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Diversity and distribution of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) and their pathogenicity against *Odoiporus longicollis* in Mizoram, India

HRANG CHAL LALRAMNGHAKI[1], LALRAMLIANA[1,♥], VANLALHLIMPUIA[1,2], HMINGTHANZUALA[3], MARY LALRAMCHUANI[1], VANRAMLIANA[1], HMAR TLAWMTE LALREMSANGA[4], SA THING LALZARZOVI[2]

[1]Department of Zoology, Pachhunga University College. Aizawl 796001, India. Tel.: +91-389-232-7095, ♥email: lrl_zoo@yahoo.co.in
[2]Department of Environmental Science, Mizoram University. Aizawl 796004, India
[3]Department of Life Sciences, Pachhunga University College. Aizawl 796001, India
[4]Department of Zoology, Mizoram University. Aizawl 796004, India

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Abstract. *Lalramnghaki HC, Lalramliana, Vanlalhlimpuia, Hmingthanzuala, Lalramchuani M, Vanramliana, Lalremsanga HT, Lalzarzovi ST. 2025. Diversity and distribution of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) and their pathogenicity against* Odoiporus longicollis *in Mizoram, India. Biodiversitas 26: 5371-5384. Odoiporus longicollis* (Coleoptera: Curculionidae), commonly known as the banana pseudostem weevil (BPW), is a significant pest that reduces banana yield. Entomopathogenic nematodes (EPNs), recognized for their robust biocontrol ability, can control economically important insect pests and can be integrated into Integrated Pest Management (IPM) strategies. To identify EPNs, a survey was conducted across Mizoram, Northeastern India. Of all the 538 soil samples collected, 19 (3.53%) were positive for EPNs, representing13 of 36 collection sites (36.11%). The ITS and 28 rDNA sequence analyses identified 13 EPN isolates: seven *Heterorhabditis indica*, one *H. baujardi*, three *Steinernema sangi*, and two *S. surkhetense*. Pathogenicity tests were performed by exposing *O. longicollis* larvae to various concentrations of nematodes in Petri dish assay. Each EPN isolate was tested twice, with eight replicas per concentration (IJs/larva). BPW larval mortality ranged from 25-100% for both *H. indica* and *S. sangi*, 18.75-100 and 12.5-100% for *H. baujardi* and *S. surkhetense*, respectively. Based on LC_{50} and LT_{50} values, *H. indica* was the most lethal, followed by *S. sangi*, *H. baujardi*, and *S. surkhetense*. All EPN isolates successfully colonized dead larvae, with heterorhabditids multiplying more effectively than steinernematids. *H. indica* showed the highest multiplication rates, producing 77.5±14.44 x 10^3 and 125.97±31.49 x 10^3 IJs emerging at 10 and 200 IJs/insect, respectively. Nevertheless, the nematode multiplication rates, measured by the number of offspring produced, varied significantly among species [$F_{(3, 28)} = 67.57$, $p \leq 0.05$]. These findings on EPNs distribution enhance the knowledge on their geographical diversity and support their potential use in field management.

Keywords: Banana pseudostem weevil, biocontrol, diversity, insect pests, parasite

INTRODUCTION

The development of insecticide resistance among pests and the potential negative impacts of synthetic pesticides on the environment are worldwide issue that needs immediate action. Biological control, which has low cost and minimal negative impact on the environment, is one of the most effective approaches for managing insect pest, and moreover, biopesticides could eventually replace synthetic insecticides.

Entomopathogenic nematodes (EPNs) belonging to the Steinernematidae and Heterorhabditidae families have excellent bio-control capability and effective insect pest management (Koppenhöfer et al. 2020; Sharma et al. 2022; Ramakuwela et al. 2025). EPNs have a widespread occurrence, with over 114 species of *Steinernema* and 22 species of *Heterorhabditis* listed globally, except for Antarctica (Bhat et al. 2020; Machado et al. 2025; Půža et al. 2025). In India, Rao and Manjunath (1966) initiated work on EPN by assessing the potential of an exotic *Steinernema carpocapsae* DD-136 strain against insect pests on apple, rice, and sugarcane. Subsequently, experiments were conducted to evaluate the efficacy of exotic EPN species against selected important insect pests (Rao and Manjunath 1966; Kaya et al. 2006). However, the results were deemed inconsequential due to poor ability of the nematodes to adapt to their new surroundings and the potential negative impacts on non-target species (Kaya et al. 2006). Therefore, several workers have focused on local EPN isolates for better adaptation and management of pests leading to listing of 12 species of *Steinernema* and 2 species of *Heterorhabditis* from India (Patil et al. 2023). A substantial number of EPN identifications have conventionally relied on morphological techniques, which are often not straightforward with complications and ambiguities. Globally, molecular methodologies, particularly polymerase chain reaction (PCR)-based DNA sequencing analysis, have augmented traditional techniques in the identification of EPNs (Londono-Caicedo et al. 2023). In this context, nuclear DNA genes are the most suitable genetic markers for delimitation of nematodes across various taxonomic hierarchies (Chan et al. 2021).

The Banana Pseudostem Weevil (BPW), *Odoiporus longicollis* (Olivier 1807), is a severely damaging and notorious pest of bananas (Alagesan et al. 2016; Kannan et al. 2021, 2022). The pest, widespread in Asia, is recognized for infesting the banana plant during different stages of its life cycle (Krishnan et al. 2015). The pre-oviposition phase of BPW lasts approximately 15 to 30 days. The adult female, having a lifespan of approximately 200 days, deposits eggs in the pseudostem air chamber, and the emerging larvae subsequently consume the leaves (Krishnan et al. 2015). Both the grub (larva) and the adult feed on the pseudostem; however, the grub is the most destructive phase, as it bores tunnels within the stem, compromising the plant's health. The extent of crop loss caused by the pest can range from 10 to 90%, particularly pronounced when the infestation occurs during the early vegetative phase of the crop. In India, the incidence of BPW has been documented across 28 states, encompassing all eight Northeastern states, and is notably more significant in Southern India (Krishnan et al. 2015).

The increasing resistance of *O. longicolli*s to conventional insecticides has led to a renewed emphasis on biological control strategies (Alagesan et al. 2019). Lopes et al. (2011) demonstrated that biocontrol agents can effectively enhance infection rate and facilitate the spread of pathogens within weevil populations, a process influenced by the insect's mating and feeding behaviors. Despite all the positive attributes of EPNs, their application against aerial insect pests is limited by certain challenges related to production cost, a significant knowledge gap on their susceptibility to environmental factors, including UV radiation, several pesticides, and soil chemistries (Sharma et al. 2022). So far, three EPN species viz. *Heterorhabditis indica*, *Steinernema carpocapsae*, *Steinernema siamkayai* have been evaluated against *O. longicollis* (Ling 2002; Padmanaban et al. 2002; Giribabu et al. 2020). Their findings indicate that these EPN isolates can induce substantial pest mortality and have introduced innovative formulation methods to enhance their field performance. The study herein aims to investigate the diversity and distribution of EPNs in Mizoram, part of the biodiversity hotspot in Northeastern India, and to evaluate their pathogenicity against *O. longicollis* under laboratory conditions.

MATERIALS AND METHODS

Study area

Mizoram, located in Northeastern India, is recognized as a part of the biodiversity hotspots within the Eastern Himalayan region having a forest cover of 88.93% of its total land area (Forest Survey of India 2015). The study area, Mizoram, India (Figure 1), is characterized by extensive mountainous terrain that gradually descends into deep valleys interspersed with rivers and streams. Over 70% of the population relies on agriculture for their livelihood, and the prevailing moderate climatic conditions owing to its geographical location (23.1645N, 92.9376E) favor the cultivation of banana, an important fruit crop of Mizoram.

