MOLECULAR CHARACTERIZATION AND EVALUATION OF LOCALLY ISOLATED ENTOMOPATHOGENIC NEMATODES AGAINST MAJOR INSECT PESTS IN MIZORAM, INDIA

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Molecular Characterization and Evaluation of Locally Isolated Entomopathogenic Nematodes against Major Insect Pests in Mizoram, India

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In partial fulfilment of the requirement of the degree of Doctor of Philosophy in Zoology of Mizoram University, Aizawl.

SUPERVISOR'S CERTIFICATE

I certify that the thesis entitled "Molecular Characterization and Evaluation of Locally Isolated Entomopathogenic Nematodes against Major Insect Pests in Mizoram, India" submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by H.C. LALRAMNGHAKI is a record of research work carried out during the period of 2018-2022 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this University or any other University or institution of higher learning.

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DECLARATION

I, H.C. Lalramnghaki, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any other University or Institute.

This is being submitted to the Mizoram University for the degree of Doctor of Philosophy in Zoology.

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(H.C. LALRAMNGHAKI)

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Abbreviations

@	at the rate of
cm	centimeter
et al.	et alii/alia (Latin: and others)
Fig.	Figure
g	gram
hrs	hours
Kg	Kilogram
mg	milligram
ml	mililiter
min	minute
NE	northeast
ng	nanogram
sec	second
SEM	Standard error of mean
SPSS	Statistical Programme for Social Science
w/w	weight by volume
v/v	volume by volume
%	Percentage

General introduction

Agriculture pests have been controlled by humans for thousands of years. In addition to the insects and mites that feed on plants, pests can also include weeds, plant pathogens (specific fungi, bacteria, and viruses), rodents, nematodes, plant pathogenic fungus, bacteria and viruses. They are thought to be responsible for up to one-third of all agricultural productivity losses. Cultural practises like weeding, the use of pesticides, and the selective breeding of plants for pest resistance are only a few examples of the extensive range of pest management strategies available. Agricultural pests, particularly plant pathogenic microbes and insects that feed on crops are essential components of agroecosystems where they have evolved alongside crops for centuries **(McCann *et al*., 2013)**. Additionally, there are a number of intricate relationships between the pests (and diseases) and their host plant that could affect them in various ways **(Berger *et al*., 2007)**.

With the rising world population, global production of agriculture needs to double by 2050 to fulfil this kind of escalating need, since the world's population will have increased demand for crop production **(Tilman *et al*., 2011)**. Numerous studies have suggested that the best sustainable strategy for ensuring food security is to increase crop output as opposed to clearing more land surface for agricultural production **(Godfray *et al*., 2010)**.

Chemical pesticides have been widely used in agriculture and have significantly altered agroecosystems long time back, turning them from typically small-scale, reasonably diversified natural groups into expansive monocultures. Since early 1940s, significant advances in synthetic organic chemistry paved the way

for a new era of pest control which leads to initial application of DDT to combat insect-borne diseases. Ever since then, the majority of the world's agricultural sectors have used significantly more pesticides than they did previously, and yields have increased significantly.

With the increasing concern on resistance that several insect pests have developed against certain chemical insecticides and the substantial risks posed to the environment, non-targeted creatures, and public health, it is necessary to look for different biological control agents as well as a search for alternative biological control agents is necessitated. The best approach for controlling insect pests is biological control because it is affordable and has minimal effect on human (and other non-target organisms) and the environment. Biological control offers a promising strategy against a wide range of insect pests, and biopesticides being environmentally safer are potential substitutes for chemical insecticides. EPNs of the families steinernematidae and heterorhabditidae, like other biological control agents, have strong bio-control potential with which to manage economically significant insect pests and incorporate them into insect pest management **(Lacey and Georgis, 2012)**. EPNs are natural inhabitant of soils and have been utilized to manage a variety of insect pests as they have several characteristics of prospective biological control agents **(Koppenhöfer, 2007)**.

EPNs are obligate and fatal parasites of insects that live in symbiotic relationships with bacteria, such as *Xenorhabdus* spp. in steinernematids and *Photorhabdus* spp. in heterorhabditids **(Poinar, 1990)**. The life cycle of both *Steinernema* and *Heterorhabditis* includes a third infective juvenile (IJ3) called dauer

juvenile. The dauer is a free-living diapause or dormant stage and possesses a relatively resistant cuticle to avoid dehydration, low metabolic rates **(O'Riordan and Burnell, 1989, 1990)** and likely food-storing intestinal granules **(Riddle, 1988)**. When the IJs come in contact with a suitable host, it enters the body through body openings through the mouth, anus and spiracle **(Triggiani and Poinar, 1976; Glazer and Salame, 2000)**, or directly through the soft integuments **(Koppenhöfer *et al*., 2000)**. The bacteria feed on insect haemolymph, rapidly divides, releases toxins and exoenzymes that usually kill the insect host within 24–48 hrs due to septicemia **(Poinar, 1983, Han and Ehlers, 2000)**. The nematode undergoes development for about two to three generations depending on the degree of parasitism and the level of host nutrient contents, and the adult development is stopped which results in the accumulation of IJs. The free-living IJs emerged from the host cadaver into the soil and may live for several months before they infect a new host **(Hominick, 1990)**.

Due to their high level of safety for mankind, non-target organisms, and the environment, tEPN have been virtually exempted from pesticide registration regulations in many countries (**Ehlers, 2005; Piedra Buena *et al*., 2015**). Immense works have been conducted for the isolation of climatically adapted indigenous species of EPNs or isolates with the intention of formulating and commercialising them later **(Hominick, 2002)**.

Rationale of the study

For the effective application of EPN as a biological control agent, proper identification and documentation of locally adapted species are necessary. This is

because isolates of EPNs from different geographical areas exhibit variances in their behavioural and physiological adaptations **(Stock *et al*., 1999)**.

Northeast India, situated at the Indo-Burma region is one of the 4 biodiversity hotspot regions in India harbouring various forms of flora and fauna. In India, a number of indigenous steinernematids and heterorhabditids have been documented. However, works on the EPNs in Northeast India is very limited, and moreover, less is known about their occurrence and potential in the region.

In light of the aforementioned account, it was deemed important to carry out a survey on the occurrence of EPN from Mizoram and assess their bioefficacy against major insect pests in Mizoram, Northeast India. The main aim of the study is:

1. To study the occurrence of EPNs from different areas across the state of Mizoram by collecting soil samples.

2. Molecularly characterizing the isolated EPNs in addition to basic morphological characterization for proper identification.

3. Analysing the bioefficacy of isolated indigenous EPN species against six agricultural pests in a laboratory.

Chapter 1

Isolation of Entomopathogenic nematodes (EPNs) from soil samples.

Introduction

EPNs species of the genera, *Steinernema* Travassos, 1927 and *Heterorhabditis* Poinar, 1976 (Rhabditida: Nematoda) have been an important concern for years due to the possibility of using them to biologically control insect pests. About 23 families of nematodes that parasitize insects have been described **(Koppenhöfer, 2007)**. Like other biological control agents, EPN offers a great bio-control potency with which the management of economically important insect pests can be accomplished **(Lacey and Georgis, 2012)** and to be integrated into insect pest management. EPNs are nematodes that naturally occur in soil and parasitize insects and possess several attributes of potential biological control agents and have been used against several insect pests **(Grewal *et al*., 2005a; Kaya *et al*., 2006; Koppenhöfer, 2007).**

EPNs of the families steinernematidae and heterorhabditidae are obligate and lethal parasites of insects **(Poinar, 1979)** that are characterized by symbiotic association with bacteria, *Xenorhabdus* spp. in steinernematids and *Photorhabdus* spp. in heterorhabditids **(Poinar, 1990)**. The general life cycle of EPNs as given in Fig.1.1 involves the eggs, four stages of juveniles (IJ1–IJ4) and adult nematode. The life cycle of both *Steinernema* and *Heterorhabditis* includes a third infective juvenile (IJ3) called dauer juvenile. The dauer is a free-living diapause or dormant stage induced by a pheromone produced constitutively in the population (only in the first and early second juvenile stage) and food limitation **(Riddle, 1988)**. It possesses a relatively resistant cuticle to avoid dehydration, low metabolic rates **(O'Riordan and**

Burnell, 1989, 1990) and likely food-storing intestinal granules **(Riddle, 1988)**. In steinernematids, the IJs harbour the symbiotic bacteria within an altered ventricular region of the gut, and mainly in the anterior region of the intestine in heterorhabditids **(Endo and Nickle, 1994; Bird and Akhurst, 1983; Boemare *et al*., 1996)**. When the IJs come in contact with a suitable host, it enters the body through body openings through the mouth, anus and spiracle **(Triggiani and Poinar, 1976; Glazer and Salame, 2000)**, or directly through the soft integuments **(Koppenhöfer *et al*., 2000)**. In addition, the IJs of heterorhabditids possess a dorsal tooth in the anterior region of the head **(Bedding and Molyneux, 1982),** and therefore could make entry into the hemocoel by penetrating the soft cuticle of the insect **(Bedding and Molyneux, 1982; Cui *et al*., 1993; Peters and Ehlers, 1994)**, wall of the midgut and peritrophic membranes **(Forschler and Gardner, 1991)**. On entering the hemocoel of the insect, the bacterial cells are released from the intestine of the IJs **(Akhurst, 1982)** by regurgitation **(Ciche and Ensign, 2003),** while the haemolymph provides a growth medium for the bacteria. The bacteria feed on insect haemolymph, rapidly divides, releases toxins and exoenzymes that usually kill the insect host within 24–48 hrs due to septicemia **(Poinar, 1983, Han and Ehlers, 2000)**. In addition, the symbiotic bacteria of steinernematids and heterohabditids produce metabolites that protect the insect cadaver from infection by other soil microbes for their survival with their host nematode **(Webster *et al*., 2002)**. The developmentally arrested IJ3 was activated by food signals from either bacteria or nutrients from the bacterial digestion of insects **(Strauch and Ehlers, 1998)**, and further developed to IJ4 by feeding on them. The

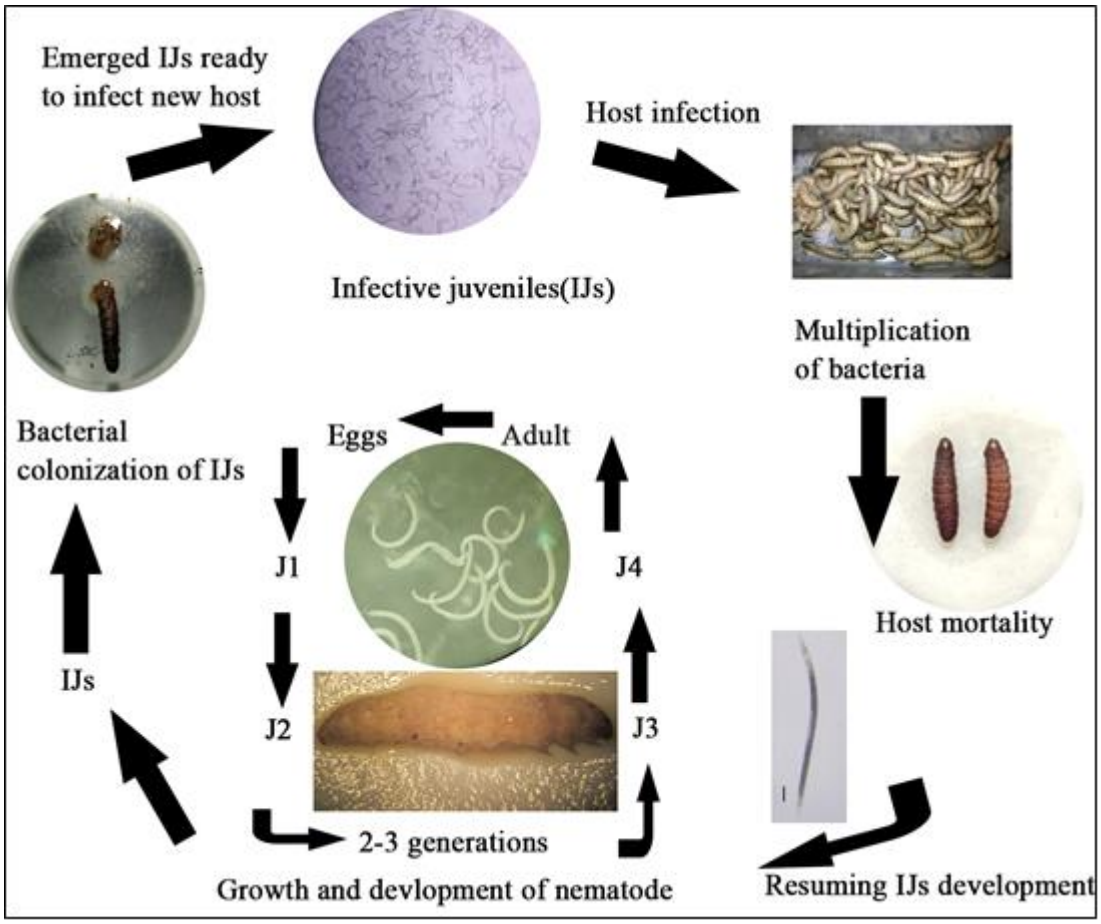

Fig. 1.1. Life cycle of entomopathogenic nematodes on larvae of greater wax moth, *Galleria mellonella* (Linnaeus, 1758).

IJs attain adulthood within 24–48 hrs where each individual IJs developed into first-generation hermaphroditic females in *Heterorhabditis* spp., whereas, in *Steinernema* spp., each individual IJs develop into amphimictic females or males **(Poinar, 1990)**. However, in both genera, the second generation consists of amphimictic females and males. The nematode undergoes development for about 2–3 generations depending on the degree of parasitism and the level of host nutrient contents, and the adult development is stopped which results in the accumulation of IJs. The free-living IJs emerged from the host cadaver into the soil and may live for several months before they infect a new host **(Hominick, 1990)**.

The biological control potential of EPNs for several insect pests has attracted the attention of several nematologists and entomologists as they:

- are environmentally safer and acceptable over chemical insecticides **(Akhurst, 1990)**.
- are highly pathogenic against a variety of harmful insects
- and usually kill the host within 24–48 hrs of infection **(Poinar, 1983)**.
- can actively find their hosts and recyclable in the soil environment **(Kaya and Gaugler, 1993)**.
- are harmless to other organisms **(Bathon, 1996)**.
- could be formulated with many chemical pesticides and applied with conventional apparatus **(Georgis, 1990; Rovesti and Deseo, 1991).**

- are superior over chemicals pesticides in finding their hosts in the soil and cryptic habitats to control insect pests **(Gaugler, 1981)**.
- are suitable for large-scale production by culturing them *in vivo* (in insects) or *in vitro* (on solid or liquid culture media) **(Dutky *et al*., 1964; Shapiro-Ilan *et al*, 2012)** and obtainable as commercially formulated.

Taxonomy of EPNs

The taxonomic classification of EPNs has undergone several changes like numerous other systematic classifications of organisms. The first described EPN was *Aplectana kraussei* by Steiner in the year 1923 **(Steiner, 1923)**. **Travassos (1927)** then later placed the species in the genus *Steinernema* as *S. kraussei*. In the year 1929, Steiner described the species *Neoaplectana glaseri* **(Steiner, 1929)** which was first discovered by **Glaser and Fox (1930)** as a parasite attacking larvae of the Japanese beetle larvae, *Popillia japonica* in US state of New Jersey. Furthermore, the superfamily Steinernematidae under which the two genera, *Aplectana* and *Neoaplectana*, were placed by **Filipjev (1934)** has undergone changes to a family. **Wouts *et al*. (1982)** stated that after careful comparison of both genera, *Neoplectana* was a junior synonym of *Steinernema*. With a comprehensive examination of both genera, **Poinar (1990)** then synonymised the genus *Neoplectana* under *Steinernema* in the year 1990. A nematode parasitizing the termite, *Reticulitemes flavipes* was later described as *Neosteinemema longicuruicauda* and placed under the family Steinernematidae **(Nguyen and Smart, 1994)**. The family Heterorhabditidae created by **Poinar (1976)** consists of the species *Heterorhabditis bacteriophora* as the type specimen. Like in the case of steinernematids, several changes have occurred in the

taxonomic classification of Heterorhabditidae **(Mason et at., 1996)**. With extensive surveys on the occurrence of EPNs from different regions of the world, around 107 and 17 species of *Steinernema* and *Heterorhabditis* respectively have been described (Table 1.1 and 1.2).

Table 1.1. List of species described from the family Steinernematidae, Chitwood and Chitwood, 1937

Sl no.	**Species**	**References**	**Country of origin**
1.	*S. abbasi*	Elawad *et al*., 1997	Asia (Oman)
2.	*S. aciari*	Qiu *et al*., 2005a	Asia (China)
3.	*S. affine*	Wouts *et al*., 1982	Europe (Denmark)
4.	*S. akhursti*	Qiu *et al*., 2005b	Asia (China)
5.	*S. anatoliense*	Hazir *et al*., 2003	Asia (Turkey)
6.	*S. apuliae*	Triggiani *et al*., 2004	Europe (Italy)
7.	*S. arasbaranense*	Nikdel *et al*., 2011	Asia (Iran)
8.	*S. arenarium*	(Artyukhovsky *et al*., 1997) Wouts *et al*., 1982)	Central Russia/Europe (Ukraine)
9.	*S. ashiuense*	Phan *et al*., 2006b	Asia (Japan)
10.	*S. asiaticum*	Anis *et al*., 2002	Asia (Pakistan)
11.	*S. australe*	Edgington *et al*., 2009b	South America (Chile)
12.	*S. backanense*	Phan *et al*., 2006	Asia (Vietnam)
13.	*S. beddingi*	Qiu *et al*., 2005	Asia (China)
14.	*S. beitlechemi*	Cimen *et al*., 2016	South Africa
15.	*S. bicornutum*	Tallosi *et al*., 1995	Europe (Serbia)
16.	*S. biddulphi*	Cimen *et al*., 2016	South Africa
17.	*S. bifurcatum*	Shahina *et al*., 2014	Asia (Pakistan)
18.	*S. boemarei*	Lee *et al*., 2009	Europe (France)
19.	*S. borjomiense*	Gorgadze *et al*., 2018	Europe (Georgia)
20.	*S. brazilense*	Nguyen *et al*., 2010	South America (Brazil)
21.	*S. cameroonense*	Kanga *et al*., 2012	Africa (Cameroon)
22.	*S. carpocapsae*	Wouts *et al*., 1982	Europe (Czechoslovakia)
23.	*S. caudatum*	Xu *et al*., 1991	Asia (China)
24.	*S. ceratophorum*	Jian *et al*., 1997	Asia (China)
25.	*S. changbaiense*	Ma *et al*., 2012a; Ma *et al*., 2012b; Ma *et al*., 2012c	Asia (China)

26.	*S. cholashanense*	Nguyen *et al*., 2008a	Asia (China)
27.	*S. citrae*	Malan *et al*., 2011	South Africa
28.	*S. colombiense*	Lopez-Nunez *et al*.,2008	South America (Colombia)
29.	*S. costaricense*	Uribe-Lorío *et al*., 2007	Central America (Costa Rica)
30.	*S. cubanum*	Mráček *et al*., 1994	North America (Cuba)
31.	*S. cumgarense*	Phan *et al*., 2006a	Asia (Vietnam)
32.	*S. diaprepesi*	Nguyen and Duncan 2002	North America (USA)
33.	*S. eapokense*	Phan *et al*., 2006a	Asia (Vietnam)
34.	*S. ethiopiense*	Tamirou *et al*., 2012	Africa (Ethiopia)
35.	*S. fabii*	Abate *et al*., 2016	South Africa
36.	*S. feltiae*	(Filipjev, 1934); Wouts *et al*., 1982	Europe (Denmark)
37.	*S. glaseri*	(Steiner, 1929) Wouts *et al*., 1982	North America (USA)
38.	*S. goweni*	San-Blas *et al*., 2016	South America (Venezuela)
39.	*S. guangdongense*	Qiu *et al*., 2004	Asia (China)
40.	*S. hebeiense*	Chen *et al*., 2006	Asia (China)
41.	*S. hermaphroditum*	Stock *et al*., 2004	Asia (Indonesia)
42.	*S. huense*	Phan *et al*., 2014	Asia (Vietnam)
43.	*S. ichnusae*	Tarasco *et al*., 2008	Europe (Italy)
44.	*S. innovationi*	Çimen *et al*., 2015	South Africa
45.	*S. intermedium*	Poinar, 1985	North America (USA)
46.	*S. jeffreyense*	Malan *et al*., 2015	South Africa
47.	*S. jollieti*	Spiridonov *et al*., 2004	North America (USA)
48.	*S. karii*	Waturu *et al*., 1997	East Africa (Kenya)
49.	*S. khoisanae*	Nguyen *et al*.,2006	South Africa
50.	*S. khuongi*	Stock *et al*., 2019	North America (USA)
51.	*S. kraussei*	Steiner, 1923a, b	Europe (Germany)
52.	*S. kushidai*	Mamiya, 1988	Asia (Japan)
53.	*S. lamjungense*	Khatri-Chhetri *et al*., 2011a	Asia (Nepal)
54.	*S. leizhouense*	Nguyen *et al*., 2006b	Asia (China)
55.	*S. litchii*	Steyn *et al*., 2017	South Africa
56.	*S. litorale*	Yoshida, 2004	Asia (Japan)
57.	*S. loci*	Phan *et al*., 2001c	Asia (Vietnam)
58.	*S. longicaudum*	Shen and Wang, 1991	Asia (China)
59.	*S. minutum*	Maneesakorn *et al*., 2010	Asia (Thailand)
60.	*S. monticolum*	Stock *et al*., 1997	Asia (Republic of Korea)

61.	*S. neocurtillae*	Nguyen and Smart, 1992	North America (USA)
62.	*S. nepalense*	Khatri-Chhetri *et al*., 2011b	Asia (Nepal)
63.	*S. nguyeni*	Malan *et al*., 2016	South Africa
64.	*S. nyetense*	Kanga *et al*., 2012	Africa (Cameroon)
65.	*S. oregonense*	Liu and Berry, 1996	North America (USA)
66.	*S. pakistanense*	Shahina *et al*., 2001	Asia (Pakistan)
67	*S. papillatum*	San-Blas *et al*., 2015	South America (Venezuela)
68.	*S. populi*	Tian *et al*., 2022	Asia (China)
69.	*S. phyllophagae*	Nguyen and Buss KB, 2011	North America (USA)
70.	*S. poinari*	Mráček *et al*., 2014	Europe (Czech Republic)
71.	*S. puertoricense*	Román and Figueroa, 1994	North America (Puerto Rico)
72.	*S. pui*	Qiu *et al*., 2011	Asia (China)
73.	*S. puntauvense*	Uribe-Lorío *et al*., 2007	Central America (Costa Rica)
74.	*S. pwaniensis*	Půža *et al*., 2017	East Africa (Tanzania)
75.	*S. ralatorei*	Grifaldo-Alcantara *et al*., 2017	North America (Mexico)
76.	*S. rarum*	de Doucet, 1986	South America (Argentina)
77.	*S. riobrave*	Cabanillas *et al*., 1994	North America (USA)
78.	*S. riojaense*	Půža *et al*., 2020	Europe (Spain)
79.	*S. ritteri*	de Doucet and Doucet, 1990	South America (Argentina)
80.	*S. robustispiculum*	Phan *et al*., 2005	Asia (Vietnam)
81.	*S. sacchari*	Nthenga *et al*., 2014	South Africa
82.	*S. sangi*	Phan *et al*., 2001a	Asia (Vietnam)
83.	*S. sasonense*	Phan *et al*., 2006a	Asia (Vietnam)
84.	*S. scapterisci*	Nguyen and Smart, 1990	South America (Uruguay)
85.	*S. scarabaei*	Stock and Koppenhöfer, 2003	North America (USA)
86.	*84 S. schliemanni*	Spiridonov *et al*., 2010	Europe (Germany)
87.	*S. siamkayai*	Stock *et al*., 1998	Asia (Thailand)
88.	*S. sichuanense*	Mráček *et al*., 2006	Asia (China)
89.	*S. silvaticum*	Sturhan *et al*., 2005	Europe (Germany)
90.	*S. surkhetense*	Khatri-Chhetri *et al*., 2011b	Asia (Nepal)
91.	*S. tami*	Luc *et al*., 2000	Asia (Vietnam)
92.	*S. taiwanensis*	Tseng *et al*., 2018	Asia (Taiwan)

93.	*S. texanum*	Nguyen *et al*., 2007	North America (USA)
94.	*S. thanhi*	Phan *et al*., 2001b	Asia (Vietnam)
95.	*S. thermophilium*	Ganguly and Singh, 2000	Asia (India)
96.	*S. thesami*	Gorgadze *et al*.,1988, 2016	Europe, Asia (Georgia)
97.	*S. tielingense*	Ma *et al*., 2012c	Asia (China)
98.	*S. tophus*	Çimen *et al*., 2014	South Africa
99.	*S. unicornum*	Edgington *et al*., 2009a	South America (Chile)
100.	*S. xinbinense*	Ma *et al*., 2012b	Asia (China)
101.	*S. xueshanense*	Mráček *et al*., 2009	Asia (China)
102.	*S. weiseri*	Mráček *et al*., 2003	Europe (Czech Republic)
103.	*S. yirgalemense*	Nguyen *et al*., 2004b	Africa (Ethiopia)
104.	*S. bertusi*	Katumanyane *et al*., 2019	South Africa
105.	*S. batswanae*	Linda *et al*., 2021	South Africa
106.	*S. sandneri*	Magdalena *et al*., 2021	Europe (Poland)
107.	*S. vulcanicum*	Clausi *et al*., 2011	Europe (Italy)

Table 1.2. List of species described from the family Heterorhabditidae, Poinar (1976)

Sl no.	**Species**	**References**	**Country of origin**
1.	*H. amazonensis*	Andaló *et al*., 2006	South America (Brazil)
2.	*H. atacamensis*	Edgington *et al*., 2011	South America (Chile)
3.	*H. bacteriophora*	Poinar, 1976	Australia
4.	*H. baujardi*	Phan *et al*., 2003	Asia (Vietnam)
5.	*H. beicherriana*	Li *et al*., 2012	Asia (China)
6.	*H. downesi*	Stock *et al*., 2002	Europe (Ireland)
7.	*H. floridensis*	Nguyen *et al*., 2006a	North America (USA)
8.	*H. georgiana*	Nguyen *et al*., 2008b	Europe, Asia (Georgia)
9.	*H. indica*	Poinar *et al*., 1992	Asia (India)
10.	*H. marelatus*	Liu and Berry, 1996	North America (USA)
11.	*H. megidis*	Poinar *et al*., 1987	North America (USA)

12.	*H. mexicana*	Nguyen *et al*., 2004a	North America (Mexico)
13.	*H. noenieputensis*	Malan *et al*., 2014	South Africa
14.	*H. safricana*	Malan *et al*., 2008	South Africa
15.	*H. sonorensis*	Stock *et al*., 2009	North America (Mexico)
16.	*H. taysearae*	Shamseldean *et al*., 1996	Africa (Egypt)
17.	*H. zealandica*	Poinar, 1990	Oceania (New Zealand)

Table 1.3. Taxonomic classification of *Steinernema* and *Heterorhabditis*

Taxonomic rank	*Steinernema*	*Heterorhabditis*
Phylum	Nematoda	Nematoda
Class	Secernentia	Secernentia
Order	Rhabditidae	Rhabditidae
Suborder	Rhabditina	Rhabditina
Superfamily	Rhabditoidea	Rhabditoidea
Family	Steinernematidae	Heterorhabditidae
Genus	*Steinernema*	*Heterorhabditis*

Occurrence of EPNs

Surveys on the occurrence of EPNs have been conducted in all inhabited countries around the world **(Kaya, 1990)** following the baiting technique of **Bedding and Akhurst (1975)**. EPNs were described from different continents of the world except

for Antarctica **(Kaya, 1990; Griffin *et at.*, 1990)**. With the growing concerns over the negative impact of exotic species on native organisms, immense works have been conducted for the isolation of climatically adapted indigenous species of EPNs or isolates with the intention of formulating and commercialising them later **(Hominick, 2002)**.

Europe

Around 14 species of *Steinernema* and 1 species of *Heterorhabditis* were described in Europe, with *S. kraussei* being the first to be described **(Steiner, 1923)**. In addition, several indigenous species of EPNs were isolated from Europe that includes *S. intermedium*, *S. feltiae*, *S. vulcanicum*, *S. arenarium*, *H. bacteriophora*, *H. indica*, and *H. megidis* **(Mráček and Bečvář, 2000; Tarasco *et al*., 2015)**.

America

In America, a survey on the occurrence and diversity of EPN was initiated by **Glaser and Fox (1930)** covering different areas of both continents that resulted in the discovery of new nematode species from the infected Japanese beetle *Popillia japonica* (*Steinernema glaseri*, **Steiner, 1929**) in New Jersey.

North America

With extensive surveys conducted on the occurrence and diversity of EPNs across the continent, several new species of EPN species had been described including *H. hepialius* (syn. with *H. marelatus*). Among steinernematids, new species such as *S. glaseri* **(Glaser and Fox, 1930)**; *S. intermedium* **(Poinar, 1985)**; *S. neocurtillae* **(Nguyen and Smart Jr, 1992)**; *S. cubanum* **(Mráček *et al*. 1994)**; *S. riobrave*

(Cabanillas *et al.* 1994); *S. puertoricense* **(Román and Figueroa, 1994)**; *S. oregonense* **(Liu and Berry, 1996)**; *S. diaprepesi* **(Nguyen and Duncan, 2002)**; *S. scarabaei* **(Stock and Koppenhöfer, 2003)**; *S. jollieti* **(Spiridonov *et al.*, 2004)**; *S. costaricense* and *S. puntauvense* **(Uribe-Lorío *et al.*, 2007)**; *S. texanum* **(Nguyen *et al.*, 2007)**; *S. phyllophagae* **(Nguyen and Buss, 2011)** were described from North America. Among heterorhabditids, the first described species was *H. megidis* **(Poinar *et al.* (1987)**, followed by *H. marelatus* **(Liu and Berry, 1996)**; *H. mexicana* and *H. floridensis* **(Nguyen *et al.*, 2006a)**; *H. georgiana* **(Nguyen *et al.*, 2008b)**.

Central America

In Central America, surveys have been conducted to study the occurrence and distribution of EPNs that resulted in the isolation of *S. carpocapsae* and *H. indica* from Mexico **(Poinar, 1990, Cortez-Madrigal *et al.*, 2003)**. **Nguyun *et al.* (2004a)** reported *H. mexicana* from Tamaulipas, Mexico. In addition, several isolates of *Steinernema* and *Heterorhabditis* which requires identification at the species level were also reported in different areas of Central America **(Bhat *et al.*, 2020)**.

South America

Pereira (1937) isolated *Rhabditis hambletoni* which was later changed to the genus *Heterorhabditis*. Meanwhile, *Neoaplectana glaseri* which was later called as *S. glaseri* was isolated from a *Migdolus fryanus* infected egg of in São Paulo, Brazil **(Pizano *et al.*, 1985)**. Some of the EPN species that were described from the same continent includes *S. rarum* **(de Doucet, 1986)**, *S. ritteri* from Argentina **(de Doucet**

and Doucet, 1990), *S. scapterisci* **(Nguyen and Smart Jr, 1990)**, *S. colombiense* **(Lopez-Nunez *et al*., 2008)**, *S. unicornum* **(Edgington *et al*., 2009a)**, *S. australe* **(Edgington *et al*., 2009b)**, *S. brazilense* **(Nguyen *et al*., 2010)**, and both *S. papillatum* and *S. goweni* **(San-Blas *et al*., 2015, 2016)**. Other EPN species reported from South America includes *S. carpocapsae*, *S. scapterisci*, *H. indica*, *S. feltiae* and *H. bacteriophora*.

Asia

China

Surveying the occurrence of EPNs across various geographical areas of China was initially conducted during the mid-1980s and early 1990s. **Li and Wang (1989)** have reported the natural occurrence of two EPN species, *S. glaseri* and *H. bacteriophora*. From the country, about 16 species of *Steinernema* and 1 species of *Heterorhabditis* were described. **Wang *et al*. (2014)** reported the natural occurrence of 14 species of steinernematids such as *S. litorale, S. silvaticum, S. feltiae, S. bicornutum, S. affine, S. riobrave, S. yirgalemense, S. kushidai, S. scapterisci, S. carpocapsae, S. ritteri, S. tami, S. rarum,* and *S. sasonense* and 5 species of heterorhabditids such as *H. megidis, H. zealandica, H. brevicaudis, H. indica,* and *H. baujardi* from Liaoning, Northeast China.

Vietnam

In Vietnam, surveys have been conducted with several new species of EPNs described from the country such as *S. tami* **(Luc *et al*., 2000)**, *S. loci*, *S. sangi*, *S. thanhi*, *H. baujardi*, *S. robustispiculum, S. backanense, S. cumgarense, S. eapokense,*

S. sasonense, and *S. huense* **(Phan *et al*., 2001a, 2001b, 2003, 2005, 2006a, 2014)**. In addition, 4 species of indigenous EPN isolates were reported from Vietnam.

Thailand

Two new species of EPN, *S. siamkayai* **(Stock *et al*., 1998)** and *S. minutum* **(Maneesakorn *et al*., 2010)** were described from the country. In addition, several indigenous species of EPNs reported from Thailand include *S. websteri*, *S. khoisanae*, *H. indica*, *H. baujardi*, *H. bacteriophora*, *S. kushidai S. scrabaei*, *H. somsookae* (=*H. baujardi*), *H. gerradi* (syn. with *H. indica*), and *H. zealandica* **(Thanwisai, *et al*., 2012; Bhat *et al*., 2020)**.

Japan

While surveying the occurrence of EPNs from soil samples obtained from different collection sites in Japan, several species of EPN have been reported. **Mamiya (1988)** described a new species of EPN, *S. kushidai* while **Yoshida (2005)** isolated another new species, *S. litorale* from the same country. Later, **Phan *et al*. (2006b)** described another new species, *S. ashiuense* from Japan. Several EPN species reported from Japan that have already been described include *H. indica*, *H. megidis*, *S. carpocapsae*, and *S. feltiae*, along with some unidentified steinernematids **(Yoshida *et al*., 1998)**.

Republic of Korea

Surveys have been conducted across the country for the isolation of EPNs. The only new species described from Republic of Korea remains to be *S. monticolum* **(Stock *et al*., 1997)**. In addition, several indigenous species of EPNs *viz*. *H. bacteriophora*,

S. carpocapsae, *S. glaseri*, *S. longicaudum*, *S. intermedia*, *S. feltiae*, and *H. megidis* **(Hang *et al*., 2007)** were reported from the country as well.

Nepal

Khatri-Chhetri *et al*. (2011a, b) described new species of EPNs *viz*. *S. nepalense*, *S. surkhetense*, *S. lamjungense*, and *S. everestense*. In addition, **Khatri-Chhetri *et al*. (2010)** have reported the natural occurrence of 5 species of EPNs *viz*. *S. abbasi*, *S. cholashanense*, *S. feltiae*, *S. siamkayai*, and *H. indica* from the country.

Jordan

From Jordan, **Stock *et al*. (2008)** conducted the first survey for EPNs along with their symbiotic bacteria and reported 3 steinernematids *viz*. *S. anatoliense*, *S. carpocapsae*, *S. feltiae* and one heterorhabditids species, *H. bacteriophora*.

Sultanate of Oman

Elawad *et al*. (1997) reported a new species, *S. abbasi* and there are no further reports on the occurrence and diversity of EPN from the country.

United Arab Emirates (UAE)

From the survey that have been conducted in United Arab Emirates (UAE), 3 species of EPN *viz*. *S. abbasi*, *S. riobrave* and *H. bacteriophora* were isolated from the country **(Abbas *et al*., 2001a, b).**

Australia

In Australia, survey on the occurrence and diversity of EPN have been conducted across the country several years back. Two heterorhabditids, *H. bacteriophora* and

H. zealandica and 5 steinernematids *viz*. *S. feltiae*, *S. longicaudum*, *S. glaseri*, *S. kraussei* and *S. bibionis* (syn. with *S. feltiae*) were reported from the country **(Wouts *et al*., 1982; Akhurst and Bedding, 1986)**.

New Zealand

From New Zealand, **Ali and Wharton (2017)** have isolated 3 indigenous species of EPNs such as *H. zealandica, S. feltiae* and *S. kraussei*

Saudi Arabia

Saleh and Alheji (2003) have reported the natural occurrence of *H. indica* from Eastern Province of Saudi Arabia.

India

The initial work of EPNs in India was started in the year using exotic species of *S. carpocapsae* (DD-1136 strain) against insect that infests rice, sugarcane and apple **(Rao and Manjunath, 1966).** Apart from this, other exotic species of EPNs *viz*. *S. glaseri*, *S. feltiae* and *H. bacteriophora* were imported to study their biocontrol potential against insect pests in India **(Rao and Manjunath, 1966; Mathur *et al*., 1971; Sundarababu *et al*., 1984)**. However, the outcome was considered as insignificant due to the limited capacity of the nematodes to adapt to their new habitat, and there is a believe that these may have harmful effects on other organisms **(Kaya *et al*., 2006)**. Since then, surveys have been focussed on the isolation of locally adapted indigenous EPN species that will serve as valuable resources from biodiversity and environmental point of view.

The extensive surveys have resulted in the recovery of numerous species and strains of EPNs from different geographical areas of India **(Ganguly, 2003)**. So far, about 13 species of EPNs (12 steinernematids and 1 heterorhabditids) have been isolated from the country **(Bhat *et al*., 2020)**. **Sivakumar *et al*. (1989)** reported *H. bacteriophora* from Burliar, the Nilgiris and performed further studies on their life history and insect host range. Till date, *H. indica* isolated from Tamil Nadu remains to be the only new EPN species described from the country (**Poinar *et al*., 1992)** and, was later reported from Coimbatore and Kanyakumari districts of the State **(Ambika, 1995)**. In 1992, Singh have isolated *Steinernema* sp. from the survey they have conducted at ICRISAT Center from Patancheru city (Andhra Pradesh). In the year 1995, **Ambika (1995)** have isolated *H. indica* from the soil samples collected from Coimbatore and Kanyakumari districts of Tamil Nadu. **Kaushal *et al*. (2000)** have conducted surveys on the occurrence of EPN of soil samples collected from various regions of India out of which 17 samples tested positive for EPNs which contain 10 *Steinernema* and 7 *Heterorhabditis* isolates. In a survey conducted across central northern region of Gujarat, **Vyas *et al*. (1998)** have reported 16 different EPN isolates that includes 4 species of the genera *Heterorhabditis* and 11 species of *Steinernema* along with 1 unidentified species. **Banu *et al*. (1998)** also have reported the natural occurrence of *H. indica* from Kerala. **Ganguly *et al*., 2002** conducted surveys on the occurrence of EPN around Anand of Anand District of Gujarat and reported 4 isolates of *Steinernema* sp. Based on the survey conducted by **Prasad *et al*. (2001a, b)**, *Heterorhabditis* sp. was isolated from different agro- ecosystems of South Andamans. In a survey conducted around cotton plantation areas of north, south and central parts of India, **Gokte-Narkhedkar *et al*. (2001)** have reported the

occurrence of 16 isolates of the genera *Steinernema* and *Heterorhabditis* by baiting *Corcya cephalonica* and *Galleria mellonella* larvae. **Sivakumar *et al*. (2002)** conducted surveys on the occurrence of EPNs from western ghat area of Tamil Nadu and have recorded 9 samples that have tested positive for steinernetatids. In the same year, across several districts of Rajasthan, **Parihar *et al*. (2002)** have reported 26 (out of 477) positive soil samples comprising 7 isolates (3 isolates of *Steinernematids* and 4 isolates of *Heterorhabditids*) from Udaipui and 19 isolates of *Heterorhabditids* from Jaipui, Rajasthan. In the same year, **Ganguly *et al*. (2002)** have reported *S. riobrave* from Gujarat, India. Three isolates of EPNs comprising *H. indica*, *S. glaseri* and *Steinernema* sp. were reported from soil samples in Kerala **(Banu *et al*., 2005)**. **Yadav and Lalramliana (2012)** have reported the natural occurrence of *S. glaseri* from survey conducted on forest areas of Meghalaya, India. With extensive surveys conducted across different areas of Uttar Pradesh, India, *S. surkhetense* was reported from Bijnor district **(Bhat *et al*., 2017)**; *S. pakistanense* was reported from Meerut district **(Bhat *et al*., 2019)**, and *S. hermaphroditum* from West Uttar Pradesh **(Bhat *et al*., 2019)**. Furthermore, **Mhatre *et al*. (2017)** have reported the occurrence of *S. cholashanense* in India. Bhat *et al*. (2020) have reported *H. bacteriophora* from Kashmir Valley of India.

Furthermore, no new species of *Steinernema* have been described from India till date. The 3 formerly described steinernematids species from India *viz*. *S. thermophilum* by **Ganguly and Singh (2000)**, and *S. meghalayensis* **by Ganguly *et al*. (2011)** were synonymized with *S. abbasi* **(Elawad *et al*., 1997)** and *S. carpocapsae* **(Wouts *et al*., 1982)**, respectively. Meanwhile, the previously described

species, *S. dharanai* **(Kulkarni *et al.*, 2012)** later become a junior synonym of *S. hermaphroditum* **(Stock *et al.*, 2004; Hunt, 2007)**.

Materials and methods

Study area

Mizoram is one of the North-Eastern States of India with a geographical area of 21,087 sq. km and is surrounded by Myanmar (Burma) in the east and Bangladesh in the west. The topographical features of the state include large mountainous area that gradually slopes to form deep valleys that culminates into rivers and streams. The state lies at 21° 58' N – 24° 35' N Latitude and 92° 15' – 93° 29' E Longitude. Mizoram is inhabited by about 94% tribal people and is a part of the biodiversity hotspots in the eastern Himalayan region (NE India). Majority of the people are indulged in shifting cultivation form of agriculture and indigenous forestry products largely support livelihood of the rural people. With advance in technology and depletion of natural resources, the people become conscious of the value and importance of protection of biodiversity and the genetic resources.

The state consists of 11 districts and exhibits a moderate and pleasant climate ranging from tropical climate towards the Assam border to the temperate climate in the areas connecting Myanmar and Manipur. During summer season, especially during the month of May to July of the year, a hot and humid weather prevails in the bordering areas of Assam with an average temperature of 35 °C. However, areas from Champhai and Saiha districts experience severe cold weather during winter

season and a pleasant warm weather in the summer months. The State have a moderate climate throughout the year and have experienced rise in temperature from the past few years. According to the 2015 Forest Survey of India Report, the State has a forest cover area of 18,748 km, or 88.93% of its total area.

Insect source

Culturing of greater wax moth, *G. mellonella* (L.)

The larvae of *G. mellonella* were collected from local bee keeper and were maintained in their natural diets (honeycombs). A 1:1 ratio of male to female mothswas kept in the oviposition cage for egg laying. Honey-water mixture and drinking water at a ratio of 1:1 was provided on cotton swabs, which were replaced every day. The cages were kept at 30 °C in a dark place. The eggs were transferred to the artificial diet and reared by modification of **Singh (1997)**.

Preparation of artificial diet

Corn meal, wheat flour, wheat bran, milk powder and yeasts tablets were properly mixed thoroughly. Honey and glycerin were mixed separately. The initially prepared mixtures were then mixed slowly by thorough stirring with glass rod. The diet was made in a plastic jar four or five days before used.

Entomopathogenic nematodes source

Collection of soil samples

Surveys on the occurrence of EPNs were conducted across different areas covering the 11 districts of Mizoram, North-Eastern India. Soil samples were collected from

different locations at a depth of 10–15 cm and approximately 1kg of soil was pooled in a polythene bag from each site covering an area about 1 sq. m. The collected samples were then transported in well-labelled plastic bags to the laboratory, Research and Instrumentation Center, Pachhunga University College Campus of Mizoram University, Aizawl, Mizoram. Information on sampling months and geographical locations were noted for each sample.

Baiting of collected soil samples

The collected soil samples were baited with larvae of *G. mellonella* within 1–3 days from collection time. The baiting technique of **Bedding and Akhurst (1975)** was followed with slight modifications where the samples were baited in a 500 ml plastic container. For each container, ten individuals of *G. mellonella* were used as baiting agents to isolate EPNs and three replicates were kept for each soil sample. The containers were covered sealed with lids, turned upside down and held at room temperature. Larval mortality for each soil samples was observed for 10 consecutive days and the dead larvae if any were cleaned with distilled water. The dead larvae were observed for the presence of EPNs by colour change and smell emanating from the insect cadaver. The dead larvae were then put in modified White traps **(Kaya and Stock, 1997).**

Re-infection

Re-infection of extracted nematodes was done on petri plates lined with moist filter paper and re-isolated to confirm their pathogenicity through Koch's postulates. The extracted nematodes were re-infected to the larvae of *G. mellonella* at 25 ± 2 °C and

the freshly emerged IJs of the nematodes were kept at 12–15 °C in an incubator with aerated water.

Maintenance of EPNs in the laboratory

Sterilization, disinfection and maintenance of equipment

In the study, all the equipments used in handling of the isolated nematodes were thoroughly cleaned with distilled water and then sterilized with 75% (v/v). The materials after use were thoroughly washed, properly dried, sterilized and stored them for the next use. The pipettes, needles and syringes used for handling each nematode isolate were properly labelled and stored separately to avoid contamination and sample mixing.

Baiting, collection and storage of EPNs in the laboratory

For all the EPN isolates, laboratory cultures were maintained in the laboratory by using last instar larvae of *G. mellonella* as bait. The White trap method of **Woodring and Kaya (1988)** was followed with slight modifications to multiply the nematodes. The method involves introduction of healthy last instar larvae to a petri dish, double lined with Whatman #1 filter paper pre-inoculated with nematode suspension. The petri dishes were sealed with a parafilm and kept in an incubator at 25 ± 2 °C.

Upon infection, the dead larvae were moved to modify White traps **(Woodring and Kaya, 1988)** and incubated at 25 ± 2°C. The freshly emerged IJs were harvested for each nematode isolates and cleaned with distilled for storage. The nematodes were then transferred to clean petri dish partially filled with distilled water and stored in an incubator at 10 ± 2 °C. All the isolates were kept separately,

passed through the host larvae every 30 days and the nematodes used for further studies were obtained from fresh culture plates only.

Nematode identification

All the nematode isolates from positive soil samples were identified at the genus level that were further characterized with the selected molecular gene markers. For each of the nematode isolates, 0.5 ml of distilled water containing 1000 freshly emerged IJs were introduced to a petri dish (35×10 mm) double lined with Whatman filter paper No. 1. The introduced nematodes were allowed to settle for 30 mins at 28 ± 2 °C in an incubator. Then, 5 individual larvae of *G. mellonella* were introduced into individual petri plate, each pre-inoculated with the nematodes and placed in in an incubator at 28 ± 2 °C. Larval mortality rate was check at 24 hrs interval time where colour change, no movement and the smell emanating from the dead body of larvae were used for primary confirmation of death due to EPNs. For steinernematids, both adult male and female individuals were obtained by dissecting the larval cadaver 2–3 days post-mortality. In the case of heterorhabditids, hermaphrodite adults were obtained by dissecting the larval cadaver 3–4 days post-mortality. However, to obtain second generation male and female, insect cadavers were individually dissected under an Olympus CX41 microscope at 5–7 days post-mortality. For morphological identification of the IJs of both steinernematids and heterorhabditids, the freshly emerged individuals from the larval cadaver placed on a modified white trap were used. Generally, the larval cadaver turns brick red if killed by the heterorhabditids and pale yellowish if killed by the steinernematids.

Phase-contrast microscopy (PCM)

All the nematodes collected for identification were transferred to Ringer's solution and killed at 60 °C. The sacrificed nematodes were then transferred to tri-ethanolamine-formalin (TAF) fixative **(Courtney *et al,* 1955; Kaya and Stock, 1997)** and processed to anhydrous glycerine dehydration following **Seinhorst (1959)**. Permanent mounts were made on glass slide with glycerine, cover with coverslips with careful precautions to avoid air bubbles and flattening of specimens. For all the nematode isolates, at least 30 individuals of IJs, male and female were used for microscopic observation with 10X and or 40X. A bright field microscope Evox XL Core with microscope image analyser Nikon Eclipse CiE DSRi2 Cam + NIS was used to perform measurements and photography.

Results

Nematode isolation and identification

Soil samples were collected from different areas that cover agro-forest, riparian, jhumland, foreland and roadside. The primary confirmation of insect mortality due to the EPN isolates was based on nature of the infected host (colour) and smell emanating from the insect cadaver. From the present study, the isolated nematodes with serial numbers 1 to 7 were distinguished by the presence of hermaphroditic stage i.e., first generation hermaphrodite females, which are absent in the case of the nematode isolates from serial number 8 to 12. Therefore, the recorded nematode isolates from series 1 to 7 belong to the Genus *Heterorhabditis* while the isolates 8 to

12 belong to the Genus *Steinernema*. In addition, the occurrence of bursa in males distinguished the isolate of the genus *Heterorhabditis* from *Steinernema*.

Upon infection, the larvae killed by heterorhabditids showed brick red colour but that of steinernematids showed pale yellow to dark brownish colour. Most importantly, the IJs in *Steinemema* spp. develop into amphimictic females or males, whereas in *Heterorhabditis* spp., only hermaphroditic female develops from each IJs. Simultaneously, both morphological and morphometric measurements were taken for the isolated nematodes as given in table 1.5 (isolate 1 to 6), table 1.6 (isolate 7), table 1.7 (isolate 8 to 11), and table 1.8 (isolate 12). Based on the morphological and morphometric measurements, all isolates of the of *Heterorhabditis* spp. are highly similar. However, the nematode isolates from series 1 to 6 can be separated from the isolate 7 by shape of the gubernaculum and the number of normal pairs of genital papillae.

