Distribution and diversity of native entomopathogenic nematodes in poplar-based agroforestry systems

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ABSTRACT: *The identification of native insect associated nematodes that are adapted to local climatic condition is essential for the insect pest control in forestry, agroforestry and agriculture. To find out the distribution and diversity of EPNs and parasitic nematodes, soil samples were collected from Poplar growing regions of Northern provinces of India. The nematodes were isolated from collected soil samples using larvae of greater wax moth, Galleria mellonella. Entomogenous nematodes were recovered from 7.92% soil samples. The nematode species were identified by DNA barcoding procedure using D2/D3 expanse of 28S rDNA region. Out of the recovered nematode samples 78.9% were from Rhabditidae and 21.0% were from Cephalobidae family. A total six nematode species were recovered. Among these, three species namely Metarhabditis rainai, Metarhabditis amsacte and Oscheius myriophilus were facultative EPNs, two species namely Acrobeloides saeedi and Distolabrellus veechi were insect parasitic and one Mesorhabditis sp. was insect phoretic nematode. Diversity indices such as Simpson, Shannon- Weiner, Pielou's and Margalef's index were calculated and result revealed that the species richness was the same in all the three states under study, with four species recorded in each state. The obtained native facultative EPNs viz. M. rainai, M. amsacte and O. myriophilus and insect parasitic nematode A. saeedi and D. veechi may be considered as potential bio-control agent against various insect pest in forestry, agroforestry and agriculture. However, field trials are required to validate their effectiveness in the location of their occurrence in the present case.*

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1. INTRODUCTION

Nematodes are microscopic roundworms belonging to phylum Nematoda, associated with different hosts and environment. The group of nematode species that parasitize and kill insects are known as entomopathogenic nematode (EPNs) and are powerful allies in biological pest control. There are 30 nematode families associated with insects and other invertebrates, however, members from only seven families, *viz.* Allantonematidae, Heterorhabditidae Mermithidae, Neotylenchidae, Rhabditidae, Sphaerularidae and Steinernematidae are reported as potential biocontrol agents (Stock and Blair,). In addition to these, members from family Cephalobidae are also being considered as potential insect killing nematode (Salari *et al*.). Nematodes have developed a diverse trophic relationship with insects, and this association helps nematodes to expand their ecological niche (Giblin-Davis *et al*. 2013). Nematodes enable themselves to find or attack the hosts through various chemical and sensory cues. They enter into insect body via natural openings such as mouth, spiracles and anus or wounds and often penetrate through rupturing the body cavity of insects.

The nematode- insect association are categorised into four types such as phoresy, necromeny, parasitism and entomopathogeny (Mracek, 2008). In phoresy, nematode uses insect for only shelter and dissemination and not for nutritional purpose. Necromeny refers to the nematodes that are not directly involved in killing but only feeds on dead insects. Parasitism occurs when nematodes not only feeds on insects but potentially leads to death of the insect. In entomopathogeny, nematodes have developed a symbiotic relationship with bacteria which cause insect mortality and helps in nematode development. These behaviour of nematodes causes delayed development, reduced fecundity and host death. All these characteristics are strongly recommended in insect control programmes.

Families such as Steinernematidae and Heterorhabditidae are insect parasite with unique symbiotic association

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with bacteria due to which they are effective biocontrol agents in insect control programme. However, other insect parasitic nematodes genus such as *Acrobeloides*, *Metarhabditis* and *Oscheius* are also found in close association with numerous bacterial groups. Genus *Oscheius* are facultative parasite, but have been reported to establish relationship with bacterial genera *Serratia* (Lephoto *et al.* 2015). Recently genus *Acrobeloides* have been observed to be associated with different bacterial genera including *Bacillus, Alcaligenes, Enterobacter, Klebsiella,* and *Pseudomonas* (Loulou *et al.* 2023), which make it a potential nematode to be used in insect control programme. Some nematodes come under opportunistic parasite category, where they become pathogenic under specific condition such as weakened host immunity. *D. veechi* is one such nematode where it kill its host. In the present study also *D. veechi* have been observed to infect and kill the bait insect *G. mellonella*. Rhabditid nematodes are bacteriophagous in nature and are reported to be associated with invertebrates. They show necromeny *i.e.*, obtain nutrition from dead and decaying matter (Stock *et al*. 2005). Very recently some member Rhabditid genera *i.e.*, *Metarhabditis* and *Oscheius* are considered facultative entomopathogenic nematodes, which can be used as potential bio control agents (Park *et al*. 2012, Dillman *et al*. 2012). It is observed that the infective juvenile stages own some characteristic feature like short life cycle, easy culture, high fecundity and symbiotic association with pathogenic bacteria. Apart from entomopathogenic nematodes the pest population is readily be controlled by native insect killing/parasitic nematodes (Salari *et al*. 2021).

