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Identification and Characterization of *Photorhabdus luminescens* Isolates From Eastern Dry Zone of Karnataka

KEYWORDS: *Photorhabdus*. EPN, RAPD, Cluster analysis, insect host

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ABSTRACT *Photorhabdus luminescens is a nematode symbiotic, gram negative, bioluminescent bacterium, belonging to the family of Enterobacteriaceae. Recent studies reveal the importance of this bacterium as an alternative source of insecticides. Isolation and identification of native nematode-bacterial association in the field are necessary for successful control of endemic pests in a particular location. Twenty bacterial isolates from entamopathogenic nematodes were isolated by insect bait method using fifth instar larvae of Galleria mellonella. Bacterial isolates were identified and confirmed using standard synaptic keys. Further it was amplified by 16S rDNA primer. Percentage infection of G. mellonella larvae by entomopathogenic nematodes in soil across different locations was found twenty percent. Molecular diversity of these isolates was characterized by RAPD marker analysis. Primer screening was carried out using 10 primers, out of which 6 primers were detected for RAPD analysis. A total of 179 bands were scored, out of which 134 bands are polymorphic. The cluster analysis revealed that all the bacterial isolates clustered at a linkage distance of about 2 units on the dendrogram. Dendrogram shows the isolates as three major clusters. The percent polymorphism observed in the isolates was 74.87 which suggest that the symbiotic bacterial population exhibit genetic diversity.*

Introduction: - *Photorhabdus luminescens* is an insect pathogenic bacterium that is symbiotic with entomopathogenic nematodes. *P. luminescens* promotes its own transmission among susceptible insect populations using its nematode host as vector. Its life cycle comprises a symbiotic stage in the nematode's gut and a virulent stage in the insect larvae. Entomopathogenic nematodes are widely available for use in biological control. They possess extreme virulence for insects at a non specific broad host range and are safe for mammals. *P. luminescens* secretes toxins potentially virulent factors (Waterfield and Clark, 2009) which kill the host and also convert host tissue into food for both replicating bacteria and nematodes (Ffrench constan and Dowling, 2007). These nematodes are characterized by their mutualistic relationship with bacterial symbionts. After their penetration into the body cavity of an insect, the nematodes breach the defense reactions and release their symbiotic bacteria (Boemare N., et al., 1997. After the death of the insect, the nematodes reproduce in the cadaver, which is bioconverted as the symbiont multiplies. *Photorhabdus i*s a member of the family Enterobacteriaceae that lives in a mutualistic association with a Heterorhabditis nematode worm (Gatsogiannis *et al.,* 2013). The nematode worm burrows into insect prey and regurgitates *Photorhabdus,* which goes on to kill the insect. The nematode feeds off the growing bacteria until the insect tissues are exhausted, where upon they reassociate and leave the cadaver in search of new prey. This highly efficient partnership has been used for many years as a biological crop protection agent. The dual nature of *Photorhabdus* as a pathogen and mutualist makes it a superb model for understanding these apparently exclusive activities.

Genetic diversity can be estimated at molecular level by Random Amplified Polymorphic DNA (RAPD) technique. RAPD are PCR- based molecular markers that may substantially reduce time, labour and cost required for molecular mapping (Shiva Reddy D. M. *et al.,* 2010). RAPD,s involve the use of a single DNA primer to direct amplification under PCR based amplification of random sequences, and can be used in wide variety of crops (Dharmendra and Boora, 2008). Unlike conventional PCR data on DNA sequence of the organisms are not a pre-requisite for RAPD analysis. Further this technique elucidates the biodiversity in a group of isolates. Hence the biodiversity of symbiotic bacteria isolated from eastern dry zone of Karnataka.

