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Isolation and Identification of Symbiotic Bacterium Associated With the Entomopathogenic Nematode, *Heterorhabditis* sp. (IISR-EPN 01) From India.

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ABSTRACT

The symbiotic bacterium isolated from entomopathogenic nematode, *Heterorhabditis* sp. (IISR-EPN 01) from Kozhikode (Kerala), India and identified through morphological, biochemical and molecular characterization. The morphological characteristics and various biochemical tests were done as per described procedures. DNA was extracted from bacterial isolate (IISR-EPN BC 09). The primers 16 S 5' AGAGTTTGATCCTGGCTCAG 3' (FORWARD) and 5' AAGGAGGTGATCCAGCCGCA 3' (REVERSE) were used for the amplification of the ITS region of the rDNA and purified DNA was sequenced. The phylogenetic relationship were also studied using neighbor-joining and maximum composite likelihood methods. The bacterial isolate was Gram negative, rod shaped, facultative and motile. While, the colony characters were red colour with off white margins, circular, raised and opaque. Biochemical characterization showed that the isolate was positive for citrate utilization, methyl red, urease and carbohydrate fermentation tests and negative for indole production, oxidase and Voges Proskauer tests. However, escullin showed weak reaction. On the basis of morphological, biochemical and molecular characterization, the bacterial isolate was identified as *Photorhabdus luminescens* subsp. *akhrustii*. This symbiotic bacterium associated with *Heterorhabditis* sp. (IISR-EPN 01) which isolated from ginger rhizosphere from India for the first time. The identification of the symbiotic bacteria would help in a better understanding of the relationship between these two organisms.

Keywords: entomopathogenic nematode, *Heterorhabditis* sp., bacteria, *Photorhabdus luminescens,* ginger.

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INTRODUCTION

Entomoapthogenic nematodes (EPNs) have a great potential as biological control agents against insect pests of crops due to their wide host range, easy to handle, short life cycle, economic production at large scale and environmentally safe [1,7,12-14].

EPNs have a symbiotic relationship with an enteric bacterium species. These bacteria are present in special vesicles in the intestine of the nematodes. The infective juveniles (IJs) actively seek out and penetrate into the insect body and release the bacterial cells into the insect hemolymph inducing toxemia and septicemia. The insect dies within 24-72 h. The bacterium and nematode symbionts continue to grow and multiply within the infected insect until emergence of a new generation of IJs carrying the bacterial symbionts to initiate a new infection cycle in another insect [2]. *Photorhabdus luminescens*, formerly *Xenorhabdus luminescens* , is an insect pathogenic Gram-negative bacterium belong to the family Enterobacteriaceae, with the characteristic of forming a symbiotic relationship with *Heterorhabditis* spp. [3,4].

Recently, a native entomopathogenic nematode, *Heterorhabditis* sp. (IISR-EPN 01) was isolated from ginger rhizosphere. However, knowledge of its symbiotic bacteria is lacking.

Hence, isolation and identification of the symbiotic bacterium associated with *Heterorhabditis* sp. (IISR-EPN 01) through morphological, biochemical and molecular characterization was attempted.

MATERIALS AND METHODS

Entomopathogenic nematode source

Infective juveniles (IJs) of test EPN was obtained from stock cultures which maintained in the Nematology laboratory, ICAR-Indian Institute of Spices Research, Kozhikode. EPN was cultured as per the procedure described by Kaya and Stock [10]. The IJs were surface sterilised in 0.1% hyamine solution and stored in distilled water in tissue culture flasks for study.

Insect source

Greater wax moth, *Galleria mellonella* larvae were obtained from cultures maintained on artificial diet [5]. The larvae were sorted and those of similar size were select for the study.

Isolation of bacteria

The bacterium was isolated from the hemocoel of greater wax moth, *G. mellonella* larva infected with *Heterorhabditis* sp. (IISR-EPN 01) as per method described by Poinar [17].

Morphological characterization

Colony morphology of the symbiotic bacterium was visualized by growth on NBTA. The parameters like, size, consistence, pigmentation, colour, form and margin of the bacterial colony were recorded. Gram staining was carried out using standard protocol [11,19].

