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DISTRIBUTION, VIRULENCE AND CHARACTERIZATION OF ENTOMOPATHOGENIC NEMATODES FROM VEGETABLE-GROWING REGIONS OF TELANGANA INDIA

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ABSTRACT

Entomopathogenic nematodes (EPNs) are globally distributed biological control agents capable of infecting a wide range of insect hosts. To explore native EPN diversity for eco-friendly pest management, a soil survey was conducted across four districts of Telangana. Out of 80 soil samples collected, three were positive for EPN infestation in *Galleria mellonella* larvae. The infected cadavers exhibited a characteristic brick-red coloration, confirming the presence of *Heterorhabditis* spp., with an incidence of 5% in Vikarabad and Sangareddy districts, and 3.33% in Rangareddy district. *In vitro* bioassays against *Plutella xylostella* revealed that larval mortality increased with both nematode concentration and exposure time, with isolate EPN1 exhibiting the highest virulence, achieving 100% mortality at 750–1000 IJs/ml within 72 hours. Molecular characterization identified EPN1 as *Heterorhabditis indica*, and its symbiotic bacterium as *Photorhabdus luminescens*.
***Keywords*:** Cabbage, *Heterorhabditis indica*, *Photorhabdus luminescens*, *Plutella xylostella*, management.

Introduction

Cabbage (*Brassica oleracea* var. *capitata*) belongs to family Brassicaceae is a second most important crop after cauliflower. It is a leafy winter vegetable crop which grown in almost all parts of India on all types of soils with 5.5- 6.5 pH range (Chadha, 2006). It is mostly used for culinary purposes and also consumed as salad, boiled as well as dehydrated vegetable. Diamond back moth (DBM) (*Plutella xylostella* L.) (Lepidoptera: Plutellidae) is the most serious pest of cabbage causing huge economic losses of more than one billion US dollars in terms of annual management costs (Furlong and Zalucki, 2024). There is no effective management strategy that works effectively against DBM as it became a difficult pest to manage. Therefore, the development of novel and effective management programmes has always been a priority amongst researchers working on this insect. In recent years, entomopathogenic nematodes, have emerged as potent insect controlling bio-agents.

Entomopathogenic nematodes (EPNs) are soft-bodied roundworms that function as obligate or, in some cases, facultative parasites of insects, primarily those belonging to the orders *Lepidoptera* and *Coleoptera*. They have been effectively utilized in various biological control strategies, including inundative, augmentative, and inoculative approaches, for the management of diverse agricultural pests (Feyisa, 2021). Among the different groups of EPNs, the genera *Steinernema* and *Heterorhabditis* (Rhabditida: Steinernematidae and Heterorhabditidae) are of major importance, as they are known to parasitize a wide range of insect hosts globally (Hominick, 2002). The infective juvenile (IJ) stage represents the non-feeding, free-living phase responsible for locating and infecting insect hosts.

Upon encountering a susceptible host, the nematode penetrates through natural body openings such as the mouth, anus, or spiracles, or through regions with thin cuticle, typically within 24–72 hours. The nematode's symbiotic bacteria residing in its intestinal lumen are released into the host hemocoel during infection (Laznik *et al*., 2011). Once inside, these bacteria rapidly multiply and produce a range of virulence factors, including toxins, hydrolytic enzymes, hemolysins, and antimicrobial compounds (Eleftherianos *et al*., 2010). These factors not only kill the host but also create a nutrient-rich environment that supports nematode growth, reproduction, and subsequent emergence of new infective juveniles from the cadaver. Gupta *et al*. (2011) evaluated a native strain, *Steinernema carpocapsae* collected from JMU against *Plutella xylostella* on cauliflower and recorded the larval mortality of 52.3% and 51.6% at 14 days post-application over two consecutive years applied at 2 billion IJs ha^{-1}.

Regional surveys are vital for understanding the biogeography and diversity of native entomopathogenic nematodes (EPNs). They help identify locally adapted strains suited to specific soil and climatic conditions, enhancing their biocontrol potential. Such surveys also reveal species distribution patterns and ecological preferences. Hence, localized exploration supports sustainable pest management strategies (Shapiro Ilan *et al*., 2012).

