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Ashok Kumar Chaubey
Nematology Laboratory,
Department of Zoology,
Ch. Charan Singh University,
Meerut-250004, India

Istkhar
Nematology Laboratory,
Department of Zoology,
Ch. Charan Singh University,
Meerut-250004, India

Aashana
Nematology Laboratory,
Department of Zoology,
Ch. Charan Singh University,
Meerut-250004, India

Aashaq Hussain Bhat
Nematology Laboratory,
Department of Zoology,
Ch. Charan Singh University,
Meerut-250004, India

Aasha
Nematology Laboratory,
Department of Zoology,
Ch. Charan Singh University,
Meerut-250004, India

Correspondence:
Istkhar
Nematology Laboratory,
Department of Zoology,
Ch. Charan Singh University,
Meerut-250004, India.

Multigene sequence analyses of an isolate of *Heterorhabditis indica* with a profile on their high virulence and density dependent biotic potential on an insect host

Ashok Kumar Chaubey, Istkhar, Aashana, Aashaq Hussain Bhat, Aasha

Abstract
Present study deals with the identification of an isolate of *Heterorhabditis indica* CH_{16} of District Bijnor, Uttar Pradesh, India with high virulent and reproductive potential grounded on morphotaxometry and multigene sequence analyses. Total body length (520 µm vs. 528 µm) support the close similarity of specimen with *H. indica* which was greater to *H. tysesare* (418 µm) and shorter with others. With a length of 547 µm, males of present isolate were shortest. Results obtained through the molecular phylogeny of sequences of ITS rDNA, and 28S rDNA placed the isolate CH_{16} with *H. indica* and *H. noenieputensis*. However analysis of COXI gene revealed unsatisfactory results with diverting results. Bioassay trial against *Galleria mellonella* showed greater pathogenicity of nematode isolate where it killed all the larvae within 60 hours post infection period with all the doses applied. Increment in dose lead to the increased number of progeny was recorded up to 100 IJs/ Larva (2.15 lacs IJs/ Larva) which was higher when compared to others. Results demonstrated that the multi-sequence analyses provide more reliable and accurate data for the diagnostic of *Heterorhabditis* species. Further, the high virulence and reproductivity prove that the present isolate as good candidate for future IPM program.

Keywords: *Heterorhabditis indica* CH_{16}, ITS, 28S rDNA, COXI gene, pathogenicity, reproductive potential

1. Introduction
Entomopathogenic nematodes (EPN) are used as microbial bio-pesticides in pest management and have been shown to be effective as insecticides of several insect pests. Steinernematidae and Heterorhabditidae are most commonly useful in the biological control of insect pests and are safe to most non-target organism and to environment [1, 2]. High virulence, broad host range, ability to seek host and mass production are the key factors which developed an immense interest in EPN as biological control agents [3, 1]. Recycling and persistence of entomopathogenic nematodes after application in field play an important role in overall effectiveness of pest control [4, 5], further the applied dose in a field also determine the usefulness of bio-control agents.
The natural reservoir for *Steinernematids* and *Heterorhabditids* is the soil, where they parasitize soil-inhabiting insects. This is the only habitat in which these nematodes can establish and recycle to provide long-term regulation. Surveys for EPNs conducted in various parts of the world reveal that these genera have global distributions as their two major genera *Steinernema* Travassos, and *Heterorhabditis* Poinar contributed greater than 100 and 26 species, respectively. Heterorhabditid nematodes were thought to be associated with sandy costal soil but increment in number of *Heterorhabditis* species imitates the inclination of EPN habitat towards the suitable hosts [6] and definite environmental adaptations [7] with physiological and behavioural adaptations for existence in a range of altered territories [8]. Due to increment in number of identified species from last decade, morphological differentiation is becoming difficult, therefore biological species concept in combination with morphological and molecular data, has been widely applied in EPN taxonomy for establishing the precise species status of the isolates. On the other side, species adapted to local climate and environments are also considered good candidates for the use of biological control. Hence, the objective of the present study was shaped to isolate EPN from the Bijnor district of Uttar Pradesh, India for future bio-control strategies and to identify it based on morphotaxometry, ITS, 28S rDNA and COXI gene analyses. The study was also focused on the virulence and recycling capability of the identified species at different doses applied

2. Materials and Methods

2.1. Isolation of Nematodes and Maintenance of Host Insect.

District Bjinor is located in western part of Uttar Pradesh, India at 29° 23' N, 79° 11' E and 237 meters above sea level. Generally sandy, clay-loam and light loam soils are found in this area originated from Siwalik Belt of Himalaya. The soil samples were collected from different agriculture fields and processed in laboratory. The processing of soil samples for nematode isolation, maintenance of host insect *Galleria mellonella* (L.) and isolated nematode was done as described by Istkhar *et al*. [9]. The 3rd stage juveniles (also known as infective juveniles or IJs) were stored in double distilled water and stored in BOD at 15±1 ºC for further investigations.

2.2. Processing of Nematodes for Identification

To study the morphology and morphometry of the EPN isolates, 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 2nd generations amphimictic males and females, respectively in Ringer's Solution [10]. Freshly emerged IJ were collected from White trap [11]. The IJ and adults so obtained were first killed in a hot water bath by raising the temperature up to 60 ºC and then fixed in TAF [12] for 5-7 days, dehydrated in Sienhorst solutions [13] and mounted in glycerine.

2.3. Morphology and Morphometry

In the present study, morphology and morphometry of *Heterorhabditis* sp. Isolates CH_{16} was carried out using light and phase contrast microscopy (Nikon Eclipse 50i, Japan). The morphology and morphometrical measurement of 3rd stage IJs and adults was done under 40x, 100x, 400x and 1000x magnifications using inbuilt software of phase contrast microscope (DS-L1). Drawing of nematodes was done with the help of camera lucida under 100X and 400X magnifications. Microphotography was also done with the help of inbuilt photographic attachment in phase contrast microscope on the same magnification.

2.4. Isolation and Amplification of DNA

Genomic DNA was extracted from 3rd stage of infective juveniles as suggested by Istkhar and Chaubey [14]. Agarose Gel Electrophoresis (AGE) for the detection of DNA was performed to detect the presence of DNA in the eluted solution. Three regions of taxonomic importance viz. internal transcribed spacers (ITSs), 28S ribosomal DNA (28S rDNA) and cytochrome small subunit 1 (COXI) of mitochondrial DNA were used as molecular markers to distinguish the present specimen form compared species of *Heterorhabditis* and were amplified as per suggested by Joyce *et al*. [15]. The composition 30 µL of PCR reaction volume were; Dream Taq Green master mix 2x (Thermo Scientific) 15 µL, forward primer 0.7 µL, reverse primer 0.7 µL, template DNA 5 µL, mili Q water 8.6 µL. The PCR amplification was performed for ITS regions as, no. of cycles 30; initial temperature 94 ºC for 5 minutes, denaturation at 94 ºC for 30 sec; renaturation at 55 ºC for 90 seconds; primer extension at 72 ºC for 2 minutes and final extension at 72 ºC for 5 minutes. For 28S gene, the PCR conditions were: no. of cycles 33; initial denaturation at 94 ºC for 5 minutes; denaturation at 94 ºC for 30 seconds; renaturation at 52 ºC for 30 seconds; primer extension at 72 ºC for 60 seconds and extension at 72 ºC for 7 minutes. The conditions of PCR for cytochrome small subunit 1 (COXI) were; no. of cycles: 37; initial denaturation at 94 ºC for 3 minutes; denaturation at 94 ºC for 30 seconds; renaturation at 55 ºC for 30 seconds; primer extension at 72 ºC for 45 seconds and final extension at 72 ºC for 7 minutes. The amplifications of final products were confirmed by 1% AGE.