Biochemical characterization

The biochemical tests *viz*., catalase, oxidase, indole, methyl red, voges proskauer, citrate utilization, urease, escullin hydrolysis, and carbohydrate fermentation such as glucose, lactose, sucrose, maltose and mannitol were carried out to characterize the symbiotic bacterium as per described procedures [3].

Molecular characterization

DNA was extracted from bacterial isolate (IISR-EPN BC 09). 16 S primers 5' AGAGTTTGATCCTGGCTCAG 3' (FORWARD) and 5' AAGGAGGTGATCCAGCCGCA 3' (REVERSE) were used for the amplification of the ITS region of the rDNA as per procedure described by Ehlers and Niemann [6]

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and Iraki et al. [9] (Fig. 1). After electrophoresis, the amplified products were excised from 1% TAE buffered agarose gel using a QIA-quick PCR purification kit (QIAGEN) and cloned. Purified DNA was sequenced at M/S Eurofins Genomics India pvt. ltd, Bangalore. The phylogenetic relationships of ITS sequences were studied using Neighbor-Joining [18] and Maximum Composite Likelihood methods [19].

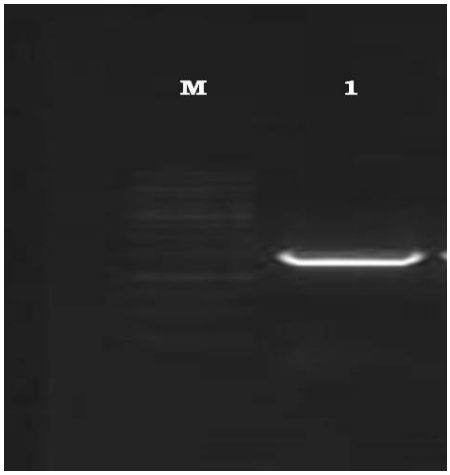

Figure 1: PCR amplification of 16S rDNA of bacterial isolate. M- 1000 bp marker, 1- IISR-EPN BC 09

RESULTS AND DISCUSSION

Morphological characterization

The bacterial isolate (IISR-EPN BC 09) was Gram negative, rod shaped, facultative and motile. The colony characters were small size, red colour with off white margins, circular, raised and opaque (Table 1).

Table 1: Morphological characteristics of symbiotic bacterium *P. luminescens* strain IISR- EPN BC 09.

Colony morphology					Cell shape	Grams reaction	Motility
Size	Shape	Elevation	Texture	Colour			
Small	Circular	Raised	Opaque	Light red with off white margin	Short rods	-	+

Biochemical characterization

Table 2: Biochemical characteristics of symbiotic bacterium *P. luminescens* strain IISR- EPN BC 09.

Biochemical tests	Reaction
Catalase test	+
Oxidase test	-
Indole test	-
Methyl red test	+
Voges Proskauer test	-
Citrate utilization test	+
Urease test	+
Esculin hydrolysis	w/k
Carbohydrate fermentation	
Glucose	+
Lactose	+
Sucrose	+
Maltose	+
Mannitol	-

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Biochemical characterization of the symbiotic bacterium (IISR-EPN BC 09) showed that, the isolate was positive for catalase, citrate utilization, methyl red, urease and carbohydrate fermentation like glucose, lactose, sucrose and maltose tests, whereas, negative for indole production, oxidase, mannitol and Voges-Proskauer tests. However, escullin test showed weak reaction (Table 2).

Molecular characterization

The DNA sequence was compared to sequences available in NCBI GenBank. The BLAST search indicated 100% similarity between the sequence of the IISR-EPN BC 09 isolate and *Photorhabdus luminescens* subsp. *akhurstii* strain 0805-P5G (EU301784.2), whereas 99% similarity with *P. luminescens* subsp. *akhurstii* strain IND (AY2786643.1) and *P. luminescens* subsp. *akhurstii* strain 0813-124 (DQ223040.2). However, 98% similarity with *P. luminescens* subsp. *thracensis* strain LB03 (HM140702.1) (Fig. 2).