Collection of soil samples

Agro-forest, riparian, jhumland, forestland, and roadsides were selected for sample collection (86.11% from riparian forest and forestland) (Figure 1). Soil samples were taken from 10 to 15 cm depth, pooling approximately 1 kg of soil (ranging from 10-15 samples) for each collection site, covering an area of about $1m^{-2}$. Soil sampling was carried out throughout the year and the samples gathered in appropriate labelled carry bags were transported to Pachhunga University College, Aizawl, Mizoram. The average minimum and maximum soil temperature recorded while sampling was from 12 and 27ºC, respectively.

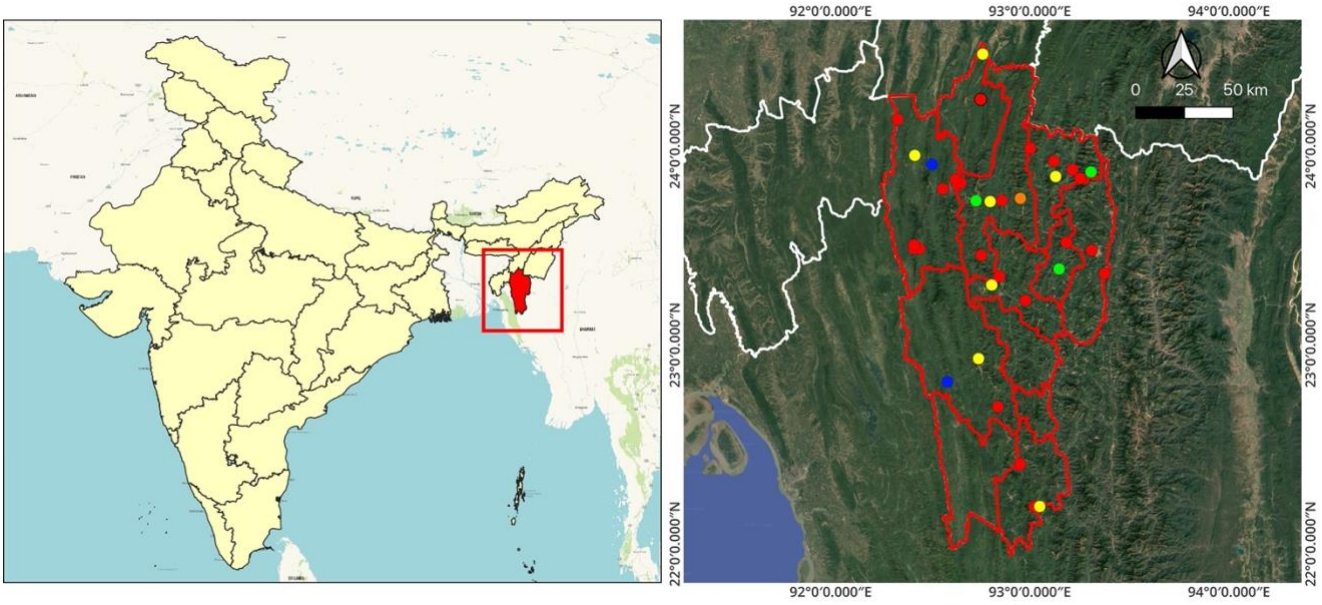

Figure 1. An illustration of the soil sample collection location (filled circle) of entomopathogenic nematodes in Mizoram, Northeastern India. ●: *Steinernema sangi,* ●: *Steinernema surkhetense,* ●: *Heterorhabditi indica,* ●: *Heterorhabditi baujardi,* ●: Negative sites

EPN isolation

EPNs were baited out from the soil using larvae of *Galleria mellonella* as per Bedding and Akhurst (1975), with slight modifications. Larval mortality was checked for at least 10 days. The dead larvae (if any) were checked, whether or not, they were infected by the EPNs through changes in color and odour. Subsequently, the infected ones were moved to a white trap to see if the nematodes emerged (Kaya and Stock 1997). The emerged nematodes were exposed to new insect hosts (Koch's postulates) to verify their pathogenicity (Kaya and Stock 1997). The pathogenic nematodes were multiplied in the final instar of *G. mellonella*, and stored in an incubator set at 10±2ºC.

EPN identification

Morphological and morphometric characterization

The EPN isolates were propagated on the final instar larvae of *Galleria mellonella*. The resulting cadavers were subsequently positioned on a white trap to facilitate the emergence of infective juveniles (IJs). The first- and second-generation adults were obtained through the dissection of the cadavers approximately two days and five days post-mortality, respectively. The thoroughly cleaned nematodes were heat-killed in Ringer's solution at 60ºC, then moved to tri-ethanolamine-formalin (TAF) fixative and processed using anhydrous glycerine dehydration (Seinhorst 1959; Kaya and Stock 1997). Permanent mounts were made on at least 25 individuals of infective juveniles (IJs), male and female for all the EPN isolates. Evos XL Core microscope attached with an image analyser (Nikon Eclipse CiE DSRi2 Cam + NIS) was used for morphometric measurement and photography.

Molecular identification

Genomic DNA was extracted from adult nematodes, and polymerase chain reaction (PCR) of the two DNA fragments, internal transcribed spacer (ITS) and 28S rDNA gene, was done with the primers TW81 (forward) and AB28 (reverse) (Joyce et al. 1994) and 391 (forward) and 501 (reverse) (Qiu et al. 2011) primer sets, respectively. The primer sequences are TW81: 5'-GTT TCC GTA GGT GAA CCT GC-3' and AB28: 5'-ATA TGC TTA AGT TCA GCG GGT-3' for the ITS region and 391: 5'- CGA TAG CGA ACA AGT ACC GAG AG -3' and 5'- CCT GCT CAG GCA TAG TTC ACC ATC-3' for the 28S rDNA gene region. The qualified PCR products were sent to Scigenom, Kochi, Kerala, India, for sequencing.

The chromatograms of nucleotide sequences were checked and analyzed using Geospiza's FinchTV 1.4.0. The sequences were aligned using Clustal X 1.64, and then compared to the known EPN sequences from the NCBI GenBank database (Thompson et al. 1997). MEGA 11 version was employed to concatenate sequences of both the marker sequences (ITS and 28S rDNA) (Tamura et al. 2021). A Bayesian phylogenetic tree (in MrBayes 3.2.6) (Ronquist et al. 2012) was generated from the concatenated sequences representing identified species of the genera *Steinernema* and *Heterorhabditis*. The analysis employed a generalized time-reversible model (nst = 2), characterized by gamma-distributed rate variation and the inclusion of a proportion of invariable positions (GTR+G+I) for the amalgamated sequences. We executed four concurrent Markov chain Monte Carlo simulations over a span of 500,000 generations, with sampling conducted every 1,000 generations. The initial 25% of samples were eliminated as burn-in. The phylogenetic trees were visualized and refined utilizing FigTree version 1.4.2. *Caenorhabditis elegans* represents the outgroup taxon. Uncorrected p-distance (with gamma distribution) was used to determine the genetic distance of the nematodes for both ITS and 28S rDNA genes.

Pathogenicity test

The efficacy of the obtained EPNs was evaluated against the larvae of *O. longicollis* using petri dish assay following Kaya and Stock (1997). The pest larvae were obtained from the infested banana plant (*Musa* sp.) of Phuaibuang Village, Saitual District, Mizoram, India (23.6889N, 92.9637E), and raised in the laboratory using their natural food. The various life stages of the pest were systematically observed to facilitate identification (Krishnan et al. 2015). Only the healthy larvae in their fourth or fifth instar were selected for the pathogenicity test.

