Molecular Characterization of Novel Symbiotic bacteria from Entomopathogenic nematodes

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Abstract: **Novel entomopathogenic nematodes (EPN) belonging to the genus *Rhabditis* (Family Rhabditidae) were isolated from different parts of the country for the first time. Each isolate is associated with a specific bacterium. About 65 isolates of such nematodes are maintained alive in CTCRI. Bacteria isolated from these are different from one another based on morphology and biochemical tests. In the present study we have isolated twelve different symbiotic bacteria from surface sterilized infective juveniles of entomopathogenic nematode isolates collected from different parts of India. Sequencing of 16S rDNA of 12 isolates indicated that all are different from one another and belong to the following seven genera viz. *Acinetobacter*, *Bacillus*, *Comamonas*, *Stenotrophomonas*, *Achromobacter*, *Klebsiella* and *Brucellaceae*. To analyze the genetic variability among the bacterial isolate DNA fingerprinting technique like ARDRA (Amplified Ribosomal DNA Restriction Analysis) was used. Nucleotide sequences of nine isolates have been submitted to NCBI database and the following accession numbers were allotted: HQ200405, HQ200406, HQ200408, HQ200409, HQ200410, HQ200411, HQ200412, HQ200414 and HQ200415.**

***Index Terms*: Entomopathogenic nematodes (EPN), *Rhabditis*, *E.coli*, phylogenetic tree, ARDRA (Amplified Ribosomal DNA Restriction Analysis).**

1. INTRODUCTION

Entomopathogenic nematodes (EPN) lead a symbiotic association with specific enterobacteria. All the species belonging to the families Steinernematidae and Heterorhabditidae numbering 76 are known as EPN. The enterobacteria mutualistically associated with Steinernematids and Heterorhabditids belong to the genus *Xenorhabdus* (Thomas and poinar, 1979) and *Photorhabdus* respectively. The genus was originally described as *Achromobacter* (Poinar and Thomas, 1965).

Mohandas *et al*., 2004 reported that the *Rhabditis (Oschieus)* spp isolated from different agriclimatic zone of kerala resemble the EPN in all respects except that they belong to Rhabditidae family. *Rhabditis (Oscheius)* spp were found to kill a number of important insect pests within 48-72 hrs in laboratory conditions. It has also been found to be effective for the control of arecanut spindle bug in the field (Mohandas *et al*., 2002).

A number of studies had been brought on the phylogenetic status of bacteria associated with EPN using 16S rRNA gene sequencing and cluster analysis. Enright *et al*., 2003 reported a novel species, *Paenibacillus nematophilus* from *Heterorhabditis* sp. based on sequencing of 16S rRNA. Brunel *et al.,* 1997 conducted a study about the fast and rapid identification of *Xenorhabdus* and *Photorhabdus* spp by restriction analysis of PCR amplified 16S rRNA gene and phylogenetic dendrogram was also constructed by the neighbour-joining method. Liu *et al.,* 1997 studied the phylogeny of the bacteria associated with the EPN based on the Restriction fragment analysis and sequencing of the 16S rRNA gene. Clarridge, 2004 studied the impact of 16S rRNA gene sequencing for the phylogeny and taxonomy of bacteria. Fischer-Le Saux *et al.,* 1998 reported about the PCR ribotyping of *Xenorhabdus* and *Photorhabdus* isolates from the Caribbean region in relation to the taxonomy and geographic distribution of their nematode host based on 16S rRNA and cluster analysis. A polyphasic approach, which is the most reliable method for distinguishing species, was applied to representative strains of the different groups inferred previously by 16S rDNA PCR-RFLP. PCR-RFLP analysis applied to 16S rDNA proved to be a rapid and sensitive typing method for distinguishing strains of the *Xenorhabdus* (Fischer-Le Saux *et al*., 1998).

2. MATERIALS AND METHODS

2.1 Isolation of Bacteria from infective juveniles

Infective juveniles (IJs) of nematodes (30 nos.) were transferred to 2 ml distilled water, treated with streptomycin (5000 units/ml) solution for one hour for surface sterilization. The nematodes were triple rinsed in sterile distilled water and transferred into a micro tube having 2 ml nutrient broth and then it was kept in a vortex shaker for 24 h. The solution was then streaked on to nutrient agar plates and kept at room temperature for 24 h.

