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Entomopathogenic Nematodes: Their Characterization, Bio-Control Properties and New Perspectives

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Abstract

The insect parasitoid nematodes are a means boon to agronomy and serve as important bio-pesticides for controlling crop damaging insect pests. These nematodes inhabit moist soils and have been to exist in all the continents excluding Polar Regions. These nematodes have 3rd larval stage infective which is the only free living stage existing outside the host. These infective stages are mutually associated with bacteria which reside in their alimentary canal and duo are responsible for mortality of the insect host. These nematodes are currently given great attention by scientific community because of their insect killing properties and can be used to replace hazardous pesticides. These nematodes include various species belonging to genus *Heterorhabditis* and *Steinernema*, and members of insectivorous group of genus *Oscheius*. Before their use as bio-control agents, these nematodes need to be properly identified. Currently, these nematodes are characterized by using morphological and morphometrical parameters and advanced molecular tools including cross hybridization and scanning electron microscope studies. Their associated bacterial partners are studied through advanced molecular and biochemical techniques. The properly characterized nematodes having more entomopathogenic properties can be easily mass produced through *in vitro* and *in vivo* methods. They can be formulated in various carrier materials and supplied to farmers for effective control of damaging insect pests. Several countries have formulated various useful products of entomopathogenic nematodes which are available in markets for use by the farmer community and some have given very effective results. India is still at the early stage in the use of these nematodes for bio-control of insects in agronomy. More research in this field needs to be carried, especially in India to produce effective indigenous nematode products which may prove a boon for agriculture.

Keywords: *Steinernema*, *Heterorhabditis*, biological control, and pathogenicity

1. Introduction

1.1 Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) range in size from 0.3 to 10 mm and they can be more or less cylindrical [1]. In Greek vocables, the term entomopathogenic

nematodes comes from “entomos”, “insects”, “pathê”, “disease” and “guenos”, “producing” means a group of nematodes which have the ability to cause disease in insects by suppressing the immune system of insects. “Entomopathogenicity clarified: “EPNs must rapidly kill their hosts with the aid of bacterial partners and must pass on the associated bacteria to future generations” [2]. They belong to the two families, the Steinernematidae consisting of two genera, i.e. *Steinernema* (100 valid species) and *Neosteinerinema* (01 species only, *N. longicurvicauda*) [3]. The other genera Heterorhabditidae comprises of one genus, *Heterorhabditis* which contains 16 well described species globally [3] (**Table 1**). These two well-known genera, *Steinernema* and *Heterorhabditis*, have the ability of infecting and killing insects with the aid of symbiotic bacteria [91]. They are receiving a lot of interest in nematological and entomological studies because of their high virulence capacities, and able to kill the insect hosts within 24–48 hours. Besides this, they are ubiquitous and reside everywhere except Antarctica [76, 115–119].

S. No	Species	Place	Reference	S. No	Species	Place	Reference
1	<i>S. kraussei</i>	Germany	[4]	60	<i>S. ichmusae</i>	Italy	[5]
2	<i>S. glaseri</i>	New Jersey	[6]	61	<i>S. australe</i>	Chile	[7]
3	<i>S. feltiae</i>	Russia	[8]	62	<i>S. unicornum</i>	Chile	[9]
4	<i>S. affine</i>	Denmark	[10, 11]	63	<i>S. boemarei</i>	France	[12]
v5	<i>S. carpocapsae</i>	Czechoslovakia	[13]	64	<i>S. xueshanense</i>	China	[14]
6	<i>S. intermedium</i>	Carolina, USA	[15]	65	<i>S. brazilense</i>	Brazil	[16]
7	<i>S. rarum</i>	Córdoba, Argentina	[17]	66	<i>S. schliemanni</i>	Germany	[18]
8	<i>S. kushidai</i>	Shizuoka, Japan	[19]	67	<i>S. minutum</i>	Thailand	[20]
9	<i>S. ritteri</i>	Córdoba, Argentina	[21]	68	<i>S. arasbaranense</i>	Iran	[22]
10	<i>S. scapterisci</i>	Uruguay	[23]	69	<i>S. citrae</i>	South Africa	[24]
11	<i>S. caudatum</i>	China	[25]	70	<i>S. nepalense</i>	Nepal	[26]
12	<i>S. neocurtillae</i>	Florida, USA	[27]	71	<i>S. surkhetense</i>	Nepal	[28]
13	<i>S. longicaudum</i>	China	[29]	72	<i>S. lamjungense</i>	Nepal	[26]
14	<i>S. cubanum</i>	Cuba	[30]	73	<i>S. phyllophagae</i>	Florida, USA	[31]
15	<i>S. riobrave</i>	Texas, USA	[32]	74	<i>S. pui</i>	China	[33]
16	<i>S. puertoricense</i>	Loiza, Puerto Rico	[34]	75	<i>S. changbaiense</i>	China	[35]
17	<i>S. bicornutum</i>	Serbia	[36]	76	<i>S. ethiopiense</i>	Ethiopia	[37]
18	<i>S. oregonense</i>	Oregon, USA	[38]	77	<i>S. tielingense</i>	China	[39]
19	<i>S. abbasi</i>	Sultanate of Oman	[40]	78	<i>S. xinbinense</i>	China	[41]
20	<i>S. arenarium</i>	Russia	[42]	79	<i>S. cameroonense</i>	Cameroon	[43]
21	<i>S. ceratophorum</i>	China	[44]	80	<i>S. nyetense</i>	Cameroon	[43]
22	<i>S. monticolum</i>	Korea	[45]	81	<i>S. sacchari</i>	South Africa	[46]
23	<i>S. kari</i>	Kenya	[47]	82	<i>S. tophus</i>	South Africa	[48]
24	<i>S. siamkayai</i>	Thailand	[49]	83	<i>S. huense</i>	Vietnam	[50]
25	<i>S. tami</i>	Vietnam	[51]	84	<i>S. poinari</i>	Czech Republic	[52]