Therefore, the nematodes isolated from positive samples were categorised into the following groups:

Table 1.4. Lists of isolated entomopathogenic nematodes from Mizoram

Nematode isolate	**Nematode isolate code**	**Genus**	**Colour of host cadaver**	**Grouping**
1.	HeM	*Heterorhabditis* sp.	Brick red	Group I
2.	HeTC	*Heterorhabditis* sp.	Brick red	
3.	HeTRE	*Heterorhabditis* sp.	Brick red	
4.	HeTR	*Heterorhabditis* sp.	Brick red	
5.	HeTS	*Heterorhabditis* sp.	Brick red	
6.	HeTV	*Heterorhabditis* sp.	Brick red	

7.	HeTD	*Heterorhabditis* sp.	Brick red	Group II
8.	StR	*Steinernema* sp.	pale pink-yellow colour	Group III
9.	StK	*Steinernema* sp.	pale pink-yellow colour	
10.	StC	*Steinernema* sp.	pale pink-yellow colour	
11.	StL	*Steinernema* sp.	pale pink-yellow colour	
12.	StPTS	*Steinernema* sp.	charcoal-grey	Group IV

Description of the isolated nematodes

Group I (Nematode isolate 1 to 6): *Heterorhabditis* sp. 1

Kingdom : Animalia

Phylum : Nematoda

Class : Secernentea von Linstow, 1905

Order : Rhabditida (Orley, 1880) Chitwood, 1933

Suborder : Rhabditina (Orley, 1880) Chitwood, 1933

Superfamily : Rhabditoidea (Orley, 1880) Travassos, 1920

Family : Heterorhabditidae Poinar, 1976

Genus : *Heterorhabditis* Poinar, 1976

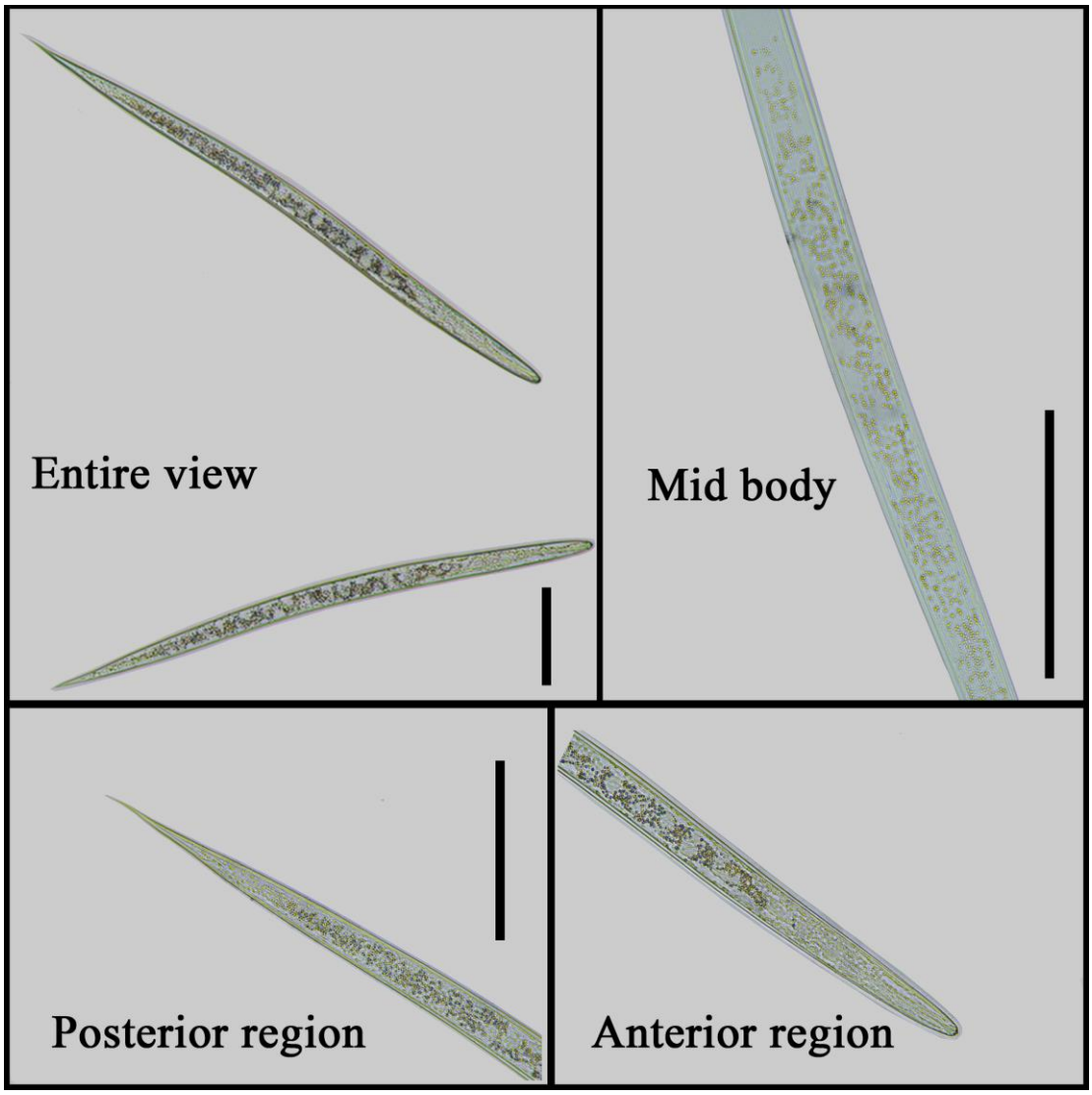

Fig. 1.2. *Heterorhabditis indica*: infective juveniles (IJ). **(Scale bars = 100µm)**

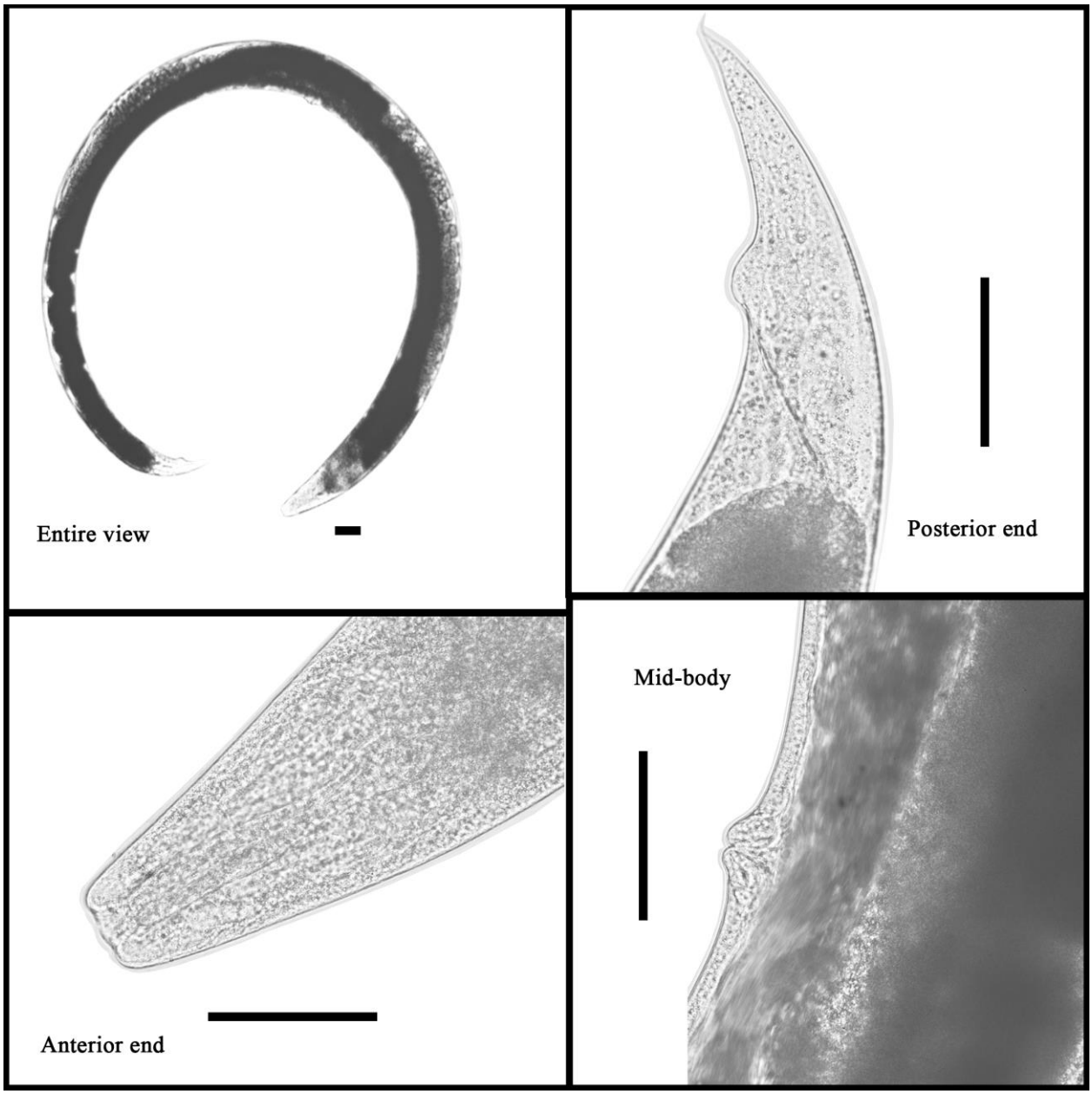

Fig. 1.3. *Heterorhabditis indica*: first generation female. **(Scale bars = 100µm)**

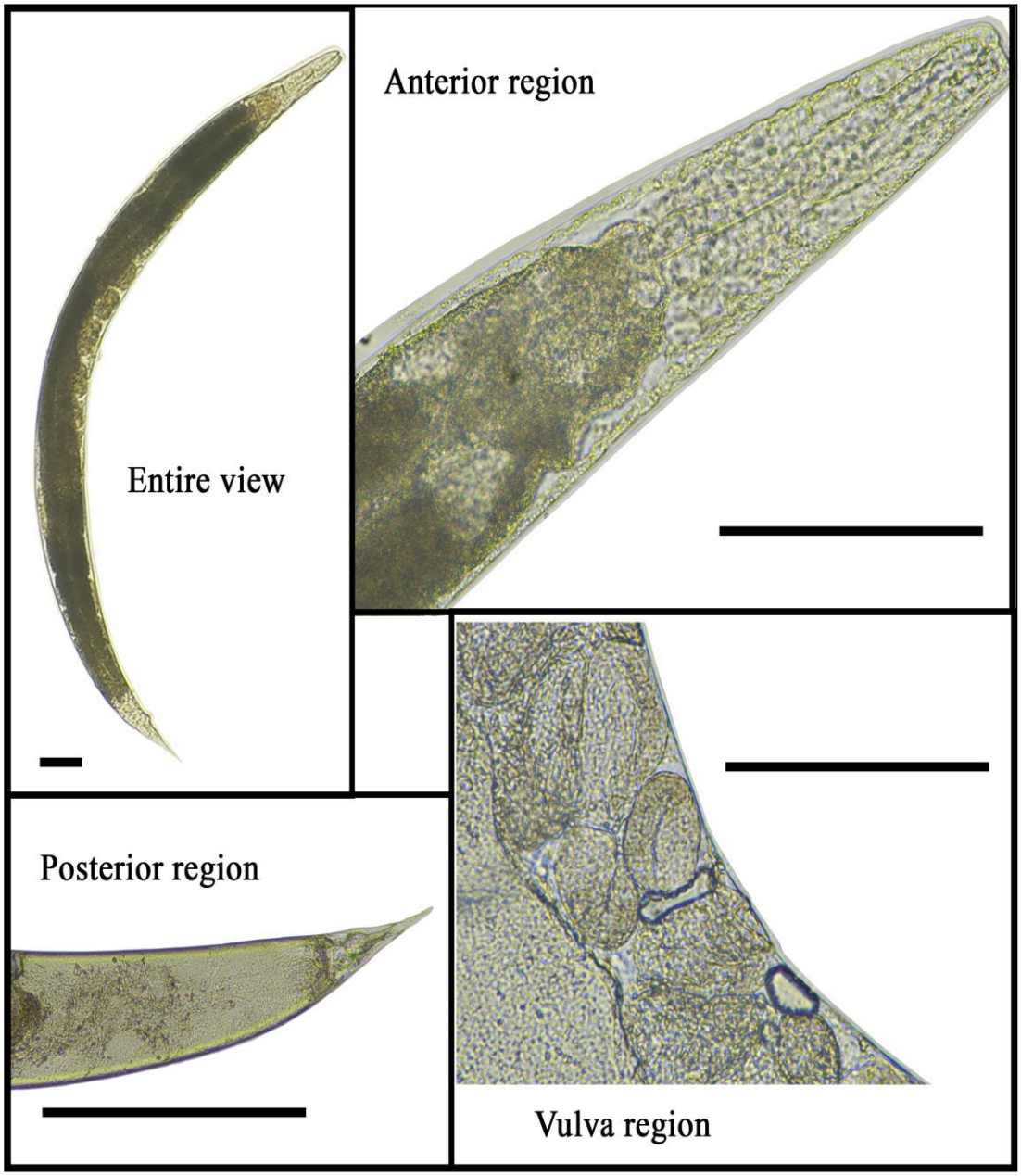

Fig. 1.4. *Heterorhabditis indica*: second generation female. **(Scale bars = 100µm)**

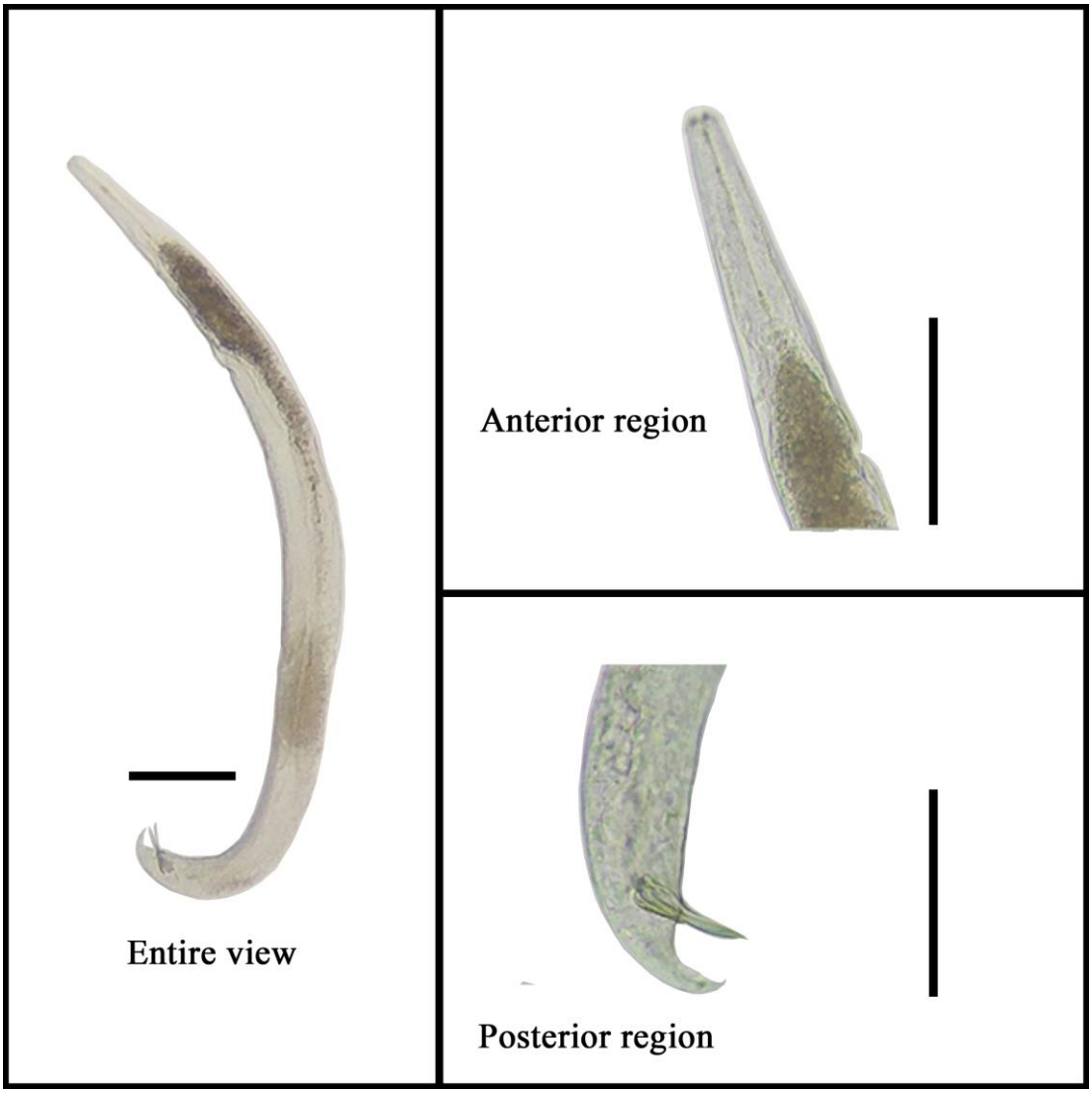

Fig. 1.5. *Heterorhabditis indica*: second generation male. **(Scale bars = 100µm)**

Group II (Nematode isolate 7): *Heterorhabditis* sp. 2

Kingdom : Animalia

Phylum : Nematoda

Class : Secernentea von Linstow, 1905

Order : Rhabditida (Orley, 1880) Chitwood, 1933

Suborder : Rhabditina (Orley, 1880) Chitwood, 1933

Superfamily : Rhabditoidea (Orley, 1880) Travassos, 1920

Family : Heterorhabditidae Poinar, 1976

Genus : *Heterorhabditis* Poinar, 1976

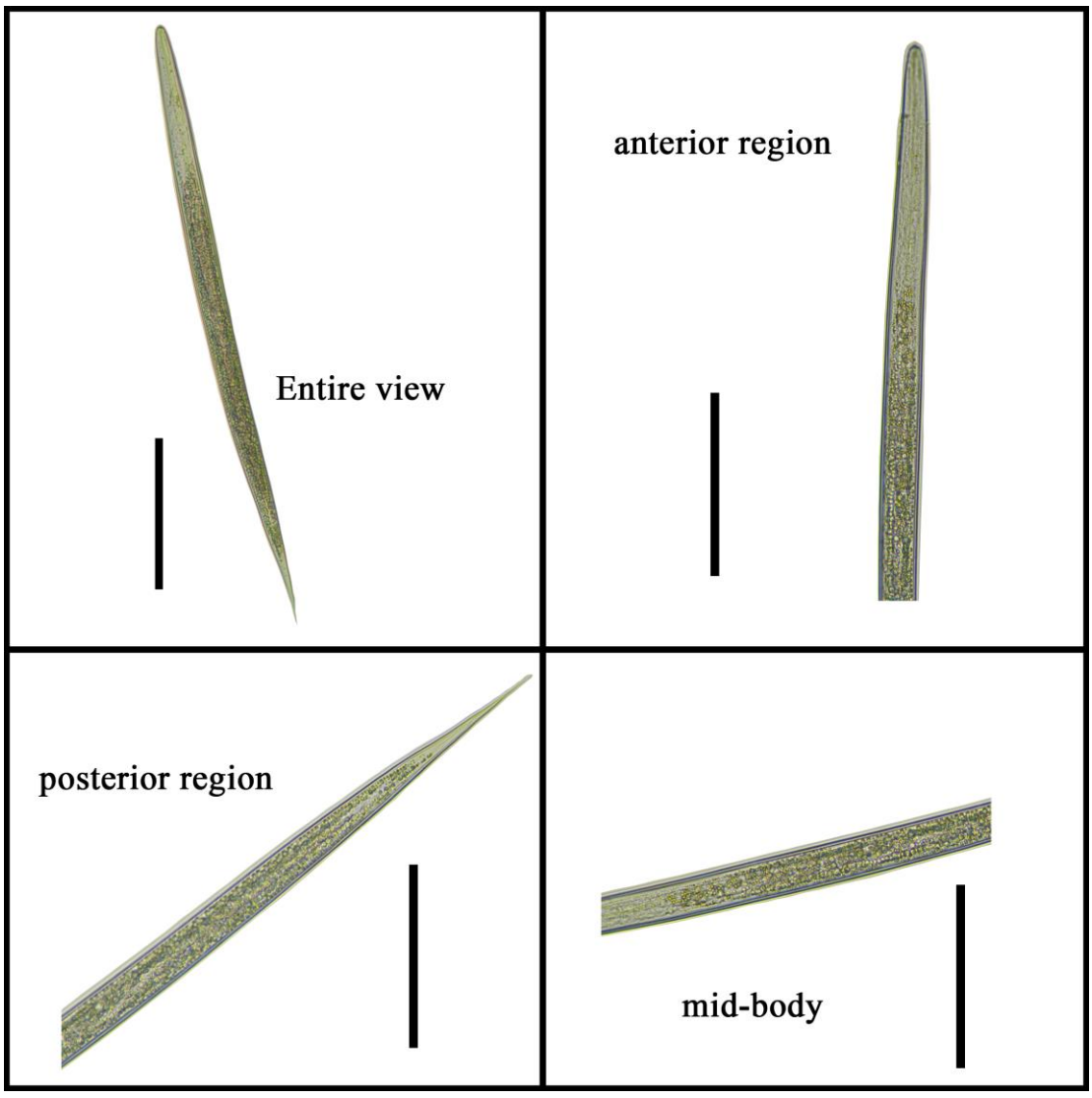

Fig. 1.6. *Hetererorhabditis baujardi*: infective juvenile. **(Scale bars = 100µm)**

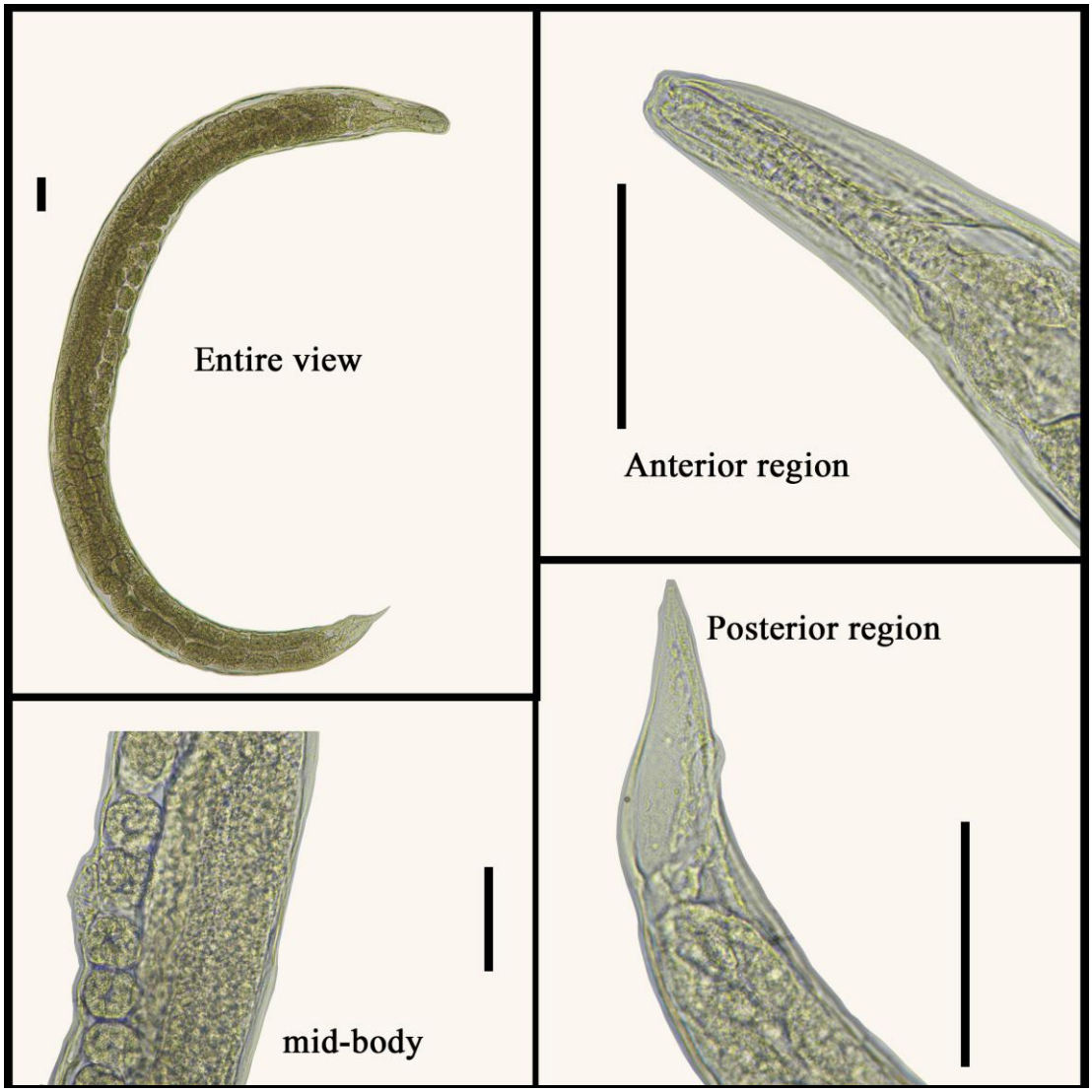

Fig. 1.7. *Hetererorhabditi baujardi*: first generation female. **(Scale bars = 100µm)**

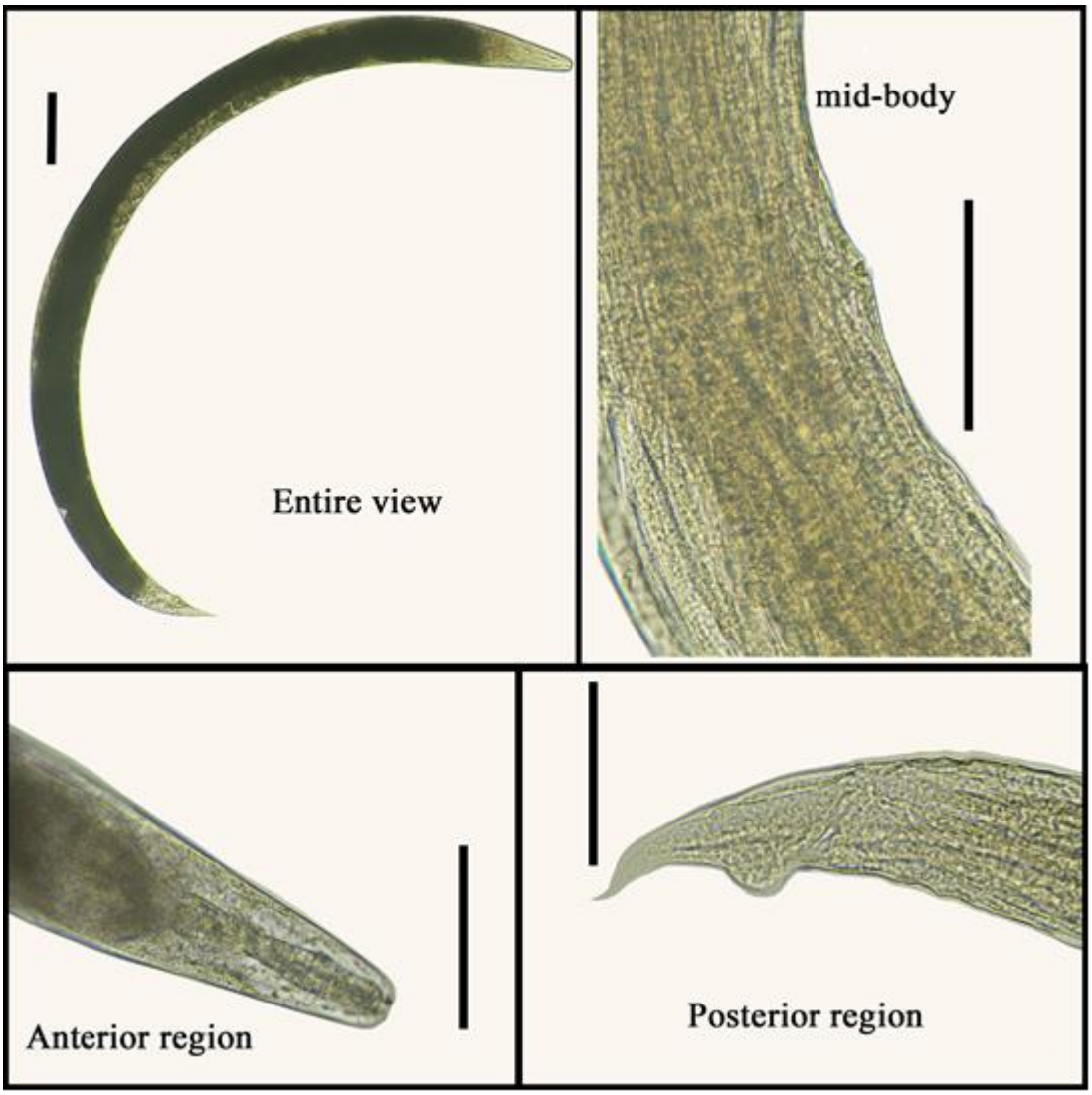

Fig.1.8. *Hetererorhabditi baujardi*: second generation female. **(Scale bars = 100µm)**

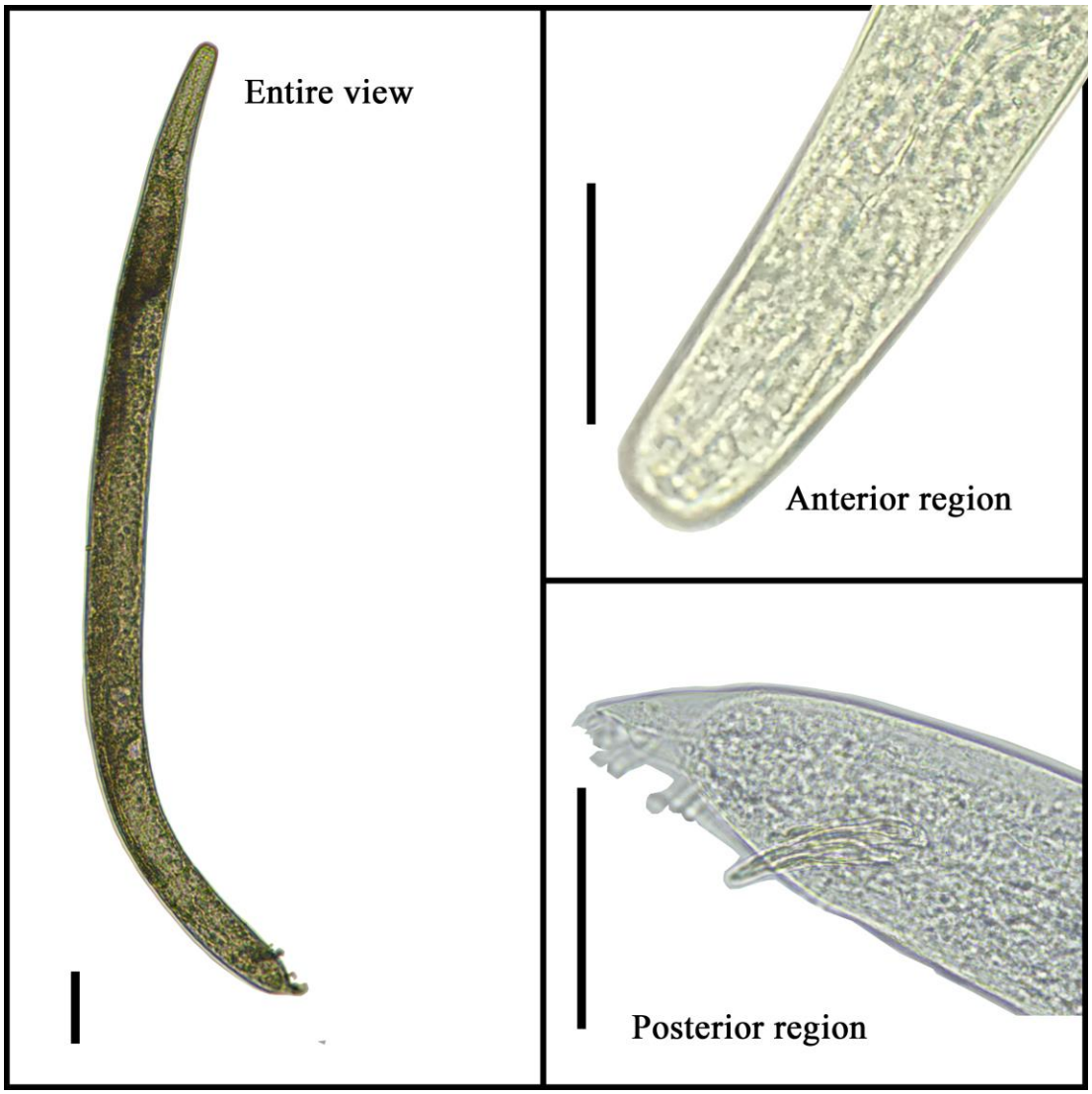

Fig. 1.9. *Heterorhabditis baujardi*: second generation male. **(Scale bars = 100µm)**

Group III (Nematode isolate 8 to 11): *Steinernema* sp.1

Kingdom	: Animalia
Phylum	: Nematoda
Class	: Secernentea von Linstow, 1905
Order	: Rhabditida (Orley, 1880) Chitwood, 1933
Suborder	: Rhabditina (Orley, 1880) Chitwood, 1933
Superfamily	: Rhabditoidea (Orley, 1880) Travassos, 1920
Family	: Steinernematidae Chitwood and Chitwood, 1937
Genus	: *Steinemema* Travassos, 1927

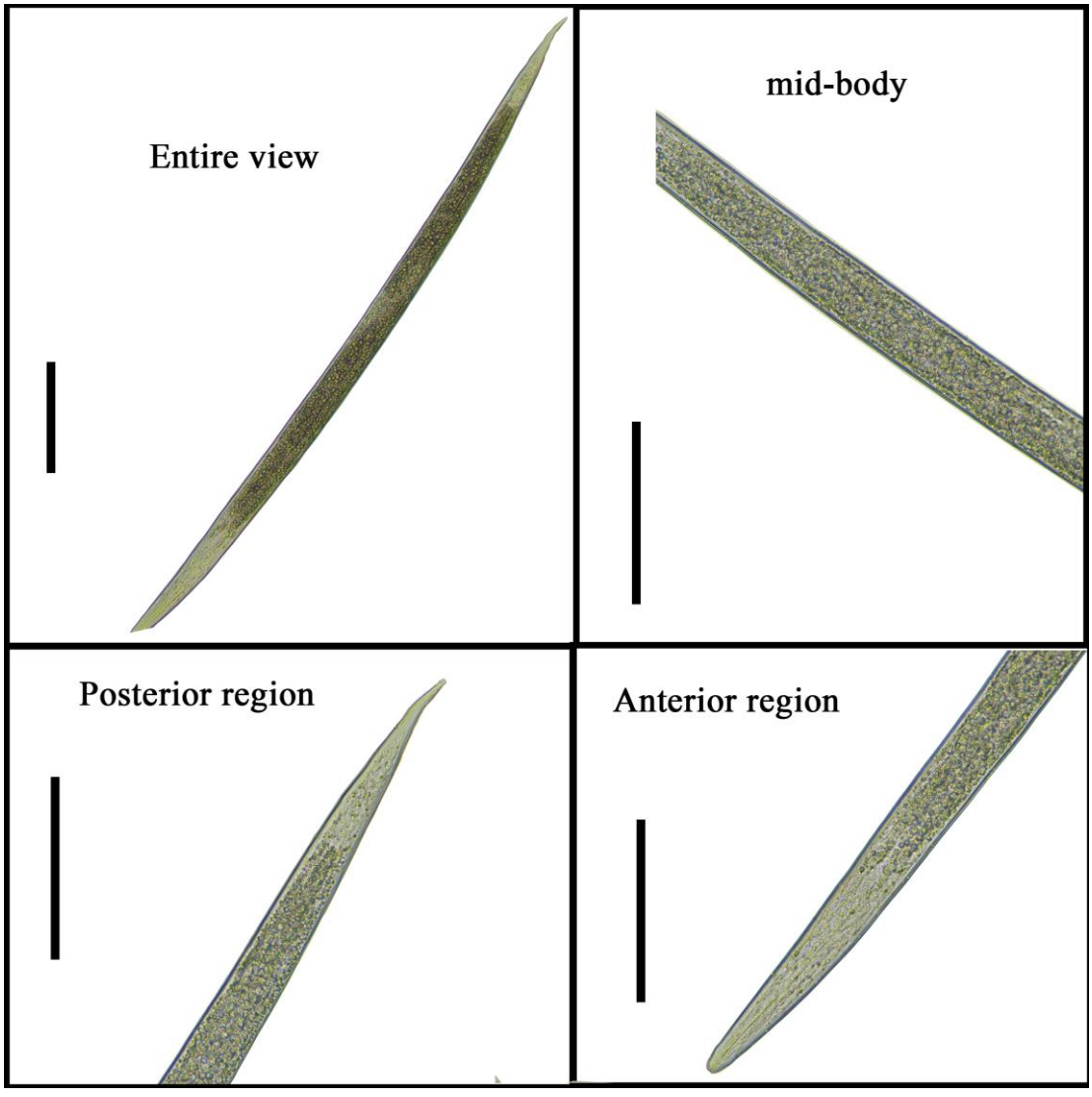

Fig. 1.10. *Steinernema sangi*: infective juvenile. **(Scale bars = 100µm)**

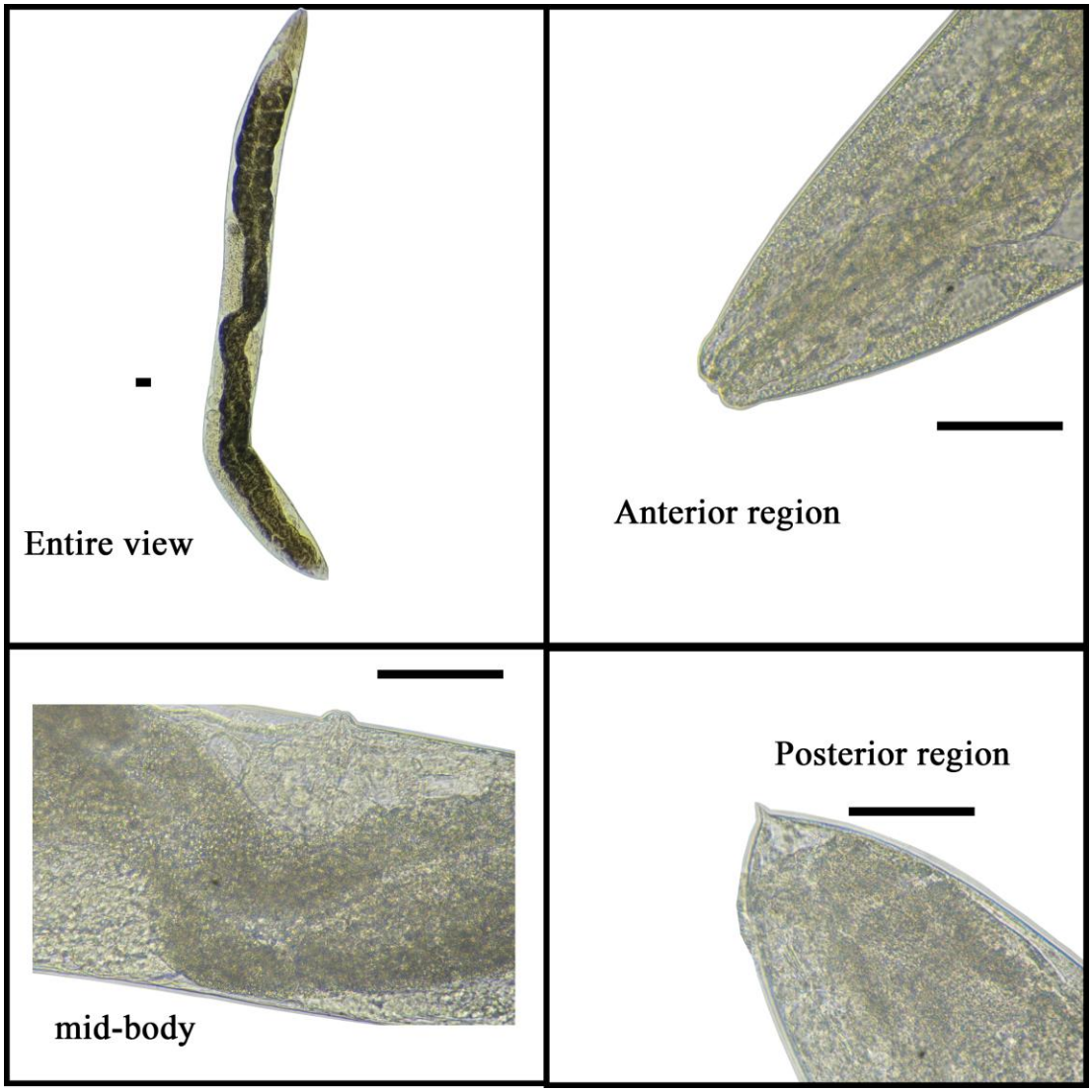

Fig. 1.11. *Steinernema sangi*: first generation female. **(Scale bars = 100µm)**

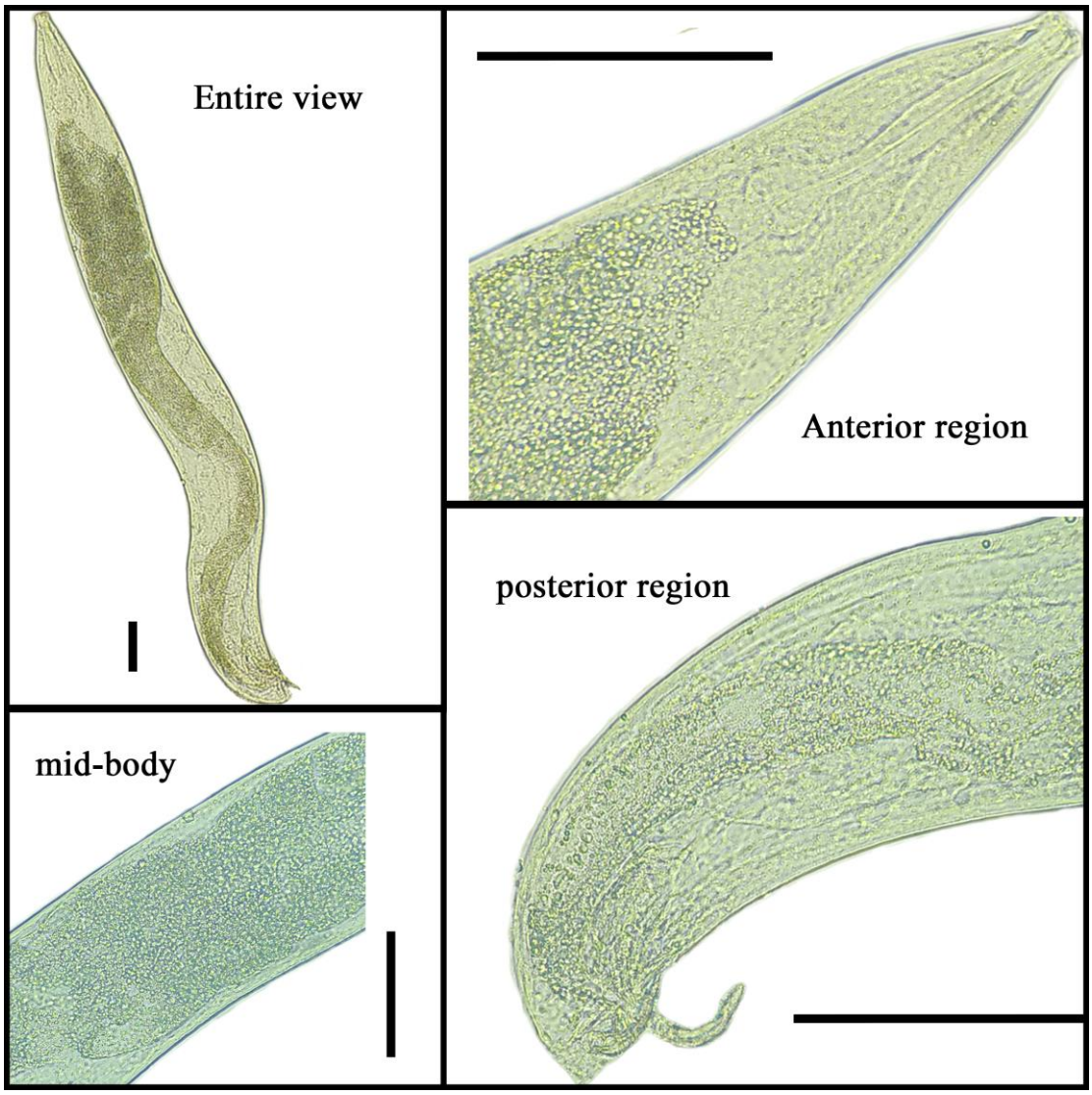

Fig. 1.12. *Steinernema sangi*: first generation male. **(Scale bars = 100µm)**

Group IV (Nematode isolate 12): *Steinernema sp.*2

Kingdom	: Animalia
Phylum	: Nematoda
Class	: Secernentea von Linstow, 1905
Order	: Rhabditida (Orley, 1880) Chitwood, 1933
Suborder	: Rhabditina (Orley, 1880) Chitwood, 1933
Superfamily	: Rhabditoidea (Orley, 1880) Travassos, 1920
Family	: Steinernematidae Chitwood and Chitwood, 1937
Genus	: *Steinemema* Travassos, 1927

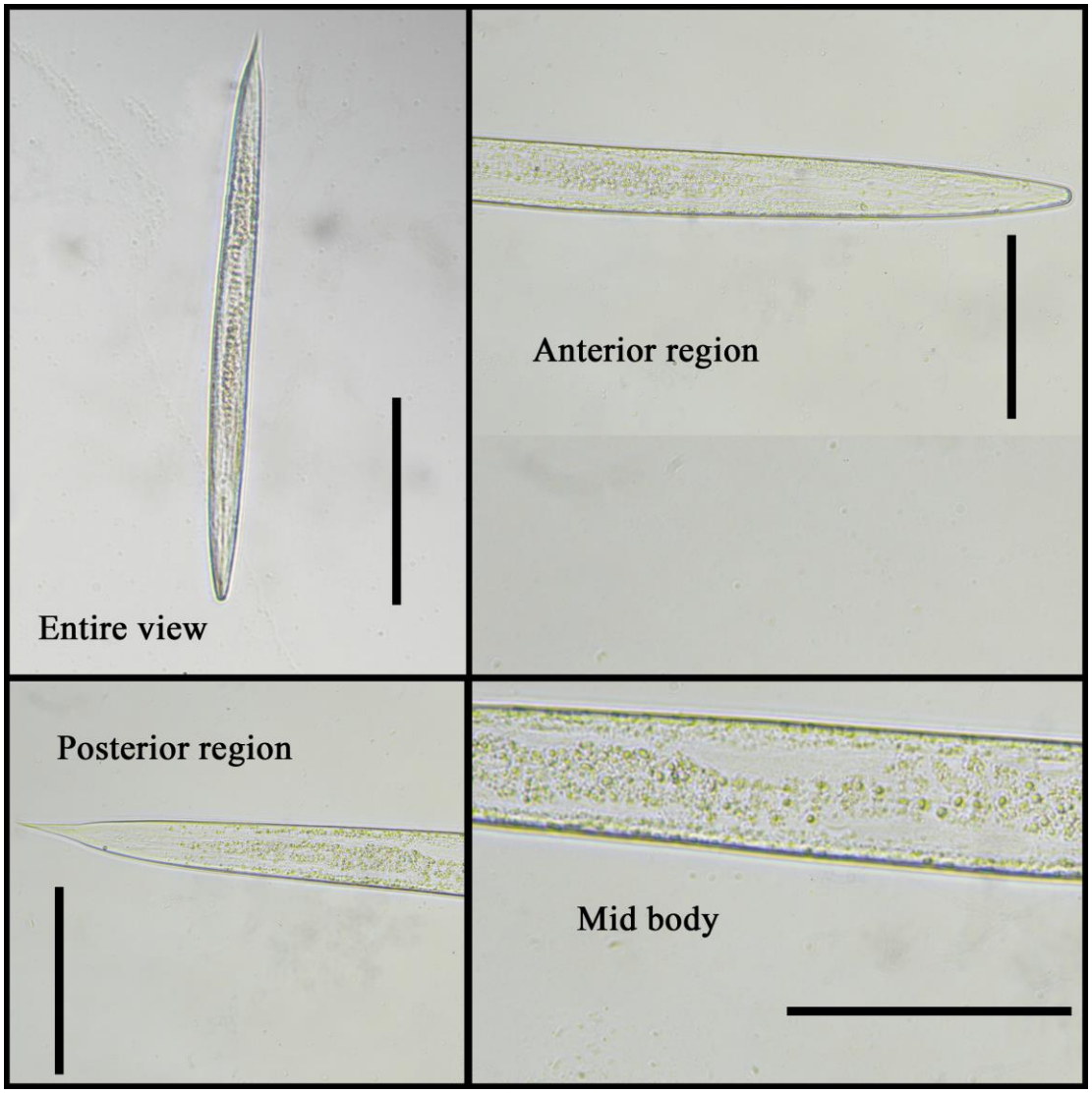

Fig. 1.13. *Steinernema surkhetense*: infective juvenile. **(Scale bars = 100µm)**

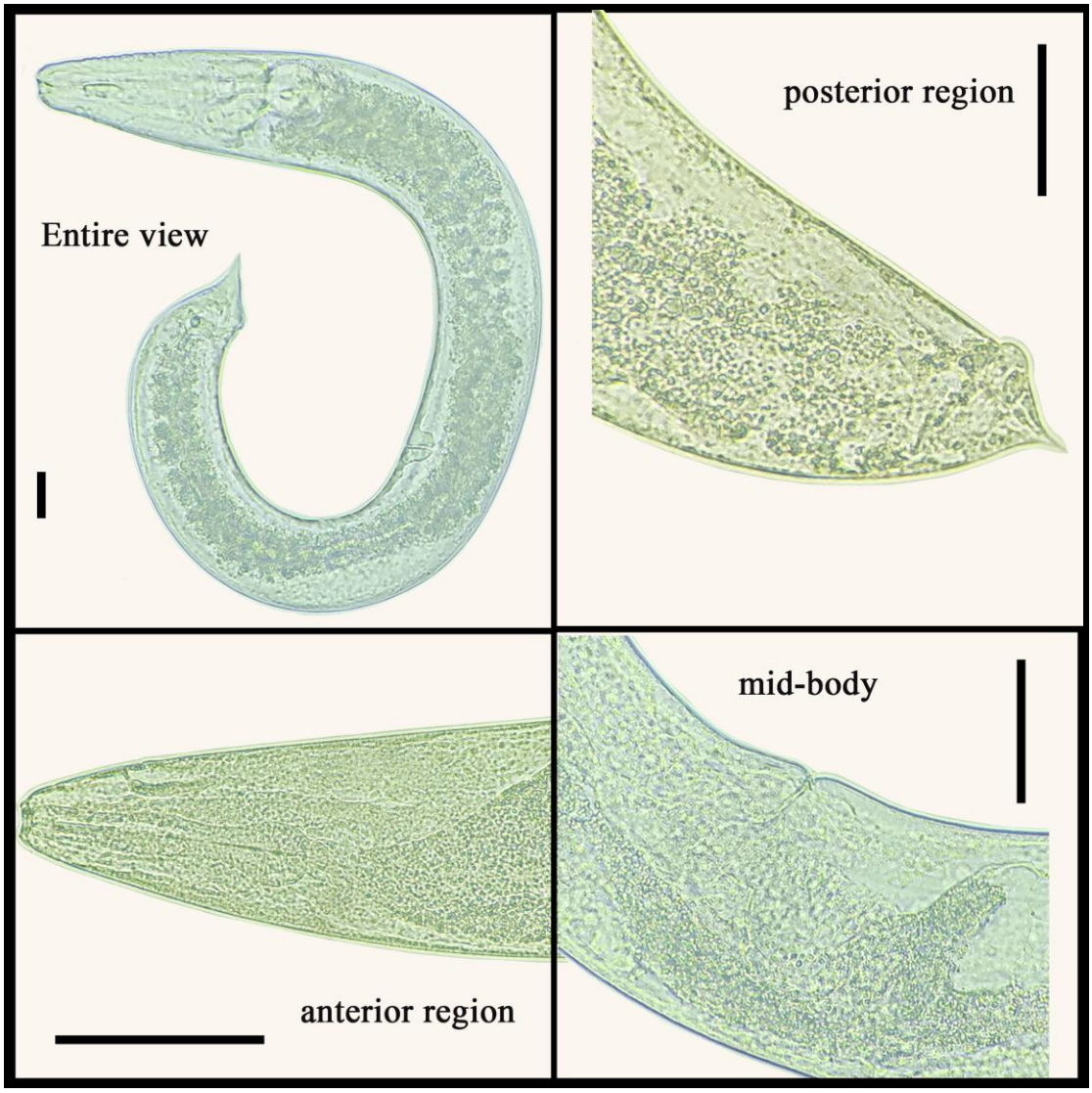

Fig. 1.14. *Steinernema surkhetense*: first generation female. **(Scale bars = 100µm)**

