

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.907305>

## Molecular Characterization of Entomopathogenic Fungal Isolates

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### ABSTRACT

#### Keywords

Entomopathogenic fungi, Molecular identification, *Beauveria bassiana*, *Metarhizium flavoviride* var. *minus*

#### Article Info

Accepted:  
22 June 2020  
Available Online:  
10 July 2020

Two most virulent local isolates, *Beauveria bassiana* (Bb 111) and *Metarhizium flavoviride* var. *minus* (Mf) were amplified using ITS1 and ITS4 primers. The amplified product length was approximately 600 bp in both isolates. The nucleotide sequences of the isolates viz., *B. bassiana* (Bb 111) and *M. flavoviride* var. *minus* (Mf) deposited in the GenBank and were allotted with the accession numbers, KR 139926 and KR 139927, respectively.

### Introduction

A great number of insects are always threatening crops of economic importance such as maize, wheat, soybean, etc. They may affect plants either directly by causing mechanical damages throughout feeding, or indirectly by transmitting and spreading plant pathogens such as viruses, mollicutes, and other bacteria. However, pesticides are not the best alternative of control, among other things, because of their undesirable effect upon the environment, altering ecological features. In addition, insects usually develop

resistance to chemical pesticides, leading in some cases to the use of chemical pesticides more frequently and at higher concentrations, which might worsen the problem (23). Based on the negative effects of pesticides and the fact that sustainable agriculture requires better strategies to maintain insect populations under threshold values, biological control appears to be a potential tool for controlling insects. Entomopathogenic fungi might be a useful tool to develop an Integrated Pest Management program. The use of entomopathogenic fungi as a microbial control agent was considered for the first time at the

end of the 19th century, and the idea was widely accepted among researchers at a time when synthetic chemical insecticides were still unknown. However, the highest number of studies was carried out during the 20th century (3, 4, 9). Since then, fungi have been found to have certain advantages over other methods of controls because they have restricted host range and are harmless to non-target organisms such as predators, parasites, and other pathogens(11-13, 15,21).

Fungal isolates from different locations will have varying virulence and adaptability to environmental conditions like temperature and humidity. Utilization of native strains of entomopathogens with greater virulence and increased persistence in the environment are need of the hour. Identification and characterization of such type of isolates will pave way for the development of biopesticides with high virulence and temperature adaptability.

Several methods have been used to identify a species of entomopathogenic fungi. Hyphomycetes are classified by their morphological characteristics of spores, conidogenous cells and colonies, their hosts, pathogenicity and growth or nutrient requirements [15]. Obviously, it is not quite possible to distinguish fungal isolates using only morphological characters. In recent years, molecular identification techniques are required as well as the traditional morphological characteristics. Different molecular techniques were used to identify a species of entomopathogenic fungi [11,16-19].

## **Materials and Methods**

The fungal pathogens, Bb 111 and *M. flavoviride* var. *minus* isolated during the survey were morphologically identified as *B. bassiana* and *M. flavoviride* var. *minus*. To

confirm the molecular level of identity, the two fungal pathogens Bb 111 and *M. flavoviride* var. *minus* were designated as Bb 111 and Mf and further investigations were made based on the internal transcribed spacer (ITS) region.

## **DNA extraction**

For the isolation of DNA, the fungus was grown in 100 ml SMAY broth and incubated for seven days. The mycelial mat was removed carefully and washed with sterile water repeatedly to remove the media constituents. 3 to 5 g of wet mycelium was freeze dried in -70°C and ground in liquid nitrogen, followed by addition of 5 to 6 ml of cetyl trimethyl ammonium bromide (CTAB) extraction buffer (10 mM tris-base (pH 8.0), 20 mM ethylene diamine tetra acetic acid (EDTA) (pH 8.0), 1.4 M NaCl, CTAB (2%), mercapto ethanol (0.1%) and PVP (0.2%). After that 20 µl of proteinase K (20 mg/ml) was added and incubated at 65°C for 1 h. This was followed by addition of 20 µl RNase A (10 mg/ml) and further incubated at 65°C for 15 minutes. To the supernatant collected after centrifugation (8,000 rpm, 10 minutes), 10µl chloroform: iso amyl alcohol (24:1) was added.

The mixture was vortexed for 5 minutes and centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred to clean tube and mixed with equal volume of ice cold isopropanol. It was incubated at 25°C for DNA precipitation. The precipitate was collected by centrifugation and the pellet was washed with 0.1 M ammonium acetate in 70 per cent ethanol.

Again incubation was given for 15 minutes to dry the pellet. The pellet was resuspended in TE buffer (10 mM Tris, 1mM EDTA, pH 8.0) and the DNA concentration was estimated spectrophotometrically.

### PCR amplification and sequencing of 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA region

Polymerase chain reaction (PCR) amplification of 18S ribosomal RNA was performed using primers of the internal transcribed spacer (ITS) region (White *et al.*, 1990).