The poplar (*Populus deltoides* Bartr. Ex Marsh) based agroforestry is one of the enriched ecosystem and emerged as one of the preferred agroforestry ecosystem in Northern India. It is cultivated as a cash crop on bund/ boundary of agricultural fields along with the agricultural crops like sugarcane, rice, wheat, fodder crops etc.

In this agro-forestry ecosystem, activities of insect pest remain limited, though about 65 insect species have been associated with *P. deltoides*. There are more than 50% tree infestation and defoliation by leaf defoliator, *Clostera* spp. leading to about 66.03 % loss in tree productivity (Singh *et al*. 2004, Kumar *et al*. 2022).

Agriculture component of this ecosystem such as wheat, maize, sugarcane, pulses, and vegetables are vulnerable to a wide spectrum of insect pests, hosts diverse insect pests like- *Chilo superssalis*, *Scirpophaga incertulus*, *Helicoverpa armigera*, aphids (*Aphis gossypii*), *Spilosoma oblique*, as well as soil-dwelling and foliar-feeding pests such as cutworms (*Agrotis ipsilon*), armyworms (*Spodoptera litura*), and shoot/stem borers (*Chilo partellus*, *Sesamia inferens*). The overlapping habitat of these insect pest is well associated with the soil and the microclimatic conditions created by tree-crop interactions in these systems often advantageous to pest dynamics and natural enemy interaction, especially entomophilic nematode. The exploration of nematode diversity of poplar based agroforestry ecosystem have potential for their diversity and entomopathogenic effect.

The utilization of nematodes as biological control agents against insect pests in poplar plantations presents a promising avenue for sustainable pest management and merits further investigation. Their ability to infect and kill a wide range of insect larval and pupal stages especially soil-dwelling stages of cutworms, borers, and root grubs, makes them highly suitable for integrated pest management (IPM) in poplar-based intercropping systems. Moreover, native nematode strains adapted to local soil and climatic conditions exhibit enhanced persistence and efficacy, offering a sustainable alternative to chemical pesticides. The search for nematode associated with insect especially insect parasitic and insect pathogenic nematodes has resulted in numerous surveys all over the globe in order to find a new potential nematode that is ecologically adapted and can be used in biological control of pest.

Therefore, this study was undertaken aiming to find out information on occurrence, distribution and diversity of entomogenous nematodes in poplar growing areas in northern region of India and providing an indication of native species.

2. MATERIALS AND METHODS

Nematode isolation

The nematode collection survey was made in the poplar growing field of the northern states of India namely Haryana, Himachal Pradesh and Uttarakhand. Soil samples were collected from two distinct poplar cultivation systems *i.e.* from the intercropping of Poplar trees with agricultural crops (agroforestry), and sole Poplar plantation. Soil samples were collected on random sampling basis, where an area of 2 m^2 was marked for each sample site and soil was taken at depth of 15 cm and about 500gm soil was collected in ziplock bags. The distance of each sampling site was at interval of 20 m and details of the sample and site along with GPS location was labelled. Two hundred and forty soil samples were collected covering the main Poplar growing regions and were brought to the laboratory for isolation of nematodes.

The larval culture of greater wax moth, *Galleria mellonella* was maintained in the laboratory and were

used as a bait insect to isolate the nematodes from soil samples as described by Bedding and Akhrust (1975). Thereafter, 250 g of soil was taken in sterilised containers and fourth instar larvae of *G. mellonella* (ten no.) were placed in the container (10 × 12cm). The container was then inverted so that the larvae were covered with soil and the samples were kept in dark place at room temperature for two weeks. Soil samples were observed every 3-4 days interval for larval mortality. The dead larvae were gathered and washed with distilled water and placed in white trap for collection of emerging nematodes. The collected nematodes culture were again used to infect fresh *G. mellonella* larvae to confirm Koch's postulates of pathogenicity. After emergence of infected juveniles (IJs), they were harvested and washed with distilled water and were stored in aerated tissue culture flasks under BOD for further investigation. The illustration of methodology has been provided in Figure 1.

