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ORIGINAL PAPER

Molecular and Phenotypic Characterization of *Heterorhabditis indica* (Nematoda: Rhabditida) Nematodes Isolated During a Survey of Agricultural Soils in Western Uttar Pradesh, India

Aashaq Hussain Bhat[1,4] · Ashok Kumar Chaubey[1] · Ebrahim Shokoohi[2] · Ricardo A. R. Machado[3]

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Abstract

Introduction Entomopathogenic nematodes (EPNs) are important biocontrol agents of insect pests. To increase the availability of locally adapted entomopathogenic nematode isolates for biocontrol programs, a survey of several agricultural soils in Western Uttar Pradesh, India was conducted.

Materials and methods Eight hundred and sixty soil samples from the districts Meerut, Bulandshahr, Baghpat, and Bijnor were collected and examined for the presence of entomopathogenic nematodos using the "*Galleria* baiting method". *Steinernema* and *Heterorhabditis* nematodes were recovered. The isolated *Heterorhabditis* nematodes were molecularly, and morphologically characterized, and their biocontrol potential was evaluated against *Spodoptera litura*. Finally, the geographical distribution of entomopathogenic nematodes was studied based on the analysis of ITS GenBank records.

Results A small proportion of the collected soil samples were positive for *Heterorhabditis* and *Steinernema* nematodes. Twelve soil samples were positive for the presence of *Heterorhabditis* nematodes, and 29 samples were positive for *Steinernema*. The *Heterorhabditis* nematodes were identified as *Heterorhabditis indica* based on morphological, morphometrical and molecular analyses. No other species of *Heterorhabditis* were isolated from the soil samples analyzed, suggesting that this species is dominant in the western part of Uttar Pradesh, India. The morphology of the nematode isolates was somewhat similar to the morphology of the *H. indica* isolate used for the original description of this species, with a notable exception mucrons were present in the hermaphrodite and female specimens we collected, but this structure was not observed in the specimens used for the original description of the species. Principal component analyses (PCA) show small inter- and intraspecific morphological variability between the nematodes species of the "*Indica*" clade. The insecticide properties of one isolate, CH7, were evaluated against *Spodoptera litura*, and the results show that this isolate effectively killed this pest under laboratory conditions, demonstrating its potential as a biocontrol agent.

Conclusion This study sets the basis for establishing new biocontrol agents to be used in future pest management programs in India.

Keywords Biological control · Crop pests · Entomopathogenic nematode isolation · Entomopathogenic nematode morphology

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✉ Aashaq Hussain Bhat
aashiqhussainbhat10@gmail.com

[1] Nematology Laboratory, Department of Zoology, Chaudhary Charan Singh University, Meerut 250004, India

[2] Green Biotechnologies Research Centre of Excellence, University of Limpopo, Private Bag X1106, Sovenga 0727, Republic of South Africa

[3] Experimental Biology Research Group, Institute of Biology, University of Neuchatel, 2000 Neuchatel, Switzerland

[4] Department of Zoology, Government Degree College Billawar-184204, University of Jammu, Billawar, Jammu, Jammu and Kashmir, India

Introduction

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae are lethal parasites of insects [33]. Nematode species from these two families are of great interest to the scientific community, because of their biocontrol attributes [32, 33, 42]. The only free-living and infective stage is the third-stage juvenile, which is associated with symbiotic bacteria, that are found throughout the alimentary canal of *Heterorhabditis* species [30] but compartmentalized in specialized structures in *Steinernema* species [17, 52]. Different formulations of these nematodes are used for safeguarding crops and forests from insect attack [75], and are currently important bio-pesticides in integrated pest mangament (IPM) programs. To maximize their biocontrol potential, the use of locally adapted isolates is though to be more suitable, as local nematodes might exhibit better performance under particular abiotic and biotic conditions than alien nematode isolates [14, 15].

The genus *Heterorhabditis* is less speciose than *Steinernema*, with only 16 species of the former and 100 species of the latter that have been identified and described [9, 38]. The genus *Heterorhabditis* is "circumtropical", or "widely distributed in equatorial and subequatorial areas". India is a mega-diverse country and has diverse niches and habitats due to its varied climatic zones and different edaphic conditions, but information on the influence of these factors on EPNs and related diversity is limited. Only three *Heterorhabditis* species and 17 *Steinernema* species have been isolated and reported from Indian soils [13, 14].

Spodoptera litura (Lepidoptera: Noctuidae) (Fabricius 1775) causes great losses to many economically important crops [15, 22, 64]. This destructive pest is widespread in almost all Indian states and has frequently been reported to cause widespread damage to soybean (*Glycine max* L.) crops (26–29%) and groundnut (*Arachis hypogaea* L.) (27.3%) at several localities in India [23, 26, 27]. Recent outbreaks of *S. litura* on soybean in Kota (Rajasthan state), and Marathwada and Vidarbha (Maharashtra state) regions of India have been reported to cause monetary losses of USD 45 million and USD 225 million, respectively [26]. To control this pest, various chemical pesticides are frequently used, but this insect species has evolved resistance to many chemical insecticides particularly pyrethroids and carbamates [5, 39, 43] and has low susceptibility to transgenic Bt cotton [87], increasing its pest significance due to the difficulty to control it. Therefore, control of this and other harmful insects using effective indigenous biocontrol agents such as entomopathogenic nematodes is a promising alternative. In this study, local entomopathogenic *Heterorhabditis* nematodes were isolated, identified and their biocontrol potential evaluated. The aims of the study were: (1) to isolate *Heterorhabditis* spp. from agricultural soils of Meerut, India; (2) to identify the isolated nematodes using morphological and molecular techniques; (3) to investigate morphological variations among *Heterorhabditis* from agricultural soils of Meerut, India using principal component analysis (PCA); and (4) to investigate the biocontrol potential of some of the isolated *Heterorhabditis* spp.

Materials and Methods

Nematode sampling and trapping

A total of 860 soil samples were collected from agricultural fields of Western Uttar Pradesh, India. Samples were collected from the district Meerut (28° 59′ N, 77° 42′ E, 225 m above sea level (m.a.s.l.), 397 samples), Bulandshahr (28° 41′ N, 77° 85′ E, 209 m.a.s.l., 197 samples), Baghpat (28° 94′ N and 77° 23′ E and 223 m.a.s.l., 164 samples) and Bijnor (29° 37′ N and 78° 38′ E and 237 m.a.s.l., 102 samples). Each sample contained 1 kg of soil, which was a mixture of five soil subsamples collected at five locations within each agricultural field (one sample from each corner of the field, and one from the center of the field). Samples were collected at 15–20 cm depth. Samples were analyzed to determine the presence of EPNs by the soil baiting technique [8]. Ten 3rd instart *Galleria mellonella* (Lepidoptera: Pyralidae) larvae were buried in 250-ml plastic containers containing 250 g of fine soil, covered with muslin cloth and stored in an incubator at 28 ± 2 °C for 7 days. Containers were inspected daily to recover nematode infested insect cadavers, rinsed with distilled water, disinfected with 0.1% sodium hypochlorite (NaOCL) solution and transferred to modified White traps [88] to obtain emerging infective juveniles (IJs). White traps were incubated in an incubator at 28 ± 2 °C and checked daily for the emergence of IJs from the cadavers. Emergence started after 5–7 days and the emerged IJs migrate to water surrounding the petri-dish. Nematode were collected regularly until nematode emergence ceased after 10–20 days [40].

Morphology and morphometry

Infective juveniles (IJs) were surface sterilized with a 1% NaOCl solution. Fifteen *Galleria mellonella* larvae were infected with 100 IJs each in sterile Petri dishes. To recover first- and second-generation adults, larvae were dissected 3–4 days or 5–7 days after infection, respectively; while IJs were recovered from White traps as

described above [88]. The different nematode generations were killed in hot water, fixed in TAF (7-ml formalin, 2-ml triethanolamine, 91-ml distilled water) [25], dehydrated using the Seinhorst method and mounted in a small drop of glycerin [70, 82]. Nematode morphological features were observed using a light compound microscope (Magnus MLX) and a phase-contrast microscope (Nikon Eclipse 50i). Twenty adults of each generation and 20 IJs were analyzed. The measurements were carried out with the help of the inbuilt software of Nikon Eclipse 50i (Nikon DS-L1).

Various morphometric traits obtained from fixed nematodes, including body length, *a*, *b*, *c*, excretory pore, nerve ring to anterior end, pharynx length, tail length, anal body diameter, spicule length, gubernaculum length, D%, SW%, GS% and greatest body diameter, were used for PCA analysis of the IJs and adult generations (Table 2). The characters used for male-based PCAs were: L, *a*, *b*, *c*, mid-body diameter, excretory pore to anterior end (EP), nerve ring to anterior end (NR), pharynx length (PS), tail length (T), anal body diameter (ABD), D%, spicule length (SL), gubernaculum length (GL), SW% and GS%. The characters for the female-based PCAs were: L, *a*, *b*, *c*, V%, mid-body diameter, excretory pore to anterior end (EP), nerve ring to anterior end (NR), pharynx length (PS), tail length (T), anal body diameter (ABD), D% and E%. The characters for the IJ-based PCAs were: L, *a*, *b*, *c*, mid-body diameter, excretory pore to anterior end (EP), nerve ring to anterior end (NR), pharynx length (PS), tail length (T), anal body diameter (ABD), D% and E%.