2.2 Molecular Characterization of Symbiotic bacteria

2.2.1 Extraction of Bacterial DNA

DNA extraction of bacteria was carried out by using DNeasy ® Blood and Tissue Kit (QIAGEN, Tokyo, Japan).

2.2.2 PCR amplification of 16S rDNA of Symbiotic bacteria

The 16S ribosomal RNA gene of the bacteria were amplified using the universal primer,16SF–5'AGAGTTTGATCCTGGCTCAG3', 16SR-5AAGGAGGTGATCCAGCCGCA. The PCR was performed in a 25µl reaction mixture having 2.5 µl of 10x Taq buffer A (Containing 15 mM $MgCl_2$), 0.5 µl 10mM dNTP's (2.5 mM each), 1.0 µl of each primer (20 ng), 2 µl of template DNA, and 0.25 µl of (1U) Taq DNA polymerase (Bangalore GeNei, India) and 17.75 µl of sterile distilled water. The reaction was carried out in an Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany) with the thermal cycle programme of 92°C for 2min,10sec (initial denaturation), 30 cycles with 94°C for 1min, 10 sec (denaturation), 49°C for 30 sec (annealing), 72°C for 2 min (extension), and final extension at 72°C for 10 min. The amplified products were resolved on a 1.5% agarose gel containing 0.5 mg ml^{-1} ethidium bromide. The DNA bands were visualized under UV transilluminator and documented through Gel Doc System (Alpha imager, Alpha Innotech, USA). 1 kb DNA ladder (Bangalore GeNei, India) was used for determining the size of the amplicon.

2.2.3 Cloning and sequencing

The amplified PCR products were purified using the Gel Extraction Kit (QIAGEN), and the product was cloned into the pGEM-T Easy vector (Promega, USA) following the manufacturer's protocols. The *E.coli* strain DH5α was transformed with the ligated mix and the resulting recombinant clones were selected on LA medium containing Ampicillin and X-gal/IPTG (Sambrook and Russal, 2001) and confirmed by colony PCR, plasmid DNA isolation and its restriction. The clones were then subjected to sequencing in Delhi University South Campus. The sequencing was performed to both directions using T7 and SP6 primers.

2.2.4 Phylogenetic analysis

The sequenced strands were then edited using the BIOEDIT software (Hall, 1999).The nucleotide sequences were compared with those in the NCBI databases using the Basic Local Alignment Search Tool (BLAST, http:// www.ncbi.nlm.nih.gov/BLAST). The sequences obtained for the bacterial isolate were aligned with each other by using Clustal W multiple alignment programme of BioEdit software (Hall, 1999). From the aligned sequences a phylogenetic tree was constructed using the neighbour-joining method (Tajima and Nei, 1984), the data sets were subjected to 100 bootstraps replicates. The tree was constructed using the TREECON software.

2.2.5 ARDRA (Amplified Ribosomal DNA Restriction Analysis)

Ribosomal-DNA fragment generated by PCR was digested to see the restriction pattern separately with restriction endonuclease (*Alu*1).

3. RESULTS AND DISCUSSION

The PCR amplification of the 16S rDNA of the bacteria with the primer 16S F and 16S R at an annealing temperature of 49°C yielded a fragment of approximately 1500 bp. Sequencing of 16S rDNA of 12 isolates indicated that all are different from one another belonging to the following seven genera viz. *Acinetobacter*, *Bacillus*, *Comamonas*, *Stenotrophomonas*, *Achromobacter*, *Klebsiella* and *Brucellaceae*. BLAST results were shown in Table 1.