2.5. Molecular and Phylogenetic Analyses

All the sequence were submitted to National Center for Biotechnology Information (NCBI) under accession numbers KU187258 (ITS rDNA), KU187259 (28S rDNA) and KU306236 (COXI). Available data of gene sequences of already described *Heterorhabditis* species were retrieved from the NCBI database. A total of 18 sequences of ITS regions, 16 sequences of 28S rDNA and 8 sequences of cytochrome small subunit 1 were downloaded from NCBI, aligned using the default parameters (gap opening penalty 15, gap extension penalty 6.66) in Clustal W programme in MEGA software version 6 [16]. Phylogenetic trees were constructed by maximum parsimony [17] method. Sequences of *Caenorhabditis elegans* for all the three genes were taken and used as out group. Nucleotide compositions of the sequences were calculated through Bio Edit software.

2.6. Virulence and Biotic Potential

To test the pathogenicity and reproductive potential of the nematode isolate, freshly emerged IJ were utilized for bioassays experiment. The efficacies of EPN against *G. mellonella* were tested as described previously [18] in sterile 6 well plates (3.5 cm diameter) lined by double filter paper. Four different concentrations viz. 25, 50, 100 and 200 IJ were prepared and poured into the wells of well plates with a total water content of 450 µl. Confirmation of the viability of nematodes was done before the application of juveniles to the larvae of *G. mellonella* under stereomicroscope. The whole experiment was replicated 10 times along with control (without juvenile with 450 µl of DDW). Mortality was recorded after each 12 hours post treatment period till the 100% mortality was achieved. After the death of the larvae, they were transferred onto the white trap for the emergence of IJs so that the confirmation of mortality can be estimated due to the nematode infection.

All the doses applied to infect the larvae were also utilized to calculate the progeny production of IJs from the cadaver viz. 25, 50, 100 and 200 IJ/Larva/450 µl DDW. The emerged IJs from each larva were collected up to their emergence which was 18-20 days after the mortality in the culture flask and stored into BOD at 15±1 ºC temperature. The IJ of nematode were counted under stereomicroscope (Nikon SMZ 645) in counting dish. The total progeny production was determined with the help of statistical tool.

2.7. Statistical Analysis

Data obtained through measurements of De Man Indices was analysed statistically where the descript analysis was performed and data was presented in measurement in µm±SD (range) except the number, ratios and percentage values. The insect larval mortality assay was analysed statistically through probit analysis and LC_{50}, LC_{90} and $LT_{50,}$ LT_{90} values were calculated at 95% confidence limit. All the mortality recorded in the form of percentage mortality and graphical presentations were made using excel. Biotic potential of the studied nematode was analysed by descriptive analysis and presented in number of IJs±SD (range). Analysis of variance (One way ANOVA) was performed to find out the significance relationship between IJ penetration and production with a

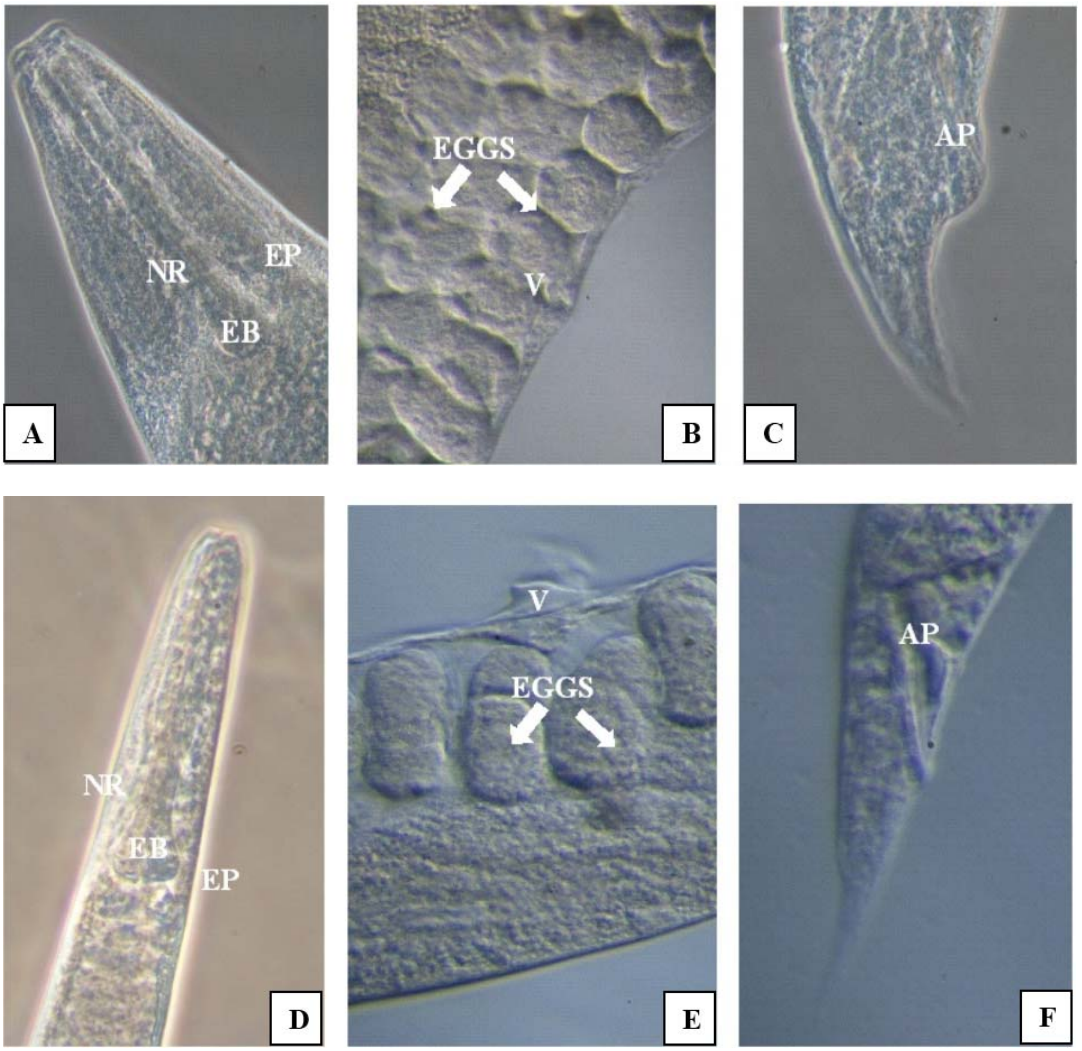
significance level of $P<0.005$.

3. Results and Discussion

3.1. Morphology and Morphometry

During the present investigation, the nematode isolate was identified as *Heterorhabditis indica* and tagged as isolate CH_{16} of Bijnor District (Fig. 1-2, Table 1). *Heterorhabditis* sp. isolate CH_{16} was compared morphologically and morphometrically with *H. tysesare* [19], *H. indica* [20], H. *baujardi* [21], *H. floridensis* [22], *H. maxicana* [23] and *H. bacteriphora* [24].

