the form: mean± SD (range)

Character	*Heterorhabditis* sp. Hermaphroditic females (n=20)	Type measurement *H. bacteriophora* Hermaphroditic female (Poinar, 1976) (n=15)	*Heterorhabditis sp.* Amphimictic female (n=12)	Type measurement *H. bacteriophora* Amphimictic female (Poinar, 1976) (n=15)
Body length (L)	2693.60±351.10 (1825-3045)	4030 (3630-4390)	2075.25±301.32 (1463-2573)	3500 (3180-3850)
Body width (W)	183.65±25.53 (156-247)	165 (160-180)	156.80±15.95 (128-180)	190 (160-220)
Anterior end to excretory pore (EP)	168.55±24.87 (134-214)	209 (189-217)	132.80±18.66 (106-174)	192 (174-214)
Anterior end to nerve ring (NR)	132.35±12.66 (98-142)	126 (121-130)	108.55±10.26 (92-127)	103 (93-118)
Anterior end to esophagus base (ES)	185.65±22.93 (156-237)	197 (189-205)	145.70±16.08 (129-181)	
Tail length(T)	94.35±14.93 (68-112)	90 (81-93)	80.65±4.03 (74-89)	82 (71-93)
Anal body width(ABW)	43.95±6.38 (36-61)	46 (40-53)	27.20±5.52 (19-43)	28 (22-31)
V%= distance from anterior end to vulva as percentage of length	47.82±3.58 (42-53)	44 (41-47)	48.10±3.97 (41-56)	47 (42-53)
a	14.74±1.61 (11.7-16.6)		13.21±1.14 (10.16-14.68)	
b	14.54±1.34 (11.7-16.5)		14.26±1.55 (10.4-16.8)	
c	28.74±2.25 (24.6-33.8)		25.65±2.89 (19-31)	
D	90.56±3.45 (81-95)		90.93±4.39 (82-96)	

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