Nematode identification

The isolated nematodes were drawn into suspension of distilled water and the process of molecular identification was carried out at Division of Nematology, Indian Agriculture Research Institute (IARI), New Delhi. Genomic DNA was obtained from each nematode isolate using metallic beads based DNA extractor (HiPurA Pre-filled Cartridges for insect DNA Extraction Kit, HiMedia Company). The 28S rDNA region was amplified by standard PCR using the primers D2 F- 5′ ACAAGTACCGTGAGGGAAA GTTG-3′ and D3 R- 5′-TCGGAAGGAACCAGCTAC TA-3′ (Kumar *et al*. 2019). PCR was performed by using a standard optimized protocol into a final reaction volume of 25 µl having 1.0 µl, 0.5 µl of each primer (forward and reverse), 5µl of PCR buffer, 0.5µl of dNTPs and 0.1µl of Taq polymerase and made up to 25µl using nucleus free water. The PCR amplification reaction was conducted for 40 cycles as follows, initial denaturation at 95°C for 2 minutes, denaturation at 94 °C for 30 seconds, annealing at 60°C for 45 seconds, extension at 72° C at 45 seconds and a final extension step at 72° C for 10 minutes. The PCR products were confirmed by resolving on 1.2 % agarose gel electrophoresis at 90V and stained with ethidium bromide for visualisation under UV light in a gel documentation unit.

For Phylogenetic analysis, the obtained sequence of nematode isolates were edited with BioEdit version 7.2, Sequence alignment editor software. Sequences were subjected to BLAST, searched and compared to sequences deposited in NCBI for closely related species. The phylogenetic analysis was inferred using the Maximum Likelihood method based on the Tamura-Nei model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 31 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 221 positions in the

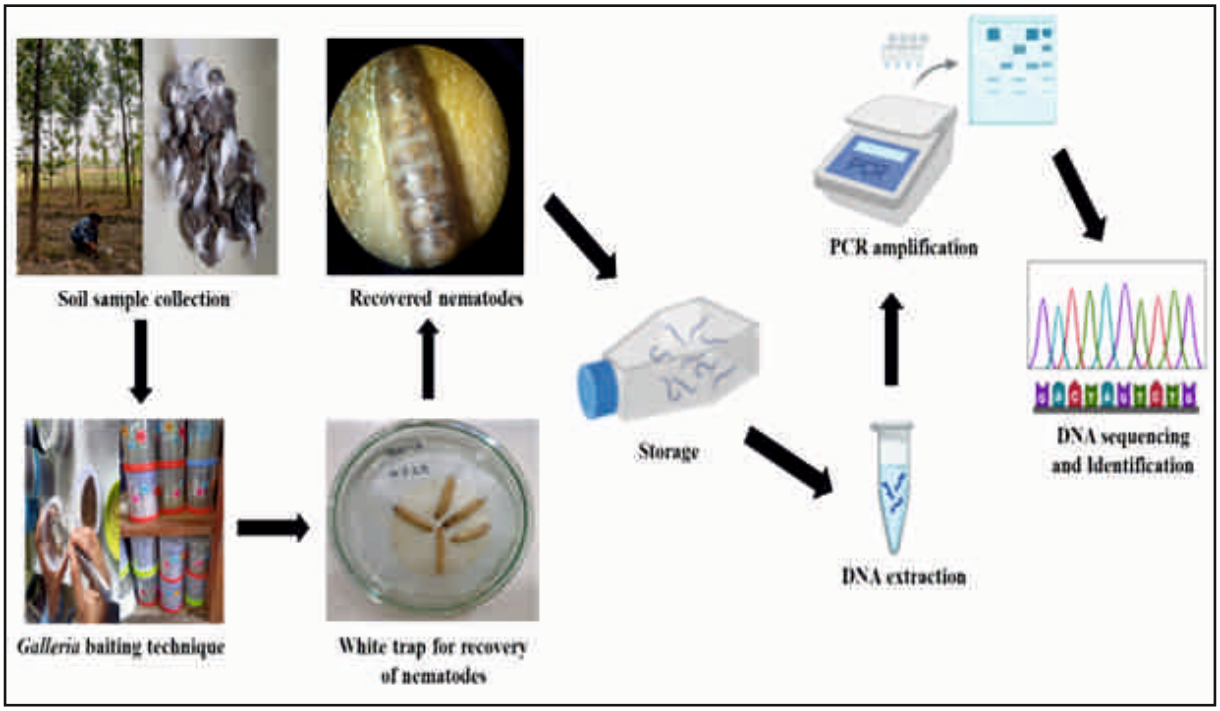

Figure 1: Illustration of methodology performed

final dataset. Tree were bootstrap 1000 times and *Caenorhabditis elagans* was selected as outgroup. Evolutionary analysis was conducted using MEGA 7.0 version.