The morphological traits of males and IJ provide most of the useful taxonomic characters for *Heterorhabditis* species [25]. Following multivariate analysis of morphometric characters of *Heterorhabditis* spp. [25] suggested that both body length and tail length of IJ and the body length and testis refexion of males contribute most in the discrimination of species. In present investigation, the means of the morphometrical characters and the ratios for several populations were used and concluded that the body length and testis reflexion of males do not contribute much to the discrimination of *Heterorhabditis* spp. Our data further indicate that morphometrical characters such as ratio E, ratio F and body diameter of IJ and spicule length, gubernaculums length and SW% of males play a significant role in discrimination (Table 3). On the basis of morphological characterization of 3rd stage juvenile, the studied specimen was showing close resemblance with *H. indica*. Total body length (520 µm vs. 528 µm) support the close similarity of specimen with *H. indica* which was greater to *H. tysesare* (418 µm) and shorter with others. Other morphometrical parameters and ratios varied in results and diverted the specimen from any compared species (Table 2). On the other hand, comparison of males showed the shortest males in isolate CH_{16} with only 547 µm as compared to others (Table 3). Number of anal papillae in males varied in numbers which were 8 pairs in isolate CH_{16} (Fig. 1-2) as compared to original description of *H indica* but reported similar in other isolates. The other parameters were also varied and diverted the studied specimen from the described ones. Consequently, these morphometrical characters and ratios should be considered when identifying and describing *Heterorhabditis* spp. The comparison of the species positions in the score plots obtained in both analyses revealed common conclusions: the clear differentiation of *H. marelatus* and *H. megidis* from other *Heterorhabditis* spp. and the high similarity of the present specimen with *H. indica* based on morphometrics of IJ, but with not good discrimination on the base of male morphometrics.



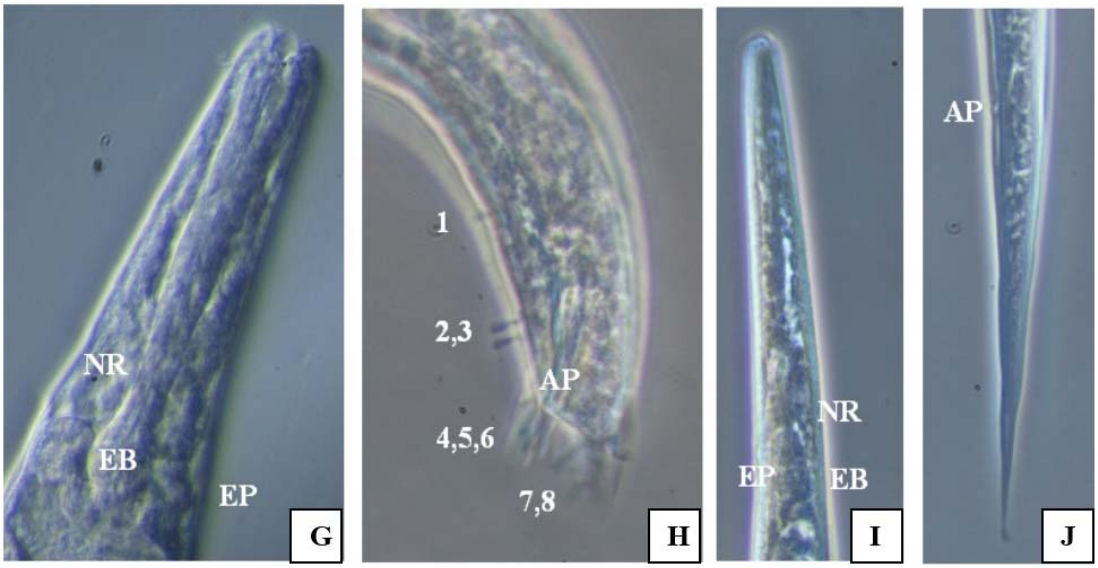


Fig A-C Hermophrodictic female; D,E,F second generation female; G-H male; I,J 3rd stage juvenile; Fig A,D,G,I anterior region; B,E middle region; C,F,H,J posterior region. NR; nerve ringh, EB; esophagial bulb, EP; excretory pore, V; vulva, AP; anal pore, number indicate anal papili.

Fig 1: Phase contrast microphotograph *Heterorabditis indica* isolate CH_{16}

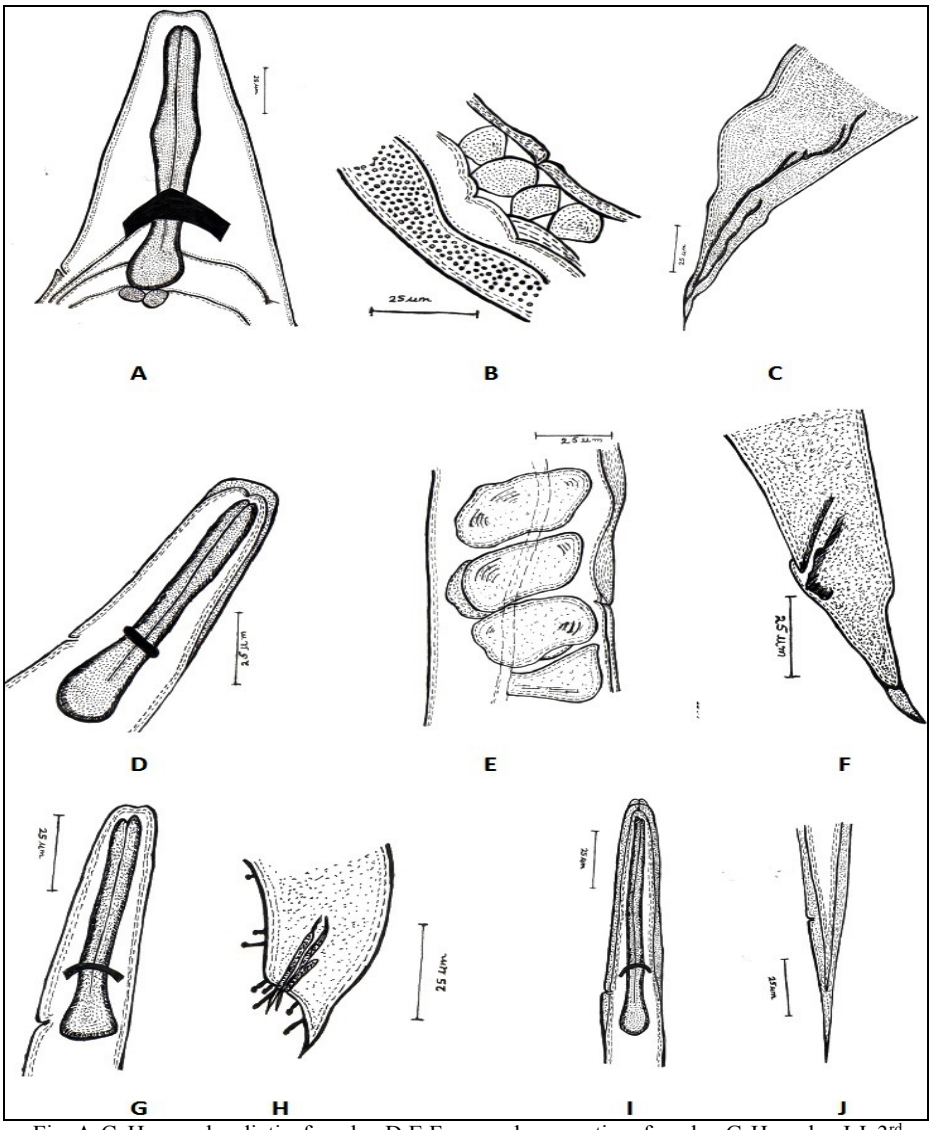


Fig A-C Hermophrodictic female; D,E,F second generation female; G-H male; I,J 3rd stage juvenile; Fig A,D,G,I anterior region; B,E middle region; C,F,H,J posterior region.