To evaluate the morphological variations between the nematodes isolated in this study and nematodes of other closely related species, a principal component analysis (PCA) with different morphological traits was conducted. PCA analysis was carried out in XLSTAT [4]. Values are shown as mean ± SD. The morphometric measurements of original populations of species of the *Indica* clade [80] were taken from their original descriptions. The measures were normalized through XLSTA software prior to their analysis [4]. The scores values were determined for each isolate based on each of the principal components, and the scores for the first two components were used to form a two-dimensional plot (PC1 and PC2) of each isolate based on eigenvalues given by the software XLSTAT.

Molecular identification

The genomic DNA was extracted from infective juveniles using DNeasy Blood and Tissue Kit (Germany) following manufacture's indications with some modifications. Internal transcribed spacer (ITS) regions of rDNA were amplified using primers 18S: 5′-TTGATTACGTCCCTGCCCTTT-3′ (forward) and 28S: 5′-TTTCACTCGCCGTTACTAAGG-3′ (reverse) [86] and partial sequence of 28S gene, D2–D3 domains were amplified using primers D2F: 5′-CCTTAG TAACGGCGAGTGAAA-3′ (forward) and 536: 5′-CAG CTATCCTGAGGAAAC-3′ (reverse) [54]. The PCR master mix consisted of nuclease-free dH2O 16.8 µl, 10 × PCR buffer 2.5 µl, dNTP mix (10 mM each) 0.5 µl, 1 µl of each forward and reverse primers, dream taq DNA polymerase 0.2 µl, and 3 µl of DNA extract. The PCR profiles used was: 1 cycle of 94 °C for 3 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s for ITS rDNA or 52 °C for 30 s for 28S rDNA, 72 °C for 60 s, and a final extension at 72 °C for 10 min [65, 10]. The ITS and D2D3 rDNA sequences were sequenced and finally deposited in the NCBI databank (Table 1). The phylogenetic trees based on the ITS and 28S rRNA gene sequences were obtained by the minimum evolution method [67] in MEGA 7.0 [44]. *Caenorhabditis elegans* was chosen as out-group taxa and to root the trees.

Table 1 List of *Heterorhabditis indica* isolates recovered from different agricultural fields, their NCBI accession numbers, locality and pH of soil where they were isolated

Isolate	Accession No		Field	GPS elevation (m.a.s.l.)	pH
	ITS	D2D3			
CH7	MF973067	–	Pepper	28° 40′ N, 77° 86′ E, 209	8.7
CH8	MH191356	–	Mango	28° 40′ N, 77° 86′ E, 209	6.9
CH9	MH191357	–	Wheat	28°40′N, 77°86′E, 209	8.5
CH10	MH191358	–	Jowar	28°40′N, 77°86′E, 209	8.8
CH11	MH191359	–	Sugarcane	28°40′N, 77°86′E, 209	8.6
CH12	MH191360	–	Mango	29°29′N, 78°57′E, 115	8.3
CH13	MH203006	–	Potato	29°29′N, 78°57′E, 115	7.7
CH14	MH203007	–	Maize	28°40′N, 77°86′E, 209	8.3
CH15	MH203008	–	Open field	29°29′N, 78°57′E, 115	7.8
CH17	MH203009	MH608352	Hemp	29°29′N, 78°57′E, 115	6.9
CH19	MH203010	MH608351	Wheat	28°98′N, 77°7′E, 225	8.6
CH20	MH203011	MH605521	Cabbage	28°98′N, 77°71′E, 225	8.7

Isolation and molecular characterization of entomopathogenic bacteria

The symbiotic bacteria associated with *Heterorhabditis indica* CH7 was obtained by crushing 500 surface-sterilized IJs in 1-ml PBS buffer (8-g NaCl, 0.2-g KCl, 1.15-g Na_2HPO_4, 0.2-g KH_2PO_4). 100 µl of the resulting suspension was spread on nutrient agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride and 0.0025% (w/v) bromothymol blue (NBTA medium) and left overnight at 28 °C [6]. Single colonies were transferred with a sterile toothpick to Luria broth [6] and cultivated in liquid media with an orbital shake (180 rpm) at 27 °C. Bacterial DNA was extracted from a 2-day-old culture using DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. 16S rRNA gene was amplified using primers 10F: 5′-AGTTTGATCATGGCTCAG ATTG-3′ (forward) and 1507R: 5′-TACCTTGTTACGACT TCACCCCAG-3′ (reverse) [68]. The PCR master mix consisted of nuclease-free H_2O 16.8 µl, bovine serum albumin 1 µl, 10 × dream Taq buffer 2.5 µl, dNTPs mix (10 mM) 0.5 µl, 0.75 µl of each forward and reverse primers, dream Taq DNA polymerase 0.2 µl and 2 µl of DNA [11]. The PCR profile was: one cycle at 94 °C for 3 min followed by 33 cycles at 94 °C for 60 s, 55 °C for 60 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min [12]. All PCR products were sequenced and deposited in the GenBank under the MK559716 accession number. Bacteria 16S rRNA gene sequences were aligned with sequences of other *Photorhabdus* species [49, 50] using default Clustal W parameters in MEGA 7.0 [44]. The evolutionary history was inferred using the Maximum Likelihood method based on the Hasegawa–Kishino–Yano model [36]. The tree with the highest log likelihood (−3288.79 is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4633. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 81.52% sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7 [44].

Phenotypic and biochemical characterization of symbiotic bacteria

Phenotypic variations were observed in symbiotic bacteria on the basis of adsorption properties towards bromothymol blue (BTB) and neutral red. The adsorption of BTB was examined on NBTA agar [6] and neutral red adsorption on MacConkey agar and were incubated for 24–48 h at 28 °C. The biochemical characterization was examined using a KB003 Hi25 Enterobacteriaceae Identification Kit from Hi-media (Mumbia, India), designed for the identification of Gram-negative Enterobacteriaceae species. A total of 13 conventional biochemical tests and 11 carbohydrate utilization tests were performed using this kit. For biochemical characterization, bacteria were cultured on NBTA media and blue–green colonies were transferred into 5-ml heart infusion broth (Hi-media). The culture was grown overnight and 50-µl aliquots were then inoculated into each of the 24 wells of the kit. The kit was incubated according to the manufacturer's instructions and changes in the color of media were recorded as positive or negative reactions as indicated by the manufacturer.

Geographical distribution

The ITS sequence was selected for the analysis, as it enables a clear distinction of the species in heterorhabditids, unlike another frequently sequenced markers as the D2D3 region of the 28S rDNA. To find *H. indica* sequences, the BLAST search was performed with the sequence of the type isolate (AY321483) as a query. The sequences that showed 97% or higher similarity scores were downloaded and their taxonomic identity was confirmed by phylogenetic analysis. The information about the site of isolation, if available, were obtained from the NCBI GenBank database, or related publications.

Virulence and reproduction on *Spodoptera litura*

The virulence of *Heterorhabditis* isolate CH7 was evaluated on fourth instar *S. litura* larvae. *Spotoptera litura* were originally purchased from ICAR- National Bureau of Agriculturally Important Insects (NBAII), Bangalore (National accession no. NBAII-MP-NOC-02) in March, 2018 and were artificially reared in the laboratory on castor leaves (*Ricinus communis*). Larvae of similar size and weight were used.

Infectivity experiments were carried in six-well plates (Tarson, India) (well size 3.5 cm). Each well was lined with a double-layered Whatman filter paper no. 1. One-week-old IJs were used in all experiments [15]. Four concentrations: 25, 50, 100 and 200 IJs were suspended in 450-µl distilled water and inoculated onto the filter paper. Controls received water only. Ten, fourth instar larvae of similar size and similar weight for each nematode concentration were used ($n = 10$). Experiments were repeated twice. Plates were incubated at 28 ± 2 °C and larval mortality was recorded every 12 h until all insects died. Ten larvae infected with

25, 50, 100 and 200 IJs/larva were transferred after seven days to modified White traps [88] to observe the persistence of infection and emergence of IJs (18–20 days). Larval mortality assay was analyzed statistically through probit analysis using SPSS software and LC_{50} values were calculated at a 95% confidence limit. Differences between percent mortalities, depending on the isolates, were assessed further using analysis of variance. Data were presented as percentage ± SD. The total number of IJs/larva of the studied nematodes was modeled by a quadratic regression and 95% confidence intervals were calculated in SigmaPlot 14.0.