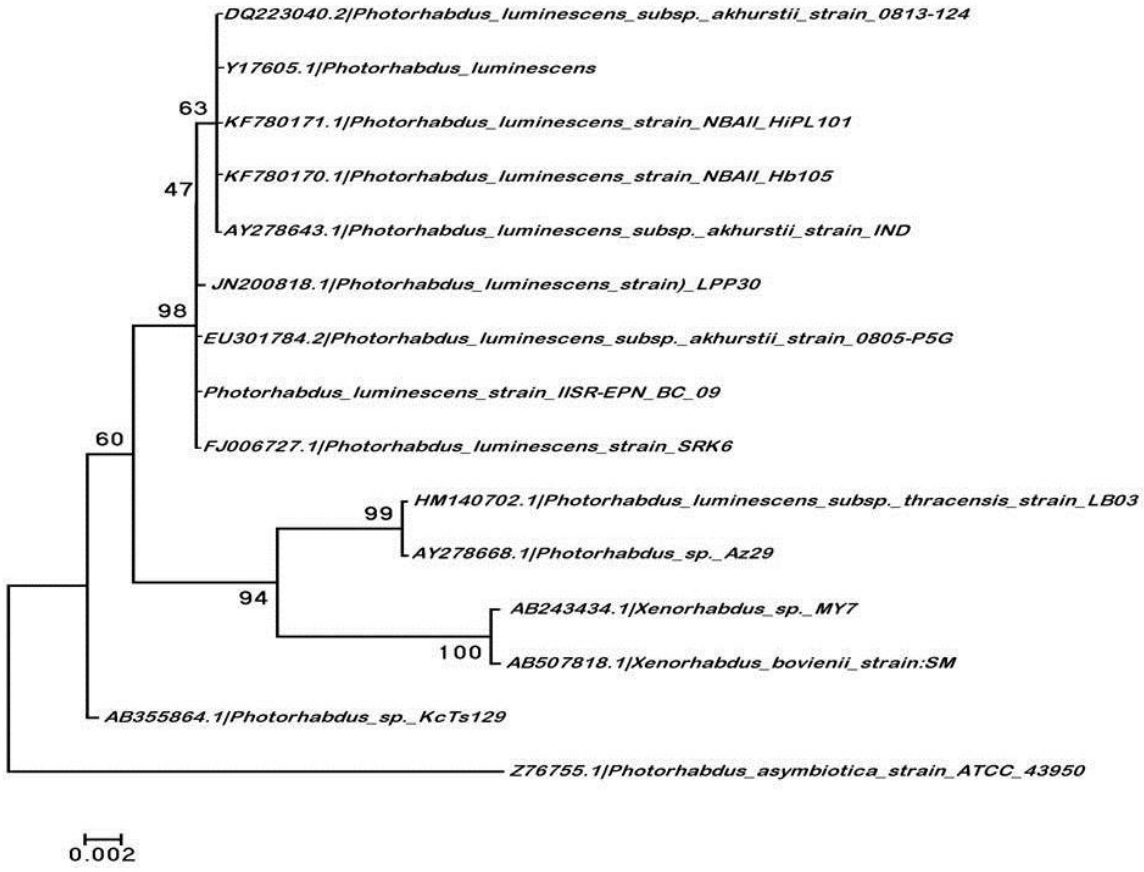

Figure 2: Dendogram for the phylogenetic relationships of *Photorhabdus luminescens* strain IISR- EPN BC 09 among closely related species based on the sequences of the ITS region.