Mortality assay

Larval mortality rates of BPW were assessed using EPN concentrations of 10, 25, 50, 100, 200, 400, 800, and 1600 IJs/insect. Each concentration of nematodes, suspended in 0.5 mL of distilled water was added to a 35 x 10 mm petri dish floored with double-layer filter paper (Whatman No. 1). A single larva was subsequently introduced to each petri dish after the nematodes had been settled in them for 30 min at 26±2ºC in an incubator. The petri dishes were sealed and incubated at 26±2ºC, and larval mortality was noted every 24 h for a total observation period of 120 h. The primary indicators of larval death caused by EPNs include changes in colour, lack of movement, and the odour released from the dead larvae. The larval cadavers were examined using an Olympus CX41 microscope, after cleaning them with distilled water, to determine if they were infected by nematodes. The experiment was conducted twice for all the EPN isolates, with eight replicas set for each nematode concentration (16 larvae per concentration). The control set up consisted of a petri dish lined with filter paper that had been moistened with distilled water. A single larva was introduced to each petri dish and the replicates were set up as in the mortality assay. The assay data were arcsine-transformed to determine larval mortality rates, the lethal concentration (LC_{50}) and lethal time (LT_{50}) of the four EPN isolates.

Reproductive potential of the EPNs

The reproductive potential (number of offspring produced) of the EPNs inside the larvae of *O. longicollis* was evaluated. The experiment was set up according to the mortality assay, including replicates and control plates. After being separately cleaned with distilled water to remove any nematodes attached to the exterior surface, the dead larva was laid on a white trap to allow the nematode reproduction within the insect's cadaver (White 1927). The total number of emerging IJs from each plate was counted

to determine the reproductive potential of the studied EPNs at different concentrations.

Data analysis

Insect larval mortality data were corrected (arcsine-transformed) and depicted as mean percentage ± standard error of the mean (SEM). Data acquired from all the assays were subjected to analysis utilizing IBM SPSS Statistics (version 20). Arcsine-transformed mortality data were subjected to Pearson correlation analysis to investigate the relationship between larval mortality rates with both EPN dosage and duration of larval exposure to nematodes. The LC_{50} and LT_{50} were calculated by probit analysis (the Pearson Goodness of Fit Test at a 0.05% significance level). A one-way analysis of variance (ANOVA) was employed to ascertain whether the larval mortality rates exhibit statistically significant differences when compared among the four entomopathogenic nematodes (at a significance level of $p \leq 0.05$). EPN multiplication was presented in the form of total progenies (IJs) emerged ±SEM per larval cadaver at different nematode concentrations for all the isolates. Pearson correlation analysis was performed to determine the relationship between the multiplication rate and the dosage of nematodes for all EPN isolates. One-way ANOVA was employed to ascertain whether multiplication rates significantly vary among the EPN isolates (at a significance level of $p \leq 0.05$).

RESULTS AND DISCUSSION

EPN isolation and identification

The survey, from several ecological sites (Figure 1), resulted in 19 positive samples (out of 538) for EPNs (3.53%). A total of 36 different sites were surveyed, and 13 sites (36.11%) tested positive (Table 1). All of the EPN-positive samples in this study originated are from sandy loam soils. Nevertheless, throughout the severe winter and rainy seasons, EPNs were not recovered. The Chite locality had the highest positivity rate (50%) among all the positive collection sites, and the heterorhabditids were more prevalent than the steinernematids.

Morphological and morphometric characterization

The isolated EPNs were characterized and further compared using morphological and morphometric studies of their various life stages (Table 2, Table 3). Of all the eight samples that tested positive for heterorhabditids, seven nematodes isolated from Tuirial, Chuhvel, Khawkawn, Khopai, Lunglei, Mat, and Chite had morphometric values within the same range and resembled *H. indica* (Poinar et al. 1992). *H. indica* was morphologically differentiated by short body length (BL) of IJs, distance of anterior end to excretory pore (EP), and body width/tail length (F). However, despite sharing morphometric and molecular similarities with the other seven isolates, the heterorhabditid isolate from Dilkhan is distinguishable by the shape of the gubernaculum (Phan et al. 2003). The heterorhabditid isolate-infected larvae turned brick red and died 24-48 h post-infection. The three steinernematid isolates from Chalrang, PUC campus, and Kawlbem exhibited morphological and morphometric resemblance to *S. sangi* (Phan et al. 2001). The diagnostic characters of *S. sangi* includes the length of the body, gubernaculum and spicule morphology in males, absence of epiptygma, presence of mucron in females, and the length of the anterior end to the excretory pore (EP), and furthermore, the infected dead larvae developed a faint pink-yellow colouration. On the other hand, the steinernematid isolates from Putlungasih and Mamit produced charcoal-grey colouring on infected larvae and were morphologically conspecific to *S. surkhetense* described by Khatri-Chhetri et al. (2011). The species of *S. surkhetense* was differentiated by BL, oesophagus length (OeL), and EP, in conjunction with spicule length of the first-generation male.

Table 1. Geographical distribution of entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*) in Mizoram, Northeastern India

Isolate code	Entomopathogenic nematodes	Locality	% Positivity	Habitat type
Sa1	*Steinernema sangi*	Chalrang (23.3825N, 93.1467E)	2.8	Forest
Sa2	*Steinernema sangi*	PUC Campus (23.7256N, 92.7297E)	2.6	Forest
Sa3 & Sa4	*Steinernema sangi*	Kawlbem (23.8703N, 93.3067E)	3.8	Roadside
PTS	*Steinernema surkhetense*	Putlungasih (22.8150N, 92.5861E)	4.2	Riparian forest
MAM	*Steinernema surkhetense*	Mamit (23.9083N, 92.5094E)	11.1	Forest
TR	*Heterorhabditis indica*	Tuirial (23.7214N, 92.8003E)	4.2	Riparian forest
CH	*Heterorhabditis indica*	Chuhvel (23.9533N, 92.4219E)	4.5	Forest
KK	*Heterorhabditis indica*	Khawkawn (23.8472N, 93.1292E)	3.7	Forest
KP	*Heterorhabditis indica*	Khopai (22.1883N, 93.0492E)	8.7	Riparian forest
LL	*Heterorhabditis indica*	Lunglei (22.9314N, 92.7419E)	7.6	Riparian forest
MAT	*Heterorhabditis indica*	Mat, Lunglei (23.3019N, 92.8083E)	9.1	Riparian forest
CT	*Heterorhabditis indica*	Chite, Aizawl (24.4622N, 92.7633E)	50	Agroforest
DK	*Heterorhabditis baujardi*	Dilkhan (23.7375N, 92.9517E)	4.2	Forest

Table 2. Morphometric measurements (Mean±SEM) of *Heterorhabditis* spp. in µm

Characters	Infective juvenile				Hermaphrodite female			
	Heterorhabditis indica		*Heterorhabditis baujardi*		*Heterorhabditis indica*		*Heterorhabditis baujardi*	
	This study	**Poinar et al. 1992**	**This study**	**Phan et al. 2003**	**This study**	**Poinar et al. 2003**	**This study**	**Phan et al. 2003**
BL	622.9±6.9 (578-694)	528±26	571±4.9 (525-615)	487-622	3958.3±85 (2510-4820)	2700±1000 (2300-3100)	3548±38.98 (3250-3970)	3105-5400
EP	97.8±0.7 (92.5-105)	98±7 (88-107)	91.5±0.6 (87.5-95.5)	90-105	160.5±1.8 (145-180)	173±8 (163-187)	164.6±2.6 (150-185)	119-197
NR	79.3±0.9 (75-87.5)	82±4 (72-85)	77.8±1.2 (67.5-85)	74-87	120.4±31.4 (107.5-135)	115±5 (104-123)	126.4±1.1 (120-135)	92-147
F	0.22±0.004 (0.2-0.3)	0.20±0.05 (0.18-0.22)	0.22±0.004 (0.2-0.3)	0.19-0.27	2.3±0.07 (1.1-2.8)	-	3.15±0.07 (2.67-3.88)	-
SpL	-	-	-	-	-	-	-	-
SpW	-	-	-	-	-	-	-	-
GuL	-	-	-	-	-	-	-	-
GuW	-	-	-	-	-	-	-	-