Results and Discussion

In this study, a total of eight hundred and sixty soil samples from several districts of the western Uttar Pradesh (India) were collected and examined for the presence of entomopathogenic nematodes. A total of 41 nematode isolates were recovered from those soil samples: 29 *Steinernema* spp. and 12 *Heterorhabditis* spp. Here, the molecular and morphological characterization of the *Heterorhabditis* isolates is reported (Table 1). The characterization of the *Steinernema* isolates is reported somewhere else [13, 14, 15, 16]. The pH of the soil where nematodes were isolated ranged from 5.8 to 9.6., and were mainly sandy loam and alluvial and the climate in these areas is mainly warm and temperate to humid subtropical with dry winters. Mounted slides and live specimens were deposited in the Nematology Laboratory of Department of Zoology, Chaudhary Charan Singh University, Meerut, India. Currently, only isolate CH7 is available as living specimens, all others were unfortunately lost.

Morphology and morphometry

The twelve *Heterorhabditis* isolates obtained during the present survey of agricultural soils were identified as *H. indica*. The morphology of the specimens isolated showed high resemblance with the specimens used for the original description of the species. Notably, the presence of mucrons in the hermaphrodite and amphimictic female specimens of this study (Fig. 1a and b) was observed, which was not the case in the adults used for the original description of the species. This mucron was, however, observed in synonymised species, such as *Heterorhabditis pakistanense* (syn. *H. indica*, Hunt and Subbotin [38]). The anal swelling of nematodes isolated during this study was very prominent in both hermaphroditic and amphimictic females (Fig. 1a and b); while in the specimens used for the original species descriptions, it was more prominent in hermaphroditic females than in amphimictic females. The rest of the morphological features were very similar between the nematodes isolated in this study and the nematodes used for the original description of the species. The morphometrical measurements of all the generations of the present heterorhabditid isolates were similar to the original population of *H. indica* [63], but some variations were observed when they were compared with each other or with the original description. A comparison in morphometric parameters in all generations is shown in Table 2.

PCA analysis

PCA results show morphometric variation between the hermaphroditic females, males and IJs of the twelve *H. indica* nematode isolates from this study, and the different developmental stages of nematodes that belong to the original

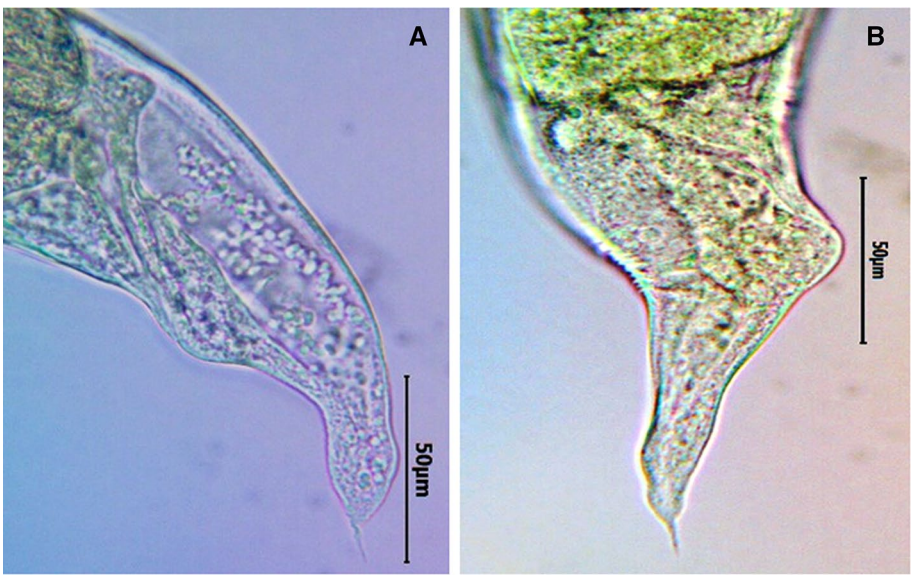

Fig. 1 Light microscopy of *Heterorhabditis indica* CH7. A and B: Tail features of first- and second-generation female, respectively, with anal swelling and mucron

Table 2 Compendium of infective juveniles and adult generations of CH7-CH15, CH17, CH19 and CH20 isolates with original *Heterorhabditis indica* populations. All measurements are in µm (except ratio and percentage) and in the form of mean ± standard deviation (range)

IJs	L	MBD	EP	NR	PS	T	*a*	*b*	*c*	D%	E%
H. indica CH7	565 ± 28 (516–598)	22 ± 4.5 (21–25)	105 ± 6.0 (98–123)	91 ± 4.6 (82–101)	117 ± 5.0 (102–129)	100 ± 7.2 (80–112)	25 ± 4.1 (24–27)	4.8 ± 0.2 (4.5–5.4)	5.7 ± 0.5 (4.9–7.5)	90 ± 4.8 (83–97)	105 ± 9.1 (93–136)
H. indica CH8	533 ± 18 (507–533)	22 ± 4.6 (20–24)	108 ± 5.8 (103–113)	89 ± 4.3 (84–93)	116 ± 3.5 (107–120)	101 ± 7.2 (81–111)	25 ± 4.1 (22–27)	4.6 ± 0.2 (4.3–5.0)	5.3 ± 0.4 (4.7–6.6)	93 ± 4.1 (88–99)	107 ± 8.8 (94–134)
H. indica CH9	532 ± 24 (480–579)	22 ± 4.9 (20–23)	103 ± 5.7 (92–109)	86 ± 4.5 (78–93)	110 ± 5.0 (94–118)	100 ± 5.1 (92–111)	25 ± 4.2 (23–27)	4.8 ± 0.3 (4.4–5.3)	5.3 ± 0.3 (4.7–6.0)	94 ± 4.1 (87–99)	103 ± 5.8 (95–113)
H. indica CH10	502 ± 18 (470–515)	21 ± 4.7 (20–22)	103 ± 5.7 (97–108)	93 ± 4.6 (57–85)	104 ± 3.4 (86–119)	95 ± 5.8 (83–105)	24 ± 3.6 (23–25)	4.9 ± 0.5 (4.2–5.8)	5.3 ± 0.3 (4.8–5.9)	100 ± 6.8 (91–114)	108 ± 8.0 (95–126)
H. indica CH11	521 ± 24 (482–551)	21 ± 4.0 (20–25)	102 ± 5.1 (93–111)	89 ± 4.0 (81–95)	111 ± 3.2 (107–117)	96 ± 4.9 (89–105)	24 ± 3.1 (21–26)	4.7 ± 0.1 (4.4–5.0)	5.4 ± 0.3 (4.9–5.9)	91 ± 4.3 (86–96)	106 ± 6.6 (92–117)
H. indica CH12	530 ± 22 (511–562)	21 ± 4.9 (19–23)	100 ± 4.9 (96–109)	86 ± 3.8 (81–95)	109 ± 3.2 (104–117)	99 ± 4.8 (93–105)	25 ± 3.7 (24–27)	4.8 ± 0.2 (4.5–5.1)	5.4 ± 0.2 (4.9–6.0)	91 ± 4.7 (86–96)	101 ± 65.6 (93–110)
H. indica CH13	521 ± 26 (480–588)	25 ± 4.7 (17–25)	96 ± 4.7 (88–103)	85 ± 4.3 (74–98)	107 ± 3.3 (93–117)	86 ± 5.7 (66–105)	25 ± 3.4 (21–30)	4.9 ± 0.3 (4.4–5.5)	6.1 ± 0.6 (5.4–7.5)	90 ± 4.6 (84–104)	113 ± 7.9 (99–139)
H. indica CH14	506 ± 24 (469–556)	19 ± 4.9 (17–25)	95 ± 7.0 (86–118)	82 ± 4.6 (75–105)	102 ± 3.2 (93–123)	93 ± 5.9 (80–102)	26 ± 3.6 (19–31)	4.9 ± 0.4 (3.9–5.6)	5.4 ± 0.5 (4.3–6.6)	92 ± 4.1 (79–98)	102 ± 8.6 (89–130)
H. indica CH15	520 ± 26 (466–557)	21 ± 4.5 (18–24)	104 ± 6.5 (94–109)	90 ± 3.8 (82–96)	112 ± 4.1 (101–120)	94 ± 4.9 (86–103)	25 ± 3.3 (23–27)	4.6 ± 0.2 (4.2–5.0)	5.5 ± 0.3 (5.0–6.0)	92 ± 4.8 (89–95)	111 ± 6.9 (102–119)
H. indica CH17	539 ± 26 (504–566)	21 ± 4.3 (19–25)	106 ± 7.3 (84–120	93 ± 5.7 (77–101)	113 ± 3.2 (94–121)	96 ± 4.8 (90–105)	25 ± 3.2 (22–27)	4.8 ± 0.3 (4.4–5.8)	5.6 ± 0.3 (5.0–6.2)	94 ± 5.8 (89–101)	111 ± 10 (80–125)
H. indica CH19	513 ± 22 (470–543)	21 ± 4.7 (19–22)	102 ± 6.8 (98–108)	89 ± 4.5 (87–92)	112 ± 3.4 (107–119)	93 ± 5.6 (89–97)	25 ± 3.3 (23–27)	4.6 ± 0.2 (4.1–4.9)	5.5 ± 0.3 (4.8–6.0)	91 ± 4.2 (87–95)	110 ± .9 (102–117)
H. indica CH20	529 ± 28 (479–569)	21 ± 4.5 (18–23)	96 ± 7.2 (90–105)	86 ± 4.1 (81–94)	113 ± 3.4 (106–121)	83 ± 7.1 (69–99)	25 ± 3.4 (22–32)	4.7 ± 0.3 (4.2–5.2)	6.4 ± 0.7 (5.6–8.0)	85 ± 4.5 (80–94)	116 ± 10 (96–136)
H. indica	528 ± 26 (479–573)	20 ± 6 (19–22)	98 ± 7 (88–107)	82 ± 4 (72–85)	117 ± 3 (109–123)	101 ± 6 (93–109)	26 ± 4 (25–27)	4.5 ± 0.3 (4.3–4.8)	5.3 ± 0.5 (4.5–5.6)	84 ± 5 (79–90)	94 ± 7 (79–90)