Materials and Method: -

Isolation and identification of bacterial isolates: - Isolation of EPN was done using *Galleria mellonella*, a host susceptible to EPN's by baiting method described by Bedding and Akhurst. To meet the requirement for isolation of entamopathogenic nematodes *Galleria mellonella* a host susceptible to EPNs was reared in the laboratory using artificial diet. Traps containing *G. mellonella* were placed in the polythene bags containing soil samples obtained from different crop fields. The traps are harvested at 4th, 7th and 12th day after placing them. The data on infestation of *G. mellonella* larvae in each trap was recorded. Infected larvae were dissected and a drop of haemolymph from insect cadaver was streaked on sterile plated NBTA medium and incubated at 28^{0} C overnight. The primary form of bacteria absorbs bromothymol blue dye and appears blue. Twenty symbiotic bacteria were isolated from these nematodes from eastern dry zone of Karnataka and named based on the source place. These bacteria were identified based on morphological, biochemical, microscopic and physiological characters like Lactose fermentation, Gelatin liquefaction, Catalase, Urease, and Motility. Further it was identified with 16S rDNA primer.

RAPD Analysis:-

DNA extraction protocol:-DNA extraction protocol was followed according to the method of Sambrook et al., 1989. The DNA was dissolved in TE buffer and stored at 4^{0} C.

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PCR amplification conditions: - PCR reactions were performed in a final volume of 25µl containing 30ng of template DNA, 0.75µl of 2mM dNTPs each, 2.5µl of 10X taq buffer, 0.36µl of 3 unit/µl of Taq DNA polymerase, 3µl of 10 picomole primers. Amplification were achieved in MWG-Biotech primus thermocycler with the program consisting initial denaturation of 94^{0}C for 3 min followed by 35 cycles each consisting of denaturation at 94^{0}C for 1 min, primer annealing temperature at 37^{0}C for 1 min, primer extension at 72^{0}C for 3 min, and final extension of 72^{0}C for 10 min. These reactions were repeated to check the reproducibility of the amplification.

Selection of primers: - To choose the RAPD primers that can amplify informative sequences, primer screening was carried out using DNA obtained from the *Photorhabdus luminescence* isolates, out of ten primers screened, finally six primers producing sharp, intense bands were selected for the RAPD analysis (Table 2).

Analysis of RAPD data: - The bands are manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis. The scored band data (presence or absence) was subjected to cluster analysis using STATISTICA.

Result and Discussion: -

Isolation and identification: Twenty isolates were isolated from different sources from eastern dry zone and named based on the sources (Table 1). All the bacterial isolates absorbed bromothymol blue dye from the NBTA media and form characteristic blue colonies on NBTA media These bacterial isolates found positive reactions towards lactose fermentation (acid gas production), Gelatin liquefaction (indication of production of yellowish or bluish green fluid on the surface of gelatin agar), urease (change of colour from yellow to red) and motility (appearance of dispersed red colour bacterial growth) while negative towards catalase test characterized by absence of bubble evolution. Further, it was confirmed with 16S rDNA primer. Later it was sent for sequencing and found that it shows homology with *P. luminescence* sub sps. *akhrustii*.

RAPD characterization: - For RAPD characterization, a total of 10 primers were used for preliminary screening (Table-2). Finally only 6 primers produced sharp bands were selected for diversity analysis of symbiotic bacterial isolates (Fig. 1). A total of 179 bands were scored. Out of which 134 bands are polymorphic with 68.72% bands are shared polymorphic and 6.15% bands are unique polymorphic (Table-3). The percentage of monomorphic bands is 19.55%. Similar kind of work was done by Shiva Reddy D.M., *et al.*, in 2010 with *Bacillus megaterium*. They carried out RAPD analysis with ten isolates isolated from different geographical areas of Karnataka. Kwon G. *et al.,* (2009) obtained the similar result with six bacillus strains isolated from Cheonggu kjang.

Cluster analysis of 20 *P. luminescence* isolates: - The cluster analysis based on RAPD bands revealed that the twenty symbiont bacterial isolates were clustered at about 2 units on dendrogram with isolate Ma-T and isolate Sa-G spanning the extremes. All the isolates form three major groups (Fig-2). It clearly indicates that isolates from geographically adjacent zones are more closely related than far away zones. Dharmendra Kumar also found similar results in 2008 with seventy isolates of *Bacillus thuringiensis* isolated from cotton fields.

Conclusion: - *Photorhabdus luminescens* is a symbiotic bacterium residing in the gut of entomopathogenic nematodes and these bacteria have many insect toxins and can become the next gen biopesticides to control the pathogenic insects from the agricultural fields biologically. Percent polymorphism observed in the isolates was high which suggest that the symbiotic bacterial population exhibit genetic diversity.

