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Description of *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae) isolated from hilly areas of Kashmir Valley

Aashaq Hussain Bhat[1*], Tarique Hassan Askary[2], Mohammad Jamal Ahmad[3], Suman[1], Aasha[1] and Ashok Kumar Chaubey[1]

Abstract

Three strains of entomopathogenic nematodes, labelled P5, P6 and PH, were isolated during surveys of agricultural soils of Pir Panjal Range, using insect baiting technique. Morpho-taxometrical studies and molecular data confirmed that these isolates belong to *Heterorhabditis bacteriophora*, making this finding the first report of this species from Jammu and Kashmir, India. Their distribution using a meta-analysis of GenBank records was attempted to assess. The morphology, morphometric studies and molecular data were conspecific to original description with minor deviations. Data analysis of the distribution showed that *H. bacteriophora* was the most ubiquitous throughout the South Africa subcontinent, but it was rarely found in Indian subcontinent having been isolated from 3 states throughout the country. As these 3 strains of *H. bacteriophora* are native to the hilly region of Kashmir Valley, they can be exploited for the control of target crop insect pests of the region. However, further studies are required regarding their life cycle, host range, virulence potential and survival capacity under extreme environmental conditions.

Keywords: Entomopathogenic nematodes, *Heterorhabditis*, ITS, Meta-analysis, Distribution

Background

Entomopathogenic nematodes (EPNs) of the genus *Steinernema* Travassos, 1927 and *Heterorhabditis* Poinar, 1976 are promising biological control agents against a variety of crop insect pests (Divya and Shankar, 2009; Kaya and Gaugler, 1993). They have short life cycle, a wide host range, capable of resisting under unfavourable conditions, easy to mass produce and apply under field conditions (Askary and Ahmad, 2017). In India, the research on EPNs was conducted primarily by exotic species/strains of some *Steinernema* species and *Heterorhabditis bacteriophora*, imported by researchers (Kaya *et al*., 2006), but these exotic EPNs often yielded inconsistent results particularly in field trials and this may be due to their poor adaptability to the local agro-climatic conditions. Besides, there was a concern that exotic EPNs might also have a negative impact on non-target organisms (Kaya *et al*., 2006). Therefore, keeping in view the biodiversity and environmental perspective, the research practitioners started paying attention to isolation of the native species of EPNs due to their adaptability and utility in biological control. Though, research on EPNs in India was initiated since the mid-1960s (Kaya *et al*., 2006), but exploration of indigenous EPNs strain started in 1990s (Sankaranarayanan and Askary, 2017) with the isolation and identification of *H. indica* (Poinar *et al*., 1992) from Coimbatore. In India, *H. indica* is the only new valid species of EPNs reported till date; however, previously described new species were either synonymized with already existing species or were included as species *inquirendae* (Bhat *et al*., 2019; Hunt and Subbortin, 2016).

The present study was undertaken for the first time in Kashmir Valley for the isolation, proper identification and taxonomic representation of EPN species with the decisive aim to exploit them in future as biopesticide against local crop insect pests.

* Correspondence: aashiqhussainbhat10@gmail.com
[1]Nematology Laboratory, Department of Zoology, Chaudhary Charan Singh University, Meerut, Uttar Pradesh 250004, India
Full list of author information is available at the end of the article

Materials and methods

Isolation and examination of nematodes

Surveys were conducted in agricultural soils of Anantnag, the hilly areas of Pir Panjal Range, Jammu and Kashmir, India, for the presence of EPNs. The surveyed area is located at 33° 72′ North, 75° 14′ East and 1601 m above sea level, where the climate is semi-arid or tropical monsoon. A total of 110 soil samples were collected from the different vegetable growing fields and walnut orchards. The soil samples were brought to laboratory in well-labelled polythene bags and EPNs were isolated from them by *Galleria* soil baiting technique (Bedding and Akhurst, 1975) followed by the White trap method (White, 1927). Emerged infective juveniles (IJs) were stored and used for further studies as described by Bhat *et al*. (2018) and Suman *et al*. (2019).