S. No	Species	Place	Reference	S. No	Species	Place	Reference
26	<i>S. loci</i>	Vietnam	[53]	85	<i>S. innovation</i>	South Africa	[54]
27	<i>S. sangi</i>	Vietnam	[55]	86	<i>S. jeffreyense</i>	South Africa	[56]
28	<i>S. thanhi</i>	Vietnam	[53]	87	<i>S. papillatum</i>	Venezuela	[57]
29	<i>S. pakistanense</i>	Karanchi, Pakistan	[58]	88	<i>S. beitlechemi</i>	South Africa	[59]
30	<i>S. asiaticum</i>	Pakistan	[60]	89	<i>S. pwaniensis</i>	Tanzania	[61]
31	<i>S. diaprepesi</i>	Florida	[62]	90	<i>S. fabii</i>	South Africa	[63]
32	<i>S. anatoliense</i>	Turkey	[64]	91	<i>S. nguyeni</i>	South Africa	[65]
33	<i>S. scarabaei</i>	New Jersey, USA	[66]	92	<i>S. biddulphi</i>	South Africa	[67]
34	<i>S. weiseri</i>	Czech Republic	[68]	93	<i>S. ralatorei</i>	Mexico	[69]
35	<i>S. apuliae</i>	Italy	[70]	94	<i>S. litchi</i>	South Africa	[71]
36	<i>S. guangdongense</i>	China	[72]	95	<i>S. borjomiense</i>	Georgia, USA	[73]
37	<i>S. hermaphroditum</i>	Indonesia	[74]	96	<i>S. khuongi</i>	Florida, USA	[75]
38	<i>S. jolietii</i>	USA	[76]	97	<i>S. taiwanensis</i>	Taiwan	[77]
39	<i>S. litorale</i>	Japan	[78]	98	<i>S. bertusi</i>	South Africa	[79]
40	<i>S. yirgalemense</i>	Ethiopia	[80]	99	<i>S. riojaense</i>	Spain	[81]
41	<i>S. aciari</i>	China	[82]	100	<i>S. sandneri</i>	Poland	[83]
42	<i>S. akhursti</i>	China	[84]	101	<i>S. batswanae</i>	South Africa	[85]
43	<i>S. beddingi</i>	China	[86]	102	<i>H. bacteriophora</i>	Australia	[87]
44	<i>S. robustispiculum</i>	Vietnam	[88]	103	<i>H. megidis</i>	USA	[89]
45	<i>S. silvaticum</i>	Germany	[90]	104	<i>H. zealandica</i>	South Africa	[91]
46	<i>S. ashiuense</i>	Japan	[92]	105	<i>H. indica</i>	India	[93]
47	<i>S. backanense</i>	Vietnam	[94]	106	<i>H. marelatus</i>	Oregon, USA	[95]
48	<i>S. cumgareense</i>	Vietnam	[94]	107	<i>H. taysearae</i>	Egypt	[96]
49	<i>S. sasonense</i>	Vietnam	[94]	108	<i>H. downesi</i>	Ireland	[97]
50	<i>S. eapokense</i>	Vietnam	[94]	109	<i>H. baujardi</i>	Vietnam	[98]
51	<i>S. khoisanae</i>	South Africa	[99]	110	<i>H. maxicana</i>	Mexico	[100]
52	<i>S. leizhouense</i>	China	[101]	111	<i>H. amazonensis</i>	Brazil	[102]
53	<i>S. sichuanense</i>	China	[103]	112	<i>H. floridensis</i>	Florida, USA	[104]
54	<i>S. hebeiense</i>	China	[105]	113	<i>H. Georgiana</i>	Georgia, USA	[106]
55	<i>S. costaricense</i>	Costa Rica	[107]	114	<i>H. safricana</i>	South Africa	[108]
56	<i>S. puntauvense</i>	Costa Rica	[107]	115	<i>H. atacamensis</i>	Chili	[109]
57	<i>S. texanum</i>	Texas	[110]	116	<i>H. beicherriana</i>	China	[111]
58	<i>S. cholashanense</i>	China	[112]	117	<i>H. noenieputensis</i>	South Africa	[113]
59	<i>S. colombiense</i>	Colombia	[114]				