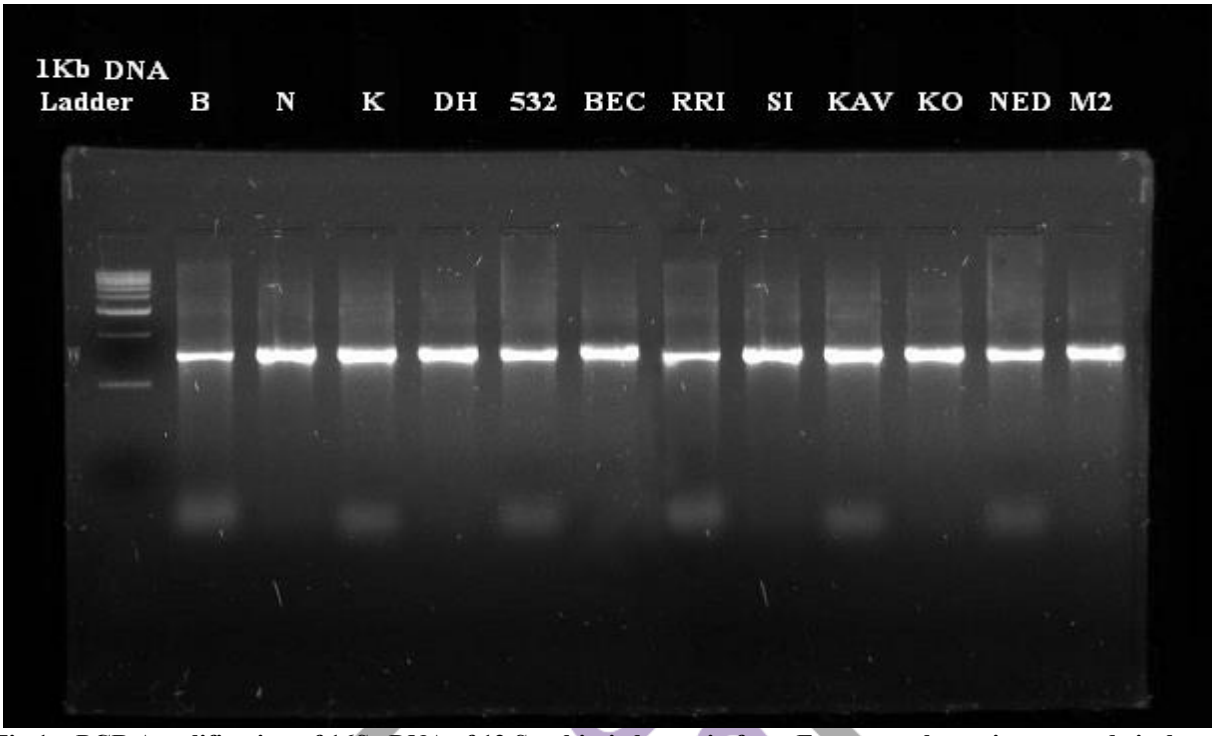

Fig.1. PCR Amplification of 16S rDNA of 12 Symbiotic bacteria from Entomopathogenic nematode isolates.

Table 1. Identification based on sequencing of 16S rDNA.

Isolate	Identification	Similarity (%)
B	*Bacillus cereus biovar anthracis* str. CI, complete genome (Accession No. CP001746)	100%
N	*Bacillus cereus* 03BB102, complete genome (Accession No.CP001407)	99%
K	*Klebsiella pneumoniae* strain HR16 16S ribosomal RNA gene, partial sequence (Accession No.EU078621)	99%
RRI	*Acinetobacter* sp. JD2(2010) 16S ribosomal RNA gene, partial sequence (Accession No.GU566314)	99%
Bec	*Comamonas testosteroni* CNB-2, complete genome (Accession No CP001220)	99%
Ned	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578, complete sequence (Accession No.CP000647)	99%
SI	*Comamonas testosteroni* CNB-2, complete genome (Accession No.CP001220)	99%
532	*Acinetobacter* sp. JD2(2010) 16S ribosomal RNA gene, partial sequence ((Accession No. GU566314)	100%
DH	*Stenotrophomonas maltophilia* strain pp5c 16S ribosomal RNA gene, partial sequence (Accession No.GQ360071)	99%
KAV	*Brucellaceae* bacterium 47211606 16S ribosomal RNA gene, partial sequence (Accession No. AY353698)	99%
KO	*Achromobacter* sp. MT-E3 16S ribosomal RNA gene, partial sequence (Accession No. EU727196)	99%
M2	*Brucellaceae* bacterium 47211606 16S ribosomal RNA gene, partial sequence (Accession No. AY353698)	97%

Nucleiotide Sequence Accession Numbers

The sequence obtained in this study have been assigned in the NCBI Gen Bank under the accession numbers HQ200405 (Isolate B), HQ200406 (Isolate K), HQ200408 (Isolate RRI), HQ200409 (Isolate KAV), HQ200410 (Isolate BEC), HQ200411 (Isolate KO), HQ200412 (Isolate SI), HQ200414 (Isolate DH) and HQ200415 (Isolate NED).

