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Biochemical and molecular characterization of *Photorhabdus akhurstii* associated with *Heterorhabditis indica* from Meerut, India

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Abstract

A population of *Heterorhabditis* (DH3) was isolated from agricultural fields from Meerut district, India by insect baiting technique. The isolate was identified as *Heterorhabditis indica* based on morpho-taxometrical and molecular analyses. The present populations were identical to the original description; however, the size of the 3rd stages was longer than the topotype populations. Analysis of ITS-rDNA sequences showed 1 nucleotide base pair difference in aligned data with type description, however, no nucleotide base pair difference was seen in the D2D3 domain. The associated bacterial symbiont of the DH3 strain was identified as *Photorhabdus akhurstii* based on phenotypic, biochemical and 16S rDNA data. Most of the biochemical tests were negative and only three tests were found positive viz., Urease, Nitrogen reduction and Oxidase. Further, we evaluated its pathogenicity against *Galleria mellonella* and *Helicoverpa armigera*. The LD50 value of strain DH3 against *G. mellonella* larvae at 48 hours was 8 IJs/larva for 50% larval mortality whereas in *Helicoverpa armigera* it needed 13 IJs/larva, to kill 50% of the larvae.

Key words: *Heterorhabditis*, D2-D3 domain, ITS-rDNA, *Galleria mellonella* and *Helicoverpa armigera.*

H*eterorhabditis* has less species when compared with *Steinernema* and are commonly found in coastal areas. They live in symbiotic relations with Enterobacteriaceae bacteria, *Photorhabdus* (Boemare *et al*., 1993) which reside in their alimentary canal and they together serve as important biocontrol agents. They kill the insect pests by penetrating the body via natural openings or also by scratching the membrane with the help of hook-like structure on their anterior region. They release the bacteria in the insect hemolymph and bacteria produce endotoxins that cause septicemia in the insect host. So far, 18 valid species in the genus *Heterorhabditis* have been reported worldwide (Arvind *et al*., 2019), but in India, only three species are known till date viz., *Heterorhabditis bacteriophora* Poinar, 1976 (Bhat *et al*., 2019) *viz*., *H. baujardi* Phan *et al*., 2003 (Vanlalhlimpuia *et al*., 2018) and *H. indica* Poinar, Karunakar & David, 1992. *H. indica* is the most commonly reported species from Indian subcontinent and has been isolated from most of the states in the country (Bhat *et al*., 2019).

During the present study, *H. indica* was reported from the agricultural soils of district Meerut. We present here its morpho-taxometrical and molecular data and the evaluation of its insecticidal property against *Galleria mellonella* (Fabricius, 1798) and *Helicoverpa armigera* (Hübner, 1808). Further, we also identified and report the associated bacterial symbiont using phenotypical, biochemical and molecular characterization.

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Materials and Methods

Collection and nematode isolation: Twenty soil samples were collected from the different agricultural fields of district Meerut India. Entomopathogenic nematodes (EPN) were isolated from soil by soil trap method (Bedding & Akhurst, 1975) with the last instar larvae of greater wax moth (*Galleria mellonella* L.) as bait. The cadavers were first disinfected with 1% NaOCl (Aasha *et al*., 2020) and placed on white traps (White, 1927), juveniles emerged from the cadaver were collected and finally kept in culture flasks at 15°C ± 1°C (Bhat *et al*., 2019) for further experiments.

Morphological characterization: For taxonomic studies, larvae infected with DH3 were dissected for hermaphroditic females and amphimictic generations on the 4th, 5th, 6th and 7th days, respectively, whereas IJs were obtained from a White trap. They were heat killed and fixed in Triethanol-amine-formalin (7ml formalin, 2ml triethanolamine, 91 ml distilled water) (Kaya & Stock, 1997), processed to anhydrous glycerin (Sienhorst, 1959) and mounted into a small glycerin drop. Microscopic studies were carried out using phase contrast microscope (Nikon Eclipse 50i) and light compound microscope (Magnus MLX) and morphometric measurements were taken with built-in software of phase contrast microscope (Nikon DS-L1).