For diversity analysis four biodiversity indices, Shannon diversity index (H), Simpson's (D), Pielou's Evenness index (J) and Margalef's index were used. The diversity was calculated by using open- access software PAST version 4.03.

3. RESULTS

The occurrence and distribution of entomogenous nematodes in the poplar growing areas of Haryana, Himachal Pradesh and Uttarakhand and entomogenous nematodes were recovered from 19 (7.9%) soil samples collected are presented in Table 1. Soil sampling sites for nematode diversity and sites with nematodes species recovered has been shown in Figure 2. Total six nematode species were identified viz. *Acrobeloides saeedi* Siddiqi et al., *Distolabrellus veechi* Anderson, *Mesorhabditis* sp. *Metarhabditis amsacte* Ali et al., *Metarhabditis rainai* Carta & Osbrink and *Oscheius myriophilus* Poinar (Figure1). Among these, *O. myriophilus* and *A. saeedi* were most diverse species observed in all three states followed by *M. rainai* (Uttarakhand and Himachal Pradesh) and *Mesorhabditis* sp. (Uttarakhand and Haryana) observed in two states followed by *M. amsactae* (Haryana) and *D. veechi* (Himachal Pradesh) observed in single state. No samples yielded more than one species of nematodes.

Sandy loamy to loamy soil was more preferred by the nematode (Figure 3). Nematode species was higher in poplar grown with agriculture crops (73.6%) than in the poplar monoculture plantation (26.3%, n = 5).). Large percentage of nematodes were observed in pH range 7.0-7.4 followed by 6.5-6.9 range. Maximum abundance of nematodes was observed in temperature range of 22.5°C-22.9°C. (Table 2).

The diversity of entomogenous nematodes was evaluated by using different diversity indices. The result revealed that the species richness was the same in all the three states under study, with four species recorded in each state. While, the state of Uttarakhand showed the highest diversity (H=1.25), followed by Haryana (H=1.22), while Himachal Pradesh (H=1.15) had the lowest. Similar pattern was also observed in Simpson index 'D' and Simpson's Diversity '1-D' (Table 3).

The phylogenetic analysis of the species of *A. saeedi*, *D. veechi*, *Mesorhabditis sp*., *M. rainai*, *M. amsactae* and *O. myriophilus* based on D2/D3 expansion rRNA gene showed a monophyly with the corresponding sequence available on GenBank, thus confirming their identification as shown in Figure 4. The edited sequence were of D2/D3 region of rDNA of nematodes and have been submitted to NCBI database. *D. veechi* NLG 39 isolate in this study have a fragment of 448 bp sequence (accession no. PQ047667.1), similarly, *Acrobeloides saeedi* isolate YNG-40 (accession no. PQ047817.1) with the

Table 1: Geographical location and habitat type of soil sample positive for entomogenous nematodes in Poplar cultivation areas of the northern region of India

