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PHYLOGENIC, PATHOGENIC AND REPRODUCTIVE CHARACTERIZATION OF *HETERORHABDITIS INDICA* FROM DISTRICT MEERUT, INDIA

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ABSTRACT

Heterorhabditis indica isolate CH_{18} was isolated from District Meerut of Uttar Pradesh, India and characterized by short juveniles and the arrangement of the terminal group of bursal ribs. The values of E and D ratio differentiate it from other described species of Heterorhabditis. It can be recognised by; L = 534 (514-548) µm, pharynx = 107 (99-111) µm; ratio 'a' = 24 (22-27); ratio 'c' = 5.4 (5-6); D% = 84 (75-91) and E%= 92 (83-101). It was further recognised by the male characters viz. L = 721 (592-884) µm, SL = 41 (32-55) µm; GL = 19 (13-27) µm; GBW = 46 (33-54) µm. H. indica isolate CH_{18} was genetically recognised by sequence lengths of the ITS region (737 bp), D2-D3 region (942 bp) and COX1 (596 bp). Pathogenicity trial based on different IJ concentration viz. 25, 50, 100, 200 IJ/larva showed that the degree of susceptibility to nematode infection varied from different dose infection and also on the exposure time. A positive relation was observed in between the doses of infective juvenile and larval mortality where increments in doses also lead to increment in mortality rate. With a maximum count of progeny ($1.79\pm0.8\times10^5$ IJs/ Larva) in lowest larval dose, a strong negative correlation was observed where the number of IJ production decreased significantly with increment in the dose of infection.

KEY WORDS

ITS region, D2-D3 region, COXI gene, pathogenicity, IJs production, negative correlation.

INTRODUCTION

Entomopathogenic nematodes, (*Heterorhabditis* and *Steinernema*) are used as potential biological control agents as they are efficient parasites of several economically important insect pests[1, 2, 3] being symbiotically associated with entomopathogenic bacteria *Photorhabdus*[4] and *Xenorhabdus*[5]. To date, twenty five species have been described for *Heterorhabditis* and more than hundred for *Steinernema*. There has been considerable debate about the proper identification of entomopathogenic nematodes[6,3,7]. Taxonomic relationships of these nematodes are usually based on morphological characters for *Heterorhabditis* species while for *Steinemema* species morphological characters are combined with cross breeding data[8,9,7]. Morphological characters cannot be used unambiguously to place new isolates into a particular species. The feasibility of using these nematodes as biological control agents depends upon the resources required for a rapid and accurate means to determine the genetic diversity among existing populations of entomopathogenic nematode species. These methods can also be used for the development of identification tools. The use of molecular approaches to study systematic relationships among entomopathogenic nematodes have been emphasized[10]. Currently regions of taxonomic importance include the internal transcribed spacer (ITS) and D2D3 region of the ribosomal DNA (rDNA). Both of these regions have been widely used for the identification of few nematodes [11, 12] and will make major contributions to the identification of entomopathogenic nematode species.

During a survey of sampling in cultivars of Meerut region, an isolate of Heterorhabditid nematode tagged as CH_{18} was isolated and based on morphological and molecular characteristics was

identified as *Heterorhabdtis indica*. In the present investigation, three regions viz. ITS, D2D3 and COX1 were used to delimit its species status in addition morpho-taxometrical investigation. The pathogenicity and reproductive prospective of this isolate was also observed in the present study.

MATERIALS AND METHODS

Nematode collection

The infective juvenile (IJ) stages of EPN mostly reside in the moist soils and have been reported in almost all type of soils except dry soils. Therefore, to get hold of these pristine indigenous, local EPN, soils were collected randomly from different agricultural fields of District Meerut using appropriate sampling methods. Recovery of EPN from soil samples was scrutinized using insect baiting with the last instars of *Galleria mellonella* (L.) (Lepidoptera:Pyralidae) [13]. Maintenance of IJ were done by recycling through *G. mellonella* larvae and stored in approximately 150 ml of sterilized distilled water in 500 ml tissue culture flasks at 15°C for subsequent identification and establishment of stock cultures.

Morphological Observations

To study the morphology and morphometry, adult stages and 3rd stage juveniles were recovered from the cadaver of *G. mellonella*. For recovery of adults, the cadaver of *G. mellonella* were dissected 2-3 days and 4-6 days after mortality for hermaphroditic females and subsequent generation of amphimictic males and females respectively in Ringer's Solution[14] while freshly emerged IJ were obtained from white trap[15] for the identification purpose. These all stages were killed with lukewarm water, fixed in TAF[16] and subsequently processed to glycerine by the Seinhorst method[17]. Nematodes were transferred to the fresh drop of glycerine on a microscope slide, crystals of paraffin wax placed around the glycerin drop and covered with a cover glass and placed on hot plate for melting of wax for sealing.

Morphometric analysis of the nematode specimens was done for 15 individuals of the adult stages of generations and infective juveniles (IJ), using light microscopy and the image analyzing software DL-S1 (Phase contrast, Japan).