Fig 2: Camera lucida scatch of *Heterorabditis indica* isolate CH_{16}

Table 1: Character table of *Heterorhabditis indica* isolate CH_{16}

Character	Male	Hermaphrodite	Female	Infective juvenile
n	15	15	15	20
L	547.3± 10.3 (482.8-614.0)	3206 ± 148 (2621-5006)	943 ± 22 (817-1085)	520.8 ± 5.06 (468.3-550.6)
a	16.1 ± 0.4 (13.4-18.8)	16 ± 0.5 (14 - 21)	14 ± 0.3 (11 - 17)	22.1 ± 0.35 (18.1 - 24.6)
b	5.9 ± 0.1 (5.5-6.8)	18 ± 0.7 (15-25)	8.5 ± 0.2 (7.2-10.1)	4.4 ± 0.05 (3.9-4.8)
c	17.4 ± 0.6 (12.1-21.0)	37 ± 1.9 (27-49)	17 ± 1.0 (13-30)	5.4 ± 0.10 (4.5-6.5)
c'	1.4± 0.05 (1.1-1.6)	1.9 ± 0.1 (1.6-2.2)	3 ± 0.1 (2.4-3.5)	5.7 ± 0.17 (4.1-6.6)
V		198 ± 9.2 (157-310)	65± 2.2 (51-80)	
GBD	34.1± 0.4 (29.9-37.5)	200 ± 9.8 (152-318)	69 ± 2.9 (57-101)	23.7 ± 0.39 (22.1-30.1)
EP	92.3± 1.0 (85.7-97.8)	172 ± 4.8 (140-206)	114 ± 2.2 (103-129)	116.3 ± 1.18 (107.8-130.6)
NR	66.8± 0.96 (60.0-72)	127 ± 2.6 (106-149)	79 ± 1.3 (73-91)	93.5± 1.02 (85.9-103.5)
ES	92.8 ± 1.1 (85.5-100.3)	188 ± 3.1 (166-204)	112 ± 1.4 (102-121)	119.3 ± 1.13 (110-127)
TR	59.4 ± 1.4 (47-70.8)			
Tail Length with sheath	31.9 ± 1.1 (26.8-41.6)	88 ± 4.2 (64-117)	57 ± 1.9 (36-67)	96.9 ± 1.65 (80.3-109.6)
Tail Length without sheath				62.4 ± 1.36 (47.4-71.2)
ABD	22.6± 0.6 (18.2-26.4)	48 ± 2.2 (31-59)	20 ± 0.8 (13-26)	17.2 ± 0.80.56 (13.6-22.8)
SP	37.8 ± 0.8 (30.2-42.1)			
GL	21.0 ± 0.5 (18.8-24.4)			
D% = EP/ES x 100	99.7 ± 1.4 (90.9-108.1)	95 ± 2.3 (82-116)	102 ± 2.1 (94-123)	97.5 ± 0.80 (89.9-102.8)
E% = EP/T x100	293.5 ± 10.3 (221.3-363.3)	201 ± 11 (139-303)	203 ± 12 (164-342)	120.5 ±1.97 (20.9-140.0)
F%	108.1 ± 2.9 (90.1-123.8)	230 ± 10 (156-288)	125 ± 12 (164-342)	24.6 ± 0.63 (20.9-32.4)
V%		46 ± 0.4 (44-48)	52 ± 0.7 (44-56)	
SW% = SP/ABD x 100	168.6 ± 5.6 (138.3-203.5)			
GS% = GL/SP x 100	56 ± 1.9 (47-74.9)			

Table 2: Comparative table of 3rd stage juveniles of *Heterorhabditis* species

Characters	*H. taysearae*	*H. indica*	*H. baujardi*	*H. floridensis*	*H. mexicana*	*H. bacteriophora*	Isolate CH_{16}
n	30	25	25	25	25	15	20
L	418(332-499)	528(479-573)	551(497-595)	562(554-609)	578(530-620)	588(512-617)	520(468-550)
a	21(18-27)	26(25-27)	28(26-30)	27(25-32)	25(23.6-28.4)	25(17-30)	22(18 - 24)
b	3.8(3.4-4.2)	4.5(4.3-4.8)	4.8(4.5-5.1)	4.3(3.9-4.9)	4.6(4.2-5.1)	4.5(4.0-5.1)	4.4(3.9-4.8)
c	7.7(6.5-8.7)	5.3(4.5-5.6)	6(6-6.7)	5.6(5.3-6.6)	5.9(5.5-6.3)	6.2(5.5-7.0)	5.4(4.5-6.5)
GBW	20(17-23)	20(19-23)	20(18-22)	21(19-23)	23(20-24)	23(18-31)	23(22.1-30.1)
EP	90(74-113)	98(88-107)	97(91-103)	109(101-122)	102(83-109)	103(87-110)	116(107-130)
NR	64(58-87)	82(72-85)	81(75-86)	86(68-107)	81(74-88)	85(72-93)	93(85-103)
ES	110(96-130)	117(109-123)	115(107-120)	135(123-142)	122(104-142)	125(100-139)	119(110-127)
Tail with sheath	55(44-70)	101(93-109)	90(83-97)	103(91-113)	99(91-106)	98(83-112)	96(80-109)
D%	82(71-96)	84(79-90)	84(78-88)	81(71-90)	81(72-86)	84(76-92)	97(89-102)
E%	180(110-230)	94(83-103)	108(98-114)	105(95-134)	104(87-111)	112(103-130)	120(20-140)

Table 3. Comparative taxometry of males of *Heterorhabditis* species

Characters	*H. taysearae*	*H. indica*	*H. baujardi*	*H. floridensis*	*H. mexicana*	*H. bacteriophora*	Isolate CH_{16}
n	20	12	14	20	20	15	15
L	703(648-736)	721(573-788)	889(818-970)	862(785-924)	686(614-801)	820(780-960)	547(482-614)
GBW	43(38-48)	42(35-46)	49(45-53)	47(43-50)	42(38-47)	43(38-46)	34(29-37)
EP	95(78-120)	123(109-138)	81(71-93)	117(104-128)	124(108-145)	121(114-130)	92(85-97)
NR	65(54-88)	75(72-85)	65(54-77)	80(73-90)	71(61-83)	72(65-81)	66(60-72)
ES	112(85-1230	101(93-109)	116(105-132)	105(97-111)	96(89-108)	103(99-105)	92(85-100)
TR	122(100-146)	91(35-144)	91(28-38)	93(78-116)	96(65-130)	79(59-87)	59(47-70)
tail without sheath	25(20-29)	28(24-32)		34(29-40)	27(21-36)	28	
ABW	25(21-30)	23(19-24)	22(20-24)	26(20-31)	24(23-27)	23(22-25)	22(18-26)
SPL	39(30-42)	43(35-48)	40(33-45)	42(36-46)	41(30-47)	40(36-44)	37(30-42)
GL	18(14-21)	21(18-23)	20(18-22)	23(17-30)	23(18-32)	20(18-25)	21(18-24)
D%	88	121		112(105-119)	129(114-149)	117	99(90-108)
SW	156	187	182(138-208)	157(133-209)	167(130-196)	174	168(138-203)
GS	46	49	50(44-61)	53(47-65)	56(43-70)	50	56(47-74)

3.2. Molecular and Phylogenetic Analyses

For ITS regions, phylogenetic analysis showed that the alignment resulted in 1030 characters of which 258 were constant, 483 were parsimony uninformative and 289 were parsimony informative. Parsimony and distance based tree building approaches produced almost identical trees.

Maximum parsimony produced 2 parsimonious trees with a total length of 726 characters. A total of 665 characters were taken in final dataset. The values of consistency index, retention index and composite index were 0.737, 0.871, and 0.642 respectively. The phylogenetic relationships between 14 *Heterorhabditis* species are presented in Figure 3. Results derived through pairwise distance matrix indicate the minor variation from *H. indica* (0.002 values) (Fig. 6).

For the D2-D3 region, phylogenetic analysis presented the alignment, resulted in 1649 characters of which 167 were constant, 1164 were parsimony uninformative and 318 were parsimony informative. Maximum parsimony produced 5 parsimonious trees with a total length of 688 characters. A total of 576 characters were taken in final dataset. The values of consistency index, retention index and composite index were 0.825, 0.871, and 0.718 respectively. The phylogenetic relationships between 14 *Heterorhabditis* species are presented in Figure 4. Distance matrix revealed no difference between isolate CH_{16} and *H. indica* (Fig. 7).