DNA extraction from nematode, amplification and sequencing: Genomic DNA was extracted from 3rd stage juveniles (n = 600) by manufacturer's protocol given in the manual using Blood & Tissue Kit (Qiagen, Hilden, Germany). For producing multiple copies, genomic DNA having the internal transcribed spacer regions (ITS1, 5.8S, ITS2) was PCR amplified using primers 18S: 5'-TTGATTACGTCCCTGCCC TTT-3' (forward), and 28S: 5'-TTTCACTCGCCGTTACTAAGG-3' (reverse) (Vrain *et al*., 1992). The flanking region D2D3 region of 28S rDNA was PCR amplified using primers D2F: 5'-CCTTAGTAACGGCG AGTGAAA-3' (forward) and 536: 5' -CAGCTATCCTGAGGAAAC-3' (reverse) (Nadler *et al*., 2006). The PCR reaction mixture (25 µl) consisted of 12.5 µl PCR master mix (Dream Taq green), 1 µl of each forward and reverse primers, 7.5 µl distilled water (nuclease) and 3 µl of DNA template. The cycling profiles used in thermo-cycler were as: the initial cycle of 95°C for 4 min and then 40 cycles of 94°C for 30 s, + 55°C for 30 s for ITS or 52°C for 30 s for D2D3, + 72°C for 60 s and an ultimate extension at 72°C for 10 min. The PCR products were scrutinized on 1% agarose gels with TAE (Tris–acetic acid–EDTA) buffered agarose gel stained with EtBr (2µl EtBr per 100 ml of gel) (Bhat *et al*., 2019). The PCR rDNA products were purified and sequenced in forward and reverse directions by Bioserve Pvt. Ltd. Hyderabad (India). The sequences results were interpreted and consensus sequences were submitted to the National Center for Biotechnology Information (NCBI) with accession numbers KY311812 and KY311814 for ITS and D2D3 regions, respectively.

Isolation and characterization of bacteria: The symbiotic bacterium of DH3 was isolated from 3rd stage juveniles by the method of Akhurst (1980). IJs were washed with ddH2O, sterilized with 0.1% NaOCl and crushed in 100 µl nutrient broth media. Crushed IJs were streaked on nutrient agar plates appended with triphenyl tetrazolium chloride (0.004% (w/v)) and bromothymol blue (NBTA medium) (0.0025% (w/v)) then incubated at 28°C for 12 hours (Akhurst, 1980). For obtaining pure culture, a single colony was shifted with a germ-free loop to Luria broth (Akhurst, 1980) and nurtured on a digital shaker (180 rpm) at 27°C in dark for 48 hours.

Phenotypic characterization in symbiotic bacteria was established based on adsorption features towards dyes bromothymol blue (BTB) and neutral red. The BTB adsorption was observed on NBTA and MacConkey agar plates (Akhurst, 1980). Enterobacteriaceae bacteria were present in two stages, viz., phase I and phase II. Phase II bacteria produced red-colored colonies as it reduced tetrazolium chloride (TTC) to red formazan while in phase I bacteria, blue-green colonies were visible as they adsorbed BTB and TTC reduction was hidden. Phase I or phase II bacterial colonies were thus characterized as blue-green or red-colored colonies. On Mac Conkey agar, Phase I variants of bacteria absorbed neutral red and appeared as

red/reddish-brown colonies, while the phase II variants appeared as light yellow.

Biochemical tests were performed with Hi Media Kit (KB003 Hi25TM Enterobacteriaceae Identification Kit) for their positive or negative response to different biochemical tests. This biochemical kit consists of 13 conventional biochemical tests and 11 carbohydrate utilization tests. A single colony of freshly cultured bacterium was inoculated in 5ml Heart Infusion Broth (HiMedia) and incubated at 28°C for overnight. 200µl cultured bacteria were added in each well of kit and Oxidase test was performed separately with oxidase reagent disc.