Heterorhabditis bacteriophora was reared on last instar larvae of greater wax moth, *Galleria mellonella* L. and hermaphroditic and amphimictic generations were obtained by dissecting 4 and 6 days infected cadaver, respectively. The IJs were collected approximately 1 week after the emergence from cadaver. All the generations were heat killed by Ringer's solution and fixed in triethanolamine formalin (Courtney *et al*., 1955). They were dehydrated by the Seinhorst method (Seinhorst, 1959) and further processed as described by Bhat et al. (2017 and 2019). The morphology and morphometric analysis of the specimens was conducted using a light compound microscope (Magnus MLX) and a phase contrast microscope (Nikon Eclipse 50i). Morphometric measurements were done by the help of inbuilt software of phase contrast microscope (Nikon DS-L1). The terminology used for the morphology of pharynx, stoma and spicules follows the proposals by De Ley *et al*. (1995) and Abolafia and Peña-Santiago (2017), respectively.

Molecular characterization

The total genomic DNA was isolated from the pool of IJ stages by using Qiagen Blood and Tissue Analysis Kit (Qiagen, Hilden, Germany). IJs were first washed separately with Ringer's solution followed by washing in PBS solution. They were then transferred into a sterile Eppendorf tube (0.5 ml) and DNA was extracted following the manufacturer's protocol. A fragment of rDNA containing the internal transcribed spacer (ITS) regions (ITS1, 5.8S, ITS2) was amplified using primers 18S: 59-TTGATTACGTCCCTGCCCTTT-39 (forward) and 28S: 59-TTTCACTCGCCGTTACTAAGG-39 (reverse) (Vrain *et al*., 1992). The PCR master mix consisted of ddH2O 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 green DNA polymerase 0.2 μl and 3 μl of DNA extract. The PCR profiles used were as follows: 1 cycle of 94 °C for 3 min followed by 40 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 7 min. PCR was followed by electrophoresis (45 min, 100 V) of 5 μl of PCR product in a 1% TAE (Tris–acetic acid–EDTA)-buffered agarose gel stained with ethidium bromide (Aasha *et al*., 2019). The amplified products were purified and sequenced in both directions at Bioserve Technologies Limited, Hyderabad India. Finally, amplified regions were annotated and submitted to the National Centre for Biotechnology Information (NCBI) under accession numbers MK256378, MK263023 and MK256358 for ITS rDNA regions of strains P5, P6 and PH, respectively.

Sequence alignment and phylogenetic analyses

The sequences were edited and compared with those deposited in GenBank by means of a Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) (Altushul *et al*., 1990). All alignments with other relevant sequences were produced by default ClustalW parameters in MEGA 7.0 (Kumar *et al*., 2016) and optimized manually in BioEdit (Hall, 1999). The phylogenetic trees of the ITS rDNA were obtained by the minimum evolution method in MEGA 7.0 (Kumar *et al*., 2016). The evolutionary distances were computed using the *p* distance method (Nei and Kumar, 2000) and were expressed as the number of base differences per site. All characters were treated as equally weighted and gaps as missing data. *Caenorhabditis elegans* was used as out-group taxa and to root the trees.

Geographical distribution

Geographical distribution of *H. bacteriophora* was assessed, using a meta-analysis of GenBank records, as natural occurrence of species facilities is use in particular areas. The ITS sequence was selected for the analysis, as it enables a clear distinction of the species in steinernematid, unlike another frequently sequenced marker D2D3 region of the 28S rDNA (Bhat *et al*., 2019). For determining the sequences of *H. bacteriophora*, BLAST search was performed with the sequence of the type isolate (AY321477) as a search query. The sequences that showed 97% or higher similarity were downloaded and their identity was confirmed by phylogenetic analysis.

Results and discussion

The morphological and morphometric studies as well as the molecular sequencing data of ITS rDNA showed that the present strains, P5 (MK256378), P6 (MK263023) and PH (MK256358), were conspecific to *H. bacteriophora* (Poinar, 1976) and hence described as the same species. The genus *Heterorhabditis* contains 16 well-described species of which only 3 have been described from India: *H. indica* (Poinar, 1992), *H. bacteriophora* (Poinar, 1976)

Sivakumar *et al.*, 1989 and *H. baujardi* (Phan *et al.*, 2003) Vanlalhlimpuia *et al.*, (2018). This is the first valid report of the existence of *H. bacteriophora* in Pir Panjal Range of Kashmir Valley. Three slides of the first-generation female bearing one female on each slide, 2 slides of first-generation males bearing 3 males on each slide, 2 slides of each second-generation females and males bearing 2 and 3 specimens, respectively, on each slide is deposited in Museum of Department of Zoology, Chaudhary Charan Singh University, Meerut.