Molecular Characterization and Phylogenetic Relationships

Genomic DNA was extracted from 3rd stage infective juveniles through Quigen DNA Blood and Tissue Kit as per manufacturer's instruction with slight modifications. Agarose Gel Electrophoresis (AGE) was performed to detect the presence of DNA in the eluted solution and the primers suggested by Joyce et al.[18] were used for PCR. The amplification of the ribosomal and mitochondrial regions was carried out using Dream Taq Green PCR Master Mix 2X (Thermo Scientific) in 100 µl eppendorf (AB Scientific) with a final reaction volume of 30 µl. Amplifications were carried out using Verti 96 well fast thermocycler with heated lid pre-set at 95 ^{0}C and subjected to the following cycling profile: For the ITS rDNA region, PCR conditions included initial denaturation at 94 ^{0}C for 5 min, followed by 33 cycles of 94 ^{0}C for 1 min, 55 ^{0}C for 1 min 30 sec and 72 ^{0}C for 2 min, followed by a final extension at 72 ^{0}C for 5 min to ensure all of the amplification products are full length.. For the 28S (D2D3) rDNA region, the parameters included denaturation at 94 ºC for 5 min, followed by 33 cycles of 94 ºC for 30 sec, 52 ^{0}C for 30 sec and 72 ^{0}C for 1 min, followed by a final extension at 72 ºC for 7 min. For the COX1 mtDNA region, the PCR protocol included denaturation at 94 ^{0}C for 3 min, followed by 37 cycles of 94 ^{0}C for 30 sec, 50 ^{0}C for 30 sec and 72 ^{0}C for 45 sec, followed by a final extension at 72 ^{0}C for 7 min. Electrophoresis of the PCR products was carried in a 1% TAE buffered agarose gel stained with EtBr. The products were analyzed on 1% agarose gel with TAE buffer.

The amplified PCR products were purified and sequenced in both directions by Bioserve Biotechnologies Pvt. Ltd, Hyderabad, India. The contig sequences were deposited in NCBI GenBank database under the following accession numbers; ITS regions (KU176726), D2-D3 regions (KU176127) of rDNA and COX1 (KU306237) for mtDNA. The sequences were edited and compared with those present in the Genbank by means of a Basic Local Alignment Search Tool (BLAST). An alignment of samples together with sequences of the closely related species of the *Heterorhabditis* was produced for each amplified DNA region using default Crustal W parameters in MEGA

6.0 (Tamura et al., 2013) and optimized manually in BioEdit. Pairwise distances and phylogenetic trees (Maximum Parsimony) were computed using MEGA 6.0[19]. Codon positions included were first + second + third + non-coding.

Bioassay experimentation for Pathogenicity and Reproductive Potential

Pathogenicity and reproductive potential of the present species was carried out at different inoculums viz. 25, 50, 100 and 200 IJ/Larvae. For evaluating pathogenicity, freshly emerged IJ were used for larval mortality against *G. mellonella* in 6 well plates (3.5 cm diameter) lined by double Whatman filter paper No. 1. Four different concentrations viz. 25, 50, 100 and 200 IJ were prepared separately in DDW. The total moisture with each IJ dose was maintained 450 µl with distilled water and was poured into each well with the help of micro pipette in the well plates separately. IJ viability was confirmed under stereo microscope (NIKON SMZ 645) before pouring into wells. Larvae of same size and weight were infected in 6 well plates and all experiments were replicated 10 times along with control for each treatment and incubated at 28±2^{0}C in BOD till the mortality of larvae. After the death, all the larvae were transferred onto the white trap for the emergence of IJ to confirm that the mortality was due to IJ infection. Mortality was recorded after each 12 h interval till 100% mortality was achieved and the same cadavers were utilized to compute the progeny production of IJ. The IJ started to emerge from the cadavers into the white trap after 5-7 days of transfer and the emerged IJ from each larva were collected till their emergence (18-20 days) stopped and were transferred from production dish to a culture flask separately in DDW. The amount of DDW was maintained to 50 ml and the nematodes were counted under stereomicroscope (NIKON SMZ 645) in 1ml suspension with the help of counting dish. The total progeny production was determined with the help of statistical tools.

Statistical Analysis

Data obtained through measurements of De Man Indices was analyzed statistically where the descriptive analysis was performed and data was presented in measurement in µm ± SD (range) except the ratios and percentage values. The insect larval mortality assay was analyzed statistically through probit analysis and LC_{50} and LT_{50} values were calculated at 95% confidence limit. All the mortality recorded in the form of percentage mortality and graphical presentations were made using excel.

Total No. of IJ/Larva of the studied nematode was analyzed by descriptive analysis and presented in number of IJ ± SE (range). Analysis of variance (One way ANOVA) was performed to find out the significance relationship between IJ penetration and production with a significance level of $P<0.05$.

RESULTS AND DISCUSSION

Morphological Observations

The *H.* indica isolate CH_{18} was isolated from the soils of sugarcane fields (*Saccharum officinarum L.*) of Baksar area of district Meerut, Uttar Pradesh, India. Present isolate was characterized by the short juveniles almost similar to the original description and the varied arrangement of the terminal group of bursal ribs. The values of E and D ratio differentiate it from other described species of *Heterorhabditis*. It can be recognised by IJ body diam. = 22 (20-24) µm, L = 534 (514-548) µm, pharynx = 107 (99-111) µm; ratio 'a' = 24 (22-27); ratio 'c' = 5.4 (5-6); D% = 84 (75-91) and E%= 92 (83-101). It was further recognised by the male characters viz. L = 721 (592-884) µm, SL = 41 (32-55) µm; GL = 19 (13-27) µm; GBW = 46 (33-54) µm.

H. indica isolate CH_{18} was compared with six closely related species of *Heterorhabditis*, and it differed in some morphological and morphometric characters with compared species but showed much similitude with *H. indica*. The comparison of the morphometrics with other species is presented in the Tables (Table 1 & 2) and is described as follows:

H. indica isolate CH_{18} was separated from *H. taysearae* by longer body of IJ 534 (514-548) vs. 418 (332-499) µm; ratio 'a' =24 (22-27) vs. 21 (18-27); ratio 'b' = 5 (4.74-5.47) vs. 3.8 (3.4-4.2) and shorter ratio 'c' = 5.46 (4.97-5.95) vs. 7 (6.5-8.7) µm; E% = 92 (83-101) vs. 180 (110-230) and tail 98 (89-106) vs. 55 (44-70) µm . Males of the present specimen can be differentiated from *H. taysearae* by SL = 41 (32-55) vs. 39 (30-42) µm; GBW = 46 (33 -54) vs. 43 (38-48) µm; EP = 74 (65-86) vs. 95 (78-120) µm; ES = 88 (78-96) vs. 112 (85-

123) µm; TR = 101 (75-131) vs. 122 (100-146) µm and SW% = 200 (113-299) vs. 156.