DNA from bacteria was extracted using a 48-hour old culture using the same kit as used for nematode DNA extraction. The 16S gene was multiplied using primers 10 F: 59-AGTTTGATCATGGCT CAGATTG-39 (forward) and 1507R: 59-TACCTTGTTACGACTTCACCCCAG-39 (reverse) (Sandstrom *et al*., 2001). The thermo-cycler reaction consisted of 7.2 µl ddH2O, PCR master mix 12.5 µl, 1 µl of each forward and reverse primers and 3 µl of DNA template. The cycling profile for amplification used was as follows: initial cycle at 94°C for 1 min; accompanied by 33 cycles at 94°C for 60 s, 55°C for 60 s and 72°C for 2 min; and ultimate extension at 72°C for 12 minutes. It was sequenced and submitted in GenBank under accession number KY311816.

Sequence alignment and phylogenetic analysis: The rDNA sequences (ITS and 28S for *Heterorhabditis* and 16S for *Photorhabdus*) were edited and matched with those already present in GenBank employing a Basic Local Alignment Search Tool (Altshul *et al*., 1990) of the National Centre for Biotechnology Information (NCBI). Their alignments were created by default Clustal W parameters in MEGA 7.0 (Kumar *et al*., 2016). Pairwise distances were figured using MEGA 7.0 (Kumar *et al*., 2016). The ITS and 28S rDNA based phylogenetic trees were produced by the minimum evolution method and 16S by NJ method, in MEGA 7.0 (Kumar *et al*., 2016). *C. elegans* and *Xenorhabdus nematophila* were used as out-group for nematode and bacteria, respectively.

Virulence tests on *Helicoverpa armigera* and *Galleria mellonella*: Virulence tests of Indian strain against targeted *Galleria mellonella* Fabricius (Lepidoptera: Pyralidae) and *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) were performed. Bioassays were performed using 7 days old IJs in six-well plates lined by filter paper (Whatman No. 1). Four different concentrations (25, 50, 100, and 200 IJs/larva) were poured on the filter paper using distilled water with a total concentration of 400µl along with control. For each concentration, larvae of identical size and weight were placed on each well and bioassays were repeated twice. The plates were kept at 27±2°C in BOD incubator and were checked for mortality of larvae after every 12 hour time lag till complete deaths of insects were observed. Progeny production was counted in cadavers infected with 100 IJs/larva doses by transferring them to White trap (18–20 days) (White, 1927). The data were analyzed with SPSS software and LC50 and LT50 values were calculated at 95% level of significance.

Results and Discussion

Soil baiting of these samples yielded one strain of *Heterorhabditis* designated as DH3. It was isolated from the soils of sugarcane fields (*Saccharum officinarum* L.).

Morphology and morphometry: *Heterorhabditis* isolates DH3 showed much similitude with the original description of *H. indica* (Poinar *et al*., 1992) and hence were considered as conspecific. Morpho-taxometric investigations and sequencing data were in such proximity to topotype population, however, some deviations were notated only in morphometric measurements. The morphometric data of present isolate and its comparative data are shown in Tables 1-3, respectively.

Table 1. Morphometric measurements of *Heterorhabditis indica* isolate DH3. All measurements are µm (except n, ratio and percentage) and in the form mean ± SD (Range).