Traditional methods for identifying EPN species have relied heavily on morphological traits, such as body length, tail shape, and reproductive structures. However, due to morphological plasticity and overlapping characteristics among closely related species, accurate identification based solely on morphology can be difficult and sometimes unreliable. Consequently, molecular tools have become indispensable in EPN taxonomy and systematics. Genetic markers such as the internal transcribed spacer (ITS) and the D2–D3 region of the 28S rRNA gene are commonly employed to delineate species boundaries and infer phylogenetic relationships among *Steinernema* and *Heterorhabditis* species (Spiridonov and Subbotin, 2016).

Materials and Methods

Survey for the collection of soil samples

A field survey was conducted over five months (August–December 2024) across four districts of Telangana-Sangareddy, Rangareddy, Vikarabad, and Medchal-Malkajgiri. Soil samples were collected from irrigated vegetable-growing regions, with two representative sites each from Sangareddy and Vikarabad, three from Rangareddy, and one from Medchal–Chevella (Fig. 1). From each selected site, 10 soil samples were randomly collected, resulting in a total of 80 soil samples (Table 1). Soil samples with 40–60 per cent moisture were collected using the zig-zag pattern, at a depth of 15–25 cm. Approximately 2 kg of soil was collected from each field, maintaining a minimum distance of 10 m between consecutive sub-samples, while avoiding a 2 m peripheral area. Sub-samples from each site were thoroughly mixed, placed in labelled polythene bags indicating the date, location, and soil and crop type.

The quadrant selection method was used to obtain a representative soil sample. Initially, the collected soil was spread on a polythene sheet and cleared of pebbles, stones, and plant debris. The soil was then divided into four equal quadrants, and two opposite quadrants were randomly selected while the remaining two were discarded. This process was repeated by subdividing the selected soil into four quadrants, again choosing two and discarding two, until a representative sample of 250–300 g was obtained. To isolate entomopathogenic nematodes (EPNs), the representative soil samples were baited using larvae of the greater wax moth, *Galleria mellonella*.

Rearing of *G. mellonella*

Greater wax moth (*G. mellonella*) larvae were used as a model insect for the isolation of EPNs. *G. mellonella* larvae were reared under laboratory conditions (27± 2℃ temperature; 65 ± 5% RH; L12: D12 photoperiod), as per the method suggested by Ellis *et al*. (2013) on artificial diet.

Soil baiting with *G. mellonella*

About 200 g of the representative soil samples was placed in a plastic box and baited with fifth-instar larvae of *G. mellonella* (Bedding and Akhurst, 1975). The boxes were stored in a dark environment at 26 ± 2 ℃ to facilitate nematode infection. After five days, the dead larvae were collected and transferred to white traps to confirm the presence of EPNs and to harvest the emerging IJs.

Isolation of Entomopathogenic Nematodes (EPNs)

Infected cadavers were identified based on characteristic colour changes. *Heterorhabditis* infections typically resulted in cadavers that were brown to dark brick-red, whereas *Steinernema* infections produced cadavers that were brown to ochre-coloured. Cadavers exhibiting these symptoms were processed using the Modified White Trap method (Dutky *et al.,* 1964) for nematode recovery. A smaller Petri dish (50–60 mm diameter) containing a single

circular Whatman no. 1 filter paper was placed inside a larger dish (90 mm diameter) filled with 20 mL of sterile distilled water. Infected cadavers were placed on the filter paper without touching each other to prevent contamination, and no water was added to the smaller dish containing the cadavers. This setup was covered and maintained at room temperature until the emergence of infective juveniles (IJs), which typically took 7–10 days depending on the species.

IJs were harvested by carefully removing the larger dish and pouring the water containing nematodes into a beaker, allowing them to settle at the bottom. This process was repeated 2–3 times with fresh water to clean the suspension. The final nematode suspension was stored in 250 mL tissue culture flasks at concentrations of 1,000–3,000 nematodes/mL in an incubator at 10–20 °C. Stored flasks were periodically monitored for their shelf life.