Table 1. Comparative morphometrics of infective juveniles of *H. indica* CH_{18} and related *Heterorhabditis* spp. (in ascending order of body length). Measurements are in µm (expect n, ratio and percentage) and in the form: mean (range). Data for *H. indica* CH_{18} in bold.

Characters	*taysearae*	*indica*	CH_{18}	*baujardi*	*floridensis*	*mexicana*	*bacteriophora*
n	30	25	**15**	25	25	25	15
l	418 (332-499)	528 (479-573)	**534 (514 -548)**	551 (497-595)	562 (554-609)	578 (530-620)	588 (512-617)
a	21 (18-27)	26 (25-27)	**24 (22-27)**	28 (26-30)	27 (25-32)	25 (23.6-28.4)	25 (17-30)
b	3.8 (3.4-4.2)	4.5 (4.3-4.8)	**5 (4.7-5.5)**	4.8 (4.5-5.1)	4.3 (3.9-4.9)	4.6 (4.2-5.1)	4.5 (4.0-5.1)
c	7.7 (6.5-8.7)	5.3 (4.5-5.6)	**5.4 (5-6)**	6 (6-6.7)	5.6 (5.3-6.6)	5.9 (5.5-6.3)	6.2 (5.5-7.0)
GBW	20 (17-23)	20 (19-23)	**22 (20-24)**	20 (18-22)	21 (19-23)	23 (20-24)	23 (18-31)
EP	90 (74-113)	98 (88-107)	**90 (82- 95)**	97 (91-103)	109 (101-122)	102 (83-109)	103 (87-110)
NR	64 (58-87)	82 (72-85)	**78 (70- 85)**	81 (75-86)	86 (68-107)	81 (74-88)	85 (72-93)
ES	110 (96-130)	117 (109-123)	**107 (99-111)**	115 (107-120)	135 (123-142)	122 (104-142)	125 (100-139)
Tail with sheath	55 (44-70)	101 (93-109)	**98 (89-106)**	90 (83-97)	103 (91-113)	99 (91-106)	98 (83-112)
ABW	-	-	**15 (13-17)**	13 (11-14)	14(12-16)	15 (12-17)	-
D%	82 (71-96)	84 (79-90)	**84 (75 -91)**	84 (78-88)	81 (71-90)	81 (72-86)	84 (76-92)
E%	180 (110-230)	94 (83-103)	**92 (83 -101)**	108 (98-114)	105 (95-134)	104 (87-111)	112 (103-130)

–, Data not available.

Table 2. Comparative morphometrics of first-generation males of *H. indica* CH_{18} and related *Heterorhabditis* spp. Measurements are in µm (expect n, ratio and percentage) and in the form: mean (range). Data for *H. indica* CH_{18} in bold.

Characters	*taysearae*	*Indica*	CH_{18}	*baujardi*	*floridensis*	*mexicana*	*bacteriophora*
N	20	12	**15**	14	20	20	15
L	703 (648-736)	721 (573-788)	**721 (592-884)**	889 (818-970)	862 (785-924)	686 (614-801)	820 (780-960)
GBW	43 (38-48)	42 (35-46)	**46 (33-54)**	49 (45-53)	47 (43-50)	42 (38-47)	43 (38-46)
EP	95 (78-120)	123 (109-138)	**74 (65-86)**	81 (71-93)	117 (104-128)	124 (108-145)	121 (114-130)
NR	65 (54-88)	75 (72-85)	**65 (56-74)**	65 (54-77)	80 (73-90)	71 (61-83)	72 (65-81)
ES	112 (85-1230	101 (93-109)	**89 (78-96)**	116 (105-132)	105 (97-111)	96 (89-108)	103 (99-105)
TR	122 (100-146)	91 (35-144)	**101 (75-131)**	91 (28-38)	93 (78-116)	96 (65-130)	79 (59-87)
SL	39 (30-42)	43 (35-48)	**41 (32S-55)**	40 (33-45)	42 (36-46)	41 (30-47)	40 (36-44)
GL	18 (14-21)	21 (18-23)	**19 (13-27)**	20 (18-22)	23 (17-30)	23 (18-32)	20 (18-25)
D%	88	121	**84 (68-93)**	-	112 (105-119)	129 (114-149)	117
SW	156	187	**200 (113-299)**	182 (138-208)	157 (133-209)	167 (130-196)	174
GS	46	49	**46 (38-56)**	50 (44-61)	53 (47-65)	56 (43-70)	50

–, Data not available.

Morphologically *H. indica* CH_{18} was separated from *H. baujardi* by the shape of gubernaculum proximal end bearing knob vs. lacking, ventrally straight vs. curved. Males of *H. indica* CH_{18} differs from *H. baujardi* by ES = 89 (78-95) vs. 116 (105-132) µm, TR = 101 (75-131) vs. 91 (28-38) µm, Tail = 27 (18-42) vs. 33 (28-38) µm, SW% = 200 (113-299) vs. 182 (138-208) and GS% = 46 (38-56) vs. 50 (44-61). The IJ of the present isolate differs from *H. baujardi* by having shorter body length 534 (514-548) vs. 551 (497-595) µm, ratio 'a' = 24 (22-27) vs. 28 (26-30), EP = 90 (82-95) vs. 97 (91-103) µm, E% = 92 (83-101) vs. 108 (98-114) and tail 98 (89-106) vs. 90 (83-97) µm.