	Male	Hermaphrodite	Second Generation	Infective Juveniles
Total number (n)	15	15	15	20
Total body length (L)	799 ± 32 (724-864)	3894 ± 440 (2751-4481)	1976 ± 166 (1713-2242)	528 ± 12 (511-546)
Anterior to anus (L')	768 ± 31 (695-831)	3806 ± 434 (2685-4373)	1903 ± 168 (1635-2180)	497 ± 12 (480-516)
Excretory pore (EP)	106 ± 5.3 (96-113)	213 ± 17 (184-238)	154 ± 8.9 (135-172)	100 ± 4 (92-108)
Width @ excretory pore	32 ± 2.7 (26-37)	126 ± 16 (92-142)	68 ± 5.1 (59-75)	19 ± 1.5 (17-23)
Nerve ring (NR)	71 ± 4.2 (63-80)	136 ± 10 (115-157)	84 ± 4.2 (77-92)	68 ± 2.9 (63-73)
Pharynx length (PS)	100 ± 5.5 (89-109)	190 ± 10 (167-204)	104 ± 3.6 (120-138)	94 ± 4.4 (86-103)
Bulb length (EBL)	23 ± 2(20-26)	43 ± 5 (36-51)	32 ± 2.2 (29-36)	20 ± 1.1 (16-21)
Bulb width (EBW)	15 ± 1.3 (12-17)	34 ± 3.5 (29-41)	23 ± 1 (22-25)	9.1 ± 0.6 (7-10)
Tail	31 ± 2 (29-36)	88 ± 11 (67-108)	74 ± 6.7 (61-83)	30 ± 2.5 (24-34)
Anal body width (ABW)	20 ± 1.1 (18-22)	47 ± 9.9 (30-71)	30 ± 2 (27-33)	7.5 ± 0.8 (6-9)
Greatest body width (GBW)	44 ± 2.6 (41-48)	237 ± 29 (168-273)	131 ± 11 (110-156)	22 ± 0.7 (21-24)
Testis reflection (TR)	120 ± 6.7 (109-133)			
Spicule length (SPL)	36 ± 3.8 (30-40)			
Gubernaculum length (GL)	27 ± 2.5 (21-31)			
Anterior to vulva length (V)	-	1697 ± 202 (1277-2026)	916 ± 81(803-1046)	
Posterior to vulva length (V')	-	2197 ± 307 (1474-2824)	1126 ± 253 (901-1983)	
Width @ vulva (WV)	-	232 ± 34 (138-274)	130 ± 11 (112-150)	
a = L/GBW	18 ± 1 (17-20)	17 ± 0.8 (16-18)	15 ± 1.2 (13-17)	24 ± 0.8 (22-25)
b = L/ES	8 ± 0.3 (7.6-8.6)	21 ± 2.2 (15-24)	14 ± 4.1 (0.1-18)	5.6 ± 0.2 (5-6)
c = L/Tail	26 ± 1.5 (22-27)	45 ± 5.2 (35-55)	27 ± 3.9 (22-36)	18 ± 1.5 (16-22)
c' = Tail/ABW	1.6 ± 0.1 (1.4-1.8)	1.9 ± 0.3 (1.4-2.5)	2.4 ± 0.2 (1.9-2.9)	4.1 ± 0.6 (2.8-5.2)
D% = EP/ES×100	105 ± 2.9 (101-111)	112 ± 8.4 (103-132)	110 ± 31 (1.2-1280)	106 ± 3.7 (100-118)
E% = EP/Tail×100	338 ± 21 (304-379)	247 ± 32 (202-297)	211 ± 26 (188-282)	335 ± 25 (301-297)
F% = GBW/Tail×100	125 ± 15 (105-158)	273 ± 31 (220-3220)	180 ± 25 (140-238)	75 ± 7.6 (65-92)
V= V'/L×100	-	44 ± 3.2 (37-48)	46 ± 1.5 (44-50)	-
SW%	183 ± 17 (155-210)	-	-	-
GS%	77 ± 6.2 (69-88)	-	-	-
EP/WEP	3.4 ± 0.4 (3-4.4)	1.7 ± 0.2 (1.5-2)	2.3 ± 0.2 (2-2.6)	5.3 ± 0.4 (4.5-5.8)
EBL/EBW	1.5 ± 0.1 (1.4-1.7)	1.3 ± 0.1 (1.1-1.6)	1.4 ± 0.1 (1.3-1.7)	2.2 ± 0.1 (1.9-2.4)

Table 2. Comparative morphometric of 3rd stage juveniles of indica group of *Heterorhabditis.*