Males	L	MBD	EP	NR	PS	T	SL	GL	SW%	GS%	D%	E%
H. indica CH7	755 ± 38 (609–916)	37 ± 6.1 (26–50)	92 ± 6.8 (78–109)	76 ± 3.6 (62–83)	101 ± 4.1 (90–116)	26 ± 2.5 (18–33)	42 ± 2.2 (37–48)	23 ± 2.7 (19–26)	214 ± 14 (164–255)	55 ± 4.5 (49–64)	91 ± 4.64 (86–106)	369 ± 34 (295–511)
H. indica CH8	751 ± 38 (706–827)	42 ± 6.3 (37–46)	94 ± 6.8 (82–108)	76 ± 3.8 (72–80)	101 ± 4.1 (96–104)	26 ± 2.5 (22–31)	43 ± 2.2 (40–47)	23 ± 2.7 (20–25)	198 ± 14 (178–243)	53 ± 4.5 (43–60)	94 ± 4.6 (83–106)	360 ± 34 (300–428)
H. indica CH9	751 ± 39 (646–807)	37 ± 5.9 (33–43)	87 ± 6.5 (77–94)	70 ± 4.1 (64–79)	94 ± 4.7 (85–102)	26 ± 2.0 (22–29)	44 ± 2.9 (41–46)	23 ± 2.3 (21–26)	232 ± 14 (209–254)	54 ± 4.4 (48–62)	93 ± 3.9 (86–102)	337 ± 32 (290–417)
H. indica CH10	816 ± 33 (759–877)	47 ± 5.9 (39–57)	93 ± 6.2 (86–101)	76 ± 3.8 (70–79)	101 ± 4.2 (94–106)	27 ± 2.0 (24–28)	44 ± 3.4 (38–50)	23 ± 2.2 (18–27)	182 ± 17 (156–213)	54 ± 4.4 (48–62)	92 ± 3.9 (86–99)	350 ± 32 (321–384)
H. indica CH11	684 ± 33 (609–732)	41 ± 6.6 (34–47)	84 ± 6/7 (61–102)	74 ± 5.3 (62–79)	92 ± 4.6 (84–107)	27 ± 2.0 (24–31)	40 ± 3.8 (33–46)	20 ± 2.2 (16–24)	204 ± 19 (174–236)	50 ± 6.2 (41–61)	92 ± 3.9 (63–103)	312 ± 32 (229–377)
H. indica CH12	819 ± 45 (697–950)	43 ± 5.2 (36–55)	88 ± 6.8 (83–97)	74 ± 3.6 (64–79)	98 ± 4.1 (91–106)	31 ± 2.5 (26–36)	44 ± 3.5 (36–50)	21 ± 2.1 (17–23)	199 ± 24 (146–234)	49 ± 5.3 (36–55)	90 ± 4.1 (80–102)	291 ± 31 (239–370)

Table 2 (continued)

Males	L	MBD	EP	NR	PS	T	SL	GL	SW%	GS%	D%	E%
H. indica CH13	823 ± 36 (748–874)	46 ± 6.2 (42–49)	91 ± 6.1 (80–100)	75 ± 3.8 (68–81)	102 ± 4.3 (91–108)	34 ± 3.3 (30–42)	47 ± 3.3 (42–53)	25 ± 2.4 (22–27)	203 ± 27 (159–259)	52 ± 5.3 (44–59)	90 ± 4.1 (81–95)	272 ± 29 (222–317)
H. indica CH14	811 ± 59 (705–886)	45 ± 6.1 (39–50)	91 ± 6.9 (81–99)	77 ± 4.7 (67–84)	104 ± 4.1 (98–111)	29 ± 2.7 (24–35)	44 ± 3.2 (24–52)	23 ± 2.9 (19–26)	193 ± 25 (91–232)	54 ± 5.6 (43–95)	88 ± 3.9 (80–94)	314 ± 36 (249–384)
H. indica CH15	832 ± 35 (767–874)	50 ± 6.3 (43–55)	95 ± 5.9 (86–105)	73 ± 4.2 (66–81)	99 ± 5.3 (90–109)	32 ± 2.2 (28–36)	45 ± 2.3 (41–50)	24 ± 5 (20–26)	220 ± 25 (175–252)	53 ± 5.7 (45–58)	96 ± 3.5 (91–104)	299 ± 30 (261–352)
H. indica CH17	823 ± 58 (684–928)	48 ± 4.7 (39–55)	96 ± 6.3 (90–105)	75 ± 4.6 (69–86)	96 ± 5.1 (84–106)	31 ± 1.6 (28–34)	45 ± 3.1 (41–54)	22 ± 2.5 (20–25)	199 ± 21 (171–243)	50 ± 4.6 (41–58)	99 ± 4.3 (92–109)	308 ± 27 (282–344)
H. indica CH19	765 ± 58 (636–916)	42 ± 4.5 (33–51)	83 ± 6.7 (73–89)	70 ± 4.1 (62–77)	97 ± 6.4 (86–110)	33 ± 2.4 (30–38)	44 ± 3.7 (37–50)	21 ± 2.9 (17–25)	197 ± 20 (155–234)	49 ± 4.7 (41–57)	86 ± 3.8 (80–95)	251 ± 23 (209–293)
H. indica CH20	801 ± 51 (696–895)	42 ± 4.7 (38–45)	81 ± 4.9 (72–86)	71 ± 4.3 (60–79)	95 ± 5.5 (84–105)	31 ± 2.3 (26–35)	46 ± 3.0 (42–53)	23 ± 2.1 (18–26)	207 ± 20 (183–259)	50 ± 4.3 (44–61)	85 ± 3.2 (79–93)	265 ± 25 (211–308)
H. indica	721 ± 64 (573–788)	42 ± 7 (35–46)	123 ± 7 (109–138)	75 ± 4 (72–85)	101 ± 4 (93–109)	28 ± 2 (24–32)	43 ± 3 (35–48)	21 ± 3 (18–23)	187	49	121	268