H. indica CH_{18} males were differentiated from *H. floridensis* males by a EP =74 (65-86) vs. 117 (104-128) µm, NR = 65 (56-74) vs. 80 (73-90) µm, ES = 89 (78 -96) vs. 105 (97-111) µm, GL = 19 (13-27) vs. 23 (17-30) µm, SW% = 200 (113-299) vs. 157 (133-209), GS%.= 46 (38-56) vs. 54 (47-65) and D% = 84 (68-93) vs. 112 (105-119). IJ were shorter than *H. floridensis* 534 (514-548) vs. 562 (554-609) µm, Tail = 98 (89-106) vs. 103 (91-113) µm and ratio 'a' = 24 (22 -27) vs. 27 (25-32), E% = 92 (83-101) vs. 101 (95-134).

Male of *H. indica* CH_{18} was separated from *H. mexicana* by GBW= 46 (33-53) vs. 42 (38-47) µm, EP = 74 (65-86) vs. 124 (108-145) µm, GL = 19 (13-27) vs. 23 (18-32) µm, D% = 83 (68-93) vs. 129 (114-149), SW% = 200 (113-299) vs. 167 (133-196), GS%.= 46 (38-56) vs. 56 (43-70). IJ can be distinguished by the body length = 534 (514-548) vs. 578 (530-620) µm, EP = 90 (81-94) vs. 102 (83-109) µm, ES = 107 (99-111) vs. 122 (104-142) µm, D% = 84 (75-91) vs. 81 (72-86) and E% = 92 (83-101) vs. 104 (87-111). The vulval pattern in

females is different in both specimens which also separated them from one another.

Males of *H. indica* CH_{18} was differentiated from *H. bacteriophora* males by SL = 41 (32-55) vs. 40 (36-44) µm, GL = 19 (13-27) vs. 20 (18-25) µm, D% = 84 (68-93) vs. 117, ABW= 21 (17-29) vs. 23 (22-25) µm, SW% = 200 (113-299) vs. 174 and GS% = 46 (38-56) vs. 50. IJ can be characterised by the shorter body length 534 (514-548) vs. 588 (512-617) µm, NR = 78 (70-85) vs. 85 (72-93) µm, EP = 90 (81-94) vs. 103 (87-110) µm, ratio 'a' = 24 (21-26) vs. 25 (17-30) µm, ES = 107 (99-111) vs. 125 (100-139) µm and E% = 92 (83-101) vs. 112 (103-130).

Molecular Characterization and Phylogenetic Relationships

H. indica isolate CH_{18} was characterised genetically by sequences of ITS (KU176726), D2-D3 regions (KU176127) of rDNA and COX1 (KU306237) mt DNA. The quality of PCR products of said genes was confirmed by agarose gel electrophoresis. The length of the internal transcribed spacer region was 737 bp with ITS1 = 369 bp, 5.8S = 154 bp and ITS2 = 214 bp and its nucleotide composition was: A = 26.46%, C = 19.95%, G = 24.83%, T = 28.77%. The length of the total sequence is the smallest among the 14 related species of *Heterorhabditis* (Table 3). *H. indica* isolate CH_{18} differed from *H. baujardi*, *H. bacteriophora*, H. *floridensis* and *H. mexicana* from its closest taxon by 25 bp, 34 bp, 23 bp and 24 bp, respectively while with already described *H. indica* (AY321483) by only 2 bp and thus not much variation, hence considered same. Pairwise distance of ITS and D2D3 regions of rDNA are shown in Table 4-5. Both the regions of taxonomic importance showed 100% percentage similarity with the original specimen.

Table 3. Sequence lengths and nucleotide composition of ITS (ITS1 + 5.8S + ITS2) and D2-D3 regions of species of *Heterorhabditis* closely related to *H. indica* CH_{18}. Data of *H. indica* CH_{18} in bold.

Species		Molecular markers							ITS(bp)	Seq. (bp)
ITS	Acc. No.	ITS1(bp)	5.8S(bp)	ITS2(bp)	A	C	G	T		
***H. indica* CH_{18}**	**KU176126**	**369**	**154**	**214**	**26.46**	**19.95**	**24.83**	**28.77**	**739**	
H. indica	AY321483	370	154	215	20.78	21.43	28.57	29.22	739	
H. noenieputensis	JN620538	371	154	216	20.78	21.43	28.57	29.22	741	
H. amazonens	DQ665222	395	154	211	21.43	21.43	28.57	28.57	760	
H. baujardi	AF548768	397	153	212	20.92	21.57	28.76	28.76	762	
H. floridens	DQ372922	393	154	213	21.43	21.43	28.57	28.57	760	
H. Mexicana	AY321478	394	154	213	22.08	21.43	28.57	27.92	761	
H. bacteriophora	AY321477	389	154	228	21.43	20.78	28.57	29.22	771	
H. Georgiana	EU099032	389	154	228	22.08	20.13	28.57	29.22	771	
H. atacamensis	HM230723	350	154	211	20.78	21.43	29.22	28.57	715	
H. safricana	EF488006	379	154	211	22.08	19.48	29.22	29.22	744	
H. marelatus	AY321479	379	154	211	21.43	21.43	28.57	28.57	744	
H. zealandica	AY321481	387	154	212	21.43	22.08	27.92	28.57	753	
H. downesi	AY321482	374	154	212	21.43	21.43	28.57	28.57	740	
H. megidis	AY321480	384	154	220	22.08	21.43	28.57	27.92	758	
D2D3										
***H. indica* CH_{18}**	**KU176127**				**27.49**	**19.43**	**28.24**	**24.84**		**942**
H. indica	JQ178379				25.85	19.39	29.13	25.63		913
H. amazonens	EU099036				26.07	19.55	29.06	25.32		936
H. floridens	EU099034				25.56	19.79	29.3	25.35		935
H. Mexicana	EU100414				25.48	19.81	29.44	25.27		934
H. bacteriophora	JQ178377				25.49	19.89	29.45	25.16		910
COX I										
***H. indica* CH_{18}**	**KU306237**				**23.83**	**12.75**	**19.3**	**44.13**		**596**
H. indica	AB355853				22.81	9.81	19.89	47.48		377