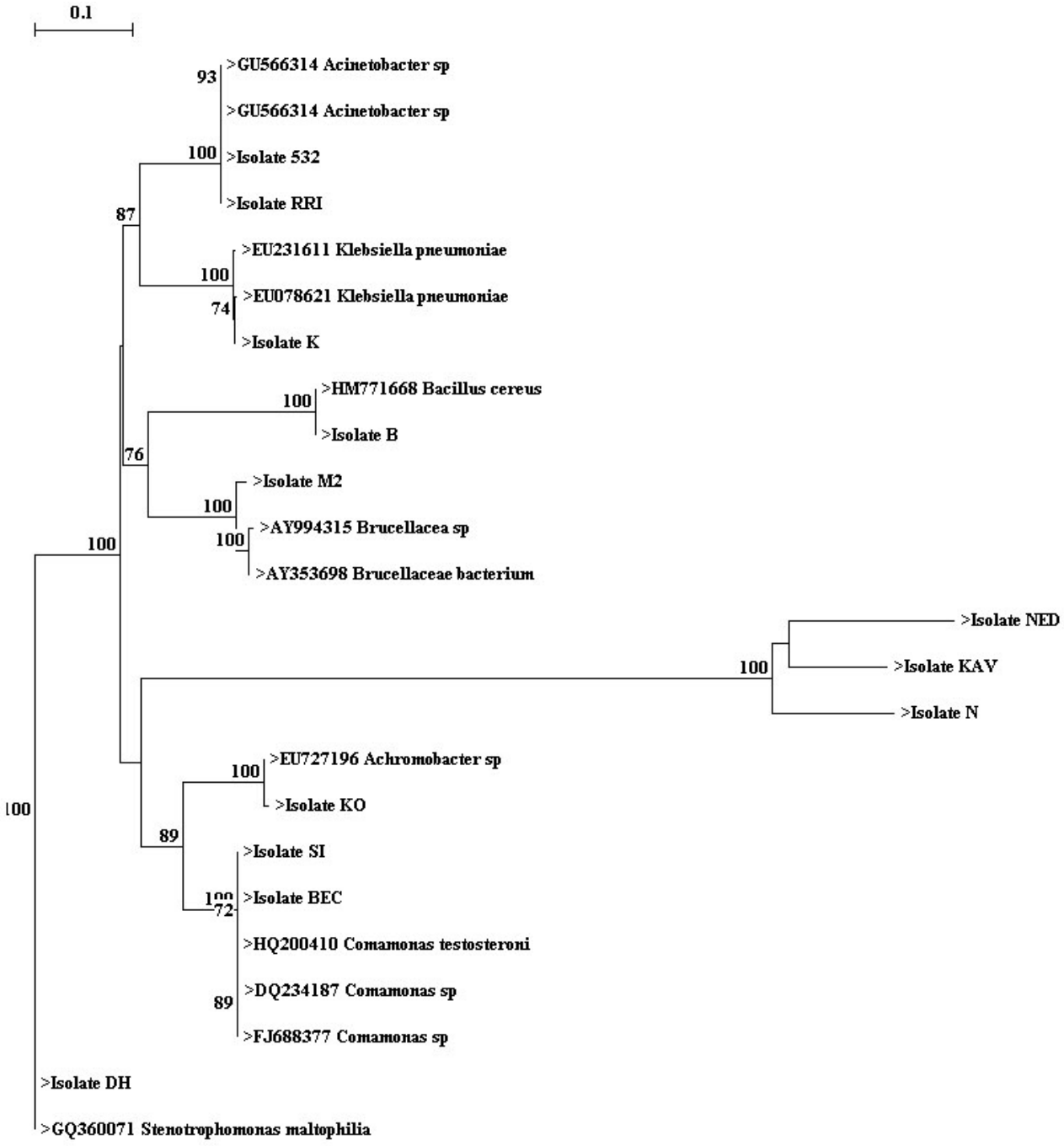

Fig.2. Phylogenetic tree of 12 symbiotic bacteria isolated from Entomopathogenic nematode isolates constructed using the neighbour-joining method of the TREECON software with 100 bootstrap replicates.