Hermaphrodites	L	MBD	EP	NR	PS	T	V%	D%	E%
H. indica CH7	3476 ± 401 (2861–4227)	245 ± 53 (140–345)	147 ± 9.0 (128–174)	131 ± 7.0 (119–146)	175 ± 6.9 (165–186)	91 ± 12 (79–114)	53 ± 2.8 (48–58)	88 ± 4.4 (81–100)	172 ± 20 (136–200)
H. indica CH8	2854 ± 304 (2312–3423)	163 ± 23 (128–208)	143 ± 15 (116–175)	124 ± 7.1 (111–136)	167 ± 6.1 (156–177)	91 ± 9.5 (67–103)	47 ± 1.7 (45–50)	86 ± 8.2 (68–105)	158 ± 24 (132–203)
H. indica CH9	2603 ± 233 (2335–3106)	130 ± 19 (106–175)	163 ± 10 (141–176)	119 ± 4.4 (109–128)	164 ± 7.7 (148–178)	92 ± 8.6 (79–105)	46 ± 0.8 (45–48)	100 ± 7.3 (91–118)	179 ± 23 (138–221)
H. indica CH10	2749 ± 305 (2065–3123)	147 ± 19 (116–192)	154 ± 8.7 (139–180)	122 ± 6.1 (110–132)	165 ± 8.2 (150–180)	81 ± 10 (69–112)	47 ± 1.6 (43–49)	93 ± 4.1 (87–100)	192 ± 19 (134–216)
H. indica CH11	2966 ± 180 (2635–3298)	150 ± 14 (121–179)	172 ± 13 (147–189)	127 ± 4.4 (117–133)	177 ± 11 (154–194)	94 ± 6 (75–108)	45 ± 2.2 (40–48)	92 ± 5 (84–104)	196 ± 32 (139–242)
H. indica CH12	2986 ± 392 (2508–3901)	169 ± 19 (139–216)	168 ± 11 (146–182)	134 ± 7.2 (118–146)	180 ± 9.3 (158–192)	103 ± 10 (80–121)	45 ± 1.4 (42–49)	94 ± 4.1 (83–99)	165 ± 18 (145–220)
H. indica CH13	3065 ± 265 (2484–3525)	168 ± 20 (131–210)	157 ± 14 (131–178)	123 ± 4.2 (114–132)	168 ± 6.6 (160–178)	95 ± 5.4 (82–103)	45 ± 2.2 (38–48)	94 ± 9.0 (77–106)	166 ± 18 (128–194)
H. indica CH14	2689 ± 263 (2151–3105)	150 ± 22 (119–194)	153 ± 8.6 (136–171)	120 ± 7.1 (110–139)	163 ± 13 (120–176)	98 ± 9.0 (79–111)	45 ± 1.8 (43–50)	94 ± 11 (87–133)	158 ± 17 (127–184)
H. indica CH15	3809 ± 495 (2962–4398)	197 ± 37 (133–262)	194 ± 12 ± (174–211)	149 ± 8.8 ± (131–166)	19 ± 1.5 (172–222)	115 ± 13 (91–132)	45 ± 1.7 (42–48)	99 ± 8.4 (79–114)	170 ± 24 (137–232)
H. indica CH17	4723 ± 391 (3957–5632)	280 ± 23 (231–307)	186 ± 12 168–221)	144 ± 9.3 (133–166)	189 ± 11 (174–208)	95 ± 10 (78–114)	44 ± 3.1 (34–48)	99 ± 4.0 (92–105)	199 ± 25 (154–246)
H. indica CH19	3115 ± 541 (2331–4224)	192 ± 35 (128–243)	154 ± 11 (136–172)	120 ± 11 (104–148)	167 ± 12 (143–195)	94 ± 13 (65–116)	46 ± 5.0 (40–63)	99 ± 4.0 (93–106)	196 ± 32 (139–242)
H. indica CH20	3945 ± 550 (2769–4799)	260 ± 50 (170–386)	171 ± 11 (155–191)	131 ± 6.7 (117–142)	192 ± 13 (171–212)	97 ± 15 (67–120)	45 ± 2.5 (40–50)	89 ± 5.3 (80–97)	180 ± 34 (137–284)

Table 2 (continued)

Hermaphrodites	L	MBD	EP	NR	PS	T	V%	D%	E%
H. indica	2700 ± 1000 (2300–3100)	132 ± 9 (107–145)	173 ± 8 (163–187)	115 ± 5 (104–123)	172 ± 6 (163–179)	92 ± 11 (72–110)	47 ± 3 (45–50)	-	-
Females	L	MBD	EP	NR	PS	T	V%	D%	E%
H. indica CH7	1434 ± 17 (1274–1993)	91 ± 17 (70–135)	115 ± 7.4 (105–129)	95 ± 6.3 (84–111)	133 ± 7.7 (124–155)	75 ± 5.9 (64–83)	49 ± 7.1 (45–52)	87 ± 5.6 (77–99)	155 ± 14 (137–186)
H. indica CH8	1042 ± 16 (917–1179)	61 ± 16 (54–74)	113 ± 7.4 (93–123)	86 ± 2.7 (82–92)	116 ± 3.6 (110–123)	66 ± 6.9 (51–78)	51 ± 8.8 (45–53)	97 ± 5.7 (79–104)	174 ± 23 (133–219)
H. indica CH9	1046 ± 18 (902–1170)	58 ± 15 (50–66)	97 ± 4.6 (93–104)	79 ± 4.0 (74–87)	106 ± 5.6 (99–121)	68 ± 6.8 (57–83)	49 ± 8.3 (48–53)	91 ± 5.9 (84–96)	145 ± 17 (124–178)
H. indica CH10	1329 ± 20 (1149–1483)	90 ± 11 (74–112)	121 ± 6.3 (105–133)	90 ± 5.1 (62–100)	125 ± 3.7 (121–134)	71 ± 6.3 (63–84)	51 ± 7.3 (49–58)	97 ± 5.0 (86–105)	172 ± 14 (150–192)
H. indica CH11	1272 ± 138 (971–1519)	86 ± 14 (61–115)	110 ± 7.3 (97–121)	86 ± 4.9 (76–94)	116 ± 5.7 (104–125)	69 ± 8.0 (60–87)	50 ± 8.6 (47–57)	88 ± 5.6 (78–96)	168 ± 18 (141–220)
H. indica CH12	1135 ± 21 (990–1327)	60 ± 16 (53–74)	102 ± 7.8 (91–115)	83 ± 6.1 (72–90)	110 ± 7.5 (98–121)	70 ± 10 (60–90)	50 ± 7.9 (47–54)	93 ± 5.8 (81–100)	147 ± 17 (121–180)
H. indica CH13	1673 ± 225 (1338–2031)	103 ± 16 (85–134)	121 ± 9.1 (111–149)	96 ± 5.2 (88–106)	129 ± 6.5 (118–142)	72 ± 7.0 (62–86)	48 ± 8.0 (42–52)	94 ± 6.6 (86–122)	170 ± 23 (139–236)
H. indica CH14	1658 ± 19 (1466–1874)	111 ± 14 (101–128)	121 ± 6.2 (110–134)	96 ± 4.4 (89–103)	129 ± 5.7 (119–138)	68 ± 9.0 (58–92)	49 ± 7.4 (45–52)	94 ± 6.5 (89–101)	181 ± 20 (138–209)
H. indica CH15	1957 ± 22 (1664–2141)	127 ± 15 (99–160)	146 ± 7.7 (130–163)	95 ± 4.7 (87–102)	131 ± 5.2 (121–137)	91 ± 6.3 (75–102)	47 ± 7.3 (42–51)	112 ± 5.3 (104–122)	161 ± 15 (144–202)
H. indica CH17	1704 ± 18 (1368–2124)	131 ± 19 (100–177)	68 ± 8.2 (56–89)	97 ± 5.7 (88–113)	128 ± 7.8 (116–147)	80 ± 6.0 (67–92)	48 ± 7.8 (40–51)	100 ± 4.9 (93–110)	162 ± 19 (139–188)
H. indica CH19	1393 ± 14 (1166–1589)	84 ± 13 (62–108)	100 ± 5.9 (85–108)	85 ± 4.9 (76–94)	120 ± 7.0 (110–132)	68 ± 5.0 (54–76)	48 ± 6.9 (43–54)	84 ± 13 (76–89)	147 ± 18 (136–167)
H. indica CH20	1654 ± 15 (1309–1990)	114 ± 21 (78–154)	104 ± 6.7 (89–113)	87 ± 4.1 (79–95)	122 ± 5.0 (111–130)	61 ± 13 (31–74)	49 ± 7.4 (46–52)	85 ± 8.8 (77–91)	184 ± 16 (136–337)
H. indica	1600 ± 12 (1200–1800)	95 ± 15 (107–145)	127 ± 4 (163–187)	92 ± 4 (104–123)	131 ± 4 (163–179)	76 ± 9 (72–110)	48 ± 9 (45–50)	–	–

L total body length, *MBD* mid-body diameter, *EP* excretory pore to anterior end, *NR* nerve ring to anterior end, *PS* pharynx length, *T* tail length, *SL* spicule length, *GL* gubernaculumn length, *a* (L/MBD), *b* (L/PS), *c* (L/T), *V%* (anterior to vulva/total body length) × 100, *D%* (EP/PS × 100), *E%* (EP/T × 100), *SW%* (SL/anal body diameter × 100), *GS%* (GL/SL × 100)

population of *H. indica* and the other six described species of the *Indica* clade [80] namely: *Heterorhabditis noenieputensis* [51], *Heterorhabditis amazonensis* [7], *Heterorhabditis baujardi* [61], *Heterorhabditis taysearae* [74], *Heterorhabditis mexicana* [58], and *Heterorhabditis floridensis* [57]. The analyzed morphological characters allowed a clear separation between the different nematode isolates of this study: the 12 isolates used in this study and the type population of *H. indica*, and other species of the *Indica* clade (Fig. 2a–c).

An accumulated variability of 62.83% was observed in the IJ-based PCA. In this study, the contribution of PC1 observed was 43.05%, and of the PC2 was 19.78% (Fig. 2a; Table 3). Two parameters: ratio *c* ($r = 0.889$) and E% ($r = 0.942$) were positively correlated across nematode isolates/species. On the contrary, three parameters: anterior end to excretory pore ($r = -0.771$), nerve ring to anterior end ($r = -0.689$) and tail length ($r = -0.725$) were negatively correlated across nematode isolates/species. Moreover, eight morphometric characters out of twelve were positively correlated across isolates and the rest displayed a negative coefficient of correlation (Fig. 2a). The highest coefficient of correlation with PC2 was observed in pharynx length ($r = 0.937$) (Table 3).