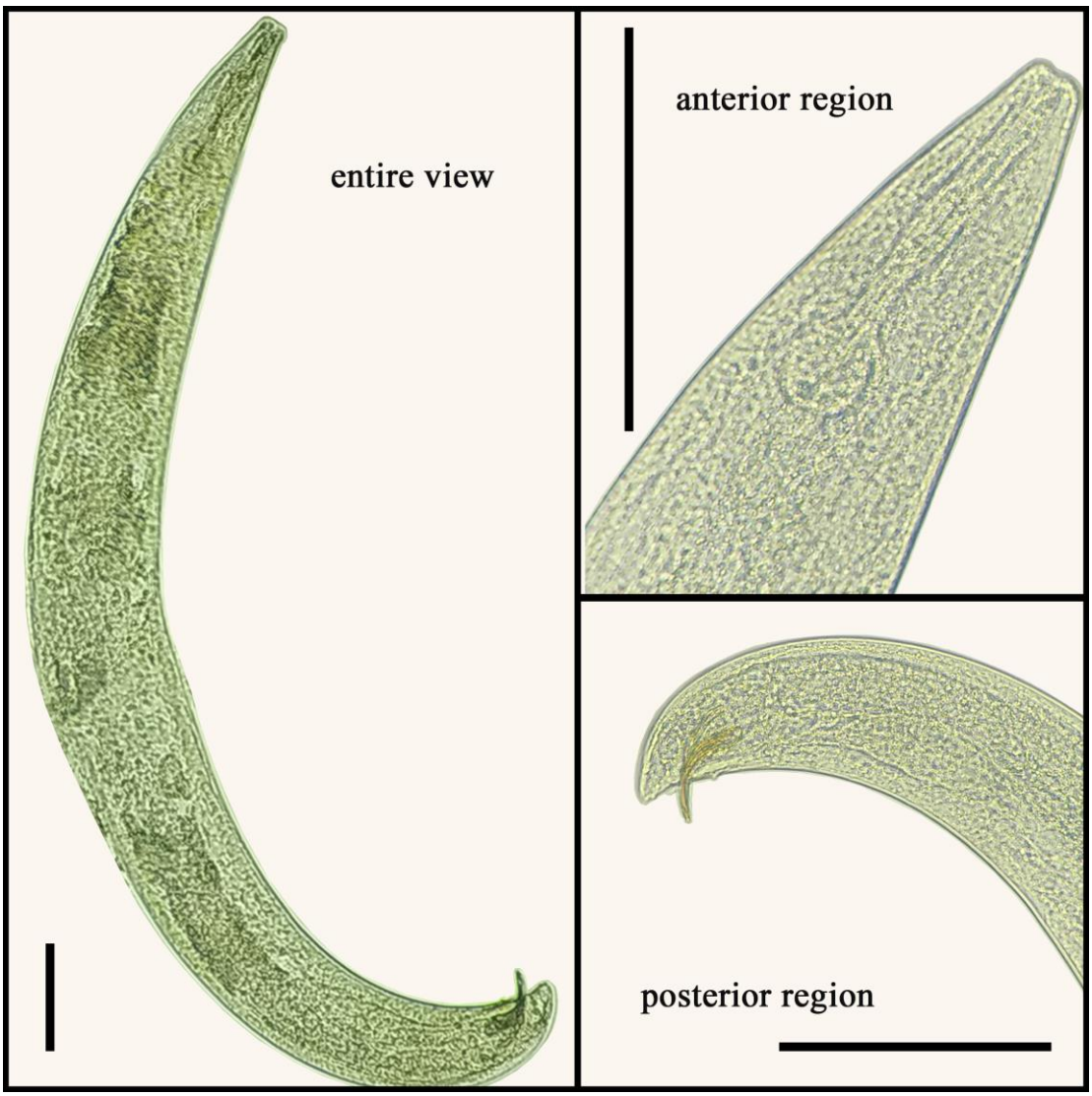

Fig. 1.15. *Steinernema surkhetense*: first generation male. **(Scale bars = 100µm)**

Table 1.5. Morphometric measurements (Mean ± SEM) of infective juveniles, hermaphrodite female, amphimictic female and male of the nematode isolate, *Heterorhabditis* sp. 1 (in μm)

Character	**Infective juvenile**	**Hermaphrodite female**	**Amphimictic female**	**Male**
Body Length	623.3±7.1 (577.5-692.5)	3958.3±85.1 (2510-4820)	2077.1±28.7 (1740-2295)	881.8±9.0 (805-947.5)
Body Width	22.9±0.3 (20-25)	224.1±6.5 (120-290)	150.8±1.7 (130-160)	53.3±0.6 (50-60)
Oesophagus Length	114.6±0.82 (107.5-120)	161.5±1.4 (150-180)	131.3±0.9 (125-140)	103.2±0.5 (100-107.5)
EP	97.8±0.7 (92.5-105)	160.5±1.8 (145-180)	122.9±1.2 (115-135)	113.5±0.5 (110-117.5)
Tail Length	103.1±1.2 (87.5-112.5)	98.8±1.8 (70-117.5)	79.6±1.1 (70-90)	29.9±0.6 (27.5-35)
E	0.98±0.01 (0.82-1.02)	1.6±0.04 (1.23-2.00)	1.6±0.04 (1.3-1.9)	3.9±0.06 (3.5-4.3)
F	0.22±0.004 (0.2-0.3)	2.3±0.07 (1.1-2.8)	1.9±0.03 (1.5-2.1)	1.8±0.04 (1.4-2.2)

EP = distance of excretory pore from anterior end; **E** = EP/Tail Length; **F** = Body Width/Tail Length

Table 1.6. Morphometric measurements (Mean ± SEM) of infective juveniles, hermaphrodite female, amphimictic female and male of the nematode isolate, *Heterorhabditis* sp. 2 (in μm)

Character	Infective juvenile	Hermaphrodite female	Amphimictic female	Male
Body Length	571.3 ± 5.3 (525.0–615.0)	3548 ± 38.9 (3250–3970)	2185 ± 14.39 (2060–2290)	814.7 ± 14.3 (710–902.5)
Body Width	20.8 ± 0.4 (17.5–25.0)	219.6 ± 3.9 (190–250)	135.6 ± 1.5 (120–150)	44.6 ± 0.6 (40.0–50.0)
Oesophagus Length	109.5 ± 1.4 (97.5–120.0)	198.2 ± 1.19 (180–205)	135.6 ± 1.4 (122.5–147.5)	102.9 ± 1.0 (97.5–110)
EP	91.5 ± 0.6 (87.5–95.5)	164.6 ± 2.6 (150–185)	108.2 ± 1.3 (97.5–115)	88 ± 0.7 (82.5–92.5)
Tail Length	101.2 ± 0.7 (95.0–107.5)	91.4 ± 1.2 (80–105)	89.8 ± 1.6 (77.5–107.5)	36.8 ± 0.6 (32.5–40)
E	0.91 ± 0.01 (0.89–0.92)	1.76 ± 0.04 (1.42–2.11)	1 ± 0.01 (0.9–1.14)	2.4 ± 0.1 (2.1–2.8)
F	0.21 ± 0.02 (0.18–0.23)	3.15 ± 0.07 (2.67–3.88)	1.9 ± 0.04 (1.6–2.2)	1.2 ± 0.0 (1.1–1.4)

EP = distance of excretory pore from anterior end; **E** = EP/Tail Length; **F** = Body Width/Tail Length

Table 1.7. Morphometric measurements (Mean ± SEM) of infective juveniles, female and male of the nematode isolate, *Steinernema* sp. 1 (in μm)

Character	**Infective juvenile**	**1st Generation Female**	**1st Generation male**
Body Length	748.8±6.2 (697.5-797.5	5959.6±147.6 (4580-7120)	1585±51.0 (1322.5-2192.5)
Body Width	32.5±0.3 (27.5-35)	321±4.02(270-360)	156.3±4.2 (127.5-202.5)
Oesophagus Length	125±1.3 (107.5-140)	221.5± 2.9 (200-250)	165±1.5 (152.5-182.5)
EP	48.8±0.6 (42.5-52.5)	98.4±1.6 (77.5-115)	76.3±1.2 (67.5-92.5)
Tail Length	77.5±0.7 (72.5-87.5)	48.8±1.0 (37.5-60)	33.8±0.8 (27.5-43.8)
E	0.63±0.01 (0.54-0.7)	2.0±0.04 (1.5-2.6)	2.32±0.05 (1.9-3.2)
F	0.42± (0.34-0.45)	6.7±0.15 (5.3-8.5)	4.8±0.1 (3.9-6.6)

EP = distance of excretory pore from anterior end; **E** = EP/Tail Length;
F = Body Width/Tail Length

Table 1.8. Morphometric measurements (Mean ± SEM) of infective juveniles, female and male of the nematode isolate, *Steinernema* sp. 2 (in μm)

Character	**Infective juvenile**	**1st Generation Female**	**1st Generation male**
Body Length	449.4±4.4 (417.5-480)	4600.9±133.6 (3690-5830)	1280.432.6 (1065-1477.5)
Body Width	26.1±0.4 (22.5-27.5)	227.3±3.4 (200-250)	121.63.7 (100-140)
Oesophagus Length	98.1±1.4 (87.5-107.5)	141.1±1.6 (130-155)	120.91.1 (112.5-127.5)
EP	34.5±0.6 (30-37.5)	85.8±1.0 (77.5-92.5)	61.6±1.0 (52.5-67.5)
Tail Length	47.9± 1.1 (37.5-55)	43.8±0.6 (40-50)	29.9±0.7 (25-35)
E	0.74±0.02 (0.59-0.88)	1.97±0.03 (1.8-2.3)	2.1±0.05 (1.83-2.5)
F	0.55±0.02(0.45-0.73)	5.2±0.07 (4.7-5.9)	4.1±0.12 (3.2-5.3)

EP = distance of excretory pore from anterior end; **E** = EP/Tail Length; **F** = Body Width/Tail Length

Discussions

Of all the constraints faced by agriculture crop production around the world, insect pests remain one of the major culprits of the loss. Besides direct losses of crops, agriculture face problems due to the contaminants leave behind by the insect pests and, by damaging packaging materials and processes products as well. In India, the losses of crops due to insect pests are relatively high and have declined to about

15.70% since a remarkable development in pest management strategies occurs including numerous biopesticides and transgenic crops **(Dhaliwal *et al*., 2015)**. Of various methods applied for pest and disease control, pesticides remain the most effective methods extensively used across developing countries. In addition to widespread insecticide resistance, many of the pesticides are linked with non-targeted organisms including environment and health issues **(Zhang *et al*., 2016)**. Therefore, the implementation of safer, and environment friendlier practices are the urgent need in agriculture system to reduce the application of agrochemicals.

One of the important attributes that EPNs possess is that the IJs could recycle in soil through infected insect cadaver which is important for extended establishment of nematode in soil. Furthermore, EPNs are compatible with various chemical pesticides, easy in mass production with low cost, and could be formulated in various forms with different biological applications **(Georgis and Horn, 1992; Grewal, 2002)**. In the year 2009, biopesticides represented about 3.50% ($1.6 billion) of the worldwide pesticide market, and with the high demand for environmentally friendly pesticides, the global market for biopesticides will reach about $5.20 billion in 2017 **(Lehr, 2010; Abd-Elgawad *et al*., 2017)**.

For the effective application of EPN as a biological control agent, proper identification and documentation of locally adapted species are necessary. This is because isolates of EPNs from different geographical areas exhibit variances in their behavioural and physiological adaptations **(Stock *et al*., 1999)**. The aim of the study, therefore, was to isolate locally-adapted EPN species from the collected soil samples in Mizoram, a north-eastern state of India.

Northeast India, situated at the Indo-Burma region is one of the 4 biodiversity hotspot regions in India harbouring various forms of flora and fauna. In India, a number of indigenous steinernematids and heterorhabditids have been documented. However, works on the EPNs in northeast India is very limited, and moreover, less is known about their occurrence and potential in the region. Mizoram is gifted with thick forests and various forms of plants and animals including diverse medicinal plants for treatment of numerous illnesses.

The present work reported the occurrence of EPNs of the family heterorhabditidae and steinernematidae, which is the first report from the state of Mizoram. From the current investigation, 2 species each of the genera Steinernema and *Heterorhabditis* were encountered based on basic morphological identification of the nematode as well as colour of host cadaver. The study thus increases additional information on the diversity of both *Heterorhabditis* spp. and *Steinemema* spp. in India. Since isolates of EPNs from different geographical area exhibit differences in their behavioural and physiological adaptations, identification and documentation of locally adapted species of a particular region is required for the successful use of EPN as a biological control agent **(Stock *et al.*, 1999)**. Furthermore, accurate identification is very important in understanding the geographical distribution and biodiversity of steinernematids **(Kerry and Hominick, 2002)**.

Soil samples were collected all year covering different habitats across the State. However, EPNs were not recovered during extreme winter and rainy seasons. One of the important ecological factors that influence the occurrence of EPN in the soil ecosystem is the soil type. The persistence, distribution and mobility of EPN is

highly influenced by texture of the soil. In the present work, most of the EPN positive samples were sandy loam soils and generally not from soil with high content of clay and silt. This may be due to low mobility and poor survivability of nematodes in clay and silt soils **(Barbercheck and Kaya, 1991)**. In conclusion, the present study therefore adds new data on the occurrence of EPNs in the state of Mizoram in particular and in India, in general.

Despite the fact that EPN has been shown to be a promising biocontrol agent against a variety of insect pests around the world, EPNs differ in their survival, search behaviour, and infectivity. As a result, there is a growing desire to locate populations with features that are suited to local conditions. Despite the fact that several EPN surveys have been undertaken around the world, little is known about the natural variety of EPNs in India. In India, a number of indigenous steinernematids and heterorhabditids have been documented. However, works on the EPNs in northeast India is very limited, and moreover, less is known about their occurrence and potential in the region. Even though the application of EPNs against insect pests in India began in the year 1966 with exotic species **(Rao and Manjunath, 1966)**, it is still in the early stages of development. Intensive surveys are required to isolate EPN species from India's various agro-climatic zones. Because they are adapted to local environmental conditions and are natural insect population controllers, indigenous EPNs are well-suited for the management of local insect pests. The findings of this study may pave the way for the use of these native EPN species in biological control efforts against insect pests in the area.

CHAPTER 2

Molecular characterization of isolated EPNs using ITS, COI and 28S rDNA markers

Introduction

Molecular techniques are frequently used to classify organisms around the world. EPNs represent one of the most prevalent insect parasitic nematodes throughout tropical and subtropical regions. Many of the EPN identifications are traditionally based on morphological methods, which are less likely straightforward **(Poinar, 1990)** and overflowed with complexities and ambiguities. In this context, accurate identification of EPN is a major concern requiring the integration of a molecular approach. Moreover, with the advance in technology, several molecular techniques were developed for accurate identification, diagnosis of genetic variability and evaluate the phylogenetic relationships between distinct EPN species **(Stock *et al.*, 2001)**. Although the EPN families of Steinernematidae and Heterorhabditidae do not share a common ancestor phylogenetically, they have convergently evolved to share comparable life cycles and morphological and ecological traits **(Blaxter *et al.*, 1998)**. Even though they have been recognised ever since the 17th century (**Nickle *et al.*, 1984)**, only in the 1930's, more researchers have focused on the biological control potency of EPNs and since then new species of EPNs are continuously discovered. However, classical identification of EPNs based on morphology and morphometric measurements is not often straightforward and raised confusion **(Reid and Hominick, 1993)**. Therefore, proper identification of EPNs plays a crucial role in their further application in pest controls and requires the involvement of molecular

methods. To identify EPNs, molecular techniques are not required to distinguish between *Steinernema* and *Heterorhabditis* since *Steinernema* do not produce hermaphrodites during their life cycle, whereas EPNs from the genus *Heterorhabditis* do **(Muthulakshmi *et al.*, 2012)**. Though phenotypic variation becomes more constrained within each genus, these methods are crucial for identifying and differentiating between species. The molecular techniques that have been used the most frequently include DNA sequencing, restriction fragment length polymorphisms (RFLP), and random amplified polymorphic DNA (RAPD).

Molecular markers have been used to examine genetic variety; they primarily reveal naturally existing polymorphisms at the DNA level, making them a potent tool to assess the impact of different causes on genetic diversity and population structure. Among many others, DNA sequencing has been reported to yield more information about intra and interspecific variations as compared to RFLP **(Szalanski *et al.*, 2000; Davis *et al*, 2007)**. In addition, the use of RAPDs has been discouraged, primarily due to the realization that a variety of factors, including the quality and concentration of DNA, PCR cycling conditions (including the type of PCR machine used), etc., can have an impact on the reproducibility of results. Many scientific studies have claimed that PCR-based DNA sequence analysis is more suitable in phylogenetic relationships assessment **(Adams *et al.*, 1998; Stock *et al.*, 2001)** with considering that nuclear DNA genes are suitable genetic markers for nematode delimitation at several taxonomic levels **(Nadler and Hudspeth, 2000)**. The 18S or Small Subunit (SSU) gene of rDNA, the internal transcribed spacer (ITS) region (covering ITS-1, 5.8, and ITS-2 genes) of rDNA, and the D2-D3 regions of 28S or Larger Subunit

(LSU) of rDNA are nuclear rDNA regions that have been widely employed for species delimitations and resolving phylogenetic relationships in EPN taxonomy **(Spiridonov and Subbotin, 2016)**. Additionally, the ITS region gene sequences have been employed for species identification, to study intra and inter-population among Steinernematids **(Nguyen *et al*., 2001; Desta *et al*., 2011)**. Furthermore, the ITS region has also been used as diagnostic markers for the delimitation of *Heterorhabditis* species, and assessment of the evolutionary relationships between the species of *Steinernema* **(Spiridonov etal., 2004)**. Furthermore, there are certain reports that the 18S or SSU gene of rDNA is too conserved in resolving relationships among *Heterorhabditis* **(Liu *et al*., 1997)** or *Steinernema* **(Stock *et al*., 2001)**. Additionally, the area of 28S or LSU of the rDNA gene is remarkably useful and reliable for *Steinernema* in terms of defining terminal taxa, assessing phylogenetic relationships and performing diagnostic tests **(Stock and Hunt, 2005).**

In India and other parts of the world, molecular approaches have supplemented conventional morphological methods for identifying EPNs due to advancements in biotechnology **(Nisa *et al*., 2022). Hussaini *et al*. (2001b)** have identified 3 *Steinernema* species isolated from soil samples in India by performing analysis on RFLP of the PCR product of ITS rDNA using 17 restriction enzymes. The combined techniques of PCR and RFLP of ITS regions of rDNA have been applied for new species descriptions of EPNs and studying phylogenetic relationships among them **(Reid and Hominick, 1992; Hazir *et al*., 2003)**. However, for the study of variations within and among nematode species, the

approach of PCR and sequence analysis of ribosomal ITS region was claimed to be more useful than PCR-RFLP approaches **(Phan et. al., 2005).**

Besides the 2 formerly mentioned gene markers, the Folmer region of the mitochondrial cytochrome oxidase c subunit 1 (COI) gene is popularly used for studying population genetics and phylogeographic in the Kingdom Animalia **(Avise, 1994, Hebert *et al*, 2003)**, including parasitic nematodes **(Elsasser *et al*, 2009)**. Furthermore, the COI gene was used for resolving the phylogenetic relationship between closely related nematode species (and or cryptic species) **(Derycke *et al*., 2005; Armenteros *et al*., 2014)**. Regarding this matter COI (M1–M6 partition) have been playing a reliable function for species delimitation due to the conserved nature of the region among species, yet variable between different species (**Hebert *et al*., 2003; Folmer *et al*., 1994)**. Nevertheless, at the levels of species resolution, in some taxa, problems have been obtained from COI (M1–M6 partition) based on DNA barcoding. However, the COI (M1–M6 partition), although incompatible with other genes like ITS and 28S rRNA in resolving phylogeny, can be a good supportive tool for species delimitation. Besides these, for assessing the evolutionary associations among species of the Genus *Steinernema* and *Heterorhabditis*, cytochrome oxidase II (COII-16S) **(Szalanski *et al*., 2000)**, 12S rDNA and cox 1 genes **(García-Varela and Nadler, 2006)** have been used as well.

From Oman, the Sultanate, *S. abbasi* was described by **Elawad *et al.* (1997)** based on the analysis of RFLP profiles of the ITS regions and morphological characters. Based on **Qiu *et al*. (2005)**, analysis of the DNA sequences of either a

partial 28S rDNA or the ITS portions of rDNA was used to differentiate the newly described species (*S. aciari*) from other *Steinernema* species.

Abate *et al*. (2018) have conducted molecular identification and phylogenetic relationship studies on 28 EPN species collected from soil samples in South Africa using ITS regions and D2-D3 regions of 28S of rDNA. They have successfully identified 27 EPN species with the exception of 1 isolate that showed low % similarity (less than 92.00%) compared to its nearest known relative. With the exception of *S. citrae*, which belongs to the "*feltiae*" group, they were able to successfully classify 24 isolates of *Steinernema* into 4 groups based on maximum likelihood (ML) analysis of the ITS and D2-D3 regions of rDNA sequences. This grouping was called the "cameroonense" group **(Stokwe *et al*., 2011)**. Also, they analysed the ITS region of rDNA of the genus *Heterorhabditis* using ML and 4 of the *Heterorhabditis* isolates were successfully grouped into two groups, where isolates of each group showed 99.00% similarity. **Aryal *et al*. (2022)** have isolated and molecularly characterized 36 isolates of EPNs from Eastern Australia. They have successfully identified 5 species of EPN using ML analysis of the ITS and D2-D3 regions of rDNA sequences. **de Brida *et al*. (2017)** conducted a survey on the occurrence of EPNs from agricultural soils in Brazil where they identified EPN species of *S. rarum*, *H. amazonensis*, *O. tipulae*, and *M. rainai* based on the analysis of the D2/D3 expansion region of the 28S rDNA. **Caoili *et al*. (2018)** carried out a survey of native EPN species throughout the Philippines' major islands and used *Ostrinia furnacalis* as bait. Four EPN species have been successfully identified that includes *S. abbasi*, *S. minutum*, *S. tami*, and *H. indica*. **Chaerani *et al*. (2018)** have

performed a search for biological control agents from East Java and Bali, Indonesia by using mealworm larvae (*Tenebrio molitor*) as bait. They have analysed the rDNA ITS 1 and 2 regions of the EPN isolates and have successfully identified 2 species, *H. indica* and *S. huense*. **Darissa *et al*. (2014)** collected 6 isolates of EPNs from various regions of ancient Palestine. They analysed and aligned sequences and identified 3 of the isolates as *S. feltiae* and the remaining 3 as *S. carpocapsae*. **Dhakal *et al*. (2020)** had 5 gene fragments: D2/D3 expansion region of the 28S rDNA, ITS region of rDNA, mitochondrial COI gene, and cmd-1 genes encoding thin filament (F-actin) of 11 *Heterorhabditis* species amplified and sequenced. The genus was confirmed to be divided into 3 group (*indica*, *bacteriophora* and *megidis*) utilising phylogenetic and sequencing analyses of these genes using Bayesian inference, ML, and statistical parsimony (groups). The study of gene sequences that were obtained from GenBank and classified as belonging to the *Heterorhabditis* species revealed numerous instances of species misidentifications as well as the existence of reading errors in some sequences. Based on their work, sequence and phylogenetic research have confirmed the synchronization of *H. somsookae* to *H. gerrardi* and *H. baujardi*, and *H. pakistanensis* to *H. indica*, and *H. sonorensis* to *H. taysearae*. They have further stated that the ITS rRNA and COI genes may serve as useful markers for species identification, barcoding, and phylogeographic research of *Heterorhabditis*. **Dzięgielewska *et al*. (2020)** have isolated EPNs from soils of several ecosystems in northern Poland and were studied for species diversity and molecular characterisation. They have utilized PCR-RFLP method for molecular characterization of the EPN isolates and distinguished 8 species in the genera Steinernematidae and Heterorhabditidae. **Emelianof *et al*. (2008)** have isolated and

molecularly characterized 2 EPN species (*S. feltiae and H. bacteriophora*) from Southern France. They have amplified, sequenced and analysed the DNA of 28S and ITS region of rDNA for *Steinernema* and *Heterorhabditis*, respectively. **Lulamba *et al*. (2020)** have compared the sequences of EPN isolates from the northernmost region of South Africa with the sequences of other known species in the genus that are currently available. The 18S rRNA gene, the ITS region, and the D2-D3 expansion region of the 28S rRNA gene were used to analyse the sequences and the EPN isolates was confirmed to be *H. zealandica*. Based on the work conducted by **Yooyangket *et al*. (2018)**, 12 EPN isolates were collected from Phetchabun Province, Thailand and molecularly characterized by using the ITS DNA region that sufficiently identified *S. websteri* and *H. baujardi*. Two EPN species, *H. bacteriphora* and *O. myriophilus* were isolated from 3 provinces of Baghdad, Iraq **(Al-Zaidawi *et al*., 2019).** The nematodes were initially sorted out by colour and shape of the infected larvae and further characterized with ITS and 18S rDNA genes sequences.

At the national level, **Ganguly and Pandey (2006)** have revealed that the analysis of RFLP profiles of the ITS rDNA gene regions sufficiently differentiates *S. thermophilium* from 2 other closely related indigenous *Steinernema* species. They have further reported that based on the length and base composition of ITS regions of rDNA, multiple sequence alignments of 667 base pairs of the sequence clearly differentiates *S. thermophilium* from other closely relates species of *Steinernema*, where *S. abbasi* showed the highest degree of resemblance (96.95%), followed by *S. ceratophorum* (80.56%), *S. bicornutum* (80.42%), and *S. riobrave* (79.94 %). Rajkumar *et al*. (2016) conducted a survey on the occurrence of EPNs in several

coconut and areca nut plantation areas of India. They have identified *S. carpocapsae* from a standard morphometric key which was confirmed with molecular analysis of the 16S ribosomal and COXII genes**. Swati *et al*. (2015)** conducted a survey on EPNs from diverse agricultural fields in the states of Uttar Pradesh, India covering Ghaziabad and Meerut districts. By employing PCR, cloning, and sequencing of the ITS DNA region, molecular studies identify and confirm the EPN species as *S. siamkayai*. **Dubey *et al*. (2010)** have purified PCR-amplified ITS rDNA of *Steinernema* sp. isolated from Chhattisgarh and performed sequencing. According to their experiment, the base sequence of ITS rDNA region exhibited maximum similarity with *S. carpocapsae* (98.00%), followed by *S. sasonense* (95.00%), *S. bakanense* (93.00%) and *S. siamkayai* (89.00%). In addition, they further concluded that the comparison of length, composition and differences in base characters of the ITS regions sufficiently differentiates the isolated *Steinernema* species from the compared sequence of *Steinernema* species.

Materials and Methods

Sample preparation for genomic DNA extraction

A petri dish (35 x 10 mm) was lined with double layer Whatman filter paper No. 1. and wetted with 0.5 ml of distilled water containing each nematode isolate. A petri dish (35 x 10 mm) was lined with double-layer Whatman filter paper No. 1. and wetted with 0.5 ml of distilled water containing each EPN isolate. A separate dish was set for the nematodes isolated for different collection sites. The petri dishes were then incubated at 28 ± 2 °C for 30 mins for the nematodes to settle down. A single last instar larva of *G. melonella* was then introduced into each petri dish pre-

inoculated with nematodes and maintained at 28 ± 2 ℃ in an incubator. Larval mortality was checked at 24 hrs intervals of time for 120 hrs. Colour change and the smell emanating from the dead body of larvae and pupae were used for primary confirmation of death due to EPNs. After 48 hrs of mortality, the dead insects were rinsed with distilled water to remove any IJs that may attach to the dead larvae, and individually dissected under an Olympus CX41 microscope in Ringer's solution to collect the adult nematodes in the cadaver. Distilled water was used to remove surface contaminants on the nematodes, transferred to 1.5 ml Eppendorf microcentrifuge tubes and stored at 70% ethanol for isolation of DNA. Five replicate tubes were set for each nematode isolate and stored in the laboratory for the isolation of genomic DNA. A total of 39 samples from different collection sites which were primarily confirmed positive by a colour change and smell emanating from dead larvae were proccesed for DNA isolation.

Genomic DNA extraction

For molecular characterization of the EPN isolates, whole genomic DNA was extracted from adult female nematodes by using an organic (phenol chloroform) extraction method **(Baker, 1998)** with slight modifications.

The nematode samples stored in 70% ethanol were washed with phosphate buffer saline (PBS) to remove any surface contaminants without disrupting the sample. For all the EPN isolates, 5–20 mg of adult female nematodes was homogenised in a 1.5 ml sterile microcentrifuge tube with 500µl of TES extraction buffer solution [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] using a sterile scissor. A sterile micro-pestle was used to further crush the sample for better homogenization. Further, 80 µl of 10% Sodium dodecyl sulfate (SDS) was added and

incubate for half an hour at 37 °C. Next, 10–15 µl of Proteinase K (25 mg/ml) was added to the sample mixture, mixed well and incubated for 1–2 hr at 55–65 °C (the samples were mixed by inverting the tubes in time intervals of 30 mins). Then, 500 µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1) (PCI) was added to each sample tube and mixed by inverting 20–25 times. The mixture was then centrifuged at 13000 rate per minute (rpm) for 10 minutes and the upper aqueous upper layer of the supernatant was collected in a new centrifuge tube, and the step was repeated once. After which, 500 µl of Chloroform Isoamyl alcohol (CI) was added to the collected supernatant, mixed by inverting the tubes 5–10 times and then centrifuged at 13000 rpm for 10 mins. The upper layer of the supernatant was then collected and transferred to a new centrifuge tube. After that, a double volume of ice-cold 100% ethanol was added to the supernatant and held overnight at -20 °C for DNA precipitation. The supernatant was then removed by centrifuging the sample tubes at 13000 rpm for 10 mins. The DNA pellet was centrifuged for 5 mins at a speed of 13000 rpm after being washed with 70% ethanol. The supernatant was discarded from the sample tubes without disturbing the DNA pellet, dried at 37 °C and resuspended in 20 µl nuclease free water. The sample tubes containing the extracted DNA was then incubated at 37 °C for 30 mins. Prior to PCR, the quality of genomic DNA was checked by 0.8% agarose gel electrophoresis and kept at -20 °C until further usage.

Polymerase chain reaction (PCR)

Internal transcribed spacer (ITS) DNA region

The amplification of ITS fragment region from isolated DNA was performed by using TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') forward primer and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') reverse primer sets **(Joyce *et al.*, 1994)**.

The PCR conditions were set as: 1 cycle of initial denaturation at 94 ℃ for 1 min followed by 35 cycles of denaturation at 94 ℃ for 60 sec, primer annealing at 55 ℃ for 60 sec, and extension at 72 ℃ for 2 min. The last step was 1 cycle of final extension at 72 ℃ for 10 min.

Mitochondrial cytochrome oxidase c subunit 1 (COI) DNA region

The amplification of COI gene region (M1–M6 partition) from isolated DNA was performed by using LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') forward and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') reverse primer sets **(Folmer *et al.*, 1994)**. The PCR conditions were set as: 1 cycle of initial denaturation at 94 ˚C for 3 min followed by 35 cycles of denaturation at 94 ℃ for 60 sec, primer annealing at 50 ℃ for 30 sec, and extension at 72 ℃ for 45 sec. The last step was 1 cycle of final extension at 72 ℃ for 7 min.

28S or Large Subunit (LSU) of rDNA region

The amplification of D2-D3 expansion regions of 28S rDNA gene region from isolated DNA was performed by using 391 (5'-CGATAGCGAACAAGTACCGAGAG-3') forward and 501 (5'-CCTGCTCAGGCATAGTTCACCATC-3') reverse primer sets **(Qiu *et al.*, 2011)**. The PCR conditions were set as: 1 cycle of initial denaturation at 94 ˚C for 1 min followed by 35 cycles of denaturation at 94 ℃ for 60 sec, primer annealing at 55 ℃

for 60 sec, and extension at 72 °C for 2 min. The last step was 1 cycle of final extension at 72 °C for 10 min.

The amplification mixtures with a 25 µl total volume contained 12.5 µl of PCR master mix, 0.5 µl of 10 pmol of each primer, 1–2 µl of template DNA (approximately 100 ng), nuclease-free water was added to keep the volume at 25 µl. The PCR was conducted in a thermal cycler (ProFlexTM 39 32-Well PCR System (Applied Biosystems).

Agarose gel electrophoresis

The quality of the PCR product was checked by 1% agarose gel electrophoresis (w/v) (SeaKem®LE agarose, USA) made in 1X Tris-acetate-Ethylenediamine tetra-acetic acid (TAE) buffer. Invitrogen™ SYBR™ Safe DNA Gel Stain was used to stain the gel for visualization of DNA in agarose gel electrophoresis. Invitrogen 100 bp DNA Ladder was used molecular weight standard to check and determine the size (base pair) of the amplified PCR product

Sequencing of PCR products

The PCR products were packed and sent for sequencing at Scigenom, Kochi, Kerala, India. The DNA sequences were directly sequenced in both directions.

Nucleotide sequence analysis and molecular phylogeny

A software package, FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) was used for editing the electropherograms generated. The final edited sequences were aligned against the known EPN sequences submitted in the NCBI GenBank database using Clustal X 1.64 **(Thompson *et al.*, 1997)**. The phylogenetic relationships were generated using the Maximum Likelihood (ML), in MEGA 7 **(Kumar *et al.*, 2016)** with all the available representative species of the

genus *Heterorhabditis* and *Steinernema* retrieved from NCBI GenBank. *Caenorhabditis elegans* was used as the outgroup taxon. All positions containing gaps and missing data were eliminated. Genetic distance was calculated by pairwise comparisons of sequences using p-distance with gamma distribution.

Results

Nucleotide analysis

The BLAST search result of NCBI GenBank using the amplified products of 3 molecular markers (ITS, 28S and COI DNA regions) showed that the 4 isolated EPNs from Mizoram exhibited high similarity (99.00–100.00%) with respective sequences available for *Steinernema sangi*, *Steinernema surkhetense*, *Heterorhabditis indica* and *Heterorhabditis baujardi*. Even though the 3 molecular markers employed in the present study yield a good result, the ITS gene region seems to be the most reliable marker for identification and distinction of the studied nematode species. The less reliability of the two molecular markers (in comparison to the ITS gene) may be attributed to inadequate submission of sequences in NCBI GenBank or misidentification of the previously submitted species that require further thorough study. The newly developed sequences have been deposited in NCBI GenBank (under the accession number as given in table 2.1.

A. Internal transcribed spacer ribosomal DNA (ITS rDNA) region

S. sangi

The generated sequences of ITS (ITS1 + 5.8S + ITS2 + 28S partial sequence) region of the nematode isolate are 759 bp long, with ITS1 = 255 bp, 5.8S = 157 bp, ITS2 =

308 bp and 28S = 38 bp. The developed sequences were deposited in NCBI GenBank (under the NCBI accession number KY861069–KY861072). The average base composition [Thymine (T); Cytosine (C); Adenine (A) and Guanine (G)] are T = 34.00%, C = 19.40%, A = 23.60% G = 23.00%, which revealed that the developed sequences of ITS gene are A + T rich (57.60%). The BLAST search result showed that the developed ITS rDNA sequences of the isolated nematode exhibit high similarity (99.00%) with that of the database sequence available for *S. sangi* (AY355441). Based on the ITS rDNA sequence similarity blast, other closely related species includes *Steinernema* sp. (GU395631) with 98.00% similarity, *S. cholashanense* (GQ377419) with 90.00% similarity, *S. kraussei* (KM016394) and *S. oregonense* with 88.00% similarity, *S. xueshanense*, *S. texanum* and *S. akhursti* with 87.00% similarity and *S. weiseri*, *S. litorale* and *S. feltiae* with 86.00%. Besides these, the sequences of all other identified *Steinernema* species available in NCBI GenBank showed ≤86.00% similarity.

S. surkhetense

The generated sequences of ITS (ITS1+5.8S+ITS2+28S partial sequence) region of the nematode isolate are 770–783 bp long (ITS1=277bp, 5.8S=157bp, ITS2=305bp, 28S=31–44bp). The developed sequences were deposited in NCBI GenBank (under the NCBI accession number MF618308–MF618312). The average base composition [Thymine (T); Cytosine (C); Adenine (A) and Guanine (G)] are T=37.60%, C=17.00%, A=24.20% G=21.20% for *S. surkhetense*, which revealed that the developed sequences of ITS gene are A + T rich (61.80%). The BLAST search result showed that the developed ITS rDNA sequences of the isolated nematode exhibit

Table: 2.1. NCBI GenBank submission of entomopathogenic nematodes from Mizoram, India

Species	Specimen Voucher	GenBank (NCBI) Accession No.		
		ITS	28S	COI
Steinernema sangi	PUC-StR	KY861069	MF620997	MF621237
Steinernemasangi	PUC-StK	KY861070	MF620998	MF621238
Steinernema sangi	PUC-StC	KY861071	MF620999	MF621239
Steinernema sangi	PUC-StL	KY861072	MF621000	MF621240
Steinernemasurkhetense	PUC-StPTS1	MF618308	MF621001	MF621241
Steinernema surkhetense	PUC-StPTS2	MF618309	MF621002	MF621242
Steinernema surkhetense	PUC-StPTS3	MF618310	MF621003	MF621243
Steinernema surkhetense	PUC-StPTS4	MF618311	MF621004	MF621244
Steinernema surkhetense	PUC-StPTS5	MF618312	MF621005	-
Heterorhabditis indica	PUC-HeM	MF618313	MF621007	-
Heterorhabditis indica	PUC-HeTC	MF618315	MF621006	-
Heterorhabditis indica	PUC-HeTRE	MF618314	MF621008	-
Heterorhabditis indica	PUC-HeTR	MF618316	MF621009	-
Heterorhabditis indica	PUC-HeTR1	-	-	MF621245
Heterorhabditis indica	PUC-HeTR2	-	-	MF621246
Heterorhabditis indica	PUC-HeTS	MF618317	MF621010	-
Heterorhabditis indica	PUC-HeTV	MF618318	MF621011	-
Heterorhabditis indica	PUC-HeTV1	-	-	MF621247
Heterorhabditis indica	PUC-HeTV2	-	-	MF621248
Heterorhabditis baujardi	PUC-HeTD1	MF618319	MF621012	MF621249
Heterorhabditis baujardi	PUC-HeTD2	MF618320	MF621013	MF621250
Heterorhabditis baujardi	PUC-HeTD3	MF618321	MF621014	MF621251
Heterorhabditis baujardi	PUC-HeTD4	MF618322	-	MF621252

high similarity (99.00–100.00%) with that of the sequences available for *S. surkhetense* (KP219886, MH822626, MH822627, HQ190042, GU395630, KR029844, MK995658, MK995659). Other closely related species includes *Steinernema* sp. (FJ418045, MG976890, HQ317503) with 99.00–100.00% similarity, *S. nepalense* (HQ190044) and *S. carpocapsae* (MF919612) with 94.00% similarity. In addition, the sequences of all other identified *Steinernema* species available in GenBank showed ≤ 93.00% similarity.

H. indica

The generated sequences of ITS (ITS1+5.8S+ITS2+28S partial sequence) region of the nematode isolate are 784–808 bp long (ITS1=371bp, 5.8S=152bp, ITS2=218bp, 28S=43–67bp). The developed sequences were deposited in NCBI GenBank (under the NCBI accession number MF618313–MF618318). The calculated average base composition [Thymine (T); Cytosine (C); Adenine (A) and Guanine (G)] were T=28.50%, C=20.30%, A=26.60% G=24.70% which revealed that the developed gene sequences are A + T rich (55.10%). The BLAST search result showed that the developed ITS rDNA sequences of the isolated nematode exhibit high similarity (99.00–100.00%) with that of the sequences available for *H. indica, including H. pakistanense* (syn. with *H. indica*). However, the database sequences of *H. indica* with accession no. MG893075, MG489821, MZ507541, MZ507542, MW654494, MW654496, MW654497, MW654499, MW654500, and MW654501 showed ≤97.00% similarity with that of the developed *H. indica* sequences. Other species showing high similarity include all database sequences of *H. noenieputensis* with 97.77–98.09% similarity while all other identified *Heterorhabditis* species available in NCBI GenBank showed ≤ 97.00% similarity.

H. baujardi

The generated sequences of ITS (ITS1+5.8S+ITS2+28S partial sequence) region of the nematode isolate are 829 bp (ITS1 = 395 bp, 5.8S = 154 bp, ITS2 = 211 bp). The developed sequences were deposited in NCBI GenBank (under the NCBI accession number MF618319–MF618322). The calculated average base composition [Thymine (T); Cytosine (C); Adenine (A) and Guanine (G)] were T=28.7%, C=20.00%, A=26.40% G=24.80% which revealed that the developed gene sequences are A + T rich (55.10%). The BLAST search result showed that the 4 developed ITS rDNA sequences of the isolated nematode exhibit high similarity (99.00–100.00%) with that of the database sequences of *H. baujardi*, with the exception of *H. baujardi* (EU363039) with 97.47% similarity. Other species showing high similarity includes *H. amazonensis* and *H. floridensis* with 98.00% similarity. Further, the database sequence of *H. mexicana*, *H. sonorensis* and *H. taysearae* showed 97.00% similarity. In fact, the database sequences of other *Heterorhabditis* species available in the NCBI GenBank showed ≤88.00% similarity.

B. D2-D3 regions of 28S or Larger Subunit (LSU) of rDNA region

S. sangi

The generated sequences of 28S rDNA (partial sequence) region of the nematode isolate are 881 bp long. The developed sequences were deposited in NCBI GenBank (under the NCBI accession number MF620997–MF621000). The average base composition [Thymine (T); Cytosine (C); Adenine (A) and Guanine (G)] are T = 26.33%, C = 18.84%, A = 24.06% G = 30.76%. Based on the 28S sequence similarity blast, other related species includes *S. akhursti*, *S. feltiae*, *S. weiseri*, *S. puntauvense*, *S. oreganense*, *S. kushidai*, *S. tielingense* and *Steinenema* sp. (identified

as *S. xinbiense*) with 96.00% similarity; *S. kraussei*, *S. silvaticum*, *S. ichnusae*, *S. jollieti*, *S. cholashanense*, *S. citrae* and *S. texanum* with 95.00% similarity. However, the only sequence available for *S. sangi* (GU569057) showed 92.41% similarity with the developed sequences and so may not be a true species. Moreover, the database sequences of other identified *Steinernema* species available in NCBI GenBank showed ≤94.00% similarity.

S. surkhetense

The generated sequences of 28S rDNA (partial sequence) region of the nematode isolate are 879 bp long. The developed sequences were deposited in NCBI GenBank (under the NCBI accession number MF621001–MF621005). The average base composition [Thymine (T); Cytosine (C); Adenine (A) and Guanine (G)] are T = 27.87%, C = 17.29%, A = 24.91% G = 29.92%. The BLAST search result showed that the developed sequences of the isolated nematode exhibit high similarity (99.00-100.00%) with the database sequences available for *S. surkhetense* (with accession no. HQ190043, KU187262, MH837096, MH837094 and MW703809). However, the database sequences of *S. surkhetense* with accession no. MZ242237, MZ242238 and MZ242239 showed 90.22–96.20% similarity with the developed sequences. Based on the sequence similarity blast, other closely related species includes *Steinernema* sp. (GU395631) with 98.00% similarity, *S. cholashanense* (GQ377419) with 90.00% similarity, *S. kraussei* (KM016394) and *S. oregonense* with 88.00% similarity, *S. xueshanense*, *S. texanum* and *S. akhursti* with 87.00% and *S. weiseri*, *S. litorale* and *S. feltiae* with 86.00%. The database sequences of all other identified *Steinernema* species available in NCBI GenBank showed ≤86.00% similarity.

H. indica

The generated sequences of the 28S rDNA (partial sequence) region of the nematode isolate are 879 bp long. The developed sequences were deposited in NCBI GenBank (under the NCBI accession number MF621006–MF621011). The average base composition [Thymine (T); Cytosine (C); Adenine (A) and Guanine (G)] are T = 25.55%, C = 18.81%, A = 26.33% G = 29.31%. The BLAST search result showed that the 4 developed sequences of the isolated nematode exhibit high similarity (98.00–100.00%) with that of the database sequences available for *H. indica*, with the exception of *H. indica* (MH029268, MW548157, MW548159, MW5481600, MW729402) with 97.00% similarity, *H. indica* (MW513451, MW513452, MW513453, MW513455) with 92.47% similarity. Meanwhile *H. pakistanense* (syn. with *H. indica* (KP096496) showed 98.49%. Other species showing higher similarity includes *H. noenieputensis* with 99.56% similarity, *H. baujardi* with 97.24% similarity, *H. amazonensis* with 97.13% similarity, *H. mexicana* with 96.68% similarity, and *H. floridensis* with 96.57% similarity. The species labelled as *H. noenieputensis* (JX624110) may not be the true species since it shows 99.56% similarity with *H. indica*. Further, it has been observed that the database sequences available for other identified *Heterorhabditis* species available in the NCBI GenBank showed ≤94.00% similarity.

H. baujardi

The generated sequences of 28S rDNA (partial sequence) region of the nematode isolate are 879 bp long. The developed sequences were deposited in GenBank (under the NCBI accession number MF621012–MF621014). The average base composition [Thymine (T); Cytosine (C); Adenine (A) and Guanine (G)] are T = 25.55%, C =

18.81%, A = 26.33% G = 29.31%. The BLAST search result showed that the four developed sequences of the isolated nematode exhibit high similarity (99.82%) with that of the GenBank species identified as *H. baujardi* (MT372503). The other isolates that showed high similarity includes *Heterorhabditis* sp. (KY024496 and KY055370) with 99.78 % similarity, *H. amazonensis* (EU099036) with 99.45 % similarity, and 98.78 % similarity with *H. floridensis* (EU099034) and *H. mexicana* (EU100414). Furthermore, other species that showed higher similarity includes *H. indica* and *H. noenieputensis* (97.00% similarity); *H. bacteriophora*, *H. beicherriana*, *H. georgiana*, *H. marelatus* and *H. safricana* (94.0%); *H. megidis* and *H. zealandica* with 93.00–94.00% similarity, and *H. atacamensis* with 93.00% similarity. In addition, it has been observed that the database sequences available for other identified *Heterorhabditis* species available in the NCBI GenBank showed ≤93.00% similarity.

C. M1–M6 regions of Cytochrome oxidase I (COI)

S. sangi

The generated sequences of COI region of the nematode isolate are 642–654 bp long. The developed sequences were deposited in GenBank under the NCBI accession number MF621237–MF621240. The average base composition [Thymine (T); Cytosine (C); Adenine (A) and Guanine (G)] are T=45.10%, C=13.90%, A=22.90% G=17.10%. The BLAST search of NCBI GenBank showed that there are no COI sequences available for *S. sangi*. All the database sequences available for other identified *Steinernema* species in the NCBI GenBank showed ≤89.00% similarity.

S. surkhetense

The generated sequences of COI region of the nematode isolate are 657 bp long. The developed sequences were deposited in GenBank (under the NCBI accession number MF621241–MF621244). The average base composition [Thymine (T); Cytosine (C); Adenine (A) and Guanine (G)] are T=47.20%, C=13.40%, A=22.40% G=17.00%. The BLAST search of GenBank showed that the four developed COI gene sequences of the isolated nematode exhibit high similarity (99.00–98.00%) with that of all the GenBank species identified as *S. surkhetense* (KU721841, KU721840 and MW287379). However, the other sequences available for *Steinernema* species in the NCBI GenBank showed ≤90.00% similarity.

H. indica

The generated sequences of COI region of the nematode isolate are 651 bp long. The developed sequences have been deposited in GenBank (under the NCBI accession number MF621241–MF621244). The average base composition T=45.70%, C=12.10%, A=22.40% G=19.80%. The BLAST search of GenBank showed the 4 developed COI sequences of the isolated nematode exhibit high similarity (98.00%) with that of all the GenBank species identified as *H. indica* as well as *H. bacteriophora* (JN572120). However, the species labelled as *H. indica* (NC040293) might not be a true species since it shows only 89.12% similarity with the rest of the sequences submitted in the NCBI GenBank. However, all the other sequences available for *Heterorhabditis* species in the NCBI GenBank showed ≤88.00.00% similarity.

H. baujardi

The generated sequences of COI region of the nematode isolate are 651 bp long. The developed sequences have been deposited in GenBank (under the NCBI accession number MF621241–MF621244). The average base composition T=45.15.0%, C=12.00%, A=22.40% G=20.60%. The BLAST search of NCBI GenBank showed that there are no COI sequences available for *H. baujardi*. In addition, all the database sequences available for other identified *Heterorhabditis* species in the NCBI GenBank showed ≤89.00% similarity.

Molecular Phylogeny

Phylogenetic tree

The phylogenetic tree constructed using ML for both the ITS rDNA and 28S rDNA data sets exhibited similar topology with a strong bootstrap support. The COI region however did not yield proper and useful phylogenetic tree which may be due to lack of comparable sequences.

A. Steinernematids

The phylogenetic relationships of the isolated steinernematids (*S. sangi* and *S. surkhetense*) and other sequences of *Steinernema* species from the GenBank are shown in the Fig.2.1 (for ITS), Fig. 2.2 (for 28S) and Fig. 2.3 (for COI). All the ITS and 28S sequences available for *S. sangi* and *S. surkhetense* in the NCBI GenBank were considered in the phylogeny. Meanwhile, one representative sequence for other *Steinernema* species that belonged to the same monophyletic group with the respective *S. sangi* and *S. surkhetense* were included in the phylogeny. For the rest of the monophyletic groups, sequences of five representative *Steinernema* species were

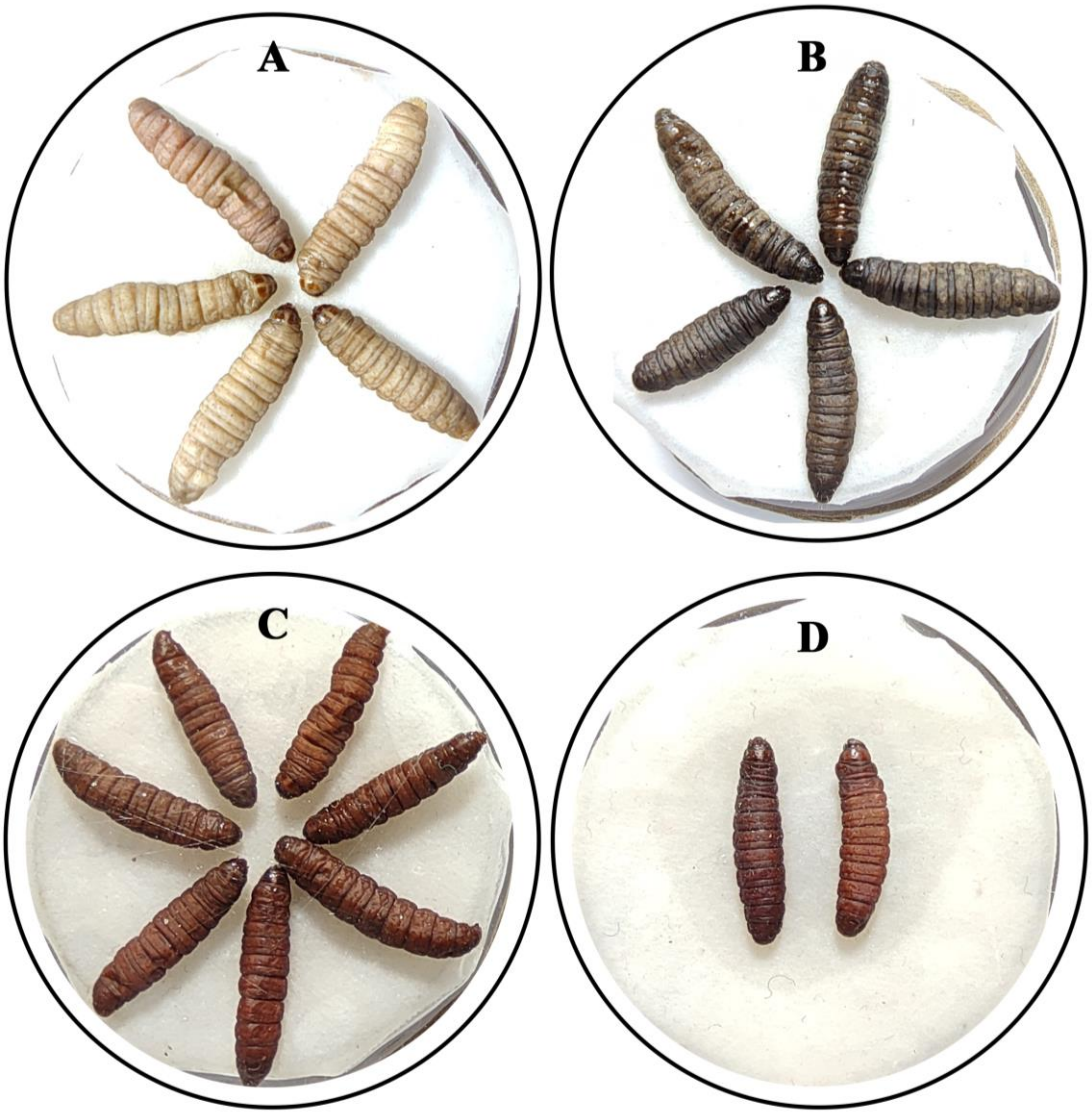

Fig.2.1. Larvae of *G. mellonella* infected with: A. *S. sangi*, B. *S. surkhetense*, C. *H. indica*, and D. *H. baujardi*.

considered. In the case of COI marker gene, all the available sequences for *Steinernema* species were considered for phylogenetic analysis.