Diagnosis of *Heterorhabditis bacteriophora*

The morphology of the 3 strains of *H. bacteriophora* P5, P6 and PH was similar to original description; however, some minor differences were observed. The anal swelling of present specimens was very prominent in both hermaphroditic and amphimictic females, while in original descriptions, it is much prominent in hermaphroditic females than in amphimictic. The rest of the morphological features were very similar. However, *H. bacteriophora* PH was selected for morphometric measurements and comparative studies as they all displayed similar morphology. The morphometric measurements of adults and IJs of *H. bacteriophora* PH (Table 1) were found similar to the topotype population of *H. bacteriophora* (Poinar *et al.*, 1976), but some deviations were seen with the original description. A comparison in morphometric parameters in all generations of strain PH with original description of *H. bacteriophora* has been depicted (Table 2).

Molecular characterization

The alignment file of ITS rDNA sequences of the present 3 strains P5 (MK 256378), P6 (MK263023) and PH (MK256358) showed 2 nucleotide differences with topotype population of *H. bacteriophora* (AY321477) at positions 324 (G in place of C) and 667 (C in place of T); however, PH in addition also showed 2 nucleotide difference at positions 16 (T in place of A) and 618 (G in place of T) with P5, P6 and topotype population. The

Table 1 Morphometric data for *Heterorhabditis bacteriophora* PH. All measurements are in μm (except *n*, ratio and percentage) and in the form: mean ± SD (range)

Characters (*n*)	Male (20)	Hermaphrodite (20)	Female (20)	Infective juvenile (20)
Body length (L)	865 ± 51 (782–927)	4952 ± 969 (3086–5492)	1874 ± 272 (1513–2290)	527 ± 268 (474–568)
L′ (L-T)	833 ± 51 (748–893)	4858 ± 970 (2963–6406)	1818 ± 267 (1468–2218)	453 ± 29 (389–492)
a (L/BD)	8 ± 0.8 (6.6–8.5)	17 ± 4.6 (9.2–28)	17.2 ± 2.9 (11–22)	22 ± 1.9 (19–25)
b (L/ES)	9 ± 0.5 (8.5–10)	31 ± 4.4 (23–37)	15.2 ± 1.9 (11–19)	5.4 ± 0.4 (4.7–6.1)
c (L/T)	27 ± 2.4 (23–32)	54 ± 12.3 (25–75)	34 ± 4.5 (26–42)	7.3 ± 1.1 (5.5–9.3)
c′ (T/ABW)	1.7 ± 0.2 (1.4–2.2)	1.9 ± 0.5 (1.2–3.7)	2.0 ± 0.3 (1.6–2.5)	5.4 ± 1.0 (3.4–7.5)
V (V′/L)×100	-	45 ± 3.6 (37–52)	45 ± 2.7 (38–51)	-
Body diameter (BD)	108 ± 8.1 (92–120)	292 ± 44 (221–352)	110 ± 16.2 (84–150)	25 ± 1.8 (22–28)
Excretory pore (EP)	122 ± 9.9 (103–139)	209 ± 33 (127–260)	152 ± 14.4 (128–181)	119 ± 4.5 (110–127)
Width at EP (WEP)	33 ± 4.2 (23–42)	110 ± 16 (72–131)	73 ± 9.1 (57–92)	20 ± 1.9 (17–23)
Nerve ring (NR)	66 ± 5.4 (58–76)	131 ± 21 (79–162)	85 ± 7.1 (71–99)	73 ± 7.2 (61–90)
Pharynx length (PL)	96 ± 6.2 (84–105)	159 ± 23 (101–200)	123 ± 6.7 (113–135)	99 ± 6.4 (90–115)
Bulb length (EBL)	20 ± 2.5 (16–26)	32 ± 4.9 (23–44)	26 ± 3.7 (21–33)	17 ± 2.1 (14–21)
Bulb width (EBW)	17 ± 1.3 (15–20)	30 ± 2.9 (22–35)	23 ± 2.7 (18–29)	9.4 ± 1.0 (7.7–10.8)
Tail	32 ± 2.2 (28–37)	94 ± 13 (71–123)	56 ± 8.2 (41–79)	74 ± 11 (57–90)
Anal body width (ABW)	18 ± 2.1 (15–23)	51 ± 8.8 (34–75)	29 ± 3.5 (24–39)	15 ± 2.3 (11–19)
Spicule length (SPL)	44 ± 4.5 (31–53)	-	-	-
Gubernaculum length, GL	21 ± 2.7 (17–26)	-	-	-
D% (EP/ES×100)	128 ± 14 (108–157)	132 ± 11.7 (112–155)	124 ± 12.1 (108–150)	122 ± 8.6 (105–139)
E% (EP/T×100)	385 ± 42 (302–465)	227 ± 43 (105–302)	278 ± 44 (187–360)	165 ± 25 (131–211)
SW% (SL/ABD×100)	242 ± 28 (194–282)	-	-	-
GS% (GL/SL×100)	47 ± 7.0 (37–57)	-	-	-
Width at vulva (WV)	-	270 ± 48 (168–341)	119 ± 21.5 (83–151)	-
Anterior to vulva (V′)	-	2241 ± 424 (1363–2903)	851 ± 132 (621–1050)	-
Posterior to vulva (V″)	-	2711 ± 611 (1684–3733)	1022 ± 156 (784–1258)	-