In vitro efficacy of EPNs against Diamond Back moth, *Plutella xylostella*

Three native EPN isolates obtained from the survey were used for the *in vitro* study. Third-instar larvae of *P. xylostella* were used for the pathogenicity assays. About 10 larvae were placed in each experimental plate, and the respective EPN isolates were added at different concentrations *viz*., 250, 500, 750, and 1000 IJs to each plate. The plates were incubated at 26±2 °C. A plate containing the *P. xylostella* alone served as a control. The experiment was laid out in a Completely Randomized Design with four replications per treatment. Observations on larval mortality were recorded at 24-hour intervals up to 72 hours.

Molecular characterisation of effective EPN isolate

The most effective EPN isolate identified from the *in vitro* study and its symbiotic bacteria was characterized at the molecular level. Individual hermaphrodites were washed with Ringer's solution and transferred to sterile Eppendorf tubes containing 20 µL of extraction buffer (17.6 µL nuclease-free water, 2 µL 5× PCR buffer, 0.2 µL 1% Tween, and 0.2 µL proteinase K). Samples were frozen at −20 °C for at least 1 h, then incubated at 65 °C for 1 h and 95 °C for 10 min in a thermocycler. The lysate was cooled, centrifuged at 6500 g for 3 min, and the supernatant used as the DNA template.

The ITS region (ITS1–5.8S–ITS2) of rRNA was amplified using primers 18S (5′-TTG ATT ACG TCC CTG CCC TTT-3′) and 28S (5′-TTT CAC TCG CCG TTA CTA AGG-3′). PCR reactions contained 1 µL DNA, 15.25 µL Emerald Amp GT Master Mix, 0.75 µL each primer, and 7.25 µL dH_2O. Amplification was performed with pre-denaturation at 95 °C for 5 min; 35 cycles of 94 °C (1 min), 55.4 °C (30 s), 72 °C (1 min); and a final extension at 72 °C for 10 min (Vrain *et al*., 1992). PCR products were visualized by agarose gel electrophoresis (1%, 100 V, 45 min) and sequenced bidirectionally (Medauxin, Bengaluru, India). The obtained sequences were curated and submitted to NCBI. Species identification was done by BLASTN analysis (≥ 97% similarity threshold). Sequences were aligned using Clustal X2, and phylogenetic relationships were inferred using the Neighbourhood-Joining method in MEGA v11.

Molecular characterization of Symbiotic bacteria

Galleria mellonella cadavers infected with *Heterorhabditis* sp. were collected 24–48 h post-infection, surface-sterilized with 70% ethanol, and air-dried for 2 min. Larvae were dissected using sterile needles and forceps without damaging the gut epithelium, and haemolymph from the ventral thoracic region was streaked onto NBTA medium (nutrient agar with 0.004% TTC and 0.00025% bromothymol blue) (Akhurst, 1980). Plates were incubated at 28 °C for 24–48 h, where *Photorhabdus* colonies appeared dark blue or green (Orozco *et al.,* 2013). Single colonies were subcultured on NBTA to obtain pure cultures, which were later grown in yeast-salts broth on an orbital shaker (180 rpm, 25 °C, 24–48 h).

Genomic DNA was extracted from 48 h-old bacterial cultures using the CTAB method (Wilson, 2001) and PCR amplification was performed using 1 µL of genomic DNA, 15.25 µL EmeraldAmp GT PCR master mix, 0.75 µL of each primer (27F: 5'-AGAGTTTGATCCTGGCTCAG -3', 1492R: 5'-ACGGCTACCTTGTTACGACTT -3'), and 7.25 µL dH_2O. PCR products were sequenced (Eurofins Advinus, Hyderabad) and submitted to GenBank for accession numbers. Species identification was carried out using BLASTN analysis (≥97% identity threshold). Sequences were aligned using Clustal X2, and phylogenetic relationships were inferred by the Neighbourhood-Joining method in MEGA v11.