S.No.	Isolate	Geographical location			Habitat type
		State	Locality	GPS coordinates	
1	PLC-5	Haryana	Panchkula	30.590997°N 77.008245°E	Poplar with intercropping
2	YNG-17	Haryana	Yamunanagar	30.361216°N 77.436894°E	Poplar with intercropping
3	YNG-28	Haryana	Yamunanagar	30.360057°N 77.437259°E	Monoculture plantation
4	YNG-38	Haryana	Yamunanagar	30.249906°N 77.423104°E	Poplar with intercropping
5	YNG-40	Haryana	Yamunanagar	30.249851°N 77.423432°E	Poplar with intercropping
6	YNG-50	Haryana	Yamunanagar	30.4358943°N 77.20717337°E	Poplar with intercropping
7	YNG-54	Haryana	Yamunanagar	30.4467559°N 77.19115532°E	Poplar with intercropping
8	YNG-62	Haryana	Yamunanagar	30.5504683°N 77.18110878°E	Poplar with intercropping
9	SRM-5	Himachal Pradesh	Sirmour	30.528437°N 77.309815°E	Monoculture plantation
10	SRM-10	Himachal Pradesh	Sirmour	30.528097°N 77.313447°E	Poplar with intercropping
11	SRM-19	Himachal Pradesh	Sirmour	30.528192°N 77.312385°E	Poplar with intercropping
12	NLG-10	Himachal Pradesh	Solan	31.041212°N 76.615546°E	Monoculture plantation
13	NLG-18	Himachal Pradesh	Solan	31.04361°N 76.617538°E	Monoculture plantation
14	NLG-39	Himachal Pradesh	Solan	31.041644°N 76.617413°E	Poplar with intercropping
15	PBS-5	Uttarakhand	Dehradun	30.343252ºN 78.006539ºE	Monoculture planation
16	GBPU-2	Uttarakhand	Udham Singh Nagar	29.127594°N 79.2842852°E	Poplar with intercropping
17	HRW-18	Uttarakhand	Haridwar	29.893608°N 78.011041°E	Poplar with intercropping
18	HRW-29	Uttarakhand	Haridwar	29.906375°N 77.992287°E	Poplar with intercropping
19	HRW-30	Uttarakhand	Haridwar	29.906032°N 77.992031°E	Poplar with intercropping

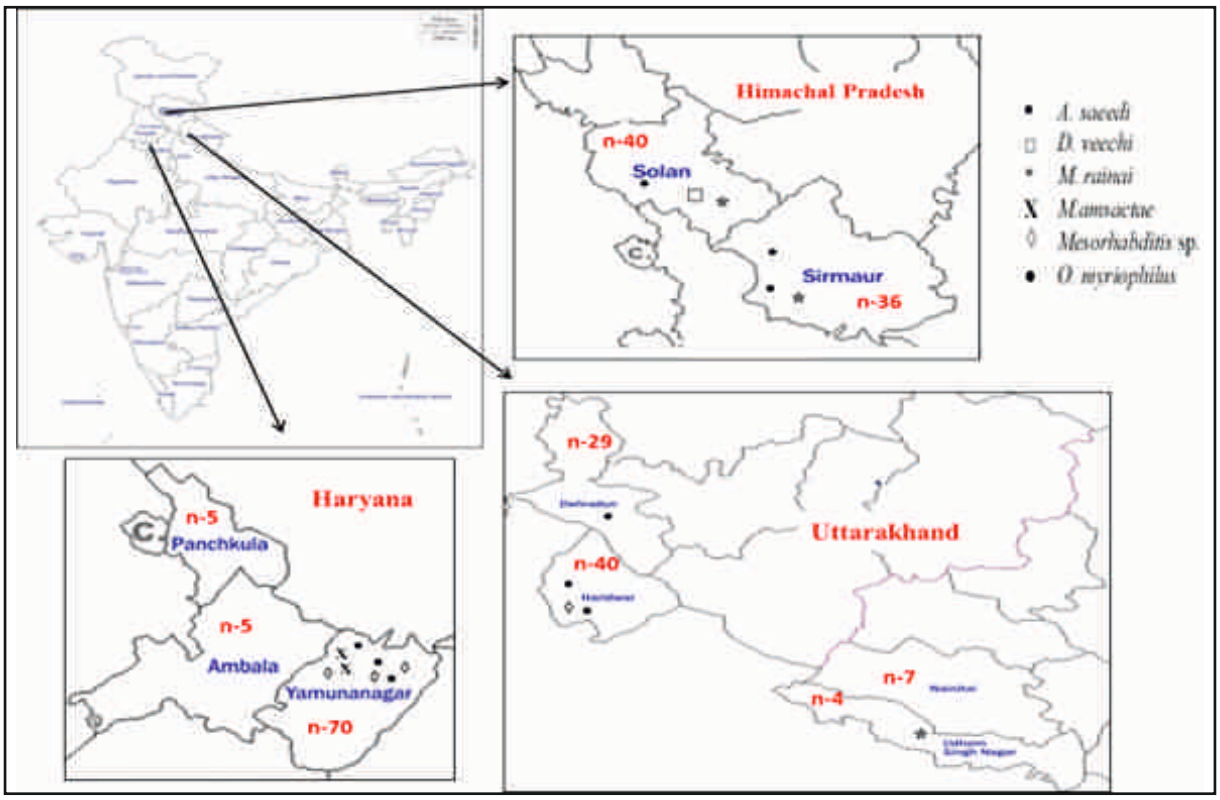

Figure 2: Soil sampling sites for nematode diversity and sites with nematodes species recovered (n= number of soil samples taken)