The phylogenetic analysis inferred from ITS rDNA region indicated that *S. sangi* (accession no. StR KY861069, StK KY861070, StC KY861071 and StL KY861072) is a species belonging to the *feltiae-kraussei-oregonense* group of **Spiridonov *et al*. (2004)** (Fig. 2.2). The isolate formed a monophyletic group with *S. sangi* (AY355441), *Steinernema* sp. (GU395631), *S. texanum, S. cholashanense, S. oregonense, S. xueshanense, S. silvaticum, S. kraussei, S. xinbiense, S. tielingense, S. jollieti, S. ichnusae, S. feltiae, S. nguyeni, S. citrae, S. hebeiense, S. litorale, S. weiseri, S. kushidai, S. akhursti* and *S. everestense*. Within this monophyletic group, the isolated *S. sangi* formed a cluster with *S. sangi* (AY355441) and *Steinernema* sp. (GU395631) and did not cluster with other species supporting the report of Nguyen *et al*. (2007, 2008). Among the group, *S. kushidai*, *S. akhursti* and *S. everestense* formed a clear sub- monophyletic group. Similarly, the phylogenetic analysis inferred from ITS rDNA region indicated that the isolated *S. surkhetense* (accession no. StPTS1 MF618308, StPTS2 MF618309, StPTS3 MF618310, StPTS4 MF618311 and StPTS5 MF618312) is a species belonging to the *carpocapsae* group (Khatri-Chhetri *et al*, 2011, Bhat *et al*, 2017) (Fig. 2.2) forming a monophyletic group with *S. surkhetense* (KP219886), *Steinernema* sp. (FJ418045), *S. surkhetense* (MH822627) *Steinernema* sp. (MW365746), *S. surkhetense* (HQ190042), *S. nepalense, S.backanense*, *S. eapokense, S. siamkayai*, *S. minutum*, *S. tami*, *S. huense*, *S. cumgarense, S. sasonense,* and *S. carpocapsae*. Within this monophyletic group, the isolated *S. surkhetense* formed a cluster with *S surkhetense* (MH522627 and

KP219886) and *Steinernema* sp. (MW365746 and JT418045) supporting the report of **Bhat *et al*. (2017)**.

The analysis of the 28S rDNA sequences indicated that the isolate, *S. sangi* (accession no. StR MF6209977, StK MF6209978, StC MF6209979, StL MF62091000) is a species belonging to the *feltiae-kraussei-oregonense* group of **Spiridonov *et al*. (2004)** (Fig. 2.3). The isolate formed a monophyletic group with the available sequences of *S. sangi* and other sequences available for the same monophyletic clade. Likewise, the phylogeny using 28S rDNA data sets revealed that the isolate, *S. surkhetense* (accession no. StPTS1 MF621001, StPTS2 MF621002, StPTS3 MF621003, StPTS4 MF621004 and StPTS5 MF621005) formed a monophyletic group with the available sequences of *S. surkhetense* (HQ190043, KU187262, MZ242237 and MH837096) and other *Steinernema* spp. of the *carpocapsae* group of **Spiridonov *et al*. (2004)** (Fig. 2.4).

B. Heterorhabditids

The phylogenetic relationships of the isolated heterorhabditids (*H. indica* and *H. baujardi*) and other *Heterorhabditis* species are shown in the fig. 2.5 (for ITS), fig. 2.6 (for 28S) and fig. 2.7 (for COI). For all the *Heterorhabditis* species, at least one sequence of ITS, 28S and COI marker genes available on the NCBI GenBank were considered in the analysis and construction of phylogenetic tree.

The phylogeny using the ITS rDNA data sets revealed that the isolated heterorhabditids, *H. indica* and *H. baujardi* belong to '*indica*' group, and formed a monophyletic group with a strong bootstrap support. In fact, the isolate *H. indica* (accession no. HeM MF618313, HeTRE MF618314, HeTC MF618315, HeTR

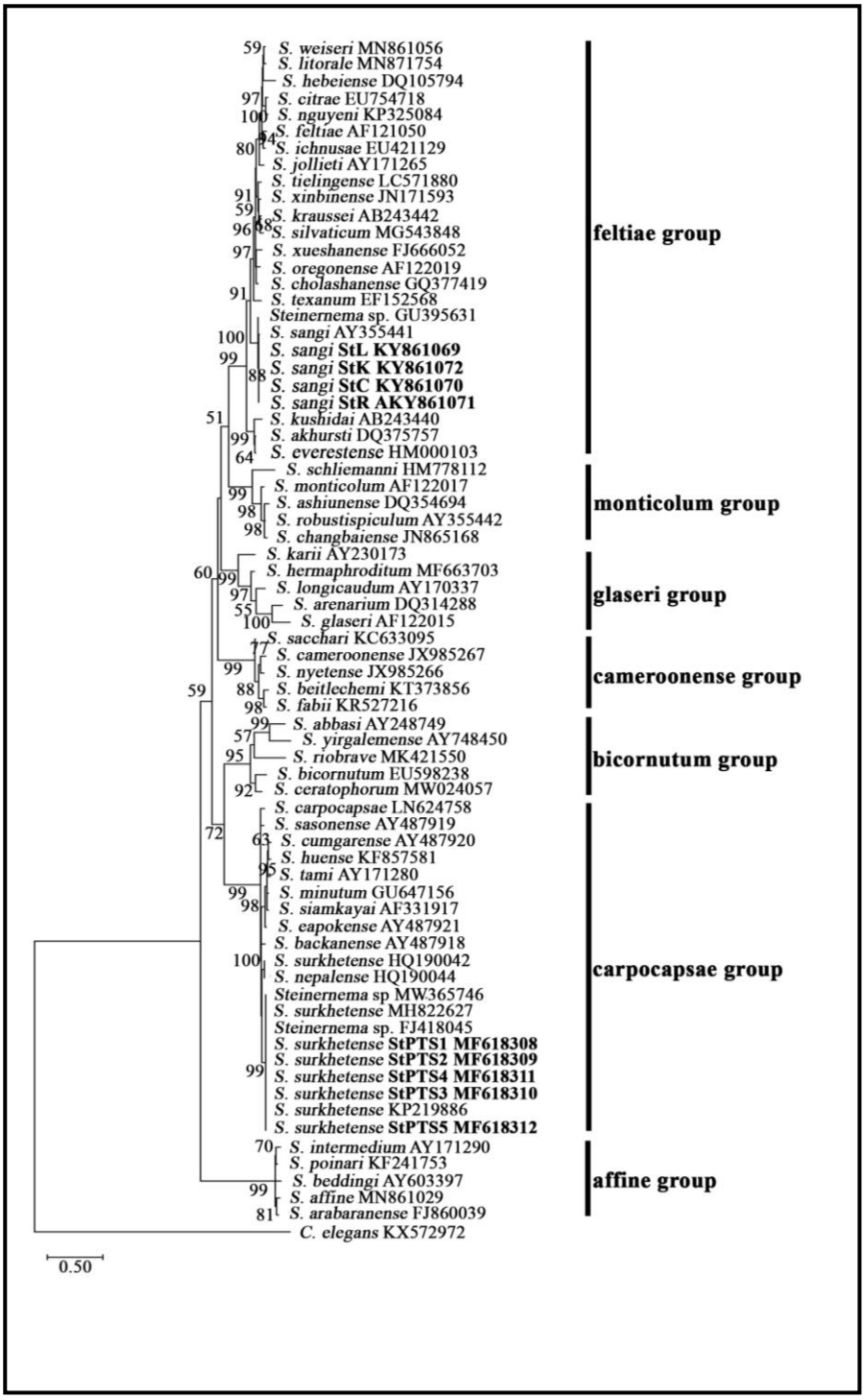

Fig.2.2. Phylogenetic relationship of *S. sangi* and *S. surkhetense* with other *Steinernema spp*. based on analysis of the ITS rDNA region. Numbers indicated at the nodes represents bootstrap proportion values (50% or more, 1000 replicates). Numbers after each species and isolate indicate the GenBank Accession numbers.

MF618316, HeTS MF618317, HeTV MF618318) further formed a sub-group with *H. indica* (AY321483), *H. pakistanense* (JX144740), and *H. noenieputensis*. Within the sub- monophyletic group, the isolated *H. indica* formed a cluster with *H. indica* (AY32148) and *H. pakistanense* (JX1447400) and did not cluster with other species. Similarly, the isolate *H. baujardi* (accession no. HeTD1 MF618319, HeTD2 MF618320, HeTD3 MF618321, HeTD4 MF618322) further formed a sub-group with *H. baujardi* (AF548768), *H. amazonensis*, *H. floridensis*, *H. Mexicana*, and *H. taysearae*. Within the sub- monophyletic group, the isolated *H. baujardi* formed a cluster with *H. baujardi* (AF548768) and did not cluster with other species.

Furthermore, the phylogeny using the 28S rDNA data sets confirmed that the isolated heterorhabditids, *H. indica* (accession no. HeTC MF621006, HeM MF621007, HeTRE MF621008, HeTR MF621009, HeTS MF621010, HeTV MF621011) and *H. baujardi* (accession no. HeTD1 MF621012, HeTD2 MF621013, and HeTD3 MF6213) belong to the '*indica*' group, and formed a monophyletic group with a strong bootstrap support. The phylogenetic tree (Figure 2.4) showed that the isolate, *H. indica* further formed a sub-group with *H. indica* (EU100415) and *H. pakistanense* (syn. with *H. indica*, KP096496). Similarly, the isolated *H. baujardi* (HeTD1 MF621012, HeTD2 MF621013, and HeTD3 MF6213) formed a sub-group with *H. baujardi* (AF548768), *H. amazonensis*, *H. floridensis*, and *H. mexicana* within the monophyletic *indica* group. Within this sub-group, the isolated *H. baujardi* further formed a cluster with *H. baujardi* (MT372503).

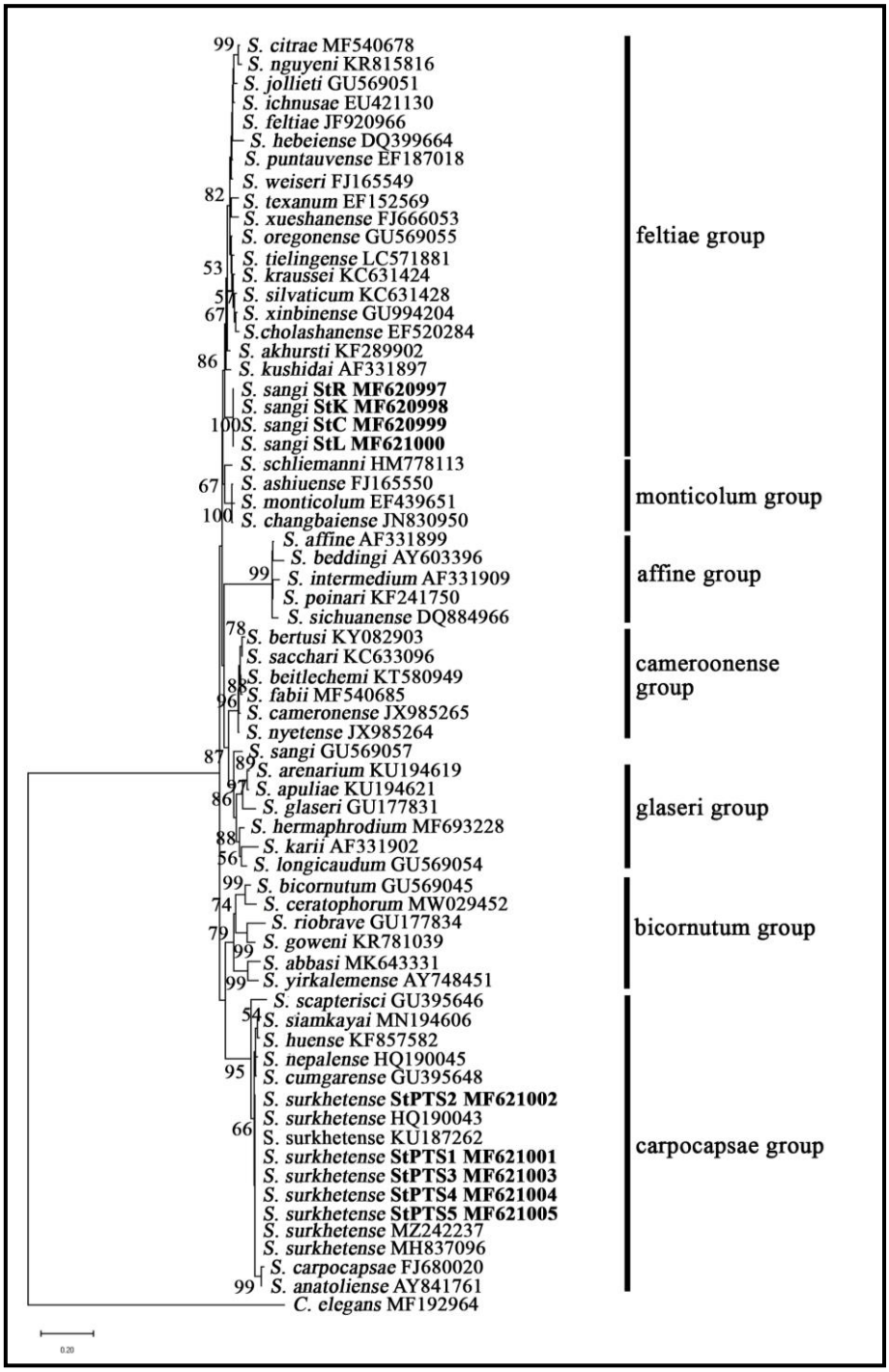

Fig.2.3. Phylogenetic relationship of *Steinernema sangi* and *S. surkhetense* with other *Steinernema spp*. based on analysis of the 28S rDNA region. Numbers indicated at the nodes represents bootstrap proportion values (50% or more, 1000 1000 replicates). Numbers after each species and isolate indicate the GenBank Accession numbers.

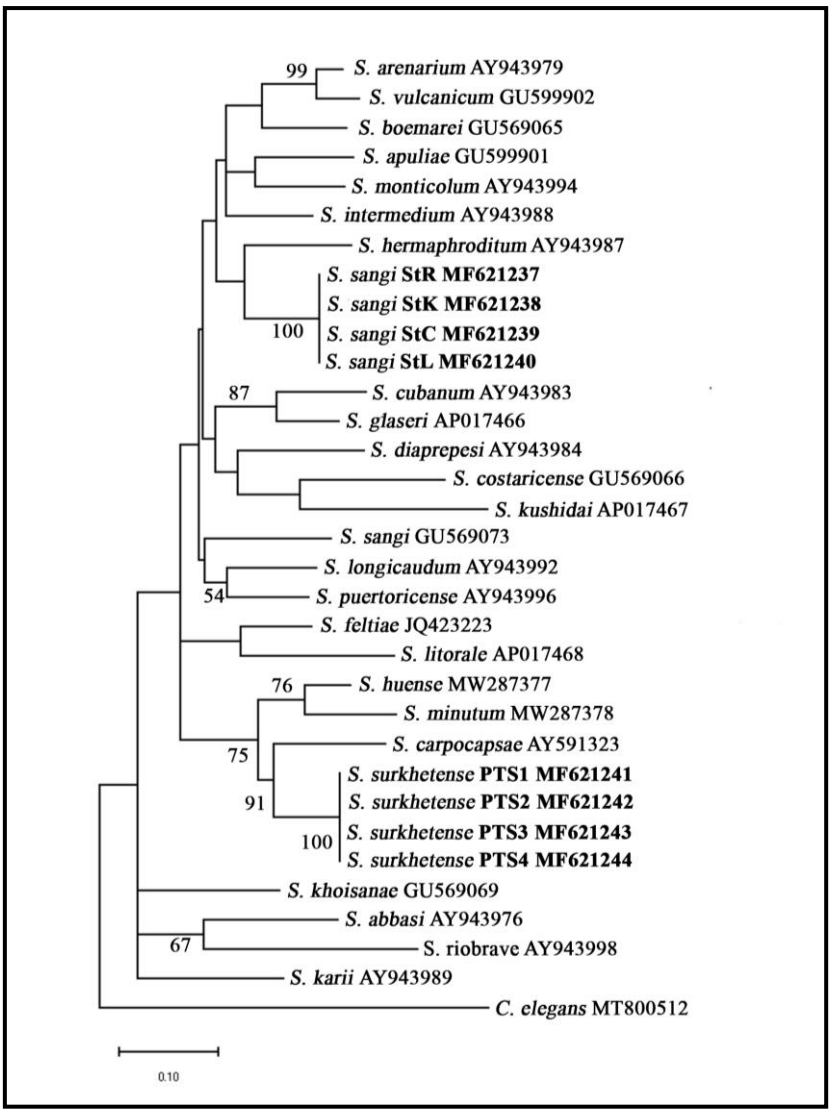

Fig.2.4. Phylogenetic relationship of *Steinernema sangi* and *S. surkhetense* with other *Steinernema spp*. based on analysis of the COI region. Numbers indicated at the nodes represents bootstrap proportion values (50% or more, 1000 replicates). Numbers after each species and isolate indicate the GenBank Accession numbers.

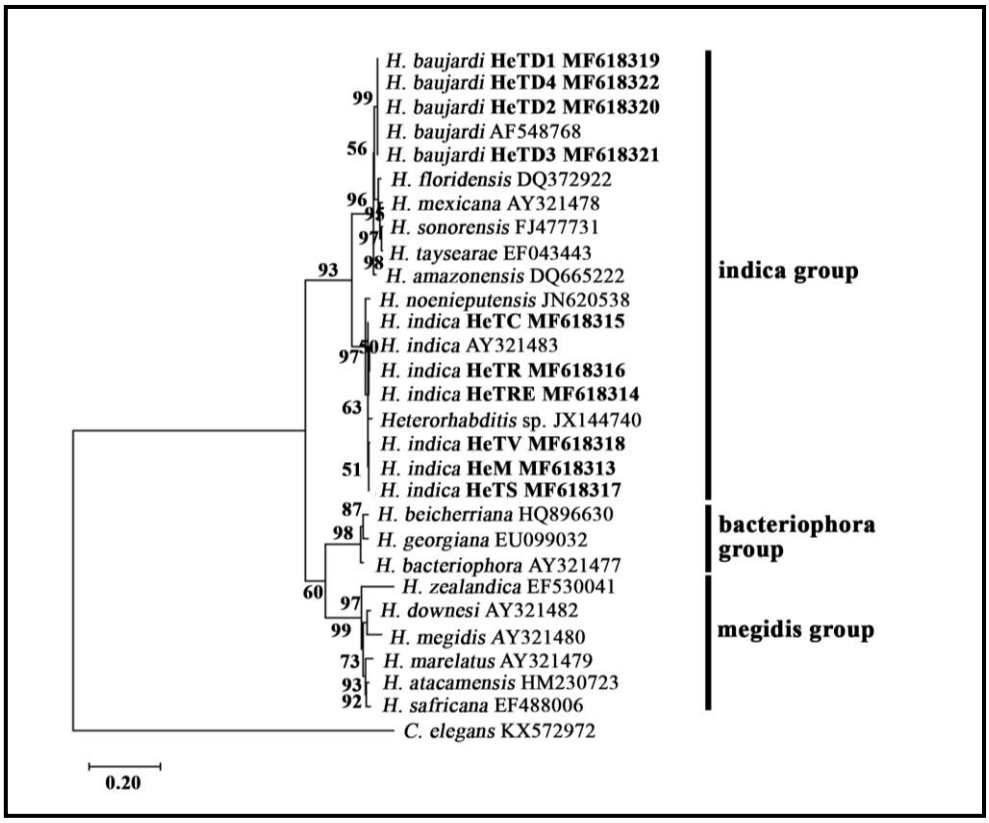

Fig.2.5. Phylogenetic relationship of *H. indica* and *H. baujardi* with other *Heterorhabditis spp*. based on analysis of the ITS rDNA region. Numbers indicated at the nodes represents bootstrap proportion values (50% or more, 1000 replicates). Numbers after each species and isolate indicate the GenBank Accession numbers.

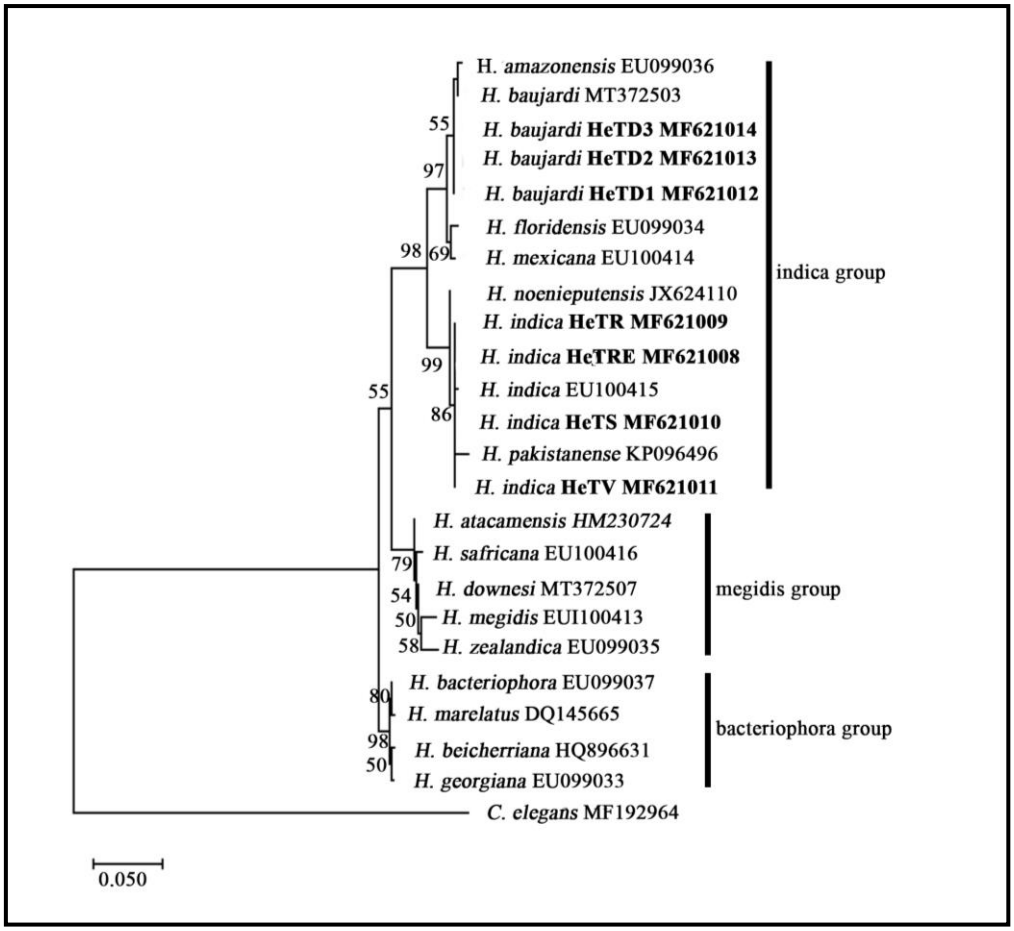

Fig.2.6. Phylogenetic relationship of *H. indica* and *H. baujardi* with other *Heterorhabditis spp*. based on analysis of the 28S rDNA region. Numbers indicated at the nodes represents bootstrap proportion values (50% or more, 1000 replicates). Numbers after each species and isolate indicate the GenBank Accession numbers.

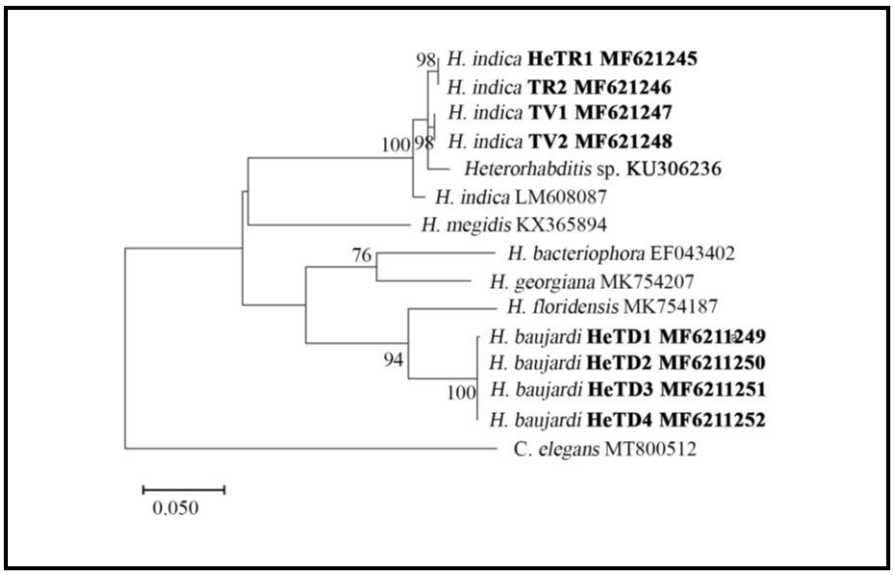

Fig.2.7. Phylogenetic relationship of *H. indica* and *H. baujardi* with other *Heterorhabditis spp*. based on analysis of the COI region. Numbers indicated at the nodes represents bootstrap proportion values (50% or more, 1000 replicates). Numbers after each species and isolate indicate the GenBank Accession numbers.

Phylogenetic distance

Being one of the most reliable tools for the distinction of species, p-distance is calculated based on the ITS and 28S rDNA regions. The sequences considered for phylogenetic distances for each of the isolated EPN species are given in table **2.2-2.9**

A. Steinernematids:

p-distance of *S. sangi* with other *Steinernema* spp.

The analysis of ITS region revealed that the 4 developed sequences of *S. sangi* and the available single database sequence of *Steinernema sangi* (AY355441) exhibit an average intraspecies distance of 0.50 ± 0.35% (0.00–1.00%) and an average interspecies distance of 13.18 ± 4.33% (9.0–51.0%) with respect to all other *Steinernema* species analysed (Table 2.2). The closest species is *Steinernema* sp. YNd37 (GU395631) with a distance of 1.00 ± 0.00%. Furthermore, based on the

analysis of 28S rDNA region, the isolated *S. sangi* exhibit an average intraspecies distance of 0.35 ± 2.40% (0.00–7.00%) and an average interspecies distance of 8.00 ± 2.22% (3.00–33.0%) with respect to all other analysed sequences (Table 2.3).

p-distance of *S. surkhetense* with other *Steinernema* spp.

The analysis of ITS sequences revealed that the 4 developed sequences of *S. surkhetense* exhibit an average intraspecies distance of 0.0 ± 0.0% (0.0–0.0%) with database sequences of *S. surkhetense* (KP219886 and MH822625) as well as *Steinernema* sp. (FJ418045 and MW365746) (Table 2.4). In the meantime, the isolated *S. surkhetense* showed 4.00 ± 0.0% intra-specific distance with the database sequence of *S. surkhetense* (HQ190042). The calculated average interspecific distance between the isolated *S. surkhetense* and other *Steinernema* species was 12.00 ± 6.65% (4.00–51.00%). Furthermore, based on the analysis of 28S rDNA region, the calculated average intraspecific distance between the isolate and database sequence of *S. surkhetense* was 1.00% ± 1.00% (0.00–1.00%). The average interspecific of the isolated *S. surkhetense* with other *Steinernema* spp. along with the outgroup species was 8.00 ± 3.33% (1.00–34.0%) (Table 2.5).

B. Heterorhabditids

p-distance of *H. indica* with other *Heterorhabditis* spp.:

The genetic distance of the isolated *H. indica* with other heterorhabditid species are given in Table 2.6 and 2.7. In case of ITS region, the analysis showed that the genetic distance between the 4 developed sequences is 0.10% with *H. indica* (AY321483) and *H. pakistanense* (JX144740) (syn. with *H. indica*); 1.00% with *H. neonieputensis* (KP096496); 3.00% with *H. mexicana*, *H. baujardi* and *H.*

amazonensis; 4.00% with *H. floridensis*; 22.00% with *C. elegans*. Therefore, the p-distance reveals that the 5 developed sequences and the database sequences of *H. indica* exhibit an average intraspecies distance of 0.50 ± 0.35% (0.00–1.00%). In addition, the calculated average interspecies distance was 16.31 ± 3.77% (1.00–51.00%). Furthermore, based on the analysis of the 28S rDNA region, the isolate *H. indica* exhibit an average intraspecies distance of 0.50 ± 0.35% (0.00-1.00%) and an average interspecies distance of 13.83 ± 4.18% (2.00–52.0%) with respect to other *Heterorhabditis* species and the outgroup species **(Table 2.7).**

Table 2.2. Estimates of genetic divergence between ITS rDNA sequences of of *S. sangi* and other *Steinernema* spp. using p-distance

S. sangi **StL KY861069**		0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. sangi **StC KY861070**	0.00		0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. sangi **StR KY861071**	0.00	0.00		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. sangi **St K KY861072**	0.00	0.00	0.00		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. sangi AY355441	0.00	0.00	0.00	0.01		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
Steinernema sp. GU395631	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. texanum EF152568	0.10	0.10	0.10	0.10	0.09	0.10		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. cholashanense GQ377419	0.09	0.09	0.09	0.09	0.09	0.09	0.08		0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. oregonense AF122019	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.05		0.01	0.01	0.01	0.01	0.01	0.02
S. xueshanense FJ666052	0.11	0.11	0.11	0.11	0.10	0.11	0.09	0.06	0.07		0.01	0.01	0.01	0.01	0.02
S. silvaticum MG543848	0.11	0.11	0.11	0.12	0.12	0.11	0.10	0.07	0.08	0.09		0.01	0.01	0.01	0.02
S. kraussei AB243442	0.10	0.10	0.10	0.10	0.10	0.10	0.08	0.06	0.06	0.08	0.03		0.01	0.01	0.02
S. xinbinense JN171593	0.10	0.10	0.10	0.10	0.10	0.10	0.08	0.06	0.06	0.07	0.04	0.04		0.01	0.02
S. tielingense LC571880	0.10	0.10	0.10	0.10	0.10	0.10	0.08	0.06	0.06	0.08	0.06	0.04	0.04		0.02
C. elegans KX572972	0.51	0.51	0.51	0.51	0.50	0.51	0.51	0.51	0.52	0.52	0.53	0.53	0.52	0.51	

Table 2.3. Estimates of genetic divergence between 28S rDNA sequences of *S. sangi* and *Steinernema* spp. using p-distance

S. sangi **StR MF620997**		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. sangi **StK MF620998**	0.00		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. sangi **StC MF620999**	0.00	0.00		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. sangi **StL MF621000**	0.00	0.00	0.00		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. sangi GU569057	0.07	0.07	0.07	0.07		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. texanum EF152569	0.05	0.05	0.05	0.05	0.07		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. cholashanense EF520284	0.05	0.05	0.05	0.05	0.07	0.04		0.00	0.01	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.02
S. oregonense GU569055	0.04	0.04	0.04	0.04	0.07	0.03	0.02		0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.02
S. xueshanense FJ666053	0.05	0.05	0.05	0.05	0.07	0.04	0.02	0.02		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. silvaticum KC631428	0.05	0.05	0.05	0.05	0.07	0.04	0.02	0.02	0.03		0.00	0.00	0.01	0.01	0.01	0.01	0.02
S. kraussei KC631424	0.04	0.04	0.04	0.04	0.07	0.04	0.02	0.01	0.03	0.01		0.00	0.00	0.01	0.01	0.01	0.02
S. xinbiense GU994204	0.05	0.05	0.05	0.05	0.07	0.04	0.01	0.02	0.03	0.01	0.01		0.01	0.01	0.01	0.01	0.02
S. tielingense LC571881	0.03	0.03	0.03	0.03	0.07	0.02	0.02	0.01	0.02	0.02	0.01	0.01		0.01	0.01	0.01	0.02
S. surkhetense StPTS1 MF621001	0.10	0.10	0.10	0.10	0.12	0.12	0.11	0.11	0.11	0.10	0.11	0.11	0.11		0.01	0.01	0.02
S. affine AF331899	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.15		0.01	0.02
S. cameroonense JX985265	0.08	0.08	0.08	0.08	0.07	0.07	0.08	0.07	0.08	0.08	0.08	0.08	0.07	0.11	0.13		0.02
C. elegans MF192964	0.33	0.33	0.33	0.33	0.34	0.34	0.33	0.33	0.33	0.34	0.34	0.34	0.34	0.33	0.35	0.32	

Table 2.4. Estimates of genetic divergence between ITS sequences of *S. surkhetense* and other *Steinernema* spp. using p-distance

S. surkhetense **StPTS1 MF618308**		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. surkhetense **StPTS2 MF618309**	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. surkhetense **StPTS3 MF618310**	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. surkhetense **StPTS4 MF618311**	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. surkhetense **StPTS5 MF618312**	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. surkhetense KP219886	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02
Steinernema sp. FJ418045	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02
Steinernema sp. MW365746	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. surkhetense MH822627	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.01	0.01	0.01	0.01	0.01	0.01	0.02
S. surkhetense HQ190042	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.05		0.00	0.01	0.01	0.01	0.01	0.02
S. nepalense HQ190044	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.00		0.01	0.01	0.01	0.01	0.02
S. backanense AY487918.	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.05	0.05		0.01	0.01	0.01	0.02
S. carpocapsae LN624758	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.06	0.06	0.06		0.01	0.01	0.02
S. eapokense AY487921.	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.07	0.07	0.08		0.01	0.02
S. siamkayai AF331917	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.07	0.08	0.08	0.04		0.02
C. elegans KX572972	0.51	0.51	0.51	0.51	0.51	0.52	0.51	0.51	0.51	0.52	0.52	0.51	0.51	0.51	0.50	

Table 2.5. Estimates of genetic divergence between 28S rRNA sequences of *S. surkhetense* and other *Steinernema* spp. using p-distance

S. surkhetense **StPTS1 MF621001**		0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.02
S. surkhetense **StPTS2 MF621002**	0.00		0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.02
S. surkhetense **StPTS3 MF621003**	0.00	0.00		0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.02
S. surkhetense **StPTS4 MF621004**	0.00	0.00	0.00		0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.02
S. surkhetense **StPTS5 MF621005**	0.00	0.00	0.00	0.00		0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.02
S. surkhetense MZ242237	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.02	0.02	0.02	0.02
S. surkhetense MH837096	0.00	0.00	0.00	0.00	0.00	0.01		0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.02
S. surkhetense HQ190043	0.00	0.00	0.00	0.00	0.00	0.01	0.00		0.00	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.02
S. nepalense HQ190045	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01		0.00	0.01	0.01	0.01	0.01	0.02	0.01	0.02
S. cumgarense GU395648	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01		0.01	0.01	0.00	0.01	0.01	0.01	0.02
S. carpocapsae FJ860020	0.02	0.02	0.02	0.02	0.02	0.04	0.02	0.02	0.02	0.02		0.00	0.01	0.01	0.01	0.02	0.02
S. anatoliense AY841761	0.09	0.09	0.09	0.09	0.09	0.11	0.05	0.12	0.13	0.06	0.01		0.02	0.01	0.01	0.02	0.02
S. siamkayai MN194606	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.03	0.16		0.02	0.02	0.02	0.02
S. sangi GU569057	0.12	0.12	0.12	0.12	0.12	0.14	0.12	0.11	0.11	0.12	0.15	0.18	0.11		0.01	0.01	0.02
S. affine AF331899	0.15	0.15	0.15	0.15	0.15	0.19	0.16	0.15	0.15	0.16	0.18	0.21	0.17	0.14		0.01	0.02
S. cameroonense JX985265	0.11	0.11	0.11	0.11	0.11	0.14	0.12	0.11	0.11	0.11	0.15	0.22	0.11	0.07	0.13		0.02
C. elegans MF192964	0.34	0.34	0.34	0.34	0.34	0.38	0.35	0.31	0.32	0.35	0.39	0.41	0.31	0.35	0.35	0.34	

Table 2.6. Estimates of genetic divergence between ITS sequences of *H. indica* and other *Heterorhabditis* spp. using p-distance

H. indica **HeTRE MF618314**		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02
H. indica **HeTR MF618316**	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02
H. indica **HeTV MF618318**	0.01	0.00		0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02
H. indica **HeM MF618313**	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02
H. indica **HeTS MF618317**	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02
H. pakistanense JX144740	0.01	0.01	0.01	0.01	0.01		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02
H. indica AY321483	0.00	0.00	0.01	0.01	0.01	0.01		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02
H. noenieputensis JN620538	0.01	0.01	0.02	0.02	0.02	0.03	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02
H. amazonensis DQ665222	0.09	0.09	0.09	0.09	0.09	0.10	0.09	0.10		0.01	0.01	0.01	0.01	0.00	0.02	0.02	0.02	0.02	0.02
H. taysearae EF043443	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.02		0.00	0.00	0.00	0.01	0.02	0.02	0.02	0.02	0.02
H. sonorensis FJ477731	0.11	0.10	0.11	0.11	0.11	0.11	0.11	0.11	0.02	0.00		0.00	0.00	0.01	0.02	0.02	0.02	0.02	0.02
H. mexicana AY321478	0.10	0.10	0.10	0.10	0.10	0.11	0.10	0.11	0.03	0.01	0.01		0.00	0.01	0.02	0.02	0.02	0.02	0.02
H. floridensis DQ372922	0.10	0.10	0.10	0.10	0.10	0.11	0.10	0.10	0.02	0.02	0.02	0.02		0.01	0.02	0.02	0.02	0.02	0.02
H. baujardi HeTD1 MF618319	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.02	0.03	0.03	0.03	0.02		0.02	0.02	0.02	0.02	0.02
H. beicherriana HQ896630	0.22	0.23	0.23	0.22	0.22	0.25	0.23	0.23	0.24	0.25	0.25	0.25	0.25	0.23		0.01	0.01	0.01	0.02
H. bacteriophora AY321477	0.22	0.22	0.22	0.22	0.22	0.24	0.22	0.22	0.23	0.25	0.25	0.24	0.24	0.23	0.03		0.01	0.01	0.02
H. zealandica EF530041	0.27	0.27	0.27	0.27	0.27	0.29	0.27	0.27	0.28	0.28	0.28	0.29	0.29	0.27	0.22	0.21		0.01	0.02
H. megidis AY321480	0.26	0.26	0.26	0.26	0.26	0.28	0.26	0.26	0.26	0.26	0.27	0.27	0.27	0.26	0.21	0.20	0.12		0.02
C. elegans KX572972	0.51	0.51	0.51	0.51	0.51	0.50	0.51	0.51	0.52	0.52	0.52	0.52	0.52	0.52	0.51	0.51	0.52	0.52	

Table 2.7. Estimates of genetic divergence between 28S rRNA sequences of *H. indica* and other *Heterorhabditis* spp. using p distance

H. indica **HeTRE MF621008**		0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. indica **HeTR MF621009**	0.00		0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. indica H**eTS MF621010**	0.00	0.00		0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. indica **HeTV MF621011**	0.00	0.00	0.00		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. indica EU100415	0.00	0.00	0.00	0.00		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. pakistanense KP096496	0.01	0.01	0.01	0.01	0.01		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
H. noenieputensis JX624110	0.00	0.00	0.00	0.00	0.00	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. mexicana EU100414	0.03	0.03	0.03	0.03	0.03	0.05	0.03		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01
H. floridensis EU099034	0.04	0.04	0.04	0.04	0.03	0.05	0.03	0.01		0.00	0.00	0.01	0.01	0.01	0.01	0.01
H. baujardi HeTD1 MF621012	0.03	0.03	0.03	0.03	0.03	0.04	0.03	0.01	0.01		0.00	0.01	0.01	0.01	0.01	0.01
H. amazonensis EU099036	0.03	0.03	0.03	0.03	0.03	0.05	0.03	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01
H. atacamensis HM230724	0.06	0.06	0.06	0.06	0.06	0.07	0.06	0.06	0.06	0.06	0.06		0.01	0.01	0.01	0.02
H. megidis EU100413	0.06	0.06	0.06	0.06	0.06	0.08	0.06	0.06	0.06	0.06	0.06	0.02		0.01	0.01	0.01
H. bacteriophora EU099037	0.05	0.05	0.05	0.05	0.05	0.08	0.05	0.04	0.05	0.05	0.04	0.04	0.05		0.00	0.01
H. beicherriana HQ896631	0.05	0.05	0.05	0.05	0.05	0.07	0.05	0.05	0.05	0.05	0.05	0.04	0.05	0.00		0.01
C. elegans MF192964	0.23	0.23	0.23	0.23	0.23	0.26	0.23	0.23	0.23	0.23	0.23	0.24	0.21	0.22	0.22	

Table 2.8. Estimates of genetic divergence between ITS sequences of *H. baujardi* and *Heterorhabditis* spp. using p-distance

H. baujardi **HeTD1 MF618319**		0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.02	0.02	0.02
H. baujardi **HeTD2 MF618320**	0.00		0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.02	0.02	0.02
H. baujardi **HeTD3 MF618321**	0.00	0.00		0.00	0.00	0.01	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.02	0.02	0.02
H. baujardi **HeTD4 MF618321**	0.00	0.00	0.00		0.00	0.01	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.02	0.02	0.02
H. baujardi AF548768	0.00	0.00	0.00	0.00		0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.02	0.02	0.02	0.02	0.02
H. mexicana AY321478	0.03	0.03	0.03	0.03	0.03		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02
H. floridensis DQ372922	0.02	0.02	0.02	0.02	0.02	0.02		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.02	0.02	0.02
H. sonorensis FJ477731	0.03	0.03	0.03	0.03	0.03	0.01	0.02		0.00	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02
H. taysearae EF043443	0.03	0.03	0.03	0.03	0.03	0.01	0.02	0.00		0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02
H. amazonensis DQ665222	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.03	0.03		0.01	0.01	0.01	0.01	0.02	0.02	0.02
H. indica HeTRE MF618314	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.12	0.12	0.11		0.00	0.02	0.02	0.02	0.02	0.02
H. noenieputensis JN620538	0.10	0.10	0.10	0.10	0.11	0.11	0.11	0.11	0.11	0.10	0.01		0.02	0.01	0.02	0.02	0.02
H. beicherriana HQ896630	0.22	0.22	0.22	0.22	0.23	0.24	0.23	0.24	0.23	0.23	0.22	0.22		0.01	0.01	0.01	0.02
H. bacteriophora AY321477	0.22	0.22	0.22	0.22	0.23	0.23	0.23	0.24	0.23	0.22	0.22	0.21	0.03		0.01	0.01	0.02
H. zealandica EF530041	0.26	0.26	0.26	0.26	0.27	0.27	0.27	0.27	0.27	0.26	0.28	0.27	0.21	0.21		0.01	0.02
H. megidis AY321480	0.24	0.24	0.24	0.24	0.26	0.25	0.25	0.25	0.25	0.25	0.26	0.25	0.20	0.19	0.11		0.02
C. elegans KX572972	0.52	0.52	0.52	0.52	0.51	0.53	0.53	0.52	0.52	0.52	0.51	0.52	0.52	0.52	0.53	0.53	

Table 2.9. Estimates of genetic divergence between 28S rRNA sequences of *H. baujardi* and other *Heterorhabditis* spp. using p-distance

H. baujardi **HeTD1 MF621012**		0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. baujardi **HeTD2 MF621013**	0.00		0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. baujardi **HeTD3 MF621014**	0.00	0.00		0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. baujardi MT372503	0.00	0.00	0.00		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02
H. amazonensis EU099036	0.01	0.01	0.01	0.00		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. floridensis EU099034	0.01	0.01	0.01	0.01	0.01		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. mexicana EU100414	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
H. noeniepurtensis JX624110	0.03	0.03	0.03	0.04	0.03	0.03	0.03		0.00	0.00	0.01	0.01	0.01	0.01	0.01
H. indica HeTRE MF621008	0.03	0.03	0.03	0.04	0.03	0.04	0.03	0.00		0.00	0.01	0.01	0.01	0.01	0.01
H. pakistanense KP096496	0.04	0.04	0.04	0.05	0.05	0.05	0.05	0.01	0.01		0.01	0.01	0.01	0.01	0.02
H. atacamensis HM230724	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.07		0.01	0.01	0.01	0.02
H. megidis EU100413	0.06	0.06	0.06	0.07	0.06	0.06	0.06	0.06	0.06	0.08	0.02		0.01	0.01	0.01
H. bacteriophora EU099037	0.05	0.05	0.05	0.06	0.04	0.05	0.04	0.05	0.05	0.08	0.04	0.05		0.00	0.01
H. beicherriana HQ896631	0.05	0.05	0.05	0.06	0.05	0.05	0.05	0.05	0.05	0.07	0.04	0.05	0.00		0.01
C. elegans MF192964	0.23	0.23	0.23	0.26	0.23	0.23	0.23	0.22	0.22	0.25	0.23	0.21	0.22	0.22	

p-distance of *H. baujardi* with other *Heterorhabditis* spp.:

The analysis of the ITS region showed that the 4 developed sequences and database sequence of *H. baujardi* (AF548768) showed an intraspecific distance of 0.00% and an interspecific distance of 13.18 ± 4.18% (2.00–52.00%) (Table 2.8). Furthermore, based on the analysis of the 28S region, the isolated *H. baujardi* exhibit an average intraspecies distance of 0.00% and an average interspecies distance of 4.80 ± 1.70% (1.00–23.0%) with respect to all other *Heterorhabditis* species along with the outgroup species (Table 2.9).

Discussions

The accurate identification of EPNs is very important prior to use as biological control agents since different species exhibited different ecological adaptations and behaviour **(Valadas *et al*. 2014).** Since isolates of EPNs from different geographical area exhibit differences in their behavioural and physiological adaptations, identification and documentation of locally adapted species of a particular region is required for the successful use of EPN as a biological control agent **(Stock *et al*. 1999).** Furthermore, accurate identification is very important in understanding the geographical distribution and biodiversity of steinernematids and heterorhabditids **(Kerry and Hominick, 2002)**. In fact, the identification of *Steinernema* to the species level based solely on morphological analysis might be risky **(Spiridonov *et al*. 2004)**. Simultaneously, the identification of *Heterorhabditis* species, in particular, based on morphology is not an easy task because of limited morphological variation **(Dolinski *et al*., 2008)**. The aim of the study, therefore, was to further identify the

isolated locally-adapted entomopathogenic nematodes at the species level by molecular analysis using the 3 selected markers such as ITS rDNA, 28S rDNA and COI gene regions for future use as an effective biological control agent.

A. Steinernematids

The phylogenetic analysis inferred from ITS rDNA, 28S rDNA and COI mitochondrial gene regions confirmed that the EPN isolate with serial no. 8 to 11 (*Steinemema* sp. that produced pale pink-yellow colour on dead larvae with short IJs) and 12 (*Steinemema* sp. that produced charcoal-grey colour on dead larvae with long IJs) belonged to *S. sangi* and *S. surkhetense*, respectively. Furthermore, the morphometric measurements of the respective nematode isolates fall within the ranges of *S. sangi* **(Phan *et al,* 2001)** and *S. surkhetense* **(Khatri-Chhetri *et al,* 2011)**. In fact, slight, but negligible variations in morphometric measurements were observed for both the isolates with their respective original descriptions. These differences may be attributed to both intraspecific genetic and environmental variations **(Bolnick *et al*. 2011)**.

In the study, the phylogenetic trees inferred from ITS and 28S rDNA in particular highly support monophyletic grouping which are in agreement with other phylogenetic studies of the genus *Steinernema* **(Stock *et al*., 2001; Spiridonov *et al*., 2004; Nguyen *et al*., 2007, 2008; Qiu *et al*., 2011;)**. Among steinernematids, *S. sangi* is a species that belongs to the *feltiae-kraussei-oregonense* group and *S. surkhetense* is a species that belongs to the *carpocapsae-scapterisci* group of **Spiridonov *et al.* (2004)**. Furthermore, the phylogenetic tree constructed from the ITS rDNA sequences showed that within the monophyletic group of the *feltiae-*

kraussei-oregonense, the isolated *S. sangi* (StR KY861069, StK KY861070, StC KY861071 and StL KY861072) formed a cluster with database sequence of *S. sangi* (AY355441) and did not cluster with other species supporting the report of **Nguyen *et al*. (2007, 2008)**. Similarly, the calculated p-distance exhibits an average intraspecies distance of 0.5 ± 0.35% (0.0–1.00%) and could differentiate the isolated *S. sangi* from other *Steinernema* species.

As shown in the fig. 2.2, the phylogenetic tree constructed by using 28S rDNA gene sequences depicted that the isolated *S. sangi* (StR MF6209977, StK MF6209978, StC MF6209979, StL MF62091000) formed a cluster and separated from other *Steinernema* species of the same monophyletic group. Nevertheless, the species labelled as *S. sangi* (GU569057) may be a case of misidentification as it clustered with other *Steinernenam*a species from *glaseri group* (rather than *feltiae-kraussei-oregonense*) and express wide genetic distance (7.00%) as well as less similarity (92.41%) with the isolated S. *sangi*. Other *Steinernema* species that showed higher similarity include *S. akhursti* (96.68%), *S. feltiae* (96.25%), *S. weiseri* (96.40%), *S. puntauvense* (96.21%), and the rest of the species expresses ≤96.00% similarities.

Based on the ITS sequences, the BLAST search result showed that the isolated *S. surkhetense* has a high similarity (99.00–100.00%) with those sequences available for *S. surkhetense* (KP219886, MH822626, MH822627, HQ190042, GU395630, KR029844, MK995658, MK995659) with an intra-specific distance of 0.00% (with the exception of the database sequence of *S. surkhetense* with accession no. HQ190042). Other closely related species include *Steinernema* sp. (FJ418045,

MW365746, and MG976890). In fact, the insufficiency is observed where the developed sequences of *S. surkhetense* exhibit p-distance of 4.00% with database sequence of both *S. surkhetense* (HQ190042) and *S. nepalense* (HQ190044). Nevertheless, the calculated p- distance exhibit an average interspecies distance of 12.00 ± 6.65% (4.00–51.00%) that could differentiate the isolated *S. surkhetense* from other *Steinernema* species. Furthermore, the phylogenetic tree inferred from the ITS region showed that within the *carpocasae-scapterisci* group, the isolated *S. surkhetense* formed a cluster with database sequences of *S. surkhetense* reported from India (KP219886, MH822627, K202R029844), the original description of *S. surkhetense* (HQ190042) including *S. nepalense* (HQ190044) reported by **Khatri-Chhetri *et al* (2011)**. Since the present study did not include morphological and molecular characterization of *S. surkhetense* and *S. nepalense* from the original description, further study is required to solve insufficiency of the analysis.

As per the phylogenetic tree constructed using 28S rDNA sequences, the isolated *S. surkhetense* is a true species since it formed a monophyletic group with the available database sequences of *S. surkhetense* (HQ190043, KU187262, MZ242237 and MH837096) and other *Steinernema* spp. of the *carpocapsae* group of Spiridonov *et al*. (2004)). However, the database sequences of *S. surkhetense* with accession no. MZ242237, MZ242238, and MZ242239 showed 96.20, 90.22, and 93.47% similarity with the isolated sequences and need further study to resolve the query. Nevertheless, the phylogenetic tree inferred from the 28S rDNA sequences showed that within the *carpocasae-scapterisci* monophyletic group, the isolated *S. surkhetense* formed a cluster with *S. surkhetense* (HQ190043, KU187262,

MZ242237, MH837098) and did not cluster with other species. Furthermore, based on the analysis of 28S region, the isolated *S. surkhetense* exhibit a relatively low intraspecific distance (0.001 ± 0.00%) with the database sequences of *S. surkhetense*. However, p-distance failed to differentiate the isolated *S. surkhetense* from the database sequences of *S. nepalense* (1.00%), *S. cumgarense* (1.00%), *S. carpocapsae* (2.00%) and *S. siamkayai* (1.00%). The average interspecific distance was 8.00 ± 3.33% (1.00–34.00%)

The study showed that all the analysed database sequences of ITS and 28S rDNA regions which represent the family Steinernematidae formed their respective monophyletic group as *affine intermedium*, *bicornutum*, *cameroonense*, *carpocapsae*, *glaseri*, *monticolum*, and *feltiae-kraussei* clades **(Nadler *et al*., 2006; Spiridonov and Subbotin, 2016; Lis *et al*., 2021)**. Therefore, the tree inferred from the present study highly support monophyletic clades which are in agreement with other phylogenetic studies of the genus *Steinernema* **(Nguyen *et al*., 2007, 2008; Qiu *et al*., 2011; Spiridonov *et al*., 2004; Stock *et al*., 2001)**.