Characters	*H. taysearae*	*H. baujardi*	*H. floridensis*	*H. mexicana*	*H. noeniputensis*	*H. indica*	Isolate DH_3
n	30	25	25	25	25	25	20
L	418(332-499)	551(497-595)	562(554-609)	578(530-620)	536 (484–578)	528(479-573)	528(511-546)
a	21(18-27)	28(26-30)	27(25-32)	25 (23.6-28.4)	24 (21–27)	26(25-27)	24(22-25)
b	3.8 (3.4-4.2)	4.8(4.5-5.1)	4.3 (3.9-4.9)	4.6 (4.2-5.1)	4.9 (4.3–5.2)	4.5(4.3-4.8)	5.6(5-6)
c	7.7(6.5-8.7)	6 (6-6.7)	5.6(5.3-6.6)	5.9(5.5-6.3)	6.2 (5.5–6.8)	5.3(4.5-5.6)	18(16-22)
MBD	20 (17-23)	20(18-22)	21(19-23)	23(20-24)	23 (21–25)	20(19-23)	22(21-24)
EP	90 (74-113)	97(91-103)	109(101-122)	102(83-109)	97 (88–105)	98(88-107)	100(92-108)
NR	64(58-87)	81(75-86)	86(68-107)	81 (74-88)	81 (69–96)	82(72-85)	68(63-73)
PS	110(96-130)	115(107-120)	135(123-142)	122(104-142)	106 (79–115)	117(109-123)	94(86-103)
Tail	55(44-70)	90(83-97)	103 (91-113)	99(91-106)	86 (78–95)	101(93-109)	30(24-34)
ABW	-	13(11-14)	14(12-16)	15(12-17)	14 (12–16)	-	7.5(6-9)
D%	82(71-96)	84(78-88)	81(71-90)	81(72-86)	89 (81–95)	84 (79-90)	106(100-118)
E%	180(110-230)	108(98-114)	105(95-134)	104(87-111)	113 (99–125)	94 (83-103)	335(301-297)

Table 3. Comparative morphometric of 3rd stage juveniles of indica group of *Heterorhabditis.*

Characters	*H. taysearae*	*H. baujardi*	*H. floridensis*	*H. mexicana*	*H. noeniputensis*	*H. indica*	IsolateDH_3
N	20	14	20	20	20	12	15
L	703(648-736)	889(818-970)	862(785-924)	686(614-801)	649 ((530–775)	721(573-788)	799(724-864)
MBD	43(38-48)	49(45-53)	47 (43-50)	42(38-47)	41 (34–46)	42(35-46)	44(41-48)
EP	95(78-120)	81(71-93)	117(104-128)	124(108-145)	86 (75–102)	123 (109-138)	106(96-113)
NR	65(54-88)	65(54-77)	80(73-90)	71(61-83)	67 (64–75)	75(72-85)	71(63-80)
ES	112 (85-123)	116(105-132)	105(97-111)	96(89-108)	95 (88–106)	101(93-109)	100(89-109)
TR	122(100-146)	91(28-38)	93(78-116)	96(65-130)	-	91(35-144)	120(109-133)
Tail	25(20-29)	-	34(29-40)	27(21-36)	25 (21–32)	28 (24-32)	31(29-36)
ABW	25(21-30)	22(20-24)	26(20-31)	24(23-27)	19 (15–22)	23(19-24)	20(18-22)
SPL	39(30-42)	40(33-45)	42(36-46)	41(30-47)	43 (37–49)	43(35-48)	36(30-40)
GL	18(14-21)	20(18-22)	23(17-30)	23(18-32)	20 (17–24)	21(18-23)	27(21-31)
D%	88	-	112(105-119)	129(114-149)	90 (81–108)	121	105(101-111)
SW	156	182(138-208)	157(133-209)	167(130-196)	231 (202–301)	187	183(155-210)
GS	46	50(44-61)	53(47-65)	56(43-70)	47 (38–56)	49	77(69-88)

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Molecular characterization and phylogenetic analysis: ITS sequence of *H. indica* (DH3) Indian strain varies from the already described ITS gene sequences by 1 bp. In the flanking D2D3 domain of the 28S rDNA gene sequences also, the present strain DH3 do not show any variation with type populations of *H. indica*. NBlast analysis also showed that present isolates showed 99.9% with type population.