Table 1.
List of valid *Steinernema* and *Heterorhabditis* species identified worldwide with geographical location and respective authority.

1.2 Life cycle of EPNs

Steinernema and *Heterorhabditis* share the similar life cycles. Both genera balance between a free-living stage and a parasitic stage (**Figure 1A-J**). The free-living form of EPNs is protected from the environment by an external cuticle. Being encapsulated, the invasive EPN stage, called infective juvenile (IJ) corresponding to J3, are unable to feed because their mouth and anus are sealed [120]. They rather possess huge lipid storage to be able to survive outside a host for several months [121]. With comparable lipid reserves, it has been shown that the IJs of *Steinernema* survive longer in the environment than *Heterorhabditis* IJs, which can be explained by the motile behavior of IJs. It has been found that IJs of *Steinernema* nictate between 50 and 80% of their life time while *Heterorhabditis* IJs nictate between 70 and 90% [122]. As a result of which lipid reserves are consumed faster in the IJs of *Heterorhabditis* as compared to *Steinernema*. These IJs wait for insect larvae up to 20 cm deep in soil [123]. In case of *Steinernema*, IJs invade the insect larvae through natural openings such as the mouth, anus, spiracles and wounds [124]. However, in case of *Heterorhabditis*, the IJs penetrate the insect body by directly scratching their cuticle as they are equipped with a large anterior tooth [125, 126]. Once inside the host, IJs lose their cuticle and release the entomopathogenic bacteria (EPB) and this nematode- bacterium complex together is lethal for the insect host.

The IJs feed on the dead insect cadaver and mature into the fourth stage juveniles (J4) which differentiate into males and females, generally 3 days post insect infestation. After mating, the first generation (G1) females lay eggs, either in the external