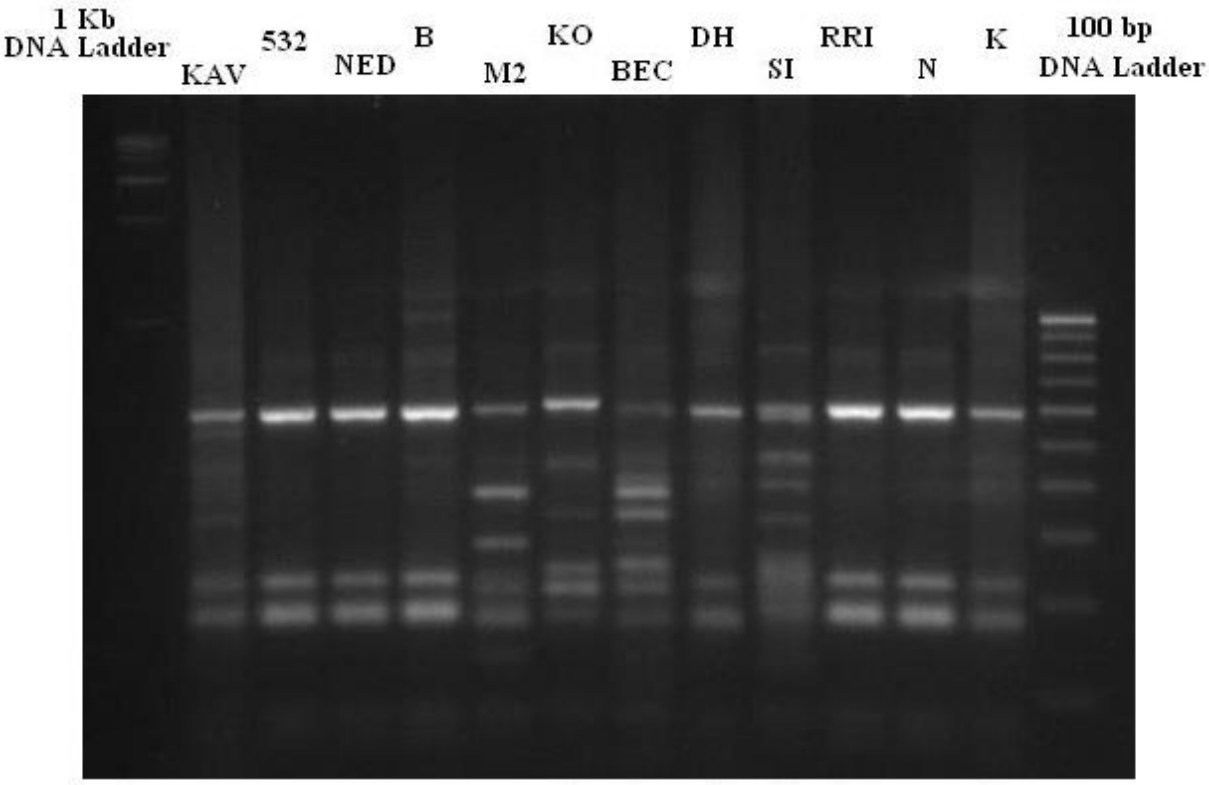

Fig.3. The restriction patterns of PCR-amplified 16S rDNA from 12 Symbiotic bacterial strains digested with the enzyme *Alu*1.

Lagace *et al.,* 2004 studied the identification of bacterial community by using ARDRA analysis and 16S rRNA gene sequencing. Georgieva *et al.,* 2008 demonstrated the genetic diversity and identification of EPN bacterial symbionts based on RFLP-PCR of 16Sr DNA analysis. Nobuhito *et al.,* 2005 reported about the Genetic Diversity on 16S rDNA Sequence and Phylogenetic Tree Analysis of bacteria.

The bacteria associated with EPN are isolated and identified based upon the biochemical test and 16S rDNA (Feng-Chia *et al*., 2009). The *Xenorhabdus* have primary and secoundary variants defining the phenotypic characterization of the variants (Akhurst and Boemare, 2005). The bacteria can be identified based on morphological, biochemical and molecular characterization (Irum Naz *et al*., 2009). The bacteria isolated from nematode are closely related genera based upon the sequence of 16S rDNA and phylogenetic analysis (Babic *et al*., 2000). Because the nucleotide sequences found in 16S rDNA vary in an orderly fashion throughout the phylogenetic tree, these sequences have been useful for the study of molecular evolution (Woese, 1987).

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