ITS and D2D3 evolutionary trees showed that the species of the 'indica' group and present strain DH3 formed a sister clad with all known species of 'indica' group and formed a clear monophyletic clade of the group formed by the isolate DH3 and original *H. indica* and several other, probably conspecific isolates (Figs. 2 and 3). *H. noenieputensis* (Malan *et al*., 2014) is much close to *H. indica* and in both trees sequences of *H. indica* populations formed a monophyletic group with *H. noenieputensis*. Thus, based on the morphological and morphometrical studies supplemented with ITS and D2D3 rDNA gene sequences proved the position of the isolate DH3 as species of *H. indica*.

Phenotypical, biochemical and molecular characterization of strain DH3: Phenotypical, biochemical and microscope photographic observations revealed that, the studied *Photorhabdus* sp., types are gram negative and rod-shaped bacteria. The isolated bacterium was found bioluminescent (bioluminescence was observed on the LB agar plates after 24-48 h of growth) and was highly unstable outside the host (*in vitro*), culturing of bacteria did not succeed to get Phase I variant and only Phase II colonies were obtained. Some of the colonies showing Phase I were not successfully sub cultured as Phase I and transformed into Phase II. In this way, all the biochemical and molecular tests were performed using Phase II variant of the bacterium. The results obtained through biochemical tests are shown in Table 4. Isolate DH3 showed urease, nitrate and oxidase positive. But rest of the results including all sugar reduction tests (except Saccharose weakly positive) were found negative. Biochemically and phenotypically, mutualistic bacteria stated by Shahina *et al*., (2004) were indistinguishable to the outcomes found here. However, two differences were prominent in that indole test was observed to be negative in the present experiments, while Shahina *et al*., (2004) testified it as positive. Nitrate reduction was positive in present isolate vs. negative in Pakistani isolates.

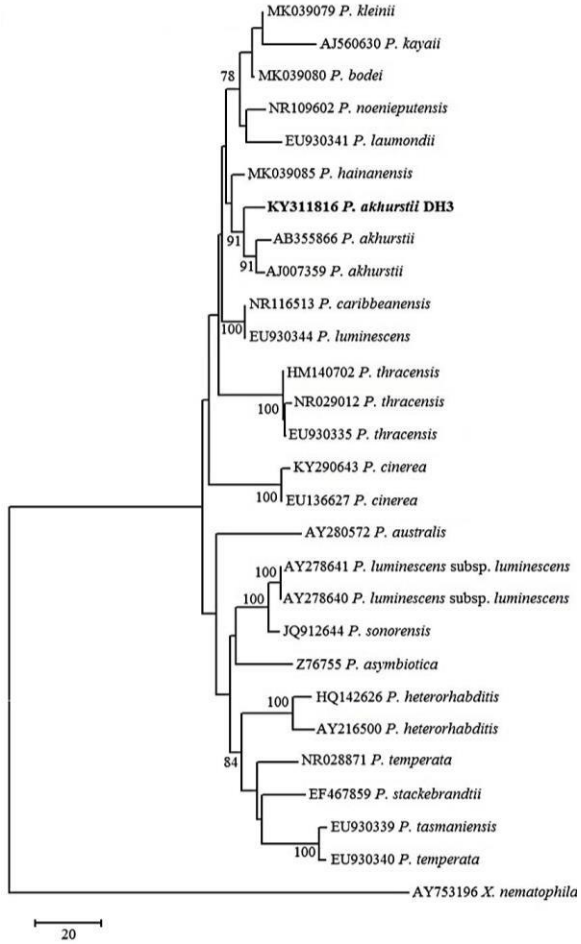

Fig. 1. Phylogenetic relationships in the genus *Photorhabdus* based on analysis of 16SrDNA regions. *X. nematophila* was used as the out-group taxon. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of number of base differences per site.