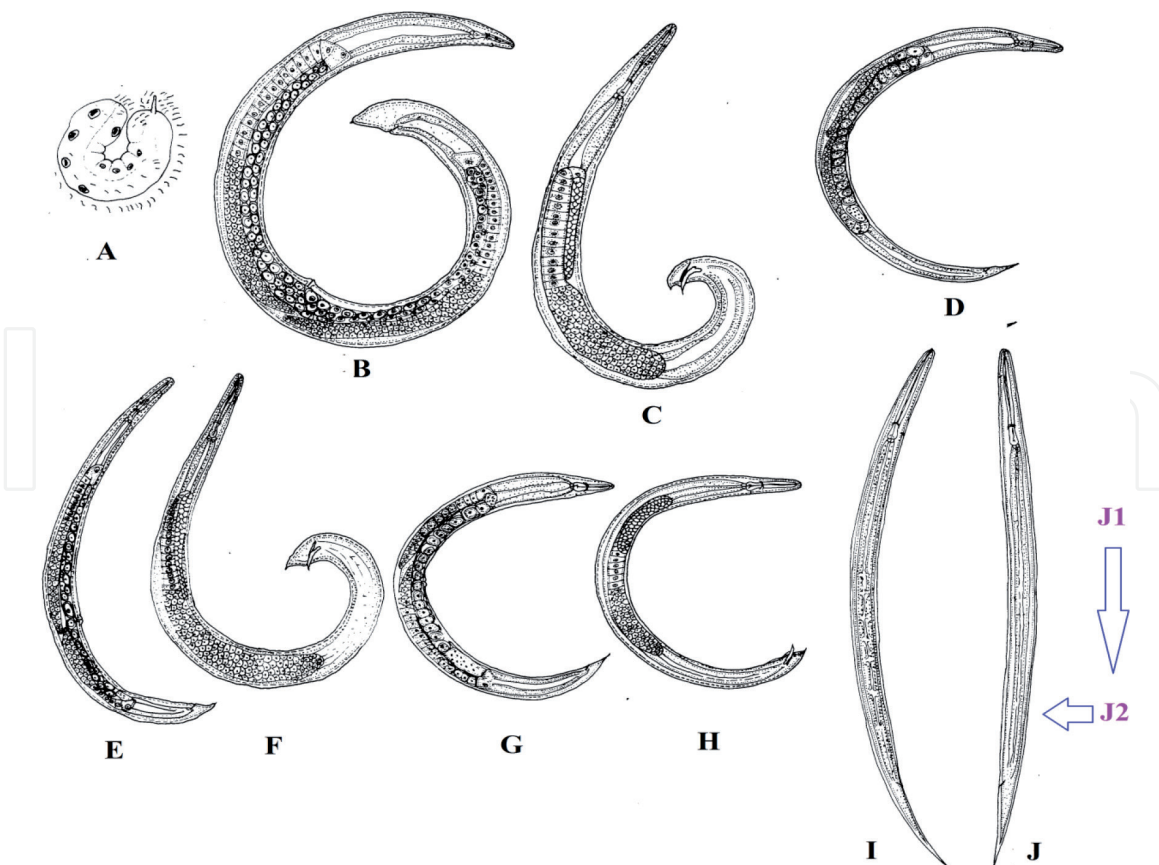


Figure 1.

A-Nematodes enters into host insect; B,C- First generation female of Steinernematidae; D- First generation hermaphrodite female of Heterorhabditidae; E,F-Second generation female and male of Steinernematidae; G,H-Second generation of Amphimictic female and male of Heterorhabditidae; I,J-Infective juvenile (IJ) stage of Heterorhabditidae and Steinernematidae.

medium or remaining in the maternal body, which hatch into the first-stage juveniles (J1). At that point, two scenarios are possible depending on the amount of food available in the insect cadaver. In case of scarce food, J1 molts into the second-stage juvenile (J2) within 2 or 3 days. Then J2 ceases to feed and molts into pre-infective stage juvenile, also called immature IJs, before becoming infective juvenile. Then the newly generated IJ emerge from the depleted insect cadaver to actively look for another susceptible insect prey. On the contrary, if food is abundant in the cadaver, then several generations of males and females can be produced in the same cadaver. After hatching from the G1 females' eggs, J1 molt successively into J2, non-infective J3 and J4 developing into the second generation (G2) adults. After mating, G2 females produce eggs that mature into J1, thereby initiating a new cycle. EPNs usually reproduce 2 or 3 generations before total depletion of the food resources in the insect cadaver occurs [124]. The entire reproductive cycle lasts between 7 and 14 days, mainly depending on temperature, after insect invasion by IJs. Both *Steinernema* and *Heterorhabditis* females lay eggs in the insect cadaver after mating with males. Juveniles hatched from released eggs often develop into amphimictic adults [127].

The reproductive life cycle of most *Steinernema* involves both sexually differentiated partners, G1 males and females whilst all *Heterorhabditis* IJs develop into self-fertilizing hermaphrodite females after insect infection [91]. However the second generation produces amphimictic *Heterorhabditis* adults. Interestingly, IJs from the species of *S. hermaphroditum* can develop into self-fertilizing hermaphrodite females just like *Heterorhabditis* IJs do. It has been argued that the uncommon feature of this *Steinernema* species supports the independent but convergent evolution with *Heterorhabditis* postulated by Poinar and described before [128]. As a consequence of the hermaphrodite reproduction of *Heterorhabditis* EPNs, the genetic diversity of offspring is highly decreased or impaired. The hermaphrodite behavior of *Heterorhabditis* allows infection by a single IJ molting into a hermaphrodite female while at least two *Steinernema* IJs have to invade an insect larva and develop into male and female [129]. Certainly, this provides a real advantage to the survival of *Heterorhabditis* species over *Steinernema* species.

Mating between males and females consists in introducing sperm to fertilize the female's eggs. Male introduces its spicule to the vulva of female and produces spermatozooids and release them in vulva. The male's sperm fertilizes female's eggs in the uterus. For hermaphrodites, sperm is produced and stored into the spermatogenic vesicles described as distal swelling of the uterus. When the female starts laying eggs, they are automatically fertilized by the sperm contained within the spermatogenic vesicles [127, 130]. Since the females are larger in size, males have to find a way to scan the entire female body to be able to find the vulva. Male finds the vulva of the female body by the two ways. These two reproductive behaviors point out another distinction between *Steinernema* and *Heterorhabditis* *i. e* males stick to a female and slips all along the female body until it finds the vulva *viz.* both *Heterorhabditis* female and male heads are pointing in opposite direction [131]. The males act like a ring around the female body *viz.* *Steinernema*. The male coils around and all along the female body until it reaches the vulva [132]. Some mechanisms do exist to avoid several males copulating with the same female. In *Heterorhabditis* species, male leaves a mating plug closing the vulva after mating preventing other males to mate with the same female [93]. In *Steinernema* species, it has been shown that virgin females produce some chemical attractants for males and their production decreases after mating [132]. However, in *S. longicaudum*, males need the presence of virgin conspecific females in their close environment [133].