H. taysearae	EF043421	24.42	11.06	19.47	45.05	606
H. Mexicana	EF043422	24.92	10.4	18.81	45.87	606
H. bacteriophora	JQ423214	23.77	8.62	18.76	48.85	917

The sequence of the D2-D3 region was 942 bp long with nucleotide composition as: A = 27.49%, C = 19.43 %, G = 28.24%, T = 24.84%. The sequence of the COX1 mtDNA region was 596 bp with nucleotide configuration: A = 23.83%, C = 12.75%, G = 19.30%, T = 44.13% (Table 3).

For ITS regions, maximum parsimony analysis showed that the alignment resulted in 1019 characters, of which 267 were constant, 467 variable characters were parsimony uninformative and 285 characters were parsimony informative. Parsimony and distance based tree building approaches produced almost identical trees. The phylogenetic relationships between 16 *Heterorhabditis* species are presented in Fig. 1, with MP Tree length = 710, Consistency Index = 0.74498, Retention Index = 0.875, Composite Index = 0.718486 (0.651857). In this consensus tree, two species, *H. indica* (AY321483) and *H. indica* isolate CH_{18} form a monophyletic group with bootstrap support of 98%. All positions containing gaps and missing data were eliminated. There were a total of 574 positions in the final dataset.

For D2-D3 region, maximum parsimony analysis showed the alignment resulted in 1034 characters, of which 174 are constant, 530 variable characters are parsimony uninformative and 330 characters are parsimony informative. The phylogenetic relationships between 17 *Heterorhabditis* species are presented in Fig. 2, with MP Tree length = 687, Consistency Index = 0.814126, Retention Index = 0.862637, Composite Index 0.799854 (0.702296). There were a total of 574 positions in the final dataset. In the consensus tree, *H. indica* and the present specimen along with two undescribed species form a monophyletic group.

For the COX1 gene, maximum parsimony analysis showed that the alignment resulted in 1034 characters, of which 105 are constant, 609 variable characters are parsimony-uninformative and 217 characters are parsimony-informative. The phylogenetic relationships between 17 *Heterorhabditis* species are presented in Fig. 3, with MP Tree length = 555, Consistency Index = 0.679878, Retention Index = 0.562500, Composite Index = 0.456081 (0.382431). There were a total of 354 positions in the final dataset. The present species showed deviation from the above results which might be due to non-utility of CO1 gene for identification purpose of EPN.

Table 4. Pairwise distances of the ITS regions between *H.indica* CH_{18} and closely related species of *Heterorhabditis*. Below diagonal: % age similarity; above the diagonal: no. of base substitutions per site between sequences, according to the Jukes-Cantor model. Data for isolate CH_{18} in bold.

ITS region	CH_{18}	*ind*	*bac*	*bau*	*noe*	*ama*	*flo*	*mex*	*geo*	*ata*	*saf*	*mar*	*zea*	*dow*	*meg*
CH18		**0.003**	**0.031**	**0.023**	**0.008**	**0.021**	**0.024**	**0.024**	**0.031**	**0.033**	**0.034**	**0.036**	**0.046**	**0.039**	**0.040**
H. indica	**100**		0.032	0.023	0.008	0.021	0.024	0.025	0.031	0.034	0.035	0.037	0.047	0.039	0.041
H. bacteriophora	**77**	77		0.034	0.032	0.033	0.035	0.035	0.006	0.024	0.024	0.026	0.035	0.027	0.028
H. baujardi	**86**	86	74		0.024	0.008	0.009	0.011	0.034	0.035	0.036	0.037	0.044	0.039	0.038
H. noenieputensis	**98**	98	76	86		0.022	0.024	0.025	0.031	0.033	0.034	0.036	0.045	0.039	0.040
H. amazonensis	**88**	87	75	98	87		0.010	0.012	0.033	0.035	0.036	0.037	0.044	0.040	0.039
H. floridensis	**85**	85	73	97	85	97		0.008	0.035	0.037	0.038	0.039	0.047	0.043	0.042
H. Mexicana	**85**	84	73	96	84	96	98		0.035	0.038	0.039	0.040	0.048	0.044	0.043
H. Georgiana	**78**	77	99	74	77	75	73	98		0.025	0.025	0.027	0.035	0.028	0.029
H. atacamensis	**74**	73	85	71	74	72	69	98	85		0.007	0.010	0.029	0.016	0.018
H. safricana	**73**	72	86	71	73	71	68	97	85	98		0.010	0.029	0.016	0.018
H. marelatus	**70**	70	84	69	71	70	66	99	83	96	97		0.029	0.017	0.019
H. zealandica	**61**	60	73	62	61	62	58	98	73	79	79	79		0.030	0.030
H. downesi	**68**	68	82	68	68	68	64	99	81	93	93	91	79		0.014
H. megidis	**66**	65	79	68	66	67	64	99	78	90	90	89	78	94	

Table 5. Pairwise distances of the D2D3 regions between *Heterorhabditis indica* CH_{18} and closely related species of *Heterorhabditis*. Below diagonal: %age similarity; above the diagonal: no. of base substitutions per site between sequences, according to the Jukes-Cantor model. Data for isolate CH_{18} in bold.