An accumulated variability of 55.83% was observed in the hermaphroditic female-based PCA. Specifically, the contribution of PC1 observed was 35.99%, and of PC2 was 19.84% (Fig. 2b; Table 3). Eleven out of thirteen morphometric characters were positively correlated across nematode isolates/species, except D% ($r = -0.114$) and V ($r = -0.309$) (Fig. 2b). Body length ($r = 0.951$) had the highest coefficient of correlation within the PC1 (Fig. 2b). Regarding the PC2, six characters were positively correlated and the remaining six were negatively correlated. The c ratio exhibitted the highest coefficient of correlation ($r = 0.904$) (Table 3).

An accumulated variability of 49.78% was observed in the male-based PCA. In this case, it was observed that the contribution of the PC1 was 27.35%, and of the PC2 component was 22.34%. Among the fifteen morphometric variables, eight were positively correlated and seven were negatively correlated (Fig. 2c). Spicule length ($r = 0.598$) and SW% ($r = 0.880$) exhibit the highest correlation of coefficient within the PC1 (Fig. 4). In the case of PC2, all characters except c ratio ($r = -0.249$) and SW% ($r = -0.141$) were negatively correlated (Table 3).

It was observed in the PCAs that some nematodes isolates grouped together, but we did not observe the same groups in all the PCAs, or on nematodes isolated from the same regions. These results indicate that there is intraspecific morphological variation across nematode isolates, and it does not depend on the nematode developmental stage or sampling location (Table 1; Fig. 2a–c). Additionally, a clear separation between species/isolates was not

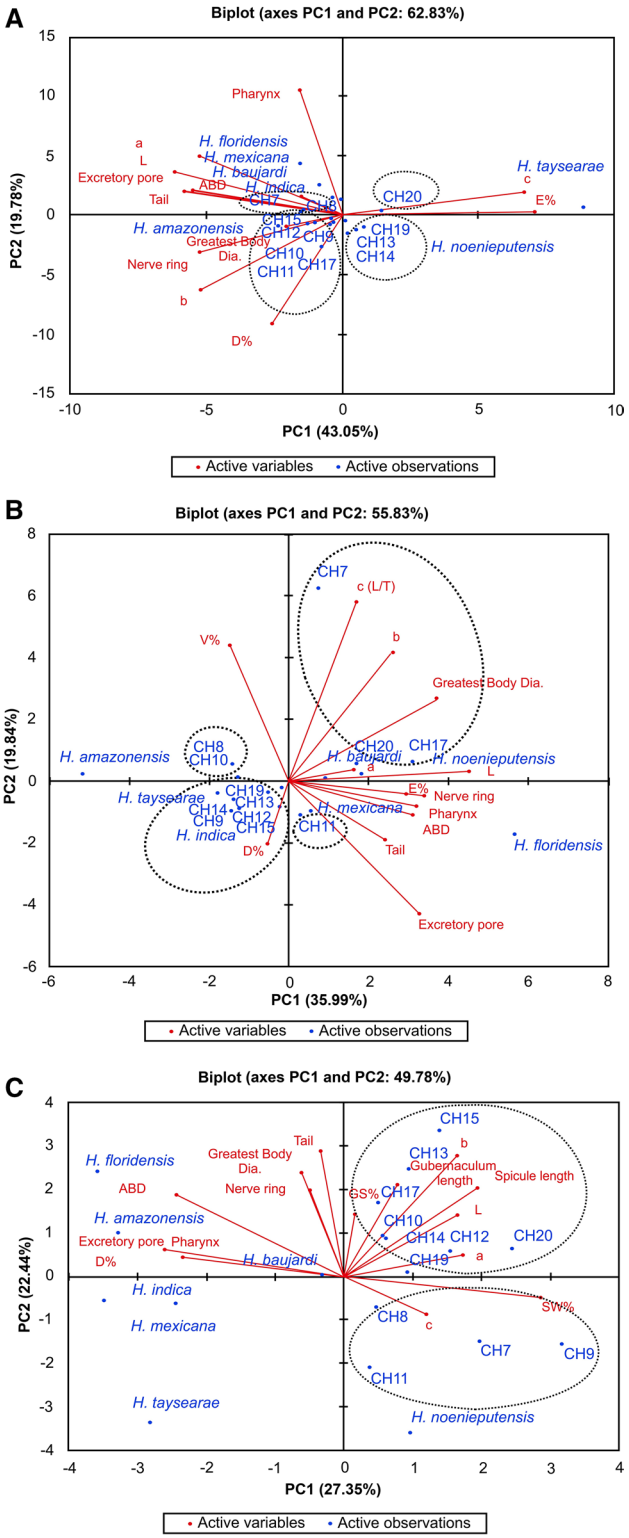

Fig. 2 Plot score of the principal component analysis (PCA) of different populations of *Heterorhabditis indica* based on infective juvenile (**a**), hermaphroditic female (**b**) and male (**c**) specimens

Table 3 Loading score of the variables and factor score of the observations for IJs, hermaphroditic female and males populations

Species	Factor score of the observations						Characters	Loading score of the variables					
	Infective juveniles		Hermaphrodites		Males			Males		Hermaphrodites		Infective juveniles	
	PCA1	PCA2	PCA1	PCA2	PCA1	PCA2		PCA1	PCA2	PCA1	PCA2	PCA1	PCA2
CH7	0.753	6.249	−1.417	0.395	1.979	−1.505	Body length (L)	0.509	0.39	0.951	0.048	−0.815	0.326
CH8	−1.41	0.565	−1.464	0.237	0.477	−0.71	*a* (L/BD)	0.53	0.133	0.342	0.057	−0.694	0.438
CH9	−1.434	−0.946	−1.275	−0.746	3.17	−1.57	*b* (L/PS)	0.51	0.768	0.551	0.649	−0.689	−0.563
CH10	−1.281	0.131	−0.725	−2.677	0.569	0.939	*c* (L/T)	0.37	−0.249	0.359	0.904	0.889	0.173
CH11	0.294	−1.092	−0.317	−0.637	0.376	−2.1	V% (AV/L×100)	–	–	−0.309	0.688	–	–
CH12	−0.253	−0.822	−0.418	−0.819	1.545	0.572	Mid-body diam. (MBD)	−0.189	0.667	0.778	0.418	−0.274	−0.088
CH13	−0.502	−0.351	0.535	−1.267	0.936	2.46	Excretory pore (EP)	−0.796	0.172	0.691	−0.669	−0.771	0.177
CH14	−1.37	−0.579	0.21	−1.525	0.617	0.867	Nerve ring (NR)	−0.151	0.552	0.713	−0.073	−0.689	−0.282
CH15	−0.213	−0.83	−0.373	−0.292	1.393	3.351	Pharynx length (PS)	−0.45	0.074	0.619	−0.064	−0.207	0.937
CH17	3.116	0.634	−0.965	−0.68	0.502	1.696	Tail length (T)	−0.105	0.802	0.51	−0.294	−0.725	0.187
CH19	−0.179	−0.199	0.133	−0.509	0.931	0.097	Anal body diam. (ABD)	−0.745	0.522	0.674	−0.123	−0.198	0.139
CH20	1.697	0.576	1.453	0.296	2.446	0.638	D% (EP/PS×100)	−0.715	0.123	−0.114	−0.314	−0.34	−0.823
H. indica	−1.227	−0.885	−0.35	1.429	−2.445	−0.639	E% (EP/T×100)	–	–	0.653	−0.17	0.942	0.025
H. noenie-putensis	1.821	0.245	0.825	−1.034	0.968	−3.593	Spicule length (SL)	0.599	0.562	–	–	–	–
H. amazon-ensis	−5.158	0.234	−2.34	−0.94	−3.274	0.99	Gubernaculum length (GL)	0.24	0.581	–	–	–	–
H. baujardi	0.915	0.125	−0.017	1.309	−0.313	0.038	SW% (SL/ABD×100)	0.88	−0.141	–	–	–	–
H. taysearae	−1.776	−0.385	8.873	0.594	−2.81	−3.371	GS% (GL/SL×100)	0.051	0.394	–	–	–	–
H. mexicana	0.552	−0.952	−0.838	2.53	−3.487	−0.564							
H. floridensis	5.655	−1.719	−1.53	4.336	−3.581	2.405							

observed, which indicates that the nematodes that belong to the *Indica* clade are morphologically very similar. Several studies have observed large intraspecific morphological variability across nematode isolates, which is consistent with our findings [1, 2, 20, 29]. Many external factors as food source, climate conditions, and environmental toxins cause morphometric variation in nematodes. Recently, for instance, studies found large variations in the morphology of *Steirnenema feltiae* nematodes upon exposure to cucurbitacin-containing phytonematicides, which was explained as morphological adjustments to avoiding hydrostatic pressure damage in the pseudocoelom [53].