After mating, a lot of eggs are retained inside the EPN maternal body, offspring hatch and start feeding inside their maternal body. This phenomenon is known as *endotokia matricida*, the term comes from two Greek words “endo”, inside and “tocos”, birth and two Latin words “mater”, mother and “caedere”, kill. *Endotokia matricida* promotes in the scarce food condition supply, then, this condition occurs for the first generation of juveniles. It becomes then obvious that the size of the susceptible insect will affect the development and survival of EPNs. Few authors reported the inefficiency of *Steinernema* IJs to control micro-insect pests [134, 135]. *Steinernema*, and *Heterorhabditis*, nematodes cannot persist for a long time in the environment if no larger insects are available to them for completing their life cycle [136].

1.3 Nematode movement and host location

The 3rd stage infective juveniles of *Steinernema* and *Heterorhabditis* move freely in soil in search of the host and have been distinguished into three categories on the basis of their host finding behaviors: (i) cruisers- species whose IJs actively move through a substrate to find a host (ii) ambushers- species that employ a ‘sit and wait’ strategy that involves little displacement and active searching and (iii) intermediates- show both the types of behaviors [137]. All *Heterorhabditis* species are cruisers [138]; however, *Steinernema* genus displays all three behaviors. *S. carpocapsae* displays ambush behavior and *S. feltiae* shows intermediate behaviors [132]. Some ambushers have the characteristic feature to stand on the substrate. At the time of standing, IJs raise a portion of the anterior section of their body off the substrate, sometimes waving it back and forth, a process referred to as ‘nictation’. This process of nictation and standing is of varying duration, commonly observed in *S. carpocapsae* and showing this phenomenon over protracted periods of time [139]. Besides this, in some species of *Steinernema*, IJs have been observed to jump which helps them in traveling longer distances [140]. This jump behavior is utilized in dissemination and might sometimes serve as a search mechanism of these EPNs to attack at passing hosts [137, 141, 142]. This jumping and/or standing behavior is advantageous in ambushers to disperse easily and bridge large pores found in some substrates (loose, porous soils or organic litter) than cruisers that do not nictate and instead move across the surface of soil particles [140, 143]. As far as cruisers are concerned, they are thought to be attracted towards host by the host volatiles and host cues from a distance [144]. Ambushers like *S. carpocapsae* do not show any change in behavior to host cues, while cruisers like *S. glaseri* does show varying behaviors [145, 146].

Only 3rd stage of EPNs is considered as infective and pathogenic which is called the infective juvenile (IJ). Infective juveniles are the only free-living stage of EPNs, while other developmental stages are only found inside infected insect hosts. The IJs are stress tolerant, non-feeding, bacterial vectoring stages that seeks out insects to infect and kill. The IJs penetrate the host insect either through natural openings like spiracles, mouth, and anus or in some species through intersegmental membranes of the cuticle, and then enter into the hemocoel [110, 125, 147]. *Heterorhabditis* species apart from natural openings also penetrate the insect host by abrading the skin. After penetrating into the skin, the IJs release cells of their symbiotic bacteria from their alimentary canal into the hemocoel (**Figure 2**). The bacteria multiply in the insect hemolymph, secrete toxins and targeted immune depressors that suppress the insect’s immune system resulting in death with 24–48 hours [148, 149]. *Photorhabdus* and *Xenorhabdus*, two well-known bacterial symbionts of EPNs, are not only lethal to entomic fauna but also prevent opportunistic bacteria and fungi from utilizing the nutrient rich cadaver, sequestering