Results and Discussion

Distribution of EPNs in the growing regions of Telangana

A total of 80 soil samples representing different soil types were collected from four districts of Telangana. Out of these, three samples showed *Galleria mellonella* infection by entomopathogenic nematodes (EPNs) (Table 2). The infected cadavers exhibited a characteristic brick-red coloration,

confirming the presence of *Heterorhabditis* sp. Region-wise, one positive sample each was recorded from Pasthapur–Nadikudi (Sangareddy district), Gollapally–Rajendranagar (Rangareddy district), and Kothapally–Kothagudi (Vikarabad district), whereas no EPNs were recovered from the soil samples collected from Shamirpet (Medchal-Malkajgiri) and Chevella (Rangareddy) regions.

The overall occurrence of EPNs in the surveyed regions was highest in Vikarabad and Sangareddy districts (5%), followed by Rangareddy (3.33%). These results are comparable with previous findings of Lorio *et al.* (2005), who reported 20.5% EPN occurrence, while Negrisoli *et al.* (2010) and Myers *et al.* (2015) recorded 15.7% and 21%, respectively. In Telangana, Sunanda *et al.* (2016) observed a lower incidence of 1.38% across different crops. Other studies from India (Singh *et al*., 2015; Chand *et al.,* 2016) have also confirmed a wide distribution of EPNs as natural biocontrol agents against lepidopteran pests. Similarly, Dobariya *et al.* (2022) reported 12% positive samples dominated by *Steinernema* sp., and Prajapati and Maru (2022) found 15% samples positive for both *Steinernema* and *Heterorhabditis* spp. in Gujarat.

