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MOLECULAR CHARACTERIZATION OF THE FIRST RECORD OF ENTOMOPATHOGENIC FUNGI *BEAUVERIA BASSIANA* AND *METARHIZIUM RILEYI* ISOLATES FROM JHARKHAND, INDIA

Cecilia K. Barla — University Department of Zoology, Vinoba Bhave University, Hazaribagh-825301, India.

Ajay K. Sharma — University Department of Zoology, Vinoba Bhave University, Hazaribagh-825301, India.

S. Maurya — ICAR-Indian Institute of Vegetable Research, Banaras Hindu University, Varanasi-221005, India.

Rahul Kumar* — University Department of Zoology, Vinoba Bhave University, Hazaribagh-825301, India. Department of Zoology, Sheodeni Sao College (Magadh University), Kaler-824127, India. *Corresponding Author

ABSTRACT Two fungal isolates, resembling *Beauveria bassiana* and *Metarhizium rileyi* were collected and cultured from the fields of okra and groundnut at Ranchi, Jharkhand, India. This is the first record of these fungi from Chotanagpur Plateau region. Their DNA was isolated and amplified using the universal ITS1 and ITS4 primers. The amplified product obtained was of the size of 500 bp for *Beauveria bassiana* and 550 bp for *Metarhizium rileyi*. Sequencing was performed using ITS1 primer. Comparing the respective sequences showed 97% resemblance to *Beauveria bassiana* and *Metarhizium rileyi* respectively. Multiple sequence alignment of ITS sequence of both the newly isolated strains along with its homologous sequences was conducted and a phylogenetic tree was built following the Neighbor-Joining method.

KEYWORDS : *Beauveria bassiana*, DNA Sequencing, Entomopathogenic fungi, Jharkhand, *Metarhizium rileyi*

INTRODUCTION

Jharkhand is a state which is also known as the Eastern Plateau and Hill region due to presence of extensive Chotanagpur Plateau over entire area of the state. It comprises of tribal people as a big portion of population, where agriculture is the main source of income for majority of the families. Increase in dependence on agriculture has led to an increase in the use pesticides to improve the quality of crops plus protection against harmful insect pests. In Jharkhand, use of pesticides has increased in the last three decades, which has been a blessing but a silent killer also. The World Vegetable Centre (AVRDC) had also reported the indiscriminate use of chemical pesticides among the farmers of Jharkhand. Farmers are mostly untrained about how to use these pesticides. They are unaware of what precautions to be taken while spraying pesticides. Most of them cover only their faces with a towel while spraying without wearing any goggles and gloves. 70-80% farmers purchase pesticides based on local seller's recommendation, without having knowledge about it. For example, Cypermethrin, which is not recommended for paddy, is still being used in some paddy fields of Deoghar district and Jamshedpur block of East Singbhum district.

Using an environment friendly method of destroying the harmful pests is the need of the hour in Jharkhand. What could be a better way than using a natural enemy (microorganism) to destroy these harmful pests? Studies have reported certain bacteria and fungi which can prove a good alternative of these chemical pesticides in reducing the population of pests out of which entomopathogenic fungi are promising bio-control agents and have its own advantages over other microorganisms. The use of entomopathogenic fungi was used first in the late 19th century, after which it was widely accepted all over the world. In the recent years, several morphological techniques and molecular techniques have been developed to identify different genera and species of entomopathogenic fungi.

Scientists in early times used morphology for identifying a fungus but this method alone is not sufficient for species identification as many of these microbes look alike. DNA sequence based molecular identification can be used for more accurate identification of fungal species. In case of fungi, molecular markers used are Internally Transcribed Spacer (ITS) gene which is present in between the highly conserved ribosomal DNA genes (18S, 5.8S, 28S). An ITS region is hypervariable and unique among species but shows moderate similarity among individuals of same species. It can be used to differentiate between genera, species and even strains of fungi. It has been widely used in fungal ecology and phylogenetic studies because it shows a low level of intraspecific variation and a high level of interspecific variation (**Horton and Bruns, 2001; Irinyi*et al*., 2016**). ITS sequencing chosen for sequence analysis has proven to be phylogentically informative for most of the fungal species (**White *et al*., 1990, Driver *et al*., 2000**).

In a previous work, we collected fungal infected insect cadavers from the fields of okra and groundnut at Ranchi, Jharkhand, India. Based on the macroscopic and microscopic examination of spore attributes under the phase contrast microscope, the isolated entomopathogenic fungal genera were determined to be *Beauveria bassiana* and *Metarhizium rileyi* (**Barla *et al.*, 2021**). This is the first record of these fungi from Chotanagpur Plateau region. The fungal pathogens isolated during the survey from different areas of Ranchi district, Jharkhand were morphologically identified as *Beauveria bassiana* and *Metarhizium rileyi* based on previous reports (**Rehner *et al.* 2011**, **Zhang *et al*., 2012**, **Chen *et al*., 2013**, **Agrawal *et al*., 2014**). Further investigations were based on the universal internal transcribed spacer (ITS) region (**White *et al*., 1990, Irinyi *et al*., 2016, Driver *et al*., 2000, A.Y.Kl Kichaoui *et al*., 2017, Lee *et al*., 2012).** In present work, we have performed DNA sequence based molecular characterization of these fungi to confirm their specific identities.