Characters	Second generation male				Second generation female			
	Heterorhabditis indica		*Heterorhabditis baujardi*		*Heterorhabditis indica*		*Heterorhabditis baujardi*	
	This study	**Poinar et al. 1992**	**This study**	**Phan et al. 2003**	**This study**	**Poinar et al. 2003**	**This study**	**Phan et al. 2003**
BL	881.8±9 (805-947.5)	(721±64) 573-788	814.7±14 (710-902.5)	641-1072	2077.1±28 (1740-2295)	1600±0.12 (1200-1800)	2185±14.39 (2060-2290)	1335-2385
EP	113.5±0.5 (110-117.5)	123±7 (109-138)	88±0.7 (82.5-92.5)	71-107	122.9±1.2 (115-135)	127±4 118-138	108.2±1.3 (97.5-115)	98-156
NR	70.8±1.3 (62.5-80)	75±4 (72-85)	60.6±1.4 (52.5-67.5)	54-83	96±1.3 (85-105)	92±4 (88-96)	89.5±1.2 (77.5-107.5)	75-123
F	1.8±0.04 (1.4-2.2)	-	1.2±0.0 (1.1-1.4)	-	1.9±0.03 (1.5-2.1)	-	1.9±0.04 (1.6-2.2)	-
SpL	43±0.6 (37.5-47.5)	(43±3) 35-48	45±0.4 (42.5-47.5)	31-47	-	-	-	-
SpW	5.6±0.23 (5-7.5)	4-6 (5±1.3)	6.1±0.3 (5-7.5)	-	-	-	-	-
GuL	23.1±0.4 (20-25)	0.9±0.1 (0.6-1.2)	24.1±0.6 (20-27.5)	14-26	-	-	-	-
GuW	2.5±0.0	0.6-1.2 (0.9±0.1)	-	-	-	-	-	-

Note: BL: Body Length, EP: Distance of anterior end to excretory pore, NR: Distance of anterior end to nerve ring, F: Body width/tail length, SpL: Spicule Length; SpW: Spicule Width, GuL: Gubernaculum Length, GuW: Gubernaculum Width

Table 3. Morphometric measurements (Mean±SEM) of *Steinernema* spp. in µm

Characters	Infective juvenile			
	Steinernema sangi		*Steinernema surkhetense*	
	This study	**Phan et al. 2001**	**This study**	**Khatri-Chhetri et al. 2011**
BL	749.0±5.9 (698.0-798.0)	753±18 (704-784)	449.4±4.4 (418.0-480.0)	415±15 (393-450)
EP	48.8±0.6 (42.5-52.5)	51±1.8 (46-54)	34.5±0.6 (30-37.5)	32±1.5 (28-34)
NR	95.0±0.7 (87.5-100)		64.7±1 (57.5-72.5)	63±3.1 (57-70)
SpL	-		-	
SpW	-		-	
GuL	-		-	
GuW	-		-	

Characters	First generation male			
	Steinernema sangi		*Steinernema surkhetense*	
	This study	**Phan et al. 2001**	**This study**	**Khatri-Chhetri et al. 2011**
BL	1585.0±51 (1322.5-2192.5)	1674±220.5 (1440-2325)	1280.4±32.6 (1065.0-1477.5)	1224±194
EP	76.3±1.2 (67.5-92.5)	82±9.2 (67-99)	61.6±1.0 (52.5-67.5)	55±10 (43-78)
NR	-	126±13 (109-166)	-	92 ±19 (59-140)
SpL	6±0.7 (55-72.5)	63±4.4 (58-80	69.1±0.8 (65-75)	70±5.2 (58-78)
SpW	12.5± 0.3 (10-15)	12±1.1 (10-14)	9.7±0.4 (7.5-12.5)	230±38 (168-330)
GuL	37.5±0.8 (30-45)	40±3.1 (34-46)	44.6±0.9 (40-52.5)	52±5.1 (42-63)
GuW	7.5±0.2 (5-10)	7±0.8 (5-9)	4.7±0.2 (2.5-5)	

Characters	First generation female			
	Steinernema sangi		*Steinernema surkhetense*	
	This study	**Phan et al. 2001**	**This study**	**Khatri-Chhetri et al. 2011**
BL	5959.6±147.6 (4580.0-7120.0)	6030±679.8 (4830-7200)	4600.9±133.6 (3690.0-5830.0)	2970±922 (1894-5692)
EP	98.4±1.6 (77.5-115)	101±10.4 (80-121)	85.8±1 (77.5-92.5)	53±13 (33-86)
NR	158.0±8.2 (140-170)	158±8.2 (140-170)	144.1±1 (137.5-152.5)	93±17 (65-114)
SpL	-		-	
SpW	-		-	
GuL	-		-	
GuW	-	5±12.2 (43-53)	-	

Note: BL: Body Length, EP: Distance of anterior end to excretory pore, NR: Distance of anterior end to nerve ring, F: Body width/tail length, SpL: Spicule Length; SpW: Spicule Width, GuL: Gubernaculum Length, GuW: Gubernaculum Width

Molecular characterization of Steinernematid isolates

The final ITS and 28S rDNA sequences of the EPN isolates were deposited to NCBI GenBank and used for phylogenetic analysis (Table 4). The phylogenetic tree of the isolated steinernematids was generated utilizing the concatenated nucleotide sequences derived from both the sequences produced in this study and the available databases for the two genes (Figure 2). Furthermore, the phylogenetic analysis included representative sequence for all *Steinernema* species that are members of both the '*feltiae*' and '*carpocapsae*' clades. However, only one representative sequence for each of the remaining *Steinernema* clades (*glaseri*, *cameroonense*, *monticolum*, *bicornutum*, and *affine*) was considered in the phylogenetic analysis.

Through comparative analysis, the ITS rDNA sequences we have developed for the steinernematids (accession numbers PQ776601, PQ783621, PQ780099, and PQ780100) demonstrated a remarkable 99% similarity with the corresponding sequences in the database for *S. sangi*. In contrast, for the same species, our generated 28S rDNA sequences (PV521984-PV521987) shared 92.41% similarity with available database sequences of *S. sangi* (GU569057). Meanwhile, our developed sequences shared 96% similarity with *S. akhursti*, *S. feltiae*, *S. kushidai*, *S. puntauvense*, *S. oreganense*, *S. tielingense*, and *S. xinbiense*; however, the sequences of all other recognized *Steinernema* species documented in GenBank showed ≤95% similarity.

Concurrently, the developed ITS rDNA sequences of accession no. MF618308-MF618312 exhibited a similarity range of 99-100% with the GenBank sequences of *S. surkhetense*. In addition, the developed sequences displayed 94% similarity with *S. nepalense* and *S. carpocapsae*; however, they exhibited ≤93% similarity with sequences available for other *Steinernema* species in the GenBank. Simultaneously, the developed 28S rDNA sequences (accession no. MF621001-MF621007) exhibited 100% similarity with GenBank sequences of *S. surkhetense*. Furthermore, the developed sequences exhibited a close similarity (98-99%) with GenBank sequences of *S. carpocapsae*, *S. huense*, *S. nepalense*, *S. siamkayai*, and *S. cumgarense*, while showing a similarity of ≤90% with the other compared GenBank sequences.