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Table 1: - Percent infection of *Galleria mellonella* larvae by Entomopathogenic Nematodes in soil across different locations

Location	Village	No. of traps	Crop	Total no. sampled	Infected			Total no. infected	% infection
					4th day	7th day	12th day		
Devanahalli	Mandibele	M-30	Tomato	30	-	2	2	4	13.33
	Dharmapura	D-30	Cabbage	30	1	1	2	4	13.33
Doddaballapur	Oddarahalli	O-30	Pigeon Pea	30	-	2	3	5	16.70
	Kantanakunte	K-30	Tomato	30	1	2	3	6	20.00
Hoskote	Ramapura	R-30	Cabbage	30	1	1	2	4	13.33
	Avalahalli	A-30	Ground nut	30	-	1	1	2	06.70
Whitefield	Samethanahalli	S-30	Pigeon Pea	30	-	2	1	3	10.00
	Sarjapura	M-30	Cowpea	30	-	-	1	1	03.33
Sarjapura	Bellary road	M-30	Tomato	30	1	2	2	5	16.70
GKVK	Nelmangala	C-30	Field bean	30	-	1	1	2	06.70
Nelamangala	Mandibele	H-30	French bean	30	-	2	1	3	10.00

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Table 2: - RAPD primers with sequences had chosen for analysis.

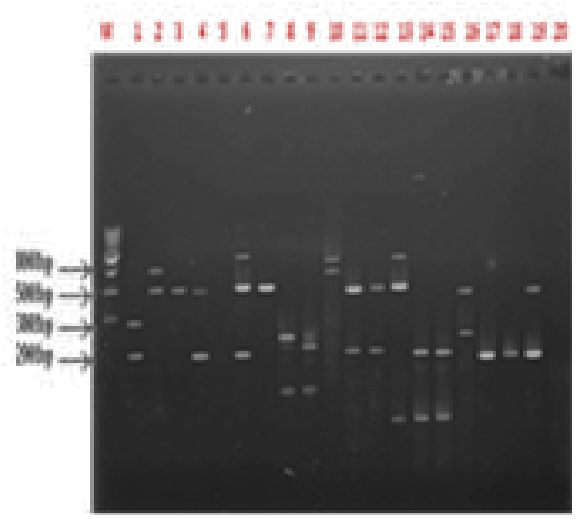
S. No.	Primer no.	Sequence
1	Random Primer 1	5′-GAG AGC CAA C-3′
2	Random Primer 2	5′-GTT TCG CTC C-3′
3	Random Primer 3	5′-GTA GTC ATA T-3′
4	Random Primer 4	5′-AAG AGC CCG T-3′
5	Random Primer 5	5′-GGC TGC TGG C-3′
6	Random Primer 6	5′-CCC GTC AGC A-3′
7	Random Primer 7	5′-GAA CGG ACT C-3′
8	Random Primer 8	5′-GGT GCG GGA A-3′
9	Random Primer 9	5′-TTG GAG GGC A-3′
10	Random Primer 10	5′-CTT CCG TCA A-3′

Table 3:- Oligonucleotide primers that showed genetic variation among the *Photorhabdus* isolates.

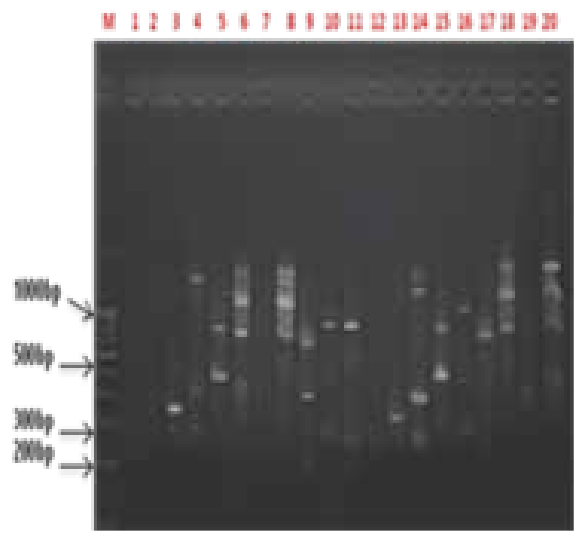
Primers	No. of amplified fragments	No. of Polymorphic bands		No. of Monomorphic bands.
		Shared	Unique	
Random primer 1	34	28	2	4
Random Primer 2	37	25	1	11
Random Primer 5	20	9	3	8
Random primer 6	30	23	2	5
Random Primer 8	22	10	3	9
Random Primer 10	36	28	0	8
Total	179	123	11	35
Percentage	100	68.72	6.15	19.55