MATERIALS AND METHODS

DNA Isolation

For the isolation of genomic DNA, standard protocol of **Sambrook and Russell (2001)** was followed. The fungus was grown in PDA agar plate incubated at 25ºC. **Chi *et al*., 2009**, **Amir *et al*., 2015** reported TE method as an efficient way for fungal DNA extraction which yields a good quality and quantity of DNA. **T.A Pfeifer** and **G.C Khachatourians (1991)** used TE method for extraction of NA from *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces*. For this method, fungal isolates were allowed to grow on PDA media until its diameter reached upto 5-10mm under appropriate condition. Single colony was removed and suspended in 1ml distilled water along with 1g of CsCl and centrifuged. Bulk of mycelium settled on the surface was removed and washed with distilled water and dried. 15mg of freeze-dried mycelium grounded into a fine powder in liquid nitrogen followed by addition of 400µl of TE buffer (100mM Tris-HCl, pH 8.0, 10mM EDTA). After that 40µl of 10% SDS, 5µl of Proteinase K (20mg/ml) was added and incubated for 3-4 hours at 55ºC. This was followed by adding 400µl of Tris saturated Phenol (pH 8) and centrifuged. To the supernatant collected (10,000 rpm, 10min) 400µl Phenol:chloroform, chilled absolute ethanol was added, incubated (20ºC, 30min) and centrifuged (13,000rpm, 10min, 4ºC). DNA pellet formed was washed, dried under laminar air flow chamber, and resuspended in 50µl TE buffer (10mM/l Tris-HCl, 0.1mM/l EDTA, pH 7.8), RNAse (50µg/ml), incubated at 37ºC, 30min in a water bath and

the DNA concentration was quantified spectrophotmetrically using NanoDrop spectrophotometer.

PCR Amplification

Polymerase Chain Reaction was done using primers of the internal transcribed spacer (ITS) region (**White *et al*., 1990**). The primers used were pair of ITS1 (forward) and ITS4 (reverse).

ITS1: 5'- CTT GGT CAT TTA GAG GAA GTAA -3'
ITS4: 5'- TCC TCC GCT TAT TGA TAT GC -3'

For Polymerase chain reaction, a reaction volume of 25µl was prepared for each isolate. The reaction mixture contained 10X PCR buffer 2.5µl, dNTP mix 2.5µl, *Taq* DNA polymerase 0.2µl, 25mM $MgCl_2$ 2.0µl, 2µl upstream and 2µl downstream primer, 1µl (1:10) diluted template DNA and nuclease free water to make up the final volume to 25µl. PCR reaction was performed on Master cycler Veriti 96 Well Thermal Cycler (Applied BioSystems) with the following conditions: initial denaturation at 95ºC for 4min 30sec, followed by 30 cycles of denaturation at 95ºC for 30 sec, annealing at 56ºC for 30 sec, extension at 72ºC for 1 min and a final extension at 72ºC for 20 min. the PCR product was verified by running on 1% agarose gel electrophoresis, visualized under UV transilluminator (BioVis), photographed and documented under Gel Documentation system (Flur Chem HD2 Protein Simple). PCR products were purified using Qiagen QIA quick purification kit and sent for Sanger sequencing to AgriGenome Labs Pvt. Ltd., Kerala. Sequencing was performed using ITS1 primer.

Sequence Submission

The acquired sequences were submitted to NCBI GenBank and accession numbers were recorded.

Sequence Analysis

To confirm the identity of isolates *B. bassiana* and *M. rileyi*, the sequences were subjected to BLAST analysis. These sequences were compared with five nearest homologous sequences from NCBI GenBank database using multiple sequence alignment using MUSCLE programme, followed by construction of phylogenetic tree by Neighbor Joining method at 100 bootstrap values using Molecular Evolutionary Genetics Analysis (MEGA-X-11) software (**Tamura *et al*., 2013**, **Sayed *et al*., 2018**).