On the other hand, the phylogenetic tree inferred from M1– M6 region of COI (region), even though delimitate the species, did not yield useful monophyletic clades and failed to resolve a clear phylogenetic relationship. The problems may be due to the fact that only a few COI gene sequences of *Steinernema* species are available for comparison. In fact, 12 new COI gene sequences of *S. sangi* and *S. surkhetense* were added from the present study to the NCBI GenBank. The phylogenetic tree inferred from COI gene showed that the respective developed

sequences of *S. sangi* and *S. surkhetense* clustered separately from other *Steinernema* species. However, the database sequence of *S. sangi* with NCBI accession no. GU569073 formed a cluster with other species the of *arenarium-glaseri* monophyletic group. This may be due to the wrong submission of sequence, misidentification of the species, poor resolution of the selected gene sequences, etc. and need further study. The study, therefore, indicated that the phylogenetic analysis using the COI gene region provides overall less resolution with fewer consistently supported clades in the phylogenetic tree.

B. Heterorhabditids

The phylogenetic analysis inferred from sequences of ITS rDNA, 28S rDNA and M1-M6 region of COI regions confirmed that the *Heterorhabditis* sp. coded as HeM, HeTC, HeTRE, HeTS, and HeTV (series 1–6) that both produce brick red colour on dead larvae belonged to *H. indica* **(Poinar *et al.*, 1992)**. However, the isolate coded as HeTD (series 7) both produces brick red colour on dead larvae belonged to *H. baujardi* **(Phan *et al.*, 2003)**. Furthermore, the respective morphometric measurements of the nematode isolates in accordance with the molecular characterization fall within the ranges of *H. indica* and *H. baujardi*. Meanwhile, negligible slight variations observed in the morphometric measurements may be attributed to intraspecific genetic variations **(Rolston *et al.*, 2009; Bolnick *et al.*, 2011)**.

Reconstruction of molecular phylogeny of the genus *Heterorhabditis* has been built by several workers. Phylogenetic analysis of *H. indica* and *H. baujardi* inferred from sequences of ITS rDNA and D2-D3 region of 28S rDNA regions

showed similar topology, where both the species belong to *'indica'* group of **Nguyen *et al*. (2008)** and **Spiridonov and Subbotin (2016)**. The phylogenetic analysis showed that both the markers' gene sequences revealed a strong bootstrap support for constructing phylogenetic relationships of *Heterorhabditis* species.

The phylogenetic tree constructed from the ITS rDNA sequences showed that within the monophyletic '*indica*' group, the isolated *H. indica* and *H. baujardi* formed a cluster with their respective database sequences. Likewise, the isolated *H. indica* exhibits an average interspecific distance of 16.31 ± 3.77% (1.00–51.00%) and could differentiate it from other *Heterorhabditis* species (except *H. noenieputensis*). The ITS rRNA region is a useful and widely accepted genetic marker for species delimitation and assessing phylogenetic relationships in *Steinernema* species. However, the spanning 5.8S region is too conserved to resolve the connection among the species (Nguyen *et al*. 2001). This can be correlated with the lack of significant difference between the isolated *H. indica* from the database sequences of *H. noeniputensis* which showed 97.00–98.00% similarity. Meanwhile, the BLAST search result showed that ITS rDNA sequences of the isolated nematode exhibit high similarity (99.00–100.00%) with that of the sequences available for *H. indica,* with the exception of the database sequences with accession no. MG893075, MG489821, MZ507541, MZ507542, MW654494, MW654496, MW654497, MW654499, MW654500, and MW654501 (≤ 97.00 % similarity).

In the case of *H. baujardi*, the database sequences of ITS region showed 99.00 –100.00% similarity with that of the developed sequences with p-distance of 0.00%. Further analysis of the sequence showed that even though the calculated p-

distance could differentiate *H. baujardi* from other *Heterorhanditis* species with an average interspecific distance of 13.18 ± 4.18% (2.00–52.00%), could not discriminate *H. baujardi* from *H. amazonensis* and *H. floridensis* with a distance of 2.00%, *H. Mexicana*, *H. sonorensis*, and *H. tayserea* with a distance of 3.00%.

Furthermore, the phylogenetic tree constructed by using 28S rDNA sequences showed that the isolated *H. indica* and *H. baujardi* formed a monophyletic group with other *Heterorhabditis* species from the *indica* group. The further analysis of the sequences could clearly differentiate the isolated *H. indica* and *H. baujardi* from other *Heterorhabditis* sp. However, it is interesting to state that the calculated p-distance based on the 28S rDNA region could not clearly differentiate *H. indica* from *H. neonieputensis* (1.00%) that belong to the '*indica*' group. Likewise, the insufficiency of the analysis was noted where the p-distance of the same gene failed to clearly differentiate *H. baujardi* from *H. amazonensis*, *H. floridensis*, and *H. mexicana* with 1.00% difference. Therefore, a further thorough study is needed to solve and resolve the insufficiency of the phylogenetic analysis.

The mitochondrial gene, cytochrome c oxidase I (M1– M6 partition), is a reliable tool for Metazoan species delimitation, including helminths **(Hebert *et al.*, 2003)**. However, **Derycke *et al*. (2010)** reported the dominance of M3–M11 partition over M1–M6 partition of COI in terms of resolving taxonomic ambiguities and uncovering cryptic diversity among marine nematodes. From the present study, phylogenetic analysis using the COI region could discriminate all the isolated EPN species from other species, but unlike the other two markers exhibit a different topology with no clear formation of phylogenetic clades. Therefore, it is difficult to

make a concrete conclusion regarding the reliability and use of the COI sequences (M1–M6 partition) as a tool for EPN species delimitation.

In India, a number of indigenous steinernematids and heterorhabditids have been documented. However, works on the EPNs in northeast India is very limited less is known about their occurrence and potential in the region. So far, five species of EPNs *viz*. *H. indica*, *H. bacteriophora*, *S. glaseri*, *S. abbasi* (=*S. thermophilum*) and *S. carpocapsae* (=*S. meghalayense*), have been documented from north-eastern India where the bioefficacy and ecological characters of only some species have been studied **(Lalramliana *et al*., 2005; Lalramliana and Yadav, 2010, 2016; Ganguly *et al*., 2011; Devi *et al*., 2016)**. Therefore, with the isolation of *S. sangi*, *S. surkhetense*, *H. indica* and *H. baujardi* from Mizoram, north-eastern region of India, 13 valid EPN species are presently known from India.

Chapter 3: Evaluation of Entomopathogenic nematodes (EPNs) against major insect pests in Mizoram

Introduction

EPNs of the families Steinernematidae and Heterorhabditidae have been known to infect a wide range of insects and were shown to be the most efficient biological control agents for soil and above-ground insect pests (**Kaya and Gaugler, 1993; Laznik *et al*, 2010**). Generally, EPN species of the genera *Steinernema* and *Heterorhabditis* have a wide range of hosts, but to date, many of the recorded species are with their host range unknown (**Peters, 1996; Shapiro-Ilan *et al*., 2017, 2018)**. Due to their high level of safety for mankind, non-target organisms, and the environment, they have been virtually exempted from pesticide registration regulations in many countries (**Ehlers, 2005; Piedra *et al*., 2015**) but exhibit poor infection of other hosts and are only well adapted to a small range of host species. The host range of indigenous isolates may have been narrowed due to specific ecological adaptation of the nematodes and may exhibit poor infection of other hosts. In laboratory tests, numerous EPN species have been shown to infect a variety of insect species. However, following field applications, the host range may be significantly reduced based on the nematode's ecology and possible hosts. Several EPN species that have been isolated from their normal hosts have a special affinity for a small subset of host species, yet they exhibit poor infection of other hosts**.**

For the successful application of EPN as an effective biological control agent, it is essential to identify naturally-adapted species in a specific area (**Stock *et al*. 1999**). Indigenous EPN isolates exhibit extensive variations with respect to insect

host range, multiplication and infectivity rate in a host, and environmental conditions for persistence **(Bedding, 1990)**. The pathogenicity of EPNs is influenced by various environmental and biotic factors, mainly by the intraspecies variations, the host insect, the life cycle of a targeted insect, and soil fauna (**Gaugler and Kaya, 1990; Kaya and Gaugler, 1993; Koppenhofer *et al*, 1995; Shapiro-Ilan *et al*., 2012; Grewal *et al*., 2005a; Georgis *et al*., 2006**). Several factors need to be considered for the selection of EPN species to control a particular insect pest, out of which the most critical factors include pathogenicity of the EPN species against the targeted insect, foraging behaviour of the EPN, moisture, and temperature (**Kung *et al*., 1991; Campbell *et al*., 2003; Grewal *et al*., 2005a).**

With the growing concern over pesticide resistance in insects, effects on non-targeted organisms (**Aktar and Chowdhury, 2009**) including humans, residues in the environment, and bioaccumulation (**Crinnion, 2009**), environmentally safer biopesticides are potent alternatives to conventional pesticides (**Gupta and Dikshit, 2010)**. Therefore, in comparison to chemical insecticides, biological control offers a high significant approach to insect pest management in terms of safety, effectiveness, and sustainability (Lou *et al*, 2014). Like other biological control agents, EPNs are potential and promising organisms for the control of several insect pests (**Lacey and Georgis, 2012)**. In addition, EPNs can be formulated with a wide variety of chemical and organic pesticides in Integrated Pest Management (IPM) programs **(Lacey and Georgis, 2012)**. In this regard, EPNs of the family Steinernematidae and Heterorhabditidae have been successfully utilised as biocontrol agents to manage insect pests in a wide variety of crops (**Grewal *et al*., 2005a)**. Although some species

of the genera *Oscheius* have recently been included in the group of EPNs (**Dillman *et al.* 2012**), the only genera that have been exclusively produced as biological control agents for commercial pest control are *Heterorhabditis* and *Steinernema* (**Kaya and Stock 1997**; **Abd-Elgawad *et al.,* 2017**). Furthermore, numerous researchers have reported the application of several indigenous EPN isolates with better adaptation to the prevailing local climatic conditions in comparison to exotic strains **(Bedding, 1990).**

Since crops cannot be grown without the ecological services provided by insects, and are thus essential for human existence. Vegetation is a major factor influencing the diversity and composition of insects, therefore, any change in the habitat is likely to have an effect on the relative abundance and distribution (**Kerchev *et al*., 2012**; **Patil *et al*., 2016**). The food plants of the world are damaged by diverse groups of animals and about 1000 species of insects have been estimated to be involved in crop damage, besides others **(Dhaliwal *et al*, 2007)**. According to **Schoonhoven *et al***. (2005), about 50% of insect species are pests, and 18% of these are herbivorous species that eat plants in one manner or another according to **Losey and Vaughan** (2006). However, altogether the pests are not of economic importance unless pest population density reaches the economic threshold (ET) level and the economic injury level (EIL). Therefore, the main requirement is the protection of the crop to reduce the losses with a low impact on the environment. Globally, about one-fifth of the loss of total crop production was believed to be caused by herbivorous insect pests **(Pimentel and Peshin, 2014)**. In India, sustained loss of crops due to insect pests and diseases remains one of the most important constraints in agriculture

production. Extensive surveys have been conducted on the losses of crops since long time back and have been assessed from time to time. During 2007–2008, about US$ 21.5 billion loss in major agricultural crops was recorded (**Dhaliwal *et al*., 2010**). According to **Dhaliwal *et al*. (2015),** Indian agriculture currently experiences an annual loss of roughly US$. 36 billion due to insect infestation. In the process of addressing this problem, the dependency of agriculture on chemical pesticides must be reduced to the minimum level and more emphasis should be taken towards an eco-friendly crop management approach involving bio-pesticide pest control strategies in IPM programs.

EPNs of the genera *Steinernema* and *Heterorhabditis* can infect a wide range of hosts in the laboratory where host interaction is guaranteed. In laboratory tests, for instance, 250 different insect species have been infected with *S. carpocapsae* (**Peters, 1996**). Their safety as biocontrol agents is increased by the fact that they have a smaller range of hosts in the field than in the laboratory (**Akhurst, 1990**; **Bathon, 1996).**

In the 1930s, the value of EPNs and natural plant protection was initially set up in the USA and their application as biocontrol agents required a methodical approach, technological, and scientific advancement. For instance, the massive production of nematodes was crucial to the commercially successful development of pest control for insects. Various techniques are currently used to produce EPNs using solid and liquid culture media, both in vivo and in vitro (solid and liquid culture) (**Friedman, 1990**). Each strategy has certain advantages and drawbacks in terms of production cost and quality, the level of technical expertise required, and economies

of scale. For instance, *in vivo* production required the lowest amount of capital expenditure and scientific knowledge input (**Shapiro-Ilan *et al.*, 2014b**), perhaps with problems such as the financial and labour costs of insect host maintenance and production. With the advancement in technologies and further scientific works that have been conducted, there are also numerous formulation and application options. Glaser developed a technique for *in vitro* production of EPNs and the first field trial was conducted against *P. japonica* in New Jersey using these nematodes **(Kaya and Gaugler, 1993)**. EPNs are thought to be remarkably safe biological control agents (**Akhurst and Smith, 2002**) and the negative impact that they can impose on the environment is far lower than that of chemical agents for having a specific activity. No instances of environmental harm caused by these bioagents have been documented in Newman, USA since the first field application of EPNs against *P. japonica* (**Glaser *et al.*, 1935**). **Akhurst and Smith, 2002** also stated that EPNs and their associated bacteria are exceptionally safe for mammals and plants.

Among insect pests, scarabs are immensely studied and were reported from all continents (except for Antarctica). Several works have been conducted on the efficacy of certain species of EPN against scarab and, *P. japonica* was reported to be one of the most-susceptible species (**Grewal *et al.*, 2005b**). *S. glaseri* was the first EPN species to be widely applied for the control of scarabs and its effectiveness, particularly against the larvae has been reported by numerous researchers (**Gaugler *et al.*, 1992**). Among the EPN species, *H. zealandica* and *H. bacteriophora* were used commercially for controlling grubs **(Grewal *et al.*, 2005b; Koppenhöfer *et al.*, 2006)**.

Several workers have reported the successful control of weevils using EPNs. **Kakouli-Duarte *et al*. (1997)** have demonstrated the field trial application of *S. carpocapsae* and *H. megidis* against *Otiorhynchus sulcatus* (black bine weevil) in strawberry cultivated land and, a comparative reduction number of the weevil larvae was recorded. In addition, based on **Haukeland and Lola-Luz (2010)**, the EPN species *H. megidis* showed high efficacy against *O. sulcatus* at a temperature above 10 ℃.

Several studies have reported the successful application of EPN against fungal gnats **(Scheepmaker *et al*., 1998a; Grewal, 2007)**. **Grewal and Richardson (1993)** have reported that *S. feltiae* showed comparatively good efficacy with successful multiplication in gnat larvae that helps to maintain longer effectiveness than certain insecticides. In addition, the commercially available *S. feltiae* are frequently used against *Lycoriella* spp. in the USA and Europe (**Grewal and Georgis**, 1998; **Grewal, 2007**). Further, pathogenicity of EPN species against larvae of the diamond black moth, *Plutella xylostella* has been conducted and demonstrated by several works which further proposed that EPNs might be useful components in management of the pest (**Lello *et al*., 1996; Baur *et al*., 1997)**. In the case of soil surface application, the management of root weevil, *Diaprepes abbreviatus* using EPNs in citrus is among the best instances of successful control. In Florida, EPN species most notably *S. riobrave* and *H. indica* have been commercialised for the prevention and control of weevils for almost 20 years (**McCoy *et al*., 2002; Stuart *et al*., 2008)**.

Some *Steinernema* spp. and *Heterorhabditis* spp. have been employed to certain levels of success against larvae and adults of certain cutworms (Lepidoptera: Noctuidae) **(Capinera *et al*., 1988, Ebssa and Koppenhöfer, 2011)**. However, they have not yet been widely applied since further implementation of scientific techniques is needed. Application of EPNs for the control of cricket pests has been documented by several workers, among which *S. scapterisci* showed effective activity against *Scapteriscus vicinus* (**Parkman *et al*, 1993**). There was a significant reduction in the cricket populations at the release sites three years after *S. scapterisci* was first introduced. The application of the EPN species against the pest was facilitated by the commercial availability of the control agent, *S. scapterisci* (**Grewal *et al*., 2005b**).

Spodoptera litura

Tobacco cutworm, *S. litura* (Fabricius, 1775) is one of the most destructive and serious pests of agricultural crops with widespread distribution throughout tropical and temperate Asia, Australia and Pacific Islands **(Feakin, 1973; Kranz *et al*., 1977)**. In India, it has a widespread distribution across the land, covering almost all the states **(CABI, 2019)**, and responsible for heavy damage to economically important crops like groundnut **(Patil *et al*., 1996)**, soybean **(Choudhary and Srivastava, 2007; Punithavalli *et al*., 2014; Sharma *et al*., 2014)**, cotton **(Gedia *et al*., 2008)**. The larva is a polyphagous pest **(Singh and Jalali, 1997)** and reported to infest different varieties of plants, including major crops such as tobacco, groundnut, taro, and castor **(Wu *et al*., 2004; Qing *et al*., 2006)** causing an economic loss of

about 26–100% on the field **(Dhir *et al*., 1992; Rao *et al*.,1993; Isman *et al*., 2007; Satyagopal *et al*., 2014).**

Spodoptera frugiperda

Fall armyworm (FAW), *S. frugiperda* (J.E. Smith, 1797) (Family: Noctuidae) is an invasive lepidopteran pest native to America **(Todd and Poole, 1980)**. It is a polyphagous pest of more than 350 species of plants, causing serious damage to economically important cultivated crops such as maize, rice, sorghum, sugarcane, cotton, and other vegetable crops **(CABI, 2020)**. Subsequently, FAW was reported to invade Central and Western Africa in the year 2016 **(Goergen *et al*., 2016)**, the Indian subcontinent in Asia in the year 2018 **(Sharanabasappa *et al*., 2018)**, and currently with worldwide distribution **(CABI, 2020)**. In India, FAW was first reported in maize fields in Shivamogga, Karnataka in May 2018 **(Sharanabasappa *et al*., 2018)** with subsequent scientific reports from other regions of the country **(Repalle *et al*., 2020).**

Pieris brassicae

The cabbage butterfly, *Pieris brassicae* (Linnaeus, 1758) (Lepidoptera: Pieridae) is a serious pest of cruciferous crops. The larvae primarily feed on the mustard plant, cabbage, broccoli, cauliflower, and other related crops as well. The life cycle of *P. brassicae* includes light yellowish freshly laid eggs (in a cluster of 20–100) on the underside of the leaves. Before hatching, the tips of the eggs turn greyish and after 1–3 weeks, the eggs hatch depending on the plant and temperature **(Ramadhane and Ihsan, 1999; Chahil *et al*., 2013)**. The larva went through four moults, containing

five instars that last for about 14–24 days depending on the host plant and prevailing environmental conditions **(Chahil *et al.*, 2013; Kumar *et al.*, 2020)**. The young larvae feed gregariously on the leaves of the cabbage plant and scrape away the lower epidermis of the leaves. The grown-up larvae get dispersed and feed vigorously which cause severe and whole defoliation of the crop thereby killing the plant in severe infestations **(Kumar *et al.*, 2020)**. In addition, the crops are also dirtied with a huge quantity of larval feces. The fully grown-up larvae stopped feeding and pupate (10–12 days) into whitish colour pupae which later turn greyish with many spots **(Kumar *et al.*, 2020)**. The butterflies emerged from the pupa and live for about 7–8 and 8–11 days in males and females, respectively **(Kumar *et al.*, 2020)**. The total development period of *P. brassicae* lasts for about 37–53 and 38–55 days respectively for male and female **(Kumar *et al.*, 2020)** the second generation begins.

Pieris canida

Cabbage white butterflies (*Pieris* spp.) are serious and destructive polyphagous pests around the world that are known to feed on plants of the family Brassicaceae and other related plants as well **(Chen *et al.*, 2004; Ali and Rizvi, 2007; CABI, 2020)**. The larvae of the pests feed on the leaves, damage the crop by creating holes, and skeletonizing the leaves which may resulted in low market value of the crops **(Mazurkiewicz *et al.*, 2017)**. Of the pests, the Indian cabbage white butterfly, *P. canidia* **(Sparrman, 1768)** is a destructive polyphagous pest of agricultural crops in many countries of Asia **(Evan, 1932; Chen *et al.*, 2004)**. The larvae primarily feed

on foliage of cabbage, cauliflower, broccoli, and other crops of the Brassicacea family, thereby causing a reduction in crop production.

Aplosonyx chalybaeus

The corm borer, *A. chalybaeus* (Hope) (Coleoptera: Chrysomelidae) is a regular and serious endemic insect pest of Colocasia (*Colocasia esculenta*) in North Eastern Region of India **(Korada, 2012)**. The pest is responsible for causing 20–30% and 80–90% damage to the foliage and corms, respectively of the crop that results in severe losses to the tribal farmers of the region. The pest attacks both the leaf sheath and the corm that may cause the plant to wither and turns yellow, and emit a foul smell thereby destroying the plant. The grubs primarily feed on the corm while the adult beetles feed on the leaves and damaged the crop by creating holes of different sizes (about half to one-inch size). A group of about 80–100 eggs is laid per the female beetle in a leaf sheath that hatched after 3–5 days. The young emerged grubs migrated to the shoot and feed on the corm. A total of about 70–80 grubs were commonly observed per plant, and the developing corm upon heavy infestation with the pest ultimately result in the death of the plant. The beetle is highly active during the day and mainly hide at night in the leaf sheaths at ground level that harboured about 3–4 individuals (9–12 individuals per plant). In addition, the pest was reported in several South Asian countries as well **(Maddison, 1993)**.

Odoiporus longicollis

Banana pseudostem weevil (BPW), *O. longicollis* (Olivier, 1807) (Coleoptera: Curculionidae) is one of the most harmful monophagous and or oligophagous

(Kannan *et al*, 2021) pest of banana limiting the production and productivity of the crop **(Visalakshi *et al*., 1989, Shukla & Kumar, 1970, Azam *et al.*, 2010)**. The pest is known to infest the host plant in the field at its multiple life stages and is cosmopolitan in Asia (**Azam *et al.*, 2010**). A major infestation of banana plantations occurs in South and South East Asia, particularly in India covering several areas across the states (**Reghunath *et al*., 1992; Justin *et al*., 2008**). In BWP, the pre-oviposition period last for about 15–30 days; the adult female lays egg of about nine numbers (**Justin *et al.*, 2008**) in the air chamber of the pseudostem and, the emerging larvae feed on the leaf sheath **(Padmanaban *et al*., 2001)**. The larva goes through five moulting and eventually pupates in a cocoon made of banana fibres. The life span of the adult weevil is about 200 days (**Ravi and Palaniswami report, 2002**) and may vary with temperature and other environmental factors. The feeding grub is the most damaging stage which creates extensive tunnels on the stem and deteriorates the plant. Depending on the growth stage of the banana plant and management efficiency, the pest could cause a total loss of about 10–90% of crop yield, especially with an infestation of the crop at the early vegetative stage (**Padmanaban and Sathiamoorthy, 2001**).

Rapidly increasing knowledge regarding biology, host range, and epidemiology has laid the groundwork for the eventual use of EPNs as effective biological control agents worldwide. In certain developed countries, commercial nematode-based products are available and are being utilized for the biological control of insects **(Grewal *et al.*, 2005a)**. EPNs have emerged as excellent candidates for biological control of insect pests. However, new developments in technology for

the formulation, production and application strategy are necessary to make an EPN-based products comparable with conventional insecticides at the market level. One of the fundamental steps in the development of an EPN for biocontrol is choosing an appropriate strain. Virulence against the target pest is a basic factor of a biological control program. The characterization of traits related to the control potential of species (strains) of EPN is therefore the key for successful biological pest control in an area.

The aim of the current research was to offer the fundamental knowledge needed for the development of isolated indigenous EPNs into biological control agents. The bio-efficacy of four EPN species, *H. indica*, *H. baujardi*, *S. sangi*, and *S. surkhetense* was tested against six (6) insect pests, *S. litura*, *S. frugiperda*, *P. canidia*, *P. brassica*, *A. chalybaeus*, and *O. longicollis* in a laboratory

Materials and methods

Laboratory evaluation of EPNs against insect pests

Larvae of the insect pests were collected from infested host plantation sites and reared in the laboratory at Pachhunga University College using their natural diet. Different life stages of the pest were obtained for morphological and molecular identification of the pest. Healthy pest larvae and pupae were chosen for the experiment in order to assess their susceptibility to the isolated EPN species.

Mortality test for larvae

The experiment was performed using a Petri dish assay, following **Kaya and Stock (1997)**. The selected nematode concentrations were 10, 25, 50, 100, 200, and 400

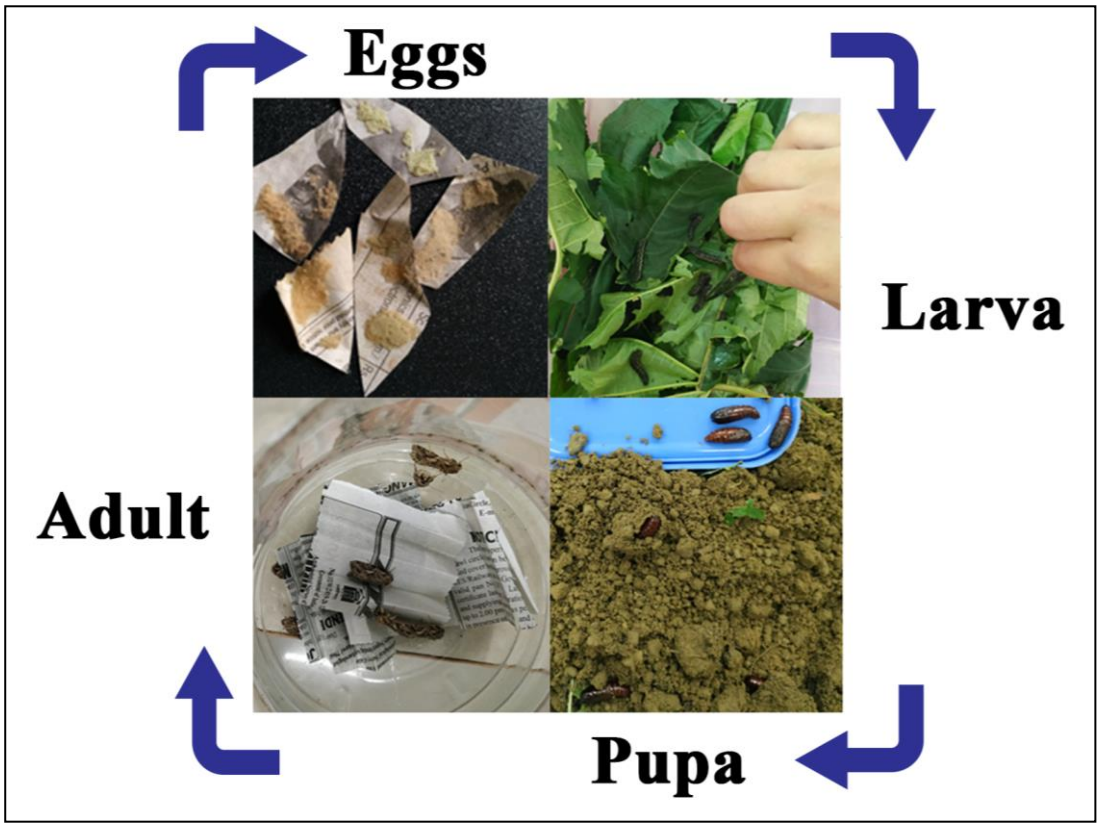

Fig. 3.1. Life cycle of tobacco cutworm, *Spodoptera litura*.

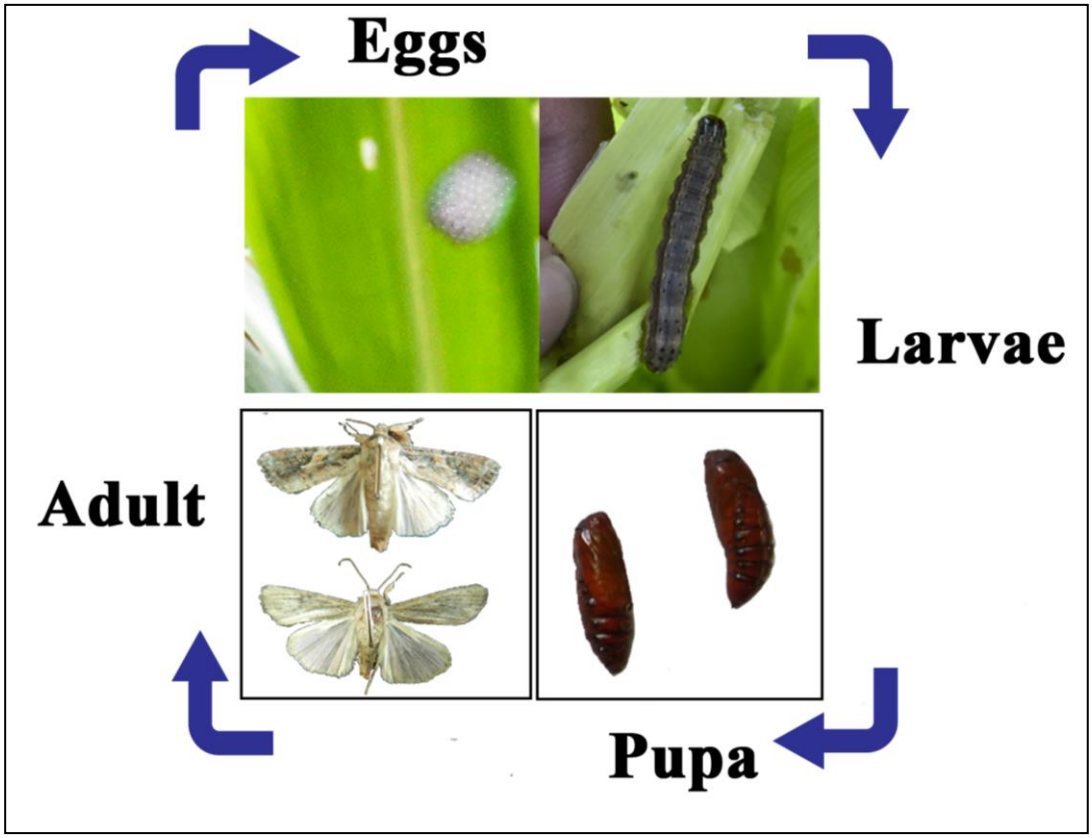

Fig. 3.2. Life cycle of fall armyworm, *Spodoptera frugiperda*.

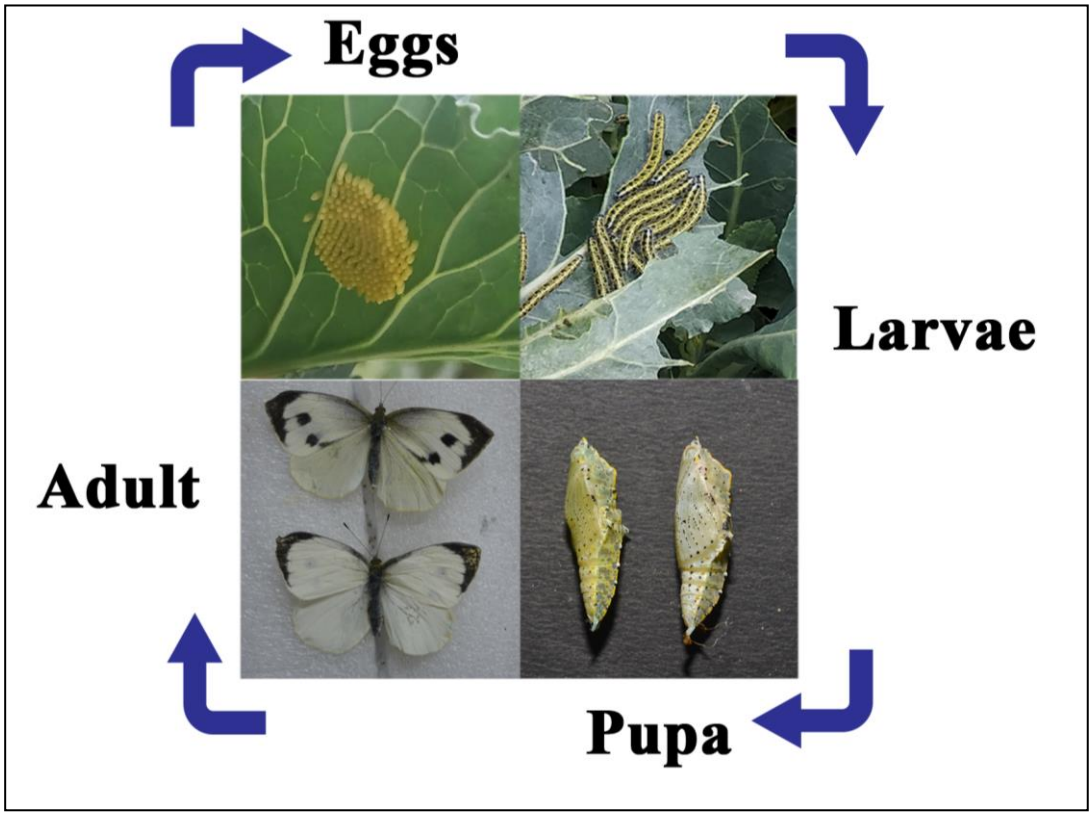

Fig. 3.3. Life cycle of the cabbage butterfly, *Pieris brassicae*.

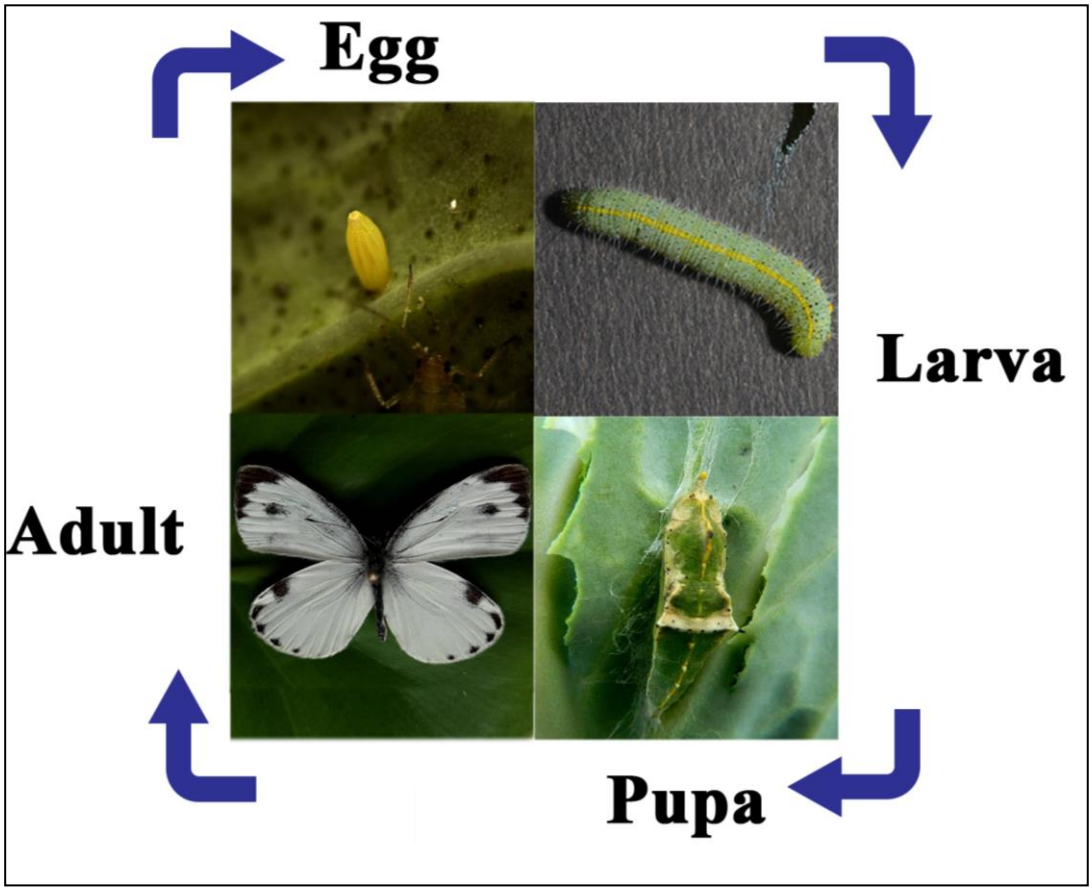

Fig.3.4. Life cycle of the Indian white butterfly, *Pieris canidia*.

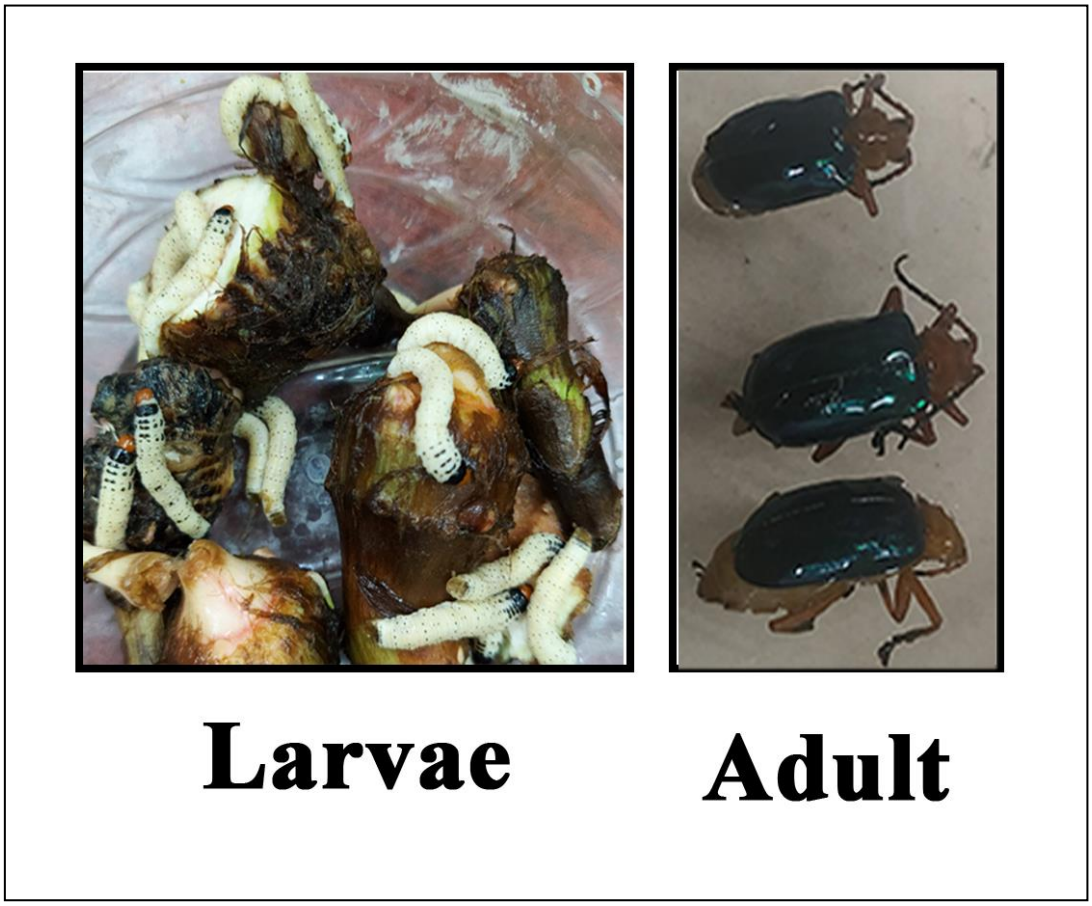

Fig. 3.5. Larvae and adults of the corm borer, *Aplosonyx chalybaeus*.

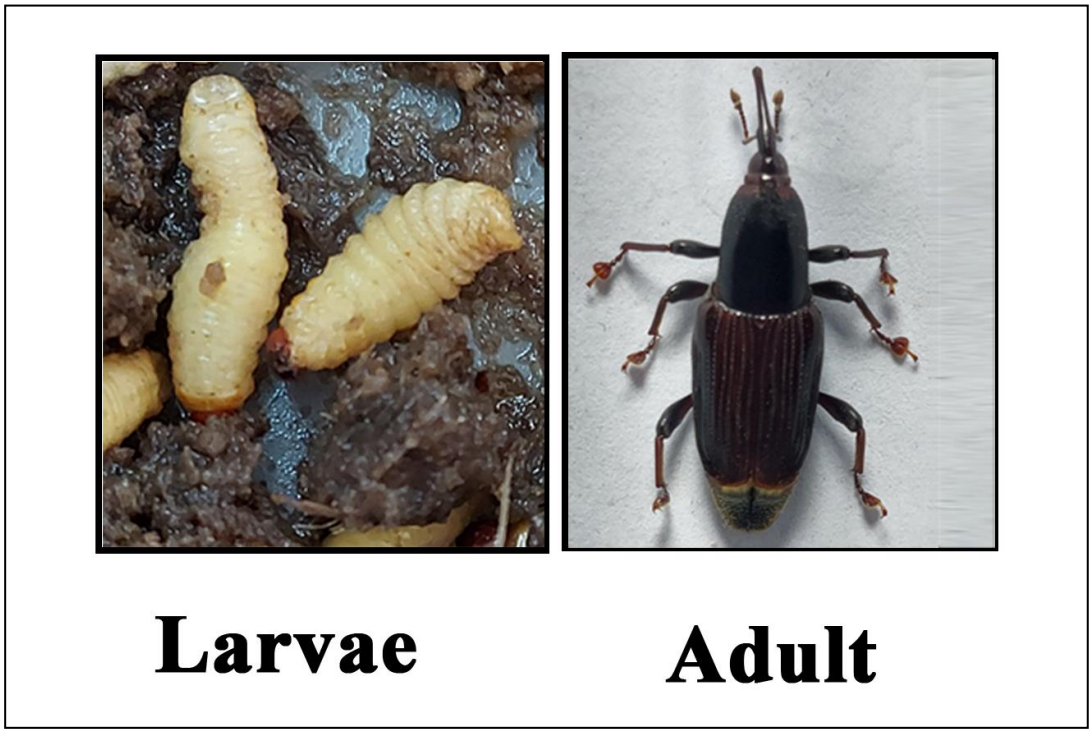

Fig.3.6. Larvae and adult of the banana pseudostem weevil, *Odoiporus longicolis.*

IJs/larva for *P. canidia* and *P. brassicae*; 10, 25, 50, 100, 200, 400, and 800 IJs/larvafor *S. litura* and *S. frugiperda*; 10, 25, 50, 100, 200, 400, 800, and 1600 IJs/larva for *A. chalybaeus* and *O. longicollis*. A petri dish (35×10 mm) was lined by double-layer Whatman filter paper No. 1. Each nematode concentration was added to a separate petri dish using 0.5 ml of distilled water and incubated for 30 min. Then, a single larva was introduced into individual petri disk pre-inoculated with nematodes.

Mortality test for pupa

Nematode concentrations of 200, 400, 800, and 1600 IJs/pupa were used to test the susceptibility of the pupa to the EPN isolates**.** The assay was performed by heating sandy loamy soil to dry for 24 hrs and the moisture content was then adjusted at 15–20% (w/w) by adding distilled water (including pipetted water in nematode suspension). For all the EPN isolates, each of the nematode concentrations was added to an individual petri dish (35 × 10 mm) filled with pre-wetted soil samples containing burrowed one-day-old pupa of insect pest.

For both the assays, eight (8) replicates for each concentration of nematodes were set and the experiment was repeated twice. A petri dish lined with filter paper and wetted only with distilled water was set as a control plate for each nematode concentration. The experiments were carried out in an incubator at 28 ± 2 °C and the mortality was checked every 24 hrs up to 120 hrs. Colour change, no movement and the smell emanating from the dead body of larvae and pupae were used for primary confirmation of death due to EPNs. After 48 hrs of mortality, the dead insects were rinsed with distilled water and individually dissected under an Olympus CX41 microscope for further confirmation.

Progeny production

To determine multiplication rate of the EPN isolates, nematode concentrations of 10, 25, 50, 100, 200, and 400 IJs/larva were used against *P. canidia* and *P. brassicae*; 10, 25, 50, 100, 200, 400, and 800 IJs/larva against *S. litura* and *S. frugiperda*; 10, 25, 50, 100, 200, 400, 800, and 1600 IJs/larva against *A. chalybaeus and O. longicolis.* Replicates and negative control plates were set and maintained in the laboratory like in the mortality assays. Mortality of the hosts were checked for the first 120 hrs. To allow the nematode to multiply and emerge from the host cadaver, the dead larva and pupa were individually placed on a modified white trap after being individually cleansed with distilled water to eliminate any attached external nematodes **(White, 1927)**. The total number of emerged IJs were collected and counted under a stereomicroscope to record the multiplication rate of the EPN isolates.

Statistical analysis

The data obtained from the assays on larval mortality and nematode multiplication were presented in the form of Mean ± SEM. Before statistical analysis, both larval and pupal mortality was corrected following **Abbott (1925)** and presented in percentage. Arcsine -transformed data were used to examine the relationship between mortality and the concentration and duration of exposure to EPNs. The values were used to calculate the lethal concentration $(LC)_{50}$ and lethal time $(LT)_{50}$ using Probit analysis (Probit model of Pearson Goodness of Fit Test at =0.05% significant level) using SPSS 20.0 version. Regression analysis was used to establish the correlation between the parameters. For each insect pests, one-way analysis of variance (ANOVA) was conducted to determine the significant differences (at the level of

$p \leq 0.05$) of host mortality against the four EPN isolates and multiplication rates among the EPN isolates. To compare the variations in host mortality and nematode multiplication assays, a two-way ANOVA (EPN and pest species, with time as blocking) was utilised.

Results

Evaluation of EPNs against major insect pests

S. litura

The EPN isolates, *H. indica*, *H. baujardi*, *S. sangi*, and *S. surkhetense* showed high efficacy against larvae and pupa of the pest, *S. frugiperda*. The mortality rates at different nematode concentrations and incubation periods varied from 6.25–100.00% in the larvae and 6.25– 68.75% in the pupal stage. In addition, the concentrations of IJs and incubation periods were positively correlated with the mortality of both larvae and pupae of *S. litura* (Table 3.1 and 3.2). For all the EPN isolates, the mortality rate showed significant differences with the life stages of the host insect ($p<0.05$) However, the insect mortality did not show significant variations among the EPN isolates ($p>0.05$).

Larval mortality

The larval mortality rates are presented in Fig. 3.1 (A–D). All the EPN isolates were capable of causing mortality of the *S. litura* larvae from the lowest nematode concentration that further increased at higher nematode concentration and incubation period.

At a concentration of 10 IJs/larva, all the isolated EPN species failed to cause larval mortality at 24 hrs post-incubation. The initial mortality was observed at 48 hrs post-incubation for *H. indica* (6.25%) while the rest of the isolates caused the first mortality (25.00%) only at 72 hrs post-incubation. For the same nematode concentration, a total mortality of 93.75% was observed after 120 hrs of incubation in case of *S. sangi*, *H. baujardi*, and *S. surkhetense* while in the case of *H. indica*, total larval mortality of 87.50% was recorded.

At a concentration of 25 IJs/larva and 24 hrs post-incubation, a total of 6.25% larval mortality was observed for *H. baujardi* which further increased to 12.50% at 48 hrs post-incubation. However, for the same nematode concentration, no larval mortality was observed until 48 hrs post-incubation in case of *H. indica* (6.25%), *S. sangi* (6.25%), and *S. surkhetense* (12.50%). After 120 hrs of incubation, a total of 93.75% and 100.00% larval mortality occurred in case of the heterorhabditids (*H. indica* and *H. baujardi*) and steinernematids (*S. sangi* and *S. surkhetense*), respectively. Further, within the total observation periods (120 hrs) for different nematode concentrations, both *S. sangi* and *S. surkhetense* caused 100.00% mortality starting from 25 IJs/larva, while *H. indica* and *H. baujardi* caused the same mortality starting from 50 IJs/larva.

At a concentration of 50 IJs/larva, initial mortality of larvae occurred after 24 hrs of incubation for *H. baujardi* (6.25%). However, for the rest of the isolates, larval mortality larval mortality started to occur at 48 hrs post-incubation. All the EPN isolates could cause 100.00% mortality after 120 hrs of incubation.

At a concentration of 100 IJs/larva, initial larval mortality occurred after 24 hrs of incubation for all the EPN isolates. The total larval mortality increased to 100.00% at 96 hrs post-incubation for *S. sangi* but the rest of the EPN isolates caused the same rate of mortality at 120 hrs post-incubation.

At a concentration of 200 IJs/larva, total mortality observed after 24 hrs of incubation was 25.00% in *S. sangi*, 18.75% in *S. surkhetense*, and 6.25% in both *H. indica* and *H. baujardi*. After 120 hrs of incubation, a total of 100.00%% larval mortality was observed at 96 hrs post-incubation for all the EPN isolates.

At a concentration of 400 IJs/larva, a total of 12.50, 31.25, 16.67 and 37.50% larval mortality was caused by *H. indica*, *S. sangi*, *H. baujardi* and, *S. surkhetense*, respectively at 24 hrs post incubation. In contrast, for the same incubation period, both *H. indica* and *H. baujardi* caused 25.00% mortality at a concentration of 800 IJs/larva while *S. sangi* and *S. surkhetense* caused 37.50% mortality. Meanwhile, for both the nematode concentrations (400 and 800 IJs/larva), a complete larval mortality was observed at 96 hrs post-incubation for all the EPN isolates.

For the control group, no mortality was observed. Table 3.3 and 3.4 displays the calculated values for LC_{50} and LT_{50}, respectively.

Pupal mortality

The pupal mortality rates of *S. litura* are given in Fig.3.2(A–D). All the EPN isolates were capable of causing pupal mortality at all the selected nematode concentrations and higher rates of mortality were observed with an increased in nematode concentration and incubation period.

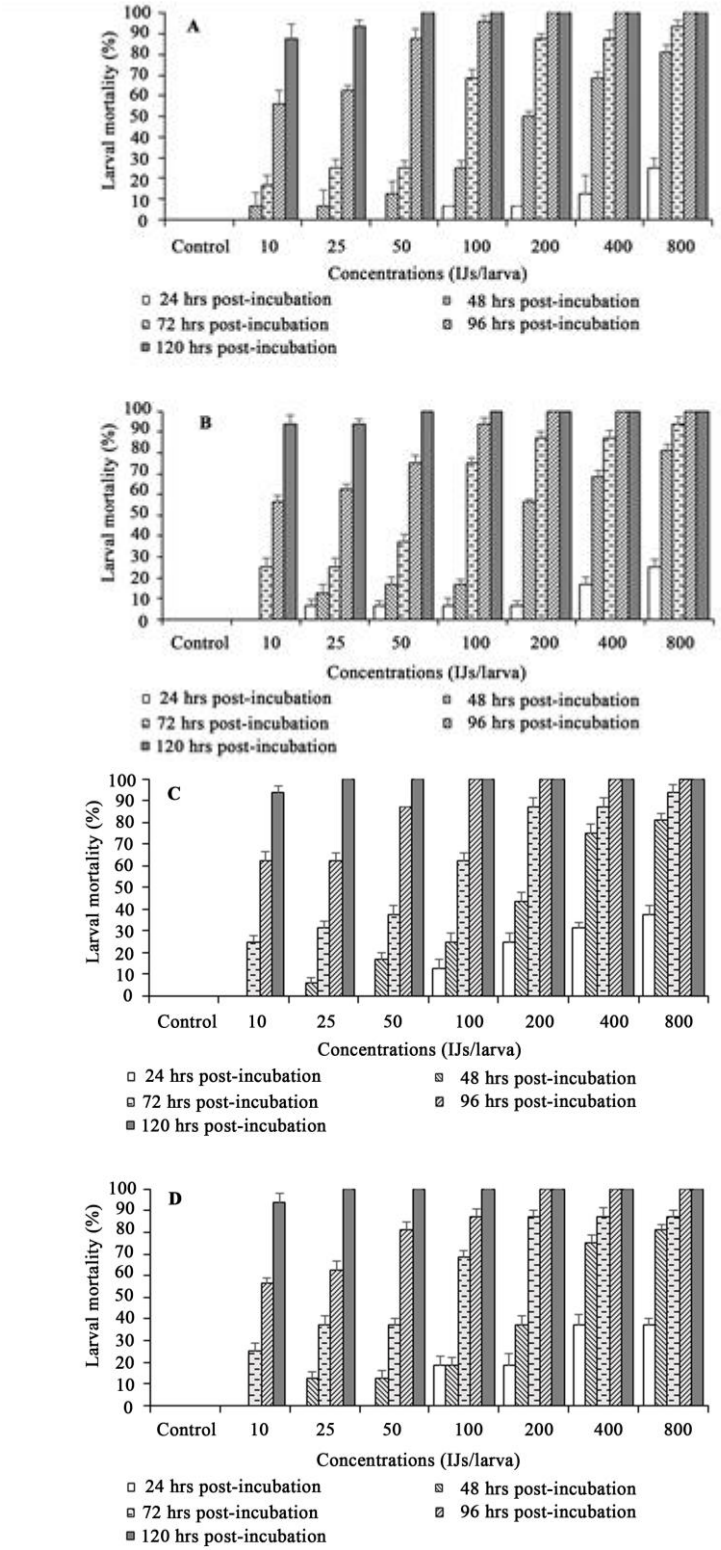

Fig. 3.7. A–D: Bio-efficacy of EPNs against larvae of *S. litura*.
Larval mortality: A. *H. indica* B. *H. baujardi*
C. *S. sangi* D. *S. surkhetense*

At a concentration of 200 IJs/pupa, a total of 12.25% pupal mortality was observed after 24 hrs of incubation with the isolates *H. indica*, *S. sangi*, and *H. baujardi*. However, the lowest mortality rate (6.25%) was observed in the case of *S. surkhetense*. After 120 hrs of incubation with the same nematode concentration, a total of 43.75% pupal mortality was observed in case of *H. indica*, while the rest of the isolates caused 37.50% mortality.