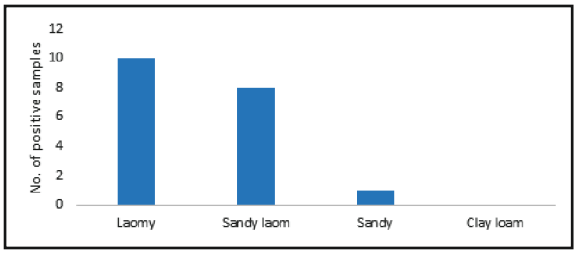

Figure 3: Recovery of entomogenous nematodes from different soil texture

fragment of 764bp, *Mesorhabditis sp*. isolate HRW29 (accession no. PQ740530.1) with the fragment length of 590 bp, *Metarhabditis rainai* isolate NLG-18 (accession no.PQ048221.1) with the fragment length of 532 bp, *Oscheius myriophilus* isolate SRM 19 (accession no. PQ740527.1) with the fragment length of 589bp.

4. DISCUSSION

Our study has documented the natural occurrence, distribution and diversity of entomogenous nematodes in Poplar growing region of Northern Province India. A total of six nematode species were identified namely *Metarhabditis rainai, Metarhabditis amsacte, Oscheius myriophilus*, *Acrobeloides saeedi*, *Distolabrellus veechi* and *Mesorhabditis* sp. The use of 28S rDNA region was helpful in identifying the nematode isolates and this may be used for detection of new native nematode species. The phylogenetic tree obtained from the 31 nucleotide sequences of the expansion of D2/D3 with six distinct groups support the molecular identification of the nematodes isolates from soil. The identification of native entomogenous nematodes associated with poplar plantations and agroforestry systems is critically important for the development of effective biological control strategies targeting insect pests, particularly those affecting poplar trees and associated agricultural crops. The native nematodes provide new insight to the ongoing control strategies as these nematodes are ecologically adapted to the environment when compared to the non-native nematodes.

Soil texture is crucial factor for the distribution of nematodes in soil. In our survey the majority of positive sample were recovered from loam followed by sandy loamy and there was no recovery from clay-loam soils. This might be due to lager pore size in loamy and sandy loamy soil that provides better aeration, physical mobility and moisture condition. Similar results have been reported by other researchers (Lankin *et al.* 2020). Entomogenous nematodes exhibit differential tolerance of pH and temperature range depending upon the environmental conditions (Khathwayo *et al.* 2021). In our study insect phoretic nematode *Mesorhabditis* sp. was observed in soil with pH 7.4 and temperature 24°C. While insect killing nematode, *D. veechi* was observed at 22°C.

G. mellonella insect baiting trap is useful in detection of species in families Steinernematidae,

Table 2: Recovery of entomogenous nematodes at different variables

Category	Percent positive samples	Number of species identified
Habitat type		
Poplar with intercropping	73.6	6
Monoculture Plantation	26.3	3
Nematode family		
Rhabditidae	73.6	4
Cephalobidae	21.0	1
Mesorhabditidae	5.2	1
Soil pH		
5.0-5.4	10.5	2
5.5-5.9	0	0
6.0-6.4	10.5	2
6.5-6.9	31.5	2
7.0-7.4	47.3	5
Soil temperature		
≤22.4	5.2	1
22.5-22.9	47.3	4
23.0-23.4	15.7	3
23.5-23.9	26.3	3
≥24.0	5.2	1

*Percent positive samples = number of positive sample per category/ total positive samples

Table3: Index for nematode diversity isolated from Poplar growing regions of North western part of India

Index	State		
	Haryana	Himachal Pradesh	Uttarakhand
Simpson index 'D'	0.321	0.329	0.312
Simpson's Diversity '1-D'	0.678	0.670	0.688
Shannon Diversity Index 'H'	1.22	1.15	1.25
Evenness J	0.853	0.793	0.873
Margalef's M	0.365	0.356	0.384