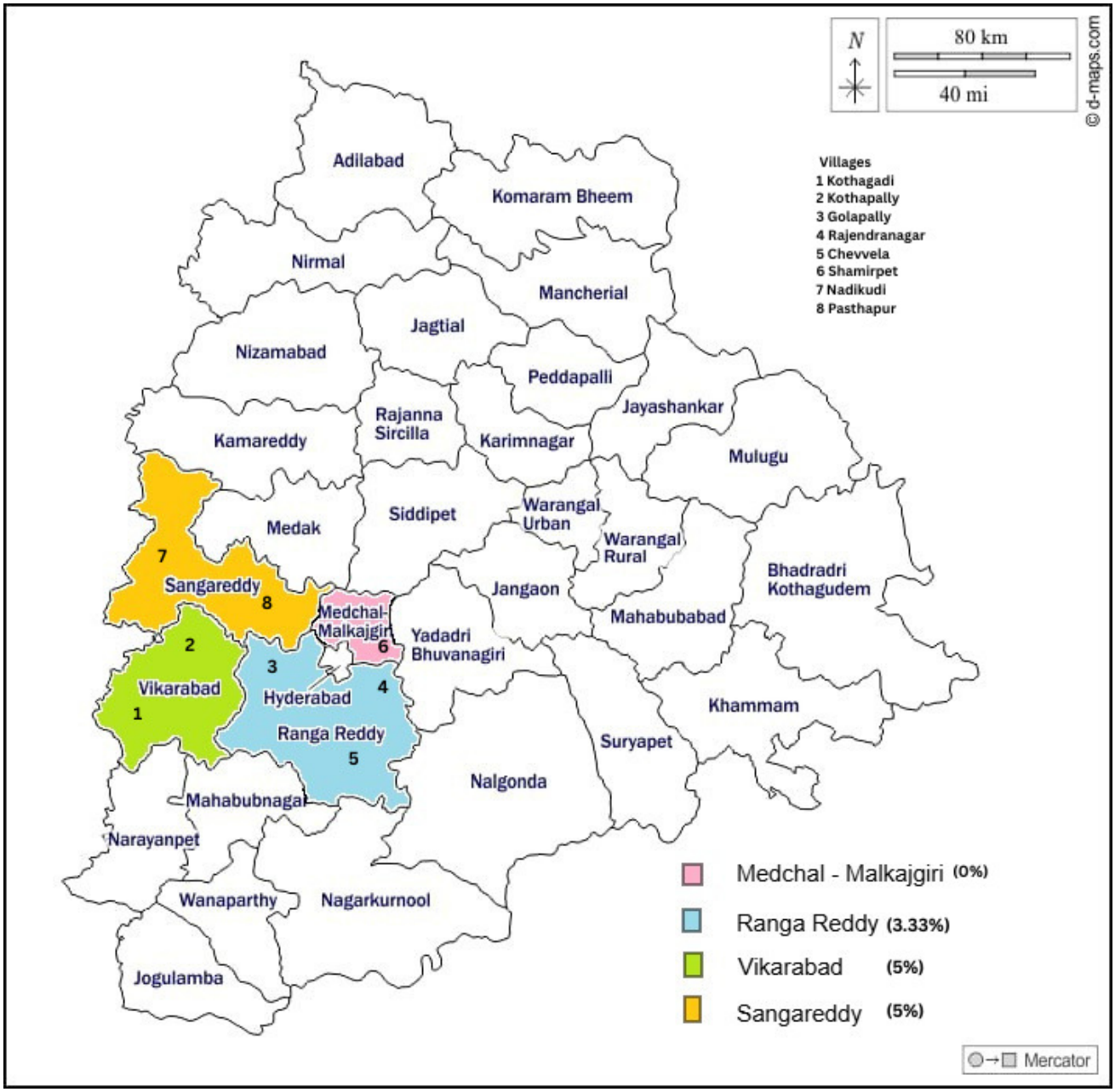

Fig. 1 : Distribution pattern of Entomopathogenic Nematodes across vegetable-growing regions of Telangana, indicating surveyed villages (1 to 8) and their per cent occurrence

Table 1 : Survey for the isolation of Entomopathogenic Nematodes in different vegetable growing areas of Telangana

S. No.	District	Village	No. of soil samples collected	Crop	Samples positive for the presence of EPN	Per cent occurrence of EPNs in each district	EPN species based on visual observations
1.	Vikarabad	Kothagadi	10	Chilli	0	5	*Heterorhabditis* sp.
		Kothapally	10	Tomato	1		
2.	Rangareddy	Gollapally	10	Cabbage	0	3.33	*Heterorhabditis* sp.
		Chevvela	10	Cabbage	0		
		Rajendranagar	10	Jasmine	1		
3.	Medchal Malkajgiri	Shamirpet	10	Tomato	0	0	-
4.	Sangareddy	Nadikudi	10	Ginger	0	5	*Heterorhabditis* sp.
		Pasthapur	10	Potato	1		

Total 80

Table 2 : Positive samples detected areas

S. No.	District	Village	Crop	Samples positive for the presence of EPN	EPN species based on visual observations
1.	Vikarabad	Kothapally	Tomato	1	*Heterorhabditis* sp.
2.	Rangareddy	Rajendranagar	Jasmine	1	*Heterorhabditis* sp.
3.	Sangareddy	Pasthapur	Potato	1	*Heterorhabditis* sp.

In vitro* effect of EPN isolates against *Plutella xylostella

The *in vitro* bioassay results (Table 2) revealed that all three native EPN isolates caused varying levels of mortality in *Plutella xylostella* larvae, depending on the concentration and exposure time. Among the isolates, EPN1 showed the highest virulence, achieving 75% mortality at 250 IJs/ml after 72 h and 100% mortality at concentrations of 750 and 1000 IJs/ml within 72 h. EPN2 exhibited moderate efficacy, with 75% mortality at 250 IJs/ml after 72 h and complete mortality at 750 and 1000 IJs/ml after 72 h. EPN3 was comparatively less virulent, causing 45% mortality at 250 IJs/ml and 90% mortality at the highest concentration (1000 IJs/ml) after 72 h. No mortality was observed in the control treatments. Overall, mortality increased with both nematode concentration and exposure time, with EPN1 emerging as the most effective isolate against *P. xylostella*.

Sunanda *et al*. (2014) reported that laboratory bioassays using various formulations of *Steinernema carpocapsae* against fourth instar larvae of *Plutella xylostella* resulted in 100% larval mortality within 72 hours at an inoculum level of 1000 IJs. Similarly, Zolfagharian *et al*. (2016) evaluated the efficacy of *S. carpocapsae* and *Heterorhabditis bacteriophora* against *P. xylostella* larvae and found that larval mortality increased with increase in nematode concentration, recorded 54 and 14% mortality at 50 IJs/ml and reached 84 and 100% mortality at 1600 IJs/ml for *S. carpocapsae* and *H. bacteriophora*, respectively. These findings indicate a positive correlation between inoculum density and larval mortality, demonstrating the high pathogenic potential of both EPN species, particularly *H. bacteriophora*, against DBM larvae.