The molecular characterization using 16S rDNA gene studies proved that the present bacterium *Photorhabdus* sp., DH3 was closely related to *P. akhurstii* (Fisher-Le-Saux *et al*., 1999). Here the 16S rDNA sequences formed a monophyletic clade with *P. akhrustii* (Fisher-Le-Saux *et al*., 1999) with 91% bootstrap values and hence was confirmed as the same (Fig. 1).

Virulence tests: The pathogenicity results of *H. indica* strain DH3 against *H. armigera* and *G. mellonella* are presented in Fig. 4. The virulence data showed that this strain (DH3) is effective against the tested insect larvae. The isolate was able to kill all the infected larvae with all the doses applied within 60 hours and 200 IJs/larvae dose was able to kill just after 36 hours of infection. No mortality was observed in control groups even after 72 hours (Fig. 4). In case of *G. mellonella*, LD50 values at 36 hours confirmed that the DH3 strain was able to cause larval mortality with 13 IJs/larvae; however, at 48 hours the quantity of IJs required for 50% larval mortality was 8 IJs/larvae.In case of *H. armigera*, these values were 18 and 13 IJs/larvae, respectively. The progeny production data showed that maximum production of IJswas found in greater wax moth larvae as compared with the cotton bollworm larvae. The progeny production showed an increasing trend from a lower dose to a higher dose in the case of *G. mellonella* (107653<<164366<193648<219357). In the case of *H. armigera*, the IJ production was highest at 50 IJs/larvae doses (59080), while the least was observed at other applied doses (29029 (25IJs/larvae) < 39909 (100 IJs/larvae) < 39280 (200 IJs/larvae).

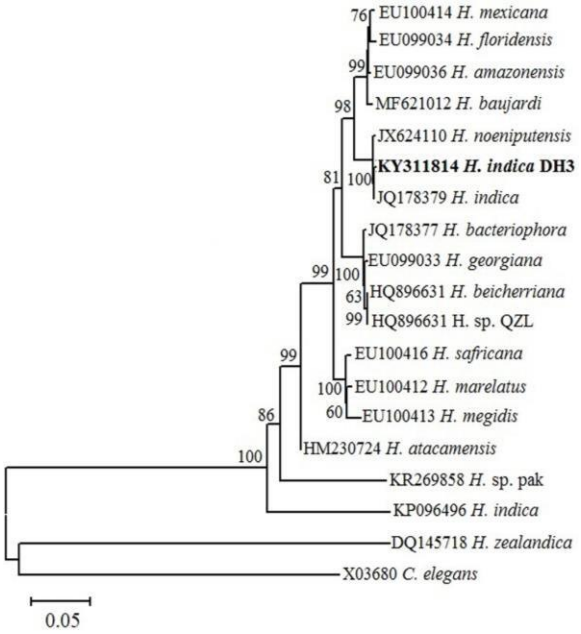

Fig. 2. Phylogenetic relationships in the genus *Heterorhabditis* based on analysis of flanking region D2D3 rDNA. *C. elegans* was used as the out-group taxon. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of number of base differences per site.

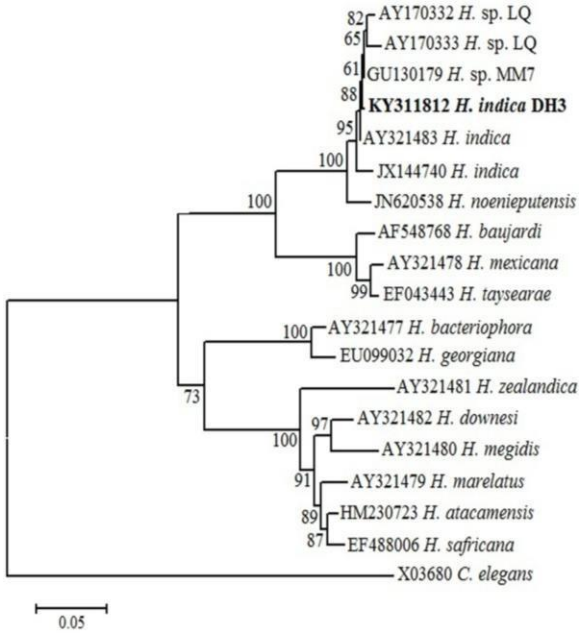

Fig. 3. Phylogenetic relationships in the genus *Heterorhabditis* based on analysis of ITS rDNA regions. *C. elegans* was used as the out-group taxon. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of number of base differences per site.