Table 4. GenBank submission of entomopathogenic nematodes Mizoram, Northeastern India

Entomopathogenic nematodes	Voucher no.	GenBank (NCBI)	
		ITS	28S
S. sangi	Sa1	PQ776601	PV521984
S. sangi	Sa2	PQ780099	PV521985
S. sangi	Sa3	PQ780100	PV521986
S. sangi	Sa4	PQ783621	PV521987
S. surkhetense	StPTS1	MF618308	MF621001
S. surkhetense	StPTS2	MF618309	MF621002
S. surkhetense	StPTS3	MF618310	MF621003
S. surkhetense	StPTS4	MF618311	MF621004
S. surkhetense	StPTS5	MF618312	MF621005
H. indica	HeTR	MF618316	MF621009
H. indica	HeTC	MF618315	MF621006
H. indica	HeTV	MF618318	-
H. indica	HeTRE	MF618314	MF621008
H. indica	HeTS	MF618317	MF621010
H. indica	HeM	MF618313	MF621007
H. indica	CT	PV533785	-
H. baujardi	Hb1	PQ780169	PQ780168
H. baujardi	Hb2	PQ780170	PQ783622
H. baujardi	Hb2	PQ780172	PQ783623

Molecular characterization of Heterorhabditid isolates

The phylogenetic tree of the heterorhabditids was generated based on the concatenated sequences of the two genes (Figure 3). The generated ITS rDNA sequences (accession no. MF618313-MF618318 and PV533785) of heterorhabditid isolate exhibited 99-100% similarity with the GenBank sequences of *H. indica,* including *H. pakistanense* (syn. with *H. indica*). Further, it showed ≤98.50% similarity (15 bp difference) with *H. noenieputensis*, whereas, the sequences of all other recognized *Heterorhabditis* species documented in GenBank exhibited <90%. Simultaneously, the developed 28S rDNA sequences (accession no. MF621006MF621010) revealed 99-100% similarity with the GenBank sequences of *H. indica*. The sequences further exhibited <97% similarity with all the other compared GenBank sequences (except for *H. noenieputensis* MT372505 and JX624110, which had 99.65% similarity).

In contrast, the developed ITS rDNA sequences (PQ780169, PQ780170, and PQ780172) of another heterorhabditid isolate exhibited 99.47-99.62% similarity with the GenBank sequences of *H. baujardi* (except for *H. baujardi* EU363039 which may be a case of potential misidentification). Further, the obtained sequence exhibited 98% similarity with *H. amazonensis*, *H. floridensis*, and *H. mexicana*; 96% similarity with *H. sonorensis* and *H. taysearae*; ≤90% similarity with all the other compared GenBank *Heterorhabditis* species. The 28S rDNA sequence of *H. baujardi* is not available in the GenBank, however, the developed 28S rDNA sequences (PQ780168, PQ783622, and PQ783623) exhibited 99.45% similarity (5 bp difference) with *H. amazonensis* (EU099036), 98.79% similarity (11 bp difference) with *H. mexicana* (EU100414), 98.79% similarity (11 bp difference) with *H. floridensis* (EU099034), and 97.23% similarity (26 bp difference) with *H. noenieputensis* (JX624110).

Genetic distance

Steinernema sangi exhibited a wide gap from other *Steinernema* species of the '*feltiae* clade' (p-distance = 10.66±0.67% and 4.39±0.27% for ITS and 28S rDNA respectively) and no gap was observed with the GenBank sequences of *S. sangi* (p-distance = 0.0±0.0%). The generated gene sequences of *S. surkhetense* revealed that it is distinct from all other *Steinernema* species of the '*carpocapsae* clade' (p-distance = 7.07±0.45% and 2.21±0.67% for ITS and 28S rDNA respectively) but exhibited 0.34±0.19% genetic distance with GenBank sequences of *S. surkhetense*. Furthermore, the isolated *H. indica* exhibited 9.82±1.39% and 2.7±0.59% genetic distance for ITS and 28S rDNA, respectively from other species of the '*indica* clade' but no gap was observed with the GenBank sequences of *H. indica* (p-distance = 0.0±0.0%).

Similarly, another isolated *Heterorhabditis* sequence seems conspecific to *H. baujardi* (p-distance = 0.0±0.0%) since no gap was observed between them. However, it exhibited 4.76±1.53% and 2.27±0.75% genetic distance for ITS and 28S rDNA respectively with other *Heterorhabditis* species of the '*indica* clade'.

Pathogenicity of EPN isolates against *Odoiporus longicollis*

Larval mortality assay

All the EPN isolates exhibited high pathogenicity against *O. longicollis* with larval mortality rates ranging from 6.25 to100% (Figures 5.A, 5.B, 5.C, 5.D), indicating successful infection and proliferation at varying nematode concentrations. Nevertheless, among the studied EPN species, no discernible variations in the mortality rates were found [$F_{(3, 60)} = 1.36$, $p = 0.26$] In addition, *O. longicollis* mortality positively correlates with the applied nematode concentrations (r = 0.71, 0.89, 0.83, and 0.84, respectively for *S. sangi*, *S. surkhetense*, *H. indica*, and *H. baujardi*), and with the incubation period.

At the lowest concentration (10 IJs/insect), only *S. sangi* and *H. indica* induced larval mortality (6.25%) during the first 24 h of incubation. This mortality increased to 25.5% at 120 h post-incubation. In the same concentration of nematodes, *H. baujardi* and *S. surkhetense* caused initial larval mortality at 48 and 72 h of incubation, respectively. The lowest nematode concentration at which 100% larval mortality was observed was 800 IJs/insect for both *H. indica* and *S. sangi* (after 120 h of incubation). Further, after 120 h of incubation, both *H. baujardi* and *S. surkhetense* induced 100% larval mortality at 1600 IJs/insect.