For the COXI, phylogenetic analysis displayed that the alignment resulted in 940 characters of which 110 were constant, 608 were parsimony uninformative and 222 were parsimony-informative. Maximum parsimony produced 1 parsimonious tree with a total length of 546 characters. A total of 355 characters were taken in final dataset. The values of consistency index, retention index and composite index were 0.658, 0.553, and 0.364 respectively. The phylogenetic relationships between 14 *Heterorhabditis* species are presented in Figure 5. Distance matrix was varied when plotted and produced diversified results (Fig. 8).

Results obtained through the molecular phylogeny of sequences of ITS rDNA, and 28S rDNA placed the *Heterorhabditis* species isolate CH_{16} with *H. indica* and *H. noenieputensis* (Malan *et al.*, 2014). For ITS regions, it was near to *H. indica* with 88% bootstrap values. For 28S rDNA, the species was sharing a common clad with *H. indica*, a new isolate of *Heterorhabditis* sp. n (KR096494) and an isolate from Pakistan (KR269858) along with *H. noenieputensis*, whereas the COXI gene phylogeny showed the divergence of isolate from the other specified species. High difference were observed while plotting the distance matrix of COXI gene where it was showing almost difference pattern which indicate that the isolate CH_{16} supposed to be a different one.

The length of the ITS1 + 5.8S + ITS2 sequence is 740 bp and its molecular composition was: A=26.49% C=19.86%, G=24.86% T=28.78%. *Heterorhabditis* isolate CH_{16} was similar with *H. indica* as its length of the ITSI + 5.8S + ITS2 sequence is 740 bp molecular composition A =20.78% C =21.43%, G=28.43% T=28.22% and differ with other *Heterorhabditis* species length of the ITS1 + 5.8S + ITS2 sequences *H. amazonens* (762bp), H. *baujardi* (760bp), *H. floridens* (761bp) *H. Mexicana* (771bp), *H. bacteriophora* (771bp), *H. Georgiana* (715), *H. atacamensis* (715), *H. safricana* (744bp), *H. marelatus* (744bp), *H. zealandica* (753bp), *H. downesi* (740bp) and *H. megidis* (758bp). Minute differences were observed in the nucleotide composition where ITS2 regions of isolate CH_{16} was 1 nucleotide long from *H. indica* while the ITS1 and 5.8S were with similar nucleotide composition (Table 4).

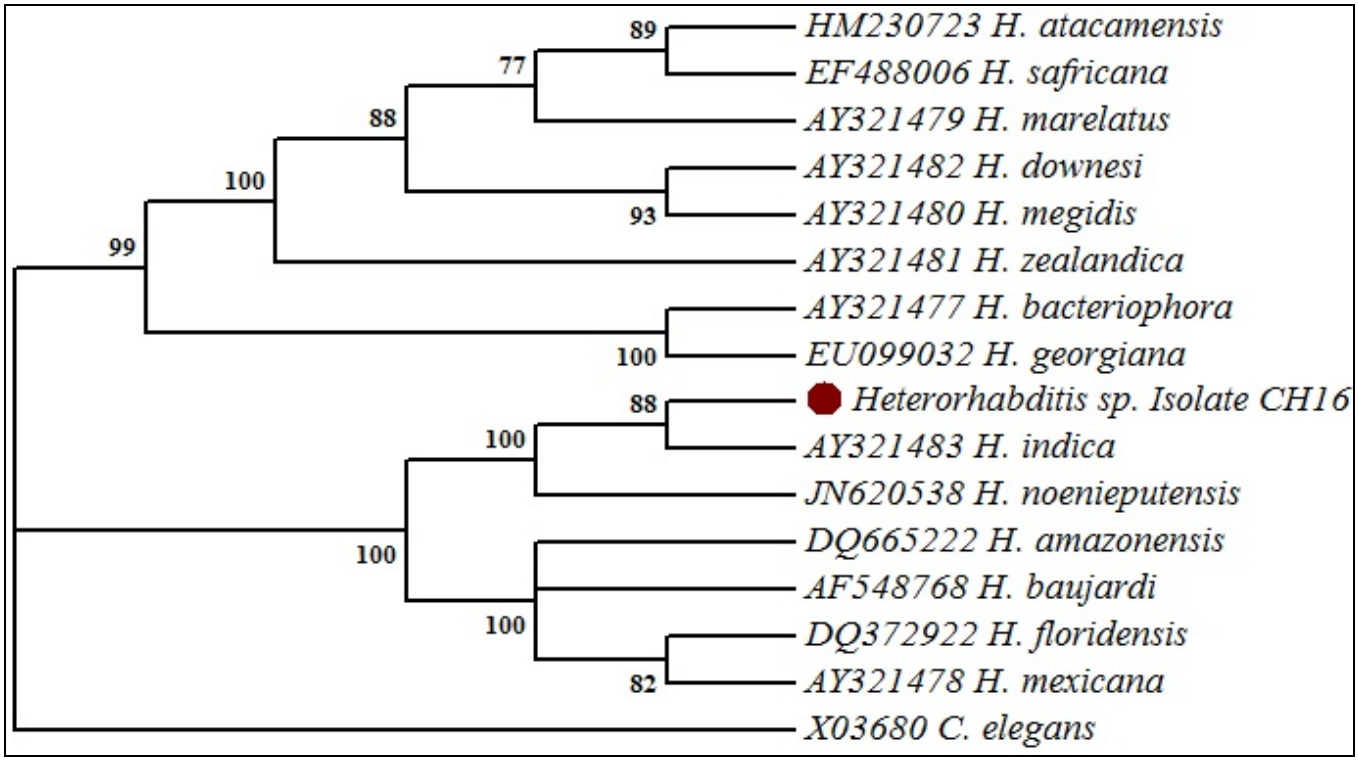


Fig 3: Phylogenetic relationships by the Maximum Parsimony method of isolate CH_{16} with 14 *Heterorhabditis* species based on ITS-rDNA sequences. *Caenorhabditis elegans* was used as out-group. The analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 665 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