Fig 1: - RAPD gel profile of *Photorhabdus luminescens* isolates generated using 10-mer random primers. (M: marker lane and lane 1 to 20 represents isolates from different sources).

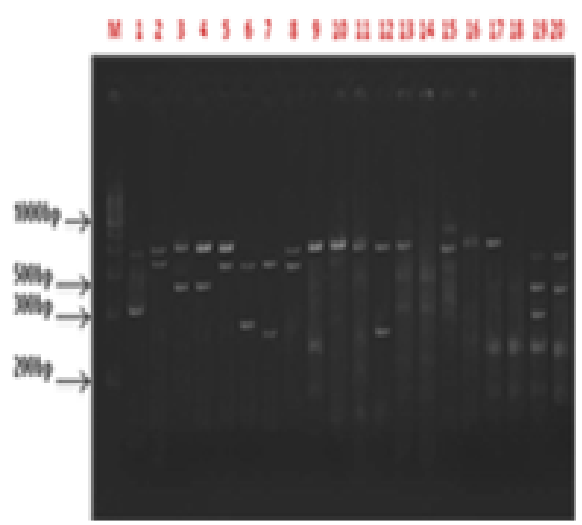
Primer No. 1



Primer No. 2



Primer No. 5



Primer No. 6

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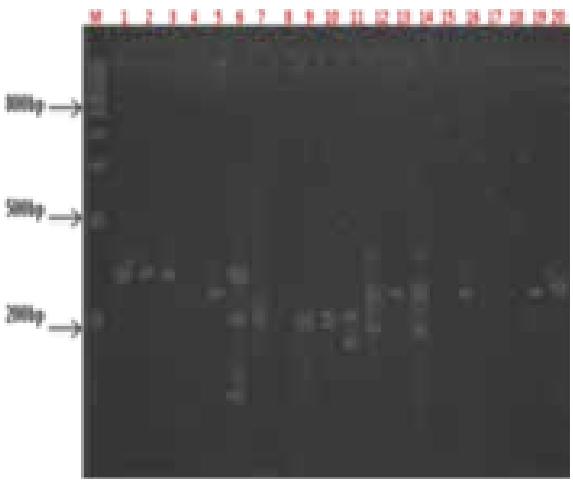
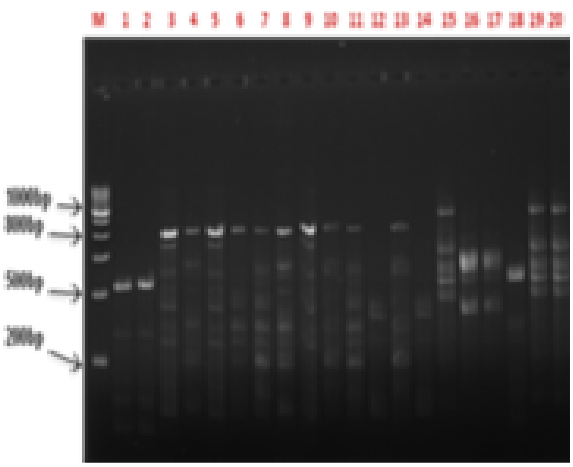
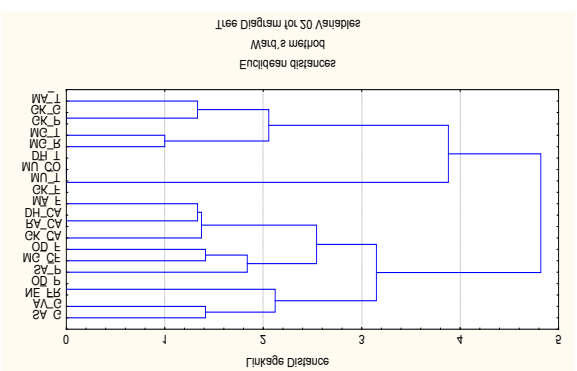


Primer No. 8



Primer No. 10

Fig. 2: - Dendrogram based on RAPD profile of 20 *Photorhabdus luminescens* isolated from different sources.