Molecular characterization

ITS sequences of the 12 Indian *Heterorhabditis indica* isolates (CH7–CH15, CH17, CH19 and CH20) isolated in this study showed two nucleotide differences with the sequences of the topotype population of *H. indica* (NCBI accession number: AY321483) at position 331 (g.331 T > A), and at position 663 (g.663delT) (Supplementary Fig. 1). The ITS rDNA sequences of present isolates of *H. indica* are separated from those of other described *Heterorhabditis* species by 10–197 bp. No sign of intra-individual variability in the ITS rRNA gene sequence was observed. Regarding the D2/D3 region of the 28S rRNA gene sequence, no differences were observed. The D2 and D3 expansion fragments of the 28S rRNA gene sequence of all the nematode isolates isolated in this study were separated by 2–55 bp from other described *Heterorhabditis* species.

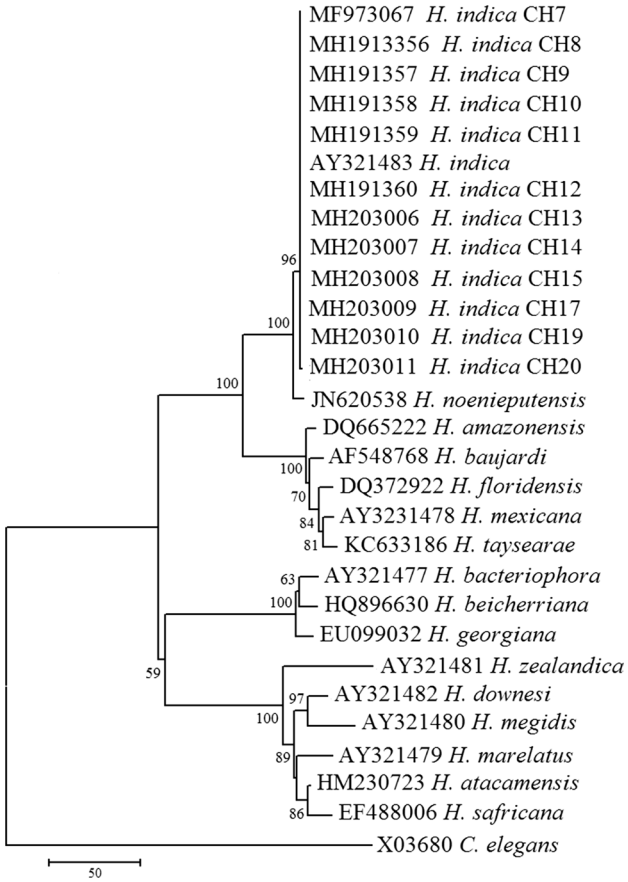

Fig. 3 Phylogenetic tree from known and the newly sequenced *Heterorhabditis indica* based on the sequences of ITS rDNA sequences. *Caenorhabditis elegans* (X03680) was used as the out-group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches

Phylogenetic analysis

The ITS rRNA gene sequence-based phylogenetic analyses of all *Heterorhabditis* species show that the present 12 nematode isolates form a monophyletic clade with the originally described *H. indica*, thus confirming their taxonomic identity (Fig. 3). Sequences of *H. indica* formed a monophyletic group with other members of the *Indica* clade: *Heterorhabditis noenieputensis* Malan, Knoetze and Tiedt [51], *Heterorhabditis amazonensis* Andaló, Nguyen and Moino [7], *Heterorhabditis baujardi* Phan, Subbotin, Nguyen and Moens [61], *Heterorhabditis floridensis* Nguyen, Gozel, Köppenhöfer & Adams [57] and *Heterorhabditis mexicana* Nguyen, Shapiro-Ilan, Stuart, Mccoy, James and Adams [58] and together formed a sister clade with the members of the *Bacteriophora* clade and *Megidis* clade (Fig. 3). Similar results were observed in D2/D3-based phylogeny. The nematode isolates from this study formed a monophyletic group with *H. indica*. In turn, *H. indica* forms a monophyletic group with all described members of the *Indica* clade and together formed a sister clade with the members of the *Bacteriophora* clade and the *Megidis* clade (Fig. 4). Thus, based on the phylogenetic reconstruction of ITS and D2/D3 sequences, the 12 *Heterorhabditis* isolates belong to the nematode species *H.indica*.

Symbiont bacteria: phenotypical, biochemical and molecular diagnosis

Bacteria isolated from *H. indica* CH7 are Gram-negative rods. On nutrient agar, colonies have a brownish pigmented center, appear shiny and opaque, and are circular to irregular and convex. Phase I colonies adsorb neutral red, forming red colonies on MacConkey agar, and adsorb bromothymol blue, forming blue colonies on NBTA agar plates [6]. They are motile and catalase positive, facultatively anaerobic, utilized citrate and used myo-inositol in low concentration. Saccharose was weakly hydrolyzed.

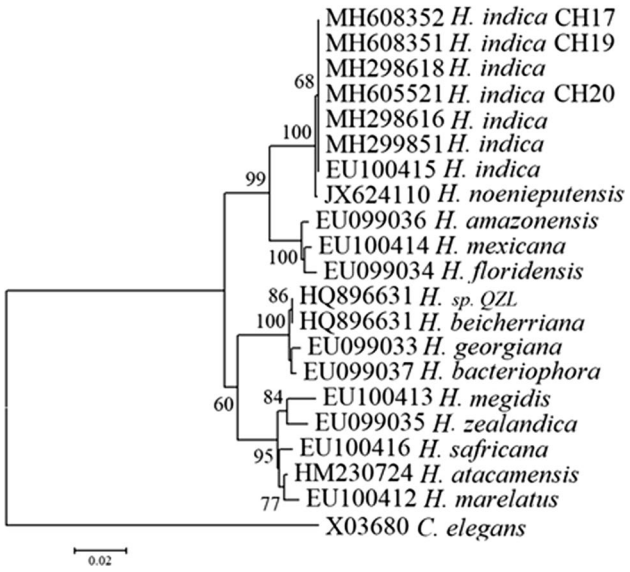

Fig. 4 Phylogenetic tree from known and the newly sequenced *Heterorhabditis indica* based on the sequences of D2/D3 domain of the 28S rDNA region. *Caenorhabditis elegans* (X03680) was used as the out-group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches

Urease, oxidase and nitrate reduction were positively assimilated (Table 4). Mostly negative for KB003 Hi25 Enterobacteriaceae Identification Kit of Hi-media tests (Table 4). Bioluminescence, as assessed by observation in the dark, was visible in 3–6-day cultures of the primary forms of the symbiotic bacteria of *H. indica* CH7.

Based on the 16S rRNA gene sequences, the bacterial isolate CH7 is closely related to *Photorhabdus akhurstii* (Fischer-Le Saux et al. 1999) [50] and share 98.6% sequence similarity. Phylogenetic relationship reconstructions confirm this observations and suggest that the bacterial isolate CH7 belongs to the *Photorhabdus akhurstii* species (Fig. 5). Given the observed ITS sequence similarity scores (98.6%), it might be that CH7 bacteria constitute a different subspecies within *Photorhabdus akhurstii*. Full genome sequences are required to confirm this hypothesis [49, 50].

Geographic distribution of species of the Indica clade

The specimens of *H. indica* used to describe the species were collected in Tamil Nadu, India and the description was based only on morphology and morphometry, but not on a molecular data. Using the NCBI database, we found that *H. indica* isolates have also been isolated from the USA (15), Pakistan (14), India (110), Thialand (59), China (9), Nepal (7), Switzerland (9), Vietnam (3), Brazil (3), Benin (4), Lebanon (2), Egypt (10), South Africa (2), Czech Republic (1), Mexico (2), Philippines (1), Turkey (2), Peru (3), France (1), Taiwan (6), Ireland (1) and Palestine (2) (Supplementary Table 1a). The majority of isolates have been recovered from Thailand (59) and India (110). Based on the NCBI GenBank records, the species seems to be widespread in India as it has been isolated from 9 states throughout the country. In South India, it has been reported from Karnataka (7), Kerala (2), Tamil Nadu (39), Telangana (3), and Maharashtra (11). In North

Table 4 Biochemical characterization of *Photorhabdus akhurstii* CH7 associated with *H. indica* CH7 nematodes