The primers used was

ITS1-F 5'- TCGGTAGGTAGGTGAACCT  
GCGG -3'

18s-ITSRV1-R 5'- TCCTCCGCTTATTGAT  
ATGC -3'

The polymerase chain reaction (50 µl) was set to amplify the 18S rRNA region by using the genomic DNA as template. The reaction mixture typically contained genomic DNA 20 to 40 ng, 1X PCR reaction buffer 2.50 µl, 2.5 mM dNTPs 1.0 µl, forward and reverse primers 100ng each, and 3 unit of Taq DNA polymerase and finally the volume was made up to 50 µl using distilled water. All the additions were done on ice and the PCR reaction was performed on gradient Mastercycler (Eppendorf, Germany). The PCR conditions for 18S rRNA amplification were initial denaturation at 94°C for 3 min; followed by 35 cycles of 94°C for 1 min, 56°C for 30 sec, 72°C for 1 min and final extension at 72°C for 10 min. The PCR products were resolved by electrophoresis in a 1.5 per cent agarose gel, visualized under UV light and photographed and documented with an Alpha Imager. The PCR products were sequenced following Sanger sequencing chemistry using universal primer pair and sequencing work has been done at SciGenom Labs Private Ltd., Kerala.

To confirm the identity of strain *B. bassiana* (Bb 111) and *M. flavoviride* var. *minus* (Mf)

and to investigate their phylogenetic relationship with other strains from different geographical region, partial sequence of 18S rRNA ITS1-5.8s-ITS4-28s region of the two strains were obtained as detailed above and compared with ten nearest homologous search in GenBank of NCBI database following BLAST analysis and the phylogenetic tree was constructed using Neighbour joining analysis. To identify the intra geographic variation and host specificity, further *B. bassiana* (Bb 111) and *M. flavoviride* var. *minus* (Mf) strain was compared with sequences of indigenous *B. bassiana* and *M. flavoviride* strains downloaded from genbank. Nucleotide sequence alignments were made using CLUSTALW2 with the multiple alignment parameters. Phylogenetic tree was constructed and the sequential agglomerative hierarchical non overlapping clustering was done using unweighted pair groups with arithmetic averages (UPGMA) method.

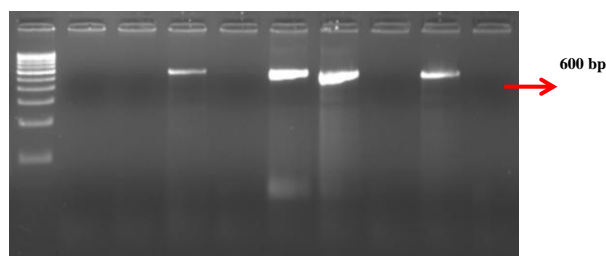
The correct identity of the isolates viz., Bb 111 as *B. bassiana* and Mf as *M. flavoviride* var. *minus* was confirmed through *B. bassiana* and *M. flavoviride* var. *minus* through molecular methods (ITS region analysis). Similar conformity studies for *B. bassiana* and *M. flavoviride* var. *minus* were documented by several workers (Shih *et al.*, 1995; Coates *et al.*, 2001; Kim *et al.*, 2008). In the present study, amplification of internal transcribed spacer region (ITS) of *B. bassiana* (Bb 111) and *M. flavoviride* var. *minus* isolates with ITS 1 and ITS 4 primers revealed that both isolates were amplified at approximately 600bp and was confirmed with identity of other *B. bassiana* and *M. flavoviride* var. *minus* (Fig 1 &2).

The results are in accordance with the findings of Neuveglise *et al.*, (1994), De Muro *et al.*, (2012) and Sabbahi *et al.*, (2012) where they differentiated the *Beauveria*

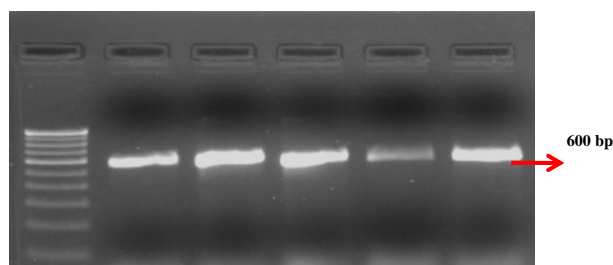
isolates by targeting 28S rDNA sequences as well 18S rRNA, ITS regions and 5.8S of rDNA. De Muro *et al.*, (2012) followed combination of different techniques to differentiate isolates of *Beauveria* species collected from litter material and insect samples. They also found that isolates having

high similarity at morphological observation were also found to show similarity with ITS sequence analysis. Similar studies with ITS 5.8S- ITS2 sequence analysis were conducted in different continents to study the genetic diversity of *B. bassiana* isolates (Ghikas *et al.*, 2010; Bhana *et al.*, 2011).

**Fig.1** Characterization of *B. bassiana* (Bb 111) based on ITS region



**Fig.2** Characterization of *M. flavoviride* var. *minus* based on ITS region



In conclusion, identification and detailed description of the fungal strain as an active ingredient of biopesticide is required environmental and epizootiological information. In this study, we conducted a molecular study of entomopathogenic fungi isolated from the plants. Additional research is required to test pathogenicity of these fungi to other pests and to determine the effectiveness in the field conditions.

### Acknowledgement

The authors thankfully acknowledge the Department of Biotechnology (DBT), New Delhi, for financial support to conduct the experiments.

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**How to cite this article:**

Sumaiya Parveen, S., K. Ramaraju and Jeyarani, S. 2020. Molecular Characterization of Entomopathogenic Fungal Isolates. *Int.J.Curr.Microbiol.App.Sci.* 9(07): 2588-2593.  
doi: <https://doi.org/10.20546/ijcmas.2020.907.305>