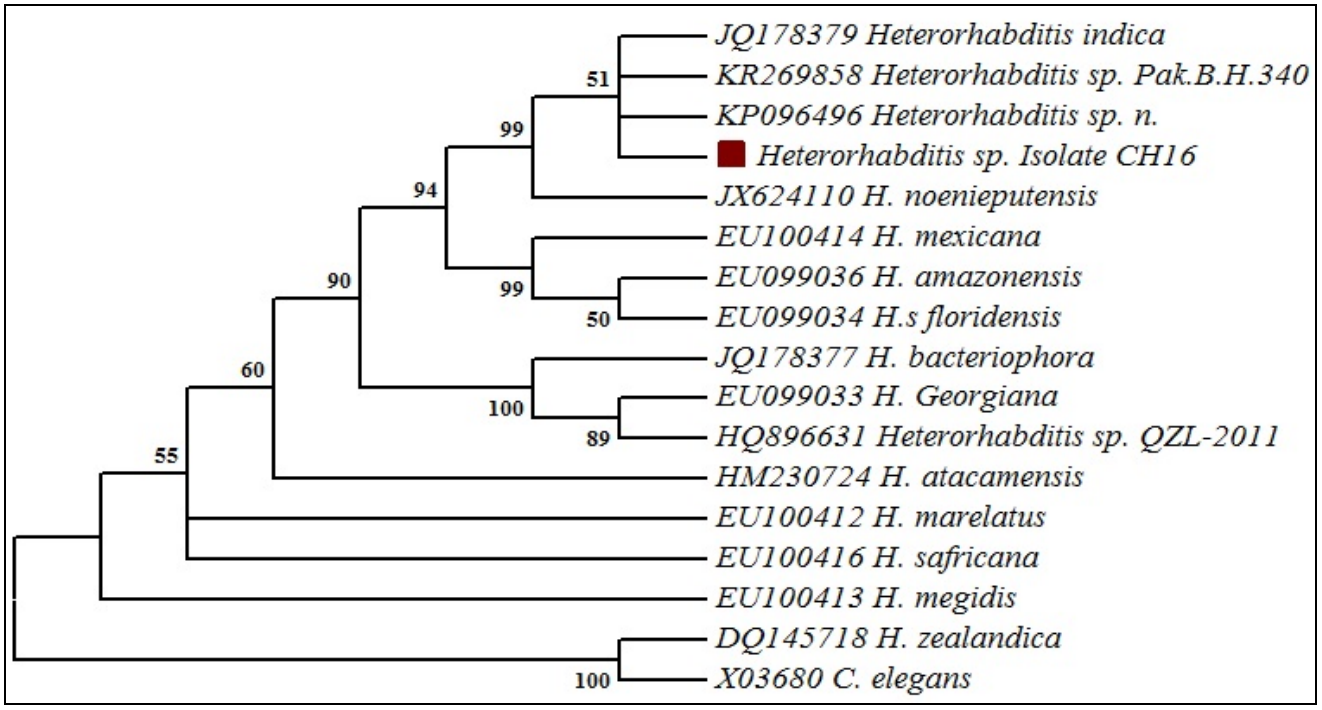


Fig 4: Phylogenetic relationships by the Maximum Parsimony method of isolate CH16 with 14 *Heterorhabditis* species based on D2D3sequences. The analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 576 positions in the final dataset. Evolutionary analyses were conducted in MEGA6

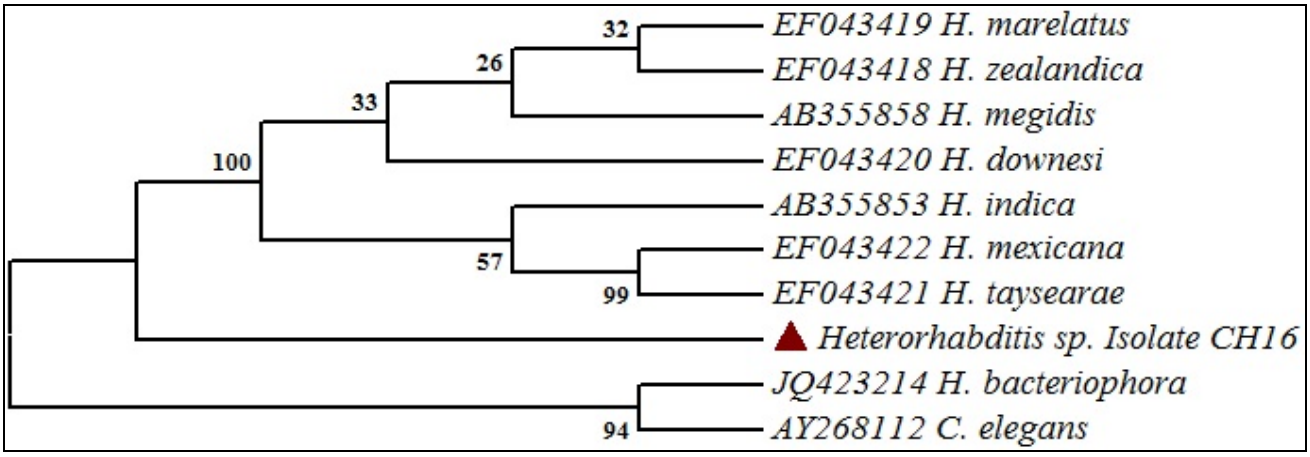


Fig 5: Phylogenetic relationships by the Maximum Parsimony method of isolate CH_{16} with 8 *Heterorhabditis* species based on COXI sequences. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 355 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Heterorhabditis sp. Isolate CH16		0.004	0.001	0.013	0.013	0.013	0.013	0.024	0.024	0.023	0.024	0.025	0.027	0.025	0.027	0.073
2. JN620538 H. noenieputensis	0.012		0.005	0.013	0.014	0.014	0.014	0.024	0.024	0.024	0.025	0.026	0.028	0.025	0.027	0.073
3. AY321483 H. indica	0.002	0.014		0.013	0.014	0.014	0.013	0.024	0.024	0.024	0.025	0.026	0.027	0.025	0.027	0.073
4. DQ665222 H. amazonensis	0.102	0.107	0.104		0.005	0.005	0.006	0.025	0.026	0.025	0.026	0.025	0.027	0.026	0.028	0.075
5. AF548768 H. baujardi	0.109	0.114	0.110	0.017		0.005	0.006	0.025	0.026	0.025	0.026	0.025	0.027	0.026	0.028	0.075
6. DQ372922 H. floridensis	0.114	0.116	0.116	0.020	0.020		0.005	0.026	0.026	0.026	0.027	0.026	0.028	0.027	0.028	0.077
7. AY321478 H. mexicana	0.114	0.119	0.116	0.026	0.031	0.018		0.026	0.027	0.026	0.027	0.026	0.028	0.026	0.028	0.076
8. AY321477 H. bacteriophora	0.275	0.283	0.277	0.304	0.306	0.319	0.320		0.006	0.019	0.020	0.020	0.023	0.020	0.022	0.084
9. EU099032 H. georgiana	0.277	0.286	0.280	0.307	0.310	0.323	0.325	0.025		0.020	0.020	0.020	0.024	0.020	0.022	0.083
10. HM230723 H. atacamensis	0.264	0.273	0.266	0.281	0.288	0.299	0.302	0.191	0.200		0.005	0.007	0.013	0.007	0.011	0.078
11. EF488006 H. safricana	0.280	0.289	0.282	0.297	0.303	0.315	0.319	0.202	0.209	0.020		0.007	0.013	0.008	0.010	0.079
12. AY321479 H. marelatus	0.288	0.297	0.290	0.295	0.300	0.313	0.317	0.196	0.205	0.031	0.032		0.012	0.008	0.011	0.080
13. AY321481 H. zealandica	0.329	0.339	0.332	0.336	0.342	0.353	0.354	0.258	0.268	0.113	0.118	0.109		0.013	0.016	0.087
14. AY321482 H. downesi	0.290	0.300	0.293	0.299	0.301	0.318	0.319	0.200	0.207	0.039	0.042	0.047	0.111		0.009	0.081
15. AY321480 H. megidis	0.312	0.321	0.314	0.313	0.316	0.326	0.330	0.224	0.233	0.072	0.074	0.077	0.141	0.055		0.083
16. X03680 C. elegans	0.860	0.864	0.866	0.897	0.901	0.912	0.905	0.964	0.955	0.905	0.921	0.913	0.957	0.924	0.956	