Thomas and Poinar (21) described *Xenorhabdus luminescens* bacterium which isolated from *Heterorhabditis bacteriophora*. Later Boemare et al. (4) described *X. luminescens* as *Photorhabdus luminescens*. *P. luminescens* subsp. *akhurstii* was isolated from *H. indica*, whereas, *P. luminescens* subsp. *luminescens* and *P. luminescens* subsp. *laumondii* from *H. bacteriophora*. However, *P. temperata* was isolated from NC strain of *H. bacteriophora, H. zealandica* and *H. megidis* (2). In this report symbiotic bacterium associated with *Heterorhabditis* sp. (IISR-EPN 01) was identified on the basis of morphological, biochemical and molecular characterization as *P. luminescens* subsp. *akhurstii.*

Heterorhabditis spp. and its bacterial symbiont *Photorhabdus* spp. have been shown to be effective agents in the biological control of many insect pests (8). In present study used EPN, *Heterorhabditis* sp. (IISR-EPN 01) is able to penetrate and kill the shoot borer, *Conogethes punctiferalis*, hairy caterpillar, *Euproctis* sp. and *Lema* sp. infesting ginger (15,16). This symbiotic bacterium associated with *Heterorhabditis* sp. (IISR-EPN 01) which isolated from ginger rhizosphere from India for the first time. This indigenous EPN will be suitable

for managing the local insect pests of ginger because of their adaptation to local climate and population regulators. Further studies are required to know the exact behaviour, pathogenicity, mode of action and multiplication of the symbiotic bacteria. This bacterial isolate open the prospect for using them in biological control programs against insect pests of ginger in future.

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REFERENCES

[1] Ali S S, Ahmad R, Hussain M A and Pervez R. Pest management of pulses through entomopathogenic nematodes. Indian Institute of Pulses Research, Kanpur, Army press, Lucknow (India) 2005; pp. 59.
[2] Boemare N E. Biology, taxonomy and systematics of Photorhabdus and Xenorhabdus, Entomopathogenic Nematology, CABI Press, New York 2002; pp. 35–56.
[3] Boemare N E and Akhurst R J. J Gen Microbiol 1988; 134 (3):751-761.
[4] Boemare N E, et al. Int J Syst Bacteriol 1993; 43: 249–255.
[5] David H et al. Techniques for mass production of Sturmiopsis inferens Tns., Biocontrol Technology for Sugarcane Pest Management, Sugarcane Breeding Institute, Coimbatore, India. 1988; pp. 87–92.
[6] Ehlers R U and Niemann I. Syst Appl Microbiol 1998; 21:509–519.
[7] Gaugler R and. Kaya H K. Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, Florida, 1990; pp. 365 .
[8] Hsieh F C, Tzeng C Y and Kao S S. Taiwan Plant Prot Bull 2005; 4: 263–271.
[9] Iraki N, Salah N, Sansour M A, Segal D, Glazer I, Johnigk S A, Hussein M A and Ehlers R U. J App Entomol 2000; 124: 375–380.
[10] Kaya H K and Stock S. P. Manual of techniques in insect pathology, Techniques in insect Nematology, Academic Press, San Diego, CA. 1997; pp. 281–324.
[11] Murray RGE, et al. Determinative and cytological light microscopy, Methods for General and Molecular Bacteriology Vol. 1, American Society for Microbiology, Washington DC 1994; pp. 22–41.
[12] Pervez R and. Ali SS. Entomopathogenic nematodes as a potent biopesticides against insect pests, Newer approaches to Biotechnology, Narendra Publishing House Publishers and Distributors New Delhi, 2013; pp. 213-235.
[13] Pervez R, Ali S S and. Ahmad R. Int J Nematol 2007; 17 (1): 55-58.
[14] Pervez R, Ali S S and. Ahmad R. Int J Nematol 2008; 18 (1): 25-28.
[15] Pervez R, Eapen S J, Devasahayam S and Jacob T K. Nematol Medit 2012; 40 (1): 39-44.
[16] Pervez R, et al. Journal of Spices and Aromatic Crops 2014; 23(1): 71-75.
[17] Poinar G G and Thomas G M. Parasitol 1966; 56.
[18] Saitou N and Nei M. Mol Biol Evol 1987; 4: 406-425.
[19] Smibert R M and. Krieg N R Phenotypic characterization, Methods for General and Molecular Bacteriology Vol. 5, American Society for Microbiology, Washington DC 1994; pp. 611–654.
[20] Tamura K, et al. Proc Natl Acad Sci (USA) 2004; 101:11030-11035.
[21] Thomas G M and G O. Poinar Jr. Int J Syst Bacteriol 1979; 29: 352-360.