Heterorhabditidae and Rhabditidae as well as Cephalobidae. In our survey Rhabditidae was most dominant family with 73.6% followed by Cephalobidae with 21.0% and least was Mesorhabditidae with 5.2%. However, species from Steinernematidae and Heterorhabditidae were not recorded. The absence of *Steinernema* and *Heterorhabditis* may be due to the low soil sample collected. The other reason may be that *Steinernema* and *Heterorhabditis* are more host specific or the failure of these to parasitize and infect the insect host *Galleria*. Therefore, this study also recommends the incorporation of alternative bait insect apart from *Galleria*, like other lepidopteran and coleopteran host, which may improve the recovery, detection, and assessment of the diversity and distribution of entomopathogenic nematodes functioning as natural biological control agents of insect pests. The presence of facultative nematodes *M. amsactae*, *M. rainai*, and *O. myriophilus* may play an important role in insect control particularly in areas where *Steinernema* and *Heterorhabditis* species are absent. Rhabditid nematodes have been recovered from different habitat such as insect carrion, insect tunnels and associated with insects like beetles, moths, grasshoppers etc. (Mahboob and Tahseen, 2024). However, apart from these habitat they are widely distributed in soil as well. In this study *M. rainai* have been recovered from soil of Himachal Pradesh and Uttarakhand. *M. rainai* has wide distribution as they have been recovered from broad habitat such as fruit orchards, agricultural field and forest area (Bhat *et al.* 2020, Morais *et al.* 2020, Danso *et al.* 2024). *M. amsactae* have been detected from agricultural field in Punjab (Kour *et al.* 2024). *O. myriophilus* has been reported from gastropods (Lazink *et al.* 2023). Besides *O. myriophilus* was also