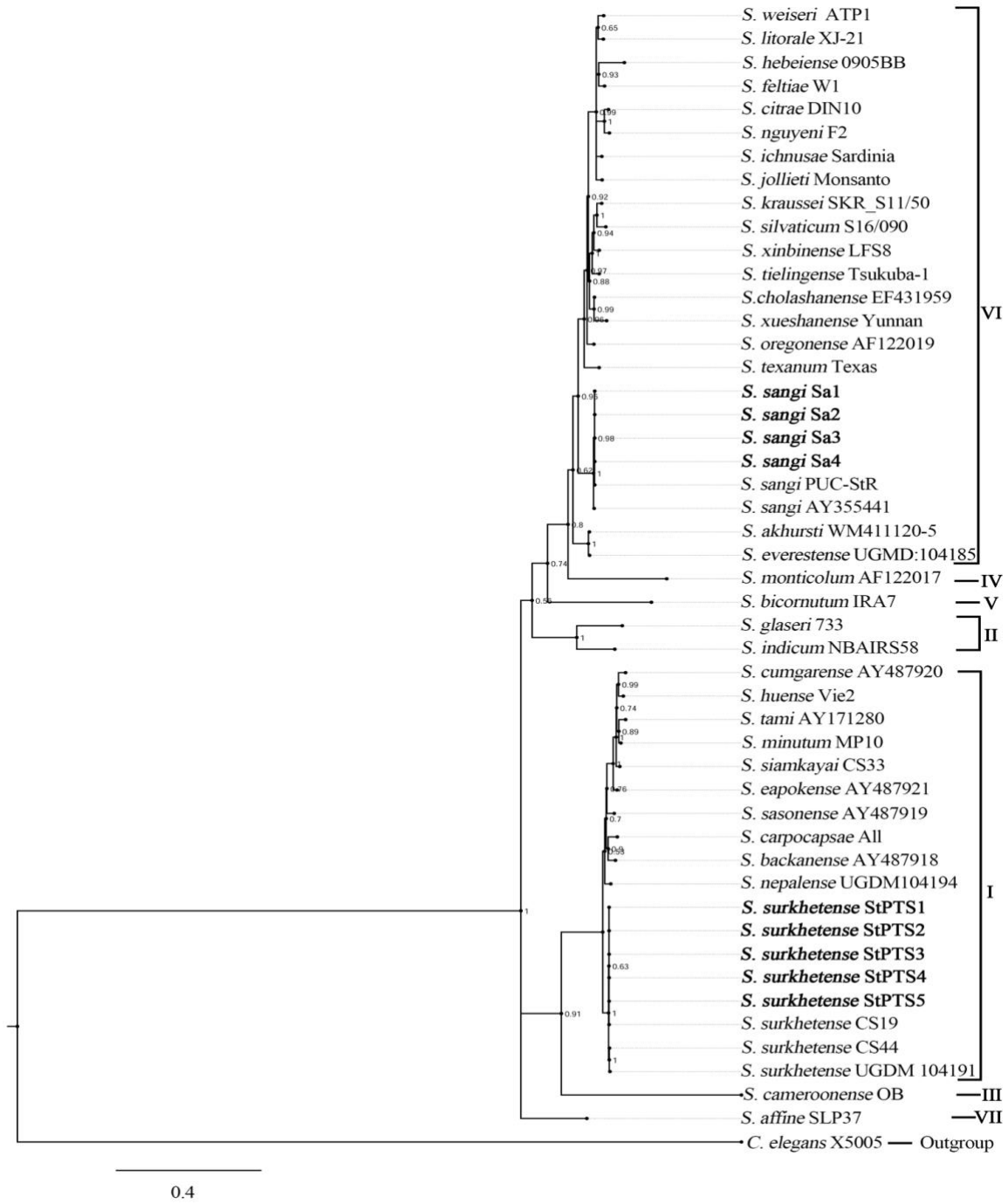

Figure 2. Bayesian phylogenetic tree of *Steinernema* species reconstructed from ITS and 28S rDNA sequences. Numbers at the nodes show the branch probability values. Clades of various *Steinernema* sp. such as *carpocapsae* (I), *glaseri* (II), *cameroonense* (III), *monticolum* (IV), *bicornutum* (V), *feltiae* (VI), and *affine* (VII) are shown in the figure. The bold letters represent the Mizoram EPN isolates.

LC_{50} and LT_{50}

Heterorhabditis indica was the most virulent, showing the lowest LC_{50} and LT_{50} values while *S. surkhetense* was the least virulent among the EPN isolates. The LC_{50} values of *H. indica* were 172.66 and 18.88 IJs/insect at 24 and 120 h of incubation respectively, while those of *H. baujardi* were 379.72 and 33.17 at 24 and 120 h of incubation respectively. However, the LC_{50} values for the steinernematids were significantly higher during the first incubation period; at 120 h, the values for *S. sangi* and *S. surkhetense* were 33.19 and 71.34, respectively. In terms of median lethal time (LT_{50}), the lowest obtained values were 17.76, 14.75, 6.50 and 8.26 for *S. sangi*, *S. surkhetense*, *H. indica* and *H. baujardi,* respectively at 1600 IJs/insect. On the other hand, the highest obtained values were 290.05, 286.32, 273.53, and 337.53 for *S. sangi*, *S. surkhetense*, *H. indica* and *H. baujardi,* respectively at 10 IJs/insect.

EPN multiplication in Odoiporus longicollis

The total number of offspring produced per *O. longicollis* larva varied at different nematode concentrations (Figure 6). This study showed that the two heterorhabditid isolates exhibited higher reproductive rates than the steinernematid isolates. The nematode concentration (IJs/insect) was positively correlated with the total number of offspring produced by the three isolates viz. *H. baujardi*, *S. sangi*, and *S. surkhetense*, but varied significantly between the studied EPNs [$F_{(3, 60)} = 144.80$, $p = 0.01$]. The offsprings produced, in the case of *H. indica*, were 77.5±14.44 x 10^3 and 125.97±31.49 x 10^3 IJs at a concentration of 10 and 200 IJs/insect, respectively, however, it decreased subsequently as the EPN concentrations increased. As for *H. baujardi*, the offspring production was 77.0±19.3 x 10^3 at 10 IJs/insect and 126.0±32.4 x 10^3 at 200 IJs/insect. Simultaneously, *S. sangi* achieved its maximum reproduction rate at 1600 IJs/insect (45.0±7.64 x 10^3 offspring) and produced 28.0±69.0 x 10^3 offspring at 10 IJs/insect. *S. surkhetense*, on the other hand, has the lowest multiplication rate among the studied EPNs, producing 5.52±1.13 x 10^3 IJs and 11.41±2.53 x 10^3 IJs at 10 and 800 IJs/insect, respectively.

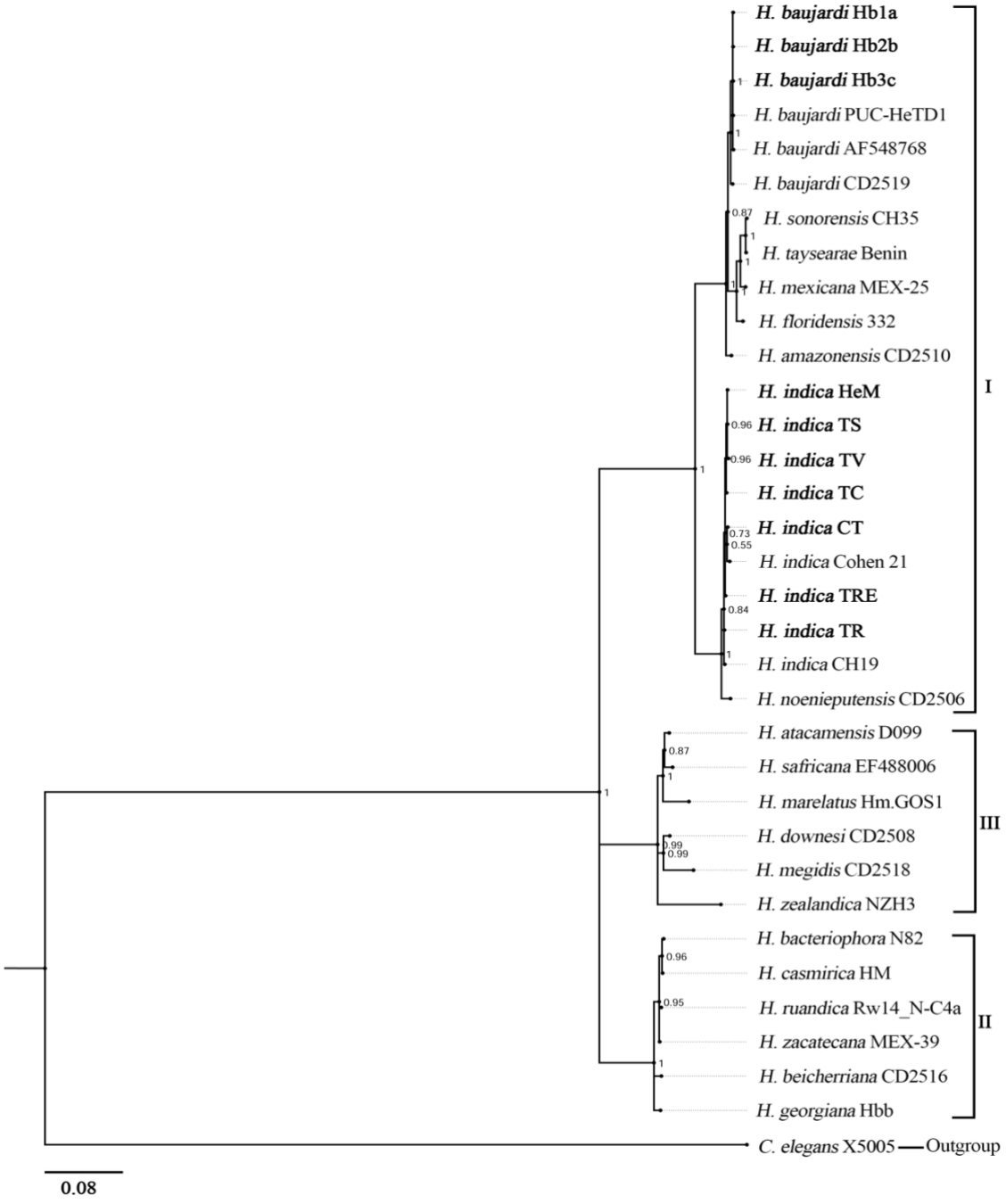

Figure 3. Bayesian phylogenetic tree of *Heterorhabditis* species reconstructed from ITS and 28S rDNA sequences. Numbers at the nodes show the branch probability values. Clades of various *Heterorhabditis* spp. such as *indica* (I), *bacteriophora* (II), *megidis* (III) are shown in the figure. The bold letters represent the Mizoram EPN isolates