S. no	Tests	Result	S. no	Tests	Result
1	O-Nitrophenyl-β-D-galactopyranoside (ONPG)	–	18	Arabinose	Weakly +
2	Lysine utilization	–	19	Xylose	Weakly +
3	Ornithine utilization	–	20	Adonitol	–
4	Urea hydrolysis	+	21	Rhamnose	–
5	Phenylalanine deaminase	–	22	Cellobiose	Weakly +
6	Nitrate reduction	+	23	Melibiose	–
7	H_2 S production	–	24	Saccharose	Weakly +
8	Citrate utilization	+	25	Raffinose	–
9	Voges Proskauer's	–	26	Trehalose	–
10	Methyl red	–	27	Glucose	Weakly +
11	Indol	–	28	Lactose	–
12	Malonate utilization	–	29	Oxidase	+
13	Esculin hydrolysis	–	30	Ribose	–
14	Myo-inositol	+	31	Bioluminescence	+
15	Dye absorption BTB from NBTA	0–20%	32	Pigmentation	Yellow
16	Neutral red MaConkey agar	Red	33	Motility	+
17	Tryptophan deaminase	–	34		

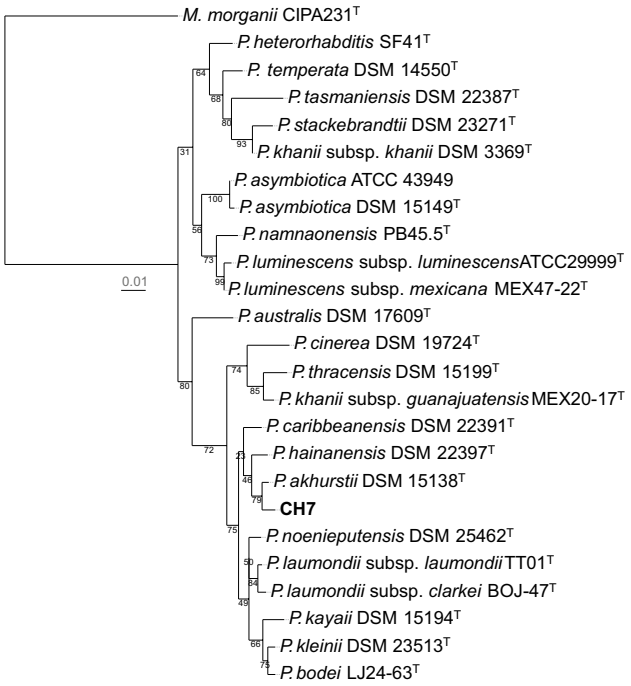

Fig. 5 Phylogenetic relationships of *Photorhabdus* species based on the analysis of 16S rRNA gene sequences. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of the number of base differences per site

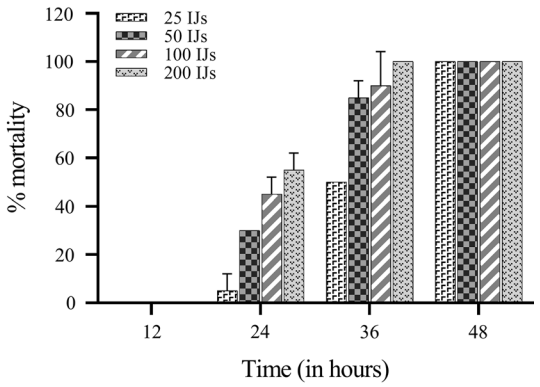

Fig. 6 Percentage mortality (mean and SD) of *Spodoptera litura* larvae infected with *Heterorhabditis indica* CH7

India, it has been reported from Uttar Pradesh (38). From the North West of India, it has been reported in Haryana (2) and Gujarat (1) and from the North East of the country, it has been reported in Mizoram (6) (Supplementary Table 1a). The number of the sequences in GenBank from a particular region reflects not only the abundance of the organism within the area, but also the actual sampling effort. However, the species seems to be present in almost all continents except Australia and Antarctica, but widely spread throughout the Indian subcontinent. In India, two other species of *Heterorhabditis* have been reported, *H. bacteriophora* from Kashmir, Tamil Naidu and Haryana [11, 77] and *H. baujardi* from Mizoram [84]. No other species of *Heterorhabditis* have been reported from India till date. *Heterorhabditis indica* is the most prevalent species of the *Heterorhabditis* genus in India followed by *H. bacteriophora*, while most of the other species are apparently endemic. For instance, *H. beicherriana* has been reported only from China, *H. georgiana* and *H. floridensis* from USA, *H. noenieputensis* and *H. safricana* from South Africa, *H. amazonensis* from Brazil, and *H. atacamensis* from Chile. This distribution may perhaps be related to distribution of suitable insect hosts, soil temperature and moisture, pH, oxygen, soil texture, soil type, crops and to the species of nematode involved [45, 46, 66]. It is also surprising that in the present study only *H. indica*, was isolated and no other *Heterorhabditis* species, in spite of the relatively high number of sampled soils. A potential explanation is that *H. indica* might be a strong intraspecific competitor and could supress other *Heterorhabditis* species. Soil metagenomic studies might answer this question.

Heterorhabditis indica was the first species of the genus recorded from India [63]. Since then, various surveys showed that *H. indica* is the most predominant species of *Heterorhabditis* in India and is found in almost all the geographical parts of the country [79]. The abundance of *H. indica* is obvious in comparison with other species of the heterorhabditid group (Supplementary Table 1b). In the NCBI GenBank database, there are more than 266 records for *H. indica*. Other closely related species have less frequently been reported. A possible explaination for this observation might be that *H. indica* nematodes are able to survive in different habitats and are less affected by changes in abiotic conditions [21, 24, 81]. This distribution pattern suggests that dispersal mechanisms can be highly effective and probably occur by a combination of active and passive dissemination mechanisms [3].

Pathogenicity tests

Laboratory pathogenicity tests showed that *H. indica* isolate CH7 is highly pathogenic against *Spodoptera litura* (Fig. 6). *Heterorhabditis indica* CH7 killed 100% of the tested hosts even at very low IJ concentrations within 48 h. The nematode dose required to kill 50% of the insect host (LD_{50}) within 24 h is 159.48, while only 24.27 nematodes are required to kill the same number of insects within 36 h, demonstrating the high killing capacity of this nematode isolate (Fig. 6). Using similar amount of nematodes, *S. pakistanense* and *S. abbasi* killed 100% of *S. litura* larvae within 48–192 h, suggesting that isolate CH7 is more effective [13, 41]. Differences in virulence against these

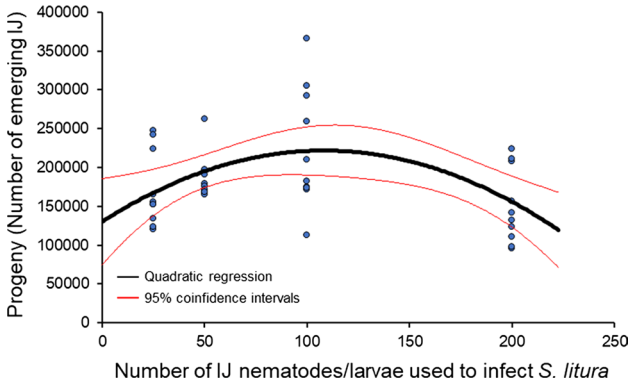

Fig. 7 Number of emerging *Heterorhabditis indica* CH7 IJ nematodes as a function of the initial number of IJ nematodes used to infect *Spodoptera litura* larvae. Quadratic regression was modeled in SigmaPlot 14.0. 95% confidence intervals and quadratic regression are shown ($p = 0.01$)

pests might be explained by nematode adaptations to specific hosts [15, 41, 76]. In addition, many other factors can explain these results, such as the rate of penetration, reproductive potential, type of bacterial symbiont carried by the nematode, doses applied and several other biotic and abiotic factors [31, 35, 42]. The reproductive potential of isolate CH7 is also very high (Fig. 7). It was observed that the number of emerging IJs is optimal when 100 IJs/larva were used to infect *S. litura* larva (Fig. 7). Susurluk and Ehlers (2008) also observed highest nematode reproduction ouput at doses of 100 IJs/larva. The present result was also in accordance with Selvan *et al*. [71] who observed that the production of IJs of *H. bacteriophora* increased with increasing the initial nematode dose up to approximately 100 IJs/larva and suggested that decrease in production rate at high inoculum level is due to an instraspecific competition.

In conclusion, *H. indica* is the dominant *Heterorhabditis* species in agricultural soils of the Western Uttar Pradesh districts in India. Morphological traits might provide little information to determine their taxonomic position, as there is large intra- and inter-specific variation. Molecular indentification tools are, therefore, recommended for future studies. *Heterorhabditis indica* isolate CH7 show great potential to control *S. litura* larvae under laboratory conditions and, therefore, future efforts should be focused to evaluate its virulence and pathogenicity against different agricultural pests throughout the country under field conditions. This may lead to incorporate isolate CH7 as a regular biological control agent in integrated pest management programs in the future.

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Compliance with ethical standard

Conflict of interest There is no conflict of interest.

Ethics approval This article does not contain any studies with human participants or animals.

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