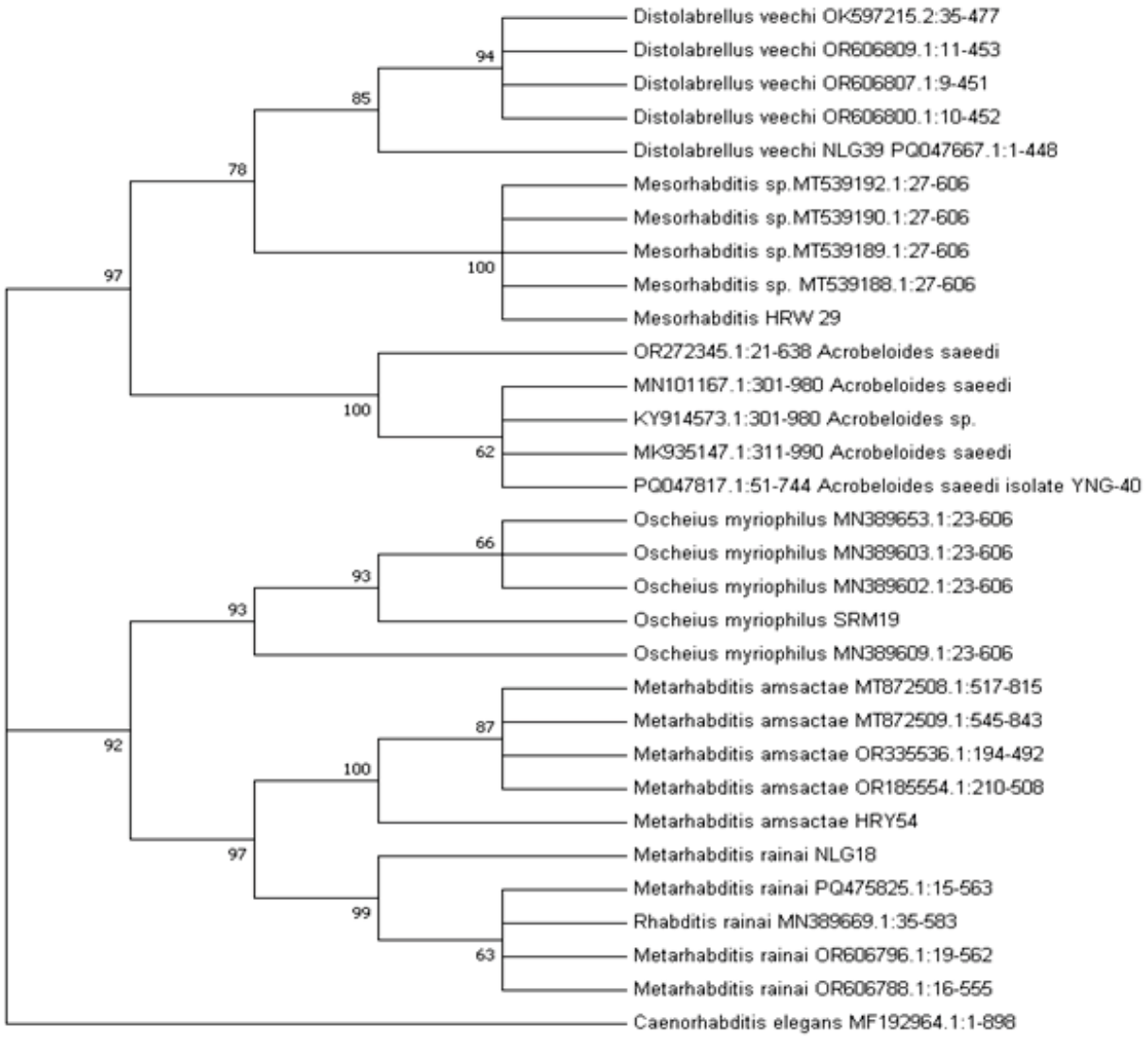

Figure 4: Phylogenetic tree showing relationship between the nematode isolated and their similarity with those from the GenBank based on partial rDNA sequences of 28S region

undisturbed soil in Thailand (Ghavamabad *et al.* 2021, Onwong *et al.* 2023). In the present study, *Oscheius myriophilus* was one of the most encountered species and was isolated from all three states with a preference for loamy to sandy-loamy soil, similar with the study conducted by Castro-Ortega et al (2020). *Acrobeloides* is widely is distributed nematode group recovered from various habitat such as agricultural fields, forest soil as well as from xeric conditions (Kim *et al.* 2021, Thakar *et al.* 2022, Abolafia *et al.* 2024). And in the present study *Acrobeloides* is also recovered from all the states. *D. veechi* is bacteriophagous free-living nematode which also could parasitize insects. In the present study, insect killing nematode *D. veechi* was observed from Himachal Pradesh. However, it has been recovered from soils of Kishanpura, Punjab (Parihar *et al.* 2016, 2019), agricultural soils in Uttarakhand and Uttar Pradesh, India (Bhat *et al.* 2020) forested areas in Guilan province, northern Iran (Jalainasab *et al.* 2025). It is to emphasise that the occurrence of insect phorectic nematode *Mesorhabditis* sp. was also observed in Uttarakhand and Haryana. Species of *Mesorhabditis* are reported to be associate with elytra and genitalia of various insects (Mahboob and Jahan, 2021).

Diversity indices offer crucial details regarding the prevalence and rarity of species within a population. The species diversity varied in the study because the diversity indices consider not only the number of species, but how evenly the individuals are distributed. The higher species diversity of nematodes in Uttarakhand may be due to availability of suitable host, and their ecological interaction. Haryana, though having same richness, exhibit moderate diversity which may be due to intensive agricultural practices in the field which favour only certain nematode population. Himachal Pradesh,

while having the same number of species, exhibited lower diversity may be due to higher elevation could reduce insect abundance, limiting nematode populations. This similarity is likely attributed to comparable edaphic and climatic conditions poplar growing areas which contribute to a consistent and favourable habitat supporting similar nematode communities. The results also revealed a higher proportion of nematodes recovery from the Poplar–agriculture intercropping system compared to the Poplar monoculture plantation. This may be due to more favourable soil conditions, elevated organic matter content, and the increased availability of suitable insect hosts within the diversified cropping environment. The nematode diversity collected so far may not cover exhaustively all the geographical areas of poplar growing regions. Though, still make significant contribution to knowledge of insect associated nematode occurrence and distribution in this region. Also, the native nematodes (three facultative EPNs *M. amsacte*, *M. rainai* and *O. myriophilus* and two insect killing nematodes *A. saeedi* and *D. veechi*) obtained from the study may be further evaluated as bio agent to control the insect pests under local conditions.

5. CONCLUSION

The present work is important as it documents the occurrence and diversity of entomopathogenic nematodes, insect killing nematodes and phoretic nematodes from poplar based agroforestry. The soil and climatic conditions in these areas creates a favourable habitat for different nematode species. Moreover, further surveys are still required to ascertain full spectrum of nematode species present in the studied areas with aim to identify more number of ecologically adapted native nematode species. The reported nematode may be screened against different pests of agroforestry and agriculture crops for their potential efficacy as biological control agent.

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