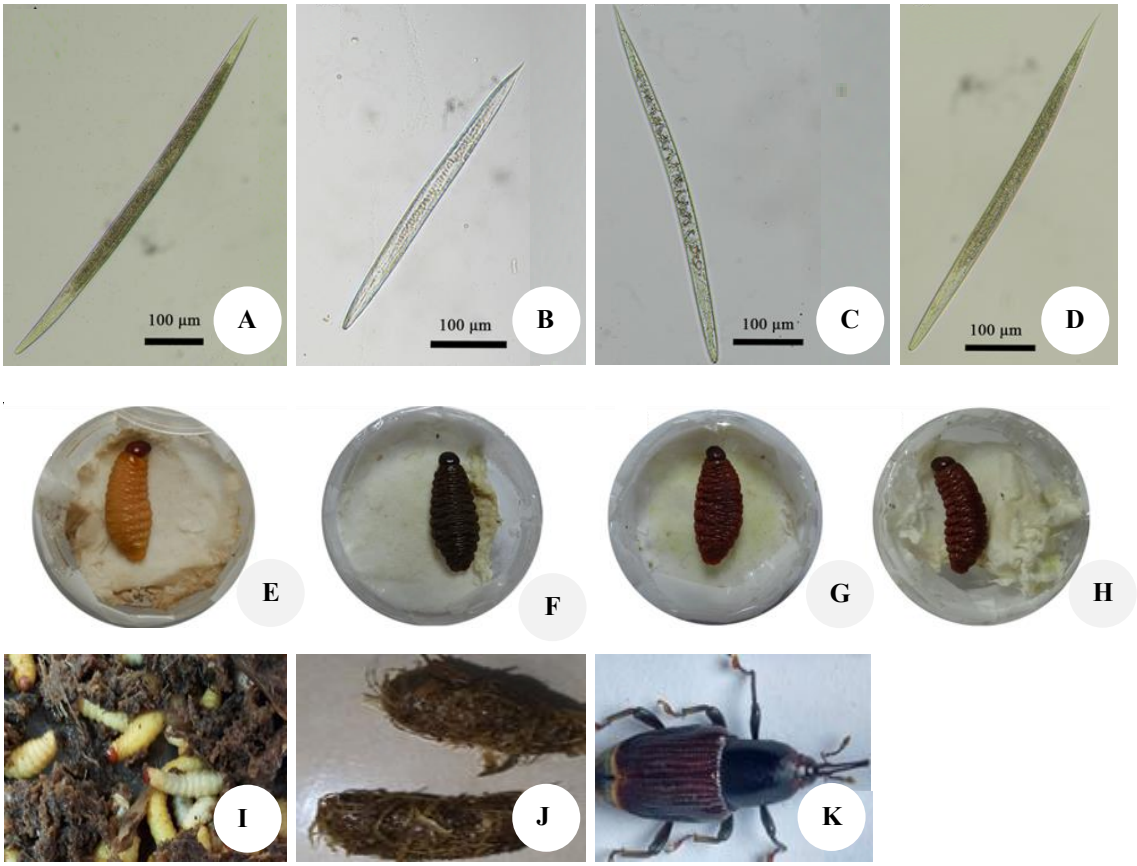

Figure 4. A. Infective juveniles of *S. sangi*, B. Infective juveniles of *S. surkhetense*, C. Infective juveniles of *H. indica*, D. Infective juveniles of *H. baujardi*, E. *S. sangi* infected larva showing faint pink-yellow colouration, F. *S. surkhetense* infected larva showing charcoal-grey colouration, G. *H. indica* infected larva showing brick-red colouration, H. *H. baujardi* infected larva showing brick-red colouration I. Larvae of *O. longicollis*, J. Pupae of *O. longicollis*, K. Adult of *O. longicollis*

Discussion

The study reports the occurrence of four species of the EPNs viz. *Steinernema sangi*, *S. surkhetense*, *Heterorhabditis indica*, and *H. baujardi* in Mizoram, Northeastern India. Among the reported species, the occurrence of *S. sangi* and *H. baujardi*, in Mizoram, India has already been reported (Lalramnghaki et al. 2017; Vanlalhlimpuia et al. 2018). Given the limited variations between closely related species and genera, particularly among nematodes, the integration of morphological and molecular analyses is strongly recommended to resolve taxonomic ambiguities (Nisa et al. 2022). When comparing the two gene markers, ITS rDNA probed more effective than 28S rDNA in resolving the identity of the EPN isolates. While the 28S rDNA gene prove some clarity for differentiating steinernematid isolates, it failed to distinguish *H. indica* and *H. baujardi* and *Heterorhabditis* species (p-distance ≤0.03). However, a notable mismatch was observed between the obtained 28S rDNA sequences of *S. sangi* and other database entries for the same species, with *S. sangi* (GU569057) showing only 92.41% similarity, which may indicate a possible misidentification of the later species.

Several studies have reported that EPN are more prevalent in agricultural fields than in natural habitats, which aligns with the findings of Consabo et al. (2025). In the present study, the higher recovery of EPNs from the natural ecosystems (specifically Forest and Riparian Forest) may be attributed to the greater diversity and composition of soil biotic communities, particularly, vegetation type and presence of insect hosts, temperature, soil type, and precipitation, and further, due to extensive sampling conducted in the natural ecosystem. Furthermore, the adaptability of EPNs to the prevailing environmental conditions could potentially determine their pathogenicity against insect hosts and their suitability for pest control strategies (Ghoneim and Hassan 2024; Fatimah et al. 2025). The implementation of another survey considering both abiotic parameters and soil biota at the EPN collection sites is required for deriving scientific conclusions on their geographical occurrence across Mizoram.