Table 2: *In vitro* effect of EPN isolates against *Plutella xylostella*

Isolate	Concentration (IJs/ml)	Per cent mortality		
		24h	48h	72h
EPN1	250	25	50	75
	500	73	85	90
	750	90	95	100
	1000	100	100	100

	Control	0	0	0
EPN2	250	15	35	75
	500	30	70	90
	750	80	90	100
	1000	90	100	100
	Control	0	0	0
EPN3	250	0	25	45
	500	15	35	55
	750	35	57	78
	1000	50	66	90
	Control	0	0	0
CD(p≤0.01)	**-**	9.7	11.2	12.4

Molecular Characterization of effective EPN and its symbiotic bacteria

Molecular characterization identified the effective EPN isolate as *Heterorhabditis indica*, exhibiting characteristic developmental traits of this well-known entomopathogenic nematode group. Its symbiotic bacterium was confirmed to be *Photorhabdus luminescens* (Table 3). BLAST analysis (NCBI BLAST 2.16.0) supported these identifications, showing 100% sequence similarity with reference sequences, thereby validating the molecular confirmation.

Accurate identification is essential for understanding the geographical distribution and habitat specificity of organisms. While EPNs have traditionally been identified through morphological traits, molecular techniques now offer faster and more reliable alternatives. PCR-based methods eliminate the need for culturing, providing rapid, sensitive, and versatile identification (Lee *et al.,* 1993). DNA sequence analysis of nuclear genes, such as the internal transcribed spacer (ITS1) and large subunit (28S) regions of rDNA, has been effectively used to determine evolutionary relationships among *Steinernema* and *Heterorhabditis* species (Nguyen *et al.,* 2001; Stock and Hunt, 2005; Nadler *et al.,* 2006). Phylogenetic analysis further aids in understanding the evolutionary and ecological patterns of EPNs, often revealing species clustering by geographic origin or host association, indicative of localized adaptation.

The symbiotic bacteria associated with EPNs are known to exhibit a high degree of host specificity (monoxeny), with each nematode strain typically harboring a single, unique bacterial partner (McMullen *et al*., 2017). It is widely proposed that EPNs and their symbiotic bacteria have coevolved, and that the nematodes rely on their specific bacterial associates to achieve effective biological control (Maneesakorn *et al*., 2011).

Chowdhury and Meena (2018) and Ahuja *et al*. (2020) carried out molecular analyses using 16S rRNA and whole-genome sequencing and confirmed that *Heterorhabditis bacteriophora* and *Heterorhabditis indica* maintain specific symbioses with *Photorhabdus luminescens*. RNA-sequencing studies revealed key genes in *H. bacteriophora* involved in immune and symbiotic regulation with *P. luminescens* (Bhat *et al*., 2022). Whole-genome data also identified *Photorhabdus africana* sp. nov. from *Heterorhabditis* isolates (Machado *et al*., 2024). These findings demonstrated the species-specific bacterial partnerships within *Heterorhabditis* spp., underscoring their evolutionary stability and functional interdependence.

Table 3 : Details of molecular identification of effective EPN isolate and its symbiotic bacteria

S. No.	Isolate name	Host	Morphological identification	Accession number
1.	EPN1	*G. mellonella*	*Heterorhabditis indica*	PX488260
2.	BAC1	*G. mellonella*	*Photorhabdus luminiscences*	PX485203