Table 2 Comparative morphometric of infective juveniles (IJs) and adult generations from populations of *Heterorhabditis bacteriophora* with original description. All measurements are in μm (except ratio and percentage) and in the form of mean (range)

IJs	**L**	**BD**	**EP**	**NR**	**PL**	**T**	***a***	***b***	***c***	**D%**	**E%**
PH	527 (474–568)	25 (22–28)	119 (110–127)	73 (61–90)	99 (90–115)	74 (57–90)	22 (19–25)	5.4 (4.7–6.1)	7.3 (5.5–9.3)	122 (105–139)	7165 (131–211)
H. bacteriophora (Poinar, 1976)	558 (512–671)	23 (18–31)	103 (87–110)	85 (72–93)	125 (10–139)	98 (83–112)	25 (17–30)	4.5 (4–5.1)	6.2 (5.7–7)	84 (76–92)	112 (103–130)
Males	**L**	**BD**	**EP**	**NR**	**PL**	**T**	**SL**	**GL**	**SW%**	**GS%**	**D%**
PH	865 (782–927)	108 (92–120)	122 (103–139)	66 (58–76)	96 (84–105)	32 (28–37)	44 (31–53)	21 (17–26)	242 (194–282)	47 (37–57)	128 (108–157)
H. bacteriophora (Poinar, 1996)	820 (780–960)	43 (38–46)	121 (114–130)	72 (65–81)	103 (99–105)	28 (22–36)	40 (36–44)	20 (18–25)	174	50	117
Hermaphrodites	**L**	**BD**	**EP**	**NR**	**PL**	**T**	**V%**	**D%**	**E%**	**ABW**	
PH	4952 (3086–5492)	292 (221–352)	209 (127–260)	131 (79–162)	159 (101–200)	30 (22–35)	45 (37–52)	132 (112–155)	227 (105–302)	51 (34–75)	
H. bacteriophora (Poinar, 1996	4030 (3630–4390)	165 (160–180)	209 (189–217)	126 (121–130)	197 (189–205)	90 (81–93)	44 (41–47)	106	232	46 (40–53)	
Females	**L**	**BD**	**EP**	**NR**	**PL**	**T**	**V%**	**D%**	**E%**	**ABW**	
PH	1874 (1513–2290)	110 (84–150)	152 (128–181)	85 (71–99)	123 (113–135)	56 (41–79)	45 (38–51)	124 (108–150)	278 (187–360)	29 (24–39)	
H. bacteriophora (Poinar, 1976)	3500 (3180–3850)	190 (160–220)	192 (174–214)	103 (93–118)	168 (155–183)	82 (71–93)	47 (42–53)	114	234	28 (22–31)	

ITS rDNA sequences of the three strains of *Heterorhabditis* showed zero total character difference with each other and with AY321477; however, they were separated from other described *Heterorhabditis* species by 19–199 bp (Table 3).

Phylogenetic analysis

Phylogenetic analyses of the species of Heterorhabditidae based on ITS rDNA region showed a clear monophyly of the group formed by the 3 isolates (*H. bacteriophora* MK256378, *H. bacteriophora* MK263023 and *H. bacteriophora* MK256358) and original *H. bacteriophora* and several others, probably conspecific isolates (Fig. 1), thus confirming their identification. Sequences of *H. bacteriophora* formed a monophyletic group with '*bacteriophora*' clade viz., *H. georgiana* Nguyen *et al*. (2008) and *H. beicherriana* Li *et al*. (2012), and together, they formed a sister clade with species of '*megidis*' clade. The ITS rDNA region is conservative to resolve phylogenetic relations among closely related species.