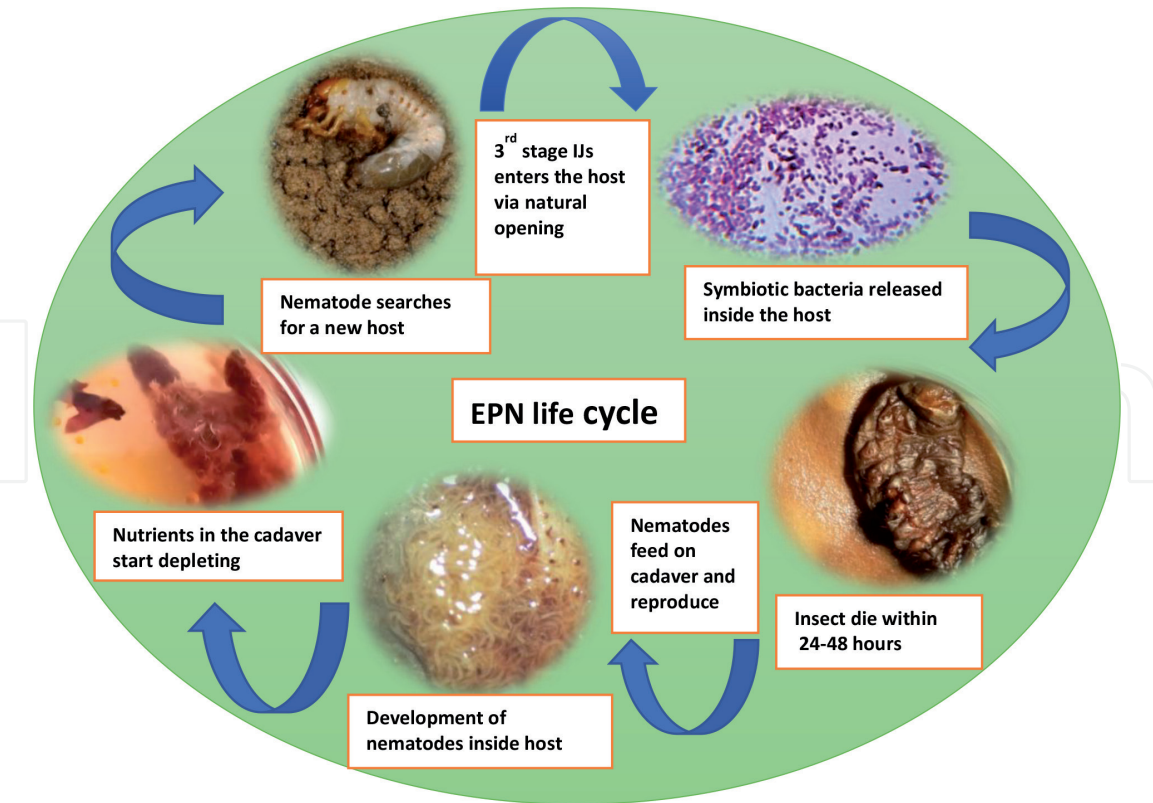


Figure 2.
Life cycle of the Entomopathogenic nematodes inside the host insect.

the resources for themselves and their nematode partners. The pathogenic bacteria always contribute to the virulence of the duo, and usually contribute the lion's share. In some species, nematodes are believed to serve as carriers of bacteria and play very little role in the death of the host, while in others, nematodes are itself responsible for the death of the host by secreting a variety of protein products that degrade and digest the host tissues, in addition to weakening the host immune system. Till date, no nonbacterial associated EPNs are known to science even if some nematodes appear lethal on their own. The nematodes, after the death of the host, continue to feed upon the bacteria and liquefy cadaver tissues, develop into mature adults (male and female) and reproduce. If there is plenty of food, the IJs develop into second generation adults and continue their life cycle. One or more generations may develop within the cadaver depending on the availability of food resources and once the food is depleted in the cadaver, a large number of IJs are eventually released into the environment to infect other hosts and continue their life cycle [150–152]. The IJs can live for weeks on stored food reserves and for months by entering a near-anhydrobiotic state. Their persistence in the soil depends on two key features *i.e.* the availability of an insect host and their progeny production in that host.

The process of reproduction in heterorhabditid and steinernematid nematodes shows few differences. The IJ of steinernematids develop into amphimictic males and females in all the adult generations (gonochorism) while in heterorhabditid nematode IJ develop into self-fertilizing hermaphrodites in the first generation and in second generation, produce males, females and hermaphrodites [153]. The insect cadaver becomes red if the insects are killed by heterorhabditids and brown or tan if killed by Steinernematids [150]. The color of the host body is indicative of the pigments produced by the monoculture of mutualistic bacteria growing in the hosts.

1.4 Taxonomy of entomopathogenic nematodes

Morphology is one of the major key components of classical taxonomy. It briefs out the genetic organization of organisms as genes themselves are expressed in the form of phenotype. *Steinernema* and *Heterorhabditis* are closely related genera under Rhabditida. Based on their morphology, they are very similar to each other, making them undistinguishable for a non-expert eye. However, detailed systematic feature keys have been developed by various nematologists and are currently used for the identification of EPN species [154]. Nematologists have provided detailed morphological differences between these two genera, including their families' that are *i.e.* used in their precise identification. These include: (i) position of excretory pore (EP) anterior to nerve ring (NR) in *Steinernema* and posterior to NR in *Heterorhabditis*, (ii) color variation in infected cadavers which appears black or no color change in *Steinernema*, while brick red in *Heterorhabditis*, (iii) cadaver showing of bioluminescence when infected with *Heterorhabditis*, whereas in *Steinernema* bioluminescence is absent, (iv) *Heterorhabditis* associated with *Photorhabdus* bacterial partners and *Steinernema* associated with *Xenorhabdus* [155].