RESULTS AND DISCUSSION

The genomic DNA of the two fungal isolates was quantified by using NanoDrop spectrophotometer. 1µl of the genomic DNA in TE buffer was analyzed and following result was obtained:

Fungi	A260 nm	A280 nm	DNA conc. (µg/ml)	DNA purity
B. bassiana	0.382	0.201	760	1.82
M. rileyi	0.087	0.038	200	1.80

The exact identity of isolates as *Beauveria bassiana* and *Metarhiziumrileyi* was confirmed through molecular technique (ITS sequence analysis). The size of amplified DNA of *Beauveria bassiana* was approximately **500 bp** (Figure 1) which is in accordance with the findings of **Das *et al*., 2020**; **Lincy *et al*., 2017**; **Ghikas *et al*., 2010**; **Bhana *et al*., 2011**; **De Muro *et al*., 2012**. The size of amplified DNA of *Metarhizium rileyi* was approximately **550bp** (Figure 2)

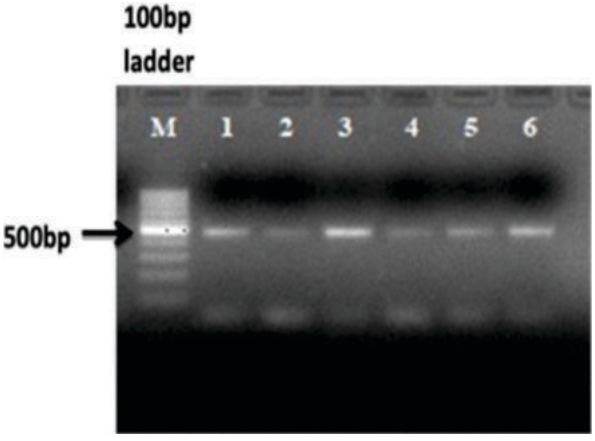

Figure 1: PCR amplification of *Beauveria bassiana* isolate using ITS primer: A product length of 500bp was observed. M-100bp ladder, 1-6 samples.

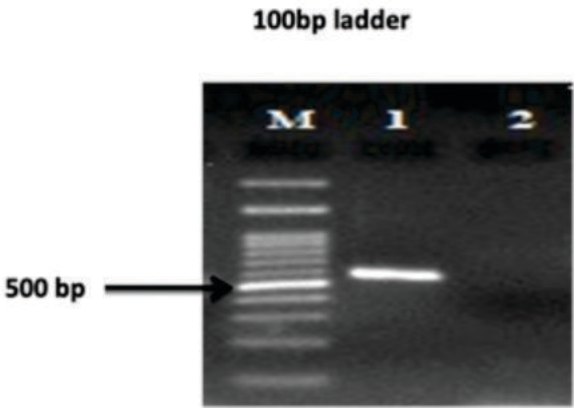

Figure 2: PCR amplification of *Metarhizium rileyi* isolate using ITS primer: A product length of 550bp was observed.

The NCBI-GenBank accession numbers of the submitted sequences are as follows:

1. *Beauveria bassiana* sequence: NCBI-GenBank accession number OR793987

2. *Metarhizium rileyi* sequence: NCBI-GenBank accession number OR793966

The NCBI-BLAST analysis of partial genome sequence amplified by ITS1 and ITS4 has confirmed the entomo-pathogenic fungi as *Metarhizium rileyi* and *Beauveria bassiana*. Fasta sequences were BLAST searched against the non-redundant nucleotide database of NCBI. Multiple sequence alignment of ITS sequence of the newly isolated strain of *B. bassiana* as well as *M. rileyi* along with its homologous sequences was constructed using the MUSCLE program. The phylogenetic tree was built following Neighbor-Joining method with 100 bootstrap values using MEGA X software. v11 (**Kumar *et al.* 2018**) (Figure 3 & Figure 4).

Figure 3: Phylogenetic tree of isolate *Beauveria bassiana* with five homologous sequences obtained from NCBI GenBank

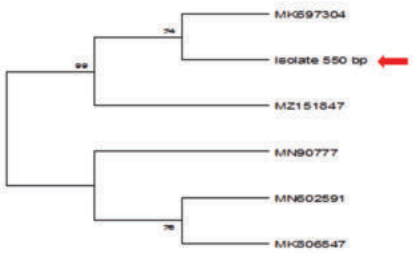

Figure 4: Phylogenetic tree of isolate *Metarhizium rileyi* with five homologous sequences obtained from NCBI GenBank

Phylogenetic trees reveal minor regional variations among all homologous sequences and tendency to cluster together due to negligible genetic distance.

CONCLUSION

Molecular technology does not replace the need of morphological method of characterization, but instead it complements the studies by

providing a complete, accurate and descriptive identification (**Hyde *et al*., 2010**). The present work gives information of the two most potent entomopathogenic fungi which were isolated from insect cadavers. This is the first record of these fungi from Chotanagpur Plateau region. According to their morphological characters, one isolate corresponded to *Beauveria bassiana* and other to *Metarhizium rileyi* species. PCR amplification, DNA sequencing, comparison of their sequence with other homologous sequences and the analysis of phylogenetic tree confirmed the taxonomic identity of both the strains of entomopathogenic fungi. These isolates can be used later to evaluate its efficacy in reduction of harmful pest population which are destroying our crops. It can be used as a good bio-formulation in place of chemical pesticide which will not harm the lives of humans and animals as well.

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