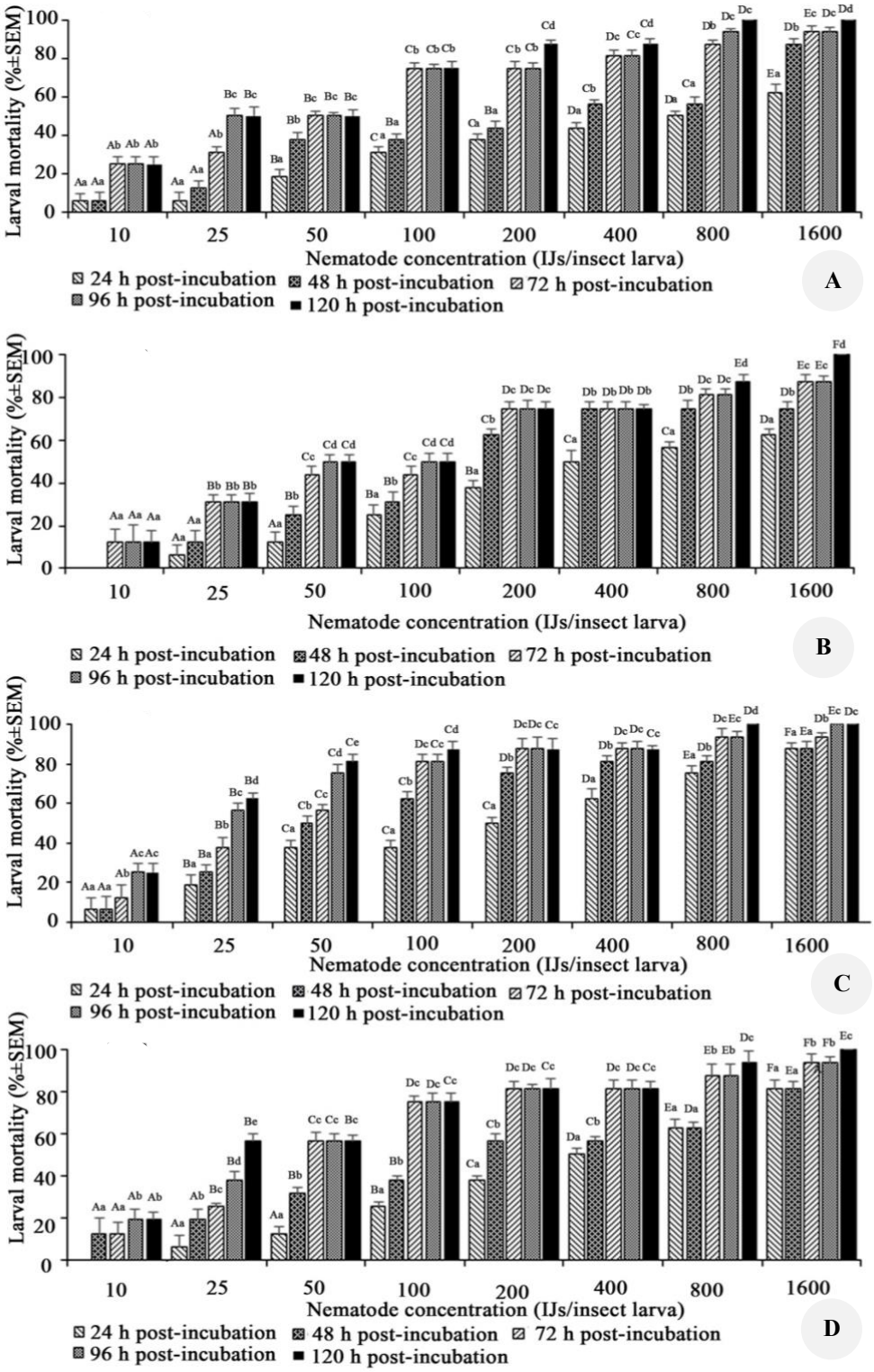

Figure 5. Insect larval mortality rates (Mean±SEM) following incubation with: A. *Steinernema sangi*, B. *Steinernema surkhetense*, C. *Heterorhabditis indica*, D. *Heterorhabditis baujardi*, are presented. The vertical lines denote the standard error of the mean values. For the same incubation time, vertical bars with the same uppercase letter denote the absence of statistically significant differences ($p>0.05$) among the nematode concentrations affecting *Odoiporus longicollis* mortality. For the same nematode concentration, vertical bars with the same lowercase letter indicate the absence of significant differences ($p>0.05$) in *O. longicollis* mortality between different incubation time

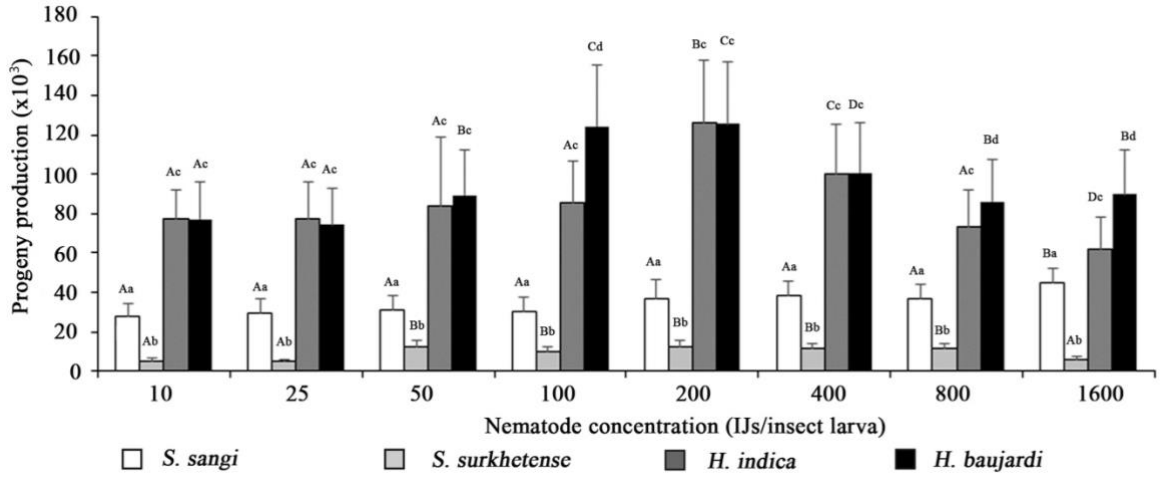

Figure 6. EPN multiplication on larvae of *Odoiporus longicollis*. The vertical lines denote the standard error of the mean values. For the same nematode isolate, bars sharing the same uppercase letter indicate the absence of significant differences between the nematode concentrations. Bars sharing the same lowercase letter indicate the absence of significant differences among the EPN isolates at the same nematode concentration

The banana pseudostem weevil, *Odoiporus longicollis*, was effectively controlled by all four EPN species studied, and further, the EPNs can be reproduced successfully inside the pest larva. The LC_{50} and LT_{50} values revealed that the two heterorhabditids were more pathogenic than the steinernematids. Our results slightly differ from Giribabu et al. (2020), who reported that the steinernematid (*S. siamkayai*) was more pathogenic than the heterorhabditid (*H. indica*) in terms of the host mortality rate. In their study, *S. siamkayai* and *H. indica* caused 74.6% and 69.20% larval mortality respectively, at a dosage 200 IJs/insect and 72 h of incubation. In concordance with their study, the present study recorded 81.25-87.5% and 75% larval mortality for steinernematid and heterorhabditid isolates, respectively at 200 IJs/insect dosage and 72 h of incubation. Mwaitulo et al. (2011), while studying the pathogenicity of nine EPN isolates against both larval and adult stages of the banana root borer, *Cosmopolites sordidus*, reported that all the EPN isolates could kill and get successfully established in the larvae, whereas adult mortality remained low after 96 h of exposure. They also observed that 50 IJs caused up to ≤50% larval mortality, increasing to 100% at higher concentrations (500 and 1000 IJs). Similarly, in our study, the EPN isolates produced about 50% larval mortality (50 IJs/insect concentration) against *O. longicollis* after 48, 72, 96, and 96 h of incubation, with mortality reaching 100% at higher concentrations despite differences in banana weevil species used, our results were comparable to those of Mwaitulo et al. (2011). In addition, Treverrow et al. (1991) reported the significant larval and adult mortality of *C. sordidus* upon exposure to two isolates of *S. carpocapsae*. Regarding the progeny production, all four studied nematodes reproduced successfully in the host cadaver, resulting in the successful emergence of IJs. Several studies have also reported higher progeny production in heterorhabditids than in steinernematids, which may be due to variations in behaviour, size differences between nematodes and their hosts, and the hermaphroditic nature of heterorhabditids (Ngoma et al. 2016; Shamseldean et al. 2024).

In conclusion, the isolation of four EPN species from Mizoram implies a potential expansion of their distributional range into neighbouring countries, including Bangladesh, Myanmar, and other regions of Northeastern India. Based on our study, we concluded that *H. indica* is the most pathogenic against the pest *O. longicollis*, while *S. surkhetense* exhibits the lowest pathogenicity among the EPN isolates. The proven effectiveness of the local EPNs isolates against *O. longicollis*, coupled with the advancement of formulation strategies, will render them appropriate for practical application in field conditions against the pest. It is thus envisaged that this study may advance the isolation of additional EPN species from the region and facilitate comprehensive investigations into their effectiveness against insect pests. This study represents the only scientific investigation of the susceptibility of *O. longicollis* to EPNs viz. *S. sangi*, *S. surkhetense*, and *H. baujardi*.

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