Geographic distribution

The geographical dissemination of *H. bacteriophora* was evaluated by means of ITS rDNA records in NCBI database, although it has obviously many limits. The lack of the record from some areas does not mean that the organism is not present. On the other hand, the existing record means the presence of the species in that locality.

The original description of *H. bacteriophora* was from Brecon, South Australia by Poinar (1976) based on morphological characters only. Therefore, type sequence (AY321477) from the USA was used as a search query. *H. bacteriophora* strains were isolated in the USA (46); Pakistan (3); China (3); Switzerland (25); India (6); Argentina (6); Bulgaria (1), Lebanon (3); Egypt (1); South Africa (67); Iran (38); Portugal (11); Poland (3); Turkey (25); Nigeria (1); France (3); Hungary (1); Croatia (3); Jordan (5); New Zealand (1); Australia (1); Syria (5); UK (4); Slovenia (1); Iraq (1); Ireland (1) and Palestine (4). The majority of the sequences originate from the strains isolated in South Africa (67) followed by the USA (46). Based on the NCBI GenBank records, the species seems to be less reported in India, having been isolated from 3 states throughout the country. The records originate from North India (Jammu & Kashmir (3), Uttar Pradesh (2) and Haryana (1)). 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 cosmopolitan, reported in almost all the continents except Antarctica but widely spread throughout the South Africa.

The abundance of *H. bacteriophora* was clear in comparison with other species of the '*bacteriophora*' group

Table 3 Pairwise distances of the ITS rDNA regions between *Heterorhabditis* species. Below diagonal: percentage similarities; above diagonal: total character differences

	ITS region	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	MK256378 *H. bacteriophora* P5		0	3	3	19	20	130	136	137	138	151	168	175	179	191	192	194	196	199
2	MK256358 *H. bacteriophora* PH	100		3	3	19	20	130	136	137	138	151	168	175	179	191	192	194	196	199
3	MK263023 *H. bacteriophora* P6	100	100		0	20	21	128	134	135	136	151	166	174	178	188	190	191	194	197
4	AY321477 *H. bacteriophora*	100	100	100		20	21	128	134	135	136	151	166	174	178	188	190	191	194	197
5	HQ896630 *H. beicherriana*	97	97	97	97		20	130	136	134	137	155	168	178	182	191	189	193	197	200
6	EU099032 *H. georgiana*	97	97	97	97	97		134	140	140	141	157	172	173	177	189	189	191	195	198
7	HM230723 *H. atacamensis*	79	79	79	79	79	78		23	27	14	49	73	171	173	180	184	182	186	190
8	AY321479 *H. marelatus*	79	79	79	79	79	78	97		35	25	55	73	184	186	188	194	191	196	198
9	AY321482 *H. downesi*	78	78	79	79	79	78	96	95		30	36	72	186	187	190	195	195	200	201
10	EF488006 *H. safricana*	78	78	79	79	79	78	98	97	96		51	77	183	185	191	197	194	199	203
11	AY321480 *H. megidis*	76	76	76	76	75	75	93	92	95	93		89	201	202	202	206	207	211	212
12	AY321481 *H. zealandica*	73	73	73	73	73	72	89	89	89	89	87		210	211	215	221	218	223	224
13	AY321483 *H. indica*	70	70	70	70	69	70	69	68	67	68	64	62		11	77	85	73	83	83
14	JN620538 *H. noenieputensis*	69	69	69	69	68	69	69	67	67	67	64	62	98		78	87	74	82	84
15	AF548768 *H. baujardi*	68	68	68	68	68	68	68	68	67	67	65	62	89	88		27	16	16	23
16	KC633186 *H. taysearae*	66	66	67	67	67	67	66	65	65	65	63	59	87	87	96		23	19	13
17	DQ665222 *H. amazonensis*	67	67	67	67	67	67	67	67	66	66	64	61	89	89	98	97		17	21
18	DQ372922 *H. floridensis*	66	66	67	67	66	67	67	66	65	65	63	60	88	88	98	97	98		14
19	AY321478 *H. mexicana*	66	66	66	66	66	66	66	66	65	65	63	60	88	87	97	98	97	98	