D2D3 egion	**CH_{18}**	*ind*	*ama*	*mex*	*noe*	*flo*	*saf*	*mar*	*bac*	*ata*	*geo*	*zea*	*meg*
CH18		**0.002**	**0.008**	**0.008**	**0.002**	**0.008**	**0.011**	**0.012**	**0.011**	**0.011**	**0.011**	**0.297**	**0.012**
H. indica	**100**		0.008	0.008	0.002	0.008	0.011	0.012	0.010	0.011	0.011	0.296	0.012
H. amazonensis	**96**	96		0.003	0.008	0.003	0.011	0.011	0.010	0.011	0.010	0.288	0.012
H. Mexicana	**96**	96	99		0.008	0.003	0.012	0.012	0.010	0.011	0.011	0.287	0.012
H. noenieputensis	**100**	100	96	96		0.008	0.011	0.012	0.010	0.011	0.011	0.295	0.012
H. floridensis	**96**	96	99	99	96		0.012	0.012	0.010	0.011	0.010	0.287	0.012
H. safricana	**94**	94	93	93	94	93		0.004	0.009	0.004	0.009	0.272	0.006
H. marelatus	**93**	93	93	93	93	93	99		0.009	0.004	0.009	0.273	0.006
H. bacteriophora	**93**	94	94	94	94	94	95	95		0.008	0.003	0.303	0.010
H. atacamensis	**93**	93	94	94	93	93	99	99	96		0.008	0.272	0.006
H. Georgiana	**93**	93	94	94	93	94	95	95	99	96		0.305	0.010
H. zealandica	**49**	50	48	48	48	50	41	42	53	42	55		0.273
H. megidis	**92**	92	93	25	92	92	98	98	94	98	94	42	

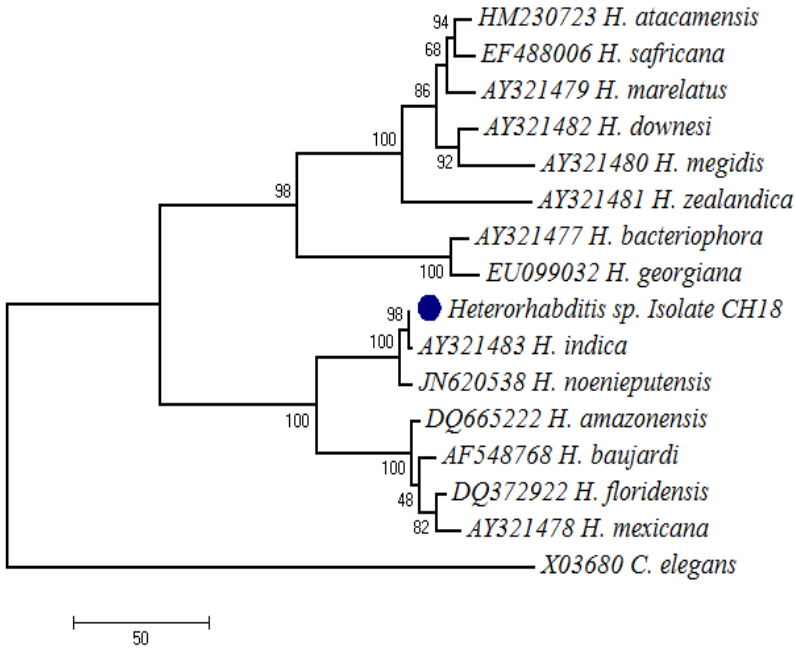

Fig. 1. Phylogenetic analyses of ITS region of isolate CH_{18} and other species of *Heterorhabditis*. The dendrogram was constructed by Maximum Parsimony method. Bootstrap values are included.

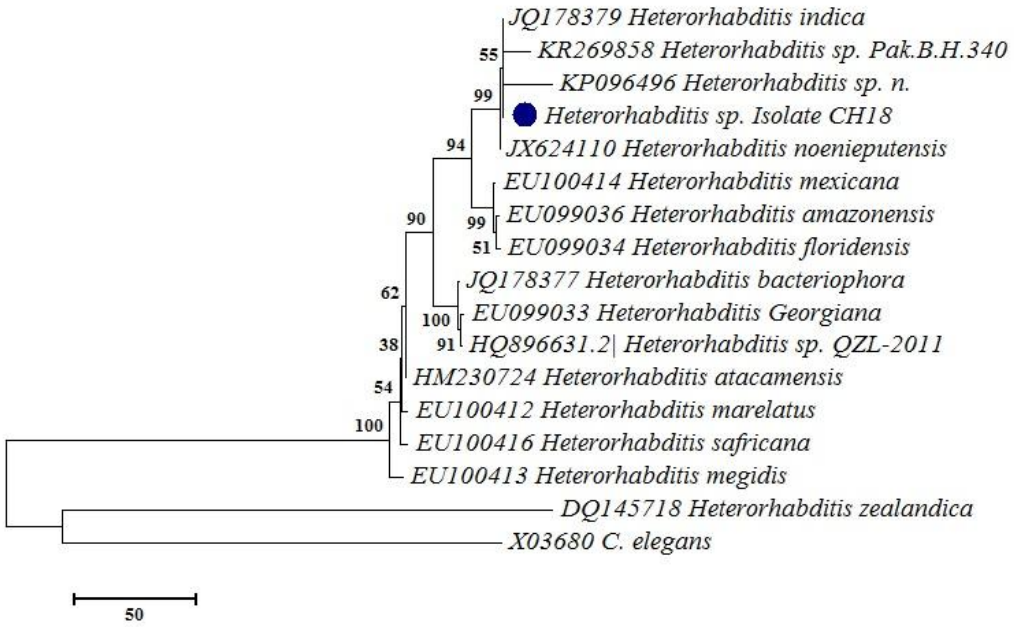

Fig. 2. Phylogenetic relationships based on Maximum Parsimony between 17 *Heterorhabditis* species and isolate CH_{18} with bootstrap analysis of D2D3 regions.