At a concentration of 400 IJs/pupa, the isolates *H. indica*, *H. baujardi*, and *S. surkhetense* caused 18.78% pupal mortality at 24 hrs post-incubation, while *S. sangi* caused 12.50% mortality. Meanwhile, for the same nematode concentration, 50.00% pupal mortality occurred at 96 hrs post-incubation in the case of *S. surkhetense* and at 120 hrs post-incubation in the case of *S. sangi* and *H. baujardi*. However, in the case of *H. indica*, only 43.75% mortality was observed at 120 hrs post-incubation for the same concentration.

At a concentration of 800 IJs/pupa, 25.00% pupal mortality was recorded after 24 hrs of incubation with *H. baujardi* and *S. surkhetense*, and 18.75% with the isolates *H. indica* and *S. sangi*. Meanwhile, for the same nematode concentration, the pupal mortality further increased to 62.50% at 120 hrs post-incubation in the case of *S. sangi* while a total of 56.35% mortality was observed for the rest of the isolates.

At a concentration of 1600 IJs/pupa, *H. baujardi* caused 37.50% mortality after 24 hrs of incubation and reached 68.75% after 120 hrs of incubation. Under the same conditions, a total of 31.25% mortality was observed in the case of *H. indica*, *S. sangi*, and *S. surkhetense* after 24 hrs of incubation that reached 68.75% (61.25% in *S. surkhetense*) after 120 hrs of incubation.

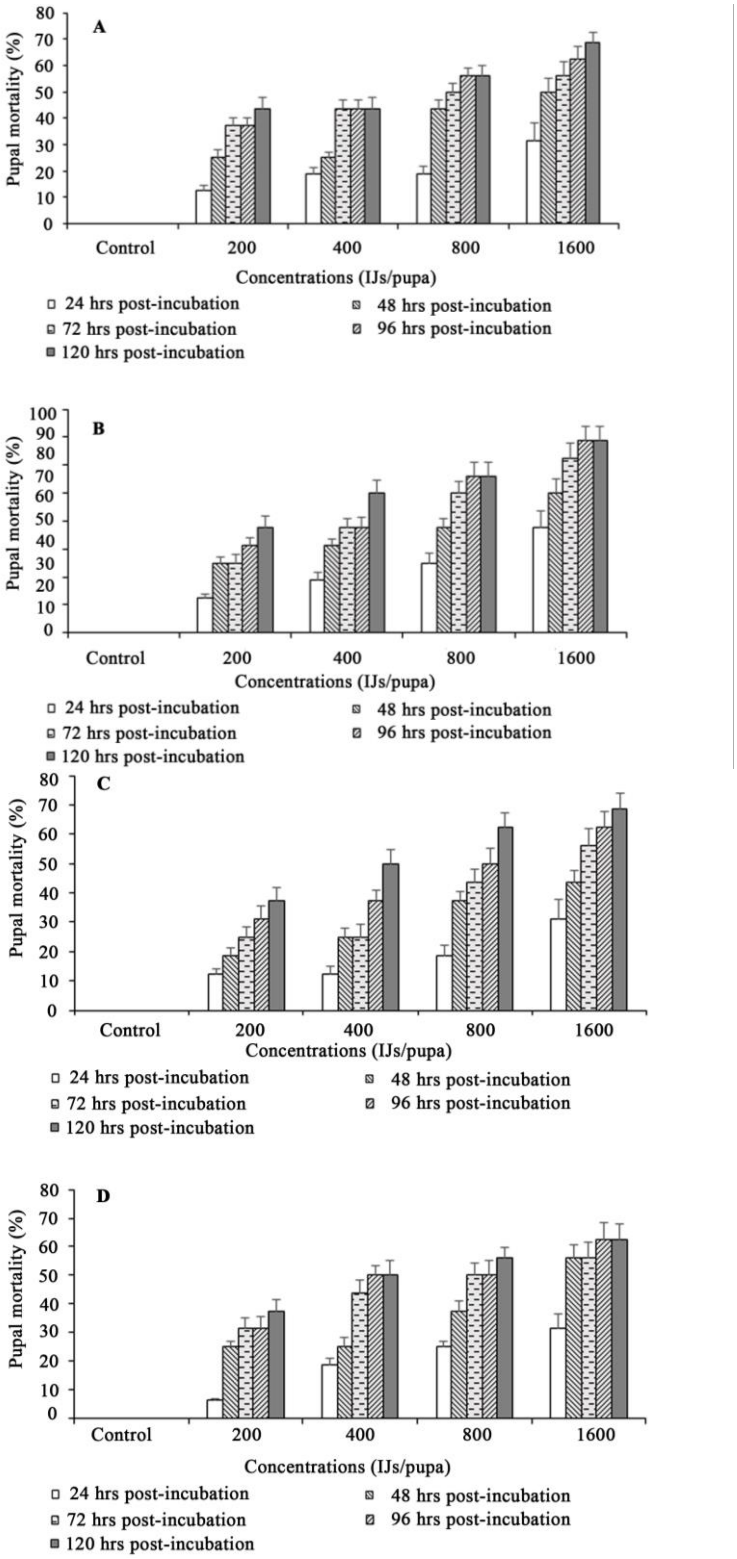

Fig. 3.8 A–D: Bio-efficacy of EPNs against pupa of *S. litura*.
Pupal mortality: A. *H. indica* B. *H. baujardi*
C. *S. sangi* D. *S. surkhetense*

Table 3.1. Correlations between nematode concentrations and larval mortality time of *S. litura.*

Concentrations IJs/larva	Time(hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	0.96**	0.97	0.96	0.97
25	0.97**	0.95	0.96	0.94
50	0.98**	0.95	0.92	0.94
100	0.97**	0.94	0.92	0.92
200	0.94*	0.93	0.91	0.91
400	0.90*	0.91	0.88	0.89
800	0.86	0.89	0.95	0.83

Time (hrs.)	Concentrations (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	0.89**	0.91**	0.88**	0.92**
48	0.91**	0.89**	0.89**	0.87**
72	0.80*	0.79*	0.79*	0.81*
96	0.59	0.61	0.63	0.62
120	0.33	0.35	0.33	0.35

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

For the control group, no mortality was observed. Table 3.5 and 3.6 displays the calculated values for LC_{50} and LT_{50}.

Table 3.2. Correlations between nematode concentrations and pupal mortality time of *S. litura.*

Concentrations IJs/pupa	Time(hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
200	0.69	0.94*	0.87	0.87
400	0.84	0.95*	0.95*	0.94*
800	0.86	0.94*	0.93*	0.94*
1600	0.95*	0.94*	0.72	0.82

Time (hrs.)	Concentrations (IJs/pupa)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	0.96*	0.95*	0.99**	0.97**
48	0.92	0.89	0.93	0.91
72	0.95*	0.94*	0.96*	0.94*
96	0.94	0.91	0.98*	0.99*
120	0.98*	0.96*	0.91	0.89

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 3.3. LC_{50} values of the EPN isolates against *S. litura* larva.

Hours post-incubation	LC_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	1003.31	1016.20	995.70	816.86
48	369.28	354.69	350.65	374.19
72	-	-	-	67.78
96	-	-	-	-
120	-	-	-	-

Table 3.4. LT_{50} values of the EPN isolates against *S. litura* larva.

Concentrations (IJs/larva)	LT_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	91.89	90.15	88.77	88.19
25	87.20	85.97	83.63	80.72
50	77.20	74.35	74.69	76.205
100	63.30	61.32	59.03	60.50
200	51.54	48.19	45.29	60.50
400	43.47	41.42	35.17	30.60
800	-	35.26	30.38	-

Table 3.5. LC_{50} values of the EPN isolates against *S. litura* pupa.

Hours post-incubation	LC_{50} (IJs/pupa)			
	H. indica		*H. indica*	
24	2791.35	24	2791.35	24
48	1474.04	48	1474.04	48
72	1000.54	72	1000.54	72
96	747.66	96	747.66	96
120	581.14	120	581.14	120

Table 3.6. LT_{50} values of the EPN isolates against *S. litura* pupa.

Hours post-incubation	LT_{50} (hrs)			
	H. indica		*H. indica*	
200	127.63	200	127.63	200
400	122.13	400	122.13	400
800	86.06	800	86.06	800
1600	61.61	1600	61.61	1600

Progeny production

The isolated EPNs successfully reproduced inside the cadaver of *S. litura*. The total number of progeny (IJs) produced per larva and pupa of *S. litura* at different nematode concentrations are given in Fig.3.9 A and B. The production showed significant differences among the EPN isolates in both the larval and pupal stages of the pest [$F_{(3, 24)} = 40.96$, $p \leq 0.05$ in larvae; $F_{(2, 13)} = 154.66$, $p = \leq 0.05$ in pupae). Furthermore, the rate of progeny production was observed to be positively correlated with nematode concentrations for all EPN isolates tested (larvae: r=0.39 in *H. indica*, r=0.50 in *H. baujardi*, r=0.67 in *S. sangi* and r=0.85 in *S. surkhetense*; pupa: r=0.96 in *H. indica*, r=0.88 in *H. baujardi*, r=0.39 in *S. sangi* and r=0.89 in *S. surkhetense*).

In the larvae of *S. litura*, *H. indica* showed the highest progeny production in the cadaver while *H. baujardi* was with the lowest production. The total progeny production increased from $39.21 \pm 6.12 \times 10^3$ at 10 IJs/larva to $59.36 \pm 6.63 \times 10^3$ at 800 IJs/larva for *H. indica*; $23.45 \pm 4.38 \times 10^3$ to $52.08 \pm 6.74 \times 10^3$ for *S. sangi*, and $19.50 \pm 6.50 \times 10^3$ to $48.30 \pm 7.99 \times 10^3$ for *S. surkhetense*. However, for *H. baujardi*, the highest progeny production in the pest cadaver was observed at 25 IJs/larvae ($1.82 \times 10^3 \pm 0.56$ IJs/larva).

In the case of pupal stage, the heterorhabditids (*H. indica* and *H. baujardi*) showed a comparatively higher rate of progeny production in comparison to the steinernematids (*S. sangi* and *S. surkhetense*). At 200 IJs/pupa, the total number of IJs produced per pupa is $54.65 \pm 10.62 \times 10^3$, $57.80 \pm 5.94 \times 10^3$, $36.38 \pm 8.02 \times 10^3$, and $33.56 \pm 6.40 \times 10^3$ IJs/pupa for *H. indica*, *S. sangi*, *H. baujardi* and *S. surkhetense*, respectively. The highest progeny production was recorded at 1600

IJs/pupa as 59.71 ± 8.34 x 10^3, 62.65 ± 9.89 x 10^3, 37.86 ± 7.51 x 10^3 and 37.02 ± 7.07 x 10^3 IJs/pupa in *H. indica*, *H. baujardi*, *S. sangi* and *S. surkhetense*, respectively.

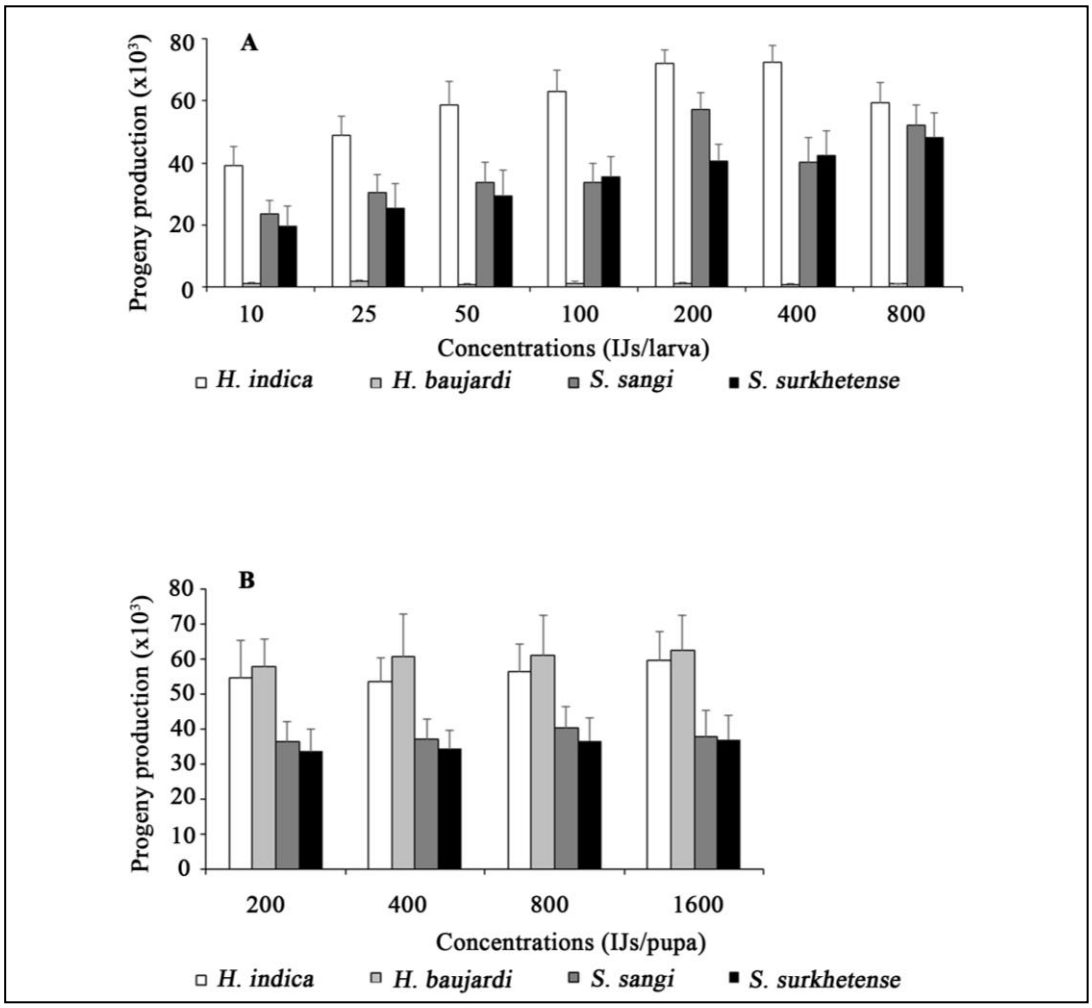

Fig. 3.9. Bio-efficacy of EPNs against *S. litura*.

Progeny production in: A. Larva B. Pupa

2. *Spodoptera frugiperda*

The EPN isolates showed high efficacy against larvae and pupa of *S. frugiperda*. The mortality rates at different nematode concentrations and incubation periods varied from 25.00–100.00% in the larvae and 37.50– 68.75% in the pupal stage. In addition, the concentration of IJs and incubation period were positively correlated with the mortality of *S. frugiperda* (Table 3.7 and 3.8). For all the EPN isolates, the mortality rate did not show significant differences with the life stages of the host insect ($p>0.05$). In addition, the insect mortality did not show significant variations among the EPN isolates ($p>0.05$).

Larval mortality

The larval mortality rates of *S. frugiperda* are presented in Fig. 3.10 (A–D). All the EPN isolates were capable of causing mortality of the *S. frugiperda* larvae from the lowest nematode concentration (10 IJs/larva), and higher rates of mortality were observed with an increased in nematode concentration and incubation period.

At a concentration of 10 IJs/larva, the heterorhabditids were responsible for 6.35% larval mortality at 24 hrs post-incubation which further increased to 25.00% in *H. indica* and 31.25% in *H. baujardi* at 120 hrs post-incubation. Meanwhile, *S. sangi* and *S. surkhetense* were able to cause 6.25% larval mortality at 24 hrs post-incubation which further increased to 25.00% at 120 hrs post-incubation.

At a concentration of 25 IJs/larva and 24 hrs post-incubation, a total of 12.50% larval mortality was caused by *H. indica* and *S. sangi*, while *H. baujardi* and *S. surkhetense* caused 6.25% larval mortality. For the same nematode concentration

at 120 hrs post-incubation, both *H. indica* and *S. sangi* caused 43.73% mortality while *H. baujardi* and *S. surkhetense* caused 37.50% mortality.

At a concentration of 50 IJs/larva, mortality of 31.25 and 75.00% respectively at 24 and 120 hrs post-incubation was observed with both *S. sangi* and *H. baujardi*. Meanwhile, at the same nematode concentration, *H. indica* and *S. surkhetense* caused 25.00% larval mortality at 24 hrs post-incubation which further increased to 68.75 and 75.00% in the case of *H. indica* and *S. surkhetense* respectively at 120 hrs post-incubation.

At a concentration of 100 IJs/larva and 24 hrs post-incubation, both *S. sangi* and *S. surkhetense* caused 43.75% mortality which further increased to 81.25% at 120 hrs post-incubation. Meanwhile, *H. indica* and *H. baujardi* caused 50.00 and 43.75% mortality at 24 hrs post-incubation which further increased to 68.75 and 75.00% at 120 hrs post-incubation respectively in the case of *H. indica* and *H. baujardi*.

At a concentration of 200 IJs/larva, the four EPN species were capable of causing 56.25% larval mortality at 24 hrs post-incubation which reached 100.00% larval mortality at 120 hrs post-inoculation.

At a concentration of 400 IJs/larva and 24 hrs post-incubation, total larval mortality of 68.75% was observed with all the EPN isolates. With the same nematode concentration, the larval mortality rate increased to 100.00% after 72 hrs of incubation with both the isolates *H. indica* and *S. sangi*. However, the same

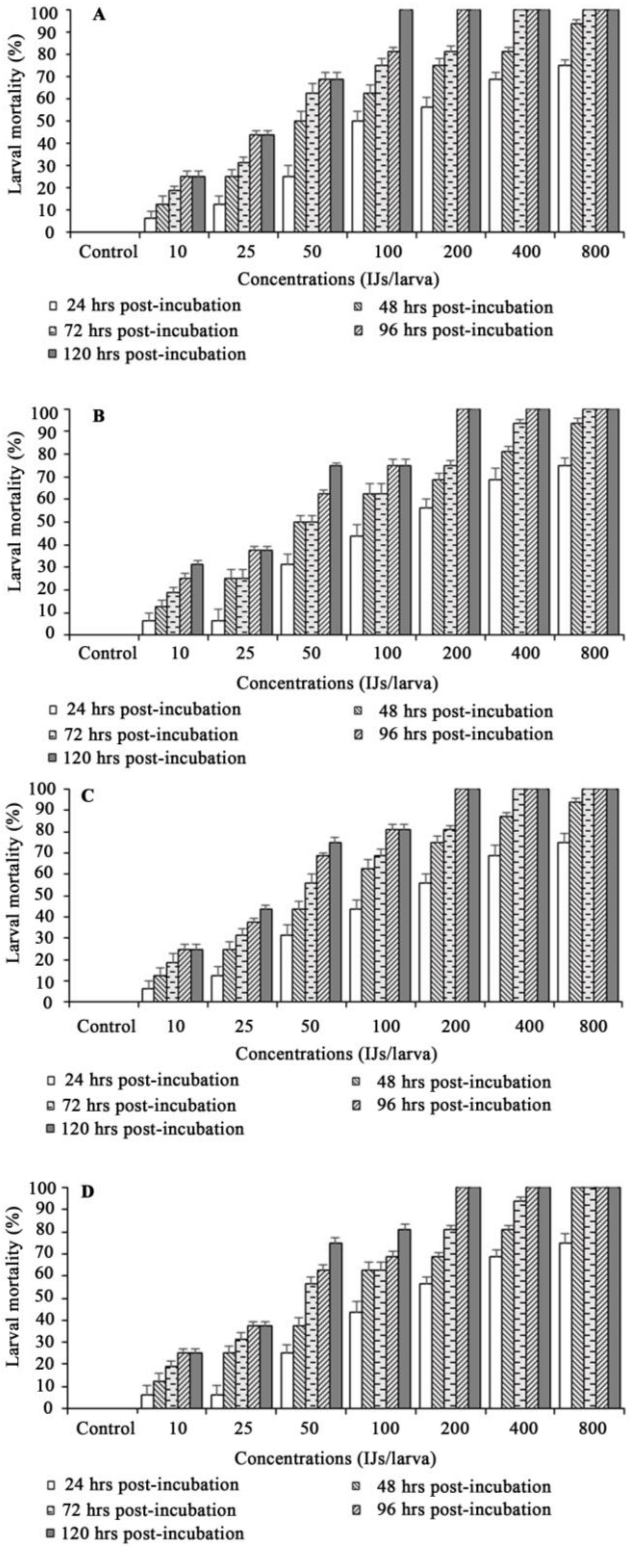

Fig. 3.10. A–D: Bio-efficacy of EPNs against larvae of *S. frugiperda*.
Larval mortality: A. *H. indica* B. *H. baujardi*
C. *S. sangi* D. *S. surkhetense*

mortality rate occurred at 96 hrs post-incubation in the case of *H. baujardi* and *S. surkhetense*.

At a concentration of 800 IJs/larva, total mortality of 75.00% was observed with all the EPN isolates at 24 hrs post-incubation. Admittedly, for the same nematode concentration, 100.00% larval mortality was observed at 48 hrs post-incubation with *S. surkhetense* and at 72 hrs post-incubation with the rest of the isolates.

There was no mortality in the control group. The calculated values for LC_{50} and LT_{50} are shown in tables 3.9 and 3.10, respectively.

Pupal mortality

The pupal mortality of *S. frugiperda* are presented in Fig. 3.11 (A–D). All the EPN isolates were capable of causing mortality of the pupa from the lowest nematode concentration and higher rates of mortality were observed with an increase in nematode concentration and incubation period.

At a concentration of 200 IJs/pupa and 24 hrs post-incubation, the highest mortality rate was observed with *H. baujardi* (12.50%) which increased to 37.50% at 120 hrs post-incubation. Meanwhile, for the same nematode concentration, the rest of the isolates caused 6.25% pupal mortality at 24 hrs post-incubation which further increased to 43.75% in *H. indica* and 37.50% in both *S. sangi* and *S. surkhetense* at 120 hrs post-incubation.

At a concentration of 400 IJs/pupa, the three EPN isolates *H. indica*, *H. baujardi*, and *S. surkhetense* caused 18.75 and 50.00% at 24 and 120 hrs post-

incubation. Meanwhile, a lower rate of pupal mortality (12.50%) was observed in the case of *S. sangi* at 24 hrs post-incubation which further reached 50.00% mortality like in the case of other isolates.

At a concentration of 800 IJs/pupa and 24 hrs post-incubation, the highest mortality rate i.e., 25.00% was observed with *H. indica* which increased to 56.25% at 120 hrs post-incubation. Meanwhile, for the same nematode concentration, the rest of the isolates caused 18.75% pupal mortality at 24 hrs post-incubation which further increased to 62.50% in *H. baujardi* and 56.35% in both *S. sangi* and *S. surkhetense* at 120 hrs post-incubation.

At a concentration of 1600 IJs/pupa, the heterorhabditids *H. indica* and *H. baujardi* caused 37.50% mortality at 24 hrs post-incubation and increased to 68.75% at 120 hrs post-incubation. Under the same conditions, a total mortality of 31.25% was observed at 24 hrs post-incubation in the case of *S. sangi* and *S. surkhetense* which reached 68.75% in *S. sangi* and 61.25% in *S. surkhetense* at 120 hrs post-incubation. There was no mortality in the control group. The calculated values for LC_{50} and LT_{50} are shown in tables 3.11 and 3.12, respectively.

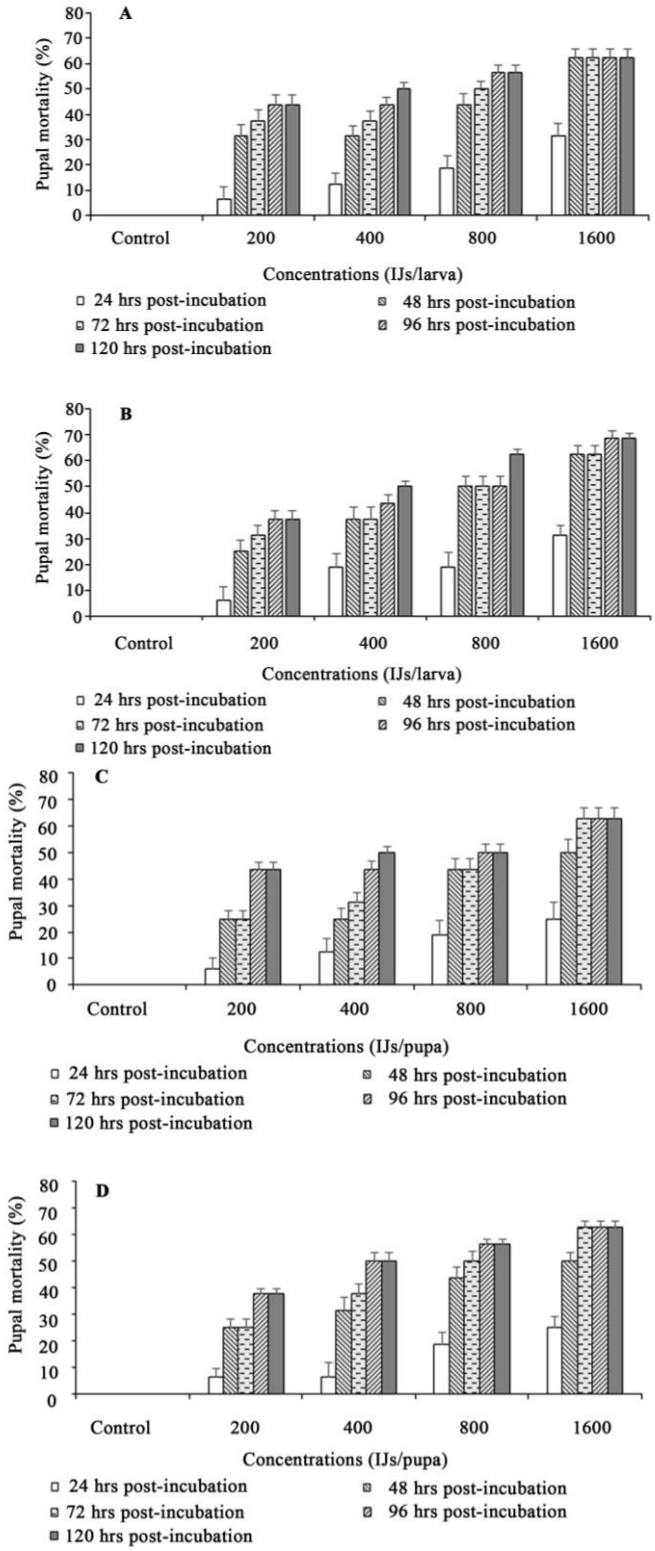

Fig. 3.11. A–D: Bio-efficacy of EPNs against pupae of *S. frugiperda*.

Pupal mortality: A. *H. indica* B. *H. baujardi*

C. *S. sangi* D. *S. surkhetense*

Table 3.7. Correlations between nematode concentrations and larval mortality time of *S. frugiperda.*

Concentrations IJs/larva	Time (hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	0.95*	0.94*	0.97**	0.95*
25	0.95*	0.89*	0.95*	0.95*
50	0.89*	0.87*	0.87*	0.89*
100	0.99**	0.97**	0.91*	0.99**
200	0.95*	0.96*	0.89*	0.95*
400	0.87*	0.88*	0.87*	0.87*
800	0.82	0.89*	0.87	0.79

Time (hrs)	Concentrations (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	0.83*	0.87*	0.93*	0.88*
48	0.83*	0.91*	0.88*	0.83*
72	0.84*	0.83*	0.91*	0.84*
96	0.72	0.77	0.81	0.69
120	0.69	0.71	0.66	0.75

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 3.8. Correlations between nematode concentrations and pupal mortality time of *S. frugiperda.*

Concentrations IJs/pupa	Time (hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
200	0.85	0.95*	0.89*	0.89*
400	0.97**	0.91*	0.99*	0.98*
800	0.90*	0.86	0.88*	0.88*
1600	0.83	0.90*	0.91*	0.91*

Time (hrs)	Concentrations (IJs/pupa)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	0.91	0.96*	0.97*	0.88
48	0.98*	0.84	0.92	0.93
72	0.98*	0.98**	0.99**	0.94
96	0.98*	0.92	0.94	0.88
120	0.98*	0.90	0.94	0.81

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 3.9. LC_{50} values of the EPN isolates against *S. frugiperda* larva.

Hours post-incubation	LC_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	321.88	330.94	322.67	339.26
48	127.76	136.40	128.74	137.52
72	62.07	96.44	70.07	80.50
96	37.29	45.04	40.00	47.78
120	32.40	34.51	34.85	37.61

Table 3.10. LT_{50} values of the EPN isolates against *S. frugiperda* larva.

Concentrations (IJs/larva)	LT_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	163.40	163.40	185.91	185.91
25	123.66	138.12	130.80	135.43
50	55.83	62.78	61.03	69.56
100	21.74	28.98	28.90	32.39
200	21.14	24.36	21.14	23.81
400	13.19	8.62	12.85	8.62
800	8.304	8.304	8.30	-

Table 3.11. LC_{50} values of the EPN isolates against *S. frugiperda* pupa.

Hours post-incubation	LC_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	2139.538	2224.204	2297.901	2440.526
48	1083.38	1155.905	1474.040	1464.70
72	913.341	963.477	1106.720	1111.753
96	581.138	604.356	747.630	646.824
120	474.106	516.3879	590.003	646.824

Table 3.12. LT_{50} values of the EPN isolates against *S. frugiperda* pupa.

Hours post-incubation	LT_{50} (hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
200	121.230	138.767	138.120	135.426
400	114.726	114.521	114.690	108.260
800	84.241	75.303	89.427	86.060
1600	37.291	43.991	59.972	63.995

Progeny production

The isolated EPNs successfully reproduced inside the cadaver of *S. frugiperda*. The total number of progeny (IJs) produced per larva and pupa of *S. frugiperda* at different nematode concentrations are given in Fig.3.12 A and B. The progeny production showed significant differences among the EPN isolates in both the larval and pupal stages of *S. frugiperda* [Larvae: $F_{(3, 20)} = 39.74$, $p=\leq 0.05$; pupae: $F_{(3,12)} =$

24.46, p=≤0.05]. Furthermore, the total number of IJs produced per larvae of *S. frugiperda* showed a positive correlation with nematode concentrations for all the EPN isolates (r=0.84 in *H. indica*, r=0.91 in *H. baujardi*, r=0.85 in *S. sangi* and r=0.82 in *S. surkhetense*). However, the study recorded a negative correlation between IJs production in pupa and nematode concentrations for all EPN isolates tested. Among the 4 EPN species, the two heterorhabditids, *H. indica* and *H. baujardi* showed comparatively higher rates of progeny production in the pest cadaver.

In the larval stage, the total progeny production increased from 4.40 x 1.31 at 10 IJs/larva to 9.80 x 0.92 x 10^3 at 800 IJs/larva for *H. indica*; 3.95 x 1.21 x 10^3 to 9.62 ± 0.95 x 10^3 for *H. baujardi*; 1.96 ± 0.73 x 10^3 to 6.08 ± 1.13 x 10^3 for *S. sangi*, and 3.16 ± 1.01 x 10^3 to 6.04 ± 0.75 x 10^3 for *S. surkhetense*. In the pupal stage, a comparatively high multiplication rate was recorded in comparison to the larval stage. Among the studied EPN species, the highest progeny production was recorded for *H. baujardi* as 78.00 ± 9.70 x 10^3, 79.07 ± 18.38 x 10^3, 84.81± 14.53 x 10^3, 100.92 ± 19.02 x 10^3 IJs at a concentration of 200, 400, 800 and 1600 IJs/pupa, respectively. In the case of *H. indica*, a total of 79.15 ± 15.42 x 10^3, 79.85 ± 7.02 x 10^3, 80.12 ± 7.59 x 10^3, and 90.76 ± 11.71 x 10^3 IJs were produced at a concentration of 200, 400, 800 and 1600 IJs/pupa, respectively. The total number of progenies produced by *S. surkhetense* was 41.13 ± 7.32 x 10^3, 49.34 ± 5.04 x10^3, 53.92 ± 5.40 x10^3, and 70.15 ± 7.04 x10^3 IJs at a concentration of 200, 400, 800 and 1600 IJs/pupa, respectively. The lowest progeny production was observed in the case of *S*

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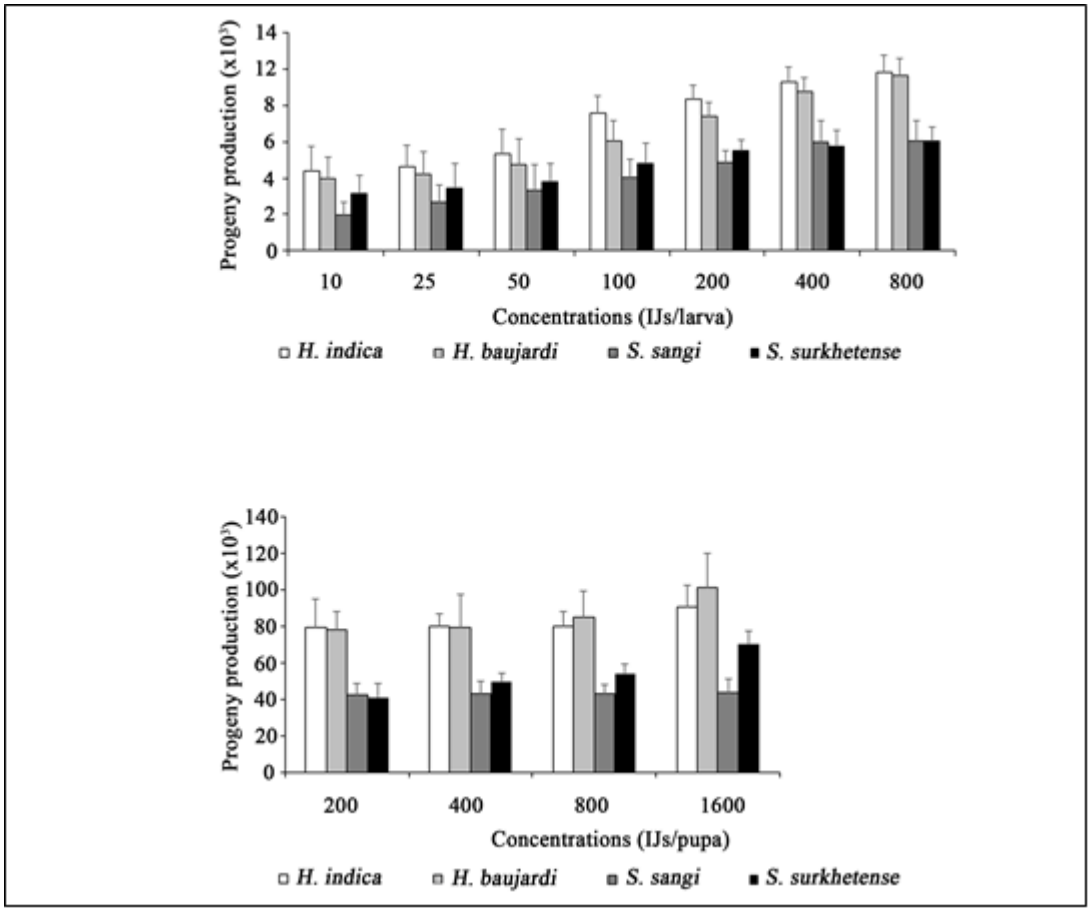

Fig. 3.12. Bio-efficacy of EPNs against *S. frugiperda*.

Progeny production in: A. Larva B. Pupa

sangi as 42.75 ± 5.69 x10^3, 42.91 ± 6.90 x 10^3, 43.07 ± 4.98 x 10^3 and 43.48 ± 8.03 x 10^3 at a concentration of 200, 400, 800 and 1600 IJs/pupa, respectively.

3. *Pieris brassicae*

The study showed that all the EPN isolates are highly pathogenic against the pest, *P. brassicae* with successful infection and production of progenies. The mortality rates at different nematode concentrations and incubation periods varied from 31.25–

100.00% in the larvae and 25.00– 100.00% in the pupal stage. In addition, the concentrations of IJs and incubation periods showed a positive correlation with larval and pupal mortality of the pest (Table 3.13 and 3.14). Despite the fact that the isolated nematodes displayed different levels of pathogenicity, the four EPN species did not have a statistically significant effect on the larval mortality rate ($p > 0.05$). Furthermore, the rate of larval and pupal mortality of *P. brassicae* did not show significant variations for all the EPN isolates ($p > 0.05$).

Larval mortality

The larval mortality rates of *P. brassicae* are presented in Fig. 3.13 (A–D). All the EPN isolates were capable of causing mortality of the larvae from the lowest nematode concentration and a higher rate of mortality was observed with an increased in nematode concentration and incubation period.

At a concentration of 10 IJs/larva, mortality was first recorded at 24 hrs post-incubation and the highest mortality occurred in the case of *S. surkhetense* (93.75%) and reached 75.00% at 120 hrs post-incubation. Meanwhile, at 24 hrs post-incubation, a total of 35.50% mortality occurred for the rest of the EPN isolates and reached 75.00% in *H. indica* and 62.50% in both *S. sangi* and *H. baujardi*. At a concentration of 25 IJs/larva, the total rate of mortality was 43.75% in *H. indica*, *H. baujardi*, and *S. surkhetense* at 24 hrs post-incubation. However, for the same nematode concertation, a complete larval mortality was observed in all the EPN isolates.

At a concentration of 50 IJs/larva, a total mortality of 50.00% occurred at 24 hrs post-incubation in *H. indica*, while the rest of the isolates caused 43.75% larval

deaths. At 120 hrs post-incubation, larval mortality of 93.75, 81.25, 75.00, and 87.50% was observed in the case of *H. indica*, *S. sangi*, *H. baujardi*, and *S. surkhetense*, respectively.

At a concentration 100 IJs/larva, the isolates, *H. indica*, *H. baujardi* and *S. surkhetense* caused 100.00% mortality after 96 hrs of incubation, but S. *sangi* caused 93.75% larval mortality under the same condition. At the same concentration, 100.00% larval mortality was observed at 96 hrs post-incubation in *H. baujardi* and at 72 hrs post-incubation for the rest of the isolates.

At a concentration of 400 IJs/larva, the total mortality was 87.50% in the case of the steinernematids (*S. sangi* and *S. surkhetense*) at 24 hrs post-incubation. With the same nematode concentration and incubation period, the heterorhbditids (*H. indica* and *H. baujardi*) caused 81.25% larval mortality. With the same nematode concentration, the steinernematids caused a complete mortality at 72 while the isolate *H. indica* and *H. baujardi* caused the same mortality at 72 and 96 hrs post-incubation, respectively.

In the control group, there was no mortality. Tables 3.15 and 3.16, respectively, display the estimated values for LC_{50} and LT_{50}.

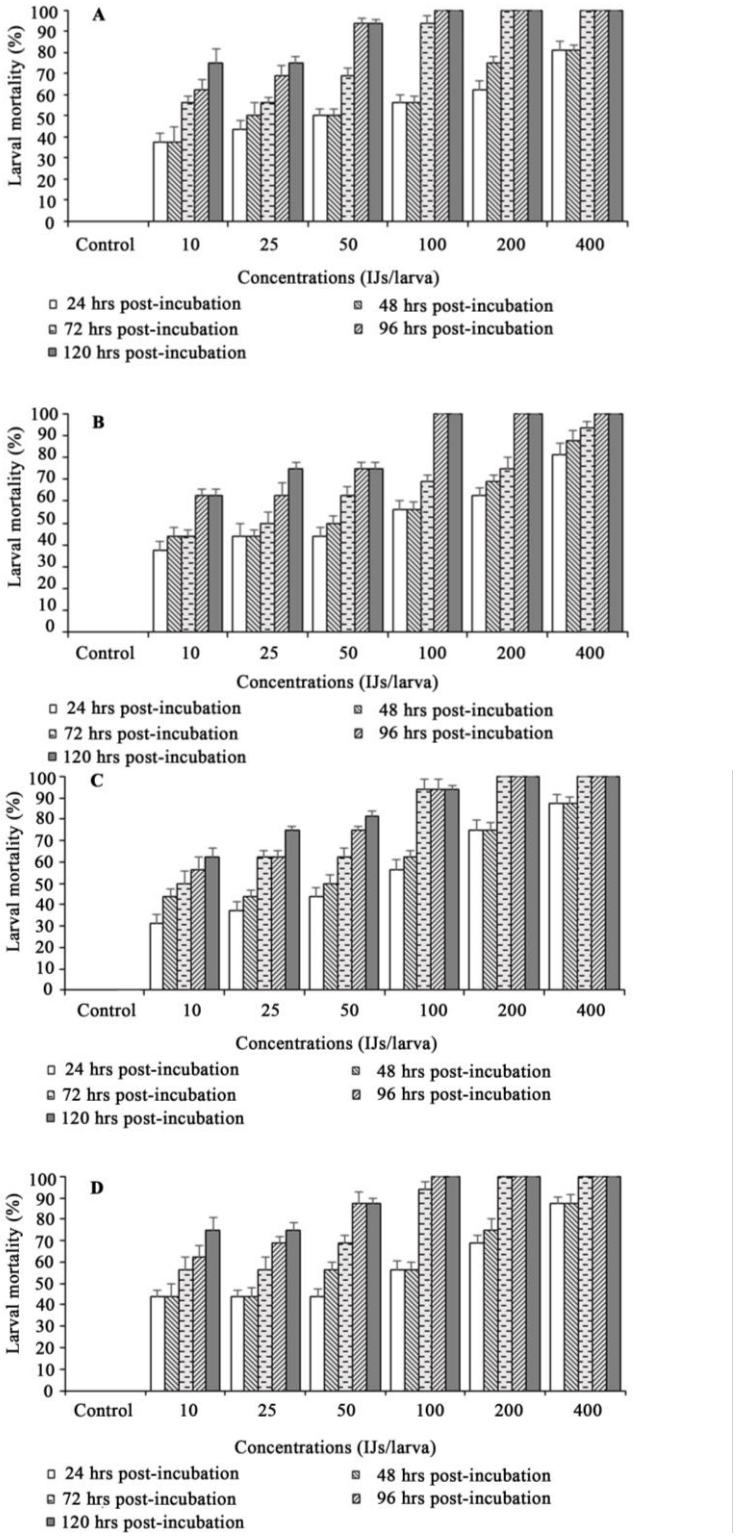

Fig.3.13. A–D: Bio-efficacy of EPNs against *P. brassicae*.
Larval mortality: A. *H. indica* B. *H. baujardi*
C. *S. sangi* D. *S. surkhetense*

Pupal mortality

The pupal mortality rates of *S. litura* are given in Fig.3.14 (A–D). All the EPN isolates were capable of causing pupal mortality at all the nematode concentrations. Higher rates of mortality were observed with an increase in nematode concentration and incubation period.

At a concentration of 200 IJs/pupa, the highest mortality rate at 24 hrs post-incubation was observed with *S. surkhetense* (18.73%) which increased to 68.75% at 96 hrs post-incubation. In contrast, for the same nematode concentration, a total mortality of 12.50% was observed in the case of *H. indica*, *S. sangi* and *H. baujardi* at 24 hrs post-incubation.

At a concentration of 400 IJs/pupa, the highest mortality at 24 hrs of incubation was observed with *S. sangi* (31.25%). With the same nematode concentration, a total of 81.25% mortality was observed at 96 hrs post-incubation in *S. sangi* while the rest of the isolates caused 75.00% mortality at the same incubation period. However, the pupal mortality did not further increase after 96 hrs of incubation for all the EPN isolates.

At a concentration of 800 IJs/pupa, the initial 100.00% pupal mortality occurred after 96 hrs of incubation in *S. sangi*, *H. baujardi*, and *S. surkhetense*. However, for the same nematode concentration and incubation period, a total of 93.75% mortality was observed in the case of *S. surkhetense*.

At a concentration of 1600 IJs/pupa, a total of 62.50% mortality occurred at 24 hrs post-incubation for the isolates, *S. sangi*, *H. baujardi*, and *S. surkhetense*

while *H. indica* caused 56.25 % mortality. With the same nematode concentration, a complete mortality was observed at 96 hrs post-incubation for all the EPN isolates. In the control group, there was no mortality. Tables 3.17 and 3.18, respectively, display the estimated values for LC_{50} and LT_{50}.

Progeny production

The total number of IJs produced per larva and pupa of *P. brassicae* at different nematodes concentrations are given in Fig 3.9 A and B. In the larval stage, a positive correlation was found between IJs production and concentrations of nematodes for all EPN isolates (r=0.90 in *H. indica*, r=0.72 in *H. baujardi*, r=0.36 in *S. sangi* and r=0.91 in *S. surkhetense*). However, the rate of progeny production in the pupa showed negative correlation with nematode concentrations for all EPN isolates. Meanwhile, the progeny production showed significant variations among the EPN isolates in the larval stage [$F_{(3, 20)} = 39.74$, $p \leq 0.05$]. In contrast, the rate of multiplication in the pupal cadaver did not show significant variations among the four EPN species ($p > 0.05$).

In the larval stage, *H. indica* and *S. surkhetense* showed comparatively higher rate of progeny production in the pest cadaver. The total number of progenies produced per larva increased from $7.21 \pm 2.16 \times 10^3$ at 10 IJs/larva to $10.17 \pm 2.20 \times 10^3$ at 400 IJs/larva for *H. indica*; $6.21 \pm 3.06 \times 10^3$ to $9.97 \pm 2.30 \times 10^3$ for *S. surkhetense*; $4.29 \pm 0.51 \times 10^3$ to $5.03 \pm 0.70 \times 10^3$ for *S. sangi*, and $2.03 \pm 0.42 \times 10^3$ to $3.26 \pm 0.60 \times 10^3$ IJs/larva for *H. baujardi*.

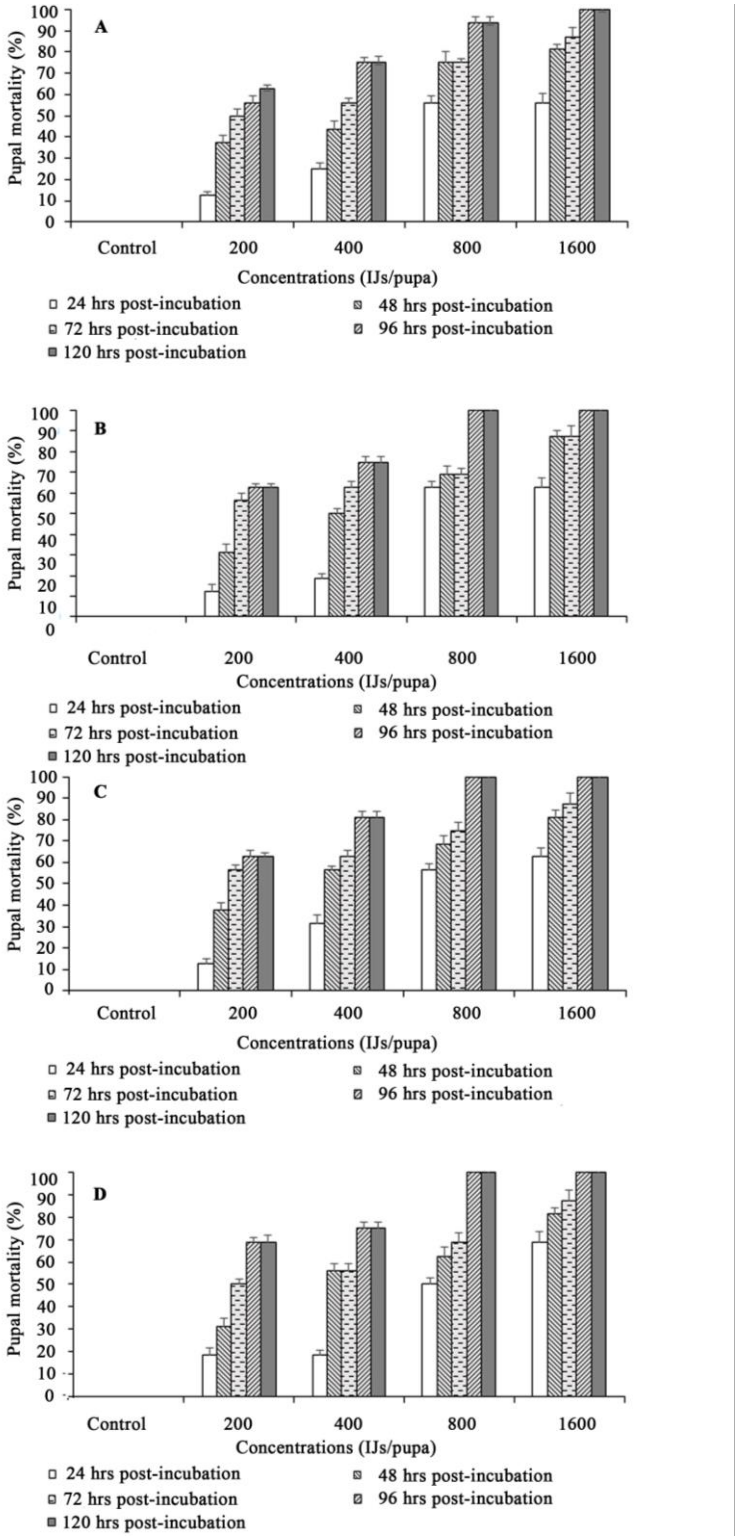

Fig.3.14. A–D: Bio-efficacy of EPNs against *P. brassicae*.
Pupal mortality: A. *H. indica* B. *H. baujardi*
C. *S. sangi* D. *S. surkhetense*

Table 3.13. Correlations between nematode concentrations and larval mortality time of *P. brassicae*

Time (hrs)	Concentrations (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	0.97**	0.98**	0.95**	0.98**
48	0.93**	0.99**	0.96**	0.89*
72	0.80	0.95**	0.79	0.69
96	0.65	0.75	0.79	0.78
120	0.67	0.74	0.79	0.66

Concentrations IJs/larva	Time (hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	0.97**	0.93*	0.99**	0.97**
25	0.99**	0.95*	0.97**	0.97**
50	0.95*	0.97**	0.99**	0.97**
100	0.90*	0.93*	0.89*	0.90*
200	0.89*	0.95*	0.86	0.89*
400	0.87	0.97**	0.86	0.86
800	0.97**	0.93*	0.99**	0.97**

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 3.14. Correlations between nematode concentrations and pupal mortality time of *P. brassicae*

Concentrations IJs/pupa	Time(hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
200	0.92*	0.94*	0.97*	0.98*
400	0.96**	0.92*	0.97**	0.91*
800	0.94*	0.89	0.93*	0.94*
1600	0.92*	0.93*	0.91*	0.93*

Time (hrs.)	Concentrations (IJs/pupa)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	0.84	0.86	0.86	0.90
48	0.90	0.91	0.90	0.98*
72	0.96*	0.94*	0.98*	0.97*
96	0.87	0.83	0.81	0.76
120	0.90	78.00	0.81	0.76

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 3.15. LC_{50} values of the EPN isolates against *P. brassicae* larva

Hours post-incubation	LC_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	77.94	88.99	88.85	67.84
48	51.15	57.92	43.97	42.10
72	9.49	11.37	12.84	9.49
96	3.28	3.84	2.01	1.18
120	-	-	-	-

Table 3.16. LT_{50} values of the EPN isolates against *P. brassicae* larva

Concentrations (IJs/larva)	LT_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	62.70	71.98	76.10	53.31
25	46.09	56.95	57.06	51.19
50	35.31	41.24	43.29	35.45
100	27.77	30.73	16.66	27.77
200	19.25	19.07	8.07	14.35
400	-	-	-	-

Table 3.17. LC_{50} values of the EPN isolates against *P. brassicae* pupa.