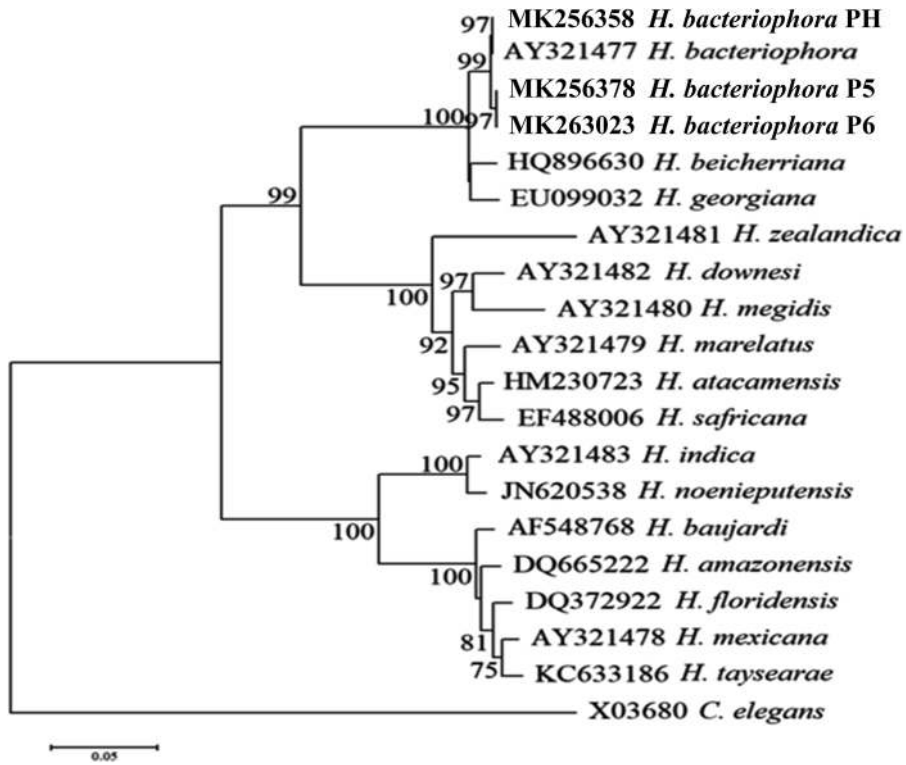

Fig. 1 Phylogenetic relationships of *Heterorhabditis* strains (P5, P6 and PH) with 16 *Heterorhabditis* spp. based on ITS rDNA sequences. *Caenorhabditis elegans* (X03680) was used as the out-group taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches

of *Heterorhabditis* (Table 4). Based on the records in NCBI GenBank database, *H. bacteriophora* was the most frequently sequenced member of the 'bacteriophora' group (Table 4). The species with a worldwide distribution, *H. bacteriophora* has 269 records. Other closely related species have much lower number of records. The explanation for these differences is unclear, and further research in this field could bring interesting results. On the basis of above findings, it can be summarized that 3 strains of *H. bacteriophora* were indigenous to hilly areas of Jammu and Kashmir, which may be utilized as biological control tool against a variety of local crop insect pests. However, prior to this, further investigations are needed such as virulence and reproductive potential inside the host as well as survival of nematode under environmental extremes.

Table 4 Number of ITS sequences belonging to the group of *Heterorhabditis bacteriophora*, present in the GenBank database (August 2018)

Species	No. of records	Country
Heterorhabditis bacteriophora	269	Cosmopolitan
Heterorhabditis georgiana	29	USA
Heterorhabditis beicherriana	1	China

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Authors' contributions
The design of the study was done by all authors, but second author (TAA) isolated the nematodes from soils samples. All molecular work and the analysis, interpretation of the data and writing were done by the first author (AHB). Both S and A calculated morphometric measurements and reference setting; then, the manuscript was finally revised by TAA and MJA. All authors read and approved the final manuscript.

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Author details
[1]Nematology Laboratory, Department of Zoology, Chaudhary Charan Singh University, Meerut, Uttar Pradesh 250004, India. [2]Division of Entomology, Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology, Wadura Campus, Sopore, Jammu and Kashmir 193201, India. [3]Division of Entomology, Faculty of Horticulture, Sher-e-Kashmir University of

Agricultural Sciences and Technology, Shalimar, Srinagar, Jammu and Kashmir 190025, India.

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