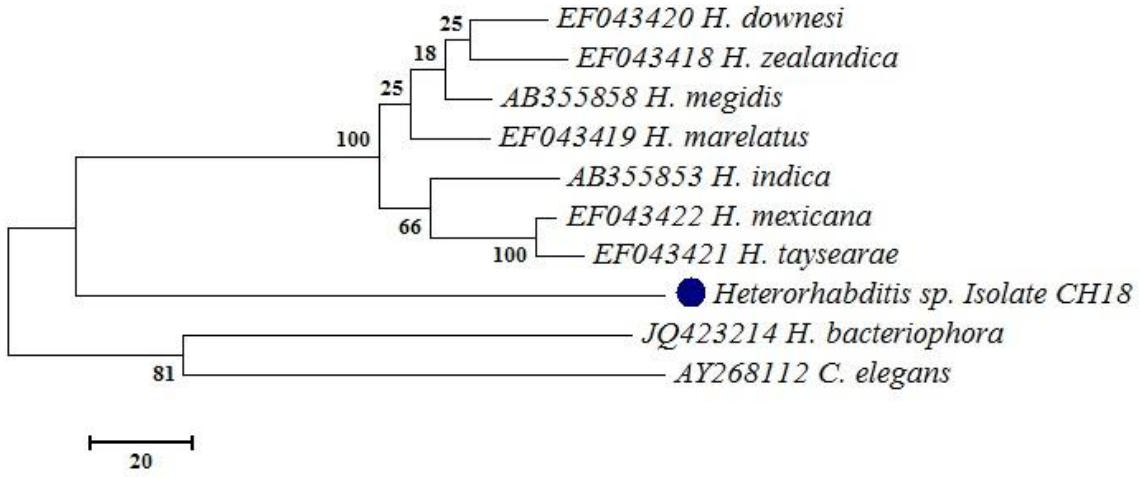

Fig. 3. Phylogenetic relationships based on Maximum Parsimony between 10 *Heterorhabditis* species and isolate CH_{18} with bootstrap analysis of COI regions.

Pathogenicity

The evaluation of the pathogenicity and reproductive potential of *H. indica* isolate CH_{18} was carried out in laboratory condition against *G. mellonella* as target host using different IJ concentration viz. 25, 50, 100, 200 IJ/larva. At 12 h post infection period, no mortality was recorded in all infected larva against the applied doses, while 10, 30, 30 and 90% mortality was observed in 25, 50, 100, 200 IJ/larva, respectively after 24 h post infection period (Fig. 4). At 36 h post infection period 100% mortality was noticed in 200 IJ/larva dose followed by 70, 60, and 40% respectively in 100, 50 and 25 IJ/larva. At 48 h post infection period, 90% mortality was observed in 100 IJ/larva while 80% was shown in 25 and 50 IJ/larva. At 60 h post infection period, 100% was discerned in all the concentrations. However, the degree of susceptibility to nematode infection varied from different dose infection and also on the exposure time. No mortality was recorded in control group even after 60 h during the experiment. All the dead larvae were transferred on the modified white trap to confirm their death was due to EPN where the emergence of IJ from the cadaver was the sign of nematode infection. A positive relation was observed in between the doses of infective juvenile and larval mortality where increments in doses also lead to increment in mortality rate.

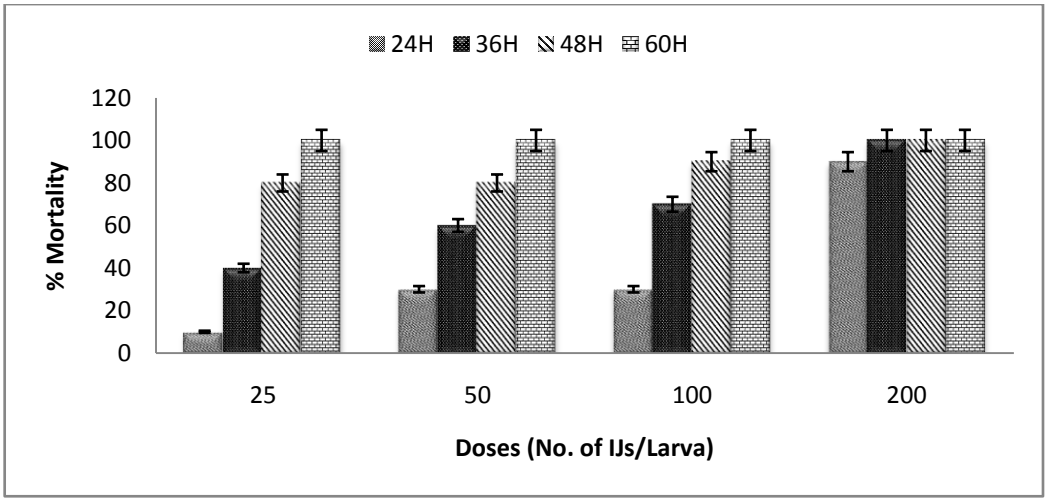

Fig. 4. Percentage mortality of *G. mellonella* larvae treated with *H. indica* isolate CH_{18}.