Fig 6: Distance matrix of ITS region of isolate CH_{16} compared with described *Heterorhabditis* species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. Heterorhabditis sp. Isolate CH16		0.000	0.008	0.008	0.002	0.008	0.010	0.011	0.011	0.010	0.011	0.249	0.011	0.008	0.011	0.006	0.144
2. JQ178379 Heterorhabditis indica	0.000		0.008	0.008	0.002	0.008	0.010	0.011	0.011	0.010	0.011	0.249	0.011	0.008	0.011	0.006	0.144
3. EU099036 H. amazonensis	0.037	0.037		0.003	0.007	0.003	0.011	0.011	0.010	0.010	0.011	0.240	0.011	0.011	0.011	0.010	0.144
4. EU100414 H. mexicana	0.037	0.037	0.007		0.007	0.003	0.011	0.011	0.010	0.010	0.011	0.241	0.012	0.011	0.011	0.010	0.150
5. JX624110 H. noenieputensis	0.002	0.002	0.036	0.036		0.008	0.010	0.011	0.011	0.010	0.012	0.249	0.012	0.008	0.011	0.006	0.144
6. EU099034 H.s floridensis	0.041	0.041	0.007	0.007	0.039		0.011	0.011	0.010	0.011	0.011	0.243	0.012	0.012	0.011	0.010	0.151
7. EU100416 H. safricana	0.063	0.063	0.065	0.067	0.064	0.071		0.005	0.010	0.004	0.010	0.225	0.006	0.013	0.010	0.012	0.147
8. EU100412 H. marelatus	0.071	0.071	0.065	0.067	0.071	0.071	0.012		0.010	0.004	0.010	0.226	0.006	0.014	0.009	0.013	0.148
9. JQ178377 H. bacteriophora	0.067	0.067	0.058	0.060	0.067	0.060	0.050	0.048		0.009	0.003	0.257	0.011	0.014	0.003	0.013	0.151
10. HM230724 H. atacamensis	0.065	0.065	0.060	0.062	0.065	0.065	0.010	0.009	0.039		0.009	0.226	0.005	0.013	0.009	0.012	0.147
11. EU099033 H. Georgiana	0.071	0.071	0.062	0.064	0.071	0.064	0.050	0.048	0.007	0.043		0.258	0.011	0.014	0.002	0.014	0.151
12. DQ145718 H. zealandica	1.469	1.469	1.452	1.452	1.455	1.468	1.383	1.391	1.514	1.394	1.529		0.226	0.281	0.258	0.252	0.201
13. EU100413 H. megidis	0.079	0.079	0.073	0.075	0.079	0.079	0.023	0.021	0.058	0.019	0.058	1.391		0.014	0.010	0.013	0.148
14. KP096496 Heterorhabditis sp. n.	0.038	0.038	0.075	0.075	0.040	0.079	0.101	0.109	0.105	0.103	0.109	1.551	0.117		0.014	0.009	0.157
15. HQ896631 Heterorhabditis sp. QZL-2011	0.069	0.069	0.062	0.064	0.069	0.064	0.050	0.048	0.005	0.041	0.003	1.514	0.058	0.107		0.013	0.151
16. KR269858 Heterorhabditis sp. Pak.B.H.340	0.021	0.021	0.060	0.060	0.023	0.064	0.087	0.095	0.091	0.089	0.095	1.518	0.103	0.055	0.093		0.154
17. X03680 C. elegans	1.131	1.131	1.135	1.159	1.140	1.165	1.137	1.131	1.168	1.140	1.152	1.331	1.149	1.192	1.162	1.165	

Fig 7: Distance matrix of 28S rDNA of isolate CH_{16} compared with described *Heterorhabditis* species.

	1	2	3	4	5	6	7	8	9	10
1. Heterorhabditis sp. Isolate CH16		0.184	0.158	0.167	0.155	0.158	0.154	0.167	0.164	0.355
2. JQ423214 H. bacteriophora	1.292		0.143	0.152	0.153	0.155	0.136	0.147	0.153	0.127
3. AB355858 H. megidis	1.023	0.997		0.023	0.022	0.023	0.015	0.018	0.017	0.116
4. AB355853 H. indica	1.110	1.061	0.149		0.022	0.023	0.024	0.025	0.025	0.143
5. EF043422 H. mexicana	1.028	1.089	0.139	0.152		0.010	0.022	0.022	0.022	0.129
6. EF043421 H. taysearae	1.060	1.140	0.149	0.170	0.037		0.022	0.023	0.022	0.132
7. EF043420 H. downesi	1.027	0.977	0.078	0.164	0.135	0.139		0.019	0.018	0.126
8. EF043419 H. marelatus	1.066	1.017	0.097	0.165	0.138	0.148	0.111		0.018	0.119
9. EF043418 H. zealandica	1.031	1.073	0.101	0.168	0.142	0.149	0.105	0.104		0.119
10. AY268112 C. elegans	1.877	0.969	0.880	0.990	0.940	0.968	0.915	0.873	0.892	

Fig 8: Distance matrix of COXI gene of isolate CH_{16} compared with described *Heterorhabditis* species.

Table 4: Comparative table of nucleotide composition with Heterorhabditis species with *H. indica* isolate CH_{16}

Species	Molecular markers								Length(bp)
	Accession No.	ITS1(bp)	5.8S(bp)	ITS2(bp)	A	C	G	T	
Isolate CH_{16}	KU187258	370	154	216	20.78	21.43	28.43	28.22	740
H. noenieputensis	JN620538	371	154	216	20.78	21.43	28.57	29.22	741
H. indica	AY321483	370	154	215	20.78	21.43	28.57	29.22	739
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

3.3. Virulence and Biotic Potential

Mortality bioassays experiment performed on G. *mellonella* larvae and mortality was recorded at 12 intervals till the 100% mortality achieved. Isolate CH_{16} showed greater pathogenicity

and killed all the larvae within 60 hours post infection period respectively with all concentrations (Fig. 9). After 12 hrs post infection period, no mortality was recorded with any concentration. Mortality was initiated after 24 hrs post infection period with all concentrations *viz*. 25, 50 100 and 200 IJs/ larva with 20, 50, 70, and 80 percent mortality. After 36 hours post infection period the mortality was increased and observed as 60, 70, 90 and 90 percent respectively in all the four concentrations applied. Mortality recorded after 48 hours was 80, 80, 90 and 100 percent in all the concentrations, respectively. 100 percent mortality was achieved after 60 hours. Control group showed no mortality during the experimental duration and even after the larvae pupate and emerged in adult stages. All the cadavers of larvae were transferred on white trap to observe the emergence of IJs from them. Emergence of IJs from all the cadavers proved the death of all the insect larvae were due to nematode infection.

LC_{50} and LC_{90} value was also calculated to judge the pathogenicity of isolate by probit analysis (Table 5) at three different post infection periods *viz.* 24, 36 and 48 hours and presented in IJs/ Larva. Lowest LC_{50} value was reported after 48 hours which was only 8 IJs/Larva whereas it was 59 IJs/Larva at 24 hours post infection period. LT_{50} and LC90 values were also figured out at different concentrations viz. 25, 50, 100 and 200 IJ/larva and for mean doses too through same analysis and presented in hour in Table 6. The lowest time (LT_{50}) was taken with high dose which was only 21 hours followed by 22.6 hours, 27.6 hours and 33 hours respectively in 100, 50 and 25 IJs/Larva. The mean LT_{50} was calculated for all the doses applied was 25.7 hours.

Based on Caroli *et al.* [27], penetration rates are different between EPN species and are influenced by different hosts and substrates. This difference in the percentage of mortality may be determined by the capacity of the IJs entering the strains/species studied to which the hosts are exposed, the host species, and the ratio of the number of IJs present in the substrate. Besides the aforementioned, the competition by the IJs to enter, the immune and behavioral response of the host are other characteristics that may probably influence on the variability in the percentage of mortality in the assays of high dosages (100-200 IJs) and low dosages (5, 10, 15, and 50 IJs) [28, 29]. Similar results were obtained from the study of present isolates where increment in the mortality was increased with penetration rate of IJs.

Reproductive potential at different concentration of *H. indica* isolate CH_{16} was calculated individually for all the four doses applied and for mean IJs production (Fig. 10). The maximum IJs production was reported in 100 IJs/Larva dose where it was calculated as $2.15\pm0.24\times10^5$ (112800-365600) and minimum with 50 IJs/Larva dose with $1.5\pm0.15\times10^5$ (96000-224000). In 25 IJs/larva and 200 IJs/ Larva doses the IJs production was $1.7\pm0.16\times10^5$ IJs/ Larva (120000-262400) and $1.83\pm0.08\times10^5$ IJs/ Larva (165600-247200) respectively. Significance value of 0.063 obtained through one way ANOVA indicate no relationship between IJ production and dose applied.