Based on the length of IJs four 'species groups' have been created: *glaseri* group (IJ >1000 μm); *feltiae* group (IJ = 700–1000 μm); *intermedium* group (IJ = 600–700 μm); *corpocapsae* group (IJ <600 μm). Another group '*bicornutum*' have also been created, which is diagnosed by the presence of horn like structures on their labial region. The male reproductive apparatus spicule is the most discriminative features in identification of steinernematids, however in second generation males, spicules are more separated from each other [156, 157].

Adults (1st and 2nd generations) and IJs of *Steinernema* and *Heterorhabditis* show some distinctive morphological features which are important for the taxonomic point of view. These characteristics are tail length; position of excretory pore (EP) and nerve ring (NR), pharynx and neck length (PL), beside these, male acquires spicule and gubernaculum. The SEM studies of the 1st generation males reveal the comprehensive structure of gubernaculums and spicules [123, 158]; presence or absence of caudal mucron, disposition of the copulatory papillae, spermatozoon morphology [159] and presence or absence of small cuticular projections *i.e.* the epiptygmata, guarding the entrance of the of the female vagina. In case of IJs, lateral field, tail shape and length, head contour, cephalic horns etc. are some of the important characteristic of taxonomic importance [160]. Measurements and analysis of these characters play an important role in proper identification of EPNs. For example, structure of vulva gives the taxonomists a comprehensible way in recognition of species.

Now-a-days, morphological characterization does not give reliable outcomes as there has been an increase in the number of species which makes the molecular characterization mandatory for the identification of species. Morphology is entirely dependent on the external features of the specimen; however, some genes have the tendency to not express themselves in the form of phenotype although they possess some conserved regions which are very important from the taxonomic point of view. Furthermore, morphology is a tedious task and requires good skilled taxonomists with the expertise in this area. This creates a demand for the molecular identification and validation of a particular species. Advancements in the molecular techniques help in the precise identification and placement of the species in its appropriate position in the classification. A number of molecular techniques are being used for more precise identification of EPNs like immunological techniques [161]; isoenzyme patterns [162]; total protein patterns [6] and RFLP detection within total genomic DNA [163–165]. Nowadays, regions of taxonomic importance which include the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA)

repeat unit, 18S and 28S rDNA and the cytochrome oxidase subunit II (COII) are widely used for nematode identification [166–169].

With the advancement in molecular identification, techniques like polymerase chain reaction, amplification and sequencing of the amplified products of the conserved areas became possible. 28S- and 18S rDNA are used compare the distant taxa that had diverged a long time ago. Besides this, IGS, ITS1, ITS2 and ETS are being used to compare the phylogeny of closely related species as compared to 28S and 18S rDNA genes [170]. D2D3 is highly variable expansion segment of 28S rDNA, which have been used for molecular taxonomy and phylogenetic relationship of the nematodes species [171]. 18S ribosomal DNA sequences are used to find out the unknown as well as new species of the nematodes by correlating sequence variations with the genetic differences among the nematode populations [172]. Comparison of the small ribosomal RNA (18S rRNA) nucleotide sequence allows distinguishing steinernematidae from heterorhabditidae [173–175]. Due to its high variability, the ITS sequence lying between the 18S and 28S rRNA genes can be used to distinguish between *Steinernema* and *Heterorhabditis* at the species level [176, 177]. However, ITS sequence analysis is not always sensitive enough and other molecular markers may be required for better identification. The 28S rRNA gene [178], the mitochondrial cytochrome oxidase II (COII)-16S rDNA region and the ND4 mitochondrial gene have been used for that purpose [175, 177, 179].