Hours post-incubation	LC_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	1170.31	1056.290	1040.91	1043.53
48	450.66	485.740	363.38	487.57
72	165.32	168.430	-	204.55
96	124.74	138.48	130.05	-
120	57.65	138.48	130.05	84.473

Table 3.18. LT_{50} values of the EPN isolates against *P. brassicae* pupa.

Hours post-incubation	LT_{50} (hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
200	85.224	81.913	79.867	76.803
400	62.563	60.874	47.718	60.362
800	18.762	19.710	24.360	32.221
1600	11.700	10.234	13.142	6.095

In the pupal stage, a comparatively higher multiplication rate was recorded in comparison to the larval stage. Among the four EPN species, the highest progeny production was recorded for *H. baujardi* as 74.49 ± 11.00 x 10^3, 76.82 ± 9.78 ± x 10^3, 100.95 ± 9.78 x 10^3, and 118.73 ± 13.24 x 10^3 IJs/pupa at a concentration of 200, 400, 800 and 1600 IJs/pupa, respectively. Meanwhile, the production of IJs increased from 71.50 ± 7.23 x 10^3 at 10 IJs/ pupa to 102.53 ± 13.43 x 10^3 at 1600

IJs/l pupa for *H. indica*; 64.59 ± 13.16 x 10^3 IJs/pupa at 10 IJs/pupa to 101.13 ± 10.73 x 10^3 IJs/pupa for *S. surkhetense*; 55.03 ± 6.99 x 10^3 IJs/pupa at 10 IJs/pupa to 94.62 ± 11.44 x 10^3 IJs/pupa for *S. sangi*.

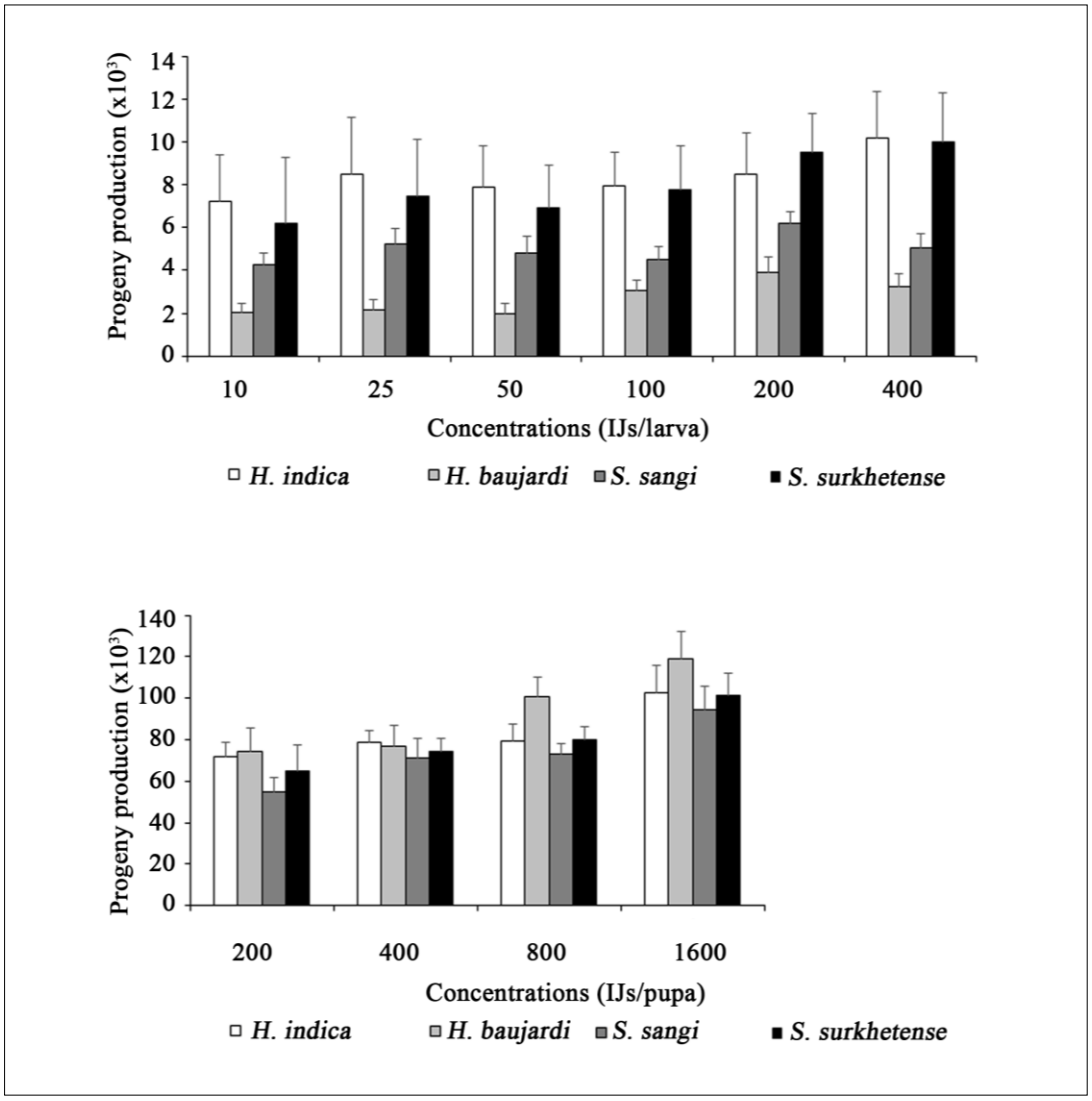

Fig.3.15. Bio-efficacy of EPNs against.*P. brassicae*.

Progeny production in: A. Larva B. Pupa

4. *P. canidia*

According to the study, the isolated EPN species are extremely pathogenic to *P. canidia*, with successfully infection and multiplication at different nematode concentrations. The mortality rates at different nematode concentrations and incubation periods varied from 18.75–100.00% in the larvae and 12.50–100.00% in the pupal stage. In addition, the concentrations of IJs and incubation periods were positively correlated with the mortality of *P. canidia* (Table 3.19 and 3.20). For all the EPN isolates, the mortality rate did not show significant differences with the life stages of the host insect ($p>0.05$). In addition, the insect mortality did not show significant variations among the EPN isolates ($p>0.05$).

Larval mortality

The mortality rates at different concentrations of nematodes and incubation periods are given in Fig 3.16 (A–D). From the lowest nematode concentration, all EPN isolates were able to kill the larvae, and higher rates of mortality were seen with increasing nematode concentration and incubation time.

At a concentration of 10 IJs/larva, 31.25% larval mortality was observed at 24 hrs post-incubation in the case of *S. surkhetense* which reached 50.00% at 72 hrs post-incubation. Meanwhile, both *H. indica* and *H. baujardi* caused 25.00% mortality at 24 hrs post-incubation, while *S. sangi* caused 18.75% mortality for the same incubation time. The same nematode concentration resulted in 50.00% mortality in *H. indica* at 72 hrs post-incubation, 96 hrs post-incubation in *S. sangi*, and 43.75% mortality in *H. baujardi* at 72 hrs post-incubation.

At a concentration of 25 IJs/larva, both *H. indica* and *S. surkhetense* caused a total of 37.50% mortality at 24 hrs post-incubation while *H. baujardi* and *S. sangi* caused 25.00 and 31.25% mortality respectively for the same incubation period. *S. surkhetense* induced 31.25% larval mortality after 120 hrs of incubation, compared to 62.50% for the other isolates during the same incubation period.

At 24 hrs post-incubation, the total larval mortality rates were 56.25% at 50 and 100IJs/larva for *S. surkhetense*; 50.00% at 50 IJs/larva and 56.25% IJs/larva at 100 for *H. indica* and *H. baujardi*; 43.75at 50 IJs/larva and 56.26 % at 100 IJs/larva for *S. sangi*. However, after 120 hrs of post-incubation, both the isolates *H. baujardi* and *S. surkhetense* caused 68.75 and 93.75% mortality at a concentration of 50 and 100 IJs/larva, respectively. In case of *H. indica*, the overall mortality was 75.00% at 50 IJs/larva and 87.50% at 100 IJs/larva at 120 hrs post-incubation. For the same incubation period, *S. sangi* caused a total of 75.00 and 93.75% larval mortality at a concentration of 50 and 100 IJs/larva, respectively.

At a concentration of 200 IJs/larva, *S. surkhetense* and *H. baujardi* caused a complete larval mortality at 72 and 120 hours, respectively. Meanwhile, both the isolate *H. indica* and *S. sangi* caused the same mortality at 96 hrs post-incubation. At a concentration of 400 IJs/larva, a complete larval mortality was recorded with all of the EPN isolates after 96 hrs of incubation.

For the control group, no mortality was observed. Table 3.21 and 3.22 displays the calculated values for LC_{50} and LT_{50}, respectively.

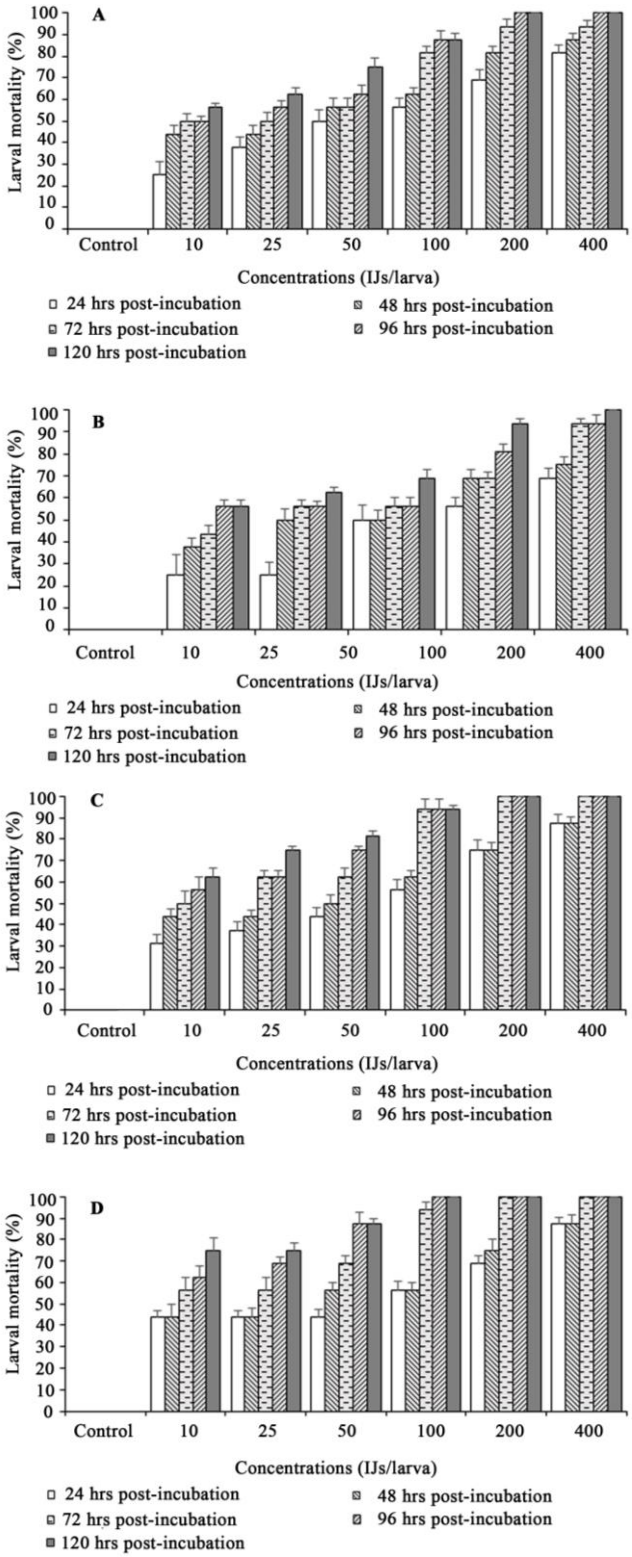

Fig.3.16. A–D: Bio-efficacy of EPNs against pupal of *P. canidia*.
Larval mortality: A. *H. indica* B. *H. baujardi*
C. *S. sangi* D. *S. surkhetense*

Pupal mortality

The results of the pupal mortality assay are presented in Fig.3.17 (A–D). All the EPN isolates were able to kill the pupa at the lowest nematode concentration, and increased rates of mortality were seen with increasing nematode concentration and incubation duration.

At a concentration of 200 IJs/pupa, both *S. sangi* and *S. surkhetense* caused 25.00 and 62.50% pupal mortality at 24 hrs and 96 hrs post-incubation, respectively. With the same concentration, *H. baujardi* caused a pupal mortality of 12.50 and 68.75% at 24 and 96 hrs after incubation, whereas *H. indica* caused a mortality of 18.75 and 62.50% at the same time points.

At a concentration of 400 IJs/pupa, 31.25% of the pupae were dead after 24 hrs of incubation with *H. baujardi* and *S. sangi*, but *H. indica* and *S. surkhetense* both resulted in 25.00% mortality under the same conditions. With the same nematode concentration, all the EPN isolated caused complete pupal mortality at 96 hrs post-incubation.

At a concentration of 800 IJs/larva, *H. baujardi* was the only isolate that could cause 100.00% mortality at 96 hrs-post incubation. In contrast, the rest of the isolates caused 93.75% pupal mortality for the same nematode concentration and incubation period. At a concentration of 1600 IJs/larva, the highest pupal mortality after 24 hrs of incubation was observed with *S. surkhetense* (68.75%). Furthermore, 120 hours after incubation, all of the EPN isolates resulted in 100.00% pupal mortality for the same nematode concentration. For the control group, no mortality

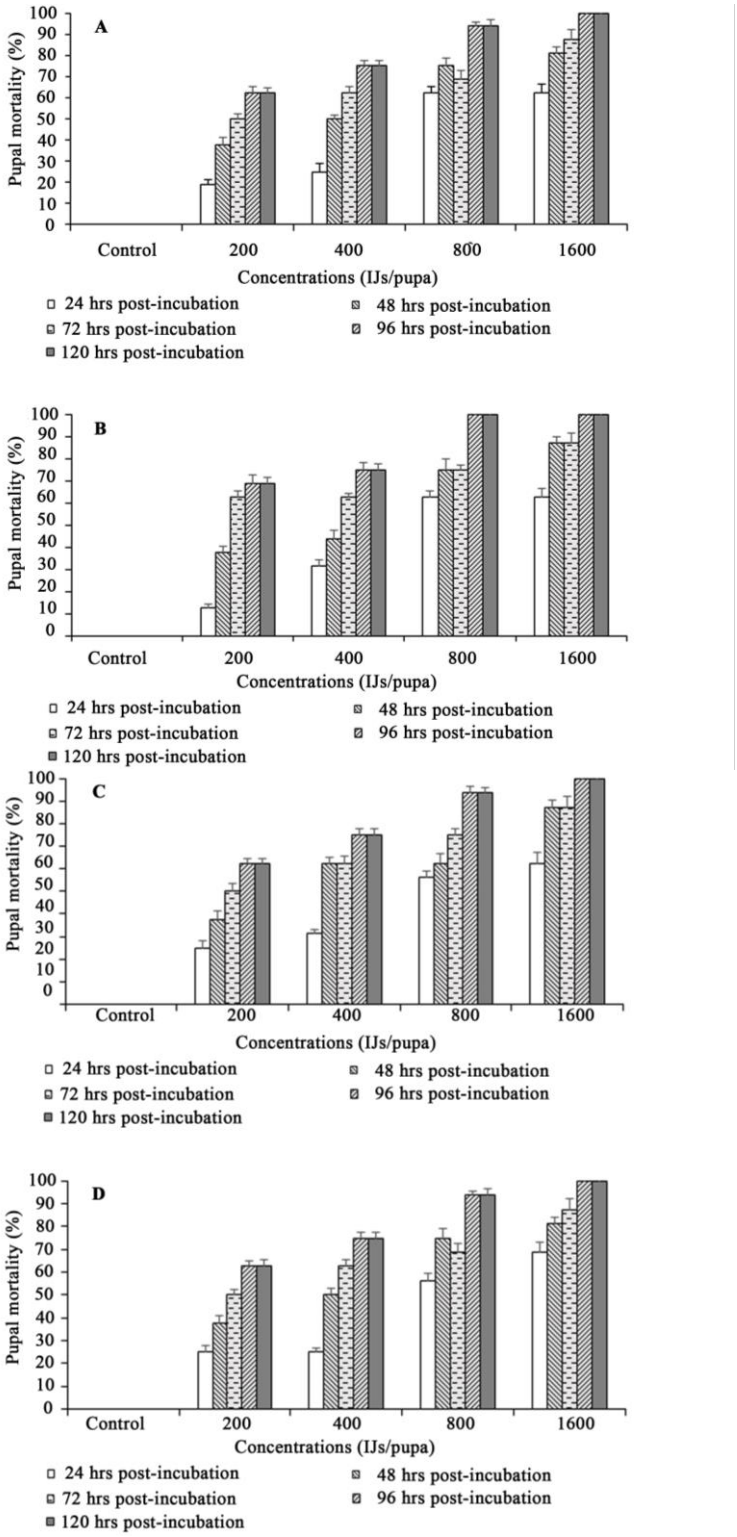

Fig.3.17. A–D: Bio-efficacy of EPNs against pupal of *P. canidia*.

Pupal mortality: A. *H. indica* B. *H. baujardi*

C. *S. sangi* D. *S. surkhetense*

was observed. Table 3.23 and 3.24 displays the calculated values for LC_{50} and LT_{50}, respectively.

Table 3.19. Correlations between nematode concentrations and larval mortality time of *P. canidia*

Concentrations IJs/larva	Time (hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	0.97**	0.93*	0.99**	0.97**
25	0.99**	0.95*	0.97**	0.97**
50	0.95*	0.97**	0.99**	0.97**
100	0.90*	0.93*	0.89*	0.90*
200	0.89*	0.95*	0.86	0.89*
400	0.87	0.97**	0.86	0.86
800	0.97**	0.93*	0.99**	0.97**

Time (hrs.)	Concentrations (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	0.97**	0.98**	0.95**	0.98**
48	0.93**	0.99**	0.96**	0.89*
72	0.80	0.95**	0.79	0.69
96	0.65	0.75	0.79	0.78
120	0.67	0.74	0.79	0.66

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 3.20. Correlations between nematode concentrations and pupal mortality time of *P. canidia*

Concentrations IJs/pupa	Time (hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
200	0.94*	0.95*	0.93*	0.96*
400	0.93*	0.94*	0.93*	0.91*
800	0.89*	0.91*	0.92*	0.90*
1600	0.95*	0.93*	0.96*	0.90*

Time (hrs.)	Concentrations (IJs/pupa)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	0.85	0.90	0.88	0.91
48	0.89	0.88	0.89	0.85
72	0.97*	0.91	0.95	0.98*
96	0.90	0.96*	0.92	0.97*
120	0.90	0.89	0.92	0.91

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 3.21. LC_{50} values of the EPN isolates against *P. canidia* larva

Hours post-incubation	LC_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	101.78	118.06	127.02	108.64
48	21.89	23.37	34.02	29.13
72	12.75	17.55	28.65	18.94
96	7.68	12.75	4.08	6.23
120	3.00	7.27	4.08	-

Table 3.22. LT_{50} values of the EPN isolates against *P. canidia* larva

Concentrations (IJs/larva)	LT_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	89.61	92.17	96.10	88.11
25	67.18	76.58	77.45	78.07
50	30.01	37.86	38.56	40.12
100	16.16	7.49	-	17.78
200	-	3.15	-	6.66
400	-	-	-	-

Table 3.23. LC_{50} values of the EPN isolates against *P. canidia* pupa

Hours post-incubation	LC_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	998.329	991.131	983.087	936.109
48	432.445	442.658	349.526	387.8
72	118.519	-	79.428	118.519
96	57.647	84.473	57.647	57.647
120	57.65	84.473	57.647	57.65

Table 3.24. LT_{50} values of the EPN isolates against *P. canidia* pupa.

Hours post-incubation	LT_{50} (hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
200	80.461	72.492	78.283	78.283
400	57.277	56.447	44.97	57.277
800	13.142	15.674	20.944	13.300
1600	9.76	10.234	10.234	6.100

Progeny production

The total number of progenies produced per larva and pupa of *P. canidia* at different concentrations of nematodes are given in Fig.3.18 A and B. Among the EPN isolates, *H. baujardi* showed the highest multiplication rate both in the larval and pupal stages of *P. canidia*. A positive correlation was found between production of IJs and concentrations of nematodes (larvae: r=0.51 in *H. indica*, r=0.42 in *H.*

baujardi, r=0.56 in *S. sangi* and r=0.70 in *S. surkhetense*; pupa: r=0.87 in *H. indica*, r=0.91 in *H. baujardi*, r=0.87 in *S. sangi* and r=0.98 in *S. surkhetense*). The progeny production showed non-significant variations between the EPN isolates in the pupal stage ($p>0.05$) but significant differences between the EPN isolates in the larval stage [$F_{(3, 23)} = 4.26$, $p \leq 0.05$].

At a concentration of 10 IJs/larva, a total number of $51.64 \pm 3.96 \times 10^3$, $87.81 \pm 4.23 \times 10^3$, $25.58 \pm 3.35 \times 10^3$, and $25.55 \pm 3.13 \times 10^3$ IJs/larva were produced in *H. indica*, *H. baujardi*, and *S. sangi*, and *S. surkhetense*, respectively. Meanwhile, at 400 IJs/larva, a total of $70.34 \pm 12.30 \times 10^3$, $145.60 \pm 22.00 \times 10^3$, $39.68 \pm 22.50 \times 10^3$ and $71.94 \pm 23.00 \times 10^3$ IJs/larva were produced in *H. indica*, *H. baujardi*, *S. sangi*, and *S. surkhetense*, respectively. The results, therefore, revealed that the nematode multiplication rate increased from the lowest to the highest concentration of nematodes. In the pupal stage, the total number of IJs produced at different nematode concentrations increased from $50.30 \pm 5.67 \times 10^3$ at 10 IJs/ pupa to $81.75 \pm 7.87 \times 10^3$ at 1600 IJs/l pupa for *H. indica*; $54.65 \pm 8.83 \times 10^3$ at 10 IJs/ pupa to $86.75 \pm 8.92 \times 10^3$ at 1600 IJs/pupa for *H. baujardi*; $51.86 \pm 6.69 \times 10^3$ IJs/pupa at 10 IJs/pupa to $72.42 \pm 10.07 \times 10^3$ IJs/pupa for *S. surkhetense*; $49.58 \pm 8.10 \times 10^3$ IJs/pupa at 10 IJs/pupa to $65.36 \pm 10.40 \times 10^3$ IJs/pupa.

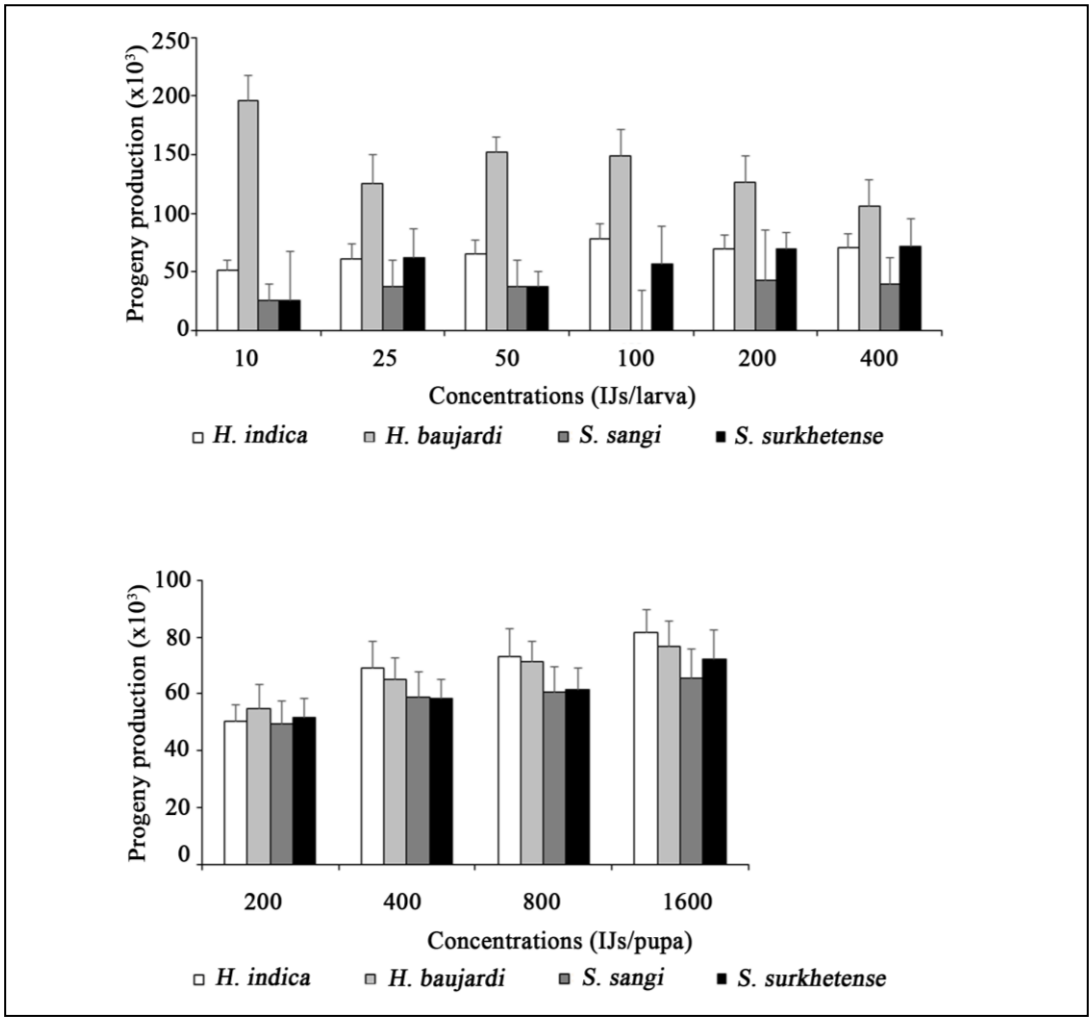

Fig.3.18. Bio-efficacy of EPNs against. *P. canidia*.

III. Progeny production in: A. Larva B. Pupa

5. *A. chalybaeus*

Larva mortality

The bio-efficacy of the isolated EPN species was evaluated against the larvae of *A. chalybaeus*. The mortality rates of the larvae at different nematode concentrations and incubation periods varied between 6.25–100.00% as given in Fig. 3.19 (A-D).

According to the study, the isolated EPN species showed high efficacy against the pest with successful infection and multiplication. However, during the observation period, none of the EPN isolates were able to cause larval mortality at the lowest nematode concentration. In addition, the concentrations of IJs and incubation periods showed positive correlation with the larval mortality of *A. chalybaeus* (Table 3.25). The larval mortality rate, however, did not differ significantly ($p>0.05$) among the EPN isolates.

At a concentration of 25 IJs/larva, mortality first occurred at 24 hrs post-incubation for all the EPN species with highest mortality rate in the case of *H. baujardi* (12.50%). After 48 hrs of incubation, the insect mortality increased to 25.00% in the case of *H. indica* and *S. sangi* while a total of 18.75% larval mortality was caused by *H. baujardi* and *S. surkhetense*. After 120 hrs of post-incubation, the same nematode concentration resulted in a total of 31.25, 43.75, and 25.00% larval mortality against *H. baujardi*, *S. sangi*, and *S. surkhetense*, respectively. In contrast, in the case of *H. indica*, there was no further increase in the larval mortality rate after 48 hrs of incubation.

Both the heterorhabditids, at a concentration of 50 and 100 IJs/larva caused a total of 81.25% to 87.24% larval mortality, respectively at 120 hrs post-incubation. However, at a concentration of 50 IJs/larva, larval mortality of 68.75 and 62.50% was observed against *S. sangi* and *S. surkhetense* respectively at 120 hrs post-incubation. Meanwhile, at a concentration of 100 IJs/larva with the same incubation period, both the steinernematids could result in 81.25% larval mortality.

At 200 IJs/larva, the total larval mortality rate increased from 31.25 to 81.25% at 24 and 72 hrs post-incubation, respectively against the isolates *H. indica*, *S. sangi*, and *H. baujardi*. However, in the case of *S. surkhetense*, a total of 25.00 and 75.00% larval mortality occurred at 24 and 72 hrs post-incubation, respectively.

At a concentration of 400 IJs/larva, the isolates, *H. indica*, *S. sangi*, and *H. baujardi* could all cause 87.25% insect mortality at 120 hrs post-incubation, whereas *S. surkhetense* caused a lesser mortality of 81.25% under the same circumstances.

At a concentration of 800 IJs/larva, the maximum mortality rate was 93.75%, and it happened 96 hrs after incubation in *H. baujardi* and 120 hrs after incubation in *H. indica* and *S. sangi*, respectively. However, at 120 hrs after incubation, the maximum larval mortality rate against *S. surketense* was 87.25%.

At a concentration of 1600 IJs/larva and 24 hrs post-incubation, the total larval mortality observed against for both *S. sangi* and *H. baujardi* was 68.75%, and 62.50% for both *H. indica* and *S. surkhetense*. With the same nematode concentration, larval mortality at 120 hrs post-incubation was 100.00% in the case of *S. sangi* and *H. baujardi*. However, in *H. indica* and *S. surkhetense*, respectively, the maximum larval mortality rates under the same conditions were 87.25 and 93.75%.

No mortality was observed for the control group. Table 3.26 and 3.27 displays the calculated values for LC_{50} and LT_{50}, respectively.

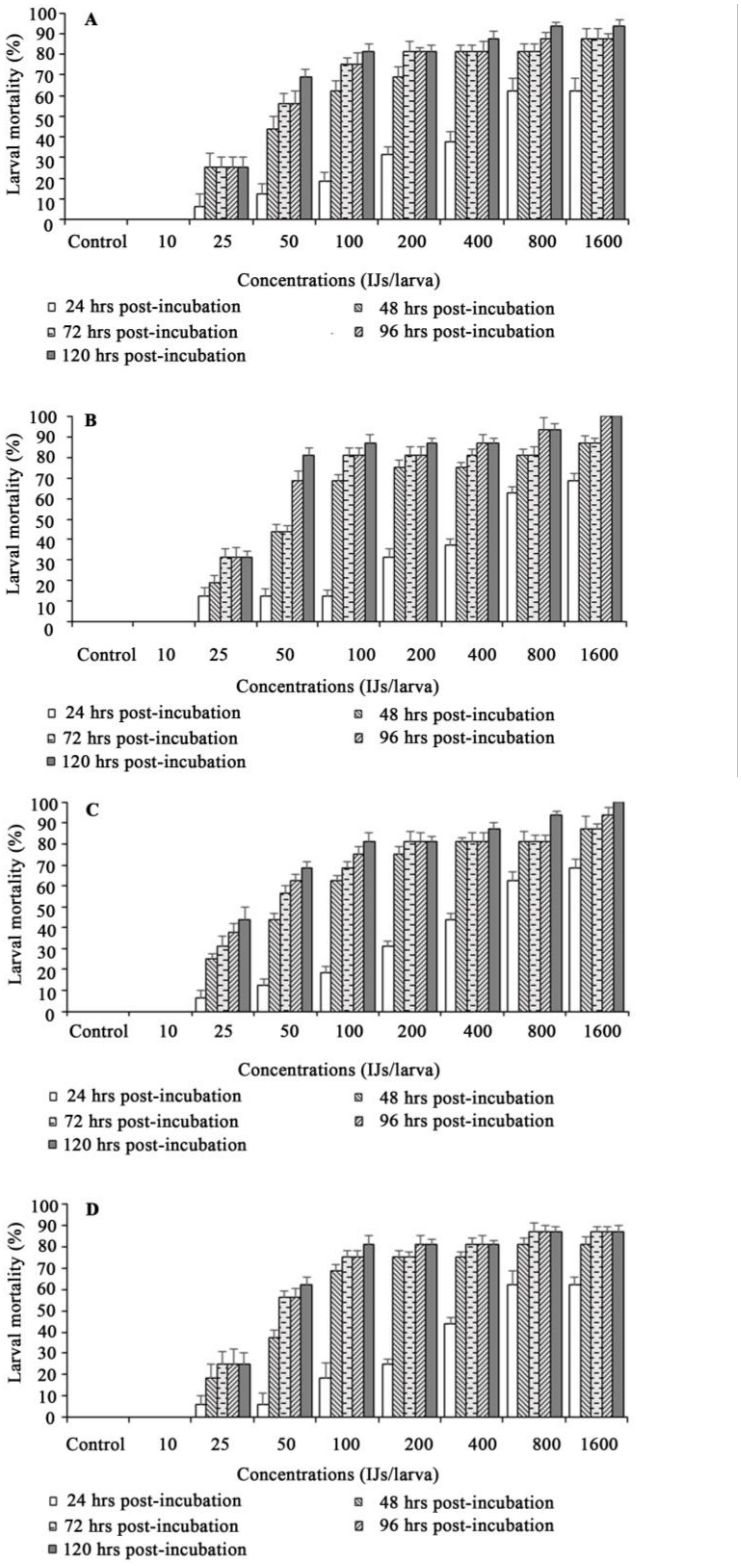

Fig.3.19. A–D: Bio-efficacy of EPNs against larvae of *A. chalybaeus*. Larval mortality A. *H. indica* B. *H. baujardi*

C. *S. sangi* D. *S. surkhetense*

Table 3.25. Correlations between nematode concentrations and larval mortality time of *A. chalybaeus.*

Concentrations IJs/larva	Time(hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	-	-	-	-
25	0.70	0.89*	0.96**	0.84
50	0.92*	0.96**	0.93*	0.90
100	0.85	0.83	0.87	0.81
200	0.82	0.82	0.77	0.78
400	0.77	0.84	0.78	0.78
800	0.92*	0.92*	0.88*	0.81
1600	0.81	0.92*	0.93*	0.81

Time (hrs.)	Concentrations (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	0.87	0.89*	0.88*	0.86
48	0.67	0.81	0.71	0.77
72	0.56	0.66	0.76	0.75
96	0.57	0.71	0.81	0.81
120	0.57	0.56	0.66	0.8

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 3.26. LC_{50} values of the EPN isolates against *A. chalybaeus* larva.

Hours post-incubation	LC_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	965.836	899.380	876.431	965.272
48	216.639	215.793	197.638	248.880
72	85.247	85.856	83.372	98.453
96	77.458	66.125	60.204	77.458
120	25.570	25.732	16.250	26.633

Table3.27. LT_{50} values of the EPN isolates against *A. chalybaeus* larva

Concentrations (IJs/larva)	LT_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	-	-	-	-
25	216.22	173.93	124.71	198.10
50	77.29	72.12	74.76	84.26
100	49.92	47.71	52.06	46.78
200	33.14	31.24	28.64	37.30
400	19.91	25.32	10.86	10.67
800	-	-	-	-
1600	-	-	-	-

Progeny production

All the EPN isolates were able to successfully proliferate within the larval cadaver of *A. chalybaeus*. The total numbers of IJs produced per larva at different concentrations of nematodes are shown in Fig. 3.21 (A). Among the EPN isolates, the highest progeny production was observed in *H. indica* while *S. surkhetense* had the lowest. A positive correlation was found between IJs production and concentrations of nematodes (r=0.75 in *H. indica*, r=0.74 in *H. baujardi*, r=0.81 in *S. sangi* and r=0.95 in *S. surkhetense*).

The nematode multiplication rate showed significant variations among the EPN isolates [$F_{(2, 28)} = 3.03$, $p \leq 0.05$]. The total progeny production increased from 25.00 ± 5.05 x 10^3 at 10 IJs/larva to 61.00 ± 7.59 x 10^3 at 1600 IJs/larva for *H. indica*; 23.00 ± 4.53 x 10^3 to 59.00 ± 6.87 x 10^3 IJs/larva for *H. baujardi*; 21.00 ± 3.63 x 10^3 to 60.25 ± 5.53 x 10^3 IJs/larva for *S. sangi*; 13.00 ± 4.09 x 10^3 to 49.00 ± 7.11 x 10^3 for *S. surkhetense* IJs/larva.

6. *Odoiporus longicolis*

Larval mortality

The bioefficacy of the isolated EPN species was evaluated against the larvae of *O. longicollis*. According to the study, mortality rates of the larvae ranged from 6.25 to 100.00% at various nematode concentrations and incubation times (3.20. A-D). The study shows that the isolated EPN species are highly pathogenic against the pest with successful infection and multiplication. Further, the nematode concentrations and incubation periods showed positive correlation with the larval mortality rates (Table

3.28). However, no significant differences in mortality rates were observed between the examined EPN species ($p>0.05$).

At a concentration of 10 IJs/larva, *H. indica* was responsible for causing the highest host mortality (12.50%) at 24 hrs post-incubation which increased to 25.50% mortality at 120 hrs post-incubation. Meanwhile, a total mortality of 6.25% was observed with the rest of isolates at 24 hrs post-incubation. After 120 hrs of incubation, a total mortality of 31.25, 18.75, and 25.00% was recorded with the isolates *S. sangi*, *H. baujardi*, and *S. surkhetense*, respectively.

At a concentration of 25 IJs/larva, all the EPN isolates caused an equal rate of mortality i.e., 12.50% at 24 hrs post-incubation. With the same nematode concentration, the total rate of larval mortality over 120 hrs of incubation was 50.00%, 43.75, 31.25, and 37.50% with *H. indica*, *S. sangi*, *H. baujardi*, and *S. surkhetense*, respectively.

At a concentration of 50 IJs/larva, the highest larval mortality was recorded in the case of the isolate *S. sangi*. At a concentration of 100 IJs/larva, the total rate of larval mortality over 120 hrs of incubation was 68.75 and 75.00% with *H. indica* and *S. sangi*, respectively. However, a total mortality of 62.50% was recorded in the case of *H. baujardi* and *S. surkhetense*.

At a concentration of 200 IJs/larva, both the isolates *H. baujardi* and *S. surkhetense* 75.00% mortality while *S. sangi* and *H. indica* caused 81.25 and 68.75% mortality respectively at 120 hrs post-incubation. Furthermore, at 400 IJs/larva and 120 hrs post-incubation, the highest larval mortality was recorded against the isolates

H. indica and *S. sangi* as 87.59%. However, a total of 81.25 and 75.00% host mortality was observed in the case of *H. baujardi* and *S. surkhetense* respectively with the same nematode concentration and incubation period.

At a concentration of 800 IJs/larva, *S. sangi* was the only isolate that could cause 100.00% larval mortality after 120 hrs of post-incubation. However, a total host mortality of 93.75% was recorded with *H. indica* while both the isolate, *H. baujardi* and *S. surkhetense* caused 87.50% mortality. Furthermore, at a concentration of 1600 IJs/larva, a total mortality of 100.00% was observed after 120 hrs of incubation of the larvae with all the EPN isolates.

No mortality was observed for the control group. Table 3.29 and 3.30 displays the calculated values for LC_{50} and LT_{50}, respectively.

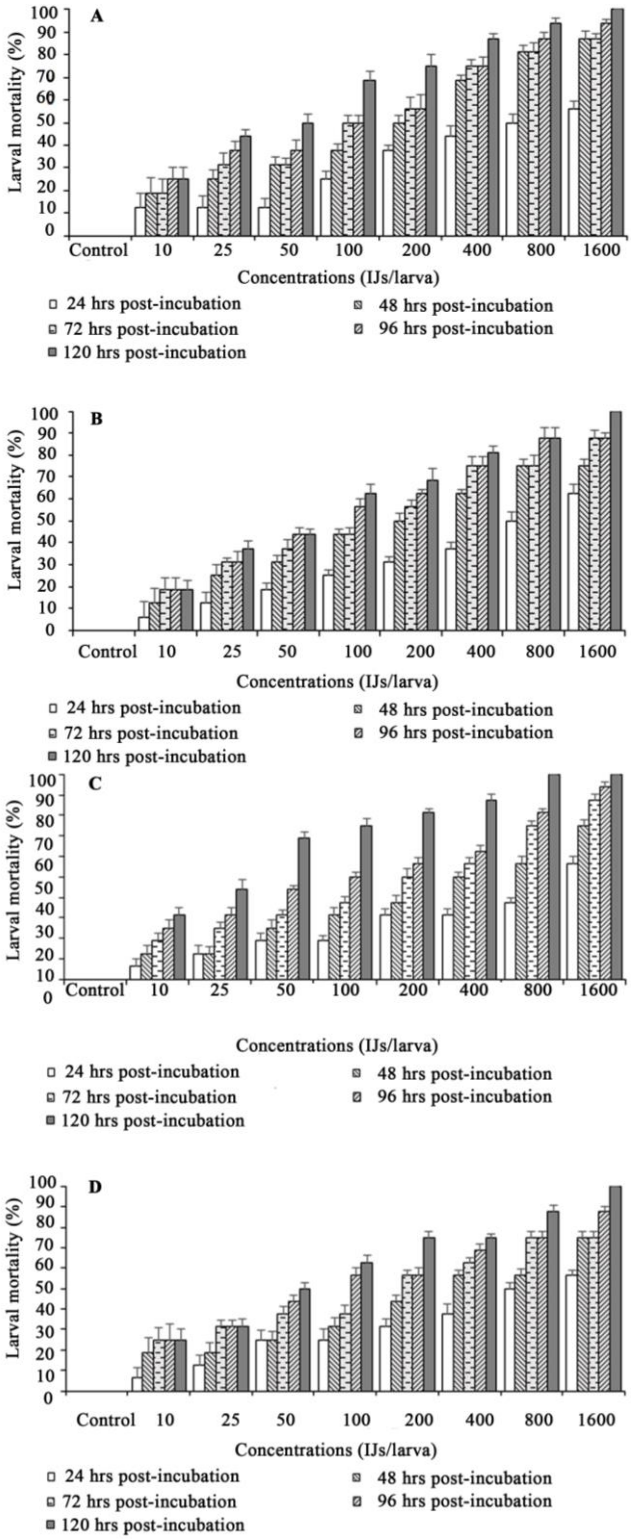

Fig.3.20. A–D: Bio-efficacy of EPNs against larvae of *O. longicolis*.
Larval mortality: A. *H. indica* B. *H. baujardi*

C. *S. sangi* D. *S. surkhetense*

Table 3.28. Correlations between nematode concentrations and larval mortality time of *O. longicolis.*

Concentrations IJs/larva	Time (hrs)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	0.94*	0.96*	0.91*	0.97**
25	0.97**	0.98**	0.89*	0.94*
50	0.93*	0.91*	0.89*	0.97**
100	0.97**	0.89*	0.76	0.98**
200	0.75	0.89*	0.90*	0.81
400	0.90*	0.76	0.86	0.76
800	0.86	0.81	0.81	0.81
1600	0.86	0.66	0.66	0.81

Time (hrs.)	Concentrations (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	0.87**	0.84**	0.86**	0.90**
48	0.78*	0.83*	0.86**	0.83*
72	0.81*	0.78*	0.83*	0.78*
96	0.85**	0.85**	0.87*	0.87*
120	0.67*	0.73*	0.68	0.78*

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Table 3.29. LC_{50} values of the EPN isolates against *O. longicolis* larva

Hours post-incubation	LC_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
24	1085.354	1022.62	1271.21	1121.77
48	358.343	472.43	733.62	665.792
72	269.830	287.68	416.62	382.66
96	185.960	171.17	231	221.15
120	55.392	60.48	80.56	103.66

Table 3.30. LT_{50} values of the EPN isolates against *O. longicolis* larva

Concentrations (IJs/larva)	LT_{50} (IJs/larva)			
	H. indica	*H. baujardi*	*S. sangi*	*S. surkhetense*
10	252.110	253.11	201.727	198.1
25	130.796	156.82	136.68	173.93
50	120.829	127.03	97.42	118.732
100	81.220	82.51	86.11	90.31
200	57.050	61.61	69.2	65.84
400	29.225	33.95	57.1	44.58
800	15.944	11.39	40.98	26.65
1600	10.474	10.965	17.520	16.490

Progeny production

The total number of IJs produced per larva of *O. longicolis* at different concentrations of nematodes is given in Fig. 3.16 B. The heterorhabditids, *H. indica* and *H. baujardi* showed greater progeny production in the larval cadaver than that of the steinernematids, *S. sangi* and *S. surkhetense*. A positive correlation was found between IJs production and concentrations of nematodes in the case of *H. baujardi*, *S. sangi* and *S. surkhetense* (r=0.27 in *H. baujardi*, r=0.93 in *S. sangi*, r=0.34 in *S. surkhetense*). Furthermore, significant differences were seen in the total number of progenies produced per larva among the EPN isolates [$F_{(3, 28)} = 67.57$, $p \leq 0.05$]. The total progeny production increased from 77.49 $x10^3 \pm 14442$ at 10 IJs/larva to 125.97 $x10^3 \pm 31491.67$ at 200 IJs/larva for *H. indica*, which then declined with higher nematode concentrations. In the case of *H. baujardi*, a total of $77.04 \pm 19.26 \times 10^3$ and $125.50 \pm 31.38 \times 10^3$ were produced at 10 and 200 IJs/larva respectively, and declined from with higher nematode concentrations. In the case of steinernematids, *S. sangi* produced $27.61 \pm 69.01 \times 10^3$ IJs at 10 IJs/larva and reached the highest multiplication rate at 1600 IJs/larva as $44.99 \pm 7.64 \times 10^3$. Meanwhile, the isolate *S. surkhetense* showed the lowest multiplication rate and produced a total of 5.52 ± 1.13 $x10^3$ IJs at 10 IJs/larva that reached the highest rate at 800 IJs/larva (11.41 ± 2.53 $x10^3$IJs).

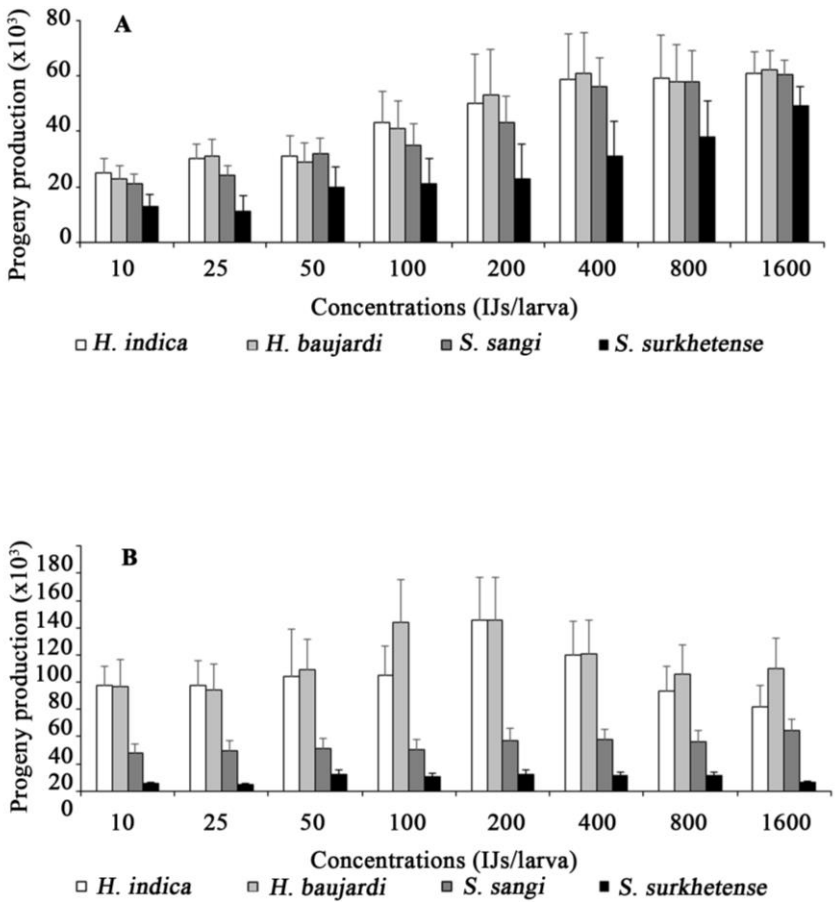

Fig.3.21. Bio-efficacy of EPNs against *A. chalybaeus and O. longicolis*.
Progeny production in: A. Larva of *A. chalybaeus*

B. Larva of *O. longicolis*

Discussion

Classical, preventative, and supplemental biological controls have all used entomopathogenic nematodes (EPNs) for the management of insect pests that have inhabited below and above ground **(Lacey and Georgis, 2012)**. Among the insect pathogenic nematodes, *Heterorhabditis* and *Steinernema* remain to be the most

commonly addressed and studied genus, primarily attributable to their diverse host range, short life cycle, ease of mass production both in vitro and in vivo, and capacity for resistance, particularly in underground environments **(Kaya and Stock, 1997; Abd-Elgawad *et al.*, 2017)**. EPNs hold great promise as future biopesticides with the advancement in formulation, application tools or methods, and strain improvements of the nematodes. Furthermore, the widespread use of EPNs bio-management is encouraged by ongoing upgradation in analysis and research on them along with an additional focus on lowering costs as well as enhancing availability of the product, improving the usability of the product, and enhancing effectiveness and persistence.

EPNs based on where they originated from showed varying environmental requirements **(Griffin, 1993; Koppenhöfer *et al.*, 2000)**, which is crucial to comprehend the ideal environmental conditions needed for each species. Furthermore, the bio-efficacy of nematodes must also be better understood beforehand to successfully formulated EPN against a specific host **(Glazer, 1992)**.

The main aim of the current study was to evaluate the bio-efficacy of the locally isolated EPNs from Mizoram, North-Eastern India, considering their possible use in the local management of economically significant insect pests. The present study highlighted the potential application of the four species of EPNs *S. sangi, H. baujardi, S. surkhetense*, and *H. indica* to control the selected local agricultural pests such as *S. litura, S. frugiperda, P. brassicae, P. rapae, A. chalybaeus*, and *O. longicolis*. The study thereby investigated (1) variations in the pathogenicity of the four EPN isolates against the pests (2) the effect of different nematode

concentrations on the mortality rate and time of the pests (3) the reproductive capability of the EPN isolates in the host cadaver (4) lethal concentrations and lethal time.

Indigenous isolates of EPN are considered more effective than exotic species for pest control in the local environment **(Bedding, 1990; Kasi *et al*., 2021)**. Furthermore, different EPN species or strains may have varying degrees of success in controlling a specific insect pest **(Kondo and Ishibashi, 1988).** The difference in size and behaviour of isolated nematode species may determine the successful reproductive ability of nematodes in the host **(Loya and Hower, 2003)**. Several scientists working in this field came up with different methods for assessing the bio-efficacy of several EPN species against insect pests **(Caroli *et al*., 1996).**

S. litura

This is the first scientific study on the bio-efficacy of locally isolated EPNs against *S. litura* from Mizoram, India. The mortality of both the larvae and pupae varied with different nematode concentrations and incubation periods and showed a positive correlation with both parameters. However, this study showed that the 4 locally isolated EPN species though highly efficient against the pest failed to cause a significant difference in the mortality of the host. Among the isolated EPN species, the steinernematids were more potent than the heterorhabditids by showing comparatively lower values of LC_{50} and LT_{50}. At 48 hrs post-incubation, the LC_{50} values were 350.65, 354.69, 369.28, and 374.19 IJs/larva for *S. sangi*, *H. baujardi*, *H. indica*, and *S. surkhetense*, respectively. In addition, the LT_{50} values at a concentration of 10 IJs/larva were 88.77 and 88.19 hrs in the case of S. sangi and S.

surkhetense respectively. However, LT_{50} values of the host were 91.89 and 90.16 hrs in the case of H. indica and H. baujardi, respectively for the same nematode concentration. Based on the study conducted by **Gokte-Narkhedkar *et al*. (2019)**, *H. indica* showed an LC_{50} value of 7.37–17.91 IJs at 48 hr of post-incubation. Upon comparison, the higher LC_{50} values may be due to differences in the larval stages selected for the experiment. The present study further recorded that against the pupal stage of *S. litura*, the heterorhanditids are more potent against the pest than the steinernematids as they showed lower LC_{50} and LT_{50} values. **Phan *et al*. (2005)** also reported the high efficacy of *S. sangi* against larvae of *S. litoralis* with successful multiplication in the insect cadaver as per the present study.