References: -

1. Akhurst R.J., (1982). Antibiotic activity of Xenorhabditis spp., bacteria associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. J. Gen. Microbiol., 128: 3061-3065.
2. Bedding R.A. and Akhurst R.J., (1975). A simple technique for the detection of insect parasitic nematodes in soil. Nematologica, 21:109-110.
3. Boemare N.E. and Akhurst R.J., (1988). Biochemical and Physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). Journal of Genetic Microbiology, 134; 751-761.
4. Boemare N., Brunel B., Givaudan A., Lanois A., Akhurst R.J., (1997). Fast and Acurate Identification of *Xenorhabdus* and *Photorhabdus* species by restriction analysis of PCR amplified 16S rRNA Genes. Appl. and Environ. Microbiol, 63(2): 574-580.
5. Bowen D.J. and Ensign J.C., (1998). Purification and characterization of a high molecular weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminescens*. Appl. Environ. Microbiol, 64: 3029-3035.
6. Dharmendra K., Chudhary K and Boora K.S., (2008). Characterization of native *Bacillus thuringiensis* strains by PCR-RAPD based fingerprinting. Indian Journal of Microbiology, 8: 1-6.
7. Ffrench- Constant R.H., Dowling A. and Waterfield N.R., (2007). Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. Toxicon Review, 49: 436-451.
8. Forst S., Dowds B., Boemare N. and Stackebrandt E., (1997). *Xenorhabdus* and *Photorhabdus* spp.: bugs that kills bugs. Annu. Rev. Microbiol., 51: 47-72.
9. Gatsogiannis C., Lang A.E., Meusch D., Pfaumann V., Hofnagel O., Benz R., Aktories K., Raunser S., (2013). A syringe - like injection mechanism in *Photorhabdus luminescens* toxins. Nature, 195: 520-523.
10. Heidi G.B., David J.C., (2007). Mutualism and Pathogenesis in *Xenorhabdus* and *Photorhabdus:* two roads to the same destination. Molecular Microbiology, 64: 260-268.
11. Kumar S.V., Mulla S.R., Suresh C.K., (2011). Isolation and molecular characterization of symbiotic bacterial isolates associated with entomopathogenic nematodes in agro climatic zone 5 of Karnataka. Journal of Phytology, 3(11): 25-29.
12. Kwon G., Lee H., Park J., Kim J.S., Lim J., Park C., Kwon D.Y., Kim J.H., (2009). Development of a RAPD-PCR method for identification of *Bacillus* species isolated from Cheonggukjang. Tnt. Jou. Of food Microb., 129: 282-287.
13. Sambrook,J., Fristch E.F., Maniatis T.,(1989). Molecular Cloning: A Laboratory manual, 2nd edition, CSH laboratory press. Vol. 1, section 6.
14. Sicard M. et al., (2008). Isolation and identification of entomopathogenic nematodes and their symbiotic bacteria from Herault and Gard (South France). Journal of invertebrate Pahology, 98: 211-217.
15. Shiva Reddy D.M., Mohan B.K., Nataraja S., Krishnappa M., Abhilash M., (2010). Isolation and molecular characterization of *Bacillus megaterium* isolated from different agroclimatic zones of Karnataka and its effect on seed germination and plant growth of *Sesamum indicum*. Res. Jou. Of Pharm., Biol., and Chem. Sci.,1(3): 614-625.
16. Waterfield, N.R.; Ciche, T.; Clark, D., (2009). *Photorhabdus* and a host of hosts. Annu. Rev. Microbiol., 63: 557–574.