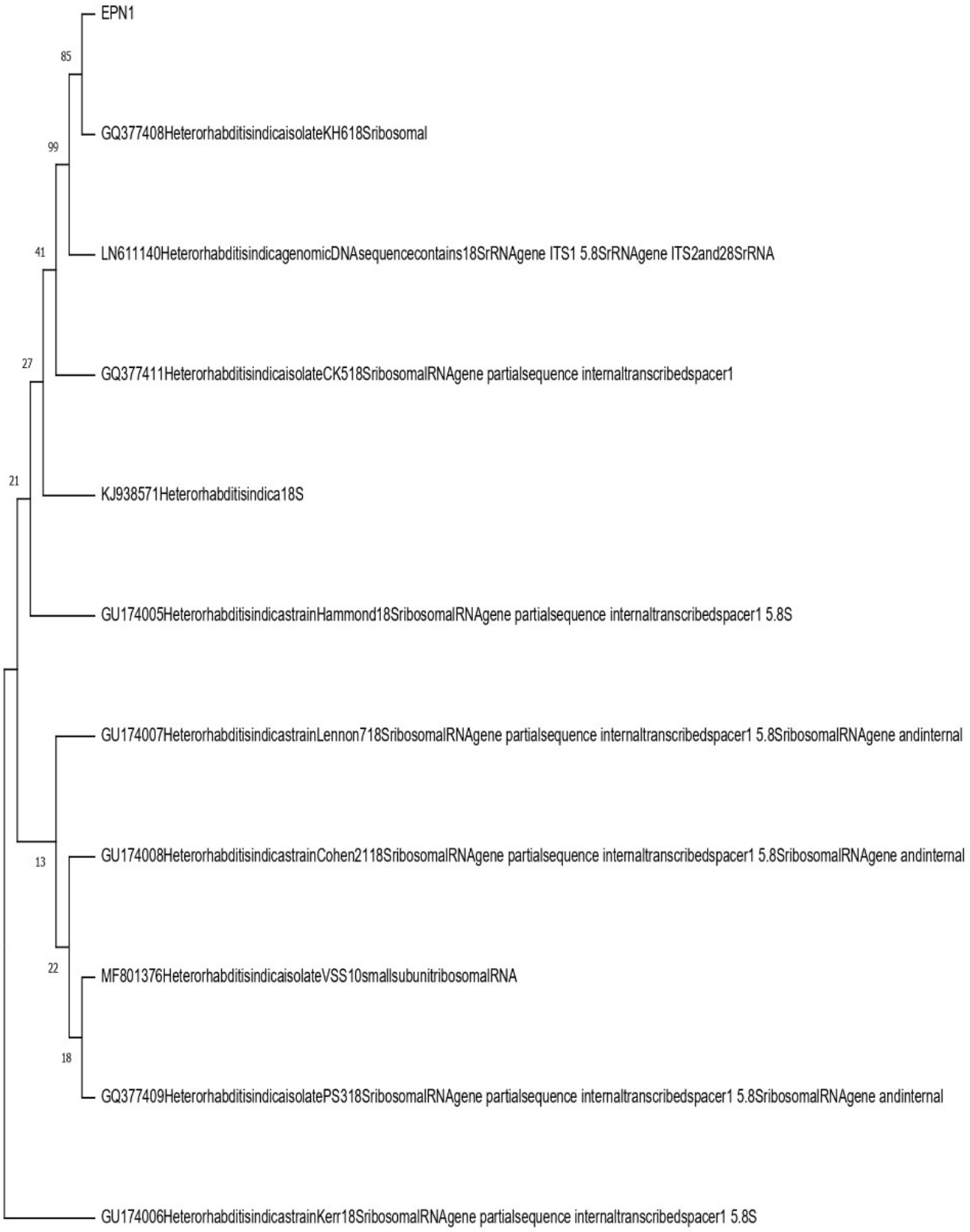

Fig. 2 : Phylogenetic tree of EPN1

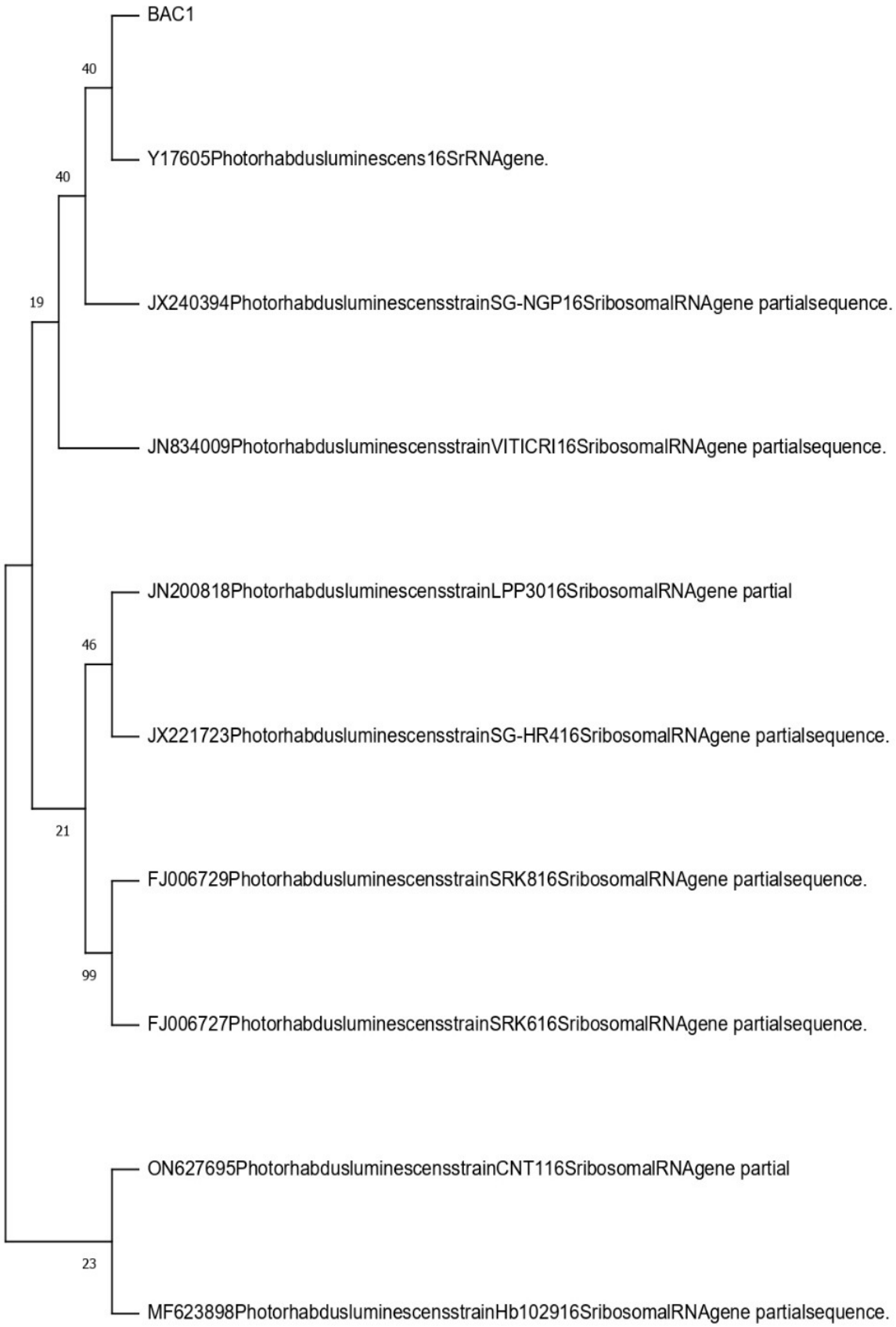

Fig. 3 : Phylogenetic tree of Symbiotic bacteria of EPN1

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Supplementary data

S.No.	District	Village	Soil type	Crop	GPS Location (Latitude, Longitude)
1	Vikarabad	Kothagadi	Red, sandy	Chilli	17.374869, 77.908001
		Kothapally	Deep red, loamy	Tomato	17.270144, 77.996260
2	Rangareddy	Gollapally	Deep red, loamy	Cabbage	17.364523, 78.170787
		Chevvela	Red	Cabbage	17.313671, 78.149401
		Rajendranagar	Deep red, loamy	Jasmine	17.325875, 78.401910
3	Medchal Malkajgiri	Shamirpet	Red Sandy	Tomato	17.582437, 78.593819
4	Sangareddy	Nadikudi	Red, sandy	Ginger	18.185346, 79.642111
		Pasthapur	Black, clay	Potato	17.671484, 77.547523