Table 4. Biochemical Tests performed for the confirmation of *Photorhabdus* sp.

S. No.	Tests	Results	S. No.	Tests	Results
1.	ONPG	-	14.	Arabinose	-
2.	Lysine utilization	-	15.	Xylose	-
3.	Ornithine utilization	-	16.	Adonitol	-
4.	Urease	+	17.	Rhamnose	-
5.	Phenylalanine deamination	-	18.	Cellobiose	-
6.	Nitrate reduction	+	19.	Melibiose	-
7.	H_2 S production	-	20.	Saccharose	W.P.
8.	Citrate utilization	-	21.	Raffinose	-
9.	Vogesroskauer's	-	22.	Trehalose	-
10.	Methyl red	-	23.	Glucose	-
11.	Indole	-	24.	Lactose	-
12.	Malonate utilization	-	25.	Oxidase	+
13.	Esculin hydrolysis	-			

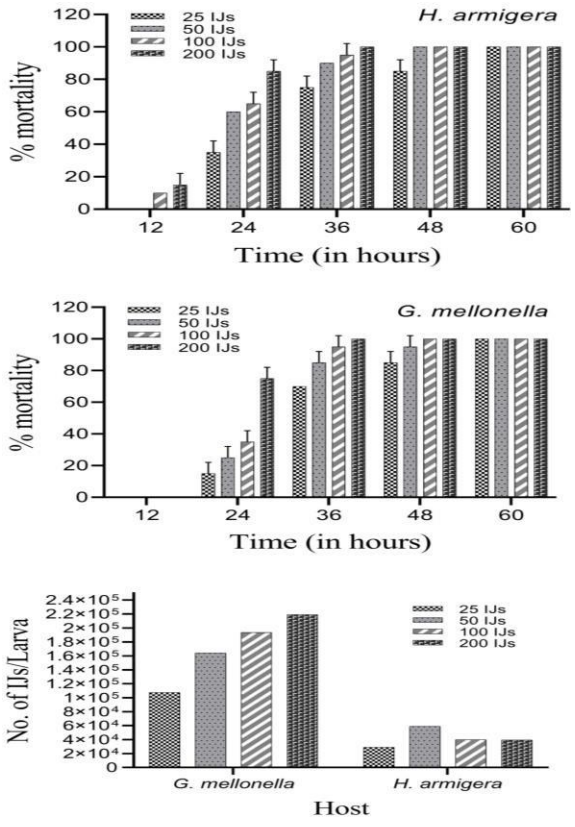

Fig. 4. Percentage of mortality (mean and SD) of *Helicoverpa armigera* (A) and *Galleria mellonella* (B) larvae with different doses of *Heterorhabditis indica* DH3. (C) Mean IJs production in *G. mellonella* and *H. armigera*.

Our results are consistent with the results of Sankar *et al*., (2009) and Hara & Kaya (1982) who also observed fast mortality (at 36 to 48 h) in insects using EPNs. The highest mortality of *G. mellonella* was observed by Divya *et al*., (2010) within 24 hours at 300 IJslarvae-1 using *H. indica*, but in our case, complete mortality was recorded in the host after 36 hours with 200IJs/larvae-1 applied. This variation may be due to reasons that pathogenicity depends on several biotic and abiotic factors, existence/non existence of the firm host, acclimatization to abiotic factors, and changes in their niche and habitats (Kaya & Gaugler, 1993). DH3 is native to India; further tests should be done to check its pathogenicity towards harmful insects affecting Indian agriculture throughout India.

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