The hermophrodatism may be a factor of the the high reproductive properties as was noted for *H. bacteriophora* [30, 31]. Another reason for higher reproduction rate for *H. bacteriophora* was observed by Loya & Hower [32] where the host larvae size and behaviour of EPN species also suggested as the factors which affect the progeny production rate. Boff *et al.* [33] studied on a reproduction examination of *H. megidis* (strain NLH-E87.3) at following doses: 10, 30, 100, 300, 1000 and 3000 IJs. Results indicated that total production of the species nearly 28000 and 30000 IJs per larva at the doses of 10 and 100 IJs, respectively. Hussaini *et al.* [34] reported progeny production of *H. indica* IJs from infected *C. partellus* to be 77954 IJs per larva. Gupta *et al.* [35] (2008) who reported gradual increase in progeny production up to 160 IJs of *Steinernema carpocapsae* per larva of *Pieris brassicae*, after which a sudden decrease from $2.20x10^5$ $larva^{-1}$ to $1.70x10^5$ was noticed. However, in the present study, the highest reproduction was observed around 8 fold in 100 IJs/Larva dose with 2.15 lacs IJs/Larva production as compared to other researchers with different/ same species in different/ same insect host. Moreover, the present result was accordance with Selvan *et al.* [36] (1993) who observing that the production of IJs of *H. bacteriophora* increased with increasing the initial density up to approximately 100 IJs/larva.

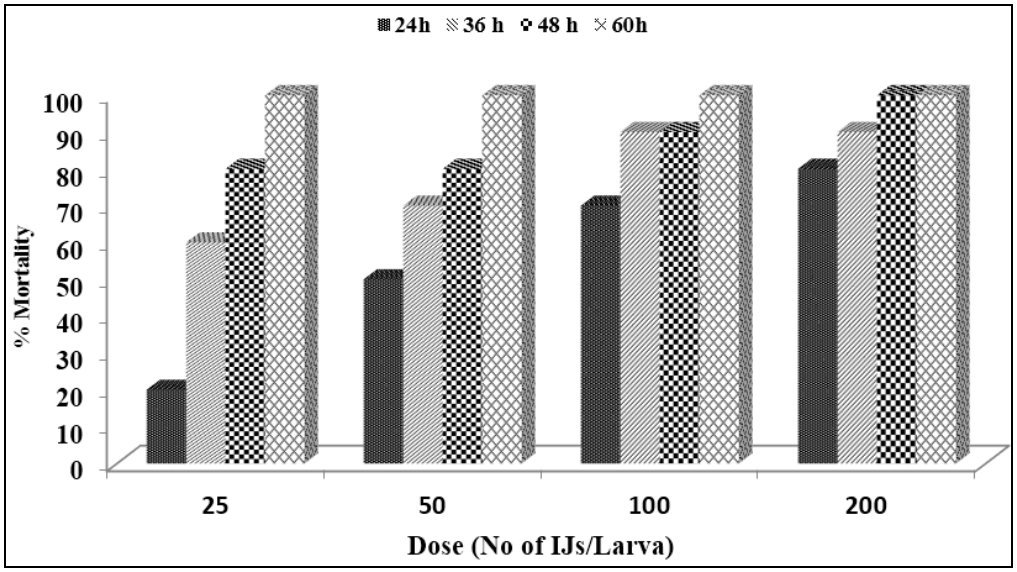


Fig 9: Percentage mortality of *G. mellonella* larvae treated with *Heterorhabditis indica* isolate CH_{16}

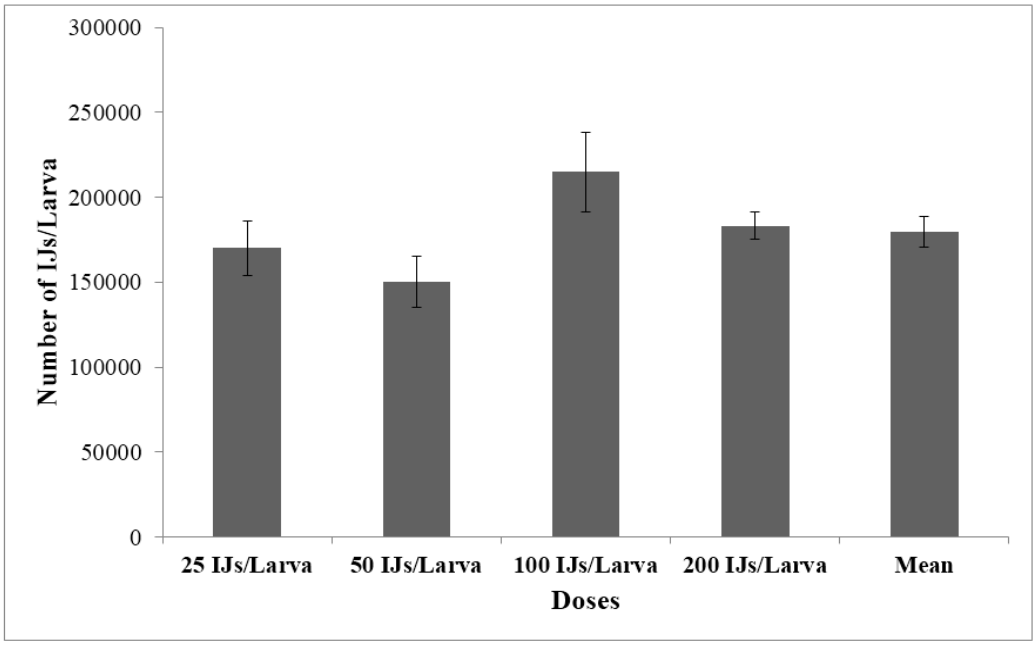


Fig 10: Graphical presentation of progeny production of *Heterorhabditis indica* isolate CH_{16} in *Galleria mellonella* larvae at different doses of infective juveniles

Table 5: LC_{50} and LC_{90} values of *Heterorhabditis indica* isolate CH_{16} against larvae of *Galleria mellonella* calculated at different time intervals.

Time (in hours)	LC_{50}	LC_{90}	Chi square	Df	Sig.
24h	59	290	0.379	2	0.827
36h	16	158	0.35	2	0.839
48h	8	73	0.848	2	0.654

Table 6: LT_{50} and LT_{90} values of *Heterorhabditis indica* isolate CH_{16} against larvae of *Galleria mellonella* calculated at different doses.

Dose (IJs/Larva)	LT_{50}	LT_{90}	chi square	df	Sig.
25	32.9	51.	0.833	3	0.849
50	27.6	49.	2.276	3	0.517
100	22.6	37.6	3.288	3	0.349
200	21	30.9	2.246	3	0.523
Combined	25.7	43.6	6.279	3	0.081

4. Conclusion

With no doubt, the applicability of traditional data is an important requisite to compare the species but the applications of multisequece analysis provide more reliable and accurate data for the diagnostic of *Heterorhabditis* species. Internal transcribes spacers are useful to resolve the difference among the related *Heterorhabditis* species and the joining region of 5.8S gene is too preserved to resolve all Heterorhabditid connections. In this context addition of 28S gene data provide a clearer picture in resolving the difference among the species. Use of COXI gene is not suitable for EPN taxonomy as contrary results obtained. Moreover, pathogenicity with high reproductivity against the host insect *G. mellonella* in this study also provide the quality information for *H. indica* isolate CH_{16} being as good candidate for biological control in future program.

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