2. Entomopathogenic nematodes as bio-agents against insect pests

India is a power house of agriculture and has made a great improvement in agriculture, but the crops are damaged by more than 10, 000 species of insects, 30, 000 species of weeds, 1, 00, 000 diseases (caused by fungi, viruses, bacteria and other microorganisms) and 1, 000 species of nematodes [180, 181]. To reduce global crop losses, it has been estimated that around US \$ 40 billion are used annually worldwide for the application of 3 million metric tons of pesticides, plus the use of various biological and other non-chemical controls worldwide [182, 183]. Out of total 70,000 estimated pests destroying 35–40% crops, insects are contributing around 14% [183]. To feed a large population of our country, the surge for production of horticultural crops is increasing day by day, due to indiscriminate, unfettered, nonjudicious and rampant use of chemical pesticides and fertilizers and without their use, it is very likely that pests would consume higher percentage and cause huge losses to productivity. A recent United Nations report (2017) assessed that 2, 00, 000 people across the world die per year from toxic exposure of pesticides and cancer problems are increasing from past few years which are directly or indirectly linked to pesticide poisoning (<https://www.aljazeera.com/news/2017/03/200000-die-year-pesticide-poisoning-170308140641105.html>). Currently, agronomists search for alternate approaches of pest control which are eco-friendly and cost effective like the use of biocontrol agents. One of the earliest examples of classical biological control targeting an insect pest in an agricultural setting is the use of the vedalia beetle, *Rodolia cardinalis*, which was introduced to citrus groves in California from Australia in the late 19th century to counteract the cottony cushion scale, *Icerya purchasi* [184]. Since then, biological control organisms such as fungi, bacteria and EPNs have been used against various insect pests [185–187]. EPNs are important biological control agents and their potential as alternatives to chemical pesticides for controlling pesky insects was recognized early on and they have been subjected to extensive laboratory and field testing [188]. EPNs are safe to most non-target organisms and the environment, are easy to apply, and are compatible

with most agricultural chemicals [149]. They also have a broad host range, ability to search for pests, and a potential to reproduce after application [149]. EPN based formulations are commercially available for pest control in home gardens and are commonly marketed as 'beneficial nematodes'. Several species of EPNs were evaluated for their pathogenicity against different pests like *Heterorhabditis bacteriophora* was noticed good control agent for controlling *Ceratiti scapitata* [189] while *H. zealandica* was tested for its ability to control *Planococcus citri*, the citrus mealy bug [190–192].

The species specific EPNs are being used worldwide as biocontrol agents under different trade names *viz.* Ecomask, Savoir Weevil larvae, Guardian, J-3 Max, Heteromask, Lawn Patrol, Scanmask, Entonem, Nemasys etc. and have provided excellent results against the entomic fauna. In United Kingdom and Europe, Bionema company and E-nema company respectively are commercially producing formulations of EPNs so as use them in biological control of different pests and earn millions of US \$ every year.

3. The future of EPN systematics: integrating molecules and morphology

Important contribution by various workers seems to be low because nematodes belong to the phylum which is taxonomically, ecologically and geographically diverse group. Nematodes usually comprise 90% of metazoan fauna and a very large number of these creatures are waiting for their discovery. Because the number of species is far from the identified species, progress in this field is still continue and new species are being added but it need tremendous research effort to know the "monopolized kingdom of nematode very well". Lack of adequate taxonomic expertise and non-availability of literature on various described species have been major constraints to identify the species of nematode parasites of insects [193].

The taxonomy of EPNs using molecular tools has made EPN systematics a lot more exciting, and probably will continue to do so in future. The rapid development of molecular techniques promoted the description of several new species and has become the technique of choice for diagnosing EPNs [194]. But morphological investigation too is important and therefore, it would be a mistake to replace traditional (morphological) methods with molecular techniques. The better procedure therefore, is the use of combination of both the approaches which offers a more resourceful perspective for resolving a variety of questions in nematode taxonomy, and particularly for EPNs. The molecular tools should be supplemented with morpho-taxometrical and hybridization tests for validation of a new species. It was found that the combined dataset of molecular and morphology represented the best working of evolutionary history for *Steinernema* [180]. It has been suggested that most morphological features are not phylogenetically informative because they represent plesiomorphic (ancestral) states or are highly homoplasious (caused by convergent or parallel evolution) [195]. For example, presence of less than 8 ridges in the lateral field of infective juveniles, a feature that has been emphasized as indicator of relationship among species, represents an ancestral state. Likewise, the absence of an epiptygma in the 1st generation female vulva is also a plesiomorphic state. Other features such as presence/absence of a tail mucron, spicule morphology, or presence of a velum in the male spicules, were depicted as highly homoplasious. Only two features, presence/absence of lamina notch and presence/absence of tail spines, had significance from a phylogenetic perspective as they were depicted as autapomorphies (unique derived characters). Most nematologists preferred molecular and morphological tools to be complementary tools in EPN systematics. Both approaches present advantages and disadvantages, and also reflect different evolutionary mechanisms,

but together provided a more comprehensive view of EPN evolution. The best approach to studying the relationship between EPN species and to determine new species is to integrate both morphological and molecular techniques [76, 196].

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Conflict of interest

“The authors declare no conflict of interest.”

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