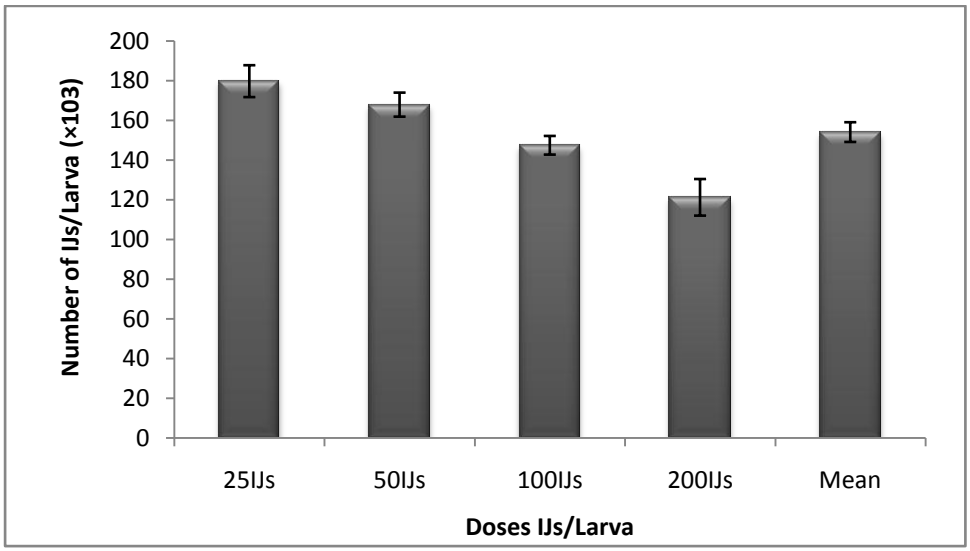

Fig. 5. Progeny productions *H. indica* isolate CH_{18} in *G. mellonella* larvae at different doses of IJ.

LC_{50} and LC_{90} value were also calculated to judge the pathogenicity of isolate by probit analysis at three different post infection periods viz. 24, 36 and 48 h and presented in IJ/Larva. Higher LC_{50} value was recorded at 24 h post infection period which was about 96 IJ/Larva followed by 37 IJ/Larva at 36 h post infection period where as lowest LC_{50} value was reported after 48 h which was only 8 IJ/Larva confirming the high pathogenicity of present isolate.

LT_{50} and LT_{90} values were also figured out at different concentrations viz. 25, 50, 100 and 200 IJ/Larva and for mean doses too through same analysis. The lowest time (LT_{50}) was taken with high dose which was only 19.3 h followed by 29.5, 31.4 and 36.8 h with 100, 50 and 25 IJ/Larva respectively. The mean LT_{50} calculated for all the doses applied was 25.7 hours.

The above findings showed similitude with that of the Sankar et al.[20] and with that of Hara and Kaya[21] which reported that nematodes are symbiotically associated with mutualistic bacteria, which allow them to kill their hosts quickly (36 to 48 h) and thus give them an advantage over other parasitoids and pathogens. Divya et al.[22] revealed highest death rate of the greater wax moth larvae at concentration levels of 300 IJ/larva within 24 h exposure of *H. indica* but the present *Heterorhabditis* isolate showed 100% mortality with different concentrations viz., 25, 50, 100 and 200 only after 36 h of exposure. This may be due to the fact the pathogenicity being complex process, depends upon many biotic and abiotic factors as was reported by Kaya and Gaugler[23].

Reproductive potential

In vivo progeny production of infective juveniles was also performed to assess the reproductive potential at different concentrations for same larvae used in pathogenicity bioassay. The IJ production was recorded as number of IJ/Larva. Descriptive analysis for IJ production revealed that the population count of IJ decreased with increase in increment of dose. The highest progeny production was recorded 1.79×105 (140800-217600) at 25 IJ/Larva infection whereas lowest was recorded at 200 IJ/Larva 1.21×105 (86400-172800). At 50 and 100 IJ/Larva, progeny count calculated were 1.68×10^5 (147200-211200) and 1.47×10^5 (121600-169600), respectively (Fig. 4).

The overall effectiveness of pest control program is directly related to the reproductive capability of EPN within the host and determines the time and dose applied in the field[24,25]. In conjugation with this, it is far too expensive to rear EPN by in vitro media as they required a lot of care, instruments and subsequent labor practices. Hence the species/strain of high yielding progeny is a requisite of the biological control program. Reproductive capability of different nematode species to produce infective juveniles was different[26,27]. Apart from this, the size of the nematode and their behaviour directly influenced the reproduction of nematode species within the body of

host[28] as reported by Bhatnagar et al.[29] in *H. bacteriophora*. Availability of food also influenced the number of eggs in the uterus of hermaphrodite by delaying the *endotokia matricida*[30].

Analysis of variance (ANOVA) indicated that there was negative correlation in between the production of IJ and doses applied where the significance value was zero. The positive relationship between the dose of IJ and host mortality found in the present examination has also been documented in several other investigations[31,32,33]. Gupta et al.[34] reported gradual increase in progeny production up to 160 IJ of *S. carpocapsae* per larva of *Pieris brassicae*, after which a sudden decrease from 2.20 ×105 larva to 1.70 ×105 was noticed. Small size of *H. indica* isolate CH_{18} and hermaphroditism could be the reason of the high yield of progeny as compared to others and supported by several findings[7,35,36]. However, in the present study, the highest reproduction was in low dose (25 IJ/Larva dose with 1.79 lacs IJ/Larva) as compared to other researchers with different/ same species in different/ same insect host.

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