In terms of multiplication, all the nematode species can successfully reproduce inside the infected host at different concentrations of IJs. In addition, the reproductive rate of the EPN isolates varied significantly and a greater number of juveniles were produced in the case of heterorhabditids. In the larval stage, the highest multiplication rate was noted in the isolate *H. indica* where nematode concentrations in the range 100-400 IJs/larva were observed as the suitable concentrations for optimum reproduction. In contrast, the isolate *H. baujardi* failed to show successful reproduction in the larval cadaver and needs further study to address the problem. Similarly, **Gokte-Narkhedkar *et al*. (2019)** reported that some species of EPN isolates failed to reproduce successively inside larvae of *S. litura* and concluded that phase change of bacterial symbionts is a reason for primary failure. In addition, **Han and Ehlers (2001)** concluded that lack of important nutrients in host and/or failure of toxins production in phase II bacterial symbiont may not affect the

development of *H. bacteriophora*. It is therefore suggested that further study on the impact of phase variation of the symbiotic bacteria on nematode reproduction is necessary.

This study further showed that all the EPN species were highly virulent against the pupal stage. Based on the LC_{50} and LT_{50} values, *H. indica* was the most pathogenic among the isolates, as it showed lower values than the rest of the isolates. Furthermore, in contrast to the results obtained in the larval stage, *H. baujardi* showed successful reproduction in the pupal stage and produced the highest number of progenies among the EPN isolates. Likewise, the reproductive potential of six isolates of *Steinernema* on *S. littoralis* ranged from 19 x10 ^{3} to 47 x 10^{3} infective juveniles per larva **(Phan *et al.*, 2005)**. **Safdar *et al.* (2018)** reported that *H. bacteriophora* (1000IJs/ml) was capable of successful reproduction (25.78 x 10^{3} IJs/larva) on the 5^{th} instar larvae of *S. litura*. However, a higher rate of multiplication was seen in the current investigation, where a total of 59.35 x 10^{3} IJs/larva were produced in the case of H. indica. Meanwhile, **Park *et al.* (2001)** have studied the bio-efficacy of several EPN species and have recorded a higher multiplication rate in the heterorhabditidae, with the highest multiplication in the 5-6 instar (1.3 x 10^{6} IJs/larva). **Phan *et al.* (2005)** reported that *S. sangi* was capable of successive multiplication inside larvae of *G. mellonella* but failed to do so on larvae of *S. litura*. Similarly, **Ali *et al.* (2008)** have documented variations in the production of infective juveniles for EPNs on host insects (*H. armigera*, *G. mellonella*, and *Corcyra cephalonica*). Differences in pathogenicity and reproduction potential among EPN isolates may be attributed to species variation, susceptibility of the host, symbiotic

bacteria, and ecological characteristics of EPN. This study showed that the isolated EPN species are good candidates for the biological control of *S. litura* in the local area.

S. frugiperda

The current study showed that larvae and pupae of *S. frugiperda* were susceptible to the isolated EPN species in terms of mortality and reproduction. Several workers have conducted scientific studies to evaluate the efficacy of different EPN species against *S. frugiperda* under laboratory and field conditions. According to several studies, the susceptibility of FAW larvae to various EPNs varies. **Andaló *et al*. (2010)** reported that at a concentration of 200 IJs/5th instar larva of *S. frugiperda*, 96.07 and 100.00% mortality rates occurred when treated with *Heterorhabditis* sp. and *S. arenarium*, respectively. The present study however showed that after 48 h of post-incubation, *H. indica* and *S. sangi* caused 75.00% mortality, while *H. baujardi* and *S. surkhetense* caused 68.75% host mortality at a concentration of 200 IJs/larva. In addition, **Caccia *et al*. (2014)** informed that at a concentration of 50 and 100 IJs, *S. diaprepesi* caused 93.00 and 100.00% mortality of the last instar larvae of *S. frugiperda* after 144 hrs of incubation. However, at the concentration of 50 IJs/5th instar larva, the present study recorded 68.78% host mortality in the case of *H. indica*, while *S. sangi*, *H. baujardi*, and *S. surkhetense* caused 75.00% mortality at 120 hrs post-incubation. Meanwhile, at a concentration of 100 IJs/5th instar larva, both *S. sangi* and *S. surkhetense* caused 81.25% mortality, while *H. indica* and *H. baujardi* caused 100.00 and 75.00% mortality, respectively for the same incubation period. Recently, **Sayed *et al*. (2022)** reported that at a concentration of 80 IJs/ml, *S.*

carpocapsae and *H. bacteriophora* caused 78.80 and 50.00% mortality of the 5th instar larvae of *S. frugiperda*, respectively. The present study showed that *H. indica* was the most pathogenic in terms of LC_{50} and LT_{50} values and reproduction in the host cadaver. That might attribute, in part, to the fact that *H. indica* showed generally the highest prevalence among the locally isolated EPN isolates, thereby predicting its high adaptability in the prevailing climatic conditions.

Reproductive efficacy inside the host insect plays an important role in the effectiveness of EPNs as a biological control agent. The nematode upon infecting the host undergoes growth and reproduction inside the cadaver for multiple generations after which the IJs emerged and seek new hosts. The progeny production data of the present study showed a successful host infection and reproduction of the EPN isolates, thereby revealing their potency as effective biopesticides. **Caccia *et al.* (2014)** evaluated the reproductive capacity of *S. diaprepesi* on the larvae of *S. frugiperda* and reported that a total of 11.33 x 10^3 and 27.15 x 10^3 IJs were produced at a concentration of 50 and 100 IJs/larva, respectively. The obtained findings on progeny production by EPN isolates showed comparatively significant differences among the 4 species, which agree with the results of **Rahoo *et al.* (2018)**, where reproductive rate significantly varied among EPN isolates. Besides, a high rate of multiplication in the pupal stage as per the obtained data may be correlated with the mass and nutrient content of the host **(Loya and Hower, 2003)**. Moreover, the heterorhabditids, *H. indica* and *H. baujardi* showed comparatively higher production of progeny than that of the steinernematids, *S. sangi* and *S. surkhetense*. Hermaphroditic reproduction of the first generation in heterorhabditids **(Glazer *et***

***al*., 1994)** may be attributed to the higher reproductive rate in comparison with steinernematids that reproduce sexually in the first generation **(Kondo and Ishibashi, 1987)** as this may strongly correlate with an initial establishment in the host.

P. brassicae

The study shows that the large cabbage white, *P. brassicae* was highly susceptible to the isolated EPN species. With varying nematode concentrations and incubation periods, the larval and pupal mortality varied and shown a positive correlation with both parameters. However, this study demonstrated that despite being effective against the pest, the host mortality failed to show significant difference among the EPN isolates. The study further highlighted that the selected nematode concentrations largely determine the extent and intensity of *P. brassicae* infectivity and showed a positive correlation. Based on the work conducted by **Gorgadze *et al*. (2018)**, the larval mortality rate of *P. brassicae* at different nematode concentrations (25, 50, and 100 IJs/larva) varied from 50.50 to 97.00% in the case of the heterorhabditid, and 26.00 to 97.00% mortality in case of the steinernematids. In the present study, the obtained larval mortality rates were 43.75% to 100.00% and 37.50 to 100.00% in the case of the Heterorhabditids and Steinernematids, respectively. In the laboratory test conducted by **Abbas *et al*. (2021)** evaluated the pathogenicity of 7 EPN species against different larval stages of *P. brassicae* and have reported that *H. bacteriophora* and *S. glaseri* caused 100.00% mortality at a concentration of 1500 IJs/ml and 48 hrs-post incubation. However, the highest nematode concentration used in the present study was 400 IJs/larvae, and from 100 IJs/larva to progressively

higher concentrations, 100.00% mortality was noted. In addition, **Lalramliana and Yadav (2009)** evaluated the pathogenicity of 3 indigenous species of EPN (*S. thermophilium*, *S. glaseri*, and H. indica) from Meghalaya, NE India against the larvae of *P. brassicae*. Based on their experiment, *S. thermoplilium* was the most potent isolate as it showed the lowest LC_{50} value of 30.21 IJs/larva and the other isolates, *H. indica* and *S. glaseri* showed LC_{50} values of 41.10 and 104.90 IJs/larva at 48 hrs of incubation. In the present study, the recorded LC_{50} values were 51.15, 57.92, 43.97, and 42.20 IJs/larva at 48 h rs-incubation for H. indica, H. baujardi, S. sangi, and S. surkhetense, respectively.

All nematode species successfully proliferate inside the host cadaver following exposure of *P. canidia* larvae and pupae to various nematode concentrations. In addition, the reproductive rate of the EPN isolates varied significantly among the EPN isolates, and the maximum IJs emerged in the case of H. baujardi. However, the isolate H. baujardi though effectively reproduced in the pupal cadaver but failed to reproduce successfully in the larval cadaver which needs further investigation.

P. canidia

Following the exposure to different concentrations of IJs, the larvae and pupae of *P. canidia* were found to be extremely vulnerable to the EPN isolates. Although the 4 isolated EPN species showed high efficacy against the pest, their levels of infectiveness varied. In the larval stage, *H. indica* seems to be the most efficacious as it caused the highest mortality and showed lower LC_{50} and LT_{50} values. At a concentration of 10, 25, and 50 IJs/larva, *H. indica* resulted in 25.00, 37.50, and

50.00% larval mortality, respectively after 24 hrs of incubation. Meanwhile, the respective concentrations of H. indica resulted in 43.75, 43.75, and 56.25 % larval mortality after 48 hrs of incubation. In terms of lethal concentration, *S. surkhetense* exhibits a similar level of pathogenicity against the host larvae. At 24 hrs post-incubation, the LC_{50} values were 101.78 and 108.64 IJs/larva, respectively for *H. indica* and *S. surkhetense*. However, the LT_{50} values at a concentration of 10 IJs/larva were the lowest in *S. surkhetense* (88.11 h), followed by *H. indica* (89.61 h), *H. baujardi* (92.61 h) and highest for *S. sangi* (96.10 h).

In the pupal stage, all the EPN isolates showed a high and same level of efficacy against the pest. Based on the pupal mortality assay, *H. baujardi* was the most efficacious among the isolates as it was the only isolate that could cause 100.00% mortality at a concentration of 800 IJs/larva.

All nematode species successfully proliferate inside the host cadaver following exposure of *P. canidia* larvae and pupae to various nematode concentrations. In addition, a greater number of juveniles were formed in the case of heterorhabditids, and the rate of progeny production in the *P. canidia* larvae differed significantly among the EPN isolates. In the larval stage, *H. baujardi* produced the highest number of progenies (87.81×10^3 at 10 IJs/larva and 145.60 at 400 IJs/larva) while *S. sangi* produced the least number of progenies (25.08×10^3 at 10 IJs/larva and 39.68×10^3 at 400 IJs/larva) for the selected nematode concentrations. Meanwhile, the rate of multiplication in the pupal stage did not significantly vary among the EPN isolates. However, the heterorhabditids showed comparatively highest rate of multiplication than that of the steinernematids at the higher nematode

concentrations. As a result, all the EPN isolates showed high efficacy against larvae and pupa of *P. canidia*, thereby indicating their potency to control the pest in the future.

A. chalybaeus

The laboratory bio-efficacy tests showed that the indigenous EPN isolates from the study area were effective against the larvae of *A. chalybaeus*. The data showed that all four species could successfully infect the host and produce a huge number of progenies that emerged from the cadaver. Although the larval mortality rate did not significantly vary among the 4 isolated species, their levels of infectiveness varied. Based on the rate of larval mortality and values of LC_{50} and LT_{50}, the isolates *H. baujardi* and S. sangi seem to be the most efficacious among the isolates. At 24 hrs post-incubation, the LC_{50} values were 899.38 and 876.43 IJs/larva, respectively for *H. baujardi* and *S. sangi*. However, comparatively higher LC_{50} values of 965.84 and 965.27 IJs/larva were obtained for *H. indica* and *S. surkhetense*, respectively. Among the four species, *H. baujardi* was the only isolate that caused complete mortality of the larvae (at 1600 IJs/larvae) after 120 h of incubation.

Upon exposure to varied nematode concentrations against larvae of A. chalybaeus, the four EPN isolates propagate within the larval cadaver, and numerous IJs successfully emerged from the nutrient- depleted host cadaver. Like in the cases of other insect pests, the heterorhabditids showed a higher multiplication rate than that of the steinernematids. However, the isolate *S. sangi* showed a similar level of reproduction to that of the heterorhabditids. Besides the present study, **Yadav and Lalramliana (2012)** have conducted a laboratory assay on the bio-efficacy of three

indigenous EPNs from Meghalaya, India against larvae of *A. chalybaeus*. Based on their study, the three isolates *viz*. H. indica, S. thermophilium, and S. galseri (200 IJs/larva) from Meghalaya caused complete larval mortality at a concentration of 200 IJs/larva and 120 h rs-incubation. In our findings, at a nematode concentration of 200 IJs/larva, the highest larval mortality (87.50%) was observed with *H. baujardi* after 120 h of incubation. With further comparison, the EPN isolates from Mizoram exhibited lower LC_{50} values than those isolates from Meghalaya though lower LC_{50} values were recorded during initial incubation in that study. Furthermore, **Sanda *et al*. (2022)** have reported the higher effectiveness of the heterorhanditids (*H. bacteriophora*) in comparison to the steinernematids (*S. carpocapsae*) against a beetle larva of *Octodonta nipae*. The data showed that all nematode species successfully proliferate inside the host cadaver. At a concentration of 200 IJs/larva, H. baujardi produced the highest number of progenies (53.00 x 10^3 IJs, followed by H. indica (50.00 x 10^3). In case of S. sangi and S. surkhetense, a total of 43.00 x 10^3 and 23.00 x 10^3 IJs/larva were produced. **Yadav and Lalramliana (2012)** previously reported successful multiplication of 3 EPN isolates from Meghalaya, India and the highest progeny production was recorded in H. indica (168.90 x 10^3 IJs/larva). Appreciably, all four indigenous EPN isolates from Mizoram were proven to be effective against the larvae of *A. chalybaeus* and, thereby are promising control agents in the future.

O. longicolis

In the present study, the bio-efficacy of four indigenous EPN isolates from Mizoram was evaluated against larvae of banana stem borer, *O. longicollis*. The data showed

that all four species could successfully infect the host and produce a large number of progenies. At a concentration of 800 IJs/larva, *S. sangi* was the only isolate that caused complete mortality of the larvae after 120 h of incubation. However, with an increase in nematode concentration to 1600 IJs/larva, all four EPN species caused complete mortality of the larva after 120 h of incubation. Based on LC_{50} and LT_{50} values, the two heterorhabditids *H. indica* and H. baujardi were more pathogenic than the steinernematids. Furthermore, the four tested nematodes successfully propagate in the host cadaver and successful emergence of infective juveniles. Like in the previous assays, the heterorhabditids produced more progenies than the *Steinernema*tids. Besides our study, **Giribabu *et al*. (2020)** evaluated the bio-efficacy of native EPN species from Tamil Nadu, India using larvae of *O. longicolis*. In accordance with our study, they have reported that in terms of host mortality rate, the steinernematid (*S. siamkayai*) was more pathogenic than the heterorhabditid (*H. indica*). They further stated that at a concentration of 200 IJs/larva, *S. siamkayai* and *H. indica caused* 74.6.% and 69.20% larval mortality, respectively, after 72 hrs of incubation. However, with the same nematode concentration and incubation period, the present study recorded 50.00-56.25% larval mortality. In addition, **Treverrow *et al*. (1991)** and **Mwaitulo *et al*. (2011)** have performed laboratory and field evaluations of several EPN species against the banana weevil *C. sorsidus*. In conclusion, all four EPN isolates were proven effective against the larvae of *O. longicolis*, therefore adding more information on the potency of EPNs to control the pest in bananas.

Summarizing the results, *H. indica* showed the highest bio-efficacy against the tested agricultural pests collected from Mizoram, India, and thereby, represents the most potent candidate of biological control agents in pests' management. However, the overall larval mortality rate did not show significant variations with the EPN isolates and the insect pests [$F_{(1, 167)}=1.39$; $p\leq0.05$]. In contrast, the overall pupal mortality rate shows significant variations with the EPN species and the insect pests [$F_{(1, 63)}=4.57$; $p\leq0.05$]. Several investigations have reported that the bio-efficacy of EPNs could be affected by several factors and thus were found to show different levels of effectiveness against insect pests **(Kaya and Gaugler, 1993; Forschler and Nordin, 1988)**. In addition, the data from our study showed successful multiplication occurred and varies in the four EPN isolates and that higher rates of multiplication mostly occurred in the heterorhabditids i.e., *H. indica* and *H. baujardi*. Based on the statistical analysis, the overall multiplication rate showed significant variations with the EPN species and insect pests [$F_{(1, 167)}=46.09$; $p\leq0.05$ for larval stage, and $F_{(1, 63)}=10.58$; $p\leq0.05$ for pupal stage]. The variations in nematode reproduction have been reported by several investigators and were found to be affected by the size of the nematodes and insect host, behaviour of the nematode species, hermaphroditic nature of heterorhabditids **(Poinar, 1990; Mannion and Jansson, 1992; Stuart *et al*. 1996; Karunakar *et al*., 1999; Bhatnagar *et al*., 2004; Jothi and Mehta, 2006)**.

SUMMARY

With the increasing concern about the resistance that several insect pests have developed against chemical insecticides and the substantial risks posed to the environment, non-targeted creatures, and public health, it is necessary to look for new and or alternative biological control agents. Therefore, in comparison to chemical insecticides, biological control offers a highly significant approach and potential substitute for insect pest management in terms of safety, effectiveness, and sustainability. Like other biological control agents, EPNs are potential and promising organisms for the control of several insect pests. In addition, EPNs can be formulated with a wide variety of chemical and organic pesticides in Integrated Pest Management (IPM) programs. EPNs of the families steinernematidae and heterorhabditidae, like other biological control agents, have strong bio-control potential with which to manage economically significant insect pests and incorporate them into integrated pest management. They are obligate and lethal parasites of insects that are characterized by symbiotic association with bacteria, *Xenorhabdus* spp. in steinernematids and *Photorhabdus* spp. in heterorhabditids.

Since works on the EPNs in northeast India is very limited, and moreover, less is known about their occurrence and potential in the region. Since no scientific work on EPNs has been conducted from Mizoram, the present study was set up to isolate indigenous species from the State.

The topic that is covered in this study can be roughly divided into three chapters:

Chapter 1. Isolation of Entomopathogenic nematodes (EPNs) from soil samples collected in Mizoram, North-Eastern India.

Chapter 2. Molecular characterization of isolated EPNs using ITS, COI, and 28S rDNA markers.

Chapter 3. Evaluation of the bio-efficacy of EPNs against major insect pests in Mizoram.

Surveys on the occurrence of EPNs were conducted across different areas covering the 11 districts of Mizoram, North-Eastern India. Soil samples were collected from different locations and were baited with larvae of *G. mellonella* following Bedding and Akhurst (1975). The dead larvae were confirmed by colour change and smell emanating from the insect cadaver and transfer in modified White traps following Kaya and Stock (1997). Secondary confirmation was done through Koch's postulates and the confirmed EPNs were stored with aerated water in an incubator at 12 - 15 °C

The primary confirmation of insect mortality due to the EPN isolates was based on the nature of the infected host (colour) and the smell emanating from the insect cadaver. The isolated nematodes with serial numbers 1 to 7 (coded as HeM, HeTC, HeTRE, HeTR, HeTS, HeTV, and, HeTD) were distinguished by the presence of only hermaphroditic female adults in the first generation, which were absent in the case of nematode isolates 8 to 12 (coded as StR, StK, StC, StL, and StPTS). Therefore, the recorded nematode from series 1 to 7 belongs to the Genus *Heterorhabditis* while the isolates 8 to 12 belong to the Genus *Steinernema*.

Based on the morphological and morphometric measurements, all the isolates of the of *Heterorhabditis* spp. are highly similar. However, the nematode isolates 1 to 6 (Group I) which shared similar morphological and morphometric measurements can be further separated from the isolate 7 (Group II) by shape of the gubernaculum and the number of normal pairs of genital papillae. In addition, the occurrence of a bursa in males further distinguished the isolate of the genus *Heterorhabditis* from *Steinernema*. The EPN isolates were further molecularly identified at the species level using the selected markers *viz*., internal transcribed spacer ribosomal DNA (ITS rDNA) region, D2-D3 regions of 28S or larger Subunit (LSU) of rDNA region and M1–M6 region of Cytochrome oxidase I (COI).

The NCBI BLAST search result of the sequencing products (amplified using the molecular markers ITS, 28S, and COI) showed that the EPN isolates *viz*., *Heterorhabditis* sp. 1(series 1 to 6), *Heterorhabditis* sp.2 (series 7), *Steinernema* sp.1 (series 8 to 11), and *Steinernema* sp. 2 (series 12) exhibited high similarity (99.00–100.00%) with respective sequences available for *H. indica*, *H. baujardi S. sangi*, and *S. surkhetense*. The developed sequences were deposited in NCBI GenBank (*H. indica*: under the NCBI accession number MF618313-MF618318, MF618313- MF618318, and MF621245- MF621245, respectively for ITS, 28S, and COI; *H. baujardi*: MF618319- MF618323, MF621012-MF621014, and MF621249- MF621252, respectively for ITS, 28S, and COI; *S. sangi* KY861069-KY861072, MF620997-MF621000, and MF621237- MF621240,

respectively for ITS, 28S, and COI; *S. surkhetense*: MF618308-MF618312, MF621001-MF621005, MF621241-MF621244, respectively for ITS, 28S, and COI).

The phylogenetic analysis inferred from ITS and 28S rDNA regions indicated that the isolated *S. sangi* belongs to the "*feltiae-kraussei-oregonense*" group. Within this monophyletic group, the isolate formed a cluster with previously deposited sequences of *S. sangi* (including *Steinernema* sp., GU395631) and did not cluster with other species. In the case of *S. surkhetense*, the phylogenetic analysis inferred from the previously mentioned markers indicated that the isolate belongs to the "*carpocapsae*" group and a separate cluster within the group was formed with previously deposited sequences of *S. surkhetense*. In the case of the heterorhabditids isolates, the phylogeny using the ITS and 28S rDNA data sets revealed that the isolated *H. indica* and *H. baujardi* belong to "*indica"* group with a strong bootstrap support. Within the monophyletic group, both species formed a separate cluster with their respective deposited sequences of *H. indica* and *H. baujardi*. The COI region, however, did not yield a proper and useful phylogenetic relationship which may be due to a lack of comparable sequences.

Being one of the most reliable tools for the distinction of species, p-distance is calculated based on the ITS and 28S rDNA regions. Based on the analysis of ITS region, an average intraspecies and interspecies distance exhibited by *S. sangi* was 0.50 ± 0.35% (0.00–1.00%) and 13.184.33% (9.0–51.0%), respectively; *S. surkhetense* was 2.00±1.40% (0.00–4.00%) and 12.00±6.65% (4.00–51.00%), respectively; *H. indica* was 0.50 ± 0.35% (0.00–1.00%) and 16.31±3.77(1.00–

51.00%), respectively; *H. baujardi* was 0.00 and 13.18±4.18% (2.00–52.00%), respectively.

Baseb on the analysis of 28S rDNA region, S. sangi exhibited an averarege intraspecies and interspecies distance of 3.50±2.40% (0.00–7.00%) and 8.00 ± 2.22 0% (3.00–33.0%), respectively. Also, an average intraspecies and interspecies distance of 0.60±1.20% (0.00–1.00%) and 8.33±3.33% (1.00–34.00%) was obsereved in *S. surkhetense*. Furthermore, an average intraspecies and interspecies distance was *H. indica was* 0.50 ± 0.35% (0.00–1.00%) and 13.83±4.18% (2.00–52.00%), respectively in *H. indica*, and 0.00% and 4.80±1.70% (1.00–23.00%), respectively in *H. baujardi*.

Even though the three molecular markers employed in the present study yield a good result, the ITS gene region seems to be the most reliable marker for the identification and distinction of the studied nematode species. The less reliability of the two molecular markers (in comparison to the ITS gene) may be attributed to inadequate submission of sequences in NCBI GenBank or misidentification of the previously submitted species and require further thorough study.

The present work represents the first report on the occurrence of EPNs from Mizoram, and, therefore, adds new data on diversity of EPNs in the world. Hopefully, the findings of this study may pave the way for the use of these native EPN species in biological control efforts against insect pests. In India, a number of indigenous steinernematids and heterorhabditids had been documented. However, works on the EPNs in northeast India are very limited, and less known about their occurrence and potential in the region. Therefore, with the isolation of *S. sangi*, *S.*

surkhetense, *H. indica* and *H. baujardi* from Mizoram, the north-eastern region of India, 13 valid EPN species are presently known from India.

EPNs of the families Steinernematidae and Heterorhabditidae have been known to infect a wide range of insect pests and are one the most efficient biological control agents for soil and above-ground insect pests. In various laboratory tests, many EPN species have been shown to infect many insect species. However, following field applications, the host range may be significantly reduced based on the nematode's ecology and possible hosts. Furthermore, the pathogenicity of EPNs is influenced by various environmental and biotic factors, mainly by the species of EPN, the host insect, the life cycle of a targeted insect, and soil fauna.

The study was taken up to evaluate the bioefficacy of the locally isolated EPNs against major insect pests in the State of Mizoram. Six agricultural pests selected for the study includes tobacco cutworm, *S. litura*, fall armyworm (FAW), *S. frugiperda*, the Indian cabbage white butterfly, *P. canidia*, the cabbage butterfly, *Pieris brassicae*, the corm borer, *A. chalybaeus* (Hope), and banana pseudostem weevil (BPW), *Odoiporus longicollis*.

The bioefficacy test was performed to evaluate the insect mortality and nematode multiplication rate in host cadavers using a Petri dish assay. The different concentrations of nematodes (IJs) used for the experiment were 10, 25, 50, 100, 200, and 400 IJs/larva for *P. canidia* and *P. brassicae*; 10, 25, 50, 100, 200, 400, and 800 IJs/larva for *S. litura* and *A. chalybaeus*; 10, 25, 50, 100, 200, 400, 800, and 1600 IJs/larva for *S. frugiperda* and *O. longicollis*. For both the assays, eight (8) replicates were set for each nematode concentration, and were repeated twice. The experiment

was carried out in an incubator at 28±2 °C, and insect mortality was checked every 24 hrs up to 120 hrs. Colour change, no movement, and the smell emanating from the dead body of larvae and pupae were used for primary confirmation of death due to EPNs. After 48 hrs of mortality, the dead insects were rinsed with distilled water and individually dissected under an Olympus CX41 microscope for further confirmation. Negative control plates were set for all the experiments.

To determine the multiplication rate of the EPN isolates, nematode concentrations, replicates, and negative control plates were set as in the case of the mortality assay. The mortality of the hosts was checked every 24 hrs up to 120 hrs. To allow the nematode to multiply and emerge from the host cadaver, the dead larva and pupa were individually placed on modified white traps after cleansing with distilled water to eliminate any attached external nematodes. The total number of emerged IJs on individual white traps was collected and counted under a stereomicroscope.

The EPN isolates *H. indica*, *H. baujardi*, *S. sangi*, and *S. surkhetense* showed high efficacy against the selected agricultural pests with successful host infection and multiplication. Among the isolates, *H. indica* showed the highest bio-efficacy against the pests, and thereby, represents the most potent candidate for future biological control agents in pest management. However, the overall larval mortality rate did not show significant variations with the EPN isolates and the insect pests [$F_{(1,167)} = 1.39$; $p<0.05$]. In contrast, the overall pupal mortality rate shows significant variations with the EPN species and the insect pests [$F_{(1, 63)} = 4.57$; $p<0.05$].

The study showed that the 4 EPN isolates successfully multiply and that higher rates of multiplication mostly occurred in the heterorhabditids i.e., *H. indica* and *H. baujardi*. Based on the statistical analysis, the overall multiplication rate showed significant variations with the EPN species and insect pests [$F_{(1,167)}$ =46.09; $p<0.05$ for the larval stage], and [$F_{(1, 63)}$ =10.58; $p<0.05$ for the pupal stage]. The variations in nematode reproduction have been reported by several investigators and were found to be affected by the size of the nematodes and insect host, behaviour of the nematode species, the hermaphroditic nature of heterorhabditids, and others.

In conclusion, the study reported the occurrence of indigenous EPNs species from Mizoram and details on extensive distribution of *H. indica*, *H. baujardi*, *S. sangi*, and *S. surkhetense* in the world. Additionally, the isolation of 4 EPN species in Mizoram suggests that the distributional range may expand farther into Bangladesh and Eastern India, and other regions of north-eastern India. Hopefully, the study may raise the possibility of isolation of more EPN species from the State and further deep study on their potency against regional insect pests.

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Nam Nao National Park in Thailand and larvicidal activity of symbiotic bacteria against *Aedes aegypti* and *Aedes albopictus*. *PloS one*, *13*(4): e0195681.

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BRIEF BIO-DATA OF THE CANDIDATE

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Educational qualifications:

Exams	**Board**	**Subject**	**Percentage**	**Division**	**Year**
HSLC	MBSE	General	82.40%	Distinction	2007
HSSLC	MBSE	Science	61.20%	First	2009
B. Sc	MZU	Zoology	79.13%	First	2012
M. Sc	NEHU	Zoology	73.10%	First	2014
SLET (NE)	SLET	Life Sciences		Lecturship (LS)	2017
NET	UGC-CSIR	Life Sciences		LS &JRF	2019
GATE		Life Sciences			2023
GATE		Biotechnology			2023

AWARDS & DISTINCTIONS

1. Secured First Position in the Bachelor of Science (Zoology) Examination conducted by Mizoram University in the year 2012.

2. Awarded Rector's Gold Medal for securing First Position in Master of Science (Zoology) Examinations conducted by North Eastern Hill University held in the year 2014.

3. Awarded Professor M.K. Khare Memorial Prize for securing First Position in Master of Science (Zoology) Examinations conducted by North Eastern Hill University held in the year 2014.

4. Awarded Young Scientist Award in Oral Presentation-Below 30 Years (III Prize) in the International Conference on Biodiversity, Environment and Human Health: Innovations and Emerging Trends, Mizoram University, Aizawl in 2018.

5. Awarded Young Scientist Award in Poster (III Prize) in the International Conference on "Chemical Ecology, Environment and Human Health: Emerging Frontiers and Synthesis (ICCEEHH, 2019) held at Department of Zoology, Sikkim University, Gangtok, Sikkim on August 9-10, 2019".

6. Awarded the Second Prize as Oral Presenter at the International Conference on Recent Advances in Animal Sciences-2019 (ICRAAS, 2019) held at Pachhunga University College, Aizawl, Mizoram, India on November 6 -8, 2019.

7. Awarded Best Oral Presenter Award at the Indian Academy of Tropical Parasitology, TROPACON N.E., 2022 organized by Zoram Medical College (ZMC) under the auspices of Indian Academy of Topical Parasitology, Northeast Chapter on November 9-10, 2022.

PAPER PRESENTED

1. "Evaluation of four species of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from Mizoram, India against a coleus pest, *Pycnarmon cribrata* (L)".in the 12th Annual Convention of Association of

Biotechnology and Pharmacy (ABAP) and International Conference on Biodiversity and Human Health: Innovations and Emerging Trends (BEHIET 2018) organised at the School of Life Sciences, Mizoram University, Aizawl, Mizoram during 12th-14th, November, 2018.

2. "Pathogenicity of two species of locally isolated entomopathogenic nematodes (Steinernematidae) from Mizoram, India against cloudless sulphur butterfly (*Phoebis sennae*) under laboratory conditions". in the Mizoram Science Congress 2018, a National Conference, held at Pachhunga University College during 4th -5th October, 2018.

3. "First report on the outbreak of fall armyworm, *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) in Mizoram, NE India, and its susceptibility to locally isolated entomopathogenic nematodes, *Heterorhabditis baujardi"* in the International Conference on "Chemical Ecology, Environment and Human Health: Emerging Frontiers and Synthesis (ICCEEHH, 2019)" held at Department of Zoology, Sikkim University, Gangtok, Sikkim on August 9th-10th August, 2019.

4. "Evaluation of the pathogenicity of indigenous entomopathogenic nematodes (Steinernematisae and Heterorhabditidae) from Mizoram, India against tobacco cutworm, *Spodoptera litura* (Fabricius, 1775)" in the International Conference on Recent Advances in Animal Sciences (ICRAAS) held at Pachhunga University College, Aizawl, Mizoram, India on November 6th-8th November, 2019.

5. "International Webinar on Recent Advances on Science and Technology (ISRAST). Organised by North East (India) Academy of Science & Technology (NEAST), Mizoram University from 16th -18th November, 2020.

6. "Antibacterial activity of indigenous entomopathogenic fungus Beauveria malawiensis from Mizoram, India against eight selected pathogenic bacteria". in the Indian Academy of Tropical Parasitology, TROPACON N.E., 2022 organized by Zoram Medical College (ZMC) under the auspices of Indian Academy of Topical Parasitology, Northeast Chapter on November 9th-10th, 2022.

7. “Bio-efficacy of indigenous isolates of entomopathogenic nematodes (EPNs) from Mizoram, India against banana pseudostem weevil Odoiporus longicollis Oliver (Coleoptera: Curculionidae)” in the International Conference on Biodiversity and Conservation) ICBC) organised by the Department of Zoology &Fishery Science, St. Anthony’s College, Shillong in collaboration with the Bio ResourcesDevelopment Centre (BRDC), Govt. of Meghalaya, the Zoological Survey of India (ZSI), Govt. of India, the Meghalaya Biodiversity Board (MBB), Govt. of Meghalaya on 14th-15th December, 2022.

POSTER PRESENTED

1. “DNA Barcoding: A tool for Identification of entomopathogenic nematodes from Mizoram” on the occasion of Aizawl Science Conclave 2016, organized by Pachhunga University College on 13th February 2016 at PUC Auditorium, Aizawl, Mizoram in commemoration of 30th years of DBT, New Delhi.

2. “Natural Occurance on Four Entomopathogenic Nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Mizoram, NE India” in the International Conference on “Chemical Ecology, Environment and Human Health: Emerging Frontiers and Synthesis (ICCEEHH, 2019)” held at Department of Zoology, Sikkim University, Gangtok, Sikkim on 9th -10th August, 2019.

CONFERENCE/SEMINAR/WORKSHOP PARTICIPATED

1. The Outreach Program on Human Health and Biological Timing organized at Department of Zoology, Mizoram University on May, 2017.

2. State Level Workshop on Research Methodology and Monitoring of Research Projects held at PUC Seminar Hall, Aizawl on 11th August, 2017 organised by Project Committee, Pachhunga University College, Aizawl, Mizoram.

3. State level Seminar on Challenges in Biosystematics in Mizoram organized by Directorate of Science and Technology, Mizoram, Mizo Academy of Sciences, Aizawl and Project Committee, Pachhunga University College, Aizawl, Mizoram held on 29th September, 2017 at Pachhunga University College, Aizawl, Mizoram.

4. Seminar on Climate Change Adaptation and Disaster Risk Reduction on 3rd November, 2017 at ATI Auditorium Secretariat Complex, Aizawl, Mizoram organised by Mizo Academy of Sciences in collaboration with MISTIC.

5. Seminar on Science and Technology for a Sustainable Future catalysed and supported by the National Council for Science and Technology Communication, Department of Science and Technology, New Delhi held on 30th April, 2018 at Pachhunga University College, Aizawl, Mizoram.

6. Seminar on Science for the People and The People for Science organized by Mizo Academy of Sciences (MAS and Mizoram Science, Technology & Innovation Council IMISTIC), Directorate of Science and Technology, Governement of Mizoram held on 3rd May, 2019 at Central YMA Hall, Tuikhuatlang, Aizawl, Mizoram.

7. Seminar on Science and Technology for a Sustainable Future catalysed and supported by the National Council for Science and Technology Communication, Department of Science and Technology, New Delhi held on 30 April, 2018 at Pachhunga University College, Aizawl, Mizoram.

PUBLICATIONS

1. Lalramnghaki HC, Lalramliana, Lalremsanga HT, Vanlalhlimpuia, Lalramchuani M, Vanramliana (2021). Susceptibility of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae), to four species of entomopathogenic

nematodes (Steinernematidae and Heterorhabditidae) from Mizoram, North-Eastern India. *Egypt J Biol Pest Control* 31: 110. **(IF- 2.055).**

2. Lalramnghaki HC, Lalramliana, Lalremsanga HT, Vanlalhlimpuia, Vanramliana, Lalzarzovi ST (2021). In-vitro Study on the Efficacy of Entomopathogenic Nematodes (Heterorhabditidae and Steinernematidae) for the Control of the Indian Cabbage White, *Pieris canidia* (Lepidoptera: Pieridae). *Science and Technology Journal* 92: 140-147. (UGC-Care list **Peer reviewed journal).**

3. Lalramnghaki HC, Lalremsanga HT, Vanramliana, Lalramliana, Vanlalhlimpuia, Lalliansanga S (2020). Evaluation of pathogenicity of indigenous entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from Mizoram, India, against tobacco cutworm, *Spodoptera litura* (Fabricius, 1775). *J Environ Biol*. 41: 851-860. **(IF- 0.78)**

4. H.C. Lalramnghaki, Vanlalhlimpuia, Vanramliana, Lalramliana (2017). Characterization of a new isolate of entomopathogenic nematode, *Steinernema sangi* (Rhabditida, Steinernematidae), and its symbiotic bacteria *Xenorhabdus vietnamensis* (c-Proteobacteria) from Mizoram, northeastern India. *J Parasitic Dis*. 41(4):1123-1131. **(Scopus Indexed Journal)**

5. Vanlalhlimpuia, Lalramliana, **H.C. Lalramnghaki**, Vanramliana (2018). Morphological and molecular characterization of entomopathogenic nematode, *Heterorhabditis baujardi* (Rhabditida, Heterorhabditidae) from Mizoram, north-eastern India. *J Parasitic Dis*. 42:341-349. **(Scopus Indexed Journal)**

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DEPARTMENT : ZOOLOGY

TITLE OF THE THESIS : Molecular Characterization and Evaluation of Locally Isolated Entomopathogenic Nematodes Against Major Insect Pests In Mizoram, India

DATE OF ADMISSION : 20.09.2017

APPROVAL OF RESEARCH PROPOSAL:

DRC : 07.04.2018

B.O. S : 27.04.2018

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Ph. D. REGISTRATION NO. & DATE: MZU/Ph.D./1130 of 03.5.2018

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ABSTRACT

MOLECULAR CHARACTERIZATION AND EVALUATION OF LOCALLY ISOLATED ENTOMOPATHOGENIC NEMATODES AGAINST MAJOR INSECT PESTS IN MIZORAM, INDIA

AN ABSTRACT SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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PH. D. REGN NO.: MZU/PH.D./1130 OF 03.5.2018

DEPARTMENT OF ZOOLOGY

SCHOOL OF LIFE SCIENCES

JANUARY, 2023

MOLECULAR CHARACTERIZATION AND EVALUATION OF LOCALLY ISOLATED ENTOMOPATHOGENIC NEMATODES AGAINST MAJOR INSECT PESTS IN MIZORAM, INDIA

BY

H.C. LALRAMNGHAKI

Department of Zoology

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Submitted

In partial fulfilment of the requirement of the degree of Doctor of Philosophy in Zoology of Mizoram University, Aizawl.

ABTSRACT

Preface and Consolidated Abstract

Agriculture pests are thought to be responsible for up to one-third of agricultural productivity losses and have been controlled by humans for thousands of years. Cultural practices like weeding, the use of pesticides, and the selective breeding of plants for pest resistance are only a few examples of the extensive range of pest management strategies available. With the rising world population, global production of agriculture needs to double by 2050 to fulfill this kind of escalating need. Chemical pesticides have been widely used in agriculture and significant advances in synthetic organic chemistry paved the way for a new era of pest control. Ever since then, the majority of the world's agricultural sectors have used significantly more pesticides than they did previously, and yields have increased significantly as a result.

With the increasing concern about the resistance that several insect pests have developed against certain chemical insecticides and the substantial risks posed to the environment, non-targeted creatures, and public health, it is necessary to look for new and or alternative biological control agents. Therefore, in comparison to chemical insecticides, biological control offers a highly significant approach and potential substitute for insect pest management in terms of safety, effectiveness, and sustainability. Like other biological control agents, EPNs are potential and promising organisms for the control of several insect pests. In addition, EPNs can be formulated with a wide variety of chemical and organic pesticides in Integrated Pest

Management (IPM) programs. EPNs of the family steinernematidae and heterorhabditidae, like other biological control agents, have strong bio-control potential with which to manage economically significant insect pests and incorporate them into integrated pest management.

The topic that is covered in this study can be roughly divided into three chapters:

Chapter 1. Isolation of Entomopathogenic nematodes (EPNs) from soil samples collected in Mizoram, North-Eastern India.

Chapter 2. Molecular characterization of isolated EPNs using ITS, COI, and 28S rDNA markers.

Chapter 3. Evaluation of the bio-efficacy of EPNs against major insect pests in Mizoram.

Chapter 1: Isolation of Entomopathogenic nematodes (EPNs) from soil samples collected in Mizoram, North-Eastern India.

EPNs of the families steinernematidae and heterorhabditidae are obligate and lethal parasites of insects that are characterized by symbiotic association with bacteria, *Xenorhabdus* spp. in steinernematids and *Photorhabdus* spp. in heterorhabditids. The life cycle of EPNs includes a third infective juvenile (IJ3) called dauer juvenile which is the only infective stage of the nematode. The IJs attain adulthood within 24–48 hrs where each IJs developed into first-generation hermaphroditic females in *Heterorhabditis* spp., whereas, in *Steinernema* spp., each IJs develop into amphimictic females or males. However, in both genera, a second generation consists of amphimictic females and males.

With the growing concerns over the negative impact of exotic species on native organisms, immense works have been conducted for the isolation of climatically adapted indigenous EPN species with the intention of formulating and commercialising them later. In this regard, extensive surveys have been carried out on the occurrence of EPNs from different regions of the world, and about 107 and 17 species of *Steinernema* and *Heterorhabditis,* respectively were described. In India, about 13 species of EPNs (12 steinernematids and 1 heterorhabditids) were reported from the country. However, works on the EPNs in northeast India is very limited, and moreover, less is known about their occurrence and potential in the region. Since no scientific work on EPNs has been conducted from Mizoram, the present study was set up to isolate indigenous species from the State.

Surveys on the occurrence of EPNs were conducted across different areas covering the 11 districts of Mizoram, North-Eastern India. Soil samples were collected from different locations and were baited with larvae of *G. mellonella* following Bedding and Akhurst (1975). The dead larvae were confirmed by colour change and smell emanating from the insect cadaver and transfer in modified White traps following Kaya and Stock, 1997. Secondary confirmation was done through Koch's postulates and the confirmed EPNs were stored with aerated water in an incubator at 12 –15 °C

The primary confirmation of insect mortality due to the EPN isolates was based on the nature of the infected host (colour) and the smell emanating from the insect cadaver. The isolated nematodes with serial number 1 to 7 (coded as HeM, HeTC, HeTRE, HeTR, HeTS, HeTV, and, HeTD) were distinguished by the

presence of only hermaphroditic female adults in the first generation, which were absent in the case of nematode isolates 8 to 12 (coded as StR, StK, StC, StL, and StPTS). Therefore, the recorded nematode from series 1 to 7 belong to the Genus *Heterorhabditis*, while the isolates 8 to 12 belong to the Genus *Steinernema*. In addition, the occurrence of a bursa in males further distinguished the isolate of the genus *Heterorhabditis* from *Steinernema*.

The present work represents the first report on the occurrence of EPNs from Mizoram, primarily based on basic morphological identification of the nematode as well as the colour of the host cadaver. Since isolates of EPNs from different geographical area exhibit differences in their behavioural and physiological adaptations, identification and documentation of locally adapted species of a particular region is required for the successful use of EPN as a biological control agent. The study, therefore, adds new data on the occurrence of EPNs in the state of Mizoram in particular, and in India, in general. Hopefully, the findings of this study may pave the way for the use of these native EPN species in biological control efforts against insect pests.

Chapter 2. Molecular characterization of isolated EPNs using ITS, COI, and 28S rDNA markers.

With the advance in technology, molecular identification techniques were frequently used to classify organisms in different parts of the world. EPN identifications are traditionally based on morphological methods, which are less likely straightforward (Poinar, 1990) and overflowed with complexities and ambiguities. The molecular techniques that have been used the most frequently include DNA sequencing,

restriction fragment length polymorphisms (RFLP), and random amplified polymorphic DNA (RAPD). Among many others, the use of molecular methods with the advent of polymerase chain reaction (PCR) and DNA sequencing has greatly contributed to the advancement of EPN identification. The Three commonly used molecular markers such as Internal transcribed spacer ribosomal DNA (ITS rDNA) region, D2-D3 regions of 28S or Larger Subunit (LSU) of rDNA region and M1–M6 region of Cytochrome oxidase I (COI) were selected for identification of the EPN isolates.

The NCBI BLAST search result of the sequencing products (of amplified ITS, 28S, and COI marker regions) showed that the EPN isolates viz., *Heterorhabditis* sp. 1(series 1 to 6), *Heterorhabditis* sp. 2(series 7), *Steinernema* sp.1 (series 8 to 11, and *Steinernema* sp. 2 (series 12) exhibited high similarity (99.00–100.00%) with respective sequences available for *Heterorhabditis indica*, *Heterorhabditis baujardi Steinernema sangi*, and *Steinernema surkhetense*. The developed sequences were deposited in NCBI GenBank (under the NCBI accession number MF618313– MF618318, MF618313– MF618318, and MF621245–MF621245, respectively for ITS, 28S, and COI in *H. indica*; MF618319–MF618323, MF621012–MF621014, and MF621249–MF621252, respectively for ITS, 28S, and COI in *H. baujardi*; KY861069–KY861072, MF620997–MF621000, and MF621237–MF621240, respectively for ITS, 28S, and COI in *S. sangi*; MF618308–MF618312, MF621001–MF621005, MF621241–MF621244, respectively for ITS, 28S, and COI in *S. surkhetense*).

Even though the three molecular markers employed in the present study yield a good result, the ITS gene region seems to be the most reliable marker for the identification and distinction of the studied nematode species. The less reliability of the two molecular markers (in comparison to the ITS gene) may be attributed to inadequate submission of sequences in NCBI GenBank or misidentification of the previously submitted species and require further thorough study. The phylogenetic analysis inferred from ITS and 28S rDNA regions indicated that the isolated *S. sangi* belongs to the *feltiae-kraussei-oregonense* group. Within this monophyletic group, the isolate formed a cluster with previously deposited sequences of *S. sangi* (and *Steinernema* sp., GU395631) and did not cluster with other species. In the case of *S. surkhetense*, the phylogenetic analysis inferred from the previously mentioned markers indicated that the isolate belongs to the *carpocapsae* group and formed a separate cluster within the group with previously deposited sequences. In the case of the heterorhabditids isolates, the phylogeny using the ITS and 28S rDNA data sets revealed that the isolated *H. indica* and *H. baujardi* belong to '*indica*' group with a strong bootstrap support. Within the monophyletic group, both species formed a separate cluster with their respective deposited sequences of *H. indica* and *H. baujardi*. The COI region, however, did not yield a proper phylogenetic tree which may be due to a lack of comparable sequences.

In India, a number of indigenous steinernematids and heterorhabditids had been documented. However, works on the EPNs in northeast India are very limited, and less known about their occurrence and potential in the region. So far, five species of EPNs viz. *H. indica, H. bacteriophora, S. glaseri, S. abbasi* (=*S. thermophilum*),

and *S. carpocapsae* (=*S. meghalayense*) have been documented from north-eastern India where the bio-efficacy and ecological characters of only some species have been studied. Therefore, with the isolation of *S. sangi*, *S. surkhetense*, *H. indica* and *H. baujardi* from Mizoram, the north-eastern region of India, 13 valid EPN species are presently known from India.

Chapter 3. Evaluation of the bio-efficacy of EPNs against major insect pests in Mizoram.

EPNs of the families Steinernematidae and Heterorhabditidae have been known to infect a wide range of insect pests and are one the most efficient biological control agents for soil and above-ground insect pests. In various laboratory tests, many EPN species have been shown to infect many insect species. However, following field applications, the host range may be significantly reduced based on the nematode's ecology and possible hosts. Furthermore, the pathogenicity of EPNs is influenced by various environmental and biotic factors, mainly by the species of EPN, the host insect, the life cycle of a targeted insect, and soil fauna.

The study was taken up to evaluate the bio-efficacy of the locally isolated EPNs against major insect pests in the State of Mizoram. Six agricultural pests selected for the study includes tobacco cutworm, *S. litura* (Fabricius, 1775), fall armyworm (FAW), *S. frugiperda* (J.E. Smith, 1797), the Indian cabbage white butterfly, *P. canidia* (Sparrman, 1768), the cabbage butterfly, *P. brassicae* (Linnaeus,

1758), the corm borer, *A. chalybaeus* (Hope), and banana pseudostem weevil (BPW), *O. longicollis* (Olivier, 1807).

The bio-efficacy test was performed to evaluate the insect mortality and nematode multiplication rate in host cadavers using a Petri dish assay. The different concentrations of nematodes (IJs) used for the experiment were 10, 25, 50, 100, 200, and 400 IJs/larva for *P. canidia* and *P. brassicae*; 10, 25, 50, 100, 200, 400, and 800 IJs/larva for *S. litura* and *S. frugiperda*; 10, 25, 50, 100, 200, 400, 800, and 1600 IJs/larva for *A. chalybaeus* and *O. longicolis*. For both the assays, eight (8) replicates were set for each nematode concentration, and was repeated twice. The experiment was carried out in an incubator at 28±2 °C, and insect mortality was checked every 24 hrs up to 120 hrs. Colour change, no movement, and the smell emanating from the dead body of larvae and pupae were used for primary confirmation of death due to EPNs. After 48 hrs of mortality, the dead insects were rinsed with distilled water and individually dissected under an Olympus CX41 microscope for further confirmation. Negative control plates were set for all the experiments.

To determine the multiplication rate of the EPN isolates, nematode concentrations, replicates, and negative control plates were set as in the case of the mortality assay. The mortality of the hosts was checked every 24 hrs up to 120 hrs. To allow the nematode to multiply and emerge from the host cadaver, the dead larva and pupa were individually placed on modified white traps after cleansing with distilled water to eliminate any attached external nematodes. The total number of emerged IJs on individual white traps was collected and counted under a stereomicroscope.

The EPN isolates *H. indica*, *H. baujardi*, *S. sangi*, and *S. surkhetense* showed high efficacy against the selected agricultural pests with successful host infection and multiplication. Among the isolates, *H. indica* showed the highest bio-efficacy against the pests, and thereby, represents the most potent candidate for future biological control agents in pest management. However, the overall larval mortality rate did not show significant variations with the EPN isolates and the insect pests [$F_{(1,167)} = 1.39$; $p<0.05$]. In contrast, the overall pupal mortality rate shows significant variations with the EPN species and the insect pests [$F_{(1, 63)} = 4.57$; $p<0.05$]. Several investigations have reported that the bio-efficacy of EPNs could be affected by several factors and thus were found to show different levels of effectiveness against certain insect pests. In addition, the study showed that the 4 EPN isolates successfully multiply and that higher rates of multiplication mostly occurred in the heterorhabditids i.e., *H. indica* and *H. baujardi*. Based on the statistical analysis, the overall multiplication rate showed significant variations with the EPN species and insect pests [$F_{(1,167)} = 46.09$; $p<0.05$ for the larval stage], and [$F_{(1,63)} = 10.58$; $p<0.05$ for the pupal stage]. The variations in nematode reproduction have been reported by several investigators and were found to be affected by the size of the nematodes and insect host, behaviour of the nematode species, the hermaphroditic nature of heterorhabditids, and others.

In conclusion, the study reported the occurrence of indigenous EPNs species from Mizoram and add details on the extensive distribution of *H. indica*, *H. baujardi*, *S. sangi*, and *S. surkhetense* in the world. Additionally, the isolation of 4 EPN species in Mizoram suggests that the distributional range may expand farther into Bangladesh and Eastern India, and other regions of north-eastern India.

Hopefully, the study may raise the possibility for more isolation of EPN species from the State, and paving way further deep study on their potency against regional insect pests.